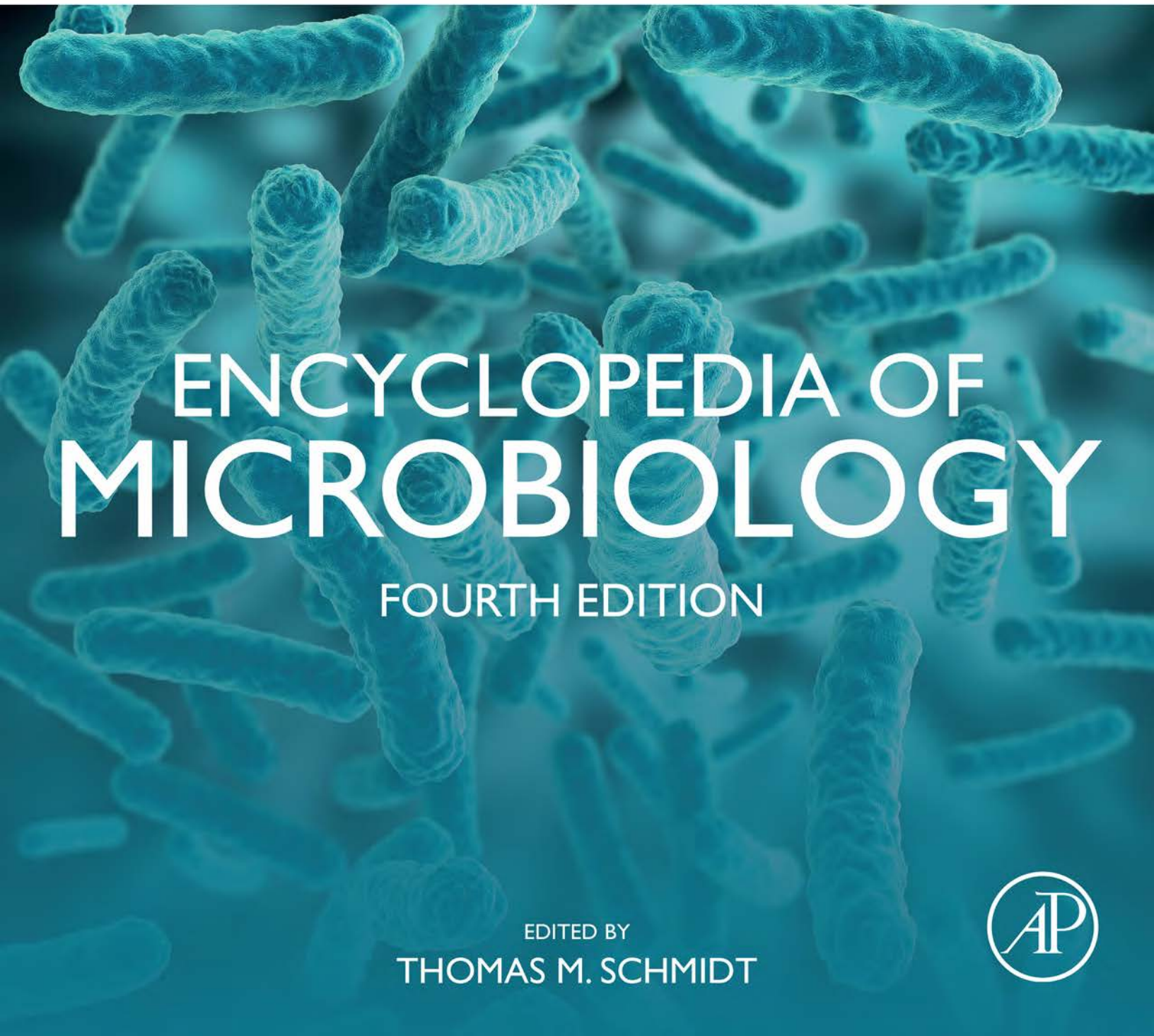
VOLUME 1



ENCYCLOPEDIA OF MICROBIOLOGY

FOURTH EDITION

EDITED BY
THOMAS M. SCHMIDT

ENCYCLOPEDIA OF
MICROBIOLOGY

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VOLUME 1

A-C

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ENCYCLOPEDIA OF
MICROBIOLOGY

Fourth Edition

EDITOR IN CHIEF

TOM SCHMIDT

Departments of Internal Medicine and Ecology & Evolutionary Biology
University of Michigan

VOLUME 1

A-C

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EDITOR-IN-CHIEF

Thomas (Tom) Schmidt is a microbial physiologist and ecologist who studies diverse microbes and microbial communities. Tom received a PhD from The Ohio State University and conducted postdoctoral research at Scripps Institute of Oceanography and Indiana University. He spent much of his career studying the ecology of microbes in soil that are responsible for the exchange of greenhouse gases with the atmosphere. More recently, he joined the University of Michigan and focused his research on the human microbiome. His joint appointment in the Departments of Internal Medicine and Ecology & Evolutionary Biology reflects his expertise in applying ecological and evolutionary principles to understand the functioning of complex microbial communities.

Tom is a fellow of the American Academy for Microbiology and was director of the Marine Biological Laboratory's Microbial Diversity Course in Woods Hole. Through that course and in his laboratory, he has helped numerous scientists incorporate molecular approaches into traditional strategies for studying the microbial world. He currently teaches a university course that merges his research and teaching goals by engaging students in a coordinated study of the effects of diet on the gut microbiome, and he directs a graduate program that combines laboratory sciences and modeling. Tom was a section editor for previous editions of the *Encyclopedia of Microbiology* and was delighted to assume the role as editor-in-chief to help bring the microbial world to an expanding audience.

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EDITORIAL BOARD

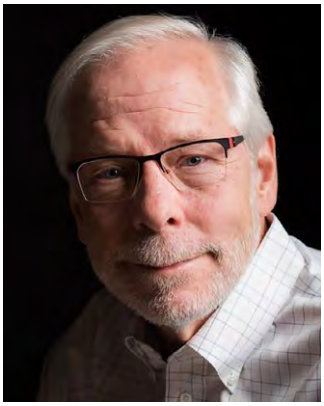
Bianca Brahamsha is a research microbiologist at Scripps Institution of Oceanography at the University of California, San Diego. She was a biology and history major at Goucher College, earned her PhD in microbiology at Cornell University, and carried out postdoctoral work in molecular genetics as an American Cancer Society fellow at the University of Chicago. Her research interests have centered on bacterial motility, the genetics and physiology of marine cyanobacteria, and on the interactions between cyanobacteria and their eukaryotic predators.

James (Jim) Brown was born in Atlanta, Georgia, and lived in Dade City (Florida), Bloomington (Indiana), Lima (Peru), and Muncie (Indiana) while growing up. From the beginning, he had an intense interest in nature, including anything found in the woods, rivers, beach or ocean that were always nearby.

Jim attended Ball State University beginning in 1976. A single lecture on microbial diversity in a general microbiology class, followed by the announcement of the discovery of the Archaea (George Fox and Carl Woese, 1977), sparked his lasting interest in microbiology. This led to undergraduate research examining *Beggiatoa* in a sulfur spring in French Lick, Indiana. After receiving his BS in biology in 1980, he joined the graduate program in microbiology at Miami University, where he worked on plant tissue culture mRNAs with Prof. Ronald Treick. After obtaining an MS degree in 1982, he moved to the MCD Biology Program at The Ohio State University to work on the molecular biology of methanogenic Archaea in the Department of Microbiology with Prof. John Reeve. While there, Jim worked on polyadenylation of mRNAs, RNA polymerase, and promoters in Archaea, and received his PhD in 1988. Jim then went to Indiana University for 5 years of postdoctoral work in Prof. Norman Pace's lab on the comparative analysis of the structure of a bacterial ribozyme, RNase P.

In January of 1994, Jim started as an assistant professor in the Department of Microbiology and moved to North Carolina State University. Research in Jim's lab focused on the comparative sequence and biochemical analysis of RNA, and in particular RNase P in Archaea. Students in the Brown lab developed a high-resolution model for the structure of this RNA, how it changed over the diversification of the Archaea, the protein subunits associated with the RNA, and how they contribute to the function of the holoenzyme. Jim developed, teaches, and wrote the textbook for a senior-level undergraduate lab course in microbial diversity. Jim is now Professor Emeritus in the Department of Biological Sciences at NC State University.

Jim has a son and two daughters, and is married to Melanie Lee-Brown, professor of biology and director of Research and Creative Endeavors at Guilford College. For fun, Jim enjoys skin and scuba diving, driving (i.e., working on) his 1968 Lotus Super Seven, and restoring vintage racing bicycles. Jim and Melanie have been renovating an abandoned fishing lodge on North Caicos Island (Turks and Caicos Islands, BWI), which will be reopened soon as a bed and breakfast.



Larry Forney is a university distinguished professor and a member of the American Academy of Microbiology with academic appointments in the Department of Biological Sciences and Bioinformatics and Computational Biology at the University of Idaho. Dr. Forney is an evolutionary ecologist who conducts research on the complex array of factors that influence the function, composition, structure, and temporal dynamics of bacterial communities in a wide array of habitats. In recent years he has largely focused on the community ecology of the human vagina across a woman's lifetime with an eye on understanding how changes in community structure and function affect a woman's risk to bacterial vaginosis, sexually transmitted infections, and pre-term birth, and how host factors shape these communities. His research extends to understanding the mutation-selection processes that govern the occurrence and persistence of genetic diversity of bacterial populations in spatially structured environments such as microbial biofilms and porous media. These studies have shown that spatial structure creates conditions in which mutation frequencies are high and selective sweeps are protracted, which leads to extraordinary within species diversity that increases the resilience of populations to environmental changes.

Robert Haselkorn was the F. L. Pritzker distinguished service professor of molecular genetics and cell biology at the University of Chicago, retiring several years ago. He was an undergraduate at Princeton, a graduate student at Harvard, and a postdoctorate in Cambridge, England. He started his teaching career at Chicago in 1961 in biophysics, extending later to microbiology, biochemistry, and chemistry. His research interests have centered on heterocyst differentiation in nitrogen-fixing cyanobacteria, in bacterial genomics, and in the enzyme acetyl-CoA carboxylase in plants, parasites, and people. He is a member of the National Academy of Sciences, a fellow of the American Academy of Arts and Sciences, and a member of the American Philosophical Society. Among other external activities he was a founder and adviser for 20 years to the International Center for Genetic Engineering and Biotechnology, located in Italy and India. For the past 15 years Haselkorn and his wife Margot have been working on selection and supporting a speaker for the Haselkorn Lecture at the University of Chicago, an award they have endowed. Many of the Haselkorn Lecturers have been Nobel Prize winners or will be soon.

Jennie C. Hunter-Cevera received her PhD from Rutgers University in 1978 (microbial physiology and biochemistry), an MS in microbial ecology in 1972, and a BS in biology from West Virginia University in 1970. She is the founder of Hunter and Associates, a consulting firm focusing on finding integrative solutions to complex problems involving sustainability in the life sciences arena. From July of 2009 to August 2012, she was the executive vice president of Discovery and Analytical Sciences (DAS), Corporate Development and Government Relations. She has 22 years of experience in the pharmaceutical and biotechnology industries (E. R. Squibb and Sons, Cetus Corporation, GeoBiotics, and Universal Foods). She was the co-founder of The Biotic Network and BlueSky Laboratory that contracted with biotechnology and pharmaceutical companies on basic and applied natural product research. Dr. Hunter-Cevera was an

employee for 5 years with the Department of Energy as the head of the Lawrence Berkeley National Laboratory's Center for Environmental Biotechnology. She worked in academia for 10 years as the president of the University of Maryland Biotechnology Institute and 2 years as the Interim Provost of Mount St. Mary's University. She also served as project manager for UC Davis's CIFAR (Center for Industrial Food and Agricultural Research). She has published many papers and chapters, and served as senior editor of the *Journal of Industrial Microbiology and Biotechnology* for 10 years. She is a co-section head of microbiology for Faculty of 1000. She holds 15 patents and specializes in areas of screen design for the discovery of natural compounds in the area of human therapeutics, biodefense, sustainable agriculture, bioremediation, and biocatalysis for industrial processes in the food and clothing industries.

While in Maryland, she served as a member of the Executive Committee for Governor Erhlich's transition team, his Committee on science, technology, engineering, and maths, and was the Technology Representative for Governor Glendenning on the Southern Governor's Association, and she served on the Technology Economic Development Corporation, TEDCO (Board of Directors for 4 years and was Chair for 2 years). Dr. Hunter-Cevera also served on the Advisory Board for the Maryland Industrial Partnerships, the MDBio's Board of Directors, and the MDBio Foundation for 10 years and on the BioIT Coalition for 5 years. She served as an Entremed Board Member for 10 years and served on the Executive Committee for 2 years. She was a board member for Patients with Power, a software company that designs programs for cancer patients to make the best decisions for their treatments. Jennie also served as acting secretary for Maryland's Higher Education Commission in 2015.

She is a member of several professional societies and has served as president of the Society for Industrial Microbiology, the International Marine Biotechnology Association, and the United States Federation of Culture Collections. Dr. Hunter has served on many national committees and commissions and was Chair of the National Research Council's Committee on Large Scale Production of Biofuels from Algae. She also chaired two other NRC Committees: Standing Committee on DOD's Translational Medicine and the DOE's Genome to Life for Biofuels.

She is the recipient of several awards and honors including Maryland's Top 50 Influential People (2007, 2009) and Maryland's Top 100 Women (2003, 2006, 2009), American Society for Microbiology's Porter Award for distinguished research in microbial systematics and taxonomy, elected as a SIM fellow, a member of the ASM Academy of Microbiology, and an AAAS fellow. She is also a WVU Distinguished Alumni Awardee and Nath lecturer and served as an entrepreneurial coach for the UNC Executive MBA program. She was awarded an Honorary Doctorate from West Virginia University, May 2013, and the Rutgers Cook College Dennis M. Fenton Distinguished Graduate Alumni Award in 2014.

She currently serves on the International Advisory Council for Brazil's Fundação Dom Cabral which is a world-class Brazilian business school that develops strategic thinking skills of executives, entrepreneurs, and public-sector managers. In addition, Jennie served 6 years on the Edison Awards Steering Committee and currently serves on the Edison Awards Advisory Board.

Stanley Maloy is a professor of microbiology and associate vice president for research and innovation at San Diego State University. Stanley's research has focused on bacterial and phage genetics and physiology, evolution of infectious diseases, and development of antimicrobials and vaccines. In addition, he has consulted with large and small companies in multiple sectors, played a role in starting several Biotech companies, and served leadership roles in multiple start-up companies.

Dr. McCormick earned her PhD in microbiology in the topic area of intestinal ecology, and completed postdoctoral training at Harvard Medical School. She remained on the faculty of Harvard Medical School where she was an associate professor of pediatric gastroenterology, and director of research for the Mucosal Immunology and Biology Research Center at Massachusetts General Hospital. In 2008, she joined the University of Massachusetts Medical School where she is professor and vice chair of the Department of Microbiology and Physiological Systems. Dr. McCormick is also the founding executive director of the University of Massachusetts Center for Microbiome Research, which she established in 2014. Dr. McCormick is one of the original pioneers in the field now known as cellular microbiology. Her work provided the first evidence that epithelial cells in response to pathogen contact orchestrate a pro-inflammatory program, which recruits inflammatory cells. Dr. McCormick has since identified new, previously unidentified and unexpected virulence mechanisms that are key to the inflammatory response, leading to both novel biological principles of host–microbe interactions and therapeutic intervention strategies for the treatment inflammatory bowel disease, and cancer. Her work continues to identify novel ways in which microbes interact with the intestinal epithelium, publishing over 100 original research papers and opinion pieces in this area. Dr. McCormick is an elected fellow of the American Academy of Microbiology, is on the Board of Editors for *Gastroenterology*, and *Gut Microbes*, and serves as a member of four Editorial Review Boards.

Dr. Mobley received his BS degree in biology from Emory University in 1975 and his PhD degree in microbiology and immunology from University of Louisville in 1981. He conducted postdoctoral training in biological chemistry and then bacterial genetics in the Center for Vaccine Development at the University of Maryland School of Medicine. He served on the faculty at the University of Maryland School of Medicine from 1984 until 2004 in the Division of Infectious Diseases (1984–97) and then the Department of Microbiology and Immunology (1997–2004) where he led the graduate program. During that time, he held a joint appointment in the Department of Biochemistry and Molecular Biology and trained graduate students in that program. In 2004, Mobley moved to the University of Michigan to chair the Department of Microbiology and Immunology and was installed as the inaugural Frederick G. Novy Collegiate Professor of Microbiology and Immunology.

Dr. Mobley's research interests focus on the molecular mechanisms of bacterial pathogenesis and on the fundamental basic research that will lay the groundwork for future therapeutics and vaccines. His lab studies virulence mechanisms of uropathogenic *Escherichia coli* and *Proteus mirabilis* that cause uncomplicated and complicated urinary tract infections, respectively, and, in the recent past, *Helicobacter pylori* that causes gastritis and peptic ulcer disease. For *E. coli* and *Proteus mirabilis*, his lab is focused on identifying surface-exposed proteins that are both synthesized by the bacteria during a urinary tract infection and conserved among uropathogenic *E. coli and Proteus* strains. Using these conserved antigens, his lab is determining the efficacy of candidate proteins as components of a multivalent subunit vaccine to protect against urinary tract infection.

Dr. Mobley is a fellow of the American Academy of Microbiology and a fellow of the American Association for the Advancement of Science and a member and past president of the Association of Medical School Microbiology & Immunology Chairs. He was the recipient of the inaugural University of Michigan Postdoctoral Association Excellence in Mentorship Award in 2012. He is a member of the editorial review boards of *Infection and Immunity and Microbiology Spectrum* and has served as a study section member for the National Institutes of Health. Dr. Mobley was awarded and named a distinguished university professor in 2015. This is the University's most prestigious professorship established to recognize senior faculty with exceptional scholarly achievements, national and international reputations for academic excellence, and superior records of teaching, mentoring, and service.

Dr. Mobley has published 255 peer-reviewed articles which have been cited in the literature over 18,000 times as of June 2019, as well as 49 book chapters and 5 books. He has trained 29 PhD students and 38 postdoctoral fellows, and has delivered 232 invited lectures in 21 countries.

Carlos Pedrós-Alió graduated in biology at the Autonomous University of Barcelona and got his PhD in bacteriology at the University of Wisconsin–Madison. After a postdoctoral stay at the Autonomous University he became an assistant professor of microbiology. He moved to the Marine Sciences Institute (CSIC, Barcelona) in 1989 where he was a research professor since 2000. In 2016 he moved to the National Center for Biotechnology (CSIC, Madrid). Dr. Pedrós-Alió's interest is to understand the ecology of aquatic microorganisms. Around 2005 he started to use genomics as a tool to generate hypotheses that could later be tested experimentally. He also likes to study extreme environments such as hypersaline systems, thermal springs, or polar waters. Another interest is in finding the mechanisms maintaining a large number of rare bacteria in aquatic ecosystems. He is also interested in outreach, relationships between art and science, biology of spirituality, fiction writing, and birding.

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SUBJECT CLASSIFICATION

HISTORICAL

AIDS, Historical
Cholera, Historical
Historical Plague
Historical Smallpox
History of Microbiology
History of Virology
Methods, Philosophy of
Spontaneous Generation
Syphilis, Historical
Typhoid, Historical
Typhus Fevers and Other Rickettsial Diseases, Historical

MICROBIAL DIVERSITY

Acidobacteria
Amitochondriate Protists (Diplomonads, Parabasalids and Oxymonads)
Amoebozoa
Aquificae
Archaea – An Introduction
Aspergillus: A Multifaceted Genus
Bacillus Thuringiensis: Mechanisms and Use
Bacteriophage: Overview
Brochothrix thermosphacta
Caulobacter
Chlamydia
Chloroflexi
Clostridia
Cyanobacteria
Dictyostelium
Dinoflagellates
Escherichia coli
Green Algae: Chlorophyta and Streptophyta
Haemophilus Influenzae
Halophilic Archaea
Helicobacter pylori
Legionella and *Bartonella*
Leishmania
Listeria monocytogenes
Mollicutes
Nanoarchaeota
Phylum *Verrucomicrobia*
Picoeukaryotes
Planctomycetes
Plant Pathogens, Minor (Phytoplasmas)
Protozoa
Rhizobia
Spirochetes
Staphylococcus

Streptococcus Pneumoniae Evolving – Impact of Antibiotics and Vaccines
Streptomyces
Trypanosomes
Viroids/Virusoids

PHYSIOLOGY AND GENOMICS

Archaellum
Autotrophic CO2 Metabolism
Bacterial and Archaeal Cell Membranes
Bacterial and Archaeal Cell Structure
Bacterial Bioluminescence
Bacterial Cell Cycles and Division
Bacterial Chemotaxis: Conservation and Variation on a Theme
Bacterial Development
Bacterial Flagella
Bioluminescence in Eukaryotic Microbes
Chromosome Replication and Segregation
Circadian Rhythmicity in Prokaryotes
Conjugation, Bacterial
CRISPR–Cas9
Crystalline Cell Surface Layers (S-Layers)
Energy Transduction Processes
Fundamentals of Metabolic Systems Biology
Gene Transfer Agents
Genetically Modified Organisms: Guidelines and Regulations for Research
Glycogen Biosynthesis
Horizontal Gene Transfer: Uptake of Extracellular DNA by Bacteria
Intracellular Structures of Prokaryotes: Inclusions, Compartments, and Assemblages
Iron Metabolism
Lipid Biosynthesis
Magnetotaxis
Methanogenesis
Methylation and other Modifications of Nucleic Acids and Proteins
Microbial Solute Transporters
Nitrogen Assimilation in Bacteria
No Bones About It: The Bacterial Cytoskeleton
Nonflagellar Bacterial Motility
Outer Membrane, Gram-Negative Bacteria
Peptidoglycan (Murein)
Phototaxis in Archaea and Bacteria
Phototrophy and Phototrophs
Pili, Fimbriae
Posttranscriptional Regulation
Regulation of Carbon Assimilation in Bacteria
Regulation of Replication Origin Firing
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PREFACE

As with previous editions of the *Encyclopedia of Microbiology*, the 4th edition takes on the challenge of providing a contemporary overview of microbes—the most abundant and diverse forms of life on Earth. These single-celled organisms were the first life forms to inhabit Earth and they transformed the planet and its atmosphere. They continue to maintain Earth's atmosphere, drive essential processes in terrestrial and aquatic ecosystems, and form intimate relationships with all plants and animals. Microbes can reproduce by doubling every 20 minutes or can remain dormant for many thousands of years. They colonize every known habitat on Earth, accounting for approximately half of the living biomass on our planet. Microbes also cause the most devastating diseases known to humans and are winning the antibiotic war we wage against them. Yet, we cannot live without microbes as symbionts of humans and drivers of Earth's biosphere.

Despite the central roles of microbes, we are only just becoming aware of our limited knowledge of interactions among microbes, other organisms, and the environment. Molecular techniques continue to lead a revolution in our understanding of microbial diversity and function, with the new "big data" sciences of genomics, transcriptomics, proteomics, and metabolomics now being applied to communities of microbes. These techniques provide the ability to identify and study complex communities of microbes in diverse environments including the human gut, forest soil, water-treatment biofilters, and the open ocean.

Given the remarkable physiological and phylogenetic diversity of microbes, their capacity for rapid evolution, and the pivotal role of host-associated and environmental microbiomes, it is difficult, if not impossible, to identify contemporary questions in biology that are not influenced by microbes. It is also increasingly difficult to assemble an encyclopedia that provides comprehensive coverage of the vast universe of microbes. Despite these challenges, we have engaged the expertise of scientists around the globe to provide an exceptional overview of and perspective on the microbial world. This edition of the *Encyclopedia of Microbiology* will help readers develop a framework for understanding microbes and will provide references directing readers to the primary literature that is needed for a more thorough appreciation of specific topics.

We have included a number of articles which provide a historical perspective of microbiology that is focused on disease—one of humanity's earliest acknowledgment of microbes. These help frame more contemporary issues in microbiology that have moved far beyond the relatively small collection of microbes that cause disease. Rather than simply alphabetize topics as in traditional encyclopedias, articles in the 4th edition are also listed under the following themes in a Subject Classification: Historical, Microbial Diversity, Physiology and Genomics, Ecology and Evolution, Pathogenesis and Immunology, and Technological Advances and Applied Microbiology. We hope this helps readers navigate the work more effectively.

On behalf of an insightful and collegial editorial team, I hope that you emerge from your forays into the *Encyclopedia of Microbiology* with some of the awe that we share for the elegance and power of the microbial world.

Thomas M. Schmidt
Editor-in-Chief

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INTRODUCTION: MICROBIAL DIVERSITY

It is what we think we know already that often prevents us from learning.

—Claude Bernard

One of the difficult lessons for any professional scientist is that, as much as we know, as good as our tools and questions are, our knowledge is crude and far smaller than we imagined when we were students. Particularly troublesome is that most fundamental aspects of various scientific fields are simply not very well understood. The physicist wonders what, exactly, "time" is, how space is structured, and why the Planck Constant is $6.62607004 \times 10^{-14}$ m^2 kg/s instead of any other number you might care to choose. Chemists argue about the nature of a covalent bond, or whether or not transition states exist in reality. Biologists are at a loss to describe how life originated, or even what exactly life (in a general sense) actually is. In the most important book of modern times, "On the Origin of Species," Charles Darwin clearly (but not concisely) deconstructs the idea of the biological "species," and despite what many of us learned in school, our view of the "species" even for multicellular sexually reproducing creatures remains murky. The modern reader of "On the Origin of Species" might be forgiven for concluding that the reason for this is that it is the actual *biology* of species, and by extension "life," our origin, etc., rather than our concepts of them, that are fundamentally disordered.

The subject of this section of the Encyclopedia is Microbial Diversity. What does this mean? What *is* "microbial diversity?"

Let us start with "microbial." Literally speaking, the term "microbial" means small living things, usually understood to mean life too small to see with the unaided eye. The scientific field of microbiology, however, mostly emerged from our need to solve crucial health dangers created by a tiny fraction of microbes. As a result, "microbiology," and so "microbes," refer to Bacteria (and in modern times the Archaea), fungi, a scattering of problematic single-celled or small multicellular eukaryotes, and the physiological systems of the human body that manages our interactions with these creatures. This sells the microbial world very short. For 85% of the history of our planet, life was *entirely* microbial, and it remains *predominantly* microbial. In the words of Stephen J. Gould, "*We live now in the 'Age of Bacteria.' Our planet has always been in the 'Age of Bacteria,' ever since the first fossils—bacteria, of course—were entombed in rocks more than 3 billion years ago. On any possible, reasonable or fair criterion, bacteria are—and always have been—the dominant forms of life on Earth.*" Another view of this comes from the phylogenetic perspective. If you scrutinize any objective/quantitative "Tree of Life," based on molecular sequence analysis or any other criterion of choice, you will quickly discover that nonmicrobial life is limited to the tips of a small number of otherwise un-noteworthy branches. Only from our self-centered anthropocentric viewpoint is microbiology distinguishable from biology as a whole.

And what of "diversity?" Living things and their morphology, structure, physiology, ecology, internal mechanisms, behavior, interactions, etc., are far more diverse than most of us have been led to believe. For example, if you think you know how mitosis works, look it up in *Giardia* and be amazed. The notion of what constitutes a "gene" in the kinetoplast of *Trypanosoma* requires you to throw almost everything you think you know about molecular genetics away. Then have a look through the genome of the archaeon *Nanoarchaeum.* These are just the tips of icebergs; these and many more can be found within the chapters that follow. And yet . . . all of this diversity rests over an almost entirely uniform biochemistry that speaks to the shared ancestry of all living things on Earth. How, then, do we assess the "diversity" of living things? Historically, this meant comparing the morphology of different living things; this was the origin of Linnaean taxonomy and, to be fair, is very useful for organisms on the human size scale. More recently molecular phylogenetics has allowed us to more clearly understand how creatures are related, and draw an objective and quantitative graph of these

relationships; the molecular "Tree of Life" is a more useful roadmap of biological diversity. But it is clear that an even more sophisticated view of biological diversity is required, for example to describe how horizontal gene transfer impacts "diversity," and many of these questions emerge from microbiology. Perhaps the most important fundamental question in microbiology today is "What is a microbial species?" In other words, how do we conceive of a "species" in organisms that do not reproduce sexually? This is not a trivial or parochial question; remember that the concept of biological evolution, and so the foundation of the science of biology, emerged directly from exploration of what a "species" is and how it originates, in plants and animals. But this was over 150 years ago. Broadening our view of microbial diversity, how it is structured and its history and origins, is the key to new and deeper biological insight.

And so, as is usual in science, attempt to answer a simple question that raises many more questions and provides few answers. What *is* microbial diversity? I dunno, but let us have a look. . ..

James Brown

INTRODUCTION: PHYSIOLOGY AND GENOMICS

The first two editions of EOM were edited entirely by Dr. Joshua Lederberg. The third edition was also planned largely by Dr. Lederberg, but he died during that phase, in 2008, and was replaced by Dr. Moselio Schaechter, promoted from the ranks of associate editors. Dr. Thomas Schmidt was also an associate editor for that edition and moved up to editor-in-chief for the fourth edition.

The time that has passed from the first to the fourth edition has seen remarkable advances in understanding the variety of microbes. Complete genome sequences were determined for 18 microbes by October 1998 and published in the second edition in 2000. By 2019, a single company had determined the complete annotated genome sequences of several thousands of microbes. Comparative genomics, including the ancillary "omics," as well as the application of systems biology to reconstruct metabolic networks, have provided new insights into many aspects of microbial physiology and genetics. The advent of cryo EM and of single particle cryo EM has revolutionized how we understand the structure of cells and macromolecules. For this fourth edition, we were guided by the splendid organization of the third and additional areas in which considerable advances have been made. The fourth edition now includes articles describing additional features of microbiology: cryo-electron microscopy, CRISPR/*cas*, and metabolic systems biology, as examples of completely new methods to study microbes, circadian rhythms as an important aspect of the physiology of at least one group of microbes, and new developments in our understanding of the many ways in which bacteria and archaea move. We have also made use of updates of chapters that were included in the third edition.

Bianca Brahamsha
Robert Haselkorn

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INTRODUCTION: ECOLOGY AND EVOLUTION

Evolution

Seven articles deal with different aspects of evolution. Two articles analyze the remains of the past evolution of microbes in the fossil record. Three other look at theories, mechanisms, and experimental procedures. Two more examine specific aspects of the evolution of viruses and of a ciliate. Finally, one article bridges ecology and evolution looking at the emerging field of evolutionary ecology of microbes.

Ecology

The ecology section contains a few general articles about what microbial ecology is about, and the particularities of the ecology of viruses and rare microorganisms.

The remaining articles can be placed in two complementary approaches: habitat oriented or process oriented. In the first approach there is a lot of attention devoted to extreme environments. These environments are usually exclusively microbial and show the capacity of microbes to deal with really difficult conditions. Besides, the relative simplicity of such ecosystems allows somewhat easier manipulations than in "normal" ecosystems. Several of the latter are also examined in articles ranging from the deep ocean to plastics. Special attention is given to animal and plant habitats and how microbes interact with their hosts.

The process-oriented approach shows, on the one hand, adaptive traits of microorganisms such as the ability to live in diluted environments or to use chemotaxis to find the optimal conditions. One promising and novel avenue to study adaptations is the analysis of the genomes of uncultured microorganisms. On the other hand, several articles review processes relevant at the ecosystem level, from feeding strategies such as myxotrophy to element cycles such as methane formation and oxidation and, finally, to the use of models in microbial ecology.

We hope this collection of articles provides an overview of the exciting times that microbial ecology is going through, with many new discoveries appearing every few years, and a fascinating and invisible world to be explored.

Carlos Pedrós-Alió
Larry Forney

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INTRODUCTION: PATHOGENESIS AND IMMUNOLOGY

This section of the encyclopedia focuses on the relatively narrow slice of all microbes that infect living hosts and how the hosts combat these microbes using innate and adaptive immunity. In addition, we examine how antimicrobial agents including unique metabolites and even bacteriophages have been enlisted to combat these infections. This section takes a broad look at infectious agents ranging from prions, viroids, and satellites to the more traditional etiologic agents like bacteria, viruses, fungi, and selected parasites. Also addressed are epidemiologic and diagnostic techniques used to identify and track the spread of infectious microbes including the most advanced diagnostic methods used by the clinical microbiology laboratory. A closer look at pathogenic mechanisms includes groups of virulence factors such as adhesins, iron acquisition, exotoxins of bacteria and fungi, and bacterial endotoxin (lipopolysaccharide). Strategies adopted by bacteria and fungi to colonize implanted foreign bodies such as catheters are also discussed. Plus, infections of the gastrointestinal tract, lung, oral cavity, and the skin are examined. In addition to pathogenic microbes, some articles examine the protective effect of the microbiome and commensal organisms. With respect to immunity this section also focuses on the interplay of the host immune system with pathogenic microbes covering evasion immune strategies in humoral and cellular immunity as well as innate immunology and the complement immune system.

Beth McCormick
Harry Mobley

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INTRODUCTION: TECHNOLOGICAL ADVANCES AND APPLIED MICROBIOLOGY

This section focuses on the development and refinement of the tools commonly used for both basic and applied microbiology. The articles in this section are written by authors who personally have worked on developing and fine-tuning technology for research in microbiology. Many of the products and processes described have multiple uses in a variety of disciplines such as health, the environment, food and agriculture, and industrial biocatalysis. The advances made in sequencing, protein analysis, and bioinformatics have given microbiologists new insights into old problems. These advances have also enabled the rapid identification of microorganisms in studies of biowarfare, infectious disease, and pollution of our water sources. At the same time the integration of mathematics and physics with biology and chemistry has provided instrumentation that enables a greater depth of analysis than ever before. Never in the history of microbiology have so many technological advances been developed and commercialized for use by scientists. Microbiologists today are equipped with improved "tools" within the toolbox to push the boundaries of not only elucidating the relationship of microbes to humans, plants and animals but also moving basic research much faster from the bench to development of new products and processes that benefit society and the planet. The renaissance of applied microbiology powered by technological advances has been both exciting and promising in all fields of study.

Jennie C Hunter-Cevera
Stanley Maloy

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A

Acidobacteria

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Abbreviations

C:N	Carbon and Nitrogen ratio
EPS	Exopolysaccharide
GYE	Glucose-Yeast Extract medium
MM	Minimal Medium
NanoSIMS	Nanoscale Secondary Ion Mass Spectrometry
PSYL5	Phosphate-Sucrose-Yeast Extract
R2A	Reasoner's 2A agar
SL10	Trace element solution
SSE	Soil Solution Equivalent
TSA	Tryptic Soy Agar

Defining Statement

The phylum *Acidobacteria* is one of the most abundant bacterial phyla in soil, yet little information is available on their physiology, ecological function, and impact on the soil environment. This lack of clarity is mainly due to the low number of cultured *Acidobacteria* representatives and their slow growth *in vitro* under standard laboratory conditions.

Overview

Acidobacteria is a ubiquitous and abundant bacterial phylum in soil, but the factors underlying their ecological advantage in the soil ecosystem remain unclear, as isolation difficulties and slow growth *in vitro* have limited the number of cultured representatives. However, non-culturable approaches, mainly 16S rRNA gene surveys, have revealed that *Acidobacteria* are metabolically diverse and widely distributed. Most *Acidobacteria* are aerobes, but some can grow under reduced oxygen conditions (1%–2% O_2). The diversity and abundance of *Acidobacteria* have been reported in a variety of sites, such as diverse agricultural and contaminated soils, sediments, forest soils, peatland, various water systems, acid mine drainage and surfaces of Palaeolithic caves and catacombs. The few sequenced genomes of *Acidobacteria* indicate a broad substrate range of ABC transporters for nutrient uptake, suggesting an advantage of *Acidobacteria* in complex environments and adaptation to oligotrophic conditions, such as nutrient-limited soil conditions.

Acidobacteria form as much as 50% of the total soil bacterial community based on 16S rRNA gene phylogenetic sequence surveys and compose on average 20% of the total microbial community in soils around the world. Three subdivisions are particularly abundant in soils: class *Acidobacteriia* (former subdivision 1), class *Blastocatellia* (former subdivision 4) and subdivision 6. At present, members of class *Acidobacteriia* are the most readily culturable under laboratory conditions. Together with subdivision 3, *Acidobacteriia* are the most abundant groups in soils.

The existence of the phylum *Acidobacteria* was first recognized in 16S rRNA gene sequence-based studies, which revealed that *Acidobacteria* and *Proteobacteria* were the predominant phyla in diverse soil environments. It was predicted that *Acidobacteria* would be as diverse as the widely studied phylum *Proteobacteria*. Only 4–5 subdivisions were initially described in 1997, a number that increased to 8 subdivisions in 1998 and 11 in 2005. The diversity and phylogeny of *Acidobacteria* currently encompasses 26 known subdivisions (Fig. 1) belonging to seven families: *Acidobacteriaceae*, *Bryobacteraceae* (within class *Acidobacteriia*),

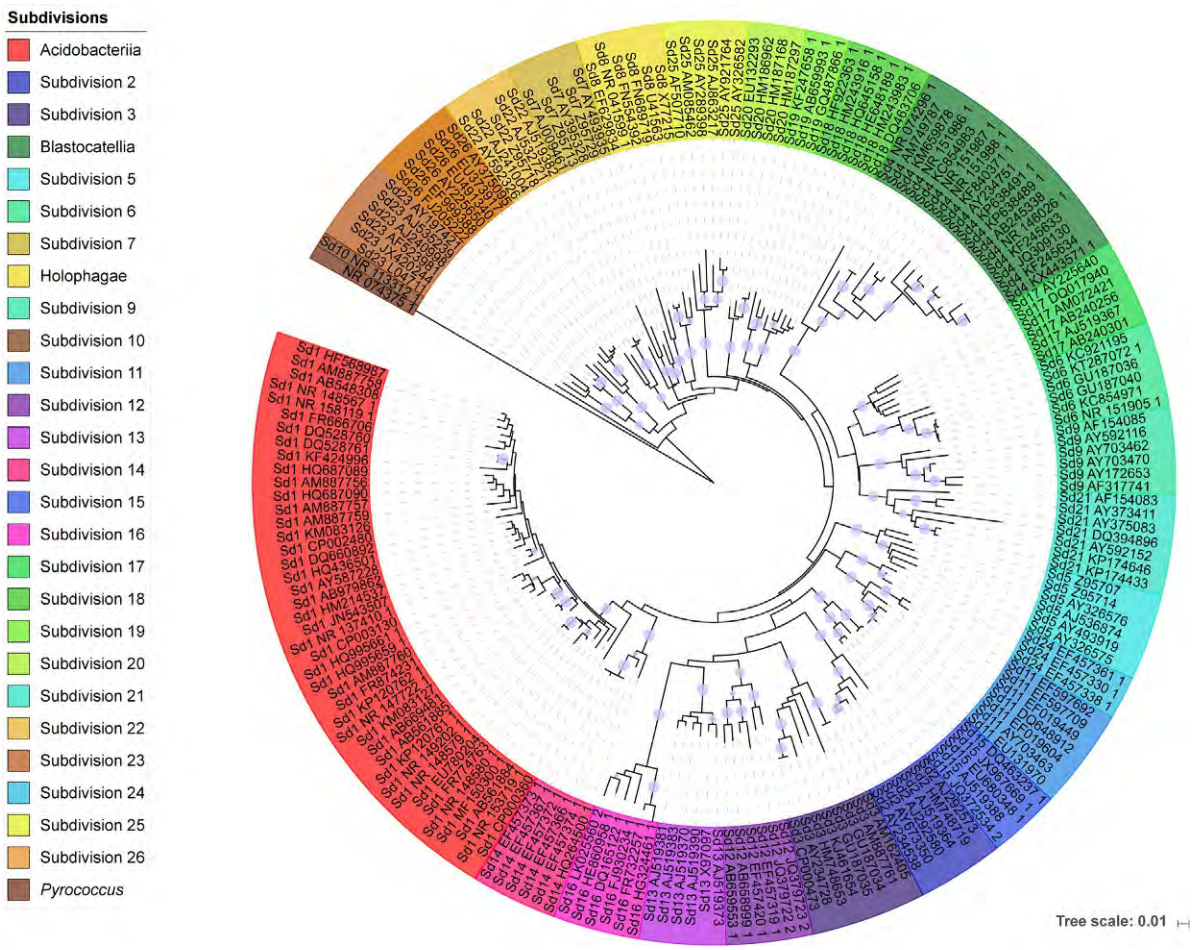

Fig. 1 Dendogram of *Acidobacteria* subdivisions. The dendrogram was constructed using 16S rRNA gene sequences from the 26 *Acidobacteria* subdivisions downloaded from the RDP 11 and NCBI databases. The sequences were aligned using the tool align.seqs in the software Mothur against Silva database version 132. The software Mega 7 was used to build the dendrogram based on the neighbour-joining method with 1000 bootstraps. The circles represent bootstrap values above 0.75. *Pyrococcus furiosus* was used as an outgroup.

Blastocatellaceae, *Pyrinomonadaceae* (within class *Blastocatellia*), *Acanthopleuribacteraceae*, *Holophagaceae* (within class *Holophagae*) and *Vicinamibacteraceae* (within subdivision 6).

Despite their high abundance in several environments, only 60 *Acidobacteria* species have been described so far (Fig. 2). The first isolate belonging to this phylum was *Acidobacterium capsulatum*, from which the name of the phylum was derived; the genus *Acidobacterium* was first proposed in 1991 for acidophilic, chemoorganotrophic bacteria isolated from an acidic mineral environment. Most *Acidobacteria* isolates belong to two classes: *Acidobacteriia* (39 species) and *Blastocatellia* (13 species), while three species belong to *Holophagae*, three to subdivision 3, two to subdivision 6, one to subdivision 10 and one to subdivision 23. Two *Acidobacteria* isolates belong to *Candidatus* genera: '*Ca. Koribacter*' (*Acidobacteriia*) and '*Ca. Solibacter*' (subdivision 3). In 2018, three new *Candidatus* genera with features of dissimilatory sulphur metabolism were proposed based on metagenome-assembled genomes: '*Ca. Sulfotelmatobacter*', '*Ca. Sulfotelmatomonas*' (Class *Acidobacteriia*) and '*Ca. Sulfopaludibacter*' (subdivision 3).

The genus *Acidobacterium* belongs to the family *Acidobacteriaceae* (class *Acidobacteriia*), which also contains the genera *Edaphobacter*, *Terriglobus*, *Acidicapsa*, *Acidipila*, *Bryocella*, *Granulicella*, *Occallatibacter*, *Telmatobacter*, *Terracidiphilus*, *Silvibacterium* and '*Candidatus Koribacter*'. These bacteria are gram-negative chemoorganotrophs with prevalent capsule formation and variable motility. They are aerobic or facultatively anaerobic and mostly mesophiles, although some are cold adapted. The members of this genus prefer sugars as a source of carbon and energy and are able to degrade complex carbohydrates. Their genomic G+C content varies from 51.7% to 62.1%.

The family *Bryobacteraceae* (subdivision 3) is formed by the genera *Bryobacter*, *Paludibaculum* and '*Candidatus Solibacter*'. These bacteria are chemoheterotrophic, gram-negative, non-spore-forming rods that are aerobes and facultative anaerobes and can use various sugars as growth substrates. In addition, members are mildly acidophilic, mesophilic and psychrotolerant. Their genomic G+C content varies from 55.5% to 61.9%.

The family *Blastocatellaceae* (subdivision 4) contains the genera *Blastocatella*, *Aridibacter*, *Tellurimicrobium* and *Stenotrophobacter*. The members of this family are gram-negative, non-spore-forming, non-capsule-forming bacteria. In addition, these aerobic

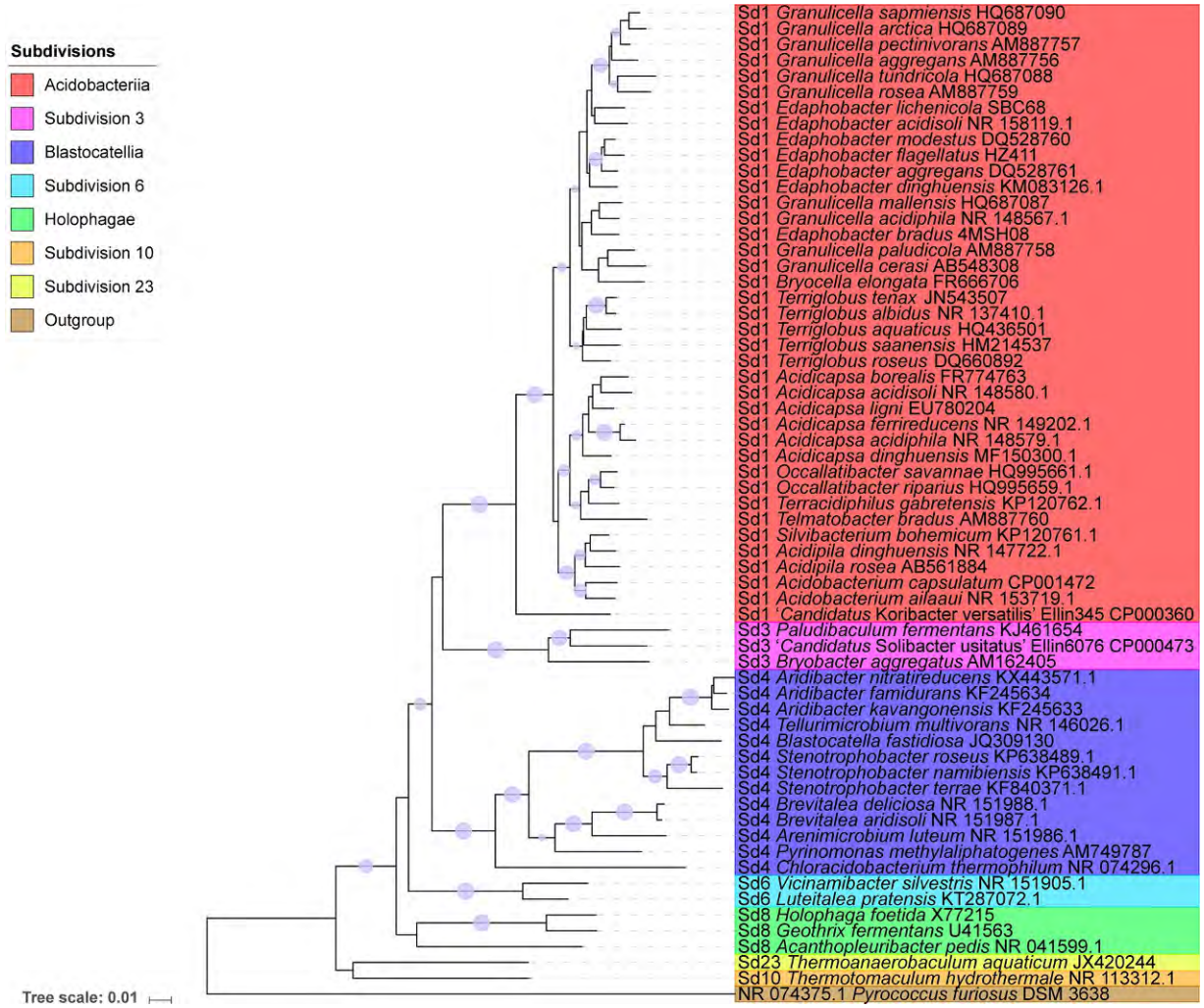

Fig. 2 Dendrogram of *Acidobacteria* isolates based on nearly full-length 16S rRNA gene sequences from the RDP 11 and NCBI databases, aligned using the tool align.seqs in the software Mothur against Silva database version 132. The software Mega 7 was used to build the dendrogram based on the neighbour-joining method with 1000 bootstraps. The circles represent bootstrap values above 0.75. The dendrogram includes sequences from the 60 described species, 2 candidate genera and *Pyrococcus furiosus* as an outgroup.

chemoorganotrophs are unable to reduce nitrate or ferment glucose and are slightly acidophilic to neutrophilic mesophiles with a preference for complex proteinaceous growth substrates, although a few complex carbohydrates can be used. Their genomic G+C content ranges from 46.5% to 59.4%.

The family *Pyrinomonadaceae* (subdivision 4) is composed of the genera *Brevitalea*, *Arenimicrobium* and *Pyrinomonas*. Members of this family are gram-negative, non-spore-forming, non-capsule-forming aerobic chemoorganoheterotrophs that are unable to grow phototrophically, reduce nitrate or ferment glucose. They are mesophiles or thermophiles and tolerate a broad range of pH. Furthermore, they prefer complex proteinaceous growth substrates and have a variable capability to break down polymers. Their genomic G+C content varies from 54.7% to 66.9%.

The family *Acanthopleuribacteraceae* contains only the genus *Acanthopleuribacter*. Cells belonging to this genus are gram-negative, motile, strictly aerobic rods that are able to use α-D-glucose, L-alanine, hydroxy-L-proline, L-serine, L-threonine, inosine, uridine and thymidine for growth. The genomic G+C content of the type strain *Acanthopleuribacter pedis* is 56.7%.

The family *Holophagaceae* is formed by the genera *Holophaga* and *Geothrix*. Both are strictly anaerobic chemoorganotrophs that are non-spore-forming, gram-negative, mesophilic, neutrophilic, and non-motile. The genomic G+C content of the type genus *Holophaga* is 62.5%.

The family *Vicinamibacteraceae* contains the genera *Vicinamibacter* and *Luteitalea*. These bacteria are gram-negative, non-spore-forming, aerobic chemoorganoheterotrophs that are capable of growth on organic/nucleic acids and simple sugars but prefer complex proteinaceous compounds. They are neutrophiles that tolerate a wide range of pH and can be psychrotolerant or mesophiles. Their genomic G+C content varies from 64.7% to 65.9%.

A few genera are not associated with any of the described *Acidobacteria* families. The genus *Thermotomaculum* belongs to subdivision 10 and is an anaerobic heterotrophic thermophile that was isolated from a deep-sea hydrothermal vent. *Thermoanaerobaculum* is another strictly anaerobic, thermophilic and chemo-organotrophic genus belonging to subdivision 23 that was isolated from a freshwater hot spring. Finally, *Chloracidobacterium* is a thermophilic, anoxygenic, chlorophototrophic member of class *Blastocatellia* isolated from a hot spring.

The number of isolates and described genera of *Acidobacteria* has gradually increased due to insights into the metabolism of these bacteria provided by genomic and metagenomics studies, as well as improvements in cultivation methods. Currently, there are 43 complete genomes (29 *Acidobacteriia*, 3 *Blastocatellia*, 3 *Holophagae*, 6 *Solibacteres* and 2 unclassified *Acidobacteria*) in the NCBI database (NCBI Resource Coordinators, 2018) (Table 1). Further efforts to unravel the metabolism of uncultured microorganisms through modern technologies, such as high-throughput sequencing and nanoscale secondary ion mass spectrometry (NanoSIMS),

Table 1 *Acidobacteria* complete genomes listed in NCBI (https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=57723, December 2018)

Genome	*Accession number*	*Genome size Mb*	*Number of proteins*
Acidobacteriia			
Acidipila sp. 4G-K13 / strain:4G-K13	NZ_QVQT00000000	5.0	3,980
Acidipila sp. EB88	NZ_QWEV00000000	4.5	3,437
Acidobacteria bacterium KBS 146 / strain:KBS 146	NZ_JHVA00000000	5.0	4,026
Acidobacteriaceae bacterium KBS 83 / strain:KBS 83	–	12.5	10,240
Acidobacteriaceae bacterium KBS 89 / strain:KBS 89	–	12.0	9,760
Acidobacteriaceae bacterium KBS 96 / strain:KBS 96	–	13.3	11,212
Acidobacteriaceae bacterium SBC82 / strain:SBC82	–	7.6	6,259
Acidobacteriaceae bacterium TAA166 / strain:TAA 166	–	12.3	10,096
Acidobacteriaceae bacterium URHE0068 / strain:URHE0068	–	2.2	1,706
Acidobacterium ailaaui / strain:PMMR2	NZ_JIAL00000000	3.7	3,082
Acidobacterium capsulatum ATCC 51196 / strain:ATCC 51196; culture-collection: ATCC:51196	–	8.2	6,640
Bryocella elongata / strain:DSM 22489	NZ_FNVA00000000	5.7	4,505
Candidatus *Koribacter versatilis* Ellin345 / strain:Ellin345	–	11.3	9,800
Candidatus *Sulfotelmatobacter kueseliae* / strain:Peat soil MAG SbA1	NZ_OMOD00000000	5.4	5,047
Candidatus *Sulfotelmatobacter* sp. SbA7 / strain:Peat soil MAG SbA7	NZ_OKRE00000000	2.8	2,943
Candidatus *Sulfotelmatomonas gaucii* / strain:Peat soil MAG SbA5	NZ_OKRB00000000	5.3	4,933
Edaphobacter aggregans DSM 19364 / strain:DSM 19364; culture-collection:DSM:19364	NZ_JQKI00000000	0.9	701
Edaphobacter dinghuensis / strain:EB95	NZ_RBIF00000000	4.5	3,734
Granulicella mallensis MP5ACTX8 / strain:MP5ACTX8	–	12.5	9,558
Granulicella pectinivorans / strain:DSM 21001	NZ_FOZL00000000	5.3	4,260
Granulicella rosea / strain:DSM 18704	NZ_FZOU00000000	5.3	4,309
Granulicella tundricola MP5ACTX9 / strain:MP5ACTX9	–	11.0	9,202
Occallatibacter savannae / strain:AB23	NZ_QFFY00000000	6.3	5,231
Silvibacterium bohemicum / strain:S15	NZ_LBHJ00000000	6.5	5,195
Terracidiphilus gabretensis / strain:S55	NZ_LAIJ00000000	5.3	4,315
Terriglobus roseus / strain:GAS232		9.7	7,958
Terriglobus roseus DSM 18391 / strain:DSM 18391; culture-collection:DSM:18391		10.5	8,478
Terriglobus saanensis SP1PR4 / strain:SP1PR4		10.2	8,364
Terriglobus sp. TAA 43 / strain:TAA 43	NZ_JUGR00000000	4.9	4,107
Blastocatellia			
Chloracidobacterium thermophilum / strain:OC1	NZ_LMXM00000000	3.6	2,883
Chloracidobacterium thermophilum B / strain:B		7.4	5,938
Pyrinomonas methylaliphatogenes / strain:type strain:K22	NZ_CBXV000000000	3.8	3,180
Holophagae			
Geothrix fermentans DSM 14018 / strain:DSM 14018; culture-collection:DSM:14018			1,750
Holophaga foetida DSM 6591 / strain:DSM 6591; culture-collection:DSM:6591	NZ_AGSB00000000	4.1	3,488
Holophagae bacterium / isolate:FeB_10	NZ_PQAJ00000000	4.2	3,545
Solibacteres			
Bryobacter *aggregatus* MPL3 / strain:MPL3	NZ_JNIF00000000	5.7	4,952
Candidatus *Solibacter usitatus* Ellin6076 / strain:Ellin6076	NZ_OKRF00000000	19.9	16,008
Candidatus *Sulfopaludibacter* sp. SbA3 / strain:Peat soil MAG SbA3		8.5	8,481
Candidatus *Sulfopaludibacter* sp. SbA4 / strain:Peat soil MAG SbA4	NZ_OMOG00000000	10.0	9,749
Candidatus *Sulfopaludibacter* sp. SbA6 / strain:Peat soil MAG SbA6	NZ_OKRH00000000	3.5	3,529
Solibacteres bacterium SbA2 / strain:Peat soil MAG SbA2	NZ_OKRG00000000	2.7	3,050
Unclassified Acidobacteria			
Luteitalea pratensis / strain:DSM 100886; HEG_-6_39; culture-collection:DSM:100886	NZ_CP015136	7.4	6,103
Thermoanaerobaculum aquaticum / strain:MP-01		5.3	4,506

may provide additional insights on how to cultivate novel genera, thereby increasing present knowledge on the characteristics and functions of members of the phylum *Acidobacteria*.

Isolation Methods

Culturing *Acidobacteria* from soils is not trivial and requires a combination of low nutrient concentrations and/or environmental extracts, complex polysaccharides (carboxymethylcellulose, microcrystalline cellulose, chitin, gellan gum, xylan and starch) as a carbon source, amendment of the growth medium with quorum-signalling molecules or catalase, and long incubation periods under non-standard CO_2 atmospheric conditions. The combination of those factors and conditions mimics the environmental conditions where *Acidobacteria* is found and thus will influence the recovery of acidobacterial isolates. Several low-nutrient or dilute culture media are used to isolate *Acidobacteria* strains, such as 1/10 diluted R2A, VL55, GYE, MM, PSYL 5 and SSE (soil solution equivalent). Frequently, the medium is composed of a mineral base, supplemented with yeast extract, trace elements, vitamin solutions and other growth factors, and solidified with either agar or gellan gum. For some strains of the genus *Granulicella*, the addition of SL-10 trace element solution can significantly enhance bacterial growth (Fig. 3). After isolation under low-nutrient conditions, *Acidobacteria* strains can often be transferred to richer media (e.g., TSA and R2A) for more convenient propagation.

Phages and Mobile Elements

Acidobacteria genomes vary greatly in size (Table 1). Many factors explain the variation of the size and plasticity of genomes, including gene duplication, rearrangement of genetic elements by phages, the presence of transposable and mobile elements and the presence of horizontally transferred genes. These factors are advantageous for the survival of *Acidobacteria* in different environments. The presence of prophages is common in acidobacterial genomes, including in *Acidobacteriaceae bacterium* KBS 146 and *A. ailaaui* (subdivision 1), *Pyrinomonas methylaliphatogenes* K22 and *Chloracidobacterium thermophilum* B (subdivision 4), and *Thermoanaerobaculum aquaticum* (subdivision 23). However, these prophages differ from previously described microbial prophages. The prophage is typically located in the same region of the genome as mobile elements encoding transposases and integrases, suggesting that these genomic islands were acquired by horizontal transfer events. The presence of these extra genetic elements impacts the evolution and metabolic capabilities of *Acidobacteria* to cope with environmental conditions.

Carbohydrate Metabolism

Carbon usage is one of the physiological requirements for the description of new species in taxonomic studies, and therefore carbohydrate metabolism in *Acidobacteria* has been widely studied. Depending on the species and subdivision, *Acidobacteria* are able to use D-glucose, D-xylose, lactose, maltose, fucose, sorbose, cellobiose, glucose and xylose as carbon sources and can degrade simple and polymeric carbohydrates. The genomes of *Acidobacteria* include genes encoding pathways for the degradation of various polysaccharides (starch, cellulose, hemicellulose, laminarin, xylan, xyloglucan, and gellan gum), and physiological

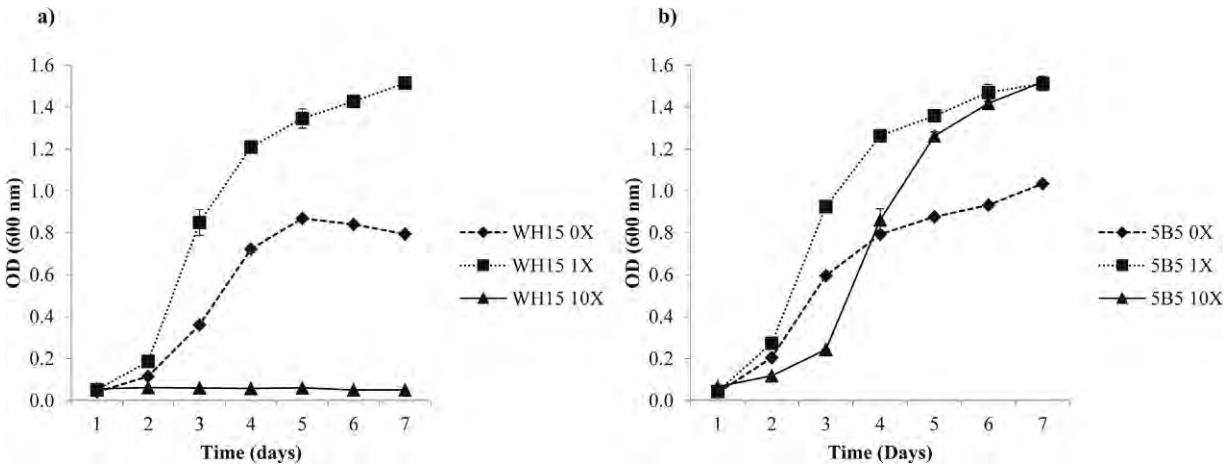

Fig. 3 Growth curves of *Granulicella* (a) WH15 and (b) 5B5 strains on PSYL 5 liquid culture medium with different concentrations of SL-10 trace element solution: 1 ml/L (1X), 10ml/L (10X) and 0 ml/L (0X). The error bar is the standard error of the mean and indicates differences in the response variable between different treatments.

studies have observed enzymatic activities such as β-D-glucosidases, endo-β-1,4-xylanase, gellan lyases, unsaturated glucuronyl hydrolases, and α-rhamnosidases. Metatranscriptome analyses have revealed the expression of functional acidobacterial glycosyl hydrolase (GH) families involved in the degradation of cellulose, hemicellulose and xylan, compounds that are rich in environments with high C:N ratios, such as tropical and temperate forests, tundra and peatland, where *Acidobacteria* are abundant and dominant.

Genes related to the biosynthesis, transfer, breakdown and/or modification of carbohydrates represent 5%–9% of acidobacterial genomes. The genomes of the non-soil isolates *G. fermentans*, *H. foetida* and *C. thermophilum* B have the lowest percentages of genes related to carbohydrate metabolism. The GH families related to polymeric carbohydrate degradation with the highest percentages across different acidobacterial genomes are GH109 and GH74. GH109 is an alpha-N-acetylgalactosaminidase (EC: 3.2.1.49) that acts on O-linked oligosaccharides, which are typically found in chitin, bacterial peptidoglycan and lipopolysaccharide, whereas GH74 is an enzyme family that targets the beta-1,4-linkages of glucans (polysaccharides of glucose). Various GH families involved in the degradation of beta-glycosidic bonds, typical cellulosic bonds, are also present in acidobacterial genomes. More specifically, GH5 is found across subdivisions 1, 3, 4 and 6, while GH8, GH9, GH44 and GH12 have only been detected in a few genomes of subdivision 1 (*T.* sp., 'Ca. *K. versatilis*', *T. gabretensis*, and *G. mallensis*) and subdivision 3 (GH9, 'Ca. *S. usitatus*'). The GH3 family includes beta-glucosidase, an enzyme that hydrolyses glycosidic bonds in oligosaccharides to produce glucose, and is present in acidobacterial genomes, and the GH18 family includes putative chitinases that are present in all of *Acidobacteria* subdivision 1, two members of subdivision 3 ('Ca. *S. usitatus*' and *Acidobacteria bacterium* KBS 96), one member of subdivision 4 (*P. methylaliphatogenes* K22), one member of subdivision 6 (*L. pratensis*) and one member of subdivision 8 (*H. foetida*). The GH19 family of chitinases is found in *G. fermentans*, *P. methylaliphatogenes* K22, *L. pratensis*, *T. saanensis* and *E. aggregans*. The presence of chitinase genes indicates that chitin is used not only as a carbon source but also a nitrogen source.

Nitrogen Metabolism

All known acidobacterial genomes (Table 1) except *Thermoanaerobaculum aquaticum* have homologue genes for ammonia uptake, such as the ammonium channel transporter family (*amtB*), glutamate dehydrogenases (*gdhA* and *gdh2*), glutamine synthetase (*gltBD*) and glutamate synthase (*glnA*). Putative genes encoding assimilatory nitrate reductases (*NaR*), nitrite reductases (*NiR*) and nitrate/nitrite transporters are present in *T. roseus*, *Granulicella mallensis*, *Granulicella tundricola*, *Acidobacterium ailaaui*, 'Ca. *Solibacter usitatus*', *Pyrinomonas methylaliphatogenes*, *Luteitalea pratensis*, *Acidobacteria bacterium* KBS 96, *Acidobacteriaceae bacterium* KBS 146 and KBS 89 and *Terriglobus* sp. *NiR* genes have only been detected in *Acidobacteriaceae bacterium* KBS 83.

Among *Acidobacteria* subdivision 1, *G. mallensis* performs nitrate reduction, and *Acidipila rosea* and *Bryocella elongata* are also able to reduce nitrate to nitrite. Among other subdivisions, only *Geothrix fermentans* in subdivision 8 is able to reduce nitrate; this organism is an iron reducer that can use nitrate as an alternative electron acceptor. All of these *Acidobacteria* are able to use yeast extract and ammonium as preferred nitrogen sources. The genomes of subdivision 1 contain the *nirA* gene, which encodes nitrate reductase, suggesting that members of this subdivision may reduce nitrate to nitrite by the assimilatory pathway, followed by further reduction to ammonia and assimilation into glutamate. Nevertheless, direct uptake of ammonium seems likely as all genomes described to date (Table 1) appear to contain genes for the ammonia transporter channel (*Amt*) family (TC 1.A.11). Nitric oxide reductase genes (*norB* but not *norC*) have been identified in the *Koribacter versatilis*, *S. usitatus* and *G. fermentans* genomes. The *narG* operon encoding a membrane-bound respiratory nitrate reductase is present in the genome of *G. fermentans*. The genomes of *Luteitalea pratensis* (subdivision 6), *G. fermentans* (subdivision 8) and *Holophaga foetida* (subdivision 8) harbour genes encoding a dissimilatory nitrite reductase (*nrfHA*) that catalyses the reduction of nitrite to ammonia. Genes encoding dinitrogenase, a heterotetramer of the proteins NifD and NifK (genes *nifD* and *nifK*, respectively), and dinitrogenase reductase, a homodimer of the protein NifH (gene *nifH*), are only present in the genome of *H. foetida*. *Thermoanaerobaculum aquaticum* contains the *napA* operon, which is involved in dissimilatory nitrate reduction, and *G. mallensis* and *Acidobacteria bacterium* KBS 96 have similar assimilatory nitrate reductase genes (*nasA* genes) involved in dissimilatory nitrogen metabolism. The denitrification pathway cytochrome cd1-containing nitrite reductase gene (*nirS*) is absent in acidobacterial genomes, but the dissimilatory copper-containing nitrite reductase gene is present as a single copy, with the exception of two copies of *nirS* in the *Acidobacteria bacterium* KBS 96 genome. Although genes related to the nitrogen cycle are present in the genomes of *Acidobacteria*, there is no experimental evidence for a denitrification pathway in *Acidobacteria*. However, *Acidobacteria* can use both inorganic (ammonia and/or nitrate/nitrite) and organic (amino acids and other high-molecular-weight compounds) N sources. In addition, genes encoding specific transporters for amino acids, polyamines and organocations are overrepresented in genomes from terrestrial environments, in which bacteria use inorganic but also organic N sources via mineralization.

Sulphur Metabolism

Seven draft genomes recovered from metagenomic data from acid peatland suggest that *Acidobacteria* is involved in dissimilatory sulphur metabolism. Novel recovered *Acidobacteria* species show potential for dissimilatory sulphite (*dsrAB*, *dsrC*, *dsrD*, *dsrN*, *dsrT*, *dsrMKJOP*) or sulphate respiration (*sat*, *aprBA*, *qmoABC*, *dsr* genes) or encode *DsrL*, which is only found in sulphur-oxidizing microorganisms. The expression of acidobacterial DsrAB sulphur metabolism genes indicates new fundamental niches

for facultative anaerobic *Acidobacteria* in wetlands. In addition, *Acidobacteria* from wetlands encode enzymes that liberate sulphite from organosulphonates, suggesting that organic sulphur compounds can serve as complementary energy sources.

Exopolysaccharides

The *Acidobacteria* strains *Granulicella paludicola*, *G. pectinivorans*, *G. aggregans*, *G. rosea*, *Acidicapsa borealis*, *A. ligni* and *Terriglobus tenax* are capable of producing exopolysaccharide (EPS) in culture medium. In addition, acidobacterial genomes belonging to subdivision 1 (with the exception of *K. versatilis* strain Ellin345) contain genes involved in EPS biosynthesis, specifically those involved in cellulose synthesis. To date, acidobacterial EPSs have been isolated and chemically characterized only from two strains of *Acidobacteria* subdivision 1, *Granulicella* sp. strain WH15 and strain 5B5. These EPSs comprise heteropolysaccharides composed of 7 different monosaccharides. One of the functions of EPS is probably related to acidobacterial cell protection for long-term survival in soil. The production of large amounts of *Acidobacteria* EPS is related to abiotic stress, i.e., dominance of *Acidobacteria* in acidic environments and resistance to pollutants like uranium, petroleum compounds, linear alkylbenzene sulfonate and p-nitrophenol. In addition, acidobacterial EPS can emulsify different oils. *Granulicella* sp. WH15 and 5B5 strains, and the type strain of the genus *Acidicapsa*, *A. ligni* WH120T, produce EPS in the adhesion of bacteria to the plant root surface via biofilm formation and promote plant growth. The underlying mechanisms include acidobacterial production of the plant hormone-like auxin indole-3-acetic acid (IAA) and a siderophore that removes iron from the environment. Iron accumulation has been observed in *B. elongata* and was confirmed by the identification of genes encoding transport systems involved in translocation across the outer and cytoplasmic membranes, i.e., cobalamin/Fe^{3+}-siderophore uptake transporters and Fe^{3+} hydroxamate transporters. Genomic analyses also suggest that *Acidobacteria* may release siderophores to scavenge iron from soil minerals by forming Fe^{3+} complexes that can be taken up by those transporters or use siderophores from other microorganisms.

Acidobacteria and Soil Ecosystems

Acidobacteria have the *qlcA* gene, which encodes a lactonase that degrades N-acylhomoserine lactones (NAHLs). Similarly, genes involved in polyketide synthesis (PKS) pathways were identified in *S. usitatus*. The identification of *mtaD* gene homologs encoding proteins involved in myxothiazol biosynthesis in isolates belonging to subdivisions 3, 4 and 6 suggests the widespread distribution of PKS pathways among *Acidobacteria*. Genes involved in PKS biosynthesis have also been identified in sequenced acidobacterial genomes, and the ubiquity of such pathways in this phylum suggests a role in the persistence, resistance and abundance of these bacteria in soil ecosystems.

Acidobacteria are predominant in low pH conditions, particularly members of subdivision 1. By contrast, some acidobacterial subdivisions have an aversion to low pH conditions in soil, whereas others, such as subdivision 6, can be either positively or negatively correlated with soil pH. The abundance of subdivisions 1, 2, 3, 12, 13, and 15 is negatively correlated with soil pH, whereas subdivisions 4, 6, 7, 10, 11, 11, 16, 17, 18, 22, and 25 have positive correlations. A possible explanation may be increased cell specialization at more extreme pH, where closely related *Acidobacteria* may share similar cellular strategies to deal with discrepancies between intra- and extracellular pH.

Acidobacteria subdivisions 4 and 6 are abundant in soils with high Mg and Ni content, such as serpentine tropical savanna soils; subdivisions 4, 6, and 7 respond to decreases in soil aluminium in tropical soils; subdivisions 6 and 7 respond to high contents of soil Ca, Mg, Mn, and B; subdivision 10 correlates with soil factors linked to soil acidity such as pH, Al, and Al saturation; and subdivision 13 correlates with soil P, B, and Zn. Although subdivision 1 negatively correlates with P, C and N, members of subdivisions 5, 6 and 17 appeared to be highly abundant in more nutrient-rich soils.

The high number of ABC transport systems facilitates the high-affinity acquisition of a broad range of substrate categories, including not only sugars but also polyols, drugs, amino acids, peptides, siderophores and anions.

Conclusions

The high abundance and ubiquity of *Acidobacteria* in different environments, especially soils, raises questions about the physiological traits underlying this marked success. Genome sequences have provided important information, especially about *Acidobacteria* subdivision 1, the group for which the most pure cultures are available. However, for other subdivisions, information is scarce due to the difficulty of isolation and cultivation. The increased high throughput of shotgun metagenomic studies and associated postgenomic analyses have enabled the assembly of acidobacterial genomes from environmental datasets and yielded important genome trait information. However, cultivation efforts remain a top priority to provide the necessary material for physiological studies and confirmation of genomic predictions. The cultivation of *Acidobacteria* might be enhanced by the use of different techniques like micro-cultivation and single-cell sequencing as steps toward obtaining a more representative range of acidobacterial genomes. Despite their limitations, current genome sequences and the recovery of 16S rRNA genes from the environment provide important clues about the factors responsible for the successful adaptation of this phylum to harsh conditions, particularly in soils.

Increased knowledge of the genomic features of different *Acidobacteria* subdivisions is critical for understanding their survival, resistance, and persistence in soil as well as possible interactions of members of this phylum with other soil microorganisms. Therefore, additional experimental approaches combined with molecular studies, such as metatranscriptomic and stable isotope probing (SIP) analyses, are needed to obtain a fundamental understanding of the ability of *Acidobacteria* to utilize a wide variety of simple and complex carbohydrates as substrates, their roles in the C, N and S biogeochemical cycles, the production of EPS and secondary metabolites, their resistance to antibiotics, and the diversity of high-affinity transporters. In addition, further studies are needed to better understand the role of genetic mobile elements and prophages in shaping the genomes, metabolic versatility and evolution of *Acidobacteria* through the acquisition of novel metabolic genes.

Further Reading

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Adaptations of Microorganisms to Low Nutrient Environments: Managing Life in the Oligotrophic Ocean

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Introduction

Inorganic and organic nutrients are essential for life as they are required for the biosynthesis of macromolecules and the construction of cellular structures. In nature, biological demand often exceeds nutrient supplies, resulting in the depletion of nutrients and a reduction of cellular processes. Already in 1905, Blackman defined nutrient limitation as the restriction of the growth rate of individual cells by low nutrient concentrations (Blackman, 1905), and much earlier Liebig's law of the minimum stated that it is the scarcest resource (limiting factor; Liebig, 1840), and not the total resources available, that defines the maximum amount of new biomass that can be formed (Cullen, 1991). As of today, it remains essential to distinguish between the influence nutrients have on defining growth rates as compared to determining standing stocks (defined by abundance or biomass) of organisms. Yet, it is important to consider not only the absolute concentrations but also the relative proportions of particular nutrients. In marine biology, the identification of elemental composition (stoichiometry) of phytoplankton cells as C/N/P = 106/16/1 (the Redfield ratio, Redfield, 1958) represented a breakthrough in understanding the nutritional demand of phytoplankton. Analyses of elemental composition of seawater and microorganisms remain essential for interpreting microbial growth and constraining fluxes in global biogeochemical cycles (Anderson and Sarmiento, 1994). If an element is present in substantially lower relative abundance than in the Redfield ratio it is highly likely to become a nutrient limiting microbial growth. Physical, chemical and biological factors collectively define the accessibility of nutrients to the living world.

A variety of habitats on Earth can be defined as low nutrient, or oligotrophic, environments, including lakes (Logue et al., 2012), soils (Hobbie and Hobbie, 2013), deep subsurface, groundwaters (Reed et al., 2010), deep oceanic waters (Arrieta et al., 2015) and most of the offshore surface ocean (Moore et al., 2013). Each of these systems merits a case study in itself. Throughout this article we will explain physiological and molecular adaptations that allow microbes to prosper in low nutrient environments. The article will focus on microbes in low-nutrient marine environments, particularly heterotrophic bacteria and photosynthetic cyanobacteria in the open ocean surface waters, but similar adaptations are widespread in microorganisms in other environments.

The Surface Ocean: The Coast-Open Ocean Continuum

The Physics Behind Nutrient Availability

The world ocean covers >70% of Earth's surface and with an average depth of ~3700 m the marine biome is the largest on our planet. In order to understand the biology of microorganisms inhabiting low nutrient (oligotrophic) open ocean regions, it is useful to consider that the open ocean represents one extreme on a continuum where the other extreme is the coastal nutrient rich waters (Fig. 1).

Physical forcing on the ocean sets the stage for ocean productivity, where land runoff, atmospheric deposition, ocean currents and wind driven water mixing determine the flux of inorganic nutrients. Particularly in the eastern boundary of oceanic gyres, winds displace surface water from the coast allowing dense, cold, nutrient-rich deep water to reach the surface. This process, called upwelling, "fertilizes" surface waters and promotes extraordinary biological productivity. Such productive regions include for example, the Peruvian coast and the coast off Northwest Africa. Coastal upwelling regions occupy only 1% of the ocean but account for an important fraction (up to 20%) of global fisheries (Kämpf and Chapman, 2016).

In comparison to coastal waters and upwelling zones, the subtropical gyres are stable environments located far from nutrient sources (continents). In the gyres temperature-driven stratification of the water column prevents nutrient-rich deep water to ascend, resulting in low nutrient concentrations in surface waters and therefore low biological production (Fig. 1). Nutrient inputs to these ecosystems do occur but are scarce and usually related to atmospheric deposition (Duce et al., 1991), mesoscale hydrographic structures like oceanic eddies (McGuillicuddy, 2016) and lateral advection processes (Letscher et al., 2016).

Nutrient Availability and Marine Microplankton

Nutrient availability determines the rate of solar energy-capture through photosynthesis, resulting in the organic matter production that fuels the marine ecosystem. In coastal surface waters, microbial biomass ranges from 30 to 100 mg C m^{-3}, as compared to 6–20 mg C m^{-3} in open ocean waters (<0.02 mg C m^{-3} in the deep sea) (Karl and Letelier, 2009). As a consequence of the different levels of nutrient availability, the food webs in the open ocean and coastal environments are different (Legendre and Rassoulzadegan, 1995). Low-nutrient conditions in the open ocean select for very small phytoplankton primary producers and a dominance of microbial food webs in which the transfer of organic carbon to higher trophic levels is relatively inefficient. In such systems, most production is sustained by nutrients like ammonium (NH_4) from remineralization of autochthonous dissolved organic matter

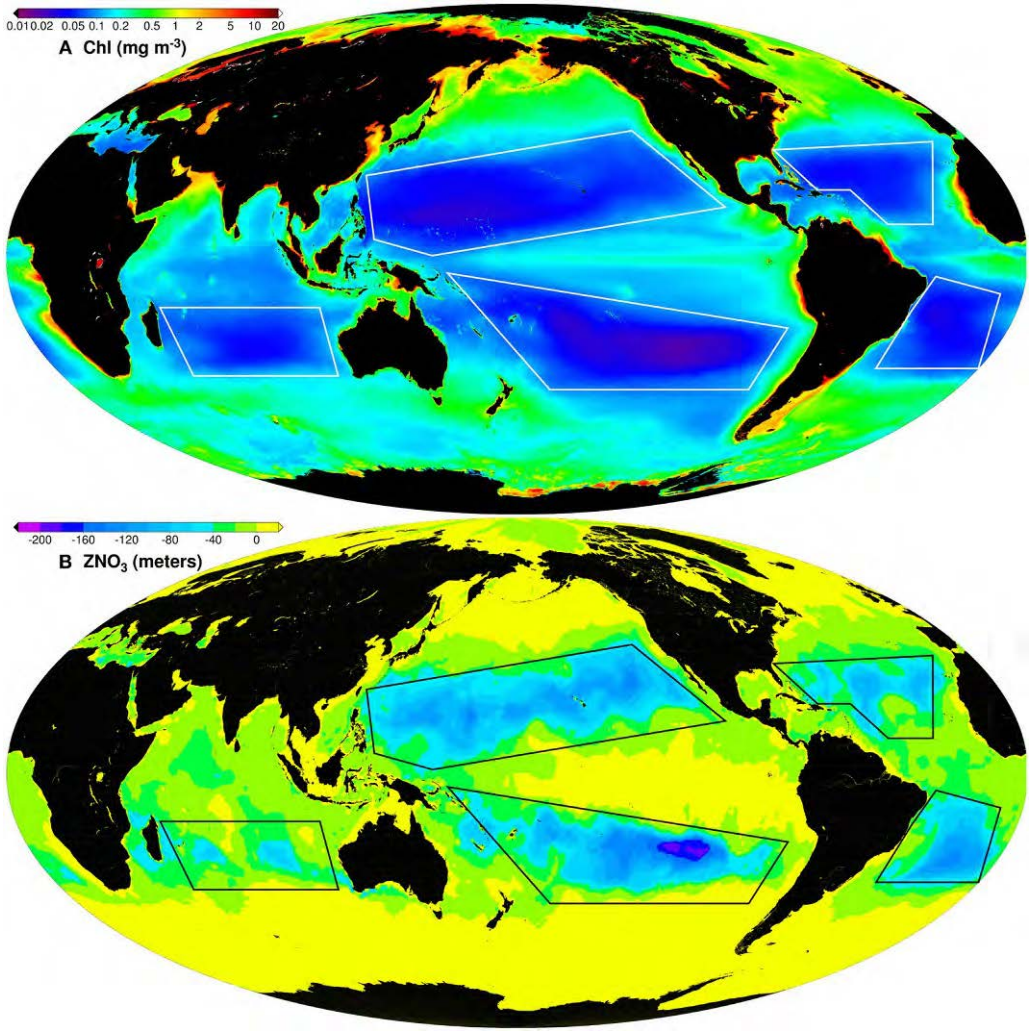

Fig. 1 Global maps of (A) Surface chlorophyll *a* (Chl) concentrations (9 km MODISA OCI Chla mission composite) and; (B) Depth at which nitrate concentration equals 0.2 μM (ZNO_3). Polygons represent the 5 oceanic gyres. Figure from Signorini, S.R., Franz, B.A., McClain, C.R., 2015. Chlorophyll variability in the oligotrophic gyres: mechanisms, seasonality and trends. Frontiers in Marine Science 2, 1; licensed under CC BY 4.0.

(DOM) (often referred to as "regenerated production"). An alternative source of reduced nitrogen (N) and carbon (C) in open ocean oligotrophic environments is atmospheric N_2 fixation by cyanobacteria (Karl and Letelier, 2008; Fig. 2).

High-nutrient conditions, in contrast, select for large phytoplankton cells and efficient herbivore food chains where phytoplankton are consumed by zooplankton which in turn are preyed upon by fish. These large phytoplankton, often heavily silicified diatoms, contribute importantly to the sinking of organic particles and thus to organic carbon export and burial at the bottom of the ocean – the so-called biological carbon pump – ultimately influencing climate through the sequestration of CO_2 from the atmosphere (Boyd and Newton, 1999). Photosynthetic production in coastal nutrient-rich systems is mainly supported by nitrogen in the form of nitrate (NO_{3^-}) (not immediately regenerated from DOM), driving so-called "new production". It is in these rich costal/upwelling areas where much of marine life as we often picture it thrives, with whales, sharks and dolphins and important fisheries (Fig. 2).

Thus, from coast to open ocean environments, a continuum of life spreads from areas dominated by large microalgae (e.g., diatoms and dinoflagellates) to open ocean environments dominated by small phytoplankton (e.g., *Prochlorococcus*) (Fig. 2). Similarly, heterotrophic bacteria inhabiting each of these environments can be expected to differ substantially with respect to physiological and ecological adaptations and evolutionary trajectories. In general, oligotrophic microorganisms, defined as those living in low nutrient environments and adapted to uninterrupted nutrient limitation, are able to maximize the import of inorganic and organic nutrients and utilize these resources conservatively (usually growing slowly). By contrast, copiotrophic microorganisms are those that are exposed to high nutrient concentrations and consume them rapidly, but are inefficient in the uptake of substrates at low concentration.

In the next section, we will focus on the description of bacterial adaptations to life in the oligotrophic surface ocean.

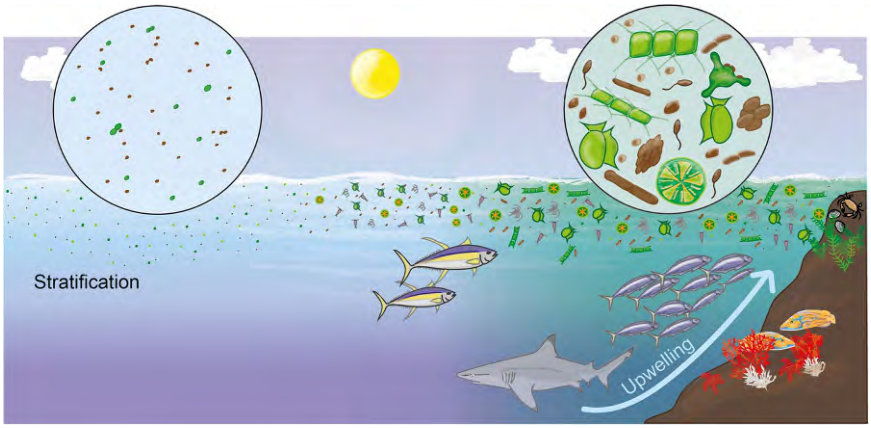

Fig. 2 Conceptual diagram displaying the coast-open ocean continuum. Alongshore, wind stress results in coastal upwelling, supporting production of large phytoplankton, zooplankton and fish. Abundance and size of microplankton decreases from coast to open ocean where intense stratification prevents mixing and nutrient input. Image courtesy of Carina Bunse.

Managing Life in the Oligotrophic Ocean

The Influence of Resource Limitation on Marine Bacteria

Microorganisms in the low-nutrient surface ocean are limited both in growth rates and in standing stocks of biomass by the supply of inorganic nutrients and organic carbon, often referred to as bottom-up control. Accordingly, microorganisms in these ecosystems devote major portions of their energy to the acquisition of nutrients (Lauro et al., 2009). Moreover, the low nutrient concentrations strongly influences the composition of the microbial community inhabiting these environments, with major portions of photosynthesis carried out by tiny cyanobacteria in the genus *Prochlorococcus* and strong dominance of SAR11 clade among the heterotrophic bacteria (Partensky et al., 1999; Morris et al., 2002).

Over the course of evolution, marine bacteria have developed a multitude of strategies to maintain viable populations and successfully compete for resources in low nutrient environments. The most obvious adaptation is the small cell sizes of microbes in the open ocean as compared to coastal representatives. This leads to higher surface to volume ratios that allow relatively more efficient nutrient uptake. Less obvious, but not less important, is that marine bacteria that are dominant in oligotrophic areas have greatly reduced genome sizes (down to below 2000 genes per genome), achieved through the loss of several metabolic pathways in order to increase metabolic efficiency. This process is known as genome streamlining (Lynch, 2006). In contrast, the larger genomes found among (larger-sized) bacteria in high nutrient environments encodes significantly more genes (reaching 3000 genes per genome and upwards). This provides a high metabolic flexibility, which is beneficial in environments with repeated blooms of phytoplankton and irregular but often repeated supply of resources (Luo and Morán, 2014).

The importance of light as an energy source in low-nutrient environments is evident, both for organic matter production through chlorophyll based photosynthesis – primarily by cyanobacteria but also by eukaryotic microalgae – and for light energy harvesting through rhodopsins and bacteriochlorophyll (i.e., photoheterotrophy; see Section "Light as an Energy Source to Photoheterotrophic Bacteria"). A tight coupling of light and bacterial metabolism (through primary production) was suggested early on as an adaptive advantage in the oligotrophic ocean (Gasol et al., 1998). Accordingly, the life of osmotrophic heterotrophs in the oligotrophic ocean (i.e., essentially bacteria and archaea) is tightly coupled with primary producers – Their main source of nutrition – much because of the extreme scarcity of bioavailable dissolved organic carbon (Morán et al., 2002; see Section "Dissolved organic carbon" on DOC). Indeed, recent high-resolution metatranscriptomics analyses in the Pacific show coordinated diel patterns in gene expression across multiple microbial species. Such diel periodicity appears more pronounced in oligotrophic than in nutrient-rich coastal environments (because of generally higher nutrient levels and/or allochtonous inputs of nutrients in the latter). Differences in diel dynamics in activity likely influence the timing and mode of energy and organic matter processing in the sea (Ottesen et al., 2014).

Different nutrients may restrict microbial growth in different regions of the surface ocean (Fig. 3). N and phosphorous (P) are often referred to as macronutrients to microorganisms, as they are the elements besides carbon that are required in highest amounts to build biomass. Macromolecules like proteins and DNA contain large portions of the cellular N and P, respectively. Heterotrophic bacteria also require a supply of organic carbon for growth (recall that photosynthetic microbes acquire C from fixation of CO_2). N availability limits primary production in major open ocean regions, for example the open Atlantic Ocean (Fig. 3). Still, in some areas of the ocean P is the primary limiting macronutrient, as in the Sargasso Sea and the Mediterranean Sea (Fig. 3). Although less is known about specific nutrient limitation of heterotrophic bacterial activity and growth throughout the world oceans, several studies have highlighted the importance not only of macronutrients but also of organic carbon limitation for marine bacterioplankton (Church et al., 2000; Pinhassi et al., 2006; Martínez-García et al., 2010). Also, co-limitation, the simultaneous limitation by two

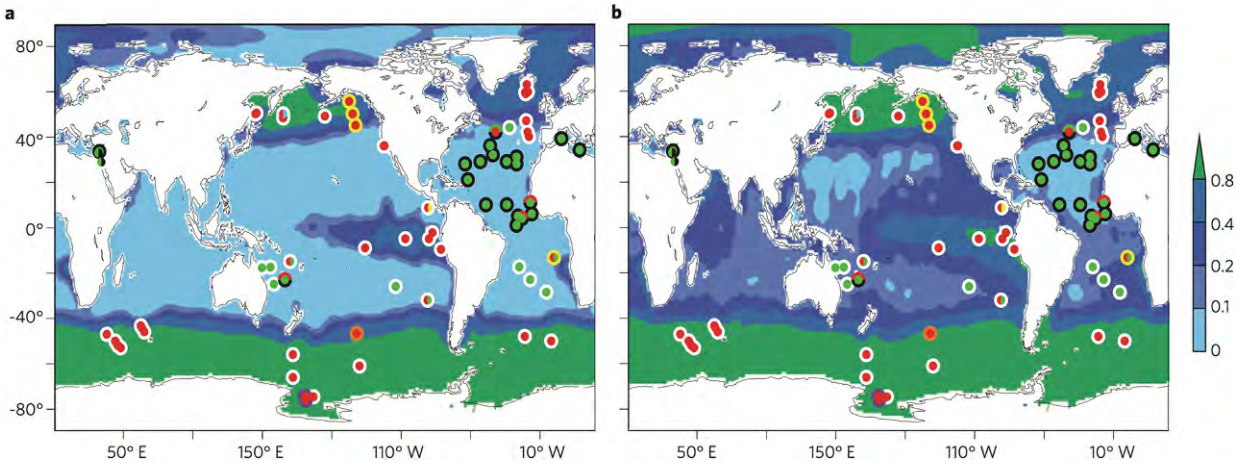

Fig. 3 Global maps showing patters of microbial nutrient limitation in the surface ocean. Backgrounds indicate annual average surface concentrations (μmol kg^{-1}) of (a) nitrate (divided by 16, the mean N:P ratio of organic matter) and; (b) phosphate. Symbols represent nutrient limitation (as inferred from chlorophyll and/or primary production increase after nutrient amendments): primary and secondary limiting nutrients are represented by the central and outer circles, respectively. Divided circles indicate potentially co-limiting nutrients. Distinct colours represent different nutrients: nitrogen (green), phosphorus (black), iron (red), silica (orange), cobalt (yellow), zinc (cyan) and vitamin B12 (purple). Figure from Moore, C.M., Mills, M.M., Arrigo, K.R., 2013. Processes and patterns of oceanic nutrient limitation. Nature Geoscience 6, 701–710; reprinted by permission from Springer Nature.

different elements, e.g., any pairwise combination of carbon, nitrogen or phosphorus, is widespread throughout tropical and subtropical environments (Saito et al., 2008). On the other hand, iron (Fe) limitation is crucial for phytoplankton growth in regions at higher latitudes like the Southern Ocean (Fig. 3; Moore et al., 2013). It should be kept in mind that the primary limiting nutrient can change across seasons as a function of hydrographic conditions – such changes impose a challenge on the microbial community that can contribute to structuring bacterioplankton succession patterns (Pinhassi et al., 2006).

In the following, we present the principal nutrients that often limit bacteria in low-nutrient marine environments. We will describe microbial adaptations to the acquisition of scarce nutrients retrieved from both laboratory experiments and field studies, and include studies that use gene expression profiling to interrogate bacterial strategies for growth and survival in low-nutrient environments.

Dissolved organic carbon

The main source of dissolved organic carbon (DOC) in the upper ocean is photosynthetic activity, released directly during photosynthesis or lost through food web interactions like viral lysis or zooplankton grazing and excretion. Through these processes around half (range between 30%–90%) of photosynthetically fixed organic C becomes DOC and is used by bacteria (Ducklow, 2000). In fact, the oxidation of DOC via respiration represents the main source of energy for heterotrophic bacteria in the ocean.

The highest DOC concentrations are found in the sun-lit surface waters (~60–90 μmol/L in the open ocean) and decrease with depth (to ~40 μmol/L in the deep ocean) (Hansell, 2002). However, most DOC is resistant to microbial degradation on time scales of days to months/years, possibly due to its intractable chemical structure, and/or low energy and nutritional value (Hansell, 2013). The DOC pool can be tentatively separated into three fractions based on its spectrum of reactivity and bioavailability: labile (a few μmol/L), semilabile (20–30 μmol/L), and refractory (ca. 40 μmol/L) (Hansell, 2013). Note that, although the concentration of DOC in the labile fraction is very low, this fraction turns over very rapidly (i.e., high production and consumption rates), and thus accounts for an important fraction of bacterial carbon demand.

Recent data suggest that over the course of evolution, marine heterotrophic bacteria in the oligotrophic open ocean have developed a coordinated, cooperative activity for the utilization of marine DOC. This activity consists of a precise resource partitioning of DOC by different bacterial "specialists", so that DOC turnover is mediated by a temporal succession of taxa, metabolic pathways, and chemical transformations (McCarren et al., 2010; Sosa et al., 2015; Teeling et al., 2012). For example, bacterial gene expression analysis in the North Sea showed pronounced dynamics in carbohydrate-active enzymes, especially TonB-dependent transporters, and in phosphate acquisition genes, "indicating that distinct populations of *Bacteroidetes*, *Gammaproteobacteria*, and *Alphaproteobacteria* are specialized for successive decomposition of algal-derived organic matter" (Teeling et al., 2012). Also in oligotrophic marine systems, polysaccharide utilization is important for nourishing marine bacteria (e.g., NOR5/OM60 clade *Gammaproteobacteria*) (Sharma et al., 2014). Experiments with water from the North Pacific Subtropical Gyre (NPSG) show that opportunistic *Idiomarina* and *Alteromonas* species initiate the processing of DOM by using TonB-associated transporters, nitrogen assimilation genes, and fatty acid catabolism genes. The resulting DOM is then attacked by *Methylophaga* using a suite of assimilatory and dissimilatory single-carbon metabolic pathways (McCarren et al., 2010). Single-carbon (C1) compounds may originate from polysaccharides containing a large fraction of methylated sugars (Quan and Repeta, 2007) and have gained increasing attention as being key for the turnover of DOC in marine environments.

Prochlorococcus can use the Pro1404 transporter to take up significant portions of glucose at nanomolar concentrations in the Atlantic Ocean (del Carmen Muñoz-Mar, 2012). Members of the SAR11 clade, being a cosmopolitan bacterial clade with some of the most abundant organisms on Earth (Morris et al., 2002), play a key role in the carbon cycle in low-nutrient seas. Their streamlined genomes encode membrane transporters (likely with high affinity) and metabolic pathways for a number of low molecular weight carbon compounds, including amino acids, sugars, carboxylic acids, and osmolytes (Giovannoni et al., 2005). SAR11 bacteria also utilize one-carbon compounds (e.g., methyl groups from methylated compounds) as a source of energy via the C1 tetrahydrofolate (THF) oxidation pathway (Sun et al., 2011). Experiments with the SAR11 clade model organism *Candidatus* Pelagibacter ubique show that the utilization of methylphosphonic acid (an organophosphorus compound shown to be produced by marine microbes (Metcalf et al., 2012)) results in the release of methane, providing an explanation for unexpected accumulations of methane in surface waters limited by phosphorus (Carini et al., 2014a). This indicates one of surely many dependencies of the carbon cycle on inorganic nutrients in the oceanic environment.

Nitrogen

N is available to marine microorganisms in several inorganic and organic forms. Nitrite and nitrate, the more oxidized forms of N, are usually found in very low concentrations in surface waters (<0.1 μmol kg^{-1}; Fig. 3 from Moore et al., 2013), although it is abundant in deep waters (below the photic zone). Ammonia is an important source of N in the surface oligotrophic ocean as it results from excretion processes and remineralization of DOM. Marine microbes in the oligotrophic ocean also acquire N directly from DOM (e.g., proteins dissolved in seawater), some of which is in highly labile forms such as urea, dissolved free amino acids (DAA), and nucleic acids (Sipler and Bronk, 2015). Urea, for example, is a potentially important nitrogen source in the ocean, and is used by marine cyanobacteria (Moore et al., 2002). Concentrations of dissolved organic nitrogen (DON) in open ocean surface waters are ca. 3–5 μmol kg^{-1} (Letscher et al., 2013) and rapidly decrease with depth below the photic zone.

Gene expression analyses show that a suite of N metabolism genes (ammonium transporter, *amt*; dissimilatory nitrite reductase, *nirK*; urea transporter, *urt*; ammonia monooxygenase subunits, *amoABC*) are among the most highly expressed genes in low-nutrient environments (Shi et al., 2011). The expression of these genes is usually related to N stress in the cell. Interestingly, successful dominant oligotrophic microbes like *Prochlorococcus* and SAR11 clade bacteria can alleviate N stress by utilizing a variety of available DON compounds (and consequently their expression of ammonium acquisition genes is strongly reduced) (Sharma et al., 2014). In fact, bacteria in the SAR11 clade show pronounced gene expression responses to N-limitation, with a redirection of metabolism to take up and utilize organic nitrogen compounds (Smith et al., 2013). N limitation can also be dealt with by modifying the elemental composition of cells. Thus, preferential use of certain amino acids for protein synthesis to reduce N content in proteins, is an adaptation to N limitation (Grzymski and Dussaq, 2012). Reductions in the net metabolic N fluxes and the N content in cytoplasmic pools represent an adaptation of *Roseobacter* clade bacteria to N starvation (Chan et al., 2012). Moreover, these bacteria may utilize methylated amines (Chen, 2012), potentially contributing to their N budget.

DON can represent an important source of N also for picocyanobacteria, and it has been suggested that the ability to utilize amino acids could contribute to the success of *Prochlorococcus* in the oligotrophic ocean (Zubkov et al., 2004; Mary et al., 2008). Analysis of closely related strains of *Prochlorococcus* and *Synechococcus* ecotypes that differ in spatiotemporal patterns of appearance show that N sources may contribute to niche partitioning (Fig. 4; Moore et al., 2002). Similar to roseobacters and SAR11 clade bacteria *Prochlorococcus* ecotypes display distinct expression patterns for genes in N and C metabolism, depending on N availability (Tolonen et al., 2006). Genetic inventories show that urea and ammonium assimilation genes are widespread in naturally occurring *Prochlorococcus* populations. In fact, the *amt* gene (an ammonium transporter in bacteria) is the most highly transcribed N-related gene in *Prochlorococcus* populations in the open ocean, likely an adaptive mechanism to efficiently scavenge N (Shi et al., 2011). In addition, genes for growth on nitrate (e.g., *narB*) are present in some *Prochlorococcus* strains, indicating that nitrate at times could be important to these bacteria in the open ocean (Fig. 4) (Berube et al., 2015). Also *Synechococcus* strains, which form dominant

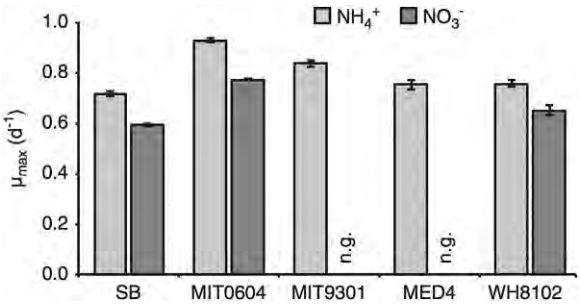

Fig. 4 Maximum specific growth rates (μmax) of *Prochlorococcus* strains SB, MIT0604, MIT9301, MED4 and WH8102 when growing on ammonium (NH_4^+) or nitrate (NO_3^-). Error bars represent standard deviation; n.g., no growth. Growth rates were significantly different ($p<0.05$) between different strains when growing on the same nitrogen source and also between biological replicates of the same strain growing in different nitrogen sources. Figure from Berube, P.M., Biller, S.J., Kent, A.G., 2015. Physiology and evolution of nitrate acquisition in Prochlorococcus. The ISME Journal 9 (5), 1195-1207; reprinted by permission from Springer Nature.

populations in coastal environments, utilize nitrate as N source. Thus, adaptations to different N-sources contribute to explaining differential spatiotemporal distributions of *Synechococcus* and *Prochlorococcus* populations in the sea (Moore et al., 2002).

Phosphorus

The main inorganic source of P in the ocean is the oxidized form: phosphate. As for nitrate, phosphate concentrations are low in surface waters (<0.4 μmol L^{-1}) (Fig. 3; Moore et al., 2013) and increase below the photic zone. A suite of uptake systems and metabolic pathways has evolved in marine bacteria to efficiently utilize different forms of phosphorus (both inorganic and organic), as seen from genome analyses and metatranscriptomics. Gene transcripts typically enriched in bacteria under P starvation include the phosphate-binding (*pstS*), permease (*pstC*), and ATP-binding (*pstB*) components of high affinity phosphate-specific ABC transporters (Martínez et al., 2013; Dyhrman et al., 2007). In addition, and as exemplified by *Prochlorococcus*, bacteria adjust their P uptake kinetics in response to P stress by increasing cell-specific affinity and maximum velocity of uptake for both inorganic and organic P (e.g., PO_4 and ATP) (Krumhardt et al., 2013). Interestingly, *Prochlorococcus* shows complicated acclimation and regulatory mechanisms to maximize growth and survival under P starvation and the expression of P stress related genes differs between ecotypes depending on the particular environment they thrive in (Martiny et al., 2006, 2009). Regarding utilization of more specific P-containing compounds, genes for utilization of phosphite (a reduced P compound) have been detected in *Prochlorococcus* and other marine bacteria (Martinez et al., 2012).

Besides inorganic P, P in organic compounds is also available to marine microorganisms (Karl and Björkman, 2015; Gómez-Pereira et al., 2013). A wide range of compounds is included in the dissolved organic phosphorus (DOP) pool: monomeric and polymeric esters, phosphonates and organic condensed phosphates (e.g., nucleic acids, phospholipids, phosphoproteins, sugar phosphates, and phosphoamides) (Karl and Björkman, 2015). DOP concentrations in surface oligotrophic oceans ranges from ca <0.1 to 0.3 μmol L^{-1}, with extremely low values (50 nmol L^{-1}) in the eastern Mediterranean Sea, one of the most oligotrophic seas in the world (Karl and Björkman, 2015). Under P-limitation, marine bacteria frequently induce the transcription of genes encoding alkaline phosphatases (e.g., *phoA* and *phoX*; Martínez et al., 2013) which hydrolyze a broad spectrum of DOP compounds (Sebastián and Ammerman, 2009; Duhamel et al., 2010).

Recent research on P cycling by marine bacteria shows that phosphonates (Pn; reduced organic compounds containing P that represent one third of high molecular weight DOP) are an important source of P for marine microorganisms. Pn utilization genes are widespread and abundant among marine bacteria (including *Proteobacteria*, *Planctomycetes*, *Cyanobacteria*, and *Pelagibacter*) suggesting that Pn may be a key P source in P-limited surface oceans, as well as in the more P-rich deep-water column (Dyhrman et al., 2006; Sowell et al., 2009; Martinez et al., 2010). Different strategies have been suggested for the use of phosphonates including high-affinity transport and hydrolysis of phosphonate compounds by a C-P lyase pathway (Dyhrman et al., 2006; Martínez et al., 2013), a phosphonatase pathway (Martinez et al., 2010) and a periplasmic substrate-binding protein for Pn acquisition (Sowell et al., 2009).

To reduce their dependence on P, some bacteria have evolved the ability to adjust their elemental requirements through modification of macromolecular composition. One example is the synthesis of sulfolipids instead of phospholipids in *Prochlorococcus* in oligotrophic environments (Bertilsson et al., 2003; Van Mooy et al., 2006). Similarly, some marine bacteria can produce phosphorus-free membrane lipids as a strategy to cope with P starvation, as exemplified by SAR11 clade bacteria and several other marine bacterial groups (Fig. 5; Carini et al., 2015; Sebastián et al., 2016). Thus, the spatial and temporal variability in availability of distinct P sources, the different adaptations to utilize different forms of (in)organic P, and the ability to alter the cellular P content all contribute to regulate the cycling of P by microbes in the world ocean.

Micronutrients

The availability of trace metals (e.g., iron, manganese, cobalt, zinc, nickel and cadmium) and vitamins is crucial for determining the productivity in marine ecosystems (Falkowski et al., 1998; Morel and Price, 2003; Saito et al., 2002). Trace metals typically play a critical catalytic role in the reactive sites in enzymes, thus influencing redox reactions (Price and Morel, 1990; Morel et al., 1994). In the open ocean most of these vital metals are found embedded within organic compounds, which hinders the direct measurement of their concentrations and bioavailability (Wells, 2002). As compared to C, N and P, micronutrients have received fairly little attention regarding how their availability contributes to structuring microbial communities and their activity.

The importance of iron (Fe) as a nutrient is appreciated since long due to its critical role as a redox catalyst in photosynthetic and respiratory electron transport chains of phototrophic and heterotrophic prokaryotes, respectively (Tortell et al., 1999), as well as being required for enzymes in N_2 fixation (Falkowski, 1997). The main allochthonous input of Fe to the surface ocean is through atmospheric dust deposition, following long distance wind transport (primarily from continental deserts; Jickells et al., 2005). Fe concentrations in oceanic surface waters are extremely low, averaging 0.07 ± 0.04 nmol L^{-1}, with ca. 99% of it found as organic complexes (Johnson et al., 1997; Turner and Hunter, 2001; Parekh et al., 2004). Fe limitation of marine productivity is extremely relevant in the so called High Nutrient Low Chlorophyll (HNLC) areas (e.g., the Southern Ocean), where macronutrients such as N and P are readily available for phytoplankton but growth is limited by the availability of Fe (Fung et al., 2000; Moore et al., 2013). In such areas also Fe limitation of marine bacteria has been observed (Church et al., 2000; Kirchman et al., 2000; Cochlan, 2001; Arrieta et al., 2004).

Interestingly, the Fe to C quota of bacterioplankton is about 1.5–2 times higher than that of phytoplankton (Maldonado and Price, 1999) and bacterioplankton may account for up to 70% of the biological Fe uptake in environments like the Subarctic Pacific (Tortell et al., 1996; Maldonado and Price, 1999). To cope with Fe limitation, marine bacteria synthesize siderophores - high-affinity

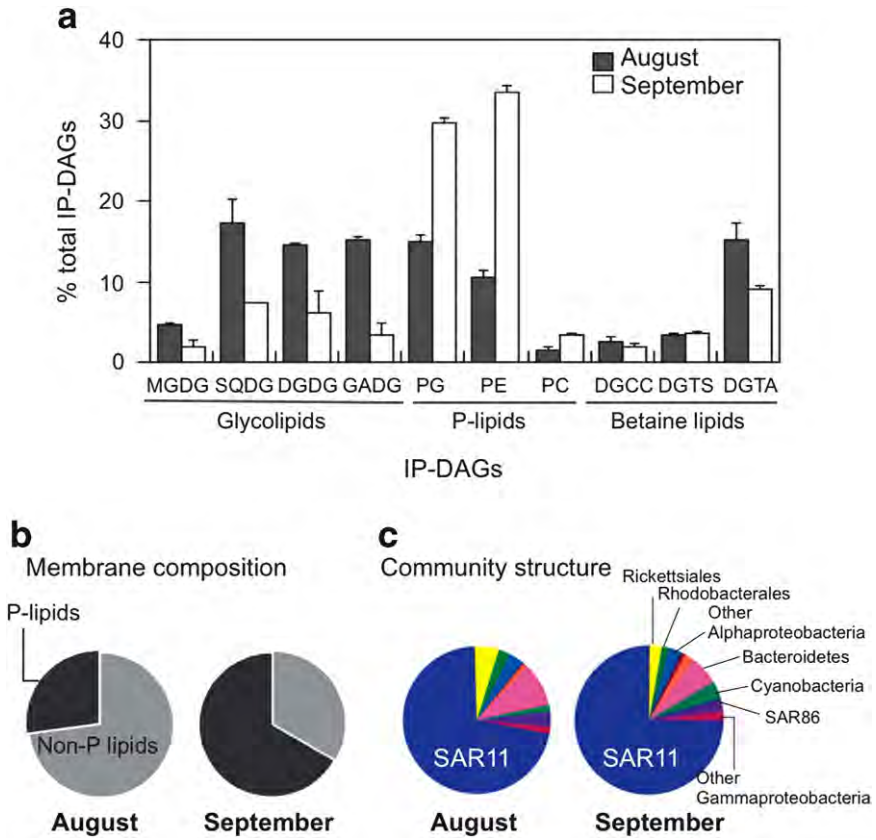

Fig. 5 Membrane lipid remodelling in heterotrophic bacterial communities from environmental samples in the Eastern Mediterranean in August (severe P-limitation) and September (alleviated P-limitation). (a) Contribution of intact polar diacylglycero lipids (IP-DAGs) to the membrane composition in P-starved (black) and P-replete (white) *mesocosms*. Contribution of P-lipids was higher during September while contribution of non P-lipids (glycolipids and betaine lipids) was higher during August. (b) Contribution of non-P versus P-lipids during severe P-limitation (August) and alleviated P-limitation (September). (c) Bacterioplankton community structure at the time of sampling. Figure from Sebastián, M., Smith, A.F., Gonzalez, J.M., 2016. Lipid remodelling is a widespread strategy in marine heterotrophic bacteria upon phosphorus deficiency. The ISME Journal 10, 968–978; licensed under CC BY 4.0.

Fe-chelating compounds – that are released into the water (Granger and Price, 1999). The siderophores with bound Fe, in turn, are taken up by outer membrane proteins, the TonB-dependent outer membrane transporters (TBTs) (Moeck and Coulton, 1998). Both SAR11 clade bacteria and *Prochlorococcus* show significant changes in gene expression upon Fe deficiency, including genes for periplasmic iron binding proteins and iron transporters (SAR11) and ferredoxin and flavodoxin genes (involved in Fe-regulated responses in cyanobacteria) (Smith et al., 2010; Thompson et al., 2011).

The importance of vitamins and other organic molecules for the ecology of microbes in the ocean is being increasingly recognized. The availability of vitamins can be a limiting factor for growth of microorganisms in open ocean waters (Panzeca et al., 2006; Bertrand et al., 2007; Koch et al., 2011). Most of current knowledge on the role of vitamins in marine environments concerns B-vitamins (Sañudo-Wilhelmy et al., 2006; Panzeca et al., 2008, 2009; Bonnet et al., 2013), which generally speaking serve as cofactors for enzymes catalyzing biochemical reactions in central metabolism. Still, different vitamins play distinct roles in microbial physiology (e.g., B12 is required for methionine synthesis, whereas vitamins B1 and B7 are required for carbon fixation, fatty acid metabolism and amino acid metabolism) (Sañudo-Wilhelmy et al., 2014). Many microbes lack the ability to synthesize vitamins (as is the case for us humans) and need to obtain them from the environment – such organisms are referred to as vitamin auxotrophs. Recent evidence shows that only a fraction of marine bacteria counts with biosynthetic pathways for B1, B7 and B12 (76%, 78% and 37% of the more than 400 bacterial isolates studied, respectively) (Sañudo-Wilhelmy et al., 2014). It is likely that much smaller portions of natural bacterioplankton are vitamin synthesizers, given that for example SAR11 clade bacteria, one of the dominant bacterial groups in the open ocean, are B1 auxotrophs (Carini et al., 2014b). Yet, some heterotrophic bacteria crucially determine vitamin availability to the microbial food web by turning B1 precursors into B1 (Paerl et al., 2015). These findings suggest that an important portion of marine bacteria may eventually compete with phytoplankton for the uptake of these growth factors from the environment.

The number of recognized micronutrients that ultimately affect metabolism and growth of microorganisms in the open ocean is constantly increasing through new research on microbial interactions. In a recent ground breaking experiment, analysis of cocultures of the diatom *Pseudo-nitzschia multiseries* with the *Roseobacter* clade bacterium *Sulfitobacter* sp. SA11 revealed that bacterial excretion of the hormone indole-3-acetic acid triggered diatom growth stimulation; and diatom release of tryptophan in turn signaled

improved growth conditions to the bacterium (Amin et al., 2015). New knowledge is much needed of the spatial distribution of different vitamins and growth factors, their concentrations, and their dynamics over different temporal scales (from days to months). Along with knowledge on which microbes are key producers of growth factors, this will bring a deeper understanding of how microbial interactions allow microorganisms to manage life in the low-nutrient open ocean.

Light as an Energy Source to Photoheterotrophic Bacteria

In addition to driving chlorophyll *a*-based photosynthesis (as found in most plants and phytoplankton), solar energy is a key energy source also to microorganisms with the light harvesting photosystems rhodopsin and bacteriochlorophyll (Fig. 6). The wide distribution and potential importance of bacteria with rhodopsin and bacteriochlorophyll in the marine environment was first highlighted in 2000 (Béjà et al., 2000; Kolber et al., 2000). Subsequent analysis of worldwide metagenomic datasets showed that on average 48% and 18% of bacteria contain rhodopsin and bacteriochlorophyll, respectively (Finkel et al., 2013). Obviously, photoheterotrophy - the ability of "heterotrophic" bacteria to complement their energy requirements with light - is a tremendous advantage in oligotrophic surface waters where growth and survival is often limited by availability of energy-rich DOC (Pinhassi et al., 2016; Koblizek, 2015). It should be noted that photoheterotrophs obtain energy from light but still rely on DOC for carbon (i.e., they cannot use carbon dioxide as their sole carbon source). Thus, light exposure allows ATP synthesis in these photoheterotrophs (i.e., photophosphorylation), which significantly reduces their dependence on respiration of DOC. Since a grand majority of DOC is typically respired to generate energy for metabolism rather than for building biomass, light harvesting can have a major impact on the fitness of bacteria.

Metatranscriptomics analyses confirm the ecological importance of rhodopsin- and bacteriochlorophyll containing bacteria (the latter carrying out aerobic anoxygenic phototrophy, AAP) (Frias-Lopez et al., 2008; Shi et al., 2011). In fact, rhodopsin genes – primarily proton pumping proteorhodopsins that ultimately generate ATP – are consistently among the most highly expressed genes in the surface ocean (Shi et al., 2011; Satinsky et al., 2014). Moreover, detailed analyses show pronounced variability in rhodopsin gene expression in many different bacterial groups over day-night cycles (Ottesen et al., 2013). A further indication of the importance of rhodopsins for obtaining energy is the high incidence of lateral gene transfer (LGT) of this photosystem: among Bacteria, between Bacteria and Archaea, and even between prokaryotes and eukaryotes. This is facilitated by the extraordinary genetic simplicity of the of rhodopsin photosystems (<10 genes). A major remaining challenge in understanding the life and success of microbes in low-nutrient environments is to obtain quantitative estimates of the energy harvested by photoheterotrophs (Pinhassi et al., 2016). Ultimately, such estimates could profoundly influence our understanding of how oceanic carbon cycling is regulated.

Deep Ocean Adaptations

Mesopelagic (200–1000 m) and bathypelagic (1000–4000 m) layers of the ocean are characterized by high pressure and relatively low temperatures, and whereas inorganic nutrient concentrations are high the availability of DOC is importantly limited. This DOC limitation arises due to strongly decreasing DOC concentrations with depth (i.e., with distance from the sun-lit surface ocean where DOC is freshly produced). Moreover, the DOC pool in deep waters is less bioavailable as it is composed of increasingly recalcitrant DOC after microbes have consumed the easily accessible and more highly energetic compounds (Jiao et al., 2010). There is ongoing discussion if this limited access to DOC is because it consists of recalcitrant materials or if it is due to the low concentration of the different DOC compounds (Arrieta et al., 2015). Similar to DOC concentrations, microbial abundance and production decline

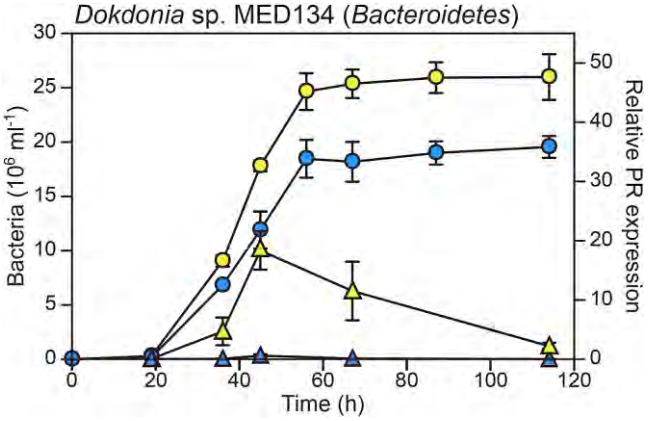

Fig. 6 Growth of the *Bacteroidetes* isolate *Dokdonia* sp. MED134 in the light (yellow circles) and in the dark (blue circles). Also shown is relative expression of the proteorhodopsin gene in the light (yellow triangles) and in the dark (blue triangles). Error bars represent standard deviations. Figure slightly modified from Pinhassi, J., DeLong, E.F., Béjà, O., González, J.M. Pedrós-Alió, C., 2016. Marine bacterial and archaeal ion-pumping rhodopsins: genetic diversity, physiology and ecology. Microbiology and Molecular Biology Reviews 80 (4), 929–954, reproduced by permission from ASM Press and the author.

exponentially from surface to bathypelagic waters by ~2–3 orders of magnitude (Reinthaler et al., 2006); yet, microbial diversity is surprisingly high in these environments (Sogin et al., 2006). The relative contribution of Bacteria to total prokaryotes decreases with depth as the proportion of Archaea, particularly chemoautotrophic Chrenarchaeota, increases (Teira et al., 2006). Important work on the genomics and ecology of natural communities and model deep-sea prokaryotes has contributed understanding of adaptations to the apparent lack of organic nutrients in these environments (Könneke et al., 2005; Vezzi et al., 2005; DeLong et al., 2006; Martín-Cuadrado et al., 2007; Konstantinidis et al., 2009; Walker et al., 2010).

In deep waters the absence of DOC production (ultimately from photosynthesis) is partly compensated for by chemolithoautotrophy. Chemoautotrophic metabolism (deriving energy from chemical reactions and synthesizing all necessary organic compounds from carbon dioxide) is suggested as a principal source of energy in deep waters. In fact, genes related to nitrate reduction and ammonium oxidation are commonly found in marine archaea (Könneke et al., 2005; Shi et al., 2011) and studies on the archaeon *Nitrosopumilus maritimus* have considerably broadened our knowledge on nitrification (i.e., ammonia oxidation) in these environments (Könneke et al., 2005; Martens-Habbena et al., 2009; Walker et al., 2010).

Recent studies have emphasized the importance to deep sea microbiota of sinking organic matter in the form of particles/aggregates produced in the euphotic zone (Baltar et al., 2009a; Shi et al., 2011; Swan et al., 2011). In general terms, genomes of several bacteria thriving in the deep-sea are larger and encode more rRNA gene copies, gene regulatory features, membrane transporters and a larger variety of metabolisms than the average surface ocean bacterium, features that are related to an opportunistic life style (Lauro et al., 2007; Lauro and Bartlett, 2008). Genetic analyses of deep-sea microorganisms further indicate the importance of a surface-attached life strategy, suggesting that deep-water prokaryotic activity to a large extent is associated with particles (López-López et al., 2005; DeLong et al., 2006; Lauro et al., 2007; Martín-Cuadrado et al., 2007).

Work with the deep-sea model bacterium *Photobacterium profundum* highlights the utilization of complex polymers for growth (e.g., chitin, pullulan, xenobiotics, and cellulose) and the presence of an amino acid fermentation pathway previously known only in anaerobes (Vezzi et al., 2005). Additionally, carbon monoxide oxidation has been suggested as an important additional energy source in the deep ocean (Martín-Cuadrado et al., 2007) and some deep-sea bacteria (the SAR324 clade) have the ability to utilize C1 compounds (Swan et al., 2011). In deep-sea bacteria, a number of membrane transporters are induced under high pressure, including ABC permeases related to nucleotide and inorganic ion transport and TonB type proton motive force-dependent transporters (Campanaro et al., 2005, 2008). Analyses of *Alteromonas macleodii* deep-sea ecotypes suggest they are adapted to rapidly exploit marine particles (García-Martínez et al., 2002; López-López et al., 2005; Ivars-Martinez et al., 2008; López-Pérez et al., 2013) and produce antibiotics-like metabolites to fight off competitors (Kimes et al., 2014; Mizuno et al., 2013). Interestingly, also free-living prokaryotes in the deep ocean have swimming abilities using flagella to approach particles or animals for subsequent attachment or colonization (Wang et al., 2008; Eloe et al., 2008). Furthermore, marine prokaryotes from deep waters are known to utilize extracellular enzymes that can be liberated as free dissolved enzymes and which are mainly utilized to access C in POM and DOM (Baltar et al., 2009b, 2010), possibly reflecting adaptations to rapidly respond to environmental change.

Conclusions

As shown in this article, there are plenty of distinct and complementary strategies that help microbes to survive in the low nutrient marine environment. Some microbes are clearly winners in this so-called hostile environment, like members of the SAR11 clade of *Alphaproteobacteria* and the cyanobacterial *Prochlorococcus* that are widespread dominants in the oligotrophic ocean. However, one may ask - is the oligotrophic environment really that "hostile" or "extreme" from these microbes's perspective? In fact, a large majority of organism life – highly adapted to their environment – may see this as a perfectly "comfortable" environment. It appears like such "comfort" in oligotrophic waters is in part provided by a blurring of the classical distinction between hetero- and autotrophic life strategies, i.e., a wide spread of mixotrophy, where an important portion of heterotrophic bacteria are in fact photoheterotrophs relying on solar energy harvesting and photosynthetic cyanobacteria also utilize organic nutrients.

Undoubtedly, critical understanding of microbial oceanography and of microbial life in one of the largest low-nutrient biomes on Earth has been provided by international programs like the Joint Global Ocean Flux Study (JGOFS, see "Relevant Websites section") and the establishment of regular long-term microbial observatories in open oceanic waters like the Hawaii Ocean Time Series (HOTS, see "Relevant Websites section") and the Bermuda-Atlantic Time Series (BATS, see "Relevant Websites section"). Such time series are also necessary to provide knowledge on the effects of climate change on marine microbes, as climate change may affect nutrient cycling in the ocean by altering nutrient inputs (e.g., aeolian inputs, stratification, release from sediments) and metabolic processes key for the cycling of specific nutrients (e.g., denitrification, ammonia oxidation). The combination of field studies and experimental work with natural microbial assemblages and model marine bacteria, along with sequencing techniques like (meta)genomics and transcriptomics, constitutes a flexible platform to test hypotheses on microbial strategies for growth and survival in low nutrient ecosystems, casting light on microbial and biogeochemical process in the ocean.

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Adaptive Radiation in Microbes

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Glossary

Adaptive radiation The rapid diversification of a lineage into several distinct genotypes or species that use different ecological niches.
Character displacement More extreme phenotypic divergence in traits associated with resource acquisition when individuals of different genotypes or species occur in the same place than when they occur in separate places.
Disruptive selection Selection against types that have intermediate phenotypes. Individuals with extreme trait values have higher reproductive output than those with intermediate trait values. Also called diversifying selection.
Diversification The formation of two or more lineages from a single common ancestor.
Ecological opportunity The availability of previously inaccessible ecological niches or resources. Requires that an alternative abiotic or biotic resources exist, that the ancestral lineage is not excluded from the resource by its spatial or temporal distribution or by more efficient species, and that the ancestral lineage has or can evolve traits that permit use of the novel resource.
Niche A property of a genotype or species that describes the range of habitats or conditions that it can tolerate.

Defining Statement

This article provides an overview of adaptive radiation in microbes. We discuss the advantages of using microbial experimental evolution as a tool to explore adaptive radiation, present the main components of the theory used to explain this process, and evaluate the contributions of particular experiments to elucidate the ecological and genetic mechanisms that influence adaptive radiation in microbes.

Introduction

Adaptive radiation (AR) is the rapid diversification of a lineage into a range of phenotypically distinct genotypes or species that each occupy a different ecological niche. Evolutionary biologists have long been interested in AR because it is thought to be a major mode through which diversification happens. Iconic examples in multicellular organisms include sticklebacks in post-glacial lakes, the Hawaiian silversword alliance, Darwin's finches in the Galapagos islands, and cichlid fishes of the African Rift Valley lakes. The long generation times of these organisms preclude the direct study of evolutionary processes in real time, making it challenging to uncover the ecological and genetic mechanisms driving AR. A growing number of examples of AR in microbes are now available (discussed below) and these offer an outstanding opportunity to study the causes of AR because generation times are short enough, and population sizes large enough, to track evolutionary diversification directly in the laboratory. When coupled with whole genome sequencing to identify the genetic changes responsible for diversification, microbial systems provide an invaluable tool for gaining insight into the ecological processes and genetic mechanisms driving evolutionary diversification.

The Ecological Theory of Adaptive Radiation

The ecological theory of adaptive radiation, formalized by Schluter (2000), is the leading explanation for AR. Central to the theory is the concept of ecological opportunity, that is, the range of unused or underutilized niches or resources. Ecological opportunities become available when a lineage gains access to a novel environment where competitors are absent or when other species modify the environment to create new niches. When ecological opportunity is available, AR proceeds in three steps. First, differences in the conditions of growth across environments causes divergent natural selection leading to the evolution of locally-adapted specialist types (niche specialists), which by definition, trade-off fitness across alternative environments. Second, strong competition for resources generates disruptive selection against intermediate phenotypes that further exaggerates phenotypic and ecological divergence. The third step, unique to sexually reproducing organisms, is the creation of reproductive barriers as a consequence of ecological specialization that contributes to forming new species (speciation). In the predominantly asexual taxa used in microbial AR experiments, no such barriers exist and ecological divergence is directly linked to lineage diversification.

Advantages and Limitations of Using Microbial Systems to Test Adaptive Radiation

Advantages of microbial systems: The great advantage of microbial systems for studying evolutionary diversification is the ability to track the origins and fate of genetic variation associated with niche specialization directly through experiment. Measures of niche specialization come from estimating the fitness of an evolved strain against the ancestral strain from which it is derived through competition experiments, or pure culture measures of growth rate or yield across a range of environmental conditions. The large population sizes and rapid generation times of most microbes under laboratory conditions means that mutations are rarely in short supply and natural selection can be effective at generating adaptation. Niche specialization and ecological diversification can thus evolve very rapidly, within tens to hundreds of generations in some experiments. Identifying the genetic targets of adaptive diversification is now accessible to most labs through genome sequencing technologies, where the whole-genome DNA sequence of evolved populations or isolates is compared directly with that of the common ancestor. The repeated evolution of mutations at the same site in multiple independent replicates, or parallel evolution, is strong evidence that site is a target of selection.

Limitations of microbial systems: The study of AR in microbial systems suffers from an overrepresentation of particular species, especially *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Escherichia coli*, that limits our ability to make strong, generalizable inferences. Others have criticized the use of microbial systems as models of AR in more complex organisms on two grounds. *First*, the majority of experiments are performed in lineages that reproduce asexually where mutation is the sole source of genetic variation, making it difficult to extrapolate results to sexual multicellular organisms where standing genetic variation is often more abundant, population sizes smaller and recombination is a key source of genetic variation. *Second*, and as we noted above, most studies of AR in microbes have not used sexually reproducing organisms, which means that the impact of ecological divergence on speciation in AR remains an open question. Some microbial systems capable of sexual reproduction, such as yeast, and species with moderate to high levels of horizontal gene transfer, such as *E. coli* Hfr strains, could be used to evaluate the role of recombination in ecological diversification.

Environmental Variation That Leads to Divergent Natural Selection

A necessary condition for ecological diversification is divergent natural selection generated by environmental variation, where different phenotypes are favored in different patches of the environment. Prolonged divergent selection leads to specialization, which means that the organism has high fitness in the patch it normally encounters but lower fitness in other patches. A typical experiment involves selecting an initially isogenic lineage in contrasting abiotic conditions of, for example, temperature, salinity, antimicrobial (antibiotic/antifungal) concentrations, or resource availability (differences in the amount or types of sugars) for hundreds or thousands of generations and then evaluating the degree of specialization in the evolved populations relative to each other and to the ancestor. Manipulations of the biotic environment, such as the presence vs. absence of predators and parasites, can also generate divergent selection.

Ecological Opportunity

Divergent selection can only be effective at driving diversification if ecological opportunity is available. This requires that alternative abiotic or biotic resources capable of generating divergent selection exist, the focal lineage has traits that allow the novel resource(s) to be used, and other species like competitors or predators that might prevent access to ecological opportunities are absent. In general, as the range of ecological opportunities increases, so too does the number of distinct types and the extent of phenotypic divergence among them in microbial AR.

Abiotic ecological opportunities can be present as a collection of distinct nutrients. For example, *P. fluorescens* and *E. coli* experiments show that increasing the number of distinct carbon resources in growth media leads to more genetic variation in resource use than evolution under a single carbon substrate. Spatial segregation of habitats can also play a role in AR, as Rainey and Travisano's (1998) seminal experiment illustrates. A single isolate of *Pseudomonas fluorescens* cultivated in a spatially homogeneous environment (created by agitating rich media on an orbital shaker) fails to diversify whereas the same genotype allowed to grow in the same medium without agitation, which affords spatial structure to the microcosm primarily through the formation of an oxygen concentration gradient, leads to the evolution of at least three ecologically and genetically distinct types over the course of ~1 week. These types are easily distinguished by their colony morphology (Fig. 1(A)): the ancestral broth-specialist 'smooth' (SM) morphotype, the 'wrinkly-spreaders' (WS) which produce high levels of a cellulose-like polymer and form a biofilm mat in the broth-air interface, and the 'fuzzy-spreaders' (FS) which are transient broth-air specialists that form loosely adhering rafts of cells that fall to the bottom after a WS biofilm collapses. Spatial structure is also necessary for the maintenance of diversity, as its removal (by shaking previously static cultures) leads to the loss of diversity within a few generations.

Some of the best examples of AR in the fossil record of multicellular species occur when ecological opportunities become available as a result of the absence of the usual suite of competitors faced by a lineage, as can occur following mass extinctions or colonization of species-poor islands. A similar process is seen in microbial ARs. When *P. fluorescens* diversifies in soil microscosms, which should more closely resemble their natural environment, they achieve a three-fold higher diversity in the absence of the resident soil-bacteria community. Despite the evolved diversity in soil being lower than in nutrient-rich broth, and mostly composed of the WS type and two SM types, the absence of interspecific competitors allows a more even distribution of distinct

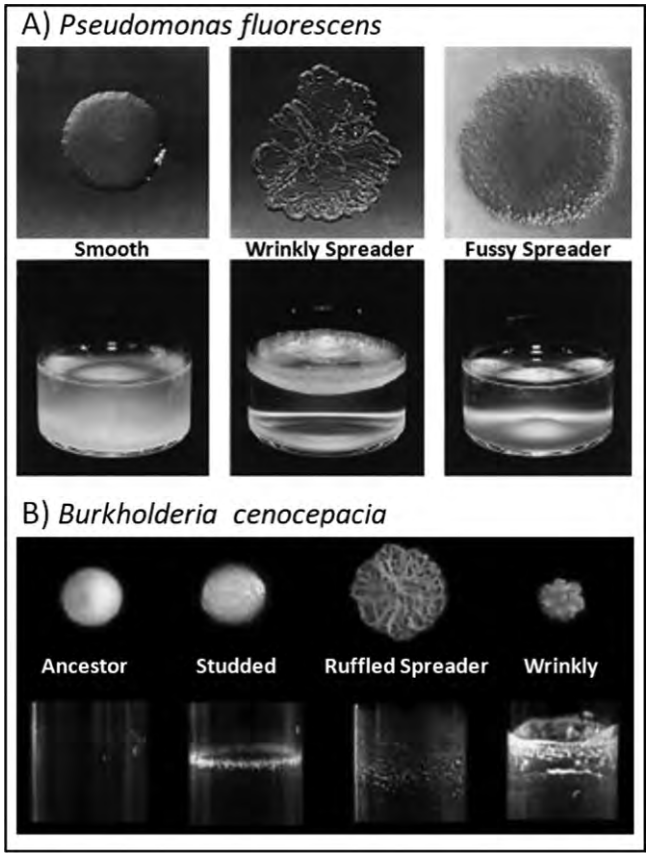

Fig. 1 AR in the laboratory in two microbial model systems, as revealed by colony morphology on agar plates (top rows). (A) Morphotype diversity in *Pseudomonas fluorescens* evolved under static conditions in nutrient rich media; bottom row shows niche occupation by each morphotype grown on its own in static microcosms. (B) *Burkholderia cenocepacia* evolved on plastic beads in a flow-through system composed of nutrient rich media; bottom row shows each morphotype growing as a biofilm on test tube walls. In both systems spatial differences in distribution reflect difference in niche preferences. Figures from (A) Rainey, P.B., Travisano, M., 1998. Adaptive radiation in a heterogeneous environment. Nature 359, 69–72. and (B) Poltak, S.R., Cooper, V.S., 2011. Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. The ISME Journal 5, 369–378. doi:10.1038/ismej.2010.136. Reprinted with permission of Springer Nature.

morphotypes than when the soil-bacterial community is present. Competitors can also prevent diversification by reducing ecological opportunity. SM morphotypes of *P. fluorescens* inoculated into static microcosms fail to diversify, for example, if WS morphotypes occupying the air-broth interface are already present.

Ecological Interactions That Promote Diversification

The striking phenotypic diversification associated with niche specialization in AR, and the unusually high rate at which diversification occurs, could be due to the combined action of divergent selection and disruptive selection (i.e., selection against intermediate forms) generated by ecological interactions. Competition is an ecological interaction that is the inevitable result of population growth on limited resources. Diversification results because lineages using a shared resource can reduce the antagonistic effects of competition for that resource by specializing on distinct resources. The tell-tale sign of diversification driven by resource competition is character displacement, where divergence in traits associated with resource acquisition is more pronounced in individuals that have evolved together compared to when they evolved separately. For example, Tyerman et al. (2008) selected *Escherichia coli* in minimal media supplemented with glucose and acetate. After 1000 generations genotypes that differ in the efficiency and dynamics with which they use the two carbon resources evolved. When these genotypes are further evolved separately for 200 generations their growth profiles become more convergent. When they are subsequently allowed to co-evolve together again for another 200 generations, the divergent phenotypes associated with resource use re-evolve. Similarly, *P. fluorescens* evolved in multi-carbon substrates show higher divergence in their metabolic phenotypes and fitness than among populations evolved in single-carbon substrates.

Enemies like predators and parasites can also generate disruptive selection leading to diversification in prey or hosts by generating selection for types that can avoid being eaten or parasitized. The predatory ciliate *Tetrahymena thermophila*, for example, can cause diversification of its *P. fluorescens* prey in minimal media by promoting the evolution of WS types that form a biofilm,

which provides a refugia against predation, at the air-broth interface. More commonly, however, predators and parasites tend to slow the rate of diversification by reducing victim density, and so weakening the strength of resource competition driving disruptive selection. In rich media, *T. thermophila* reduces *P. fluorescens* density by approximately an order of magnitude, slowing the rate of diversification.

Commensalistic or mutualistic ecological interactions can also create novel ecological opportunities and influence AR. One example is niche construction or facilitation, where the activities of organisms create novel niches and so generate the opportunity for divergent selection. Cross-feeding, where one type feeds on the metabolic by-products of another, is a well characterized form of niche construction in microbial systems. For example, in *E. coli* populations selected on minimal media with glucose as a sole carbon source a small 'S' morphotype sometimes evolves. The S morphotype feeds on acetate by-products of the ancestral large 'L' morphotype, and the two can stably co-exist. Niche construction is also seen during the formation of diverse biofilm communities in the opportunistic pathogen *Burkholderia cenocepacia*. Three distinct morphotypes (Fig. 1(B)) evolve following prolonged selection for cycles of colonization and growth on plastic beads in agitated nutrient media that show a regular colonization dynamic reminiscent of ecological succession. Following transfer to fresh media, 'wrinkly' morphotypes act as early surface colonizers, followed by the subsequent growth of 'ruffled' types, and then 'studded' morphotypes grow over-top the other two. Each type also distributes differently within the biofilm structure, 'ruffled' cells adhere well to the surface and spread sideways whereas 'wrinkly' cells adhere and grow mostly vertically. Interestingly, the total yield of diverse communities of all three types is higher than that expected from their combined yield when grown in pure culture, indicating that coevolved communities can be more productive.

The Genetics of Adaptive Radiation

Missing from the ecological theory is an explanation of how divergence in phenotype and ecology occurs at the genetic level and whether there are any general patterns and mechanisms that drive this process. Any genetic theory of AR must provide an explanation for how a lineage that gains access to novel ecological opportunities diversifies into ecologically distinct specialists from a founding lineage whose population size is likely to be small initially. The small size of the population is a consequence of it being mal-adapted to prevailing conditions, making it susceptible to extinction and limiting the amount of genetic variation accessible to selection. Models for the evolution of novel gene function, either through the modification of existing genetic material through mutation or the acquisition of novel (to the lineage) genes through horizontal gene transfer, provide a helpful starting place to explain how AR occurs at the genetic level.

Modification of Pre-Existing Genes

New genes and genetic functions are thought to evolve through a three-step process involving exaptation, amplification, and divergence (EAD). Most enzymes are exapted to some degree, meaning their native function also allows some fortuitous side-functions to be performed that allows a lineage to at least persist in a novel environment. Increases in fitness, and so population size, are caused by amplifying the production of limiting enzymes through either gene duplication or regulatory mutations that result in constitutive expression. The additional genetic material resulting from gene duplication and/or the expansion of the population allows functional divergence leading to niche specialization. A somewhat more complex version, the PEAD model, posits an additional step before exaptation called potentiation (the P in PEAD). Potentiation is the evolution of a genetic background that allows a lineage to access genetic variation relevant to the novel ecological conditions. Most lineages that undergo AR are likely to be 'pre-potentiated' in the sense that the genetic variation required for diversification is readily accessible when ecological opportunities become available. Key innovations, traits that contribute to diversification, may be the result of potentiation that occurs long before the ecological opportunities allowing diversification become available.

In the *P. fluorescens* AR, the transition from SM to WS involves often just a few nucleotide changes that cause loss-of-function mutations in one of several operons (*wsp* 7 genes; *aws* 3 genes; or *mws* 1 gene) that comprise the first of a two-component signal transduction pathway allowing the cell to sense and respond to environmental signals. That these operons are present in the genome means they are, in effect, exaptations. Mutations in these operons, the most common being *wsp*, cause amplification by constitutively expressing the signaling molecule c-di-GMP and causing a downstream operon, *wss*, to produce a cellulose-like polymer that is the key structural component of the WS biofilm. Functional divergence occurs because growth as a biofilm is traded-off against growth in the broth. The fact that several operons that influence the expression of c-di-GMP can be targeted helps explain the high repeatability of WS morphotype evolution in this system. Moreover, the fact that just one loss-of-function mutation is sufficient to cause the transition from SM to WS suggests the founding lineage is pre-potentiated and explains why this AR occurs so rapidly.

Horizontal Gene Transfer

The asexual nature of bacterial reproduction ensures that primary mode of inheritance is vertical, from parent to offspring. Nevertheless, novel genetic functions can be transferred 'horizontally' between lineages as well through conjugation (physical connections between bacterial cells), transduction (phage- or vesicle-mediated transfer), and transformation (uptake of extracellular DNA). Horizontal gene transfer (HGT) is known to be an important mode by which novel gene function can be acquired by a

lineage, as the spread of antimicrobial resistance on plasmids attests. However, the importance of HGT in generating ecological divergence associated with AR has been little studied in laboratory experiments, although, as we shall see in an upcoming section, it may be important in driving AR in nature.

Divergence via Niche Specialization

Fitness trade-offs across environments are the hallmark of niche specialization. Trade-offs evolve because selection is effectively 'blind' to the fitness effect of a mutation in any environment other than the one in which it is selected. Since many genes can have environment-specific effects, the increase in fitness in the environment of selection will usually exceed that in any other environment, resulting in specialization. Two genetic mechanisms can cause environment-specific effects: antagonistic pleiotropy resulting from functional interference, where a gene beneficial in one environment is deleterious in another, and mutation accumulation, where genes neutral in the environment of selection disrupt function in alternative environments. The rapidity of most ARs suggests that the niche specialization that evolves is most often underlain by antagonistic pleiotropy, as the WS example from the *P. fluorescens* radiation illustrates.

We can formalize the contribution of divergent selection to the evolution of niche specialization by extending Fisher's geometric model of adaptation, which sees adaptation as a series of mutation-driven steps in multidimensional trait space towards an optimal combination (the fitness optimum), to multiple optima (i.e., – environments; Fig. 2). In populations far from both optima, mutations that have beneficial effects in both environments are likely to be selected and fixed. As adaptation occurs to one environment, it will bring the lineage closer to its local optimum and farther from the alternate optimum, leading to specialization. Antagonistic pleiotropy leads to specialization when the lineage is located between two optima, since progress towards one optimum necessarily takes it further away from the other. Schick et al. (2015) tested this model by measuring the fitness effects of all mutations that arose in *P. fluorescens* during selection in three distinct carbon sources. Genome sequencing showed approximately three mutations per treatment for the end populations in each carbon source (xylose $= 3.7 \pm 0.2$, mannose $= 2.7 \pm 0.8$, glucose $= 2.3 \pm 0.8$) after ~1000 generations. Consistent with the multi-optima version of Fisher's model, the pleiotropic effects of later stage mutations fixed in a focal environment were more often negative in the alternate environments than those of earlier stage mutations.

Do Laboratory Experiments Reflect Adaptive Radiation in Natural Microbial Systems?

While a growing number of examples of AR in microbes have been documented in various kinds of laboratory environments, the prevalence of AR in natural populations of microbes remains largely unknown. There is no reason why microbes would not frequently undergo AR outside the lab when ecological opportunities become available, as they might following, for example, a course of antibiotics that eliminates much of the competing microbiota alongside the targeted pathogen. However, comparative

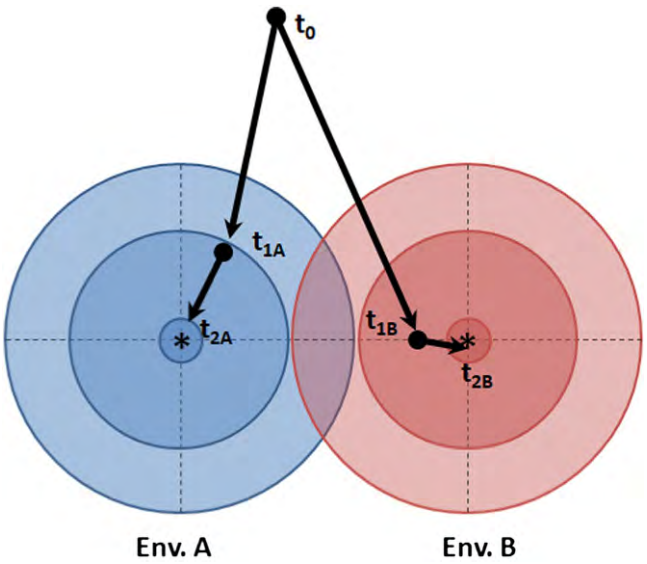

Fig. 2 Mutational steps depicted by time sequences "t". Degree of phenotypic change depicted by arrow length. Initial mutations might be more general adaptations to laboratory conditions (t_0 to t_1) and can lead to increased fitness in alternative environments (arrow moves closer to both Env. A and Env. B fitness optima '*') although fitness increase is higher in the selection environment than the alternative environment (e.g. t_{1A} in Env. A); subsequent mutations that move the population closer to the optimum in the selective environment will decrease fitness in the alternative environment (e.g., t_{2B} moves further away from Env. A optimum as it approaches the Env. B optimum).

data supporting AR in wild microbial systems is rare, and the genomic tools to distinguish between lineages among recently diverged assemblages of co-occurring bacteria are still being developed. The most compelling example of a natural AR is the work of Hehemann et al. (2016) who documented genetic and ecological divergence of marine *Vibrionaceae* into at least three types that differ in their ability to degrade alginate, a glycan derived from algae. The 'pioneer' ecotype produces enzymes that diffuse into the alginate gel, breaking down polymers into more soluble forms and small oligomers. These by-product compounds constitute ecological opportunities that are exploited by two additional ecotypes, the 'scavengers' and 'harvesters'. 'Scavengers' are cheats that cannot degrade alginate directly and instead take up the small oligomeres produced by pioneers. 'Harvesters' degrade larger alginate polymers, like pioneers, but their enzyme is attached to the cell rather than simply released into the surrounding media. Harvesters can also use small oligomeres like scavengers. Interestingly, the lack of broadcast enzymes in harvesters means that they can capture more of the products of their enzymatic activity for themselves but at the cost of slower growth relative to pioneers, a trade-off that likely is important in supporting diversity in this system. Unusually, the genetic components allowing ecological differentiation seems to have evolved via extensive HGT rather than mutation-driven modification of pre-existing gene function. This observation suggests that we may need to refocus attention on the role of HGT in microbial ARs.

Another approach is to ask if laboratory AR experiments can recapitulate the spectrum of diversity observed in natural microbial populations. Schick and Kassen (2018) have demonstrated that, in one case at least, the answer is yes. The opportunistic pathogen *Pseudomonas aeruginosa* is known to diversify rapidly following colonization of the airways of cystic fibrosis (CF) patients into a range of phenotypic and genetically distinct lineages in a way that bears a striking resemblance to an AR. Schick and Kassen (2018) reasoned that the CF airway represents an ecological opportunity for a colonizing *P. aeruginosa* strain because of the spatial structure associated with different lung compartments, reduced dispersal due to the thick mucous layer in the lung lumen, and the rich spectrum of nutrients available. In an experimental test of this hypothesis, the authors showed that *P. aeruginosa* strain (Pa14) diversified rapidly (within ~220 generations) in environments that resembled the nutrient profile of the CF lung but not in simpler environments containing just a single carbon source. Importantly, diversification was accompanied by trait changes that recapitulate those often seen in isolates from CF patients, including increased biofilm formation, a loss of motility, and a reduction in some virulence factors. Given that these experiments were done in the absence of an active immune system and competing microflora that often characterize the CF lung, the results suggest that nutrient complexity is an ecological opportunity capable of driving *P. aeruginosa* diversification in the CF lung. Whether this diversity also contributes to the formation of chronic infections remains to be seen.

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Adhesins During Infection

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Introduction: The Function of Adhesion During Infection

Just as our macroscopic world is not static but in constant movement and flux, this is even more apparent at a microscopic level, where Brownian motion, the laws of mass action and forces such as shear and sedimentation guide the movement and fate of microbes.

In the context of the human host, bacteria can only succeed in colonizing and establishing an infection if they manage to overcome the body's attempt to remove them. In doing so, they have to resist mechanical forces associated with the body's physical clearance mechanisms in the form of fluid movement, including gastrointestinal movements, blood flow, or bladder filling and emptying, or air movement, including coughing and sneezing. While secretory shear stresses, such as in the intestine, are generally low (0.5–5 dynes cm^{-2}), and blood flow generates moderate shear stresses (between 15 and 60 dynes cm^{-2} in human arteries), some clearance mechanisms generate intense forces. The act of coughing, for example, can generate airflow rates of more than 500 L min^{-1}, producing shear forces of up to 1700 dynes cm^{-2}. It is intuitive that, to withstand removal by these physiological processes, microbes have to adhere efficiently to host cell surfaces in order to colonize tissues. However, in addition to providing adhesive forces counteracting bacterial removal, the process of adhesion plays a number of roles during colonization and infection that are perhaps less intuitive: First, the sensing of physical forces and chemical features of adhesive surfaces deliver a unique set of environmental cues to the adherent microbe, allowing the organism to undergo an adaptive response, priming it for colonization and infection. Second, direct contact to host cell surfaces is often required to deploy secreted effectors and toxins that play important roles during pathogenesis. Third, adhesins can, in some cases, directly modulate host cell signaling in a way that facilitates bacteria-host interactions. We outline examples for each of these cases below, and refer readers to a recent, review for a more comprehensive discussion of these mechanisms.

Surface Sensing and Adhesion Lead to Bacterial Adaptations Facilitating Infection

Phenotypically, motile and sessile bacteria are distinct, and there are further adaptions that can be observed when comparing sessile bacteria on abiotic surfaces to those colonizing host tissues. So, how do bacteria distinguish between these environments to adapt their physiology? When a bacterium first encounters a surface, features of its cell wall, such as flagella, fimbriae and curli (Table 1 gives an overview of different pilus structures), act as probes that perceive both chemical and mechanical cues. Flagella have been implicated in initial surface sensing in a number of systems, although the sensory mechanisms involved are still not fully characterized and seem to differ between species. Type I and type IV pili also play a role in transitions to a sessile lifestyle upon initial attachment. Differences in pilus tension during retraction may transmit information about surface properties, resulting in changed levels of secondary messengers, such as cAMP and cyclic-di-GMP, and thus changes in transcriptional programs. The phenotypic features affected by surface sensing are specific to each microbe, but commonly include pathways involved in strengthening adhesion and optimizing nutrient salvaging within the host, or induction of secretion systems and toxins.

Contact-Dependent Secretion Systems Require Intimate Cell Contact

Many microbes use bacterial effectors to modulate host cell signaling in a way that promotes bacterial infection, for example by triggering or dampening inflammatory responses, manipulating intracellular trafficking pathways, or cytoskeletal dynamics. Most are deployed through specialized secretion systems which rely on direct host cell contact in order to function. Type three secretion systems (T3SS) for example, consist of a hollow channel and a tip, which forms a pore in the host cell membrane upon contact, allowing passage and direct transfer of effector proteins from the bacterial cytoplasm into the host cell. Although effector secretion can be artificially induced in some cases, effector translocation into host cells and full effector function are strictly contact-dependent. Due to requirements for high local concentrations to exert full activity, secreted toxins can show similar contact-dependence.

Adhesins as Modulators of Host Signaling During Infection

In addition to facilitating the translocation of bacterial effectors into host cells, adhesins themselves can act as "extracellular effectors" and can modulate host signaling processes to facilitate infection by binding to surface-exposed host receptors. Examples of this include integrins, which regulate diverse cellular functions, such as cell-substrate interactions and inflammatory responses, and are coopted by bacterial adhesins such as Yersinia invasin or FimH of uropathogenic *E. coli* (UPEC) to achieve bacterial invasion and spread. Another example is the V. parahamolyticus adhesin MAM7, which modulates GTPase-driven host cytoskeletal remodeling and ultimately bacterial transmigration by engaging the host membrane.

Table 1 A brief overview of pilus structures, their assembly and function in Gram-negative and Gram-positive bacteria

	Gram-negative chaperone-usher pili (fimbriae)	*Gram-negative type IV pili*	*Gram-negative curli*	*Gram-positive pili*
Prominent examples	Type 1 pili in UPEC, P pili in Pseudomonas	Pseudomonas PAK pilus; V. cholerae TCP	*E. coli* and *Salmonella curli*	*C. diphteriae* and Lactobacillus Spa pili
Morphology	Rigid helical OM anchored structure, 1–2 μm in length	Flexible, sometimes retractable structure, both IM and OM anchored, 1-several μm in length	Coiled, amyloid structure; 1–2 μm in length	Flexible rod, 0.3–3 μm in length
Subunits	1 major pilin forming the helical body; 3–4 different minor pilin subunits	>10 and up to 30 different subunits	2 curlins: major (amyloid forming) CsgA, minor (nucleator) CsgB, 4 assembly factors (CsgCEGF)	2–3 pilins, 1–4 assembly factors
Biogenesis	Through the chaperone-usher pathway (Sec- dependent type V secretion); noncovalent assembly through polymerization of >1000 pilin subunits into a helical body; capping by minor pilins and an adhesive tip protein	Type II secretion system	Extracellular nucleation-precipitation (type VIII secretion) of the major pilin, CsgA, into a cross-β-structure	Sec-dependent secretion, folding and membrane insertion of pilins by the thiol-disulfide oxidoreductase MdbA, sortase-dependent polymerization (covalent cross-linking) and anchoring of the pilus to the peptidoglycan layer
Adhesin	Lectins: e.g., FimH (type 1 pili), PapG (P pilus)	Both major and minor pilins are adhesive, but only distal subunits are functional adhesins	CsgA	Lectin-like tip adhesin (e.g., SpaC)
Host receptors	Mannosylated glycolipids and glycoproteins	Asialo-GM1, asialo-GM2 glycolipids	Extracellular matrix proteins (e.g., fibronectin, laminin, plasminogen)	Glycoproteins (e.g., mucin)

Throughout this chapter, we will discuss a variety of examples for specific adhesion/receptor pairs, highlighting mechanisms of adhesion from a biochemical perspective as well as downstream consequences of adhesion on regulation of host and bacteria cellular function and how these phenotypic changes facilitate colonization and infection. We will herein focus on bacterial adhesion to the human host and refer the reader to other sources for adhesion to abiotic surfaces or viral and protozoan adhesion mechanisms.

We attempted to organize the chapter by cell types, rather than bacteria, to highlight preferred targets for bacterial adhesion within a tissue. We think this will highlight how microbes have at times evolved common strategies to target a certain tissue, while at other times they purposefully target distinct receptors within a tissue to achieve different outcomes (invasion versus evasion, attack versus subversion, etc.). At the same time, we will try to point out that, while some host receptors are distinct, there is some overlap in receptors, or more generally, functional groups recognized by specific adhesins, in different cell types. Our understanding of these shared and distinct features and mechanisms of adhesion-receptor interactions helps to make sense of key characteristics of infectious agents, such as mode of infection, host and tissue tropism, mechanisms of spread and disease symptoms, and ultimately, ways to prevent and treat infections.

Epithelial Cells

Epithelial cells, such as keratinocytes, barrier epithelial cells, or brush border cells, form a protective lining on many body sites, including the skin, respiratory tract, urogenital tract and gastrointestinal tract. Beyond their role as a physical barrier, they fulfill important roles in relaying information derived from environmental cues that result in physiological responses, such as assimilation, secretion and immune regulation. Glycoproteins are abundant on epithelial surfaces, and the O-linked glycans within them exhibit a tremendous variety both in terms of their chemical identity and their sequence and structure. As such, they are frequently coopted as adhesion receptors by both fimbrial and nonfimbrial lectin-like bacterial adhesins. Below we will discuss the spectrum of their binding modalities and the role this plays in determining tissue and host tropism.

Bladder Epithelium

The bladder epithelium lines the urinary bladder, a hollow muscular organ that collects urine from the kidneys and regularly purges it. This creates a dynamic force environment, with regular changes between quasi-static and high-shear stress conditions and pH

ranging from 4.8–8.0. One of the best characterized bacterial adhesins, the adhesive tip protein FimH located at the tip of type I pili, is a key determinant of bacterial adhesion to the urothelium. Type I pili are wide-spread across Gram-negative bacteria. These pili are assembled via a dedicated chaperone-usher pathway consisting of a periplasmic chaperone that delivers pilin subunits to the outer membrane usher protein, which assembles them into a filamentous structure protruding from the bacterial cell surface. FimH consists of a C-terminal pilin domain required for polymerization and assembly, and an N-terminal jelly-roll domain with lectin function. FimH specifically recognizes D-mannosylated receptors, which allows binding of uropathogenic *E. coli* (UPEC) to uroplakin 1a, a glycoprotein expressed on the urothelium displaying high levels of terminally exposed mannose residues (located on Man(6)GlcNAc(2) to Man(9)GlcNAc(2)). FimH forms a unique catch-bond with mannose and undergoes conformational changes under high shear stresses, increasing the affinity for its receptor and allowing bacteria to withstand physical clearance upon urine purging. Uroplakin 1a is a tetraspanin (TSPAN21) associated with membrane lipid rafts, and UPEC have been shown to invade bladder epithelial cells in a caveolin and raft-dependent manner. FimH also cooperates with pathovar-specific pili, such as CF (colonization factor) pili of enterotoxigenic *E. coli* (ETEC), to facilitate colonization of the intestinal tract, and the fim operon encoding for type I pili is upregulated during GI tract infection.

Kidney Epithelium

UPEC encode for a second type of fimbrial adhesin, PapG on the tip of P pili. P pili are largely analogous to type I pili and are also assembled by the chaperone-usher pathway (Table 1). They are encoded by the pap locus. In contrast to FimH, PapG specifically recognizes galabiose containing glycosphingolipids, primarily found on the kidney epithelium, and has a shear-sensitive slip-bond, adapted to the low-shear environment in the kidneys and allowing for better bacterial dissemination.

Intestinal Epithelium

The intestinal epithelium is made up of enterocytes, which are the most prominent cell type and function mainly in nutrient absorption, as well as different types of secretory cells, including goblet cells (mucus secreting), enteroendocrine cells (hormone secretion), paneth cells (antimicrobial peptide secretion) and cells with immunomodulatory functions (e.g., M cells, Tuft cells). Below, we describe only adhesive mechanisms ascribed to cell-associated receptors, while a whole subsequent section is dedicated to adhesion to secreted mucins. Although most intestinal bacteria are restricted to the intestinal lumen and loose mucus layer covering the intestinal tract, some bacteria are capable of migrating through or degrading the mucus layer and adhering to the underlying intestinal epithelium. As is the case with other epithelial cells, this adherence is largely mediated by interactions between bacterial lectins and glycosylated host receptors. Enteroaggregative *E. coli* (EAEC) produce aggregative adherence fimbriae (AAF), which assemble via the chaperone-usher pathway and belong to the Afa/Dr. family of adhesins. Their host receptor is the epithelial transmembrane mucin Muc1, and sialoglycosylation of Muc1 is important for AAF adherence. Adherence of EAEC to Muc1 induces host cellular signaling which increases Muc1 expression, leading to positive feedback for bacterial adherence. On the other hand, the AAF-Muc1 interaction also increases pro-inflammatory signaling and enhances neutrophil recruitment and migration across the epithelium. Salmonella express a nonfimbrial lectin-like adhesin, SiiE, which recognizes *N*-acetyl-glucosamine and α2,3-linked sialic acid on intestinal epithelial cells. SiiE is secreted in a type I secretion system-dependent manner, contains 53 repetitive bacterial immunoglobulin (BIg) domains, and with 595 kDa represents the largest protein expressed by Salmonella. Recent studies by Kohler and colleagues have demonstrated the receptor for cholera toxin B subunit adhesion involves binding to glycoproteins, not proteins, and that fucosylated structures promote entry of cholera toxin into colonic epithelial cells.

Gastric Epithelium

The gastric epithelium is composed of columnar and a variety of secretory epithelial cells, overlaid by a mucus layer. Together, the secretions of the gastric epithelium create a pH gradient, ranging from extremely acidic conditions (pH ~1.5) in the gastric lumen, to a neutral pH at the epithelial surface. Helicobacter pylori, which infects half the world's population and is a leading cause of gastric ulcers and gastric cancers, adheres to the gastric epithelium via the mucin Muc5AC. Approximately 20% of *H. pylori* adhere directly to the gastric epithelium, while the residual population colonizes the mucin layer. They do so via a range of adhesins, including the autotransporters LabA, which recognizes a GalNAcβ1–4GlcNAc motif in Muc5AC, and BabA, which recognizes terminal Fuc, GlcNAc and Gal residues of Lewis(b) antigens on Muc5AC. In contrast to other lectins, such as FimH or PapG, no structural changes are induced upon carbohydrate binding. Instead, binding of BabA is pH sensitive; binding is strong at neutral pH conditions close to the epithelium but is of low affinity under acidic pH conditions, meaning *H. pylori* can escape upon shedding of mucus or cells into the gastric lumen. Interestingly, *babA* expression can be transiently lost, either by phase variation or by gene conversion, in which the paralog *babB* recombines into the *babA* locus. It is not known what selective pressures drive these expression changes, since it is independent of binding to Lewis(b) antigens, but they are recognized as a mechanism to adapt to host-specific variations in the intensity of pro-inflammatory responses to gastric infection. Not all adhesins mediating attachment to epithelial cells rely on lectin-carbohydrate interactions. *H. pylori* HopQ, for example, uses host receptors of the carcinoembryonic antigen-related cell adhesion molecule family (CEACAMs). HopQ specifically binds to the N-terminal IgV-like domain of CEACAM1, CEACAM3, CEACAM5 or CEACAM6 proteins, thereby enabling translocation of the major *H. pylori* virulence factor CagA into gastric epithelial cells. The HopQ-CEACAM interaction is characterized by a remarkably high affinity (K_D from 23 to

268 nM), which is independent of CEACAM glycosylation. HopQ deletion reduces CagA translocation, and inflammation and colonization in vivo.

Keratinocytes

Keratinocytes are epidermal-derived epithelial cells, which permanently exit the cell cycle and migrate to the epidermal surface, where they fulfill their function as the body's outermost barrier against environmental insults. They distinguish themselves from other cell types by the expression of keratins, which are often used as receptors by bacterial adhesins. The *S. epidermidis* serine-aspartate repeat (Sdr) family adhesin SdrF is a multi-ligand MSCRAMM (microbial surface components recognizing adhesive matrix molecules) and binds both keratin 1 and 10 (via it's A and B domains), and α1 and α2 chains of type I collagen (via its B domains). Group A Strep, such as S. pyogenes, produce sortase-dependent cell wall-anchored pili displaying a minor adhesive pilin, Spy0125, that binds keratinocytes in a very distinctive, covalent manner. Spy0125 is a four-domain protein, comprised of two Ig-type CnaB domains and two thioester domains (TEDs). The folded adhesin forms an intramolecular thioester bond, which enables the protein to distinguish between binding of soluble and surface-immobilized ligands: Binding to soluble amine containing ligands, such as histamine, leads to attachment of the thioester via the ligand's amine and formation of an intermolecular isopeptide bond. This bond, however, is extremely transient, since solute binding does not exert force on the adhesin and the Spy0125's cysteine, which remains in close proximity, rapidly reforms the thioester bond and rejects the ligand. In contrast, binding of a surface-attached amine containing ligand, such as a host surface protein, distorts the adhesin upon covalent binding, leaving the cysteine unable to re-establish the original thioester and making the interaction extremely stable.

Mucus and Mucin-Producing Cells

Mucins are high molecular weight glycoproteins produced by certain subtypes of epithelial cells that are specialized in exocrine secretion and produce the mucus layer. To date, 21 different mucins have been identified—seven of them (including the adhesin target Muc1 mentioned above) are integral transmembrane proteins, while the rest are excreted. Excreted mucins are the main structural components of mucus, a highly hydrated gel consisting of structural proteins (mainly mucins), salts, and antimicrobial enzymes and peptides. Mucus is a continuously turned-over layer covering the gastrointestinal, respiratory, and urogenital epithelium, and serves both as a protective barrier keeping microbes at a distance from the epithelial surface, as well as a diverse niche supporting microbial growth. The entire gastrointestinal tract is covered by a protective mucus layer, but its composition and thickness vary dramatically. While the stomach mucus layer is approximately 1 mm thick in humans, the mucus layer is thinner in the intestinal tract, with an average thickness of 100–150 μm in the colon and rectal region.

Muc5, Muc7, and Muc19 are the predominant structural salivary mucins, and play an integral role in protection of the oral cavity against colonization by cariogenic bacteria. *Streptococcus mutans*, the main agent of tooth decay, produces several surface glycoproteins (e.g., the serine-rich repeat (SRR) proteins GspB and human salivary antigen, Hsa) which in the absence of a capsule can mediate adhesion to salivary mucins and mucin-mediated bacterial aggregation, keeping the bacteria from adhering to the enamel. The interactions are mediated by sialoglycan modifications on the mucins: While GspB selectively binds sialyl-T antigen, Hsa displays broader specificity, including to sulfated glycans. Sialoglycan binding is mediated by a conserved domain that is structurally similar to the glycan-binding module of mammalian Siglecs (sialic acid-binding immunoglobulin-like lectins), including an arginine essential for sialic acid recognition.

The mucus layer of the stomach consists mainly of Muc5 and Muc6. BabA mediates binding to Muc5 both on epithelium associated mucin and shed mucus and has low affinity at low pH conditions (as discussed above). *H. pylori* also expresses a sialic acid binding adhesin (SabA), and the contribution of SabA to *H. pylori* adhesion increases in gastritis. While intimate association of *H. pylori* with the gut epithelium is necessary for translocation of type IV secreted effectors, such as CagA, into host cells, sensing of mucins by *H. pylori* creates positive feedback by upregulating expression of BabA and CagA. There is also evidence that *H. pylori* infection changes the glycosylation pattern of infected individuals displaying intestinal metaplasia while asymptomatically infected individuals had glycosylation patterns similar to uninfected controls, suggesting that *H. pylori* and the human host are engaged in a tug-of-war resulting either in alteration or adaptation of gastric mucus upon infection.

Intestinal mucus consists of two structurally distinct layers, an inner, epithelium associated layer and an outer luminal layer that is looser, more hydrated and subject to rapid turnover (in the order of 4–6 h). The structural mucin Muc2 forms the basis of the mucus layer covering the small and large intestine and is produced by goblet cells; goblet-, mucosal and absorptive epithelial cells synthesize the apical membrane-bound mucins Muc1, Muc3, Muc4, Muc12, Muc13, and Muc17. While the inner layer is devoid of microbes in healthy individuals, the outer mucus layer provides the main adhesive substrate and a growth niche for the intestinal microbiota. As such, most intestinal commensals express adhesins that recognize mucins.

One of the best characterized mucus adhesins in commensal bacteria is the canonical mucus binding protein MUB expressed by Lactobacilli, such as *L. reuteri*. MUB is a ~350 kDa modular protein forming extended fibrils and consists of an N-terminal domain of unknown function and 14 Mub1 and Mub2 type repeats at the C-terminus involved in mucin binding. Although both types have divergent sequences, their overall structures are well conserved. Binding of Mub domains to mucin is mediated by individual interactions by multiple Muc repeats and terminal sialoglycan modifications on mucin. Gram-positive pili (Table 1) also adhere to mucins via the adhesive pilus protein, SpaC. *Lactobacillus rhamnosus* express 10–50 pili per cell, with lengths up to 1 μm. Their major

structural component is SpaA, while SpaB and SpaC are minor pilus components integrated into the pilus at a ratio of 5:1:2 for SpaA:B:C. Unlike in Gram-negative pili, SpaA, B, and C are covalently cross-linked via isopeptide bonds giving them exceptional mechanical strength, while the Ig-type CnaA domains within individual pilins allow the pili to dissipate mechanical shocks without breaking. SpaC contains additional adhesive domains, including a von Willebrand factor A (vWFA) domain, and SpaC is found over the entire pilus length as well as a tip protein. Interactions between SpaC and mucin are likely mediated by lectin-glycan interactions; this is supported by the fact that SpaC itself is glycosylated and also mediates cell aggregation through homophilic recognition. While individual SpaC molecules display fast dissociation from mucin, multiple SpaC molecules along the pilus engage mucin in a zipper-like fashion, allowing bacteria to dynamically explore their niche but also to persist in the mucus layer. Enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are a main cause of diarrheal disease as a result of food-borne infection in the Western world or in children without access to sanitized water, respectively. Both EHEC and EPEC adhere to Muc2 and adhesion depends on the presence of mucin-type core 2 O-glycan. Muc2 secretion is a double-edged sword for the host: On the one hand, Muc2 is a receptor that enables EHEC and EPEC attachment. On the other hand, bacterial adhesion to Muc2 also acts as a decoy keeping epithelial surface colonization at bay, and in the absence of Muc2, EHEC or EPEC exposure leads to high host morbidity and mortality. Both types of infection have the potential to stimulate mucin overproduction. EHEC adherence to intestinal epithelial cells stimulates the production of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-8), which stimulates Muc2 expression and enhances EHEC clearance. However, the translocation of EHEC T3SS effectors into epithelial cells attenuates inflammatory signaling through ERK1/2 and PI3K/Akt, which partially counteracts Muc2-mediated clearance. Some atypical EPEC strains also induce hyperexpression of mucins, particularly the secreted Muc2 and Muc5 as well as the membrane associated Muc3 and Muc4, which act as receptors as well as growth substrate for those strains.

Several adhesins have been described in EHEC and EPEC but recently, the multivalent adhesion molecule (MAM) YebT expressed by commensal as well as pathogenic *E. coli* was shown to recognize 3-O-sulfogalactosyl moieties on host receptors, including Muc2. Interestingly, sulfatase production by some commensals such as *Bacteroides thetaiotaomicron*, depletes sulfoglycan modifications on mucin, thus making the mucus layer more permeable to *E. coli*. Enterotoxigenic *E. coli* (ETEC) express several adhesins that enable them to bind mucins, including the conserved two-partner secreted adhesin EtpA that localizes to the tip of ETEC flagella, creating a bridge between the bacterial surface and its host receptors, Muc2 and Muc3. EtpA recognizes a conserved, GalNAc containing glycosylation pattern on Muc2 and Muc3. Hypoglycosylation in a glycosyltransferase deficient host background producing only core but not extensively glycosylated mucins led to decreased ETEC colonization of the small intestine. Interestingly, secretion of the heat-labile toxin, LT, by ETEC increases Muc2 secretion, creating positive feedback and increasing colonization.

Extracellular Matrix and Connective Tissue

The extracellular matrix (ECM) is an interstitial substance present in all tissues and organs. Although its exact composition and properties vary depending on its location within the body, it is generally composed of fibrous proteins, such as fibronectin, collagens, laminins, and proteoglycans. The ECM is secreted by the cells it surrounds, including fibroblasts and osteoblasts, and is attached to them via ECM receptors, such as integrins and syndecans, which enables feedback between ECM properties and cell functions, including adhesion, migration, proliferation, and differentiation. The ECM is a key site of bacterial adhesion, and interactions between bacterial adhesins and ECM components are the most extensively characterized in adhesin biology. Adhesins that are not lectins, and recognize one or more ECM proteins by means of protein–protein, rather than protein–sugar interactions, are referred to as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). There are also many adhesins that classify as lectins, and recognize glycopeptides or glycans within ECM proteins and/or ECM proteoglycans. Glycan epitopes occurring on multiple ECM components may partially explain the multiligand binding specificities observed with some bacterial adhesins. This is the case with some Gram-negative adhesins belonging to the family of multivalent adhesion molecules (MAMs), such as the *E. coli* HS MAM YebT, which recognizes the ECM proteins laminin, fibronectin and collagen IV, among other ligands, via a shared 3-O-sulfogalactosyl moiety.

Fibrinogen (Fgn) is a glycoprotein found mostly in plasma, but also deposited as fibrin in the ECM, where it serves a role in scaffolding. The *S. epidermidis* protein SdrG is a cell wall anchored protein that binds the β chain of Fgn via a dock, lock and latch mechanism. SdrG consists of N-terminal N domains (N1–3) responsible for Fgn binding, and C-terminal serine aspartate repeat (Sdr) region. While the N1 repeat appears to be proteolytically cleaved in vivo, the N2 and N3 repeats, which each assume a DEvariant-IgG fold, bind the fibrinogen polypeptide chain and lock it in place with the C-terminal extension of N3, which assumes a β-strand conformation and extends towards the N2 subdomain where it acts like a latch by complementing one of the β-sheets in N1. The staphylococcal proteins Clumping factor (Clf) A and ClfB are similarly organized, as are the A regions of the multi-ligand MSCRAMMs FnbpA and B (see below), but differ in terms of the epitope they target on fibrinogen. ClfA, for example, interacts with the γ chain of Fgn by a dock, lock and latch mechanism, but also binds residues in the Fgn D domain at a distinct site located in N3. ClfA has been implicated as a major virulence factor in *S. aureus* infection models of septic arthritis and infective endocarditis.

Collagens are the main constituents of extracellular matrices and most abundant protein in mammals, with 28 types of collagen described to date and giving rise to matrices that vary widely in terms of their stiffness, from rigid structures, such as bone, to compliant structures, such as tendons. One of the best characterized collagen binding proteins is *S. aureus* CNA, which consists of a ligand binding N-terminal A region (IgG-like domains N1–3), followed by 1–3 N-terminal repeat motifs (B1–3) and a sorting signal. CNA binds collagen I using a variant of the dock, lock and latch mechanism called the "collagen hug," whereby the N1 and

N2 domains are connected via a long loop and cooperate to wrap around and "hug" the rope-like structure of a collagen monomer, and the C-terminal extension of N2 forms a β-strand that complements one of the β-sheets in N1 and locks the bound collagen in place. CNA is required for *S. aureus* adhesion to cartilage and has been identified as a virulence factor in staphylococcal infections including arthritis, endocarditis, osteomyelitis, mastitis and keratitis. The repetitive B region functions as a spring, projecting the A region away from the cell surface and sustaining adhesion under high mechanical forces.

Fibronectin (Fn) is deposited in the form of fibrils and forms a key structural component of the ECM but also occurs as a soluble plasma protein. *S. aureus* expresses two structurally related fibronectin binding proteins, FnbpA and FnbpB. The C-terminal domain contains tandem repeats (FnBRs) that recognize the type I modules at the N-terminus of Fn via a tandem β-zipper mechanism. In their unbound state, FnBRs are intrinsically disordered, but bound to Fn, they form β-strands which extend the antiparallel β-sheets of adjacent Fn type I modules. The repetitive nature of both type I modules and FnBRs means this binding mechanism leads to a unique stabilized fold (the β-zipper) and exceptionally high binding affinities. This domain arrangement and mode of binding is also conserved in a range of other adhesins, including the streptococcal adhesins SfblI/F1, SfbII, Sof22, and F2, *Borrelia burgdorferi* Fn-BA and others. Binding of Fn by *S. aureus* FnBRs also allows the bacteria to interact with the α5β1 integrin, which initiates bacterial invasion of host cells. Recent work on the interaction of *S. gordonii* adhesin CshA with Fn revealed a two-state catch-clamp binding mechanism distinct to the β-zipper. Fn binding is associated with two regions (NR1 and NR2) localized in the nonrepetitive CshA N-terminal domain. NR1 is an intrinsically unstructured region and its interaction with Fn is highly dynamic, while NR2 is a discrete Fn-binding domain with a lectin-like fold and high affinity for Fn, suggesting a bipartite, cooperative mechanism involving a low affinity but high capture radius domain (catch) and a high affinity interaction with a carbohydrate binding domain (clamp). The N-terminal A region of Fnbps contains three independently folded N domains (N1–3) with divergent binding specificity depending on protein variant and strain, but all also bind fibrinogen via a dock, lock and latch mechanism. This organization and mechanism of fibrinogen binding is shared with the *S. aureus* clumping factor A (ClfA). In contrast to ClfA, Fnbp N domains can also bind elastin, which is important for connective tissue colonization, and can capture plasminogen from plasma, which plays an important role in the formation of bacteria-containing clumps and endocarditis.

MAM7 is found on the surface of many bacterial pathogens. It has been identified as a bacterial adhesion molecule that binds two ligands, fibronectin and phosphatidic acid. Using microbial genetics and various infection models, this outer membrane receptor has been shown to contribute to virulence for many Gram-negative bacteria. Further studies using a recombinant form of MAM7, demonstrated that MAM7 can be used as an antimicrobial by attenuating the binding and colonization of pathogens, including MRSAs, as they also use fibronectin for adhesion. In a recent study, it was shown that the MAM7 treatment could attenuate infections by Pseudomonas aeruginosa in a burn model without affecting vascularization or promoting an inflammatory response. Furthermore, a molecule with intermediate binding, such as MAM7, does not affect wound healing, whereas antimicrobials that bind with a much higher affinity to fibronectin, have a detrimental effect on would healing.

Laminins are high molecular weight (400–900 kDa) heterotrimeric proteins consisting of cross-linked α-, β-, and γ-chains forming a cross-like structure. They can assemble into higher-order sheet like structures and cross-link the ECM. Several laminin binding adhesins have been identified, including the *Haemophilus influenzae* autotransporter adhesin Hap, the EHEC autotransporter EhaB, and the leptospiral proteins OmpL47, Lsa24, Lsa27 and Lsa63. However, structural and mechanistic details of these interactions remain to be determined.

Elastin is a fibrous protein found in connective tissues, and forms a cross-linked highly elastic matrix than can be stretched to several times its length but recoils upon force relaxation. Several *S. aureus* proteins, including Fnbps, bind elastin, but *S. aureus* also expresses a separate elastin binding protein, EbpS, which binds both soluble elastin and insoluble fibrous elastin (tropoelastin) via its N-terminal domain. The leptospira IgG-like protein B (LigB) also binds elastin, which likely facilitates adherence to fibroblasts and lung infection. However, structural details regarding the mechanism of elastin binding by adhesins have yet to be uncovered.

Endothelial Cells

The endothelium forms the inner lining of the vascular system, including blood vessels and the heart, and lymphatic vessels. They also form a part of the blood-brain barrier and protect the central nervous system from invading pathogens. Bacteria have evolved many ways to interact with the endothelial cell surface, allowing them to modulate vascular clotting and to reach and persist in the central nervous system, including the brain, blood and lymphatic vessels, niches typically inaccessible to microbes.

S. aureus and Streptococcus species can cause vascular infections, including endocarditis and sepsis, and many receptors are involved in causing these diseases, including circulating ECM proteins that act in strengthening bacterial–endothelial adhesion and platelet receptors important for bacteria induced clotting, discussed elsewhere in this chapter. The interactions they form to specifically mediate endothelial adherence are described here. *S. aureus*'s ability to bind Fn, thereby engaging the α5β1 integrin, not only allows it to invade cells but also triggers pro-inflammatory responses in endothelial cells and positive feedback by upregulating α5β1 integrin expression on the endothelium in turn. Adhesion-promoting pro-inflammatory responses are triggered to recruit phagocytes and activate epithelial migration as part of normal wound healing, making wounded tissues susceptible to colonization. Vascular damage as a result of infection and inflammation also leads to exposure of collagen, which is readily exploited by both Staphylococcus and Streptococcus as an adhesion substrate and further increases bacterial attachment to the endothelium. Adhesins such as the *S. aureus* extracellular adhesion protein (Eap) of the SERAM (secretable extended

repertoire adhesion molecule) family exploit the inflammation triggered upregulation of the intercellular adhesion molecule (ICAM)-1 on endothelial cells by competitively blocking the adhesion to and transmigration of phagocytes through the endothelium.

CEACAMs (carcinoembryonic antigen-related cell adhesion molecules) are expressed by endothelial and phagocytic cells and involved in cell-cell adhesion. They are exploited by several pathogens including Neisseria species, which can cause vascular infections and meningitis by crossing the blood-brain barrier. Neisseria species express opacity associated (Opa) proteins, which interact with CEACAMs with nanomolar affinity, outcompeting their native interactions involved in cell-cell adhesion and triggering bacterial invasion. Opa-mediated binding to CEACAMs triggers increased CD105 (endogline) expression and activation of β1 integrin, which increases host cell adhesion and inhibits infection-induced cell shedding. The platelet endothelial cell adhesion molecule (PECAM)-1 is expressed by endothelial and phagocytic cells as well as platelets, and it is involved in phagocyte transmigration, angiogenesis and integrin activation. PECAM-1 is also recognized by Streptococcal pili, via the adhesive protein RrgA. RrgA colocalizes with its receptor PECAM-1 at the blood-brain barrier endothelium and is required for bacterial entry into the brain and development of meningitis as a result of bacteremia. Interestingly, PECAM-1 expression is downregulated following bacterial adhesion, leading to loss of PECAM-1 from intercellular junctions, increased intercellular gap formation and enhanced permeability of endothelial barriers.

Borrelia burgdorferi, a spirochete and causative agent of Lyme disease, expresses several adhesins, including DbpA and B, which both bind to glycosaminoglycans, and BBK32. BBK32 recruits soluble fibronectin via its N-terminal region and undergoes binding-induced folding into a tandem β-zipper, similar to *S. aureus* Fnbps. Under vascular shear stresses, recruited Fn extends but binds BBK32 more tightly via a catch-bond mechanism. The transfer of mechanical load along a series of Fn-BBK32 complexes on the bacterial surface allows the spirochete to maintain adherence to the vessel wall under fluid shear, as well as movement along the vasculature and extravasation.

Nerve Cells

Although bacterial infections of the peripheral nervous system are relatively rare, they are associated with high morbidity and mortality. *Mycobacterium leprae*, the causative agent of leprosy, is able to adhere to many tissues, including endothelium, macrophages, dendritic cells and myelinating Schwann cells, which wrap around and form the myelin sheath protecting the axons of motor and sensory neurons. The attachment to and invasion of Schwann cells by *M. leprae* triggers demyelination, resulting in sensory motor loss and long-term disability. Adhesion to Schwann cells was long thought to be mediated by alpha-dystroglycan and ErbB-2. Both were later shown to be associated with 9-O-acetly GD3 ganglioside, the real host receptor which is recognized by glycolipid-I on the surface of *M. leprae*. The protein Hlp binds laminin as well as the glucosaminoglycans heparin and heparin sulfate and also contributes to Schwann cell adhesion. Attachment of *M. leprae* leads to increased expression of 9-O-acetly GD3, activation of the neuregulin receptors, ErbB-2 and ERK 1/2, cell proliferation, demyelination and nerve damage. Demyelination also allows *M. leprae* to invade Schwann cells, which is necessary for proliferation and persistence of this obligate intracellular pathogen. Spirochetes such as *Borrelia burgdorferi* and *B. garinii* can also colonize glial cells and cause neuropathies. The host receptor galactocerebroside (GalCer) has been implicated in *Borrelia* adhesion via its interaction with the bacterial surface protein OspA. The sialogangliosides GD1a and GT1b and acidic glucosaminoglycans have also been identified as *Borrelia* receptors, but do not rely on OspA for binding. Adhesion to the nerve fiber modulates the amplitude and conduction velocity of the action potential, likely due to adhesion-triggered desorption of myelin bound calcium rather than demyelination.

Bacterial Adherence to Blood Cells

Blood cells include red blood cells (erythrocytes), white blood cells (platelets) and immune cells such as neutrophils, macrophages, microglial cells, dendritic cells, T and B cells. Erythrocytes are a popular system to study bacterial adhesins in hemagglutination assays and have been used in this capacity for over hundred years, little is known about their specific role in bacterial adhesion in vivo. In contrast, platelets and immune cells have been described as specific targets of bacterial adhesion and thus we will focus on these two cell types below.

Platelets

Despite their structural simplicity compared to other cells, platelets play important functions during infection, especially in infectious endocarditis as a result of bacterial infections in the oral cavity (e.g., *Streptococcus gordonii*, *Streptococcus sanguinis*, *Porphyromonas gingivalis*) and sepsis (e.g., through *S. aureus* blood-stream infection). There is a clear link between bacterial adhesion to platelets and prothrombic activation, but they are also discussed as part of the innate immune response since bacterial adhesion can trigger antimicrobial peptide secretion and enhance the activity of neutrophils and neutrophil extracellular trap (NET) formation.

Platelets carry a number of adhesin receptors on their surface, including the hemostatic integrins GPIbα and GPIIβ-IIIα, Toll-like recptors, complement receptors and the immunoglobulin (Ig) G receptor FcγRII. Bacterial adhesion to platelets occurs either via

direct interaction between adhesins and surface receptors, or indirectly via binding of adhesins to circulating plasma proteins, such as von Willebrand factor (vWF), fibronectin, or fibrinogen, which in turn are ligands of platelet receptors. The leucine-rich repeat containing glycoprotein GPIbα is an integrin involved in hemostasis and is usually involved in binding shear-activated von Willebrand Factor (vWF) to induce aggregation. Streptococcus directly binds GPIbα via highly glycosylated serine-rich repeat (SRR) proteins such as SrpA, GspB, SspA/B and the hemagglutinin salivary antigen, Has. Homologous SRRs, such as the GspB homolog SraP, are found in *S. aureus*, and disruption of SRR adhesin- GPIbα interactions has been shown to inhibit platelet aggregation and protect from infective endocarditis in vivo. Structural and functional studies have shown direct adhesion is mediated by the interaction of Siglec-like domains of SRRs with sialoglycan modifications in GPIbα. Interestingly, *S. oralis* has evolved a sialoglycan-independent SRR, Fap1, which mediates platelet adhesion, and produces neuraminidase to inhibit SRR-mediated adhesion of other Streptococcal species. Other adhesins bind to soluble vWF, which when bound to bacteria, does not require shear force as an activator to induce binding to GPIbα and aggregation. The glycoprotein GPIIβ-IIIα is also an integrin involved in hemostasis, and a receptor for both direct bacterial adhesion and indirect adhesion, mediated by adhesin binding to circulating fibronectin and fibrinogen. Several bacterial MSCRAMMs, including *Staphylococcus aureus* clumping factors ClfA and ClfB and fibronectin binding proteins FnbpA and FnbpB, as well as Streptococcus M1, bind to GPIIβ-IIIα via such indirect interactions. The *Staphylococcus epidermidis* serine-aspartate repeat (Sdr) protein SdrG binds IIβ-IIIα both indirectly via fibrinogen and directly. The *S. aureus* iron-regulated surface determinant (Isd) proteins usually scavenge heme iron, but can also directly bind GPIIβ-IIIα in the absence of plasma proteins, as can the *Streptococcus gordonii* platelet adherence protein PadA. Binding sites for directly bound adhesins overlap those required for fibrinogen binding. Bacterial adhesion via GPIIβ-IIIα induces outside-in platelet signaling via the cyclooxygenase and Tx pathways, which leads to prothrombotic activation and platelet aggregation observed in infectious endocarditis.

The immune receptor FcγRII recognizes the Fc domain of IgG, and bacteria bound by IgG can interact with platelets. Bacterial adhesion to FcγRII can cause cross-talk with GPIIβ-IIIα and outside-in signaling involved in prothrombotic platelet activation. In the case of *S. aureus* and *P. gingivalis*, bacterial adhesion to platelets via FcγRII leads to bacterial internalization, but little is known about the role of this during infections. While some view bacterial internalization by platelets as an escape-route into a replicative niche, others view platelets as a part of antibacterial innate immunity. Although platelets do not form phagolysosomes, it is thought that endosomes containing internalized bacteria fuse with α-granules, leading to bacterial clearance.

Immune Cells

Innate immune cells including neutrophils, macrophages and dendritic cells form the first line of defense against bacterial infections. They display a wide range of receptors involved in recognition and binding of pathogens, including toll-like receptors (TLRs), Fc receptors, lectins, scavenger receptors, and integrins. However, many bacterial pathogens adhere to these receptors in a way that allows them to subvert immune cell signaling, either to block phagocytic function and antibody production, or to use them as a protective niche for proliferation, persistence, and dissemination to distal colonization sites.

Yersinia species are well adapted to evade immune cell mediated clearance, allowing them to colonize lymphoid tissues. During infection of the human host, enteropathogenic Yersinia express two major adhesins, invasin and YadA, which are both implicated in adhesion to and invasion of epithelial and phagocytic cells, contributing to serum resistance. Yersinia also express several minor adhesins, including Ail, pH 6, and Pla, some of which are cryptic but play important roles in immune cell subversion in the absence of one or both major adhesins. Invasin is an autrotransporter adhesin expressed during the initial contact with the host, and has a modular architecture consisting of an N-terminal OM embedded β-barrel domain and periplasmic α-domains, and an extracellular Ig-like (BIG) domains (D1–4) capped by a lectin-like head domain (D5). D4 and D5 form a functional module that binds β1 integrins with approximately 100 times higher affinity than Fn. While this facilitates bacterial attachment and, given a high enough concentration of invasion-integrin complexes, a zipper-like invasion mechanisms, the invasin β1 interaction serves additional functions. Engagement of β1 on neutrophils, for example, triggers PI3K activation, production of reactive oxygen species, and release of chromatin, highlighting invasin's contribution to neutrophil extracellular trap formation. The trimeric autotransporter adhesin Yersinia adhesin A (YadA) and its interactions with macrophages are among the best studied in the field. YadA is composed of an N-terminal passenger domain and a C-terminal translocation domain. The N-terminus contains a distal β-roll head region that is connected to the translocation domain via a central coiled-coil stalk region. Three YadA C-termini contribute to form a 12-stranded β-barrel, which inserts into the outer membrane and exposes the passenger domain on the extracellular face of the membrane. YadA is essential for *Yersinia enterocolitica*, and YadA-deficient bacteria are avirulent in rodent infection models. YadA mediates adherence to many tissues via ECM proteins, and in the absence of tight attachment via YadA, T3SS effectors are not efficiently translocated into host cells. It also interacts with integrins via an ECM bridge, allowing it to invade immune cells. Beyond that, YadA contributes to serum resistance by adhering to the bacterial cell surface via complement factor H, which acts as a negative regulator of the alternative pathway and counteracts deposition of C9, a component of the terminal lytic complex. YadA also recruits C4 binding protein, a negative regulator of both classical and lectin pathways of complement recruitment.

Infective endocarditis caused by oral streptococci is thought to depend on interactions between streptococci via sialic acid-binding SRR adhesins and monocytes. Attachment of the *S. gordonii* adhesin Hsa to human monocytes depends on sialylation and leads to an up-regulation of the dendritic cell markers CD83, CD1a, CD86 and interleukin-12, causing differentiation into mature dendritic cells. Attachment of SRRs to monocyte-derived dendritic cells is also required for their full activation, which contributes to

disease development. The periodontal pathogen *Porphyromonas gingivalis* attaches to complement receptor-3 (CR3) via its type-1 fimbriae. Binding of the tip adhesin FimE stimulates TLR2 inside-out signaling, which induces conformational changes in CR3 leading to monocyte adhesion to the endothelium and extravasation. The proadhesive conformation of CR3 also leads to cellular invasion by *P. gingivalis* and avoidance of phagolysosomal degradation.

Targeting Adhesion to Prevent Infection

As the above-mentioned examples highlight, the surface structures, host receptors and mechanisms underpinning bacterial adhesion appear incredibly diverse, but also, in many cases, appear redundant. So, is it possible to interfere with bacterial infection by targeting adhesion? Many approaches to achieve this are currently under investigation and development. Some, particularly vaccines and therapeutic antibodies targeting adhesins, are undergoing clinical trials and show great promise as future supplements or alternatives to antibiotics in the face of rising drug-resistance. For a more comprehensive summary of current approaches we refer the reader to our recent review of antiadhesion therapy.

Targeting adhesion as a defense strategy is, however, not a human invention. Many naturally occurring molecules have antiadhesive properties associated with innate immunity against bacterial infections. A prominent example are human milk oligosaccharides and glycoconjugates, which exert a protective function against infantile diarrheal diseases through binding and neutralizing bacterial adhesins by acting as receptor decoys. Another group of human defense molecules with antiadhesive properties are collectins. While they have far-reaching activities in immunoregulation and disease modulation, they also directly impede colonization by neutralizing adhesive carbohydrates on pathogens or tissue surfaces. The surfactant protein A (SP-A), for example, is an abundantly expressed collectin that binds both UPEC and the UPEC FimH receptor uroplakin 1a in the bladder. In the absence of SP-A, host susceptibility to bladder infections is increased. There are many more naturally occurring adhesion inhibitors, including in plant-derived foods, and characterizing and harnessing these often highly complex mixtures may allow us to treat infections more effectively.

By leveraging our knowledge of adhesion structures and mechanisms it is also possible to purposefully design and produce compounds that target either a broad range or specific subset of adhesive microbes (Fig. 1). The detailed structural and functional characterization of FimH-carbohydrate interactions, for example, has led to the development of synthetic mannosides which target host colonization by fimbriated *E. coli* with high efficacy and specificity and good bioavailability. Oral administration of antibodies targeting fimbrial adhesins of ETEC has proven effective in the prevention of diarrheal disease, providing the first clinical evidence that fimbrial adhesins function as protective antigens. In addition to compounds neutralizing adhesins on the bacterial surface, compounds blocking adherence by protecting host receptors are also under investigation. For example, treatment of burn wounds with a bacteriomimetic material (MAM7) that targets fibronectin and phosphatidic acid receptors on the exposed tissue has been shown to decrease bacterial burden. Since many microbes adhere via glycosphingolipids exposed on the host cell surface, the use of GSL inhibitors to deplete these receptors from host tissues, which has been used as a last resort to treat systemic infections and osteomyelitis in patients, has led to complete convalescence. Targeting virulence rather than survival could reduce evolution of drug resistance pathogens to these applications. Although so far there are only a few examples of adhesion inhibitors undergoing clinical trials or allowed for clinical use as a last resort, the above studies highlight that this is a worthwhile area for further discovery and development, which may eventually replenish our arsenal to fight drug-resistant infections.

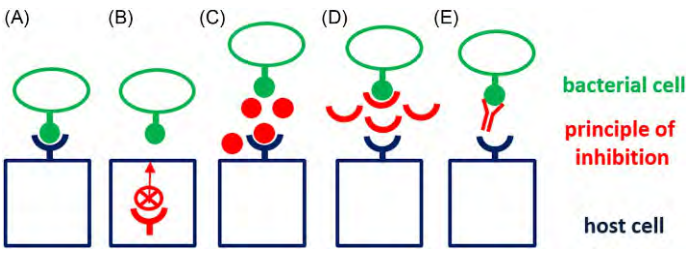

Fig. 1 *An overview of strategies used to prevent bacterial adhesion to host cells.* (A) In the absence of inhibitor, bacteria (*green*) employ surface localized adhesins to engage receptors on the host cell surface (*blue*). (B) Inhibition of biogenesis or surface display of nonessential host receptors prevents bacterial attachment, for example the use of glycosphingolipid inhibitors has been used as a strategy of last resort to treat systemic infections. (C) Host-derived or artificial molecules that bind to host receptors with high affinity competitively inhibit the binding of bacterial adhesins that share the same receptor. For example, materials based on MAM7 fragments competitively block adhesion of MAM7-expressing bacteria to host tissues. (D) Host-derived or exogenous molecules that act as host receptor decoys bind to bacterial adhesins, thereby interfering with efficient attachment to the host cell surface. For example, mannosides bind to the fimbrial adhesin FimH with high affinity, a strategy used to prevent UPEC attachment and urinary tract infections. (E) Host-synthesized or exogenous antibodies targeting bacterial adhesins can block interactions between adhesin and host receptor either through steric hindrance, or by preventing a conformational change in the adhesin necessary for high affinity binding to the host receptor.

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AIDS, Historical

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Glossary

ART (anti-retroviral therapy) Broadly defined, these include any medications active against HIV. In practice, ART usually refers to a combination of a protease inhibitor and two reverse transcriptase inhibitors that have powerful inhibitor effects against HIV replication. Also known as highly active anti-retroviral therapy (HAART).
HIV (human immunodeficiency virus) An RNA retrovirus, related to a series of simian immunodeficiency viruses, which is the cause of AIDS.
opportunistic infections `Infections that do not cause significant disease in nonimmunocompromised hosts, but can cause severe disease in people with AIDS; for example, Kaposi's sarcoma (KS) and pneumocystis carinii pneumonia (PCP).
stigmatization The process by which the stigma attached to certain behaviors, notably homosexuality and intravenous drug use, is transferred to a disease, such as AIDS, having a substantial impact on theories of, and responses to, the disease.
viral load A measure of the concentration of HIV RNA in a person's blood. It can be used to screen patients for HIV and to monitor the progress of therapy.

Abbreviations

3TC	Lamivudine
ACT UP	AIDS Coalition to Unleash Power
AIDS	Acquired immune deficiency syndrome
ART	anti-retroviral therapy
AZT	azidothymidine
CDC	Centers for Disease Control and Prevention
CMV	cytomegalovirus
ELISA	enzyme-linked immunosorbent assay
HPA-23	Heteropolyanion-23
HAART	highly active anti-retroviral therapy
HIV	human immunodeficiency virus
HTLV-III	human T-lymphotropic virus type III
IDUs	intravenous drug users
KS	Kaposi's sarcoma
LAV	lymphadenopathy virus
PCP	pneumocystis carinii pneumonia
PCR	polymerase chain reaction
PEPFAR	President's Emergency Plan for AIDS Relief
SIV	simian immunodeficiency virus
STDs	sexually transmitted diseases

Defining Statement

The appearance and rapid globalization of the AIDS pandemic demonstrate the social and economic processes that shape the distribution of diseases at present. These same processes also determine access to life-saving treatment which, as of yet, has reached only a minority of people who would benefit from it.

This article is a reprint of D.S. Jones, A.M. Brandt, AIDS, Historical, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 1–16.

Introduction

Acquired immune deficiency syndrome (AIDS), first identified in 1981 is an infectious disease caused by the human immunodeficiency virus (HIV). The virus attacks the host's immune system, causing its eventual failure. This failure leaves affected individuals vulnerable to many infections and cancers, leading inexorably to severe morbidity and high mortality. HIV emerged in the middle of the twentieth century, following the infection of humans with simian immunodeficiency viruses (SIV). Spread sexually and through blood, it penetrated populations in Africa, Europe, and the United States in the 1970s. AIDS appeared in the 1980s caused considerable fear and provoked dramatic social responses. Despite rapid progress in scientific understanding and medical treatment of the disease, and despite the existence of valuable preventive technologies, HIV continued to spread rapidly throughout the world in the 1980s and 1990s. Decisive treatments appeared in the late 1990s, triggering debates about whether or not these could be implemented on a global scale. Although dramatic progress has been made, disparities in risk of infection and in access to treatment continue to expose critical inequities in the distribution of social and medical resources in developed and developing countries.

Pathophysiology of HIV and AIDS

HIV is an RNA retrovirus with fewer than 10 000 bp. These code for three structural genes and six regulatory genes that are, in turn, transcribed and spliced in a variety of ways to produce a total of 15 proteins. The viral particles, or virions, have an icosahedral structure. Two identical strands of RNA and the virus's crucial proteins – protease, integrase, and reverse transcriptase – are enclosed within a bullet-shaped core, composed of the capsid protein (p24). This, in turn, is surrounded by the viral envelope, composed of an inner layer, made up of the matrix protein (p17), and an outer lipid layer. Seventy-two spikes, made up of proteins gp120 and gp41, protrude from this envelope. The virus can attack any cell that expresses the CD4 molecule and a coreceptor, often the chemokine receptors CCR5 or CXCR4. The virus's gp120 proteins bind to CD4, allowing the viral envelope to fuse with the cell membrane, releasing the core into the cell's cytoplasm. Once there, viral reverse transcriptase translates the viral RNA into DNA. This viral DNA is then inserted, by viral integrase, into the genome of the infected cell. The cell's own machinery then transcribes and translates the viral DNA, producing a single long protein. This is cleaved, by the viral protease, into the 15 proteins. New virions form and bud from the infected cell. A chronically infected person can produce more than 10 000 000 000 virions each day.

HIV primarily targets the CD4+ subset of T lymphocytes, known as the helper T cells, which regulate crucial aspects of the normal immune response. But any cell that expresses CD4 can be infected. Infection has been reported in macrophage, epithelial cells in the gastrointestinal and urinary tracts, astrocytes, microglial cells, cardiac myocytes, and a variety of other cell types. Infected individuals can transmit infection through blood, semen, and other genital secretions. Heterosexual transmission is responsible for most of the infections worldwide, possibly as many as 85%. However, substantial transmission has also occurred through maternal–fetal infection (during pregnancy, birth, or breast-feeding), homosexual contact, needle-sharing by intravenous drug users (IDUs), and contaminated blood products. Transmission has also been reported through organ donation, renal dialysis, artificial insemination, and acupuncture. HIV cannot be transmitted by insect bites, and it is almost never transmitted through casual contacts at home or in public.

The predominant mode of transmission varies substantially from country to country. In sub-Saharan Africa, most cases have occurred from heterosexual contact. In the United States, in contrast, roughly 45% of the cases have occurred among men who have sex with men, 25% among IDUs, and just over 10% among heterosexuals. Meanwhile, in India, transmission through drug use predominates in the northeastern states, while heterosexual transmission is most common in the south. Since modes of transmission vary so widely from place to place, public health campaigns to prevent transmission must be targeted to reflect local circumstances.

When HIV enters a body, initial infection and replication produce a surge in viral load in the victim's blood (viremia). The viremia triggers a vigorous immune response: antibodies are generated, especially against p24 and gp41, and the infection is initially contained, largely within the body's lymphoid tissues. This first stage of infection often goes unnoticed, though some individuals do experience a flulike prodromal syndrome characterized by fever, rash, and malaise. After this initial stage, an individual may remain free of symptoms for many years. During this time, the virus continues to attack the immune system, causing rapid turnover of CD4+ lymphocytes. In most people, the virus eventually gains the upper hand: the CD4+ count slowly but steadily declines and immune functions become increasingly compromised. This latent period, in which infected individuals are generally healthy, typically lasts for 10 years. Other outcomes are also possible. In roughly 10% of people, for instance those with mutations to CCR5, the immune system contains the virus and there is no progressive loss of immune function. These people have remained disease free for as long as 20 years after infection. In other individuals, especially in the setting of malnutrition and tuberculosis, the progression from infection to AIDS to death occurs within 1 year.

In the majority of cases, HIV eventually cripples the host's immune system, leaving the infected individual vulnerable to other disease-causing agents in the environment. Clinical signs of infection generally begin to appear when the person's CD4+ count, normally around 1000 cells ml^{-1}, drops below 500. As the immune system deteriorates further, a series of opportunistic infections begin to appear, including thrush, tuberculosis, pneumocystis carinii, histoplasmosis, toxoplasmosis, herpes, cytomegalovirus (CMV), and *Mycobacterium avium* complex. According to the current criteria, established in 1993, the diagnosis of AIDS is given to patients who are HIV+ when either their CD4+ count drops below 200 or they develop opportunistic infections or cancers (especially Kaposi's sarcoma (KS)), AIDS encephalitis, or AIDS wasting syndrome. Clinical presentations of AIDS have varied over the course of the epidemic and in different countries. In the United States, the first cases presented with pneumocystis carinii pneumonia (PCP) or KS. In sub-Saharan African countries, a syndrome of chronic diarrhea and severe weight loss ('Slim disease')

was common; many people also presented with fulminant tuberculosis. Now that anti-retroviral therapy (ART) controls many HIV infections in the United States, patients often have greater problems with medication side effects than with opportunistic infections.

HIV is accepted as the causative agent of AIDS by an overwhelming majority of physicians, scientists, and public health experts. Despite this, opposition to the HIV explanation appeared early in the epidemic and has persisted in a vocal minority. Citing the existence of clinical syndromes that resembled AIDS in HIV individuals (known as idiopathic CD4+ lymphocytopenia), some observers have argued that HIV is an insufficient explanation for AIDS. One theory argued that HIV and other factors together trigger an autoimmune response that produces immunodeficiency. Another replaced the infection model with a pollution model: recreational drug use, overuse of antibiotics, multiple sexually transmitted infections, exposure to impure blood products, and malnutrition somehow converged to produce AIDS. The most prominent skeptic of HIV has been South African President Thabo Mbeki, who long refused to acknowledge HIV as the cause of AIDS. His advocacy of alternative theories proved to be a major obstacle to the implementation of ART in South Africa in the early 2000s.

Origins and Spread of HIV

HIV was first isolated in January 1983 by Francis Barre´Sinoussi and Luc Montagnier at the Pasteur Institute in Paris. They named it lymphadenopathy virus (LAV) and suspected that it might be the cause of AIDS. In April 1984 Robert Gallo and his team at the National Institutes of Health announced the discovery of another virus that they believed to be the cause of AIDS. Recognizing the virus as a retrovirus, related to two retroviruses that Gallo had previously shown to cause lymphoma, they named it human T-lymphotropic virus type III (HTLV-III). A third group, led by Jay Levy at the University of California at San Francisco, independently isolated the virus as well. After a bitter priority dispute between Montagnier and Gallo, researchers realized that LAV and HTLV-III were the same virus. In 1986 an international commission officially assigned the name HIV.

Subsequent study revealed that many different strains of HIV circulate in human populations. In 1985 a second virus, now named HIV-2, was identified in some cases of AIDS in West Africa. In 1994 two subgroups of HIV-1 were described: HIV-1 group M (main) and HIV-1 group O (outlier). In 1998 a third subgroup was described, HIV-1 group N (non-M/non-O, or new) in two individuals in Cameroon. HIV-1 M counts for the vast majority – over 90% – of all cases of AIDS. Many subtypes of HIV-1 M now exist, which vary in their geographic distribution. While subtype B dominates in the Americas and western Europe, A and B can be found in eastern Europe, B and C in North Africa and East Asia, and A, D, F, G, C, H, J, K, and others in sub-Saharan Africa. Analysis of these subtypes has shown that a single individual can become infected with multiple subtypes and that these can recombine to produce novel forms of the virus.

Careful analysis of viral genetics and host phylogeny has revealed the origins of the different subgroups of HIV. The family of retroviruses has two main divisions. The first division, the oncoviruses, had been described in animals early in the twentieth century; Gallo described the first human oncogenic retrovirus in 1980, HTLV-1. HIV belongs to the second division, the lentiviruses, which are cytopathic. They have been found in a variety of animal species, including horses, goats, humans, and other primates. The various strains of SIV are the closest relatives of HIV. The first SIV was identified in 1985 in a rhesus monkey, in captivity, which suffered from an AIDS-like illness. Subsequent study has found over 20 species-specific strains of SIV. Recombination between these lineages obscures the details of their phylogeny. However, genetic analysis of the strains suggests a common origin, in Africa, in the Pleistocene epoch. Each species-specific strain is harmless in its natural host, a fact consistent with the ancient association between the strains and their hosts. But when a strain infects an individual from another species, an AIDS-like illness results. For instance, the rhesus money from which SIV was first identified was infected with a strain from a sooty mangabey.

HIV reflects a similar phenomenon. At some point in the past, a single subspecies of chimpanzee, *Pan troglodytes troglodytes*, was infected with two different strains of SIV, one from red capped mangabeys, and one from spot nosed monkeys. These two strains recombined in chimpanzees to form a novel strain, SIVcpz. This strain then jumped to humans and evolved into HIV-1 M. When and where did this jump take place? Chimpanzees infected with SIVcpz have been found in Cameroon, Gabon, Congo, Central African Republic, and Equatorial Guinea, an area that corresponds well with the epicenter of the human epidemic. Transmission from chimpanzees to humans likely occurred repeatedly, wherever humans hunted chimpanzees for food. The three different subgroups of HIV-1 (M, N, O), for instance, represent infections of humans with three different strains of SIVcpz. HIV-2, in contrast, came from strain of SIV found in sooty mangabeys in West Africa, where the monkeys are hunted and kept as household pets. Some researchers argue that humans were infected hundreds or thousands of years ago. Most, however, believe that HIV first emerged in the 1930s or 1940s.

Many theories have been offered to explain why significant spread of HIV among humans began at this time. During the final decades of French colonial rule in Africa, policies of resettlement, forced labor, and urbanization destabilized African populations. Malnourished and overworked, some people turned to chimpanzees as a food source. This increasingly brought SIV into contact with human populations who had reduced resistance to infection. This foothold may have facilitated the evolution of SIV into HIV. Once human-to-human transmission became possible, HIV spread during the turbulent social changes that followed decolonization after World War II. Civil wars produced migrations of refugees. Urbanization disrupted traditional social practices, marginalized women, and promoted prostitution. Governments increased spending on military programs and sacrificed social services and education. Poorly funded health care programs had to reuse needles, something that accelerated the spread of HIV. Major vaccination campaigns in the 1950s and 1960s might have played a crucial role. Highway construction and improving transportation allowed the virus to spread from its central African origins throughout sub-Saharan Africa, and then throughout the world. International aid workers and tourists who indulged in commercial sex industries became infected and brought the virus back to

their home countries. Once HIV gained access to developed countries, it found many routes of spread. Needle sharing spread HIV among IDUs in New York City in the 1970s. The 1970s also saw the emergence of gay identity, especially in San Francisco where, for some gay men, promiscuous anonymous sex became an important part of their identity. Blood sharing technologies, particularly transfusion and fractionated blood products, had also become more widespread. These varied factors transformed a local practice – hunting of chimpanzees for food – into a global pandemic. This rapid evolution of HIV over several decades may be a harbinger of further changes yet to come.

Researchers have scrutinized medical records to detect HIV or make retrospective diagnoses of AIDS to reconstruct a detailed prehistory of the epidemic. Collections of old blood samples have been tested to find direct evidence of the presence of HIV in human populations. The oldest match comes from a 1959 sample from Leopoldville (Kinshasa), Congo. This strain is the common ancestor of three of the current subtypes of HIV-1 M. The earliest definitive clinical evidence of AIDS comes, surprisingly, from a Norwegian family. In 1961 a Norwegian teenager worked on a merchant ship that sailed down the west coast of Africa, stopping in many ports from Senegal to Cameroon. During this trip, he was treated for gonorrhea. He subsequently returned to Norway and began to show symptoms of AIDS in 1966. His wife fell ill in 1967 and their daughter, born that year, became sick in 1969. All three died in 1976. Subsequent testing of blood samples taken from them between 1971 and 1973 have tested positive for HIV-1 O, a subgroup found most often in Cameroon. The earliest case in the United States has been identified in a 15-year old from St. Louis. Diagnosed with a severe chlamydial infection in 1968, he died in 1969. Autopsy revealed extensive, aggressive KS. Tests on surviving blood samples, performed in 1987, confirmed the diagnosis of HIV. Dozens of other likely cases of AIDS, that lack confirmatory blood tests, have been found in Africa, Europe, and the United States in the 1970s. How prevalent was the infection? Screening of surviving blood samples has revealed its insidious spread. Samples from sub-Saharan Africa show a prevalence of 0.1% in 1959, 0.3% in 1970, and 3% in 1980. Data from clinics for IDUs in New York City show that HIV had entered the city by 1976. Infection had reached 9% of IDUs by 1978, 39% by 1980, and over 50% by 1984. These data suggest that HIV existed, but was rare, in human populations in the 1950s. By the 1970s, however, parallel epidemics had begun in sub-Saharan Africa, the United States, and Europe.

Emergence and Recognition of AIDS

In the late 1970s, physicians in New York and California noted the increasing occurrence of KS, PCP, and other rare infections among previously healthy young men. Because of the unusual nature of these diseases, which are typically associated with a failure of the immune system, epidemiologists began to search for characteristics that might link the cases. In the summer of 1981, Michael Gottlieb described an outbreak of PCP among five homosexual men in Los Angeles. By the end of the year, similar cases, as well as unusual cases of KS, had been described among homosexuals, IDUs, and female sexual partners of IDUs, in Los Angeles, San Francisco, New York City, and Europe. Researchers traced these opportunistic infections to a specific deficit in CD4 + cells. Although several risk groups had been identified, etiological hypotheses focused on particular aspects of gay culture which might explain the outbreak of the disease, including use of amyl nitrite ('poppers') and steroid skin creams, antigen overload, and pathogenic sperm. Some researchers suggested a link to CMV.

Other risk groups were identified in 1982. Researchers traced cases of immunodeficiency and opportunistic infections to contaminated blood products, especially the clotting factors used to treat hemophilia. Although the risk posed by clotting factor concentrates was soon recognized, many people with hemophilia, tragically, had no choice but to continue taking them. Meanwhile, during the summer of 1982, deaths from opportunistic infections, notably toxoplasmosis, were described among Haitians at detention camps in Florida. The occurrence of cases in diverse risk groups suggested infection with a bloodborne agent. In September 1982, the Centers for Disease Control and Prevention (CDC) replaced the range of early labels (gay plague, gay cancer, and gay-related immune deficiency) with a more neutral term: AIDS. By the end of the year, over 1000 cases had been described.

By early 1983, the CDC had defined AIDS as the result of infection with an unknown transmissible agent, characterized its mode of transmission, and defined four risk groups, the '4H Club': homosexuals, heroin addicts, hemophiliacs, and Haitians. Recognizing that many European cases had links to Africa, researchers speculated that Africa might be the source of the virus. This suspicion was strengthened by reports of slim disease and KS beginning to emerge from Uganda, Zaire, and Zambia. The identification of HIV by Barre´-Sinoussi, Montagnier, and Gallo enabled intensive scientific scrutiny of the epidemic. The fortuitous emergence of HIV at exactly the moment when scientists had developed the techniques needed to study it allowed rapid progress in understanding the biology and pathophysiology of the virus. The problems posed by the virus – biologically, clinically, and epidemiologically – quickly moved virology and molecular immunology onto the center stage of scientific research. The first crucial innovation came in March 1985 with the licensing of the first blood screening tests. The enzyme-linked immunosorbent assay (ELISA) and the Western Blot did not detect the virus itself. Instead, both identified the high levels of antibody produced by most infected individuals within several months of infection. Although the tests could miss people who had recently been infected, they allowed clinicians to test patients, epidemiologists to survey populations, and officials to screen donated blood to protect the blood supply from HIV.

With both a clinical definition of AIDS and a screening test for HIV, officials could map the spread of the epidemic more accurately. Through the 1980s the United States had the greatest number of cases of AIDS, with 100 000 by 1989 and 200 000 by 1991. By the mid-1980s, however, concern had begun to shift to Africa. The first cases of AIDS in Africa had been described in Uganda in 1982. Reports from Zaire, Zambia, and Rwanda of patients with slim disease, KS, thrush (oral candidiasis), and meningitis emerged in 1983 and 1984. By 1985, with additional cases in Kenya and Tanzania, the WHO began to fear a vast epidemic in Africa. These fears have been realized: fully two-thirds of all cases of HIV have been in sub-Saharan Africa. The first cases

of AIDS in Asia were reported in Thailand in 1984. Reported cases of AIDS, however, remained rare for several years. Rapid spread began in 1988 with prevalence among IDUs and commercial sex workers quickly approaching 50%. By 1991 HIV had spread into the general Thai population, with 10–15% of military conscripts testing positive for the virus. In contrast to the rapid spread in these areas, many regions were largely spared during the 1980s. There were few cases of AIDS in Japan in the 1980s and those were largely limited to transmission from blood products, which were often imported from the United States. The Soviet Union and eastern European countries reported few cases before the breakup of the Soviet empire, and most of the acknowledged cases came from iatrogenic transmission. For instance, in 1990, Romania reported HIV among 2000 children who had been abandoned by their parents. According to folk practice, many newborns received small blood transfusions to increase their strength; this practice exposed the infants to unsterilized needles and a contaminated blood supply. However, even countries that were spared during the 1980s experienced accelerating epidemics in the 1990s. China, for instance, reported very few cases in the 1980s, mostly among foreigners or people who had traveled abroad. However, in the mid-1990s, the virus began to spread among IDUs and commercial blood donors. India similarly saw few cases in the 1980s, but by the mid-1990s, HIV was spreading through drug use, the blood supply, and heterosexual contact.

AIDS Before Effective Treatment: Fear and Blame

As scientists struggled to characterize AIDS and HIV, the epidemic caused considerable suffering and generated a worldwide health crisis. The epidemic began at a moment of relative complacency, especially in the developed world, concerning epidemic infectious disease. Not since the influenza epidemic of 1918–20 had an epidemic with such devastating potential struck. Developed countries had experienced a health transition from the predominance of infectious to chronic disease and had come to focus their resources and attention on systemic, noninfectious diseases. In this respect, AIDS appeared at a historical moment in which there was little social or political experience in confronting a public health crisis of its dimension. The epidemic fractured a widely held belief in medical security.

Not surprisingly, early sociopolitical responses were characterized by blame and discrimination. AIDS first appeared among homosexuals and IDUs, some of the most marginalized groups in society. Echoing earlier assessments of other sexually transmitted diseases (STDs), observers divided victims of HIV into categories: the 'innocent victims', who acquired their infections through transfusions or perinatal exposure, and the 'guilty perpetrators', who engaged in high-risk, morally condemnable behaviors. Since the disease was associated with 'voluntary' behaviors considered to be immoral, illegal, or both, individuals were typically blamed for their disease (Figure 1). Some religious groups in the United States, for example, saw the epidemic as an occasion to reiterate particular moral views about sexual behavior, drug use, sin, and disease. AIDS was viewed as 'proof' of a certain moral order. Hostile commentators called for a series of regressive, even punitive, measures to control the epidemic, including universal testing and quarantine. While such measures were never implemented in the United States, victims did suffer from discriminatory behavior, including loss of jobs, housing, and insurance. Homophobia became more visible as violence against gays and lesbians increased. The Food and Drug Administration (FDA) barred blood donations by members of the risk groups, including all Haitians. As the global extent of HIV became clear in the mid-1980s, the nature of blaming and bias also globalized. Early commentators condemned African behaviors that had supposedly spread the disease from monkeys to humans, such as the reported use of monkey blood as an aphrodisiac. Others blamed ecological disruption, particularly the destruction of tropical rain forests. In 1985 Soviet and Indian media reported that HIV had been created by biological warfare experts in the United States and released in Zaire in 1978. This theory received extensive international attention until it became clear that it had its origins in rumors actively spread by the KGB starting in 1983.

This early period of the epidemic in the United States also saw considerable fear of casual transmission despite reassurances that HIV could only spread through blood or sexual contact. A 1985 poll showed that 47% of Americans felt that infection could be transmitted through shared drinking glasses and 28% feared infection through toilet seats. The link between AIDS and receiving blood transfusions became transformed into belief that one could be infected by donating blood. Realtors in California were instructed to warn customers if a house had previously been owned by someone with AIDS. In some communities, parents

Figure 1 In the early years of the acquired immune deficiency syndrome (AIDS) epidemic in the United States, AIDS was associated with a series of stigmatized behaviors, as in this poster indicating the risk of shared needles among intravenous drug users. Such stigmatization blamed victims for their disease and hindered early public health efforts to contain the spread of HIV.

protested when HIV-infected school children were permitted to attend school. In one instance, a family with an HIV infected child – an 'innocent victim' – was driven from a Florida town when their home was burned down. As the disease, deadly and poorly understood, continued to spread, governments and victims' advocacy groups struggled to make difficult decisions. The debates about bath houses are particularly revealing. In the 1970s an outbreak of hepatitis B revealed that these bath houses, and the sexual behaviors they facilitated, had become dangerous sites of disease transmission. In the early 1980s they were recognized as a dominant site of transmission of HIV. Many public health officials demanded that they be closed. The gay community was torn between those who saw bath houses as an essential expression of gay identity and those who feared the growing impact of AIDS. San Francisco shut down its bath houses in 1984 but similar efforts failed in Los Angeles.

The United States government initially showed little interest in AIDS. The defined risk groups had little political clout, and even less political appeal to the conservative administration of President Ronald Reagan. Failing to display leadership crucially needed in the early years of the epidemic, his administration repeatedly denied the need to make special appropriations for HIV and AIDS. However, as the potential ramifications of the epidemic became evident, national institutions began to mobilize. Congressional appropriations for research and education rose steeply. The National Academy of Sciences and a Presidential Commission both issued prominent reports. Meanwhile, international programs proliferated. Medical researchers organized a series of International AIDS Conferences, beginning in 1985. The World Health Organization established a Global Program on AIDS in 1986 to coordinate international efforts at epidemiologic surveillance, education, prevention, and research. The WHO also tried to guide responses to the epidemic, lobbying against the use of coercive measures, such as Cuba's experiment with mandatory isolation of HIV-infected individuals. Instead, it advocated for the use of less restrictive measures to contain the epidemic.

Circumstances changed dramatically in 1985 with the development and wide implementation of screening tests. Just as the CDC definition of four risk groups shaped the early years of the epidemic, the ELISA and Western Blot generated a new range of responses to the epidemic. The tests quickly became a crucial method of reassurance. For the first time, people could know whether or not they were infected, without the uncertainty of the long latency that preceded the symptoms of AIDS. The tests also allowed public health officials to reestablish the safety of the blood supply. Prior to this, blood banks could only reduce risk by restricting donations from defined risk groups and testing for various surrogates, such as hepatitis B, a virus that shared many of the epidemiological characteristics of HIV. But despite these methods, over 10 000 people were infected by blood products in the United States by 1985. However, with the implementation of universal screening, the safety of the blood supply quickly improved. Since then, there have been very few transfusion-related cases of AIDS in the United States.

The advent of screening, however, raised difficult issues about the balance of individual rights and public health. The ability to screen for HIV made it likely that people who were infected, but still healthy, would suffer the full burden of discrimination faced by people with AIDS. Many questions were debated. Should public health officials require mandatory reporting and case tracing of HIV? Should states demand premarital testing, as they did for syphilis? Should employers be able to screen prospective employees? Should health insurers be able to screen prospective customers? Should hospitals be able to test all patients, and should patients be able to demand testing of their doctors? The Department of Defense, citing the risks posed by its vaccination programs and by potential battlefield transfusions, began screening all recruits in 1985. Critics of this policy saw it as a thinly veiled effort to enforce the military's ban on homosexuals. Heated controversy also arose in 1987 when Congress forced the CDC to add HIV to the list of 'dangerous contagious diseases' which would be used to ban immigrants from the United States, despite evidence that HIV was not easily communicable.

For the most part, the rights of privacy won out in the United States. Few states required reporting of HIV at a time when reporting of other diseases, from tuberculosis to syphilis, was routine. In other countries the situation was quite different. The Soviet Union, for instance, implemented a massive compulsory testing program. Travelers, homosexuals, soldiers, pregnant women, prisoners, and those thought to engage in casual sex were all tested, 142 000 000 altogether. Infected foreigners were banned from entering the country. The advent of screening also set the stage for a series of tragic scandals. In the United States, public and private blood banks implemented screening relatively quickly. In other countries, notably France and Japan, the tests were not immediately implemented. Thousands of patients, particularly hemophiliacs, were infected. Subsequent investigations have ended in both civil and criminal convictions, with prison sentences for negligent officials and substantial payments to infected victims.

Although the ability to screen for HIV reduced many of the fears associated with the epidemic, many new fears quickly emerged. By 1986, two factors fueled increasing fear in developed countries that heterosexual spread would bring HIV into the general population. First, public health officials began to shift emphasis from the stigmatized 'risk groups' to more precise 'risk behaviors', stressing that dangerous behaviors such as needle sharing and anal intercourse could be found among people not identified as IDUs or homosexuals (Figure 2). Second, epidemiologists realized that heterosexual transmission was the dominant mode of spread in Africa and Haiti. Since these epidemics were believed to have begun earlier than in Europe and the United States, they were perceived as glimpses into the future of AIDS in Europe and the United States.

The fears were exacerbated by slow progress in developing treatments for HIV and AIDS. Many treatments were hailed initially, including interleukin-2, alphainterferon, bone marrow transplants, heteropolyanion-23 (HPA-23), and various immune stimulants. However, subsequent work inevitably showed them to be both toxic and of little value. Vaccines proved similarly elusive. When Gallo announced the discovery of his virus in 1984, federal officials optimistically predicted that a vaccine would be available within 2 years. By the end of the decade, however, researchers had little to show for their efforts other than skepticism and pessimism. In the absence of treatment, physicians and patients had little to offer people with HIV. Patients tried diets and alternative medicines to delay the progression to AIDS. Doctors could only work to prevent, and then treat, the devastating opportunistic infections.

Figure 2 During the late 1980s, public health officials increasingly recognized the threat of spread of HIV through heterosexual contact. Campaigns such as this one, moving outside of the traditional risk groups, encouraged women to protect themselves from incurable infection with HIV.

Although there was little that could initially be done to help people infected with HIV, public health officials knew everything that they needed to know to prevent the spread of the epidemic. Once the two primary modes of transmission had been identified – sexual intercourse and needlesharing by IDUs – two modes of prevention became obvious: widespread distribution of condoms and sterile needles. These techniques, combined with screening programs, could have slowed the epidemic. Some countries, notably the Netherlands, England, and Canada, did implement such programs. However, it quickly became clear that a diverse range of obstacles prevented effective deployment of public health programs. In the United States, for instance, the conservative federal government criticized condom and needle distribution programs as condoning morally and legally sanctioned behaviors. In 1988 Congress criminalized needle distribution programs. In the absence of federal support, grassroots needle exchange programs were begun in Boston, New Haven, New York, and San Francisco. Some programs received support from local governments, while others faced opposition and arrests. Such marginalization seriously impaired efforts to slow the spread of HIV.

By the end of the decade, the panic created by the emergence of AIDS had settled into a new equilibrium. Incidence rates began to level off in Europe and the United States. Public faith in the safety of the blood supply had been restored. Education efforts curtailed the spread of HIV among homosexuals. Fears that HIV would spread into the general population diminished. New cases remained confined to marginalized groups, notably urban minority populations. AIDS was no longer seen as a wildfire epidemic that would sweep the globe and threaten civilization. Once compared to the great epidemics of the past – plague, cholera, and influenza – AIDS now evoked comparisons to chronic diseases, particularly cancer. It had become routinized. Some observers feared that this new complacency would have adverse consequences. Public health officials in the United States, for instance, feared that increased public apathy would lead to decreased vigilance and allow a resurgence of the epidemic (Figure 3). Meanwhile, the view in developing countries was much bleaker. HIV continued to spread quickly in sub-Saharan Africa. Health officials faced difficult choices. Should they recommend that HIV+ mothers breast feed their children, which risked transmitting HIV, or should they encourage use of infant formulas, which exposed the children to contaminated water supplies? Seeing no good solution, the WHO supported breast feeding in the late 1990s. HIV also began to spread in countries, such as Thailand and India, and then China, that had initially been spared. Despite this, developed countries, no longer fearing a global pandemic, had become less supportive: international assistance for AIDS actually dropped between 1991 and 1992. Although some progress had been made, the epidemic continued.

The Development of Effective Treatments

As soon as HIV was identified, pharmaceutical companies began screening drug compounds for antiviral activity. In 1985 researchers realized that azidothymidine (AZT), which had been developed as an anticancer drug in 1964, blocked reverse transcriptase, an enzyme essential to the viral life cycle. On the basis of promising results from clinical trials, Burroughs Wellcome received authorization to market AZT in 1987. However, initial enthusiasm that AZT might be a magic bullet for HIV quickly gave way to frustration. The drug had many toxic side effects. Long-term studies showed that AZT conferred no survival benefit, in part because patients quickly developed resistance to the drug. Meanwhile, the research process became highly politicized. AIDS activists protested cumbersome FDA regulations that slowed the approval of new drugs. AIDS Coalition to Unleash Power (ACT UP), founded in 1987 by playwright Larry Kramer, issued scathing critiques, sought increased funding for drug research, and demanded more rapid access to promising treatments. In October 1988, ACT UP took over the FDA offices in Washington, DC, to bring attention to the plight of patients who died while the FDA worked to decide whether or not to approve new drugs. ACT UP also

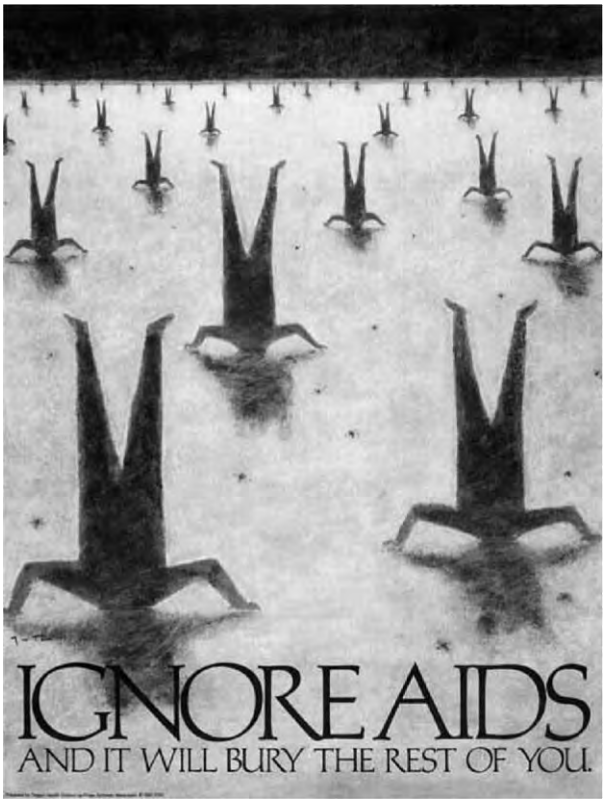

Figure 3 Belief that HIV would not spread widely beyond traditional risk groups, or beyond marginalized urban populations, generated considerable apathy among many people. Public health officials tried to overcome this apathy by highlighting the general threat that would be posed if HIV was not acknowledged and managed.

organized drug sharing, buyers clubs, and basement laboratories to provide underground access to potentially valuable treatments. Such advocacy eventually led to major reforms in FDA policy, facilitating the testing and licensing of new treatments for HIV and AIDS. Another problem with AZT quickly rose to the forefront. When the drug was approved by the FDA in 1987, it costed roughly $10 000 per patient per year of treatment. While this might have been within reach of people with HIV in Europe and the United States, international health activists realized that few developing countries had the resources to provide such an expensive treatment. At the Fourth International AIDS Conference in Stockholm in 1988, officials debated ways of getting treatment to the poor worldwide.

Realizing that AZT would not solve the challenges posed by HIV, researchers and drug companies continued to seek new and better treatments. By 1991 several new reverse transcriptase inhibitors became available (ddI, ddC, Lamivudine (3TC)). Clinicians hoped that combination therapy would be more powerful than AZT alone. While this proved to be true, even a combination of these drugs did not stop the infection. Resistance continued to emerge quickly. Moreover, clinical experience showed that even when patients had an excellent clinical response to therapy, the virus could survive, dormant, in various parts of the body (e.g., the testes). There seemed to be no way to completely eradicate the infection and cure patients. New hope came in 1994 when a major research trial (ACTG 076) showed that AZT could reduce the risk of transmission of HIV from pregnant women to their children by over 60%, from 20 to 8%. This gave patients greater hope that they would not transmit the infection to their children, and it provided a method for preventing a significant burden of new cases of HIV. Efforts to implement campaigns against maternal-to-child transmission, however, also became mired in controversy. The 6-month course of AZT used in the research study cost $800 per patient, a sum beyond the reach of most patients in developing countries. Researchers wanted to know whether or not a shorter course, that cost only $50, would be as effective. The quickest way to learn this would be to compare a short course of AZT to a placebo control group. A 1997 trial of a such shorter, cheaper regimen became embroiled in fierce controversy over what ethical guidelines should regulate international health research. Angry confrontations among researchers united by their desire to reduce the suffering created by HIV demonstrated the complicated scientific, social, and moral problems raised by the epidemic.

While health officials attempted to optimize the implementation of reverse transcriptase inhibitors, researchers continued to study the biology of HIV intensively. By 1991 they hoped that they would soon be able to block the HIV protease, crippling the activity of HIV. The first such protease inhibitor, Saquinavir, was approved by the FDA in December 1995. To maximize its efficacy and to minimize the emergence of resistance, clinicians combined the protease inhibitor with two reverse transcriptase inhibitors. The flexibility of such regimens increased in 1996 when researchers developed a third class of drugs, the non-nucleotide reverse

transcriptase inhibitors (e.g., nevirapine). Combination 'triple therapy' (initially called HAART, for highly active antiretroviral therapy, but now generally referred to as ART proved to be a powerful weapon against HIV. Patients who had been near death from AIDS were miraculously revived: not cured, but restored to health, an effect that quickly became known as the Lazarus syndrome. AIDS mortality dropped quickly in western Europe, the United States, Canada, Australia, and New Zealand (Figure 4). AIDS, which had been the leading cause of death in young men in the United States in 1995, fell to fifth place. The incidence of HIV began to decrease as well. Physicians hoped that protease inhibitors might succeed where earlier drugs had failed and actually eliminate HIV from patients, curing them of their disease. At the 11th International AIDS Conference in Vancouver in 1996, protease inhibitors were celebrated as a 'quantum leap' in the treatment of HIV. Another major advance also appeared in 1996: viral load testing. The initial blood tests for HIV relied on detection of antibodies produced by the body in response to infection. While both sensitive and specific, these tests often did not become positive until several months after infection. The viral load test, in contrast, used polymerase chain reaction (PCR) technology to detect directly viral RNA in the patient's blood. This had two advantages. First, it could detect the virus much earlier in the infection. Second, it could be quantified to measure the activity of the infection. Changes in viral load could then be used to gauge prognosis and response to treatment.

Motivated by the optimism that followed the development of protease inhibitors, the United Nations created a new program devoted to AIDS. However, as had happened with the advent of AZT, a series of obstacles quickly emerged that threatened to undermine the efficacy of combination ART. First, the initial ART regimens were complex, requiring up to 16 pills taken at various times during the day. Outcomes in clinical practice were inferior to those attained in clinical trials, presumably because patients struggled to adhere to the complex regimens. Physicians feared that imperfect adherence to drug regimens would facilitate the emergence of resistant strains. Second, it became clear that the virus could become resistant to each class of medications, even when they were used carefully in combination. Third, patients developed a series of side effects in response to protease inhibitors, particularly lipodystrophy, a disruption of fat metabolism that could increase the risk of diabetes and heart disease. Fourth, researchers increasingly realized that the ability of HIV to insert its genetic material into the genome of host cells made eradication of HIV from an individual nearly impossible. Reservoirs of infected cells persisted, even when viral load became undetectable in patients' blood. If treatment was ever stopped, these internal reservoirs of HIV could become seeds of reinfection. Finally, the regimens were extremely expensive, initially costing $10 000–$20 000 per patient per year, a cost beyond the means of the developing countries where most infections occurred. By the late 1990s a familiar pattern had been established. Public health officials knew that powerful methods could prevent the spread of HIV, but these were rarely utilized to their full potential. Physicians had developed promising new treatments, from AZT to ART, but these faced difficult obstacles, including drug resistance, side effects, and cost. While better treatments could always be developed, the greatest challenge became making good use of existing technology.

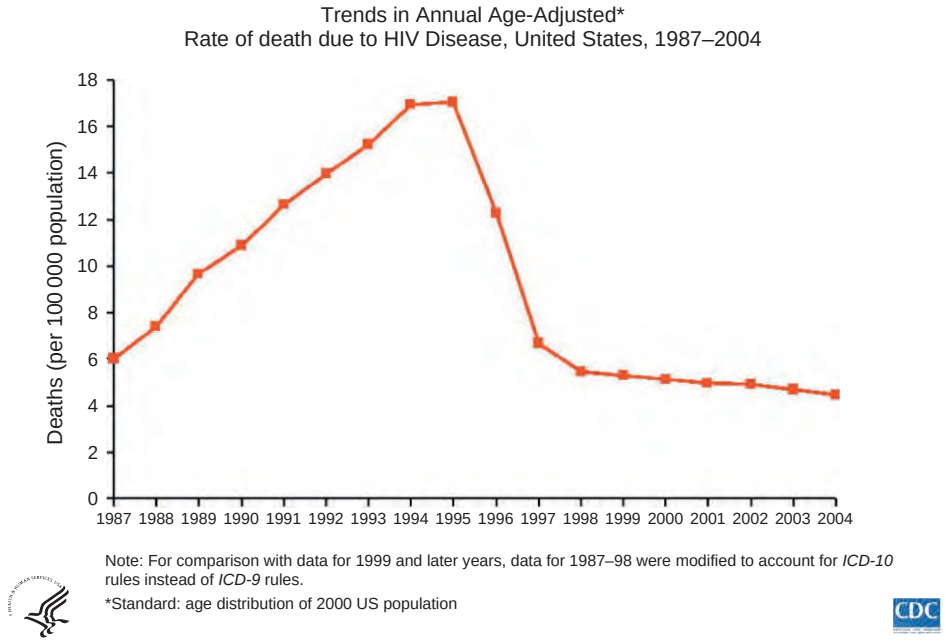

Figure 4 Rate of death from human immunodeficiency virus (HIV). Death rates from HIV in the United States climbed steadily through the 1980s and early 1990s, peaking in 1995. The introduction of protease inhibitors and combined anti-retroviral treatment led to a dramatic reduction in HIV death rates. Bringing this treatment to all populations in the United States, and worldwide, has proven exceedingly difficult. Reproduced from CDC, Divisions of HIV/AIDS Prevention, Slide Series L285, Slide #4.

The Challenge of Providing Care

Throughout the history of AIDS, the nature of the epidemic had depended on where it took place. Modes of transmission varied significantly from country to country, as did the most prevalent opportunistic infections. These geographic variations persisted over the first three decades of AIDS. By 2000, however, a new disparity had emerged, one in many ways more troubling. For the first time a technology existed that could control, though not cure HIV. Patients in developing countries had good prospects for accessing ART and experiencing substantial gains in life expectancy after infection. Meanwhile, ART initially remained out of reach in developing countries, creating enormous disparities in the experience of infection.

The advent of ART led to substantial changes in the nature of the AIDS epidemic in developed countries. Since 1981, the United States had seen over one million cases of AIDS with over 500 000 deaths, the eighth highest total for a country worldwide. Mortality rates had climbed steeply from the 1980s to the 1990s, reaching over 16/100 000 in 1995. With ART, however, the United States witnessed an 80% reduction in mortality, with the rate falling to under 6/100 000. With mortality declining, a diagnosis of HIV no longer seemed to be the life-ending event it had been in the 1980s. Testing became much more widespread. In 1996 the FDA licensed home test kits for HIV. Although testing services were regulated to ensure that patients who received positive test results by telephone also received adequate counseling, HIV testing became nearly as simple as home pregnancy tests. Testing technology continued to improve, allowing clinics to provide results within 30 min. Treatment, even though expensive, was well funded by both insurers and government programs. As a result, everyone with HIV in the United States had access, in theory, to ART. Pharmaceutical companies also produced complex pill formulations, allowing a one-pill-a-day regimen for people infected with nonresistant strains. Widespread access to treatment led to a dramatic increase in life expectancy for people with AIDS: over 13 years by 2006, a number expected to grow with continuing use of the powerful medications.

Decreased mortality, easier testing, and better access to treatment all contributed to the routinization of HIV in the United States. Health officials, physicians, and patients became more willing to accept control measures that, during the height of AIDS fear, would have been dismissed as unacceptable infringements of civil liberties. By 1998, 30 states (but representing only 30% of all cases) had established some form of mandatory reporting. The CDC worked hard to increase the coverage of its HIV surveillance efforts. In 1999 and again in 2005, the CDC pushed all states to report all cases of HIV to the CDC. The CDC argued that this was already done routinely for many communicable diseases and that there was no longer any reason to treat HIV differently. Eventually, tying mandatory reporting to over $1 400 000 000 in federal aid, the CDC achieved this goal: by the end of 2007, the United States finally had a system of nationwide mandatory reporting in place, something that had taken 30 years to implement. Working in parallel, physicians pushed to make HIV testing a routine part of annual physical exams and prenatal care. Officials heralded the end of 'AIDS exceptionalism': while special measures to protect privacy had been appropriate during the early years of AIDS, such protections were increasingly seen as missed opportunities for controlling the epidemic.

However, some old problems persisted. Strong moral judgments about patients and modes of infection continued to disrupt efforts to prevent and contain HIV. Condom and clean needle distribution programs remained mired in political debate. In 1998 President William Clinton refused to lift the ban on federal funding for needle exchange programs. Political opposition to needle exchange programs continued under President George W. Bush, despite evidence that they were the most effective means of preventing HIV among IDUs, and that they might actually decrease the prevalence of drug use, presumably because the site of needle exchange programs became a valuable site for educational programs. Public health officials also feared that declining mortality and routinization would lead to complacency and carelessness about prevention. Although mortality dropped, the incidence of HIV remained stable, at roughly 40 000 new cases each year since 1992. Most men (58%) continued to be infected through homosexual contact; incidence even began to increase among young homosexual men. Among women, however, most cases (71%) came through heterosexual transmission.

Even more worrisome was the accelerating spread of HIV among the most marginalized communities in the United States. By the mid-2000s, African Americans made up 50% of all cases, even though they represented only 12% of the population. The incidence among African Americans compared to whites was 7 times higher for men, and 21 times higher for women. Women also made up an increasing share of the cases, especially among younger age groups. Among HIV+ teens, for instance, nearly two-thirds were women, mostly African Americans, infected through heterosexual contact. Enormous disparities existed not just for incidence of HIV, but also for outcomes after infection. Treatment failure was especially prevalent in minority populations, with 50% of AIDS deaths occurring in these populations. The AIDS mortality rate in Boston and New York City, for instance, was roughly 15 times higher in black women than in white women. Many obstacles prevented successful implementation of ART in these populations. One study found that only 37% of patients in Baltimore had undetectable viral loads after 1 year of treatment. Such data suggested that in the United States, where adequate resources existed to control HIV and AIDS, failures of social and political will disrupted responses to the epidemic.

The situation outside of the United States was even more complex. Some developing countries, notably Brazil, successfully implemented ART to reduce mortality from AIDS substantially. Many more countries, however, were devastated by HIV, a testimony to the global failure to contain the epidemic. By the end of 2006, an estimated 33 200 000 people lived with HIV, nearly 0.8% of the world's population. Despite the ability, in theory, to prevent infection through abstinence, condom use, and clean needle distribution, over 2 500 000 people were newly infected in 2006. Despite the ability of ART to extend life expectancy dramatically, nearly 2 100 000 died of HIV in 2006. Much of the burden of the epidemic remained in sub-Saharan Africa. Over 60% of people living with HIV, and over 70% of deaths from AIDS, were in sub-Saharan Africa, where overall prevalence of HIV among adults reached 5.9%. Many countries in southern Africa had adult prevalence rates over 20%. Swaziland, suffering the most intense

epidemic in 2006, had an estimated prevalence rate of 33.4%. South Africa continued to have the highest number of cases, 5 500 000, without evidence that spread of infection had slowed. Most of the people living with HIV continued to be unaware of their infection.

In contrast to southern Africa, many other regions of Africa experienced much less severe epidemics. In eastern and western Africa, many countries had prevalence rates below 5%, and some remained below 1%. Outside of Africa, rates also remained low, but with concerning evidence of rapid increases in the late 1990s and early 2000s. Rates of HIV increased by 70% in eastern Europe and central Asia between 2004 and 2006, with 90% of this increase confined to Ukraine and Russia, where most spread occurs among IDUs. Rates have also increased in East Asia, south Asia, the Middle East, and north Africa. Although China reported only 650 000 cases overall, nearly half among IDU, prevalence in that group rose in the early 2000s, reaching over 50% in some Chinese provinces. Increased rates have also been seen among pregnant women. India, poised to displace South Africa as the country with the highest number of cases, experienced a highly varied epidemic, with most cases confined to industrial centers in the south, west, and northeast. Preliminary data suggest that prevention programs had begun to slow transmission. A few countries, notably Cuba, have been largely spared from the epidemic, with prevalence less than 0.1%.

The HIV epidemic has had striking demographic impacts. AIDS has orphaned 15 000 000 children, of whom 80% live in sub-Saharan Africa. Many sub-Saharan Africans have also seen marked decreases in overall life expectancy. Although life expectancy had reached 65 years in Botswana by the late 1980s, it fell to less than 36 years because of HIV. Similar drops occurred in Zimbabwe and Swaziland. Throughout southern Africa, AIDS became a major barrier to economic development and political stability. Tragically, in the absence of development and stability, AIDS will remain difficult to control. How has this happened, 20 years after the development of successful preventive strategies and 10 years after the development of effective treatment?

When ART became available in the late 1990s, many leading health officials believed that it could not be implemented in developing countries. In 1998, for instance, many prominent officials argued that ART was inappropriate for poor countries. The treatment was too expensive, far exceeding the annual per capita health expenditures of every country. It was too difficult to implement, requiring sophisticated testing services and health infrastructure. If implemented imperfectly, it would facilitate the emergence of resistant strains, which would then threaten populations worldwide. Instead of treatment, health officials emphasized prevention. One influential comparison of treatment and prevention concluded that prevention was 28 times as cost effective as ART and argued that funding ART would, overall, lead to a greater loss of life. The focus on prevention achieved a powerful consensus. At the 13th International AIDS Conference, held in South Africa in 2000, over 5000 attendees signed the 'Durban Declaration': while ongoing research might lead to cheaper treatments, even a vaccine, AIDS campaigns needed to focus on prevention of sexual transmission.

Proponents of prevention had evidence that such approaches could be successful. Even amidst the many calamities of the HIV pandemic, some countries achieved remarkable success at slowing the spread of HIV through preventive programs including health education, drug treatment, employment, and distribution of condoms and clean needles. In Uganda, for instance, education programs emphasized abstinence and condom use among sexually active teens. Such programs decreased prevalence, which peaked in the late 1980s and fell through the 1990s. Knowing that the presence of other STDs substantially increased the risk of transmitting HIV, officials in Tanzania implemented an STD treatment program that led to a 42% reduction in the incidence of HIV. Prevalence rates have also declined in Zambia, Kenya, Ghana, Rwanda, and Burkina Faso. Countries in southern Africa generally had less success, with Zimbabwe standing out as an exception. Despite substantial poverty and civil unrest, the prevalence of HIV among pregnant women fell from over 30% in 2000 to 24% in 2004. There have been successes outside of Africa as well. Aided by WHO, the Thai government began a major campaign for public education and condom use by prostitutes. As a result, prevalence among commercial sex workers declined significantly over the 1990s. Overall incidence in Thailand has decreased from 143 000 in 1991 to only 18 000 in 2005.

Critics, however, argued that strict emphasis on prevention consigned the 30 000 000 people living with HIV to death. They insisted that large-scale treatment programs must be implemented, both because of belief in equity and social justice and because treatment could potentially be a highly effective form of prevention: if reducing viral loads to undetectable levels decreases the infectiousness of HIV+ individuals, then comprehensive treatment would substantially slow the spread of HIV. Arguing that no developed country would tolerate withholding treatment from a large sector of its own population, treatment activists called on developed countries to make resources available for global treatment programs. Noting that the roughly $8 000 000 000 needed each year for global AIDS control represented only 0.044% of the GDP of the 22 wealthiest countries, they argued that resources for AIDS treatment clearly existed. The challenge was simply one of priorities. Treatment advocates were especially critical of the use of costeffectiveness analysis to support prevention at the expense of treatment, arguing it would be better to mobilize the resources needed for both. When Lee Jong-Wook took over as director general of the WHO in 2003, he pushed aggressively for treatment. With only 2% of HIV+ people having access to ART, he argued that a worldwide health emergency existed.

Although such advocacy of treatment was initially seen as naïve and unrealistic, remarkable progress was made quickly. Success came from several directions. First, a series of reforms substantially reduced the cost of HIV treatment. In the late 1990s, pharmaceutical companies in Thailand, Brazil, and India began producing generic versions of HIV medications, in violation of international patent law. Brazilian companies, for instance, reduced the cost of ART in 1996 from $15 000 per person per year to only $4000. In 1998 South Africa attempted to import generic ART from these companies. Fearing that this would serve as a precedent for violations of patent rights worldwide, 39 pharmaceutical companies sued South Africa, initially with the support of the United States government. This lawsuit proved to be immensely embarrassing for the United States, which was seen as protecting the interests of profitable corporations while millions of people died from AIDS. As a result, in 2000, President Clinton

issued an executive order forcing the companies not to enforce their patents in South Africa. Nearly simultaneously, those companies agreed to slash prices for their drugs in developing countries by over 90%. This created a two-tiered system in which developed countries paid full price while developing countries received steep discounts. Such arrangements were formalized in 2001 by the World Trade Organization's agreement on Trade-Related Aspects of Intellectual Property Rights. Known as the Doha Declaration, this agreement gave governments the right to take certain measures to protect public health. In the midst of a health crisis, governments may impose compulsory licensing and allow local companies to produce medications in violation of international patents. Seen as a recognition of the primacy of public health over private property, the Doha Declaration enabled further reductions in the cost of ART. The nongovernmental organization Me´decins Sans Frontie`res worked with Cipla, an Indian pharmaceutical company, to produce combined ART at a price of only $350 per person per year. Following this lead, the Clinton Foundation negotiated further price reductions in 2003, to only $140 per person per year.

The marked reduction in the price of ART motivated the second major development, a dramatic increase in funding for global AIDS control. When ART became available, the United States provided limited funding for international AIDS programs, only $121 000 000 in 1998. Activists asserted that much greater resources existed and that corporations had the knowledge needed to deploy treatment effectively worldwide. As prices for ART fell by 99% in the early 2000s, resistance to treatment programs dissipated and donor countries began to develop funding mechanisms. In 2002 the Global Fund to Fight AIDS, Tuberculosis and Malaria was created as a partnership between governments, communities, and industry to direct resources to areas suffering from those diseases. Although it received only a fraction of the funding it needed, the Global Fund made progress in many areas. In 2003 President Bush increased the commitment of the United States, promising $15 000 000 000 in AIDS assistance over 5 years; in 2007 he called for another $30 000 000 000 to extend this 5 years further. This initiative, which became the President's Emergency Plan for AIDS Relief (PEPFAR), significantly increased ART treatment worldwide (Figure 5). Private donors, notably Bill Gates and Warren Buffett, also made substantial contributions. All told, funding available for global AIDS treatment increased from only $292 000 000 in 1996 to over $8 000 000 000 in 2005.

Decreased cost of ART and increased funding for global AIDS made both prevention and treatment possible on a previously unprecedented scale. Actual implementation proved difficult. One crucial challenge was ensuring that treatment, once started, was never interrupted, something that would facilitate the emergence of resistant strains. This required careful attention to planning and logistics. Experience also showed that simply making treatment available, through voluntary counseling and testing, did not get enough patients into treatment. The most successful HIV control programs instead tied HIV services more broadly to the provision of primary health care services, especially STD prevention and treatment, as well as maternal and child health programs. Wraparound services, including nutrition assistance, substance use treatment, and social support, made it possible to achieve high rates of treatment compliance even in the world's poorest regions.

However, as happened in the United States, a variety of political factors conspired to undermine optimal implementation of HIV prevention and AIDS treatment. President Thabo Mbeki's opposition to ART delayed its widespread introduction into South Africa until 2004. Meanwhile, critics argue that domestic social politics in the United States interfered with AIDS programs funded by the United States. Specifically, the Bush administration mandated that one-third of the funding it gave to prevention programs be used for advocacy of abstinence and monogamy, efforts that were not always well suited to local regions. As has happened since the 1980s, ideology and pragmatism remained in tension, disrupting HIV prevention.

One of the most dramatic efforts to increase AIDS treatment worldwide came in 2003 when the WHO launched its 3 by 5 campaign, an effort to get 3 000 000 people in developing countries on ART by 2005. This program met fierce resistance from critics and skeptics. Some complained that the target of 3 000 000 represented only half of the people who needed treatment. Others

Figure 5 The PEFPAR testing center in Migori, Kenya. In the early to mid-2000s, a variety of programs made anti-retroviral therapy (ART) available to people in developing countries on an unprecedented scale. The most effective programs combined voluntary counseling and testing with access to ART and a broad range of primary care services. Reproduced from The Power of Partnerships: Third Annual Report to Congress on PEPFAR (2007), p. 103 (http://www.pepfar.gov/press/c21604.htm).

worried that infrastructure did not exist in enough countries and that the WHO did not control enough funding to make this possible. The outcome of 3 by 5 was a mixed success. By the end of 2005, only 1 300 000 people were on ART in developing countries. Few of the target countries had reached the goal of getting 50% of eligible patients on treatment. However, this still reflected a threefold increase over 2 years. Moreover, some countries, notably Botswana, made enormous progress, bringing ART to 85% of the people in need. Such successes demonstrated the feasibility of extensive treatment in developing countries and fueled growing enthusiasm for ART. Countries acting on their own, such as Brazil, or in collaboration with either PEPFAR or WHO brought ART to millions of patients. By the end of 2006, the Global Fund reported that it had 770 000 on ART, PEPFAR 822 000, and WHO 1 800 000. South Africa alone claimed over 1 000 000 people, a tenfold increase since 2003. Despite such progress, a majority of people who would benefit from ART – over 70% – still did not have access. World leaders repeatedly pledged to do better. In 2006, for instance, the General Assembly of the United Nations committed member nations to provide universal access to prevention and treatment by 2010. While most global health leaders supported this goal, no one produced a clear road map for how to make it possible amidst daunting obstacles.

Health officials believe that vaccines will ultimately provide the best solution for the long-term control and prevention of HIV in developing countries. An ideal vaccine would be safe, cheap, and stable enough for mass vaccination campaigns; it would provide long-lived protection; and it would be active against the diverse subtypes of HIV. Such a vaccine remains the Holy Grail of AIDS research. Despite more than 25 years of research, there has been little success. The first clinical trial of an AIDS vaccine began in 1998, but nothing promising enough for widespread use has been developed. Researchers predict that a vaccine will not be available for many years. Some even confess that the task might be impossible because of the ways in which HIV sabotages the immune system. But research continues with the hope that a partial success would still be valuable. Even a 'therapeutic vaccine', one that did not prevent infection but instead strengthened an individual's response to infection, would help. Efforts to develop other measures to prevent infection have also been disappointing. Researchers have long tried to develop an anti-HIV microbicide that women would insert into their vaginas to protect themselves against infection by HIV+ partners. A series of clinical trials, however, have shown that each proposed microbicide actually increased rates of infection, presumably because of irritation of the vaginal mucosa.

New hope came from an unexpected direction. Epidemiologists had recognized by the 1990s that rates of HIV transmission were lower in countries where male circumcision was widely practiced than in countries where it was not. This led to the suggestion that circumcision somehow protected against HIV transmission. Several clinical trials were begun to test prospectively whether circumcision of adult men could be an effective means of reducing the spread of HIV. In 2006 researchers stopped two randomized trials prematurely because the outcomes were so dramatic: circumcision reduced transmission rates by over 50%. Since even a 50% reduction could have a substantial impact on the epidemic, circumcision could work as a powerful, if imperfect, vaccine. However, as with condoms and needle distribution, circumcision is tied to social beliefs and practices that might limit its popularity as a means of preventing HIV.

As AIDS approaches the end of its third decade, it has attained a complicated status, characterized by a series of striking disparities. Medical research and public health campaigns have produced some dramatic successes. Condom and clean needle distribution, as well as male circumcision, can substantially reduce the transmission of HIV. Such measures have been implemented in some countries. Furthermore, people who have become infected with HIV can control their illness with ART, increasing their life expectancy dramatically. However, a variety of social, economic, and political obstacles have disrupted successful implementation of these powerful technologies. Although AIDS mortality has decreased significantly in developed countries, marginalized minority populations experience marked disparities in both rates of infection and mortality. Although some developing countries have successfully deployed both prevention and treatment, AIDS continues to devastate sub-Saharan Africa, where the millions of people now infected with HIV will likely develop and die from AIDS. The impact on other areas, notably Russia, China, and India remains unclear. These countries have a limited window for containing the epidemic before beginning to suffer the decrease in overall life expectancy seen in southern Africa.

At every level, AIDS has been an epidemic created by, and starkly reflecting, inequalities within and between countries. Poverty creates dangerous vulnerabilities to HIV, and AIDS exacerbates poverty. The same inequalities that create the epidemic also hinder responses to it. While patients in wealthy countries have access to powerful treatments, the vast majority of victims do not yet. As a result, scientific success to date has served to expose economic inequality. In the mid-2000s, a strong consensus emerged that called for bringing access to effective prevention and treatment to all countries worldwide. Will it be possible to make universal access a reality? If such efforts contain the epidemic, will it be possible to maintain support for lifelong treatment for everyone now infected with HIV? Will a vaccine emerge that will someday replace ART as the cornerstone of global HIV campaigns? Unprecedented efforts will be needed, over decades, to implement the necessary medical, public health, and social reforms. Such efforts could have an enormous reward, preserving the health of populations worldwide and restoring health to the many areas already devastated by HIV and AIDS.

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Airborne Infectious Microorganisms☆

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Glossary

Aspergilloma Pulmonary fungus ball consisting of a solid mass of hyphae growing in a lung cavity.
Aspergillosis Disease caused by the inhalation of some species of the genus *Aspergillus* resulting in growth of the fungus in the lungs.
Bioaerosol An airborne suspension of biological particles and microbial by-products.
Blastomycosis A symptomatic infection caused by the inhalation of the fungus *Blastomyces dermatitidis*.
Chickenpox Disease caused by the virus varicella-zoster.
Coccidioidomycosis Disease caused by the inhalation of *Coccidioides immitis* arthroconidia (also called San Joaquin Valley fever).
Cryptococcosis Disease caused by the fungus *Cryptococcus neoformans*.
Endotoxin A lipopolysaccharide component of the Gram-negative bacteria cell released during active cellular growth and after cell lysis that can cause respiratory distress when inhaled.
Enteric viruses Group of viruses related with the digestive system of hosts, including the genera Adenovirus, Calicivirus, Enterovirus, Rotavirus, etc.
Fomite An inanimate object that transmits infectious agents to a new host.
H5N1, H7N2, H9N2, and H7N3 Strains of avian influenza A viruses that have been linked to respiratory disease in humans.
Histoplasmosis Disease caused by the infectious fungus *Histoplasma capsulatum* that can manifest as mild flu-like symptoms to chronic lung disease that resembles tuberculosis.
HIV Human immunodeficiency virus
HPS Hantavirus pulmonary syndrome.
Legionnaires' disease A severe respiratory disease resulting from the inhalation of *Legionella pneumophila*.
Measles Term for the disease caused by the virus of the same name.
MERS Middle east respiratory syndrome
Mycobacterium tuberculosis The causative agent of human tuberculosis.
NLVs Norwalk-like viruses.
Pontiac fever A mild flu-like syndrome following inhalation of nonviable *L. pneumophila*.
Rubeola Another name for the disease measles.
SARS Severe acute respiratory syndrome.
SARS-CoV Severe acute respiratory syndrome associated coronavirus.
Sin nombre virus Causative agent of the majority of hantavirus pulmonary syndrome cases in the United States.

Defining Statement

Inhalation of airborne pathogenic microorganisms can elicit adverse human health effects including infection, allergic reaction, inflammation, and respiratory disease. This article discusses viruses, bacteria, endotoxin, and fungi that are dispersed through the air and can result in infection following inhalation.

Introduction

Inhalation exposes the upper and lower respiratory tracts of humans to a variety of airborne particles and vapors. Airborne transmission of pathogenic microorganisms to humans from the environment, animals, or other humans can result in disease. The lung is more susceptible to infection than the gastrointestinal tract as ingested microorganisms must pass through the acidic environment of the stomach before they can colonize tissue, while inhaled microorganisms are deposited directly on the moist

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surfaces of the respiratory tract. Inhalation of microbial aerosols can elicit adverse human health effects including infection, allergic reaction, inflammation, and respiratory disease.

Bioaerosols

A bioaerosol is an airborne collection of biological material. Bioaerosols can be comprised of bacterial cells and cellular fragments, fungal spores and fungal hyphae, viruses, and by-products of microbial metabolism. Pollen grains and other biological material can also be airborne as a bioaerosol. Microbial aerosols are generated in outdoor and indoor environments as a result of a variety of natural and anthroprogenic activities. Wind, rain and wave splash, spray irrigation, wastewater treatment activity, cooling towers and air handling water spray systems, and agricultural processes such as harvesting and tilling are examples of activities that generate bioaerosols outdoors. Indoors bioaerosols are generated and dispersed by mechanical and human activity. Industrial and manufacturing practices and biofermentation procedures can generate high concentrations of microbial aerosols. Heating, ventilation, and air conditioning (HVAC) systems, water spray devices (e.g., showerheads and humidifiers), and cleaning (e.g., dusting, sweeping, vacuuming, and mopping) result in the transport of microbial materials in the air. Talking and coughing generate bioaerosols from individuals, some of which may be infectious. Facilities with medical, dental, or animal care practices can generate infectious microbial aerosols.

The individual particle size of particulate material in bioaerosols is generally 0.3–100 μm in diameter; larger particles tend to settle rapidly and are not readily transported in the air. Virus particles are nanometer in size, bacterial cells are approximately 1 μm in diameter, and fungal spores are >1 μm. These microorganisms can be dispersed in the air as single units, but are often present as aggregate formations. The larger aggregates have different aerodynamic properties than single-cell units; therefore their dispersal may be different than single-unit particles. Aggregates of biological material also afford protection from environmental stresses such as desiccation, and exposure to ultraviolet radiation ozone and other pollutants in the atmosphere. Often bacterial cells and virus particles are associated with skin cells, dust, and other organic or inorganic material. During agricultural practices (e.g., during harvesting, and tilling), fungus spores are released from plant surfaces and the soil and raft on other particulate matter. This "rafting" affects the aerodynamic characteristics and the survival of the cells in the bioaerosol. When biological material is dispersed from water sources (e.g., splash, rainfall, or cooling towers and fountains), it is generally surrounded by a thin layer of water. This moisture layer also changes the aerodynamic properties and aids in the survivability of the microorganisms while airborne.

Airborne particulate will remain airborne until settling occurs or they are inhaled. Following inhalation, large airborne particles are lodged in the upper respiratory tract (i.e., nose and nasopharynx). Particles <6 μm in diameter are transported to the lung where the 1–2 μm particles have the greatest retention in the alveoli. The average person inhales approximately 10 m^3 of air per day, which may result in an infective dose for some airborne pathogens. Diseases resulting from inhalation of bioaerosols include allergic reaction, irritation, inflammation, infection, and respiratory disease.

The by-products of microbial metabolism and foreign proteins of microorganisms and fragments of cells can result in allergic reactions and irritant responses. The inflammatory response and allergic reactivity can be debilitating to sensitized individuals in indoor and outdoor environments, and it has been demonstrated following exposure to airborne bacterial fragments present in airborne dust. However, the greatest adverse health impact following exposure to airborne microorganisms is associated with inhalation of infectious agents. These infectious agents include viruses, bacteria, and fungi. The following sections introduce representative pathogens of each grouping that are associated with the airborne route of exposure. Table 1 provides a summary of the pathogens presented in this article.

Infectious Airborne Viruses

Viruses are noncellular units consisting of either DNA or RNA. They do not have self-directed biosynthesis and, therefore, require a living host cell for replication. Following inhalation, infectious viruses can establish in host cells of the respiratory tract. Some are translocated and infect the gastrointestinal tract and other tissues.

Influenza

Influenza is a contagious respiratory illness caused by flu viruses A, B, and C. There are subtypes of influenza type A viruses based on two proteins on the surface proteins of the virus, hemaglutinin (H) and neuraminidase (N). Many animals carry influenza A viruses, while influenza B viruses circulate among humans. Both influenza types A and B viruses cause epidemics of disease almost every winter. Influenza type C infections cause a mild respiratory illness and are not thought to cause epidemics. While symptoms can be mild, influenza may result in severe illness and death. The Centers for Disease Control and Prevention reports that there are more than 200,000 hospital admissions and 36,000 deaths per year in the United States due to influenza. All ages can have serious illness from influenza, but those more likely to have complications are people aged 65 years and older, people with chronic medical conditions, and very young children. Transmission of influenza viruses is from person to person in respiratory droplets expelled during coughing and sneezing. The droplets are propelled usually less than 3 feet through the air and deposited on people in close proximity. The presence of the virus has been confirmed in aerosol particles within the breathable fraction in a study performed in a hospital emergency room (Blachere et al., 2009). Fomites contaminated with settled infectious droplets can also serve as a vehicle for transmission of influenza.

Table 1 Characteristics of selected infectious microorganisms transmitted via the airborne route of exposure

Organism	*Characteristics*	*Disease*	*Reservoir*
Aspergillus spp.	Fungus, a mold with spherical spores ~1 μm in diameter, variety of species including *Aspergillus fumigatus, Aspergillus flavus, Aspergillus nidulans, Aspergillus terrus*, and *Aspergillus niger*	Aspergillosis, aspergilloma (fungus ball), allergic sinusitis, and allergic bronchopulmonary disease	Environment, compost and plant, material, soil, dust, building materials
Blastomyces dermatitidis	Fungus; 8–15 μm spherical yeast cells	Flu-like illness, chronic lung disease	Moist soil enriched with decomposing organic debris
Coccidioides immitis	Fungus; 5 μm alternating barrel-shaped arthrospores	Coccidioidomycosis/ San Joaquin Valley fever	Desert soils with intermittent wet and hot dry cycles
Cryptococcus neoformans	Fungus; vegetative form (yeast cell) 2.5–10 μm, basidiospores 1.8–3 μm	Cryptococcosis	Pigeon droppings debris and soil associated with birds
Enteric viruses	Virus; RNA/DNA, ~20–100 nm	Gastrointestinal symptoms, meningitis, encephalitis, respiratory infections	Infected persons, surfaces
Histoplasma capsulatum	Fungus; dimorphic 2–5 μm oval cells	Flu-like symptoms, chronic lung disease, dissemination to other organ systems	Environment
Influenza viruses	Virus; single-stranded RNA, three distinct types (i.e., A, B, and C), 80–120 nm	Influenza, pneumonia, acute respiratory distress	Human respiratory secretions; avian flu associated with birds
Legionella pneumophila and *Legionella* spp.	Bacterium; vegetative Gram-negative bacillus, 0.5–1 μm by 2 μm	Legionnaires' disease and Pontiac fever	Ubiquitous in fresh and waste water environments, an intercellular parasite of protozoa
Measles virus (MeV)	Virus, enveloped, negative – sense, single-stranded RNA hantavirus, 100–250 nm	Rubeola: acute, febrile disease with an exanthematous rash, dry cough, sore throat, headache	Human – only known reservoir
Middle East respiratory syndrome – associated coronavirus (MERS-CoV)	Virus, enveloped, positive-sense single-stranded RNA	Acute respiratory distress syndrome, multi-organ failure, septic shock, death	Infected persons, bats and camels reservoirs
Mycobacterium tuberculosis	Bacterium; acid-fast, 0.2–0.6 μm by 1–10 μm	Tuberculosis	Infected persons
Severe acute respiratory syndrome – associated coronavirus (SARS-CoV)	Virus; RNA with helical symmetry, positive-sense single-stranded RNA	Acute respiratory distress	Infected persons
Sin nombre virus (SNV)	Virus; negative – sense, single-stranded RNA hantavirus	Hantavirus pulmonary syndrome (HPS)	Rodent urine, saliva, and feces especially *Peromyscus maniculatus* (deer mouse)
Varicella-zoster virus (VZV)	Virus; double-stranded DNA, 150–200 nm	Chickenpox	Humans – only known reservoir; virus may be dormant until reactivates as shingles

Avian influenza A viruses usually do not infect humans. However, cases of human infection with avian influenza viruses have been reported since 1997. Illness caused by several strains of avian influenza A viruses (e.g., H5N1, H7N2, H9N2, and H7N3) have been reported since 2004. The majority of human avian influenza infections have resulted from inhalation during direct contact with infected poultry or contaminated surfaces such as avian cages, or from the ingestion of uncooked blood of infected birds. Therefore, this influenza has been popularized as "bird flu." The illnesses resulting from avian influenza infection in humans range from typical mild influenza-like symptoms (e.g., fever, sore throat, cough, and muscle aches) and conjunctivitis to more serious cases of pneumonia, acute respiratory distress, and other severe and life-threatening complications. To date, there have been no sustained human-to-human transmission of avian influenza A viruses, but because of the potential for these viruses to gain the ability to spread easily between people, monitoring for human infection and person-to-person transmission is important. In addition, recent studies have addressed the possibility of long-range transport of both, Influenza and Avian influenza viruses, using dust storms as a vehicle to be dispersed at farther distances (Chen et al., 2009, 2010).

Severe Acute Respiratory Syndrome

Severe acute respiratory syndrome (SARS) is a newly reported respiratory illness. It was first reported in February 2003 in Asia. Since then, it has also been reported in North America and Europe. It is a viral respiratory illness caused by a coronavirus, termed SARS-associated coronavirus (SARS-CoV). Exposure to SARS-CoV results from close personal contact with infected individuals. Transmission is generally the result of touching the skin of an infected person or objects contaminated with infectious droplets and then

transferring the virus to the new host's eyes, nose, or mouth. Coughing or sneezing by an infected individual also releases droplets into the air that are propelled a short distance (<1 m) and deposited on the mucous membranes of the mouth, nose, or eyes of persons who are nearby. It is possible that SARS can be spread further through the air by very small particles, and although it may be unusual, it has been confirmed in different environments (housing complex, aircraft, hospital facilities) (Wong et al., 2004; Olsen et al., 2003; Yu et al., 2004). Hand hygiene, use of gown, gloves, and eye protection when in contact with an infected individual, and use of an appropriate respirator minimizes the spread of SARS.

Middle East Respiratory Syndrome

Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012 in Saudi Arabia. Since then, a major outbreak occurred in 2015 in the Republic of Korea, and many other countries have reported positive cases (WHO, 2015). Human-to-human contact seem to be the principal way of transmission, however, its survival in aerosols has been positively tested, pointing to the airborne route as an alternative form of dispersal (van Doremalen et al., 2013). Genetic analyses of patients' specimens showed similarities between MERS-CoV and other coronavirus isolated from hedgehogs, bats and camels, suggesting their possible role as reservoirs (WHO MERS-CoV Research Group, 2013). Despite all these data, the exact way of transmission remains on debate. Pathology may vary from asymptomatic to have a fatal outcome. It starts with mild symptoms (fever, cough, etc.) followed by pneumonia with acute respiratory distress and multi-organ failure that may lead to death in 60% of the cases (WHO MERS-CoV Research Group, 2013; de Groot et al., 2013).

Enteric Viruses

Enteric viruses primarily cause gastrointestinal infections, but may also be responsible for more serious diseases such as respiratory infections, meningitis or encephalitis (Fong and Lipp, 2005). Adenovirus, enterovirus, norovirus, hepatovirus and rotavirus are some of the genera considered within this group (Bosch, 1998). They are typically transmitted by fecal contaminated water or food, but airborne dispersion has been considered in some major outbreaks (Kuo et al., 2009; Marks et al., 2000, 2003; Xu et al., 2013; Dick et al., 1987; Ho et al., 1999; Lin et al., 2002). In the case of norovirus, which causes diarrhea and sudden projectile vomiting, it has been estimated that over 30 million virus particles can be liberated during vomiting. Since the infectious dose is very low, 10^1–10^2 particles, airborne transmission within close contact is highly likely (Caul, 1994; Baert et al., 2008).

Hantaviruses

Hantavirus pulmonary syndrome (HPS) is a life-threatening disease caused by rodent-borne hantaviruses. It is characterized by severe pulmonary illness and a mortality rate of 30–40%. The sin nombre virus (SNV) causes the majority of HPS cases in the United States, and *Peromyscus maniculatus* (the deer mouse) is its predominant reservoir. The virus found in the rodent urine, saliva, and feces becomes aerosolized as mist from urine and saliva or dust from feces. Inhalation is the most common route of infection. However, touching the mouth or nose after handling contaminated materials or being bitten by an infected rodent also result in infection. Hantavirus is not spread from person to person. Classic symptoms of disease include fever, fatigue, and muscle aches. Symptoms progress in 4–10 days to cough and shortness of breath.

Measles

The disease measles, also known as rubeola, is contracted by inhalation of infectious droplets expelled as respiratory secretions by an infected individual. The only known reservoir of the measles virus (MeV) is humans. All strains of this virus belong to a single antigenic type and immunity is long lasting following infection.

Fever, malaise anorexia, and respiratory symptoms mimic other respiratory infections, but the appearance of Koplik's spots precedes a rash on the face that proceeds down the body to the soles and palms. Severe complications may occur including a chronic degenerative neurologic disease that occurs many years after a measles infection.

Measles is worldwide, but the success of vaccination programs in developed countries has limited the number of cases of childhood measles, decreasing the prevalence over 90% in Europe. However, there are still sporadic outbreaks, which cause more than 100,000 deaths, especially in patients from developing countries. An eradication program from the World Health Organization initiated in 2001 is promising if the appropriate measures are implemented (Holzmann et al., 2016). This program will not only have to assure the availability of the vaccines to developing countries, but also counteract the anti-vaccination movements that have increase the number of unvaccinated children in high-income countries in the recent years (Whelan, 2016; Dube et al., 2015; Kajetanowicz and Kajetanowicz, 2016).

Varicella

Chickenpox is a highly contagious disease caused by the varicella-zoster virus (VZV). Chickenpox is usually a self-limited disease lasting 4–5 days with fever, malaise, and a generalized vesicular rash of blister-like lesions. The rash covers the body, but it is usually more concentrated on the face, scalp, and trunk. Serious complications including bacterial infections of skin lesions, pneumonia, cerebellar ataxia, and encephalitis may occur, but are rare. Disease is spread by aerosol dissemination of the virus during coughing

and sneezing by an infected person or it may become airborne directly from the skin lesions. Incidence of chickenpox is highest among children 1–6 years of age, but the licensing of a vaccine in 1995 and vaccination requirements for daycare and school children greatly reduced the impact of this virus in the United States in around 75% in a 10-year frame (2000–10) (WHO, 2014).

Following varicella infection, the virus becomes latent in sensory nerve ganglia and may reactivate later in life resulting in shingles. Airborne dispersion from herpes zoster patients may also occur and spread the virus to new varicella outbreaks (Saidel-Odes et al., 2010; Suzuki et al., 2004; Lopez et al., 2008).

Infectious Airborne Bacteria

Unlike viruses that require a host cell, bacteria can colonize and grow on a variety of surfaces and in liquids when physical and nutritional factors are suitable. Therefore, bacteria can be released into the air from a variety of natural and anthropogenic environments. When infectious bacteria are released into the air, they can be readily transported via bioaerosols to susceptible individuals.

Legionella

The first report of Legionnaires' disease followed the outbreak of respiratory illness among American Legion conventioneers in Philadelphia in 1976. Currently an estimated 10,000–15,000 cases of Legionnaires' disease are reported in the United States per year. The causative agent, *Legionella pneumophila*, is a vegetative Gram-negative bacterium that is ubiquitous in freshwater environments worldwide. It survives as an intercellular parasite of protozoa thereby resulting in protection for the bacterium while in the environment. Infective aerosols can be transported up to 200 m from the source and can be generated from contaminated water spray devices such as showerheads, evaporative condensers, humidifiers and fountains, and also from cooling towers or waste treatment plants (Blatny et al., 2008, 2011).

Following the inhalation, *L. pneumophila* replicates in host macrophage resulting in severe pneumonia. While *L. pneumophila* accounts for the majority of disease cases caused by this genus, 19 other *Legionella* species are also human pathogens, often causing disease in immunosuppressed patients. *Legionella* bacteria that cannot replicate in the lung are cited as the cause of Pontiac fever, a mild flu-like syndrome. Often these organisms are released in aerosols from whirlpool tubs. Pontiac fever results in many more cases of illness, but fewer deaths; while Legionnaire's disease has a much higher mortality, but lower morbidity.

No human-to-human transmission has been documented. For an outbreak of Legionnaires' disease to occur, a series of events must happen. A reservoir of infectious bacteria must be present, the environmental conditions must be right for the concentration of organisms to increase, the organisms must be infectious and the infectious bacteria must be dispersed resulting in human exposure, organisms must be deposited in the appropriate site in the human host, and the host must be susceptible to infection by the *Legionella*.

Other Gram-Negative Bacteria

Escherichia coli, *Pantoea* (*Enterobacter*) *agglomerans*, *Pseudomonas* spp., and *Acinetobacter* spp. commonly isolated in cow barns, pig houses, and poultry barns are among the airborne Gram-negative bacteria associated with animal facilities that can result in human disease. Special interest has been focused in the spread of antibiotic-resistance genes by these bacteria, not only among livestock, but also to humans. Alarming results have been observed and effective measures should be applied to stop their dispersal (Gandolfi et al., 2011; Gao et al., 2014; Li et al., 2016). Disease-causing Gram-negative bacteria are also dispersed into the air from wastewater treatment plants, vegetable and herb processing, recycling facilities and within hospital facilities.

These organisms can cause a variety of health problems, especially to the young, elderly, and immunocompromised persons.

Endotoxin

Endotoxin is a lipopolysaccharide component of the Gram-negative bacteria cell and is released during active cellular growth and after cell lysis. While it is not an infectious particle and people are exposed to them daily, endotoxin is biologically active material derived from bacteria that can affect many human organ systems and disrupt humoral and cellular host mediation systems. Symptoms of exposure to airborne endotoxin include chest tightness, cough, shortness of breath, fever, and wheezing. Obstruction of airflow and exacerbation of asthma has been related to airborne endotoxin concentrations, and atopic and nonatopic asthma have been associated with endotoxin exposure because of its ability to induce inflammatory reactions. Repeated inhalation exposure has been shown to induce allergen-specific airway inflammation. The organic dust toxic syndrome (ODTS) is an acute inflammatory condition caused by high levels of endotoxin, usually resolves within 48 h, and has a prevalence of more than 20% among farmers. Severity of hypersensitivity pneumonitis (HP) can be increase under the influence of endotoxin exposure (Thorne and Duchaine, 2007).

Airborne endotoxin exposure is a concern in dusty occupational facilities such as cotton processing facilities and agricultural settings, and in industrial environments that utilize water spray components (e.g., machining fluids). Indoor airborne endotoxin is a

concern with humidified mechanical air conditioning systems. The presence of dogs, moisture, and settled dust increase the likelihood of airborne endotoxin in the home. Despite the evidences that high concentrations of endotoxin has over human health, no limits have been established to prevent it and no international standard protocol is available to obtain comparable results (Smets et al., 2016).

Mycobacterium Species

Mycobacterium tuberculosis is recognized for its role as the causative agent of human tuberculosis. Infected individuals can spread pathogenic bacilli when coughing, talking, speaking, laughing, and sneezing. Aerial dissemination of pulmonary tuberculosis was confirmed back in 1959, and recent studies have shown the need for implementing prevention measures to avoid the spread of the disease and ultimately, eradicate it (Riley et al., 1959; Escombe et al., 2007; Fennelly, 2007).

Overcrowding and low-socioeconomic status are often contributing factors to the spread of tuberculosis as individuals are in close association and hygiene conditions are usually poor. The confined spaces of airplane cabins have been implicated in transmissions to passengers and flight crew members. Infectious aerosols resulting from the operation of suctioning instruments during medical treatment, manipulation of tuberculosis ulcers, drainage of necrotic tissue, and exposure during autopsy have also been cited as causes of nosocomial *M. tuberculosis* infections.

Also, nontuberculosis mycobacterial species can be transported via the air and result in disease. Respiratory infection in immunocompromised patients has been associated with members of the *Mycobacterium avium* complex. *Mycobacterium bovis* can also affect humans, especially in low-income countries, and airborne transmission has been suggested as a main form of dispersion (Gannon et al., 2007; Lan et al., 2016). *Mycobacterium leprae* bacilli have been reported from the nose blow of an untreated person with lepromatous Hansen's disease (leprosy), and *Mycobacterium terrae* isolated from a water-damaged building demonstrated inflammatory responses in mouse lung during laboratory experiments.

Gram-Positive Non-Spore-Forming Bacteria

Numerous Gram-positive bacteria are usually disseminated from the skin, oral and nasal surfaces, and hair of humans. This group is more frequently isolated from air samples than Gram-negative bacteria, with *Staphylococcus* being the most common genus of non-endospore-forming Gram-positive bacteria isolated in indoor air samples (Okten and Asan, 2012; Gilbert et al., 2010; Yu et al., 2015). The presence of these cells in the air increases the likelihood of nosocomial infections in healthcare settings. Nosocomial infections by airborne pathogens and with antibiotic-resistant bacteria in hospital environments are transmitted among patients through aerosol dispersal from patients, staff, surfaces, and aspiration from respiratory instrumentation. Airborne dispersal of pathogens is also a concern in home care and assisted living environments.

Additionally, the Gram-positive actinomycetes have been associated with illness in occupants of water-damaged buildings. Often they are associated with fungi that colonize on surfaces and building materials following water intrusion. High concentrations of actinomycetes have been obtained in air samples from wastewater treatment plants, entailing an exposure risk, not only for workers, but for the nearby population (Awad and El Gendy, 2014; Li et al., 2012).

Infectious Airborne Fungi

Fungi are ubiquitous in nature. They utilize organic matter for their metabolism. Depending on the genus, they can readily disperse spores, cellular fragments, or cells into the air. Infectious fungi can cause serious mycoses of the respiratory tract and some are disseminated to the bone and other systems in the body.

Histoplasma capsulatum

Inhalation of spores of the fungus *H. capsulatum* can result in histoplasmosis. This disease is not transmitted from an infected person or animal to someone else but is the result of airborne transport from environmental surfaces and it is the most frequent mycosis in the United States (Cano and Hajjeh, 2001). Histoplasmosis primarily affects a person's lungs, and its symptoms vary greatly. Infected people are generally asymptomatic or they experience mild flu-like symptoms not requiring medical attention. Chronic lung disease resulting from infection with *H. capsulatum* resembles tuberculosis, and can worsen over months or years. The most severe and rarest form of histoplasmosis occurs when it is disseminated involving spreading outside the lungs. If untreated, disseminated histoplasmosis is fatal and has emerged as an opportunistic pathogen in HIV (human immunodeficiency virus) positive people (Cano and Hajjeh, 2001).

H. capsulatum is a dimorphic fungus with a mold (mycelial phase) when it is growing in soil at ambient temperatures, and a yeast phase after being inhaled by humans or animals. It has a global distribution in temperate zones, usually closely to rivers (Baumgardner, 2012; Chakrabarti and Slavin, 2011). The mold spores are either microconidia ranging from 2 to 5 μm in diameter or macroconidia ranging from 8 to 15 μm. The yeast cells of *H. capsulatum* are oval to round in shape with diameters ranging from 1 to 5 μm.

Coccidioides immitis

Inhalation of airborne *C. immitis* arthroconidia may result in the disease coccidioidomycosis (also called San Joaquin Valley fever). A cryptic species, *Coccidioides posadasii*, presents a different distribution and characteristics, but it can also cause coccidiomycosis and its dispersion it through the airway route (Welsh et al., 2012). Within 48–72 h after inhalation and deposition in the lung, the arthroconidia change into spherules and develop numerous endospores. When spherules rupture, they release endospores which have the capacity to develop into a mature spherule.

Approximately 40% of those who become infected are symptomatic with flu-like illness with fever, cough, headaches, rash, and myalgia. Some individuals fail to recover and suffer chronic pulmonary infection. It accounts for 29% of pneumonia cases in endemic areas (Baumgardner, 2012). Around 5% will develop widespread disseminated infection that may affect the soft tissues, joints and bone, or meninges (Welsh et al., 2012). Coccidioidomycosis meningitis may lead to permanent neurologic damage. The immunocompromised and HIV-infected persons suffer severe pulmonary conditions and diffuse lung disease.

C. immitis grows in the soil of semiarid areas. It is primarily found in the Lower Sonoran life zone and is endemic in the southwestern United States; it is also found in parts of Mexico and South America. Exposure to airborne arthrospores occurs after disturbance of contaminated soil by humans or natural disasters (e.g., dust storms and earthquakes). Association with dust storms has been well documented, with significant outbreaks following a dust storm, with a marked seasonality during the drier and dustier months of the year (Griffin, 2007; Goudie, 2014). Persons in areas with endemic disease who have occupations exposing them to dust (e.g., construction or agricultural workers, and archeologists) are at risk. African-Americans and Asians, pregnant women during the third trimester, and immunocompromised persons have a higher risk than others.

Blastomyces dermatitidis

Blastomycosis is an infection caused by the dimorphic fungus *B. dermatitidis*. This fungus grows in moist soil that is enriched with decomposing organic debris, and it is endemic in parts of the southcentral, southeastern, and mid-western United States. It is also present in some areas of Central and South America and parts of Africa and India. Exposure is via the inhalation of airborne spores, generally after disturbance of contaminated soil. Generally, individuals who frequent wooded areas with endemic disease (e.g., farmers, forestry workers, hunters, and campers) are the most exposed ones.

There are <1 to 100 per 100,000 population in areas with endemic disease. The infection may be asymptomatic or result in a flu-like illness with fever, chills, productive cough, myalgia, arthralgia, and pleuritic chest pain (Baumgardner, 2012). However, some infected individuals fail to recover and develop chronic pulmonary infection and permanent lung damage. Other individuals develop widespread disseminated infection that may involve the skin, bones, and genitourinary tract, and occasionally the meninges of the brain are affected. The mortality rate for blastomycosis is approximately 5%.

Cryptococcus neoformans

C. neoformans var. *neoformans* causes most cryptococcal infections in humans. Infection with this yeast-like fungus can cause disease in apparently immunocompetent and immunocompromised persons, but most people do not get sick with cryptococcosis. Patients with T-cell deficiencies are the most susceptible to infection, and in the United States 85% of cases occur in HIV-infected persons. While the initial pulmonary infection is usually asymptomatic, cryptococcosis can cause serious symptoms of brain and spinal cord disease, such as headaches, dizziness, sleepiness, and confusion. Disseminated infection, especially meningoencephalitis, occurs in most patients.

C. neoformans var. *neoformans* is found worldwide, and its habitat includes debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings. Exposure generally occurs when dried bird droppings are disturbed and dust containing *Cryptococcus* disperses in the air. The exact nature of the infectious particles of *C. neoformans* is not known, but they are likely to be dehydrated yeast cells or basidiospores of the appropriate size range to be deposited in the lungs. Once in the lung, the yeast cells become rehydrated and acquire the characteristic polysaccharide capsule. Virulence of this organism is associated with the ability to produce the large capsule and shed great amounts of capsular material into the body fluids of the host.

Aspergillus

Aspergillosis is an invasive pulmonary infection with fever, cough, and chest pain. A localized pulmonary infection occurs for immunocompetent persons who have an underlying lung disease. The fungus infection may disseminate to other organs, including brain, skin, and bone. Exposure may also result in allergic sinusitis and allergic bronchopulmonary disease. A variety of *Aspergillus* species have been implicated.

Aspergillus fumigatus and *Aspergillus flavus* most often have been cited, but *Aspergillus terreus*, *Aspergillus nidulans*, and *Aspergillus niger* have also been associated with the disease. These fungi are ubiquitous in the environment, and their conidia (spores) are disseminated from soil, compost piles and other decomposing plant matter, household dust, building materials, and ornamental plants. Among with other genera (*Cladosporium*, *Penicillium* or *Alternaria)*, *Aspergillus* is one of the most frequent funguses isolated from air samples, both in indoor and outdoor environments (Mahieu et al., 2000; Cao et al., 2014; Hwang et al., 2016; Oberle et al., 2015; Oh et al., 2014; Grishkan et al., 2012). Water-damaged buildings can also be a source for exposure, and infections have been

associated with dust exposure during building renovation or construction. To decrease workers and nearby population exposure to high levels of *Aspergillus* spores, certain construction works can be restricted to cooler seasons, which seem to influence the growth of the fungus (Pilmis et al., 2016).

Airborne Bioterrorism

The threat of purposeful transmission of airborne pathogenic microorganisms and microbial toxins as weapons of mass destruction (WMD) has increased the awareness of the importance of bioaerosols. The Centers for Disease Control and Prevention and the Department of Health and Human Services have listed several pathogenic microorganisms as select agents. Of these, *Francisella tularensis, Yersinia pestis, Bacillus anthracis*, and *Variola virus*, the causative agents of tularemia, plague, anthrax, and smallpox, respectively, are ones that can be dispersed via aerosol and, therefore, are a concern for inhalation infection. Moreover, availability of new technologies, such as genetic engineering, may provide the tools to modify innocuous microorganisms or the transmission routes of other pathogens, involving a higher biosafety risk for the population (MacIntyre, 2015). Continued researches and awareness by public health professionals are needed to recognize these diseases and minimize the risk of exposure of the population. For example, the uses of biosensors to detect airborne pathogens are being developed and installed in official buildings and strategic sites, that allow a rapid detection and intervention (Fronczek and Yoon, 2015).

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Algal Blooms[☆]

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Glossary

Cladocerans These small crustaceans dominate the biomass of zooplankton in shallow eutrophic lakes. They graze on phytoplankton by filtering water and can rapidly build up their populations by parthenogenesis.
Copepods Small crustaceans ranging in size from 0.2 to 10 mm that form the bulk of metazooplankton biomass of large lakes and the sea. Their complex life cycles involve six naupliar and five copepodite (larval) stages that require, depending on temperature and intrinsic timing, a few weeks to a year to complete.
Euphausiids A class of shrimp-like zooplankton ranging in size from 1 to 6 cm that are major grazers of shelf and open-ocean blooms.
Neritic The relatively shallow part of the ocean extending from the low-tide mark to the edge of the continental shelf at *c*. 200 m depth.
Nutricline An abrupt gradient in nutrient concentrations at the bottom of the nutrient-impoverished mixed layer.
Phytoplankton Unicellular algae belonging to many different lineages that inhabit the surface layer of lakes and oceans and grow by converting dissolved plant nutrients taken up from their surroundings into biomass by means of photosynthesis, which requires adequate light energy and the green pigment chlorophyll.
Protists Unicellular eukaryotic organisms including autotrophic, mixotrophic and heterotrophic taxa from many different lineages.
Zooplankton The collective term for planktonic grazers that can migrate vertically but are too small to swim against ocean currents. They include larger multicellular organisms such as copepods, cladocerans and their predators as well as smaller unicellular protozoa.

Abbreviations

ASP	Amnesic shellfish poisoning
CFP	Ciguatera fish poisoning
DMS	Dimethylsulfide
DMSP	Dimethylsulfoniopropionate
HABs	Harmful algal blooms
HNLC	High-nutrient, low-chlorophyll
PCD	Programmed cell death
PSP	Paralytic shellfish poisoning
SML	Surface mixed layer

Defining Statement

Dense aggregations of phytoplankton cells of one or more species are loosely referred to as algal blooms. They play a central role in the ecology and biogeochemistry of all water bodies from ponds to the ocean, but a mechanistic understanding of the factors leading to the rise and fall of blooms is still lacking.

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Introduction

'Algal bloom' is a term of convenience applied to an outbreak of phytoplankton cells well above the average for a given region or water body. Blooms show up as peaks in the annual cycle of phytoplankton biomass and chlorophyll concentrations. They are ephemeral phenomena that arise when growth rates of one or more species of the phytoplankton assemblage exceed their mortality rates. As a result, cells of these species accumulate in the water column until their growth is checked by resource depletion, generally a nutrient such as phosphorus, reactive nitrogen, or iron. The magnitude of the bloom peak is determined by the nutrient concentrations prior to outbreak of the bloom. Since all phytoplankton contain chlorophyll *a*, which is easily assessed by a range of methods, the size of a bloom is conveniently expressed in units of chlorophyll. In productive water masses with high-nutrient availability, such as eutrophic lakes and coastal seas, chlorophyll concentrations at bloom peaks can reach >20 mg Chl m^{-3}, but over much of the ocean tenfold lower values are also commonly referred to as blooms because their concentrations again are five- to tenfold higher than the concentrations prevailing there over much of the year.

Phytoplankton blooms develop in the surface mixed layer (SML), so the total biomass or standing stock of a bloom is expressed as the concentration (cell numbers or biomass m^{-3}) multiplied by the depth of the mixed layer (cell numbers or biomass m^{-2}). The latter varies from less than a few meters in lakes to tens of meters in the ocean, so standing stocks of blooms in both regions are broadly similar because the higher concentrations of algae generally found in shallower mixed layers are compensated by the greater depth of the surface layer where concentrations are lower. It is the standing stock of the bloom that determines how much carbon is fixed per unit area and hence how much food is available to the larger organisms of higher trophic levels and to the microbes and animals (benthos) of the underlying sediments. It follows that the highest standing stocks (largest blooms) develop in SMLs of intermediate depths.

Algal blooms are short-lived phenomena superimposed on the ubiquitous microbial food web, which is the characteristic pelagic ecosystem prevailing in the surface layer of most of the oceans and large lakes (Fig. 1). The system maintains itself by recycling regenerated nutrients (ammonia, phosphate, and iron) between phytoplankton, comprising <2 μm solitary cyanobacteria and small (<10 μm) autotrophic flagellates, and heterotrophs, comprising bacteria, their nanoflagellate grazers, and larger protozoa in particular ciliates and dinoflagellates that feed mainly on the eukaryotic components of the food web. They in turn are grazed by copepods and other suspension-feeding zooplankton. Algal biomass is generally <20 mg Chl m^{-2}, that is, concentrations of <2 mg Chl m^{-3} and <0.5 mg Chl m^{-3} in coastal and open-ocean regimes, or eutrophic and oligotrophic lakes, respectively. Heterotrophic biomass is generally several fold that of the autotrophs. The opposite situation prevails during blooms: additional or new nutrients, generally in the form of nitrate, are introduced by admixture of nutrient-rich water, which disrupts the quasi-steady state of the microbial food web. The microbial community also profits from the new nutrients, but the bulk of the biomass increase is due to larger phytoplankton inaccessible to smaller grazers. These are the bloom-forming species.

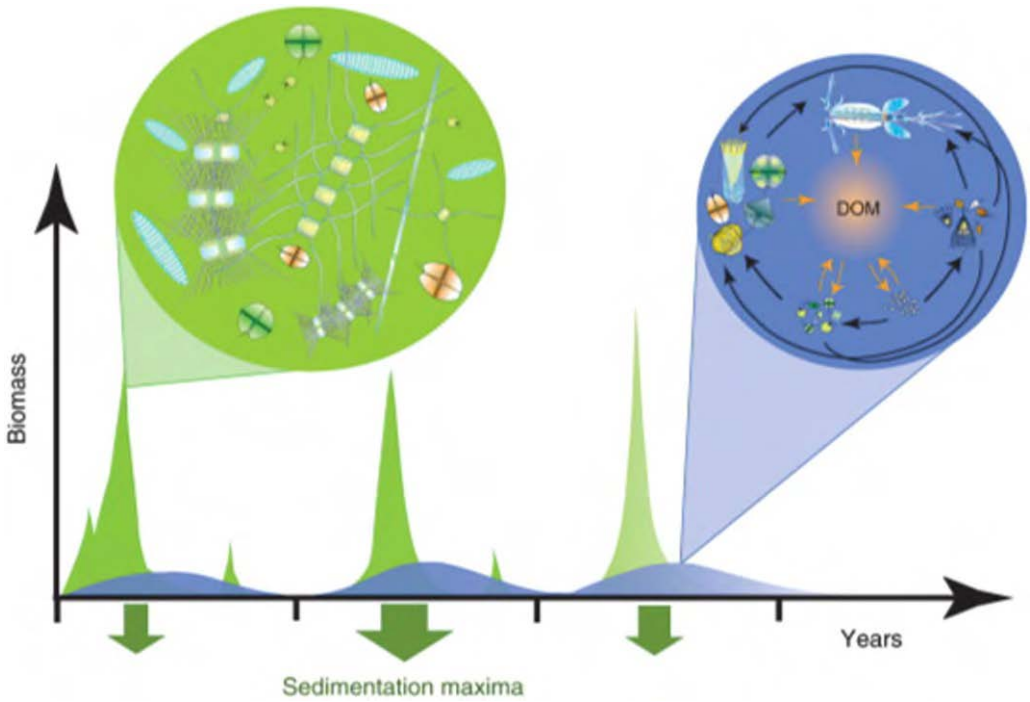

Fig. 1 A schematic representation of the relationship between seasonality of the microbial network (regenerating system) and superimposed blooms. The latter are followed by sedimentation pulses. DOM, dissolved organic matter. Modified by Christine Klaas from Smetacek, V., Scharek, R., Nöthig, E,M., 1990. Seasonal and regional variation in the pelagial and its relationship to the life history cycle of krill. In: Kerry, K.R., Hempel, G. (Eds.), Antarctic Ecosystems: Ecological Change and Conservation, first ed. Berlin, Heidelberg: Springer. pp. 103–114. (© Christine Klaas).

In the following we first deal with the so-called bottom-up factors that influence bloom formation, i.e., the physical and chemical properties of the environment. After an overview of the major genera and species contributing to blooms, we deal with the top-down factors – i.e., viruses, parasites, and grazers – that kill phytoplankton cells. The various types of algal blooms – recurrent and sporadic – are discussed followed by a brief account of harmful algal blooms (HABs). We conclude with some future research avenues that need to be explored to further our mechanistic understanding of the rise and fall of algal blooms.

Physical Environment of Blooms

The depth of the SML is generally determined by wind mixing, which in turn is a function of wind force and fetch, which is why the SML is shallowest in small, protected lakes and deepest in the open ocean. The density difference between surface and deeper layers constrains the depth of mixing because the greater the gradient the more energy required to mix across it. Generally, temperature determines density, so the SML is at its shallowest during the summer and in low latitudes. The steep density gradient between the warm SML and the underlying colder water is known as the thermocline. Exceptions are found in estuaries, brackish seas, and at the sea-ice edge during melting where the vertical salinity gradient has a greater effect than temperature in constraining depth of the SML. Here the boundary between the fresher SML and the saltier underlying water is known as the halocline. Pycnocline is the collective term for a density gradient whether caused by temperature, salinity or both.

The depth and rate of vertical mixing of the SML is of critical importance in bloom formation because it determines the light climate experienced by the phytoplankton suspended in it. Light intensity decreases exponentially with depth because light is absorbed by water and converted into heat. Suspended particles and some classes of dissolved organic matter such as humic acids also absorb light or scatter it, contributing to attenuation of light intensity with depth. In the clear blue water of the open ocean, living phytoplankton are the major light-absorbing particles because detritus plays a minor role and most protozoa and zooplankton are transparent to avoid visual predators. At the other extreme, in the green waters of turbid, shallow lakes and tidally mixed shallow seas, suspended particles (detritus and lithogenic particles) scatter and absorb a far greater percentage (>90%) of incoming light than the phytoplankton. So with increasing depth and turbidity of the SML, the light climate experienced by the phytoplankton population as a whole declines.

Net growth of an algal cell occurs only when carbon fixed by photosynthesis exceeds remineralization by respiration. The light intensity at which the two opposing processes balance each other is termed as the compensation light intensity and is in the order of 0.1% of incoming radiation at the surface. Its location in the water column sets the lower boundary of the euphotic zone within which net growth occurs. The depth of the euphotic zone can range from decimeters to meters at low solar angles (winter of high latitudes) and in highly turbid or ice-covered waters, to >50 m in the open ocean. The relationship between the depths of the euphotic zone and the SML determines the rate of bloom growth. If the SML is much deeper, then loss processes outweigh gains by photosynthesis, and phytoplankton biomass cannot increase. In the reverse case, given the presence of adequate nutrients, growth can be sufficiently rapid to attain bloom proportions.

Multi-year satellite records from the Subarctic Atlantic have now challenged the traditional view that blooms are caused by enhanced growth rates in response to improved light climate, rising temperatures and increased stratification. The satellite record showed that bloom initiation coincides with maximum mixed layer depth in winter despite the fact phytoplankton instantaneous growth rates (μ) are minimal in winter. This apparent discrepancy can be explained by decoupling of phytoplankton growth and mortality rates during deep winter mixing. Phytoplankton cells and their grazers are diluted thus lowering encounter rates between predator and prey and decoupling net population growth rates (r) from grazing pressure.

The relationship between SML and euphotic zone depths on bloom development is further rendered complex by the biology of plankton organisms. Thus, phytoplankton can adjust their photosynthetic machinery to work efficiently at low light levels (shade adaptation) of a deepwater column, but the cells suffer damage at high light intensities prevailing closer to the surface if transported there by vertical mixing. A day or two of full sunshine in otherwise cloudy weather can have the same negative effect on shade-adapted cells. Clearly, the species with the highest growth rates will have achieved the optimal trade-off respective to the inherently fluctuating physical environment. On the other hand, loss rates due to pathogens and grazers also vary widely and are generally of much greater importance than respiratory losses of the phytoplankton.

Satellite observations of chlorophyll distribution indicate that the occurrence of algal blooms is related to nutrient rather than light fields. Light limitation of growth rate is restricted to the few winter months at high latitudes and the perennially ice-covered Arctic Ocean. It should be mentioned that the daily photon flux in midsummer at the poles is equivalent to that in the tropics because low solar angle is compensated by the much greater day length. Temperature also has a secondary effect on algal growth rates because of cold adaptation. Unlike land plants that are exposed to widely fluctuating temperatures in the transition phase from dormant to growth seasons, aquatic plants can adapt to a narrow temperature range. Thus, maximum division rates close to the freezing point can be as high as one per day, which is more than half that attained in the tropics. Indeed among the highest rates of primary production measured anywhere (>10 g C m^{-2}) were recorded in dense blooms developing in the nutrient-rich, shallow SML of the Bering Sea inflow to the Arctic Ocean.

Chemical Environment of Blooms

A prerequisite for the growth of blooms is the availability of essential elements of which the macronutrients phosphorus and reactive nitrogen and the micronutrient iron can limit build-up of blooms. Phytoplankton grow by taking up dissolved nutrients and incorporating them into biomass. Upon cell death, microalgae sink out of the SML, which is thus nutrient-impoverished. Nutrients are supplied back by mixing of deep, nutrient-rich water to the SML. In high and mid latitudes, this is invariably due to

cooling and breakdown of thermal stratification by convective mixing during autumn and winter. In the tropics, upwelling of deeper water due to large-scale lateral advection of the surface layer is the major source of nutrients although runoff from land is also locally important. The passage of storms also mixes nutrients into the SML.

Bloom biomass is limited by the concentration of the nutrient in shortest supply. There is now general consensus that it is phosphate in lakes and some oligotrophic seas (e.g., the Mediterranean), nitrate in iron-rich coastal waters, and iron in the open ocean with the exception of most of the North Atlantic. The concentrations of phosphate and iron are limited by their geochemical properties. Thus, maximum winter concentrations of $\sim$1 μmol PO_4 L^{-1} seem to be the rule in coastal regions where surface water is in interaction with oxygenated sediments. The corresponding iron concentrations are >1 nmol Fe L^{-1}, which are well in excess of the P:Fe ratio required by phytoplankton. In contrast, dissolved molecular nitrogen is abundant and can be fixed to reactive forms by cyanobacteria, which results in maintenance of N:P ratios commensurate with the demands of phytoplankton. Because of the extreme insolubility of ferric hydroxide (rust), iron is selectively lost from the SML and becomes the limiting nutrient in land-remote regions. The subarctic North Pacific Ocean and the entire Southern Ocean harbor perennially high concentrations of nitrate and phosphate because of iron limitation of phytoplankton growth. The same is true of the equatorial Pacific. Phytoplankton growth is not continuously iron-limited in the high latitude and equatorial Atlantic presumably due to iron input by dust emanating from the adjacent arid regions.

As unicellular algae are too small to experience shear stress caused by the kinetic energy of water they do not require investment in supporting structures such as carbohydrates to maintain shape and position as do all larger metaphytes. They also have less storage capacity for reserves such as starch or lipids, which, in sessile plants, enable spurt growth at the beginning of the growth season as a means to overgrow competitors for space and light. As a result, the C:N:P ratio of aquatic unicellular organisms resembles that of living cytoplasm and varies <20% around the global average of 106:16:1. This is known as the Redfield ratio after the scientist who first discovered its relative constancy not only in suspended matter but also in dissolved nutrients in the deep ocean. It should be pointed out that variation in the Redfield ratio within species as a result of adaptation to the growth environment can be as great as between the algal classes.

Major Contributors to Algal Blooms

Of the several thousand phytoplankton species described so far <5% have been reported to contribute significantly to algal blooms in lakes and oceans, which implies that the ability to form blooms is not an indication of evolutionary success. Many species are widely distributed and regularly contribute to blooms throughout their range, implying that adaptation to a specific set of environmental conditions, such as nutrient ratios or light conditions, is not a prerequisite. Bloom-forming species differ widely from one another in shape, size, and behavior, and – because they belong to different phylogenetic groups – also differ in their pigment composition and biochemistry. Not surprisingly, their blooms also have very different impacts on the ecosystem and biogeochemistry. Their degree of recurrence also varies. Some species bloom every year in the same water body and are an integral part of the seasonal cycle. Others reach bloom proportions only in some years but always in the same season. Yet others form blooms seemingly haphazardly. It is thus not possible to formulate generalizations about bloom-forming species other than that their size (cell diameter, chain length, and colony diameter) tends to be larger than 10 μm. Since smaller cells tend to have higher growth rates, the ability to grow fast per se cannot be regarded as a prerequisite for bloom formation. Apparently, the ability to withstand attack by pathogens and herbivores by means of larger size and other defenses is hence of greater importance in enabling accumulation of cells. Here, we refer to species differentiated under the microscope, that is, on the basis of their morphology (Fig. 2). In the last decades, the use of molecular approaches coupled with detailed ultrastructural studies showed the presence of several 'cryptic' species, i.e., one morpho-species includes genetically distinct species that have exactly the same morphology or differ only for some minor ultrastructural details not visible in light microscopy. This is the case of several important bloom-forming species, which are the better studied ones and which have been often split in a number of species with distinct biogeography. In order to achieve a morphology-independent assessment of species diversity, molecular markers such as the V4 or V9 region of 18S rDNA, are used as barcodes in studies based on the analysis of high-throughput environmental sequencing data. In the following we deal with the bloom-forming species according to taxonomic groupings with some brief notes on their ecology and impact.

Cyanobacteria

This group, formerly known as blue-green algae, are prokaryotes and the progenitors of the chloroplasts of all eukaryotes. They possess essentially the same photosynthetic machinery as the algae but, unlike all algae, some species of cyanobacteria are able to fix molecular nitrogen and channel it into the ecosystem. Another unique property of the larger species is their ability to secrete gas bubbles within minute intracellular vesicles, which renders them positively buoyant and results in formation of conspicuous scum on the surface. Cyanobacteria are present in freshwaters, soil, as endosymbionts in some plants and lichens; in the oceans there are unicellular species (*Synechococcus*, *Prochlorococcus*) and the large filamentous *Trichodesmium* in tropical waters.

Cyanobacteria tend to be rare when fixed nitrogen in the form of nitrate or recycled ammonia is available, but increase their numbers when nitrogen becomes limiting for algal growth but other nutrients, such as phosphate and iron, are still available. This situation invariably arises in the summer months when the N:P ratio, normally at 16:1, reaches its annual minimum. In oxygenated waters, characteristic of most of the ocean as well as oligotrophic deep lakes, this happens because P is recycled more rapidly than N.

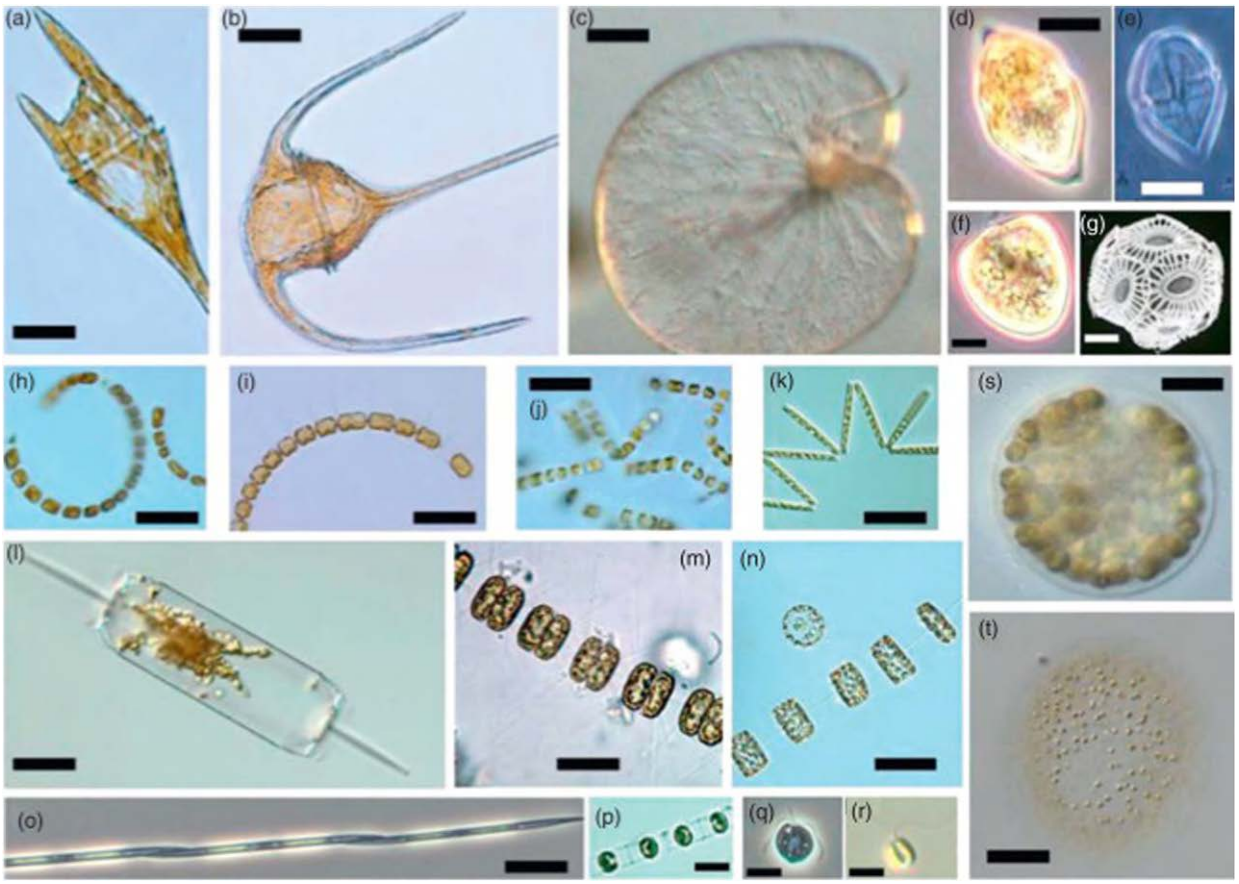

Fig. 2 Pictures of prominent bloom-forming species. (a) *Tripos furca*, (b) *Tripos mülleri*, (c) *Noctiluca scintillans*, (d) *Heterocapsa triquetra*, (e) *Scrippsiella trochoidea*, (f) *Prorocentrum minimum*, (g) *Emiliania huxleyi*, (h) *Chaetoceros debilis*, (i) *Chaetoceros curvisetus*, (j) *Chaetoceros socialis*, (k) *Thalassionema nitzschioides*, (l) *Ditylum brightwelli*, (m) *Thalassiosira nordenskioeldii*, (n) *Thalassiosira rotula*, (o) *Pseudo-nitzschia lineola*, (p) *Skeletonema costatum*, (q) *Chrysochromulina polylepis*, (r) *Phaeocystis antarctica* solitary cell, (s) *P. antarctica* compact small colony, and (t) *P. antarctica* large colony. Scale bars = 2 μm (g), 5 μm (f), 10 μm (d, e, m, and p–r), 15 μm (a), 20 μm (l, n, o, and s), 30 μm (b), 50 μm (h, j, and k), 60 μm (i), 100 μm (t), 200 μm (c). Light micrographs (o), (r), (s), and (t) by Marina Montresor. Scanning electron micrograph (g) was taken by Philipp Assmy. All other light micrographs were taken from the open access repository for plankton-related information PLANKTON*NET (URL: http://planktonnet.awi.de) of which (a), (b), (h–k), (m), and (p) were kindly provided by Mona Hoppenrath; (d) and (f) by Regina Hansen; (l) and (n) by Tanya Morozova; (c) by Susanna Knotz; (q) by http://www.algaebase.org/index.lasso; and (e) by Alexandra Kraberg.

In shallow eutrophic lakes with suboxic deep water, nitrate is oxidized to N_2 by denitrifying bacteria, which further lowers the N:P ratio. The lowest N:P ratios (<10:1) are found in water bodies overlying anoxic sediments that mobilize not only P but also Fe. It follows that the highest concentrations of cyanobacteria occur in highly eutrophic lakes and ponds.

The most common bloom-forming genera in freshwater, but also brackish seas such as the Baltic, are dealt with next. The ubiquitous genus *Microcystis* builds spherical colonies, but all the other genera occur either as solitary cells (such as the bacteria-sized *Synechococcus* and *Prochlorococcus*) or as chains (*Nodularia*, *Aphanizomenon*, and *Anabaena*). The latter generally form aggregates in the shape of bundles or globular clumps of chains. These species have specialized cells known as heterocysts in which nitrogen is fixed. Cyanobacteria are generally well defended and some species, particularly *Microcystis*, *Anabaena*, and *Nodularia*, sometimes secrete potent toxins. Blooms of these species can pose a serious problem in reservoirs and lakes. The only genus that regularly forms blooms in the ocean is *Trichodesmium*, which grows in sheathed chains (trichomes) that form large aggregates causing a reddish-brown coloration of surface waters in many warm ocean regions.

Diatoms

Most algal blooms in oceans and lakes are dominated by a broad range of species belonging to this group (Fig. 2(h–p)). Diatoms are characterized by their silica shells comprising two half-boxes that fit tightly into one another. The shell surfaces are dotted with minute pores that only allow dissolved substances to enter into the cells. Diatoms can also regulate buoyancy despite the ballast effects of the shell. Shape and size of bloom-forming diatoms vary widely from long-needle-shaped cells up to few millimeters long to small cylindrical cells in long chains. Some species have long silica spines or chitin threads that protrude outward from the cells.

Bloom-forming species are scattered over all diatom lineages (raphid and araphid pennates and various families of centrics) and many genera. Pennate diatoms often dominate algal biomass in sea ice habitats and the microphytobenthos.

The most prominent recurrent feature of the seasonal plankton cycle in temperate and boreal systems is the spring bloom, which is generally dominated by comparatively few species of unrelated genera of diatoms. Typical examples of recurrent bloomers are species of the cosmopolitan diatom genera *Skeletonema*, *Thalassiosira*, and *Chaetoceros* (Fig. 2(h–j, m, n, and p)). *Skeletonema* blooms occur throughout the world with the exception of the polar oceans. They are prominent not only in the spring blooms of the brackish Baltic and the open Atlantic, but also in upwelling blooms of the tropics. *Skeletonema costatum* (Fig. 2(p)), was considered to be a cosmopolitan species but it has been shown that genetically distinct species, which differ in their geographic distribution and seasonal occurrence, share the same gross morphology. A wide variety of species within the genus *Thalassiosira* form blooms worldwide. A few examples include *Thalassiosira antarctica* responsible for blooms in layers underlying sea ice in the Antarctic; *Thalassiosira weissflogii*, *Thalassiosira nordenskioeldii*, and *Thalassiosira rotula* (Fig. 2(m and n)) that contribute to blooms in temperate and boreal areas; and *Thalassiosira partheneia* that builds large (>1 cm diameter) colonies and is found in the upwelling region of northwestern Africa.

The bloom-forming *Chaetoceros* species belong to the subgenus *Hyalochaete* that includes species without chloroplasts in the setae. Most bloom-forming *Chaetoceros* species are capable of converting vegetative cells into thick-walled resting spores that overwinter on the sediment surface in shallow environments, inside the sea ice in the seasonal sea-ice zone, and in the pycnocline in the open ocean. The widespread and consistently high accumulation rates of *Chaetoceros* resting spores in the Southern Ocean throughout the last glacial and their restricted occurrence during the Holocene provide compelling evidence for substantially higher productivity and organic carbon export into the deep ocean during the last glacial and highlight the potential of resting spores as biological proxies for paleoceanography. Three prominent species are *Chaetoceros socialis*, *Chaetoceros debilis*, and *Chaetoceros curvisetus* (Fig. 2(h–j)), with the former two species showing a more polar-to-cold temperate distribution and the latter species a warm temperate distribution. However, also in the genus *Chaetoceros* there are several cases of 'cryptic diversity': as an example, it is *C. gelidus* and not *C. socialis* that is abundant in the Arctic and sub-Arctic region. The two species share the same gross morphology and the distinctive globular arrangement of their chains but differ in their genetic profile, the morphology of spores and physiological traits.

The cosmopolitan needle-shaped pennate genus *Pseudo-nitzschia* comprises many species that are difficult to differentiate under the light microscope but some of them can contribute substantially to both open-ocean and coastal blooms. This is one of the best studied diatom genera because it includes several species capable of producing the toxin domoic acid, harmful to humans and other vertebrates. Pennate diatoms have a heterothallic mating system, i.e., cells belong to two different mating types, and this enabled to test reproductive isolation, i.e., the 'biological species concept' amongst different cryptic *Pseudo-nitzschia* species.

The needle-shaped, thin-shelled centric genus *Rhizosolenia* commonly forms blooms later in the year. A dense bloom in which the cells were aggregated into mats was reported from the equatorial Pacific where the mats apparently carried out vertical migration between the surface and the nutrient-rich deeper layer. Another widespread species that is common in the summer months is *Ditylum brightwelli* (Fig. 2(l)), which has, however, once formed an almost monospecific spring bloom in the southern North Sea.

Haptophytes (Prymnesiophytes)

This group comprises small flagellates equipped with a prehensile organ, the haptonema, which is used to feed on small particles such as bacteria (Fig. 2(g and q–t)). The cell of these microalgae is surrounded by thin organic scales that, in coccolithophores, bear a mineral layer made of calcium carbonate. These calcareous platelets are called coccoliths. A common characteristic of this group is the copious production of dimethylsulfoniopropionate (DMSP), which is split by an enzyme into volatile dimethylsulfide (DMS) and the noxious acrylic acid. Several functions of the widespread molecule DMSP have been suggested such as a compatible solute in osmoregulation, defense against predators, and as an antioxidant. DMS released from haptophyte blooms is a major global source of sulfur to the atmosphere where it is oxidized to hygroscopic sulfate, which can act as a cloud condensation nucleus. Large amounts of sulfur in the atmosphere result in smaller droplets, that is, whiter clouds that scatter sunlight back into space, thus contributing to cooling. The global significance of haptophyte blooms in cooling the planet is under debate.

The calcareous coccolithophores are second in importance to the diatoms in their contribution to extensive oceanic blooms. These normally arise in the aftermath of the spring diatom bloom and, because of the high reflectance of their coccoliths, are visible from space. The species *Emiliania huxleyi* (Fig. 2(g)) is the most prominent member of this group and forms blooms in both coastal and open-ocean regions. Extensive genome variability within this species seems to explain its ecological success across large latitudinal and environmental gradients. Coccolithophores exhibit highly complex life cycles in which haploid and diploid, stages, some bearing different types of coccoliths, alternate with one another. Blooms of *E. huxleyi* are often terminated by the spread of viral infection within the population. Interestingly, despite its numerical dominance in the surface layer, *E. huxleyi* contributes only a minor fraction of the total calcareous material accumulating in the deep North Atlantic. Less abundant but more heavily calcified coccolithophores like *Calcidiscus leptoporus* contribute the bulk of accumulated calcareous sediments.

Another group of haptophytes involved in extensive blooms in both temperate and polar waters are species of the genus *Phaeocystis*. At least six species have been formally identified worldwide based on morphological and genetic characteristics but some of these taxa may hide cryptic diversity. Three species – *Phaeocystis pouchetii* (Arctic), *Phaeocystis globosa* (temperate and tropical waters), and *Phaeocystis antarctica* (Antarctic, Fig. 2(r–t)) – dominate blooms. These species form colonial stages comprising many hundreds of cells. The life cycle however includes solitary nanoflagellates and coccoid stages. The flagellate stage suffers much higher

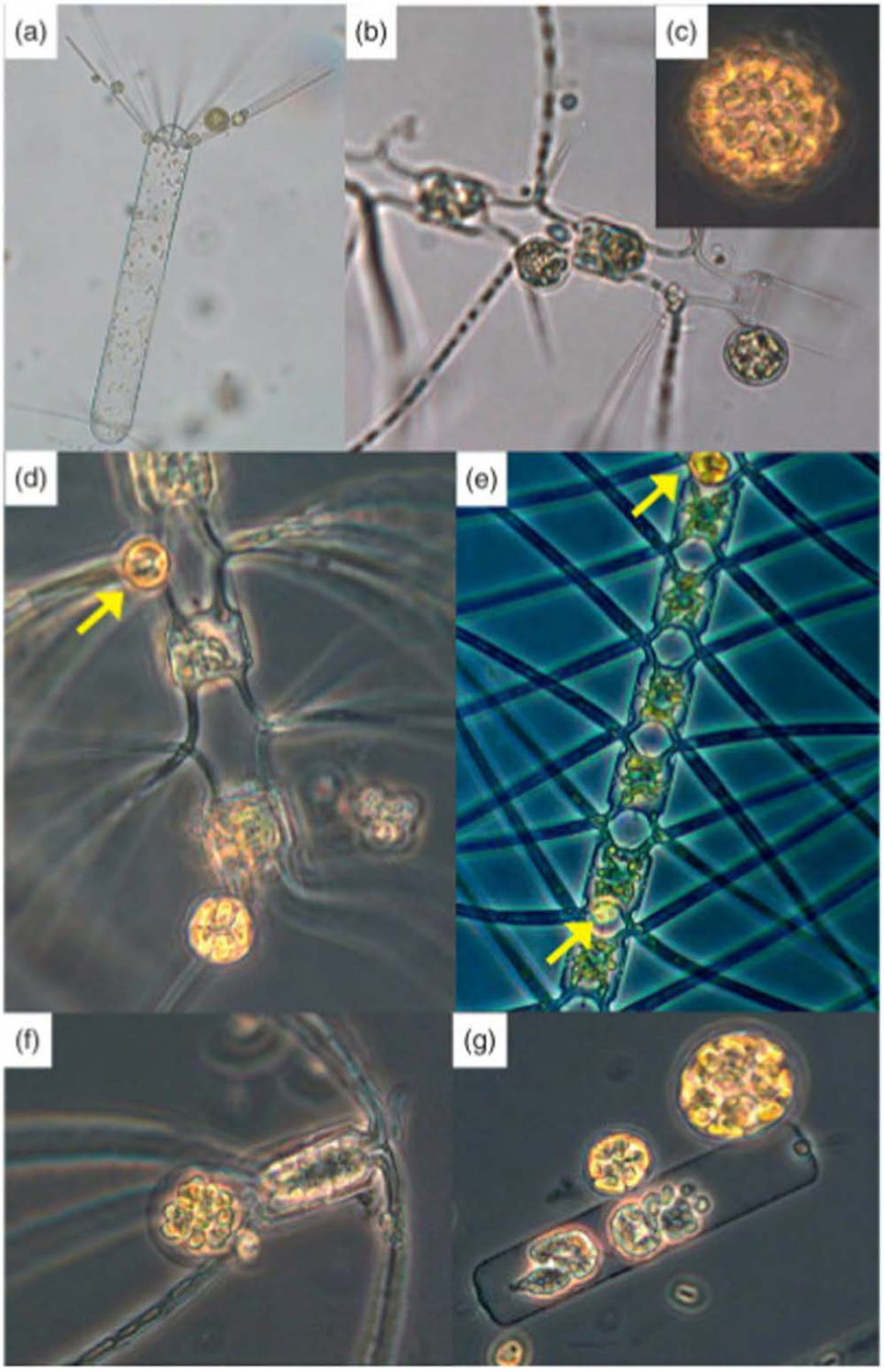

Fig. 3 Colonies of *Phaeocystis antarctica* attached to different diatom species (from field material): (a) Spines of *Corethron pennatum*; (b) setae and cell wall of *Chaetoceros dichaeta*; (c) close-up of colony; (d) *Chaetoceros dichaeta*; (e) *Chaetoceros atlanticus*; (f) *Chaetoceros peruvianus*; (g) *Guinardia cylindrus*. Light micrographs by Philipp Assmy.

mortality by both viral infection and protozoan grazing than the colonial one, indicating that colony formation is a defense strategy. The fact that colonies in their early stages are often found attached to the spines of diatoms (e.g., *Chaetoceros*, *Corethron*) also suggests that small colonies are more vulnerable and find protection from protozoan grazing on these large diatoms (Fig. 3). In the North Sea, *P. globosa* tends to attain dominance in the later spring, in the aftermath of the diatom bloom following Si exhaustion. Extensive

blooms dominated by *P. antarctica* are a regular feature of the Ross Sea but, for unknown reasons, are more sporadic elsewhere around Antarctica. On rare occasions dense blooms of the solitary flagellate genera *Chrysochromulina* and *Prymnesium* occur in some regions.

Dinoflagellates

This is an ancient, heterogeneous group comprising autotrophic, mixotrophic and heterotrophic modes of nutrition across a wide range of shapes and sizes (Fig. 2(a–f)). Interestingly, the morphology of photosynthetic and predatory forms does not differ, signifying that their shapes and sizes have not evolved to maximize resource acquisition. The life cycle of several dinoflagellates includes the formation of resting cysts, non-motile stages surrounded by a thick cell wall that can fossilize thus leaving a fingerprint of these organisms over extended time scales. Small-celled dinoflagellates (~10 μm) encased in cellulose armor (e.g., *Prorocentrum*, *Heterocapsa*, *Scrippsiella*) (Fig. 2(d–f)) commonly form blooms in the aftermath of the spring diatom bloom in coastal and shelf areas. Larger, often unarmored forms such as *Gymnodinium* form sporadic blooms not only in the summer months but also in upwelling regions. Spectacular blooms of the heterotrophic bioluminescent *Noctiluca scintillans* (Fig. 2(c)), colored by pigments from their algal food, cause discoloration of large stretches of surface waters mainly in sheltered bays or estuaries. Because of their motility and comparatively large size, some dinoflagellates have been observed to migrate vertically to take up nutrients below a shallow SML. Many species also form dense layers at density discontinuities, which reach the surface along hydrographic fronts where they are highly conspicuous. The standing stocks of such blooms are low, despite their dense concentrations because they are restricted to thin layers.

The largest dinoflagellate blooms in both lakes and the ocean are formed by species of the large, armored genera *Ceratium* and *Tripos* (new genus name for the marine *Ceratium*) during the autumn. For unknown reasons, grazing pressure on this genus is low, suggesting that survival rather than fast growth rates is the major reason for bloom build up over the late summer (Fig. 2(a and b)).

Other Groups

The phototrophic, cosmopolitan ciliate *Mesodinium rubrum* (also known as *Myrionecta rubra*) is an active swimmer that, like dinoflagellates, carries out vertical migration and sometimes forms dense reddish layers at the surface. Its cells are packed with cryptophyte chloroplasts that still carry the cryptophyte nucleus. Blooms of this species are common and have been reported from habitats as diverse as tropical upwelling regions, the inner Baltic and the Arctic shelf. Large blooms of this ciliate tend to be sporadic but patchy blooms occur regularly in the North and Baltic seas in early summer. Other groups reported to contribute significantly to algal blooms are the euglenophytes, cryptophytes, and raphidophytes; their blooms are only of local importance. In lakes, various species of chlorophytes and chrysophytes form blooms under eutrophic conditions.

Pathogens and Grazers of Algal Blooms

The important role of viruses in the sea has only come to light over the past three decades. It is now known that bacterial populations are susceptible to viral infection and that their density is partly regulated by this factor. Less is known about viral infection of algae but blooms of the coccolithophore *Emiliania huxleyi* can be terminated by mass viral infection. It has been demonstrated that viruses can re-shape the antioxidant metabolic network of the host cell during infection, which is essential for a successful viral replication cycle. Viruses of *E. huxleyi* have been detected in copepod guts and can be dispersed by these crustaceans and by their fecal pellets over relatively long distances. An even more effective dispersal can be provided by aerosol, where *E. huxleyi* viruses can remain infective allowing potential dispersal and infectivity over hundreds of kilometers. So far, only few viruses have been found to infect diatoms and dinoflagellates, and the viruses affecting other groups have barely been studied.

Another relevant but poorly known source of mortality for phytoplankton is represented by parasites. They belong to various protistan lineages (e.g., chytrids, various stramenopiles and syndiniales) and many of them share the presence of small flagellates (zoospores) that represent the infective vector. Zoospores can attach themselves to large diatoms and insert feeding tubes through pores in the frustule or between the girdle bands or through the sutures between thecal plates in dinoflagellates. The parasite multiplies while feeding on the microalgal plasma, releasing large numbers of flagellates that can infect other cells. Parasites can be species-specific in prey selection and have the potential to decimate prey populations during blooms. However, the diversity of parasites, their infection modality, and their impact on natural microalgal populations is still largely unexplored.

A wide range of grazers is present in the plankton: puncturing and ingesting protists (i.e., unicellular eukaryotes) that prey on individual microalgal cells and chains, suspension-feeding copepods that select and ingest particles individually, and filter-feeding larger metazooplankton such as euphausiids (krill) that collect particles *en masse* and ingest them indiscriminately. Protistan grazers have growth rates equivalent to their phytoplankton prey and many have evolved techniques to cope with prey sizes as large as or even larger than themselves. Complexity is introduced by the tendency of copepods to selectively feed on protistan grazers of phytoplankton, in particular ciliates and heterotrophic dinoflagellates, which results in a trophic cascade favoring accumulation of algal cells.

Dinoflagellates have evolved the broadest range of feeding techniques: some ingest prey cells up to their own size, others pierce the cell wall and suck out the contents with a feeding appendage known as the peduncle. Several genera of armored dinoflagellates are able to extrude a feeding veil known as the pallium, which can envelope an entire chain of diatoms many times the size of the

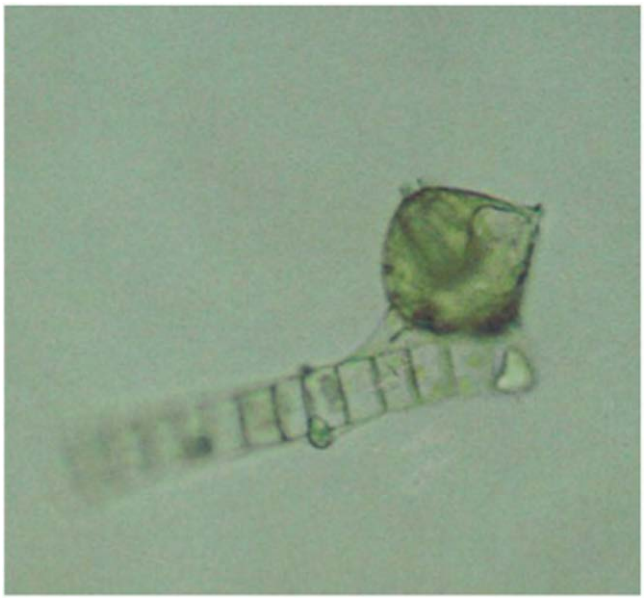

Fig. 4 A pallium-feeding dinoflagellate of the genus *Protoperidinium* capturing a pennate chain-forming diatom. Light micrograph by Christine Klaas.

predator and digest it outside the cell wall (Fig. 4). The pallium is retracted after digestion is completed. The entire process of prey capture and pallium retraction lasts for 10–20 min. Again, decimation of algal blooms by dinoflagellates is rarely reported although they have the potential to do so. In all likelihood, the ubiquitous small copepods selectively feed on large heterotrophic protists, which releases grazing pressure on the algae, particularly diatoms.

Metazooplankton, in particular copepods and euphausiids, have complex life cycles involving a range of larval stages that take weeks or months to complete, depending on temperature in opportunistic species, or innate life cycle traits in species with pronounced seasonality. The latter generally have larval stages that overwinter at depth and ascend to the surface in spring. It is widely believed that blooms arise because copepod populations are unable to match their appearance at the surface with the timing of bloom initiation and subsequently gear their population size to the growing bloom. However, this neither explains why other grazers, in particular unicellular herbivores, are unable to suppress blooms, nor does it explain why copepods, if they are indeed so inefficient at utilizing potential food sources, nevertheless manage to maintain the bulk of zooplankton biomass in oceans and oligotrophic lakes.

Mortality is not just caused by an external agent but autocatalytic cell death or apoptosis, analogous to programmed cell death (PCD) in multicellular organisms, does occur in unicellular algae and has already been reported from cyanobacteria, green algae, coccolithophores, dinoflagellates, and diatoms. PCD seems to play a crucial role in the coordinated collapse of phytoplankton blooms but the mechanisms are still not fully understood. This cellular self-destruction process in phytoplankton is triggered by specific environmental stressors that range from cell age, nutrient limitation, high light and/or UV exposure, and reactive oxygen species. Furthermore, it has been shown that lytic viral infection and autocatalytic PCD in the coccolithophore *E. huxleyi* work in tandem.

Mortality or inhibition of growth can be also attributed to allelochemical compounds that are produced by many unicellular microalgae. This is the case of dinoflagellate species of the genus *Alexandrium* that can produce lytic compounds active on a broad range of taxa and it has been hypothesized that those allelochemical compounds may play a role in the formation and maintenance of *Alexandrium* blooms.

It has long been appreciated that, unlike terrestrial vegetation, the bulk of phytoplankton cells are grazed. So, given the diversity of phytoplankton grazers and their potential to overgraze their food, it is indeed surprising that blooms develop at all. However, it should be remembered that only a few of the species present contribute significantly to algal blooms. The other species either do not respond with high growth rates to the advent of favorable conditions or are grazed faster. The conclusion is that understanding bloom dynamics requires basic knowledge of the ecophysiology and species-specific regulatory mechanisms inherent to the bloom formers as well as a deeper understanding of the complex interactions and potential trophic cascades within pelagic food webs. Given the great variability inherent to complex biological systems, it is indeed surprising that there is any degree of recurrence in the annual cycles of species composition and biomass in lakes and the sea.

Recurrent and Sporadic Algal Blooms

Algal blooms can be broadly differentiated into seasonally recurrent and sporadic stages in the annual cycles of plankton of a given water body or region. The former tend to be initiated by seasonal change in the physical regime. At higher latitudes, this is due to

warming and shallowing of the SML by thermal stratification in the spring, and its breakdown by convective mixing during the autumn. The former process improves the light supply of the cells trapped in the SML and the latter reintroduces nutrients lost by sinking from it. Spring and autumn blooms, respectively, are the result of these physical processes (Fig. 1). Blooms are also a regular feature of the upwelling regions of lower latitudes. Long-term data series on the annual cycles of phytoplankton gathered from coastal monitoring sites have provided valuable information on many of the recurrent and sporadic aspects of bloom phenomena outlined in the following paragraphs.

Spring Blooms

Algal blooms, almost always dominated by diatoms, are regular events in the annual cycles of all lakes and coastal oceans that arise when deep mixing during the winter is stopped by the advent of surface stratification during spring (Fig. 1). The timing of the spring bloom is influenced as much by the hydrography of the region as its latitude (light intensity). Thus, spring blooms along the melting ice edge off Greenland occur well before the bloom on the Norwegian shelf 1000 km ($\sim 10^{\circ}$ latitude) further south. This fact was first noticed in the 1930s and attributed to earlier improvement of the light climate by shallow stratification due to meltwater in the Arctic as compared to the slower establishment of thermal stratification in the stormy northern North Sea. Similarly, blooms start about a month earlier in protected bays and fjords than in the adjacent, open shelf. Turbidity of the water also plays an important role in spring bloom initiation, which occurs much later in the shallow, highly turbid water overlying the tidal mud flats of the German Bight in the southern North Sea than in the clearer water of the tide-free Baltic Sea on the other side of the Danish Peninsula.

The dynamics and composition of the spring bloom varies widely from region to region and also from year to year in the same region, depending on weather conditions, phytoplankton seeding stocks, and prevailing grazing pressure. Thus, when bloom initiation coincides with prolonged calm, sunny weather the bloom peak is reached within 2–3 weeks and is often dominated by only a few diatom species with a high initial seeding stock. At the other extreme, bouts of stormy weather alternating with calmer periods at intervals of a few days can prolong the life time of the bloom by many weeks. In such cases different species dominate biomass across a succession of smaller peaks until nutrient exhaustion is reached and the recycling community established.

The fate of bloom biomass varies depending on its dynamics. In the first case, algal biomass is much higher than that of the heterotrophs and the bloom is terminated by mass sinking of ungrazed cells within a few days. Although the termination phase of blooms is less well studied than the longer growth phase, it is likely that morphology and behavior of the species involved influence the depth and extent to which bloom biomass reaches the sea floor. Thus diatom species tend to sink out quantitatively and the long chains of the spiny genus *Chaetoceros* clump into aggregates, aided by sticky mucilage that entrains other cells in the large, rapidly sinking flocs. Such boom-and-bust-type blooms transfer a large amount of organic matter to the benthos and, when they overlie deeper water, play a significant role in sequestering carbon in the deep water column and sediments.

Consensus is emerging that diatoms are ubiquitous in spring blooms not only because they have high growth rates but also because they are better defended than algae from other groups and hence do not represent an ideal food source. This is at odds with the long-held view of diatoms representing the pastures of the sea. The first and most obvious line of defense constitutes the silica shell that provides mechanical protection against a range of crustacean predators, wards off protistan grazers through long and spiny cell appendages and restricts access to the encased cell for piercing predators and parasites. Although copepods have evolved silica-edged mandibles to crack diatom shells, laboratory experiments and field observations suggest that they prefer protozoa over diatom food. Why this is so is still under debate. One line of explanation suggests that diatoms are nutritionally less valuable than protozoa; another explanation is that diatoms produce substances noxious to copepods, either directly or by reducing their reproductive efficiency. Thus, it has been shown that some diatom species contain enzymes that cleave structural lipids into toxic aldehydes when the shells are crushed. Diatom aldehydes are teratogens, that is, the feeding adults are not affected but egg viability is reduced and the larvae develop malformations. However, only some diatoms possess the enzymes that form aldehydes, and some copepod species are more susceptible to its effect than others. So this is by no means a universal defense but merely one of the weapons in an arsenal of unknown diversity.

The dominant spring bloomers in lakes also tend to be diatoms whereby the centric genera *Melosira* and *Cyclotella* and the pennate species *Asterionella formosa* often dominate. Indeed the latter species has always dominated the spring bloom of Lake Windermere over the past 60 years of continuous observation. In other lakes, its presence fluctuates annually as a result of infection by a parasite.

A pronounced spring bloom, generally dominated by diatoms, was long considered an invariable feature of high and midlatitude seasonal cycles but recent analyses of long-term data indicate otherwise. Thus, a winter–spring bloom of *Skeletonema* used to occur every year over decades in Narragansett Bay, Rhode Island, USA, but is absent since about a decade. Blooms now occur later in the year and have different characteristics to the spring bloom. A similar situation has been reported from San Francisco Bay and could be related to the spread of an invasive filter-feeding bivalve that prevented the bloom from developing. The situation changed when a basin-scale shift in the climate regime led to more input of shelf water to the Bay, which introduced fish predators that checked benthic filter feeders from grazing down the phytoplankton. These examples indicate the complex interactions between bottom-up and top-down factors in shaping the annual cycles of pelagic ecosystems. Long-term data are normally recorded adjacent to research institutes and it is likely that similar variation occurs in deeper, offshore water.

Summing up, there is still a lot we do not understand about the dynamics and fate of spring blooms, particularly at the species level.

Autumn Blooms

Autumn blooms arise when enhanced vertical mixing due to stormy weather and cooling deepens the summer thermocline, thereby introducing new nutrients to the SML (Fig. 1). The biomass and species composition of autumn blooms vary regionally more than those of the spring and they do not occur in all high- and midlatitude oceans. However, much less effort has been invested in their study as compared to spring blooms. In many regions, the autumn bloom is dominated by diatoms that, in the Kiel Bight, can reach their peak as late as mid-November. The species involved are similar to the spring bloom but genetic studies have revealed that the spring *Skeletonema* species is different to the autumn one. Although the characteristic spring genera are present in autumn diatom blooms, the species assemblage tends to be more diverse and includes dinoflagellates and larger ciliates. The latter is probably due to the low number of copepods. Where studied, the bulk of bloom biomass sinks out of the water column.

In regions where dinoflagellates dominate late summer plankton, an early autumn bloom comprising species belonging to this assemblage develops in the initial stages of thermocline erosion. Most prominent are species of the armored genus *Tripos* (= the marine *Ceratium*), in particular the cosmopolitan species *T. muellerii*, *T. fusus*, and *T. furca*. These species are apparently unpalatable although their defense mechanisms, other than their cellulose armor plating, are not known. They have comparatively low growth rates but because their populations accumulate over the summer months, a few divisions over several weeks in the nutrient-rich, autumn water column suffices to build up large biomasses that, in some regions, can rival those of the spring bloom. Growth of their bloom is generally accompanied by declining zooplankton populations, which can be interpreted as a sign of success in the evolutionary arms race.

Tripos blooms regularly occur in many, but not all, coastal environments from the tropics to the Arctic but they are absent around Antarctica. They are apparently terminated by mass cell death followed by sinking, but the sinking rates and the depth to which they sink are not known. Recurrent large *Tripos* blooms regularly lead to anoxic events in the Laholm Bay of southern Sweden. A massive *Tripos muelleri* (formerly *Ceratium tripos*) bloom, which for unknown reasons survived through the winter and sank the following spring, caused widespread anoxia and collapse of the commercial scallop fishery in New York Bight in 1976. This was a one-time event. The same can be said of a massive *Tripos furca* bloom that caused extensive anoxia in the late summer of the North Sea in 1981, but has not occurred since.

Blooms in Upwelling Regions of Low Latitudes

Upwelling of nutrient-rich, cold, deep water occurs seasonally or for longer periods along the western continental margins. The intensity of upwelling varies regionally and over periodic cycles of several years of which the El Nino oscillation is the best known. Dense blooms of diatoms develop in regions with intense upwelling but, because of the high sun angle and rapid warming of the newly replenished SML, algal growth rates are much higher and the bloom peak is reached faster than in spring blooms. Nevertheless, as mentioned in the section on diatoms, much the same diatom genera typical of the midlatitude spring blooms – *Skeletonema*, *Thalassiosira*, *Chaetoceros* – are prominent contributors to biomass, although *Phaeocystis* blooms have only been reported from the Arabian Sea. There is considerable regional and interannual variability in bloom magnitude and composition depending on the intensity of upwelling. Thus, the depth from which the upwelling water emanates can favor either diatom or dinoflagellate blooms. Extensive blooms of *M. rubrum* have also been reported off Peru.

The structure and response of the food web is generally similar to that of spring blooms and again, much the same genera of protozoa and copepods are represented. However, in some upwelling regions, the local sardine-like fishes have developed fine-meshed gill rakers to graze directly on the diatoms, thus short-circuiting the food chain. Upwelling regions harbor large stocks of pelagic fishes indicating that a significant proportion of primary production is retained in the surface pelagic system. However, in regions where upwelling is prolonged over many months, the sinking flux to deeper layers and the underlying sea floor can be substantial. Thus, suboxic conditions can develop in deeper layers over extensive regions. These zones play a crucial role in oceanic nutrient cycles because denitrifying bacteria reduce nitrate to N_2, thus lowering the N:P ratio. Nitrous oxide (N_2O), which is 300 times more potent as a greenhouse gas than CO_2, is also formed. In the underlying anoxic sediments methane and hydrogen sulfide are produced, which reach such high concentrations off the Namibian coast that methane bubbles, together with H_2S gas, reach the surface and cause mass death of the local benthos. H_2S is oxidized to elemental sulfur in surface water and the resulting reflective particles can be seen in satellite images. Since global warming steepens the temperature gradient between land and sea, winds are expected to be stronger and upwelling more intense in the future. This will result in greater input of methane and N_2O to the atmosphere further exacerbating global warming.

Sporadic Algal Blooms

Blooms caused by vagaries of the weather acting on hydrography are generally singular and local events. For instance, the passage of storms over stratified, nutrient-poor waters of summer months causes deep mixing and upward transport of nutrients, often resulting in a bloom. Generally the standing stock of these blooms is lower than in seasonal ones, but where motile forms that concentrate their populations along hydrographic gradients (nutricline) are involved, algal densities in thin layers can be very high. Tilting of the pycnocline along hydrographic fronts introduces this layer to the surface resulting in conspicuous stretches of discolored waters. The spatial extent of the discolored patches or streaks ranges from meters in protected bays to many kilometers in the open sea, where they appear as meandering structures in satellite images.

In many regions characterized by surface fronts between water masses with different properties, such as at the entrance of the English Channel, frontal blooms are regular features. Most often dinoflagellates dominate frontal blooms but in estuaries cryptophyte and haptophyte blooms have also been reported.

Harmful Algal Blooms

The term HAB is in common usage to denote proliferations of algal species that have a detrimental effect on human use of lakes and the sea, from drinking water reservoirs, and recreation to aquaculture. Some species produce toxins that reach humans through farmed fishes or molluscs, others harm directly farmed fishes or molluscs, still others produce toxins that reach directly humans through e.g., drinking water, as the toxins produced by freshwater cyanobacteria, and others build-up massive biomass resulting in oxygen depletion in deeper water layers. The spread of artificial fertilizers in agriculture in the second half of the last century led to increased nutrient input to lakes and estuaries, often resulting in algal blooms. Because aquaculture has intensified and spread over the past decades, the incidence of HABs in coastal waters has accordingly increased. Thus, they can be regarded as the aquatic equivalent of terrestrial weeds, which by definition are plants that interfere with human usage of the region. It follows that the number of HABs increases with the intensity and diversification of usage.

Algal blooms caused by nutrient runoff from fertilized fields, referred to as eutrophication, and particularly prominent in lakes, rivers, estuaries, and coastal regions, are being brought under control in Europe and North America by improving agricultural practices and installing sewage treatment plants. In fresh- and brackish waters these blooms are at times constituted by cyanobacteria that can produce potent toxins such as the hepatotoxic microcystin, the neurotoxins anatoxins and saxitoxins, and the cytotoxin cylindrospermopsin, which pose a serious threat for freshwater supplies.

HABs can be produced by phytoplankton species belonging to almost all phylogenetic lineages that differ in size, life history, ecology and produce a range of chemically different toxins or harmful compounds. Here we provide a brief overview of the major types illustrated with some examples.

The occurrence of toxic algal blooms was first brought to the attention of the European public by the notorious bloom of a little-known, small haptophyte flagellate, *Chrysochromulina polylepis*, in May 1988. This species caused extensive fish kills in aquaculture farms along the Swedish and Norwegian coasts. It built up biomass as a dense layer in the pycnocline of the Belt Sea, which moved north with outflowing Baltic water and reached the surface along the Swedish and Norwegian coasts. The bloom of this toxic species was associated with increasing eutrophication at the time but the absence of a similar bloom in subsequent years despite similar weather conditions to those of May 1988 cast doubt on the explanation of a single cause-and-effect relationship.

The dinoflagellates include the greatest number of harmful species some of which produce potent toxins. Many of the species are responsible for fish kills as well as human poisoning via shellfish. Saxitoxin was the first algal toxin to be described; it blocks the sodium channels of neurons causing muscular paralysis and, at high dosage, death. The condition is termed paralytic shellfish poisoning (PSP). Some related genera of dinoflagellates, particularly various species of *Alexandrium*, cause PSP. Not only mussel farms but also wild mussel beds are now closely monitored for toxins, but in the past human deaths due to PSP occurred regularly. A number of other toxic molecules have since been identified in dinoflagellates. Toxins produced by the dinoflagellate *Karenia brevis* escape to the atmosphere and cause respiratory problems in humans. Blooms of this species have been reported for the Gulf of Mexico where they also cause massive fish kills.

Diarrhetic shellfish poisoning is caused by okadaic acid and its derivatives produced by some species of the common dinoflagellate genera *Prorocentrum* and *Dinophysis*. Although not fatal, the toxins can cause severe discomfort to the digestive system. Most of *Dinophysis* species escape detection by standard quantitative methods due to their typically low cell density (10–40 cells L^{-1}). Blooms of unarmoured dinoflagellates of the genus *Cochlodinium* are responsible of massive fishkills in Southeast Asia and North America and their geographic distribution has considerably expanded in the last decades. Dinoflagellates in the genus *Gambierdiscus* that live in close association with macroalgae produce toxins that bioaccumulate in tropical and sub-tropical fishes causing ciguatera fish poisoning (CFP).

Species of the genus *Pseudo-nitzschia* – and a few *Nitzschia* – are the only diatoms known to produce the potent neurotoxin domoic acid, which bioaccumulates in shellfish and causes amnesic shellfish poisoning (ASP). Long-lasting blooms of *Pseudo-nitzschia* species, related to anomalously warm ocean conditions, resulted in the largest outbreak of domoic acid along the North American west coast. Marine mammals have been affected by domoic acid, and clam and crab fisheries experienced prolonged closures.

Ecosystem-disruptive blooms of the small-celled pelagophyte *Aureococcus anophagefferens* appeared suddenly along the northeast coast of the USA in the 1980s and spread southward in subsequent years reaching the Gulf of Mexico. The extremely dense blooms produced unsightly brownish colored water known as brown tides. The increase in light attenuation decreased the biomass and diversity of benthic invertebrates and seagrasses. Copious amounts of mucus produced by the large, centric diatom *Coscinodiscus wailesii* that invaded the North Sea from the North Pacific during the late 1970s caused damage to the fisheries by clogging and tearing fishing nets. However, although this species is now established in the North Sea, blooms comparable to those of the 1970s have not since been reported.

Future Research Avenues

What more we have to investigate in order understand the mechanisms that regulate algal blooms? We have to gain a better understanding of the diversity of species or populations not only in terms of their morphological or genetic diversity but also their physiology, life histories and capability to perceive environmental signals and respond to them. We have to augment our observation capabilities of the plankton community, both in terms of visualizing the organisms in their environment as it is nowadays allowed by cell-imaging technology such as the Imaging FlowCytobot but also extending the temporal scale of observations through long term monitoring programs.

The phenology of bloom-forming species, that is, their mode of gearing to seasonality and hydrography of their environment together with their specific defense strategies and capabilities to send and perceive 'signals', need to be addressed if we are to make headway in understanding the driving forces shaping algal blooms. We conclude by briefly illustrating marine genomic approaches that are shedding light on the biological functioning of unicellular microalgae and on large scale *in situ* experiments that represent a unique opportunity to follow bloom dynamics under natural conditions.

Marine Genomics

Recent advances in genomics hold promise for further understanding of phytoplankton biology. In the last decade, next generation sequencing (NGS) has allowed to move from the expensive and time-consuming methods based on Sanger sequencing to cheaper and faster protocols, coupled with improved bioinformatics pipelines for data managing. This is leading to a steady increase in the number of fully sequenced microalgal genomes (Table 1), including two toxic *Pseudo-nitzschia* species.

The complete genomes of two diatom species, the centric diatom *Thalassiosira pseudonana* and the pennate diatom *Phaeodactylum tricornutum*, have shed light on the evolutionary origins of diatoms and their ecological success. Red algal genes have been found in both the *T. pseudonana* and the *P. tricornutum* genomes providing evidence for a red algal endosymbiont that subsequently became the chloroplast, but the predominance of green algal genes suggests that the green algal endosymbiont preceded the red alga. These findings indicate that diatoms derive from a serial secondary endosymbiosis and not a single event. Diatoms not only inherited genes from their different endosymbionts and the exosymbiont but also a number of bacterial genes through horizontal gene transfer. This unique combination has permitted novel metabolic pathways for these microalgae, e.g., carbon concentrating mechanisms (CCMs), urea cycle, genes encoding the iron storage protein ferritin, which contributed to their ecological success.

Table 1 Sequenced genomes of marine microalgae

Species Name	*Class*	*Reference*
Cyclotella cryptica	Mediophyceae	Traller *et al.* (2016)
Fistulifera solaris	Bacillariophyceae	Tanaka *et al.* (2015)
Fragilariopsis cylindrus	Bacillariophyceae	Mock *et al.* (2017)
Phaeodactylum tricornutum	Bacillariophyceae	Bowler *et al.* (2008)
Pseudo-nitzschia multiseries	Bacillariophyceae	https://genome.jgi.doe.gov/Psemu1/Psemu1.home.html
Pseudo-nitzschia multistriata	Bacillariophyceae	Basu *et al.* (2017)
Thalassiosira oceanica	Mediophyceae	Lommer *et al.* (2012)
Thalassiosira pseudonana	Mediophyceae	Armbrust *et al.* (2004)
Emiliania huxleyi	Coccolithophyceae	Read *et al.* (2013)
Pavlovales sp. CCMP2436	Haptophyte	https://genome.jgi.doe.gov/Pavlov2436_1/Pavlov2436_1.home.html
Bigelowiella natans	Chlorarachniophyceae	Curtis *et al.* (2012)
Dunaliella salina	Chlorophyceae	Polle *et al.* (2017)
Guillardia theta	Cryptophyceae	Curtis *et al.* (2012)
Cryptophyceae sp. CCMP2293	Cryptophyceae	https://genome.jgi.doe.gov/Crypto2293_1/Crypto2293_1.home.html
Nannochloropsis gaditana	Eustigmatophyceae	Corteggiani Carpinelli *et al.* (2014)
Nannochloropsis oceanica	Eustigmatophyceae	Vieler *et al.* (2012)
Picochlorum sp.	Related to *Nannochloropsis* and *Chlorella*	Foflonker *et al.* (2015)
Bathycoccus prasinos	Mamiellophyceae	Moreau *et al.* (2012)
Micromonas commoda	Mamiellophyceae	Worden *et al.* (2009)
Micromonas pusilla	Mamiellophyceae	Worden *et al.* (2009)
Ostreococcus lucimarinus	Mamiellophyceae	Palenik *et al.* (2007)
Ostreococcus sp. RCC809	Mamiellophyceae	https://genome.jgi.doe.gov/OstRCC809_1/OstRCC809_1.home.html
Ostreococcus tauri	Mamiellophyceae	Derelle *et al.* (2006)
Aureococcus anophagefferens	Pelagophyceae	Gobler *et al.* (2011)
Pelagophyceae sp. CCMP2097	Pelagophyceae	https://genome.jgi.doe.gov/Pelago2097_1/Pelago2097_1.home.html
Ochromonadaceae sp. CCMP2298	Chrysophytes	https://genome.jgi.doe.gov/Ochro2298_1/Ochro2298_1.home.html

With more genomes available, comparative genomics is allowing to appreciate a great level of diversity even within the same phylogenetic groups, revealing very different genetic makeups and indicating that congeneric species can have up to 20% of unique sequences, often with completely unknown function. This will hopefully provide the basis for a mechanistic understanding of the distinctive features at the level of species or populations. In the genome of the recently sequenced polar diatom *Fragilariopsis cylindrus* an unexpectedly high level of allelic divergence has been reported. The differential expression of the divergent alleles under different environmental conditions is probably part of the mechanism that determines the impressive adaptive potential of this species to the extreme and variable environment in which it evolved. Other species are being sequenced for the exploration of specific features, such as lipid accumulation for biotechnological purposes or for understanding the mechanisms that regulate sexual reproduction in diatoms; novel details and insights are emerging from the vast metabolic potential, mechanisms of adaptation, evolutionary history and phylogenetic relationships.

In addition to genome sequencing, transcriptome sequencing (RNA-seq) provides us with complementary tools to explore the gene content of any given species and to approach global gene expression studies in non-model species. The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) has generated over 650 transcriptome sequences from phytoplankton species, including harmful algae. For species with large and complex genomes, such as dinoflagellates, transcriptomic approaches provide a cost-effective alternative.

Much attention has been devoted to the study of changes in light and nutrient conditions, as these are two of the main bottom-up environmental factors affecting growth and regulating blooms. Transcriptomics datasets exist for different light conditions (day/light cycle, intensity, spectrum), from different growth phases, for cells grown under nutrient starvation, and general metabolic states have been linked to gene modules that co-vary and are similarly coordinated even between distantly related species of a given group.

Trophic relationships appear to be as important in shaping blooms as nutrient and light availability, and phytoplankton cells perceive and actively respond to cues from other organisms. Diffusible cues from zooplankton predators can trigger changes in key traits in phytoplankton. Transcriptomics has provided a few insights into the genetic control of morphological or physiological responses to predators: the increased toxicity of the dinoflagellate *Alexandrium minutum* in the presence of copepods was linked to a very small set of genes changing, while a study of the chain-forming diatom *Skeletonema marinoi* exposed to copepods indicated a stronger response, with activation of stress genes, changes in the lipid and nitrogen metabolism, in cell cycle regulation and in frustule formation. *Alexandrium fundyense* strains have been examined in the presence of the dinoflagellate grazer *Polykrikos kofoidii*, and greater differences have been observed among ungrazed strains than between grazed and ungrazed treatments. This result corroborates other observations suggesting a large variability among strains of the same species and calls for attention when drawing conclusions from studies based on a single strain.

Viral infections are also able to induce profound changes in their host metabolism, as highlighted by studies in infected *Emiliania huxleyi* in which a modulation of host metabolic genes involved in life cycle and of the sphingolipid and antioxidant metabolisms occurs. The role of viruses, bacteria and zooplankton grazing in affecting algal blooms, and the genetic basis for the production of toxins and allelochemicals will need to be explored in dedicated future studies.

In case of toxic algae, transcriptomics has been used to investigate and compare the gene expression of mixotrophic protists under light and dark conditions, and in the presence of prey, and in toxic diatoms to explore responses to changes in nutrients or to chemical cues produced by neighboring cells. Studies of the interactions between toxic *Pseudo-nitzschia* and bacteria have revealed that bacterial compounds can affect diatom growth.

While the number of detected algal genes increases, molecular tools to explore their function are still scarce, although some approaches are routinely applied in a few model species, mostly diatoms. Genetic transformation, possible in a range of species, is exploited to increase, reduce or eliminate a gene product and to look for the phenotypic consequences of the change to infer the role. An exciting new avenue is represented by the application of the recent CRISPR/Cas9 technology, an effective and precise genome editing method that is revolutionizing the way in which genes are manipulated, and that has recently been applied in the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*. Most of the unexplained features of phytoplankton, possibly accounting for many of the differences between groups, are likely linked to the vast amount of genes with unknown function. Steadily, functional approaches will allow to assign a role to those that are prominent in specific processes (highly overexpressed in transcriptomics studies, or recurrently appearing in gene models).

Finally, whole genomes of individual species are now being supplemented by metagenomic and metatranscriptomic approaches that aim at circumscribing the genomic variation of natural communities, in case of the former, and the gene expression profile of environmental mRNA in case of the latter. To give some idea of the power of these approaches, the recent TARA Oceans expedition produced and released 116 million eukaryotic transcripts from plankton samples collected from 210 stations during a 2.5-year circumnavigation of the world oceans. The combination of genomic approaches with conventional oceanography will improve our understanding of the factors determining species dominance and succession in phytoplankton blooms and thus help to understand how different algal classes will respond to future climate change.

Large-Scale *In Situ* Experiments

Large-scale *in situ* perturbation experiments are a powerful tool to unravel the complexity of factors enabling bloom formation under natural physical and biological conditions. Their advantage over single-species and microcosm experiments is that they

encompass the entire pelagic community, in particular an intact mortality environment comprising the natural gamut of pathogens, parasites, predators and grazers.

Whole lake experiments in North America and Northern Europe date back to the 1960s and have provided important insights into the limiting nutrient (phosphate) in temperate lakes, eutrophication and toxic bloom remediation, acid rain and trophic cascading effects and lake restoration. These insights have played a key role in the implementation of water management practices to control some of the negative man-made impacts.

In the ocean the equivalent of whole lake experiments are mesoscale fertilization experiments (initial patch size on the order of 25–300 km^2) to study the response of the plankton community to nutrient addition and to follow the ensuing biogeochemical fluxes. Large expanses of the open ocean have perennially high macronutrient concentrations (nitrate and phosphate) but low phytoplankton biomass. These are the so-called high-nutrient, low-chlorophyll (HNLC) ocean regions – the subarctic and equatorial Pacific and the Southern Ocean – where phytoplankton growth rates are limited by low iron availability. Thus far 12 artificial ocean iron fertilization (OIF) experiments and three natural iron fertilization studies have been carried out in the HNLC regions. Provided that silicate was in ample supply, the iron-induced blooms were dominated by diatoms. The species composition of the blooming diatoms varied widely and depended on the seeding stock present at the time of fertilization. In some OIF experiments many different species accumulated biomass in about equal proportions, others were dominated by one or a few species with exceptionally high accumulation rates. The smaller phytoplankton of the microbial food web were also stimulated by iron addition but their biomass failed to increase, indicating that the increase in growth rates was compensated by a corresponding increase in mortality rates presumably due to herbivores. This is supported by results from two of the three OIF experiments conducted in high-nutrient, low-chlorophyll, low-silicate waters (HNLCLSi) of the Southern Ocean where the phytoplankton assemblage was dominated by pico- and nanophytoplankton (largely flagellates) and bloom biomass was kept in check by micro- and mesozooplankton grazers. Thus, while production of the microbial community was largely channelled to higher trophic levels, only the mortality rates of the diatoms were sufficiently low to enable accumulation of biomass. A phosphorous addition experiment carried out in low-nutrient, low-chlorophyll (LNLC) waters of the Eastern Mediterranean elicited a response in the microbial food web that was subsequently transferred to copepods. While all OIF experiments increased the productivity of the plankton community and channelled energy to higher trophic levels, only one OIF experiment could show deep export of the iron-induced diatom bloom. Most OIF experiments were however too short to follow the fate of the iron-induced bloom and thus more research is needed to investigate the effectiveness of OIF experiments for carbon sequestration. The use of ocean pipes to induce artificial upwelling of deep nutrients to nutrient-impoverished surface waters was also put forward as a means for carbon sequestration and sustainable fisheries and aquaculture. Given its size, the ocean seems to be a promising place to cure some of the anthropogenic pressures facing our planet. Research into ocean fertilization approaches is thus highly warranted to improve our understanding of the functioning of pelagic ecosystems on the one hand and to investigate the risks and their potential for ocean sequestration of anthropogenic CO_2 and restoration of dwindling fish stocks on the other.

Acknowledgments

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Amino Acid Production[☆]

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Glossary

Carriers/transporters Membrane proteins that function to transport substances into or out of the cell through the cytoplasmic membrane.
Fermenter A large growth vessel used to culture microorganisms on a large scale for the production of commercially valuable products.
Immobilized cell A cell attached to a solid support over which substrate is passed and is converted into product.
Metabolic engineering The improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the application of recombinant DNA technology.
Regulation Processes that control the activities or synthesis of enzymes.
Selection Placing organisms under conditions where the growth of those with a particular genotype will be favored.

Introduction

Amino acids are simple organic compounds that contain one or more amino groups and one or more carboxyl groups. They are the building blocks of peptides, proteins, and they form many complex polymers. There are 20 protein-forming amino acids, all of which except glycine, are optically active and occur as L-enantiomers. Eight of these protein-forming L-amino acids are essential for mammals. There are large demands for amino acids for use in the areas of food and feed additives (Table 1). In 2015 the largest volume of the total amino acid market is occupied by the so-called feed amino acids L-lysine, DL-methionine, L-threonine, and L-tryptophan (Leuchtenberger *et al.*, 2005). Also substantial is the share of the food sector (34%), which is determined essentially by L-glutamate in the form of the flavor-enhancer monosodium glutamate (MSG) and the amino acids L-aspartate and L-phenylalanine, the latter two used as starting materials for the peptide sweetener L-aspartyl L-phenylalanyl methyl ester (Aspartame). Furthermore, amino acids are used in medicine for infusions and as therapeutic agents, they are also required in the chemical industry, such as derivatives in synthetic leathers, surface-active agents, fungicides, and pesticides. The total amino acids made represent a value of more than $11 billion. The market is growing steadily by about 5–10% per year. The production methods to date are: (1) microbial production, (2) protein hydrolysis, (3) chemical synthesis, and (4) enzymatic synthesis. While chemical synthesis produces a racemic mixture, which may require additional resolution, the other procedures give rise to optically pure L-amino acids.

Microbial Production

Development of Amino Acid Overproducing Strains

Many bacteria are capable of growing on a simple mineral salt medium containing ammonium, phosphate, further salts and glucose as the carbon and energy source. These bacteria are able to synthesize all the compounds necessary for the living cell from these simple nutrients. The dry matter of the bacterial cell consists of about 60% protein, 20% nucleic acids, 10% carbohydrates, and 10% fat. Therefore, the cell must be able to synthesize amino acids rapidly and efficiently. However, as a rule, only as much of the various amino acids as required for growth are synthesized in the bacterial cell, ie, normally, the bacteria do not overproduce and excrete amino acids into the culture medium. The reason is that bacteria have a number of sophisticated regulatory mechanisms, like repression and feedback inhibition through end products, which economically control the overproduction of metabolites.

Thus, classical mutagenesis has been applied to obtain mutants which are able to overproduce a specific amino acid in large amounts. Regulatory strains were obtained by selecting mutants which are resistant to amino acid analogs. Some of these commonly used lysine-, threonine-, and tryptophan-analogs are shown in Table 2. Amino acid analog resistance may be due to derepression of the enzymes involved in the biosynthesis of amino acids or in elimination of the allosteric control of biosynthetic key enzymes. Furthermore, the amount of amino acids synthesized via a branched pathway can be significantly increased by selecting strains auxothrophic for the competing branch. The application of mutagenesis and selection belong to the most important techniques in the development of amino acid overproducing microorganisms, and such mutants still form the basis to apply powerful modern techniques for the development of superior production strains.

[☆]*Change History*: June 2016. L. Eggeling updated the Sections "Introduction," "L-Glutamate" and "References." He also added the new Table 1.

This is an update of L. Eggeling, H. Sahm, Amino Acid Production, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 150–158.

Table 1 Quantities of selected amino acids produced in 2015 and their major use

Amino acid	*Amount (t/a)*	*Main use*
L-Glutamate	4,100,000	Food (MSG, umami)
L-Lysine	2,300,000	Feed
DL-Methionine	1,200,000	Feed
L-Threonine	450,000	Feed
L-Tryptophan	25,000	Feed
L-Aspartate	25,000	Food (Aspartame)
L-Phenylalanine	25,000	Food (Aspartame)

Table 2 Analogues of amino acids used for the selection of L-lysine-, L-threonine-, or L-tryptophan-overproducing strains

Lysine	*Threonine*	*Tryptophan*
S-(2-aminoethyl)-L-cysteine (AEC)	α-Amino-β-hydroxyvaleric acid	5-Methyltryptophan
4-Oxalysine	β-Hydroxyleucine	4-Methyltryptophan
2,6-Diamino-4-hexenoic acid	N-Lauryl leucine	6-Fluorotryptophan
ε-C-Methoylysine (2,6-diamino-heptanoic acid)	Norvaline	DL-7-Azatryptophan
δ-Hydroxylysine	N-2-Thienoylmethionine	2-Azatryptophan
α-Chlorocaprolactam	2-Amino-3-methylthiobutyric acid	3-Inolacrylic acid

The current repertoire of strain development encludes amongst others: (1) Identification of mutations in classical strains by genome sequencing and their introduction via recombinant DNA techniques into a wild type thus re-building the producer. The reason is that due to undirected mutagenesis disadvantageous mutations might have been accumulated thus reducing growth or speed of sugar conversion. (2) Combination of beneficial mutations of different strains. (3) Gene overexpression to overcome limiting steps. (4) Identification of genes by global (microarray) DNA analysis whose expression correlates with favorable producer strains or fermentation conditions, and the subsequent controlled expression of such genes by genetic means. (5) Application of intracellular flux quantifications using ^{13}C-labeled substrate and ^{13}C-NMR (Niedenführ *et al.*, 2015). (6) Manipulation of export activity to increase the export of amino acid (Bellmann *et al.*, 2001; Dassler *et al.*, 2000). The improvement of cellular activities by directing the enzymatic, transport, and regulatory functions of the cell by use of recombinant DNA technology is now a standard method for obtaining highly productive amino acid producing strains, often based on strains derived by undirected mutagenesis.

L-Glutamic Acid

Following the increasing demand for monosodium glutamate as a flavoring agent in the mid-1950s, a bacterium was isolated in Japan that excreted large quantities of the amino acid L-glutamic acid into the culture medium. This bacterium, *Corynebacterium glutamicum*, is a short, aerobic, Gram-positive rod capable of growing on a simple mineral salt medium with glucose, provided that biotin is also added. A recent monograph collects a great number of the exiting features of *C. glutamicum* leading to amino acid production (Eggeling and Bott, 2005). The production of L-glutamic acid by *C. glutamicum* is maximal at a critical biotin concentration of 0.5 μg/g of dry cells, which is suboptimal for growth. Despite being effectively produced for over 50 years in amounts of currently 4.1 million tons/year the molecular features resulting in L-glutamate excretion are still poorly understood. However, besides biotin limitation a surprising large variety of other treatments also results in L-glutamate excretion, for example, addition of selected detergents like Tween-40, addition of penicillin, use of fatty acid auxotrophic strains, or addition of ethambutol inhibiting arabinogalactan synthesis. Interestingly, all these conditions have more or less in common to target the cell wall. Only recently exciting discoveries were made disclosing new features of *C. glutamicum*, which are linked to cell wall metabolism and L-glutamate production. It was found that *C. glutamicum* possesses a regulatory OdhI protein, together with a eucaryotic-type serine/threonine protein kinase PknG and a phosphatase Ppp, the latter two controlling the phosphorylation status of OdhI (Niebisch *et al.*, 2006). Unphosphorylated OdhI inhibits ketoglutarate dehydrogenase activity and the OdhI protein was shown to be necessary for high L-glutamate production. Importantly, Ppp is apparently membrane bound, and the genes controlling the phosphorylation status are adjacent to genes of cell wall synthesis and cell division. Excretion of L-glutamate occurs through the mechanosensitive channel MscCG by passive diffusion (Nakamura *et al.*, 2007). This channel is a homolog of *Escherichia coli* MscS, which serves as an osmotic safety valve in *E. coli* cells. Thus a disordered cell wall influences the osmotic properties enabling L-glutamate efflux, and the regulatory properties of OdhI inhibit ketoglutarate dehydrogenase activity. As a consequence α-oxoglutarate is not further metabolized in the citric acid cycle, but instead preferably converted into L-glutamic acid, whose efflux into the surrounding medium is mediated by MycCG.

In *C. glutamicum*, glucose is mainly metabolized via the glycolytic pathway into C_3 and C_2 fragments. Oxaloacetate is formed via the phosphoenolpyruvate carboxylase and the pyruvate carboxylase (Fig. 1). Thus, *C. glutamicum* has two anaplerotic reactions for

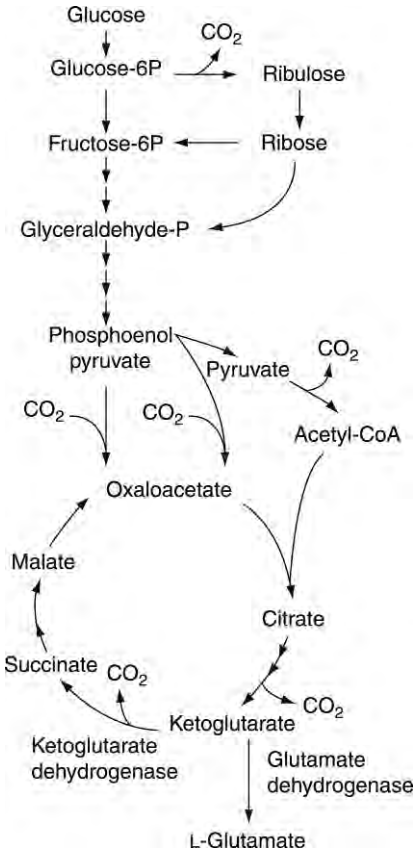


Fig. 1 Biosynthesis of L-glutamic acid in *Corynebacterium glutamicum* using glucose as the carbon source.

the conversion of the C_3-intermediates into oxaloacetate. These reactions are important to always have sufficient oxalaoacetate available for condensation with acetyl-CoA and conversion of citrate to α-oxoglutarate. The overall reaction for L-glutamic acid production from D-glucose is:

$$C_6H_{12}O_6 + NH_3 + 3\ NAD^+ \rightarrow C_5H_9O_4N + 3\ NADH + 3\ H^+ + CO_2$$

Thus, the theoretical maximal yield is 1 mol of L-glutamic acid per mol of glucose metabolized. This represents a 100% molar conversion or 81.7% weight conversion of D-glucose to L-glutamic acid.

The L-glutamic acid production is carried out in stirred fermenters up to a size of 500 m^3. Provision for cooling, dissolved oxygen measurement, pH measurement and control (usually with ammonium) are required. A temperature between 30 and 35°C and a pH between 7.0 and 8.0 are optimal. The oxygen transfer rate is fairly critical: a deficiency leads to poor glutamate yields, with lactic and succinic acid being formed instead, while an excess causes accumulation of α-oxoglutaric acid. The yield of L-glutamic acid obtained after 2–3 days of incubation is in the order of 60–70% (by weight) of the sugar supplied, and the final concentration is approximately 150 g/L.

L-Lysine

L-Lysine, an amino acid essential for human and animal nutrition, is mainly used as a feed supplement as it is present in most plant proteins only in low concentrations. The wild-type of *C. glutamicum* does not secrete L-lysine into the culture medium. However, excellent high-yield production strains were developed through mutation and selection to antimetabolite resistance together with modern recombinant DNA-techniques (Eggeling and Bott, 2015).

The pathway for the biosynthesis of L-lysine in *C. glutamicum* is illustrated in Fig. 2. A remarkable feature of *C. glutamicum* is its split pathway for the synthesis of L-lysine. At the level of L-2,3,4,5-tetrahydrodipicolinate there are two pathways for the conversion of this precursor into DL-2,6-diaminopimelate. The first enzyme in L-lysine biosynthesis, aspartokinase, is regulated by concerted feedback inhibition by L-threonine and L-lysine. Hence, a homoserine auxotroph or a threonine and methionine double auxotroph of *C. glutamicum* diminishes the intracellular pool of threonine, reduces its marked feedback inhibitory effect on aspartokinase, and promotes lysine overproduction (15–30 g/L). Another effective technique for obtaining L-lysine-producing strains is the selection of regulatory mutants. Growth of *C. glutamicum* is inhibited by the L-lysine analogue aminoethyl-L-cysteine (AEC). This inhibition is

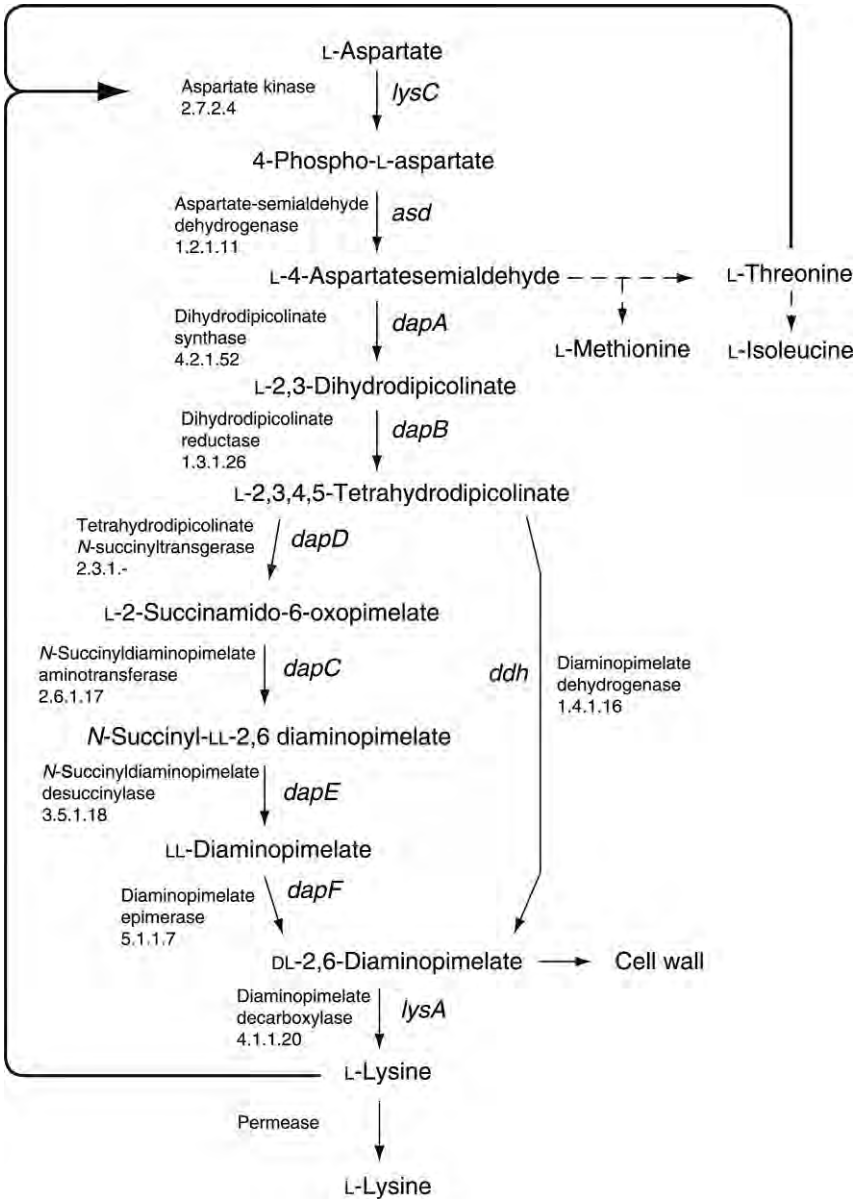


Fig. 2 The split pathway of L-lysine biosynthesis and its regulation in *Corynebacterium glutamicum*; *rastered arrows*=feedback inhibition.

markedly enhanced by L-threonine, but reversed by L-lysine. This implies that AEC behaves as a false feedback inhibitor of aspartokinase. Some mutants, which are capable of growing in the presence of both AEC and L-threonine, contain an aspartokinase that is insensitive to the concerted feedback inhibition; therefore, L-lysine is overproduced (30–35 g/L). L-Aspartate as the precursor of L-lysine synthesis is formed from oxaloacetate by the anaplerotic reaction of phosphoenolpyruvate and pyruvate carboxylation (Fig. 1). By combined overexpression of aspartokinase and dihydrodipicolinate synthase, L-lysine production can be further increased by 10%. Also the ample supply of NADPH is important which is achieved by an increased flux via the pentose phosphate shunt.

In addition to all steps considered so far, the active export of L-lysine into the culture medium is important. Export is mediated by the specific exporter LysE, a small membrane protein of 25.4 KDa, with five transmembrane spanning helices (Fig. 3). Expression of this exporter is controlled by the LysR-type transcriptional regulator LysG. At elevated intracellular L-lysine concentrations of 35 mM, LysG binds L-lysine and drives expression of the exporter gene to result in an about 20-fold increased induction. As a consequence effective export occurs, enabling high export rates of up to 7 nmol min^{-1} mg(dry weight)$^{-1}$. The components driving L-lysine export are the electrochemical proton potential and the chemical concentration gradient of L-lysine. A formal description of the energetic steps during translocation involves symport of the positively charge L-lysine with two OH^- (Fig. 3). For the substrate translocation step the pH gradient, and the L-lysine gradient are important, whereas reorientation of the carrier involves the membrane potential. This transporter is a genetic and biochemical system well-designed for excretion purposes:

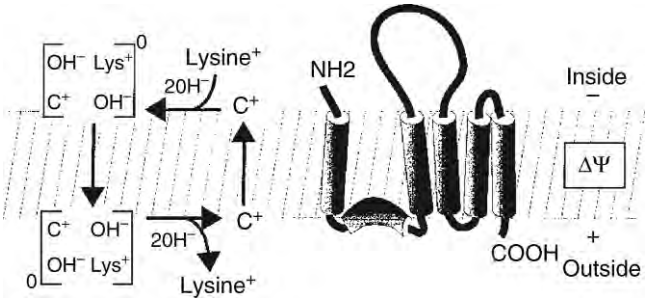


Fig. 3 Structure of the L-lysine exporter and the putative mechanism of L-lysine excretion in *Corynebacterium glutamicum*.

- It is only induced at elevated intracellular L-lysine concentrations of about 40 mM.
- It has a high K_m value for L-lysine (20 mM) at the internal (cytoplasmic) side, thus preventing efflux under low internal lysine concentration.

In fed-batch culture L-lysine production strains are able to reach under the optimized culture conditions final concentration of at least 170 g/L L-lysine, and the conversion rate relative to sugar used is about 50%. A typical L-lysine production curve is shown in Fig. 4; together with sugar also ammonium has to be fed. The conventional route of lysine down-stream processing is characterized by

- removal of the bacterial cells from the fermentation broth by separation or ultrafiltration,
- absorbing and then collecting lysine in an ion exchange step, and
- crystallizing or spray drying as L-lysine hydrochloride.

An alternative process consists of biomass separation, concentration of the fermentation solution, and filtration of precipitated salts. The liquid product contains up to 50% L-lysine base that is stable enough to be marketed.

Recently, a new concept for lysine production was introduced, in which the entire L-lysine containing fermentation broth is spray dried, and granulated to yield a feed-grade product which contains L-lysine sulfate corresponding to at least 60% of L-lysine hydrochloride. Waste products usually present in the conventional L-lysine hydrochloride manufacture are thus avoided in this process.

In 2015, the price for L-lysine×HCl feed-grade was approximately $2 per kg. With the benefits provided by modern techniques, such as genetic engineering, and with the potentials of the fermentation technology, additional improvements in the L-lysine process should be realized. L-lysine will continue to be the most attractive feed additive produced by fermentation.

L-Threonine

Until 1986, L-threonine was used mainly for medical purposes, in amino acid infusion solutions, and nutrients. It was manufactured by extraction of protein hydrolyzates or by fermentation using mutants of coryneform bacteria in amounts of some hundred tons per year worldwide. The production strains were developed by empirical classical breeding, introduction of auxotrophies and resistance to threonine analogs such as α-amino-β-hydroxy-valerate, and reached product concentrations up to 20 g L^{-1}. Although the pathway of L-threonine biosynthesis in *E. coli* is much more regulated compared to that in *C. glutamicum*, *E. coli* strains have proved to be superior to *C. glutamicum*. The reason is that *E. coli* has much more effective excretion systems for L-threonine and

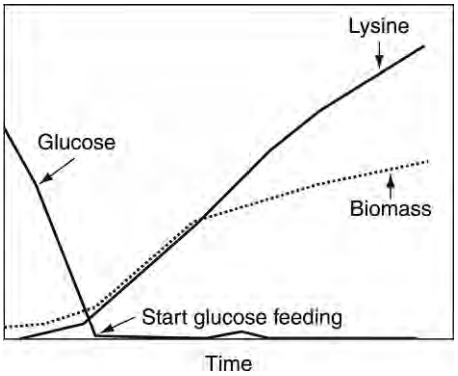


Fig. 4 Production of L-lysine with a mutant strain of *Corynebacterium glutamicum*; glucose is fed together with ammonium.

related amino acids, which are not present in *C. glutamicum*. *E. coli* strains with excellent threonine yield and productivity are now available.

In *E. coli* the regulation of the L-aspartate derived amino acids involves several isoenzymes. As shown in Fig. 5 the phosphorylation of L-aspartate, the first reaction in the biosynthetic pathway of L-threonine, is catalyzed by three different aspartokinases. One of these isoenzymes is inhibited by L-threonine and its synthesis is repressed by L-threonine and L-isoleucine. The second aspartokinase isoenzyme is repressed by L-methionine, and the third one is inhibited and repressed by L-lysine. Threonine biosynthesis takes place from aspartate-β-semialdehyde in three enzymatic steps which are encoded in *E. coli* by the *thrABC* operon. The *thr*A gene encodes a bifunctional enzyme with aspartokinase and homoserine dehydrogenase activities. The *thr* operon is under the control of a single promoter, which is bivalently repressed by L-threonine plus L-isoleucine. Additionally, L-threonine synthesis is regulated by feedback inhibition of the homoserine dehydrogenase and homoserine kinase. L-threonine ranks third in production volume among the biotechnologically produced amino acids behind L-lysine and L-glutamic acid.

Based on the synthesis pathway there is a clear focus on two major targets for the design of L-threonine overproducing strains, ie, the prevention of L-isoleucine formation, and stable high-level expression of *thrABC* operon. Therefore, in one of the first steps of strain development chromosomal mutations were introduced to result in an isoleucine leaky strain, which requires L-isoleucine only at low L-threonine concentrations, but at high L-threonine concentrations growth is independent of the addition of L-isoleucine. This mutation has several advantageous consequences: firstly, it prevents an excess formation of the undesired byproduct L-isoleucine; secondly, it prevents the L-isoleucine-dependent premature termination of the *thrABC* transcription. Furthermore, the isoleucine leaky mutation has a positive selection effect on all the cells containing the plasmid with the threonine operon. To obtain very high activities of the *thrABC*-encoding enzymes, this operon was cloned from a strain in which the aspartokinase and homoserine dehydrogenase activities are resistant to L-threonine inhibition and was overexpressed. To prevent the degradation of L-threonine the gene *tdh* was inactivated which encodes the threonine dehydrogenase.

By continuous sugar feeding of such *E. coli* strain L-threonine concentrations of more than 80 g/L are obtained with a conversion yield of about 60%. The recovery of feed-grade L-threonine is rather simple, after the cell mass has been removed from the culture broth by ultrafiltration, the filtrate is concentrated and the amino acid is isolated by crystallization. Currently, L-threonine has been successfully marketed as a feed additive with a worldwide demand of more than 450,000 t per year, with a price of approximately $3 per kg.

L-Serine

The engineering of an efficient L-serine producing bacterium is a rather recent example where a combination of cellular analyses and engineering methods resulted in a breakthrough in microbial L-serine production (Eggeling, 2007). L-Serine is used as all the other proteinogenic amino acids in infusion solutions but has no major other application. The market is therefore smaller, but the price per kilogram higher than for feed additive amino acids, for example. The current price is approx. $25/kg. The microbial production of L-serine originally occurred with methanol utilizing organisms taking advantage of the serine hydroxymethyltransferase (SHMT) reaction. In the biosynthesis reaction the SHMT catalyzes the glycine condensation with the C1-unit activated by a tetrahydrofolate

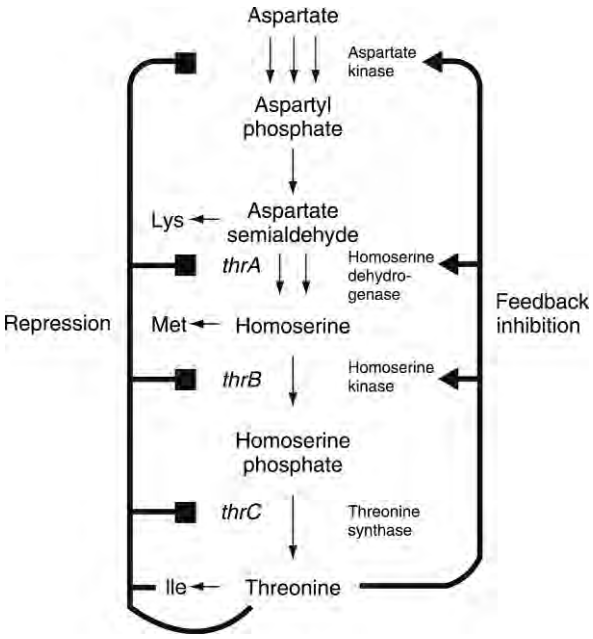


Fig. 5 Regulation of L-threonine biosynthesis in *Escherichia coli*. Only the regulation by L-threonine and L-isoleucine is shown.

derivative (THF) to form L-serine. However, this method suffered from the external addition of glycine and low conversion yields. As will be seen below the SHMT reaction plays a key role in microbial L-serine production. It catalyzes the interconversion of 5,10-methylenetetrahydrofolate+glycine+H2O=tetrahydrofolate+L-serine, with the interconversion and dissociation of reactants within the same order of magnitude.

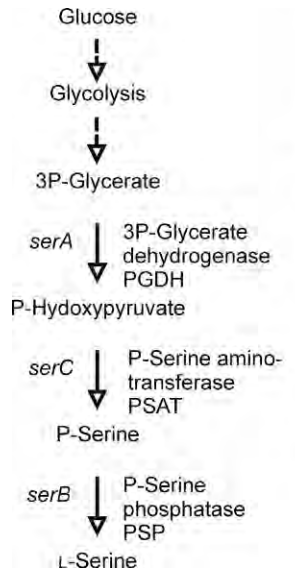
Using sugars like glucose as a carbon source L-serine derives directly from glycolysis in just three enzymatic steps (Fig. 6), and a systematic study revealed that besides removing bottlenecks in its synthesis, a major issue of L-serine accumulation is to prevent its degradation. Indeed L-serine has a key position in central metabolism, since as much as 8% of the glucose carbon flux is via L-serine. The reason is, that L-serine besides being incorporated into protein is required for a number of purposes, like synthesis of phospholipids, L-tryptophan, and L-cysteine. Moreover, the SHMT reaction in its biodegradative reaction provides glycine required for purine, protein and heme synthesis, and more importantly, provides the activated C-1 compound 5,10-methylene-THF which in turn can be further converted into 5,10-methenyl-THF, and other activated C-1 units like 10-formyl-THF, 5-formyl-THF, and 5-methyl-THF to serve different demands in metabolism, like 5-methyl-THF for L-methionine synthesis, 5,10-methenyl-THF for D-pantothenate synthesis, or 10-formyl-THF for N-formylmethionyl-tRNA and purine synthesis. It is obvious that only due to the requirement for purine synthesis and provision of $tRNA^{fMet}$ for translation initiation a high L-serine degradative flux via the SHMT reaction occurs.

To derive from the "work horse" of amino acid production, *C. glutamicum*, an L-serine producer a number of steps were undertaken. These were as follows: At first the feedback control of the 3P-glycerate dehydrogenase (PGDH) was removed, which is allosterically controlled by L-serine. Sequence comparisons revealed that the C-term of the *serA*-encoded PGDH represents the domain involved in allosteric control. Therefore, a comprehensive set of truncated *serA* versions was made, with the most prominent mutation being *serA*Δ197, where as much as 197 amino acyl residues from the C-term were deleted and which resulted in an activity almost unsensitive to L-serine inhibition but with catalytic activity largely retained (Fig. 6). However, overexpression of the mutant allel *serA*Δ197 in *C. glutamicum* either alone or in combinations with *serC* and *serB* overexpressed did not result in significant L-serine accumulation pointing to an intracellular conversion of L-serine.

Therefore, in the second step, the intracellular fate of L-serine was studied with 13-C labeled L-serine. Tracing of the label revealed that L-serine as an entity is converted to pyruvate and a genomic screen revealed furthermore that an *sdaA*-encoded serine dehydratase is present in *C. glutamicum*, likely to be responsible for degradation. The serine dehydratase contains an [4Fe-4S] cluster involved in the pyridoxal-5′-phosphate independent deamination of L-serine to pyruvate. When the enzyme was deleted and then the *serA*Δ197, *serC* and *serB* genes overexpressed a slight transient L-serine accumulation was observed.

The study with 13-C labeled L-serine also showed that glycine was formed from L-serine added, and this was not surprising considering that there is no preference of the SHMT for the forward or backward reaction. As mentioned, L-serine conversion catalyzed by SHMT serves to provide amongst others activated C-1 units and this cellular demand cannot entirely be bypassed by external metabolite addition. Therefore SHMT is essential. However, the metabolic engineers used a trick to deplete the SHMT activity. As mentioned, the SHMT requires THF for function. Therefore, the *pabAB* and *pabC* genes were deleted, encoding the aminodeoxychorismate synthase and aminodeoxychorismate lyase catalyzing two steps of THF synthesis. SHMT activity and growth of the resulting strain was dependent on THF, or its intermediate p-aminobenzoate.

Thus the strain eventually made was *C. glutamicum* 13032Δ*sdaA*Δ*pabABC* p*serACB*. This is derived from the type strain ATCC13032, deleted of serine dehydratase (*sdaA*), and of the folate synthesis genes (*pabABC*), and overexpressing the three genes



Construct	Strain	Specific activity	Residual activity %
	WT pZ1	0.13	38
	WT p*serA*	2.15	37
	WT p*serA*Δ79	0.19	15
	WT p*serA*Δ188	0.44	99
	WT p*serA*Δ197	1.32	92
	WT p*serA*Δ205	0.39	<1
	WT p*serA*Δ211	0.15	<1

Fig. 6 On the left is shown L-serine synthesis as derived from 3-phosphoglycerate from glycolysis. On the right is shown a series of plasmid constructs and their resulting 3-phosphoglycerate dehydrogenase activity in absence and presence of L-serine.

of the L-serine synthesis pathway (p*serACB*) with the *serA* gene carrying the deletion at its C-term. Under industrial relevant conditions the performance of this strain was demonstrated in a 20-L reactor based on corn steep liquor (CSL) medium. CSL provides amongst others some folate to enable restricted growth and reduced SHMT activity of the strain. The medium contained 35 g L^{-1} solid CSL plus initially 15 g L^{-1} glucose, 15 g L^{-1} fructose and salts. The minimum dissolved oxygen concentration was set to 50% saturation to ensure no oxygen limitation. As can be seen in Fig. 7, inoculation of the reactor with cells enabled growth up to a maximum specific growth rate of 0.25 h^{-1}, which is about 60% that of the wild type. L-Serine formation occurred already from the beginning, suggesting a suitable folate supply in the culture due to CSL, which can be assumed to contain at least traces of this vitamin. The maximum oxygen uptake rate, OURmax, was about 110 mol L^{-1} h^{-1}, which was present at the end of the logarithmic growth of the culture. The maximal specific productivity was 1.45 mmol g^{-1} h^{-1}, and the volumetric productivity about 1.4 g L^{-1} h^{-1}. In this experiment a final concentration of 345 mM L-serine was reached, but significantly higher concentrations can be obtained, and further derivatives of this basic strain have been developed.

Enzymatic Production

L-Aspartic Acid

L-Aspartic acid is industrially produced by an enzymatic process in which aspartase (L-aspartate ammonia lyase EC 4.3.1.1) is used. This enzyme catalyzes the reversible interconversion between L-aspartate and fumarate plus ammonia. The equilibrium constant of the deamination reaction catalyzed by the enzyme is 20 mmol/L at 39°C and 10 mmol/L at 20°C, thus the amination reaction is favored. Aspartase purified from *E. coli* is a tetramer with a molecular weight of 196 kDa and it has a strong requirement for divalent metal ions. As the isolated enzyme is very unstable in solution an immobilized cell system based on *E. coli* cells entrapped in a polyacrylamide gel matrix was developed. Using this system the half-life of the aspartase activity could be increased to 120 days. Immobilization of the cells in κ-carrageenan, resulted in remarkably increased operational stability; thus, a biocatalyst with a half-life of approximately 2 years was obtained (Table 3). In addition, recombinant DNA-techniques helped to improve aspartase-containing strains. A plasmid with the *aspA* gene elevated aspartase formation in *E. coli* approximately 30-fold.

The production of L-aspartate by means of immobilized cells has been industrialized by using a fixed bed reactor system. A continuous process enables automation and efficient control to achieve high conversion rates and yields. A column packed with

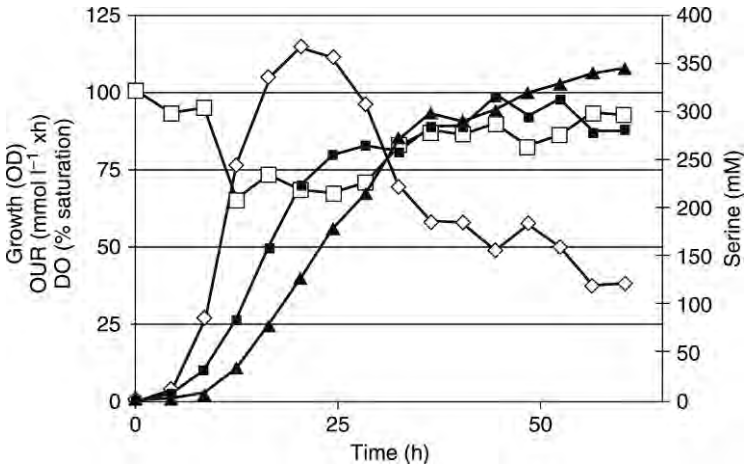


Fig. 7 Performance of an L-serine producer strain in a fermenter showing growth (■), the L-serine concentration in the medium (▲), the dissolved oxygen, DO (□), and the oxygen uptake rate, OUR (◊).

Table 3 Half-life of aspartase in *E. coli* cells immobilized using various methods

Immobilization method	*Aspartase activity (U/g cells)*	*Half-life (days)*	*Relative productivity (%)*[a]
Polyacrylamide	18,850	120	100
Carrageenan	56,340	70	174
Carrageenan (GA)a	37,460	240	397
Carrageenan (GA+HA)[b]	49,400	680	1498

[a]Considers the initial activity, decay constant, and operation period.
[b]GA=glutaraldehyde, HA=hexamethylene diamine.

the κ-carrageenan-immobilized cells produces 200 mmol L-aspartate per hour and gram of cells; thus, in a 1-m^3 column about 100 t of L-aspartate can be produced in 1 month. In comparison to the microbial amino acid production the advantages of this enzymatic production method are higher product concentration and productivity. Furthermore less by-products are formed; thus, L-aspartic acid can be easily separated from the reaction mixture by crystallization. In recent years the market for L-aspartic acid has increased to approximately 30,000 t per year due to the fact that this amino acid is a precursor for the production of the dipeptide sweetener aspartame (methyl ester of aspartyl-L-phenylalanine).

Future Prospects

For the synthesis of the proteinogenic amino acids microbial fermentation represents the key position among the production methods in the amino acid industry. Due to modern techniques such as genome sequencing, high-throughput screening based on amino acid sensors, DNA chip technology, and metabolomics, further improvement of the microbial processes are constantly achieved. Bottlenecks in the L-amino acid synthesis can be removed by amplification of genes coding for the limiting enzymatic steps. The recent discovery of amino acid secretion carriers opens up an entirely new field for increasing the overproduction of various L-amino acids. Furthermore, a thorough understanding of the various elements and mechanisms controlling the biosynthesis of an amino acid should make it possible to still further influence its production rate in a predictable way.

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Amitochondriate Protists (Diplomonads, Parabasalids and Oxymonads)[☆]

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Glossary

Anthropozoonotic disease A disease that is able to circulate between humans and animals.
Axostyle A large bundle or hollow rod of microtubules that runs down the central axis of the cell, providing structural support. Present in oxymonads and most parabasalids, but of independent origin in the two groups.
Flagellate A unicellular eukaryote that has flagella in the main growth and division phase of the life cycle (trophozoite). Usually refers specifically to heterotrophic eukaryotes.
Giardia A group of intestinal parasites of humans and other vertebrates. Belongs to the taxon Diplomonadida.
Hydrogenosome A form of mitochondrion-related organelle that generates ATP under anaerobic conditions, generating hydrogen gas as a waste product.
Karyomastigont A subcellular complex consisting of a discrete cluster of flagella, with associated cytoskeleton, and a nucleus. The term is also used to refer to some organisms in which the flagellar cluster and nucleus are tightly associated.
Mastigont A subcellular complex consisting of a discrete cluster of flagella and associated cytoskeleton.
Metamonads In its current usage, the group consisting of diplomonads, retortamonads, parabasalids, oxymonads, and their relatives.
Mitosome A small organelle that is homologous to mitochondria, but has no role in generating ATP.
Monophyletic group A group of organisms consisting of an ancestor and all of its descendents. Also known as a clade.
Trichomonas A group of commensal or parasitic flagellates belonging to the taxon Parabasalia. One species, *Trichomonas vaginalis*, infects the human urogenital tract.
Trophozoite Feeding form of a protist (in contrast to other possible lifecycle stages, e.g., cysts).
Undulating membrane In parabasalids, a flap-like connection between the cell membrane and a flagellum that moves with the beating flagellum.

Abbreviations

PFO Pyruvate: ferredoxin oxidoreductase
TCA Tricarboxylic acid
TEM Transmission electron micrograph

Defining Statement

The article covers the diversity, systematics, life history, cell organization, and evolutionary history of diplomonads, parabasalids, oxymonads, and their relatives. It gives an overview of some important diseases they cause, and symbioses with animals they are involved in. The diverse mitochondrion-related organelles of these anaerobes are also examined.

Introduction

Mitochondria are often considered to be a defining feature of eukaryotic cells; however, it is now clear that a large number of species lack canonical mitochondria. These organisms do not form a single taxonomic group, and they are referred to collectively as 'amitochondriate eukaryotes' or 'amitochondriate protists', though as discussed below, almost all have retained the mitochondrial organelle in a highly modified form. An alternative moniker is 'anaerobic eukaryotes'. These protists belong to a number of different

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lineages that have independently become adapted to environments where little or no free oxygen is present and the primary energy-generating function of canonical mitochondria – oxidative phosphorylation using oxygen as a terminal electron acceptor – is not feasible.

Instead of mitochondria, almost all well-studied amitochondriate eukaryotes have 'mitochondrion-related organelles' (MROs) that are bounded by two membranes but that lack characteristic features of mitochondria, most notably those associated with oxidative phosphorylation. These often-missing features include cristae (infoldings of the inner bounding membrane), most proteins of the tricarboxylic acid (TCA) cycle and of the electron transport chain, and the mitochondrial F_0F_1 ATPase (ATP synthase). In most cases these organelles also lack a genome. Some mitochondrion-like organelles also display positive properties that are not found in canonical mitochondria, such as the production of molecular hydrogen. Since the early 2000s it had been suspected that all living eukaryotes have retained some form of mitochondrion-related organelle, however it was recently shown that the oxymonad *Monocercomonoides* has, most likely, completely discarded the whole organelle (see below).

Amitochondriate/anaerobic organisms are scattered across the eukaryote tree of life, and many have well-established phylogenetic placements within groups that ancestrally have canonical aerobic mitochondria. For example, there are important amitochondriate clades within the heterolobosean amoeboflagellates (Psalteriomonadidae), the amoebozoan amoebae (Archamoebae: pelobionts and entamoebae), and the ciliates (several taxa). There are also amitochondriate fungi and fungi-relatives, the most prominent of which are certain chytrids that inhabit the rumen of ruminant animals, and the microsporidia, which are intracellular parasites. The best-known collection of amitochondriate eukaryotes, however, is the assemblage made up of diplomonads, parabasalids, oxymonads, and their relatives. This group, collectively called Metamonada or 'metamonads', is the most diverse in terms of cell organization and life history. These metamonads are the major focus of this article.

Diplomonads, parabasalids, and oxymonads collectively contain a broad range of symbiotic and parasitic species. These include the important human parasites *Trichomonas vaginalis* and *Giardia intestinalis* (Fig. 1). These organisms are also important from an evolutionary point of view. In the past diplomonads and parabasalids have often been proposed as the earliest diverging branches among living eukaryotes. While this idea is now obsolete, the exact phylogenetic placement of Metamonada within the eukaryote tree of life is still not fully resolved (Fig. 2). Of course, their highly modified mitochondrial organelles, and attendant changes in the biology and biochemistry of the rest of the cell, have made them compelling subjects for study by evolutionary cell biologists.

Amitochondriate Protists and Eukaryote Evolution

The mitochondrion was derived from a prokaryotic cell, specifically an α-proteobacterium. At some point, perhaps 1.5–2 billion years ago, this bacterium entered into a symbiotic association with a eukaryotic or 'proto-eukaryotic' host cell. The symbiont became a resident of the host cell, and over time, most genes were lost from the symbiont genome. Many of these genes were dispensable in the new environment, and therefore lost entirely, while others were transferred to the host nuclear genome. The protein products of

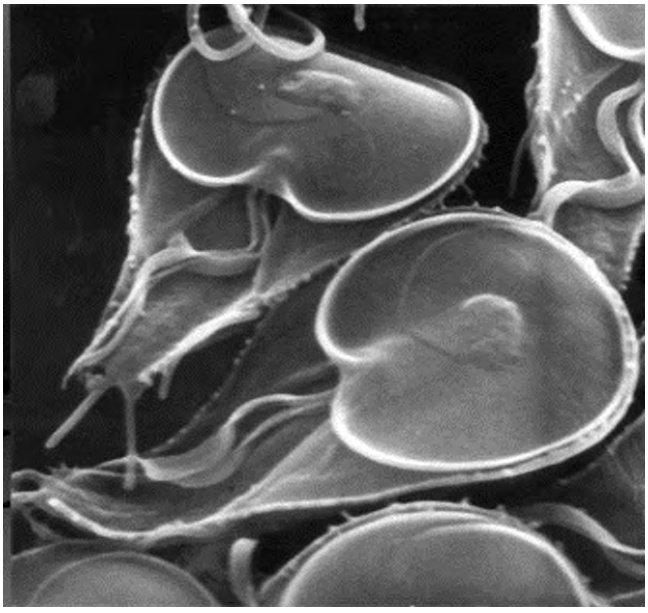

Fig. 1 Scanning electron micrograph of *Giardia intestinalis*, an amitochondriate eukaryote belonging to the group Diplomonadida and a significant human intestinal parasite. The cells are seen from the ventral face, much of which forms a large, flanged disk that is used for adhesion to the intestinal wall. Several of the flagella are visible (there are eight in total per cell), including two that bear vanes and beat within a ventral channel posterior to the disk. Image courtesy of K. Vickerman.

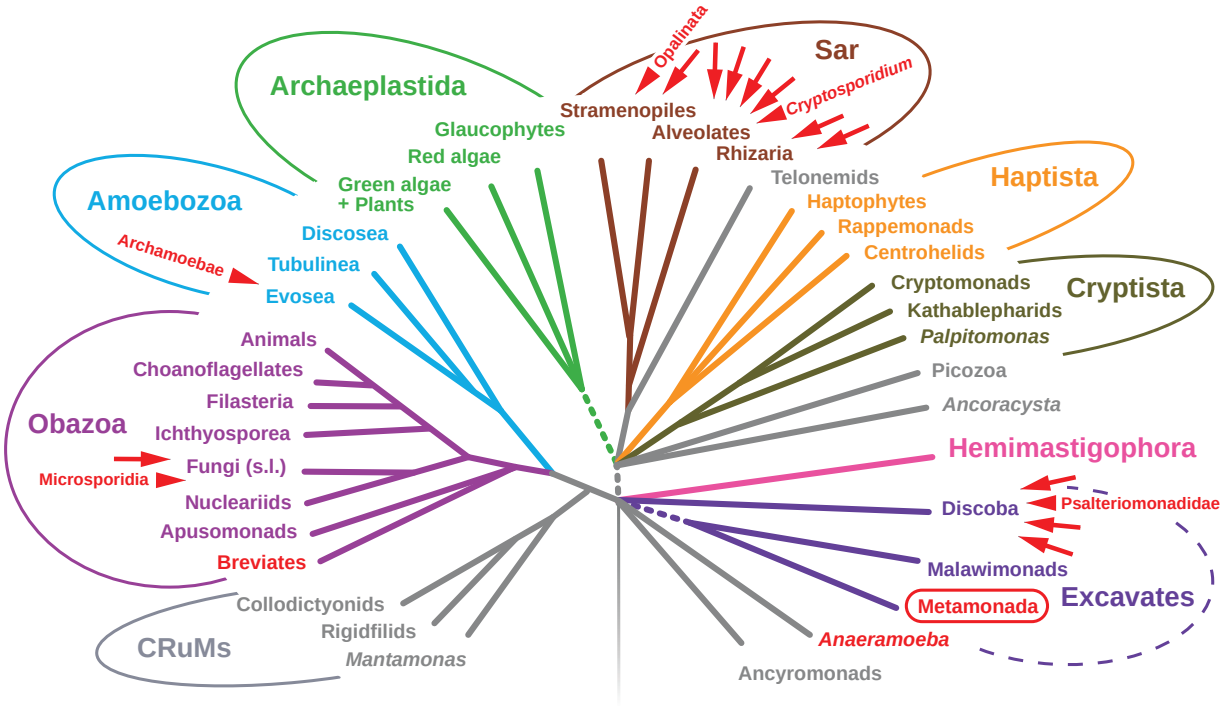

Fig. 2 Summary view of the eukaryote tree of life, as inferred (mainly) from recent phylogenomic analyzes, highlighting the phylogenetic positions of various better-documented amitochondriate/anaerobic protists (red text and arrows). Each red name or arrow represents a separate inferred evolutionary transition to the amitochondriate/anaerobic condition. Metamonada represents the most diverse of these amitochondriate/anaerobic protist groups, and includes all three of the diplomonads, parabasalids and oxymonads discussed further is this article. Polytomies and dashed lines both represent substantial phylogenetic uncertainty. 's.l.' – *sensu lato* (i.e., in the broad sense).

some transferred genes (together with many genes of non-symbiont origin) are now imported back into the organelle via a complex protein import apparatus that evolved to enable this transport. The evolution of this system cemented a total integration of the erstwhile symbiont into the eukaryotic cell as an organelle.

In the 1980s, it was proposed that some of the living groups of amitochondriate eukaryotes lacked mitochondria because they never had them. These taxa might be early branches from the 'main line' of eukaryotic descent that had diverged prior to the acquisition of mitochondria, making them 'primitively amitochondriate'. In groups with a conspicuous mitochondrion-like organelle, this structure was usually interpreted as the result of a different endosymbiosis involving a different prokaryotic symbiont. The general idea of late acquisition of mitochondria seemed to be supported in the late 1980s by molecular phylogenies that placed several groups of amitochondriate protists, including diplomonads and parabasalids (as well as Microsporidia), as the very earliest branches of the eukaryotic tree.

By the end of the last century, however, this 'mitochondria late' hypothesis had been essentially rejected. Studies of species that were proposed to be primitively amitochondriate showed that these organisms did, in fact, contain genes that likely originated from the genome of the α-proteobacterial symbiont. Actual mitochondrion-related organelles have now been found in nearly all of these taxa. In several cases, the products of nucleus-encoded genes of symbiont origin are known to be targeted to mitochondrion-like organelles, further confirming that these organelles do indeed stem from the same endosymbiotic event as canonical mitochondria. Furthermore, it is no longer inferred that amitochondriate organisms such as diplomonads and parabasalids are uniquely early branches on the eukaryotic tree. Under certain circumstances, molecular sequences that have experienced very large amounts of evolutionary change can cluster together artificially in a phylogenetic tree through the phenomenon of 'long-branch attraction'. Fast-evolving sequences are often drawn toward 'outgroup' sequences, which frequently act as 'long branches'. The fast-evolving sequences may thus appear 'deeper' in the phylogenetic tree than they actually belong. Genes from diplomonads and parabasalids, as well as Microsporidia, are often very divergent, and several studies showed that their deep evolutionary positions could be a long-branch attraction artifact. For example, a variety of evidence demonstrates that Microsporidia are actually closely related to true fungi.

Nonetheless, the true phylogenetic placement of diplomonads, parabasalids, and oxymonads is still not fully resolved (except that they are closely related to each other as 'Metamonada' – see below), and is the subject of ongoing research. Metamonads are usually inferred to be related to some or all other 'Excavates', namely

the taxa Discoba and/or Malawimonadida (Fig. 2). Discoba includes Euglenozoa (e.g., *Euglena* and *Trypanosoma*), the heterolobosean amoebae/amoeboflagellates (e.g., *Naegleria*), and jakobid flagellates. Malawimonads are a small group of free-living flagellates. Since both Discoba and Malawimonadida ancestrally have classical mitochondria, this phylogenetic inference further supports that metamonads evolved from fully mitochondriate ancestors.

Systematics

Diplomonads (Diplomonadida) are flagellated cells, most of which share the characteristic feature of having two nuclei and two near-identical clusters of flagella per cell (see Section "Cell Organization"). There are two major subgroups of diplomonads: Giardiinae and Hexamitinae. The Giardiinae (*Giardia*, *Octomitus*) are all parasites or commensals. The best known is *Giardia intestinalis* (synonyms: *G. lamblia*, *G. duodenalis*), which causes a highly prevalent diarrheal disease in humans – giardiasis or 'beaver fever' (see Section "Important Pathogenic Species"). The hexamitines (e.g., *Spironucleus*, *Hexamita*, *Trepomonas*) are more diverse and consist of a mixture of free-living, parasitic, and commensal species (Table 1). Some hexamitines (collectively referred to as 'enteromonads' e.g., *Enteromonas* and *Trimitus*) instead have a single nucleus and one flagellar cluster per cell, and look somewhat like half of a typical diplomonad cell.

Parabasalids (Parabasalia or Parabasala) are a diverse assemblage, containing a few hundred described species, almost all of which are parasites, commensals, or beneficial symbionts of animals. There are two basic morphological types. Parabasalids of the first type have a single nucleus associated with a single cluster of a few (usually 4–6) flagella. These are mostly relatively small cells (<20 μm), although there are some much larger forms, and they are found in different types of associations with a diversity of animal hosts. There are also a few free-living species. Parabasalids of the second type have dozens to thousands of flagella. These cells are usually large (tens to hundreds of μm) and are found almost exclusively in the hindguts of wood-eating termites and cockroaches, where they act (or are suspected to act) as beneficial symbionts. Traditionally, most of the multiflagellated species were referred to as 'hypermastigids' or 'hypermastigotes', while the simple forms were referred to as 'trichomonads'. Molecular phylogenetic studies confirm, however, that neither hypermastigotes nor trichomonads represent monophyletic groups (clades). It is clear

Table 1 Example amitochondriate protists from Metamonada

Group	*Subgroup*	*Example*	*Comments*
Diplomonadida	Giardiinae	*Giardia intestinalis*[a]	Prevalent human intestinal parasite
	Hexamitinae	*Spironucleus salmonicida*	Virulent parasite of salmonid fish
		Hexamita	Many free-living
		Trepomonas	Most/all free-living
		Enteromonas hominis	Enteromonad; human commensal (?)
Retortamonadida		*Chilomastix mesnili*	Human gut commensal/parasite (?)
Caviomonadidae		*Caviomonas*	Rodent commensal
Carpediemonas		*Carpediemonas*	Free-living, marine
Dysnectes		*Dysnectes*	Free-living, marine
Kipferlia		*Kipferlia*	Free-living, marine
Parabasalia	Trichomonadida	*Trichomonas vaginalis*	Prevalent human urogenital parasite
		Trichomonas tenax	Commensal (?) of human oral cavity
	Honigbergiellida	*Honigbergiella*	Commensal of cattle, free-living
	Hypotrichomonadida	*Trichomitus*	Commensal of various animals
	Tritrichomonadida	*Tritrichomonas suis*[b]	Causes spontaneous abortion in cattle
		Histomonas meleagridis	Poultry pathogen ('blackhead')
		Dientamoeba fragilis	Amoeba; human gut commensal/parasite (?)
	Cristamonadida	*Mixotricha paradoxa*	Termite symbiont; with motility symbionts
		Calonympha	Termite symbiont; with many karyomastigonts
	Trichonymphida	*Trichonympha*	Termite/wood-eating roach symbiont; multiflagellated
	Spirotrichonympha	*Spirotrichonympha*	Termite symbiont; multiflagellated
	Lophomonadida	*Lophomonas*	Cockroach symbiont; multiflagellated
Oxymonadida		*Monocercomonoides*	Small; diverse vertebrate and insect hosts
		Oxymonas	Medium-large; with holdfast and rostellum
		Saccinobacculus	With actively flexing axostyle
		Pyrsonympha	Large, attaches with holdfast
Trimastix		*Trimastix*	Free-living, marine
Paratrimastix		*Paratrimastix*	Free-living, freshwater

[a]Synonyms: *Giardia lamblia, Giardia duodenalis.*
[b]Synonym: *Tritrichomonas foetus.*

that the large multiflagellated forms arose from simpler trichomonad-like ancestors on multiple occasions in the evolutionary history of Parabasalia.

As of 2017, the high-level classification of parabasalids was based on a combination of morphological characters and molecular phylogenies. This system divides parabasalids into eight major taxa: Trichomonadida, Honigbergiellida, Hypotrichomonadida, Tritrichomonadida, Cristamonadida, Spirotrichonymphida, Trichonymphida, and Lophomonadida (with Honigbergiellida and Tritrichomonadida being possibly paraphyletic). The first four of these taxa are mostly small flagellates with 4–6 flagella. Some species are parasites, including the causative agents of human trichomoniasis (*T. vaginalis*) and of several diseases of domestic animals (see Section "Important Pathogenic Species"). Cristamonadida includes often quite large cells with only a few flagella (e.g., *Devescovina*), as well as multiflagellated species (e.g., *Joenia*), some of which have numerous small clusters of flagella, each usually connected to a nucleus (e.g., *Calonympha*). The Trichonymphida are all multiflagellated (e.g., *Trichonympha*), as are the Spirotrichonymphida (e.g., *Spirotrichonympha*). The latter three subgroups are symbionts of wood-eating insects. Lophomonadida contains only two species of *Lophomonas* with small cells and several dozen flagella; both live in cockroaches.

Oxymonads (Oxymonadida) are not immediately related to parabasalids, but share several superficial similarities. They are all commensals or perhaps beneficial symbionts of animals, and they range in morphology from simple species around 10 μm long to elaborate cells up to 200 μm. The moderate-to-large-sized forms, which constitute the bulk of the described species, are found only in wood-eating insects. A few of the largest oxymonads are also multinucleated and multiflagellated, but most species have only one nucleus and four flagella. Examples of oxymonads include the small, simple *Monocercomonoides* and *Blattamonas*, and larger organisms such as *Oxymonas*, *Saccinobaculus*, and *Pyrsonympha*.

The three groups described above are related to several more obscure lineages of amitochondriate eukaryotes. The immediate relatives of diplomonads include the retortamonads (Retortamonadida) and Caviomonadidae, both of which are usually commensals or parasites of animals, and a series of free-living forms such as *Carpediemonas*, *Dysnectes*, and *Kipferlia*. Retortamonads are divided into two genera: *Retortamonas*, with two flagella, and *Chilomastix*, with four. Caviomonadidae have a single flagellum (in the past, some caviomonads were thought to be 'enteromonad' diplomonads–see above). Meanwhile, *Carpediemonas*, *Dysnectes*, *Kipferlia*, and similar taxa are small free-living cells with two flagella, which have been shown to be related to diplomonads and retortamonads mainly through molecular phylogenies (see Fig. 3). A different set of free-living flagellates, *Trimastix* and *Paratrimastix*, turn out to be the closest relatives of oxymonads. Despite their names, *Trimastix* and *Paratrimastix* species have four flagella. We will not consider these obscure lineages in detail here.

Most recent molecular phylogenies demonstrate that the metamonads are monophyletic. Parabasalids and diplomonads (plus the obscure relatives of diplomonads) are almost always now inferred to be closely related to each other. Oxymonads, *Trimastix*, and *Paratrimastix* (collectively Preaxostyla) typically form the immediate sister group of the diplomonad–parabasalid assemblage. Therefore, it is likely that diplomonads, parabasalids, and oxymonads all descended from a common ancestor that already lacked canonical mitochondria (Fig. 3).

Habitats

Amitochondriate eukaryotes are characteristically found in habitats with little free oxygen. Many do not withstand high oxygen levels, but some (including *Giardia* and especially *T. vaginalis*) at least tolerate or even grow in microoxic conditions and are therefore not strict anaerobes.

Free-living amitochondriate protists are frequently reported from highly eutrophic (nutrient-enriched) sites where high levels of microbial activity have depleted the free oxygen. Natural environments for these species include freshwater swamps, many marine sediments, and intertidal mudflats. Similar but artificial environments in which amitochondriate eukaryotes are observed include sewage sludges and waste ponds from certain industrial processes such as sugar refining. Amitochondriate eukaryotes also occur in noneutrophic anoxic water masses, such as those that can form at depth in deep fjords due to poor mixing with surface water. Most free-living forms eat prokaryotes.

Many commensal and parasitic amitochondriate eukaryotes live within the intestinal tracts of animals. *Giardia* in humans is one of the many examples. Some parasites, however, have other sites of infection. The parabasalid *T. vaginalis*, for example, infects the human urogenital system, while a related commensal species, *Trichomonas tenax*, lives in the human mouth. Some species of *Spironucleus* that infect fish can cause systemic infections that spread to numerous tissues in the host.

Certain large parabasalids and oxymonads are beneficial symbionts of wood-eating insects, namely, many termites and the related wood-eating roach *Cryptocercus*. These symbionts live in the insect's hindgut, together with a diverse biota of prokaryotes (the hindgut also hosts other protists, such as small parabasalids). The larger species produce cellulases, enzymes that degrade cellulose, a major component of woody plant material. These cellulases, together with those produced by the prokaryotic symbionts, are a significant supplement to the endogenous cellulases produced by the termite, which are most important before the hindgut. Larger parabasalids and oxymonads are able to engulf large particles of wood by phagocytosis, and partially digest them. Under the generally anaerobic conditions of the hindgut some end products of their metabolism (e.g., acetate and other organic acids) can then be absorbed and utilized by the insect for aerobic energy production. Other products of their metabolism are substrates for various prokaryotes, which in turn release products useable by the insect.

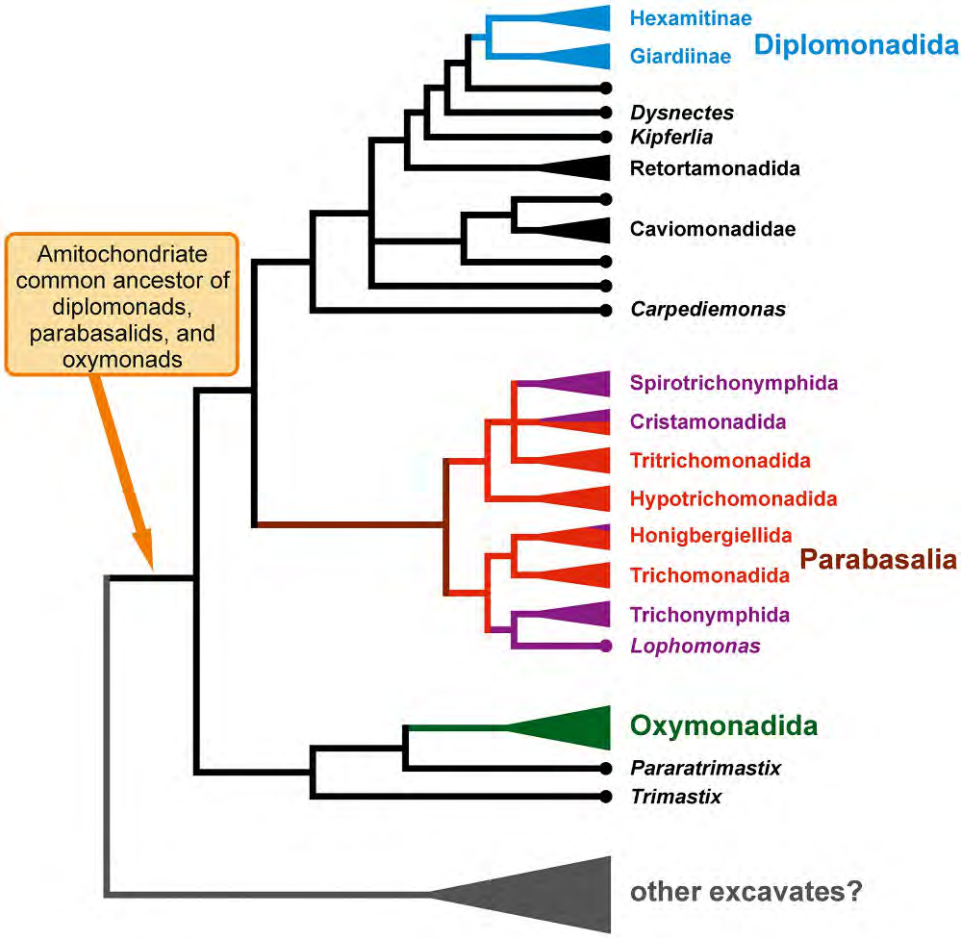

Fig. 3 Phylogenetic diagram for Metamonada, inferred from molecular phylogenetic analyses, illustrating the relationships among diplomonads, parabasalids, oxymonads and related minor taxa, as currently understood. Unlabeled branches represent obscure genera not discussed further in the text. Also shown is the current higher systematics of Diplomonadida and Parabasalia, as discussed in the text. The two colors within Parabasalia distinguish small forms (red) from large multiflagellated forms (purple) – see text.

Cell Organization

Diplomonads

Diplomonads are small cells, usually around 10 μm in length and often less. With the exception of the 'enteromonad' organisms (see Section "Systematics"), diplomonads have a characteristic 'doubled' organization. Each cell has two identical-sized nuclei located alongside each other in the anterior half of the cell (Fig. 4). Associated with each nucleus is a flagellar apparatus consisting of a single cluster of flagella and a series of 'microtubular roots' formed by parallel microtubules that originate in association with the basal bodies of the flagella and extend into the cytoplasm in different directions. Each flagellar cluster and associated cytoskeleton is sometimes referred to as a 'mastigont', and the complex of the mastigont and its associated nucleus is termed the 'karyomastigont'. There are usually four flagella per cluster, one of which is directed posteriorly, another of which is generally directed laterally, and the final two may be directed anywhere from laterally to posteriorly (Fig. 4). There are three basic microtubular roots recognized in diplomonads: one that travels posteriorly, usually alongside the posteriorly directed flagellum, and two that pass over the top of and underneath the nucleus respectively, although the last of these roots is absent in Giardiinae (see below). The microtubular roots determine much of the basic shape of the cell. In all species except *Giardia* spp. (see below), the two flagellar apparatuses are more-or-less identical to each other, and the cell has a rotational symmetry.

The other conspicuous cell component in many diplomonads is the endoplasmic reticulum, which often forms stacks within the cell. Hexamitine diplomonads may also contain food vacuoles, which tend to lie in the posterior half of the cell. A classical stacked Golgi apparatus is not observed by electron microscopy, although individual vesicles appear in encysting *Giardia* cells (see below), and these are involved in Golgi-like posttranslational modifications of cyst wall proteins. To date, mitochondrion-related organelles have been observed in only a couple of species, and these organelles are tiny compared with canonical mitochondria. In *Giardia*, where the mitochondrion-related organelles are ~100 nm in size and are definitively identified as 'mitosomes' (see Section "Hydrogenosomes and Mitosomes", below), they are generally located in the center of the cell, posterior to the nuclei,

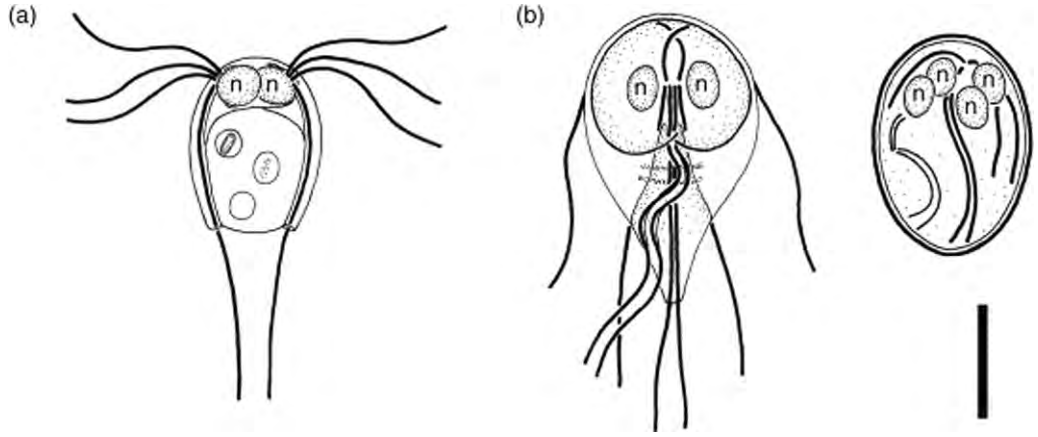

Fig. 4 Representative diplomonads. (a). *Hexamita* (Hexamitinae). (b). *Giardia* (Giardiinae) in trophozoite form (seen from the ventral side) and in cyst form. *n* – nucleus (note that the *Giardia* cyst contains four nuclei). Scale bar = 5 μm for all images.

and between the flagellar apparatuses. In *Spironucleus* the organelles function as unusual hydrogenosomes (see below). There are often conspicuous glycogen granules in the cytoplasm of diplomonads.

As in most flagellate eukaryotes, each flagellar apparatus duplicates prior to mitosis, and the flagellar apparatuses serve as the microtubular organizing centers for the mitotic spindle. Mitosis in diplomonads is 'semi-open', meaning that the nuclear envelope remains largely intact, but the spindle microtubules penetrate through holes in the envelope to attach directly to the chromosomes. In diplomonads, of course, there are two nuclei, and each must undergo mitosis before actual cytokinesis (cell division). Like many unicellular eukaryotes, some diplomonad species form cysts that are resistant to chemical insult and/or desiccation (Fig. 4(b)). In some parasitic forms (e.g., *Giardia*), the cyst is the infectious form that is transmitted between hosts.

In hexamitine diplomonads, the two flagellar apparatuses are located apart from each other, usually toward the 'outside' of the cell. The nuclei, or at least parts of each nucleus, are located between the two flagellar clusters (Fig. 4(a)). Each posterior flagellum is either associated with an open groove on the cell surface or is enclosed by a tube that opens near the posterior end of the cell. The grooves or tubes are feeding structures where material (e.g., prokaryotic prey) is phagocytosed.

In Giardiinae, the two flagellar apparatuses are located close to each other, more-or-less in the center of the cell, and between the two nuclei (Fig. 4(b)). The proximal part of each flagellum is intracytoplasmic, that is, it runs some distance through the cytoplasm before emerging as a distinct structure surrounded by a flagellar membrane (this arrangement is also seen in some hexamitines, but to a much more limited extent). In particular, one flagellum from each flagellar apparatus runs down the central axis of the cell and emerges only at the extreme posterior end. The other flagella emerge at various locations around the cell. There are no distinct feeding-related structures and the cells do not perform phagocytosis.

The best-known member of Giardiinae is *Giardia*. *Giardia* is unusual among diplomonads because the cell has a superficial bilateral symmetry, rather than rotational symmetry. The 'ventral' side of the cell is flattened and the anterior half of the ventral face is shaped into a large, flanged 'adhesive disk' that the cell uses to attach to the intestine wall of its host (Figs. 1 and 4(b)). Two of the flagella (one from each flagellar apparatus) lie within a wedge-shaped channel that extends posterior to the disk. These flagella bear vanes (Fig. 1) and beat within the channel during attachment. The exact method by which this parasite attaches to the host epithelium may involve several mechanisms. It has often been assumed that the disk acts like a suction cup. Alternatively, or in complement, contraction within the cytoskeleton that supports the disk may allow the rim of the disk to mechanically grip the intestine wall. The disk flange also appears to be capable of actual adhesion to surfaces.

Parabasalids

As discussed above, there is a wide range of cell complexity in parabasalids. The smaller, simpler cells include *Trichomonas* and the other species in the taxa Trichomonadida, Tritrichomonadida, Honigbergiellida, and Hypotrichomonadida (Fig. 5(a)). These cells almost always have a single nucleus, which is located near the anterior end of the cell. The nucleus is associated with a flagellar apparatus, usually in the form of a single cluster of 4–6 flagella and a cytoskeletal system (i.e., the cell has a single karyomastigont). One flagellum is directed posteriorly while the others are directed anteriorly or laterally. In many species, the posteriorly directed flagellum is unusual in that the flagellar membrane is physically connected to the cell membrane for some of its length. The cell membrane is extended as a flap or broad projection along this zone of attachment (in some cases the attached flagellum is also expanded). This creates a fin-like region that moves with the flagellum as it beats, and is referred to as an 'undulating membrane'.

There are two main microtubular structures within the parabasalid cell – the pelta and the axostyle – which are both associated with the flagellar basal bodies and the nucleus. The pelta is a sheet of spaced microtubules that curves over the anterior side of the nucleus and gives structure to the anterior end of the cell. The axostyle is a column of microtubules that forms the central supportive axis of the cell, and usually extends from the posterior end of the cell into a point. There are also conspicuous nonmicrotubular

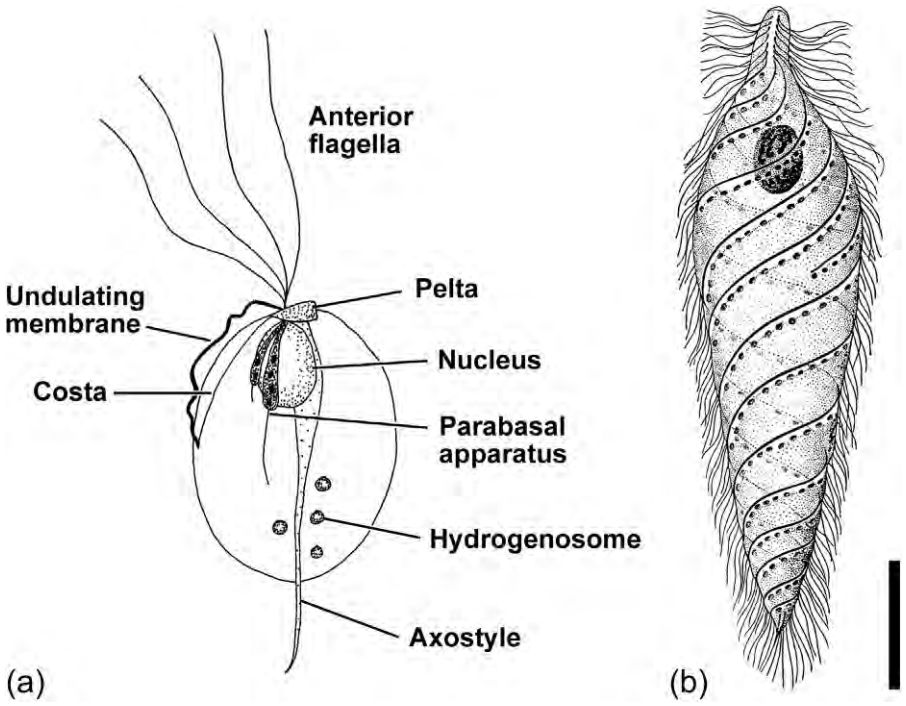

Fig. 5 Representative parabasalids. (a). *Trichomonas vaginalis* (Trichomonadida), a small form. (b). *Spirotrichonympha* (Spirotrichonymphida), a moderately large multiflagellated parabasalid. Scale bar = 5 μm for (a) and 20 μm for (b). B – after Duboscq, O., Grassé, P.-P., 1933. L'appareil parabasal des Flagellés, avec des remarques sur le traphosponge, l'appareil Golgi, les mitochondries et le vacuome. Archives de Zoologie Expérimentale et Générale 73, 381–621.

elements to the cytoskeleton such as the striated 'parabasal fibers' that extend posteriorly from the flagellar apparatus into the cytoplasm. These parabasal fibers are associated with the Golgi apparatus, which is conspicuous and shows the classical 'stacked' organization of membrane sacs. The fibers and the Golgi collectively form the 'parabasal apparatus', which is characteristic for the group (and inspired its name). Some species also have a 'costal fiber' or 'costa', a striated fiber that runs under the cell membrane, in association with the undulating membrane. The costal fibers vary in appearance, and are contractile in some cases. There are a few species (e.g., *Histomonas meleagridis*, *Dientamoeba fragilis*) in which the flagellar apparatus and cytoskeleton are reduced or completely absent; these species are highly variable in shape and usually behave as amoebae.

Simple parabasalids usually phagocytose host cellular debris, prokaryotic cells, and even entire host cells in some cases (*T. vaginalis*). Thus, in addition to endoplasmic reticulum, many cells also contain food vacuoles. The parabasalid mitochondrion-related organelles are referred to as hydrogenosomes (see Section "Hydrogenosomes and Mitosomes"). They are roughly the same size as mitochondria, are quite numerous, and are usually found loosely associated with the axostyle and/or costal fiber. Granules of glycogen also preferentially accumulate in association with the axostyle. Curiously, the formation of cysts seems to be uncommon in parabasalids, though some small forms do produce cysts with a distinct extracellular wall. *Trichomonas vaginalis* and some other species form somewhat resistant 'pseudocysts', which are compact, but lack a cyst wall.

Large parabasalids are quite different in overall appearance. In some species each cell has many individual flagellar apparatus–cytoskeleton complexes, each usually attached to a nucleus (i.e., the cell has dozens to thousands of karyomastigonts). The karyomastigonts may be 'bundled together' by their axostyles. In most species, however, there is a single large nucleus. The numerous flagella are then either organized in a single field near the anterior end of the cell (some cristamonads) or formed into two or more plates (trichonymphids) or rows (spirotrichonymphids) that originate at the anterior end of the cell, and run down most of its length, often in spirals (Fig. 5(b)). The parabasal apparatuses may be aligned to these rows. Cells generally phagocytose objects such as wood particles at their posterior end. Many of the large multiflagellated species are more than 100 μm in length, and some reach 0.5 mm. The complement of cell organelles is similar to that of simple parabasalids, although undulating membranes are absent, and the form of the pelta and axostyle can be modified. The parabasal apparatus may have numerous branches and takes many complex forms.

In parabasalid mitosis, the nuclear envelope does not break down, and the mitotic spindle is formed external to the nucleus, with noncentriolar regions called 'attractophores' serving as the microtubular organizing centers for the spindle. The form of the spindle is distinctive. The attractophores begin mitosis next to each other on one side of the nucleus. Some of the spindle microtubules attach to the chromosomes indirectly through the intact nuclear envelope, while others form a solid rod directly between the attractophores. It is this rod that pushes apart the two attractophores, thereby separating the attached chromosomes.

Some larger parabasalids have substantial numbers of prokaryotic ectosymbionts. One remarkable example is the unusual termite-inhabiting cristomonad *Mixotricha paradoxa*, which has only four flagella, yet is very large – up to 0.5 mm. Most of the cell surface is

covered by several species of epibiotic bacteria, including spirochetes that attach to distinct docking sites on the surface of the parabasalid cell. The spirochetes are motility symbionts, and *Mixotricha* is propelled through its environment by the locomotory action of these attached spirochetes rather than by its own flagella. Prokaryote–eukaryote motility symbiosis is also seen in another cristamonad, *Caduceia*.

Oxymonads

Oxymonads generally have a single nucleus located in the anterior portion of the cell. This nucleus is associated with four flagella arranged as two separated pairs connected by a broad sheetlike microtubular root, the 'preaxostyle'. The preaxostyle is also the site-of-origin of a two-dimensional array of linked microtubules, called the axostyle (Fig. 6), that runs down the longitudinal axis of the cell. This structure is only superficially similar to the axostyle of parabasalids and presumably evolved independently. In the oxymonad *Saccinobaculus* the axostyle is highly motile: active sliding of microtubules within the axostyle causes the structure to flex dramatically, such that the entire cell performs a squirming motion.

In many species, the extreme anterior end of the cell forms a microfibrillar structure called a holdfast. This is used to attach the cell to the gut wall of its host. In some groups this holdfast is located at the end of a long columnar structure supported by microtubules, called the rostellum (Fig. 6(b)). The oxymonad cell contains conspicuous endoplasmic reticulum, while a discrete Golgi apparatus is not observed. Most oxymonads can feed by phagocytosis, and cells of smaller species typically ingest prokaryotes, while larger species may ingest wood particles, as per large parabasalids. Many species have prokaryotic symbionts either as endosymbionts within the cytoplasm or as ectosymbionts that attach to the cell surface, as in some large parabasalids. Some oxymonads are known to produce cysts, which are presumably used in transmission to new hosts.

The smallest oxymonads are less than 10 μm long, while the largest uninucleate forms can be up to 200 μm in length, and may have eight flagella rather than the usual four (*Pyrsonympha*). A few large species have numerous nuclei and flagella. In these cases, each nucleus within the cell is connected to a cluster of four flagella, in a manner analogous to some cristamonadid parabasalids (see 'Parabasalids').

Hydrogenosomes and Mitosomes

The capabilities of the mitochondrion-related organelles of amitochondriate eukaryotes vary widely, however many can be classified as some variant of one of two types: hydrogenosomes and mitosomes (Fig. 7). Hydrogenosomes were first identified in parabasalids, where they seem to be universal. Similar organelles are also found in some other amitochondriate eukaryotes not considered in detail in this article. These include, among others, anaerobic ciliates, some rumen-dwelling fungi, and some heteroloboseans.

The hydrogenosomes of parabasalids are about the same size as mitochondria and, like mitochondria, are organelles that generate energy for the cell. However, they lack most components of the mitochondrial electron transport chain, they do not have a functioning TCA cycle, and they do not use oxygen as an electron acceptor when generating energy. They do, however, contain

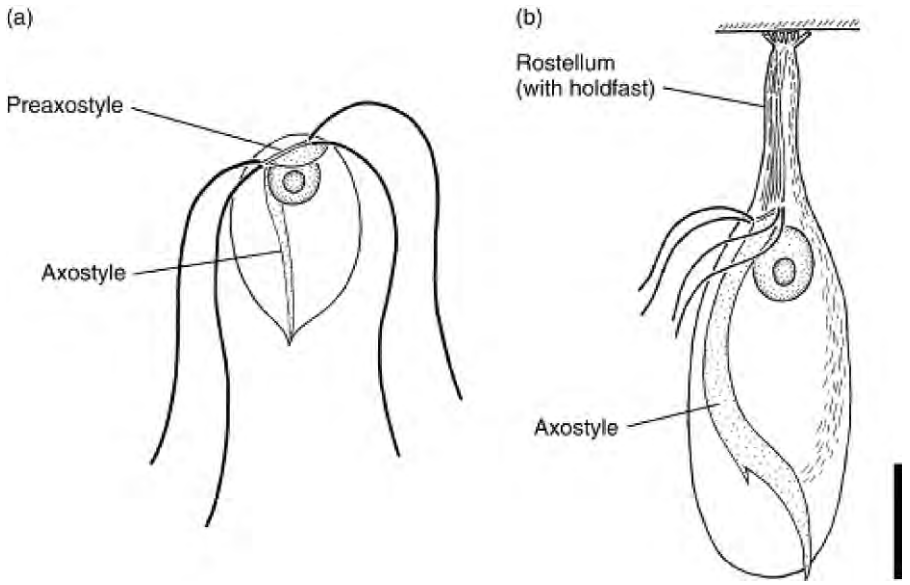

Fig. 6 Representative oxymonads. (a) *Monocercomonoides*, a small free-swimming form. (b). *Oxymonas*, an attached form. Scale bar = 5 μm for (a) and 10 μm for (b).

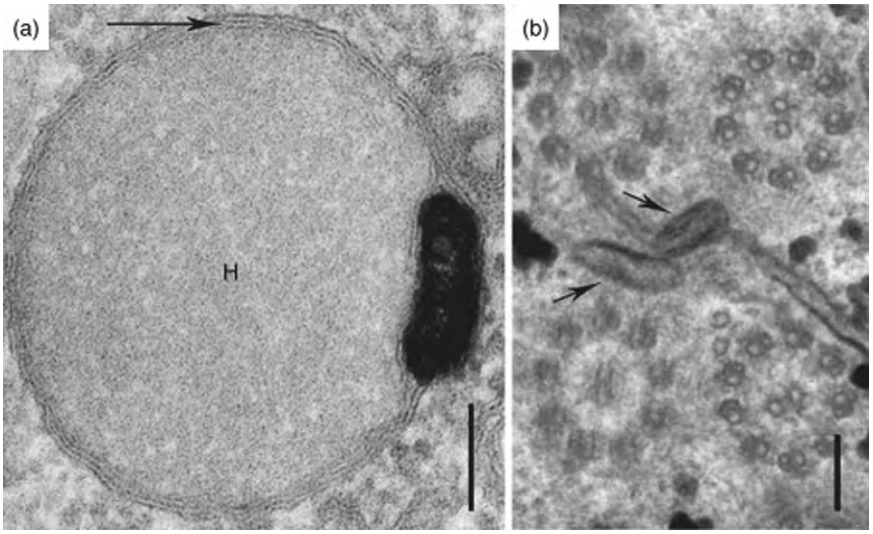

Fig. 7 Hydrogenosomes and mitosomes. (a). Transmission electron micrograph (TEM) of a hydrogensome of the trichomonad parabasalid *Tritrichomonas suis*. Note the presence of two closely adpressed bounding membranes (see arrow), but the lack of cristae. (b). TEM showing two mitosomes of *Giardia intestinalis* (arrows), located in the center of the cell, between the intracytoplasmic portions of some of the flagella. Scale bars = 100 nm. (a) Image courtesy of M. Benchimol, from Benchimol, M., 2008. Structure of the hydrogenosome. In: Tachezy, J. (Ed.), Hydrogenosomes and Mitosomes: Mitochondria of Anaerobic Eukaryotes. Berlin: Springer-Verlag, copyright 2008 Springer-Verlag Berlin Heidelberg, reproduced with kind permission of Springer Science and Business Media. (b) Image reprinted with permission from Macmillan Publishers Ltd: Nature, Tovar, J., León-Avila, G., Sánchez, L.B., et al., 2003. Mitochondrial remnant organelles of *Giardia* function in iron-sulfur protein maturation. Nature 426, 172–175, copyright 2003.

several enzymes that are absent in canonical mitochondria, including Pyruvate:Ferredoxin Oxidoreductase (PFO) and [FeFe] hydrogenase.

The core of hydrogenosomal metabolism is depicted in Fig. 8. Like mitochondria, hydrogenosomes can utilize pyruvate as a primary substrate. Within hydrogenosomes, PFO catalyses the decarboxylation and oxidation of pyruvate, forming acetyl-CoA and carbon dioxide. The electrons lost from pyruvate are accepted by the carrier ferredoxin. CoA is recycled via a succinate-to-succinyl-CoA cycle, releasing acetate as a waste product (via an Acetate:Succinyl CoA Transferase – ASCT) and yielding one ATP molecule by substrate-level phosphorylation (via Succinyl-CoA Synthase – SCS, which is actually an enzyme of the TCA cycle of canonical mitochondria). Oxidized ferredoxin is then recycled by donating electrons to H^+ ions (protons), forming H_2 gas. It is this last reaction that is catalyzed by the enzyme hydrogenase. All the classical hydrogenosomal reactions take place in the matrix of the hydrogenosome, and movement of protons across the inner membrane is not required to generate ATP. However, this partial oxidation yields one ATP per pyruvate molecule, many times lower than the yield in mitochondria with a full TCA cycle and electron transport chain, and using oxygen as the terminal electron acceptor.

The waste products of hydrogenosomal metabolism, acetate and H_2, can be utilized by some prokaryotes. For example, H_2 is a substrate for many methanogenic Archaea, and methanogens and hydrogenosome-bearing parabasalids coexist in the hindguts of termites. Other hydrogenosome-bearing eukaryotes (e.g., some anaerobic ciliates) harbor endosymbiotic methanogens that reside within the eukaryote's cytoplasm and are usually situated immediately alongside the hydrogenosomes. Others support ectosymbiotic sulfate-reducing proteobacteria.

Another form of mitochondrion-related organelle is the 'mitosome'. Mitosomes have no role in energy generation, even under anaerobic conditions. The extremely small (~100 nm) mitochondrion-related organelles of the diplomonad *Giardia* (Fig. 7(b)) are identified as mitosomes. Organelles classed as mitosomes are also found in Microsporidia (Fungi, *sensu lato*) and *Entamoeba* (Archamoebae), neither of which is closely related to diplomonads and parabasalids (see Fig. 2).

Canonical mitochondria perform several important functions in eukaryotic cells that are not directly related to ATP synthesis, opening the possibility that amitochondriate protists could rely on their mitochondrion-related organelles primarily for one or more of these other functions. One such process that is performed by canonical mitochondria is the synthesis of iron–sulfur clusters, which are embedded in the active sites of various enzymes involved in electron transfer reactions. Proteins from the mitochondrial iron–sulfur cluster synthesis pathway (ISC) have in fact been localized to the mitosome of *Giardia*, and their activity has been demonstrated *in vitro*. This indicates that this mitochondrial function is indeed retained by this highly reduced organelle. Iron–sulfur cluster synthesis is also a known function of *Trichomonas* hydrogenosomes. Interestingly, it appears that the mitosomes of *Entamoeba* do not harbor the ISC pathway, demonstrating that there is no universal function for mitosomes (and mitochondria) across eukaryotes.

Canonical mitochondria still contain a small genome that is a remnant of the genome of the original α-proteobacterial symbiont. This genome usually encodes some ribosomal RNAs and a few (up to ~70) proteins, especially certain components of the electron transport chain. By contrast, both parabasalid hydrogenosomes and *Giardia* mitosomes have completely lost their genomes, and presumably, their translation machinery. Thus, all of the proteins that function in these hydrogenosomes or mitosomes must be encoded by the nuclear genome and imported to the organelle post-translationally.

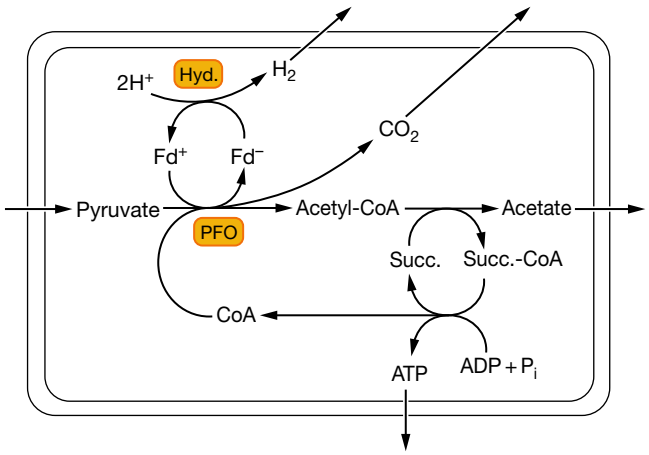

Fig. 8 Diagram showing the core metabolic pathway of the parabasalid hydrogenosome. Pyruvate is partially oxidized, yielding acetate and carbon dioxide. Electrons are ultimately accepted by protons (rather than by oxygen, as is typical of mitochondria), yielding hydrogen gas. 1 ATP is generated per molecule of pyruvate processed. PFO, pyruvate:ferredoxin oxidoreductase; Hyd., hydrogenase; Fd, ferredoxin; Succ., succinate; Succ.-CoA, succinyl-CoA. Redrawn from several sources, including Hrdy, I., *et al.*, 2008. Metabolism of trichomonad hydrogenosomes. In: Tachezy, J. (Ed.), Hydrogenosomes and Mitosomes: Mitochondria of Anaerobic Eukaryotes. Berlin: Springer-Verlag, copyright 2008 Springer-Verlag Berlin Heidelberg, reproduced with kind permission of Springer Science and Business Media.

Convincing mitochondrion-related organelles have never been observed in electron microscopy studies of oxymonads. Recently, -omic-level investigations of oxymonad molecular biology were conducted, including genome sequencing of *Monocercomonoides exilis*. These studies have shown that oxymonads lack the mitochondrial ISC system for iron-sulfur cluster assembly, and instead encode proteins of the SUF system, an iron-cluster assembly machinery found in plastids (chloroplasts), some prokaryotes, and in the cytoplasm of just a couple of other groups of eukaryotes. This SUF machinery was acquired by horizontal gene transfer from a prokaryote, very likely in a common ancestor of Preaxostyla (since *Trimastix* and *Paratrimastix* also have SUF, and seem to lack ISC). However, in addition to lacking the ISC system, no trace of any other signature mitochondrial proteins, for example protein import machinery components and mitochondrial chaperones, could be found in the *Monocercomonoides* genome. It is most likely that *Monocercomonoides*, and perhaps all oxymonads, have completely lost the mitochondrial organelle. If so, oxymonads are the only living eukaryotes known to be completely amitochondriate in the literal sense.

Genetics and Genomics

The genome of *Giardia* is small and compact for a eukaryote. At ~12 Mb in size and with ~5000–6500 protein-coding genes it is similar in size and coding capacity to the genome of brewer's yeast, *Saccharomyces cerevisiae*. The intergenic regions in *Giardia* are short, and adjacent genes sometimes even overlap. A comparable organization was found in the slightly larger genome of the hexamitine diplomonad *Spironucleus salmonicida*. The *Trichomonas vaginalis* genome is much larger (~160 Mb), with at least 25,000 genes interspersed with numerous repetitive elements, and there is evidence of recent and dramatic genome expansion. Interestingly, spliceosomal introns are very sparse in both diplomonads and parabasalids; there are ~65 in *Trichomonas* and just eight identified in *Giardia*, for example. The recently sequenced genome of the oxymonad *Monocercomonoides* is ~75 Mb in size and, unlike the genomes of parabasalians and diplomonads, contains many spliceosomal introns. Metamonad genomes seem to have been shaped significantly by lateral gene transfer. The bulk of the transferred genes are metabolic genes that have been acquired from prokaryotes, probably more than 100 in the case of *Giardia*. Most of these are probably stable long-term transfers, as a large fraction of the genes of foreign origin in diplomonads are shared by *Giardia* and *Spironucleus*.

One issue of interest in diplomonads is whether or not their two nuclei are genetically equivalent. Prior to cell division both nuclei undergo mitosis, and one copy of each of the parent nuclei is passed on to each daughter cell. In other words, each nucleus could, in principle, act as a separate lineage and diverge in sequence from the other over evolutionary time. Nonetheless, most of the evidence to date from *Giardia* suggests that the nuclei are, in fact, equivalent (although karyotypic differences have been recorded too). A process of internuclear genetic transfer has been documented in *Giardia* cysts, providing a possible mechanism for homogenization of the nuclear genomes. If diplomonads are truly sexual (see below) this may also act as a homogenization mechanism.

Various lines of evidence indicate that at least some diplomonads, parabasalids, and/or oxymonads are sexual. Over the 1940–1960s L.R. Cleveland examined and described sexual cycles in several large parabasalids and oxymonads from wood-eating insects. These light-microscopy-based accounts record gamete formation, meiosis, fertilization events, and autogamy (i.e., fusion of gametic nuclei within a single cell). However, these phenomena await reexamination with modern microscopic techniques. Recent attention has focused on small parasitic forms, and molecular approaches. Diplomonads and parabasalids both

have genes that encode proteins required for meiosis, and evidence suggests that there was genetic recombination in the ancestry of various contemporary strains of both *G. intestinalis* and *T. vaginalis*. Interestingly, while the nuclei of *G. intestinalis* appear to be diploid, the strain WB (from which the first genome sequence was derived) is almost completely homozygous. This is not what would be expected from a long history without recombination, over which the homologous chromosomes should diverge from one another in sequence.

Important Pathogenic Species

Diplomonads, parabasalids, and their relatives are mostly harmless commensals or beneficial symbionts found in the digestive tracts of both vertebrates and invertebrates. However, some species are pathogenic and cause various diseases of the intestine, urogenital tract, or other internal organs. The following is a brief discussion of some of the diseases caused by these organisms, especially those affecting humans and domestic animals.

Giardia intestinalis (synonyms: *G. duodenalis*, *G. lamblia*) is a parasite of the small intestine of humans and many animals, and several genetic lineages (assemblages) exist. Two of these lineages, assemblages A and B, are anthropozoonotic; humans may be infected from a wide variety of both domestic and wild animals. In the developing world there is also extensive person-to-person transmission due to sewage contamination of drinking water, and prevalence is high (~20%–30%). The disease caused by *G. intestinalis*, giardiasis, is also called beaver fever or backpacker's diarrhea. Humans contract giardiasis by ingestion of water or food that contains *Giardia* cysts. Occasionally, transmission may be via fecal–oral or sexual routes.

After the passage of tetranucleate cysts through the stomach the binucleate trophozoites are released. These adhere to enterocytes of the small intestine, thereby blocking nutrient absorption. Following an incubation period of one to a couple of weeks, the main symptom of giardiasis manifests – bloodless and non-festering slimy diarrhea that may be accompanied by stomach ache, nausea, vomiting, and loss of appetite. As lipids are not effectively absorbed by the infected small intestine, the diarrheic feces are often white and oily, and float in water. The disease usually abates spontaneously in a few weeks, but may persist for years without treatment in some cases (although a large proportion of infections are asymptomatic). Giardiasis is treated with 5-nitroimidazole derivates (metronidazole, tinidazole). *Giardia intestinalis* also causes an intestinal disease in some domestic animals that is similar to human giardiasis. Three nonpathogenic or slightly pathogenic relatives of *Giardia* also live in the human large intestine: *Enteromonas hominis* (an 'enteromonad' species belonging to Hexamitinae), *Chilomastix mesnili*, and *Retortamonas intestinalis* (the latter two are retortamonads).

Spironucleus is a genus of hexamitine diplomonads that includes several species that cause enteritis, skin disease, or systemic infections of fish, birds, and rodents. *S. salmonicida* has caused serious mortality in farmed salmonid fish.

Trichomonas vaginalis causes human urogenital trichomoniasis, perhaps the most prevalent sexually transmitted disease. There are estimated to be more than 170 million infections worldwide. The incubation period lasts from a few days to a few weeks. The infection is asymptomatic or accompanied by only mild symptoms in most men and approximately half of women, and these people serve as carriers of the disease. Rarely, epididymitis and prostatitis, or even sterility, may develop in some men. The infection is typically more severe in women, and can include inflammation of the vagina and womb accompanied by 'strawberry cervix'. In these cases, the normal vaginal microbiota is perturbed and a fetid discharge develops. Although the symptoms usually disappear spontaneously after some time, *Trichomonas* cells persist in the vagina and inflammations may reappear in the future. Acute trichomoniasis during pregnancy may cause premature birth, and in some long-term untreated cases it has caused sterility. Persons with trichomoniasis are also considerably more susceptible to HIV infection due to inflammation of the genital mucosa. Human urogenital trichomoniasis is treated with 5-nitroimidazole derivates (usually metronidazole or ornidazole), and it is necessary to treat both sexual partners even if the symptoms have developed in only one of them. A closely related species, *T. tenax*, lives in the human oral cavity and is usually considered to be nonpathogenic, although its harmlessness has been disputed by some workers. At least two trichomonad parabasalids live in the human large intestine. Of these, *Pentatrichomonas hominis* is a probably nonpathogenic commensal whereas the aflagellated, ameboid *D. fragilis* may be involved in some intestinal disorders.

Tritrichomonas suis, more commonly known by its synonym ***Tritrichomonas foetus***, lives in the nasal cavity of pigs and the urogenital tract of cattle. Although harmless to pigs, *T. suis* is highly pathogenic to cattle and causes bovine urogenital trichomoniasis. The epidemiological role of pigs has not been elucidated yet and transmission between pigs is probably fecal–oral. In cattle, however, *T. suis* is transmitted sexually. In infected bulls, the disease has only mild symptoms and they serve as lifelong carriers of the parasite. In cows, *T. suis* causes serious inflammation of the vagina and womb, and the infected cow may abort and/or become sterile. Bovine urogenital trichomoniasis was effectively treated with metronidazole; however, the application of metronidazole to domestic animals has been prohibited in several countries including the United States and European Union countries. As a result the disease is now virtually untreatable and the infected animals have to be destroyed. Artificial insemination is an effective prevention.

Histomonas meleagridis and ***Trichomonas gallinae*** are pathogens of poultry. The uniflagellated *H. meleagridis* lives in the large intestine of chickens and turkeys and is transmitted between birds by the eggs of the intestinal nematode *Heterakis gallinarum*. *Trichomonas gallinae* occurs in the beak and crop of pigeons and is transmitted by water or by predation (to birds-of-prey). Under some circumstances, these parasites are capable of invading the intestinal mucosa (*H. meleagridis*) or oral mucosa (*T. gallinae*) and may then be transported via the blood to the internal organs. The resulting diseases are then often lethal for the hosts.

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Amoebozoa

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Glossary

Amitochondriate Lacking mitochondria.
Anaerobic In absence of oxygen.
Bicellular Consisting of two cells.
Biflagellated Carrying two flagellae.
Binucleate Containing two nuclei.
Dictyosomes Membrane stacks of the Golgi apparatus.
Dispersal Spreading over specific distances.
Dormancy Resting period.
Heterotroph Feeding on other organisms.
Keratitis Inflammation of the cornea.
Macroscopic Scale large enough to see with naked eye.
Methanogenic Producing methane.
Microaerobic In presence of small amounts of oxygen.
Mixotroph Using a combination of feeding and photosynthesis.
Monophyletic Group sharing a single last common ancestor.
MRSA Methicillin-resistant *Staphylococcus aureus*.
Multiciliated Bearing multiple cilia.
Multinucleate Containing several nuclei.
Phagocytically Eating by engulfing food particles into membrane-bound vesicles.
Plasmotomy Pinching off from the multinucleate cytoplasm without mitosis.
Proteinaceous Made from protein.
Protoplasm All internal fluids and organelles of a cell.
Pseudopodium Temporary cytoplasmic protrusion.
Sensu strictu In the strict sense.
Solitary In isolation, alone.
Sorocarp Fruiting body consisting of a spore mass and stalk.
Sporocarp Fruiting body made of one or very few spores on top of a secreted stalk.
Synapomorphy Group-defining trait inherited from the last common ancestor.
Tetranucleated Containing four nuclei.
Trophozoite Feeding stage of a cell.
Unicellular Consisting of a single cell.
Uninucleate Containing a single nucleus.
Xenogenous Of foreign origin.

Introduction

Amoebas are generally known as a unicellular organisms that have no defined shape and are constantly shifting form. From the early beginnings of microscopic studies those shapeshifting cells have captured the interest of observers and for a long time have resisted consistent classification. Amoeboid protists with pseudopodia or locomotive protoplasmic flow have been placed in artificial classes like Rhizopoda (von Siebold, 1848) or Sarcodina (Levine et al., 1980). However, these morphologically similar groups of organisms form very different branches of the eukaryotic tree, which are not necessarily related. With the availability of molecular data those systems had to be abandoned. In this review I will concentrate on Amoebozoa *sensu strictu*, *i.e.*, as a group of related eukaryotic organisms displaying typical amoeboid movement (Fig. 1) that descended from a common ancestor.

The origin of Amoebozoa is estimated to lie about 1.2 billion years ago. Even though some Amoebozoa are large enough to be observed by the naked eye, it was only after the invention of proper functioning microscopes in the 1670s that they sparked the curiosity of many researchers. With his seminal observations of moving 'animalcules' in pond samples Antonie van Leeuwenhoek perceived the idea of a world of microbes beyond our eyesight. So much so, that almost every biology class nowadays includes a demonstration of live amoebas. Even though the cells have no fixed shape, moving amoebas possess a more or less dynamically

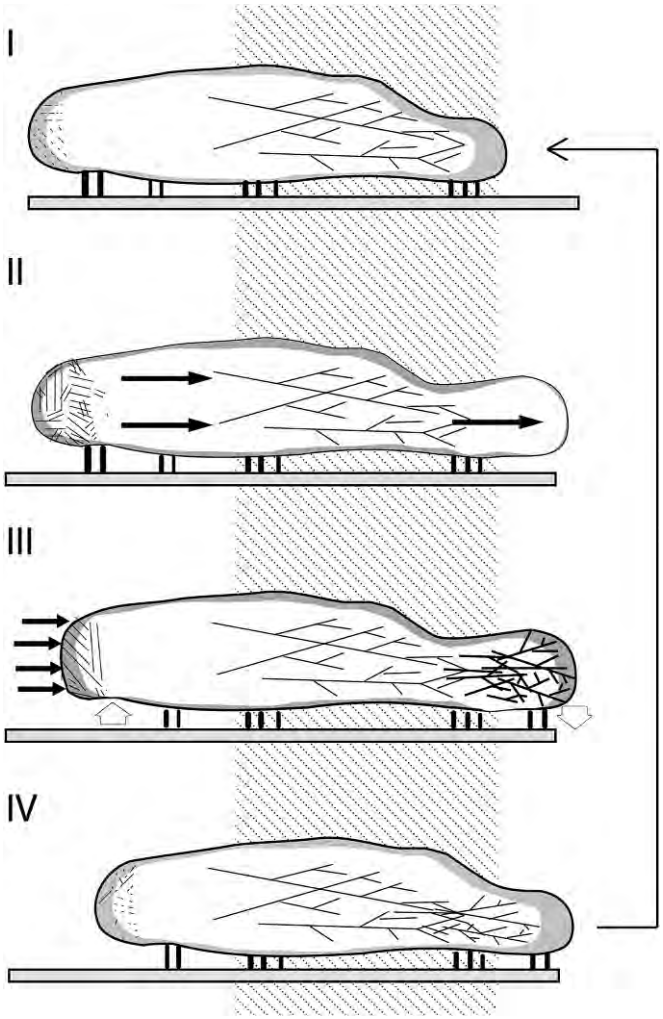

Fig. 1 Amoeboid movement. The characteristic amoeboid movement involves the formation of dynamic cell protrusions termed pseudopods. This type of movement is common in many Amoebozoa, other amoeboid organisms and even some cells in our body, for instance macrophages and sarcoma cells. Importantly, in Amoebozoa pseudopods are never supported or stiffened by microtubules. The movement is caused by action of an actin-myosin network and different viscosity of the cell cortex (ectoplasm) and inner mass (endoplasm). The ectoplasm is more solid (gel-like) than the fluid endoplasm (sol-like). A pseudopod is formed when the anterior part of the cell becomes more fluid (sol-like) and the hydrostatic pressure exerted by the myosin network at the uroid (arrows) presses the endoplasm forward (II). When migrating in confined spaces this can also cause the formation of membrane blebs where the membrane locally detaches from the cell cortex at the leading edge. The pseudopod then stabilizes due to fresh actin polymerization at the leading edge (III; thick lines), but myosin function, membrane uptake and localized exocytosis also contribute to the process. The pseudopod forms new adhesion sites (III; open arrows) with the substratum and locomotion of the cell occurs due the sol-gel conversion of the cytoplasm within the cell, a process, which is not yet fully understood. Note that established actin fibers (thin lines) don't move with respect to the substratum. Myosin filaments (hatched lines) at the uroid contract and substrate-adhesion sites in the rear are dissolved allowing the cell to move forward (III; open arrows). The movement in many cases can appear random, or non-directed, however often movement is directed with regards to a signal source (chemotaxis, phototaxis, thermotaxis).

stable shape that makes different species recognizable to the trained eye (Fig. 2 panel I). Especially the form of the pseudopods, the shape of the posterior end (uroid), the way the cytoplasm streams and how the cell body as a whole moves are very characteristic and have been used to diagnose species (Fig. 2 panel II). The presence or absence as well as the structure of a cell coat or shell is another diagnostic trait (Fig. 2 panel III). Additionally, when amoebas form dormant cysts, their shape was also used for species diagnosis. Using histochemical staining, amoebas were also differentiated by their specific shape of the nucleolus, and often only a combination of characters gave reliable morphological information to allow species determination. Presently, the 18S small subunit RNA sequence and, where available, the concatenated sequences of many protein coding genes are used to reliably identify and place species.

Individual amoebas can range in size from 3 to 5 μm for the fittingly named *Parvamoeba* ('small amoeba') up to a respectable 5 mm in large specimens of *Pelomyxa palustris*. The multicellular fruiting bodies of some multicellular Dictyostelia can reach over 7 cm length (*Dictyostelium giganteum*), and the syncytial plasmodia of some myxogastrids can cover areas of up to several square meters (Fig. 3(A)) making them the largest unicellular organisms. Several branches of Amoebozoa independently evolved the ability to differentiate encapsulated spores or cysts on a secreted stalk (sporocarpy). Others evolved different levels of aggregative multicellularity where many spores are lifted up together in a fruiting body (sorocarpy).

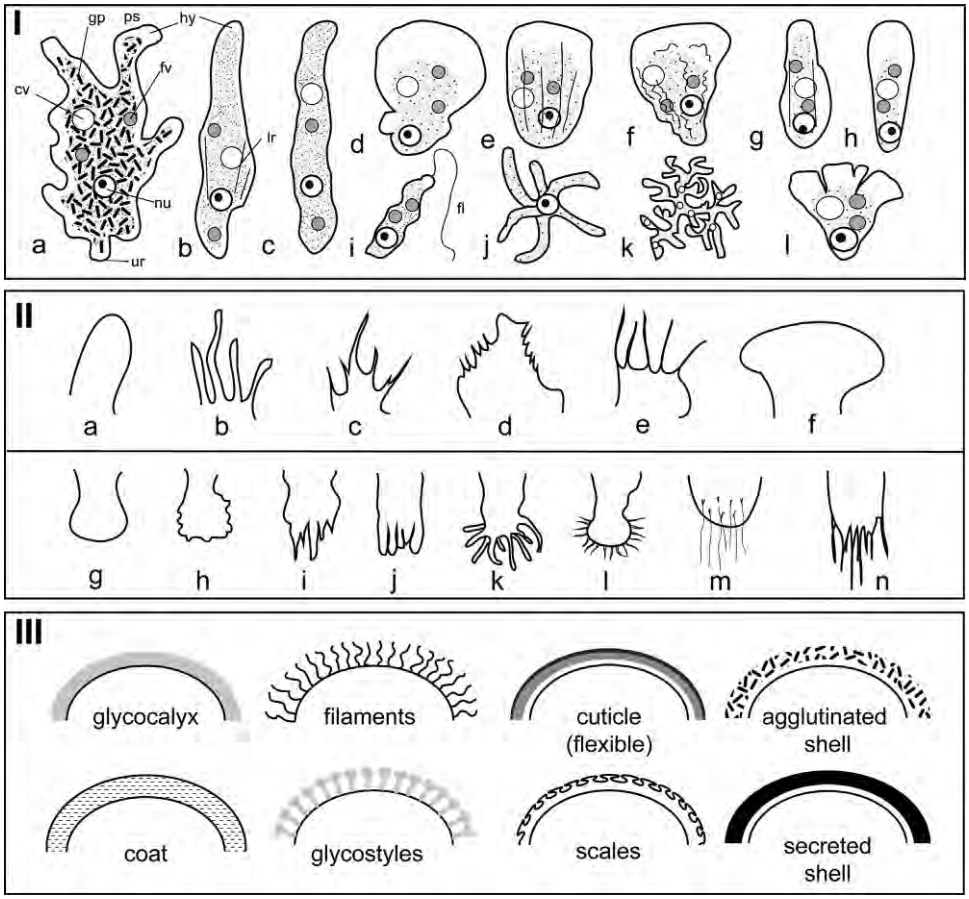

Fig. 2 Panel I. Basic amoeba morphotypes (after Smirnov, 2009). (a) polypoidal polytactic (pseudopods can be formed in any direction, sometimes involving subpseudopods); cv – contractile vacuole, fv – food vacuole or other vacuole, gp – granuloplasm with organelles and inclusions, hy – frontal hyaline area, nu – nucleus (with nucleolus), ps – pseudopod, ur – uroid; (b) monopodial orthotactic (single long pseudopod, cell bell-shaped in cross-section with lateral wrinkles); (c) monopodial monotactic (cell tubular in cross-section without wrinkles); (d) spatulate (fan-shaped flattened); (e) striate (flattened cell body with almost parallel dorsal folds); (f) rugose (flattened with irregular dorsal folds and wrinkles); (g) lanceolate (lancet-shaped with distinct lateral flattening); (h) lingulate (flattened, oblong, without any folds); (i) amoeboflagellate (with one or two flagella); (j) floating form (with protruding pseudopods increasing surface area); (k) syncytial (flat, branching, with several or many nuclei); (l) flabellate (flattened with clear anterior hyaline hyaloplasm and uneven frontal edge; no subpseudopodia). Panel II. Basic pseudopod (a–f) and uroid (g–n) shapes**.** (a) smooth pseudopod; (b) dactylopodia (finger-like protrusions); (c) acanthopodia (stiff spiky pseudopods that are internally supported by microtubules); (d) echinopodia (short spiky pseudopods running in ridges); (e) filopodia (very fine and long pseudopods. not supported by internal microtubules); (f) lobopodia (broad and flat single pseudopod per cell); (g) smooth; (h) morulate; (i) fasciculate; (j) plicate; (k) fingered; (l) villous-bulbous; (m) and (n) trailing cytoplasmic filaments or threads. Panel III. Cell surface coatings**.** Not to scale.

Diversity

Amoebozoa are unicellular eukaryotes containing the typical set of organelles apart from plastids. They derive from a last common ancestor amoebozoan (LCAA) that had asexual as well as sexual life phases and carried a single flagellum of the eukaryotic $9\times2+2$ microtubule structure at least at some life cycle stage. The LCAA might also have had some kind of walled resting structure (cyst). Cyst formation, sexual recombination, the flagellum and mitochondria have subsequently been lost in some individual groups. Amoebozoa are situated on the unikont branch of the eukaryotic tree and constitute a sister group to the Obazoa, which comprise Fungi, Metazoa (animals) and a number of other protozoan relatives. Other protists with amoeboid movement are found within Rhizaria and Percolozoa. Amoebozoa have previously been defined by the specific shape of their uroid, their nuclear fine structure and division pattern and histochemical staining patterns. Sequencing of individual genes and whole genomes of Amoebozoa has made it possible to infer phylogenetic relationships from molecular data. As a molecular synapomorphy all Amoebozoa have fused mitochondrial *coxI* and *coxII* genes. Amoebozoa are subdivided into the subphyla Discosea, which have a flattened appearance, move mostly with a single broadly lobed pseudopodium and have branched tubular mitochondrial cristae, and Tevosa, which display more varied pseudopod and mitochondria membrane morphology (Fig. 3(B)). The subphylum Tevosa (Tubulinea + Evosa) comprises the classes Tubulinea, Cutosea, Variosea, Archamoebea and Eumycetozoa. Flagellated stages are known in Variosea, Archamoebea and Eumycetozoa and the presence of a specialized microtubular cone-shaped structure connecting the mostly single basal body of the flagellum to the nucleus called karyomastigont has been used to group those together as Conosa. The subphylum Discosea can be divided into the superclasses Flabellinia and Centramoebia. A number of protosteloid amoebas are now also classed under Discosea, but do not form a monophyletic group.

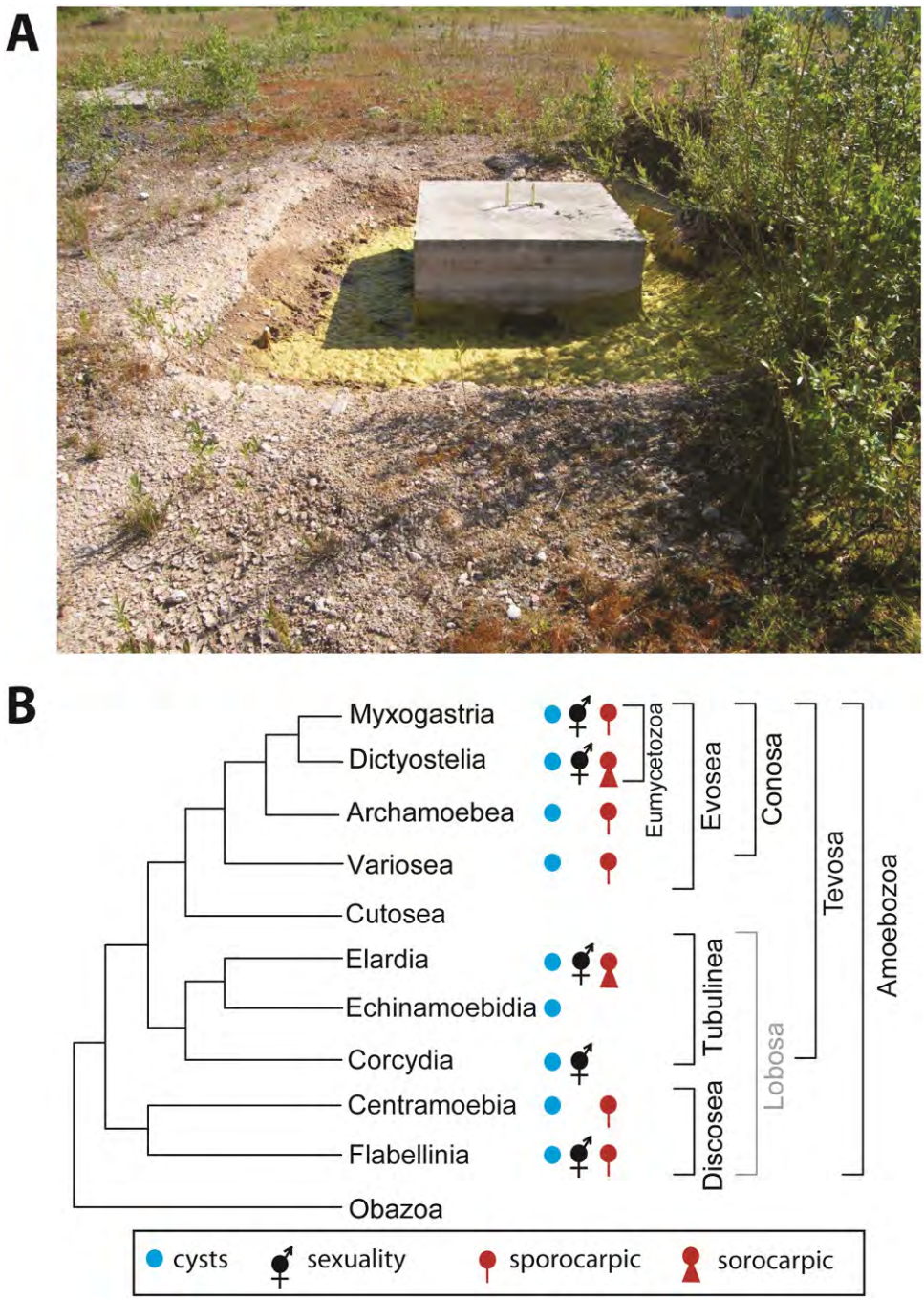

Fig. 3 (A) Myxogastrid plasmodium of undetermined species covering the whole area of a concrete building foundation pit measuring several square meters. Ludwigsau, Germany, 2008. (B). Phylogenetic relationships between major groups of Amoebozoa. After Kang, S., Tice, A.K., Spiegel, F.W., et al., 2017. Between a pod and a hard test: the deep evolution of amoebae. Molecular Biology and Evolution 34 (9), 2258–2270. doi:10.1093/molbev/msx162. This synthesis is based on multi-gene phylogenies and previous groupings into the now polyphyletic Lobosa (indicated in grey) and Conosa is also indicated. The occurrence of cysts, sexual processes, sporocarpic (single cyst/spore on acellular stalk) and sorocarpic (aggregation of several cells) fruiting body formation is indicated next to the groups.

Subphylum Tevosa

Superclass Tubulinea

Tubulinea are characterized by tubular pseudopods lacking subpseudopodia. Tubulinea comprises naked amoebae (*Amoeba, Chaos, Copromyxa, Hartmanella, Leptomyxa, Gephryamoeba, Echinamoeaba, Vermamoeba*) as well as shell-bearing (testate) amoebae (Arcellinida). *Amoeba proteus* is a frequently observed and large fresh-water amoeba and is often used in classroom-demonstrations.

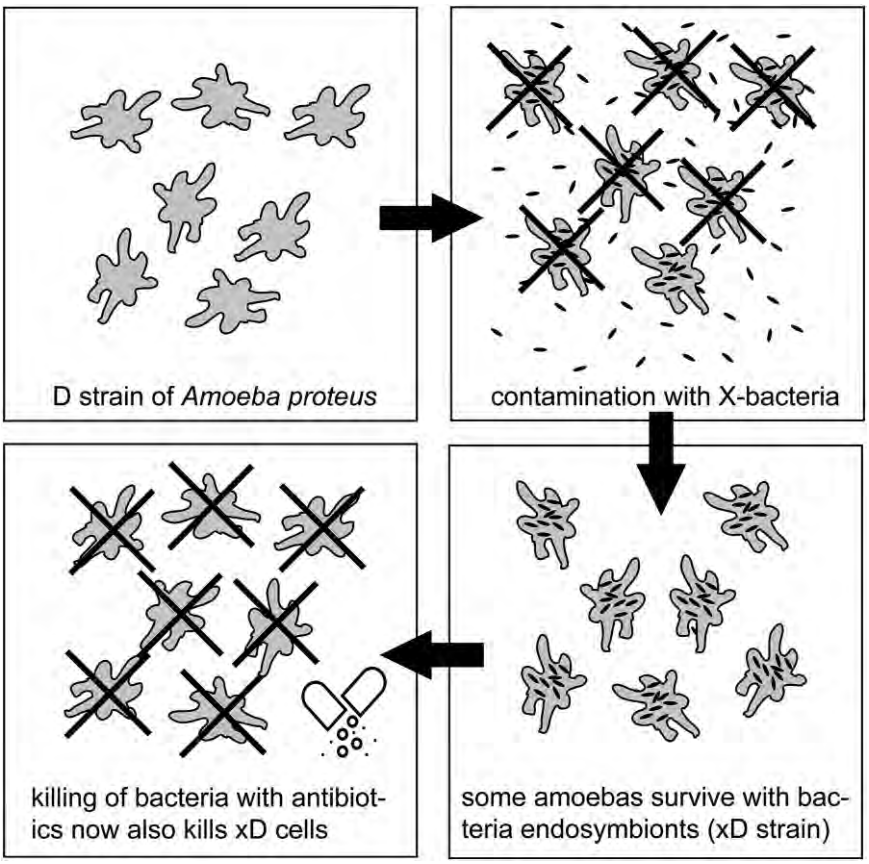

Fig. 4 Kwang W. Jeon accidentally contaminated a culture of the D strain of *Amoeba proteus* with a lethal bacteria strain ('x-bacteria'; possibly *Legionella*). Whilst most of the infected amoebae died, some survived and became dependent on the x-bacteria (xD strains). Killing the bacteria with antibiotics also caused the xD amoebae to die. This newly established endosymbiosis happened reproducibly within only 200 generations. The endosymbiotic bacteria which number about 42,000 per amoeba cell are not digested by the host amoebae, but held in specialized symbiosomes. The exact mechanism for the development of host dependence remains unknown, but it seems to depend on the presence of two plasmids in the x-bacteria and mutual changes in gene regulation of both partners. Interestingly, xD amoebae also show a nucleocytoplasmic incompatibility with symbiont-free D strains in that xD strain nuclei produce a toxic substance similar to the mate-killer strains of the ciliate *Paramecium* which kills non-symbiont bearing cells.

Also known as 'Jeon's Amoeba' it is a famous example for an endosymbiotic event taking place in the laboratory (Fig. 4). *Amoeba* and *Chaos* species can also contain two morphologically distinct types of mitochondria within the same cell. *Copromyxa protea* is the only species within this group to have evolved aggregative multicellularity. *Copromyxa* amoebas normally feed on bacteria within dung, but when they starve, single cells start to encyst either individually into jigsaw piece-shaped cysts called microcysts or after fusion of two amoebas into round double-walled sphaerocysts. Microcysts can remain solitary, or, depending on local amoeba density, other starving amoebas can attach to founder microcysts, climb on top of them and encyst themselves. This results in small tree-like structures of encysted cells that are then called sorocysts. Upon germination, each sorocysts or microcyst releases one single limax-type uninucleate amoeba. The ecological benefit of this form of encystation, or if this aids species dispersal, remains unclear.

Testate amoebae possess an outer shell (test) with one or two openings, which consists entirely of secreted organic material (proteinaceous; *Arcella*-type) or a mix of secreted organic material and xenogenous material, like diatom shells (frustules), sand particles *etc*., (agglutinate; *Difflugia*-type). In rarer cases the tests are built of secreted inorganic material (*Quadrulella*). All testate amoebae are free-living and common in fresh-water, soils and mosses and feed using their pseudopods on bacteria, protists, fungi, algae and even small animals. Under unfavorable conditions encystation inside the shell is frequently observed. Sexual recombination is known or suspected in *Copromyxa protea*, *Arcella* and *Trichosphaerium*.

Superclass Cutosea

The Cutosea are a species-poor group and are characterized by a non-adhering cell surface coat containing small scales. The shape and size of those scales are species-specific. There are only three marine species so far representing this group, *Sapocribrum chincoteaguense*, *Squamamoeba japonica* and *Armaparvus langoidus*.

Superclass Variosea

Variosea contain species with very diverse shapes, like the marine flagellate *Phalansterium solitarium*, the multiciliated amoeba *Multicilia marina*, and the reticulate shaped *Acramoeba dendroida*. This is a class containing many sporocarpic species, which produce spores or cysts sitting on a secreted stalk. Those were previously grouped as Eumycetozoa together with the cellular and acellular slime molds (Dictyostelia and Myxogastria), but molecular phylogenies showed that the protosteloid sporocarp originated multiple times in the Amoebozoa. Sporocarps usually only contain a single spore or very few spores (2−8) on top of a thin secreted, hollow stalk of 5–250 μm length. The amoeboid stages of many Variosea are characterized by thin and sometimes branched pseudopods like *Acramoeba*, *Grellamoeba*, *Filamoeba* and *Flamella.* The formation of cysts is frequently observed in Variosea.

Superclass Archamoebea

The Archamoebea contain some species that have adapted to anaerobic and microaerobic environments by secondary loss of mitochondria, Golgi stacks and peroxisomes. They were previously believed to be basal eukaryotes, however are now placed within Tevosa. The superclass Archamoebea contains the anaerobic amitochondriate orders Mastigamoebida (*Mastigamoeba*, *Endolimax*, *Iodamoeba*), Entamoebida (*Entamoeba,*) and Pelobiontida (*Pelomyxa*, *Mastigella*). The marine *Mastigamoeba* have a long forward facing flagella and the nucleus is physically attached to its basal body by microtubule ribbons so that the nucleus can be protracted. The life cycle of *Mastigamoeba* can also display amoebae with simple or branched pseudopods, multinucleate cells and cysts. *Endamoeba* and *Endolimax* are found in the guts of animals. Most are harmless commensals and spread as cysts.

Pelomyxa palustris is one of the largest amoebas usually reaching 500–5000 μm size and feeds phagocytically in anaerobic freshwater sediments. It apparently indiscriminately takes up material of the upper layer of substratum and digests usable constituents and is known to even ingest cotton wool and filter paper. *P.palustris* is multinucleate containing up to several thousand nuclei and lacks mitochondria and typical dictyosomes. Besides organic and inorganic inclusions, like sand grains and diatom shells, and storage bodies the cytoplasm harbors several bacterial endosymbionts, some of which are methanogenic. The life cycle of *P.palustris* is complex involving binucleate cells derived by plasmotomy from larger cells or hatched from cysts and cysts with four nuclei. Until recently, the genus *Pelomyxa* was thought to be monospecific with *P.palustris* as the only member and many other described 'species' are actually life stages of the same species. However, a second species, *Pelomyxa corona* has been described, which differs in the morphology of the anterior pseudopod. Multinuclearity evolved several times independently in Amoebozoa: In the Myxogastria, *Pelomyxa*, *Trichosphaerium*, *Schizoplasmodiopsis* and *Tychosporium acutostipes*.

A number of *Entamoeba* species (*E.coli*, *E.hartmanni*, *E.polecki*, *E.dispar*) are harmless commensals residing in the large intestine of humans and, like *Endolimax nana*, are transmitted between hosts by desiccation resistant cysts. Lacking mitochondria, they can only survive outside the body as dormant cysts. The same holds true for the human pathogen *E.histolytica*, the causal agent of amoebiasis, where cysts are transmitted by fecally contaminated hands, food and water. From the tetranucleated cyst of *E.histolytica*, which passages through the stomach unharmed, eight trophozoites emerge in the small intestine, move to the large intestine and multiply there by binary fission. Most *E.histolytica* infections are asymptomatic and trophozoites remain in the intestinal lumen and feed on gut bacteria, but about 10%–20% of the infections develop into invasive amoebiasis which causes around 70,000 deaths per year. In the pathogenic phase, *E.histolytica* trophozoites invade tissues of the gut causing bloody diarrhea and can be distributed over the blood stream and colonize other internal organs. A notable exception entirely lacking encystation is *Entamoeba gingivalis*, another commensal living on bacteria in the human oral cavity, where no cyst stage has ever been found and which is transmitted directly as trophozoites by saliva contact.

Superclass Eumycetozoa

The Eumycetozoa, characterized by spore-bearing fruiting bodies, comprise the orders Dictyostelia and Myxogastria. Both are monophyletic and produce macroscopic fruiting bodies containing a large number of encapsulated spores.

Dictyostelia are soil amoebas often isolated from leaf litter of forest soils, which form multicellular sorocarps by aggregation of numerous 'myxamoebae'. To date, more than 150 species have been described and Dictyostelia have been isolated globally, from the Arctic to the tropics, but there are no marine species known. The haploid free-living amoebae normally phagocytose bacteria and small yeasts in the soil and divide by binary fission. They occasionally engulf each other whole, but the amoebae of *D.caveatum* have the capacity to ingest amoebae of other dictyostelid species by nibbling off pieces. Upon starvation, amoebae secrete a chemoattractant (cAMP, the di-peptide glorin, folate or other substances) and form an aggregate consisting of fewer than ten to thousands of cells. An intermediate, migratory pseudoplasmodium ('slug') can occur in some species that can move for extended time periods responding to heat and light to find the soil surface. In the aggregate, cells differentiate into prespore and prestalk cells giving rise to a stalk consisting of dead cells that lifts a sorocarp containing several thousand encapsulated spores above the substratum. In one genus of Dictyostelia, *Acytostelium*, the cellular stalk has been replaced by a secreted thin cellulose tube and their sorocarps are relatively small. Auxiliary structures holding the spore mass in place (upper and lower cup) and anchoring the stalk to the substratum (basal discs, crampons) may be present in some species. The spores are surrounded by a thick coat of glycoproteins and cellulose and remain dormant until food is available again. Unlike fungal spores, Dictyostelid spores are not hydrophobic and require either running water or motile creatures for dispersal. Since no sexual processes take place during multicellular development of the Dictyostelia, spores are haploid and hatch one haploid myxamoeba each. Many species can also encapsulate solitarily as

microcysts or engage in sexual fusion to form macrocysts, a process during which the zygote attracts a number of other myxamoebae before surrounding itself with a thick wall and ingesting them. Specific environmental conditions trigger the pathways for either sorocarp, microcyst or macrocyst formation. Sorocarp formation is favored by relatively dry and light conditions. For microcyst formation wet and dark conditions, combined with solute stress are favorable. Macrocyst formation requires the presence of compatible mating types and high cell density in wet and dark conditions. After a period of dormancy, the macrocyst undergoes one meiotic and several mitotic divisions causing several haploid myxamoebae to hatch from the macrocyst. Rare self-fertile homothallic strains are also known.

The Myxogastria are the most diverse group of Amoebozoa and divide into two clades containing either dark spores containing melanin (Collumellidia: Physarales and Stemonitales) or brightly colored spores containing various organic pigments and only little melanin (Lucisporidia: Liceales and Trichiales). The Echinosteliales with hyaline spores comprise a fifth, more basal order with some affinity to the dark-spored group. In Myxogastrids, a diploid plasmodium forms from the zygote through repeated nuclear divisions without cell division. The plasmodium is often brightly colored and can reach macroscopic dimensions (Fig. 2(A)). Nuclear divisions in the plasmodium are synchronous and often a rhythmic cytoplasmic shuttle streaming in the cytoplasmic strands is observed. This is based on actin-myosin contractions and aids the intracellular nutrient transport and communication within the large plasmodium. The streaming is also responsible for the high speed of movement of the plasmodium of up to 500 mm h^{-1}. External factors, like change in humidity, pH, temperature or starvation can trigger the formation of sporocarps. In some species with colored plasmodia, light is necessary for sporocarp formation and a phytochrome-like light receptor has been described from the genome of *Physarum polycephalum*. Spores give rise to haploid amoebae which may transform into biflagellated amoeboflagellates under wet conditions. If partners of compatible mating types (of which there are 13 or more in *P.polycephalum*) are present, two amoebae or amoeboflagellates can fuse to give rise to a zygote and start a new plasmodium. The macroscopic fruiting bodies of Myxogastria are often found on decaying wood and myxogastrids can also produce cysts confusingly also called macrocysts. *Ceratiomyxa* has recently been established as a sister group to Myxogastria and its large hyaline plasmodium is probably homologous to the plasmodia of myxogastrids, even though its mode of external spore production resembling protosteloid sporocarps is different from the sporulation within an enclosed structure (peridium) of Myxogastria. *Physarum polycephalum* is one of the best-studied myxogastrids and because it is easy to grow in the laboratory it has yielded important and surprising insights into amoeboid movement and cell motility. It even has been shown to possess a primitive "intelligence" being able to solve maces, learning to anticipate events and finding the most efficient network connecting oat flakes representing Japanese towns with Tokyo.

Subphylum Discosea

Flabellinia

Flabellinia contain some strongly flattened amoebas with amorphous glycocalyx, like *Vannella* and *Mayorella*, but their life cycles as well as within-group relationships are mostly unknown. *Vannella* typically have fan-shaped frontal pseudopodia, but there are also species with finger-like pseudopodia (*Vexillifera*). Lingulate (tongue-shaped) and lanceolate (lancet-shaped) forms and species with rigid dorsal surface ridges (*Stenamoeba, Thecamoeba*) or a thick cuticle (*Dermamoeba*) also occur. Some *Vannella* species harbor intracellular bacteria that are pathogenic to humans. Cysts have been reported to be formed by *V.cutleri* and *V.peristens. Neoparamoeba* spp., free-living marine amoebas with unknown life cycle and opportunistic parasites, are the cause of amebic gill disease in salmon and other farmed fish. *Sappinia diploidea* forms large binucleate trophozoites and bicellular cysts, which can fuse to a single cell and probably represent a sexual stage. *Sappinia pedata* forms a 'standing amoeba', the reason why it was sometimes included in the protostelids, however, no spore formation takes place, the stalk remains an integral part of the cell and the process is reversible. Aggregation of cells has been reported in *S.pedata*, but no sorocarp formation has ever been observed.

Centramoebia

The genus *Parvamoeba* is the smallest amoeba known and consists of only two known species (*P.rugata* and *P.monoura*) that are at 3–5 μm diameter almost too small to observe by light microscopy. Whereas most Centramoebia are present as solitary amoebae, *Endostelium* spp., *Luapelamoeba hula, Luapelamoeba arachisporum* and *Acanthamoeba pyriformis* form minute sporocarpic fruiting bodies of the protosteloid type.

The *Acanthamoeba* life cycle consists of feeding trophozoites carrying spike-like pseudopodia (acanthopodia), which upon starvation transform into double-walled dormant cysts. No sexual stages are known in *Acanthamoeba*, however, a number of sex-genes have been identified in the genome of *A.castellanii*. *Acanthamoeba* spp. have been isolated from many environments including freshwater, seawater, soil, sediments, salt water lakes, Antarctica ice and even from air samples indicating their ubiquitous distribution. They have also been found in many man-made environments like bottled mineral water, factory water discharges, air-conditioning units, sewage, hot water systems and contact lens solutions. The latter two are important, because *Acanthamoeba* can form a reservoir for pathogenic bacteria (MRSA, *Legionella*) and are also themselves opportunistic human pathogen. *Acanthamoeba*-keratitis is most commonly found in contact-lens wearers, who do not observe strict contact-lens hygiene, but can also affect other people. Here, *Acanthamoeba* is introduced into the eye as trophozoites that first feed on the bacterial biofilm on the contact lenses and later can enter the cornea through pre-existing micro-injuries and start to ingest

corneal cells leading to painful infection and sometimes even blindness. The extremely rare, but more severe condition granulomatous amebic encephalitis (GAE) is caused by *A.castellanii*, *A.culbersoni*, *A.polyphaga*, *Hartmanella* and *Balamuthia mandrillaris*. GAE occurs mostly in immunocompromised individuals, but is almost always fatal. Here, *Acanthamoeba* enters the blood-stream over cuts in the skin or the oral-nasal route and may colonize other inner organs (lungs, liver, and kidneys) first. Those infections can be asymptomatic until the trophozoites cross the blood-brain barrier and infect the brain causing lethal neurological damage and typical granulomas.

Ecology and Distribution

Amoebozoa are typically heterotrophs with the exception of some mixotrophic species of testate amoebae (*Amphitrema flavum*, *Difflugia oblonga*, *Hyalosphenia papilio*) which harbor numerous photosynthetic *Chlorella* endosymbionts in their cytoplasm. Most are free-living in soil, freshwater, on plants, in brackish or sea water and, considering the large number of over 1500 described species, only a small number of these are parasites of humans and other animals. Amoebozoa live in aerobic or anaerobic habitats and the loss of mitochondria of parasitic or anaerobic species is a secondary adaptation to those environments. Amoebozoa play an important role as primary grazers on bacteria in soil and freshwater and are important for nutrient cycling as they are the prey of larger animals, like nematodes. Most Amoebozoa divide clonally by binary division and sexual processes are rarely observed, within most individual species and within the whole group. Life cycles of many Amoebozoans involve durable cyst stages, which can be a means of species dispersal and be deposited as seed banks to repopulate the habitat after periods of dormancy. Some cysts have even been recovered from 8,580-year old permafrost soil samples and hatched viable actively dividing amoebas. The cysts of some species can be airborne and distributed with wind currents over vast distances.

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Amylases☆

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Abbreviations

CD	cyclodextrin
DE	dextrose equivalence
DP	degree of polymerization
GH	glycoside hydrolase
HFCS	high-fructose corn syrup

Defining Statement

The catalytic properties of α-amylase, β-amylase, glucoamylase, debranching enzymes, CGTase, pullulanases and pullulan hydrolases, and other enzymes are described to clarify the differences among them and their microbial sources. The structures of Taka-amylase, CGTase, β-amylase, glucoamylase and pullulanase are explained with a focus on their domain structures, catalytic site structures and identification, and position of two catalytic residues.

Definition of Amylase

In this article, amylases are defined as a group of enzymes that act on α-1,4- and α-1,6-glucosidic bonds present in starch and glycogen, a starch-related polysaccharide. Amylases, in most simple terms, are a group of glycosyl hydrolases, which hydrolyze α-1,4- and/or α-1,6-glycosidic bonds of starch and glycogen. The structural information on the enzymes has expanded the concept of amylase and classified the enzymes in GH-13 family. It is sometimes difficult to discriminate certain enzymes from amylase, depending on their action specificity. Here, enzymes that act only on oligosaccharides derived from starch were excluded from amylase. Although some phosphorylases act on starch or glycogen, they are not included in amylases.

Substrate of Amylases

The Substrate Starch

Starch is the storable form of energy produced by all green plants and is utilized by almost all living organisms on this planet as their energy source. Plants store starch in the form of semi-crystalline granules in fruits, seeds, rhizomes and tubers. The diameter of the granules is in the range of 1–100 μm and the characteristics of the starch depend mainly on the location in the plant where the starch is present. High starch content is found in potatoes, maize, wheat, rye, sorghum, rice, barley, peas and tapioca. Starch, also called amylum, is a polysaccharide of glucose/α-D-glucopyranose in the $^4C_1$ conformation. Starch is stored in the plants as granules composed of amylose and amylopectin. Amylose is a water insoluble polyglucan which constitutes about 20%–27% of starch and is essentially linear with 100–10,000 α-D-1,4-linked anhydrous glucose units but with a few (∼10) branch points per molecule. Amylopectin with a much larger molecule (DP 104-105) constitutes the bulk of starch consisting of α-D-1,4-linked glucose units and joined by α(1→6) linkages (Fig. 1(a–b)) after every 24–30 glucose units. Regular branching in amylopectin molecule allows the formation of crystalline structure. Amylopectin accounts for 75%–85% of normal starch. Starch molecules are specific in structure and composition (the length of glucose chains or the amylose/amylopectin ratio), as well as the protein content of the storage organs. Starch has, therefore, various industrial uses depending on the agricultural raw material from which it is extracted. Waxy starch found in some waxy plants such as waxy corn or waxy rice is composed solely of amylopectin. In nature, starch is present as insoluble starch granules (raw starch), which have a crystalline structure. Most of the research on amylases, whether basic or application oriented, has been conducted on gelatinized starch, which is a starch solution obtained by heating starch granules in water.

☆*Change History*: 2017. Nisha Mohanan and Tulasi Satyanarayana introduced small edits in the abstract, defining statement and classification of amylases; added the sections: 'Pullulan hydrolases' and 'References', subsections, 'Pullulan', 'Starch liquefaction and saccharification', 'Antistaling agent in making of bread and other baked products', 'Detergent application', 'Preparation of cyclodextrins', 'Preparation of resistant starch' and 'Preparation of panose and isopanose containing syrups'; and updated the sections: 'Definition of Amylase', 'Substrate of Amylases', 'Glucoamylase', 'Debranching enzymes', 'Neopullulanase', 'CGTase', 'Glucoamylase', 'Engineering of Amylases' and 'Industrial applications of Amylases'.

This article is an update of H. Taniguchi, Y. Honnda, Amylases, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 159–173.

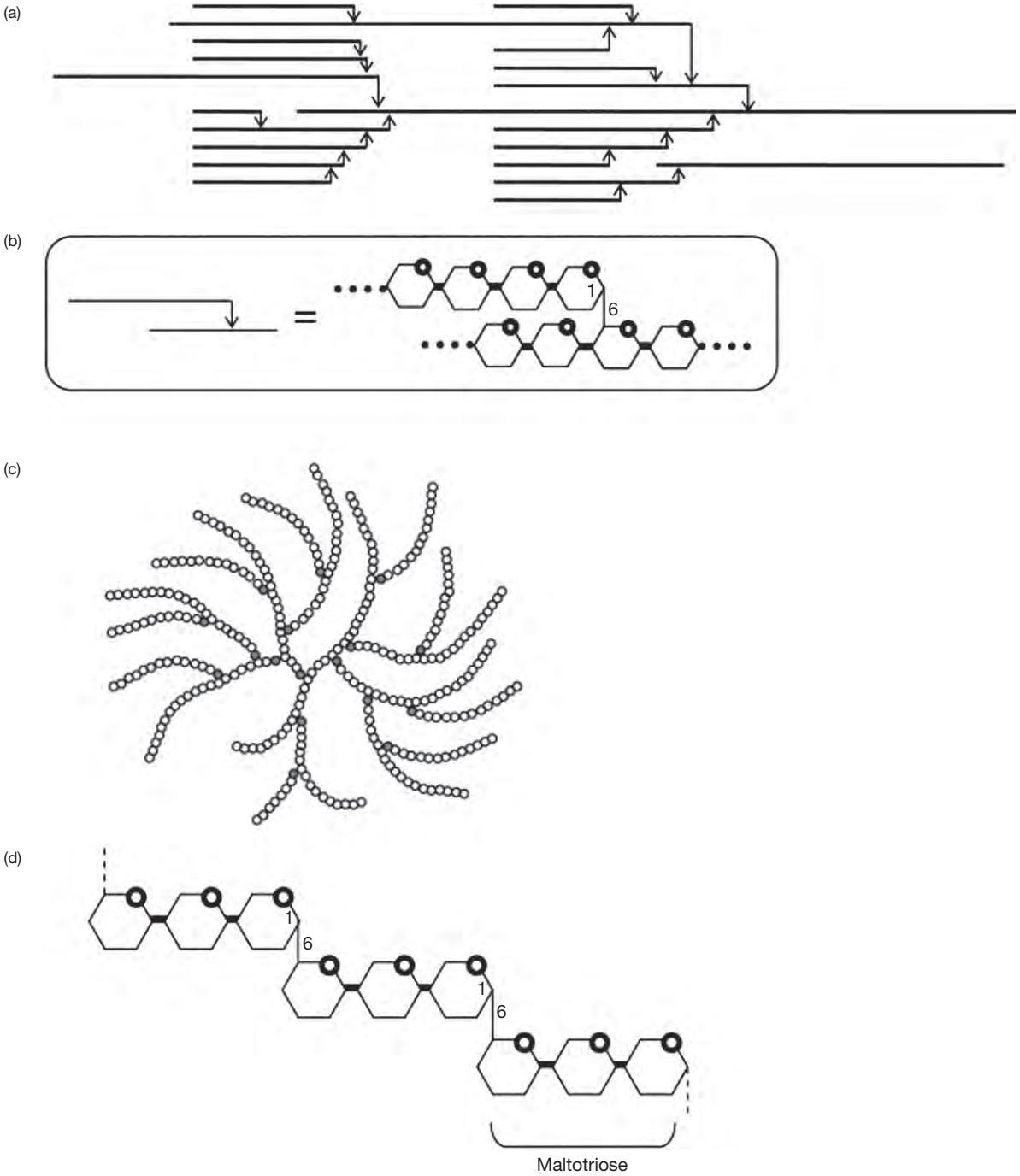

Fig. 1 Schematic representation of amylopectin, glycogen, and pullulan. (a) Horizontal lines represent α-1,4-linked glucan chains and arrow heads represent α-1,6 linkage at the branch points. (b) Enlarged structure around the branching point where hexagon with a small ring stands for glucose residue. (c) Open circles stand for glucose residues linking through α-1,4 linkage and closed circles stand for glucose residues linking through α-1,6 linkage at the branch points. (d) Same symbols as in (b).

Glycogen is a storage α-glucan found in animals and microorganisms. Like amylopectin, it consists of α-1,4-linked glucans connected via the α-1,6 linkage (branching). This branching occurs frequently and is distributed evenly throughout the glycogen molecule as shown in Fig. 1(c), and makes glycogen soluble in water. Amylase acts on both of these α-polyglucans.

The Substrate Pullulan

Pullulan is an extracellular glucan synthesized by the fungus *Aureobasidium pullulans*, formerly known as *Pullularia pullulans*. It is composed of linearly polymerized maltotriose units linked by α-1, 6-glucosidic bonds (Fig. 1(d)), although the same can occur as a

polymer of isopanose. It is a highly ordered and branched polysaccharide with 2:1 ratio of α-1, 4-glycosidic to α-1, 6-glycosidic bonds. The α-1, 6 linkages in pullulan are considered to mimic partially the α-1, 6-branch points of amylopectin. Pullulan plays an important role as a model substrate for assaying starch-debranching enzymes. Pullulan is soluble in water and forms a colorless and viscous adhesive solution but insoluble in solvents such as ethanol, methanol and acetone. Pullulan has potential applications in food, pharmaceutical, and biomedical industries and is often used to determine the substrate specificities of amylases.

Classification of Amylases

Amylases are classified based on the catalytic properties of enzymes, such as substrate and product specificities. Microbial amylases with their EC numbers (IUBMB Enzyme Nomenclature) are shown in Table 1. These enzymes fall into three EC classes: transferases (EC 2), hydrolases (EC 3), and isomerases (EC 5), and most of these enzymes belong to the EC 3 class. The enzymes can be divided into two groups, retaining and inverting based on their action mechanism. The anomeric configuration in the substrate is retained after the catalytic action in retaining enzymes, whereas it is inverted after the catalytic action in inverting enzymes. β-Amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3) are inverting enzymes, whereas all other enzymes including α-amylase (EC 3.2.1.1) are retaining enzymes.

Microbial amylases are further classified on the basis of their action specificity towards α-glucan chains. These are classified as either exo-acting or endo-acting. β-Amylase and glucoamylase are exo-acting enzymes as they release maltose or glucose, successively from the non-reducing end of α-glucans. In contrast, α-amylase is an endo-acting enzyme because it cleaves the inner glycosidic bonds of α-glucans to produce oligosaccharides with various DPs.

Based on the vast amount of information on primary and tertiary structures of enzymes, Henrissat proposed a new classification method for carbohydrate-active enzymes. Currently, all glycoside hydrolases (GHs) are classified into 110 GH families (CAZy database at See Relevant Websites section, October 2007), and all microbial amylases are classified into 5 GH families: GH 13, 14, 15, 31, and 77. As shown in Table 1, enzymes with various catalytic specificities, therefore, various EC numbers, belong to the GH 13 family. The enzymes belonging to the GH 13 family possess seven conserved amino acid sequences and a $(\beta/\alpha)_8$ barrel structure. Recently the GH 13 family was further subdivided into 35 subfamilies based on detailed structural differences. This makes it possible to approximately correlate enzymes with specific EC numbers to those with specific GH families or subfamilies as summarized in Table 1.

Catalytic Properties of Amylases

α-Amylase (EC 3.2.1.1, GH 13-1, 2, 5, 6, 7, 15, 19, 20)

This enzyme plays a central role in the hydrolysis of starch, both in nature and in the starch industry. It is found in a wide range of living organisms because starch is the most important energy source for known living organisms. Large amounts of data on enzymes

Table 1 Enzymes belonging to amylase

Enzyme name	*EC number*	*GH family*	*GH 13 subfamily*	*Mechanism*	*Mode*	*Fold*	*Base*	*Acid*
α-Amylase	3.2.1.1	13	1, (2), 5, 7, (19), 27, 28, 32	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
β-Amylase	3.2.1.2	14		Inversion	Exo	$(\beta/\alpha)_8$	Glu	Glu
Glucoamylase	3.2.1.3	15		Inversion	Exo	$(\alpha/\alpha)_6$	Glu	Glu
Oligo-1,6-glucosidase	3.2.1.10	13	31	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
α-Glucosidase	3.2.1.20	13	21, 29	Retaining	Exo	$(\beta/\alpha)_8$	Asp	Glu
		31					Asp	Asp
Amylo-1,6-glucosidase	3.2.1.33	13	25	Retaining	Exo?	$(\beta/\alpha)_8$	Asp	Glu
Pullulanase	3.2.1.41	13	12, 13, 14	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
Cyclomaltodextrinase	3.2.1.54	13	(20)	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
Glucan-1,4-α-maltotetraohydrolase	3.2.1.60	13	NC	Retaining	Exo?	$(\beta/\alpha)_8$	Asp	Glu
Isoamylase	3.2.1.68	13	11	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
Glucan-1,4-α-maltohexaohydrolase	3.2.1.98	13	(19)	Retaining	Exo?	$(\beta/\alpha)_8$	Asp	Glu
Glucan-1,4-α-maltotriohydrolase	3.2.1.116	NC	NC	Retaining	Endo	NK	NK	NK
Glucan-1,4-α-maltohydrolase	3.2.1.133	13	(20)	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
Neopullulanase	3.2.1.135	13	(20)	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
4-α-D-glucanotrehalose trehalohydrolase	3.2.1.141	13	10	Retaining	Exo?	$(\beta/\alpha)_8$	Asp	Glu
Branching enzyme	2.4.1.18	13	9	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
Cyclomaltodextrin glucanotransferase	2.4.1.19	13	2	Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
4-α-Glucanotransferase	2.4.1.25	77		Retaining	Endo	$(\beta/\alpha)_8$	Asp	Glu
4-α-Glucan 1-α-D-glucosylmutase	5.4.99.15	13	26	Retaining	Exo?	$(\beta/\alpha)_8$	Asp	Glu

Note: NC, not classified; NK, not known.

of this group from animals, plants, and microorganisms have been accumulated. As a microbial amylase, the enzyme from *Aspergillus oryzae* has been studied most extensively, and the information on this enzyme constitutes the majority of our knowledge on α-amylase as described in the following section.

α-Amylase hydrolyzes the α-1,4 linkages in α-glucan but cannot hydrolyze the α-1,6 linkages. In the early stage of hydrolysis, dextrins with relatively higher molecular weights are produced, with a rapid decrease in the viscosity of starch solution. As the hydrolysis proceeds, the average DP of dextrins decreases gradually, and in the final stage of hydrolysis, large amounts of maltose, maltotriose, glucose, and oligosaccharides (α-limit dextrins) with the α-1,6 linkage constitute the hydrolysis products. All hydrolysis products have an α-configuration. Profiles of product composition during hydrolysis differ greatly depending on the origin of the enzyme. Some show a sharp decrease in viscosity in the early stage of hydrolysis, whereas others show a relatively slow decrease in the same stage. Enzymes of this group have been purified and characterized from a wide range of microorganisms, including archaea such as *Sulfolobus*, prokaryotes such as *Bacillus*, and eukaryotes such as *Aspergillus*. Most of them have maximum activity at 30–37°C at a neutral pH; however, some exhibit maximum activity at pH values as low as 3 or as high as 10 and at temperatures more than 100°C. Enzymes from *Aspergillus niger*, *A. oryzae*, *Bacillus amyloliquefaciens*, *B. circulans*, *B. licheniformis*, *B. stearothermophilus* and *B. subtilis* are of special importance from the standpoint of both basic research and industrial application. All enzymes of this group are classified into the GH 13 family, and their structure contains a $(\beta/\alpha)_8$ barrel with Glu as a proton donor and Asp as a nucleophile on its catalytic sites. They are further classified into eight subfamilies, as shown in Table 1.

β-Amylase (EC 3.2.1.2, GH 14)

Enzymes of this group were discovered long after α-amylase had been believed to be present only in the plant kingdom. In 1974, *Bacillus megaterium* was found to produce β-amylase for the first time in the microbial kingdom. Since then, several microorganisms such as *Bacillus*, *Pseudomonas*, and *Streptomyces* have been reported to produce these enzymes. Enzyme proteins were purified from several strains, and their catalytic and structural properties were studied and compared with those of plant β-amylase. There is no major difference in the properties of plant and microbial enzymes, and both enzymes are classified into the EC 3.2.1.2 and GH 14 groups. β-Amylase hydrolyzes the α-1,4 linkage next to the non-reducing end of α-glucan, which successively yields maltose in a β-configuration as the sole low-molecular-weight product. It cannot hydrolyze the α-1,6 linkages. When amylopectin is subjected to the action of this enzyme, α-1,4glucan chains in the highly branched molecule are trimmed down from the chain end towards the α-1,6 branching points. This action completely stops at four glucose units before the branching points, which produces β-maltose and the high-molecular-weight branched dextrin (β-limit dextrin). Thus, β-amylase contrasts sharply with α-amylase in terms of its action mechanism (inverting versus retaining) and action specificity (exo-acting versus endo-acting). Structurally, it has the same $(\beta/\alpha)_8$ barrel as α-amylase; however, two Glu residues act as a proton donor and a nucleophile.

Glucoamylase (EC 3.2.1.3, GH 15)

Intensive investigations of fungal amylase by Japanese researchers in the 1950s yielded a new type of amylase. This new amylase produced β-glucose from starch as the sole reaction product and was named glucoamylase. As it was the third amylase discovered following α- and β-amylases, it was once called γ-amylase or amyloglucosidase. It catalyzes the hydrolysis of successive α-1,4 linkages in the non-reducing ends of glucans, thereby producing β-D-glucose as the sole hydrolysis product. Thus, glucoamylase is an inverting type and exo-acting enzyme, similar to β-amylase. It also hydrolyzes the α-1,6 linkage, although at a rate lower than that of the α-1,4 linkage hydrolysis. This is a remarkable feature of glucoamylase compared with α- and β-amylases and makes it the only enzyme that can hydrolyze starch completely into glucose. In industrial starch saccharification, glucoamylase plays an important role together with α-amylase. At high starch (and therefore, glucose) concentrations (e.g., 40%), this enzyme catalyzes a condensation reaction to yield maltose and isomaltose. This reaction prevents 100% yield of glucose in industrial starch saccharification. Glucoamylase is produced mainly by fungi such as *A. oryzae*, *A. niger*, *Rhizopus delemar*, *Rhizopus oryzae* and *Rhizopus niveus*. Bacteria seldom produce this type of enzymes. Enzymes from *Aspergillus* and *Rhizopus* are widely produced by enzyme manufacturers. Glucoamylase has an $(\alpha/\alpha)_6$ structure, different from the $(\beta/\alpha)_8$ structure of α- and β-amylases. Both the proton donor and the nucleophile in the catalytic sites of this enzyme are Glu residues.

Most of the fungi and some yeast produce more than one form of enzyme, and these forms may have different molecular mass, amino acid composition, glycosylation, ability to digest raw starch and stability (Jensen and Olsen, 1999). The two forms of glucoamylases have been reported from the commercially used *A. awamori* and *A. niger* (Takahashi et al., 1981; Svensson et al., 1982; Yasuda et al., 1989; Hatada et al., 1997), a type-I strain of *R. oryzae* NRRL 395 (Mertens and Skory, 2007). While *Rhizopus* sp., *R. delemar*, *A. oryzae*, *A. niger* Bo-1 and *Arthrobotrys amerospora* ATCC 34468 produce three different forms of the enzyme (Takahashi et al., 1978; Mitsue et al., 1979; Abe et al., 1985; Norouzian et al., 2000) and the five and six different forms of glucoamylases have been reported from *Corticium rolfsii* AHU 9627 and *Chalara paradoxa*, respectively (Monma et al., 1989). The starch metabolising yeast, *S. cerevisiae* var. *diastaticus* and *Saccharomycopsis fibuligera* produce three and two different glucoamylase forms, respectively (Pretorius et al., 1991; Hostinova, 2002). An amylolytic yeast, *Pichia subpelliculosa* ABWF-64 (Kumar and Satyanarayana, 2001) and thermophilic mould, *Thermomucor indicae-seudaticae* (Kumar et al., 2007) are known to produce

glucoamylases. Glucoamylases have also been reported from the thermoacidophilic Archaea, *Thermoplasma acidophilum*, *Picrophilus torridus* and *Picrophilus oshimae* (Serour and Antranikian, 2002) and the hyperthermophilic archaeon, *Sulfolobus solfataricus* (Kim et al., 2004).

Debranching Enzymes

Five enzymes can be grouped in this category: oligo-1,6-glucosidase (EC 3.2.1.10), amylo-1,6-glucosidase (EC 3.2.1.33), isoamylase (EC 3.2.1.68), pullulanases (type I and II, EC 3.2.1.41 and EC 3.2.1.1/41) and pullulan hydrolase type III. All these enzymes, except amylopullulanases and pullulan hydrolases type III, hydrolyze only the α-1,6 linkage in starch, glycogen, and the oligosaccharides derived from them to produce sugars with an α-configuration. They cannot hydrolyze the α-1,4 linkage.

Oligo-1,6-glucosidase is present mainly in the animal kingdom (often called isomaltase), although some bacterial species, such as *Bacillus*, can also produce this enzyme. It acts on oligosaccharides with anα-1,6 linkage to produce α-glucose as the main product. Amylo-1,6-glucosidase is usually present as a part of a bifunctional enzyme. This single protein has another catalytic center with 4-α-glucanotransferase (EC 2.4.1.25) activity. When starch or glycogen are digested by phosphorylase (EC 2.4.1.1), glucan chains in these polysaccharides are trimmed down from their nonreducing ends, and phosphorylase-limit dextrins with maltotetraose stubs attaching to other chains through α-1,6 linkages are produced. 4-α-Glucanotransferase (EC 2.4.1.25) activity of the bifunctional protein transfers the maltotriose unit to the non-reducing ends of other chains to form α-1,4 linkage, and its amylo-1,6-glucosidase activity hydrolyzes the remaining α-1,6 glucosyl stubs to release α-glucose. This protein is usually found in animal muscles together with phosphorylase. In the microbial kingdom, *Saccharomyces cerevisiae* is known to contain this enzyme.

In the hydrolysis of starch, pullulanase type I and isoamylase play important roles in both living cells and in the starch industry because they are the only enzymes that selectively hydrolyze α-1,6 linkages in glucans. Both enzymes are endo-acting and produce α-1,4-glucan chains or maltooligosaccharides. Pullulanase type I hydrolyzes pullulan and amylopectin completely or nearly completely; however, it can hydrolyze glycogen only to a limited extent. On the other hand, isoamylase hydrolyzes amylopectin and glycogen completely; however, it cannot act on pullulan. Isoamylase is being used as a powerful tool in the analysis of the fine structure of amylopectin, whereas pullulanase, in combination with β-amylase, is widely used in the starch saccharification industry to produce maltose. Enzymatic properties of isoamylase from *Pseudomonas amyloderamosa* have been extensively studied, and an enzyme preparation from *Pseudomonas* sp. is commercially available. Pullulanase preparations from *Klebsiella* sp., *Bacillus brevis*, *B. licheniformis* and *B. acidopulluliticus* are available from several enzyme manufacturers. The enzyme preparation from *B. acidopulluliticus* is thermostable and thus widely used in the starch industry.

The pullulanase type II (amylopullulanases and/or α-amylase-pullulanase) is capable of hydrolyzing both α-1, 4- and α-1, 6-linkages in starch, pullulan, limit dextrins and other polysaccharides. The action of the enzyme on the α-1, 4-linkages of amylose forms DP2-4 products, while pullulan is generally cleaved at α-1,6-linkages to form maltotriose (Nisha and Satyanarayana, 2013b). The amylopullulanases from *Geobacillus thermoleovorans* NP33 (Nisha and Satyanarayana, 2014), *Lactobacillus amylophilus* GV6 (Vishnu et al., 2006) and *Geobacillus stearothermophilus* L14 (Zareian et al., 2010), however, hydrolyze α-1, 4-linkages in maltotriose (pullulan hydrolysate) to release glucose. Because of its action on multiple substrates, the enzyme action results in the complete hydrolysis of polysaccharides to simple sugars without requiring other amylolytic enzymes like α-amylase and β-amylase (Antranikian, 1992; Nisha and Satyanarayana, 2013c). The enzyme is termed as amylopullulanase (Saha et al., 1989) or α-amylase-pullulanase (Kim and Kim, 1995) based on the presence of a single catalytic domain or dual active sites in separate catalytic domains for cleaving α-1, 4- and α-1, 6-linkages. The pullulanase type II from thermophilic anaerobes possess single active site (Mathupala et al., 1993; Ramesh et al., 1994), while that of the aerobic microbes contain either one (Lee et al., 1994; Brunswick et al., 1999) or two active sites for hydrolyzing α-1, 4- and α-1, 6-linkages (Hatada et al., 1997; O'Connell Motherway et al., 2008). While pullulan hydrolase type III cleaves both α-1, 4- and α-1, 6-glycosidic linkages of pullulan to form panose, maltotriose, maltose and glucose. The enzyme also hydrolyzes related polysaccharides like starch, amylopectin, amylose, glycogen and cyclodextrins, and forms maltose as the major product.

All the debranching enzymes have a $(\beta/\alpha)_8$ barrel forming the catalytic domain and are classified into the GH 13 family. The debranching enzymes, excluding pullulanases, are classified into three different subfamilies as shown in Table 1.

Enzymes That Form Maltooligosaccharides With Specific DPs

Glucan-1,4-α-maltotetraohydrolase (EC 3.2.1.60), glucan-,4-α-maltohexaohydrolase (EC 3.2.1.98), glucan-1,4-α-maltotriohydrolase (EC 3.2.1.116), and glucan-1,4-α-maltohydrolase (EC 3.2.1.133) produce maltotetraose, maltohexaose, maltotriose, and maltose, respectively, as sole low-molecular-weight products from starch. All these maltooligosaccharides are formed in the α-configuration. All four enzymes are classified in the GH 13 family, and into four specific subfamilies as shown in Table 1. Although glucan-1,4-α-maltopentaohydrolase is not shown in Table 1 and no EC number has been assigned to it, it is found in several microorganisms and is classified in the GH subfamily 13-19 based on its amino acid sequence. This enzyme produces α-maltopentaose from starch. Although all of these enzymes appear to be exo-acting, a detailed study of these enzymes revealed that most of them are actually endo-acting.

These enzymes are produced by a limited number of bacteria. For example, glucan-1,4-α-maltotetraohydrolase is produced by *Pseudomonas stutzeri* and *B. circulans*, glucan-1,4-α-maltohexaohydrolase by *Klebsiella pneumoniae* and *Bacillus* sp.,

glucan-1,4-α-maltotriohydrolase by *Streptomyces griseus* and *Microbacterium imperiale*, glucan-1,4-α-maltohydrolase by *B. stearothermophilus*, *Thermus* sp., and *Streptomyces hygroscopicus*, and glucan-1,4-α-maltopentaohydrolase by *B. licheniformis* and *Pseudomonas* sp. These enzymes are used in the commercial production of maltotriose, maltotetraose, maltopentaose, and maltohexaose produced for food applications in Japan. Glucan-1,4-α-maltohydrolase is often called maltogenic α-amylase, and the enzyme from *B. stearothermophilus* is widely used in baking. The maltogenic α-amylase from *Thermus* sp. has unique characteristics, that is, this enzyme hydrolyzes the α-1,6 and α-1,4 linkages and transfers cleaved oligosaccharides to C3-, C4-, or C6-hydroxyl groups of acceptor sugars.

α-Glucosidase (EC 3.2.1.20, GH 13-21, GH 13-30, GH 31)

Enzymes belonging to this group hydrolyze non-reducing end glucosidic linkage to release α-glucose, in contrast to glucoamylase, which releases β-glucose. These enzymes are found in the animal, plant, and microbial kingdoms. The microbial enzymes from *A. niger*, *Bacillus*, *Penicillium*, *S. cerevisiae*, and *Thermotoga maritima* are studied extensively. They have long been considered to act on low-molecular-weight substrates such as oligosaccharides and glycosides. Recent studies have shown that some of these enzymes act on starch as well. These enzymes hydrolyze not only the α-1,4 linkage but also the α-1,6-, α-1,2-, and α-1,3-glucosidic linkages. A remarkable feature of α-glucosidase is that it has strong transglycosylation activity at higher maltose concentrations. Isomaltooligosaccharides are produced as a food ingredient via the activity of *A. niger* α-glucosidase. Based on amino acid sequences, enzymes of this group are classified in the GH 13 and GH 31 families, the former being further classified into the GH 13-21 and GH 13-30 subfamilies. The GH 31 family also has a $(\beta/\alpha)_8$ barrel; however, it is different from that of GH 13. Both the proton donor and the nucleophile in the catalytic sites of these enzymes are Asp residues.

Cyclomaltodextringlucanotransferase (Cgtase, EC 2.4.1.19, GH 13-2)

Cyclodextrins (CDs) are mixtures of cyclomaltohexaose (α-CD), cyclomaltoheptaose (β-CD), and cyclomaltooctaose (γ-CD). They form inclusion complexes with guest compounds and are used as food ingredients in Japan. Enzymes belonging to CGTase are produced by several species of *Bacillus* such as *Bacillus macerans* and *B. megaterium*. They act on α-glucans such as starch and glycogen to produce a mixture of CDs; the composition varies depending on enzyme sources and reaction conditions. The enzyme from *B. macerans* has been studied extensively. CGTase catalyzes three kinds of transglycosylation reactions: intramolecular transglycosylation, intermolecular transglycosylation, and disproportionation reactions. In the intramolecular reaction (cyclization reaction), the enzymes cleave the α-1,4 linkage of glucans at a certain distance (6, 7, or 8 glucose units) from their non-reducing ends and transfer the newly formed reducing end glucose unit to the C-4 hydroxyl group of the non-reducing end glucose unit of the same glucan chain. CDs are produced by this reaction. In the presence of glucose together with starch or CDs, the enzymes catalyze intermolecular transglycosylation (coupling reaction) to form a series of maltooligosaccharides. Glucose can be replaced with other sugars or glycosides. This reaction of CGTase is also often used in the food industry to add glucose units to other sugars or glycosides for the synthesis of new sugars or for the modification of glycosides. When incubated with maltooligosaccharides of certain DPs, the enzymes disproportionate them to produce a series of maltooligosaccharides. Enzymes of this group cleave the α-1,4 linkages exclusively and form α-1,4 linkages exclusively in their reactions. These enzymes show a hydrolytic reaction, although with low activity, with starch and CDs.

Cyclomaltodextrinase (EC 3.2.1.54, GH 13-20)

Enzymes of this group act preferentially on CDs compared to starch and produce linear maltooligosaccharides. They also hydrolyze linear maltodextrin. Some enzymes of α-amylase (EC 3.2.1.1) can also hydrolyze CDs. The only difference between cyclomaltodextrinase and α-amylase is the high affinities of the former to CDs. Enzymes of this group are produced by several species of *Bacillus* and some other bacteria. They are classified in the same subfamily (GH 13-20) as maltogenic α-amylase (EC 3.2.1.133) and neopullulanase (EC 3.2.1.135).

Pullulan Hydrolases

Pullulan hydrolase type I (neopullulanase, 3.2.1.135, GH 13-20)

Neopullulanases hydrolyze α-1, 4- linkages of pullulan to release panose (Antranikian, 1992; Kuriki et al., 1988) and cleaves α-1, 4- and α-1, 6- linkages of starch and other polysaccharides with much less efficiency. Other amylases known to attack α-1, 4-glycosidic linkages of pullulan to form panose are *Thermoactinomyces vulgaris* R-47 α-amylases (TVA I (Tonozuka et al., 1995) and TVA II (Tonozuka et al., 1993)) and *Bacillus licheniformi* α-amylase (Kim et al., 1992), which efficiently hydrolyzes starch more than pullulan to release maltose as major product. These enzymes are therefore designated as neopullulanase-α-amylase (Svensson, 1994; EC 3.2.1.1/135). These are polyspecific enzymes capable of hydrolyzing at least two of the three substrates, cyclomaltodextrins, starch and pullulan, and known to catalyze transglycosylation reaction (Park et al., 2000, 2007; Nisha and Satyanarayana, 2016). The neopullulanase of *Bacillus subtilis* NA-1 forms α-1,4-and α-1,6-glycosidic linkages using maltose as the substrate by

transglycosylation (Takata et al., 1992). The neopullulanases act on starch, CDs, and maltooligosaccharides to produce large amounts of maltose and small amounts of glucose, both with an α-configuration. The activity pattern of the enzyme from *B. Stearothermophilus* has been extensively studied. It catalyzes hydrolysis of both the α-1,4 and α-1,6 linkages in branched oligosaccharides. Furthermore, it catalyzes glucosyl transfer to C-4 or C-6 hydroxyl groups of acceptor sugars to form maltooligosaccharides or branched oligosaccharides such as isomaltose and panose. In this context, some of the maltogenic α-amylases (EC 3.2.1.133) have both hydrolyzing and transferring activities to form both the α-1,4 and α-1,6 linkages as described above.

Kuriki and Imanaka, based on their research on the *B. stearothermophilus*neopullulanase mutations to modify its action specificity, proposed the following concept. According to this proposal, α-amylase (EC 3.2.1.1), debranching enzymes such as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), CGTase (EC 2.4.1.19), and a branching enzyme (EC 2.4.1.18) can be classified into a single family – the α-amylase family – based on the structural similarities and interchangeability of four reactions among these enzymes. All these enzymes share the common $(\beta/\alpha)_8$ barrel and two catalytic amino acid residues: Glu as a proton donor and Asp as a nucleophile. Additional domains present in respective enzyme groups modify the basic catalytic function of the $(\beta/\alpha)_8$ barrel to provide specificities to four enzyme groups. Enzymes of this group are produced by many *Bacillus* strains, including*B. stearothermophilus*, *Thermoactinomyces vulgaris* and other bacteria.

Pullulan hydrolase type II (isopullulanase, 3.2.1.57, GH 13-20)

Isopullulanases cleave α-1, 4- linkages of pullulan to produce isopanose (Glcα1-4Glcα1-6Glc) and hydrolyzes panose (Glcα1-6Glcα1-4Glc) to release glucose and isomaltose. The enzymes, however, do not hydrolyze starch and dextran unlike that of neopullulanases (Aoki and Sakano, 1997; Nisha and Satyanarayana, 2016), therefore, it is not included as a member of the amylase family in this article.

4-α-Glucanotransferase (EC 2.4.1.25, GH 77)

Enzymes of this group cleave α-1,4 linkages in glucan and transfer the newly formed reducing end glucose residue to the C-4 hydroxyl group of nonreducing end glucose residues. When the glucose residue is transferred to other glucan chains (intermolecular transglycosylation), the reaction results in disproportionation of glucan chains, and the plant enzyme catalyzing this reaction is often called disproportionating enzyme. Microbial enzymes with the same action specificities are sometimes called amylomaltase. Recently, some of these enzymes were found to catalyze intramolecular transfer to form cyclic glucans consisting of only the α-1,4 linkage. The enzyme from *Thermusaquaticus* produces cycloamyloses from starch with DPs greater than 17. Besides *T. aquaticus*, microorganisms such as *Aquifexaeolicus*, *Escherichia coli*, and *Streptococcus pneumoniae* also produce this type of enzyme. Although enzymes of this group have a common (β/α)8 barrel with Glu as a proton donor and Asp as a nucleophile, they are classified as GH 77.

Branching Enzyme (EC 2.4.1.18, GH 13-9)

Enzymes of this group exist in the plant kingdom and are known to play an important role in starch synthesis. They catalyze the cleavage of α-glucan chains and the transfer of the cleaved chains to the C-6 hydroxy group of other glucan chains to form the α-1,6 branch linkage. Enzymes that can catalyze the same reaction are found in microorganisms such as *Bacillus*, *Escherichia*, and *Pseudomonas*. The enzyme from *B. stearothermophilus* was found to catalyze the intramolecular transfer reaction to form highly branched cyclic dextrins (Fig. 8(e)) from amylopectin. Enzymes of this group have the same $(\beta/\alpha)_8$ barrel structure as that of α-amylase and are classified into the GH 13-9 subfamily.

Enzymes Forming Trehalose From Starch

Two enzymes of remarkably unique specificities, maltooligosyltrehalose synthase (EC 5.4.99.15, GH 13-26) and maltooligosyltrehalosetrehalohydrolase (EC 3.2.1.141, GH 13-10), convert starch into α-1,1′-trehalose by a cooperative process. The former enzyme acts on the α-1,4 linkage in the non-reducing end of starch or maltooligosaccharides and mutates into the α-1,1′ linkage, which results in the formation of maltooligosyltrehalose. The second enzyme hydrolyzes the α-1,4 linkage adjacent to the trehalose moiety, thus releasing trehalose from maltooligosyltrehalose. Consequently, the two enzymes act on maltooligosaccharides, yielding a high amount of trehalose. These enzymes are found together in several microorganisms such as *Arthrobacter* and *Pseudomonas*. They are also found in some thermophilic archaea such as *Sulfolobus*. The industrial production of trehalose is described in a later section. Trehalose synthase (EC 5.4.99.16) catalyzes the conversion of maltose into trehalose in a manner similar to that of maltooligosyltrehalose synthase. This enzyme does not act on starch; therefore, it is not considered a member of the amylase family.

Reaction Mechanism of Amylases

As mentioned previously, most enzymes are of the retaining type of amylase, in which the α-configuration in the substrates is retained in the products. Other enzymes catalyze by means of an inverting type reaction, in which the α-configuration in the

substrates is inverted in the products. β-Amylase and glucoamylase follow the latter reaction, which yields β-maltose or β-glucose, respectively. Both types of enzymes have two acidic catalytic amino acid residues, one acting as a proton donor (general acid) and the other as a nucleophile (base).

Two theories are proposed to explain the molecular mechanisms of the two reactions: covalently bound intermediate theory and carbonium ions intermediate theory. According to the former theory, the catalytic reactions proceed via the formation of a covalently bound intermediate between substrate and enzyme protein. According to the latter theory, reactions proceed via carbonium ions without forming covalent bonds. The mechanism of the two reactions according to the covalently bound intermediate theory is depicted in Fig. 2. In the retaining reaction (Fig. 2(a)), the proton donor (general acid) donates its proton to the glycosyl oxygen, engaging the α-linkage to be cleaved, and, in cooperation with this, the nucleophile directly attacks the anomeric carbon next to the oxygen from the opposite side to form a covalently bound intermediate with a β-configuration (first Walden inversion). In the next step, the deprotonated proton donor, in turn, attacks the hydrogen in the water molecule. The activated water molecule attacks the anomeric carbon on the intermediate from the opposite side to form α-glucose (second Walden inversion). As a result of two Walden inversions, the anomeric configuration in the substrate is retained in the product. In the retaining enzyme, the water molecule is located near the proton donor in the catalytic site, whereas in the inverting enzyme, it is positioned near the nucleophile. These different positions of the water molecules in catalytic sites have been confirmed for some enzyme proteins belonging to the α-amylase, β-amylase, and glucoamylase groups, as shown below.

In the inverting reaction, the nucleophile attacks water molecules close to this residue and activates it as shown in Fig. 2(b). The activated water molecule then attacks the anomeric carbon in the substrate from the opposite side to form β-glucose. As a result, α-configuration in substrates was converted into β-configuration in the products.

Because the reaction of amylase is usually conducted in aqueous solution, the reaction is practically irreversible if a water molecule participates. Enzymes catalyzing the retaining reaction can perform a tranglycosylation reaction because the covalent intermediate can be attacked not only by water but also by the hydroxyl group of sugars and alcohols. Contrary to this, enzymes catalyzing the inverting reaction do not form a covalently bound intermediate because the activated water molecule directly forms covalent bond with substrates.

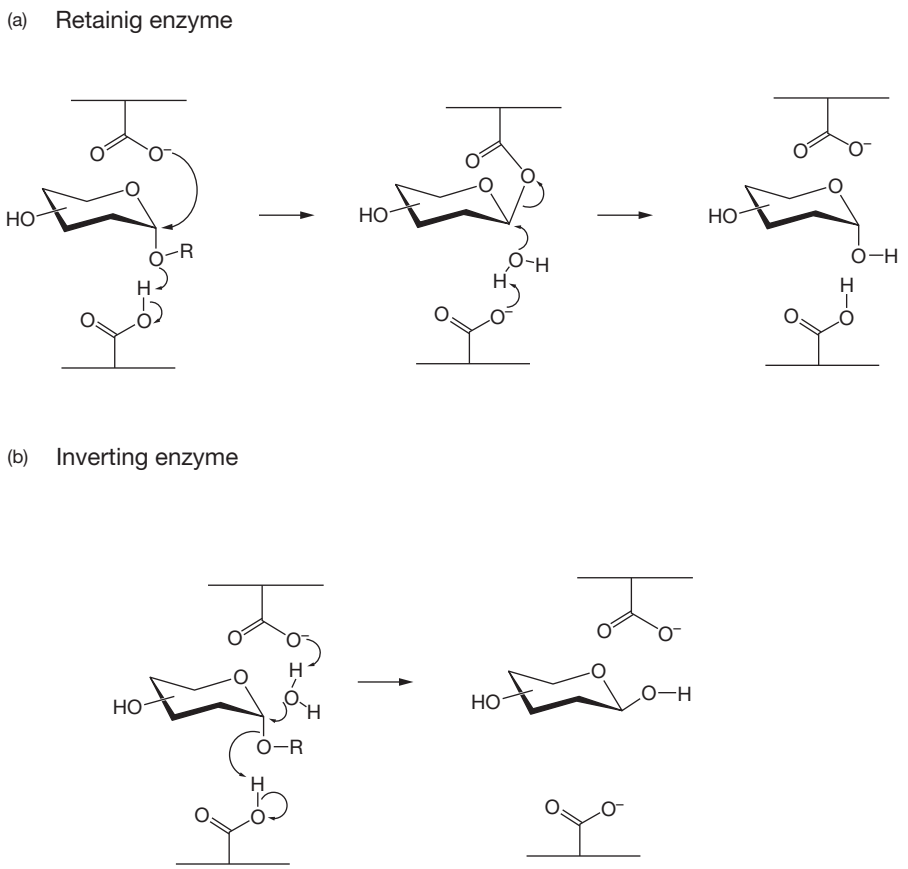

Fig. 2 Illustration of retaining and inverting mechanisms. (a) Retaining mechanism. (b) Inverting mechanism. Nucleophilic residue is at the top and proton donor is at the bottom in both mechanisms. Reactions proceed from left to right.

Structure–Function Relationships of Amylases

Taka-amylase (EC 3.2.1.1, GH 13-1)

Crystal structures of Taka-amylase (α-amylase from *A. oryzae*), β-amylase, CGTase, and glucoamylase are shown in Fig. 3. They have distinct catalytic properties and distinct three-dimensional (3D) structures. The structure of Taka-amylase was determined by X-ray diffraction analysis in 1984; this was the first structure determination case in amylases. To date, more than 200 3D structures have been reported for the enzymes classified as amylases. Taka-amylase is a single peptide consisting of 478 amino acid residues, and its 3D structure is composed of three domains called A (aa 1–376), B (aa 128–201), and C (aa 385–478) as illustrated in Fig. 4. Domain A (catalytic domain) has a $(\beta/\alpha)_8$ barrel (TIM barrel) common to all enzymes of the GH 13 family, which consists of eight identical pairs of β-sheet and α-helix. Domain B is composed of three β-sheets and is inserted between the third β-sheet and the third α-helix as a short loop extruding from the large domain A. Domain C has eight antiparallel β-sheets and follows after the eighth α-helix of the $(\beta/\alpha)_8$ barrel. The catalytic site of this enzyme is located at the cleft (catalytic cleft) formed between domains A and B as shown in Fig. 3(a). This cleft has a size that can accommodate just seven glucose units of α-glucans and is gently curving to fit the helical structure of the glucans. About 20 amino acid residues are aligned on the surface of this catalytic cleft, as illustrated in Fig. 5. Two catalytic amino acid residues, Asp206 and Glu230 in the fourth and fifth β-sheets, respectively, are located near the third glucosidic linkage of the substrate. Other residues located throughout the cleft are involved in fixing glucose residues by forming hydrogen bonds with their hydroxyl group. These interactions affect substrate distortion and help the concerted hydrolytic reaction of catalytic amino acid residues. In the catalysis, Asp206 acts as a nucleophile (base), and Glu230 acts as a proton donor (acid). A water molecule is known to present near Glu230 at the catalytic site. Although most of these amino acid residues are on the $(\beta/\alpha)_8$ barrel in domain A, some are on domain B. The Ca^{2+} ion is located inside the catalytic cleft, as shown in Fig. 3(a), and coordinates with several amino acid residues, thus stabilizing the cleft architecture essential for catalytic reaction. The function of domain C is not fully understood.

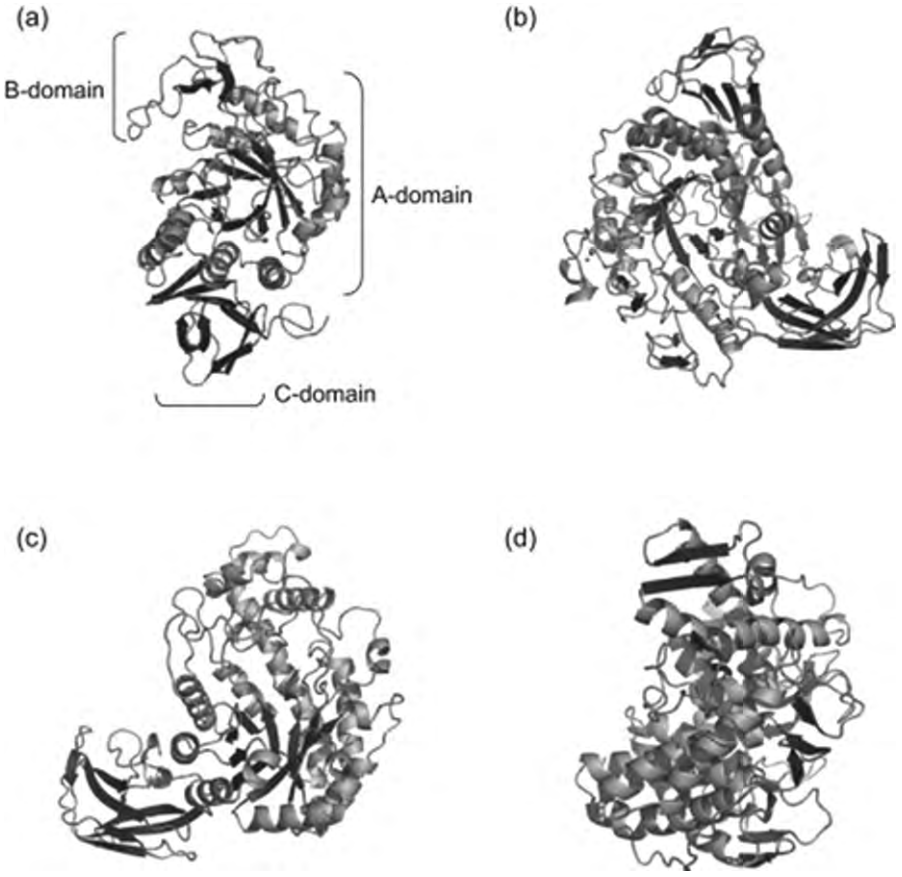

Fig. 3 Crystal structures of representative amylase proteins. (a) Taka-amylase from *Aspergillus oryzae*. (b) CGTase from *Bacillus circulans*. (c) β-Amylase from *Bacillus cereus* var. mycoides. (d) Domain A of glucoamylase from *Aspergillus awamori*. Drawings were done based on the PDB (2TAA, 1CXE, 5BCA, 1AGM) using PyMOL program (http://pymol.sourceforge.net/). Helical ribbon stands for the α-helix and broad arrow stands for the β-sheet.

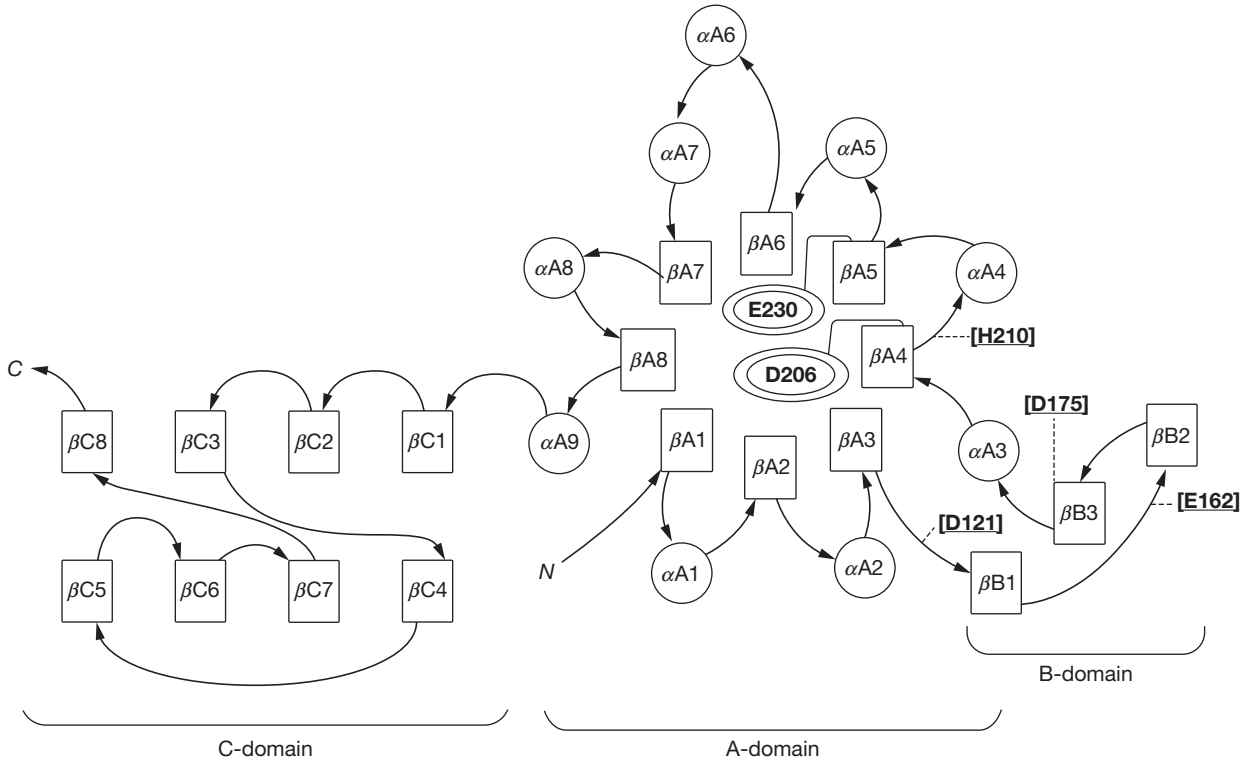

Fig. 4 Topological diagram of Taka-amylase. The drawing was done based on the Matsuura, Y., Kusunoki, M., Harada, W., Kakudo, M., 1984. Structure and possible catalytic residues of Taka-amylase A. J. Biochem. 95, 697–702 (2TAA). Circles and squares indicate α-helices and β-sheet, respectively. Numbers in these symbols indicate their sequence order in each domain. Positions of the two catalytic amino acid residues are shown within double circles and residues binding to calcium ion are shown within square brackets. Amino acids are expressed by single letters.

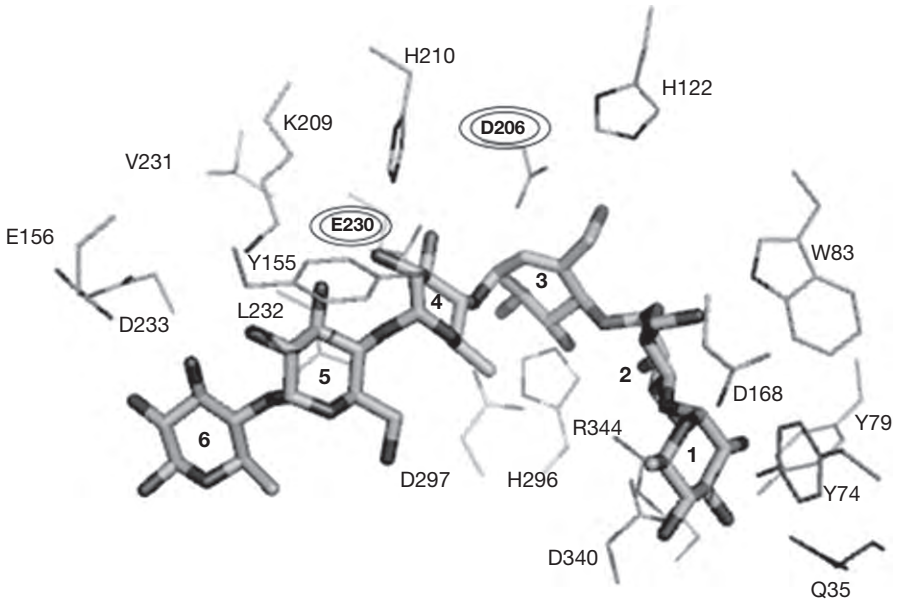

Fig. 5 Catalytic site structures of Taka-amylase binding acarbose glucose residues in the acarbose are numbered from the nonreducing end residue. Positioning of two catalytic residues, Glu230 and Asp206, are indicated by double circles. Residues involved in substrate binding are also shown. Amino acids are expressed by single letters.

Cyclomaltodextringlucanotransferase (Cgtase, EC 2.4.1.19, GH 13-2)

This enzyme has catalytic properties distinct from those of α-amylase; however, it is classified in the same GH 13 family. The 3D structure of CGTase from *B. stearothermophilus* strain TC-91 is shown in Fig. 3(b). It is composed of five domains: A (aa 1–403), A′ (aa 136–196), B (aa 404–493), C (aa 494–576), and D (aa 577–680). Domain A has a $(\beta/\alpha)_8$ barrel, and domain A′ is extruded from this barrel between the third β-sheet and the third α-helix of the barrel. Thus, domains A, A′, and B of CGTase roughly correspond to the three domains of Taka-amylase. A large cleft present between the $(\beta/\alpha)_8$ barrel and domain A′ forms the catalytic site (catalytic cleft). This enzyme has two calcium ions, one of which is present in the cleft, as is Taka-amylase. As in the case of the catalytic amino acid residues of Taka-amylase, Asp225 and Glu253 of CGTase are presumed to be the nucleophile and the proton donor, respectively. The structural features explaining the transglucosylation reaction of CGTase are not fully understood. Domains C and D are the structures specific for CGTase. Domain D consists of eight antiparallel β-sheets and acts as a starch-binding site, which plays an important role in the digestion of raw starch (starch granules). Domain C also contains β-sheets, although its function is not fully understood. The crystal structure of the CGTase from alkaliphilic *Bacillus* sp. 1011 with the substituted His233N revealed the change of active site conformation, such that the mutated variant does not produce α-cyclodextrin, while the wild type forms a mixture of α-, β- and γ-cyclodextrins (Ishii et al., 2000). The three dimensional structure of CGTases reveals that the Tyr/ Phe residues play an important role in the formation of CD (Leemhuis et al., 2010).

β-Amylase (EC 3.2.1.2, GH 14)

The 3D structure of β-amylase from *Bacillus cereus* is shown in Fig. 3(c). It consists of large domain A (aa 1–417) and small domain B (aa 418–516). Although this enzyme belongs to GH 14, its domain A has a $(\beta/\alpha)_8$ barrel like that of α-amylase. The catalytic site of this enzyme is formed on the barrel by the assembly of short loops present between β-sheets and α-helices. The catalytic site has a pocket-like structure, as illustrated in Fig. 6. Glu367 and Glu172 are present near the center of this pocket and serve as a nucleophile and a proton donor, respectively. A water molecule is known to be present near Glu367. A short peptide extending between the third β-sheet and the third α-helix is located around the catalytic site as a flexible loop, which is a unique structure to β-amylase. Four or five glucose units at the non-reducing end of α-glucans can enter into this pocket. The flexible loop holds the substrate tightly during catalysis and moves away to release the product (β-maltose) from the catalytic pocket after the reaction completes. The pocket-like structure of β-amylase is in sharp contrast to the cleft-like structures found in α-amylase and CGTase and explains the exo-acting style of this enzyme. Domain B has a structure similar to that of domain D of CGTase and has starch-binding activity.

Glucoamylase (EC 3.2.1.3, GH 15)

The 3D structures of glucoamylase from *A. niger* and related strains have been extensively studied. The enzyme from *A. niger* consists of three domains: domain A (aa 1–440), B (aa 441–512), and C (aa 513–616). As illustrated in Fig. 7, domain A of this enzyme has an $(\alpha/\alpha)_6$ barrel, which is clearly different from the $(\beta/\alpha)_8$ barrel found in almost all of the other enzymes listed in Table 1.

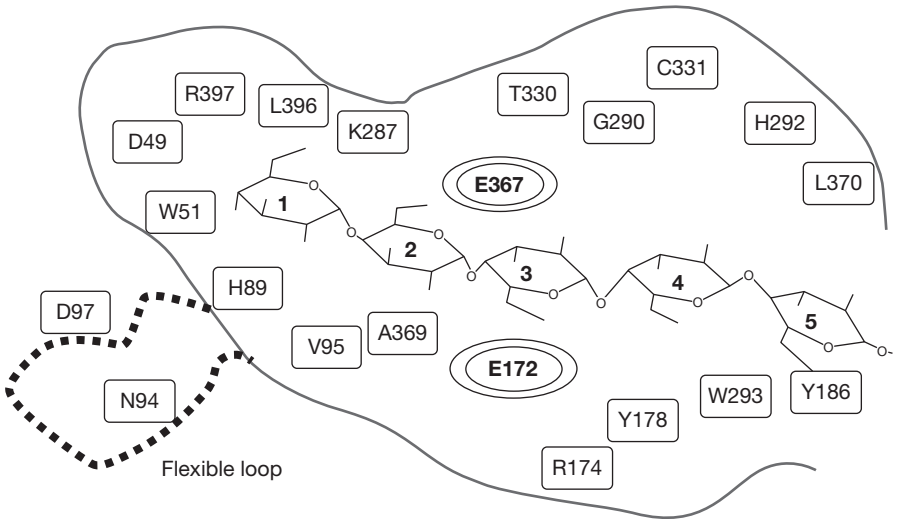

Fig. 6 Catalytic site structure of *Bacillus cereus* β-amylase. Image of pocket-like structure of catalytic site is drawn with broad solid line based on PDB(1ITC). Glucose residues in glucans are numbered from the residue at the nonreducing end. Positions of the two catalytic amino acid residues, Glu367 and Glu172, are shown with double circles. Residues involved in substrate binding are also shown. Amino acids are expressed by single letters. Image of a flexible loop in closed form is shown by a dotted line.

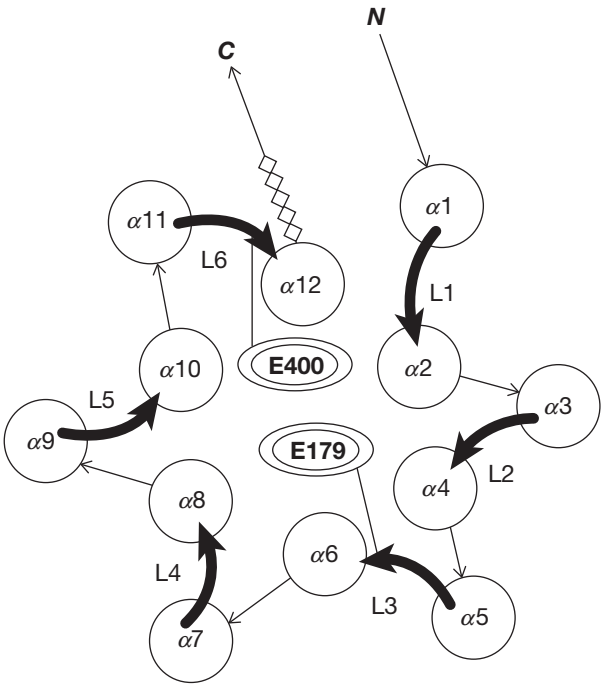

Fig. 7 Schematic representation of $(\alpha/\alpha)_6$ barrel in comparison with $(\beta/\alpha)_8$ barrel. (a) $(\alpha/\alpha)_6$ barrel of *Aspergillus awamori* glucoamylase. (b) $(\beta/\alpha)_8$ barrel of Taka-amylase. Circles indicate α-helices with numbers ordered from N-terminal. Large arrows indicate loops connecting outer and inner helices with numbers ordered from N-terminal. Small arrows indicate loops connecting inner and outer helices. Two catalytic amino acid residues, Glu400 and Glu179, are shown with double circles. Residues blocking one end of the catalytic cavity are shown with boxed line. Amino acids are expressed by single letters.

The even-numbered six helices form the inner core of the barrel, whereas the odd-numbered helices form the outer part of the barrel. The catalytic site (catalytic cavity) is composed of six inner α-helices and six loops connecting the C-terminal residues of the outer helices and the N-terminal residues of the inner helices. One end of this catalytic cavity is blocked off by the residue following the last inner helix, and only the opposite end is open to the substrates. Two catalytic amino acids, Glu400 in the sixth loop and Glu179 in the third loop, act as a nucleophile and a proton donor, respectively. As in the case with β-amylase, a water molecule is oriented near Glu400 during the catalytic cavity. Domain B is a linker domain rich in Ser and Thr residues. Domain C is a starch-binding domain with a sequence resembling that of the starch-binding domains in CGTase and β-amylase.

The crystal structure of the starch binding domain of *R. oryzae* glucoamylase and its complex with the substrates, β-cyclodextrin and maltoheptaose identified two carbohydrate binding sites, Trp47 and Tyr32 as well as the asparagine residues in polyN loops (Tung et al., 2008). The *Saccharomycopsis fibuligera* HUT 7212 glucoamylase, which lacks the raw starch binding domain, displayed the presence of two substrate binding sites within the catalytic domain upon crystal structure analysis of the complex of the enzyme with acarbose (Sevcik et al., 2006).

Engineering of Amylases

Directed evolution and site-directed mutagenesis have been employed upon amylases to improve their properties like activity, substrate specificity, thermal and pH stability and solubility (Rubin-Pitel and Zhao, 2006). Thermostability, Ca^{2+}-independence, raw starch degrading ability and acid stability of α-amylases are desirable for their use in the starch saccharification process, and oxidative stability as well as pH optima in alkaline range is a prerequisite for the applicability of amylases in detergent industry.

In general, enzymatic reactions are performed under moderate conditions (temperature and pH), because most enzyme proteins cannot maintain active structures under extreme conditions. However, in the industrial application of enzymes, enzymatic reactions at higher temperatures are advantageous because of the higher solubility of substrates and protection from contamination. Thus, screening of thermostable enzymes or microorganisms has been actively carried out in extreme circumstances, such as hot springs and seabed volcanoes. The enzymes are genetically engineered to generate thermostable enzymes. Random mutagenesis and gene shuffling are used most often to increase the heat stability of known enzymes. The thermostability of *Thermus* sp. maltogenic amylase, for instance, was greatly improved by four rounds of DNA shuffling. The variant of *Bacillus thermalkalophilus* ET2 maltogenic amylase formed by three rounds of DNA shuffling and the recombination of the selected mutations displayed 10°C higher temperature optimum compared to the native enzyme. One of the variants carrying substitution exhibited a 20-fold longer half-life in comparison to the wild-type (Tang et al., 2006). The maltogenic amylase, Novamyl, displayed improved thermostability (10°C upward shift in melting temperature as compared to the wild type at pH 4.0) upon triple mutation and therefore enhanced its use as an antistale for breads made at low pH (Jones et al., 2008). A significant increase in half-life was observed for

the *Bacillus* sp.TS-23 α-amylase when the deletion was made of Arg210-Ser211 sequence (Lin et al., 2008). The site directed mutagenesis of the calcium binding sites (Asn-75, Ser-76 and His-77) of α-amylase from *B. megaterium* resulted in improved thermostability (Ghollasi et al., 2013). The chimeric enzyme formed by the fusion of the *Bacillus acidicola*α-amylase and the gene fragments encoding partial N- and C- terminal region of *G. thermoleovorans* α-amylase displayed higher thermostability than the wild type *Bacillus acidicola* α-amylase (Parashar and Satyanarayana, 2016).The N- domain deletion of the maltogenic amylases of *G. thermoleovorans* and *Thermus* sp. identified its role in thermostability and substrate affinity (Mehta and Satyanarayana, 2013; Kim et al., 2001).

The protein engineering using site-directed mutagenesis resulted in a significant increase in thermostability and catalytic efficiency as well as starch saccharification efficiency in case of pullulanases from *Bacillus deramificans* (Duan et al., 2013) and *B. acidopullulyticus* (Chen et al., 2015). The N-terminal truncated *B. deramificans* pullulanase mutant displayed improved extracellular secretion and thermostability (Duan and Wu, 2015). The amylopullulanases of *T. ethanolicus* 39E (Lin et al., 2008), *T. saccharolyticum* (Lin et al., 2011), *L. plantarum* L137 (Kim et al., 2009) and *G. thermoleovorans* NP33 (Nisha and Satyanarayana, 2013a) showed an improved catalytic efficiency and thermostability by C-terminus truncation. The N- and C-terminal truncated variants of the *G. thermoleovorans* exhibited reduced random coils compared to the wild type, suggesting improved conformational and thermal stability of the enzyme with the deletion (Nisha and Satyanarayana, 2015a,b). An increased stability towards temperature and detergents was achieved by mutation of some bulkier side chain amino acids in neopullulanase of *B. stearothermophilus* (Ece et al., 2015).

Improved acid stability was achieved in case of *B. licheniformis* α-amylase, when Leu134 andSer320 were mutated (Liu et al., 2008). In another example, single, double and triple mutations at four histidine residues (His222, His275, His293 and His310) in the catalytic domain significantly increased the acid stability compared to the wild type (Yang et al., 2013).

Specificities to the α-1,4 or α-1,6 linkages of enzymes belonging to the amylase group are generally considered to be very strict, except for glucoamylase and α-glucosidase. Recently, endo-acting enzymes such as neopullulanase and maltogenic α-amylase were shown to have specificities not only to the α-1,4 linkage but also to the α-1,6 linkage in the substrates. The specificity ratio of α-1,4/α-1,6 of neopullulanase was modified by replacing the special amino acid residues responsible for substrate binding with other residues. Currently, creation of mutated enzymes by substituting special amino acid residues with other residues based on the 3D structures of enzymes has become one of the most popular enzyme engineering methods. Site-directed mutagenesis of the catalytic site residues resulted in the loss of *B. stearothermophilus* neopullulanase activity towards both α-1, 4- and α-1, 6- glycosidic linkages, thereby suggesting the presence of one active site for the dual activity. Further, altered substrate specificity towards α-1, 4- and α-1, 6-glycosidic linkages was observed by mutating the substrate binding sites (Kuriki et al., 1992). The protein engineering of the active subsites -3/-6/-7 altered cyclodextrin specificity of CGTases (Kelly et al., 2008a,b; Nakagawa et al., 2006).

Transglycosylation reactions of α-amylase and α-glucosidase have been widely used for the production of useful oligosaccharides. A great deal of effort has been spent on the screening of enzymes with high transferring activities. In the field of enzyme engineering, many studies on modification of the catalytic properties of enzymes such as substrate and product specificities and action modes have been reported. In some cases, successful results were obtained, with special enzymes tested. However, no versatile method of modification has had successful results. Withers and colleagues reported that a mutant of β-glucosidase (EC 3.2.1.21) from *Agrobacterium* sp., with a mutation at its nucleophilic E358, catalyzed the synthesis of various β-glycosides using α-glucosyl fluoride as a donor and various p-nitrophenyl-β-glycosides as acceptors. They called the mutated enzyme glycosynthase. By replacing one of the catalytic (nucleophile) amino acid residues with other amino acids, hydrolase was successfully changed into synthase. To date, 19 hydrolases with retaining or converting mechanisms have been converted into glycosynthases by substituting their nucleophilic residues. α-Glucosidase from *Schizosaccharomyces pombe* was successfully converted into glycosynthase using this method.

The protein engineering of the maltogenic amylase (MAG1) of *Bacillus lehensis* G1 by the mutations of the residues, W359F, Y377F, and M375I minimized steric interference and changed the active site conformation to accommodate larger donor/acceptor molecules for transglycosylation, thereby improving the transglycosylation to hydrolysis ratio of upto 4.0-fold (Manas et al., 2015). The maltogenic amylases with high hydrolysis to transglycosylation ratio are used to decrease the content of maltotriose in high maltose syrups. Transglycosylation activity of the *B. stearothermophilus* maltogenic amylase was decreased and the increased maltose production was attained by site-directed mutagenesis of the W177 residue (Sun et al., 2016). Tran et al. (2014) reported an improved transglycosylation activity by site directed mutagenesis of the His235 in the catalytic domain of the enzyme. The mutations in two residues, Ser-77 and Trp-239, on the outer region of the active site of *Thermoanaerobacterium thermosulfurigenes* cyclodextrin glucanotransferase revealed 15-fold lower hydrolytic activity over cyclization activity, while the hydrolytic rates were unaffected by mutations generated within the active site of the enzyme (Kelly et al., 2008b).

Industrial Application of Amylases

Industrial Production of Amylases

The history of the industrial production of amylase dates back to 1894, when Jhokichi Takamine began the commercial production of a digestive enzyme preparation, Taka-diastase, from a wheat bran culture of *A. oryzae*. In 1905, a textile desizing agent containing α-amylase from *A. oryzae* was produced in Japan. The industrial production of α-amylase for its application in starch liquefaction started as early as 1939 with the use of *B. amyloliquefaciens*. In 1959, *Rhizopus* sp. began to be used in the industrial production of

glucoamylase. In those days, enzymes were produced in solid culture using wheat bran, although this cultivation method was replaced with submerged cultures.

Besides α-amylase and glucoamylase, many enzymes, such as β-amylase, pullulanase, and CGTase, are commercially available from enzyme manufactures such as Novozyme, Danisco, and Amano Enzyme Inc. The value of the industrial enzyme market was estimated at USD 4.2 billion in 2014 and to reach approximately USD 6.2 billion by 2020. The starch hydrolyzing enzyme finds potential application in the preparation of HFCS (high fructose corn syrup), production of ethanol as well as in detergent and baking industries. The starch enzyme market is estimated to a value of $2.24 billion by 2018 (see "Relevant Websites section"). Enzymes belonging to the amylase group are estimated to account for 30% of world enzyme production. Recombinant enzymes are also released in the market for industrial application. The following list shows the enzymes that are currently available for industrial applications and their origins.

α-Amylase: *A. oryzae*, *A. niger*, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *Bacillus* sp.
Glucoamylase: *A. niger*, *R. niveus*, *Rhizopus* sp.
Pullulanase: *Bacillus acidpullulyticus*, *B. brevis*, *B. licheniformis*, *Klebsiella* sp.
α-Glucosidase: *A. niger*, *B. stearothermophilus*, *S. cerevisiae*
CGTase: *B. macerans*
Maltotriohydrolase: *Microbacterium* sp.
Maltopentaohydrolase: *Bacillus* sp.

Application of Amylases in Food Industries

Starch liquefaction and saccharification

The global enzyme market was dominated by the food and beverage industry in 2010, with the major market share occupied by the baking enzymes. Starch industry approximately accounts for 15%–20% of the global sales of industrial enzymes. The industrial starch hydrolysis requires the action of different endo- and exo-acting enzymes including α-amylase, pullulanase, glucoamylase, glucose isomerase and others.

Starch, especially corn starch, is the most abundant and cheapest carbohydrate commercially available. For the production of glucose or oligosaccharides, raw starch (starch granules) is first gelatinized to disrupt the starch granules; to this, α-amylase is added to hydrolyze starch into low-molecular-weight dextrins. Thermostable liquefying α-amylase, such as that from *B. subtilis* and *B. licheniformis* is added to the starch slurry, and the mixture is heated quickly to around 100°C for a suitable period to obtain products of desired dextrose equivalence (DE). Dextrins with a DP of 3-25 are commercially produced for various applications, such as to improve viscosity or to be a filler or ingredient of food. The dextrins are then saccharified by debranching enzymes to products of DP2-5. Maltose is often used as a sweetener or to improve the quality of food. Part of the highly purified maltose is used in the medical field. For the industrial production of maltose, starch is liquefied as described above, α-amylase is inactivated by heating the solution at 120–140°C, the pH is adjusted to around 4.5, and then β-amylase and isoamylase from *P. amyloderamosa* or pullulanase from *Bacillus* sp. are added. This saccharification process is run for 2 or more days at 60°C to yield a high amount of maltose. Currently, β-amylase from plants is used; however, this will be replaced by bacterial β-amylase in future. In Japan, about 3 million tons of starch is produced annually, mainly from corn. More than 60% of the starch is hydrolyzed to glucose, which is consequently converted into fructose to obtain a commercial product, high-fructose corn syrup (HFCS). More than 8 million tons of HFCS is produced annually in the United States, whereas about 1 million tons is produced annually in Japan. HFCS is a mixture of glucose and fructose and is widely used as a sweetener for beverages. Conversion of glucose into fructose is carried out by the action of glucose isomerase. This enzyme (xylose isomerase; EC 5.3.1.5) plays an important role in industrial starch saccharification; however, it cannot be classified as an amylase and, thus, will not be discussed further. Thus, production of glucose from starch is the main goal of the starch industry. The enzymatic process used to produce glucose from starch consists of two steps: liquefaction and saccharification. The starch granules are heated (gelatinized) in a jet cooker to 105–110°C for 5 min (pH 5.8–6.5) and then partially hydrolyzed (liquefaction) using *B. licheniformis* α-amylase (24,000 U g^{-1}), for example, is added to a starch slurry (30%–40%) at an enzyme–starch (dry weight) ratio of 0.6 kg ton^{-1}. Then, the mixture is kept at 90–95°C for 1–2 h to obtain a final DE of 5–15. In the saccharification step, the pH of the liquefied solution is adjusted to 4.5 and the temperature lowered to 55–60°C. The *A. niger* glucoamylase (200 AGU ml^{-1}) and *Bacillus* sp. pullulanase (200 U g^{-1}) are added at enzyme–starch (dry weight) ratios of 0.8 l ton^{-1} and 0.3 kg ton^{-1}, respectively. The mixture is kept at 60°C for 2–3 days to obtain the final DE of 97–98.5. Most of the obtained glucose syrup is converted into HFCS, and the remaining part is purified to produce crystalline glucose. In the production of alcoholic beverages, fungal α-amylase preparations are often used to promote the digestive action of malt or Koji enzymes in the saccharification of starch. Amylase preparations are occasionally used together with pectinases in juice clarification.

The pullulanases are widely used in the industrial starch saccharification process for the production of maltose, maltotriose and maltotetraose syrups. The currently employed pullulanase in starch saccharification process increases the glucose or maltose concentration by 2% and 20%–25%, respectively and minimizes the use of glucoamylase by 50% and reduces the total reaction time (Jensen and Norman, 1984). The enzyme preparation containing both glucoamylase and pullulanase are commercially available (OPTIMAX® from Genencor). The acid stable, Ca^{2+}-independent and thermostable amylopullulanase from extreme thermophile, *Geobacillus thermoleovorans* NP33 has potential application in the starch processing industry as these are thermostable and Ca^{2+}-independent with raw starch degrading ability (Nisha and Satyanarayana, 2013a, 2014). Because of the debranching

ability and debranching action of the enzymes, thermostable amylopullulanases can be employed as biocatalysts in one-step starch liquefaction-saccharification for producing different sugar syrups by replacing amylolytic enzymes like glucoamylase and α-amylases, thereby reducing the cost of sugar production. The *G. thermoleovorans* NP33 amylopullulanase pretreated raw starches have been saccharified to a greater extent by the glucoamylase in comparison to the starch saccharification achieved with the α-amylase pretreated starches and glucoamylase alone (Nisha and Satyanarayana, 2013a, 2014). An improved thermostability and catalytic efficiency achieved with the protein engineering effort via C-terminal truncation has enabled the efficient use of the enzyme in starch saccharification. An improved thermostability and catalytic efficiency achieved with the protein engineering effort via C-terminal truncation has enabled the efficient use of the enzyme in starch saccharification (Nisha and Satyanarayana, 2013a).

Preparation of maltooligosaccharides

Maltooligosaccharides are composed of glucopyranosyl units linked by an α-1,4 linkage, while the ones containing both α-1,4 and α-1,6 glucosidic linkages are called branched oligosaccharides or isomaltooligosaccharides. According to the International Union of Pure and Applied Chemistry, linear maltooligosaccharides are defined as polymers of monosaccharides with degree of polymerization between 3 and 10. The maltooligosaccharides are widely used in beverage, food and pharmaceutical industries (Fogarty and Kelly, 1990). The amylases that efficiently produce maltooligosaccharides have gained considerable attention in the past few decades. Maltooligosaccharides are characterized by high solubility, less sweetness, low calories, less water activity, good moisture level, acid resistance and heat stability. These can be made into various healthcare and nutritional foods including baby foods, confectionary items, healthcare candies, beverages, healthcare beers and ice creams. Maltotriose, maltotetraose and maltopentaose minimize retrogradation by inhibiting realigning of amylose and amylopectin chains, thus prevent loss of moisture from starch and interfere with starch–gluten interaction; these properties make them useful as antistaling agent in the bread industry (Min et al., 1998; Nagarajan et al., 2006; Plácido Moore et al., 2005).

Maltooligosaccharide syrups serve as the feed of high nutrition to infants and the aged (Stahl et al., 2013). Maltooligosaccharides come under soluble non-digestible carbohydrates (Roberfroid and Slavin, 2000), which stimulate the intestinal microbiota, especially the bifidobacteria and form fermentation products in the colon (Nakakuki, 2002; Gibson and Roberfroid, 1995). These non-digestible oligosaccharides are called prebiotics (Mussatto and Mancilha, 2007), and are categorized as functional food ingredients which can escape from low pH stomach fluids and digestive enzymes, and are metabolized in large bowels. Maltooligosaccharides can be gradually absorbed in human body, and thus, provide energy continuously for a long period. Furthermore, maltose and maltooligosaccharides have low calories, thus regulating blood glucose levels, and decreasing post-prandial blood glucose levels (Kayode et al., 2009). Therefore, it is beneficial for the cardiovascular, diabetes and obese patients. In addition, maltooligosaccharides provide increased body endurance, immunity and induce good therapeutic effect to persons with hypoglycemia and brain disorder associated with overconsumption of energy (Zhu et al., 2011). Maltooligosaccharides are commercially available as Fuji Oligo syrups, a product of Nihon Shokuhin Kako Kogyo Kabushiki Kasha (Tokyo, Japan).

Antistaling agent in making of bread and other baked products

The amylases can be employed in the bread industry as an antistale. Staling refers to all the undesirable changes such as loss of the moisture content in the bread crumb, reduced crispness of the crust and diminished bread flavor that occurs upon storage of bread. Staling occurs because of retrogradation of the amylopectin of starch (Kulp and Ponte, 1981; Champenois et al., 1999). An antistaling agent acts by shortening the amylopectin chain. In making bread, *A. oryzae* α-amylase, for instance, is added to dough to degrade damaged starch into dextrin, which is utilized by yeast during fermentation. The increase in fermentable sugars eventually results in an increase in loaf volume. Another role of added amylase is to produce surplus amounts of maltose in the dough, some of which lasts after baking and prevents baked bread from becoming stale by interfering with the retrogradation of starch molecules on the shelf. For this purpose, plant β-amylase or maltogenic α-amylase (EC 3.2.1.133) from *B. stearothermophilus* are widely used.

The α-amylase presently used in making bread results in sticky bread when added in excess because of the production of branched maltodextrins (De Stefanis and Turner, 1981). The *G. thermoleovorans* NP33 amylopullulanase has been reported to act as an antistale, which in turn improves the shelf life of bread and other baked products (Nisha and Satyanarayana, 2014). The debranching action of amylopullulanase enables the hydrolysis of the branched maltodextrins, thereby eliminating the stickiness of the α-amylase treated bread and other bakery products. The supplementation of whole wheat dough with the *G. thermoleovorans* NP33 amylopullulanase markedly improves the quality of the bread by enhancing the shelf-life, texture of bread and the loaf volume (Nisha and Satyanarayana, 2014). Moreover the maltooligosaccharides, generated in the bread using enzymes, will act as prebiotics in ameliorating human health.

Detergent applications

The alkalitolerant amylopullulanase of *G. thermoleovorans* NP33 finds application as a detergent additive (Nisha and Satyanarayana, 2014). The enzyme exhibits significant stability in different commercially available detergents and efficiently removes the starch stain as revealed from the wash performance analysis of the stained cotton fabric. An increase in reflectance, when washed with the combination of enzyme and the detergent as compared to the detergent alone, suggested the potential use of the enzyme as detergent additive.

Preparation of cyclodextrins

Cyclodextrins are cyclic oligosaccharides with six to eight α-1, 4-linked glucose units. CDs comprising six, seven, and eight α-D-glucose residues are called α-, β- and γ-CD, respectively. CDs can form an inclusion complex with hydrophobic compounds with suitable molecular sizes and are being used in the food, pharmaceutical, cosmetic and plastic industries as emulsifiers, stabilizing agents and antioxidants. An inclusion complex with a volatile compound (allyl isothiocyanate) of wasabi (a Japanese spice) is widely used as a food ingredient to keep the volatile compound from evaporating while serving on the table. The annual production of CDs is around 2000 tons. For the industrial production of CDs, a gelatinized starch solution is incubated with cyclodextrin glucanotransferase (CGTase) from *B. macerans* in a membrane reactor using intramolecular transglycosylation (cyclization reaction). Ratios of α-, β-, and γ-CDs in the reaction product vary depending on the sources of CGTase to be used. Branched cyclodextrins possesses one or more saccharide chains such as maltose linked to cyclodextrins by anα-1, 6 bond (French et al., 1965). Maltosyl-CDs (CDs with maltose linked though the α-1,6 linkage) are industrially produced using a reverse reaction of thermostable pullulanase at a high concentration of maltose and CDs. Pullulanases are known to produce branched cyclodextrins. Branched cyclodextrins such as 6-*O*-α-maltosyl-CDs (G_2-CDs) and 6-*O*-α-D-glucosyl-CDs (G_I-CDs) are generally more soluble than linear cyclodextrins and thus result in more soluble inclusion complexes (Okada et al., 1988). The enzyme forms the condensation product of CDs with malto-oligosaccharides (Kitahata et al., 1987). Watanabe et al. (1997) used pullulanase from *Klebsiella pneumoniae* for condensation of α-CD and maltose (G_2) by reverse synthesis and the hydrolysis of the resulting Q-*O*-α-maltosyl-α-CD (G_2-α-CD) by glucoamylase, which formed 6-*O*-α-maltosyl-CDs (G_2-CDs) and 6-*O*-α-D-glucosyl-CDs (G_I-CDs).

Preparation of resistant starch

Pullulanases have been used for improving resistant starch content (Vatanasuchet et al., 2010; Perera et al., 2010; Zhang and Jin, 2011). Resistant starch forms because of reversion of gelatinized starch to a form highly resistant to α-amylase hydrolysis (Annison and Topping, 1994).The resistant starch is fermented in the large intestine by the gut microbiota and escapes digestion in the small intestine. The resistant starches have gained importance in the health care sector as these produce short-chain fatty acids, and low levels of plasma glucose and insulin (Mun and Shin, 2006). Resistant starch functions as dietary and functional fibre based on whether these occur naturally in foods or are added. An increased ratio of the amylose/amylopectin is the most important factor in the resistant starch preparation. The linear amylose chains condense to form double helices after gelatinization as a result of its retrogradation and make the α-1, 4-glucosidic linkages accessible to amylase.

Preparation of panose and isopanose containing syrups

The pullulan hydrolase is employed for the preparation of panose and/or isopanose containing sugar syrups. These syrups act as proliferating agents of the intestinal *Bifibacterium* and *Lactobacillus* species and are commercially used as functional foods. The panose and isopanose containing sugar syrups are being used as dietary supplements in Japan and USA (Machida et al., 1991). As compared to other prebiotic sugar syrups (lactulose), these selectively stimulate the proliferation of *Bifidobacterium* and prevent the growth of other microbes which produce harmful substances like amines and ammonia in the intestines. These are also known to display anticariogenic activity.

Application of Amylases in Non-Food Industries

Amylase preparations, together with other hydrolases such as β-glucanases, xylanases, and proteinases, are added to animal feed to increase the absorption efficiency of nutrients. These enzymes need to be thermostable because the feed is usually heated during manufacturing. In textile weaving, starch paste (gelatinized starch) is usually used as a sizer to provide strength to textiles. The starch must be removed from the textile before the next process, such as dyeing. Amylases facilitate the process of desizing (removal of sizing materials) without affecting the fabric quality. *B. subtilis*α-amylase is widely used for this purpose. For example, sized cloth is steeped in an α-amylase solution (15,000–40,000 U l^{-1}) at 70–80°C for less than 1 min and then the enzyme and hydrolyzed products are removed by washing the cloth with hot and cold water successively. The use of hydrolases in detergents to remove stains on cloth and tableware is the biggest application field for bacterial enzymes. α-Amylases are added together with other hydrolases, such as proteinase, lipase, and cellulase, to hydrolyze starchy stains. Enzymes stable under alkaline conditions and under the presence of surfactants have been developed for this purpose. Most papers are coated with starch granules to improve their strength, stiffness, and erasability. Starch suspensions must have sufficient viscosity in the coating process, which can be achieved by adding amylase to this suspension. Because of the explosive increase in oil prices, there is a strong demand for petroleum substitutes worldwide. Increasing amounts of bioethanol are produced from starch or sucrose to meet these demands. *Saccharomyces cerevisiae* and *Zymomonas mobilis* (ethanol fermenting microbes) do not possess enzymatic system for the complete hydrolysis of starch into simple sugars, thus, they are not capable of converting starch into ethanol directly. Starch, therefore, needs to be hydrolyzed enzymatically using α-amylase and glucoamylase into simple sugars (Uma Maheswar Rao and Satyanarayana, 2007).

Discovery and Application of New Enzymes

The discovery of microorganisms (or enzymes) that convert starch directly into trehalose, by Hayashibara Biochemicals in 1990, started the industrial production of trehalose. In 2006, the annual production of trehalose reached 30,000 tons; its main

application is to improve the quality of food. Besides protecting the retrogradation of gelatinized starch, this sugar is known to have unique chemical and physiological functions. Several microorganisms, such as *Arthrobacter* sp. and *Brevibacterium helvolum*, produce two unique enzymes responsible for this conversion: maltooligosyltrehalose synthase (4-α-glucan 1-α-D-glucosylmutase, EC 5.4.99.15) and maltooligosyltrehalosetrehalohydrolase (4-α-D-glucanotrehalosetrehalohydrolase, EC 3.2.1.141), as described above.

A series of new enzymes or new actions of known enzymes, discovered recently, act on starch and produce various types of cyclic glucans (Fig. 8). Cyclic nigerosyl α-1,6-nigerose (Fig. 8(a)) is produced from starch by the cooperative action of two transferases produced by *Bacillus globiformis*: 6-glucosyltransferase (EC 2.4.1.24) and 3-isomaltosyltransferase (EC 2.4.1.–). The first enzyme is responsible for the formation of isomaltosyl residue, and the second enzyme catalyzes the formation of the α-1,3 linkage and cyclization. Another cyclic tetrasaccharide, maltosyl α-1,6-maltose (Fig. 8(b)), is produced from starch by the action of an enzyme obtained from *Arthrobacter globiformis*. This single enzyme was found to catalyze both intermolecular maltosyl transfer and intramolecular tetrasaccharide transfer. Isocyclicmaltopentaose, which has one α-1,6 linkage and four α-1,4 linkages (Fig. 8(c)), is produced from starch by a unique transferase from *B. circulans*. The enzyme isocyclomaltooligosaccharide glucanotransferase catalyzes the disproportionation reaction of maltooligosaccharides and intramolecular transfer reactions such as CGTase. However, differently from CGTase, this enzyme transfers the cleaved oligosaccharides to the α-1,6- hydroxyl group. Cycloamyloses (Fig. 8(d)), which have DPs of 17 or higher, are produced from starch by the newly discovered intramolecular transfer reactions of *T. aquaticus* amylomaltase (4-α-glucanotransferase, EC 2.4.1.25) or by a glycogen-debranching enzyme from *S. cerevisiae*, which is known to have both transferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33) activities. Highly branched cyclic dextrins (Fig. 8(e)) with narrow molecular weight distributions are produced from amylopectin by an intramolecular transfer reaction of the unique branching enzyme (EC 2.4.1.18) from *B. stearothermophilus*. Most of these cyclic sugars are commercially produced in Japan.

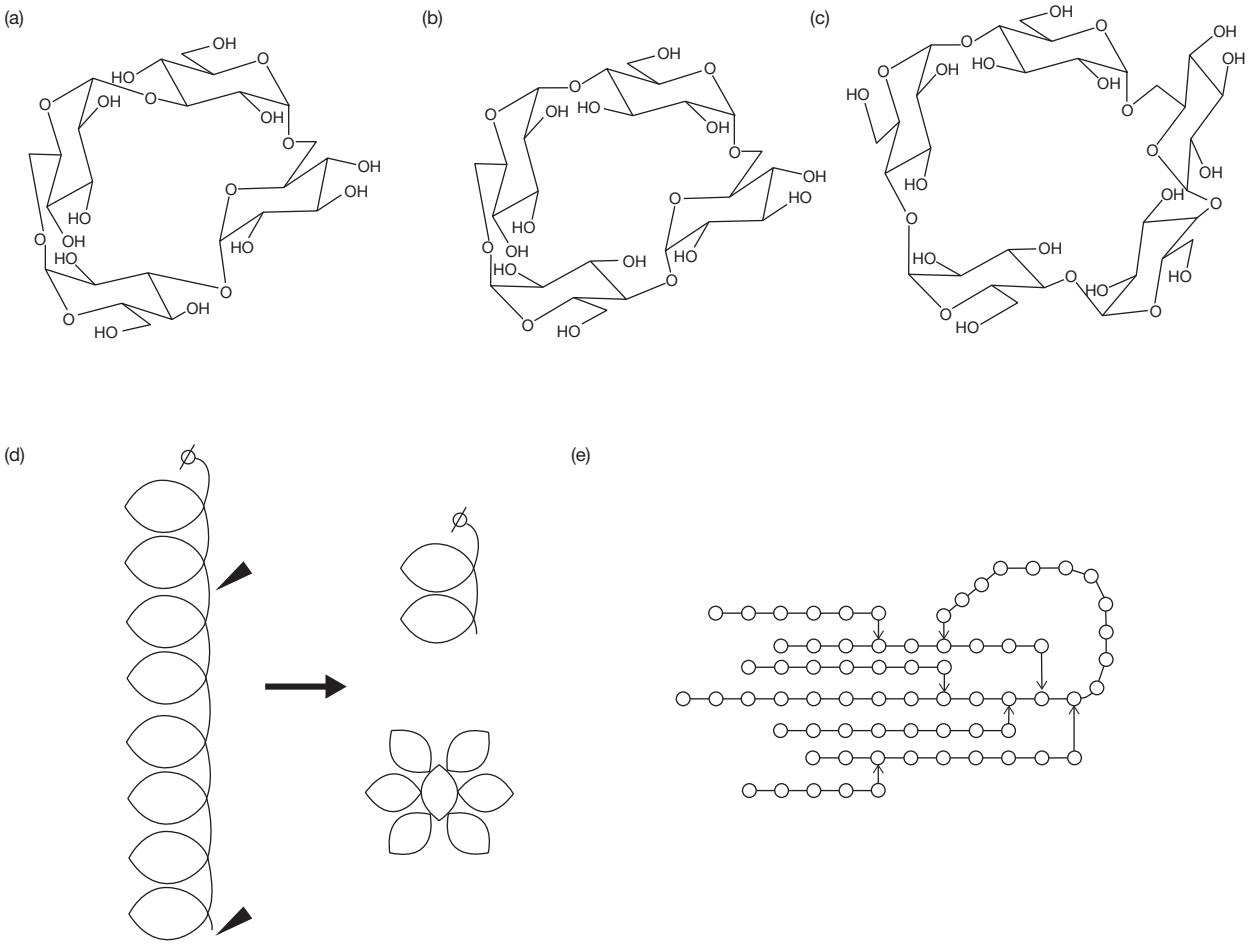

Fig. 8 Newly developed cyclic glucans. (a) Cyclic nigerosyl-α-1,6-nigerose. (b) Cyclic maltosyl α-1,6-maltose. (c) Isocyclicmaltopentaose. (d) Cycloamylose produced from amylose, α-1,4-glucan chain is expressed by a solid line. (e) Highly branched cyclic dexrin, α-1,4-glucan chain is expressed with circles with attached solid lines. Arrows indicate α-1,6-linked branching points.

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Antibiotic Resistance☆

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Glossary

Antibiotic molecule of microbial origin able to inhibit the growth or to kill other microorganisms.
Antimicrobial agent substance active against microorganisms but not obligatory of microbial origin. It could be synthetic, semi-synthetic or originate from plants or mammals.
Chromosome DNA molecule which contains all the genetic information necessary for the life of the bacterium. Most often double-standed, covalently closed circular, and self-replicating.
Conjugation unidirectional transfer of genetic information (in this chapter a plasmid) involving direct cellular contact between a donor (male) and a recipient (female).
Integron DNA element that acquire open-reading frames embedded in gene cassette units and convert them to functional genes by ensuring their correct expression.
Mutation any inheritable alteration of DNA.
Operon adjacent genes co-ordinately expressed.
Plasmid minichromosome encoding accessory genetic information, such as antibiotic resistance.
Replicon DNA molecule that can replicate autonomously (chromosome or plasmid)
Resistance when a strain can grow in the presence of higher concentrations of the antibiotic compared to other strains of the same species.
Transposon (transposable genetic element) DNA segment able to migrate from replicon to replicon (plasmid or chromosome) while retaining its physical integrity. A transposon can isert into non homologous DNA, exit, and relocate independently of the general recombination function of the host.

Resistance of bacteria to antibiotics, in particular multiple resistance, has become a major health problem worldwide. Antibiotics are defined as secondary metabolites produced by microorganisms in the environment (generally the soil) active against other microorganisms because of their interaction with, and inhibition of, a specific target. In this chapter, the term "antibiotic" is used to designate natural, but also semisynthetic (e.g. certain aminoglycosides) or entirely synthetic (e.g. quinolones) molecules with antibacterial activity.

Antibiotic Classes

Antibiotics are grouped in classes (or families) on the basis of their chemical structure for examples, ß-lactams (penicillins, cephalosporins), aminoglycosides (streptomycin, kanamycin, gentamicin), the tetracyclines, etc. As a consequence, members of a given class are closely related and generally share the same target in the cell and are thus substrates for the same mechanism of resistance. As we will see below, this implies that the reasoning in terms of resistance should be in classes rather than in individualized antibiotics.

The main classes of antibiotics act on four different targets (Table 1).

Inhibition of Cell Wall Biosynthesis

The cell wall protects prokaryotes from the environment and from osmolysis. The bacterial cell wall is characterized by the presence of a peptidoglycan located outside the cytoplasmic membrane; this peptidoglycan is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape. Synthesis of the cell wall requires several steps in the cytoplasm, such as synthesis of the muramyl pentapeptide, which is then translocated to the external face of the cell membrane by a carrier. Crosslinking by transglycosylases and transpeptidases are responsible for the complete synthesis of the peptidoglycan outside of the cell.

The β-lactam antibiotics, such as penicillins and cephalosporins, block the transpeptidation events by binding to the transpeptidases, also named penicillin-binding proteins (PBPs).

☆*Change History:* February 2015. Authorship was changed. Authors added new text to most sections and added new references.

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Table 1 Targets of the main classes of antimicrobial drugs

Cell wall synthesis	*Protein synthesis*	*DNA replication*	*Membrane*
Bacitracin	Aminoglycosides	Coumarines	Polymixins
β-lactams	Chloramphenicol	Quinolones	
Glycopeptides	Fusidic acid	Rifampin	
Fosfomycin	Ketolides	Sulphonamides	
	Lincosamides	Trimethoprim	
	Macrolides		
	Oxazolidinones		
	Streptogramins		
	Tetracyclines		

Glycopeptides act by binding, in a noncovalent fashion, to the C-terminal D-alanine-D-alanine dipeptide of the peptidoglycan precursors, preventing their incorporation into the growing wall.

Fosfomycin, an antibiotic active against both Gram-negative and Gram-positive bacteria, inhibits the activity of MurA, an enzyme implicated in the conversion of the nucleotide diphosphosugar UDP-*N*-acetylglucosamine to UDP-muramyl pentapeptide, by binding to its active-site cysteine and thus blocking the formation of muramyl pentapeptide. Consequently, the pool of peptidoglycan precursors is reduced.

Inhibition of Protein Biosynthesis

Bacterial ribosomes, which translate the mRNA in amino acid sequences, are constituted of two subunit nucleoprotein particles, named 50S and 30S. The large 50S subunit contains proteins and two rRNA, 23S and 5S, whereas the small 30S subunits are composed of proteins and of the 16S rRNA. The translation events start with the binding of mRNA to the 30S subunit. A formylmethionyl-tRNA is then attached to the peptidyl donor (P) site face of the AUG initiator codon. The 50S subunit is then added, and the adequat aminoacyl-tRNA enters the aminocyl receptor (A) site which is adjacent to the P site. A specific peptidyl transferase mediates a peptide bond between the *N*-formylmethionine and the adjacent amino acid.

Macrolides, such as erythromycin, bind to the 23S rRNA, near the peptidyl transferase center, block the entrance of the ribosomal tunnel, and thus stop the elongation of the peptide chain.

Tetracyclines bind in the vicinity of the A site of the 30S subunit and block the moving of the tRNA along the ribosome that impede the formation of the first peptide bond.

Chloramphenicol binds to the A site and prevents binding by tRNA.

Lincosamides (lincomycin and clindamycin), by interacting with both, the A-site and P-site, inhibit peptide bond formation.

The ribosome is also the specific target of aminoglycosides that act by causing translational errors and by inhibiting translocation.

Inhibition of DNA Replication

Topoisomerases are essential for cell viability. DNA gyrase is implicated in the control of DNA topology, in DNA replication, recombination and transcription. Topoisomerase IV is involved in DNA replication and decatenation of the chromosome. Interaction of quinolones with enzyme-bound DNA complexes is responsible for conformational changes and accumulation of complexes that could block the replication fork.

Rifampin is a RNA polymerase inhibitor that hinders protein transcription of DNA into mRNA.

Other Targets

Folic acid is an essential precursor in nucleic acid synthesis. Trimethoprim-sulfamethoxazole inhibit the folic acid metabolism pathway: the first molecule blocks the dihydrofolate reductase (DHFR), an essential enzyme for DNA synthesis, whereas the second blocks the dihydropteroate synthase.

Polymixins increase the permeabilty of the cell membrane.

Antibiotic Resistance

There are two major types of resistance to antibiotics: intrinsic and acquired.

Intrinsic Resistance

Intrinsic (or natural) resistance is present in all the bacteria of a given species or genus and could thus be better considered as insensitive rather than resistant. Intrinsic resistance delineates the spectrum of activity of an antibiotic. This type of resistance could

be the result of the physiological characteristics of the bacterial species or of the presence of a structural gene. Natural resistance is often due to (i) inaccessibility of the target by antibiotics, (ii) low affinity of the antibiotics for the target or (iii) absence of the target. For example, presence of an external membrane in Gram-negative bacilli (such as *Escherichia coli*) leads to resistance to various drug classes by impermeability (glycopeptides, macrolides, lincosamides, streptogramins, etc.).

Pseudomonas aeruginosa is a typical organism that exhibits a high broad substrate range intrinsic resistance resulting from a particularly low permeability of its outer-membrane associated with a number of endogeneous multidrug efflux systems (such as MexAB-OprM and MexXY-OprM) and a chromosomally encoded β-lactamase (AmpC).

Enterococcus faecium produces an intrinsic low-affinity penicillin-binding protein (PBP 5) responsible for high-level resistance to cephalosporins, oxacillin, and monobactams and for an increase in resistance to the penicillins and carbapenems. *Enterococcus* spp. are also intrinsically resistant to low-levels of aminoglycosides, due to inefficient uptake of this class of antibiotics. As already mentioned, glycopeptides, vancomycin and teicoplanin, inhibit cell wall synthesis in Gram-positive bacteria by binding to the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residues of late pentapeptide peptidoglycan precursors. *Enterococcus gallinarum* and *E. casseliflavus-E. flavescens* are intrinsically resistant to low-levels of glycopeptides by synthesis of modified peptidoglycan precursors ending in D-Ala-D-serine (D-Ala-D-Ser), for which glycopeptides have a low affinity, and elimination of the D-Ala-D-Ala ending precursors. These two concomittent events are due to a chromosomally-encoded *vanC* gene cluster.

Resistance to both β-lactams and cyclines in Mycobacteria is due to the combination of reduced permeability of the bacterial cell wall, presence of modifying enzymes, and low affinity for the target (such as PBP and DNA gyrase).

Acquired Antibiotic Resistance

Acquired resistance is present only in some strains of the same species or genus. In certain instances, it can be highly prevalent such as, for example, penicillinase production in staphylococci that is present in more than 90% of the strains. Intrinsic and acquired resistances do not differ in their mechanisms; both can employ the four major pathways depicted in Figure 1.

Mechanisms of Resistance

From a biochemical point of view, bacteria have developed four major mechanisms of resistance (Figure 1): (1) modification of the target; which leads to loss or decrease affinity of the drug for its target or synthesis of a new target; (2) production of an enzyme that will inactivate or modify the drug; (3) impermeability, in particular by loss of a porin (pore in the external membrane) or by diminution of its diameter in Gram-negative bacteria, and (4) efflux of antibiotics outside of the cells by energy dependent pumps. The common motif of these various mechanisms is to impede interaction of the antibiotic with its target.

Alteration or Synthesis of a New Target

A mechanism frequently used by bacteria to prevent the action of antimicrobial agents is the alteration of specific targets that have a necessary role in microbial growth. Target modification often results in decreased affinity for the drug. Enzymes involved in several steps of peptidoglycan synthesis, such as PBPs, or assembly represent targets of choice for antibiotics. Alteration of PBPs resulting in low-affinity for β-lactams or acquisition of new PBPs are responsible for β-lactams resistance. As an example, resistance to this antibiotic class in *Streptococcus pneumoniae* is mainly due to alteration of PBPs. *S. pneumoniae* has six PBPs (1a, 1b, 2a, 2b, 2x, and 3) in which point mutations could be responsible for β-lactam resistance in mutants obtained *in vitro*. In clinical isolates, resistance is

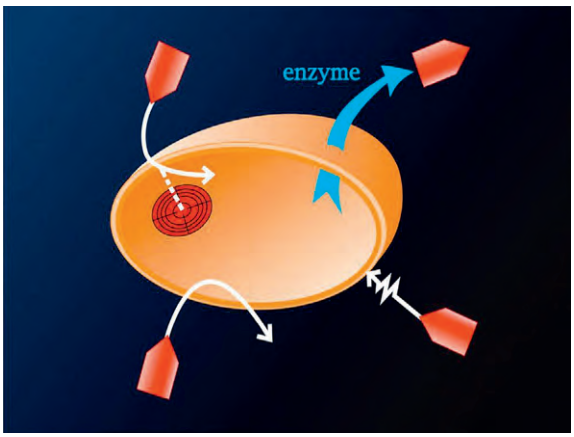

Figure 1 Major mechanisms of resistance to antibiotics. From top, counterclockwise: alteration of the target; production of an enzyme inactivating the drug; impermeability by mutation in a porin channel; impermeability by active efflux of the drug.

due to low-affinity variants of PBPs 2b, 2x, and 1a that are encoded by mosaic genes that result from DNA acquisition by transformation from related species of streptococci that are intrinsically less susceptible to β-lactams followed by homologous recombination. Genesis of these mosaic PBP genes is facilitated by the fact that *S. pneumoniae* is naturally transformable. Competence, which is the ability to take up DNA from the environment is due in *S. pneumoniae* to a specific protein, the competence-stimulating peptide (CSP), that acts as a pheromone. In response to a certain population density, the production of CSP is increased by the upregulation of the *comC* or *comA* gene. A typical example of a mosaic-PBP-mediated resistance is the mosaic PBP 2x which is the result of the replacement of a portion of PBP 2x of *S. pneumoniae* by the corresponding portion of the gene from *Streptococcus oralis* or *S. mitis*. This mosaic PBP 2x is more resistant to cefotaxime, despite the fact that the donor strain and the recipient *S. pneumoniae* were cefotaxime susceptible. Other mosaic structures responsible for synthesis of low-affinity PBP variants have been described (PBP 1a, 1b, 2a and 2b). Alteration of three or more PBPs are found in highly resistant isolates.

High-level resistance to methicillin and all other β-lactams in *Staphylococcus aureus* (Methicillin Resistant *S. aureus* strains, MRSA) is due to acquisition of a gene, *mecA*, responsible for synthesis of a new PBP, PBP2a, with reduced affinity for β-lactams. In the absence of *mecA*, low-level resistance could be due to overproduction of PBP4 or to modification of PBP2.

Acquired resistance to glycopeptides in *E. faecium* and *E. faecalis* detected in 1986 is due to acquisition of operons encode enzymes producing modified peptidoglycan precursors terminating in D-Ala-D-lactate (D-Ala-D-Lac) (VanA, VanB, VanD, VanM, and VanO-type resistance) or in D-Ala-D-Ser (VanC, VanE, VanG, VanL, and VanN-type). Interestingly, the base composition mol% G+C of the genes in the *van* operons suggest that these clusters are composed of genes from various sources (Figure 2). The first clinical isolates of MRSA that have acquired a *vanA* gene cluster were detected in 2002. Vancomycin resistance in vancomycin-intermediate *S. aureus* (VISA strains) is not due to acquisition of a *van* gene cluster but to synthesis of a thicker cell wall that traps vancomycin, leading to a reduced number of molecules that reach the transglycosylase targets located in the cytoplasmic membrane.

Since most ribosome-targeting antibiotics interact with rRNA, nucleotide mutation of rRNA can lead to resistance by alteration of the conformation of the drug-binding site. Due to the presence of multiple copies of rRNA operons in most bacteria, high-level resistance requires mutation in most of these operons.

The macrolide, lincosamide and streptogramin B antibiotics act by binding to the 50S ribosomal subunit and thus prevent protein synthesis. Resistance to these molecules is due to the methylation of an adenine residue (A2058 in *Escherichia coli*) in 23S rRNA by a methyltransferase specified by an *erm* (erythromycin ribosome methylation) gene. Addition of a methyl group reduces the affinity of the rRNA for these three groups of antibiotics that have very different structures. Enzymatic methylation of 16S rRNA by other methylases such as those of the Rmt family (RmtA, B, C, D, D2, E, F, G, and H), NpmA, or ArmA confer high level resistance to aminoglycosides. Rmt enzymes and ArmA methylate residue G1405 and confer resistance to the 4,6-disubstituted 2-deoxystreptamine (DOS), such as amikacin, tobramycin, arbekacin, and gentamicin, whereas NpmA methylates residue A1408 and confer resistance to the 4,6-disubstituted DOS and to the 4,5-disubstituted DOS, such as neomycin, and apramycin. RmtB and ArmA seem to be the predominant 16SrRNA methyltransferases.

Monomethylation of A2503 of the 23S rRNA confers resistance to chloramphenicol, lincosamides, oxazolidinones, some macrolides, and streptogramine A antibiotics.

Mutations in ribosomal proteins are also responsible for antibiotic resistance. For example, macrolide resistance could be due to mutations of L22 protein.

Alteration of the targets of fluoroquinolones type II topoisomerases, DNA gyrase and topoisomerase IV, constitute the main mechanism of resistance to these antimicrobial agents. These two enzymes, implicated in bacterial DNA synthesis, are composed of two subunits (GyrA and GyrB for DNA gyrase and ParC and ParE for topoisomerase IV). Mutations in a specific region of these subunits, the QRDR (Quinolone Resistance Determining Region), prevent the fixation of the quinolones on the DNA-enzyme complex. The levels of resistance conferred by mutations in the subunits of DNA gyrase or topoisomerase IV depend both on the bacterial species and the quinolone. The primary target is the DNA gyrase in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria. Mutations in the GyrA or ParC subunits are more common than mutations in GyrB or ParE. Another resistance mechanism to fluoroquinolones is mediated by the plasmid-borne *qnr* (quinolone resistance) gene. The Qnr gene product, which belongs to the pentapeptide repeat family, protects gyrase and topoisomerase IV from quinolone inhibition by binding these enzymes directly. To date, three types of *qnr* genes have been described, *qnrA*, *qnrB*, and *qnrS*. Within each type, several variants have been reported.

Antibiotics of the rifamycin family, such as rifampin, interact with the β subunit of RNA polymerase, which is encoded by the *rpoB* gene, and block transcription initiation. Mutations, including point mutations, insertions, and deletions, responsible for rifampin resistance are located in highly conserved regions, notably between the residues 507–534. Mutations in *rpoB* have been detected in mycobacteria, *S. pneumoniae*, *S. aureus*, and *Neisseria meningitidis*.

Mutations in the *dhfr* gene, encoding a dihydrofolate reductase, are responsible for trimethoprim resistance in staphylococci.

Alteration of other targets, such as iso-leucyl-tRNA synthetase, elongation factor G (EF-G), or NADH-enoyl-ACP-reductase/β-ketoacyl-ACP-synthase are responsible for resistance to mupirocin, fusidic acid, and isoniazid, respectively.

Enzymatic Modification

This is a major mechanism of resistance to antibiotics such as β-lactams, macrolides, aminoglycosides, and chloramphenicol.

The most important antibiotic resistance mechanism in Gram-negative bacteria is due to β-lactamases that hydrolyze the four-membered β-lactam ring in penicillins, cephalosporins, carbapenems, and monobactams. The enzymes can be classified in a

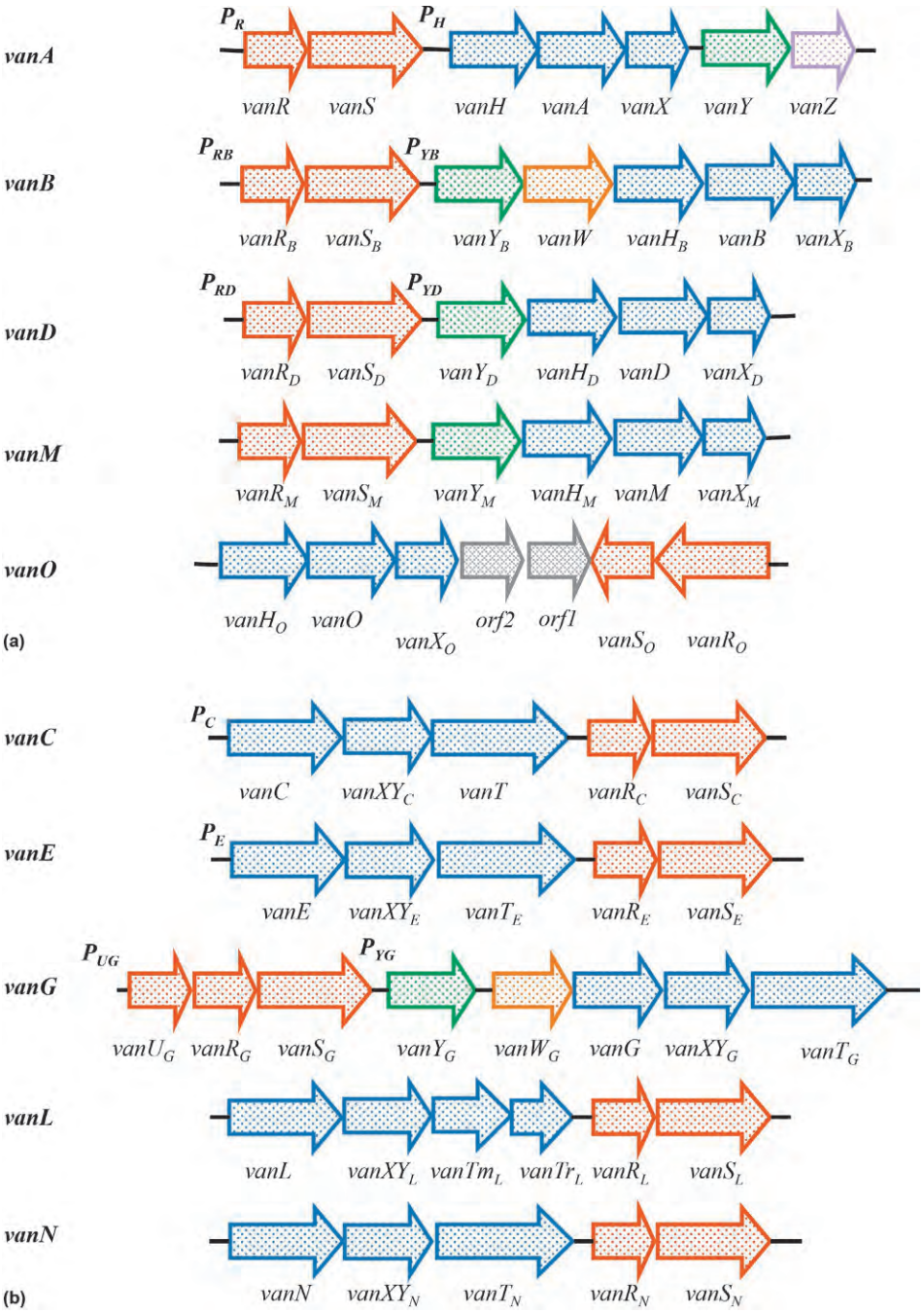

Figure 2 Comparison of the *van* gene clusters. Comparison of the D-Ala-D-Lac (A) and DAla-D-Ser (B) type *van* operons. Arrows indicate coding sequences and sens of transcription.

number of ways, such as by their amino acid sequences or by their enzymatic activity spectrum. In the latter classification, four groups have been defined: : group 1, cephalosporinases on which the β-lactamase inhibitor clavulanic acid has a weak activity; group 2, penicillinases sensitive to clavulanic acid and extended spectrum β-lactamases; group 3, metallo-β-lactamases; and group 4, other β-lactamases weakly sensitive to clavulanic acid.

The macrolide esterases, such as EreA and EreB, inactivate macrolides by cleaving the macrocycle ester.

The transferase group (phospho-, nucleotidyl-, acetyl-, thiol-, ADP-ribosyl, and glycosyl-transferases) constitute a large family of modifying enzymes. The phosphotransferases catalyze the transfer of a phosphate group (generally from ATP) to a substrate. The aminoglycoside phospho-transferases (APH) confer a higher level of resistance than aminoglycoside acetyl-transferases (AAC) or aminoglycoside nucleotidyl-transferases (ANT). Depending on the aminoglycoside modification, more than fifty antibiotic-inactivating enzymes have been reported. Furthermore, various aminoglycoside inactivating enzymes can be present in the same

host. A remarkable example of this co-existence is the bifunctional enzyme AAC(6′)-APH(2″), which is the fusion product of two genes and possesses an acetyl and a phosphatase activity in the same protein. This enzyme is responsible for high-level resistance in Gram-positive bacteria to all the aminoglycosides, except streptomycin and spectinomycin. Resistance to chloramphenicol is due to a large variety of chloramphenicol acetyltransferases, which are widely distributed among bacterial pathogens of all genera. Fosfomycin is modified by thiol transferases such as FosA, FosB, or FosX. FosA, found in Gram-negative bacteria, is present on the chromosome of *P. aeruginosa* whereas FosB is found in Gram-positive bacteria and, notably, on the chromosome of *Bacillus subtilis*. Three genes, *mph*(*A*), *mph*(*B*), and *mph*(*D*), encoding macrolide 2′- phosphotransferases have been reported in *E. coli* and *Pseudomonas*.

Reduced Uptake of Antibiotics

Gram-negative bacteria possess an outer membrane (OM) external to the peptidoglycan and composed of the lipopolysaccharide (LPS) and phospholipids. This OM functions as an effective barrier, the LPS being responsible for impermeability of the OM to many molecules. Thus, some outer membran proteins (Omp), also called porins and acting as aqueous channels, are used by several antibiotics, such β-lactams, chloramphenicol, or fluoroquinolones, to permeate the OM. Resistance to these families of antibiotics could be due to the diminution of the porin copy number or reduction in the size of the pore.

The outer membrane of *P. aeruginosa* has a very low permeability to small hydrophilic molecules, allowing resistance of this organism to fluoroquinolones. Resistance to imipenem in *P. aeruginosa* is caused by a loss of the OprD porin in response to exposure to this antibiotic. Other antibiotic resistances associated with loss of a porin have been documented in *S. marcescens*, *E. coli*, *Enterobacter aerogenes*, *E. cloacae*, *K. pneumoniae* . . .

Increased Efflux of Antibiotics

Chromosomally encoded efflux pump could be responsible for intrinsic resistance to different class of antibiotics, initially suspected to be the effect of the OM, and consequently of reduced permeability. The widespread active export or efflux of antibiotics outside bacteria limits intracellular accumulation of toxic compounds such as antibiotics. This mechanism is mediated by the activity of membrane-based efflux proteins acting as pumps. Efflux pumps are present in the chromosome of all organisms and may have a narrow (such as tetracycline pumps) or a broad specificity, the latter conferring a multidrug resistance (MDR) phenotype to chemically and structurally unrelated compounds. Generally, drug-specific efflux pumps are encoded by mobile genetic elements, whereas MDR efflux pumps are specified by the chromosome. To date, five families of MDR efflux systems have been described: the Major Facilitator Superfamily (MFS), the ATP-binding cassette (ABC), the Resistance-Nodule-cell Division (RND), the Small Multidrug Resistance (SMR) and the Multidrug And Toxic compound Extrusion (MATE) (Table 2).

Drug efflux systems act in an energy-dependent manner by using ATP hydrolysis (ABC) or an ion antiport mechanism (MFS, RND, SMR, and MATE). The expression of multidrug transporters is commonly controlled by specific regulatory proteins. Antibiotic resistance in an efflux mutant is due to overexpression of an endogeneous pump or to a mutation in a protein pump that enhance the potential of export of this protein.

As opposed to ABC transporter that usually mediates the export of specific antimicrobial classes, the MFS, RND, SMR, and MATE pumps, also designed as secondary drug transporters, are generally responsible for resistance to multiple antimicrobial agents.

Efflux pump specific for one substrate

Tetracyclines inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. Tetracycline-specific efflux pumps, which are members of the MFS family, are found in both pathogenic Gram–negative and –positive bacteria. The *tet* efflux genes code for membrane-associated proteins that reduce the intracellular drug concentration and thus protect the ribosome. Most of the *tet* determinants are found on mobile elements.

Macrolides also inhibit bacterial growth by binding to ribosomes. Efflux is also implicated in macrolide resistance. The *mef* genes, initially described in *S. pneumoniae* and *S. pyogenes*, are implicated in the specific efflux of 14- and 15-membered macrolides.

Table 2 Typical substrates of the five classes of antibiotic efflux pumps

MFS	*RND*	*SMR*	*MATE*	*ABC*
Aminoglycosides	Aminoglycosides	Aminoglycosides	Aminoglycosides	Aminoglycosides
Chloramphenicol	β-lactams	Chloramphenicol	Fluoroquinolones	Chloramphenicol
Erythromycin	Chloramphenicol	Erythromycin		β-lactams
Fluoroquinolones	Erythromycin	Tetracyclines		Erythromycin
Lincosamides	Fluoroquinolones			Fluoroquinolones
Novobiocin	Novobiocin			Lincosamides
Rifampin	Rifampin			Macrolides
Tetracyclines	Tetracyclines			Novobiocin
	Trimethoprim			Tetracyclines

The *msr*(*A*)/*msr*(*B*) and *msr*(*C*) genes, detected in *Staphylococcus* spp. and *E. faecium*, respectively, are responsible for macrolide/streptogramin efflux.

The *cmlA* genes, which are also widespread among Gram-negative bacteria, encode exporters of the MFS family that confer chloramphenicol resistance.

Efflux pump associated with multiple drug resistance

Major facilitator superfamily

Overproduction of NorA, a chromosomally-encoded protein of the MFS family, is responsible for quinolone resistance in *S. aureus*. NorA is homologous to Bmr of *Bacillus subtilis* and PmrA of *S. pneumoniae*, the latter being responsible for an increase of the norfloxacin MIC. A fluoroquinolone efflux gene, named *qepA* and located on a plasmid detected in *E. coli*, specifies an MFS transporter that confers low level of resistance to hydrophilic quinolones norfloxacin and ciprofloxacin.

An efflux pump, Tap, conferring resistance to aminoglycosides and the tetracyclines, has been detected in mycobacteria, such as *Mycobacterium fortuitum* and *M. tuberculosis*.

The chromosomal-encoded effux pump EmrAB confers in *E. coli* resistance to nalidixic acid and to other toxic compounds. An homologous pump, VceAB, was found in *Vibrio cholerae*, a Gram-negative enteric pathogen.

An endogenous gene of *Listeria monocytogenes*, *lde*, encodes a protein of the MFS family is responsible for fluoroquinolone resistance.

Multidrug and toxic compound extrusion

Two homologous pumps, NorM (*V. parahaemolyticus*) and YdhE (*E. coli*), are implicated in fluoroquinolone and aminoglycoside resistance.

Small multidrug resistance

Substrates of these smallest multidrug resistance pumps, detected in *S. aureus* (Smr), *E. coli* (EmrE), include disinfectants and antiseptics. Genes coding for QacE and QacEΔ1, responsible for quaternary ammonium compounds resistance, are found in Gram-negative and in both Gram-negative and –positive bacteria, respectively, and are located in integrons.

ATP-binding cassette

Most bacterial ABC drug transporters are implicated in the export of specific antibiotics and have been described in various species. Expression of *lmrA* from *Lactococcus lactis* in *E. coli* is responsible for resistance to aminoglycosides, lincosamides, macrolides, quinolones, streptogramins, tetracyclines, and chloramphenicol. Similarly, the *E. faecalis efrAB* gene is responsible for norfloxacin and ciprofloxacin resistance; expression of *vcaM* of *Vibrio cholerae* renders bacteria resistant to tetracycline, norfloxacin and ciprofloxacin.

Resistance-nodule cell division

The RND family constitutes the most important multidrug efflux systems for clinically important antimicrobials. RND systems, composed of an inner membrane protein (RND pump) linked by a membrane fusion protein (MFP) to an outer membrane factor (OMF), are able to extrude a wide range of substrates, often unrelated in structure, from the cytoplasm to the environment.

In addition to intrinsic resistance by impermeability, *P. aeruginosa* also expresses efflux systems of the RND family. As already mentioned, some of them (such as MexAB-OprM or MexXY-OprM), participate in the intrinsic resistance. MexAB-OprM contributes to resistance to quinolones, chloramphenicol, β-lactams, novobiocin, trimethoprim, macrolides, tetracycline, and the biocide triclosan. The MexCD-OprJ system is implicated in fluoroquinolone resistance but also accomodates β-lactams, chloramphenicol, macrolides, tetracycline and trimethoprim.

In *E. coli*, the AcrAB-TolC system is homologous to MexAB-OprM. Substrates of AcrAB-TolC include macrolides, chloramphenicol, fluoroquinolones, tetracycline, rifampin, lipophilic β-lactams, fusidic acid, ethidium bromide and triclosan. AcrD and AcrEF also encode efflux pumps. AcrA, B, D, and F are also present in the *Salmonella enterica* genome.

In *Acinetobacter baumannii*, overexpression of AdeABC, a RND tripartite efflux pump, is responsible for resistance to aminoglycosides and decreased susceptibility to chloramphenicol, fluoroquinolones, erythromycin, tetracycline, trimethoprim, meropenem, and the dye ethidium bromide. Expression of the *adeABC* operon is regulated by a two-component regulatory system, *adeRS*. Mutations in *adeR* or *adeS* are responsible for the constitutive expression of the AdeABC pump, which is otherwise cryptic in wild-type *A. baumannii*. Two other efflux systems have been described in *A. baumannii*. AdeIJK contributes to intrinsic resistance to β-lactams, fluoroquinolones, tetracyclines, tigecycline, lincosamides, rifampin, chloramphenicol, cotrimoxazole, novobiocin, and fusidic acid and is regulated by AdeN, a TetR-type protein. AdeFGH confers, when overexpressed, high-level resistance to fluoroquinolones, chloramphenicol, trimethoprim, and clindamycin and decreased susceptibility to tetracyclines, tigecycline, and sulfamethoxazole. AdeFGH is regulated by a LysR-type transcriptional regulator, AdeL.

Active efflux is generally responsible for low-level antibiotic resistance. High-level of resistance is most often due to the combined effect of the impermeability of the outer membrane and of the active efflux of the antibiotics. However, intrinsic resistance is not only due to these two mechanisms. It has been demonstrated that in certain pathogens, such as *Acinetobacter baylyi*, *P. aeruginosa*, *E. coli*, and *S. aureus*, the intrinsic resistance could be associated with other chromosomally encoded elements implicated in various cellular functions.

Acquisition of Resistance

On a genetic point of view, resistance can be acquired by two totally distinct events. Either occurrence of a mutation in the genome leading to vertical inheritance of resistance to the progeny of the bacterium or acquisition of foreign genetic information, from other bacteria, by horizontal transfer.

There is a multiplicity of definitions of resistance and we have already considered the intrinsic and acquired types. Genetic resistance when the daughter cell differs from the parental cell by a genetic event (following a mutation or horizontal acquisition of genetic information). Biochemical, in bacteria that differ by the presence or the absence of a resistance mechanism. Microbiologic, when a strain can tolerate a significantly higher concentration of antibiotic (generally expressed as the Minimal Inhibitory Concentration, MIC in mg/L). Clinical, which is based on the clinical outcome: success or failure of antibiotic therapy in a patient suffering from a bacterial infection. For the sake of clarity, we will consider only the genetic dimension of resistance; in other words, the resistant bacterium (daughter cell) has suffered a genetic alteration relative to its parent (mother cell).

Biochemistry of Resistance

Cross-Resistance

Cross-resistance corresponds to resistance to all the antibiotics belonging to the same class due to a single mechanism. As we have seen above, drugs assigned to a same class are chemically related, have thus the same target of action in the cell, and are therefore subject to cross-resistance: bacteria that are resistant to one member of the class are generally resistant to the other members. However, there are degrees in cross-resistance: the more active the drug, the lower level of resistance. In general, drugs recently developed are more active than old molecules of the same class. For example, among quinolones, ciprofloxacin is much more active than nalidixic acid. As a consequence, Gram-negative bacteria that have suffered a mutational event in the target of quinolones, the type II topoisomerases (DNA gyrase and topoisomerase IV) become much more resistant to nalidixic acid (that has high MICs) than to ciprofloxacin (which retains lower MICs). This observation stresses that a resistance mechanism has no absolute value. The level of resistance also depends on the degree of susceptibility of the host bacterium. Resistance by a given mechanism will be much higher if the bacterial species is poorly susceptible. For example, the same mechanism will confer to *Pseudomonas aeruginosa*, a species naturally poorly susceptible to antibiotics, a resistance level much higher than in *Neisseria meningitidis*, a species exquisitely susceptible to drugs. Importantly, cross-resistance implies cross-selection: use of a given antibiotic can select resistance to other members of the class but not to drugs belonging to other classes.

Co-Resistance

In co-resistance, various mechanisms are associated in the same bacterial host, sometimes stabilized by integration into the genome. Each confers (by cross-resistance) resistance to a class of antibiotics which, *in fine*, can result in a broad spectrum of resistance (multidrug resistance). Again, the consequence of co-resistance is co-selection. Use of a member of a drug class can co-select resistance to another class of antibiotics with a totally distinct mode of action. This is, for example, the case for *Streptococcus pneumoniae* (Table 3).

In France in 1999, among clinical isolates of pneumococci, 46% of the strains were susceptible to penicillin G whereas 54% were resistant to this antibiotic. If one compares the rates of resistance of these two groups of bacteria to other drug classes (two top lines of Table 3), it is apparent that the strains resistant to penicillin G are much more often resistant to the other classes of antibiotics. If now one considers exclusively the penicillin G resistant strains (considered as 100%, left column in Table 3), one realises that resistance to other classes of antibiotics is extremely high. For example, administration of the combination trimethroprim-sulfamethoxazole (Bactrim®, the antibiotic most prescribed worldwide) has 88% chances to co-select a pneumococcus strain resistant to penicillin G, although these two drugs have totally different structures and targets of action.

Table 3 Antibiotic resistance in *S. pneumoniae*

	Resistant to (%)				
S. pneumoniae *(%)*	*PenG*	*Em*	*Cm*	*Tc*	*Tp-Su*
PenS (46%)	0	20	14	15	10
Pen^R (54%)	100	80	38	51	66
Em^R	82	100			
Cm^R	77		100		
Tc^R	80			100	
Tp-Su^R	88				100

Cm, chloramphenicol; Em, erythromycin; PenG, penicillin G; R, resistant; S, susceptible; Su, sulfonamides; Tc, tetracycline; Tp-Su, trimethoprim-sulfamethoxazol (Bactrim).

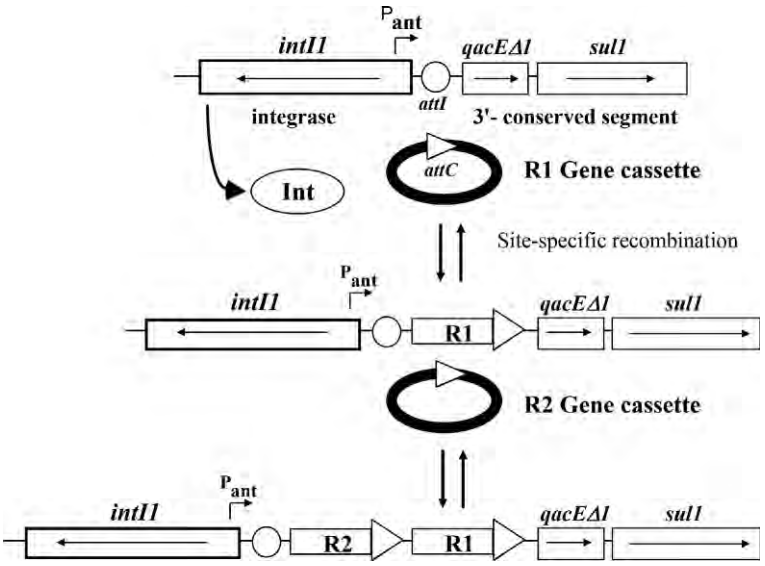

Figure 3 Model for site-specific gene cassette integration-excision in an integron. *attI*, Attachement site of the integron; *attC*, attachment site of the cassette; *intI1*, gene for the integrase; Int, integrase; P_{ANT}, outward-oriented promoter for the cassettes. Horizontal arrows indicate sense of transcription (after D. Mazel).

Integrons are a most efficient way to achieve co-resistance (Figure 3). These elements, that represent a very elegant genetic system for capture and expression of resistance genes, are not mobile and spread using mobile elements. Integrons, that can be located either in the chromosome or in plasmids, are composed of genes that have been acquired by site-specific recombination. They possess the machinery necessary to capture exogeneous genes: an integrase (*intI*) that allows recombination of circularized DNA (gene cassettes), a recombination site (*attI*), and a promoter (P_{ant}) that directs transcription of the captured genes. The resistance gene cassettes inserted in the integron contain a single gene and, downstream from it, a specific *attC* site, which is an imperfect inverted repeat. In presence of the integron-encoded integrase, a gene cassette containing *attC* inserts by site-specific recombination at the *attI* site and the gene is transcribed from the P_{ant} promoter. After integration of a gene cassette, another one can be inserted at the *attI* site. This integrative process is reversible. There is a clear relationship between the position of a cassette in the integron and the level of resistance: the closer of Pant, the higher level of expression of the resistance gene. To date, five classes of integrons implicated in the dissemination of antibiotic-resistance genes have been reported. Class 1 integrons, in which most of the antibiotic-resistance-gene cassettes can be found, have been detected in many Gram-negative and, less frequently, in Gram-positive bacteria. Gene cassettes in class 1 integrons confer resistance to β-lactams, aminoglycosides, erythromycin, chloramphenicol, trimethoprim, fosfomycin, lincomycin, and antiseptics of the quaternary-ammonium-compound family. The resistance determinants are tightly linked, because they are not only adjacent, but co-expressed from the same promoter. Since the genetic organization of integrons results in co-expression of the genes that have been integrated, use of any antibiotic that is substrate for one of the resistance mechanisms will co-select for resistance to all the others. The emergence of new gene cassettes in class 1 integrons, such as *qnr* implicated in resistance to fluoroquinolones, is of concern.

Extended Cross-Resistance

This type of resistance is due to a single mechanism (we are therefore dealing with cross-resistance) that can confer resistance to various drug classes and is thus designated as "extended" cross-resistance. As in co-resistance, but with differences in genetic and biochemical organization, a class of antibiotics can select for resistance to other drug classes. A typical example is the methylation of a specific adenine residue in 50S rRNA that confers high-level resistance to macrolides, lincosamides, and streptogramins B although these three classes have different chemical structure.

Another example of this type of resistance is overexpression of efflux pumps that can have very broad substrate ranges. The pumps, that are grouped in superfamilies, use energy provided by the proton motive force or hydrolysis of ATP. In Gram-negative bacteria the RND pumps can export a large array of antibiotic molecules, with very different structures, but also biocides such as triclosan. This accounts for the fact that detergents, that are increasingly used in house hold products, can select multiresistant bacteria. The pumps should be considered as the kidneys of the bacteria since they export molecules that are toxic, in particular products of the cellular catabolism. The chromosomal structural genes for the pumps are positively or negatively regulated and are generally expressed at low level. A mutation in one of the genes involved in regulation (activator, repressor, or two-component regulatory system) or in the operator will result in overexpression of the pump and to resistance to its various substrates. Thus, the smallest genetic event, a point mutation, can lead in one step to resistance to a large set of antibiotics, the so-called multidrug resistance.

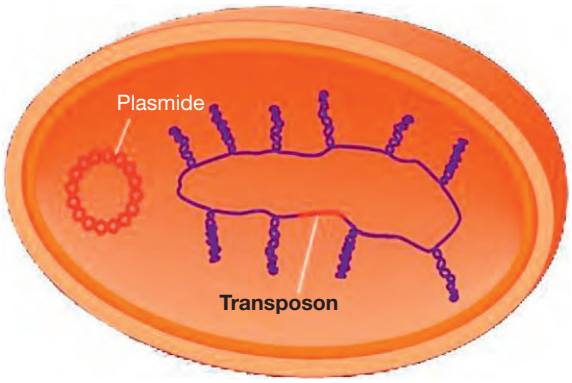

Figure 4 Schematic representation of the bacterial genome.

Genetics of Resistance

The genome of bacteria is constituted of the chromosome and of mobile genetic elements such as plasmids and transposons (Figure 4). Antibiotic-resistance genes can spread using these structures but also using insertion sequences, integrons, and other complex structures such as genomic islands, integrative conjugative (ICE) and mobilizable elements (IME). The chromosome contains all the genetic information required for the life cycle of the bacteria. In general, the chromosome is not self-transferable horizontally to other bacteria. Chromosomal resistance genes and mutated genes involved in drug resistance, are inherited vertically by the next generation of bacteria. Plasmids and transposons encode functions that are not strictly required for bacterial life but that can provide advantages to the host. Antibiotic resistance genes are only transiently useful to bacteria and it thus makes sense that they are often transferable and part of mobile genetic elements. In fact, any gene can be part of a volatile structure as long as it provides intermittent selective advantage to the host and that adequate selective pressure exerts. These mobile genetic elements can be inherited horizontally and vertically.

Plasmids

Plasmids are extrachromosomal elements that can be transferred laterally by conjugation or by mobilization. They may carry resistance to several antibiotics. Conjugative plasmids are self-transferable from cell to cell by conjugation, a mechanism that requires physical contact between the donor and the recipient bacterium (Figure 5). Mobilizeable plasmids can be transferred with the help of a conjugative plasmid co-resident in the same cell. A plasmid can carry multiple, easily up to seven, resistance determinants. Because of this physical linkage, selection for resistance to any of them will lead to co-transfer of the others. Thus in a single genetic event, conjugation, a bacterium can acquire "en bloc" a multiplicity of resistances.

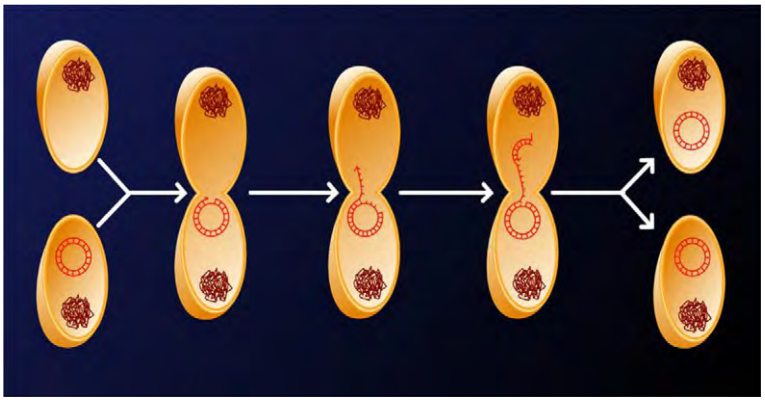

Figure 5 Schematic representation of plasmid conjugation. Bottom, donor bacterium; top, recipient bacterium. The chromosomes are represented in a condensed state. After a single nick on one of the two complementary DNA strands, one strand is transferred from the donor to the recipient. During this process, the complementary strand of the remaining DNA strand in the donor is synthesized while the complementary strand of the incoming DNA is synthesized in the recipient. After transfer, each bacterium contains a copy of the plasmid and can therefore act, in turn, as a donor.

Transposons

Transposons are DNA fragments that can migrate from one replicon to another while retaining their structural integrity (Figure 6). Transposons encode a transposase that allows site-specific insertion and excision. They can transfer actively, in the case of conjugative transposons of Gram-positive cocci, or passively when they are borne by a transferable plasmid. Integrons, as described above, are found frequently as part of transposons, and transposons are frequently carried on plasmids. Numerous plasmids and transposons carry antibiotic resistance genes; often several of them. Furthermore, mobile genetic elements carrying antibiotic resistance genes often encode resistance to heavy metals and detergents. Thus, selection pressure exerted by biocides may select for antibiotic resistance.

As already mentioned, there are two major pathways to antibiotic resistance: mutational events in the chromosome or acquisition of foreign genes.

Mutations can occur not only in a structural gene for the target of an antibiotic (as discussed for quinolones) but also in regulatory regions of genes (for example efflux pumps).

Resistance to an antibiotic is often inducible by the antibiotic itself. In this case, the drug should be considered as having two types of activities; induction of resistance and killing of the bacteria, that act on distinct targets. Acquired resistance to vancomycin is a typical example of inducibility. Expression of the resistance genes of the *van* operon is controlled by a two-component regulatory system VanS/VanR, in which VanS acts as a sensor and VanR as a transcriptional regulator (Figure 2). In the presence of vancomycin in the environment, the signal is transduced from the sensor domain to the catalytic domain of VanS, leading to autophosphorylation of VanS followed by transfer of the phosphoryl group to the VanR response regulator. The phosphorylated regulator binds to the promoter regions resulting in transcriptional activation of the regulatory (*vanR*/*vanS*) and of the resistance genes, allowing expression of the resistance pathway (synthesis of modified peptidoglycan precursors) and elimination of the normal precursors ending in D-Ala-D-Ala. Under non-inducing conditions, that is in the absence of vancomycin, VanS acts as a phosphatase, dephosphorylates VanR resulting in arrest of expression of the resistance genes. Since antibiotic resistance usually corresponds to a gain of function, there is an associated biological cost resulting in the loss of fitness of the bacterial host. It therefore appears that modulation of gene expression probably reflects a good compromise between energy saving and adaptation to a rapidly changing environment.

Antibiotics Could act as Pheromones

Antibiotics provide selective pressure for resistant bacteria to maintain and disseminate but they can also induce transfer of resistance genes. For example, it has been reported that (i) use of subinhibitory concentrations of penicillins increase the conjugal transfer of plasmid DNA from *E. coli* to *S. aureus* and *L. monocytogenes*, (ii) oxacillin increased the frequency of *in vitro* transfer of Tn*916*, an enterococcal conjugative transposon, from *E. faecalis* to *Bacillus anthracis*, (iii) transfer frequency of conjugative transposons belonging to the Tn*916*/Tn*1545* family, that contain a tetracycline resistance determinant, is increased 10- to 100-fold *in vitro* and *in vivo* in the presence of low concentrations of tetracycline, and (iiii) tetracycline also increases the transfer of a *Bacteroides* conjugative transposon.

Thus, several antibiotics can behave like pheromones: they are synthesized by specific cells (such as the *Actinomycetes* producers) and they act on another cell, at low concentrations, on very specific targets to promote DNA exchange.

Biological Cost of Antibiotic Resistance

The frequency of appearance of resistant strains in a bacterial population depends on several factors such as the volume of antibiotic use, the biological cost of resistance, and the ability of bacteria to compensate for the fitness cost. Acquisition of antibiotic resistance

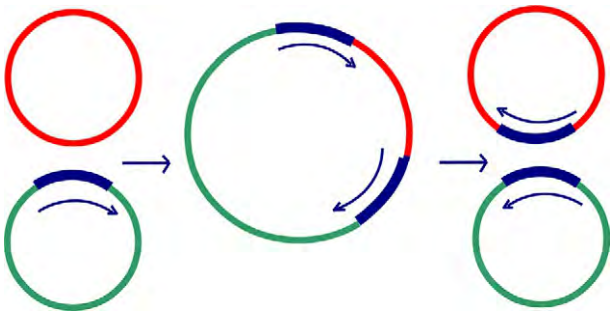

Figure 6 Replicative transposition. The donor replicon (lower left) contains a copy of the transposon (close bar; the direction of replication is indicated by an arrow) whereas the acceptor replicon (upper left) does not. Selective replication of the transposon and replicon fusion generate a bireplicon that contains two copies of the element, in the same orientation, at the borders of the replicons. Following recombination between the two copies of the element, after completion of transposition, each replicon contains a copy of the element and can thus, in turn, act as a donor.

is often associated with a biological cost because (i) bacteria acquire a new gene or set of genes responsible for new functions, (ii) the resistance mutations occur in genes with essential functions or (iii) of the replication and maintenance of extrachromosomal elements that bear the resistance genes. The biological cost allows determination of the stability and the potential reversibility of resistance. It can affect the growth rate *in vitro* or the survival in the host or in the environment and can modify the virulence capacity of the strain. Thus, the fitness cost of antibiotic resistance could be assessed by measuring, in isogenic (susceptible and resistant) strains, the growth rate *in vitro* or in animals. A compensatory evolution could occur to reduce the biological cost, allowing the stabilization of the resistant bacteria in a natural population. This stabilization may allow resistant strains to compete with susceptible strains in an antibiotic-free environment. The compensatory evolution could be the result of (i) a true reversion of the resistance mutation or loss of the resistant element or (ii) an acquisition of a second mutation (reverse mutation) located in the same (intragenic) or in another (extragenic) gene. Reversion mutations are more common than reversion to the susceptible phenotype. Once the resistant and compensated mutants are fixed in the bacterial population, a reversion to susceptibility is unlikely. Chances of reversibility of the resistance are also reduced in case of co-resistance to several antibiotics.

It was observed by competition experiments in *Helicobacter pylori* that clarythromicin resistance confers a biological cost in mice. Reduction of this cost, which was observed in clinical isolates, suggests that compensation is a clinically relevant phenomenon.

Comparison of the growth of a fosfomycine sensitive uropathogenic *E. coli* strain with that of an isogenic mutant resistant to this antibiotic obtained *in vitro*, revealed that the growth of the latter is altered in a medium that does not contained fosfomycin.

The biological fitness was also partially or totally restored in fusidic acid resistant mutants of *S. aureus* and *S. typhimurium* and in rifampin resistant mutants of *E. coli*. Similar results were obtained in *S. enterica* that were resistant to a deformylase inhibitor, an antibiotic that targets peptide deformylase. Resistance mutations, that occur in the *fmt* or *folD* gene, confer a fitness cost in the absence of antibiotics. Intragenic mutations in the *fmt*/*folD* genes or extragenic mutations (such as amplification of genes encoding tRNAi) partially reduce the fitness cost.

Some resistant bacteria could have a normal growth suggesting that they have acquired a no-cost resistance mutation. A specific substitution in the *rpsL* gene (which encodes ribosomal protein S12), responsible for streptomycin resistance in several enteric bacteria, is a typical exemple of a no-cost high-level resistance mutation.

Regulation of gene expression plays an important role. It has been demonstrated that, in the absence of induction by vancomycin, carriage of the *vanB* locus by the conjugative transposon Tn*1549* has no biological cost in *E. faecalis*. However, induced or constitutively resistant strains have a reduced fitness and are impaired in colonization ability and dissemination among mice. Thus, tight regulation of gene expression could be responsible for the modulation of the biological cost and, consequently, of the dissemination of the resistant strains.

Finally, the fitness cost of resistance depends on several factors such as the environmental conditions, the bacterial species, the specific resistance mutation or the growth conditions. The methods used to determine the fitness cost are also crucial: one study demonstrates that no fitness cost was associated with vancomycin resistance in enterococci whereas another group found that vancomycin-susceptible enterococcal strains were more fit than their resistant counterparts.

Conclusion

Intrinsic or acquired resistance to antimicrobial drugs could be the result of different mechanisms. Resistance could (i) be limited to one class of antibiotics or (ii) involve several classes by extended cross-resistance or co-resistance. Resistance determinants that are chromosomally located could be vertically inherited whereas those that are part of mobile genetic elements can be vertically and horizontally acquired. There are three levels of exponential dissemination of antibiotic resistance: epidemics of resistant bacteria among mammals, resistance-plasmid epidemics due to the broad host range of conjugation, and gene epidemics among bacteria (transposons, integrons). Thus, resistance genes can easily disseminate under natural conditions. The biological cost associated with resistance, when it exists, is frequently reduced by a compensatory evolution that allows the stabilization of the resistant bacteria in the population. It is thus necessary to develop (i) strategies to reduce resistance dissemination (such as prudent use of antibiotics, increase of surveillance resistance) and (ii) new antibiotics addressing novel targets and thus escaping to cross-resistance with already developed drug. However, many *in vitro* and *in vivo* studies indicate that several pathways will confer, soon or later, resistance to every new antibiotic.

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Antifungal Agents☆

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Glossary

Emerging fungal infections Infections caused by new or uncommon fungi.
Granulocytopenia/neutropenia Acquired or chemically induced immunosuppression caused by low white blood cell counts.
Immunocompromised Having a defect in the immune system.
In vitro and in vivo Describing or referring to studies carried out in the test tube and in animals, respectively.
Mycoses and mycotic infections Diseases caused by yeasts or molds.
Nephrotoxicity Damage to the kidney cells.
Opportunistic infections Infections caused by saprophytic fungi or not true parasites.

Abbreviations

AMB	Amphotericin B
CSF	Cerebrospinal fluid
DMPC	Dimyristoyl phosphatidylcholine
DMPG	Dimyristoyl phosphatidylglycerol
ECV	Epidemiological cutoff values
GVHD	Graft-versus-host disease
HSCT	Hematopoietic stem cell transplant
IS	Isavuconazole, rezafungin (CD101IV), (R) ibrexafungerp (SCY-078), APX001 (E1210)
MEC	Minimal effective concentration
MIC	Minimal inhibitory concentration
NYS	Nystatin
OPC	Oropharyngeal candidiasis

Defining Statement

Antifungal agents are naturally occurring or synthetically produced compounds that have in vitro or in vivo activity against yeasts, molds, or both. Fungi and mammalian cells are eukaryotes, and antifungal agents that inhibit synthesis of proteins, RNA, and DNA are potentially toxic to mammalian cells.

Introduction

Fungi can be unicellular (yeasts) and multicellular or filamentous (molds) microorganisms. Some medically important fungi can exist in both of these morphologic forms and are called dimorphic fungi. Of the estimated 250,000 fungal species described, fewer than 150 are known to be etiologic agents of disease in humans. Most fungi associated with disease are considered opportunistic pathogens (especially the yeasts) because they live as normal flora in humans, lower animals and plants, and rarely cause disease in otherwise healthy individuals. Many fungi, on the contrary, are important plant and lower animal parasites and can cause damage to crops (wheat rust, corn smut, etc.) and to fruit (banana wilt), forest (Dutch elm disease), and ornamental trees and other plants. Historically, the potato famine, which was the reason for the great migration from Ireland to the Americas, was caused by a fungal infection (potato blight). At the same time, fungi and their products play an important economic role in the production of alcohol, certain acids, steroids, antibiotics, and so on.

Because, the number of fungal diseases caused by both yeasts and molds has significantly increased during the past 20 years, especially among immunocompromised patients at high risk for life-threatening mycoses, 15 antifungal agents are currently licensed for the treatment and prevention of systemic fungal infections: the polyene amphotericin B and its three lipid

☆*Change History*: May 2019 A. Espinel-Ingroff updated the text figures and references.

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formulations, the pyrimidine synthesis inhibitor 5-fluorocytosine (flucytosine), the imidazoles miconazole and ketoconazole, the triazoles fluconazole, isavuconazole, itraconazole, posaconazole, voriconazole, and the echinocandins caspofungin, micafungin, and anidulafungin. However, the number of fungal diseases caused by both yeasts and molds and the continued incidence of resistance and cross-resistance has significantly increased during the past years, especially among the larger number of immunocompromised patients. More recently, *Candida auris* has emerged as a new species causing candidemia infections that appear to be resistant to most available agents. All those factors have prompted the current development of two new echinocandins (Ibrexafungerp [SCY-078] and rezafungin [CD101IV] and the cell wall inhibitor APX001 [E1210]); these three new agents are undergoing Phase II to III clinical trials. There are other agents for topical treatment as well as agriculture and veterinary use, and more importantly, several compounds are under investigation for the management of severe and/or refractory fungal infections in humans. Table 1 only lists the antifungal agents that are useful in the clinical setting or currently under development (Phase II to III clinical trials) or in the veterinary/agriculture world. In addition, a brief description is provided for a variety of compounds with potential clinical use, as well as for those that have been discontinued due to toxicity and other issues.

The Polyenes

The polyenes are macrolide molecules that target membranes containing ergosterol, which is an important sterol in the fungal cell membranes. Traces of ergosterol are also involved in the overall cell cycle of fungi.

Nystatin

Nystatin was the first of the polyenes to be discovered when it was isolated from *Streptomyces noursei* in the early 1950s. It is an amphoteric tetrane macrolide that has a similar structure (Fig. 2A) and identical mechanism of action to those of amphotericin B. Although it has an in vitro spectrum of activity similar to that of amphotericin B, this antifungal is used mostly for the therapy of gastrointestinal (orally) and mucocutaneous candidiasis (topically). This is not only due to its toxicity after parenteral administration to humans and lower animals but also to its lack of effectiveness when given i.v. to experimental animals. It is used for candidiasis in small animals and birds and for otitis caused by *Microsporum canis*.

Amphotericin B

Amphotericin B is the most important of the 200 polyenes. Amphotericin B replaced 2-hydroxystilbamidine in the treatment of blastomycosis in the mid-1960s. Two amphotericins (A and B) were isolated in the 1950s from *Streptomyces nodosus*, an aerobic bacterium, from a soil sample from Venezuela's Orinoco River Valley. Amphotericin B (the most active molecule) has seven conjugated double bonds, an internal ester, a free carboxyl group, and a glycoside side chain with a primary amino group (Fig. 1A). It is unstable to heat, light, and acid pH. The in vitro spectrum of activity of amphotericin B includes yeasts, dimorphic fungi, and most of the opportunistic molds. The fungistatic (inhibition of fungal growth) and fungicidal (lethal or killing) activity of amphotericin B is due to its ability to combine with ergosterol in the cell membranes of susceptible fungi. Pores or channels are formed causing osmotic instability and loss of membrane integrity. This effect extends to mammalian cells. The drug also binds to cholesterol, creating the high toxicity associated with all conventional polyene agents. A second mechanism of antifungal action has been proposed for amphotericin B, which is oxidation-dependent. Amphotericin B is highly protein bound (91–95%). Peak serum of 1–3 $\mu g\ mL^{-1}$ and trough concentrations of 0.5–1.1 $\mu g\ mL^{-1}$ are usually measured after the intravenous (i.v.) administration of 0.6 mg/kg doses. Its half-life of elimination is 24–48 h, with a long terminal half-life of up to 15 days.

Since amphotericin B is insoluble in water, aqueous solubility of amphotercin B is achieved by formulation with deoxycholate resulting in the amphotericin B deoxycholate clinical formulation; the variety of lipid formulations will be discussed below. Although amphotericin B penetrates poorly into the cerebrospinal fluid (CSF), amphotericin B is effective in the treatment of invasive *Aspergillus*, *Candida* (alternative therapy), Mucorales and *Cryptococcus* meningitis (alone and/or in combination with 5-fluorocytosine for the latter infections) as well as other mold infections (Table 1). Toxicity is the limiting factor during amphotericin B therapy and has been classified as acute or delayed (Table 2). Nephrotoxicity is the most significant delayed adverse effect and initial therapy is not recommended with this drug when the patient is at high nephrotoxicity risk. Therefore, close monitoring of renal function tests, bicarbonate, electrolytes including magnesium, diuresis, and hydration status is recommended during amphotericin B therapy. Current recommendations regarding daily dosage, total dosage, duration, and its use in combination with other antifungal agents are based on the type of infection and the status of the host (Pappas et al., 2016; Perfect et al., 2010; Patterson et al., 2016). Because severe fungal infections in the granulocytopenic host are difficult to diagnose and cause much mortality, empirical antifungal therapy with amphotericin B and other agents has improved patient care. Systemic prophylaxis for patients at high risk for invasive mycoses has also evolved. Amphotericin B adverse drug interactions can occur with the administration of electrolytes and other concomitant drugs. This drug is also used in these animals for the treatment of systemic infections (e.g., blastomycosis in dogs), but it is not effective against aspergillosis. Side effects (especially in cats) and drug interactions are similar to those in humans.

Table 1 Antifungal agents, mechanisms of action, and their use.

Antifungal class	*Antifungal target of action*	*Agent*	*Use*
Polyenes	Membranes containing ergosterol	Amphotericin B (AMB)	Systemic mycoses[a,b]
		Nystatin (NYS)	Superficial mycoses[a,b]
		AMB lipid formulations: Ambisome Abelcet Amphocet	Alternative use for invasive mycoses (candidiasis and aspergillosis included) refractory and/or in patients intolerant to conventional AMB (for other specific indications, see below).
		Ambisome, Liposomal AMB, LAMB	Empirical, febrile neutropenic patients; cryptococcal meningitis in AIDS patients; prophylaxis in liver transplant patients; severe disseminated histoplasmosis
		Abelcet, AMB lipid complex, ABLC	See above
		Amphotec, AMB colloidal dispersion, ABCD	See above
		Pimaricin	Topical keratitis[a]
Phenolic cyclohexane	Microtubule aggregation and DNA inhibition	Griseofulvin	Dermatophytic infections[a]
Natural glutarimide	Protein synthesis inhibition	Cycloheximide	Laboratory and agriculture
Phenylpyrroles	Unknown	Fenpiclonil	Agriculture
Synthetic pyrimidines	Fungal cytosine permease and deaminase	Flucytosine	In combination with AMB, cryptococcal meningitis in HIV patients[a]
	Ergosterol inhibition	Triarimol	Agriculture
Azoles	Ergosterol biosynthesis inhibition	*Imidazoles*	
		Clotrimazole	Topical, oral troche[a,b]
		Econazole	Skin infections (ring worm, Athlet's foot, tinea versicolor, etc.)
		Isoconazole	Topical infections
		Oxiconazole	Skin infections (ring worm, Athlet's foot, etc.)
		Tioconazole	Ointment for vaginal yeast infections
		Fenticonazole	Mixed *Candida* and bacterial infections.
		Miconazole	Topical and veterinary[b]
		Ketoconazole	Second-line drug for nonmeningeal systemic mycosesand veterinary[a,b]
		Enilconazole	Veterinary
		Epoxiconazole	Agriculture (pesticide)
		Fluquinconazole	
		Triticonazole	
		Prochoraz	
		Triazoles	
		Fluconazole	Candidiasis, cryptoccosis, coccidioimycosis, prophylaxis[b]
		Itraconazole	Candidiasis, aspergillosis, endemic mycoses, superficial diseases, long-term prophylaxis[a]
		Posaconazole	Prophylaxis (invasive *Aspergillus* and *Candida* infections), oropharyngeal candidiasis (OPC)
		Voriconazole	*Aspergillus* and *Candida* infections, salvage therapy (*S. apiospermum* and *Fusarium* infections)[a]
		Terconazole	Intravaginal
		Isavuconazole	Invasive *Aspergillus* and Mucorales infections
Allylamines		Terbinafine	Superficial infections
Benzylamines		Butenafine	Topical
Thiocarbamates		Tolnaftate	Topical
Dithiocarbamates	Nonspecific	Mancozeb Thiram	Agriculture
Benzimidazoles and methylbenzimidazole carbamates	Nuclear division	Carbendazim	Pesticide
		Benomyl Thiophanate	

Table 1 Continued

Antifungal class	*Antifungal target of action*	*Agent*	*Use*
Morpholines	Ergosterol biosynthesis inhibition	Amorolfine	Topical
		Fenpropimorph	Agriculture
		Tridemorph	Pesticide
Pyridines		Buthiobate	Agriculture
		Pyrifenox	Pesticide
Echinocandins	Fungal (1,3)-glucan synthase inhibition	Anidulafungin	Invasive *Candida* infections, including candidemia, esophageal candidiasis[a]
		Caspofungin	Invasive *Candida* infections, including candidemia; empirical therapy (febrile neutropenic patients unresponsive to antibacterial therapy); salvage therapy (aspergillosis)[a]
		Micafungin	Invasive *Candida* infections including candidemia and esophageal candidiasis in adult patients. Prophylaxis for HSCT patients and liver transplants[a]
Under development		Ibrexafungerp (SCY-078)	*Candida* and *Aspergillus* infections: Phases II to III clinical trials for vaginal infections, invasive candidiasis and aspergillosis (oral formulation). IV formulation under development.
Under development		Rezafungin (CD101 IV)	*Candida*, *Aspergillus*, and *Pneumocystis* infections: Phase II to III clinical trials for invasive candidiasis, including candidemia, and prophylaxis of fungal infections (IV formulation).
Other fungal cell wall inhibitor: Under development	Glycosylphosphatidylinositol biosynthesis inhibition	APX001 (E1210)	*Candida*, *Aspergillus*, *Fusarium* and other mold infections. Phase I to II clinical trials of murine disseminated candidiasis (including oral infections), pulmonary aspergillosis and fusariosis.
Cinnamic acid	Cell wall	Dimethomorph	Agriculture
Oomycete fungicide	Oxidative phosphorylation	Fluazynam	Agriculture
Phthalimides	Nonspecific	Captan	Agriculture, horses (dermatophytic infections)

Only licensed, commonly used, and antifungals under clinical investigation are listed; see older versions of this review for other antifungal agents.
[a]Clinical and veterinary use; other applications for use in humans only.
[b]A human product used in veterinary.

Although resistance to amphotericin B is rare, both in vitro and in vivo resistance has been reported. Information regarding the mechanisms of amphotericin B resistance is mostly available for *Candida albicans*, *Aspergillus terreus*, and *A. flavus*. Alterations of the fungal cell wall composition, a lower ergosterol content and a higher catalase production have been reported to be responsible for in vitro resistant in *A. flavus* and *A. terreus*. Clinically, resistance to amphotericin B has become an important problem, particularly with certain yeast and mold species for which high MICs (minimal inhibitory concentrations) are usually obtained, such as *Candida lusitaniae (Clavispora lusitaniae)*, *A. terreus* (poor response to different formulations of amphotericin BMICs >1 μg/mL), *Fusarium* spp., *Malassezia furfur*, *Sedosporium apiospermum* complex (*Pseudallescheria boydii*), *Lomentospora* [*Scedosporium*] *prolificans*, *Trichosporon beigelii*, and other emerging fungal pathogens, such as *C. auris* (30–40% resistance) (Clancy and Nguyen, 2017). Although clinical breakpoints are not available for any fungal species and amphotericin B, species-specific epidemiological cutoff values (ECVs) have been defined for the more prevalent *Candida* spp., *Aspergillus* spp., and the *Cryptococcus neoformans-C. gattii* complex (Espinel-Ingroff and Turnidge, 2016; CLSI M59 document, 2018). These ECVs will distinguish wild-type (WT, isolates without mechanisms of resistance) from non-WT isolates (mutants or isolates with known mechanisms of resistance and potential reduced susceptibility to the agent being evaluated). In addition, amphotericin B MICs >0.5 and > 1 μg/mL are found in the literature as default cutoffs for resistance or nonsusceptibility to this drug and its lipid formulations, but this cutoff is mostly based on obtaining peak serum concentrations of 2 μg/mL. However, correlations of in vivo versus in vitro results using the available MIC methodology have not been established, but MICs above 2 μg/mL for *Aspergillus* spp. (or above the ECV) have been associated with treatment failures and MICs below 2 μg/mL with clinical cure.

Amphotericin B liposomal, lipid complex and colloidal formulations

With the outcome of azole resistance associated with treatment failure, amphotericin B lipid formulations have become useful alternatives for the treatment of invasive azole-resistance aspergillosis and candidiasis (Pappas et al., 2016; Patterson et al., 2016). The three lipid formulations of amphotericin B are indicated in the treatment of invasive fungal infections (candidiasis and aspergillosis included) caused by susceptible fungal pathogens that are refractory to/or in patients intolerant to conventional

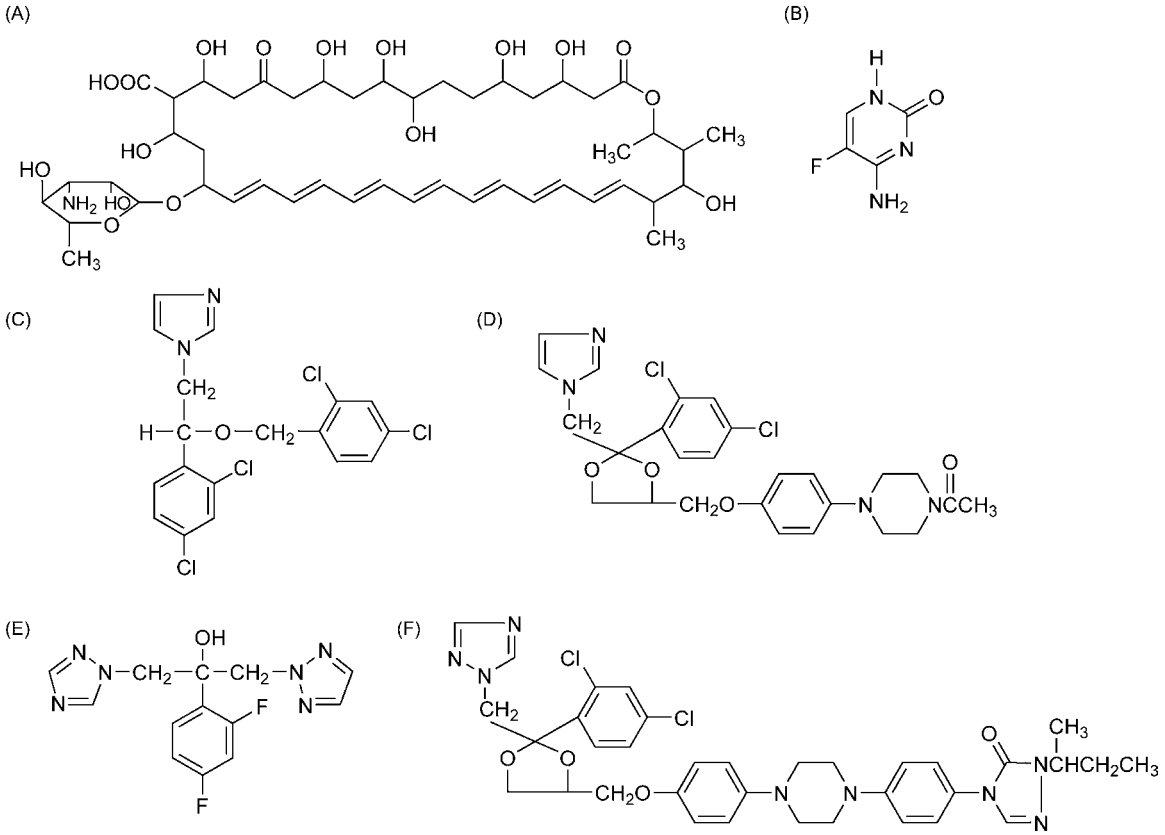

Fig. 1 Chemical structures of some systemic licensed antifungal agents: (A) amphotericin B, (B) 5-fluorocytosine, (C) miconazole, (D) ketoconazole, (E) fluconazole, and (F) itraconazole.

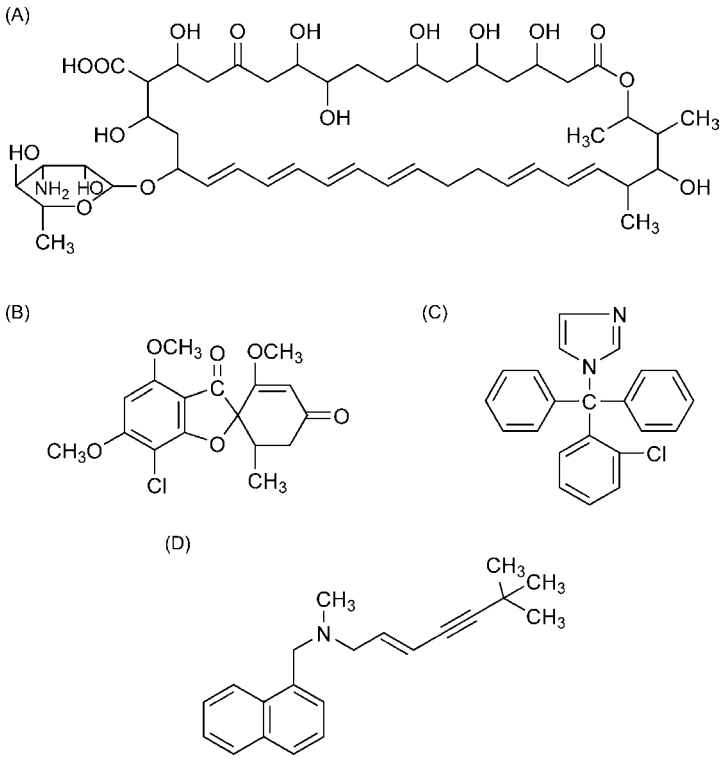

Fig. 2 Chemical structures of the most commonly used topical antifungal agents: (A) nystatin, (B) griseofulvin, (C) clotrimazole, and (D) terbinafine.

Table 2 Adverse side effects of the licensed systemic antifungal agents.

Side effect	*Drug*
Non-melanoma skin cancer-prolonged therapy	V
Fever, chills, flushing	A, IS, K, V, Fl, An, C, R
Rash,	FC, K, I, FL, C, V, P
Nausea, vomiting, constipation	A, FC, K, I, IS, FL, R, V, P, An, C
Abdominal pain	FC, K, IS, V, An, M
Anorexia	A, K, An
Diarrhea	FC, IS, V, C, M
Elevation of transaminases	FC, K, I, V, IS, C, An (rare)
Hepatitis (rare); hepatobiliary	FC, K, I, FL IS
Anemia	A, FC, C
Leukopenia, thrombocytopenia	FC, Fl, An(rare)
Decreased renal function (azotemia, acidosis, hypokalemia, decreased plasma creatinine, etc.)	A, C, V
Decreased testosterone synthesis	K (I, rare)
Adrenal insufficiency, menstrual irregularities, female alopecia	K
Syndrome of mineralocorticoid excess, pedal edema	I
Headache	A, FC, K, I, FL, R, V, An, C
Photophobia	K, V
Transient vision disturbances	V (30%), IS
Dizziness	I, V
Seizures	FL
Confusion	FC, V, An
Arthralgia, myalgia, thrombophlebitis	A, R
Ventricular tachycardia	C, V
Alopecia (rare)	Fl
Hypokalemia	Fl, C, V, An
Dyspnea and hypotension (rare)	An
Wheezing, chest tightness, trouble breathing, low potassium (uneven heart rate, QT interval shortening, extreme thirst, increased urination, leg discomfort, muscle weakness or limp feeling),	An, Is,
Dark urine, clay-colored stools, jaundice (yellowing of the skin or eyes), dysuria	An, M, R
EaEasy bruising, unusual bleeding (nose, mouth, vagina, or rectum), purple or red pinpoint spots.	M
Skin/eye disorders, pyrexia	Is

A, amphotericin B; An, anidulafungin; C, caspofungin; FC, flucytosine; K, ketoconazole; M, micafungin; Fl, fluconazole; I, itraconazole; IS, isavuconazole; V, voriconazole, rezafungin, (R).

From Sandison, T., Ong, V., Lee, J., Thye, D. (2017). Safety and pharmacokinetics of CD101 IV, a novel echinocandin, in healthy adults. *Antimicrobial Agents and Chemotherapy* **61,** e01627–16. https://doi.org/10.1128/AAC.01627-16; Keirns, J., Desai, A., Kowalski, D. et al. (2017). QT interval shortening with isavuconazole: In vitro and in vivo effects on cardiac pepolarization. *Clinical Pharmacology & Therapeutics* **101**, 282–290. https://doi.org/10.1002/cpt.620.; NIH antifungal agents.

agent are: (i) liposomal amphotericin B (3–5 mg/kg q.d. approved dose, LAMB, *ambisome*); (ii) amphotericin B lipid complex (5 mg/kg q.d. approved dose; ABLC, *abelcet*); and amphotericin B colloidal dispersion (3–4 mg/kg q.d. approved dose, ABCD, *amphotec*). Other specific indications are as follows: (i) *ambisome*, for cryptococcal meningitis in AIDS patients (6 mg/kgq.d. approved dosage), empirical treatment in febrile neutropenic patients with persistent fever despite antibacterial treatment for 5–7 days (approved dose: 3 mg/kg q.d.), disseminated histoplasmosis and prophylaxis in liver transplant patients at risk of fungal systemic infections; (ii) *amphotec*, for invasive aspergillosis in patients with renal impairment, unacceptable toxicity that precludes the use of amphotericin B deoxycholate in effective doses. A brief description of these three agents and other less used lipid formulations can be found below.

In general, these preparations have selective toxicity or affinity for fungal cell membranes and promote the delivery of the drug to the site of infection while avoiding the toxicity of supramaximal doses of conventional amphotericin B. The result is a reduction of human erythrocytes lysis and higher doses of amphotericin B can be safely used. Although most of these formulations have been approved for the treatment of invasive fungal infections, not enough information is available regarding their pharmacokinetics, drug interactions, long-term toxicities, and the differences in both efficacy and tolerance among the three formulations.

Ambisome: liposomal amphotericin B

Ambisome (LAMB; Gilead Sciences, UK [marketed in Europe], Astellas Pharma, formerly Fujisawa Pharmaceuticals [marketed in USA], Sumitomo Pharmaceuticals [marketed in Japan and Australia] is perhaps the most widely used amphotericin B lipid preparation. In this formulation, amphotericin B is incorporated into small unilamellar, spherical vesicles (60- to 70-nm liposomes) in a 2: 0.8:1: 0.4 M ratio. Ambisome is also recommended for the treatment of visceral leishmaniasis.

Abelcet: amphotericin B lipid complex

Abelcet (ABLC, Sigma-Tau Pharmaceuticals) is a preparation of amphotericin B and two phospholipids (DMPC/ DMPG) in a 1:1 drug-to-lipid molar ratio.

Amphotec: amphotericin B colloidal dispersion

Amphotec (ABCD, Ben Venue Laboratories and InterMune, Inc. [manufacturer and distributor, respectively], USA) is a colloidal complex of amphotericin B and cholesteryl sulfate in a 1:1 M ratio.

Amphocil

Amphocil (Samaritan Pharmaceuticals, UK) is another disk shaped colloidal complex of amphotericin B where the drug is also stabilized with cholesteryl sulfate.

Fungisome

Fungisome is the latest and cheapest addition to the lipid formulations of amphotericin B. It is marketed in India by Lifecare Innovations.

Pimaricin (Natamycin)

Pimaricin is a tetraene polyene produced by *Streptomyces natalensis*. It has a higher binding specificity for cholesterol than for ergosterol and, therefore, it is highly toxic for mammalian cells. The therapeutic use of pimaricin is limited to the topical treatment of keratitis (eye infections; also in horses) caused by *Fusarium* spp., *Acremonium* spp., and other mold species.

Phenolic Cyclohexane: Griseofulvin

Griseofulvin is a phenolic, benzyfuran cyclohexane agent (Fig. 2B) that binds to RNA. It is a product of *Penicillium janczewskii* and was the first antifungal agent to be developed as a systemic plant protectant. It acts as a potent inhibitor of thymidylate synthetase and interferes with the synthesis of DNA. It also inhibits microtubule formation and the synthesis of apical hyphal cell wall material. With the advent of terbinafine and itraconazole, the clinical use of griseofulvin as an oral agent for treatment of dermatophytic infections has become limited. However, it is frequently used for these infections in small animals, horses, and calves (skin only) as well as for equine sporotrichosis. Abdominal adverse side effects have been noted, especially in cats.

Natural Glutarimide: Cycloheximide

This is a glutaramide agent produced by *S. griseus*. This agent was among the three antifungal agents reported between 1944 and 1947. Although cycloheximide had clinical use in the past, it is currently used as a plant fungicide and in the preparation of laboratory media.

Phenylpyrroles: Fenpiclonil

Although this agent is used in agriculture, the mechanism of action is unknown.

The Synthetic Pyrimidines

5-Fluorocytosine (Flucytosine)

The synthetic 5-fluorocytosine has only fungistatic activity against yeasts and some dermatophytes. It is an oral, low-molecular-weight, fluorinated pyrimidine related to 5-fluorouracil and floxuridine (Fig. 1B). It acts as a competitive antimetabolite for uracil in the synthesis of yeast RNA. Two enzymes are involved in the mode of action of 5-fluorocytosine: a membrane-bound *permease* (drug uptake) and a *deaminase* (conversion to the main active form, 5-fluorouracil). Flucytosine resistance may arise from a mutation of an enzyme necessary for the cellular uptake or metabolism of the drug or from an increased synthesis of pyrimidines, which compete with the drug metabolites, e.g., mutations in the *FCY2*, *FCY1*, and *FUR1* genes encoding cytosine permease, cytosine deaminase, and uracil phosphoribosyltransferase, respectively (Table 4). Resistance to flucytosine has been shown to emerge after monotherapy and prolonged exposure to the drug. *C. krusei* should be considered to be resistant to flucytosine. Although clinical breakpoints and ECVs are not available for *Candida* spp., ECVs have been approved for the *C. neoformans-C. gattii* complex (Espinel-Ingroff and Turnidge, 2016; CLSI M59 document, 2018).

Clinically, the major therapeutic role of 5-fluorocytosine is its use in combination with amphotericin B in the treatment of meningitis caused by *C. neoformans-C. gattii* complex in AIDS patients (Table 1; Perfect et al., 2010). 5-Fluorocytosine should not be used alone for the treatment of any fungal infections. The most serious toxicity associated with 5-fluorocytosine therapy is bone marrow suppression (6% of patients), which leads to neutropenia, thrombocytopenia, or pancytopenia (Table 2). Therefore, monitoring of the drug concentration in the patient's serum (serial 2-h levels post-oral administration) is highly recommended to adjust dosage and maintain serum levels between 40 and 60 g mL^{-1}. Because the drug is administered in combination with amphotericin B, a decrease in glomerular filtration rate, a side effect of the latter compound, can induce increased toxicity to 5-fluorocytosine. Adverse drug interactions can occur with other antimicrobial and anticancer drugs, cyclosporine, and other therapeutic agents. Given its toxic potential, 5-fluorocytosine should not be administered to pregnant women or animals. This drug has been used in combination with ketoconazole for cryptococcosis in small animals (very toxic for cats) and also for respiratory aspergillosis and severe candidiasis in birds.

Triarimol, Fenarimol, Pyrimethanil, and Cyprodinil

Triarimol and fenarimol are pyrimidines that inhibit lanosterol demethylase, an enzyme involved in the synthesis of ergosterol, while the anilinopyrimidines, pyrimethanil and cyprodinil, inhibit the secretion of the fungal enzymes that cause plant cell lysis, which leads to the inhibition of this biosynthesis pathway. However, the activity is lost after several applications. Only Triarimol is used in agriculture in some countries.

The Azoles

The azoles are the largest single source of synthetic antifungal agents; the first azole was discovered in 1944. As a group, they are broad spectrum in nature and mostly fungistatic. The broad spectrum of activity involves fungi (yeasts and molds), bacteria, and parasites. This group includes fused ring and N-substituted imidazoles and the N-substituted triazoles. The mode of action of these compounds is the inhibition of lanosterol demethylase, a cytochrome P450 enzyme.

Fused-Ring Imidazoles (Flubendazole)

The basic imidazole structure is a cyclic five-member ring containing three carbon and two nitrogen molecules. In the fused-ring imidazoles, two carbon molecules are shared in common with a fused benzene ring. Most of these compounds have parasitic activity (anthelmintic); two have limited antifungal activity: 1-chlorobenzyl-2-methylbenzimidazole and thiabendazole (previously used for superficial yeast and dermatophyte infections and aspergillosis and penicilliosis in dogs, respectively).

Recently, the amorphous solid compound flubendazole is a benzimidazole (Janssen Pharmaceutica) that was developed for the oral treatment of some tropical diseases, including onchocerciasis. It also has in vitro activity (low MICs) against *C. neoformans* by binding to cryptococcal ß-tubulin. Although flubendazole showed oral bioavailability in rabbits, there was no quantifiable drug concentrations or antifungal activity in either the CSF or cerebrum. These results indicated that for flubendazole to be useful for cryptococcal meningitis treatment, further studies are needed to increase the drug gut absorption and the optimal drug levels into the different CNS areas (Nixon et al., 2018).

N-Substituted (Mono) Imidazoles

In this group, the imidazole ring is intact and substitutions are made at one of the two nitrogen molecules. At least three series of such compounds have emerged for clinical and agricultural use. In the triphenylmethane series, substitutions are made at the nonsymmetrical carbon atom attached to one nitrogen molecule of the imidazole ring. In the second series, the substitutions are made at a phenethyl configuration attached to the nitrogen molecule. The dioxolane series is based on a 1,3-dioxolane molecule rather than on the 1-phenethyl molecule. These series vary in spectrum, specific level of antifungal activity, routes of administration, and potential uses.

Clotrimazole

Clotrimazole is the first member of the triphenylmethane series of clinical importance (Table 1; Fig. 2C). It has good in vitro activity at very low concentrations against a large variety of fungi (yeasts and molds). However, hepatic enzymatic inactivation of this compound, after systemic administration, has limited its use to topical applications (1% cream, lotion, solution, tincture, and vaginal cream) for superficial mycoses (nail, scalp, and skin infections) caused by the dermatophytes and *M. furfur*, for initial and/or mild oropharyngeal candidiasis (OPC; 10-mg oral troche), and for the intravaginal therapy (single application of 500-mg intravaginal tablet) of vulvovaginal candidiasis. Other intravaginal drugs require 3 to 7-day applications. This drug is also used for candidal stomatitis, dermatophytic infections, and nasal aspergillosis (infused through tubes) in dogs.

Econazole, isoconazole, oxiconazole, tioconazole, and fenticonazole

Other frequently used topical imidazoles include econazole (1% cream), isoconazole (1% cream), oxiconazole (1% cream and lotion), and tioconazole (6.5% vaginal ointment) (Table 1). As with clotrimazole, a single application of tioconazole is effective in

the management of vulvovaginal candidiasis and as a nail lacquer for fungal onychomycosis (nail infections). Mild to moderate vulvovaginal burning has been associated with intravaginal therapy. Oxiconazole and econazole are less effective than terbinafine and itraconazole in the treatment of onychomycosis and other infections caused by the dermatophytes. Although topical agents do not cure onychomycosis as oral drugs do, they may slow down the spread of this infection. However, the recommended drugs for the treatment of onychomycosis are terbinafine (by dermatophytes) and itraconazole.

On the other hand, fenticonazole was synthesized and developed in Italy in the 1990s and has been used for the topical treatment of infections caused by dermatophytes and especially for *Candida* vaginitis. Recently, based on an in vitro study, the use of this agent was recommended for the treatment of vaginal infections caused by a combination of *Candida* and bacteria agents or with different etiology in order to minimize the possibility of antimicrobial resistance (Sanguinetti et al., 2019).

Miconazole

Miconazole (Fig. 1C) was the first azole derivative to be administered intravenously for the therapy of systemic fungal infections. Clinically is only used as a topical agent for dermatophytic infections in humans and other large animals, fungal keratitis and pneumonia in horses, in birds, and aspergillosis in raptors. However, safety and efficacy data are not available (veterinary use). It is also available as vaginal suppository.

Ketoconazole

Ketoconazole was the first representative of the dioxolane series (Table 1; Fig. 1D) to be introduced into clinical use and was the first orally (tablet) active azole. This drug has a broad spectrum of in vitro activity, but due its adverse side effects, drug interactions and the high rate of relapses, it been replaced by itraconazole and other triazoles in most countries. However, creams (for tinea, cutaneous candidiasis, and pityriasis versicolor) and shampoos (for dandruff or seborrhoeic dermatitis of the scalp) are available. Therapeutic failure with ketoconazole has been associated with low serum levels; monitoring of these levels is recommended in such failures. Ketoconazole also has been used for a variety of systemic and superficial fungal infections in cats and dogs. Ketoconazole requires a normal intragastric pH for absorption, for example an increase in pH will decrease its absorption in patients with gastric achlorhydria or treated with antacids or H_2-receptor antagonists (Table 3). This drug should be taken with either orange juice or a carbonated beverage. Ketoconazole pharmocokinetics corresponds to a dual model with an initial half-life of 1–4 h and a terminal half-life of 6–10 h, depending on the dose. This drug highly binds to plasma proteins and penetrates poorly into the CSF, urine, and saliva. Peak plasma concentrations of approximately 2, 8, and 20 $\mu g\,mL^{-1}$ are measured 1–4 h after corresponding oral doses of 200, 400, and 800 mg. The most common and dose-dependent adverse effects are nausea, anorexia, and vomiting in 10% of the patients receiving a 400 mg dose and in approximately 50% of the patients taking 800 mg or higher doses (Table 2).

Table 3 Adverse interactions of the licensed systemic antifungal agent with other drugs during concomitant therapy.

Azole	*Concomitant drug*	*Adverse side effect of interaction*
V		Non-melanoma skin cancer
K, FL, I	Nonsedating antihistamines, cisapride, terfenadine, astemizole	Fetal arrhythmia
K, Fl, I, V, C	Rifampin, isoniazid, phenobarbital, rifabutin, carbamazepine, and phenyton	Reduces azole or C plasma concentrations
K, Fl, I, V	Phenytoin, benzodiazepines, rifampin	Induces the potential toxicity levels of co-compounds
K, I	Antacids, H_2 antagonists, omeprazole, sucralfate, didanosine	Reduces azole absorption
K, Fl, I	Lovastin, simvastatin	Rhabdomyolysis
I	Indinavir, vincristine, quinidine, dicyclosporine, tacrolimus, methylprednisolone, and ritonavir	Induces potential toxicity co-comgoxin, compounds
Fl, I, V	Warfarin, rifabutin, sulfonylurea	Induces potential toxicity of co-compound
K	Saquinavir, chlordiazepoxide, methylprednisone	Induces potential toxicity of these compounds
K	Protein-binding drugs	Increases the release of fractions of free drug
K, C, V	Cyclosporine A	Nephrotoxicity
C, V	Tacrolimus	Tacrolimus can be decreased
C	Efavirenz, nevirapine, phenytoin, dexamethasone	C can be reduced (70 mg of C should be considered)
V	Omeprazole	Reduces omeprazole dose to one half
P	Phenytoin, cimetidine	Reduces P exposure Induces C clearance
P	Cyclosporine A, tacrolimus	Increases concomitant drug exposure
C	Carbamazepine, rifampin, dexamethason	Induces C clearance
P	Midazolam	
P	Rifabutin	Two-way interactions
M	Sirolimus, nifedipine or itraconazole	Increases concomitant drug exposure

K, ketoconazole; Fl, fluconazole; I, itraconazole; P, posaconazole; V; voriconazole; C, caspofungin.

See Clinical pharmacology of systematic antifungal agents: *Advances in Pharmacology* and the NIH website for antifungal drugs.

Table 4 Reported molecular mechanisms of resistance for the most important systemic antifungal agents.

Antifungal agent and molecular mechanism	*Resulting effect*
Azoles and *Candida* spp.: CDR1/CDR2 and MDR1 upregulation due to point mutations in TAC1 and MRR1 transcription factors	Upregulation of drug transporters
Gene (*ERG11*) point mutations	Decreased lanosterol 14-α-demethylase binding affinity for the drug
ERG11 upregulation due to gene duplication and transcription factor regulation	Increased concentration of lanosterol 14- α -demethylase
Gene (*ERG3*) point mutations	Inactivation of C5 sterol desaturase leading to alterations in the synthetic pathway
Azoles and Aspergillus spp.: Gene (*cyp51*) single or multiple point mutations	Increased level of gene expression
Echinocandins: Gene point mutations (FKS1 and FKS2)	Decreased glucan synthase or drug affinity
Polyenes: Gene (ERG3 and ERG6) point mutations	Decreased cell ergosterol content
Flucytosine and other nucleoside analogs	
Gene (FCY2) point mutations	Inactivation of cytosine permease affecting drug uptake
Gene (FCY1) point mutations	Inactivation of cytosine deaminase leading to alterations in 5-fluorocytosine metabolism
Gene (FUR1) point mutations	Inactivation of uracil phosphoribosyl transferase leading to alterations in the metabolism of 5-fluorocytosine

Enilconazole

This is the azole most widely used in veterinary practice for the intranasal treatment of aspergillosis and penicilliosis as well as for dermatophytic infections. The side effects are few.

Epoxiconazole, fluquinconazole, triticonazole, and prochloraz

Epoxiconazole, fluquinconazole, and triticonazole are important agricultural fungicides, which have a wider spectrum of activity than that of the earlier triazoles, triadimefon, and propiconazole, and the imidazole, prochloraz, as systemic cereal fungicides. However, development of resistance to these compounds has been documented.

The Triazoles

The triazoles are characterized by a more specific binding to fungal cell cytochromes than to mammalian cells due to the substitution of the imidazole ring by the triazole ring. Other beneficial effects of this substitution are (1) an improved resistance to metabolic degradation, (2) an increased potency, and (3) a superior antifungal activity. Several triazoles, including isavuconazole, are licensed for antifungal systemic therapy (Table 1) and others are under clinical investigation.

Triazole resistance, molecular resistance mechanisms

The wide use of fluconazole and other triazoles led to in vitro resistance among *Candida* and other yeast isolates (See below for listing of some species). The molecular mechanisms associated with in vitro resistance to the triazoles among *Candida* spp. are: (i) modifications in the quality or quantity of the target enzyme, reduced access of the drug to the target, mutations in the *ERG* genes participating in ergosterol biosynthesis, or a combination of these mechanisms, and (ii) an active efflux of azole out of the cell through the activation of multidrug efflux transporters encoded by the *MDR* and *CDR* genes; these mechanisms have been linked to in vitro and clinical resistance. On the other hand, acquired azole resistance among *Aspergillus* spp. (mostly investigated in *A. fumigatus* and *A. flavus*) is associated with single or multiple point mutations of the CYP*51* gene that encodes the enzymes 14-α-sterol demethylases A and B (Table 4; Espinel-Ingroff et al., 2018). These mutations have been linked with multiazole resistance or cross-resistance (high MICs) as well as patient failure during triazole treatment. Not much genetic information is available for other *Aspergillus* spp.

Reduced susceptibility to fluconazole and other triazoles has been widely reported for *Candida* non-*albicans*, such as *C. glabrata* (>15% resistant), *C. krusei* (intrinsically resistant to fluconazole), *C. lusitaniae*; triazole use for the treatment of such infections is precluded. *C. auris* has been recently reported as the cause of candidemia hospital outbreaks in various countries among neonates, elderly or patients having known risk factors for invasive candidiasis. The majority of *C. auris* isolates are fluconazole resistant or resistant to other agents (Clancy and Nguyen, 2017). Resistance and cross-resistance also has increased among *Aspergillus* spp., especially in Europe. Overall, antifungal susceptibility testing is recommended since species-specific clinical breakpoints are now available for fluconazole and voriconazole (but not for posaconazole, itraconazole, or isavuconazole) and most common *Candida* spp., but not for *C. glabrata* (CLSI document M60, 2017). Susceptibility testing should be also performed when the patient is failing therapy to rule out that is due to in vitro resistance. In the absence of clinical breakpoints, CLSI ECVs have been defined for *Aspergillus* spp. versus itraconazole, isavuconazole, posaconazole and voriconazole, some *Candida* spp. and the *C. neoformans-C. gattii* complex (Espinel-Ingroff and Turnidge, 2016; CLSI document M59). However, for the first time, the CLSI has recently

proposed breakpoints for *A. fumigatus* and voriconazole as follows: Susceptible: <0.5 μg/mL; intermediate: 1 μg/mL; and resistant: ≥ 2 μg/mL (CLSI Annual meeting minutes, January, 2019). The EUCAST has also established both ECVs and breakpoints for a variety of species/drug combinations (http://www.eucast.org/ast_of_fungi/). Although ECVs will distinguish WT from non-WT or mutant isolates when clinical breakpoints are not available, but should not be used when breakpoints are available for the drug/species combination being evaluated. More recently, ECVs have been defined for the widely used SYO commercial method for *S. cerevisiae* and 9 *Candida* spp. including prevalent (*C. parapsilosis* sensu stricto) and less prevalent species (*C. dubliniensis*, *C. guilliermondii*, *C. orthopsilosis*) and *S. cerevisiae* (Espinel-Ingroff et al., 2019). The posaconazole SYO ECV of 0.06 μg/mL for *C. albicans* and the Etest itraconazole. ECV of 2 μg/mL were the best predictors of non-wild type (mutant) isolates by this commercial method (Espinel-Ingroff et al., 2019).

Fluconazole

Fluconazole is a relatively small molecule (Fig. 1E) that is partially water soluble, minimally protein bound, and excreted largely as an active drug in the urine. It penetrates well into the CSF and parenchyma of the brain and the eye, and it has a prolonged half-life (up to 25 h in humans). Fluconazole pharmacokinetics are linear and independent on the route of administration and the drug formulation. This drug is well absorbed orally (its total bioavailability exceeds 90%), and its absorption is not affected by food or gastric pH. Plasma concentrations of 2–7 μg mL^{-1} are usually measured in healthy subjects after single doses of 100 and 400 mg. After multiple doses, the peak plasma levels are 2.5 times higher than those of single doses. The CSF to serum fluconazole concentrations are between 0.5 and 0.9% in both healthy human subjects and laboratory animals.

Fluconazole does not have in vitro or in vivo activity against most molds. Both oral and i.v. formulations of fluconazole are available for the treatment of candidemia in nonneutropenic and other nonimmunosuppressed patients, mucosal candidiasis (oral, vaginal, and esophageal), and chronic mucocutaneous candidiasis in patients of all ages (Pappas et al., 2016). Fluconazole is the current drug of choice for maintenance therapy of AIDS-associated cryptococcal and coccidioidal meningitis (Table 1; Perfect et al., 2010). However, since resistance to this drug can develop during therapy, fluconazole prophylaxis should be reserved for HIV-infected individuals or AIDS patients, or for patients with prolonged (2 weeks) and profound neutropenia (500 cells). Although the recommended dosage of fluconazole for adults is 100–400 mg each day (q.d.) higher doses (800 mg q.d.) are required for the treatment of severe invasive infections and for infections caused by a *Candida* spp. that exhibit a MIC of 8 μg mL^{-1}.

Intraconazole

In contrast to the imidazoles and itraconazole, fluconazole does not exhibit major toxicity side effects (2.8–16%). However, when the dosage is increased above 1200 mg, adverse side effects are more frequent (Table 2). Fluconazole interactions with other concomitant drugs are similar to those reported with other azoles, but they are less frequent than those exhibited by ketoconazole and itraconazole (Table 3). Fluconazole has been used to treat nasal aspergillosis and penicilliosis in small animals and birds when topical enilconazole is not feasible.

In contrast to fluconazole, itraconazole is insoluble in aqueous fluids; it penetrates poorly into the CSF and urine but well into skin and soft tissues; and it is highly protein bound (90%). Its structure is closely related to that of ketoconazole (Fig. 1F), but itraconazole has a broader spectrum of in vitro and in vivo antifungal activity. Similar to ketoconazole, itraconazole is soluble only at low pH and is better absorbed when the patient is not fasting. Absorption is erratic in cancer patients or when the patient is taking concomitant H_2-receptor antagonists. Plasma peak (1.5–4 h) and trough concentrations between 1 and 2.2 μg mL^{-1} and 0.4 and 1.8 μg dosages (per orally, p.o.) or twice daily (b.i.d.) or after i.v. administration (b.i.d.) for 2 days and q.d. for more days; these concentrations are also obtained in cancer patients receiving 5 mg kg^{-1}.

Clinically, itraconazole (200–400 mg/day, capsule) is indicated for endemic, non-life-threatening mycoses and as a prophylactic agent (Table 1); it is one the agents used for the treatment of sporotrichosis infections. For more severe mycoses (invasive yeast and mold infections), higher doses are recommended and clinical resistance may emerge. Monitoring of itraconazole plasma concentrations is recommended during treatment, drug concentration 0.5 μg mL^{-1} by high performance liquid chromatography and 2 μg mL^{-1} by bioassay appear to be critical for favorable clinical response (Pappas et al., 2016; Perfect et al., 2010). In summary, a lower through concentration of 0.25 mg/L (prophylaxis target) or 0.5 mg/L (treatment target) could be the lower effective limits. On the other hand, trough of 1.0 mg/L is associated with increased hepatotoxicity and other adverse events.

But, both i.v. and oral solution formulations have improved its solubility and enhance drug absorption and bioavailability (consistent plasma concentrations) versus capsule formulation. The additional flexibility offered by the different routes of administration means that itraconazole treatment can be used in all patient populations, including children and those requiring intensive care. The oral solution is useful for the treatment of HIV-associated oral and esophageal candidiasis. Treatment with itraconazole has been associated with 10% adverse and mostly transient side effects (Table 2), and these effects are usually observed when the patient takes up to 400 mg during several periods of time.

In animals, itraconazole has prescribed for the treatment of aspergillosis, crytococcosis, blastomycosis (especially in dogs), equine sporotrichosis, and osteomyelitis caused by *Coccidiodis immitis* in large animals, but its use is minimal. No data are available regarding its side effects or drug interactions in animals.

Itraconazole in combination with Pulmazole™ (PUR1900)

Recently, Pulmazole™ (PUR1900) is an inhaled dry powder and oral itraconazole combination that was evaluated in an immunocompromised guinea-pig model of aspergillosis. The animals received either placebo, or PUR1900, or oral itraconazole.

After 10 days of treatment, which began the day after the animals were infected, the drug exposure and animal survival were better among the animals treated with PUR1900 than those treated only with oral itraconazole. So this combined therapy is promising for the treatment of pulmonary aspergillosis and perhaps more advantageous than oral itraconazole (Curran et al, 2018; Advances Against Aspergillosis, Lisbon, Portugal).

Voriconazole (UK-109496)

Voriconazole is a triazole related to fluconazole obtained by replacement of one triazole moiety by fluoropyrimidine and α-methylation groups (Fig. 3A); it is insoluble in aqueous fluids. As do the other azoles, voriconazole acts by inhibiting fungal cytochrome P450-dependent, 14-α-sterol demethylasemediated synthesis of ergosterol. Voriconazole pharmacokinetics in humans are nonlinear and dose dependent. Following single oral doses, peak plasma concentrations were achieved after 2 h and multiple doses resulted in a higher (eight times) accumulation. The mean half-life of elimination is about 6 h. Voriconazole binds to proteins (65%), is extensively metabolized in the liver (80%), and is found in the urine (78–88%) practically unchanged after a single dose. Voriconazole has good oral bioavailability, distributes widely into tissues including those of the central nervous system. It does not have activity against the Mucorales, certain isolates of *Candida rugosa*, *Sporothrix schenckii* and a significant number of *Rhodotorula* strains, especially *R. muciloginosa*. Clinically, it has been approved for the primary treatment of invasive aspegillosis, salvage therapy for other mold infections caused by *S. apiospermum* and *Fusarium* spp., candidemia and other infections caused by *Candida* spp. (Table 1; Pappas et al., 2016; Patterson et al., 2016). Breakthrough infections caused by the members of the Mucorales have been reported.

Although the drug is generally well tolerated with only few side effects (hepatic [10–15%], transient visual [10–15%] and skin rash [1–5%]); non-melanoma skin cancer has been associated with voriconazole exposure shortly after the drug was approved by the US Food and Drug Administration, among organ (especially lung) transplant recipients taking voriconazole for prophylaxis and/or treatment of invasive fungal infections. (Table 2; Williams et al., 2014). The control of underlying patient characteristics is essential and patients should be educated about their increased risk of skin cancer. Screening for skin cancer and considering alternatives to voriconazole for patients at risk are highly recommended. For reported drug interactions see Table 3.

Posaconazole (SCH 56592)

Posaconazole is the product of a modification of the n-alkyl side chain of SCH 51048, which included a variety of chiral substituents (Fig. 3B). Posaconazole absorption from the intestinal tract is slow and peak serum concentrations are achieved 11–24 h after the actual dose. Posaconazole exhibits dose-proportional absorption up to 800 mg; dividing the dose and food or liquid supplements

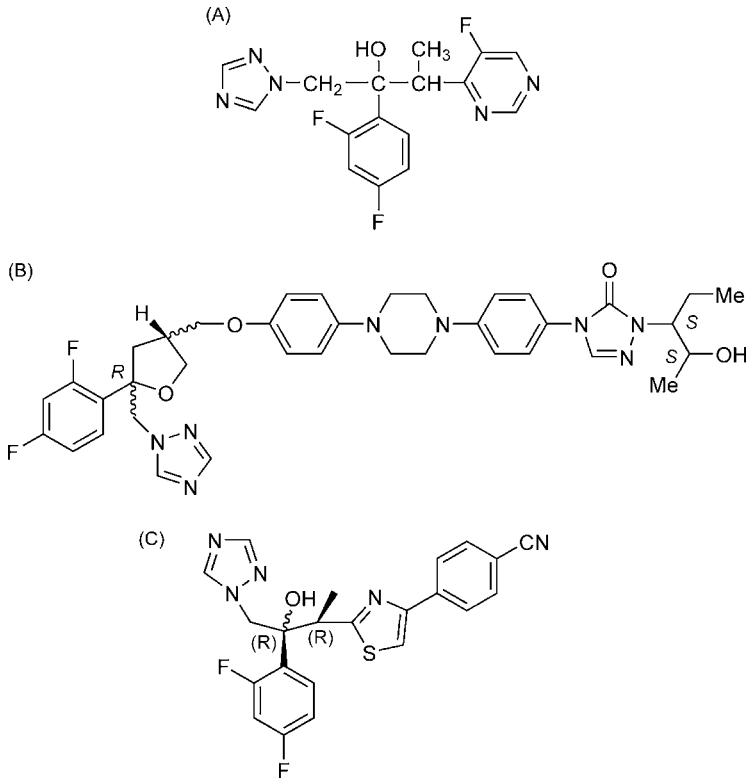

Fig. 3 Chemical structures of three triazoles: (A) voriconazole, (B) posaconazole, and (C) ravuconazole (BMS-207147; ER-30346).

increase exposure. It has a large volume distribution (1774 L) and it is highly protein bound (>98%). Posaconazole has a prolonged elimination half-life (20–66 h); it is not extensively metabolized (approximately 14%) and is eliminated unchanged in the feces (66%). The in vitro fungistatic and fungicidal activities of posaconazole are similar to those of voriconazole and amphotericin B against yeasts, the dimorphic fungi, most opportunistic molds including the Mucorales, certain phaeoid fungi, and the dermatophytes.

Three posaconazole formulations (NOXAFIL, developed by Merck) have been approved by the FDA in USA for prophylaxis against invasive *Aspergillus* and *Candida* infections in high-risk patients (severely immunocompromised hematopoietic stem cell transplant [HSCT]) recipients with graft-versus-host disease (GVHD) or those with hematologic malignancies with prolonged neutropenia (low white blood cell counts) from chemotherapy: (i) an oral delayed-release tablet (100 mg; loading dose of 300 mg [three 100 mg tablets] twice daily on the first day, followed by a once-daily maintenance dose of 300 mg [three 100 mg tablets]); (ii) an i.v. formulation (18 mg/mL; for patients 18 years of age and older); and (iii) an oral suspension (40 mg/mL, dosed three times daily for patients 13 years of age and older). The tablet has higher bioavailability and more importantly it can be administered independent of food ingestion. The i.v. formulation will be beneficial for patients who are intolerant to oral posaconazole or patients who may need i.v. administration for other reasons. However, the incidence of fungal infections was not significantly different between patients receiving the tablet versus the oral suspension formulations (>500 patients) (Foruno et al., 2018). The most frequently reported adverse reactions are diarrhea (32%), hypokalemia (22%), fever (21%), increased hepatic enzymes and nausea (19%) (Table 2). Inducers of the UDP glucuronidation pathway may affect posaconazole plasma concentrations (Table 3). Posaconazole is generally well tolerated and serious adverse effects (most common, altered drug level, nausea, rash, and vomiting) have occurred in 8% of patients (http://www.mercknewsroom.com/news-release/prescription-medicine-news/fda-approves-mercks-noxafil-posaconazole-injection-18-mgml-I#sthash.kxV0QM7t.dpuf).

Isavuconazole (BAL8557)

Isavuconazole (isavuconazonium sulfate, Cresemba®, Astellas Pharmaceuticals US, Inc., Northbrook, IL, USA) is a water soluble triazole that has been recently approved for fungal treatment. Isavuconazole inhibits ergosterol biosynthesis (enzymes 14-α-sterol demethylases A and B which are encoded by the CYP*51A* and *B* genes) and has a similar in vitro activity as the other triazoles against *Aspergillus* spp., other molds, including the Mucorales (zygomycetes), as well as *Candida* spp. and *C. neoformans* (Table 1). The global double-blind randomized clinical trial (516 patients) evaluated the safety and efficacy of once-daily isavuconazole versus twice-daily voriconazole for the primary treatment of invasive infections caused by *Aspergillus* spp. and other molds. Although isavuconazole was found to be non-inferior to voriconazole for the treatment of invasive fungal infections, it was documented that it had fewer drug-related adverse effects. Based on these results, the US FDA approved isavuconazole for the primary treatment of invasive aspergillosis and mucormycosis in adults; the European Medicines Agency (EMA) also approved this agent for the same infections in patients for whom amphotericin B is inappropriate. The licensed dosage is 200 mg three times a day for the first 2 days, then 200 mg daily (either i.v. or oral delivery). At steady state, the Cmin is 3.9 ± 2 mg/L. Because it has activity against the Mucorales molds, e.g., the species of *Rhizopus*, *Mucor* and *Cunninghamella*, it could be used as a supplement of the medical-surgical treatment therapy for these infections https://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/ucm430748.

The oral formulation has 98% bioavailability and its absorption is not influenced by food; the i.v. formulation lacks the solubilizing agent used for other azoles, cyclodextrin, which is associated with nephrotoxicity in patients with renal insufficiency. Although and unlike the other azoles, isavuconazole does not appear to exacerbate QT prolongation, "it can shorten the cardiac QT interval in a dose-related manner" and it is contraindicated in patients with familial/congenital short QT syndrome. Compared to voriconazole, isavuconazole has linear pharmacokinetics and is less prone to be the perpetrator drug or victim drug in the setting of drug–drug interactions with concomitantly administered drugs. Isavuconazole adverse events were statistically fewer than those of voriconazole (hepatobiliary, skin and eye disorders) and the most common events were nausea, vomiting, pyrexia (fever) and diarrhea (Desai et al., 2016; Keirns et al., 2017) (Table 2). Reports of cross reactivity of isavuconazole in patients with a history of acute liver injury from another azole are few but it is recommended that therapy should be started with caution as well as appropriate monitoring (NHI website, antifungal agents).

Due to the similarity of voriconazole and isavuconazole MICs, it has been concluded that the use of high doses of isavuconazole might be an option in patients infected with an *A. fumigatus* isolate for which an isavuconazole MIC of 2 μ/mL has been obtained, a value that is above the EUCAST susceptible endpoint of 1 μ/mL (Buil et al., 2018).

Terconazole

Terconazole was the first triazole marketed for the topical treatment of vaginal candidiasis and superficial dermatophyte infections (Table 1). Currently, it is only used for vulvovaginal candidiasis (0.4% and 0.8% vaginal creams and 80 mg vaginal suppositories).

Investigational triazoles

PCI244

The new triazole PC1244 was designed for topical or inhaled administration against *A. fumigatus* infections. It has potent in vitro activity against a variety of fungal pathogens including *A. fumigatus* WT and mutant isolates, *R. oryzae*, and *C. neoformans*. In vivo, when given once daily via intranasal administrations (3.2–80 μg/mL) to temporarily neutropenic mice infected with *A. fumigatus*,

reduction of fungal load in the lung, galactomannan in serum and circulating inflammatory cytokines were observed. Given those results, it has potential as a novel treatment of lung *A. fumigatus* infections (Colley et al., 2018).

Other triazoles

The following triazoles were under clinical investigation, but are not longer being developed: saperconazole (R 66905), BAY R 8783, SCH 39304, SCH 51048, BAY 3783, and SDZ 89-485 were discontinued from further development due a variety of adverse side effects. Although lbaconazole (CAS 187949-02-6) reached Phase II clinical trials, it does not appear to be under further development.

Cell Membrane as Antifungal Target: VT-1129, VT-1598 and VT-1161

VT-1129, VT-1598 and VT-1161 (Viamet Pharmaceuticals) are novel class of metalloenzyme inhibitors that, like the azoles and triazoles, inhibit the enzyme responsible for the final step of ergosterol biosynthesis, the fungal 14α-ergosterol demethylase (CYP51). The idea was to have molecules than are more specific for the fungal than the human CYP51 enzyme. It is expected that the selectivity for fungal cells may decrease the risk for toxicity at high doses of drug. VT-1129 is more selective for *Cryptococcus* cells and VT-1161 is for *Candida* cells. On the other hand, VT-1598 is in preclinical development for treatment of coccidioidomycosis. VT-1161 has completed Phase 2b clinical trials for the treatment of recurrent vulvovaginal candidiasis (RVVC) and onychomycosis (www.viamet.com).

The Allylamines

The allylamines act by inhibiting squalene epoxidase, which results in a decrease in the ergosterol content and an accumulation of squalene.

Terbinafine and Naftifine

Terbinafine (Fig. 2D) is the most active derivative of this class of antifungals. It has an excellent in vitro activity against the dermatophytes and other filamentous fungi, but its in vitro activity against the yeasts is controversial. It follows linear pharmacokinetics over a dose range of 125–750 mg; drug concentrations of 0.5–2.7 μg mL^{-1} are detected 1 or 2 h after a single oral dose. Terbinafine has replaced griseofulvin and ketoconazole for the treatment of onychomycosis and other infections caused by dermatophytes (oral and topical). It is also effective for the treatment of vulvovaginal candidiasis. It is usually well tolerated at oral doses of 250 and 500 mg per day and the side effects (10%) are gastrointestinal and cutaneous. The metabolism of terbinafine may be decreased by cimetidine and increased by rifampin. Pharmacokinetics and poor activity have limited the use of naftifine to topical treatment of dermatophytic infections.

The Benzylamines, Thiocarbamates, and Dithiocarbamates

The benzylamine, butenafine, and the thiocarbamates, tolnaftate, tolciclate, and piritetrade, also inhibit the synthesis of ergosterol at the level of squalene. Their clinical use is limited to the topical treatment of superficial dermatophytic infections. The Bordeaux mixture (reaction product of copper sulfate and lime) was the only fungicide used until the discovery of the dithiocarbamate fungicides in the mid-1930s. Of those, mancozeb and thiram are widely used in agriculture, but because they are only surface-acting materials frequent spray applications are required. Ferbam, maneb, and zineb are not used as much.

The Benzimidazoles and Methylbenzimidazole Carbamates

A great impact on crop protection was evident with the introduction of the benzimidazoles and other systemic (penetrate the plant) fungicides. These compounds increased spray intervals to 14 days or more. The methylbenzimidazole carbamates (MBCs; carbendazim, benomyl, and thiophanate) inhibit nuclear division and are also systemic agricultural fungicides. However, since MBC-resistant strains of *Botrytis cinerea* and *Penicillium expansum* have been isolated, these compounds should be used in combination with *N*-phenylcarbamate or agents that have a different mode of action.

The Morpholines

The morpholines interfere with 14 reductase and 7,8 isomerase enzymes in the ergosterol biosynthetic pathway, which leads to an increase in toxic sterols and an increase in the ergosterol content of the fungal cell.

Amorolfine

Amorolfine, a derivative of fenpropimorph, is the only morpholine that has a clinical application for the topical treatment of dermatophytic infections and candidal vaginitis.

Fenpropimorph, Tridemorph, and Other Morpholines

Protein binding and side effects have precluded the clinical use of these morpholines, but they are important agricultural fungicides.

The Pyridines (Pyrifenox and Buthiobate)

The pyridines are another class of antifungal agents that inhibit lanosterol demethylase; pyrifenox and buthiobate are agriculture pesticides.

The Echinocandins, Pneumocandins, and Papulocandins

The echinocandins and papulocandins are naturally occurring metabolites of *Aspergillus nidulans* var. *echinulate* (echinocandin B), *A. aculeatus* (aculeacin A), and *Papularia sphaerosperma* (papulocandin). They act specifically by inhibiting the synthesis of fungal (1,3)-glucan synthetase, which results in the depletion of glucan, an essential component of the fungal cell wall.

The Papulocandins

The papulocandins A–D, L687781, BU4794F, and chaetiacandin have in vitro activity only against *Candida* spp. but poor in vivo activity, which precluded clinical development.

The Echinocandins and Pneumocandins

Echinocandin resistance, molecular mechanisms of resistance

The echinocandins include echinocandins, pneumocandins, aculeacins, mulundo- and deoxymulundocandin, sporiofungin, vWF 11899 A–C, and FR 901379. Among them, the echinocandins have better in vitro and in vivo antifungal activity than the papulocandins and pneumocandins. Anidulafungin, caspofungin and micafungin are indicated for the treatment and/or prophylaxis of candidemia in nonneutropenic patients, esophageal candidiasis, and/or salvage therapy for aspergillosis, especially in patients who had been treated with an azole and/or for infections caused by *C. glabrata* and *C. krusei*. Currently, another two echinocandins are under development for vulvovaginal, invasive candidiasis and aspergillosis (Ibrexafungerp [SCY-078] and rezafungin [formerly CD101]) as well as potential prophylactics for aspergillosis in immunocompromised patients (rezafungin) (Table 1) (Sandison et al., 2017; Sofjan et al., 2018; https://www.scynexis.com/pipeline). Other applications are provided under the description of each agent when applicable, but higher doses are recommended for patients with endocarditis and other cardiovascular infections, e.g., caspofungin 50–150 mg/day, micafungin 100–150 mg/day, or anidulafungin 100–200 mg/day. Although *C. parapsilosis* and *C. guilliermondii* have less in vitro susceptibility to the three echinocandins than other *Candida* species, the concern that *C. parapsilosis* infections may be less responsive to echinocandin therapy has not been clearly demonstrated. These three agents are not effective for the treatment of infections caused by *C. neoformans*, *T. beigelii*, *Fusarium* spp., and the Mucorales.

Echinocandin resistance has been reported in patients infected with *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei*. Echinocandin resistance to *C. glabrata* has increased from 4.9% to 12.3% between 2001 and 2010 including reports of cross-resistance to both echinocandins and azoles. Such in vitro and clinical resistance has been associated with point mutations in the *FKS1* and/or *FKS2* genes (Table 4; Spampinato and Leonardi, 2013) which encode the glucan synthase complex. In contrast to *Candida* spp., the relationship between *FKS*1 gene mutations and high caspofungin and other echinocandins MECs (minimal effective concentrations) for *Aspergillus* spp. is yet to be determined. Caspofungin MECs of ≥ 1 μg/mL for *A. fumigatus* were obtained from breakthrough infections in patients receiving this agent. Although EUCAST clinical breakpoints and/or CLSI ECVs are available for the three licensed echinocandins and most common *Candida* spp. (including *C. glabrata*), antifungal susceptibility testing of caspofungin by both reference methods is not recommend for *Candida* spp. due to interlaboratory variability problems. However, ECVs for the widely used Sensititre YeastOne colorimetric broth microdilution method have been proposed for most common *Candida* species and the three echinocandins (Espinel-Ingroff et al., 2015). As far as the filamentous fungi, EUCAST and CLSI categories are available for some *Aspergillus* spp. (CLSI document M59, 2018; Espinel-Ingroff and Turnidge, 2016; www.eucast.org).

Anidulafungin (VER-002; LY303366, V-Echinocandin)

Anidulafungin is a semisynthetic cyclic lipopeptide which resulted from an increase of aromatic groups in the cilofungin sidechain (Fig. 4A). It has high potency and oral and parental bioavailability. In laboratory animals, peak levels in plasma (5 or 6 h) of

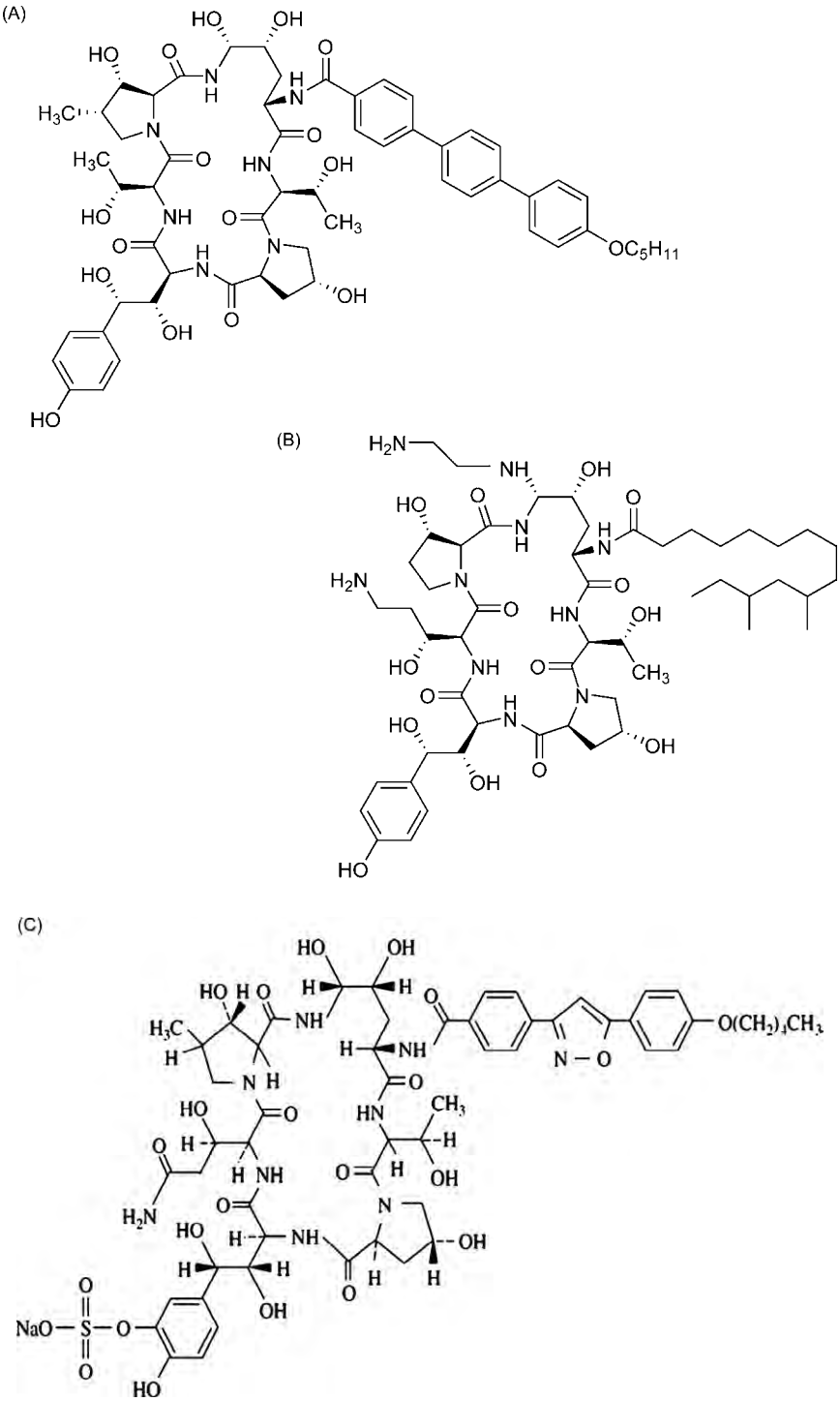

Fig. 4 Chemical structures of (a) anidulafungin (VER-002; (b) V-echinocandin LY303366), (c) caspofungin (L 743872 or MK-0991), micafungin.

0.5–2.9 μg mL^{-1} have been measured after single doses of 50–250 mg/kg. In humans, peak levels of 105–1624 ng mL^{-1} are measured after oral administrations of 100–1000 mg/kg. Anidulafungin pharmacokinetics are linear with a short distribution half-life (0.5–1 h) and a volume distribution of 30–50 L. The terminal elimination half-life is about 40–50 h and clearance is about 1 L/2 h. Tissue concentrations are usually higher than those in plasma in animals. Systemic exposures of anidulafungin (post i.v. doses) are dose proportional and this agent is moderately protein bound in humans.

Anidulafungin has good in vitro activity against a variety of yeasts, including isolates resistant to itraconazole and fluconazole, and *Aspergillus* spp. MECs for certain molds are higher than those of the azoles, but its fungicidal activity against some species of *Candida* is superior to those agents. Laboratory abnormalities in liver functions have been observed, but no clinically relevant drug–drug interactions have been observed with drugs likely to be co-administered with anidulafungin.

Caspofungin (MK-0991 or L743872)

Caspofungin (Fig. 4B) is the product of a modification of L733560 and was selected for evaluation in clinical trials in humans. Caspofungin is water soluble, highly protein bound (97%) with a half-life that ranges from 5 to 7.5 h and drug concentrations are usually higher in tissue than in plasma. As for anidulafungin and micafungin, caspofungin exhibits favorable dose dependent linear pharmacokinetics and only i.v. formulations are available. Caspofungin has fungistatic and fungicidal activities similar to those of anidulafungin against most *Candida* spp. and lower activity against the dimorphic fungi. It also has fungistatic in vitro activity against some of the other molds, especially *Aspergillus* spp. The drug is mostly well tolerated, but histamine release, mild hepatotoxicity, fever, nausea, vomiting, and rash have been reported (Table 2). Caspofungin (70 mg loading and 50 mg maintenance doses) has been approved for refractory invasive aspergillosis or for patients intolerant to other agents and empiric therapy for febrile neutropenic patients unresponsive to antibacterial therapy (Table 1). Caspofungin has been evaluated for the prophylaxis of invasive candidiasis; the differences between the drug and placebo were 16.7% (14/84 patients) versus 9.8% (10/102 patients), respectively. The three echinocandins have been recommended for this purpose only as an alternative to fluconazole (*Candida* infections). (Pappas et al., 2016).

Micafungin (FK 463)

Micafungin is a semisynthetic derivative of a naturally occurring lipoprotein that was synthesized by a chemical modification from a product of the mold *Coleophora impedir*. As the other echinocandins, it has in vitro activity against *Candida* and *Aspergillus* species. The drug is well tolerated (Table 2). It is protein binding (99%) and plasma concentrations attain a steady state by day 4 with repeated doses. Micafungin has been approved for the prophylaxis of *Candida* infections in patients undergoing HSCT (50 mg daily maintenance) (Table 1). There have been reports of clinical failures and breakthrough infections and high MICs in patients receiving micafungin therapy due to the development of drug resistance associated in some cases with specific mutations in the FKS protein component of the glucan synthase enzyme.

Ibrexafungerp

Ibrexafungerp (Scynexis, NJ, USA; formerly SCY-078) is a triterpenoid agent that also inhibits the beta 1,3 glucan synthase enzyme. It is the first oral glucan synthase inhibitor derivative of the natural product enfumafungin under development for the treatment of systemic candidiasis, including candidemia, and aspergillosis. Glucan synthase inhibitors have been effective in treating invasive fungal infections in a hospital setting, but currently are only available as an intravenous dosing option. Ibrexafungerp has in vitro activity against *Candida* spp. (including FKS mutants and *C. auris* biofilm). In a model of neutropenic rabbits, this agent compared well with isavuconazole for the treatment of experimental invasive aspergillosis. In vivo activity was also demonstrated in a murine model of invasive aspergillosis when animals were infected with both wild type and mutant *A. fumigatus* isolates. Phase II clinical trial for vulvovaginal candidiasis (VVC) indicated that the 600 mg dose of ibrexafungerp (given as two doses of 300 mg 12 h apart) is the optimal dose and it was a well-tolerated regimen that provided overall strong clinical and mycological outcome effect when compared to fluconazole. Preliminary data of a second Phase II clinical trial indicated that this agent also has good activity against invasive candidiasis with few drug interactions as compared with fluconazole (no effects on tacrolimus or rosiglitazone); data are not yet available for the comparison with micafungin. In addition, it has demonstrated synergistic activity in combination with isavuconazole for the treatment of invasive pulmonary in neutropenic rabbits ("Advances against aspergillosis", Lisbon, Portugal, February 2018; https://www.scynexis.com/pipeline). However, the development of the IV formulation has been delayed.

Rezafungin

Rezafungin acetate is another new echinocandin under clinical evaluation (Cidara Therapeutics; formerly CD101 IV). The safety and pharmacokinetic of rezafungin acetate was evaluated in a dose-escalation study (50 to 400 mg once a week) in healthy adults who were given the drug over 1 h. A higher incidence of mild and transient adverse side effects and transient infusion reactions in the 400-mg dose group than those in 200-mg dose were reported (Table 2). The efficacy of rezafungin versus daily standard caspofungin is being evaluated in a Phase II study for candidemia and/or invasive candidiasis-bridging extension (STRIVE: IV 400 mg rezafungin once weekly for up to 4 weeks or 400 mg on week 1 and 200 mg thereafter). The data indicated that rezafungin is safe and effective at separate doses regarding both clinical and microbiological outcome. In a model of *C. auris* candidiasis, this agent also showed antifungal activity as compared to those of amphotericin B and micafungin. Due to the in vitro activity against azole-resistant *Aspergillus*, the high concentrations of rezafungin (including isolates harboring TR34/L98H mutations) distributing into the lungs and the rationale for dose selection also indicated the potential use of this agent for the prevention of *Aspergillus*, *Candida* and

Pneumocystis infections in BMT patients (Sandison et al., 2017; Sofjan et al., 2018; Hager et al., 2018; clinicaltrials.gov/ct2/show/study/NCT02734862). The Phase III program of rezafungin clinical trials will address the efficacy of this agent for difficult-to-treat, invasive *Candida* infections.

Glycosylphosphatidylinositol Biosynthesis Inhibition Inhibitors (*GPI*) for Clinical Use

E1210/APX001

E1210/APX001, discovered by Eisai, Ltd., Japan, has entered Phase 1 to Phase II clinical trials under Amplyx Pharmaceuticals, Inc. It inhibits the inositol acylation step in fungal GPI biosynthesis, interfering with the fungal cell wall growth, hyphal elongation, and fungal attachment to the biological substrates. It has a broad spectrum of in vitro activity against both species of *Fusarium* and *Scedosporium* (including *Lomentospora. prolificans*), *Paecilomyces lilacinus* and other species usually resistant to triazoles and polyenes. It also has demonstrated efficacy in murine models of oropharyngeal and disseminated candidiasis and fusariosis (*F. solani*) as well as pulmonary aspergillosis (*A. fumigatus* or *Aspergillus flavus*). In rats, it was well tolerated at 100 mg/kg and up to 300 mg/kg (Osherov and Kontoyiannis, 2017; McCarthy et al., 2017).

Gepinacin

Gepinacin is a monocarboxylic amide that inhibits acyltransferase, an enzyme that anchors GPI during its biosynthesis producing stress to the endoplasmic reticulum. Gepinacin does not affect the viability of mammalian cells and it has been reported that increases the immunogenicity against *C. albicans* (Osherov and Kontoyiannis, 2017).

Fungal Mitochondria Inhibitor

T-2307

T-2307 (Toyama Chemical Co., Tokyo, Japan) is an arylamidine small molecule similar to pentamidine. Its target is the fungal mitochondria (*Candida* cells) leading to membrane loss. T-2307 exhibits potent in vitro as well as in vivo activity in murine models of *C. albicans* systemic infection (100% two-week survival, 0.02 mg/kg), *C. neoformans* (>70% two-week survival, 0.3 mg/kg), and *A. fumigatus* (>80% two-week survival, 1 mg/kg). In Phase I clinical trials in healthy volunteers, T-2307 demonstrated good safety and tolerability, and pharmacokinetics (https://clinicaltrials.gov/ct2/show/NCT02289599; Osherov and Kontoyiannis, 2017).

Zinc Inhibitors

ZAC307 and ZAC989

Two novel zinc27 attenuating compounds, ZAC307 and ZAC989, have shown both in vitro and in vivo antifungal activity in a kidney candidiasis model. Genetic analysis suggested that these ZAC molecules interfered or obstructed the expression of the genes encoding the major components of *A. fumigatus* zinc uptake system, suggesting the disruption of zinc homeostasis as their likely mode of action (Cohrt et al., 2018).

The polyaminocarboxylate molecule

Of a total of 116 molecules that had inhibitory effects on fungal growth, six molecules were further screened. Although all had good in vitro antifungal activity, only one of the polyaminocarboxylates molecules significantly improved in vivo survival in an immunosuppressed murine model of pulmonary aspergillosis. This small molecule also showed low toxicity and it could become a new treatment for invasive fungal infections. (See Laskaris et al., 2018).

Acylhydrazones

The BHBM D13 derivative

The acylhydrazone BHBM derivatives target the fungal sphingolipid pathway. Of the total of 19 derivatives of BHBM that have been screened, three of them had specific in vitro activity against *C. neoformans* and toxicity was low against mammalian cells. Of those three, the molecule denominated D13 showed better in vivo activity in various experimental models (cryptococcosis, candidiasis and pulmonary aspergillosis), suitable pharmacokinetic properties and was able to pass through the blood brain barrier (See Lazzarinia et al., 2018).

The Orotomides

Olorofim (F901318)

Olorofim (F901318; F2G Limited, UK) is the first in a new class of antifungal agents called the orotomides. It inhibits the enzyme dihydroorotate (*DHODH*/*URA1*) catalyzing the fourth enzymatic step of pyrimidine biosynthesis. Although it has shown good

in vitro activity (MICs mostly below 1 μg/mL) against azole-resistant molds, *Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., and the dimorphic fungal species, it did not show activity against the Mucorales or *Candida* spp. It was effective in a neutropenic model of murine *A. terreus* infection. Phase I clinical trials indicated that a single dose of olorofim administered IV at up to 4 mg/kg was well tolerated and no adverse effects were observed. Although it was announced that this agent could be moving to Phase IIa studies for the prophylaxis (in combination with caspofungin) of neutropenic patients with acute myeloid leukemia, no data are available yet (Osherov and Kontoyiannis, 2017; Lackner et al., 2018).

Other Approach to Antifungal Drug Development

Salicylanilide and niclosamide compounds

Recently, a new approach to antifungal therapy was investigated. A total of 678 small molecules were evaluated for their potential antifungal activity against the invasive hyphal form of *C. albicans*. Among those, the halogenated salicylanilide (N1-[3,5-dichlorophenyl]-5-chloro-2-hydroxybenzamide) and niclosamide compounds exhibited antifungal activity against resistant *C. albicans* hyphae and biofilms, as well against *C. auris*. In addition, niclosamide protected the intestinal epithelial cells from *C. albicans* tissue invasion. Since the safety of niclosamide is well established in humans as an FDA-approved anthelmintic, this therapeutic could become the first clinically approved fungal anti-virulence agent (Garcia et al., 2018).

Dimethomorph and fluazinam

Dimethomorph is a cinnamic acid derivative for use against *Plasmopara viticola* on vines and *Phytophthora infestans* on tomatoes and potatoes; it is not cross-resistant to phenylamides (systemic controllers of *Phycomycetes* plant infections). Fluazinam is used in vines and potatoes but also acts against *Botrytis cinerea* as an uncoupler of oxidative phosphorylation.

The phthalimides

The discovery of captan in 1952 and later of the related captafol and folpet initiated the proper protection of crops by the application of specific fungicides. Captan is also used to treat dermatophytic infections in horses and cattle, but it causes skin sensitization in horses.

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Antigenic Variation in Bacterial Pathogens

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Glossary

Antigenic variation The process by which pathogens change the sequence of their antigenic molecules to evade clearance by the host immune response during the course of an infection.
Epigenetic modification Alterations made to DNA, usually by methylation or alteration of chromatin structure, which effect the expression of a gene but do not change its genetic sequence. These changes in expression patterns are heritable.
Gene conversion Whole or partial gene sequences, termed donor sequences, are copied from one unexpressed location in the genome to another expressed location, termed the acceptor gene. The donor gene sequence remains unchanged. Also called duplicative homologous recombination or duplicative transposition.
Gene inversion The reversible switching of a promoter element by specific recombinases that either turn expression of a gene "on" or "off", depending on the orientation of the promoter.
Phase variation The reversible switch between "on" and "off" expression phases by genetic or epigenetic mechanisms in which the expression phase in heritable by daughter cells.
Reciprocal recombination The mutual exchange of genetic sequences between donor and expression sites.

Mechanisms of Antigenic Variation

Gene Conversion

Gene conversion is the best studied and most widely used genetic mechanism that microorganisms employ to achieve antigenic variation of their surface proteins. Microorganisms that undergo this mechanism can encode up to hundreds of copies of one gene, each of which has nucleic acid differences in what are termed "variable regions" of their nucleotide sequence. Typically, one copy of the gene is expressed at a time under the control of a single promoter, which is termed the acceptor sequence. Silent copies of the gene or smaller variable regions of the gene, which lack a promoter and are termed functional pseudogenes or donor sequences, are encoded at another location in the genome. Recombination events occur in which the acceptor copy of the gene is replaced in whole or in part by one of the donor copies by the process of duplicative homologous recombination. The genetic exchange is unidirectional in that the sequence of the expressed acceptor gene copy is changed while the sequence of the silent donor copy remains unchanged. Specifically, the donor strand invades the acceptor sequence and a heteroduplex is formed. Mismatch repair and synthesis of the complementary sequence then occurs to replace the acceptor sequence with the new donor sequence, resulting in a new expressed copy of the gene and leaving the donor site unchanged (Fig. 1(A)). A few examples and variations of the gene conversion mechanism in different pathogenic organisms are illustrative of the process.

A classic example of antigenic variation in bacteria is pilin antigenic variation in *Neisseria gonorrhoeae*. *N. gonorrhoeae* infects the urogenital tract to cause gonorrhea, and the type IV pilus is important for successful infection by mediating adherence to epithelial cells and its role in host cell signaling and twitching motility. The major subunit of the type IV pilus is encoded by *pilE*, which displays one of the highest rates of reported antigenic variation in bacteria. PilE antigenic variation occurs by the mechanism of segmental gene conversion. The *N. gonorrhoeae pilE* gene is expressed from one to two loci and undergoes unidirectional exchange of genetic sequence with silent truncated variant copies found elsewhere in the chromosome within what are called *pilS* loci. There are 5 different *pilS* loci, each of which contains 17–19 silent copies of *pilE*. The conserved N-terminal domain of *pilE* is not encoded in the silent *pilS* cassettes and remains unchanged after the recombination event. The hypervariable loop region and the hypervariable tail region encoded further downstream in *pilE* display the highest rate of sequence variation in the silent *pilS* copies, with 1–200 bp of sequence flanked by 3–68 bp of homologous regions transferred during each recombination event. In addition to recombination between the *pil*E gene and *pilS* copies elsewhere on the chromosome (intragenic recombination), *intergenic* recombination may also occur between *pilE* and *pilS* copies within genomic DNA taken up from lysed gonococci.

Further investigation of the mechanism of *pilE* antigenic variation revealed that components of the homologous recombination machinery as well as several other elements are required for recombination between *pilE* and the *pilS* copies. The RecA, RecO and RecR proteins, which are involved in homologous recombination, are required for antigenic variation of *pilE* as are the two *cis*-acting sequences termed the Sma/Cla repeat, a 65 bp sequence that may provide an extended region of homology for the recombination event, and the G4-forming sequence, which is a 16 bp G-rich sequence that forms a guanine quartet structure that may initiate the homologous recombination event at the *pilE* locus. Formation of the G4-quartet structure requires transcription of a small RNA that initiates its transcription within the G4 structure-forming sequence to activate formation of the G4-quartet structure. Antigenic variation of the *pilE* gene sometimes results in pilus phase variation (the turning 'on' or 'off' of gene expression or functional protein

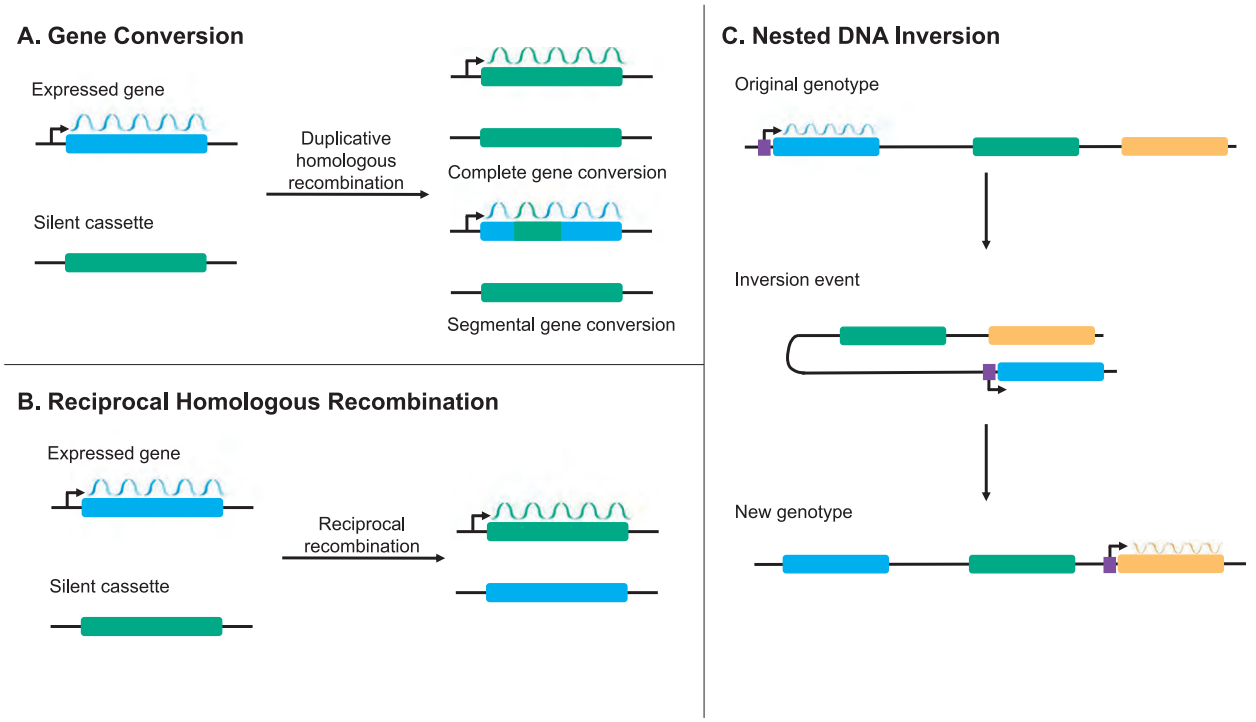

Fig. 1 Mechanisms of antigenic variation. Antigenic variation of bacterial immunogens can be achieved through several different mechanisms. (A) Gene conversion uses unexpressed gene cassettes located elsewhere in the genome to copy a new sequence variant of a gene, either wholly are partially, into the expression site with a promoter (arrow). (B) Reciprocal homologous recombination is related to gene conversion, except the silent variant gene and the original expressed gene switch places in the expression and silent sites after the recombination event. (C) Nested DNA inversion transposes the promoter instead of the coding region of the gene to a new site within a contiguous array of gene variants to achieve expression of a new variant. A recombinase site is encoded within the conserved sequence (purple box), which also contains the promoter (arrow), upstream of each oppositely oriented gene variant. To achieve transfer of the conserved sequence with the promoter, the recombinase sites are aligned and the DNA between the two recombinase sites is reversed, which results in the rearrangement of the cassettes and the inversion of the promoter to drive expression of a new gene variant. Portions of panel C were modeled on information provided in Fig. 3 of Dworkin, J., Blaser, M.J., 1997. Nested DNA inversion as a paradigm of programmed gene rearrangement. Proceedings of the National Academy of Sciences of the United States of America 94 (3), 985–990.

production), in that PilE subunits that cannot be assembled into functional pilin can result due to the introduction of missense or nonsense mutations during the recombination event.

Antigenic variation by whole gene conversion occurs in the *vlp* and *vsp* genes of *Borrelia hermsii* and *Borrelia recurrentis*, respectively, which cause the tick-borne disease known as relapsing fever. The *vlp* and *vsp* genes encode variable large and small surface lipoproteins that are likely involved in the host-pathogen interactions. A single expression site for each gene is found on a plasmid, which can recombine with 38 *vlp* and 21 *vsp* pseudogene copies encoded in 10 genetic clusters on 5 different plasmids. Each pseudogene copy encodes the complete gene and ribosome binding site sequence and shares upstream and downstream homology sequences that allow the recombination event to occur. Expression of new *vsp* and *vsl* variants occurs at a high frequency, and each new wave of the relapsing infection is mostly dominated by bacteria that express one *vsp* or *vsl* variant. The *vsp* and *vsl* genes are only expressed in the mammalian bloodstream, suggesting an additional layer of genetic regulation that is host-restricted. Likewise, *Anaplasma phagocytophilum*, another tick-borne pathogen and the causative agent of human granulocytic anaplasmosis, uses gene conversion as a mechanism of antigenic variation. The Msp2 protein of *A. phagocytophilum* is a major surface protein involved in host cell attachment, and *A. phagocytophilum* uses segmental gene conversion to replace the *msp2* "acceptor sequence", which is expressed, with the whole sequence of one of ~100 pseudogenes encoded elsewhere in the genome.

Another *Borrelia spp.* and another spirochete, *Treponema pallidum*, undergo antigenic variation of key surface proteins. The *vslE* gene of *Borrelia burgdorferi*, the causative agent of Lyme disease, shares similarity with the *vsl* and *vsp* genes, but, like *N. gonorrhoeae pilE*, undergoes antigenic variation via a segmental gene conversion mechanism based on an array of contiguous silent pseudogenes encoded on a linear plasmid. The silent pseudogenes only encode the middle segment of *vslE*, and variable lengths of the expressed *vslE* gene are replaced during the segmental recombination events. Unlike relapsing fever, Lyme disease is a chronic disease in which the infection is maintained at low levels. The chronic nature of the disease coupled with the generation of new *vslE* variants through continuous recombination events results in an array of bacterial clones, each of which produces an antigenically different *VslE* protein. Like *B. burgdorferi*, *Treponema pallidum*, the causative agent of syphilis, causes chronic disease. *T. pallidum tprK* also employs a

segmental gene conversion system in which only 7 variable regions of the acceptor gene are replaced by one of 47 variable region variants encoded in another gene, *tprD*.

Reciprocal Homologous Recombination

Although much less frequently observed in microorganisms, reciprocal homologous recombination is similar to gene conversion in that homologous recombination is used to exchange a gene at a single expression site with variant pseudogene sequences located elsewhere in the genome. However, the recombination event is reciprocal, meaning that the pseudogene variant sequence is not merely used as a template to copy the sequence into the expression site, but is exchanged with the gene sequence originally encoded in the expression site. The result is that the original gene variant is now located in the genetic position that the pseudogene formerly occupied and the former pseudogene sequence is now the gene in the expression site (Fig. 1(B)).

The surface-exposed proteins MgpB and MgpC of *Mycoplasma genitalium*, which are involved in adhesion, motility and cell division, undergo antigenic variation using the reciprocal homologous recombination mechanism within their genes. The *mgpB* and *mgpC* genes have alternating conserved and variable regions and are located in a single expression site. Partial pseudogenes termed MgPa repeats are located in nine different loci in the genome and share homology to 3 regions of the *mgpB* gene and 2 regions of the *mgpC* gene. Segments of the MgPa repeats are exchanged with the variable regions in *mgpB* and *mgpC*, which leads to increased genetic diversity. Because the *M. genitalium* genome is relatively small (580 kb, 470 genes) compared to other microorganisms, this mechanism of homologous recombination offers a compact solution for allowing genetic diversity and antigenic variation while maintaining a small genome that is not amenable to hosting an array of full-length pseudogenes. The dedication of 4.7% of the space-limited genome to the MgPa operon, however, highlights the importance of the antigenic variation of MgpB and MgpC to the survival of *M. genitalium* in the host.

Nested DNA Inversion

The nested DNA inversion mechanism of antigenic variation involves the inversion of a promoter along with one or more tightly clustered variations of a gene flanking the promoter element (Fig. 1(C)). The surface layer protein (SLP) encoded by *sapA* of *Campylobacter fetus* is involved in the resistance of the host innate immune response by inhibiting the binding of the complement system protein C3b and is one of the best-studied examples of nested DNA inversion. The 6.2 kb promoter element is flanked by 5–9 variants of *sapA* in opposite orientations. One or more of the *sapA* variants are inverted along with the promoter element, which can ultimately result in the expression of all of the *sapA* variants. The promoterless *sapA* variants contain a 626 bp 5′ conserved region and a 26 bp 3′ conserved region, which allows each variant to be recognized and inverted with the promoter element. Intragenic recombination is also possible between some variants of *sapA*, which can lead to new *sapA* variant formation.

Hypermutation

The accumulation of point mutations in a gene at a greater frequency than that which occurs in the rest of the genome can also lead to antigenic variation. The *vsp* and *vsl* genes of *B. hermsii* accumulate mutations at a rate of 0.04 per cell per generation but only in the expressed gene variants and not in the silent pseudogenes. M proteins involved in adhesion and invasion of host cells by *Streptococcus spp*. have hypervariable regions encoded in the N-terminal portion of the gene that sustain frequent point mutations that ultimately result in the generation of new M serotypes. The exact mechanism(s) by which the organisms direct the hypermutation of these specific regions remains unclear (Table 1).

Table 1 Mechanisms of antigenic variation and example organisms

Species	*Gene*	*Surface antigen*
Whole gene conversion		
Neisseria gonorrhoeae	*pil*	Type IV pili
Borrelia hermsii Borrelia recurrentis	*vsp, vlp*	Variable small protein Variable large protein
Anaplasma phagocytophilum	*msp2*	Outer membrane protein
Segmented gene conversion		
Borrelia burgdorferi	*vlsE*	Variable major protein-like sequence expressed
Treponema pallidum	*tprK*	Putative outer membrane protein
Reciprocal homologous recombination		
Mycoplasma genitalium	*mgpB, mgpC*	Adhesion proteins
Nested DNA inversion		
Campylobacter fetus	*sapA*	Surface layer protein
Hypermutation		
Borrelia hermsii	*vsp, vlp*	Variable small protein, Variable large protein
Streptococcus spp.		M protein

Mechanisms of Phase Variation

Gene Inversion

Phase variation through sequence inversion most often occurs at loci that encode pilin or flagellar structural units and is accomplished by inversion of the promoter element between 'on' and 'off' states. One or several site-specific recombinases that recognize a specific sequence in the promoter element are employed to invert the DNA region (Fig. 2(A)). For example, the promoter upstream of *fimA*, which encodes the major structural unit of type I fimbriae of uropathogenic *Escherichia coli*, is part of the *fimS* invertible element. The *fimS* sequence is flanked by inverted repeats whose sequence is recognized the FimB and FimE recombinases. FimB can flip *fimS* to either the 'off' or 'on' orientation to prevent or drive expression of *fimA*, respectively. FimE can only switch *fimS* from the 'on' to 'off' position. Because FimE exhibits higher activity than FimB, *fimA* is typically not expressed until adhesion is required and signals from the environment alter the activity or expression of FimB and FimE to cause a shift in the balance from the 'off' to 'on' position. These environmental signals include growth on solid or in liquid medium, osmolarity, and pH, which all affect the expression of *fimB* and *fimE*, and temperature, which differentially affects the activity of FimB and FimE.

A second example of phase variation by gene inversion is the flagellar locus of *Salmonella spp*. Two sequentially distinct flagellin subunits, FliC and FljB, are alternately expressed due to the Hin recombinase-mediated inversion of an 800 bp region containing the *fljB* promoter. When the *fljB* promoter is oriented in the 'on' position, both *fljB* and *fljA* are transcribed to produce type B flagella, and FljA represses expression of *fliC*. When the *fljB* promoter is in the 'off' position, *fliC* is transcribed to produce type C flagella. Coordinated phase variation of pili and flagella allow bacteria to adapt to environmental cues by using the 'stick or swim' switch mechanism. In a favorable environment, the bacteria may promote adherence through increasing expression of pili genes and decreasing expression of flagellar genes so that the bacteria can colonize a host surface and perhaps form biofilms that are resistance to host defenses and antibiotics. If the environment becomes unfavorable, the bacteria can then increase production of flagella expression and decrease production of adherent pili, thus promoting a planktonic lifestyle in which new host sites can be encountered and colonized.

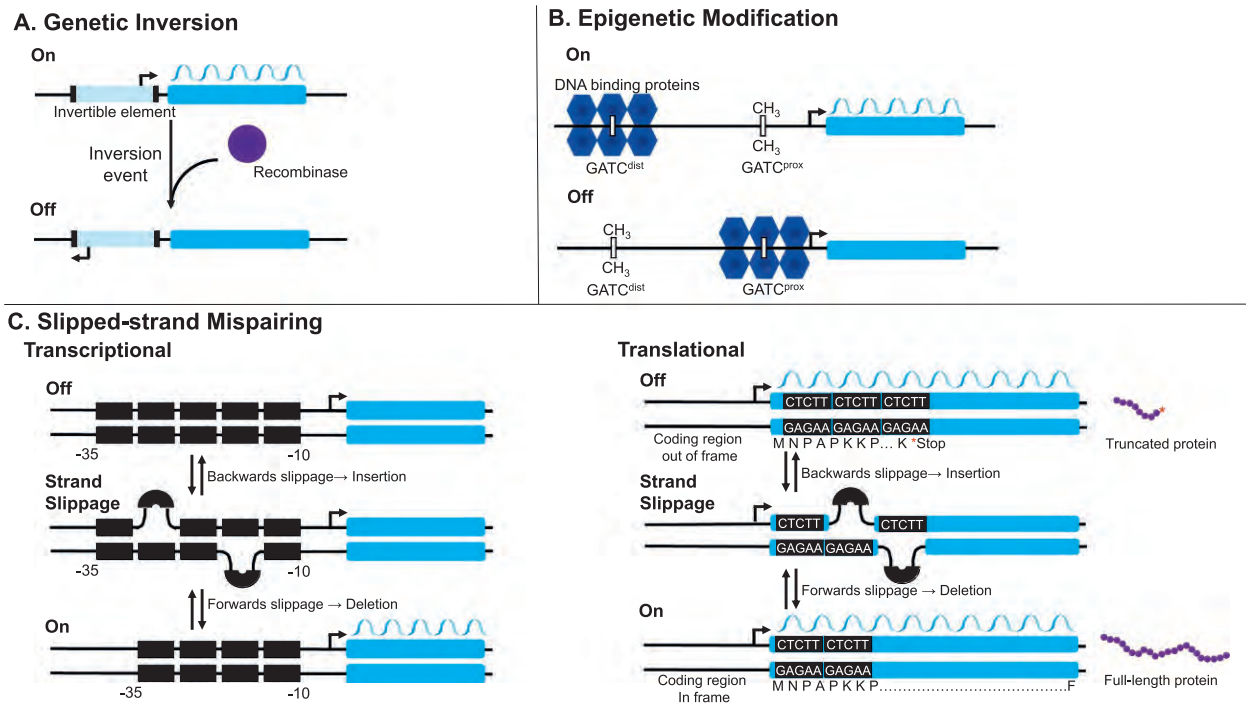

Fig. 2 Mechanisms of phase variation. Phase variation is related to antigenic variation but involves turning gene expression 'on' or 'off' instead of recombination with silent variant gene copies. (A) Genetic inversion uses a recombinase that recognizes specific repeat sequences on the edges of an invertible element, which includes a portion of the promoter (arrow), to flip the promoter into an orientation that either allows or inhibits expression of the gene. (B) Epigenetic modification, such as through methylation of GATC sites in the promoter region, alters the ability of DNA binding proteins to recognize their binding sites. In the example, methylation of the base pairs prevents binding of a transcriptional inhibitor and allows RNA polymerase to bind the promoter and initiate transcription. (C) Slipped-strand mispairing alters expression and translation by 'slipping' of the DNA polymerase during DNA replication. Transcriptional changes occur when the DNA polymerase slips either backwards or forwards on multiple repeats found in the promoter region before the transcriptional start site of a gene. The RNA polymerase is only able to transcribe the gene when the promoter is the appropriate length. Translational changes occur when the DNA polymerase slips either backwards or forwards on multiple repeats found in the coding region of a gene. Depending on the insertion or deletion of base pairs, the RNA polymerase will then transcribe either the correct, in frame sequence of the gene or the incorrect, out of frame sequence of the gene that will result in either a truncated or nonfunctional protein. Portions of panel C were modeled on information provided in Fig. 2 of Deitsch, K.W., Lukehart, S.A., Stringer, J.R., 2009. Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. Nature Reviews Microbiology 7 (7), 493–503. https://doi.org/10.1038/nrmicro2145.

Epigenetic Modification

Phase variation can also be achieved by epigenetic modifications of the DNA sequence surrounding the pilin gene. This mechanism is common to a large group of pili operons. For example, expression of the mutligene *pap* operon in uropathogenic *Escherichia coli* that encodes Pap pili (pyelonephritis-associated pili) is controlled by the methylation status of two GATC sites distal and proximal to the promoter that drives expression of the Pap pili assembly and structural genes. When *pap* gene expression is in the phase 'off' state, the transcriptional regulator Lrp is bound to the promoter region at sites that prevent methylation of the proximal GATC sequence, thus preventing transcription. In the phase 'on' state, Lrp binds to the distal portion of the *pap* promoter, occluding the distal GATC site and allowing methylation of the proximal GATC site. Methylation of the proximal GATC site then allows transcription of the *pap* genes to proceed (Fig. 2(B)). Phase variation of Pap pili, which mediate bacterial adhesion to the α-D-galactopyranosyl-(*1–4*)-β-D-galactopyranoside moiety present on cells lining the upper urinary tract, allows the bacteria to colonize the kidneys to cause pyelonephritis.

Slipped-Strand Mispairing

Deletion or insertion of short sequence repeats (SSR) in the promoter or coding region of a gene can cause phase variation through a mechanism called slipped-strand mispairing. The SSR are repetitive DNA segments of either a single nucleotide or multiple nucleotides. When the number of SSR is altered within a promoter region, the mechanism is specified as transcriptional slipped-strand mispairing because the ability of RNA polymerase to bind the promoter to initiate transcription is either enhanced or inhibited. Alteration of a SSR located in the coding region is termed translational slipped-strand mispairing due to the insertion or deletion of base pairs that, when translated by the ribosomal machinery, result in a protein that is either functional, nonfunctional, or truncated (Fig. 2(C)). The number of these repeats is determined during DNA replication when the DNA polymerase 'slips' while copying the repeated tracts because the DNA strands are denatured and displaced. The outcome of the replication error is the incorporation of more or fewer of the repeats in the promoter or coding regions of the affected genes.

The opacity proteins (Opa) of *N. gonorrhoeae*, which mediate adhesion, epithelial cell invasion, tissue tropism and host cell responses, undergo translational phase variation. A total of 11–12 *opa* loci in *N. gonorrhoeae* are dispersed throughout the genome, and a single bacterium can display a single Opa protein, multiple Opa proteins, or no Opa proteins. A repetitive 5′-CTCTT-3′ sequence, termed the coding repeat, is present in the leader sequence-encoding region of each *opa* gene. The number of these coding repeats determines whether the coding sequence of a particular *opa* gene will be shifted in or out of frame relative to the ATG start codon. Each *opa* gene will be successfully translated into a functional Opa protein as long as 6, 9, or 12 of these coding repeats are present, which is referred to the phase 'on' position. If the number of coding repeats differs from a multiple of three, the gene is in the phase 'off' position, and an aberrant or truncated protein will be produced.

Phase variation by transcriptional slipped-strand mispairing occurs in the *opc* gene of *Neisseria meningitidis*, a pathogen that causes meningitis and sepsis. The *opc* gene encodes an outer membrane protein that facilitates attachment to the host epithelium. The promoter of the *opc* gene contains a repeated stretch of cytosines (poly(C) tract) where strand slippage can occur to produce a promoter of varying length. When there are 12 or 13 cytosine bases present, the gene is in the 'on' phase, transcription is initiated, and an abundance of Opc is produced. Transcription is also initiated when there is a tract of 11–14 cytosine bases in the *opc* promoter, although at a lower rate. However, when the cytosine bases number more than 15 or less than 10, the gene is in the 'off' phase because initiation of transcription does not occur and no Opc is produced. *T. pallidum* uses a similar strategy to regulate the expression of the outer membrane proteins encoded by the *tpr* gene family. The number of repeated tracts of guanosine bases (poly-G tract) encoded just upstream of the transcriptional starts sites of the *tprE* gene and the *tprG*/*F* and *tprJ*/*I* operons control the transcription of these genes. Poly-G tracts with 8 or fewer encoded guanosine bases allows transcription to proceed, while longer poly-G tracts inhibit transcriptional initiation (Table 2).

Table 2 Mechanisms of phase variation and example organisms

Species	*Gene*	*Surface antigen*
Gene inversion		
Escherichia coli	*fimA*	Type I fimbriae
Salmonella typhimurium	*fliC*, *fljB*	Flagella
Epigenetic modification		
Escherichia coli	*papBA*	Pap pili
Translational slipped-strand mispairing		
Neisseria gonorrhoeae	*opa*	Opacity proteins
Transcriptional slipped-strand mispairing		
Neisseria meningitidis	*opc*	Outer membrane protein
Treponema pallidum	*tpr*	Putative outer membrane proteins

Importance of Antigenic Variation in Evasion of the Immune Response

The end goal of antigenic variation is to avoid clearance by the host immune response and promote transmission to new hosts. Phase variation, which as discussed can lead to antigenic variation, is also used to conserve energy by turning off expression of unneeded genes in response to environmental conditions (*e.g.*, expression of *E. coli* pili and flagella are reciprocal and pilin genes are only expressed when attachment to cell surfaces is required for colonization of the host). For most examples of antigenic variation, the systematic switching of a major surface protein from variant to variant helps the pathogen to avoid antibody-mediated clearance via complement activation, neutralization, or opsonization for engulfment by host phagocytes. Even small sequence changes in some surface-exposed proteins on the bacterial surface can prevent antibody recognition and binding to the surface of the bacteria. Avoidance of innate host factors by antigenic variation has also been shown, such as variation of lipopolysaccharide to avoid killing by cationic peptides. These examples are far fewer than examples of how antigenic variation allows escape from the adaptive immune response, the most striking of which is illustrated by blood-borne infections in which cyclic waves of bacteremia are followed by periods of recovery in patients as the host antibody response clears one variant, only to be challenged again as the population of the next variant grows in number. Ultimately, antigenic variation increases the duration of the infection, thus increasing the chance that the pathogen will be transmitted to a new host, whether by carriage by an arthropod vector to a new host in the case of species such as *B. burgdorferi* and *A. phagocytophilum* or by sexual transmission of the pathogen to a new host in the case of species such as *T. pallidum* and *N. gonorrhoeae*.

Beyond the evolutionary advantage of increasing the duration of the infection to increase the possibility of transmission to a new host, antigenic variations also allows for reinfection of the same host by the same pathogen as well as superinfection in which one host is persistently infected with several genetically distinct populations of the same pathogen. When protective immunity is not established during the initial infection, that host remains open to reinfection or superinfection. This lack of the ability to mount a sterilizing immune response effectively keeps the susceptible host population large and increases the chance of successful transmission and survival of the pathogen. Superinfection of the host also provides the additional advantage of possible genetic exchange of genes between genetically distinct populations of pathogens, which may contribute to the rapid rise of antibiotic resistance in some bacterial species.

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Antimicrobial Susceptibility Testing☆

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Glossary

Antibiogram The antimicrobial susceptibility pattern of a bacterial isolate, indicating the antimicrobial agents to which the organism is susceptible and resistant.

Breakpoints The standardized criteria that enable disk diffusion and minimal inhibitory concentration (MIC) test results to be interpreted as susceptible, intermediate, or resistant.

Broth microdilution assays A minimal inhibitory concentration (MIC)-based susceptibility testing method in which a standardized inoculum of bacteria is transferred to all but one well of the 96-well tray containing growth media and doubling dilutions of the antimicrobial agents to be tested. After overnight incubation, the MICs of each antimicrobial agent are determined and the results are interpreted using standard guidelines (breakpoints). The last well in the plate serves as a sterility control for the broth growth medium.

Disk diffusion assay A susceptibility testing method in which paper disks, each saturated with a different antimicrobial agent, are placed on the surface of an agar medium that has been inoculated with a bacterial isolate. After overnight incubation, the zones of inhibition around the disks are measured and the results interpreted using standard guidelines (breakpoints).

Empiric therapy Antimicrobial therapy that is given to a patient with an infection before the biochemical identification of the organism and its standardized antimicrobial susceptibility patterns are known.

Hospital antibiogram An annual or semiannual report issued by the microbiology laboratory in a hospital that summarizes the percentage of organisms, usually by individual bacterial species, which are susceptible to various antimicrobial agents. These data are typically used to guide empiric therapy.

Minimal inhibitory concentration (MIC) The lowest concentration of an antimicrobial agent that prevents visible growth of a bacterial isolate on an agar plate or in a broth-based susceptibility testing assay.

Abbreviations

AM	Ampicillin
CC	Clindamycin
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
E	Erythromycin
ERT	Ertapenem
ESBLs	Extended-spectrum β-lactamases
EUCAST	European Union Committee for Antimicrobial Susceptibility Testing
IMI	Imipenem
MDR	Multidrug resistance
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NI	Noninterpretable
NS	Nonsusceptible
PCR	Polymerase chain reaction
TP	Teicoplainin
VA	Vancomycin
VISA	Vancomycin-intermediate *S. aureus*
VRE	Vancomycin-resistant enterococci

Defining Statement

One of the major functions of a clinical microbiology laboratory is to test bacterial isolates recovered from infections for susceptibility to a variety of antimicrobial agents. The results of the antimicrobial susceptibility testing are provided to physicians to guide their choice of antimicrobial therapy for patients with infections.

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Introduction

One of the major functions of a clinical microbiology laboratory is to test bacterial isolates recovered from patients with infections for susceptibility to a variety of antimicrobial agents. The results of the antimicrobial susceptibility testing are provided to physicians to guide their choice of antimicrobial therapy. The role of antimicrobial susceptibility testing of bacteria for guiding therapy for infectious diseases is becoming more and more important as resistance continues to spread among a wide variety of bacterial species, impacting both initial (empiric) and definitive therapy. Susceptibility testing (previously referred to as 'sensitivity testing') has long been one of the major functions of the clinical microbiology laboratory. In fact, it is not unusual to hear physicians in hospitals still request a 'C and S' (culture and sensitivity) for a variety of clinical specimens, including blood, urine, and sputum. The antimicrobial susceptibility data indicating which antimicrobial drugs are likely to be effective and those that are not are critical particularly in seriously ill patients, such as those with bloodstream infections, infections of heart values (endocarditis), or meningitis. The value of the antimicrobial susceptibility data that are generated in a clinical microbiology laboratory goes beyond the individual patient. Cumulatively, the data are used to aid physicians in the selection of empiric therapy, that is, antimicrobial therapy for an infection in a patient before the biochemical identification of the organism and its standardized antimicrobial susceptibility patterns are known. The presentation of the cumulative data by year and usually by bacterial species is often referred to as the hospital's 'antibiogram.' The techniques by which the microbiology laboratory generates antimicrobial susceptibility testing data will be the subject of this article.

Testing for Antimicrobial Resistance in the Modern Era

Antimicrobial resistance among bacterial pathogens is no longer a rare event. Unfortunately, many of the novel resistance phenotypes that are emerging among bacterial species are not easily detected by the automated susceptibility testing methods frequently used in clinical laboratories today. Approximately 90% of clinical microbiology laboratories in the United States use an automated instrument to identify organisms to the species level and determine the susceptibility of that organism to various antimicrobial agents. The remaining laboratories typically use a disk diffusion method. The ability of the clinical laboratory to detect emerging resistance profiles is often directly related to the extra efforts that are expended to identify novel resistance mechanisms (Table 1). This includes inoculating agar plates containing a specific concentration of an antimicrobial agent to detect borderline resistance, molecular tests to detect specific genes encoding mechanisms of antimicrobial resistance or mutations associated with a resistance phenotype, or tests designed to detect inducible resistance that may otherwise go undetected. Thus, susceptibility testing has become a complex and often costly endeavor as the laboratory seeks to guide the physician in choosing the best antimicrobial agent to treat an infection.

Although antimicrobial resistant bacteria were for many years considered to be confined to intensive-care units of hospitals where antimicrobial agents are used most heavily, resistance to multiple antimicrobial agents (also called 'multidrug resistance' (MDR)) has emerged among strains of community-acquired pathogens, such as *Escherichia coli*, Salmonella, *Mycobacterium tuberculosis* (the causative agent of tuberculosis), and *Neisseria gonorrhoeae* (the causative agent of gonorrhea). To complicate matters further, resistant organisms that arise in the community, such as the novel community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains, are now also spreading into healthcare settings. Thus, it is imperative that changes in resistance patterns of a wide range of bacterial pathogens be monitored continually to insure optimal treatment both of individual patients and for maintaining the efficacy of empiric therapy regimens.

Antimicrobial Susceptibility Testing Methods

It is rare for a clinical microbiology laboratory to use a single method to determine the antimicrobial susceptibility patterns of bacterial isolates, given the variety of bacterial species that need to be tested and their varying growth requirements. The two major

Table 1 Phenotypic screening and confirmation tests

Test name	*Resistance phenotype detected*	*Organism groups*
Aminoglycoside resistance, high level	Synergistic activity with ampicillin, penicillin, or vancomycin	Enterococci
Brain heart infusion vancomycin agar screen test	Vancomycin resistance	Staphylococci, enterococci
Cefoxitin disk test	*mecA* and *mecC*-mediated oxacillin resistance	Staphylococci
D-zone test	Inducible clindamycin resistance	Staphylococci, streptococci
Extended spectrum β-lactamase screening test	Penicillin and cephalosporin resistance	*Escherichia coli*, *Klebsiella* species, *Proteus mirabilis*
Carbapenem inactivation test (Modified Hodge Test)	Carbapenem resistance	Enterobacteriaceae
Oxacillin screen test	Penicillin susceptibility	*Streptococcus pneumoniae*

phenotypic methods of determining the susceptibility of a bacterial isolate to an antimicrobial agent are disk diffusion and minimal inhibitory concentration (MIC) testing. As noted above, in the United States, approximately 90% of susceptibility test results are produced using automated or semi-automated instruments that generate MIC data, often together with bacterial species identification, while the remainder of results are produced primarily by disk diffusion testing. Susceptibility testing results may be available in as little as 6–8 h for some antimicrobial agents, while others require a full 24 h to insure detection of resistance. However, clinical laboratories also utilize a series of screening and confirmation tests to detect subtle resistance mechanisms (those that produce low MIC values near the breakpoint that defines susceptibility) and insure the accuracy of antimicrobial susceptibility test reports (Table 1). More recently, molecular methods to detect antimicrobial resistance genes and mutations associated with resistance phenotypes have been introduced into clinical microbiology laboratories and are frequently used to test for resistant organisms directly in clinical samples, rather than be used on bacteria grown in culture.

Disk Diffusion

The disk diffusion method is among the most flexible susceptibility testing methods in terms of antimicrobial agents that can be tested. The method consists of placing paper disks saturated with antimicrobial agents on a lawn of bacteria seeded on the surface of an agar medium, incubating the plate overnight, and measuring the presence or absence of a zone of inhibition around the disks (Figure 1). Studies conducted at the University of Washington in the mid-1960s resulted in the technique often referred to as the 'Kirby–Bauer method,' which was published by Bauer and colleagues in 1966. This method standardized the variables of disk size, inoculum size, temperature, and time of incubation. Results are reported qualitatively as susceptible, intermediate, or resistant. The disk diffusion method described by Bauer and colleagues has been continually expanded and improved by the Clinical and Laboratory Standards Institute (CLSI) in the United States. Several other international societies (e.g., the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST), and the British Society for Antimicrobial Chemotherapy) use similar techniques. Alternative disk-based methods, including the Roscoe NeoSensitabs and the Australian CDM method, are also used in some countries. Instruments that measure the zones of inhibition using cameras can speed the process of reading disk diffusion plates. These instruments can also transform the zone diameter readings into approximate MIC values.

MIC Testing

The goal of MIC testing is to provide a quantitative result (usually in $\mu g\ ml^{-1}$) along with a categorical interpretation (susceptible, intermediate, or resistant) that can guide antimicrobial therapy more precisely, particularly for infections in body sites where antimicrobial agents achieve lower concentrations than in serum (e.g., cerebrospinal fluid and bone). MIC testing can be performed

Figure 1 Disk diffusion test for *Staphylococcus aureus*. No zone of growth inhibition is observed around the ampicillin (AM) disk. Photo courtesy of Janet Hindler.

by one of several methods including agar dilution, broth microdilution, agar gradient dilution, or by one of several automated or semiautomated methods. Quantitative MIC results are also useful when long-term therapy is required, as for bacterial endocarditis.

Agar dilution

The agar dilution method involves preparing a series of agar plates containing the antimicrobial agent to be tested in increasing concentrations, usually in doubling dilutions (i.e., 1, 2, 4, 8, 16, 32 μg ml^{-1}, etc.). A suspension of the organism to be tested is prepared to equal the turbidity of a 0.5 McFarland standard ($\sim 1 \times 10^8$ colony forming units (CFU) ml^{-1}), and 1–5 μl of this suspension is placed on each of the series of plates with increasing concentrations of the antimicrobial agent using a replicator device (final inoculum is $\sim 5 \times 10^4$ CFU/spot). Thirty different bacterial isolates (plus quality control organisms) can be tested simultaneously on each agar plate. Nonfastidious organisms are incubated at 35 °C for 16–18 h usually in ambient air, while fastidious organisms, such as *Streptococcus pneumoniae*, are tested on blood-containing media, incubated from 18 to 24 h, typically in a CO_2-enriched atmosphere. The agar dilution method, while laborious due to the time required to prepare each set of agar plates for each antimicrobial agent to be tested, is often cost-effective for laboratories that test large numbers of bacterial isolates against a limited set of antimicrobial agents. The testing medium is usually Mueller–Hinton agar for nonfastidious organisms and Mueller–Hinton agar containing 5% sheep blood for fastidious organisms. The exceptions are *Haemophilus influenzae* isolates, which require HTM (CLSI) or MH-F (EUCAST), and *N. gonorrhoeae*, which requires GC medium.

Broth microdilution

Broth microdilution is the standard method used in most reference laboratories in the United States and abroad. The method typically tests twofold dilutions of multiple antimicrobial agents in 96-well disposable plastic trays (Figure 2). The test medium is typically cation-adjusted Mueller–Hinton broth, or for fastidious organisms, cation-adjusted Mueller–Hinton broth containing 5% lysed horse blood. A suspension of the organism to be tested is prepared in saline or Mueller–Hinton broth to the turbidity of a 0.5 McFarland standard ($\sim 1 \times 10^8$ CFU ml^{-1}). The suspension is diluted 1:20 in saline, and 1–5 μl of this suspension is transferred to all but one well of the 96-well tray containing doubling dilutions of the antimicrobial agents to be tested (usually between 8 and 12 antimicrobial agents per tray) using a disposable plastic inoculator (the remaining well is a broth sterility control). The final inoculum size is 5×10^5 CFU ml^{-1} or 5×10^4 CFU/well.

Automated susceptibility testing methods

A series of commercially available automated and semiautomated methods are used in clinical microbiology laboratories to test and report the results of antimicrobial susceptibility tests. Most of the methods combine bacterial identification and susceptibility testing reagents in a single panel or card to enhance the speed with which antimicrobial susceptibility testing results can be reported to physicians. Many systems also incorporate software programs to interpret the results and prepare reports that can be linked readily to laboratory information systems, which in turn deliver the results to the patient's medical record. The goal of the automated methods is to reduce the time necessary to produce accurate identification and susceptibility test results. Indeed, results may be available for some bacterial species in as little as 6 h, versus the 16–24 h often required for disk diffusion testing or standard MIC tests. For staphylococci, the results of oxacillin and vancomycin tests often require prolonged incubation times (up to 24 h) to

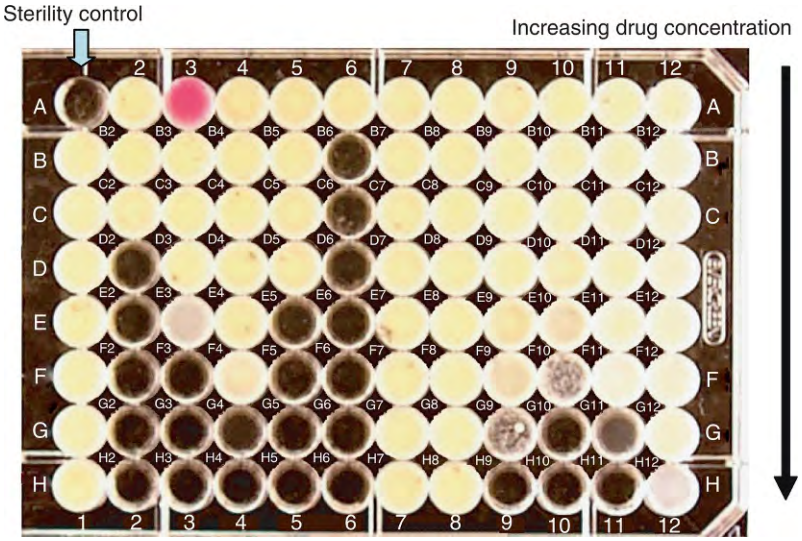

Figure 2 Minimal inhibitory concentration (MIC) test with *Staphylococcus aureus*. The first well where complete inhibition of growth is observed in considered the MIC for that antimicrobial agent. The lowest concentrations of the antimicrobial agents are on the top of the plate and the concentrations of the drugs increase in doubling dilutions (1, 2, 4, 8 μg ml^{-1}, and so on) toward the bottom of the plate (there are 12 drugs tested in this plate (columns 1, 2, 3,. . . each are different antimicrobial agents)). The pink well is the growth control. Photo courtesy of Janet Hindler.

achieve accurate results. Some software programs employ 'expert systems' to enhance reporting by recognizing and flagging unusual results, such as ampicillin-susceptible *Klebsiella pneumoniae*, where the bacterial identification and susceptibility pattern are conflicting with typical results for wild-type *K. pneumoniae* populations, or less common results, such as carbapenem-resistant Enterobacteriaceae or vancomycin-resistant *S. aureus* isolates.

Overall, automated systems work well, although they have traditionally shown problems with certain resistance phenotypes, including oxacillin-resistant *S. aureus* isolates and *Pseudomonas aeruginosa* isolates that are resistant to β-lactam agents, such as piperacillin. Inducible resistance, such as clindamycin resistance caused by *erm*-type genes in staphylococci, is difficult to detect in automated systems, although some systems now incorporate wells containing both erythromycin and clindamycin to detect this phenotype. Finally, the detection of resistance due to induction of β-lactamases present among some strains of Enterobacteriaceae, and the related problem of detection of resistance to third-generation cephalosporins due to extended-spectrum β-lactamases (ESBLs), has been addressed in many of the automated systems by use of low concentrations of β-lactams as screening tests. When positive, these tests indicate to the user that additional testing outside of the automated system may be necessary to achieve accurate results.

Agar gradient dilution

Agar gradient dilution allows the generation of MIC data in a testing format similar to that of disk diffusion testing (Figure 3). The antimicrobial agent is microencapsulated on the back of a plastic strip, and when placed on the surface of an agar plate, the antimicrobial agent diffuses from the strip into the agar medium in a rapid and predictable fashion forming a gradient. Agar gradient strips, which are available from at least two commercial sources, evaluate the inhibitory potential of a single antimicrobial agent over a large range of concentrations (e.g., 0.03–256 μg ml^{-1}). Several test strips, each containing a different antimicrobial agent, can be arranged on a single agar plate. The agar gradient method is particularly useful for testing fastidious microorganisms such as campylobacters, pneumococci, and anaerobic bacteria, where only a limited number of antimicrobial agents need to be tested.

Molecular Diagnostic Testing Methods

Over the last several years, a series of molecular assays using nucleic acid amplification methods, microarrays, line probes, and several other methods have been developed to detect antimicrobial resistance genes and mutations associated with resistance phenotypes. The most commonly used molecular tests for antimicrobial resistance are real-time polymerase chain reaction (PCR) assays to detect the *mecA* methicillin resistance gene associated with MRSA isolates directly in clinical samples (including positive blood culture bottles, wounds specimens, and nasal swabs for surveillance), often in <2 h. Several commercial methods for identifying gram-positive cocci in blood culture bottles also test for the presence of the vancomycin resistance genes, *vanA* and *vanB*, among enterococci, while other assays test for the presence of the bla_{KPC} carbapenem resistance gene in gram-negative

Figure 3 Agar gradient diffusion (Etest method is pictured) analysis of *Staphylococcus aureus* showing results for vancomycin (VA) and teicoplainin (TP), two glycopeptides drugs. The MICs are read off of the strip where the bottom portion of the ellipse intersects with the strip (see white arrows). (VA MIC = 1 μg ml^{-1}, TP MIC = 1.5 μg ml^{-1}).

organisms causing sepsis. PCR assays using molecular beacon technology and line probe assay have been developed to detect mutations in the ribosomal RNA polymerase gene *rpoB* associated with rifampin resistance in *M. tuberculosis*. Rifampin resistance is frequently a marker of multidrug-resistance in *M. tuberculosis*. The PCR assays can be performed directly on expectorated sputum samples or concentrated pellets prepared for mycobacterial culture, produce results in <2 h, and are now used in over 100 countries worldwide, including many that have been designated as high burden, developing countries (HBDC) by the World Health Organization. Line probes that utilize reverse hybridization also can be used to assess both rifampin and isoniazid resistance.

Interpretive Guidelines

Once a disk diffusion zone of inhibition has been measured or an MIC for an antimicrobial agent has been determined, and the microbiologist has affirmed that the quality control results indicate that the testing system has performed appropriately, the results of the susceptibility tests have to be interpreted. For most antimicrobial agents, the results transmitted to the patient's chart will be reported as either 'susceptible,' 'intermediate,' or 'resistant.' If an MIC method was used, the results transmitted may include the quantitative MIC results, usually reported in $\mu g\,ml^{-1}$, as well (see Table 2 for an example). However, for some antimicrobial agents, such as linezolid when testing staphylococci, the results transmitted will be either 'susceptible' or 'nonsusceptible.' At the time the drug was approved for use by the United States Food and Drug Administration, and when interpretive criteria (i.e., breakpoints) were established by the CLSI, there were inadequate numbers of resistant strains available with associated clinical outcome data on which to establish intermediate and resistant breakpoints. The lack of intermediate and resistant breakpoints often poses a challenge for the automated methods which, depending on the system, will either leave the interpretation field blank for a nonsusceptible result, or place an 'N' or 'NS' (for nonsusceptible), or an 'NI' (for noninterpretable) in the interpretation field – a result that may be confusing to the physician reading the laboratory report. Some microbiology laboratories will override these 'NS' results and report them as resistant to avoid confusing physicians, even though technically, resistance has not been defined. Recently, a new interpretive category called 'susceptible dose dependent' (SDD) was introduced by CLSI for reporting results for cefepime for bacterial infections. Although this category has been used for reporting results for antifungal susceptibility testing for several years, its application for cefepime results for bacterial infections is novel. It is meant to bring clarity to the intermediate category by indicating to the physician that the organism causing the infection may still respond to cefepime if high doses of the drug are used.

The categorical interpretations used for disk diffusion and MIC test results are developed by a number of organizations around the world. In the United States, this is done initially by the FDA, with follow up criteria established by CLSI (the two sets of criteria may or may not be the same). The reference disk diffusion method for the United States is defined in the CLSI M2 document (i.e., Performance Standards for Antimicrobial Disk Susceptibility Tests). The M2 document is revised every 3 years. The next version of the M2 document (M2-A12) will be published in January 2015. The agar and broth dilution reference (MIC) methods are described in the CLSI M7 document (Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that grow aerobically). The next version of the M7 document (M7-A10) will be published in January 2015. A separate document containing the interpretive criteria for both disk diffusion and MIC testing, quality control ranges, and methods for preparing and diluting antimicrobial agents, is published each year in January (the M100 series). A separate document outlining testing methods and interpretive criteria for fastidious bacteria and organisms that are isolated only infrequently in clinical laboratories, CLSI document M45 is also available. Similar documents describing susceptibility testing methods and interpretive criteria are published by the EUCAST (http://www.srga.org/), the British Society for Antimicrobial Chemotherapy (http://www.bsac.org.uk/), and other international organizations.

Resistance Phenotypes That Require Specialized Testing

Penicillin-Resistant Pneumococci

For many years, before the introduction of the agar gradient diffusion method and commercially available MIC panels for fastidious organisms, performing MIC tests on isolates of *S. pneumoniae* was too difficult and costly for many clinical laboratories, particularly

Table 2 Antimicrobial susceptibility test results for an *Escherichia coli* isolate from a blood culture

Antimicrobial agent	*Minimal inhibitory concentration ($\mu g\,ml^{-1}$)*	*Interpretation*
Ampicillin	>128	Resistant
Cefazolin	>32	Resistant
Cefotaxime	1	Susceptible
Ciprofloxacin	2	Intermediate
Gentamicin	1	Susceptible

those in developing countries. Yet, even as early as 1977, penicillin resistance was emerging around the world and multiply drug-resistant pneumococci were reported in South Africa. One simple test for identifying strains with the potential for penicillin resistance, which is still used in many laboratories today, is the oxacillin disk screening test. A 1-μg oxacillin disk is placed on a Mueller–Hinton agar plate containing 5% sheep blood that has been inoculated with the strain of *S. pneumoniae* to be tested. A zone of growth inhibition of $\geq$20 mm around the oxacillin disk indicates that the *S. pneumoniae* strain is susceptible to penicillin. If the zone of inhibition around the oxacillin disk is $\leq$19 mm, this indicates that the organism has undergone some change in its penicillin-binding proteins; however, the organism could still be penicillin-susceptible. Thus, an MIC test is required to differentiate penicillin-susceptible from penicillin-resistant organisms if the zone of inhibition is $\leq$19 mm. *S. pneumoniae* strains that are susceptible to penicillin are considered susceptible to all β-lactam agents.

Oxacillin Resistance Among Staphylococci

Although the term MRSA is thoroughly entrenched in the medical literature, it is often oxacillin that is tested in lieu of methicillin due to its greater stability during testing procedures. Oxacillin MICs are still determined in many commercial AST systems. However, more recently, the cephamycin cefoxitin has replaced oxacillin in both disk diffusion and MIC testing. Cefoxitin is a better predictor of strains of staphylococci that carry the *mecA* and *mecC* methicillin resistance genes. Even so, detection of oxacillin resistance in staphylococci can be difficult because oxacillin-resistant strains, particularly of *S. aureus*, grow more slowly than susceptible strains and often show heteroresistance, that is, only a portion of the population of organisms (i.e., 1 in every 100 000 cells) show oxacillin resistance. Incubating staphylococci at 35 °C instead of 37 °C, adding 2% NaCl to the broth testing medium, and incubating the test for a full 24 h improves the detection of oxacillin-resistant strains. For disk diffusion testing, CLSI recommends using a 30-μg cefoxitin disk instead of a 1-μg oxacillin disk to predict the presence of *mecA* or *mecC* among both *S. aureus* and coagulase-negative staphylococcal isolates. The cefoxitin disk diffusion test can be read in 16–18 h and shows levels of sensitivity and specificity that are higher than those of the oxacillin disk, particularly for testing coagulase-negative staphylococci.

Extended-Spectrum β-Lactamase Producing Enterobacteriaceae

Among the Enterobacteriaceae, a major susceptibility testing challenge is detecting the presence of ESBLs. These β-lactamases, which are typically the result of mutations in bla_{TEM}, bla_{SHV} or $bla_{\text{CTX-M}}$ β-lactamase genes, have a much wider spectrum of activity than the wild-type (unmutated) β-lactamase and can hydrolyze monobactams (such as aztreonam), most third-generation cephalosporins (such as cefotaxime, ceftriaxone, and ceftazidime) and, in some cases, even fourth-generation cephalosporins (such as cefepime and cefpirome). Since the ESBLs do not hydrolyze all of the extended-spectrum cephalosporins at similar rates, some organisms may show resistance to one or two third-generation cephalosporins but susceptibility to others in laboratory tests. Clinical data, however, show that patients infected with ESBL-producing organisms will fail therapy when treated with any of the cephalosporin drugs even if the organisms appear susceptible *in vitro*. The dilemma was what to do with an organism that is resistant to one or two third-generation cephalosporins, but susceptible to other cephalosporins. For almost a decade the answer was to determine if the organism was an ESBL producer, and if so, to use this information to modify the results for most of the beta-lactam drugs (except carbapenems) to non-susceptible (either I or R depending on laboratory policy). Isolates of *Escherichia coli*, *K. pneumoniae*, and *Proteus mirabilis* that were suspected ESBL producers were tested against cefotaxime and ceftazidime, either by disk diffusion or by a broth microdilution assay, in the presence and absence of clavulanic acid – a β-lactamase inhibitor. If the zones of inhibition increase by 5 mm or more in the presence of clavulanic acid, or the MICs decrease by three or more doubling dilutions in the presence of clavulanic acid when compared to the results in the absence of clavulanic acid, the organism was presumed to harbor an ESBL and the results for all penicillins, cephalosporins, and aztreonam were reported as resistant, since those infections will not respond to those drugs. The reports for other antimicrobial agents, including β-lactam/β-lactamase inhibitor combinations (e.g., piperacillin-tazobactam) and cephamycins (cefotetan and cefoxitin) are not affected by ESBL testing and the susceptible or intermediate results obtained by routine testing were not modified. The β-lactam/β-lactamase inhibitor combinations were presumed to still be effective against some ESBL-producing strains. Similar strategies to identify plasmid-mediated AmpC β-lactamases using boronic acid were described, but were not incorporated into CLSI documents and were not routinely used in the United States.

More recently, this approach of determining the presence of ESBLs to modify susceptibility testing results has fallen on disfavor. The additional testing delays the time it takes to finalize AST results and the method often suffers from technical problems. In lieu of additional testing, both CLSI and EUCAST lowered the susceptibility beakpoints for most cephalosporins making then more sensitive for the detection of ESBL and AmpC producing organisms. ESBL testing is now used primarily for epidemiologic purposes. Although this approach has its critics, it is now in use globally.

Detecting Carbapenemase-Producing Gram-Negative Bacilli

The carbapenems, that is, doripenem, ertapenem, imipenem, and meropenem, are highly potent β-lactam agents that are often held in reserve for the sickest patients in the hospital with the most difficult to treat Gram-negative organisms. Carbapenems often are

Figure 4 A carbapenem inactivation assay using imipenem (IMI) and ertapenem (ERT) disks. The growth of the indicator organism toward the disk at the top of the zone of inhibition around the streak of growth indicates a positive test (arrow). The lack of indentation of the zone of inhibition on the bottom of the zone is a negative test.

active even against bacteria that contain ESBLs or AmpC β-lactamases. Organisms, such as *K. pneumoniae*, that are carbapenem-resistant are often resistant to all available antimicrobial agents. However, as with the ESBLs, not all organisms that contain carbapenemases, such as the KPC (Klebsiella pneumoniae carbapenemase), VIM (Verona integron mediated β-lactamase), or NDM (New Delhi metallo β-lactamase), are detected as resistant to each of the carbapenems by automated susceptibility testing methods. Thus, additional *in vitro* assays were often required to identify those stealth resistance mechanisms. The most common test was a carbapenem inactivation assay in which a sensitive indicator strain of *E. coli* was inoculated onto an agar plate and one or more disks containing carbapenem drugs were placed on the plate (Figure 4). The organism to be investigated was then streaked from the disk out to the edge of the agar plate. A control organism that does not inactivate carbapenems is often streaked on the opposite side of the disk. If a carbapenemase type β-lactamase was present, the drug was inactivated and the susceptible *E. coli* grew in from the edge of the zone of inhibition toward the disk. If the test was positive, all carbapenem results were reported as resistant.

As with the ESBLs, detecting carbapenemase-producing organisms using the Modified Hodge Test was not always effective as some organisms, especially those containing an NDM carbapenemase, escaped detection. False positive test results, often with AmpC containing organisms, coupled with tests that yielded indeterminate results further undermined the utility of the assay. Thus, CLSI and EUCAST adopted the strategy of establishing lower breakpoints for the carbapenem drugs to enhance detection of resistant strains, especially those that clustered at the susceptible/intermediate breakpoints. This strategy has not been met with universal acceptance, although it does appear to be increasing the accuracy of results for carbapenem testing globally.

Detecting Inducible Clindamycin Resistance Among Gram-Positive Cocci

Staphylococci and streptococci that are resistant to erythromycin (a macrolide class drug) are often also resistant to clindamycin (a lincosamide drug) because resistance to both drugs can be mediated by the same mechanism, that is, methylation of the 50S bacterial ribosome. This is referred to as the MLS_B-resistance phenotype (for macrolide–lincosamide–$streptogramin_B$) and is mediated by one of the *erm* family of resistance genes. However, some isolates of staphylococci and streptococci that are erythromycin-resistant due to carriage of an *erm* gene, appear clindamycin-susceptible in laboratory tests because the *erm* gene is inducible and clindamycin itself is not a good inducer (i.e., the gene is not activated during the time it takes to complete the susceptibility test). Because spontaneous mutations can occur in the *erm* gene during clindamycin therapy, which lead to high-level clindamycin resistance and thus treatment failure, it is important to identify organisms that contain an inducible *erm* gene. Such isolates must be differentiated from other isolates of staphylococci and streptococci that contain an efflux-type macrolide resistance gene, which does not mediate clindamycin resistance, and can be treated successfully with clindamycin. Examples of erythromycin efflux mechanisms that do not mediate clindamycin resistance are *msrA* (in staphylococci) or *mefA* (in streptococci). Thus, for strains of staphylococci or streptococci that show an erythromycin-resistant, clindamycin-susceptible phenotype, it is important to differentiate between those strains that carry an inducible *erm* gene (which may cause clindamycin treatment failure) and those that harbor an efflux gene (which will not result in clindamycin failure). The D-zone test, which is a disk diffusion-based assay, uses a 15-μg erythromycin disk that is placed 15–26 mm away from a 2-μg clindamycin disk on an agar plate that has been inoculated with the test organism. Blunting of the zone of inhibition between the erythromycin and clindamycin disks (which forms a 'D' shape) indicates the presence of an inducible *erm* gene (a positive test) (Figure 5). A circular zone of inhibition (normal zone) indicates a negative test. If the D-zone test is positive, the results for clindamycin are reported as resistant to avoid a potential treatment failure.

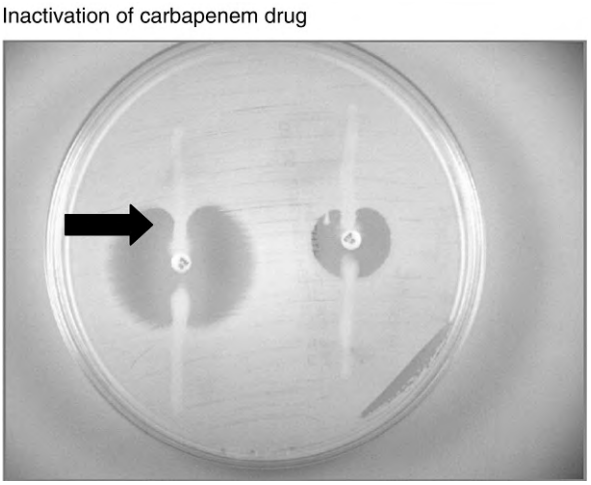

Figure 5 Positive D-zone test showing blunting of the zone of inhibition between the erythromycin (E) and clindamycin (CC) disks. Photo courtesy of Janet Hindler.

High-Level Aminoglycoside Resistance Among Enterococci

Serious enterococcal infections, such as endocarditis, are usually treated with a combination of antimicrobial agents that are synergistic (i.e., have enhanced killing activity when used in combination), such as a cell wall-active agent and an aminoglycoside. To determine whether there is likely to be synergy between the cell wall-active agent (usually ampicillin, penicillin, or vancomycin) and the aminoglycoside (i.e., gentamicin or streptomycin) special disk diffusion and MIC tests to detect high-level aminoglycoside resistance have been established by CLSI. The presence of high-level resistance to either aminoglycoside will negate the likelihood of synergistic activity with ampicillin, penicillin, or vancomycin, which is important information for the physician treating the patient.

Detecting Resistance to Vancomycin Among Staphylococci

Vancomycin is a member of the glycopeptide class of drugs, which are large molecules that diffuse poorly through agar media. Thus, disk diffusion testing with vancomycin and other glycopeptides often does not discriminate very well between susceptible isolates and those with intermediate or reduced susceptibility to the glycopeptides tested, such as the vancomycin-intermediate *S. aureus* (VISA) strains. While detection of *S. aureus* strains with high-level vancomycin resistance (i.e., vancomycin MICs >32 μg ml^{-1}), such as those rare strains that contain the *vanA* resistance gene from enterococci, can still be detected as resistant by disk diffusion, VISA isolates cannot be distinguished from vancomycin-susceptible *S. aureus* isolates using the disk diffusion method. Thus, laboratories often inoculate 10^6 CFU of the staphylococcal strain to be tested onto a Brain Heart Infusion agar plate containing 6 μg ml^{-1} of vancomycin to check for reduced susceptibility to vancomycin. Those organisms with reduced susceptibility to vancomycin will grow within 24 h. This technique is very sensitive (>99%) for detecting isolates for which the vancomycin MICs are 8 μg ml^{-1} or higher, but only about 75% sensitive for isolates for which the vancomycin MICs are 4 μg ml^{-1}.

Fluoroquinolone Resistance in Salmonella Typhi and Other Extraintestinal Salmonella Infections

Clinical failures have been reported when treating *S. typhi* infections, that is, typhoid fever, and other extraintestinal salmonella infections, with fluoroquinolones when the fluoroquinolone MICs (such as for levofloxacin) are in the upper end of the susceptible range (i.e., MICs of 0.25–1 μg ml^{-1}; the levofloxacin susceptible breakpoint is <2 μg ml^{-1}). Interestingly, such salmonella strains usually show nalidixic acid MICs that are resistant (MIC >16 μg ml^{-1}), so a nalidixic acid disk diffusion test was used to try and identify low level resistant strains. However, not all fluoroquinolone resistance was detected by this method, so lower ciprofloxacin breakpoints were instituted by CLSI.

Molecular Tests for Identification of Patients Colonized with Resistant Bacteria

Rapid tests to identify patients colonized with MRSA

Screening patients being admitted to hospitals or other healthcare institutions for nasal colonization with MRSA is becoming widespread in the United States and elsewhere around the world as part of enhanced infection control programs. Screening for MRSA can be accomplished by plating material from nasal swabs directly on selective agar media that inhibit the growth of most organisms, while allowing MRSA to produce clearly identifiable colonies. However, this often requires 18–72 h depending on the medium used, whether an overnight broth enrichment step is included, and the number of confirmatory tests undertaken by the laboratory to prove that the organism growing on the agar is MRSA. A more rapid approach uses molecular amplification tests, such

as PCR, that simultaneously target the *mecA* gene and a chromosomal DNA sequence that is unique to *S. aureus*, thereby linking the resistance gene specifically to the *S. aureus* strain that carries it. PCR-based assays for MRSA detection often can be completed in <2 h from the time the nasal swab specimen arrives in the laboratory, versus the 18–72 h often required to complete an agar-based identification test. Amplification-based assays are more expensive to perform but the rapid turnaround time can be important when trying to control the spread of MRSA in a hospital setting.

Molecular assays for detecting colonization with VRE and carbapenem-resistant organisms (CRO)

Molecular assays for VRE and CROs to identify colonized patients to aid in infection control efforts have been instituted in a variety of healthcare settings. These assays, which target both *vanA* and *vanB* vancomycin resistance genes, or multiple carbapenem resistance genes including bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA48}, and bla_{IMP}, are often more sensitive than conventional agar screening media for detecting resistant organisms, with results available often in <1 h. While there is a high correlation between the results of molecular assays for *vanA* in perirectal samples and positive cultures for VRE containing *vanA*, the correlation of molecular assays and cultures for *vanB* is lower, that is, several *vanB*-positive samples have negative cultures for enterococci containing the *vanB* gene. This probably reflects the fact that organisms in bowel flora other than enterococci, such as *Clostridium* species, can harbor the *vanB* resistance determinant. Correlation of results for detection of CROs by culture and molecular methods, however, is very high.

Conclusion

As antimicrobial resistance becomes more widespread among bacterial species, the role of the clinical laboratory to produce accurate and timely antimicrobial susceptibility test results becomes more critical to the physician who has the responsibility of treating the infected patient and controlling the spread of the disease to others.

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http://clsi.org/standards/micro/sub-ast/ – Clinical and Laboratory Standards Institute.

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http://www.srga.org/ — The Swedish Reference Group for Antibiotics (SRGA) and its subcommittee on methodology (SRGA-M).

Antiviral Agents☆

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Glossary

Alanine aminotransferase An enzyme found in the liver and blood serum, the concentration of which is often elevated in cases of liver damage.
Antiretroviral agent Any drug used in treating patients with human immunodeficiency virus (HIV) infection.
Antiviral resistance The developed resistance of a virus to a specific drug.
Bilirubin A greenish compound formed in the liver from the degradation of the hemoglobin from degraded red blood cells.
Bioavailability The property of a drug to be absorbed and distributed within the body in a way that preserves its useful characteristics; for example, it is not broken down, inactivated, or made insoluble.
Chemoprophylaxis Preventive treatment with chemical agents such as drugs.
Codon A triplet of three consecutive nucleotide components in the linear genetic code in DNA or messenger RNA, which designates a specific amino acid in the linear sequence of a protein molecule.
Creatinine An end product of energy metabolism found in the blood in uniform concentration, which is excreted by the kidney at a constant rate. Alterations of this rate are considered an indication of kidney malfunction.
Cytokine One of a variety of proteins which has a regulatory effect on a cell.
Drug resistance Decreased susceptibility to antiviral usually due to changes in the amino acid residues of target enzyme. For example, a substitution of valine for methionine at residue 184 of the reverse transcriptase enzyme of HIV confers resistance to lamivudine (M184V mutation, see Table 1 for letter codes of amino acids).
EC_{50} Concentration of a drug that produces a 50% effect, for example, in virus yield.
Hemagglutinin Specific glycoprotein molecules on the surface of some viruses, which have the property of binding to the surface of the red blood cells of some animal species. Because there are multiple binding sites, one virus can bind to two red cells causing them to clump (agglutinate).
Hepatotoxicity Liver toxicity.
Interferon Any group of glycoproteins with antiviral activity.
Lipoatrophy Redistribution/accumulation of body fat including central obesity, dorsocervical fat enlargement (buffalo hump), peripheral wasting, facial wasting, breast enlargement, and 'cushingoid appearance' observed in some patients receiving antiviral agents for HIV treatment.
Maintenance therapy Drug treatment given for a long time to maintain its effect after the condition has been controlled or to prevent recurrence.
Monotherapy Treatment with a single drug, contrasted with combination therapies with more than one drug at the same time.
Mutations Changes to the base pair sequence of the genetic material of an organism.
Nephrolithiasis The presence of kidney stones.
Nephrotoxicity Kidney toxicity.
Neuraminidase An enzyme, present on the surface of some viruses, which catalyzes the cleavage of a sugar derivative called neuraminic acid.
Peptidomimetic A molecule having properties similar to those of a peptide or short protein.
Pharmacokinetic Refers to the rates and efficiency of uptake, distribution, and disposition of a drug in the body.
Phase III The final stage in testing of a new drug, after determination of its safety and effectiveness, in which it is tested on a broad range, and large population of patients for comparison to existing treatments and to test for rare complications.
Placebo An agent used as a 'control' in tests of drugs. The placebo is an agent without the specific effects of the drug under test and is used to determine to what extent any observed effects of the drug are due to psychological effects or expectations. It is usually given to some patients while the test drug is given to others, but neither group knows which agent it is receiving (the so-called 'blind' design).
Prodrug A drug that is given in a form that is inactive and must be metabolized in the body to the active form.
Prophylaxis Prevention.

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Protease An enzyme that catalyzes the cleavage of proteins. In the case of HIV, a virus-specific protease is needed to cleave some of the virus coat proteins into their final, active form.
Protease inhibitor A substance that inhibits the action of protease enzyme.
Replication cycle The series of steps that a virus or cell goes through to multiply.
Shingles Eruptive rash, usually in a girdle (L. cingulus; hence 'shingles') distribution on the trunk, resulting from infection with varicella zoster virus.
$T_{1/2}$ The time for reduction of some observed quantity, for example, the blood concentration of a drug, by 50%.
Teratogenesis Production of fetal abnormalities by some agent.
Therapeutic index The numerical ratio of the concentration needed to achieve a desired effect in 50% of the patients and the concentration that produces unacceptable toxicity in 50% of the patients.
Thymidine kinase An enzyme that catalyzes the transfer of a phosphoryl group from a donor such as adenosine triphosphate to the sugar (deoxyribose) component of the thymidine molecule, a building block of DNA.
Viremia The presence of virus in the bloodstream.
Virion A complete virus, including the coat and nucleic acid core.

Abbreviations

ALT	Alanine aminotransferase
CMV	Cytomegalovirus
CNS	Central nervous system disease
CoV	Coronavirus
CPK	Creatinine phosphokinase
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
dATP	Deoxyadenosine triphosphate
dGTP	Deoxyguanosine triphosphate
EBV	Epstein–Barr virus
FDA	Food and Drug Administration
FEV1	Reduced forced expiratory volume
HAART	Highly active antiretroviral therapy
HBeAg	Hepatitis B e antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV-6	Human herpes virus-6
HHV-7	Human herpes virus-7
HHV-8	Human herpes virus-8
HIV	Human immunodeficiency virus
HPMPC	(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine
HPV	Human papillomavirus
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
IFNs	Interferons
MNR	Multinucleoside resistance
NA	Neuraminidase
NAMs	Nucleoside-analog-associated mutations
NK	Natural killer
PEG	Polyethylene glycol
Pgp	P-glycoprotein
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
SEM	Skin, eye, or mouth
SPAG	Small-particle aerosol generator
TAMs	Thymidine analog resistance mutations
TK	Thymidine kinases
UGT	UDP-glucuronosyl transferase
VZV	varicella zoster virus

Defining Statement

Antiviral agents are drugs approved in the USA by the Food and Drug Administration (FDA) for the treatment or control of viral infections. They target stages in the viral life cycle. An ideal antiviral agent should be effective against both actively replicating and latent viruses; however, most of the available antiviral agents are effective against only replicating viruses.

Introduction

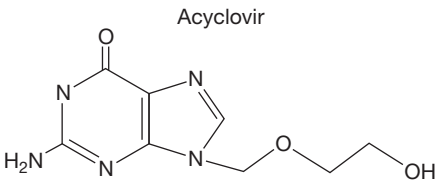
Antiviral agents are drugs approved in the USA by the Food and Drug Administration (FDA) for the treatment or control of viral infections. The development of antiviral agents is not trivial as viral replication is intricately linked with the host cell that any antiviral drug that interferes even to a lesser extent with host cell factors may be toxic to the host depending on the duration and dosage used. Available antiviral agents mainly target stages in the viral life cycle. The target stages in the viral life cycle are; viral attachment to host cell, uncoating, synthesis of viral mRNA, translation of mRNA, replication of viral RNA and DNA, maturation of new viral proteins, budding, release of newly synthesized virus, and free virus in body fluids. Antiviral agents used to treat viral diseases are currently limited, and at least half of the available agents are for the treatment of human immunodeficiency virus (HIV) infections. The others are used for the management of herpes simplex virus (HSV), varicella zoster virus (VZV), cytomegalovirus (CMV), hepatitis B virus (HBV), hepatitis C virus (HCV), respiratory syncytial virus (RSV), human papillomavirus (HPV), and influenza virus-related diseases.

Viruses could stay in the cells in an episomal form, or are incorporated into host chromosomal DNA without engaging in active viral replication (i.e., viral latency state). An ideal antiviral agent should be effective against both actively replicating and latent viruses; however, most of the available antiviral agents are only effective against replicating viruses. The goals for treating acute viral infections in immunocompetent patients are to reduce the severity of the illness and its complications and to decrease the rate of transmission of the virus. The therapeutic index, or ratio of efficacy to toxicity, must be extremely high in order for the therapy to be acceptable. For chronic viral infections, the goal is to prevent viral damage to visceral organs, and therefore efficacy becomes paramount. Antiviral agents can be used for prophylaxis, suppression, preemptive therapy, or treatment of overt disease. Two important factors that can limit the utility of antiviral drugs are toxicity and the development of resistance to the antiviral agent by the virus. In addition, host phenotypic behaviors toward antiviral drugs because of either genomic or epigenetic factors could limit the efficacy of an antiviral agent in an individual. This article summarizes the most relevant pharmacologic and clinical properties of the available antiviral agents.

Therapeutics for Herpesvirus Infections

There are eight members of the human herpesviridae family: HSV-1, HSV-2, VZV, Epstein–Barr virus (EBV), CMV, human herpes virus-6 (HHV-6), human herpes virus-7 (HHV-7), and human herpes virus-8 (HHV-8). The hallmark of the herpesviruses is their ability to establish latency within the neuronal ganglia of the nervous system or cells of the immune system and reactivate during periods of stress, trauma, or immune suppression.

Acyclovir



Chemistry, mechanism of action, and antiviral activity

Acyclovir [9-(2-hydroxyethoxymethyl) guanine] is a synthetic acyclic purine nucleoside analogue that lacks the 3′-hydroxyl group of nucleosides. Acyclovir is phosphorylated to the active triphosphate metabolite that inhibits viral DNA synthesis (Figure 1). Viral encoded thymidine kinases (TK), present in only herpesvirus-infected cells, catalyze the phophorylation to acyclovir monophosphate. Host cell TK or other kinases cannot phosphorylate acyclovir to its monophosphate metabolite efficiently. Acyclovir is highly selective for cells engaged in active viral replication and does not affect noninfected cells. The monophosphate is subsequently phosphorylated to the di-, and triphosphate by cellular kinases, resulting in acyclovir triphosphate concentrations much higher in HSV-infected than in uninfected cells. Acyclovir triphosphate inhibits viral DNA synthesis by competing with deoxyguanosine triphosphate (dGTP) as a substrate for viral DNA polymerase, as illustrated in Figure 1. Since acyclovir triphosphate lacks the

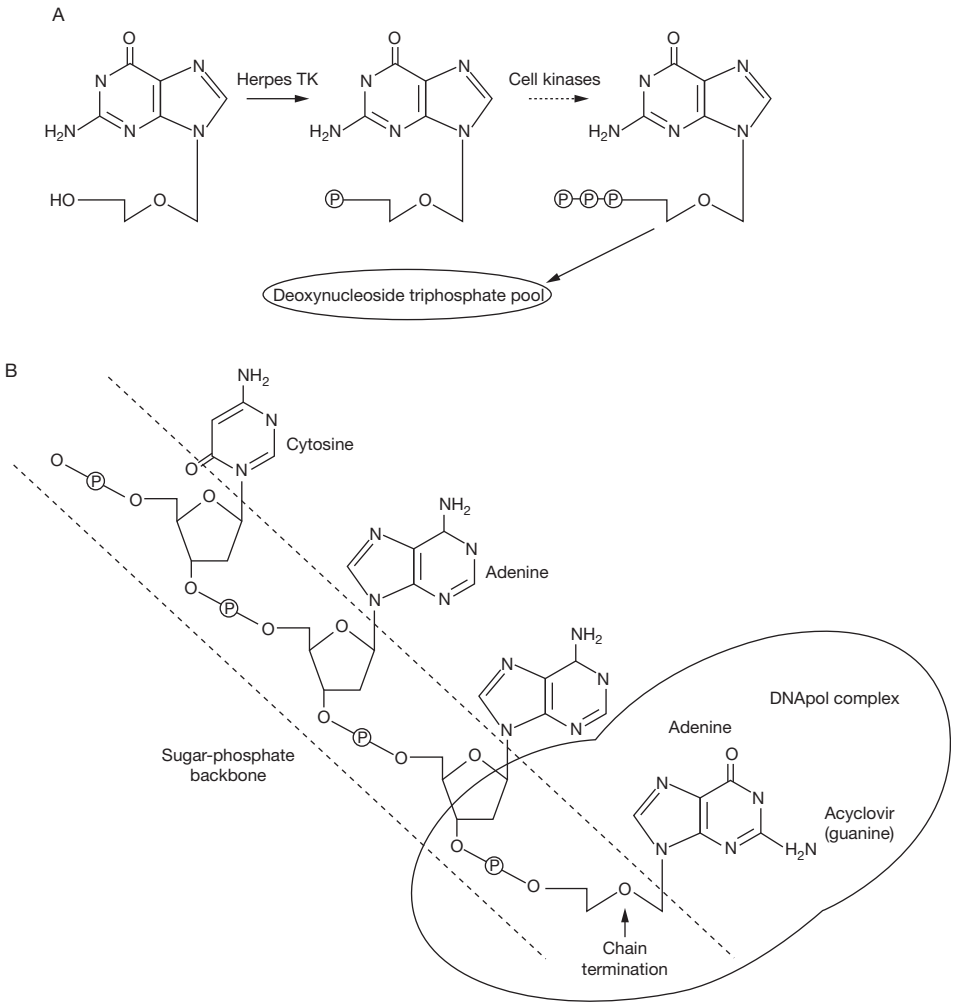


Figure 1 The mechanism of action of acyclovir: (a) activation; (b) inhibition of DNA synthesis and chain termination.

3′-hydroxyl group required for DNA chain elongation, the growing chain of DNA is terminated. In addition, the incorporated acyclovir can trap viral DNA polymerase and prevent it from initiating other viral DNA replication. The viral polymerase has a greater affinity for acyclovir triphosphate than cellular DNA polymerase, resulting in little incorporation of acyclovir into cellular DNA. *In vitro*, acyclovir is most active against HSV-1 (average $EC_{50} = 0.04$ μg ml^{-1}), HSV-2 (0.10 μg ml^{-1}), and VZV (0.50 μg ml^{-1}). Varicella virus is much less susceptible to acyclovir than is HSV, and hence, higher doses of acyclovir are required in the treatment of VZV infections. EBV TK has poor efficiency to utilize acyclovir as substrate, therefore, higher acyclovir concentrations are required for EBV inhibition. CMV, which lacks a virus-specific TK, is relatively resistant.

The bioavailability of oral formulations of acyclovir is 15–30%. Peak concentrations of approximately 0.57 and 1.57 μg ml^{-1} are attained after multidose oral administration of 200 or 800 mg of acyclovir, respectively. Higher plasma acyclovir levels are achieved with intravenous administration. The plasma half-life is 2–3 h in older children and adults with normal renal function and 2.5–5 h in neonates with normal creatinine clearance. The elimination of acyclovir is prolonged in individuals with renal dysfunction, with a half-life of approximately 20 h in persons with end-stage renal disease. Acyclovir is minimally metabolized and approximately 85% is excreted unchanged in the urine via renal tubular secretion and glomerular filtration.

Clinical indications

For most of the clinical indications of acyclovir, valacyclovir and famciclovir are as effective, safe, and convenient alternatives. The clinical applications of valacyclovir and famciclovir are detailed in section 'Clinical indications' under 'Valacyclovir' and in section 'Clinical indications' under 'Penciclovir and Famciclovir,' respectively.

Genital herpes

Initial and recurrent episodes of genital HSV infection can be treated with acyclovir, and recurrent episodes can be suppressed with acyclovir. Topical acyclovir is not an effective treatment for genital HSV. Intravenous acyclovir (15 mg kg^{-1} day^{-1} in three divided

doses for 5–7 days) is the most effective treatment for a first episode of genital herpes and results in a significant reduction in the median duration of viral shedding, pain, and time to complete healing (8 vs. 14 days) but is reserved for patients with systemic complications. Oral therapy (200 mg five times daily) is the standard treatment.

Recurrent genital herpes is less severe and resolves more rapidly than primary infection. Orally administered acyclovir (200 mg five times daily or 400 mg three times daily) for 7–10 days shortens the duration of signs and symptoms, virus shedding, and time to healing by 2, 7, and 4 days, respectively, when initiated within 24 h of onset of symptoms.

Oral acyclovir administration effectively suppresses recurrent genital herpes. Daily administration of acyclovir reduces the frequency of recurrences by up to 80%, and 25–30% of patients have no further recurrences while taking the drug.

Herpes labialis

Topical therapy for HSV-1 mouth or lip infections is of no clinical benefit. Orally administered acyclovir (200 or 400 mg five times daily for 5 days) reduces the time to loss of crust by approximately 1 day (7 vs. 8 days) but does not alter the duration of pain or time to complete healing.

Immunocompromised host

Immunocompromised individuals, such as those infected with HIV or transplant recipients, are afflicted with frequent and severe HSV infections. Clinical benefit from intravenous or oral acyclovir therapy is documented as evidenced by a significantly shorter duration of viral shedding and accelerated lesion healing. Oral acyclovir therapy in high doses in immunocompromised patients with herpes zoster is effective but valacyclovir is superior.

Herpes simplex encephalitis

HSV infection of the brain is the most common cause of sporadic fatal encephalitis in the United States. HSV-1 is predominantly the causative agent of herpes simplex encephalitis (HSE) (Whitley and Kimberlin, 2005). Acyclovir at a dose of 10 mg kg^{-1} every 8 h (30 mg kg^{-1} day^{-1}) for 10–14 days is the therapy of choice and reduces mortality from 70 to 19%. Furthermore, 38% of acyclovir recipients returned to normal neurologic function.

Neonatal HSV infection

HSV infection of the neonate is rare; with an estimated 1500 cases diagnosed annually in the United States from a birth cohort of more than 4 million (Kimberlin et al., 2013). HSV infection of the neonate is classified as: (1) skin, eye, or mouth (SEM) disease, (2) central nervous system (CNS) disease, and (3) disseminated (if there is evidence of visceral involvement) HSV disease. This classification system is predictive of severity and outcome of disease (Kimberlin et al., 2001; Whitley et al., 1991). The recommended treatment for neonatal herpes infection is 20 mg kg^{-1} every 8 h of parenteral acyclovir with duration dictated by the extent of disease; 14 days for SEM disease, 21 days for CNS and disseminated disease. Oral acyclovir suppressive therapy for 6 months is recommended after parenteral acyclovir treatment (Kimberlin et al., 2013). For babies with SEM disease, 98% of acyclovir recipients developed normally 2 years after infection. For babies surviving encephalitis and disseminated disease, 43 and 57% of acyclovir recipients, respectively, developed normally.

Varicella

Varicella or chickenpox, is a common, highly contagious illness caused by VZV. It is primarily a disease of early childhood with 90% of cases occurring in children 1–14 years of age. Chickenpox is generally self-limiting in young children and is manifested by fever, mild constitutional symptoms, and an itchy, vesicular rash (Arvin, 2002). The disease is more severe in neonates, adults, and immunocompromised individuals. Oral acyclovir therapy when initiated within 24 h of the onset of the rash reduces the duration of fever, and the number of maximum lesions in immunocompetent children. At present, the clinical importance of acyclovir treatment in otherwise healthy children, in whom chicken pox is self-limiting and results in few complications, remains uncertain. Furthermore, the widely use of the varicella vaccine to protect against VZV will make the use of acyclovir for immunocompetent children with chickenpox obsolete.

Acyclovir therapy of chicken pox in immunocompromised host substantially reduces morbidity and mortality. Intravenous acyclovir treatment (500 mg m^{-2} of body surface area or 10–12 mg kg^{-1} every 8 h for 7–10 days) improved the outcome, as evidenced by a reduction of VZV pneumonitis from 45 to $<5\%$. Oral acyclovir therapy is not indicated for immunocompromised host with chicken pox. The bioavailability of valacyclovir makes it an attractive alternative.

Herpes zoster

Herpes zoster or singles is caused by the reactivation of VZV, which resides in a latent state in the sensory ganglia following primary varicella (chicken pox) infection (Dworkin et al., 2007). Acute herpes zoster is a painful, debilitating condition, especially in older adults. The risk of zoster-associated pain persisting after the healing of the rash correlates with increasing age. Intravenous acyclovir therapy of herpes zoster in the normal host produces some acceleration of the healing of the rash, and resolution of pain (both acute neuritis and zoster-associated pain). Oral acyclovir (800 mg five times a day) administration results in accelerated healing of the rash and reduction in the severity of acute neuritis. Oral acyclovir treatment of zoster ophthalmicus reduces the incidence of serious ocular complications such as keratitis and uveitis. Intravenous acyclovir therapy

significantly reduces the frequency of cutaneous dissemination and visceral complications of herpes zoster in immunocompromised adults. Acyclovir is the standard therapy at a dose of 10 mg kg^{-1} (body weight) or 500 mg m^{-2} (body surface area) every 8 h for 7–10 days.

Resistance

The most common mechanism for conferring acyclovir resistance is mutations in the HSV genome resulting in a deficiency or alteration in viral TK activity. Occasionally, HSV strains are TK altered and maintain the ability to phosphorylate the natural substrate, thymidine, but selectively lose the ability to phosphorylate acyclovir. Mutation of the viral DNA polymerase gene resulting in failure to incorporate the acyclovir triphosphate in progeny DNA molecules is an alternate, but infrequent, mechanism that may result in HSV resistance to acyclovir.

Resistance to acyclovir is uncommon, with prevalence of 0.1–0.4% and 5–6% in immunocompetent and immunocompromised patients, respectively (Englund et al., 1990; Morfin and Thouvenot, 2003). Acyclovir-resistant isolates of VZV have been identified much less frequently than acyclovir-resistant HSV but have been recovered from marrow transplant recipients and AIDS patients (Lyall et al., 1994). The acyclovir-resistant VZV isolates all had altered or absent viral TK function but remained susceptible to vidarabine and foscarnet, which do not require viral TK for their activity.

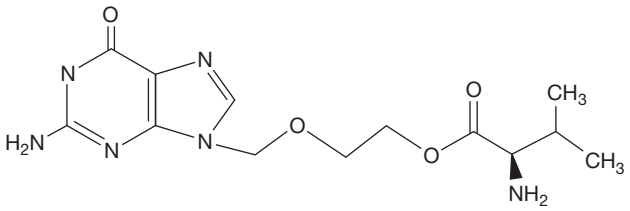
Adverse effect

Acyclovir therapy is associated with few adverse effects. The most common complaints associated with acyclovir therapy include nausea, diarrhea, and headache. Rapid infusions of intravenous acyclovir can result in reversible crystalline nephropathy. A few reports have linked intravenous acyclovir use with CNS disturbances, including agitation, hallucinations, disorientation, tremors, and myoclonus (Cohen et al., 1984; Wade and Meyers, 1983).

Data on outcomes from pregnant mothers exposed to acyclovir during pregnancy showed that the rate of birth defects did not exceed that expected in the general population and the pattern of defects did not differ from those in untreated women.

Valacyclovir

Valacyclovir



Chemistry, mechanism of action, and antiviral activity

Valacyclovir, is a prodrug of acyclovir (the L-valyl ester of acyclovir). Valacyclovir is rapidly metabolized into acyclovir and valine by the enzyme valacyclovir hydrolase (esterase) found in the brush border of the gastrointestinal tract, and the liver. Valacyclovir provides a high bioavailability of acyclovir, threefold to fivefold higher than that obtained with oral acyclovir, and is equivalent to plasma levels achieved with doses of intravenous acyclovir. The mechanism of action and antiviral activity spectrum of valacyclovir are similar to that as described for acyclovir.

Clinical indications

The antiviral spectrum of valacyclovir encompasses HSV-1, HSV-2, VZV, and CMV. It is effective for treatment of HSV-1 and HSV-2 infections in immunocompetent individuals; initial episode of genital herpes (1 g, twice daily for 10 days); episodic therapy for recurrent herpes labialis (2 g, twice a day for 1 day) and recurrent genital herpes (1 g or 500 mg, twice a day for 3–5 days); and suppression of recurrent genital herpes (1 g or 500 mg, once a day).

For immunocompromised patients, valacyclovir is effective for episodic therapy (1 g, twice a day for >5 days) and suppression of recurrent genital herpes (500 mg, twice a day, or 1 g, once a day). Valacyclovir (1 g, three times daily for 7–10 days) is superior to acyclovir for the reduction of pain associated with shingles.

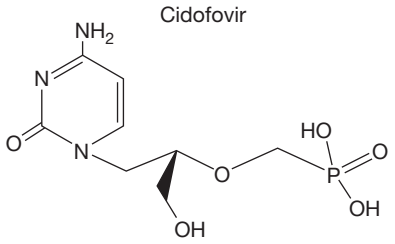
Resistance

The mechanism of resistance to valacyclovir is similar to that of acyclovir.

Adverse effects

Valacyclovir has similar side effect profile as acyclovir; however, no crystalline nephropathy has been reported with its use.

Cidofovir



Chemistry, mechanism of action, and antiviral activity

Cidofovir, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC), is an acyclic phosphonate nucleotide analogue of deoxycytidine monophosphate. Cidofovir has a single phosphate group attached therefore it does not require viral enzymes for conversion to the monophosphate, cellular kinases sequentially phosphorylate the monophosphate to its active triphosphate metabolite. The triphosphate metabolite then serves as a competitive inhibitor of DNA polymerase. The active form of the drug exhibits a 25- to 50-fold greater affinity for the viral DNA polymerase, compared with the cellular DNA polymerase, thereby selectively inhibiting viral replication. Owing to its unique phosphorylation requirements for activation, cidofovir usually maintains activity against acyclovir- and foscarnet-resistant HSV isolates, as well as ganciclovir- and foscarnet-resistant CMV mutants. Cidofovir is less potent than acyclovir *in vitro*; however, cidofovir persists in cells for prolonged periods, increasing drug activity. In addition, cidofovir produces active metabolites with long half-lives (17–48 h), permitting once weekly dosing. Cidofovir has *in vitro* activity against VZV, EBV, HHV-6, HHV-8, HPV, polyomaviruses, orthopoxviruses, and adenovirus. Unfortunately, cidofovir concentrates in kidney cells 100 times greater than in other tissues and produces severe proximal convoluted tubule nephrotoxicity when administered systemically. Cidofovir has limited and variable oral bioavailability (2–26%), therefore, it is administered intravenously.

Clinical indication

Cidofovir is licensed for treatment of CMV retinitis and has been used to treat acyclovir-resistant HSV infection. The dosing regimen is 5 mg kg^{-1} per week during the first 2 weeks, then 5 mg kg^{-1} every other week, with sufficient hydration and coadministration of oral probenecid to prevent nephrotoxicity. There are anecdotal reports that dividing the 5 mg kg^{-1} weekly dose into three doses given alternate days in a week may reduce renal toxicity substantially.

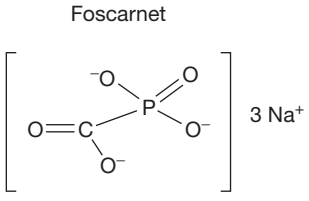
Resistance

The development of resistance with clinical use is uncommon; however, mutations in CMV DNA polymerase can mediate altered susceptibility.

Adverse effects

Nephrotoxicity is the principal adverse event associated with systemic administration of cidofovir, occurs in 30–50% of recipients. Other reported side effects include neutropenia, fever, diarrhea, nausea, headache, rash, anterior uveitis, and ocular hypotonia.

Foscarnet



Chemistry, mechanism of action, and antiviral activity

Foscarnet, is an inorganic pyrophosphate analogue of phosphonoacetic acid that inhibits all HHVs, including most ganciclovir-resistant CMV isolate and acyclovir-resistant HSV and VZV strains. It inhibits DNA polymerase by blocking the pyrophosphate-binding site and preventing cleavage of pyrophosphate from deoxynucleotide triphosphates. Unlike acyclovir, which requires activation by a virus-specific TK, foscarnet acts directly on the virus DNA polymerase. Thus, TK-deficient, acyclovir-resistant herpesviruses remain sensitive to foscarnet.

The oral bioavailability of foscarnet is about 20%. The cerebrospinal fluid (CSF) concentration of foscarnet is approximately two-thirds of the plasma level. Renal excretion is the primary route of clearance of foscarnet with >80% of the dose appearing in the urine. Bone sequestration also occurs, resulting in complex plasma elimination.

Clinical indications

The principal indications are CMV retinitis in AIDS patients, and mucocutaneous acyclovir-resistant (viral TK-deficient) or penciclovir-resistant HSV and VZV infections in immunocompromised patients.

Mucocutaneous HSV infections and those caused by VZV in immunocompromised host can be treated with foscarnet at dosages lower than that for the management of CMV retinitis.

Resistance

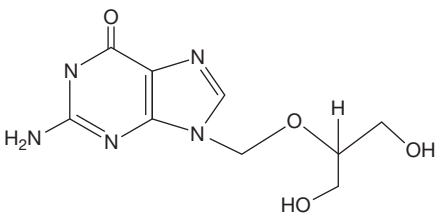
Isolates of HSV, CMV, and VZV resistant to foscarnet develop both in the laboratory and in the clinical setting (Safrin et al., 1994; Wagstaff and Bryson, 1994). These isolates are all DNA polymerase mutants.

Adverse effects

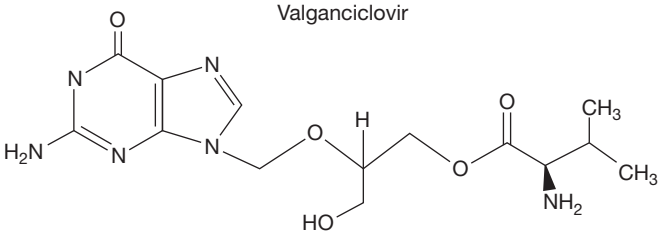
Foscarnet toxicity includes mainly nephrotoxicity (acute tubular necrosis and interstitial nephritis), metabolic and hematologic abnormalities, and CNS side effects (Deray et al., 1989; MacGregor et al., 1991). Patients may develop isolated or combined hypomagnesemia, hypocalcemia, hypokalemia, and hypophosphatemia. CNS side effects include headache, seizures, irritability, tremor, and hallucination. Other reported side effects include fever, rash, painful genital ulcerations, diarrhea, nausea, and vomiting.

Ganciclovir and Valganciclovir

Ganciclovir



Valganciclovir



Chemistry, mechanism of action, and antiviral activity

Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl) guanine] is a nucleoside analogue that differs from acyclovir by having a hydroxymethyl group at the 3′ position of the acyclic side chain. It has 8–20 times greater *in vitro* activity against CMV, and as active as acyclovir against HSV-1 and HSV-2 and almost as active against VZV. Like acyclovir, the first step of phosphorylation to ganciclovir monophosphate in herpesvirus-infected cells depends on virus-encoded enzymes. In cells infected by HSV-1 or HSV-2, TK catalyzes the phosphorylation of ganciclovir to ganciclovir monophosphate. Because CMV lacks the gene for TK, the enzyme that catalyzes the initial phosphorylation of ganciclovir in CMV-infected cells is the phosphotransferase encoded by *UL97* gene. The final phosphorylation steps to the di- and triphosphate is by cellular kinases. Ganciclovir triphosphate serves as a competitive inhibitor of herpes viral DNA polymerase and inhibits the incorporation of guanosine triphosphate into viral DNA. Incorporation of ganciclovir triphosphate into the growing viral chain results in slowing and subsequent cessation of DNA chain elongation. Intracellular ganciclovir triphosphate concentrations are at least tenfold higher in CMV-infected cells than uninfected cells.

The oral bioavailability of ganciclovir is poor (5–7%). Concentrations of ganciclovir in biologic fluids, including aqueous humor and CSF, are less than plasma levels. The plasma elimination half-life is 2–4 h for individuals with normal renal function.

The kidney is the major route of clearance of ganciclovir, and therefore, impaired renal function requires adjustment of dosage. The pharmacokinetics of ganciclovir in neonates is similar to that in adults.

Valganciclovir, L-valine ester of ganciclovir, serves as oral prodrug of ganciclovir. Valganciclovir is orally bioavailable (approximately 60%) and is rapidly converted to ganciclovir after absorption. Its mechanism of action and spectrum of activity are similar to that of ganciclovir. Oral valganciclovir can be given in doses that result in serum levels that approximate ganciclovir serum levels achieved with intravenous ganciclovir. Oral valganciclovir is convenient to use and may replace intravenous ganciclovir for initial and maintenance treatment.

Clinical indications

Ganciclovir is approved for treatment and chronic suppression of CMV retinitis in AIDS or other immunocompromised patients, and prophylaxis or preemptive treatment of CMV infection in high-risk transplant recipients. It is also effective for CMV syndromes, including CMV pneumonia, CMV colitis, and gastrointestinal infection in AIDS and transplant patients. In immunocompromised patients, therapy with ganciclovir requires an induction and maintenance phases. The induction dose is 10 mg kg^{-1} day^{-1} in two divided doses given for 14–21 days, and a maintenance dose of 5 mg kg^{-1} day^{-1} given once daily for 5–7 days per week. Oral valganciclovir dosage is 900 mg twice a day, and 900 mg once a day for induction and maintenance therapy, respectively (Balfour, 1999).

Ganciclovir is now recommended for treatment of neonates congenitally infected with CMV. In a phase III randomized controlled trial, ganciclovir therapy (6 mg kg^{-1} per dose administered twice a day for 6 weeks) protected infants from hearing deterioration beyond one year of life and reduced incidence of neurodevelopmental delays (Kimberlin et al., 2003; Oliver et al., 2009). Oral valganciclovir may become available for treatment of congenital CMV disease in the near future. In a randomized trial, valganciclovir treatment (16 mg kg^{-1} per dose every 12 h for six months) of newborns with congenital CMV disease was associated with improved head circumference and developmental milestones, and decreased risk of hearing loss or progression of hearing loss.

Resistance

In immunocompromised patients receiving prolonged therapy, the prevalence of resistance exceeds 8%. There are two mechanisms of resistance by CMV to ganciclovir: (1) The alteration of the CMV phosphonotransferase (coded by CMV *UL97* gene) (Markham and Faulds, 1994) reduces intracellular phosphorylation of ganciclovir, and (2) mutations in the CMV DNA polymerase (coded by CMV *UL54* gene). Resistance is associated with decreased sensitivity up to 20-fold. Occasionally, strains of HSV that are resistant to acyclovir because of TK deficiency are also much less sensitive to ganciclovir.

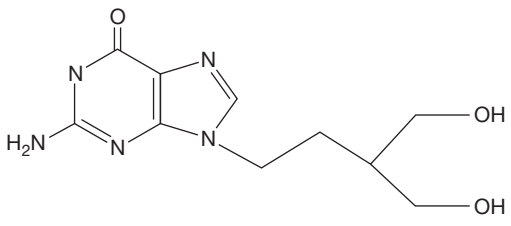
Adverse effects

The most important side effects of ganciclovir therapy are the development of neutropenia, and thrombocytopenia. Neutropenia occurs in approximately 24–38% of patients. The neutropenia is usually reversible with dosage adjustment of ganciclovir, or withholding of treatment. Thrombocytopenia occurs in 6–19% of patients.

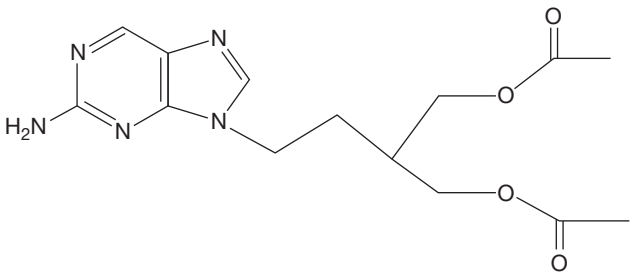
Ganciclovir has gonadal toxicity in animal models, most notably as a potent inhibitor of spermatogenesis. It causes an increased incidence of tumors in the preputial gland of male mice, a finding of unknown significance. As an agent affecting DNA synthesis, ganciclovir has carcinogenic potential.

Penciclovir and Famciclovir

Penciclovir



Famciclovir



Chemistry, mechanism of action, and antiviral activity

Penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)] a guanine nucleoside analogue is structurally similar to ganciclovir, differing only by the substitution of a methylene bridge for the ether oxygen in the acyclic ribose part of the molecule. Its metabolism and mechanism of action are similar to those of acyclovir, except that it is not an obligate DNA-chain terminator. The *in vitro* inhibitory effects of penciclovir on HSV-1, HSV-2, and VZV are similar to those of acyclovir. The oral bioavailability of penciclovir is poor (<5%). Famciclovir, a prodrug of penciclovir with improved bioavailability (approximately 77%), is the diacetyl ester of 6-deoxy penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)-6-deoxyguanine]. It is well absorbed after oral administration and is rapidly metabolized to penciclovir by deacetylation in the gastrointestinal tract, and liver, after which it is oxidized by the liver at the position 6 of the purine ring. Penciclovir is phosphorylated more efficiently than acyclovir in HSV- and VZV-infected cells. Host cell kinases phosphorylate both penciclovir and acyclovir to a small but comparable extent. The preferential metabolism in HSV- and VZV-infected cells is the major determinant of its antiviral activity. Penciclovir triphosphate has, on average, a tenfold longer intracellular half-life than acyclovir triphosphate in HSV-1-, HSV-2-, and VZV-infected cells after drug removal. Because penciclovir is more stable, it has longer antiviral activity, allowing for less frequent dosing. Both compounds have good activity against HSV-1, HSV-2, and VZV. Penciclovir, like acyclovir, is relatively inactive against CMV and EBV. Penciclovir is active against hepatitis B.

Penciclovir is eliminated rapidly and almost unchanged by active tubular secretion and glomerular filtration by the kidneys. The elimination $T_{1/2}$ in healthy subjects is approximately 2 h.

Clinical indications

Famciclovir is available in an oral preparation for treatment of HSV-1, HSV-2, and VZV infections. It is used in the treatment of the following conditions: initial episodes of genital herpes (250 mg, three times a day for 10 days), episodic treatment of recurrent genital herpes (125 mg, twice a day for 5 days), suppression of recurrent genital herpes (250 mg, twice a day), and for shingles (500 mg, every 8 h for 7 days). For immunocompromised patients, famciclovir is efficacious for episodic treatment of recurrent genital herpes (500 mg, twice a day for 7 days). Compared with acyclovir, famciclovir is as effective, safe, and well tolerated in the treatment of HSV infections in HIV-infected individuals. Famciclovir is also at least as effective as acyclovir for ophthalmic zoster and for shingles and acute zoster pain in immunocompromised patients. Compared with valacyclovir, famciclovir is as effective, safe, and convenient in the treatment of zoster.

Penciclovir is available as a 1% cream for topical therapy of mucocutaneous HSV infections, particularly recurrent herpes labialis (cold sores). Topical penciclovir 1% is approved for episodic therapy of herpes labialis and applied every 2 h during waking hours for 4 days. It accelerates lesion healing and resolution of pain by about 1 day. It is available over-the-counter in many countries.

Resistance

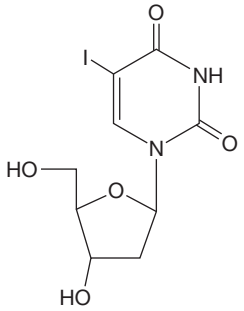
HSV and VZV isolates resistant to penciclovir have been identified in the laboratory. In clinical trials, penciclovir-resistant HSV was isolated from 0.22% immunocompetent patients and 2.1% of immunocompromised patients (Sarisky et al., 2003). Resistance is attributed to alterations or deficiencies of TK and DNA polymerase.

Adverse effects

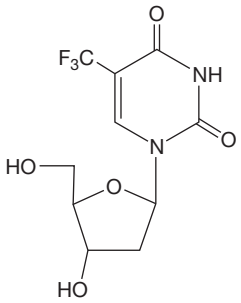
Therapy with oral famciclovir is well tolerated, being associated only with headache, diarrhea, and nausea. Preclinical studies of famciclovir indicated that chronic administration was tumorigenic (murine mammary tumors) and causes testicular toxicity in other rodents.

Idoxuridine and Trifluorothymidine

Idoxuridine



Trifluorothymidine



Chemistry, mechanism of action, and antiviral activity

Idoxuridine (5-iodo-2′-deoxyuridine), and trifluorothymidine (5-trifluoromethyl-2′-deoxyuridine) are thymidine analogues. When administered systemically, these nucleosides are phosphorylated by both viral and cellular kinases to active triphosphate derivatives, which inhibit both viral and cellular DNA synthesis. Parenteral administration results in potent antiviral activity but also sufficient host cytotoxicity to prevent the systemic use of these drugs. The toxicity of these compounds is not significant when applied topically to the eye in the treatment of HSV keratitis. Both idoxuridine and trifluorothymidine are effective and licensed for treatment of HSV keratitis. Topically applied idoxuridine or trifluorothymidine will penetrate cells of the cornea. Low levels of drugs can be detected in the aqueous humor.

Clinical indications

Trifluorothymidine is the most efficacious of these compounds, and the treatment of choice for HSV keratitis (1 drop of 1% ophthalmic solution instilled in each eye, up to nine times a day). Idoxuridine was the first antiviral compound to receive FDA approval in 1963 for treatment of HSV keratitis.

Resistance

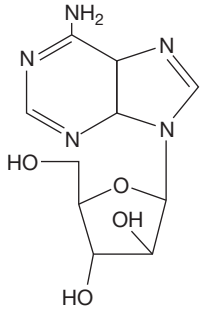
Trifluorothymidine-resistant HSV strains with altered TK substrate specificity have been selected for *in vitro*. However, clinical significant resistance has not been established.

Adverse effects

The ophthalmic preparation of idoxuridine and trifluorothymidine causes local discomfort, irritation, photophobia, edema of the eyelids, and less commonly, hypersensitivity reactions as well as superficial punctate or epithelial keratopathy.

Vidarabine

Vidarabine



Chemistry, mechanism of action, and antiviral activity

Vidarabine (vira-A, adenine arabinoside, and 9-D-arabinofuranosyl adenine) is active against HSV, VZV, and CMV. Vidarabine is a purine nucleoside analogue that is phosphorylated intracellularly to its mono-, di-, and triphosphate derivatives. Thus, unlike acyclovir, conversion of vidarabine to its active intracellular derivative does not require viral enzymes at any of the phosphorylation

steps. The triphosphate derivative competitively inhibits DNA dependent DNA polymerases of some DNA viruses approximately 40 times more than those of host cells. In addition, vira-A is incorporated into terminal positions of both cellular and viral DNA, thus inhibiting elongation. Viral DNA synthesis is blocked at lower doses of drug than is host cell DNA synthesis, resulting in a relatively selective antiviral effect. However, large doses of vira-A are cytotoxic to dividing host cells.

The use of vidarabine was replaced by acyclovir because of poor solubility and toxicity. It is no longer available as an intravenous formulation. However, vidarabine should be recognized historically as the first antiviral agent licensed in 1977 for systemic treatment.

Clinical indications

Although trifluorothymidine is the antiviral agent of choice for the topical treatment of HSV keratitis, in patients in whom trifluorothymidine cannot be used vidarabine is a suitable alternative. Topical vidarabine is superior to idoxuridine in the treatment of HSV ocular infections.

Resistance

Resistance to vidarabine is conferred by mutations in the viral DNA polymerase gene. The degree of maximal resistance to vidarabine is fourfold, much lower than the 100-fold resistance to acyclovir with similar DNA polymerase resistant mutations. Acyclovir-resistant clinical HSV isolates are always susceptible *in vitro* to vidarabine.

Adverse effects

Ocular toxicity consists of occasional hyperemia and increased tearing, both of low incidence.

Fomivirsen

Chemistry, mechanism of action, and antiviral activity

Fomivirsen is a 21-nucleotide phosphorothioate oligonucleotide that inhibits CMV replication through an antisense mechanism. Its oligonucleotide sequence (5′-GCG TTT GCT CTT CTT CTT GCG-3′) is complementary to a sequence in mRNA transcripts of the major immediate early region 2 (IE2) of CMV, which encodes for several proteins responsible for the viral gene expression that are essential for the production of infectious viral particles. Binding of fomivirsen to the target mRNA results in inhibition of IE2 protein synthesis, with subsequent inhibition of viral replication. *In vitro*, fomivirsen inhibits CMV replication in a dose-dependent manner, with a mean IC_{50} of between 0.03 and 0.2 μmol l^{-1}. Pharmacokinetic assessment of fomivirsen in humans after intraocular administration is limited. In a rabbit model, intraocular administration revealed first-order kinetics with half-life of 62 h. Fomivirsen is cleared from the vitreous in rabbits during the course of 7–10 days by a combination of tissue distribution and metabolism. No systemic absorption has been observed after intravitreal administration in humans.

Clinical indications

Fomivirsen is indicated for use in HIV patients with CMV retinitis who are intolerant of or have contraindication to other treatment for CMV retinitis or in whom the disease is recalcitrant to ganciclovir or cidofovir treatment. It has activity against cidofovir- and ganciclovir-resistant strains of CMV.

Resistance

CMV strains with tenfold decreased susceptibility have been selected *in vitro*. However, no resistant clinical isolates have been reported.

Adverse effects

Adverse events of fomivirsen are uveitis, including iritis and vitritis, occurring in approximately 25% of patients. These reactions are usually transient or reversible with topical corticosteroids treatment.

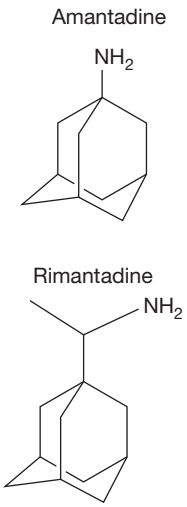
New Prospects for Therapy of Herpesvirus Infections

All the licensed antiviral agents for herpes infections ultimately target the viral HSV DNA polymerase, which is essential for virus replication. Recent drug discovery efforts have not only focused on improving the efficacy and safety profile of current antivirals but also explored new viral targets (Kleymann et al., 2002). A prodrug of cidofovir (CMX001) has demonstrated that the prodrug approach can not only improve oral absorption but also reduce the toxicity of the parent compound. Pritelivir (AIC316),a helicase–primase inhibitor, with preclinical pharmacological profile that outperforms the current standard HSV treatment has

shown promise in a Phase II trial (Vere Hodge and Field, 2013; Wald et al., 2014; Weller and Kuchta, 2013). Recent discovery of antiviral activity of letermovir (AIC246), a terminase inhibitor, is a proof of concept that herpes virus terminase enzyme is a viable target for new HSV therapies (Goldner et al., 2011). The ultimate breakthrough for the treatment of herpes virus infection will be the discovery of agents or innovative strategies to eliminate herpes latency.

Therapeutics for Respiratory Virus Infections

Amantadine and Rimantadine



Chemistry, mechanism of action, and antiviral activity

Amantadine (1-adamantanamine hydrochloride) and rimantadine (α-methyl-1-adamantanemethylamine hydrochloride) are symmetric tricyclic amines with narrow spectrum of activity, being useful only against influenza A infections. Rimantadine is fourfold to tenfold more active than amantadine. The mechanism of action of these drugs relates to the influenza A virus M2 protein, an integral transmembrane protein that functions as an ion channel for this virus and is activated by pH. The drop in pH accompanying the hydrogen flux facilitates the dissociation of the M2 protein from the ribonucleoprotein complexes so that the ribonucleoprotein can enter the cell nucleus and initiate replication. By interfering with the function of the M2 protein, amantadine and rimantadine inhibit the acid-mediated dissociation of the matrix protein from the ribonuclear protein complex within endosomes. This event occurs early in the viral replication cycle. The consequences of these drugs are the potentiation of acidic pH-induced conformational changes in the viral hemagglutinin during its intracellular transport.

Absorption of rimantadine is slower compared with that of amantadine. Amantadine is excreted unchanged in the urine by glomerular filtration and, likely, tubular secretion. The plasma elimination $T_{1/2}$ is approximately 12–18 h in individuals with normal renal function. However, the elimination $T_{1/2}$ increases in the elderly with impaired creatinine clearance. Rimantadine is extensively metabolized following oral administration, with an elimination $T_{1/2}$ of 24–36 h. Approximately 15% of the dose is excreted unchanged in the urine.

Clinical indications

Amantadine and rimantadine are licensed for the chemoprophylaxis and treatment of influenza A infections. Prophylaxis with either drug prevents approximately 50–60% of infections and 70–90% of clinical illnesses caused by type A influenza virus. Because of a lower incidence of side effects associated with rimantadine, it is used preferentially. Rimantadine can be given to any unimmunized member of the general population who wishes to avoid influenza A, but prophylaxis is especially recommended for control of presumed influenza outbreaks in institutions housing high-risk persons. High-risk individuals include adults and children with chronic disorders of the cardiovascular or pulmonary systems. Prophylaxis also is indicated if the vaccine may be ineffective because the epidemic strain differs substantially from the vaccine strain of influenza A, and for the 2 weeks after vaccination if influenza A already is active in the community.

Amantadine and rimantadine also have been shown to be effective in treatment of influenza A infections in adults and children if treatment is initiated within 48 h of the onset of symptoms (Couch, 2000). Drug therapy results in reduction in the duration of viral excretion, fever, and other systemic complaints, as well as earlier resumption of normal activities, in comparison with placebo. On average, the duration of illness is shortened by about 1 day. Amantadine and rimantadine are given orally at 200, and 300 mg day^{-1}, respectively.

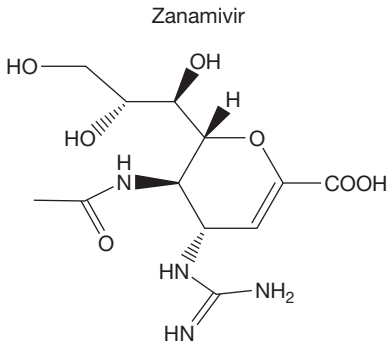
Resistance

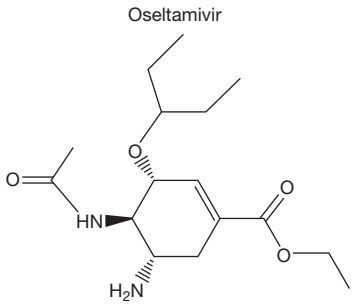
Resistance to amantadine and rimantadine results from point mutations in the RNA sequence encoding the M2 protein transmembrane domain. Resistance typically appears in the treated subjects within 2–3 days of initiating therapy. About 25–35% of treated patients shed resistant strains by the 5th day of treatment (2006). The clinical significance of isolating resistant strains from the treated subject is not clear; infection and illness in immunocompetent people infected with a drug-resistant virus are similar to those in patients infected with drug-sensitive virus.

Adverse effects

Although the spectrum of adverse events associated with amantadine and rimantadine are qualitatively similar, they are less frequent and less severe with rimantadine. Amantadine is reported to cause side effects in 5–10% of healthy young adults taking the standard adult dose of 200 mg day^{-1}. These side effects are usually mild and cease soon after amantadine is discontinued, although they often disappear with continued use of the drug. CNS side effects, which occur in 5–33% of patients, are most common and include difficulty in thinking, confusion, lightheadedness, hallucinations, anxiety, and insomnia. More severe adverse effects (e.g., mental depression and psychosis) are usually associated with doses exceeding 200 mg daily. About 5% of patients complain of nausea, vomiting, or anorexia. CNS adverse effects associated with rimantadine administration are significantly less; however, rimantadine has been associated with exacerbations of underlying seizure disorders.

Zanamivir and Oseltamivir





Chemistry, mechanism of action, and antiviral activity

Zanamivir (4-guanidino-2,3-dideoxy-2,3-didehydro-*N*-acetylneuraminic acid), and oseltamivir [ethyl ester of (3*R*,4*R*,5*S*)-4-scetamido-5-amino-3-(1-ethylpropoxyl)-1-cyclohexane-1-carboxylic acid] are sialic acid analogue that competitively inhibit influenza virus neuraminidase (NA). Influenza virus NA is located on the surface of the virus and is responsible for cleaving terminal sialic acid residues, which are essential for the release of the virus from infected cells, viral aggregation, and spread within the respiratory tract. Influenza NA also decreases viral inactivation by respiratory mucous. The lipophilic side chain of zanamivir and oseltamivir binds to the influenza virus NA, blocking its ability to cleave sialic acid residues. Zanamivir and oseltamivir are effective against both influenza A and influenza B.

Zanamivir has poor oral bioavailability and therefore it is administered by oral inhalation. More than 75% of an orally inhaled dose of zanamivir is deposited in the oropharynx, approximately 13% of this is distributed to the airways and lungs. Local respiratory mucosal concentrations of zanamivir exceeds 1000 ng ml^{-1} in sputum for 6 h after inhalation (i.e., over and above the concentration required to inhibit influenza A and B viruses). Approximately 10% of inhaled dose is absorbed systemically; peak serum concentrations range from 17 to 142 ng ml^{-1} within 2 h of administration of a 10 mg dose. The plasma $T_{1/2}$ is between

2.5 and 5 h. Systemically absorbed zanamivir is excreted unchanged in the urine. Although serum concentrations of zanamivir increase with decreasing creatinine clearance, no adjustment in dosing is necessary for renal insufficiency because of the limited amount of systemically absorbed drug.

Oseltamivir is an ethyl ester prodrug that, following hydrolysis by hepatic esterases, is converted to the active compound, oseltamivir carboxylate. Approximately 75% of orally administered oseltamivir reaches the systemic circulation in the form of oseltamivir carboxylate. Oseltamivir carboxylate is eliminated unchanged by renal excretion through glomerular filtration and tubular secretion. The elimination $T_{1/2}$ of oseltamivir carboxylate is 6–10 h. Serum concentrations of the drug increase in the presence of declining renal function, and dose adjustment is recommended in patients with renal insufficiency.

Clinical indications

Zanamivir and oseltamivir are used for treatment and prevention of influenza A and B infections. Treatment of otherwise healthy adults and children with zanamivir and oseltamivir reduces the duration of symptoms by 0.4 and 1 days, and provides 29–43% relative reduction in the odds of complications when given within 48 h of onset of symptoms. These drugs also significantly diminish viral replication in respiratory secretions. Zanamivir is available as dry powder for inhalation using a breath-activated Diskhaler delivery system. The recommended dose of zanamivir in patients >7 years is 10 mg twice daily for 5 days, while oseltamivir is given at 75 mg twice a day for 5 days.

Inhaled zanamivir, 10 mg once daily given for 4 weeks as seasonal prophylaxis, reduces the likelihood of laboratory confirmed influenza (with or without symptoms) by 34%, influenza disease by 67%, and influenza disease with fever by 84%. Oseltamivir administered for 6 weeks during the peak of influenza season significantly reduces the risk of contracting influenza. The protective efficacy of oseltamivir in preventing culture-proven influenza is about 90%.

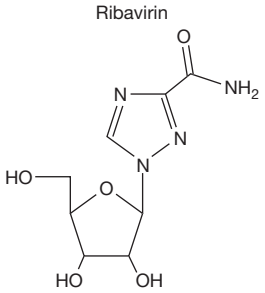
Resistance

Viruses resistant to zanamivir and oseltamivir have been generated after *in vitro* passage in cell culture. Clinical influenza virus isolates with reduced susceptibility to both NA inhibitors have been reported (de Jong et al., 2005). There are two mechanisms of resistance: mutations in the hemagglutin receptor-binding site, and mutations in the conserved portions of the NA enzyme active site. In general, resistant viruses with mutations in the NA enzyme are thought to have decreased infectivity and fitness and therefore less likely to be transmitted.

Adverse effects

Both NA inhibitors are generally well tolerated. Adverse events following administration of oseltamivir have primarily been gastrointestinal with nausea and vomiting occurring in some patients. Inhalation of zanamivir has resulted in bronchospasm and reduced forced expiratory volume (FEV1). Zanamivir should be used with caution in individuals with reactive airway diseases or chronic obstructive pulmonary diseases.

Ribavirin



Chemistry, mechanism of action, and antiviral activity

Ribavirin (α-methyl-1-adamantane methylamine hydrochloride) has antiviral activity against a variety of RNA and DNA viruses. Ribavirin is a nucleoside analogue with poorly understood mechanism of action and probably virus-specific mechanism of action. However, its ability to alter nucleotide pools and the packaging of mRNA appears important. This process is not virus specific, but quiet selective, in that infected cells produce more mRNA than noninfected cells. A major action is the inhibition by ribavirin-5′-monophosphate of inosine monophosphate dehydrogenase, an enzyme essential for DNA synthesis. This inhibition may have direct effects on the intracellular level of GMP and other nucleotide levels may be altered. The 5′-triphosphate of ribavirin inhibits the formation of the 5′-guanylation capping on the mRNA of vaccinia and Venezuelan equine encephalitis viruses. In addition, the triphosphate is a potent inhibitor of viral mRNA (guanine-7) methyltransferase of vaccinia virus.

The capacity of viral mRNA to support protein synthesis is markedly reduced by ribavirin. Ribavirin may inhibit influenza A RNA-dependent RNA polymerase.

Ribavirin can be administered orally (bioavailability of approximately 40–45%) or intravenously. Aerosol administration has become standard for the treatment of RSV infections in children. Oral doses of 600 and 1200 mg result in peak plasma concentrations of 1.3 and 2.5 μg ml^{-1}, respectively. Intravenous dosages of 500 and 1000 mg result in 17 and 24 μg ml^{-1} plasma concentrations, respectively. Aerosol administration of ribavirin results in plasma levels that are a function of the duration of exposure. Although respiratory secretions will contain milligram quantities of drug, only microgram quantities (0.5–3.5 μg ml^{-1}) can be detected in the plasma.

The kidney is the major route of clearance of drug, accounting for approximately 40%. Hepatic metabolism also contributes to the clearance of ribavirin. Notably, ribavirin triphosphate concentrates in erythrocytes and persists for a month or longer. Likely, the persistence of ribavirin in erythrocytes contributes to its hematopoietic toxicity.

Clinical indications

Respiratory syncytial virus

Ribavirin is licensed for the treatment of carefully selected, hospitalized infants and young children with severe lower respiratory tract infections caused by RSV. Use of aerosolized ribavirin in adults and children with RSV infections reduced the severity of illness and virus shedding. However, placebo controlled trials have failed to demonstrate a consistent decrease in need for mechanical ventilation, duration of stay in intensive care unit, or duration of hospitalization among ribavirin recipients. The use of ribavirin for the treatment of RSV infections is controversial and remains discretionary. The most common adverse events following aerosol administration of ribavirin include bronchospasm and malfunction of ventilator delivery systems. The usual dosage of aerosolized ribavirin is 20 mg ml^{-1} of drug instilled in a small-particle aerosol generator (SPAG) and administered for 12–22 h day^{-1} for 3–6 days. To avoid potential exposure of health care workers to ribavirin, special containment delivery system in an isolation room with negative pressure is used.

Hepatitis C

Oral ribavirin in combination with interferon-α (IFN-α) and a direct-acting antiviral agent (DAA) is recommended for hepatitis C infection.

Lassa fever and hemorrhagic fever

Systemic ribavirin has demonstrated efficacy in the management of life-threatening infections caused by Lassa fever and hemorrhagic fever with renal syndrome. Oral ribavirin is recommended for prophylaxis against Lassa fever in exposed contacts.

Resistance

Treatment failures with ribavirin occur in some patients; however, resistance to ribavirin has not been identified as a clinical problem.

Adverse effects

Adverse effects attributable to aerosol therapy with ribavirin of infants with RSV include bronchospasm, pneumothorax in ventilated patients, apnea, cardiac arrest, hypotension, and concomitant digitalis toxicity. Pulmonary function test changes after ribavirin therapy in adults with chronic obstructive pulmonary disease have been noted. Reticulocytosis, rash, and conjunctivitis have been associated with the use of ribavirin aerosol. When given orally or intravenously, transient elevations of serum bilirubin and the occurrence of mild anemia have been reported. Ribavirin has been found to be teratogenic and mutagenic in preclinical testing. Its use is contraindicated in women who are or may become pregnant during exposure to the drug. Concern has been expressed about the risk to persons in the room of infants being treated with ribavirin aerosol, particularly females of childbearing age. Although this risk seems to be minimal with limited exposure, awareness and caution are warranted and, therefore, the establishment of stringent precautions for administration of ribavirin.

New Prospects for Therapy of Respiratory Viruses

While influenza pandemics have long posed a threat to humankind, a threat realized to varying extents in 1918, 1957, and 1968, particular concern has mounted of late due to continued sporadic human cases of H5N1 avian influenza in Southeast Asia, Eastern Europe, and Africa. Amantadine and rimantadine are not recommended for seasonal or avian influenza because circulating influenza A viruses as well as the H5N1 strains affecting humans in Southeast Asia are resistant to these medications. Zanamivir and oseltamivir are the only available options. To expand the antiviral drug arsenal against influenza, researchers have been testing a number of investigational agents, including peramivir and T-705. Although peramivir is a NA inhibitor administered intramuscularly. Preclinical studies in mice and ferrets revealed that the drug could protect 80% or more of animals exposed to pathogenic H5N1 influenza virus compared with 36% of untreated animals. A pyrazine derivative, T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide), is a viral RNA polymerase inhibitor in the preclinical testing stage.

The outbreaks of novel coronavirus infections such as severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV)(Drosten et al., 2003) and Middle East respiratory syndrome coronavirus (MERS-CoV)(Guery et al., 2013), underscore the need for novel antiviral drugs for the control of future outbreaks of coronavirus and other respiratory viruses infections.

Therapeutics for Hepatitis

Interferons

Chemistry, mechanism of action, and antiviral activity

IFNs are glycoprotein cytokines (intercellular messengers) with a complex array of immunomodulating, antineoplastic, and antiviral properties. IFNs are currently classified as α, β, or γ, the natural sources of which, in general, are leukocytes, fibroblasts, and lymphocytes, respectively. Each type of IFN can be produced through recombinant DNA technology. The binding of IFN to the intact cell membrane is the first step in establishing an antiviral effect. IFN binds to the cellular receptors and activates secondary messengers to initiate production of multiple proteins, which are pivotal for the defense of the cell against viruses. The mechanism of action is rather complex (Thomas et al., 2003). The antiviral effects of IFN include degradation of viral mRNA, inhibition of viral protein synthesis, and prevention of the viral infection of cells. The immunomodulating effects of IFN include enhancement of antigen presentation by HLA I and II to the immune system, activation of natural killer (NK) cells and other immune cells, and increased cytokine production. IFNs are active against a wide spectrum of viruses with RNA viruses being more sensitive than DNA viruses.

IFNs are not orally bioavailable and are administered intramuscularly or subcutaneously (including into a lesion). There appears to be some variability in absorption between each of the three classes of IFN and, importantly, resultant plasma levels. Absorption of IFN-α is more than 80% complete after intramuscular or subcutaneous injection. Plasma levels are dose dependent, peaking 4–8 h after administration and returning to baseline between 18 and 36 h. However, the antiviral activity peaks at 24 h and then slowly decreases to baseline over about 6 days. IFN is eliminated by inactivation in various body fluids and metabolism in a number of organs. Negligible amounts are excreted in the urine unchanged.

IFN-α molecule covalently bonded to polyethylene glycol (PEG) improves the pharmacokinetic profile of IFN markedly. The pegylated forms of IFN-α (Peg-IFN-α) offer a more convenient once weekly instead of daily administration, are licensed for the treatment of hepatitis B and C.

Clinical indications

Hepatitis B

The major goals of therapy for hepatitis B are to prevent progression of the disease to cirrhosis, end stage liver disease or hepatocellular carcinoma (Chiaramonte et al., 1999; Yang et al., 2002). Three generally accepted indications for treatment are: (1) positive test for HBV DNA, (2) positive hepatitis B e antigen (HBeAg), and (3) alanine aminotransferase (ALT) level greater than two times normal. Treatment end points differ in HBeAg positive, and HBeAg negative disease. However, suppression of HBV replication to levels less than 1×10^4 copies per ml (2000 IU ml^{-1}) is regarded as a surrogate of treatment success with a resultant improvement in serum ALT and hepatic necroinflammatory disease.

Hepatitis B DNA polymerase level, a marker of replication, is reduced with standard IFN therapy. Clearance of serum HBeAg and HBV DNA polymerase occurs with treatment (30–40%). The use of pegylated forms of IFN has become common with the convenience of weekly dosing. Genotype and other baseline factors affect the response to PEG-IFN-α2a in HBeAg-positive chronic hepatitis B. Patients with genotypes A and B have a better response in comparison with genotypes C and D patients.

Hepatitis C

The aim of therapy for chronic HCV infection is to decrease and ultimately prevent progressive liver damage leading to cirrhosis, liver failure, or hepatocellular carcinoma. Therapy for chronic HCV infection is indicated in patients with detectable HCV RNA viral load and persistently elevated ALT. Findings of cirrhosis, fibrosis, or even moderate inflammation on liver biopsy support the choice of therapeutic intervention; however, biopsy is not mandatory prior to treatment initiation. Standard IFN, either as monotherapy or in combination with ribavirin, has been used extensively for HCV infections. Combination therapy for 40 weeks resulted in sustained responses in more than 60% of patients. The standard treatment of HCV infection is either PEG-IFN-α2a or PEG-IFN-α2b given alone or in combination with ribavirin and a direct-acting antiviral agent (DAA). Genotypes 1 and 4 infections are associated with lower sustained virologic response than other HCV genotypes.

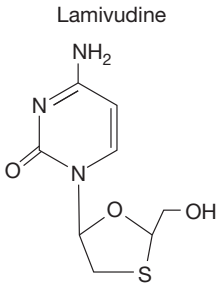
Resistance

Resistance to administered IFN has not been documented although neutralizing antibodies to recombinant IFNs have been reported. The clinical importance of this latter observation is unknown.

Adverse effects

Side effects are frequent with IFN (both standard and pegylated) administration and are usually dose limiting. Influenza-like symptoms (i.e., fever, chills, headache, and malaise) commonly occur, but these symptoms usually become less severe with repeated treatments. Leukopenia is the most common hematologic abnormality, occurring in up to 26% of treated patients. Leukopenia is usually modest, not clinically relevant, and reversible upon discontinuation of therapy. Increased ALT levels may also occur as well as nausea, vomiting, and diarrhea. At higher doses of IFN, neurotoxicity is encountered, as manifested by personality changes, confusion, attention deficits, disorientation, and paranoid ideation.

Lamivudine



Chemistry, mechanism of action, and antiviral activity

Lamivudine is the (−) enantiomer of a cytidine analogue with sulfur substituted for the 3′ carbon atom in the furanose ring [(−) 2′,3′-dideoxy, 3′-thiacytidine]. It has significant activity *in vitro* against both HIV-1 and HIV-2 as well as HBV. Lamivudine is phosphorylated to the triphosphate metabolite by cellular kinases. The triphosphate derivative is a competitive inhibitor of the viral reverse transcriptase (Chang et al., 1992).

The oral bioavailability in adults is >80% for doses between 0.25 and 8.0 mg kg^{-1}. Peak serum concentrations of 1.5 μg ml^{-1} are achieved in 1–1.5 h and the plasma $T_{1/2}$ is approximately 2–4 h. Lamivudine is eliminated by the kidneys unchanged by both glomerular filtration and tubular excretion, and dosages should be adapted to creatinine clearance.

Clinical indications

Lamivudine is effective as monotherapy for the treatment of chronic HBV infection (Dienstag et al., 1999) and in combination with other antiretroviral drugs for treatment of HIV infection. It is the first line drug for the treatment of HBeAg and anti-HBe positive disease. Elevated serum ALT levels have been shown to predict a higher likelihood of HBeAg loss in patients with chronic hepatitis B treated with lamivudine. Lamivudine is administered orally at 100 mg day^{-1} in the treatment of HBV infections, though the ideal dose could be higher.

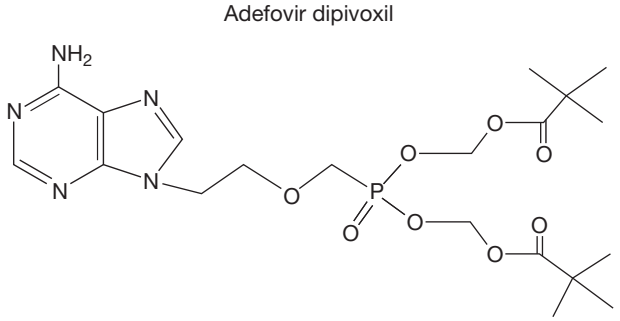
Resistance

Resistance to lamivudine monotherapy develops within 6 months of therapy. The incidence of lamivudine resistance is 15–20% per year, with 70% patients becoming resistant after 5 years of treatment (Lok et al., 2003; Pawlotsky, 2003). It will be curious to know if lamivudine at higher doses will affect the incidence of resistance. Lamivudine resistance to HBV is conferred through HBV strains with mutations in the viral polymerase, within the catalytic domain (C domain), which includes the YMDD motif (e.g., M204V or M204I), and within the B domain (e.g., L180M or V173L) (Das et al., 2001). These mutants have a reduced replication capacity compared with the wild type HBV virus. Lamivudine resistance is managed by sequential treatment with either adefovir or entecavir. However, the advantage of sequential treatment compared to *de novo* combination therapy is questionable.

Adverse effects

Lamivudine has an extremely favorable toxicity profile. This may be partly because lamivudine does not affect mitochondrial DNA synthesis and its poor inhibition of human DNA polymerases (Chang et al., 1992). At the highest doses of 20 mg kg^{-1} day^{-1}, neutropenia is encountered but at a low frequency.

Adefovir Dipivoxil



Chemistry, mechanism of action, and antiviral activity

Adefovir dipivoxil, bis(pivaloyloxymethyl)ester of 9-(2-phosphonylmethoxyethyl) adenine, is an orally bioavailable prodrug of adefovir, a phosphonate acyclic nucleotide analogue of adenosine monophosphate. Adefovir is monophosphorylated and is not

dependent on initial phosphorylation by viral nucleoside kinases to exert its antiviral effect. The phosphorylation to the di- and triphosphate metabolites is by cellular kinases (Merta et al., 1992). The triphosphate competes with endogenous deoxyadenosine triphosphate (dATP) in incorporation to the nascent viral DNA resulting in premature termination of viral DNA synthesis due to the lack of a 3′ hydroxyl group (De Clercq, 2004). It has activity against HIV, hepadnaviruses and herpesviruses. The bioavailability of adefovir dipivoxil in humans is about 40%. It has a long intracellular half-life of 18 h allowing for a once-daily dose. Clearance of adefovir is by renal excretion. Its pharmacokinetics is substantially altered in subjects with moderate and severe renal impairment.

Clinical indications

The efficacy of adefovir has been assessed in patients with HBeAg positive and negative disease and other settings in the spectrum of chronic hepatitis B infection. At the recommended dose of 10 mg once a day, adefovir resulted in significant improvement when compared with placebo (Marcellin et al., 2003): improvement in liver histology (53% vs. 25%), reduction in HBV DNA (3.52 vs. 0.55 log copies ml^{-1}), normalization of ALT (48% vs. 16%), and HBeAg seroconversion (12% vs. 6%). It is also useful for the treatment of lamivudine-resistant HBV infection (Schiff et al., 2007; Zoulim et al., 2009).

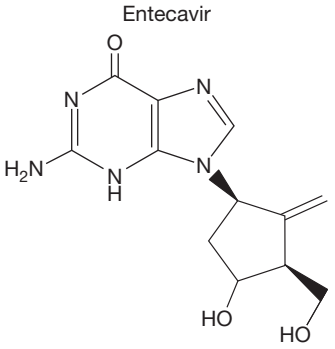
Resistance

Adefovir resistance occurs in approximately 6% of patients 3 years after adefovir monotherapy (Hadziyannis et al., 2005). Mutations in the HBV polymerase B domain (A181V/T) and the D domain (N236T) confer resistance to adefovir (Lacombe et al., 2006; Osiowy et al., 2006).

Adverse effects

Nephrotoxicity is the major side effect of higher doses of adefovir (Hadziyannis et al., 2006; Schiff et al., 2007). It causes a proximal convoluted tubule lesion characterized by a rise in urea and creatinine. Other dose-related clinical adverse events have been gastrointestinal events, including nausea, anorexia and diarrhea. These are usually mild, intermittent and self-limited without the need for concomitant medications or dose interruption.

Entecavir



Chemistry, mechanism of action, and antiviral activity

Entecavir (2-amino-1,9-dihydro-9-[(1*S*,3*R*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one), monhydrate is a guanosine nucleoside analogue. Entecavir is efficiently phosphorylated by cellular kinases to the active triphosphate metabolite. It affects three-steps in the replication of HBV: (1) prevent the priming of the HBV reverse transcriptase, (2) prevent reverse transcribing of the HBV pregenomic mRNA, and (3) inhibits DNA-dependent DNA synthesis (i.e., terminating viral DNA synthesis) (Seifer et al., 1998; Zoulim, 2006). The HBV polymerase binds preferentially to entecavir triphosphate, and entecavir triphosphate does not affect human mitochondrial DNA synthesis. The effect of entecavir on human cellular polymerase is minimal. Studies prior to approval of entecavir for HBV treatment suggested that entecavir did not have anti-HIV activity at clinical relevant concentrations. However, recent studies have suggested an anti-HIV activity of entecavir at drug concentrations in the low nanomolar range (McMahon et al., 2007; Sasadeusz, 2007).

Entecavir is well absorbed after oral administration achieving peak plasma concentrations between 0.6 and 1.5 h. Entecavir is not a substrate of the cytochrome P450 (CYP) enzyme system. It is eliminated primarily in the urine through glomerular filtration and

tubular secretion. The mean elimination $T_{1/2}$ of entecavir varies from 77 to 149 h in patients with normal function. The intracellular half-life of the triphosphate metabolite *in vitro* studies is about 15 h (Yamanaka et al., 1999).

Clinical indications

Entecavir was approved in March 2005, for the management of adult patients with chronic HBV infection who have active viral replication and/or elevation in liver transaminases or signs of active disease on histological examination. In phase III trials, responses achieved with entecavir surpassed previously published response rates for IFN-α-2b, lamivudine, and adefovir dipivoxil. With recent reports of an anti-HIV activity of entecavir, entecavir monotherapy probably should not be used in individuals with HIV–HBV coinfection who need HBV but not HIV treatment.

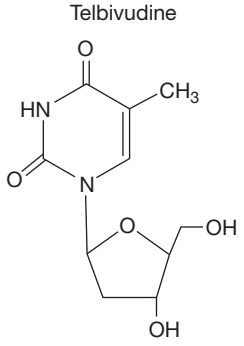
Resistance

The prevalence rate of resistance to entecavir in HBV-treatment naive is about 1.2%. However, virologic rebound and resistance have been reported in 43% of lamivudine-resistant patients after 4 year of switching treatment to entecavir. Entecavir resistance requires the following amino acid sequence changes in the reverse transcriptase domain of HBV; M204V/I+L184G, S202I, or M250V (Baldick et al., 2008).

Adverse effects

Most adverse events in the phase III studies were mild and comprised of headache, upper respiratory tract infections, cough, fatigue, pharyngitis, abdominal pain, and gastrointestinal upset. The most common laboratory abnormality was ALT level greater than five times the upper limit of normal. Monitoring for long-term toxicity is needed.

Telbivudine



Chemistry, mechanism of action, and antiviral activity

Telbivudine (β-L-2′-deoxythymidine) is an L-configured nucleoside with potent and specific activities against HBV and other hepadnaviruses. Telbivudine is a competitive inhibitor of both HBV viral reverse transcriptase and DNA polymerase. Telbivudine is phosphorylated by cellular kinases to the triphosphate metabolite, which competes with naturally occurring thymidine triphosphate for viral DNA elongation. The incorporation of telbivudine into the viral DNA terminates viral DNA chain elongation (Kim et al., 2006). In contrast to other nucleoside analogue, such as lamivudine, telbivudine preferentially inhibits anticomplement or second-strand DNA, whereas lamivudine triphosphate preferentially inhibits the complement DNA synthesis.

Preliminary studies have shown a potent inhibition of HBV replication with a safe profile and no effect on mitochondrial metabolism. Telbivudine triphosphate does not inhibit human cellular polymerase α, β, or γ. In addition, telbivudine triphosphate is not a substrate for human DNA polymerase and thus will not induce genotoxicity.

Telbivudine is rapidly absorbed after oral dosing with peak plasma concentration achieved within 1–3 h, the absolute oral bioavailability of telbivudine is not known. Over an 8-h period, telbivudine exhibits an apparent single-phase decline, with $T_{1/2}$ of 2.5–5 h. However, a presence of a second, slower elimination phase was observed with intensive sampling in healthy volunteers up to 168 h post-dosing. The second phase starts approximately 16–24 h after dosing, with a long observed terminal-phase $T_{1/2}$ of approximately 40 h. The long plasma $T_{1/2}$ of telbivudine is consistent with the long intracellular $T_{1/2}$ (14 h) of its triphosphate *in vitro* studies. The elimination $T_{1/2}$ of telbivudine increases with renal dysfunction, therefore, dosage reduction of telbivudine is recommended in individuals with renal dysfunction.

Clinical indications

Telbivudine was approved in October 2006 by the FDA for treatment of chronic HBV infection. In clinical trials with primary end point of therapeutic response (a composite of suppression of HBV DNA and either loss of serum HBeAg or ALT normalization) after one year, in HBeAg-positive patients a therapeutic response occurred in 75% of patients treated with telbivudine and 67% of those treated with lamivudine (Lai et al., 2005). In HBeAg-negative patients, the response was 75 and 77% for telbivudine and lamivudine, respectively. In the second year of the study, telbivudine was found to be superior to lamivudine. Using the two drugs in combination was no more effective than telbivudine monotherapy.

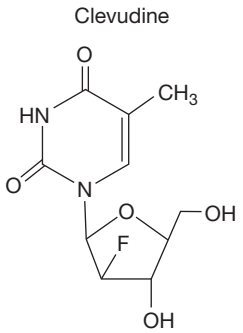
Resistance

HBeAg-positive, 21.6%, and HBeAg-negative, 8.6%, recipients of telbivudine had HBV DNA rebound that was associated with resistance mutations. Lamivudine-resistance HBV strains have a high level of cross resistance to telbivudine. The mutations in the RT domain of HBV associated with telbivudine resistance are M204I or M204I + L180I/V (Seifer et al., 2009).

Adverse effects

Most of the adverse effects of telbivudine reported in clinical studies were mild to moderate. The most common were elevated creatinine phosphokinase (CPK), an enzyme present in muscle tissue and a marker for the breakdown of muscle tissue, upper respiratory tract infection, fatigue, headache, abdominal pain, and cough (Lai et al., 2005; Liaw et al., 2009).

Clevudine



Clevudine was approved in South Korea and in the Philippines in 2006 and 2009, respectively, for the treatment of hepatitis B after demonstration of potent anti-hepatitis B activity in phase II and III clinical trials (Yoo et al., 2007a,b). It is likely to be licensed for hepatitis B treatment in other countries.

Chemistry, mechanism of action, and antiviral activity

Clevudine [1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl) thymidine] is a nucleoside analogue of the unnatural β-L configuration with potent activity against HBV and some activity against EBV. Clevudine is efficiently phosphorylated by cellular kinases to clevudine-triphosphate in target cells. The mechanism of action is mainly inhibition of viral plus-strand DNA synthesis (Balakrishna Pai et al., 1996; Chong and Chu, 2002). Preclinical studies revealed that human cellular DNA polymerases α, β, γ, and δ could not utilize the 5′-triphosphate of clevudine as a substrate and, hence, the lack of cytotoxicity. The EC_{50} of clevudine for HBV inhibition values ranges from 0.02 to 0.84 μmol l^{-1}. Clevudine is well absorbed after oral administration with estimated long half-life of 44–60 h.

Clinical indications

Clevudine is approved for treatment of chronic hepatitis B infection in South Korea and in the Philippines. In a randomized, placebo-controlled phase III study in South Korea, chronic HBeAg-positive patients who received 30 mg of clevudine once daily for 24 weeks maintained a 3.73 $\log_{10}$ and 2.02 $\log_{10}$ viral suppression at 34 and 48 weeks, respectively. A unique characteristic of clevudine is the slow rebound of viremia after cessation of treatment.

Resistance

In vitro studies suggest that there may be cross-resistance with lamivudine-resistant HBV mutants. The A181T mutation, which is associated with resistance to lamivudine and adefovir, was selected for after 24 weeks of clevudine treatment (Yoo et al., 2007a).

Adverse effects

In clinical trials, clevudine was well tolerated without any serious adverse events reported. However, further development of clevudine is on hold due to associated severe myopathy and mitochondrial toxicity occurring several months after cessation of clevudine treatment in patients (Kim et al., 2009; Seok et al., 2009).

Tenofovir disoproxil fumarate

Chemistry, mechanism of action, and antiviral activity

The chemistry and mechanism of action of tenofovir disoproxil fumarate have been described in section 'tenofovir disoproxil fumarate' under 'Anti-HIV Agents.' Tenofovir has significant activity *in vitro* against both HIV-1 and HBV. Tenofovir was approved by the FDA for the treatment of HIV in 2001 and for the treatment of chronic HBV infection in 2008.

Clinical indications

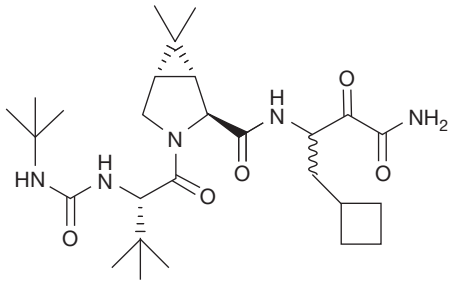
Tenofovir is given orally at 300 mg day^{-1} (one 300 mg tablet once a day) for the treatment of HBV infection. It is effective against both wild type and lamivudine-resistant HBV strains. Tenofovir is more potent than adefovir dipivoxil in the treatment of HBV infection (Del Poggio et al., 2007; Tan et al., 2008).

Adverse effects

In HIV-infected patients treated with tenofovir, there have been reports of nephrotoxicity, bone mineral density loss, and osteomalacia (Gafni et al., 2006; Grund et al., 2009; Lee and Marosok, 2003). Therefore, monitoring of both renal and non-renal adverse effects of tenofovir in HBV patients is essential.

Boceprevir

Boceprevir



Chemistry, mechanism of action, and antiviral activity

Boceprevir [(1R,5S)-N-[3-amino-1-(cyclobutylmethyl)-2,3-dioxopropyl]- 3-[2(S)-[[[(1,1-dimethylethyl)amino]carbonyl]amino]-3,3-dimethyl-1-oxobutyl]- 6,6-dimethyl-3-azabicyclo[3.1.0]hexan-2(S)-carboxamide] is a linear peptidomimetic keto-amide serine protease inhibitor that binds reversibly to the HCV nonstructural 3 (NS3) active site (Venkatraman et al., 2006). Boceprevir has good binding ($K_i = 14$ nM) and cellular activity (EC50 = 350 nM) (Venkatraman, 2012). In an HCV replicon model, treatment with boceprevir resulted in 2 and 4 $\log_{10}$ reduction in HCV RNA by 72 h and 15 days, respectively (Malcolm et al., 2006). In the initial phase I trial, combination of peginterferon with boceprevir resulted in the greatest reduction in HCV RNA and was additive (Sarrazin et al., 2007). There was no drug-drug interaction; the area under the curve (AUC) for each drug was comparable to AUC of each drug when given alone.

Bioavailability in animals ranges from 12 to 37%, indicating incomplete absorption. However, boceprevir displayed a rather high liver/plasma average ratio of 30 in rats, indicating good uptake by the target tissue. Boceprevir is metabolized primarily by aldo-keto reductase (AKR); it is reduced from ketoamide to hydroxyl amide, which is less active (Venkatraman, 2012). In addition, it undergoes oxidative metabolism by the CYP 3A4/5 enzymes to a lesser extent.

Clinical indications

Boceprevir was approved by the FDA in 2011 as the first direct-acting antiviral drug against HCV genotype 1. Boceprevir is administered in combination with peginterferon alfa and ribavirin; the dose of boceprevir is 800 mg three times a day with food. Therapy with peginterferon alfa and ribavirin for 4 weeks (lead-in period) is recommended before adding boceprevir; this is based on results from clinical trials. The lead-in period allows peginterferon and ribavirin to reach steady-state concentrations to avoid a period of functional monotherapy with boceprevir and reduce the development of drug resistance HCV (Kwo, 2012; Poordad et al., 2011). A response-guided duration of therapy is recommended. If HCV RNA levels are undetectable at weeks 8 and 24, patients may be treated with triple therapy (boceprevir, peginterferon and ribavirin) for 28 weeks. If HCV RNA is detected at week 8 and undetected at week 24, then the triple therapy should continue for 36 weeks followed by a 12-week combination of peginterferon and ribavirin tail for a total of 48 weeks of therapy (Kwo, 2012).

Resistance

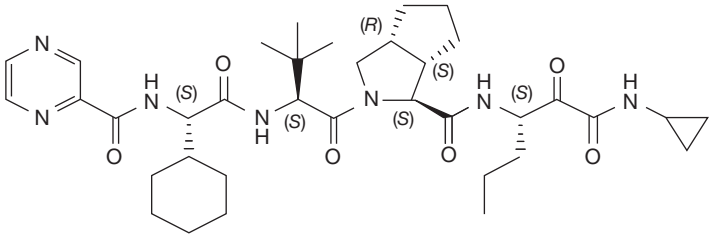
In vitro resistance mutation selection for boceprevir in replicon cells revealed the following mutations – A156S/T, R155K, T54S, and V36M (Tong et al., 2006). Population sequencing of the NS3 domain of isolates from the clinical trials revealed major mutations (V36M, T54S, and R155K) and minor mutations (T54A, V55A, R155T, A156S, V158I, and V170A) (Kwo, 2012).

Adverse effects

The most commonly reported adverse effects were fatigue, anemia, nausea, headache, and dysgeusia when boceprevir was used in combination with peginterferon and ribavirin (Poordad et al., 2011).

Telaprevir

Telaprevir



Chemistry, mechanism of action, and antiviral activity

Telaprevir ((1*S*,3a*R*,6a*S*)-2-[(2*S*)-2-({(2*S*)-2-cyclohexyl-2-[(pyrazin-2-ylcarbonyl)amino]acetyl}amino)-3,3-dimethylbutanoyl]-*N*-[(3*S*)-1- (cyclopropylamino)-1,2-dioxohexan-3-yl]-3,3a,4,5,6,6a-hexahydro-1H-cyclopenta[c]pyrrole-1-carboxamide) is a reversible HCV NS3/4A protease inhibitor; an example of direct-acting antiviral agents (DAAs) for the treatment of HCV (Perni et al., 2006). Telaprevir first binds weakly to the NS3/4A protease and then forms a covalent bond between the hydroxyl group of the catalytic serine and the keto-carbonyl group of telaprevir. The $T_{1/2}$ dissociation of this complex is 58 min. Telaprevir is selective for HCV NS3/4A and does not inhibit other serine proteases.

Telaprevir is readily absorbed orally with a T_{max} between 2.5 and 5.0 h (Foster et al., 2011). The Cmax, Cmin, and AUC for telaprevir are higher when telaprevir is given in combination with peginterferon and ribavirin than when given as monotherapy. A high-fat breakfast increased the the AUC by about 20% compared with a standardized meal (Matthews and Lancaster, 2012). Telaprevir is metabolized in the liver through reduction, hydrolysis, and oxidation. Telaprevir is a substrate and an inhibitor of CYP 3A4 isozyme. Telaprevir is both a substrate and an inhibitor of P-glycoprotein. Agents that induce CYP 3A4 or P-glycoprotein may reduce plasma concentration of telaprevir. Telaprevir is eliminated mainly through the feces and to some extent through expired air and urine.

Clinical indications

Telaprevir was approved in May 2011 by the FDA for treatment of HCV genotype 1 infected patients based on its efficacy and tolerability in clinical trials (McHutchison et al., 2010; Zeuzem et al., 2011). Telaprevir is administered in combination with peginterferon and ribavirin. The recommended dosage of telaprevir is 750 mg 3 times a day. A respond-guided duration of treatment is recommended for telaprevir combination therapy. The recommended duration of treatment with telaprevir is 12 weeks in combination with peginterferon and ribavirin. HCV RNA levels should be monitored at weeks 4 and 12 to determine combination treatment duration and assess for treatment futility. Recommendations for treatment-naïve and patients with relapse of infection: (1) if HCV RNA is undetectable at week 4, triple therapy (telaprevir/peginterferon/ribavirin) is given for 12 weeks and an additional 12 weeks of dual therapy (peginterferon/ribavirin) for a total of 24 weeks of therapy; and (2) if HCV RNA is detectable at weeks 4 and/or 12, triple therapy (telaprevir/peginterferon/ribavirin) is given for 12 weeks and an additional 36 weeks of dual therapy (peginterferon/ribavirin) for a total of 48 weeks of therapy. For prior partial and null responsder patients, triple therapy (telaprevir/peginterferon/ribavirin) is given for 12 weeks and an additional 36 weeks of dual therapy (peginterferon/ribavirin) for a total of 48 weeks of therapy.

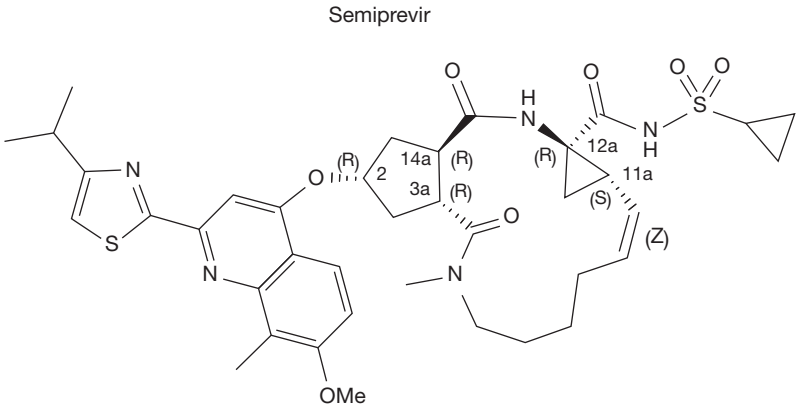
Resistance

Mutations associated with telaprevir resistance are: minor mutations, conferring low level resistance, (V36A/M/L, T54S/A, R155K, and A156V/T) and major mutations, conferring high level resistance, (V23A + V36M, and V36M + R155K).

Adverse effects

In clinical trials, the prevalence of adverse effects commonly associated with telaprevir in combination with peginterferon/ribavirin versus peginterferon/ribavirin were rash (56% vs 34%); pruritus (47% vs 28%); nausea (39% vs 28%); diarrhea (26% vs 17%) and anemia (36% vs 17%) (Hezode et al., 2009; McHutchison et al., 2010).

Simeprevir



Chemistry, mechanism of action, and antiviral activity

Simeprevir ((2*R*,3a*R*,10*Z*,11a*S*,12a*R*,14a*R*)-*N*-(cyclopropylsulfonyl)-2-[[2-(4-isopropyl-1,3-thiazol-2-yl)-7-methoxy-8-methyl-4-quinolinyl]oxy]-5-methyl-4,14-dioxo-2,3,3a,4,5,6,7,8,9,11a,12,13,14,14atetradecahydrocyclopenta[*c*]cyclopropa[*g*] [1,6]diazacyclotetradecine-12a(1*H*)-carboxamide) is an HCV NS3/4A protease inhibitor, a direct-acting antiviral agent, for the treatment of chronic HCV infection. The K_i value of simeprevir on HCV NS3/4A protease of genotypes 1a and 1b was 0.5 and 1.4 nM, respectively (Lin et al., 2009; Rosenquist et al., 2014). Simeprevir is highly selective (>1000-fold) for NS3/4A versus most of the evaluated human proteases. *In vitro* combination studies of simeprevir with interferon, ribavirin, NS5A or NS5B inhibitors resulted in additive or synergistic effects (54.) Simeprevir has activity against HCV genotypes 1, 2, 4, 5, and 6. In combination with ribavirin and interferon, simeprevir showed additive and synergistic effects, respectively (Lin et al., 2009).

The PK profile of simprevir varied in different animals. In dogs, simeprivir achieved 100% bioavailability after oral administration of 6.5 mg kg^{-1}; with high C_{max} (4.72 μM), AUC (14 986 ng·h ml^{-1}), and a long half-life ($T_{1/2}$ = 5.1 h) (Rosenquist et al., 2014). After oral administration in rats, simeprevir was well distributed with a high concentration observed in the liver and with a liver/plasma ratio of 32. Achieving high drug concentrations in the liver is critical for HCV DAAs given that viral replication of HCV occurs almost exclusively in hepatocytes. Simeprevir binds extensively to plasma proteins (>99%). However, only a 2.4-fold shift of the replicon EC_{50} value was observed with the addition of 50% human serum albumin. Simeprevir inhibits CYP3A4 and P-glycoprotein; with a potential for drug-drug interaction.

Clinical indications

Simeprevir was approved in November 2013 by the FDA for treatment of chronic HCV infected patients. The recommended dose of simeprevir is 150 mg once a day administered in combination with peginterferon and ribavirin. All treatment-naïve and prior relapser patients, including those with cirrhosis, should receive an additional 12 weeks of peginterferon alfa and ribavirin after completing 12 weeks of treatment with simeprevir, peginterferon alfa and ribavirin (total treatment duration of 24 weeks). All prior non-responder patients, including those with cirrhosis, should receive an additional 36 weeks of peginterferon alfa and ribavirin after completing 12 weeks of treatment with simeprevir, peginterferon alfa and ribavirin (total treatment duration of 48 weeks).

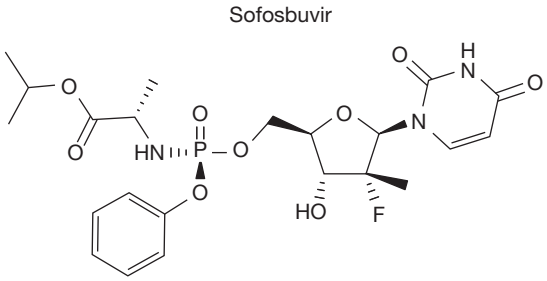
Resistance

In vitro drug resistance selection studies revealed that mutations at the NS3 positions 43, 80, 155, 156 and 168, either alone or in combination conferred varying degrees of resistance to simeprevir (Lenz et al., 2010). In clinical trials, patients with breakthrough viremia or relapse had the D168V mutation alone or in combination with other NS3 mutations HCV (Izumi et al., 2014).

Adverse effects

The most common adverse effects were headache, fatigue, pyrexia, and influenza-like illness; these were similar in the simeprevir and placebo arms. However, rash and photosensitivity reactions were higher in the simeprevir arm (Manns et al., 2014).

Sofosbuvir



Chemistry, mechanism of action, and antiviral activity

Sofosbuvir ((*S*)-Isopropyl 2-((*S*)-(((2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)-(phenoxy)phosphorylamino)propanoate) is a nucleotide analogue NS5B polymerase inhibitor and a direct-acting antiviral agent against the hepatitis C. Sofosbuvir is phosphorylated by host cellular kinases to a uridine triphosphate analogue, which is responsible for its antiviral activity.

Following oral administration of sofosbuvir, the peak plasma concentration was observed at 0.5–2 h post-dose, regardless of dose level. Based on population pharmacokinetic analysis in subjects with genotype 1–6 HCV infection who were coadministered ribavirin (with or without pegylated interferon), geometric mean steady state AUC_{0-24} of sofosbuvir and GS- 331007 (predominant circulating metabolite of sofosbuvir) were 828 ng●h ml^{-1} and 6790 ng●h ml^{-1}, respectively. Sofosbuvir is approximately 61–65% bound to human plasma proteins and the binding is independent of drug concentration over the range of 1–20 μg ml^{-1}. It is renally eliminated via glomerular filtration and active tubular secretion as the metabolite GS-331007, with a median $T_{1/2}$ of 0.48–0.75 h (Rodriguez-Torres et al., 2013). Sofosbuvir is a substrate of P-glycoprotein and breast cancer resistance protein (BRCP) drug transporters. Sofosbovir is not metabolized by cytochrome P450 (CYP) isoenzymes, nor does it induce or inhibit the metabolism of agents that are substrates of these enzymes.

Clinical indications

Sofosbuvir was approved in December 2013 by the FDA for treatment of chronic HCV infected patients. It is recommended for use in combination with ribavirin for genotypes 2 and 3 infections or in combination with peginterferon and ribavirin for genotypes 1 and 4 infections. The recommended dose of sofosbuvir is 400 mg once daily in combination with ribavirin or ribavirin and peginterferon. The recommended regime and treatment duration is: for patients with HCV genotype 1 or 4, sofosbuvir is given with peginterferon and ribavirin for 12 weeks; for genotypoe 2 patients, sofosbuvir in combiantion with ribavirin for 12 weeks; and for genotype 3 patients, sofosbuvir in combination with ribavirin for 24 weeks.

Resistance

Sofosbuvir has a high barrier to resistance. *In vitro* drug resistance selection studies revealed that S282T mutation could reduce susceptibility to sofosbuvir. Subsequently, V321A and L159F mutations have been observed in minority of patients receiving sofosbuvir treatment during clinical trials (Zeuzem et al., 2014). The clinical significance of these mutations is not known.

Adverse effects

The most common adverse effects when sofosbuvir was given in combination with peginterferon and ribavirin were fatigue, headache, nausea, insomnia, and anemia.

Future Prospects

Current antiviral agents either inhibit hepatitis B replication, or invoke an immune response, which may be necessary but not sufficient to effect viral control (Dusheiko and Antonakopoulos, 2008). Moreover, antiviral resistance remains a concern with long-term therapy, the search for novel agents, and treatment strategies with minimal or no resistance and good long-term safety profile are the focus of ongoing research (Cheng et al., 2005). Emtricitabine, licensed for the treatment of HIV infections, also has activity against HBV, but are not yet FDA-approved for this indication. There are a number of new nucleoside and nucleotide analogue in the pipeline; elvucitabine, valtorcitabine, amdoxovir, racivir, MIV 210, β-L-FddC, alamifovir and hepavir B may soon be part of the armamentarium for hepatitis B treatment. Another challenge is the management of hepatitis B in individuals with HIV coinfection. Appropriate combination regimens for individuals with coinfections are expected in the near future; target treatment of HBV to alter the outcome and take into account the impact of HBV treatment on HIV.

The last decade has ushered in unparalleled advances in the treatment of HCV. The advent of DAAs has provided highly effective, well tolerated and shorter duration of HCV therapy. There are several next generation DAAs in clinical development that will soon make interferon-based HCV regimens a thing of the past (Afdhal et al., 2014a,b; Feld, 2014; Feld et al., 2014; Kowdley et al., 2014a,b; Poordad et al., 2014). Future goals of HCV therapy are to improve tolerability, shorten duration of therapy, discovery of agents with pan genotypic activity, overcome issues of resistance, and availability of cost-effective regimens.

Therapeutics for Papillomavirus

HPVs are small DNA viruses with strict epithelial tropism. HPV infection induces the hyperproliferation of epithelial cells, leading to a broad spectrum of human diseases, ranging from benign warts (self-limiting) to malignant neoplasms. In general, there is no virus-specific effective systemic therapy available. Furthermore, treatment of disease with current therapies has not been shown to reduce the rates of transmission.

The recently FDA-approved quadrivalent prophylactic vaccine (HPV6/11/16/18) has been shown in clinical trials to be effective in preventing high-grade vulval and vaginal lesion associated with HPV 16 and 18. With time, this prophylactic vaccine is expected to reduce the incidence of HPV infections, particularly, infections due to the vaccine types (HPV6, 11, 16, and 18).

Interferon

IFNs have antiproliferative, antiviral, and immunomodulatory properties. IFNs have been administered (mostly IFN-α) topically, systemically, and intralesionally with variable results. They are more effective if used in combination with either local surgery or podophyllotoxin. Several large controlled trials have demonstrated inconsistent clinical benefits of the use of standard IFN-α therapy of condyloma acuminatum (caused by HPV) that was refractory to cytodestructive therapies. Intralesional therapy is painful, systemic therapy is associated with influenza-like symptoms such as fever and myalgia. Furthermore, IFN treatment is expensive and there is limited efficacy.

Imiquimod

This is an immunomodulator approved by the FDA for topical treatment of external and perianal genital warts. It acts as a ligand for Toll-like receptor 7 and activates macrophage and dendritic cells to release IFN α and other proinflammatory cytokines. With imiquimod application, gradual clearance of warts occurs in about 50% of patients over an average of 8–10 weeks. The adverse effects are; application site reactions (irritation, pruritus, flaking, and erosion), and systemic effects including fatigue and influenza-like illness.

Podophyllotoxin

Podophyllotoxin is the main cytotoxic ingredient of podophyllin, a resin used for many years for topical treatment of warts. The exact mechanism of action is unknown. Podophyllotoxin 0.5% solution or gel is similar in effectiveness to imiquimod but may have more adverse effects. Adverse effects include irritation of the adjacent skin, local erosion, ulceration and scarring.

Trichloroacetic acid, podophyllotoxin, and cryotherapy (with liquid nitrogen or a cryprobe) remain the most widely used treatments for external genital warts, but response rate is only 60–70%, and at least 20–30% of responders will have recurrence.

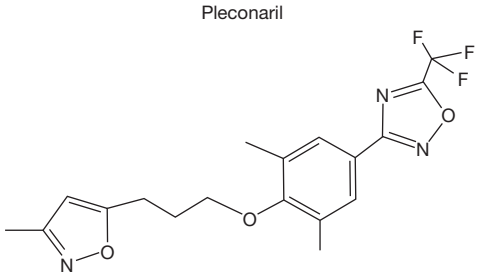
Future Prospects

The current therapies are not targeted antiviral therapies. They result in the physical removal of the lesion or the induction of nonspecific inflammation, thereby inducing a bystander immune response. There is urgent need to develop specific and effective antiviral agents for HPV infections.

Therapeutics for Enteroviral Infections

The enteroviruses include nearly 70 serotypes of closely related pathogens that cause a wide spectrum of human illness, from mild nonspecific fever to common upper respiratory infections, aseptic meningitis, severe myocarditis, encephalitis, and paralytic poliomyelitis. Certain patients, including antibody-deficient individuals, bone marrow recipients, and neonates, may develop potentially life-threatening enterovirus infections for which therapeutic options have been limited. There are case series of the use of immune serum globulin and pleconaril for serious enteroviral infections. Pleconaril failed to secure FDA approval because of its induction of CYP 3A enzyme activity, and the potential for drug interactions, particularly the interference with oral contraceptives.

Pleconaril



Chemistry, mechanism of action, and antiviral activity

Pleconaril (3-(Poordad et al., 2011)propyl]phenyl]-5[trifluoromethyl]-1,2,4-oxadiazole) exerts its antiviral effect by integrating into a hydrophobic pocket inside the virion, and prevents viral replication by inhibiting viral uncoating and blocking viral attachment to host cell receptors, thus interrupting the infection cycle. The viral capsid structure, which is the target of pleconaril, is relatively conserved among the picornaviruses. Pleconaril has broad spectrum and potent activity against enteroviruses and rhinoviruses.

Pleconaril is 70% bioavailability when given orally. This high level of bioavailability was achieved by the substitution of trifluoromethyl on the oxadiazole ring that reduces its degradation in the liver by enzymes involved in oxidative processes. The metabolic stabilization is reflected in the drug's long serum half-life (about 6.5 h) after oral dosing. Pleconaril also readily penetrates the blood–brain barrier.

Clinical indications

Common cold

In a phase I trial of pleconaril for treatment of common cold, there was a significant reduction in rhinorrhea of about 1.5 days in those on 400 mg three times daily, and a reduction in a severity score as compared to the placebo. Subsequent trials confirmed a modest reduction in length of symptoms for common cold in patients treated with pleconaril (Hayden et al., 2003).

Immunocompromised host

Patients with compromised humoral immunity, such as those with agammaglobulinemia, who contract enteroviral infections may develop chronic meningitis and meningoencephalitis, often with a fatal outcome. There are case reports of the efficacy of pleconaril in these patients.

Enteroviral meningitis

For treatment of enteroviral meningitis, two large studies showed a marginal statistical improvement in a clinical score in the pleconaril-treated groups (Desmond et al., 2006). A subsequent small study of 21 infants with proven enteroviral meningitis in the United States did not have enough power to show unequivocal benefit with pleconaril (Abzug et al., 2003).

Resistance

Resistance to pleconaril has been reported in some serotypes of enteroviruses, however, the mechanism is not well understood.

Adverse effects

Pleconaril is generally well tolerated. The most common adverse events are headache, diarrhea, and nausea. Long-term use of pleconaril is associated with an increase in menstrual irregularities in women.

Future Prospects

Pleconaril has not been licensed for treatment of enteroviral infections; there is an urgent need to identify alternative drugs that might be effective. There are several investigational compounds; however, none has reached phase I clinical trial. Combinations of drugs are likely to offer the best chance of cure and protection from enterovirus infections in the future.

Anti-HIV Agents

The combination of three or more anti-HIV agents into multidrug regimens, often termed highly active antiretroviral therapy (HAART), can efficiently inhibit HIV viral replication to achieve low or undetectable circulatory HIV-1 levels. This is the start-of-the-art treatment of AIDS or HIV-infected individuals. Drug combinations are, in principle, aimed at obtaining synergism between the compounds, while reducing the likelihood of the development of drug resistance virus, and minimizing toxicity. The available anti-HIV drugs are categorized according the step they target within the HIV viral life cycle (Figure 2); (1) binding inhibitors, for example, coreceptor antagonist (maraviroc); (2) fusion inhibitors (enfuvirtide); (3) reverse transcriptase inhibitors (nucleoside/nucleotide (zidovudine,

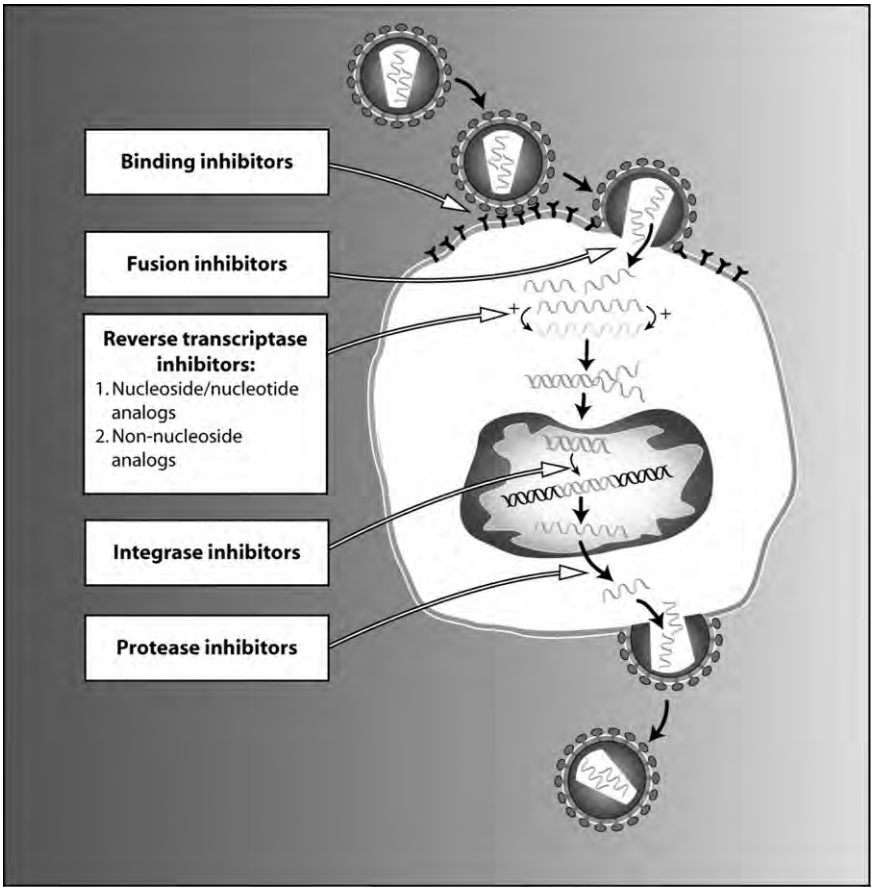


Figure 2 HIV life cycle showing the stages of intervention of available anti-HIV agents.

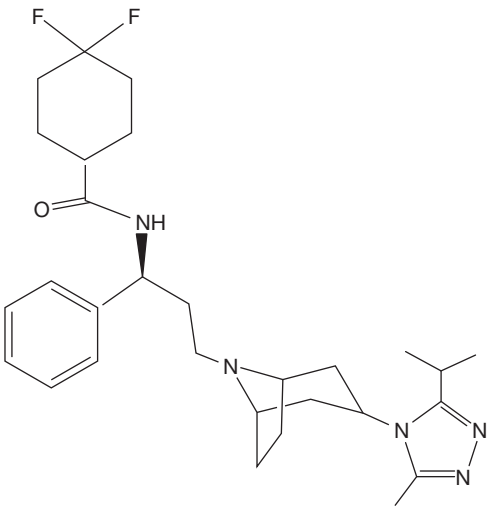
didanosine, zalcitabine, stavudine, lamivudine, abacavir, emtricitabine, and tenofovir), and non-nucleoside (nevirapine, delavirdine, and efavirenz) analogue); (4) integrase inhibitors (raltegravir); and (5) protease inhibitors (saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, fosamprenavir, lopinavir, atazanavir, tipranavir, and darunavir).

Fixed-dose combinations and once-daily dosage forms of many anti-HIV agents are available. There are fixed-dose combinations for zidovudine/lamivudine, zidovudine/lamivudine/abacavir, abacavir/lamivudine, tenofovir/emtricitabine, and tenofovir/emtricitabine/efavirenz.

Coreceptor Antagonist

Maraviroc

Maraviroc



Chemistry, mechanism of action, and antiviral activity

Maraviroc (4,4-difluoro-*N*-{(1*S*)-3-[*exo*-3-(3-isopropyl-5-methyl-4*H*-1,2,4 triazol-4-yl)-8-azabicyclo(3,2,1]oct-8-yl]-1-phenylpropyl}cyclohexanecarboxamide) is the first of the class of CCR5 coreceptor antagonists licensed (August 2007) for HIV treatment. Maraviroc selectively binds to the human chemokine receptor CCR5 present on the cell membrane, preventing the interaction of HIV-1 gp120 and CCR5 necessary for CCR5-tropic HIV-1 to enter cells. It inhibits the replication of CCR5-tropic laboratory strains and primary isolates of HIV-1 *in vitro*. The mean EC_{50} for maraviroc against various strains of HIV-1 ranges from 0.1 to 1.25 nmol l^{-1} (0.05–0.64 ng ml^{-1}) in cell culture. Maraviroc was not active against CXCR4-tropic and dual-tropic viruses (EC50 value >10 μmol l^{-1}). The antiviral activity of maraviroc against HIV-2 has not been evaluated.

The absolute bioavailability for 100 and 300 mg doses are 23 and 33%, respectively. Peak plasma concentrations of maraviroc are attained at 0.5–4 h following single oral dose of 1200 mg administered to uninfected volunteers. Maraviroc is bound (approximately 76%) to human plasma proteins. It is principally metabolized by the cytochrome P450 system to metabolites that are essentially inactive against HIV-1. Maraviroc is a substrate of CYP3A and the efflux transporter P-glycoprotein (Pgp), and therefore, its pharmacokinetics are likely to be modulated by inhibitors and inducers of these enzymes/transporters. The terminal half-life in healthy subjects is 14–18 h.

Clinical indications

Maraviroc is approved for use in combination with other anti-HIV agents for the treatment of adults with CCR5-tropic HIV-1, who are treatment-experienced with evidence of viral replication and HIV-1 strains resistant to multiple antiretroviral agents.

Resistance

The resistance profile in treatment-naive and treatment-experienced subjects has not been fully characterized. HIV-1 variants with reduced susceptibility to maraviroc have been selected in cell culture, following serial passage of two CCR5-tropic viruses (CC1/85 and RU570). The maraviroc resistant viruses remained CCR5-tropic with no evidence of a change from a CCR5-tropic virus to a CXCR4-using virus. Two amino acid residue substitutions (Table 1, letter codes of amino acids) in the V3-loop region of the HIV-1 envelope glycoprotein (gp160), A316T and I323V were shown to be necessary for the maraviroc-resistant phenotype in the HIV-1 isolate CC1/85. In the RU570 isolate, a 3-amino acid residue deletion in the V3 loop, ΔQAI (HXB2 positions 315–317), was associated with maraviroc resistance. The clinical relevance of these mutations is not known.

Adverse effects

The most common adverse events reported with maraviroc were cough, fever, upper respiratory tract infections, rash, musculoskeletal symptoms, abdominal pain, and dizziness. The product label includes a warning about liver toxicity (hepatoxicity) and a statement about the possibility of heart attacks.

Table 1 The three-letter and one-letter codes for amino acid residues

Amino acid	*Three-letter code*	*One-letter code*
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Fusion Inhibitors

Enfuvirtide

Chemistry, mechanism of action, and antiviral activity

Enfuvirtide, a linear 36-amino acid synthetic peptide with the N-terminus acetylated and the C-terminus is a carboxamide, is the first licensed agent in the class of fusion inhibitors. Enfuvirtide interferes with the entry of HIV-1 into cells by inhibiting fusion of viral and cellular membranes (Figure 2). Enfuvirtide binds to the first heptad-repeat (HR1) in the gp41 subunit of the viral envelope glycoprotein and prevents the conformational changes required for the fusion of viral and cellular membranes. The IC_{50} of enfuvirtide for baseline clinical isolates ranged from 0.089 to 107 nmol l^{-1} (0.4–480 ng ml^{-1}). Enfuvirtide is active against R5, X4, and dual tropic viruses, but has no activity against HIV-2.

Enfuvirtide is administered twice daily by subcutaneous injection. Single-dose vials contain 108 mg of enfuvirtide for the delivery of approximately 90 mg ml^{-1} when reconstituted. The absolute bioavailability is $84.3 \pm 15.5\%$. Following 90 mg bid dosing of enfuvirtide subcutaneously in combination with other antiretroviral agents in HIV-1 infected subjects, the median T_{max} was 4 h (ranged from 4 to 8 h). Enfuvirtide is catabolized by proteolytic enzymes. It is not metabolized by hepatic CYP450 isoenzyme systems. There are no known clinically significant interactions between enfuvirtide and other medications.

Clinical indications

Enfuvirtide was approved by the FDA in March 2003 for use in adults, and in children aged 6 and older, with advanced HIV infection. Enfuvirtide is used with other anti-HIV agents to treat HIV-1 infection in patients who are treatment-experienced and have detectable viral loads even though they are taking anti-HIV agents.

Resistance

HIV-1 isolates with reduced susceptibility to enfuvirtide have been selected *in vitro*. Genotypic analysis of these resistant isolates showed mutations that resulted in amino acid substitutions at the enfuvirtide binding HR1 domain positions 36–38 of the HIV-1 envelope glycoprotein gp41. In clinical trials, HIV-1 isolates with reduced susceptibility to enfuvirtide have been recovered from subjects failing enfuvirtide-containing regimen. Most of the isolates with decreased in susceptibility to enfuvirtide of greater than fourfold exhibited genotypic changes in the codons encoding gp41 HR1 domain amino acids 36–45.

HIV-1 clinical isolates resistant to nucleoside analogue reverse transcriptase inhibitors, non-nucleoside analogue reverse transcriptase inhibitors, and protease inhibitors are susceptible to enfuvirtide in cell culture.

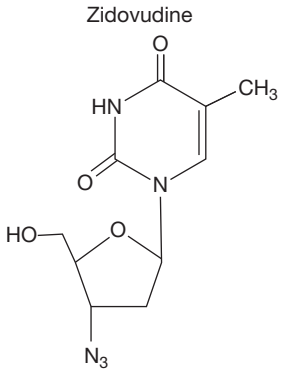
Adverse effects

The most common adverse effects of enfuvirtide are injection site reactions. Other symptomatic side effects may include insomnia, headache, dizziness, and nausea. Several cases of hypersensitivity have been described. In phase III studies, bacterial pneumonia was seen at a higher rate in patients who received enfuvirtide than in those who did not receive enfuvirtide. Eosinophilia is the primary laboratory abnormality seen with enfuvirtide administration.

Reverse Transcriptase Inhibitors

Nucleoside/nucleotide reverse transcriptase inhibitors

Zidovudine



Chemistry, mechanism of action, and antiviral activity

Zidovudine (3′-azido-2′,3′-dideoxythymidine) is a pyrimidine analogue with an azido group substituting for the 3′ hydroxyl group on the ribose ring. Zidovudine is initially phosphorylated by cellular TK and then to its diphosphate by cellular thymidylate kinase. The triphosphate derivative competitively inhibits HIV reverse transcriptase, and functions as a chain terminator. Zidovudine inhibits HIV-1 at concentrations of approximately 0.013 μg ml^{-1}. In addition, it inhibits a variety of other retroviruses. Synergy has been demonstrated against HIV-1 when zidovudine is combined with didanosine, zalcitabine, lamivudine, nevirapine, delavirdine,

saquinavir, indinavir, ritonavir, and other compounds. It was the first drug to be licensed for the treatment of HIV infection, and still is used in combination with other drugs as initial therapy for some patients.

Zidovudine is available in capsule, syrup, and intravenous formulations. Oral bioavailability is approximately 65%. Peak plasma levels are achieved approximately 0.5–1.5 h after treatment. Zidovudine penetrates cerebrospinal fluid, saliva, semen, and breast milk and it crosses the placenta. Drug is predominately metabolized by the liver through the enzyme uridine diphosphoglucuronosyltransferase to its major inactive metabolite 3′-azido-3′-deoxy-5′-*O*-β-D-glucopyranuronosylthymidine. The elimination $T_{1/2}$ is approximately 1 h; however, it is extended in individuals who have altered hepatic function.

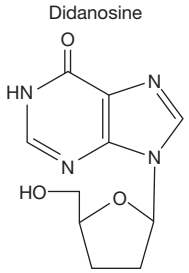
Clinical indications

Zidovudine is used in combination with other anti-HIV agents. It is administered orally at 600 mg day^{-1} (300 mg tablet, twice a day). The single most important usage of zidovudine in the last decade has been the peripartum three-part zidovudine regimen, which has decreased the incidence of transmission of HIV infection from pregnant women to their infants.

Adverse effects

The predominant adverse effect of zidovudine is myelosuppression, as evidenced by neutropenia and anemia, occurring in 16 and 24% of the patients, respectively. Zidovudine has been associated with skeletal and cardiac muscle toxicity, including polymyositis. Nausea, headache, malaise, insomnia, and fatigue are common side effects.

Didanosine



Chemistry, mechanism of action, and antiviral activity

Didanosine (2′,3′-dideoxyinosine) is a purine nucleoside with inhibitory activity against both HIV-1 and HIV-2. Didanosine is activated by intracellular phosphorylation. It is first converted to 2′,3′-dideoxyinsine-5′-monophosphate by 5′nucleotidase and inosine 5′-monophosphate phosphotransferase and subsequently to 2′, 3′-dideoxyadenosine-5′-monophosphate by adenylsuccinate synthetase and lyase. It is then converted to diphosphate by adenylate kinase and subsequently by creatine kinase or phosphoribosyl pyrophosphate synthetase to the triphosphate. The triphosphate metabolite is a competitive inhibitor of HIV reverse transcriptase and a chain terminator. The spectrum of activity of didanosine is enhanced by synergism with zidovudine and stavudine as well as the protease inhibitors.

Didanosine is acid labile and has poor solubility. A buffered tablet results in 20–25% bioavailability. A 300 mg oral dose achieves peak plasma concentrations of 0.5–2.6 μg ml^{-1} with a $T_{1/2}$ of approximately 1.5 h. It is metabolized to hypoxanthine and is cleared primarily by the kidneys.

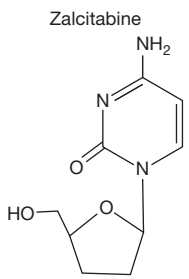
Clinical indications

Didanosine is used in combination with other anti-HIV agents as part of HAART. It is given as two 100 mg tablets (buffered tablets) twice a day or as one 400 mg capsule (delayed-release capsule) once a day.

Adverse effects

The most significant adverse effect associated with didanosine therapy is the development of peripheral neuropathy (30%) and pancreatitis (10%). Lipoatrophy, lactic acidosis and diabetes have been observed in patients on antiretroviral regimens containing didanosine.

Zalcitabine



Chemistry, mechanism of action, and antiviral activity

Zalcitabine (2′,3′-dideoxycytidine) is a pyrimidine analogue, which is activated by cellular enzymes to its triphosphate derivative. The enzymes responsible for activation of zalcitabine are cell cycle independent, and therefore this offers a theoretical advantage for nondividing cells, specifically dendritic and monocyte/macrophage cells. Zalcitabine inhibits both HIV-1 and HIV-2 at concentrations of approximately 0.03 μmol l^{-1}.

The oral bioavailability following zalcitabine administration is more than 80%. The peak plasma concentrations following an oral dose of 0.03 mg kg^{-1} range from 0.1 to 0.2 μmol l^{-1} and the $T_{1/2}$ is approximately 20 min. The drug is cleared mainly by the kidneys, and therefore, the presence of renal insufficiency leads to a prolong plasma $T_{1/2}$.

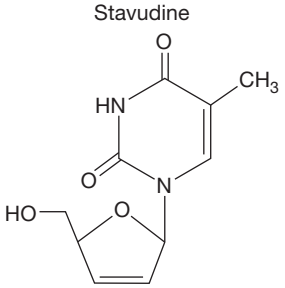
Clinical indications

Zalcitabine is used as part of HAART regimen for HIV-1 infections. It is administered orally at 2.25 mg day^{-1} (one 0.75 mg tablet every 8 h).

Adverse effects

Peripheral neuropathy is the major toxicity associated with zalcitabine administration, occurring in approximately 35% of individuals. Pancreatitis can occur, but does so infrequently. Thrombocytopenia and neutropenia are uncommon (5% and 10%, respectively). Other zalcitabine-related side effects include nausea, vomiting, headache, hepatotoxicity, and cardiomyopathy.

Stavudine



Chemistry, mechanism of action, and antiviral activity

Stavudine (2′,3′-didehydro, 3′-deoxythymidine) is a thymidine analogue with significant activity against HIV-1, having inhibitory concentrations, which range from 0.01 to 4.1 μmol l^{-1}. Its mechanism of action is similar to that of zidovudine.

The oral bioavailability of stavudine is more than 85%. Peak plasma concentrations of approximately 1.2 μg ml^{-1} are reached within 1 h of dosing at 0.67 mg kg^{-1} per dose. Stavudine penetrates CSF and breast milk. It is excreted by the kidneys unchanged and, in part, by renal tubular secretion.

Clinical indications

Stavudine is used for HIV infection in combination with other anti-HIV agents. Stavudine is a highly potent inhibitor of HIV-1 replication *in vitro*. However, its use has been limited by delayed toxicity, notably peripheral neuropathy and myopathy caused by mitochondrial damage. It is administered orally at 80 mg day^{-1} (one 40 mg capsule every 12 h).

Adverse effects

The principal adverse effect of stavudine therapy is the development of peripheral neuropathy. The development of this complication is related to both dose and duration of therapy. Inhibition of mitochondrial DNA synthesis is proposed to induce depletion of cellular mitochondrial DNA and it is ultimately responsible for the delayed toxicity observed with the use of stavudine and other nucleoside reverse transcriptase inhibitors (NRTIs). Neuropathy tends to appear after 3 months of therapy and resolves slowly with medication discontinuation. Other side effects are uncommon. Fatal and nonfatal pancreatitis have occurred during therapy when stavudine was part of a combination regimen that included didanosine. Redistribution and accumulation of body fat (lipoatrophy) have been observed in patients receiving stavudine as part of their antiretroviral regimen.

Lamivudine

Chemistry, mechanism of action, and antiviral activity

The chemistry and mechanism of action of lamivudine have been described previously in section 'Lamivudine' under 'Therapeutics for Hepatitis.' Lamivudine has significant activity *in vitro* against both HIV-1 and HIV-2, as well as HBV. Lamivudine is a competitive inhibitor of the viral reverse transcriptase.

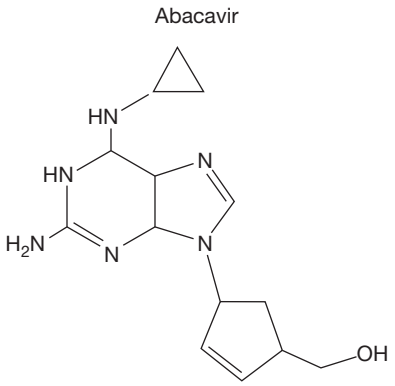
Clinical indications

Lamivudine is used in combination with other anti-HIV agents. Lamivudine is given orally at 300 mg day^{-1} (one 150 mg tablet twice a day, or one 300 mg tablet once a day). It is also formulated in combination with zidovudine, or with zidovudine and abacavir as fixed-dose combination tablet.

Adverse effects

Lamivudine has an extremely favorable toxicity profile. This may largely be attributed to the low affinity of lamivudine for human DNA polymerases, and the lack of active lamivudine metabolites in the mitochondrial compartment of cells. At the highest doses of 20 mg kg^{-1} day^{-1}, neutropenia is encountered but at a low frequency. In pediatric studies, pancreatitis and peripheral neuropathies have been reported.

Abacavir



Chemistry, mechanism of action, and antiviral activity

Abacavir sulfate, (1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol, is a structural analogue of the purine guanine. The phosphorylation pathway of abacavir differs from that of all other nucleoside analogues. The first step in the conversion of abacavir to its active metabolite, carbovir triphosphate, is phosphorylation to abacavir monophosphate by adenosine phosphotransferase. This step is followed by deamination by a cytosolic enzyme to form carbovir monophosphate, which undergoes two subsequent phosphorylations, to the diphosphate by guanylate kinase and to the triphosphate by nucleoside diphosphate kinase and other enzymes. Carbovir triphosphate competes with endogenous 2′-dGTP for incorporation into the nucleic acid chain, and after incorporation, terminates DNA chain elongation. Abacavir exhibits potent *in vitro* antiviral activity against wild-type HIV-1 (IC_{50} 4.0 μmol l^{-1}), but this activity is lower than the activity of zidovudine (IC_{50} 0.040 μmol l^{-1}). However, there is no significant difference between the levels of activity of abacavir (IC_{50} 0.26 μmol l^{-1}) and AZT (IC_{50} 0.23 μmol l^{-1}) against clinical isolates of HIV-1.

Abacavir is well absorbed after oral administration with a bioavailability between 76 and 96%. After single or multiple doses, C_{max} is attained after a mean of 0.7–1.7 h, and the mean half-life is 0.8–1.5 h. However, at a dose of 300 mg twice daily as part of a combination regimen, levels of intracellular carbovir triphosphate ranged from <20 to 374 fmol per 10^6 cells. The intracellular carbovir triphosphate was measurable throughout the 24-h study period, with the highest levels found between 6 and 8 h. This finding suggests a long half-life for carbovir triphosphate within cells. The main route of excretion is renal.

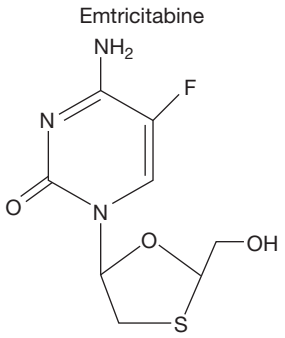
Clinical indications

Abacavir is used in combination with other anti-HIV agents. It is given as a 300 mg tablet twice a day, or two 300 mg tablets once a day. A fixed-dose combination with lamivudine is available (600 mg abacavir, plus 300 mg lamivudine).

Adverse effects

The abacavir hypersensitivity reaction is a potentially fatal syndrome occurring in approximately 5% of HIV-infected patients exposed to this nucleoside analogue after a median of 11 days (range: 1–318 days). Systemic manifestations can include fever, rash, fatigue, and gastrointestinal or respiratory symptoms. Rechallenge with abacavir in individuals presumed to have abacavir hypersensitivity reaction is avoided due to reports of fatal reactions caused by repeated administration of abacavir following a hypersensitivity reaction. The presence of HLA-B*5701 has been associated with elevated odds of developing abacavir hypersensitivity reaction.

Emtricitabine



Chemistry, mechanism of action, and antiviral activity

Emtricitabine, 5-fluoro-1-(2*R*,5*S*)-[2-(hydroxymethyl)-1,3-oxathiolan 5-yl]cytosine, is a fluorinated nucleoside analogue of cytosine. Emtricitabine, similar in many ways to lamivudine, has *in vitro* activity against HIV-1 that is similar to or approximately fourfold to tenfold more potent than that of lamivudine. The EC_{50} for emtricitabine is in the range of 0.0013–0.64 μmol l^{-1} (0.0003–0.158 μg ml^{-1}) for laboratory and clinical isolates of HIV-1 in cell culture. Against HIV-2, the EC_{50} ranges from 0.007 to 1.5 μmol l^{-1}. The cellular enzymes involved in the phosphorylation of emtricitabine are similar to that of lamivudine. The active triphosphate competitively inhibits reverse transcriptase by being incorporated into the viral genome, and causing termination in DNA chain elongation.

The bioavailability of the capsules and oral solution are 93 and 75%, respectively. Emtricitabine is rapidly and extensively absorbed following oral administration with peak plasma concentrations occurring at 1–2 h post dose.

The mean plasma elimination half-life of emtricitabine after a single dose is about 8–10 h in HIV-infected patients. However, after multiple doses of the drug at a dose of 200 mg daily, the intracellular half-life is approximately 39 h. The high intracellular levels of emtricitabine triphosphate achieved are associated with better suppression of plasma HIV RNA. Emtricitabine is not an inhibitor of human CYP450 enzymes. Emtricitabine is eliminated by a combination of glomerular filtration and active tubular secretion.

Clinical indications

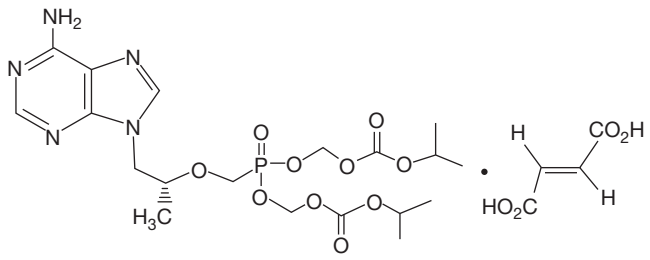
Emtricitabine is used with other anti-HIV agents. It is administered orally at a once-daily 200 mg capsule. Emtricitabine is a component of Truvada (a fixed-dose combination of emtricitabine and tenofovir disoproxil fumarate), and Atripla (a fixed-dose combination of efavirenz, emtricitabine, and tenofovir disoproxil fumarate).

Adverse effects

The most common adverse events are headache, diarrhea, nausea, and rash, which are generally of mild to moderate severity. Approximately 1% of patients discontinued participation in clinical trials due to these events.

Tenofovir disoproxil fumarate

Tenofovir disoproxil fumarate



Chemistry, mechanism of action, and antiviral activity

Tenofovir disoproxil fumarate is (a prodrug of tenofovir), 9-[(*R*)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy] propyl]adenine fumarate (1:1), converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analogue of adenosine 5′-monophosphate. Tenofovir disoproxil fumarate requires initial diester hydrolysis for conversion to tenofovir and subsequent phosphorylations by cellular enzymes to form tenofovir triphosphate. Tenofovir triphosphate inhibits the activity of HIV-1 reverse transcriptase by competing with the natural substrate deoxyadenosine 5′-triphosphate and, after incorporation into DNA, by DNA chain termination. Tenofovir triphosphate is a weak inhibitor of mammalian DNA polymerases α, β, and mitochondrial DNA polymerase γ. Tenofovir has antiviral activity *in vitro* against HIV-1 with IC_{50} values ranging from 0.5 to 2.2 μmol l^{-1}. The IC_{50} values of tenofovir against HIV-2 range from 1.6 to 4.9 μmol l^{-1}.

The oral bioavailability of tenofovir in fasting patients is approximately 25%. Following oral administration of a single dose of 300 mg to HIV-1 infected patients, maximum serum concentrations are achieved in 1.0 ± 0.4 h. C_{max} and AUC values are 296 ± 90 ng ml^{-1} and 2287 ± 685 ng h ml^{-1}, respectively. The oral bioavailability increases when tenofovir is administered after a high-fat meal (40–50% fat). *In vitro* studies indicate that neither tenofovir disoproxil nor tenofovir are substrates of CYP450 enzymes. Tenofovir is primarily excreted by the kidneys by a combination of glomerular filtration and active tubular secretion. The pharmacokinetics of tenofovir is altered in patients with renal impairment.

Clinical indications

Tenofovir is used in combination with other anti-HIV agents for the treatment of HIV-1 infection. It is administered orally once daily, 300 mg tablet.

Adverse effects

The most common adverse reactions seen in a double-blind comparative controlled study were mild to moderate gastrointestinal events and dizziness. However, the following adverse events have been identified during post-approval use of tenofovir; allergic reaction, hypophosphatemia, lactic acidosis, dyspnea, abdominal pain, increased amylase, pancreatitis, increased liver enzymes, hepatitis, renal insufficiency, renal failure, fanconi syndrome, proximal tubulopathy, proteinuria, increased creatinine, acute tubular necrosis, and nephrogenic diabetes insipidus.

Resistance to nucleoside/nucleotide analogue

Nucleoside analogue-associated mutations (NAMs) develop by at least three pathways: (1) accumulation of zidovudine or thymidine analogue resistance mutations (TAMs), (e.g., 41 L, 67 N, 70R, 210 W, 215Y/F, and 219Q/E); (2) selection of the key 151 M mutation, followed by the mutations 62 V, 75I, 77 L, and 116Y, referred to as the 151 complex; and (3) the 69 insertion complex, consisting of a mutation at codon 69 (typically Ser), followed by an insertion of two or more amino acids (e.g., Ser-Ser, Ser-Arg, or Ser-Gly) and generally accompanied by other NAMs. In clinical isolates, two TAM pathways have been observed: 41 L, 210 W, 215Y/F and 67 N, 70R, 219Q/E/N/R; of these, the 41–210–215 combination is the most prevalent. The cytidine analogue select for the M184V mutation (lamivudine, and emtricitabine), while the K65R is seen with tenofovir selection pressure.

Resistance patterns with earlier nucleoside analogue combinations (zidovudine or stavudine with lamivudine)

The resistance profiles seen with earlier nucleoside analogue combinations are well characterized. With thymidine-based NRTIs (lamivudine/zidovudine or stavudine/lamivudine), the M184V mutation emerges rapidly, whereas TAMs are slower to arise. The use of emtricitabine in place of lamivudine would presumably yield similar results, although clinical data are limited. The M184V mutation has been shown to increase zidovudine susceptibility in the absence or presence of zidovudine resistance mutations and without regard to which TAM combination is present. The M184V mutation may increase sensitivity to tenofovir.

The most commonly observed mutations with zidovudine/didanosine or stavudine/didanosine are TAMs. TAMs and multinucleoside resistance (MNR) mutations (Q151M, T69 insert) are more prevalent in didanosine-containing regimens than in lamivudine-containing regimens.

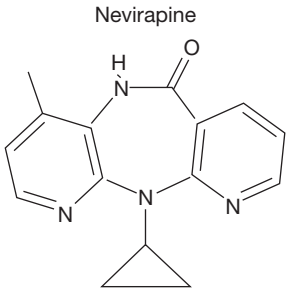
Resistance patterns with new nucleoside/nucleotide analogue combinations

With abacavir/lamivudine as backbone, the most common mutation selected for is M184V/I followed by the L74V mutation at treatment failure. The K65R is the major mutation selected *in vitro* by tenofovir alone or in combination with abacavir or lamivudine while abacavir appears to favor L74V and K65R.

In triple-nucleoside/nucleotide regimens (tenofovir/lamivudine/didanosine or tenofovir/lamivudine/abacavir) that lack a thymidine analogue, early treatment failure has been associated with a high frequency of M184V. In addition, 50% of the patients with M184V also harbor the K65R.

Non-nucleoside reverse transcriptase inhibitors

Nevirapine



Chemistry, mechanism of action, and antiviral activity

Nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-b:2′,3′-e]; [1,4]diazepin-6-one) is a reverse transcriptase inhibitor of HIV-1. However, its mechanism of action is different from nucleoside analogue. It binds to a hydrophobic pocket adjacent to the active site of the reverse transcriptase and causes conformational changes that affect replication. Nevirapine has a bioavailability of approximately 65%. Peak serum concentration of 3.4 μg ml^{-1} is achieved approximately 4 h after a 400 mg oral dose. Nevirapine is metabolized by liver microsomes to hydroxymethyl-nevirapine.

Clinical indications

Nevirapine is used in combination with other anti-HIV agents. It is administered orally at 200 mg day^{-1} for the first 14 days (one 200 mg tablet per day), then 400 mg day^{-1} (two daily 200 mg tablets). Single-dose nevirapine is used widely in resource-limited settings to prevent mother-to-child transmission of HIV infection.

Adverse effects

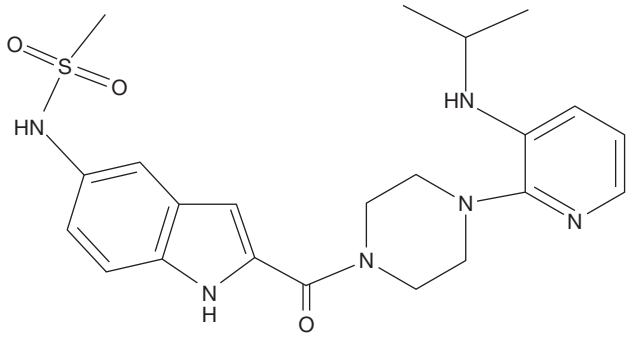
The most common adverse effects include the development of a nonpruritic rash in as many as 50% of patients who received 400 mg day^{-1}. In addition, fever, myalgias, headache, nausea, vomiting, fatigue, and diarrhea have also been associated with administration of drug.

Resistance

Changes in two sets of amino acid residues (100–110 and 180–190) in the reverse transcriptase gene confer resistance to nevirapine. Nevirapine monotherapy is associated with resistance most frequently appearing at codon 181.

Delavirdine

Delavirdine



Chemistry, mechanism of action, and antiviral activity

Delavirdine (1-[5-methanesulfonamido-1*H*-indol-2-yl-carbonyl]-4-[3-(1-methylethylamino) pyridinyl] piperazine) is a second-generation bis (heteroaryl) piperazine licensed for the treatment of HIV infection. Its mechanism of action is similar to that of nevirapine. It is absorbed rapidly when given orally with bioavailabilty of >60%. Delavirdine is metabolized by the liver with an elimination $T_{1/2}$ of approximately 1.4 h. It has an inhibitory concentration against HIV-1 of approximately 0.25 μmol l^{-1}. Inhibitory concentrations for human DNA polymerases are significantly higher.

Clinical indications

Delavirdine is used in combination with other anti-HIV agents. It is administered at 1200 mg day^{-1} (two 200 mg tablets three times a day).

Adverse effects

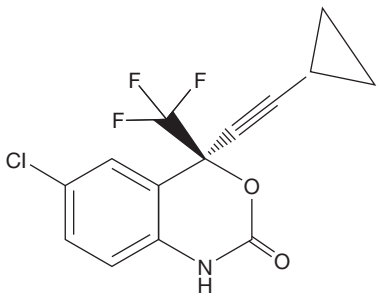
Delavirdine administration is associated with a maculopapular rash. Other side effects are less common.

Resistance

Delavirdine resistance can be generated rapidly both *in vitro* and *in vivo* with the codon change identified at 236, resulting in an increase and susceptibility to >60 μmol l^{-1}. Delavirdine resistance can be conferred by mutations at codons 181 and 188, as seen with other non-nucleoside analogue.

Efavirenz

Efavirenz



Chemistry, mechanism of action, and antiviral activity

Efavirenz [(*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4 (trifluoromethyl)-2*H*-3,1-benzoxazin-2-one] is a non-NRTI that can be administered once daily. Activity is mediated predominately by noncompetitive inhibition of HIV-1 reverse transcriptase. HIV-2 reverse transcriptase, and human cellular DNA polymerases α, β, γ, and δ are not inhibited by efavirenz. The 90–95% inhibitory concentration of efavirenz is approximately 1.7–25 nmol l^{-1}.

Clinical indications

Efavirenz is used in combination with other antiretroviral agents for the treatment of HIV-1 infection. Combination therapy has resulted in a 150-fold or greater decrease in HIV-1 RNA levels.

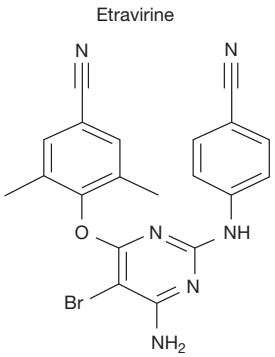
Adverse effects

The most common adverse events are skin rash (25%), which is associated with blistering, moist desquamation, or ulceration (1%). In addition, delusions and inappropriate behavior have been reported in 1 or 2 patients per 1000.

Resistance

Resistance to efavirenz is caused by mutation in the reverse transcriptase gene as with other non-nucleoside analogue, and appears rapidly.

Etravirine



Chemistry, mechanism of action, and antiviral activity

Etravirine (4-[[6-amino-5-bromo-2-[(4-cyanophenyl) amino]-4-pyrimidinyl]oxy]-3,5-dimethylbenzonitrile) is a non-NRTI with activity against HIV-1 and HIV-2. Etravirine has activity against NNRTI-resistant viruses in the nanomolar range (Andries et al., 2004). Activity is mediated predominately by noncompetitive inhibition of HIV-1 reverse transcriptase. Etravirine does not inhibit human DNA polymerases α, β, γ, and δ.

Following oral administration, etravirine was absorbed with a T_{max} of about 2.5–4 h. The absolute bioavailabilty of etravirine is unknown. Administration of etravirine during fasting conditions decreases the AUC by about 50%. Etravirine is bound to plasma proteins, predominantly to albumin (99.6%). However, its antiviral activity was not affected in the presence of 50% human serum (Andries et al., 2004). Etravirine is metabolized by the liver with an elimination $T_{1/2}$ of approximately 41 (±20) h. Etravirine is a substrate of cytochrome P450 isoenzymes. Drug that induce or inhibit cytochrome P450 isoenzymes may alter the therapeutic effect or side effects of etravirine.

Clinical indications

Efavirenz is used in combination with other antiretroviral agents for the treatment of HIV-1 infection. The recommended dose is 200 mg twice daily following a meal.

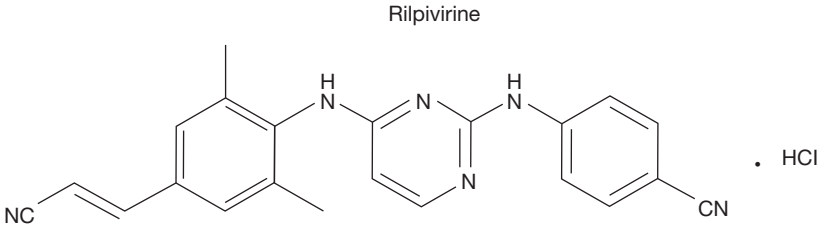
Adverse effects

The most common adverse events of moderate to severe intensity (at least 2%) are skin rash and peripheral neuropathy (Lazzarin et al., 2007).

Resistance

Resistance to etravirine is caused by mutation in the reverse transcriptase gene as with other NNRTIs. However, unlike other NNRTIs concurrent presence of three or more of these mutations are required to substantially reduce susceptibility to etravirine (Lazzarin et al., 2007).

Rilpivirine



Chemistry, mechanism of action, and antiviral activity

Rilpivirine (4-[[4-[[4-[(E)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile monohydrochloride) is diarylpyrimidine NNRTI of HIV-1) and inhibits HIV-1 replication by non-competitive inhibition of HIV-1 RT. Rilpilvirine does not inhibit human cellular DNA polymerases α, β, γ, and δ.

After oral administration, the maximum plasma concentration of rilpivirine is generally achieved within 4–5 h; the absolute bioavailability is unknown. Rilpivirine is approximately 99.7% bound to plasma proteins *in vitro*, primarily to albumin. Rilpivirine

is primarily metabolized by cytochrome P450 (CYP3A), and drugs that induce or inhibit CYP3A may thus affect the clearance of rilpivirine. The elimination $T_{1/2}$ of rilpivirine is approximately 50 h.

Clinical indications

Rilpivirine is used in combination with other antiretroviral agents for the treatment of HIV-1 infection. The recommended dose is 25 mg orally once a day with a meal. Rilpivirine is as effective as efavirenz in HIV treatment-naïve patients. However, development of resistance and virologic failure occur more frequently with rilpivirine than with efavirenz (Cohen et al., 2011; Molina et al., 2011).

Adverse effects

Adverse effects of rilpivirine include depression, insomnia, headache, and rash. Fewer patients discontinued rilpivirine containing therapy due to adverse effects than with efavirenz contiainign therapy (3% vs. 8%).

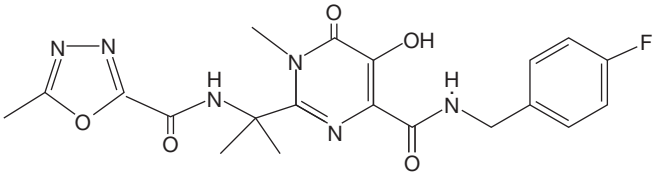
Resistance

Resistance to rilpivirine is caused by mutation in the reverse transcriptase gene as with other NNRTI. Cross-resistance to efavirenz, etravirine and/or nevirapine is likely after virologic failure and development of rilpivirine resistance.

Integrase Inhibitors

Raltegravir

Raltegravir



Chemistry, mechanism of action, and antiviral activity

Raltegravir, a structural analogue of a class of compounds with a distinct diketo acid moiety, is a novel HIV-1 integrase inhibitor with potent *in vitro* activity against HIV-1 (IC_{95} of 33 nmol l^{-1}) in the presence of 50% human serum. It is active against a wide range of wild-type and multidrug-resistant HIV-1 clinical isolates and has potent activity against viruses that use CCR5 and/or CXCR4 coreceptors for entry.

Raltegravir is absorbed rapidly, with median T_{max} values in the fasting state of about 1 h; plasma concentrations decrease from C_{max} in a biphasic manner, with a half-life of approximately 1 h for the initial (α) phase and an apparent half-life of approximately 7–12 h for the terminal (β) phase. The pharmacokinetic data for raltegravir are supportive of twice daily administration. It is metabolized by hepatic glucuronidation and has no effect on CYP3A4. Approximately 7–14% of the raltegravir dose is excreted unchanged in urine.

Clinical indications

Raltegravir received priority approval from the FDA (October 2007) for treatment of HIV-1 infection in combination with other antiretroviral agents in treatment-experienced patients with evidence of HIV-1 replication despite ongoing antiretroviral therapy. The dosage of raltegravir is 400 mg administered orally, twice daily with or without food.

Adverse effects

Side effects (mostly mild to moderate) were seen with similar frequency in the raltegravir and placebo arms; the rate of serious adverse events was less than 3% across arms. No lipid abnormalities have been reported so far with raltegravir.

Resistance

Several mutations have been identified in patients failing raltegravir containing regimen including S230R, G163R, N155H, Q148K/R/H, Y143R/C/H, G140S/A, T97A, and L74M (da Silva et al., 2010).

Elvitegravir

Chemistry, mechanism of action, and antiviral activity

Elvitegravir is a first generation HIV-1 integrase inhibitor with potent *in vitro* activity against HIV-1. It is active against a wide range of wild-type and multidrug-resistant HIV-1 clinical isolates and has potent activity against viruses.

Elvitegravir is rapidly absorbed and achieves the maximum concentration in 2–4 h. As elvitegravir is metabolized by CYP3A4, its half-life is significantly increased by co-administration with a pharmaco-enhancer like ritonavir, which inhibits the CYP3A4 metabolic pathway. Twice daily 800 mg dosing of elvitegravir alone to steady-state conditions achieved an AUC_{0-24h} of 3570 μg*h ml^{-1} (37 %CV) and a C_{24h} of 48.0 μg ml^{-1} (33 %CV). However, when 50 mg of elvitegravir was boosted with

100 mg of ritonavir the AUC_{0-24h} and C_{24h} increased to 8840 μg*h ml^{-1} (26 %CV) and a C_{24h} of 135.0 μg ml^{-1} (37 %CV), respectively (Mathias et al., 2009).

Clinical indications

Elvitegravir was approved by the FDA in August 2012 as part of a fixed-dose daily tablet containing tenofovir, emtricitabine, and the cytochrome P450 isoenzyme 3A (CYP3A) inhibitor cobicistat for treatment of HIV-1 infection (Sax et al., 2012).

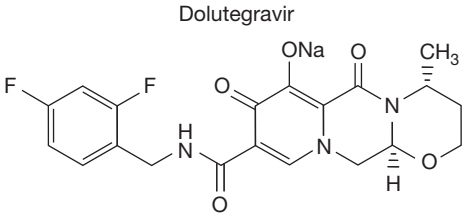
Adverse effects

Adverse effects of elvitegravir appear to be few but may include diarrhea and rash. Laboratory abnormalities include elevations in hepatic transaminases.

Resistance

Resistance to elvitegravir is associated with the selection of 1 or more resistance mutations. *In vitro* and *in vivo* studies show the emergence of a number of integrase mutations, including T66I, E92Q, Q148R, and N155H.

Dolutegravir



Chemistry, mechanism of action, and antiviral activity

Dolutegravir (4*R*,12a*S*)-9-{[(2,4-difluorophenyl)methyl]carbamoyl}-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2*H*-pyrido[1′,2′:4,5]pyrazino[2,1-*b*][1,3]oxazin-7-olate) is a second generation HIV-1 integrase inhibitor with subnanomolar EC_{50} antiviral activity *in vitro* (Kobayashi et al., 2011). 50 mg daily monotherapy of dolutegravir for 10 days in HIV-1 infected adults resulted in a 2.48 mean log_{10} reduction in HIV-1 RNA (Min et al., 2011).

Dolutegravir is absorbed rapidly, with a dose-dependent kinetics and low apparent clearance with oral terminal $T_{1/2}$ of about 15 h; supporting once daily dosing without the need for a boosting agent (Min et al., 2010). 50 mg dosing of dolutegravir to steady-state conditions achieved a geometric mean C_{max} of 3.34 μg ml^{-1} (16 %CV), an AUC_{0-24h} of 43.4 μg*h ml^{-1} (20 %CV), a $T_{1/2}$ of 12.0 h (22 %CV) and a C_{24h} of 0.83 μg ml^{-1} (26 %CV). It is metabolized primarily via UGT1A1 with minor contribution by CYP3A and is a substrate for P-glycoprotein. It is not an inducer or inhibitor of CYP3A (Min et al., 2010). Approximately 64% and 32% of the dolutegravir dose is recovered unchanged in feces and urine, respectively (Castellino et al., 2013).

Clinical indications

Dolutegravir received priority approval from the FDA in August 2013 for treatment of HIV-1 infection in combination with other antiretroviral agents in adults and children aged 12 years and older and weighing at least 40 kg. The dosage of dolutegravir is 50 mg administered orally once daily with or without food. The dosage of dolutegravir is 50 mg twice daily when coadministered with the following potent UGT1A/CYP3A inducers: efavirenz, fosamprenavir/ritonavir, tipranavir/ritonavir, or rifampin.

Adverse effects

Dolutegravir was generally well tolerated effects during clinical trials. Adverse effects of at least moderate intensity (grade 2–4) occurring in $\geq$2% of treatment-naïve participants on dolutegravir were insomnia (3%) and headache (2%) (Walmsley et al., 2013). In some treatment-naive participants, small mean elevations in serum creatinine ranging from 0.1 to 0.2 mg dl^{-1} were observed.

Resistance

Dolutegravir has a higher barrier to resistance than raltegravir and elvitegravir. *In vitro* as well as clinical data indicate that HIV-1 with primary mutations at codon 155 or 143, and the T66I and E92Q mutants remain susceptible to dolutegravir, whereas mutations at codon 148 in the presence of other secondary mutations (L74I/M, E138A/D/K/T, G140A/S, Y143H/R, E157Q, G163E/K/Q/R/S, or G193E/R) can lead to decreased dolutegravir efficacy (Hare et al., 2011).

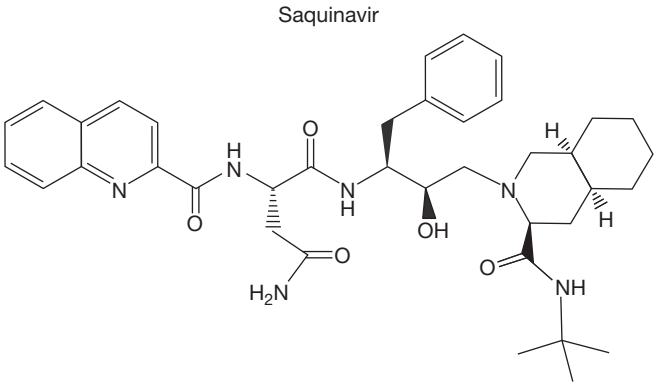
Protease Inhibitors

Protease inhibitors are used in combination with other anti-HIV agents for treatment of HIV infection. They are a potent component of HAART regimens. Protease inhibitors are used in combination with ritonavir as the boosting protease inhibitor. The concept of boosting involves pharmacokinetic drug interactions; currently available protease inhibitors are metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. Ritonavir is the most powerful enzyme inhibitor in the protease inhibitor class. The combination with ritonavir allows the boosted protease inhibitor to maintain

prolonged blood levels. This allows for decreased dosage, and reduces a three times a day schedule to a twice daily or even a once daily regimen.

Long-term HAART containing protease inhibitors has been most strongly associated with syndromes characterized by dyslipidemia, peripheral lipodystrophy, and insulin resistance.

Saquinavir



Chemistry, mechanism of action, and antiviral activity

Saquinavir (*cis-N*-tert-butyl-decahydro-2[2(*R*)-hydroxy-4-phenyl-3-(*S*)-([*N*-(2 quinolycarbonyl)-Lasparginyl] amino butyl)-4a*S*, 8a*S*]-isoquinoline-3[S]-carboxyamide methanesulfonate) is a hydroxyethylamine-derived peptidomimetic HIV protease inhibitor. Saquinavir inhibits HIV-1 and HIV-2 at concentrations of 10 nmol l^{-1} and is synergistic with other nucleoside analogue as well as selected protease inhibitors.

Oral bioavailability is approximately 30% with extensive hepatic metabolism. Peak plasma concentrations of 35 mg μl^{-1} are obtained following a 600 mg dose.

The clinical efficacy of saquinavir is limited by poor oral bioavailability but improved formulation (soft-gel capsule) enhances efficacy. Saquinavir is boosted with 100 mg twice a day of ritonavir to improve its bioavailabilty and efficacy even against saquinavir-resistant HIV strains.

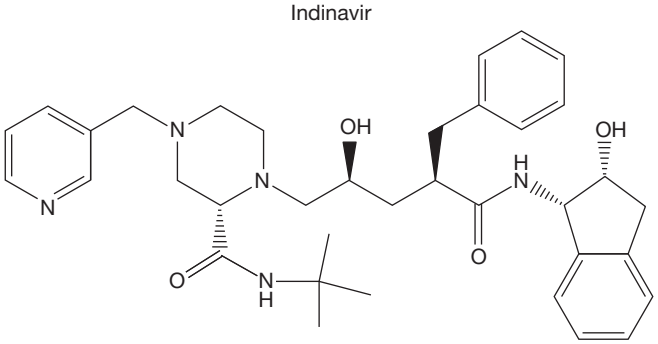
Adverse effects

Adverse effects are minimal with no dose-limiting toxicities. Abdominal discomfort, including diarrhea and nausea, has been reported infrequently.

Resistance

Mutations at codon sites 90 and 48 of the protease gene result in approximately a 30-fold decrease in susceptibility to saquinavir.

Indinavir



Chemistry, mechanism of action, and antiviral activity

Indinavir {*N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[2(*S*)-(1,1-dimethylethlaminocarbonyl)-4-(pyridin-3-yl) methylpiperazin-1-yl]-4[*S*]-hydroxy-2[*R*]-phenylmethyl pentanamide} is a peptidomimetic HIV-1 and HIV-2 protease inhibitor. At concentrations of 100 nmol l^{-1}, indinavir inhibits 90% of HIV isolates. Indinavir is rapidly absorbed with a bioavailability of 60% and achieves peak plasma concentrations of 12 µmol l^{-1} after a 800 mg oral dose.

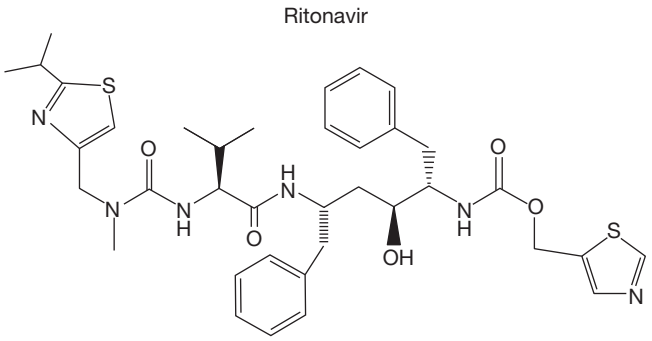
Adverse effects

Although indinavir is well tolerated, commonly encountered adverse effects include indirect hyperbilirubinemia (10%) and nephrolithiasis (5%).

Resistance

Indinavir resistance develops rapidly with monotherapy and occurs at multiple sites. The extent of resistance is directly related to the number of codon changes in the HIV protease gene. Codon 82 is a common mutation in indinavir-resistant HIV isolates.

Ritonavir



Chemistry, mechanism of action, and antiviral activity

Ritonavir (10-hydroxy-2-methyl-5[1-methylethyl]-1[2-(1-methylethyl)-4-thiazo lyl]-3,6,dioxo-8,11-bis[phenylmethyl]-2,4,7,12-tetra azatridecan-13-oic-acid, 5 thiazolylmethylester, [5S-(5R, 8R,10R, 11R)]) is an HIV protease inhibitor with activity *in vitro* against HIV-1 laboratory strains (0.02–0.15 μmol l^{-1}). It is synergistic when administered with nucleoside analogue. Oral bioavailability is approximately 80%, with peak plasma levels of approximately 1.8 μmol l^{-1} after 400 mg administered every 12 h. The plasma half-life is approximately 3 h.

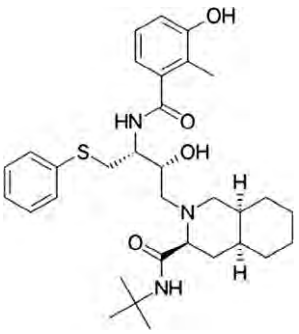
Adverse effects

Adverse effects include nausea, diarrhea, and headache, but all occur at a low frequency.

Resistance

Ritonavir has cross-resistance to indinavir. Mutations at codon 82 are the most common.

Nelfinavir



Chemistry, mechanism of action, and antiviral activity

Nelfinavir [3*S*-(3*R*, 4a*R*, 8a*R*, 22′*S*, 3′*S*)]-2-[2″-hydroxy-3′-phenylthiomethyl-4′-aza-5′-ox-o-5′-(2″methyl-3′-hydroxyphenyl) pentyl]-decahydroiso-quinoline-3-*N*-(tert-butyl-carboxamide methanesulfonic acid salt) is another peptidomimetic HIV protease inhibitor. Inhibitory concentrations of HIV-1 are in the range of 20–50 nmol l^{-1}. It has anti-HIV-2 activity. Nelfinavir is orally bioavailable at approximately 40%, achieving peak plasma concentrations of 2 or 3 mg following a 800 mg dose every 24 h. The drug is metabolized by hepatic microsomes.

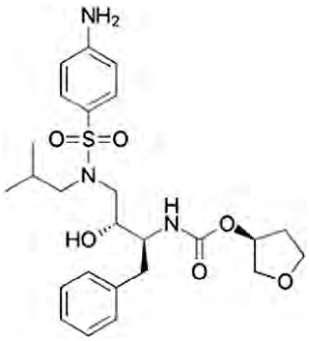
Adverse effects

Nelfinavir is well tolerated with mild gastrointestinal complication reported.

Resistance

Cross-resistance to other protease inhibitors, particularly saquinavir, indinavir, or ritonavir, is not common. The most frequently demonstrated site of mutation is at codon 30.

Amprenavir



Chemistry, mechanism of action, and antiviral activity

Amprenavir is a hydroxyethylamine sulfonamide peptidomimetric with a structure identified as (3*S*)-tetrahydro-3-furyl *N*-(1*S*,2*R*)-3-(4-amino-*N* isobutylbenzenesulfonamido)-1-benzyl-2-hydroxypropylcarbamate. It is active at a concentration of 10–20 nmol l^{-1}. The oral bioavailability is >70% and peak plasma concentrations of 6.2–10 μg ml^{-1} are achieved after dosages of 600–1200 mg. The plasma half-life is 7–10 h. CSF concentrations are significant. Amprenavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system.

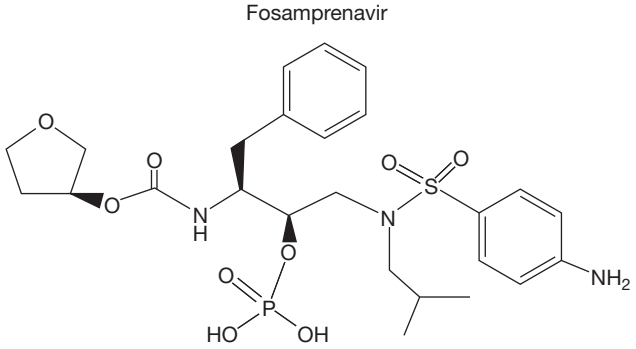
Adverse effects

The most common adverse events are gastrointestinal events (nausea, vomiting, diarrhea, and abdominal pain/discomfort), which are mild to moderate in severity. Also, skin rash can occur in patients on amprenavir.

Resistance

Genotypic analysis of isolates from treatment-naive patients failing amprenavir-containing regimens showed mutations in the HIV-1 protease gene resulting in amino acid substitutions primarily at positions V32I, M46I/L, I47V, I50V, I54L/M, and I84V, as well as mutations in the p7/p1 and p1/p6 Gag and Gag-Pol polyprotein cleavage sites.

Fosamprenavir



Chemistry, mechanism of action, and antiviral activity

Fosamprenavir, a prodrug of amprenavir [(3*S*)-tetrahydrofuran-3-yl (1*S*,2*R*)-3-[[(4-aminophenyl) sulfonyl](isobutyl) amino]-1 benzyl-2-(phosphonooxy) propylcarbamate monocalcium salt], is an inhibitor of human HIV protease. Fosamprenavir is rapidly hydrolyzed to amprenavir by enzymes in the gut epithelium. After administration of a single dose of fosamprenavir to

HIV-1-infected patients, the peak concentration occurs between 1.5 and 4 h (median 2.5 h). Amprenavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. The plasma elimination half-life of amprenavir is approximately 7.7 h.

Adverse effects

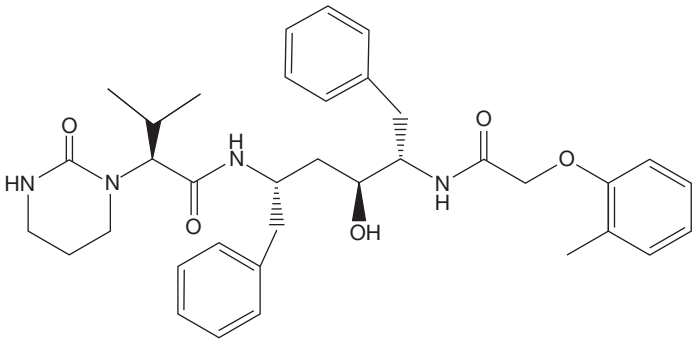
Side effects profile is similar to that of amprenavir.

Resistance

Fosamprenavir selects for amprenavir-associated mutations on treatment failure, though, at a much lower incidence.

Lopinavir

Lopinavir



Chemistry, mechanism of action, and antiviral activity

Lopinavir [N-(4(S)-(2-(2,6-dimethylphenoxy)-acetylamino)-3(S)-hydroxy-5-phenyl-1(S)-benzylpentyl)-3-methyl-2(S)-(2-oxo(1,3-diazaperhydroinyl)butanamin)] is an inhibitor of the HIV protease, prevents cleavage of the Gag-Pol polyprotein, resulting in the production of immature, noninfectious viral particles. It is coformulated with ritonavir at 4:1 ratio (Kaletra). In the presence of 50% human serum, the mean EC_{50} values of lopinavir against HIV-1 laboratory strains ranges from 65 to 289 nmol l^{-1} (0.04–0.18 μg ml^{-1}). It has some activity against HIV-2 strains. Lopinavir peak plasma concentration occurs approximately 4 h after administration. Lopinavir is metabolized by CYP3A, and ritonavir inhibits the metabolism of lopinavir, thereby increasing the plasma levels of lopinavir.

Adverse effects

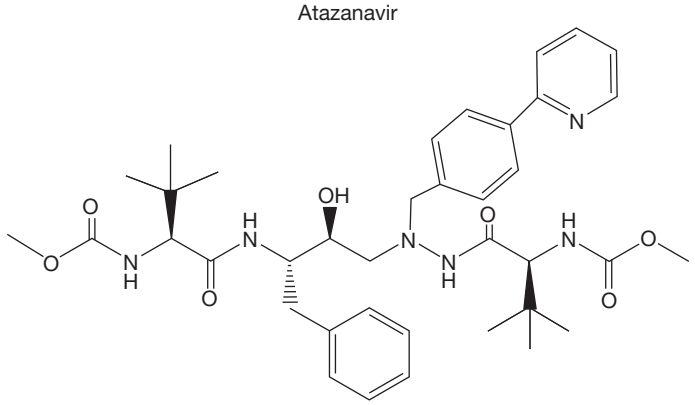
Most common adverse events are nausea, diarrhea, increased cholesterol and triglycerides, and lipodystrophy.

Resistance

Virologic response to lopinavir/ritonavir has been shown to be affected by the presence of three or more of the following amino acid substitutions in protease at baseline: L10F/I/R/V, K20M/N/R, L24I, L33F, M36I, I47V,G48V, I54L/T/V, V82A/C/F/S/T, and I84V.

Atazanavir

Atazanavir



Chemistry, mechanism of action, and antiviral activity

Atazanavir [(3*S*,8*S*,9*S*,12*S*)-3,12-Bis(1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9 (phenylmethyl)-6-[[4-(2-pyridinyl)phenyl] methyl]-2,5,6,10,13-pentaazatetradecanedioic acid dimethyl ester, sulfate (1:1)] is an azapeptide inhibitor of HIV-1 protease.

Atazanavir exhibits anti-HIV-1 activity with an EC_{50} in the absence of human serum of 2–5 nmol l^{-1} against a variety of laboratory and clinical HIV-1 isolates *in vitro*.

Atazanavir is rapidly absorbed with a T_{max} of approximately 2.5 h. Atazanavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. The mean elimination half-life of atazanavir in healthy volunteers and HIV-infected adult patients is approximately 7 h.

Adverse effects

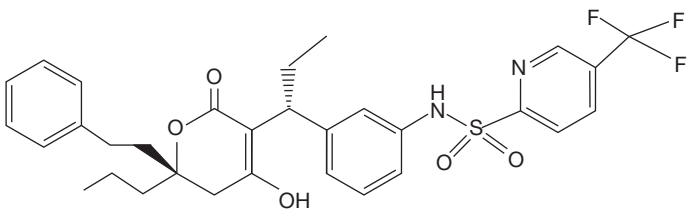
The most common adverse event in patients is the asymptomatic elevations in indirect (unconjugated) bilirubin related to inhibition of UDP-glucuronosyl transferase (UGT). The hyperbilirubinemia is reversible upon discontinuation of atazanavir. Atazanavir may cause abnormal electrocardiogram findings, increased serum glucose, and lipodystrophy in some patients.

Resistance

HIV-1 isolates with a decreased susceptibility to atazanavir have been selected *in vitro* and obtained from patients treated with atazanavir or atazanavir/ritonavir. The mutations associated with resistance to atazanavir are I50L, N88S, I84V, A71V, and M46I. Atazanavir-resistant clinical isolates from treatment-naive harbored the I50L mutation (after an average of 50 weeks of atazanavir therapy), often, in combination with an A71V mutation. However, the viral isolates with the I50L mutation are phenotypically resistant to atazanavir but show *in vitro* susceptibility to other protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir).

Tipranavir

Tipranavir



Chemistry, mechanism of action, and antiviral activity

Tipranavir [2-Pyridinesulfonamide, N-[3-[(1R)-1-[(6R)-5,6-dihydro-4-hydroxy-2-oxo-6-(2-phenylethyl)-6-propyl-2H-pyran-3-yl] propyl]phenyl]-5-(trifluoromethyl)] is a nonpeptidic HIV protease inhibitor belonging to the class of 4-hydroxy-5,6-dihydro-2-pyrone sulfonamides. Tipranavir inhibits the replication of laboratory strains of HIV-1 and clinical isolates *in vitro*, with EC_{50} ranging from 0.03 to 0.07 μmol l^{-1} (18–42 ng ml^{-1}).

The effective mean elimination half-life of tipranavir/ritonavir in healthy volunteers and HIV-infected adult patients is approximately 4.8 and 6.0 h, respectively, at steady state following a dose of 500/200 mg twice daily with a light meal. Tipranavir is predominantly metabolized by the CYP 3A4 enzyme system.

Tipranavir, coadministered with 200 mg of ritonavir, is used in combination with other anti-HIV agents for the treatment of HIV-1 infected adult who are highly treatment-experienced with evidence of viral replication, or have HIV-1 strains resistant to multiple protease inhibitors. Response rates are reduced if five or more protease inhibitor-associated mutations are present at baseline and patients are not given concomitant enfuvirtide with tipranavir/ritonavir.

Adverse effects

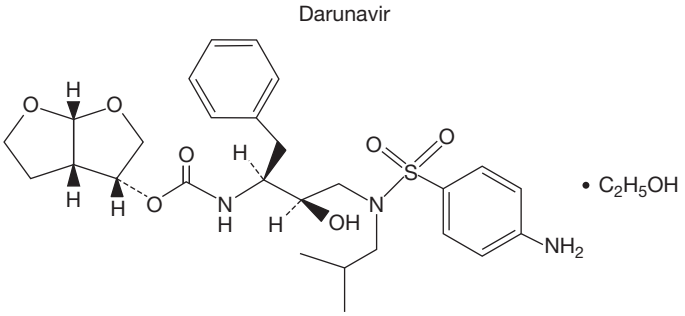
Adverse events include rash, increased cholesterol, increased triglycerides, lipodystrophy, and hepatitis. There have been reports of both fatal and nonfatal intracranial hemorrhage with the use of tipranavir/ritonavir. Tipranavir/ritonavir should be used with caution in patients who may be at risk of increased bleeding from trauma, surgery or other medical conditions, or who are receiving medications known to increase the risk of bleeding such as antiplatelet agents or anticoagulants.

Resistance

HIV-1 isolates that were 87-fold resistant to tipranavir were selected *in vitro* by 9 months and contained 10 protease mutations that developed in the following order: L33F, I84V, K45I, I13V, V32I, V82L, M36I, A71V, L10F, and I54V/T. In clinical trials

tipranavir had less than fourfold decreased susceptibility against 90% of HIV-1 isolates resistant to amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, or saquinavir. Tipranavir-resistant viruses selected for *in vitro* have decreased susceptibility to the protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, and ritonavir but remain sensitive to saquinavir.

Darunavir



Chemistry, mechanism of action, and antiviral activity

Darunavir, in the form of darunavir ethanolate, has the following chemical name: [(1*S*,2*R*)-3-[[(4-aminophenyl)sulfonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl) propyl]-carbamic acid (3*R*,3a*S*,6a*R*)-hexahydrofuro[2,3-*b*]furan-3-yl ester monoethanolate. It is an inhibitor of the HIV protease. Darunavir exhibits activity against laboratory strains and clinical isolates of HIV-1 and laboratory strains of HIV-2 with median EC_{50} values ranging from 1.2 to 8.5 nmol l^{-1} (0.7–5.0 ng ml^{-1}). Darunavir, coadministered with 100 mg ritonavir twice daily, was absorbed following oral administration with a T_{max} of approximately 2.5–4 h. The absolute oral bioavailability of a single 600 mg dose of darunavir alone and after coadministration with 100 mg ritonavir twice daily was 37 and 82%, respectively. Darunavir is primarily metabolized by CYP3A. Ritonavir inhibits CYP3A, thereby increasing the plasma concentrations of darunavir when given in combination.

Darunavir, coadministered with 100 mg ritonavir, and with other anti-HIV agents, is indicated for the treatment of HIV infection in antiretroviral treatment-experienced adult patients, such as those with HIV-1 strains resistant to more than one protease inhibitor.

Adverse effects

The most common treatment-emergent adverse events (>10%) reported in the *de novo* subjects, regardless of causality or frequency, were diarrhea, nausea, headache, and nasopharyngitis. Other side effects are increased triglycerides, increased cholesterol, lipodystrophy, increased glucose, and increased liver enzyme levels.

Resistance

Darunavir-resistant virus derived in cell culture from wild-type HIV had 6- to 21-fold decreased susceptibility to darunavir and harbored three to six of the following amino acid substitutions S37N/D, R41E/S/T, K55Q, K70E, A71T, T74S, V77I, or I85V in the protease. In phase IIb trial, the amino acid substitution V32I developed on darunavir/ritonavir (600/100 mg twice a day) in greater than 30% of virologic failure isolates and substitutions at amino acid position I54 developed in greater than 20% of virologic failure isolates. Other substitutions that developed in 10–20% of darunavir/ritonavir virologic failure isolates occurred at amino acid positions I15, L33, I47, G73, and L89.

Future Prospects in HIV Therapeutics

The simplification of HAART regimens has been a high priority for many years. As the number of effective drugs increases, so does the number of possible effective regimens. The trend toward fixed-dose combinations and once-daily dosage forms of many antiretroviral drugs has provided welcome relief to patients. The following multi-class fixed dose combination agents have been approved by the FDA: Atripla (efavirenz, emtricitabine and tenofovir); Complera (emtricitabine, rilpivirine, and tenofovir); and Stribild (elvitegravir, cobicistat, emtricitabine, and tenofovir). Not only is their medication burden simplified, but as a consequence of improved adherence to therapy, they should experience better control of HIV and thus reduced morbidity.

New drug discovery strategies attempt at circumventing the current drug resistant problem by focusing on either novel targets or new compounds capable of suppressing HIV strains that are resistant to current inhibitors. There are several nucleoside analogues in preclinical and clinical studies. Notably are the novel 4′-substituted thymidine analogues with potent antiviral activity and less cytotoxic (Yang et al., 2006). An example is the recently discovered 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine, structurally related to stavudine, is a more potent inhibitor of HIV-1 replication and is much less inhibitory to mitochondrial DNA synthesis and cell growth in cell cultures than its progenitor stavudine. The triphosphate metabolite accumulates in cells much longer than stavudine, and exerts persistent antiviral activity even after removal of drug from culture (Paintsil et al., 2007). It also has a unique resistance profile when compared to other thymidine analogues and maintains activity against multidrug resistant HIV strains. There is increasing number of compounds discovered as anti-HIV agents targeted at virtually any step in the replicative cycle of the virus and novel targets in development.

Summary

It is anticipated that new and effective treatments for viral infections will be available with the advent of modern and improved technology, based on molecular biology, combinatorial chemistry, and computer-aided design of compounds with greater specificity targeting on viral life cycle.

Acknowledgement

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Aquificae

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Glossary

crcB The *crcB* RNA motif is now called the fluoride riboswitch. A *crcB* motif from *Pseudomonas syringae* was shown to be capable of selectively sensing the very small and highly charged fluoride ion.
Chemolithoautotroph Organism that uses anorganic compounds as electron donors and carbon source.
Chemolithoheterotroph Organism that uses anorganic compounds as electron donors, but organic compounds as carbon source.
Chemoorganoheterothroph Organism that uses organic compounds as electron donors and carbon source.

Abbreviations

Co	Cobalt
Fe	Iron
HGT	Horizontal gene transfer
kbp	Kilo (10^3) base pairs
Mn	Manganese
Moco	Molybdenum cofactor
ORF	Open reading frame
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SRP RNA	Signal recognition particle RNA
tmRNA	Transfer-messenger RNA

Introduction

The bacterial phylum Aquificae comprises the orders *Aquificales* (families *Aquificaceae* and *Hydrogenothermaceae*) and *Desulfurobacteriales* (family *Desulfurobacteriaceae*) (Gupta and Lali, 2013). Members of the Aquificae inhabit terrestrial and marine hydrothermal systems around the planet. For example, genera from all three families can be isolated at deep-sea vents such as the Eastern Lau Spreading Center and Valu-Fa Ridge (ELSC/VFR) in the Southwestern Pacific (Ferrera et al., 2014), where they inhabit hydrothermal, mineral and sulfur-rich deposits that form when the cold oxygenated seawater mixes with the high temperature hydrothermal fluid. Such geochemical habitats are dominated by mesophilic ε-proteobacteria, but Aquificae complement these bacterial communities, evidently by thriving in the higher temperature areas (Ferrera et al., 2014). The phylum members *Aquifex aeolicus* and *Aquifex pyrophilus* are the most temperature-resistant bacteria known; they grow up to 95°C, whereas other *Aquificales* have upper growth temperature limits of ~ 80°C. The known Aquificae are Gram-negative, non-sporulating and motile bacteria that grow at slightly acidic (~ pH 5) to neutral pH and which are adapted to the salinity of their habitats (Guiral et al., 2012). They are primarily chemolithoautotrophs that utilize anorganic compounds as energy source (electron donor) and obtain carbon by CO_2 fixation using the reductive tricarboxylic acid (rTCA) cycle. Electron donors are H_2 (with the exception of some *Hydrogenothermaceae* species), elemental sulfur (S^0) or thiosulfate ($S_2O_3^{2-}$); electron acceptors are O_2 in the case of the microaerobic *Aquificaceae* and *Hydrogenothermaceae* (some organisms can also use NO_3^- or S^0), whereas the strictly anaerobic *Desulfurobacteriaceae* only utilize sulfur compounds like S^0 or NO_3^- as electron acceptors. Some characteristics of the eleven Aquificae strains with completely assembled genome sequence (or of related strains if the sequenced strains have not been characterized specifically) plus *A. pyrophilus* as a founding member of the phylum (Huber et al., 1992) are summarized in Table 1.

Energy Metabolism – Idiosyncrasies, Commonalities and Differences

Aquifex stands for "water-maker" to indicate H_2 oxidation and terminal electron transfer to O_2 ("Knallgas" reaction). However, some *Sulfurihydrogenibium* strains such as sp. Y03AOP1, *S. yellowstonense* and sp. UZ 3–5 are unable to oxidize H_2

Table 1 Physiological characteristics of members of the phylum Aquificae

	Growth T (°C)min-opt-max	Electron donor	Electron acceptor	Products of energy metabolism	Carbon source	Other requirements	Cell morphology	Growth style[a]	References
Family Aquificaceae									
Aquifex aeolicus VF5	58-85-95	H_2, $S_2O_3^{2-}$, S^0	O_2 (low), S^0	H_2O, SO_4^{2-}, H_2S	CO_2	mineral salts/trace elements	Gram$^-$, rods, motile	cla, ma	(Deckert *et al.*, 1998; Guiral *et al.*, 2005; Huber and Eder, 2006)
Aquifex pyrophilus Kol5a	67-85-95	H_2, $S_2O_3^{2-}$, S^0	O_2 (low), NO_3^-	H_2O, SO_4^{2-}, H_2S, $NO_2^- \rightarrow N_2$	CO_2			cla, ma, aa	(Huber *et al.*, 1992)
[b]*Hydrogenivirga caldilitoris* IBSK3	55-75-77.5	H_2, S^0	O_2 (low), NO_3^-	H_2O, N_2O, SO_4^{2-}	CO_2			cla, ma, aa	(Nakagawa *et al.*, 2004; Reysenbach *et al.*, 2009)
[c]*Hydrogenobacter thermophilus* TK-6	70 50-┤-78 75	H_2, $S_2O_3^{2-}$, HCO_2^-, $HCONH_2$	O_2 (low), NO_3^-	H_2O, SO_4^{2-}, H_2S, NO_2^-, NO, $N_2O \rightarrow N_2$	CO_2			cla, coh, ma, aa	(Kawasumi *et al*, 1984; Suzuki *et al.*, 2001; Eder and Huber, 2002)
[d]*Hydrogenobaculum acidophilum*	~45-65-70	H_2, $S_2O_3^{2-}$, S^0	O_2 (low)	H_2O, SO_4^{2-}	CO_2			cla, ma	(Shima and Suzuki, 1993; Stöhr *et al.*, 2001; Reysenbach *et al.*, 2009)
[e]*Thermocrinis ruber* DSM 23557	44-80-89	H_2, $S_2O_3^{2-}$, S^0, HCO_2^-, $HCONH_2$, AsO_3^{3-}, $AsSO_3^{3-}$	O_2 (low), S^o	H_2O, SO_4^{2-}, H_2S, NH_3, AsO_4^{3-}	CO_2			cla, coh, ma	(Huber *et al.*, 1998; Härtig *et al.*, 2014)
Thermocrinis albus DSM 14484	44-80-89	H_2, $S_2O_3^{2-}$, S^0, HCO_2^-, $HCONH_2$	O_2 (low), S^o	H_2O, SO_4^{2-}, H_2S, NH_3	CO_2			cla, coh, ma	(Huber *et al.*, 1998)
Family *Hydrogenothermaceae*									
[f]*Sulfurihydrogenibium sp.* Y03AOP1	~70 (optimal)	$S_2O_3^{2-}$, S^o	O_2 (low)[g]	SO_4^{2-}	CO_2			cla, clh, ma, aa	(Takai *et al.*, 2003; Nakagawa *et al.*, 2005; O'Neill *et al.*, 2008; Reysenbach *et al.*, 2009)
[h]*Sulfurihydrogenibium azorense* Az-Fu1	50-68-73	H_2, $S_2O_3^{2-}$, S^0, SO_3^{2-}, Fe^{2+}, ASO_3^{3-}	O_2 (low), S^o, Fe^{3+}, SO_3^{2-}, SeO_4^{2-}, AsO_4^{3-}	H_2O, SO_4^{2-}, Fe^{2+}, AsO_4^{3-}, AsO_3^{3-}, Se^0	CO_2 yeast extract, bacto peptone,trypticase peptone, casamino acids			cla, clh, ma, aa	(Aguiar *et al.*, 2004; Nakagawa *et al.*, 2005)
Persephonella marina EX-H1	55-73-80	H_2, $S_2O_3^{2-}$, S^0	O_2 (low), NO_3^-, S^o	H_2O, H_2S, N_2	CO_2			cla, ma	(Götz *et al.*, 2002)
Family *Desulfurobacteriaceae*									
[i]*Desulfobacterium thermolithotrophum* DSM 11699	40-70-75	H_2	S^o, $S_2O_3^{2-}$, SO_3^{2-}	H_2S	CO_2			cla, aa	(L'Haridon *et al.*, 1998)
Thermovibrio ammonificans HB-1	60-75-80	H_2	S^o, NO_3^-	H_2S, NH_4^+	CO_2			cla, aa	(Vetriani *et al.*, 2004)

[a]cla: chemolithoautotroph (uses anorganic compounds as electron donors and carbon source); coh: chemoorganoheterothroph (uses organic compounds as electron donors and carbon source); clh: chemolithoheterotroph (uses anorganic compounds as electron donors, but organic compounds as carbon source); ma: microaerob; aa: anaerobic.

[b]*Hydrogenivirga caldilitoris* IBSK3 is related to strain *Hydrogenivirga sp.* 128-5-R1-1 (Ferrera *et al.*, 2014) that was subjected to genome sequencing (NCBI, NZ_ABHJ00000000.1); another related strain is *Hydrogenivirga okinawensis* LS12-2 (Nunoura *et al.*, 2008) with the following determined features: a growth temperature range of 65-85°C (optimal 70-75°C); use of S^o and $S_2O_3^{2-}$, but not H_2 as electron donors; N_2, $S_2O_3^{2-}$ and SO_4^{2-} as products of energy metabolism; strictly chemolithoautotrophic, microaerobic and anaerobic growth (Nunoura *et al.*, 2008).

[c]unable to grow anaerobically with nitrate in the presence of formate, formamide or formaldehyde (Eder and Huber, 2002).

[d]*Hydrogenobaculum acidophilum* (optimal pH: 3-4) is related to *Hydrogenobaculum sp.* Y04AAS1 (optimal pH: 4; Ferrera *et al.*, 2007), the strain for which the genome sequence is available. The latter was reported to be unable to reduce nitrate under laboratory conditions (Reysenbach *et al.*, 2009), although genes for nitrate respiration are encoded (Reysenbach *et al.*, 2009; Romano *et al.*, 2013). The optimal growth temperature for strain Y04AAS1 is 58°C (Ferrera *et al.*, 2007). A substantial microdiversity of *Hydrogenobaculum* in Yellowstone National Park samples was observed; ~ half of the genes unique to strain Y04AAS1 relative to *Hydrobaculum sp.* isolates reported in (Romano *et al.*, 2013) were predicted to be acquired by HGT. *Hydrobaculum sp.* can further grow on H_2S as energy source (Romano *et al.*, 2013).

[e]*Thermocrinis ruber* isolate OC14/7/2 (DSM No. 23557) was used as authentic type strain instead of the deposited isolate DSM No. 12173 (Huber *et al.*, 1998) which turned out to be non-authentic based on 16S rRNA sequence analysis (Härtig *et al.*, 2014).

[f]No specific strain characterization available; the given data are based on descriptions of the genus *Sulfurihydrogenibium* (Takai *et al.*, 2003; Aguiar *et al.*, 2004; Nakagawa *et al.*, 2005; O'Neill *et al.*, 2008).

[g]optimally microaerobic conditions, but aerobic conditions (> 10 volume % O_2) tolerated by some species.

[h]Some of the products of energy metabolism were inferred from analysis of *Sulfurihydrogenibium subterraneum* (Takai *et al.*, 2003) based on the utilization of the same electron donors/acceptors by *Sulfurihydrogenibium azorense* Az-Fu1 (Aguiar *et al.*, 2004; Nakagawa *et al.*, 2005).

[i]A novel species, *Desulfurobacterium indicum*, was characterized recently and compared with four other *Desulfurobacterium* species including *D. thermolithotrophum*, which differ in their utilization of terminal electron acceptors (Cao *et al.*, 2017). With H_2 as electron donor, *D. indicum* can utilize S^o, $S_2O_3^{2-}$ and NO_3^- as electron acceptors, but not SO_3^{2-}.

(O'Neill et al., 2008; Reysenbach et al., 2009) which is consistent with the failure to identify hydrogenase genes in the corresponding genomes. A commonality of all Aquificae is their capability to fix CO_2 by the rTCA cycle to synthesize acetyl-CoA. While the *Aquificaceae* utilize the presumedly ancestral "B-type" rTCA cycle (cleavage of citrate to oxaloacetate and acetyl-CoA by successive action of two enzymes, citryl-CoA synthetase and citryl-CoA lyase), the "A-type" rTCA cycle (cleavage of citrate to oxaloacetate and acetyl-CoA by the single two-subunit enzyme ATP citrate lyase) is implemented in the other two Aquificae families (Hügler et al., 2007). The *Desulfurobacteriaceae* additionally encode an incomplete reductive acetyl-CoA pathway thought to be the most ancient carbon fixation pathway on earth. Together with their ability to utilize sulfur as electron acceptor and their strictly anaerobic growth, this family was proposed to have retained primordial metabolic components that evolved before the emergence of photosynthesis and the rise of oxygen in the atmosphere (Giovannelli et al., 2017). A common trait of members of the order *Aquificales* is their ability to utilize elemental sulfur or inorganic sulfur compounds as electron donors (Table 1).

Oxidative Stress

Aquificae that grow under microaerobic conditions have to cope with reactive oxygen species (ROS) such as superoxide and peroxide. Three genes encoding superoxide dismutase (one Fe/Mn and two Co/Zn family enzymes) were identified in the *A. aeolicus* genome. However, no catalase gene could be identified, although *Aquifex* cells react with H_2O_2 as inferred from rapid gas production (Huber et al., 1992; Swanson, 2001). Catalase activity, detected in the insoluble fraction of *A. aeolicus* cell lysates, was resistant to acid treatment and could not be extracted by organic solvents from insoluble lysate pellets. Further analysis identified phosphate salts of Fe as a major component of such insoluble fractions. As Fe alone has catalase activity, it has been speculated that *A. aeolicus* may promote the intracellular precipitation of such inorganic Fe-phosphate salts to protect cells against damage by H_2O_2 (Swanson, 2001).

Thermovibrio ammonificans, which grows strictly anaerobic, also encodes proteins that protect against damage by ROS. This includes a catalase/peroxidase, a putative superoxide reductase, a cytochrome c peroxidase and a cytochrome bd complex. The latter was shown to contribute to oxygen tolerance in anaerobic bacteria and to detoxification of nitrous oxide radicals (Giovannelli et al., 2017).

Gluconeogenesis

The Aquificae synthesize pentose and hexose monosaccharides from products of the rTCA cycle by the Embden-Meyerhof-Parnas pathway. The key gluconeogenic enzyme fructose-1,6-bisphosphatase (*fbp*) was identified in *T. ammonificans*, *Desulfobacterium thermolithotrophum*, *Sulfurihydrogenibium* sp. Y03AOP1, *Persephonella marina*, *Hydrogenivirga* 128-5-R-1-1, but not in *Hydrogenobacter thermophilus* and *A. aeolicus*, suggesting a pathway variation in the latter two bacteria (Deckert et al., 1998; Giovannelli et al., 2017). Genes encoding enzymes of the pentose-phosphate pathway and for glycogen synthesis and catabolism were also found in the *A. aeolicus* genome (Deckert et al., 1998).

Motility

All Aquificae are motile, forming one or two (mono- or polytrichous) monopolar flagella. Some members of the phylum, such as *T. ammonificans* and *D. thermolithotrophum*, also encode homologs of the bacterial chemotaxis system (Giovannelli et al., 2017); corresponding genes were not identified in the *A. aeolicus* genome (Swanson, 2001). As the well-characterized bacterial chemotaxis systems respond to the presence of sugars and amino acids, chemolithoautotrophs like *A. aeolicus* may sense other changes, such as concentration of dissolved CO_2, H_2, O_2 or temperature, by other mechanisms (Swanson, 2001).

Sizes and G/C Content of Aquificae Chromosomes

To date (end of 2018), the NCBI genome browser lists 23 Aquificae genome projects. Ten represent complete genome assemblies, all others are in premature assembly states. We also included *Hydrogenivirga* in our comparative genome analyses, as done in a recent study to which we refer here (Lechner et al., 2014; see phylogenetic tree in Fig. 1). The eleven strains are specified in Table 1; if the strain with sequenced genome was not characterized in detail in the literature, we instead describe the features of a closely related strain. In addition, we included the founding strain *A. pyrophilus* with unsequenced genome for comparison of metabolic features (Table 1). Genome sizes are close to the assumed lower limit of genomes in free-living bacteria, ranging from 1.5 (*T. albus*) to $\sim$ 2 Mbp (*P. marina*). The current annotation file of the *Hydrogenivirga* 128-5-R-1-1 genome contains a sequence of 3.04 Mbp, which is roughly double the size as found in other Aquificae. However, the *Hydrogenivirga* sequence file only represents the lowest contig assembly level and there is evidence that the genome assembly is erroneous, likely a blend of two genomes of related bacteria, one from a member of the *Aquificaceae* and the second from a *Hydrogenothermaceae* species. This is inferred from the finding of two 6S

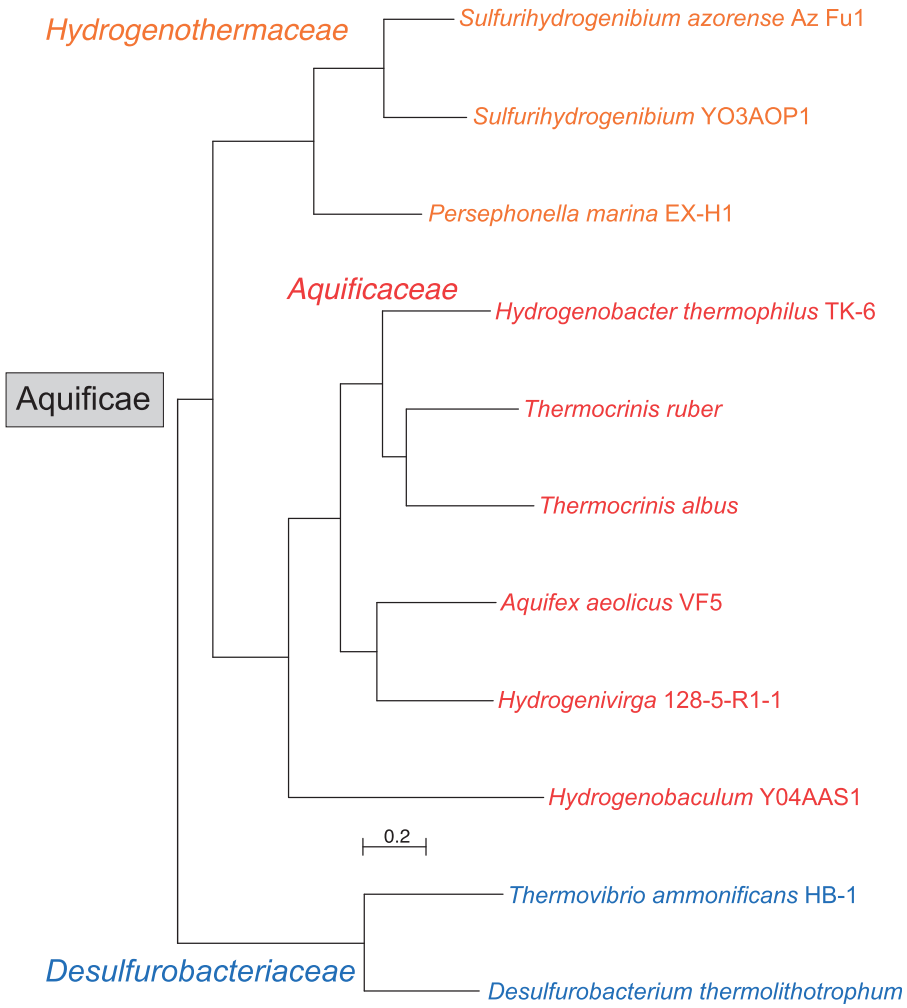

Fig. 1 Maximum likelihood tree of Aquificae based on whole genome alignments. Strains: *Thermocrinis ruber* DSM 23557; *Thermocrinis albus* DSM 14484; *Desulfurobacterium thermolithotrophum* DSM 11699. Reproduced from Lechner, M., Nickel, A.I., Wehner, S., 2014. Genomewide comparison and novel ncRNAs of *Aquificales*. BMC Genomics 15, 522. doi:10.1186/1471-2164-15-522.

RNA candidates and two different subtype tmRNAs – rather than single loci as in the other Aquificae – as well as an increased number of tRNA genes and CRISPR loci (Lechner et al., 2014). The genome G/C content varies between 32% (*Sulfurihydrogenibium* sp. Y03AOP1) to 52.1% (*T. ammonificans*).

Extrachromosomal Plasmids

Plasmids were isolated and sequenced for *A. aeolicus* (39 kbp, 32 genes), *T. ammonificans* (77 kbp, 97 genes) and *P. marina* (54 kbp, 74 genes). In *A. aeolicus*, on average two plasmid copies were estimated to be present per chromosome. Except for a transposase, ORFs were classified as hypothetical proteins; one plasmid ORF is also present in two identical copies on the chromosome (Deckert et al., 1998). In *T. ammonificans*, the plasmid encodes mostly ORFs with unknown hypothetical functions, except for a putative RNA polymerase sigma factor, a DNA topoisomerase I, a type II secretion protein E and an ArsR-like helix-turn-helix transcription regulator. Furthermore, the plasmid contains highly repeated DNA (Giovannelli et al., 2017). In *P. marina*, apart from mostly hypothetical proteins, the plasmid encodes, among others, an antitoxin, several proteins involved in conjugal plasmid transfer, a site-specific integrase, a type IA DNA topoisomerase, a plasmid replication initiator and segregation proteins.

Protein Genes and mRNA Decoding

The first published Aquificae genome of *A. aeolicus* revealed several idiosyncrasies (Deckert et al., 1998). In the densely packed genome, most genes are part of polycistronic transcription units as in other Bacteria. There are many operons encoding genes of the

same functional context, which is pronounced for ribosomal protein genes, but also seen for many genes involved in energy metabolism, cell envelope biogenesis and processes such as flagella synthesis. Yet, there are also many genes of the same functional context, such as those of the tryptophan and histidine biosynthesis pathways, that are not organized in the same operons as in other Bacteria, but are dispersed in the *A. aeolicus* genome or appear in novel operons. This also pertains to genes for subunits of the same enzyme, for example *gltB* (Aq_1565) and *gltD* (Aq_2064; glutamate synthase), the α and β subunit genes of glycin and phenylalanine tRNA synthetases (Aq_945/2141 and Aq_953/1730), *ribH*/*ribC* (Aq_132/1707) encoding the α and β subunits of riboflavin synthase and *nrdA*/*nrdF* (Aq_94/1505) coding for the α and β chains of ribonucleotide reductase. It has been surmised that the lack of cocistronic arrangement of biosynthetic genes may reflect the chemolithoautotrophic life style of Aquificae that is committed to CO_2 fixation. In contrast, chemoorganoheterotrophic microorganisms can metabolically switch between different energy and carbon sources. This is achieved by coordinated up- and down-regulation of transcription units that encode the genes participating in the respective metabolic pathways (Swanson, 2001).

It is also not clear if transcription in the Aquificae may differ from what is known from other bacteria. The RNA polymerase and sigma factors in the genus *Aquifex* have functional properties very similar to those of other bacteria (Klenk et al., 1999; Studholme and Buck, 2000; Studholme et al., 2000), but *A. aeolicus* was reported to lack hairpin structures for transcription termination (Washio et al., 1998). The latter observation might suggest that transcript ends/operon boundaries are less defined, possibly pointing to read-through transcription of longer genomic segments. However, this may not pertain to the entire genome, as potential (though not very stable) hairpin structures were identified at the end of the consecutive *rplAJL* (encoding ribosomal proteins) and *rpoBC* (encoding the ß and ß′ subunits of RNA polymerase) operons in *A. pyrophilus* (Klenk et al., 1999). Many convergently transcribed ORFs in *A. aeolicus* overlap, ranging from simple stop codon overlaps to overlaps of tens of base pairs (Swanson, 2001). In RNA-Seq experiments, high sense and antisense read levels were found in genes encoding validated and putative non-coding RNAs. The same pertains to overlapping 5′-upstream regions of two protein-coding genes located on opposing strands, as well as areas where two 3′-untranslated regions overlap on opposing strands (Lechner et al., 2014). Such RNA reads might be vestiges of extensive transcription of both genome strands, although this remains to be validated.

Whole genome alignments using the software tools Pomago (v. 1.0) and TBA (v. 11.2) revealed that individual genomes have gaps of 10.5%–13% (Pomago) or 8.4%–9.6% (TBA) when aligned to the other ten genomes. The original protein annotations of the genome files were recently extended, predicting for example ∼ 6% more proteins with assigned or hypothetical function in *A. aeolicus*. In addition, the 5′- and 3′-ends of ORFs were partially refined. The Shine-Dalgarno sequences in the 11 organisms are quite conserved (5′-GGAGG, always 5′-NGAGN), -10 promoter signatures are more variable though recognizable (A/T-rich). The number of tRNA genes is minimized to 39–46 (*Hydrogenivirga* excluded) compared with > 80 tRNA genes in strains of *Escherichia coli* and *Bacillus subtilis*. With respect to codon usage, the two Arg codons with 5′-A and the Ile AUA codon are preferentially utilized in the *Aquificaceae* and *Hydrogenothermaceae*. The preference for AUA correlates with expression of tRNA-Ile lysidine synthetase (TilS) in the Aquificae (a crystal structure of *A. aeolicus* TilS is available). TilS converts the 5′-C residue in the CAU anticodon of specific tRNA-Ile molecules to lysidine (2-lysyl cytidine; abbreviated as *L* or k^2C) to allow decoding of 5′-AUA (Ile) instead of 5′-AUG (Met) codons. Apparently, this anticodon modification enables reliable decoding at high temperatures. Consistent with the reduced number of tRNA genes (39–46) in the Aquificae, multiple copies of tRNA isoacceptor genes are avoided. Almost no tRNAs with 5′-A in the anticodon are encoded and anticodons with 5′-C are reduced in the non-*Aquificaceae* within the Aquificae. The streamlining of the tRNA repertoire goes along with expression of the tRNA modification enzyme tRNA adenosine deaminase (TadA; crystal structure of the *A. aeolicus* protein available) that converts the anticodon 5′-A residue to inosine in the single $tRNA^{Arg}$ (anticodon 5′-ACG). This allows decoding of codons 5′-CG(U,C,A), while each of the three other $tRNA^{Arg}$ isoacceptors recognize one of the remaining three Arg codons through Watson-Crick base-pairing at all three codon positions. The maintenance of wobble decoding by $tRNA^{Arg}$ (5′-ICG) while decreasing the fraction of 5′-CG(U,C,A) codons in the genome may be a compromise resulting from constraints to downsize the tRNA repertoire and to minimize the number of labile codon:anticodon interactions. The *Aquificaceae* (except for *Hydrogenobaculum*) and *Desulfurobacteriaceae* encode selenocysteine-specific tRNAs decoding 5′-UGA codons; those are absent from the *Hydrogenothermaceae*. The *Aquificaceae*, but not *Hydrogenivirga* and the other Aquificae, express the lysine tRNA isoacceptor with the anticodon 5′-CUU to stabilize AAG decoding, a feature shared with other thermophilic bacteria (*Thermus thermophilus*, *Thermotoga maritima*). In mesophiles, AAG codons are decoded by isoacceptors harboring the anticodon 5′-UUU.

Non-Coding RNAs (ncRNAs) in the Aquificae

Apart from tRNAs and 1–3 copies of 5S/16S/23S rRNA, bioinformatics identified genes for 6S RNA (except for uncertain candidates in *D. thermolithotrophum* and *T. ammonificans*), a regulator of bacterial RNA polymerase, the signal recognition particle RNA (SRP RNA), and a tmRNA of type A or B; riboswitch candidates were found sporadically in a few species [TPP, Moco, Cobalamin and *crcB* (Lechner et al., 2014)]. Of note, 6S RNA is present in the *Aquificaceae* and *Hydrogenothermaceae*, as well as in *Thermodesulfatator indicus* of the Aquificae sister phylum Thermodesulfobacteria, but is absent from the next related phyla (according to 16S rRNA phylogeny), Thermotogae and Deinococcus-Thermus. RNase P RNA is not encoded in the genomes of *Aquificaceae*, but is present in the two other families. Instead, the *Aquificaceae* encode a novel type of protein-only RNase P, a PIN_5 domain metallonuclease consisting of a single 23-kDa polypeptide. The genes for the ancient RNA-based enzyme (*rnpB* encoding the RNA and *rnpA* the protein subunit), present in the vast majority of Bacteria, were lost in the *Aquificaceae*, which represents a novel hallmark feature of

this family. Homologs of *Aquifex* RNase P (HARP) were identified in many Archaea and in a few other bacteria of different phyla; all these Archaea encode an RNA-based RNase P as well. Combined with the assessment that at least 10% of genes in *A. aeolicus* were acquired by horizontal gene transfer (HGT) from Archaea (Aravind et al., 1998), it is very likely that the progenitor of the *Aquificaceae* acquired the HARP gene by HGT from an archaeon (Nickel et al., 2017). In accordance, bootstrap analysis groups all bacterial HARPs with those of archaeal Thermocci, suggesting that bacterial HARPs originate from this class of Euryarchaeota (Nickel et al., 2017).

The *A. aeolicus* genome was analyzed for ncRNA candidates, applying constraints such as a minimum length of 25 nt and no overlap with protein-coding sequences, rRNA or tRNA genes. Since the housekeeping ncRNAs discussed above have a G/C content of 66% in *A. aeolicus*, an elevated G/C content (relative to the 43% average G/C content of the genome) was taken as an additional criterion for ncRNA identity. The analysis identified a candidate list of ~ 100 ncRNAs in *A. aeolicus* (Lechner et al., 2014), including the housekeeping ncRNAs, such as SRP RNA, tmRNA or 6S RNA. As mentioned before, RNA-Seq data for *A. aeolicus* suggest a substantial level of antisense transcripts for known ncRNAs and novel ncRNA candidates, which could be partly confirmed by Northern blot analyses (Lechner et al., 2014).

Mechanisms of Thermostabilization

Adaptations to growth at high temperatures are evident at the level of cell envelope, genome, RNAs and proteins. The cell membranes of several *Aquificaceae* were found to contain the more stable glycerol-ether phospholipids in addition to acyl glycerides (Jahnke et al., 2001). *A. aeolicus* expresses two reverse gyrases (genes Aq_886 and Aq_1159), which are topoisomerases originally acquired form Archaea by HGT and which introduce positive supercoilings into the DNA. Potential functions include prevention of excess local unwinding of the double helix or protection of DNA against damage (e.g., depurination, strand breakage) at high temperature (Brochier-Armanet and Forterre, 2007; Ogawa et al., 2015). Another adaptation to (hyper)thermophilic conditions is an elevated G/C content of non-coding RNAs (ncRNAs). The ribosomal RNAs of *A. aeolicus* have 65% G/C compared with an average G/C content of ~ 43% in the bacterium's genomic DNA (Deckert et al., 1998). This G/C-richness, which correlates with enhanced RNA secondary and tertiary structural stability, is also implemented in other ncRNAs of *A. aeolicus*, such as tRNAs, tmRNA or 6S RNA, the thermodynamically most stable 6S RNA known so far (Deckert et al., 1998; Willkomm et al., 2005; Köhler et al., 2015). Increased thermostability of proteins is achieved by multiple adaptations that are not specific for the Aquificae. Of note, alterations relative to mesophilic Bacteria and Archaea differ between moderately and extremely thermophilic organisms, and different protein families have evolved individual strategies to achieve high thermal stability (Szilágyi and Závodszky, 2000). Hyperthermophiles (Archaea and Bacteria such as *A. aeolicus*) have increased proportions of charged amino acids (Lys, Glu) at the expense of polar non-charged amino acids (Asn, Gln, Ser, Thr), indicative of an increase in ion pairs including salt bridges between N- and C-termini (Cambillau and Claverie, 2000; Szilágyi and Závodszky, 2000). Disulfide bonds can also contribute to stabilization, such as in an *Aquifex* serine protease containing eight Cys residues (Choi et al., 1999), ferredoxins (Meyer et al., 2002) or rhodanese (Aq_1599, (Giuliani et al., 2010)). There is a trend to increase the fraction of α-helices and β-strands and to decrease flexible regions (e.g., surface loops) in thermophilic proteins. An example is a thermostable cytochrome *c* equipped with an extra α-helix (Obuchi et al., 2009). C-terminal ends of proteins are frequently folded into the protein core to avoid flexible and accessible regions.

Apart from increases in the number of ion pairs, increases in the number of hydrogen bonds or enhanced core hydrophobicity have been questioned as substantial contributors to extreme thermostability (Szilágyi and Závodszky, 2000). Yet, protein oligomerization is often enhanced. In an archaeal histone dimer, improved intermolecular hydrophobic interactions and additional favorable ion pairs at or near the dimer interface were found to be associated with thermostability, along with the preference for large hydrophobic side chains to reduce cavity sizes and to increase packaging density in the hydrophobic dimer core; in addition, C-terminal protein extension by two Lys residues was proposed to shield the dimer core from solvent exposure (Li et al., 2000). As another example, an *A. aeolicus* rhodanese enzyme (Aq_477) involved in sulfur transfer forms tetramers as a prerequisite for thermostability and enzymatic function. Formation of supercomplexes is another strategy to enhance thermostability. The thermostable sulfide-oxidase and oxygen-reductase supercomplex of *A. aeolicus* was found to be resistant to denaturation/inactivation by up to 8 M urea, 6% Triton X-100 and 1% SDS (Guiral et al., 2012).

Phylogeny

The phylum Aquificae has been defined by 16S rRNA phylogeny, yet its member organisms show phylogenetic, ecological, morphological and metabolic diversity. Several protein-based phylogenies have supported 16S RNA-based phylogenies that place the Aquificae as a deep branching sister phylum of the Thermotogae in the bacterial phylogenetic tree. Other analyses have indicated a closer affiliation with other bacterial groups such as the ε-proteobacteria. This is further complicated by bioinformatic evidence that *A. aeolicus* and other Aquificae as well as Thermotogae have acquired many of their protein-coding genes by HGT from Archaea (Aravind et al., 1998; Zhaxybayeva et al., 2009). Two major hypotheses have been formulated to explain the findings of various studies: (a) the Aquificae and the Thermotogae represent the deepest-branching bacterial phyla containing many hyperthermophiles,

with strong affiliations to the ε-proteobacteria owing to large-scale gene sharing, and further shaped by acquisition of genes from archaeal thermophiles; (b) the Aquificae are essentially ε-proteobacteria or a sister to this group that extensively exchanged genes with other thermophilic lineages such as Thermotogae and Archaea. The large extent of genetic mosaicism, also observed in other Aquificae such as *T. ammonificans* (Giovannelli et al., 2017), led Eveleigh et al. (2013) to conclude that "The evolutionary history of the Aquificae implicates different partner lineages, most notably the Archaea, Thermotogae, δ-proteobacteria, and thermophilic members of Nitrospirae, Clostridia and ε-proteobacteria, with different lineages making disproportionate contributions to different molecular subsystems." Indeed, bioinformatic analysis of *A. aeolicus* proteins from selected functional subsystems (cell wall, flagella, oxidative phosphorylation, ribosomal proteins) revealed different "closest neighboring phyla or classes", which even differed between the various complexes participating in oxidative phosphorylation (Eveleigh et al., 2013). This mosaic-like origin of genes in a single metabolic pathway also pertains to enzymes involved in the putative reductive acetyl-CoA pathway in *T. ammonificans* (Giovannelli et al., 2017). Eveleigh et al. (2013) favored a scenario according to which most genes with closest relatedness to homologs in thermophiles are derived from HGT, whereas genes related to those in ε-proteobacteria are relics of a mesophilic past that preceded colonization of high temperature environments. This takes evidence into account that ε-proteobacteria developed diverse strategies to colonize many deep-sea hydrothermal habitats, favored by their high growth rates, rapid adaptations to changing geochemical conditions and metabolic versatility. Finally, it cannot be excluded that the extensive genetic mosaicism in the Aquificae might have erased the traces of their real ancestry.

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Arboviruses☆

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Glossary

Arbovirus Arthropod-borne virus, a virus that is transmitted between its animal hosts by arthropod vectors.
Serogroup A group of antigenically related viruses.
Viremia Presence of a virus in blood.
Zoonosis An infectious disease episode characterized by the transmission of a pathogen from animals to humans.

Abbreviation

BFV	Barmah Forest virus
CCHFV	Crimean-Congo Hemorrhagic Fever virus
CHIKV	Chikungunya virus
CSF	Cerebrospinal fluid
CT	Computed tomography
DEN	Dengue
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
EEEV	Equine encephalitis virus
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
KFDV	Kyasanur forest disease virus
MRI	magnetic resonance imaging
MVE	Murray Valley encephalitis virus
NSDV	Nairobi Sheep Disease virus
OHFV	Omsk hemorrhagic fever virus
ONNV	O'nyong-nyong virus
POWV	Powassan virus
RRV	Ross River virus
RVFV	Rift Valley fever virus
SIADH	Syndrome of inappropriate antidiuretic hormone
SINV	Sindbis virus
SLEV	St. Louis encephalitis virus
TBEV	Tick-borne encephalitis virus
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus
WNV	West Nile virus
YF	Yellow fever
YFV	Yellow fever virus
ZIKV	Zika virus

☆*Change History*: August 2016. David Warrilow changed nomenclature to either formal or informal virus usage as appropriate, updated taxonomic information based on the latest ICTV 2015 report, introduced Zika virus and added a section on this, expanded the arbovirus syndromes to incorporate ZIKV birth defects, made mention of the recent licensed dengue vaccine, added more viruses in the bunyavirus section, added two previously unmentioned arbovirus families infecting humans, overall made multiple minor grammatical changes to improve readability.

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Defining Statement

The arboviruses are a heterogeneous group of viruses transmitted by arthropods. The clinical manifestations of infections in humans by arboviruses range from asymptomatic infections, to a variety of diseases including arthritis, encephalitis, and hemorrhagic fever. The pathogenesis and disease caused by human arboviruses are summarized in this article.

Introduction

The arbovirus group of viruses includes a diverse group of almost exclusively RNA viruses that are transmitted by arthropods to vertebrate human and animal hosts. This group includes positive-strand RNA viruses belonging to the families *Togaviridae* and *Flaviviridae*; negative-strand RNA viruses belonging to the families *Bunyaviridae* and *Orthomyxoviridae*; and a double-stranded RNA virus family *Reoviridae*. Arthropod vectors such as mosquitoes, ticks, sandflies or midges facilitate arbovirus transmission and infection of vertebrate hosts following a blood meal, usually required as a source of protein for egg development. Arboviruses can be maintained in a continued transmission cycle as new uninfected arthropod vectors feed on infected reservoir vertebrate hosts. Virus replication and amplification within the infected arthropod vector usually occurs in the midgut, followed by dissemination and infection of the salivary glands. This allows transmission of the virus into a new vertebrate host when the infected vector bites again. Replication of the virus in the vertebrate host starts at the local site of injection, and viruses are systemically spread via the lymphatic system and the blood to different organs. Subsequent cycles of replication and the presence of virus in the blood (viremia) can result in host viral levels which are sufficient to infect a susceptible vector when it bites, thereby allowing the transmission cycle to continue. However, some arboviruses can be maintained in their arthropod vectors without the need of a vertebrate host through vertical transmission to eggs, allowing survival of the virus even during times when the vertebrate–arthropod cycle is disrupted due to environmental or other ecological factors for prolonged periods of time.

In addition to reservoir vertebrate hosts, there are some species, which although susceptible to arbovirus infection and disease, are unable to sustain viremia levels sufficient for the infection of new vectors. These are dead-end hosts in effect, and represent a spillover of incidental virus transmission from the normal amplification cycle. Some species may participate as reservoir hosts for some arboviruses, but not others. For example, humans are part of the transmission cycle for dengue virus (DENV) and yellow fever virus (YFV), but are dead-end hosts for other flaviviruses such as West Nile virus (WNV) and Japanese encephalitis virus (JEV), whose natural hosts are avian species. Thus, humans infected with DENV or YFV can potentially reach viremia levels required for infection of the mosquito species that transmit these viruses, and therefore are intrinsically important for ongoing transmission of DENV or YFV which can lead to unprecedented outbreaks and widespread epidemics. However, in most instances, humans are considered dead-end hosts in the transmission cycles of many arboviruses.

Although humans can develop severe disease such as encephalitis, as may occur following WNV or JEV infections, generally they do not attain viremia levels necessary to infect mosquito vectors. Transmission of an arbovirus from one human to another can also occur occasionally in the absence of an arthropod intermediate, as is the case if a transplant recipient receives an organ from an infected donor, via blood transfusions or indeed transfer of other bodily fluids as in the case of sexual transmission. Regardless of these factors, the complex interactive transmission cycles of arboviruses require that these viruses adapt to two evolutionary different and distinct hosts: vertebrates and arthropods. This adaptation can be very exquisite, and therefore the occurrence and prevalence of arbovirus infections depends on the presence of the particular arthropod/vertebrate species that can be infected. Because of this, most arbovirus infections are zoonotic, with humans becoming accidentally infected by an infected vector that usually bites a different vertebrate species. However, in cases when humans are natural hosts and viremia levels are sufficient, arbovirus infections have the potential to become endemic and even epidemic if the arthropod vector is abundant. This is evident in many DENV endemic tropical regions, where the main mosquito vector, *Aedes aegypti*, is highly abundant.

Arboviral Diseases

Arboviral infections can produce a spectrum of infections from asymptomatic and mild febrile illnesses to severe clinical manifestations and disease. For certain arboviruses, severe disease can develop, manifesting as arthritis, encephalitis, or hemorrhagic fever in a minority of patients. Encephalitis and hemorrhagic forms are associated with a high mortality. The development of a clinical illness following an arboviral infection is a result of a complex interaction between viral and host factors. Host factors determine the onset and manifestations of the infection, and as such, follow a temporal sequence with the early clinical presentation often protean in nature. Fever is a universal manifestation of all symptomatic arboviral infections and often the initial clinical presentation. As the illness progresses and the host response matures, classic signs and symptoms present and severe disease may develop. As part of the initial evaluation, an estimate of the day of onset of clinical illness should be determined from the patient where possible based on the first day of febrile illness. The initial date of exposure may not be known and may vary between viruses; however, the incubation period is usually 2–15 days prior to the onset of symptoms, on average. Viremia occurs during the acute phase of illness which is within the first 5–10 days of symptoms appearing.

Early knowledge of exposure and symptom onset can be critical for determining appropriate diagnostic tests, effective treatments and disease control and management, particularly if the threat of more severe disease or further outbreaks exists. Virus isolation and molecular detection of viral nucleic acid by polymerase chain reaction are effective tests early during the viremic phase and can provide a definitive diagnosis. Serology-based technologies can be useful once antibodies appear and levels and types produced (e.g. IgM and IgG) can be dependent on whether the infection is primary or secondary. For serology, it is preferable to compare levels of antibody species between two serum samples tested in parallel, each collected in the respective acute and convalescent phases of illness, approximately 10–14 days apart.

In humans, the clinical syndromes that arboviral infections generally manifest, together with their representative viruses fall into five categories: (1) generalized febrile illness (e.g., chikungunya, O'nyong-nyong, Ross River, dengue (DENV)); (2) fever with rash and arthritis (eg chikungunya, Ross River, O'nyong-nyong) or myalgia (eg DENV); (3) encephalitis (eg JEV, WNV, St. Louis virus (SLV), tick-borne encephalitis (TBEV), equine encephalitis viruses; (4) hemorrhagic fever (e.g., YFV, DENV, Rift Valley fever (RVF), and Crimean-Congo hemorrhagic fever); and (5) congenital microcephaly or Guillain–Barré syndrome (ZIKV). Within a family of viruses, there are generally shared viral factors and thus an overlap of clinical presentations. Understanding the clinical presentations of a family of viruses will aid the clinician in determining the clinical course and etiology of the infection.

Alphaviruses

In the International Committee on Taxonomy of Viruses (ICTV) 2015 release, the genus *Alphavirus* (family *Togaviridae*) consists of 31 virus species. All are transmitted by arthropods and, in general, geographically restricted individually; however, as a genus, they are widely distributed globally. Traditionally, alphaviruses that produce human illness are broadly classified into those that produce rash and polyarthritis and are primarily found in the Old World, and those that produce encephalitis, which are primarily found in the New World.

The alphaviruses associated with polyarthritis and rash include chikungunya virus (CHIKV), O'nyong-nyong virus (ONNV), Igbo Ora virus, Ross River virus (RRV), Sindbis virus (SINV), Barmah Forest virus (BFV), and Mayaro virus. Though geographically diverse, all present with a similar clinical picture. Following the bite of an infected mosquito and a 3–12 day incubation period, a high fever develops with headache, nausea, vomiting, and severe joint pain. A diffuse maculopapular rash develops 4–8 days after the initial illness. Joint pain in the form of arthralgia, can continue for months after the acute illness without evidence of joint damage or arthritis. CHIKV is representative of this family and is widely distributed throughout India, Southeast Asia, and sub-Saharan Africa. It is transmitted to humans by the bite of infected *Aedes* and *Culex* mosquitoes, and primarily by *Aedes aegypti* and *Aedes albopictus*. Mother-to-child CHIKV transmission resulting in severe neonatal encephalopathy has also been documented. First isolated in 1952–53 during an epidemic in Tanzania, CHIKV has emerged from sylvan transmission cycles between nonhuman primates and arboreal mosquito vectors in sub-Saharan Africa to cause human outbreaks of endemic and epidemic polyarthritis throughout Africa and Southeast Asia. To date, the largest known outbreak of CHIKV began in March 2005 in the Indian Ocean islands of Mayotte, Mauritius, Réunion, and the Seychelles. The outbreak strain, belonging to the central/east African genotype, was shown to have mutated during the course of the outbreak, which is believed to have greatly influenced its explosive transmission via *Ae. albopictus*. The mutated CHIKV strain further spread to Sri Lanka and then to neighboring India. By October 2006, there were reports of chikungunya in 151 districts in 8 states/provinces of India, the affected states being Andhra Pradesh, Andaman & Nicobar Islands, Tamil Nadu, Karnataka, Maharashtra, Gujarat, Madhya Pradesh, and Kerala. It is estimated that more than 1.25 million suspected cases have been reported in the country, 752,000 from Karnataka and 260,000 from Maharashtra provinces with some areas reporting an attack rate of 45%. Following an incursion of the Indian Ocean CHIKV strain via a viremic traveler, there was an outbreak in Italy in late 2007, resulting in 205 cases. This represents the first reported cases of CHIKV in Europe, and underscores the potential of this arbovirus to spread to different regions of the world. Whilst this same CHIKV strain continues to cause outbreaks in the Pacific, recent emergence of CHIKV, firstly in the Caribbean in late 2013 and later in the Americas, has surprisingly involved an Asian lineage of CHIKV and *Ae. aegypti* mosquitoes.

O'nyong-nyong is antigenically related to CHIKV and found in East Africa. Outbreaks of ONNV have been reported throughout eastern Africa in Uganda, Kenya, Tanzania, Zaire, Malawi, and Zambia. First isolated in Queensland in 1959, RRV is the most common cause of epidemic polyarthritis in Australia, the disease being first recognized clinically in New South Wales in 1928. RRV is endemic throughout Australia and several Pacific island nations and has caused epidemics in both Australia and Fiji. BFV, like RRV, is another important cause of polyarthritis in Australia. First isolated in the Barmah Forest region in the Murray River Valley of southeastern Australia, it is now recognized as an important cause of fever and arthritis in both eastern and western regions of Australia. SINV was first isolated from Sindbis, Egypt, and is now recognized as one of the most widely distributed alphaviruses causing rash and polyarthritis from Northern Europe to South Africa. In Northern Europe, it is known as Ockelbo in Sweden, Pogosta fever in Finland, and Karelian fever in Russia. Like CHIKV, all the alphaviruses are spread by mosquitoes specific to their geographic region. All produce fever, rash, and acute polyarthritis that can continue for months after the initial infection. A glomerulonephritis has been reported as a potential complication of infection but in general the mortality rate is very low with few deaths reported. CHIKV has been associated with hemorrhagic disease especially in Southeast Asia where it was reported in the 1960s as a cause of hemorrhagic fever in children hospitalized for a presumptive dengue hemorrhagic fever (DHF).

Unlike the alphaviruses that produce arthritis, the alphaviruses associated with human encephalitis occur as a result of spillover from an epizootic episode. Equine and human cases occur through a bridging mosquito vector, and mortality is high in cases of encephalitis in both horses and humans. These alphaviruses include eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), and western equine encephalitis virus (WEEV). Eastern equine encephalitis virus is maintained in an enzootic cycle between ornithophilic mosquitoes, primarily *Culiseta melanura* in North America and passerine birds. Horse and humans are dead-end hosts producing severe encephalitis with 30–40% mortality. In the United States, EEEV has been prevalent in coastal states along the Gulf of Mexico and the Atlantic seaboard, as well as in inland foci in regions near the Great Lakes, including Upstate New York. By contrast, WEEV is distributed in the western plains of the United States and Canada and parts of South America. Enzootic transmission of WEEV is maintained between domestic and passerine birds and its mosquito vector, primarily *Culex tarsalis*. In contrast to EEEV, encephalitis caused by WEEV is associated with a lower mortality of approximately 10% for humans and 30% for horses. VEEV was first isolated in 1934 in Venezuela and found to be the primary cause of equine and human encephalitis in South and Central America, the Caribbean Islands, and the southern parts of the United States including Florida and Texas.

Clinically, EEEV, WEEV, and VEEV all produce similar illnesses with fever, headache, and myalgias as the first presenting symptoms. Encephalitis is associated with severe symptoms of headache and myalgia, along with signs of central nervous system infection including restlessness, neck stiffness, seizures, obtundation, and coma. Focal neurologic defects can develop as cranial nerve deficits and hemiparesis. Laboratory findings can be abnormal with a hyponatremia secondary to a syndrome of inappropriate antidiuretic hormone (SIADH) secretion and cerebrospinal fluid (CSF) consistent with viral encephalitis (high protein, low glucose, high WBC, primarily lymphocytes). Radiographic findings with computed tomography (CT) scan or magnetic resonance imaging (MRI) may demonstrate edema and/or hemorrhage in the thalamus, basal ganglia, and brainstem. Death can occur 2–10 days after the onset of encephalitis. Neurologic sequelae postinfection with seizures or hemiparesis are common.

The different disease manifestations in humans affected by alphavirus infections are believed to be mainly associated with the target tissue where the virus replicates. Thus, alphaviruses that cause rash replicate in the skin, those that cause arthritis replicate in the joints, and encephalitis is associated with virus replication in the nervous system. However, the specific cells that support alphavirus replication in joints are not well characterized; the mechanisms by which alphaviruses that cause encephalitis enter the central nervous system are also not well characterized. Within the central virus system, these viruses replicate in neurons, microglial and oligodendroglial cells. Disease is accompanied by infiltration in the infected tissue of inflammatory cells. These inflammatory cells, while having a role in clearing virus infections, may also contribute to tissue damage and fatal disease.

The severity of disease induced by alphavirus infections is influenced by both host and viral factors. Strains with high susceptibility to type I interferon, one of the first lines of innate immune defense, are in general less virulent. Different viral products from this group of viruses have recently been described to attenuate the type I interferon response, and therefore, are likely to play a role in severity of disease. These include the nsp2 protein of Old World alphaviruses, such as SINV, and the capsid protein of New World alphaviruses, such as EEEV. These viral products inhibit expression of cellular genes, including the type I interferon. Another major virulence determinant of alphaviruses that cause encephalitis is their ability to invade the central nervous system, which depends on multiple poorly understood virus and host factors.

Bunyaviridae

The family *Bunyaviridae* is one of the most diverse arboviral families and contains three genera of human-infecting arboviruses: *Orthobunyavirus* (48 distinct species), *Nairovirus* (7 distinct species), and *Phlebovirus* (10 distinct species). *Hantavirus*, an important genus of human disease, is rodent-borne.

Orthobunyaviruses

The genus *Orthobunyavirus* consists of different virus serogroups. The Bunyamwera serogroup includes the representative virus Bunyamwera, and the Cache Valley, Fort Sherman, Germiston, Ilesha, Kairi, and Shokwe viruses. The Bunyamwera viruses are distributed in sub-Saharan Africa, Uganda, Nigeria, and South Africa and produce acute febrile illnesses in humans. Cache Valley virus is the exception to the group and is distributed in the United States, Canada, and Mexico. It produces embryonic and fetal death, stillbirths, and congenital malformations in sheep and other ruminants and a generalized febrile illness with reported cases of encephalitis in humans. The Simbu serogroup viruses are globally distributed and include Akabane, Ingwavuma, and Oropouche viruses. Oropouche virus is the only one of the Simbu serogroup to produce illness in humans and manifests as a generalized febrile illness. Oropouche is found in South America and is responsible for large epidemics of febrile illnesses. It is spread primarily by the biting midge, *Culicoides paraensis*. After a 4–8 day incubation period, fever develops accompanied by joint and muscle pain, severe headache, chills, and photophobia. Rash and meningitis have been described.

The California serogroup viruses all produce encephalitis in humans and are spread by mosquitoes. This group includes California encephalitis, Jamestown Canyon, La Crosse, and Snowshoe hare found in North America; Guaroa in South and North America; and Inkoo and Tahyna in Europe. La Crosse virus is found throughout the northern Midwest and in northeastern United States primarily in the Mississippi and Ohio River basins, with the majority of cases in Wisconsin, Minnesota, Iowa, Indiana, Ohio, and Illinois. Cases from 28 US states have been reported so far. Jamestown Canyon is also widely distributed throughout North

America. All the California serogroup viruses produce encephalitis though disease severity is age-dependent, with La Crosse virus producing more severe disease in children and Jamestown Canyon being more severe in adults. The incubation period is 3–7 days followed by fever, stiff neck, headache, lethargy, nausea, and vomiting. Seizures are a common manifestation and up to 30% can develop a coma. Epilepsy can develop in 10–15% of children, with permanent neurologic disorders in 2%. The California serogroup viruses can be confused with bacterial meningitis since meningitis is a common clinical presentation of infection in the case of these viruses, with both mononuclear and polymorphonuclear cells being found in the CSF. As with the New World alphaviruses, encephalitis is associated with viral invasion of the central nervous system, and with viral replication in neurons and glial cells. La Crosse virus is maintained in nature in a cycle that involves chipmunks and hares as vertebrate hosts and the mosquito vector *Aedes triseriatus*.

Globally, there are a number of other mosquito-transmitted orthobunyavirus serogroups associated with human disease. These are the Anopheles A (Americas), Bwamba (Africa), Group C (Americas), Guama (Americas), and Nyando (Africa) groups. Although not much is known about the determinants of virulence of orthobunyaviruses, the nonstructural protein NSs encoded by these viruses appears to play an important role in virulence by inhibiting host antiviral responses. This is achieved through its ability to inhibit cellular mRNA transcription, preventing host gene expression and dampening the induction of antiviral host genes, such as type I interferon.

Nairoviruses

The *Nairovirus* genus contains two important human pathogens: the Crimean-Congo hemorrhagic fever group, which includes Crimean-Congo hemorrhagic fever virus (CCHFV) and Hazara virus, and the Nairobi sheep disease group, which includes Nairobi sheep disease virus (NSDV) and Dugbe virus. Both CCHF and NSD group viruses are transmitted primarily by ticks though the virus has been isolated from culicoides flies and mosquitoes. CCHFV is widely distributed throughout Central Asia and Africa and NSDV in parts of Africa. Both are maintained in the environment by domestic animals as sheep, goat, and cattle, with humans becoming infected either through the bite of an infected tick or by inoculation from the slaughtering of infected animals. CCHF is transmitted to humans by the bite of infected *Hyalomma* species ticks. Domestic animals serve in the transmission cycle as the viral amplifying host or reservoir. Ticks maintain the virus in the environment and can remain infected for long periods and infect their progeny by transovarial transmission. Risk for human infection primarily involves behavior that increases the potential exposure of bites from infected ticks, handling of infected carcasses, and nosocomial transmission. In a study conducted in northern Senegal, antibody prevalence to CCHFV was similar among the sexes, increased with age, and was related to herding activities, sleeping outdoors, bite by a tick, or contact with sick animals. Abattoir workers are at particular risk. An investigation of an outbreak largely associated with workers in Saudi Arabia and the United Arab Emirates demonstrated both clinical disease and high antibody seroprevalence rates; Pakistan has experienced 13 outbreaks of CCHF since 1976 that were associated with significant nosocomial transmission among health care professionals due to contact with infected body fluids and blood. CCHF in humans is associated with hemorrhagic fever with a 3–7 day incubation period, and the time between the onset of fever to hemorrhagic manifestations is brief. The time from the onset of fever, chills, headache, and muscle pains to the time of severe hemorrhage can be 3 days, with death occurring on the sixth day of illness. Other signs and symptoms associated with CCHF are vomiting, diarrhea, and throat pain. Mortality from CCHF can be as high as 30%, with human to human transmission through contact with infected body fluids responsible for nosocomial outbreaks.

The high mortality rate associated with CCHFV infections and the lack of vaccines and antivirals against this pathogen restrict the research with this virus to laboratories that have high biocontaiment facilities (BSL4). In addition, there is no animal model of the disease caused in humans by CCHFV. Thus, very little is known on the molecular determinants of pathogenesis of CCHFV. Recently, a protease domain in the N-terminal region of the viral-encoded RNA polymerase has been characterized and shown to deconjugate ubiquitin and ubiquitin-like molecules from target proteins. Since ubiquitin and ubiquitin-like molecules are involved in many diverse cellular processes, including host defense, it is likely that this viral protease domain disarms host antiviral responses and contributes to virulence. In contrast to CCHFV, which usually causes inapparent infections in vertebrate hosts different from humans, NSDV causes disease in sheep and goats that is associated with virus replication in endothelial cells and necrosis of capillary walls. NSDV has been isolated from febrile patients in Uganda, but its impact on human health is unclear.

Phleboviruses

The genus *Phlebovirus* contains the sandfly fever group, which includes the viruses Candiru, Punta Toro, RVF, sandfly fever Naples, Toscana, sandfly fever Sicilian, and Chagres. Also, there are a number of tick-transmitted human pathogens in the Bhanja group including severe fever with thrombocytopenia syndrome virus (China), Heartland virus (United States of America) and Bhanja virus (Eurasia and Africa) that are associated with acute febrile illness. Most other phleboviruses are transmitted by the phlebotomine sandfly, with the exception of RVFV which is transmitted by *Aedes* species mosquitoes. They are widely distributed throughout the world including the Middle East, sub-Saharan Africa, and Africa.

RVFV is a human pathogen, also of economic importance, that has emerged from Africa to produce epidemics throughout sub-Saharan Africa and the Middle East. RVF is an acute zoonotic disease that infects both ruminant animals and humans. Human infections occur as an epizootic, with transmission occurring primarily from infected mosquitoes (*Culex*, *Aedes*, and *Anopheles* species) and secondarily from the handling of infected animal carcasses. RVFV was originally isolated in 1930 in the Rift Valley of

Kenya, East Africa, and was responsible for over 30 large outbreaks of animal and human disease in East Africa since the 1930s. Age-specific mortality for RVF is greatest for the elderly, 57%≥90 years, 22% in ages 80–89 years, 19% in 70–79 years, with the overall mortality being 14%. RVF produces considerable economic loss among domestic animals and also causes human disease. In a study of 124 patients, the major clinical characteristics for RVF included hepatocellular failure (75%), acute renal failure (41%), and hemorrhagic manifestations (19%). Development of retinitis, unique to RVF, was seen in 16 patients, and 7 experienced meningoencephalitis as a late complication of the disease. A total of 56 patients (34%) died. The development of hepatorenal failure, shock, and severe anemia were all factors associated with patient death. Sicilian, Naples, and Toscana viruses are spread by infected sandflies and are found throughout the Mediterranean basin, the Middle East, and the Arabian Peninsula. Following an incubation period from 2 to 6 days, there is fever and malaise accompanied by headache, photophobia, back and joint pain. Most cases are self-limited though acute lymphocytic meningitis and meningoencephalitis occur in more severe cases.

Like the *Orthobunyaviruses*, but in contrast to *Nairoviruses*, RVFV is known to encode a nonstructural NSs protein. Despite the lack of amino acid identity between the NSs proteins of Bunyamwera and of RVFV, the NSs of RVFV also inhibit cellular gene expression of antiviral genes, including type I interferon. The attenuated clone13 strain of RVFV has a defect on its NSs gene that is associated with viral attenuation, underscoring the importance of the NSs protein of RVFV in its virulence.

Flaviviruses

The family *Flaviviridae*, genus *Flavivirus*, contains a number of important human arboviruses that have both historical and ongoing significance and which have produced large epidemics of disease ranging from severe febrile illnesses to encephalitis and hemorrhagic fever. The *Flavivirus* genus is organized into antigenic groups and includes Kyasanur forest disease virus (KFDV); Omsk hemorrhagic fever virus (OHFV); Powassan virus (POWV); TBEV (European, Far Eastern, Siberian, TBE); Aroa (Aroa, Bussuquara, Iguape, Naranjal); (DENV 1–4); Rio Bravo (Rio Bravo virus); JEV, Murray Valley encephalitis virus (MVE), St. Louis encephalitis virus (SLEV); West Nile (Kunjin virus, WNV); Kokobera (Kokobera virus); and YF (Banzi virus, YFV). All are either tick-borne (KFDV, OHFV, POWV, TBEV) or mosquito-borne (DENV, JEV, MVEV, SLEV, WNV, YFV, and ZIKV). Clinically, the flaviviruses produce a range of symptoms from acute febrile illness to hemorrhagic fever to encephalitis. Most are associated with high morbidity and mortality and are geographically widely distributed. DENV, JEV and WNV have emerged as important human pathogens that have spread geographically in the last 50 years.

Dengue Virus

Dengue fever is the most prevalent mosquito-borne viral disease affecting subtropical and tropical regions of the world. Much of its global spread is attributed to the resurgence of its primary mosquito vector, *Ae. aegypti*. Acute DENV infection can be asymptomatic or manifest in a spectrum of illnesses ranging from a subclinical mild febrile illness, to a more severe non-hemorrhagic illness termed dengue fever (DF), to DHF and dengue shock syndrome (DSS). DENVs have been antigenically classified into one of four individual serotypes (DENV 1–4). Depending on the immune status and other demographic factors of the existing human population and the serotype responsible for the outbreak, the incidence of asymptomatic dengue infections can range from 1.4% to 4.3% per year or approximately 1-2 times the incidence of symptomatic dengue infection. During the initial febrile illness, patients with both DF and DHF present with fever, headache, myalgia, arthralgias, fatigue, and anorexia. Saddle-back fever is typically present and heralds defervescence, onset of rash, and convalescent period in the DF patient. Convalescence can be prolonged and associated with postinfection depression and lethargy. The presenting symptoms of both DF and DHF are similar to other viral hemorrhagic fevers and are often protean in nature. These syndromes are also often difficult to distinguish from each other and other causes of non-dengue febrile illnesses. Common laboratory findings during the initial phase of illnesses for both DF and DHF include leukopenia, mild anemia, mild thrombocytopenia, and elevations in liver function tests. DHF/DSS is the most severe form of acute DENV infection and clinically distinguished from DF by the development of plasma leakage and hemorrhage. The World Health Organization case definition of DHF (requiring all of the following) is as given: fever or a history of acute fever lasting from 2 to 7 days; hemorrhagic tendencies as manifested by a positive tourniquet test or petechiae, ecchymoses, purpura or external bleeding or hematemesis or melena; thrombocytopenia (100,000 cells per mm^3 or less); evidence of plasma leakage by a rise in the hematocrit equal to or greater than 20% above average for age, sex, and population or a drop in the hematocrit following volume replacement equal to or greater than 20% of baseline or signs of plasma leakage as demonstrated by pleural effusion, ascites, or hypoproteinemia. Children with DHF prior to the onset of plasma leakage are more likely to manifest higher liver enzyme abnormalities than children with DF and a higher erythrocyte sedimentation rate. The acute febrile phase lasts between 2 and 7 days with hemorrhagic manifestations limited to petechiae hemorrhage or easy bruising. The liver may be enlarged; the spleen is frequently normal and generalized lymphadenopathy may be present. Defervescence is the hallmark sign for impending plasma leakage and the patient is frequently restless with cold extremities. Thrombocytopenia, coagulopathy, and hemorrhage occur during this time period and the onset of shock is acute. Plasma leakage manifests as a pleural effusion, ascites, or swollen extremities and is indicated by an intravascular hemoconcentration and an increase in the hematocrit of greater than or equal to 20% from baseline levels. The World Health Organization criteria define DHF in increasing severity from grades I to IV, with grade I as fever with constitutional symptoms and hemorrhagic manifestation, a positive tourniquet test or easy bruising, to grade IV, with profound shock with undetectable blood pressure or

pulse. Unusual manifestations of DENV infection can occur and include severe hepatitis with jaundice, metabolic acidosis, and disseminated intravascular coagulation producing a hepatic encephalopathy and a Reyes-like syndrome. Encephalitis and a neurological disorder similar to poliovirus infection can also occur during acute DENV infection.

The development of DHF/DSS, the most severe disease manifestation of DENV infection, is well known to be associated with secondary DENV infections. The four different DENV serotypes circulating in nature can inherently complicate the development of disease and clinical outcomes for the susceptible human host. Whilst infection with one serotype confers neutralizing host immunity that protects against reinfection with the same serotype, previous infection with a given serotype can increase the risk of severe disease should a subsequent infection occur with a different DENV serotype. The lack of an animal model of DHF has hampered our understanding of the molecular and immunological mechanisms responsible for this severe clinical manifestation of the disease. Cross-reactive antibodies elicited against the first-infecting DENV serotype are suspected to play a major role in DHF, as they cannot efficiently neutralize a different DENV serotype, but can enhance infectivity by facilitating viral entry in target cells through the Fc receptor. However, DHF is a very complex disease that is influenced by many factors, including previous exposure to a different DENV serotype, viral factors, and host factors, most of which are still poorly understood. The onset of severe clinical manifestations coincides at a time when viral replication and viremia are subsiding, and therefore plasma leakage is probably not caused by direct infection of endothelial cells by DENV. Higher activation of DENV-specific immune cells has been associated with DHF, and the presence of memory cross-reactive T cells elicited by previous infections with a different serotype might have a role in facilitating DHF. Thus increased levels of T cell activation in secondary DENV infections can result in elevated levels of cytokines, chemokines and other host factors that could lead to plasma leakage, platelet dysfunction and DHF. High levels of circulating tumor necrosis factor, soluble tumor necrosis factor receptors, soluble CD8, soluble interleukin-2 receptors, interleukin-10, macrophage migration inhibition factor, interferon-alpha, interferon-gamma, interleukin-13, interleukin-18, CCL2, and complement components 3a and 5a, among other factors, have been correlated with severity of dengue disease. However, the specific contributing role of cross-reactive antibodies and T cells in facilitating DHF is unclear. In addition, several DENV genotypes have also been associated with increased virulence, but the molecular basis for these observations is not completely elucidated. The predisposition that preexisting immunity against one DENV serotype increases the risk of severe infection with a different serotype is one of the major challenges faced by DENV vaccines. Alternative vaccination approaches are being considered, but essentially, in order for any DENV vaccine candidate to be considered, it will need to provide protective immunity against all four DENV serotypes and not a partial immune response that could predispose toward more severe infection with any of these serotypes. Following several clinical trials, a chimeric, live-attenuated vaccine to all four serotypes has been licensed in a number of endemic countries. Its efficacy in broad-scale field application is yet to be determined.

YFV and Other Hemorrhagic Flaviviruses

YFV is the prototypic virus of the *Flavivirus* genus and, like dengue, produces a spectrum of clinical illnesses from febrile illness to severe hemorrhagic fever. It is also spread by *Ae. aegypti* mosquitoes but, unlike dengue, has been geographically restricted to parts of South America and Africa. Following an incubation period of 3–6 days, illness begins with fever, chills, headache, back and muscle pain, and loss of appetite, nausea and vomiting. Fever can then remit with the patient's symptoms improving within 24 h followed by a reoccurrence of fever, vomiting, epigastric pain, and jaundice. Hemorrhagic illness can then occur with bleeding as hematemesis, melena, petechiae, ecchymoses, and bleeding from the gums. Dehydration and renal failure are potential complications, with death resulting in 20–50% of cases of severe illness. Experimental YFV infection in macaques has given insights on the pathogenesis of this virus. The virus first replicates in lymph nodes, and then spreads to the liver, spleen, bone marrow, and muscle. Infection of the liver and the spleen results in hepatocelullar damage and lymphocytic necrosis, respectively. However, the bases of the hemorrhagic manifestations and of renal dysfunction are less understood, and these clinical manifestations are attributed to a generalized circulatory collapse and altered platelet function. YFV infections can be prevented by vaccination, but the disease is still highly prevalent in some areas of the world due to the lack of widespread vaccination.

Other flaviviruses that produce hemorrhagic illness include the KFD virus, which is tick-borne transmitted and geographically restricted to Karnataka state, India. Clinical illness in humans is characterized by fever, headache, muscle pain, cough, gastrointestinal symptoms, and hemorrhage. A biphasic course may develop with fever and hemorrhage lasting 6–11 days followed by an afebrile period of 9–21 days. Fever reappears and a meningoencephalitis may develop. OHFV is also tick-borne and geographically restricted to the Omsk and Novosibirsk areas of the former Soviet Union. Infection is as in KFD, resulting in fever, muscle pain, and hemorrhage. Hearing loss, hair loss, and neuropsychiatric complaints are common.

WNV, JEV, and Other Encephalitic Flaviviruses

Encephalitis is a feature of the TBEV group (European, Far Eastern, Siberian, TBE), JEV group (JEV, MVEV, SLEV), WNV group (Kunjin virus, WNV), and Rocio virus. All differ in their geographic distribution, arthropod vector (ticks for TBEV and mosquitoes for JEV and WNV), but are similar in having complex life cycles, with animal reservoirs including birds in the case of JEV and WNV. Humans are considered a dead-end host.

The clinical syndromes produced by these viruses are similar and range from an acute febrile illness with or without headache, encephalitis, and aseptic meningitis. In general, after a 6–16 day incubation period, clinical illness develops with fever, chills, anorexia, nausea, vomiting, dizziness, and drowsiness. Clinical progression to encephalitis can occur, manifesting with nuchal rigidity, photophobia, altered consciousness, and neurologic signs such as muscular rigidity, cranial palsies, tremors, involuntary

movements, focal or generalized paralysis, and seizures. Atypical presentations can occur with focal lower motor neuron deficits leading to extremity paralysis similar to acute paralytic poliovirus infection. Mortality is high if disease progresses to encephalitis (5–40%), and occurs on the fifth to ninth day of illness. Poor prognostic signs include respiratory compromise, seizure frequency and duration, duration of fever, albuminuria, high levels of viral replication in the brain, and low IgM antibodies in the CSF. Laboratory findings associated with encephalitis are elevation in the CSF pressure, CSF pleocytosis, primarily consisting of monocytes or lymphocytes, low CSF glucose, and high protein. Brain imaging may demonstrate focal inflammation and/or hemorrhage in the thalamus and basal ganglia. Long-term sequelae following acute infection can occur in severe cases and include paralysis, seizure disorder, learning disabilities, mental retardation, and psychiatric disorders. Different viral strains are known to have different levels of virulence, with the best example corresponding to the virulent New York strain of WNV, as compared to the low virulent Kunjin virus strain of WNV. Multiple viral genes are likely to be responsible for differences in virulence. Host factors also play a major role in the outcome of the disease. The elderly are more likely to develop severe encephalitis after WNV infection. Deficiencies in the chemokine receptor CCR5 in humans, associated with resistance to HIV infection, have been correlated with increased incidence of severe WNV infections. During WNV encephalitis, neurons are infected, and immune-mediated damage contributes to the disease. Access to the central nervous system by the virus is an important determinant of disease, and factors that facilitate this process are likely to play a major role in pathogenesis. Interestingly, TLR3, a host factor that helps to trigger cytokine production in response to WNV infection, appears to contribute to disease by inducing tumor necrosis alpha production that in turn increases the permeability of the blood–brain barrier, facilitating viral entry in the brain.

Zika Virus

ZIKV is a flavivirus which is related to DENV, and was originally isolated in the Zika forests of Uganda, Africa, in 1947 from a sentinel monkey. The virus circulates globally in two main lineages: African and Asian. Whilst >80% of infections are believed to be asymptomatic, the disease mostly causes mild dengue fever-like symptoms. However, a recent incursion of an Asian strain of ZIKV into Brazil in 2015 which has led to ongoing transmission and major epidemics in the Americas, has been associated with Guillain–Barré syndrome and an increased risk of congenital microcephaly in babies whose mothers are infected during pregnancy.

Inhibition of Host Antiviral Responses by Flaviviruses

Among the host antiviral responses, type I interferon appears to contribute to control flavivirus infections, as better evidenced by the increased susceptibility to DENV infection of mice deficient in the type I interferon response. Interestingly flaviviruses, as with many other viruses, have also evolved specific mechanisms that contribute to attenuate the type I interferon response, and are likely to play a role in pathogenesis. For WNV and DENV, multiple nonstructural proteins have been shown to have an impact in decreasing type I interferon signaling, most likely allowing these viruses to replicate even in the presence of interferon. The NS5 protein of other flaviviruses, including JEV and TBEV, has been shown to prevent type I interferon signaling by preventing phosphorylation of transcription factors induced by interferon. Another host immune component that is known to be inhibited by flaviviruses, at least in the case of WNV, is the complement system. The nonstructural protein NS1 of WNV has been shown to inhibit complement activation by binding the regulatory protein factor H. This protein is known to be circulating at high levels in the sera of infected patients, and by inhibiting complement-mediated antiviral defenses, is likely to contribute to viral disease. However, many of the specific interactions between the host and the virus that modulate disease outcome are still unknown, and further investigation in this area is needed to uncover the molecular basis of the pathogenesis of arboviruses and to provide with new rational strategies to treat and prevent severe infections caused by this diverse group of viruses.

Orthomyxoviridae

In the family *Orthomyxoviridae*, only the genus *Thogotovirus* is associated with arthropod transmission. Two tick-transmitted virus species are currently recognized: the type species *Thogoto virus* and *Dhori virus*. These viruses are geographically widespread, infect a variety of animal species, and can cause disease in humans.

Reoviridae

This is the only known family of double-stranded RNA virus that is transmitted by arthropods. Of the 15 recognized genera, three (*Orbivirus*, *Coltivirus*, and *Seadornavirus*) are able to cause infection of humans and animals by arthropod transmission. Colorado tick fever virus is a tick-transmitted coltivirus which causes disease in humans. It is found in the western United States of America and Canada.

Further Reading

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Archaea – An Introduction

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Archaea: Definition and General Characteristics

Archaea are prokaryotic microorganisms that possess molecular characteristics distinctive from both bacteria and eukaryotes. They were described as a separate domain of life by C.R. Woese and coworkers in 1977 following a comparison of small ribosomal RNAs (rRNA).

Archaea are peculiar in various aspects. While their molecular machineries (replication, transcription and translation) resemble the homologous eukaryotic systems, they possess unique membrane lipids, which are characterized by an isoprenoid hydrocarbon side chain linked via an ether-bond to the *sn*-glycerol-1-phosphate backbone. In contrast to bacteria, their cell walls do not contain peptidoglycan, although some members have pseudomurein. Like bacteria, archaea do not possess organelles or a nucleus, and their ribosomes are 70S-type ribosomes. The DNA of many archaeal representatives is organized with histones similar to eukaryotes. Moreover, while archaea cannot form spores to escape temporarily harmful situations or endure over time, they have been described to be able to grow extremely slowly, or to remain dormant, which allows them to survive for thousands of years under certain conditions.

Similar to bacteria, archaea are metabolically diverse and include both auto- and heterotrophs as well as organo- and lithotrophs. For instance, archaea include methanogens, which produce methane as a major end product of their metabolic activity, a trait which has been described only rarely for fungi or bacteria. On the other hand, currently known members of the Archaea do not include *bona fide* photosynthetic organisms, although some halophilic archaea are able to exploit sunlight for energy-yielding processes.

Originally, archaea have been thought to comprise organisms thriving predominantly in extreme environments (e.g. high-salt, low or high pH, and high temperature conditions), from which numerous archaeal isolates have been obtained. Recent progress in cultivation-independent sequencing technologies has however revealed that archaea are much more widespread and of greater ecological importance than assumed previously. Yet, most of these recently described archaea, which inhabit moderate environments, such as marine or freshwater systems, sediments and soils, or occur in association with other organisms, have so far resisted cultivation.

Until today, many archaea are considered to be specialists in energy exploitation, which are well adapted to nutrient-limiting ecological niches. For example, some members of the Archaea, such as the anaerobic methane oxidizing euryarchaeota, are able to exploit metabolic strategies that operate close to the thermodynamic limit of life. The unique metabolic characteristics of some archaeal groups such as the methanogens place them at the end of complex food chains, where they play a regulatory role in numerous ecological settings.

The Recognition of Archaea as a Separate Domain of Life

Archaea have been known to microbiologists for more than 150 years. The first investigated genera included strictly anaerobic, methanogenic species (methanogens), halophiles that are able to tolerate very high salt concentrations, and hyperthermophilic archaea with optimal growth temperatures above 80°C. It was A. Béchamp who first described the role of microbes in methanogenesis in 1868, a process that is (almost) exclusively associated with archaea today. However, due to their prokaryotic nature, archaea were initially regarded as bacteria and were not recognized as a separate domain of life until the end of the 20th century. The recognition of Archaea is closely linked to the development of molecular classification systems, developed by Carl R. Woese and his co-workers.

Until the 1970s, concepts of taxonomic assignment relied on morphological and metabolic traits, such as differences in the basic morphology and structure of cells. For example, the separation of eukaryotes and prokaryotes was based on the presence and absence of membrane-bound nuclei, respectively. In fact, it was common practice to describe all representatives of 'small germs' lacking a nucleus as 'bacteria', a scientific term originally introduced by C. G. Ehrenberg in 1838. From this standpoint, it comes as no surprise that the first isolates of archaea were also regarded as bacteria, considering their morphological similarities. Even today this bias is apparent in the nomenclature of archaeal taxa. Since the 1930s, pure cultures of methanogenic archaea were acquired by the microbiologists H. A. Barker, K. Schnellen, and T. C. Stadtman, terming the first methanogens *Methanobacillus omelianskii*, *Methanobacterium formicicum*, *Methanosarcina barkerii*, and *Methanococcus vannielii*. With *Bacillus halobius ruber* and *Bacterium*

halobium, the nomenclature of subsequently isolated haloarchaea also continued to include the stem 'bacterium' in their description of order, family, and certain genera.

The pioneering work of Carl Woese and George Fox in 1977, however, changed the entire view on the diversity of life. For the first time, Archaea were recognized as a major and separate domain of life besides Bacteria and Eukarya. Woese and his colleagues were working on methods to infer phylogenetic relationships by using molecular markers instead of morphological traits or other characteristics that are either subjective or too simplistic for a reliable taxonomic assignment. This was not a novel approach, as biochemists were aiming to infer phylogenetic relationships by analyzing amino acid sequences of proteins such as cytochrome C (Fitch and Margoliash) or blood-clotting fibrinopeptides (Doolittle and Feng). However, the primary problem with the usage of specific amino acid sequences as phylogenetic marker was their lack of universality. Woese and his colleagues on the other hand, focused on a biopolymer that turned out to be highly conserved and a universal and defining feature of cellular life: the ribosomal RNA (rRNA). All cellular life encodes rRNA components (ribozymes), which form, together with ribosomal proteins, the large (LSU) and small subunit (SSU) of the ribosome. The rRNA represents the catalytic center of the protein synthesis machinery, thus carrying out a core process of cellular life. Importantly, the ribosomal RNA and protein components are thought to evolve slowly (as compared to proteins involved in other cellular functions including metabolism) and to be mainly inherited vertically, i.e., from parent to daughter. In particular, the SSU rRNA (also called 16S [prokaryotes] or 18S rRNA [eukaryotes]) turned out to be a suitable phylogenetic marker and was described as "the ultimate molecular chronometer": The nucleotide sequence of the SSU rRNA can be compared and used to infer the evolutionary relatedness of the respective organisms.

Based on the finding that the 16S rRNA of methanogenic organisms not only differed fundamentally from the 18S rRNA of eukaryotes but also from to 16S rRNA of bacteria, Woese and Fox proposed the existance of a third domain of life. This domain was originally termed "Archaebacteria" (Greek *arkhaîos*; ancient, antique, primitive), in reference to the suspected antiquity of the methanogenic phenotype. Later, Woese and his colleagues revised the nomenclature to Archaea, as the former terms "Archaebacteria" and "Eubacteria" (now Bacteria) implied a close but not justified relatedness. They proposed a tripartite tree of life consisting of Archaea, Bacteria, and Eucarya, and thereby provided an important framework for the study of members of the Archaea and the diversity of life.

Archaeal Ecology – From Extremophiles to Symbionts

The predominant isolation of archaea from samples characterized by extreme environmental conditions led to the misconception that archaea mainly comprise extremophilic members. Indeed, representatives of the archaeal domain are still record holders with respect to the highest maximum growth temperature (e.g., *Pyrococcus fumarii* [113°C]) or the lowest pH for growth (e.g., *Picrophilus torridus* [pH 0]). It is assumed that in particular their specific cell wall/membrane architecture allows members of this group to survive and thrive under these extreme conditions. Until to date, the best-characterized archaeal representatives are halophiles, hyperthermophiles and methanogens.

All halophilic (salt-loving) archaea (haloarchaea) belong to the phylum Euryarchaeota and form the class of Halobacteria, which includes diverse lineages. Haloarchaea have been isolated from numerous environments, such as shore mud of the Dead Sea (e.g., *Haloferax volcanii*), alpine salt deposits (e.g. *Halococcus dombrowskii*, *Halobacterium noricense*), or salted foods (e.g., *Natrinema pallidum*). Haloarchaea include members with peculiar cell shapes, such as *Haloquadratum walsbyi*, a square-shaped, flat archaeon. Notably, this archaeon had been discovered more than 20 years before its successful cultivation in 2004. All haloarchaea require high salt concentrations for growth. Most species grow optimally above concentrations of 15% salt, and even lyse at concentrations below 10%. Moreover, they are resistant to a number of physical and chemical stressors, such as low water activity, desiccation and low nutrient availability. They depend on organic matter as a carbon source, and some use bacteriorhodopsin for a light-driven synthesis of ATP. It has been reported, that some haloarchaea have survived millions of years in salt-rich sediments, however, contamination by extant microorganisms could not be completely ruled out. Members of the *Natronomonas* genus are multi-extremotolerant/philic, as they withstand not only extremely high salt concentrations, but also high pH (e.g., in soda lakes in Africa).

Methanogenic archaea are a diverse and polyphyletic group of microbes with different (growth) characteristics that include psychrophiles and hyperthermophiles, many of which are widespread in anoxic environments (such as sediments and bogs, as well as the gastrointestinal tract of cattle, termites and many other holobionts). By definition, their metabolism results in the formation of methane gas, which represents a potent climate gas contributing to global warming. It has been predicted, that the melting of permafrost areas (25% of Earth's terrestrial surface) will further increase methane production by methanogens residing in these areas. Methanogenic archaea can use a variety of different carbon substrates, including carbon dioxide, methyl or other C1 compounds, but also acetate. Notably, many members of this group exist in syntrophy with bacteria, living off the products of the bacterial partners and supporting fermentative processes by lowering hydrogen partial pressures inhibiting for many other microorganisms.

Hyperthermophiles are defined as microorganisms with optimal growth temperatures above 80°C. The best characterized hyperthermophiles in the archaea belong to the phyla Euryarchaeota and Crenarchaeota. Many members of these groups have

been isolated from volcanic solfatara fields, hot springs or hydrothermal vent systems from Iceland, Italy, Japan, US, Russia and many other places. They either grow chemolithoauto- or chemolithoheterotrophically on a number of geologically important substances such as sulfur and nitrogen compounds.

The upper limit of growth is highly debated. *Pyrolobus fumarii*, isolated from a black smoker hydrothermal vent was described to actively divide at 113°C in 1997, ceasing growth at temperatures below 90°C. Other studies report metabolic activity at 121°C (Strain 121, *Geogemma barossii*) or even 122°C (*Methanopyrus kandleri*, under specific cultivation conditions). In general, growth at higher temperatures is accompanied by adaptations of various biomolecules, such as preferred use of a certain amino acid composition for the stabilization of proteins (for increased number of disulfide bonds, larger hydrophobic core), an increased proportion of tetraether lipids in the membranes and increased protection of DNA.

First indications about a more global distribution and role of archaea in non-extreme environments were obtained from 16S rRNA gene-based surveys that revealed a high archaeal abundance in cold sea water. In 2001, Karner et al. reported on the archaeal dominance in the mesopelagic zone of the Pacific Ocean, and thereby opened a series of discoveries indicating an archaeal omnipresence in all biotopes on Earth. The universal distribution of archaea was recently confirmed by results derived from the direct sequencing of DNA isolated from various microbial communities. Currently, 250,000 archaeal 16S rRNA gene sequences from a large diversity of environments are available in curated databases such as SILVA (2019). In contrast, only approximately 120 archaeal genera have been cultivated and validly described thus far (source: see "Relevant Website section"). The NCBI Genome database and the JGI Gold (genomes online database) currently host more than 1500 entries for complete archaeal genomes, with overwhelming predominance of Euryarchaeota (in particular Halobacteria, Methanomicrobia), Crenarchaeota (Thermoprotei) and Thaumarchaeota sequences. However, the majority of the recently sequenced archaeal genomes (often represented by metagenome bins or single amplified genomes) originates from uncultivated representatives. To put this into perspective: more than 100,000 bacterial genomes have been generated until today, the majority of which are derived from uncultivated organisms as well.

It is meanwhile well accepted that archaea represent a considerable fraction of Earth's ecosystems, and it is estimated that they comprise approx. 10%–30% of all microbial life. Notably, archaea seem to be particularly abundant in subsurface ecosystems, in which they have been predicted to be involved in carbon fixation or organic compound turnover. However, most of these taxa are uncultivated and only (partial) genomic information is available. Archaea also play an important role in soil, marine and freshwater systems. For instance, the phylum Thaumarchaeota comprises aerobic ammonia-oxidizing archaea, which have an important role in the global nitrogen cycle. The activity of members of this group in soil environments has direct impact on the available nitrogen content of agricultural ecosystems. Thaumarchaeota, however, have also been found in anoxic and acidic, thermophilic and iron/sulfur-rich sediments.

Furthermore, numerous archaea have been found to be involved in symbiotic interactions. For instance, they can be part of microbial syntrophic communities (such as the anaerobic methane oxidation consortium) and complex microbiomes of sponges, ruminants or plants. Notably, archaea have recently even been detected in the human microbiome, in which they may comprise up to 10% of the microbial community in the gastrointestinal tract and on skin. Due to methodological limitations, the archaeal fraction of the microbiome (the archaeome) remains poorly characterized, although the presence of methanogenic archaea in the human gut has been known for decades. Methanogenic archaea are important components of the human gastrointestinal tract, as they support bacterial fermentation processes by acting as a sink for hydrogen, CO_2, and methyl compounds. Curiously, pathogenic archaea have not been identified thus far, but involvement in pathogenic processes cannot be excluded.

Notably, archaeal parasites that depend on other archaea for growth are however known. For instance, the ultrasmall archaeon *Nanoarchaeum equitans* lives on the surface of its archaeal host *Ignicoccus hospitalis* and is not able to proliferate independently. *N. equitans* and related lineages assigned to the DPANN archaea (see below), have a limited metabolic gene repertoire and lack various genes that encode enzymes of diverse anabolic and catabolic pathways (e.g., carbon, amino acids, lipid, nucleoside, and/or vitamin metabolism). This suggests that various members of this group may depend on microbial or eukaryotic partners to acquire essential metabolites.

Due to their peculiar characteristics, selected halophilic and methanogenic archaea serve as microbial models for the exploration of the limits of life and the assessment of whether life could exist outside of Earth. For instance, brine streamlets were detected on the surface of Mars, fueling the debate on whether halophilic microorganisms could potentially thrive therein. Furthermore, the presence of methane in the Martian atmosphere has raised the question whether methanogenic archaea could represent a biogenic source of this gas. In these regards it is interesting to note, that several halophilic archaea have been shown to be able to survive exposure to space conditions, when shielded against direct radiation, and the ongoing ExoMars mission (orbiter 2016 and lander 2020) aims to track methane production and search for signs of (archaeal) life (Fig. 1).

Archaeal Taxonomy and Evolution

Recent progress in the use of cultivation-independent sequencing methods, such as metagenomics and single cell genomics, has revealed a large diversity of additional archaeal lineages that significantly expand the phylogenetic tree of archaea (Fig. 2). While initial trees only included two potential major archaeal divisions, namely the Euryarchaeota and Crenarchaeota, various additional

Fig. 1 Archaeal biotopes. Color intensity of the circles indicate high salt content (yellow), temperature (red), oxygen content (blue), moderate conditions (green) and symbiosis (purple). Biotopes (from top, clock-wise): Terrestrial volcanic areas (Grand Prismatic Spring, Yellowstone National Park); aquatic environment (freshwater streamlet); soil; mammalian anaerobic gastrointestinal tract; bog; hydrothermal vents in deep-sea marine trenches; and hypersaline aquatic environments (salt pans). Picture credits: Salt pans (figure cropped): Tony Hisgett from Birmingham, UK [CC BY 2.0], cow (figure cropped): Pikaluk [CC BY 2.0 (https://creativecommons.org/licenses/by/2.0)].

lineages of high taxonomic rank have been added recently. The domain Archaea is now thought to be comprised of the Euryarchaeota, the TACK superphylum or Proteoarchaeota as well as the DPANN and Asgard archaea.

The Euryarchaeota likely forms two major sub-divisions both of which comprise a diversity of archaeal lineages including the previously cultivated methanogens, hyperthermophiles and halophiles. The TACK archaea include in addition to the Thaumarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota, a variety of recently proposed phylum- or order-level lineages, such as for example the Bathyarchaeota, Geoarchaeota, Geothermarchaeota, Verstraetearchaeota and Marsarchaeota (Fig. 2). The Asgard archaea represent a group with currently four recognized sub-lineages referred to as the Loki-, Thor-, Odin- and Heimdallarchaeota (Fig. 2). Finally, the DPANN (originally an acronym for Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, and Nanohaloarchaeota) includes a large variety of sub-lineages many of which comprise organisms with small cell and genome sizes. The cellular and genomic features of members of these DPANN archaea, which include known ectoparasites such as *Nanoarchaeum equitans* (see previous paragraph), comprise putative host dependent organisms.

While the taxonomy and exact phylogenetic placement of these archaea as well as the root in the archaeal tree is still debated, the investigation of the genomes of these only recently described lineages has unveiled many interesting details about the metabolic potential of archaea as well as widened our perspective on their role in key evolutionary events. First of all, it was recently shown that the hallmark enzymes involved in methanogenesis and methane oxidation, previously thought to be exclusively found in few members of the Euryarchaeota, are encoded in the genomes of various additional archaeal lineages, including in representatives of the TACK archaea. This much broader distribution of methanogenesis marker proteins has reinvigorated discussions on the early evolution of archaea and raised the possibility that the last archaeal common ancestor (LACA) may have been an anaerobic chemolithoautotroph that used the Wood-Ljungdahl pathway for carbon fixation and methanogenesis for energy generation. Yet, the patchy distribution of the key enzymes of these pathways in archaea points towards a complex evolutionary history involving several independent gene losses as well as horizontal gene transfers. Furthermore, the evolutionary origins of the potentially deep-branching DPANN archaea, many of which have very restricted metabolic gene sets and do not encode enzymes involved in methanogenesis, remain to be investigated.

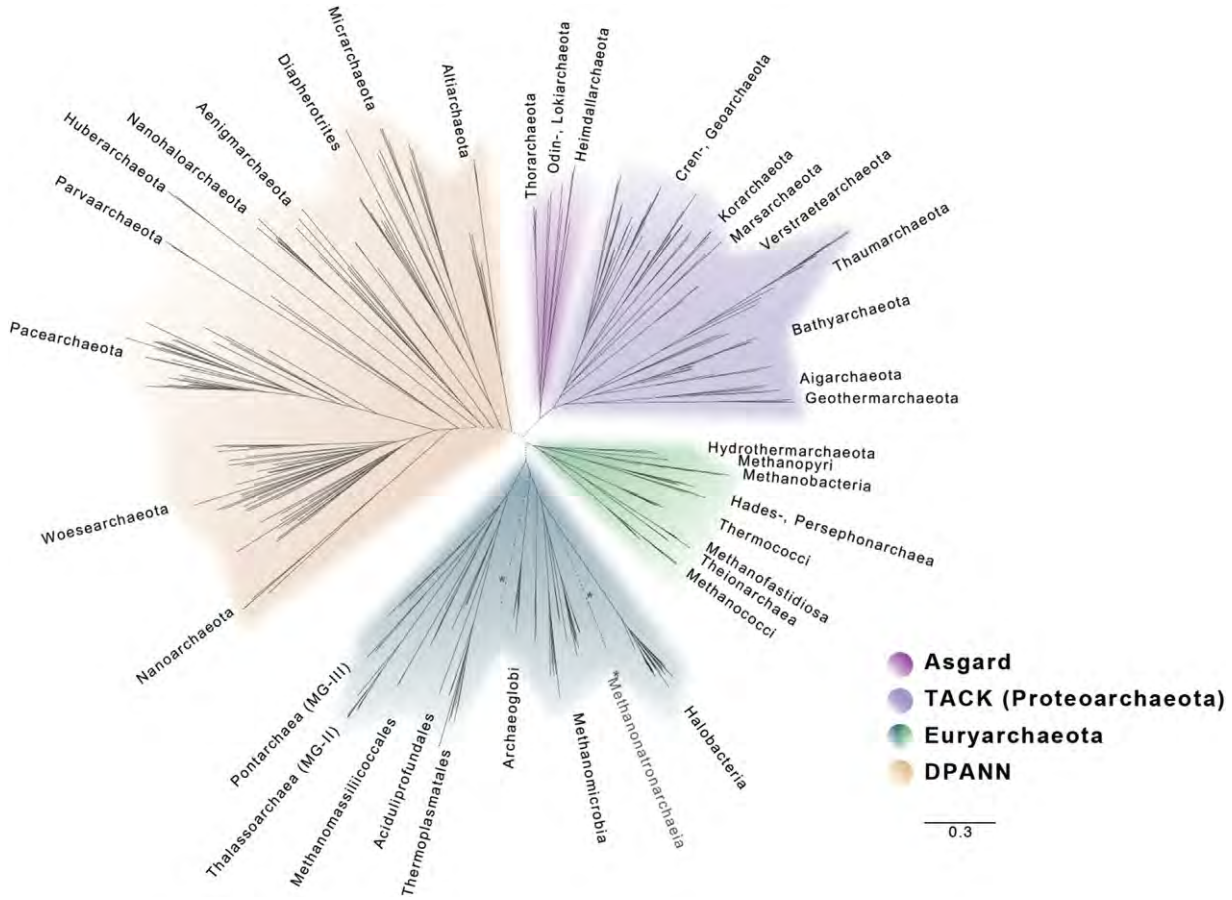

Fig. 2 Schematic tree representing major currently recognized lineages of the Archaea. The tree has been adapted and modified from a phylogenetic analysis generated using a maximum likelihood approach implemented in IQ-tree (model of evolution: LG + C60) with an amino acid alignment consisting of 34 conserved marker proteins. The relationship of the various different lineages to each other is poorly established and deeper nodes are therefore represented by dashed lines. Furthermore, the exact placement of various lineages such as Altiarchaeota and Methanonatronarchaeia is currently unresolved. For instance, the asterisks point out two alternative placements recently suggested for the Methanonatronarchaeia.

Further interesting insights into a key evolutionary event came from the discovery of the Lokiarchaeota, which themselves were subsequently shown to belong to the Asgard superphylum. The Asgard archaea are currently known to include the four major archaeal lineages, referred to as the Loki-, Thor-, Odin-, and Heimdallarchaeota, all of which were named after figures of the Norse mythology. The first genome of a member of the Lokiarchaeota has been obtained from sediment samples taken from about 3300 m below sea level in proximity to the active venting site Loki's Castle, while additional genomes of members of this group have been reconstructed from metagenomes derived from sediment samples all over the world. Notably, phylogenetic analyzes of a set of marker genes has suggested that Asgard archaea may represent the closest archaeal sister lineage of Eukaryotes. Furthermore, members of these archaea were shown to contain several so-called eukaryotic signature proteins (ESPs), i.e., proteins that have not previously been found in archaeal or bacterial genomes but represent important components of eukaryotic proteomes or proteins that are most similar to eukaryotic homologs. Altogether, these findings support the hypothesis that eukaryotes evolved from a host cell of archaeal decent and an alphaproteobacterial symbiont. In this respect, Archaea and Bacteria may be regarded as primary domains of life, while eukaryotes form a secondary domain of life. However, the origin of the eukaryotic cell and the nature of the tree of life remain a matter of ongoing debate and subsequent research will be needed to further unveil the relationship of the major divisions of life.

Genomic and Molecular Characteristics of Archaea

In 1996, Craig Venter and co-workers reported the first full genome sequence of the archaeon *Methanocaldococcus jannaschii* (formerly *Methanococcus jannaschii*), only one year after the completion of the first bacterial genome (*Haemophilus influenzae*). Similar to bacterial genomes, archaeal genomes are characterized by the presence of a main circular chromosome, with high gene

density, and short intergenic spaces. Besides the circular chromosome, some archaea also contain plasmids, which can be transferred from one cell to another.

Although bacterial and archaeal genome architectures appear overall similar, some general differences can be found. For instance, the proportion of the genome containing intergenetic regions is consistent across different genome sizes in bacteria, whereas larger archaeal genomes possess a greater proportion of intergenic regions. Additionally, the range of archaeal genome sizes is much narrower than that of bacteria (0.11 Mb, *Nasuia deltocephalinicola* to 16.04 Mb, *Minicystis rosea*). Within archaea, the smallest genomes are found in the DPANN superphylum (~0.5–1.5 Mb) with *N. equitans* having a reduced genome of only 0.49 kb in size (550 protein coding genes). Overall, DPANN genomes are characterized by a high coding density and few pseudogenes, which is in contrast to the small genomes of bacterial symbionts. *Methanosarcina acetivorans* possesses the largest completed archaeal genome (5.75 Mb) known to date (4528 protein coding genes). This methanogenic archaeon is ecologically widely distributed, and has a complex cell morphology and life style.

Microorganisms may be seen as highly integrated self-sustaining metabolic entities (the phenotype), and it remains to be elucidated how these entities are controlled by their underlying genes (the genotype). An important link between these two types is the information processing system of a cell. The process of archaeal replication was already studied for a long period of time, due to the superior (thermostable) properties of archaeal DNA polymerases and their biotechnological application for PCR (e.g., *Pfu* from *Pyrococcus furiosus*) and DNA sequencing (specific variants of *Thermococcus sp.* 9°N PolB DNA polymerase) as well as their high capacity for DNA repair even under extreme conditions.

Besides the family B DNA polymerase and the primase, archaea share many other components of the replisome with eukaryotes, many of which are not homologous to the bacterial counterparts, such as the replicative minichromosome maintenance (MCM) helicase, proliferating cell nuclear antigen (PCNA)-like DNA sliding clamp, Orc/Cdc6-like initiation complex, and replication factor C (RFC). Thus, the archaeal replication apparatus is more similar to the one of Eukaryotes and, due to its simplicity, has been extensively studied as a surrogate to understand the basic mechanisms of eukaryotic DNA replication.

The archaeal transcription apparatus is likely to resemble the ancestral version of the eukaryotic RNA polymerase II (RNAPII). The core contains five universally conserved subunits (Rpo1, 2, 3, 6 and 11) and includes almost all critical elements for transcription. In contrast to Bacteria, which use sigma factors to initiate transcription, Archaea need three general transcription initiation factors: TBP (TATA-binding protein), TFB (Transcription Factor B) and TFE (Transcription Factor E). These initiation factors are homologous to eukaryotic TBP, TFIIB and TFIIE, respectively. Finally, these promoter elements include a TATA-box, B-recognition (BRE) and an initiator (Inr) element on sequence level to initiate the transcription process.

Insights from archaeal genomes have revealed that archaea represent a much more genetically versatile group of organisms than assumed previously. Horizontal gene transfer (HGT) represents a major source of innovation in the evolution of bacterial and archaeal genomes and seems to have had a significant impact in the evolution of some archaeal linages. In particular, gene acquisitions from bacteria may have contributed to the adaption of various archaeal lineages to a larger variety of habitats and lifestyles and thereby aided in the diversification of the archaeal domain of life.

Archaeal Cell Structure

The basic cell morphology of archaea is comparable to that of bacteria and varies from rods, cocci, spirals to irregular shaped cells. The total cell volume ranges from 0.5 to 2.5 μm^3, which is ideal for maximizing the surface area-to-volume ratio, optimizing the exchange of nutrients and reaction products via diffusion.

Coccoid cells are found within the Desulfurococcales, whereas lobed cocci are characteristic for members of the Sulfolobales as well as for *Nitrososphaera viennensis*. Members of the genera *Thermoproteus*, *Pyrobaculum* (Crenarchaeota) and *Methanothermus* (Euryarchaeota) depict short rods with a length in the range of usually 2–5 μm. In contrast, rod-shaped *Thermofilum* cells can exhibit a length of up to 100 μm with a diameter of just 150 nm. *Methanospirillum* (as the name already indicates) as well as *Methanosaeta* form long spiral chains. *Picrophilus*, *Hyperthermus* or the ultraflat cells typically found in *Pyrodictium* species are characterized by pleomorphic cell shapes. *Haloquadratum walsbyi*, is remarkable in that it forms an almost perfect quadrate flattened cell. An overview of different archaeal cell structures and morphologies is given in Fig. 3.

All known archaea are surrounded by a cytoplasmic membrane, which is composed of variations of di- or tetra-ether lipids. Apart from members of the Thermoplasmatales, which do not harbor a cell wall, the majority of archaea described so far have a variety of cell walls or surface layers. For instance, the S-layer, which is characteristic for the cell walls of members of the *Desulfurococcales* and the *Sulfolobales*, is often composed of just one protein, which self-arranges in a 2D-pseudocrystalline lattice with p1-, p2-, p3-, p4- or p6- symmetry on the cell surface and which is usually anchored in the cytoplasmic membrane or another cell wall component.

Other cell wall types such as pseudomurein (polymeric analog of murein found in bacteria), glutaminylglycan, halomucin or simple protein sheets are present in methanogens and halophiles. Finally, some archaeal representatives such as *Ignicoccus hospitalis* as well as *Methanomassillicoccus luminyensis*, *Cand.* Altiarchaeum hamiconexum, or the ARMAN archaea have two membranes. With an inner and outer(most) membrane, they resemble the architecture of the Gram-negative cell wall.

Similar to bacteria, there are also several different cell apendages described in archaea. While archaea do not encode flagella homologous to those of bacteria or eukaryotes, they have a functional counterpart referred to as the archaeal flagellum or

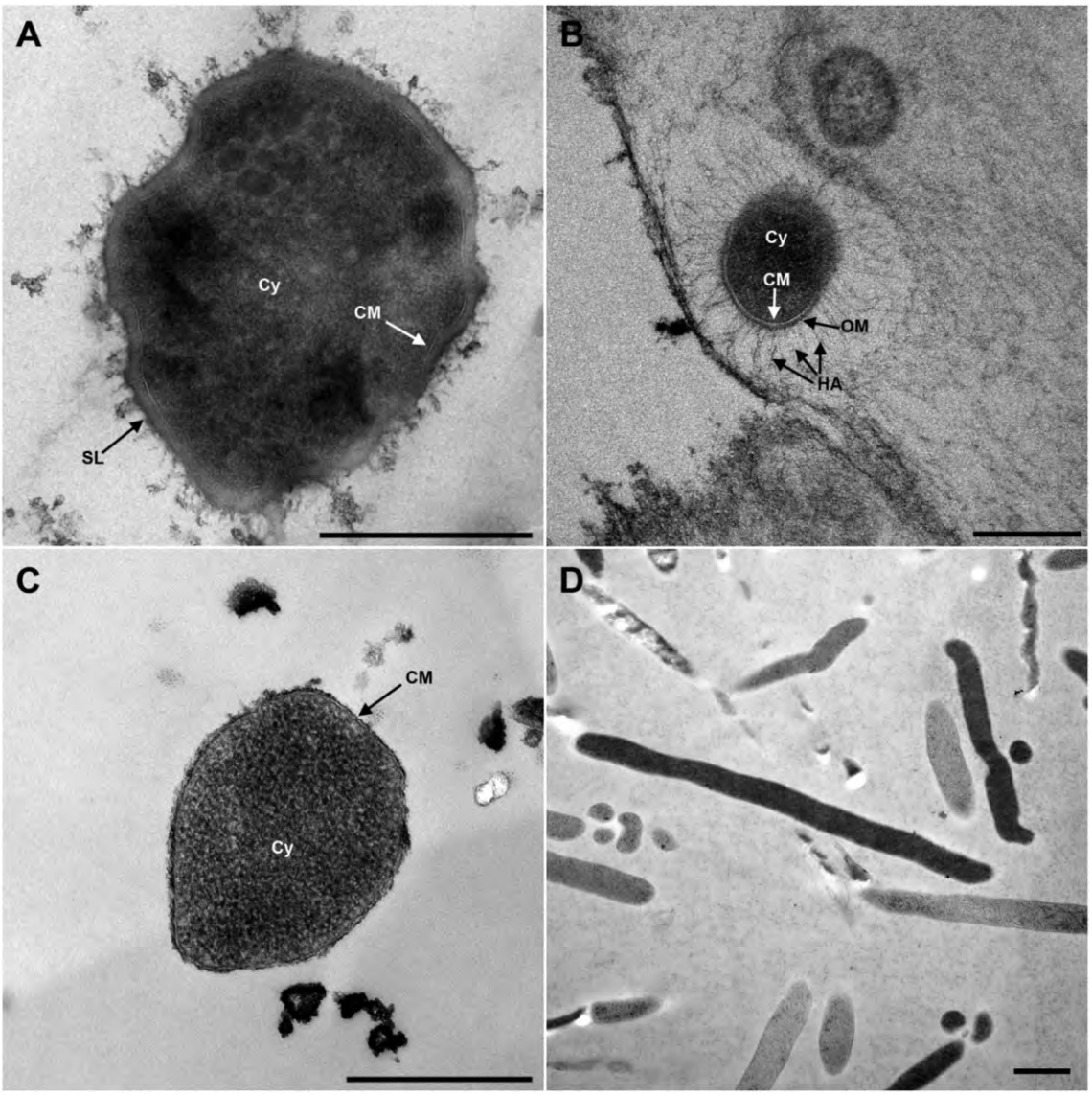

Fig. 3 Transmission electron micrographs of ultrathin sections of different archaeal cells and structures. (A) Lobed, coccoid cell of thermoacidophilic *Sulfolobus metallicus*. The cell is surrounded by a cytoplasmic membrane and a S-layer. (B) A biofilm of *Cand.* Altiarchaeum hamiconexum. The cells are typically surrounded by a double membrane and reveal numerous appendages called hami. (C) Irregular, coccoid *Thermoplasma acidophilum* does not possess a cell wall outside the cytoplasmic membrane. (D) Rod-shaped cells of the methanogen *Methanothermobacter thermautotrophicus*. CM, cytoplasmic membrane; Cy, cytoplasm; HA, hami; OM, outer membrane; SL, S-layer. Scale bars: 500 nm.

archaellum. These archaeal flagella have a diameter of 10–14 nm and show high similarities to bacterial and archaeal type IV pili. Further cell appendages include pili or fimbriae, as well as the grappling hook-bearing hami (Fig. 4), cannulae and fibers.

Biotechnological Applications of Archaea

Archaea are considered an excellent source for nano-/biotechnological applications. Due to their specific biological features, they serve as interesting and valuable tool box for e.g., novel biopolymers and extremozymes. However, only a few aspects of the archaeal biology have thus far been exploited, which to some extent is due to the lack or inefficiency of hosts for gene expression.

Extremozymes are enzymes from microbial extremophiles. For instance, these include the *Pfu* DNA polymerase, which was isolated from the hyperthermophilic archaeon *Pyrococcus furiosus*. The *Pfu* polymerase has a high fidelity at high temperatures, a

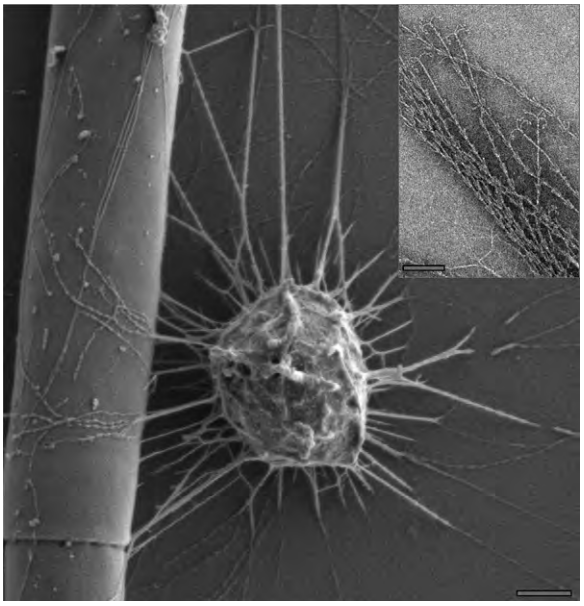

Fig. 4 *Cand.* Altiarchaeum hamiconexum (coccoid cell) with cell appendages ("hami") for attachment (here: bacterial cell) (scanning electron micrograph, bar: 200 nm; credit: Gerhard Wanner), and grappling-hook bearing hami in higher magnification (upper picture, transmission electron micrograph, bar: 100 nm).

precise proof-reading ability, a low error rate, a high-processivity, and a high extension rate, resulting in more efficient DNA target amplification when compared to the traditional *Taq* polymerase. Numerous additional archaeal DNA polymerases have been isolated and are being engineered. Other enzymes of interest include DNA and RNA ligases as well as amylases, proteases and lipases, which among others find applications in the beer and paper industry.

In addition, archaea are of relevance with regard to their photopigments and in the production of biogas (methane), ethanol and polyhydroalkanoates (for medical plastics). Some of their unique cellular features, such as the hami (nano-grappling hooks), could prove useful for nanobiotechnology while their liposomes and cell wall compounds are exploited in the development of vaccines. Finally, archaea are being used for metal biooxidation and biomining. The recent finding that archaea (such as *Cand*. Nitrosocosmicus oleophilus) can have positive impact on plant growth and trigger the systematic resistance in *Arabidopsis thaliana* have raised interest in agricultural applications as well. There, archaeal activity could support fixation, solubilization or mobilization of nutrients, growth hormone production or protection in general.

Viruses of Archaea

Archaea are associated with an extremely diverse virosphere. The vast majority of isolated archaeal viruses infect hyperthermophiles and halophiles from the phyla Crenarchaeota and Euryarchaeota, respectively. However, a handful of viruses has also been described for methanogenic archaea.

Archaeal viruses are currently classified into 18 families, whereas several additional virus groups await official classification. Informally, archaeal virus diversity can be categorized into archaea-specific and cosmopolitan fractions (Fig. 5). The archaea-specific viruses, by definition, are unique to archaea and in their genomic and morphological features do not resemble viruses of bacteria or eukaryotes. Most of these viruses infect hyperthermophilic archaea and are characterized by unique virion morphologies such as bottles (family *Ampullaviridae*), spindles (e.g., family *Fuselloviridae*) or droplets (family *Guttaviridae*). Specific to archaea are also viruses with ovoid, coil-shaped and pleomorphic virions. By contrast, the cosmopolitan archaeal viruses, such as head-tailed viruses of haloarchaea and methanogens, are structurally and genomically related to bacterial (tailed phages of the order *Caudovirales*) and eukaryotic (*Herpesvirales*) viruses.

All archaeal viruses identified thus far carry DNA genomes, which can be either linear or circular. The majority of viruses have double-stranded genomes, although single-stranded (ss) genomes have also been characterized. Notably, a virus with the largest ssDNA genome among all known ssDNA viruses infects a hyperthermophilic archaeon, *Aeropyrum pernix*. Comparative genomics analyses have shown that ~75% of archaeal virus genes do not have homologs in sequence databases (outside of related viruses), whereas some archaeal virus genomes remain completely refractory to functional annotation based on sequence anayses. Thus, archaeal virus genomes represent a rich source of unknown genes, which may underlie unique mechanisms of virus-host interactions and could possess unexpected properties useful for biotechnological applications.

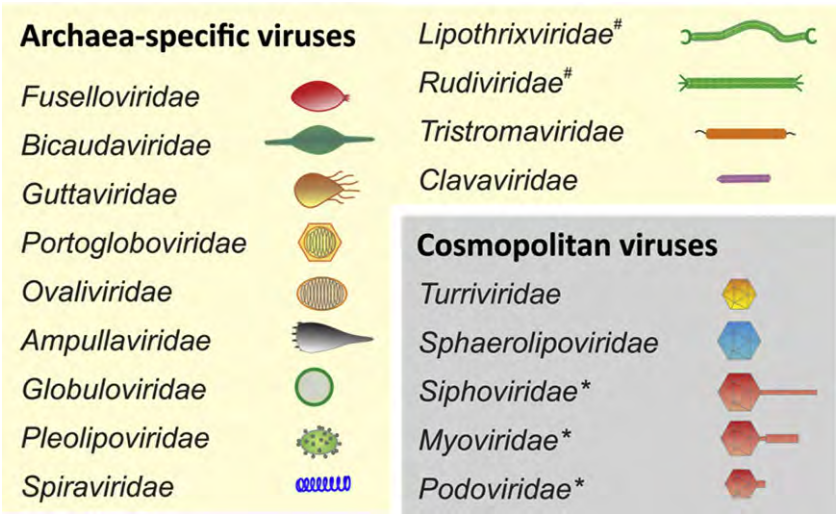

Fig. 5 Viruses of archaea. Archaea-specific and cosmopolitan archaeal viruses are shown in light yellow and gray backgrounds, respectively. The schematics of virion morphologies are shown next to the corresponding family names. * – members of the order *Caudovirales*; # – members of the order *Ligamenvirales*.

Archaeal viruses have evolved a range of mechanisms for efficient penetration of the cell envelope, subversion of the cellular machineries for viral genome replication and expression as well as virion assembly and release. Although some of the stages of the reproduction cycles are likely to mechanistically resemble those of viruses infecting bacteria and eukaryotes (especially in the case of cosmopolitan viruses) other aspects have evolved in the context of archaeal cells and are unique to archaeal virus-host systems. For instance, enveloped viruses, such as fuselloviruses, are assembled and released via a budding mechanism highly similar to that employed by enveloped eukaryotic viruses such as HIV-1 and Ebola virus. By contrast, rod-shaped virions of rudiviruses and icosahedral virions of turriviruses are assembled inside the cytoplasm and are released through pyramidal portals.

Although for most environmentally widespread and ecologically important archaeal lineages, such as Thaumarchaeota, viruses are yet to be isolated, metagenomics and single-cell genomics studies have shown that these archaea are also associated with a diverse virome. Importantly, it has been demonstrated that virus-mediated turnover of archaea, primarily thaumarchaea, in surface deep-sea sediments accounts for up to one-third of the total microbial biomass killed, resulting in the release of ~0.3–0.5 gigatons of carbon per year globally. Thus, archaeal viruses are not mere curiosities of the viral world, as has been considered previously, but are prominent players in the biosphere, with measurable impact on global biogeochemical cycles.

Archaea – Quo Vadis?

During the last four decades, Archaea moved into the spotlight of modern microbiology. Their wide distribution in Earth's ecosystems, their involvement in processes of global relevance, their unique properties and their role in the evolution of cellular life are increasingly recognized. In this article, a number of archaeal peculiarities have not been addressed, which include their complex cell division, or the presence of CRISPR-Cas. Furthermore, numerous questions remain to be answered in future, including the role of Archaea in (human) health and disease, and as components of microbiomes in general. The field of archaea research is still young and dynamic, and many surprises and critical insights can be awaited.

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Relevant Website

www.bacterio.net–List of Prokaryotic names with Standing in Nomenclature.

Archaellum

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Glossary

Archaea Unicellular microorganisms lacking a nucleus (prokaryotic) that are one of the three primary Domains of life. Cultivated members, such as hyperthermophiles, extreme halophiles and methanogens, are often from extreme environments although their distribution in nature if actually cosmopolitan.
Methanogen One of the major subgroupings of Archaea, methanogens are strict anaerobes that produce methane gas as an end-product of their metabolism.
N-linked glycosylation The attachment of oligosaccharides to proteins specifically at asparagine residues that are found within the sequence asparagine- any amino acid except proline-serine/threonine.
Signal peptide A short stretch of amino acids initially present at the amino terminus of a secreted protein that helps to guide it to its final destination outside of the cytoplasm. During transport across the cytoplasmic membrane, the signal peptide is removed through the action of a signal peptidase.
Sulfolobus A genus of Archaea found often in acidic hot springs that grows at elevated temperatures and low pH (thermoacidophile).
Type IV pili Surface appendages widespread in bacteria and archaea that can be used for attachment or, in bacteria, for a type of motility across solid surfaces called twitching.

Introduction

Archaea are prokaryotic organisms that were originally thought to be unusual extremophilic bacteria. Now, based on the pioneering studies of small ribosomal RNA by Carl Woese in the 1970s it is believed that Archaea represent a third Domain of life, in addition to Bacteria and Eucarya. While prokaryotic like Bacteria, they share certain traits with Bacteria, other traits with Eukarya and have a number of fundamentally unique attributes in addition to their signature 16S rRNA features. It is now known that Archaea are not limited to the extreme environments like hydrothermal vents, hypersaline niches and solfataras where they were originally isolated but are found in most "non-extreme" environments in which they have been looked for. The major cultivatable archaea include extreme halophiles (capable of growth in concentrated NaCl), methanogens (extreme anaerobes), thermoacidophiles (growth at 60–80°C and pH 2), hyperthermophiles (capable of growth in excess of 100°C) and ammonia-oxidizing organisms.

The three Domains of life all contain members that are motile by cellular appendages that were all known previously as flagella, despite the fact that the organelles from eukaryotes, bacteria and archaea are unrelated to each other and only share the one common trait of providing swimming motility to the cells. Unexpectedly, data accumulated on the archaeal appendages revealed their uniqueness compared to bacterial flagella (as outlined below) and, to denote the fundamental difference between the two prokaryotic motility structures, we proposed in 2012 that the archaeal organelle be given a unique designation. We proposed archaellum (pl. archaella), a fabricated name that importantly allowed for use of terms analogous to ones employed by bacterial flagella researchers. Thus, archaea possessing these organelles are called archaellated cells and the main structural proteins of the archaellum are archaellins.

The Archaellum is a Rotating Type IV Pilus

A large bank of data all clearly pointed to the two prokaryotic swimming organelles being distinct, evolutionarily unrelated organelles and that the archaella were not just unusual adaptations of the bacterial flagella structure to cope with the extreme environments that archaea often inhabit. It became apparent that archaella were not homologous to bacterial flagella but, quite unexpectedly, homologous to type IV pili systems common in bacteria and archaea (Table 1). While archaeal type IV pili are involved primarily in adhesion and biofilm formation, they are used in bacteria for additional processes such as DNA transfer and a kind of surface motility called twitching. Key points that indicate that archaella are not a modified version of bacterial flagella come from bioinformatic, biochemical, structural and genetic studies. As sequenced genomes for archaea became available, it could be shown that none of them contained genes associated with the bacterial flagellum structure. Additionally, while archaella and bacterial flagella are both rotating organelles, the energy source to drive rotation differs fundamentally in the two systems: Bacterial flagella utilize the energy in ion gradients (i.e., either the proton-motive force or less frequently the sodium-motive force) to drive

Table 1 Major similarities and differences of archaella, bacterial flagella and type IV pili

Trait	*Archaella*	*Bacterial flagella*	*Type IV pili*
Filament diameter	10–14 nm	18–22 nm	6 nm
Filament structure	No central channel	Central channel	No central channel
Growth point	Base	Distal tip	Base
Anchoring structure	Ill-defined, knoblike	Basal body, rod and rings	None observed
Major structural proteins	Usually multiple archaellins, some minor	Typically single flagellin, sometimes multiple	One major pilin, several minor
Signal peptides	Class III type on archaellins	None on flagellins	Class III type on pilins
Signal peptidase for subunit processing	FlaK/PibD (homologue of type IV pilus PilD)	None	PilD (homologue of FlaK/PibD)
Glycosylation	N-linked to archaellins is common, O-linked rarely	O-linked to flagellins, but rare	O-linked to pilins
Main functions	Swimming, adhesion	Swimming, adhesion	Surface motility (twitching), adhesion
Mechanism of movement	Rotation, switchable	Rotation, switchable	Extension/retraction of pilus
Energy for movement	ATP	H^+/Na^+ gradient	ATP
Genes	Some homologs to type IV pilus genes, none to bacterial flagella genes	Assembly system is homologous to type III secretion systems	Some homologs to archaella genes, not to bacterial flagella genes
Interaction with chemotaxis system	In some archaea, mainly Euryarchaeota	Yes	Sometimes

rotation while archaella use ATP hydrolysis. Finally, archaellins are almost universally modified by attachment of N-linked glycans, a posttranslational modification not found in bacterial flagellins but one that is key for archaellation and motility.

While these data indicated what archaella were not (i.e., bacterial flagella), other data began to accumulate that showed what they were; i.e., structures homologous to type IV pili. Key to this was the realization that archaellins, the main structural proteins in the archaellum filament were initially made with N-terminal signal peptides that had to be cleaved from the archaellins in an essential step for archaellins to assemble into filaments, while bacterial flagellins are never made as preproteins. The signal peptides of archaellins were, in themselves, atypical in being usually very short and with overall similarity to signal peptides found in type IV pilins. In type IV pilin systems, signal peptide cleavage is the function of a dedicated signal peptidase called prepilin peptidase (or signal peptidase III). Different archaea were shown to possess a homologue of the prepilin peptidase (designated FlaK or PibD depending on the species) that carried out signal peptide removal from archaellins and this enzyme was necessary for archaellation. Analysis of archaellins themselves showed amino acid sequence similarity in the mature N-terminus to the same region in type IV pilins, a conserved hydrophobic region important for subunit-subunit interaction in the assembled structures. In addition, other genes in the *fla* operon required for archaellation were clear homologs to genes important for type IV pilus assembly. These archaeal genes were *flaI* and *flaJ*. FlaI is a homologue of ATPases necessary for the incorporation or removal of pilin subunits from the base of the structure, a process that results in extension or retraction of the pilus from the cell surface. FlaJ is a conserved cytoplasmic membrane protein homologous to a type IV pilin protein that interacts with the pilin ATPases to form a platform for pilus assembly.

The type IV pilus-like characteristics of the archaellum led to the prediction that archaella were assembled by a type IV pilus-like mechanism. A key important piece of supporting evidence for this assembly model was the first 3D reconstruction of the archaellum structure which was shown to share features with the type IV pilus and, most importantly for assembly model predictions, demonstrated that archaella did not possess a hollow centre that would allow the passage of archaellin subunits. In bacterial flagella growth, flagellins, made without signal peptides and with the aid of a flagellum-specific type III secretion system located at the flagellum base, enter the lumen present in the flagellum, travel through this lumen to its distal tip and incorporate here under a filament capping protein complex. Thus, the lack of a central channel in archaella seemingly ruled out any possibility that the assembly of new archaellin subunits could occur at the distal tip. The proposal of incorporation of new subunits at the base of the archaellum was a radical idea for a prokaryotic "flagellum". The idea of the archaellum as a rotating type IV pilus was thus supported, although this idea is likely a simplification as more structural data about the archaellum motor was later revealed (see below).

Distribution

Motility is a very common attribute of members of all the well-studied archaeal phyla, including Euryarchaeota, Crenarchaeota, Thaumarchaeota and most recently Nanoarchaeota, a phylum composed of very small obligate symbionts or ectoparasites. With the exception of gas vesicles which allow movement of a limited number of archaeal species by floating, the sole motility apparatus described for archaea is the archaellum. Non-archaellated cells are always non-motile. The large number of archaea that grow at low pH and/or high temperatures (even above 100°C) and have archaella attest to the extreme stability of these structures. Many different arrangements as well as number of archaella have been reported depending on the species (Fig. 1). Some species may

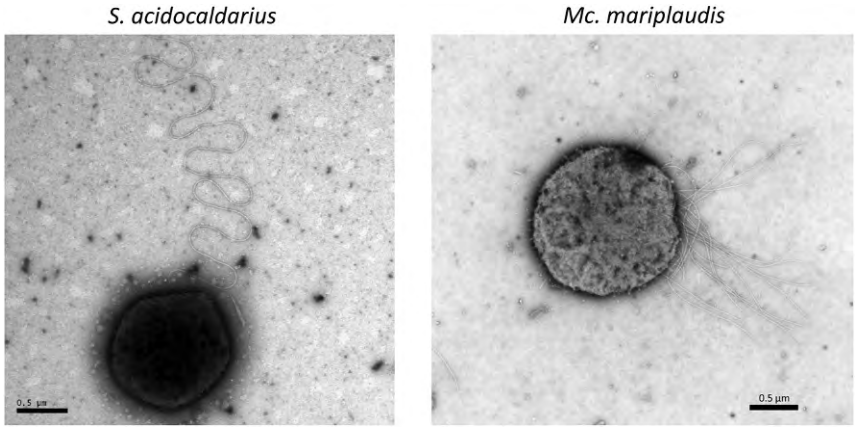

Fig. 1 Electron micrographs showing archaella in two model organisms, *Sulfolobus acidocaldarius* (A) with a single archaellum and *Methanococcus maripaludis* (B) with many archaella. (B) is from Ding, Y., Uchida, K., Aizawa, S.I., et al., 2015. Effects of *N*-glycosylation site removal in archaellins on the assembly and function of archaella in *Methanococcus maripaludis*. PLOS ONE 10, e0116402. Bars=0.5 μm.

possess only 1 or 2 archaella (e.g., *Sulfolobus acidocaldarius*) while others can have upwards of 50 (e.g., *Methanococcus voltae, Methanocaldococcus villosus* and *Pyrococcus furiosus*) and archaella can be found at a single cell pole, both poles or peritrichously around the cell surface.

Functions Other Than Swimming

While the archaellum is the sole motility apparatus reported in archaea, archaella are not necessarily limited to providing motility. In several species, archaella have additional functions. In *Mc. maripaludis*, *P. furiosus* and *Mcc. villosus*, archaella are known to establish cell to cell contacts and help in the adhesion of the cells to abiotic surfaces (Fig. 2(a)) although this is not a role for archaella in *Hfx. volcanii*. In the case of *Mc. maripaludis*, both type IV pili and archaella are needed for surface attachment. Archaella are also known to play a role in biofilm formation. In the case of *S. acidocaldarius*, the archaellum is needed at the late stages of biofilm formation for cells to leave the mature biofilm.

Structure and Rotation

Archaella are typically thinner than flagella, usually 10–14 nm in diameter, although there are rare reports of much thicker archaella, as in the case of *Haloarcula marismortui* where archaella can reach 20–22 nm in diameter. Typically, archaella are many cell lengths long. While flagella have a conserved curved hook region and complicated basal body composed of a series of stacked rings around a rod substructure, archaella structure is much simpler. The presence of a curved hook-like region is variable, reported for some species like *Mc. maripaludis*, *Mc. voltae* and *Hbt. salinarum*, but absent in *Sulfolobales* and *Hfx. volcanii*. No evidence for a flagellum-like basal body is present in archaella, although knob-like structures have been reported in some species at the cell proximal end (Fig. 2(b)). In the limited number of cases where it has been determined, archaella exhibit a right-handed helicity. Like flagella, archaella are

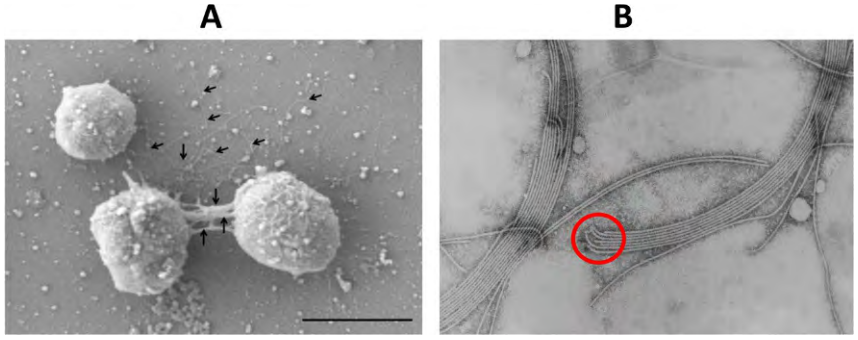

Fig. 2 Archaella of *Methanococcus sp.* (A) Scanning electron micrograph showing *Mc. maripaludis* cells interacting with each other and the underlying surface through bundles of archaella (arrows). Scale bar=1 μm. Courtesy of Meg Stark and James Chong, Department of Biology, University of York, UK. (B) Purified archaella from *Methanococcus voltae* isolated by detergent extraction. Hook-like regions ending with a knob, representing the cell proximal end are observed (e.g., in circled area). Archaella are 12 nm in diameter. Courtesy of Shin-Ichi Aizawa, Department of Life Sciences, Prefectural University of Hiroshima, Japan.

rotating organelles that can switch the direction of rotation. However, the rotation of archaella is driven by ATP hydrolysis and not ion gradients as in flagella. Recent elegant biophysical studies determined that in the case of *Hbt. salinarum*, the archaella motor torque was estimated to be 50 pN nm and the motor efficiency to be 6%–10%. Remarkably, tethered cells were observed to pause during rotation, which may be the moments when FlaI hydrolyzes ATP to power rotation. The maximum swimming speeds for many well-studied archaea have been reported with a large range from 10 μm/s for *Hbt. salinarum* to near 600 μm/s for *Methanocaldococcus* species. In terms of body lengths per second, these hyperthermophilic methanogens are the fastest organisms on Earth. These extremely fast swimming speeds may be necessary for survival in their hydrothermal vent habitats. Importantly, unlike flagella, archaella do not possess a central channel that would allow subunits to travel through to the distal end to incorporate.

Archaella usually contain multiple archaellins, sometimes one or more major archaellins that dominate the structure, and additional minor archaellins that comprise a much smaller proportion of the structure. However, at the other extreme, certain crenarchaea like *Sulfolobus sp* have fully functional archaella with only a single archaellin gene present. In the case of *Haloarcula marismortui*, the major archaellin that forms the filament can vary depending on growth conditions, leading to the designation of the different archaellins as ecoparalogs.

Archaellum Genes

Almost all of the genes required for archaellum structure and function reside in a single cluster called the *fla* operon. The *fla* operon is best studied in the crenarchaeon *Sulfolobus acidocaldarius* and the euryarchaeon *Mc. maripaludis* (Fig. 3). *S. acidocaldarius* contains a minimum set of only 7 genes, a single archaellin and the *fla* accessory genes *flaX, flaF, flaG, flaH, flaI* and *flaJ*. In euryarchaeotes, the *fla* operon begins with multiple archaellin genes (*Mc. maripaludis* has 3; *flaB1, flaB2* and *flaB3*) followed by *flaC, flaD, flaE, flaF, flaG, flaH, flaI* and *flaJ*. FlaX is not found in euryarchaeotes (or Nanoarchaeotes or Thaumarchaeotes) while *flaC/D/E* are not found in crenarchaeotes. In some species *flaC, flaD* and *flaE* are not distinct ORFs but fused to form *flaCD, flaDE* or *flaCDE*. Deletion of any *fla* operon gene, other than some of the multiple archaellin genes in some archaea, leads to nonarchaellated cells. One key gene required for archaellum formation that is typically not found as part of the *fla* operon encodes the pre-archaellin peptidase, called FlaK in methanogens and PibD in other archaea, like *Sulfolobus sp* and *Hfx. volcanii*, an enzyme that removes the signal peptide from archaellins. Signal peptide cleavage is a necessary step in the assembly of the archaellins into the archaellum filament as mutants deleted for *flaK* are nonarchaellated. FlaK/PibD are members of the same family of unusual aspartic acid proteases as the prepilin peptidases that remove signal peptides from type IV pilins, although the archaeal enzymes do not methylate the resulting N-terminal amino acid as found for the bacterial enzymes. Site-directed mutagenesis studies have demonstrated that the pre-archaellin peptidases possess two aspartic acid residues that are critical for enzymatic activity. FlaK seems to have a substrate range limited to archaellins while PibD processes all substrates possessing type III signal peptides, including type IV pilins and bindosome (sugar binding proteins) components. FlaK from *Mc. maripaludis* was the first member of this aspartic acid protease family to be crystallized and its structure was determined at 3.6 Å.

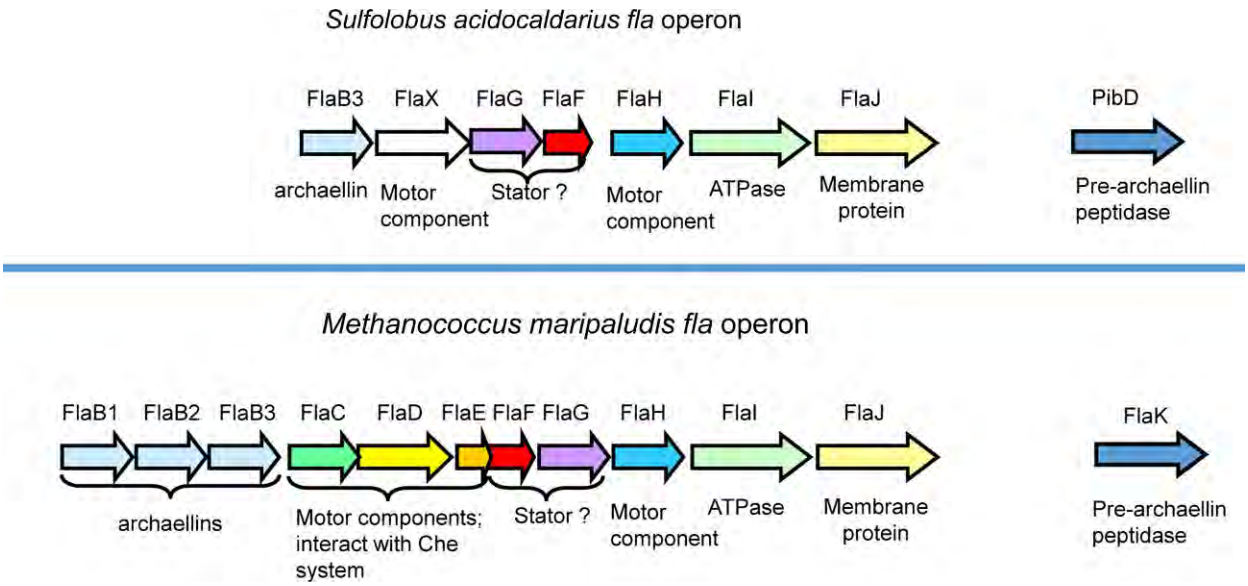

Fig. 3 Archaella operons from a typical crenarchaeote *S. acidocaldarius* and a typical euryarchaeote *Mc. maripaludis*.

Regulation and Regulation Crosstalk

Depending on the archaeon, production of archaella may not be constitutive but can be dependent on the availability of certain nutrients, growth phase and temperature. Molecular dissection of the regulation is available for only a very limited number of archaea, mainly *S. acidocaldarius* and *Mc. maripaludis*. In the latter organism, a transcriptional regulator EarA was shown to be essential for transcription of the *fla* operon and mutants deleted for *earA* were nonarchaellated. In *S. acidocaldarius*, a much more complicated archaellum regulatory network, has been unveiled including phosphorylation-dependent repressors and activators controlled by different kinases and phosphatases. Interestingly, there is crosstalk in the regulation of archaella and adhesive pili in *S. acidocaldarius* as deletion of genes encoding the adhesive pili lead to hyperarchaellated cells. Cross regulation of pili and archaella synthesis also occurs in *Hfx. volcanii*, where pilins are important regulators of archaella-mediated motility. Such crosstalk presumably allows for the production of only one type of organelle depending on whether the conditions call for adherence or motility.

Importance of N-Linked Glycosylation for Archaellation and Motility

Besides genes encoding the Fla proteins, *agl* genes involved in the biosynthesis and assembly of glycans that are N-linked to various cell surface proteins including S-layer proteins, pilins and archaellins have been shown to be important or essential for archaellation and motility. Until recently, only N-linked glycosylation was identified on archaellins but a recent study of archaellins of *Methanospirillum hungatei* identified both O- and N-linked glycosylation. The critical importance of N-glycosylation was demonstrated by the deletion of *aglB* (encoding the oligosaccharyltransferase), the signature enzyme of the N-linked glycosylation pathway, which catalyzes the transfer of the assembled glycan from its lipid carrier to the target protein. Mutants deleted for *aglB* possess archaellins completely devoid of N-linked glycans and these cells are nonarchaellated in *Mc. maripaludis* and *Hfx. volcanii*. Deletion of other *agl* genes lead to mutants able to make and transfer only a truncated glycan. Depending on the severity of the truncation, these mutants may be nonarchaellated or archaellated but with motility defects. Direct evidence for the importance of N-glycosylation of archaellins for assembly of archaella and motility was sought by genetically removing N-glycosylation sites on archaellins in *Mc. maripaludis*, *Hfx. volcanii* and *S. acidocaldarius*. Interesting, three different results were obtained. In *S. acidocaldarius*, all archaellin N-glycosylation sites could be removed with little effect on motility while in *Hfx. volcanii*, removal of even one glycosylation site resulted in complete loss of motility. For *Mc. maripaludis*, at least a single glycosylation site had to be present for archaellation to occur. Whether the role of glycosylation is for filament stability or whether it plays a role in the assembly mechanism or both is still under investigation. Glycosylation has been reported to play a role in protein stability under harsh conditions.

Biochemical Studies of Fla Proteins

Biochemical studies on targeted archaellar proteins have been initiated in efforts to understand how the various Fla proteins interact within the archaellum motor complex, how the motor works and how the archaellum is inserted into the archaeal cell envelope. Early studies reported the expression and purification of *S. solfataricus* FlaI and confirmed that it is an active ATPase. Later, the crystal structure of *S. acidocaldarius* FlaI showed that it assembles into a hexameric conformation induced by ATP binding. The hexamer adapted a crown-like shape where the tips of the crown were formed by the flexible N-terminus of the FlaI monomers. When the N-terminus of FlaI was removed in *S. acidocaldarius*, the cells were still archaellated but unable to swim, showing that FlaI plays two roles: First, it is required for assembly of the filament and secondly, it is required for rotation of the filament by ATP hydrolysis. Based on the homology to the bacterial type IV pili system, FlaI is expected to interact with FlaJ, an integral membrane protein. However, so far no biochemical data has be reported for any FlaJ homologs as the expression of the full-length version of this protein is very difficult. Depending on the species, FlaJ has 7–13 predicted transmembrane domains and most have 1 or 2 predicted large, cytoplasmic loops. It is thought that these large loops serve as an interaction platform for FlaI to form the heart of the archaellum motor. FlaH, highly conserved in all archaellum operons, has a Walker A motif but a non-canonical Walker B motif. FlaH has been shown to bind nucleotides with high affinities, but it does not actively hydrolyze ATP, as was shown for FlaH from both *Mcc. jannaschii* and *S. acidocaldarius*. In vivo, ATP binding by FlaH is essential for archaellum assembly as the FlaH Walker A and Walker B mutants in *S. acidocaldarius* were unable to assemble an archaellum filament. Both mutant forms of FlaH were also unable to interact with FlaI in vitro. In the case of *Mcc. jannaschii*, the addition of ATP lead to a decrease in the affinity of the wildtype versions of FlaH and FlaI. It was proposed that FlaH is important in the archaellum motor complex to regulate the activity of FlaI during assembly and rotation.

FlaX-like proteins are only found in crenarchaeal archaellum operons. FlaX of *S. acidocaldarius* is a monotopic membrane protein that shows no homology to any known proteins. Only expression of the soluble domain, which contains a coiled-coiled domain, was successful. This truncated protein formed very stable ring-like oligomers with an approximate diameter of 20–30 nm, as analyzed by single particle analysis. In vitro assays showed that FlaH, FlaI and FlaX from *S. acidocaldarius* interact with high affinities and probably form a complex in vivo in the archaellum motor complex with FlaX acting as a scaffold during motor assembly (Fig. 6, right panel). Single particle analysis showed that FlaH formed an inner ring inside the FlaX ring implying

that the *S. acidocaldarius* archaellum motor is organized like this. However, so far, a complex including FlaH, FlaX and FlaI could not be formed. In euryarchaea, FlaX is replaced by FlaC/D/E, but for these proteins no structural or biochemical information is available yet.

All archaellum components discussed so far are localized at the cytoplasmic side of the cytoplasmic membrane. FlaF and FlaG both contain a predicted archaellin domain and a hydrophobic stretch at the N-terminus. It is not clear whether the membrane domain is part of a signal peptide that is cleaved during archaellum assembly or whether this domain anchors the proteins in the membrane. The crystal structure of *S. acidocaldarius* FlaF showed an extended β-sheet-containing archaellin domain which was very similar to the recently solved cryo EM structures of the archaellins of *Msp. hungatei* and *P. furiosus* (see below). *S. acidocaldarius* FlaF bound to isolated S-layer proteins confirming that the FlaF archaellin domain is extracellularly localized. FlaG can be modelled on FlaF, therefore it is expected that it also exhibits a β-sheet structure. Consistent with FlaF binding to the envelope protein, it is proposed that FlaF/G anchor the archaellum to the S-layer (acting as the stator), or they might interact with the growing filament itself.

Recent Archaella Atomic Models

Early structural studies on the archaellum revealed a helical symmetry and the lack of a central channel. More recently, atomic models of the archaella filament and motor have been published. In the case of *Msp hungatei*, the filament was shown was be composed of a single archaellin (FlaB3) even though the organism has three *flaB* genes. An atomic model of the archaellum filament was presented at 3.4 Å resolution. Each archaellin monomer has a tadpole shape with two domains: An N-terminal domain that forms a long hydrophobic α-helix and forms the inner core of the filament and a C-terminal domain that forms a hydrophilic β-barrel exposed to the aqueous environment. In the C-terminal domain, posttranslational modifications of FlaB3 were identified: 6 sites of potential O-glycosylation (the first case of O-glycosylation of archaellins reported) and one atypical site of N-linked glycosylation. A further site has an unidentified modification that is unlikely to be glycan. Each archaellin monomer interacts with eight neighboring archaellins presumably contributing greatly to the rigidity and stability of the thin filament.

For *P. furiosus*, the filament was resolved to 4.2 Å. As with *Msp. hungatei*, three archaellin genes are present in *P. furiosus* but the filament was found to be predominantly composed of only one, FlaB0. Like the *Msp. hungatei* filament, that of *P. furiosus* was shown to have a hydrophobic core composed of the archaellin N-terminal α-helices with an outer globular domain of β strands. Each FlaB0 monomer interacts with 6 neighbours. Sites of likely N-glycosylation were detected at consensus N-glycosylation sequons in the surface exposed C-terminal domain of FlaB0. A problem seldom addressed with archaella is how they emerge through the S-layer that cover most archaeal species. The data presented here showed that the pores of the S layer were not large enough to accommodate passage of the 11 nm archaellum filament but it was hypothesized that removal of a single S-layer subunit would be enough to accommodate the diameter of the filament. Excitingly, this study also was able to visualize the archaellum motor complex in situ using sub-tomogram averaging of densities at the base of the archaella, allowing the creation of a model of the entire archaellum machinery (Fig. 4). The motor was revealed as a bell-shaped structure extending 19 nm into the cytoplasm. It is narrowest (9.5 nm diameter) at the membrane where it is linked by six protrusions of 3 nm. At the membrane distal end, the diameter widens to 18 nm and it is connected to a cytoplasmic ring of 26 nm. It is believed that FlaJ is fully embedded in the cytoplasmic membrane while the bell shaped cytoplasmic extension represents FlaI and FlaH. The cytoplasmic ring is hypothesized to be formed from the poorly understood FlaC and FlaD/E. The archaella motors are co-localized with a so-called polar cap, a sheet-like cytoplasmic structure which may serve as an anchor for the archaella (Fig. 5).

Archaella motors were also visualized in *T. kodakarensis*. Similar to the finding in *P. furiosus*, archaella in *T. kodakarensis* were found to associate with a large cytoplasmic conical structure which was hypothesized to serve as a massive anchor for the filaments. This conical structure also associates with large arrays of chemoreceptors.

The association of archaella motors with cytoplasmic structures (polar cap or cytoplasmic cone) in *P. furiosus* and *T. kodakarensis* is reminiscent of cytoplasmic discoid lamellar structures (DLS) that were observed in thin sections of *Hbt salinarum* under archaella insertion sites decades ago. Subsequently, isolated archaella from *Hbt. salinarum* were found attached to structures called polar caps which may be the previously observed DLS. Sub-cytoplasmic membrane structures were prominent as potential archaella anchoring mechanisms in the earliest archaella assembly models. Similar polar membrane-like structures were also observed in thin sections of various *Methanococcus* species, suggesting that in the Euryarchaeota, at least, such a cytoplasmic anchoring structure may be widespread.

Chemotaxis System Interactions

Unexpectedly, while the archaellum and the flagellum of bacteria are unrelated, both can interact with a conserved bacterial chemotaxis system to detect attractants and repellents in their environment. Both bacteria and many archaea possess CheY which in its phosphorylated form binds to the switch complex at the base of bacterial flagella causing a change in rotation, but archaea lack a discernible homologue of this bacterial switch protein. To integrate the bacterial chemotaxis system with the archaellum, a number of archaea-specific Che and Fla proteins may be involved. FlaX proteins are only found in crenarchaeotes which lack *che* genes. In other phyla like the Euryarchaeota, FlaX is replaced by what are believed to be the functional equivalents FlaC/D/E that appear to facilitate interaction with the chemotaxis system. An archaeal-specific Che protein called CheF is thought to directly

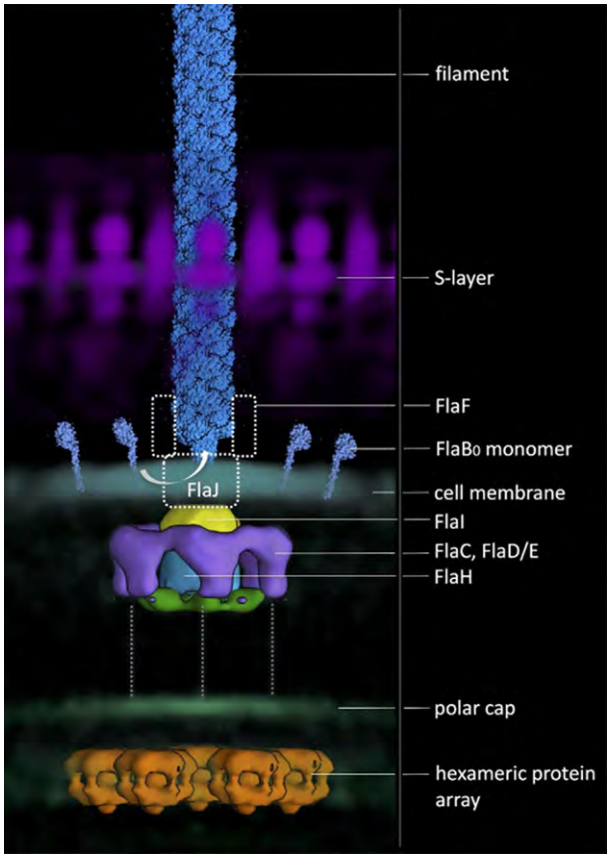

Fig. 4 Composite model of the archaellum machinery in the euryarchaeon *Pyrococcus furiosus*. The archaellum filament is composed of multiple copies of a single archaellin $FlaB_0$. Light blue, $FlaB_0$ monomers and filament (from helical reconstruction); hazy magenta, S-layer; solid yellow, blue, green and purple, motor complex; hazy blue, cell membrane; hazy green, polar cap; solid orange, hexagonal protein array (from different sub-tomogram averages). Putative positions of protein subunits are indicated. Dashed grey lines, putative interaction with polar cap. From Daum, B., Vonck, J., Bellack, A., et al., 2017. Structure and in situ organisation of the *Pyrococcus furiosus* archaellum machinery. eLIFE 6, e27470.

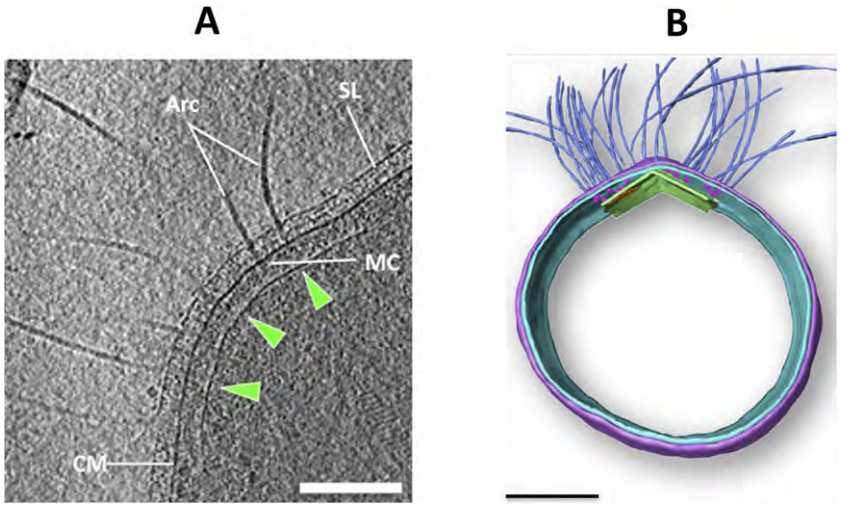

Fig. 5 *P. furiosus* archaella with associated polar cap. A. Electron cryo-tomography of *P. furiosus* showing polar cap (green arrowheads) underneath a bundle of archaella extruding from the cell pole. B. Segmented 3D representation of a tomogram of a *P. furiosus* cell. Motor complexes (magenta) have been repositioned into the original tomogram using coordinates from sub-tomogram averaging. Medium blue, archaella; purple, S-layer; cyan, cell membrane; green, polar cap. From Daum, B., Vonck, J., Bellack, A., et al., 2017. Structure and in situ organisation of the *Pyrococcus furiosus* archaellum machinery. eLIFE 6, e27470.

interact with CheY and the CheF-CheY complex is believed to interact with the FlaC/D/E at the archaellum motor to change the direction of rotation. There are several examples of archaea sensing environmental changes and adapting their swimming. For example, certain hydrogenotrophic methanogens have been shown to upregulate the *fla* operon upon H_2 depletion, *Hbt. salinarum* cells switch rotation of their archaella upon unfavorable UV-blue light stimulus and *S. acidocaldarius* induces swimming under nutrient starvation. Unexpectedly, however, there are examples of archaellated members of the archaea, such as *Sulfolobus sp* and *Mcc. jannaschii,* that can detect temperature changes and nutrient levels but which lack *che* genes. How this is done is currently unknown.

Assembly Model

Recent data has identified the location and possible functions of most of the Fla proteins within the archaellum, including interaction partners (Fig. 6). Given the simplicity of the archaellum structure vis-à-vis the bacterial flagellum, these studies have provided remarkable progress in the current understanding of the structure and assembly of the archaellum in the past few years, although much is yet to be learned about the order of assembly of the component proteins. The archaellum structure may be considered as composed of two major substructures: The filament and the motor. Since FlaJ and FlaI at minimum are needed to incorporate new archaellins into a filament, these two proteins at least seem to be the initial ones to start the assembly process, although it is possible the entire motor is assembled prior to archaellin incorporation. In the *S. acidocaldarius* system, it is believed that FlaJ assembles in the membrane after which FlaI comes into the complex. FlaH will then bind to FlaI, which will initiate the ring formation of FlaX around the motor complex. This is based on the result that in the FlaH deletion mutant the FlaX protein was degraded. In phyla other than Crenarchaea, the FlaC/D/E take the place of FlaX.

With at least FlaJ and FlaI in position, the formation of the filament can occur. Archaellins are made as preproteins and transported, perhaps with the aid of chaperones, to the cytoplasmic membrane. Here two posttranslational events must occur; the signal peptide must be cleaved through the actions of the pre-archaellin peptidase FlaK/PibD and the archaellins typically are modified with N-linked glycans through the activity of AglB. The order of the two events is unknown but each can occur in the absence of the other, as shown in studies in *Mc. maripaludis* using strains deleted for either *flaK* or *aglB*. The final step involves the incorporation of the modified archaellins into the base of the structure by the ATP-hydrolyzing activity of FlaI.

The stage at which FlaF/FlaG, the possible stators, are assembled during this process is not known and how the archaella interact with the proposed cytoplasmic anchoring structure, the polar cap, is likewise completely unknown.

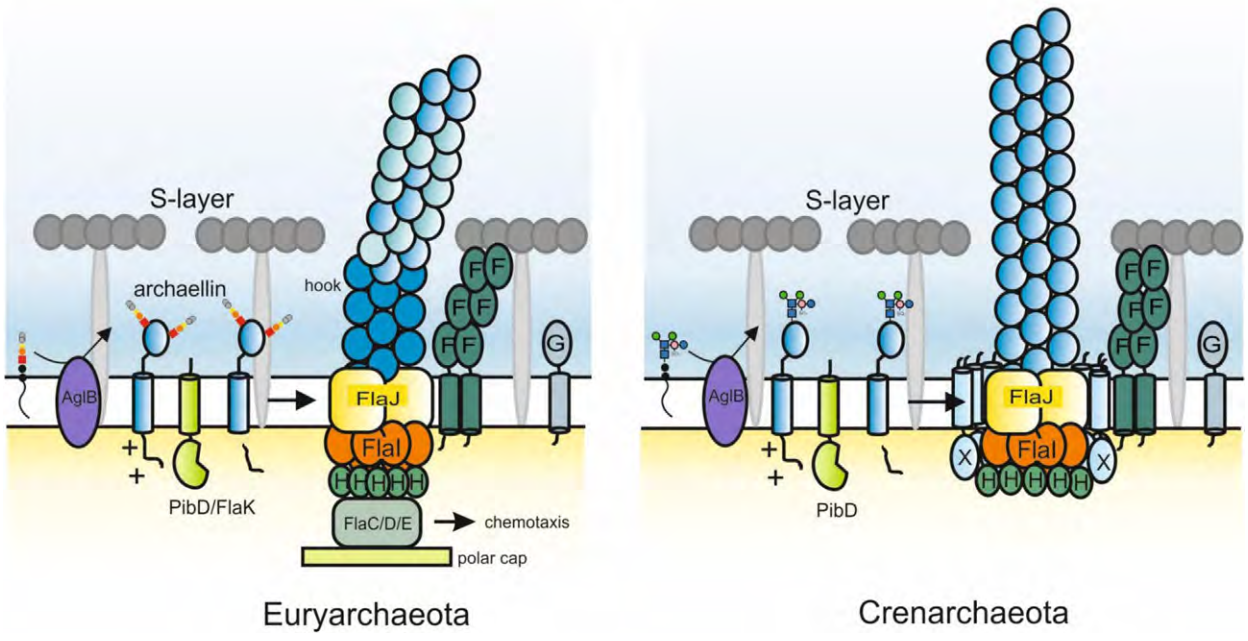

Fig. 6 Current models of euryarchaeal (*Mc. maripaludis*) and crenarchaeal (*S. acidocaldarius*) archaellum structures, showing location of known Fla proteins as well as processing steps (signal peptide removal and N-linked glycosylation) for the archaellins prior to their incorporation into the filament. Glycan structures are not shown on the assembled archaellins for clarity. CM, cytoplasmic membrane; AglB, oligosaccharyltransferase that transfers glycan from lipid carrier to select asparagine residues in target proteins, including archaellins; FlaK/PibD, pre-archaellin peptidase that remove the signal peptides from archaellins.

Future Outlook

It is clear that the archaellum is a remarkable motility apparatus. With a complexity far less than that seen in bacterial flagella, it nevertheless is an extremely stable and efficient rotating organelle that can impart swimming speeds that far exceed that observed for even the fastest bacteria. Despite its apparent simple protein composition and significant strides made in the biochemical analysis of its component proteins, much remains to be discovered about how and in what order the apparatus is assembled, how it is stabilized in the unusual archaeal envelope (vis a vis potential stators and cytoplasmic anchoring devices), how it interacts with the chemotaxis apparatus to alter its rotation and how its regulation is interconnected with the control of genes involved in adhesion and biofilm formation that allow the cells to best adapt to their changing environments. Even though there are only few data available on different archaella proteins and in situ archaellum complexes, it is clear that, similar to the bacterial flagellum, this archaeal molecular device has been adapted differently in different archaeal phyla. It is important to get structural data of many more archaella complexes to be able to compare these different structures.

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Aspergillus: A Multifaceted Genus☆

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Abbreviations

APC	Anaphase-promoting complex
HMG CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ORF	Open reading frame
ROS	Reactive oxygen species

Defining Statement

In this article, different aspects of ongoing work in the genus *Aspergillus* are discussed, ranging from toxin production, pathogenicity to humans and animals, traditional and modern biotechnological uses, genomics, and the use of *Aspergillus nidulans* as a model organism to study fundamental problems of cell and molecular biology.

Note

For *Aspergillus* genes and proteins, the standard nomenclature is followed, for example, the *benA* gene encodes the BenA protein, which is a β-tubulin. When genes or proteins of other species are mentioned, the standard nomenclature for each species is used.

What is *Aspergillus*?

An *Aspergillum* is an instrument used in the Roman Catholic mass to sprinkle holy water over the heads of the faithful. *Aspergillus* is a genus of the ascomycete fungi (see below). In 1729, Pietro Antonio Micheli, priest and botanist, described the asexual spore heads (conidiophores; see below) of a number of common molds. The heads of some of these molds showed rows of spores radiating from a globular central structure, which he thought resembled the *Aspergillum* he was familiar with. The morphology of the conidiophore is still an essential taxonomic marker. Fig. 1 compares the original Micheli's drawing with modern observations of conidiophores.

The classification of the kingdom fungi into major groups (Phyla, such as Ascomycota and Basidiomycota) is based on the morphology of the sexual reproductive structures. The Aspergilli should be placed among the ascomycetes (see below), those fungi that have the products of meiosis placed in a sac or ascus. However, most of the fungi we call Aspergilli (see below) do not have sexual reproduction, and thus no asci. To solve this problem, mycologists created the group fungi imperfecti or otherwise called deuteromycetes in which they placed all fungi without known sexual reproduction. This is a mixed bag without any phylogenetic significance. This provokes ridiculous situations, by which a fungus would change genus, and in fact phylum, every time sexual reproduction is detected. Thus the 'imperfect' fungus *Aspergillus nidulans* (see below) becomes the 'perfect' fungus *Emericella nidulans*, and it is placed in a different phylum from *Aspergillus sydowii*, in spite of the fact that morphological and molecular data show the two organisms to be close relatives. Names like *Emericella*, *Eurotium*, and *Neosartorya* design Aspergilli with a sexual cycle (also called teleomorphs). Thus, *E. nidulans* is the teleomorph (perfect form) of *A. nidulans* (anamorph, imperfect form). No one but a mycologist would know that we are talking about one and same organism.

This situation exists for many other genera. The only solution to this conundrum is to completely abandon the division 'fungi imperfecti' and choose in each case one and only one name for a given genus. As early as in 1926, Thom and Church, Thom and Raper (1945), and Raper and Fenell (1973) proposed that "the generic name *Aspergillus* should be applied to all these fungi whether or not an ascosporic (sexual) stage was produced". The main morphological characteristics of the genus, drastically abbreviated from Raper and Fenell (1973), are "vegetative mycelium consisting of septate branching hyphae ... Conidial apparatus developed as condiophores ... conidiophores ... broadening into turbinate elliptical, hemispherical, or globose fertile vesicles ... bearing fertile cell or sterigmata ... conidia (asexual spores) ... produced successively from the sterigmata. Ascocarps (asci, containing

☆*Change History*: April 2019. Claudio Scazzocchio has made minor changes to the reference and text.

This article is an update of C. Scazzocchio, Aspergillus: A Multifaceted Genus, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 401–421.

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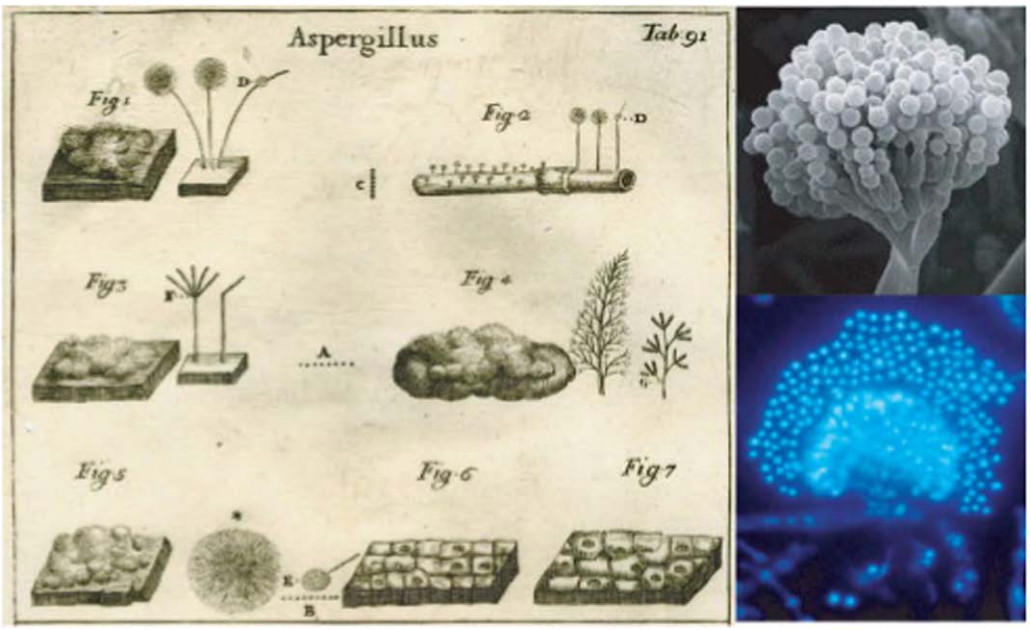

Fig. 1 The left panel shows a scan of copper-engraving 91 from Micheli's *Nova plantarum genera*, showing his drawings of *Aspergillus* conidiophores. The description in Micheli's text suggest that figure of the engraving, called *Aspergillus capitatus* (*muffa turchina*, blue mold) by Micheli, may correspond to *Aspergillus fumigatus* or a close relative. The right panel shows on the top a scanning electron micrograph of the conidiophore of *Aspergillus nidulans* and at the bottom an epifluorescence micrograph. The preparation is stained with DAPI which reveals the nuclei of the conidia and of the subjacent structures of the conidiophore. Both pictures on the right panel have kindly provided by Reinhard Fischer. Reproduced with permission from Kues, U., Fischer., R. (Eds.), 2006. The Micota I, Growth Differentiation and Sexuality. Berlin: Springer-Verlag. Micheli describes in his text the condiophore as been formed by a stalk, and a head, which he called 'placenta' carrying the conidia. See legend to Fig. 13 for the correspondence with the modern terminology.

sexual spores) found in certain groups only, unknown in most species". Recent work shows that the Aspergilli are as a whole a monophyletic group, where loss of sexual reproduction has occurred many times independently.

Not everyone agrees with the reasonable proposal of Raper and Fenell. A recent classification (2005) of the ascomycetes places the *Aspergillus*-related teleomorph genus names in the kingdom Fungi, subphylum Pezizomycotina, class Pezizomycetes, family Trichocomaceæ. The genera *Aspergillus* and the related *Penicillium* do not appear in this list as they are considered imperfect forms! Throughout this article, the generic name *Aspergillus* is used, as a genus comprising all the related 'teleomorphs' together with the forms where sexual reproduction is absent. The teleomorph name is also indicated when appropriate, as this is used in some important databases (as NCBI). The kiss of death to the concept of 'fungi imperfecti' was delivered by recent molecular data that show that the genomes of 'imperfect' Aspergilli, include the genes that determine mating types, these genes being clearly homologous to, and even placed in the same place in the chromosome as, the ones found in the 'perfect forms' ("The Genus Aspergillus in the Genomic Era").

Fungi of the genus *Aspergillus*, which includes about 200 species, are important in public health as toxin-producing food contaminants, as human and animal pathogens, as useful fungi in traditional and modern biotechnological processes, and finally one species has been used as a model to study a number of cellular processes. A recent development is the availability of eight complete genomes within the genus, a matter of obvious practical, taxonomical, and evolutionary importance.

Food Contamination by Aspergilli

Many organisms, including bacteria, fungi, and plants, produce secondary metabolites. These are molecules that can be very complex and are not obviously necessary for the viability of the organism. In fungi, they are produced during the stationary phase and their synthesis is usually coordinated with asexual sporulation (see below). Some secondary metabolites are extremely toxic, and when fungi grow on stored foods, they secrete them, provoking food spoilage and eventually intoxications that may be fatal. Among the Aspergilli, the two main culprits are strains of *Aspergillus flavus* and *Aspergillus parasiticus*, which secrete aflatoxins, a group of highly substituted coumarins. Strains that are closely related may vary drastically in their ability to produce the toxin. These saprophytic fungi can grow on a variety of foodstuffs, or even on plants before harvesting. In fact, *A. flavus* can be considered a weak opportunistic, nonspecific plant pathogen. The aflatoxins were discovered in 1960 when thousands of turkeys died in an English hatchery. The contaminated food was a ground peanut meal. The most serious contamination is that of maize. While this contamination results in loss of hundreds of million dollars every year to farmers in developed countries, the impact on human health is extremely serious in developing countries. Of the related compounds called aflatoxins, Aflatoxin B1 is one of the most toxic and carcinogenic compounds

known, as judged by tests on laboratory animals. Maize stored under warm and humid conditions becomes contaminated with aflatoxigenic Aspergilli, and when consumed by humans or animals, this can lead to liver failure and death. Periodic outbreaks of acute aflatoxin poisoning occurred in East Africa, the latest in 2004, leading to 125 deaths. It is more difficult to assess the damage caused by chronic aflatoxin poisoning and the correlation of the toxin in food with the frequency of liver cancer. Controls on aflatoxin levels are tight in developed countries; they are, however, impossible to be enforced in developing countries, where people would store grains in their homes and the stored grain may be the only available food. Human aflatoxicosis is a disease of poverty.

The Aspergilli can contaminate food with other toxic molecules. Only the ochratoxins, produced by a number of Aspergilli and Penicillia will be discussed below. The ochratoxins comprise an isocumarin moiety and a phenylalanine ring joined by an amide bond. Ochratoxin contamination has been reported in many foodstuffs, including grapes, nuts, cacao, coffee beans, and spices. In poultry, and laboratory animals, ochratoxins provoke serious kidney lesions. It is difficult to assess damage to human health caused by chronic exposure to ochratoxins. They have been implicated as a cause of testicular cancer. The similarity of symptoms of porcine mycotoxin nephropathy with that of Balkan endemic nephropathy, a disease localized to regions of Bulgaria, Romania, and former Yugoslavia, has implicated ochratoxins as causal agents of the disease. A similar case can be made for chronic interstitial nephropathy of northern Africa. A case of acute renal failure, almost certainly due to the exposure to an ochratoxin, has revived the hypothesis that exposure to this mycotoxin is the cause of the 'mummy curse', which is alleged to have killed archaeologists who have braved the prohibition to open royal tombs.

Aspergillus as Pathogens

The common fungus diseases are mild and superficial, while those that are deep-seated and endanger life are so rare that one man can seldom see enough cases to make any extensive study of them. (Henrici, Presidential Address to the Society of American Bacteriologists, 1939).

Aspergillus as Human Pathogens

The emergence of species of the genus *Aspergillus* as, in many cases, intractable human pathogens, has gone hand in hand with the progress of medicine. All Aspergilli encountered as causal agents of human or animal diseases are opportunistic pathogens. The Aspergilli are all saprophytes, usually growing in decomposing vegetal material. The main pathogen, *Aspergillus fumigatus*, thrives on compost.

Before the transplant era, *Aspergillus* infections were only encountered sporadically. Farmer's lung is a general name for an allergic disease that could be due to different causal agents, bacteria or fungi, of which the Aspergilli are the main culprits. It is an occupational disease associated with high exposure of spores, in environments such as grain silos. In the nineteenth century, two exotic occupational diseases associated with *A. fumigatus* were described: the *maladie de gaveurs de pigeons* and the *maladie de peigneurs de cheveux*. These pulmonary diseases were associated with people who force-fed pigeons and with people who sorted hair for wigs, respectively. A perusal of the Pathogenesis article by Austwick, included in Raper and Fennell's monograph of 1973, leaves the impression that a large number of *Aspergillus* species could be opportunistic pathogens, that pulmonary disease was basically an occupational hazard, that virtually every organ could be colonized by one or other *Aspergillus* species, and that once the fungus was established the prognosis was bleak. Henrici, compared invasive fungal diseases to autocatalytic processes, sluggish to start, but eventually becoming unstoppable. The comparison still holds today, except that immunodepression gives the fungus a head start. *A. fumigatus*, was then as now, the prevalent species, followed by *A. flavus*.

Three types of respiratory pathologies are associated with the Aspergilli. Exposure to the fungus can result in allergic diseases, such as farmer's lung and allergic bronchopulmonary aspergillosis, encountered mainly in asthmatic and cystic fibrosis patients. *Aspergillus* spores can germinate in preexisting cavities such as the sinuses or those present in the lung as a result of tuberculosis. This leads to localized Aspergillomas in immunocompetent subjects, which can be treated surgically and/or with appropriate drugs. Finally, the most threatening form is the invasive Aspergillosis, associated, in most but not in every case, with a depression of the immune system.

The ability to perform grafts of bone marrow cells in leukemic patients, of solid organs such as kidney, liver, and lung, has been accompanied by the emergence of invasive Aspergillosis. AIDS patients are also at risk, but *Aspergillus* spp. are encountered less frequently in these patients than *Pneumocystis carinii, Candida* spp., or *Cryptococcus neoformans*. Susceptible patients include those affected by neutropenia. Neutropenia can result from leukemia or from the chemotherapy used to control it, or be subsequent to treatment with immunosuppressant used in bone marrow, stem cell, or organ transplants. Patients of systemic diseases treated with immunodepressing drugs, mainly corticosteroids, are also at danger. In all these patients, germination of *Aspergillus* spores leads to invasive aspergillosis, usually of the lung, which breaking through the blood vessels can infect other organs. There seems to be no organ in which the fungus cannot grow in the absence of an appropriate immune response. In almost all cases, spores enter through the respiratory tract and germinate in the parenchyma of the lung, leading to invasion of the bronchiolar walls and the adjacent blood vessels. Invasive Aspergillosis has been classified into angioinvasive and bronchio-invasive forms, but this classification is somewhat artificial, as invasion of both bronchioles and arterioles can be seen in the same patient. This leads eventually to respiratory failure and death. Fig. 2 shows an *Aspergillus* mycelium grown in lung tissue.

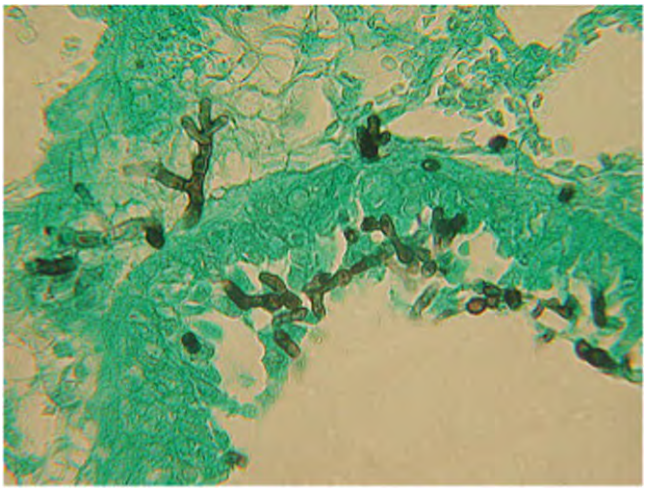

Fig. 2 A neutropenic mouse lung tissue, experimentally infected with *Aspergillus nidulans* is shown at 20 h postinfection. The fixed sections were stained with Grocotts Methenamine Silver. The pictures show hyphae (stained brown) actively growing in the lung tissue (stained green). Photograph kindly provided by Elaine Bignell.

A very recent review estimates an eight-fold increase in *Aspergillus*-disseminated infections, from the 1970s to the present day. Between 9% and 17% of all deaths in transplant recipients are due to *Aspergillus* infections according to recent data. The prognosis of invasive aspergillosis is grim; mortality in transplant patients infected with *Aspergillus* sp. is never lower than 60% for patients treated with antifungals and 100% in nontreated patients.

The most common encountered species in all *Aspergillus*-related pathologies is *A. fumigatus*, *A. flavus*, *Aspergillus niger*, *A. nidulans*, and *Aspergillus ustus* have also been recorded. One recent study of nosocomial infection reports that of 458 patients 154 were infected with *A. fumigatus* and 101 with *A. flavus*. The same and other studies establish a link between construction or renovation work in the vicinity of the hospital and frequency of invasive aspergillosis and conclude that even very low spore counts (1 spore per m^3) are dangerous to immunocompromised patients. Genotyping has shown that there are no specific pathogenic strains and suggests that every *A. fumigatus* strain present in the environment is a potential risk for immunodepressed patients. Recently, an upsurge of *Aspergillus terreus* infections has been observed. This is particularly worrying, as the organism is resistant to Amphotericin B, the drug most widely used to treat invasive Aspergillosis. The prevalence of *A. fumigatus* infection has not been explained. We are all continuously exposed to fungal spores and two obvious factors can be considered to explain the prevalence of one or other species. The first is the spore density in specific environments. Unfortunately, many early studies simply report the density of '*Aspergillus*' without any further species discrimination, let alone genotyping. It is generally accepted that the high frequency of *A. fumigatus* infections cannot be explained by a prevalence of the organism in the environment. The second parameter to be considered is spore size. The smaller the spores, the most likely they are to reach the alveolar tissue of the lung, as they will be less susceptible to removal by the mucociliary tissue of the respiratory tract. *A. fumigatus* spores are usually about 2–3 μm in diameter, at the lower end of the genus. Specific gravity of spores has, to my knowledge, never been measured. Another obvious parameter is thermotolerance, especially in relation to spore germination. However, it is unlikely that the combination of small spores and ability to germinate rapidly at 37°C be sufficient to explain the prevalence of *A. fumigatus*. Both characteristics are shared by *A. fumigatus* and *A. nidulans*, the latter being rarely encountered as an opportunistic pathogen. Another possibly interesting parameter is spore hydrophobicity. This is determined by a family of proteins called hydrophobins. Strains of *A. fumigatus* lacking a specific hydrophobin become more sensitive to macrophage killing. Sensitivity of different species to neutrophil and macrophage killing has been sporadically, but not systematically, assessed.

It is important to distinguish putative specific virulence determinants from essential metabolic processes, even if the latter can be potential drug targets. Only those processes, that when blocked, by mutation or otherwise result in reduced virulence but do not affect the growth of the fungus outside infected tissues, can be considered proper virulence determinants. This is of course conditional to the media in which the fungus is tested, my feeling is that the more we know about the metabolism of the fungus in the wild, the less we will be inclined to call a specific metabolic step a 'virulence determinant'. It is not surprising that engineered strains, deficient in essential biosynthetic pathways, or cell wall biosynthesis show reduced or no virulence. As an example, strains blocked in lysine biosynthesis show reduced virulence, but this tells nothing about virulence, it reveals that lysine is limiting in the alveolar environment. However, as some of these processes are fungal-specific, they are potential targets for antifungal drugs.

Secondary metabolites and nonribosomal peptides vary considerably from one fungal species to the other, and thus they represent an interesting avenue of research bearing on virulence. These metabolites may have evolved in saprophytic organisms in response to the presence of competing organisms in a common environment. As such, they may be cytoxic and eventually

involved in pathogenicity. One of these metabolites, gliotoxin, a substituted diketopiperazine, has received considerable attention. It been implicated in the suppression of the innate immune response, including apoptosis of neutrophils. However, specific inability to synthesize gliotoxin does not affect the virulence of *A. fumigatus* in the neutropenic mouse model. However, deletion of *laeA*, a gene necessary for the transcription of a large number of genes encoding enzymes of secondary metabolite synthesis (see 'Medically useful secondary metabolites' and 'Regulation of secondary metabolism'), including gliotoxin, does affect the virulence of *A. fumigatus*. Absence of LaeA leads to a pleiotropic phenotype, and the decreased virulence may result from a combination of factors. It seems that at present we simply do not know why *A. fumigatus* is the prevalent pathogen and why other Aspergilli are occasional pathogens. It is likely that a complex combination of characters is responsible for triggering the autocatalytic process proposed by Henrici. Opportunistic pathogens have not evolved as such, in a coevolutionary relationship with a host organism, it thus may be completely fortuitous that one or other of them be able to thrive in the tissues of immunocompromised patients.

It is important to determine which are the barriers that prevent fungal infections in immunocompetent subjects. Alveolar macrophage would get rid of ungerminated conidia, while polymorphonuclear neutrophils destroy hyphae mainly through the action of reactive oxygen species (ROS). One proposed mechanism involves the recognition of fungal cell wall constituents, such as β 1-3-glucans, by macrophage membrane receptors, leading to phagocytosis. Recent studies, however, imply a less clear-cut distribution of labor, with neutrophils also having an important role in preventing conidial germination, which correlates with the susceptibility of neutropenic patients. Dendritic cells are able to ingest *Aspergillus* spores, thus being able to present specific antigens to T cells, a role shared with macrophage. Both $CD4^+$ T and $CD8^+$ T cells respond to fugal antigens, $CD4^+$ T cells produce cytokines, which further recruit neutrophils. The protective role of specific antibodies is subject to discussion, as they are found in infected patients, which they fail to protect. Recently, protective roles have been postulated for surfactants secreted by epithelial cells that interact with conidia and may facilitate phagocytosis. A crucial role in innate immunity to opportunistic fungi is carried out by PTX3, a protein belonging to the pentraxin family of secreted, soluble proteins. PTX3 is essential in conidial recognition by macrophage and dendritic cells, and homozygous knocked-out mice genes are highly susceptible to experimental infection. The study of the immune response to infection by *Aspergillus* spores has made considerable progress in recent years and may lead to treatments, which promote the recovery of the immune response of the patient as an alternative or in association with antifungal drugs.

Fungi are eukaryotes, more closely related to metazoans than to plants, that is why ascomycetes such as *Saccharomyces cerevisiae* and *A. nidulans* are useful models in molecular and cell biology. Many cell processes are common to the fungal and the animal cell, and that, in order to find effective antifungal agents, is necessary to identify those processes that will inhibit growth of the fungal cell without damaging the host. Flucytosine (5-fluorocytosine) has been used as an antimycotic since 1968. In clinical practice, it is used mainly in candidiasis. It affects nucleic acid synthesis and thus can hardly be considered a specific antifungal agent. Four other classes of compounds are currently used to treat fungal infections in clinical practice. The polyenes, such as Amphotericin B, interact directly with ergosterol in the fungal cell membranes leading to leakage of potassium ions and cell death. Amphotericin B, one of the most used antifungals, interacts with animal cell membranes also, which can lead to acute kidney failure. The azoles, such as fluconazole or the newly developed voriconazole, inhibit specifically lanosterol demethylase, blocking the synthesis of ergosterol. They are less toxic than Amphotericin B, and a case has been made to use voriconazole as a first line, rather than a second line, drug for the treatment of invasive Aspergillosis. However, they are not free of secondary effects. The allylamines such as Terbinafine also result in ergosterol depletion by inhibiting squalene epoxidase. Finally, the echinocandins are really specific antifungal drugs, as they affect the fungal-specific process, the synthesis of the glucans of the fungal cell wall by inhibiting noncompetitively β-1,3-glucan synthase. Better knowledge of fungal development and metabolism, the search for genes essential for the pathogen, but absent in, or not essential for the host should lead to the development of new specific antifungal drugs. A different and complementary approach is to reinforce the immunological response of the host. This includes the possible development of an antifungal vaccine. Besides the uncertainty as to whether protective antibodies can be produced, the large variety of fungi that can affect immunodepressed patients posits an additional difficulty. Recently, a whole roaster of new fungi appeared as opportunistic pathogens, such as *Fusarium*, black molds, and zygomycetes. Success against *Candida* has been followed by an increase of *Aspergillus* infections. Preventive treatment with voriconazole, effective against *Aspergillus*, has been followed by infections by a whole variety of zygomycetes. It has been proposed that a vaccine using β-1,3-glucan as antigen, which is a universal component of fungal cell walls, may be worth exploring. An early diagnosis is essential in the successful treatment of invasive fungal infections. Immunological detection of cell wall components such as galactomannan and 1-3-β-D-glucan and detection of fungal DNA by PCR are being developed and evaluated.

Aspergillus in Veterinary Medicine

Aspergilli are encountered, even if uncommonly, in veterinary practice. Here, as in the human disease, *A. fumigatus* is the most frequently encountered pathogen, followed by *A. flavus*. In mammals, canine sinonasal Aspergillosis, guttural pouch mycosis of horses, and bovine mycotic abortion are the most common diseases, but infection of other species and pulmonary and generalized aspergillosis has also been described. The horse disease is correlated with the presence of an extension of the Eustachian tube, the guttural pouch, an organ of uncertain physiological significance exclusive of horses, other Equidæ, and rhinos and tapirs. This organ could provide temperature and humidity conditions suitable for the growth of *Aspergillus*. Bovine mycotic abortion is correlated with

confinement to sheds, which leads to exposure to high concentrations of spores. More surprising is the finding of pulmonary Aspergillosis in free-range dolphins. If the finding of *Aspergillus* infections in mammals is sporadic and infrequent, birds are at a much higher risk. The main pathogen is *A. fumigatus* and the route of entry is the respiratory tract. In a large postmortem study, 4% of more than 10,000 birds showed fungal infection of the respiratory tract, probably in most cases due to *A. fumigatus*. Aspergillosis affects both free-ranging and domestic birds. Turkeys, poultry, and waterfowl are commonly affected but fatal infections of penguins, ostriches, and rheas have also been reported. The susceptibility of birds to Aspergilli has been explained by both anatomical characteristics of the respiratory system and cellular differences related to innate immunity such as the absence of alveolar macrophage.

A. sydowii, a Specific Pathogen for Gorgonian Corals

An ecologically menacing new Aspergillosis affects Gorgonian (fan) corals, mainly but not exclusively *Gorgonia ventalina* (infections of *Gorgonia flabellum* and one outbreak affecting *Pseudopterogorgia americana* have been reported). Up to the present time, it has been recorded only in the Caribbean Sea, first identified in Saba in 1996 and studied intensively in the Florida Keys. In an epizootic starting in 1997, more than 50% of the sea fan corals were lost. A subsidence of the epizootic has been since reported. The organism responsible is exclusively *A. sydowii*. The restricted host–pathogen specificity contrasts with the situation described above for mammals and birds. This species is a common saprophyte, which can be isolated from a number of environments. Cultures isolated from diseased *G. ventalina* are infectious, while strains isolated from nonmarine environments are not. As only three and two strains respectively were analyzed, this experiment is not definitive. The pathogenic and nonpathogenic strains do not form separate clades when molecular markers are analyzed. As *A. sydowii* does not sporulate in seawater, aerial dissemination has been suspected. One hypothesis is that the spores are carried by dust storms, originating in the North Africa. While fungal spores are surely carried by dust storms, no genotyping work confirming this hypothesis has been reported. Warming and nutrient effluents, including nitrates, have also been blamed for the outbreak. Obviously, these possible causes are nonexclusive. It is possible that the decrease of the epizootic is due to selection for resistant strains of *G. ventalina*. Thus, sea fan infection by *A. sydowii*, besides being an ecological menace, provides an interesting opportunity to study a specific host–parasite interaction involving an *Aspergillus*, and the elucidation of the mechanism of resistance could lead to the discovery of new antifungal compounds, for which there is a crying need. An infected sea fan coral is shown in Fig. 3.

Fig. 3 A specimen of the fan coral Gorgonia ventalina infected with *Aspergillus sydowii*. The infected areas are deep purple. The purple gall-like growths may be a result of the infection. A necrotic area surrounded by a deep-purpled ring can be seen at the bottom left of the colony. Bar 5 cm. The photograph has been kindly provided by Kiho Kim.

Useful Aspergilli

Aspergillus biotechnology ranges from the first steps of sake fermentation to the production of recombinant mammalian proteins. These processes are briefly summarized below.

Oriental Food Uses of *Aspergillus*

The use of Aspergilli in the food industry in the far East relies on the extracellular enzymes secreted by the fungus when grown on solid or semisolid substrates. These technologies originated in China more than 2000 years ago. An old review cites more than a hundred such different fungal fermentations. The main products are shoyu (soy sauce), miso (fermented soybean paste), and sake (rice wine). The production of soy sauce involves the fermentation of a mixture of cooked soybeans and wheat. The mixture is inoculated in traditional production by 'koji', which derives from a previous fermentation, or in more modern procedures by a spore suspension of specific strains of *Aspergillus oryzae* or *Aspergillus sojae*. A second fermentation is carried out by lactic acid bacteria and yeasts. In the production of sake, the Japanese wine derived from rice, the steamed rice is inoculated with spores of *A. oryzae*, and the hydrolyzed product is used as the substrate for alcoholic fermentation by *Saccharomyces sake*. The *Aspergillus* strains used in the soy sauce production differ from those used for sake production, the former are selected for high protease, the latter for high amylase titers. Both *A. oryzae* and *A. sojae* belong to the *A. flavus* groups, and genomic analysis has confirmed the very close relationship between *A. oryzae* and *A. flavus*, while *A. sojae* is considered a domesticated strain of *A. parasiticus*. Through the centuries, the organisms have been in use, they have been selected for both high extracellular enzyme titers and nil toxin production, at least under fermentation conditions. *Aspergillus* fermented food products represent, according to a recent source, 2% of the gross national product of Japan.

Extracellular Enzymes Produced by Aspergilli: Aspergilli as Hosts for Recombinant Proteins

The Aspergilli are major producers of enzymes such as carbohydrate hydrolases, lipases, and proteases, used in a variety of industries such as food, beverages, detergent, and animal food additives industries. The first microbial enzyme to be marketed (1894) was an amylase, 'takadiastase', produced from *A. oryzae*. At least 27 different enzymes are produced industrially by the Aspergilli. Different species, mainly but not exclusively, of the *A. niger*, *A. oryzae*, and *A. sojae* groups have been optimized for the production of specific enzymes. In some cases, increased production has been achieved through proprietary recombinant procedures, which allows an increase in the copy number of homologous and in a few cases heterologous enzyme genes. Chymosin (rennin) is an enzyme essential for cheese production, which prior to its heterologous production by *A. niger var. awamori* (and other microorganisms), had to be extracted from calf's stomach. The stunning efficiency of some of the Aspergilli in the process of enzyme secretion (>20 $g\,l^{-1}$), the considerable experience of the fermentation industry, and the fact that many procedures involving Aspergilli are generally regarded as safe (GRAS) had suggested that the Aspergilli could be used as 'cell factories' for the production of heterologous proteins. This has been successful for some recombinant enzymes (chymosin, lipase, and phytase), but not for high valued, medically important mammalian proteins. Lactoferrin is produced in commercial quantities by recombinant strains of *A. awamori*. More research is needed to understand why some filamentous fungi are so efficient at secreting many fungal proteins but are inefficient as heterologous hosts. Tissue plasminogen activator and interleukin have been experimentally produced at a rate of 12–25 $mg\,l^{-1}$ in a protease-less mutant of *A. niger*. A number of bottlenecks, such as specificity of glycosylation and the onset of the unfolded protein response by the translation of foreign proteins, are under active investigation.

Aspergillus and Production of Organic Acids

Depending on culture conditions, strains of *A. niger* are able to excrete a number of organic acids such as oxalic (used in metal leaching), citric, and itaconic acids and are thus used in their industrial production. Citric acid, a tricarboxylic acid, is an intermediate of the Krebs cycle. It is used in the food, beverage, and pharmaceutical industries. The annual production of citric acid, quoted for 2001, was 1 million tons. The main producing organisms are strains of *A. niger*. Since the ability of the organism to divert its metabolism to the production of citric acid was detected, industrial strains, that can convert over 90% of the carbon source in the culture media (carbohydrates) into citric acid were selected. Industrial carbon sources are low-grade molasses (typically sugar beet), but in principle many other residues of industrial process could be used. Specific culture conditions such as high concentrations of carbon source, low pH, and limitation of ions such as manganese are essential. It is not clear how the metabolism of the organism is diverted to citric acid overproduction. The production of citric acid implies that there is a bottleneck in the Krebs cycle, so that much more citric acid is produced than that is utilized in the cycle. Citrate itself inhibits phosphofructokinase I, the enzyme that catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, a crucial step in glycolysis. It has been proposed that under the culture conditions used, this inhibition is counteracted by other metabolites, thus removing this feedback inhibition of citrate production. Alternatively or additionally, tricarboxylic acid mitochondrial transporters leak out citrate from the mitochondrion, thus depleting the cycle.

Itaconic acid is a dicarboxylic acid, which is used in industry as a precursor of polymers used in plastics, adhesives, and coatings. New uses of itaconic acid-derived polymers are under active investigation. The production of itaconic acid for 2001 was quoted as 15,000 tons. There is a renewed interest in this chemical as industry searches for substitutes of petroleum-derived chemicals. Virtually all itaconic acid produced is by fermentation by specific strains of *A. terreus*. Itaconic acid production is a further perversion of the Krebs cycle, citrate is converted as normally into *cis*-aconitate, which for reasons unknown is, in some organisms, decarboxylated into itaconitate, which has no known metabolic role in the cell.

The fact that different strains of *Aspergillus* and more generally of fungi can divert metabolic pathways to the overproduction and secretion of useful chemicals, coupled with the fact that these organisms can grow on residues of processes such as sugar and ethanol production, open the possibility of engineering pathways to produce high value chemicals through 'green', low polluting, waste-eliminating procedures.

Medically Useful Secondary Metabolites

Of the useful fungal secondary metabolites, the most well-known are the β-lactam antibiotics, penicillin and cephalosporin, and their derivatives. Some Aspergilli, such as *A. nidulans*, produce low titers of isopenicillin N. This has been useful in the elucidation of the genomic organization and regulation of the pathway. It has already been mentioned above (see Section "Aspergillus as Human Pathogens") that the echinocandins are specific antifungal drugs. Anidulafungin is a semisynthetic derivative of echinocandin B_0, produced by *A. nidulans* var. *echinulatus*. Anidulafungin has been recently introduced in clinical practice and it is specifically indicated to treat *Candida* infections of the digestive tract. The most widely used secondary metabolite produced by an *Aspergillus* is lovastatin, produced by *A. terreus*. This metabolite, as other statins, is used in medical practice to reduce cholesterol levels. The market for statins has been estimated at more than 12 billion US$ annually. Statins are specific inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which reduces HMG-CoA to mevalonate. Statins are built around a common polyketide skeleton, have a structure similar to HMG, and act as competitive inhibitors of the HMG-CoA reductase. As other secondary metabolites, statins are synthesized by complex sequential steps, involving polyketide synthases, the genes coding for the cognate enzymes map in a 64 kb gene cluster. Gene clustering and its possible role in the synthesis of secondary metabolites is discussed in 'Regulation of secondary metabolism'.

A. (Emericella) nidulans as a Model Organism

The *A. nidulans* Genetic System

In 1953, Guido Pontecorvo published a 97-page review on the 'Genetics of *Aspergillus nidulans*'. Why did Pontecorvo and coworkers spend a considerable amount of time and energy to develop a genetic system for what was then an exotic organism? One of the key problems of biology was, at the time, that of the nature of the gene. The classical image of the gene was that of a discrete element, and mutations were considered to be alternative states of this element. The gene was an abstract concept whose molecular nature was elusive. The image of the gene as an indivisible, discrete unit was based on the experimental fact that mutations that did not complement were not separable in recombination experiments. That is, crosses of individuals carrying different alleles of the same gene, wild-type progeny were never obtained. 'Never' was a few dozen progeny in mice, a few thousands in *Drosophila melanogaster*. The modern reader may not grasp how fundamental the problem was at the time. In the 1940s, there were already exceptions to the 'nonrecombination' rule. In *D. melanogaster*, a few noncomplementing mutations could recombine in crosses to yield rare wild-type individuals. These mutations were called 'pseudo-alleles'. Pontecorvo was looking for a system where hundreds of thousands progeny could be scored. *A. nidulans* happened to be such an organism. By early 1950s, it became clear in Pontecorvo's laboratory, through the work of Alan Roper, followed by Bob Pritchard, that the gene was divisible. The paradigmatic work on the divisibility of the gene was carried out by Seymour Benzer using the bacteriophage T4., "That is, that the gene as a working unit in physiological action is based on a chromosome segment larger than the unit of mutation or recombination" Roper (1953) and "The classical 'gene' which served at once as the unit of recombination, of mutation, and of function, is no longer adequate. These units require separate definition. A lucid discussion of this problem has been given by Pontecorvo" Benzer (1957). The phage system of Benzer was so powerful and elegant that *A. nidulans*, as a system to study the fine structure of the gene, seemed redundant. Nevertheless, a beautiful genetic system was there, ready for the taking. I dare say that in 1953 no system afforded the same degree of sophistication.

This system allows conventional meiotic genetics, carried out by analyzing the progeny contained in a fruiting body (cleistothecium). The cleistothecium may contain as many as 100,000 thousand asci, each containing eight ascospores, the product of a single meiosis and an additional mitosis. In the standard genetic analysis, the products are analyzed 'in bulk', without isolating single asci, as those are small and difficult to dissect. However, tetrad analysis is possible and was employed in early work. The power of resolution of the 'in bulk' genetic analysis has permitted fine structure mapping to the extent that mutations separated by 11 nucleotides have been resolved by recombination. *A. nidulans* strains carrying different markers can form heterokaryons. Nuclei in heterokaryons can rarely fuse, giving origin to stable diploids, which allow another layer of genetic analysis, developed by Pontecorvo and Etta Käffer, the parasexual cycle. Diploids can revert to haploids in which all markers in one chromosome segregate as a unit, allowing the rapid assignment to any new mutation to one of the eight chromosomes of the organism. Mitotic

recombination also occurs and can be selected for in diploids, permitting the mapping of markers in relation to the centromere. The discovery of the parasexual cycle led to two developments. The first one was the possibility to carry out genetic analysis in the Aspergilli and the related Penicillia where a sexual cycle was not described. It is not known why stable diploids, different from the transient diploids that occur during the sexual cycle, can be obtained in these organisms and not in other filamentous ascomycetes. The second was the analogous development of systems, initiated by Pontecorvo, and based on cell fusion to carry out somatic genetics in mammalian cells. *A. nidulans* entered the molecular era when relatively efficient transformation techniques were worked out in 1983 followed by the development of replicating plasmids. We are witnessing a second methodological revolution, with the development of techniques and modified strains that allow to inactivate genes, introduce point mutations, change promoters, or add tags in a very simple and rapid way, opening the possibility of a high throughput reverse genetics. This, together with the availability of a complete genome and microarrays is changing again the prospects for this model organism. Usually a technique is first worked out in *A. nidulans*, and then that is applied to the other Aspergilli, such as the pathogenic or industrially important organisms mentioned in previous sections. The life cycle of *A. nidulans* is shown in Fig. 4.

Work carried out with *A. nidulans* has served as a model mainly in three aspects of cell and molecular biology (but a few others will be briefly discussed below). Historically, each of these three aspects can be related to a specific scientific school. David Cove and John Pateman initiated the investigation on control of gene expression in *A. nidulans*. The cell biology work derives from an early article by Ron Morris (1976) where he isolated mutations blocked in the cell cycle and in nuclear migration. Bill Timberlake initiated an analysis of the development of the conidiophore, work which profited from the early genetic work of John Clutterbuck, himself a product of the Glasgow school of genetics. Work on secondary metabolism stems from a confluence of this work with work carried out in other species of Aspergilli producing noxious chemicals.

The Mitochondrial DNA of *A. nidulans*

The possibility to construct heterokaryons allows the genetic study of mutations that occur in the mitochondrial genome, as these are cytoplasmically inherited. A few markers were characterized and a circular genetic map was established. In the late 1970s, two groups, led respectively by Hans Künzel and Wayne Davies, attempted to sequence the whole 33,000 bp mitochondrial DNA of *A. nidulans*. This was almost accomplished, except for a gap of around 200 bp. At the time, where the longest DNA sequenced was

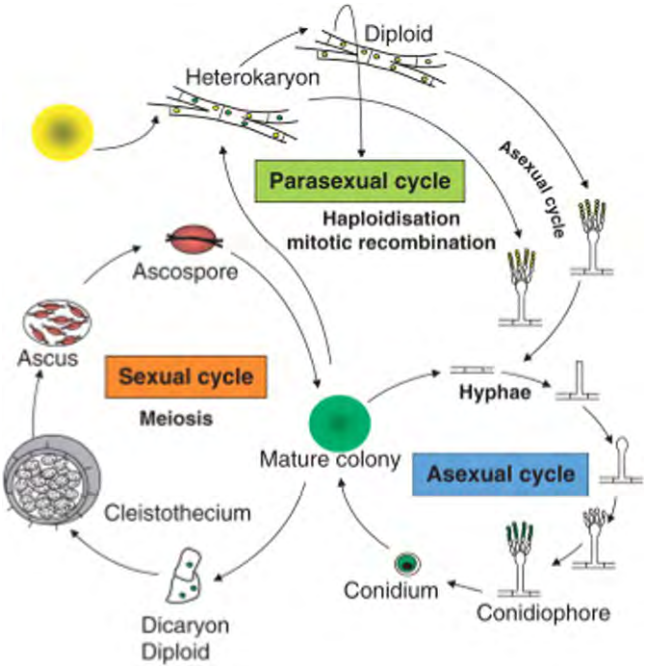

Fig. 4 Schematized life cycle of *Aspergillus nidulans.* Three cycles are shown, the asexual cycle, which has been described in the text (see A. *nidulans* as a model for cell biology and *A. nidulans* developmental pathways). In the sexual cycle, two nuclei divide synchronously as a dikaryon in a specialized structure. Eventually the nuclei fuse to give a diploid, which does not divide as such but undergo meiosis. No differentiated mating types exist; any given mycelium can generate fertile cleisthotecia. To cross two strains, nutritional and color markers are used. Classical genetics procedures are facilitated by the fact that one cleisthotecium derives from only one fertilisation event. In the parasexual cycle nuclei fuse in mycelia, outside the specialized developmental structure to yield a diploid, which does not undergo meiosis (at variance with diploids produced during the sexual cycle) but divides as such. Breaking up of the diploid (haploidization) and mitotic recombination are additional genetic tools. For clarity we have supposed that the heterokaryon and diploid formation are carried out between green (yA^{+}) yellow strains (yA^{-}) and nuclei are colored accordingly. Both diploids and heterokaryons can undergo the asexual cycle. The different structures are not shown to scale. Scheme kindly provided by Stéphane Demais and modified by the author.

the 16,000 bp mitochondrial DNA of *Homo sapiens*, this was a less than trivial enterprise. A number of important results derived from this sequencing effort. It was possible to compare the whole DNA organization with that of the completely sequenced human mitochondrial DNA, and the ongoing sequence of the *S. cerevisiae* mDNA, an organism where sophisticated genetic and molecular studies were actively carried out. These comparisons were extended to other mitochondrial DNA sequencing projects such as those of *Neurospora crassa*, *Podospora anserina*, and *Schizosaccharomyces pombe*. A number of unidentified open reading frames (ORFs) were found in the mitochondrial DNA of *A. nidulans* and the human mitochondrial DNA, but not on that of *S. cerevisiae* or of *S. pombe*. These reading frames correspond to genes coding for complex I, the NADH dehydrogenase complex, which is absent in both model yeasts. The highlight of this work was the elucidation of the structure of class I introns. Metazoan mitochondrial genes have no introns, while introns of two different classes are present in the mitochondria of fungi and plants. Introns on the nuclear genome of the fungi are small, typically of >100 bp. Mitochondrial fungal introns are large, usually >1000 bp, and can contain ORFs. A comparison of the sequences of the mitochondrial introns of *A. nidulans* with those of wild-type and mutant strain of *S. cerevisiae* led to a model of the secondary structure of class I introns, including the proposal of a mechanism of intron splicing. Around the same time, it was published that the intron of the precursor of the ribosomal 26s gene of *Tetrahymena thermophila* was self-splicing. It was noticed that pairings that were postulated by us and by Bernard Dujon and François Michel for mitochondrial group I introns were conserved in this self-splicing intron; thus the model for splicing of mitochondrial class I introns became a model of self-splicing, which was confirmed experimentally. Self-splicing of introns was essential to the concept of ribozyme and eventually to that of a primeval RNA world. Thus, the sequence of the mitochondrial DNA of *A. nidulans* contributed, albeit somewhat indirectly, to present ideas on the origin of life.

A. nidulans as a Model for Genetic Metabolic Diseases

The metabolic versatility of the Aspergilli led a group of Spanish scientists to use mutants blocked in amino acid degradation to identify the enzymes and the genes of human metabolic diseases, including those of aromatic and branched amino acid catabolism. As stated in a review article "The metabolic capacity of *A. nidulans* for amino acid degradation largely resembles that of human liver". Fig. 5 shows the breakdown of phenylalanine and the cognate blocks in human diseases affecting this metabolism. The gene of *A. nidulans* coding for the fumaryl-acetoacetate hydrolase was cloned as a cDNA highly expressed in the presence of phenylacetic acid (*fahA*, Fig. 5). Mutations in the human homologue result in the serious disease tyrosinemia I, and the *A. nidulans* ORF shows 47% identity with the human gene. Satisfactorily, the growth of *A. nidulans* is strongly inhibited by the accumulation of this metabolite in *fahA*-deleted strains. In a second step, suppressor mutations of this inhibited phenotype were isolated.

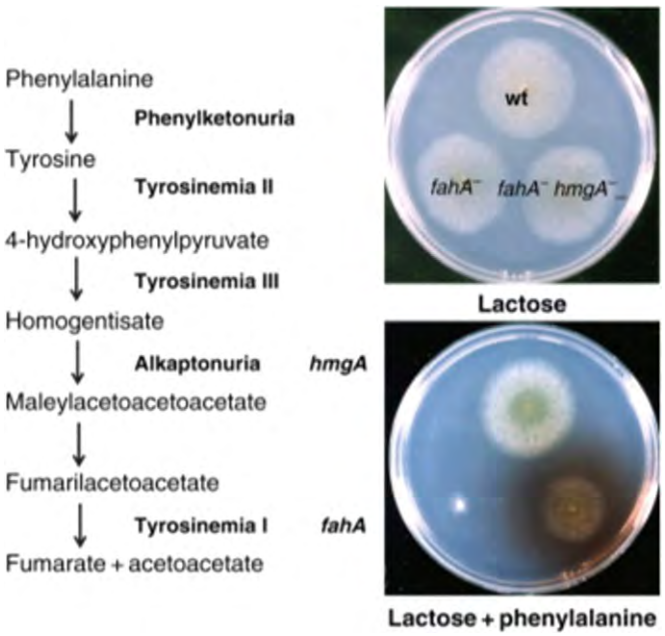

Fig. 5 *Aspergillus nidulans* as a model for human metabolic diseases. To the left the degradation of phenylalanine is shown, to the right of the pathway, the steps blocked in the cognate human metabolic diseases. In italics the relevant corresponding genes of *A. nidulans* are shown. To the right the toxicity of fumaryl-acetoacetate and the suppression of the toxicity by the upstream *hmgA* null is shown together with the secretion of the purple oxidation product of homogentisic acid. Lactose is used as a poor, nonrepressing carbon source, as phenylalanine catabolism is subject to carbon catabolite repression (see Section "Nitrogen and Carbon Utilization"). See text for details. Photographs of plates were kindly provided by Miguel Peñalva.

These pinpointed the gene (*hgmA*) coding for homogentisate dioxygenase. Not only these mutations suppressed the toxicity of phenylalanine seen in *fahA* nulls, they also resulted in the accumulation of a purple pigment (see Fig. 5). This is exactly the pigment that is accumulated in the urine of patients affected by a milder disease, alkaptonuria. The identification of the gene was straightforward and its sequence served to identify the hitherto unknown human gene and to identify the loss-of-function mutations present in a number of patients. Alkaptonuria was identified by Garrod in 1902 as an 'inborn error of metabolism' and shown to be inherited as single Mendelian gene. The work of Beadle and Tatum in *N. crassa*, and the one gene-one enzyme proposal arising from it, can be seen as a completion of Garrod early proposals. The identification of the human gene through the cloning of the *A. nidulans* gene is of more than historical importance and underlines the necessity of choosing the correct model system for a particular problem.

Control of Gene Expression

Nitrogen and Carbon Utilization

The Aspergilli can utilize a large number of metabolites as nitrogen and/or carbon sources. Early work with *A. nidulans* has established some fundamental concepts pertaining to the control of gene expression and metabolic regulation. Some of the first eukaryotic pathway-specific regulatory genes (*nirA*, *uaY*; see below) were characterized in the 1960s. The genes encoding the key regulators for nitrogen (*areA*) and carbon catabolite repression (*creA*) were the first such genes to be described in any eukaryotic organism. Their mode of action was established by formal genetic analysis long before recombinant DNA technology came into existence.

In general, the genes coding for the enzymes involved in the utilization of a specific metabolite are only transcribed in the presence of a specific inducer. Inducers act by activating specific transcription factors, which in turn elicit the transcription of specific catabolic genes. Thus, nitrate activates the NirA protein, necessary for the transcription of the genes encoding nitrate and nitrite reductase and the nitrate transporters, acetaldehyde activates AlcR, regulating ethanol utilization, uric acid activates UaY, regulating at least eight scattered genes encoding enzymes and transporters involved in purine utilization, proline activates PrnA, regulating all the other genes of the proline utilization gene cluster, while β-alanine activates AmdR/IntA a protein that positively regulates the *amdS* gene (encoding acetamidase) and the *gabA* gene (encoding the γ-aminobutyrate transporter).

Almost all the pathway-specific transcription factors belong to a group of proteins that bind DNA through a specific fungal motif, the Zn binuclear cluster (Cys6Zn2). Most bind DNA as dimers, including the paradigmatic *S. cerevisiae* protein GAL4. AlcR is an exception, which uniquely binds as a monomer. NirA and UaY are localized in the nucleus as a result of induction. Nitrate induces by breaking the association of NirA with KapK (the orthologue of the mammalian and *S. cerevisiae* exportins Crm1P and CRM1). PrnA and AlcR are always nuclear, PrnA necessitating induction to bind its cognate sequences in the promoter.

The induction of genes involved in the utilization of nitrogen sources does not occur in the presence of preferred sources such as ammonium and glutamine, while the induction of genes involved in the utilization of carbon sources is strongly diminished in the presence of glucose. These processes, nitrogen metabolite repression and carbon catabolite repression, involve two additional regulators, AreA and CreA. AreA is a GATA factor, acting positively in synergy with the specific regulators (such as NirA or UaY). Ammonium and glutamine negate AreA function at a number of levels, including the stability of its cognate mRNA. The dependence on AreA is absolute for the *niiA-niaD* bidirectional promoter, driving the genes encoding nitrate and nitrite reductases, less marked for some of the genes of the purine utilization pathway.

CreA acts as a genuine repressor in the presence of favored carbon sources, negating the activation by or competing with the binding of the pathway-specific factors such as AlcR.

CreA is a Zn finger protein, with a Zn finger sequence extremely similar to Mig1p, the repressor mediating carbon catabolite repression in *S. cerevisiae* and related organisms. However, the similarity between CreA and Mig1p stops there. Little sequence conservation can be seen outside the DNA-binding domain. Neither the glucose signaling mechanism nor the downstream mechanism of transcriptional repression seems to be shared by Mig1p and CreA. Mig1 represses transcription by recruiting the Tup1/Ssn6p co-repressor complex, which is not the case in *A. nidulans* and most likely in the fungi where a CreA, rather than a Mig1p orthologue, is present.

The Aspergilli can use a number of metabolites as both carbon and nitrogen sources, the mechanism of regulation having been elucidated for the *prn* gene cluster (comprising five genes involved in the utilization of proline) and the *amdS* gene. Repression occurs only when both repressing carbon (glucose) and nitrogen sources (ammonium or glutamine) are present. This can be rationalized by thinking that if a repressing nitrogen source is present, it will be advantageous for the organism to use proline or acetamide as a carbon source, while if only a favored carbon source is present, it will still be advantageous to use proline or acetamide as a nitrogen source. Carbon metabolite repression requires the CreA repressor, while nitrogen metabolite repression operates through the inactivation of the AreA GATA factor. While, for example, in the nitrate assimilation pathway AreA is always essential for transcription to occur, for *prn* and *amdS*, it is only necessary when the CreA repressor is activated by a repressing carbon source. These regulatory patterns are conserved in the Aspergilli and more generally in the filamentous ascomycetes and are schematized in Figs. 6–8, while the nuclear-cytoplasmic shuffling of NirA is illustrated in Fig. 9. The *gabA* gene, encoding the γ-aminobutyrate transporter, is subject to an even more complex pattern of regulation. It is induced by ω-amino acids and subject to concomitant repression by nitrogen, carbon, and alkaline pH.

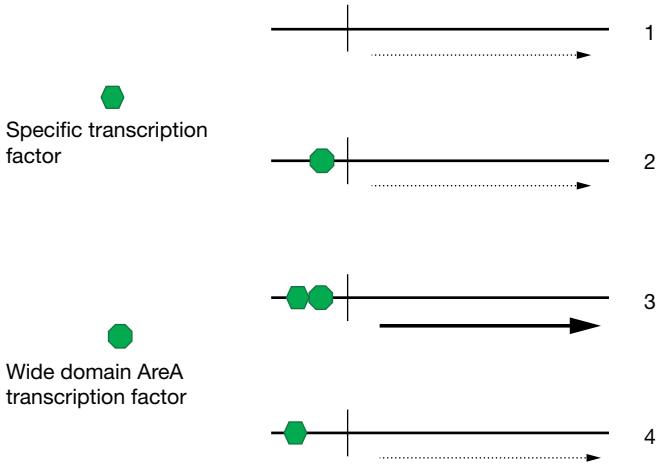

Fig. 6 General scheme of the transcriptional regulation of genes involved in the utilization of nitrogen sources. (1) In the absence of a specific inducer (such as nitrate) and in the presence of a preferred, repressing nitrogen source (ammonium, glutamine), neither the specific transcription factor nor the broad-domain GATA factor AreA is activated. No or only basal transcription is seen. (2) In the absence of a specific inducer in the presence of a nonrepressive nitrogen source, only AreA is active. No or only basal transcription is seen. (3) In the presence of a specific inducer and in the absence of a repressing nitrogen source, both transcription factors are active, full transcription is seen. (4) In the presence of both a specific inducer and a repressing nitrogen source, the specific transcription factor is active, but the AreA factor is inactive and no transcription is seen. In the nitrate utilization pathway a further mechanism is in act, as AreA is necessary both indirectly through its regulation of transporters for the uptake of the specific inducer (nitrate) and for the binding of the specific transcription factor (NirA) to DNA. Thus, the situation will be identical to that seen in Scheme 1, at the top of this figure.

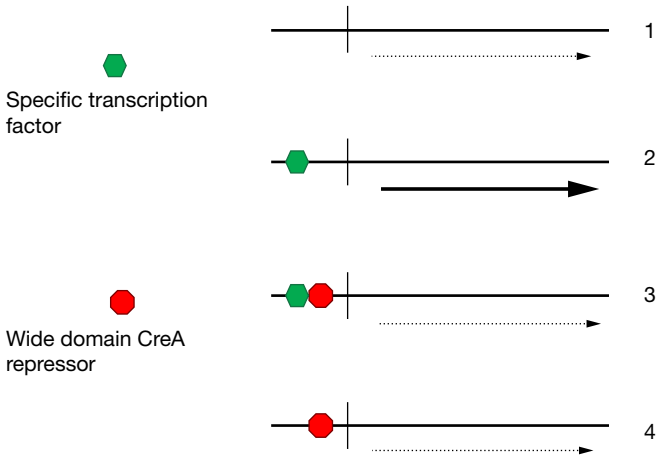

Fig. 7 General scheme of the transcriptional regulation of genes involved in the utilization of carbon sources. (1) In the presence of 'neutral' carbon source (such as glycerol) and the absence of an inducing carbon source, neither the specific positive-acting transcription factor nor the CreA repressor are bound to the promoter. No or only basal transcription is seen. (2) In the presence of an inducer carbon source, the specific transcription factor (such as AlcR in the ethanol utilization pathway) is bound to DNA and active, full transcription is seen. (3) In the presence of both inducing and repressing carbon sources, the specific transcription factor is active but the CreA repression partially or totally negates its effect. No or only basal transcription is seen. (4) In the presence of only a repressing carbon source, only the CreA repressor is bound to DNA, no or only basal transcription is seen.

Regulation of Gene Expression by External pH

Soil organisms, such as the Aspergilli, respond to a variety of environments and it is not surprising that a system that regulates gene expression as a function of external pH has evolved. External pH regulates genes coding for extracellular enzymes or transporters or those encoding steps in the synthesis of exported metabolites. In neutropenic mice experimentally infected with *A. nidulans*, this process is necessary for virulence. Penicillin is synthesized by some Aspergilli but only at alkaline pH. The synthesis and uptake of siderophores is also regulated by pH.

The elucidation of the mechanism of pH regulation is a superb scientific achievement of the groups of Herb Arst and Miguel Angel Peñalva. The signal transduction pathway described below is conserved throughout the ascomycetes. The key actor is PacC, a transcription factor of the classical Zn finger type. In its active form, PacC acts as a positive transcription factor of alkaline-expressed genes (such as alkaline phosphatase or isopenicillin synthase) and as a repressor of acid-expressed genes (such as

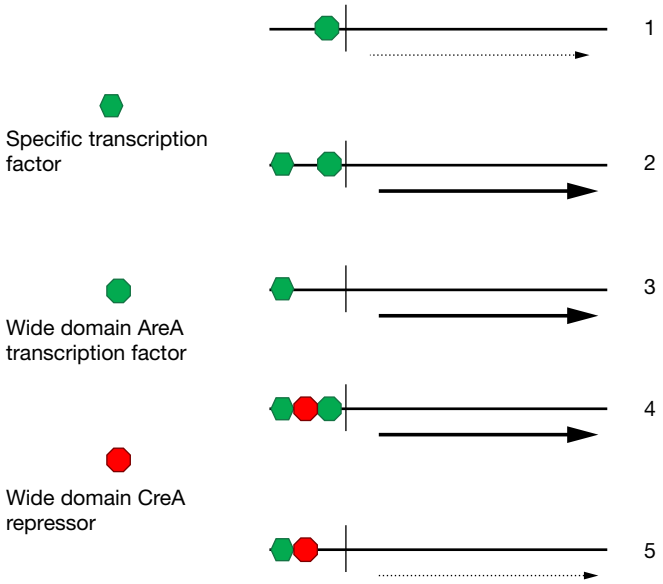

Fig. 8 General scheme of the transcriptional regulation of genes involved in the utilization of metabolites that can serve as both nitrogen and carbon sources. (1) In conditions where the inducer is not present the specific transcription factor (PrnA in the proline utilization gene cluster) is not bound to the promoter. In the scheme shown, under neutral conditions (e.g., urea as nitrogen source, lactose as carbon source) AreA would be bound, and CreA would not be bound. No or only basal transcription is seen. This applies to every other combination (not shown) where the specific inducer is absent. (2) Same conditions but in the presence of the inducer (proline in the example given in the text), both the specific transcription factor (such as PrnA) and AreA are bound, full transcription is seen. (3) In the presence of the inducer and a repressing nitrogen source (ammonium or glutamine) but no repressing carbon source. Only the specific transcription factor is bound. Full or almost full transcription. (4) In the presence of the inducer and a repressing carbon source (glucose), but no repressing nitrogen source. The three regulatory proteins are bound; AreA negates the repressing action of CreA. Full or almost full transcription. (5) In the presence of inducer (such as proline) and both carbon and nitrogen repressing metabolites (ammonium or glutamine and glucose). The specific transcription factor (such as PrnA) is bound and CreA negates its action. Efficient repression. No or only basal transcription is seen.

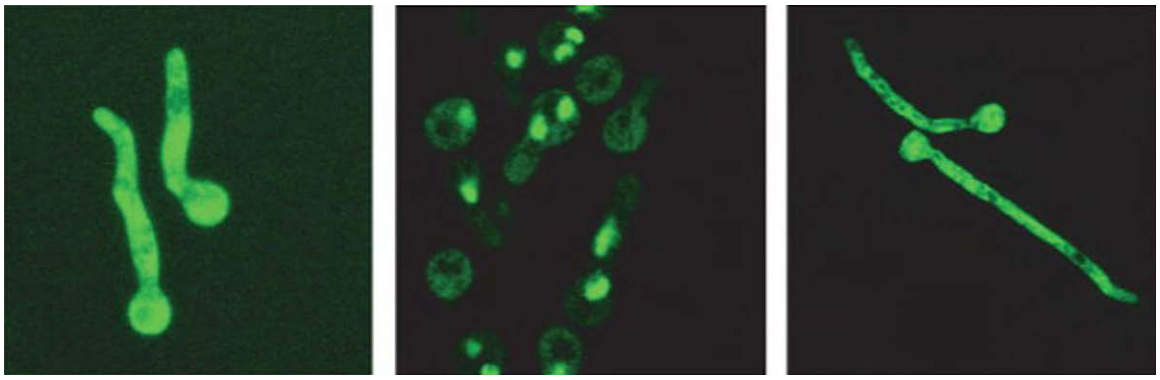

Fig. 9 Cytoplasmic and nuclear localization of the NirA transcription factor. A construction where the whole NirA transcription factor is fused to green fluorescent protein (GFP) substitutes the NirA wild-type gene. This construction is competent to mediate induction by nitrate. NirA is localized in the cytoplasm in the presence of a noninducing, nonrepressing nitrogen source (arginine), of a repressing nitrogen source (ammonium) and localizes in the nucleus only when an inducing nitrogen source is present. See text for details. This figure illustrates also the technology of gene fusions and epifluorescence microscopy, which is been extended to all other Aspergilli and many filamentous fungi, including animal and plant pathogens. The original pictures were kindly provided by Joseph Strauss.

acid phosphatase or the γ-aminobutyrate transporter). At acidic pH (pH usually tested 4.0), there is no activation signal, and the protein is in an inactive form. In the full-length PacC ($PacC^{72}$), intramolecular interactions hold the protein in a folded inactive form, which is largely excluded from the nucleus. At alkaline pH values (usually 8.0), the protein is activated by two proteolytic steps. The *palA*, *palB*, *palC*, *palF*, *palH*, and *palI* genes encode proteins involved in pH sensing and in signal transduction. Mutations in all these *pal* genes have an acidity-mimicking phenotype, while mutation in the transcription factor *pacC* can lead to acidity mimicking (loss-of-function mutations), alkalinity-mimicking or neutrality-mimicking phenotypes, where both 'alkaline' and 'acidic' genes are expressed. The pH sensor is probably PalH assisted by PalI, both of which are plasma membrane proteins.

The C-terminus cytoplasmic tail of PalH interacts directly with PalF, a member of the arrestin family, which is, similarly to the mammalian arrestins, phosphorylated and ubiquinated. These modifications occur at alkaline pH and are dependent on the PalH and PalI proteins. Under alkaline pH conditions, PalA binds to motifs flanking a specific protease-sensitive sequence in the C-terminus of the full-length PacC ($PacC^{72}$). PalA interacts directly with the A. nidulans orthologue of Vps32, a protein involved in the formation of multivesicular endosomes. PacC/PalA interaction renders PacC sensitive to a specific cleavage, catalyzed by PalB, a protease of the calpain family. $PacC^{72}$ is cleaved to $PacC^{53}$. The cleaved form of PacC becomes susceptible to further processing by the proteasome, yielding $PacC^{27}$. This is the active form of PacC, strictly localized in the nucleus, where it activates genes expressed at alkaline pH and represses genes expressed at acid pH. This account leaves open the mechanism of pH sensing and the connection between the arrestin-like PalF and PalA-PalB. The interactions of PalA with components of the mature endosome, and recent work on the related signal transduction pathway in *S. cerevisiae*, strongly suggests that endocytosis provides this connection. PalC, the unplaced actor of the process, has a functionally important Bro1 domain (also present in PalA), a domain of possible interaction with Vps32, strengthening the endosomal connection of the pH signaling pathway. The YPXL/I motif recognized by PalA is also recognized by its putative mammalian orthologue, AIP1/Alix, a protein involved in a variety of functions, including the budding of the human HIV virus from infected cells. The whole process has tantalizing similarities with the Hedgehog signaling pathway in metazoans, leading to the proteolytic activation of the Zn finger transcription factor *cubitus interruptus*/*Gli*, posing the question of whether these pathways are evolutionarily related. A simplified version of the pH signaling process is shown in Fig. 10.

Specific Regulatory Mechanisms Acting at the Level of Transporters

The control of transporter synthesis and activity is a key step in metabolic regulation, as the activity of specific transporters modulates the entry of metabolites that serve as inducers or repressors of specific pathways. Work with *A. nidulans* has led to the identification of two new control processes affecting transporters, besides their tight specific transcriptional regulation. The transcription of a number of transporters is activated during the isotropic phase of conidial germination (see below). This is a developmental control, which bypasses other specific control systems. Recent transcriptomic work suggests that this mechanism occurs for many transporters and is general for the filamentous ascomycetes. It can be proposed that germinating fungal spores explore an unknown environment by expressing a whole range of transporters, to progress to specific induction once the spore has germinated. The second mechanism is posttranslational. In the presence of a favored nitrogen source such as ammonium, both purine and amino acid transporters are internalized to the vacuole, where they are possibly destroyed. This posttranslational mechanism is synergistic with but independent from the nitrogen metabolite repression mechanism (described above). Fig. 11 illustrates this process.

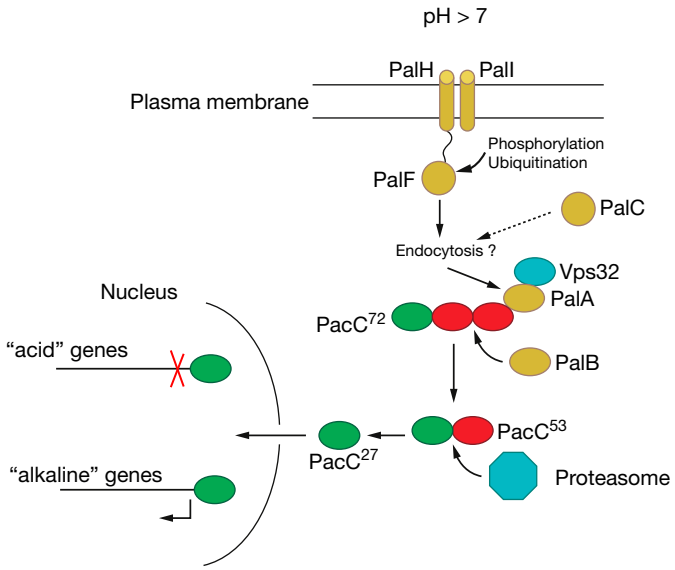

Fig. 10 Simplified scheme of the regulation of gene expression by external pH, redrawn from a number of articles of the groups of Arst and Peñalva. Alkaline pH is sensed by the PalH and PalI proteins, the signal is transduced to the PalF arrestin via the C-terminus of PalH. PalF is phosphorylated and ubiquinated and signals PalA, which has been shown to interact with the endosomal protein Vps32. The role of PalC is hypothetical. PalA leads to the opening of PacC72, which is cleaved by PalB at a specific site. PacC53 is further processed by the proteasome, leading to the active form PacC27. In this simplified scheme both proteolytic processing steps are shown in the cytoplasm, in fact the second step may occur in either or both the cytoplasm and the nucleus. In yellow, proteins of the pH signal transducing pathway, in blue cellular proteins or complexes nonspecific for the pathway. The 'active' portion of PacC is shown in green, the inhibitory, cleaved portions are shown in red.

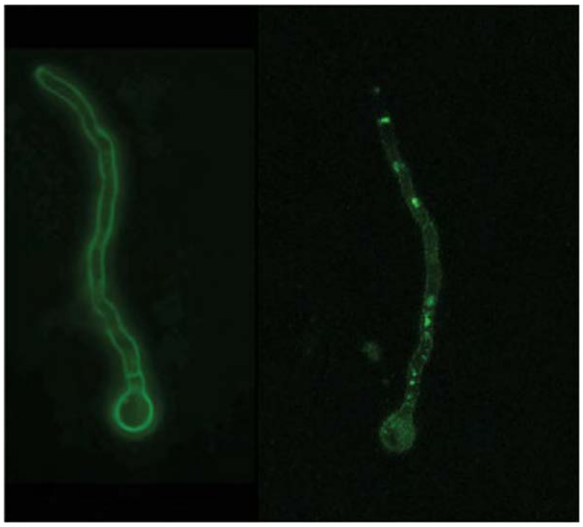

Fig. 11 Posttranslational regulation of transporters. The left panel shows the membrane localization of a fusion with the green fluorescent protein of the proline transporter (PrnB-GFP). The cognate gene *prnB* maps in the proline gene cluster, its transcriptional regulation is schematized in Fig. 8 (see Section "Nitrogen and Carbon Utilization"). Conidiospores were grown for 16 h at 25°C in the presence of urea as nitrogen source, and induced with L-proline after 10 h. In the right-hand panel ammonium was added for the last 2 h. In the left panel, the PrnB-GFP fusion strongly stains also the basal septum. Localization in septa is a characteristic of all membrane proteins studied up to now. Confocal microscope images were kindly provided by Vicky Sophianopoulou.

Regulation of Secondary Metabolism

Fungi produce an astonishing variety of secondary metabolites. Fungal toxins, the β-lactam antibiotics and lovastatin, have already been mentioned. In the pregenomic days, conventional genetic analysis led to the identification, cloning, and sequencing of a number of genes encoding biosynthetic steps for a number of secondary metabolites, while many more metabolites were identified as secreted by a variety of Aspergilli. As many secondary metabolites involve nonribosomal peptide or polyketide synthases, putative fungal metabolite gene clusters have been identified in a number of fungal genomes. In *A. fumigatus*, the estimate is that of 22 secondary metabolite gene clusters. The best studied pathways are those leading to the biosynthesis of isopenicillin in *A. nidulans*, aflatoxin in *A. flavus*, and the aflatoxin precursor sterigmatocystin in *A. nidulans*. Secondary metabolism synthesis occurs late during mycelial growth and is generally correlated with conidiation and shares with this process some of its signaling pathway. Pathway-specific transcription factors have been characterized for the aflatoxin, sterigmatocystin, and gliotoxin pathways. The clusters of aflatoxin and sterigmatocystin biosynthesis include the regulatory gene *aflR*, necessary for the expression of the rest of the genes of the cluster. AflR belongs to the Cys_6Zn_2 family of specific fungal activators. It is not known whether the activation of AflR involves a specific metabolite or if it is only activated by the 'fluffy' signaling pathway to be described below (*A. nidulans* developmental pathways). No pathway-specific activator has been described for isopenicillin biosynthesis, which is regulated by a number of environmental parameters, including extracellular pH.

Clustering of genes is variable for genes involved in primary metabolism. In contrast, the genes of secondary metabolism biosynthesis are as a rule organised in large clusters. The 70 kb aflatoxin cluster comprises 25 coregulated genes. The gliotoxin gene cluster comprises 12 genes. Does the clustering of secondary metabolism genes have an evolutionary and/or functional significance? Possibly the two divergently transcribed genes responsible for isopenicillin-N synthesis have been horizontally transferred from a *Streptomyces* to an ancestor of the Aspergilli and Penicillia. There is no evidence for horizontal transfer for any other secondary metabolite gene cluster. Comparative genomics is providing some clues, even if not yet an answer, to the significance of secondary metabolite gene clustering. There is a significant bias toward the location of secondary metabolite clusters in subtelomeric regions. The fact that species of Aspergilli differ widely in the secondary metabolites they produce correlates with the mapping of the cognate genes in genomic regions where synteny between species is broken.

A fundamental advance in the understanding of the regulation of secondary metabolism arises from the discovery of the global regulator LaeA in the laboratory of Nancy Keller. LaeA is conserved in filamentous fungi, but not in yeasts. LaeA shows a domain typical of histone methyltransferases, the SAM domain, while lacking a second domain found in these enzymes, the SET domain. LaeA regulates positively the synthesis of isopenicillin, sterigmatocystin, gliotoxin, and lovastatin. The global role of LaeA has recently been investigated by transcriptomic studies with *A. fumigatus*. Of the 22 gene clusters, a deletion of *laeA* diminishes clearly the transcription of 13. Thus LaeA is a broad, but not a universal regulator of secondary metabolism. Recent work points to a role of LaeA in remodeling chromatin structure. In *A. nidulans*, deletion of a number of genes universally involved in gene silencing in heterochromatin result in premature secondary metabolite production. More strikingly, these deletions act as partial suppressors of a *laeA* deletion. Thus the exciting possibility arises that LaeA acts by reversing a heterochromatic state of the secondary metabolite gene clusters. Thus the study of the regulation of secondary metabolism may lead to an understanding of the role and genomic distribution of heterochromatin in filamentous ascomycetes.

A. nidulans as a Model for Cell Biology

The life cycle of the Aspergilli includes a number of tightly regulated developmental pathways, from the germination of conidia or ascospores to the formation of complex structures involved in sexual (cleistothecia) or asexual (conidiophore) spore formation. The germination of conidiospores, but not that of ascospores, has been well studied. Conidiospores can stay dormant and viable for many years and contain (in *A. nidulans*) one nucleus arrested in the G1 phase. When plated on suitable media, they go through a phase of isotropic growth, where the conidium swells. The first mitosis may occur in this phase or after the emergence of the germ tube (Fig. 12). Mitosis occurs synchronically, up to the eight nuclei stage when a perforated septum appears basally (Fig. 11). Other septa are laid during hyphal growth out every – three to four nuclei. Only the nuclei comprised between the septum and hyphal tip are competent to divide and they do so synchronously. A second germ tube can arise from the conidiospore at 180° from the first one. Nuclei in nonapical compartments became again competent to divide when the conidiophore is developed (see below) and when branches arise from subapical compartments. Thus a highly coordinated process occurs, involving the regulation of mitosis, the establishment of a primary polar axis, the establishment of secondary polar axes in branches, nuclear migration, the laying down of septa and finally the appearance of another highly polarized structure, the conidiophore. Some processes, such as hyphal polar growth and the deposition of septa, are specific of fungi, while others are common to all eukaryotes, and the *A. nidulans* work matches and has added considerable information to the work carried out in *S. pombe* and *S. cerevisiae*. In both yeasts and most cells in higher eukaryotes, mitosis is followed by cytokinesis, where the two daughter cells separate. This is not exactly the case in filamentous fungi, where the whole mycelium is one syncytium, subdivided by perforated septa. It must be stressed that during conidiogenesis the situation resembles budding, with proper cytokinesis, as metullae, phialides, and conidia are uninucleate cells, while ascospores are binucleate (see '*A. nidulans* developmental pathways'). Thus an understanding of *Aspergillus* cytokinesis involves understanding the generation of these different patterns. The determination of hyphal polarity and the related problem of the relationship of mitosis with septum formation are active fields of research at present, and recent work has shown that while some of the determinants of polarity and cytokinesis are common with the yeasts, some are entirely novel. In particular, a specific ceramide synthase is essential for polarity, probably by generating specific lipid rafts at the growing tip, which in turn would be involved in the localization of other polarity determinants such as formin, an actin nucleating protein. Particular to filamentous fungal growth is the Spitzenkörper, a subapical organelle that acts as a vesicle supply center. The challenge for future research is to understand the coordination of signaling pathways, the polarization of the actin cytoskeleton, the formation of lipid rafts, and the activity of the Spitzenkörper to reach a complete understanding of polarity determination.

Some of the highlights of the work relating to the cell cycle are indicated below, where *A. nidulans* has served as an eukaryotic model, while some specific aspects of *Aspergillus* development are summarized in '*A. nidulans* developmental pathways'.

The judicious use of mutants resistant to the tubulin inhibitor benomyl led to the identification of the first α- and *β*-tubulin-encoding genes in any organism. It was then shown that the tubulins are involved in nuclear and chromosomal movement. The crowning of this work was the discovery of *γ*-tubulin by Berl and Liz Oakley. A *benA* (encoding one of the isoforms of β-tubulin) temperature-sensitive mutant, *benA33*, results in microtubules that are hyperstable (rather than non-functional) at the nonpermissive temperature. Three suppressors of *benA33* mapped in a gene that when cloned and sequenced was shown to code for a new tubulin. This tubulin is critical for the nucleation of microtubules in all eukaryotes where it has

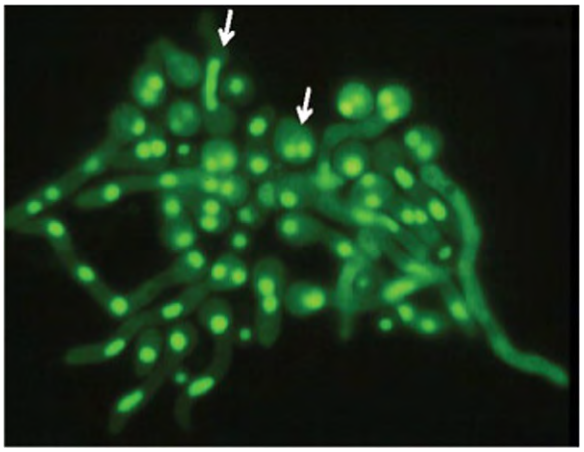

Fig. 12 Conidial germination. A group of germinating conidia from A. nidulans are shown. They are stained with the green fluorescent protein (GFP) fused to a strong nuclear localization signal, driven by a strong constitutive promoter. One white arrow indicates a conidia where the first mitosis has occurred before the production of the germinal tube, another mitosis occurring concomitantly with germination. Note that the signal is not lost during mitosis, which as in other fungi is closed. Photograph by Ana Pokorska in the laboratory of the author.

been studied. The establishment of the function of γ-tubulin illustrates the use of *A. nidulans* as a model organism. While the inactivation of the cognate gene is lethal, the mutation could be maintained in a heterokaryon (see Section "The *A. nidulans* Genetic System"). As conidia are uninucleate, heterokaryons will produce two types of conidia, one of which carries the disrupted allele, where the phenotype caused by the mutation during conidial germination can be assessed microscopically. The disruption does not affect germination, but blocks nuclear division and to some extent nuclear migration. DNA is replicated, chromosomes condense, but spindles are not assembled. Thus, work that started with the isolation of tubulin inhibitor-resistant mutants led to the discovery of a new tubulin, which in all organisms is crucial for microtubule nucleation in centrosomes and in fungi (which have a closed mitosis) in spindle polar bodies.

In the seminal Morris article of 1976, a large number of conditional mutants were characterized. These were temperature-sensitive mutants, which either failed to enter mitosis at the nonpermissive temperature (*nim*, never in mitosis), were blocked at different stages (*bim*, blocked in mitosis), or where the nuclei failed to migrate (*nud*, nuclear distribution), while *sep* mutants are defective in septum formation. Eventually, the cognate genes were cloned and sequenced, suppressors were isolated and identified, to give a growing picture of the genes involved in basic processes of cell biology.

Cellular motors of the myosin class associate with actin filaments, while kinesis and dyneins move cargo (vesicles and organelles) along microtubules. *nudA* encodes the dynein heavy chain, *nudG* the dynein light chain, while other *nud* mutants defined hitherto undescribed regulatory proteins of the dynein complex. In particular, *nudF* encodes a close homologue of the human protein LIS1, which is mutated in Miller–Dicker lysencephaly, a human hereditary disease of the nervous system where neurons fail to migrate in the hemizygote. NudC, a protein that interacts with NudF is also conserved from fungi to mammals. It is likely that the primary effects of NudC/NudF in organisms with an open mitosis are in cytokinesis, a role obviously that can only be partially conserved in a syncytial organism with a closed mitosis such as *A. nidulans*. This pioneering work, which exploited both the *A. nidulans* genetic system and its specific morphology, has guided the work leading to the understanding of the function of the dynein complex in the nervous system.

At variance with dyneins, kinesin genes are highly redundant and only one was identified through mutant screens. This is *bimC*, which defines a specific class of plus-end conserved kinesins. Mutants in this gene are defective in spindle pole separation and are thus blocked in nuclear division and provided the first direct evidence the kinesins are involved in mitosis.

In the genetic screen, no mutants blocked in G1 were found, mutants blocked in the S-phase map at five loci, others blocked in the transition of G2 to mitosis map at six loci. Among the genes so defined, some are orthologues of genes previously known from *S. pombe*. *nimX* (not identified in the screen) encodes the orthologue of the cyclin-dependent *S. pombe* cdc2 kinase. The homologue of the cdc13 cyclin B is encoded by *nimE*, while the phosphatase activity necessary for the activation of $NimX^{cdc2}$ is encoded by *nimT*. NimA, on the contrary, is a newly discovered serine/threonine kinase, which defines a whole class of proteins conserved throughout the eukaryotes. NimA functions downstream of $NimX^{cdc2}$/cyclin B, which would then have two independent functions, one to promote spindle formation, through the activation of other kinases, the second to activate NimA, which in turn is necessary for chromosome condensation. NimA is necessary for entry into mitosis, mutants showing duplicated spindle polar bodies, while its destruction by proteolysis is necessary for exit from mitosis. There is a considerable evidence for similar roles in mitosis for NimA homologues in higher eukaryotes. A human protein, Pin1, interacting with NimA was identified in a two-hybrid screen. Pin1 mutants have a phenotype reciprocal to that of NimA mutants, suggesting that Pin1 (PinA in *A. nidulans*) is involved in the inactivation of NimA. Pin1 is a universally (in eukaryotes) conserved peptidyl-prolyl isomerase that catalyzes specifically the isomerization of prolyl bonds in a P-Ser/Thr-Pro dipeptide, increasing its rate by about 1000 times, thus allowing a drastic change in the peptide backbone conformation, NimA is only one of its substrates, another one being cdc2/cyclin B. It has recently been shown in HeLa cells that Pin1 is necessary for entry into mitosis, associates with mitotic chromosomes, and it strongly stimulates cdc2 phosphorylation. The discovery of Pin1 and its involvement in mitosis has led to flurry of activity, concerning its possible role in cancer, but more cogently in the onset of Alzheimer's disease. Mice homozygously deleted for the *Pin1* gene develop a neuronal degeneration with many of the histological characteristics of Alzheimer's. Both *tau*, a microtubule-associated protein, and APP (amyloid precursor protein) are phosphorylated at Ser/Thr-Pro motifs. These proteins are hyperphosphorylated and insoluble in Alzheimer's. It had been proposed that the key regulator of the state of these proteins is actually Pin1, which would displace the equilibrium toward the nonphosphorylated, soluble forms.

The anaphase-promoting complex (APC) is an ubiquitin ligase that targets key mitotic proteins such as cyclins and directs them to the proteasome. Mutants in its components will be expected to be blocked in metaphase and to show a *bim* phenotype. Two such components were first identified among the *bim* mutants. BimE was identified first as a negative regulator of mitosis. Biochemical work in *Xenopus* oocytes showed that a protein that copurified with APC (APC1) is the orthologue of BimE. *bimA* encodes the APC3 component. Once all chromosomes are attached to microtubules, APC activation results in degradation of securin. This releases and activates separase, a protease that cleaves cohesin. As cohesin keeps sister chromosomes together, this cleavage is the prerequisite for anaphase. *bimB* encodes separase. A component of cohesin, *sudA*, was identified as a suppressor of a *bimD* allele, which itself results in an anaphase block characterized for defective chromosome separation. Finally, mutations in *bimG* result in large, polyploid nuclei that fail to complete anaphase. Nuclei are clumped and conidia fail to germinate highlighting the link between the regulation of mitosis and the establishment of polarity. BimG is a phosphatase, showing striking identity with mammalian phosphatases of the PP1 class. BimG is localized to the spindle polar bodies, to the nucleolus, to the tip of the hypha, and transiently in the septum. There is no hint as to what are the substrates of BimG in the mitosis, septum formation, and polarity establishment.

A. nidulans Developmental Pathways

In the sexually reproducing Aspergilli, the mycelial mat can follow two different developmental pathways. Meiosis and the formation of asci that contain ascospores, occurs in specialized structures, the cleistothecia. In *A. nidulans*, mature cleistothecia are globose, darkly pigmented structures of 100–200 μm in diameter. Ascospores have a characteristic bivalve morphology (about 4 μm×3.5 μm) showing two equatorial crests. Ascospore ornamentation is a valuable taxonomical character in the sexually reproducing Aspergilli. The protocleistothecium is generated from vegetative hyphae, which coil in a spherical structure developing into a cleistothecium, surrounded by specialized, modified hyphal cells called hülle cells. *A. nidulans* is homothallic; two genetically identical nuclei can fuse to give diploids which, as in all other filamentous ascomycetes, are immediately committed to meiosis. In heterothallic Aspergilli, the sexual cycle occurs only when nuclei of opposite mating types meet in heterokaryons. Some nonsexual Aspergilli (*A. flavus and A. parasiticus*) form structures, sclerotia, which may be developmentally related to the cleistothecium. Conceptually, we can distinguish two processes in the development of the mature cleistothecium. One is the morphological process that leads to cleisthotecia, surrounded by hülle cells. The second is the behavior of nuclei, which in the primordium of the cleisthotecium form dikaryons, in which two nuclei divide synchronously. Dicaryotic nuclei fuse into transient diploids, which undergo immediate meiosis, followed by two mitoses leading to eight binucleate haploid ascospores per ascus. These processes can be experimentally separated, as it is possible to obtain morphologically perfect cleisthothecia that do not contain asci. Many genes, including transcription factors and G-coupled receptors, have been implicated in either or both processes. The availability of the genome and possibility of following tagged proteins through the developmental processes should lead to an understanding of the sexual maturation process, including the roles of mating types in homothallic and also heterothallic species. Very recent work has established that both α and HMR mating type genes (see Section "The Genus *Aspergillus* in the Genomic Era") are necessary for fertility but not for cleistothecial formation. At present, we cannot yet draw a scheme of the developmental pathway leading to the formation of sexually mature, fertile cleistothecia.

The second developmental pathway is the formation of asexual conidia, which is present in all Aspergilli. These are formed from a specific structure, the conidiophore, which is the taxonomic marker of the genus. Conidiophores sizes range from 50 to 70 μm long in *A. nidulans* as much as 5 cm in *Aspergillus giganteus*. The structure of the conidiophore is shown in Fig. 13. From the flat mycelial mat, a stalk grows from a foot compartment at a right angle from the mat. The stalk then swells into a multinucleate vesicle. From the vesicle a first series of cells arise, the metulae or primary sterigmata. About 60 metulae are formed in each vesicle. Each metula buds at its tip to give two or three uninuclear phialides, also called secondary sterigmata. From the phialide, uninuclear

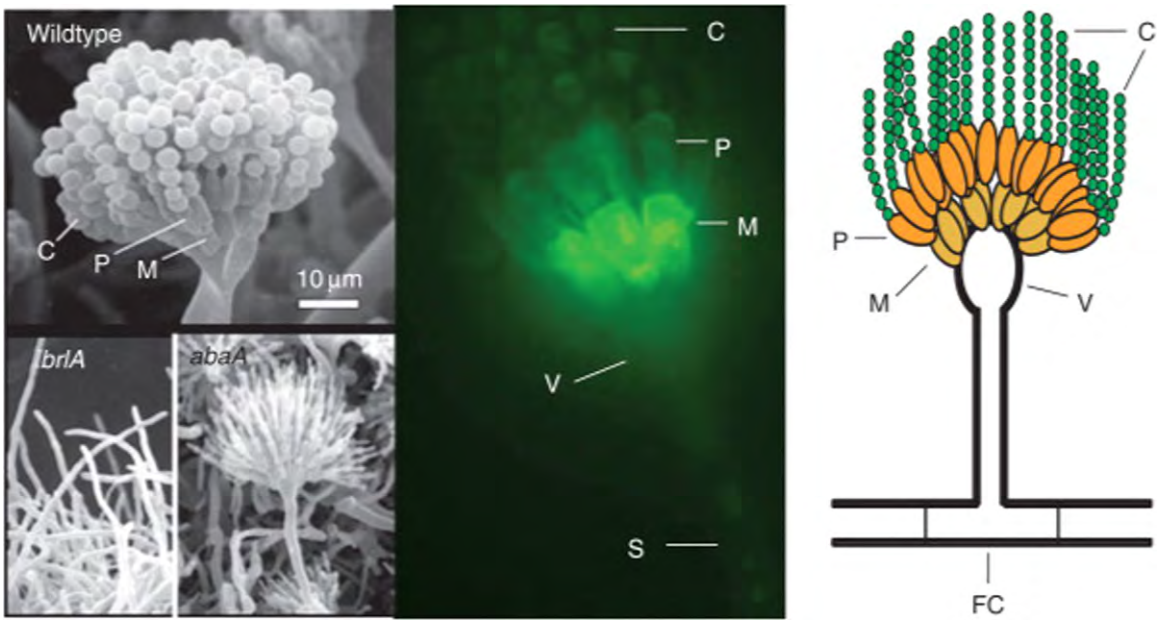

Fig. 13 The condiophore of *Aspergillus* nidulans. In the left panel scanning electron microscopy images of condiophore of the wild-type and two mutant strains are shown, these carry loss-of-function mutations in the *brlA* (bristle) and *abaA* (abacus) gene respectively. The center panel illustrates the developmental process by showing the expression of a membrane protein (the UapA transporter fused to the green fluorescent protein (GFP)), which is specifically expressed in the metula stage, which is then diluted on in the phialides and conidia, the same transporter is then expressed again during conidial germination (see text). The right panel show a schematic representation of the conidiophore of *A. nidulans*, metulae and phialides are arbitrarily colored to facilitate identification. FC, foot compartment; S, conidiophore stalk; V, Vesicle; M, metula; P, phialide; C, conidia. Notice that for some metulae in the left and center panels the two cognate phialides can be clearly seen. The pictures in the left hand panel has been kindly provided by Reinhrad Fischer. Reproduced with permission from Kues, U., Fischer., R. (Eds), 2006 The Micota I, Growth Differentiation and Sexuality. Berlin: Springer-Verlag. One of the center panel by George Diallinas and Areti Pantazopoulou.

conidia bud, only one nucleus enters each conidium. The process is repeated, in such a way that clonal rows of conidia are formed, the last conidium to be formed is adjacent to the metula, the first and oldest being the most distal one. This process is not identical in all Aspergilli; some species, called uniseriate (such as *A. fumigatus*), have only one series of sterigmata from where conidia arise directly, while in some Aspergilli, conidia contain more than one nucleus (such as *A. oryzae*).

Two approaches were used to study this process. In the first one, mutants were isolated and blocked in different steps of conidiophore development; in the second, mycelia were synchronized, and by the technique of 'cascade hybridization', an early methodology to define a transcriptome, it was determined which genes were expressed at different stages of conidiophore development. John Clutterbuck published in 1969 the seminal article of the study of the conidiophore developmental pathway, while the first cascade experiment in any organism was published by the Timberlake Laboratory in 1980. Clutterbuck described a number of mutants blocked in different steps of conidiophore development. In *bristle* mutants (*brlA*), conidiophore stalks that fail to complete the developmental pathway originate from the mycelial mat. In *abacus* mutants (*abaA*), sterigmata continue to give row after row of additional sterigmata, without ever terminally differentiate phialidae or conidia (Fig. 14). *Stunted* mutants (*stuA*) result in short conidiophores with conidia being made directly from the vesicle, while in *medusa* (*medA*), metulae do not immediately differentiate and produce series on metulae before giving origin to phialides. Finally, *wet* mutants (*wetA*) do not affect the development of the conidiophore, but result in defective conidia that autolyze. Once these genes were cloned, their function was analyzed by inactivating them, following their expression pattern and overexpressing them conditionally using the tightly regulated *alcA* promoter (see above). BrlA is a Zn finger transcription factor, which directly regulates the expression of *abaA*. AbaA is also a transcription factor, which regulates *brlA* in a feedback loop and *wetA*. WetA regulates late-expressed and conidial-specific genes such as cell wall genes, and it is supposed to be a transcription factor. StuA has the characteristics of a transcription factor and limits in some unknown way the spatial distribution of the BrlA and AbaA proteins as well as being involved in conidiophore elongation and wall thickening, while MedA regulates the temporal expression of *brlA* along the developing conidiophore. Downstream of the three core regulators, BrlA, AbaA, and WetA, there are target genes, which are activated at different stages of the conidial developmental process. Some of these, as genes involved in spore pigmentation, were known from the earlier days of *A. nidulans* genetics, others were identified by the cascade hybridization methodology. These target genes show a variety of regulation patterns, some like *wA* (see Fig. 14) being under the control of WetA, some like *yA* under the control of AbaA, some others requiring, in order to be expressed, various combinations of the three transcription factors. The number of downstream genes has been estimated <100 by genetical procedures and at about 1200 by cascade hybridization. This huge difference could be partly explained by the

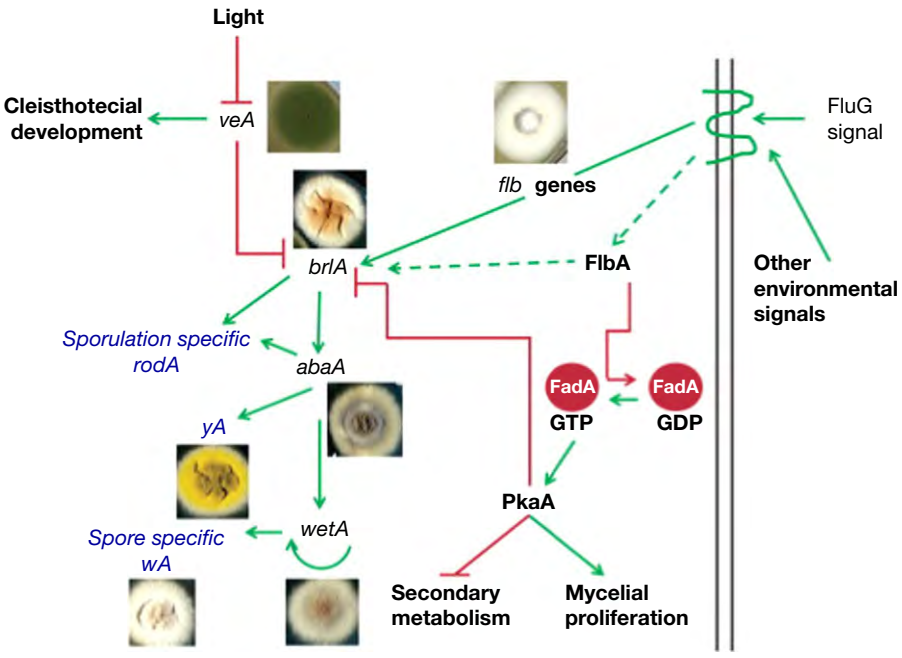

Fig. 14 Simplified scheme of the development of the conidiophore. The central developmental pathway, involving the products of the BrlA, AbaA, and WetA transcription factors is shown. In blue, target genes. Three are specifically shown, rodA encoding a conidial specific hydrophobin (see Section "Aspergillus as Human Pathogens"), *yA* encoding the last step of the synthesis of the wild-type green pigment and *wA*, encoding a polyketide synthase which is the first step in the synthesis of the conidial pigment. The insets show the morphological phenotypes of *veA1*, *brlA*, *abaA*, *wetA*, *yA*, and *wA* mutations. To the right a simplified version of the signalization pathway defined by dominant and recessive fluffy mutations. The inset shows the morphological phenotype of a fluffy mutant. The transmembrane receptor of the FluG signal has not been identified. The insets correspond approximately to the size of colonies grown on complete medium at 37°C for 48 h. Pictures from the laboratory of the author or kindly provided by Reinhard Fischer. All signals are shown as affecting the expression of *brlA*, but this is for simplicity's sake, as we do not know if these are direct or indirect. PkaA may affect the BrlA protein posttranslationally. To simplify the scheme the β and γ subunits interacting with FadA are not shown.

fact that the inactivation of at least some sporulation-specific genes does not lead to any visible phenotype and that overexpression of many metabolic genes occurs during sporulation.

This scheme does not account for the signaling pathway that leads to the onset of conidiation, that is, the formation from the mycelial mat of the conidiophore stalk. All the early work was carried out in a standard Glasgow strain, which is really a hyperconidiating constitutive mutant carrying the *veA1* mutation. This partial loss-of-function mutation leads to profuse conidiation in the dark. In strains carrying this mutation, when the mycelia are transferred from submerged culture to an air interphase, conidiation occurs synchronously in the dark, which allows the monitoring of the expression of relevant genes. The veA^{+} (wild-type) strains behave quite differently. In these strains, conidiation is light inducible, and veA^{+} strains produce profusely cleisthothecia in the dark. *veA* null mutants do not produce cleisthotecia at all. Blue light irradiation result in exclusion of the VeA protein from the nucleus, whereas the VeA1 mutant protein lacks precisely the nuclear localization signal and is always present in the cytoplasm. VeA mediates also, directly or indirectly, the response to polyunsaturated fatty acids, which have been shown to provide a sporogenic signal that is alternative or additive to light, the ratio of different unsaturated fatty acids driving development toward the sexual or the asexual cycle. The *veA* gene product behaves formally in the absence of light as a repressor of conidiation and an activator of cleistothecial development. Thus, VeA must directly or indirectly repress the expression of *brlA*. Fig. 13 and Fig. 14 illustrate and summarize the process of conidiophore development.

In order to study the signalization of *brlA*, another set of mutants was isolated. These are strains where a conidiophore does not develop, 'fluffy' mutants, which make a fast growing, colorless, undifferentiated mycelium. The study of fluffy mutations has revealed a complex signaling pathway, which determines both the conidiation pathway and the production of secondary metabolites. Recessive mutations of the *fluG* gene lead to a fluffy phenotype. FluG codes for an enzyme that is responsible for the synthesis of a diffusible product, which activates, through an unknown receptor, a signaling cascade specified by several *flb* genes. Dominant mutations in the *fadA* gene also lead to a fluffy phenotype. FadA is a subunit of a trimeric G-protein, which in its GTP-bound form activates a protein kinase (PkaA) that through phosphorylation of target proteins represses secondary metabolism, promotes growth, and represses conidiation. The FlbA protein, also characterized by fluffy loss-of-function mutations, acts downstream of the FluG signal to shift FadA to the GDP-bound inactive form. Fig. 14 summarizes the 'fluffy' signaling pathway and its possible relationship with the developmental pathway leading to conidiation. Recent work has shown that the 'fluffy' class of mutations has not been saturated and that there are at least three concurrent signaling pathways. This complex signaling pathway (which has been drastically simplified here and in Fig. 14) conceals, however, a conceptual problem. Fluffy mutants are not only aconidial but they are also foremost, fast proliferating mutants. By studying fluffy mutants, we are studying the signals that control and limit mycelial proliferation, and the complete or partial loss of conidiation could be a necessary result of the enhanced proliferative activity. In fact, if we slow down the growth of fluffy mutants by using very poor carbon sources, quite good conidiation can be seen. Mutants that are specifically altered in the signals upstream of bristle should not be fluffy, but 'bald', where condiophores do not arise from a normally proliferating mycelial flat mat. However, 'bald' mutants were not reported in the early mutant screens, and it will be most interesting to know if they could be isolated at all.

The Genus *Aspergillus* in the Genomic Era

In the last few years, complete genomic sequences have been established for *A. nidulans*, *A. oryzae*, *A. fumigatus*, *A. terreus*, two different strains of *A. niger*, *A. flavus*, *Aspergillus clavatus*, and *Neosartorya fischeri* ('perfect' name for *Aspergillus fischerianus*). Articles have been published describing the genomes of *A. nidulans*, *A. fumigatus*, *A. oryzae*, and *A. niger*. Dedicated Web sites exist for all the species except for the ongoing projects of *A. clavatus* and *N. fisheri*; the predicted genes and proteins of the latter can be found in the NCBI database. For *A. nidulans*, where a detailed genetic map exists, a correlation of the genetic and physical maps is available, even if the *in silico* identification of many of the classically mapped genes with autocalled genes is far from complete. Genome annotation is in different stages of completion in different species and so is the availability of microarrays. The availability of complete genomes has stimulated technological developments that allow high throughput gene inactivation and substitution. The genomes reflect the high metabolic versatility of the genus and have highlighted the evolutionary divergence of the different species. Telomeres have been assigned to each *A. nidulans* chromosome and this can be carried out for all the other member of the genus. Putatively active transposons of different families have been identified, including the interesting finding that helitrons, a newly described family of rolling-circle replicating eukaryotic transposons, are present and active in *A. nidulans* but not in the other species. The last release of the *A. nidulans* genome predicts 10,701 proteins as coded by the genome of this species, while for *A. niger*, the predicted number is 14,165. Interestingly, all the genomes sequenced contain mating type genes and other genes known or presumed to be involved in the sexual cycle. The homothallic *A. nidulans* and *N. fischeri* contains unlinked genes for both the α and HGM mating types, but their location in the genome suggest that homothallism has evolved independently in these species. Interestingly, for *A. fumigatus*, *A. oryzae*, and *A. flavus*, strains of opposite mating types have been found in nature, opening the possibility that in fact all (or at least many) Aspergilli can undergo the sexual cycle. Another interesting finding is the variability and phylogenetic scatter of the genes putatively involved in the production of secondary metabolites. As an example, the genome of *A. niger* contains a gene cluster similar to that involved in the synthesis of fumonisin in *Giberella* (*Fusarium*) *moniliformis*, while these genes are absent in other Aspergilli, thus positing the question of whether convergent evolution or horizontal transfer is involved. The considerable knowledge accumulated in *A. nidulans* may permit to extrapolate to other Aspergilli, for example, by asking which

are the genes regulated by transcription factors such as NirA, AreA, or PacC (see Section "Control of Gene Expression"), investigate promoter structure *in silico*, or more subtly to investigate the factors that make the conidiophore of *A. nidulans* biseriate, that of *A. fumigatus* uniseriate and that of *A. clavatus* characteristically elongated and uniseriate. At present, genome data have grown faster than the capacity of the research community to make use of them, a general problem of the genomic era. It is hoped that the comparison of genomes will lead to a better understanding of problems such as the evolution of metabolism, the evolution of the sexual and parasexual cycles, the evolution of silencing mechanisms, the isolation of useful new secondary metabolites, the possible engineering of pathways to produce new metabolites, and the identification of specific fungal essential genes that could lead the development of highly specific antifungal agents.

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Relevant Websites

http://www.fgsc.net/—Fungal Genetics Stock Center
http://www.aspergillus.org—Fungal Research Trust
http://docterfungus.org—Mycoses Study Group
http://www.fieldmuseum.org—The Field Museum

Appendix A: *Aspergillus*: A Multifaceted Genus. 2018 Update

Introduction

In 2007 (3rd edition of the Encyclopaedia of Microbiology, published 2009), there were 26,641 Pub Med entries for "*Aspergillus*", today (October 2018), there are 46,233. In 2007 the genomes of *A. nidulans*, *A. oryzæ*, *A. fumigatus*, *A. flavus*, *A. fischerianus* and *A. niger* were available. A recent article reports the genome analysis of 19 species. The Joint Genetic Institute includes 85 genomes of *Aspergillus* species, together with genomes of many other related organisms such as *Penicillium* and *Monascus spp*. Species Fungorum, a taxonomical site maintained by Kew Gardens, has 823 records for different *Aspergillus* species, from *A. acanthosporus* to *A. zutongii*. In 2017, 37 new species of *Aspergillus* were described. Thus, to update our knowledge of the genus *Aspergillus* is a daunting task. I thus apologise if some of my choices of the work reported appear somewhat subjective.

Contrary to what I have stated, Pier Antonio Micheli was not ordained as a catholic priest. Poor, and with no formal education, several priests and friars stimulated his vocation as a naturalist. Besides being considered "the father of Mycology" Micheli devised (1729), following Redi on flies (1668) but preceding Spallanzani (1765–1767) and Pasteur (1864) ingenious experiments leading to the disproving of spontaneous generation. His contribution went far beyond descriptive mycology and has a place in the history of the experimental method. In his experiments, he used a *Botrytis* sp. and a *Mucor* sp., together with "*Aspergilli capitati*, *capitulo glauco*", which could have been *A. fumigatus* or *A. glaucus*. This work was the very first use of *Aspergillus* (and other fungi) as model organisms to investigate a general biological problem. The most accessible account of the experimental work of Micheli is G.C. Ainsworth, *Introduction to the History of Mycology* (1976), reissued by Cambridge University Press. Even for us who don't read Latin, the *Nova plantarum genera*... of Micheli, 1729 (an example on Fig. 1, left panel, chapter printed above, third edition), can now be downloaded from the library of the Royal Botanical Gardens in Madrid, I will follow up some of the sections of the 2009 publication, and try to point out major advances for each of these.

What are the Aspergilli, taxonomy

The dual nomenclature of "perfect" and "imperfect" forms has almost died out. On the whole, the term *Aspergillus* is used for all species. In 2012, the international commission on *Aspergillus* and *Penicillium* decided: "Option 5: Compromise: Keep the name *Aspergillus* (as option 2), and treat other names as optional when it has a meaning (but always together with the *Aspergillus* name)." In practice, almost everyone uses *Aspergillus*. *Aspergillus* is, within the fungi, a genus in the phylum Ascomycota, sub-phylum Pezizomycotina, Class Eurotiomycetes, Order Eurotiales, Family Trichomomaceæ. All evidence points out to the genus being monophyletic.

Food contamination by Aspergillus

Aflatoxin B1 is metabolised in the liver to an 8,9-exo-epoxide derivative. It is this derivative which is mutagenic and carcinogenic. Hepatitis viruses (mainly Hepatitis B) and chronic exposure to aflatoxin are strongly synergic in the development of hepatocarcinoma. In 2013 a major scare followed the finding of levels of aflatoxins above the permitted threshold in milk in Holland and Germany. Apparently, this originated from animal feed imported from Serbia. No further negative consequences for human health were reported. Aflatoxin poisoning may not only be a disease of poverty, but also a result of globalisation.

Aspergillus *(mainly* fumigatus*) as human pathogens*

While concentrating below on invasive pulmonary aspergillosis, the actual number of cases of the non-life-threatening Allergic Pulmonary Aspergillosis and Chronic Pulmonary Aspergillosis conditions (the latter affecting mainly patients with a previous, underlying lung disease) is actually far higher than those with invasive pulmonary aspergillosis. Invasive Pulmonary Aspergillosis continues to be a major pathology for immunocompromised patients, the mortality rate being >50%. It is still not clear why *A. fumigatus* is the major species involved. However, patients suffering from chronic granulomatous disease (a genetic condition resulting from mutations affecting any of 5 sub-units of NADPH-oxidase) are uniquely sensitive to invasive infection by *A. nidulans*. Ability of *A. fumigatus* to form biofilms contributes to pathogenicity and drug resistance. Melanin presence in the conidiospores, hydrophobicity of the latter and early production of the toxic gliotoxin are still considered contributing factors. The exopolysaccharide galactosaminogalactan (GAG, a linear polysaccharide composed of galactose bound to N-acetyl galactosamine in no specific arrangement) has shown to be essential for adherence of *A. fumigatus* hyphae to both cells and artificial substrates, while also masking cell wall antigens, thus suppressing host inflammatory responses. Mutants deleted for the epimerase gene necessary for GAG synthesis (*uge3*) are critically impaired in virulence in mice. Orthologues of *uge3* are present throughout the Aspergilli (84% identity with *A. nidulans*) and GAG is present in many fungal species, including *A. nidulans*. However, the amounts of mycelial cell-wall bound GAG are significantly higher in *A. fumigatus* and the relatives amounts of N-acetyl-galactosamine in the polymer are also higher. An early expression of *uge3* during conidial germination, could be actually a long-searched for specific pathogenicity factor. A consensus seems to have been reached that there are no specific pathogenic factors, but rather a serendipitous constellation of characteristics in this species has pre-disposed it to become an opportunistic pathogen.

I correct an inaccuracy in the 2009 version of this article. The specificity of 5-flourocytosine to inhibit fungal growth is based on its conversion to 5-fluorouracil by cytosine deaminase, an enzyme absent in human cells. However, toxicity affecting both liver and

bone marrow has been reported. It is not widely used in the treatment of Aspergillosis, as *A. fumigatus* is not very sensitive to it, due to the repression of the cognate transporter at physiological pHs.

It is essential to develop new antimycotic agents, given the appearance of azole resistant strains. *A. fumigatus* resistant strains are present in the environment, in all probability due to the widespread use of azoles in agricultural practice. Nevertheless, *in patient* resistance can also arise. The most common genotype of pan-azole resistance strains includes a tandem duplication of the promoter region of *cyp51*(encoding lanosterol 4-α-demethylase) leading to eight-fold overexpression of the gene, together with a mutation in the ORF, L98H being the most frequent. Advances in treatment have relied mostly in developing new azoles and echinocandins and/or new ways to deliver already currently employed drugs. Nevertheless, new agents targeting specific aspects of fungal metabolism are being developed, among these specific inhibitors of cell wall. Some newly developed Nikkomycins, inhibitors of chitin synthases are in Phase 2 clinical trials. The most promising new antifungal drug, VL2397, produced by *Acremonium perscinum* is effective against *A. fumigatus*, and is undergoing Phase 2 clinical trials. It was discovered by a brute force screen, which used infected silk-worms (larvae of *Bombix mori*) as the test-organism. The mechanism of action of VL2397 is not known, but it is structurally similar to siderophores (Fe^{3+} chelators) and is incorporated into the mycelium by siderophores transporters. Siderophore synthesis is well known and essential for *A. fumigatus* pathogenicity. The latter has suggested a possible "trojan horse" approach where an antimycotic drug is conjugated with a siderophore, which then delivers it to the fungal cell.

Aspergillus sydowii *as a gorgonian coral pathogen*

Recent work has disproved the existence of specific pathogenic strains of *A. sydowii*. A study including both morphological and molecular markers did not find any evidence favouring the "African dust hypothesis". The snail *Cyphoma gibbosum*, a specialist predator of gorgonian corals, may be responsible from spreading the disease. While the prevalence of the disease was quite alarming shortly after its discovery in 1995, the epizootic outbreak has now subsided to relatively low endemic levels. Selection of resistant gorgonian corals, is supposed to have been the main mechanism for this outcome.

Extracellular enzymes produced by Aspergilli

An exciting development is the very recent isolation and characterisation of a strain of *A. tubingensis* (a member of the *A. nigri* -black Aspergilli- group) able to degrade polyester polyurethane (PU). It was estimated (2006) an annual global production of 8 million tons of PU increasing by 4%–5% year. PU is usually disposed in land-fills (forbidden in several European countries) or burned with the production of both carbon monoxide and hydrogen cyanide. To determine the detailed enzymology and the regulation of the process, together with the possible use of *A. tubingensis* in PU bio-degradation, alone or within a microbial community, would be of obvious importance.

Aspergillus nidulans *as a model organism*

The reverse genetics of the Aspergilli has seen a number of methodological advances. Transformation can be carried out now directly with composite DNA molecules assembled from PCR products. Arguably the most useful advance has been the availability of strains (first obtained for *Neurospora crassa*) inactivated for the *nkuA* gene, necessary for non-homologous end-joining recombination, thus virtually eliminating the problem of non-homologous integration in transformation experiments. Transformation frequencies were improved for a number of Aspergilli by direct transfer of the Ti plasmid of *Agrobacterium tumefaciens,* which can also compete with or complement transposon-based methods for random inactivation of genes. Recently CRISPR-Cas9 methods have been adapted to a variety of Aspergilli. The advances on reverse genetics had the effect of displacing research -at least in quantitative terms- from the model organism *A. nidulans* to species of industrial or medical importance, as these methods partially by-pass the need for sophisticated classical genetics. For 2018 there are in PubMed (up to October), 112 entries for *A. nidulans* compared with 486 for *A. fumigatus* and 369 for *A. niger*.

A number of fluorescent proteins are available to generate fusions to investigate cell trafficking and intracellular protein-protein interactions. This, coupled with striking advances in cell imagining has allowed extremely sophisticated studies of the cell biology of filamentous fungi with *A. nidulans* an *N. crassa* as the key model organisms. The articles of many of the groups working on *Aspergillus* cell biology are routinely accompanied by high-quality movies showing intracellular traffic.

I will detail below some recent contributions to fundamental cell and molecular biology based on work with *A. nidulans*. Progress has been achieved in understanding the signaling of some of the regulatory proteins mentioned in the 2009 version of this article (see Figs. 6, 7 and 8 of the 2009 edition printed above, and related text pages). The activation of the CreA repressor, mediating carbon catabolite repression is complex and surely different form that of that of the isofunctional Mig1p *of Saccharomyces cerevisiæ*. A ubiquitination/de-ubiquitination cycle is involved in the activation of CreA in *A. nidulans*. The positive-acting GATA factor AreA responds to the presence of favored nitrogen sources through several mechanisms. An important one is RNA stability, intracellular glutamine destabilising the *areA* mRNA. The protein mediating this process, and also the degradation of other mRNAs involved in the utilization of nitrogen sources, RrmA has been identified. RrmA is quite conserved even in very basal fungal species such as *Rozella allomyce*s (Cryptomycota) and even in Oomycetes (Stramenopiles, related to brown algae), organisms with a "fungal lifestyle", some of which are important plant pathogens.

Fig. 9 of the 2009 edition printed above shows the nitrate-induced localization to the nucleus of NirA, the specific transcription factor necessary for the expression of the three genes of the nitrate utilization gene cluster. Strauss and co-workers have shown that in

the absence of nitrate, a conserved methionine in the Nuclear Export Sequence is oxidised to methionine sulfoxide by a specific enzyme. Nitrate elicits the reduction of the oxidised methionine, nuclear localization and activation of NirA. While the intramolecular structural changes resulting from this oxidation/reduction cycle are not yet elucidated, sulphoxidation of methionine is a novel signaling mechanism, probably conserved throughout the nitrate induction mechanism in the Pezizomycotina.

The continuing work of the laboratories of Miguel Peñalva and Herb Arst have led to a more profound understanding of the signaling pathway leading to the activation by proteolysis of the transcription factor PacC, illustrated in Fig. 10 of the 2009 edition. Endocytosis, as it was then proposed, is not involved, but nevertheless, the ESCRT complexes (Endosomal Sorting Complexes Required for Transport) participate in PacC activation. The ESCRT multi-protein complexes, conserved throughout eukaryotes, mediate multi-vesicular body biogenesis, a process which internalise membrane proteins into endosomes leading to their eventual degradation in vacuoles. They are involved in other processes such as membrane excision during cell division and viral budding. In alkaline conditions, the ubiquitinylated PalF binds to PalH and recruits proteins of the ESCRTI and ESCRTII complexes. These, in turn recruit the ESCRTIII protein Snf7/Vps32 (*S. cerevisiæ* nomenclature, shown in Fig. 9 of the 2009 edition, printed above to bind PalA in the cytoplasm, see below). The polymer of Snf7 interacts with a PalA/PalC complex that in turn recruits PalB, the protease which catalyzes the first proteolytic step leading to the activation of PacC. The whole process occurs in a plasma membrane-bound rather than a cytoplasmic location as proposed previously and shown in Fig. 9 of the 2009 edition, printed above. Data from related work in *S. cerevisiæ* and the basal yeast species *Yarrovia lypolitica* point to an overall conservation of this signaling pathway in the ascomycetes. An unresolved problem is how PalH senses extracellular pH, leading to its binding of PalF. The signaling of pH regulation in fungi, provides a further example of the multiple cellular functions of the ESCRT complexes. This work led to the discovery of a novel signaling system involved in cation homeostasis. Mutations in genes encoding proteins of the ESCRT complexes lead to severely impaired growth. Suppressors of this phenotype map in two genes, *sltA* and *sltB*. These encode respectively a Zn-finger transcription factor and protease/pseudokinase self-activating protein, required for SltA activation. SltA and SltB are required for cation tolerance; mutations in either *slt* gene also result in oversized vacuoles. The range of targets of SltA includes both vacuolar and plasma membrane cation transporters. Another transcription factor CrzA, regulated by the calcineurin/calmodulin system also participates in cation homeostasis. CrzA is widely conserved among eukaryotes, while the SltA system is specific to the Pezizomycotina, a sub-phylum of the Ascomycota (see above).

Post-translational regulation of transporter fate is now well established (Fig. 11 of the 2009 edition, printed above). A new phenomenon, endocytosis as a direct result of transport activity has been shown for the uric acid-xanthine transporter of *A. nidulans*. Several *A. nidulans* transporters have been dissected by mutagenesis coupled with molecular modelling of docked substrates. The highpoint of this work has been the determination of the actual structure of the UapA xanthine/uric acid transporter complexed with xanthine. Substrate specificity is determined by the interaction of the substrate binding domain with gate domains along the substrate translocation pathway. The structure confirms predictions based on mutational evidence and strongly suggests an "elevator model" where in a UapA dimer, a core domain which binds the substrate sliding down a dimerisation/gate domain, thus leading to the internalisation of the substrate (cartoon in Fig. A1).

Work with *S. cerevisiæ* has defined specific compartments in the cell membrane. One of these (MCC), containing the arginine transporter Can1p is organised thanks to a number of other proteins, some tetraspan membrane proteins such as Sur7 and Nce102, others localized immediately below the membrane. These are two BAR domain proteins which generate furrows in the membrane called eisosomes, which typically appear as punctate structures. Eisosomes were described as sites of endocytosis in *S. cerevisiæ*, but

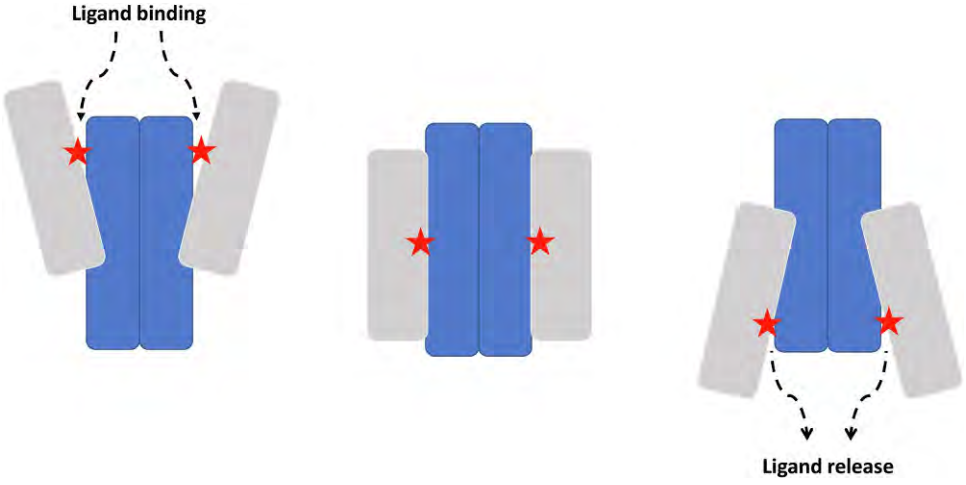

Fig. A1 A very schematic cartoon of the elevator model which accounts for all the structural and mutational data obtained for the UapA transporter. Grey, core domain which includes substrate binding residues. Blue, gate/dimerisation domain which includes both gate residues, which limit substrate specificity, and the dimerisation surface. Red star, substrate of the transporter. The core domain slides and twists around the dimerisation domain, transporting the ligand from the extracellular space (up, left scheme) to the cytosol (down, right scheme). A similar model accounts for the properties of the bacterial uracil transporter UraA, a member of the same transporter family (NAT) as UapA. I thank Sotiris Amillis and George Diallinas for help with this figure.

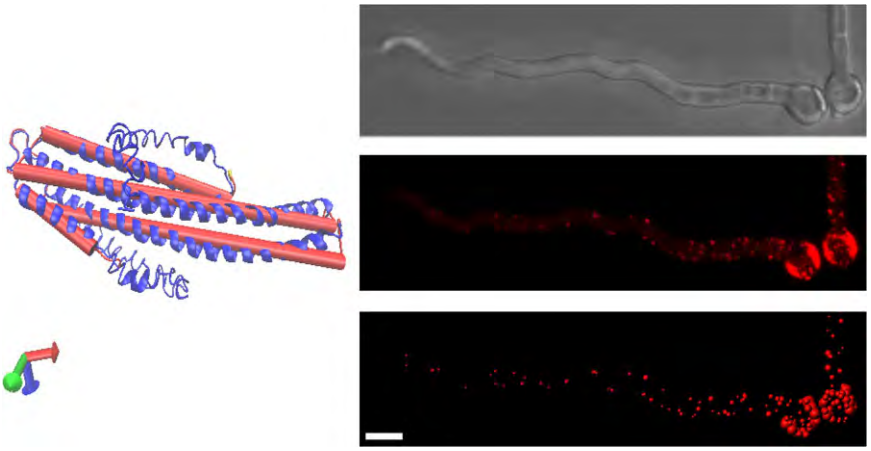

Fig. A2 Left panel: A model of the PilA eisosomal protein of *A. nidulans* (blue) superposed to the solved cartoon structure of the LspA protein of *S. cerevisiæ* (red) showing the typical BAR domain structure. Model obtained with I-Tasser (Yang, J., Yan, R., Roy, A., et al., 2015. The I-TASSER Suite: Protein structure and function prediction. Nature Methods 12, 7–8) and visualised with VMD 1.9.4 (Humphrey, W., Dalke, A., Schulten, K.,1996. VMD - Visual Molecular Dynamics. Journal of Molecular Graphics 14, 33–38). Right panel: Eisosomes in a 14 h germling of *A. nidulans* grown at 25°C. Top, DIC, Middle, PilA-mRFP fusión protein fluorescence, Bottom PilA-mRFP three dimensional reconstruction. Scale bar 5 μm. Picture courtesy of Alexandros Atanasopoulou and Vicky Sophianopoulou. Detailed methods to be found in the recent publication of these authors.

recent work points out rather to a protective role in relation to endocytosis. Eisosome organization has been studied sparingly in a few other species, but in detail in *Aspergillus nidulans*. There are two BAR domain eisosomal paralogues in the Aspergilli, PilA and PilB. Eisosomes in conidia and ascospores contain both PilA and PilB, while mycelial eisosomes include PilA but not PilB. Eisosomes are quite an enigma, as the cognate proteins are almost universally conserved in ascomycetes, but their deletion has virtually no phenotype (Fig. A2).

The laboratory of Steve Osmani has established the fate of all nuclear pore components during mitosis in *A. nidulans*, his results challenging the traditional drastic opposition of closed and open mitosis processes. Further collaborative work with the laboratory of Eduardo Espeso has aimed to establish the role and fate during mitosis of the 14 putative nuclear transport (importins and exportins) proteins found in the genome of *A. nidulans*. This work identified six essential nuclear genes involved respectively in a general protein import pathway, a general nuclear export pathway, an mRNA/nucleoprotein export pathway and a possible specific carrier of the RanA GTPase. Hydrolysis of GTP, catalyzed by RanA, provides the energy for all nuclear entry/exit transactions. The essential proteins studied remain partially attached to the nucleus or nuclear envelope during mitosis. The KapK/CrmA export pathway had been previously shown to mediate NirA and AreA localization while the importin α1/β1is responsible for the import of VeA. The detailed knowledge of nuclear import/export pathways coupled with that of the role transcription factors involved in metabolism, cell cycle and morphogenesis would allow a unique thorough description of nuclear entry/exit processes coupled with gene expression in this organism.

The Golgi apparatus of *A. nidulans*, studied in detail in the laboratory of Miguel Peñalva, is a dynamic network, which is polarized early during apical extension and remains intact during mitosis. They have also established that the biogenesis of secretory vesicles fuelling apical extension is governed by the recruitment to the trans-Golgi network of the RAB11 GTPase, which is mediated by the TRAPPII oligomeric GTPase exchange factor (GEF). This latter finding is of general importance as RAB11 function in the establishment and maintenance of polarity during development has been established in several animal systems, including human cells, *Xenopus* and *Drosophila*.

The filamentous nature of the Aspergilli (and in general all Pezizomycotina as opposed to the single celled yeast models) and their growth by apical extension implies an active directional intracellular traffic. *A. nidulans* has been, together with *N. crassa* the model to study these processes. Molecular motors (dynein, kinesins, myosin-5) are involved in the long-distance movement of organelles, such as secretory vesicles, early endosomes and peroxisomes. Classical and molecular genetic analysis together with newly developed imaging techniques is leading to a complete analysis of the growing hyphae, defining the role of exocytosis and endocytosis at the hyphal tip. One substantial advance is the molecular and functional definition of the Spitzënkorper. This German term, coined in 1924, referred to dark staining organelle at the tip of hyphae. The Spitzënkorper results from an accumulation of secretory vesicles involved in exocytosis and hyphal expansion at the apex before fusing with the plasma membrane, and thus can be considered as the organelle determining hyphal apical extension.

Aspergillus *mitochondrial genomes*

Total genome sequencing necessarily includes mitochondrial DNA and thus it is possible to assemble and compare these genomes within and outside the genus. A systematic approach for all available *Aspergillus* mitochondrial genomes has not been carried out. The comparison of mitochondrial genomes of six species of *Aspergillus* and three of *Penicillium* showed a variation in size mostly due to class I introns, proteins encoded in their open reading frames and "accessory genes", that is mitochondrial encoded genes of

unknown function. Curiously, *A. nidulans* where very early work on the mitochondrial genome, intron positions and structure was carried out, was not included in this survey.

Secondary metabolites and their synthesis

The availability of whole genome sequences led to the realisation that only a small minority of fungal secondary metabolites (SM) had been detected by standard chemical methods. The genes encoding SM synthetic enzymes are usually clustered and they include as a key enzyme a polyketide synthase and/or a non-ribosomal peptide synthase (such as that catalysing the condensation of L-α-aminoadipic acid, L-cysteine and L-valine into a tripeptide in the synthesis of the penicillins). The presence of SM gene clusters is extremely variable even between different strain of the same species. This had led to the development of informatic programmes to detect secondary metabolism gene clusters. Genome analysis has led to the identification of 56 SM gene clusters in *A. flavus*, 26 in *A. fumigatus* and 56 in *A. nidulans*. This is probably the tip of the iceberg. The potentiality of Aspergilli to produce novel compounds is illustrated by the identification in 2017 of *A. hancockii*, (a member of the *flavus* group) which when cultured on rice, produces, besides a number of hydrolytic enzymes, 69 secondary metabolites, of which 11 were novel. Gene clusters involved in SM metabolite synthesis can be organised in different patterns. In *A. fumigatus* trichothecene synthesis genes are split in two clusters, while a single super- cluster, regulated by LaeA encodes the enzymes involved in the synthesis of the unrelated, fumitremorgin, fumagillin and pseurotin, the latter two in an interwoven (Fig. A3).

Under standard laboratory conditions most of SM gene clusters are not expressed. We are faced here with two problems, one conceptual and one practical. The first is to identify and dissect the signaling which leads to the expression of each secondary metabolite gene cluster. The second, is to elicit the expression of each cluster under controlled conditions, such as to be able to assay the biological activity of each of the many yet undescribed secondary metabolites. These are problems not specific to the *Aspergilli*, as secondary metabolites gene clusters are present in fungi, bacteria and plants, and perhaps in other poorly studied microbial eukaryotes. The most obvious method is to try as many culture conditions as feasible and monitor the metabolites produced by standard chemical methods. Transcriptome analysis could then be used to identify the cognate genes or gene clusters. Many secondary metabolite gene clusters include a specific transcription factor gene. Its overexpression being a straightforward way to activate the gene cluster. The overexpression of *laeA* led to expression of several secondary metabolite gene clusters. Heterologous expression of SM genes, coupled with the substitution of native promoters by inducible, well characterized ones, has been used to investigate the products of several polyketide synthases and has led to the elucidation of the biosynthetic pathway of the *A. fumigatus* meroterpenoid pyripyropene, a potentially useful inhibitor of cholesterol synthesis. A promising avenue is the construction of gene libraries of ≈ 100 Kb in a multi-copy, self-replicating plasmid, comprising potentially each of the SCM gene clusters in a given genome, followed by transformation and expression in *A. nidulans*. Using this methodology (FAC-MS, Fungal Artificial Chromosomes-Metabolic Scoring) seventeen SC metabolites from different Aspergilli were identified and assigned to their cognate gene clusters. Finally, the expression of some SM clusters was achieved serendipitously. In a forward mutation screen, *laeB* (not a paralogue of *laeA* see above) was identified, where a deletion results in loss of stergimatocystin synthesis but also unexpectedly in eight new secondary metabolites being produced. The deletion of a protein kinase (*mpkA*, see below, genomic section) resulted in the activation of the Asperindin A gene cluster in *A. nidulans*. Life-saving (penicillin) or deadly (aflatoxin B) fungal secondary metabolites were at first discovered serendipitously. Statins were discovered after a systematic search of HMG-CoA reductase inhibitors in the culture broths of 3800 strains of fungi. This illustrates the problem of trying to find a function and/or application of the plethora of secondary metabolites produced by fungi and revealed by genome screens. A combination of methods, both bioinformatic and experimental high-throughput screening will be necessary to address this challenge.

The importance of chromatin organization in secondary metabolism gene expression is under active investigation and has revealed a role for the Compass complex (involved in methylation of H3K4), histone deacetylation, H3K4 and K9 methylation and heterochromatin protein I in secondary metabolite gene cluster silencing. The link between secondary metabolism and developmental regulation has been brought to the forefront with the discovery that LaeA, a global regulator of secondary metabolism, forms a complex with VeA and a VeA-like protein, VelB. As established previously, VeA is enriched in the nucleus in the light, but the distribution of LaeA and VelB does not seem to be affected by light/dark conditions. This physical interaction was unexpected and opens a new avenue of research. In a thorough screen several new positive regulators of secondary metabolism cluster were discovered. A global negative-acting putative DNA binding protein, McrA was detected in *A. nidulans* and is conserved in many other ascomycetes. McrA seems to be a global regulator, involved in the repression of other genes beside secondary metabolite gene clusters. A cogent model linking the function of LaeA in secondary metabolism production with the light-regulated roles of VeA in

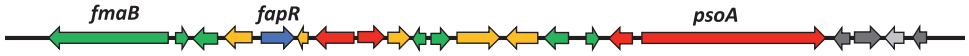

Fig. A3 A complex secondary metabolism gene cluster of *A. fumigatus*. Partial view of a sub-telomeric region of chromosome VIII of *A. fumigatus*. All the genes in this cluster and the near-by cluster involved in the biosynthesis of fumitremorgin are regulated by LaeA, and those shown in the figure also by the specific transcription factor encoded by *fapR* (in blue in the scheme). In red, genes encoding enzymes involved in fumagillin biosynthesis; in green genes, involved in pseurotin biosynthesis, interspersed yellow genes involved in neither, but nevertheless FapR-regulated. I have indicated the names of only the two "signature" genes, *fmaB* encodes a polyketide synthase, *psoA* a hybrid polyketide synthase non-ribosomal peptide synthase. Redrawn and simplified from Wiemann, P., Guo, C.-J., Palmer, J.M. et al., 2013. Prototype of an intertwined secondary-metabolite supercluster. Proceedings of the National Academy of Sciences of the United States of America 110 (42), 17065–17070.

the shift from the asexual to the sexual cycle is not yet available, neither is a comprehensive scheme of how the different positive and negative regulators interact.

While several environmental signals may be involved in triggering secondary metabolite production, a quite striking finding is that at least some secondary metabolite biosynthesis can be triggered by a close direct interaction of *A. nidulans* with *Streptomyces hygroscopicus*. Conversely, a non-identified *Aspergillus* species secretes a specific bacteriostatic metabolite (structure known, but biosynthetic pathway and genes involved not determined), which turns on the synthesis of nitrous oxide by a non-identified, co-cultured *Streptomyces* species, which activates the biosynthesis by the bacterium of a fungistatic agent, heronapyrrole B. These two studies underline the importance both conceptual and practical of studying secondary metabolism production in relevant ecological settings.

Aspergillus *asexual sporulation*

The pathway leading to the differentiation of the conidia in *A. nidulans* as shown in Fig. 14 of the main text still holds with some additional complications and refinements.

Research has proceeded in two directions. In the first, the details of the pathway signaling the key transcription factor BrlA were ironed out. To somewhat simplify, the central role of BrlA is confirmed, different paths leading of conidiogenesis are different ways of turning on *brlA* transcription. The Velvet complex responds to environmental stimuli, such as blue and red light, carbon levels, exposure of the hyphae to the atmosphere, osmotic and/or oxidative stresses, which lead to VeA cytoplasmic as opposed to nuclear localization, with resulting de-repression of the asexual sporulation pathway.

The "FluG factor" (Fig. 14) can be bypassed by an adduct of dehydroaustinol (a meroterpenoid) and diorcinol (a derivative of the secondary metabolite orsellinic acid). As the adduct is lypophilic, it may go through the membrane without the need of a specific receptor or transporter, which would leave open the identity of the intracellular interactor of the adduct.

In Fig. 14 of the main text it is indicated that a number of genes called *flb* (for fluffy, low *brlA* expression) are involved in the signaling of *brlA*. FlbB is a basic-leucine zipper transcription factor which is targeted to the hyphal apex, where it complexes with FlbE. The latter is essential for the apical localization of FlbB. In response to an unknown signal (perhaps mediated by the hypothetical receptor of the FluG signal) FlbB detaches itself from FlbE, travels in a retrograde fashion and eventually into the nuclei of aerial cells. FlbB transit through the apex is a prerequisite for its transcriptional competence, which was unexpected. Once inside the nucleus, FlbB elicits the synthesis of FlbD, a transcription factor of the cMyb-type. A dimer of both cooperate with a third transcription factor, of the Cys2His2 type, FlbC, to turn on *brlA* transcription. Uniquely among the Flb and Flu proteins, FldD is also required for the formation of the peridium, the wall of the sexual fruiting bodies. In addition to the central regulators showed in Fig. 14 a number of negative regulators of asexual sporulation have been identified. SfgA, is $Zn^{++}2$ Cys6 factor (motif called Cys_6 Zn_2 in page 411, shared by a large number of fungal transcription factors). SfgA represses the FlbB/D/C pathway, epistasis relations indicate that FluG acts relieving SfgA repression. VosA belongs to the same family as VeA and acts in vegetative growth by repressing *brlA*, finally NsdD is a repressive GATA factor, which on the other hand is necessary for sexual development. The latter two factors act by repressing *brlA*. A simplified scheme is shown in Fig. A4. A number of viruses (see below) affect conidiation of different species of *Aspergillus*. The interaction of these viruses with the asexual sporulation pathway is an unexplored area of research.

The second direction is to investigate how general is the conidiation induction pathway worked out for *Aspergillus nidulans*; within the Aspergilli and beyond. The first approach is obviously genomic, that is, to investigate which components of the conidiation cascade are present in which species. Some of these genes are not limited to the Aspergilli, or indeed to the Pezizomycotina. Twentyfive genomes species of *Aspergillus* show a complete conservation of the genes involved in asexual sporulation. The central regulatory network composed by *brlA*, *abA* and *wetA* is conserved almost without exception throughout the searched *Aspergillaceæ*. Once relevant genes are identified in a given species, standard methods could be used to check if their function is conserved. An example of this work is the investigation of the role of transcriptional activator WetA in *A. nidulans*, *A. flavus* and *A. fumigatus*. This work included RNAsec and Chip-sec (see below). The repression feed-back loop of *brlA* by WetA (not shown in Fig. 14 of the main text) is common to the three species, as it is the conidial color-less and autolysed phenotype. However, a number of downstream target genes are species specific. Previous work had established a pervasive role for WetA in *A. flavus*, including in vegetative growth. Travelling far away from the Aspergilli, in the wheat pathogen *Zymosteria tritici* (Pezizomycotina, Dothideomycetes) genome we find homologues of many of the genes involved in the sporulation pathway in the Aspergilli, including the central regulator *brlA*. Systematic deletion of these genes revealed that the orthologues of *abaA*, *brlA* and *flbB* do not have a crucial role in asexual sporulation, while *stuA* deletions failed to produce spores. This illustrates the limits of model systems and that apparently orthologous genes can be used in different ways in developmental pathways.

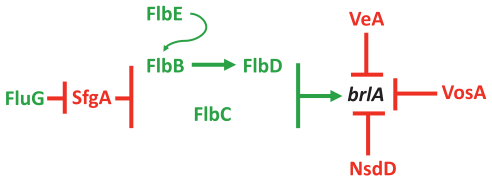

Fig. A4 The complex regulation of the expression of the *brlA* gene. This figure complements Fig. 14 of the 2009 edition, printed above. In red negative regulators and in green positive regulators of expression. Red connectors, repression, green arrows induction of expression. The squiggle arrow indicates the role FlbE in FlbB apical localization.

Aspergillus Sex

For an old-fashioned mycologist, this title would be an oxymoron, as Aspergilli by definition don't have sex, and when they have, they also have to change name. Following the cogent phylogenetic evidence that the apparently non-sexed Aspergilli and their sexual relatives form a monophyletic clade, I will discuss them together. In the original article I mentioned sex in two contexts, first while describing the sexual cycle of *A. nidulans*, which led to its development as a model organism, secondly in the context of developmental pathways. The mutational approach, which was a resounding success in the dissection of asexual sporulation has not delivered such a cogent picture for the process of fruiting body (cleisthotecial) formation, in spite of some interesting mutations having been characterized. It seems clear, however, that the formation of fruiting bodies, differentiation of hülle cells and fertilisation/nuclear fusion and meiosis are separable processes. It may be necessary to summarize again the process of sexual reproduction in the Pezizomycotina, the sub-phylum of the ascomycetes to which the Aspergilli belong. This has clear differences with other ascomycetes, the "yeasts" (properly Saccharomycotina and Taphrinomycotina). In specialised structures, two nuclei, which could be of the same or of different strains, divide synchronically in a common cytoplasm. This stage is called the dikaryon. Fertilisation occurs when the two nuclei fuse, giving origin to a diploid nucleus. This nucleus never divides mitotically, but goes immediately into meiosis resulting in four haploid cells. These may divide once more mitotically, typically resulting in eight ascospores, contained within an ascus (sack). Asci of *A. nidulans* comprise eight bi-nucleate ascospores (Fig. A5). *A. nidulans* is a homothallic species, no strains of different mating types are extant. Heterothallic ascomycetes came in two mating types and only strains of opposite types can mate. This is the case for *S. cerevisiæ* and *Schizosaccharomyces ponbe*, yeasts, which follow a different path to meiosis without the dicaryon stage (see above). Two heterothallic Aspergilli *A.* (*Neosartoria) fennelliæ* and *A.* (*Emericella*) *heterothallicus* were described about 50 years ago. In heterothallic ascomycetes mating type is determined by alternative genes (called idiomorphs), which are non-homologous but occupy, however the same chromosomal location (I am ignoring here the silent mating type loci, present in some yeasts, and which underlie mating type switching as they are not relevant to this discussion). These two genes encode DNA binding proteins comprising two different domains. One mating type gene encodes an α-box protein (MAT1-1 abbreviated MAT1). The alternative mating type gene (MAT1-2, abbreviated MAT2) encodes a protein including a HMG-box (high mobility group-box). There are usually several paralogues of HMG proteins in the genome (e.g., 7 in *A. nidulans*) of which one is concerned with determining the mating type. In the genome of the homothallic *A. nidulans*, both HMH-box and α-box encoding genes are present in different chromosomes and necessary for the sexual cycle, while in the "asexual" *A. fumigatus* and *A. oryzae* only the HMG-box encoding gene and only the α-box encoding gene were detected respectively. This was coherent with *A. nidulans* being homothallic, but suggested that the other two species were heterothallic rather than asexual, and it was a serendipitous occurrence which mating type was found in the single sequenced strain. Indeed, targeted sequence of many isolates of *A. fumigatus* yielded strains with either one or other mating types. In *N. fischeri* (*A. fisherianus*) a homothallic species of the *Fumigati* group, both mating type genes are present. In a more recent survey comprising 18 *Aspergillus* species, all species were found to have either one or the other mating type (thus possibly being heterothallic),while in addition to *A. nidulans* and *A. fischerianum* *A. glaucum* also has both. All species genomes included the pheromone and pheromone receptor encoding genes first described in *A. nidulans*. Thus, probably none of the species involved in this survey is asexual. Population genetics studies had suggested

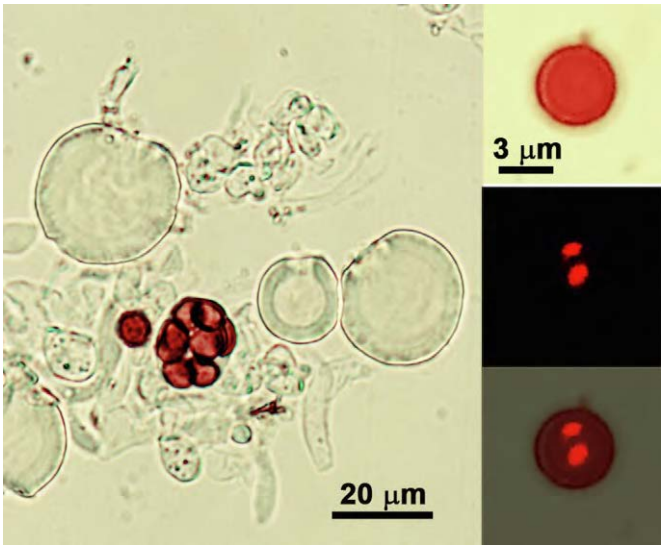

Fig. A5 Left panel, shows partial contents of a crushed cleistothecium. In the center an entire ascus with eight brown-red pigmented ascospores. To the left of it, an ascospore from a broken ascus. The large pale round cells are hülle cells. Right panel: Isolated ascus, top light microscopy, center epifluorescence of nuclei labelled with histone H1 fused to a red-fluorescent protein, bottom merge of top and central panels. Photographs courtesy of Eszter Bokor and Zszsanna Hamari.

recombination in *A. fumigatus*, *A. flavus* and *A. parasiticus*. The discovery of strains of the same species with complementary mating types led directly to attempts to obtain sexual crosses in the laboratory. This was strikingly successful for *A. fumigatus* (which lead to a suggested name change to *Neosartoria fumigata*, unfortunate in my opinion) and successively for a few other species, including members of the biotechnologically important black *Aspergilli*, such as *A. tubingensis*. We should start doubting whether asexual Aspergilli exist at all. Similar work has been carried out with Penicillia, and some other members of the Pezizomycotina. There has been an unresolved debate, as to which of the modes of sexual reproduction (homothallic or heterothallic) is primitive and which derivative. The two mating types should not be confused with the fact, that when crossing two strains of *A. nidulans*, distinguished by genetic markers, in any one cleisthotecium either nucleus can behave as a "female" or "male" nucleus. Both mitochondrial markers and nuclear markers with a maternal effect have been used to establish this fact, which was known from classical genetical work, but has not been investigated further.

Viruses of *Aspergillus*

I did not address this subject in the original article, but recent work prompts me to include it here. Following work with *Penicillium*, it was published in 1970 that both *A. niger* and *A. fœtidus* (a close relative and probably a strain of *A. niger*) contain viral particles and that double stranded RNA extracted from these was able to elicit interferon production in mice. Double stranded RNA viruses were reported in a number of *Aspergillus* species. No extracellular transmission seems to occur. Transmission was achieved through heterokaryons, and also between species through protoplast fusion. Most viral infections are asymptomatic. This contrasts with the well-studied killer phenotype of *S. cerevisiæ*, which is caused by a dsRNA. Interspecies transfer to *A. nidulans* showed that one specific RNA virus supressed the dicer-argonaut silencing pathway, while a second one actually was degraded to siRNAs, presumably though the action of dicer. Recently there has been a revival in the search and study of double stranded viral RNAs, using multiplex RNAseq. Most of the recent work concerns *A. fumigatus*. Many strains of *A. fumigatus* were screened for dsRNAs which revealed viruses belonging to several different families. Some alterations of morphology were reported, but no glaring correlation with pathogenicity or fungicide resistance was found. The virology of the Aspergilli is an underexplored area, mainly in respect of the phenotypic effects of viral infection. Old work (some of it of the 50s) showed cytoplasmic transmissible morphological phenotypes in a number of Aspergilli. The correlation of these with viral infections deserve a re-investigation.

Aspergillus *in the post-genomic era*

In the original article I referred to the "genomic" era of *Aspergillus* research. It has now become customary to refer to "post genomics" to highlight the challenges posited by an accumulation of data that goes beyond the capabilities of a research community. I have referred throughout this update to the contribution that genome availability has made to different research areas (SM, Asexual and sexual sporulation, fate of nuclear envelope proteins). *Aspergillus* is arguable the genus with most genomes sequenced and annotated. The availability of a genome does not automatically lead to the knowledge of the proteome. Gene models, derived from genome sequencing are based on algorithms that detect the start and end of open reading frames and the intron/exon structure of each gene in the genome. The latter is a particularly vexed problem. In spite of the existence of algorithms trained on different species, it is not uncommon to find erroneous gene models in data bases. I have personally encountered spuriously fused genes, promoters interpreted as introns, dubious start codons and incorrect intron/exon organization. While alternative splicing is less frequent in fungi than in other eukaryotes; it does occur, including in the Aspergilli, and to detect it is essential to establishing a complete correct proteome. Correct gene models are essential to interpret proteomes obtained under different conditions analyzed by mass spectrometry. The corollary of genome availability is transcriptomics, and "next generation" sequences techniques (RNAsec) have displaced other methods to investigate global gene expression. The application of RNAsec to the complete transcriptome has two objectives, firstly, to obtain correct gene models for all genes in a given genome. Secondly, to establish the transcriptome under different growth conditions or at different developmental stages. Whole transcriptomes are available for *A. nidulans A. flavus*, *A. oryzae*, *A. niger*, *A. fumigatus* among others. These were used to investigate general metabolic competence, secondary metabolism gene expression, developmental switches, resting conidia, response to drugs and alteration of expression in response to specific mutations and the transcriptional competence of specific transcription factors. As an example, FlbB mentioned in the context of asexual sporulation (see above), has other functions including the positive regulation of four secondary metabolism gene clusters. It is also possible to draw the complete chromatin landscape of a specific genome, combining chromatin immunoprecipitation (ChIP) microarray hybridization (Chip-Chip) or by parallel sequencing of precipitated DNA (Chip-Seq). Chip-Seq has been used to study the histone H3 acetylation profile of *A. nidulans* co-cultivated with *S. rapamycinicus* (see above Section "Regulation of Secondary Metabolism"), which led to the discovery of BasR a Myb-like transcription factor, which mediates the response to co-cultivation.

In both *S. certevisiæ* and *N. crassa*, knock-out strains are available for every gene. In *A. nidulans* knock-out cassettes can be obtained from the Fungal Genetics Stock Center. Using this methodology, all kinases of *A. nidulans* were deleted, and the phenotypes of their deletions studied. In principle such throughput methods can be used for any single gene family or groups of genes.

The availability of whole genomes has contributed to establish phylogenetic relations within the Aspergilli and of the Aspergilli with other fungi. A very recent article reports whole genomes for 23 species of *Aspergillus* section *nigri*. The availability of genomes opens new areas of *in silico* research. The evolution of gene clustering goes beyond secondary metabolism. Pre-genomic work has shown clustering in a number of primary metabolism pathways in *A. nidulans*, usually absent in *S. cerevisiæ* and *N. crassa*. Comparative genomics allows the study of how clustering arises, changes and vanishes within and beyond the genus.

An example of this is provided by recent work on the nicotinate utilisation gene cluster of *A. nidulans*. Horizontal gene transfer (HGT) within eukaryotes is subject to controversy. There is no question that, notwithstanding our ignorance of its mechanism, transfer within the fungi, to the fungi and beyond has occurred. Already mentioned was the transfer of genes involved β-lactam synthesis from a Streptomyces to an ancestor of Aspergilli and Penicillia. Examples derived from genome analysis are the acquisition by *A. niger* fumonisin gene cluster from a *Fusarium* sp. and the acquisition by *Podospora anserina* (Sordariomycetes) of the sterigmatocystin gene cluster from an *Aspergillus*. Algorithms have been developed to detect HGT and applied specifically to *Aspergillus* species (HGT-finder). This predicts, as an example, 715 possible HTG events for *A. nidulans*. SM genes are over represented in this sample which confirms previous proposals. However, specific, individual phylogenies are necessary to validate the findings of HGT-finder. As for other taxa, the availability of whole genomes reveals a surprising role for HGT in genome evolution and metabolic capabilities. An untapped area is the evolution of transposable elements, limited recently to an investigation of LINE (long interspersed nuclear elements) retroposons in strains of *A. fumigatus*.

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Autotrophic CO_2 Metabolism☆

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Glossary

Autotrophy Self-feeding, is the ability of an organism to synthesize all cell carbon constituents exclusively from inorganic carbon. Therefore, if an autotrophic organism was to be grown in the presence of labeled CO_2, every single carbon in the cell would become labeled (except the ones derived from essential growth factors added to the medium).
Carboxylation is the addition of CO_2 or bicarbonate to another carbon-containing molecule, resulting in a carbon-carbon bond.
CO_2 assimilation Describes the biosynthesis of cell carbon constituents (biomass) starting from carbon dioxide or bicarbonate.
CO_2 fixation Describes the conversion of CO_2 (a gas) to an organic compound containing carbon-carbon bonds as well as the assimilation of this CO_2 fixation product.
Heterotrophy Organic compounds are used as carbon sources.
Inorganic carbon species Used in autotrophy are carbon dioxide (CO_2), bicarbonate (HCO_3^-), but also carbon monoxide (CO) or cyanide (CN^-).
Primary production Is the formation of organic compounds from inorganic carbon species using light or chemically-derived energy.

Introduction

Over the past years, since the publication of the last edition of the *Encyclopedia of Microbiology* in 2009, there have been several advances in our knowledge of microbial autotrophy. A sixth pathway for autotrophic carbon fixation, the dicarboxylate/4-hydroxybutyrate, has been described. New tools have been developed to access the distribution of the different pathways for CO_2 fixation. This aids in assigning the contribution of specific autotrophic organisms in biomass production for a given habitat.

This chapter, however, not only focuses on recent progress in the field of autotrophic CO_2 metabolism, but aims also to present a comprehensive overview of this area of microbiology.

Autotrophic Modes of Life

The biosynthesis of organic carbon starting from inorganic carbon species by autotrophic organisms is a prerequisite to sustain life. The complete oxidation of organic carbon compounds to CO_2 by heterotrophic organisms allows the maximum gain of reducing equivalents, which can be transferred to a terminal electron acceptor such as oxygen, nitrate, and sulfate for energy conservation. CO_2 fixation by autotrophic organisms refills the organic carbon pool. When CO_2 assimilation is viewed as the reversal of carbon oxidation to CO_2 this process then requires: reducing equivalents and some input of energy. Because CO_2 is the sole source of carbon for autotrophic organisms, reducing equivalents and energy cannot be obtained by oxidation of an organic carbon substrate. (Formate and methanol oxidation and re-assimilation of carbon dioxide by some aerobes can be viewed as an exception of autotrophy). Instead the source of reducing power is provided by inorganic compounds such as water, hydrogen, reduced sulfur compounds, reduced metal ions or ammonium. Likewise, energy is provided by photosynthesis or by the reduction of oxidized inorganic compounds such as oxygen, nitrate, oxidized metals or sulfate. Primary production (using light or chemical energy), therefore, occurs in aerobic as well as in anaerobic environments. Members of all three domains of life, *Bacteria*, *Archaea*, and *Eukarya* are able to thrive autotrophically. It is important to remember, however, that CO_2 assimilation in *Eukarya* (terrestrially quantitatively most important in land plants) is of microbial origin, as the chloroplasts from such organisms are thought to have arisen from a cyanobacterial endosymbiont.

Conversion of Inorganic Carbon to Cell Carbon

Overall Equation

The general equation for cell carbon biosynthesis from CO_2 is the same for any given organism, because the average oxidation level of the carbon of any cell is close to zero (equal to oxidation state of formaldehyde, CH_2O). However, the amount of adenosine

☆*Change History:* August 2014. BE Alber introduced small edits in the text of the article including citations, added the sections 'Applications' and 'References', and added Figures and Table.

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triphosphate (ATP) required is dependent on the mechanism used for CO_2 assimilation as well as the electron donor used (see below). Therefore, autotrophic CO_2 fixation follows the general reaction scheme:

$$CO_2 + 4\,H + nATP \leftrightarrows [CH_2O] + H_2O + nADP + nP_i$$

Synthesis of Precursor Metabolites

When considering the biosynthesis of all cell constituents from CO_2, it is sufficient to understand the synthesis of specific intermediates of central carbon metabolism that are the starting molecules leading to all building blocks of the cell. These so-called precursor metabolites include acetyl-CoA, oxaloacetate, 2-oxoglutarate, pyruvate and 3-phosphoglycerate. There are presently six CO_2 assimilation sequences known: the reductive pentose phosphate cycle (Calvin-Bassham-Benson cycle), the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway), the reductive citric acid cycle (Arnon-Buchanan cycle), the 3-hydroxypropionate/malyl-CoA bicycle (Holo-Fuchs bicycle), the 3-hydroxypropionate/4-hydroxybutyrate cycle, and the dicarboxylate/4-hydroxybutyrate cycle. These autotrophic pathways account for the net synthesis of acetyl-CoA (reductive acetyl-CoA pathway, reductive citric acid cycle, 3-hydroxypropionate/4-hydroxybutyrate cycle, and the dicarboxylate/4-hydroxybutyrate cycle), pyruvate (3-hydroxypropionate/malyl-CoA bicycle) or 3-phosphoglycerate (reductive pentose phosphate cycle) from either CO_2 or bicarbonate. The further conversion of acetyl-CoA to C_3- or C_4-coumponds is not discussed in detail here, but in each case the reductive carboxylation of acetyl-CoA to pyruvate, catalyzed by pyruvate synthase (pyruvate:ferredoxin oxidoreductase), has been proposed. The conversion of pyruvate to the C_4-compound oxaloacetate proceeds either by direct carboxylation (pyruvate carboxylase) or via carboxylation of phosphoenolpyruvate (PEP) catalyzed by PEP carboxylase. The C_5-compound 2-oxoglutarate is formed from acetyl-CoA and oxaloacetate by enzymes of the oxidative citric acid cycle or via a (complete or incomplete) reductive citric acid cycle.

CO_2 Concentrating Mechanisms

The concentration of dissolved CO_2 at pH 7 under an air atmosphere is only about 10 μM. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the major CO_2 fixation enzyme, has K_m values for CO_2 that range from 10 to 300 μM. In addition, RuBisCO has a rather slow turnover even at saturating levels of CO_2; this enzyme also catalyzes an apparent wasteful competing oxygenation reaction in the presence of oxygen. Carboxysomes provide a microenvironment for RuBisCO with locally elevated CO_2 (and most likely decreased oxygen) concentrations. The function of these protein microcompartments were first suggested for *Halothiobacillus neapolitanus* (formerly: *Thiobacillus neapolitanus*), an aerobic thiosulfate oxidizer and obligate autotroph. Similar cellular inclusions, albeit with different structural components, were found in oxygenic phototrophic cyanobacteria. Carboxysomes consist of several different proteins, among them specific shell proteins, carbonic anhydrase (catalyzing the interconversion of bicarbonate and CO_2), and the majority of the cellular RuBisCO enzyme. In addition to carboxysomes, there are various CO_2/bicarbonate transporters involved in the CO_2 concentration mechanism which deliver bicarbonate into the cell. The rather recently recognized protein-facilitated transport of CO_2 and/or bicarbonate across membranes adds a fascinating aspect to autotrophic CO_2 metabolism. Currently there are at least five such systems known in cyanobacteria. Independent of the CO_2 assimilation mechanism used by a given organism, CO_2 or bicarbonate has to first enter the cell. Furthermore, the different carboxylating enzymes are specific for either CO_2 or bicarbonate. The rapid interconversion of bicarbonate and CO_2 catalyzed by carbonic anhydrases is at issue as long as carbon flux rates are high enough to make the uncatalyzed interconversion of CO_2/HCO_3^- rate-limiting. These aspects of CO_2 metabolism have not been addressed in most autotrophs.

Evolutionary Aspect

The question as to whether autotrophy is a primitive trait has been controversial. That is, did heterotrophic life processes evolve after the establishment of autotrophy or did heterotrophic life forms capable of metabolism of low molecular compounds present in the 'primeval soup' precede autotrophy? The speculations, hypotheses, and presentations of the various controversial views on this subject will not be part of this overview.

Mechanisms for CO_2 Assimilation

The assimilation of CO_2 cannot mechanistically be simply a reversal of the oxidation of central metabolic intermediates to CO_2, because (nearly) irreversible steps are usually involved in these oxidative routes. Presently six different pathways for CO_2 assimilation are known. The pathways are listed and described in the order of their discovery. For the figures, each pathway is divided into carboxylation (red), reduction (green), and regeneration (blue) steps. The reductive acetyl-CoA pathway is the only linear, non-cyclic route and, therefore, does not include a regeneration step. The energy requirement, in form of ATP hydrolysis, is not indicated in the figures.

The Reductive Pentose Phosphate Pathway (Calvin-Bassham-Benson Cycle)

In terms of overall CO_2 assimilated, the reductive pentose phosphate pathway for CO_2 fixation is the most important. This is based on the fact that oxygenic phototrophs, like cyanobacteria, algae, and plants, use the reductive pentose phosphate pathway and such organisms generate the majority of the biomass on land and possibly on earth. The primary carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), can comprise up to 50% of the soluble cellular protein in organisms using this cycle and is, therefore, considered the most abundant protein on earth. The reason for the high abundance of RuBisCO in the cell is its rather slow turnover number of 3 to 5 molecules per second (for comparison: carbonic anhydrase, albeit one of the fastest enzymes known, turns over one million molecules per second). In addition, various important mechanistic considerations must be accounted for. During catalysis, an enediol of ribulose-1,5-bisphosphate is formed. This intermediate can not only react with CO_2 but also with oxygen. Carboxylation and subsequent hydrolysis of the C_6 carboxylation intermediate produces two molecules of 3-phosphoglycerate whereas in the case of oxygenation, one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate are formed. Two molecules of the C_2 compound 2-phosphoglycolate generate one molecule of 3-phosphoglycerate but one molecule of CO_2 is lost. This process is called photorespiration and requires additional CO_2 to be fixed by RuBisCO in order to compensate for the loss of CO_2 during photorespiration. The ability to discriminate between CO_2 and O_2 as substrates is a characteristic feature of individual RuBisCO enzymes. The so-called specificity factor, which describes the ratio of the efficiency of carboxylation and oxygenation of ribulose-1,5-bisphosphate, varies significantly between RuBisCO enzymes from different sources. There has been great effort in understanding how the two substrates, CO_2 and O_2, are discriminated by RuBisCO with the hope to engineer an enzyme which is more efficient towards the carboxylation reaction.

For every three rounds of the reductive pentose phosphate cycle, one molecule of 3-phosphoglycerate is generated from three molecules of CO_2 (Figure 1). The cycle starts by carboxylation of three molecules of the C_5 compound ribulose-1,5-bisphosphate catalyzed by RuBisCO. Besides one molecule of 3-phosphoglycerate which is taken out of the cycle as the primary CO_2 fixation product, five more molecules of 3-phosphoglycerate are formed. These five C_3 compounds are used to regenerate three C_5 acceptor molecules in form of ribulose-1,5-bisphosphate.

Regeneration starts by the activation of the second carboxyl group of 3-phosphoglycerate to its phosphate-ester, followed by reduction to the level of the aldehyde catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Triosephosphate isomerase catalyzes the equilibrium between glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The transformation of these five C_3 molecules to two molecules of xylulose-5-phosphate and one molecule of ribose-5-phosphate involves transaldolases and transketolases which catalyze the transfer of C_3- and C_2-fragments between various activated C_4- (erythrose-4-phosphate), C_5- (xylulose-5-phosphate, ribose-5-phosphate), C_6- (fructose-6-phosphate), and C_7- (sedoheptulose-7-phosphate) sugar molecules. Ribose-5-phosphate and xylulose-5-phosphate are converted to ribulose-5-phosphate. Phosphoribulokinase, aside from RuBisCO the second unique enzyme of the reductive pentose phosphate cycle, activates ribulose-5-phosphate to the CO_2 acceptor molecule ribulose-1,5-bisphosphate and the cycle is closed.

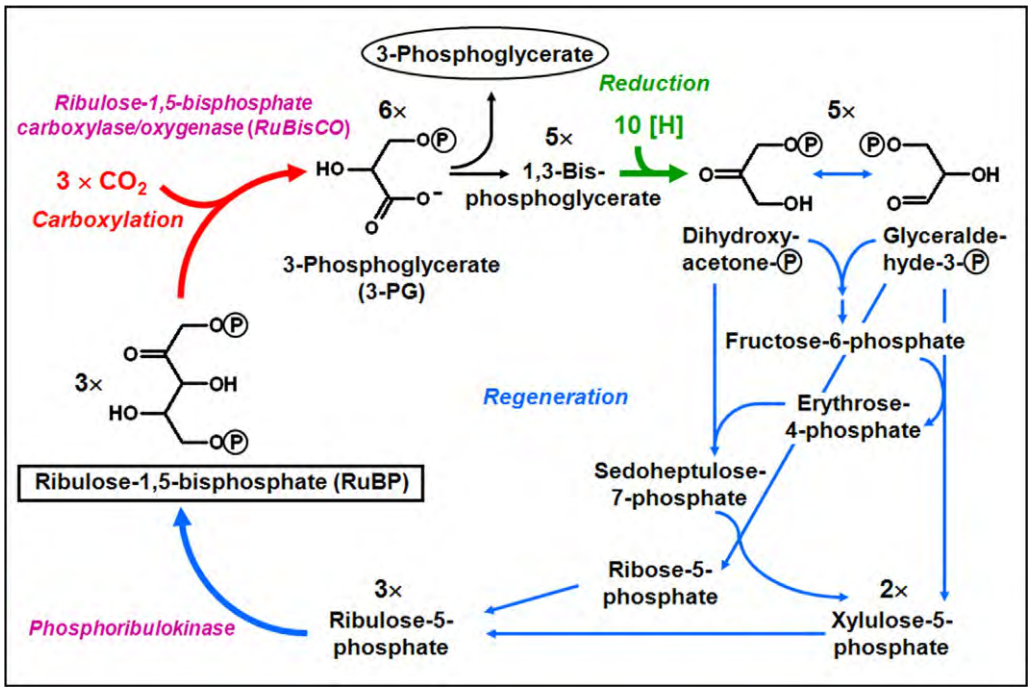

Figure 1 The reductive pentose phosphate cycle (Calvin-Bassham-Benson cycle). The primary fixation product of the reductive pentose phosphate cycle is 3-phosphoglycerate.

Four forms of RuBisCO enzymes have been recognized. Members of form I, II, III or IV RuBisCOs have sequence identities of greater than 35% to members of the same class, but less than 30% sequence identity to members of the other classes. Only form I and form II RuBisCO enzymes are involved in autotrophic CO_2 fixation. Form IV RuBisCOs are also referred to as RuBisCO-like proteins, because these enzymes are unable to catalyze carboxylation of ribulose-1,5-bisphosphate and their physiological role is still under investigation. Form III enzymes are bonafide RuBisCOs and occur in some Archaea; again the physiological role of the form III enzymes is not in autotrophic CO_2 fixation. Form I RuBisCO consists of eight small and eight large subunits and it is the form exclusively present in plants, most algae, and cyanobacteria. In the case of other autotrophic bacteria which use the reductive pentose phosphate cycle for autotrophic CO_2 fixation, form I, form II, or both forms I and II of RuBisCO are present. Form II RuBisCO consists only of one type of subunit, similar to the large subunit of form I enzymes.

The Reductive Citric Acid Cycle (Arnon-Buchanan Cycle)

As the name implies, the reductive citric acid cycle for autotrophic CO_2 fixation is the reversal of the oxidative pathway (Krebs cycle, tricarboxylic acid cycle) for conversion of acetyl-CoA to two molecules of CO_2. The reductive citric acid cycle has been discovered and initially studied for the green sulfur bacterium *Chlorobium*. More recently, the thermophilic hydrogen-oxidizing bacterium *Hydrogenobacter thermophilus* has become a focus of studying the enzymology of the reductive citric acid cycle. Steps considered essentially irreversible have to be catalyzed by enzymes different from those of the oxidative citric acid cycle. An outline of the pathway is shown in Figure 2.

The reducing potential of NADH is not sufficient for the reductive carboxylation of succinyl-CoA to 2-oxoglutarate. 2-Oxoglutarate dehydrogenase is, therefore, replaced by 2-oxoglutarate synthase (2-oxoglutarte:ferredoxin oxidoreductase). In addition to using ferredoxin with a more negative redox potential than $NADH/NAD^+$ (which makes the reaction reversible), 2-oxoglutarte synthase is also unrelated to the 2-oxoglutarate dehydrogenase enzyme complex. The same is true for pyruvate synthase (involved in the further assimilation of acetyl-CoA via reductive carboxylation of acetyl-CoA to pyruvate) and the pyruvate dehydrogenase complex. 2-Oxoacid dehydrogenases and 2-oxoacid synthases also use different mechanisms. Whereas the 2-oxoacid dehydrogenase complex catalyzes one oxidation step with two electrons transferred, 2-oxoacid synthase catalysis involves two electron transfer steps with a radical intermediate. The later enzyme contains iron-sulfur clusters, which can accept one electron at a time and the enzyme is, therefore, oxygen sensitive. However, the enzyme is found and is functional in some aerobic organisms.

The second (nearly) irreversible step in the oxidative citric acid cycle is the condensation of acetyl-CoA and oxaloacetate to form citrate. The reaction catalyzed by citrate synthase is exergonic, because the activation of the carboxyl group from acetyl-CoA is lost and free CoA released. The ATP- and CoA-dependent cleavage of citrate to acetyl-CoA and oxaloacetate during autotrophic CO_2 fixation by the reductive citric acid cycle, consists of two enzymatic activities: first citrate is activated to citryl-CoA (citryl-phosphate as an intermediate is also involved), which—in a second step—is cleaved into acetyl-CoA and oxaloacetate. For the thermophilic

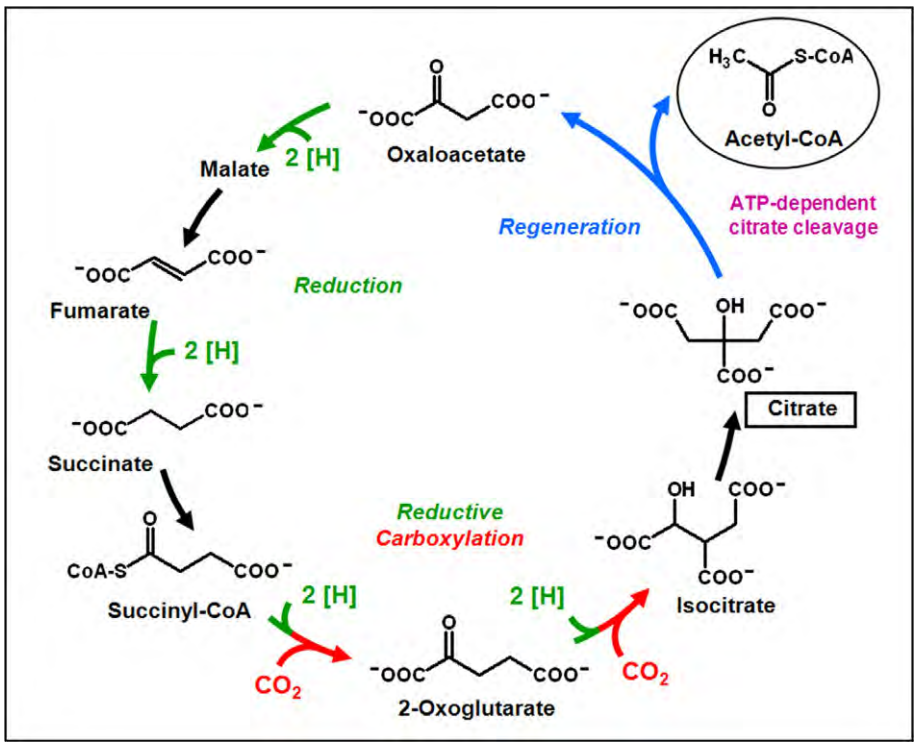

Figure 2 The reductive citric acid cycle (Arnon-Buchanan cycle). The free intermediates of the pathway for members of the *Aquificaceae*, citryl-CoA and oxalosuccinate, are not shown.

hydrogen-oxidizing bacterium *H. thermophilus* these two activities are confined to two separate enzymes: the first, citryl-CoA synthetase, consists of two different subunits and catalyzes the first step in the citrate cleavage reaction; the enzyme is related to succinyl-CoA synthetase: it requires ATP for CoA transfer and releases adenosine diphosphate (ADP) and inorganic phosphate. A second enzyme, citryl-CoA lyase, cleaves citryl-CoA and forms acetyl-CoA and oxaloacetate. Citryl-CoA lyase is related to citrate synthase. For the green sulfur bacterium *Chlorobium limicola* and *C. tepidum* both steps are catalyzed by a single enzyme. ATP citrate lyase consists of two different subunits; however, these subunits do not correspond directly to the two separate enzymes of *H. thermophilus*. The large subunit of ATP citrate lyase of *Chlorobium* contains the citrate synthase-related citryl-CoA lyase domain as well as an N-terminal part corresponding to the small subunit of the citryl-CoA synthetase enzyme. The second subunit represents the large subunit of citryl-CoA synthetase. Both subunits of the *C. tepidum* enzyme contribute to the active site of ATP citrate lyase. A fusion protein combining the citrate activating and citryl-CoA cleaving domains on a single subunit is found in animals, where ATP citrate lyase plays an important role in fatty acid biosynthesis in the cytosol.

The reversibility of the isocitrate dehydrogenase catalyzing the oxidative decarboxylation of isocitrate to 2-oxoglutarate has been demonstrated for the enzyme from *C. limicola*, even though the equilibrium of the reaction lies on the side of 2-oxoglutartate. In the case of *H. thermophilus* an additional enzyme is required to catalyze the reductive carboxylation of 2-oxoglutarate during CO_2 fixation: 2-oxoglutarate carboxylase is a biotin-containing two-subunit enzyme requiring ATP to form oxalosuccinate (and ADP + P_i) from 2-oxoglutarate and CO_2 (or more likely: bicarbonate). 2-Oxoglutarate carboxylase (formerly named: carboxylating factor for isocitrate dehydrogenase or CFI) is related to pyruvate carboxylase, the enzyme which allows conversion of the C_3-compound pyruvate to the C_4-compound oxaloacetate for the replenishment of the citric acid cycle. A second protein from *H. thermophilus* with almost 50% sequence identity to isocitrate dehydrogenase from *Escherichia coli* has been named oxalosuccinate reductase to indicate its specific role in autotrophic CO_2 fixation. The reductive carboxylation of 2-oxoglutarate to isocitrate by *H. thermophilus* is, therefore, catalyzed by two enzymes: 2-oxoglutarate carboxylase and oxalosuccinate reductase.

The membrane-bound succinate dehydrogenase complex is replaced by soluble fumarate reductase in the reductive citric acid cycle. Even though, depending on the reducing potential of the electron donor, the reduction of fumarate to succinate could be used in energy conservation (fumarate respiration is coupled to the generation of an electrochemical gradient), this has not been observed for autotrophic organisms. An exception may be *Desulfobacter hydrogenophilus*; this sulfate reducer uses the cycle in both directions and fumarate reductase appears to be membrane-bound.

In summary: succinate is formed from the reduction of oxaloacetate and is then activated to its CoA-ester (Figure 2). Two, mechanistically completely different, reductive carboxylation steps follow and isocitrate is formed from succinyl-CoA and two molecules of CO_2. Oxaloacetate is regenerated and acetyl-CoA released as the primary CO_2 fixation product. The last step is brought about by an ATP-dependent citrate cleavage system, which is viewed as the key reaction (sequence) in the reductive citric acid cycle.

The Reductive Acetyl-CoA Pathway (Wood-Ljungdahl Pathway)

The reductive acetyl-CoA pathway is the only linear (non-cyclic) pathway for CO_2 fixation. The pathway is linear because acetyl-CoA, the primary CO_2 fixation product is formed from the direct but independent reduction of two CO_2 molecules—one to the level of a carbonyl-, the other to the level of a methyl group (Figure 3). Therefore regeneration of a primary CO_2 acceptor molecule is not required.

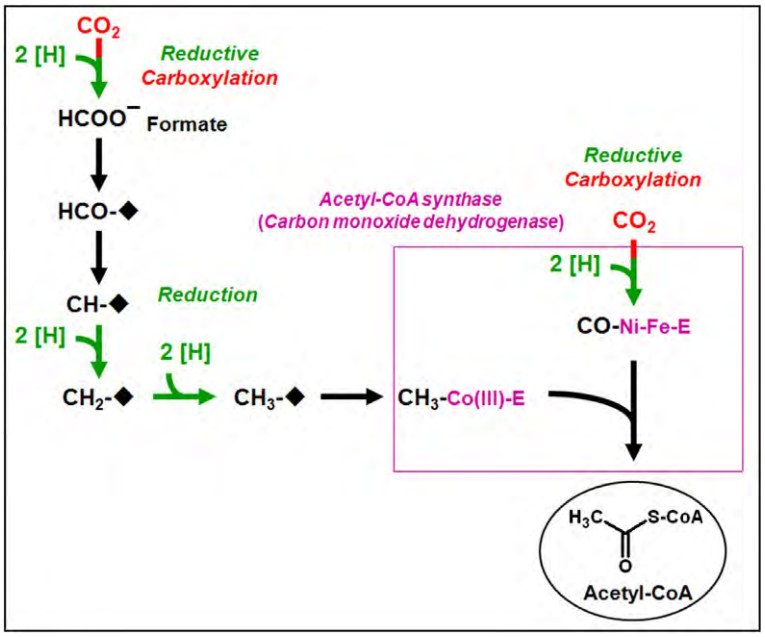

Figure 3 The reductive acetyl-CoA pathway (Wood-Ljungdahl pathway). Due to its metal centers, the key enzyme of the pathway, acetyl-CoA synthase/carbon monoxide dehydrogenase, is extremely oxygen sensitive. ◊ represents a C_1 carrier.

One molecule of CO_2 is reduced to an enzyme-bound carbonyl group. This one-step reduction is catalyzed by an enzyme formerly named carbon monoxide dehydrogenase due to this initially observed activity. The same enzyme, however, also impressively catalyzes carbon-carbon bond formation between this enzyme-bound carbonyl group and an enzyme-bound methyl group, and the carbon sulfur ester bond formation by addition of coenzyme A and was, therefore, renamed acetyl-CoA synthase.

The methyl group is derived from an independent three-step reduction of a second molecule of CO_2. After the first reduction step, catalyzed by formate dehydrogenase, formate is generated as a free intermediate. The subsequent transfer of the formyl group to a C_1 carrier requires the input of energy. Once bound to the C_1 carrier, water is released and the methenyl group reduced in two steps to the methyl group (which corresponds to the oxidation level of methanol). A methyltransferase passes the methyl group from the C_1 carrier on to the corrinoid cofactor of acetyl-CoA synthase, where it will become the methyl group of acetyl-CoA. The nature of the C_1 carriers as well as the electron donors for the reduction steps differ in various organisms using the reductive acetyl-CoA pathway.

Acetogens are eubacteria which form acetate as the main or only product of metabolism. They use the reductive acetyl-CoA pathway for *(i)* acetyl-CoA synthesis for carbon assimilation from C_1 compounds (including CO_2, carbon monoxide, and formate) as well as for *(ii)* acetate synthesis and energy conservation from a variety of substrates. Autotrophic growth of acetogens is possible, which means that acetyl-CoA synthesis from CO_2 (with hydrogen as electron donor) requires less than 1 ATP, because only 1 ATP is gained by conversion of acetyl-CoA to acetate. The pathway was discovered for acetogens during heterotrophic growth with glucose, where CO_2 is formed and then used as an electron acceptor (see scheme below). In the case of acetogens the C_1 carrier is tetrahydrofolate.

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,CH_3COOH + 2\,CO_2 + 8\,H^+ + 8\,e$$
$$2\,CO_2 + 8\,H^+ + 8\,e \rightarrow CH_3COOH + 2\,H_2O$$
$$C_6H_{12}O_6 \rightarrow 3\,CH_3COOH$$

Methanogens are archaea which form methane as a metabolic product. Many of them also use CO_2 as a carbon source as well as an electron acceptor. For methanogens, reduction of CO_2 results in the formation of methane instead of acetate as for acetogens. The reductive acetyl-CoA pathway is also required for non-autotrophic methanogenic growth with methylated compounds (methanol, methylamines, etc.) for cell carbon biosynthesis. During methanogenic growth on acetate, acetyl-CoA synthase functions in reverse for acetyl-CoA cleavage, the methyl group is reduced to methane, whereas the carbon group is oxidized to CO_2. For archaeal methanogens different pterin-based C_1 carriers have been described (e.g methanopterin, sarcinopterin). In addition, formate is not a free intermediate of the pathway, but instead reduction of CO_2 in the methyl branch of the pathway leads to formyl-methanofuran. The formyl group is subsequently transferred to the pterin-based C_1 carrier and further reduced to the methyl group.

Some sulfate reducers also use the reductive acetyl-CoA pathway for CO_2 assimilation. The same pathway is used in reverse during growth on acetate; acetate is oxidized completely to CO_2 and the electrons are transferred to sulfate (and hydrogen sulfide is formed).

The 3-Hydroxypropionate/Malyl-CoA Bicycle (Holo-Fuchs Bicycle)

The primary carboxylating enzymes of the 3-hydroxypropionate/malyl-CoA bicycle are acetyl-CoA and propionyl-CoA carboxylase. These biotin-dependent enzymes require ATP for catalysis (forming ADP and inorganic phosphate) and use bicarbonate as the inorganic carbon species instead of CO_2.

Carboxylation of acetyl-CoA forms malonyl-CoA. The activated carboxyl group of malonyl-CoA is reduced completely to form the methyl group of propionyl-CoA. This conversion formally requires five enzymatic reactions: malonyl-CoA reduction to malonate semialdehyde, malonate semialdehyde reduction to 3-hydroxypropionate, activation of 3-hydroxypropionate to its CoA-ester, dehydration of 3-hydroxypropionyl-CoA to acrylyl-CoA, and finally reduction of acrylyl-CoA to propionyl-CoA. Amazingly, for *C. aurantiacus* these steps are catalyzed by only two proteins. Malonyl-CoA reductase is an unusual protein with only limited sequence identity (and only over a short stretch of the protein) to short chain alcohol dehydrogenases related to FabG (3-ketoacyl-(acyl-carrier-protein) reductase). Malonyl-CoA reductase catalyzes the two-step reductive conversion of malonyl-CoA to 3-hydroxypropionate using NADPH as the source for reducing equivalents. Propionyl-CoA synthase is a fusion protein containing three functional domains: an acyl-CoA synthetase domain responsible for the activation of 3-hydroxypropionate to its CoA-ester, an enoyl-CoA reductase domain eliminating water from 3-hydroxypropionyl-CoA thereby forming acrylyl-CoA, and an enoyl-CoA reductase domain reducing acrylyl-CoA to propionyl-CoA using NADPH as the source for reducing equivalents.

Carboxylation of propionyl-CoA forms (2*S*)-methylmalonyl-CoA. Carbon skeleton rearrangement involves methylmalonyl-CoA epimerase and (2*R*)-methylmalonyl-CoA mutase and yields succinyl-CoA. In the 3-hydroxypropionate/malyl-CoA bicycle, succinyl-CoA is oxidatively converted to L-malyl-CoA (therefore the name of the pathway) involving enzymes of the citric acid cycle as well as succinyl-CoA:malate CoA transferase (Figure 4). L-Malyl-CoA lyase, a key enzyme of this pathway, regenerates acetyl-CoA and yields glyoxylate as the primary CO_2 fixation product. Glyoxylate has to be converted to central biosynthetic intermediate for further assimilation. The assimilation of glyoxylate for *C. aurantiacus* requires a second cycle. Propionyl-CoA is formed by carboxylation of acetyl-CoA and reductive conversion of malonyl-CoA via 3-hydroxypropionate as described

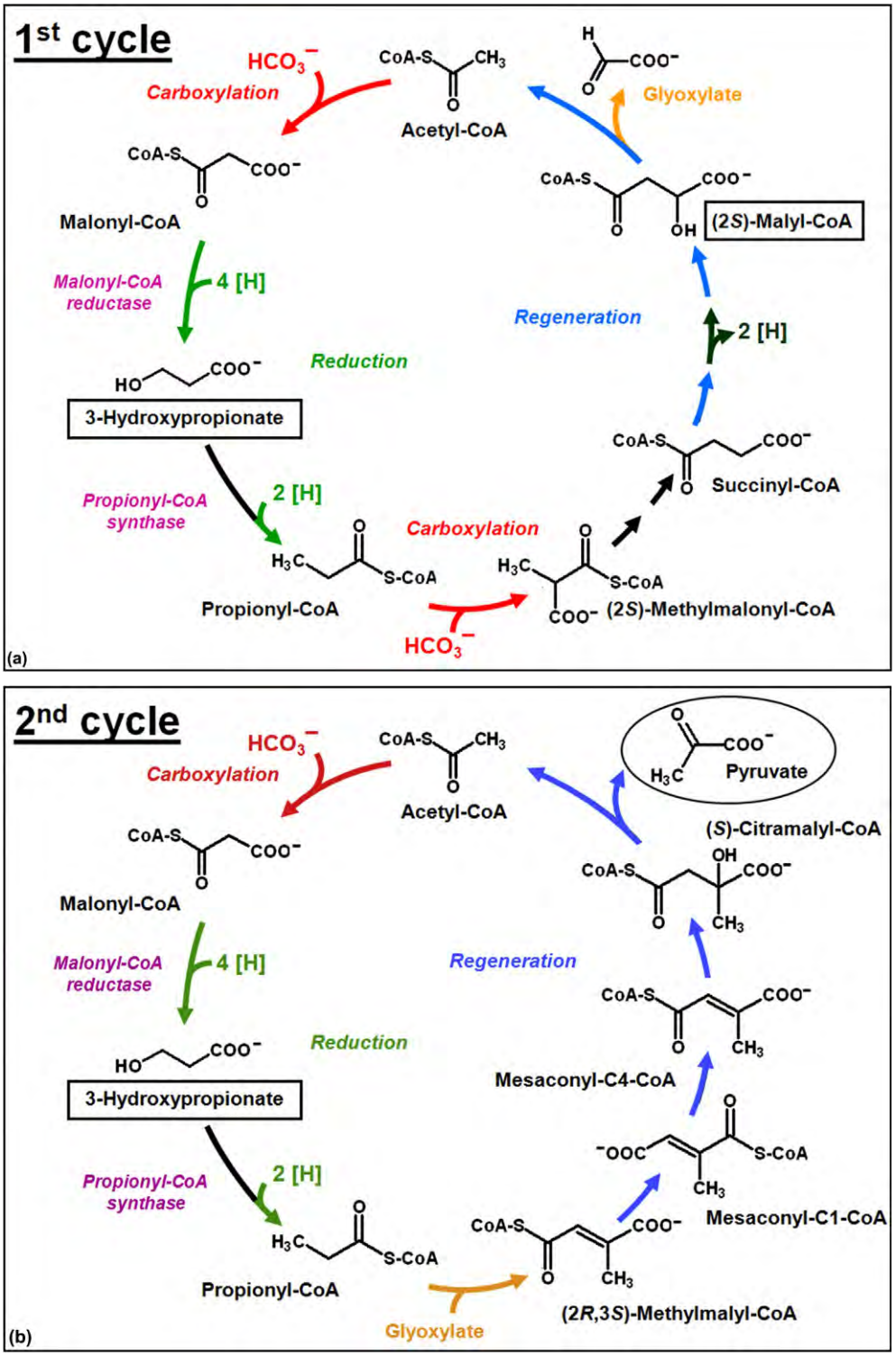

Figure 4 The 3-hydoxypropionate/malyl-CoA bicycle (Holo-Fuchs bicycle). The primary fixation product of the first cycle is glyoxylate which is further converted to pyruvate in the second cycle. The first half of both cycles is the same.

(Figure 4). Glyoxylate then condenses with propionyl-CoA to form erythro-ß-methylmalonyl-CoA, a step which is catalyzed by the L-malyl-CoA lyase, the final enzyme of the first cycle. ß-Methylmalonyl-CoA is dehydrated to mesaconyl-C1-CoA (2-methylfumaryl-CoA) and a CoA transferase transfers the CoA moiety from one end of mesaconyl-C1-CoA to the other side of the molecule, forming mesaconyl-C4-CoA (3-methylfumaryl-CoA). Hydration of mesaconyl-C4-CoA yields (*S*)-citramalyl-CoA,

which is cleaved into acetyl-CoA and pyruvate. Therefore, in the second cycle, propionyl-CoA and glyoxylate have been converted to acetyl-CoA (from which propionyl-CoA is formed) and the CO_2 fixation product pyruvate, a central intermediate from which cell carbon biosynthesis can proceed by conventional reactions.

The 3-Hydroxypropionate/4-Hydroxybutyrate Cycle

The 3-hydroxypropionate/4-hydroxybutyrate assimilation pathway shares intermediates with the 3-hydroxypropionate/malyl-CoA bicycle—most notably the intermediate 3-hydroxypropionate (Figure 5). The enzymes involved in the transformation of these common intermediates, however, are different for the two pathways. In the case of *M. sedula*, using the 3-hydroxypropionate/4-hydroxybutyrate cycle, five individual enzymes are required for the reductive conversion of malonyl-CoA to propionyl-CoA compared to only two (fusion) proteins for *C. aurantiacus*. Furthermore, NADPH-dependent malonyl-CoA reductase of *M. sedula* (catalyzing only a one-step reduction of malonyl-CoA to malonate semialdehyde and succinyl-CoA to succinate semialdehyde) is unrelated to the enzyme from *C. aurantiacus* (catalyzing a two-step reduction of malonyl-CoA to 3-hydroxypropionate). Instead, malonyl-CoA reductase from *M. sedula* is homologous to aspartate semialdehyde dehydrogenases. Likewise, acrylyl-CoA reductase of *M. sedula* shares very limited sequence identity (centered around a conserved NADPH-binding site) with the enoyl-CoA reductase domain of the trifunctional propionyl-CoA synthase from *C. aurantiacus* catalyzing the same reaction. Carboxylation of propionyl-CoA is catalyzed by a biotin/ATP-dependent and bifunctional acetyl-CoA/propionyl-CoA carboxylase. Succinyl-CoA is formed by carbon skeleton rearrangement of (2*R*)-methylmalonyl-CoA.

From here on out the 3-hydroxypropionate/4-hydroxybutyrate cycle differs from the 3-hydroxypropionate/malyl-CoA bicycle. The reductive conversion of succinyl-CoA to two molecules of acetyl-CoA regenerates the primary CO_2 fixation acceptor molecule, acetyl-CoA; the second acetyl-CoA molecule represents the primary CO_2 fixation product (Figure 5). This reaction sequence involves several interesting reactions: succinyl-CoA is reduced with NADPH to succinate semialdehyde, a reaction catalyzed by the same enzyme that reduces malonyl-CoA to malonyl-CoA semialdehyde. Succinate semialdehyde is further reduced to the characteristic intermediate 4-hydroxybutyrate (therefore the name of the pathway) which is activated to its CoA-ester. The 3-hydroxypropionate/4-hydroxybutyrate cycle was discovered after detection of 4-hydroxybutyryl-CoA dehydratase activity in cell extracts of autotrophically-grown *M. sedula*. The enzyme catalyzes the challenging elimination of water from an activated 4-hydroxyacid by a and had been discovered and previously only been described during fermentation by certain clostridia. 4-Hydroxybutyryl-CoA dehydratase yields crotonyl-CoA. The oxidative conversion of crotonyl-CoA to two molecules of acetyl-CoA via 3-hydroxybutyrate and acetoacetyl-CoA involves reactions known from other common metabolic routes.

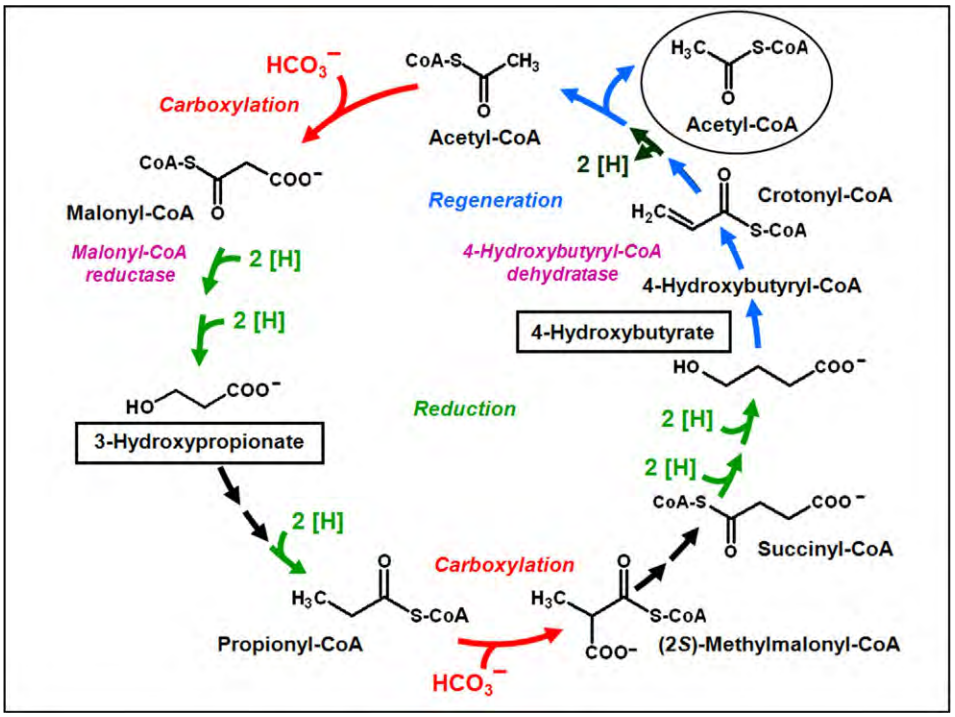

Figure 5 The 3-hydroxypropionate/4-hydroxybutyrate cycle. Although the reaction sequence of the first half of the cycle is shared with the 3-hydoxypropionate/malyl-CoA bicycle, most enzymes of the reductive conversion of malonyl-CoA to propionyl-CoA are unrelated in the two pathways, as studied for *Chloroflexus aurantiacus* (3-hydoxypropionate/malyl-CoA bicycle) and *Metallosphaera sedula* (3-hydroxypropionate/4-hydroxybutyrate cycle.

The Dicarboxylate/4-Hydroxybutyrate Cycle

The dicarboxylate/4-hydroxybutyrate and 3-hydroxypropionate/4-hydroxybutyrate cycles have in common the reaction sequences regenerating acetyl-CoA from succinyl-CoA and forming a second acetyl-CoA molecule as the CO_2 fixation product. Therefore, 4-hydroxybutyrate is also the characteristic intermediate of this CO_2 fixation pathway and 4-hydroxybutyryl-CoA dehydratase, is also the key enzyme.

The formation from succinyl-CoA from acetyl-CoA and two molecules of inorganic carbon, however, differ between the two 4-hydroxybutyrate cycles and both employ different carboxylases (Figure 4 and 5). In the dicarboxylate/4-hydroxybutyrate cycle (Figure 5), acetyl-CoA is reductively carboxylated by pyruvate:ferredoxin oxidoreductase (=pyruvate synthase). Pyruvate is phosphorylated by pyruvate water dikinase. The product, phosphoenolpyruvate (PEP), is carboxylated by PEP carboxylase to form oxaloacetate. Reactions of the reductive citric acid cycle convert oxaloacetate to succinyl-CoA. Therefore, in the dicarboxylate/4-hydroxypropionate cycle, one molecule of acetyl-CoA is formed from one molecule of CO_2 and one molecule of bicarbonate. Parts of the first half of the cycle are also used for synthesis of pyruvate (or succinyl-CoA) from acetyl-CoA, CO_2 (and bicarbonate).

Other CO_2 Assimilation Pathways

It is very likely that other mechanisms for CO_2 fixation will be described, for example for bacteria and archaea which will be discovered in the future. So far a considerable number of extremophiles have turn out to be autotrophs using chemical energy (mainly anaerobic respiration) for growth. The description of the 3-hydroxypropionate/4-hydroxybutyrate cycle also shows that parts of one CO_2 fixation pathway may be combined with other reaction sequences to create a new cycle. Also, variations of the known six CO_2 assimilation pathways are expected to exist.

Assessment of the Distribution of the Different Pathways

Key Enzymes

Key enzymes of pathways catalyze reactions yielding or using unique intermediates in metabolism that are characteristic for that particular pathway. Key enzymes can, therefore, be used as markers and their detection provide an excellent indication for the presence of an entire reaction sequence involving other enzymes that are shared with more common pathways of central carbon metabolism.

The key enzymes of the reductive pentose phosphate cycle are phosphoribulokinase and RuBisCO (Figure 1). Phosphoribulosekinase catalyzes the ATP-dependent activation of ribulose-5-phosphate to ribulose-1,5-bisphosphate, which is the primary CO_2 acceptor molecule of the reductive pentose phosphate cycle. RuBisCO, of course, is the carboxylating enzyme that uses ribulose- 1,5-bisphospate as its substrate, forming two molecules of 3-phosphoglycerate.

The key reaction sequence of the reductive citric acid cycle is the ATP-dependent conversion of citrate to acetyl-CoA and oxaloacetate (Figure 2). This reaction is catalyzed by ATP citrate lyase or two enzymes: citryl-CoA lyase and citryl-CoA synthetase, depending on the organism. Enzymes of both ATP-dependent citrate cleavage systems are related. In addition, pyruvate and 2-oxoglutarate synthase are required for the reductive carboxylation of acetyl-CoA and succinyl-CoA.

The key enzyme of the reductive acetyl-CoA pathway is acetyl-CoA synthase/carbon monoxide dehydrogenase, which catalyzes the reduction of CO_2 to an enzyme-bond CO intermediate (Figure 3). The enzyme also catalyzes formation of acetyl-CoA from the enzyme-bound carbonyl group, an enzyme-bound methyl group, and coenzyme A. In addition, pyruvate synthase is required for the functioning of the reductive acetyl-CoA pathway for further assimilation of the primary CO_2 fixation product acetyl-CoA.

There are several reaction sequences which appear to be unique but also common to the 3-hydroxypropionate/malyl-CoA bicycle and 3-hydroxypropionate/4-hydroxybutyrate cycle (Figure 4 and 5). Therefore, the enzymes involved in these reactions can all be referred to as key enzymes. The two-step reduction of malonyl-CoA leads to the formation of 3-hydroxypropionate. This characteristic intermediate is further reduced to propionyl-CoA via acrylyl-CoA. Specifically for the 3-hydroxypropionate/malyl-CoA bicycle, L-malyl-CoA lyase is the key enzyme, whereas 4-hydroxybutyryl-CoA dehydratase is characteristic for the 3-hydroxypropionate/4-hydroxybutyrate cycle. 4-Hydroxybutyryl-CoA dehydratase is also the characteristic enzyme for the dicarboxylate/4-hydroxybutyrate cycle (Figure 6); however, organisms using this CO2 fixation pathway lack the biotin-dependent carboxylase as well as a malonyl-CoA reductase.

Detection of Key Enzymes

The detection of key enzymatic activities in cell extracts of autotrophically grown cells of a particular organism is a prerequisite to assign a particular CO_2 fixation pathway used by a certain species. The specific activity of the key enzyme must be high enough to account for the doubling time of the organism during autotrophic growth. It is likely that the activity is down-regulated during growth on an organic substrate (heterotrophic growth) instead of CO_2 (autotrophic growth). Regulation of an activity in facultative autotrophs in response to growth substrate is an excellent indication of the enzyme's proposed role in a particular pathway. In some instances it is also possible to follow an entire reaction sequence using cell extracts. Similarly, short-term labeling studies with $^{14}CO_2$ or other ^{14}C-labeled (suspected) intermediates and cell extracts or cell suspensions have been found to be extremely helpful.

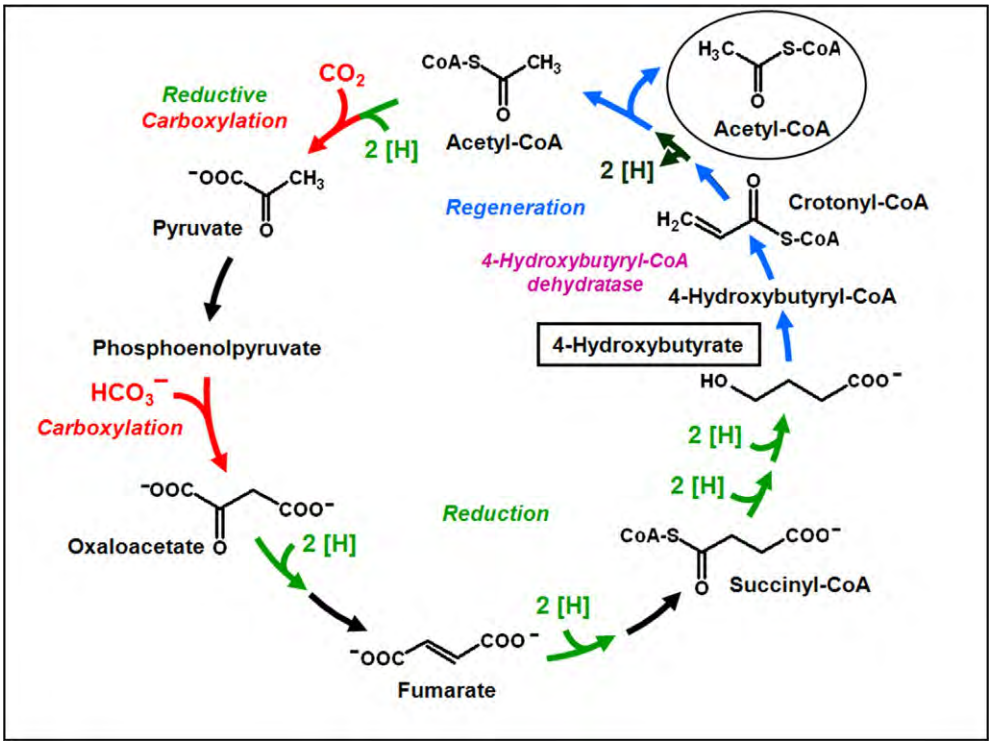

Figure 6 The dicarboxylate/4-hydroxybutyrate cycle. The second half of the cycle is shared with the 3-hydroxypropionate/4-hydroxybutyrate cycle. The first half of the cycle is used by other organisms to assimilate acetyl-CoA.

As confirmation of a particular CO_2 fixation mechanism used by a given organism, activities of key enzymes of alternate CO_2 assimilation pathways are expected to be absent.

With the advent of complete genome sequences there is the temptation to assign a specific mechanism for CO_2 fixation of an autotrophic organism based on genomic analysis alone. Furthermore, one may even wish to uncover the prevailing CO_2 fixation pathway in a particular habitat, analyzing sequences derived from entire communities (metagenomics). The assignment of the gene encoding for the key enzyme of a pathway is not sufficient. Candidates for all genes involved in the pathway must be assigned. It is not uncommon to find several enzymes of a pathway encoded by genes that are clustered on the genome. In order to confirm the proposed role of an assigned gene, it is usually cloned, heterologously expressed, and the recombinant enzyme analyzed for its predicted activity. In some cases, enzymes catalyzing subsequent reactions in a pathway are even fused; for example, propionyl-CoA synthase from *C. aurantiacus* of the 3-hydroxypropionate/malyl-CoA bicycle consists of three domains corresponding to enzymes catalyzing the activation of 3-hydroxypropionate to its CoA-ester, dehydration of 3-hydroxypropionyl-CoA, and reduction of acrylyl-CoA to propionyl-CoA. For *Roseiflexus* species the gene encoding propionyl-CoA synthase is found clustered with genes encoding malonyl-CoA reductase and the subunits of acetyl-CoA/propionyl-CoA carboxylase; other genes encoding for enzymes required for the 3-hydroxypropionate/malyl-CoA bicycle are found elsewhere on the genome. It, therefore seems clear that *Roseiflexus* use the 3-hydroxypropionate/malyl-CoA bicycle for CO_2 assimilation. This, however, was surprising because isolates of *Roseiflexus* species, which are filamentous anoxygenic phototrophic bacteria related to *C. aurantiacus* (83% sequence identity on the 16SrRNA level), had not been shown to grow autotrophically. It is possible that *Roseiflexus* species might use the 3-hydroxypropionate/malyl-CoA bicycle to fix CO_2, while coassimilating organic carbon compounds at the same time (mixotrophic growth).

More often than not, the assignment of a particular CO_2 assimilation mechanism or the prediction of the capability of a given organism to grow autotrophically, based on genomic data alone, is not straightforward. The following situations are often encountered:

1. Specific genes can be assigned but are not involved in autotrophic growth. A good example is the presence of a gene encoding RuBisCO in many methanogenic archaea. The enzyme represents a form III RuBisCO and is not involved in autotrophic CO_2 assimilation. These organisms use the reductive acetyl-CoA pathway for CO_2 fixation. ATP citrate lyases are present in a variety of organisms including mammals and play a role in fatty acid biosynthesis. Genes for 2-oxoacid synthases (2-oxoacid:ferredoxin oxidoreductase) are found in many genomes, especially in genomes of strict anaerobic—and at the same time strict heterotrophic-bacteria. The reductive acetyl-CoA pathway and the reductive citric acid cycle can be used exclusively in reverse by some organisms for acetyl-CoA oxidation during heterotrophic growth. 4-Hydroxybutyryl-CoA dehydratase one of the key enzymes of the 3-hydroxypropionate/4-hydroxybutyrate cycle and dicarboxylate/4-hydroxybutyrate cycle was first discovered in *Clostridium aminobutyricum* where it is involved in fermentation.

2. Not all (required) genes of a CO_2 assimilation pathway can be assigned. Although ATP citrate lyase activity (albeit low) was detected in cell extracts of *Thermoproteus tenax* and *Magnetococcus* sp. MC-1 and additional evidence was presented for the functioning of the reductive citric acid cycle in these bacteria, a corresponding gene for ATP citrate lyase is absent in the completed genome sequences. It is not clear, if another CO_2 assimilation pathway is operating in these organisms or if the enzyme catalyzing the cleavage of citrate is unrelated to ATP citrate lyase or citryl-CoA lyase. An alternate mechanism for citrate conversion to acetyl-CoA and oxaloacetate has been proposed but experimental evidence is missing. In case of the 3-hydroxypropionate/malyl-CoA bicycle, the conversion of malonyl-CoA to propionyl-CoA involves different and in some instances completely unrelated enzymes compared to the 3-hydroxypropionate/4-hydroxybutyrate cycle. Catalysis of identical reactions by unrelated enzymes (convergent evolution) is not unexpected, especially with members of general enzyme classes, like dehydrogenases, hydratases, acyl-CoA synthetases, etc.
3. Genes specific for several pathways appear to be present. The genome of *Archaeoglobus fulgidus* harbors genes encoding proteins related to RuBisCO (form III), acetyl-CoA synthase/carbon monoxide dehydrogenase, and 4-hydroxybutyryl-CoA dehydratase. The reductive acetyl-CoA pathway is thought to function as the autotrophic CO_2 assimilation pathway.

Confirmation (or refutation) of a proposed pathway for CO_2 assimilation is possible by long-term ^{13}C-labeling studies as discussed below. This, however, requires rather robust growth of an isolated organism. In case of bacteria and archaea not amenable for mass cultivation, additional evidence might be obtained by studying ^{13}C isotope contents in habitats or microbial consortia which might be indicative for the presence of a specific CO_2 assimilation pathway.

Qualitative Assessment (^{13}C Isotopic Depletion)

Differences in stable ^{13}C isotope content (relative to ^{12}C) between inorganic carbon and organic matter synthesized from CO_2 by autotrophs may be used to suggest a specific mechanism for CO_2 fixation. For example, the depletion of ^{13}C in organic matter relative to CO_2 from which the carbon was derived via the reductive pentose phosphate pathway is due to the preference of RuBisCO for $^{12}CO_2$ relative to $^{13}CO_2$. An isotopic signature of sedimentary organic matter of -20 to -30% is typical for CO_2 assimilation via the reductive pentose phosphate pathway. In contrast CO_2 fixation via the reductive citric acid cycle only leads to a depletion of -2 to -12%. For the 3-hydroxypropionate/malyl-CoA bicycle a depletion value of -13% has been reported. The depletion of ^{13}C carbon of organic matter formed by the reductive acetyl-CoA pathway relative to the $^{13}CO_2$ assimilated is greater than -30%; for the 4-hydroxybutrate cycles these values are much closer to zero.

The analysis of stable carbon isotopes can provide insights into carbon fixation mechanisms used (and the type of organisms involved) in a given habitat. This concept has been applied to microbial mats present in the effluent of sulfur-containing hot springs. The ^{13}C isotope content of organic matter of mats close to the source pool was diagnostic for the 3-hydroxypropionate/malyl-CoA bicycle, consistent with the fact that those mats were constructed mainly by *Chloroflexus* species. Mats further downstream formed by both cyanobacteria and *Chloroflexus* had a more typical reductive pentose phosphate cycle signature. However, compound-specific isotope analysis of unique lipids of *Chloroflexus* pointed mainly to CO_2 assimilation (via the 3-hydroxypropionate/malyl-CoA bicycle) in addition to some cross-feeding of photosynthetic products from cyanobacteria by *Chloroflexus*.

Qualitative Assessment (Long-term *in vivo* ^{13}C-tracer Labeling Studies)

A very elegant method has been introduced to analyze the specific fate of fixed CO_2 into specific positions of central precursor metabolites, reflecting the mechanism of CO_2 assimilation in a given autotrophic organism. Limited amounts of differentially ^{13}C-labeled intermediates of the proposed CO_2 fixation pathway are added as tracers to cultures growing autotrophically. After several generation times, the cells are harvested and major cell constituents are isolated. The ^{13}C-labeling patterns of several building blocks (sugars, amino acids, nucleotides, etc.) are determined by quantitative nuclear magnetic resonance (NMR) spectroscopy. Based on known biosynthetic pathways, ^{13}C-labeling patterns of central metabolic metabolites are retraced. It is important to note that the labeling pattern of a particular central metabolite is determined multiple times, because all the different building blocks are derived from one or several of these central metabolites. Therefore, variations in specific biosynthetic pathways in a particular organism can be accounted for. These results are compared with predicted labeling patterns based on the proposed CO_2 assimilation pathway which either leads to the confirmation or falsification of the suggested pathway.

Distribution and Physiological Restrains

There is no clear distribution of the different autotrophic CO_2 fixation pathways according to phylogenetic groups (Table 1). However, *Archaea* as well as strict anaerobic bacteria appear to use a mechanism for CO_2 fixation distinct from the reductive pentose phosphate cycle. The 3-hydroxypropionate cycles do not seem to be used by strict anaerobes.

The diversity of different pathways for the assimilation of inorganic carbon is at first unexpected. However, an alternate way to fix CO_2, other than the very energy-demanding reductive pentose phosphate pathway, was expected for organisms that are only able to gain rather limited energy through their metabolism (e.g. strict anaerobic microorganisms). At the same time, an anaerobic version for CO_2 fixation would not be feasible for aerobic organisms, because some of the enzyme involved in the reductive citric acid cycle

Table 1 Distribution of the different pathways for CO_2 fixation among various phylogenetic and physiological relevant groups

CO_2 assimilation pathway	*Phylogenetic groups*	*Physiological groups*	*Examples*
Reductive pentose phosphate pathway (Calvin-Bassham-Benson cycle)	chloroplasts cyanobacteria purple non-sulfur bacteria, purple sulfur bacteria, α-/γ-proteobacteria, etc.	oxygenic phototrophs, anoxygenic phototrophs, hydrogen-/sulfur-/ ammonium-oxidizers nitrate reducers	plants, algae, *Synechococus* sp. , *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Ralstonia eutropha*, *Xanthobacter autorophicus*, *Thiomicrospira crunogena*
Reductive citric acid cycle (Arnon-Buchanan cycle)	green sulfur bacteria, δ-/ε-proteobacteria, *Desulfobacteriaceae*, *Aquificaceae*, etc.	anoxygenic phototrophs, hydogen-/sulfur oxidizers, sulfur reducers, sulfate reducers	*Chlorobium tepidum*, *Hydrogenobacter thermophilus*, *Desulfobacter hydrogenophilus*, *Suflurimonas denitrificans*
Reductive acetyl-CoA pathway (Wood-Ljungdahl pathway)	*Desulfobacteriaceae*, Methanobacteria, etc.	homoacetogens, methanogens sulfate reducers	*Moorella thermoacetica*, *Methanothermobacter thermoautotrophicus*, *Desulfobacterium autotrophicum*, *Ferroglobus placidus*
3-Hydroxypropionate/malyl-CoA bicycle	green non-sulfur, bacteria,	anoxygenic phototrophs,	*Chloroflexus aurantiacus*
3-Hydroxypropionate/ 4-hydroxybutyrate cycle	Sulfolobales	thermophilic hydrogen-/ sulfur- oxidizers	*Metallosphaera sedula, Stygiolobus azoricus*
Dicarboxylate/ 4-hydroxybutyrate cycle	Thermoproteales, Desulfococcales		*Ignicoccus hospitalis, Pyrocolobus fumari*
unknown			e.g., *Pyrodictium*

and especially the reductive acetyl-CoA pathway are oxygen-sensitive. (The reductive citric acid cycle, however, is also used by some microaerophilic and even aerobic bacteria with high O_2 respirations rates). In addition to theses two 'anaerobic' versions (reductive acetyl-CoA pathway/reductive citric acid cycle), at least three versions of high-energy-demanding CO_2 assimilation pathways are now known: the reductive pentose phosphate cycle and the 3-hydroxypropionate cycles. The 3-hydroxypropionate/4-hydroxybutyrate cycle uses 4-hydroxybutyryl-CoA dehydratase, an enzyme containing oxygen labile iron-sulfur clusters and so far the pathway has been described for microaerophiles and may also occur in strict anaerobes. Clearly, the presence of oxygen demands adaptation of the CO_2 mechanism employed; this is mainly due to the fact, that the use of reducing equivalents with low redox potentials makes assimilation of CO_2 more energy efficient, reversible, but at the same time prevents their use in the presence of high oxygen tensions.

So why do even related organisms, for example different species of sulfur-reducers of the Desulfobacterales, use one 'anaerobic' version for CO_2 fixation over the other (Table 1)? Why is the 3-hydroxypropionate/malyl-CoA bicycle generally used by anoxygenic phototrophic green nonsulfur bacteria and the reductive pentose phosphate cycle by phototrophic purple nonsulfur bacteria capable of anaerobic CO_2 fixation? Clearly, there appear to be additional constraints for a particular microorganism to use one pathway rather than another, which are not understood at this point.

The differences between the six (and there might be more) CO_2 assimilation pathways extents beyond the energy requirements and type of electron carriers used. As a matter of fact, those two variables can even differ for the same CO_2 fixation mechanism; for example, the reductive citric acid cycle requires up to two more ATPs to synthesize one molecule of acetyl-CoA from two molecules of CO_2 in the case of *H. thermophilus* compared to *Desulfobacter hydrogenophilus*. In addition, different electron carriers for the reductive acetyl-CoA pathway are used by methanogens and acetogens. Additional differences between the various CO_2 assimilation pathways are also: (a) the requirement for specific cofactors and metals, (b) the type of inorganic carbon species (CO_2 or bicarbonate) assimilated, and (c) and perhaps most importantly, the type of metabolic intermediates through which the carbon passes.

It has been speculated that the 3-hydroxypropionate cycles allow for the simultaneous assimilation of fermentative products, such as acetate or propionate. Likewise the reductive acetyl-CoA pathway can be used for (co)assimilation of C_1-compounds (carbon monoxide, formaldehyde, formate, methanol, and methyl groups of methylamines). The latter pathway is also reversible and is used for acetate fermentation by methanogens or even some acetogens, provided that the hydrogen partial pressure is kept low. The assimilation of organic compounds and inorganic carbon by parts of the same pathway then would allow for metabolic flexibility such that some or all of the same enzymes may be used for autotrophic, heterotrophic or mixotrophic growth. It would be very exciting then, if there were examples of organisms capable of using different CO_2 fixation pathways depending on overall carbon availability. Even for purple nonsulfur bacteria (which use the reductive pentose phosphate cycle) where autotrophic and heterotrophic growth appeared to be clearly separated at first, RuBisCO has been shown to act in redox-balancing by using CO_2 as an electron sink during photoheterotrophic growth on various organic carbon compounds. The unique integration of a particular CO_2 assimilation pathway into the overall carbon metabolism of an organism is therefore an exciting field for further study.

Quantitative Assessment

In terms of total biomass generated, the reductive pentose phosphate pathway is the most important CO_2 assimilation pathway, because it is used by land plants, algae and cyanobacteria, which are also responsible for maintaining the oxygen level in the atmosphere. With regard to bacterial CO_2 fixation, the contribution of the individual pathways appears to be much more difficult to assess. However, considering the ubiquitous occurrence of cyanobacteria, again there is no doubt that the reductive pentose phosphate pathway contributes the most. Even more importantly, oxygenic photosynthesis allows the synthesis of extensive biomass.

In specific habitats, for example anaerobic or hypothermal, one particular pathway – other than the reductive pentose phosphate cycle might become dominant. Recent studies, for example, point to the prevalence of the reductive citric acid cycle as the main CO_2 assimilation pathway at hypothermal vents. This pathway is employed by proteobacteria of the ε-group, like *Sulfurimonas denitrificans* or an epibiont of the marine worm *Alvinella pompejana*, contributing significantly to primary production at such sites. Also, it has been suggested that the sulfide-oxidizing uncultured endosymbiont belonging to the γ-proteobacteria, which supplies the deep-sea tube worm *Riftia pachyptila* with fixed carbon, uses the reductive citric acid cycle for CO_2 assimilation, although RuBisCo is also present in the organism and might be used under conditions where the energy supply is plentiful.

Regulation

Facultative autotrophic organisms are able to also use organic substrates as their carbon source if these are available. Organic carbon compounds usually become the preferred carbon source over CO_2, because less energy and possibly no exogenous electrons are required for their assimilation. Therefore, the enzymes needed for autotrophic CO_2 fixation are under tight regulation in these facultative autotrophs. Even during mixotrophic growth, when CO_2 and organic carbon are assimilated simultaneously, flow through the CO_2 fixation pathway must be controlled and this is depended on the availability of energy and reducing equivalents.

It is clear that the type of regulation depends on the organism studied as well as the CO_2 fixation mechanism employed. The reductive citric acid cycle and the reductive acetyl-CoA pathways may be used in reverse for the oxidation of acetyl-CoA by some bacteria (sulfate reducers, acetogenic/methanogenic consortia) and even the same enzymes might be involved. Such a scenario would then make posttranslational regulation most likely under those conditions. However, this has not been established at this time.

Limitations to study regulation of the 3-hydroxypropionate/malyl-CoA bicycle, the 3-hydroxypropionate/4-hydroxybutyrate cycle and the dicarboxylate/4-hydroxybutyrate cycle at present revolve around the lack of genetic tools for organisms using these pathways for CO_2 assimilation. The activities of key enzymes of the 3-hydroxypropionate/malyl-CoA bicycle are up-regulated during autotrophic versus heterotrophic growth. Of particular interest is the branch point at the level of malonyl-CoA; this C_3 compound is either used for fatty acid synthesis or reduced to 3-hydroxypropionate for further conversion to other central precursor metabolites. The pathway is considered irreversible, making acrylyl-CoA reduction to propionyl-CoA the committed step of the pathway.

Regulation of the reductive pentose phosphate cycle is, therefore, the only CO_2 assimilation pathway studied in some detail. Here the current state of knowledge on the regulation of CO_2 fixation by purple non-sulfur bacteria is briefly discussed. Purple non-sulfur bacteria represent an excellent group of organisms to study carbon metabolism and the molecular basis for its regulation: their enormous metabolic versatility allows them to grow under a variety of different growth modes (anaerobically in the light, aerobically in the dark and even fermentatively that is anaerobically in the dark) using many organic substrates as their carbon source as well as CO_2. In addition, the complete genome sequences of several purple non-sulfur bacteria (*Rhodobacter sphaeroides*, *Rb. capsulatus*, *Rhodospirillum rubrum*, and *Rhodopseudomonas palustris*) have been determined over the last years and genetic tools are in place.

RuBisCO catalyzes the irreversible carboxylation step in the reductive pentose phosphate cycle and is regulated during autotrophic versus heterotrophic growth. A regulator protein, called CbbR (for Calvin-Benson-Bassham cycle regulator), controls the transcription of genes encoding RuBisCO enzymes in all purple non-sulfur bacteria studied and also other bacteria, including non-phototrophic bacteria. As mentioned before, some purple non-sulfur bacteria produce two forms of RuBisCO (e.g. *Rb. sphaeroides*) and the genes encoding both forms I and II are regulated by CbbR. The genes for either form are found in different gene clusters or operons located on distinct chromosomal loci where they are cotranscribed (and coregulated) with additional genes, especially those that encode enzymes required for the regeneration of ribulose-1,5-bisphosphate, the substrate of RuBisCO. CbbR is a transcriptional regulator belongs to a class of DNA-binding proteins (LysR-type regulators) that often require the binding of small molecules effectors or coinducers in order to be active in controlling transcription. It is likely that CbbR binds a metabolite which is present under conditions where CO_2 fixation is desirable but the nature of various positive and negative effector molecules might be different for different bacteria. For *Rp. palustris*, an additional level of regulatory control is added: a three protein two-component system that is thought to influence the activity of CbbR, the details of this are only beginning to be understood. Oxygen is also sensed and the signal transmitted through the two-component RegAB (also called PrrAB) system in *Rhodobacter*. Furthermore there is differential regulation of the form I and II enzymes and their cognate operons, suggesting additional signals or regulatory elements. Finally, there is also indication of posttranslational regulation and modulation of activity of RuBisCO by certain metabolites, among them the substrate ribulose-1,5-bisphosphate, which is thought to bind tightly to the active site of form I enzymes. Studies on the overall regulation of RuBisCO have already revealed the need for CO_2 fixation by RuBisCO, not only during autotrophic CO_2 assimilation, but also for balancing the redox state of the cell during photoheterotrophic growth.

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Bacillus Thuringiensis: Mechanisms and Use☆

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General Characteristics

Bacillus thuringiensis (Bt) is a member of the *Bacillus cereus* group that also includes *B. cereus*, *B. anthracis* and *B. mycoides* (Helgason et al., 2000). The feature that distinguishes Bt from the other members of the *Bacillus cereus* group is its entomopathogen properties. Bt produces insecticidal proteins (Cry and Cyt toxins) during sporulation phase as parasporal crystals. These crystals are predominantly comprised of one or more proteins, also called δ-endotoxins or Cry proteins. These toxins are highly specific to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable. Therefore, Bt is a viable alternative for the control of insect pests in agriculture and disease vectors of importance in public health.

Numerous Bt strains have been isolated that show activity towards lepidopteran, dipteran or coleopteran insects (Schnepf et al., 1998). In recent years Bt strains active against Hymenoptera, Homoptera, Orthoptera and Mallophaga insect orders and to other non-insect organisms like nematodes, mites and protozoa have been isolated (Crickmore et al., 1998; Wei et al.,2003). The entomopathogenic activity of Bt is mainly due to Cry toxins. One feature that distinguishes Cry proteins is their high specificity for their target insect. More than 300 different *cry* genes have been isolated. This constitutes an important arsenal for the control of a wide variety of insect pests. In this article we will summarize the knowledge on the pathogenic properties of Bt, the mode of action of Cry toxins and their application in agriculture and disease-vector control.

Since the publication of the article *Bacillus thuringiensis*, with Resistance mechanisms, in Comprehensive Molecular Insect Science (2005) there have been important developments in the understanding the mode of action of these toxins in different insect orders, in resistance mechanisms and gene discovery. Different recent reviews that revise recent literature regarding gene discovery and toxin evolution (Bravo et al., 2013; Adang et al., 2014), toxin mode of action (Vachon et al., 2012; Pardo-López et al., 2013; Adang et al., 2014), resistance evolution and ways to counter resistance (Bravo and Soberón, 2008; Tabashnik et al., 2013) have been published. Following we highlight the most important developments on *Bacillus thuringiensis* Cry toxins in recent years.

Virulence Factors and the Plcr Regulon

The *B. cereus* group is characterized by its pathogenic capabilities in diverse type of organism as mammals in the case of *B. anthracis* and *B. cereus* or to insects in the case of Bt. The pathological characteristics of the different members of the *B. cereus* group is mainly due to the presence of specific toxin genes that are most of the time encoded by large, self transmissible extrachromosomal plasmids (Schnepf et al., 1998). However, other chromosomal encoded virulence factors contribute to the pathogenic effect of the different members of the *B. cereus* group (Dubois and Dean, 1995; Lereclus et al., 1996; Agaisse et al., 1999; Guttmann and Ellar, 2000). In the case of Bt, it has been known for some time that Bt spores synergies the effect of Cry proteins (Dubois and Dean, 1995). This synergistic effect was shown to be due to the production of several virulence factors, such as α-exotoxins, β-exotoxins, chitinases, phospholipases and enterotoxins (Lereclus et al., 1996; Agaisse et al.,1999; Guttmann and Ellar, 2000).

Mutation of genes encoding some of these virulence factors showed that they contribute to the pathogenicity of Bt to insects (Lereclus et al., 1996; Fedhila et al.,2002). In the case of phosphatidylinositol specific phospholipase C (PI-PLC), the disruption of the structural gene *plcA* diminished the synergistic effects of spores indicating that at least this virulence factor was necessary for an efficient virulence of the bacterium (Lereclus et al., 1996). This is also the case for the *inhA*2 gene that codes for a zinc-requiring metalloprotease (Fedhila et al., 2002). The characterization of the mechanism controlling *plcA* gene expression led to the discovery of a pleiotropic transcriptional regulator, PlcR. Mutations in the *plcR* gene abolished *plcA* gene expression and the virulence of the bacterium (Lereclus et al., 1996; Agaisse et al., 1999). A genetic screening of PlcR regulated genes identified several extra cellular virulence factors including a secreted RNase, a S-layer protein, phopholipase C and enterotoxins indicating that the PlcR-regulon

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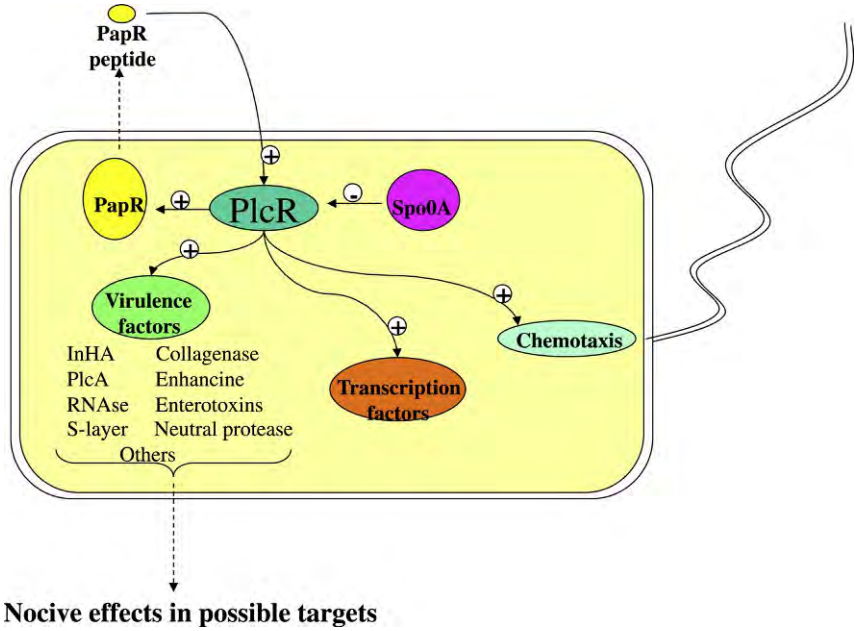

Fig. 1 PlcR regulon in *Bacillus thuringiensis*. Positive (+) and negative (−) regulation in the PlcR regulon.

include a diverse set of virulence factors (Agaisse et al., 1999). Also, the *inhA*2 gene was shown to be under the control of PlcR (Fedhila et al., 2003). A proteome analysis of secreted proteins from *B. cereus* strain ATCC 14,579 showed that disruption of the *plcR* gene reduced the levels at least 56 exported proteins (Gohar et al., 2002). Alignment of the promoter regions of the PlcR-regulated genes identified a 20-nucleotide palindromic sequence ("PlcR box") that is required for PlcR activation (Agaisse et al., 1999). Although, several PlcR regulated genes are also present in *B. cereus* and *B. anthracis* (Guttmann and Ellar, 2000), only the *B. cereus* PlcR protein is functionally equivalent to that of Bt since the *B. anthracis plcR* gene is disrupted by a transposon-like sequence indicating that production of virulence factors in *B. anthracis* is blocked or may be different from that of Bt and *B. cereus* (Agaisse et al., 1999). In fact, expression of a functional PlcR in *B. anthracis* leads to the activation of a large set of genes encoding enzymatic and toxin activities including protease, phospholipase and haemolysis (Mignot et al., 2001).

PlcR regulated genes are expressed on vegetative cells in the stationary phase of growth (Lereclus et al., 1996). PlcR expression is negatively regulated by the sporulation key factor Spo0A indicating that this gene is only temporally expressed on vegetative cells before the onset of sporulation (Lereclus et al., 1996). PlcR protein senses cell density and is therefore a quorum sensing mechanism (Slamti and Lereclus, 2002). The *papR* gene, encoding a 48 amino acid peptide and located downstream of *plcR*, is activated by PlcR and the gene product is secreted. Outside the cell, PapR is degraded by extra cellular proteases and the carboxi-terminus end pentapeptide is internalized to the bacterium cytoplasm by the oligopeptide permease Opp (Slamti and Lereclus, 2002). Inside the cell, the pentapeptide binds PlcR activating the capacity of PlcR to bind to the "PlcR box" sequence activating gene expression (Slamti and Lereclus, 2002). It was also shown that the PlcR activating- peptide confers strain-specificity to the activation of the PlcR-regulon, single amino acid changes on the activating PapR peptide were enough for strain specific induction of the PlcR regulon (Slamti and Lereclus, 2002). These results suggest that quorum sensing of related Bt strains is restricted by the sequence homology of the c-terminal pentapeptide of PapR. Fig. 1 shows the PlcR regulon network including the gene targets and the regulation of PlcR activation by PapR protein.

Insecticidal Toxins

Classification and Nomenclature

The mayor determinants of Bt insecticidal properties are the δ-endotoxins (Schnepf et al., 1998). The δ-endotoxins form two multigenic families, *cry* and *cyt*. Cry proteins are specifically toxic to orders of insects, *Lepidoptera*, *Coleoptera*, *Hymenoptera* and *Diptera*. In contrast, Cyt toxins are mostly found in Bt strains active against *Diptera*, although few exceptions of *Coleopteran* active strains containing Cyt proteins have been documented (Guerchicoff et al., 2001). Currently more than 74 Cry groups and 3 Cyt with more than 700 members have been characterized and classified (Crickmore et al., 2014). Besides these crystals proteins, some Bt and *B. cereus* strains produce a third group of insecticidal proteins called Vip (Estruch et al., 1996). In contrast to crystal proteins, Vip proteins are synthesized during the vegetative growth-phase of the bacterium and secreted into the medium. Including Cyt and VIP toxins, Bt produces at least six different families that are not related in structure, the three-domain (3D) family, the Bin-family and the MTX family. Classification and diversity of Cry, Cyt and Vip toxins has been reviewed recently (Adang et al., 2014).

The definition of Cry proteins is rather broad: a parasporal inclusion protein from Bt that exhibits toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein (Crickmore et al., 1998). This definition includes the Cyt toxins although it is agreed to give the mnemonic Cyt to Cry proteins that are structurally related to Cyt toxins (Crickmore et al., 1998). The nomenclature of Cry and Cyt proteins is based on primary sequence identity among different gene sequences. Crystal proteins receive the mnemonic Cry or Cyt and four hierarchical ranks consisting of numbers, capital letters, lower case letters and numbers (e.g., Cry1Ab1). The ranks are given depending on the sequence identity shared with other Cry or Cyt proteins. A different number (first rank) is given to a protein if it shares less than 45% identity with all the others Cry proteins (Cry1, Cry2 etc.). The capital letter (second rank) is given if the protein shares less than 78% but more than 45% identity with a particular group of Cry proteins (e.g., Cry1A, Cry1B etc.). The third rank gives a lower case letter to distinguish proteins that share more than 78% but less than 95% identity with other Cry proteins (e.g., Cry1Aa, Cry1Ab etc.). Finally a number is given to distinguish proteins that share more than 95% identity but which are not identical and that should be consider variants of the same gene (Crickmore et al., 1998). Although this system does not take into account the insect specificity of Cry toxins, the subgroups of Cry toxins show toxicity against a particular genus of insects (Cry1 to lepidopterans, Cry3 to coleopterans etc.). In the case of dipteran active toxins, it is surprising the high amount of different subgroups active against mosquitoes that represent Cry proteins that share low sequence similarity (e.g. Cry1C, Cry2Aa, Cry4, Cry11 etc.).

Different proteins not related phylogenetically comprise the Cry family. Among these, the 3-Domain Cry family, the bigger group of Cry proteins, binary toxins and Mtx toxins related to toxins produced by *Bacillus sphericus* have been described (Crickmore et al., 1998). The members of the 3-Domain family are globular molecules containing three distinct domains connected by single linkers. The alignment of their protein sequences revealed the presence of five conserved sequence blocks, although, some blocks are absent in some sub-groups of the 3-Domain family. One particular feature of the members of this family is the presence of protoxins with two different lengths. One large group of protoxins is approximately twice as long as the majority of the toxins. The C-terminal extension found in the longer protoxins is dispensable for toxicity but is believed to play a role in the formation of the crystal inclusion bodies within the bacterium (de Maagd et al., 2001).

Cyt toxins comprise three highly related gene families (Cyt1, Cyt2 and Cyt3) (Crickmore et al., 1998, 2002). Analysis of amino acid sequences shows that the different Cyt versions show a high degree of conservation in predicted α-helices and β-sheets (Guerchicoff et al., 2001). Cyt toxins are also synthesized as protoxins and small portions of the N-terminus and C-terminus are removed to activate the toxin (Li et al., 1996). Cyt proteins are almost exclusively found on dipteran active strains although a few exceptions have been found (Guerchicoff et al., 2001). Cyt toxins synergies the toxic effect of some Cry proteins and also that of the *B. sphaericus* binary toxin (Wu et al., 1994; Wirth et al., 2001). In the case of Cry and Cyt proteins it is widely accepted that the primary action of these toxins is the formation of lytic pores on the midgut cells of susceptible insects. Fig. 2 shows the phylogenetic dendogram of Cry and other Bt toxins.

Finally, Vip proteins comprise three families with seven members. The mechanism of action of these proteins has not been clearly described. Nevertheless, the three dimensional structure of Vip2 protein suggests that it may have ADP-rybosilating activity (Craig et al., 1999). Vip3A is a pore forming toxin of 88-kDa that form ion channels in target membranes and in planar lipid bilayer (Lee et al., 2003). It has a broad lepidopteran spectrum and binds to different protein receptors that are different from Cry1A or Cry1C toxins receptors (Lee et al., 2003; Mahon et al.2012; Abdelkefi-Mesrati et al., 2011).

The Bin-like toxins named Cry35 and Cry36 are active against some coleopteran larvae. They are named Bin like since they share some similarities with the dipteran specific binary toxins (Bin) produced by *Lysinibacillus sphaericus*. These are also pore-forming toxins that form ionic channels in black lipid bilayer at acidic conditions, which mimics the pH of the Coleopteran larvae (Masson et al., 2004).

The Mtx-like Cry family contains members with similarities to the Mtx toxin from *L. sphaericus* that shows mosquitocidal activity. Some of the Mtx-like Cry are active against Coleopteran insects (Cry15, Cry23, Cry33, and Cry38); others are toxic to Dipteran larvae (Cry60); or show dual activity against Coleopteran and Hemipteran (Cry51) (Crickmore et al., 2014). The mechanism of action of these toxins is not completely understood.

Structure of Toxins

The crystal structure of Cry1A (PDB 1CIY for Cry1Aa, 4ARX for Cry1Ac) (lepidopteran specific); Cry3 and Cry8 (PDB 1DLC for Cry3Aa, 1JI6 for Cry3Bb, 3EB7 for Cry8Ea) (coleopteran specific); Cry4 (PDB 2C9K for Cry4Aa, 1W99 for Cry4Ba) (dipteran specific) and Cry5 (PDB 4D8M) (nematode specific) trypsin activated toxins and Cry2Aa (PDB 1I5P) (dipteran-lepidopteran specific) protoxin have been solved (Grochulski et al., 1995; Li et al., 1991; Galitsky et al., 2001; Morse et al., 2001; Boonserm et al., 2005, 2006; Guo et al., 2009; Hui et al., 2012; see "Relevant Websites section").

Although the sequence similarity between these toxins is very low (20% and 17% identity of Cry2Aa with Cry3Aa and Cry1Aa respectively) the overall structural topology of these proteins is very similar. Fig. 3 shows the three-dimensional structure of Cry1A, Cry3Aa toxins and Cry2Aa protoxin (Morse et al., 2001). The structure is composed of three structural domains. Domain I is a seven α-helix bundle in which a central helix α-5 is surrounded by six outer helices. This domain has been implicated in the membrane channel formation. The six α-helices are amphipathic and are long enough to span the 30-Å thick hydrophobic region of a membrane bilayer. Cry2Aa protoxin has an extra 49-amino acid region forming two extra α-helices in the amino-terminal end

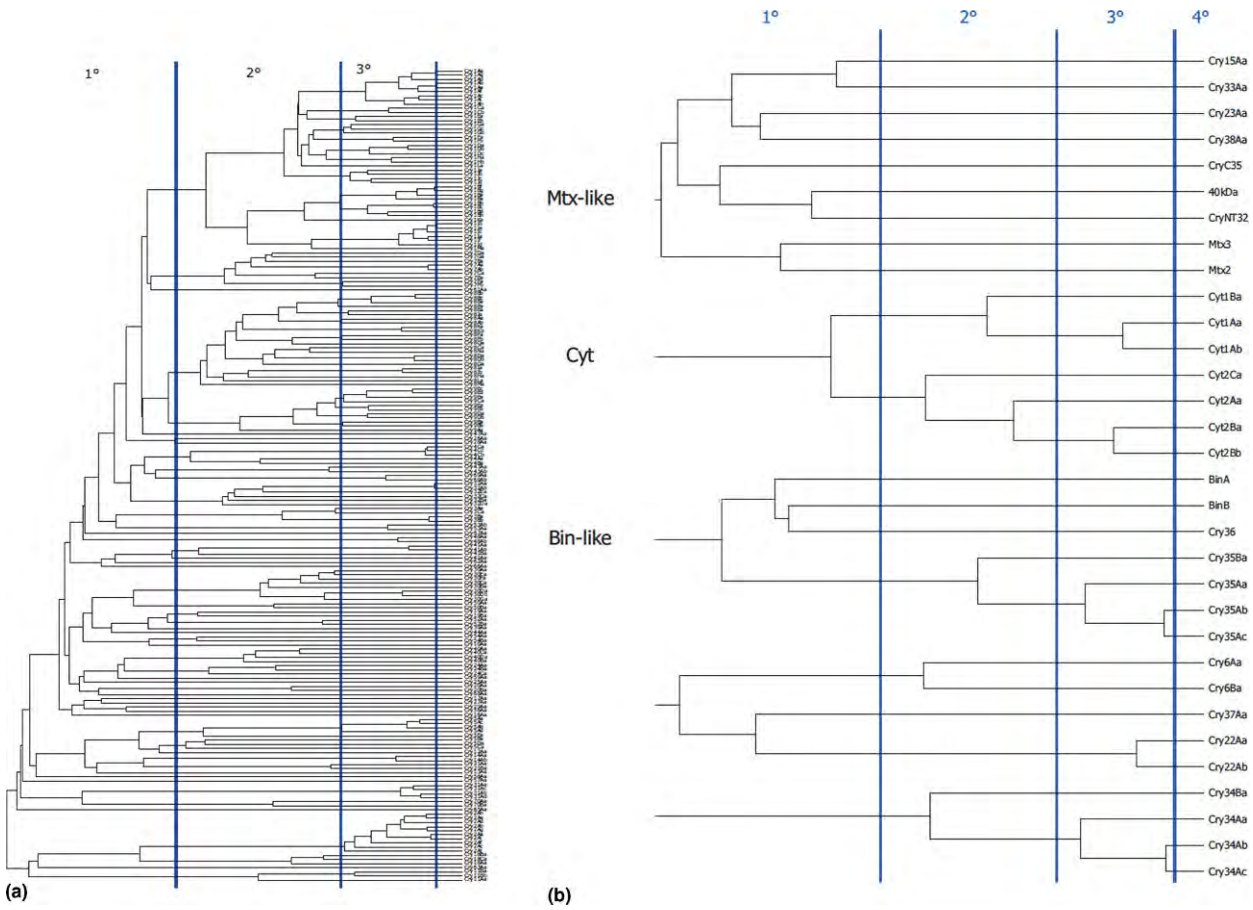

Fig. 2 Dendogram of Cry protein sequences. Three domain (left panel) and other Cry protein sequences (right panel). Update and dendogram analysis can be found in http://www.btnomenclature.info/.

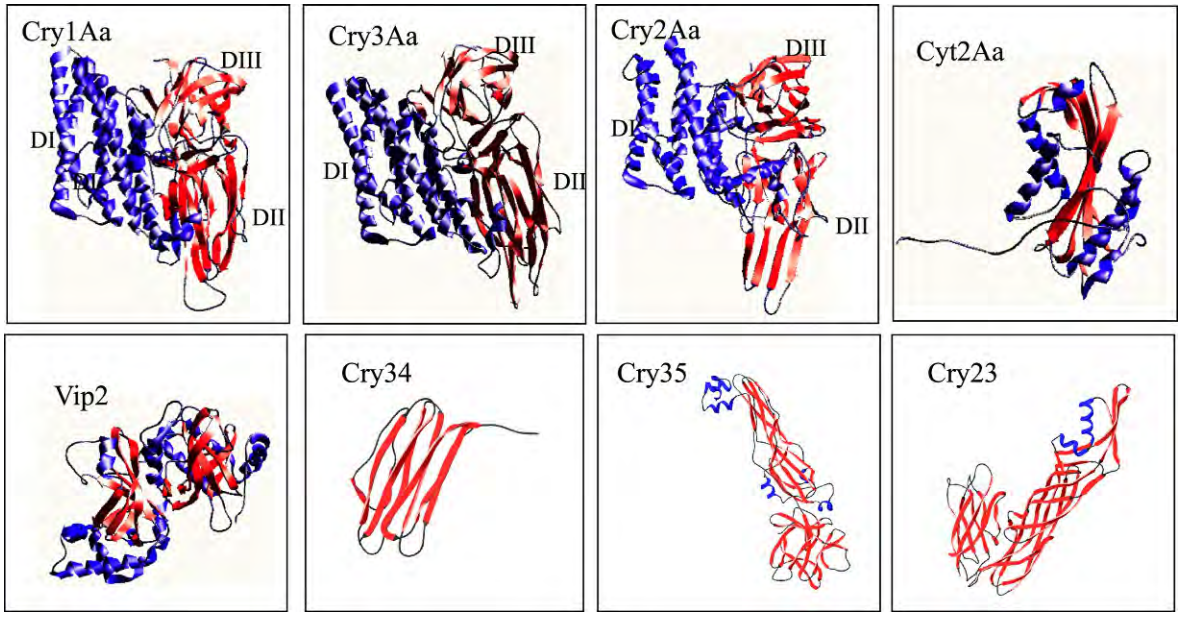

Fig. 3 Three dimentional structures of insecticidal toxins produced by *Bacillus thuringiensis*.

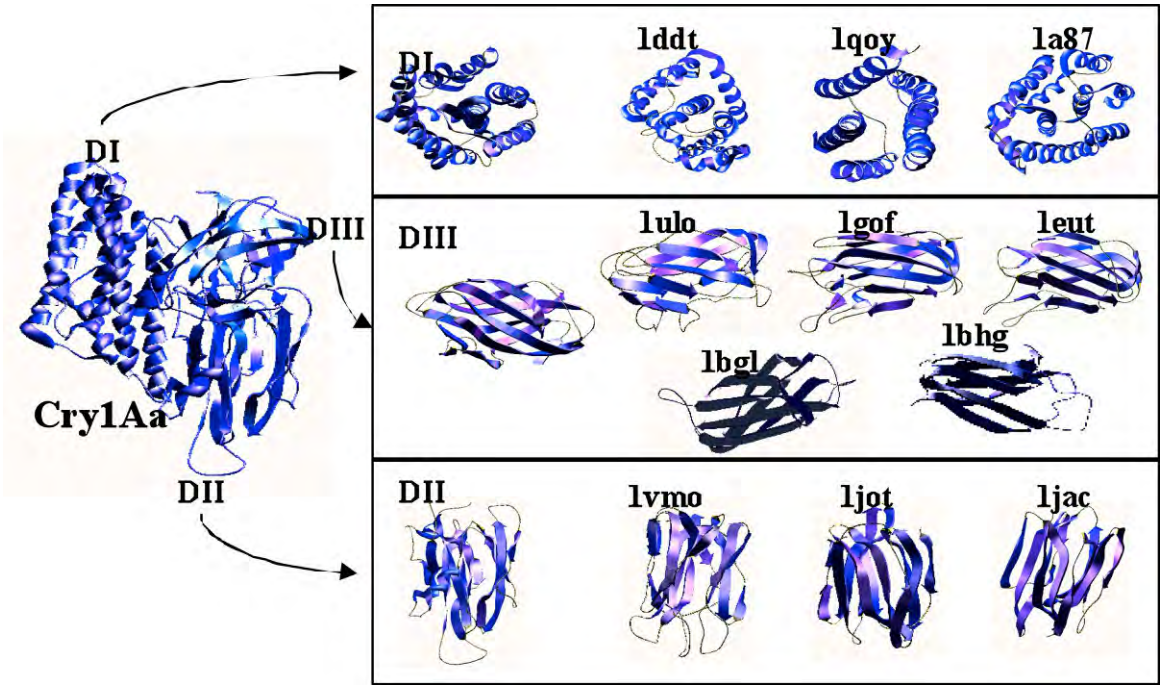

Fig. 4 Proteins with structural similarity to the three domains of Cry toxins.

that are cleaved out after proteolytic activation of the toxin (Fig. 3, 21). Domain II consist of three anti-parallel β-sheets packed around a hydrophobic core in a "beta-prism". This domain represents the most divergent part in structure among Cry toxin molecules (de Maagd et al., 2001) and has been described as the specificity-determining domain. Finally, domain III is a β-sandwich of two antiparallel β-sheets. Several lines of evidence indicate that domain III is implicated insect specificity and therefore involved in receptor binding. Although additional roles of this domain, as pore-formation have been suggested (Schwartz et al., 1997c; de Maagd et al., 2001).

Fig. 4 shows the analysis of structural similarities of the three domains of Cry toxins with other proteins. This analysis revealed interesting features that could give hints on the possible role of these domains in the mode of action of Cry proteins. Domain I shares structural similarities with other pore forming toxins like colicin Ia and N (PBB codes: 1cii, 1a87), haemolysin E (1qoy), and diphteria toxin (1ddt), supporting the role of this domain in pore-formation. In the case of domain II, structural similarities with several carbohydrate-binding proteins like vitelline (1vmo), lectin jacalin (1jac), and lectin Mpa (1jot). For domain III, also carbohydrate-binding proteins with similar structures were identified as the cellulose binding domain of 1,4-*b*-glucanase CenC (1ulo), galactose oxidase (1gof), sialidase (1eut), b-glucoronidase (1bgh), the carbohydrate-binding domain of xylanase U (1gmm) and b-galactosidase (1bgl). These results suggests that carbohydrate moieties could have an important role in the mode of action of 3-Domain Cry toxins. As will be discussed later the involvement of carbohydrate moieties in the mode of action of some Cry toxins has been demonstrated.

Fig. 3 shows also the ribbon representation of the three dimensional structure of Cyt2Aa toxin (PDB 1CBY) (Li et al., 1996). The structure of other Cyt toxins have also been solved and reported (PDB 3RON for Cyt1Aa, 2RCI for Cyt2Ba). (Cohen et al., 2008, 2011; see "Relevant Websites section"). Cyt2Aa has single domain of α/β architecture comprising two outer layers of α-helices hairpins wrapped around a β-sheet. Cyt2Aa protoxin is organized as a dimer. Proteolytic activation releases the active Cyt2Aa toxin monomer (Gazit et al., 1997). Based on the length of the secondary structures it was proposed that the inner core of β-sheets (β-5, β-6, β-7 and β-3) could span the cell membrane (Gazit et al., 1997). However, analysis of membrane insertion capabilities of synthetic peptides corresponding to the different secondary structural elements suggested that α-helices A and C, rather than the β-sheets, could be the elements responsible for membrane interaction (Gazit et al., 1997). In the case of Cyt1Aa it was shown that the N-teminal part of the toxin containing the α-helix hairpins is involved in toxin oligomerization while the β-sheets are involved in membrane insertion (Rodriguez-Almazán et al., 2011). Also, it was shown that certain mutations in the Cyt1Aa α-helix C affected oligomerization, membrane insertion and toxicity suggesting also that oligomerization of Cyt1Aa is important for membrane insertion (López-Diaz et al., 2013).

Vip2A and Vip1A were isolated and characterized from a *B. cereus* strain (Warren, 1997). The structural genes *vip2* and *vip1* form an operon and both proteins are required for toxicity. Therefore Vip1 and Vip2 form a binary toxin. Fig. 3 shows the three-dimensional structure of Vip2 protein (PDB 1QS1) (Han et al., 1999). Vip2 is a mixed α/β protein and is divided into two domains. The domains are structurally homologous although they share low amino acid identity. The overall fold of each domain resembles

the catalytic domains of classical A-B toxins that have two components, a binding component (Vip1 presumably) and a catalytic ADP-rybosilating component (Vip2) (Warren, 1997). It is porposed that Vip2A attaches a ADP-ribose moiety to a monomeric G-actin, affecting its polymerization and the cytoskeleton integrity (Jucovic et al., 2008).

The structure of Bin-like Cry34 and Cry35 proteins have been solved. Fig. 3 shows the three-dimensional structure of these proteins (PDB: 4JP0 and 4JOX) (Kelker et al., 2014). Cry35 has similarities with BinB toxin from *L. sphaericus* and Cry34 shows similarities with other pore forming toxins such as Aegerolysin family of proteins (Kelker et al., 2014).

Finally, regarding to the Mtx-Cry toxins only the structure of Cry23 was solved. Fig. 3 also shows the three-dimensional structure of Cry23 protein (PDB 4RHZ). This protein associates with Cry37, which has no similarities with other Mtx-like Cry toxins but together form a pore forming toxin (de Maagd et al., 2003).

Evolution of 3-Domain Toxins

The increasing number of Cry toxins and the wide variety of target organisms that these proteins have, puts forward the question as how evolution of this protein family create such a diverse arsenal of toxins. It has been proposed that toxins co-evolved with their target insects (de Maagd et al., 2001). However, there are no studies that correlate the geographical distribution and Cry protein content of Bt strains and the distribution of their target insect species in nature. In one particular study, a Bt strain collection obtained from different climate regions of Mexico revealed that putative novel *cry* genes were more frequently found on Bt strains isolated from tropical regions where the insect diversity is high (Bravo et al., 1998). These results suggest that Cry toxins and insects may co-evolve although more comprehensive studies are needed to support this assumption.

The results of phylogenetic analysis of the complete Cry toxin or protoxin protein showed a broad correlation with toxicity (Bravo, 1997). However, the analysis of the phylogenetic relationships of the isolated domains revealed interesting features regarding the creation of diversity in this family of proteins (Bravo, 1997; de Maagd et al., 2001). The analysis of phylogenetic relationships of isolated domain I and domain II sequences revealed that these domains coevolved. In the case of domain II, phylogenetic relationships showed a topology clearly related to the specificity of the toxin proteins (Bravo, 1997). This result is not surprising in spite of the fact that domain II is involved in receptor recognition and is therefore a determinant of insect specificity (Bravo, 1997; de Maagd et al., 2001). Surprisingly, phylogenetic relationships of domain I sequences, that is involved in the pore formation of the toxin, showed a topology also clearly related to the specificity of the toxin proteins (Bravo, 1997; de Maagd et al., 2001) suggesting that different types of domain I have been selected for acting in particular membrane conditions of their target insect (Bravo, 1997; de Maagd et al., 2001). The analysis of domain III sequences, revealed a different topology due to the fact that several examples of domain III swapping among toxins occurred (Bravo, 1997; de Maagd et al., 2001). Some toxins with dual specificity (coleopteran, lepidopteran) are clear examples of domain III swapping among coleopteran and lepidopteran specific toxins (Bravo, 1997; de Maagd et al., 2001). To illustrate domain III swapping in natural toxins, Cry1B toxins are a good example. Five different Cry1B toxins have been described. These toxins share almost identical domain I and domain II sequences. However, Cry1B domain III sequences cluster with other Cry genes suggesting a very active process of domain III exchanges: Cry1Ba domain III sequence is closely related to domain III sequences of Cry1Jb, Cry8Aa and Cry9Da (see Fig. 5). In contrast Cry1Bb and CryBc share similar domain III sequences between them. In the case of Cry1Be, domain III clusters with domain III from Cry1Cb and Cry1Eb. Finally Cry1Bd domain III is similar to domain III of Cry1Ac toxin. For some Cry1B toxins bioassays show that some of these toxins have different insect specificities, Cry1Ba is active against the coleoptera insect Colorado potato beetle, while Cry1Bd has higher activity against the lepidoptera *Plutela xylostella* than Cry1Ba and Cry1Bb toxins. A more comprehensive analysis of toxicity of Cry1B toxins could establish the relevance of domain III swapping in toxicity. However, this result suggests that domain III swapping could create novel specificities. Indeed, *in vitro* domain III swapping of certain Cry1 toxins results in changes in insect specificity (Bosch et al., 1994; de Maagd et al., 2000, 2001). Fig. 5 shows the topology of the phylogenetic relationships of domain III sequences and the representation of the natural Cry toxins in which domain III swapping occurred.

The phylogenetic analysis of the Cry toxin family shows that the great variability in the biocidal activity of this family has resulted from two fundamental evolutionary process: (1) Independent evolution of the three structural domains and (2) domain III swapping among different toxins. These two processes have generated proteins with similar modes of action but with very different specificities (Bravo, 1997; de Maagd et al., 2001).

Two different types of 3D protoxins have been described, short protoxins of around 70 kDa and long protoxins of 130 kDa. The three dimensional structure of two protoxins one short, Cry2Aa, and one long, Cry1Ac, have been solved (Morse et al., 2001; Evdokimov et al., 2014). In the case of Cry2Ab the N-terminal fragment corresponds to two small α-helices that are hiding domain II regions presumably involved in receptor interaction (Morse et al., 2001). In the case of Cry1Ac protoxin, the C-terminal end is composed of four additional domains IV to VII (Evdokimov et al., 2014). It is remarkable that C-terminal region is extremely conserved among all long Cry protoxins. The role of the C-terminal domains in toxin action still remains to be analyzed. Domains IV and VI are composed of α-helices, they resemble structural/interaction domains. Domains V and VII are form of β-strands and resembled carbohydrate-binding modules (Evdokimov et al., 2014). The conserved structure of different 3D-Cry toxins that share low sequence identity and that show different insect specificity suggests that the mode of action is conserved in different insect orders (Pardo-lópez et al., 2013).

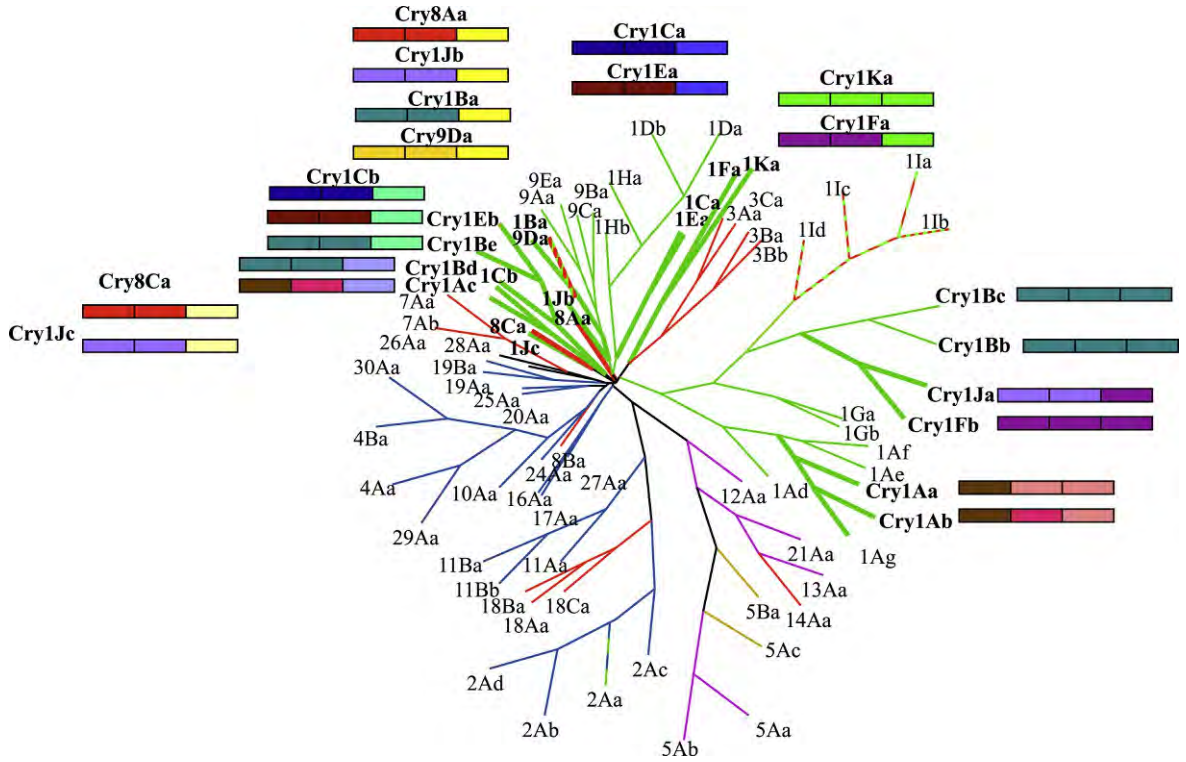

Fig. 5 Phylogenetic relationships of Cry domain III sequences. Genes with similar domain III sequences are shown and depicted with the same color.

Mode of Action of 3-Domain Cry Toxins

Intoxication Syndrome of Cry Toxins

As mentioned previously, it is widely accepted that the primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming lytic pores in the apical microvilli membrane of the cells (Schnepf et al., 1998; Aronson and Shai, 2001; de Maagd et al., 2001). In order to exert its toxic effect, Cry proteins pass from crystal inclusion protoxins into membrane-inserted oligomers that causes ion leakage and cell lysis. The crystal inclusions ingested by susceptible larvae dissolve in the environment of the gut, and the solubilized inactive protoxins are cleaved by midgut proteases yielding 60–70 kDa protease resistant proteins (Choma et al., 1990). Toxin activation involves the proteolytic removal of an N-terminal peptide (25–30 amino acids for Cry1 toxins, 58 for Cry3A and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells (Schnepf et al., 1998; de Maagd et al., 2001) before inserting into the membrane (Schnepf et al., 1998; Aronson and Shai, 2001). Toxin insertion leads to the formation of lytic pores in microvilli apical membranes (Schnepf et al., 1998; Aronson and Shai, 2001). Cell lysis and disruption of the midgut epithelium releases cell content providing spores a germinating medium leading to a severe septicemia and insect death (Schnepf et al., 1998; de Maagd et al., 2001).

Solubilization and Proteolytic Activation

Solubilization of long protoxins (130 kDa) depends on highly alkaline pH that is present in *Lepidoptera* and *Diptera* guts, in contrast to the *Coleoptera* guts that have a neutral to slightly acidic pH (Dow, 1986). In few cases protoxin solubilization has been shown to be a determinant of insect specificity. Cry1Ba is toxic to the Coleopteran *Leptinotarsa decemlineata* only if the protoxin is previously solubilized *in vitro*, suggesting insolubility of the toxin at the neutral pH of Coleopteran insects (Bradley et al., 1995). C-terminal portion of protoxins contain many cystein residues that form disulphide bonds on the crystal inclusions, therefore reducing the disulphide bonds is a necessary step on the solubilization of long Cry protoxins (Du et al., 1994). Differences on midgut pH between *Lepidopteran* and *Coleopteran* midguts resulted on a bias in the utilization of arginine as basic amino acid over lysine in the Lepidoptera-specific toxins Cry1, Cry2, and Cry9 with exception of the Cry1I toxin that is also active against coleopteran insects (Grochulski et al., 1995; de Maagd et al., 2001). The higher pK_a of arginine compared with lysine might be required for maintaining a positive charge even at the high pH of *Lepidoptera* guts resulting in soluble toxins at alkaline pH.

Proteolytic processing of Cry toxins is a critical step involved not only on toxin activation but also on specificity (Haider and Ellar, 1989; Haider et al., 1989) and insect resistance (Oppert et al., 1997; Shao *et al*., 1998). Besides pH, Lepidopteran and

Coleopteran insects differ on the type of proteases present on the insect gut; serine proteases are the main digestive proteases of *Lepidoptera* and *Diptera*, whereas cystein and aspartic proteases are abundant in *Coleopteran* midguts (Terra and Ferreira, 1994). It has been reported that enhanced degradation of Cry toxins is associated with the loss of sensitivity of fifth instar *S. litoralis* larvae to Cry1C (Keller et al., 1996) and that serine protease inhibitors enhanced the insecticidal activity of some Bt toxins up to 20 fold (MacIntosh et al., 1990). More recently, it was found that the low toxicity of Cry1Ab toxin to *Spodoptera frugiperda* could be explained in part by rapid degradation of the toxin on the insect midgut (Miranda et al., 2001). For several Cry proteins inactivation within the insect gut involves intramolecular processing of the toxin (Choma et al., 1990; Lambert et al., 1996; Audtho et al., 1999; Pang et al., 1999; Miranda et al., 2001). However, for several other toxins, intramolecular processing of Cry toxins is not always related to loss of toxicity and sometimes is required for proper activation of the toxin (Dai and Gill, 1993; Zalunin et al., 1998; Yamagiwa et al., 1999). Therefore, in some cases, differential proteolytic processing of Cry toxins in different insects is a limiting step in the toxicity of Cry toxins (Miranda et al., 2001).

During activation by midgut proteases all protoxins are processed in the N-terminal end loosing short peptides of around 40 amino acids while only long protoxins are processed also at the C-terminal end loosing more than 500 amino acid fragment. It was proposed that the C-terminal region of the long protoxins could be involved in crystal formation and that it could be dispensable for toxicity since it is cleaved out during toxin activation. However, the recent structure of this C-terminal region suggest that this region may be playing an additional role in toxin action that remains tp be analyzed in the future. After protease activation a 65 kDa toxic fragment is produced that is composed of three domains (de Maagd et al., 2001).

One interesting feature of Cry toxin activation is the processing of the N-terminal end of the toxins. The 3-dimentional structure of Cry2Aa protoxin showed that two α-helices of the N-terminal region occlude a region of the toxin involved in the interaction with the receptor (Morse et al., 2001; Fig. 3). Several lines of evidences suggest that the processed N-terminal peptide of Cry protoxins might prevent binding to non-target membranes (Martens et al., 1995; Kouskoura et al., 2001; Bravo et al., 2002b). *E. coli* cells producing Cry1Ab or Cry1Ca toxins lacking the N-terminal peptide were severely affected in growth (Martens et al., 1995; Kouskoura et al., 2001). It was speculated that the first 28 amino acids prevented the Cry1A toxin from inserting into the membrane (Martens et al., 1995; Kouskoura et al., 2001). Recently, it was found that a Cry1Ac mutant that retains N-terminus end after trypsin treatment binds nonspecifically to *M. sexta* membranes and was unable to form pores on *M. sexta* brush border membrane vesicles (Bravo et al., 2002b). Therefore, processing of the N-terminal end of Cry protoxins may unmask a hydrophobic patch of the toxin involved in toxin-receptor or toxin-membrane interaction (Morse et al., 2001; Bravo et al., 2002b).

Receptor Identification

It is generally accepted that 3D-Cry toxins bind to receptors located in brush border membranes of midgut cells of susceptible insects and exert their toxicity by inserting into the membrane forming pores that lyse cells by osmotic shock (Vachon et al., 2012; Pardo-lópez et al., 2013).The major determinant of Cry toxin specificity is the interaction with specific receptors on the insect gut of susceptible insects (Jenkins and Dean, 2000). Therefore, receptor identification is fundamental for determining the molecular basis of Cry toxin specificity and also in insect resistance management that in many cases has been shown to correlate with defects on receptor binding (Ferré and Van Rie, 2002). Different insect gut molecules that bind Cry toxins have been characterized in different insect species (Pigott and Ellar, 2007; Likitvivatanavong et al., 2011). These include aminopetidase-N, alkaline phosphatase and cadherins (Pigott and Ellar, 2007). However, other Cry binding molecules as ABCC2 transporters in lepidopteran insects (Heckel, 2012), amylases or glucosidases in mosquitoes (Fernández-Luna et al., 2010; Zhang et al., 2013) or sodium solute symporter in coleoptera (Contreras et al., 2013) have been recently described.

In *M. sexta*, the Cry1Aa, Cry1Ab and Cry1Ac proteins bind to a 120 kDa aminopeptidase-N (APN) (Knight et al., 1994; Garczynski and Adang, 1995; Denolf et al., 1997) and to a 210 kDa cadherin-like protein (Bt-R_1) (Belfiore et al., 1994; Vadlamudi et al., 1995). Cadherins represent a large family of glycoproteins that are responsible for inter-cellular contacts. These proteins are transmembrane proteins with a cytoplasmic domain and an extra cellular ectodomain with several cadherin repeats (12 in the case of Bt-R_1). The ectodomain contain calcium-binding sites, integrin interaction sequences and cadherin binding sequences. In *Bombyx mori*, Cry1Aa binds to a 175 kDa cadherin-like protein (Bt-R_{175}) (Nagamatsu et al., 1998, 1999) and to a 120 kDa APN (Yaoi et al., 1997). *In Heliothis virescens* Cry1Ac binds to two proteins of 120 kDa and 170 kDa both identified as APN (Gill et al., 1995; Oltean et al., 1999). Also, a cadherin-like protein is involved in the mode of action of Cry1Ac toxin in *Heliothis virescens* (Gahan et al., 2001). In *Plutella xylostella* and *Lymantria dispar* APNs were identified as Cry1Ac receptors (Valaitis et al., 1995; Lee et al.1996; Denolf et al., 1997; Luo et al., 1997). In *Lymantria dispar*, besides APN and cadherin-like receptors, a high molecular weight anionic protein (Bt-R_{270}) that binds Cry1A toxins with high affinity was identified (Valaitis et al., 2001). For Cry1C toxin an APN-receptor molecule was identified in *Spodoptera litura* (Agrawal et al., 2002). Sequence analysis of various APN from lepidopteran insects suggests that APN´s group into at least four classes (Oltean et al., 1999). *P. xylostella* and *B. mori* produce the four classes of APN and it is anticipated that several other lepidopteran insects may also produce the four isoforms (Nakanishi et al., 2002). Cry1A binding to the different *B. mori* APN isoforms revealed that this toxin only binds the 115 kDa isoform in ligand–blot binding analysis (Nakanishi et al., 2002).

Surface plasmon resonance experiments showed that the binding affinity of Cry1A toxins to the *M. sexta* APN is on the range of 100 nM (Jenkins and Dean, 2000), while that of cadherin-like receptors (Bt-R_1) is on the range of 1 nM (Vadlamudi et al., 1995).

The differences of binding affinities between APN and Bt-R_1 suggest that binding to Bt-R_1 might be the first event on the interaction of Cry1A toxins with microvilli membranes and, therefore, the primary determinant of insect specificity.

Different experimental evidence suggests the involvement of both Cry1A toxins receptors (APN and cadherin-like) in toxicity. Expression of the *M. sexta* and *B. mori* cadherin-like proteins, Bt-R_1 and Bt-R_{175} respectively, on the surface of different cell lines cells render these cells sensitive to Cry1A toxins (Nagamatsu et al., 1999; Dorsch et al.,2002; Tsuda et al., 2003). Cry1Aa toxin was shown to lyse isolated midgut epithelial cells, this toxic effect was inhibited if the cells were preincubated with anti-Bt-R_{175} antisera in contrast with the treatment with anti-APN antisera, suggesting that cadherin-like protein Bt-R_{175} is a functional receptor of Cry1Aa toxin (Hara et al., 2003). Also, a single-chain antibody (scFv73) that inhibits binding of Cry1A toxins to cadherin-like receptor, but not to APN, reduced the toxicity of Cry1Ab to *M. sexta* larvae (Gómez et al., 2001). Moreover, disruption of a cadherin gene by a retrotransposon-mediated insertion and its linkage to high levels of resistance to Cry1Ac toxin in *H. virescens* YHD2 larvae (Gahan et al., 2001). Overall, these results suggests that binding to cadherin-like receptor is an important step in the mode of action Cry1A toxins.

Regarding APN-receptor, several reports argue against the involvement of this protein in toxin activity. Mutants of Cry1Ac toxin affected on APN-binding retained toxicity levels to *M. sexta* as the wild-type toxin (Burton et al., 1999). Expression of APN in heterologous systems did not resulted in Cry1A sensitivity (Denolf et al., 1997) and, as mentioned above, an APN-antibody did not protect *B. mori* midgut cells from Cry1Aa toxic effect in contrast to a cadherin-antibody (Hara et al., 2003). However, two recent reports clearly demonstrate the importance of this molecule on the mode of action of Cry1 toxins. Inhibition of APN production on *S. litura* larvae by dsRNA interference showed that insects with low APN levels became resistant to Cry1C toxin (Rajagopal et al., 2002). Also, heterologous expression of *M. sexta* APN in midguts and mesodermal tissues of transgenic *Drosophila melanogaster* caused sensitivity to Cry1Ac toxin (Gill and Ellar, 2002). Additionally, previous reports demonstrated that incorporation of *M. sexta* APN into black lipid bilayers lower the concentration of toxin needed for pore formation activity of Cry1Aa toxin (Schwartz et al., 1997b). Finally, all Cry1A APN-receptors are anchored to the membrane by a glycosyl phosphatidylinositol (GPI) (Knight et al., 1994; Garczynski and Adang, 1995; Denolf et al., 1997; Oltean et al., 1999; Nakanishi et al.,2002; Agrawal et al., 2002). The phosphatidylinositol specific phospholipase C (PIPLC) treatment of *Trichoplusia ni* brush border membrane vesicles (BBMV), resulting in cleavage of GPI-anchored proteins from membrane, reduced Cry1Ac pore-formation activity (Lorence et al., 1997). These reports suggest that APN binding is also an important step in the mode of action of Cry1 toxins. Fig. 6 shows the representation of the APN and cadherin Cry1A receptors.

In the case of the dipteran specific Cry11A and Cry4B toxins, two binding-proteins of 62 and 65 kDa were identified on brush border membrane vesicles from *Aedes aegypti* larvae (Buzdin et al., 2002). The identity Cry11A and Cry4 receptors in mosquitoes include cadherin, ALP, APN, alpha-amylases and glucosidases (Fernández-Luna et al., 2010; Zhang et al., 2013; Likitvivatanavong et al., 2011)

One interesting feature of the Cry1 receptor molecules identified so far is that all these proteins are glycosylated. As mentioned before, it is interesting to note that domain II and domain III of Cry toxins have structural homology to proteins domains that interact with carbohydrates. In the case of Cry1Ac, Cry5 and Cry14 toxins different experimental evidence suggest a role of carbohydrate recognition in the mode of action of these toxins (Masson et al., 1995; Griffitts et al., 2001). However, the structural similarities of domain II and III with carbohydrate-binding proteins does not exclude the possibility that these domains may have protein-protein interactions with the receptor molecules. In fact some loop regions of domain II of Cry1Ab and the Bt-R_1 receptor have protein-protein interaction (Gómez et al., 2002a). The interaction between toxin and its receptor can be complex. Interestingly, binding of Cry1Ac to two sites on the purified APN from *M. sexta*, and the interaction is inhibited by N-acetyl-galactosamine, which do not inhibit the binding of Cry1Aa and Cry1Ab to its receptors (Masson et al., 1995). Only one Cry1Ac binding site on APN is also recognized by Cry1Aa and Cry1Ab toxin (Masson et al., 1995).

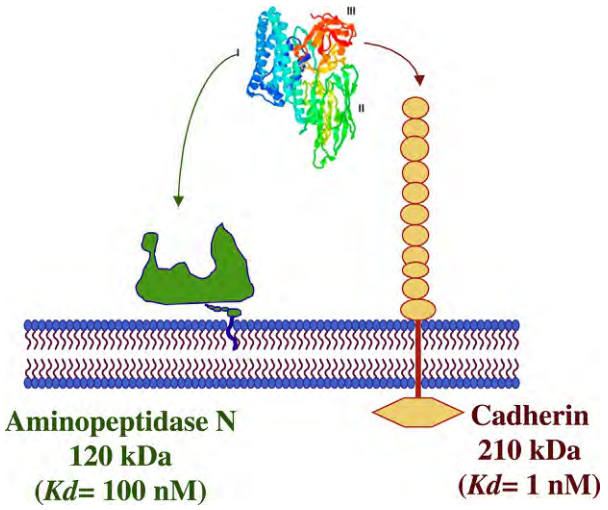

Fig. 6 Receptor molecules of Cry1A proteins. *Kd* values are average affinity values of Cry1A toxins to Aminopeptidase-N and Cadherin receptors.

Toxin Binding Epitopes

The identification of epitopes involved in Cry toxin-receptor interaction will provide insights on the molecular basis of insect specificity and could help in the characterization of resistant insect-populations in nature as to develop strategies to design toxins that could overcome receptor point mutations leading to Cry toxin-resistance.

The toxin binding epitopes have been mapped for several Cry toxins. Domains II and III are most variable regions of Cry toxins and had therefore been subject to mutagenesis studies to determine their role on receptor recognition. Domain II was first recognized as an insect specificity determinant domain based on hybrid-toxin construction using *cry* genes with different specificity (Ge et al., 1989). Site-directed mutagenesis studies of Cry1A toxins showed that some exposed loop regions, loop α-8, loop 2 and loop 3, of domain II are involved on receptor recognition (Rajamohan et al., 1996a,b; Jenkins et al., 2000; Lee et al.2000, 2001). Mutagenesis of amino acid residues present in loop 2 region of Cry1Ab toxin and characterization of these mutants with respect to binding to insect BBMV, showed that RR368–9 residues have an important role in the reversible binding of the toxin to BBMV, while residue F371 is involved in the irreversible binding of the toxin (Rajamohan et al., 1996b, 1995; Jenkins and Dean, 2000). Reversible binding is related to the initial interaction of the toxin to the receptor while irreversible binding is related to the insertion of the toxin into the membrane (Rajamohan et al., 1995). This result was interpreted as if residue F371 was involved on membrane insertion (Rajamohan et al., 1995, 1996b). Also, evidence has been provided showing that loop 2 residues RR368–9 of Cry1Ab are involved on APN binding since this mutant showed no binding to purified APN in surface plasmon resonance experiments (Jenkins and Dean, 2000; Lee et al., 2000). In this regard it is interesting to note that a truncated derivative of Cry1Ab toxin containing only domain II-III was still capable of receptor interaction but was affected on irreversible binding (Flores et al., 1997). Therefore it is likely that mutations in F371 residue affect membrane insertion probably by interfering a conformational change, necessary for membrane insertion, after initial recognition of the receptor or as originally proposed this residue could be directly involved in membrane interaction (Rajamohan et al., 1995). Phylogenetic relationship studies of different Cry1 loop 2 sequence show that there is a correlation between the of loop 2 amino acid sequence and cross-resistance with several Cry1 toxins in resistant insect populations, implying that this loop region is an important determinant of receptor recognition (Tabashnik et al., 1994; Jurat-Fuentes and Adang, 2001). Overall these results suggest that loop 2 of Cry1A toxins play an important role in the recognition of receptors. Regarding loop α-8 and loop 3, mutations of some residues in these regions of Cry1A toxins affected reversible binding to BBMV or purified APN-receptor (Rajamohan et al., 1996a; Lee et al., 2001). The close related toxins Cry1Ab and Cry1Aa have different loop 3 amino acid sequences even though they interact with the same receptor molecules (Rajamohan et al., 1996a). Analysis of binding of Cry1Ab and Cry1Aa to the cadherin-like Bt-R_1 receptor in ligand blot experiments and competition with synthetic peptides corresponding to the toxin exposed loop regions, showed that, besides loop 2, loop 3 of Cry1Aa toxin was important for Bt-R_1 recognition (Gómez et al., 2002a). Although Cry1A loop 1 region seems not to play an important role in receptor recognition this is certainly not the case for other Cry toxins. Mutagenesis of Cry3A loop 1 residues showed that this region, besides loop 3, is important for receptor interaction in *Coleopteran* insects (Wu and Dean, 1996). Loop 1 residues of two Cry toxins with dual insecticidal activity (Cry1Ca and Cry2Aa) are important for dipteran toxicity but not for lepidopteran activity (Widner and Whiteley, 1990; Smith and Ellar, 1994; Morse et al., 2001). Besides loop1 of Cry1C toxin, loops 2 and 3 are important for toxicity to dipteran and lepidopteran insects (Abdul-Rauf and Ellar, 1999). In the case of Cry2Aa toxin an amino acid region involved on lepidopteran activity was not located in the loop regions but was located to a different part of domain II (Morse et al., 2001). These results show that the loop regions of domain II are important determinants for receptor interaction although other regions of this domain, in certain toxins, could also participate in receptor recognition.

As mentioned previously, domain III swapping showed that this domain is involved in receptor recognition (Bosch et al., 1994; de Maagd et al., 2000), and has been used as an evolutionary mechanism of these toxins (de Maagd et al., 2001; Bravo, 1997). The domain III swapping of Cry1Ac to Cry1Ab toxins showed that Cry1Ac domain III was involved in APN recognition (Lee et al., 1995; de Maagd et al., 1999a). The interaction of Cry1Ac domain III and APN was dependent on N-acetylgalactosamine residues (Burton et al., 1999; Lee et al., 1999). Mutagenesis studies of Cry1Ac domain III identified residues ^{509}QNR^{511}, N506 and Y513 as the epitope for sugar recognition (Burton et al., 1999; Lee et al., 1999). The three dimentional structure of Cry1Ac in the presence of the sugar confirmed that these residues are important for Gal-Nac recognition (Li et al., 2001). In the case of Cry1Ac toxin, a sequential binding mechanism to APN receptor has been proposed (Jenkins et al., 2000). The interaction of domain III to a N-acetyl galactosamine moiety in the receptor precedes the binding of loop regions of domain II (Jenkins et al., 2000).

Receptor Binding Epitopes

In regard to the receptor binding epitopes, a region of 63 residues (I135-P198) involved in Cry1Aa binding was identified by analysis of truncated derivatives of *B. mori* APN. This site was specific for Cry1Aa toxin since it was not involved in Cry1Ac binding (Yaoi et al., 1999; Nakanishi et al., 2002). Nevertheless, this binding region is shared with APN molecules that do not bind Cry1Aa toxin when assayed by toxin overlay assays (Nakanishi et al., 2002). This result can be explained if the epitope mapped was not accessible in native conditions. In fact it has been shown that denaturation of *M. sexta* APN exposes binding epitopes hidden under nondenaturating conditions (Daniel et al., 2002). Therefore, the role of the mapped binding epitope in *B. mori* APN in toxicity remains to be analyzed.

Regarding cadherin-like receptors, a Cry1A binding epitope was mapped in Bt-R_1 and Bt-R_{175} receptor molecules by the analysis of truncated derivatives of these receptors in toxin overlay assays (Nagamatsu et al., 1999; Dorsch et al., 2002). In the case of Bt-R_1

and Bt-R_{175} 70 amino acid residues toxin binding region were mapped to cadherin repeat number 11 that is close to the membrane spanning region (Nagamatsu et al., 1999; Dorsch et al., 2002). Using a library of single chain antibodies displayed in M13 phage, a second Cry1A toxin-binding region was mapped in Bt-R_1 receptor (Gómez et al., 2001). An scFv antibody (scFv73) that inhibited binding of Cry1A toxins to the cadherin-like receptor Bt-R_1, but not to APN, and reduced the toxicity of Cry1Ab to *M. sexta* larvae was identified (Gómez et al., 2001). Sequence analysis of CDR3 region of scFv73 molecule led to the identification of an eight amino acid epitope of *M. sexta* cadherin-like receptor, Bt-R_1, (^{869}HITDTNNK^{876}) involved in binding of Cry1A toxins. This amino acid region maps in the cadherin repeat number seven (Gómez et al., 2001). Using synthetic peptides of the exposed loop regions of domain II of Cry1A toxins, loop 2 was identified as the cognate binding epitope of the *M. sexta* receptor Bt-R_1 ^{869}HITDTNNK^{876} site (Gómez et al., 2002a). This finding highlights the importance of ^{869}HITDTNNK^{876} binding epitope since extensive mutagenesis of loop 2 of Cry1A toxins has shown that this loop region is important for receptor interaction and toxicity (Rajamohan et al., 1995, 1996b; Jenkins and Dean, 2000; Jenkins et al., 2000). Accumulating evidence indicate that proteins can interact through amino acid sequences displaying inverted hydropathic profiles (Blalock, 1995). The interaction of loop 2 with Bt-R_1 ^{869}HITDTNN^{875} region was shown to be determined by hydropathic complementarity and that the binding epitope (^{865}NITIHITDTNN^{875}) could be larger than the epitope identified by sequence similarity to the scFv antibody (Gómez et al., 2002a). As mentioned previously, it is generally accepted that the toxic effect of Cry proteins is exerted by the formation of a lytic pore. However, it has also been shown that Cry1A toxins target molecules involved in cell–cell interactions (cadherin) within susceptible hosts as is the case of several other pathogens (Dorsch et al., 2002). Targeting cell junction molecules seems to be representative of those bacteria that disrupt or evade epithelial barriers in their hosts. In this regard it is remarkable that Cry1A toxins interact with at least two structural regions that are not close together in the primary sequence of Bt-R_1 (cadherin repeats 7 and 11). Although we cannot exclude that both sites could be located near by in the three dimensional structure of Bt-R_1, we speculate that binding of Cry1A toxins could cause a conformational change in cadherin molecule that could have a consequence with the interaction with other cell-adhesion proteins and consequently on the disruption of the epithelial cell layer. Fig. 7 shows a schematic representation of the interaction of Cry1A toxins with the Bt-R_1 receptor.

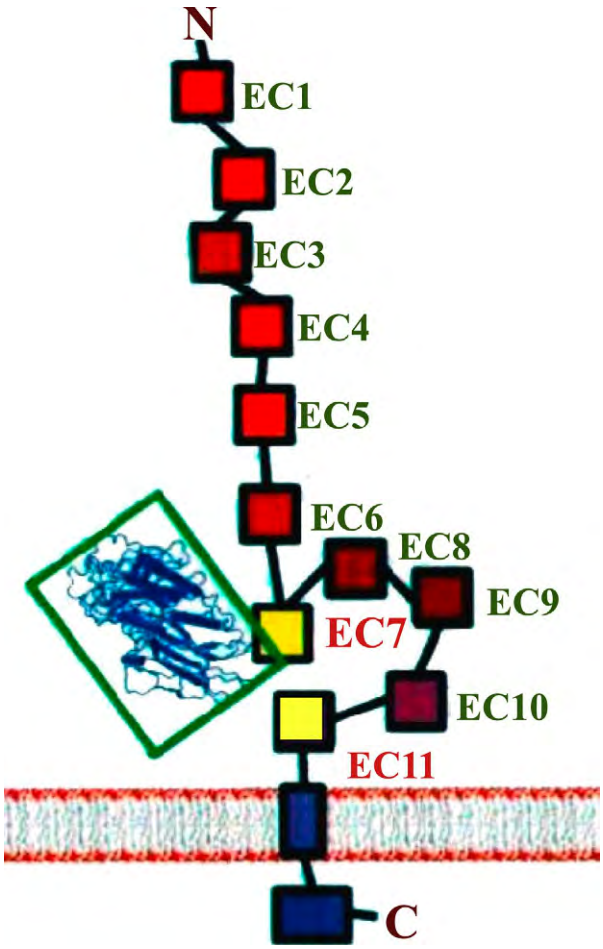

Fig. 7 Structural regions on *M. sexta* cadherin receptor (Bt-R_1) involved in Cry1A binding. Ectodomains (EC) 7 and 11 contain Cry1A binding epitopes.

As mentioned previously, mutagenesis studies have shown that besides domain II loop 2, loop α-8 and loop 3 of Cry1A toxins are important for receptor interaction and toxicity (Rajamohan et al., 1996a; Lee et al., 2001). The Bt-R_1 and the APN epitopes involved in binding loop α–8 and loop 3 regions still remains to be identified.

Cry Toxin-Receptor Binding Function in Toxicity

Three different models of the mode of action have been proposed, the signal transduction model, the pore formation model that involves insertion of monomeric toxin to the membrane before oligomerization and a pore-formation model that involves the formation of a pre-pore oligomer that is responsible for membrane insertion (Zhang et al., 2006; Vachon et al., 2012; Pardo-López et al., 2013). The three models share the initial steps that include protoxin solubilization and activation by gut proteases to release the toxin core.

The signal transduction model was put forward based on the characterization of the death pathway of an insect cell line (H5 cells from *T. ni*) that expressed a cadherin molecule cloned from *M.sexta* larvae, which is a well-known receptor of Cry1Ab toxin (Zhang et al., 2006). The signal transduction pathway that was shown to be involved in cell death included activation of protein G after Cry1Ab binding to cadherin resulting in activation of adenylate cyclase increasing the cellular levels of cAMP. High cAMP levels activate a protein kinase A leading to cell death by oncosis (Zhang et al., 2006). In the signal transduction pathway oligomerization of Cry1Ab toxin and binding other molecules as aminopetidase-N (APN) or alkaline phosphatase (ALP) is not important for toxin action (Zhang et al., 2006).

The two other models propose that toxicity is exerted by pore-formation that leads to osmotic cell lyisis of the midgut cells (Vachon et al., 2012; Pardo-López et al., 2013). However, both models differ in that one model proposes that oligomerization of Cry toxin follows after insertion of monomer toxin to the insect gut membrane while the other model involves oligomerization of the toxin before membrane insertion (Vachon et al., 2012; Pardo-López et al., 2013). The first model proposes that binding of monomeric Cry toxin to any of the characterized midgut receptors as cadherin, APN or ALP is sufficient to facilitate toxin insertion into the membrane (Vachon et al., 2012). This model is supported by the fact that incubation of monomeric toxin with brush border membranes from susceptible insects results in irreversible binding of the monomeric toxin to the membrane (Vachon et al., 2012). This has led to propose that monomers insert into the membrane before toxin oligomerization (Vachon et al., 2012). Nevertheless, it was recently shown that Cry1A toxin is able to form oligomers in solution in the absence of lipid membranes after binding to cadherin (Gómez et al., 2014). Also it was shown that after interaction of monomeric toxin with brush border membranes that contain toxin receptors (including cadherin) the irreversible interaction of the activated toxin with BBMV correlated with insertion of a heat sensitive oligomer into the membrane (Ihara and Himeno, 2008; Gómez et al., 2014), implying that the observed monomers inserted into the membranes are indeed disassembled oligomers that are heat labile (Gómez et al., 2014).

Finally, the third proposed model of the mode of action of Cry1A toxins involves the sequential interaction of Cry1A toxins with different receptor molecules, as GPI-anchored ALP and APN and cadherin protein leading to toxin oligomerization, membrane insertion and pore-formation (Pardo-López et al., 2013). It was proposed that monomeric toxin binds with low affinity to the highly abundant ALP or APN approaching the toxin to the target membrane (Pacheco et al., 2009; Arenas et al., 2010). Then Cry1A monomers bind with high affinity to cadherin facilitating the removal of the N-terminal α−helix 1 by an uncharacterized protease triggering toxin oligomerization (Gómez et al., 2002b; Pacheco et al., 2009). Oligomers bind again ALP and APN with high affinity leading to membrane insertion and pore-formation (Pacheco et al., 2009; Arenas et al., 2010). Domain II exposed loops and Domain III β-16 and β-22 were shown to be involved in the sequential interaction of Cry1Ab toxin with the different receptor molecules (Gómez et al., 2006; Pacheco et al., 2009; Arenas et al., 2010). In favor of this model of the mode of action, Cry1Ab and Cry1Ac were engineered to lack the N-terminal α- helix 1 creating Cry1AMod toxins (Soberón et al., 2007). The Cry1AMod toxins were shown to form oligomers in the absence of cadherin and to counter resistance to *P. gosypiella* Cry1Ac resistant population linked to cadherin mutations. These data shows that cadherin is involved in Cry1A toxin oligomerization and that oligomerization is a key step in Cry1A mode of action (Soberón et al., 2007). Interestingly, Cry1AMod toxins were shown to counter resistance in other insect species where resistance is not linked to cadherin mutations like in *Plutella xylostella* where Cry1Ac resistance is linked to mutations on an ABCC2 transporter (Tabashnik et al., 2011).

It was shown that helix α-3 from Domain I is involved in into oligomerization since certain Cry1Ab or Cry11Aa mutations in this region are affect on toxin oligomerization and toxicity to *Manduca sexta* or *Aedes aegypti* respectively showing that oligomerization is necessary for Cry toxicity in different insect orders (Jiménez-Juárez et al., 2007; Muñóz-Garay et al., 2009). Moreover, characterization of Cry1Ab and Cry11Aa α-helix 4 non-toxic mutants showed that these mutations did not affect toxin oligomerization but oligomers were affected in membrane insertion (Rodríguez-Almazán et al., 2009; Carmona et al., 2011). These results show that oligomerization is a prerequisite step in toxin membrane insertion.

In other pore-forming toxins, receptor binding facilitates a complete proteolytic activation of the protoxins resulting in the formation of functional oligomers that re membrane insertion competent (Abrami and Van der Goot, 1999). In the case of Cry1A toxins, the differences of binding affinities between APN and Bt-R_1 (100 nM *vs* 1 nM K_d respectively) suggest that binding to Bt-R_1 is the first event on the interaction of Cry1A toxins with microvilli membranes.

Evidences that interaction of Cry toxin with its cadherin-like receptor is a necessary step for a complete proteolytic of the toxin were provided (Gómez et al., 2002b). Incubation of Cry1Ab protoxin with the single chain antibody scFv73, that mimics the cadherin-like receptor, and treatment with *M. sexta* midgut juice, resulted in toxin preparations with high pore-formation activity

in vitro in contrast to toxin preparations activated with proteases in the absence of the antibody that showed very poor pore-formation activities (Gómez et al., 2002b). The high pore formation activity correlated with the formation of a 250 kDa oligomer composed of four Cry toxins that lacked the helix α-1 of domain I. The oligomer, in contrast with the 60 kDa monomer, has higher hydrophobicity as judged by 8-anilino-1-naphthalenesulfonate binding (Gómez et al., 2002b). The oligomer was membrane insertion competent in contrast with the monomer as judged by measuring toxin membrane insertion using the intrinsic fluorescence of tryptophan residues (Raussel and Bravo unpublished results). The 250 kDa oligomer structure could also be obtained by incubation of the Cry1Ab protoxin with brush border membrane vesicles, presumably by the action of a membrane associated protease (Gómez et al., 2002b). Therefore the proposed role for cadherin binding is to facilitate the complete proteolytic activation of the toxin and the formation of a pre-pore structure of four monomers. The characterization of the membrane insertion capabilities of the pre-pore, the kinetic characterization of the ionic pore formed by this structure and solving its three-dimensional structure could be important steps towards understanding the role of this insertion-intermediate structure in the mode of action of Cry toxins.

As mentioned previously APN receptors are also key molecules involved in the toxicity of Cry1 toxins. What is the role of APN binding on the mode of action of Cry1 toxins? An indication on its possible role came from the characterization of membrane microdomains (lipid rafts) from microvilli membranes of *M. sexta* and *H. virescens* (Zhuang et al., 2002). Lipid rafts are detergent-insoluble lipid microdomains enriched in cholesterol, sphingolipids, and GPI-anchored proteins (Zhuang et al., 2002). Lipid rafts have been implicated in membrane and protein sorting and in signal transduction (Simons and Toomre, 2000). Also, they have been described as portals for different virus, bacteria and toxins. The interaction of different bacterial toxins with their receptors located in lipid rafts is a crucial step in the oligomerization and insertion of toxins into the membrane (Cabiaux et al., 1997; Abrami and van der Goot, 1999; Rosenberger et al., 2000). Like their mammalian counterparts, *H. virescens* and *M. sexta* lipid rafts are enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol anchored proteins (Zhuang et al., 2002). Several Cry1A receptors, including the GPI anchored proteins, 120- and 170 kDa APN´s from *H. virescens* and the 120 kDa APN from *M. sexta* were preferentially partitioned into lipid rafts. After toxin exposure, Cry1A toxins were associated with lipid rafts and the integrity of these microdomains was essential for *in vitro* Cry1Ab pore forming activity (Zhuang et al., 2002). Therefore the possible role of APN-binding could be to drive Cry1A toxins, probably the pre-pore, to lipid rafts microdomains where the toxin inserts and forms pores. The participation of lipid rafts in the mode of action of Cry toxins could suggest a possible role of signal transduction events and/or the internalization of Cry toxins, since lipid rafts have an active role in these cellular processes. In the case of Cry5 toxin evidence was provided showing that toxin is internalized into the epithelial midgut cells after exposure *C. elegans* nematodes to the toxin (Griffitts et al., 2001).

Binding of Cry1A toxins to cadherin and APN receptors are key steps on the mode of action of these toxins. However, the participation of other uncharacterized molecules on the process of membrane insertion and pore formation of these toxins cannot be excluded. It was proposed that ABCC2 transported could participate in the insertion of the oligomeric structure into the membrane (Heckel, 2012), but this hypothesis requires to be proven. Heterologous expression of the *B. mori* ABCC2 transporter in SF9 cells conferred binding capacity of these cells with the Cry1A toxin and also induced susceptibility to the toxin in toxicity assays suggesting that ABCC2 is a functional receptor of Cry1A toxins (Tanaka et al., 2013). An alternative hypothesis is that ABCC2 may be involved in oligomer formation, this idea was porposed since the Cry1AMod toxins counter resistance to resistant insect populations linked to ABCC2 mutations (Franklin et al., 2009; Tabashnik et al.,2011) and Cry1AMod toxins are able to make oligomers in absence of toxin receptors. Thus, it is possible that in different insects cadherin and ABCC2 may play similar roles.

In an extension of the sequential binding model of toxin action it was reported that Cry1Ab and Cry1Ac protoxins are also able to bind with high affinity cadherin from *M. sexta or P. gosypiella* respectively (Fabrick and Tabashnik, 2006; Gómez et al., 2014). In the case of Cry1Ab it was shown that protoxin binding to cadherin in the presence of midgut juice resulted in Cry1Ab oligomerization. Both oligomers, the one formed from activated toxin or the one formed from protoxin are assembled with monomeric toxins as shown by disassembling oligomers by heat treatment (Gómez et al., 2014). The two oligomers are somehow different they can be distinguished by their membrane insertion capabilities, apparent molecular size, heat sensitivity and pore-formation (Gómez et al., 2014). Also, it was shown that both pre-pore oligomers are involved in toxicity of Cry1Ab to *M. sexta* larvae (Gómez et al., 2014). Therefore, it was proposed that two distinct modes of action involving toxin binding or protoxin binding to receptors are involved in Cry1A toxicity. In favor of this dual-mode of action model, it was recently shown that different resistant lepidopteran strains that evolved resistance to Cry1Ac were more sensitive to Cry1Ac protoxin than to activated Cry1Ac toxin indicating that protoxins have an independent pathway to exert toxicity (Tabashnik et al., 2015).

Toxin Insertion

Insertion of Cry toxins into membranes may require a major conformational change in the toxin to expose hydrophobic surface that could interact with the membrane bilayer. Domain I has been recognized as the pore forming domain based on mutagenesis studies (Wu and Aronson, 1992; Cooper et al., 1998), and the similarity of the structure of this domain with other pore-forming domains of bacterial toxins as colicin Ia, diphteria translocation domain and hemolysin (Aronson and Shai, 2001). The α-helices of domain I are long enough to span the membrane and have a amphipathic character (Aronson and Shai, 2001). Helix α-5 is highly hydrophobic and conserved (conserved block 1) among all 3-domain Cry toxin family (de Maagd et al., 2001). Cross-linking experiments done in Cry1Ac mutants that were genetically engineered to create disulphide bridges between some α-helices of domain I showed that domain I swings away from the rest of the toxin exposing α-7 helix for the initial interaction

with the membrane resulting in the insertion of the hairpin formed by α-helices 4 and 5 into the membrane (Schwartz et al., 1997b). Analysis of the insertion capabilities of synthetic peptides corresponding to the seven α-helices of domain I from Cry3A showed that α-helix 1 is the only helix that does not interact with the membrane in contrast to the other helices (Gazit et al., 1998). Helices α-4 and 5 were the only helices capable of adopting a transmembrane orientation (Aronson and Shai, 2001; Gazit et al., 1998). These results suggest that α-helices 4 and 5 insert into the membrane while the rest of the helices remain on the membrane surface. These data agree with the proposed "umbrella model" of toxin insertion in which helices α-4 and α-5 insert into the membrane leaving the rest of the α-helices in the interface of the membrane (Schwartz et al., 1997b). In view of the proposed role of the pre-pore structure in toxin insertion (Gómez et al., 2002b), it is tempting to speculate that in the three dimensional structure of the pre-pore, four α-helices 7 are exposed and form an hydrophobic surface that participates in the initial interaction of the tetramer with the membrane. Following, four hairpins formed by α-4 and α-5 helices inserts into the membrane. The location of domains II-III in the membrane inserted state is unknown. However, with exception of α-helix 1, membrane inserted Cry1Ac toxin resist proteinase K treatment suggesting that domains II-III might be also, somehow, inserted into the membrane (Aronson, 2000).

A different model of the inserted toxin, based on calorimetric determinations, proposed that the three-dimensional structure of the toxin does not change dramatically compared to the structure of the soluble monomer (Loseva et al., 2001). In this model, pore lumen is provided by domain II and domain III residues while domain I hydrophobic surface (without helices α-1 to α-3) faces the lipid bilayer in a oligomeric structure (Loseva et al., 2001). In this regard it is interesting to note that mutagenesis of conserved arginine residues in Cry1Aa domain III affected the characteristics of the pore-formation activity of Cry1Aa toxin suggesting that domain III participates in the channel activity (Schwartz et al., 1997b). Analysis of pore-formation activity of several Cry1 quimeric proteins established that the characteristics of the pore is influenced by domain II and domain III, combination of domain III from Cry1Ab with domains I and II of Cry1C gave a protein that was more active than Cry1C in Sf9 cells. (Rang et al., 1999). Solving the structure of Cry toxins in the membrane inserted state will be important to determine the possible roles of domains II-III in pore-formation.

Analysis of fluorescence spectroscopy of different single Cys Cry1Ab mutants located each one in different regions of Cry1Ab and labeled with different fluorescent probes showed that the three domains of the toxin remain in the surface of the membrane and only a discrete region of domain I, composed by the α-helices 4 and 5 hairpin, is inserted into the lipid bilayer supporting the umbrella model of toxin insertion (Zavala et al., 2011).

Pore Formation

Based on the observation of large conductance states formed by several Cry toxins in synthetic planar lipid bilayers (Lorence et al., 1995; Peyronnet et al.,2002) and the estimation of a pore size between 10 and 20 Å (Von-Tersch et al., 1994), it has been proposed that the pore could be formed by an oligomer of Cry toxins containing four to six toxin monomers. Evidences showing that the intermolecular interaction between Cry1Ab toxin monomers is a necessary step for pore formation and toxicity were provided (Soberón et al., 2000). Two Cry1Ab mutant proteins affected in different steps of their mode of action (binding and pore formation) recovered pore-formation activity and toxicity against *M. sexta* larvae when mixed, showing that monomers affected in different steps of their mode of action can form functional hetero-oligomers and that oligomerization is a necessary step for toxicity (Soberón et al., 2000). Recently, the structure of the pore formed by Cry1Aa toxin was analyzed by atomic force microscopy showing that the pore is a tetramer (Vie et al., 2001). This data is in agreement with the proposition of a pre-pore structure composed of four monomers (Gómez et al., 2002b). The regions of the toxin involved in oligomerization have not been determined, however, based on mutagenesis studies and analysis of toxin aggregation it has been suggested that some residues of α-helix 5 are implicated in this process (Vie et al., 2001; Aronson et al., 1999).

The pore activity of Cry toxins has been studied by a variety of electrophysiological techniques (Schwartz and Laprade, 2000). Pore formation activity of Cry toxins has been studied in synthetic membranes without receptor or in isolated brush border membrane vesicles containing natural receptors (Peyronnet et al., 2001, 2002; Bravo et al., 2002a). As mentioned previously, the presence of receptor (APN) diminished the concentration, more than 100 fold, of Cry1Aa toxin required for pore-formation activity in synthetic planar bilayers (Schwartz et al., 1997a). Cry toxins form pores that are poorly selective to cationic ions including divalent cations (Lorence et al., 1995; Kirouac et al., 2002). High pore conductances are induced by Cry toxins in synthetic planar bilayers (Lorence et al., 1995; Peyronnet et al., 2002). These high conductances are probably related to clusters of various number of identical size pores operating synchronously rather than pore oligomer structures of different sizes (Peyronnet et al., 2002). In contrast to other pore forming toxins, pore-formation activity of Cry1 proteins is not regulated by low pH, suggesting that Cry toxins are not internalized into acidic vesicles for insertion as other pore forming toxins (Tran et al., 2001).

Overall the mode of action of Cry toxins is depicted in Fig. 8 and can be visualized as follows: (1) Crystal solubilization resulting in 130 kDa protoxin; (2) activation of the protoxin by midgut proteases resulting in the monomer 60 kDa activated toxin production; (3) binding of toxin monomer to GPI-anchored receptors, ALP or APN, located in the apical membrane of midgut cells; (4) binding of toxin monomer or protoxin to the cadherin receptor also located in the apical membrane of midgut cells. Conformational change and oligomer formation of a molten globule state of the monomer with exposure of hydrophobic regions; (5) formation of an oligomeric structure; (6) Binding of the toxin oligomer to APN or ALP receptors; (7) mobilization of receptors and Cry toxin to membrane microdomains and insertion into the membrane for pore formation (Fig. 9).

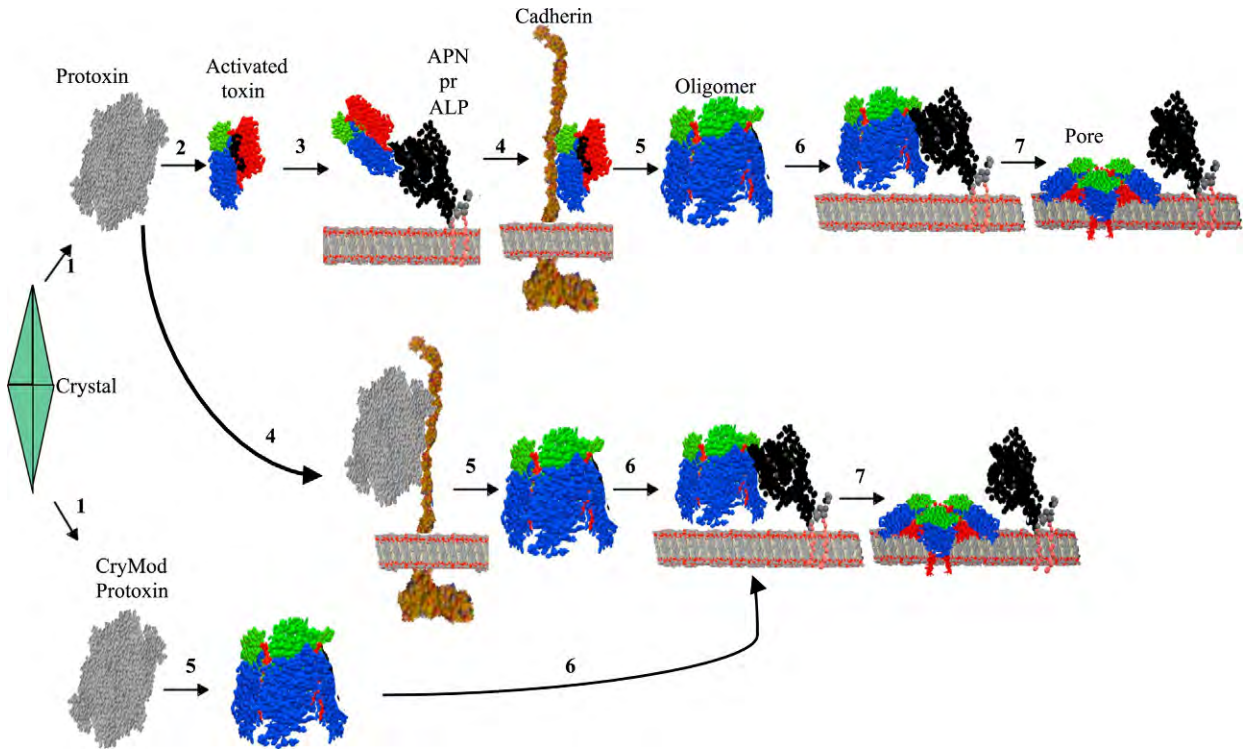

Fig. 8 Mode of action of Cry1A toxins. (1) Toxin solubilization (2) Toxin activation by midgut proteases. (3) Monomer binding to ALP or APN. (4). Cadherin binding of activated toxin or protoxin. (5) Toxin oligomerization. (6) Oligomer binding to ALP or APN. (7) TOlogomer membrane insertion and pore-formation. Upper panel shows the action of activated toxin, middle panel the mode of action from protoxin and lower panel mode of action of Cry1AMod toxins.

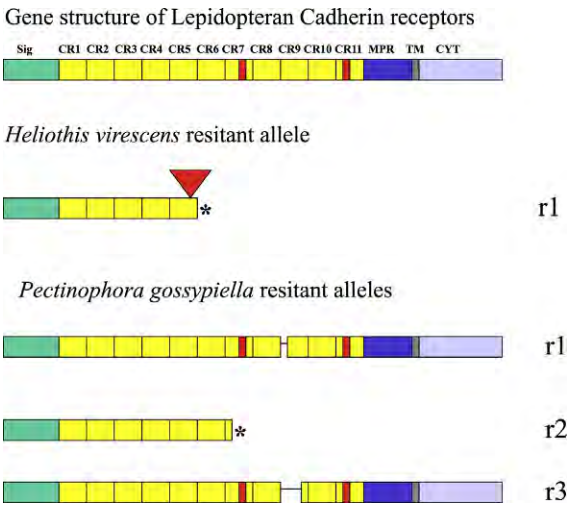

Fig. 9 Gene structure of resistant cadherin-alles of *Heliothis virescens*. Reproduced from Gahan, L.J., Gould, F., Heckel, D.G., 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293, 857–860. And *Pectinophora gossypiella*. Reproduced from Morin, S., Biggs, R.W., Shriver, L., et al., 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. 100, 5004–5009. Sig corresponds to signal sequence for protein export; CR, cadherin repeat or repeated Ectodomains; MPR, membrane proximal region; TM, transmembrane region and CYT, cytosolic domain. * stands for stop codons; red triangle, transposon insertion and deletions are depicted as solid lines. Red sections on CR 7 and 11 are Cry1A binding epitopes mapped on *M. sexta* Bt-R_1 cadherin receptor.

Synergism of Mosquitocidal Toxins

As previously mentioned Cyt toxins synergies the insecticidal effect of some Cry toxins. *Bacillus thuringiensis* subsp. *israeliensis* (Bti) is highly toxic to different mosquito species like *Aedes spp.*, *Culex spp.*, *Anopheles spp.* and black fly. (Margalith and Ben-Dov, 2000). This bacterium produces a crystal inclusion composed of at least four toxins: Cry4A, Cry4B, Cry11A and Cyt1A (Guerchicoff et al., 1997). The toxicity of the crystal inclusion is, by far, greater than the toxicity of the isolated Cry and Cyt components. Bti Cry toxins are

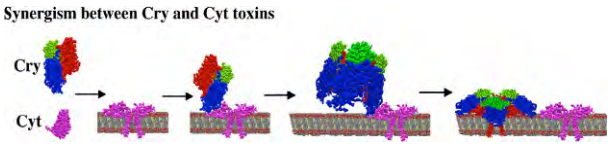

Fig. 10 Mechanism of synergism between Cyt and Cry toxins in mosquitoes. Cyt1Aa toxin functions as a membrane bound receptor of Cry11Aa and Cry4Ba facilitating Cry toxin oligomerization and membrane insertion.

more toxic against mosquito larvae than Cyt1A with a difference in toxicity of at least 25-fold (Wirth et al., 1997). However, the Cyt1A toxin synergies the effect of the other Cry toxins. Toxicity of different combinations of Cry toxins with CytA1 was higher than the addition of expected toxicities of the isolated components (Crickmore et al., 1995; Khasdan et al., 2001). The mode of action of Cyt toxins resembles in some aspects the mode of action of Cry toxins: solubilization of the toxin under the alkaline pH and reducing conditions of the midgut, proteolytic processing of the protoxin, binding of the toxin to the epithelium surface of midgut cells and pore-formation leading to cell lysis (Li et al., 1996, 2001). An important difference between Cyt and Cry toxins is the lack of a protein receptor, Cyt toxins binds phospholipids and are capable of forming pores in cell lines of different origin (Li et al., 1996, 2001). Besides the synergistic effect of Cyt toxins, these proteins overcome or suppress resistance of mosquitoes to the Cry toxins (Wirth et al., 1997). *Culex quinquefasciatus* populations resistant to Cry4A, Cry4B or Cry11A recovered sensitivity when assayed in the presence of Cyt toxin (Wirth et al., 1997). Even more, it has not been possible to select resistant mosquitoes against Cry toxins when selection is performed in the presence of Cyt toxin (Wirth et al., 1997). As mentioned previously, Cyt toxins are principally found in dipteran active strains. However, a coleopteran-active strain containing a Cyt toxin has been described, although the possible synergistic effect of this toxin to other Cry proteins has not been analyzed (Guerchicoff et al., 2001).

The mechanism of synergism of Cyt1Aa and Cry11Aa or Cry4Ba depends on the specific interaction between these toxins since single point mutations on specific epitopes of Cyt1Aa affected Cry11Aa or Cry4Ba binding and synergism (Pérez et al., 2005; Cantón et al., 2011). Also, it was shown that Cry11Aa or Cry4Ba domain II loop regions involved in receptor binding were also involved in binding and synergism with Cyt1Aa (Pérez et al., 2005; Cantón et al., 2011). These data led to the hypothesis that Cyt1Aa functions as a membrane bound receptor of Cry11Aa or Cry4Ba (Pérez et al., 2005; Cantón et al., 2011) (Fig. 10). To enhance the toxicity of Cry11Aa, Cyt1Aa facilitates oligomerization of Cry11Aa as has been shown for cadherin in *A. aegypti* (Pérez et al., 2007). These results indicate that Cyt1Aa is a surrogate receptor of Cry11Aa and Cry4Ba explaining its capacity to counter resistance to these toxins. This is the first example of an insect pathogenic bacterium that carries toxins and also their functional receptor, promoting toxin binding to target membranes and toxicity. Interestingly the Cyt1Aa α-helix C mutants affected in oligomerization still synergize the toxicity of Cry11Aa indicating that membrane insertion is not necessary for the synergistic activity of Cyt1Aa (López-Diaz et al., 2013).

Finally the insect specificity of Cyt2Ab toxin was modified to kill aphids by inserting a peptide sequence in exposed regions of the toxin (Chougule et al., 2013). The peptide sequence was identified by its binding capacity to an aphid APN (Chougule et al., 2013). This suggests that toxicity of Cyt2Ab in aphids is limited by the number of binding sites in the membrane. This is in agreement with the analysis of the mode of action of Cyt1Aa in the non-susceptible lepidopteran *M. sexta* in comparison with *A. aegypti* that showed that toxicity of Cyt1Aa in *M.sexta* is limited by the number of Cyt1Aa binding sites in brush border membranes (Cantón et al., 2014).

Genomics

The genome size of different Bt strains is in the range of 2.4–5.7 million bp (Carlson et al., 1994). Comparison of physical maps with that of *B. cereus* strains suggests that all *B. cereus* and Bt have chromosomes with very similar organization (Carlson et al., 1996). In May 2003 the complete genome sequence of type strains of the close Bt relatives *B. anthracis* (Gene Bank: AE016879) and *B. cereus* (Gene Bank: AE016877) were completed (Read et al., 2003; Ivanova et al., 2003). The *B. cereus* strain ATCC 14,579 strain could be considered a Bt strain based on ribosomal 16S sequence, but does not produce crystals inclusions. 5.427 million bp corresponds to the complete sequenced genome of strain *B. cereus* ATCC 1479 (Ivanova et al., 2003). Analysis of putative coding regions and comparison to the sequence of the *B. anthracis* type strain showed that this group of bacteria lack the capacity of metabolizing diverse types of carbohydrates that characterize typical soil bacteria like *Bacillus subtilis*. Carbohydrate catabolism in the *B. cereus* group is limited to glycogen, and starch, chitin and chitosan (Ivanova et al., 2003). In contrast, *B. cereus, B. anthracis*, and presumably Bt, have a wide variety of peptide, amino-acid transporters and amino-acid degradation pathways indicating that peptides and amino-acids may be the most preferred nitrogen and carbon sources for these bacteria. These data led to the speculation that probably the habitat of the common ancestor of the *B. cereus* group was the insect intestine (Ivanova et al., 2003). The presence in the genome of several hydrolytic activities support this speculation, chynolitic activities could participate in the degradation of chitin present in the peritrophic membrane, while the zinc metalloprotease enhancing could degrade the major component of the peritrophic membrane mucin. Also, several genes related to possible invasion, establishment and propagation in the host were identified in both genome sequences (Read et al., 2003; Ivanova et al., 2003). Finally, the analysis of putative PLcR regulated genes gave a figure of 55 "PlcR boxes" controlling over 100 genes including four other transcriptional activators implying that the PlcR regulon is more complex than expected (Ivanova et al., 2003).

Sequence of Plasmid Pbtoxis

Bt isolates usually harbor large plasmids, which are self-transmisible, containing δ-endotoxin genes that distinguish them from *B. cereus* strains (Schnepft et al., 1998). Therefore, the sequencing of plasmids containing *cry* genes could help to understand the evolution and spread of *cry* genes in nature. In the year 2002, the complete sequence of a toxin encoded plasmid from Bti (pBtoxis) was released (Berry et al., 2002). One hundred twenty eight kilobases were sequenced. This plasmid contains 125 putative coding sequences with an average gene length of 725 bp (Berry et al., 2002). Sequence analysis showed that besides the known Cry toxins genes (*cry4Aa*, *cry4Ba*, *cry11A*, *cry10Aa*, *cyt2Ba*, *cyt1Aa*) a putative novel *cry* gene was identified. Based on phylogenetic relationships with other Cyt toxins, the novel gene was named *cyt1Ca* (136). In contrast to the Cyt toxins, Cyt1Ca, contains an additional domain located on the C-terminus (280 residues) that shares similarity with domains present in other bacterial toxins that are involved on carbohydrate recognition (Berry et al., 2002). Therefore it was proposed that Cyt1Ca could be recognizing carbohydrate moieties in the cell surface (Berry et al., 2002). Based on sequence analysis, 8 pseudogenes were identified. Among these, several gene fragments that shared significative sequence identity to described *cry* genes were identified, suggesting that the ancestors of plasmid pBtoxis contained other *cry* genes that were lost afterwards (Berry et al., 2002). Interestingly the *cry* gene fragments are located nearby transposon related sequences indicating that gene transposition may have caused these genetic rearrangements and could be an important mechanism for toxin-gene spread and evolution (Berry et al., 2002). Beside *cry* genes, plasmid pBtoxis contains several putative genes coding for virulence factors (Berry et al., 2002). A second *plcA* gene, coding for phosphatidyl-inositol phospholipase C, was identified. However, this gene is probably not translated and no "PlcR box" was found in the promoter region of this gene. Interestingly, a "PlcR box" was identified in front of a peptide antibiotic production and export system, this peptide antibiotic may be a novel virulence factor (Berry et al., 2002). Other genes related to sporulation and germination were identified, suggesting that plasmid could influence the sporulation and germination of the host strain (Berry et al., 2002). An interesting feature of plasmid pBtoxis is that it shares similarity with a limited number of genes of plasmid pXO1, the virulence plasmid of *B. anthracis*; 29 of 125 open reading frames show amino acid sequence similarity to open reading frames present in pXO1 (Berry et al., 2002).

Mechanism of Insect Resistance

One of the major concerns regarding the use of Bt is the generation of insect populations resistant to Cry toxins. Resistance to Cry toxins could be obtained by interfering any of the steps involved on the mode of action of Cry toxins. The analysis of the molecular mechanism of insect resistance to Bt toxins have been mostly based on the study of insect resistant populations selected in the laboratory. These studies could provide knowledge leading to design rational procedures for resistant management in nature. However, resistance mechanisms of natural insect populations are likely not to compromise significantly the fitness of the insects to guarantee the fixation of resistant mutant alleles in the population. Therefore, the mutant alleles found on laboratory selected insect populations may not necessarily be found in nature although alleles found in nature could be present in laboratory selected lines.

Selection of insects resistant to Bt toxins in the laboratory has been performed for a variety of insect species. *Lepidopteran* insects resistant to Bt toxins include, *Plodia interpunctella*, *Plutella xylostella*, *Heliothis virescens* and *Spodoptera exigua* (Ferré and Van Rie, 2002). Coleopteran insects species include *Leptinotarsa decemlineata* and *Chrysomela scripta* (Ferré and Van Rie, 2002). As mentioned above, selection of Cry toxin resistant dipteran insects has been performed for *Culex quinquefasciatus* (Wirth et al., 1997). Finally resistance to Cry toxins has also been achieved for the, non-insect, nematode *C. elegans* (Griffitts et al., 2001). Different insect species have developed resistance to Cry toxins in nature, such as the diamondback moth (*P. xylostella*) to Bt- spray applications (Ferré and Van Rie, 2002) and at least 6 different insect species to different Bt-crops, *Spodoptera frugiperda* resistant to Cry1Fa-corn in Puerto Rico and Brazil (Storer et al., 2010; Farias et al.,2014), *Busseola fusca* resistant to Cry1Ab-corn in South Africa (Van Rensburg, 2007), *Pectinophora gossypiella* resistant to Cry1Ac-cotton in India (Dhurua and Gujar, 2011), *Helicoverpa zea* resistant to Cry1Ac-cotton (Tabashnik et al., 2008) and *Diabrotica virgifera virgifera* resistant to Cry3Bb-corn in the USA (Gassmann et al., 2011).

Resistance to Cry toxins can evolve by blocking any of the steps of the mode of action. Following, we will summarize recent findings on the mechanisms involved in resistance to Bt toxins.

Proteolytic Activation

As mentioned previously Cry protoxins are activated by the action of midgut proteases. A Bt toxin-resistant line of *P. interpunctella* had lower protease and protoxin activating activities. These differences were shown to be due to the lack of a major trypsin-like proteinase in the gut. Genetic analysis showed a co-segregation of the lack of the major protease and insect resistance to Cry1Ab toxin (Oppert et al., 1997). The consequence of reduced gut protease was a lower concentration of the activated toxin in the gut (Oppert et al., 1997). The resistant strain showed altered growth and morphology suggesting that in particular this allele is unlikely to be selected in natural populations.

Receptor Binding

The most frequent mechanism of resistance to Cry toxins is defects on receptor binding (Ferré and Van Rie, 2002). Different *Lepidopteran* resistant populations from different species show an effect on toxin binding (Ferré and Van Rie, 2002). In a *P. interpunctella* strain selected for resistance to Cry1Ab, toxin binding affinity (K_d) was reduced 50-fold. Interestingly, this strain

showed an increased susceptibility to Cy1C toxin that correlated with an increase number of binding sites to this toxin (Ferré and Van Rie, 2002). In the case of four *P. xylostella* strains that developed resistance in the field, reduced Cry1A binding correlated with resistance (Schnepf et al., 1998; Ferré and Van Rie, 2002).

In the case of the laboratory selected *H. virescens* Cry1Ac-resistant line YHD2 it was shown that a single mutation was responsible for 40%–80% of Cry1Ac resistance levels. The mutation was mapped and shown to be linked to a retrotransposon insertion in the cadherin-like gene (Gahan et al., 2001). As mentioned previously, this result show that binding of Cry1A toxins to cadherin-like receptors is an important event in the mode of action of these toxins. Based on these results, the group of Tabashnick suggested that if mutations of cadherin genes are the primary basis of resistance in the field, the characterization of cadherin-alleles could be helpful in monitoring field resistance (Morin et al., 2003). The characterization of cadherin alleles in field-derived and laboratory selected strains of the cotton pest *Pectinophora gossypiella* (pink bollworm) revealed three mutated cadherin alleles that were associated with resistance in this lepidopteran insect (Morin et al., 2003).

Resistance has been associated or linked to mutations that affect receptor expression or production. Examples of resistance to Cry1Ac linked to cadherin gene mutations have been shown in *Heliothis virescens* (Gahan et al., 2001), *Pectinophora gossypiella* (Morin et al., 2003) and *Helicoverpa armigera* (Xu et al., 2005). In the case of field-evolved resistance of *Pectinophora gossypiella* in India to Cry1Ac-cotton it was shown that resistance was associated with different cadherin allele mutations (Fabrick et al., 2014). Resistance associated with low ALP or APN expression has been documented in *Trichoplusia ni* (Tiewsiri and Wang, 2011), *H. armigera* (Zhang et al., 2009) or *H. virescens* (Jurat-Fuentes et al., 2002, 2011) to Cry1Ac and also in *S. frugiperda* to Cry1F-Cotton (Jurat-Fuentes et al., 2011) or in *S. exigua* to Cry1Ca (Herrero et al., 2005). In recent years a novel mechanism of resistance to Cry1Ac in different lepidopteran species was shown to be linked to mutations in an ABCC2 transporter gene (Heckel, 2012). ABC transporters mediate the import or export of molecules against their concentration gradient by coupling transport to ATP hydrolysis (Heckel, 2012). Resistance linked to ABCC2 mutations have been characterized in *H. virescens* (Gahan et al., 2010), *Plutella xylostella* (Baxter et al., 2011), *T. ni* (Baxter et al., 2011) and *Bombix mori* (Atsumi et al., 2012). In the case of *T. ni* and *P. xylostella* resistance associated with ABCC2 mutations was evolved in filed conditions in response to Bt formulations (Tabashnik et al., 1994; Janmaat and Myers, 2003). The precise role of ABCC2 in Cry1A toxin mode of action is not understood but expression of *B. mori* ABCC2 gene in insect cell line conferred binding and susceptibility to Cry1A toxins suggesting that ABCC2 is a functional receptor of Cry1A toxins (Tanaka et al., 2013). In the case of *B. mori* resistance to Cry1Ab toxin was linked to a single tyrosine insertion in a predicted exposed loop region suggesting that this loop may be a binding site for Cry1Ab toxin (Atsumi et al., 2012).

Oligosaccharide Synthesis

As mentioned previously, domain II and domain III of Cry toxins show structural similarity to carbohydrate binding domains and in the case of Cry1Ac toxin, binding to the APN receptor involves carbohydrate moieties (de Maagd et al., 1999a; Burton et al., 1999). Nevertheless, the most compelling evidence of the involvement of carbohydrate moieties on the mode of action of Cry toxins came from the selection and characterization of nematode *C. elegans* Cry5B-resistant lines (Griffitts et al., 2001). Five independent *C. elegans* resistant strains were selected by feeding with *E. coli* cells expressing Cry5B toxin. Cloning and sequence of one gene responsible for the resistant phenotype (*bre-5*) showed that the mutated gene shares high sequence similarity to β-1,3-galactosyltransferase enzyme involved in carbohydrate synthesis (Griffitts et al., 2001). The *bre-5* mutation also confers resistance to the Cry14A toxin that also has been shown to be toxic to insects (Griffitts et al., 2001). *bre-5* mutation had no effect on the fitness of the resistant nematodes (Griffitts et al., 2001). These results suggest that carbohydrate moieties could be important in the mode of action (probably binding) of some Cry toxins.

In the case of the *H. virescens* YHD2 Cry1Ac-resistant line, an altered pattern of glycosylation of two microvillar proteins of 63 and 68 kDa was shown to correlate with resistance (Jurat-Fuentes et al., 2002). As mentioned above, YHD2 strain contains several mutations responsible for its Cry1Ac-resistant phenotype. Although the most important resistant allele is the disruption of the cadherin-like gene, altered glycosylation of microvilli membrane proteins increases its resistant phenotype (Jurat-Fuentes et al., 2002).

Applications of Cry Toxins

Forestry

One of the most successful applications of Bt has been the control of *Lepidopteran* defoliators pests of coniferous forests mainly in Canada and United States. Strain HD-1, producing Cry1Aa, Cy1Ab, Cry1Ac and Cry2Aa toxins, has been the choice for controlling forests defoliators. Use of Bt for the control *Choristoneura fumiferana* (Spruce worm) started in the mid 1980s (Van Frankenhuyzen, 2000). Afterwards Bt has been used for the control of *Ch. pinus pinus* (Jackpine budworm), *Ch. occidentalis* (Western spruce worm), *Lambdina fiscellaria fiscellaria* (Eastern hemlock looper) *Lymantria dispar* (gypsy moth) and *Orgyia leucostigma* (whitemarked tussock moth) (Van Frankenhuyzen, 2000). In Canada, for the control of spruce worm, up to 6 million hectares have been sprayed with Bt while up to 2.5 million hectares have been sprayed for the control of other defoliators. In the case of United States up to 2.5 million hectares have been sprayed with Bt. In these countries, the control of forests defoliators relies 80%–100% on the use of Bt. In Europe, Bt use for the control of defoliators greatly increased in the 1990s reaching up to 1.8 million hectares of forests treated

with Bt (van Frankenhuyzen, 2000). Successful application of Bt for the control of defoliators is highly dependent of proper timing, weather conditions and high dosage of spray applications. These factors combine to determine the probability of larvae ingesting a lethal dose (Van Frankenhuyzen, 2000). The use of Bt in the control of defoliators has resulted in a significant reduction on the use of chemical insecticides for pest control in the forests.

Control of Mosquitoes and Black Flies

As mentioned previously, Bti is highly active against disease vector mosquitoes like *Aedes aegypti* (vector of dengue fever) *Simulium damnosum* (vector of Onchocearciasis) and certain *Anopheles* species (vector of malaria). The high insecticidal activity, the lack of resistance to this Bt subspecies, the lack of activity to other organisms and the appearance of insect resistant populations to chemical insecticides made a fast development of Bti as an alternative control method of mosquitoes and black flies populations.

In 1983, very soon after the discovery of Bti, a control program for the eradication of Onchocearciasis was launched in eleven countries of Western Africa since the *Similimum damnosum* populations developed resistance to the larvicidal organophosphate (Guillet et al., 1990). Now days, more than 80% of the region is protected by Bti applications and 20% with temephos (Guillet et al., 1990). The disease has been controlled protecting over 15 million children without the appearance of resistance to Bti (Guillet et al., 1990).

In the Upper Rhine Valley in Germany, Bti along with *B. sphaericus*, has been used to control *Aedes vexans* and *Culex pipiens pipiens* mosquitoes. Each year, approximately 300 river kilometers and 600 km^2 inundation areas are treated with Bti. Since 1981, more than 170,000 hectares of mosquito breeding sites have been treated with different Bti formulations. This program (KABS) has resulted in the reduction of mosquito populations by over 90% each year (Becker, 2000). These results are comparable to the use of Bti for the control of *Aedes vexans* in the United States of America and in Southern Switzerland (Becker, 2000). Again, no resistance to Bti has been observed after more than 20 years of the launch of the program (Becker, 2000).

High Malaria incidences in Hubei Province in China were reduced by more than 90% by the application of Bti and *B. sphaericus* into the Yangtze River. The vector mosquitoes *Anopheles sinensis* and *Anopheles anthropophagus* populations were reduced 90% by weekly applications of fluid formulations of two Bti and *B. sphaericus* strains (Becker, 2000). In Brazil the outbreak of *A. aegypti* resistant populations to chemical insecticides in Rio de Janeiro and Sao Pablo led to the application of Bti formulations for mosquito control (Regis et al., 2000).

The success of vector control using Bti will certainly spread the use of this Bt subspecies around the world. Still the low activity of Bti to certain vector mosquitoes will require the isolation of novel strains with novel cry genes effective against different disease vectors.

Transgenic Crops

Bt is the leading biopesticide used in agriculture. However only 2% of the insecticidal market is cover by Bt sprayable products, the rest is cover mostly by chemical insecticides (Navon, 2000). Since the discovery of the HD-1 strain, formulations for lepidopteran pests control were released. Now days Bt products for the control of coleopteran insects are also available. However, the higher costs of commercial Bt products than chemical insecticides, lower efficacy of Bt compared to chemical insecticides, the limited persistence of Bt products and the narrow insect spectrum limited the number of crops on which Bt is used for plant protection. Moreover, sucking and borer insects are poorly controlled by the spray of Bt products (Navon, 2000).

The creation of transgenic crops that produce crystal Bt proteins has been a major break through on the substitution of chemical insecticides by environmental friendly alternatives. In transgenic plants Cry toxin is produced continuously protecting the toxin from degradation and making it reachable to sucking and borer insects. Cry protein production has been improved by engineering *cry* genes with a codon usage compatible with that of plants, by removal of putative splicing signal sequences and deletion of the c-terminal region of the protoxin (Schuler et al., 1998). In the year 1995 the first Bt-crop (Bt-potato) was commercialized. These transgenic plants expressed the Cry3A protein for protection against Colorado potato beetles resulting in 40% reduction in insecticidal use in the year 1997 (Schuler et al., 1998). In the year 1996 Bt-cotton, producing the Cry1Ac toxin, was released to protect cotton from tobacco budworm, cotton bollworm and pink bollworm. Also in the year 1996 Bt-maize, producing Cry1Ab toxin, was released for the control of European corn borer larvae (Schuler et al., 1998). In the year 2000, transgenic crops were grown on 44.3 million hectares globally. Of this, 23% were insect resistant Bt-maize and 74% were herbicide resistant crops (James, 2001). Yield effects of insect resistant crops in USA are less than 10% on average, nevertheless use of insect resistant crops has diminished considerably the use of chemical pesticides (Qaim and Zilberman, 2003). However, the use of Bt-cotton in countries like China, Mexico and India showed that the use of this Bt-crop had a significant effect on yields and also in the use of chemical pesticides since in underdeveloped countries yield loss is mainly due to technical and economical constrains which are overcome in part by the use of insect resistant crops (Qaim and Zilberman, 2003; Toenniessen et al., 2003).

Recently the pyramided expression of Vip3A with Cry1Ab in Bt- plants have shown that this practice increases the efficacy of insect control of some pests such as *H. zea*, *O. nubilalis* and *S. frugiperda* (Burkeness et al., 2010). In addition, the binary toxin Cry34/Cry35 was expressed alone or in combination with Cry3Bb in corn plants showing efficient control of *D. virgifera* larvae. Hybrids containing both events received less root damage than a single trait, supporting that pyramided traits is a viable strategy for delaying resistant to individual traits (Prasifka et al., 2013).

Public Concerns on the Use of Bt-Crops

Three major concerns have been raised on the use of Bt-crops: Insect resistance, a direct effect on non-target insects and transgene flow to native landraces.

The large scale introduction of Bt-crops endangers the durability of Bt as insecticide in crops and in sprays since continuous exposure to Bt-plants will lead to selection for Cry toxin resistant insect populations. In response to this threat, resistance management strategies associated with the release of Bt-crops have been developed. The predominant approach involves the combination of high production levels of the Cry protein with the establishment of refuge zones (de Maagd et al., 1999b; Conner et al., 2003). Refuges are areas of non-transgenic crops that are within close proximity of the Bt-plants. This procedure aims to maintain a population of susceptible insects that contain wt alleles. In most cases studied, resistance to Cry toxins is conferred by recessive mutations (Ferré and Van Rie, 2002; de Maagd et al., 1999b; Conner et al., 2003). Thus mating of homozygotes individuals with susceptible alleles with heterozigotes individuals with a resistant allele will give rise to heterozigotes susceptible progeny, delaying the development of resistance. Also, second generation transgenic-crops are now being developed that produce two different Cry proteins that bind to different receptor molecules in the same insect. Thus, the possibility of selecting mutation affected on receptor-toxin interaction are diminished exponentially (de Maagd et al., 1999b).

The massive spray of Bt for forest defoliators raised concern on the effect of Bt toxins on non-target insects (Scriber, 2001). More recently, public concerns over the impact of Bt-crops on non-target insects scaled up based on results obtained on the toxicity of Bt-maize pollen on the Monarch butterfly (Losey et al., 1999). Pollen from a commercial variety of Bt-maize (N4640) expressing *cry1Ab* gene in the whole plant including pollen, spread onto milkweed leaves (*Asclepias syriaca*), was lethally toxic to Monarch butterfly caterpillars in the laboratory (Losey et al., 1999). However, follow-up studies showed that low expression of Bt toxin genes in pollen of most commercial transgenic hybrids, lack of toxicity at expected field rates, no overlap of pollen shed and larval activity and the limited overlap in distribution of Bt-maize and milkweed, made field risk to the Monarch populations negligible (Gatehouse et al., 2002; Hellmich et al., 2001; Oberhauser et al., 2001; Pleasants et al., 2001; Sears et al., 2001; Stanley-Horn et al., 2001; Zangerl et al., 2001).

Concerns were raised about the effects of transgene introduction, or gene flow, on the genetic diversity of crop landraces and wild relatives in areas of crop origin. In the year 2001 Quist and Chapela (UC Berkeley) reported the presence of transgenic DNA (from Bt-maize) constructs in native maize landraces in Oaxaca, México (Quist and Chapela, 2001). Oaxaca is consider part of the Mesoamerican center of origin and diversification of maize. Transgenic DNA was identified by PCR based methodology, the 35S promoter sequence was found in five of seven different Criollo maize samples analyzed (Quist and Chapela, 2001). Moreover, inverse PCR reactions of genomic DNA of native landraces showed a high frequency of transgene insertion into a range of genomic contexts (Quist and Chapela, 2001). Nevertheless, based on criticisms on the interpretation of the data and possible false priming in the PCR reactions (Metz and Futterer, 2002; Kaplinsky et al., 2002), *Nature* reconsider its previous judgment on the acceptability of the results reported by Quist and Chapela and concluded that the evidence provided was not sufficient to justify publication (Editor Nature, 2002). Independent studies carried out by the Mexican government (National Institute of Ecology, INE and National Commission of Biodiversity, Conabio) claim to have evidences of the presence of transgenic DNA in native maize landraces although publication of these results is still pending. In any case, gene flow is not restricted to transgenic plants but naturally selected hybrids could also impact the populations and gene content of native landraces. Even if gene flow is confirmed, further studies on the possible impact of the gene flow from commercial hybrids (transgenic or non-transgenic) to native landraces should include long term studies on the integrity of the transgenic constructs (or genes from non-transgenic hybrids) and the maintenance of these genes in the native landraces. These studies should provide a rational framework for taking decisions regarding the introduction and commercialization of transgenic crops in center of origin and diversification of crops. The final goal should be to assure the use of this environmental friendly technology without affecting the genetic diversity of natural landraces.

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Bacterial and Archaeal Cell Membranes☆

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Glossary

Archaea One of the three domains of living organisms: archaea, bacteria, and eukarya. Although archaea share a basic morphology with bacteria and they are also prokaryotes (*i.e.*, they lack a true nucleus); in many molecular details they resemble eukaryotes more than bacteria. Previously called archaebacteria.

Cell membrane A phospholipid bilayer that surrounds all cells. Also called cytoplasmic membrane or plasma membrane.

Cell wall The tough envelope surrounding many cells, including nearly all bacteria and archaea. Located outside the cytoplasmic membrane.

Channel proteins Proteins that form aqueous pores or channels through membranes.

Crystalline surface layer A surface layer (S-layer) of some bacteria and archaea consisting of protein arrays, usually quite resistant to chemicals and proteases.

Domain (1) A discrete, independently folded region of a protein. Different subfunctions of a multidomain protein are usually localized in separate domains. (2) One of the three major taxons: bacteria, archaea, or eukarya.

Electron transport chain The sequential oxidation/reduction of compounds embedded in a membrane that creates proton/sodium gradients across membranes.

Facilitated diffusion Movement of molecules across a membrane from higher to lower concentration mediated by proteins that permit the passage of specific molecules only.

Glycerol ether lipid A type of lipid, characteristic of the archaea, containing isoprenoid lipids that are ether linked to glycerol.

Gram-negative bacteria A group of bacteria with cell envelopes composed of two membranes, the inner and the outer, lipopolysaccharide-containing membranes as well as thin peptidoglycan cell wall layers.

Gram-positive bacteria Two groups of bacteria, both with thick peptidoglycan cell layers. One group, the low G+C Gram-positive bacteria, lacks an outer membrane. The other, the high G+C Gram-positive 'acid-fast' bacteria, has a thick outer cell membrane overlying the cell wall.

Lipid A A phosphorylated glycolipid common to all Gram-negative bacterial lipopolysaccharides.

Lipopolysaccharides Major components of the outer monolayers of the outer membranes of most Gram-negative bacteria. Abbreviated LPS.

Lipoprotein A protein containing covalently bound fatty acids.

Mycolic acid A long-chain organic acid found in the waxy cell envelope of mycobacteria and related acid-fast high G+C Gram-positive bacteria.

Outer membrane The outer lipid bilayer of many prokaryotes and some eukaryotic organelles. In Gram-negative bacteria, they consist of an outer lipopolysaccharide leaflet and an inner phospholipid leaflet plus proteins. In high G+C Gram-positive bacteria, the outer membranes incorporate mycolic acids.

Passive transport Diffusional passage of a compound across a membrane.

Permease A proteinaceous system functioning in the transport of specific substances through a membrane.

Phospholipid bilayer A membrane consisting of two leaflets, each composed of phospholipid.

Proton motive force (PMF) Potential energy stored in the form of an electrochemical gradient of protons across a cell or organellar membrane.

Secondary active transport Active transport of substances using the sodium or proton motive force as the energy source driving substrate accumulation or efflux.

Sodium motive force (SMF) Potential energy stored in the form of an electrochemical gradient of sodium ions across a cell or organellar membrane.

Symbiosis The living together of two different kinds of organism in a nonharmful fashion.

Symport The coupled movement of two molecules together across a cell membrane. Usually, the concentration gradient of one of them drives the movement of the other.

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Abbreviations

ATP	Adenosine triphosphate
CMC	Cytoplasmic membrane
DMSO	Dimethyl sulfoxide
GTP	Guanosine triphosphate
KDO	keto deoxy octulosonate
LPS	Lipopolysaccharides
OM	Outer membrane
OMP	Outer membrane protein
PEP	Phosphoenolpyruvate
PIM	Phosphatidylinositol mannoside
PMF	Proton motive force
SAM	Sorting and assembly machinery
SMF	Sodium motive force
Tat	Twin arginine targeting/translocating
TCDB	Transporter Classification Database
TMAO	Trimethylamine *N*-oxide
TMS	Transmembrane segment
UDP-GlcNAc	Uridyl-diphospho-*N*-acetylglucosamine

Defining Statement

In addition to cytoplasmic membranes, shared by all living cells, many prokaryotes possess protective outer membranes that have compositions very different from those of their inner membranes as well as from those of the outer membranes of other organismal types. This article describes these membranes.

Introduction

Bacterial cytoplasmic membranes (CMs) consist primarily of amphipathic phospholipids with lesser amounts of glycolipids. In the case of Gram-negative bacterial outer membranes (OMs), phospholipids are present in the inner leaflet of the bilayer while lipopolysaccharides (LPS) predominate in the outer leaflet. Most Gram-positive bacteria lack an OM. This is true of almost all low G+C Gram-positive bacteria, called 'firmicutes', although acid-fast, high G+C Gram-positive bacteria, such as *Mycobacterium* species, have mycolic acid-containing OMs that are structurally very different from the OMs of Gram-negative bacteria. Like Gram-positive bacteria, most archaea lack an OM, but recent studies have revealed the occurrence of archaea with these structures. The lipid constituents of both the inner and OMs of archaea are very different from those of bacteria. The OMs of all of these organisms provide a degree of protection from toxic substances, not found in organisms having a single membrane.

Almost all bacterial membranes are assembled in bilayers with embedded integral and associated peripheral membrane proteins. Lesser amounts of carbohydrates (glycolipids and glycoproteins) extend outward from these membranes. Properties of bacterial membrane proteins and lipid–protein interactions have been studied in detail as have those of archaeal membranes. The latter unique membranes often consist of hydrophobic tails linked by ether rather than ester bonds to the glycerol-containing lipid backbone. A few archaea have complex envelopes which, like those in some bacteria, consist of inner and outer membranes that are of different lipid and protein contents.

Our understanding of prokaryotic cell membrane dynamics has advanced considerably since the fluid mosaic model was proposed by Singer and Nicolson in 1972, and links between CM structure and function have been extensively elucidated. Lipid and protein membrane compositions are central to survival since prokaryotes in general are subject to extreme physical and chemical stresses. The CM should be thought of as the primary boundary between the external environment and the living cell while the OM serves a primary protective function. Flexibility in the adaptive capacity of the envelope and its components to environmental conditions is a primary determinant of cell survival.

The selectively permeable envelope allows appreciable diffusion of small neutral molecules such as H_2O, NH_3, CO_2, and O_2, although transport proteins may increase diffusional rates or allow accumulation against concentration gradients. However, this envelope presents high-energetic barriers to the permeability of moderately sized and large polar molecules. The CM serves the cell in numerous capacities providing functions including active transport, macromolecular synthesis, energy generation, maintenance of electrochemical gradients, and cell division.

Membrane Fluidity

Microbial membranes provide a fluid matrix for embedded proteins. The membrane state is frequently defined in terms of degree of fluidity. Fluidity reflects the lipid order and microviscosity which in turn are determined by lipid shape and packing. A composite measure of the lateral and rotational movement of lipids seems to determine membrane lipid phase behavior.

Static as well as dynamic properties are characteristic of all biological membranes. Changes in CM fluidity may reflect physical and chemical interactions with environmental factors such as temperature, pH, osmotic pressure, internal and external ion compositions, and the presence or absence of various chemicals. Membrane perturbations elicit adaptive responses that must compensate for suboptimal conditions. Membrane alterations represent only one type of response, but these membrane responses, required to maintain cell function, guarantee a degree of fluidity that allows transmembrane transport as well as lateral lipid and protein diffusion without jeopardizing membrane stability.

Lipids exhibit polymorphism; each lipid has a distinctive headgroup and two dissimilar fatty acids. They aggregate into different structures such as bilayers and micelles, and assume structurally distinct phases. In a bacterial cell, integral membrane proteins largely exist in the bilayers of the cell, but a smaller portion of these proteins are present as small micelles with a range of phospholipids layered on the hydrophobic belt of the protein, replacing the bilayer. The phases of a bilayer depend on forces including exclusion volume, headgroup interactions, and van der Waals interactions between hydrocarbon chains. Lipid behavior within bacterial membranes is complicated by the huge variety of lipid types present. This diversity makes lipids among the most flexible and versatile of the various types of biological macromolecules.

The dynamic phase behavior of membranes balances the gel-to-liquid crystalline and the lamellar-to-nonbilayer phase transitions. Cumulative evidence suggests that mechanisms of cellular homeostasis are integrated elements of the dynamic range of membrane behavior. Methods used to study fluidity include X-ray diffraction, differential scanning calorimetry, electron spin resonance, nuclear magnetic resonance using ^{2}H or ^{31}P, and fluorescence polarization. Visualization of the membrane has been achieved by electron microscopy and X-ray diffraction. Fluorescence polarization and several of the other cited methods can be used to measure the static versus dynamic characteristics of the membrane. The first of these techniques has the advantage that it can be used with viable bacteria under a variety of normal and stressed conditions as well as *in vitro* with isolated membrane preparations.

Molecular studies have revealed the types of lipid order and phase alterations employed by prokaryotes to maintain CM fluidity and function. The use of extracted bacterial and archaeal lipids has revealed differences of *in vitro* versus *in vivo* properties resulting from membrane adaptations. CM polarization data for bacteria have been obtained under normal and a variety of environmental stress conditions, revealing the responses of membranes to these conditions.

Bacterial CMs

The CM of any bacterium is an ~80-Å-thick structure separating the interior of the cell from the environment. It prevents the diffusion of most substances into and out of the cytoplasm and acts as a selective barrier to concentrate metabolites and nutrients within the cell while secreting waste products and toxins. These structures will be reviewed here while OMs of bacteria will be described in the sections titled 'Gram-negative bacterial OMs' and 'OMs of acid-fast Gram-positive bacteria'. Archaeal membranes will be discussed in the section Archaeal membranes.

Structure, Composition, and Function of CMs

The CMs of most bacteria consist of roughly equal amounts of phospholipid and protein (see Fig. 1(a)). They contain ~70% of the cellular phospholipids and 25% of the cellular proteins. The phospholipids are amphipathic, having hydrophobic tails and hydrophilic heads. The glycerol backbone contains two bound fatty acids and a phosphoryl headgroup. Three major types of phospholipids are present in *Escherichia coli*, about 2×10^7 molecules per cell: 75% phosphatidylethanolamine, 20% phosphatidylglycerol, and 5% cardiolipin (diphosphatidylglycerol). All of these phospholipids contain α-glycerol-phosphate esterified with fatty acids at the one and two positions. The predominant fatty acids in *E. coli* are palmitic acid (16:0), palmitoleic acid (16:1), and *cis*-vaccenic acid (18:1). Different bacteria have different ratios of these lipids, and many have others as well. Sterols, glycolipids, amino acid-containing lipids, and other hydrophobic and amphipathic molecules can be present, depending on the species.

The CM is stabilized by hydrophobic interactions and hydrogen bonds; the former are also known as van der Waals interactions. In addition, divalent cations such as Mg^{2+} and Ca^{2+} stabilize the membrane by neutralizing the negative charges of the phospholipids on both sides of the bilayer, serving as 'salt bridges'. The asymmetric bilayer, with different lipid and protein compositions for the two apposed monolayers, is thus a stable structure that serves as an encapsulating 'bubble' for the cell cytoplasm.

As noted above, the fatty acid composition of the phospholipids that comprise the CM (*e.g.*, chain length, substitutions, and degree of saturation) are determined during biosynthesis, dependent on internal and environmental conditions such as stage of growth, temperature, and composition of the external milieu. The CM maintains a fluid state to allow conformational flexibility and lateral diffusion of proteins and protein complexes. Fluid membranes also have higher transmembrane permeabilities to small molecules than do more rigid bilayers. As noted above, the phospholipids occur primarily in a bilayer, forming a hydrophobic barrier, but micellar structures and 'lipid rafts' of unusual composition may be present, illustrating their dynamic nature.

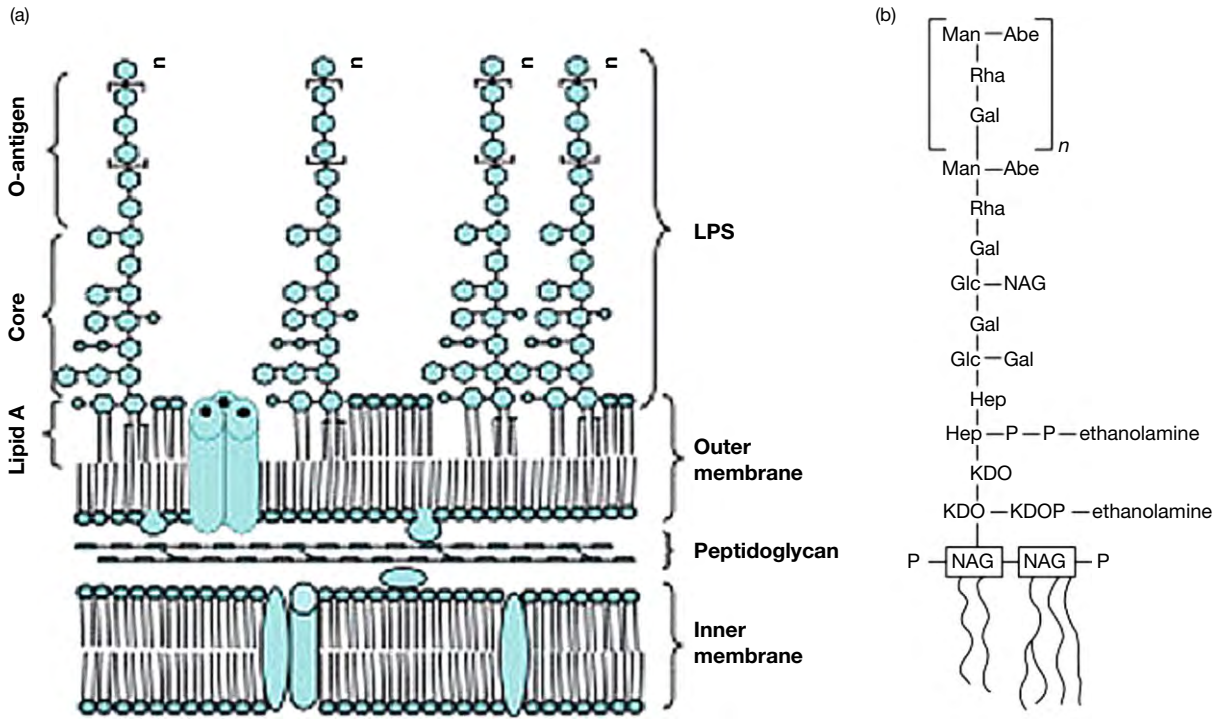

Fig. 1 (a) Schematic view of the *Escherichia coli* cell envelope. Lipopolysaccharide (LPS), embedded within and extending from the outer surface of the outer membrane, consists of three moieties: lipid A, core polysaccharide, and the O-antigen polysaccharide side chains. A trimeric porin in the outer membrane and integral membrane proteins in the inner membrane are depicted schematically. The peptidoglycan cell wall in the periplasm separates the two membranes. (b) Structure of the *E. coli* lipopolysaccharide (LPS) showing the sugar residues. KDO, keto deoxy octulosonate; Hep, heptose; Glc, glucose; Gal, galactose; NAG, *N*-acetylglucosamine; Rha, rhamnose; Man, mannose; Abe, abequose; *n*, a variable number of repeat units.

The membrane prevents the unregulated transmembrane movement of polar molecules and allows the selective retention of ions, essential metabolites, and macromolecules. Due to the presence of specific transport systems, it also catalyzes the active extrusion from the cell of end products of metabolism, drugs, and toxins.

Integral membrane proteins in the CM are anchored into the membrane with one or more transmembrane segments (TMSs). Others, peripheral membrane proteins, are loosely bound and interact transiently, often due to ionic attractive forces. Analyses of the *E. coli* protein content (the proteome) indicate that about one quarter of the predicted gene products are integral membrane proteins in the CM. Many are critical for cellular functions (*e.g.*, transport and cell division). The largest functional class is the transport proteins which comprise 5%–15% of the total proteome, depending on the organism. Owing to their hydrophobic and amphiphilic characters, membrane proteins are much more difficult to study than soluble (cytoplasmic and extracellular) proteins. They account for less than 2% of the known high-resolution protein structures, solved by either X-ray crystallography, multidimensional nuclear magnetic resonance, or cryo-electron microscopy. Topological models have been derived that depict the number of TMSs and the orientation of the proteins in the lipid bilayer, and in numerous cases, these structural predictions have been verified experimentally. However, in relatively few cases do we know how the various TMSs interact with each other to form compact, functional proteins and protein complexes.

Amino acid residues of the portions of proteins that are embedded in the membrane have hydrophobic character, as do residues in the cores of soluble proteins. Residues in the same membrane proteins that are exposed to the aqueous environment, however, are much more polar. Residues in membrane proteins that are exposed to lipid acyl side chains have greater hydrophobic character than do residues in the protein interior. The latter are often semipolar and are important for maintaining correct conformation of the lipid bilayer. In general, TMSs in the CM are hydrophobic α-helices.

In addition to transport, CM proteins can be involved in transmembrane electron flow, energy generation and conservation, biosynthesis of hydrophobic substances, synthesis of cell envelope constituents, and translocation of cell wall and envelope macromolecules from the inside of the cell to an extracytoplasmic locale. This last mentioned function can include translocation and insertion of proteins through and into the one or two membranes of the bacterial cell envelope. Thus, there are at least five compartments in the Gram-negative bacterial cell: the cytoplasm, the inner membrane, the periplasm between the two membranes, the OM, and the extracellular milieu. Moreover, in both membranes, the inner and outer leaflets of these bilayers can be considered as distinct compartments. Specific, dissimilar, and evolutionarily distinct protein insertion complexes are responsible for integration of inner and outer membrane proteins (OMPs) into the envelope. It is also important to note that certain bacteria have been shown to possess a variety of membrane-bounded organelles such as magnetosomes which allow bacteria to orient in the Earth's magnetic

field, chromatophores where photosynthesis occurs, gas vacuoles that provide the function of flotation, and sulfur granules that house elemental sulfur. The complexity of the prokaryotic cell is thus far greater than was initially believed.

Energy Generation and Conservation

Many biosynthetic and CM transport processes are driven by the hydrolysis or transfer of the high-energy phosphoryl moieties of adenosine triphosphate (ATP), guanosine triphosphate (GTP) or phosphoenolpyruvate (PEP). In many cases, the phosphoryl group is transferred transiently to a protein or solute, whereas in a few other cases, phosphoryl bond hydrolysis drives the formation of high-energy protein conformational states; yet other transport processes are energized by transmembrane ion gradients. Cells growing under fermentative conditions, in the absence of oxygen or another inorganic electron acceptor, produce ATP by substrate-level phosphorylation reactions as in the glycolytic pathway. Generally, bacteria that generate energy primarily by substrate-level phosphorylation (fermentation) use ATP or PEP to drive the majority of their transport processes, whereas bacteria that generate energy primarily *via* electron transfer (respiration) use ion (proton and sodium) gradients to drive most of their transport processes. In the former case, the ATP synthesized by substrate-level phosphorylation can also be used to form transmembrane ion gradients by using the F_1F_0 proton-translocating ATPase, while ion gradients generated *via* respiration can be used to synthesize ATP in the reverse process catalyzed by the same enzyme complex. Marine bacteria more frequently use Na^+ gradients to drive transport than observed for fresh water or terrestrial bacteria.

For cells growing under respiratory conditions, the passage of electrons through an electron transfer chain to suitable electron acceptors (oxygen, fumarate, nitrate, nitrite, dimethyl sulfoxide (DMSO), trimethylamine *N*-oxide (TMAO) or hydrogen (H_2)), can be coupled to the extrusion of protons or sodium ions and the creation of transmembrane electrochemical gradients. The resultant proton motive force (PMF, $\Delta\mu_H^+$) or sodium motive force (SMF, $\Delta\mu_{Na}^+$) is essential for life and can be used to drive transport. However, the transmembrane electrochemical gradient is also important in maintaining protein conformations, opening channels, and influencing transmembrane enzyme activities.

Bacterial respiratory chains consist of a series of physically separate protein complexes. Commonly, membrane-bound dehydrogenases transfer two electrons and/or hydrogen atoms from their substrates to the pool of quinones. Electron donors in bacteria include reduced molecules such as NADH, succinate, α-glycerol phosphate, nitrite, and sulfides. Quinones serve as mobile hydride carriers diffusing through the membrane. These quinones shuttle reducing equivalents from the dehydrogenases to terminal reductases or oxidases that oxidize the electron acceptors listed in the preceding paragraph. While ubiquinone-8 is the predominant quinone species in aerobically grown *E. coli* cells, menaquinone-8 is the major species in cells grown anaerobically. Several, but not all of these respiratory/electron transfer complexes, catalyze proton or sodium ion export during electron flow. This proves to be one of the primary mechanisms for generating ion motive forces (the PMF and SMF).

Translocation of Proteins

Integral CM proteins need to be integrated into the membrane, and hydrophilic proteins need to be translocated through the CM from the inside of the cell where they are made, to the external cell surface where they function. For these purposes, the general secretory (Sec) pathway and the twin arginine targeting/translocating (Tat) pathway which act on unfolded and folded proteins, respectively, are usually used. A protein, YidC, assists insertion of proteins into the CM, alone or in conjunction with the Sec pathway. Especially in Gram-negative bacteria, but also in other prokaryotes, other protein complexes are integrated into the CM, working in conjunction with outer-membrane complexes destined to secrete proteins into the medium. Altogether, 18 distinct protein insertion/secretion systems have been identified in Gram-negative bacteria: nine each for transport across or into the inner and outer membranes, respectively. Some of these systems translocate their protein substrates across the two membranes in a single energy-coupled step (See the Transporter Classification Database (TCDB; www.tcdb.org)).

Solute Transport

Nonpolar substances such as fatty acids, neutral alcohols, and simple aromatic compounds enter and exit the cell to some extent by dissolving in the lipid bilayer. By contrast, charged molecules such as organic acids and inorganic salts must be specifically transported. Water penetrates the membrane fairly freely, being small and uncharged, but aquaporins may facilitate the process, allowing more rapid water fluxes than would otherwise be possible in response to osmotic stress conditions. Similarly, passage of NH_3 and CO_2 through the membrane can be stimulated by the presence of 'gas channels'. Most polar molecules are transported *via* specific membrane transporters, but there are many different types and hundreds of families of these proteins as tabulated in the Transporter Classification Database (TCDB). Active transport mechanisms allow the accumulation and extrusion of solutes against concentration gradients, while facilitated diffusion merely allows the energy-independent equilibration of the substrate across a membrane.

Most solutes are transported across prokaryotic CMs by energy-dependent mechanisms, and prokaryotes possess a remarkable array of active transport systems. These systems usually exhibit high substrate affinity and stereospecificity; the affinities reflect the concentrations of the solutes in the natural environments of these organisms. Mechanisms of energy-coupling to transport include: symport with and/or antiport against ions, ATP or GTP hydrolysis, phosphoryl transfer from phosphoenolpyruvate to sugar substrates (group translocation), organic acid decarboxylation, methyl transfer, light absorption, and electron flow. Group

translocation involves the simultaneous transport and modification of substrates, often involving the expenditure of phosphoryl bond-type energy.

Gram-Negative Bacterial OMs

Gram-negative bacteria, and some Gram-positive bacteria, and archaea are surrounded by OMs which serve as selective permeation barriers. They prevent the entry of noxious compounds while allowing the influx of nutrients. They contain β-structured porins and other proteins that allow selective permeability and catalyze specific reactions. These membranes in Gram-negative bacteria will be considered in this section. Those in Gram-positive bacteria will be discussed in the section titled 'OMs of acid-fast Gram-positive bacteria', and those in archaea will be considered in the section titled 'Archaeal membranes'.

Structure and Composition of the OM

The OMs of most Gram-negative bacteria are asymmetric lipid bilayers where the inner leaflet contains phospholipids while the outer leaflet contains a preponderance of LPS (Fig. 1(a) and (b)). Gram-negative bacteria lacking LPS may instead have sphingolipids and/or various glycolipids. These bilayers show low permeability to many solutes. Amino acids, most vitamins, short peptides, sugars, *etc.*, can cross the OM by diffusion through porin channels if smaller in mass than 600 Da. These channel proteins form β-barrel structures with transmembrane spanning segments consisting of amphipathic antiparallel β-strands. α-Helical proteins in the OMs of these organisms are rare, just as are β-structured proteins in the inner membranes. β-Barrel porins, in general, do not concentrate their substrate solutes across the membrane; they catalyze facilitated diffusion.

Other compounds such as vitamin B_{12} and iron siderophore complexes use substrate-specific, high-affinity active transporters to cross the OM. The energy required to allow these transporters to accumulate their substrates in the periplasm is derived from a complex of proteins (*e.g.*, TonB, ExbB, and ExbD in *E. coli*) that use the PMF across the inner membrane to energize uptake. Since these receptors are exposed to the cell surface, infective agents such as some colicins and bacterial viruses (phage) can parasitize TonB-dependent systems or their homologues to enter and kill the host bacteria. Exactly how these protein complexes accumulate their substrates in the periplasm of the Gram-negative bacterial cell and allow passage of toxic proteins or DNA from phage into the cell is still an intense area of research.

The OMs of Gram-negative bacteria contain at least five major classes of proteins: (1) structural lipoproteins, (2) membrane-integrated β-barrel porins, (3) solute-specific receptors, (4) membrane-anchored enzymes, and (5) multicomponent surface structures such as fimbriae (organelles of adhesion), pili (organelles of conjugation or twitching motility), and flagella (organelles of motility). Lipoproteins usually have lipids and/or fatty acids covalently attached to an N-terminal cysteine. These tails, embedded in the OM, anchor these proteins within the membrane. β-Barrel proteins consist of β-sheets that are wrapped into cylinders. Many of these proteins (the porins referred to above) form channels allowing the free flow of nutrients and waste products. Porins can be nonspecific or specific for a particular class of substrates. Nonspecific porins act as 'molecular sieves', but the more specific porins may restrict permeation to a class of sugars, amino acids, ions, or other nutrient types. β-Barrel proteins may also possess enzymatic activities such as hydrolase activities. Moreover, as mentioned in the preceding paragraph, receptor proteins, embedded in the OMs *via* β-barrel structures, can accumulate their solutes in the periplasm against considerable concentration gradients using the PMF across the CM to drive uptake.

OM Lipopolysaccharides

LPSs (Fig. 1(a) and (b)) are found uniquely in most Gram-negative bacterial OMs. They are composed of three parts: the proximal, hydrophobic lipid A region which is embedded in the outer leaflet of the OM; the distal, hydrophilic O-antigen polysaccharide region that protrudes into the medium; and the core oligosaccharide region that connects lipid A to the O-antigen repeat units (Fig. 1(a)). Lipid A is a polar lipid of unusual structure in which a backbone of glucosaminyl-β-(1 → 6)-glucosamine is substituted with six or seven saturated fatty acyl residues.

In *E. coli*, LPS biosynthesis begins in the bacterial cytoplasm with the acylation of uridyl-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) with β-hydroxymyristate. After deacetylation, the product of this reaction is further modified with a second β-hydroxymyristate to generate UDP-2,3-diacylglucosamine. Cleavage of the pyrophosphate bond and displacement of the nucleotide, UMP, produces 2,3-diacylglucosamine-1 phosphate. After condensation of this compound with another molecule of UDP-2,3-diacylglucosamine and 4′ phosphorylation, the intermediate, lipid A, is formed. Two keto deoxy octulosonate (KDO; 3-deoxy-D-manno-oct-2-ulosonic acid) residues are then transferred, and two acyltransferases add lauroyl and myristoyl groups (Fig. 1(b)). The core sugar residues are added onto this intermediate, and an export system translocates them from the cytoplasmic side to the periplasmic surface of the CM. The O-antigen, which is assembled and polymerized separately, is added in the periplasm, completing the biosynthetic process (Fig. 1(b)). Subsequent transport reactions probably move the LPS molecules across the periplasmic space into the inner leaflet and finally to the outer leaflet of the OM (Fig. 1(a)). Lipid A is the biologically active component of LPS which causes inflammation and septic shock in animals.

Three kinds of LPS modifications have been observed: (1) substitution of the phosphate groups in lipid A with phosphoethanolamine, (2) decoration of the basic structure with additional sugar residues, and (3) addition of palmitate by esterification.

Derivatization with phosphoethanolamine renders the bacteria resistant to a lipid A-binding, cyclic, cationic peptide antibiotic, polymyxin, while palmitoylation provides resistance against cationic antimicrobial peptides induced by the innate immune system in response to bacterial infections. These modifications may occur alone or in combination on a single LPS molecule, yielding multiple LPS species in a single bacterium.

Gram-negative bacteria produce OM blebs or vesicles of 0.5–1.0 μm in diameter. These vesicles that can contain enzymes and signaling molecules are released into the culture medium to be delivered to other bacteria, where the vesicles again fuse to the OM of the recipient bacterium. These vesicles can also be used to deliver bacterial protein toxins to mammalian cells. They provide a novel mechanism of prokaryotic communication.

OM Proteins

OMPs are usually synthesized in the bacterial cytoplasm as precursors with N-terminal signal peptides and are translocated, often co-translationally, across the CM *via* the general Sec pathway. After removal of the signal peptides by a signal peptidase, many of the mature proteins insert into the OM and assume β-barrel structures with hydrophobic, membrane-embedded outer surfaces suitable for interaction with LPS and membrane lipids. The insertion process is mediated by a complex of proteins called the BAM complex where Bam A serves as the outer membrane receptor and translocator (see OMP insertion). OM proteins can be classified based on their functions: (1) lipoproteins, (2) general porins, (3) substrate-specific receptors, (4) enzymes, and (5) various other OM proteins.

Lipoproteins

Dozens of lipoproteins have been described. The murein lipoprotein, Lpp of *E. coli*, is the most prominent and best-studied member. Lpp is a small protein (7200 Da) present in about a million copies per cell. Its N-terminal cysteine is modified at two sites. The cysteyl sulfhydryl group is substituted with a diglyceride, and the α-amino group is derivatized by a fatty acyl residue. This allows penetration into and anchoring to the inner leaflet of the OM. About one-third of these molecules are bound covalently to the underlying peptidoglycan cell wall layer, thereby attaching the OM to the wall. Deletion of the *lpp* gene results in numerous defects such as leakage from the periplasm, increased susceptibility of the cell to toxic compounds, and increased blebbing of membrane vesicles from the OM with the release of vesicles into the external milieu. These lipoproteins thus serve important structural roles.

Porins

Porins allow the diffusion of fairly small hydrophilic (and occasionally hydrophobic) molecules. They exhibit varying degrees of substrate specificity. They can be nonspecific or show selectivity only for the charge of the substrate, either anionic or cationic. Some are even specific for certain types of molecules – oligosaccharides, peptides, amino acids – or anions. They generally form OM water-filled channels. Many are either monomeric or homotrimeric, the latter being formed by three hollow β-barrels. However, other quaternary porin structures have been reported. They can be small or large, having 8–24 transmembrane β-strands per polypeptide chain, and they frequently have extra hydrophilic protein domains on one or both sides of the membrane. Their pore sizes vary; several of their three-dimensional structures have been determined, allowing visualization of the permeation pathway. A conspicuous structural feature is the presence of an 'eyelet' region, a narrow constriction in the pore, lined with charged residues. These charged residues determine in part the specificity of the porin for the substrates.

As an example, the trimeric phosphoporin, PhoE of *E. coli*, is produced under conditions of phosphate starvation. The channel-forming motif of PhoE is a 16-strand antiparallel β-barrel. Short β-hairpin turns define the periplasmic side of the barrel, whereas long irregular loops are found at the cell surface. PhoE functions primarily in anion transport due to the presence of positively charged residues near the mouth of the channel. OMPs probably fold in the periplasm before being inserted into the OM in the presence of LPS. The insertion of proteins into these membranes is generally poorly understood, but it depends on a multicomponent protein insertion apparatus which is essential for the process (see 'OM protein insertion').

Substrate-Specific Receptors

While most nutrients gain access to the periplasm by diffusion through porins, a few substrates are too large to enter by this route. Large receptor/transport systems form energy-dependent gated channels which take up these compounds. The TonB/ExbB/ExbD type systems energize transport using the PMF- and TonB-dependent receptors. Examples of such receptors in *E. coli* include BtuB for vitamin B_{12} uptake and several receptors for the uptake of different iron–siderophore complexes. Iron–siderophore complexes are high-affinity iron chelators of microbial origin. Transport requires an interaction with the periplasmic protein TonB, which may shuttle between the inner and outer membranes. The action of TonB requires an energized CM in the form of a PMF. Energy is transferred to the receptors with the assistance of the two cytoplasmic H^+ channel-forming membrane proteins ExbB and ExbD, which energize TonB by transporting protons down their electrochemical gradient, across the CM. Energized TonB then transmits its energy to the receptors.

These siderophore receptors and BtuB are β-barrel monomeric proteins that consist of 22 transmembrane β-strands each. The N-terminal domain consists of a globular structure that inserts itself into the barrel from the periplasmic side, forming a plug. Binding of a ligand induces a conformational change in the protein so that the most N-terminal portion containing a short motif, called the 'TonB box', can interact with the TonB protein. This first step is followed by a large-scale conformational change caused by the energized TonB. The molecular details of this process are not yet fully understood.

OMP Insertion

Gram-negative bacterial OMPs are assembled from the periplasm into the OM in a process that is a current area of research. Large (~800 aas) OMPs, complexed with several others, play a crucial role. These bacterial proteins are very distantly related to the chloroplast import-associated channel proteins, IAP75, constituents of the chloroplast envelope protein translocase. IAP75 has been shown to be a β-barrel porin in the OM of plant chloroplasts. Another homologue is the yeast mitochondrial sorting and assembly machinery (SAM) constituent, SAM50. The SAM complex in yeast mitochondria consists of at least three proteins and is required for the assembly of OM β-barrel proteins in mitochondria. It seems clear that these organellar protein complexes were derived from bacterial proteins when endosymbiotic α-proteobacteria and cyanobacteria became permanent residents of eukaryotic cells as mitochondria and chloroplasts, respectively.

The functionally characterized homologue in the Gram-negative bacterium *Neisseria meningitidis*, Omp85, is essential for bacterial viability. It has a two-domain structure with an N-terminal periplasmic domain rich in hydrophilic repeat sequences and a C-terminal domain that forms an integral OM β-barrel. Unassembled forms of various OMPs accumulate when Omp85 is depleted. Homologues of Omp85 are present in all Gram-negative bacteria examined, but not in other prokaryotes. The *E. coli* homologue functions as a principal constituent of a complex that catalyzes protein insertion into the OM.

Normally OMPs are translocated into the periplasm *via* the Sec translocase. They are believed to fold in the periplasm before being inserted into the OM. Folding is stimulated by small periplasmic chaperone proteins. In *E. coli*, these chaperones feed a substrate protein to the OM integrated multiprotein BAM (β-barrel assembly machinery) complex required for OM biogenesis. The activities of the constituents of this system are absolutely required for proper OMP assembly. In fact, the BAM complex functions to insert both outer membrane β-barrel proteins such as porins and outward facing lipoproteins. The BAM complex consists of five proteins that form a heteromeric complex. These five constituents are Bam A, B, C, D and E. Bam A is a large multi domain transmembrane protein that serves as the primary receptor and channel. The channel has a lateral "gate" between the first and last β-strands (β-TMSs1 and 18) in the barrel, allowing a β-strand in a substrate protein to escape into the OM lipid bilayer. BamB – E, on the other hand, are peripheral membrane proteins that facilitate the folding and insertional events.

OMs of Acid-Fast Gram-Positive Bacteria

Acid-fast actinobacteria belong to a distinctive suprageneric actinomycete taxon, which includes mycobacteria, corynebacteria, nocardia, rhodococci, and other related genera. All of these bacteria share the property of having an unusual cell envelope composition and architecture (Fig. 2(a)). Based on available published data, the envelope layers consist of a typical CM of phospholipid and protein, a characteristic wall of unusual structure, and a complex outer layer. Although studies with mycobacteria are more detailed than with other related genera, it is evident that the envelopes of these bacteria are all similar, especially in terms of ultrastructure and cell-wall composition.

The cell walls are formed by thick *meso*-diaminopimelic acid-containing peptidoglycan layers covalently linked to arabinogalactan. The arabinogalactan is in turn esterified with long-chain α-alkyl, β-hydroxy fatty acids. These fatty acids in mycobacteria are called mycolic acids (Fig. 2(b)). They possess very long chains (C_{60-90}) and may contain various branches, oxygen functions such as hydroxyl, methylated hydroxyl, and keto groups as well as unsaturations. Mycolic acids found in other actinomycetes consist of mixtures of saturated and unsaturated acids, but they contain shorter chains. Nocardomycolic acids are of length C_{40-50} while corynomycolic acids are of C_{22-36}. Thus, mycobacterial OMs are thicker than nocardial OMs which, in turn, are thicker than corynebacterial OMs.

Acid-fast high G+C Gram-positive microbes share with Gram-negative bacteria the property of possessing OMs that are very different in composition from the plasma membranes. While the outer barrier in Gram-negative bacteria is a typical bilayer of phospholipid and LPS, in mycobacteria, nocardia, and corynebacteria, the cell wall-linked mycolates comprise much of this barrier. The lengths and structures of mycolic acids are important in determining not only the membrane width, but also the envelope fluidity and permeability. The existence of OM diffusion barriers in actinobacteria is reinforced by the characterization of cell envelope proteins with pore-forming abilities. The outer permeability barrier consists primarily of a monolayer of mycoloyl residues covalently linked to cell wall arabinogalactans (Fig. 2(a)). Other lipids may be arranged in an outer leaflet to form a complex asymmetric bilayer.

Freeze-fractured samples of mycobacteria and other related bacteria have revealed details of the envelope structures of these organisms with distinct lipid domains. Freeze-fracture electron microscopy also revealed the presence of ordered arrays on the surfaces of these envelopes consisting of surface layer proteins (S-layers) that overlie the OMs. There may therefore be five layers: (1) the inner CM, (2) the cell wall, (3) the arabinogalactan/arabinomannan polysaccharide layer, (4) the OM, and (5) the external proteinaceous S-layer. All have protective functions.

The five layers of the actinobacterial envelope are believed to be integrated to form the protective envelope. Because the amounts of cell wall-linked mycolates are insufficient to cover the entire bacterial surface, other types of noncovalently bound lipids must play

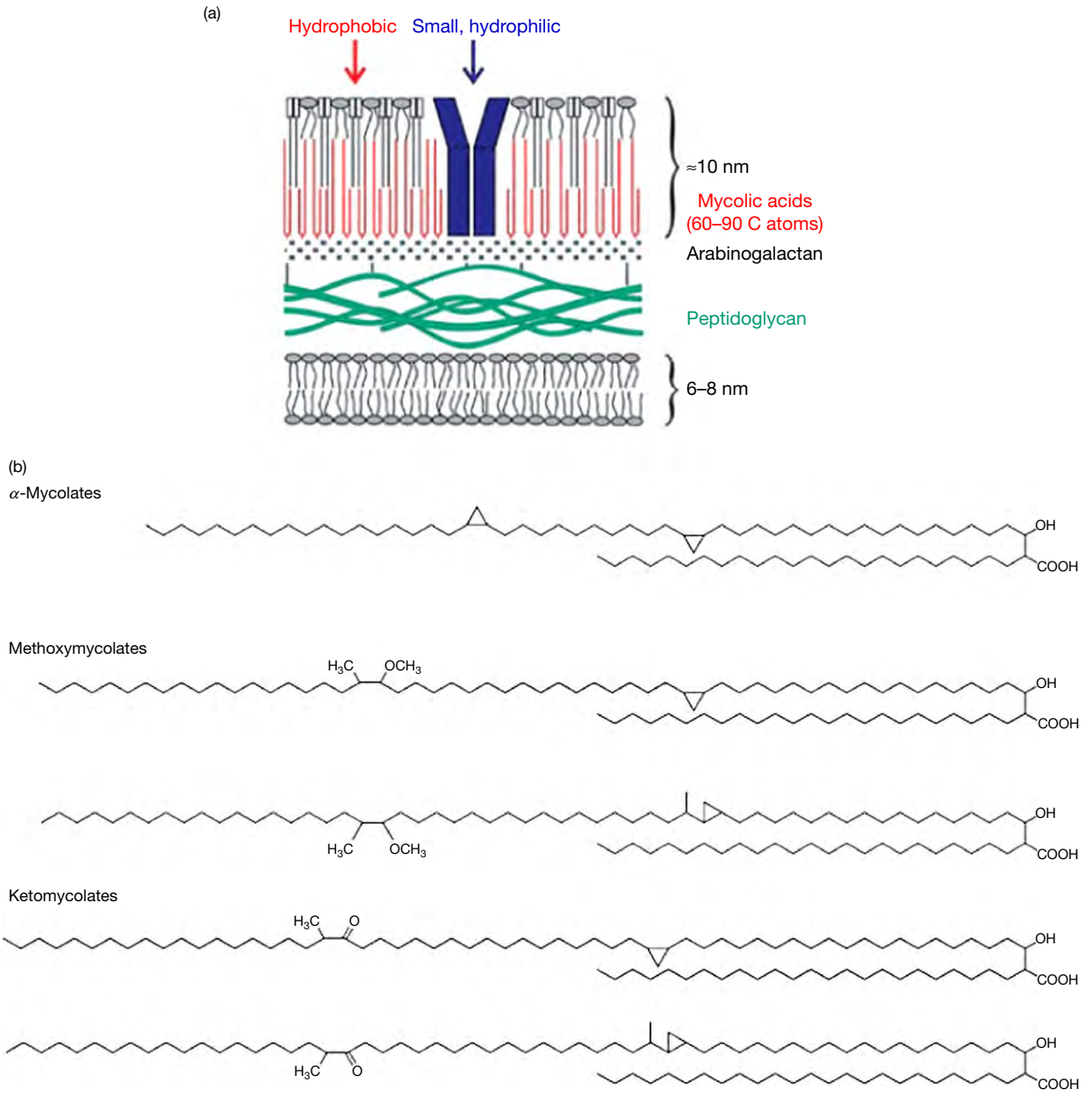

Fig. 2 (a) The most important structural components of the mycobacterial cell envelope. While small hydrophobic molecules may diffuse through the outer lipid bilayer, small hydrophilic molecules require the involvement of outer membrane porins as indicated at the top of the diagram. The figure illustrates the covalent linkages between cell wall peptidoglycan, arabinogalactan, and mycolic acids. (b) Structures of mycolic acids in *Mycobacterium tuberculosis*. α-Mycolates: the meromycolate chains contain two *cis*-cyclopropanes; methoxymycolates: their meromycolate chains contain an α-methyl-ether moiety in the distal position and a *cis*-cyclopropane or an α-methyl *trans*-cyclopropane in the proximal position; ketomycolates: their meromycolate chains contain an α-methyl ketone moiety in the distal position and proximal functionalities as in the methoxy series. Unsaturations are present in some meromycolate chains of *M. tuberculosis* (not shown).

roles in forming the OM. In fact, these lipids have been shown to form bilayer structures spontaneously. Thus, the cell wall permeability barriers in these bacteria involve both covalently wall-linked mycolates and noncovalently bound lipids. These molecules together with various proteins comprise the bulk of the cell envelopes.

OMs of Mycobacteria: Function, Structure, and Composition

As noted above, the permeability of mycobacteria, and other bacteria related to them, to substances in their environments is determined by the properties of their envelopes. Current models depicting the structural organization of the mycobacterial cell wall

assume that peptidoglycan and arabinogalactan strands overlie the CM forming horizontal layers beneath perpendicularly oriented mycolic acids. The mycolate layer prevents entry of small hydrophilic molecules which gain access to the cell only *via* porins (Fig. 2(a)). Some small lipophilic molecules may diffuse through the lipid layer. The capsule prevents passage of virtually all macromolecules unless specific transport systems mediate their entry or exit. The structure of the outer lipid barriers is similar in all mycobacteria, but the capsule is more abundant in slow-growing species than in fast-growing species. The slow-growing organisms comprise the group that includes most mycobacterial pathogens.

Mycobacteria secrete proteins that are important to the pathogenesis of the many human and animal diseases caused by these microbes. Information about how the secreted proteins and the polysaccharides of the capsule cross the outer lipid barrier is fragmentary and is only now coming to light. It is possible that proper knowledge of mycobacterial envelope permeability will enable new approaches to the treatment of mycobacterial diseases.

The cell envelopes of mycobacteria substantially contribute to their resistance to therapeutic agents. This is largely due to the presence of the C_{60-90} mycolic acids that are covalently linked to the large arabinogalactans as well as the acylated and nonacylated arabinomannans. Recent studies have clarified the unusual structures of arabinogalactans as well as extractable cell wall lipids such as phenolic glycolipids, glycopeptidolipids, and trehalose-based lipooligosaccharides called 'cord factor'. Most of the hydrocarbon chains of these lipids assemble to produce the exceptionally thick, asymmetric OM. Structural considerations suggest that the fluidity is unusually low in the innermost parts of the bilayer, gradually increasing toward the outer surfaces. Differences in mycolic acid structure may affect the fluidity and permeability of the bilayer and explain the different sensitivities of various mycobacterial species to lipophilic compounds. Hydrophilic nutrients, vitamins, minerals, toxins, and growth inhibitors, in contrast, traverse the OM exclusively *via* porin channels.

The detailed molecular structures of representative mycobacterial cell envelopes and their lipids are illustrated in Fig. 2(a) and (b). The cell wall architecture resembles a massive 'core' comprised of peptidoglycan covalently attached *via* a linker unit (L-Rha-D-GlcNAc-P) to a linear galactofuran. This in turn is attached to several strands of a highly branched arabinofuran, which is attached to mycolic acids. The mycolic acids are perpendicularly oriented relative to the plane of the membrane (Fig. 2(a)). They create a lipid barrier responsible for many of the physiological, disease-inducing, and drug resistance properties of *Mycobacterium tuberculosis*, *leprae*, *bovis*, *avium*, and other mycobacterial pathogens.

Intercalated within this envelope are the lipids that have intrigued biochemists for over five decades: the phthiocerol dimycocerosate, cord factor (dimycolyltrehalose), the sulfolipids, the phosphatidylinositol mannosides (PIMs) and others. The lipomannans and lipoarabinomannans also play important roles in the physiology and pathogenesis of mycobacteria. These molecules have functions of signaling to the host and stimulating immune responses of infected humans and animals. Mycolic acids are recognized by CD1-restricted T-cells, and antigen 85 – one of the most powerful protective antigens of *M. tuberculosis*– is a mycolyltransferase. Moreover, lipoarabinomannans, when 'capped' with short mannose oligosaccharides, promote phagocytosis of the bacteria by animal cells, an important phase of pathogenesis.

Sequencing of the *M. leprae*, *M. tuberculosis* and other pathogenic and nonpathogenic mycobacterial genomes has aided efforts to define the biosynthetic pathways for all of these exotic lipid and complex carbohydrate-containing molecules. These include mycolic acids, the mycocerosates, phthiocerol, lipidated arabinomannans and arabinogalactans, and the polyprenyl phosphates. We now know that synthesis of the entire core is initiated on a decaprenyl-P with synthesis of linker units. There seems to be concomitant extension of the galactan and arabinan chains while these intermediates are transported through the CM. The final steps in these events, the attachment of mycolic acids, and ligation to peptidoglycan, must occur in the periplasm.

Mycolic Acids and Other Unusual Mycobacterial Lipids

As noted above, mycolic acids of mycobacteria are long-chain fatty acids, many of which vary in size and structure (Fig. 2(b)). Arabinogalactan mycolates are covalently linked *via* phosphodiester linkages to the underlying peptidoglycan cell wall polymer (Fig. 2(a)). Mycolic acids together with other cell wall lipids (trehalose-based lipooligosaccharides, phenolic glycolipids, and glycopeptidolipids) comprise part of the OM and contribute to the low permeability of these envelopes. They account in part for the remarkable drug resistance of mycobacteria rendering treatment of mycobacterial diseases difficult. This is particularly important to human health since one-third of the world's population is infected with mycobacteria, and millions die from mycobacterial diseases every year. The hydrophobic hydrocarbon chains of these lipids comprise the OMs which may be the thickest of all biological membranes yet identified. The asymmetric OM consists largely of long-chain mycolic acids comprising most of the inner leaflet with a diversity of other lipids contributing to the outer leaflet.

Some lipids and lipidated glycans unique to mycobacteria appear to be present in both the inner and the outer membranes. These are the PIMs, their hypermannosylated derivatives, lipomannans, and lipoarabinomannans. They are important virulence factors in pathogenic species. These facts reveal a surprising degree of lipid diversity in mycobacterial envelopes and show that fatty acyl esters linked to complex carbohydrates contribute to the rigidity of these structures.

Mycobacterial OMPs

OMPs of mycobacterial species are far less well characterized than those of Gram-negative bacteria; however, substantial progress has been made. The most important observations are summarized here.

Lipoproteins

The availability of complete genome sequences of mycobacterial species has greatly facilitated the identification of OM lipoproteins. The occurrence of genes encoding these lipidated proteins in the reduced-size genome of *M. leprae* provides a guide to the minimal mycobacterial gene set. Surprisingly, perhaps, these lipoproteins are superficially similar to those of Gram-negative bacteria.

The consensus sequence at the N-terminal region of these proteins includes the cysteine residues to which the lipid moiety becomes attached. This sequence provides clues for the identification of these proteins. More than 20 potential lipoprotein genes have been identified in the *M. leprae* genomic sequence. Lipoprotein LpK, for example, encodes a 371 amino acyl precursor protein which becomes lipidated after it is synthesized, exported from the cytoplasm and proteolytically processed. The purified lipoprotein induces production of interleukin-12 (IL-12) in humans. This implies that LpK is involved in protective immunity against leprosy. The pursuit of such lipoproteins is likely to reveal details of the pathogenesis of a variety of mycobacterial diseases.

Mycobacterial OM Porins

Pore proteins in Gram-negative bacterial OMs that span the membrane mediate the diffusion of small hydrophilic nutrients such as sugars, amino acids, anions, cations, and vitamins (see 'Porins'). *M. tuberculosis* possesses at least three porins, one which is the low-activity NH_4^+ secretion channel protein, OmpATb. OmpATb is essential for adaptation of *M. tuberculosis* to low pH and survival in macrophage. The channel activity of oligomeric OmpATb probably plays a role in the defense of *M. tuberculosis* against acidification within the phagosomes of macrophage. While the C-terminal domain is similar to that of *E. coli* OmpA, the N-terminal channel-forming domain has an $\alpha + \beta$ sandwich class fold unlike the fold of β-barrel porins of Gram-negative bacteria. There is still controversy regarding the porin activity of OmpATb.

Another porin, MspA, is the main porin of the related, fast-growing, nonpathogenic species *M. smegmatis*. It forms a tetrameric and an octameric complex with the former having a central pore of 10 nm length and a cone-like structure. This structure is entirely different from that of the trimeric porins of Gram-negative bacteria suggesting that these two porin types evolved independently. In agreement with this conclusion, MspA shows no significant sequence similarity with any of the Gram-negative bacterial OMPs. The decreased numbers of porins in acid-fast bacteria compared with Gram-negative bacteria and the increased lengths of mycobacterial pores are two primary determinants of the low permeabilities of outer mycobacterial membranes to small hydrophilic solutes. Slow transport through porins needs to be considered when designing novel drugs against mycobacterial diseases.

All actinobacteria with mycolic acid-containing OMs contain porins that allow passage of nutrients, salts, and other small molecules across the OM as noted above. Several of these, in addition to those cited above, have been identified and characterized. Some are very small, the smallest being only 37 amino acyl residues in length, but it forms oligomeric pore structures. Others are much larger. Surprisingly, it appears that all or most of these porins contain transmembrane α-helical structures rather than the β-barrel structures found in Gram-negative bacteria. Future studies will reveal if this observation proves to be generally applicable to the actinobacteria. If so, it clearly suggests the actinobacterial porins evolved independently of those in Gram-negative bacteria. By extrapolation, and in view of their very different hydrophobic lipid compositions, it seems reasonable to suggest that outer membranes in these two groups of bacteria evolved independently of each other.

Compartmentalization of Lipid Biosynthesis in Mycobacteria

The plasma membranes of *Mycobacterium* species are the sites of synthesis of several distinct classes of lipids. Some of these lipids are retained in the inner membrane while others are exported to the overlying cell envelope. Enzymes involved in the biosynthesis of different major lipid classes, the PIMs and aminophospholipids, for example, are compartmentalized within the CM and the cell wall containing envelope fractions. Enzymes involved in the synthesis of early PIM intermediates are localized to a plasma membrane subdomain, while later stages of synthesis seem to be associated with external parts of the envelope. This suggests that like the LPSs in Gram-negative bacteria, complex outer envelope lipids may be synthesized in several stages that occur in different compartments of the cell envelope.

Molecular Action of Antimycobacterial Agents

There is evidence that many drugs exert their antimycobacterial activities by interacting with classical bacterial proteinaceous targets. This possibility is supported by both direct and indirect evidence. Mechanistic studies have been performed for drugs such as fluoroquinolones, macrolides, rifampicin, and streptomycin. Although the modes of action of many agents with antimycobacterial activities are not well understood, it seems likely that many drugs will prove to inhibit specific molecular targets involved in the biosynthesis of mycobacterial cell envelope constituents such as its many unique lipids and carbohydrate polymers. The recent reemergence of tuberculosis as an important human pathogen has prompted the development of improved methods for exploring the structures, biochemistry, and genetics of mycobacterial envelopes. These advances should be useful in gaining a better understanding of the molecular basis of drug action in mycobacteria.

Archaeal Membranes

Archaea are similar to bacteria in many aspects of cell structure, but they differ radically with respect to the lipid compositions of their membranes (Fig. 3(a) and (b)). The structures of their cell surfaces and their protein, lipid, and carbohydrate constituents are also unique. Archaeal membranes contain glycerol ether-linked isoprenoidal lipids rather than ester-linked fatty acyl lipids as are found in bacteria and eukaryotes (Fig. 3(b)). Only the archaeal lipids are based on isoprenoid side chains. In addition, bacterial-type peptidoglycan cell walls are altogether lacking, and in their place, cell walls consisting of surface layer proteins are present (Fig. 3(a)). OMs are not found in the better-characterized archaea, although they have been identified in one class of these organisms and are probably present in others. Because the existence and the unique properties of these organisms have been recognized for a relatively short period of time, much less is known about the OMs of archaea than about those of Gram-negative and acid-fast Gram-positive bacteria as well as those of eukaryotic organelles.

Archaeal Lipids

Polar ether lipids of archaea account for 80%–90% of the total membrane lipids in these organisms. The remainder are neutral squalenes and other isoprenoids. Many such unique lipids have been discovered in recent years. Genus-specific combinations of various lipid core structures include diether-tetraether, dietherhydroxydiether, and diether-macrocyclic diether-tetraether lipid moieties. The basic structure of a representative archaeal ether lipid and its comparison with a bacterial eukaryotic ester lipid are shown in Fig. 3(b).

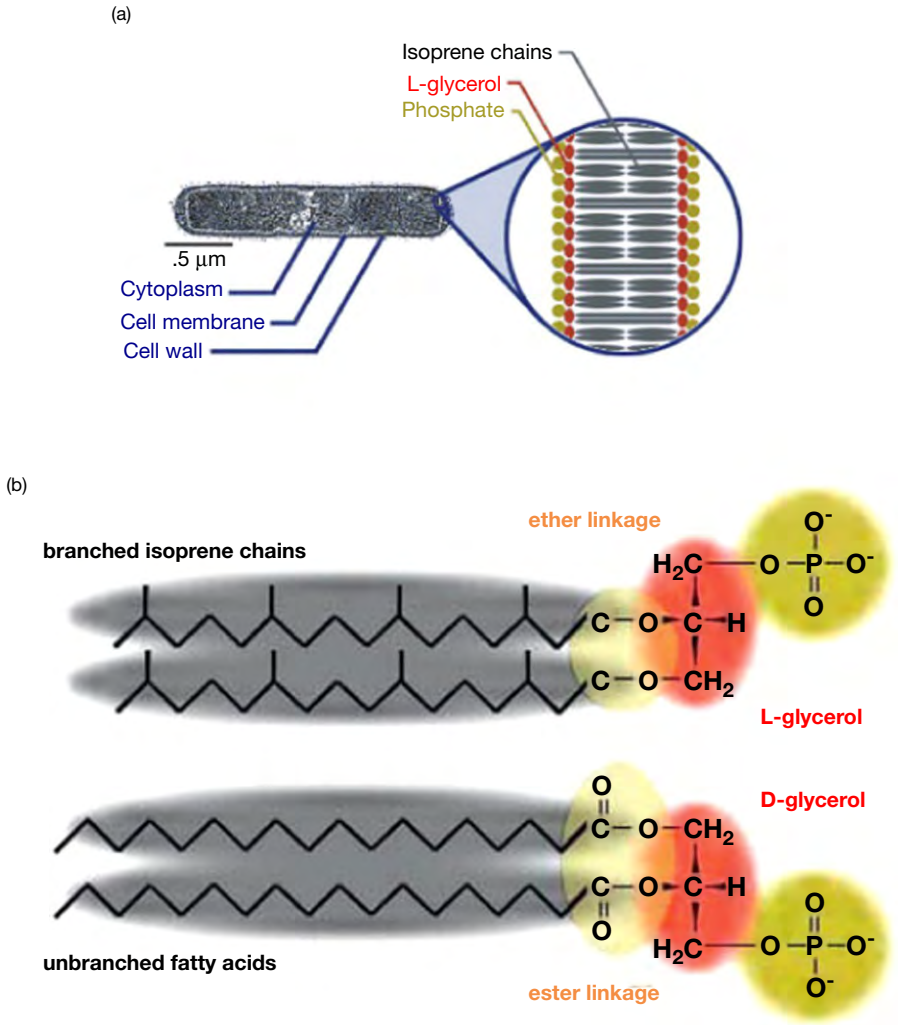

Fig. 3 (a) A typical archaeal cell illustrating the positions and structural features of the ether-linked lipids in the cytoplasmic membrane. The illustrated membrane is a 'blow-up' of the archaeal cell as visualized by electron microscopy. (b) Structure of an archaeal ether lipid (top) compared with that of a typical bacterial ester lipid (bottom). Primary structural differences are illustrated.

Some archaeal species have only the standard diether core lipids. None is known with predominantly tetraether lipids. The relative proportions of these lipid cores are known to vary with growth conditions in some archaea such as *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*. The ether-linked isoprenoids can exist as either monolayers or bilayers. Polar headgroups in glycosidic or phosphodiester linkage to glycerol consist of polyols, other carbohydrates, or amino compounds. These lipid structures have been shown to provide protection from extreme temperatures, pH values, and a range of salinities. Recently, a radical S-adenosylmethionine (SAM) protein in *Sulfolobus acidocaldarius* has proven to be required for the synthesis of a unique cyclopentyl head group, known as calditol. Calditol-linked glycerol dibiphytanyl glycerol tetraethers (GDGTs) are membrane-spanning lipids in which calditol is ether-bonded to the glycerol backbone. They are found in a subset of thermoacidophilic archaea of the Sulfolobales order within the Crenarchaeota phylum, but they are also found in other archaeal phyla including the Korarchaeota and Marsarchaeota. These unusual lipids are required for growth in extremely low pH environments.

The available structural data indicate close similarities between the polar lipids found in species of the same genus. Thus, the closer the phylogenetic relationships of the organisms, the more similar the lipid compositions of their membranes. These ether-containing lipid structures are more stable than the ester-containing lipids of bacteria and eukaryotes. This may have resulted in part through evolution, from the extreme environments inhabited by many archaea.

The extreme environments that some archaea thrive in include hot springs and strongly acidic, salty, and/or alkaline lakes. For example, *M. jannaschii* grows optimally at 85°C and pH 6, *Thermoplasma acidophilum* at 55°C and pH 2, and *Halobacterium salinarum* in near-saturated salt brines. Archaea that can not only survive but also grow at temperatures above 100°C are known.

A primary role of any cell CM is to provide a selective barrier between the external environment and the cytoplasm. Given the extreme environmental conditions conducive to archaeal growth, it is not surprising that their membranes contain lipids that differ markedly from those of bacteria and eukaryotes. The presence of ether rather than ester bonds contributes to their chemical stability, particularly at high temperatures and extreme pH values. Surprisingly, the glycerol ethers of archaea contain an *sn*-2,3 stereochemistry that is different from that of the *sn*-1,2 stereochemistry of glycerophospholipids of the other domains of life. The unique basic lipid core structures of these two lipid types are depicted in Fig. 3(b).

Two major classes of archaeal lipids include the archaeol lipids (diphytanyl glycerol diethers) and the caldarchaeol lipids (dibiphytanyl diglycerol tetraethers). The caldarchaeol lipids span the membrane, and liposomes made from these lipids preferentially form monolayers rather than the bilayers formed from conventional glycerophospholipids. Many of the tetraether lipids are phosphoglycolipids containing one or more sugar residues at one pole, most commonly gulose, glucose, mannose, and/or galactose, and a phosphopolyol moiety, such as phosphoglycerol or inositol, on the other. The more bulky sugar residues probably face outward, and the phosphate residue may face toward the cytoplasmic side of the membrane. Depending on the growth temperature, certain thermophilic archaea are capable of controlling membrane fluidity by altering the number of cyclopentane rings (from 0 to 8 in caldarchaeol lipid chains).

Archaeal OMs

Many hyperthermophilic *Crenarchaeota* have two-dimensional crystalline arrays of (glyco-)protein subunits (the S-layer) as the most rigid component of their cell walls. Thus, the typical cell wall of archaea consists of a pseudo-crystalline S-layer. Within the crenarchaea, the S-layer is usually the only cell wall component, but glycosylated S-layers are found in (hyper)thermophilic cren- and euryarchaea as well as halophilic archaea. Moreover, other cell wall structures such as proteoglycan-like S-layers (Halobacteria), glutaminylglycan (Natronococci), methanochondroitin (*Methanosarcina*) and double layered cell walls with pseudomurein (*Methanothermus* and *Methanopyrus*) have also been identified. The euryarchaeal methanogen, *Methanomassiliicoccus luminyensis*, cells of the ARMAN group, and the SM1 euryarchaeon provide further examples of two-layered cell envelopes. Subunits of the S-layer are directly anchored to the CM by stalk-like structures. The space between the CM and the S-layer is called the 'quasi-periplasmic space' by analogy to the equivalent structures in Gram-negative bacterial cell envelopes.

Ignicoccus is a hyperthermophilic archaeon belonging to the *Desulfurococcales* subdivision of the *Crenarchaeota*. It is a chemolithoautotrophic organism that obtains its energy by the reduction of elemental sulfur with molecular hydrogen. Cells of *Ignicoccus* have been examined ultrastructurally following cultivation in cellulose capillaries and processing by high-pressure freezing. They consistently showed a cell envelope structure previously unknown among the archaea: CM and OM separated by a periplasmic space of variable width (20–200 nm) containing membrane-bound vesicles. The outer sheath, approximately 10 nm wide, seemed to resemble the OMs of Gram-negative bacteria. The *Ignicoccus* sheath contains three types of particles: (1) numerous irregularly packed single particles, about 8 nm in diameter; (2) putative pores with a diameter of 24 nm; and (3) tiny particles arranged in a ring with a diameter of 130 nm surrounding the pores. Clusters of up to eight particles, each particle 12 nm in diameter, were conspicuous. Freeze-etched cells exhibited a smooth surface without a regular pattern, with frequent fracture planes through the outer sheath. This observation indicated to the researchers the presence of an OM and the absence of an S-layer. The study illustrated the novel complex architecture of the cell envelope of *Ignicoccus*. Comparative studies suggest that OMs of prokaryotes have evolved independently at least three times, once in Gram-negative bacteria, once in high G+C Gram-positive bacteria, and once in *Crenarchaeota*.

Membrane Transfer Between Cells

Ignicoccus lives in symbiosis with another archaeon, a very small, single-celled organism called *Nanoarchaeum equitans* (see Fig. 4). The *Nanoarchaeum* cell has one of the smallest genomes yet sequenced (less than 500,000 bp). In fact, too few genes are present to

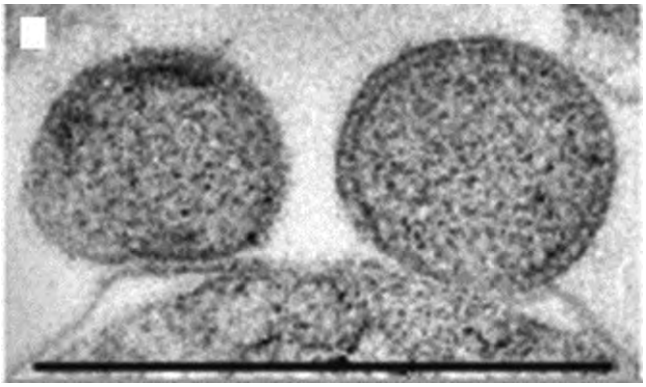

Fig. 4. An electron microscopic depiction of an *Ignicoccus* cell (bottom), showing the inner and outer membranes, in symbiotic association with two *Nanoarchaeum* cells (top).

code for all of the biological functions thought to be essential for life. It can live only together with *Ignicoccus*. Among the missing functions are the enzymes that catalyze lipid biosynthesis. If these enzymes are really absent from this organism, then how does *Nanoarchaeum* get its lipids for construction of its CM?

Ultrastructural analyses reveal not only the two-membrane envelope of *Ignicoccus*, but also the presence of intraperiplasmic vesicles. Because the lipid and protein compositions of the inner and outer membranes are different, it has been possible to establish that these vesicles derive from the inner membrane of *Ignicoccus*. Moreover, the lipids in the nanoarchaeal membrane are very similar, if not identical, to those in the CM of *Ignicoccus*.

These observations led to the postulate that one organism makes the lipids for both. Some of the membrane transport proteins in the nanoarchaeal membrane of one organism may also derive from its symbiotic partner cell. The details of the transfer process still need to be confirmed. However, it already seems likely that these symbiotic archaea have developed mechanisms for intercellular communication and molecular transfer involving periplasmic vesicles that are unique to them. Alternatively, elucidation of such mechanisms may lead to the discovery of analogous processes in bacteria and eukaryotes.

Conclusions

Prokaryotes, including bacteria and the much less well-studied archaea, possess cell envelopes of extremely varied compositions and structures. In both prokaryotic domains of living organisms, as in organelles of eukaryotes, the envelopes can possess one or two membranes. The two membranes always consist of different combinations of lipids and proteins. We are now coming to appreciate the complexities of the assembly machineries that function to construct these envelopes. Many are present in specific membranes while others span the entire cell envelope structures. Moreover, completely different transport apparati are found in the inner and outer membranes of organisms that have both. Evaluation of the structural data available for the OMs of Gram-negative bacteria, high G+C Gram-positive bacteria, and archaea leads to the conclusion that prokaryotic OMs have probably evolved independently in these three organismal types. It is clear that further research will be required to clarify the many important but poorly understood issues dealing with basic aspects of the functions, structures, biogenesis, and evolution of prokaryotic OMs. Moreover, novel processes and mechanisms are likely to come to light. This article thus serves as a progress report of prokaryotic membrane research efforts that will hopefully provide a basis for future advances.

Further Reading

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Bacterial and Archaeal Cell Structure

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Cell Boundaries of Microorganisms

Most bacteria and archaea constantly face changing and often hostile environments. Therefore, their cell envelopes must provide adequate protection against physical, chemical and biological stresses while allowing an adequate influx of nutrients and efflux of waste products and signaling molecules. As bacterial and archaeal cell envelopes differ fundamentally in their composition and structure, they will be discussed separately.

Bacterial Envelope Structure

Traditionally, bacterial envelopes are classified into one of two groups using the Gram strain. Gram-negative bacteria possess a cytoplasmic membrane (CM), a thin peptidoglycan (PG) layer and an outer membrane (OM), while Gram-positive bacteria lack the OM and produce a thicker PG cell wall (Fig. 1, upper panel). Some notable exceptions of bacteria that lack a continuous PG layer exist, such as in *Mycoplasma*. The CM of bacteria is a phospholipid bilayer, its two leaflets are clearly distinguishable using electron microscopy at high magnification (Fig. 1, lower panel). The CM is water-permeable but impermeable to polar organic solutes and inorganic ions. This allows the cells to generate an inward-directed proton motive force, which is used to facilitate essential processes such as ATP syntheses and motility. Accordingly, a multitude of proteins involved in energy production, transport and secretion are embedded in the CM.

The major stress-bearing component of most bacteria is the PG network that envelopes the cell. PG is composed of long glycan strands of alternating *N*-acetyl glucosamine and *N*-acetyl muramic acid molecules which are crosslinked by short peptide chains. The CM and the PG are separated by a 10–20 nm wide space. In Gram-positive and -negative bacteria, the PG is similar in chemical composition and synthesis, but the PG layer of Gram-positive bacteria is substantially thicker (~40 nm) than in Gram-negatives. Lipoteichoic acids associated with the CM, teichoic acids and polysaccharides, which are linked to the PG, extend through the peptidoglycan network and form a negatively charged, loose outer layer. In Gram-negative bacteria, the PG is a ~4 nm thin single layered mesh. The glycan strands run in a circumferential fashion around the cell body and the peptides roughly parallel to the long axis of the cell (see Fig. 2). While the PG of Gram-positive cells is generally much thicker than that of Gram-negatives, the thickness does not serve as a reliable phylogenetic marker as several species possess intermediate thicknesses, and Gram-negative and -positive staining organisms may be found in the same class. Furthermore, the thick PG layer of *Bacillus subtilis* and *Acetonema longum* is converted to a thin layer reminiscent of the PG of Gram-negative bacteria during then initial stages of sporulation. The PG layer in Gram-negative bacteria is enclosed by the OM. The OM itself differs chemically from the CM as it is composed of an inner leaflet and an exterior leaflet of lipopolysaccharides (LPS) that provide the cell with a negatively charged barrier. Some Gram-positive and -negative bacteria produce an additional proteinaceous surface layer on top of the PG layer or OM, respectively. This S-layer is mostly composed of one protein that self-assembles into oblique, square, or hexagonal lattice symmetries. Since the S-layer proteins arrange into a two-dimensional crystal *in vitro*, they can readily be studied by electron microscopy (Fig. 3). Finally, the outmost layer of many Gram-positive and -negative bacteria is a thick capsule of vastly variable polysaccharide associated with the OM or PG. This capsule provides additional protection against desiccation and is of clinical relevance as it facilitates cell attachment and evasion of the host immune defense.

Archaeal Envelope Structure

While the chemical composition of CM, PG and OM is conserved among the bacteria, archaea produce a more diverse and species-specific set of envelope components (Fig. 4(a)). The lipids comprising the CM are unique for the archaea. Some species produce diether lipids that arrange into two leaflets whereas others possess tetraether lipids with up to 8 cyclopentane rings, thus forming a monolayer. Archaea also produce a variety of further structural components that envelope the CM. Some Euryarchaeota possess a cell wall composed of sugar polymers. However, unlike the PG of bacteria, archaeal cell walls differ vastly regarding their chemical composition and properties. Methanochondroitin for example is a fibrillar polymer that forms a rather loose matrix around aggregated cells. In contrast, pseudomurein has a thickness of approximately 15–20 nm and resembles PG in general architecture and rigidity. In most archaea that lack polymeric cell walls, an additional proteinaceous layer provides protection and maintains the cell shape. Most commonly, this is an S-layer with similar properties as their bacterial counterparts. However, while bacterial S-layers are only five to 20 nm thick, they may reach a thickness of up to 70 nm in archaea (Fig. 4(b)). Another type of protein layer is the tubular sheath of the filamentous growing *Methanospirillum* and *Methanosaeta* species. This sheath is composed of circumferential rings that envelope the entire filament. The circumferential hoops can clearly be seen using transmission electron microscopy (TEM). Capsule-like structures have been described for several archaea and genomic analysis suggests that many archaea encode genes for capsule systems.

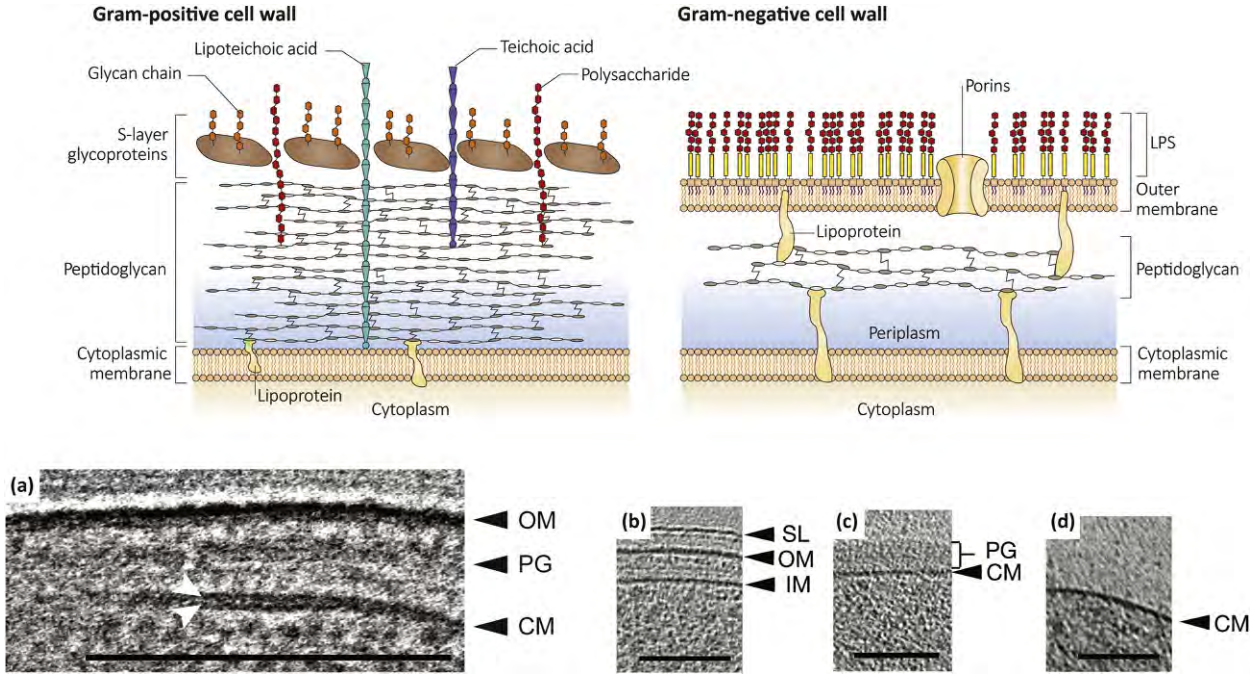

Fig. 1 (Upper panel) Schematic side view of cell envelopes of exemplarily Gram-negative and -positive bacterial cells. LPS, Lipopolysaccharides. (Lower panel) Electron micrographs show the architecture of a Gram-negative cell wall without S-layer showing the two leaflets of the cytoplasmic membrane (white arrows): (a) *Vibrio cholera*; a Gram-negative envelope with S-layer: (b) *Caulobacter crescentus*; Gram-positive cell wall: (c) *Listeria monocytogenes*; cell-wall less: (d) *Mycoplasma pneumonia*. IM, inner membrane; OM, outer membrane; PG, peptidoglycan; SL, S-Layer; CM, cytoplasmic membrane; LPS, Lipopolysaccharides. Scale bars, 100 nm. Upper Panel reproduced from Albers, S.-V., Meyer, B.H., 2011. The archaeal cell envelope. Nature Reviews Microbiology 9 (6), 414–426. https://doi.org/10.1038/nrmicro2576. Lower panel (a) courtesy of Wen Yang; (b-d) reproduced from Skennerton, C.T., Haroon, M.F., Briegel, A., et al., 2016. Phylogenomic analysis of Candidatus "*Izimaplasma*" species: Free-living representatives from a Tenericutes clade found in methane seeps. The ISME Journal 10 (11), 2679–2692.

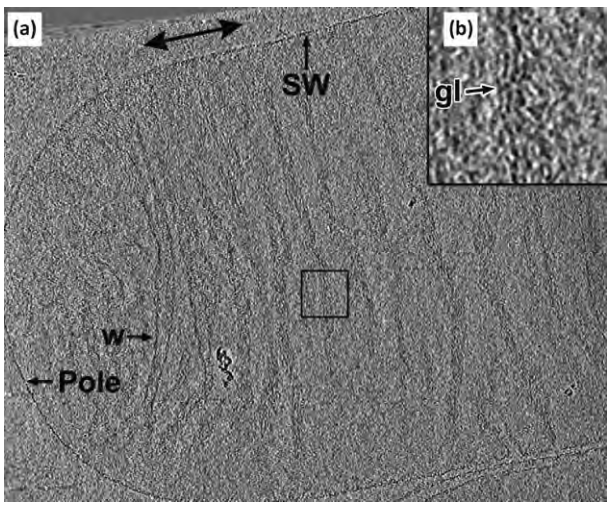

Fig. 2 (a) Tomographic slices of *Escherichia coli* XL-10 sacculus. Abbreviations: (gl) glycan strand; (SW) side wall; (w) wrinkle. (b) A four-fold enlarged view of the boxed region showing the glycan strands of the sacculus. The double-headed arrow denotes the saccular polar axis. Reproduced from Gan, L., Chen, S., Jensen, G.J., 2008. Molecular organization of Gram-negative peptidoglycan. Proceedings of the National Academy of Sciences of the United States of America 105 (48), 18953–18957.

Cell Shape

Bacteria and archaea exhibit a broad diversity of cell shapes and cell sizes (Fig. 5). These features are tightly controlled through elaborate cell division machineries and structural features that maintain their intrinsic morphology. While some archaea share both structural and cell division components with bacteria, others have systems homologous to eukaryotic division machineries.

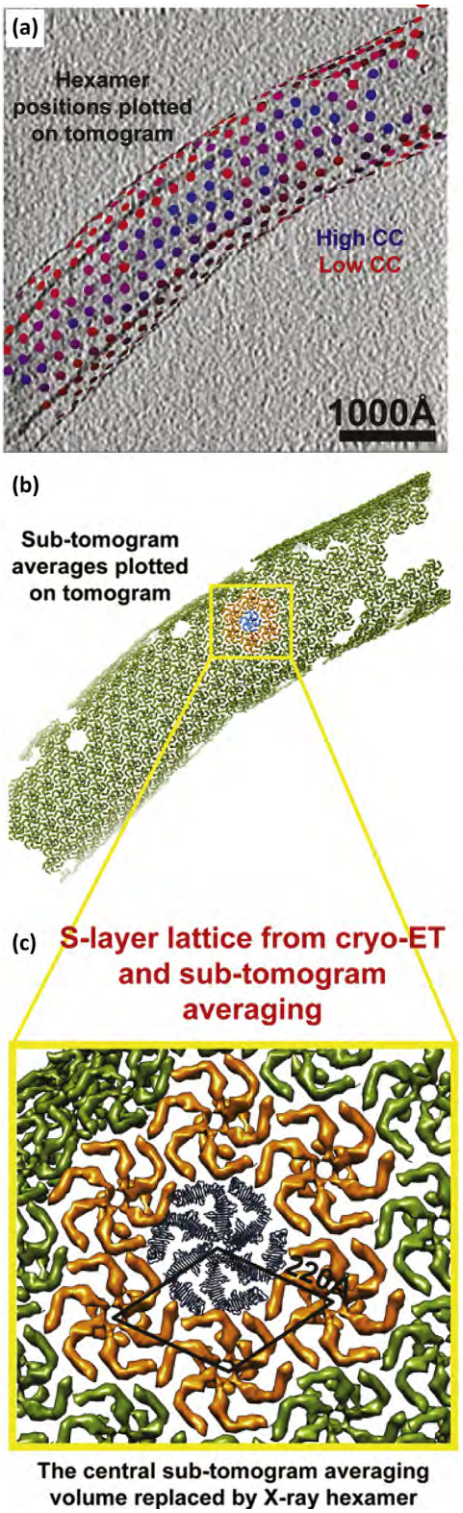

Fig. 3 (a) Final refined positions of sub-tomograms of S-layer proteins plotted back onto a tomogram of a *C. crescentus* cell stalk with the corresponding refined orientations. Positions have been coloured from blue (high cross-correlation of alignment) to red (low cross-correlation). (b) The same plot as panel A, except each hexamer position is illustrated with the sub-tomogram average (green volumes). One hexamer is highlighted in blue, and the hexamers directly contacting it are shown in orange. (c) A zoomed view of the hexameric lattice revealed by cryo-ET and sub-tomogram averaging. The central blue hexamer from panel B is replaced by one copy of the X-ray hexamer. Reproduced from Bharat, T.A.M., Kureisaite-Ciziene, D., Hardy, G.G., et al., 2017. Structure of the hexagonal surface layer on *Caulobacter crescentus* cells. Nature Microbiology 2, 17059.

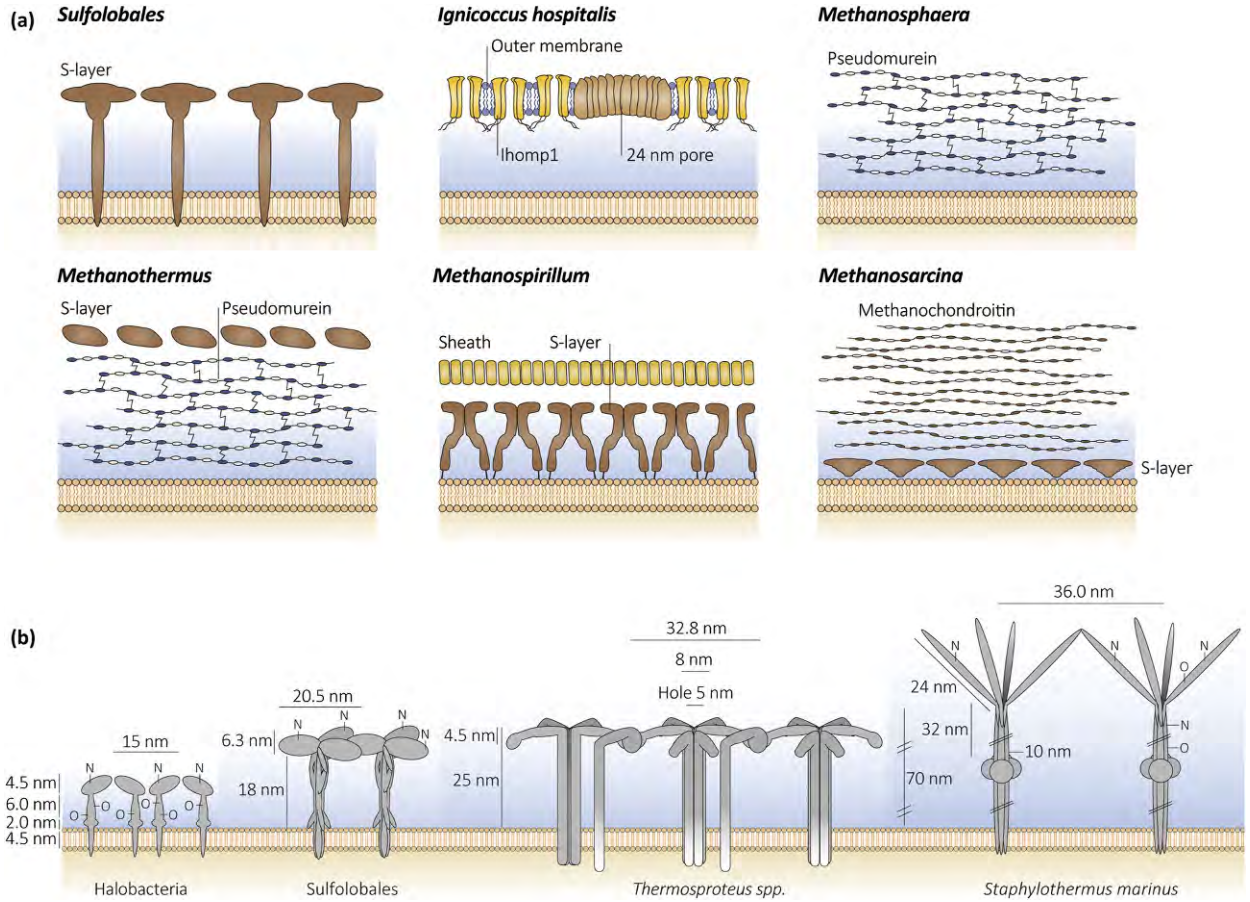

Fig. 4 (a) Schematic side view of cell envelopes of exemplarily archaea. LPS, Lipopolysaccharides. (b) Models of the archaeal S-layer inside view. N, N-linked glycosylation; O; O-linked glycosylation. Reproduced from Albers, S.-V., Meyer, B.H., 2011. The archaeal cell envelope. Nature Reviews Microbiology 9 (6), 414–426. https://doi.org/10.1038/nrmicro2576.

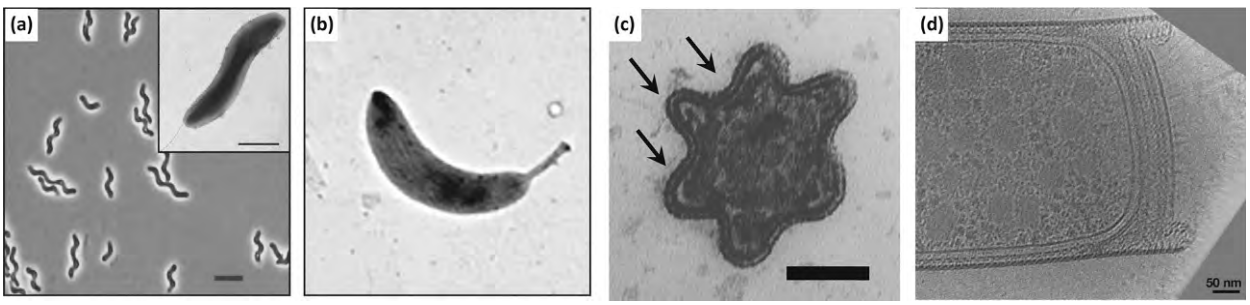

Fig. 5 Diverse bacterial and archaeal morphologies. (a) Uncharacterized spiral-shaped methanotroph. Phase contrast with inset electron micrograph. (b) Transmission electron micrograph of *C. crescentus* (c) ultrathin section TEM micrograph of a six-pointed starshaped bacterium. (d) Cell tip of *Methanospirillum hungatei* cells imaged by cryo-electron microscopy. Modified from (a) Danilova, O.V., Suzina, N.E., Van De Kamp, J., et al., 2016. A new cell morphotype among methane oxidizers: A spiral-shaped obligately microaerophilic methanotroph from northern low-oxygen environments. ISME Journal 10 (11), 2734–2743. (b) Jiang, C., Brown, P.J., Ducret, A., Brun, Y.V., 2014. Sequential evolution of bacterial morphology by co-option of a developmental regulator. Nature 506 (7489). (c) Wagner, G., Onstott, T.C., Southam, G., 2008. Stars of the terrestrial deep subsurface: A novel "star-shaped" bacterial morphotype from a South African platinum mine. Geobiology 6 (3), 325–330. (d) Briegel, A., Ortega, D.R., Huang, A.N., et al., 2015. Structural conservation of chemotaxis machinery across Archaea and Bacteria. Environmental Microbiology Reports 7 (3), 414–419.

For most bacteria, the PG is both essential and sufficient to regulate and maintain their shape. However, how the PG synthesis is orchestrated to result in a particular shape is not well understood for most morphologies and organisms. In general, it is believed that cytoskeletal elements direct the PG synthesis machinery to the appropriate positions. In *E. coli*, the actin-like cytoskeletal protein MreB coordinates localization of the PG synthesis machinery and, consequently, the insertion of PG precursors. MreB itself preferentially localizes to regions of negative curvature. The insertion of PG at these sites results in the growth of *E. coli* as a straight rod. In some cases, the cytoskeleton may play a more active role in shaping a cell. The vibrioid cell shape of *Caulobacter crescentus* is the result of crescentin,

a homologue to eukaryotic intermediate filaments. This protein coats the inner curvature of the cytoplasmic side of the CM. In contrast to coccoid spheres, alternative cell shapes offer the possibility of subcellular organization. In rod shaped cells for example the cell can distinguish between polar and midcell regions. In some bacteria, structures required for motility and chemosensing are predominantly found at one or both poles of the cell. The differently curved membrane at the pole can establish polarity of bacterial cells, and landmark proteins can recruit further proteins to the pole or protein gradients along the axis of the cell.

The cell shape maintenance in archaea is less well understood. CetZ, a protein related to eukaryotic tubulin and bacterial FtsZ, was shown to be essential for the development of rod-shaped *Haloferax volcanii* cells. This protein forms an additional layer underneath the membrane in cells producing high levels of this protein.

Cell Division

With few exceptions, the cell division machinery is necessary to reliably divide a cell into two daughter cells of similar volume and content. In nearly all bacteria, cell division is orchestrated by the tubulin homologue FtsZ. This specialized cytoskeletal element assembles into circumferentially orientated, overlapping filaments at the cytoplasmic side of the CM, which marks the division site and provides an assembly platform for the cell division machinery (Fig. 6). In Gram-positive bacteria, cell division occurs by septation. Here, the CM is pulled inwards and two new PG layers grow in parallel into the septum. At the outer rim of the septum, a PG bridge connects the old cell wall material of the mother cell both to each other as well as to new PG layers. Once septation is complete, the two daughter cells are fully separated and split from each other. Division of Gram-negative bacteria follows a similar scheme with some variations. The constriction of the Gram-negative cell envelope starts either symmetrically, or asymmetrically at one side of the cell, before occurring circumferentially. Additionally, instead of forming a compact thin septum, the OM and PG lag behind the CM that moves into the center of the cell first, forming a V-shaped constriction. Here, the hydrolysis and synthesis of the PG bridge is speculated to be more controlled as it needs to move inward following the FtsZ ring while preventing premature rupture.

Archaea also divide by binary fission, but exhibit a higher diversity of systems and modes of cell division than bacteria. Most Euryarchaeota, Thaumarchaeota, Nanoarchaeota and Korarchaeota encode an FtsZ-based division machinery, while the Crenarchaeota possess a system homologous to the eukaryotic endosomal sorting complex required for transport III (ESCRT-III). Cell division of these archaea may occur either symmetrically or asymmetrically.

Interaction With the Environment

Bacteria and archaea have developed an extraordinary arsenal of molecular machinery to interact with, as well as influence, the environment around them. The cells are able to sense their surroundings, determine the levels of nutrients, change the environmental milieu, and provide defenses for themselves and their neighbors. The following sections describe how bacteria and archaea interact with their environment, highlighting the roles of motility apparatus, membrane vesicles, secretion systems, pili and, hami.

Motility

Some of the most striking structural features of bacteria and archaea are the proteinaceous ultrastructures required for cellular motility. Several distinct types of motility machineries have evolved in microorganisms, each adapted to a certain life style and environment.

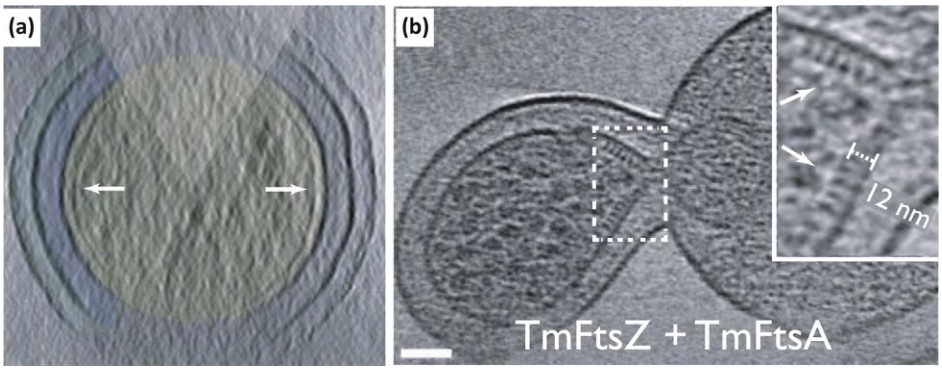

Fig. 6 (a) FtsZ forms bands of filaments completely encircling *C. crescentus* NA1000/CB15N division site with filaments near the inner membrane IM. The Z ring (arrow) is continuous. The cytoplasm (beige), periplasm (blue), and space between the OM and S layer (cyan) have been coloured for clarity. (b) 10-nm thick electron cryotomographic slice of an *E. coli* minicell formed from cells expressing *Thermotoga maritima* FtsZ and FtsA proteins, with a deeply constricted area showing cross-sections of FtsZ and FtsA filaments (black dots marked with white arrows). Distance between FtsZ filaments and IM is around 12 nm (inset in b). (a & b). Modified from Szwedziak, P., Wang, Q., Bharat, T.A.M., Tsim, M., Löwe, J., 2014. Architecture of the ring formed by the tubulin homologue FtsZ in bacterial cell division. eLife, 3.

Swimming Motility

Due to their small size, viscous forces are predominantly influencing microorganisms that move through water bodies. Therefore, reciprocal movements do not propel the cell. Bacteria and archaea solve this problem by using long rotating filaments that generate a fluid flow. Surprisingly, the bacterial flagellum and the archaeal archaellum have evolved separately, although they facilitate the same task.

The overall architecture of the flagellum can be divided into three major parts: The long helical filament extending from the cell body, a flexible hook and the envelope embedded basal body which comprises the rotary motor. Although the principal flagellar components are conserved among all flagellated bacteria, further proteins can modify the flagellar motor properties. Several additional periplasmic discs and ring structures are found to provide an increased structural support as well as a scaffold that allows more stators to be included in the motor. This results in increasing widths of the C- and stator ring (Fig. 7(a-i)). Both features allow cells to swim at a higher speed and torque.

Due to a similar assembly mechanism and homology of some components, the archaellum structurally resembles bacterial type IV pili (T4P) rather than the flagellum. Contrary to the flagellar filament, the archaellum is not hollow. Here, the prepilins are assembled into the pilus at the base of the growing archaellum. The motor is a multiprotein complex, however, rotation of the archaellum is powered by ATP hydrolysis and not by an ion-flux. While recent advances have elucidated the structure of the archaellum, several components and functions remain to be defined (Fig. 7(k and l)).

Surface Motility

Besides being required for attachment and biofilm formation, several bacteria possess retractile T4P that can attach to a surface and pull the cell towards the attachment point. This form of motility is referred to as twitching motility. Assembly of the pilus results in its extension, while the disassembly results in its retraction. Both processes depend on ATP hydrolysis. In several organisms, a specialized ATPase facilitates the depolymerization. Non-retractile and retractile T4P are structurally very similar.

An additional surface motility mechanism has been described for *Myxococcus xanthus*. The structural requirements of the so-called gliding motility, known as adventurous or A-motility, are not entirely understood. The current model of this mode of motility is based on the consecutive adhesion of OM lipoproteins to a surface, followed by the movement of the cell relative to

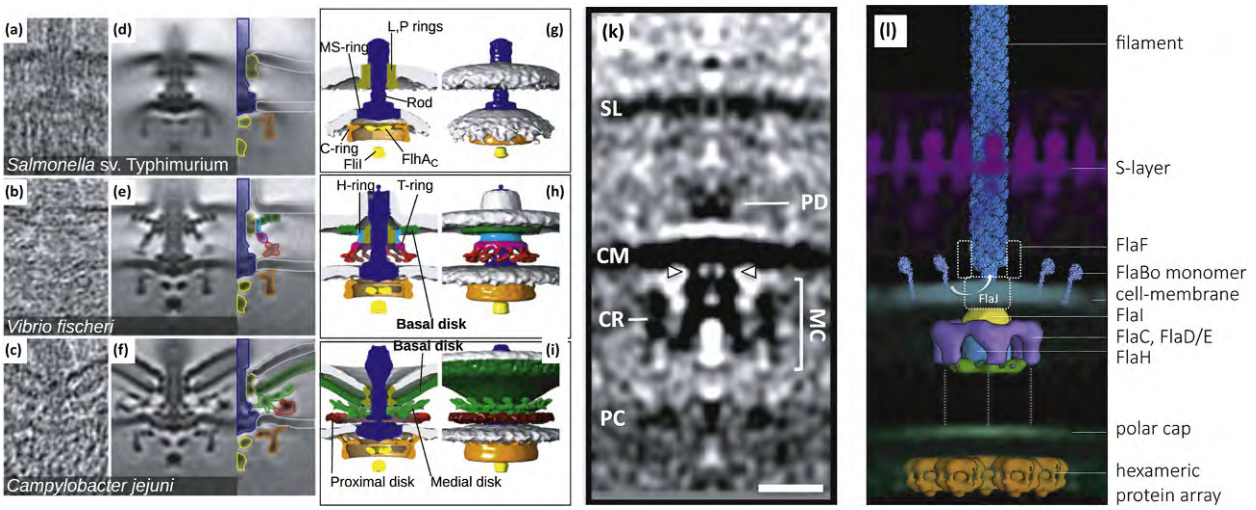

Fig. 7 High-torque bacterial flagellar motors assemble large periplasmic disk complexes. (a–c) Tomographic slices through intact cells of *Salmonella* (a), *V. fischeri* (b), and *C. jejuni* (c) showing individual flagellar motors. (d-f) Slices (100×100×0.81 nm) through subtomogram averages of hundreds of motors. Color keys indicate the regions of the motor (named in g-i), (g-i) Isosurface renderings of motors shown in (d-f). FliI and $FlhA_C$ are components of the flagellar type III secretion system. (k) Tomographic slice through the sub-tomogram average of the motor complex of the archaellum of *Pyrococcus furiosus*. SL, S-layer; PD; periplasmic densities; CM, cell membrane; MC, motor complex; CR, cytosolic ring; PC, polar cap. Arrowheads indicate two of six narrow connections between MC and CM. (l) Composite model of the archaellum machinery of *P. furiosus*. Light blue, $FlaB_0$ monomers and filament; hazy magenta, S-layer; solid yellow, blue, green and purple, motor complex; hazy blue, cell membrane; hazy green, polar cap; solid orange, hexagonal protein array. Putative positions of protein subunits are indicated. Dashed grey lines, putative interaction with polar cap. (a-i). Modified from Beeby, M., Ribardo, D.A., Brennan, C.A., et al., 2016. Diverse high-torque bacterial flagellar motors assemble wider stator rings using a conserved protein scaffold. Proceedings of the National Academy of Sciences of the United States of America 113 (13), E1917–E1926. (k & l) Daum, B., Vonck, J., Bellack, A., et al., 2017. Structure and in situ organization of the *Pyrococcus furiosus* archaellum machinery. eLife, 6. doi:10.7554/elife.27470.

that adhesion point, and the final release and disassembly of the lipoprotein complex at the lagging end of the cell. The involved protein complex appears to span the entire cell envelope. The release of a slime further facilitates the movement across the substratum.

Chemotaxis

In order to optimally benefit from the ability to move through the environment, many bacteria and archaea can sense their chemical environment and control their motility accordingly. This allows the cells to seek out their preferred environment and evade potentially harmful conditions.

Chemotaxis is best understood in the model organism *Escherichia coli*. Here, the cells can sense environmental cues such as sugars, amino acids or toxins via chemoreceptors that are anchored in the CM. The receptors, or MCPs (for Methyl accepting chemotaxis proteins), form trimer-of -receptor dimers, and these trimers are in turn arranged in highly ordered arrays, where six trimers form the corners of a hexagon (Fig. 8). This receptor packing, and thus the distance between the centers of the hexagons of 12 nm in the lattice, is highly conserved and universal across the bacteria and archaea.

In *E. coli*, the cytoplasmic tips of the receptors are networked by rings formed by the histidine kinase CheA and the linking protein CheW. CheA autophosphorylates and transfers this phosphoryl group to a messenger protein CheY upon an increase of repellents or a removal of attractants. The phosphorylated messenger protein CheY binds to the flagellar motors and biases the direction of flagellar rotation from the default counter clockwise (CCW) to clockwise (CW). This results in a tumbling behavior where the cells randomly reorient their cell axis. Once all flagella of the cell return to CCW, the cells swim smoothly forward (run) in a new direction. The control of frequency and duration of these tumbles result in a biased random walk, which ultimately provides the means to find their preferred environment.

While *E. coli* only possesses one chemotaxis system, more than half of all chemotactic bacteria have additional chemotaxis systems encoded in their genome. Some of these systems lack the transmembrane and periplasmic regions of the receptors, and thus form purely cytoplasmic chemoreceptor arrays. Structurally, these arrays consist of two layers, where each layer is hexagonally packed like their membrane-bound counterparts. The receptors of both layers overlap and are sandwiched in

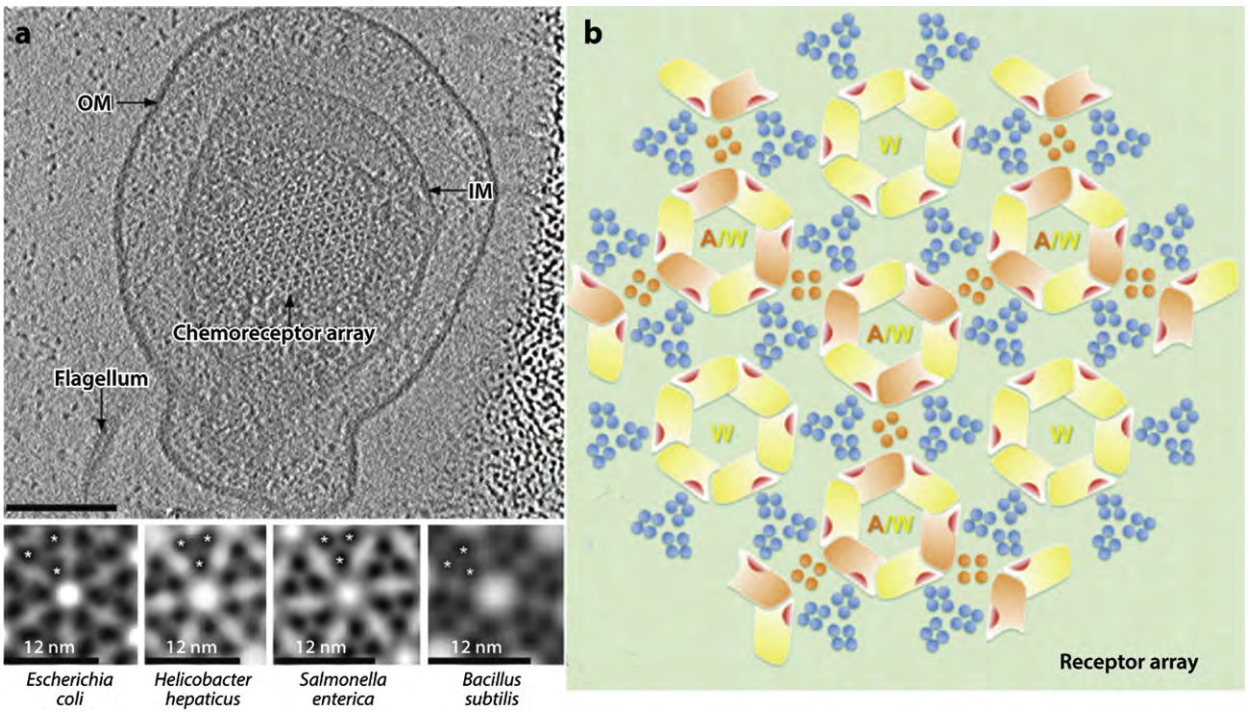

Fig. 8 Visualization of the highly structured chemotaxis array by cryogenic electron microscopy (cyro-EM) in *Salmonella enterica* mini cells. (a) Viewed from the top, the chemotaxis array assumes a lattice formation (top) that is universal among bacteria (bottom). (b) Illustration of the chemotaxis array highlighting key proteins and their interacting partners that maintain the array: CheA (A, orange), methyl-accepting chemotaxis protein trimers (blue) and CheW (W, yellow). Scale bar in A (top) is 100 nm. Outer membrane (OM), inner membrane (IM). Modified from Briegel, A., Jensen, G., 2017. Progress and potential of electron cryotomography as illustrated by its application to bacterial chemoreceptor arrays. Annual Reviews of Biophysics 46, 1–21. https://doi.org/10.1146/annurev-biophys-070816-033555.

between two layers composed of CheA and CheW. While we are beginning to understand the architecture of such cytoplasmic arrays, their function remains largely unclear.

Membrane Vesicles

Membrane vesicles are an essential component found in both Gram-positive and Gram-negative bacteria, as well as in archaea (also referred to outer membrane vesicles in gram negative bacteria). These vesicles are typically spherical, ranging in size from 20 to 100 nm in Gram-positive bacteria and archaea to 100–300 nm in Gram-negative bacteria (Fig. 9). The lipid bilayer composition of the vesicles resembles that of the membrane where the vesicle originated. They can contain cell-wall material, as well as a variable content enclosed by the membrane. In addition, the content of membrane vesicles can be enriched with a diverse set of molecules including LPS, peptidoglycan, nucleic acids, metabolites, and signaling molecules. The mechanism by which membrane vesicles are generated is still under investigation.

Membrane vesicles have many proposed functions: bacterial communication, modulation of stressful environments, components for biofilm formation, and defense strategies. The membrane vesicles can be filled with specialized cargo that elicit specific types of response, including the recognition of self and non-self organisms. For the acquisition of nutrients, outer membrane vesicles (OMVs) can be filled with proteases and glycosidases, or with DNA and proteins that can be a source of food for the community. Recent studies have also demonstrated that interactions with specific hosts can bias the proteins found in membrane vesicles to those that are important to the bacteria-host relationship, including adhesions for binding to specific cell types. For instance, the Gram-positive bacteria *Staphylococcus aureus* produce membrane vesicles that contain beta lactamase as a way to protect the local environment from the antibiotic penicillin. In addition, membrane vesicles have also been shown to provide a defense against bacteriophage infection by acting as a decoy. Thus, membrane vesicles play an essential role in the bacterial and archaeal life cycle by modulating the environment in favor of their survival.

Secretion Systems

In addition to releasing membrane vesicles into the environment, bacteria and archaea have developed various mechanisms to transport unfolded or folded proteins across their membrane(s) and into the local environment or across the membrane of a target cell. Some systems allow a protein to move from the cytoplasm directly to the extracellular space in a one-step process. Others require several steps for the target protein to exit the cell via a transport system through the CM, followed by a second transport system that spans the OM and/or cell wall. Upon translocation, the proteins remain either attached to the cell envelope, are released into the extracellular space, or are transferred directly into the target cell. Thus far, as many as sixteen unique complexes have been shown to be involved in protein transport across the membrane. Each system plays a specific role in bacterial and archaeal physiological processes. Furthermore, these systems are the primary method for the release of bacterial effector proteins that act on other cells in the environment. Some secretion systems are universal among bacteria and archaea, such as the Sec and Tat pathways, while others are restricted to a specific phylum or species. The same is true for the cargo of each system, some transporting a wide range of proteins and others are specific to just a small number of proteins. The secreted proteins have many roles in the bacterial and archaeal life cycle, including uptake of nutrients, expression of virulence factors, and attachment to target cells. Gram-negative bacteria have multiple secretion systems: Sec, Tat, type 1 secretion (T1SS), T2SS, T3SS, T4SS, T5SS, T6SS, and T9SS. Gram-positive bacteria have at least four systems, Sec, Sec2A, Tat, and T7SS, and only Sec and Tat have been identified in archaea so far.

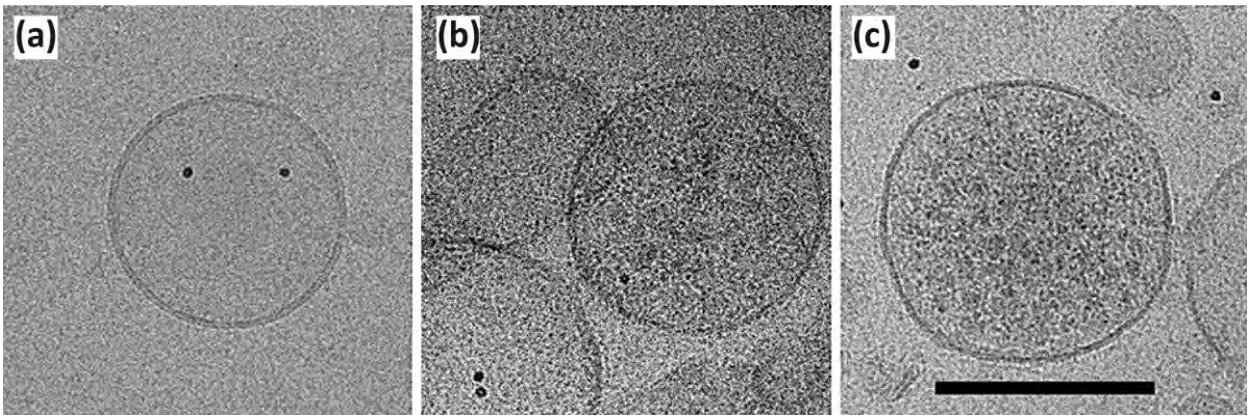

Fig. 9 Cyro-EM of membrane vesicles isolated from *Streptomyces*, demonstrating the variety of sizes and variability in contents. Three types of vesicles are noted: Empty (a), partially filled, with or without external membrane complexes (b), or heavily filled with material (c). Scale bar 200 nm. Modified from Schrempf, H., Koebsch, I., Walter, S., Engelhardt, H., Meschke, H., 2011. Extracellular *Streptomyces* vesicles: Amphorae for survival and defense. Microbial Biotechnology 4 (2), 286–299.

Because of the large number of secretion systems across bacteria and archaea, this section will highlight only those systems with significant ultrastructural information.

Type II Secretion System

The type II secretion system (T2SS) is only found in Gram-negative bacteria where it is responsible for secreting folded proteins from the periplasm through the OM. Proteins that are destined for the T2SS are first transported in an unfolded state across the CM by the Sec or Tat systems. Once in the periplasm, the protein is folded and then transported into the extracellular space by the T2SS. This secretion system is an important component in pathogenesis, and many pathogens utilize this pathway to deliver toxins to target cells. A well-studied example of this mechanism is the transport of the cholera toxin during *Vibrio cholerae* infection. The T2SS is thought to work by a piston mechanism, where the cholera toxin is mounted to a platform on the periplasmic side of the CM and then pushed through the OM pore. This export is powered by the retraction of the pseudopilins (Fig. 10).

Type III Secretion System

The type III secretion system (T3SS) is also known as the injectisome because its structure resembles a needle and syringe and acts in a similar fashion. This system is found exclusively in Gram-negative bacteria, where it spans the whole cell envelope, as well as the membrane of its target cell. This provides the opportunity for the transport of unfolded proteins directly into the target cell. This transfer of bacterial effectors typically facilitates the creation of a more favorable environment for the delivering bacteria. Three main components of the T3SS have been characterized: the basal body, the needle and the translocon. The needle extends from the base into the extracellular space and consists of a hollow channel that permits transport of unfolded proteins. The translocon is responsible for the transport of effector proteins upon contact with the target cell. The T3SS has been structurally well characterized, and recent studies provided detailed insight into the structural variations of the T3SS in *Chlamydia* and *Salmonella* (Fig. 11).

Type IV Secretion System

The function of the type IV secretion (T4SS) system is used for bacterial conjugation, where it enables the cells to secrete a wide variety of molecules including single stranded (ss) DNA and ssDNA-protein complexes directly into a target cell. There are two types of T4SS: the retractable T4aSS and the non-retractable T4bSS. The T4SS spans the CM and OM of Gram-negative bacteria as well as the membrane of the target cell. The secretion system provides the means to export and import DNA, and thus contributes to spread of antibiotic resistance genes. The structure of the T4SS is similar across all identified T4SS variants, though the role of an extracellular pilus in some systems is still unknown. This system is important for virulence in many bacteria, such as in the pathogen *Legionella pneumophila*, where the structure of this T4bSS has been recently solved (Fig. 12). In this organism, the T4bSS transports effectors into the target cells in order to disrupt the host's defense, and thus enabling bacterial colonization.

Type VI Secretion System

Similar to the T3SS, the type VI secretion system (T6SS) transports proteins from the bacterial cytoplasm directly into the target cell. Found only in Gram-negative bacteria, this system is thought to be both a form of communication and competition

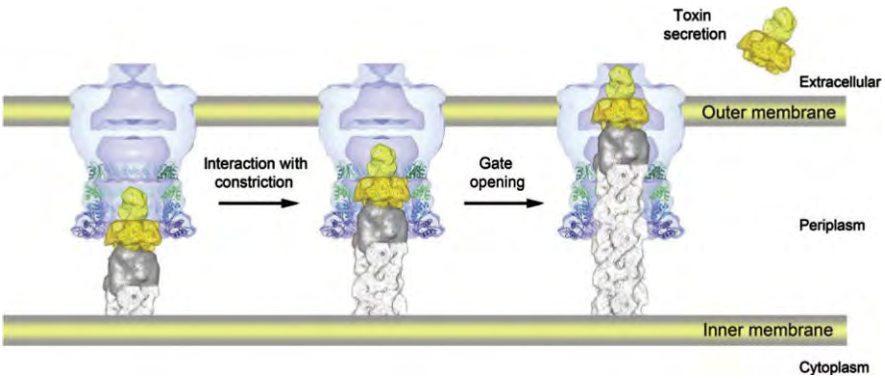

Fig. 10 Schematic representation of the secretion of *Vibrio cholerae*'s cholera toxin by a piston-driven mechanism of the T2SS. Cholera toxin (gold) is loaded on the pseudopilus tip (grey). The toxin is then pushed into the periplasmic portion of the membrane channel by the extension of the pseudopilus. Secretion of the toxin is permitted by the opening of the membrane channel and continued growth of the pseudopilus. Modified from Reichow, S.L., Korotkov, K.V., Hol, W.G.J., Gonin, T., 2010. Structure of the cholera toxin secretion channel in its closed state. Nature Structural and Molecular Biology 17 (10), 1226–1232. doi:10.1038/nsmb.1910.

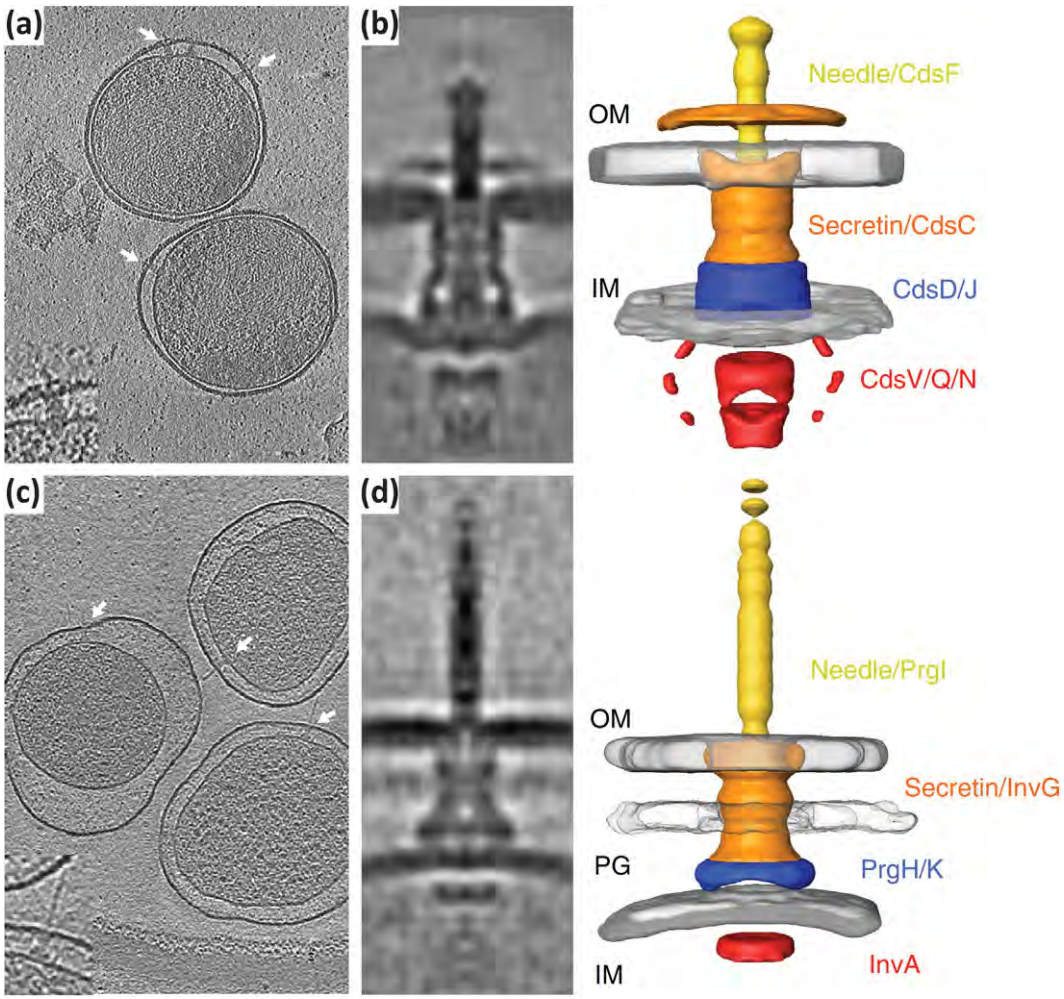

Fig. 11 Cyro-EM visualization of the T3SS obtained from *Chlamydia trachomatis* elementary bodies (a, b) and *Salmonella enterica* minicells (c, d). The T3SS apparatus spans the inner and outer membranes (a, c; white arrows). Further image processing provides a detailed view of the T3SS structure (b, d) and can be represented by both electron micrographs (left) and 3D surface rendering (right). OM – outer membrane, PG – peptidoglycan, IM – inner membrane. Scale bars, 200 nm (a,c) and 15 nm (b,d). Modified from Nans, A., Kudryashev, M., Saibil, H.R., Hayward, R.D., 2015. Structure of a bacterial type III secretion system in contact with a host membrane in situ. Nature Communications 6, 10114. https://doi.org/10.1038/ncomms10114.

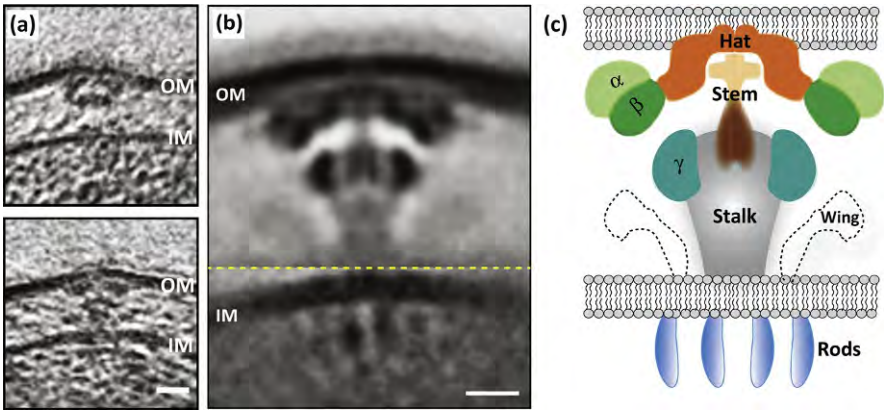

Fig. 12 Cryo-EM and advanced image processing reveal the in vivo structure of the T4bSS of *Legionella pneumophila*. The T4bSS spans the outer and inner membrane (OM, IM) of the cell (a) Subtomogram averaging clarifies the structural features (b) and is fitted with known protein components that comprise the complete T4bSS structure (c) Scale bars: 20 nm (a) and 10 nm (b) Modified from Ghosal, D., Chang, Y., Jeong, K.C., 2017. In situ structure of the *Legionella* Dot/Icm type IV secretion system by electron cryotomography. EMBO Reports 18 (5), 726–732. https://doi.org/10.15252/embr.201643598.

among various bacteria. The T6SS secretes bacterial toxins either into other bacterial cells in the local environment, or into eukaryotic cells. The T6SS is highly efficient in protein transport, and it provides the cells with both defensive and offensive protection against other bacteria that also employ the T6SS. In addition, this system has been implicated in bacterial response to stress and for self-recognition. Furthermore, it contributes to horizontal gene transfer, since it uses this system to kill opponent cells. The resulting lysis frees the DNA that can subsequently be taken up by other secretion systems.

The T6SS is homologous to the tail structure found in bacteriophages. Structurally, the base of the T6SS is anchored to the CM, and upon contraction of a sheath-like structure, the inner tube is propelled into a target cell, puncturing the target cell's membrane with a spike-like protein tip. A well-studied structural model for the T6SS comes from *M. xanthus*. In this organism, the T6SS is characteristic tube-shaped structure within the cell (Fig. 13(a)). The tube structure is extended into the cytoplasm and anchored to the CM. Upon external trigger the sheath of the tube constricts propelling bacterial effectors into the target cell. In the bacterium *Pseudoalteromonas luteoviolacea*, a structured termed the MAC complex induces metamorphosis in tube worms. Here, the T6SS form a large array of tubes held together by a protein mesh, that are released into the environment and act as 'land mines' that induce the metamorphosis of the tube worm.

External Appendages

Pili (Also Known as Fimbriae)

Bacteria and archaea have evolved a number of external appendages that can be used for attachment, secretion, electron uptake, and motility. Such structures include four types of pili. Gram-positive and Gram-negative bacteria have similar types of pili, however their fine structures differ. The chaperone-usher pili are mainly found in Gram-negative bacteria and are used for host attachment and virulence. Chaperone-usher pili are typically 1–2 μm in length with the pilin subunits arranged in a helical pattern. This pilus architecture provides the ability to stretch.

In addition to the functions mentioned for the chaperone-usher pilus, the type 4 pilus (T4P) is also involved in twitching and gliding motility. T4P can be several microns in length with a diameter of 6–9 nm. T4P are unique among the pili because some variants have the ability to extend and retract, which provides the means for the cell's motility. During assembly, pilins are removed from the CM and assembled in the periplasmic space by the T4P basal body, causing the filament to extend out into the extracellular space. There are two types of T4P, Type IVa (T4aP) and Type IVb (T4bP). T4aP has a range of functions including cell motility and DNA transfer, while T4bP is specifically used for host colonization. The Gram-negative organism *V. cholerae* has a unique, well-studied form of the T4bP called the toxin co-regulated pili (TCP). The TCP is essential to the *V. cholerae* infection cycle, providing a means of attachment for the bacterium when it enters the mucosal regions of its host. Additionally, it enables the cells to form microcolonies. The different T4P types are remarkably similar in composition and structure, though only T4aP have the ability to retract (Fig. 14). TcpA is the major pilin subunit for both systems, which forms the extracellular appendage.

The conjugative T4P pilus, also called F-pilus or F-like pilus, is the primary method used between bacteria for the transfer of genetic information. This structure is present only in Gram-negative bacteria and requires a specific plasmid that codes for the essential components of this conjugative pilus. This system is unique as it requires the use of a T4SS for assembly. The current model of this system is that once two cells are connected, the pilus is retracted in order to bring the two cells together, thus allowing for the transfer of genetic material.

Lastly, the type 5 pili (T5P) are unique to the *Bacteroides* phylum, consisting of two types of pilin appendages: a long pilus that extends approximately 0.3–1.5 μm from the cell body, whereas the short pilus is restricted to 80–120 nm. This pilus type has several roles including adhesion, aggregation, biofilm formation, and virulence.

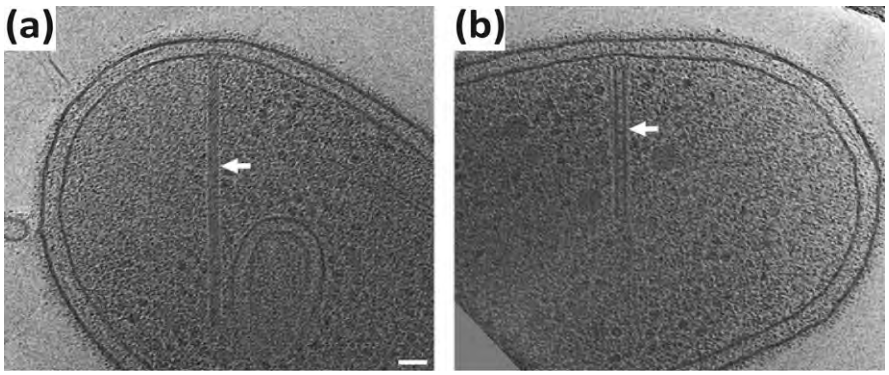

Fig. 13 Visualization of the T6SS in vivo by cryo-EM. The T6SS in the extended (a) and contracted state (b) in *Myxococcus xanthus*. The extended version spans a significant portion of the cell and once triggered contraction delivers effectors to the target cell. Scale bars: 50 nm. Modified from Chang, Y., Rettberg, L.A., Ortega, D.R., Jensen, G.J., 2017. In vivo structures of an intact type VI secretion system revealed by electron cryotomography. EMBO Reports 18 (7), 1090–1099. https://doi.org/10.15252/embr.201744072.

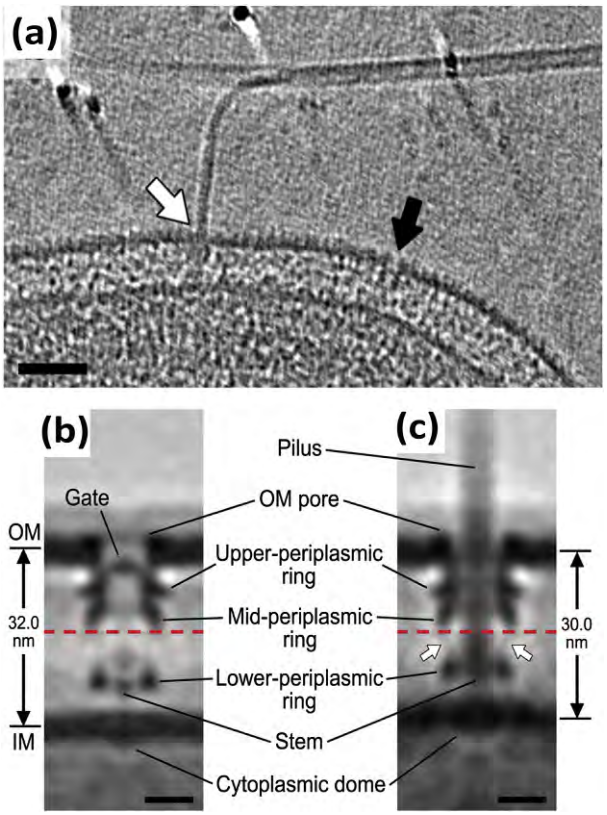

Fig. 14 T4P. *Vibrio cholerae* exhibits a unique type IV pilus apparatus called the toxin-*co*-regulated pilus machine (TCPM). A. Using cryo-EM, the piliated TCPM (white arrow) and non-piliated basal body (black arrow) are identifiable on the cell surface. Image processing enables the user to gain greater detail of the TCPM allowing the comparison of the piliated (b) and non-piliated states (c). Scale bars: 50 nm (A), 10 nm (B, C). Modified from Chang, Y., Kjaer, A., Ortega, D., et al., 2017. Architecture of the *Vibrio cholerae* toxin co-regulated pilus machine revealed by electron cryotomography. Nature Microbiology 2, 16269. https://doi.org/10.1038/nmicrobiol.2016.269.

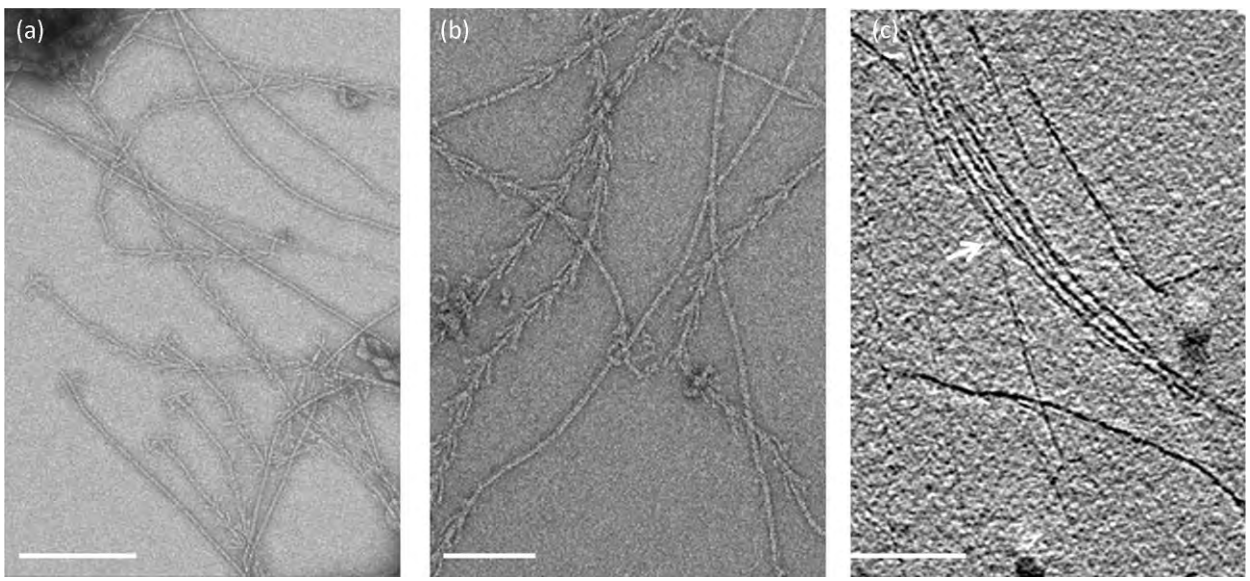

Fig. 15 The archaeal hami of *Candidatus* Altiarchaeum *hamiconxeum* visualized by negative stain EM (a, b) and cryo-electron tomography (c) demonstrating the characteristic barbed structure filament with tripartite tips. Scale bar: 500 nm. Reproduced from Perras, A.K., Daum, B., Ziegler, C., et al., 2015. S-layers at second glance? Altiarchaeal grappling hooks (hami) resemble archaeal S-layer proteins in structure and sequence. Frontiers in Microbiology 6, 543.

Other Cell Attachment Structures

Hami

Some archaea, such as the *Candidatus* Altarchaeum *hamiconexum*, have an additional, unique appendage that is used for attachment to surfaces, other cells, as well as important in biofilm formation in extreme environments. Upwards of a hundred of these pilus-like structures extend from the cell surface, with each hamus having a diameter of 6–8 nm and protruding several microns into the environment. The hami are made of three intertwined filaments. The hamus terminates in a characteristic tripartite structure that resembles a grappling hook (Fig. 15). Many hami have three barbs emerging approximately 47 nm apart along the main thread. The assembly of this structure is thought to be mediated by the Sec pathway, moving the three subfilaments into the periplasm for hamus assembly. However, the filaments are not related to known bacterial filaments and recent research suggests that it may have evolved from modified S-layer proteins.

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Bacterial Bioluminescence

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Introduction

The remarkable phenomenon of bioluminescence no doubt intrigued the first humans, and it still fascinates today. By the dawn of recorded history, the ability of certain living things to make light had been observed and pondered by different cultures. It would have been obvious that many different organisms produced light, including fireflies, glowing mushrooms, and a variety of marine animals. However, microbial bioluminescence must have posed a particular puzzle, as the producers themselves were too small to be seen as individuals and awaited discovery in later centuries. Nonetheless, the glow of bacteria from a dead caterpillar or from a rotting fish would have been familiar well before the microbial nature of the phenomenon was understood.

The advances in our understanding of bacterial bioluminescence in many ways parallel the progress in the field of microbiology itself. In the 1600s, microorganisms or "animalcules" were discovered and observed microscopically, and by the 1700s it was proposed that some microbes produced bioluminescence. In the 1800s, breakthroughs in the ability to grow and isolate bacteria as purified clonal colonies allowed scientists to connect certain properties with specific bacteria. In this way, several species of bioluminescent bacteria were isolated, characterized, and catalogued. In the late 1800s and early 20th century, as the germ theory of disease was being developed, so too was it suggested that the light from some bioluminescent animals might actually be produced by symbiotic microbes, a notion that was confirmed in the early 20th century. The mid twentieth century saw great advances in our biochemical understanding of bacterial physiology, and the biochemistry of bacterial bioluminescence was likewise elucidated. As the field of bacterial genetics grew and its tools became more sophisticated in the late twentieth century, so too were the bacterial genes responsible for bioluminescence characterized first by mutational analysis and later as an early example of successful cloning.

Bioluminescent bacteria were also at the forefront of the field of "sociomicrobiology", a term coined in 2005 to connote that some microorganisms exhibit group behaviors; emergent properties that are absent in isolated individuals. This concept of sociomicrobiology very much has its roots in observations made decades earlier that most bioluminescent bacteria use pheromone signaling to coordinate their decision to turn on their luminescence.

Standing on the shoulders of this long research history, modern scientists continue to add new discoveries. This article summarizes discoveries both old and new relating to bacterial bioluminescence, and highlights some mysteries that remain.

Bacteria That Produce Light

To date, the naturally bioluminescent bacteria described have belonged to three families, all within the gamma-proteobacteria class of Bacteria; the Vibrionaceae, the Shewanellaceae, and the Enterobacteriaceae. Bioluminescent bacteria are often found in mixed microbial communities, and the experimental introduction of a single gene cluster, the *lux* genes (discussed below), can confer bioluminescence on a wide range of bacteria, so it is noteworthy that relatively few bacteria have acquired and retained this trait. By contrast, many useful innovations found on similarly compact genetic loci have been shared broadly across the Bacteria domain, suggesting that perhaps the relative fitness costs and benefits of bioluminescence have constrained the range of bacteria for which it has a net positive value. This section will list and briefly describe the known bioluminescent bacteria, while their habits and ecology will be discussed further in a later section.

Bacterial bioluminescence is most often seen in members of the Vibrionaceae, which encompasses a number of marine and estuarine species. Bioluminescent bacteria are frequently found in coastal seawater, but they can also be isolated from deeper water, as well as from animal gut tracts, light-emitting organs, and surfaces. Such isolates are almost invariably representatives of the Vibrionaceae, and can be classified into the genera *Vibrio* and *Photobacterium*. It has been suggested that a clade representing a phylogenetic intermediate between *Vibrio* and *Photobacterium* warrants a separate genus, named *Aliivibrio*, but this nomenclature has not been adopted universally. Best known among this clade of bioluminescent bacteria is *Vibrio fischeri*, which taxonomists had suggested moving from *V. fischeri* to *Photobacterium fischeri*, and then back to *V. fischeri*, prior to the proposed *Aliivibrio fischeri* nomenclature. For the purpose of this article, the designation *V. fischeri* will be used when referring to this popular model bioluminescent bacterium.

Current knowledge is consistent with the hypothesis that an ancestral member of the Vibrionaceae was bioluminescent and that the *lux* genes underlying this trait were passed down through this lineage, with some branches losing the genes and occasionally re-acquiring them horizontally. Today, only a relatively small fraction of species and strains in this family are luminous. In most of the rare instances of horizontal gene transfer, the *lux* genes have been kept within the Vibrionaceae, but as noted above two other bacterial families include bioluminescent members. Additional bioluminescent bacterial species that have been isolated from seawater are members of the Shewanellaceae family; *Shewanella hanedai* and *Shewanella woodyi*. The former was also isolated from marine sediment and the latter isolated at least once from a squid ink sac.

It appears that at some point the *lux* genes made the leap from the ocean to the land, and into the Enterobacteriaceae family where they now reside in the genus *Photorhabdus*, which remains the only known genus of terrestrial bioluminescent bacteria. Indeed, with the exception of rare bioluminescent isolates of *Vibrio cholerae* from freshwater environments, the *Photorhabdus* species are unique as non-marine bioluminescent bacteria. Although the taxonomy of these bacteria is somewhat unsettled, at least three bioluminescent species have been described: *Photorhabdus luminescens*, *Photorhabdus temperate*, and *Photorhabdus asymbiotica*. These bacteria are symbionts of insect-killing nematodes, and are responsible for the phenomenon of glowing dead insects. At least *P. asymbiotica* also appears to be an opportunistic pathogen responsible for glowing wounds.

Although much effort has been devoted to sampling and searching for bioluminescent bacteria, it is possible that other species with the capacity to produce light exist in nature and await discovery. Given the vast scale of the marine environment and its various microhabitats, including potential host species, perhaps sampling has been insufficient and the isolation techniques used in the past will reveal additional bioluminescent bacteria in the future. On the other hand, some host-dependent bioluminescent bacteria have resisted culturing, while other bioluminescent bacteria do not produce bioluminescence when they are cultured and thus may escape notice. Skirting the need for culturing, metagenomic studies that analyze the sequences of DNA isolated directly have reported *lux* gene sequences, suggesting they came from bacteria with the ability to produce light. As these culture-independent data sets expand it will be interesting to investigate more thoroughly the origins of these sequences and their genetic contexts, as this information may reveal previously unknown bioluminescent bacteria.

It is easy to find bacterial species and genera not mentioned in this article that are described or listed elsewhere as bioluminescent, but in many cases these references simply reflect taxonomic name changes. As noted above, *V. fischeri* has been given different genus designations, and confusion can result from several other name changes as well. For example, *S. hanedai* was formerly *Alteromonas hanedei*, *Photorhabdus luminescens* has been called *Xenorhabdus luminescens*, some *Photorhabdus* isolates have been alternately considered subspecies or distinct species, several bioluminescent *Vibrio* species were put in the proposed genus *Beneckea* until that fell out of usage, etc. While experimentalists and taxonomists debate the relative merits of name changes, students of any particular bioluminescent bacterium would be well advised to know the history of its nomenclature or risk overlooking many good studies in the literature that were published during different taxonomic regimes.

Biochemistry of Bacterial Bioluminescence

The core proteins responsible for bacterial bioluminescence are well conserved among the bacteria that produce light. Throughout the biological world, the enzymes generating bioluminescence typically are called luciferases, and bacterial luciferase is a heterodimer of the two proteins LuxA and LuxB, which are sometimes referred to as alpha and beta luciferase subunits, respectively. The LuxAB luciferase sequentially binds reduced flavin mononucleotide ($FMNH_2$), O_2, and an aliphatic aldehyde (RCHO), and these substrates are converted to FMN, water, and the corresponding aliphatic acid (RCOOH) with the concomitant production of bluish light at around 490 nm wavelength (Fig. 1). Thus, luciferase catalyzes the reduction of oxygen while both the $FMNH_2$ and the aldehyde substrate are oxidized. The quantum yield of bioluminescence from the turnover of this reaction has been estimated at 0.1–1 photons. Luciferase is an unusually slow enzyme that forms a relatively stable complex with its bound substrates before catalyzing the light-generating reaction. Interestingly, bacterial luciferases seem to fall into two groups that have distinctively "fast" or "slow" luciferase reaction kinetics, although the significance of this observation is unclear.

Besides catalyzing the production of bioluminescence, in the absence of its aldehyde substrate, bacterial luciferase can generate reactive hydrogen peroxide (H_2O_2) through what is called the "dark reaction". Light itself seems to be the important

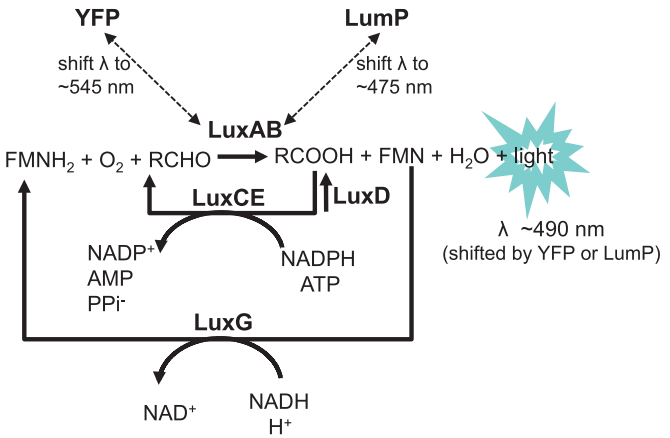

Fig. 1 The biochemistry of bacterial bioluminescence. Bold font indicates proteins. Dotted lines indicated protein–protein interactions between LuxAB and YFP or LumP that alter luciferase kinetics and the wavelength of emitted light. See Text for details.

product of Lux system at least in most circumstances, and the "dark reaction" may be simply an unintended side reaction akin to the production of H_2O_2 by aerobic respiratory chains. However, some bacteria, including *Vibrio salmonicida*, are naturally aldehyde-limited with respect to bioluminescence, and it may be the case that Lux-derived H_2O_2 is an important, if often forgotten, product of this system.

Additional core Lux proteins, LuxC, LuxD, and LuxE, act as a complex to (re)generate the aldehyde substrate for luciferase (Fig. 1). LuxD is a thioesterase responsible for redirecting fatty acid metabolism toward generation of the luciferase substrate. LuxD can release a fatty acid from either an acyl-carrier protein (ACP) or from acyl-coenzyme A. The liberated fatty acid is subsequently acted on by LuxE and then LuxC. LuxE, which is referred to as the synthetase, binds the fatty acid substrate and activates the reaction with ATP hydrolysis, while LuxC is the reductase responsible for using NADPH to reduce RCOOH to RCHO. Multiple lines of evidence point to RCOOH and RCHO being the fourteen-carbon molecules myristic acid and tetradecanal, respectively; however, the system is not particularly specific with respect to acyl chain length, and aldehydes longer than six to eight carbons generally will serve as substrates for luciferase. It seems likely that tetradecanal is a primary substrate for luciferase *in vivo*, at least in the bacteria that have been examined in detail, but it remains possible that other aldehydes contribute to bioluminescence.

Some, but not all, light-producing bacteria have additional Lux proteins that contribute to bioluminescence. One of these is LuxG, which shuttles reducing power from NADH to FMN to (re)generate the $FMNH_2$ substrate for luciferase. Other flavin reductases can substitute for LuxG, which explains why not all bioluminescent bacteria have *luxG*. The role of another auxiliary Lux protein, LuxF, only became clear relatively recently. LuxF protects luciferase by binding to myristylated FMN (myrFMN), which is a byproduct of the bioluminescence reaction that can inhibit LuxAB. LuxF has higher affinity for myrFMN than does LuxAB, so the presence of LuxF will effectively titrate myrFMN before it reaches concentrations that would inhibit LuxAB.

Two additional proteins have been described that interact with luciferase and shift the wavelength of emitted light longer or shorter than the typical 490 nm. The *lumP* gene in *Photobacterium leiognathi* encodes lumazine protein (LumP), which has the unusual effect of shifting the emitted light to a shorter wavelength (~475 nm) bluer light. The opposite effect is seen with the *luxY* gene from *Vibrio* strain Y1, which encodes a yellow fluorescent protein (YFP) that shifts the bioluminescence emitted to a longer wavelength (~545 nm) yellowish light. Strain Y1 is referenced in most reports as *V. fischeri*, but both phenotypic and multi-locus sequencing analyses suggest that this is actually a distinct species, *Vibrio* (or *Aliivibrio*) *sifiae*. Both LumP and YFP interact directly with LuxAB, and these complexes alter both the kinetic and the spectral properties of luciferase. Each of these proteins contains a ligand derived from the riboflavin biosynthetic pathway, 6,7-dimethyl- 8-(1′-D-ribityl) lumazine for LumP and FMN in the case of YFP. Other wavelength-shifting proteins that are not as well characterized as LumP or YFP probably exist, and a preliminary report suggests one such example is responsible for the bluish hue (~472 nm) emitted by *Vibrio azureus*.

Genetics of Bacterial Bioluminescence

The genes encoding the core enzymes responsible for bioluminescence usually are found clustered together in the conserved order of *luxC*, *luxD*, *luxA*, *luxB*, *luxE*, and *luxG*, with a few additions and minor variations (Fig. 2). These genes are typically expressed on a single transcript, often referred to as the *luxCDABEG* operon. Exceptions to this operon organization include the observations that *luxG* is absent in *P. luminescens*, and that in some *Photobacterium* strains *luxF* is found in between *luxB* and *luxE* (Fig. 2). In addition, at the distal end of the operon, downstream of *luxG* but presumably transcribed with *luxCDABEG*, some strains carry *ribB* (also called *luxH*), or a *ribEBHA* cluster (Fig. 2). These *rib* genes appear to encode enzymes related to riboflavin biosynthesis. FMN and lumazine, whose roles in luminescence are described above, are generated as offshoots of riboflavin metabolism, and the role of the *lux*-associated *rib* genes may be to augment levels of one or both of these.

In some bacteria, genes that are co-transcribed with, or adjacent to, the *luxCDABEG* operon are involved in its regulation or function. In *V. fischeri*, the regulatory combination of LuxI and LuxR are encoded with *luxI* at the beginning of the main *lux* operon and *luxR* adjacent and divergent from it (Fig. 2). In some *Photobacterium* strains, *lumP*, which encodes the lumazine protein described above, is found adjacent to the *lux* operon along with *lumQ*, which appears to encode a companion regulator. It is possible that other bacteria cluster luminescence-related non-*lux* genes near the *lux* locus as well, a possibility that will be tested as the genome sequences of more bioluminescent bacteria become available.

The specific sequences of the *lux* genes in different bacteria have provided interesting evolutionary insights. In particular, relatedness between *lux* operons in distinct bacteria has implicated the sources in instances of horizontal gene transfer of this locus. As just one example, an unusual bioluminescent strain of the human pathogen *Vibrio vulnificus* has been isolated, and the sequence of its *lux* operon suggests it was acquired from *Vibrio harveyi* or a related species. Intraspecies *lux*-sequence comparisons have provided additional if more mysterious evidence of selective pressures on luminescence. For example, in two isolates of *V. fischeri*, one noticeably brighter than the other, the *luxCDABEG* genes and the proteins they encode have diverged significantly more than the surrounding housekeeping genes, suggesting relatively recent selective pressure and rapid evolution of Lux even in strains of the same species. In another interesting discovery, some *Photobacterium* strains are merodiploid with respect to *lux*, carrying two similar but genetically distinct copies of this locus, possibly reflecting two subtly different functional roles. Finally, non- or partially functional *lux* loci have been documented in *Vibrio* strains. Thus, the sequences of the *lux* loci have provided insight into their acquisition by some strains, their degradation and loss in others, and what appears to be their relatively recent functional honing.

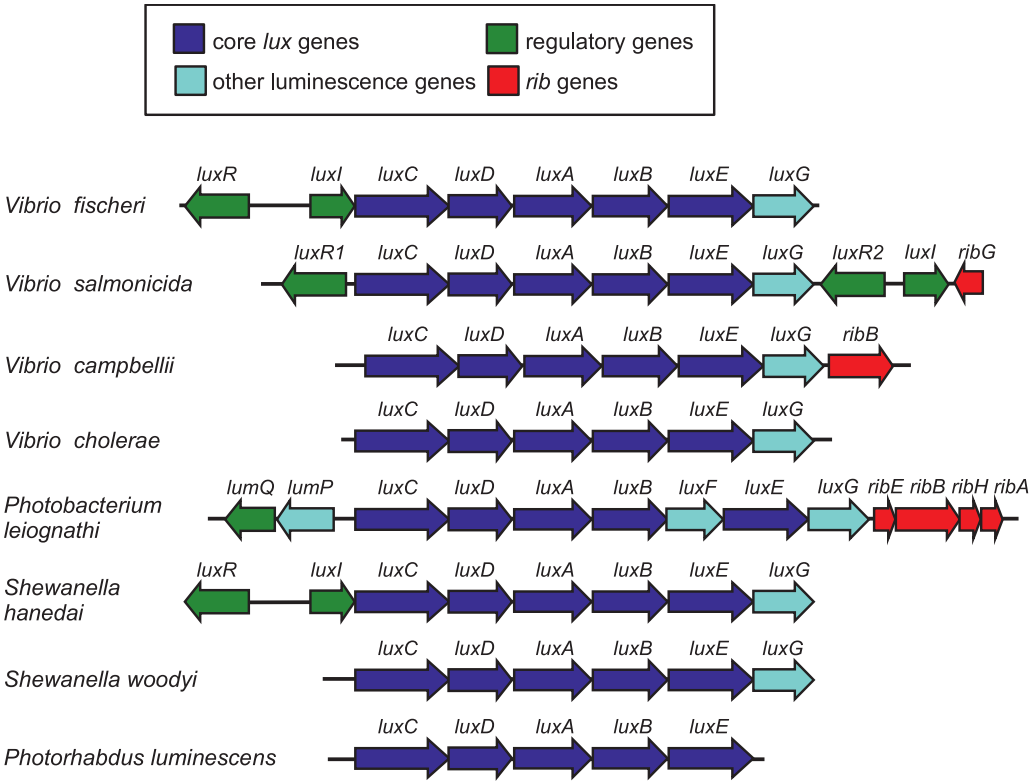

Fig. 2 Genetic organization of the lux locus in different bioluminescent bacteria. The core *lux* genes, *luxC*, *luxD*, *luxA*, *luxB*, *luxD* and *luxE* are found in that in each of the bioluminescent bacteria, while the auxiliary lux genes are found in only some.

Regulation of the *lux* Genes

The regulation of the *lux* genes, in other words the conditions under which they are transcribed and translated to produce bioluminescence, has been the focus of considerable research. Although the benefits of bioluminescence and its ecological role(s) for bacteria are covered in a separate section below, this section on the regulation of bioluminescence is intertwined with the discussion of bioluminescence's functional role for the simple reason that one expects bioluminescence to be turned on when it is beneficial for the bacteria and turned off when it is disadvantageous. There may not be a regulatory mechanism common to all bioluminescent bacteria, but there are intriguing common themes.

One clear theme is that many bioluminescent bacteria produce light only when they are in a relatively dense population. As noted above this discovery led to the concept of "sociomicrobiology", which acknowledges that certain emergent properties of bacteria are seen in groups but not in lone cells far from others of their kind. In the case of bioluminescence (and many other bacterial group behaviors), pheromones called "autoinducers" released by the cells must reach a critical concentration to activate gene expression. This phenomenon has been termed "quorum sensing" to connote that a behavior is induced upon cultures reaching some minimum critical population density. A more accurate term might be "quorum-dependent regulation", because in some instances a quorum is necessary but not sufficient. Moreover, a number of complexities in the pheromone-signaling circuitry make it doubtful that the sole purpose of these systems is to census local population density. Many bacteria have multiple pheromones, their synthesis and their receptors are regulated in response to the environment, and in most cases pheromone synthesis is subject to positive-feedback regulation. These elements could allow pheromones to transmit information about the environment across populations. At the same time there must be a population present with which to communicate, and the requirement of a minimum cell density to induce bioluminescence has been widely interpreted from the viewpoint that bioluminescence is meant to be seen, and so it is a waste of resources for a single cell, which does not produce enough light to be visualized.

Quorum-dependent pheromone-mediated control of bioluminescence was first described in *V. fischeri*, which led to the discovery of the LuxR/LuxR signaling system. In *V. fischeri*, LuxI synthesizes the acyl-homoserine lactone (acyl-HSL) pheromone *N*-3-oxohexanoyl homoserine lactone (3OC6-HSL), which can diffuse through cell membranes and thereby becomes a shared extracellular signal. Upon reaching a critical threshold concentration, 3OC6-HSL binds LuxR, which can then activate gene expression. In *V. fischeri*, *luxI* is cotranscribed with the *luxCDABEG* operon, and *luxR* is adjacent and divergently transcribed (Fig. 2). Among other targets, 3OC6-HSL-LuxR activates transcription of *luxICDABEG*, which results in both induction of bioluminescence and, in a positive feedback loop, more LuxI and 3OC6-HSL. These functions of LuxI and LuxR are an archetype for similar systems with a diverse array of HSL signals in a wide range of Proteobacteria.

Although LuxR and LuxI are synonymous with the cell-density dependent regulation discovered in bioluminescent bacteria, another form of pheromone signaling is more common in the Vibrionaceae. In most vibrios, a core pheromone-receiving circuit transduces signal perception through a phosphorylation cascade and eventually activates a "master regulator" that controls many genes, including the *lux* operon in most bioluminescent vibrios. *V. fischeri* has this conserved core system in addition to LuxR and LuxI, and its master regulator is named LitR. Unlike similar vibrio master regulators, LitR does not regulate the genes for bioluminescence directly, but instead LitR activates transcription of *luxR*. Homologs of LitR are found throughout the Vibrionaceae, the best known of which are HapR in *V. cholerae* and LuxR in *V. harveyi*, which despite its name is structurally unrelated to LuxR in *V. fischeri*. Interestingly, both *V. fischeri* and *V. harveyi* use AHL signals to control their core pheromone-signaling circuit, but these signals are produced by proteins that are very different from LuxI and apparently arose through convergent evolution. The presence of other non-AHL signals, including a widespread "autoinducer-2" or AI-2 signal, also affects this core signaling circuit and can likewise control bioluminescence. The complexity of this signaling is interesting in its own right and is covered in some of the suggested further reading below.

Some strains do not appear to control bioluminescence with pheromone-mediated signaling, although arguably such exceptions are most notable for their rarity. One such bacterium is *Photobacterium leiognathi* KNH6, which displays dazzlingly bright bioluminescence but does not appear to control bioluminescence based on cell density or pheromone signaling. This observation is more remarkable given that the KNH6 genome encodes the core pheromone-signaling circuitry common to most of the *Vibrionaceae*, so the regulatory capacity is present but the *lux* genes are not controlled in this common way. Whether this difference in regulation reflects a different use of bioluminescence is unknown.

The regulation of bioluminescence in response to environmental conditions can vary between species, but again *V. fischeri* has emerged as an experimental model, and at least some of the findings in *V. fischeri* reflect observations in other bacteria as well. *V. fischeri* and many other bioluminescent bacteria are found interacting with hosts, either in gut-tract consortia or dedicated symbiotic tissues, and some regulation of *lux* can be interpreted as turning on bioluminescence in a host-like environment. For example, in *V. fischeri* the *lux* operon is controlled by the regulators Fur and CRP such that bioluminescence is induced when access to iron is restricted and complex carbon sources other than glucose support growth, presumably reflecting conditions common in animal tissues.

The *lux* system is also regulated in response to redox conditions, which is germane to discussion below of its possible costs and benefits for the bacteria. Although one report indicates that bioluminescence in *V. fischeri* is maximally expressed under microaerobic conditions, other studies have suggested it is most induced under more oxidative conditions. More than one control mechanism has been implicated in this regard, but especially noteworthy is the ArcA/ArcB two-component regulatory system, which represses bioluminescence in *V. fischeri* and is thought to be activated by reducing conditions. Mutants lacking *arcA* or *arcB* can be a hundred- to a thousand-fold brighter than their parent strain in batch culture, underscoring their importance. One important open question is the relative importance of oxygen *per se* versus other oxidants in *lux* regulation. Reactive oxidants and/or alternative respiratory electron acceptors may play regulatory roles, although these distinctions are not yet clear.

Ecological Role(s) of Bacterial Bioluminescence

The question of why bacteria produce bioluminescence is both centrally important and difficult to answer. Many interesting hypotheses have been proposed, and it is difficult to rule many out entirely. It is important to keep in mind that the answer is likely to be different for different bioluminescent bacteria and that even for a single bacterium there may be more than one selective advantage for producing bioluminescence. Indeed, as discussed below, bioluminescence appears advantageous to *V. fischeri* on more than one level. The selective pressures on bioluminescence are likely to vary depending on context, and while one can argue that regulation should tune *lux* expression to the contexts where it is beneficial, most studies of bioluminescence and its regulation have been conducted under artificial experimental conditions (e.g., batch cultures growing on rich media in shake flasks). Taken together, our understanding of the real-world significance of bacterial bioluminescence is certainly incomplete, yet it remains a topic of great interest.

Before considering the selective advantages for bacteria to express bioluminescence, it is useful to consider the costs that must be balanced against any benefits. LuxAB can comprise 5% or more of the protein in bright cells, regenerating the aldehyde substrate requires hydrolysis of ATP (Fig. 1), and the consumption of oxygen and reducing equivalents might compete with energy-generating aerobic respiratory pathways. Consistent with luminescence coming at a price, researchers have long noticed that dim or dark mutants arise during prolonged culture, leading to speculation that in the absence of natural selection to maintain luminescence, it is evolutionarily lost for being energetically too expensive. It now appears that many such spontaneous dim mutants were probably regulatory in nature, thus simultaneously changing multiple phenotypes other than luminescence; however, a defined dark Δ*luxCDABEG*, mutant outcompeted its isogenic wild-type parent in a carbon-limited chemostat, demonstrating a disadvantage to luminescence in at least one condition. On the other hand, bioluminescent bacteria, particularly members of the Vibrionaceae, seem well suited to lifestyles of feast and famine, growing rapidly in relatively nutrient-rich conditions and surviving through low-nutrient oligotrophic waters long enough to find another feast. It is possible that when bioluminescence is induced, its energetic costs are not all that important to the bacteria given an abundance of growth substrates.

The benefits of the Lux system could derive from any of its products. Understandably, most attention has focused on the light generated, but luciferase also consumes oxygen and reducing equivalents, and hypotheses centered on the potential importance of these activities have been proffered. For example, it has been proposed that by consuming reducing equivalents, luminescence may effectively scavenge small amounts of oxygen to help support fermentative growth by recycling NAD^+ without the need to give up a valuable organic fermentation product. This concept of supporting fermentation is consistent with the observation that luciferase has a very high affinity for oxygen, and can reduce it in low-oxygen environments where aerobic respiration is relatively ineffective anyway. At least in *V. fischeri* however, regulation is the opposite of what one would predict based on this hypothesis, as the *lux* genes are repressed by the same regulatory system that promotes many fermentative pathways. Rather, in *V. fischeri*, the *lux* genes appear up-regulated under highly aerated conditions, which is more consistent with the proposal that luciferase benefits cells as a defense against oxidative stress, either by minimizing the production of reactive oxygen species by or near the cell or by generating a reduced cytoplasmic environment that is more resistant to oxidative damage.

Complicating matters, as noted above luciferase can generate H_2O_2 when it is limited for aldehyde, and thus in some conditions luciferase might prime a response to H_2O_2 and other reactive oxygen species. Similarly LuxAB-generated H_2O_2 might be a virulence factor, doing more damage to nearby host cells than to the bacteria. However, even if the "dark reaction" production of H_2O_2 is important in some situations, it does not explain the evolution of luciferase's light-generating capacity.

To find the importance of light-production *per se* we might ask where bioluminescence is produced in nature. For members of the Vibrionaceae, bioluminescence has been observed in several "light-organ" symbioses with animals, and here an understanding of host ecology can explain benefits to the bacteria. Squid and fish with symbiotic light-emitting tissues employ the bioluminescence for a range of uses, including lures for prey, intra-species signals, and a camouflaging strategy called counter-illumination. The simple argument is that these symbioses represent mutualisms, and if hosts are supporting symbiotic bacteria in exchange for bioluminescence, then by benefiting their hosts the bacteria could effectively be benefiting themselves. Although in most cases the bacterial symbionts from the Vibrionaceae have retained an environmental lifestyle outside the host, symbionts of certain anglerfishes have reduced genomes and appear to have evolved a dependence on the host, underscoring the support that hosts may provide.

The light-organ symbioses between sepiolid squids and *V. fischeri* or *V. logei* have received considerable attention, particularly the model of *V. fischeri* and the Hawaiian bobtail squid *Euprymna scolopes*. This nocturnal shallow-water host squid has a bioluminescent "light organ" with light provided by *V. fischeri*, and it is thought to use the light in a counter-illumination strategy, matching the down welling moon and star light with its own dim ventrally oriented light. In this way, the squid may obscure their silhouette from other animals beneath them in the water column. Moreover, the squid expel some of the bacteria back into the environment each day, resulting in higher *V. fischeri* populations where the squid live. In this case, the benefit of bioluminescence for the bacteria can be rationalized as a contribution to a mutualistic symbiosis; helping a host that supports the bacterial population in return.

Interestingly, there is another layer of advantage for *V. fischeri* symbionts of *E. scolopes*, illustrated by the observation that dark mutants are attenuated in the ability to colonize the light organ. This colonization defect is apparent even when a Δ*luxCDABEG* mutant co-colonizes a host with a luminescent wild-type strain, so it seems unlikely that this effect could be explained entirely by the host sanctioning a dark infection. This relative fitness defect of a dark mutant in the light organ is the opposite of what has been observed in a carbon-limited chemostat, where (as noted above) a dark mutant can outcompete the bioluminescent wild type. Understanding the light-organ environment and the physiology of the symbionts in this environment may hold a key to understanding growth conditions in which the Lux system is inherently advantageous, which would provide enormous insight into the evolution of bioluminescence.

In addition to light-organ symbioses, *Vibrio* species are frequently found in the enteric tracts of hosts, which typically contain mixed consortia of many bacteria and provide them with a relatively rich set of resources compared to the surrounding waters. If populations of bioluminescing bacteria are high enough in gut tracts, then the fecal matter released by hosts may actually glow, and it has been proposed that this property might attract other marine animals to eat the feces and thus return the bacteria to the privileged environment of a host gut. This simple idea is compelling and could explain the widespread distribution of luminescent bacteria in fish guts even in areas without known light-organ symbioses.

Another large-scale but intermittent and mysterious phenomenon called "milky seas" appears to result from large swaths of bioluminescent bacteria near the ocean surface. Covering areas measuring up to thousands of square kilometers, the persistent luminescence from "milky seas" has been reported by ships and corroborated by satellite imaging. The duration of these luminescent patches, which can extend for hours or days, seems more consistent with bacterial bioluminescence than with other known sources of marine luminescence. How bioluminescent bacteria could achieve high enough populations to produce the amount of light emitted is not known, but speculation and anecdotal evidence have suggested blooms of phytoplankton (or some other organism) that can serve as a host for a bioluminescent bacterium. *Vibrio campbellii* has been suggested as a candidate bioluminescent bacterium able to exploit phytoplankton blooms, but whether this is the case and whether bioluminescence is in some way beneficial in this context require further exploration.

What of bioluminescent bacteria other than members of the Vibrionaceae? As noted above, bioluminescent *Photorhabdus* species are symbionts of insecticidal nematodes and are responsible for glowing insect cadavers. *Photorhabdus* symbionts play important roles for their nematode hosts, helping to kill and digest insect victims and contributing to normal development in their host's life cycle; however, their bioluminescence is not required for this symbiosis in at least one symbiont-host combination, and its purpose is unclear. It has been proposed that the luminescence from a dead insect might attract other potential insect victims, or alternatively it might deter larger organisms from eating the dead insect, which would potentially represent a dead end for the *Photorhabdus* and

nematode. Bioluminescence in *Photorhabdus* is subject to regulation, and notably it is among the properties controlled by a bi-stable regulatory switch that results in two distinct phenotypic variants. The bioluminescent variant retains its symbiotic properties with insecticidal nematodes, implicating a role in this context.

The ecological importance of luminescence in *Shewanella* species is perhaps even less clear. *Shewanella hanedai* and *Shewanella woodyi* have been isolated from seawater, sediments, and from the surfaces of some marine animals, but there are no known light-organ symbioses, nor is there a natural environment in which their bioluminescence has been documented. Unlike members of the Vibrionaceae, which tend to be facultative anaerobes and are often found in animal enteric tracts, *S. hanedai* and *S. woodyi* appear to rely on respiratory growth and are associated with animal or abiotic surfaces rather than guts. Thus, the concept of luminescence serving to attract animals to eat fecal matter, thereby returning the bacteria to an enteric tract, does not seem to apply. The *lux* genes appear to have moved into *S. hanedai* and *S. woodyi* from the Vibrionaceae relatively recently, and perhaps the *lux* operon conferred a physiological advantage unrelated to the light emission that has been selected for in the vibrios.

Although many proposed roles for bioluminescence appear viable in at least some systems, a few appear unlikely. For example, a series of indirect observations led to the hypothesis that luciferase could allow ATP generation in the absence (or inhibition) of cytochromes, and this hypothesis has led to the occasional claim that luciferase can replace cytochromes. Although this is thermodynamically feasible, an ATP-generating mechanism theorized by this model has never been shown, and thirty years after it was suggested, clear experimental evidence that luciferase can replace cytochromes remains lacking. In another example, it has been proposed that luminescence might provide cells a benefit by activating light-dependent photolyase-mediated DNA repair. Although experimental data suggest luciferase-generated light *can* activate photolyase in this way, the arguments against this process being ecologically relevant seem overwhelming. In these cases and others however, even if the proposed role of luminescence is doubtful, the observations merit further exploration.

Applications of Bacterial Bioluminescence in Research and Biotechnology

The bioluminescence produced by LuxAB can be detected with great sensitivity, making it attractive for a number of applications. For example, bioluminescent bacteria are used in toxicity tests wherein compounds are added to cultures and the effect on bioluminescence is measured. This approach has advantages of simplicity and rapid output over other assessments of toxicity that measure effects on metabolic activity or loss of viability. If a compound kills bacterial cells or broadly blocks their metabolism, the loss of luminescence may be the fastest possible measure of this effect. Most often, cultures of *V. fischeri* are sold and used for this purpose.

Bioluminescence has also been exploited by introducing the *luxCDABE* operon into non-native bacteria. For example, in one general approach, phages (viruses that infect bacteria) have been engineered to carry the *luxCDABE* operon, such that when the phage injects its DNA into a specific target bacterium, that bacterium will bioluminescence. In order to produce light, the target bacterium must be capable of expressing the *lux* genes and able to provide LuxAB with substrates, and as a result such engineered phages can be used to detect metabolically active cells of a specific bacterium in a sample, whereas other (e.g., antibody-based) approaches may detect both live and dead cells. This technology has been combined with the addition of various antibiotics to test whether particular antimicrobials affect a target bacterium. This luminescence-based strategy holds potential to be much faster than traditional antibiotic-sensitivity testing that is based on growth inhibition.

The *luxCDABE* operon or simply the *luxAB* genes also have been used in a wide range of bacteria as reporter genes fused to transcriptional promoters of interest. Researchers probing the transcriptional regulation of a particular gene may fuse its promoter to *luxCDABE*, allowing them to use bioluminescence as a convenient readout of promoter activity. Sometimes *luxAB* is simply used rather than the entire operon, and in this case aldehyde substrate must be provided at the time bioluminescence is to be measured, and consideration should be given to the possibility of H_2O_2 production from the "dark reaction" in aldehyde-limited cells. Through such reporter studies, the environmental conditions or transcription factors controlling expression of a particular gene or operon can be discovered and assessed. Viewed from another angle, the same basic approach can be used to generate bioluminescent "bioreporters", wherein a particular environmental condition can be assessed based on known effects on a transcriptional reporter. For example, if the *lux* genes are placed in a bacterium under control of a transcriptional promoter that is activated in response to phosphate limitation, cells will induce luminescence when available phosphate is low. In this way, bacteria have been engineered to bioluminescence only in response to a particular pollutant, stress, antibiotic, or other cue. This technology can be applied in complex samples and has advantages when biochemical detection of a particular compound is difficult or somewhat ambiguous. Because bioreporters are based on living cells, they have the advantage that they can be used to assess not only the presence of particular molecules but also their bioavailability.

Another application of the *luxCDABE* operon is to constitutively express it in otherwise dark bacteria to make them luminescent, thereby allowing the cells to be detected and tracked in a sample based on their bioluminescence. This approach became much more attractive with the advent of intensified charged-coupled device (ICCD) camera detectors, which allow researchers to visualize the location of light-producing bacteria even in what appears visually to be an opaque tissue. For example, when mice are infected internally with transgenic *Salmonella enterica* carrying the *lux* operon, the mice do not become visibly bioluminescent, but an ICCD camera can detect the photons emitted and track the location of the pathogen in host tissues. This use of *luxCDABE*, along with its use as a reporter, further illustrates that acquisition of a single locus is sufficient to render many phylogenetically diverse bacteria bioluminescent.

While most of the above applications have used naturally bioluminescent bacteria or their *lux* genes, in some approaches the Lux system has been engineered to maximize its utility in other organisms. For example, the *lux* genes have been reconstructed to better match normal codon usage in various hosts of interest, the *luxA* and *luxB* genes have been fused to encode a single protein, the *lux* genes have been expressed on smaller transcripts in organisms that typically do not express genes as operons, and alternative or additional pathways to $FMNH_2$ synthesis have been added to certain hosts. Such tweaks to the original *lux* system have made it more useful in a broader range of organisms.

Bioluminescent Bacteria in Education

Given the tractability of bacteria as research subjects and the intriguing attraction of bioluminescence, it is not surprising that bioluminescent bacteria have found their way into classrooms and laboratory demonstrations. In classes where specific types of bacteria are targeted for isolation from the environment, bioluminescent strains are a favorite. As noted above, many bioluminescent bacteria are found in marine waters or associated with marine animals such as fish. As a general approximation, coastal seawater contains about one bioluminescent colony-forming unit per ml when plated on a rich salty medium, and potential environmental isolates can be concentrated by passing several milliliters of coastal seawater through a filter with a 0.2–0.4 μm pore size. Alternatively, students can isolate bioluminescent bacteria from fresh marine fish or squid acquired a local market, provided the meat has not been disinfected. Fortunately, many bioluminescent marine bacteria are relatively fast growing on rich media when incubated at temperatures similar to the environments from which they came, so while a plate may be crowded with various microbes the bioluminescent bacteria typically are not overgrown by others. By taking isolation plates into a darkroom, students are able to pick out target colonies, sometimes with slightly different shades and intensity of blue light. Students can readily narrow the identity of their isolates using traditional microbiological techniques or molecular phylogenetics.

Bioluminescence is also used in experiments that demonstrate molecular cloning methods. In this case, instructors take advantage of the fact that all the *lux* genes necessary for bioluminescence are clustered together on a single locus and therefore can be cloned on a single DNA fragment. The *lux* operon from *V. fischeri* is a favorite for cloning, because in many strains the sequences flanking the boundaries of the *lux* locus contain recognition sites for the restriction enzyme *Sal*I, such that digestion of *V. fischeri* genomic DNA with *Sal*I liberates a distinct *lux*-specific DNA fragment with short single stranded ends suitable for cloning into a plasmid that has likewise been digested with *Sal*I. Moreover, the workhorse bacterium for molecular cloning, *E. coli*, is able to bioluminescence once transformed with *lux*-containing plasmids, provided it is grown at temperatures compatible with *V. fischeri* growth (e.g., 22–28°C).

Conclusions

Extensive research has unraveled many of the mysteries of bacterial bioluminescence. The bacteria, genes and enzymes underpinning this phenomenon have been elucidated in many respects, and our understanding has enabled us to exploit bacterial bioluminescence in basic research, biotechnology, and education. Yet new details continue to be filled in and many important questions remain open and unanswered. Among these, the central question of the role bioluminescence plays in bacterial ecology still invites speculation and further testing. While the central features of the *lux* system are well conserved, differences at the biochemical and regulatory level suggest that there may not be a single answer to this question, and bioluminescence may confer a fitness advantage on different bacteria for different reasons. Studying the expression and impact of bioluminescence in natural environments for the bacteria is a promising approach for further exploration. Future studies of bioluminescent bacteria in ecologically relevant contexts will be bolstered both by model systems amenable to laboratory experimentation and by methods for studying bacteria in the environment. Such approaches along with deeper molecular insights into the evolution and biochemistry of the Lux system will add to our already rich understanding of bacterial bioluminescence.

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Relevant Websites

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Bacterial Cell Cycles and Division☆

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Glossary

Cell division genes Genes that encode for proteins that specifically function during the division process.
Constriction, septation Mode of cell envelope invagination during division. During constriction all envelope layers move inwards simultaneously and the daughter cells move gradually apart; septation involves the ingrowth of the cell envelope forming a T-like structure.
Cytokinesis In prokaryotes, the process of cell division. By contrast, in eukaryotic cells division also includes mitosis.
***dcw* cluster** The cluster of genes involved in division (*d*) and cell wall (*cw*) synthesis. In many bacteria this cluster is evolutionary conserved.
Divisome The macromolecular complex that carries out division at the cell center.
Fts proteins Cell division proteins encoded by *fts* genes. In temperature-sensitive (ts) cell division mutants, division is blocked and, because cells continue to grow, filaments (f) are formed.
Minicells Small DNA-less cells that arise through divisions at the poles of rod-shaped bacteria.
Multifork replication More than one round of DNA replication ongoing in one and the same chromosome. It arises when the doubling time of the culture is shorter than the duration of the DNA replication period.
Penicillin binding proteins Proteins involved in peptidoglycan assembly outside the cytoplasmic membrane. They bind specific antibiotics.
Peptidoglycan Covalently closed structure that has the shape of a bacterium. It is composed of glycan chains, which have peptide side chains. Cross-linking through the peptide side chains provides for a strong network.
Potential division sites Cellular sites beyond the nucleoid areas. They occur at cell pole or in the cell center, provided that the nucleoids have segregated.

Introduction

Proliferation of cells, whether pro- or eukaryotic, requires duplication of cytoplasm and genetic material (DNA) and the subsequent distribution of the new genomes over two daughter cells. All molecular components should have been duplicated before fission. Though the above description applies to all cells, some prokaryotes (bacteria) show features that are essentially different from their eukaryotic counterparts. It is important to appreciate these differences because, as will be shown below, they bear on concepts pertaining to the progression of the cell cycle. For instance, is a newborn cell completely new or even is there a cell cycle?

According to the standard view, the eukaryotic cell cycle is punctuated by four sequential periods: the G1 period in which cells grow and prepare for DNA replication, the S-period in which DNA replication takes place, the G2-period where cells prepare for mitosis, and finally, the M-period during which chromosomes become compact, align in the cell center, and segregate producing new daughter cells. The fission of cells is termed cytokinesis. Note that in the eukaryotic field cell division encompasses mitosis and cytokinesis, whereas in prokaryotes cytokinesis is commonly designated as cell division alone. Of the many prokaryotic species, only a few bacteria have been or are being studied in detail. However, cell cycle studies on archaea are strongly emerging. For bacterial cell division, a distinction should be made, though not fundamentally, between Gram-positive species [*Bacillus subtilis*, *Enterococcus hirae* (formerly called *Streptococcus faecalis* and *S. faecium*), *Streptococcus pneumoniae*] and Gram-negative species (*Caulobacter crescentus* and *Escherichia coli*).

To date, *E. coli* has been studied most intensively with respect to the cell cycle, though closely followed by *B. subtilis* and *C. crescentus*. Here we will focus mainly on *E. coli* (Figure 1), because *B. subtilis* and *C. crescentus* have been treated elsewhere (specify). Some interesting features have emerged from the study of archaea, which differentiate them from bacteria and which sheds a broader light on prokaryotes in general.

The *E. coli* cell cycle is marked by three main events: the DNA replication cycle, cellular growth and the division cycle. Increase in cell size in rod-shaped organisms is manifested as cell elongation, which is terminated by fission, that is, the ingrowth of the cell envelope. As envelope growth is a dominant feature of cell growth the focus will be on its shape-maintaining peptidoglycan layer. Below the employed terminology and the temporal relationship between the DNA replication cycle and the division cycle will be described.

☆*Change History:* August 2014. N Nanninga introduced small edits in the text of the article including Figures and Further Reading.

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Figure 1 Electron microscopical image of a thin section of dividing *Escherichia coli*. Cytokinesis involves contriction of the envelope layers (cf. Figure 12). Scale = 0.5 μm. Courtesy of Dr. CL. Woldringh.

The Cell Cycle of *Escherichia coli*

The Cell Cycle Periods of *E. coli* Differ from Those of Eukaryotes

How do the standard eukaryotic cell cycle periods compare with those of a bacterium like *E. coli*? In both cases, the lengths of the periods are affected by growth conditions. In eukaryotes the duration of G1 is directly related to the richness of the growth medium, the other periods being somewhat independent; however, in *E. coli*, the growth conditions specifically affect the position of the DNA replication period within the division cycle. In slowly growing cells, that is, cells growing in a relatively poor medium, an equivalent of a G1-period can be distinguished. This is followed by an S-like period denoted as C. However, a striking difference with eukaryotes can now be observed: in *E. coli* DNA replication and DNA segregation go hand in hand. So in fact, S = M. Consequently, bacteria lack classical mitosis. In *E. coli* the C-period is followed by the D-period, being defined as the period between termination of DNA replication and cell division. During the D-period cells prepare for and carry out cytokinesis. Thus, the G2- and M-periods have no direct counterparts in *E. coli*. As will be shown below archaea are to some extent reminiscent of eukaryotic cells.

The Division Cycle and the DNA Replication Cycle Do Not Coincide

The duration of C is affected by growth conditions. *E. coli* B/r cells that grow with doubling times (Tds) of 20–60 min at 37 °C have a C-period of 40 min and a D-period of 20 min. This length of D appears to be the minimal time span required to build the division apparatus or divisome (see below). Similarly, the shortest duration of C is about 40 min. Thus, under given growth conditions at least 60 min (C + D) have to elapse before fission is completed. Application of this rule for a range of doubling times is graphically presented in Figure 2.

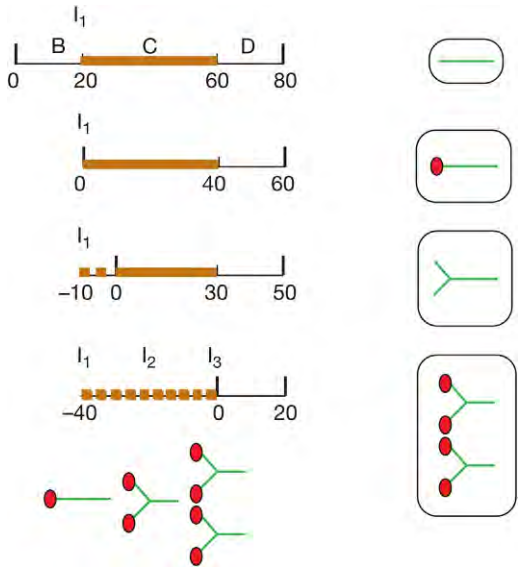

Figure 2 Temporal relationship between DNA replication period (C) and the division cycle in *Escherichia coli* B/r at doubling times (Tds) from 20 to 80 min at 37 °C. B, period between cell birth and initiation of DNA replication. D, period between termination of DNA replication and cell division. The C-period is shown as a brown bar. An interrupted brown bar denotes DNA replication before cell birth at the extant cycle. In this scheme C = 40 min and D = 20 min. I_1, initiation of DNA replication belongs to the extant divison cycle. I_2 and I_3, subsequent initiations of DNA replication. The frequency of initiation equals Td. The green line depicts an unreplicated chromosome. The red dot represents an activated origin of replication. At Td = 50 min, one quarter of the chromosome has been replicated at birth. At Td = 20 min, a newborn cell contains two chromosomes that have been replicated halfway. Initiation of DNA replication follows immediately after birth at the four origins. This happens before the ongoing round of replication has been completed. This results in multifork replication (cf. Figure 3). How one arrives at the chromosome configuration at cell birth is shown below. At Td = 20 min, 20 min are needed for the preparation of division (D-period), which is preceded by C = 40 min. Thus I_1 begins at −40 min as indicated. After 20 min at −20 min the chromosome is replicated halfway. At the same time (after one Td) a new round of DNA replication starts at I_2. One Td later chromosomes have segregated and are at the same time replicated halfway. New initiations start at I_3. Note that cells become bigger as Tds decrease. C and D values apply for *E. coli* B/r grown at 37 °C. For K12 strains they can be different.

Let us start with a Td of 60 min. Since C + D equals Td, DNA replication must start at cell birth. At slower growth rates (Td > 60 min) a period without DNA synthesis after birth is seen, and it becomes longer as Td increases. In the example, the period is 20 min (Td = 80 min; Figure 2). When C + D < Td, for instance, when Td is 50 min the duration of the division cycle is too short to complete DNA replication and division. The problem is solved by DNA replication beginning in the previous cycle. It can be seen that DNA replication has to start 10 min before cell birth (Figure 2). It will be appreciated that this leads to a newborn cell whose chromosome is one quarter replicated. Conceptually, this has an important consequence: a newborn c ell does not start out with a new round of DNA replication.

Multifork Replication

What happens if C ≤ Td? For example, if Td equals 20 min (when C + D = 60 min). To solve this problem DNA replication initiates at cell birth two cycles earlier as indicated (I_1 in Figure 2). A new initiation takes place every 20 min in line with Td. These subsequent initiations have been indicated as I_2 and I_3 in Figure 2. As a result, the newborn cell of the extant cell cycle has its chromosome not only fully replicated (two chromosomes in a newborn cell) it also has been engaged in DNA replication for 20 min. Moreover, a new round of DNA replication starts at birth (I_3 in Figure 2). Thus 5 min after birth, the bacterial chromosome is involved in two DNA replication cycles at one and the same time. This phenomenon has been termed multifork replication (Figure 3).

In eukaryotes multifork replication does not occur, presumably because chromosome compaction during mitosis precludes DNA replication. It is also for this reason that in eukaryotes the DNA replication cycle is contained within the ongoing division cycle. In bacteria, as outlined above, this is not obligatory. The fact that in bacteria the DNA replication cycle and its division cycle do not always coincide implies (as mentioned above) that a newborn bacterial cell is not completely new. Clearly, bacteria can also do without an identifiable G1-period. This has led to the notion that bacteria continuously prepare for the initiation of DNA replication, not only during DNA replication but also during D. It implies that the bacterial G1-period has no specific functional meaning; it simply arises because C + D < Td. It has been argued that the same reasoning would apply to the G1-period in eukaryotes. This common concept for pro- and eukaryotes has been called the continuum model by Cooper. The division cycle can be conceived of as a means for a cell to maintain and enlarge its molecular fabric to allow replication of its genome. Thus, while mass continues to be made, the bacterial chromosome replicates, independently of division. So one may ask, do cells really cycle?

As implied above (Figure 2), cellular DNA content increases with the growth rate. Roughly, there is a relationship between cell size and the number of chromosomes that initiate DNA replication. Thus, dependent on growth conditions there can be a dramatic difference in cell mass and DNA content (Figure 4).

DNA Replication Cycle

DNA Replication Cycle in *Escherichia coli*

In the standard description of the eukaryotic cell cycle the G1-period encompasses protein synthesis including preparation for DNA replication during the S-period (see however, the continuum model above). A separate description of the bacterial equivalent (B-period) does not seem warranted because it often is not distinguishable (dependent on growth conditions; see above).

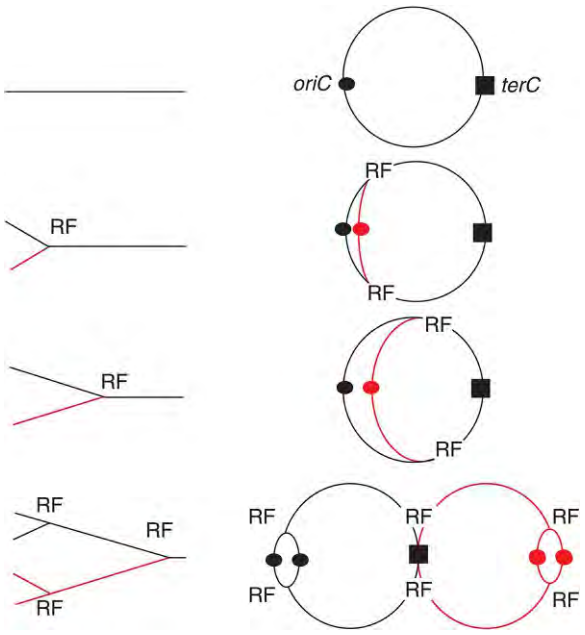

Figure 3 Linear (left, cf. Figure 2) and circular (right) representation of a replicating chromosome. *oriC*, origin of DNA replication; *terC*, terminus of DNA replication. RF, replication fork. The circle shows bidirectional replication. Below, multifork replication is shown.

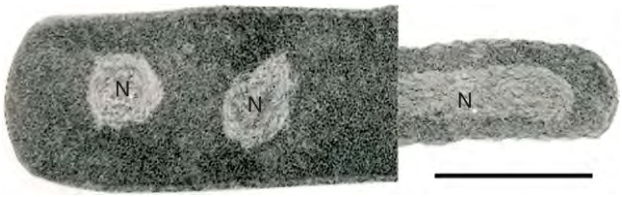

Figure 4 Electron microscopical images of thin sections of two *Escherichia coli* cell halves. The left and right cells have been grown at Td's of 21 and 150 min, respectively. The mean respective genome equivalents are 4.6 and 1.2. The left cell contains four replicating chromosomes at division, the right cell one. Scale = 1 μm. N, nucleoplasm.

It has been early recognized that initiation of DNA replication takes place at a defined cell mass (initiation mass). Of course, the challenge has been to translate this rather vague notion into molecular terms. In *E. coli*, the protein DnaA has been invoked as a positive regulator of initiation of DNA replication. DNA replication in *E. coli* starts at a fixed DNA sequence on the chromosome (*oriC*) and progresses bidirectionally toward the terminus *terC* (Figure 3). *OriC* is a 258-bp sequence that is flanked by other regions required for the onset of DNA replication. Of particular importance are so-called DnaA boxes that recognize the DnaA protein. DnaA becomes active when it binds ATP. When sufficient DnaA-ATP is available for occupation of the DnaA boxes, melting of the DNA double strands of the origin takes place, allowing replication proteins to bind. There are additional DnaA boxes on the chromosome but their function is not known. Conceivably, these boxes titrate away excess DnaA to prevent premature reinitiation of *oriC*. Continued protein synthesis serves to provide new DnaA molecules for a next round of replication.

An additional mechanism has been invoked that prevents premature initiation of DNA replication. This is based on the fact that newly synthesized DNA is hemimethylated in contrast to mature DNA, which is fully methylated. Hemimethylated DNA containing GATC sites becomes sequestered by a protein appropriately called SeqA, thus preventing reinitiation of DNA replication. The duration of the re-replication block is in the order of one third of Td. Presumably, this period in combination with a limited amount of DnaA is sufficient to prevent reinitiation prematurely.

DNA Replication Cycle in Archaea

Bacteria and archaea, though prokaryotes, differ from one another with respect to DNA replication and possibly DNA segregation. This supports the inference that archaea are closer to eukaryotes than to bacteria. It should be emphasized, however, that this similarity is on the level of genes and macromolecules and not on the level of cellular organization. Though a limited number of archaea have been investigated some interesting features have emerged. For instance, in hyperthermophile *Sulfolobus* spp., which belong to the archaeal phylum Crenarchaeota the chromosome contains three different origins. These initiate simultaneously, though termination appears variable in time. This is reminiscent of the multiple origins in a eukaryotic chromosome. In *S. solfataricus* pairing of replicating as well as postreplicating chromatids have been observed. This suggest the presence of genuine G2-period in this organism. Whether chromosome compaction precedes DNA segregation remains to be seen. However, it seems likely that DNA replication and DNA segregation do not run in parallel as in *E. coli*.

Division Cycle

Rod-shaped cells like *E. coli* elongate before they divide. This implies growth of the covalent peptidoglycan layer and insertion of new components in the non-covalent inner and outer membrane. This in turn is followed by division-specific peptidoglycan synthesis.

Peptidoglycan Synthesis During Cell Elongation

The cell envelope of *E. coli*, as in all Gram-negatives, is composed of three layers. From outside to inside these are the outer membrane, the peptidoglycan or murein layer, and the inner- or cytoplasmic membrane. Because of its rigid nature the peptidoglycan-containing cell wall serves as an exoskeleton, which maintains the shape of the cell. Its strength prevents cellular disruption due to osmotic pressure and also plays an active role during the constriction process. Recent research has revealed the occurrence of the actin-like cytoplasmic protein MreB directly underneath the inner membrane. Impairment of MreB leads to spherical cells, suggesting a functional interplay between MreB and peptidoglycan assembly in establishing and maintaining cell shape.

Precursors of building blocks for the peptidoglycan layer are produced stepwise in the cytoplasm. The final cytoplasmic products are lipid I and II, which are made by the enzymes MraY and MurG, respectively. MraY, a translocase, is an integral membrane protein that binds UDP-MurNAc-pentapeptide to undecaprenyl phosphate forming lipid I. MurG, a transferase is tightly associated with the cytoplasmic side of the inner membrane. It adds UDP-GlcNAc to lipid I producing lipid II. An unknown flippase activity transfers the disaccharide moiety of lipid II to the periplasmic side of the inner membrane, where it serves as a substrate for penicillin binding proteins (PBPs). Elongation of the glycan chains of existing peptidoglycan is carried out by a transglycosylase activity and cross-linking of peptide side chains is due to a transpeptidase activity. Some PBPs such as PBP1a and PBP1b are bifunctional and perform

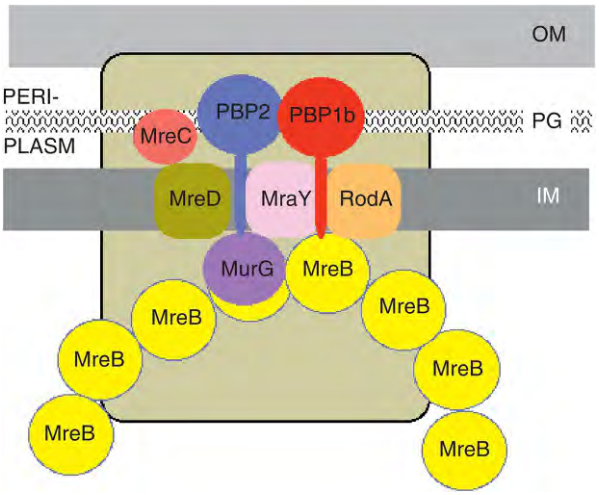

Figure 5 Protein machine (elongasome) involved in cell elongation. It encompasses components in cytoplasm, inner membrane, and nascent peptidoglycan. IM, inner membrane; OM, outer membrane; PG, peptidoglycan layer. The peptidoglycan synthesizing machinery including MurG, MraY, PBP1b and PBP2 is positioned by MreB located underneath the inner membrane. This model integrates data from *Escherichia coli* and *Caulobacter crescentus*.

both activities. PBP2 is monofunctional with only transpeptidase activity. PBP2 is essential for the cells' rod shape, because mutations or binding by the PBP2-specific antibiotic mecillinam turns rods into spheres. Light microscopic fluorescent studies have shown that PBP1b and PBP2 are dispersed along the lateral wall (like MreB), with additional label at the site of constriction (see also below). Another rod-shape determinant is RodA, a transmembrane protein, whose biochemical function is not yet known. Recently, an additional rod-shape determinant, RodZ, has been found. It is a membrane protein with cytoplasmic and periplamic domains. Possibly, connecting peptidoglycan-associated activities across the inner membrane (Figure 5).

Increasing evidence points to the presence of macromolecular complexes that connect cytoplasm and periplasm in order to carry out peptidoglycan synthesis at many site along the cells' length. These complexes seem to be randomly located with the actin-like MreB protein(s) serving as a scaffold (Figure 5). Such an arrangement can explain why a diffuse incorporation of peptidoglycan precursors has been found in earlier studies. Older studies have also shown that outer membrane assembly is a random process along the cell length. Originally, it was thought that MreB polymers are arranged in helical fashion underneath the inner membrane. However, recent evidence indicates that the helical arrangement has been caused by the presence of a fluorescent protein tag at N-terminal end of MreB. Whether this outcome bears on other studies with fluorescent protein tags remains to be seen.

Though we have focused on PBPs with synthetic activities, there are numerous other enzymatic activities that lead to remodeling and recycling of peptidoglycan. In this sense, Figure 5 represents only a first approximation of the *in vivo* situation.

The Divisome and Divisome Subassemblies

Most cell division proteins have been discovered through the phenotypes of temperature sensitive cell division mutants. At the nonpermissive temperature cells grow as filaments, thus revealing defects in the division process. Many cell division proteins have the prefix Fts, meaning filamentation-thermosensitive. Genetic studies have been complemented by microscopic labeling studies, both by electron microscopical immunogold labeling (Figure 6) and fluorescent light microscopy (Figure 7).

Cytokinesis is carried out by a protein machine called the divisome, which contains about 20 different proteins. The majority of them are specific for the divisome, others are also active during cell elongation. These proteins are located in different cellular compartments and are cytoplasmic proteins, integral transmembrane proteins, or periplasmic proteins with a membrane anchor. Recently, a protein complex has been identified (Tol-Pal), which connects the divisome to the *E. coli* outer membrane.

The first protein to arrive at the site of division is the cytoplasmic protein FtsZ (Figure 6). It is a tubulin homologue with GTPase activity. FtsZ occurs in thousands of copies per cell. Potentially, FtsZ forms one or more ring-like polymers underneath the cytoplasmic membrane in the cell center. The exact *in vivo* conformation of the FtsZ polymer(s) is (are) not known. It clearly is a dynamic structure, as demonstrated by photobleaching experiments. The Z-ring must decrease in circumference during the constriction process, without losing its integrity. Whereas FtsZ occurs in thousands of copies in a cell, other proteins like FtsQ (Figure 7) are present in only about 50 copies per cell. Thus, such proteins cannot participate in forming a ring that spans the circumference of the cell. Presumably, FtsQ and other low-copy number proteins are grouped into divisome subassemblies, which decorate extended FtsZ polymers (Figure 8).

Recent investigations have revealed the temporal sequence of divisome biogenesis. The assembly of a functional divisome takes place in two steps. In the first step, the cell division proteins FtsA, ZipA (Z-interacting protein A) and ZapA (Z-associated protein A) bind and stabilize an initial FtsZ-ring. In the later stage, the other cell division proteins are recruited, including FtsK, FtsQ, FtsL, FtsB,

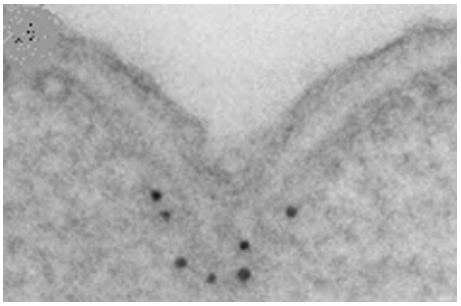

Figure 6 Electron microscopic image of immunogold labeled FtsZ in a thin section. Reproduced from Nanninga N (1998) Morphogenesis of *Escherichia coli*. *Microbiology and Molecular Biology Reviews* 62: 110–129.

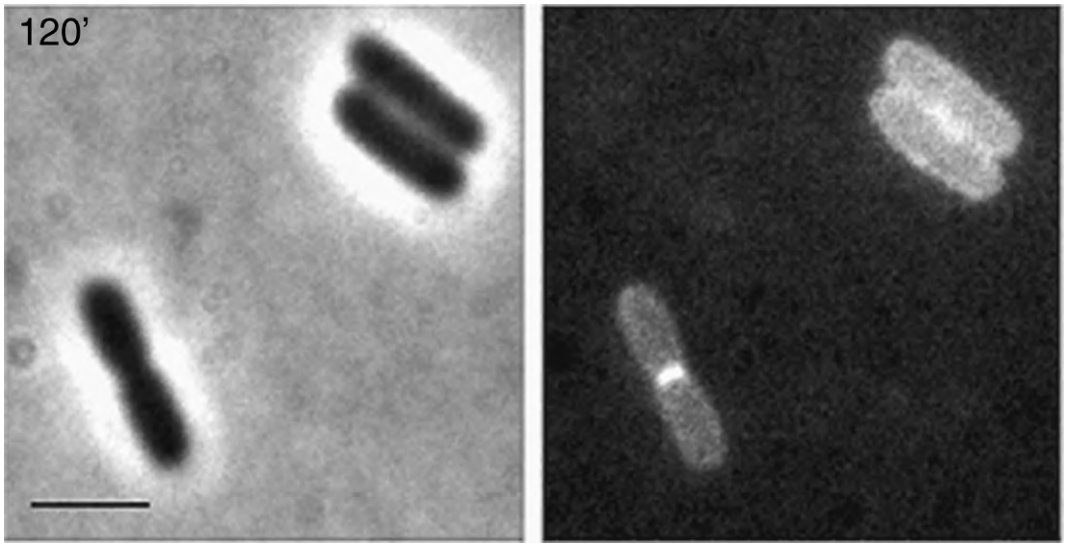

Figure 7 Fluorescent image (right) of FtsQ-GFP labeled live *Escherichia coli*. Left: phase contrast image. See attached film clip GFP-FtsQ. Courtesy of Dr. T den Blaauwen.

FtsW, FtsI (PBP3; penicillin binding protein 3), FtsN, and AmiC. Presumably, a subassembly is composed of these proteins (Figure 9). Remarkably, a complex of FtsB, C and L, can exist outside the divisome. The function of the various proteins is largely unknown. FtsA is a member of the actin superfamily, FtsK has a role in separating chromosomes after termination of DNA replication, FtsI is a transpeptidase, and AmiC an amidase. The latter two proteins emphasize the importance of peptidoglycan synthesis during division.

Potential Division Sites and Site Selection

Although it is customary to think that cells split in the middle, additional potential division sites exist at the cell poles (Figure 10). As depicted here, division takes place at regions that are not near the nucleoid (nucleoid occlusion; see '**Cell Cycle Regulation**'). Interestingly, polar divisions occur in thermosensitive *min* mutants and result in the so-called minicells. These are devoid of DNA, though not of ribosomes, thus can still carry out some residual protein synthesis. The phenomenon of minicell production indicates the existence of a system to prevent polar divisions. It is known as the Min system and includes different proteins. The protein MinC inhibits polymerization of FtsZ at the cell poles. MinC is recruited to the membrane by the ATPase MinD in its ATP-bound form. Binding of MinD to the membrane is released by interaction with MinE, which causes ATP hydrolysis. MinE is specifically active at the cell center, thus preventing inhibition of FtsZ polymerization by MinC. Remarkably, the polar topology of these reactions is achieved by oscillation of MinD from pole to pole. The time course of one oscillation is in the order of one minute. The Min system as described for *E. coli* is not found in *B. subtilis* or *C. crescentus*. Interestingly, Min proteins (and FtsZ) have been found in chloroplasts as well as in some mitochondria, which emphasizes the endosymbiontic origin of the latter organelles.

Though the mechanism of division site selection is not fully understood, regulating elements include the sensing of the cellular position of the nucleoid (see also below) and the inhibition of polar divisions by the Min system.

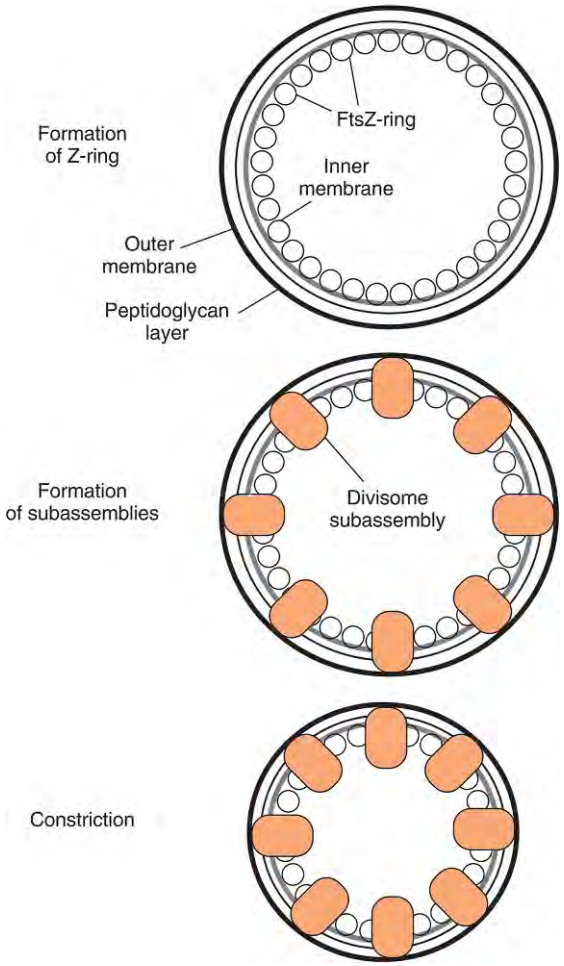

Figure 8 The division process in *Escherichia coli*. First the FtsZ ring with associated proteins (FtsA, ZapA and ZipA; not shown) is formed. Next divisome subassemblies are positioned. During constriction the subassemblies approach each other, whereas FtsZ leaves the ring. Note that the various components have not been drawn to scale.

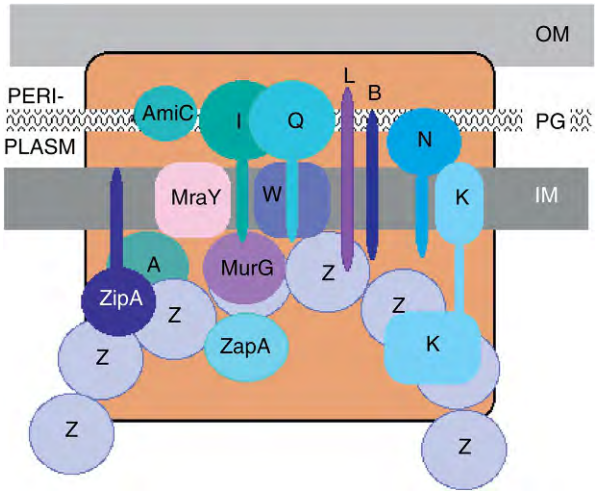

Figure 9 Protein composition of a divisome subassembly. The divisome subassembly spans cytoplasm, inner membrane (IM), peptidoglycan layer (PG), periplasm and outer membrane (OM). It interacts with an FtsZ scaffold, which is reinforced by the cytoplasmic proteins FtsA, ZapA, and ZipA. The latter is anchored to the inner membrane. Specific components of the divisome subassembly are FtsB, FtsI, FtsK, FtsL, FtsN, FtsQ, and FtsW. Most of the proteins have their main domain in the periplasm as depicted. Note that FtsW is membrane-embedded. The cytoplasmic domain of FtsK functions in DNA segregation. Tol-Pal connects the subassembly to the outer membrane. Also present are MraY, MreB, and MurG, which can also be found in the elongasome (cf. Figure 5).

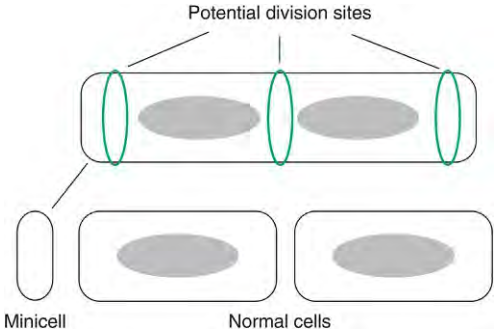

Figure 10 Potential division sites in a rod-shaped cell. Division takes place where there is no physical obstruction of the nucleoid, that is at poles and in the cell center. Normally division only occurs in the cell center. However, when the Min system is impaired a minicell is formed at a pole. Division starts by the positioning of a Z-ring (green) composed of polymerized FtsZ.

Peptidoglycan Synthesis at the Divisome

Constriction is not simply the pulling inwards of the envelope by an FtsZ-ring of a decreasing circumference. The constriction process requires local envelope synthesis at least on the level of the peptidoglycan layer. Early electron microscopic autoradiographic studies have shown that the peptidoglycan precursor ^{3}[H]-*meso*-diaminopimelic acid is especially incorporated at the site of constriction. FtsI (PBP3) is involved in divisome-specific peptidoglycan synthesis. It is the most intensively studied of all cell division proteins. Certain antibiotics, such as cephalexin, furazlocillin and aztreonam, act on this protein specifically. Inhibition of FtsI by these antibiotics or inactivation of FtsI in a thermosensitive *ftsI* mutant at the nonpermissive temperature produces filaments with aborted blunt constrictions. This is in contrast to filaments of thermosensitive *ftsZ* mutants, which have a smooth morphology. Peptidoglycan assembly does not take place at the blunt constrictions. Remarkably, inhibition of FtsI does not prevent peptidoglycan synthesis at new division sites flanking the blunt constrictions (Figure 11). This has been demonstrated by electron microscopic autoradiography and by viewing the dilution of immunogold label attached to -SH groups of D-Cys incorporated into the peptidoglycan layer. It is plausible that initial peptidoglycan synthesis at the division site is carried out by PBP2 and PBP1B as remnants of the cell elongation system. The phenotype of FtsI-impaired cells has led to an early suggestion that FtsI plays a role in a later stage of cell division. It also fits the idea that FtsI becomes recruited to the assembling divisome in a second step (see above).

The localized function of FtsI requires the presence of enzymes, which prepare the substrate prenylated disaccharide peptide (lipid II) for PBPs to act upon in the periplasm. During cell elongation these enzymes are MraY and MurG. Most likely these proteins are also present in the divisome (Figure 9). An interaction between FtsZ and MreB has recently been described.

A Gram-negative organism like *E. coli*, as outlined above, divides by constriction in the cell center (Figure 12). In a Gram-positive like *B. subtilis*, a circumferential inward-growing septum divides the new daughter cells, whereas in the latter, the cell constricts in the cell center (Figure 12). *B. subtilis* lacks an outer membrane (which affects the Gram-stain) and has a thick cell wall composed of peptidoglycan and teichoic acid as the main components. Yet, the arrangement of genes involved in peptidoglycan assembly and cell division on the respective chromosomes reveals similarity (see below).

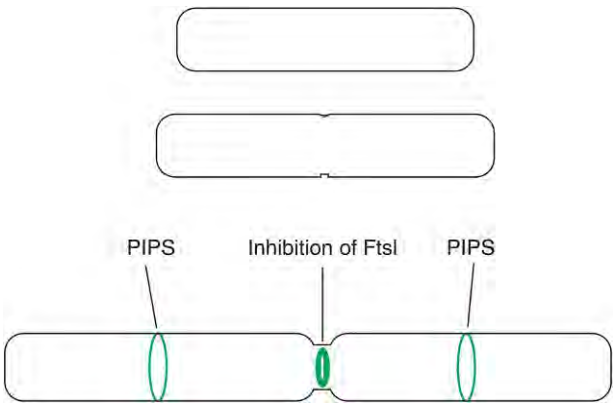

Figure 11 Inactivation of FtsI (PBP3) halts constriction and produces blunt constrictions. Incipient division remains possible at future division sites flanking the aborted constriction. FtsZ rings (green) form and PBP3-independent peptidoglycan synthesis (PIPS) proceeds.

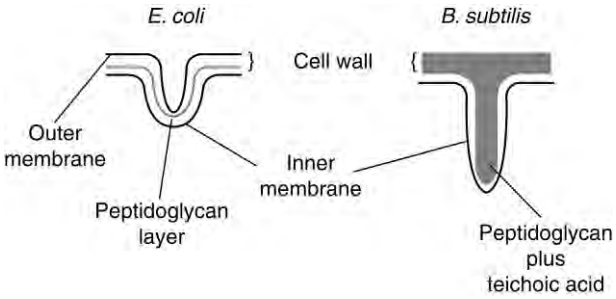

Figure 12 Division by constriction in *E. coli* and by septation in *B. subtilis*. During the constriction process the envelope layers invaginate together; during septation a T-structure is formed.

Figure 13 Genes involved in cell divison (*d*) and cell wall (*cw*) synthesis are grouped together (*dcw* cluster) in a wide range of bacteria. In *Escherichia coli* they are located at the 2-min region of the chromosome. Genes encoding for cell division proteins are shown in red; those involved in cell wall synthesis in green. Reproduced from Mingorance J, Tamames J, and Vicente M (2004) Genomic chanelling in bacterial cell division. *Journal of Molecular Recognition* 17: 481–487.

Organization of Cell Division Genes on the Chromosome

The *E. coli* chromosomal map is divided into 100 min. In this organism most cell division genes are located at 2 min. Remarkably, many genes encoding enzymes involved in the formation of peptidoglycan precursors and in peptidoglycan assembly are also situated in this region. The 2-min region is therefore also denoted as the *dcw* cluster, where *d* stands for division and *cw* for cell wall (Figure 13). This clustering suggests the existence of a global regulatory mechanism that coordinates expression of the many genes and which directs the transformation of elongation-specific to division-specific peptidoglycan synthesis. To date, little is known about division-specific gene expression. Recent genomic sequences studies have shown that the *dcw* cluster is conserved amongst bacteria. Note that archaea have no peptidoglycan and consequently, no PBPs.

Cell Cycle Regulation

The tight coordination between DNA replication and cell division requires that cell division takes place at the right time and at the right place. In the *E. coli* cell cycle the frequency of initiation of DNA replication equals Td. If $C+D<Td$ newborn cells contain a replicating chromosome, which exemplifies the fact that a newborn cell is not completely new. The fastest division cycle in *E. coli* in rich medium at 37 °C is about 20 min, which is the same as the minimal D–period. Consequently, cells growing with a Td of 20 min are continuously involved in cell division. As outlined above such cells initiate DNA replication every 20 min, which makes multifork replication ($C>Td$) a must. Cells have to 'know' when to initiate DNA replication and when to start cell division. As already indicated above this has to do, at least in part, with the accumulation of the Dna A protein.

Potential division sites (Figure 10) exist where there is no spatial obstruction by the nucleoid. This implies that the divisome is assembled after nucleoids have segregated upon completion of DNA replication. Thus, a regulating aspect seems to reside in the cells' perception of the location of its nucleoid, thus prohibiting the cutting of its DNA by the constricting envelope. This has become known as the nucleoid occlusion model. So far little is known about the sensing mechanism that links termination of DNA replication (and segregation) with initiation of division. However, recently DNA binding proteins (Noc and SlmA), which seem to interfere with divisome assembly have been detected in *B. subtilis* and *E. coli*, respectively. It is intriguing that cells are capable to adjust for DNA damage via the so-called SOS response, that is, cell division is postponed.

Progression of the cell cycle, as outlined above, is accompanied by segregation of replicating DNA, and the cellular redistribution of proteins involved in envelope synthesis. It includes the organized dynamic positioning of gene products in the growing cell. The combination of molecular genetics and fluorescence microscopy has contributed immensely to these insights. Yet, the underlying mechanism guiding growth of the individual cell remains to be elucidated.

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Bacterial Chemotaxis: Conservation and Variation on a Theme☆

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Many bacterial species move through their environment. Their generally small size means they are unable to sense a gradient in space, but must sense it in time, comparing current conditions with those a few seconds ago. They then bias their random pattern of movement towards an improving environment (Fig. 1A). Remarkably, most species can respond to a change of around 1% in the concentration of a compound over a background of 5–7 orders of magnitude. This mechanism of responding to changes requires sensing, signal amplification, response, signal termination, and receptor adaptation, plus a memory of past concentrations. The advantages of being chemotactic are clearly seen in the simple experiments carried out by Julius Adler in the 1960s. *Escherichia coli* inoculated onto the middle on a soft, low-nutrient agar plate will metabolize the nutrients, creating a gradient, and then move down that gradient, filling the petri-dish with rings of growing cells after overnight incubation. However, if the cells are motile, but not chemotactic, they will spread a little as they randomly swim, but not be able to bias that movement towards an optimum environment. If non-motile, the colony will stay where it was inoculated with limited growth. Mutagenesis showed that the rings form as the colony spread down the concentration gradient because *E. coli* has four types of chemoreceptor, with each ring on the petri dish being a population of cells following a specific chemical gradient sensed by one of those receptors. Using transport mutants it was also shown that the chemoreceptors were not involved in either transport or metabolism, but connected to a dedicated sensory system. As we will discover later, it can be much more complex in other species.

Despite the different mechanisms used by bacteria to move: swimming, gliding, swarming, twitching in bacteria, and even the very different swimming of Archaea, all prokaryotes use a variation on the same histidine protein kinase system to control movement in response to chemical changes.

Core Components

E. coli is, of course, the best studied of all chemotaxis systems in bacteria and the core pathway identified in this species applies to most other species (Fig. 2A). Indeed it was fortuitous that this species was chosen by Julius Adler for chemosensory studies, as we now know that it has one of the simplest chemosensory systems, and therefore allowed the basic principles to be set in place in the 1960s and 70s. A decade earlier, Rod Clayton had investigated chemosensory responses in *Rhodospirillum rubrum* and despite heroic efforts and some exceptional papers, he changed fields after obtaining results which were really too complex to interpret in the days before molecular genetics and live cell imaging. *R. rubrum* chemosensing is exceptionally complex when compared with *E. coli*

(a) *Chemoreceptors*. Bacteria must sense a change in concentration of a chemoeffector, usually a metabolite or a toxin. It was clear from early research that *E. coli* and its relative *Salmonella enterica* have different, but related, 55 kDa chemoreceptors named Methyl-accepting Chemotaxis Proteins (MCPs). Each of the four different MCPs (Tsr, primarily sensing serine and Tar sensing aspartate and maltose are high copy number while Trg sensing galactose and Tap, dipeptides are low copy number) of *E. coli* form transmembrane dimers, inserted into the cytoplasmic membrane and operating as trimers of dimers (Fig. 1B). Each monomer is a very long protein, around 30 nm, consisting of a periplasmic domain, which interacts with the chemoeffector, two transmembrane domains, and a long coiled-coil cytoplasmic domain. The periplasmic binding domain consist of a four-helix bundle with an effector-binding site on each. Depending on the MCP, the effector might be an amino acid or it could be a large sugar-binding periplasmic-binding-protein, with affinity for both the MCP and the transporter. Binding to one of the periplasmic monomer domains reduces the affinity for the site on the second monomer. In the case of Tar, binding of the attractant aspartate causes a small piston movement in the protein which is transmitted across the transmembrane domain. For other receptors, for example Tsr, binding of serine induces a helix-clutch mechanism to signal across the membrane. In each case these structural changes result in the same overall outcome, with a change in activity of the CheA kinase associated with the cytoplasmic tip of the MCP.

This change in structure in the transmembrane domains has to be communicated, through a long coiled-coil region over 20 nm long to the CheA kinase bound to the distant tip. Just below the membrane the MCPs contain a HAMP domain which is involved in transforming this transmembrane movement into that distant structural change. In *E. coli* each MCP monomer also has four conserved glutamate residues in the coiled-coil domain, two encoded as glutamines. These are the sites of adaptation (see later).

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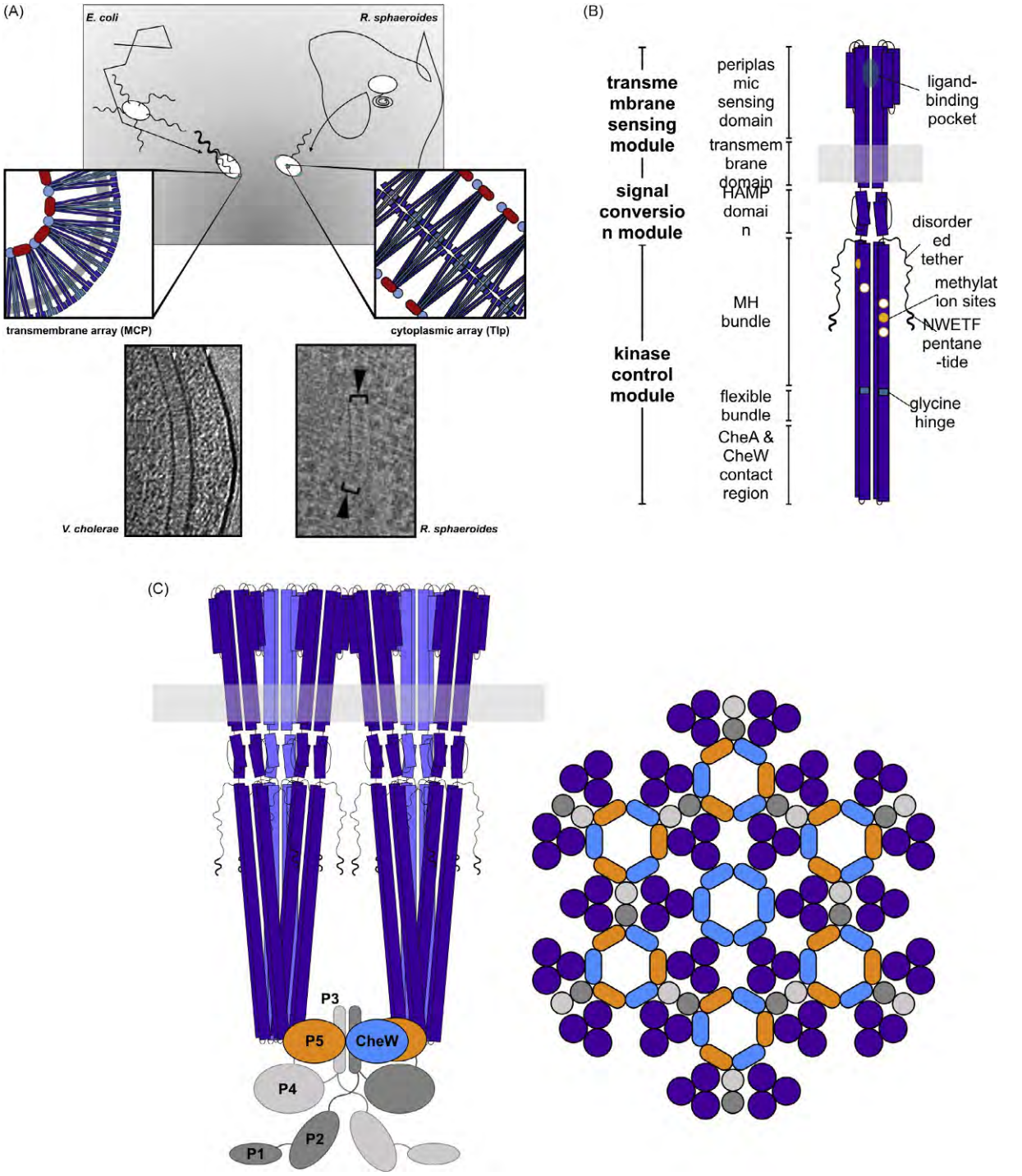

Fig. 1 Shows the biased random walk of *E. coli* and *R. sphaeroides* and the organization of the chemosensory clusters and chemoreceptor structure. (A) *Top panel* shows the swim and tumble pattern of *E. coli* and the run and stop pattern of *R. sphaeroides*. Expanded cartoons show the arrangement of the chemosensory proteins in large membrane-associated and cytoplasmic clusters. *Dark blue* structures are chemoreceptors, *red* are CheA kinases and light blue CheW. Lower images are cryo-electron micrographs of the membrane and cytoplasmic clusters. (B) Shows the structure of an *E. coli* chemoreceptor dimer and (C) shows how homodimers forming two heterotrimers of dimers interacting with the CheA, shown as domains P1-P5, and CheW. These form large hexagonal arrays shown from the cytoplasmic side of cell. Dark *blue* circles are the dimers, *orange* and *gray* the CheA domains and light *blue* CheW.

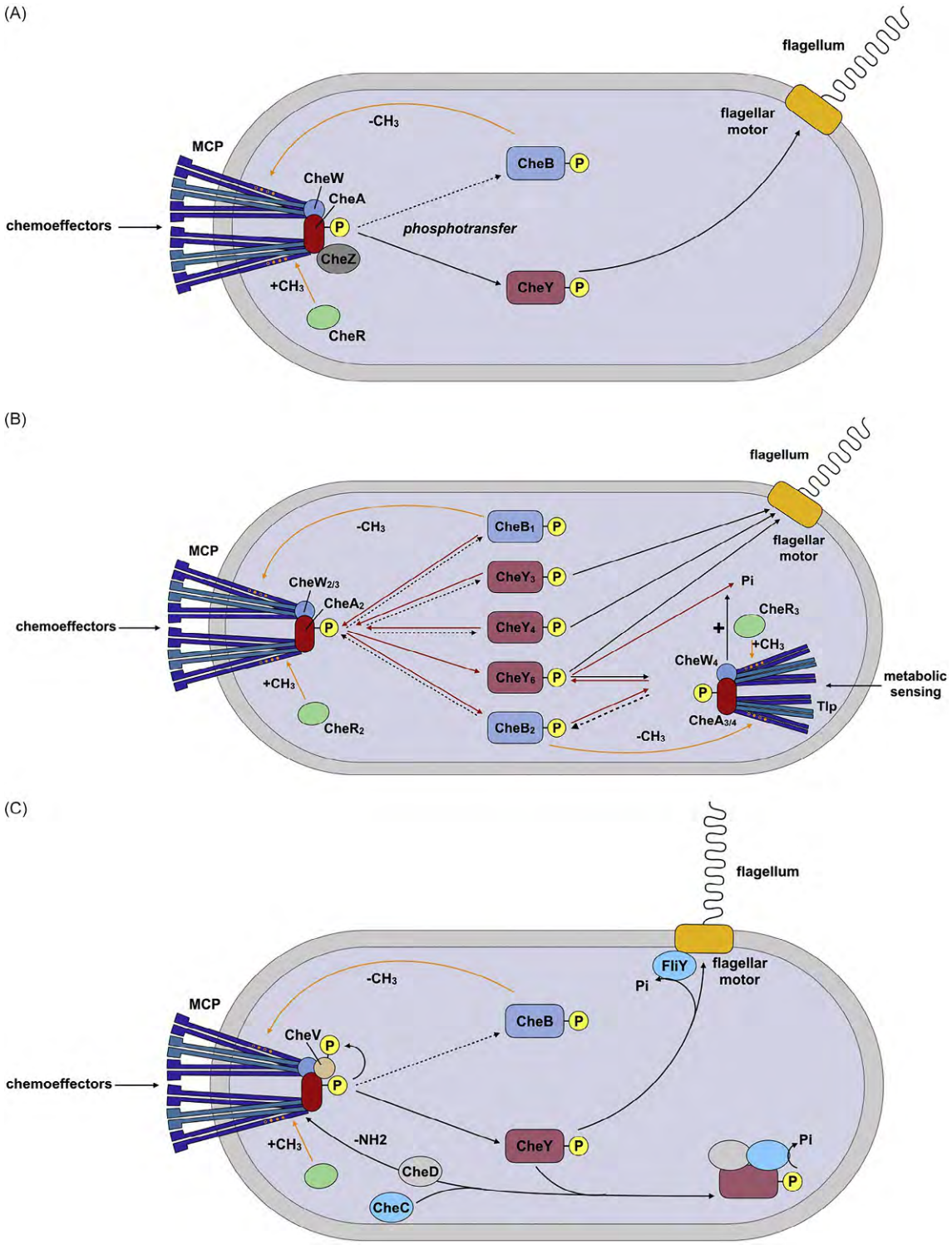

Fig. 2 Shows the chemosensory pathways of (A) *E. coli*, (B) *R. sphaeroides*, and (C) *Bacillus subtilis*. Details of the pathways can be found in the text.

Chemoeffectors bind to specific sites between the periplasmic domains of the dimers and cause one of the monomers' transmembrane domain to move or twist. This movement is transmitted through the cytoplasmic domain to the tip. Analyses of isolated monomers, dimers and trimers of dimers in nanodiscs show that while signaling requires at least a dimer to activate the kinase, but two trimers of dimers are required for optimal signaling. While the dimers are homomeric, the trimers of dimers can be composed of different MCPs, even though their transmembrane signaling mechanism might be different. All four MCPs and the oxygen sensor Aer have identical contact residues at the receptor tip enabling interaction with the signaling proteins.

(b) *Receptor networks.* Immunogold electron microscopy and fluorescent labelling of receptors and associated proteins showed that receptors tend to form large clusters, often in specific cellular sites, such as the poles or, late in the cell cycle, at midcell. Cryo-electron tomography revealed that these are not clusters of free trimers of dimers, but massive interconnected networks of hundreds to thousands of receptor trimers forming 12 nm hexagonal repeats connected by CheA and CheW proteins linking across trimers (Fig. 1C). It is thought that these massive protein complexes allow the sensitivity of the chemosensory system (see later).

(c) *Signaling* two proteins are directly associated with the MCP tips: CheW, a small protein structurally very similar to the conserved tip domain, often called a linker protein as its precise role is unclear, and CheA, an unusual Histidine Protein Kinase (HPK). CheA is a soluble HPK which, unlike other HPKs, does not have an integral sensing domain, but instead responds to signals received by the associated MCPs, causing a two state transition between kinase-on and kinase-off. It has the conserved His site (P1) of an HPK separated from the ATP binding site (P4) by an additional, P2, domain. Two response regulator (RR) proteins compete for binding to the P2 domain. One, CheY, is a 15 kDa small, diffusible, single domain signaling protein and the other, CheB, has an adaptation methyl esterase enzyme domain linked to the RR domain. CheA functions as a homodimer. On the LOSS of an attractant, ATP binds to the Walker box on the P4 domain of CheA and a phosphoryl residue is transferred to the conserved His on the P1 domain of the partner monomer. The phosphoryl is then taken by the conserved Asp of one of the two RRs that might be bound to the P2 domain. Once phosphorylated the RR is released and, in the case of CheY$\sim$P diffuses to the flagellar motor where it binds to a FliM protein in the rotor C-ring to induce a switch in rotational direction of the motor and thus the cell to tumble. Calculations suggest that the binding or release of a single chemoeffector can alter the On-Off state of around 35 CheA kinases in the array and associated with the activated receptor, allowing for signal amplification. When CheB is phosphorylated its esterase activity is increased by up to 100-fold enabling it to remove methyl groups from the conserved glutamates on the MCPs and reset the signaling state of CheA and thus bring about adaptation.

(d) *CheY signaling and signal termination.* CheY is one of the most studied RR proteins, as it is an easily crystallized, single domain RR protein. It has a classical RR fold, with five alpha helices surrounding a central five-stranded beta sheet, and an acidic pocket containing the phosphorylatable Asp. On phosphorylation of Asp37, the affinity for the P2 domain of CheA is decreased and the affinity for the FliM protein of the flagellar motor increased. Several hundred to thousand CheY$\sim$P molecules are therefore released from the chemosensory clusters and diffuse, in around 100 ms, to the motors. There are around 34 FliM proteins per motor and around six flagella per *E. coli*, depending on growth phase. The actual mechanism of switching rotational direction in a motor where the driving force (the ion motive force) is always inward remains unclear, but it is thought that CheY$\sim$P binding drives a cooperative change in the ring of FliM molecules and, after a threshold is reached, this conformational change is transferred to the rotor protein proper, FliG. The resulting alteration in charge interface with the ion transporting stator proteins induces a switch in rotational direction. The structural changes that occur in CheY when phosphorylated are also unclear, as CheY$\sim$P is unstable. Structural studies of the phosphorylated form rely on mimicking the phosphorylated state, either with beryllium fluoride or by mutations thought to lock the protein in the active conformation.

To be able to continue to respond to a changing environment, the signal must be terminated, that is, CheY$\sim$P must dephosphorylate. RRs do spontaneously dephosphorylate at a rate determined by the protein structure. Although CheY$\sim$P spontaneously dephosphorylates with a half time of about 14 s, that is too slow for bacterial chemotaxis. The size of bacteria means that in liquid they are constantly being buffeted by the environment and only swim smoothly for a few seconds. The CheY$\sim$P signal must therefore be "switched off" within a second or so. In *E. coli* this is achieved by a protein CheZ localized close to the sensory cluster, which binds CheY$\sim$P and accelerates the rate of dephosphorylation a 100-fold, switching off the tumbling signal. CheZ turns out to be confined to the gamma proteobacteria and other species have evolved very different mechanisms for rapid signal termination (see below).

(e) *Adaptation* not only must the CheY$\sim$P signal be terminated, the chemoreceptors must also adapt, allowing the cell to sense and respond to future changes against a new background concentration. This is achieved by the addition and removal of methyl groups to the four conserved glutamate residues at specific sites on the long coiled-coil cytoplasmic domain of the MCPs. These sites are modified in any order and the extent of modification depends of the signal strength and the steady state background chemoeffector concentration. Given the thousands of receptors that might form part of a cluster, each with four adaptation sites, the number of adaptation states is extremely large. Adaptation was initially characterized when it was identified that methionine, and specifically *S*-adenosyl methionine (SAM), is required for adaptation. When tritiated SAM was added to *E. coli* and an attractant added or removed, four radiolabelled bands were seen on SDS PAGE gels at apparently different molecular weights. The weights increased by 1 kDa or so on addition of attractant and reduced on removal, reflecting the addition and removal of methyl groups to and from the glutamates. Each MCP has four conserved glutamate residues (Fig. 1B). Two are encoded as glutamines, ensuring that new MCPs are in a neutral signaling state when expressed and inserted into the membrane. The methylesterase is also a specific deamidase, and deamidates the two glutamines, presumably after the MCPs are inserted into the membrane.

To allow adaptation to occur, CheB works in concert with a methyltransferase, CheR. This is constitutively active and tends to localize to a specific sequence at the C-terminus of the two most abundant MCPs; in *E. coli*, Tsr (a serine responsive receptor) and Tar (an aspartate and maltose receptor). The two low abundance receptors, Trg (ribose and galactose receptor) and Tap (dipeptide) lack the CheR binding domain but still have the conserved glutamates. The mixed trimers of dimers ensures a methyl transferase on the

high abundance receptors can interact with the low abundance receptor if it is activated. If an attractant binds, the activity of CheA is reduced, resulting in a reduction in CheB∼P and therefore CheR can add methyl groups to exposed glutamates, neutralizing the charge. This serves to alter the cluster packing and to reset the structural state of the receptor and consequently the signaling state of CheA. Removal of the attractant, or addition of a repellent, causes CheA autophosphorylation and release of CheY∼P and CheB∼P. The esterase is now more active and removes methyls from the glutamates, releasing them as methanol, and resetting the signaling state of CheA and the receptor. The activation time of CheB and subsequent adaptation time is longer than the time taken to terminate the CheY∼P signal, resulting in a memory of the recent change. In addition the sequential resetting of the responding receptors allows the cluster state to act as a memory of the past environment.

(f) *Sensitivity, gain and adaptation* experiments measuring the responses to different signal strengths of mutants unable to adapt, but expressing single receptors locked into different methylation states revealed that the sensitivity range of the receptors is remarkable, with a Hill coefficient of over 11. Recently cryo-electron microscopy has shown structural changes in regions of the chemoreceptor array, suggesting that signaling and adaptation may occur within neighborhoods of receptors. This has led to a large number of papers developing models to explain this exceptional sensitivity of the bacterial chemosensory system.

(g) *CheV*. *E. coli* and *S. enterica* encode homologous chemosensory pathways, except for one additional protein, CheV, found in *S. enterica*, but not *E. coli*. CheV resembles a CheW fused to a CheY-like RR domain and in some enteric species CheV is thought to act as an adaptor protein, linking chemoreceptors to CheA. It may have additional roles in other species as it is found in ∼60% of genomes sequenced from motile species (see later). Genome analysis shows that species with few different chemoreceptors (on average five) tend to lack CheV, while those with more (on average 23) encode CheV. *S. enterica* has four more chemoreceptors than *E. coli*. This has led to the idea that CheV is a preferential adaptor allowing the clusters to accommodate certain chemoreceptors that cannot be accommodated into the cluster by CheW and exemplified by the *S. enterica* McpC chemoreceptor.

Numbers of Chemosensory Pathways and When is a Chemotaxis Pathway Not a Chemotaxis Pathway?

Given the conservation of the above core pathway over the many millions of years of evolution, it is unsurprising that different species have evolved modifications to this chemosensory pathway for survival in their own niches, some of which may be much more complex and varied than that of *E. coli*. There appears to have been both gene duplication and horizontal gene transfer, sometimes of whole pathways, tuning each species for its niche.

The advances in genome sequencing, combined with methods for looking at proteins within living cells has revealed that things are much more complex than the *E. coli* system in the majority of bacterial species. It was originally assumed that there were only four MCP types in *E. coli* because this conserved membrane usage and a change in just one or two amino acids or sugars probably reflected a more general changing environment. However, we now know that the majority of bacterial species have around 20 different chemoreceptors, with some having over 50. These are not only found in the membrane, but many species have both membrane spanning and soluble chemoreceptors, suggesting intracellular as well as periplasmic sensing. There are also chemoreceptors with different numbers of heptad repeats in the cytoplasmic coiled-coil domain, leading to different length cytoplasmic domains. Different length cytoplasmic domains might not readily form mixed trimers or dimers or pack into arrays, suggesting patches of receptors with different organizations.

Not only are there more chemoreceptors, presumably sensing a much wider range of chemoeffectors than expected, but many species have multiple chemosensory pathways. Chemosensory pathways are identified from genome sequences by genes encoding receptors, CheY, CheA, and CheB and CheR, the latter almost always being encoded together. It now seems probable that not all pathways identified through genome sequence analysis actually encode chemosensory proteins. For example, *Pseudomonas aeruginosa* has three putative pathways, but one (Wsp) is certainly involved in EPS production and biofilm formation, and of the eight putative pathways identified in *Myxococcus xanthus* only three seem to control motile behaviour. The pathway Che1 controls chemosensory reversals of single cells of this gliding bacterium using a mechanism similar to *E. coli* chemotaxis, but employing soluble chemoreceptors, while Che4 and Che7 appear to be involved in social motility and fruiting body development, respectively.

Rhodobacter sphaeroides encodes three putative pathways and there is evidence that all of these are involved in motility. *R. sphaeroides* has two different flagellar systems. Analysis suggests one flagellar system, forming polar tufts, was the original flagellar system controlled by one of the chemotaxis pathways (CheOp1), but neither this flagellar system nor its chemotaxis pathway are expressed under normal laboratory conditions. The other two pathways control the other single, randomly positioned flagellum. One pathway localizes to the cell poles with transmembrane chemoreceptors, as seen in *E. coli*, while the other localizes to midcell (in a newly divided cell) with a cluster of soluble chemoreceptors. The CheA associated with the cytoplasmic cluster is split into two proteins, with the kinase domain (CheA4) separate from the P1 histidine domain (CheA3). CheA3 controls the phosphorylation state of the dominant CheY, CheY6, through phosphorylation via CheA4 and dephosphorylation through a domain of CheA3. CheA3 and CheA4 have different P5 receptor-binding domains, suggesting different input signals. This would allow phosphorylation via CheA4 to P1 of CheA3 and dephosphorylation via the phosphatase domain to be independently controlled, and to tune the level of CheY6∼P in the cell in response to metabolic signals. It seems probable that the dominant chemosensory pathway is the cytoplasmic pathway, controlling a background level of CheY6∼P, and only if the level is below a threshold do the CheY3∼P or CheY4∼P proteins released from the membrane chemoreceptors in response to extracellular stimuli cause a change in behaviour.

By tuning the level of the motor-stopping CheY6∼P to the metabolic state, the strength of the response to changes in the external conditions will depend on the metabolic needs of the cell (Fig. 2B). Interestingly, *R. sphaeroides* only responds to the removal of chemoeffectors, not their addition.

Different Methods of Termination and Adaptation

It is clear that the CheY∼P signal needs to be terminated, but CheZ is only found in the gamma proteobacteria. Other groups have different termination mechanisms. *R. sphaeroides* has a large internal domain, between the CheA3P1 and P5 domains, that acts as a specific phosphatase for CheY6∼P, but not CheY3∼P or CheY4∼P. CheY3∼P and CheY4∼P may themselves use a different mechanism to terminate. In many related alpha proteobacteria, one CheY acts as a phosphate sink. In *Rhizobium meliloti*, one CheY is phosphorylated and can bind the motor, but can also transfer phosphoryl groups back to the CheA, and the second CheY, which does not bind the motor, spontaneously dephosphorylates, acting as a phosphate sink. The flux of phophoryls thus regulates signal strength and rate of termination.

Similarly, while adaptation is a key component essential for chemotaxis, and most species encode CheB and CheR, almost always in the same operon, most do not have a CheR-binding domain in the MCPs. Although the adaptation glutamates in *E. coli* are readily identifiable from their positions relative to each other on the receptors, they are harder to identify in other species. Glutamates suggested to be involved in adaptation on the cytoplasmic chemoreceptor TlpT of *R. sphaeroides* using bioinformatic methods proved to not to be involved, but mass spectroscopy of peptides isolated from receptors stimulated in backgrounds lacking either CheR3 or CheB2 identified four methylatable glutamates in different positions on the receptors to those of *E. coli* MCPs. As with *B. subtilis*, mutating these sites produced populations which did not adapt, but with each site producing a different swimming phenotype when mutated (unpublished results, this lab).

Variation in Signaling

Bacillus subtilis has many of the same chemosensory proteins as *E. coli*, but has a number of additional proteins, and many of the signaling mechanisms are the reverse of those of *E. coli*. *B. subtilis* has a CheA and a CheY, but in this case the addition of an attractant activates the kinase and increases CheY∼P levels resulting in continued smooth motor rotation, rather than motor reversals (Fig. 2C).

Adaptation is also more complex. Unlike *E. coli*, where methylation/demethylation of the MCP glutamates does not occur in any specific order, in *B. subtilis* the methylation sites are regulated and the methylation of specific glutamates has different effects on behaviour. For example, methylation of an MCP at position 371 will activate the kinase, while methylation of the glutamate at position 630 will inhibit the kinase, suggesting much more complex structural changes in different regions within the receptor cluster causing different kinase outputs. In addition to receptor methylation, *B. subtilis* has two other adaptation systems, CheC/CheD/CheY∼P and CheV. CheD tunes kinase activity relative to the adaptation state of the receptor while the activity of CheV depends on whether or not position 630 is methylated, allowing this species to very finely tune its behaviour to current conditions.

Conservation of Cluster Geometry and Role in Signaling

One of the surprising recent discoveries is that the cytoplasmic clusters found in species as diverse as *R. sphaeroides* and *Vibrio cholerae* are arranged in exactly the same pattern as each other and the cytoplasmic receptors and kinases are arranged in the same hexagonal arrangement as that seen in the membrane chemoreceptor clusters. The major difference is that the cytoplasmic receptor arrangement is organized as a sandwich of receptors with the equivalent of the MCP periplasmic domains overlapping slightly in the middle of the sandwich. The CheW and CheA kinases then form on the outer surface of the sandwich (Fig. 1). This suggests that for chemosensory signaling, sensitivity and gain, the array provides the optimum, critical geometric arrangement.

Positioning of Clusters

It was originally thought that the receptor clusters of *E. coli* were at the poles to allow the lateral membrane to be used for other functions. This now seems unlikely, given the numbers of receptors and sizes of the clusters. The clusters may form by diffusion-capture: as new receptors are inserted into the membrane they diffuse and either are "captured" by a forming cluster or, if too far, nucleate their own cluster. They may end up at the poles because the shape of the trimer of dimers slows diffusion from the more highly curved poles. This is certainly the case for the membrane clusters of *R. sphaeroides* where clusters of 800 or so receptors diffuse freely in sphaeroplasts, but are mainly at the poles of normal cells.

The positioning of the cytoplasmic clusters is more complex. The receptors of polarly flagellate species such as *V. cholerae* and *V. parahaemolyticus* are actively positioned through a protein targeted to the cell pole, HupP. This protein, which may anchor to the peptidoglycan, recruits ParA-like ATPases, required to recruit the *OriC* of the major chromosome, the polar flagellum and the CheII

chemotaxis proteins controlling flagellar activity. In the case of the Che cluster the ATPase is ParC, which forms a cluster at the flagellate pole, recruiting ParP, which has a CheW domain and interacts with CheA, helping form a complete chemosensory cluster. Similar systems may operate in species such as *Shewanella*.

In *M. xanthus* the cytoplasmic FrzCD chemoreceptors (Che1) localize as multiple clusters along the nucleoid, interacting directly with the nucleoid through their N-termini and probably being stochastically positioned. The FrzCD chemoreceptors recruit the partner chemotaxis proteins to form the functional cluster arrays. It is thought that nucleoid-dependent formation of multiple clusters ensures inheritance of functioning chemotaxis systems on division. In the much smaller *R. sphaeroides* there is only one cytoplasmic cluster after division. As the cell grows this cluster splits and one part moves to each of the daughter cells. Without a ParA-like protein, PpfA, the cluster forms, but is not associated with the nucleoid, does not divide and only one daughter inherits a cluster. PpfA localizes to the nucleoid surface and interacts with the N-terminal domain of TlpT, the dominant cytoplasmic chemoreceptor. TlpT is required for the formation of a chemosensory cluster, but requires PpfA to piggy-back on the chromosome surface and divide and segregate as the cell grows and divides. Unlike plasmid inheritance, which uses a related system, the process for segregating the chemosensory cluster using chromosome segregation appears passive rather than active. TlpT binds PpfA and nucleates a chemosensory cluster. The cluster splits when the chromosome divides with one cluster going with each chromosome. There are no specific binding domains and PpfA does not seem to actively move the cluster.

Interactions With Other Pathways

Chemotaxis does not operate in isolation of other sensing systems or the growth state of the cell. Although membrane spanning chemoreceptors do not transport chemoeffectors and the chemoeffectors do not need to be metabolized, it seems that the strength of the response to different amino acids by *E. coli* does depend on its suitability as a nutrient. However *B. subtilis* responds equally to all amino acids, suggesting they simply reflect an increase or decrease in overall nutrient levels.

Oxygen levels in the atmosphere have obviously changed greatly during the evolution of bacteria. Reflecting the very different lifestyles of different species, a wide range of mechanisms for sensing oxygen have evolved, from cytoplasmic haem-containing receptors in the strictly anaerobic *Desulfovibrio* to the well-studied FAD Aer receptor in *E. coli*. In *E. coli*, Aer is a membrane anchored protein with a PAS domain and a bound FAD below the membrane followed by the conserved coiled-coil of an MCP, but without adaptation sites. The redox state of FAD changes with the rate of electron transfer through the respiratory electron transport chain, which alters the redox state of the quinones. This feeds through to CheA and regulates the activity of CheY. In the soil alpha proteobacterium *Azospirillum*, which prefers a microaerophilic environment, the response to oxygen is closely linked to cyclic di-GMP levels. Cyclic di-GMP is a molecule identified across a wide range of species as regulating the switch between a free living state and biofilm formation. In *Azospirillum brasilense*, *c*-di-GMP binds to the oxygen chemoreceptor, Tlp1, and tunes the strength of the response to oxygen. As the intracellular levels of *c*-di-GMP change with the level of available carbon, this directly links the response to oxygen to the current metabolic state of the cell.

Oxygen sensing also influences the behaviour of magnetotactic bacteria. Many species of bacteria show responses to the Earth's magnetic field. They are able to synthesis intracellular magnetite, which is organized into magnetosomes along the length of the cell body, giving the cells a magnetic dipole. The majority of identified species are microaerophiles living in estuarine mud and show negative aerotaxis. It is thought that the magnetosomes orient the bacteria along the Earth's field lines then negative aerotaxis rapidy returns the bacteria to an optimum oxygen level. In *Magnetospirillum gryphiswaldense*, the magnetotactic direction of swimming polarity can be changed by a change in oxygen gradient sensed through one of its four chemosensory operons (CheOp1), suggesting a direct link between aerotaxis and magnetotaxis.

While most investigations into *E. coli* behaviour have concentrated on the MCPs, which only require binding to the periplasmic domain of the chemoreceptor, it has been known for many decades that *E. coli* also responds to PTS sugars, and the response is dependent on transport. PTS sugars are transported through specific transporters via a PEP-dependent transport system. PEP transfers a phosphoryl to an EI protein which, through a common HPr protein, phosphorylates an EII protein responsible for phosphorylating the specific sugar. Transport therefore directly affects the phosphate pools in the cell. EI and EII can directly interact with CheA and CheW to affect the signaling state of CheA. Thus, as the sugar levels increase, the activity of CheA decreases and the length of smooth swimming increases.

The serine receptor, Tsr, of *E. coli* not only responds to serine levels, but can respond to the autoinducer AI-2. This quorum sensing molecule is involved in biofilm formation in a range of species, where it diffuses freely through the cell membrane. When the population reaches a critical threshold, the level of intracellular AI-2 is high enough to activate transcription of a range of specific pathways. In *E. coli* it also binds Tsr and on binding it promotes autoaggregation. Similar effects have been seen in *A. brasilense* although in both cases the connection between autoaggregation, chemotaxis and biofilm formation is unclear.

Conclusion

The similarity between the core components of the chemosensory pathways identified across bacterial, and archaeal, species suggests an ancient ancestry. The additional complexities of some pathways reflec the duplication and adaptation of the individual pathways to the niches of those species. All pathways studied to date have diffusible CheYs being released from large hexagonal

arrays of chemoreceptors and binding to the cytoplasmic face of the motor to control its activity. The signals are terminated and the receptors adapt, resetting the signaling state of the kinase. These feedback systems, with signaling, termination and receptor adaptation occurring through large dynamic interactive sensory arrays allow bacteria to show exquisite sensitivity to changes in their background, with most able to respond to ~1% changes in concentration over 5–7 orders of magnitude, and enable motile bacteria to reach or maintain themselves in their optimum environment for growth.

Acknowledgments

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Bacterial Development

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Introduction

All developmental model organisms perform some complicated, multi-step activity that goes above and beyond the normal growth and division performed by all bacteria. These developmental systems integrate multiple individual processes into a larger complex physiological scheme. Therefore, the challenge in understanding bacterial development is one of understanding how all the moving parts of a machine work in concert to accomplish a given task. As development involves the coordination of multiple processes, signaling systems and regulatory networks figure prominently in developmental models. In fact, coordination is a key principle in bacterial development. It is not enough simply for a process to occur, it must occur at the right time and at the right place. Whether it's the subcellular protein localization and temporal progression of global regulators in *Caulobacter crescentus*, the communication between subcellular compartments during endospore formation in *Bacillus subtilis*, or the multi-day accumulation and organization of *Myxococcus xanthus* cells into a fruiting body, all the model developmental bacteria require precise spatial and temporal control of activities. In this article, some of the major model developmental organisms will be presented, and examples of regulatory systems used to coordinate activities in each model will be highlighted. It is worth noting that the regulatory pathways presented here are often simplified versions, and many intricate details are abridged for the sake of simplicity. The reader is encouraged to seek out more specific literature on a given topic to truly appreciate the subtle nuances of these systems.

Cellular Asymmetry and the Dimorphic Life Cycle of *Caulobacter crescentus*

Caulobacter crescentus is a freshwater aquatic microbe of the Alphaproteobacteria, and is a model developmental bacterium because of its unusual dimorphic life cycle. Instead of producing two identical cells after binary fission, *C. crescentus* produces two cells that differ in both their morphology as well as their replication capability (Fig. 1). The cell cycle begins with a stalked cell, a crescent shaped cell with a thin extension of the cell body from one pole called a "stalk". The stalk is tipped with an adhesive polysaccharide termed the "holdfast". As the stalked cell grows in size and prepares for division, it is termed a "predivisional cell". During the predivisional cell stage, a single flagellum is synthesized at the pole opposite the stalk, though it is held in an inactive state. Once cell division occurs, flagellum rotation is activated and pili are produced at the same pole as the flagellum. The asymmetric positioning of cell structures (stalk vs. flagellum/pili) is critical to the developmental program of *C. crescentus*, as the two progeny cells inherit different structures and are therefore different cell types. One cell inherits the stalk and holdfast, is non-motile, and is the same as the progenitor stalked cell; the other cell inherits the flagellum and pili and is a motile "swarmer cell". The cell types are also different in their reproductive capability. The progeny stalked cell is able to immediately enter the replication cycle, however the progeny swarmer cell cannot replicate and must spend a portion of its life cycle swimming. Eventually, either as a function of time or due to contacting a surface, the swarmer cell differentiates into a stalked cell. It does so by ejecting the flagellum, retracting the pili, and producing a stalk and holdfast from the same pole. Only when it has differentiated into a stalked cell is that cell able to replicate and divide. *C. crescentus* typically inhabits oligotrophic (i.e., very low nutrient) environments and it is thought that the swarmer cell stage is an obligate dispersal stage, forcing *C. crescentus* to constantly seek new environments to colonize.

Progression through the predivisional stage is tightly controlled by a sequential series of global transcriptional regulators, each regulator controlling the expression of dozens of genes, and is intertwined with the progression of chromosome replication. Chromosome replication is initiated by DnaA which also serves as a global regulator. One of the regulatory targets of DnaA is another global regulator GcrA. A regulatory target of GcrA is CtrA, a third global transcriptional regulator. CtrA is perhaps the most important regulator in *C. crescentus* development, regulating over 100 genes, including those involved in the flagellum biosynthesis cascade, pilus synthesis genes, chemotaxis genes, and cell division genes. CtrA also binds to five places in the origin of replication and prevents chromosome replication. Not only does the activity of one regulator lead to the activity of the next, each regulator also faces checkpoints from other physiological activities to ensure that it does not become active before a critical step has become accomplished. An example of this is in the regulation of CtrA by GcrA.

GcrA is an unusual DNA binding protein whose activity is, at least in part, regulated by the methylation state of the chromosome. At the beginning of the cell cycle, the chromosome is fully methylated on both strands, but the methyltransferase CcrM is not active. Newly synthesized DNA strands are unmethylated, so two hemimethylated chromosomes are produced during replication (where the old strand is methylated and the new strand is not). GcrA induces CtrA production by binding to the first of two promoters (P1) of the *ctrA* gene, but GcrA is unable to activate transcription from this promoter while the chromosome is fully methylated. Only once the replication fork has passed through this region and the *ctrA* promoter becomes hemimethylated is GcrA able to induce transcription from this promoter. Thus the expression of CtrA is tied not only to GcrA activity, but to successful chromosome replication as well. The use of chromosome replication as a timing and quality control measure requires an unusual feature of

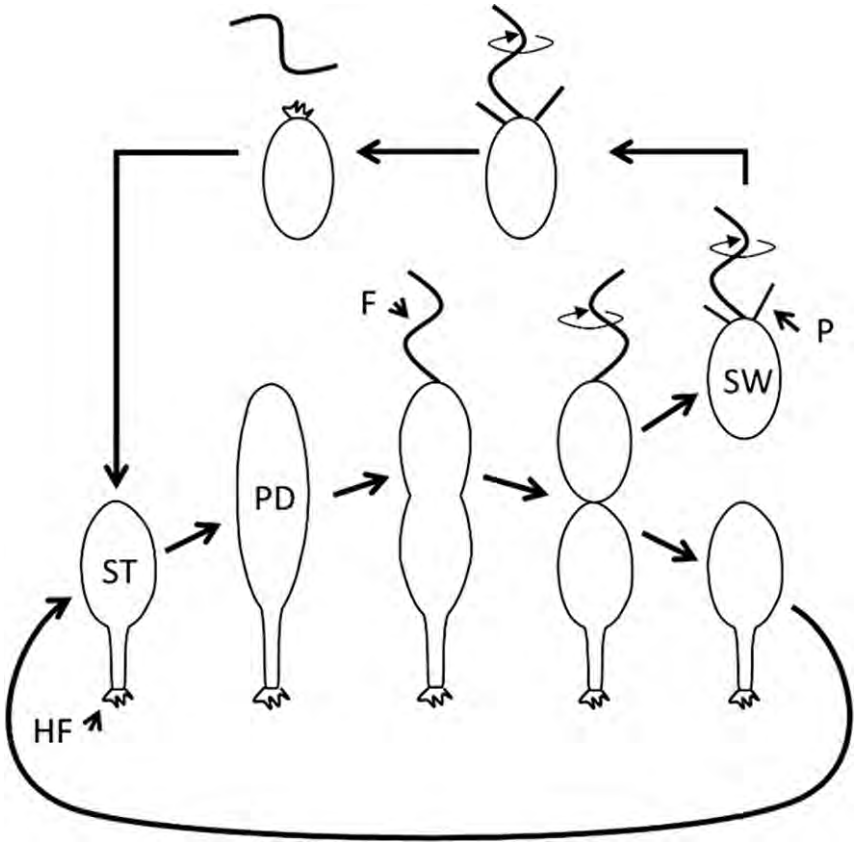

Fig. 1 Diagram of the *C. crescentus* cell cycle. The cycle begins with a stalked cell (ST). The stalk is tipped with an adhesive polysaccharide called the holdfast (HF). As the cell grows larger in preparation for cell division, it is called a predivisional cell (PD). During this stage, a flagellum (F) is made on the pole opposite the stalk. At the time of cell division, flagellum rotation is activated. The daughter stalked cell is able to re-enter the replication cycle. The other daughter cell inherits the flagellum and is motile, so is called a swarmer cell (SW). At this time pili (P) are also extended at the flagellar pole. The swarmer cell is unable to replicate until it differentiates into a stalked cell by ejecting the flagellum, retracting pili, and synthesizing a stalk and holdfast at that pole.

C. crescentus in that chromosome replication is initiated once and only once each cell cycle. Most bacteria, when rapidly growing in exponential phase, will initiate a round of chromosome replication before the previous round is finished, which sometimes lead to multiple chromosomes in different stages of replication completion.

The critical activity of CtrA is not just controlled by the timing of its expression. CtrA is also spatially controlled such that its activity is maintained in the swarmer cell but not the stalked cell. This spatial control prevents chromosome replication in the swarmer cell while permitting it in the stalked cell. CtrA is a response regulator and is activated by a phosphorelay beginning with the hybrid histidine kinase CckA and using the histidine phosphotransfer protein ChpT. Activation of CtrA in the swarmer cell and not the stalked cell is accomplished by specific activation of CckA in the swarmer cell. This spatial activation of CckA is accomplished by a very unusual signaling network composed of several other two-component signaling proteins (Fig. 2). The two component histidine kinase protein DivJ is localized to the stalked pole of the predivisional cell. Unlike most histidine kinases, DivJ does not recognize a specific signal but instead constitutively phosphorylates its target response regulator DivK. At the flagellar pole of the predivisional cell is the histidine kinase protein PleC that also does not recognize a signal but in this case constitutively dephosphorylates DivK (many histidine kinases can switch between kinase and phosphatase activities depending on the circumstances). How each protein becomes targeted to its specific pole is not well understood. This spatial arrangement means that after cell division, the progeny stalked cell inherits DivJ activity resulting in all DivK in that cell becoming phosphorylated. Conversely, the progeny swarmer cell inherits PleC resulting in all its DivK becoming dephosphorylated. It is the phosphorylation state of DivK in the newly born cells that ultimately controls CtrA activity in those cells as described below.

The hybrid histidine kinase CckA does not respond to a chemical signal. Instead, its activity is regulated through interaction with a histidine kinase DivL. DivL is unusual in that instead of a histidine that accepts phosphoryl groups, it has a tyrosine and most likely does not participate in phosphotransfer. Instead, it interacts with CckA, promoting kinase activity of that protein. When DivK is phosphorylated, such as in the progeny stalked cell, it disrupts functional DivL-CckA interaction and actually causes CckA to switch to phosphatase activity, reversing phosphate flow through the pathway, causing CtrA to become dephosphorylated and inactive, ultimately relieving repression of the origin of replication and allowing chromosome replication to initiate. In the progeny swarmer cell, DivK is non-phosphorylated, DivL-CckA interaction is not disrupted, phosphate flows toward CtrA and thus its activity is maintained, preventing chromosome replication initiation. It is this amazing

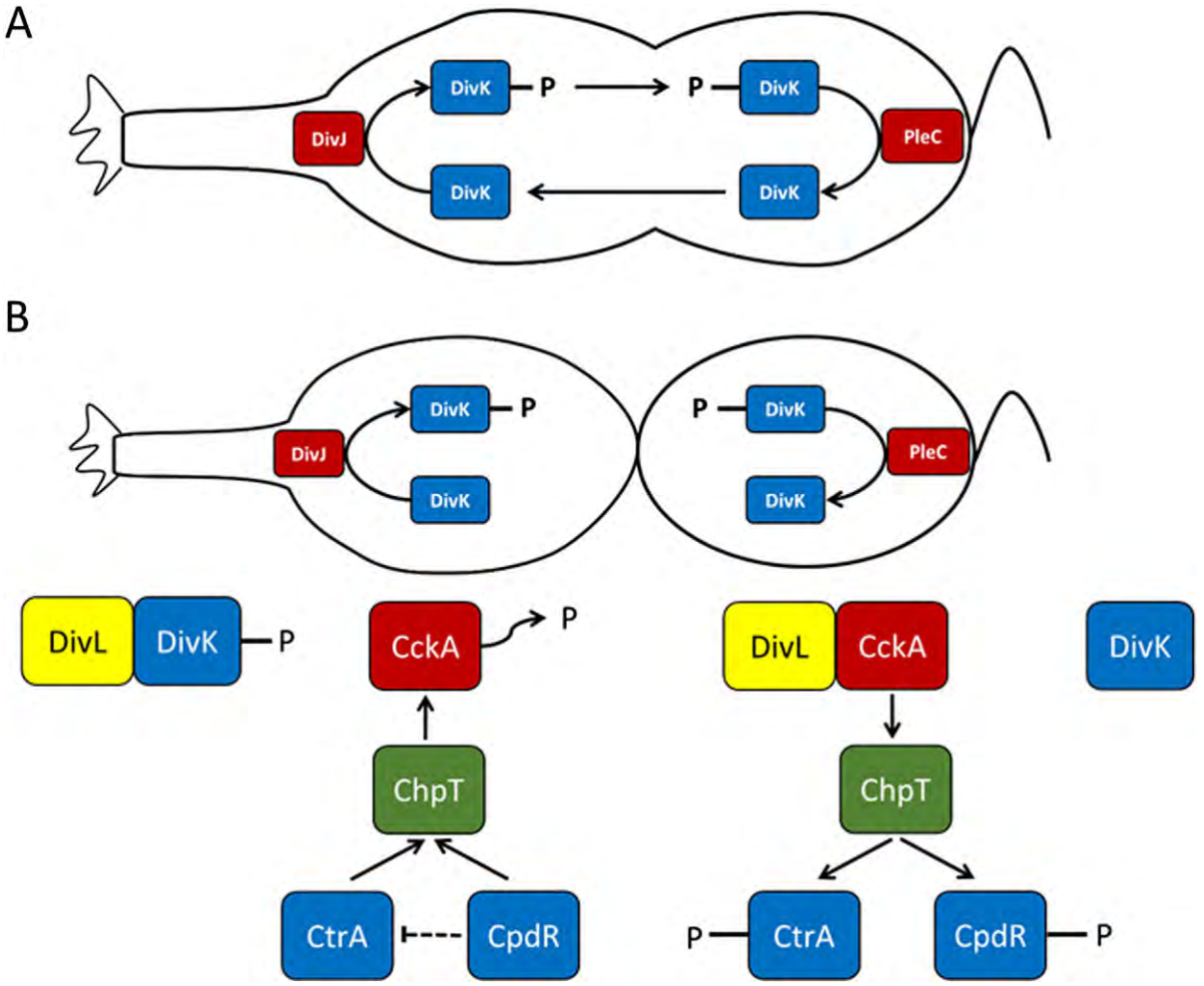

Fig. 2 Spatial control of CtrA regulation in *C. crescentus*. (A) In the predivisional cell, stalked-pole-localized DivJ phosphorylates DivK while flagellar-pole-localized PleC dephosphorylates DivK. (B) After cell division, in the stalked cell DivK becomes phosphorylated and disrupts a functional interaction between DivL and CckA, which reverses phosphate flow through the CckA-ChpT-CtrA/CpdR pathway, leading to CtrA dephosphorylation (deactivation) and proteolysis induced by dephosphorylation of CpdR. In the swarmer cell, DivK becomes dephosphorylated, permitting functional interaction between DivL and CckA, inducing phosphate flow through the CckA-ChpT-CtrA/CpdR pathway, leading to CtrA activation and protein stability by deactivation of CpdR by phosphorylation. Spatial control of CtrA leads to the stalked cell being able to replicate the chromosome while the swarmer cell cannot. In this diagram, histidine kinases are red, response regulators are blue, the Hpt protein ChpT is green, and the unusual histidine kinase DivL is yellow.

combination and integration of signaling proteins that leads to asymmetric activation of the master regulator between two progeny cells. CtrA is so important to cellular function that *C. crescentus* has redundant mechanisms to ensure its appropriate activity/inactivity. While ChpT can pass phosphates to CtrA to activate it, ChpT can also pass phosphates to a single domain response regulator CpdR. CpdR controls the stability of the CtrA protein by targeting it for proteolysis by the housekeeping ClpXP protease. When CckA is in the kinase state, CtrA is phosphorylated and active while CpdR is phosphorylated and inactive, leading to CtrA protein stability. When CckA is in the phosphatase state, CtrA is dephosphorylated and inactive and CpdR is dephosphorylated and active, targeting CtrA for degradation. Thus when CtrA is active, it is stabilized, but when it is dephosphorylated the protein itself is also removed from the cell, ensuring there is no residual CtrA activity. CtrA is a prime example of the massive amounts of regulation and coordination involved in performing developmental processes.

Endospore Formation of *Bacillus subtilis*

Bacillus subtilis is a Gram positive member of the Firmicutes phylum and best known for the ability to produce stress-resistant, metabolically-quiescent spores that can remain dormant but viable for many, many years. These spores are formed inside the cell and thus are referred to as "endospores". Spores are produced in response to starvation, but the process of sporulation is costly in

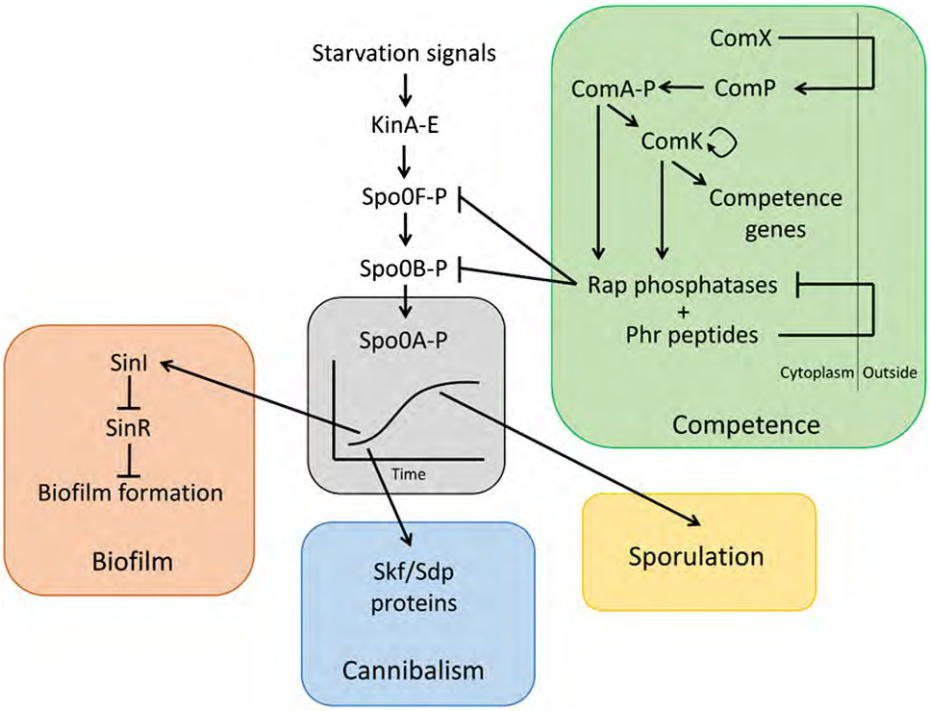

Fig. 3 Regulation of events during *B. subtilis* starvation. Starvation signals induce signaling through five histidine kinases (KinA-E), inducing phosphate flow through the Spo0F-Spo0B-Spo0A phosphorelay. Spo0A (gray box): Spo0A-P levels increase over time. Competence (green box): Vegetatively produced ComX quorum signaling peptide, if accumulating to sufficient levels, induces signaling through the ComP-ComA two-component system. ComA-P induces expression of ComK, which positively regulates itself. ComK production leads to expression of competence genes. Both ComA-P and ComK lead to production of Rap phosphatases which dephosphorylate the main phosphorelay, preventing Spo0A phosphorylation. Each Rap phosphatase has a cognate Phr quorum peptide that is expressed and exported. If these accumulate to sufficient levels they inhibit Rap phosphatase activity, permitting phosphate flow through the phosphorelay. Biofilm (orange box): Low levels of Spo0A-P induce expression of SinI, an inhibitor of SinR, which itself inhibits biofilm formation. Therefore, low levels of Spo0A-P induce biofilm formation. Cannibalism (blue box): Low levels of Spo0A-P induce production of *skf* and *sdp* gene products, which include toxins and associated resistance proteins that induce lysis of other cells, including neighboring *B. subtilis*. Released cellular contents can be utilized for nutrients. Sporulation (yellow box): Only if starvation conditions persist long enough for Spo0A-P to reach high levels is sporulation finally induced.

both time and energy. Additionally, while the spore itself is viable, the cell that produces the spore is killed in the process. Therefore, the decision to sporulate is a very important one, and *B. subtilis* has multiple responses to starvation prior to initiating sporulation that are all integrated in a signaling network (Fig. 3). The backbone of this signaling network is composed of the phosphorelay proteins Spo0F, Spo0B, and the master regulator Spo0A. Phosphorylation through this pathway is initiated by any one of five histidine kinases (KinA-E). While kinase activity is stimulated by starvation signals, for the most part the exact nature of the signal for each kinase is not well understood. Once kinase activity is induced, phosphates are passed along the relay to the terminal response regulator Spo0A, which induces sporulation when phosphorylated. However, there are many processes that can occur before sporulation is ultimately initiated.

One process that can occur before sporulation is competence, whereby the *B. subtilis* cell can take up and assimilate foreign DNA from the surrounding environment. During vegetative growth (i.e., when cells are actively growing), *B. subtilis* cells produce the quorum signaling peptide ComX which can build up in the extracellular environment to sufficient levels to activate its target receptor, the histidine kinase ComP. ComP then phosphorylates the response regulator ComA, which is then able to induce production of the transcriptional regulator ComK. ComK stimulates its own expression, leading to a positive feedback loop and a burst of ComK production, which induces expression of the competence genes. In addition, both ComA~P and ComK induce expression of a number of phosphatases (including RapA, RapB, and RapH) that remove phosphates from the Spo0F-Spo0B-Spo0A pathway. Therefore, while competence is active, sporulation cannot occur. Presumably this is because the uptake of foreign DNA may provide proteins that can open up a previously untapped nutrient source and relieve the starvation. However, competence does not provide a permanent blockage to sporulation. When Rap phosphatases are produced, corresponding quorum signaling Phr pheromone peptides are also produced and excreted. Once these peptides build up to sufficient levels, they re-enter the cell and inhibit their cognate phosphatase either directly or indirectly.

Once Rap phosphatase inhibition has been relieved, Spo0A~P levels begin to rise. Yet again, there are still more processes that can occur before sporulation. Low Spo0A~P levels induce the production of SinI, an antagonist of the protein SinR, which itself inhibits biofilm formation. Thus low Spo0A~P levels induce biofilm formation, which can provide many physiological advantages to a starving cell. Low Spo0A~P levels also induce expression of *skf* (sporulation killing factor) and *sdp* (sporulation delaying

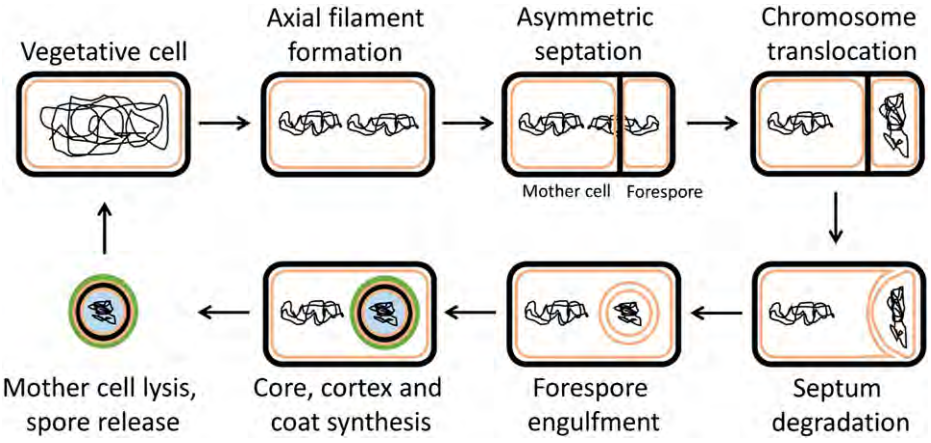

Fig. 4 Developmental progression of *B. subtilis* spore formation. Once the vegetative cell enters sporulation, the chromosome is duplicated and both copies are condensed to axial filaments along the horizontal length of the cell. Asymmetric septation occurs across one chromosome, making the larger mother cell compartment and smaller forespore compartment. The remainder of the chromosome is translocated into the forespore compartment. Degradation of the peptidoglycan septum allows the mother cell to engulf the forespore, giving the spore two membrane bilayers. A thick peptidoglycan cortex is assembled between the bilayers and a complex spore coat is assembled on the exterior of the spore by the mother cell. The spore core undergoes changes to protect the chromosome. The mother cell lyses to release the spore, which can germinate under favorable conditions to become a vegetative cell.

protein) genes. Some of the products of these genes are secreted toxins that kill and lyse neighboring *B. subtilis* cells. The components of the lysed cells provide nutrients and resources to the toxin-producing cells; this process is referred to as "cannibalism" and serves to (at least temporarily) relieve starvation. Thus, competence, biofilm formation, and cannibalism all can occur as starvation responses prior to sporulation, and only if these processes fail to relieve starvation and Spo0A∼P rises to high levels is sporulation finally induced.

The process of sporulation begins with the replication of the host chromosome such that there are two copies (Fig. 4). The chromosomes are then condensed along the long axis of the cell as a linear "axial filament". A septum is produced at an asymmetric point of the cell. The placement of the septum is the first committed step of sporulation; after this point, sporulation will complete even if nutrients are introduced to the cell. Septation causes the formation of two cellular compartments: the larger mother cell compartment and the smaller forespore compartment. Septation also temporarily traps a portion of one chromosome in the forespore compartment. At this point, the two compartments begin to have different patterns of gene expression. After a time, the remainder of the trapped chromosome is translocated into the forespore compartment by the DNA pump SpoIIIE, the septum is completed, and the septum peptidoglycan is degraded, bringing the mother cell and forespore membranes into direct contact. Further peptidoglycan degradation is coupled to a ratchet system that leads to the mother cell engulfing the forespore, thereby giving the forespore a second membrane. A thick peptidoglycan layer called the "cortex" is produced between the membranes. The core of the spore undergoes several changes, including protection of the DNA by small acid-soluble proteins and dehydration. Additionally, over 80 different proteins are produced and assembled by the mother cell onto the forespore, creating the inner and outer layers of the spore coat. It is the combination of spore core, cortex, and coat that give the *B. subtilis* spore its amazing stress resistance properties. Sporulation ends when the mother cell lyses and releases the mature spore.

During sporulation, each compartment has a progression of alternative sigma factors that drive expression of their own regulons and the distinct patterns of gene expression. High levels of Spo0A∼P lead to the production of two sigma factors: σ^E and σ^F; however, both sigma factors are held in an inactive state. σ^E is in a proteolytically unprocessed and inactive form (pro-σ^E), and σ^F is sequestered by the anti-sigma factor SpoIIAB. Both sigma factors are produced prior to septation and thus are found in both compartments once septation occurs. However, septation leads to compartment specific activation. SpoIIAA is an anti-anti-sigma factor that binds the anti-sigma factor SpoIIAB, but is prevented from doing so by a covalently attached phosphate group. Septation leads to activation of the SpoIIE phosphatase in the forespore compartment, which removes the phosphate from SpoIIAA. SpoIIAA then binds to SpoIIAB, thus releasing σ^F and allowing it to initiate transcription from σ^F-dependent promoters. Therefore, σ^F only becomes active in the forespore compartment. One of the regulatory targets for σ^F is the gene encoding SpoIIR, a protein that enters the inter-compartmental space and activates the membrane-bound SpoIIGA protease in the mother cell membrane, which processes pro-σ^E into the active σ^E. Therefore, activation of σ^F in the forespore leads to activation of σ^E in the mother cell. Another regulatory target of σ^F is the gene encoding σ^G. σ^G is inactive until engulfment of the forespore occurs, but the mechanism of activation is unclear. Activation of σ^G requires the expression of the *spoIIIAA-AH* operon driven by σ^E in the mother cell. These proteins have similarity to Type III secretion complexes and it is thought that they form a channel from the mother cell to the interior of the engulfed spore (the "feeding tube hypothesis"). What it unknown is how exactly this channel leads to σ^G activation. One theory is that the nascent spore is metabolically diminished because nutrients have no way to enter that compartment, and σ^G-dependent transcription occurs once the feeding tube provides energy to that compartment. Alternatively, activation of σ^G seems to strongly depend on SpoIIIAG and SpoIIAH, so it is possible that the feeding tube provides a mechanism for these proteins to reach into the

forespore compartment and relieve some type of specific inhibition on σ^G. Regardless, both engulfment and σ^E activity are necessary for proper σ^G activity.

Another regulatory target of σ^E is the gene encoding σ^K, which, like σ^E, is produced as an inactive pro-protein. Activation of σ^G in the forespore leads to expression of the forespore protein SpoIVB, which leads to activation of the mother cell protease SpoIVFB, which processes pro-σ^K into the active σ^K form. In total, each compartment has its own sigma cascade, with σ^F to σ^G in the forespore, and σ^E to σ^K in the mother cell. Each cascade functions in parallel, but there is extensive cross-compartment communication and checkpoint control to ensure that the next stage in the process only occurs once a previous stage was successful. As seen here and in the *C. crescentus* system as well, checkpoints are vital to coordinating multiple processes.

Myxococcus xanthus Fruiting Body Formation

A member of the Deltaproteobacteria, *Myxococcus xanthus* is most commonly found in soil. That is because *M. xanthus* is a predatory microbe and prefers to be in environments with the highest prey density. *M. xanthus* secretes hydrolytic enzymes that degrade prey cells and then metabolizes the contents. However, the effectiveness of a single cell secreting these enzymes is limited, so *M. xanthus* travels in multicellular swarms, thereby pooling the hydrolytic enzymes from multiple cells to increase feeding efficiency. This has caused *M. xanthus* to be referred to as "wolf pack predators". When a swarm of *M. xanthus* is starved, the cells aggregate together to form a 3-dimensional, spore-filled structure called a "fruiting body". It is thought that the fruiting body serves to keep the spores together so that when they germinate they are able to immediately re-establish a swarm. Fruiting body formation is a potent example of cellular differentiation because different cells have different destinies during the process (Fig. 5). Only one in ten cells actually becomes a spore inside the fruiting body. Most cells die and are cannibalized for energy during the process, which can take days to complete. Some cells never enter the fruiting body but instead stay outside as "peripheral rods", potentially acting as guards to prevent other bacteria from invading the fruiting body.

Fruiting body formation is a concert of cellular organization which requires constant intercellular communication, where the end result is a careful arrangement of cells into a structure with varying levels of spatial intricacy. While the *M. xanthus* fruiting body is a relatively simple mound shape, other Myxobacteria form more elaborate fruiting body structures. Regardless of the exact

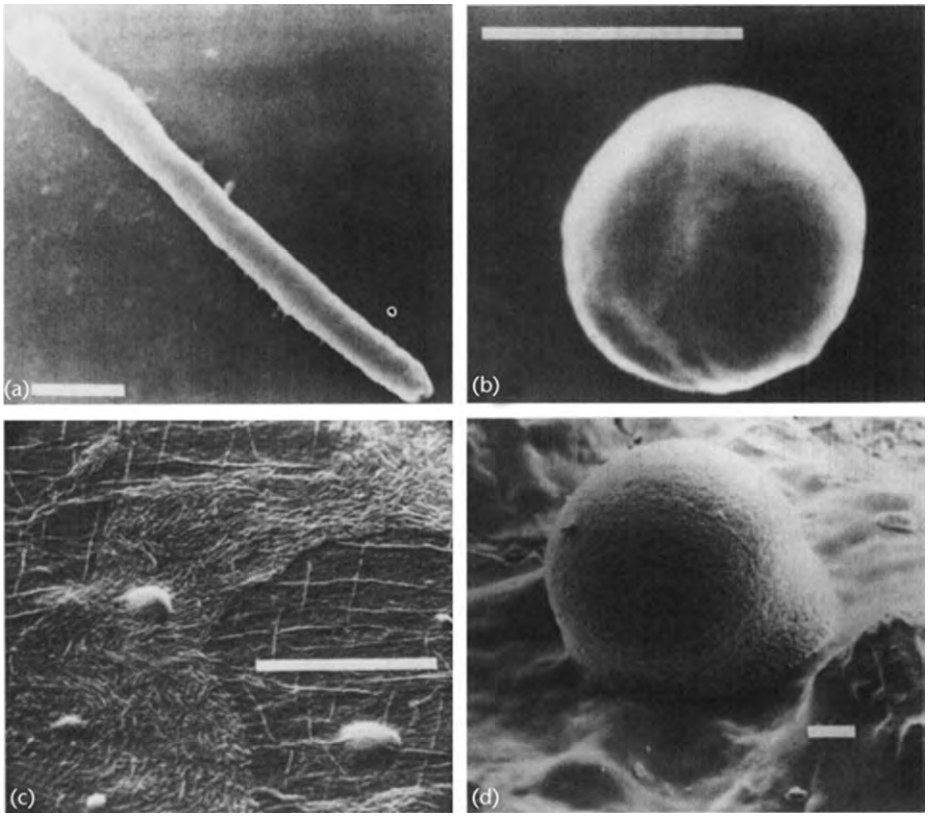

Fig. 5 Cell types and forms of *M. xanthus*. (A) a single vegetative cell (bar=1 μm). (B) a myxospore (bar=1 μm). (C) cells aggregating into fruiting bodies (bar=20 μm). (D) a *M. xanthus* fruiting body (bar=20 μm). From Shimkets, L.J., 1999. Intercellular signaling during fruiting-body development of *Myxococcus xanthus*. Annu. Rev. Microbiol. 53, 525–549. Copyright 2006 Annual Reviews.

organization, intercellular communication is a key aspect of the process. Much of the research into fruiting body formation has been driven by the examination of extracellular complementation groups. These are groups of mutants that are unable to form fruiting bodies on their own but can form fruiting bodies when mixed with wild-type cells or mutants from another group. Presumably this complementation is performed by some extracellular signal. Five major complementation groups have been characterized (A, B, C, D, and E signaling mutants), and the mechanisms behind these phenotypes are understood to greater or lesser extent. The A and C signals are the most well-characterized and will be presented here. The A signal is one of the earlier signals during fruiting body formation. *M. xanthus* responds to starvation much like other bacteria by producing the alarmone ppGpp, synthesized by the RelA enzyme when tRNAs uncharged with their respective amino acid enter the ribosome (known as the "Stringent Response"). The buildup of ppGpp somehow induces signaling through the unusual two component signaling protein AsgA, which is a hybrid histidine kinase with an N-terminal response regulator receiver domain. Signaling through this protein leads to production and export of protease enzymes, which degrade extracellular proteins and thus release small peptides and amino acids. While it may seem like this response serves to produce more nutrients in response to starvation (and it may do so to a certain extent), this mixture of amino acids actually serves as the extracellular A signal and functions as a quorum sensing system. The mixture of amino acids builds up over time and leads to the next stage of fruiting body formation by inducing signaling through the SasS/R two component system. An interesting facet of the A signal is that the signal is not a specific molecule, but instead a mixture of amino acids with Tyr, Pro, Phe, Trp, Leu, and Ile amino acids having more stimulatory activity.

A signaling occurs ~2 h after starvation, while the next signal (C signal) occurs ~6 h after starvation. C signaling is driven by the protein CsgA which has homology to short chain alcohol dehydrogenase enzymes. The molecular mechanism for C signaling has been extensively studied and is the subject of some controversy. Some evidence suggests that the enzymatic activity of CsgA is critical, as fruiting body formation in *csgA* mutants can be restored by over-expressing a different short chain alcohol dehydrogenase SocA. Other evidence suggests that a proteolytically processed, enzymatically inactive form of CsgA that is anchored to the outer membrane serves as the actual C signal through direct cell contact. Perhaps both activities figure into CsgA function. Regardless, CsgA is one of the most important proteins during fruiting body formation, and much like Spo0A in *B. subtilis* sporulation, is able to induce different processes at different levels of expression. Low levels of CsgA induce a response known as "rippling", where *M. xanthus* swarms appear to be rippled like the surface of disturbed water. Rippling is caused by a change in motile behavior and likely functions in scavenging nutrients from dead cells. Intermediate CsgA levels induce aggregation of cells into mounds, and high CsgA levels induce sporulation. CsgA levels are known to rise steadily during fruiting body formation, and exogenously supplying CsgA at high levels causes *M. xanthus* to turn into spores before aggregation has occurred. Thus, regulated accumulation of CsgA is necessary for the proper timing of events during fruiting body formation. While the exact nature and sensing mechanism of the C signal is not known, C signaling is needed for phosphorylation of FruA, an orphan response regulator with a DNA binding domain. Strains lacking FruA cannot ripple, aggregate, or sporulate, though how FruA specifically impacts these processes is not clear. *M. xanthus* fruiting body formation is a wonderful model to study fundamental principles of multicellularity, and spatial and temporal regulation are critical to the process.

Spore Formation of *Streptomyces coelicolor*

Streptomyces coelicolor is a member of the Actinobacteria, and more specifically of the Actinomycetes. While perhaps best known as a major source of clinical antibiotics and other beneficial secondary metabolites, the Actinomycetes are a group of bacteria that share many characteristics in common with fungi. They are principally soil-dwelling, and grow in long, filamentous, branching cells called "hyphae" in the soil subsurface, with the total subsurface hyphal mat referred to as a "mycelium". Under nutrient limitation, "aerial hyphae" are produced that grow vertically and exit the substrate. The ends of aerial hyphae then develop into chains of spores.

Whether growing in the soil or in a laboratory agar medium, the mycelium grows in an aqueous environment. When aerial hyphae are produced, they must exit the aqueous environment. To do so, they must pass through the air-liquid interface, which presents a significant physical barrier. So potent is this barrier that *S. coelicolor* has two semi-redundant systems to penetrate the air-liquid interface, and mutants defective in these systems are unable to produce aerial hyphae. The first system is the production of an oligopeptide SapB. SapB is a lantibiotic, which are ribosomally synthesized oligopeptide antibiotics. SapB is made by the *ramCSAB* cluster of genes, where *ramS* encodes the SapB oligopeptide, RamC processes and matures the oligopeptide into the finished form, and RamAB form an ABC transporter for extracellular export. SapB has no demonstrated antibiotic activity and instead appears to be a surfactant, which serves to reduce surface tension at the air-liquid interface, making it easier for aerial hyphae to break that barrier. The *ramCSAB* cluster is regulated by the RamR response regulator, but how RamR is regulated is unknown. The second system for penetrating the air-liquid interface is the production of the "chaplin" proteins. The *S. coelicolor* chaplins are an unusual family of 8 proteins with conserved hydrophobic domains. These proteins are exported and anchored to the exterior of aerial hyphae, forming a hydrophobic sheath around the cell. Filaments of chaplins are organized into a beautiful basket-like network of filaments by proteins called "rodlins" (Fig. 6). This hydrophobic sheath allows the cell to exit the aqueous environment, and may also protect aerial hyphae against desiccation while in the air. A number of "bald" mutants have been found, so-called because they do not produce the fuzzy aerial hyphae and the colonies appear smooth, yet all of these mutations seem to culminate in SapB/chaplin production because overexpression of RamR leads to SapB and aerial hyphae formation in all bald mutants. Many bald mutations are in predicted transcription factors, and some extracellular signaling function may be involved, but how all these factors link together is still unknown.

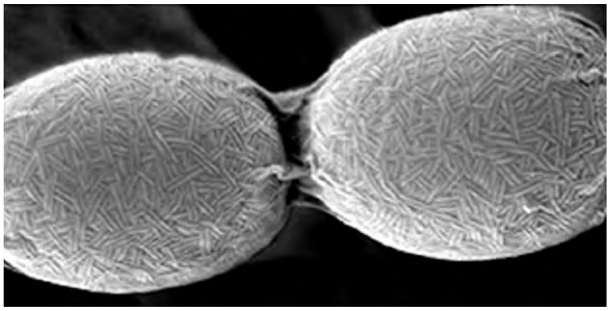

Fig. 6 Electron micrograph of basket-like networks of chaplin filaments on the surface of *Streptomyces* spores. Modified from Di Berardo, C., Capstick, D.S., Bibb, M.J., *et al.*, 2008. Function and redundancy of the chaplin cell surface proteins in aerial hypha formation, rodlet assembly, and viability in *Streptomyces coelicolor*. J. Bacteriol. 190, 5879–5889. Copyright 2008 American Society for Microbiology.

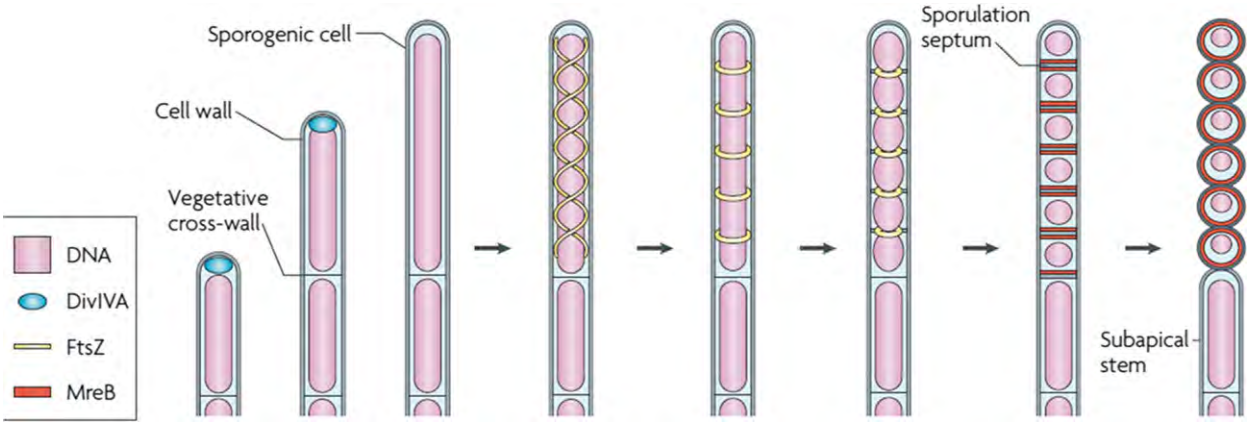

Fig. 7 Morphogenesis of *S. coelicolor* aerial hyphae into spores. Modified from Flärdh, K., Buttner, M.J., 2009. *Streptomyces* morphogenetics: Dissecting differentiation in a filamentous bacterium. Nat. Rev. Microbiol. 7, 36–49. Copyright 2009 Macmillan Publishers Limited.

As aerial hyphae grow, they become tipped with a long, non-septated compartment that has been referred to as a "sporogenic cell" (Fig. 7). Inside this cell, the *S. coelicolor* chromosome is extensively replicated, resulting in often more than 50 copies. Genome copies then become evenly spaced throughout the sporogenic cell. This is then followed by multiple, synchronous cell divisions between spaced chromosomes, producing individual spore compartments, each with a copy of the chromosome, which are then matured into full spores. The cell division events that produce spore compartments represents a massive switch in the growth/cell wall synthesis pattern of the organism. Most rod-shaped cells that divide by binary fission grow by adding peptidoglycan along the full lateral length of the cell wall, then divide by directing peptidoglycan synthesis inward at the midcell. Growth of *S. coelicolor* is very different. Vegetative hyphae grow by a process of tip extension, where new peptidoglycan synthesis occurs only at the tips of hyphal branches (a process mediated by the DivIVA protein), and cells are infrequently septated. One of the major proteins responsible for septum formation, FtsZ, is essential in almost every bacterium but not in *S. coelicolor*. However, FtsZ is necessary for spore formation. FtsZ forms a helical filament along the length of the sporogenic cell and is then separated out in a series of evenly spaced rings. How these rings achieve even spacing is not clear, but probably involves the ParAB chromosome partitioning system. The FtsZ rings direct peptidoglycan synthesis inward, creating septa and thus spore compartments. A thick peptidoglycan layer is produced around each compartment (mediated by the bacterial actin homolog MreB), with a coincident change in spore compartment shape from cylindrical to ovoid. A number of mutants have been found that produce aerial hyphae but cannot complete sporulation. As a result, the colonies do not produce a gray spore pigment and instead remain white in color, leading to these mutants being referred to as "white mutants". Like the bald mutants, a number of white mutations have been identified in predicted transcription factors, but if/how they are organized into regulatory networks is not clear. Nor is the interplay between bald and white systems well characterized. Clearly the regulatory systems of *S. coelicolor* require further investigation.

Heterocyst Formation in *Anabaena*

Anabaena sp. strain PCC 7120 is a member of the Cyanobacteria, which are Gram negative bacteria that perform oxygenic photosynthesis and are major players in the global cycling of carbon, nitrogen, and oxygen. Some groups of cyanobacteria (including *Anabaena*) grow in a filamentous fashion, where each cell division occurs along the same axis, leading to chains that

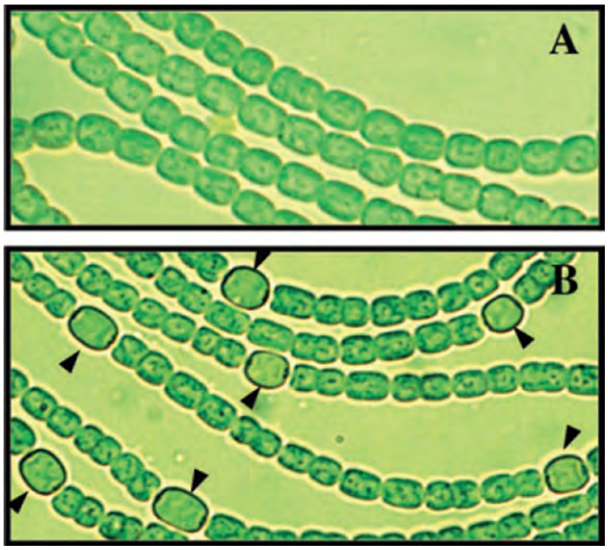

Fig. 8 Morphological differentiation of Anabaena. (A) *Anabaena* filament grown with excess combined nitrogen. (B) *Anabaena* filament after 24 h combined nitrogen starvation. Arrows indicate heterocysts. Modified from Zhang, C., Laurent, S., Sakr, S., Peng, L., Bédu, S., 2006. Heterocyst differentiation and pattern formation in cyanobacteria: A chorus of signals. Mol. Microbiol. 59 (2), 367–375. Copyright 2005 Blackwell Publishing Ltd.

can be hundreds of cells long. However, the outer membrane is not included in the cell division process, so the filament of cells has one continuous outer membrane which results in a single, shared periplasmic space. Additionally, the cytoplasms of adjacent cells are connected by tiny channels called microplasmodesmata, which may be proteinaceous tubes formed of SepJ (FraG) protein. When grown in the presence nitrate or ammonia, the chain of cells appears uniform. However, when these nitrogen sources are depleted, some cells in the filament will differentiate into a specialized cell type called a "heterocyst" (Fig. 8). Heterocysts are terminally differentiated cells specializing in fixing atmospheric N_2 into bioavailable nitrogen.

Fixing nitrogen poses a mechanistic problem for cyanobacteria. These bacteria use Photosystem II during photosynthesis, so O_2 is given off as a waste product. However, O_2 permanently inactivates the nitrogenase enzymes that fix nitrogen. To solve this problem, heterocysts undergo several physiological changes to protect the cell from O_2 contamination. First, Photosystem II in heterocysts is degraded. Second, thick glycolipid and exopolysaccharide layers are added to the cell to limit exogenous O_2 from diffusing in. Lastly, the shape of the heterocyst cytoplasm is altered to minimize the junction area with adjacent cells, likely to minimize diffusion of O_2 generated by adjacent cells into the heterocyst. All these processes serve to create a micro-oxic environment inside heterocysts conducive to nitrogen fixation. Vegetative cells perform photosynthesis and provide carbon and reductants (principally in the form of sucrose) to the heterocyst, while the heterocyst provides nitrogen compounds to the vegetative cells. Clearly molecular sharing between cells is critical, and it is likely microplasmodesmata aid this function.

Amination of the TCA cycle intermediate α-ketoglutarate to glutamate is the main nitrogen assimilation mechanism in bacteria. Heterocyst formation begins with the sensing of "combined nitrogen" (i.e., nitrate and/or ammonia) depletion by the build-up of α-ketoglutarate. α-ketoglutarate binds to the transcriptional regulator NtcA, increasing its affinity for DNA. Activated NtcA induces expression of the response regulator NrrA, which then induces expression of the master regulator of heterocyst development HetR. HetR is a serine protease that also has DNA binding activity. By 3.5 h after depletion, *hetR* expression is seen in a pattern of cells resembling those that will become heterocysts, and mutants of *hetR* arrest heterocyst formation at early stages.

The pattern of heterocyst formation in cyanobacterial chains is particularly fascinating. Heterocysts arise within chains with almost mechanical regularity; one heterocyst every ten-to-twenty vegetative cells. As the cells in the chain grow and divide, new heterocysts arise to maintain this pattern. Presumably this maximizes the efficiency of molecular exchange between heterocyst and vegetative cells and does not wastefully commit cells to a terminal process that could otherwise remain viable. The major mechanism of determining heterocyst pattern formation is centered around the product of the *patS* gene and is consistent with a longstanding model wherein heterocyst cells produce an inhibitor of heterocyst formation that diffuses into the cell chain in a gradient, thus preventing cells adjacent to a heterocyst from differentiating. All heterocyst producing cyanobacteria have a *patS* gene, encoding proteins varying in length from 13 to 90 amino acids, but all end with the amino acid sequence RGSGR. Proteolytic processing and release of the carboxyl terminus as a pentapeptide is thought to be the diffusible inhibitor. A *patS* null mutant produces many heterocysts, often adjacent to each other, while *patS* overexpression completely inhibits heterocyst formation. Exogenous addition of synthetically made pentapeptide also completely inhibits heterocyst formation. Proper heterocyst patterning can be achieved in a *patS* mutant by expressing *patS* from a different heterocyst-dependent promoter. However, expressing just the pentapeptide from that same promoter does not restore the pattern, suggesting there are other facets to this system. The *patS* pentapeptide has been shown to inhibit HetR's ability to bind DNA in a very specific manner, suggesting that pattern formation of heterocysts is accomplished by specifically inhibiting HetR in heterocyst-adjacent cells. There also may be other unknown regulatory

factors participating in heterocyst pattern formation. *Anabaena* and other filamentous cyanobacteria are tremendous systems to study how biological processes create patterns, which is an important concept in biology from the molecular level all the way up to ecosystems.

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Bacterial Flagella☆

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Structure

Filament

The flagellum is a complex structure composed of many different kinds of proteins. However, the term flagellum often indicates the flagellar filament only since the filament is the major portion of the entire flagellum. Especially, in earlier papers, the term flagella always denoted filaments. In this section, I am going to describe the filament and may occasionally call the filament flagellum.

Number of Flagella Per Cell

The number and location of flagella on a cell is one of the readily discernible traits for the classification of bacterial species. The number ranges from one to several hundreds depending on the species, and hence the nomenclature: monotrichous (one) or multitrichous (two or more). Occasionally, the term "amphitrichous" is used for two flagella.

There are three possible locations on a cell body for flagella to grow: polar (at the axial ends of the cell body), lateral (at the middle of the cell body), or peritrichous (anywhere around the cell body). In some cases, "lateral" is used as the counterpart of "polar," as in the two flagellar systems of *Vibrio alginolyticus*: polar sheathed flagellum and lateral plain flagella. The "lateral" flagella have been sometimes mistaken as "peritrichous" flagella, but now there are a several lines of evidence that these two are different from each other: (1) they belong to different flagellar families (see "Three Flagellar Families" section); (2) the gene organization of the two is different (see "Flagellar Genes" section); and (3) lateral flagella are inducible in higher viscosity environments, but peritrichous flagella are not. Thus, lateral flagella seem to have their own mechanism for localization on the cell.

A tuft of flagella growing from a pole is called lophotrichous. In most cases, flagella can be named by a combination of number and location; for example, the polar lophotrichous flagella of *Spirillum volutans*.

Although ordinary flagella are exposed to the medium, some flagella are wrapped with a sheath derived from the outer membrane (e.g., *Vibrio cholerae*, *Helicobacter pylori*). In an extreme case such as spirochaetes, flagella are confined in a narrow space between the outer membrane and the cell membrane and thus are called the periplasmic flagella or axial filaments. The flagella still can rotate; the helical cell body works as a screw, and the flagella counterbalance the torque on the cell body.

Filament Shape and Polymorphic Transition (Fig. 1)

Filament shape is helical. In theory, there are two types of helices, right-handed and left-handed; for example, *Salmonella* spp. have a left-handed filament and *Caulobacter crescentus* has a right-handed filament under physiological conditions. However, it should be noted that shapes of these two helices are not mirror images of each other.

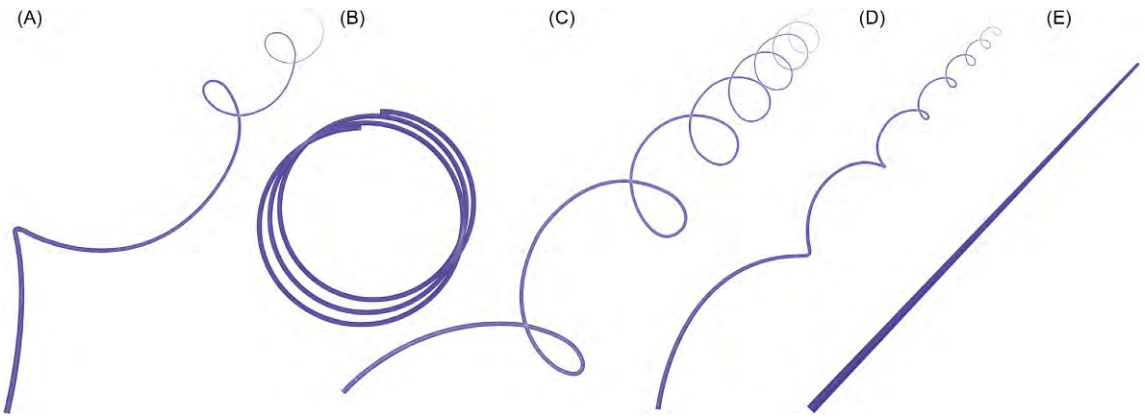

Fig. 1 Helical forms of flagellar filaments. Helices are seen from a position slightly off its axial direction so that the handedness can be easily visualized. The figure shows five typical forms with their helical parameters (*p*: pitch, *d*: diameter). (a) Normal (*p*: 2.55 mm, *d*: 0.6 mm), (b) Coiled (*p*: 0, *d*: 1.0 mm), (c) Semicoiled (*p*: 1.26 mm, *d*: 0.5 mm), (d) Curly (*p*: 1.20 mm, *d*: 0.2 mm), and (e) Straight filament (*p*: 1, *d*: 0).

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There are several detailed filament shapes, and it will be convenient to use the names of typical shapes found in *Salmonella* spp.: Normal (left-handed), Curly (right-handed), Coiled (left-handed), Semicoiled (right-handed), and Straight. Note that we often indicate the names of filament shapes by adjectives starting with capital letters. The helical parameters of these helices are discrete and distinguishable from one another.

Flagella can switch between a set of helical shapes under appropriate conditions; not only helical pitch but also helical handedness is interchangeable. The transformation of shapes can be induced by physical or chemical perturbation (torque, temperature, pH, salt concentration of medium, etc.). Genetic changes such as point mutations in the flagellin (the component protein of the flagellar filament) gene also result in transformation of helices, but some mutant flagella such as straight flagella have no freedom to transform into another helix.

This phenomenon called "polymorphism" of flagella is a visible example of conformational changes in proteins and, therefore, has evoked an idea of a functional role in motility; could polymorphism of the flagellum by itself cause the motion? The answer is No. Flagella are passive in terms of force generation. Polymorphism of flagella is observed to occur naturally on actively motile cells with peritrichous flagella. The helical transformation is necessary for untangling a jammed bundle of tangled flagella.

When Normal flagella in a jammed bundle are transformed into Curly flagella, knots of tangled flagella run toward the free end of each flagellum to untangle the jammed bundle.

Models that explain the polymorphism were first introduced by Sho Asakura in 1970 and theoretically strengthened by Chris R. Calladine in 1978. Twisting and bending a cylindrical rod give rise to a helix. Models predict 12 shapes, and 8 of them have been found in existing filaments: Straight with a left-handed twist, f1, Normal, Coiled, Semicoiled, Curly I, Curly II, and Straight with a right-handed twist. Only a small energy barrier seems to lie between two neighboring shapes. Polymorphic transition occurs from one shape to its neighbors; for example, in a transition from Normal to Curly I, the filament briefly takes on Coiled and Semicoiled forms.

Three Flagellar Families (Figs. 2 and 3)

The bacterial flagellum transforms its typical shape into several distinguishable helical shapes (polymorphs) under various environmental conditions as mentioned above. Therefore, we have regarded flagella from other species as one of those polymorphs

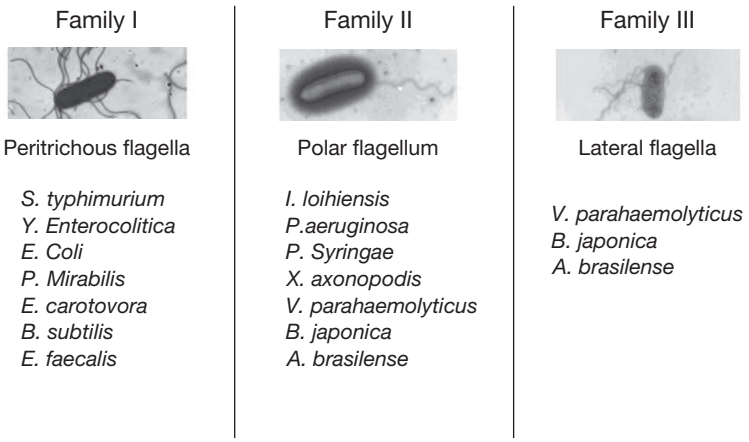

Fig. 2 Flagellar family. According to the helical parameters, flagella are divided into three families: Family I for peritrichous flagella, Family II for polar flagella, and Family III for lateral flagella.

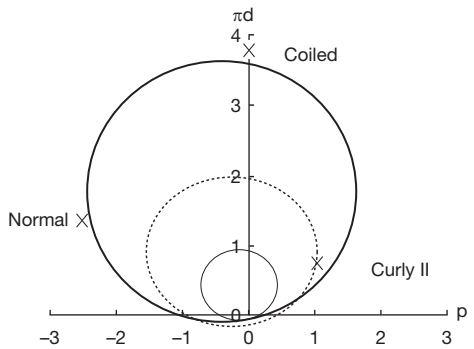

Fig. 3 The pitch–diameter (p–πd) plot. In the p–πd plot, a set of polymorphs of one type of flagella is placed on a circle. The handedness was expressed as + (for right handed) or − (for left handed) of the pitch value. Polymorphs of *Salmonella* flagellum stay on a circle (*thick circle*) in the p–πd plot: Normal, Coiled, and Curly II are shown (X). Polymorphs of some other species which have peritrichous flagella also stay on the same circle and thus belong to Family I (F1). Family II (F2, *dotted circle*) and Family III (F3, *thin circle*) show smaller circles in the p–πd plot.

defined for *Salmonella typhimurium*. In 2005, we found that the polar flagellum of *Idiomarina loihiensis* appeared "Curly with left-handed helix," which urged us to reexamine all flagella shapes known by then. Indeed, it turned out that polymorphs of polar flagella form a family distinguished from those of peritrichous flagella. A helix is uniquely defined by three parameters: the pitch (p), the helix diameter (d), and the handedness. If the handedness was expressed as + (right handed) or − (left handed) of the pitch value, any helices will be plotted on the pitch–diameter (p–πd) plane. For example, the parameters of the Normal filament are written as (−2.55, 1.88) and those of the Curly filament as (+1.20, 0.63).

Note that πd is better than d; if a tube was flattened, a unique d disappears but πd (periphery) remains constant. Polymorphs of the *Salmonella* flagellum stay on a circle in the pitch–diameter (p–πd) plot (Fig. 3), indicating that they all belong to one family (Family I) predicted by the Calladine model. The flagellar polymorphs of *I. loihiensis* (Family II) are smaller than those of Family I. Precisely to say, the pitch and diameter of Family II flagella are half of the same of Family I flagella. Furthermore, lateral flagella had helical parameters much smaller than those of the two families and thus belonged to a new family (Family III).

Flagellin

The component protein of the filament is called flagellin. Although the flagellum of many bacteria is composed of one kind of flagellin, some flagella consist of more than two kinds of closely related subspecies of flagellin. The molecular size of flagellin ranges from 20 to 60 kDa. Enterobacteria tend to have larger molecules, while species living in fresh water have smaller molecules.

The three-dimensional structure of the flagellin in the filament of *S. typhimurium* has been solved at the atomic level.

One of the most characteristic features of flagellin is evident even in the primary structure of the molecule; the amino acid sequences of both terminal regions are well conserved, whereas that of the central region is highly variable even among species or subspecies of the same genera. As a matter of fact, this hypervariability of the central region gives rise to hundreds of serotypes of *Salmonella* spp.

The terminal regions are essential for binding of each molecule to another to polymerize into a filament. Complete folding of flagellin occurs during assembly; although the terminal regions do not take on any specific secondary structure in solution, they are converted into an α-helix upon polymerization.

In the filament, the terminal regions are located at the innermost radius of a cylindrical structure, while the central region is exposed to the outside. It should be noticed that the filament is extremely stable; it does not depolymerize in water, in contrast to actin filaments or tubulin filaments which depolymerize in the absence of salts.

The description of the flagellin molecule is not applicable to that found in archaea. Archaeal flagella seem to have a system totally different from bacterial flagella in several aspects; (1) archael flagellins have signal sequences; (2) there is no channel in the filament; and (3) the filament structure resembles that of the type IV pilus.

Flagellin can be posttranslationally modified. *Salmonella* flagellin is methylated at several Lys residues by a methylase encoded by the *fliB* gene neighboring the *fliC* gene. The role of methylation of flagellin is not clear, because *fliB* mutants behave like the wild-type under ordinary conditions. *Pseudomonas syringae* flagellin is glycosylated at six Ser residues. Deglycosylation of the flagellin results in the loss of virulence to the host plant rice. Two types of flagellins of *Pseudomonas aeruginosa* are also glycosylated, but the meaning of modification is not clear. Archaeal (*Methanococcus voltae*) flagellins are highly glycosylated, which is important for flagellar assembly.

Cap Protein

Flagella have been regarded as a self-assembly systems. Indeed, flagellin can polymerize into flagella under conditions that commonly promote protein crystallization in vitro. However, in vivo, flagellin assembly requires another protein, without which the flagellin is secreted into the medium as monomers. The protein that helps filament formation is located at the tip and is thus called the cap protein or HAP2 or FliD.

The three-dimensional structure of the cap has been solved at the atomic level. The cap proteins assemble in a pentamer, forming a star-shaped structure. The star hands fit in the grooves of flagellin subunits at the tip of flagellum, leaving a small gap for a nascent flagellin to insert.

Hook

Shape

The hook, as the name suggests, is more sharply curved (almost right-angled) than the filament and is much shorter. The curvature indicates the flexibility of the hook, though it has to be stiff enough to transmit torque generated at the basal structure to the filament. From these physical properties, the hook has been regarded as a universal flexible joint. The physical property of the hook is important in understanding the behavior of the tethered cells (see "Function" section).

The length of the hook is 55 nm with a standard deviation of 6 nm, which is rather well controlled at aconstant size when compared with the length of filament. However, this large standard deviation, 10% of the mean, indicates that the hook length control is not deterministic but probabilistic. Polyhook is a mutant hook of indefinite length, obtained in *fliK* mutants. There are several factors that affect hook length: FliK, the hook protein FlgE, the hook cap protein FlgD, and C-ring components (FliG, FliM, and FliN). Among them, FliK is a major factor for hook length control, because hook length in various species is proportional to the molecular length of the corresponding FliK (see section "Morphological Pathway").

It is not clear whether the polyhook is a tandem polymer of the wild-type hook. Its shape is a right-handed superhelix. The wild type hook has the same superhelix but consists of about one-fourth of the helical pitch, which is 55 nm.

Hook protein

The hook is a tubular polymer made of a single kind of protein: hook protein or FlgE. The molecular size of hook protein varies from 264 amino acids (*Bacillus subtilis*) to 718 amino acids (*H. pylori*), but is around 400 amino acids for most species.

The architecture of hook protein resembles that of flagellin: the amino acid sequence in both terminal regions is well conserved, but in the central region it is variable. Hook protein folding also completes on assembly. The three-dimensional structure of the hook protein in the hook has been solved at the atomic level.

The amino acid sequences of the terminal regions of FlgE is homologuous with that of FlgG,or the distal rod (see "Rod" section). There is an extra sequence of 18 amino acids in the N-terminal region of FlgG. Insertion of the amino acid fragment into FlgE causes the change of hook curved shape into straight shape. Consequently, the physical properties of the hook change; it is not flexible anymore but stiff so that flagellar function as a propeller is deteriorated.

Spirochete flagella are confined in the periplasmic space and are called periplasmic flagella. There must be enormous friction between the periplasmic flagella and membranes when the flagella rotate. Although the hook looks ordinarily curved, the architecture is quite different from that of any other hooks. The hook protein subunits are covalently cross-linked to each other in a self-catalyzing manner, resulting in unusually stable hooks.

Scaffolding Protein FlgD

The hook does not self-assemble in vivo; it requires a helper protein, FlgD, which functions in a similar way as FliD does for filament formation. FlgD sits at the tip of the nascent hook to polymerize hook protein coming out from the central channel. When the hook length reaches around 55 nm, FlgD is replaced by HAP1 (FlgK), which remains in the mature flagellum. Because of its temporary existence, FlgD is regarded as a scaffolding protein.

Hook-associated proteins

There are two minor proteins between the hook and filament. They are called hook-associated proteins (HAPs), because they were found at the tip of the hook in several filament-less mutants. Originally, there were thought to be three HAPs, called HAP1–3 in the order of their molecular size. HAP2 (FliD) turned out to be located at the tip of the filament as described above, leaving HAP1 (FlgK) and HAP3 (FlgL) between the hook and filament. They are, therefore, hook–filament junction proteins.

The number of subunits of HAP1 and HAP3 in a filament are estimated to be five or six, indicating that they form one-layer rings sitting one on another. The roles of these two HAPs have been ambiguous. The idea of a connector to smooth the junction between the two polymers is blurred by the question, 'why are not one but two kinds necessary.' In a mutant of HAP3, filaments undergo polymorphic transitions so easily that cells cannot swim smoothly, suggesting a specific role of HAP3 as a stabilizer of filament structure.

Basal Structure

Flagella are anchored in the cell wall. The structural entity for the anchoring was originally called the basal structure or basal granule, hinted at by vague images by electron microscopy. Since DePamphilis and Adler in 1974 defined the details of the basal structure, it has been called the basal body. The basal body typically consists of four rings and one rod in Gram-negatives.

The basal body, when isolated from the cellbody, contains some stable components necessary for flagellar function, but fragile and soluble components were detached from the basal body during purification. In 1985, one such fragile structure was found attached to basal bodies purified by a modified method; it was named as the cytoplasmic ring (C ring). In 1990, another rod-like structure was found in the center of the C ring and named as the C rod. In 2006, flagellar export ATPase (FliI) was found at the periphery of the C ring as a complex with the supporter protein FliH. In 2011, the electron cryomicroscopy revealed that those soluble proteins actually form a dome-like structure beneath the C ring. Therefore, the in situ basal structure is a gigantic structure consisting of the basal body, the C ring, the C rod, export ATPase, and a few accessary proteins.

Basal body

The basal body contains rings and a rod penetrating through them. The number of rings varies depending on the membrane systems: four rings in most of Gram-negatives and two rings in Gram-positives exemplified by *B. subtilis*. Some variations in the number (such as five rings in *C. crescentus*) have been occasionally seen. The fifth ring might be a ghost image of electron microscopy or could be erroneously added during ring formation (see "Rod" section).

The structure of the basal body of *S. typhimurium* has been extensively analyzed. The physical and biochemical properties of the substructures of the basal body described below are from *S. typhimurium*, unless otherwise stated.

MS-ring complex

Earlier studies on flagellar motor function assumed that torque would be generated between the M and S rings, which face each other on the inner membrane. However in 1990, it was shown that a single kind of protein, FliF, self-assembles into a complex consisting of the M and S rings and a part of the rod. FliF is 65 kDa, the largest of the flagellar proteins. It contains no cysteine

residues. Overproduction of FliF in *Escherichia coli* gives rise to numerous MS-ring complexes packed in the inner membrane, indicating that the central channel is physically closed. Image analysis revealed that the MS-ring complex is composed of 24–26 subunits of FliF.

The S ring has been seen in the basal bodies from all the species studied so far (>30 examples). It stays just above the inner membrane and has no apparent interaction with any other structures, thus named S (supramembrane) ring. Besides, it is very thin and the role of the S ring remains mysterious.

Although the MS-ring complex is no longer regarded as the functional center of the flagellar motor, it is still the structural center of the basal structure and, as will be seen later (see "Genetics" section), plays an important role in flagellar assembly.

Rod

The rod is not as simple as its name suggests; it is structurally separated into two parts: the proximal rod and the distal rod of 10 nm length each. The proximal rod consists of four proteins (FliE, FlgB, FlgC, and FlgF), while the distal rod consists of only one kind of protein FlgG. Filaments, when sheared off from the cell body by external physical force, often retain the hooks and the distal rods at the proximal ends, indicating that the rod breaks at the midpoint between FlgF and FlgG.

Rod formation seems complicated because of the five component proteins. No intermediate rod structure has been observed; either there is a whole rod or no rod at all. Two *flgG* point (G65V, G183R) mutants produce distal rods ca. 60 nm long. Double mutants of a *flgG* point (G65 V) mutation and *fliK* deletion give rise to polyrods, rods of undefined length, indicating that FliK controls the polyrod length by the same mechanism for the hook-length control. In 2017, it was shown that intrinsic rod-length is limited by the width of the periplasmic space as short as 10 nm, indicating that the physical barrier of the outer membrane stops further elongation of the rod.

Since many P rings can be formed on the long distal rod, FlgG is the target for interaction with FlgI, the subunit of the P ring (see below).

LP-ring complex

The outermost ring, the L ring, interacts with the lipopolysaccharide layer of the outer membrane, and the P ring just beneath the L ring may bind to the peptidoglycan layer. The LP-ring complex works as a bushing, fixed firmly enough to hold the entire flagellar structure stably in the cell surface.

The component proteins, FlgH for the L ring and FlgI for the P ring, have signal peptides, indicating that they are secreted through the general secretory pathway (GSP), which is the exception for flagellar proteins as will be seen later (see "Genetics" section). FlgH undergoes lipoyl modification.

P ring formation precedes L ring formation; without the P ring, L ring formation does not occur. Once the L and P rings have bound together to form the LP-ring complex by an unknown mechanism, this complex is extraordinarily resistant against extremes of pH or temperature. Treatments with pH 11, pH 2, or boiling for a minute do not destroy the complex, confirming that the complex serves as a rigid bushing in the outer membrane. However, essential roles of the complex are still ambiguous because no corresponding structure has been found in Gram-positive bacteria and spirochaetes.

C ring

The C ring is missing from the basal structure when biochemically isolated. It is resistant to the nonionic detergent Triton X-100 but is destroyed by the alkaline pH or high concentration of salts employed by the conventional purification method. The shape of the C ring is easily flattened on a grid during preparation for electron microscopy. By electron cryomicroscopy, the C ring shows a cup shape, whose structure is solved at 2-nm resolution.

Genetic studies revealed that the C ring plays important roles in chemotaxis, torque generation, and flagellar formation. The C ring consists of the switch proteins (FliG, FliM, and FliN) necessary for changing the rotational direction of the motor, and so is called the switch complex. FliG directly binds to the cytoplasmic surface of the M ring. FliM binds to FliG, and FliN to FliM. Stoichiometry of these molecules in the C ring is determined from the high-resolution image of the C ring: 24–26 copies of FliG, 32–36 copies of FliM and FliN. The copy number of FliN can be increased up to 100 without affecting the motor function. FliM in the C ring directly binds signal molecules, CheY, produced in the sensory transduction system, but the mechanism of the switching is still ambiguous.

The role of the C ring in flagellar formation is clear in deletion mutants of one of the three genes; the mutant cells are non-flagellated. Point mutations in one of these genes result in short hooks, indicating the C ring is involved in regulation of secretion of at least hook proteins (see "Hook" section).

Export apparatus

Flagella have been regarded as a self-assembly system, similar to that of bacteriophage. However, flagellar assembly is quite different from phage assembly in many ways. First, the flagellum, being an extracellular structure, assembles not in the cytoplasm but outside the cell. Second, the component proteins have to be transported from the cytoplasm to the outside. Third, assembly proceeds in a one-by-one manner at the distal end of the nascent structure.

For this kind of assembly, a protein secretion system must play an important role in an ordered secretion consistent with cell growth and cell division. As a matter of fact, among 14 genes required in the very first step of flagellar assembly, more than half of

the gene products are necessary to form a protein complex called an export (or secretion) apparatus. One of them, FliI, has an ATPase activity, suggesting that one step in the export process requires ATP hydrolysis as an energy source. However, transport of substrates through the channel in the axial structures (the rod, hook, and filament) of the flagellum utilizes PMF as energy source. Thus, FliI may be used at the first step of the process, that is, insertion of substrates into the secretion gate. However, flagellin, the most abundant substrate, does not use FliI for secretion.

The physical body of the secretion gate has not been identified yet; the C rod is a strong candidate, judging from its location at the center of the C ring. Genetic analysis indicates that at least five components are required for the C rod: FliP, FliQ, FliR, FlhA, and FlhB, all of which have membrane-spanning region(s). How these component proteins are inserted in the small space of the central area of the MS-ring complex is still mysterious (see "Morphological Pathway" section).

Function

The function of flagella is described here briefly so that the meaning of structure can be understood. Bacterial flagella rotate. There is no correlation between bacterial flagella and eukaryotic flagella either in function or in structure; the type of movement, the energy source, and the number of component proteins differ greatly between the two. No evolutionary correlation between these two types of flagella has been shown. Among motile bacterial species, swimming by flagellar rotation is the most common. However, the flagella of *Spirochetes* are an exception; flagella rotate in the periplasm and accordingly the helical cell body rotates to move in a viscous medium or glide on the surface. Several families such as *Myxococcus*, *Mycoplasma*, and *Cyanobacteria* do not have flagella but move on a solid surface by a gliding motion; *Myxococcus* moves by means of type IV pili and *Mycoplasm* has motor proteins on the cell surface as the locomotive organ.

Torque

Rotational force (torque) of the flagellar motor is difficult to measure directly but can be estimated from the rotational speed of flagella. The method most widely employed is the tethered cell method, developed by Silverman and Simon in 1974. The rotation of a cell body caused by a tethered filament can be observed with an ordinary optical microscope. Rotating flagella on a cell can be observed by dark-field microscopy, developed independently by Robert M. Macnab and Hirokazu Hotani in 1976. Using laser as the light source of a dark-field microscope, the rotational speeds of a flagellum on a cell stuck on the glass surface were measured at the time resolution of milliseconds. A more sophisticated method employs the fluorescent microscope to detect the rotation of fluorescent beads attached to the hook. Most of the experiments for measuring torque have been done by Howard C. Berg and his colleagues, followed by several Japanese groups.

Rotational direction

Flagella of many species (e.g., enterobacteria) can rotate in both the directions: clockwise (CW) and counterclockwise (CCW).

Under ordinary circumstances, around 70% of the time is occupied by CCW rotation, which causes smooth swimming. A brief period of CW rotation causes a tumbling motion of the cell. There is no perceptible pause in switching between the two modes.

In some bacterial species such as *Rhodobacter sphaeroides*, a lateral flagellum on a cell rotates in the CW direction only with occasional pauses. During pauses, the filament takes on a Coiled form and curls up near the cell surface. Upon application of torque on this filament, the Coiled form extends to a semistable, right-handed form that closely resembles a Curly form. The CW rotation of this right-handed helix causes a forward propulsive force on the cell.

Rotational speed

Flagella on a stuck cell (the cell body attaches to the glass surface but flagella freely rotate) rotate at ca. 200 Hz, which is comparable with that of a free-swimming cell. In contrast, tethered cells (one flagellum attaches to the glass surface but the cell body freely rotates) rotate only at ca. 20 Hz. It has been long believed that the cell body is too large and thus hydrodynamically too heavy for a tiny motor to rotate faster. However in 2007, we have shown that tethered cells almost always interact with the surface; the distance between the cell and the glass surface is only 55 nm on average, which is determined by the hook length. The hook seems to play an active role as a flexible joint in the interaction.

The rotational speeds of flagella correlate directly with the torque and inversely with the viscosity of the solution. The correlation appears as a straight line in a speed–torque diagram; the higher the viscosity, the slower the speed is. This indicates that the torque of the flagellar motor is constant over a wide range of speed.

The highest speed so far measured is 1700 Hz for *V. alginolyticus*. However, only 10% of the torque derived from the speed is used as a propulsive force, the rest being lost in slippage of flagella in the medium. In general, cells with single polar flagellum swim ($>100\ \mu m\ s^{-1}$) faster than cells with peritrichous flagella ($<20\ \mu m\ s^{-1}$).

Energy source

The energy source of torque generation in the flagellar motor is not ATP but proton motive force (PMF). PMF is the electrochemical potential of the proton and results in the flow of protons from outside to inside the cell. PMF consists of two different forms of energy: membrane potential and entropy caused by a difference in pH between outside and inside the cell. Since these two parameters are independent and separable from each other, either one can, in principle, be abolished without affecting the other.

It is not known whether protons directly flow through the flagellar motor to generate torque or flow nearby to induce conformational changes of the component proteins in the C ring. In *V. alginolyticus*, sodium motive force (SMF) is used for the polar flagellum, whereas the lateral flagella use PMF.

One of the goals of flagellar research is the elucidation of the mechanism by which PMF or SMF is converted into torque in the motor.

Switching of rotational direction

Switching of the rotational direction of flagella is the primary basis of chemotaxis, one of the most important behaviors shown by bacteria. Damage in the switching mechanism results in a rotation biased to either CCW only or CW only. Although cells that rotate only CCW swim smoothly in liquid, they cannot form a swarm (chemotaxis) ring on a semisolid agar plate.

In a strict sense, the switching mechanism will not be solved until the mechanism of rotation is solved. However, factors involved in the mechanism are known; an effector binds to FliM of the switch complex in the flagellar motor. The effector is the phosphorylated form of CheY, a signaling protein within the sensory transduction system.

Genetics

Flagellar genetics has been most extensively studied in *S. typhimurium*, especially using the enormous number of SJW (Salmonella Japan Waseda) strains that Shigeru Yamaguchi has collected for >30 years. Once the fundamental genetics was established, molecular biology has been serving powerful tools to reveal the details. Topics regarding the *fla* genes in this section are mostly based on results obtained from *Salmonella* strains, whereas chemotaxis (*che*) genes and motility (*mot*) genes were more extensively analyzed using mostly *E. coli* and other strains (e.g., *Rhizobium* spp., *Vibrio* spp., etc.).

Flagellar Genes

There are >50 flagellar genes divided into three types according to null mutant phenotype (Fig. 4).

The fla genes

Defects in the majority of the flagellar genes result in flagellar-deficient (Fla^-) mutants. These genes were originally called *fla* genes.

In 1985 when the number of genes exceeded the number of letters in the alphabet, a unified name system for both *E. coli* and *S. typhimurium* was proposed by Robert M. Macnab and introduced in the field with approval by all contemporary geneticists. They are *flg*, *flh*, *fli*, and *flj*; each for one of the clusters of genes scattered in several regions around the chromosome (Fig. 4). When a particular gene does not have the corresponding gene in *Salmonella*, historical names are used, for example, *flaF*, *flaG*, *flbS*, and *flbT*.

The mot genes

Mutants that produce paralyzed flagella are called motility-deficient (Mot^-) mutants. There are only two *mot* genes (*motA* and *motB*) in *S. typhimurium*. In *V. alginolyticus*, there are two sets of flagella—polar and lateral. The energy source for each motor is

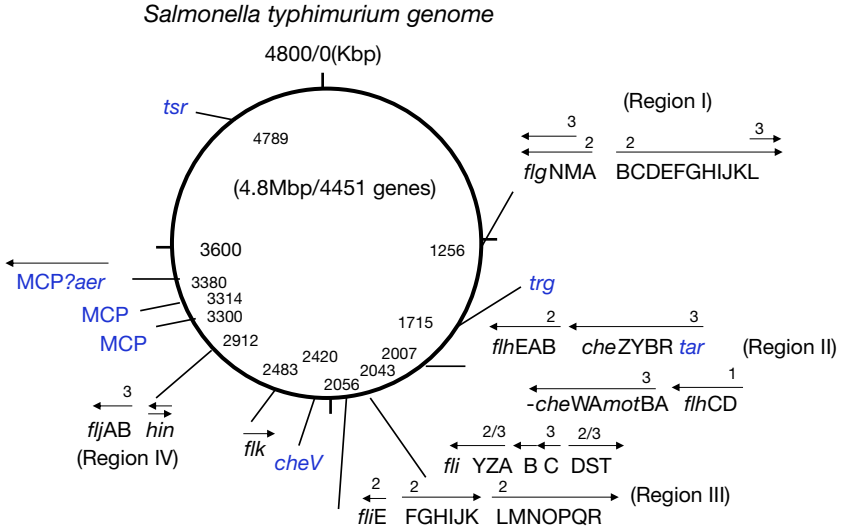

Fig. 4 Genetic map of *Salmonella typhimurium*. Flagellar genes distribute in several clusters on the chromosome. *Arrows* over genes indicate the size of operons and their transcriptional directions. The numbers on the *arrows* indicate classes of transcription. The regulation of class 2 and 3 is not simple; some operons are expressed twice in class 2 and class 3. Chemotaxis receptors (Tsr, Trg, and Tag) and receptor homologues (MCP) are scattered on the chromosome.

distinguishable; SMF for the polar flagellum and the PMF for the lateral flagella. *Mot* genes for the polar flagellum are called *pomA* and *pomB*. A certain sequence of a few amino acids in Mot proteins is responsible for selection of ions. There are four more *mot* genes (*motA*, *motB*, *motX*, and *motY*) in *V. alginolyticus*, *R. sphaeroides*, and *Aeromonas hydrophila*. *V. alginolyticus* MotX and MotY are associated with the basal body of sodium-driven polar flagellum and required for stator formation.

P. aeruginosa retains one set of *mot* genes (*motA* and *motB*) and another set (*motC* and *motD*). Although it is indicated that the *motC* and *motD* genes play an important role in pathogenicity, their roles in motor function are not known. There was renaming of some mot genes; MotD of *Sinorhizobium meliloti* and related alpha-proteobacteria turned out to be the flagellar-hook-length regulator FliK, indicating that original naming was wrong. MotE in *S. meliloti* is a chaperone specific for the periplasmic motility protein, MotC.

The che genes

Mutants that can produce functional flagella but cannot show a normal chemotactic behavior are called chemotaxis-deficient (Che^-) mutants. These are divided into two types—general chemotaxis mutants (authentic Che^-) and specific chemotaxis mutants. The former involve the proteins working in the sensory transduction (CheA, CheW, CheY, CheZ, CheB, and CheR), and the latter involve the receptor proteins for attaractants such as amino acids and sugars (e.g., Tsr, Tar, Trg, and Tap).

In some species, there are multiple homologues of the *E. coli* chemotaxis genes. For example, *R. sphaeroides* possesses $5 \times cheA$, $2 \times cheB$, $3 \times cheR$, $4 \times cheW$, and $6 \times cheY$ arranged in several operons. Thirteen chemoreceptors, including both membrane-spanning and cytoplasmic or transducer-like proteins (Tlps), have been identified (as of 2017). These are differentially expressed according to the environmental conditions. It is not known whether the products of the *che* operons operate through independent, linear pathways or there is significant cross-talk between the components of these operons.

In *V. cholerae*, there are five *cheY* genes including one putative *cheY*. It was shown that only one of them directly switches flagellar rotation. In *E. coli*, CheY dephosphorylation by CheZ extinguishes the switching signal. But instead of CheZ, many chemotactic bacteria contain CheC, CheX, and FliY for dephosphorylation.

Gene Clusters in Four Regions

Most flagellar genes are found in gene clusters on the chromosome. They are in four regions: the *flg* genes in region I, the *flh* genes and *mot* and *che* genes in region II, and the *fli* genes in regions IIIa and IIIb (Fig. 4). The *flj* operon (including *fljA* and *fljB*) in region IV involves an alternative flagellin gene to *fliC* and is only found in *Salmonella*. Either FliC flagellin or FljB flagellin is produced at one time. The *hin* gene inverts the transcriptional direction at a certain statistical frequency; if the *flj* operon is being expressed, FljA represses *fliC*, allowing FljB flagellin alone to be produced. This alternate expression of two flagellin genes is called "phase variation."

This clustering of flagellar genes is also observed in other peritrichously flagellated species; grouping of particular genes and gene order are similar to those in *S. typhimurium*. Flagellar genes for the polar flagellum in *Pseudomonas* spp. and *Vibrio* spp. form clusters in a few regions, but the gene order is different from that of peritrichous flagella. The polar flagellum requires special regulatory genes (such as *fleP*, *fleQ*, *flhF*, *flhG*) to localize one flagellum at the pole of the cell, which peritrichous flagella do not require them. Flagellar genes of some species (*C. crescentus* or *Campylobacter jejuni*) are scattered in more than seven regions; the extreme case is *H. pylori*, in which single flagellar genes, or at most three-gene clusters, are scattered all over the chromosome. It is interesting to see that a cluster, FliC (flagellin)–FliD (the capping protein)–FliS (chaperone for FliC)–FliT (chaperone for FliD), is ubiquitously found in all species but not in *H. pylori* and *Buchnera* spp. Since *H. pylori* flagellum is covered by the membranous sheath, the mechanism of flagellar assembly could be different from that of *Salmonella*. For example, *H. pylori* FlgM is not secreted but stay in the cytoplasm, and it interacts with FlhA (a component of the secretion gate).

Transcriptional Regulation

Flagellar construction requires a well-ordered expression of flagellar genes not only because there are so many genes, but also because flagellar assembly requires only one kind of component protein at a time, as described in previous sections. There is a strict hierarchy of expression among the flagellar genes. The hierarchy is controlled or maintained by a few prominent regulatory proteins.

Hierarchy: Three Classes

The hierarchy of flagellar gene expression is divided into three classes: class 1 regulates class 2 gene expression, and class 2 regulates class 3. Class 1 contains the master genes: only two genes in one operon, *flhD* and *flhC*.

Class 2 consists of 35 genes in eight operons. There are two regulatory genes, *fliA* and *flgM*; the rest are component proteins of the flagellum or the export apparatus.

Class 3 genes code flagellin, MotA and MotB, and all the Che proteins. Flagellin is one of the most abundant proteins in a cell, suggesting that the tight regulation in the hierarchy guarantees the economy of the cell.

There is another class of regulation pathway in species that produce polar flagellum. In *P. aeruginosa*, there are four classes; class 1 contains the master gene *fleQ*, class 2 contains 25 genes necessary for constructing the export apparatus, class 3 contains 12 genes necessary for completing the hook-basal body, and class 4 contains 13 genes to produce the filament and the chemotaxis system.

Master Genes, flhDC

Master gene products form a tetrameric complex of FlhD/FlhC, which works as a transcriptional activator of the class 2 operons.

The master operon (*flhDC*) is probably transcribed with the help of the "housekeeping" sigma factor, sigma70. The master operon is also activated by a complex of cyclic AMP and catabolite activator protein (cAMP–CAP), which binds to a site upstream of the promoter.

In *P. aeruginosa*, *fleQ* is the master gene instead of *flhCD*. However, *fliA* is out of the control of *fleQ*; the controlling element of *fliA* is not known yet.

Sigma Factor F (sigma F: FliA) and Antisigma Factor (FlgM)

The FliA and FlgM proteins expressed from the operon competitively regulate the class 3 operons. FliA is the sigma factor that enhances the expression of the class 3 operons, while FlgM is an antisigma factor against FliA.

If the hook and basal body have been constructed normally, FlgM is secreted into the medium through the basal body and the complete hook, allowing free FliA proteins to work on the class 3 operons. However, if the hook and basal body construction is somehow halted in the middle of process, FlgM stays in the cytoplasm in a complex with FliA, maintaining shut-off of the expression of the class 3 operons. FliK plays an important role in switching of the secretion modes (see "Hook Growth" section).

A combination of *fliK–fliA–flgM* genes has been found in genomes of all species so far studied except *Buchnera* spp. that lacks all of the *fla* genes necessary for filament formation.

Global Regulation Versus Internal Regulation

There are several external genes or factors that affect the flagellar gene expression through the master operon *flhDC*. Some of the factors show pleiotropic effects on many cellular events such as cell division, suggesting that flagellation would be finely tuned with the cell division cycle due to well-organized tasks of global regulation systems.

As described above, the master operon (*flhDC*) is probably transcribed using the "housekeeping" sigma factor, sigma70. In the last decades, other factors regulating or modulating *flhDC* expression have been identified, mainly in *E. coli*. The motility of *E. coli* cells is lost at temperatures higher than 40°C as a result of reduced *flhDC* expression. It has been shown that some of the heat-shock proteins are involved in both class 1 and class 2 gene expressions. This strongly suggests that flagellar genes are under global regulation in which the heat-shock proteins play a major role; probably the proper protein folding (or assembly) mediated by these chaperons is essential for flagellar construction.

Other adverse conditions such as high concentrations of salts, carbohydrates, or low-molecular-weight alcohols also suppress *flhDC* expression, resulting in lack of flagella. The regulation by all these factors is independent of the cAMP–CAP pathway.

Flagellar gene expression is also controlled by the ubiquitous bacterial second messenger cyclic diguanosine monophosphate (*c*-di-GMP). In response to changing environments, fluctuating levels of *c*-di-GMP inversely regulate flagellar formation and thus cell motility.

The factors and mechanism of directly turning on the master *flhDC* operon in accordance with the cell cycle are yet unknown. Another goal of the flagella research is the elucidation of the roles of global regulators on flagellar gene expression to uncover the complex regulatory network connecting flagellation and cell division.

Morphological Pathway

The order of the steps toward the construction of a flagellum (the morphological pathway) was analyzed in the same way as was used for bacteriophage—analyzing intermediate structures in various flagellar mutants and aligning them in size from small to large ones. Flagellar construction starts from the cytoplasm, progresses through the periplasmic space, and finally extends to the outside of the cell (Fig. 5).

In the Cytoplasm

The smallest flagellar structure recognizable by electron microscopy is the MS-ring complex. Since the MS-ring complex self-assembles from FliF protein alone in the cytoplasmic membrane, it has been regarded as the first structure in the pathway of the flagellum construction. However, it is not functional by itself but requires five other membrane proteins (FliP, FliQ, FliR, FlhA, and FlhB) to form the secretion gate for external structure of the flagellum (see "Export apparatus" section). It is not clear at the moment

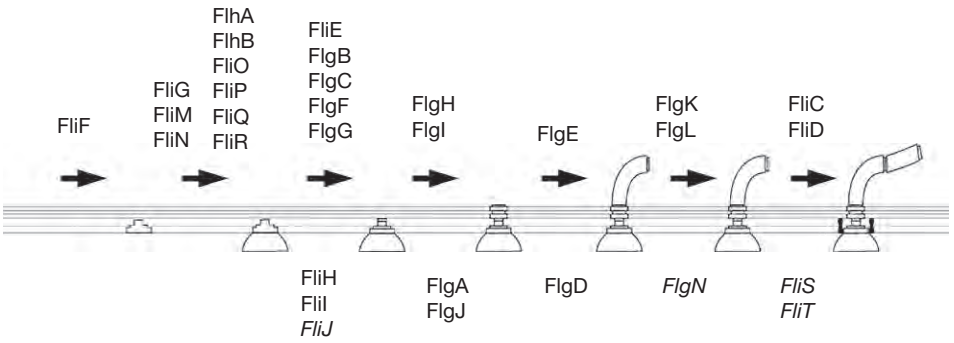

Fig. 5 Morphological pathway of flagellation. Flagellar construction proceeds from *left* to *right*. Gene products shown above the membranes are incorporated in the flagellar structure at each step. The gene products shown under the membranes are chaperones for the component protein just above the membrane (shown in *italics*) or enzymes: FliI is an ATPase, and FlgJ is muramidase.

whether the MS-ring complex comes first or a complex of the secretion-gate proteins comes first. Nevertheless, the MS-ring complex works as the construction base of the flagellum. When the C ring attaches on the cytoplasmic side of the M ring, the gigantic complex starts secreting other flagellar proteins to construct the extracellular part of the flagellum.

In the Periplasmic Space

The first extracellular structure constructed on the MS-ring complex is a rod. The proximal rod grows short but the distal rod may grow longer than 10 nm (see "Rod" section). The distal rod growth stops when it reaches the outer membrane, and then the hook starts growing. However, the outer membrane physically hampers again the hook growth until the outer-ring complex makes a hole in it. Among flagellar proteins, FlgH and FlgI, the component proteins of the outer-ring complex, are exceptional in terms of the manner of secretion; these two proteins have cleaved signal peptides and are exported through the GSP. However under special conditions, filaments can grow in the absence of the outer rings but stay in the periplasm to form the periplasmic flagella as seen in spirochaetes.

Outside the Cell

Once the physical block by the outer membrane has been removed, the hook resumes growth with the aid of FlgD until the length reaches about 55 nm. Then, FlgD is replaced by HAPs, which is followed by the filament growth. The filament growth proceeds only in the presence of FliD (HAP2 or filament cap protein); without this cap, exported flagellin molecules are lost to the medium.

Flagellar Protein Export as a Type III Secretion System

There are several ways to transport proteins outside bacterial cells. The best-known pathway is the GSP. However, many flagellar proteins cannot pass through this system, since they do not have the signal sequences that are necessary for the recognition by GSP.

To date, there are six characterized bacterial protein secretion systems (type I–VI) that are grouped according to their function in pathogenesis. Here I will briefly explain the major three types of export systems.

Type I secretion system (T1SS) secretes proteins without modification through the secretion apparatus consisting of a few component proteins that span both the inner and the outer membranes, for example, hemolysin in *E. coli*.

Type II secretion system (T2SS) secretes proteins retaining the signal peptide, which is cleaved upon secretion by GSP, for example, pullulanase in *Klebsiella oxyto*ca. Thus, GSP is a secretion machinery in T2SS.

Type III secretion system (T3SS) secretes proteins without cleavage through the gigantic secretion apparatus spanning both the inner and the outer membranes, for example, virulence factors (or effectors) from many pathogenic *Enterobacter* spp. The flagellar protein export system is now regarded as a T3SS. The secretion gate of the flagellar export apparatus consists of five components (FlhA, FlhB, FliP, FliQ, and FliR). The amino acid sequences of these proteins share homology with those for secretion of effectors in many pathogenic bacteria. The structures between these two distinguishable systems resemble each other; the secretion apparatus for effectors is the needle complex, which looks like the flagellar basal body, consisting of several ring structures and a needle. It is now suspected that flagellum and pathogenesis might be derived from a common ancestor.

The two secretion systems are superficially independent from each other in *S. typhimurium*. However, it is now known that the *Salmonella* pathogenesis island (SPI) 1 gene expression is regulated by a flagellar gene *fliZ* in serovar Typhimurium and is dependent on flagellar sigma factor FliA in serovar Typhi. Note that *fliA* and *fliZ* sit next to each other in an flagellar operon.

The Kinetics of Morphogenesis

The morphological pathway of the flagellum described above indicates the order of the construction steps but ignores the time to be consumed at each step. In order to achieve coherent cell activities, flagellar construction has to be synchronized with cell division. The most time-consuming step of flagellation seems to be the first step, the construction of the export apparatus, which takes almost one generation to complete. The filament elongation also takes time; filaments grow over generations in peritrichously-flagellated cells. The growth processes of the filament and the hook have been carefully analyzed. By taking a closer look at elongation modes of these two polymers, we will get a glimpse of the whole kinetic process of flagellar construction.

Filament Growth

In bacteria with peritrichous flagella, the number and the length of flagella are, if not exactly, fairly well defined; there are 7–10 flagella per cell and the average length of filament is 5–8 μm.

A defined number of flagella have to be supplied at each cell division. A large deviation from this number will cause disastrous results to the cell—either no flagella at all or too many to swim. The number of flagella must be genetically controlled, but the gene(s) for this role has not been identified.

On the other hand, filament growth is dependent on physiological conditions and it continues over generations. Although filament length seems free from genetic control in peritrichous flagellated bacteria, it is genetically controlled in polarly-flagellated species; the *flaG* gene is suggested to control of flagellar length in *C. jejuni*.

From statistical analysis of the length distribution, the elongation rate of filaments is inversely proportional to the length; thus, a filament grows rapidly in the beginning and gradually slows down to a negligible rate. Direct observations of growth rate of indivisual filaments have been attempted using fluorescent labeling method. In a short range, the growth rate is constant, whereas in a long range the rate changes from quick to slow, agreeing with the stastical data.

Hook Growth

In contrast to the wide distribution of filament lengths, hook length is rather well controlled at 55 nm with a deviation of 6 nm. Hook length is controlled by a secreted protein FliK; deletion of the *fliK* gene results in hooks with undefined length, called polyhooks. Engineered FliK, either elongated by insertion of foreign sequences or shortened by internal deletions, gives rise to hooks whose length is proportional to the molecular size of the mutant FliK. Thus, FliK is a molecular ruler. In 2014, we showed that FliK determined the minimal length of the hook; hooks shorter than 30 nm have been rarely observed. On the other hand, polyhooks (in a *fliK*-deletion mutant) are ready to form filament immediately after FliK is expressed from a plasmid. Therefore, FliK does not have to measure hook length but simply switches the secretion gate into the mode for FlgM to be secreted. Next, we want to know how FliK passes through the gate without switching to occur when hook length is still short.

Statistical analysis of the length distribution of polyhooks reveals that the hook grows in a similar manner as the filament does; it starts outgrowing at 40 nm min^{-1} and exponentially slows down to reach a length of 55 nm. After the length is 55 nm, the hook grows at a constant rate of 8 nm min^{-1}. It takes many generations for polyhooks to grow as long as several micrometers. We have also known that mutations in switch proteins (FliG, FliM, and FliN) gave rise to short hooks with a defined length. Therefore, kinetics of substrate secretion would be involved in this event, though relationship between FliK and the C ring cup has been unsolved. Studies of the correlation between flagellation and cell division are under way, but no definite schemes have been found.

Conclusion

In previous editions, I wrote, “Analysis of the flagellar structure has been coming to an end”; but since then many unexpected facts were revealed. We are proud of the brilliant results of our research; most components of the flagellum were identified, some of the substructures including the filament and the hook were sloved at the atomic resolution, the pathway of flagellar construction has been revealed, and roles of ca. 40 flagellar genes in the flagellar construction are now known. However, I now realize that nature is not so shallow to reveal everything in the short life of a man. We have to keep asking “Why?” with indefatigable enthusiasm to unveil mysteries of the flagellum.

We do not yet know the physics principles of flagellar rotation. One of the immediate goals is to answer a simple but important question; what is rotating against what and how? This question stems from the controversy that has started from the beginning of flagellar research. Without knowing the rotor and the stator in detail, the mechanism of motor function will never be understood. And then, we want to answer a more intriguing and difficult question: what is the ancestor of the flagellum? The question arose from the recent discovery of similarity between the flagellum and pathogenicity: (1) the supramolecular structures of the flagellum and the needle resemble each other, and (2) the gene sequences of the major components in their basal structure are homologous. This also leads us to the most primitive question: what is the flagellum?

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Relevant Website

micro, n.d.—http://www-micro.msb.le.ac.uk/–Microbiology@ Leicester.

Bacterial Iron Acquisition Strategies

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Introduction

Iron is a fundamental building block for life. The dynamic oxidation states of this transition metal are required for the catalysis of many essential biochemical reactions. Consistent with this, iron typically exists in the ferric (+3) or ferrous (+2) oxidation states. However, the ease with which iron transitions between these oxidation states and its low solubility at neutral pH are two problems organisms must solve. The solution is to complex iron within cofactors or proteins. Thus, this highly reactive atom is rarely "freely" available to react with sensitive molecules within organisms. Iron-containing cofactors and proteins allow cells to harness the chemical dynamism of iron without the associated toxicity or insolubility. Numerous chemical reactions involved in central metabolism, nucleotide synthesis, and the detoxification of reactive oxygen and nitrogen species are catalyzed by enzymes that contain one or more iron atom, underscoring the essentiality of iron to cellular physiology.

An additional benefit of maintaining low levels of "free" iron is the challenge that the limitation imposes on invading bacterial pathogens. Most pathogens, like their hosts, require iron to perform essential biochemical reactions, but pathogens are completely dependent on sources of iron present within the host environment to fulfill this requirement. The host exploits this requirement and utilizes multiple mechanisms to impede pathogen iron procurement. The process of inhibiting the proliferation of invading bacteria by obstructing access to essential nutrients, such as iron, is called "nutritional immunity". To overcome nutritional immunity, bacterial pathogens have evolved sophisticated systems to procure iron from the host environment. The focus of this article includes the sources of iron available to pathogens within the host and the strategies bacterial pathogens employ to acquire and maintain iron homeostasis.

Sources of Iron Within the Host

Low solubility at physiological pH and a high propensity for oxidation necessitate that iron be complexed to a molecule. Numerous iron-binding molecules present in the host achieve this goal. In vertebrates, iron is predominantly bound to proteins or the tetrapyrrole heme. Iron-binding and heme-binding proteins also sequester iron from invading bacteria. Additionally, iron is principally contained within host cells, further obstructing the accessibility of this metal to pathogens. Extracellular iron is bound by high-affinity iron-binding proteins such as ferritin, transferrin, and lactoferrin. Extracellular heme is sequestered by hemopexin. Together, these mechanisms of iron sequestration ensure that the host environment is relatively limited in "free" iron. However, pathogens have evolved elegant systems to extract iron from these proteins.

The most abundant iron-containing molecule in vertebrates is the tetrapyrrole heme. Consequently, many pathogens encode heme acquisition systems to steal heme-iron from the host. Heme is predominantly complexed to the protein hemoglobin within erythrocytes. In this context, heme functions to transport oxygen to and carbon dioxide away from host tissues. Consistent with this, erythrocyte lysis is a virulence trait associated with many bacterial pathogens, and this trait functions to liberate a great deal of iron in the form of heme-bound hemoglobin. Pathogens express elaborate systems composed of dedicated hemoglobin and heme receptors, as well as, transport proteins that import and degrade host heme, fulfilling the iron requirement. However, because iron can yield highly reactive hydroxyl radicals through the Fenton reaction, which are toxic at high concentrations, the systems that function to scavenge iron or heme from the host environment are under strict transcriptional control. As such, pathogens express iron acquisition systems only when the cell resides within iron deplete environments. In most bacteria this transcriptional control is achieved by the **f**erric **u**ptake **r**egulator, Fur.

Transcriptional Control of Iron Acquisition Systems

Despite the abundance of iron within vertebrates, iron-binding proteins and the vast intracellular reservoirs are not readily available to invading pathogens. Therefore, upon breach of protective barriers and invasion into tissues, the pathogen encounters an iron-deplete environment. Concomitantly, the microbial invaders upregulate genes encoding iron acquisition systems. Fur is the master transcriptional regulator of iron acquisition systems and Fur-dependent transcriptional control is achieved via a canonical mechanism of repression. Within the bacterial cytoplasm, Fur binds excess iron and represses the expression of iron acquisition systems. Repression of target transcripts is mediated by the high affinity of iron-bound Fur for consensus DNA sequences present within the promoter sequences of genes encoding iron acquisition systems. Fur binding blocks RNA polymerase, resulting in transcriptional repression. When iron becomes limiting, the amount of intracellular iron decreases. This causes the release of iron from Fur and subsequent reduced DNA-binding affinity for the Fur consensus sequence. Consequently, the target promoter becomes accessible to RNA polymerase that transcribes iron acquisition genes. Fur is conserved in both Gram-positive and

Gram-negative pathogens, and locating the Fur consensus sequences within bacterial genomes has led to the identification of many of the iron acquisition systems discussed below. The importance of this transcriptional regulator is demonstrated by the profound virulence defects of *fur* mutants. Additionally, Fur activity ensures that iron acquisition systems are expressed exclusively in iron-deplete environments. Unregulated expression of these systems is energetically costly to the bacterium in iron-replete conditions when iron import is not needed. Constitutive import of iron is also detrimental to bacterial cells as excessively high levels of iron can lead to production of damaging reactive oxygen species through the Fenton reaction. Therefore, while iron is crucial to bacterial proliferation, regulation of these systems is equally as important.

Siderophore-Mediated Iron Acquisition

A major of class of Fur-regulated genes encode enzymes that synthesize low molecular weight (<1 kDa), ferric (Fe^{3+}) iron-binding compounds called siderophores. The high affinity siderophores have for ferric iron allows these molecules to outcompete most host proteins. For example, the ferric binding constants of siderophores have been reported to be as high as 10^{-52} M, which is much greater than the binding constant of transferrin, 10^{-23} M. Siderophore-mediated iron acquisition is a common iron procurement strategy that is widely conserved among pathogenic and non-pathogenic bacteria but also fungi, yeast, and animals. Consistent with this, over 500 different siderophores has been described. Often, different species of bacteria produce identical siderophores; however, some pathogens produce unique siderophores to prevent competing microbes from stealing their siderophore. In bacteria, the genes involved in siderophore biosynthesis and import are typically present within a single operon (Fig. 1). Specifically, these operons usually consist of biosynthetic enzymes, siderophore efflux pumps, siderophore-iron complex importers, and accessory proteins that aid in siderophore function. The operons present in Gram-negative bacteria also contain outer membrane-localized receptors specific to a particular siderophore. The importance of siderophores to bacterial pathogenesis has been established in numerous studies demonstrating that mutant strains devoid of siderophore production are severely attenuated in many different models of infection. Additionally, a number of pathogens synthesize more than one siderophore and mutant strains that are restricted to the synthesis of a single siderophore often demonstrate organ-specific virulence defects. These findings demonstrate the temporal and spatial specificity between different siderophores and have prompted investigators to evaluate the potential of siderophores as vaccine candidates. Thus far, two reports demonstrate that immunization with siderophores mounts an effective host immune response that reduces bacterial burdens within the vaccinated host. Thus, host antibodies that bind the siderophore can be generated that obstruct siderophore-mediated import of host iron into the pathogen.

Siderophores are classified into five different families based on the chemical signature of the iron-binding moiety. Catecholate, phenolate, hydroxymate, and α-hydroxycarboxylate represent four of the five classifications. The fifth classification represents mixed siderophores that contain more than one iron-binding moiety. Siderophore synthesis occurs via one of two mechanisms. The first

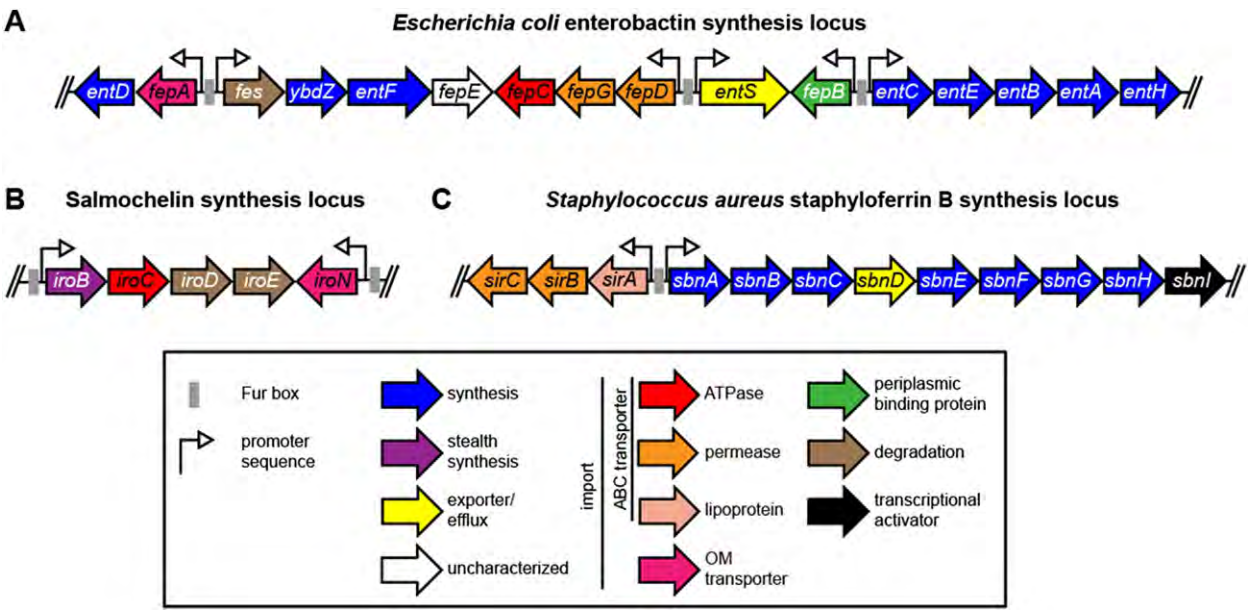

Fig. 1 The genes encoding siderophore-mediated iron acquisition systems are often located in close proximity within the genome. (A) An illustration of the genes required for enterobactin synthesis in *E. coli*. (B) Proteins encoded by the *iro* genes synthesize, import, and degrade the stealth siderophore salmochelin. (C) Production of the siderophores staphyloferrin A and staphyloferrin B is conserved in the Gram-positive pathogen *S. aureus*. The *sbn* operon encodes the proteins needed to facilitate staphyloferrin B synthesis and import. The SirABC complex is an ABC transporter. The ATPase that generates the energy needed for staphyloferrin B import is FhuC, which is encoded elsewhere in the genome. The functions of the proteins encoded by the genes are based on reference material or UNIprot analysis. The genes not drawn to scale.

mechanism is dependent on large multi-enzyme complexes called non-ribosomal peptide synthetases (NRPSs) that bind precursor molecules and catalyze siderophore synthesis using energy generated from ATP hydrolysis. Specific genes within the siderophore operon create the precursor molecules needed for the NRPS. Next, the precursor molecules are assembled into the siderophore product with the aid of other enzymes present within the NRPS complex. Alternatively, siderophore biosynthesis can occur independently of the NRPS. The NRPS-independent pathways are not as well studied; however, recent reports have shown NRPS-independent siderophores contain their own synthetases within the siderophore operon that catalyze the formation of amide or ester bonds between the precursor molecules via condensation reactions. In both systems, the final product is then effluxed from the bacterium using dedicated, membrane-bound exporters.

Upon siderophore binding of iron in the extracellular space, the molecule undergoes a conformational change, allowing the iron-siderophore complex to be recognized by specific bacterial cell surface receptors expressed within the outer membrane of Gram-negative bacteria. This interaction facilitates import. The outer membrane-localized receptors typically consist of a beta-barrel structure with a domain inside the barrel that creates a "plug". Studies have shown that reconfiguration of the "plug" domain allows for the passage of the siderophore-iron complex through the barrel. The energy needed to traffic the siderophore across the outer membrane and into the periplasm is provided by the TonB complex. This complex, consists of three proteins, TonB, ExbB, and ExbD, utilizes the proton motive force (PMF) to couple energy from the inner membrane to drive import of the substrate. Once the iron-siderophore complex is transported into the periplasm, transporters of the ATP-binding cassette (ABC) superfamily are responsible for passage of the complex across the inner membrane and into the cytoplasm. The energy required for this transport is generated via ATP hydrolysis. Gram-positive bacteria also use ABC transporters to import the siderophore-iron complex. ABC transporters typically consist of three distinct functional units: a lipoprotein that binds the siderophore-iron substrate in the periplasm (or within the cell envelope in the case of Gram-positive bacteria), a permease composed of two proteins located within the cytoplasmic membrane that function as the channel through which the siderophore-iron complex traverses, and a cytoplasmic ATP hydrolysis enzyme. Typically, the genes encoding a siderophore ABC transporter are located in close proximity within the genome to the genes encoding the siderophore biosynthesis enzymes (Fig. 1). However, some pathogens express orphan ABC transporters that promiscuously import siderophores produced by other bacteria.

Once in the cytoplasm, iron must be liberated from the siderophore for it to be utilized in various enzymatic processes. Two unique mechanisms facilitate the release of iron from the siderophore. The first mechanism is mediated by enzyme called ferrisiderophore reductases that catalyzes the reduction of ferric iron, Fe^{3+}, to ferrous iron, Fe^{2+}. Because siderophores bind Fe^{2+} with a lower affinity than Fe^{3+}, the Fe^{2+} is easily extracted. Alternatively, some bacteria release iron through enzymatic hydrolysis of the siderophore; however, this disrupts the integrity of the complex and is costly because the siderophore is unable to be reused. Therefore, an advantage of the reductase-mediated strategy of iron release is that the siderophore can be recycled to retrieve another Fe^{+3} atom.

In the continuous battle between host and pathogen, vertebrates are equipped to impede siderophore-mediated iron acquisition. The lipocalin family of proteins function to bind and sequester catecholate- and phenolate-type siderophores. For instance, lipocalin 2 (also referred to as 24p3, NGAL, or siderocalin) binds enterobactin, a siderophore commonly produced by species within the family *Enterobacteriaceae*. The importance of this siderophore countermeasure is demonstrated by the observation that lipocalin 2-deficient mice are more susceptible to infections caused by Gram-negative bacteria. In response to the selective pressure applied by the lipocalin family of proteins, pathogens adapted by evolving mechanisms to chemically modify their siderophores, blocking recognition by host siderophore-binding proteins. These so called stealth siderophores contain slight chemical modifications to an existing siderophore, maintaining the high affinity for iron but abolishing recognition by lipocalin family of host proteins (Fig. 1(B)). For example, *Escherichia coli* synthesizes the siderophore enterobactin, which is bound by lipocalin 2. However, some strains of *E. coli* also encode the enzyme, *iroB*, which glycosylates enterobactin, resulting in a modified enterobactin called salmochelin. Lipocalin 2 fails to bind salmochelin, allowing the stealth siderophore to bypass host sequestration of enterobactin such that *E. coli* can achieve unperturbed acquisition of host iron. In addition to the synthesis of stealth siderophores, many pathogens are also equipped with the capacity to scavenge siderophores produced by other bacterial species. Together these mechanisms enhance the iron acquisition strategies of invading pathogens ensuring that the requirement for iron is satisfied during infection.

Iron Acquisition Through Targeting Host Heme

Heme represents the most abundant iron-containing molecule in vertebrates. Not surprisingly, many pathogens, especially those capable of proliferating with the vasculature, target host heme to fulfill the iron requirement. Heme acquisition begins with the lysis of erythrocytes, liberating the enclosed hemoglobin. Each hemoglobin binds four heme molecules and each erythrocyte contains approximately 270 million hemoglobins. Therefore, lysis of one erythrocyte results in a tremendous potential yield of iron for the pathogen. Hemolytic activity via hemolysins is a virulence trait associated with many Gram-positive and Gram-negative pathogens.

Some pathogens are equipped with additional mechanisms that facilitate acquisition of heme from reservoirs less abundant than hemoglobin. One example is the heme-binding protein hemopexin that functions to alleviate heme-mediated damage to host tissues. The high reactivity of heme requires that the molecule be complexed to proteins to prevent illicit damage to other host molecules. Consequently, the amount of 'free' heme is kept low in order to protect tissues from heme-induced redox damage. Hemopexin functions in this capacity and is a viable source of heme-iron for Gram-positive and Gram-negative pathogens such as

Bacillus anthracis and *Serratia marcescens*, respectively. Not all pathogens are capable of stealing heme from hemopexin. A notable exception is the important human pathogen *Staphylococcus aureus*. For the pathogens that do, a common strategy is to procure the hemopexin heme using a siderophore-like mechanism that outcompetes hemopexin for heme. In the case of *S. marcescens*, the protein that facilitates this competition is a hemophore called HasA. HasA has a higher affinity for heme than hemopexin. This allows HasA to retrieve heme from host hemopexin. The production of hemophores is not limited to Gram-negative pathogens as the Gram-positive pathogen, *B. anthracis* also utilizes hemophores to scavenge host heme form hemopexin and other sources.

The Gram-Positive Iron-Regulated Surface Determinant System

Gram-positive pathogens acquire host heme utilizing the **i**ron-regulated **s**urface **d**eterminant (Isd) system. Isd function can be divided into four distinct steps (Fig. 2(A)). Generally, these steps include (i) binding of extracellular heme or heme-containing proteins, (ii) stripping the heme from the host protein and transporting it across the cell wall, (iii) importing the heme into the bacterial cytoplasm, and (iv) degradation of heme, releasing free iron. Each step is performed by one or more proteins that together support pathogen heme acquisition. The importance of the Isd system to pathogenesis is underscored by conservation of the system in numerous Gram-positive pathogens, as well as, the virulence defects observed in multiple species genetically inactivated for any one of the Isd proteins.

The process of Isd-mediated heme-iron acquisition is initiated by extracellular receptors covalently bound to the surface of the peptidoglycan in the cell wall. These receptors bind heme or heme-containing host proteins. Consistent with their localization at the cell surface, the amino acid sequence of the receptors contains canonical Sec translocon signal sequences and conserved sortase A (SrtA) recognition domains. The receptors are endowed with distinct affinities for heme or hemoglobin (Fig. 2(A)). Haptoglobin, a host protein that binds hemoglobin to initiate hemoglobin recycling, is also a target of Isd receptor proteins (Fig. 2(A)). *S. aureus* expresses several Isd receptors that bind specific heme reservoirs. These receptors function in the sequential transport of heme to other Isd proteins embedded within the cell wall. For example, IsdH and IsdB bind haptoglobin and hemoglobin, respectively

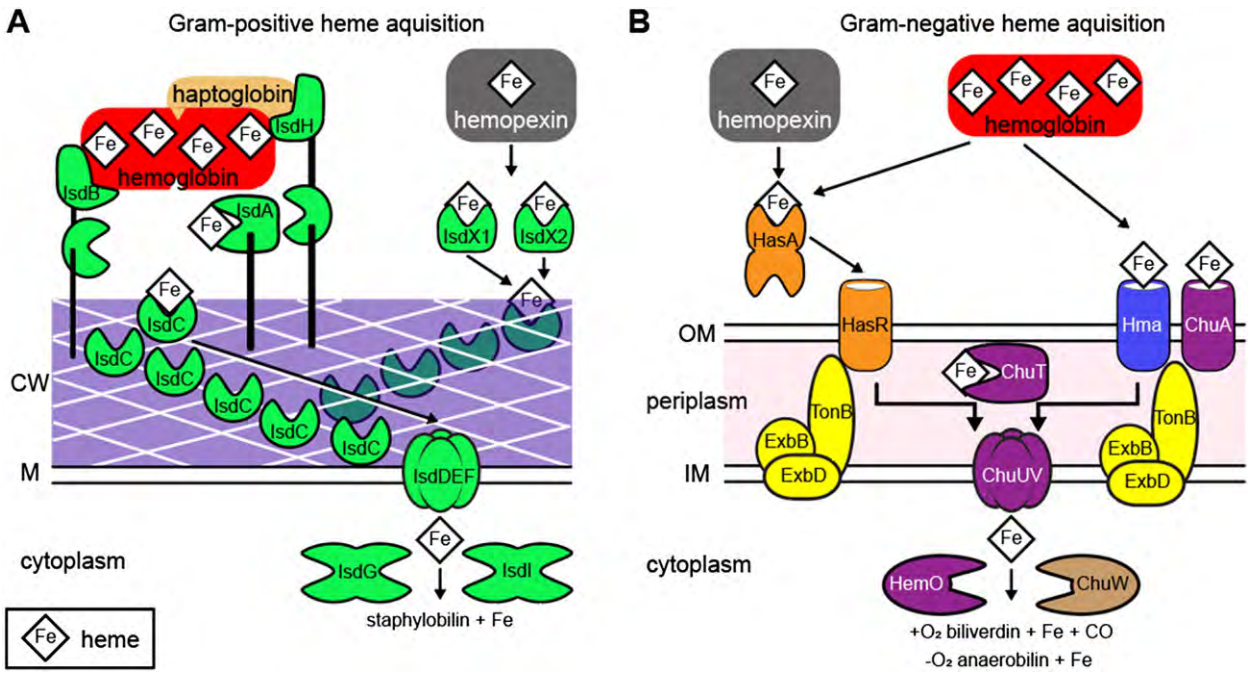

Fig. 2 Heme acquisition strategies employed by bacterial pathogens. (A) The iron-regulated surface determinant system (Isd) expressed by Gram-positive pathogens is required for heme acquisition from the host environment. Heme or heme-containing proteins are bound by the surface-exposed receptors IsdH, IsdB, or IsdA. Heme is subsequently transferred between these proteins via near-iron transporter (NEAT) domains to the cell wall (CW)-embedded protein IsdC. In some Gram-positive species, the hemophores, IsdX1 or IsdX2, steal heme from host hemopexin and transfer the heme directly to IsdC. IsdC transfers heme to the membrane (M)-localized ABC transporter IsdDEF. The heme is then imported into the cytoplasm where is it a substrate for the Isd-family of heme oxygenases, IsdG or IsdI which liberate the iron atom. (B) This illustration depicts the Gram-negative heme acquisition systems. Hemophore-dependent heme acquisition is also a strategy employed by some species of Gram-negative pathogens. In this depiction, the hemophore, HasA, retrieves host heme from hemopexin and transfers the heme to its receptor, HasR. Heme can also be captured by outer membrane (OM)-localized heme receptors. In this model the receptors are Hma and ChuA, but over 30 OM-localized heme receptors have been described. Typically, Gram-negative species employ either hemophore-mediated or receptor-mediated heme acquisition. Using energy derived from the PMF, the TonB complex facilitates import of heme to the bacterial periplasm. ChuT functions as a periplasmic chaperon that traffics the heme to the ChuUV ABC transporter. In the cytoplasm, heme is degraded by heme oxygenases to liberate iron. This degradation results in unique products depending on the presence or absence of oxygen.

(Fig. 2(A)). Binding of the host-derived substrate is mediated by a unique domain referred to as the near iron transporter or NEAT domain, which binds heme or heme-containing proteins with high affinity. In keeping with this, IsdH contains three NEAT domains while IsdB has two. The NEAT domains coordinate binding and trafficking of heme-containing proteins or heme between the Isd receptor proteins (Fig. 2(A)). IsdA expressed on the surface of *S. aureus* contains a single heme-binding NEAT domain. Consistent with this, IsdA functions as an independent heme receptor but can also receive heme from IsdB or IsdH. IsdB is sufficient for *S. aureus* utilization of heme or hemoglobin as sources of iron, but the redundant heme-binding activities of IsdH, IsdB, and IsdA allows for maximal flexibility of pathogen heme-iron acquisition. Some Gram-positive species, such as *B. anthracis*, encode hemophores that allow the pathogen to target heme bound to hemopexin. *B. anthracis* secretes two hemophores, IsdX1 and IsdX2 that steal heme from hemopexin (Fig. 2(A)). Once the hemophores and receptor proteins have isolated heme, the molecule is trafficked through the cell wall, a hallmark of the next phase of Isd-mediated heme-iron acquisition.

The two steps that follow receptor binding of host heme are transporting heme through the thick peptidoglycan and importing the molecule into the cytoplasm (Fig. 2(A)). Heme is transferred across the peptidoglycan layer by IsdC. In keeping with its function as a heme-binding protein, IsdC also contains a NEAT domain. A feature that distinguishes IsdC from the surface-exposed receptors is the enzyme that covalently links IsdC to the peptidoglycan, Sortase B (SrtB). The current model suggests that SrtB-mediated anchoring of IsdC facilitates localization of IsdC throughout the peptidoglycan. Consistent with this, IsdC is the only known SrtB substrate. In addition, both IsdC and SrtB are encoded within the *isd* operon, providing further evidence that these proteins function in a concerted effort to aid transport of host-derived heme through the Gram-positive cell wall. This model implies that heme is sequentially passed between multiple IsdC proteins that work in a bucket brigade-like fashion to transport heme through the peptidoglycan to the membrane-localized heme importer. Once at the membrane, IsdC passes heme to a conical ABC transporter consisting of the IsdD, IsdE, and IsdF proteins. These proteins are also encoded within the *isd* operon and use energy derived from the hydrolysis of ATP to import heme into the cytoplasm. Once in the cytoplasm, the Isd system contains dedicated enzymes that liberate iron from heme.

The final step of heme-iron acquisition is heme degradation. Heme is degraded by enzymes called heme oxygenases that cleave the tetrapyrrole ring, liberating the iron atom. In *S. aureus* heme degradation is catalyzed by two oxygenases, IsdG and IsdI (Fig. 2(A)). Until recently, bacterial heme degradation was thought to proceed in a similar fashion as mammalian heme degradation. However, studies that focused on the mechanistic details of IsdG and IsdI enzymatic activity, including the discovery of a novel heme degradation product, revealed that heme degradation in Gram-positive species is distinct from heme degradation in mammalian and Gram-negative bacteria. The distinguishing characteristic between the two mechanisms are the products resulting from the cleavage of the tetrapyrrole ring. Heme oxygenases expressed in mammals and Gram-negative species degrade heme to a molecule called biliverdin and carbon monoxide. However, IsdG and IsdI degrade the heme yielding a molecule called staphylobilin and formaldehyde. In mycobacterial species, yet another heme degradation product has been discovered called mycobilin. That different bacterial species degrade heme to unique products supports the intriguing hypothesis that the heme degradation products also have a function. Consistent with this, in vertebrates, biliverdin is converted to bilirubin, a potent antioxidant. However, specific functions of the heme degradation products in bacterial species has yet to be elucidated.

Gram-Negative Heme Acquisition Systems

Gram-negative pathogens also target heme-containing hemoglobin as a source of iron. First, the pathogen lyses erythrocytes to liberate hemoglobin from these cells and initiate heme acquisition. To accomplish this, *S. marcescens* and *E. coli* rely on the hemolysins ShlA and HylA, respectively. Gram-negative bacteria capture the heme liberated via hemolysis utilizing two distinct mechanisms: direct heme binding to outer membrane receptors or secretion of hemophores that scavenge for host heme, subsequently passing the heme to their cognate receptor (Fig. 2(B)). Some Gram-negative bacteria possess both hemophore-mediated and receptor-mediated systems; however, it is more common for the pathogens to employ one or the other. Two heme receptors have been extensively characterized in uropathogenic *E. coli*, ChuA and Hma, but more than 30 heme receptors have been described in Gram-negative species (Fig. 2(B)). Outer membrane heme receptors are similar to the iron-siderophore complex receptors described above both in terms of structure and the utilization of the proton motive force (PMF) to drive import. Heme receptors are β-barrels that encode specific residues within extracellular domains that bind heme. Two of these domains, FRAP and NPNL, contain conserved amino acid motifs in the extracellular loops of outer membrane receptors that are critical for heme import. These domains function to bind heme and transport it through the outer membrane. The β-barrel heme receptors rely on the TonB protein complex to couple energy (PMF) from the inner membrane to power heme import.

Gram-negative pathogens also utilize hemophores to retrieve host heme. One of the best-described hemophores is HasA produced by *S. marcescens*. The **h**eme **a**cquisition **s**ystem (Has) consists of the hemophore and its outer membrane receptor, HasR. Analogous systems have been characterized in *Pseudomonas aeruginosa* and *Yersinia pestis*. HasR is structurally similar to the β-barrel heme receptors but heme binding is significantly enhanced when it functions in concert with HasA. HasA is secreted as a dimer and conserved tyrosine and histidine residues coordinate within a pocket to facilitate heme binding. These residues are critical for heme binding. HasR recognizes monomeric HasA; therefore, the function of the HasA dimer is not well understood. Once HasR binds HasA, both proteins undergo conformational changes leading to the exchange of heme from HasA to HasR.

Periplasmic binding proteins (PBPs) are responsible for shuttling heme from the outer membrane through the periplasm to the inner membrane (Fig. 2(B)). PBPs that bind heme, such as ChuT, have been identified in *P. aeruginosa*, *Shigella dysenteriae*, *E. coli* and *Y. pestis*. Similar to HasA, PBPs coordinate heme binding using a conserved tyrosine residue within a cleft connecting the N- and C-termini. The heme-binding PBPs then pass the heme to dedicated ABC transporters. Analogous to siderophore import, ABC transporters hydrolyze ATP to mediate heme delivery to the cytoplasm. While the outer membrane receptors for both siderophores and heme are incredibly substrate specific, the ABC transporters do not seem to be. For example, *P. aeruginosa* encodes approximately 34 TonB-dependent outer membrane receptors, but only four ABC transporters function in iron import. Therefore, it is likely that the same ABC transporters used for siderophore-iron import are also used to import heme.

Gram-negative bacteria employ heme oxygenases to degrade heme. However, the enzymes expressed in Gram-negative species are distinct from the IsdG-family heme oxygenases expressed in Gram-positives. Consistent with this, the products of the Gram-negative heme degradation reaction are iron, biliverdin and carbon monoxide (Fig. 2(B)). A new mechanism of heme degradation has recently been reported in the Gram-negative pathogen, enterohemorrhagic *E. coli* O157:H7. In aerobic environments canonical heme oxygenases catalyze the release of iron from heme, using oxygen as a cofactor. However, bacteria capable of growing in anaerobic environments such as the intestines, must have alternative mechanisms to liberate iron from heme without oxygen. *E. coli* O157:H7 encodes an operon for heme uptake that includes three additional uncharacterized genes, *chuW*, *chuX*, and *chuY*. Elegant biochemical experiments demonstrated that ChuW uses the oxidizing potential of a carbon radical to free heme-bound-iron through a methyl transfer reaction leading to the rearrangement of the heme porphyrin ring. The complete mechanism of this reaction is currently not known; however, ChuW catabolizes the porphyrin ring of heme yielding free iron and a tetrapyrrole called anaerobilin (Fig. 2(B)). ChuY breaks down and reduces anaerobilin, a potentially toxic product of heme degradation. *chuW* and *chuY* are co-transcribed, suggesting a comprehensive mechanism of anaerobilin synthesis by ChuW after iron liberation from heme followed by anaerobilin reduction by ChuY.

Circumventing Heme Toxicity

To harness the redox potential of heme, organisms complex the tetrapyrrole to proteins. Thus, the quantity of 'free' heme within cells is low. However, in the absence of protein binding, the unharnessed redox potential of 'free' heme is reactive with proteins and other molecules within cells. Moreover, as a hydrophobic molecule, 'free' heme aberrantly interacts with membranes potentiating the resulting toxicity. Thus, organisms that encounter 'free' heme must have strategies to counteract this toxicity. The host protein hemopexin is one such example, but how do bacterial pathogens that target host heme to satisfy their iron requirement deal with heme associated toxicity? In general, Gram-negative pathogens are more resistant to the toxic effects of heme than Gram-positive pathogens. One such example is *S. dysenteriae*, which rapidly degrades heme to reduce its toxic effects. Gram-positive pathogens, on the other hand, encode systems such as the **h**eme-**r**egulated **t**ransporter, HrtAB that protects the bacterial cell from heme toxicity. The HrtAB transporter is a canonical ABC transporter, composed of an ATPase, HrtA, and a permease, HrtB. The current model of HrtAB-mediated heme detoxification predicts that these proteins function to efflux heme from the bacterial cytoplasm. HrtA hydrolyzes ATP to generate the energy needed to pump heme from the bacterial cytoplasm across the plasma membrane through HrtB, allowing the cell to reduce intracellular levels of 'free' heme.

HrtAB is widely conserved in Gram-positive pathogens, as well as, non-pathogenic species such as *Lactococcus lactis*. Notably, pathogens and commensals have evolved unique ways to regulate HrtAB expression. Pathogens utilize a dedicated two-component system (TCS) called the **h**eme-**s**ensing **s**ystem, HssRS that regulates expression of HrtAB efflux pump. HssRS functions as a canonical TCS. Phosphorelay from the membrane-bound sensor, HssS, to the response regulator, HssR, induces expression of HrtAB. The precise molecular signal that stimulates HssRS activation and phosphorelay, remains elusive, however, heme is an absolute requirement. An alternative mechanism of HrtAB regulation was discovered in *L. lactis*, a bacterium typically associated with plants that is used extensively in the production of cheese and other dairy products. In *L. lactis*, a cytoplasmic-localized transcriptional regulator, HrtR induces HrtAB expression. HrtR represses expression of HrtAB in the absence of heme, but upon heme binding, the affinity of HrtR for DNA decreases relieving HrtAB repression. Why Gram-positive species evolved two different mechanisms to regulate the HrtAB heme efflux pump transporter remains to be elucidated. However, it is clear that HrtAB, and HssRS or HrtR act in a concerted manner to effectively manage heme homeostasis ensuring that Gram-positive bacteria circumvent heme toxicity.

Coordinating the Iron Acquisition Systems

Heme and iron are exceedingly toxic at high levels. Therefore, bacteria that target both siderophore-mediated and heme-mediated iron acquisition systems must coordinate expression of the acquisition systems to maintain iron homeostasis without corresponding toxicity. It is likely that the abundance of different iron-containing molecules is dramatically altered in different host tissues. In keeping with this, siderophore-mediated iron acquisition may be better suited for colonization of distinct organs or tissues compared to heme-mediated acquisition systems and vice versa. Together these facts require that bacteria coordinate the expression of the acquisition systems to maximize fitness during infection of distinct niches. We are just beginning to understand the interplay

between the iron acquisition systems but a recently described transcriptional regulator expressed in *S. aureus* has emerged that serves as the paradigm for coordination of iron acquisition systems.

SbnI is a transcriptional regulator encoded within one the *S. aureus* siderophore synthesis operons (Fig. 1(C)). *S. aureus* produces three siderophores, a nicotinamine-like broad spectrum metallophore called staphylopine and two α-hydroxycarboxylate siderophores: staphyloferrin A and staphyloferrin B. The *sbn* operon encodes the genes required for staphyloferrin B synthesis. *sbnI* is the terminal gene of a nine gene *sbn* operon. The function of SbnI remained elusive, but SbnA-H function to synthesize or efflux staphyloferrin B (Fig. 1(C)). Recent, elegant work demonstrated that SbnI enhances the expression of *sbnD-H* in conditions of low iron. Notably, SbnI-dependent expression of *sbnD-H* is affected by of the presence of heme. SbnI is capable of binding heme and this reduces the affinity SbnI has for its DNA promoter sequence within the *sbnD* gene. In the presence of heme the expression of *sbnD-H* is decreased, reducing the production of staphyloferrin B. Therefore, SbnI coordinates the production of staphyloferrin B in iron-deplete environments devoid of heme. However, in iron-deplete environments containing heme, staphyloferrin B is not produced. This elegant coordination of heme- and siderophore-mediated iron acquisition maximizes *S. aureus* fitness by ensuring that the iron requirement of the pathogen is satisfied.

The EfeUOB/FepABC Iron Acquisition System

The EfeUOB, and analogous FepABC system facilitate iron acquisition in both Gram-positive and Gram-negative pathogens. However, the mechanism by which this system functions in iron acquisition is debated. Much of the debate centers on the function of EfeB, or FepB, as two competing models have emerged. In the first model, EfeB functions as a deferrochelatase. That is to say that EfeB/FepB extracts iron from heme leaving the porphyrin ring intact. Therefore, this model predicts that heme is the target of the EfeB/FepB. Alternatively, it has been demonstrated that the FepB protein in *Listeria monocytogenes* reduces ferric iron to ferrous iron. This study serves as the foundation for the second model which implies the target for the Efe/Fep system is ferric iron. Additional investigation is needed to define how FepB and EfeB function in iron acquisition. The two other components of the tripartite system, EfeU/FepC and EfeO/FepA are more clearly defined. EfeO/FepA chaperones the ferrous molecule that results from the EfeB/FepB reaction to the permease encoded by EfeU/FepC. EfeU/FepC then transports the iron atom into the cytoplasm.

Concluding Remarks

As iron is a requirement for bacterial proliferation and survival, it is not surprising that pathogens evolved multiple mechanisms that target the most abundant sources of iron within their hosts. One mechanism utilizes the production of low molecular weight siderophores that outcompete host iron-binding proteins to procure the essential metal. A second mechanism, often also employed by bacteria that synthesize siderophores, targets the most abundant iron-containing molecule in vertebrates, heme. To acquire heme-iron, Gram-positive and Gram-negative pathogens secrete hemolysins that liberate the molecule from erythrocytes. The heme is then targeted by proteins that facilitate its import and degradation resulting in the release of nutrient iron (Fig. 2). This multifaceted strategy ensures that pathogens are able to fulfill their iron requirement during infection. Maintaining more than one system dedicated to the procurement of iron seems redundant. However, the quantity of iron and the chemical moieties that sequester iron within the host varies between sites of infection. Therefore, encoding multiple iron acquisition systems allows pathogens to fine-tune iron acquisition in the diverse and dynamic environments encountered during infection. There is much to be learned about how these two strategies are regulated and how they can be exploited to advance novel therapies that combat antibiotic-recalcitrant bacterial infections.

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Bacterial Targeting of Tumors

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Origins of Live Bacteria for the Treatment of Cancer

The concept that bacteria might have beneficial effects against cancer has several potentially independent origins. In 1813, Vautier reported that tumor regressions sometimes occurred patients with gas gangrene, the disease now known to be caused by anaerobic members of the genus *Clostridium* (as cited by Mowday et al., 2016). Live *Clostridium* were later explored in several early studies as antitumor agents (e.g., Parker et al., 1947; Möse and Möse, 1964; Carey et al., 1967), and more recently were genetically engineered to improve safety and antitumor activity (e.g., Minton et al., 1995; Fox et al., 1996; Dang et al., 2001; Nuyts et al., 2002). An attenuated *Clostridium novyi* strain is currently undergoing clinical trials (Roberts et al., 2014). Because tumors have irregular vasculature, they are often hypoxic or anoxic, which provides a suitable habitat for these anaerobic bacteria. Another anaerobic bacteria that has also been explored as antitumor agents is *Bifidobacterium longum* (e.g., Taniguchi et al., 2016), which is non-pathogenic.

Following the early report by Vautier, antitumor effects were noted in association with erysipelas (Busch, 1866; Fehleisen, 1883; Bruns, 1887), the disease now known to be caused by Group A *Streptococcus*. Based on the studies of Fehleisen and Bruns, the American surgeon William B. Coley began experimenting with administering live erysipelas organisms (then referred to as *Streptococcus erysipelatis*) to patients with sarcoma, and noted that there was a strong inflammatory reaction including hyperthermia that accompanied a reduction in tumor size (Coley, 1891, 1991; Nauts, 1976). Subsequently, Coley experimented with heat-killed bacterial extracts that were safer than live bacteria yet resulted in similar inflammatory and antitumor effects. The bacterial extracts were known as Coley's toxin and were included in early US Pharmacopeias for the treatment of cancer, and were marketed by the Park Davis company. The results achieved with Colery's toxin were considered variable by the cancer therapy community, which might have been due to variation in its manufacturing, and its use was discontinued in the 1960s when other chemotherapeutics became available. Coley's work is widely regarded as the origin of immunotherapy and immunooncology (IO).

Another immunostimulatory bacterium, Bacillus Calmette-Guerin (BCG), a mycobacterial strain derived from *Mycobacterium bovis* that was developed for vaccination against tuberculosis, has also found use as an anticancer immunotherapy agent (Fuge et al., 2015; Herr and Morales, 2008). Tuberculosis patients had been noted to have lower incidences of cancer as early as 1929 by Pearl. Studies by Old et al. (1959) and then Zbar et al. (1971) ultimately led to the recognition of BCG as an antitumor agent, and it became established as the standard of care therapy for superficial bladder cancer.

The number of bacteria being explored as tumor-targeting has been steadily growing, and collectively includes *Bifidobacterium*, *Caulobacter*, *Clostridium*, *Escherichia*, *Listeria*, *Proteus*, *Salmonella*, and *Streptococcus* (see Forbes, 2010; Zhou et al., 2018 for reviews). Continued investigations of the antitumor properties of these bacteria my prove that each have particular advantages in regard to safety and/or efficacy characteristics, or be of specific use against certain subtypes of cancer. In this discussion, some of the many studies with *Salmonella* are used to illustrate aspects of the technological approaches to developing microbes that target cancerous tumors and enhancing their anticancer activities.

Development of Tumor Targeted *Salmonella*

Early work on the use of *Salmonella* as a delivery vector for biologically functional proteins includes the study by Carrier et al. (1992) on oral and intravenous delivery of bacteria producing IL-1β. IL-1β biological activity was inferred by demonstrating that it induced radiation resistance in mice pretreated with the bacteria and then exposed to high doses of radiation. Use of *Salmonella* as an anticancer immunotherapy agents, either injected intralesionally or intraperitoneally, was also investigated and considered to be similar to BCG (Eisenstein et al., 1995). Subsequent work on the use of *Salmonella* as a cancer therapeutic agent utilized bacteria that produced a genetically engineered interleukin 2 (IL-2; Saltzman et al., 1996, 1997). Following oral or intraperitoneal delivery the bacteria colonized the liver and spleen. When hepatic tumors were implanted, the IL-2 producing bacteria localized in proximity to the tumors due to liver tropism, and resulted in an anti-tumor and anti-metastasis effects.

The approach for development of tumor-targeted *Salmonella* was based on intentionally delivering the bacteria systemically and assessing direct colonization of the tumor by harvesting the tumor and comparing it with normal tissues, such as the liver, thereby defining a tumor to normal tissue preference, or tumor-targeting ratio (Pawelek et al., 1995, 1997). The notion of using *Salmonella* or other microbes as direct tumor-cell targeting agents was inspired by the publication of Munzarova et al. (1992) who described the macrophage characteristics of melanoma. As a pathogen that specializes in invasion and survival within macrophages, *Salmonella* was speculated to potentially have innate tumor specificity. It was somewhat surprising when it was later revealed by electron microscopy that most of the bacteria were in the interstitial spaces of the tumor as well as inside phagocytic cells, with only a few intracellular bacteria within the melanoma cells (Pawelek et al., 1997). Although this initial result did not seem to support the original hypothesis of cell type specificity as a mechanism for tumor tropism toward macrophage-like tumor cells, it did suggested a complex interaction that might be mediated by extracellular bacteria combined with intracellular bacteria and possible

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contributions from the immune system (Pawelek et al., 1997), concepts that have been bourn out by many times in subsequent studies (Zhou et al., 2018), and described further below. Later it was recognized that autophagy of *Salmonella* is involved in the antitumor mechanism (Lee et al., 2014), and that this may account for the absence of large amounts of intracellular bacteria within tumors.

The initial study of *Salmonella* tumor targeting utilized a hyperinvasive mutant, "clone 72" (Yale *Salmonella* clone 72; YS72), which had been selected for enhanced internalization into melanoma cells. Fortuitously, YS72 also a purine auxotroph due to the UV and nitrosoguanidine exposure used to generate the mutational pool of bacteria from which the hyperinvasive phenotype was selected (Pawelek et al., 1997). When injected systemically into mice with Cloudman S91 melanoma tumors, YS72 had an apparent tumor-homing ability, with a tumor targeting ratio greater than 1000:1 (Pawelek, Low and Bermudes, unpublished data). This ratio was a result of more than 10^9 colony forming units (CFU) per gram of tumor tissue after only 1 day, whereas only 10^6 bacteria had been injected into the mice, indicating that the result was due to selective replication within the tumor. And while the wild type *Salmonella* also preferentially replicated within tumors, indicating a natural tropism for tumors, its accumulation was to a lesser degree than clone 72, and rapidly caused death of the mice. Importantly, while the hyperinvasive clone 72 also had a slightly higher CFU within the tumor than wild type, it was approximately 10 times lower in the liver, and did not result in death of the immunocompetent mice, establishing that it was attenuated relative to the wild type strain. Melanoma tumor growth was delayed in the mice treated with clone 72 and their overall survival times increased, which was correlated with tumor growth delay. Thus, in mice, attenuated bacteria are safe, target tumors from a distant inoculation site, selectively replicate within tumors, and cause tumor suppression of tumor growth.

The results of these studies also established that attenuation could serve multiple purposes. As tumors are rich in purines due to their fast-growing nature as well as containing necrotic tissue due to irregular vasculature, purine auxotrophy provided both the safety necessary for administration to a live animal and a targeting enhancement mechanism, via a nutritional requirement, to increase specificity toward tumors. In addition, a third contribution of purine auxotrophy is competition of the bacteria with the tumor for available purines, and therefore also provides a mechanism to contribute toward tumor growth inhibition. Among the other features of these bacteria, it was also noted that *Salmonella* were facultatively anaerobic, as compared to the mostly anaerobic genus *Clostridium*, which could potentially provide an advantage for targeting smaller, more aerobic tumors. Furthermore, the pathogenic origin of the bacteria was thought to contribute to the general ability of the bacteria to temporarily evade the immune system to establish tumor colonization, although this ability is also shared with some non-pathogenic bacteria such as *Bifidobacterium*.

In subsequent, studies when partially immunodeficient mice were examined as the host for menanoma tumors, clone 72 was not sufficiently attenuated and resulted in death of the mice (Pawelek et al., 1997). Additional attenuating mutations that provided sufficient safety were added that included auxotrophy for isoleucine/valine, arginine, aromatic amino acids, and uracil. These strains retained both the tumor targeting properties and the antitumor effects of the clone 72 in immunodeficient mice. The suitability of these mutations was notable, as they further established the utility of mutations in biosynthetic pathways for the basic building blocks of amino acids and nucleotides for simultaneously providing attenuation and tumor selectivity while maintaining the bacteria's ability to delay tumor growth, similar to the results obtained with purine auxotrophy.

At the time of these initial studies, live attenuated vaccines, especially orally delivered *Salmonella* based vaccines, also provided a framework of both attenuation and protein delivery to draw from in order to develop tumor-targeted *Salmonella*. In fact, both purine auxotrophy (Bacon et al., 1950a,b, 1951) and aromatic amino acid auxotrophy (Hoiseth and Stocker, 1981) were known as attenuating mutations. A typhoidal strain, Ty21A, had been developed as a vaccine against typhoid fever that had multiple mutations including purine auxotrophy (Schödel, 1992). However, distinct differences in their effects on tumor-targeted bacteria were found among these known attenuating mutations. For example, aromatic amino acid auxotrophy was compatible with tumor targeting and antitumor activity (Pawelek et al., 1997), but while *purI* mutations were compatible with both large and small tumors (Pawelek et al., 1995, 1997; Low et al., 1999), *purA* mutations only worked with very large tumors (Bermudes, unpublished). In addition, other attenuated genetic backgrounds that seemed particularly useful for vaccines, such as PhoP/Q mutations (Hohmann et al., 1996), resulted in bacteria that targeted tumors but lack significant antitumor activity (John Pawelek, Brooks Low, David Bermudes and Sean Murray, unpublished observations).

The initial study of *Salmonella* tumor targeting also explored the potential of the bacteria to delivery a heterologous enzyme, the herpes simplex thymidine kinase (HSVTK). HSVTK is known as a prodrug-converting enzyme because it has the ability to activate a non-toxic prodrug (acyclovir or gancyclovir) into its active phosphorylated form that has the ability to inhibit DNA synthesis and cause cell death (Pawelek et al., 1995, 1997). The HSVTK was constructed with a Type II bacterial secretion signal to export the HSVTK into the periplasm, and was demonstrated to be enzymatically active. When melanoma-bearing mice were administered these bacteria, there was a gancyclovir-dependent enhancement of antitumor activity, resulting in the proof of concept that genetically engineered *Salmonella* could selectively deliver an enzymatically active therapeutic protein directly to the tumor and activate an anticancer drug.

Salmonella is a gram-negative bacterium with a typical lipid A in the outer leaflet of the outer membrane. Lipid A is a pathogen associated molecular pattern (PAMP) that is recognized by Toll-like receptor 4, and through signal transduction results in production of TNFα by monocytes and macrophages. As with most gram-negative bacteria, excessive exposure to lipid A via bacteremia results in TNFα-mediated septic shock in the host, which is often lethal. However, a notable exception occurs in mice (Keith Joiner, personal communication; Fink, 2014). Even normally responsive mice have a lethal dose of LPS that is substantially less than the toxic does in normal human volunteers. For example, the LD_{50} dose of LPS in mice can be 10 to 1000-fold or more

greater than the dose of LPS that is required to induce acute illness in humans (Taveira da Silva et al., 1993). Thus, the potential for use of a tumor-targeted *Salmonella* that functions safely in mice could be severely limited by toxicity in humans.

Several potential solutions to the lipid A mediated septic shock problem were investigated. Earlier studies of lipid mutant bacteria had shown that not all forms of lipid A were equally stimulatory of TNFα. Examples included monophosphoryl lipid A and lipid X, which both occur in the deep rough mutant *Salmonella minnesota* R595 (Danner et al., 1987), and have even been shown to prevent induction of septic shock. However, it was found that this strain of *Salmonella* does not inhibit tumor growth (Bermudes unpublished observations). Other mutants that were assessed included *firA*, which had some tolerance to physiological temperature and was of low pyrogenicity, but only colonized tumors at a rate of about 1000-fold less than other mutants (Pawelek et al., 1995). Generally, among the other lipid mutants, the attendant problem is that they result in a temperature sensitive phenotype, whereas the requirement for a tumor-targeted vector is to be functional at physiological temperature.

A series of investigations on temperature sensitive mutants and their suppressor mutations led to the publication of the first non-temperature sensitive lipid mutant in *E. coli* (Somerville et al., 1996), which had a deletion in the *msbB* gene that is responsible for myristolation of lipid A (Clementz et al., 1997). Deletion of *msbB* in *Salmonella* also retained low pyrogenicity in monocyte assays of TNFα production, approximately 10,000 times lower than induced by wildtype, but retained the ability to grow at physiological temperatures (Bermudes and Low, 1997; Khan et al., 1998; Low et al., 1999). When *msbB* mutants were assessed in murine tumor models, tumor targeting and antitumor activity were both retained (Bermudes and Low, 1997; Low et al., 1999). Faster growing variants of *msbB Salmonella* were isolated which proved to be suppressor mutations for other physiological sensitivities that were induced by the *msbB* mutation (Low et al., 2016), including sensitivity to salt (Murray et al., 2001), chelating agents and MacConkey media containing bile and crystal violet (Murray et al., 2004), polymyxin (Murray et al., 2007) and CO_2 (Karsten et al., 2009).

Although mice are relatively insensitive to TNFα mediated septic shock, the *msbB* bacteria were found to be highly attenuated, and generally safe without additional attenuating mutations. This was somewhat surprising considering that the lipid A did not have toxic consequence for the mice. The primary reason for this difference appears to be due to enhanced ability of macrophages to eliminate these bacteria. Interestingly, *msbB Salmonella* retained a tumor targeting ratio of greater than 1000:1 despite the enormous alteration in the ability to be eliminated from normal tissues. Furthermore, without an auxotrophic mutation generating a requirement for nutrients within the tumor, tumors remained the site of preferential replication. These data were interpreted as indicating the tumor targeting phenomenon (preferential replication) within tumors might also involved the immune privileged nature of the tumors (Low et al., 1999), and that forms of attenuation other than auxotrophy were compatible with the tumor targeting system, and in fact, offered an alternative to requiring a metabolite provided by the tumor in order to enhance the tumor targeting ratio.

The proof of concept that the *msbB* mutation would provide safety against septic shock for non-murine animals was demonstrated in Sinclair swine (Low et al., 1999). At a high dose (10^9 CFU), 9 of 10 swine died within five days, whereas all of the *msbB*-treated swine survived. Purified LPS from *msbB Salmonella* injected into swine resulted significantly lower respiration rates compared to injection of wild type LPS, consistent with the cellular assays described above. Collectively, both the lipid A and the whole bacteria were significantly reduced in their inflammatory properties, and significantly reduced in virulence.

The development of the *msbB Salmonella* was widely viewed as an advancement that could facilitate human clinical studies (Less Shocking Antitumor Agents?, Marshall, 1999), and several human clinical trials were subsequently sponsored by Vion Pharmaceuticals in New Haven, CT. Although there had been earlier investigations of *Clostridium* in humans for the treatment of cancer, there had never been a review by the Food and Drug Administration (FDA) of any of the prior studies of intravenously injected bacteria, which had all been Gram-positive. Thus, intravenous injection of live Gram-negative *Salmonella* faced significant scrutiny in order to allow the studies to be initiated. However, it was subsequently shown in two separate publications that live bacteria can be subjected to many of the same principles as chemical entities, biologics and orally delivered bacterial vaccines, which helped guide the regulation of this precedent of a live intravenously administered bacterium (Clairmont et al., 2000; Lee et al., 2000; Lee, 2002).

VNP20009, an *msbB* strain of *Salmonella* derived from clone 72 that was a Tn*10* derived purine auxotrophy combined with hyperinvasiveness and was antibiotic sensitive (later described by Low et al., 2004), was used for the clinical studies. VNP20009 also contains a large deletion that acts as a suppressor for salt and EGTA sensitivity referred to as the Suwwan mutation (Murray et al., 2004; Broadway et al., 2014). VNP20009 was evaluated in mice, dogs and monkeys for its biodistribution properties and safety (Clairmont et al., 2000; Lee et al., 2000). It was estimated that VNP20009 is approximately 50,000 times less toxic than the wildtype *Salmonella enterica* serotype Typhimurium.

Salmonella Intratumoral Administration in Humans

VNP20009 underwent its first human clinical study at the Cleveland Clinic, administered by direct intra-tumoral (IT) injection (Mier et al., 2001). The results of this study are of significance in contrasting the differences observed in mice and human treated with VNP20009, and have provided some guidance for the development of new vectors with improved properties. Nine patients were enrolled representing melanoma (4), renal (3), prostate (1) and merkel cell (1) tumors. After 12-19 days following intratumor injection (conducted as a dose escalation with different patients) excisional biopsies or fine needle aspirates (FNA) were performed and assessed for CFU/g tumor tissue. The bacteria were found in 6 of 9 patients of these tumors, with 10^6 CFU/g as the highest-level colonization within the study (compared with 10^9 CFU/g in mice), which was therefore substantially lower than was observed in preclinical models. Inflammation, necrosis and fibrosis were observed in most patients, with most patients being stable at 30-days

follow-up, although two of the tumors grew more rapidly directly following injection. No toxicities were reported. Three of the observations from this study were of particular interest: (1) the bacteria were shown to be safe, (2) the bacteria persisted in tumors at least 12 days in most of the tumors injected and (3) the CFU/g tumor tissue was substantially lower in humans than in mice.

Salmonella Intravenous Administration in Humans

The second clinical study of VNP20009, which reported the results of intravenous (IV) administration, was conducted at the National Cancer Institute, primarily (24/25) on melanoma patients that were no longer responsive to chemotherapy or immunotherapy (Toso et al., 2002). These results have significantly shaped the on-going development of *Salmonella*-based vectors in terms of establishing differences in VNP20009's translation from mice to humans, and have helped define many of the goals of on-going research.

The study was conducted as a bolus intravenous administration, dose escalation, with evaluation of toxicities, tumor colonization (CFU/g) and possible antitumor effects. The dose began at 1×10^6 CFU/m^2 and was escalated as a semi-log function, with a total of seven different dose levels administered. The study established that 3×10^8 CFU/m^2 was the maximum tolerated dose; four patients were administered one does higher (1×10^9 CFU/m^2), which resulted in unacceptable toxicities consistent with disseminated bacteremia. Of the 25 patients administered in the seven dose escalations, only 7 received the maximum tolerated dose (MTD) or higher. Of note in the study was the overall tolerability of VNP20009, which reached up to 200,000 CFU/ml of blood at the MTD. At the MTD, only 1 of 3 patients was documented to have bacteria localized within their tumors. The presence of the bacteria in that patient went undetected by fine needle aspiration (FNA), but was later established by excisional biopsy as 1×10^4 CFU/g; a finding of CFU/g of tissue similar to the intra-tumoral study in humans and substantially lower than occurred in mice. The variation from undetected by FNA to detectable by excisional biopsy was also of significance, as it suggested that the distribution within tumors is heterogeneous, and likely occur as small foci. This is also consistent with the finding that the initial entrapment of the bacteria within tumors is sparse (Forbes et al., 2003). In the cohort that received the highest dose (1×10^9/CFU/m^2), two patients were documented to have bacteria in their tumors by FNA; one with 2×10^2 CFU/g and one that progressed to levels 7×10^9 CFU/g. Overall, the findings showed that VNP20009 was had acceptable toxicity, and targeted tumors in 1/3 patients at the MTD, and that the CFU/g was substantially lower compared to mice. The only high-level tumor targeting with CFU/g equivalent to that observed in mice occurred in humans who had received a dose above the MTD.

Other finding of the intravenous study included cytokine analysis, with IL-1β, TNFα, IL6, and IL12, results that are consistent with the immunooncology effects of bacteria. However, no objective clinical responses were found in the study, even in patients that were documented to have had bacteria that colonized their tumors.

Salmonella Continuous Intravenous Infusion in Humans

The mode of drug delivery is known to have the potential to alter the distribution, and therefore a 4-hour continuous infusion of VNP20009 was studied in four patients with metastatic melanoma, with tumor colonization measured exclusively by excisional biopsy within two weeks of administration (Heimann and Rosenberg, 2003). However, only one patient was found to be positive for tumor colonization, with only 58 CFU/g tumor tissue, with no objective antitumor responses.

Intratumoral Administration of *Salmonella* Expressing Cytosine Deaminase in Humans

Following the intravenous clinical trials of VNP20009, evaluation of a derivative of VNP20009 engineered to express the *E. coli* cytosine deaminase (King et al., 2002, 2009) was initiated in head and neck tumor patients (Cunningham and Nemunaitis, 2001). The *E. coli* cytosine deaminase acts as a prodrug-converting enzyme that converts the non-toxic 5-fluorocytosine (5-FC) to the known cytotoxic anti-cancer metabolite 5-fluorouricil (5-FU). Thus, by virtue of the bacterial selective localization within tumors, the non-toxic prodrug could be preferentially activated within tumors and therefore selectively toxic toward tumors. The study, which was reported after treating only three patients (Nemunaitis et al., 2003), escalated intratumoral doses beginning at 3×10^6/m^2, ending in 3×10^7/m^2. The patients also received six cycles of 5-FC. The results indicated that two of the three patients had their tumors colonized by the bacteria, which persisted for at least 15 days, ranging from 84 to 2.6×10^6 CFU/g tumor tissue. There was a measurable difference in the blood to tumor ratio for the metabolite 5FU, which coincided with the colonized tumors and not the un-colonized tumor. This study established for the first time the *in vivo* conversion of a prodrug by an intratumoral bacterium. The patient with the higher level of bacteria had evidence of stabilized disease in the injected tumor, while disease progression occurred in the uninjected tumors. The study represented an important proof of concept for bacterial colonization by intratumoral injection and activation of a cytotoxic agent. However, due to financial constraints, Vion Pharmaceuticals discontinued funding the study.

Summary of the *Salmonella* Clinical Studies in Humans

Intravenous (IV) and intratumoral (IT) administration of the live VNP20009 bacteria to humans was generally well tolerated, and a maximum tolerated dose of the bacteria was established for IV administration. In the IT study, the bacteria were documented to persist for 12 days or longer. In the IV study at the MTD, the bacteria targeted the tumors in one of three patients, and no tumors were documented to have been targeted below the MTD. FNA was shown to be inaccurate in assessing tumor colonization, and

suggests that the distribution of the bacteria within the tumors is heterogeneous. The percentage of tumors targeted by the bacteria following IV administration in humans was substantially lower than in preclinical models. In the intratumoral and intravenous administration studies, the CFU/g of tumor tissue was also usually lower than in preclinical models. Continuous infusion did not improve the targeting percentage or CFU/g tumor tissue. In these studies, no objective antitumor responses were observed. TAPET-CD was administered to three patients by intratumoral administration. Two of the patients treated with TAPET-CD were documented to have tumor colonization and conversion of 5FC to 5FU, and one patient had evidence of stable disease in the injected tumor. Overall, these findings suggest that improvements in (1) the percentage of tumors colonized, (2) the number of CFU/g of tumor tissue, and (3) the antitumor effects of the bacteria, represent areas that could lead to development of more effective live tumor targeting *Salmonella* vectors. In addition, murine tumor models that give results similar to those from the human studies with lower percentages of tumors targeted, and with lower CFU/g tumor tissue would aid in assessing the effects of efforts to improve the *Salmonella* tumor-targeting properties.

Salmonella Veterinary Clinical Trials for the Treatment of Cancer

VNP20009 was assessed in veterinary trials of pet dogs with naturally occurring tumors. VNP20009 was administered to forty-one dogs with a range of different tumor types where there was no established standard therapy, or had failed or declined standard therapy in a Phase I study (Thamm et al., 2005). The study included excisional biopsies to assess tumor colonization and monitoring of clinical signs to determine the maximum tolerate dose (MTD). Bacteria were detected from 42% of the tumors. Of 35 evaluable patients, there were 4 complete responses and 2 partial responses, and stable disease lasting at least 6 weeks occurred in 10%, with overall response rate of 15%. Although these results were encouraging, there has been no follow-up study with VNP20009. Other studies of live bacteria for the treatment of canine cancer have included *Clostridium* (Roberts et al., 2014) and a *Salmonella* expressing IL-2, described below.

An IL2-expressing *Salmonella* was recently evaluated in a Phase I study in pet dogs with osteosarcoma (Fritz et al., 2016). Dogs were treated on day 0, followed by amputation of the affected bone at day 10, with concurrent chemotherapy. The IL2 expressing *Salmonella*-treated dogs had extended disease-free interval (DFI) compared to those not receiving the bacteria. Overall the treatment was well tolerated and supports the potential for *Salmonella* expressing IL2 as an adjuvant therapy for dogs with osteosarcoma.

In addition to the potential of these canine studies to result in significant antitumor effects in dogs, they also point out that, at least to date, the murine studies are more similar to those in dogs than they have been in humans. However, as also noted, the human clinical studies were primarily limited to pretreated melanoma patients, whereas the canine studies did not all receive pretreatment and included melanoma as well as several other types of tumors, with complete responses in two with metastatic melanoma, one with soft tissue sarcoma, and one with cutaneous epitheliotropic lymphoma. Therefore, additional studies are needed to further correlate murine studies with those of dogs and humans.

Mechanisms of *Salmonella* Antitumor Effects

Multiple mechanisms associated with *Salmonella* antitumor effects have been described, most of which involve immune responses stimulated by the bacteria. Other mechanisms involve *Salmonella*-induced apoptosis through type III secretion and through autophagy.

Antitumor Effects of Tumor-Targeted *Salmonella*

Tumor necrosis factor alpha (TNFα), formerly known as cachectin, was discovered as the antitumor cytokine that is elicited by bacterial lipopolysaccharide (LPS; Beutler and Cerami, 1989), work that was inspired by the findings of William B Coley. LPS is detected by macrophages and dendritic cells through CD14 and TLR4 receptors whose signal is transduced through MyD88 (Medvedev et al., 2002). TNFα elicited by *Salmonella* through TLR4 has been shown to participate in the *Salmonella* antitumor effect (Lee et al., 2008) and to result in a hemorrhagic effect with marked disruption of the tumor vasculature (Leschner et al., 2009), although this effect is exhaustible in experimental models (Kocijancic et al., 2017).

Tumor associated macrophages (TAMS) and similar M2-like macrophages produce the immunosuppressive cytokine IL10 (see Chávez-Galán et al., 2015 for a review), and have reduced iNOS and TNFα expression and collectively limit antitumor immune responses. TLR4 (Lee et al., 2008) and MyD88-TLR (Kaimala et al., 2014) are required for a *Salmonella*-induced antitumor responses, and result in increased iNOS, IFNγ and S100A9 that have antitumor effects, with reduced ARG1, IL4 TGFβ and VEGF that would otherwise promote tumor growth. The presence of *Salmonella* within tumors has the outcome of shifting macrophages to an M1-like phenotype, altering TAMS away from their suppressive state. The presence of *Salmonella* also results in the conversion of immunosuppressive myeloid-derived suppressor cells (MDSCs) into TNF-α producing myeloid cells (Hong et al., 2013). *Salmonella*, but not *E. coli*, induce IPAF, NLRP3 and P2X7 and secretion of IL-1β in response to LPS and damaged cancer cells (Phan et al., 2015).

Macrophage phagocytosis of bacteria results in MHC Class II antigen processing, which induces CD4+ T-cells to stimulate B-cells to produce antibodies. B-cells contribute to the tumor-targeting, or selective tumoral replication effect through production of

antibodies, resulting in limiting *Salmonella* within normal tissues (Lee et al., 2011b). Proliferation within tumors may be due to the poor penetration of antibodies within tumors as well as other immune components that would otherwise control *Salmonella* replication. This differential effect thereby contributes to the apparent tumor targeting that is the result of preferential growth within the tumor compared to normal tissues. Macrophages may also produce nitric oxide (NO) in response to *Salmonella* that has an antitumor effect that can be enhance by deletion of the *Salmonella* genes involved in NO detoxification (Barak et al., 2010).

The presence of bacteria within tumor cells also results in cytotoxic T-cell responses from *Salmonella* (Avogadri et al., 2005; Saltzman, 2005; Lee et al., 2011a), which is believed to contributed to through immunogenic cell death (Kroemer et al., 2013; Galluzzi et al., 2016). The presence of intracellular bacteria or cytoplasmic introduction of proteins through secretion of Type III effector systems effectively targets the cells for elimination by cytotoxic T-cells through MHC Class I antigen presentation. The presence of *Salmonella* inhibits indoleamine 2, 3-dioxygenase 1 (IDO) expression (Kuan and Lee, 2015; Lin et al., 2017) and increases the T cell-dependent immune response.

The presence of the bacteria results in upregulation of connexin 43 which also inhibits IDO (Lin et al., 2017) and increases gap junctions that promote tumor antigen presentation through adjacent dendritic cells and results in activation of cytotoxic CD8+ T-cells (Saccheri et al., 2010). Bacterial upregulation of connexin 43 has also been found to suppress tumor angiogenesis through hypoxic-induced factor-1α mediated downregulation of VEGF (Wang et al., 2015), and also inhibits phospho-protein kinase B (AKT)/phospho-mammalian target of rapamycin (P-mTOR; Tu et al., 2016). Increased gap junctions also increase sensitivity to chemotherapeutics (Chang et al., 2013), and the Type III effector protein SipA downregulates the P-glycoprotein multidrug resistance pump, and also increases cancer cell sensitivity to chemotherapeutics (Mercado-Lubo et al., 2016). *Salmonella* treatment also results in quiescent tumor cells altering their cell cycle status, and become sensitive to chemotherapeutics (Yano et al., 2014).

Bacterial flagellin is a potent TLR5 agonist who's activation by bacteria is associated with tumor cell growth delay (Sfondrini et al., 2006; Cai et al., 2011), enhanced CD8+ T cells (Nguyen et al., 2013), a perforin-dependent response against tumor cells by natural killer (NK) cells (Leigh et al., 2014), and IFN-γ release mediated by the inflammasome intracellular danger sensor NLRC4 (Kupz et al., 2014). *Salmonella*, but not *E. coli*, induce an inflammasome response through secretion of IL-1β in response to LPS and damaged cancer cells (Kim et al., 2015; Phan et al., 2015). NK1.1+CD3- cells have also been associated with *Salmonella*-induced antitumor effects (Feltis et al., 2002).

Autophagy also plays a role in inhibiting the antitumor effect of bacteria. A blockade of autophagy using siRNA has shown enhanced tumor targeting and cancer-cell killing (Liu et al., 2016). Blocking autophagy using chloroquine through increasing bacterial targeting also enhances the antitumor effect of the bacteria and promoting immune infiltrates into the tumor (Zhang et al., 2016b).

Salmonella are known to be able to directly induce apoptosis of macrophages (Gálan et al., 2014; Monack et al., 1996), and there is evidence for direct apoptosis induction of tumor cells (Jia et al., 2007; Li et al., 2012; Uchugonova et al., 2015; Mercado-Lubo et al., 2016). Necrosis and cell lysis varies among different cell lines exposed to *Salmonella* A1-R (Uchugonova et al., 2015) indicating that cancers are not uniform in their response to the effects of *Salmonella*.

Improved *Salmonella* Genetic Backgrounds Tumor Models and Assessments of Antitumor Effects

Stemming from the human clinical trials demonstrating safety and tumor targeting in some humans, areas for potential improvement and augmentation have become a focus of many recent and on-going studies. Owing to lack of antitumor activity in humans, lower percentage of tumors targeted, and generally lower CFU/g, there have been many different approaches aimed at improving on these results as well as new applications that utilize tumor-targeting *Salmonella*. A few of the many studies are described below.

Improved Murine Tumor Models

A murine model that yields results with VNP20009 or other *Salmonella* similar to those obtained in human studies might represent an improved model. Recently it was shown that VNP20009 had 4-logs less innate tumor targeting in autochthonous BALB-neuT tumors compared to the conventional murine breast tumor model 4T1 (Drees et al., 2015). This finding could indicate that these tumors more closely match the physiology of human tumors in regard to their permissiveness to initial entrapment and subsequent bacterial replication properties (Lezzi et al., 2012). Thus, this could be correlated with the lower colonization rates and lower CFU/g tumor results of the intravenously administered VNP20009 in humans. Intriguingly, pre-treatment with the vascular disrupting agent combretastatin A-4 significantly increased the presence of the bacteria 2-days post treatment. These finding suggest autochthonous tumor models combined with vascular targeting agents may facilitate translational studies of tumor-targeting bacteria.

Mutational Backgrounds

Different mutational backgrounds are thought to hold the potential to improve the tumor-targeting and antitumor effects of attenuated *Salmonella*. Several different mutational backgrounds are currently being explored in preclinical models, and may represent improvements that can be translated from mice to humans. *Salmonella* strain A1-R, which is a leucine and arginine auxotroph that was selected by reisolating the bacteria directly from murine tumors, has shown strong antitumor activity with complete regressions in multiple tumor models (Zhao et al., 2006, 2007), and has shown advantages in antitumor effects over

VNP20009 (Zhang et al., 2017). Another strain of *Salmonella* that is currently being evaluated has a mutation guanosine pentaphosphate (ppGpp) synthesis, and has recently been shown to be highly attenuated and effective as an anti-tumor vector (Yun et al., 2012). Reduced toxicity may lead to the ability to deliver higher doses that may improve the tumor targeting percentage and/or the CFU/g tumor tissue.

Isolation and identification of mutations that enhance tumor tissue levels and decrease normal tissue levels has also been explored (Arrach et al., 2010). Mutation in *STM3120* were identified as avirulent and improved compared to a parental *aroA/aroD Salmonella* in their ability to target tumors. In another study (Zhang et al., 2016a) however, *STM3120* was identified as a key factor contributing to the antitumor effect of VNP20009 in melanoma along with *slyA* and *htrA*, underscoring that mutations affecting virulence may sometimes interfere with antitumor effects. Mice treated with *Salmonella* containing these mutations had reduced proinflammatory cytokines TNFα and IL-1β that associated with antitumor effects, and thus it is believed these genes contribute to the immune-oncology effects of *Salmonella*.

Salmonella with a mutation in the ZnuABD transporter has also been studied for its antitumor properties (Chirullo et al., 2015). These bacteria replicate in mammary adenocarcinomas, delay tumor growth and increase survival. These bacteria also retain their ability to cause an inflammatory response that includes recruiting innate and adaptive immune cells to the tumor.

Modulating Bacterial Entry Into Tumors

Penetration of drugs into tumors has long been understood as a limitation of cancer chemotherapeutics (Jain, 1994), and is the subject of active investigation. Decreased initial entry of bacteria into tumors, which is already low in mice (Forbes et al., 2003), would be consistent with the observed differences in tumor targeting between studies in murine tumor models and the human clinical studies. Initial entry of the bacteria into tumors has been modulated using several different approaches.

Pretreatment with TNFα (Sheppard et al., 1989) or by endotoxin-induced release of TNFα (Bloksma et al., 1982) can result in tolerance (tachyphylaxis) to the potentially lethal effects of TNFα, and thus pretreatment could enhance both efficacy and safety. The potential for pretreatment with lipid A as a modulator of tumor penetration has been investigated (Zhang et al., 2014). Because treatment with *Salmonella* results in TNF-α mediated vascular hemorrhage (Leschner et al., 2009), lipid A pretreatment may also increase influx into the tumor mediated by TNF-α. Pretreatment with lipid A resulted in a measurable increased in the fraction of well-colonized tissues (>25%) within a mouse model of breast cancer. Primer dosing of *Salmonella* has also been used to enhance efficacy (Tome et al., 2013). Following an initial low does, a second dose ten times higher was given four hours later, and resulted in increased TNFα, enhanced destruction of tumor vasculature and smaller Lewis lung tumors compared to the high dose only.

The investigational agent PEGPH20 is a long-circulating (pegylated) hyluronidase that increases blood flow into tumors that have elevated interstitial fluid pressures (IFP) and vascular collapse due to the presence of extracellular hyaluronan (Yu and Tannock, 2012; Provenzano et al., 2012). Pretreatment of autochthonous and orthotopic pancreatic tumors in mice with PEGPH20 depleted extracellular matrix hyaluronan, resulted in significantly higher levels of *Salmonella* colony forming units within the tumors, and resulted in complete regressions in combination with an IDO shRNA (Manuel et al., 2015).

Cell-Surface Binding Ligands and Antibodies

Bacterial expression of antibodies on the cell surface increases tumor targeting and efficacy. Surface display of an anti CEA-specific antibody enhanced the antitumor activity of VNP20009 against a MC38 murine adenocarcinoma transduced with CEA (Bereta et al., 2007). Similarly, surface expression of an anti-CD20 antibody results in significantly enhanced cell binding to CD20-positive cells (Massa et al., 2013). Coexpression with the prodrug converting enzyme herpes simplex virus thymidine kinase (HSVTK), which activated gancyclovir and conferred ganciclovir sensitivity in combination therapy against CD20 human xenografts, resulted in complete eradication of the implanted lymphomas. This approach therefore represents a favorable combination for using *Salmonella* engineered to include both increased targeting and a controlled cytotoxicity mechanism.

Bacterial cell-surface display of a ligand (RDG) for tumor associated αvβ3 integrin over-expressing cancer cells has utilized to enhance tumor accumulation compared to the parental *Salmonella* strain (Park et al., 2016). The strain is improved in both *in vitro* binding to cancer cells and in vivo initial and longer-term targeting levels with up to a 1000-fold increase in solid tumors, which correlated with improved antitumor efficacy.

Modulation of Motility and Chemotaxis

The potential for motility to play a positive role in *Salmonella* antitumor effects and targeting ability was suggested early in their development (Bermudes et al., 2001). Modulation of motility and chemotaxis have been explored in regard to tumor accumulation and antitumor activity in several different tumor models. Collectively, the results support a role for motility and chemotaxis shaping the biodistribution within the tumor, especially with respect to location near blood vessels and quiescent cells. However, the bulk of the tumor entry and CFU/g tumor tissue within murine tumor models apparently does not require motility or chemotaxis. For example, Stritzker et al. (2010) found that chemotaxis and flagellar mutants did not alter the apparent tumor biodistribution as assessed by fluorescent microscopy, and *cheY* (chemotaxis$^-$) and *fliGHI* (motility$^-$ and chemotaxis$^+$) mutations in *Salmonella* SL1344 did not numerically alter tumor or liver CFU/g or the apparent biodistrubution as assessed by fluorescent microscopy for either chemotaxis$^-$ or motility$^-$ mutants.

VNP20009 is deficient in chemotaxis, containing a P110S mutation in *cheY*, a key response regulator involved in flagellar motility that was not recognized at the time of the human clinical study (Broadway et al., 2014, 2015). The *cheY* mutation can be suppressed by mutations in *fliM*, whose gene product is known to interact with CheY (Broadway et al., 2017). Restoring the wild type CheY amino acid sequence partially restores chemotaxis in VNP20009. However, the $CheY^+$ VNP20009 did not accumulate in tumors at higher levels, and neither the $CheY^+$ VNP20009 nor VNP20009 had significant antitumor properties against the murine 4T1 breast cancer primary tumors, or on the formation of metastases. Both the parental and $CheY^+$ strain had significant liver toxicity in this model (Coutermarsh-Ott et al., 2017). Although this study also did not find significant improvement in tumor colonization by VNP20009 $CheY^+$, it did find an improvement in the targeting ratio, with slightly lower levels in normal tissue.

Despite the apparent lack of influence on the bulk biodistribution of *Salmonella* within tumors, it is of potential importance to note that *Salmonella* chemotax toward larger cylindroids that contain quiescent cells as compared to smaller cylindroids that lack them (Kasinskas and Forbes, 2006) and is increased in ribose receptor deficient *Salmonella* (Kasinskas and Forbes, 2007). Because quiescent cells are more difficult to treat therapeutically than actively growing cells, this observation provides a potential mechanism for improved treatment against this tumor cell subpopulation by using tumor-targeted bacteria that switch quiescent cells from G0/G1 to S/G2/M and makes them sensitive to chemotherapeutics (Yano et al., 2014). Therefore *Salmonella* combinations with standard chemotherapies may offer the potential to treat quiescent tumor cells that could otherwise persist and result in subsequent tumor regrowth.

Improved *Salmonella* Gene Expression and Antitumor Efficacy

Improved tumor-targeting properties, as described above, may enhance the colonization efficiency in terms of the percentage of tumors colonized, the maximum CFU within tumors, and the tumor-targeting ratio. However, in an effort to augment or surpass the innate antitumor effects of bacteria, a variety of different approaches have been taken to develop antitumor effector systems. These systems generally involve the combination of one or more effector proteins, a gene expression cassette, and a diffusion, secretion or release systems for the effector component that delivers it outside of the bacteria.

Antitumor Effector Systems

A variety of systems have been explored as antitumor effectors for expression by tumor targeted bacteria. These systems fall within several broader categories as listed below and in Table 1.

Prodrug converting enzymes metabolize non-toxic precursors into toxic metabolites that have effects at the site of conversion. Localization of a prodrug converting enzyme to a specific site results in localized conversion with higher concentration at that site, and has been explored widely as a form of anticancer therapy (Lehouritis et al., 2013).

Cytokines are immune signaling molecules with the potential to recruit an immune response against the cancerous tissue. Cytokines have been studied intensively as cancer therapeutics (Lee and Margolin, 2011).

siRNA Delivery. Small interfering RNA (siRNA) delivery makes use of *Bactofection*, the transfer of DNA from a bacterium to a eukaryotic cell. Transfer of DNA with eukaryotic promoters results in RNA and/or protein expression by the eukaryotic cell (Pálffy et al., 2006; Chung et al., 2015).

Antivascular/antiangiogenic factors block neovascularization, which is required for tumor growth, and therefore inhibit tumor growth (Vasudev and Reynolds, 2014).

Cytotoxic proteins directly result in tumor cell death. Proteins secreted by bacteria have the potential to result in a "bystander" effect, whereby cells only adjacent to the site of production are also killed.

Protease Inhibitors halt the action of proteases, which are abundant in tumors and are involved in tumor metastasis and degradation of protein-based therapeutics. In the context of delivery by tumor-targeted bacteria, they have the ability to halt proteolytic activity, thereby enhancing the activity of proteins therapeutics delivered by the bacteria or administered independently, as well as inhibiting metastasis (Quintero et al., 2018).

Apoptosis inducing peptides are not directly toxic, but activate the cellular system for self-destruction, and can result in death of tumor cells.

Immune activator peptides have immunostimulatory effects that activate and/or recruit immune cells to the site of cancerous tissues and result in anticancer effects.

Enhanced nitric oxide production is an approach that uses the anaerobic respiration of *Salmonella* to produce NO, combined with deletion of the genes encoding nitric oxide detoxification proteins. NO is associated with anticancer effects, although its mechanism is not clearly defined.

Bacterial Cell Lysis and Inducible Delivery

Bacterial delivery systems have been engineered using phage and colicin lysis proteins, and cell wall biosynthesis mutations to generate empty immunogenic shells in order to release internal contents or preferentially release periplasmic contents. Lysis of

Table 1 Effector system used in tumor targeted bacteria

System or category	*Examples*	*References*
Prodrug converting enzymes	Herpes simplex virus thymidine kinase (HSV-TK); activation of gancylcovir	Pawelek et al. (1995, 1997), Tjurajev et al. (2001), and Massa et al. (2013)
	Cytosine deaminase; activation of 5FC	Fox et al. (1996) Nemunaitis et al. (2003), King et al. (2002, 2009), and Mesa-Pereira et al. (2015)
	Purine nucleoside phosphorylase; activation of 6MePdR	Fu et al. (2008a,b) and Chen et al. (2013)
	Carboxypeptidase G2; prodrug activation	Friedlos et al. (2009)
Cytokines	IL-2	Saltzman et al. (1996), Feltis et al. (2002), Sorenson et al. (2008), and al-Ramadi et al. (2009)
	TNFα	Yoon et al. (2011)
	LIGHT	Loeffler et al. (2007)
	IL-18	Loeffler et al. (2008a,b)
	TRAIL	Ganai et al. (2009)
	CCL21	Loeffler et al. (2009)
Antiangiogenesis	Endostatin	Lee et al. (2004) (gene delivery); Jia et al. (2012)
	Thrombospondin (gene delivery)	Che-Hsin Lee et al. (2005)
shRNA/siRNA	β-catenin	Xiang et al. (2006)
	Stat3-siRNA	Zang et al. (2007), Manuel et al. (2011), Jia et al. (2012), Tian et al. (2012), and Li et al. (2013)
	IDO	Blache et al. (2012), Manuel and Diamond (2013), and Manuel et al. (2015)
	bcl2	Yang et al. (2008)
Cytotoxic proteins	ClyA	Ryan et al. (2009)
	ToxA	Swofford et al. (2014)
	SAH	St. Jean et al. (2014)
	PE-38K	Quintero et al. (2016), and Lim et al. (2017)
	Shiga toxin	Flentie et al. (2012)
Protease inhibitor	SFTI	Quintero et al. (2018)
Apoptosis inducer	Apoptin	Guan et al. (2013)
	FasL	Loeffler et al. (2008a, b)
	Hemagglutinating virus envelope transfer	Lee et al. (2017)
	Noxa	Jeong et al. (2014)
Immune activator peptides	Flagellin	Zheng et al. (2017)
	Flt3	Yoon et al. (2007)
	PD-L1	Binder et al. (2013)
Enhanced nitric oxide production	Deletion of *hmp* and *norV*	Barak et al. (2010)

bacterial host cells by bacteriophage ΦX174 is controlled by the phage-encoded lysis protein E (Henrich et al., 1982; Young and Young, 1982). Lysis protein E is used as a biotechnological tool to generate non-replication competent bacterial-based vaccines with nearly intact antigenic bacterial cell walls that lack cytoplasmic contents, known as bacterial ghosts (Langemann et al., 2010). Programmed loss of bacterial cell wall structural integrity can also be achieved in live replication competent vaccines by disruption of the *asd* gene, which is essential for cell wall peptidoglycan synthesis (Kong et al., 2008). Through utilization of arabinose inducible control (Loessner et al., 2007) to keep the gene on, cell lysis occurs in the absence of the inducer. Programmed lysis can be further enhanced by arabinose control of the cell wall biosynthesis genes *murA* and *asd* coupled with expression of the p22 phage c2 gene (Kong et al., 2012). Subsequent to removal of the arabinose inducer caused by introduction of the bacteria into an arabinose-free host, *murA* and *asd* are further inhibited through loss of repression of P_R, which then drives an antisense *murA* and *asd*. The combined result of this two fold control of *murA* and *asd* is not only highly efficient at release of an antigenic payload, but also results in complete loss of viability, and thereby provides biological containment.

High lysis efficiencies can also be engineered using the bacteriophage lambda lysis gene cluster SRRz for tumor-targeted delivery with anhydrotetracycline induction coupled in parallel with salicylate induction for therapeutic gene expression has also been further provided (Camacho et al., 2016). This study further combined the system with a *sifA* mutation that results in liberating the bacteria from their location within host cell vacuoles into the host cytoplasm. Efficiency of bacterial autolysis systems continues to be improved for biological delivery by combinations such as inclusion of the λ phage holin-endolysin with the ΦX174 gene E (Won et al., 2017). Selective release of periplasmic proteins has also been accomplished by use of bacteriocin release proteins (BRPs; Van der Wal et al., 1995). The colicin E3 lysis protein has been used to enhance release of a tumor-selective toxin based on *Pseudomonas* ToxA (Quintero et al., 2016).

Quorum Sensing Regulated Gene Expression Circuits

The application of synthetic biology and artificial gene circuits is being applied to bacterial delivery systems (Prindle et al., 2012). Quorum sensing is a gene expression system based on concentration-dependent induction by an autoinducer produced by the bacteria. When concentrations of the inducer are high, indirectly due to high concentrations of the bacteria, the inducer interacts with the quorum gene expression regulon to initiate expression. Because bacteria such as *Salmonella* localize within tumors to numbers exceeding 10^9 CFU/g, the preferential localization of the bacteria offers a tissue-dependent bacterial concentration phenomena that can be coupled to a quorum sensing circuit. Bacterial quorum sensing circuits have been reengineered to control bacterial invasion of host cells (Anderson et al., 2006). An *E. coli* modified to express the *Yersinia* invasion gene under inducible control of the *Vibrio fisheri* quorum sensing *lux* operon was used to induce uptake of non-invasive *E. coli*, which they further tuned using a ribosomal binding site library, and showed that concentration dependent invasion occurred based on quorum-based induction (Anderson et al., 2006). Quorum sensing has also been used induce tumor-localized expression (Swofford et al., 2015; Din et al., 2016), and synchronized tumor-localized lysis combined with expression (Din et al., 2016). The *Vibrio fisheri* quorum sensing *lux* operon was used to preferentially expressing a GFP reporter within murine tumors (Swofford et al., 2015). Above a high-level threshold (4.2×10e10 CFU/g), expression was triggered to the same levels as constitutive controls, while below 0.6×10e10, expression levels were only at 3%. The colonization level within liver of these bacterially infected mice was completely below the level required to initiate expression, thereby preventing liver-associated expression and establishing that expression is tumor-selective. Coupling of expression of a therapeutic protein (HlyE; Ryan et al., 2009) and the ΦX174 gene E with a *Vibrio fisheri* quorum sensing circuit that resulted in oscillatory expression of the effector gene, individual cycles of protein expression followed by cell lysis occurred on a cyclical basis, and resulted in repeated rounds of protein expression and release that led to a therapeutic protein-dependent (HlyE) antitumor effect (Din et al., 2016). This systems have the potential to be used for expression control of other effector systems such as those outlined above.

Summary

Bacterial targeting of tumors offers a promising alternative to conventional cancer therapeutics. In mice, attenuated bacteria are safe, target tumors from a distant inoculation site, selectively replicate within tumors, and cause tumor suppression of tumor growth. These phenomena also occur in dogs treated with *Salmonella*, and in some cases resulted in complete regressions. However, based upon the studies with *Salmonella*, no significant antitumor effects have yet been shown in humans, even when the bacteria successfully targeted the tumors. Further exploring treatment of tumors other than melanoma remains of interest. Many approaches to conferring *Salmonella* with enhanced antitumor effects are being explored. Other on-going clinical studies, including those using *Clostridium* and *Listeria*, also have the potential to generate novel forms of cancer therapeutic bacteria.

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Bacteriophage Ecology☆

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Introduction

An isolated phage particle on its own is fairly simple, consisting of one or more strands of nucleic acid (either DNA or RNA) enclosed in a protein capsid, often with additional structures in the form of a tail or, less often, a membrane. As such, it is a relatively inert, non-metabolizing, non-evolving complex of macromolecules. Place this encapsidated nucleic acid into its natural environment, however, and it veritably comes alive. Such a particle now can interact with a bacterial cell, harnessing cellular metabolism to replicate and produce offspring at the cell's expense; alternatively, the phage genome may contribute gene products to the benefit of both itself and its host. Offspring viruses, including spontaneous mutants, can compete intracellularly or infect new cells, potentiating rapid evolution of the virus population. Cells killed by viruses release dissolved organic carbon and other nutrients as well as DNA into the environment, in turn altering organism competition and other ecological interactions. Further driving changes to their communities, many phages in the process of transduction can actively transfer genes from one bacterial cell to another. Some bacterial sequences have even evolved to exploit transduction and transduction-like processes, resulting in the use of phage machinery to efficiently package and deliver plasmids and pathogenicity islands, as well as other phage-like elements. Phage ecology links phage molecular and structural biology to evolutionary biology. Indeed, phages have become model organisms in the study of all of these fields.

Whether comparing phage genotypes in the lab or observing entire populations of phages directly in nature, the study of phage ecology spans all levels of biological organization (Table 1). These levels range from individual phage particles to ecosystems comprised of phages, bacteria, other microbes, macroorganisms (animals, plants, fungi, and larger protists), and abiotic factors. In this article, we review how phages interact with their environments across these levels of organization, and we discuss how phages are detected and characterized in their natural environments.

Phage Population Ecology

The study of populations is central to understanding one of the major questions in ecology: what generates and maintains the diversity of the biological world? Populations are the major unit in which evolution occurs, so uncovering the forces that influence phage population ecology allows a detailed understanding of how and when phage evolution happens. Elucidation of population dynamics, and thereby of population ecology, requires consideration of the genotypes present in populations, how those genotypes confer individual differences in phenotypic fitness, and how individuals with similar or different genotypes interact. In this section, we review the major classes of phage-phage interaction, especially with respect to competition for their most important resource: bacterial cells.

Phage reproduction strategies

Decades of research have uncovered a diversity of molecular strategies which drive both phage ecology and evolution (see Bacteriophage (Overview)). For example, phage reproduction can involve the maturation and release – either by killing the cell or chronically infecting depending on the phage – of new virus particles into the extracellular environment. Alternatively, phage lysogenic strategies involve the replication of phage nucleic acid within infected bacterial cells but without immediate production of new virus particles. Though all phages can produce new virus particles, temperate phages are ones which also can display lysogenic strategies. Nevertheless, phages are sometimes misleadingly referred to as 'lytic or lysogenic' and thus presented as if these two strategies are either mutually exclusive from one another (though temperate phages by definition can display both) or are the only two strategies phages can employ (e.g., consider also chronic release).

Production of new virus particles is often described in terms of the lytic life cycle common to many phages, such as phage T2 (obligately productive) and phage λ ('strategically productive,' i.e., temperate). During the lytic cycle, a phage undergoes the following steps: (1) Adsorption to one or more cell-surface receptors; (2) Entry of the nucleic acid into the cell; (3) Cooption of cellular machinery; (4) Nucleic acid replication; (5) Assembly of phage particles; and (6) Cell lysis and release of new phage particles into the environment. The number of particles released per infected cell is the burst size, which can vary tremendously among different phages but often numbers hundreds per phage-infected bacterium (see Bacteriophage (Overview)). As noted, the final step of the lytic cycle (lysis) is not required for all phages, and indeed phages such as M13 (Table 2) are released without killing the cell

☆*Change History*: July 2018. AR Burmeister updated the text, including moving some content within Tables 1 and 2, regular text modifications, and figure caption modifications.

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Table 1 Levels of biological organization and example ecological interactions experienced by phages

Organization level	*Definition*	*Ecological example*
Individual	A single organism	
Population	All individuals of the same species within a specific location	Within a population, individuals compete for resources, such as phages competing to infect cells.
Community	All individuals of all species within a specific location	Within a community, one species exploits another, such as lytic phage infection and killing of bacterial cells.
Ecosystem	The community plus all abiotic factors in a specific location	Within an ecosystem, phage lysis of cells causes nutrient levels to increase.
Biosphere	The set of all ecosystems on Earth	Within the biosphere, abiotic nutrients and biotic entities flow between ecosystems.

Table 2 Phage types arranged by genomic characteristics

Nucleic acid	*Strands*	*Super-coiling*	*Circularity*	*Genome sense*	*Segments*	*Morphology (%)*[a]	*Family (genus examples)*	*Genome size (kb)*
DNA	Double	+	−		−	Contractile tail (14%)	Myoviridae (Mu, P2)	34–240
						Long, noncontractile tail (59%)	Siphoviridae (λ, T5)	22–121
						Short, noncontractile tail (24%)	Podoviridae (P22, T7)	16–70
						Isometric, internal membrane (<1%)	Tectiviridae (PRD1)	15
		−	+	−	−	Isometric, internal membrane (<1%)	Corticoviridae (PM2)	9–10
						Spherical-pleiomorphic, enveloped (<1%)	Plasmaviridae (L2)	12
	Single	+	+	+	−	Elongated (1%)	Inoviridae (M13)	4.5–8.5
						Isometric (1%)	Microviridae (ϕX174)	4.5–5.5
RNA	Double	+	−		+	Isometric, enveloped (<1%)	Cystoviridae (ϕ6)	13
	Single	+	−	+	−	Isometric (1%)	Leviviridae (MS2, Qβ)	3.5–4.2

[a]Approximate relative abundances among electron micrograph-characterized cultured phage isolates.

Source: Summarized from Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2005. Virus Taxonomy. Elsevier: Oxford.

(termed 'chronic' release instead of 'lytic'). The primary ecological effect of productive infections is phage population growth, resulting in more phages in the environment.

Lysogenic infections often involve the integration of the phage genome directly into the bacterial genome (as done by phage λ) or instead replication of the phage genome as a plasmid-like element (as done by phage P1). In these forms, the hosting bacterium is referred to as a lysogen and the phage genome as a prophage, and phage replication is largely dependent on the bacterial host's replication. Sometimes, a phage genome may simply lie quiescent in the bacterial cytoplasm, replicating neither productively nor lysogenically, in a state called pseudolysogeny.

Competition

Population ecologists often consider the mechanisms by which individuals of the same species interact to compete for resources. The outcome of these interactions is that some individuals will gain more resources than others, with the difference often expressed in terms of evolutionary fitness. For phages, fitness can be quantified directly in laboratory experiments by observing the relative changes in phage genotype frequencies over time. Alternatively, fitness may be approximated by measuring specific traits such as phage growth rates.

Ecologists generally consider two broad categories of competition within a population: scramble competition and contest competition. These categories differ in the way that individuals compete for and control resources. Scramble competition involves all individuals within a population competing for resources to which they all have equivalent access, while contest competition involves a small number of individuals controlling access to one or more resources. Individuals may experience combinations of scramble and contest competition at any given time as the two types of competition are not mutually exclusive.

An example of scramble competition commonly invoked by biologists is that of finches competing for seeds. One bird may more quickly pick up seeds with its beak and thereby outcompete the slower seed-picker, even though the two birds have equal access to seed resources. For phages, scramble competition will occur as different genotypes of the same species compete for bacterial cells. One phage may more quickly adsorb to cells and thereby outcompete slower-adsorbing phages, even though the two viruses otherwise have equal access to all of the cells.

With contest competition, one individual controls a resource and another individual can be harmed when attempting to access that resource. For example, a single male elephant seal can control a beach to ensure exclusive access to all mating events with

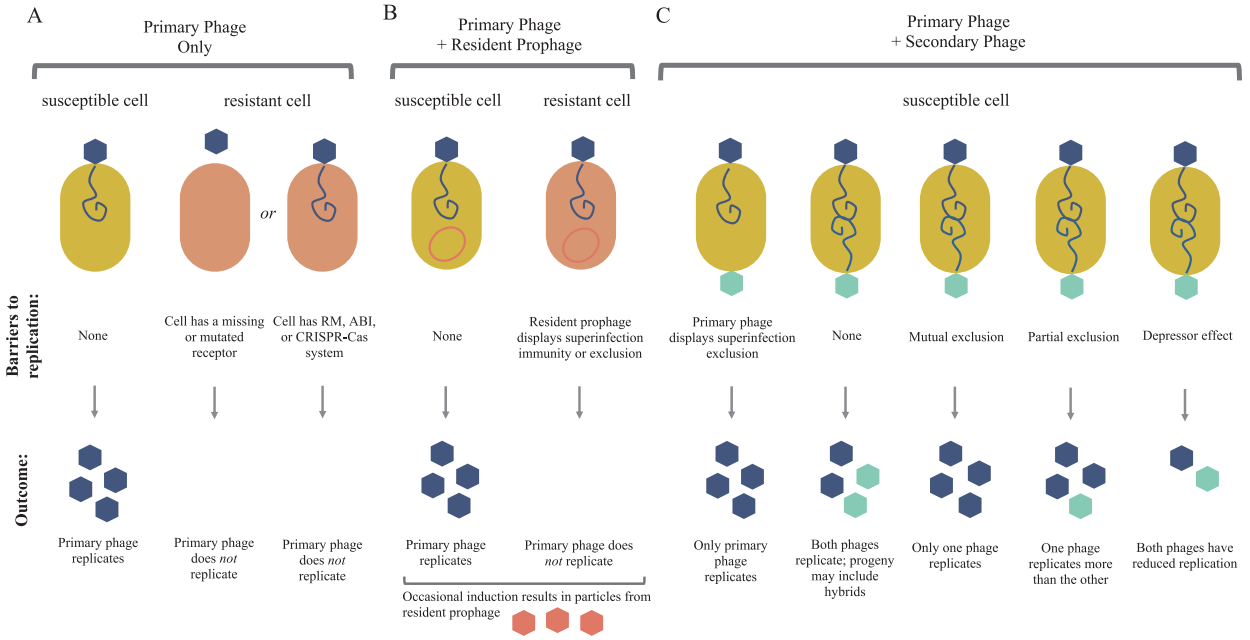

Fig. 1 Competition and outcomes of infection involving a single primary phage infection (Panel A), a primary phage infection of a cell with a resident prophage (Panel B), or both primary and secondary phage infections (Panel C). Successful infection and replication of phages depends on whether a cell is resistant to the phage, including via superinfection immunity or superinfection exclusion. When two phages initiate infections of the same cell at the same time, then mutual exclusion, partial exclusion, and the depressor effect can also alter the outcome of infection (Panel C). All barriers to replication depicted in Panels A, B, and C may occur within the same cell; individual barriers are shown here for simplicity and clarity.

females. To compete for mates, another male must fight the 'beach master,' a challenge that incurs risk of bodily harm or even death. For phages, contest competition occurs when one phage controls all of a cell's resources, often by being 'primary,' i.e., the first to infect (Fig. 1).

Relationship between reproductive strategies and competition

Successful phage infection depends on several factors; foremost, a phage-susceptible cell must be sufficiently metabolically capable of supporting phage replication. The cell must also not contain other interfering phages that prevent the new phage from replicating (Fig. 1). A primary phage is the first phage particle to encounter a cell (Fig. 1(A)). This phage, if singly infecting, has been subject only to scramble competition: if its general rate of encountering, infecting, and replicating within cells is faster than other phage particles infecting other cells, then it will be more competitive and thereby have a greater fitness. See Bacteriophage (Overview) for other steps in the phage life cycle that influence phage replication rate and fitness.

The outcome of a phage infection is more complex when multiple phage particles or types are involved, potentially resulting in contest competition. There are two common mechanisms of phage contest competition. The first occurs during a process called 'superinfection,' when a phage infects a cell that already contains a prophage (Fig. 1(B)). Prophages can confer resistance to infection through superinfection immunity, preventing the incoming virus from replicating. The second mechanism, superinfection exclusion, occurs when a primary infecting phage interferes with the genome uptake of a subsequently adsorbing phage (Fig. 1(C)). These mechanisms, and others involving multiple adsorptions to individual bacteria, tend to be limited to related phages, such as those from the same population. In the case of less closely related phages, direct competition between phages coinfecting the same cell will tend to result in a favoring of one phage over the other by means other than superinfection immunity or superinfection exclusion.

Phage Community Ecology

Communities comprise all of the species that coexist within a given area, and for phage communities, this includes at least one phage population and one bacterial population. In nature, a community is unlikely to be as simple as a single phage species and a single bacterial species, but in the following section we will discuss how even such simple communities can be complex in their types of interactions.

Model communities with single populations of phage and bacteria

Communities consisting of only a single phage type and single host type have been extensively studied using laboratory microcosms and mathematical modeling. Many such communities display distinct predator-prey cycles as also observed with macroscopic organisms (Fig. 2) and have been useful for studying ecological and evolutionary principles in general, not just those specific to microbial communities.

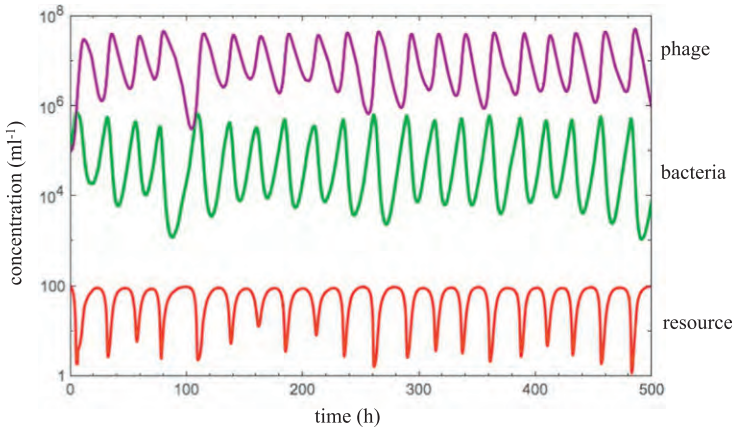

Fig. 2 Model of population dynamics in a simple community comprised of a single type of lytic phage, a single type of susceptible bacterium, and resources for bacterial growth. This simulation shows so-called 'predator-prey' cycles caused by bacterial growth and death due to lytic phage infection. As phages (purple line) consume bacteria (green line) and produce more phages, there is an increase in phage abundance and a corresponding decrease in bacterial abundance. As bacterial abundance declines, phages have fewer cells for replication, so phage abundance declines. The decline in phage abundance allows more bacteria to grow, resulting again in greater phage growth, and ongoing propagation of these community dynamics. The bacterial resource (red line) is impacted indirectly by the phage population through the killing of the bacterial consumer.

Even simple communities comprised of a single phage species and a single bacterial species can have many types of ecological and evolutionary interactions, including:

- Harm to bacteria by lytically or chronically infecting phages.
- Benefit to bacteria through the contribution of temperate phage genes, a process termed lysogenic conversion.
- Evolution of resistance to phages by at least a portion of a bacterial population.
- Evolution of more productive phages including overcoming bacterial resistance mechanisms.

All of these effects involve interactions between a bacterial population and a phage population, and they exemplify the types of dynamics that can play out in ecological communities.

In nature, it is rare for communities to consist of only a single phage strain and a single bacterial strain, and even laboratory communities can quickly evolve increased diversity in terms of bacterial resistance to phages and phages overcoming that resistance (next subsection). Such experiments provide insight into how ecological interactions can generate and maintain diversity, a central question throughout all of ecology. For example, laboratory experiments and models show that phages can mediate coexistence of two bacterial genotypes if one genotype is phage sensitive and has a higher intrinsic growth rate. In the absence of phages, phage-resistant bacteria with lower intrinsic growth rates will tend to decline to extinction.

Bacterial resistance to phages and antagonistic coevolution

Four common categories of host-encoded resistance mechanisms exist, each with a different ecological impact:

- Envelope resistance: Phages fail to adsorb so retain their fitness; bacteria survive.
- Restriction-modification: Phages adsorb and are killed; bacteria survive.
- Abortive infection: Phages adsorb and are reduced in fitness; bacteria do not survive.
- CRISPR-Cas: Phages adsorb and are inactivated by bacteria which have gained genetic memory of the phage's nucleic acid sequence.

The consequence of these mechanisms, as of phage-host incompatibilities more generally, is reduction in the number of susceptible bacteria that are available for phages to ecologically exploit.

If a cell carries a resistance mechanism, then a phage's ability to replicate may be diminished; unable to switch to a new cell, an adsorbed phage particle thereby can suffer reduced fitness and often death. Thus, there can be substantial fitness benefit to phages in overcoming bacterial resistance mechanisms. The process by which bacteria evolve resistance mechanisms to phages and phages respond by evolutionarily overcoming those resistance mechanisms is termed antagonistic coevolution.

Phage interactions with more than just bacteria

Phages can parasitize bacteria, and these bacteria may be parasites of eukaryotic hosts, such as humans. Consequently, one question of increasing importance is how do phages impact human health? Research suggests that phages may naturally play a role in protection against bacterial pathogens of humans and other animals. On the other hand, many temperate phages carry genes encoding bacterial virulence determinants; in these cases, the resulting lysogenic conversion can potentially convert benign host bacteria into virulent pathogens. These virulence determinants include cholera toxin (*Vibrio cholerae*), Shiga toxin (*Shigella* and *Escherichia coli*), diphtheria toxin (*Corynebacterium diphtheriae*), and the botulism neurotoxin (*Clostridium botulinum*).

Phages also experience interactions with other phage species. Associated modes of contest competition occur when two phages successfully initiate infection of the same cell at the same time (Fig. 1(C)). These modes include mutual exclusion (one phage replicates and the other does not), partial exclusion (one phage replicates more than the other), or the depressor effect (both phages experience reduced replication).

Phage Ecosystem Ecology

In addition to interacting with other community members, phage populations can also shape (and be shaped by) their abiotic environment. Together the biotic and abiotic components of an environment comprise an ecosystem, with organism impact on ecosystems studied by ecosystem ecology.

Ecosystem effects of phage lytic and lysogenic lifecycles

In addition to the production of toxins, phages also alter environments by simply lysing bacteria. Cell lysis releases both phages and soluble nutrients, the latter which can be used as growth substrates by other bacteria. This process, termed the 'viral shunt,' changes the flow of energy and matter through ecosystems (Fig. 3). Without viruses present, the 'microbial loop' instead moves energy and nutrients from bacteria to their predators. For example, in aquatic ecosystems cyanobacteria serve as food for their protist predators. When phages are present, cyanobacteria serve as resources for both protists and phages. While protists incorporate biomass that can be assimilated up the food chain, phages incorporate only a small portion of the bacterial biomass, the rest of which is released back into the environment as dissolved organic matter/material (DOM) via the viral shunt.

Phage Environmental Microbiology

Phage environmental microbiology largely deals with the detection and characterization of phages in their natural environments. Three methodological categories of phage environmental microbiology include culture-based techniques, direct visualization, and sequence-based techniques. Together they reveal much of what is known of phage diversity.

Culture-based techniques

Culturing typically relies on growing phages in isolation with specific bacterial hosts, often first enriching phages found in mixed communities on target bacteria. The success of these methods depends, however, on the phage types being grown and the culturability of their hosts.

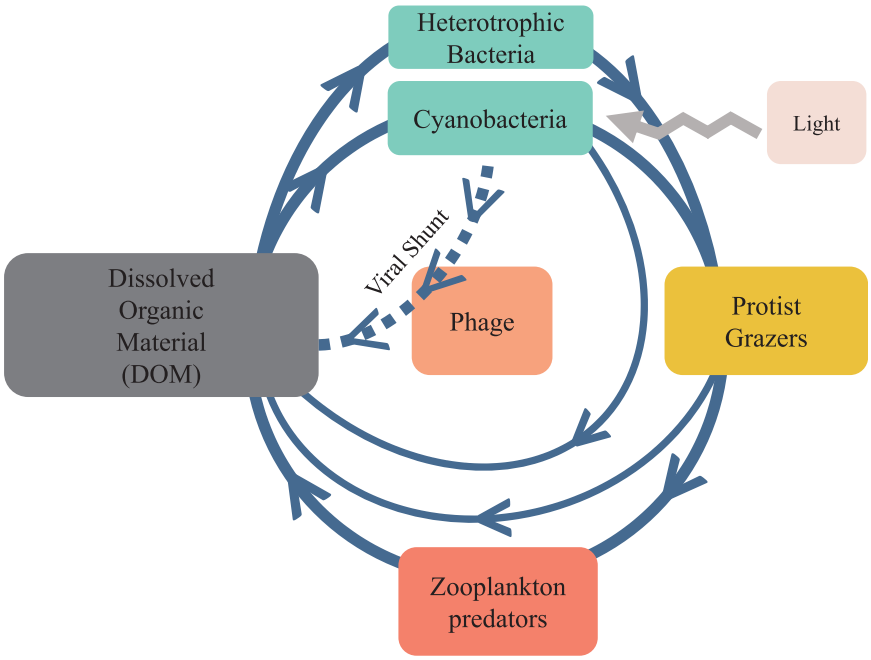

Fig. 3 Nutrient cycling and energy flow via the microbial loop and viral shunt in the ocean. Energy and matter flow through the microbial loop beginning with phototrophic carbon fixation by the cyanobacteria. The bacteria are consumed by protist grazers, which are consumed by zooplankton predators. Death and incomplete consumption release dissolved organic material into the ecosystem, providing nutrients for growth of cyanobacteria and heterotrophic bacteria. Phages increase turnover through ecosystems by breaking down bacterial biomass before it is assimilated at higher trophic levels.

Many phages of easily-cultured bacteria can often be readily grown in laboratory broth cultures. When added to bacterial cultures, the phages replicate and may lyse the bacteria sufficiently to allow detection of reduced cell densities by eye or using a spectrophotometer. The resulting product, called a 'lysate,' is then centrifuged to remove cell debris. Residual living cells can be removed with chloroform or a filter that allows the phage, but not the cells, to pass through. Once prepared, a purified phage stock can be characterized in terms of growth rate, adsorption time, lysis time, burst size, particle stability and other key fitness parameters. Such culture-based methods remain the standard for phage characterization, providing clear evidence that a substance is indeed a phage, and not something phage-like in terms of morphology, sequence identity, or effect on cells. Culture-based methods are also useful for characterizing basic ecological and evolutionary relationships among phages, including their host range, receptor use, optimal growth conditions, and fitness.

While culture based methods are useful for characterization of specific phages that can be grown in the lab, most phages are not readily propagated outside of their natural environments, in part because most host bacteria are themselves not easily cultured. Furthermore, culture-based methods reveal only a limited set of properties of phages, often one phage isolate at a time, so can be somewhat limited in their ability to characterize the diversity of the phage world. Therefore, culture-based methods are often used alongside direct visualization and sequence-based methods of phage detection.

Direct visualization

Direct visualization methods include microscopic imaging of purified or mixed phage communities. Transmission electron microscopy (TEM) is frequently used, as it allows resolution of particle morphology (Table 2) for mixed environmental samples as well as purified phages. Used on mixed communities, TEM can provide estimates of viral diversity and abundances in an ecosystem. Scanning electron microscopy (SEM) is also used but provides lower resolution, making phage particle morphology difficult to determine. Epifluorescent staining and microscopy is often more affordable than TEM and SEM, although it provides even lower resolution. On the other end of the spectrum, cryo electron microscopy (cryo-EM) can allow exquisite resolution of single phage particles, ideal for the detailed study of purified phages and their molecular interactions with bacterial host cells. One limitation of direct visualization methods is that they cannot distinguish between infectious virions and other virus-like particles, such as encapsidated bacterial genes called 'gene transfer agents,' defective viruses, and others.

Sequence-based techniques

Sequence-based methods can determine the content and diversity of phage genomes regardless of whether a phage can be grown in the laboratory. Therefore, these methods can be used independently of or in conjunction with culture-based and direct-visualization methods. Phage metagenomics – the mass sequencing of whole phage communities – is often instead called 'viral metagenomics' or 'viromics,' because preparation methods do not necessarily separate phages from the viruses of archaea and eukaryotes. Metagenomic approaches have been used to characterize phages from many environments, including aquatic systems, animal microbiomes, and terrestrial communities. Like bacterial metagenomics, phage metagenomics has been rapidly evolving from a tool of natural history to one that is used to test hypotheses, compare treatment groups, and make predictions.

For mixed communities from natural environments, many of the sequencing technologies and analysis tools used for bacterial communities are being adapted for virus communities. However, unlike bacteria that share conserved genes, such as the 16S *rRNA* genes frequently used in sequence-based diversity studies, phages have no universally shared sequences. Furthermore, phage genomes can differ according to various nucleic acid forms, included ssDNA, ssRNA, and dsRNA (Table 2), so methods that rely on, for example, only DNA sequencing may miss large portions of viral diversity. Other approaches first convert RNA genomes into DNA genomes for sequencing. Together, these methods can allow separate descriptions of the 'dsDNA virome,' the 'RNA virome,' and so on. Nevertheless, viral metagenomics is revealing an unprecedented amount of sequence diversity within virus and virus-like particles.

Conclusion

Phage ecology is a rapidly growing and changing field. Population-focused approaches study the mechanisms of ecological and evolutionary change and have made phages into model organisms not only for molecular biology, but also for ecology and evolutionary biology, with new phages discovered continuously. Meanwhile, sequence-based detection and population sequencing are revealing unprecedented snapshots of the diversity of the viral world, and these methods continue to grow more powerful and sophisticated. Combined, all of these approaches have transformed bacteriophage ecology into a highly dynamic field with many open questions and much room for discovery.

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Bacteriophage: Overview☆

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Glossary

Adsorption A series of events, culminating at a minimum in free phage attachment to a bacterium, involving virion movement (via diffusion), virion encounter with a bacterium, virion attachment, and, depending on authors, transfer of the phage genome into the bacterial cytoplasm.
Capsid The proteinaceous structure that contains the phage genome.
Chronic release Extrusion or budding of mature virions from infected bacteria without cell lysis; chronic release neither destroys the bacterium nor ends the phage infection.
Free phage A mature phage (virion) particle that is not found associated with a bacterium.
Induction (prophage induction) The transition of a lysogenic infection to a productive one, involving expression of numerous prophage genes that otherwise remain quiescent in an uninduced lysogen.
Lysis Release of mature virions from an infected bacterium via the destruction of the bacterium, ending the phage infection.
Lysogeny A phage infection in which phage virions are not produced and the phage genome replicates only so as to continue infecting bacterial progeny produced via binary fission. Typically this involves phage existence as a prophage which is integrated into the bacterial genome.
Lytic cycle A productive phage infection that ends with bacterial lysis and release of free phages.
Lytic phage A phage that displays a lytic cycle given productive infection; lytic phages appear to be the most common of phage types. An obligately lytic phage cannot display a lysogenic cycle.
Productive infection Phage infection that results in production of free phages.
Prophage The phage genome as it exists during lysogeny.
Temperate phage A phage that is capable of displaying lysogeny, though infections by temperate phages often are productive rather than leading to lysogeny.
Virion An infectious viral particle.

Introduction

Viruses are obligate intracellular parasites and may be categorized in terms of the types of cellular organisms that they infect. Three cell types exist, members of domains Eukarya, Archaea, and Bacteria. The viruses that infect the bacteria, for historical reasons, are called bacteriophages, or phages for short, meaning that they are "eaters" of bacteria. Since most environments—including our own bodies—contain numerous bacteria, phages are found almost everywhere. Phage biologists have found phages in most environments that can support bacterial growth, often in ratios of phage-to-bacteria of 10-to-1 or more. As there exist estimates of total bacterial numbers of approximately 10^{30}, there consequently is an expectation that there are 10^{31} or more total individual virus (mostly phage) particles, making phages Earth's most numerous "organisms." Bacteria are highly diverse with perhaps millions of species of these prokaryotes. Phage-bacterial interactions therefore are quantitatively vast (huge numbers of interactions) and qualitatively diverse (huge numbers of environment types, bacterial-host types, and also individual phage types). In this entry we provide an overview of this enormous diversity of phage types, variations on phage biology, and the importance of phages in the laboratory, in environments, and, potentially, in the clinical setting.

Primer on Phage Biology

F. W. Twort (in 1915) and Felix d'Hérelle (in 1917) both described phages as macroscopic phenomena: the dissolving of bacterial colonies, the clearing of turbid broth cultures of bacteria, and the formation of holes (plaques) in turbid "lawns" of bacteria. Confirmation of phages as viruses awaited the invention of the electron microscope in the early 1940s. Subsequently, phages were harnessed toward elucidating the basic molecular mechanisms of life, and are credited with extensive contribution to the formation of the scientific subdisciplines of molecular biology and molecular genetics. In this section we provide an overview of the phage life

☆*Change History*: April 2019, ST Abedon updated all the text and references.

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cycle, which by definition must include at some point an extracellular, virion state. We then discuss major criteria by which phage biologists distinguish among different phage genes and different phage types.

Phage Life Cycle

We begin our description of the phage life cycle with the formation of a free phage, which initiates the phage extracellular search for new bacteria to infect. The extracellular search culminates with adsorption. Entrance of the phage genome into the cell begins the infection period, typically described as a latent period. The latent period ends either with phage-induced bacterial destruction (lysis) or via an extruding or budding of maturing phage virions across the bacterial cell envelope (chronic release, which occurs in a manner that does not lead to immediate bacterial death). Infections that result in phage release can be described as productive. Alternatively, phage infections can enter a latent state (lysogeny or pseudolysogeny) or can fail to successfully infect (phage restriction or abortive infection). See Table 1 for a summary of phage infection types and their characteristics.

Phage Genes

Functionally, we can differentiate the genes and factors involved in a phage infection into three types: products of bacterial genes, products of phage genes that are expressed and employed during phage infection, and phage genes whose products are associated with released phage virions. Though useful when viewing phages as evolving, ecological entities, nevertheless not all of the gene products employed by phages may be unambiguously differentiated into these three categories. For example, phage virions can carry some proteins that are then employed only once subsequent infection has occurred. Also ambiguously, many phage genes encode proteins that are involved in virion assembly but which nevertheless are not represented as part of the resulting virion.

Evolutionarily, we can distinguish phage genes in terms of their potential to coevolve with other phage genes. Coevolution among phage genes is often cited as an explanation for the genetic linkage—similar location on genomes—of genes encoding interacting phage capsid proteins, such that a mutation in one gene requires a corresponding mutation in another. Alternatively, phage genes can evolve to become further refined in their interactions with smaller molecules (e.g., nucleotides), generic versions of larger molecules (e.g., as with phage exonuclease action on bacterial DNA), or with specific bacterial macromolecules, for example, RNA polymerase or the phage receptor molecule found on the surface of host bacteria. The latter interactions also can be coevolutionary should antiphage adaptations by bacteria lead to the evolution of counter adaptations by phages (antagonistic coevolution).

Phylogenetically, we can distinguish phage genes in terms of where those genes came from. Many or most genes associated with phages, for example, likely have been evolving within phage genomes for millions or even billions of years. Other genes, or portions of genes, appear to be newly acquired from bacterial genomes. Some phage genes seem to readily move from phage genome to phage genome due to genetic recombination following coinfection of individual bacteria by different phage types. Still other genes, such as virion structural genes (below), appear to move between phage genomes as multigene complexes, presumably as a consequence of the difficulty in splitting apart these coevolved genes while still retaining function (above).

Metabolically, phage genes broadly may be sorted into at least six classes: (i) phage genes involved in the metabolic takeover of host metabolism (e.g., gene products that shut down the transcription of bacterial genes), (ii) phage genes involved in establishing or maintaining a latent state (e.g., lysogeny), (iii) phage genes involved in preparing the infected cell for phage-progeny maturation (e.g., genes involved in prophage induction, nucleic acid repair, and nucleic acid replication), (iv) genes that express phage-virion

Table 1 Different types of phage infections

Type of infection	*Does the phage genome replicate?*	*Are free phages produced?*	*Do bacteria survive?*	*Do phages survive?*	*Comments*
Lytic	Yes	Yes	No	Yes	These are productive cycles that release free phages via phage-induced bacterial lysis, which destroys both bacterium and phage infection
Chronic	Yes	Yes	Yes	Yes	These are productive cycles that result in the formation of free phages without destroying either the infected bacterium or the phage infection, a.k.a., persistent infections
Lysogenic	Yes	No	Yes	Yes	With lysogenic cycles, phage genome replication occurs without production of virion progeny; replicated genomes segregate into daughter bacteria
Pseudo-lysogenic	No	No	Yes	Yes	By one definition these are infecting phage genomes that do not replicate and do not produce phage progeny, a quiescent state that can lead to productive or lysogenic cycles
Restricted	No	No	Yes	No	Passive or active bacteria-mediated inactivation of infecting phages such as via restriction endonucleases; a broader definition does not specify whether bacterial hosts survive
Abortive	No	No	No	No	Abortive infections involve the inactivation of both infected bacterium and infecting phage; a broader definition replaces infecting phage inactivation with infecting phage inability to produce plaques

Based on table presented by Abedon, S. T. (2008). Phages, ecology, evolution. In: S.T. Abedon (ed.), *Bacteriophage ecology*. Cambridge, UK: Cambridge University Press.

structural and virion-assembly proteins, (v) gene products that process and package newly replicated phage genomes into virions, and (vi) gene products required for release of assembled virions from the host cell either by lysing the host cell or by exporting the newly assembled virions through the cell envelope. Though it is traditional to view phage genes as distinct from bacterial genes, in considering these genes as models for the basic metabolic workings of life there is no fundamental difference between a phage gene versus bacterial genes encoding parts of complex molecular machines such as those involved in gene transcription.

Genetically, phage genes required for productive infections can be described as encoding essential functions. Mutations in these genes tend to be lethal or instead effectively so by substantially decreasing the number of progeny produced during infection such that phage plaques do not form. Phages also encode nonessential genes. Inactivation of these genes still allows production of viable progeny. The yield of progeny phages per infection often can be reduced, but not to the point that phage propagation is severely curtailed. Alternatively, if infection yield is not reduced, and additional phage growth parameters are not otherwise affected, then it may be that these nonessential genes encode proteins that are only needed for infection of certain hosts or under certain metabolic or environmental conditions. The number of nonessential genes found within a phage genome can be significant. Sixteen of the 50 genes of bacteriophage T7 (32%) are nonessential, for example. Lastly, it should be noted that some bacteriophage genomes contain other genetic elements such as introns or can encode inteins (self-excising protein segments).

Phage Gene Expression

The expression of phage genes typically occurs in a well-defined temporal order, one which typically "makes sense" in terms of the metabolic functions of these genes. In addition, as bacterial parasites, phages rely on the host cell for a range of metabolic functions. In the most genomically complex phages, functions supplied by the host cell may be limited to cell structural elements (membranes, cell wall), energy transduction machinery (e.g., cellular respiration), and protein synthesis apparatus (ribosomes). Large, more complex, and usually obligately lytic phages often shut down host gene expression soon after initiating an infection and may degrade the host genome. Simpler phages usually rely more on the host cell for metabolic functions. Lysogenically infecting as well as chronically released phages are reliant on host cell functions over longer periods and presumably as a consequence are less disruptive of these functions.

Upon infecting a host cell, phages begin expressing what are termed early or immediate-early genes. The promoters of these early genes—DNA sequences where RNA polymerase binds to initiate transcription—tend to more closely resemble bacterial promoters than those of later-expressing phage genes. The early genes of obligately lytic phages produce proteins involved in coopting bacterial gene expression. These include RNA polymerases and sigma factors (which modify RNA polymerase promoter recognition). Phage-encoded sigma factors favor transcription of viral genes over bacterial genes. Expression of these early genes is followed by delayed-early or middle genes (or, in some cases, late genes) that initiate phage genome replication and related functions. Phages that encode their own DNA or RNA polymerase will begin synthesizing that enzyme along with proteins involved in nucleic acid metabolism. Collectively, early and middle genes are sometimes referred to as prereplicative genes, that is, genes expressed prior to the initiation of phage genome replication.

The first proteins made by temperate phages are those that determine whether lysogeny or instead a lytic infection will immediately occur. Expression of these early genes is sensitive to the metabolic state of the bacteria or to signals from communication peptides produced by previously infecting phage that allow an estimate of the number of previous infections in a location. The resulting relative differences in levels of expression of key phage proteins commits the phage to either the lysogenic or lytic pathway by activating one set of regulatory genes and inhibiting a second set of regulatory genes. If the phage is to enter the lysogenic phase, then proteins are produced that bring about, for example, the integration of the phage genome into the host genome, or which instead stabilize the phage genome in an episomal (plasmid) form. Proteins are also produced that prevent (by inhibition or termination) expression from the remainder of the phage's genes. If the phage enters the lytic cycle, then it begins expressing early and middle lytic genes and blocks expression of genes required for lysogeny.

During the postreplicative phase of the lytic cycle, both obligately lytic phages as well as induced temperate phages begin expressing late genes, including structural genes that will make up the phage virion, processing proteins (if they encode any) that can be required to mature the virion, and packaging proteins which place newly replicated phage genomes into the phage particle. Late genes are much more dependent than earlier genes on phage-encoded functions for expression. In some phages, such as phages T3 and T7, late-gene expression is dependent on a phage-encoded RNA polymerase. In other phages, such as phage T4, the switch to late-gene expression is brought about by modification of the *Escherichia coli* RNA polymerase complex by phage proteins. In bacteriophage λ and related phages, expression of late genes begins by a combination of derepression (loss of a repressor protein allowing RNA polymerase access to promoters) and antitermination (caused by a phage protein that allows transcription to pass through transcription termination sites).

A few phages also encode unusual late genes that neither contribute to the phage virion nor to phage release. Notable examples of this are lambdoid phage strains, which both lysogenize pathogenic *E. coli* strains such as the O157:H7 serotypes and encode a type 2 Shiga toxin. In these phages, the toxin genes are located next to the genes inducing cell lysis and are expressed with those genes late in infections. The release of the Shiga toxin from the cell is also dependent on phage-induced cell lysis. There is debate as to whether these toxin genes ought to be considered phage genes or, instead, as a form of phage-located bacterial genes. As discussed further below, numerous additional phage-encoded genes that express bacterial virulence factors are known including genes producing cholera toxin (as associated with the bacterium *Vibrio cholerae* and the disease cholera), diphtheria toxin (produced by *Corynebacterium diphtheriae* and giving rise to the disease, diphtheria), and numerous additional virulence factors displayed by bacterial pathogens such as *Clostridium* spp., *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.

Phage Diversity

Phages, like viruses in general, are typically differentiated in terms of their infection type, virion morphology, or genome characteristics. Based on infection types, a phage may be described as a lytic versus chronic releaser or as obligately lytic (a.k.a., virulent) versus temperate. Based on virion morphology, phages may be described as tailed (also known as binary), cubic (including icosahedral), helical, or pleomorphic. Tails can be long or short, flexible or rigid, and contractile or noncontractile. Phage genomes can be single-stranded (ss) DNA, double-stranded (ds) DNA, ssRNA, or dsRNA. Genomes can also be segmented (multipartite) or nonsegmented (monopartite). The majority of phage isolates are tailed and so far as is known all tailed phages display dsDNA, monopartite genomes as well as lytic cycles (though many are also temperate). See Table 2 for a description of the various phage families and Fig. 1 for the basic morphologies of each phage family.

Increasingly, phages are differentiated in terms of their genome nucleotide sequences, and whole genome sequencing provides a wealth of information useful for inferring phage genomic functionality and evolutionary history. The analysis of phages in this manner is referred to, generally, as the study of phage genomics. This type of analysis is especially important in classifying phages at the genus and subfamily levels and has led to the distinguishing of many new genera. The 2008 release of virus taxonomy by the International Committee on Taxonomy of Viruses (ICTV) described a total of 18 genera in the Caudovirales (tailed phages) order. In the 2011 release, there were 37 genera with most of the increase in the Myoviridae and Podoviridae families. As of the 2013 release, there are 39 genera.

Table 2 General characteristics of major phage types infecting eubacteria

Family	*Genome*	*Morphology*	*Release*	*Examples*
Cystoviridae	dsRNA, segmented	Enveloped	Lytic	ϕ6
Inoviridae	ssDNA, circular	Helical, rod shaped or filamentous	Chronic	f1, fd, M13, CTXΦ
Leviviridae	ssRNA, linear	Icosahedral	Lytic	MS2, F2, Qβ
Microviridae	ssDNA, circular	Icosahedral	Lytic	ϕX174
Myoviridae	dsDNA, linear	Tailed, contractile	Lytic	Mu, P1, P2, T2, T4, T6, RB69
Siphoviridae	dsDNA, linear	Tailed, noncontractile, long, flexible or rigid	Lytic	λ, N15, SPP1, T1, T5
Podoviridae	dsDNA, linear	Tailed, noncontractile, short	Lytic	Gh-1, N4, ϕ29, ϕA1122, ϕYe03–12, P22, SP6, T3, T7

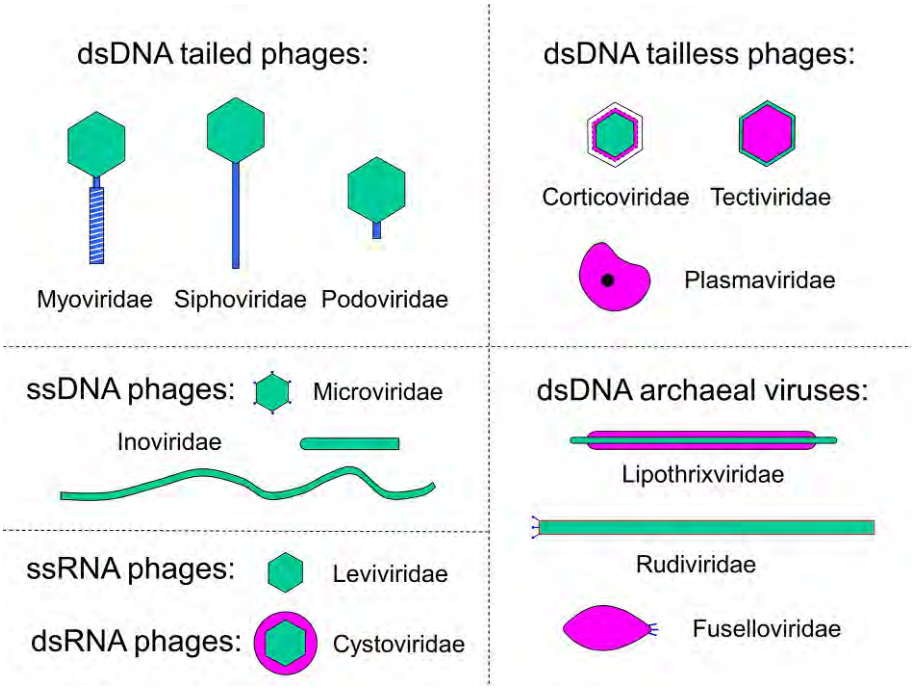

Fig. 1 Basic morphologies of different families of virions of prokaryotes. Green represents nucleic-acid encasing capsid protein. This includes the heads of tailed phages. Blue indicates tails and other adsorption organelles (tail fibers and other appendages on tailed phages, however, are not shown). Pink is lipid of various kinds. The contractile portion of the Myoviridae tail is indicated with diagonal lines. The flexibility often seen with the Siphoviridae tail is not indicated. All shown heads are isometric but in addition tailed phages often display elongated "prolate" heads. The Inoviridae are depicted as two types: short rigid rods (genus Inovirus) and long flexible rods (genus Plectovirus). Long rigid rods of genus Plectovirus also exist. Plasmaviridae are unusual budding viruses of mycoplasma bacteria, which lack cell walls. Only a subset of known archaeal virus morphologies are depicted. Adapted from that of Ackermann, H. W. (2006). Bacteriophage classification. In: R. Calendar and S. T. Abedon (eds.), *The bacteriophages* (2nd edn). Oxford: Oxford University Press, pp. 8–16.

Phages additionally may be distinguished in terms of their host range, that is, what bacterial types they are capable of infecting. In general, however, host-range based classification is less useful toward obtaining a facile understanding of common themes of phage biology. Perhaps the best illustration of the difficulty of host range-based classification are phages P1 and Mu which are each able to switch between different host ranges by utilizing alternate phage-encoded adsorption proteins. In addition, it is increasingly common to characterize phages through nucleic acid sequencing without prior phage culturing. This can be accomplished via the techniques of metagenomics whereby a sampling, often rather large, of environmental DNA (or RNA) is sequenced, resulting in what are described as viromes. In addition, even whole phage genomes can be sequenced via these related techniques, again without prior culturing. As a result of these approaches, huge amounts of phage genetic and genomic information is being generated, often with little appreciation of phage host range.

Importance of Phages

Bacteriophages play important roles—some positive, some negative, some current, some historical—in biology, in ecology, and in health. In this section we provide an overview of these roles. These include, in order of presentation: Phage use as molecular model systems; phage and phage product use as molecular tools (including phage-display technologies); phage use in bacterial identification; the problem of phages as contaminants; the promise of phage therapy (i.e., phages as antibacterial agents); use of phage-derived molecules as antibacterial agents or in antibacterial drug discovery; use of phages as gene or toxin delivery vehicles (such as for fighting cancer or as gene-therapy vectors); phage use as indicators and tracers; the study of phage interaction with animal bodies; the study of phage ecology; phage-mediated transfer of genetic material between bacteria (i.e., transduction); the role of phages in bacterial pathogenesis; and the use of phage models in the general study of organismal ecology and evolutionary biology.

Phages as Molecular Model Organisms

Phages have been used as model organisms for almost as long as their biological nature was recognized (e.g., the use of phages as model viruses by Emory Ellis in the late 1930s). In part this was and is due to their ease of culturing but also due to their amenability to chemical as well as biological manipulation. Bacteriophages were used in key experiments in the history of molecular biology including understanding the structure and composition of the gene, mutagenesis, and gene regulation. Two notable examples are a demonstration that DNA is the genetic material in phage T2, by Hershey and Chase, which served as the second and universally convincing system for which DNA was demonstrated to be the hereditary material, and the existence of mutations within a population before selection, by Luria and Delbrück, which ushered in the now obvious understanding that bacteria, like peas and fruit flies, also possess experimentally accessible genetics.

Phages were used in many other key discoveries in the history of molecular biology. A partial list includes: recombination of phage chromosomes; viral encoding of metabolic enzymes; restriction/modification enzymes; fine structure of the gene; mechanisms of mutation; existence and function of mRNA; nature of the genetic code; colinearity of genes and proteins; conditional lethal mutations, nonsense and frameshift mutations; protein assembly pathways; role of sigma factors in transcription; in vitro gene expression; noneukaryotic introns; and the identification of DNA replication fork proteins.

Phages as Molecular Tools

Just as bacteriophages played a critical role in the development of molecular biology, bacteriophages have been vital in genetic engineering technologies. The use of phages as cloning vectors (carriers of cloned genes), for example, takes advantage of the ability of phages to infect a large population of bacteria essentially simultaneously and begin expressing the cloned gene in a temporally coordinated fashion. This has allowed the expression of proteins that would otherwise be lethal to bacteria. Likewise, the ability of some phages to integrate their DNA into specific sites in the bacterial genome allows for bacterial strain modification that would prove difficult by general recombination.

Many different phages have been used to develop genetic engineering tools and techniques, but three have played especially important roles. These are the temperate phage λ (and related lambdoid phages), the chronically released filamentous phage M13 (and its cousins f1 and fd), and the obligately lytic phage T7. Phage λ along with the various filamentous phages have been used to develop a series of gene expression and cloning vectors. The organization of the λ genome is especially useful as a cloning vector since the central one-third of the 48,000 base-pair genome can be deleted, leaving ample room for inserted genes. This segment includes all of the genes responsible for lysogeny, committing the derived phage to a solely lytic life cycle. Phages M13 and T7 have been widely used in phage display technology (below). Functional elements of other phages, including the promoters of phages T3, T7 and SP6 as well as the phage f1 origin of replication, have come to be used in a wide variety of cloning vectors. Filamentous phages (M13, f1) lack a large dispensable region but have other uses since cloning vectors derived from these phages can form a plasmid-like intermediate in the infected cell and replicate and package their genomes in a ssDNA form. Single-stranded DNA is particularly useful for a number of genetic engineering procedures including oligonucleotide-directed site-specific mutagenesis, synthesis of strand-specific probes, subtractive hybridization, and DNA sequencing. Likewise, there are more specialized functional elements, such as the cin recombinase (also called cre recombinase) and recognition sites (cix-cin sites) of bacteriophage P1, which

have been adapted to make a powerful site-specific recombination system: the Cre/Lox system. As well, particular phage enzymes have proven useful in a variety of molecular biology techniques. These include DNA polymerases, RNA polymerases, DNA ligase, single-strand binding protein and others. Bacterial adaptations to resist phage infection have also provided important molecular tools. Restriction enzymes are classic examples but more recently the CRISPR-Cas system, a prokaryotic system for gene silencing of incoming bacteriophage genes, has been adapted for use in eukaryotic cells and organisms. Several temperate bacteriophage homologous recombination systems have been repurposed into systems for assembly of genetic constructs, a technique described as recombineering.

The development of some of these enzymes and other systems has been aided by advances in genome sequencing as well as metagenomic analysis of environmental samples. These both allow the rapid and inexpensive identification of useful novel versions of genes from new species of bacteriophages.

Phage Display

A powerful phage-based genetic engineering tool is phage display. Phage display is a form of high throughput screening that allows large numbers of peptide segments to be screened for binding to a target. For these systems, a library encoding short peptide segments is inserted into a gene encoding a phage-virion capsid protein. Insertion of these peptides is accomplished at a location that leaves the added peptides on the surface of the virion particle, in a manner that minimally disrupts virion functionality, and where they are able to interact with other proteins or materials. Depending on the virus vector used, the displayed peptides may be in head proteins, receptor proteins, coat proteins, etc. The resulting recombinant phages are exposed to a target protein, or other material, that is usually fixed to a surface or other solid matrix. If the inserted protein segment has affinity for the target then the phage will bind to it. Noninteracting phages are washed away and the bound phages are recovered. The recovered phages are then grown to produce a population enriched for phages whose inserted peptide binds the target. These steps, which are sometimes termed "biopanning," are repeated through several (commonly three to five) rounds to obtain phages containing the tightest binding segments under the conditions used.

Phage display has been used in finding peptides that bind to a wide variety of targets and which are useful for many purposes including the generation of antibody and other protein-binding peptides (which are useful for epitope mapping and for vaccine development); virus-receptor binding peptides (which are useful in drug discovery); novel DNA binding peptides, especially ones that show sequence specificity (which may be employed in designing novel gene regulatory proteins); and peptides that bind to inorganic materials (such as semiconductor materials which are being used to create hybrid organic-inorganic materials). Dozens of monoclonal antibody-based drugs that utilize binding-site peptides have been derived using phage display. Not all phage display systems work equally well for a given application and finding the most appropriate system for a particular target is sometimes a matter of trial and error.

Phage Use in Bacterial Identification

The treatment of bacterial disease, or otherwise dealing with bacterial contamination (including by quarantining), is eased via rapid and accurate bacterial identification. For decades phages have played important roles in such identification. Early techniques involved phage typing, which is bacterial identification (and classification) on the basis of phage susceptibility. Toward bacterial detection, phages may be added to bacteria-containing cultures, looking for phage amplification that occurs only if phage-susceptible bacteria are present. Alternatively, phages endogenous to a culture (that is, naturally occurring) and that infect specific bacteria types, may be used to infer bacterial presence. More recently, phages have been tagged with reporter molecules, such as fluorescent markers, or have been bioengineered to express reporter genes, which provide light or instead color changes within a culture only if phage infection successfully occurs. The latter approach is especially useful in identifying bacterial antibiotic susceptibility—as well as antibiotic resistance—since antibiotics capable of blocking phage infection (due to blocks on bacterial metabolism) can also block reporter-gene expression. All of the techniques are dependent in their efficacy on the relatively narrow host range displayed by many phages, that is, the potential by phages to infect only a limited number of bacterial types. Several phage-based systems are now or have been available commercially including systems for detection of *Bacillus anthracis*, *Yersinia pestis*, *S. aureus* and *Mycobacterium tuberculosis*. Phage based detection of *M. tuberculosis* is especially advantageous in providing results in a day compared to several weeks of traditional culture-based identification.

Phages as Contaminants

Biotechnology employs organisms to produce useful products and in the modern world this typically involves employing some form of recombinant DNA. As the use of microorganisms in biotechnology has become more sophisticated, especially beyond their use, for example, as naturally acquired cultures during food production, the tendency has been to employ pure cultures. A pure bacterial culture, however, is especially susceptible to inactivation by contaminating phage. That is, a single bacterial type may be wiped out by a single phage type, whereas wild microbial communities, consisting of multiple bacterial types, are more likely to contain bacterial populations which are inherently resistant to any given phage type. With the advent of modern culturing techniques, along with bacterial culturing on industrial scales, phage contamination thus can and often does lead to catastrophic fermentation failure.

Fermentation failures are especially prevalent in the production of fermented dairy products, such as cheeses, since the milk is not sterilized prior to use and therefore can contain phages specific to the starter-culture bacteria employed to create the specific characteristics of the fermented food. A variety of steps may be taken to minimize phage contamination, or its impact. These include modifications to fermentation facilities or to the bacterial strains employed. In addition, the CRISPR-Cas systems mentioned above were first recognized as antiphage mechanisms in conjunction the characterization of phage resistance in bacteria employed in dairy fermentation.

Indigenous phages have been implicated also in the failure of natural bacterial assemblies. These may include the lactobacilli, vaginal normal flora that otherwise can serve as a bulwark against bacterial vaginosis. It has been hypothesized that cigarette smoking can lead to bacterial vaginosis as a consequence of prophage induction in vaginal lactobacilli lysogens. Though long studied, the role of endogenous, naturally occurring phages in limiting growth of normal microbiota bacteria as well as pathogenic bacteria is only minimally understood or even appreciated.

Phage Therapy

While phages can be a nuisance due to their ability to infect and thereby inhibit or destroy bacterial cultures of industrial importance, that same propensity can be harnessed to reduce numbers of instead nuisance bacteria, including bacterial pathogens. This phage application as anti-bacterial agents is described as phage therapy. The history of phage therapy is almost as long as the history of phage study itself. In parts of Eastern Europe and the former Soviet Union, phage therapy has been used more or less continuously since the 1930s. But phage use as antibacterial therapeutic agents was largely discarded by Western medicine as a consequence of inconsistent results and especially after the widespread adoption of antibiotics. Phage therapy has a number of advantages over traditional antibiotics, however. Phages are self replicating, minimally toxic, and easily isolated. Furthermore, phages display a "narrow spectrum of activity" (which for phages we would describe also as their host range), meaning that only relatively few bacteria are susceptible to a given phage strain. As a consequence, unlike antibiotics with their often broader spectrum of activity, phage therapy has much less potential to cause the "collateral damage" (a.k.a., dysbiosis) of destroying beneficial bacteria along with pathogenic, target bacteria. Phage therapy therefore is less likely to give rise to side effects such as overgrowth of opportunistic pathogens, so-called superinfections, that sometimes occur such as with antibiotic-associated vaginal yeast infections or *Clostridium difficile*-associated colitis.

There are some practical limitations to phage therapy. The specificity of phage infections means that there is no equivalent to a broad-spectrum antibiotic. Identification of an infection's causative pathogen is necessary although to some extent this can be by-passed by using cocktails consisting of multiple phages possessing diverse host ranges. Also, just as bacteria can become resistant to antibiotics, they can become resistant to bacteriophages. Unlike antibiotics, however, this resistance is not difficult to overcome, either through bacteriophage mutation or substitution of another species of bacteriophage that attacks the same pathogenic host. Finally, there are some bacterial infections that are not amenable to phage therapy due to bacterial dissemination to body sites that are not be easily reached by phages, including the targeting of intracellularly infecting bacterial pathogens such as various *Mycobacterium* species. Alternatively, phages can be effective against certain bacterial infections in which antibiotics are less potent, such as against biofilm-associated bacteria.

The other limitation to use of phage therapy is regulatory. The biological nature of bacteriophages is not well suited to a regulatory pathway built to test drugs that do not have the capacity to evolve during treatment or that will need periodic reformulation to counter pathogen resistance. But the growing problem of antibiotic resistant bacteria has created renewed interest in phage therapy. Clinical trials for treatment of bacterial infections with phage are underway.

An alternative approach is the development of phages as decontaminating agents. Phage-based products are available from several companies for treating food products contaminated with *Listeria monocytogenes*, *E. coli* O157:H7 and *Salmonella*. Phage mixtures formulated for treating bacterial infections of plants are also being marketed.

Phage-Derived Enzybiotics

Another promising therapeutic use is not of whole phages but rather certain purified phage-encoded proteins that possess antibacterial properties. Most notably are phage endolysins, or simply "lysins," which are responsible for breaking down the bacterial cell wall to terminate lytic infections. They can be applied exogenously to various bacteria, especially Gram-positive organisms, to lyse and thereby kill those bacteria. This killing is similar to that effected by the animal enzyme lysozyme but with higher specificity and effectiveness.

A second category of phage enzymes that possess antibacterial properties are known as extracellular polymeric substance depolymerases, or EPS depolymerases for short. These are capable of dissolving the "glue" that binds bacterial biofilms (i.e., EPS). Like lysins, EPS depolymerases can display high specificities, in many instances so high that these enzymes can be limited in their activity to only one or a handful of strains within a bacterial species. During phage infections there also are often phage proteins that are more subtly lethal to their bacterial hosts via interactions with especially internal bacterial components. One technology that is being explored is to use these proteins, and where they bind to host molecules, as guides toward the discovery of molecules that could serve as anti-bacterial drugs which do not require the phage virion to gain access to the bacterial cytoplasm.

Phages as Delivery Vehicles

Just as phage virions can be used to deliver DNA and thereby genes into host bacteria, so too phages can be engineered to deliver DNA as well as other molecules to nonhost bacteria or nonbacterial targets. In this way phages can be used as much more broadly acting therapeutic or instead prophylactic agents. Phages thus can be engineered to deliver lethal genes to eukaryotic cells, and do so with high specificity, such as to combat cancers. Alternatively, various toxins or instead antibiotics can be linked directly to the phage virions which, again with high specificity, can deliver these agents to target cells, such as tumor or cancer cells, or instead to bacteria that otherwise cannot support infections by these same phages. The utility of these latter processes is that cytotoxic drugs can be concentrated at their sites of action, just as phage therapy as more strictly defined represents a concentration of cytopathic effects to the immediate vicinity of their target bacteria, indeed to within the cytoplasms of those bacteria.

Phages also can package noncytoxic materials, such as genes for gene therapy. In this case the phages are engineered so that they display both high specificity for target animal cells and carry DNA that is capable of being incorporated into and then expressed by those same cells, here as potentially located within our own bodies. Equivalent phages also can be employed to deliver genes that can be used to give rise to what is known as a DNA vaccine, that is, genes that are expressed within body cells that are able to elicit an immune response against their products, thereby stimulating a prophylactic immune response. As with many phage-based therapeutic technologies, key to phage-based gene therapy or vaccination is the genetic malleability of phages, which allows precise engineering of payloads as well as binding specificities.

Phages as Fecal Indicators and Environmental Tracers

Phages are found associated with fecal material because bacteria are abundant in the animal colon. As feces carry enteric pathogens capable of causing disease in otherwise healthy individuals, public health measures are directed toward both avoiding contaminating water supplies with feces and also monitoring water supplies for fecal contamination. In addition to certain bacterial types, phages can be employed as indicators in such monitoring, especially since, as viruses, they can mimic to a certain degree the survival characteristics of pathogenic enteric viruses. Also as mimics of enteric viruses, phages can be employed to test the potential of various water treatments for removal of such viruses, as well as virus removal, or containment, in a variety of nonwater contexts. Phages also are employed as relatively inert tracers of water movement, including of underground water supplies.

Phage-Based Interactions With Animal Bodies

Phages can be employed as a means of characterizing animal bodies, whether in terms of immune response, their removal from serum by nonspecific immunity, or in mapping tissues using phage display. Phage ϕX174, especially, has been used to study the human humoral immune response, serving as a neoantigen (one not previously experienced by an animal) such that primary, secondary, and tertiary immune responses may be induced simply with subsequent vaccination. Antibody fragments produced by phage-display methods can also be used to target specific tissues. A phage-display library of antibody fragments is injected into an animal or, in certain instances, into humans. Recovery of phage virions from tissues and subsequent phage growth results in amplification of antibody fragments with specific binding properties and/or a mapping of antibody binding to specific tissues. This can be thought of as an in vivo biopanning. Phages that display tissue-targeting peptides may be employed in the course of gene therapy, serving as vectors targeting specific tissues. Here, phage DNA is replaced by appropriate gene-therapy vector DNA along with the therapeutic gene of interest. Phages may also serve as platforms for the display of antigens as vaccines. Because phage proteins also provoke an immune response, the phage itself may act as an adjuvant for the displayed peptide, that is, a substance that enhances protective immune responses.

Phage Ecology

Phage ecology is the study of the interaction of phages with their environments. Environments consist of biotic components (other organisms) or abiotic components (spatial, chemical, and physical factors). Subdisciplines of phage ecology consist of organismal, population, community, and ecosystem ecologies. Organismal phage ecology considers those phage adaptations responsible for phage survival or acquisition of new bacteria to infect. Phage population ecology considers groups of similar phages (loosely: a phage species) that are located within the same environment. Of concern are demographic factors, especially births and deaths, and how these factors are affected by phage and bacterial population densities. Communities consist of more than one species found within the same environment, and phage community ecology considers especially the interaction between phages and bacteria but also among phages or between phages and other organisms that are affected by phage-associated bacterial genes such as toxin genes.

Ecosystems consist of both biotic and abiotic factors contained within a reasonably well-delineated environment, such as a pond or a field, and phage ecosystem ecology considers especially the impact of phages on the movement of energy and nutrients through and within ecosystems. The interruption of such movement by viruses is especially important in aquatic environments, which are principally based on primary production (photosynthesis) that is performed by microorganisms such as cyanobacteria

(susceptible to cyanophages). In addition, the lysis of these microorganisms provides nutrients to heterotrophic bacteria, which serve as a second trophic base of aquatic ecosystems. Phage disruption of aquatic environments is thought to have a significant impact on the global carbon budget and therefore on the global warming that results from atmospheric carbon dioxide accumulation.

In addition, under the auspices of phage ecology is phage environmental microbiology, for example, the study of what phages are located where. A large component of such phage environmental microbiology is based upon metagenomic analysis, generating phage viromes. These viromes can then be compared between environments and phage physiological characteristics can be inferred by comparing virome-defining sequenced genes with those of other phages (and other organisms) that have been previously characterized and whose sequences have been deposited in databases. Increasingly a goal of these efforts is both full-genome sequencing and matching uncultured phages with probable environmental hosts.

Horizontal Gene Transfer

Transduction is the movement of DNA from one bacterium to another via a phage-virion carrier that has picked up bacterial DNA during infection. When the virion carrying the bacterial DNA infects another bacterium, the transduced DNA enters the recipient cell following the same entry path normally used by phage DNA. The DNA can then be incorporated into the bacterial genome via recombination. Transduction appears to play important roles in bacterial gene exchange, contributing in some cases substantially to bacterial adaptation.

In generalized transduction the DNA within a phage virion comes entirely from the bacterial genome. Generalized transduction can move bacterial genes without distinguishing among them, but is mediated by what are, as a consequence of the transducing DNA, nonviable phage virions. Specialized transduction occurs when a prophage excises from a bacterial genome in a manner that incorporates adjacent genetic material. Specialized transducers can retain phage viability, though likelihood of viability is reduced as the size of the bacterial genetic material transferred is increased. Phages also can incorporate bacterial genetic material via illegitimate recombination, forming what are known as morons (for "more" DNA). The Moron-Accretion Hypothesis posits that phage evolution occurs via the gradual incorporation of morons. Through coevolution with the rest of the phage genome, these morons either are eliminated or come to take on important phage functions.

Phage Role in Bacterial Pathogenesis

As transducers of DNA, phages play important roles in bacterial pathogenesis, the potential for bacteria to cause specific diseases. Upon reduction to lysogeny, many prophages can express genes that impact bacterial phenotype, including the production of exotoxins, which are eukaryote-affecting toxic proteins released from the bacterial cell. The expression of these genes is typically described as phage conversion or lysogenic conversion. In addition, following induction of a prophage, genes may be expressed that impact the characteristics of the harboring bacterial culture. Also, via generalized transduction, phages can transfer bacterial DNA as so-called pathogenicity islands, which are contiguous strings of genes that encode factors that contribute to bacterial pathogenicity.

While not all examples of lysogenic conversion contribute to bacterial pathogenesis (superinfection immunity by temperate phage does not, for example), there are many instances where bacterial toxins are encoded by temperate phages either in the lysogenic phase or, less commonly, during the lytic cycle. *Corynebacterium diphtheriae*, for example, is converted from a nonpathogenic form to a pathogenic form after specific phage infection. Likewise, about 5% of the genomes of pathogenic strains of *Salmonella typhimurium* are composed of prophage genomes, and these prophages contain some of the bacterial arsenal of exotoxin and other virulence factor genes. Pathogenic strains of *Vibrio cholerae* also owe much of their pathogenicity to phage conversion, with cholera toxin encoded by the temperate and filamentous phage CTXΦ. Another variation on this theme is seen in the production of Shiga toxin by pathogenic strains of *E. coli* such as the serotype O157. There are several related Shiga toxins but a typical feature is that their genes are located in prophage genomes and the harboring bacteria lack a mechanism to export the toxin. Instead, Shiga toxin is released when the prophage is induced and lyses the bacterium to release phage progeny.

Phages as Evolutionary and Ecological Model Organisms

In addition to the study of the ecology and evolutionary biology of phages, researchers employ phages to test more general ecological and evolutionary biological theories. In other words, just as phages have played extremely useful purposes as models for universal molecular aspects of life (e.g., transcription, translation, and DNA replication), phages too can serve as model organisms for studying universal or at least common aspects of organismal ecology and evolutionary biology. Included are the study of predator-prey interactions, evolutionary optimization of adaptations, the generation of known phylogenies (diverging lineages of evolving populations), genetic constraints on natural selection, and the evolutionary roles played by genetic drift. The advantages associated with employing phages in these studies include their relatively simply biology, their rapid rates of reproduction, the exceedingly high numbers that phages may be grown within relatively small volumes, ease of long-term storage, high mutations rates (especially for RNA and ssDNA phages), ease of laboratory manipulation, amenability to genetic engineering (including reverse genetics, i.e., engineering in mutations), and, perhaps especially, the relative smallness of phage genomes which allows for routine whole-genome sequencing.

Closer Look at Certain Aspects of Phage Biology

Certain aspects of phage biology have been extremely well defined molecularly. In this section we provide an introduction to several of these processes, focusing on phage adsorption, virion assembly, lysis, lysogeny, and phage evolution.

Adsorption

Adsorption describes the process by which phages attach to the surface of host bacteria. This process has been especially well defined for the contractile, long tail fiber-containing T-even bacteriophages. The tips of the six long tail fibers bind to specific surface proteins or other bacterial receptor molecules (such as lipopolysaccharide). It has been shown that three of the six tail fibers are sufficient to properly orient the phage onto the surface of the cell, placing the base plate, found at the distal end of the tail, in proximity to the surface. Binding of the long tail fibers is reversible so that if the base plate is not adjacent to its receptor, then the long tail fibers can release. These reversible interactions allow the phage to "walk" across the surface of the cell until the short tail fibers, found under the phage base plate, are able to successfully bind. Short tail fiber binding is irreversible and, along with the long tail fibers, triggers the opening of the base plate. The remainder of the infection process consists of tail sheath contraction, exposure of phage enzymes that help degrade the cell wall, penetration of the tail tube through the cell envelope to the cell membrane, and subsequent ejection of the phage genome into the bacterial cytoplasm.

Virion Self Assembly

Assembly of the virion particle is often described as "self" assembly because an ordered assembly process has evolved in which complex structures are built by the individual component proteins, which assemble in a defined order, aided by only a limited number of accessory or helper proteins. In addition, different virion components, such as the head, tail, and, if present, the tail fibers, will self assemble independently and then join together. The tail is perhaps the most complex of the three main components and its assembly illustrates several principles. As outlined in Fig. 2, in T-even phages tail assembly begins with the

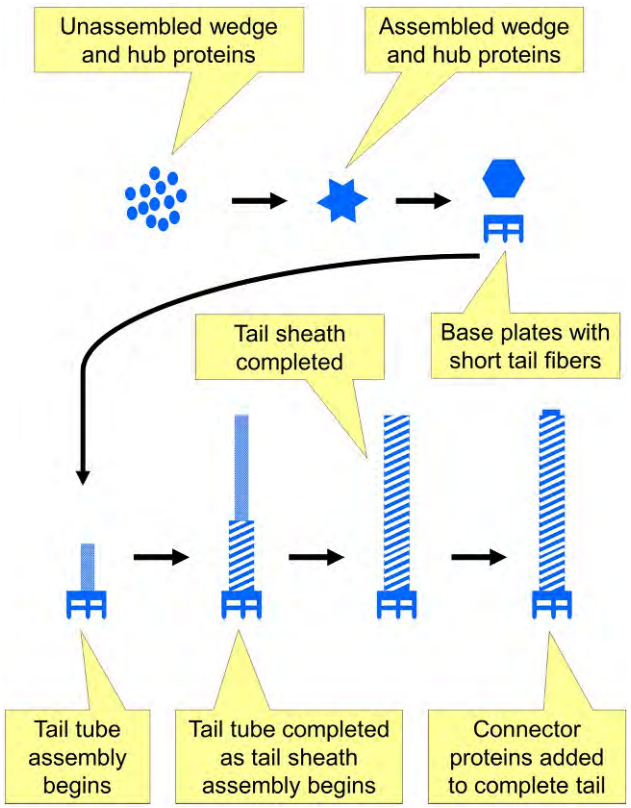

Fig. 2 T-even phage tail assembly. Self assembly of the phage tail begins as wedge and hub proteins join together to form a partial base plate. Base plate assembly is completed by the addition of the short tail fiber and joining proteins. Two completed base plates (top and side views) are shown. The base plate acts as the assembly initiator for the tail tube. As the tail tube assembles, the base plate/tail tube acts as the assembly initiator for the tail sheath. Once the tail tube and, then, the tail sheath are completed, connector proteins are added. The connector proteins will join the head and tail together after the head is filled with DNA. Note that not all steps are shown and that some steps require accessory proteins that are not part of the final structure. For example, short tail fiber assembly requires a chaperonin, which is a protein necessary for proper folding of another protein into its native conformation. Adapted from Figure 8 of Coombs, D. H. and Arisaka, F. (1994). T4 tail structure and function. In: J. Karam (ed.), *Molecular biology of bacteriophage T4*. Washington, DC: American Society of Microbiology, pp. 259–281.

base plate. This is composed of six wedges that are each made up of several different proteins and hub protein complexes. The wedges and hubs combine to form the hexagonal base plate. Once the base plate has assembled, the short tail fibers attach to it. Only after that step is completed does the tail tube form, assembling onto the base plate. Once the tail tube is formed, then the proteins that make up the contractile sheath assemble around the tail tube. Finally, the tail assembly is completed by the addition of connector proteins that will link the completed tail to the head, after its assembly (including genome packaging) is completed. Over 20 different proteins form the tail, but only two chaperone proteins (proteins that aid protein folding) are needed for tail assembly. In each step of assembly, the partially completed structure acts as the initiating complex for the next proteins to bind to and complete their folding to their final form. A similar process is followed by phage heads and tail fibers.

Filamentous phages, such as M13, have a very different assembly pathway that reflects their chronic release. Because of this type of release, completed virions do not accumulate in the infected cell. Instead, they are continually extruded from the infected bacterium and assembly is intimately tied to this extrusion. Assembly takes place at the cell membrane and all phage proteins that are involved in virion formation, including those involved in pore assembly through which virions are extruded, are integral proteins that collect in the membrane. Virion assembly begins when a phage genome, bound to a specific structural protein at the genome packaging site, enters the pore. Additional capsid proteins attach to the growing virion as it passes through the pore until the entire genome is coated with the major coat protein. The capsid is completed with minor coat proteins binding to the terminus, including the receptor binding proteins. This completion also triggers the release of the now mature virion.

Genome Packaging into Capsids

As part of the self assembly of phage virions, replicated phage genomes must be packaged into phage capsids. This process has been well studied for the tailed, dsDNA phages, which package their DNA into the virion head. The process of DNA packaging is not straightforward owing to the length of DNA that must be packaged along with the need to package that DNA into heads at a density that in many cases is nearly crystalline. Furthermore, the DNA must be packaged in such a way that DNA exits from heads both smoothly and rapidly when infecting a new host cell. This packaging involves enzymes responsible for moving DNA through ports found in what are known, prior to their maturation, as proheads. All tailed, dsDNA phages package their genomes as linear molecules either exactly one genome in length or slightly longer. In both cases the genome is packaged from a large concatemer containing many copies of the phage genome that may be linear or more structurally complex (branched concatemers). Phages that package more than one genome length of DNA package that DNA directly from this concatemer and continue packaging DNA into the prohead until it is full. Hence, it is the capacity of the phage head that determines the amount of DNA that is packaged and the mechanism is described as "headful" packaging. Because there is more than one genome length of DNA packaged, the ends of the linear molecule are duplicated and this duplicated DNA is often used to circularize the genome after the next infection. Furthermore, this mechanism means that each individual phage packages genomes that have different segments of the genome duplicated, resulting in a genomic map that is circular.

Phages that do not package DNA by headful mechanisms often employ genomic elements that determine the ends of the packaged DNA by defining where the genome is cut from the replication concatemer. Hence, these phages package exactly one genome length and the map of that genome is linear. For example, phage λ flanks the ends of its packaged genome with cos sites which are over 200 base pairs long and act as recognition sites for the packaging enzymes (terminases) that cleave near the middle of the cos sites, leaving a 12 base pair overhang. These ends are recognized by other packaging enzymes that place the DNA into the maturing phage head. The cos sites also allow for the λ genome to circularize when infecting the next cell. Other phages use similar packaging-enzyme recognition sites that are more generically described as pac sites (for packaging).

Lysis

Phage-induced bacterial lysis serves as a means by which mature phage progeny may be released from phage-infected bacteria. Lysis allows rapid release of large numbers of these progeny, but comes at the expense of ending intracellular phage-progeny production. That is, lytic phages can continue to produce progeny or release those progeny to find new cells to infect, but not both simultaneously, and only in that order. Phage-induced lysis also has the effect of destroying the infected bacterium, which is relevant with regard to nutrient cycling within ecosystems (i.e., the freeing up of nutrients locked within bacteria as well as the release of intracellular bacterial enzymes, and other molecules, which can go on to modify the extracellular environment). The best studied of phage-associated lysis mechanisms involves at least two components, a protein called a holin and a second protein that digests the bacterial cell wall (the endolysin, also known, generically, as a lysozyme or lysin).

The holin controls both the timing of lysis and the access of the endolysin to the cell wall (the cell wall is divided from the cytoplasm, where the endolysin accumulates, by the bacterial plasma membrane). Accumulating in the plasma membrane of the infected bacterium, the holin proteins create holes with precise timing to effect catastrophic exposure of the cell wall to the phage endolysin. For lytic ssDNA phages, by contrast, lysis is achieved via the production and exporting of a cell-wall synthesis inhibitor. As cells continue to grow despite phage replication, these inhibitors eventually cause a cell-wall failure, giving rise to osmotic lysis of the bacterial cell and thereupon release of intracellular phage progeny.

Another type of lysis is lysis from without, which is effected by T4-like phages. In this lysis mechanism, adsorption by multiple phages to a single cell weakens the cell wall, due to lytic enzymes found on the phage tail, contributing to cell wall failure and osmotic lysis of the cell. This mechanism of lysis may serve to augment the lysis of these phages during growth within cultures containing high densities of phage-infected bacteria. The lysis effected by purified phage endolysins, that is, lysins, as applied to bacteria as bactericidal agents is also described as a lysis from without.

Lysogeny

Temperate phages are capable of causing lysogeny. Fig. 3 outlines the relationship of lysogeny as compared to a lytic infection. The prophage is the temperate phage genome as found during lysogeny. Traditionally temperate phages are described as integrating into the host genome, and replicating along with the bacterial genome, as seen with the prototype temperate phage, phage λ. Temperate phages which effect lysogeny by forming plasmids, however, are also known (e.g., phages P1 and N15). Note that temperate phages are not properly described as lysogenic since lysogeny, traditionally, is considered to be a characteristic of bacteria rather than of phage.

Upon infection, temperate phages may be "reduced" to a prophage rather than display a productive infection. Temperate phages such as phage λ (as well as phage P22 and CTXΦ) display a site-specific reciprocal insertion into the host genome. The steps in this mechanism include a closing of the linear phage genome into a circle, which is followed by recombination over small regions of

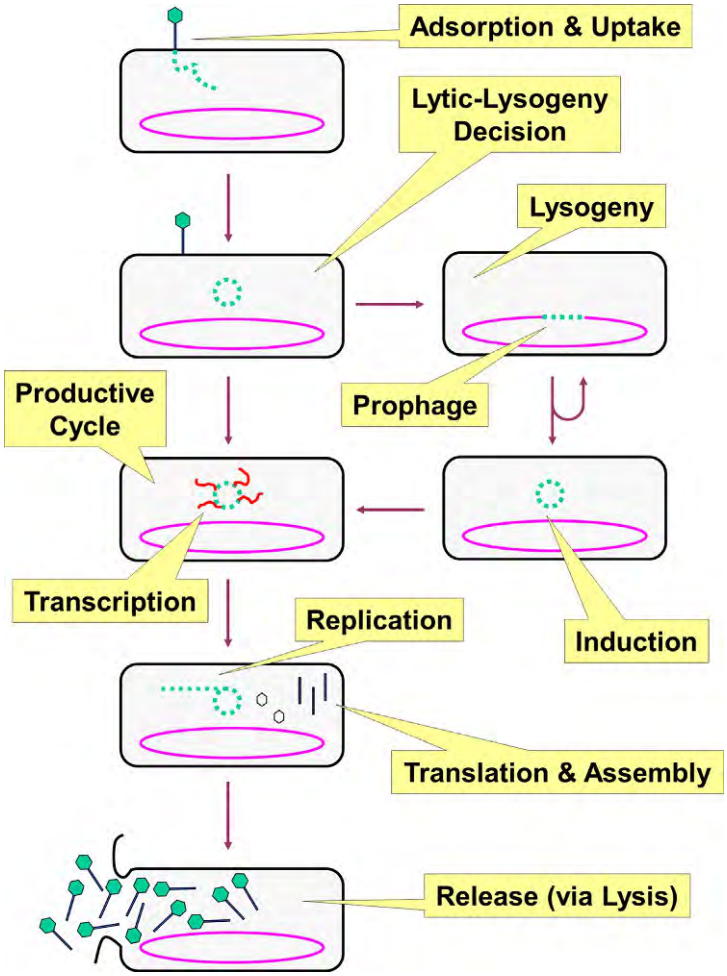

Fig. 3 Outline of the life cycles of a temperate phage. After infecting, early proteins made by the phage determine whether the phage will enter the lytic or lysogenic modes. In lysogeny, the phage genome forms a prophage, which for most temperate phages is integrated into the host genome. In this state, bacterial replication also replicates the phage genome, thereby maintaining lysogeny. Some change in bacterial metabolism, typically DNA damage that induces the bacterial SOS pathway, triggers the prophage to enter an active state via the process of prophage induction. The prophage is excised from the bacterial genome and begins expressing phage genes, just as occurs with phage infections that instead go directly into the lytic phase. Genes for genome replication as well as virion structural proteins and genome packaging are expressed. The genome is packaged into proheads, virion assembly is completed, and the cell is lysed to release the phage progeny. Note that not all details are shown and that some temperate phages do not follow these specific details.

homology between phage and bacterium at a specific site in the host chromosome. This integration mechanism is catalyzed by the phage integrase enzyme. To go from a lysogenic to a productive state, a process called induction, excision enzymes reverse this integration step. Contrasting phage λ, phage Mu displays a nonreplicative transposition mechanism to insert rather than recombine its genome into the host bacterium in a manner that is similar to that employed by transposons or retroviruses, such as human immunodeficiency virus (HIV). This transposition is not site-specific, unlike phage λ integration, and, as a consequence, results in random mutation by gene disruption of the bacterial chromosome. "Mu" in fact is named for the first two letters of the word "Mutation."

To prevent induction, which is activation of a productive infection, prophages express repressor proteins that inhibit the transcription of genes necessary for exit from the lysogenic state. In addition to maintaining lysogeny, the repressor protein is responsible for causing immunity (superinfection immunity), which is the inhibition of infection by phage of the same type. For many temperate phages, induction occurs following DNA damage, which induces the bacterial SOS system, leading to repressor inactivation by cleavage.

Mosaic Model of Phage Evolution

The earliest studies of the arrangement of genes in phage genomes showed that these genes tend to cluster by function. For example, most of the virion head protein genes tend to be adjacent to each other within the genome and may even express as a single operon. This has advantages for coregulation of genes of similar function and it also tends to increase the genetic linkage of genes of related function. As more and more phage genomes have been sequenced, it has become clear that genes of related function are often exchanged in blocks via recombination events. This recombination can be illegitimate, and thereby involving no sequence homology among the regions swapped. Alternatively it may involve minimal homology. In addition to the swapping of gene blocks, individual genes, or more rarely even parts of genes may be swapped as well.

These nonhomologous or minimally homologous exchanges are probably fairly rare on a per-infection basis, and most recognizable recombination events likely result in phage genomes that are fatally defective. The large numbers of phages and phage infections that are thought to be present and occur in natural environments, and even to some degree in the laboratory, however, presumably allow for sufficient recombination between divergent coinfecting phages such that gene exchange is a major mode of evolution for bacteriophages despite minimal homology. These exchanges should occur especially during productive phage infections of bacterial lysogens, with recombination occurring between infecting phage and prophage, or among prophages present within a single bacterial cell. The result is that phage genomes typically are mosaic, with different genes or regions found within a phage genome displaying greatest homology with a diversity of different phages.

Homology also is found with bacterial genes but, in many cases, phage genes with no homology to previously characterized gene sequences instead are found. This is seen to a substantial extent during both phage whole-genome sequencing and during the generation of viral metagenomes and suggests that there exists an enormous diversity of genetic information encoded by the world's phages. In addition, regions of genome homology between phage types that otherwise are quite distinctive (e.g., phage T4 and phage λ) are not uncommon.

Overview of Well-Studied Phage Types

A number of phage types have been extensively studied and serve as models for our understanding of phage biology. Among these phages are the famous "T" (for type) phages of *E. coli* B which were assembled by Milislav Demerec with the help of Max Delbrück: phages T1 through T7. We present an overview of these T phages first, starting with the best studied among them, phage T4. We then discuss phage λ, which also is a coliphage, and which serves as the archetypical temperate phage. Then we turn to brief synopses of a number of additional phage types and lastly discuss certain categories of phage types including marine phages, cyanobacterial phages, and phages of mycobacteria as well as archaeal viruses.

Phage T4 (Also T2, T6, RB69, etc.)

Bacteriophage T4 is a well-studied, obligately lytic phage of *E. coli*. It is a member of the family Myoviridae, meaning that it has a contractile tail as well as an icosahedral head. Phage T4 has a relatively large dsDNA genome of approximately 170,000 bases, including modified bases (glycosylated hydroxymethylcytosines) that allow the phage genome to resist digestion by numerous bacterial restriction endonucleases. As one of the best studied of phage types, the biology of phage T4, like that of phage λ, may be considered archetypical. In studies of phage evolution, phage T4 along with phages T2 and T6 form the original members of the T-even family of bacteriophages, which now includes many members that infect many other species of bacteria besides *E. coli*.

Among the interesting, additional characteristics of phage T4 biology are a highly structured self-assembly pathway for virion particles; multiple modes of DNA replication initiation that are utilized at defined times during the replication cycle; a gradual subversion of the host RNA polymerase complex by a combination of covalent modification and replacement of accessory proteins that allows sequential recognition of various classes of phage promoters; rapid breakdown of the host genome and mRNA to supply nucleotides for phage replication and transcription; a large number of nonessential gene products; unusual genetic features including overlapping genes; and the first non-eukaryotic introns identified.

Phage T1 (Also TLS, and Others)

Bacteriophage T1 is another member of the classic seven T phages. It is also an obligately lytic virus of *E. coli*, but unlike T4 it is a member of the Siphoviridae family, having an icosahedral head and a long flexible, noncontractile tail. The T1 genome consists of a single dsDNA molecule of just under 50,000 base pairs. Phage T1 and related phages are less well studied than the T-even family, in part due to T1's early reputation as a troublesome phage: T1 virion particles are notoriously durable and persistent, resistant to desiccation, and able to spread as aerosolized particles, resulting in its reputation as a contaminant. Stories of labs requiring periodic sterilization or hanging ultraviolet lamps abound, as does the (likely apocryphal) story of an early researcher recovering a sample of T1 from the letter sent by a colleague declining to send a sample. While some of these stories may be exaggerated, many commonly used laboratory strains of *E. coli* carry the *ton*A or *ton*B allele, which confers resistance to phage T1 infection.

Phages T3 and T7

Morphologically, T3 and T7 are Podoviridae, possessing an icosahedral capsid with a short tail and tail fibers. The T7 genome is just under 40,000 base pairs of dsDNA. Unlike most phages, the packaged genome only occupies about half of the virion head. The remainder of the space is taken up by a large complex of internal proteins that the genome is coiled around. Packaging of the phage genome is less precise than with other phages, and DNA of between 85% and 103% of the full genome may be packaged. The internal proteins are ejected from the phage head before genome ejection and seem to form a channel through the cell membrane for the genome. Genome entry into the cell is unusually slow for a bacteriophage and seems to require active transcription of the initially entering DNA (850 base pairs) for the remaining genome to enter the cell. It appears that it is transcription itself rather than the expression of particular genes that is the key to phage DNA internalization.

Both phages T3 and T7 produce their own RNA polymerases for late gene expression. These RNA polymerases are unusually specific for the phage promoters and have found widespread use in many commercialized gene expression vectors. Phage T7 has also found use as a phage display vehicle.

Phages T3 and T7 are two members of a larger group of phages related by genomic analysis. Not all members are coliphages but include, for example, phages infecting *Yersinia*, ϕA1122 and ϕYeO3-12, and a *Pseudomonas putida* phage, gh-1. These other phages display high degrees of homology to phage T7 genes as consistent with the mosaic model for phage evolution. The host range of phage T7 supports this family linkage: Wild type T7 can infect some species of *Salmonella* and *Shigella* but extended host range mutants that can infect some *Yersinia* species have also been isolated as have some mutants of ϕA1122 that can infect *E. coli*.

Phage T5

Bacteriophage T5 is an obligately lytic phage of *E. coli* that is classified as a member of the Siphoviridae family, having an icosahedral head and a long, noncontractile tail. Its genome is composed of a single linear piece of dsDNA, just over 121,000 base pairs. Although its life cycle has several unusual features, phage T5 is not nearly as well studied as other phage types considered here. In part this lack of study is due to the presence of a large number of unusually strong promoters that interfere with bacterial metabolism when cloning of genomic fragments is attempted. Among the unusual features are a number of nicks in the packaged genomic DNA and the excretion of nucleotides from the infected cell soon after infection. This excretion ends after the genome has completely entered the cell.

The entry of the phage genome into the cell is also different from most other families of phages in that phage T5 (and a few near relatives) do not eject their entire genomes into the cell at one time. Instead, about 8% of the genome is ejected into the cell. Expression of the genes on this segment are necessary for the remainder of the genome to enter the cell. Ten proteins appear to be expressed from this genome segment, although not all are essential for genome entry. The pause between first stage and second stage genome transfer is about 5 minutes.

Phage λ

Phage λ displays the Siphoviridae morphology, possessing an icosahedral head and a long, flexible, noncontractile tail. Phage λ is the type phage for a large number of related phages usually described as lambdoid and though phage λ infects *E. coli*, other lambdoid phages infect strains of *Salmonella*, *Shigella*, *Pseudomonas*, *Burkholderia*, etc. Though strictly speaking the term lambdoid is reserved for those phages that can form recombinants with phage λ when they enter the same cell, the term also is used more loosely to describe any phage that has the appropriate morphology and a temperate life cycle.

The genome of phage λ is a single dsDNA molecule about 48,500 base pairs long. In its intracellular circular form, the genome can be readily distinguished into a single portion of about one-third of the genome that contains genes related to lysogeny. The other segment, constituting the other two-thirds of the genome, contains structural and other lytic-phase related genes. When the genome is packaged, the lytic phase genes are separated, forming right and left arms of the genome, flanking the central lysogenic segment. The temperate life cycle and lysogenic state are described elsewhere in this entry.

Phage N4

Phage N4 is a member of family Podoviridae which infects a limited number of *E. coli* K-12 strains. Unlike other coliphages, which infect using receptors that are fairly common on the *E. coli* surface, there appear to be only five attachment sites for N4 on each cell.

The N4 genome is a single linear dsDNA molecule of about 70,000 base pairs. The left end of the genome is unusual in that it has a 5–6 base 3′ overhang while the right end varies between substrains and may be blunt or have a 1, 2 or 3 base pair 3′ overhang.

The virion of phage N4 is also unusual, not in its structure, but in its contents. In addition to the phage genome, there are one or two copies of a phage-encoded RNA polymerase, designated vRNAP. This RNA polymerase is essential for phage N4 early gene transcription. One of those early transcripts is for a second RNA polymerase, RNAPII, which mediates middle gene transcription. Surprisingly, phage N4 late gene transcription appears to be effected by the host *E. coli* RNA polymerase. Even more surprisingly, the phage- encoded ssDNA binding protein, normally employed during DNA replication, is also needed for transcription from late genes.

Phage ϕ29

Phage ϕ29 and related phages are Podoviridae that infect the Gram positive *Bacillus* species, especially *B. subtilis*, as well as other especially Gram-positive bacteria. These phages are notable for their relatively small genomes, which are linear, dsDNA, and about 20,000 base pairs long. Phage ϕ29 has served as a model system for the study of transcriptional regulation, DNA replication, and virion morphogenesis.

Phage SPP1

Phage SPP1 is a well-characterized generalized transducing phage of *B. subtilis* possessing a Siphoviridae virion morphology. It has a linear dsDNA genome of about 44,000 base pairs. Phage SPP1 and related phages are capable of effecting the transduction of plasmids. SPP1 also has been employed to study phage transfection, which is the transformation of competent bacteria with a phage genome leading to infection.

Phage P1

Phage P1 is a temperate phage of the Myoviridae family, possessing an icosahedral head and long, contractile tail. Phage P1 can mediate generalized transduction between *E. coli* strains and other Gram-negative bacteria. In addition to its generalized transducing abilities, phage P1 has two interesting features: First, though a temperate phage, P1 does not integrate its dsDNA genome into the bacterial genome. Instead, the genome circularizes and replicates in a plasmid-like state. The phage devotes several genes to controlling replication and partitioning of the plasmid so that it is stably maintained at low copy number.

Second, phage P1 also has two complete sets of tail fiber genes, each conferring a different host range. These genes are arranged in opposite orientation in the phage genome with a single control region located between. A phage-encoded inversion system switches the control region from one orientation to the other, activating one or the other set of tail fiber genes. Any single burst contains identical progeny. However, during the lysogenic phase the inversion system is active enough so that a population of lysogens will produce about 50% of phage of each host range type. It is this inversion system that has been adapted to create the Cre/Lox recombination system.

Phage P2

Phage P2, like phage P1, is a temperate Myoviridae of *E. coli* and, in fact, both phages were isolated at the same time. The phage P2 genome is a single, linear dsDNA molecule, about 33,000 base pairs in length. Unlike phage P1, phage P2 integrates its genome into the host chromosome during lysogeny and is not particularly efficient at generalized transduction. Phage P2 and related phages, however, do seem to be readily able to capture nonessential genes that have no role in phage metabolism, that is, morons (as described above. These allow the phage to confer lysogenic conversion phenotypes to host bacteria. In addition, these related phages display extensive mosaicism similar to that of the T-even and lambdoid families of phages.

Phage P4

Phage P4 is a satellite phage, only able to productively infect *E. coli* strains that are P2 or related phage lysogens. Viruses that parasitize viruses in other host systems have also been described as virophages although this term is becoming more commonly associated with satellite viruses whose host virus is a virus infecting amoeba. P4 is dependent on the P2 genome for all of its virion proteins. Consequently, morphogenetically phage P4 is also a member of family Myoviridae, although a P4-encoded protein causes the P4 heads to be smaller than normal phage P2 heads. This smaller head size reflects the smaller genome size of phage P4. The linear dsDNA genome is only 11,600 base pairs compared to the over 33,000 base pairs of phage P2.

Phage P4 is also a temperate phage and can form *E. coli* lysogens irrespective of the presence or absence of a phage P2 genome. The P4 genome can exist in the lysogenic state as a multicopy plasmid form or it can integrate into the host genome at a specific integration site, as does phage λ. Because of this dual mode of lysogeny, the genome is sometimes described as a phasmid (phage-plasmid). Alternatively, the phage P4 genome is described by some as a plasmid that uses an especially effective means of transferring between cells: transduction by phage P2. This description also reflects genetic analysis of the phage P4 genome, which indicates that it is not merely a defective form of phage P2 but rather an independently arising phage. It should also be noted that phage P4 is not unique as a satellite phage, although the number of other examples for which a bacteriophage is the host virus is still very small.

Phage N15

Phage N15 is a temperate phage displaying a Siphoviridae morphology of an icosahedral head, and long noncontractile tail. It is quite similar to phage λ in genome size and organization as well. It deviates from the lambdoid life cycle in the lysogenic phase, where the phage genome does not integrate into the host genome or form a circular, plasmid-like form. Instead it remains as a linear episomal element separate from the host genome. It does this by forming a circular intermediate upon entering the cell which is acted upon by a phage-encoded protelomerase that cuts the circular genome and covalently closes each end of the molecule by joining the two strands together. Replication is accomplished in several modes which either involve cutting of one or both of the ends for replication and resealing or which lead to the production of a circular dimer that is cut and resealed as two linear closed-monomer genomes.

Phage P22

Phage P22 is a temperate phage of *Salmonella* that is often described as lambdoid because of its temperate life cycle and similar genetic structure to phage λ. It differs from λ in that phage P22 is a member of family Podoviridae (rather than family Siphoviridae), with adsorption organelles, the tailspikes and a tail needle, attached to a short tail at one of the vertices of the phage head. Also, while λ is a specialized transducing phage, P22 is a generalized transducing phage. Phage P22 and related phages are thought to play important roles in the evolution of pathogenic strains of *Salmonella* and many P22-like phages have acquired genes that make their lysogens more virulent through phage conversion.

Phage Mu

Phage Mu is a member of family Myoviridae, with an icosahedral head and contractile tail. Its packaged genome consists of a single, linear dsDNA molecule with about 37,000 base pairs. In addition, the phage usually packages between 500 and 3000 base pairs of host DNA from random chromosomal locations, making phage Mu a generalized transducing phage. Phage Mu exhibits a temperate life cycle with its genome integrated into the chromosome of its host *E. coli*. It is after integration that the Mu genome displays an unusual alternate mode of movement, acting as a transposon that moves from one location in the host genome to another. It can do this in a nonreplicative manner leaving one location and inserting into another or it can utilize a replicative transfer mode and a second copy of the genome/transposon is created at the second location. Phage Mu is not unique in this dual existence. Mu-like prophages have been found in other bacteria including *Pseudomonas*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Deinococcus radiodurans*.

In addition to its dual prophage/transposon nature, phage Mu has other notable features including a dual set of tail fiber genes that are differentially expressed in a manner similar to those of phage P1. Each set confers a very different host range: *E. coli* and *Salmonella* strains in one mode and *Shigella*, *Enterobacter*, *Erwinia*, and *Citrobacter* strains in the other. Finally, as a transposon, Mu has been used to modify and study a wide variety of bacterial genomes, many of which phage Mu cannot naturally infect. These range as far from *E. coli* as strains of *Rhizobium* and *Agrobacterium*.

Phages Found in Marine Environments

Marine phages are notable especially in terms of their great numbers and probable impact on marine bacteria. Recently a wealth of information has accumulated on these phages, an exploration that is driven, to a great extent, by the potential impact marine phages have on global carbon cycles as important converters of bacterial biomass into dissolved organic matter. To some degree the phrase marine "phages" is a misnomer, however, as direct counts of virus particles, as marine viruses are often observed, consist not just of bacteriophages but also of archaeal as well as eukaryotic viruses. This is true to a degree as well for marine virus metagenomes (viromes) though many of the resulting sequences are relatively easily classified as bacteriophage-like versus genetically similar to archaeal or eukaryotic viruses.

Cyanophages

Among marine as well as freshwater bacteriophages are many infecting cyanobacteria, once described instead as blue-green algae. Cyanobacteria are bacteria possessing a plant-like photosynthetic apparatus and are responsible for generating a significant proportion of the Earth's atmosphere oxygen. They also are important producers in aquatic systems (that is, photosynthetic fixers of CO_2). Consequently, the lysis of these bacteria by phages (i.e., by cyanophages) redirects much of the resulting organic carbon (and energy) of cyanobacterial photosynthesis from protist predators of cyanobacteria, redirecting it instead into dissolved organic matter (that is, converting intact bacteria into smaller bacterial parts). Dissolved organic matter is mostly unavailable to eukaryotes and instead nourishes especially heterotrophic bacteria which, like cyanobacteria, also serve as prey of phagotrophic eukaryotes as well as of phages. Characterization of cyanophages of the genera *Synechococcus* and *Prochlorococcus* has been especially productive. Among the interesting observations is the encoding by certain cyanophages of proteins that play roles in host-cell photosynthesis during infection.

Mycobacterium Phages

The acid-fast mycobacteria are important disease agents (e.g., tuberculosis) but nonpathogenic mycobacteria are commonly found as soil saprobes. Mycophages, the phages of mycobacteria, are important for their role as molecular tools useful in the study of mycobacteria, especially their genetics. As an offshoot of those efforts, mycobacteriophages serve as a phage cohort that is employed in the study of comparative phage genomics, the study of entire phage genomes. A diverse group of mycophages have been identified as part of the SEA-PHAGES (originally Phage Hunters) program, an educational module used by many college and high school biology programs. This has also led to the identification of new genera of bacteriophages. Clinical roles for mycophages are in phage-based bacterial detection schemes including ones that assess antibiotic susceptibility and for phage therapy of mycobacterial infections.

Archaeal Viruses

The archaea represent one of the three cellular domains of life, the others being the bacteria and the eukaryotes. Typically researchers prefer to not describe the viruses of this lineage as phages, though archaeans are bacteria-like in the sense that the cells of both domains lack cell nuclei. Many archaeal viruses, however, are tailed (just as many or most bacteriophages are tailed), though among archaeal viruses are also a number of morphologies that are quite unlike those found among bacteriophages (Fig. 1). Presumably archaeal viruses play similar roles in archaean ecology as bacteriophages play in bacterial ecology. In addition, there appears to be distant but nonetheless structurally perceptible evolutionary kinship between certain archaeal viruses and bacteriophages.

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Bacteriophages and Rapid Detection of Bacterial Pathogens: A Novel Approach

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Introduction

Foodborne pathogenic bacteria are bacteria that contaminate food products with the potential to cause disease, transmitted through the ingestion of the contaminated food. Some of the more prominent bacterial foodborne pathogens include *Listeria monocytogenes, Campylobacter jejuni, Salmonella* spp. and *Escherichia coli*. Significant costs are incurred as a result of the distribution and consumption of foods contaminated with these bacteria, both in terms of the monetary cost to the food industry and more importantly, in terms of the cost to the wellbeing of the members of the public affected by such a disease outbreak (Zhao *et al.*, 2014).

Of the 864 outbreaks of foodborne illness in the US in 2014, 468 had confirmed or suspected etiology, for which the Centre for Disease Control (CDC) attributes 53% of these outbreaks to bacterial pathogens. *Salmonella* was the most common bacterial causative agent of foodborne disease outbreaks; 140 outbreaks resulting in 2395 illnesses were attributed to the bacterium. *Salmonella* was also responsible for the largest number of hospitalisations (395), whilst *Listeria monocytogenes* that in the US in 2014 was most likely to cause hospitalisation amongst those who had been infected (93%). It was estimated, 17 deaths were caused by bacterial foodborne pathogens, 13 of which were caused by *Listeria monocytogenes* (CDC, 2016). These death rates and rates of infection put a strong impetus on clinical bacteriology laboratories to return a diagnosis to the clinic as rapidly as possible in order to deliver the correct treatment (Gould *et al.*, 2013). Bacteriology results also play a significant role in determining the source of a disease outbreak.

Painter *et al.* (2013) estimated that of the 1451 annual deaths attributed to foodborne illness outbreaks between 1998 and 2008, 862 were caused by bacterial pathogens. 19% of deaths were attributed to poultry, the majority of which had been contaminated by *Listeria* and *Salmonella* spp.

Between 2010 and 2014, 3% of all foodborne outbreaks in the US were said to be multi-state in nature, however, significantly, these outbreaks were responsible for 54% of deaths associated with foodborne disease outbreaks. The most common pathogens associated with multi-state outbreaks were *Salmonella*, shiga-toxin producing *Escherichia coli* (STEC) and *Listeria* species. The rise of the multi-state outbreak is likely due to the centralisation of food production and its wide distribution across many states as well as the demand for ready-to-eat (RTE) foods that do not require cooking. This calls for more stringent food safety practices within the food industry and stricter implementation of quality management systems in food production facilities, with the aim to restrict the size and frequency of outbreaks (Crowe *et al.*, 2015).

In order to achieve the best standards of food safety, new methods that enable rapid, sensitive and specific detection of foodborne pathogens are required. Ideal methods must not be labour intensive, must not require significant levels of expertise and should be portable for use at the point of production. These methods must also be cost effective allowing for regular and reproducible testing at critical control points throughout the production process. Ideally, methods must also be easily adapted to suit different sample matrices (Billington *et al.*, 2014). The aim of this article is to review the current state of the art in rapid bacterial pathogen detection focusing on the potential of bacteriophage derived detection systems compared to the more traditional culture-, antibody and nucleic acid-based methods.

Pathogen Detection Methods

Culture Based Pathogen Detection Methods

Conventional methods for bacterial pathogen detection include culture based screening followed by identification using a variety of biochemical assays. These culture based methods remain the standard to which all newly developed methods are compared due to the high levels of accuracy that can be achieved. Despite being both sensitive and specific for detection of the target pathogen, there are a number of significant issues associated with culture based methods. Perhaps the most significant issue is that culture based methods are very time consuming. This process often involves sample pre-enrichment, selective enrichment and plating on agar plates, followed by the use of standard biochemical analysis for identification of the bacteria present in a sample. This process is laborious and requires a certain level of microbiological expertise and can take at least 2–3 days to get primary results with longer times needed for the identification of the contaminant. This delay between the taking of a sample and the identification of a bacterial contaminant can have major consequences due to the fact that the food from which the contaminant was isolated, may have already been distributed for sale (Law *et al.*, 2014).

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Culture based methods can lead to the release of negative result for foods that contain viable, but non-culturable cells, as these cells will not be grown during the enrichment process, only growing in their natural habitats when conditions are most suitable. This may lead to the distribution of goods for sale that contain bacterial pathogens (Zeng *et al.*, 2016).

Due to the drawbacks of culture based pathogen detection, other avenues have been explored that have the potential to reduce the time needed to identify contamination in food during the production process. These alternative methods can be grouped into three categories: nucleic acid based methods, immunoassays and biosensors (biosensors usually incorporating aspects of immunoassays or nucleic acid based methods with the addition of a signal transduction platform that allows for signal amplification) (Law *et al.*, 2014).

Nucleic Acid Based Pathogen Detection

Nucleic acid based detection methods usually involve polymerase chain reaction (PCR). The presence of a pathogen is determined by the presence of nucleic acid sequences (DNA or RNA) that are specific to the pathogen in question (Mangal *et al.*, 2016) or by the detection of toxin encoding or virulence genes (Law *et al.*, 2014).

PCR based methods work by amplifying target DNA from the pathogen to a detectable level, thus confirming the presence of DNA from that pathogen in the tested sample. The products of the PCR are then visualised on anagarose gel alongside positive and negative controls, confirming the presence or absence of the target and thus the presence or absence of the bacterial contaminant.

Multiplex PCR is used for the simultaneous detection of more than one pathogen, or multiple strains of the same pathogen in a single PCR reaction eliminating the need for numerous different tests. Kawasaki *et al.* (2009) developed a multiplex PCR system for the detection of extracted *Salmonella* spp., *L monocytogenes* and *E. coli* O157:H7 DNA from enrichment cultures. The presence of DNA from each of the bacteria is confirmed through the amplification of different sized DNA fragments, with each fragment corresponding to the presence of a different pathogen. In order for multiplex PCR to be successful, all PCR primers must be designed to work under the same conditions to ensure that all of the target bacterial species or strains are detected (Law *et al.*, 2014).

Quantitative real-time PCR (q-PCR) allows for the simultaneous detection and quantification of the target pathogen. In this method the addition of an oligonucleotide that acts as a molecular probe allows for the real time detection of the target DNA. As the target DNA is amplified, the probe fluoresces with levels of fluorescence directly proportional to the number of amplicons. This allows for the enumeration of the original template and therefore the quantification of the number of target pathogens in the initial sample (Mangal *et al.*, 2016). Boehme *et al.* (2010) described a method based on real time PCR for the point of care detection and diagnosis of *Mycobacterium tuberculosis* and its susceptibility to rifampin treatment. The method involved the direct detection of the bacterium from clinical sputum samples. The assay could identify 97% of culture-positive patients in less than two hours.

A third method based on PCR is reverse-transcription PCR (RT-PCR). In this method, RNA acts as the template. This ensures that only bacteria that are actively transcribing their DNA into mRNA are detected, thus lowering the chances of detection of dead cells. This method involves an initial step which uses a reverse transcriptase to reverse transcribe RNA to cDNA. This cDNA then acts as the template similar to a traditional PCR (Sharma, 2006). Senoh *et al.* (2014) described a method that utilised RT-PCR for the detection of mRNA transcripts of toxin-encoding genes from *Clostridium difficile* in spiked faecal samples thus distinguishing vegetative cells from dead cells or spores. The method described has a detection limit of 5×10^2 cfu in a 100 g faecal sample.

An alternative nucleic acid based method of pathogen detection is the DNA microarray. This method involves the immobilisation of DNA probes; oligonucleotides that have sequences that are specific to target DNA from the pathogen in question. Presence of a pathogen is determined by hybridisation between the probes and sample DNA. Microarrays often involve an initial amplification of the target DNA in order to increase sensitivity and eliminate interference from the matrix material (Rasooly and Herold, 2008).

Whilst nucleic acid based methods are useful there are a number of drawbacks associated with them. The need for samples to be enriched before testing can proceed adds at least 24 h to the time that it takes to get a result. Due to the complex nature of these methods, they are not amenable to use at the point of production. The technicalities of the procedures require highly trained personnel to carry them out. This raises questions about the rapidity of these methods (Zhao *et al.*, 2014).

Immunoassay Based Pathogen Detection

Immunological assays for pathogen detection work on the basis of antibody–antigen interaction. The quality of an immunoassay hinges on the level of affinity and specificity of the antibody for the antigen. In the case of the detection of whole bacteria, the antibody must bind to an epitope(s) on the cell surface. The epitope must be unique to the target pathogen in order to ensure assay specificity. Monoclonal antibodies have greatly increased the reliability of immunoassays for pathogen detection by reducing the chances of non-specific binding and thus false positive results (Zhao *et al.*, 2014).

Enzyme-linked immunosorbance assays (ELISA) are amongst the most commonly used immunoassays for pathogen detection. In a method known as a sandwich ELISA, antibodies are immobilised to the surface of the wells of a microtiter plate. The sample to be tested is then added to the wells followed by a subsequent washing. If the target antigen is present it will bind to the antibody and remain bound following the wash step. A secondary antibody that will bind to another epitope on the analyte, usually conjugated with an enzyme such as horseradish peroxidase, is then added and subsequently washed. If the antigen is present the secondary antibody will remain bound to the antigen in the well. The substrate to the enzyme which is conjugated to the secondary antibody is then added resulting in a colorimetric change that is directly proportional to the amount of secondary antibody that is bound and is therefore an indication of the presence of the pathogen. The intensity of the colour can be compared to a standard curve that relates

to known concentrations of the antigen, allowing for the inference of the concentration of the antigen in the test sample (Law *et al.*, 2014). Savolainen *et al.* (2013) used an ELISA for the detection of urinary lipoarabinomannan (uLAM), a component of the *Mycobacterium tuberculosis* cell wall that is dispersed into the urine during cell replication. When urine samples were concentrated 100×, assay sensitivity of 57% was achieved with specificity of 87%.

Lateral flow immunoassays employ immunochromatographic strips through which the sample migrates. If the antigen is present it will first bind to an antibody conjugate such as a colour labelled antibody. It then migrates to a strip of immobilised antibody, which binds to the antigen conjugate. If the antigen is present a colour change will occur at the strip of antibodies. A second strip of antibodies that specifically detect the colour labelled antibody acts as a control, ensuring that there has been adequate migration of the sample as in a pregnancy test (Law *et al.*, 2014). The use of a lateral flow immunoassay has been described for the detection of the *Staphylococcus aureus* virulence factor Panton-Valentine leukocidin (Monecke *et al.*, 2013). The assay used monoclonal antibodies that were generated through phage display with the assay achieving a lower limit of detection of 1 ng ml^{-1} in *S. aureus* cultures.

Immunoassays experience similar pitfalls to nucleic acid based methods of pathogen detection. Sample enrichment is often required in order to have desired levels of sensitivity resulting in less rapid methods. Some immunoassays such as ELISA require trained personnel to carry them out, increasing costs. Antibodies can become denatured at room temperature and therefore require specific storage conditions in order to maintain their functionality. There are also ethical issues associated with the production of antibodies due to the need to immunise animals with potentially harmful substances (Singh *et al.*, 2013).

All of these methods require sample enrichment. This involves the growth of the pathogen. Due to this proliferation of the pathogen, these methods are not amenable to use at the point of production as it will increase the likelihood of contamination. This means that any diagnostic protocols must be performed at a separate facility to that of production. This further adds to the delay in retrieving positive contamination results, and thus a delay in the implementation of measures that will prevent the distribution of potentially contaminated foods.

Biosensors

Immunosensors

To overcome limitations associated with traditional immunoassays, the concept of antibody-antigen interaction has been exploited for the development of biosensor platforms. Biosensors offer an alternate means of pathogen detection. A biosensor has four key components; a biorecognition element or bio-probe, a signal transduction platform, a signal amplifier and a display, all of which combine to make an automated system that requires little labour. The biorecognition element is responsible for detection. Any binding to the bio-probe is then converted into a signal by the signal transducer. The signal amplifier amplifies the signal to measurable levels and the display provides a read-out of the test results (Fig. 1; Singh *et al.*, 2013). The parameters to be considered when developing a biosensor include assay sensitivity and specificity, the time it takes to get reliable results, the size of the equipment, the extent of the sample preparation and the level of expertise required by the operator (Table 1).

The Biorecognition Element

Perhaps the most important component of a biosensor, the biorecognition element allows for the specific detection of the target. Biorecognition elements that have been used on these technology platforms include antibodies, oligonucleotides, bacteriophages (phage), phage derived proteins and phage display peptides. The method of immobilisation of the biorecognition element can significantly alter the quality of the assay and is therefore usually assessed during assay development (Singh *et al.*, 2013).

Li *et al.* (2015) describe an impedance based immunosensor for the detection of the foodborne pathogen *E. coli* O157:H7. Impedance based biosensors work on the principle of an analyte binding to a bioreceptor resulting in a change in the electron transfer resistance and capacitance on the surface of an electrode (Ahmed *et al.*, 2014). The method described by Li *et al.* (2015)

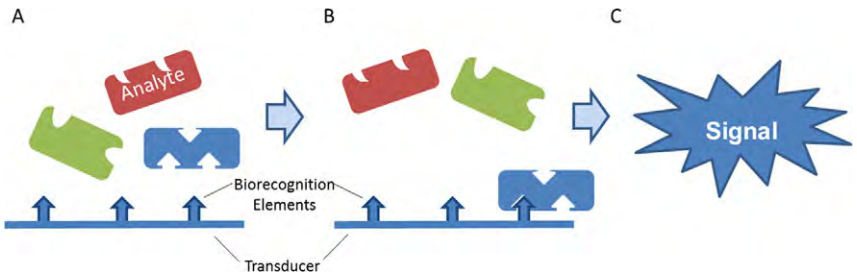

Fig. 1 A schematic diagram of a biosensor system. (a) A sample is passed over a signal transducer that has been functionalised with a biorecognition element (antibodies or antibody fragments, oligoneucleotides, bacteriophage, phage derived proteins or phage display peptides). (b) Where an analyte is present that is recognised by the biorecognition element a binding event will occur. (c) The binding of the analyte will result in the change of a property of the transducer resulting in a measurable signal or the addition of a further reagent that reacts with the analyte, following a wash step, will provide a measurable signal.

Table 1 Ideal properties of a detection system for foodborne pathogens in the food industry

Parameter	*Description*
Sensitivity	A detection system should be able to detect as few live bacteria as possible
Specificity	A detection system must be able to accurately differentiate target from non-target bacteria
Time	There should be minimal delay between sample receipt and contaminant identification
Size	The detection system should be portable and amenable for use at the point of production
Preparation	There should be minimal sample preparation
Expertise	The operator should require minimal levels of expertise

involves the immobilisation of antibodies on screen-printed interdigitated microelectrodes. The antibodies used bind to an antigen on the bacterial cell surface. This binding event results in a change in the impedance of the electrodes and thus indicates the presence of the pathogen. A detection limit of 10^2 cfu ml^{-1} was achieved using this method to detect stationary phase *E. coli* O157:H7 in phosphate buffered saline (PBS).

An electrochemical immunosensor for the detection of the uropathogenic *E. coli* was described by Gayathri *et al.* (2016). Uropathogenic *E. coli* is one of the leading causes of urinary tract infections. A detection range of 10^2–10^9 cfu ml^{-1} was observed in cells suspended in phosphate buffered saline (PBS). Clinical applicability of the sensor was tested using artificially spiked urine samples.

Barlen *et al.* (2007) describe the development of an immunosensor that employs a surface plasmon resonance (SPR) based immunoassay for detection of *Salmonella* serotypes. SPR systems use polarised light, which passes through a prism that is in contact with a transducer surface functionalised by the biorecognition element. The binding of an analyte to the biorecognition element results in a change to the refractive index of the transducer thus altering the angle at which the light exits the prism (Ahmed *et al.*, 2014). The method noted above for the detection of *Salmonella* uses an immobilised polyclonal antibody to capture all serotypes followed by specific detection using detection antibodies similar to a sandwich ELISA. The assay was shown to simultaneously detect *Salmonella typhimurium* and *Salmonella enteritidis* at lower limits of 2.50×10^5 cells ml^{-1} and 2.50×10^8 cells ml^{-1}, respectively.

Whilst antibodies are among the most widely exploited entities for biorecognition in sensor development, there are a few issues associated with their use. There are significant costs associated with antibody production and purification which can drive up the cost of the sensor. Antibodies also have a notoriously short shelf-life resulting in a reduction in binding efficiency and their stability over time. The quality of the biosensor is also dependent on the level of specificity that antibody has for its target antigen. This has led to research into alternative biorecognition elements for detection of foodborne bacteria (Ahmed *et al.*, 2014).

Bacteriophages

The Potential of Bacteriophage Based Pathogen Detection

By their nature bacteriophages (phage) and phage derived proteins have attractive characteristics for exploitation in the detection of pathogens, offering an alternative option to more traditional nucleic acid and antibody based methods (Singh *et al.*, 2013).

Phages are obligate parasitic viruses that infect bacteria with incredible levels of specificity, having co-evolved with their respective hosts; phages have been known to infect their hosts with specificity down to the strain level. They are the most diverse and populous organism on the planet, such has been their success. Phages are divided into two categories; temperate and virulent. In order for both types of phage to reproduce they must first infect their host. The first step in the infection process is host recognition. Receptor binding proteins (RBP) in the phage tail are responsible for the initial phage-host interaction. These proteins bind to a receptor on the cell surface of the host. The receptor is usually unique to that bacterium, conferring the specificity of the phage-host interaction. Upon receptor binding, the phage essentially injects its genetic material into the host cell. It will then employ the host's DNA replication machinery to replicate its genome and the host's translation machinery to translate it into the components that assemble into a whole phage, eventually resulting in the burst of the cell (Fig. 2). Temperate phages may also incorporate their DNA into the host genome where they can remain dormant, replicating and reproducing at a later time (Vinay *et al.*, 2015). Due to their harmlessness to humans and their specific interaction with their host, phages have been exploited by the food industry for both biocontrol and detection of foodborne pathogens in food matrices.

A number of phage based food additives have been approved for use by administrative bodies for the biocontrol of bacteria, harnessing the phage replication cycle for its bactericidal properties. A phage preparation, available commercially as ListShield™ (Intralytix Inc.), consisting of a preparation of six bacteriophages known to infect and kill *L. monocytogenes* cells, was approved by the Food and Drug Administration (FDA) for use in preservation of ready-to-eat (RTE) meats. Listex P100 (Micreos Food Safety) is a preparation of a single broad spectrum bacteriophage active against multiple *L. monocytogenes* serotypes, that has been generally regarded as safe (GRAS) by the FDA and is commercially available for use in cheese production as well as other areas of the food industry (Bai *et al.*, 2016). Companies with a focus on phage based biocontrol of bacteria are outlined in Table 2.

Enzyme linked fluorescent assays that employ phage proteins for the detection of a number of pathogens are commercially available under the name VIDAS (bioMérieux). These automated assays offer "next day detection", requiring a single enrichment step. A comparative study showed that the VIDAS apparatus for the detection of *E. coli* O157:H7 produced comparable results to a

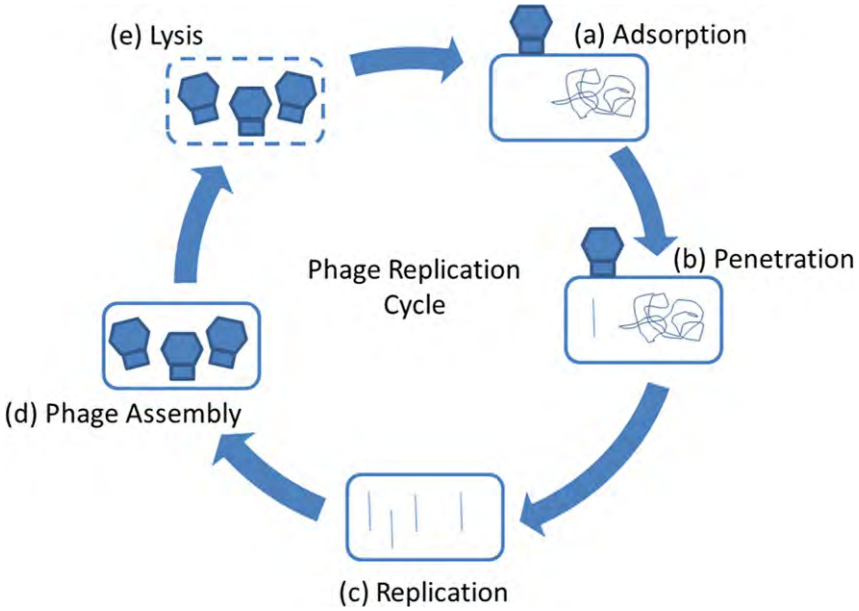

Fig. 2 Schematic diagram of the phage replication cycle. (a) The phage adsorbs to the bacterial cell surface via its receptor-binding protein (RBP). (b) The phage genome penetrates the host cell wall. (c) The phage harnesses the host DNA replication machinery to replicate its own genome. (d) The newly replicated phage genomes are translated and are then encapsulated forming fully assembled phage. (e) The host cell is lysed allowing the newly reproduced phage to adsorb to the surface of other host bacteria initiating the cycle again.

Table 2 Companies producing phage based biocontrol products for the food industry

Company	*Statement*	*Source*
EpiBiome	"A substantial opportunity exists for EpiBiome's technology, which provides. . . compatibility with organic production practices"	EpiBiome (2017)
APS Biocontrol Ltd	"Our core technology is based on bacteriophage;. . . and our lead product, Biolyse® is a world first; a bacteriophage-based solution for washed and packed fresh produce"	APS Biocontrol Ltd (2017)
Intralytix Inc.	"a biotechnology company focused on the discovery, production and marketing of bacteriophage-based products to control bacterial pathogens in environmental, food processing, and medical settings"	Intralytix Inc (2017)
Micreos	"Micreos has FDA-approved food safety products: Salmonelex™ against *Salmonella* and **Listex**™ against *Listeria monocytogenes*"	Micreos (2017)

U.S. Department of Agriculture, Food Safety and Inspection Service reference method, following an 18–24 h enrichment (Savoye *et al.*, 2011). This precedence for the safe use of phage in the food production and food safety environments opens up opportunities for further use of phage in food safety and diagnostics (Sharma, 2013).

The Current State of the Art in Phage Based Pathogen Detection

The idea of using fluorescently labelled phage or phage proteins has been widely exploited for both direct detection and biosensor based detection of bacterial pathogens (Table 3) (Jain *et al.*, 2012; Namura *et al.*, 2008; Vinay *et al.*, 2015; Oda *et al.*, 2004; Schmelcher *et al.*, 2010). Schmelcher *et al.* (2010) developed a method that allowed for detection and differentiation of different strains of *L. monocytogenes* by using different cell wall binding domains (CBD) derived from phage endolysins, tagged with different colour fluorescent markers. Endolysins are enzymes that are produced by phages which catalyse the hydrolysis of the peptidoglycan in the bacterial cell wall, rupturing the cell at the end of the virulence cycle. These proteins have a modular structure consisting of two (or more) distinct domains; most notable are the enzymatically active domain (EAD), which is responsible for lysis, and the cell wall binding domain (CBD) which directs the protein towards its peptidoglycan substrate. The CBDs of phage endolysins have been shown to bind to their respective ligands in the host cell wall with great levels of specificity and have thus been harnessed for use in the detection of Gram positive bacteria that do not have an outer membrane protecting their cell wall peptidoglycan (Schmelcher *et al.*, 2012). Schmelcher *et al.* (2010) exploited a number of CBDs that had different binding spectra in order to differentiate *L. monocytogenes* strains in mixed culture. The different CBDs were tagged with green, cyan and yellow fluorescence proteins as well as RedStar protein, allowing for the visualisation of different strains under different fluorescence microscopy conditions following incubation with the fluorescently tagged CBDs.

Table 3 Examples of phage based methods of pathogen detection

Technology	*Detection principle*	*Organism detected*	*Detection limit*	*Sample preparation*	*Time of analysis*	*Reference*
Reporter phage	Expression of a g*fp*-tagged high-intensity flourophage	*Mycobacterium tuberculosis*	N/A	12 h incubation	1 to 2 days (incl. drug susceptibility testing)	Jain *et al.* (2012)
	Expression of a *luxAB*-tagged reporter phage	*Pseudomonas syringae* pv. *maculicola*	1.3×10^3 cfu ml^{-1}	120 min incubation	4 h	Schofield *et al.* (2012a)
	Expression of a *luxAB*-tagged reporter phage	*Listeria monocytogenes*	0.1–10 cfu g^{-1} (depending on the food matrix)	20 h pre-enrichment	24 h	Loessner *et al.* (1997)
Tagged phage and phage proteins	Binding of a GFP-labelled phage to the cell surface	*Escherichia coli* O157: H7	N/A	10 min incubation with GFP tagged phage	N/A	Oda *et al.* (2004)
	Binding of fluorescently-labelled CBD's to differentiate strains	*Listeria monocytogenes*	N/A	24 h incl. selective enrichment	N/A	Schmelcher *et al.* (2010)
	Host mediated biotinylation of phage followed by conjugation to streptavidin coated quantum dots	*E. coli*	20 cells ml^{-1}	N/A	1 h	Edgar *et al.* (2006)

Another means by which phage have been used for detection of pathogens is by harnessing their virulence to make reporter phage. Schofield *et al.* (2012a) developed an engineered bioluminescent reporter phage for the detection of the blight causing bacteria *Pseudomonas cannabina* pv. *alisalensis*, which affects members of the *Brassicaceae* family including significant food crops such as cabbage, broccoli and rapeseed. Bacterial *luxAB* genes, which code for a bacterial luciferase, were incorporated into a *P. cannabina* pv. *alisalensis* specific bacteriophage. The reporter phage conferred rapid and specific identification of the target bacteria through a bioluminescence assay. Bacterial pathogens that affect *Brassica* spp, that were closely related to the target pathogen were not detected or produced a significantly smaller signal when compared to *P. cannabina* pv. *alisalensis*.

Jain *et al.* (2012) describe the development of a fluorescent reporter phage for the detection of *Mycobacterium tuberculosis* in clinical sputum samples. Pre-incubation of these samples with the reporter phage resulted in detection of *M. tuberculosis* by both microscopy and flow cytometry. Additionally this reporter phage was used for drug susceptibility testing of *M. tuberculosis* strains by observing fluorescence levels following antibiotic exposure.

Burnham *et al.* (2014) developed a method that harnesses the lytic activity of the T4 bacteriophage for the detection of *E. coli* in water samples. A recombinant *lac* Z T4 phage which carried the gene for β-galactosidase was used. Upon host lysis, β-galactosidase was released into the media allowing for its detection through the addition of chromogenic or bioluminescent substrates. A detection limit of less than 10 cfu ml^{-1} was achieved in 5.5 h through the use of the bioluminescent β-galactosidase substrate from the Beta-Glo® assay system (Promega).

A method for the detection of the causative agent of anthrax, *Bacillus anthracis*, infamous for its use by bioterrorists, has been approved by the FDA for the detection of the bacterium from clinical samples (Schofield *et al.*, 2012b). The U.S. Army Medical Research Institute of Infectious Diseases (USARMRIID) modified the method that was originally developed by the Centre for Disease Control (CDC) in the 1950s. This method employs γ phage to detect the target bacteria. This phage is highly selective for *B. anthracis* whilst having a very broad host range within the species. *B. anthracis* is identified by zones of clearing on bacterial isolate lawns as a result of exposure to the phage (Abshire *et al.*, 2005)

Phage Based Biosensors for Pathogen Detection

Phages are known to infect their respective hosts with extraordinary specificity. This level of specificity marks them as a great alternative to antibodies and nucleic acids as biorecognition elements for the detection of their hosts on biosensor platforms (Vinay *et al.*, 2015).

Phage based biosensors have shown great promise for the detection of foodborne pathogens (Table 4). Singh *et al.* (2011) harnessed the receptor binding protein (RBP) of the *Campylobacter jejuni* phage NCTC 12673 for the detection of the pathogen. Phage RBPs confer the phage's specificity for its host, binding to unique structures on the bacterial cell surface. The RBP was expressed as a glutathione-S-transferase (GST) fusion protein allowing for its immobilisation on surface plasmon resonance (SPR) surfaces via oriented attachment using glutathione self-assembled monolayers. The principle of surface plasmon resonance, as mentioned previously, is based on the change in the refractive index of the SPR transducer that is in contact with a glass prism, and

Table 4 Examples of phage based biosensors for the detection of bacteria

Detection principle	*Organism detected*	*Biorecognition element*	*Limit of detection*	*Reference*
Surface plasmon resonance	*Staphylococcus aureus*	Entact lytic phage	10^4 cfu ml^{-1}	Balasubramanian *et al.* (2007)
	Campylobacter jejuni	Phage NCTC 12673 RBP	10^2 cfu ml^{-1}	Singh *et al.* (2011)
	Bacillus cereus	Phage PBC1 CBD	10^2 cfu ml^{-1}	Kong *et al.* (2015)
	E. coli O157:H7	T4 Phage	10^3 cfu ml^{-1}	Tawil *et al.* (2012)
	MRSA	BP14 Phage	10^3 cfu ml^{-1}	Tawil *et al.* (2012)
Magnetoelastic Biosensors	*Salmonella typhimurium*	E2 Phage	1.6×10^2 cfu cm^{-2} (on the surface of egg shells)	Chai *et al.* (2012)
	Salmonella typhimurium	Gentically engineered phage	N/A	Huang *et al.* (2009)
	Salmonella typhimurium	Filamentous *S. typhimurium* phage	10^3 cfu ml^{-1}	Lakshmanan *et al.* (2007)
	Bacillus anthracis	Genetically engineered phage	N/A	Huang *et al.* (2009)
Fluorescence	*E. coli*	HK620 Phage	10 cfu ml^{-1}	Vinay *et al.* (2015)
Impedimetric	*E. coli*	T4 Phage	10^4 cfu ml^{-1}	Shabani *et al.* (2008)

thus the angle of light emitted from the prism. Prior to being subjected to the biosensor a pre-concentration step was done. Capture beads that had the RBP in question immobilised on them were used to concentrate the bacteria from sample solutions. This was necessary due to the low sample volume used on the biosensor platform. A detection limit of 10^2 cfu ml^{-1} was achieved. Tawil *et al.* (2012) developed a SPR biosensor that uses phage as biorecognition elements for the detection of *E. coli* and methicillin resistant *Staphylococcus aureus* (MRSA). The method that was developed had a detection limit of 10^3 cfu ml^{-1}.

Tawil *et al.* (2012) developed a SPR biosensor that uses phage as biorecognition elements for the detection of *E. coli* and methicillin resistant *Staphylococcus aureus* (MRSA). The method that was developed had a detection limit of 10^3 cfu ml^{-1}.

A phage based magnetoelastic biosensor was developed by Park *et al.* (2013) for the detection of *Salmonella typhimurium* on the artificially inoculated surface of tomatoes. Magnetoelastic biosensors work on the principle of altered resonant frequency of a magnetoelastic resonator upon analyte binding, when said resonator is exposed to an alternating magnetic field. In the case of Park *et al.* (2013) the resonator was functionalised with E2 filamentous phage that specifically targets *S. typhimurium*. Binding of the bacteria to the immobilised phage increases the mass of the resonator thus decreasing its resonant frequency and confirming the presence of the bacterium in the sample. The number of bacteria bound is proportional to the change in mass of the resonator.

Vinay *et al.* (2015) describe a method which employs GFP fusion proteins for the detection of faecal contamination of water through the detection of *E. coli* TD2158 and the successful adaptation of the technique for *Salmonella* detection. The test that was developed involved a 1 h infection with the recombinant phage followed by detection using a portable flow cytometry device. Successful detection of less than 10 cfu ml^{-1} was achieved in M9 supplemented media. The same detection limit was observed in artificially contaminated seawater.

Chibli *et al.* (2014) demonstrated the use of phage endolysin derived proteins for the detection of *Staphylococcus* spp. and examined their potential for immobilisation on surface plasmon resonance surfaces. This involved the immobilisation of the proteins on PEGylated silicon wafers. These wafers were then immersed in solutions containing fluorescently labelled bacteria. The bound bacteria were visualised by fluorescence microscopy and a detection limit of 5×10^3 bacteria was achieved with the authors noting that this limit may be lower with the use of a signal transduction platform.

Conclusion

Summary

The prevalence of food related disease outbreaks is placing great pressure on the food industry to improve its standards as regards food safety. The demand for convenience foods places a great pressure on manufacturers to ensure that their products are free from contaminants, and that these products measure up to the "ready-to-eat" label that is given to them. Failed detection of bacterial pathogens in food can lead to widely dispersed outbreaks of disease, that have a major impact on public health, to a significant cost to the manufacturer of that food product as well as healthcare services.

The ultimate goal of the food industry is to deliver high quality produce to customers in as fast a time as possible. In order to produce food up to the highest safety standards, food producers must be capable of detecting bacterial contamination in their produce or production facilities in a rapid manner. Bacteriophage offer a novel means to this end. Bacteriophages are (i) specific to their host and therefore may be harnessed for specific detection of that host, (ii) inexpensive to produce and (iii) more robust when compared to alternatives such as antibodies. Whilst phage derived proteins may lack the robustness of whole phage, they remove the pitfalls associated with phage virulence whilst maintaining the advantages that whole phage pose for pathogen detection (van der Merwe *et al.*, 2014).

Receptor binding proteins (RBPs) are responsible for initial phage host interaction and confer the phage's specificity for its host (Kot *et al.*, 2013). This specificity has been harnessed for the purpose of pathogen detection. Developments in the areas of genetic engineering and cloning have allowed for the tagging of RBPs resulting in the ability to orientate immobilisation of the proteins, allowing for greater capture efficiencies during detection (Singh *et al.*, 2013). Cell-wall binding domains from phage endolysins have also been exploited for the detection of Gram positive bacteria. Phage proteins have significant advantages when compared to antibodies. They are less sensitive to fluctuations in environment such as pH or temperature (Singh *et al.*, 2013).

When combined with biosensor technology, bacteriophages have the potential to be a powerful tool in microbiology laboratories in the food industry. Biosensors offer the possibility of rapid screening for bacterial contaminants with the potential to be used by personnel who lack training in molecular or microbiology skills that are required to perform immunoassays; culture or nucleic acid based diagnostic procedures. The portable nature of biosensors makes them amenable to use at the point of production, but only in cases where minimal sample preparation is required.

Bacteriophage Based Diagnostics: Future Prospects

Future research into bacteriophage based diagnostics has the potential to lead to the commercialisation of high throughput biosensor platforms, which are capable of identifying food related pathogens in food matrices in comparably fast turnaround times, when compared to methods that are currently used. The ease of integration of phage and phage derived proteins onto detection platforms, which are already in use with other bioprobes, as well as the precedence for the use of phages as biopreservatives makes them an attractive alternative for use in pathogen detection in the food industry. The broadening of the field of nanobiotechnology occurring in parallel to research into bacteriophages and the mechanisms by which they recognise their hosts, makes for great prospects in the realm of phage based nanodiagnostics.

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Beer/Brewing[☆]

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Glossary

Bottom-fermenting yeasts Yeasts used to produce the type of beer known as lager.
Chill-proofing Use of proteases to prevent haze development when beer is chilled to refrigerated temperatures.
Hops The dried flowers of the female *Humulus lupulus* plant that contribute flavor and antibacterial compounds to beer.
Malt Major raw material used in brewing that provides the appropriate substrate and enzymes needed to yield wort.
Top-fermenting yeasts Yeasts used to produce the type of beer known as ale.
Wild yeasts Yeasts that are present in the brewing process that were not introduced purposely nor tolerated for a specific purpose during brewing.
Wort Liquid that remains after mash is strained, containing soluble fermentable compounds.

Defining Statement

In the brewing of beer, grains are converted through fermentation to produce a desirable beverage with distinct sensory characteristics. This article outlines the steps involved in brewing beer and discusses the variations that can be done to produce the vast variety of beers available.

Introduction

Beer is defined in the Bavarian Purity Law of Germany, as a fermented alcoholic beverage made of malted cereals, water, hops, and yeast. This is the classical definition and has been enforced in Germany since the 16th century. Many countries, however, now allow additional substances to be used in this product. For instance, various enzymes and antifoaming agents are used by some brewers during the fermentation process. Others supplement expensive barley malt with unmalted cereals such as corn, rice, or wheat, which contribute to beer flavor while reducing processing costs.

Beer is generally subdivided into lagers and ales based on its geographic origin and history (Table 1). For centuries, the only beer known to all brewers was ale. During the fermentation of this beer, a thick yeast foam rose to the surface. All too frequently the beer went sour, especially during warmer months. In the 1800s, German brewers observed that if beer could be kept at colder temperatures by brewing in the colder months or by using natural ice or caves, the beer soured less frequently. They also noticed that the yeast settled to the bottom of casks in the cold. This cold-storage method gave birth to "lager" beer in Germany. Lager beers are now traditionally fermented cool and aged cold for several weeks to several months. Ales, on the contrary, ferment at warmer temperatures and are aged only for days. The yeast rises to the surface and is skimmed. Ales have remained the beer of choice in England. The yeast used in lagers and ales have adapted over time to ferment optimally at the temperatures used to produce the respective beer types.

Classic examples of German lager beers are Märzen, Pilsener, Dortmunder, and Bock beer. All these beers are malty with a balance of hoppiness (bitterness). Color can vary from light (Helles) to dark (Dunkel). They usually contain 4.5–5% w/v alcohol, with bock containing 6% w/v. Examples of English ales are bitter, pale ale, porter, and stout. Bitter and pale ale are characterized by a copper color, full body, and elevated bitterness. Porter and stout are dark in color, contain roasted or burnt malts, and may or may not be bitter. Examples of German ales are Weizenbier (wheat beer), Alt, and Kölsch. There are other beer styles that do not clearly fit into the ale or lager category. Most of these beers are Belgian in origin and are spontaneously fermented, for example, Lambic.

History of Brewing

Evidence shows that brewing of beer was a popular practice in Mesopotamia before 3500 BCE. Beers offered people a flavorful alternative to drinking water, were often thought to possess therapeutic properties, and were safer to drink than water in some instances because they were less likely to harbor waterborne pathogens due to the fermentation process. Brewing beer, became

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Table 1 Major beer types

Types	*Country of origin*
Ales and stouts	
Alt or altbier	Germany
Barley wine	England
Bitter ale	England
Brown ale or mild ale	England
Cream ale	United States
Imperial stout	England
India pale ale	England
Kölsch	Germany
Lambic	Belgium
Porter	England
Saison	Belgium
Stout	Ireland
Sweet stout	England
Weizenbier or wheat beer	Germany
Lagers	
Bock	Germany
Dortmunder	Germany
Dunkel	Germany
Helles	Germany
Malt liquor	United States
Märzen	Germany
Pilsener	Czech Republic
Rauchbier	Germany
Schwarzbier	Germany
Vienna	Austro-Hungarian
Zwickelbier	Germany

popular in other areas of the Middle East, including Ancient Egypt and Israel. Brewing practices had spread to Rome during Caesar's reign and into other parts of Europe. Over the centuries, it was discovered that the addition of hops and other spices would improve the flavor of beer. Since that time, hops have become essential in beer due largely to their contribution to the flavor and stability of beer.

As Europeans settled in North America, they brought brewing practices with them. During the 16th and 17th centuries, breweries were established in Virginia and New England. Barley did not prosper in the New England climate, so a variety of unusual ingredients were fermented, including pumpkins, maple sugar, persimmons, and apples. Two factors contributed to the further development of the North American brewing industry. Pennsylvania was found to be a good barley and hop production area, and the immigration of Germanic and Dutch brewmasters bolstered the American industry.

During this entire period, brewing was basically a hit-or-miss process. Individuals experienced in brewing recognized that using old brewing vessels yielded better products than new ones. It is now realized that the cracks, crevices, and pores present in the older vessels, but lacking in the newer ones, harbored the yeasts and bacteria responsible for the fermentation. Several theories were developed in the 19th century in an attempt to explain the changes that occur during fermentation. Much of the debate centered on the issue of whether fermentation was a purely chemical process or a biological process. The issue was largely settled by Louis Pasteur in the 1860s and 1870s, when he published reports (eg, "Etudes sur la Biere") concluding that fermentation was due to the actions of yeast.

In 1883, Emil Christian Hansen established the method of using pure yeast cultures to produce beer at the Carlsberg Brewery in Copenhagen, Denmark. He had demonstrated previously that the culture used in brewing was often a mixed culture and that the metabolism of wild yeasts caused many of the defects in improperly processed beer. Over the next several years, the practice of using pure yeast cultures to produce beer became more widely accepted. The purpose and function of yeast enzymes in the fermentation process was shown by Buchner in 1897. It was found that cell-free extracts contained enzymes that could ferment sugars.

Today beer remains a popular and profitable beverage. While beer is consumed worldwide, on a per capita basis, the leading beer consuming countries are the Czech Republic, Ireland, Germany, Austria, and the United Kingdom. While US per capita beer consumption in 2002 was about half of that consumed by the leading European countries, it was greater than the volume of milk and bottled water consumed in the country. In that same year, beer sales in the United States exceeded $60 billion. While US beer production is dominated by two major brewing conglomerates, there has been an explosion in the number of small breweries producing the product, often using traditional methods. These smaller operations are typically labeled as microbreweries.

The beer industry is in the midst of major changes, and nowhere is it more noticeable than with the American craft brewer. Since 2007, the total number of US breweries has grown from 1460 to more than 4600. From 2008–15, production by the largest macrobrewers declined by 23 million barrels. The declined has been attributed to small and independent brewers who in 2016

earned over $22 billion of the $105.9 billion US beer industry. With craft beer in America gaining market share from macrobrewers, big brewers have begun acquiring these once small and independent brewers in an attempt to gain back the market share they have lost. With these mergers and acquisitions, the definition of American craft beer as we know it is changing.

The Brewers Association defines an American craft brewer by three pillars: *Small*, *Independent*, and *Traditional*. The brewer must have an annual production of six million barrels or less, have less than 25% of the brewery owned or controlled by a beverage alcohol member that is not itself a craft brewer, and have a majority of its total beverage alcohol volume in beer whose flavor is produced from traditional or innovative brewing ingredients and fermentation. This definition encompasses, brewpubs, microbreweries, regional craft breweries, and contract brewing companies.

As craft beer has taken hold in America, it has created its own culture spreading to different areas of the food and beverage industry. The coinage of "craft" as a moniker to describe a business is now used to separate small, independent entities from large companies. Such is the case with craft maltsters, craft hop growers, and craft distillers.

Brewing

The conversion of cereals into beer is not a direct process. The cereals used in beer production do not contain sufficient quantities of fermentable sugars. These cereals must first undergo modification during the malting and mashing steps to yield carbohydrates that yeast can convert during the fermentation step into ethyl alcohol and carbon dioxide. Freshly produced beer can then be aged for flavor development before it undergoes finishing steps which can include filtering, pasteurization, and packaging. Each of these steps is examined more closely in the following sections and are shown in Fig. 1.

Ingredients

The basic ingredients in beer are water, malted cereals, hops, and yeast. Water comprises 90–95% of the content of finished beer, and its quality can influence the flavor of beer. Barley is the most common cereal used in the Americas and Europe to produce malt, although small volumes of beer are made from other cereal grains. Better beers are produced with clean barley that was properly dried after harvest (to about 10–12% moisture content). Overheating barley during drying can lead to its becoming unacceptable for malting, since its germination potential is adversely affected. Barley contains a high starch content suitable for conversion to fermentable carbohydrates and a sufficient protein content to support yeast growth and contribute to forming beer foam. In addition, it contributes unique flavor components. Hops are the dried flowers from the female hop (*Humulus lupulus*) plant and contribute flavor and antibacterial compounds to beer. Yeasts are the predominant fermentation organisms used to make beer worldwide. In some instances, bacteria may contribute certain characteristics to some regional beers. Malt adjuncts, such as corn, rice, wheat, sorghum grain, soybeans, cassava, potatoes, sugars, and sirups, may be used in some formulations. The adjuncts are all starch- or sugar-containing substrates that contribute fermentable carbohydrates. They also contribute flavor characteristics to produce distinctive varieties of beers. Although enzymes other than those in the malt and those contributed by the yeast are not needed to produce beer, some brewers use additional enzymes to impart desired characteristics to their product.

Malting

The main objective of malting is to produce an ample supply of enzymes that degrade starch, proteins, and other components of grain. The subsequent enzymatic changes provide fermentable sugars from starch and substances needed to support yeast growth (eg, amino acids and fatty acids) from the other substrates. Malt also is a major contributor to the final color and body characteristics of the finished beer. To produce malted barley, barley grains are first steeped in 10–15°C aerated water and then germinated at 15–20°C for 3–7 days. During this time the moisture content increases to approximately 45%. After the barley germinates, the sprouts are removed, leaving a medium rich with α-amylase, β-amylase, proteases, and their respective substrates. The malt is dried and kilned, under controlled conditions that remove the water without inactivating the desired enzymes, to approximately 5% moisture and ground. The flavor of the finished beer can be influenced by the amount of nonenzymatic browning and heat-generated flavor compounds that are formed during the drying process. Dark beers are made using darker, more flavorful malt while less roasted malt is used to brew paler beers. Grinding exposes the starchy endosperm of the grain, which makes the carbohydrates more available.

Mashing

During the mashing step, most of the nonsoluble, unfermentable carbohydrates and proteins are hydrolyzed into soluble fermentable materials, by the enzymes present in the malt. To accomplish this, the ground malt is mixed with water and placed into a mash tun. To enhance α-amylase action during the initial mashing period, the temperature of the mixture is maintained between 40 and 50°C. After a period of time, the temperature of the mix is increased to 65–70°C to enhance β-amylase activity. Within a few hours, the process is complete, and the temperature is increased to at least 75°C to inactivate the enzymes.

While the amylases are active, they degrade the starch contributed by the grain. Dextrins are produced by the action of the α-amylase. The β-amylase splits off the disaccharide, maltose, from the amylose portion of the starch. The products that result from

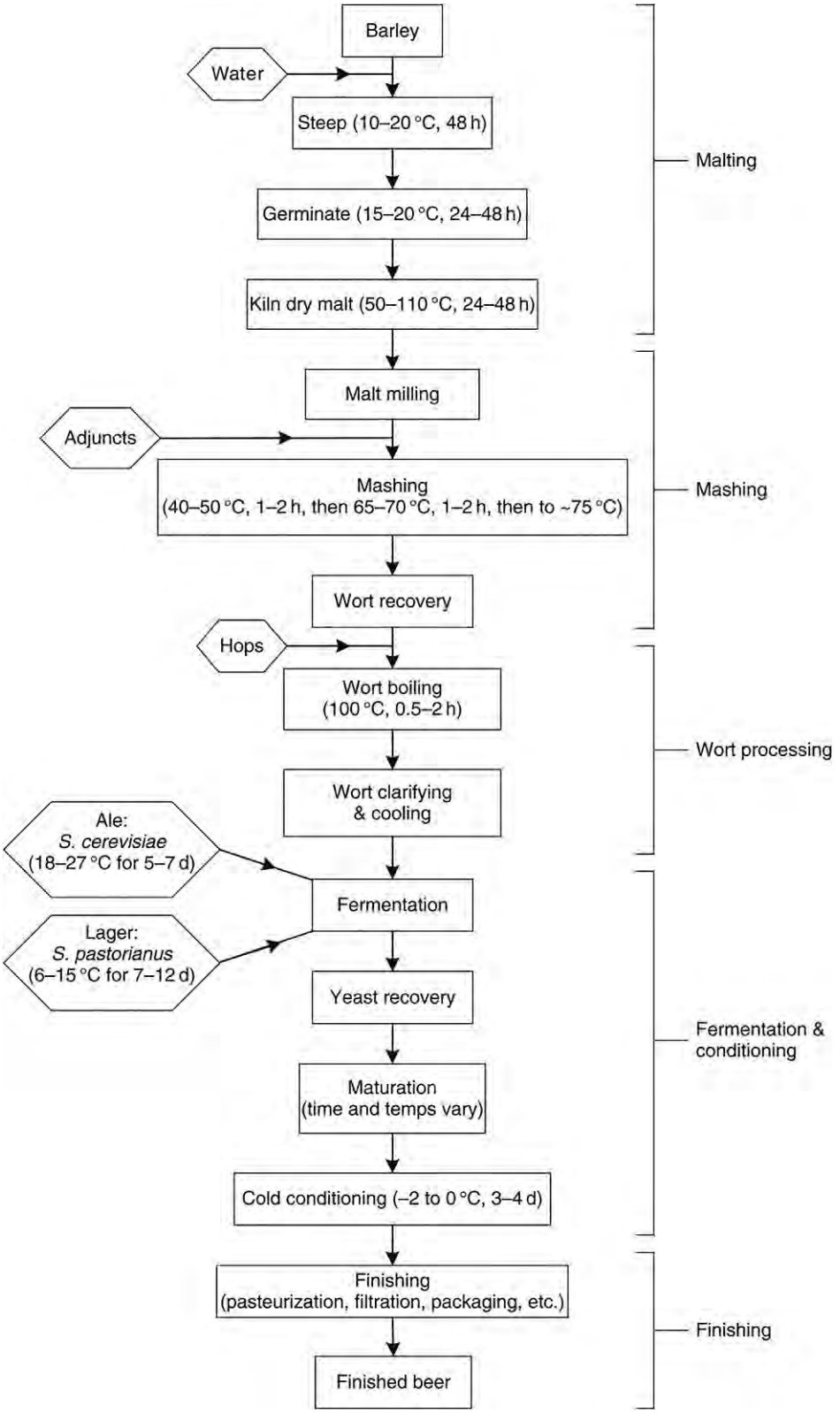

Fig. 1 Brewing process.

the action of the two amylases known as dextrins also undergo additional enzymatic changes. Branch linkages of the amylopectin portion of starch are broken by de-branching enzymes while amyloglucoside removes single glucose residues from the dextrins. Alterations in the color are also noted (changing from light to dark amber) during mashing.

The normal pH of malt is approximately 5.8 and is not acidic enough for optimum enzyme activity. To achieve optimum activity, the pH can be reduced to approximately 5.2 for lager production. The pH is adjusted to be more acidic for ale production. Adjustment of the acidity, if desired, can be accomplished by addition of acid, usually lactic acid, or by bacterial fermentation. Although the lactic acid bacteria usually are undesirable contaminants, *Lactobacillus delbrueckii* has been used to accomplish this pH reduction in the past. This thermophilic bacterium converts sugars to lactic acid efficiently at temperatures of 42–51°C. Because the variety of microorganisms that can grow at these temperatures is small, it is easier to maintain a pure culture bacterial fermentation as well as to reduce the possibility of contamination by other microbes. Processors may find, however, that the bacterial modification requires much greater supervision and, if not controlled, may contribute to beer spoilage.

After the naturally occurring and any added enzymes are inactivated, the solids settle out, leaving the wort. Wort contains the soluble compounds including the fermentable sugars and longer, nonfermentative oligosaccharides and is separated from the solids before it is transferred into the brew kettle. The spent grain can be used in animal feed. The blend of grains used and the degree of enzymatic activity will influence the composition of the wort. These differences are some of the factors contributing to the characteristics noted in beers from different breweries.

Wort Processing, Hop Addition, and Kettle Boil

After the wort is transferred into a heating tank, called the brew kettle, hops are added to the wort before the mixture is boiled. The boiling of the wort serves several functions: (1) nearly all the microorganisms remaining after mashing are killed, (2) inactivates enzymes remaining after mashing, (3) enhances extraction of essential oils and resins from the hops, (4) substances responsible for cloudiness are precipitated, (5) enhances color development, (6) undesirable volatiles are removed, and (7) water is evaporated and the wort is concentrated. The wort is boiled for 1–2 h.

Hops provide flavor and aroma compounds as well as some compounds, which possess antimicrobial activity. Bitterness is the principal flavor of note. Among the compounds extracted from the hops are essential oils humulone (α-bitter acid), lupulone (β-bitter acid), and tannin. Humulone and lupulone provide important flavor characteristics and also have some antimicrobial properties. The wort is then separated from the spent hops, cooled rapidly, and placed into a fermentation vessel. The spent hops may be used as fertilizer.

Fermentation

At this point in the process, the wort is inoculated with brewers' yeast and fermented. During fermentation, the yeast produces alcohol, carbon dioxide, and some additional flavor constituents. The inoculation step is also called by other names, such as pitching and seeding.

The fermentation room must be maintained in a clean manner to reduce possible contamination problems, and it should be kept at a constant temperature and humidity to maintain the desired growth rate for the yeast. Fermentation vats can be glass lined or constructed of wood, stainless steel, or aluminum. Wooden vats pose problems in cleaning and disinfecting that are not experienced with vessels made of any of the other materials.

The strain of yeast used depends on what type of beer is desired. Lagers are produced using bottom-fermenting yeasts, whereas ales are produced using top-fermenting yeasts. These yeasts have traditionally been referred to as *Saccharomyces pastorianus* (formerly *Saccharomyces carlsbergensis*) and *Saccharomyces cerevisiae*, respectively (Table 2).

The temperature of fermentation for lagers produced by the bottom-fermenting yeasts is usually in the range of 6–15°C and takes 2–7 days. During fermentation, the yeasts tend to flocculate and settle to the bottom of the fermentation vat. These yeasts can be collected from the bottom of the vat for reuse in subsequent fermentations. By varying the fermentation temperature, slightly different versions of lagers can be produced. The fermentation process is exothermic so the fermentation tanks must be cooled.

Ales are traditionally produced using top-fermenting yeasts and incubation temperatures of 18–27°C for 5–7 days. These yeasts tend to form small clumps of cells that are carried to the top of the fermenting liquid and adsorbed to bubbles of carbon dioxide. These yeast cells can be collected from the surface for reuse with the next fermentation batch.

Table 2 Comparison of *Saccharomyces cerevisiae* used to make ales and *Saccharomyces pastorianus* used to make lager yeasts

Characteristic	Saccharomyces cerevisiae	Saccharomyces pastorianus
Flocculation	Clumps or flocs entrap CO_2; lower density floc; floc rises to the surface of the liquid in the fermenter	Higher density floc; floc settles to the bottom of the fermenter
Minimum growth temperature	15°C	7°C
Optimum growth temperature	>30°C	<30°C
Maximum growth temperature	40°C	34°C
Ability to sporulate	Some	No

Since the mid-1800s, the use of bottom-fermenting yeasts in closed fermentation vessels has increased worldwide. A corresponding decrease has occurred for top-fermenting yeasts in open fermentation vessels. In recent times the advantages of the closed fermenter have given rise to "bottom-fermenting" ale strains. These strains maintain the ale flavor characteristics and can be harvested from the bottom, eliminating the need for an open vessel and the risk of contamination.

Regardless of the type of beer made, a rapid decrease in pH during the fermentation will increase its stability and decrease potential problems of contamination. After fermentation, the pH of most lagers decreases from approximately 5.2–5.3 to approximately 4.1–4.2, and it decreases slightly more in ales. These acidic pHs assist in preserving the final product by inhibiting bacterial growth.

Maturation and Conditioning

At the end of fermentation, the "green beer" is separated from the sediment and transferred to wooden (eg, oak) or glass-lined steel tanks. In traditional brewing, maturation of the beer occurs during storage at 0–2°C for several weeks. Lagers are normally aged for slightly longer periods of time than ales. This step allows the beer to develop its final flavor, color, and body characteristics. Many brewers include a short conditioning period after fermentation. Conditioning can be done at temperatures of approximately −1°C for at least 3 days. Clarification occurs to some extent as the yeasts, unstable proteins, and other suspended solids precipitate. Chemical changes occur, which create a more mellow and smooth flavor.

Finishing

Next, beer undergoes several processing steps in preparation for distribution to the consumer. Some of these steps are optional, and some of the steps can be done by a variety of methods. The choice of the brewer depends on what type of finished product is desired. Most beers will be chill-proofed by the addition of proteases to prevent haze development by residual proteins when the product is kept at refrigerated temperatures. Most will also be clarified and filtered to remove remaining solids. The final carbon dioxide level will be adjusted to 0.45–0.52%. The most common carbonation method is to add carbon dioxide back into the product. An alternative way is to add freshly yeasted wort to the beer and allow a natural secondary fermentation. Packaging for beer includes cans, bottles, barrels, and kegs.

To increase the shelf life of canned or bottled beer, it is usually pasteurized. The majority of beer in the past several decades has received heat pasteurization (eg, 62°C for up to 20 min). Beer flavor can be adversely affected if the product is overheated. Thus, there has been and continues to be an interest in using an alternative pasteurization method. One alternative is to flash pasteurize the beer at 71–75°C for 15–30 s before aseptically filling containers. "Cold pasteurization" offers some advantages, such as less flavor loss due to heating and better energy efficiency. Cold pasteurization involves the use of chemical agents for preservation or filtration ("cold filtration") through membrane filters followed by aseptic packaging. Beer packaged in barrels or kegs may or may not be pasteurized. If it is not pasteurized, it must be chilled and stored under refrigeration temperatures to maintain maximum quality. Typical shelf life is approximately 1 month. If heat- or filter-sterilized, then the shelf life can be up to 3 months.

Beer Properties

A myriad of beers are produced by different brewers. Properly brewed beers have flavor, color, and body characteristics dictated by the ingredients and yeast strain used, wort composition, and conditions for fermentation, aging, and finishing. Among the metabolites produced are ethanol, carbon dioxide, ethyl acetate, other esters, fusel alcohols, diacetyl, 2,3-pentanedione, various sulfur compounds, and amino acids and nucleotides from yeast cells. The type and proportion of these and other compounds in beer contribute the characteristics to a particular brew.

Beers also possess factors that aid in preservation of the product. Properly fermented and packaged beer has alcohol produced during fermentation, a relatively low pH, and a low redox potential, which inhibit a variety of spoilage microorganisms. Some compounds extracted from hops are inhibitory to Gram-positive bacteria.

Acetic acid bacteria, lactic acid bacteria, coliforms, and wild yeasts often contaminate improperly processed beers. Growth of most contaminating microbes in beer is inhibited by the preservation factors associated with the product. For example, the anaerobic environment within the packaged beer inhibits the growth of the aerobic acetic acid bacteria. Coliforms are inhibited when the pH level of the beer is 4.3 or lower.

Properties of Brewing Yeasts

Saccharomyces Characteristics

The genus *Saccharomyces* is composed of ascosporogenous yeasts that produce ovoid, spherical, or elongate cells. *Saccharomyces* strains used in commercial brewing are polyploid species. Reproduction by vegetative means occurs by multilateral budding, whereas sexual reproduction involves the formation of an ascus containing one to four spores. Members of this genus ferment sugars vigorously. Those strains that can yield significant amounts of ethanol by this metabolism are useful commercially.

This genus is a rather diverse group. Some of the growth characteristics for the species *S. cerevisiae* include minimum a_w for growth of 0.90; minimum, optimum, and maximum temperatures for growth of 0–7, 20–30, and 40°C, respectively, and minimum and optimum pH for growth of 2.0–2.4 and 4.0–5.0, respectively.

The taxonomic classification of the yeasts commonly referred to as brewers' yeasts has been debated for many years. According to the most recent taxonomic references, both bottom yeasts and top yeasts are members of the genus *Saccharomyces*. It is estimated that there are probably more than 1000 strains of *Saccharomyces*. Although the differences between strains are usually minor, they may be important to individual brewers. In older literature, the bottom yeasts were widely recognized as *S. carlsbergensis* and later as *S. carlsbergensis* (*uvarum*) or *Saccharomyces uvarum*. Today the species is known as *S. pastorianus*. The old names are still part of the terminology of the brewing industry. Top yeasts are referred to as *S. cerevisiae*.

Factors Affecting Growth of Brewing Yeasts

During brewing, the goal is to maximize the production of desired metabolites of yeast growth while minimizing production of undesirable compounds and yeast biomass. By properly controlling various factors that influence yeast behavior, the brewer can achieve the desired end product. Factors that influence yeast metabolism include wort composition, oxygen level in the wort, inoculation rate, condition of yeast at pitching, the level of microbial contamination, wort pH, and temperature during fermentation. In addition, fermentation can be influenced by the fermenter design and whether a batch or continuous process is used. For example, there is evidence that continuous fermentation may be faster than batch fermentation and may reduce problems associated with handling the yeast culture.

Yeasts growing in batch fermentations use the individual sugars in wort in a sequential order. Sucrose is the first used, followed by glucose, fructose, maltose, and maltotriose, although not all strains can fully use maltotriose efficiently. Ethanol and flavor compounds are among the compounds produced by carbohydrate metabolism. This metabolism also provides the energy to maintain yeast growth. Yeasts also utilize the 19 amino acids normally present in wort in an orderly fashion. The metabolism of the amino acids is needed to maintain yeast nutrition and to contribute flavor to the product.

Strain Development

Throughout the centuries, brewers have searched for yeast strains that will provide the best quality beer in an efficient manner. In the past, this has often been done by screening for the most suitable strain by trial and error or by forming hybrids of two strains with different desirable qualities and screening for a progeny strain with the desired capabilities. Not only are these methods time-consuming, but also the resulting yeast may have both desirable and undesirable qualities from both parental strains. For example, substrate utilization may be improved, but flavor traits may be adversely affected. Recently, use of molecular methods such as recombinant DNA techniques to improve yeast strains has become an option. The advantage of using the molecular methods is that alterations of single characteristics can be accomplished specifically.

The brewing industry is interested in modifying yeast behavior to accomplish several goals, including increasing the efficiency of fermentation, by modifying the uptake and metabolism of wort components, improving the control of fermentation (eg, the timing of late fermentation or yeast flocculation properties), and overall organoleptic quality of beer, developing new beers, and improving the value of by-products (eg, spent yeasts).

Modification of some of these traits using molecular methods has been investigated with some success. Although some of these altered strains have been developed experimentally, currently there are few, if any, used in full-scale commercial operations. In an effort to produce "light" beers less expensively, strains have been developed that ferment more of the carbohydrates present in wort not fermentable by the normal strains. This alleviates the expense of using added enzymes to degrade these carbohydrates.

A second modification that has been accomplished concerns the degradation of α-glucan. This substance causes filtration problems and forms precipitates and hazes in beer. Since use of genetically developed yeast can solve this problem, the expense of using an added enzyme to do the job is eliminated.

Strains with improved proteolytic capabilities have also been developed. These strains reduce the need to use added proteases to degrade the proteins in beer that are responsible for the haze that can develop when finished beer is refrigerated.

Spoilage Problems of Beer

Range of Problems

Concern regarding the transmission of pathogens via beer is rare. In this aspect, beer is unlike most other food and beverage items. The lack of pathogen-related problems is due to the nature of the ingredients, processing methods, and characteristics of the final product.

Spoilage problems, however, can occur. A diverse group of microorganisms can present problems at various stages of brewing (Table 3). The type of spoilage and the microorganisms responsible are influenced by the stage of processing and the characteristics related to the stage. Characteristics that affect spoilage include pH, alcohol content, the types of ingredients, the oxygen level of the product at particular stages, and the level of sanitation within the brewing environment. Wort just prior to fermentation is a rich medium for supporting microbial growth and is extremely susceptible to spoilage.

Table 3 Spoilage problems in beer and the causative microorganisms

Microorganism	*Stage of processing encountered*	*Problem*
Lactobacillus spp.	Any stage	Off-flavors Haze Ropiness Acidification
Pediococcus spp.	Inoculation Fermentation Aging	Off-flavors Haze Ropiness Acidification
Acetic acid bacteria	Open fermenters Packaging	Off-flavors Haze Surface pellicles Ropiness
Hafnia protea	Wort Fermentation	Off-flavors Acidification
Other Enterobacteriaceae	Wort	Off-flavors
Bacillus spp.	Wort	Off-flavors
Zymomonas spp.	Packaging	Off-flavors
Pectinatus spp.	Packaging	Off-flavors
Megasphaera spp.	Bottled beers with pH >4.1 and <3.5% ethanol	Off-flavors
Wild yeasts	All stages	Off-flavors Haze
Killer yeasts	Fermentation	Death of desired strain Off-flavors
Molds	Raw barley spoilage	Gushing Off-flavors

Bacterial Spoilage

Lactic acid bacteria

Species of *Lactobacillus* are among the most frequent and troublesome spoilage microorganisms of beer. They can cause spoilage problems at all stages of processing and even in the finished product. The lactobacilli can tolerate hop substances that are inhibitory to many other Gram-positive bacteria. Several species can spoil beer. Among these are those that grow relatively fast during the fermentation step, *Lactobacillus plantarum* and *Lactobacillus brevis*, and those that grow slower, *Lactobacillus lindneri*. A variety of flavor and appearance defects can be produced depending on the metabolic products that the particular spoilage organism produces. For example, *L. brevis* can produce acetic acid in addition to CO_2, ethanol, and lactic acid. Other lactobacilli species that have been noted as problems in beer include *Lactobacillus casei*, and *L. delbrueckii*.

Several lactic acid bacteria species can produce diacetyl in beer. Diacetyl is a chemical that produces a buttery flavor that is undesirable in beer. In addition to the diacetyl-producing *Lactobacillus* species, *Pediococcus acidilactici*, *Pediococcus inopinatus*, and *Pediococcus damnosus* are responsible for this defect. *P. damnosus* is probably the most notable of these and is a continuing concern for brewers. This lactic acid bacterium, like the lactobacilli, is resistant to the inhibitory substances in hops. Some strains of this species can tolerate up to 10% (w/v) ethanol.

Acetic acid bacteria

Species of *Acetobacter* and *Gluconobacter* can spoil beer primarily by converting ethanol to acetic acid. These bacteria are aerobic and are usually only associated with spoilage of beer exposed to air. The increased use of anaerobic conditions in modern breweries has decreased the frequency of problems related to these microorganisms.

Enterobacteriaceae

Although conditions suitable to support growth of most members of the Enterobacteriaceae are relatively few during the processing of beer, there are opportunities for some species to pose problems. The main concern is spoilage of wort before the decrease in pH and accumulation of ethanol that occurs during fermentation. Most enterics stop growing during early fermentation when the pH of wort decreases to <4.4 and when the ethanol content becomes >2% (w/v). Species of *Escherichia*, *Aerobacter*, *Klebsiella*, *Citrobacter*, and *Obesumbacterium* have been reported to cause spoilage problems.

Sporeformers

Although *Clostridium* species may be present on the raw ingredients of beer, these sporeforming microorganisms present no serious spoilage problems in the liquid phase once production commences. Spoilage of spent grains by *Clostridium* has been reported.

Bacillus species, however, have been encountered in spoiled beer. Since most, if not all, of the species within this genus are sensitive to hop components, the spoilage bacilli tend to be more problematic in the stages before the addition of hops to the wort.

Anaerobic gram negatives

Species of three genera of anaerobic, Gram-negative bacteria have been recognized as spoilers. *Zymomonas* was first described in the 1930s in Britain. The occurrence of spoilage problems due to this bacterium elsewhere in the world is rare. This organism produces hydrogen sulfide and acetaldehyde in beer.

Pectinatus and *Megasphaera* species were both described for the first time in the late 1970s. Both are unique to the brewing industry. *Pectinatus* species produce acetic acid, propionic acid, and hydrogen sulfide in beer, whereas species of *Megasphaera* produce primarily fatty acids and hydrogen sulfide.

Other bacteria

Species of *Achromobacter*, *Alcaligenes*, *Flavobacterium*, *Acinetobacter*, and *Pseudomonas* are some of the other bacteria that have been associated with infrequent cases of beer spoilage. Many of these miscellaneous spoilage organisms are aerobic, intolerant of acid environments, and die once fermentation starts. Thus, the problems they can cause are limited to the mash and early wort stages.

Fungal Spoilage

Wild yeasts

Wild yeasts include any yeasts that are present in the brewing process that were not introduced purposely or tolerated for specific purposes in the development of the desired product. There are probably more than 40 species that could be considered within this group. Commonly encountered genera include *Brettanomyces*, *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Torulaspora*, *Zygosaccharomyces*, and other *Saccharomyces* species. Problems due to wild yeasts include the production of undesired flavor compounds, including esters, acidic and phenolic substances, and fatty acids. They may also ferment the product beyond the desired end point, due to their ability to ferment carbohydrates, that the desired brewing yeast cannot utilize. Detection and identification of wild yeasts are important to brewers if they are to recognize the problem and take corrective action. Identification methods vary from biochemical classification to genetic methods, including DNA fingerprinting and gene probes.

Killer yeasts

Killer yeasts can produce a toxic protein, or zymocide, that adversely affects the plasma membrane of other yeast species or strains. Members of several yeast genera are capable of producing this compound, including some *Saccharomyces* species. The major problem is the death of the desired fermentative yeast strains caused by the toxic protein. Thus, the product is not properly fermented. It has often been noted that killer yeasts can also produce phenolic off-flavors. Spoilage problems due to killer yeasts can occur in either batch or continuous fermentations but tend to be more common with the continuous process. This may be due to the slightly lower pH encountered in the early stages of continuous fermentation.

Molds

Mold contamination of barley is common by a variety of species; thus, it is important to handle barley properly to prevent spoilage prior to malting. Barley spoiled by *Fusarium* can adversely affect the malting process and can cause a problem known as "gushing" in the finished product. Gushing is the sudden release of carbon dioxide when a bottle or can containing beer made from mold-spoiled barley is opened.

Nonmicrobial Chemical and Physical Problems

Skunky and stale beer are two chemical-related problems that can occur. Sunlight-induced photooxidation can result in skunky beer which is most noticeable in clear glass bottled beer. This problem can be avoided (or skunk-proofed) by using reduced forms of iso-α-acids from hop extracts. These compounds are more light-stable and nonreactive. Stale beer is a problem that develops over time during storage. The best way to avoid this problem is to limit the amount of oxygen present in the stored product. Shelf life of the finished product is determined largely by the onset time of staleness.

Haze and foam instability are two physical problems that can occur. Proteinase treatment can reduce the haze problem due to proteins, while β-glucanases degrade β-glucan-containing polysaccharides. Foam stability can be improved by increasing the viscosity and surface tension by adding proteins, gums, and hop components.

Spoilage Control

Controlling spoilage problems in the brewing industry is largely a matter of using the proper sanitation practices. Maintaining a clean, sanitized brewing environment will greatly reduce potential problems related to most of the environmental contaminants. Use of good quality water not only in the beer but also during cleaning and sanitizing is critical.

Using the proper processing practices is also important. Care must be taken to maintain the purity of the yeast strain or strains used by a brewer. The brewer should ensure that the raw ingredients are of good quality and that the processing steps proceed as

expected. It is also important for a brewer to be able to identify contamination problems early, so corrective action can be taken and damage minimized.

Other Types of Beer Processing

African Beers

The native beers of Africa have different ingredients and are brewed differently than the typical European or American beers. Rather than using barley in the malting step, sorghum, millet, or maize are used depending on the geographic location within Africa and the customary practice. Brewing in Africa originated in the home for personal or ceremonial uses, although it later expanded into commercial production. Fermentation of these beers is a two-stage process. First, lactic acid fermentation softens the malt proteins, reduces the pH sufficiently to limit growth of bacterial pathogens, regulates starch conversion to sugar, and contributes to the body of the beer. The second step is yeast fermentation that is initiated just prior to packaging the product. During distribution, there is an active yeast fermentation.

The finished product is unhopped with sour yogurt-like taste, has a pinkish-brown color, and normally contains 2 or 3% alcohol. It is opaque due to the high concentration of suspended solids and yeast cells. This product is actually thought of as a food rather than a beverage. These beers are highly susceptible to spoilage and have a shelf life that is usually less than 5 days. This limited shelf life is due to the wort not being boiled coupled with the fact that the final product characteristics (eg, pH and alcohol content) are not sufficient to limit the growth of acid-tolerant spoilage bacteria.

Home Brewing

It is possible to brew beer at home that may be of similar quality to that of commercially produced beers. Many supply stores sell the necessary ingredients and equipment, although normal kitchen utensils can be used for some of the needs. The processing steps that are used in home brewing are basically the same as those used commercially. There are naturally some limitations to home brewing that the commercial breweries are able to overcome. For example, in home brewing, excess yeast is usually not removed to the same degree and the storage temperature is usually not controlled as well to limit extended fermentation that can occur after packaging. Several books describe home brewing in detail.

Light Beer

Light beers, which were introduced to the market in the 1970s, are made by reducing the unfermentable dextrin content in wort before fermentation occurs. The enzyme glucoamylase is added to the wort to hydrolyze most of the dextrins to glucose. During fermentation the yeasts are able to ferment the glucose to alcohol. Thus, the amount of carbohydrates present that could contribute to the caloric content of the product is decreased, typically by approximately one-third. Simply watering-down regularly brewed beer would reduce the alcohol and caloric content of the beer, but the resulting product would be undesirable due to the diluted flavor and texture components.

Ice Beer

In the 1990s, ice beers attained a degree of popularity. In making ice beers, the fermented beer is chilled down to $-4^{\circ}C$ to allow for the formation of ice crystals. Removing the crystals reduces the water content and results in a slight increase in the sweetness and alcohol concentration of the finished product.

Dry Beer

Like ice beer, dry beer also became somewhat popular in the 1990s. Dry beer is less sweet than conventionally brewed beer and has less flavor, color, and body. It is made by using less malt and more readily fermentable adjuncts.

Nonalcoholic Beer

Nonalcoholic beers must contain less than 0.5% ethanol. In producing these beverages, ethanol is removed from fermented beer. Various methods are available including distillation, evaporation, dialysis, or reverse osmosis. Using the last two options that are done at low temperatures (less than $10^{\circ}C$) results in the retention of desirable flavor volatiles without the formation of undesirable heat-generated off-flavors.

Wheat Beer

By combining wheat malt with barley malt, a beer that has a thinner body and is more sour in taste than conventionally brewed beers can be made. Wheat beers have a higher phenolic content and is often cloudy in appearance.

Gluten-free Beer

Due to dietary restrictions of those affected by Celiac disease, gluten-free beer has become an option for those who cannot tolerate the protein. Gluten-free beer can be produced by either sourcing gluten-free sugar or starch or by modification of gluten-containing sources. Enzymatic modification is necessary when attempting to make gluten-free beer with normal brewing ingredients. One such way is with the use of a proline-specific endoprotease such as Brewer's Clarex. Such a technology will cleave the immunoreactive epitopes of gluten so as to cause no reaction in the body.

Sour Beer

The term "sour beer" is a blanket term used to describe many types of fermentations and styles. The history of brewing before the isolation of brewing yeast strains and the advent of sanitation is vast with wild and spontaneously fermented beers; most of which had sour qualities. In fact, many classic styles such as Gose, Berliner Weisse, Belgian Oude Bruin, Flanders Red, and Lambic beers contained bacterial and wild yeast qualities [1]. In modern craft brewing, brewers have implemented a variety of methods to achieve flavorful, sought after acidity in their final product.

Lactic acid producing *Lactobacillus* is one of the most common microbes used to sour beer. Its beer spoiling capabilities have been previously mentioned, but used by a skilled brewer, *Lactobacillus* can yield desirable acidity. The quickest use of *Lactobacillus* to gain acidity is in the brewhouse via wort kettle souring. To utilize this method, pre-boil non-hopped wort is kept at a temperature optimal to the *Lactobacillus* strain the brewer chooses to use. Total acidity is monitored by the brewer until the desired acidity is achieved. After the preferred sourness level is reached, wort is boiled with hops added, killing the *Lactobacillus*, and normal beer production commences.

Most sour beer programs involve secondary fermentation due to the yeast *Brettanomyces* or by bacteria such as *Lactobacillus*, *Pediococcus*, and *Acetobacter*. Where *Lactobacillus*, *Pediococcus*, and *Acetobacter* fermentations produce the common acidic notes, flavors derived from *Brettanomyces* fermentation are usually characterized as "funky," with barnyard, earthy, and musty notes. All of these microbes can be inoculated in wooden barrels or tanks post primary fermentation and then blended after aging to produce the desired product.

Some modern brewers use *Brettanomyces* in place of *Saccharomyces* for primary fermentation. When used, a large pitch of the yeast is needed in order for completion of fermentation in a normal production period. Higher levels of aeration will lead to higher production of acetic acid, but lactic acid will not be produced.

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Biocides

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Introduction

Biocides have been extensively used in the control of bacteria for decades, and are commonly incorporated into a variety of products including disinfectant formulations, cosmetics, preservatives, pesticides and antiseptics (McDonnell and Russell, 1999; Paulus, 2012). The Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998, concerning the placing of biocidal products on the market, provides a harmonized definition for biocides. Accordingly, biocides are classified as active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user. These are intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means. However, the term biocide is commonly used as synonym of antimicrobial agent or disinfectant/sanitizers. Gilbert and McBain (2003) differentiated and defined the three independently: Biocides (active substances that above certain concentrations and defined conditions will kill cells within specified times); Antimicrobial agents (active substances that have adverse effects on the growth or survival of microorganisms); Disinfectants/sanitizers (formulations containing active substances that are safe for the application to inanimate surfaces and which kill specified groups of disease-producing microorganisms within specified times). The Directive 98/8/EC furthermore states that a biocidal product should obey to the following characteristics: (i) Sufficiently effective with no unacceptable effects on the target organisms (i.e., resistance or cross-resistance); (ii) no unacceptable effects itself or as a result of its residues, on human or animal health, directly or indirectly (i.e., through drinking water, food or feed, indoor air or consequences in the place of work) or on surface water and groundwater; (iii) no unacceptable environmental effect itself, or as a result of its residues (i.e., its fate and distribution in the environment; particularly contamination of surface waters, groundwater and drinking water; its impact on non-target organisms); (iv) its physical and chemical properties have been determined and deemed acceptable for purposes of the appropriate use, storage and transport of the product. In terms of biocide diversity, chemical classification and possible applications, the books of Wypych and Wypych (2015), Rossmoore (1995), Paulus (2012), and the Russell, Hugo and Ayliffe's Principles and Practice of Disinfection, Preservation and Sterilization book (Fraise et al., 2013) provide an excellent amount of information.

The excessive use of biocides has considerable environmental and economic impacts and the misuse of more aggressive biocides and increased doses (as a way to overcome the resistance phenomena) constitutes an extra risk to public health. These measures can lead to the selection of pathogens insusceptible to the main available antimicrobials (Russell, 2004). Antimicrobial resistance is even more significant when cells are embedded in a biofilm. Therefore, novel biocides are required for effective disinfection. Phytochemicals (molecules from the plant secondary metabolism) are an unexploited source of novel biocides (Gomes et al., 2016; Malheiro et al., 2016). This chapter describe major biocides in current disinfection practices, with particular emphasis on industrial disinfection, and provides information on the role of phytochemical as a promising source of novel biocides.

Cleaning and Disinfection

The operations of cleaning and disinfection are essential parts of any process affected by microbial contaminations (Carpentier and Cerf, 1993). For instance, on industrial food processing equipment cleaning is an important stage for minimizing microbial colonization (Carpentier and Cerf, 1993; Meireles et al., 2016; Simões et al., 2010). It seems to be of fundamental importance to eliminate as many microorganisms as possible before applying a disinfectant. In the industry, cleaning procedures should effectively remove food debris and other soils that may contain microorganisms or promote microbial growth. Most cleaning regimes include removal of loose soil with cold or warm water followed by the application of chemical agents, rinsing, and disinfection. High temperatures can reduce the need for physical force (Carpentier and Cerf, 1993; Maukonen et al., 2003). Chemical agents, usually surface active agents or alkali compounds, used as detergents, suspend and dissolve food residues by decreasing surface tension, emulsifying fats, and denaturing proteins (Maukonen et al., 2003). These chemical agents are currently used in combination. Many situations, require the occasional use of acid cleaners to clean surfaces soiled with precipitated minerals or having high food residue/mineral content. Mechanical action, particularly water turbulence and scrubbing, are recognized as being highly effective in removing biofilms (Chmielewski and Frank, 2003; Maukonen et al., 2003). An effective cleaning procedure must break up or dissolve the biofilm extracellular polymeric matrix so that the biocide can gain access to the viable cells (Carpentier and Cerf, 1993; Gibson et al., 1999).

The cleaning process can remove around 90% of the surface colonizing microorganisms, but cannot be relied upon to kill them. Bacteria can redeposit at other locations and, given time, water and nutrients can form a new biofilm. Therefore, disinfection must be implemented (Gibson et al., 1999).

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Disinfection is the use of products (disinfectants) to destroy microorganisms. Nevertheless, microorganisms have been found in disinfectant solutions, which is due to their ability to acquire resistance and build-up protective biofilms (Simões et al., 2011). This means that microbial contamination can be spread on the surface to be disinfected instead of being disinfected.

In food plant operations, disinfection is required, since wet surfaces provide favourable conditions for the growth of microorganisms (Maukonen et al., 2003; Meireles et al., 2016). The aim of disinfection is to reduce the surface population of viable cells after cleaning and prevent microbial growth on surfaces before restart of production. Disinfectants do not penetrate the biofilm matrix left on a surface after an ineffective cleaning procedure, and thus do not destroy all the living cells in biofilms (Carpentier and Cerf, 1993). Disinfectants are more effective in the absence of organic material (fat, carbohydrates, and protein based materials). Interfering organic substances, pH, temperature, water hardness, chemical inhibitors, concentration and contact time generally control the efficacy of biocides (Cloete et al., 1998; Mosteller and Bishop, 1993). The biocides must be effective, safe and easy to use, and easily rinsed off from surfaces, leaving no toxic residues that affect the sensory values of the product. The use of biocides in food plants depends on the material used and the adhering microorganisms. The biocides to be used should be chosen based on the following statements (Troller, 1993; Wirtanen, 1995; Wirtanen et al., 2000):

- Is effective in the pH range used?
- Is stable when diluted?
- Is toxic, safe or irritating?
- What is the microbial spectrum?
- How does the temperature affect the activity?
- Is corrosive at the surface?
- Is surface active?
- Is stable when reacting with organic material?

Table 1 resumes the properties of disinfectants commonly used in industrial systems.

Table 1 Properties and uses of biocides

Type	*Applications*	*Limitations*	*Comments*
Aldehydes	Neutral/alkaline conditions	Slower, less active under acid conditions	Broad spectrum kill
	Non-corrosive to all materials	Absorbed by porous materials	Toxicity at high concentrations
	Water treatment/slime control in can/ bottle warmers, tunnel pasteurizers		
	Glycol and sweetwater systems in dairies		
	Conveyor lines		
Amphoteric surfactants	Neutral/alkaline conditions	Reduced activity under acid conditions	No odour/taste carryover
	Applicable to all materials	Selective germicidal activity	Low toxicity
	Food contact surfaces	Residual film affects cheese starter cultures	Non-irritating
	Environmental areas	Used at high concentrations	
	Spray, manual soak	Moderate to high cost	
	Fog air	Moderate to high foam	
	Foam is suitable for external surface disinfection		
Anionic surfactants at acid conditions	Acid conditions, $<$ pH 3	Effective only at low pH	Moderate to high foam production
	Carbon dioxide atmosphere	Neutralized by detergent residues	Stable at high temperature
	Stainless steel, plastics	Corrosive to soft metals, mild steel	No odour/taste carryover
	Foam on external surfaces	Some products moderate to high form upon circulation	More costly than halogens
	CIP, spray, soak, manual		
	Carbon dioxide atmosphere		
	Overnight disinfection		
	Milkstone/beerstone removal		
Chlorine	Neutral/alkaline conditions	Acid conditions	Broad spectrum kill
	Stainless steel	High temperature	Organic Cl less corrosive
	Food contact surfaces	Soft metal; mild steel	Taste/odour carryover
	Floors/walls/air	Vapor phase corrosion	Irritates eyes, skin, etc.
	CIP, spray, soak, fog		Environmentally unfriendly
			Cheap
Chlorine dioxide	Water treatment/slime/odour control	Stabilized form slower and less active under alkaline/neutral conditions	Acidified form is broad spectrum

(*Continued*)

Table 1 Continued

Type	*Applications*	*Limitations*	*Comments*
	Rinse for fruit/vegetables	More active acid form requires activating acid and mixing step	Does not form chloramines or trihalomethanes
	Acid form on food contact surfaces	More expensive than chlorine	Uncertain corrosion properties
	Stainless steel	Hazards of generating gas	
	CIP, spray, soak	Irritates human tissues	
Hydrogen peroxide	Applicable to all materials	Weak germicidal properties	Safe decomposition products, water and oxygen
	Sporicide at high concentration at high temperature	Efficacy at high temperatures/high concentration	
	Aseptic packing of beverages	Destabilized by metal contaminants (copper, iron)	
Iodine	Acid conditions, $<$ pH 3	Less effective than chlorine	Broad spectrum kill
	Stainless steel, plastics	Activity declines rapidly above pH 5	Organic soil reduces efficacy
	Food contact surfaces	Corrosive to soft metals and mild steel	More costly than chlorine but effective at lower concentration
	Floors/walls	Stains certain plastics	
	CIP, spray, soak, manual	Potential taste/odour carryover	
	Hand disinfectant	Most foam upon circulation	
	Carbon dioxide atmosphere		
	Helps dissolve mineral deposits		
Isothiazolinones	Acid, alkaline, neutral conditions	Slowly active	Broad spectrum kill
	Applicable to all materials	Use on non-food contact surfaces	More active under acid conditions
	Cooling water/towers, can/bottle warmers		
	Long-term, continuous activity		
	Conveyor lubricants		
Peracetic acid	Acid conditions	Corrosive in presence of chlorine ions	Broad spectrum kill
	Carbon dioxide atmosphere	Concentrate causes burns, irritations	Safe decomposition products
	Stainless and mild steel, soft metals, plastic, rubber	Pungent odour	Concentrate should not contact mild steel, soft metals
	Food contact surfaces	Rapid decomposition at high temperatures, generates gas and heat	No phosphates;
	CIP, spray, soak	Rapid decomposition by metals, organic matter	Moderate cost
Phenolics	Lubricants for conveyor lines; Water treatment	Variable germicidal activity	
		Toxicity	
		Irritates body tissue	
		Readily absorbed by many materials	
		Unpleasant odours	
Polymeric biguanides	Acid/alkaline conditions	Limited activity	Activity reduced by organic soil
	Applicable to all materials	Incompatible with anionic surfactants	
	Food contact surfaces	Moderate to high cost	
	Environmental areas		
	Can/bottle warmers, water treatment		
	Spray, soak, manual, circulation		
	Fog air		
Quaternary ammonium compounds (cationic surfactants)	Neutral/alkaline conditions	Selective germicidal activity	Properties vary among different QAC´s
	Applicable to all materials	Residual fil affects cheese starter cultures	Effective for wetting and penetrating soils
	Food contact surfaces	Moderate to high foam upon circulation	Effective in presence of organic soils
	Environmental areas/residue can extend activity	Toxicity at high concentrations	Non-irritating
	Mildew and odour control	Neutralized by certain surfactants	Newer QAC´s have high water hardness tolerance
	Water treatment		
	Spray, soak, manual, circulation		

Note: Troller, J.A., 1993. Sanitation in Food Processing, Academic Press Inc. Banner, M.J., 1995. The selection of disinfectants for use in food hygiene. In: Rossmoore, H.W. (Ed.), Handbook of Biocide and Preservative Use. Springer, pp. 315–333. Wirtanen, G., 1995. Biofilm Formation and its Elimination from Food Processing Equipment. VTT Publications, p. 251.

Mode of Action of Biocides

The processes involved in the antimicrobial action comprise transportation of the antimicrobial agent to the surface of the cell, adsorption, diffusion, penetration and interaction at the target site. These processes are not instantaneous, the time they take, and the correspondent killing time may differ within antimicrobial agents. The differences also depend on the mode of action, as well as on the chemical constitution and chemical–physical properties of the biocide (Paulus, 2012).

An antimicrobial effect can be defined as an interaction between an active substance and specific targets of the microbial cell. Critical governing features of these interactions are the physicochemical characteristics of the biocide, cell morphology, and the physiology status of the microorganism (Denyer and Stewart, 1998).

Biocides derive from a variety of chemical classes (Table 1). The precise mechanisms of interaction often reflect this diversity, although the final damage outcomes may show considerable similarity. Typical antibacterial events include (Denyer and Stewart, 1998; Araújo et al., 2011):

- Disruption of the transmembrane proton motive force leading to an uncoupling of oxidative phosphorylation and inhibition of active transport across the membrane
- Inhibition of respiration or catabolic/anabolic reactions
- Disruption of replication
- Loss of membrane integrity resulting in leakage of essential intracellular constituents
- Lysis
- Coagulation of intracellular material

Factors Influencing the Efficacy of Biocides

The efficacy of the application of a disinfection procedure is the result of the correct application of an efficient biocide. That means that lack of efficacy may have multicausal explanations and resistance is only one of them. There are some reasons to cause loss of efficacy in a disinfection process which may be misunderstood as resistance. They can be summarized as follows (Bessems, 1998; Heinzel, 1998; Russell, 2003; Simões et al., 2010):

- Use of an inefficient product, i.e., a biocidet which has an incomplete spectrum of activity. By this way all microorganisms which are outside the range of product efficiency will survive and eventually develop resistance to the specific antimicrobial and to related or unrelated molecules (cross-resistance).
- Application of the product without regard to the correct conditions as recommended by the supplier. This concerns mainly concentration, pH, temperature, and time of application but also inactivation by organic matter or other products.
- The prolonged application of biocides at sublethal concentrations may provoke the adaptation of microorganisms to the biocide and possibly to others.
- Insufficient contact of the biocide with the microorganisms.
- Insufficient availability of the biocide.

All these circumstances may diminish the expected action of biocides.

Nevertheless, the main reasons for the failure of a disinfection procedure are linked to the development of microbial resistance. According to Gilbert and McBain (2003), resistance is a description of the relative insusceptibility of a microorganism to a particular treatment under a particular set of conditions. For biocides, it is usually quantified as the concentration that cause sublethal effects on the population cells. Besides the environmental factors, resistance to biocides can be a natural property of a microorganism (intrinsic) or acquired by mutation or acquisition of plasmids – self-replication, extrachromosomal DNA or transposons – chromosomal or plasmid integrating, transmissible DNA cassettes (Cloete, 2003; Gilbert and McBain, 2003; McDonnell and Russell, 1999; White and McDermott, 2001).

Mechanisms of Cellular Resistance to Biocides

Reduced Susceptibility Associated With Genotypic Changes (Acquired Mechanisms)

Reduced susceptibility of microorganisms to biocides may be acquired through mutation, or by the acquisition of a plasmid or transposon (Forbes et al., 2017; Møretrø et al., 2017). Chromosomal gene mutations conferring resistance to antibiotics are relatively well-studied (McDonnell and Russell, 1999; White and McDermott, 2001). By contrast, fewer studies have been performed to determine whether mutation confers resistance to biocides (McDonnell and Russell, 1999). In fact, the advances on this topic are modest (Forbes et al., 2017). The mutation that alters the target site of an antimicrobial agent, which acts at specific sites within the bacterial cell, is likely to cause resistance. These mechanisms of resistance are observed through the existence of bypass in the bacterial ribosome or a metabolic enzyme, or overproduction of the target enzyme or of an efflux pump (Beumer et al., 2000).

Acquired resistance arises *via* mutation or as result of the acquisition of genetic elements – plasmids, transposons (White and McDermott, 2001). Acquired, nonplasmid-encoded resistance may result when bacteria are trained to grow in gradually increasing

concentrations of a biocide, although, resistance is not always stable. Temporary resistance by phenotypic adaptation is known, but is considered that, in general, a nongenetic adaptative type of resistance is unlikely to play an important role in determining the long-term survival of bacteria to biocides (Russell, 2001).

Intrinsic Properties of Microorganisms Conferring Reduced Susceptibility

Intrinsic insusceptibility is a natural property of a microorganism and is shown by bacterial spores, mycobacteria, and several Gram-negative bacteria. Gram-negative bacteria are generally relatively less susceptible to antimicrobial agents than Gram-positive bacteria because their cell walls present a more significant barrier to entry (McDonnell and Russell, 1999). The outer membrane of Gram-negative bacteria acts as a permeable barrier because the narrow porin channels limit the penetration of hydrophobic molecules and the low fluidity of the lipopolysaccharides leaflet slows down the inward diffusion of lipophilic compounds (Cloete, 2003; McDonnell and Russell, 1999). Mycobateria, which possesses a waxy envelope that inhibits the uptake of some antimicrobial agents, are even more resistant (McDonnell and Russell, 1999). The coat and the cortex of bacterial spores present a barrier to the entry of antimicrobial agents, explaining their relatively extreme insusceptibility (Cloete, 2003; McDonnell and Russell, 1999).

When spores germinate, the biochemical and structural changes result in the germinating cells becoming more susceptible to the action of some biocides (Beumer et al., 2000). So, intrinsic microbial resistance is frequently associated with cellular impermeability imparted by the outer layers of a bacterial cell that limit the uptake of antimicrobial agents, although active efflux pumps appears to be an important process (Cloete, 2003; Gilbert and McBain, 2003; Russell, 2001). Efflux is increasingly implicated as a resistance mechanism. Efflux pumps contribute to the intrinsic resistance of Gram-negative bacteria by pumping out a variety of agents, including dyes, detergents and antibiotics (Cloete, 2003). Efflux pumps are recognized as common membrane components in all cell types, from prokaryotes to superior eukaryotes (Van Bambeke et al., 2003). It confers bacteria a common and basic mechanism of resistance by extruding toxic molecules (Cui, 2004). In the context of antibiotic resistance, the term multidrug resistance (MDR) is used to describe a situation where reduced susceptibility to an antibiotic is associated with reduced susceptibility to other chemically unrelated antibiotics through an efflux mechanism. There is evidence that *Pseudomonas aeruginosa* can efflux triclosan and that this represents an important intrinsic susceptibility to bisphenol (Chuanchuen et al., 2001). The presence of the efflux systems coupled with the narrow porin channels in the outer membrane of the cell which restricts diffusion of antimicrobial agents into the cells is responsible for the very high intrinsic resistance of Gram-negative bacteria to antimicrobial agents (Cloete, 2003).

As well as impaired uptake or increased efflux, some microorganisms demonstrate intrinsic resistance through the inactivation of antimicrobial agents (Beumer et al., 2000). Some bacteria can degrade antimicrobial agents (Gilbert and McBain, 2003; Nishihara et al., 2000) and this phenomenon does not appear to be any plasmid involvement, representing another example of intrinsic insusceptibility.

Physiological (phenotypic) adaptation of microorganisms that reduces susceptibility to antimicrobial agents in response to environmental changes is also considered as intrinsic resistance (McDonnell and Russell, 1999). The cell phenotype expressed can vary significantly depending on the environmental conditions under which it is grown (Russell, 2003; White and McDermott, 2001). The resistance of these microorganisms to antimicrobial agents may derive partly from changes in outer cell layers that increase the barrier properties and prevent access to their site of action, but other changes are also involved (Cloete, 2003; McDonnell and Russell, 1999; White and McDermott, 2001). The association of microorganisms with solid surfaces leads to the formation of a biofilm, with bacteria in different zones of the biofilm experiencing different nutrient environments and displaying different physiological properties (Brown and Gilbert, 1993; Gilbert et al., 1990; McDonnell and Russell, 1999). Reduced susceptibility of bacteria in biofilms to antimicrobials can sometimes be extreme and is probably caused by a variety of factors including nutrient depletion within the biofilm, reduced access of the antimicrobial agent to cells in the biofilm, chemical interaction between the chemical agent and the biofilm, and the production of degradative enzymes and neutralizing chemicals (Brown and Gilbert, 1993). Biofilms have been reported as possessing susceptibilities towards biocides and antibiotics that are 100–1000 times less than equivalent populations of planktonic bacteria (Simões, 2011).

Mechanisms of Biofilm Resistance

There is no one answer to the question of why and how bacteria growing in a biofilm develop increased resistance to antimicrobial agents. In the following statements it will be described some of the possible mechanisms accounting for the resistance of bacteria within biofilms.

Resistance and the Extracellular Polymeric Matrix

It has been suggested that the extracellular polymeric matrix, among other functions, prevents the access of biocides to the cells embedded in the biofilm community due to the presence of a charged, hydrated exopolymer matrix around individual cells and microcolonies (Mah and O'Toole, 2001). Restricted diffusion from the surrounding medium, by a combination of ionic interaction and molecular sieving events, may occur for appropriate classes of molecules (Costerton et al., 1987). The constituents of the biofilm matrix act as would an ion exchange resin and actively remove strongly charged molecules (Gilbert et al., 2002). Total penetration failure will only occur when the reaction sites are sufficient to deplete the bulk concentration of the biocide or

replenishment of the matrix proceeds at a faster rate than does adsorption/reaction and diffusion (Stewart et al., 1998). Diffusion limitation studies have generally focused on antibiotics rather than biocides and upon medically relevant biofilm populations rather than biofilms in industrial situations (Stewart, 1996).

In addition to the potential of the biofilm matrix to react directly and chemically quench reactive moieties, retention of enzymes with the capability to inactivate biocides within the biofilm matrix will amplify its barrier properties with respect to the diffusion of suitable substrates (Araújo et al., 2014; Heinzel, 1998).

Resistance Associated With Growth Rate and Nutrient Availability

When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to biocides (Lewis, 2001; Wentland et al., 1996). Because cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents (Mah and O'Toole, 2001; Stewart and Franklin, 2008). Desai et al. (1998) compared the resistance of planktonic and biofilm cells at different stages during exponential growth up to the entry into stationary phase. They found that resistance increased as the planktonic cultures and the biofilm cells approached stationary phase. The maximal resistance of both cultures occurred in stationary phase where the biofilm cells were about 15 times more resistant than the planktonic cells. These results suggested that some determinant other than growth rate is responsible for a certain level of resistance and slow growth adds additional protection (Fux et al., 2005).

Oxygen gradients within the biofilm may also directly influence the activity of some biocides (Gilbert et al., 2002). Another phenomenon is the existence of physiological gradients across biofilms on growth and metabolism of cells at the periphery, to consume nutrients before they permeate to the more deeply placed cells. The peripheral cells will have growth rates and nutrient profiles that are similar to those of planktonic cells, allowing for the existence of heterogeneity within biofilm. Advances in technology have resulted in the ability to visualize the heterogeneity within a biofilm (Fux et al., 2005). The environmental heterogeneity that exists within a biofilm might promote the formation of a heterogeneous population of cells, such different levels of resistance can be expressed throughout the community (Fux et al., 2005; Wentland et al., 1996). So, a major contributor towards the inefficacy of biocides when applied to biofilms must, therefore, be associated with physiological heterogeneity (Mah and O'Toole, 2001; Simões et al., 2011).

Resistance Associated With the Adoption of Resistance Phenotypes

Bacteria can sense the proximity of a surface, up-regulate production of EPS and rapidly alter their biocide susceptibility after binding. In some instances, 3–5 fold decreases in susceptibility occurred immediately on attachment in the presence of biocides that exceeded the minimum inhibitory concentration for planktonic cells (Fux et al., 2005). The magnitude of the decreases in susceptibility observed immediately after bacterial attachment, but before biofilm formation, is generally far less than that observed in mature biofilms and is insufficient to account for the reported levels of resistance in biofilm communities (Gilbert et al., 2002).

The microorganisms generate physiological changes that act to protect the cell from various environmental stresses. Thus, the cells are protected from the detrimental effects of heat shock, cold shock, changes in pH and many biocides. Nevertheless, the physiological changes begin when cells attach to a surface, by expressing a biofilm phenotype that can confer resistance face to environmental stress (Mah and O'Toole, 2001; Stewart et al., 2015). This resistant phenotype might be induced by nutrient limitation, certain types of stresses, high cell density, efflux of the treatment agent or a combination of these phenomena.

The role of quorum sensing in biocide is not yet clear. It has been suggested that regulation of EPS, under the control of signal molecules such as *N*-acyl homoserine lactones is responsible for the early transcriptional events associated with biofilm formation (Davies et al., 1998). Such global regulators are responsive to increase cell density beyond critical threshold values, and may be general regulators of biofilm-specific physiology (Davies et al., 1998). In biofilms, signal molecules would become concentrated within geometric centre of biofilm, thereby increasing EPS production. This would alter the distribution and density of cells throughout the matrix and confer some level of structural organization upon the community to provide customized microniches at various points within the biofilm (Gilbert et al., 2002). Brooun et al. (2000) showed that *P. aeruginosa* mutants defective in quorum sensing were unaffected in their resistance to biocides and antibiotics. Nevertheless, Mah and O'Toole (2001) suggested that additional experiments are required to elucidate the role of quorum sensing in antimicrobial resistance.

Sublethal concentrations of biocides might act as inducers/transcriptional activators of more tolerant phenotypes, such as those expressing the multidrug resistance operons and efflux pumps in *Escherichia coli* (Ma et al., 1993; Maira-Litran et al., 2000).

A novel hypothesis for the considerable recalcitrance of biofilm relates to the potential of damaged bacterial cells to undergo apoptosis or programmed cell death. Lewis (2000, 2001, 2010, 2017) suggested that death of cells following treatment with biocides results not from direct action of the agent but from a programmed suicide mechanism and cellular lysis. Following the absence of an adverse condition, the damaged persistent cells would grow rapidly in the presence of nutrients released from their lysed community partners and the community would become restored. These cells would survive treatment phases and proliferate in the post-treatment phase, thereby stimulating considerable recalcitrance upon the biofilm community. More recently, persister cells of *P. fluorescens* were found in biofilms exposed to *ortho*-phthalaldehyde, a biocide used for high-level disinfection (Simões et al., 2011).

In addition to the recognized resistance of biofilms there is a conspicuous absence of novel and effective control strategies. In the future, the use of novel biocides will even become more critical especially in Europe as a result of the Biocidal Product Directive (BPD) (Wypych and Wypych, 2015). The most recent Regulation (EU) No 528/2012 of the European Parliament and the Council (2012), concerning the making available on the market and use of biocidal products, outlines the important aspects of the use of biocidal products and governs principles of introduction of new product to the market. The BPD will have two major consequences:

(1) Decrease in number of biocidal actives available to customers. There are estimations that this number will decrease in Europe from 1500 to 2000 actives today to less than 300–500 actives in the future (Wypych and Wypych, 2015).
(2) Decline in new product introductions, because initial cost will be much higher and the return on investment will take longer.

There will be also an increasing pressure to substitute biocides which give rise to concerns regarding their toxicological or ecotoxicological properties. In parallel, microorganisms will continue acquiring resistance to antimicrobial agents, including to biocides. For example, Simões et al. (2006) found that a *Pseudomonas* strain exposed for 30 min to the quaternary ammonium compound cetyltrimethylammonium bromide expressed the OprM protein responsible for multiple drug resistance. Therefore, new information on the action and resistance of current biocides will enable their prudent use for effective disinfection.

A strategy of potential interest to replenish the biocide pipeline is the off-label use of chemical molecules currently applied in industrial products and process. Phytochemicals, currently used as food additives and food preservatives, are an under-exploited source of antimicrobial products (Cowan, 1999; Simões et al., 2009). Pine oil is probably the only plant-derived product already used in disinfection (Dellano et al., 2009). Details of structures and sources of many antimicrobial phytochemicals have been widely compiled, and evidence of the functions of these products has also been reviewed (Simões et al., 2009; Borges et al., 2016). It is clear that phytochemical products may inhibit bacterial growth by different mechanisms than the presently used biocides (Gomes et al,., 2016; Malheiro et al., 2016; Simões et al., 2009). In fact, there is an encouragement to replace the toxic biocides for more readily antimicrobial agents, like naturally-produced compounds, such as plant extracts that will be more easily biodegraded and, consequently, environmentally acceptable (Guiamet and De Saravia, 2005).

Phytochemical Compositions Used as Disinfectants

Plants synthetize a vast range of organic compounds that are traditionally classified as primary and secondary metabolites. Primary metabolites are compounds that have essential roles associated with photosynthesis, respiration, and growth and development. These include sugars, fatty acids, amino acids and nucleic acids (Kliebenstein and Osbourn, 2012; Theis and Lerdau, 2003). Secondary metabolites, also referred to as natural products or phytochemicals, are considered to be those chemicals required for plant interactions with the environment, as pest and pathogen defense compounds, attractants for pollinators and seed-dispersing animals, allelopathic agents and ultraviolet-B sunscreens. These are structurally diverse and many are distributed among a very limited number of species within the plant kingdom, many of which accumulate in surprisingly high concentrations in some species (Bourgaud et al., 2001; Kliebenstein and Osbourn, 2012).

The natural products derived from medical plants have proven to be an abundant source of biologically active compounds, many of which might indeed be potential source of new antimicrobial agents. Plant products have been used since ancient time for flavoring foods and beverages, and also for medicinal purposes with varying success to cure and prevent diseases. As there are approximately 250,000–500,000 plant species occurring worldwide, of which a small percentage has been phytochemically explored till date, there is great potential for discovering new bioactive compounds (Borris, 1996; Negi, 2012; Palombo, 2011). Plants are potential sources of natural bioactive compounds such as secondary metabolites and antioxidants. They adsorb the sun light and produce high levels of oxygen and secondary metabolites by photosynthesis (Ghasemzadeh and Ghasemzadeh, 2011). The antimicrobial activity found in plant extracts has been attributed to some of the secondary metabolites, normally referred to as phytochemicals, which are biosynthesized to protect the plant against microbial infections and other external stress conditions (Malheiro et al., 2016).

The proven effectiveness of several phytochemicals, generally with low cytotoxicity, environmentally friendly, degradable and affordable, against microorganisms makes them a rich source of antimicrobial agents and, consequently, an interestingly alternative to commonly used benchmark biocides (Upadhyay et al., 2014). Additionally, an important benefit of using plant-derived antimicrobials is that they do not exhibit the side effects often associated with the use of synthetic chemicals (Upadhyay et al., 2014). The structural diversity of plant-derived compounds is immense and, the impact of antimicrobial action they produce against microorganisms depends on their structural configuration. Secondary metabolites can be classified based on their chemical structure, which also influences their antimicrobial properties, and the major groups that are responsible for antimicrobial activity from plants include phenolics, terpenoids and alkaloids (Fig. 1) (Gyawali and Ibrahim, 2014).

Classification of Phenolic Compounds

Phenolic compounds are the largest category of phytochemicals and the most widely distributed in the plant kingdom. These compounds are important determinants in the sensory and nutritional quality of fruits, vegetables and other plants (Balasundram et al., 2006; Saxena et al., 2013). Phenolic compounds possess an aromatic ring, bearing one or more hydroxyl groups, and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular mass polymer (Balasundram

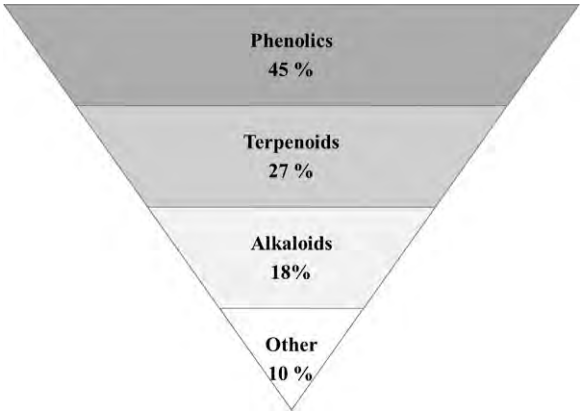

Fig. 1 Scheme presenting the major groups of plants phytochemicals. Based on Saxena, M., Saxena, J., Nema, R., Singh, D., and Gupta, A., 2013. Phytochemistry of medicinal plants. Journal of Pharmacognosy and Phytochemistry 1, 168–182.

et al., 2006). These compounds may be classified into different groups according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. The main groups of these compounds are flavonoids, phenolic acids, and tannins (D'Archivio et al., 2007; King and Young, 1999).

Flavonoids

Flavonoids are pigmented compounds found in fruits and flowers, being present in high concentrations in the epidermis of leaves and the skin of fruits. The basic structural feature of flavonoid compounds is the 2-phenyl-benzo[α]pyrane or flavane nucleus, which consists of two benzene rings linked through a heterocyclic pyrane ring (Ahmad et al., 2015; Cowan, 1999; Cushnie and Lamb, 2005; Savoia, 2012). These compounds may be divided in subclasses as a function of the type of heterocycle involved: flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins, of which flavones and flavonols are the most widely occurring and structurally diverse (Ignat et al., 2011; Manach et al., 2004). The source of variance in flavonoids includes: differences in the ring structure of the aglycone and in its state of oxidation or reduction; differences in the extent of hydroxylation of the aglycone and in the positions of the hydroxyl groups; and difference in the derivatization of the hydroxyl groups, e.g., with methyl groups, carbohydrates, or isoprenoids (Ahmad et al., 2015; Havsteen, 2002).

A marked antimicrobial property of flavonoids against a variety of bacterial pathogens has been observed (Cazarolli et al., 2008; Cushnie and Lamb, 2005; Wächter et al., 1999). Mechanism of action of flavonoids against bacteria may be attributable to up to three different ways, which include damage of cytoplasmic membrane by perforation mechanism and decrease in membrane fluidity, inhibitory effect on energy metabolism and inhibition of synthesis of nucleic acids (Ahmad et al., 2015; Cushnie and Lamb, 2011). Work with compounds in the flavonol and flavan-3-ol classes suggests that they damage the cytoplasmic membrane. Work with flavan-3-ols and isoflavones suggests that they inhibit nucleic acid synthesis. In addition, compounds in the flavonol, flavan-3-ol and flavone classes have shown to inhibit energy metabolism (Ahmad et al., 2015; Cushnie and Lamb, 2011). Evidences about another two mechanisms of flavonoid action have also been presented, which are the inhibition of cell wall synthesis and inhibition of cell membrane synthesis (Ahmad et al., 2015; Cushnie and Lamb, 2011).

Phenolic acids

Phenolic acid compounds form a diverse group that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids. These derivatives differ in the patterns of the hydroxylations and methoxylations of their aromatic rings. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring, and the type of substituents, cause significant changes on the properties of the phenolic products (Robbins, 2003; Sroka and Cisowski, 2003; Stalikas, 2007).

The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanism thought to be responsible for the toxicity of phenols and phenolic acids to microorganisms include enzyme inhibition by the oxidized compounds, possible through reaction with sulfhydryl groups or through more non-specific interactions with the proteins (Cowan, 1999; Samy and Gopalakrishnakone, 2010; Saavedra et al., 2010). Phenols can also inhibit the synthesis of nucleic acids of both Gram-negative and Gram-positive bacteria (Saavedra et al., 2010).

Hydroxycinnamic acids occur frequently in foods as simple esters with quinic acid or glucose, while hydroxybenzoic acid derivates are mainly present in foods in the form of glucosides. Glucose esters have found only occasionally (Ignat et al., 2011; Mattila and Hellstrom, 2007; Mattila et al., 2006). Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain, and caffeic, ferulic, p-coumaric and sinapic acids are the most common representatives occasionally (Ignat et al., 2011; Manach et al., 2004; Mattila et al., 2006).

Phenolic acids are commonly used as preservatives in food applications, due to their antioxidant and antimicrobial properties (Robbins, 2003). However, diverse phenolic acids are promising candidates for cleaning and disinfection due also to their antimicrobial properties and low cutaneous toxicity (Borges et al., 2012; Lemos et al., 2014; Lou et al., 2012; Phan et al., 2001).

Tannins

Tannins are water-soluble polyphenols that are commonly found in almost every plant part, like bark, wood, leaves, fruits, and roots. The properties of tannins are based on their chemical structures having two or three phenolic hydroxyl groups on a phenol ring, in a molecule of moderate large size. They can be classified into two groups: hydrolyzable and non-hydrolyzable (condensed tannins). Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tannic acid is an important gallotannin belonging to the hydrolysable class, while catechin belongs to the non-hydrolysable class (Akiyama et al., 2001; Barbehenn and Constabel, 2011; Cowan, 1999; Okuda and Ito, 2011).

The mode of antimicrobial action of tannins appear to be related to the inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation. Many microbial enzymes, such as cellulase, pectinase, xylanase, peroxidase, laccase, and glycosyl transferase were inhibited when mixed with tannins. The antimicrobial activity of tannins may also be related to their action on the microbial membranes. Complexation of metal ions by tannins could be another possible mechanism. Tannins are capable of chelating many metal ions, thus reducing the availability of essential metal ions for microorganisms (Akiyama et al., 2001; Chung et al., 1998a,b; Scalbert, 1991).

Terpenoids and Essential Oils

Essential oils (EOs) are a complex mixture of natural, volatile, and aromatic compounds obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruit and roots). EOs are secondary metabolites that are highly enriched in compounds based on an isoprene structure (Bakkali et al., 2008; Barbieri et al., 2017; Cowan, 1999; Nerio et al., 2010). These plant secondary metabolites have their greatest use in food as flavourings, in perfumes as fragrances, in aftershaves and in pharmaceuticals for their functional properties. Moreover, it has been recognized that some EOs have antimicrobial properties (Burt, 2004; Dorman and Deans, 2000; Hammer et al., 1999; Rota et al., 2004).

An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable. Therefore, the mode of action of EOs is based on their ability to disrupt cell wall and cytoplasmic membrane, leading to lysis and leakage of intracellular compounds (Bakkali et al., 2008; Ben Arfa et al., 2006; Burt, 2004; Liolios et al., 2009). In this context, oregano essential oil was reported to induce permeability alteration in *Staphylococcus aureus* and *P. aeruginosa* membranes with a consequent leakage of intracellular content (Lambert et al., 2001). Nevertheless, it has been proposed that the chemical structure, such as the presence of the functional hydroxyl group and the aromaticity are also responsible for the antibacterial effect. Generally, phenolic compounds, having the hydroxyl group attached to a phenyl ring, have the greatest activity among the phytochemicals found in EOs (Dorman and Deans, 2000; Lambert et al., 2001).

The active compounds of EOs can be divided into four groups according to their chemical structure: terpenes, terpenoids, phenylpropenes, and "others" (Hyldgaard et al., 2012). Terpenes are hydrocarbons produced from the combination of several isoprene units. Terpenes contain a hydrocarbon backbone which can be rearranged into cyclic structures by cyclases, thus forming monocyclic or bicyclic structures (Hyldgaard et al., 2012; Nazzaro et al., 2013). The main terpenes are the monoterpenes and sesquiterpenes, but hemiterpenes, diterpenes, triterpenes and tetraterpenes also exist in EOs at low concentration. Monoterpenes consist of two isoprene units, being the most representative molecules constituting 90% of EOs extracted from many plants (Bakkali et al., 2008; Hyldgaard et al., 2012; Nazzaro et al., 2013; Tongnuanchan and Benjakul, 2014). Among the terpenes, p-cymene, limonene, terpinene, sabinene, and pinene are the most well-known (Hyldgaard et al., 2012; Nazzaro et al., 2013). Most terpenes do not have high inherent antimicrobial activity (Hyldgaard et al., 2012; Nazzaro et al., 2013).

Terpenoids are terpenes with added oxygen molecules or that have had their methyl groups moved or removed by specific enzymes. Terpenoids are derived from five-carbon isoprene units; most of terpenoids have multi cyclic structures that differ from one another in their functional groups and basic carbon skeletons (Barbieri et al., 2017; Hyldgaard et al., 2012; Nazzaro et al., 2013). Carvacrol, thymol, linalool, menthol, geraniol, linalyl acetate, citronellal, and piperitone are the most common and well-known terpenoids (Hyldgaard et al., 2012; Nazzaro et al., 2013). Terpenoids are active against a broad spectrum of microorganisms, with the most active monoterpenoids identified so far being carvacrol and thymol (Dorman and Deans, 2000; Hyldgaard et al., 2012). Carvacrol is a phenolic monoterpenoid that is found primarily in the essential oil from oregano, being one of the most investigated EO constituents showing antimicrobial activity against a broad-spectrum of bacteria (Aligiannis et al,., 2001; Dorman and Deans, 2000; Knowles et al., 2005; Ultee et al., 2000). Carvacrol acts on microbial cells and causes structural and functional damage to their membranes with consequent loss of integrity and leakage of cellular material such as ions, ATP and nucleic acid (Hyldgaard et al., 2012; Nostro et al., 2007; Sikkema et al., 1995). Additionally, carvacrol is a volatile molecule that evaporates easily and its vapor phase has shown antimicrobial activity (Ben Arfa et al., 2006; López et al., 2007; Skandamis and Nychas, 2002), which suggests interesting applicative prospects. The efficacy of carvacrol vapor was demonstrated against food-borne *E. coli* O157:H7 and *Salmonella* on the surface of freshly produced vegetables, such as lettuce, spinach and tomatoes (Obaidat and Frank, 2009a,

b). Thymol presents a structure similar to carvacrol, and it is a phenolic monoterpenoid that is found in the essential oil of thyme, being also one of the most explored EO constituents. Similarly to carvacrol, thymol antimicrobial activity results in structural and functional alterations in the cytoplasmic membrane that can damage the outer and inner membranes, and the interaction of thymol with the membrane affects its permeability resulting in the release of ions and ATP (Hyldgaard et al., 2012; Nostro et al., 2007; Sikkema et al., 1995). The high antimicrobial activity of terpenoids compared to terpenes can be attributed to the functional moieties, since the chemical structure of terpenoids are closely related to that of terpenes (Hyldgaard et al., 2012).

Phenylpropenes are named as such because they contain a six-carbon aromatic phenol group and a three-carbon propene tail from cinnamic acid, which is produced in the first step of phenylpropanoid biosynthesis. These compounds constitute a relatively small part of EOs, and those that have been most thoroughly studied are eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde (Hyldgaard et al., 2012; Nazzaro et al., 2013). Eugenol, a phenylpropene present in many plants, is the main component of clove essential oil and its antimicrobial activity is related to the ability to permeabilize the cell membrane and interact with proteins. The action of eugenol on membranes occurs mainly by a non-specific permeabilization, which has been showed in various studies as increased transport of potassium and ATP out of the cells (Devi et al., 2010; Gill and Holley, 2006; Hyldgaard et al., 2012). Moreover, the hydroxyl group present in eugenol is believed to bind and affect the properties of proteins, thereby contributing to eugenol inhibitory effect at sub-lethal concentrations (Devi et al., 2010; Hyldgaard et al., 2012). Eugenol has been showing excellent antimicrobial activity against a wide range of bacteria, including *E. coli*, *S. aureus*, and *P. aeruginosa* (Gill and Holley, 2006; Walsh et al., 2003). On the other hand, the antimicrobial mode of action of cinnamaldehyde, another phenylpropene, is not clear. There are three things that are thought to occur: at low concentrations it inhibits different enzymes involved in cytokinesis; at higher but sub-lethal concentrations it acts as an ATPase inhibitor; and at lethal concentrations it perturbs the cell membrane (Hyldgaard et al., 2012). Cinnamaldehyde has also been reported to have an antimicrobial effect against a broad range of microorganisms (Sanla-Ead et al., 2012; Shen et al., 2015; Ye et al., 2013).

Alkaloids

Alkaloids are produced by a large variety of organisms such as plants, bacteria, fungi and animals and have been used in medicine since ancient times. Alkaloids can occur in any part of the plant, though specific compounds may be limited to a certain part (Barbieri et al., 2017; Cushnie et al., 2014). Alkaloids are characterized by great structural diversity, the presence of a basic nitrogen atom being the only unifying feature. Most alkaloids possess just one nitrogen atom, but some have up to five (Cushnie et al., 2014). No single taxonomic principle exists that would allow consistent classification of all alkaloids, being normally classified according to chemical structure, biochemical origin and/or natural origin. In terms of chemical structure, there are two wide divisions of alkaloids: the heterocyclic alkaloids, also known as typical alkaloids, which contain nitrogen in the heterocycle; and the non-heterocyclic alkaloids, also known as atypical alkaloids or protoalkaloids, which contain nitrogen in a side chain (Amirkia and Heinrich, 2014; Cushnie et al., 2014).

Numerous plant families are known to produce alkaloids and it has been reported that several of them possess high antimicrobial activity, including against *E. coli*, *P. aeruginosa*, *Proteus mirabilis, S. aureus, Bacillus subtilis, Raoultella planticola*, *Enterobacter aerogens, Agrobacterium tumefaciens,* and *Klebsiella pneumonia* (Saleem et al., 2010; Singh and Kumar, 2012). The mechanism of action of aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA thereby resulting in impaired cell division and cell death (Cowan, 1999; Savoia, 2012).

Conclusions

The extensive use of biocides for microbial growth control has considerable environmental and economic impacts. The misuse of more aggressive biocides and increased doses (as a way to overcome the resistance phenomena) constitutes an extra risk to public health. These measures can lead to the selection of pathogens insusceptible to the main available antimicrobials. Antimicrobial resistance is even more significant when cells are embedded in a biofilm. This has encouraged the replacement of toxic biocides with more readily antimicrobial agents, like natural-produced compounds. In this context, plants are potential sources of natural bioactive molecules. Phytochemicals have already shown great effectiveness against a broad spectrum of microorganisms making them a rich source of biocides and, consequently, an interestingly alternative to commonly used benchmark biocides.

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Biodeterioration – Including Cultural Heritage[☆]

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Glossary

Alteration Change in materials that does not necessarily involve a worsening in their conservative characteristics.
Biodeterioration Every form of irreversible alteration that causes changes/modifications in the properties of the materials due to metabolic activity and the development of one or more microbial population.
Biodeterioration (general) A process in the environment that recycles complex organic matter (and materials), and is an integral component of the life cycle.
Bioreceptivity Tendency of a material to be colonized by one or more organisms not always linked to a biodeterioration process.
Cultural heritage Term now utilized to cover the immensely diverse mass of documents and other historical objects of all types upon which societies confer a particular artistic, historic, or ethnologic value.
Decay Change in materials that always involves a worsening in their conservative characteristics.
Efflorescence Presence of a deposit of inorganic substances on stone or masonry surfaces that is not originally there in the sound material.
Foxing Deterioration of paper occurring as brownish, reddish stains because of the activity of microorganisms (majority) or the oxidation and/or formation of heavy metals deposits.
Patina Originally defined by Filippo Baldinucci (*Dictionary of Art*, 1681) as the time-dependent darkening of frescos and oil painting. Today, the term denotes the sum of all changes that the surface layers of objects of art are submitted to and inherit from environmental, biological, and substrate-related factors; in a broad sense, a term denoting all.

Abbreviations

DGGE	Denaturing gradient gel electrophoresis
EPS	Extracellular polymeric substances
MCF	Microcolonial fungi
MIC	Microbially influenced corrosion
SRB	Sulfate-reducing bacteria

Defining Statement

This article gives an overview of the biodeterioration of cultural heritage caused by biodeteriogen agents and their mechanisms and processes. Basic information on the material composition of objects of art is provided to address the problem of biodeterioration. Finally, it stresses and highlights the relevance of preservation and conservation practices.

Biodeterioration

Biodeterioration is a phenomenon that affects all materials, including those used in buildings, metals, monuments, pigments, frescoes, and so on. Stone deterioration is a natural process resulting from exposure to atmospheric agents and is referred to as stone/rock disintegration, with debris (gravel, sand, and clay) that, through transportation and sedimentation processes, can then constitute new rock, being formed.

[☆]*Change History*: August 2017. T. Lech updated section Biodeterioration, made small edits in sections Biodetriogens and small edits in section Organic Material/Parchment, added subsection Textile in the section Organic Material, made small updates in section Methods of Control and Prevention, added sections Molecular Methods in Microbial Hazard Identification, and Protection of Cultural Heritage Collections, added new figs. 5–8, updated section Further Reading.

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Table 1 Activities of main microbial deteriogens capable to use organic and inorganic substrates related to several matrices of cultural heritage

Microbial deteriogens	*Activities*	*Substrates*	*Matrices*	*Genera*
Proteolytic bacteria (ammonifying)	Hydrolyze, peptidase	Casein, egg yolk, collagen	Parchment, leather, wool, silk, frescoes	*Pseudomonas*, *Sarcina*, *Bacteroides*
Cellulolytic bacteria	Cellulase	Cellulose, tannins, resins, shellac, waxes, etc.	Textile fibers, (cotton, linen, jute, hemp), paper	*Cytophaga*, *Sporocytophaga*, *Sorangium*, *Vibrio*, *Cellvibrio*, *Cellfalcicula*
Amylolytic	Amylase	Starch	Frescoes	*Bacillus*, *Clostridium*
Lipolytic bacteria	Lipase	Fats, walnut and linseed oils, waxes	Wood, frescoes	*Bacillus*, *Alcaligenes*, *Staphylococcus*, *Clostridium*
Nitrifying bacteria	Corrosion by NO_2^- and NO_3^- yield	NH_3	Stone and metal artworks, varnish	*Nitrosomonas*, *Nitrobacter*
Denitrifying bacteria	Denitrification (anaerobiosis)	Several organic substrates	Stone artworks	*Pseudomonas*, *Bacillus*, *Vibrio*
Sulfate-oxidizing bacteria	Oxidations	S compounds	Stone artworks	*Thiobacillus*, *Thiosphaera*
Sulfate-reducing bacteria	Corrosion by H_2S (anaerobiosis)	S compounds	Stone and metal artworks	*Desulfovibrio*, *Desulfobacter*, *Desulfococcus*
Heterotrophic bacteria	Red/ox; weak corrosion	Inorganic compounds (air pollutants); cell lyses of organotrophs	Several materials	*Idrogenomonas*, *Thiobacillus*
Actinomycetes	Hydrolytic; Mechanical action	Cellulose, hemicellulose, etc.	Textile fibers (cotton, linen, jute, hemp)	*Streptomyces*, *Nocardia*, *Cellulomonas*
Yeasts	Fermentative	Carbohydrates	Frescoes, textile fibers	*Candida*, *Lypomyces Cryptococcus*, *Torulopsis*
Fungi	Several (hydrolyze, proteinase, lipase); Mechanical action by hyphae penetration	Starch, cellulose, hemicellulose, lignin	Wood and stone artworks, frescoes, mortar, plaster, varnish, paper	*Geotrichum*, *Pullularia*, *Cladosporium*, *Tricoderma*, *Aspergillus*, *Alternaria*, *Penicillium*
Algae	Photosynthetic	Inorganic compounds	Stone artworks, frescoes, mortar, varnish, metals	*Chlorococcales* order *Scenedesmus*, *Chlorella*

Outdoor artwork in particular suffers deterioration processes, especially in urban and industrialized areas where atmospheric pollutants and climatic conditions act together. Indeed, rain and pollution act directly on stone surfaces, altering their chemical composition, while wind and thermal differences tend to lead to an increase in specific types of stone surfaces, resulting in the formation of microfissures and microfractures. This can give rise to opportune conditions for the development and growth of microbes. Biodeterioration is defined as any form of irreversible alteration, implying a modification in the properties of materials due to metabolic activity and the growth of organisms called biodeteriogens. Atmospheric pollutants play a determining role in deterioration processes, and industrial development over the past century has led to an increase in the number of pollutants and caused accelerated deterioration in artwork exposed to the open air. Apart from corrosive substances present as atmospheric contaminants, other compounds can also accumulate on surfaces and favor the colonization of specific microbial populations able to utilize such compounds for growth (Table 1).

Deterioration is a phenomenon that occurs in materials of every type, including those used in buildings, metals, stones of monuments, fresco pigments, and so on. The deterioration of stone on the geological temporal scale is part of a natural cycle that leads to the disintegration of rock exposed to atmospheric agents, forming detritus material (gravel, sand, and clay) that, through processes of transportation, sedimentation, and diagenesis, can constitute new rock. Artwork out in the open air in highly industrialized areas is especially subjected to deterioration as there is direct interaction with atmospheric polluting agents as well as climatic conditions. Rain and pollutants act directly on the stone surfaces, altering the chemical composition of the material. Wind, extreme temperatures, and aggressive pollutants present in the atmosphere provoke over time a deterioration that leads to an increase in the specific surfaces of marble, with the phenomenon of decohesion and the formation of microfissures. After the surface has deteriorated, conditions become favorable for microbial attack and the development of living organisms (Fig. 1).

Biological agents such as bacteria, algae, fungi, lichens, mosses, and plants can colonize different substrates (paper, wood, stone, frescoes, paintings, etc.) and damage both surfaces and deep layers. Indeed, microflora able to grow on objects of art have a very complex ecosystem, and their development varies depending on the environmental conditions and the chemico-physical properties of the substrate. Also, climatic conditions play an important role. Actinomycetes and mycetes growing on a stone surface produce acids and substances, and these react with the substrate, causing its disintegration and allowing penetration of the hyphae into internal layers. Thus, such processes represent both chemical and mechanical deterioration. Furthermore, organisms often aggregate and produce biofilm and biological patina.

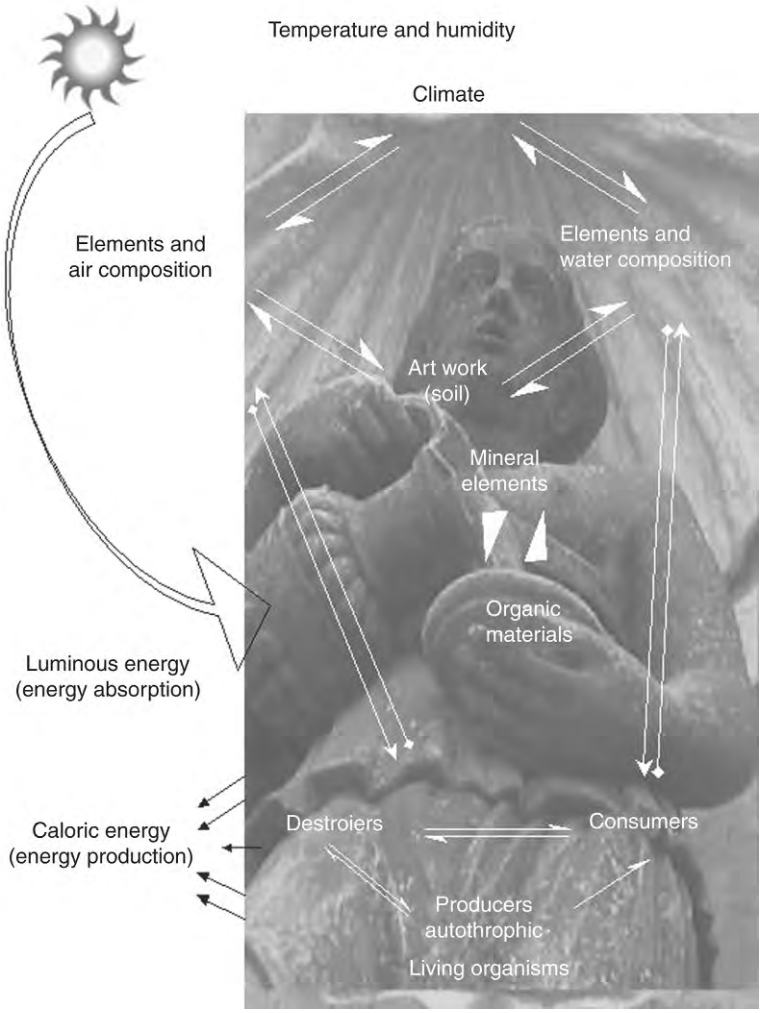

Fig. 1 Ecological succession on the artwork by autotrophic–heterotrophic microorganisms. Photo: G. Ranalli, G. Lustrato.

Biodeterioration is a complex process, usually involving simultaneous activity of various microorganisms. Microbial consortia may be less or more complex and their development frequently depends upon the appearance of microorganisms with low requirements, the presence and metabolic activity of which enable formation of a specific microclimate (frequently provision of higher relative humidity on the material surface or delivering nutrients), thereby allowing settlement of subsequent more demanding microorganisms. As has been mentioned above, the ability of forming microbial consortia is completely different in external environments (surfaces of containers, building outer walls, etc.) than in enclosed rooms where historical objects are stored. This mainly depends on the source of carbon in the environment and potential growth of autotrophic microorganisms.

As shown in recent research, biofilm formation is a multi-step process. The first stage is reversible attachment, with the greatest impact of gravitational forces between organisms and material, including the properties of the material (e.g., hydrophilic or hydrophobic area), as well as Van der Waals and Debye forces. This stage is followed by irreversible attachment which involves chemical forces and development of extracellular polymeric substance (EPS). EPS is a mix of polysaccharides, proteins, nucleic acids, lipids and other substances secreted by microorganisms, and its composition depends on the diversity of microorganisms creating the consortium. On the one hand, EPS facilitates more permanent attachment between microorganisms and the surface and stabilizes the biofilm on the surface. On the other, it ensures protection from unfavorable effects of environmental factors. Moreover, EPS is responsible for retention and accumulation of organic and non-organic substances essential for the biofilm to develop. The subsequent stage of biofilm formation is its maturation which involves development of a heterogeneous structure of cells connected with one another by channels used for transportation of nutrients and other metabolites. In a structure organized in such a way, microbial diversity is considerable, and owing to reciprocal interactions and an ability to produce a wider range of lytic enzymes, they gain greater biodeteriorative potential in both organic and non-organic materials. The final stage of biofilm

formation is its dispersion. Of note is the fact that the formation of a classical biofilm structure requires favoring environmental conditions and usually appears at the interface of phases.

The phenomenology of the microbial attack and the type of alteration depend on both the environmental conditions and the characteristics and state of the stone surface. Indeed, biodeterioration is a secondary deterioration process that occurs in substrates that have already been altered by chemical and physical damages.

Abiotic Factors

The main abiotic factors that affect the growth of biodeteriogens are humidity, temperature and their fluctuations, light (UV radiation), the type of substrate, ventilation, and air pollution. Humidity strongly favors the growth of algae and fungi. Light, both natural and artificial, is necessary for photosynthetic microorganisms (algae and cyanobacteria), lichens, mosses, and plants. Temperatures higher than 30°C together with high relative humidity accelerate all biological processes, while low temperatures (4°C) delay them. The substrate composition of artwork plays an important role in the growth of organisms. A material that is mainly composed of organic substances favors the growth of heterotrophs, particularly mycetes, while materials consisting mainly of inorganic substances, such as stone, favor the growth of chemolithotrophs and phototrophs. Also, the properties of materials, such as mineralogical composition, porosity, structural characteristics, along with the conservation state, can affect microbial colonization. This tendency, defined as the bioreceptivity of a surface, includes all the properties and characteristics of a material that contribute to, and favor, microbial growth. Ventilation is very important in the colonization process in order to reduce humidity, prevent condensate formation on the surface, avoid stagnancy of polluted air, and prevent atmospheric particulate and microorganism deposition on surfaces. The organic chemical pollutants in the atmosphere serve as an important carbon source for organisms. There is a strong correlation between aerobiology and biodeterioration. In fact, air represents the most important medium for the transportation of microorganisms (vegetative cells and spores) that can reach the surface of artistic manufacts and give rise to colonization. Other factors that can affect this process are the type of microorganism, the chemical composition of the substrate, and climatic conditions.

Mechanisms of Biodeterioration

Deterioration is the term used to define the modification of a material that results in a worsening in the material's characteristics from the point of view of conservation. Instead, the term 'alteration' indicates a modification of a material, but it does not necessarily imply a worsening in its characteristics from the conservation point of view. Indeed, the aggression of living organisms on materials can occur in two different ways that, however, act in synergy toward the disintegration of the material.

Chemical aggression

Chemical aggression occurs through the decomposition of a substrate and its transformation due to the direct action of intermediate and final products of microbial metabolism. Such deterioration, exclusive to stonework, is defined as biocorrosion. The metabolites mainly involved in this deterioration mechanism are organic and inorganic acids and chelating substances. The acids produced by the organisms react with the material of the stone, generally the insoluble salts, dissolving them and exposing them to be washed away by rainwater. All living aerobic organisms produce, through respiration, CO_2 as a final metabolism product, and when this reacts with water on limestone material, it is transformed to carbonic acid (H_2CO_3). Although weak, this acid still dissolves the relatively insoluble calcium and magnesium carbonates to form their bicarbonates that are very soluble and easily removed by heavy rain. The sulfuric acid produced biologically by the sulfur-oxidizing bacteria, particularly the *Thiobacillus* genera, and the nitric acid produced from the nitrifying *Nitrobacter* and *Nitrosomonas* bacteria act as powerful corrosives, reacting with calcium ions to form the soluble and easily washed away calcium sulfate (chalk) and calcium nitrate. All acidic substances taking part in chemical attack processes act through an acidolysis mechanism. The chelating substances, some of which are organic acids, dissolve the trivalent metallic cations of the stone, and thus provoke its disintegration. On materials containing organic substances (frescoes, tapestries, and paintings), there are biochemical processes due to the activity of exoenzymes from heterotrophic colonizers, mostly fungi, that use the organic substances present (casein, solvents, organic acids, and pigments of natural origin) as growth substrates. Indeed, bacteria developing on wall paintings can provoke variations in the color of paintings not only through pigment production but also through the excretion of metabolic products that provoke a loss of color in parts of the painting on the wall.

Aggression via mechanics

Stones exposed to the open air suffer accelerated deterioration of a mechanical type – washing by the rain, thermal excursions in the form of freezing and thawing cycles, and the action of the wind. Mechanical aggression by living organisms occurs through the growth of microorganisms or parts thereof (hyphae of fungi, rizine of licheni, and superior plant roots). Such action is manifested in the form of fissures and fractures of the attacked substrate, but there is no variation in the chemical composition of the material. This mechanical aggression becomes much more important when the colonizing organisms are

not limited to the development on the surface of the material (epiphytic organisms) but actively penetrate the stone (endolytic organisms), provoking disintegration of the material. Toward the end, there is a physical/mechanical attack by the filamentous microorganisms that taking advantage of the material's porosity penetrate and cause its disintegration. Some microorganisms cause damage due to variation in the water mass volume in the tissues; as such tissues are subject to dehydration and rehydration. A particular type of degradation, associated indirectly with biodeterioration, can be seen in restoration interventions. Indeed, damage is often due to operators who are not fully aware of the many pitfalls of dealing manually with plants anchored to walls or the cleaning of surfaces covered by biological patina.

Biodeteriogens

Biodeteriogens are organisms that participate in the biodeterioration process of materials, including historical and artistic manufacts. Such organisms are autotrophs and heterotrophs and colonize different types of inorganic (stonework, metals, and glass) and organic materials (paper, wood, parchment, leather, textile, etc.). Until the last decade, it was always considered that the first colonizers of stone were autotrophic organisms (both photoautotrophs and chemolithotrophs) able to utilize CO_2 as the carbon source, while the colonization by heterotrophs on the surface occurred only subsequently, with these organisms growing on the organic compounds released by the cellular lyses of autotrophs.

It was recently demonstrated that microbial symbiotic association can occur on stone surfaces. Under certain environmental conditions (i.e., in polluted areas), specific and adapted heterotrophic microflora have been evidenced in cometabolic processes associated with the degradation of organic atmospheric pollutants accumulated on exposed surfaces. In these cases, pollutants acting directly in the deterioration process and causing chemical damage on the superficial layers can also act indirectly as a nutritional source for microorganisms, favoring the selection of a specific heterotrophic microflora. Indeed, it has been demonstrated that some of these microorganisms growing on stonework exposed to air pollution are able to degrade aliphatic and aromatic hydrocarbons, including polycyclic compounds, as the carbon source for their growth. Furthermore, the metabolic potentiality of the colonizing microflora has been confirmed by the presence of functional genes that encode enzymes involved in the degradation of aromatic compounds, such as monoxygenases and dioxygenases. Thus, on the basis of this metabolic capability, heterotrophic microflora can also be considered the first colonizer of pollutant-exposed stonework. Note also that the microorganisms can grow using the very compounds applied to the stonework as a protective and consolidating agent (Fig. 2).

Finally, some microorganisms that grow on art objects can also exhibit particular metabolic conditions, lying dormant or in a noncultivable state under oligotrophic conditions for many years. The principal groups of microorganisms that are biodeteriogenic with regard to cultural properties are listed below. They are classified according to autotrophic or heterotrophic nutritional characteristics.

Bacteria (Eubacteria and Archaea)

Bacteria are unicellular microorganisms, principally characterized by the prokaryotic organization of their cells, that show a wide range of metabolic functions and capabilities. As per the phylogenetic tree of life, two prokaryotic domains, Bacteria and Archaea (unicellular organisms that have a nucleus but no membrane) are present. The main differences in the cellular structure of bacterial

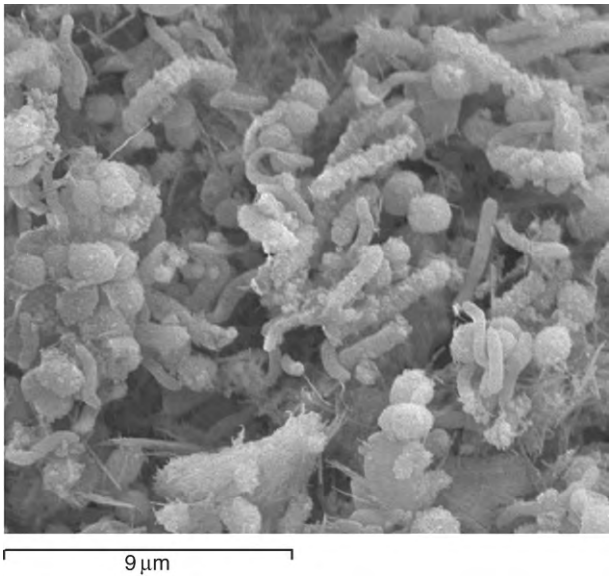

Fig. 2 Scanning electron micrograph of biofilm on altered marble surface. Photo: G. Ranalli.

and archaeal microorganisms are seen in the composition of the cell wall: Peptidoglycan in most Eubacteria and pseudomurein, polysaccharides, proteins, or glycoproteins in the Archaea, while the RNA polymerase is made up of a greater number of subunits for the bacteria, between 8 and 12, as compared to the 4 found in Eubacteria. Given the different cell wall composition, the Eubacteria are subdivided into Gram-positive and Gram-negative. The groups most frequently associated with artwork deterioration are considered here.

Chemolithotrophic bacteria

Sulfur-oxidizing bacteria are often considered one of the most dangerous groups for the conservation of stonework (chemolithotrophic) as they produce sulfuric acid, an inorganic acid that has a strong degrading action through the oxidation of hydrogen sulfide, elemental sulfur, and thiosulfates. Indeed, the most dangerous for artifacts are the thiobacilli (*Thiobacillus* and *Thiomicrospira*) as they directly oxidize sulfides to sulfates and their ecological characteristics favor a greater production of sulfuric acid.

Nitrifying bacteria include ammonia-oxidizing bacteria (*Nitrosomonas* and *Nitrosococcus*) that oxidize ammonia to nitrous acid and nitrite-oxidizing bacteria that oxidize nitrous acid to nitric acid (*Nitrobacter* and *Nitrococcus*). The metabolic process is called nitrification. Nitrifying bacteria play an important role in stone weathering because of nitric acid production.

Hydrogen bacteria use molecular hydrogen as a source of energy, converting CO_2 to organic carbon. Their importance lies in the fact that they remain active under limited environmental conditions and even in the temporary absence of the primary energy source. Almost all are facultative chemolithotrophic organisms, having the capacity to use organic compounds as the energy source (chemoorganotrophs). Hydrogen bacteria are active in both the presence and the absence of organic substances, as long as the hydrogen concentration is high. Both Gram-positive (*Bacillus*) and Gram-negative organisms are found, the most common and well known being the *Pseudomonas*, *Paracoccus*, and *Alcaligenes*.

Ferrobacteria are microorganisms that obtain their energy from the aerobic oxidation of iron: from ferrous (Fe^{2+}) to ferric (Fe^{3+}). Ferrobacteria are found on stone containing pyrites (ferric sulfide), frescos and wall paintings, and so on, where reduced iron compounds were employed, and on iron artifacts. In acid environments, *Thiobacillus ferrooxidans* is the most frequently found, growing autotrophically by utilizing both ferrous ions and compounds reduced from sulfur as electron donors.

Heterotrophic bacteria

A great number of heterotrophic bacteria (Eubacteria and Archaea) are able to colonize artwork. Such bacteria are responsible for various types of enzymatic activity that can directly affect colonized materials, especially organic material. Heterotrophic bacteria have also been found frequently on inorganic materials such as stone monuments and mural paintings, and the great majority of them have been Gram-positive bacteria belonging to the genera *Bacillus*, *Brevibacillus*, *Micrococcus*, *Kocuria*, *Clostridium*, *Frankia*, *Geodermatophilus*, *Blastococcus*, *Staphylococcus*, *Streptomyces* and related genera (such as *Nocardia*, *Rhodococcus*, *Streptoverticillium*, and *Micromonospora*), and Gram-negative bacteria (*Pseudomonas* and *Acinetobacter*). Recently, halophilous Archaeal bacteria such as *Halomonas* and *Halococcus* were found on frescos. Heterotrophic bacteria can be distinguished according to the nature of their enzymatic activity:

Proteolytic and ammonifying bacteria

These bacteria hydrolyze proteinaceous substances to peptides and the peptides to amino acids, producing extracellular hydrolytic enzymes (i.e., protease and peptidase). The amino acids are then broken down with the liberation of ammonia. The most frequently identified species belong to the genera *Pseudomonas*, *Sarcina*, *Bacteroides*, and *Streptomyces*. Proteolytic bacteria are particularly dangerous for items of animal origin, such as parchment, leather, silk and wool.

Cellulolytic bacteria

These bacteria can be distinguished as primary cellulolytic bacteria and as facultative cellulolytic bacteria. The primary cellulolytic bacteria specialize in cellulose degradation, to such an extent that they are unable to grow if this compound is not present (*Cytophaga*, *Sperocytophaga*, and *Sporangium*), while the facultative cellulolytic bacteria are able to also utilize other organic compounds (*Vibrio*, *Cellvibrio*, and *Cellfalcicula*). Cellulolytic bacteria are quite common and have the capacity to break down lignin and other wood components such as resins, gums, dye, tannic acid, waxes, and fats.

Amylolytic bacteria

They are essential for starch breakdown. Among them certain species of *Bacillus* and *Clostridium* are able to break down starches, and the speed of the breakdown will vary according to the chemical composition of the different molecules constituting the starch, particularly the percentage of amylopectin present. Amylopectin is a branched compound and is the component that is the slowest to be broken down by bacteria.

Lipolytic bacteria

Relatively few genera are able to break down lipids, resulting in the production of lipase (a specific esterase that hydrolyzes the ester bonds between glycerol and the fatty acids), but among these particular mention must be made about *Bacillus*, *Alcaligenes*, *Staphylococcus*, and *Clostridium*. These microorganisms are able to degrade artifacts in which fatty substances are present as natural components (e.g., in wood) or else introduced during artifact manufacture (e.g., pastels).

Denitrifying bacteria

These are anaerobic bacteria that are relatively easily found on the surface of artifacts, provided organic substances are present. In fact, they are facultative anaerobes and metabolize the substrate by means of aerobic respiration in the presence of oxygen; however, to reduce nitrates they require anaerobic conditions. They can also utilize other electron acceptors such as the ferrous ion (Fe^{3+}). The biodeteriogenic activity of denitrifying bacteria is, in all respects, similar to that of other heterotrophic bacteria and is always bound to a specific enzymatic activity (amylase, protease, and esterase) and the production of catabolites such as organic acids, regardless of whether metabolism occurs in aerobic or anaerobic conditions (*Bacillus denitrificans*, *Pseudomonas stutzeri*, *Achromobacter severina*, etc.).

Fungi

Eumycota

They are eukaryotic organisms that are heterotrophic and are characterized by the presence of a rigid cell wall made principally of chitin; they belong to the kingdom of Fungi or Mycota. Fungi play an important role in the degradation of cultural heritage as they act either directly on organic materials that are utilized nutritionally or indirectly on materials of an inorganic nature that, although not metabolized directly, can nevertheless have organic fractions able to support fungal growth. In addition, fungi adapt easily to varying environmental conditions, and this means that environments for the conservation of artifacts of a historical and artistic interest, for example, hypogean environments, churches, museums, libraries, and archives, can provide ideal habitats for their development. In fungi with a pigmented mycelium (e.g., *Dematiaceae*), melanin is present. This confers the fungi with a resistance to numerous chemical and physical agents such as reducing substances, UV radiation, gamma and X-rays, and attack by enzymes. This resistance can be explained by the chemical nature of melanin, a quinone and hydroquinone combination that limits the damage caused by the formation of free radicals. However, the presence of melanin in the cell wall also seems to play a fundamental role in biodeterioration processes affecting stone in that it favors the penetration of the fungus into the substrate.

Many fungi such as *Cladosporium* have wide metabolic diversity and are able to flourish even in conditions of oligotrophy (scarcity of nutrient presence). Thus, they have often been identified as the fungi responsible for fresco degradation, the particles on the fresco surface representing the sole organic source. Degradation caused by microfungi, which in the case of artifacts of an organic origin often coincides with the degradation of the artwork itself, is due to the production of intracellular and extracellular enzymes specific to the individual substances to be consumed. Furthermore, the development of mycelium on a material, namely on the artwork, can also give rise to physical and mechanical damage caused by hyphae penetration. It must be noted that the term microbially-induced corrosion, rather than biodeterioration, is used for fungal deterioration of non-organic materials where mechanical damage is caused by mycelium growth or impact of secondary metabolites. The term biodeterioration is reserved for the deterioration process induced by microbial enzymatic activity.

Meristematic fungi

Among the fungi, meristematic fungi are of particular interest in stonework biodeterioration processes. Black fungi of meristematic growth (or microcolonial fungi (MCF)) are a broad and heterogeneous group of microorganisms, grouped together because of their ability to grow meristematically and to produce a melanin pigment, generally olive-black in color.

Both the production of melanin and meristematic growth endow the cell with resistance to extreme environmental conditions such as strong sunlight and water and nutrient scarcity, conditions frequently encountered by artwork in stone that is left out in the open in Mediterranean regions. Black fungi with meristematic growth form small black colonies (60–100 μm in diameter) on rocks and grow very slowly (1–2 months) both under natural conditions and in the usual culture media. Moreover, they are extremely versatile and are able to grow on the most varied substrates, as well as in oligotrophic conditions. Such fungi are resistant to environmental stress such as high temperatures (>100°C), as well as to prolonged periods of UV radiation exposure and osmotic stress (up to 40%). It is also important to consider the deterioration actions of organisms like insects, rodents, and birds on art objects made using different materials.

Up until now, microorganisms on cultural heritage have generally been considered to be connected to actions of alteration. However, it was recently demonstrated that some microorganisms can be used in biocleaning and biorestoration procedures to remove harmful compounds from artistic objects. For example, the sulfate-reducing bacteria, *Desulfovibrio vulgaris*, has been used to remove a black crust containing sulfates and specific heterotrophic bacteria, *P. stutzeri*, to remove glue from frescoes. In addition, such bacteria can also be employed in calcite bioprecipitation for the conservation of monumental stone and for stone reinforcement.

Phototrophic microorganisms

This group includes algae and cyanobacteria and there is remarkable diversity in the organization of cells and vegetative structures. Algae are eukaryotic cells, exhibiting a full range of pigments (chlorophylls, carotenoids, and phycobilins), highly specific reserve materials, and wall components that can be used as discriminating elements during placement within systematic classification. Cyanobacteria are prokaryotic organisms linked to the kingdom of Eubacteria, and are able to carry out photometabolism similar to that of higher plants. They colonize archeological remains, stonework, frescoes, wall paintings, and mosaics, producing chromatic alterations, patinas, and biofilms of different colors (green, gray, orange, brown, and violet-red). Their deteriorative action is seen not only in aesthetical damage but also in chemical damage, because of substrate corrosion. Cyanobacteria can be divided into epilithic, growing on the surface of the stone, chasmoendolithic, in the fissures and cavities of

the stone but in contact with the surface, and endolithic, inside the stone itself. The genera of algae most frequently referred to in the biodeterioration of stone belong to the division *Chlorophyta*. The matrix may form a structured sheath called a sheath, capsule, or calyx, or else simply be an amorphous mucilage. It constitutes a fundamental characteristic for the colonization and the survival of the organisms: It permits adhesion to the substrate, functioning as a water reserve as it slowly swells with water and then gradually releases it, allowing the organism to overcome periods of adversity, inhibits predation, and, most importantly, it is the cement that holds together the microbial cells, thus giving rise to the formation of the biofilm. Investigations carried out on stone monuments have shown that most of the cyanobacteria present are forms endowed with a gelatinous matrix. The chemical nature of such a matrix, generally made up of negatively charged polysaccharides due to the presence of acid groups, can also contribute to the deterioration of the stone through complex-forming processes and leaching of the constituent elements; it also represents a source of organic material that supports the growth of the nutritionally more demanding heterotrophic microorganisms.

Lichens

The most frequent organisms found on stone monuments in an outdoor environment are the lichens. They tend to enrich environments lacking in biodiversity, for example, archeological sites and urban centers, but can also be agents of chromatic alteration and surface biodeterioration. Lichens, small symbiotic ecosystems formed by a fungus (mycobiont), depend nutritionally on photosynthetic partners (photobionts). In addition to their biodeteriogenic action, lichens can also play a bioprotective role: In fact, in the presence of high atmospheric pollution levels, they provide an effective barrier against pollutants that attack the mineral components of a substrate.

Bryophytes

Bryophytes are photosynthetic eukaryotic organisms that can play a significant role in the biodeterioration of materials, especially stonework, as they penetrate the substrate by means of rhizoidal structures that cause physical and mechanical damage. Like algae and lichens, many moss species can be considered pioneer organisms as they can colonize exposed rocky surfaces, and in a few cases are able to survive high temperature levels and radiation from the sun as well as long drought periods. Because of their capacity to survive in a latent form, and return to normal metabolic activity with the restoration of favorable conditions of humidity, Bryophytes are also considered poikilohydric organisms.

Vascular plants

They are an important consideration in biodeterioration processes. Indeed, their roots form a complex system that, during growth, can exert considerable pressure on a substrate. In the event of the substrate being ancient walls, architectural structures, monuments, or hypogeum chambers, root growth can result in severe physico-mechanical damage.

Materials Related to Cultural Heritage

Considering the most accepted and first proposed definition of biodeterioration by Hueck (1965) as "any undesirable change in the properties of a material caused by the vital activities of organisms", it is important to illustrate the different kind of materials that can be attacked (Allsopp et al., 2003).

Materials are any form of matter, with the exception of living organisms, that are used by humankind, and all materials have an intrinsic value and thus there is an important economic dimension to biodeterioration. Our cultural heritage is made up of almost all types of materials, both natural and artificial, organic (that may be natural, semisynthetic, or synthetic compounds) and inorganic, and include stone, metals, textiles, paper, wood, and so on; in more recent times, photographic documents and magnetic and optical carriers have been introduced and widely established all over the world.

The biodegradation of organic materials is an essential process in the environment that helps in recycling complex organic matter and is an integral component of life. However, biodeterioration which mechanisms are similar to biodegradation also destroys historical structures, resulting in the loss of valuable cultural properties stored in libraries, archives, and repositories. Many enzymes break the bonds of polymers; some polyesters are degraded by lipases and esterases, proteins by proteases, starch by amylases, and cellulose and cellulose derivatives by cellulases. The organisms that pose major concerns to memorable heritage objects are microorganisms (bacteria, fungi, yeasts, and algae) and insects.

The biodeterioration of inorganic materials used in the works of art is essentially a process that involves monumental rocks and archeological remains, glasses, and metals. These cultural heritage objects are influenced by environmental parameters, which can modify their structure and composition by biological mechanisms. As already cited, the main types of damage derived from metabolic activity of organisms are associated with physical, chemical, and aesthetical mechanisms, while the intensity of damage is strictly related to the type and dimension of the organism involved, the kind of material and state of its conservation, environmental conditions, microclimatic exposure, and the level and types of air pollutants (Fig. 3). Information on materials related to cultural heritage, separated on the basis of the inorganic, organic, and composite materials, are reported.

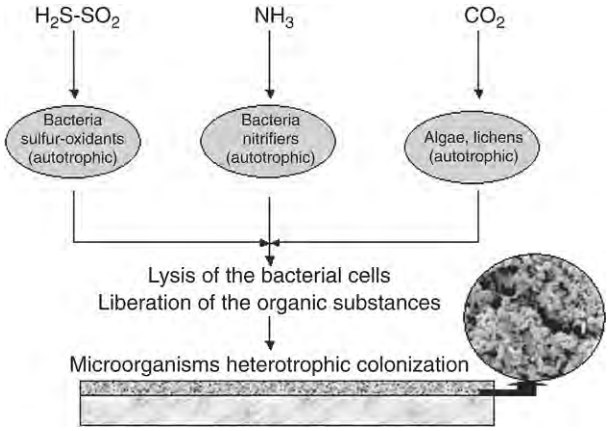

Fig. 3 Theoretical scheme of ecosystemic view of a cultural heritage. Photo: G. Ranalli, G. Lustrato.

Inorganic Materials

Historical stone objects (sculptures, buildings, and rocks) and frescoes

Deterioration of building stone begins from the moment it is quarried as a result of natural weathering processes. Other factors, sometimes acting synergistically, including a progressive dissolution–crystallization of soluble salts, pollution, and biological colonization, can accelerate natural deterioration of the mineral matrix. Decay is a consequence of the weathering action of several physical, chemical, and biological factors. In the case of calcareous stones, the materials, due to calcite leaching, increase their porosity in time and decrease their mechanical characteristics. All building walls contain soluble salts, dispersed within the porous materials or locally concentrated. These salts are solubilized and migrate with the water in and out of the stone. The drying out of the solution at the exposed surface results in the formation of efflorescences. Moreover, climate plays an important part in influencing the activity of organisms on stone in monuments and other objects of cultural value. The presence of living organisms on stone increases its susceptibility to damage through their water-binding capacity. Then, the mineralogy, porosity, surface roughness, and capacity to collect water and organic materials control its bioreceptivity and tendency to biodeterioration. However, environmental factors such as temperature, light intensity, pH, and relative humidity affect the number and type of colonizing species and hence the progress of colonization. Different hygroscopic salts (carbonates, chlorides, nitrates, sulfates, etc.) can be found on the surface of decayed monuments, which have extremely saline environments. Halotolerant bacteria and Archaea can grow in many different niches including deteriorated mural paintings; the detection and identification of an Archaeal community by DNA extraction, fragment amplification with specific primers using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified DNA encoding 16S rRNA, confirm important ecological implications of Archaea on monuments because of the considerable production of efflorescences. Water is very important in causing the mechanical degradation of stone, assessed mainly by porosity and bulk density, while its circulation through stone is controlled by its porosity size, distribution, specific surface, and capacity. Rainfall intensity and amount greatly influence erosion rates of marble and limestone, with a minor effect on sandstone. The presence and amount of water control biological activity and growth; moreover, hydrodynamic forces bring microorganisms (i.e., bacteria) close to substrates and their sorption onto solid surface induces the formation of extracellular polymeric substances (EPS), biofilms, and patina.

Patina includes many environmentally induced surface changes such as oxalate film, lacquer, crust, deposit, karst, rock varnish, microstromatolite, efflorescence, carbonate, gypsum, iron, manganese, oxalate and silica skin, and others. The formation of patina is a very complex process. Since it takes a very long period of time to develop, the patina itself is often seen as an object of historical interest. The main process can be summarized as an exchange of matter and energy between two open systems: the solid matrix and the surrounding environment (indoor or outdoor atmosphere, water soil, an intermediate cover of plaster or paint). The formation of patina can come to a standstill under environmental conditions. However, in cases in which patina formation becomes a historic evolution of the surface area of a monument, the real danger is in the addition of new layers on historical deposition on top of the original material. These deposits (crusts, skins, sinters, gypsum crust, etc.) may represent an increasingly dangerous hazard for the original material they cover; in fact, the mass increase may lead to fissures, exfoliation, and loss of the original surface material. Among the additional factors that accelerate deterioration of stone, the effects of anthropogenic sources of pollution on building stone are still to be definitively characterized. An example of particular interest is the formation and constituents of black crusts (Fig. 4).

It has long been acknowledged that burning of fossil fuels has led to an increase in the concentrations of acid gases in the atmosphere; of these, perhaps the most important is sulfur dioxide, which forms sulfurous acid when dissolved in water (acidic rain). Sulfurous acid is oxidized to sulfuric acid, which in turn reacts with calcium carbonate to form calcium sulfate, the mineralized form of which is known as gypsum. The formation of gypsum then leads to the creation of cavities below the surface as a result of migration of calcium ions to the surface. Thus, if soluble gypsum is washed away, it takes with it some of the stone itself, initially causing loss of surface details but eventually leading to loss of structural integrity. Particulate matter from the atmosphere can combine with gypsum to leave unsightly black crusts on the surface of the stone. Carbonaceous particles were

Fig. 4 Black crust patinas on marble surface lunetta (Milan Cathedral, Italy). Photo: G. Ranalli, E. Zanardini.

thought to be the most significant element in black crusts, but they also contain a complex mixture of aliphatic and aromatic carboxylic acids and polycyclic aromatic hydrocarbons. Finally, stones in urban areas act as 'passive repositories for any kind of gaseous and particulate air pollutant present in the surrounding atmosphere'.

Glass

Glass is a ceramic material (solid, inorganic, and nonmetallic) obtained by vitrification (fusion and cooling without crystallization) of primitive substrate (amorphous silicate, about 70%) and by adding several agents for fusion, stabilizing, decoloring, coloring, and so on. The exact origin of glass is still unknown, but there exist objects dated 1700 BC from Mesopotamia. The biodeterioration of glass is related to several factors, including both the glass composition and the environmental conditions on exposure. Although preliminary case studies of glass biodeterioration date back to the early nineteenth century, the phenomenon is understood only now. The presence of fungi, bacteria, lichens, and algae as biodeteriogen agents poses a serious problem for the glass surfaces found inside damp ancient structures. Free silicon bonds can react with organic acids produced by specialized microorganisms, which are capable of growing on the glass surface and finding nutrients for their metabolism (acidic attack), on glass surfaces. An hazard is the alkaline attack leading to the rapid destruction of the Na reticulum of the glass, accompanied by silicon release. This process is accelerated by temperature and exists even when glass is exposed to the air, but not for a long time. Glass may be severely damaged by biodeterioration because of initial fungal attack that leads to etching, increase in opacity, and the presence of black spots on the surface of glass. Among the bacteria capable of colonizing objects in glass, the iron bacteria (*Sphaerotilus* and *Gallinella*) and the sulfur-oxidizing bacteria play an important role in the metabolism of sulfur and manganese, respectively. In marine environment, algae and bacteria can play an important role inducing phenology of more alterations in glass as surface erosion, microfractures, and pitting. In other cases, isolated bacteria related to the genus *Flexibacter*, exhibit their capability to grow on the surface of glass and adapt to an oligotrophic environment and to develop themselves inside a thin layer of biofilm produced by other microorganisms.

Metals

Deterioration of metal materials appears as a complex matter from the physical, chemical, and biological points of view. Their specific mineralogical, structural, mechanical, and electrical properties are further influenced by the fact that metal materials utilized in the artistic fields are represented by alloys from the fusion of a single metal with almost another element (e.g., ancient bronze, made of copper and tin). All metal materials, except gold, are chemically reactive in the presence of compounds present in air, soil (O_2, CO_2, SO_2, salts, etc.), and, electrochemically, in water and moisture. Chemical corrosion of metals occurs when the structural elements undergo a chemical change, transitioning from the ground state to an ionized state. The phenomenon consist of two equivalent reactions, for example, for a ferrous metal in an aqueous oxygenated environment: $2Fe \rightarrow 2Fe^{2+}+4e$ anodic reaction, oxidation $O_2+2H_2O+4e \rightarrow 4OH^-$ cathodic reaction, reduction.

In the absence of oxygen, either hydrogen ions or water is reduced at the cathode: $4H^++4e \rightarrow 2H_2 2H_2O+4e \rightarrow H_2+2OH^-$

Microbial activities can influence the above reactions (biocorrosion), but the basic mechanism is still electrochemical. The microbially influenced corrosion (MIC), estimated as 20% of all metal corrosion, is generally associated with the microbial colonization of the metal surfaces and the formation of nonhomogeneous biofilms; the resulting surface appears as a pitting corrosion (biopitting) where the microbial metabolites, CO_2, S, H_2S, NH^{4+}, and acids (citric, oxalic, succinic, and fumaric), are the responsible agents. Aerobic and anaerobic bacteria are the major microbial groups involved in metal biocorrosion.

Under aerobic conditions, the sulfur-oxidizing bacteria related to genus *Thiobacillus* can oxidize elemental sulfur to obtain energy, solubilize metals producing sulfuric acid, which is highly corrosive. Metal biocorrosion by *T. ferrooxidans* and *Thiobacillus thiooxidans*, *Metallogenium* spp., *Sulfolobus* spp. is a serious problem, for example, in acid mine waters where, in presence of Fe^{3+} ions, the pH value drops to between 2 and 3, causing ferric sulfate deposition and finally severe corrosion in concrete and pipes.

Under anaerobic conditions and in a reduced environment with redox potential higher than −100 mV, the sulfate-reducing bacteria (SRB) related to genera *Desulfovibrio* and *Desulfotomaculum* produce H_2S, which is highly corrosive, inducing precipitation of metal sulfur salt. In the presence of iron ions, the overall reaction, where FeS and $3Fe(OH)^{2-}$ are final corrosion products, is as follows: $4Fe+SO_4^{2-}+4H_2O \rightarrow FeS+3FeOH^{2-}+2OH^-$

Organic Materials

Paper

Its invention is usually credited to a Chinese court official, Tsai Lun, in about AD 105. In the eighth century paper was produced in the Middle East by the Arabs. Papermaking techniques were slightly changed by the Arabs, who introduced linen into the raw material and the use of paper mills. The art of papermaking gradually spread and in the twelfth century paper became the most common writing material in the West as the Europeans learnt the art from the Arabs. The principal compound in paper is cellulose, which is made of vegetable fibers. Until the nineteenth century, pure cellulose was mainly obtained from rags generally made of linen, hemp, and cotton. The handmade process consisted of converting the cellulose into pulp. After dispersion in water, the pulp was drained through a mold, dried, and pressed to form sheets. As the demand for paper grew, wood became the main raw material from the nineteenth century. Fibers were then obtained either by mechanical or by chemical processes. So, the composition of paper changed from almost pure cellulose to cellulose and significant amounts of hemicellulose and lignin. However, the production of a paper sheet also needs other compounds. For example, in order to reduce the spread of ink, the paper surface needs to be filled, the most used sizing materials being starch, gelatin, resin, and various synthetic polymers. Fillers, such as clays or chalk, are added to fill the pores and make the paper opaque. Other products used during papermaking include dyes, pigments, and fluorescent whitening agents.

The scientific and conservation literature claim that paper is an easy source of organic carbon for microorganisms. Paper biodegradation is well known. A major concern related to fungal colonization is pigment that may stain the substrate. Some pigments are soluble in certain solvents and can be removed through the application of a specific solvent. In addition, some permanent discoloration may also be caused by weak acids produced by fungi. Interestingly, there is one type of degradation named foxing that is still not fully understood. Some foxing stains are claimed to have a biotic origin and others an abiotic one. Foxing is a deterioration of paper occurring as brownish, reddish stains that could be the result of the activity of microorganisms or the oxidation and/or formation of heavy metals deposits. After many years of research and discussion, it is believed that the majority of foxing stains are due to microorganisms. Some microbial growth is presented in the Fig. 5.

Fig. 5 Scanning electron micrograph of microbial growth on paper. Photo: T. Lech.

Wood

The most serious damage to wooden objects in indoor environments (museums) is caused by insects; they utilize wood as a nutrient source and for depositing eggs. Many insects (Coleoptera and Lepidoptera orders) use as substrate cellulose, as they have microorganisms in their gut that supply the cellulase enzyme. In an outdoor environment, when the moisture content of the wood is above 20%, it becomes susceptible to microbial attack by microfungi and bacteria. The predominant fungal infections, by exoenzyme production and subsequent destruction of natural polymers, are white rot (*Fomes* sp., and *Pleurotus* sp.), brown rot (*Poria* sp., *Merulis* sp., and *Coniophora* sp.), and soft rot (*Chaetomium* sp., *Alternaria* sp., and *Humicola* sp.). Because both bacteria (*Pseudomonas* sp. and *Achromobacter* sp.) and Actinomycetes (*Micromonospora* sp.) require a higher water content, their role is important in outdoor and marine environments.

Papyrus

The papyrus plant belongs to a widely distributed family of plants found in wet environments in tropical and subtropical areas. Papyrus was the material used by ancient and classical civilizations to write their texts. The raw material of papyrus comes from the pith of *Cyperus papyrus*, the largest member of the family. The papyrus pith is composed mainly of cellulose (54%–68%) and lignin (32%–24%); the proportions are principally based on age, manufacturing process, and environmental effects. It is worth noting that papyrus contains more lignin and less cellulose than paper and also contains uronic acid. The papyrus-cementing material is a polymer whose monomers are galactose, arabinose, and rhamnose.

Papyrus samples from different museums in Cairo were isolated and degrading microorganisms were identified. Some were tested for their cellulolytic activity, and it was found that the cellulose was attacked by all *Chaetomium* fungi that attack cellulose as well as by *Emericellopsis minima* and *Botryodiplodia theobromae*. In particular, *Chaetomium globosum*, *Chaetomium ochraceum*, and *Chaetomium elatum* along with *E. minima* intensely depolymerized cellulose with values ranging from 76% to 100%. The majority of microorganisms are able to hydrolyze the sugars dl-arabinose and L-rhamnose. Papyrus may be completely decomposed by fungi (genus *Chaetomium*) or Actinomycetes; the latter are especially dangerous when the conditions are unsuitable for other microorganisms, as in the desert. *Penicillium* is very often detected on paper but high temperature does not favor its growth. The order of the utilization of inorganic nitrogen sources is ammonium nitrate, ammonium phosphate, ammonium sulfate, and sodium nitrate. Ammonium nitrate represents a good source of nitrogen as nitrogen is present as ammonium and nitrate ions. The suitable temperature for fungal growth, under slightly acidic pH and relative humidity of 95%, is 24–30°C, but it is 42°C for only seven species.

Parchment

Perhaps, parchment was invented in the second century BC in Pergamum, or probably known 100 years before; parchment replaced papyrus almost completely from the third or fourth century AD. Parchment had many advantages, especially over papyrus. It was

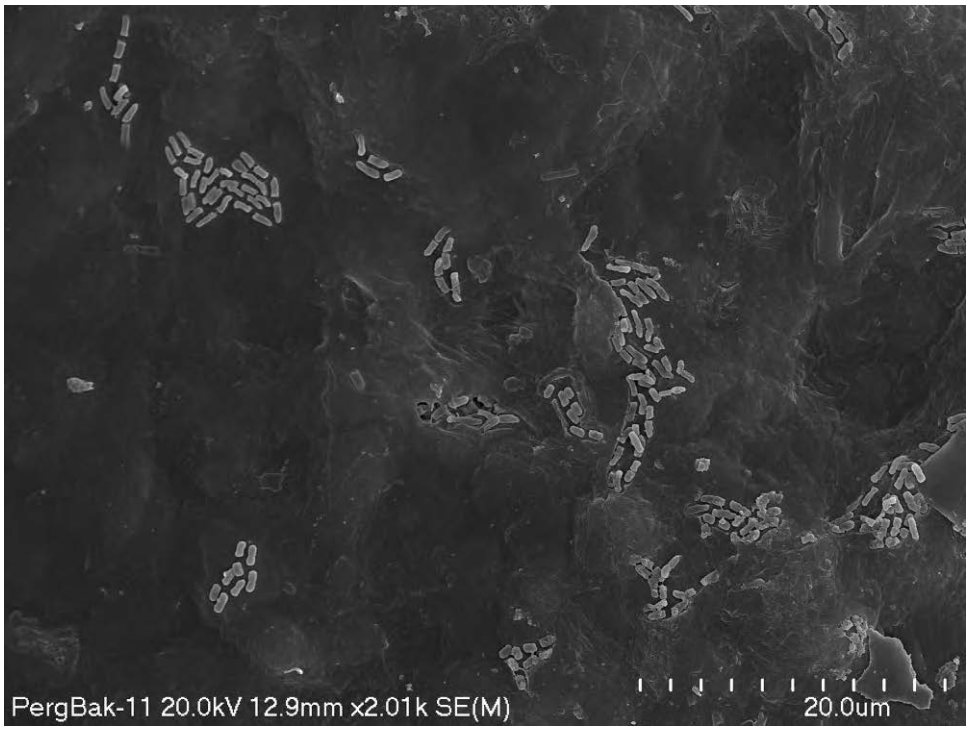

Fig. 6 Scanning electron micrograph of bacterial growth on parchment. Photo: T. Lech.

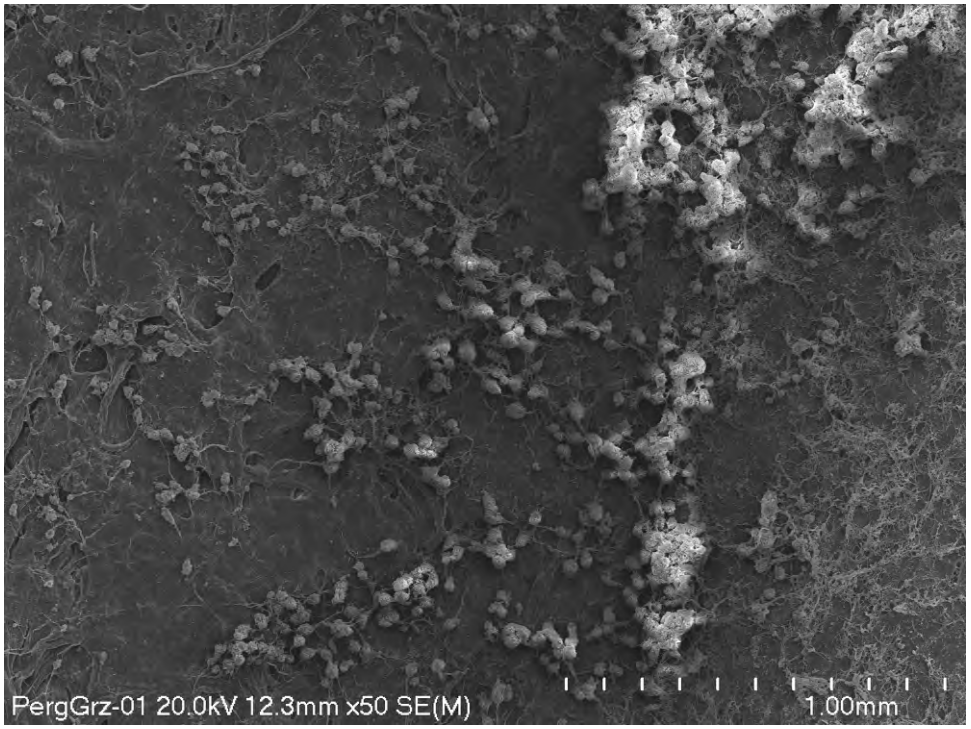

Fig. 7 Scanning electron micrograph of fungal growth on parchment. Photo: T. Lech.

stronger and more durable than fragile papyrus and the raw materials for making it, animal skins, were available everywhere and not limited to one geographic location, namely Egypt. Parchment could be written on both sides (*recto* and *verso*).

Collagen is the major component of most connective tissues in animals and therefore is the bulk material for parchment. The main distinction between parchment and leather is that parchment preparation involves drying the wet skin while under tension. In contrast, the properties of leather are mainly due to the addition of tanning agents. As for the other supports, the susceptibility of parchment to microbial deterioration is dependent on the raw material, methods of production, and conditions of preservation. The most active microbial agents that cause damage to parchment belong to the genera *Streptomyces*, *Cladosporium*, *Scopulariopsis*, *Fusarium*, *Sporendonema*, *Ophiostoma*, *Aspergillus*, *Mucor*, *Penicillium*, *Nocardia*, *Alternaria*, *Trichoderma*, *Botryotrichum*, *Acinetobacter*, *Micrococcus*, *Bacillus*, *Peanibacillus*, *Staphylococcus* and *Serratia*. The biodeterioration of these materials (parchment and leather) was mainly caused by fungal attack. In contrast, the increase in the pH during the deacidification treatment, generally necessary for parchment with a pH value lower than 5, by using ammonia greatly increased the microbiological deterioration suffered by parchment. Some bacterial and fungal growth are presented in the Figs. 6 and 7.

Biodeterioration of these materials (parchment and leather) was mainly due to fungal contamination, and less due to bacterial contamination. However, bacteria with proteolytic activity and in particular collagenolytic activity were sought and most of the isolated strains were able to grow on media containing collagen or parchment as the sole source of carbon and nitrogen. SEM observations demonstrated that the bacteria colonized subsurface layers along the collagen fibers. Among several biocides, Preventol R-80 and Catamin AB proved to be the more active biocides. In addition, mixed treatment using gamma radiation after a 3% pretreatment with Catamin AB is suggested. However, it should be noted that the list of legally permissible biocides in the conservation of cultural heritage objects is reduced from one year to another. As for historical parchments, the lack of a safe disinfection method is the greatest problem. It is therefore crucial to provide appropriate storage condition to minimize the risk of active microbial growth.

Textiles

Textiles have accompanied mankind for thousands of years and they have been used for a variety of purposes. Starting from their utilitarian aspect, they have been used as clothing, upholstery material and decorations. They have also performed a significant role in religious rites and diplomacy. As such, they constitute a significant and very specific part of our cultural heritage. The vast majority of historical textiles are made of natural fabrics, such as linen, cotton, wool or silk. The first recorded usage of natural fibers concern mainly those of plant origin, i.e., linen that dates back to the 7th century BC and cotton, the use of which was recorded in the 5th century BC. Usage of fibers of animal origin was noted slightly later: 7,000 years ago for wool in Mesopotamia and in the 3rd century BC for silk in China. However, collections of synthetic textiles, which are rather young and date back to the 20th century, are not without significance. Currently, conservators make every effort to preserve historical textiles for the future generations in the best

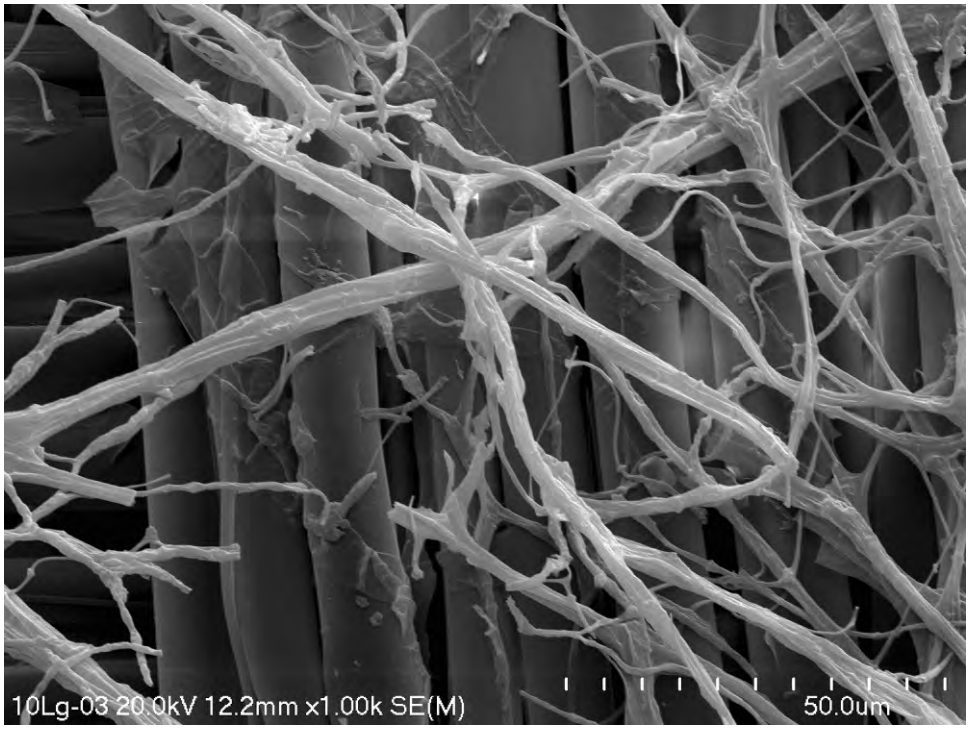

Fig. 8 Scanning electron micrograph of microbial growth on silk. Photo: T. Lech.

condition possible as they constitute unquestionable heritage and evidence of not only technological, but also artistic, religious and diplomatic activity of former communities.

As any historical materials, textiles, particularly those made of natural fibers, undergo constant deterioration. Processes of destruction and decomposition may be triggered by various environmental factors, such as UV exposure, too high or too low relative humidity and temperature, oxidation and hydrolysis processes, as well as destructive effects of microorganisms and insects. Textiles of natural fibers, most of them showing organic polymer structures, are particularly prone to biodeterioration. Microorganisms, both fungi and bacteria capable of producing appropriate enzymes, may use natural fibers as a source of carbon for their development. Initially, microbial growth on textiles might be associated with esthetic changes (staining or discoloration). Later, microorganisms may decrease fiber resistance and cause their thinning, thereby leading to their complete destruction. As has been mentioned earlier, active microbial growth is dependent upon specific environmental conditions: relative humidity, temperature, adequate pH or presence of carbon sources in the form of organic contaminations. Also, it must be underlined that any mechanical fiber defects that have occurred during their use and further storage have a significant impact on their susceptibility to be settled by microorganisms.

Silk items constitute a considerable part of historical textile collections. This is mainly caused by their lower susceptibility to biodeterioration. Silk is mainly made of fibril proteins creating long fibroin chains of a high crystallization degree. Fibroin filaments are connected with one another with sericin, which was usually discarded during silk production. Another textile of animal origin is wool. It is also composed of protein fibers derived from hair of various animal species. The structure of wool fibers is more cellular in nature, and it is mainly composed of keratin. However, the outer layer of these fibers is coated with a protein membrane that also contains waxes and fatty acids, which constitute additional protection of the fibers thanks to their hydrophobic properties. The most common bacteria isolated from animal-origin textiles include strains of the following genera: *Aeromonas*, *Bacillus*, *Micrococcus*, *Pseudomonas*, *Proteus* and *Streptomyces*, whereas the most common fungi include: *Acremonium*, *Alternaria*, *Aspergillus*, *Chaetomium*, *Chrysosporium*, *Cladosporium*, *Ctenomyces*, *Fusarium*, *Penicillium*, *Phoma*, *Torulopsis*, *Trichoderma* and *Trichophyton*. Some microbial growth on silk is presented in the Fig. 8.

The main constituent of plant-origin textiles is cellulose. It is a polysaccharide, a linear cellobiose polymer, that is made up of a crystalline region, which is significantly more resistant to biodeterioration, and an amorphous region, which is more readily attacked by microorganisms and is a site where cellulose fiber deterioration usually begins. Microorganisms that are most frequently isolated from historical textiles of plant origin or places of their storage are: *Bacillus*, *Cellulomonas*, *Cellvibrio*, *Clostridium*, *Micrococcus*, *Peanibacillus*, *Pseudomonas*, *Streptomyces*, *Alternaria*, *Aspergillus*, *Chaetomium*, *Fusarium*, *Memnoniella*, *Myrothecium*, *Neurospora*, *Penicillium*, *Scopulariopsis*, *Stachybotrys* and *Trichoderma*.

An additional difficulty in preserving textile collections lies in the fact that a large part of such textiles, especially historical ones, e.g., liturgical vestments, are made of various natural fibers, which provides a source of nutrition for a vast range of microorganisms. These microbes may cooperate with one another and rapidly lead to textile destruction. Historical textiles are

stored in museums, galleries and private collections. However, lots of such items, being a unique collection of liturgical vestments, are stored in sacristies and church treasuries that are historical buildings themselves and not always assure appropriate storage conditions.

Composite Materials

Paintings

Paintings, including easel and mural objects, contain a wide range of organic and inorganic compounds, many of which are biodegradable by a large variety of microflora. Additives such as glue, emulsifiers, and thickeners are used to prepare the support, to facilitate the application of paint layers, or to increase the final esthetic object. In easel, the organic components of the paintings are sugars, gums, polysaccharides, proteins, oils, waxes, and undefined mixtures such as egg yolk. In mural paintings, pigments are mixed in oil and water, in the presence of casein or milk, in order to guarantee better adhesion on the plaster. This implies that microorganisms can grow inside different ecological niches on several substrates. The biological attack on these materials occurs in confined environments only under poor conservation conditions such as high humidity level, soil contact, poor ventilation, and rare maintenance operations. Moreover, the differences in the materials used and the chemical nature of the substrate may influence different evolution of microbial taxa colonizing works of art. Several studies on microbial succession in mural paintings show that bacteria can be considered among the first colonizers of frescoes, able, in some cases, to oxidize inorganic elements like lead present in pigments, and leading to the formation of lead oxides that cause damage by changing color (brown-black spot production). Moreover, the most frequent bacterial species present are members of the genus *Alcaligenes*, *Bacillus*, *Flavobacterium*, and *Pseudomonas*. Fungi normally are considered secondary colonizers. However, when frescoes in hypogean sites (tombs and grottes) are excavated, the predominant species isolated are members of the order Actinomycetales (genera *Streptomyces* and *Nocardia*); then, in very short time (months), photosynthetic bacteria, fungi, and algae become associated with the first colonizers, conferring typical colored crusts (green to black stain); these phenomena are quite evident after exposure to air, on illumination, and when hypogean rooms are open to visitors. Many are the mechanisms of aggression and microbial successions on paintings. Generally, microorganisms colonizing paintings and frescoes may be effected by environmental conditions and by the presence and concentration of atmospheric pollutants, especially sulfur dioxide. The sulfuric acid present in air containing moisture dissolves calcium carbonate in works of art (fresco, plaster, and stone) and leads to the precipitation of dihydrous calcium sulfate (gypsum). Gypsum deposition, by the formation of white crystal aggregates, is usually responsible for efflorescence, and for colonization by sulfur-oxidizing bacteria that cause direct and indirect mechanical damage such as the detachment of portions of painted layers. This is true if we consider that the death and lysis of these bacteria provide the organic substrate necessary for heterotrophic bacteria and fungi.

Photography

Photography is a method that produces a visible image by the interaction of light with silver halide salts contained in a binder applied to a substrate. Photographic materials include still and moving images and microfilms; many other different methods have been introduced and used since 1831. However, from a microbiological point of view, it is very interesting to know about the materials that photographic materials are made of. Photographic supports can be made of metals, for example, a copper plate covered with a fine silver layer as in the daguerreotype or a black varnished metallic plate as in the ferrotype; glass as for the collodium plate; paper, typical of calotype and albumin print; and, finally, semisynthetic and synthetic polymers, including cellulose nitrate, cellulose acetate, and polyethylene terephthalate. The light-sensitive materials are silver halides: silver chloride, silver bromide, and silver iodide. Other important photographic materials are binders such as collodium, which contains cellulose nitrate, albumin, and gelatin, sensitizers, antifoggants, and hardenings. Gelatin is the most satisfactory binder in terms of cost, chemical processing, and physical and optical features. Fungal attack is the main form of biodeterioration that occurs in photographic materials. The presence of substances of animal origin such as albumin and gelatin represent an important source of nutrition for microorganisms, in particular fungi that cause, for example, an increase in gelatin solubility in water. Thus, water or water solutions should not be used to clean photographic materials containing gelatin when there is a fungal attack. The use of cotton soaked in adequate film cleaner is suggested. In addition, the contact between fingers and photographic documents should be avoided as salts in the fingerprints absorb moisture and create favorable conditions for fungal growth. The Krieger collection includes thousands of glass plate negatives made in the years 1880–1926: Several glass negatives showed fungal contamination, and among the isolated fungi, *Aspergillus nidulans*, *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Penicillium cyclopium*, and *Penicillium janthinellum* were the most frequent.

Photographic prints were protected against microflora by immersing the prints for 3 min in 0.5% water solution of sodium salt of 4-chloro-3-cresol. For both black-and-white and color films and prints, fungicidal treatment by immersion in 1% solution of zinc fluorosilicate yields positive results. Use of activated film coating reduces scratches, fungal growth, and color dye fading, where humidity control of storage environment is not feasible. On microfilm objects based on cellulose acetate and polyester, the susceptibility to biodeterioration and subsequent isolation and identification of biodeteriogens showed the presence of *Aspergillus niger*. Different species and strains of fungi contaminate microfilms. One way to prevent fungal and insect attack is either to hermetically seal the photographs in laminated envelopes or to laminate/encapsulate the photograph in plastic. However, the best method to prevent fungal deterioration of photographic material is to keep it in a cool, dry, and dark place.

Magnetic tape, optical disk

The magnetic tape is made of a thin magnetic layer that comprises magnetic particles, such as iron oxide and chromium dioxide suspended in a lubricant, and a polymer binder (generally a polyester- or polyether polyurethane-based system). The most commonly used tape substrate is polyethylene terephthalate. Among several sources of damage (heat, light, grit, moisture, and atmospheric pollutants), microbial growth is considered a main concern for the preservation of magnetic media. The most frequently isolated fungi belong to the genera *Alternaria*, *Aspergillus*, *Chaetomium*, *Penicillium*, and *Stemphylium*, and these have been detected on floppy disks in tropical countries and also in temperate countries under humid conditions. Many of these microorganisms are widespread fungi that are able to grow on a variety of products. For the prevention of fungal growth on videotape, it is recommended that the interior of the tape container and the edges of the tape itself be checked for black, brown, and greenish stains that might indicate the presence of fungi. Moldy tapes should not be used until the microorganisms have been eradicated. Recent experiences suggest that the material should be placed in a desiccating container and after equilibrium is reached the surface should be vacuumed. After this treatment, the film can be cleaned with a chlorinated solvent cleaning machine. However, the use a UV source, except for some black and white films, is not recommended as UV damages dyes.

Compact disk (CD-ROM) and digital versatile disks (DVDs) introduced into the market are based on an improved polymer, made of aromatic polycarbonates, more durable than aliphatic. CD producers generally do not care about biological deterioration of CDs. However, a fungus of the *Geotrichum* type was able to grow on a common CD under tropical conditions (about 30°C and UR 90%). Biodeterioration occurred as bioturbation traces. A fractal structure and the destruction of the pits, which in turn means the loss of the information stored in the disk, were observed. It is worth noting that *Geotrichum* is a very common genus commonly found in food products, water, and animals, and it has also been isolated from air. Thus, fungal growth is not related to a particular geographical area but to the environmental conditions that permitted the growth.

Methods of Control and Prevention

The microbial contamination of cultural heritage is still an underestimated concern. One reason may be the difficulty in establishing a definitive causative relation between the biological agents and the damage to materials, especially in those cases where there is damage but not much biological growth.

The prevention of biodeterioration of a cultural heritage includes all the operations needed to avoid the growth of microorganisms and a consequent biological attack by biodeteriogen microorganisms on the materials composing the heritage. These so-called indirect methods do not act directly on the microorganisms but hinder or slow down biological growth, modifying where possible the environmental conditions so that they become unsuitable for biological growth. Because of the close environmental relationship and dependence between the biological species and the environment, the most efficient and easily realized methods for impeding undesirable growth act on the causal factors, particularly humidity, temperature, ventilation, light, that can inhibit or condition their presence. These factors cannot always be controlled, whereas it is possible to control the conditions of confined environments (museums, libraries, and churches). It is decidedly more difficult to control an external environment (monuments and archeological areas). In fact, whereas some parameters (light, humidity, and temperature) can theoretically be modified, nutritional factors present in the artwork when support the biological growth, cannot be changed without damaging the nature of the object itself. On the contrary, factors encouraging biodeterioration (dirt, dust, and deposits of varying kind) can be removed. It is not necessary to act on all the parameters that restrict biological growth and it is sufficient to lower or to raise just one of these until there is no increase. 'Direct' methods of biodeterioration control provide for direct action on the organisms, and this can vary.

Biochemical

Such methods use compounds of biological origin: Antibiotics (active at very low doses, but there is loss of effectiveness over time), enzymes (used especially for cleaning) and pheromones (used to induce males to leave the structures where they nest) are used for infestations in the deep layers of the material where biocides cannot penetrate.

Biological

These are methods that use antagonist or parasitic species to limit the growth of other animal or vegetable species. In the case of flora, phytophage insects can be used, while for animals, above all avifauna, the choice lies with predator species of eggs and the young to reduce the population density of the other species. Unfortunately, such actions are very difficult and are not usually used in the case of microorganisms with deteriorative potentials that might change and pose even greater threat to historical materials.

Physical

These methods foresee the use of X-rays with biocidal action or noxious gamma radiation and UV rays. Indeed, UV rays have low penetration and are thus not effective for alterations involving the interior of objects. The most problematic aspect of UV treatment

is bound to its ability to alter, from the chemical point of view, the treated materials (pigments, cellulose, and proteic materials) and to the difficulty of transporting the irradiating source *in situ*. Note that gamma rays have a greater biocidal activity than UV, but their application is limited to treating organic materials (paper, parchment, and wood), and is aimed more at resolving microflora disinfection problems than those connected to the presence of insects.

Mechanical

These methods foresee the removal of biodeteriogen organisms by the frequent use of manual instruments like scalpels, scrapers, spatulas, and so on. Nevertheless, they have the disadvantage of failing to guarantee results over the long term, in that it is very difficult to completely remove the vegetative or reproductive structures of the species present, at least without seriously damaging the surface of the substrate. In fact, on dealing with a fungal mycelium, a lichen thallus, or even with roots rather than a plant, it is rare for the colonization to be only superficial, so to avoid damage to the surface of the stone mechanical removal ends up being only partial. However, mechanical methods do have the advantage of adding nothing that can cause further degradation. Generally speaking, these methods can be used with chemical methods and can thus be very useful.

Chemical

Chemical methods foresee the use of synthetic chemical substances like pesticides and disinfectants, more generally called biocides. The requirements of products to be used in the conservation of artwork are high efficiency against biodeteriogens, absence of interference with the constituent materials, low toxicity for human health, and low pollution risk for the environment. Biocides must be employed with caution as they can react with the substrate and cause macroscopic effects like spots, yellowing and bleaching, and increased brilliance or opacity. In fact, the recent application of more accurate investigative methodologies showed how, in reality, many of these products produce negative collateral effects, though these are not always obvious. Furthermore, biocide substances can interact with other products used in previous restoration.

For protecting monumental stones, it is practically impossible to control weather variables, while for rock sites the construction of protective structures, preventing water runoff of surfaces, can effectively reduce biological growth.

In order to reduce biological risk for the organic objects maintained in confined environments, the control of temperature and relative humidity is frequently cited as the first step in environmental control especially in museums, archives, and libraries. Poor storage conditions impede their preservation. High relative humidity, for example, above 60%, along with high temperature encourage the growth of microorganisms. Damage to historical records due to bacteria and fungi can be reduced by limiting access of visitors to storage areas and monitoring the collections environment. However, it is worth noting that not only improper storage conditions but also disasters caused by water, such as broken pipes, leaking roofs, blocked drains and fire extinguishing, favor microbial growth, if a prompt action is not taken. In indoor environments, another important goal should be the monitoring of the microflora of the air throughout the year. This can then be used as a standard reference to determine changes that had happened in a building or a room. It is worth noting that a close similarity has been found between the aeromicroflora and the library and archival materials biodeteriogens. Another important issue is maintenance, such as dust removal; dust and dirt can contain spores and bacteria and provide the nutrients required for microbial growth.

No less important is the protection of workers from risks related to exposure to biological agents at work. In the past few decades, growing concerns about the use of chemical compounds have resulted in an in-depth evaluation of the effects of pesticides on human health and safety. Any process should be safe for the operator and the environment and no chemical residues should remain in the treated materials. The awareness of pesticides hazardous to human health and the environment has led to a search for alternatives. Among the main nonchemical treatment alternatives used for collections including freezing, gamma rays, microwaves, and modified atmospheres, only high-energy radiation can kill microflora. However, high-energy radiation is generally recognized as a harmful process for library and archival materials. Possible future alternatives can include biological control to stop the growth of harmful microorganisms and bioremediation/bio-recovery of cultural heritage. Of note is the fact that disinfection treatments are not neutral to historical materials and may cause further weakening of the structure of these frequently already deteriorated textiles. Moreover, further fiber weakening in combination with the elimination of microorganisms creates a niche that can be settled by microbes of even greater biodeteriorative potential. That is why decisions about any disinfection treatments should be made with caution and be based on both microbiological tests and the preservation status of a given item.

Molecular Methods in Microbial Hazard Identification

Microorganisms are capable of adaptation to various atmospheric conditions, thanks to which they can settle various environmental niches, even those unfavorable for development/life of organisms. Moreover, microorganisms may cooperate with one another, which additionally enhances their adaptation ability. That is why gaining full knowledge about the diversity and biodeteriorative potential of microbes settling historical objects or their storage environments is so important, particularly in protection of cultural heritage collections, i.e., historical items touched by time. Currently, there are a number of research methods that enable determination of a microbiological status of a given item and its storage environment. Despite their numerous advantages,

conventional microbiological methods aided with biochemical tests and light microscopy, which have been commonly used for years, do not allow full microbiological assessment. These methods are mainly based on culture techniques requiring adjustment of various environmental factors (temperature, humidity) or application of an adequate microbiological medium (pH, content of mineral salt, sugars, amino acids, etc.) in laboratory settings in order to grow the greatest spectrum of fungi and bacteria present in a test sample. Unfortunately, this is very difficult and, as noted in the literature, only 1%–5% of microorganisms present in a given environment can be isolated using culture methods. That is why it is vital to implement more accurate test methods in order to protect valuable collections of cultural heritage. This is where methods of molecular biology, in particular metagenomics, proteomics and metabolomics, can be helpful. The greatest advantage of the omics techniques is the ability to obtain a wide spectrum of information on, among others, microbial species diversity, their deteriorative potential or metabolic activity directly on the basis of samples collected from the environment. Tested material may be derived from samples taken from a variety of items, such as paper documents, parchments, textiles, leather, ceramics, glass, stone, wood etc., as well as samples of indoor air collected from storage rooms. Microbial species identification is usually performed by methods based on the polymerase chain reaction (PCR) technique. It enables amplification of selected fragments of genetic material. Genetic markers typically used for this purpose are DNA sequences conserved within ribosomal RNA (rDNA) gene: 16S rDNA is used for bacterial identification, and 18S rDNA for fungi. As for identification of filamentous fungi and molds, the non-coding internal transcribed spacer (ITS) sequences are also frequently used as a genetic marker. Their additional asset as a genetic marker is a large number of its copies in all fungi.

A slightly different research approach is creating genetic fingerprints enabling one to analyze biodiversity, trace genotypic structure changes in an environment, also including non-culturable microorganisms, and identify quantitative changes in microbial communities in a given environment. In the event of microbiological hazard to cultural heritage collections, the most common method is DGGE/TGGE (denaturing/temperature gradient gel electrophoresis). It consists in amplification of selected marker gene sequences (16S rDNA, 18S rDNA, ITS etc.), followed by separation of a PCR product mix in polyacrylamide gel with a denaturing agent. The outcome is a pattern of bands (a fingerprint), each of them corresponding to specific genotypes present in the environment. In this technique, the most common matrix is DNA obtained from the environment. Also, RNA can be used for this purpose, in which case the results include information on the expression of selected genes, e.g., those responsible for the production of cellulolytic or proteolytic enzymes. The usage of RNA as a matrix enables initial specification of microbial deteriorative potential in a given environment. Analyses using DGGE/TGGE have been successfully implemented in studies on biodeterioration of cultural heritage objects made of various materials, for example for testing diversity of microbial communities, including bacteria, cyanobacteria and actinomycetes and fungi. Environmental fingerprints can also be created using the following methods: SSCP (single strand conformation polymorphism), ARDRA (amplified rDNA restriction analysis), T-RFLP (terminal restriction fragment length polymorphism) and ARISA (automated ribosomal intergenic spacer analysis). In the recent years, the development of the next-generation DNA sequencing (NGS) techniques has been a breakthrough in metagenomic studies. Not only do these methods enable taxonomic identification of microorganisms in a test sample, but also allow phylogenetic and functional analysis of microbial communities. It must be emphasized, however, that methods based on next-generation sequencing deliver a vast amount of valuable information, which poses considerable challenges in its analysis and interpretation since advanced bioinformatics tools are needed. Nevertheless, research based on NGS and its further modifications will become the basis for investigations in the fields of ecology and molecular microbiology.

Protection of Cultural Heritage Collections

In the recent years, institutions protecting cultural heritage collections, such as museums, archives, galleries and libraries, have completely changed their attitude towards fulfilment of their crucial task. A considerably greater emphasis is currently put on undertaking preventive measures to protect historical objects from deterioration and damage. This approach is called preventive conservation. Among other things, it encompasses legal regulations and institutional principles, visitor movement management, sharing and loaning items, maintenance of appropriate climate conditions, minimization of biological hazards as well as ensuring appropriate protective containers and proper manner of storage. The monitoring of the inner climate should include, among others: Controlling relative air humidity, temperature, UV exposure, dust level (PM10 and PM2.5) or the presence of organic contamination. Furthermore, stable climate parameters are vital for assuring proper housing conditions of cultural heritage items. Such collections must be protected against abrupt temperature and humidity alterations in a short period of time. Also, periodical observation of items in order to detect alarming changes, particularly of the microbiological origin, is essential. Compared with remedial conservation, preventive conservation requires conservators to use knowledge and methods from the field of natural and technological sciences and to see collections as a whole, rather than as single items. The currently exercised approach to the protection of cultural heritage items is consistent with the saying "better prevent than treat," and all actions, including Integrated Pest Management, create a specific management system for the protection of historical collections.

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Biofilms and Disease: A Persistent Threat

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Introduction

Bacteria are our ever-present neighbors. In fact, most current estimates show that the number of bacteria on and in our body is much greater than the number of human cells in our body (Sender et al., 2016). Though most of our bacterial neighbors are benign, or even beneficial, bacteria continue to kill millions each year across the globe despite continuous spending on the development of new antibiotics. A major factor in the fight against pathogenic bacteria is the formation of these microbes into structured communities known as biofilms. Bacteria living in a biofilm are implicated in most chronic infections and can increase resistance to disinfectants up to 600-fold (Otter et al., 2015) and resistance to antibiotics up to 1000-fold when compared to their free-floating planktonic counterparts (Luppens et al., 2002; Rasmussen and Givskov, 2006; Mah and O'Toole, 2001). The leading sources of nosocomial infections, and the cause of 100,000 deaths per year in the United States, are collectively known as ESKAPE pathogens, consisting of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp. (Santajit and Indrawattana, 2016). It is no coincidence that each of these dangerous pathogens are known to produce biofilm (Santajit and Indrawattana, 2016).

What Is Biofilm?

Biofilm is a three-dimensional community of bacteria living within a secreted matrix of hydrated excreted extracellular polymeric substances (EPS). Biofilm matrix composition is 97% water by weight with polysaccharides, proteins, lipids, and extracellular DNA making up the remainder (Jamal et al., 2015). The structure and function of the biofilm is adaptable to a vast array of environments and stimuli with heterogeneity in differentiation and function among the bacteria residing within. The secreted matrix of the biofilm creates nutrient and oxygen gradients, resulting in localized differentiation patterns within the biofilm and functional differences between bacteria at the core of the biofilm and bacteria near the outer surfaces (Flemming et al., 2016). Moreover, extracellular DNA (eDNA) binds polysaccharide molecules to form filamentous tubules and pore structures that enable nutrients and water to enter the biofilm and facilitate the movement of cells within the matrix (Flemming et al., 2007). Enzymes embedded within the matrix degrade nutrients and organic compounds, which are then captured by charged polysaccharides for use by the cells residing in the biofilm (Flemming et al., 2016). The biofilm matrix also facilitates chemical signaling between encased bacteria.

Bacteria living within a biofilm are more protected from immune cells and predation by other organisms, more resistant to desiccation and environmental stresses, and more tolerant of antibiotics (Flemming et al., 2016). In many cases, biofilm matrix components bind to antibiotics, which deactivates or slows antibiotic diffusion into the biofilm; the net effect of this action is increased antibiotic resistance (Mah and O'Toole, 2001). Moreover, localized phenotypic changes within the biofilm lead to slower growth, reduced nutrient uptake, and a biofilm-specific phenotype that is recalcitrant to antibiotics (Mah and O'Toole, 2001). These phenotypic changes can be dramatic. In a study looking at the expression of proteins in a mature biofilm of *P. aeruginosa* compared to planktonic *P. aeruginosa*, only 40% of all proteins were expressed similarly in both groups (Sauer et al., 2002). As our understanding of bacterial biofilms has grown, we have learned that phenotypic changes can lead to development of highly-resistant cells deep in the biofilm called persister cells that greatly contribute to the biofilm antimicrobial recalcitrance (Mah and O'Toole, 2001; Gominet et al., 2017; Elgharably et al., 2016; Anderson and O'Toole, 2008).

How Does Biofilm Form?

The formation of a biofilm can be viewed in stages of development and maturation. Thus, it is helpful to think of "biofilm" as a process, rather than a discreet item.

Stage 1: Initial Reversible Attachment

Planktonic or free-floating bacteria come into contact with a surface. Lewis acid-base, electrostatic, and van der Waals interactions guide the initial attachment. A variety of factors influences this process including surface hydrophobicity and charge, pH, and hydrodynamic forces in the microenvironment (Kostakioti et al., 2013). This step is known as "reversible attachment" because detachment often occurs, and it is thought that the microbe is "sampling the surface" (Hoffman et al., 2015). Initial attachment can be mediated by secreted adhesins, flagella, and other surface molecules, depending on the microorganism (Kostakioti et al., 2013). Chemotaxis toward preferred nutrients may also influence the location of initial attachment (Anderson and O'Toole, 2008; Kostakioti et al., 2013).

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Stage 2: Irreversible Attachment

In this step, a microbe makes a commitment to the surface and remains bound. Irreversible attachment is most often aided by pili. For example, *Escherichia coli* use type I pili; these are assembled via the chaperone usher pathway (CUP), and the FimH adhesin on the end of the pilus mediates attachment (Anderson et al., 2003). *P. aeruginosa*, however, uses type IV pili with adhesin CupA mediating attachment (Vallet et al., 2001).

Stage 3: Maturation I—Aggregation into Microcolonies

Adhered bacteria secrete EPS to aggregate neighboring bacteria into discrete clusters called microcolonies (Kostakioti et al., 2013). EPS also connects surrounding microcolonies into a thin layer across the adhered surface. This activity makes the surface more conducive to attachment by additional, planktonic bacteria (Huang et al., 2011).

Stage 4: Maturation II—Three-Dimensional Biofilm Formation

As the biofilm continues to grow, eDNA binds to EPS to form a three-dimensional architecture, which gives the biofilm tensile strength and increased rigidity. Many species use quorum sensing signaling between cells to direct phenotypic differentiation in a cell density dependent manner (Flemming et al., 2007; Nealson et al., 1970; Gambello and Iglewski, 1991; Jennings et al., 2015).

Stage 5: Dispersal

The last stage of the biofilm process is the dispersion of some of the cells from the existing community to ensure the survival and propagation of the bacteria. This process can be broken down into two basic modes: active and passive. In an active mode of dispersal, the bacteria respond to community and environmental cues to break down the biofilm matrix and allow the release of cells that can then transit to new locations. Passive dispersal, however, is a result of the environmental forces, such as external organism interaction, fluid shear, or abrasion, dislocating a portion of the biofilm through mechanical disruption (Kaplan, 2010).

Where Do We Find Biofilms?

Biofilms form throughout nature on living and non-living surfaces, both environmental and human-fabricated (Donlan, 2002). Of particular note, microbes often form biofilms during infection (Donlan, 2002). In fact, it is estimated that 80% of all human infections involve a biofilm component (NIH, 2002). We focus on these biofilm infections here, describing biofilms that form within and on tissues and those that form on medical devices.

Tissue-Associated Biofilms

Burn Wounds

Each year, 40,000 individuals in the United States are hospitalized with burns (McDermott et al., 2013). Studies indicate annual expenditures in the United States of $1.5 billion for burn wound-associated medical costs and $5 billion in costs related to lost work (McDermott et al., 2013). In a burn, thermal destruction of the skin leaves damaged tissue exposed and prone to infection. Moreover, massive tissue damage results in initial release of an abundance of humoral factors like cytokines, leukotrienes, and prostaglandins that cause an immunosuppressive state in the patient, further increasing the risk of wound colonization and potential systemic infection (Polavarapu et al., 2008). Infection is the leading cause of death in burn wound patients (Polavarapu et al., 2008; Gomez et al., 2009; Ramakrishnan et al., 2016) and biofilm formation is highly associated with mortality (Ramakrishnan et al., 2016). Biofilm growth within the wound bed subsequently promotes a chronic inflammatory state that further impairs wound healing by reducing re-epithelialization and inhibiting the in-growth of granulation tissue (Gurjala et al., 2011). Moreover, burns often lead to formation of eschar, a portion of tough, inelastic, necrotic tissue that forms over the wound. Plasma and wound exudate collect underneath the eschar, putting pressure on the remaining vasculature, impairing circulation to the wound bed and areas distal to the injury (Tiwari, 2012). The ischemic wound bed thus created encourages the formation of biofilm (Seth et al., 2012).

Most infections in patients with burns are caused by several major bacteria: *P, aeruginosa*, *S. aureus*, *Streptococcus* sp., and *Acinetobacter*. These bacteria are each associated with biofilm production and antibiotic resistance to one or more agents (Gomez et al., 2009; Ramakrishnan et al., 2016). *P. aeruginosa* is particularly dangerous due to the development of resistance to systemic antibiotics as well as resistance to many topical agents used to prevent or treat burn wound infections.

Treatment for burn wound infections involves an array of modalities. Primary treatment in a severe burn is the debridement of damaged tissue, including coagulated proteins, to create a clean wound bed with tissue that is vascularized; this action promotes wound healing and inhibits infection (Polavarapu et al., 2008). Next, topical antibiotics and/or silver dressings are administered to the wound bed (Metcalf and Bowler, 2013). Systemic antibiotics are not recommended initially as they are ineffective at preventing wound sepsis due to limited vascularization of damaged tissue and instead promote the development of drug-resistant infections.

Table 1 Selected antibiofilm treatment schemes

Novel treatment approach	*Prevention or intervention*	*How does it work?*
Probiotic microorganisms	Prevention	Nonpathogenic microbes out-compete pathogens for nutrients and binding sites (Polavarapu et al., 2008; Delcaru et al., 2016; Soto, 2014)
DNase treatment	Intervention	DNase is used to break down the eDNA that provides structural integrity to a biofilm (Aung et al., 2017; Gnanadhas et al., 2015)
Mucinase treatment	Intervention	Mucinase enzymatically degrades mucoid biofilms (Chandki et al., 2011; Henke and Ratjen, 2007)
Antimicrobial peptides	Both	Antimicrobial peptides can be used prophylactically to prevent microbial growth or can be administered post hoc to attack established biofilms (Sevgi et al., 2013; Wang et al., 2017)
Inorganic metal derivatives (e.g., Silver compounds)	Both	Antimicrobial properties of certain metals can be utilized to prevent infection as well as to treat established infections (Sevgi et al., 2013; Connaughton et al., 2014)
Plant extracts	Both	Extracts inhibit microbial growth and reduce the formation of biofilm (El-Ganiny et al., 2017; Delcaru et al., 2016)
Quorum sensing inhibitors	Both	Inhibitors target the signals that bacteria use to coordinate biofilm formation (Elgharably et al., 2016; Delcaru et al., 2016; Kalanuria et al., 2014)
Diazeniumdiolate compounds	Both	Diazeniumdiolate compounds release NO upon contact with bacteria resulting in biofilm dispersal (Connaughton et al., 2014)

However, such antibiotics are given if sepsis develops (Polavarapu et al., 2008). Novel treatment strategies for biofilm infections of burn wounds include administration of probiotic beneficial microorganisms (Polavarapu et al., 2008), antimicrobial and anti-biofilm peptides (Sevgi et al., 2013; Wang et al., 2017), inorganic metal derivatives, halogens, chitosan, XF porphyrins, phage therapy, and light-mediated therapies (Sevgi et al., 2013) (Table 1).

Keratitis

Keratitis is inflammation of the cornea, usually caused by a bacterial infection, but fungi, protozoa, or viruses can also induce this ailment (Collier et al., 2014; Al-Mujaini et al., 2009). Keratitis is a major cause of corneal opacity and is among the leading causes of blindness (Collier et al., 2014; Al-Mujaini et al., 2009; Bispo et al., 2015). Infection can lead to complete destruction of the cornea in 1–2 days (Al-Mujaini et al., 2009). Contact lens usage is the major risk factor, associated with 56% of bacterial keratitis cases, with poor hygiene and care also precipitating many of the infections (Collier et al., 2014; Zegans et al., 2002). Extended-wear lens users have a much higher incidence of keratitis than daily-wear users: 20.9 per 10,000 people versus 4.1 per 10,000, respectively (Poggio et al., 1989). Prevalence of bacterial keratitis is significantly higher in developing countries (e.g., 11 per 100,000 persons in the United States vs. 799 per 100,000 persons in Nepal) and incidence is rising proportionally as the number of people using contact lenses increases across the world (Al-Mujaini et al., 2009). Keratitis causes nearly 1,000,000 doctor office visits each year resulting in $175 million in associated medical costs and 250,000 h of clinician time (Collier et al., 2014).

While in the eye, proteins and lipids from the tear fluid adsorb to the surface of the lens creating a conditioning layer on the lens that facilitates the binding of bacteria to the surface (Bispo et al., 2015; Zegans et al., 2002). Bacterial keratitis is primarily caused by three genera, all known biofilm formers: *Staphylococcus, Streptococcus,* and *Pseudomonas* (Al-Mujaini et al., 2009). It is thought that biofilm formation on either the abiotic contact lens or the eye itself is what allows the bacteria to persist, thrive, and ultimately infect the eye (Bispo et al., 2015; Zegans et al., 2002). A study in rats observed that contact lenses inoculated with planktonic *P. aeruginosa* caused keratitis in approximately 7 days while contact lenses taken from another infected rat with an adherent *P. aeruginosa* biofilm caused a keratitis in 2 days (Tam et al., 2010). In vivo adaptation of the bacteria, through biofilm formation, resulted in enhanced pathogenesis of the bacteria and reduced the time from inoculation to infection.

There are a variety of ways to prevent or reduce the risk of bacterial keratitis including switching from extended-wear to daily-use contact lenses (Bispo et al., 2015), switching from soft to rigid contact lenses (Al-Mujaini et al., 2009; Bispo et al., 2015), improving hygiene (including hand washing and rubbing of lenses with contact lens solution) (Bispo et al., 2015; Wagner et al., 2014), and more frequent replacement of contact lens cases (Bispo et al., 2015). Between the 1970s and the 1990s, heat disinfection was used to clean contact lens cases. Since then, heat disinfection has become much less prominent as users have switched to contact lens solution (Willcox et al., 2012). Initial studies showed that using a combination of heat and contact lens solution to disinfect the cases significantly decreases viable biofilm bacteria and substantially improves disinfection compared to each method used individually (Willcox et al., 2012). Continued research is needed to develop optimal disinfection procedures to prevent biofilm formation.

Treatment for bacterial keratitis usually involves antibiotic therapy with 4th generation fluoroquinolones, like gatifloxacin or moxifloxacin (Al-Shehri et al., 2009). Several naturally-derived methods have been examined to remove biofilm growth from contact lenses and contact lens cases (Table 1) including using plant extracts from *Calendula officinalis* and *Buddleja salviifolia*, each of which showed nearly complete inhibition of biofilm formation (El-Ganiny et al., 2017). Another proposed approach uses DNase treatment in concert with antibiotic therapy. DNase targets eDNA within the biofilm matrix, thus reducing the structural integrity of the biofilm making the cells within more susceptible to antibiotic therapy (Aung et al., 2017).

Chronic Otitis Media

Chronic otitis media (COM) affects between 65 and 330 million people worldwide, with many under the age of 5 (Mittal et al., 2015). 80% of children less than 3 years old have had at least one incident of otitis media, a portion of which will advance to chronic otitis media (Mittal et al., 2015). Hearing loss is a common complication of COM, which can result in speech and language deficits and problems in school, and hearing loss can be permanent in some cases (Mittal et al., 2015). A study using light and transmission electron microscopy found that 100% of samples taken from patients with COM with exudate showed microbial biofilm formation (Akyildiz et al., 2013). Another study using scanning electron microscopy to analyze tissue samples from patients with COM found that 70% showed biofilm formation on tissues excised during surgery (Kaya et al., 2013). Combined, these studies indicate a pivotal role of biofilms in the pathogenesis of COM.

In cases of COM, biofilm formation is typically located in the middle ear mucosa and less frequently on the mastoid (Kaya et al., 2013; Lampikoski et al., 2012). Often, biofilms form on cholesteatomas, or abnormal skin growths in the ear that can inhibit drainage and function of the ear (Kaya et al., 2013). In COM, infection is most often caused by the biofilm-forming bacteria *P. aeruginosa*, *S. aureus*, and *E. coli* (Mittal et al., 2015; Akyildiz et al., 2013). Despite topical antimicrobial concentrations that far exceed the minimum inhibitory concentrations (MICs) of even the most resistant strains, COM is often recalcitrant to antibiotic therapy (Akyildiz et al., 2013). The interplay of biofilm formation, structural, and anatomic changes, like cholesteatoma formation due to chronic infections, and earwax accumulation may interfere with successful delivery of the antibiotics (Mittal et al., 2015; Akyildiz et al., 2013; Lampikoski et al., 2012).

Treatment for COM often involves aural toilet to remove ear wax, mucus, desquamated epithelial cells, and biofilms through mechanical disruption. This allows for better penetration and more effective delivery of topical antimicrobial agents. The most commonly used antibiotics are aminoglycosides (neomycin, gentamicin, tobramycin) or fluoroquinolones (ciprofloxacin) (Ahmad, 2013). Antibiotics are frequently combined with a cationic detergent like polymyxin B to improve penetration (Miro, 2000). Concurrent use of topical corticosteroids like dexamethasone is also common as the corticosteroids reduce inflammation and the growth of granulation tissue, allowing for greater penetration (Mittal et al., 2015). In children, tympanostomy tubes are often placed to improve drainage and ventilation of the ear canal (Mittal et al., 2015; Vlastarakos et al., 2007). Surgery can be used to correct defects in situations involving patients with abnormal anatomy of the ear (Mittal et al., 2015).

Dental Biofilms

The human oral cavity is home to over 700 different bacterial species (Huang et al., 2011). These bacteria live on many different surfaces in the mouth: from teeth, tongue, and gums, to the oral mucosal surfaces (Huang et al., 2011). Most of these bacteria are harmless and actually serve a beneficial role to the health of the mouth, while other species are connected to infection and invasive disease. It is the competition and complex interaction of healthy microbiota with pathogenic varieties within oral biofilms that can lead to disease (Huang et al., 2011; Hojo et al., 2009; Dewhirst et al., 2010). Beneficial microbes maintain good oral health by excluding more harmful bacteria through several mechanisms (Huang et al., 2011; Hojo et al., 2009). Healthy bacteria are thought to inhibit pathogens by secreting antimicrobial compounds like peroxides or bacteriocins that inhibit nearby pathogens. Additionally, these microorganisms can outcompete pathogens for nutrients and attachment sites (Hojo et al., 2009). Oral dysbiosis, characterized by proportional changes in the ratios of pathogens to non-pathogens in the mouth, has been shown to cause several disease states including periodontitis, tooth decay, tonsillitis, and endodontic infections (Huang et al., 2011; Hojo et al., 2009; Dewhirst et al., 2010). Often, pathogenesis is dictated not by the mere presence of pathogenic bacteria, but rather by the relative abundance of pathogenic bacteria compared to beneficial bacteria, with disease occurring during states of imbalance (Huang et al., 2011). Moreover, the oral microbiota can cause disease states outside of the oral cavity. There are many links between oral health and heart disease, atherosclerosis, and endocarditis (Dewhirst et al., 2010). Thus, good oral health is important for maintaining overall human well-being. Importantly, the persistence of bacterial species within the oral cavity is attributable to biofilm formation. In fact, over 90% of the bacteria in the mouth (both pathogenic and beneficial) live within a mixed-species biofilm on various oral surfaces. A greater understanding of these processes can lead to improved health.

There are interesting correlations between oral disease state and particular types of microorganisms. Cariogenic bacteria, like *Streptococcus mutans*, tend to be acidogenic and Gram-positive. In a healthy, non-cariogenic environment, levels of *S. mutans* are low, whereas cultures from active caries tend to show high levels of *S. mutans* (Chandki et al., 2011). Periopathogenic bacteria, like *Porphyromonas gingivalis*, however, are more likely to stain basophilic and Gram-negative (Chandki et al., 2011). The composition of bacteria within a dental biofilm changes as the biofilm matures. Early colonizers, or pioneers, tend to be *Streptococci* sp., *Neisseria* sp., and *Rothia* sp. (Heller et al., 2016). These bacteria have special adhesins and surface lectins that allow them to bind more readily to the enamel surface of teeth as well as to the layer of surface-bound proteins and lipids that quickly adsorb to the tooth surface when exposed to saliva (Heller et al., 2016). As a dental biofilm matures, additional species accumulate, including *Actinomyces*, *Corynebacterium*, *Fusobacterium*, and *Veillonella* (Heller et al., 2016). *P. gingivalis* is particularly adept at binding to these early colonizers in the supragingival tooth surface and spreading into the subgingival pocket where it can form a nidus of infection that allows the bacteria to persist despite attempts at eradication (Kuboniwa and Lamont, 2010).

Management of oral biofilms traditionally involves regular brushing and flossing of teeth, use of antiseptics and fluoridated mouthwash, and regular cleanings at the dentist that may involve scaling or root planing of the tooth surface to disrupt biofilm

attachment (National Institute of Dental and Craniofacial Research, 2013). In a disease state, topical antibiotics like chlorhexidine and doxycycline may be used either as a rinse or gel (National Institute of Dental and Craniofacial Research, 2013). Systemic antibiotics like penicillin, vancomycin, or erythromycin, as well as enzymatic degradation of dental calculus using mucinase, pancrease, or lactoperoxidase hypothiocyanite, are also used (Chandki et al., 2011). More recently, biofilm-disrupting synthetic peptides have been explored as a novel treatment for unhealthy biofilm growth in the mouth (Table 1) (Wang et al., 2017).

Infective Endocarditis

Infective endocarditis (IE) is a serious condition involving infection of the endocardium that can cause severe damage to the heart and heart valves (Elgharably et al., 2016). As people are living longer, and more cardiac devices and prosthetic valves are being implanted, the rate of IE has increased significantly in the last 30 years (Forestier et al., 2016). Precipitating the development IE, bacteria in the bloodstream bind to damaged cardiac tissues and create biofilm communities called vegetations (Hoen and Duval, 2013). These bacteria can come from the oral cavity (as discussed above), from infections at distal locations, and from contaminated implanted medical devices (we discuss device-related biofilm infections below), among other sources. *Streptococci*, *Staphylococci*, or *Enterococci* cause 80% of IE cases, and, importantly, each of these genera are known biofilm-formers (Elgharably et al., 2016). Studies looking at bacterial isolates from IE lesions showed that IE isolates were significantly more likely to produce biofilm than non-pathological isolates of the same species, indicating an important role for the biofilm process in cardiac infection (Nallapareddy et al., 2006). Attachment to the endocardium is mediated by fibronectin and polysaccharides (Elgharably et al., 2016). Often, biofilm is adhered to fibrin-platelet plugs that give structure to vegetations (Elgharably et al., 2016; Hoen and Duval, 2013). Cardiac vegetations can mechanically interfere with valve function. Additionally, parts of the vegetation can break off and cause infections elsewhere in the body or block the flow of blood, leading to systemic infections, stroke, heart failure, or meningitis (Elgharably et al., 2016; Hoen and Duval, 2013). IE is thus a very dangerous condition with a 20% mortality rate in year one and 40% over five years (Hoen and Duval, 2013).

Current treatments for IE consist of intravenous antibiotics. However, IE is often recalcitrant to antibiotics likely due to phenotypic changes of the bacteria within the biofilm and creation of highly-resistant persister cells deep in the biofilm (Elgharably et al., 2016). Treatment failure due to antibiotic resistance often necessitates surgical removal of infected tissue and/or prosthetic valves or other cardiac implants, as well as radical debridement of the area, imposing significant risks and costs on the patient and medical system (Elgharably et al., 2016; Hoen and Duval, 2013). New strategies to treat IE include antibiofilm peptides and quorum sensing inhibitors, among others (Table 1) (Elgharably et al., 2016; Reffuveille et al., 2014).

Urinary Tract Infections

Urinary tract infections (UTIs) affect 150 million people across the world annually, necessitating 12–14 million medical visits in the United States each year (Flores-Mireles et al., 2015). As the reason for nearly 1% of all doctor visits and costing $3.5 billion per year in the United States alone, UTIs represent a major public health issue (Flores-Mireles et al., 2015; Stamm and Norrby, 2001). Bacterial biofilms are thought to be responsible for recurrent and persistent infections, particularly nosocomial infections and catheter-associated UTIs (CAUTI; discussed further below) (Delcaru et al., 2016). Over a quarter of patients who develop a UTI will suffer recurrences (Stamm and Norrby, 2001). Moreover, many suffer additional complications, including kidney damage, pyelonephritis with sepsis, and pre-term birth in pregnant women (Flores-Mireles et al., 2015; Stamm and Norrby, 2001). Long-term antibiotic use to treat persistent UTI can result in *Clostridium difficile* infections, such as antibiotic-associated diarrhea and pseudomembranous colitis (Flores-Mireles et al., 2015).

Most UTIs (80%) are caused by *E. coli*, particularly uropathogenic *E. coli* (UPEC) strains (Anderson et al., 2003, 2004; Flores-Mireles et al., 2015; Delcaru et al., 2016). *Proteus mirabilis*, *K. pneumoniae*, *P. aeruginosa*, *Enterococcus* sp., *Enterobacter* sp., group B *Streptococcus*, and *Staphylococcus saprophyticus* are also found as causative agents, albeit less frequently (Anderson et al., 2003). Bacteria use a variety of strategies to colonize the urinary tract, including adhesin-displaying pili to mediate attachment to epithelial cells (Anderson et al., 2004). A study of *E. coli* strains collected from patients experiencing relapse and recurrence of UTI showed that 74% of bacterial isolates from relapsing UTI formed biofilms in vitro compared with 42% from other recurrent infections, indicating an important role for biofilm in the pathogenesis of UTIs (Soto et al., 2006). Moreover, in addition to extracellular biofilms on urinary catheters, it has been shown that UPEC forms intracellular biofilms in the epithelium of the bladder, which may be a key persistence mechanism that enables their recalcitrance to treatment in some patients (Anderson et al., 2003).

Treatment for UTI typically involves oral antibiotics, however many cases recur despite antibiotic treatment (Delcaru et al., 2016). Home treatment for UTIs frequently entails cranberry juice and cranberry juice extract capsules. Cranberries contain proanthocyanidins and polyphenols which have shown antibiofilm properties in vitro (Liska et al., 2016). Cranberry-based treatments reduce the risk of symptomatic, recurrent UTIs, however the exact mechanism of activity remains unclear (Wang et al., 2012). There are a variety of novel treatment strategies for difficult-to-treat UTIs (Table 1) including plant extracts like *Rosmarinus officinalis*, *Salvia officinalis*, and *Mentha piperita* which can inhibit a biofilm lifestyle while preventing antimicrobial resistance (Delcaru et al., 2016). Other approaches include using nanoparticles to penetrate biofilms, using biofilm-targeting bacteriophages, and quorum sensing inhibitors to target the signals that lead to biofilm production (Delcaru et al., 2016).

Cystic Fibrosis Lung Infection

Cystic fibrosis (CF) is a disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which forms chloride channels. This mutation causes problems throughout the body including gastrointestinal disorders, fertility problems in males, and most notably progressive lung function decline (O'Sullivan and Freedman, 2009). The decline in lung function is due to increased viscosity of lung secretions which creates a niche within the lungs for pathogens to thrive (O'Sullivan and Freedman, 2009). CF is the most common inherited lethal disorder among people with European descent, affecting 1:2500–1:3000 births in this population (O'Sullivan and Freedman, 2009; Scotet et al., 2012). Life expectancy for people with CF has nearly doubled over the last two decades due to advances in medication, physical therapy modalities, and improved understanding of the disease (O'Sullivan and Freedman, 2009; Scotet et al., 2012). However, lung infections, particularly involving biofilm-forming pathogens, remain the leading cause of death among people living with CF (O'Sullivan and Freedman, 2009).

In the CF lung, there are a variety of environmental cues that promote biofilm infections, including: thickened lung secretions leading to hypoxic conditions within pooling mucus, reduced ciliary clearance, chronic inflammatory immune response, and high antibiotic levels (O'Sullivan and Freedman, 2009; Hoiby et al., 2017; Anderson et al., 2008). Immune response is mediated by polymorphonuclear leukocytes, which release their DNA when they die, further contributing to the increased viscosity of lung secretions and providing additional structure to biofilm formations in the lungs (O'Sullivan and Freedman, 2009; Hoiby et al., 2017). High antibiotic levels have also been shown to influence biofilm formation in vitro (Anderson et al., 2008). Infections are most commonly caused by *P. aeruginosa*, *S. aureus*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, and atypical mycobacterium (O'Sullivan and Freedman, 2009; Hoiby et al., 2017). *P. aeruginosa* is one of the most concerning CF lung pathogens, and patients that successfully avoid *P. aeruginosa* infections tend to have a significantly increased life expectancy (O'Sullivan and Freedman, 2009; Hoiby et al., 2010). 98% of young children with CF show signs of infection with this microorganism (Burns et al., 2001). *P. aeruginosa* is adept at forming biofilms within the CF lung, which makes the infection dramatically more difficult to treat with antibiotic therapy. Indeed, once a chronic *P. aeruginosa* biofilm infection has taken hold, it is nearly impossible to eradicate (Hoiby et al., 2010).

Treatment and prevention of CF-related infections, including biofilms, typically involve physical therapy to improve airway clearance, bronchodilators, mucolytic therapy such as dornase alfa, hypertonic saline solution to hydrate lung secretions, molecules to potentiate or correct CFTR function (such as Ivacaftor), and antibiotic therapy (O'Sullivan and Freedman, 2009; Condren and Bradshaw, 2013; Henke and Ratjen, 2007; Cutting, 2015; Claudius et al., 2015). Novel treatment strategies for CF-related biofilms (Table 1) include L-methionine in conjunction with antibiotics; L-methionine upregulates DNase activity, which degrades the structural integrity of the biofilm by attacking eDNA (Gnanadhas et al., 2015). Another new treatment approach involves using nitric oxide to disrupt biofilms by increased phosphodiesterase activity, resulting in a decrease in the biofilm regulatory molecule cyclic-di-GMP (Howlin et al., 2017). However, one of the most effective ways to conquer *P. aeruginosa* biofilms is to prevent it in the first place. Early, aggressive antibiotic therapy during the initial acute colonization of the lungs, when the bacteria are far more susceptible to antibiotics, can prevent over 80% of chronic *P. aeruginosa* infections (Hoiby et al., 2010).

Implant- or Device-Associated Biofilms

Prosthetics

As the population ages in the developed world, the number of total joint arthroplasties (TJA) is greatly increasing (McConoughey et al., 2014; Maradit Kremers et al., 2015). Over 1 million TJA procedures are performed each year in the United States (Maradit Kremers et al., 2015). Among patients who receive TJA, infection is a major cause of surgical revision (McConoughey et al., 2014; Pandey et al., 2000). Infection of a prosthetic joint is estimated to cost $50,000 per incident (Connaughton et al., 2014).

Infection of an artificial joint stems from several sources: microbes that were on the surface of the implant, microbes introduced by the surgical team or surgical environment, and through hematogenous spread. The most common bacteria involved in orthopedic infections include: *Staphylococcus* sp., *Enterococcus* sp., *Klebsiella* sp., *Pseudomonas* sp., and *Acinetobacter* sp. (Pandey et al., 2000; Fernandes and Dias, 2013). Biofilm formation on the surface of the implant makes the constituent bacteria more resistant to sterilization pre-implantation and much more resistant to antibiotic treatment in vivo. Moreover, the lack of vascularization of the tissues at the surface of the implant dramatically reduces antimicrobial penetration into the biofilm (Pandey et al., 2000; Fernandes and Dias, 2013; Lamagni et al., 2015).

Treatment for orthopedic infections primarily involves antibiotic therapy. However, the difficulty in treating biofilm infections in an avascular environment often necessitates surgical revision to remove the infected implant (Lamagni et al., 2015). For this reason, prevention of infection is most effective. Improved aseptic surgical techniques have greatly improved outcomes (Fernandes and Dias, 2013), and several novel techniques have been explored to further reduce the risk of biofilm infections in TJA. Changes to the implant surface chemistry or pretreatment with antimicrobial substances can inhibit colonization of the surface. A variety of surface treatments are being pursued (Table 1), including a polyethylene oxide coating, silver or copper ions, metal nanoparticles or nanotubules, impregnation of the implant with antimicrobial peptides, diazeniumdiolate coatings that release nitric oxide, and altering the implant to generate a hydrophilic surface charge (Connaughton et al., 2014).

Central Venous Catheters

Central venous catheters (CVCs), also known as central line catheters, are used to deliver medications, blood products, or nutrition directly into the bloodstream of chronically ill or critically ill patients over a long period of time. CVCs can be kept in place longer than standard intravenous catheters, so they are ideal for patients receiving treatment modalities requiring long-term intravenous therapy. However, CVCs also form a portal from the environment directly to the bloodstream and are associated with a significant risk of bloodstream infection (Yousif et al., 2015). CVCs are the leading cause of bacteremia among patients who have been hospitalized (Gahlot et al., 2014). There are between 250,000 and 500,000 central line-associated bloodstream infections (CLABSIs) each year in the United States (Yousif et al., 2015; Maki et al., 2006). These infections increase length of hospitalization by 10–20 days and result in up to $2.5 billion in medical costs in the United States each year (Yousif et al., 2015; Shah et al., 2013). Importantly, CLABSIs increase mortality of hospitalized patients, to 15%–25% (Maki et al., 2006).

Most CLABSIs are caused by bacteria from the patient's skin, although contamination can occur from the hub site, hematogenous spread from another infection site, or, rarely, from administering contaminated products through the catheter (Gominet et al., 2017; Gahlot et al., 2014; Shah et al., 2013). The organisms most likely to cause infections are *Staphylococci*, Gram-negative bacilli, enterococci, and yeast, with *S. aureus* the most prevalent etiological agent (Shah et al., 2013). Biofilm formation on the catheter can begin less than 24 h after insertion and protects the bacteria from host immune attack and from antibiotic therapy (Gominet et al., 2017). As the biofilm matures, the dispersal process begins, causing bacteremia and sepsis as pathogens are shed from the community directly into the bloodstream. Many bacteria remain behind, though, including durable persister bacteria, to maintain the localized infection (Gominet et al., 2017; Shah et al., 2013).

Strategies to combat CLABSIs begin with prevention of the underlying contamination, with hygiene and maximal sterile barrier technique playing a very important role (Gominet et al., 2017). Most infections are treated with intravenous antibiotics, often through the contaminated catheter if the catheter is still in-place (Shah et al., 2013). These antibiotic lock solutions are a good strategy to salvage necessary catheters that have been contaminated. The process involves filling the catheter with highly-concentrated antibiotic solutions; the high concentration is necessary to attack biofilm-state cells (Gominet et al., 2017). However, despite attempts to eradicate already growing biofilms on catheters, removal of the catheter is still considered the most effective way to respond to CLABSIs due to the recalcitrance to any eradication efforts (Gominet et al., 2017). Other ideas to prevent CLABSIs include impregnating catheters with silver compounds, antimicrobial drugs, or antimicrobial peptides to inhibit initial biofilm formation (Table 1) (Gominet et al., 2017).

Urinary Catheters

Catheter-associated urinary tract infections (CAUTIs) are the most common nosocomial infections (Trautner, 2010). CAUTI is associated with risk of acute renal inflammation and is one of the largest drivers of antibiotic use (Trautner and Darouiche, 2004). Hospital wards that operate without catheters showed a 90% drop in antibiotic usage (Trautner and Darouiche, 2004). Furthermore, antibiotics are often ineffective in treating most CAUTIs as many infections are caused by biofilms growing on the lumen of the catheter itself, which lacks any vascular perfusion that would normally deliver antibiotics to an infection site (Trautner, 2010; Trautner and Darouiche, 2004). Catheterization, by definition, undermines the best defenses against infection by forming an open portal through which bacteria can enter the urinary tract (Hamill et al., 2007). Moreover, catheterization causes damage to adjacent epithelial tissues, which can enhance susceptibility to additional infections (Trautner and Darouiche, 2004). It is estimated that the cost of CAUTIs in the United States and Europe is nearly $3 billion per year (Hamill et al., 2007). Moreover, catheterization is strongly correlated with increased mortality (Trautner and Darouiche, 2004; Hamill et al., 2007).

CAUTIs are most commonly caused by Gram-negative species, with *E. coli* the most common (causing nearly 80% of all UTIs) (Trautner and Darouiche, 2004; Hamill et al., 2007; Mobley et al., 2009), followed by *Proteus mirabilis*, *P. aeruginosa*, and *K. pneumoniae* (Hamill et al., 2007). Gram-positive bacteria like *Staphylococcus epidermidis* and *Enterococcus faecalis* are also known to cause infections (Hamill et al., 2007). Urine is an excellent medium for *E. coli* and it enhances the growth of bacteria on catheters as the bacteria are provided a continuous supply of fresh urine (Mobley et al., 2009). Moreover, UPEC strains have developed an array of virulence factors that facilitate UTI progression. The genome for UPEC strains is up to 1 Mb larger than other *E. coli* strains (Lloyd et al., 2007), and much of this additional genome encodes for attachment factors, host defense protection mechanisms, several antimicrobial resistance elements, iron-capture mechanisms, and more that help the cells adapt to the unique environment of the urinary tract (Anderson et al., 2004; Lloyd et al., 2007).

There are a variety of strategies to combat CAUTIs, particularly biofilm infections caused by indwelling catheters. The primary method is prevention (Trautner, 2010; Trautner and Darouiche, 2004). Reducing the rate and duration of catheterization can dramatically reduce the number of infections (Trautner and Darouiche, 2004). Regular changing the catheter and moving toward a closed system with a fused tube connected to the collection bag can help reduce the risk of infection (Trautner, 2010; Hamill et al., 2007). Going further, impregnating catheters with antibiotics, silver compounds, bacteriophages, nitric oxide, or urease-inhibitors has the potential to reduce the risk of colonization of the catheter itself (Delcaru et al., 2016). Other treatments being explored include disrupting quorum sensing, using iron-scavenging materials, and even bacterial interference through non-pathogenic strains of *E. coli* that act as probiotic strains that can out-compete pathogenic strains for nutrients and attachment sites (Table 1) (Delcaru et al., 2016; Soto, 2014).

Ventilator-Associated Pneumonia

Ventilator-associated pneumonia (VAP) is pneumonia that forms in a patient who has been mechanically ventilated for more than 48 h. The chance of developing VAP increases the longer a patient is on a ventilator (Gadani et al., 2010). VAP is also associated with increased morbidity and mortality, longer hospital stays, and an increased burden on medical systems (Gadani et al., 2010; De Souza et al., 2014), costing an estimated $10,000 to $25,000 per incident (Fernandez et al., 2012). Mortality associated with VAP is estimated between 33% and 50% (Kalanuria et al., 2014). Recent research has pointed to biofilm growth on the endotracheal tube (ETT) as the most important precipitating factor in the development of VAP and a main target of strategies to prevent it (Fernandez et al., 2012; Gil-Perotin et al., 2012).

The main causative agents of VAP include *P. aeruginosa*, *K. pneumoniae*, *Acinetobacter* sp., and *S. aureus*, with *Streptococci* and *Haemophilus influenzae* occasionally implicated in early-onset VAP. The fungus *Candida albicans* is also frequently found to be involved in VAP (Khilnani and Jain, 2013). Among these, *P. aeruginosa* and *S. aureus* are the most common infecting pathogens, with *P. aeruginosa* tied to an increased risk of mortality (Kalanuria et al., 2014). Universally, biofilm forms on the ETT (De Souza et al., 2014). Lung infection occurs as increased tracheal secretions allow for microaspiration of dispersed bacteria from the ETT to colonize the lower airways (De Souza et al., 2014; Kalanuria et al., 2014).

Treatment for VAP involves intravenous or inhaled antibiotics (Kalanuria et al., 2014; Gil-Perotin et al., 2012). Some novel strategies for preventing in VAP include impregnating ETTs with antimicrobials like natural antibiotics or silver compounds, iron-scavenging materials, and quorum sensing inhibitors (Table 1) (Kalanuria et al., 2014). Other approaches focus on preventing microaspiration by addressing cuff pressure, material, and shape (Fernandez et al., 2012). Another potentially promising therapy involves photodynamic treatment in combination with photosensitizers to eliminate 99.9% of biofilm growing on ETTs (Biel et al., 2011).

Conclusion

The nature of our interactions with bacteria is complex and the roles bacteria play in our lives range from necessary and beneficial to pathogenic and deadly. With the emergence of bacteria that are highly-resistant to all antibiotics, we may be entering a post-antibiotic era (Chen et al., 2017; McGann et al., 2016). In this context, understanding the mechanisms that bacteria use to evade eradication is of vital importance. Indeed, years of research has already revealed new targets for antibiofilm therapy, leading to novel treatment strategies that could be effective against a wide range of biofilm infections (Table 1). Considering the integral role of biofilms in numerous human infections, understanding and combatting biofilm has the potential to save millions of lives across the globe.

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Relevant Website

http://www.biofilm.montana.edu/.

Biological Warfare☆

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Introduction

Definitions

In line with the current literature and regulations, biological warfare (BW, biowar, germ war) can be defined as the intentional use of harmful biological organisms such as bacteria, viruses and fungi or their products (toxins) in warfare to cause damage on humans, animals or plants. The warfare is legally the conflict between states, that is, attacks by nonstate actors would have to be considered as bio-terrorism. Biological warfare was never considered as stand-alone action, but as embedded into a wider range of military activities.

Theoretically any harmful biological agent that could be released with bad intentions is a biological weapon, but from a legal, political and military perspective, bioweapons are those biological agents which potentially could cause mass casualties as Weapons of Mass Destruction (WMD). The security institutions handle bioweapons in the CBRN (chemical, biological, radiological and nuclear) threat complex. The targeted attack on single individuals with harmful organisms or toxins would be a matter of crime or-if politically motivated-of bioterrorism, but not an act of biological warfare.

In contrast to other WMDs, with the exception of the extinct smallpox virus, all agents and their infections occur naturally as well. The agents themselves can thus not be forbidden and the research on them is medically needed, as demonstrated by the recurrent Ebola outbreaks.

Brief History

The history can be divided into two phases: In ancient times and medieval age, people were already aware that some diseases could be spread by the people who had the diseases. This resulted in defensive measures (separation of people with Lepra, quarantine of ships to prevent Black Death pandemics), but occasionally also in offensive measures, for example, throwing sick bodies into areas controlled by the enemy by catapults. In the 18th and 19th century, attempts by White Americans to intentionally infect Indians with smallpox were reported, but these incidents are still debated.

The second phase started with the understanding of the existence of infectious agents at the beginning of the 20th century. During World War I, Germany was accused for the use of glanders (*Burkholderia mallei*) and anthrax (*Bacillus anthracis*) against animals. World War I finally led to the Geneva Protocol of 1925, which banned the use of biological weapons for the first time. In World War II, there were reports of bio-attacks in the Sino-Japanese War based on the research of Japan's Unit 731 by using ceramic bombs with infected fleas to spread bubonic plague. During the Cold War, leading powers like the United States (locations in Fort Detrick in Maryland, the Dugway Proving Ground test area etc.), United Kingdom (Porton Down) and the Soviet Union (multiple locations) continued research on biological weapons. Several experiments with open air release in the late 1940s and 1950s for example, with anthrax spores and exposure of volunteers to the Q-fever agent Coxiella burnetii took place.

The United States and the Soviet Union supported the United Nations Biological and Toxin Weapons Convention from 1972 which made the development, production and stockpiling of biological and toxin weapons illegal. By defected experts, it became public that the Soviet Union continued to produce bioweapons in their Biopreparat organization, which apparently collapsed with the Soviet Union in the early 1990s.

Current Situation

There are concerns on continued bioweapon research. Since 2015, the Russian Military Scientific Committee of the Armed Forces affiliated to the National Defense Management Center (NDMC) runs Science Squadrons with staff from leading universities; their mostly classified research activities include biotechnology. However, evidence for bioweapon research is missing.

Russia expressed also concerns triggered by the targeted collection of synovial fluid/RNA samples from Russians by the US Air and Training Command 2017, but US denied bad intentions. Medically relevant genetic differences between ethnics exist (e.g., tolerance against paracetamol/acetaminophen), but no genetic variance allowing a bio-attack on a certain population is known yet, that is, a so-called ethnic bomb remains hypothetical.

Smallpox may still be stockpiled as bioweapon, so US military personnel on the Korean peninsula and staff of the US Central Command must be vaccinated with smallpox vaccine.

☆*Change History*: December 2018. KP Saalbach has made minor changes to the text and references.

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China expressed concerns about the ongoing gain of function (GoF) research which tries to increase contagiosity and damage to the infected organism by targeted virus mutations. In 2016, a researcher re-created the extinct horse pox virus by synthetic DNA. The dual use research of concern (DURC) is also an issue as the advances in genome editing (CRISPR/CAS9-technology) significantly reduce the hurdles for genetic modifications and may enhance biohacking activities.

Biosecurity Framework

The most important global regulation is the United Nations Biological and Toxin Weapons Convention from 1972 which prohibited the development, production and stockpiling of biological and toxin weapons. The use of biological weapons by nation states was already prohibited by the Geneva protocol from 1925. In response to 9/11 and to the subsequent anthrax incidents, the institutional framework was expanded to cover all aspects of biological warfare, biodefense and biothreats. Table 1 shows the biosecurity architecture of the United States in 2018.

The Biological Weapons and Anti-Terrorist Act from 1989 clarifies the penalties for those, who unlawfully try to gain or to work with bioweapons or try to gain too large portions of the smallpox genome sequence.

For military prevention, the United States set up in 1998 the Military Vaccination (MILVAX) Programs in particular for smallpox and anthrax, but also general vaccinations (e.g., influenza) under the direction of the Military Vaccine Agency. The program provides consultation services, education, safety surveillance and research support.

Table 1 Biosecurity architecture of the United States

Sector	*Institution*	*Responsibilities include*
Government (Executive Office of the President of the United States)[a]	Biological Defense Research and Development Subcommitee (BDRD) of the National Science and Technology Council (NTSC) managed by the Office of Science and Technology Policy (OSTP)	NTSC coordinates the Science and Technology Council, the BDRD provides an interagency forum for coordination and collaboration on biodefense and biothreats matters
Civil Sector US Department of Health and Human Services (HHS)[b,c]	Center of Disease Control (CDC) and Prevention	The CDC runs the Select Agents Program to protect the public from dangerous organisms and toxins. The Biosafety Level 4 laboratories are the official US repository for smallpox
	National Institute of Health (NIH) with subordinate National Biosecurity Advisory Board (NSABB)	Formulates Policies for Biosafety and Recombinant DNA; Biosecurity with Dual Use Research of Concern (DURC) and gain of function (GoF) research and Emerging Biotechnologies
	Biomedical Advanced Research and Development Authority (BARDA)	Project BioShield for funding of research, development and stockpiling of vaccines and treatments in case of CBNR emergencies BARDA procures materials for the Strategic National Stockpile (e.g., Anthrax vaccine)
	Public Health Emergency Medical Countermeasures Enterprise (PHEMCE)	Interagency cooperation to advise secretary of HHS with respect to Medical Countermeasures (MCM)
	Food and Drug Administration (FDA)	Regulation and approval of MCM, FDA is also responsible for food security
US Department of Agriculture (USDA)[c]	Environmental Protection Agency (EPA)	Biological incident response (characterization, decontamination, and waste management), water infrastructure protection. USDA is overall responsible for plant and animal protection
Intelligence (Department of Homeland Security DHS)[c]	National Biodefense Analysis and Countermeasures Center (NBACC)	NBACC is a research laboratory at Fort Detrick for threat assessments in the National Biological Threat Characterization Center (NBTCC) and bioforensics in the National Bioforensic Analysis Center (NBFAC). The DHS provides Material Threat and Bioterrorism Risk Assessments (MTAs/BTRAs), manages the early warning program BioWatch and runs the National Biosurveillance Integration Center (NBIC) for interagency cooperation
Military (Department of Defense DoD)[c]	Defense Threat Reduction Agency (DTRA) with Joint Science and Technology Office for Chemical and Biological Defense	Coordinates research by US Army Medical Research Institute of Infectious Diseases (USAMRIID) as part of the US Army Medical Research and material command (USARMRC) USAMRIID is the primary facility for defensive research against biological warfare with a top-level (Biosafety Level 4) laboratory. Research is also done by further actors

[a]OSTP (2016). Homeland Biodefense. Science and Technology Capability Review, December 2016. Executive Office of the President of the United States, Washington DC., p.iv.
[b]Website of the National Institute of Health (NIH) www.nih.gov, last retrieved 24 May 2018.
[c]GAO (2017). Biodefense - federal efforts to develop biological threat awareness. Report GAO-18-155, condensed from pp. 9–12 and Appendix II, pp. 46–50.

After 9/11 and the subsequent anthrax incidents, the US released the Patriot Act and the Public Health Security and Bioterrorism Preparedness and Response Act 2002 which was the basis for the Select Agent Rules last finalized in 2005. As shown in Table 1, new institutions were the NBACC in 2002, the NSABB in 2004 and the BARDA, which was established in 2006 through the Pandemic and All-Hazards Preparedness Act. There is an ongoing biosecurity debate whether a centralization of organizations and budgets may improve effectiveness. The National Biodefense Strategy of 2018 introduced a Biodefense Steering Committee chaired by the HHS and proposed a coordinated information collection, distribution and analysis.

In 2002, the European Medicines Agency EMEA (meanwhile EMA) released guidelines and recommendations for bioterrorism. The seven EU laboratories with the highest Biosecurity-Level (BSL) P4 were linked with each other to ensure information exchange, exercises, trainings and education. Already in 2003, the European Commission introduced strict surveillance rules for *Bacillus anthracis*, *Franciscella tularensis*, *Coxiella burnetii* (Q-Fever) and smallpox and added these rules to an EU list of critical infectious agents.

The prohibition of offensive BW does not forbid defense BW measures, so the North Atlantic Treaty Organization (NATO) still conducts BW Preparedness Exercises for example, with inactivated anthrax for defensive purposes.

Bioweapons

Practical Aspects

In biosecurity practice, bioweapons are those agents which can cause harm to a larger number of people as WMD.

Viruses dependent on blood transfer cannot be easily spread, so only agents which can be spread via the air are used as bioweapons. Too large particles would fall down to earth where many agents degrade quickly, with the exception of anthrax spores that could rest for years. So only those agents with a few microns length are of military relevance. The delivery can be done by aerosols or more targeted by missiles, but the agents need to survive the explosion/release in the target region. Mass infection requires mass production, but scale-up of such agents is complex and significantly increases the risk for detection and of accidents. Cultivation (feeding, cleaning, temperature, storage, optimization of pH values and electrolyte balance etc.) requires significant experience. Sometimes, the agents have to undergo special preparatory steps, for example, a microscopic sealing procedure of anthrax particles to improve inhalation. Altogether, bioweapon production and use is a high-tech matter and with the current state of the art, large-scale bioterrorist attacks are unlikely without financial and technical support of nation states.

Genetic engineering did not lead to new superorganisms so far. General strategies are mutations, that is, change of genetic code resulting in altered protein products; insertion of new genes into the set of genes of an organism (genome), resulting in additional proteins and functions and finally deletion of genes, resulting in loss of functions of the organism, for example, to make viruses less harmful (attenuation) and to utilize them for vaccines. Insertions and mutations could create an energetic burden and may change structure and function; these obstacles prevented the creation of superorganisms and new bioweapons.

Classification

Introduction

Current bioweapons can be differentiated into bacteria, viruses and toxins. The Center for Disease Control and Prevention (CDC) has released an internationally recognized Bioweapon Classification with the three categories A (highest risk; easily spread or transmitted with high death rates and major public impact such as smallpox and anthrax), B (high risk; moderately easy to spread, moderate illness rates and low death rates such as *Coxiella burnetii* (Q-fever) and C (emerging pathogens with future risk).

Medical Countermeasures (MCM) are preventive, that is, avoiding contact to infectious agents and vaccination, therapeutic, that is, treating the infectious agent directly, like antibiotics for bacteria, antivirals for viruses and antitoxins for toxins or supportive/symptomatic, that is, treating symptoms like shock, fever, myalgia, headache, nausea etc.

Conventional bioweapons

Considering the overall research intensity and the real military production and stockpiling in the past, the primary conventional bioweapons are the smallpox virus, the bacteria *Bacillus anthracis* (Anthrax), *Francisella tularensis* (Tularemia) and *Yersinia pestis* (Plague), the bacteria-like *Coxiella burnetii* (Q-fever) and the toxins Staphylococcal Enterotoxin B (SEB) and Botulinum toxin (BT). While Ebola has no biowarfare history, it is considered as a potentially fatal biothreat (with the risk of spreading by air travelers after an outbreak) and part of the regulatory framework.

Viruses

Smallpox (Variola major) After oral infection, a massive viral replication takes place in lymphatic tissues with subsequent swelling of lymph nodes, then it returns to body surfaces (mucosa and skin with multiple lesions) allowing infection of others. Smallpox virus was officially declared extinct in 1980 after an intense global vaccination and surveillance campaign. The vaccination against smallpox (which historically was the first vaccination) was then stopped due to side effects like brain inflammation (encephalitis) or heart inflammation (perimyocarditis).

Officially, smallpox viruses are safely stored at only two locations in the United States and Russia, the Center for Disease Control and Prevention (CDC) in Atlanta and the Center of Virology and Biotechnology (VECTOR) in Koltsovo, but for example, in 2014 the United States National Institute of Health (NIH) found forgotten frozen poxviruses in their own archive.

The key biodefense strategy is vaccination. In July 2018, the US FDA approved the first smallpox-specific antiviral drug tecovirimat, which blocks the enveloping protein p37, after efficacy tests with rabbit pox and monkey pox in animals and safety tests in healthy humans. Only very limited data are available for the antivirals cidofovir and ribavirin. Since decades, a milder (attenuated) smallpox variant, the vaccinia virus (VV) exists, which is further developed by cultivation and mutation to make it milder while keeping its efficacy (causing immunity). To reduce side effects, a new variant which lost the ability to replicate, the Modified Ankara Strain (MAV), was recently cultivated. Now a synthetic viral platform for pox vaccines is developed and the virus researcher Evans re-created the extinct horse pox virus by synthetic DNA in 2016, but the combination of DNA fragments was complex and required for example, the involvement of a helper virus system.

Viral hemorrhagic fevers (VHF)/Ebola Viral hemorrhagic fevers (VHF) including Ebola Virus, Marburg Virus and others cause severe inner bleedings (e.g., as spontaneous nose bleeding). Due to recurrent outbreaks, the World Health Organization (WHO) and others have gained a lot of experience with infection control. Treatments of Ebola with the antiviral drug ribavirin and the chimeric antibody combination ZMapp as well as vaccinations remain experimental.

Bacteria

Anthrax (*Bacillus anthracis*) can form spores which after inhalation get in contact with immune cells. Certain anthrax proteins protect the spores against destruction and cause hyperinflammation, an overactive immune system with shock-like symptoms. In nature, skin infections are more common, while bioweapons aim on inhalation. Vaccination and antibiotics such as ciprofloxacin are used.

Francisella tularensis causes Tularaemia, a highly infectious diseases which can be inhaled or transferred by contact with wild animals leading to severe fever and sepsis symptoms. Antibiotics such as streptomycin and gentamicin are used.

Yersinia pestis causes plague, typically transmitted by infected flea. The infection causes a painful lymph node swelling, known as buboes, then septic reactions follow, appearing as Black Death. Antibiotics like streptomycin are used, limited data exist for other drugs.

Q-fever is caused with *Coxiella burnetii* which may lead to pneumonia (lung infection), but also to chronic heart valve infections. Treatment is done with antibiotics of tetracycline type, gyrase blockers and others.

Toxins

Staphylococcal enterotoxin B (SEB) is a bacterial toxin released by *Staphylococcus aureus*. It overstimulates the immune system as superantigen and causes severe gastrointestinal symptoms, dizziness, vomiting, vertigo etc. It naturally occurs as food and water intoxication. Treatment is symptomatic only.

Botulinum toxin (BT) is a protein released by bacterium *Clostridium botulinum* which irreversibly blocks cholinergic synapses, causing neuromuscular blockade. Equine botulinum antitoxin is effective, if timely given.

Biodefense Considerations

Harmful biologic agents can suppress, confuse or overstimulate the immune system, which can deteriorate infections or lead to shock-like symptoms.

Some agents are suppressing the immune system by blocking certain molecules, for example, many viruses block the immune defense by interferon blockade. Some viruses like the Spanish Flu influenza virus can enhance secondary (on top) infections (infections on top with bacteria which increase morbidity and mortality, so an antibiotic treatment can sometimes be indicated influenza cases).

Bacteria may overstimulate the immune system, by circulation in blood or too many bacterial fragments after destruction by immune system or by releasing superantigenes which lead to a diffuse and untargeted massive activation of the immune system. Viruses may also cause overstimulation for various reasons.

Meanwhile, it was found that the altered immune hormone (cytokine) communication plays a role for morbidity and mortality by shock-like and septic symptoms. This was shown for many harmful agents like smallpox (by virus homologs of cytokines, called virokines), anthrax, Ebola and SEB (all causing massive cytokine release), but may also be important for weaponization of viruses by GoF Research.

So the understanding and the correction/suppression of cytokine excesses is a relevant biodefense strategy in case of new GoF-based bioweapons and for classical biological weapons like anthrax, smallpox, Ebola and SEB.

Attribution of Biological Attacks

The nature of an attack as well as the attacker identification is a matter of bioforensic attribution. There are indicators for an intentional use (compared to natural outbreak), for example, sudden outbreak of uncommon diseases with high morbidity and mortality such as a massive zoonosis outbreak only in humans.

The characteristic structure of bioweapon facilities may identify potential attackers. The order and shipment of research materials, bioreactors, feeding materials etc. can give further hints; (cyber-) intelligence may help to reconstruct such events or to track them. All distribution methods and carrier systems have a technology history which gives further directions to the background of an attack.

On the biologic level, microorganisms exist often in variants (strains) which may have been cultivated from precursor strains. The genetic code (DNA) analysis may give hints to the original strains and the sources. Sometimes, microorganisms need a special

preparation for use in attacks which can create a molecular fingerprint and could identify the original production plant. Genetic modifications of bacteria or of bacterial proteins typically result in unique microscopic variations of surface glycoproteins which could be used for production plant attribution like a fingerprint.

Potential Biothreats

Biohacking and Bioterrorism

The typical biohacker works outside established research units or companies as DYI (do it yourself, also DYIBIO) researcher and tries as a kind of ethical hacking to modify genes to invent something useful, but the biohacking scene is closely observed by government authorities. The biohacking scene has made substantial progress in creation of technical equipments (such as thermocyclers for DNA replication), but no critical biohack incident was reported so far.

The other issue is bio-terrorism. The anthrax attacks after 9/11 by sending letters with anthrax-containing powder were done by a bioweapon expert who was reported to have suffered from a psychiatric disorder. The group Aum Shirinkyo was able to conduct terrorist attacks with chemical weapons, but failed with their bio-terrorist attacks. The repeated spraying of anthrax in June and July 1993 in Tokyo apparently caused no reports of diseases or anthrax-related symptoms. In 2013, a man sent letters containing the herbal toxin ricin to parliamentarians and to President Obama. This attempt was detected and failed; the attacker was very quickly identified and imprisoned. A similar attack failed on President Trump in 2018. Another issue is the use of food and water contaminated with infectious agents for attacks, for example, with the bacteria *Salmonella typhimurium* and *Shigella dysenteriae*. In 1984, an intentionally contaminated blue cheese dressing released by two members of the Rajneesh cult led to 751 Salmonella cases in The Dalles in Oregon. Infected muffins led to an outbreak of *S. dysenteriae* type 2 in Dallas, Texas in 1996. Recently, a Shigella outbreak was reported in Seattle by an infected catering, the investigation is still going on.

Gain of Function-Research

In 2011, the researcher Kawaoka combined an Avian flu and Swine flu virus and showed that this was transmittable between ferrets (which are the animal model for humans due to similar reaction pattern) by droplet transmission. In parallel, the researcher Fouchier showed that Influenza A virus could be much more contagious to ferrets after introduction of certain mutations. Initially, there was a misunderstanding that this virus would also be more aggressive, but this was not the case. This triggered the DURC policy from 2012 and virologists agreed to suspend this gain of function (GoF) research, but 2017 US lifted the ban on GoF funding accompanied by a new regulatory framework on potentially contagious organisms. In short, there are major safety concerns like the inadvertent release from a laboratory while others argue that the nature is driving mutations anyway and that this research is the only chance to be ahead of nature. However, a certain proportion of humans will very likely survive an infection of whatever agent, due to accidental immunity by genetic disposition or genetic defects, by cross-immunity to other agents, by interference with other ongoing diseases, current medication intake or metabolic situations.

Synthetic Biology

Since 2010, Craig Venter worked on a minimal genome cell with the smallest possible genome allowing autonomous life and replication. Mycoplasma was used as model organism and in 2016 a new cell, called Syn 3.0 was created with only 473 genes with unknown function of 149 genes. If the function of these 149 genes could be clarified, this could be the breakthrough to designable artificial cells which would be lean and fast replicating.

CRISPR/Cas9

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) are DNA sequences in bacteria which help to detect virus DNA from attacking viruses. There are nearby located genes called Cas (CRISPR-associated system), of which the Cas9 type is of practical relevance, as CRISPR/Cas9 can be used in many cells, including human cells. By linking the CRISPR/Cas9 to a synthetic guide RNA, this system can be used for targeted insertion of new and/or deletion of old genes. This technique is much more precise and easier to handle than previous technologies; it allows genome editing (like a draft movie is cut and stuck together) and genome surgery, which is the targeted repair of genetic defects in humans. A biosecurity issue is the potential enhancement of biohacking. The US Defense Advanced Research Projects Agency (DARPA) is currently evaluating Horizontal environmental genetic alteration agents (HEGAAs) as tools to transfer genes into crops to defend them against biothreats, also known as Insect Allies program. With use of CRISPR/Cas9 as part of the research. While DARPA emphasizes the protective potential of this research, concerns were expressed for a potential offensive use as bioweapon or agrarian terrorism tool.

Antimaterial Weapons

The bacterium *Ideonella sakaiensis* 201-F6, discovered in 2016, is able to utilize Polyethylene terephthalate (PET) as energy and carbon source that is used worldwide in plastic products. Other plastic-degrading organisms such as fungi and moths are also

known. Due to the contamination of the environment with plastic garbage, also with microplastic particles, the research on genetically improved plastic-degrading enzymes is in progress. However, such enzymes/agents may have the potential to be antimaterial bioweapons for silent destruction of infrastructure.

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Bioluminescence in Eukaryotic Microbes

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Introduction

Bioluminescence in Eukarya occurs in diverse marine and terrestrial organisms. Light emission exists as long glows or short flashes to serve one, or several, of the many known ecological functions of bioluminescence: defense, camouflage, communication, prey detection, and prey attraction. Bioluminescence is known from ancient times, with accounts of firefly bioluminescence dating back to 1500 BCE and references to ocean bioluminescence as far back as 500 BCE. It is unique from other forms of biological light emission in that the light originates from an enzymatic chemical reaction involving a substrate luciferin and a protein, either luciferase or a photoprotein, within bacterial or eukaryotic free-living cells, or within specialized photocytes of multicellular organisms.

Bioluminescence from microscopic organisms creates sea-sparkle, a globally occurring, nocturnal phenomenon of blue sparkling light in breaking waves or around moving objects (Fig. 1). For centuries the light was attributed to physical or supernatural sources, but only later was the biological source documented; for example 'phosphorescence' in the English Channel in 1810 originated from the dinoflagellate *Noctiluca miliaris* (now *N. scintillans*), first described by naturalist Henry Baker in 1753 as 'animalcules' based on observations with an early microscope.

Eukaryotic luminescent microorganisms consist of dinoflagellates, radiolarians, and phaeodarians. Dinoflagellates are the dominant source of light in coastal and surface waters. Radiolarians contribute mainly to off shore, near surface bioluminescence, while most phaeodarians dwell in deeper water. In these organisms bioluminescence is considered to serve as a defense against predators, and may be a key factor in the success of some dinoflagellate species in competition with other plankton.

Bioluminescence of Radiolaria and Phaeodaria

Radiolarians and phaeodarians are unicellular zooplankton found in all oceans, with the exception of brackish areas. In older taxonomy phaeodaria were grouped with the radiolarians, but they are now within the phylum Cercozoa, while the radiolarians belong to the Retaria, both in the super-phylum Rhizaria. Recent changes in taxonomy affect descriptions of the distribution of bioluminescence in older literature, where no phaeodarian species where considered bioluminescent. Phaeodarians are solitary, while radiolarians live solitary or in colonies. Cells from both groups range in size from 30 μm to 2 mm, and radiolarian colonies can reach three meters in length. Radiolarian and phaeodarian cells are organized in a central and an outer capsule, separated by a membrane. Some radiolarians harbor symbiotic microalgae, e.g., dinoflagellates, in the outer capsule, and their most striking morphological characteristic are the species-specific skeletons of opal-silica or strontium-sulfate (Fig. 2(A)).

Of the 400–800 radiolarian and 400–500 phaeodarian living species, only around a dozen are known to be bioluminescent. Both radiolarian and phaeodarian bioluminescence is triggered by mechanical stimulation. Investigated radiolarians respond with a glow lasting typically 1–2 s, but glows lasting >3 s have also been detected, such as in the colonial *Rhaphidozoum acuferum* and in the solitary *Thalassicolla nucleata*. The light is blue with a relatively short peak wavelength of 440–460 nm, as compared to other bioluminescent members of the marine plankton, such as dinoflagellates, ctenophores, and crustaceans with wavelength maxima of 470–490 nm.

In radiolarians light originates from intercellular structures, "microspheres", located in the outer capsule of the organism, that have the same location as dinoflagellate symbionts. However, it is unlikely that light emission originates from the dinoflagellate symbiont, *Brandtodinium nutricula* (formerly *Zooxanthella nutricula*), which is not known to be bioluminescent, because radiolarian bioluminescence differs from dinoflagellate bioluminescence in its longer flash duration, shorter wavelength, different luciferin type, and lack of pH sensitivity and light inhibition (further described below).

The radiolarian bioluminescence reaction involves the luciferin coelenterazine in combination with a Ca^{2+}-dependent photoprotein or luciferase. Coelenterazine is the most widespread luciferin, also utilized by some crustaceans, coelenterates, squid, brittlestars, and fish. Small crustaceans (copepods) are one original producer of coelenterazine, which is then distributed throughout the food web and obtained by other organisms that have a dietary requirement for the substance. Whether radiolarians are producers or consumers of coelenterazine is not known, but they are unlikely to obtain the substance from copepod prey as these feed on bacteria and other microorganisms.

The ecological benefit of bioluminescence in radiolaria and phaeodaria has not been investigated. Because bioluminescence is mechanically stimulated, it may serve a predator defense function if it is evoked by interaction with a predator and interrupts the feeding behavior of the predator. It is even possible that the radiolarian outer capsule, containing the bioluminescent microspheres, could be shed and left as a glowing lure to confuse the predator. Shedding of capsules occurs in non-bioluminescent radiolarians, but it is not known if microspheres stay functional in a shed capsule.

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Fig. 1 Bioluminescence of a bloom of the dinoflagellate *Noctiluca scintillans*. From Gippsland Lakes National Park, Australia. Copyright 2009, Phil Hart. Used with permission.

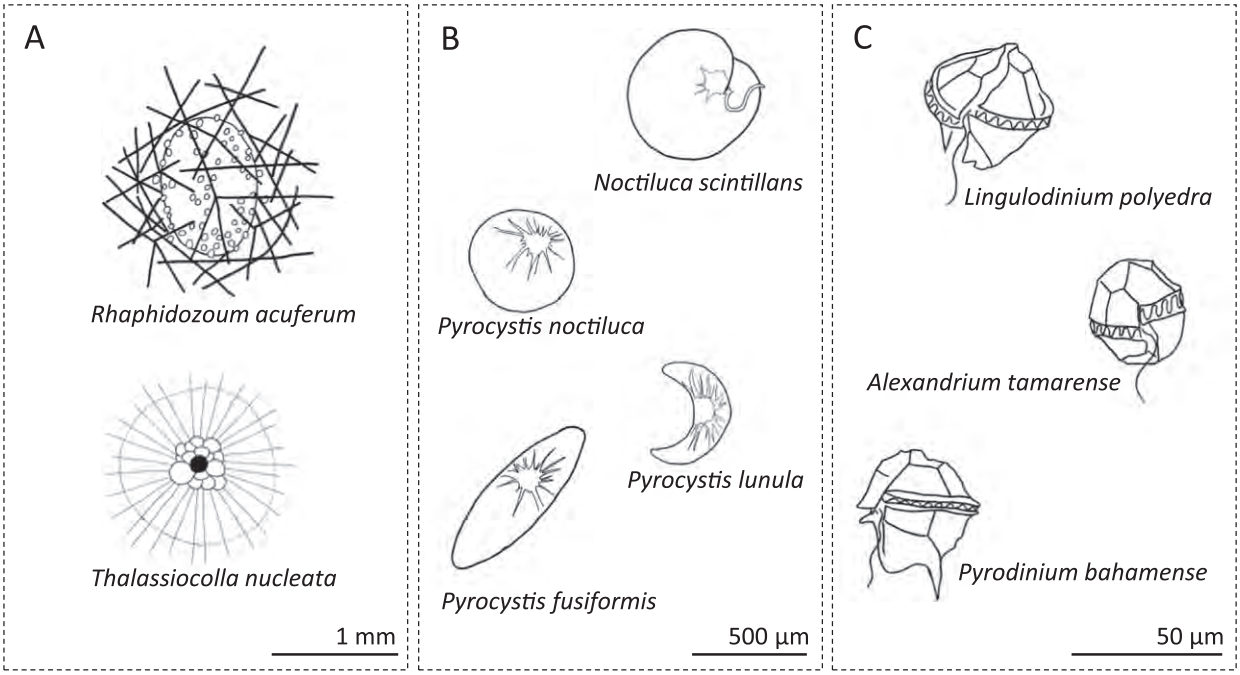

Fig. 2 Drawings of representative luminescent eukaryotic microbes. (A). Radiolarians. (B). Non-motile, large dinoflagellates. (C). Motile, thecate, small dinoflagellates.

Bioluminescence of Dinoflagellates

Dinoflagellate Characteristics

Dinoflagellates are unicellular plankton, ranging in size between 2 μm and 2 mm in diameter, with complex life cycles involving asexual, sexual, motile, and non-motile life stages as well as resting cysts, depending on species. Some species are obligate autotrophs, or obligate heterotrophs, while others are mixotrophs with the capacity for both photosynthesis and feeding on other organisms. Of the >2300 living dinoflagellate species, sixty nine are known to be bioluminescent.

Common morphological characteristics for dinoflagellates are two flagella, which may not be present in all life stages, e.g., *Pyrocystis* or *Noctiluca*; a large C-shaped nucleus, known as the dinokaryon; and the arrangement of cortical cell membranes called the amphiesma. The amphiesma consist of the outer cell membrane and a layer of flattened vesicles, the alveolae, below which is a pellicle layer. In armoured (thecate) dinoflagellates the alveolae contain cellulose plates, while these plates are lacking in the 'naked' (athecate) dinoflagellates. The dinoflagellate cell contains numerous vacuoles, and vacuolar membranes usually add to the membrane layers of the amphiesma. In bioluminescent dinoflagellates small vesicles known as scintillons, containing the bioluminescence chemistry, are associated with a large acidic vacuole (described further below).

Dinoflagellates have huge genomes of about 3–245 giga base pairs cell^{-1}, arranged in several to >100 chromosomes; for context the human genome is about 3 giga base pairs cell^{-1}. Dinoflagellate genomes are characterized by many redundant and multiple copies of genes. The large genomes, together with a lack of knowledge of dinoflagellate genetic processes, has impaired sequencing efforts, and limits the molecular toolkit available for research on bioluminescent dinoflagellates. To date only genomes of symbiotic species of the genus *Symbiodinium* are fully sequenced, although they are poorly annotated with many genes undescribed. The *Symbiodinium* genome is the smallest in dinoflagellates, perhaps because of its symbiotic life style, and the genus have no known bioluminescent representatives. Dinoflagellate genomic research is increasingly carried out using transcriptomic data.

Dinoflagellate bioluminescence is triggered by mechanical stimulation but spontaneous luminescence, independent of external stimuli, is also well documented. Mechanical stimulation causes flashes of light of 40–500 ms duration with a spectral maximum at 472–474 nm, while spontaneous luminescence can be both short flashes and persistent weak glowing. Stimulated flashes have documented ecological relevance (described below), while the mechanisms for, and potential benefits of, spontaneous luminescence are poorly known.

The bioluminescence of *Lingulodinium polyedra* has been well studied ever since the species was brought into culture by B. M. Sweeney in 1952. This temperate species is around 35 μm in diameter and may form blooms that result in red tides (see below). It is motile, armoured, and autotrophic; some *L. polyedra* strains produce yessotoxins. Other frequently studied bioluminescent species are *Alexandrium tamarense* (autotroph, saxitoxin producer), *Pyrodinium bahamense* (autotroph, saxitoxin producer), *Noctiluca scintillans* (heterotroph, non-toxic), *Pyrocystis lunula*, *Pyrocystis fusiformis* (autotroph, non-toxic) and *Pyrocystis noctiluca* (autotroph, non-toxic) (Fig. 2(B) and (C)). As bioluminescence per cell is related to cell surface area, the smaller *L. polyedra*, *A. tamarense*, *P. bahamense*, and other species of similar size, are often referred to as 'small and dim', while species from the genera *Noctiluca* and *Pyrocystis* are 'large and bright', being about 30 times larger, with 100–1000 times higher light capacity.

Bioluminescence Chemistry

Luciferin, luciferase and luciferin binding protein

Bioluminescence in dinoflagellates involves a unique dinoflagellate luciferin and dinoflagellate luciferases. Luciferin binding protein, which protects luciferin from spontaneous oxidation when light is not produced, is present in all investigated species, except within the genus *Pyrocystis*. Luciferin is highly sensitive to oxygen and reactive oxygen species, while luciferase and luciferin binding protein have pH-sensitive properties that are crucial for the bioluminescence reaction (further described below).

Dinoflagellate luciferin is a linear tetrapyrrole whose structure resembles that of krill luciferin and chlorophyll, a cyclic tetrapyrrole. Its biosynthesis is largely unknown, but like chlorophyll, it is thought to be synthesized as part of the tetrapyrrole biosynthetic pathway. There is currently no evidence for a dietary requirement for luciferin or chlorophyll as a potential precursor of luciferin in dinoflagellates, as the bioluminescence of heterotrophic species can persist when maintained on a luciferin-free non-algal diet.

Luciferase and luciferin binding protein are among the most highly expressed proteins in the cells of luminescent dinoflagellates, with each protein comprising up to 1.5% of the total protein in *L. polyedra*. Luciferase activity is pH dependent, due to histidine residues within the N-terminal region, with no activity at physiological pH and full activity at pH 6.0 or lower due to unfolding of the protein to expose the luciferin binding site. Similarly, the activity of luciferin binding protein is also pH sensitive, with binding at physiological pH and dissociation of the luciferin at pH 7.0 or lower, although the physical structure of the molecule has not been determined to identify the location of the binding site.

Luciferase and luciferin binding protein genes are present in a large number of copies within a species, and sequences can vary considerably between copies; e.g., 10% variation in nucleotide residues in *L. polyedra* or *Alexandrium funydense*. The large interspecies variation is enough to confuse the boundaries between species and even genera in phylogenetic analyses including bioluminescent genes. In *L. polyedra* the luciferin binding protein genes make up two gene families, LBPα and LBPβ, which share around 86% similarity and are equally expressed, and in *P. lunula* three distinct variants of the luciferase gene are described, *lcf*A, *lcf*B and *lcf*C.

In photosynthetic species, including *L. polyedra*, the luciferase gene is composed of three domains, each consisting of a highly conserved central region that encodes the catalytic active site, flanked by more variable N- and C-terminal regions with roles in the pH response and activity of the enzyme. These domains are repeated tandemly three times within the same gene, being the template for three equally functional luciferase mRNAs. The heterotrophic *N. scintillans* lacks the tandem repeat of domains and instead has one luciferase gene fused with the luciferin binding protein gene, a second luciferin binding protein gene is located separately in the genome. The arrangement of bioluminescence genes has the potential of providing important insights into dinoflagellate evolution, and conserved regions of the luciferase gene have proven to be useful indicators of the distribution of luminescent dinoflagellates in situ.

Scintillons and activation of the bioluminescence chemistry

Luciferin, luciferase and luciferin binding protein (when present) are densely packed in 0.5–1.5 μm diameter vesicles, known as scintillons or "micro-sources", structures that are unique to bioluminescent dinoflagellates. Scintillons are formed in association with the Golgi body and are translocated via the cytoskeleton to the cell periphery, where they associate with the vacuole membrane and end up hanging like droplets in the vacuolar space (Fig. 3). Through a thin neck of vacuolar membrane the scintillon content is continuous with the cytoplasm. Thus the scintillons, which have neutral pH, are surrounded by the acid content of the vacuole (pH ~3.5).

The pH difference between the vacuole and the scintillons is the key to the bioluminescence activation mechanism. At stimulation of the cell membrane (further described below), H^+ enter scintillons from the acid vacuole through voltage-gated proton channels, following the biochemical gradient. The resultant drop in pH results in release of luciferin by luciferin binding protein, and activation of luciferase to expose the luciferin binding site followed by oxidation of luciferin resulting in a flash of light.

Low pH caused by, e.g., addition of acetic acid to the growth medium or seawater containing dinoflagellate cells, is used experimentally to stimulate maximum light production. This method activates the bioluminescence chemistry directly and bypasses signal transduction pathways activated by mechanical stimulation of the cell, as described in the next section.

Stimulation of Dinoflagellate Bioluminescence

Mechanical forces associated with bubbles, flow or turbulence, and moving objects are sensed by the dinoflagellate cell membrane and activate a signaling pathway that, via multiple intracellular events, leads ultimately to the activation of the bioluminescent chemistry within the scintillons resulting in light emission (Fig. 4).

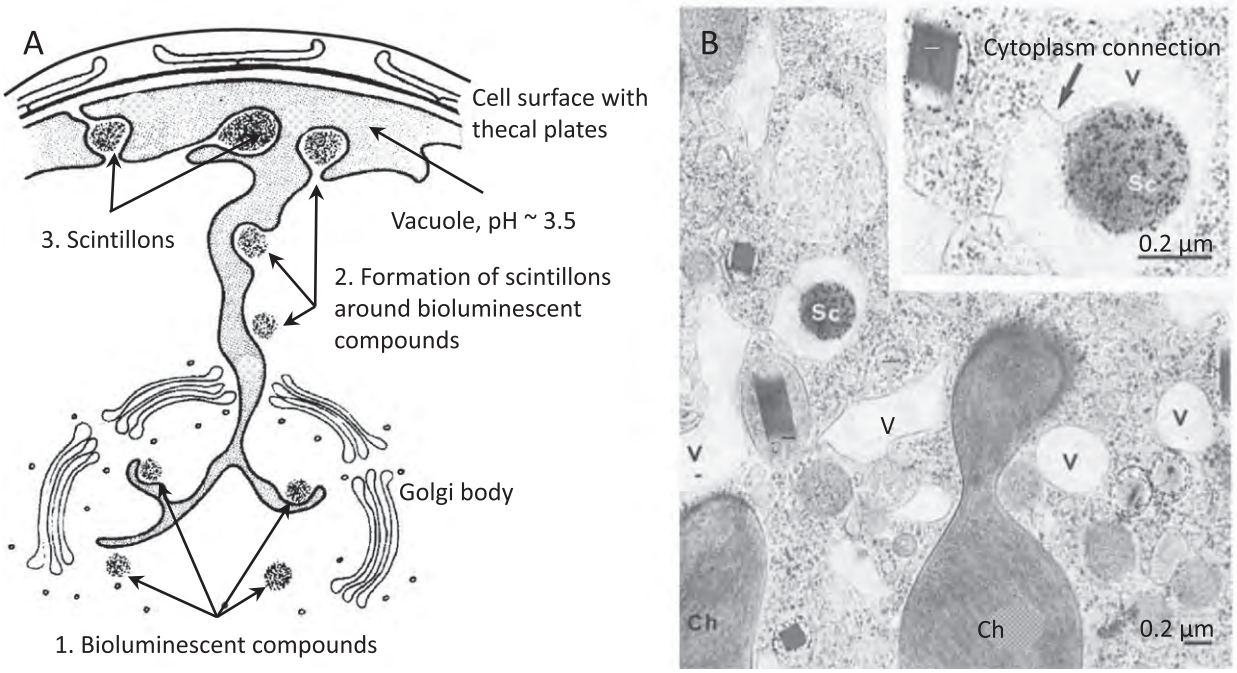

Fig. 3 Dinoflagellate scintillon formation and association with the vacuole membrane. (A). Hypothesized steps of scintillon formation. (1). Bioluminescent compounds as cytoplasmic condensations in the Golgi area. (2). Formation of scintillons in association with the vacuole membrane and migration to the cell periphery. (3). Mature scintillons. (B). Electron micrograph showing part of a *Lingulodinium polyedra* cell in cross section, with a scintillon (Sc) hanging like a drop within the vacuolar space (V) (inset). Black spots inside the scintillon are immunogold labeling of luciferase. Chloroplasts (Ch) are also shown. Nicolas, M.-T., Nicolas, G., Johnson, C.H., Bassot, J.-M., Hastings, J.W., 1987.Characterization of the bioluminescent organelles in *Gonyaulax polyedra* (Dinoflagellates) after fast-freeze fixation and antiluciferase immunogold staining. Originally published in Journal of Cell Biology 105, 723–735. https://doi.org/10.1083/jcb.105.2.723. Reprinted with permission.

Fig. 4 Light emission from scintillons in cells of the dinoflagellate *Pyrocystis fusiformis* that are mechanically stimulated. Cell length is about 600 μm. Image © Iyvone Khoo 2016. All rights reserved. Courtesy the Artist, image used with permission.

Shear stress activation of bioluminescence

Fluid shear stress associated with flow is most effective in stimulating dinoflagellate bioluminescence. Bioluminescence is not activated by typical fluid shear levels found in the ocean interior or within the feeding current of a copepod predator, but requires forces equivalent to an actual encounter with a copepod or breaking waves, >0.02–0.3 N m^{-2} depending on the dinoflagellate species. A rapid change in shear stress is more stimulating than slowly developing shear stress, which results in desensitizing of the mechanosensory system.

Cellular mechanism for bioluminescence activation

Cellular mechanisms of mechanotransduction are evolutionary well conserved and allow individual cells in multicellular and unicellular organisms to sense and respond to environmental stimuli. In luminescent dinoflagellates the response latency between mechanical stimuli and a flash of light ranges between 15 and 22 ms for investigated species, including *L. polyedra*, *P. bahamense*, *Alexandrium monilatum*, *P. fusiformis*, and *N. scintillans*, with a minimum latency of only 8 ms. Thus dinoflagellates are capable of rapid mechanosensing.

Mechanotransduction in dinoflagellates is only partly understood, but shares several characteristics with sensing of fluid shear stress due to blood flow in human endothelial cells, and other guanosine triphosphate binding protein (G protein) mediated pathways such as phototransduction in the eyes of fruit flies. Only, the response latency for stimulation of dinoflagellate bioluminescence is shorter than that for sensing of fluid shear in endothelial cells, and even operates more rapidly than photoreception in the fruit fly. Hence, it could be one of the fastest known cellular responses mediated by G proteins in any organism.

From currently available experimental evidence, the following signaling pathway is hypothesized: Mechanical stimulation increases the fluidity of the cell membrane, which activates G proteins directly or via a G protein receptor. G protein activation initiates a series of poorly understood biochemical steps that results in the opening of calcium permeable Transient Receptor Potential channels (TRP channels) located in the cell membrane, via activation of Phospholipase C (PLC), one of the three major G protein second messenger pathways. The open TRP channels allow for influx of calcium across the cell membrane, which in turn stimulates the release of more calcium from intracellular stores.

The increased cytoplasmic calcium concentration leads to depolarization of the electrically excitable membrane of the acidic vacuole, which is associated with the scintillons. Depolarization initiates a self-propagating action potential that travels over the vacuole membrane to open voltage gated H^+-channels in the scintillon membranes. Influx of H^+ from the vacuole to the scintillons and activation of the bioluminescence reaction as described above.

Intriguingly, genetic studies of dinoflagellate-related organisms suggests that G proteins are lacking in alveolates including dinoflagellates. On the contrary, pharmacological substances that activate or inhibit G protein activity affect bioluminescence (see above) and other activities of alveolates, and a ciliate G protein section has been cloned, suggesting that G proteins may be present in dinoflagellates. Evidence for several types of TRP channels, including motifs conserved from protists to mammals, in an *L. polyedra* transcriptome was only recently discovered.

The cytoskeleton, mainly F actin, serves important functions in the mechanosensing mechanism, possibly by regulating the stiffness of the cell, integrating different parts of the mechanosensing pathway, and/or anchoring the scintillons to the vacuolar membrane.

Circadian Rhythm and Light Inhibition of Bioluminescence

Circadian rhythm

Bioluminescence of the majority of luminescent dinoflagellates follows a circadian rhythm, where the cells are brightly luminescent at night and poorly mechanically excitable during the day. In the dark phase the bioluminescence response to mechanical stimulation is 60–950 times greater than during the day phase, except for a few investigated species, e.g., *N. scintillans*,

that do not have diurnal variations in the bioluminescence response. The day night variation in stimulated bioluminescence is a convenient tool for obtaining luminescent and non-luminescent cells of the same species. This is common and convenient procedures for experimental purposes, as described below in the ecology section. There are two mechanisms that regulate bioluminescence:

(1) Diel variation of scintillons, luciferin, luciferase and luciferin binding protein.
In *L. polyedra* bioluminescence capacity is diminished by the breakdown of scintillons, and their content, at the end of the dark-phase; the number of scintillons is about 10 times higher in the dark-phase as compared to the light-phase. During the light phase, luciferase and luciferin binding protein are not expressed in the cell, and production is upregulated at the end of each light phase. The expression of luciferase and luciferin binding protein is likely controlled at the translational level, as the level of mRNA templates for these proteins is constant throughout the light and dark phases. The mechanism for translational control is unknown, but there are conflicting reports on a circadian controlled translational regulator (CCTR) for luciferin binding protein in *L. polyedra*, and regulation involving miRNA has been experimentally ruled out.

(2) Change in scintillon position between light and dark phase.
Members of the genus *Pyrocystis* use a different strategy of scintillon regulation than does *L. polyedra. P. noctiluca* and *P. fusiformis* both express luciferase throughout the light and dark phase, The bioluminescent chemistry remains intact in the cell during the dark phase; instead the cellular localization of the scintillons varies with the diurnal rhythm. During the light phase the scintillons are located around the nucleus at the center of the cell. At the beginning of the dark phase, scintillons are translocated via the cytoskeleton to a peripheral position in the cell and are anchored to the membrane of the acidic vacuole. In this position, the bioluminescence chemistry can be activated to produce a flash of light in response to mechanical stimulation. During the light phase the scintillons are not associated with the vacuolar membrane, so action potentials during that phase do not stimulate bioluminescence.

Photoinhibition of bioluminescence

Light exposure during the dark phase inhibits dinoflagellate bioluminescence by as much as 99%. This mechanism of photoinhibition occurs in response to ambient light levels as low as the intensity of the dinoflagellate's own bioluminescence. It is present in heterotrophic and autotrophic dinoflagellates, but absent in some species, e.g., *N. scintillans* and *Polykrikos*. Light exposure affects some component of the mechanotransduction signaling pathway rather than the chemiluminescent reaction, as luminescence from photoinhibited cells can still be stimulated using acid treatment to directly activate the luminescent chemistry.

Inhibition of bioluminescence is complete within 10 min of light exposure, but recovery to full sensitivity takes 30–120 min depending on the level of light exposure and species investigated. Light with a blue emission spectrum is most efficient; inhibiting wavelengths are similar to the absorbance spectrum of chlorophyll, suggesting that the inhibition mechanism involves light absorbance by chlorophyll. However, light inhibition also occurs in several species of heterotrophic dinoflagellates, suggesting that other molecules are light sensitive.

Photoinhibition is one of several physiological mechanisms, including circadian regulation, cell membrane sensitivity, and predator-induced up-regulation of luminescence, all described below, that regulate light emission to minimize the energy spent on bioluminescence in situations where it would not benefit the dinoflagellate. During the dark phase, luminescence intensity in autotrophs is dependent on the energy obtained from sunlight during the previous light phase, and for heterotrophs from prey abundance. In situations when recourses are limited dinoflagellates allocate energy to bioluminescence over growth, suggesting that bioluminescence is an expensive but important trait. Existence of non-luminescent strains of otherwise luminescent species may suggest that some strains benefit from mimicry and are able to avoid the energetic cost of producing light.

Ecology of Bioluminescent Dinoflagellates

Worldwide distribution of luminescent dinoflagellates

Dinoflagellates are found in all oceans of the world, with large spatial and temporal variations in abundance. They are the most common source of near-surface displays of bioluminescence, and account for "sheet-like" displays that have been widely observed. Based on observations of *P. bahamense* from bioluminescent bays in Puerto Rico and Jamaica (described below), bioluminescence is observable at cell concentrations >1000 cells L^{-1}, the blue color of their emission is apparent at concentrations >6000 cells L^{-1}, and for concentrations >100,000 cells L^{-1} the light is bright enough to read by.

Plankton bioluminescence is assessed at night using bathyphotometers that measure flow-stimulated bioluminescence potential across a range of spatial scales. Bathyphotometer measurements has a potential as an early indicator of harmful algal blooms, as described below, as they can indicate low concentrations of the twelve luminescent dinoflagellate species known to be toxic, as well as the presence of non-luminescent toxic species when their abundance is correlated. Bathyphotometer measurements also have potential to assess the composition of phytoplankton communities, and to examine relationships between luminescent populations and oceanographic conditions.

Bioluminescence protects dinoflagellates from copepod grazing

Experimental evidence supports the hypothesis that dinoflagellate bioluminescence serves as a defense against predators. Dinoflagellates in the lower size range (10–50 μm in diameter) are a preferred prey for copepods, compared to other micro/phytoplankton.

Fig. 5 Enhancement of bioluminescence of the dinoflagellate *Lingulodinium polyedra* by copepod lipids. Total bioluminescence capacity assayed by acidifidication was enhanced 29% from controls following pre-exposure to 1 nM extract of copepod lipids. Modified from Lindström, J., Grebner, W., Rigby, K., Selander, E., 2017. Effects of predator lipids on dinoflagellate defence mechanisms – Increased bioluminescence capacity. Scientific Reports 7, 13104 (9 pages).

Larger dinoflagellates (>50 μm) are too large prey for copepods and are instead consumed by other organisms including hydromedusae, crustaceans, and fish larvae. Although the ecological benefits of bioluminescence in dinoflagellates has been focused on the interaction with copepods, the value of dinoflagellate bioluminescence is less clear against other predators, especially those without visual systems.

Copepods are the most abundant zooplankton in the ocean and thus exert top-down control on dinoflagellate population by grazing. Both *L. polyedra* and *A. tamarense* upregulate their bioluminescence capacity when exposed to lipid compounds released from copepods, while dinoflagellates kept in predator free cultures lose bioluminescence capacity. This suggests that bioluminescence in these species is an effective defense against copepods (Fig. 5).

Bioluminescence reduces grazing from copepods by 50%–80%, as compared to grazing on non-luminescent controls of the same dinoflagellate species. There are three major theories about the predator defense role of dinoflagellate bioluminescence:

(1) A flash of light from the dinoflagellate interrupt the copepod feeding behavior by startling the copepod.
Copepod swimming behavior is affected by both natural dinoflagellate bioluminescence and artificial flashes that mimic dinoflagellate flashes. Marine and estuarine copepods increase burst swimming behavior when exposed to flash intensities corresponding to flashes from "small and dim" dinoflagellates like *L. polyedra* (0.002–0.02 μE m^{-2} s^{-1}). This temporarily interrupts feeding behavior of copepods, resulting in reduced grazing. In addition to increased burst swimming, higher light intensities stimulate increased swimming speed and a straighter swimming path that would make copepods both interrupt grazing and potentially swim away from a patch with a high concentration of dinoflagellates. The copepod responses to bioluminescent flashes seem to be specific, as fresh water copepods, with no experience of dinoflagellate bioluminescence, do not respond to flashes.

(2) The dinoflagellate flash, due to interaction with a copepod, works as a 'burglar alarm' that attracts larger predators to feed on the copepod.
Flashes from bioluminescent dinoflagellates have been experimentally demonstrated to make prey (e.g., copepods, mysids, shrimp and fish) more vulnerable to their predators (e.g., fish and cephalopods), and thus are functional as a 'burglar alarm'. Small and dim dinoflagellates at low concentrations are not effective as a burglar alarm. Instead, inhibition of grazing may be due to the mechanism of aposematic coloration or startling effect. However, as *L. polyedra* and *A. tamarense* are capable of upregulating their light capacity as a response to lipid compounds released from copepods, the burglar alarm effect may be efficient even at lower cell concentrations. The 'burglar alarm' effect of the dinoflagellate flash is considered to counteract the tendency for copepods to habituate to the light flash, in which case their risk of predation from visual predators attracted by the flash would increase.

(3) Bioluminescence works as an aposematic coloration, signaling that the dinoflagellate is toxic.
Bioluminescence works as an aposematic coloration in some terrestrial bioluminescent organisms such as millipedes, signaling the inedibility of the organisms to predators. This mechanism has also been suggested for marine scale worms, brittlestars and jellyfish. As twelve bioluminescent dinoflagellate species are known to produce toxins, and some dinoflagellates also produce other compounds that affect marine organisms, bioluminescence in those species could be effective as an aposomatic signal. Copepod physiology is negatively affected by ingestion of toxic dinoflagellates. But it is unclear if bioluminescence serves as an aposomatic signal in dinoflagellates that results in rejection of toxic cells, as copepods are often capable of recognizing and rejecting toxic dinoflagellates even if they are not bioluminescent.

Red tides and harmful algal blooms

Red tides are blooms of microorganisms, commonly dinoflagellates, that discolor the water because of their high abundance. At night blooms of luminescent dinoflagellates can lead to spectacular displays of bioluminescence, observed in breaking waves, surge, and associated with moving ships and animals.

Red tides or Harmful Algal Blooms (HAB) occur worldwide and are increasing in frequency and distribution, due to increasing ocean temperatures, a higher anthropogenic load of nutrients in coastal regions, and spreading of organisms to new areas via storms and ship ballast water. Red tides of bioluminescent dinoflagellates can be harmless, but also devastating to sea life and affect human health and economy, when blooming species produce potent toxins that are passed through the food chain to be consumed by marine mammals and humans. Even blooms of non-toxic bioluminescent species, e.g., *N. scintillans*, can still have a negative impact on the ecosystem, causing oxygen depletion and increased concentrations of ammonia.

Bioluminescent bays

Unlike typical dinoflagellate blooms, which can be intermittent and unpredictable in occurrence and duration, there are locations known as bioluminescent bays, or biobays, where high abundance of bioluminescent dinoflagellates persists throughout the year. There are fourteen known biobays worldwide, with eleven located in the Caribbean region. The bays are popular sites for ecotourism, and important contributors to the local economies.

Biobays are typically small shallow bays with limited water exchange with the sea, and fringed with a mangrove forest. The understanding of which factors regulates dinoflagellate populations in the biobays are still limited, but common characteristics include; prolonged water retention time, nutrient availability, shallow basin bathymetry, prevailing wind direction that retains organisms with the biobay, and limited tidal range. Mangrove trees are believed to benefit dinoflagellates in two ways: 1. The microbial community associated with the mangroves provides vitamin B12, which is required for dinoflagellate growth, and 2. Phenolic substances derived from mangrove leaf litter inhibits the growth of phytoplankton competing with dinoflagellates for light and nutrients.

The major source of bioluminescence in biobays is the dinoflagellate *Pyrodinium bahamense*, a bloom-forming dinoflagellate found globally in tropical coastal regions and originally described from a biobay in the Bahamas (Fig. 2(C)). The success of this species is believed to be related to its high tolerance for salinity fluctuations; motility that allows cells to relocate to patches with nutrients; and production of saxitoxins that would suppress grazing from copepods. Bioluminescence would also suppress grazing from copepods, as described above, but the relationship between *P. bahamense* abundance and grazing pressure needs to be investigated, because copepods can be very abundant in biobays; for example in Phosphorescent Bay, Puerto Rico, copepod abundance ranges between 20 and 1500 individuals l^{-1}.

Blooms of *P. bahamense* are globally associated with rainfall, most likely due to increased nutrient availability caused by run-off from watersheds. However, in the biobay Laguna Grande in Puerto Rico, daytime monthly abundance of *P. bahamense* measured over three years was not correlated with salinity, nutrients, temperature, or rainfall, although storm events resulted in short-term decreases in abundance.

Applications of Dinoflagellate Bioluminescence

Flow imaging and bioassays

Dinoflagellate bioluminescence is stimulated by a change in flow velocity rather than absolute flow velocity, with an almost instantaneous response, making it useful for the visualization of hydrodynamic stresses and local velocity gradients. Dinoflagellate bioluminescence has been applied as a tool for imaging boundary flows associated with a moving dolphin (Fig. 6) and predicting the luminescent wakes of moving ships, to estimate flow conditions such as stresses within a breaking wave, over a rippled seabed in bioreactors used for cell culture, and associated with a water jet. It has also been used as a bioassay for toxicity, flow stress in microgravity simulators, and detecting magnetic fields. The development of a statistical model for the dinoflagellate flash response provides a theoretical framework for examining the relationship between flow stimulation and the bioluminescence response.

Functions and evolution of cellular mechanotransduction

Dinoflagellate bioluminescence is also a promising experimental system for investigating the evolutionary origins of mechano-transduction in eukaryotes. Mechanosensitive proteins present in dinoflagellates have been evolutionarily conserved and are present in higher organisms including mammals. Dinoflagellate bioluminescence act as a whole-cell reporter of mechanical stress

Fig. 6 Bioluminescence from naturally occurring dinoflagellates stimulated by the motion of an Atlantic bottlenose dolphin *Tursiops truncatus* gliding alongside a boat moving at approximately 0.1 m s^{-1}. The image is from a single video frame taken by a black and white image intensified camera. Reduced bioluminescence on the melon was due to a thinner boundary layer, while flow separation off the fins resulted in enhanced bioluminescence. From Rohr, J., Latz, M.I., Fallon, S., Nauen, J.C., Hendricks, E., 1998. Experimental approaches towards interpreting dolphin-stimulated bioluminescence. Journal of Experimental Biology 201, 1447–1460.

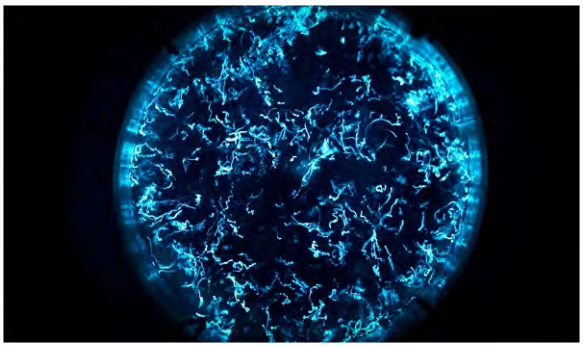

Fig. 7 Sound stimulation of bioluminescence by the dinoflagellate *Pyrocystis fusiformis*. Still image from *Tribal Waves*. Copyright 2016, Jack Smith. Used with permission.

where the effects of experimental treatments that target the mechanosensing mechanism are measured as a change in the bioluminescence response to mechanical stimulation. Pharmacological and transcriptomics approaches are providing insight into the structure and function of signaling proteins within the rapid dinoflagellate mechanotransduction pathway.

Education and art

Bioluminescence is a beautiful display of nature that is suitable for educational and artistic purposes. It engages student learning in biology, chemistry, and physics, seeking to answer questions such as: What is bioluminescence? What organisms produce bioluminescence? Where do they live? What is the chemical reaction that results in light emission? What are the functions of bioluminescence? Lessons can be accompanied by classroom demonstrations of dinoflagellate bioluminescence, which can be stimulated by flow, bubbles, or sound, creating opportunities for artistic interpretations leading to creative demonstrations of bioluminescence (Fig. 7).

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Relevant Websites

http://siobiolum.ucsd.edu. –Latz Laboratory.
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Bioreactors

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Glossary

Airlift reactor Column with defined volumes for upflow and downflow of the culture broth; vertical circulation occurs because air is bubbled into the upflow volume.
Batch bioreactor Culture broth is fed into the reactor at the start of the process; air may flow continuously.
Bubble column reactor Aerated column without mechanical agitation.
Fed batch Liquid media is fed to the reactor continuously; the broth accumulates in the reactor as there is no outflow of liquid.
Heterotrophs Microorganisms growing on an organic compound that provides carbon and energy.
Insect cell culture Cultivation of insect cells in a bioreactor to produce a protein or other product.
Photoautotrophs Microorganisms that use light for energy and carbon dioxide for their carbon source.
Plant cell culture Production of plant cells in a bioreactor to produce useful products.
Protein engineering The design, development, and production of new protein products with properties of commercial value.
Tissue engineering The design, development, and production of tissue cells (biomaterials) for use on or in humans.

Introduction

The importance of the bioreactor is recorded in early history. The Babylonians apparently made beer before 5000 BCE. Wine was produced in wineskins, which were carefully selected for their ability to produce a beverage that met the approval of the King and other members of his sensory analysis taste panel. Food and beverage product quality depended on art and craftsmanship rather than on science and engineering during the early years of bioreactor selection and utilization. Early recorded history shows that some understood the importance of the reactants and the environmental or operating conditions of the reactor. This allowed leavened bread and cheese to be produced in Egypt more than 3000 years ago.

The process of cooking food to render it microbiologically safe for human consumption as well as to improve its sensory qualities is also an ancient tradition. The process of thermal inactivation of microorganisms through the canning of food to allow safe storage was an important early achievement in bioreactor design and operation.

As humans learned to live in cities, waste management including wastewater treatment emerged as a necessity for control of disease. One of the first process engineering achievements was the biological treatment of wastes in bioreactors designed and built by humans for that purpose. Because a significant fraction of the population of a city could die from disease spread by unsanitary conditions, these early bioreactors represented important advancements.

After microorganisms were discovered, microbiologists and engineers increased their understanding of the biochemical transformations in bioreactors. Simple anaerobic fermentations for the production of ethyl alcohol, acetone, and butanol were developed. Aerobic and anaerobic treatment of wastewater became widely used. Sanitary engineering became a part of civil engineering education.

In the 1940s, the field of biochemical engineering emerged because of developments in the pharmaceutical industry that required large-scale bioreactors for the production of streptomycin and penicillin. Progress in bioreactor design and control resulted from research on oxygen transfer, air and media sterilization, and pH control. The central concern of the early biochemical engineers was the development of bioreactors that could achieve and maintain the chemical and physical environment for the organism that the biochemist/microbiologist recommended. The ability to scale up from laboratory bioreactors to large fermentors required the development of instrumentation such as the sterilizable oxygen electrode. Early courses in biochemical engineering were concerned with the analysis, design, operation, and control of bioreactors. Bioreactors for mammalian cell applications have been developed and used for health care advances. Stem cell production, tissue engineering, and bioreactors to support regenerative medicine are examples of more recent developments. The liver is a bioreactor, and there is significant progress in the development of bioartificial liver support systems. Some of the significant developments in bioreactor technology are listed in Table 1 together with the approximate date.

Classifications of Bioreactors

Several methods have been used to classify bioreactors. These include the feeding of media and gases and the withdrawal of products; the mode of operation may be batch, fed batch, or continuous. The classification may be based on the electron acceptor; the design may be for aerobic, anaerobic, or microaerobic conditions. In aerobic processes, the methods of providing oxygen have

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Table 1 Significant developments in bioreactor technology

Development	*Year*[a]
Fermented beverages	5000 BCE
Pasteur's discovery of yeast	1857
First medium designed for culturing bacteria	1860
Trickling filter for wastewater	1868
Anaerobic digester	1881
Production of citric acid using mold	1923
Production of penicillin in a Petri dish	1928
Production of penicillin in small flasks	1942
Hixon and Gaden paper on oxygen transfer	1950
Air sterilization in fermentors	1950
Continuous media sterilization	1952
Aiba, Humphrey, and Millis biochemical engineering textbook on bioreactor design	1965
Continuous airlift reactor for production of yeast	1969
Advances in instrumentation and computer control	1970
Progress in airlift bioreactor design	1973
Recombinant DNA technology	1973
Insect cells grown in suspension culture	1975
Large-scale cell culture to produce interferon	1980
Insulin produced using bacteria	1982
Bioreactors for fragile cell cultures	1988
Textbook on plant cell biotechnology	1994
Textbook on protein engineering	1996
Textbook on tissue engineering	1997
Rapidly increasing number of bioreactors for renewable energy applications	2006
Stem cell bioreactor book	2016

[a]The dates are approximate and indicative of periods of time when advances were moving from initial studies to published works or commercial use.

resulted in mechanically agitated bioreactors, airlift columns, bubble columns, and membrane reactors. The sterility requirements of pure culture processes with developed strains differ from those of environmental mixed-culture processes, which are based on natural selection. There are bioreactors in which the vessel is made by humans and natural bioreactors such as the microbial cell, the flowing river, and the field of native grass. In this article, the classification of bioreactors will be based on the physical form of the reactants and products.

Gas Phase Reactants or Products

Oxygen and carbon dioxide are the most common gas phase reactants and products. Others include hydrogen, hydrogen sulfide, carbon monoxide, and methane. Oxygen is a reactant in aerobic heterotrophic growth processes, whereas it is a product in photoautotrophic growth. Generally, the concentration of the reactants and products in the liquid phase in the microenvironment of the cell influences the kinetics of the cellular reaction. Mass transfer to and from the gas phase affects bioreactor performance in most processes with gas phase reactants or products. The anaerobic reactor is designed to exclude oxygen. In some cases, inert gases are bubbled into the anaerobic reactor to provide gas-liquid interfacial area to remove the product gases.

Because the solubility of oxygen in water is very low, the dissolved oxygen in the broth is rapidly depleted if oxygen transfer from the gas to the liquid phase is disrupted in aerobic processes. The distribution of dissolved oxygen throughout the reactor volume and the transient variation affect reactor performance. When mold pellets or biofilms are present, the diffusion of oxygen into the interior should be considered. A significant fraction of the bioreactor literature is devoted to oxygen transfer and the methods recommended for the design and operation of aerobic bioreactors. The phase equilibrium relationship is based on thermodynamic data, while the rate of oxygen transfer depends on the gas–liquid interfacial area and the concentration driving force. Mechanical agitation increases the gas–liquid interfacial area. Aeration provides the supply of oxygen, and it affects the gas–liquid interfacial area.

Oxygen has been supplied by permeation through membranes in cultures in which bubbles may damage shear-sensitive cells. The membrane area and concentration driving force determine the oxygen transfer rate in these bioreactors.

Most large-scale bioreactors have either oxygen or carbon dioxide among the reactants or products. In many anaerobic fermentations, the formation of carbon dioxide results in bubbling, and often no additional mixing is required for either mass transfer or suspension of the microbial cells. Methane is produced through anaerobic digestion of waste products. It is also a product of microbial action in landfills, bogs, and the stomach of the cow.

Packed bed bioreactors are used to biodegrade volatile organic compounds in air pollution control applications. The rhizosphere provides a natural environment where many volatile compounds in soil are transformed by microbial and plant enzymes.

Liquid Phase Reactants or Products

Many bioreactors have liquid phase reactants and products. Ethanol, acetone, butanol, and lactic acid are examples of liquid products that can be produced by fermentation. The kinetics of biochemical reactions depend on the liquid phase concentrations of the reactants and, in some cases, the products. The Monod kinetic model and the Michaelis–Menten kinetic model show that many biochemical reactions have first-order dependence on reactant (substrate) concentration at low concentrations and zero-order dependence at higher concentrations. Rates are directly proportional to concentration below 10 mg l^{-1} for many reactants under natural environmental conditions. At very high concentrations, inhibition may be observed.

Hydrocarbons that are relatively insoluble in the water phase, such as hexadecane, may also be reactants or substrates for biochemical reactions. Microbial growth on hydrocarbons has been observed to occur at the liquid–liquid interface as well as in the water phase. The airlift bioreactor is uniquely suited for this four-phase process because of the tendency of the hydrocarbon phase to migrate to the top of the fermentor. The hydrocarbons are found suspended as drops in the water phase, adsorbed to cells, and at the gas–liquid interface. The cells are found adsorbed to hydrocarbon drops, suspended in the water phase, and at the gas–liquid interface. In the airlift fermentor, the vertical circulation mixes the hydrocarbons and cells that have migrated to the top of the fermentor with the broth that enters the downflow side of the column.

One of the oldest and most widely practiced fermentations is the microbial production of ethanol and alcoholic beverages such as beer and wine. Because ethanol inhibits the fermentation at high concentrations, the process of inhibition has been extensively studied for this fermentation. Ethanol affects the cell membrane and the activities of enzymes. This inhibition limits the concentration of ethanol that can be obtained in a fermentor. Because ethanol is also produced for use as a motor fuel, there is still considerable research on ethanol production. Because the cost of the substrate is a major expense, inexpensive raw materials such as wastes containing cellulose have been investigated.

Solid Phase Reactants or Products

There are many examples of bioreactors with solid phase reactants. The cow may be viewed as a mobile bioreactor system that converts solid substrates to methane, carbon dioxide, milk, and body protein. While the cow is a commercial success, many efforts to transform low cost cellulosic solid waste to commercial products in human- made bioreactors have not achieved the same level of success because of economics.

Solid substrates such as soybean meal are commonly fed into commercial fermentations. Through the action of enzymes in the fermentation broth, the biopolymers are hydrolyzed and more soluble reactants are obtained.

Many food fermentations involve the preservation of solid or semisolid foods such as in the conversion of cabbage to sauerkraut and meats to sausage products. Cereals, legumes, vegetables, tubers, fruits, meats, and fish products have been fermented. Some fermented milk processes result in solid products such as cheeses and yogurts.

Other examples include the composting of yard wastes, leaching of metals from ores, silage production, biodegradation of crop residues in soil, microbial action in landfills, and the remediation of contaminated soil.

In many of these fermentations, mixing is difficult or expensive. Transport of essential reactants may depend on diffusion; the concentrations of reactants and products vary with position. Rates may be limited by the transport of essential reactants to the microorganisms.

Most compounds that are present as solids in bioreactors are somewhat soluble in the water phase. For reactants that are relatively insoluble, biochemical reaction rates may be directly proportional to the available interfacial area. The surface of the solid may be the location of the biochemical transformation. An example of microorganisms growing on the surface of a solid substrate is mold on bread. To design bioreactors for solid substrates and solid products, the solubility and the transport processes as well as the kinetics of the process should be addressed.

Recently, there has been considerable progress in tissue engineering. The rational design of living tissues and the production of these tissues by living cells in bioreactors are advancing rapidly because of the progress in systems design and control for both in vitro flow reactors and in vivo maintenance of cell mass. The importance of the local environment with respect to concentration of oxygen, reactants and products must be carefully controlled.

Bioreactors

The rate of reaction in bioreactors is often directly proportional to the concentration of microbial biomass. In biological waste treatment, the influent concentration of the organic substrate (waste) is relatively low, and the quantity of microbial biomass that can be produced from the waste is limited. The economy of the operation and the rate of biodegradation are enhanced by retaining the biomass in the bioreactor. In the activated sludge process, this is done by allowing the biomass to flocculate and settle; it is then recycled. The trickling filter retains biomass by allowing growth on the surfaces of the packing within the bioreactor.

A variety of immobilized cell reactors and immobilized enzyme reactors have been designed and operated because of the economy associated with reuse of cells and enzymes. In the anaerobic production of ethanol, lactic acid, and the other fermentation products, the product yield is greatest when the organisms are not growing and all of the substrate is being converted to products. Continuous processes can be designed in which most of the cells are retained and the limiting maximum product yield is approached. Ultrafiltration membrane bioreactors have been used to retain cells, enzymes, and insoluble substrates.

In nature, cells are retained when biofilms form along flow pathways. The biofilms allow microorganisms to grow and survive in environments where wash out would be expected. The excellent quality of ground-water is the result of microbial biodegradation and purification under conditions where microbial survival is enhanced by biofilm formation and cell retention on soil and rock surfaces. The ability of microorganisms to survive even after their food supply appears to be depleted is well established; this accounts for our ability to find microorganisms almost everywhere in nature. When spills occur, organic substances will often be degraded by microorganisms, if the nutritional environment is balanced. Nitrogen, phosphorus, and other inorganic nutrients often must be added.

The concentration of cells adsorbed to the surface and the concentration in the water phase depend on an adsorption phase equilibrium relationship and the operating conditions. In many environmental applications, most of the cells are adsorbed to surfaces. However, in large-scale fermentors with high cell concentrations and rich media feeds, only a small fraction of the cells are found on surfaces.

In many animal cell bioreactors, cell attachment to surfaces is beneficial and microcarriers are present in bioreactors to provide surface area for cell attachment by adsorption.

Photobioreactors

Light is the energy source that drives photoautotrophic growth processes. Because light is absorbed by the growing culture, the intensity falls rapidly as the distance from the surface increases. Photobioreactors are designed to produce the quantity of product that is desired by selecting a surface area that is sufficient to obtain the needed light. Heat transfer is an important design aspect because any absorbed light energy that is not converted to chemical energy must be dissipated as heat.

There is significant commercial production of algae in open raceway pond photobioreactors that make use of sunlight for the energy for growth. Since temperature is important, these commercial raceway ponds are often in locations where the temperature is in the range from 15C to 35C most or all of the year. Fig. 1 shows the top view of a raceway pond photobioreactor with a paddlewheel to promote flow and mixing for algal mass production. The normal depth of the fluid in the system is about 0.30m. Since the system is open, it is important to have algal biomass that grows rapidly and is able to compete well with other photoautotrophic organisms.

Principles of Bioreactor Analysis and Design

The basic principles of bioreactor analysis and design are similar to those for chemical reactors; however, many biochemical processes have very complex biochemistry. The chemical balance equations or stoichiometry of the process must be known or investigated. The yield of microbial biomass and products depends on the genetics of the strain and the operating conditions. The consistency of data from experimental measurements can be evaluated using mass balances such as the carbon balance and the available electron balance.

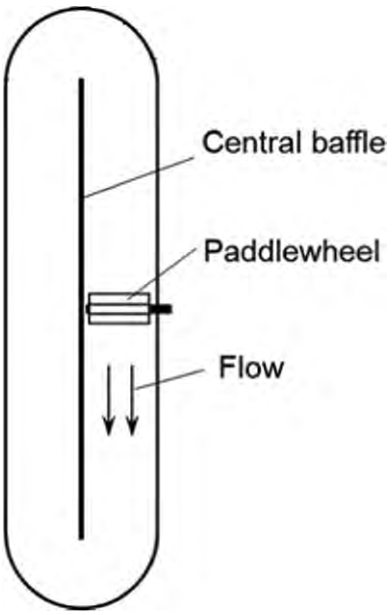

Fig. 1 A top view of a raceway pond photobioreactor for algal biomass production.

Microorganisms obey the laws of chemical thermodynamics; some heat is produced in heterotrophic growth processes. The free energy change is negative for the complete system of biochemical reactions associated with heterotrophic growth and product formation. Thus, the chemical energy available for growth and product formation decreases as a result of microbial assimilation of the reactants.

The rate of growth and product formation depends on the number of microorganisms and the concentrations of the nutrients. The kinetics of growth and product formation are often written in terms of the concentration of one rate-limiting substrate; however, in some cases, more than one nutrient may be rate limiting. The kinetics must be known for rational design of the bioreactor.

Heat is evolved in microbial bioreactors. For aerobic processes, the quantity of heat generated (heat of fermentation) is directly proportional to the oxygen utilized. Thus, the heat transfer and oxygen transfer requirements are linked by the energy regularity of approximately 450 kJ of heat evolved per mole of oxygen utilized by the microorganisms.

Transport phenomena is widely applied in bioreactor analysis and design. Many fermentation processes are designed to be transport limited. For example, the oxygen transfer rate may limit the rate of an aerobic process. Bioreactor design depends on the type of organism as well as the nutritional and environmental requirements. For example, in very viscous mycelial fermentations, mechanical agitation is often selected to provide the interfacial area for oxygen transfer. Likewise, animal cells that grow only on surfaces must be cultured in special bioreactors, which provide the necessary surface area and nutritional environment. In other cases, animals are selected as the bioreactors, because the desired biochemical transformations can best be achieved by competitively utilizing animals; cost and quality control are both important when food and pharmaceutical products are produced.

The term biopharming has been introduced to describe the production of proteins for health care applications by transgenic livestock. The product of interest may be produced and separated from milk, for example.

Sensors, Instrumentation, and Control

The ability to measure the physical and chemical environment in the fermentor is essential for control of the process. In the last 60 years, there has been significant progress in the development of sensors and computer control. Physical variables that can be measured include temperature, pressure, power input to mechanical agitators, rheological properties of the broth, gas and liquid flow rates, and interfacial tension. The chemical environment is characterized by means of electrodes for hydrogen ion concentration (pH), redox potential, carbon dioxide partial pressure, and oxygen partial pressure. Gas phase concentrations are measured with the mass spectrometer. Broth concentrations are measured with gas and liquid chromatography; mass spectrometers can be used as detectors with either gas or liquid chromatography. Enzyme thermistors have been developed to measure the concentration of a variety of specific biochemicals. Microbial mass is commonly measured with the spectrophotometer (optical density) and cell numbers through plate counts and direct microscopic observation. Instruments are available to measure components of cells such as reduced pyridine nucleotides and cell nitrogen. Online biomass measurements can be made using a flow cell and a laser by making multiangle light scattering measurements. Multivariate calibration methods and neural network technology allow the data to be processed rapidly and continuously such that a predicted biomass concentration can be obtained every few seconds.

The basic objective of bioreactor design is to create and maintain the environment needed to enable the cells to make the desired biochemical transformations. Advances in instrumentation and control allow this to be done reliably.

Metabolic and Protein Engineering

Genetic modification has allowed many products to be produced economically. With the use of recombinant DNA technology and metabolic engineering, improved cellular activities may be obtained through manipulation of enzymatic, regulatory, and transport functions of the microorganism. The cellular modifications of metabolic engineering are carried out in bioreactors. Successful manipulation requires an understanding of the genetics, biochemistry, and physiology of the cell. Knowledge of the biochemical pathways involved, their regulation, and their kinetics is essential.

Living systems are bioreactors. Through metabolic engineering, man can modify these living bioreactors and alter their performance. Metabolic engineering is a field of reaction engineering that utilizes the concepts that provide the foundation for reactor design including kinetics, thermodynamics, physical chemistry, process control, stability, catalysis, and transport phenomena. These concepts must be combined with an understanding of the biochemistry of the living system. Through metabolic engineering, improved versions of living bioreactors are designed and synthesized.

While many products are produced in microbial cells, other cell lines including insect cells, mammalian cells, and plant cells are utilized for selected applications. The science to support these various living bioreactors is growing rapidly and the number of different applications is increasing steadily. The choice of which organism to select for a specific product must be made carefully with consideration of biochemistry, biochemical engineering, safety, reliability, and cost. Both production and separation processes affect the cost of the product; however, the cost of product development, testing, regulatory approval, and marketing are substantial as well.

Proteins with specific functional properties are being designed, developed, and produced through applications of protein engineering. Through molecular modeling and computer simulation, proteins with specific properties are designed. Protein

production may involve applications of recombinant DNA technology in host cell expression bioreactors. An alternative is to produce a protein with the desired amino acid sequence through direct chemical synthesis.

Stability and Sterilization

While beneficial genetic modification has led to many industrial successful products, contamination and genetic mutations during production operations have resulted in many batches of useless broth. Batch processes are common in bioreactors because of the need to maintain the desired genetic properties of a strain during storage and propagation. Continuous operation is selected for mixed culture processes such as wastewater treatment, where there is natural selection of effective organisms.

Bioreactors that are to operate with pure cultures or mixed cultures from selected strains must be free of contamination, that is, the reactor and associated instrumentation must be sterilizable. The vessels that are to be used for propagation of the inoculum for the large-scale vessel must be sterilizable as well. Methods to sterilize large vessels, instrumentation, and connecting pipes are well developed; however, there is a continuing need to implement a wide variety of good manufacturing practice principles to avoid contamination problems.

Steam sterilization has been widely applied to reduce the number of viable microorganisms in food and fermentation media. As temperature increases, the rates of biochemical reactions increase exponentially until the temperature affects the stability of the enzyme or the viability of the cell. The Arrhenius activation energies, which have been reported for enzymatic reactions and rates of cell growth, are mostly in the range of 20–80 kJ g mol^{-1}, whereas activation energies for the thermal inactivation of microorganisms range from 200 to 400 kJ g mol^{-1}. Many of the preceding principles also apply to the thermal inactivation of microorganisms in bioreactors. When solids are present in foods or fermentation media, heat transfer to the interior of the solid is by conduction. This must be considered in the design of the process because of the increase in the required sterilization time.

Conclusions

Bioreactors are widely used for a variety of purposes. The knowledge base for their application has increased significantly because of the advances in chemical, biochemical, and environmental engineering during the last 70 years. Many different bioreactors have been designed because of the importance of optimizing the production environment within each vessel for each application.

Many pharmaceutical, biomedical, biochemical, food, beverage, fuel, and biomaterial products are produced in bioreactors. The total amount and commercial value of these bioproducts increases annually.

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Biosensors☆

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Glossary

Biochips Biochips are biosensor devices with multianalyte capability which integrate biorecognition elements into direct spatial contact with the transducer. These devices typically have biorecognition molecules absorbed/attached to a surface in some sort of array pattern, with the array coded to specific analyte detection at specific sites on the array. The transducer must be able to read out the various signals from specific sites on the array in an independent manner.

Biomimetic In this article, biomimetic refers to synthetic materials/matrices/structures, which in some manner serve to mimic or imitate biological structures found in living systems, although their level of complexity is much reduced from the actual living system. Examples would include liposomes and Langmuir–Blodgett films, which reflect cell membranes, and molecularly imprinted polymers, which imitate biorecognition elements such as antibodies and membrane receptors.

Biorecognition element A biomolecule or synthetic analogue used in a biosensor, which serves the function of recognizing and interacting/binding to the analyte molecule. The selectivity of the biosensor is imparted by the biorecognition element. Examples of biorecognition elements include antibodies, enzymes, nucleic acids, and microorganisms. In this article, biorecognition element and bioreceptor are used interchangeably.

Bioreporters In this context, bioreporters are defined as microbial cells that have been modified by genetic means to express a particular reporter function upon exposure to certain environmental conditions or chemicals/physical stresses. The reporter function may be an enzyme that produces a detectable signal or synthesis of a protein that can be readily detected. Examples of bioreporter organisms would be bacteria that have the luciferase reporter construct linked to a promoter region that responds to some environmental stimulus, and so express bioluminescence when the stimulus occurs, or bacteria that express green fluorescent protein in an analogous manner.

ELISA An acronym for enzyme-linked immunoabsorbent assays. This common type of immunological assay has as its distinctive features the use of antigen-specific antibodies as bioreceptors for the analyte of interest, and the linking of a specific enzyme to at least one of the antibodies forming the immunological sandwich. Addition of the substrate for the enzyme covalently attached to the detector antibody results in an enormous amplification of the signal (detected by optical or electrochemical means, for example) indicating binding between analyte and antibodies, due to the iterative processivity of the enzyme for its substrate. This process thus increases sensitivity of immunoassays.

FRET Abbreviation for Förster Resonance Energy Transfer, sometimes also defined as Fluorescence Resonance Energy Transfer. This process is based on a non-radiative quantum mechanical process which neither requires molecular collisions nor produces heat. FRET can be defined as the distance- and orientation-dependent radiationless transfer of excitation energy from a shorter-wavelength donor to a longer wavelength acceptor. In practical terms, if two fluorophores are close enough together in space (approximately 10 nm or less), and also oriented correctly with respect to the other, then if the higher energy fluorohore is excited, it passes its excited state energy to the lower energy fluorophore via a dipole-dipole interaction. Thus the excitation of the donor fluorophore results in emission of photons from the acceptor fluorophore.

Langmuir–Blodgett films A Langmuir–Blodgett (LB) film is a set of monolayers, or layers of organic material one molecule thick, deposited on a solid substrate. An LB film can consist of a single layer or many. In biosensor applications, biorecognition molecules may be imbedded within LB films, and changes within the film upon analyte binding can be detected by a suitable transducer.

Molecularly imprinted polymers (MIP) Molecularly imprinted polymers are polymers that are formed in such a manner so as to have cavities within them which correspond, in a complementary manner, with specific molecular structures that were used as target molecules when the polymer was formed. Thus, MIP, once formed, become sensing structures that can interact with, and bind, specific molecular species within the specific cavity sites formed during synthesis of the polymer.

Nanoparticles/nanomaterials Nanoparticles are defined as particles in the range of 1–100 nm, while more generally, the realm of nanomaterials encompasses structures in the nanometer size range, that is, submicron sized but larger than atomic dimensions. A great deal of recent interest has focused upon uses of materials in this size range for a variety of applications. For biosensors, nanoparticles with fluorescent properties (quantum dots), magnetic nanoparticles, and nanoparticles derivitized with biorecognition elements may all play increasing roles.

☆*Change History:* August 2014. GD Griffin, DN Stratis-Cullum and TE McKnight updated the text and further readings. One author was added to the authorship.

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Transducer The transducer is that portion of the biosensor that converts the interaction between the biorecognition element and analyte into a measurable signal. Changes in physical and/or chemical properties such as changes in mass, optical properties, temperature, or electrical properties can be exploited to transduce the interaction of biorecognition element and analyte into a parameter which can be measured by electronic means.
Vesicles In the context of this article, synthetic lipid bilayers in the form of spheres, encapsulating an interior space. Examples would be the many types of liposomes.

Abbreviations

BOD	biological oxygen demand
BWAs	biowarfare agents
ECL	electrochemiluminescence
ELISA	enzyme-linked immunoabsorbent assay
FETs	field effect transistors
FPW	flexural-plate wave
GFP	green fluorescent protein
GMOs	genetically modified organisms
LAP	light addressable potentiometric sensor
LB	Langmuir–Blodgett
LNAs	locked nucleic acids
LPS	lipopolysaccharide
MIP	molecularly imprinted polymers
NASBA	nucleic acid sequence-based amplification
PCR	polymerase chain reaction
PNA	peptide nucleic acid
QCM	quartz crystal microbalance
SAW	surface acoustic wave
scFv	single-chain variable fragments
SERS	surface enhanced Raman spectroscopy
SPR	surface plasmon resonance

Defining Statement

Biosensors are integrated analytical devices which use a recognition element, generally a biomolecule, to bind an analyte, and some transduction mechanism to detect this binding event. Details regarding recognition and transduction, as well as applications of such devices to areas such as biomedical applications, food safety, environmental sensing and biological warfare agent detection are discussed.

Introduction/Limitation of Scope

The subject of biosensors is voluminous, with thousands of journal articles (English language only) treating the subject in the last 10 years. Even taking into account that some of these articles use a rather loose definition of biosensors (e.g., not integrated systems), the literature is too extensive to provide an in-depth review for this article. The focus in this article will be on discussion of biosensors of relevance to biology and then on a subset of all potential applications. The overwhelming majority of the current biosensors market is made up of glucose monitors that are used by diabetics for monitoring blood sugar. Since this application has been covered so extensively in numerous reviews, it is omitted here. Also excluded are discussions of biosensors for the wine industry (ethanol sensing) and applications of biosensors to petroleum microbiology.

The biosensing of small molecules of biological importance continues to be the dominant usage in practical medical applications. Thus, sensors for glucose in blood account for ~85% of the world market for biosensors (Ngoepe et al., 2013), due to the prevalence of diabetes. Another major chronic illness, cardiovascular disease, requires monitoring of cholesterol levels. Together, these two biochemical analytes account for the vast bulk of routine biomedical biomonitoring. Nevertheless, earlier biomonitoring for a variety of biomarkers of different diseases has great benefits for the patient, since early intervention can frequently reduce pathological severity and complications. As more understanding develops of underlying pathologies of disease, a greater variety of biomarkers are identified. For some diseases, like cancer, for example, where the underlying pathological basis is still not

understood, identification of robust biomarkers becomes difficult. What is frequently done in such cases is to identify a suite of biomarkers which may be associated with a particular disease process. Integration of diagnostic biosensing and drug delivery is another area which is receiving increasing attention. This is summarized in the word 'theranostics', which incorporates the ideas of diagnosis and therapeutics, and suggests, ultimately, an implantable system which monitors pathological processes, and tailors administered drug regimens to on-going status of the pathology. Suffice it to say that this ideal diagnostic/therapeutic combination is not yet practical, except perhaps in the case of implantable glucose sensors which administer doses of insulin based upon *in vivo* sensing of blood glucose levels. In this review, we focus only on the biosensing arm of theranostics, and do not address drug delivery systems. Beside the standard biosensing technologies such as optical and electrochemical devices, one can also include bioreporter systems, and imaging based biosensors, such as nanoparticles used for defining tumor margins. Indeed, preparation of this review has indicated many ingenious concepts in regard to biosensing.

The incredibly wide range of current developments of biosensing technology, does not, unfortunately immediately translate into practical biomedical devices used in the clinic. Nor is this necessarily the only legitimate use of biosensors. Investigations of fundamental biological processes frequently entail the use of biosensors to reveal the mechanics of the process. Thus, many of the more exotic biosensors which appear in the literature may eventually find their niche for rather unique applications. But, this is not guaranteed. Sensing technologies which require fabrication of components difficult or expensive to produce (or produce in a reproducible manner), or protocols which require much expertise and experience to carry out, may not find widespread use in the biosensing community. Some sensors may remain in the category of laboratory 'curiosities', but it is impossible to predict whether, some day in the future, such 'curiosities' may become eminently practical, as technology advances. We thus attempt to include a very wide range of both biorecognition paradigms and technologies which are being developed, trusting to time and future development to sort out which will become most useful. In some cases, these capabilities are maturing to the point that the notion of 'ubiquitous' biosensing may soon be possible.

There has been an increasing emphasis, in recent years, upon label-free detection of analytes, as opposed to using labels for detection (e.g., fluorescent molecules). Label-free biosensing has become of increasing importance, which in turn has called forth new technologies, in biorecognition, transduction and readout. A very fundamental objection to label-based sensing is that the label itself may introduce changes in conformation of biomolecules, and so may alter the behavior of these molecules in the sensing process. Further, there is an increasing emphasis on *in vivo* sensing prejudices against the use of added molecules which may be toxic. Another two advances in much favor at present are the ability to multiplex (i.e., perform multi-analyte assays simultaneously) and the ability to perform the sensing in real time. The more analytes assayed, the more information, sometimes of a critical nature, is obtained. Real-time monitoring is becoming more important in, for example, diagnosis of pathogen infections, where early detection translates to improved outcomes.

Since understanding of the biorecognition and transduction processes is critical to understanding biosensors, extensive discussion of these topics is included. The various types of molecules which may serve as biorecognition elements are reviewed, and the transduction systems based on the four major categories (i.e., electrochemical, thermal, mass, and optical) are discussed. A recent review (Tamayo et al., 2013), in which the authors tracked the number of publications using various transduction technologies for biosensing, indicated very clearly that optical and electrical-based transduction techniques have dominated in the literature over the past 20 years, with electrical being used most often, followed by optical. The present article also discusses the applications of biosensors to detection of pathogenic organisms or organism toxins in the areas of food safety and biowarfare/terrorism, since these applications have become of great concern. A brief overview of biosensors for environmental monitoring is also provided, although in many such applications, the biosensor is in fact a bioreporter organism, and not integrated into a sensor device. There is also an extensive discussion of new directions in biosensor development, in which topics such as virus–based biosensors, genetically engineered fluorescent proteins as biosensors, and nanotechnology applications to biosensors are reviewed. This article is divided into five main sections: (1) Biosensor Overview, (2) Biorecognition Elements, (3) Transduction Mechanisms, (4) Applications, and (5) New Directions.

Biosensor Overview

Definition

The IUPAC definition of a biosensor is that a biosensor is a self-contained integrated device that is capable of providing specific quantitative or semiquantitative analytical information using a biological recognition element (biochemical receptor) that is retained in direct spatial contact with a transduction element. See Figure 1 for a schematic of how a biosensor works. A common

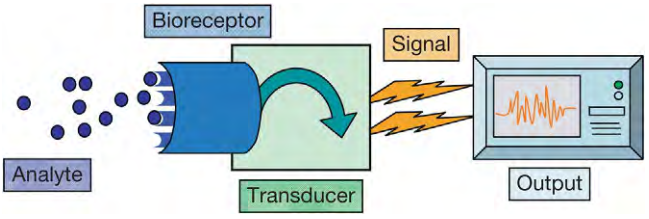

Figure 1 Schematic diagram of a biosensor.

working definition is that biosensors are analytical devices that use biological macromolecules to recognize an analyte and subsequently activate a signal that is detected with a transducer. However, this common working definition does not suggest an integrated device in which the transducing element is in spatial contact with the biorecognition element. Some authorities subdivide biosensors into affinity biosensors and catalytic biosensors, based on the activity of the biorecognition element. Thus, affinity biosensors have as their fundamental property the recognition (binding) of the analyte by the biorecognition element (e.g., antibody–antigen). Catalytic biosensors have as their biorecognition element as biomolecules, proteins (or microorganisms) that not only bind the analyte but also catalyze a reaction involving the analyte to produce a product (e.g., glucose biosensors). The above discussion does not, however, cover biosensing with bioreporter genes/proteins or bioreporter organisms, nor does it include nanomaterial-based sensors which may be introduced intracellularly to track biological pathways or metabolic processes. We feel it is important to include at least some discussion of such biosensing applications. We do not, however, include imaging techniques per se, as this is a vast field in its own right.

Ideal Biosensor Characteristics

Table 1 lists the ideal characteristics of a biosensor. In many cases biosensors are single-use, as the definitions of biosensors do not, in general, explicitly require that the biosensor be regenerable, although for many applications, this is desirable. The ideal biosensor is frequently described as being robust, selective, reproducible, and sensitive with a large dynamic range. This ideal is seldom attained. Bioanalytical systems, such as immunoassays like ELISA (enzyme-linked immunoabsorbent assay), which may well use the same elements (i.e., bioreceptors and transduction devices), but require additional processing steps, such as reagent addition and washings, and usually involve reading the completed assay on some separate piece of instrumentation, are to be distinguished from biosensors. Thus, the very large opus of experimental literature describing the use of immunoassays, nucleic acid-based assays, and so on for detection of various analytes does not necessarily describe biosensors, *per se*, although the techniques described in such assays may well have application in the development of actual biosensors.

Assay Formats

In general, a variety of assays can be employed with the many different biorecognition and transduction elements described below. There is not sufficient space to provide anything more than a very general overview of the assay formats involved in biosensing. Briefly, the assay formats can be either direct or indirect. In the direct methods, analyte binding to the biorecognition element is detected directly, by such techniques as mass changes, changes in refractive index or impedance, pH, and so on (Figure 2). For indirect techniques, an additional reaction (other than the biorecognition binding event) has to occur in order to detect the binding of analyte and bioreceptor. In a competitive indirect strategy, the analyte and a competitor molecule (labeled in some fashion) compete for limited numbers of biorecognition binding sites (Figure 3). In the noncompetitive indirect assay, a second biorecognition element with a label is added to the analyte sample, so that when the analyte binds to the immobilized biorecognition element, the second biorecognition element also binds to the analyte (Figure 4). In the case of labels for the indirect type of assay, the label can be optical-, electrochemical-, or mass-related. In the case of direct assays and noncompetitive indirect assays, the signal is directly proportional to the analyte concentration, while in the case of competitive indirect assays, the signal is inversely proportional to analyte concentration. Frequently also, biosensor assay formats involve some form of amplification, the rationale

Table 1 Ideal biosensor characteristics

Characteristic	*Description*
Analysis time	A fast analysis time is desired, with 'real-time' responses to target analytes ideally desired
Sensitivity	A sensitive analysis allows for detection of low concentrations of target analytes and leads to low false-negative analyses
Specificity	A specific analysis allows for discrimination between target analytes and other closely related species and minimizes false-positive analyses
Reproducibility	The analysis should be highly reproducible, to provide for a reliable analysis that is easy to calibrate
Accuracy	The biosensor device should be highly accurate, meaning false-positives and false-negatives are minimized
Robustness	The biosensor must be insensitive to environmental conditions (temperature, pH, electronic interferences, etc.)
Unit and operational costs	A lower unit cost and operational costs for reagents, and so on will allow for more wide spread implementation of the biosensor systems
Size and weight	The ability to miniaturize a biosensor is desired, particularly for integration into process monitoring applications as well as for field portable applications
Regeneration	The ability to regenerate the binding surface, allowing multiple measurements by the same sensor element is preferable, although single-use platforms are sometimes sufficient
Multianalyte detection	A biosensor that can detect multiple analytes simultaneously is highly desired for efficient cost, time, and size and weight utilization
Adaptable	A flexible biosensor platform is highly desired, especially when considering the need to quickly adapt sensing modalities to respond to new threats as they emerge.
User interface	Ideally, fully automated systems are desired, or at the minimum require little-to-know operator skills for routine analysis

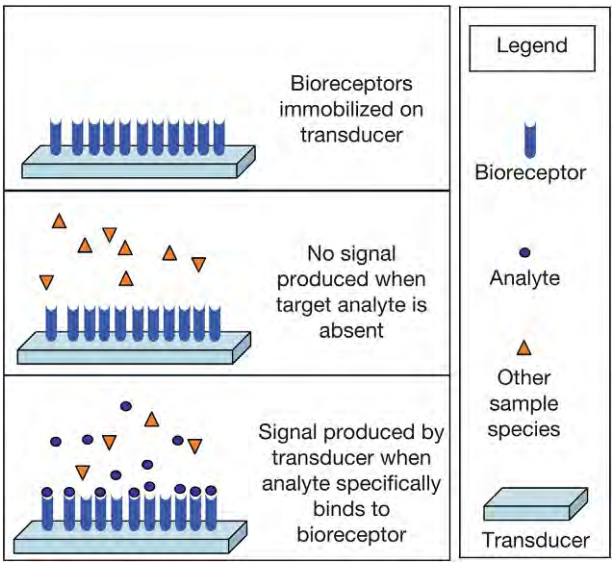

Figure 2 Schematic diagram illustrating a direct assay format.

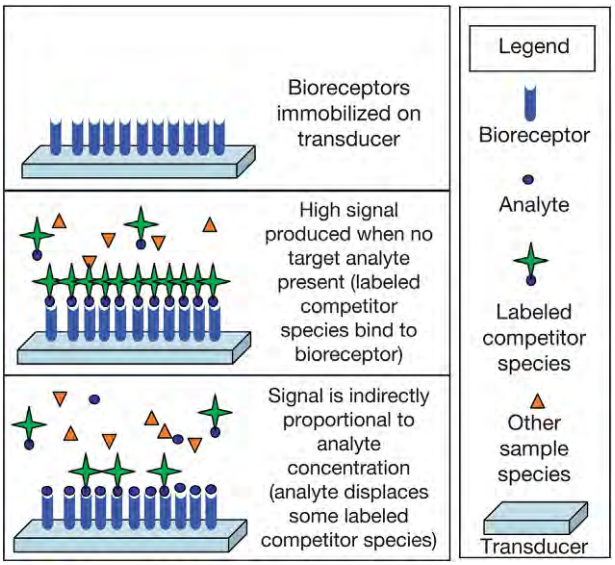

Figure 3 Schematic diagram illustrating an indirect assay with competitive binding.

for this being increased sensitivity of the assay. Either the analyte molecules themselves may be amplified (e.g., polymerase chain reaction (PCR) of DNA sequences) or the biorecognition event may be amplified (e.g., ELISA immunoassays).

Biorecognition Elements of Biosensors

The biorecognition element of biosensors has classically been some natural biomolecule, antibodies being the most common example. However, biorecognition elements also include bioreceptors such as enzymes, nucleic acids, peptides, and receptors that reside in the membranes of cells, and even larger biological units, such as whole cells. The biorecognition element plays a crucial role to the overall biosensor performance, imparting selectivity for a particular analyte. If the definition of biorecognition elements only includes natural biomolecules (up to the collection of biomolecules comprising a whole cell), such artificial recognition elements as imprinted polymers or peptide nucleic acids (PNAs) would be excluded. This rigorous definition seems too restrictive, however, and in this article, the authors include biosensors using such biorecognition elements as synthetic peptides, aptamers, PNAs, liposomes, and molecularly imprinted polymers (MIP). A brief description of the various types of biorecognition elements will now be given.

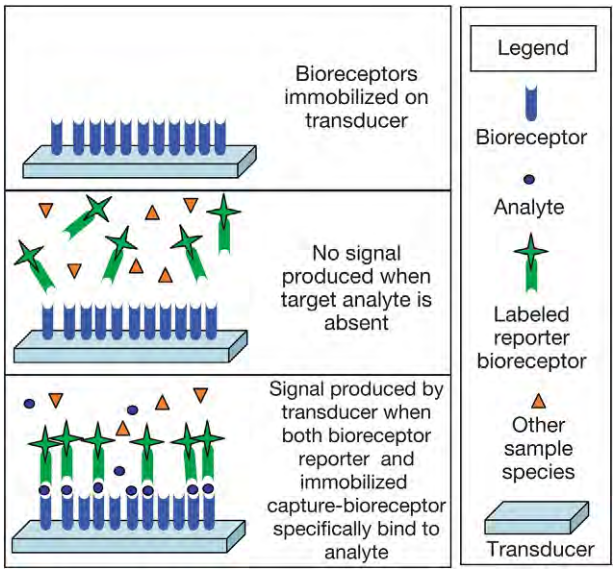

Figure 4 Schematic diagram illustrating an indirect assay with noncompetitive binding.

Antibodies

Antibodies are proteins produced by the immune system that have as their unique feature antigen recognition sites that bind antigens by noncovalent interactions in an exquisitely specific fashion and often with relatively high affinity. The antigen-binding portions of the molecule are the V_H (heavy chain variable region) and the V_L (light chain variable region) regions, which both fold to provide a sort of 'lock and key' fit for the specific antigen. Included in antibody categories would be polyclonal and monoclonal antibodies. Antibodies are probably the most commonly used of all biorecognition elements for sensor applications. Frequently, sensor design makes use of antibody 'sandwiches', where, for example, capture antibodies immobilize the analyte, while other labeled detector antibodies bind to the analyte and thus provide a sensing signal. It is also common to use some sort of amplification technique (e.g., enzymes-ELISA assays) to provide a more robust signal of the recognition process. Ingenious combinations of nucleic acid and immunological techniques (e.g., immuno-PCR and immuno-rolling circle amplification) can also be used for amplification.

Antibody fragments/engineered antibody fragments

For biosensing applications, the molecular recognition site(s) of the antibody (the antigen combining sites) are of overwhelming importance, while the function of the Fc region, of importance physiologically, may not be required for sensing applications. Also, antibodies are often immobilized on solid substrates for sensing applications, and here the orientation of the antibodies is as critical as the antigen combining site should ideally be oriented in a spatially accessible manner. Thus, smaller antibody fragments may have certain advantages, such as more defined points of immobilization through thiol groups liberated after cleavage of the whole immunoglubulin molecule, and also these fragments could be more densely packed upon a sensor surface, thus increasing the epitope density over that found with intact immunoglobulin and so perhaps enhancing sensitivity. Enzymatic cleavage of immunoglobulin molecules to produce fragments such as Fab (antigen-binding fragment) is one way to produce these smaller recognition elements. Using the techniques of phage display or ribosome display for antibody gene cloning and expression, antigen-binding fragments of antibody molecules called single-chain variable fragments (scFv) can be obtained. These fragments feature the V_H and V_L domains joined by a flexible polypeptide linker which can be engineered to have desirable properties for immobilization (e.g., a thiol group of cysteine). Some studies have found the scFv antibody fragments to be superior to either the Fab fragment or the whole immunoglobulin molecule for sensor applications.

Enzymes

Enzymes are catalytic proteins that have an active site that shares some of the features of the antigen-binding site of antibodies, that is, exquisite specificity for certain molecular structures, referred to as their substrates. Again, genetically engineered enzymes with altered properties can be used. Enzymes are not frequently used as biorecognition elements, *per se*, but are usually a component of a multiple molecular biorecognition package, where the enzyme is included to provide amplification (such as in ELISA immunoassays or coupling to an electrochemical detection transduction system (e.g., horseradish peroxidase and urease for electrochemical detection schemes)). An example where an enzyme functions as a direct biorecognition element would be the use of cholinesterase enzymes to detect certain organic phosphate pesticides/chemical warfare agents that are cholinesterase inhibitors.

Proteins/Peptides with Specific Binding Properties

Phage display

Using techniques developed in recent years, it is now possible to produce and evolve peptides/proteins with strong binding affinity for specific proteins. These techniques include phage display and ribosome display. It is outside the scope of this article to provide an in-depth review of these procedures. In each technique, iterative rounds of affinity purification enrich for proteins/peptides with desired binding properties for a specific ligand. The unique feature of phage display is that the production of 10^7–10^8 short peptides on the surface of filamentous bacteriophage can be achieved through the fusion of an epitope library of DNA sequences with the gene(s) coding for one of several coat proteins (the minor coat protein, phage gene III, was used initially). An epitope library of such cloning can display 10^7 or greater peptides that can be panned for peptides that bind specifically to an analyte of interest. Proteins made from the phage display technique have been used to detect bacteria, bacterial spores, toxins, and viruses.

Bacterial display

Although biorecognition has shown the potential to meet many of the desired features outlined in Table 1, a key advantage of many synthetic reagent technologies is the speed at which they can be discovered (Stratis-Cullum and Finch, 2013). This is particularly critical when considering rapidly changing detection needs, including having the ability to sense newly engineered threat agents to which no current biosensor or biomolecular receptor exists. For example, antibodies (including engineered antibodies) usually require several weeks if not months to isolate from living hosts, whereas recent advances have demonstrated that synthetic routes can be employed using various synthetic peptide recognition element technologies (e.g., bacterial display and phage display) to produce bioreceptors in as little as a few days to a couple of weeks (Kogot et al., 2011; Stratis-Cullum et al., 2012, 2010, 2011).

The mechanisms for specific binding with peptides (protein building blocks) are similar to antibody-antigen interactions, and synthetic peptide receptors show some of the greatest potential as synthetic antibody alternatives (Pavan and Berti, 2012). Although, several variations of combinatorial peptide technologies, including yeast, phage, and bacterial display and other chemically synthetic techniques, are currently used to isolate synthetic reagents, bacterial display with unconstrained bacterial peptide display technology shows great potential. In this work, the extremely fast replication rate of bacteria and biological components (e.g., modified proteins) on an *E. coli* cell surface are harnessed to produce the peptide library of binders (Daugherty, 2007; Kogot et al., 2011; Stratis-Cullum et al., 2012). Another unique feature is the ability to normalization for the expression level-a key enabling feature for reproducible, quantitative reagent discovery. Although discovery is rapid and easily automated using bacterial display technology, the characterization, optimization, and integration into assays and devices can still be a bottleneck to successful biosensor development using synthetic alternatives.

Protein catalyzed capture agents

More recently, advances in peptide *in-situ* click chemistry have allowed for development of highly manufacturable and highly selective synthetic antibody alternatives known as protein-catalyzed capture (PCC) Agents (Farrow et al., 2013; Millward et al., 2013, 2011). With PCC technology, the target protein is used directly as a catalytic scaffold for assembling in a stepwise fashion, synthetic anchor, and multiligand systems. PCC agents are assembled from comprehensive, chemically synthesized peptide libraries that incorporate non-natural amino acids into the starting library to produce a final product that is highly stable. Additionally they serve as synthetic 'drop-in replacements' that are highly adaptable and easily integrated into assays and sensor systems. The process is repeated to build biligand and triligand structures, or alternatively advanced libraries can be used. In either case, each subsequent ligand modification, serves to refine the binding affinity and specificity through avidity with the target. The final synthetic peptide can be scaled up on-demand by automated chemical synthesis, avoiding the problem of batch-to-batch reproducibility. Recently, this technology has been shown for protective antigen, for *Bacillus anthracis* where the remarkable temperature stability is also highlighted (Farrow et al., 2013).

Nucleic Acids

Included here are oligo- or polynucleotides such as DNA and RNA. DNA is used more frequently due to its inherent greater stability under a variety of conditions. The biorecognition process consists of noncovalent interactions between bases of complementary nucleic acid strands by Watson–Crick base pairing rules and is manifested by hybridization of two single strands having such complementary base sequences. Exquisite specificity can be obtained due to the cumulative specific interaction of many bases to their complementary units on the other polynucleotide chain. Nucleic acid sensors are frequently coupled to schemes that utilize amplification of diagnostic nucleic acid sequences (e.g., PCR, nucleic acid sequence-based amplification (NASBA), and rolling circle amplification) with the potential for extreme sensitivity, since a large number of copies of an initially low copy number sequence can be obtained by amplification techniques.

Aptamers

Aptamers can also be included as nucleic acid biorecognition elements, since they are made up of single strands of nucleic acid (DNA or RNA), but here the biorecognition is not via base pairing but by folding to form a unique structure. In contrast to base pairing as the biorecognition feature, the aptamer folds in such a manner that it forms a highly specific three-dimensional structure that recognizes a specific analyte molecule, somewhat similar to the nature of the antibody–antigen interaction. Aptamer selectivity

and binding affinity can be similar to the specificity and affinities of antibodies. An advantage of aptamers over antibodies is that the aptamer structure is inherently more robust than the antibody quaternary structure, so that aptamers can be subjected to numerous rounds of denaturation and renaturation, thus providing for easy regeneration of the sensor as well as an overall more robust biosensor. Antibodies are fundamentally products of animal immune systems, with all the limitations that implies. Aptamers are produced by chemical synthesis after selection rather than via immune system cells, and so can be readily modified to enhance stability and affinity. Also, aptamers can be selected against certain targets that can cause the immune system to fail to respond, such as bacterial toxins or prions. Because of all these advantages, aptamers are being used increasingly as biosensor elements. Aptamers have been increasingly utilized for small biomolecular sensing in recent times. Antibodies can be developed against small molecules, but are not, in general, the most effective biorecognition elements for such applications. Aptamers are smaller in molecular size and more stable than antibodies, can be modified with various tags more readily and with less chance of disrupting the recognition site than antibodies, and can be produced to recognize small molecules such as metal ions, or biomolecules such as ADP and ATP. Various transduction schemes can be utilized to signal binding of aptamer to target, including FRET-based assays, relying on aptamer conformational changes produced by target interaction, or generation of DNAzymes, or separation of the aptamer into two components, which are reunited upon target binding, and thereby produce a transducing signal.

Among interesting developments has been the design of RNA sensors, which combine aptamers with ribozymes (catalytic RNAs) to produce RNA molecules (these structures have been called aptazymes) whose catalytic activity depends in some manner upon binding of a specific ligand, the analyte. The binding of the analyte can either stabilize or destabilize the catalytic domain and so adjust the catalytic activity. Thus detection of the analyte species is signaled by changes in the enzyme's activity.

A quite unique aptamer approach described by Paige et al. (2012) is the development of what might be termed a 'dual-function' RNA aptamer. These investigators previously developed an aptamer that would specifically bind a small organic fluorophore in a specific location, and so switch on its fluorescence, somewhat analogous to the photo-active center of the naturally occurring green fluorescent protein. They were able to fuse this RNA with another aptamer which had small molecule (e.g., S-adenosyl methionine [SAM], GTP, ADP) binding properties. Upon binding of the analyte ligand, the analyte-recognizing portion of the composite aptamer folded in such a way as to stabilize the fluorophore-binding stem of the composite structure, thus turning on fluorescence(upon addition of the fluorophore) as an indicator of analyte binding. Of course, development of this composite sensing molecule required a great deal of molecular biology manipulation. The authors were able to demonstrate detection of production of the SAM metabolite in vivo, using *E. coli* which expressed the composite RNA aptamer. They noted that fluorescence increases of ~20-fold could be produced upon analyte binding to these sensors, a much more robust response than FRET-based systems for detecting small molecules such as metabolites. It should be noted that use of the sensor in vivo was only demonstrated in cells which genetically expressed the sensor, and in this case, were *E. coli*.

Peptide nucleic acids

PNA structures are not strictly natural biorecognition molecules, since they are a hybrid of a series of *N*-(2-aminoethyl)-glycine units making up the backbone structure (instead of the sugar–phosphate backbone of natural nucleic acids) with the standard bases bound to the peptide backbone. All intramolecular distances and configurations of the bases are similar to those of natural DNA. Thus, hybridization to DNA or RNA molecules is readily achieved. Since the PNA backbone is uncharged, PNA–DNA duplexes are more thermally stable than DNA–DNA duplexes, and so single-base mismatches have a more destabilizing effect in PNA–DNA hybrids. The PNA structure is stable over a wide temperature and pH range and resistant to nuclease and protease activity and so has obvious applications in the biosensor area. Also, PNAs can be used for the detection of double-stranded DNA directly, eliminating the requirement for thermal denaturation, due to the ability of PNAs to form higher order complexes (three- and four-stranded) with DNA.

Also to be mentioned are another type of nucleotide analogue called locked nucleic acids (LNAs). These nucleic acid analogues have modified RNA nucleosides where a ribonucleoside has a methylene linkage between the 2′-oxygen and the 4′-carbon. These LNAs have the usual hybridization properties expected of nucleic acids with other desirable properties such as highly stable base pairing, high thermal stability, compatibility with most enzymes, and predictable melting behavior.

Molecular beacons

This is a subset of nucleic acid biorecognition molecules, generally synthetic oligonucleotides that are designed to have a hairpin (stem-loop) structure. The loop contains a nucleotide sequence complementary to the analyte sequence to be detected, while the stem structure has only a small number of complementary bases, forming a short double-stranded portion. Fluorophore and quencher molecules are held in close proximity on the two ends of the double-stranded stem, when the molecular beacon is in the closed position. Upon binding of the analyte sequence to the loop portion, the stem opens, the quenching effect on the fluorophore is relieved and the resulting fluorescence signals the presence of the analyte sequence. Aptamers can also serve as molecular beacon biosensors, with this important distinction, that they can be used to detect non-nucleic acid analytes, such as proteins or small organic molecules. PNAs can also be used to form molecular beacon-type structures, and these can be stem-less, in contrast to the usual stem-loop structure found in natural DNA molecular beacons. This stemless structure has advantages (less sensitive to ionic strength, quenching not affected by DNA-binding proteins) as compared to the initially designed DNA beacons. Molecular beacons have generated great interest because of their potential to investigate cellular mechanisms, particularly with regard to genetic expression events. A recent review provides an in-depth look at what the authors refer to as oligonucleotide optical switches, referring to the fact that these probes switch on (or off) optical response in response to molecular interaction with specific targets

(see Giannetti et al., 2013). Besides the standard stem-loop structure, other configurations are implemented to accomplish the same optical switching effect. Thus, so-called binary probes have been used to detect mRNA expression inside cells. In this case, a probe structure is constructed such that two oligonucleotide segments, which are complementary to adjacent sequences on the mRNA target, are labeled with a donor and acceptor fluorophore respectively, and are linked by a longer PEG spacer. The spacer keeps the donor and acceptor fluorophore apart so that FRET cannot occur. If hybridization to the target mRNA occurs, however, the acceptor and donor are in close apposition, and FRET can be detected.

In an even simpler design, called nanoflares, the probe oligonucleotide is hybridized to a short complementary oligonucleotide (called the reporter) with an attached fluorophore. One free end of the probe oligonucleotide is attached to a gold nanoparticle, thus quenching the fluorophore's fluorescence. Upon hybridization of the target nucleic acid, the reporter is displaced, because of a more stable hybridization with the longer target sequence, and the fluorescence of the fluorophore attached to the reporter is restored. An analogous concept can be used with aptamers to detect protein targets. Although in principle the molecular beacon concept seems very versatile, there are problems. These exogenous nucleic acids can be susceptible to endogenous nuclease degradation, the signal intensity can be low, due to limited numbers of fluorophores (only one per probe molecule) and the quenching effects are not 100% effective.

Cell Surface Receptors/Glycoproteins, Glycolipids/Glycans

Many of the molecular recognition events having to do with cell–cell interaction, pathogen attack upon cells, and so on take place in the glycocalyx layer of the cell membrane. The glycocalyx layer consists primarily of oligosaccharide headgroups of glycoproteins and glycolipids. The lipid or protein portion of these molecules is embedded in the membrane, while the hydrophilic oligosaccharide chain is extended into the outer environment. As the molecular biology of pathogen and toxin interaction with the cell surface is elucidated at the molecular level, these receptor molecules are receiving increasing attention as biorecognition elements. One of the major difficulties heretofore with exploitation of these receptors is the complexity of the cell membrane, making mimicry of this structure a daunting task. Simpler models of cell membranes with defined chemical compositions include liposomes (vesicles) and Langmuir–Blodgett (LB) monolayers. Recent advances have formulated biorecognition molecules into these synthetic lipid structures. In some cases, pathogens or toxins produced by pathogens target specific cell surface receptor sites. These same cell-based receptors can then become biosensor recognition elements. Thus, biorecognition elements seeing use in sensors include gangliosides, glycosphingolipids that are localized upon certain cell types, as well as carbohydrate/oligosaccharide structures found on the cell surface. For example, ganglioside GT1b has been used as a recognition element for botulinum toxin, while *N*-acetylneuraminic acid and *N*-acetylgalactosamine bound cholera and tetanus toxin, respectively, in a semispecific manner. Uropathogenic *Escherichia coli* strains attach to uroepithelial cells by adhesion to the glycolipid globotetraosylceramide (globoside), and so sensors for these *E. coli* strains can be formulated using this globoside. Human gastric mucin has been used in a biosensor format to study the interaction of *Helicobacter pylori* with the extracellular matrix components. Lectins, a broad class of proteins/glycoproteins that bind with high affinity to specific carbohydrate moieties, have also been incorporated into biosensing devices.

Often, oligosaccharides form primary molecular components and markers on pathogen surfaces, and thus may form the basis for diagnostic sensors for specific bacteria or groups of bacteria. For example, the lipopolysaccharide (LPS) molecules (commonly known as bacterial endotoxin), which are found on the cell surface of Gram-negative bacteria, can serve as targets for biorecognition by lectins or other proteins. See sections 'Liposomes' and 'Biomimetic Materials' for further discussion on this topic.

Olfactory Receptors

Biosensors based upon olfactory receptors (OR) are generating increasing interest. These sensing elements are situated in the nasal epithelium and have the ability to respond selectively to thousands of low-molecular weight organic compounds. Cell and tissue-based olfactory biosensors have the advantage of retaining the complex biological structural integrity of the intact olfactory system, but have the great disadvantage of requiring demanding culture conditions to function normally. A more practical approach has been to use olfactory receptors and/or olfactory-based proteins. Probably the most practical method of generating reasonable amounts of one specific OR is, by genetic engineering, to express this OR heterologously in various cell lines, from which the active OR can be extracted, retaining its native conformation and activity. Cell-free protein synthesis is also a route to produce ORs, or, whole cells can be used as biosensors, in which the OR's are expressed, through gene transfer, on the cell membrane. So far, the fragility (ease of denaturation) of the odorant-detecting proteins themselves, has been a major obstacle to use in durable biosensors. With development of basic understanding of the olfactory process on a molecular level, synthetic peptides having OR properties will become increasingly important. Several examples of chemicals which have been detected by OR-based sensors include acetic acid, hexanal and octanal.

Whole Cells

In some ways, whole cells do not fit the strict definition of a biosensor biorecognition element, since they are most often used to function as 'bioreporters'. That is, the cells are genetically altered to synthesize a certain marker that produces a detectable signal of some sort (i. e. luminescence and fluorescence) when they encounter specific compounds/environmental conditions. Such whole cell sensors have been applied to environmental analysis and monitoring. A brief description of such applications is included later

in this article (see Section Bacterial Biosensors for Environmental Monitoring under Section Applications of Biosensors). There are, however, some examples of whole cells being used as biosensors, particularly microbial cells. Often in these instances, the whole cellular machinery functions in a sense as a biorecognition/processing system for the analyte, as, for example, using bacterial cells induced to constitutively produce an amino acid-metabolizing enzyme to detect that particular amino acid. Another innovative approach using whole cells is to genetically engineer cells of the immune system to respond to specific pathogen binding events by producing a detectable signal from some genetically inserted reporter construct.

Either microorganisms or mammalian cells can potentially be used as cell-based sensors.

Microorganism-based sensors have a variety of advantages over mammalian cells, such as much better stability in harsh environments, relative ease of mass production, and ease of genetic manipulation. The most common employment of these entities is either in optics-based or electrochemical-based biosensing. Electrochemical sensing can involve either production or consumption of electroactive species which can be detected by conductimetric, amperometric or a variety of other electrical techniques. Optical sensing usually involves the genetic modification of the sensor microbe, so that in some manner an optical signal is produced upon interaction with a specific analyte. Park et al. (2013) provide a recent review of many such optically-based sensors. One strategy is to make use of reporter genes, whose protein products are under the control of a specific regulatory pathway, which pathway can be induced by an analyte or analytes of interest. Thus, a common approach is to use a transcriptional regulator (e.g., repressor) and promotor whose induction is linked to, for example, external chemicals, or specific nutrients, or antibiotics or signaling molecules, etc. When the analyte to be detected is present, the repression of the promotor is removed and the resulting induction results in expression of the reporter gene. Beta-galactosidase is one such reporter protein: since it is an enzyme, there is great amplification attendant upon its expression, and a wide variety of substrates offer great flexibility in detection. Another widely used genetic reporter system are the various luciferases, be they bacterial, or from *Renilla reniformis* (sea pansy) or from the firefly. The introduction of the bacterial lux operon into a microbial host produces a light-generating enzymatic reaction which does not require addition of an exogenous substrate. Firefly luciferase, introduced as a reporter gene, requires addition of a substrate, luciferin, but has the advantage of a higher quantum yield than bacterial luciferase. Linking metallothionein promoters with firefly luciferase was carried out to develop a heavy metal biosensor, using *Tetrahymena thermophila*. Fluorescent proteins originally isolated from various marine organisms can also serve as valuable reporter gene constructs, and genetic engineering has produced artificial versions of these proteins which have superior optical properties compared to the natural proteins. Analogous to the reporter gene systems discussed above are ribosomal switches or riboswitches, which consist of an aptamer domain linked to a reporter gene. Binding of analyte to the aptamer results in a conformational change in the aptamer, thereby triggering expression of the protein product of the reporter gene.

Bacteriophage

Bacteriophage, by virtue of the fact that they recognize in a specific manner their host bacteria, can become biorecognition elements. The bacteriophage themselves may be used, with appropriate labels (e.g., fluorescent dyes) to detect the binding event, or the phage may be selected for binding to a specific analyte via fusion proteins on the phage coat (i.e., phage display). This latter paradigm has even been extended to expression of the biorecognition peptide as an N-terminal add-on to the major coat protein of the phage. In this case, there is a display of thousands of copies of the recognition protein on the phage surface, thus potentially greatly increasing the sensitivity of the assay by increasing the density of the epitopes displayed. Thus, the phage themselves become both biorecognition (by virtue of peptides displayed on their surface) elements and amplification (multiple copies of fluorescent dye per phage) elements. This type of biosensor could be considered a whole cell biosensor (bacteriophage) that has a bioreceptor on its surface.

Biomimetic Materials

Biomimetic strategies are becoming more varied at present. Besides molecularly imprinted polymers (MIP), which aim to generate a fully artificial, negative macromolecular mold of an analyte, polymer coatings are being investigated which change the biocompatibility of surfaces and thus permit immobilization of receptors or ligands in a more 'natural' setting. Such materials could either be membrane mimics (for example, liposomes) or self-assembled monolayers. Also, using computer simulations, it is becoming possible to identify peptides, DNA fragments, enzymes or smaller portions of receptors which make up the functional biorecognition sites of proteins, or other bioactive species, and so use these molecular fragments as biomimetic sensors. Also, nanoparticles, nanomaterials and nanocomposites may be used as biomimetics.

Liposomes

Liposomes are synthetic lipid vesicles, mimicking in some respects cell membranes, although not nearly as complex. Liposomes are frequently used as components of biorecognition systems, but usually the liposomal membrane structure is modified with a standard biorecognition element such as an antibody or oligonucleotide, and these molecules provide the specific recognition. In such cases, the liposome may have encapsulated fluorescent dyes, enzymes, electrochemically active species, and so on, which, upon biorecognition, may be released, and thus the liposome itself functions as an amplification system. More direct involvement of liposomes as sensing elements *per se* are occasionally also encountered. For example, insertion of the ganglioside GM1 into the liposomal membrane has been used to develop a sensor for cholera toxin, since the GM1 has specific affinity for cholera toxin. In a similar vein, liposomes containing cholesterol in the membrane could function as sensors for the toxin listeriolysin.

Molecularly imprinted polymers

A recent development that may, in the future, provide artificial macromolecular bioreceptors is the technique of molecular imprinting. In this procedure, a target molecule (i.e., the analyte), acting as a molecular template, is used as a pattern to direct the assembly of specific binding elements (i.e., monomeric units) that are subsequently polymerized with a cross-linker. The interaction of the monomeric units with the template molecule may be either through noncovalent (the more usual) or covalent bonding. Following polymerization, the target molecule is removed from the polymeric matrix (by suitable solvent or chemical cleavage) leaving a cavity within the polymeric matrix, which is a molecular 'negative' image of the target molecule. The monomeric subunits that interact with the target molecule in a noncovalent molecular imprinting scheme do so by interactions such as hydrogen bonding, van der Waal's forces, and hydrophobic interactions, just as antibody–antigen interactions take place. The subsequent interaction with the cross-linker freezes the binding groups in a specific orientation. Ideally, once the target molecule is removed from the polymer, a binding site now exists which is complementary in size and chemical functionality to the original target molecule. In a number of studies, MIPs have been shown to have binding characteristics similar to those of antibodies and other bioreceptors. A potentially important advantage of MIPs is enhanced stability compared to natural biomolecules. The use of MIPs for the analysis of small molecules is becoming established, but detection of larger biomolecules (e.g., proteins and protein toxins) is more problematic. Here, the formation of the template cavity to conform to the three-dimensional image of the total protein may be difficult (e.g., individual antibodies to proteins respond to one antigenic determinant or epitope).

MIP's may be very suitable sensors for whole cell-sensing, be it of yeast, bacteria, mammalian cells or even viruses. Using a technique called double imprinting, 'plastic' copies of cells or say, antibodies can be produced, In this technique, using an antibody for illustration, the first molecular imprint produces a negative copy of the conformation of the native antibody. This 'plastic' copy serves as a template for another round of molecular imprinting, generating a fully synthetic 'positive' copy of the antibody's structure. The fully synthetic antibody now takes the place of the natural antibody in analyses. Such structures have sometimes shown similar affinity as the native antibody. The same double imprinting technique can be used to generate a positive 'plastic' copy of the whole cell.

A somewhat analogous approach to MIP is to form a self-assembled monolayer (SAM) film (say, an alkane thiol on a gold-coated silicon chip), but to include analyte molecules during the formation of the SAM. Subsequent removal of the analyte molecules leaves 'footprints' of the analyte in the monolayer matrix.

Biomimetic chromic membranes

A specific biomimetic system that has been used for convenient colorimetric sensing of bacterial toxins, bacteria or viruses is the use of cell membrane-mimicking materials ('smart' materials) into which are inserted cell surface receptor residues. These systems use polydiacetylenic membranes (LB or vesicle bilayers) containing either cell surface gangliosides, sialic acid, or carbohydrate residues to detect the presence of toxins such as cholera toxin or influenza virus, the binding event being signaled by a color change (bichromic shift). The specificity of the sensor resides in the ganglioside or sialic acid residues in the polymeric assembly. Ganglioside GM1 is the primary target of cholera toxin on the intestinal cell surface, while gangliosides GT1b are located at the neuromuscular junction and form the target for botulinum toxin. In the case of influenza virus, the sialic acid residues on glycolipids form targets for the viral surface hemagglutinin of the influenza virus, by which pathway the virus is endocytosed into the cell. When these receptor molecules are incorporated into the polydiacetylenic structure, binding of the virus or bacterial toxins apparently induces a distortion of the conjugation plane of the polymer, leading to the color change.

Biomimetic recognition: new approaches

In addition to the new synthetic peptide approaches highlighted above, using a biorecognition element which only consists of the functional core of a receptor has obvious attractive features, such as ease of synthesis, ease of fabrication into a transducing system, etc, yet the requirement, in most cases, for extensive computer simulations to select the appropriate core structure can lead to extensive computer effort. Nevertheless, the effort may well be repaid, if such sensors can demonstrate similar selectivity and sensitivity as the larger receptor from which they are modeled.

Nanosized materials can potentially serve as biomimetic materials. Nano-dimensional structures have obvious advantages for biosensing operations such as biocompatibility, chemical and thermal stability, large surface-to-volume ratios, allowing for immobilization of multiple recognition molecules on their surface, unique physical and chemical properties which facilitate sensitive transduction techniques, and, finally, nanometer size which potentially allows elucidation of discrete subcellular sites where interaction is probed. Biomimetic nanomaterials, at present, comprise mainly nanocomposite structures, in which the core is a nanoparticle or similar structure, and the surface is modified with various receptors which allow biorecognition. For example, positively charged quantum dots (QDs) whose surface structure has been modified with PEG and amino groups, have been used to probe negative surface charge distribution on normal and transformed cells. A novel approach to mapping cell surface glycoconjugates on mammalian cells was to co-embed QDs and magnetic nanoparticles into an organic copolymer nanosphere, which was surface modified with agglutinins (acting as biorecognition elements for cell surface sugar moieties). Thus these composite nanoparticles had functional capability for magnetic separation of cell types, fluorescence tracking of specific biorecognition events (agglutinins binding to sugar moieties), and selective detection of N-acetylglucosamine, galactosamine, and N-acetylgalactosamine on cell surfaces (Xie et al., 2009).

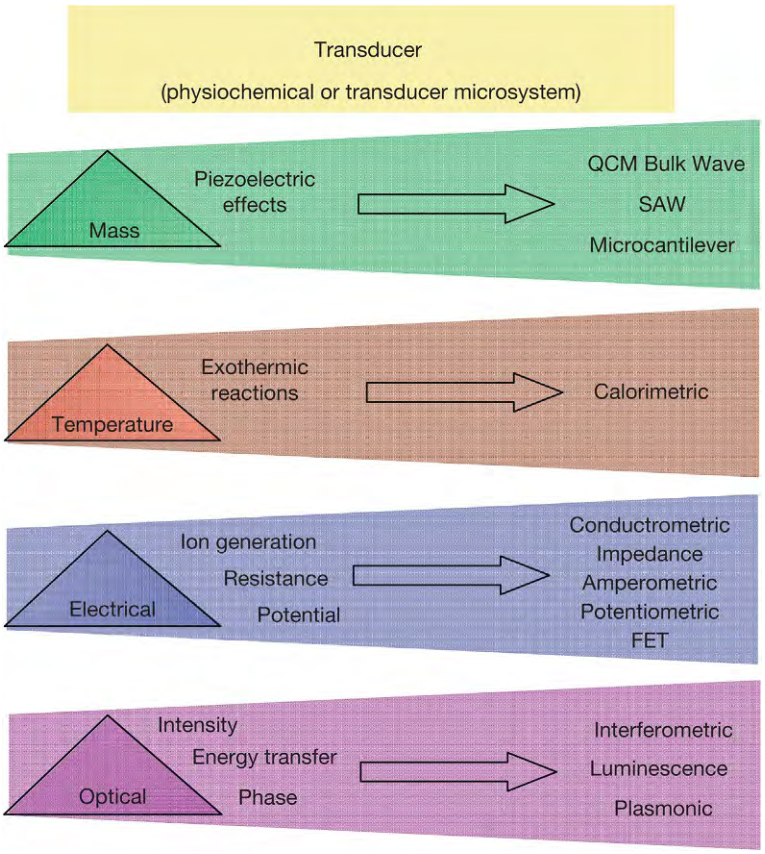

Figure 5 Schematic diagram of typical transduction formats employed in biosensors.

Bio-Amplification as Part of Recognition

Frequently it is desirable to not only specifically recognize the analyte of interest, but to also amplify, in some way, the biorecognition event, as a means of increasing sensitivity of analysis. An obvious example is the ELISA assay, in which the processive activity of an enzyme is used to amplify the recognition of an antibody for an antigenic epitope. There have been many such amplification schemes devised for biosensing applications. Here we highlight a few such techniques which seem to have earned a place in recent analysis schemes. Recent articles have discussed the use of so-called DNAzymes, as signal amplification elements in biosensors. DNAzymes (or deoxyribozymes) are catalytically functional nucleic acids which can fold into three-dimensional shapes to bind to specific targets. For example, there is a DNAzyme structure which can form a G-quadruplex (G-rich motifs) structure which can act, in the presence of hemin, to form a hemin/G-quadruplex complex which has peroxidase activity. Since a variety of substrates are already available for peroxidase reactions, the DNAzyme lends itself to ready detection by colorimetry, for example. Analogs of molecular beacon structures have been designed for DNA detection, in which the G-quadruplex sequence is in a caged configuration, and the hybridization of the target sequence to the molecular beacon loop sequence produces a functional DNAzyme. Ingenious constructs have been devised, in which an aptamer and a Pre-DNAzyme are combined in a hairpin structure. Upon binding of analyte to aptamer, the hairpin opens, and generates the G-quadruplex sequence, which then functions as the DNAzyme, upon addition of hemin and peroxidase substrate. Another approach using the same concept was to start with a functioning DNAzyme which was in the form of a molecular beacon (but without fluorophore and quencher). Two molecular beacon elements formed the intact G-quadruplex DNAzyme. Upon addition of the analyte DNA sequence, the complementary sequences on the molecular beacon surrogates hybridized to the analyte, thus disrupting the DNAzyme and producing loss of signal.

Other bio-amplification techniques for signal enhancement have also been used. For example, if molecular beacons are used for recognition of target DNA's, when the beacon unfolds, gold nanoparticles with short oligonucleotide segments complementary to a stem sequence of the beacon can be hybridized to this stem portion. Addition of nucleotides and polymerase can lead to polymerization and release of target DNA, which can recycle to amplify the amount of gold nanoparticles at the transducer surface, thus improving electrochemical detection sensitivity.

Transduction Mechanisms

As described above, the transducer is the portion of the biosensor responsible for converting the biorecognition event into a measurable signal. As shown in Figure 5, it is possible to exploit a change in a number of physical and chemical properties (mass,

temperature, electrical properties, and optical properties) to allow for different transduction formats. The four basic transduction types: electrochemical, piezoelectric, calorimetric, and optical are reviewed here, with emphasis on the advantages and disadvantages to each, and recent advances toward idealizing biosensor performance.

Electrochemical

Electrochemical transduction is one of the most popular transduction formats employed in biosensing applications. One of the main advantages of biosensors which employ electrochemical transduction is the ability to operate in turbid media and often in complex matrices. Another distinct advantage of electrochemical transduction is that the detection components are inexpensive and can be readily miniaturized into portable, low cost devices. In general, electrochemical-based sensing can be divided into three main categories, potentiometric, amperometric, and impedance. Potentiometric sensors typically rely on a change in potential caused by the production of an electroactive species that is measured by an ion selective electrode. For a biosensor system, this change in electroactive species concentration is usually brought about by an enzyme. In an amperometric sensor system, a change in current is directly measured. Electrochemical sensors based on impedance, most commonly utilize impedance spectroscopy since controlled AC electrical stimulus over a range of frequencies is used to detect variations in the sensor surface properties (i.e., charge transfer and capacitance at the interface layer). In this way, the resistance to flow of an alternating current is measured as voltage/current. For example, metabolic changes (e.g., growth and metabolism) have been shown to correspond to an increase or decrease in impedance. Some of the many variations of potentiometric, amperometric, and impedance biosensors that provide for improved biosensor performance include field effect transistors (FETs) and electrochemiluminescence (ECL).

Field effect transistors

Many researchers have recently looked to FETs as a means to miniaturize potentiometric sensors, while providing increased sensitivity due to minimal circuitry. A particularly promising FET advance includes the light addressable potentiometric sensor, or LAPS, which consists of an n-type silicon semiconductor-based sensor and an insulating layer that maintains contact with the biological solution. Detection of potential changes occurring at this silicon interface are monitored as differences in charge distribution between the insulator surface and the FET. To accomplish this, alternating photocurrents generated by a light-emitting diode are utilized, so that changes in potential can be transduced into voltage per time differentials. Successful application of the LAPS has been demonstrated with commercially available systems, for the detection of biological threats of interest to defense applications, as well as foodborne pathogen threats of concern to food safety applications.

Electrochemiluminescence

ECL combines the advantages of chemiluminescence (high sensitivity and low background) with electrochemical transduction. ECL utilizes a controlled voltage potential at the surface of an electrode to power a luminescent redox reaction. The redox reaction most commonly employs a ruthenium (II) trisbipyridal chelate coupled with a tripropyl amine, although recent studies have demonstrated success with other fluorophore species.

Mass

Biosensors that detect the change in mass due to target and biorecognition element interactions predominately rely on piezoelectric transduction. Piezoelectric transduction relies on an electrical charge produced by mechanical stress, which is correlated to a biorecognition binding event causing a change in the mass on the piezoelectric device. The main advantage to the piezoelectric transduction (i.e., mass sensor) approach includes the ability to perform label-free measurements of the binding events, including real-time analysis of binding kinetics.

Bulk wave

The most commonly employed transducer is the quartz crystal microbalance (QCM), which relies on a bulk wave effect, illustrated in Figure 6. A QCM device consists of a quartz disk that is plated with electrodes. Upon introduction of an oscillating electric field, an acoustic wave propagates across the device. The change in mass associated with bioreceptor–target interactions causes a decrease in the oscillation frequency that is directly proportional to the amount of target. This transduction format can be coupled to a wide variety of bioreceptors (e.g., antibody, aptamer, and imprinted polymer), provided that the mass change is large enough to produce a measurable change in signal. Not surprisingly, QCM transduction is not capable of small molecule detection directly, and usually requires some sort of signal amplification to be employed.

Surface acoustic wave

Changes in the overall mass of the biomolecular system due to association of the bioreceptor with the target analyte can be measured using alternative piezoelectric transducer devices that offer some advantages over bulk wave sensing. For example, surface acoustic wave (SAW) devices exhibit increased sensitivity compared to bulk wave devices and transmit along a single crystal face, where the electrodes are located on the same side of the crystal and the transducer acts as both a transmitter and a receiver. SAW devices can directly sense changes in mass due to binding interactions between the immobilized bioreceptor and target analytes and exhibit increased sensitivity compared to bulk wave devices. However, the acoustic wave is significantly dampened in biological

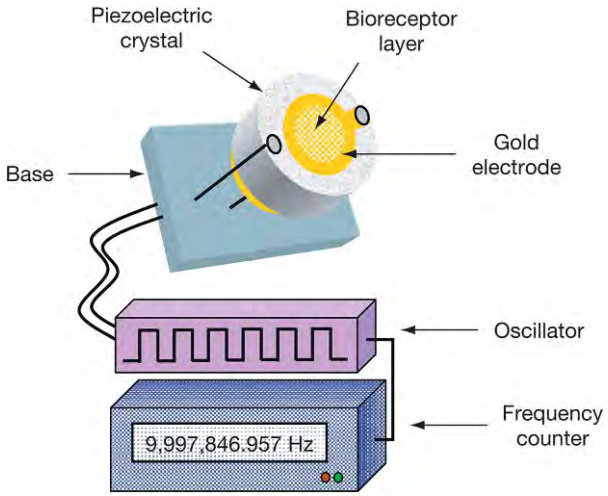

Figure 6 Schematic diagram of a biosensor based on piezoelectric/acoustic wave transduction.

solutions, limiting its utility for biosensing applications. Some improvements using dual channel devices, and special coated electrode systems allowing for noncontact SAW devices, which can function in biological solution interfaces have been produced. However, reliable biosensor application incorporating these devices is still under pursuit, as improvements in sensitivity are still required for specific microbial analyses.

Micro- and nanomechanical devices

Micro- and nanomechanical cantilevers can be manufactured through silicon fabrication techniques developed in the electronics industry. Under ambient temperature conditions, the cantilever devices naturally oscillate, and this resonant frequency can be monitored (Figure 7). Through modifications with bioreceptors, target interactions can be monitored as changes in mass, and hence the resonant frequencies, upon binding. Obvious advantages of this form of transduction include low cost and mass production. However, practical applications to biosensing are limited due to oscillation dampening in liquid solutions, compared to air- and vacuum-packed cantilever systems. To address this limitation, there have been recent advances toward incorporating hollow channels inside the microcantilever in order to immobilize the bioreceptors internal to the device. In other words, high resonant efficiencies obtained through vacuum packaging can be maintained, while specific mass changes can be monitored by flowing the sample through the inside of the device. Further signal enhancements can be obtained through use of nanoparticle and magnetic bead amplification to cause larger frequency shifts, and overall a more sensitive analysis.

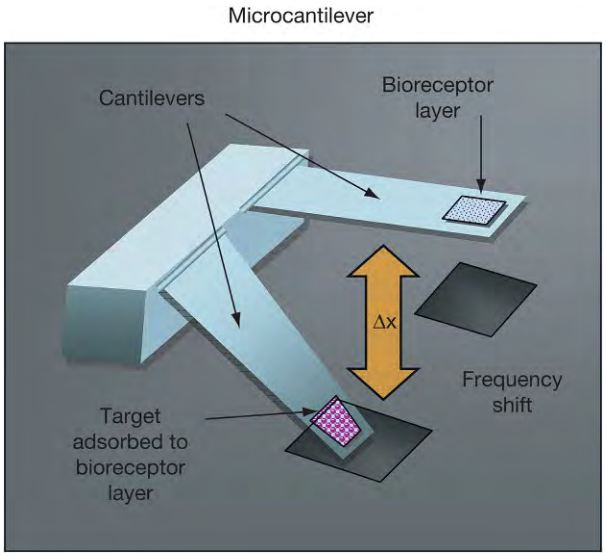

Figure 7 Schematic diagram of a microcantilever-based biosensor.

Nanomechanical Transduction Techniques

Although the fundamental principles involved (e.g., mass sensing) already are in use in micro- or meso-scale (see discussion in text above), the development of nanoscale fabrication of materials has opened paths to novel sensing devices. Only recently has the sensing of mechanical processes (e.g., deformation, stretching, bending) in biology systems begun to receive some wide-spread attention. For example, the 'lock and Key' analogy of antibody/antigen interaction implies a certain rigidity while, in fact, actual conformations may be fluid and only attain a more-or-less fixed state upon the binding event occurring. When proteins (for instance) bind to membranes, the chemical interposition within the membrane translates into mechanical membrane bending, so that mechanical stress at the membrane may be important in multiple biological and pathological processes. The mechanical properties of cells themselves may provide valuable insight into differences between malignant and normal cells: transformed cells deform more (stretch) than non-malignant cells. Also, some viruses show changes in elasticity as the viral particles mature. The tremendous advantage of nanoscale devices to analyze such processes is that the sensors themselves are not much larger than the molecules which are being probed, so that even small changes translate into detectable events in the sensor. Mechanical sensors fabricated with nanosized moving parts whose vibration and deformation are sensitively altered by molecular adsorption have great potential for mechanical sensing. Thus, nanomechanical systems which use the same principle as quartz crystal microbalances can resolve messes verging on the scale of single atoms. Biological interactions such as nucleic acid hybridizations, protein-carbohydrate interactions, conformational changes in viruses upon viral packaging, are all amenable to nanomechanical sensing.

Flexural-plate wave

A flexural-plate wave (FPW) transducer contains a thin membrane that can propagate a SAW. The FPW is in contact with a thin film of liquid that vibrates with the membrane and hence the change in mass of a vibrating element can be detected to indicate biological interactions. This technique is not very sensitive, but similar to SAW devices can provide for real-time analyses.

Calorimetric

Calorimetric sensors utilize thermometric probes, such as thermistors, resistance temperature devices, and thermocouples, to monitor changes in temperature due to exothermic chemical reactions. Many biological reactions are exothermic (e.g., enzyme reactions), and hence calorimetric detection allows for a near universal transduction format. One key disadvantage of this approach is that environmental temperature fluctuations must be shielded from the sensor system. Traditionally calorimetric biosensors have been large and bulky, although advances in silicon microfabrication technologies and microfluidics have allowed for miniaturization and improved performance.

Optical

Due to a number of advantages, optical transduction is one of the most widely used biosensor transduction formats. For example, optical transduction can be very rapid where the limiting factor for the speed of detection is often a diffusion-limited process of the biomolecular recognition event, rather than the optical transducer. Another advantage of optical transduction is the interferences that can hinder electrochemical transduction measurements such as voltage surges, harmonic induction, corrosion of electrode elements, and radio frequency interferences are not present. Some of the disadvantages of using optical transduction formats include: detection challenges when analyzing turbid samples, and the cost associated with detection system components. A wide variety of optical transduction formats have been employed, where changes of the interaction of light with the biomolecular system is used to produce a measurable signal. These changes can be based on differences in refractive index, production of chemiluminescent reaction products, fluorescence emission, fluorescence quenching, radiative and nonradiative energy transfer, temporal changes in optical emission properties, scattering techniques as well as other optical effects. These effects can be monitored using a variety of optical platforms including total internal reflectance and evanescent wave technologies, interferometric, resonant cavities, biochip devices, and so on. The following paragraphs review the most common as well as most popular emerging optical transduction formats: fluorescence, interferometric, chemiluminescence, surface plasmon resonance (SPR), and surface enhanced Raman spectroscopy.

Fluorescence

Fluorescence is the most popular form of optical transduction due to the high sensitivity that is fundamental to this type of optical process. Another advantage of fluorescence-based methods is that they generally do not have the interference issues that SPR and other refractive index-based methods possess. However, in most cases, the intrinsic sample fluorescence is not sufficient for analysis, and a fluorogenic reporter is used to label an affinity reagent to create a bioreporter. By monitoring the intensity of the fluorogenic reporter, it is possible to determine the presence and concentration of the target analytes, as illustrated in the bioassay techniques section described previously. It is also possible to monitor shifts in the wavelength of the fluorophore reporter, as well as energy transfer phenomena, and time dependence of the fluorescence emission, all of which can be related to binding interactions depending on the assay employed. The distinguishing features between fluorescence-based biosensors, besides the above mentioned properties that can be monitored, include the optical detection format used. For example, it is possible to utilize fiber optic probes, often termed optrodes, to immobilize bioreceptors at the tip of the fiber, and use total internal reflectance properties of the

fiber to transmit excited and emitted light. Total internal reflectance can also be employed in an evanescent wave format, where a residual amount of (evanescent) light at the reflectance point that escapes is used to excite immobilized bioreceptors only in close proximity to the surface, rather than in bulk solution. This format allows for controlled excitation, and can allow for minimal fluorescence background. However, a key disadvantage is the lack of evanescent excitation power, and sometimes poor coupling of the emission when using similar collection geometries. Fluorescence detection can be used with a wide variety of detection formats. For example, it is routinely coupled with flow cytometry and microfluidic platforms or imaging array systems such as biochips which utilize spatial patterning of biological recognition elements to match fluorescence location to target species.

Interferometry

Interferometers can measure biomolecular interactions that take place at a surface within an evanescent field that causes a refractive index change and a corresponding change in the phase of the propagating light. For example, a Mach-Zehnder interferometer-type of sensor can be constructed in which an optical waveguide is split into two arms of equal length and is recombined before the detector. One arm is a reference arm, which does not experience refractive index changes, while the other is the sensing arm. In the absence of analyte, light from both arms is in phase as it reaches the sensor. If analyte is bound to bioreceptors on the sensing arm, the local refractive index of the evanescent wave is altered, producing a phase shift, which results in destructive interference at the detector, resulting in an output power loss. Studies have indicated that 10^{-5} to 10^{-7} changes in refractive index units can be detected with these sensors (Washburn and Bailey, 2011). Although this approach has been in existence for quite some time, it is primarily a laboratory technique since it is not a very robust biosensor technology, and suffers from significant false-positive results.

One potential difficulty with interferometric sensing is that the length of the optical wavepath necessary to produce interference can be on the order of a centimeter, which is large compared to many other optical detection devices. A possible design for multiplexing (chip-integrated Young interferometer) is to project the light output from the optical waveguides onto a CCD device, which allows analysis of the interference patterns, which could be then extended to several different waveguides analyzing different analyte species.

Nevertheless, potentially very powerful biosensing technologies based upon evanescent waves and changes in refractive index are currently being explored. See below for in-depth discussions of these concepts (Optical Resonators, Waveguide Mode Sensors). Other procedures can be used to increase sensitivity of optical techniques employing refractive index changes as signaling events. The use of enzymes to amplify output signals of sensors is a standard technique in assays like ELISA. It is also possible to apply the same strategy for interferometric sensors. For example, using an enzyme coupled to an antibody, and with a substrate which produced a colored product in solution, it was possible to amplify the index change in an interferometric optical sensor, since the colored enzymatic product generated a larger change in index than without enzyme amplification.

Chemiluminescence

Chemiluminescence is one type of optical sensor technology which relies on a series of chemical reactions, usually employing oxidation of a luminol reactant, to produce a reaction product that gives off characteristic light. The intensity of this light is correlated to a sample target of interest, by coupling some of the reaction products to biorecognition elements, to serve as chemiluminescent reporters. The advantage of chemiluminescent transduction includes very low optical background. Theoretically the background in the absence of analyte for a chemiluminescent biosensor should be very low or non-existent, leading to a very sensitive (high signal-to-background) measurement but practically, non-specific interactions commonly occur, resulting in some low to very low level of chemiluminescence even in the absence of analyte. The reaction usually employs signal amplification and bioassays with multiple washing and labeling steps, which leads to a somewhat time-consuming analysis.

Surface plasmon resonance (SPR)

SPR is a phenomenon that can exist at the interface between a metal and a dielectric material such as air or water. A surface plasmon (commonly excited by optical radiation) of the electrons at the surface of the metal results in an associated surface bound and evanescent electromagnetic wave of optical frequency. This evanescent wave has a maximal intensity at the interface and decays exponentially with distance away from the interface. In SPR-based biosensing, changes at the interface, that is, biological recognition element and analyte binding, causes changes in the local refractive index which in turn causes changes in the resonance excitation of the surface plasmon.

SPR is a form of reflectance spectroscopy, where change in a SPR that occurs during optical illumination of a metal surface is harnessed to detect biomolecular interactions. A schematic diagram illustrating a basic SPR chip platform is illustrated in Figure 8. The SPR chip consists of a prism with a thin gold film upon which the bioreceptors are immobilized. Light is totally internally reflected from the prism face coated with metal and the changes in reflectivity measured. Surface plasmons are excited at a specific incident angle and result in a massive reduction in reflectivity at that angle. Changes in the refractive index at the interface results in a change of the optimal angle required for excitation.

Any change in the optical properties of the medium in contact with the immobilized layer will cause changes in the characteristics of the surface Plasmon wave. Specifically, these changes can be measured as changes in wavelength, intensity, phase, or angle of incidence. SPR techniques are widely popular in contemporary biosensor development because it is a surface-sensitive technique that can measure real-time interactions between unlabeled species.

Although SPR is a simple technique with a number of advantages, it is not the most sensitive. However, one variation of SPR includes a resonant mirror format which utilizes a series of polarizing filters to block light that is internally reflected. At a particular

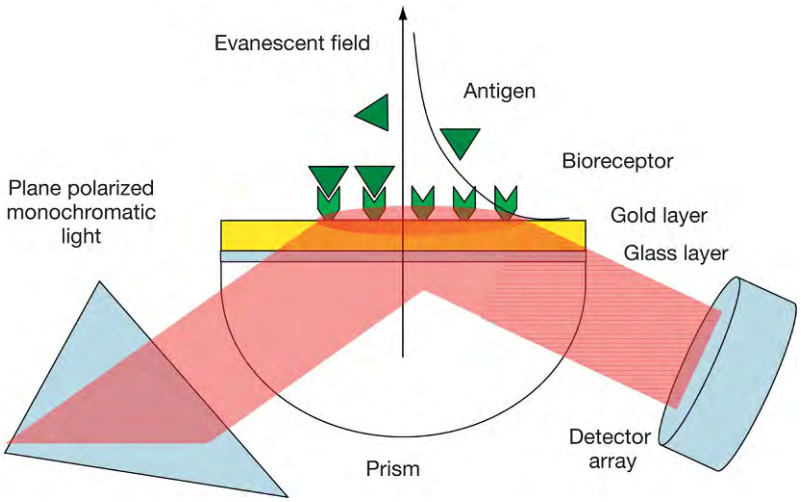

Figure 8 Schematic diagram of a biosensor utilizing surface plasmon resonance (SPR) transduction.

incident resonant angle, the light is diverted through a spacer layer that has a low refractive index, into a higher refractive index guide so that the signal peak appears on a dark background. Despite these advances, one of the primary limitations of SPR-based biosensors is that anything which alters the refractive index at the sensing surface will interfere with the analysis including nonhomogenous (complex) sample matrices, and nonspecific binding interactions.

Surface enhanced Raman spectroscopy

Another type of plasmonic spectroscopy that is gaining popularity in biosensing applications is surface enhanced Raman spectroscopy (SERS). SERS is an enhanced form of Raman spectroscopy, where a nano-roughened metal surface is used to enhance the scattered Raman signal. The SERS enhancement is thought to be the result of a combination of intense localized fields arising from SPR in metallic nanostructures and chemical effects.

Advantages of this approach include the ability to obtain a spectroscopic fingerprint, similar to infrared absorption spectroscopy, while being relatively interference-free from water. Also, since SERS is an enhanced technique, it can be used to see very low concentrations of even biological materials, as evidenced by recent interest in a variety of immuno-SERS methods under development. The difficulties often encountered with SERS biosensors, primarily stem from signal reproducibility challenges, which are directly tied to the reproducibility of nanostructured SERS substrate fabrication. Of the transduction methods covered in this article, SERS is one of the least established for biosensor development but shows much promise for continued development.

A review of recent literature finds SERS is increasingly employed in biomedical applications. A variety of factors are responsible for this surge in interest. Among these factors are the facts that SERs can be used to detect analytes in complex mixtures directly, without separation steps, that it can sample non-invasively from tissues and cells, that it potentially can sensitively detect small changes in structures of macromolecules, that it has high spatial resolution, and that it can be readily multiplexed, thus allowing analysis of multiple analytes simultaneously. Further advantages include a remarkable freedom from interference in SERS measurements due to unexpected species which might appear in complex biological samples, and the ability to suppress fluorescent backgrounds produced by common biological species by carrying out SERS analysis in the near IR. The reduction in background noise of complex biosamples possible using SERS is one of the most attractive aspects of this analytical technique. A complete description of the foundational theory behind the SERS process is beyond the scope of this review. In brief, SERS depends primarily upon electromagnetic effects on nanostructured materials. Surface charges (also called surface plasmons) on a metal surface are delocalized and flow freely and can be resonantly excited by an oscillating external electric field (i.e., a laser beam). If incident light frequency matches the natural oscillating frequency of the surface plasmons, strong electric fields can arise. Thus the fundamental process associated with SERS is the production of an enhanced electromagnetic near field at the surface of metallic nanostructures when localized surface plasmon modes are excited by laser irradiation, and this localized electromagnetic field can interact with the molecules on or near the nanostructure surface, thereby enhancing their scattering properties. It is generally accepted that the aforementioned electromagnetic enhancement is the dominating process for SERS, although chemical (i.e., charge-transfer) SERS enhancement also contributes to the overall SERS enhancement. The sensitivity associated with SERS comes from interaction of the electromagnetic field produced by the surface plasmons with the analyte, which must be in very close contact with a plasmonic surface, resulting in sensitivities even down to single molecule detection, in contrast to the inherently insensitive Raman scattering spectroscopy. It should be noted that there is another effect, very similar to SERS, which is surface enhanced resonance Raman scattering (SERRS). In this latter case, when the molecule to be detected has a chromophore which is close in energy to the frequency of the excitation used to excite the surface plasmon and generate SERS, enhancement of the scattering occurs both because of

plasmon resonance (SERS) and molecular resonance, resulting in very intense scattering and producing very large enhancement factors (sometimes in the range of 10^{13} to 10^{15}).

Much has already been written about the importance of the structural details of the plasmonic surface, and the underlying theory has also been discussed. In this brief review, we cover only recent advances of relevance to biomedical sensing. Practically, gold and silver are the two plasmonic materials of choice for SERS at present. The recent development of the field of nanomaterials has provided a rich resource for potentially novel and effective SERS substrates. Among the most important are various nanoparticle (NP) structures. It has been noted that NP's with corners such as nanocubes or the ends of nanorods or the sharp points of nanostars, nanoflowers or nanopopcorn can strongly focus the electromagnetic field generated by the laser interacting with metallic surface plasmons at such points, producing strong SERS enhancement. Also, formation of so-called 'hot spots', which are specific gaps between nanoparticles where the electromagnetic field is very high due to coupling between plasmon resonances of the NP's, can probably be controlled by engineering design in nanostructures, rather than leaving formation of such interactions to chance as would occur in colloidal NP suspensions. In fact, structures such as nano-crescent moons or nano-plasmonic split rings have been produced to utilize 'hot spots' associated with these structures.

One of the requirements for effective SERS enhancement is very close apposition of the analyte to the metallic surface. Various techniques have been devised to provide a more-or-less universal SERS detection scheme which can be applied to wide ranges of analytes. Methodologies involving molecular traps and cages have been used; one example is the use of a temperature responsive pNIPAM gel coating gold NP's to trap small molecules such as (1-napthol), which are brought into close proximity to the gold NP surface by raising the temperature, thus collapsing the gel and forcing the analyte into close contact with the NP surface. A variety of techniques for trapping/capping various analyte molecules on NP's are discussed in Alvarez-Puebla and Liz-Marzan (2012). On the other hand, various molecules, such as antibodies, can be immobilized onto NP surfaces, thus providing specificity for capture of the desired analyte. Actual detection of the analyte can be either by direct or indirect techniques. In the direct method, the analyte is detected by the SERS spectral fingerprint of the analyte molecule, and this fingerprint is generally unique to each substance, due to differences in chemical structure. Both large macromolecular structures (and even microorganisms and eukaryotic cells) and small molecules are theoretically amenable to direct sensing. Many molecules, however, have a low SERS cross-section and so the detection sensitivity for such molecules is poor. Antibody capture of an antigenic analyte can also be considered direct detection, if the SERS spectrum of the resulting antibody/antigen complex is detected. In such cases, it is necessary to subtract the antibody SERS spectrum from the composite spectrum. Analogous detection by direct spectroscopic fingerprinting of specific DNA sequences can take place following hybridization of the target DNA to DNA immobilized on NP's. In indirect detection SERS techniques, a strongly SERS-active substance (i.e., SERS dye) is incorporated into NP's (the particles may also have capture ligands attached), and the resulting NP's basically serve as barcodes for detection of the analyte. In practical terms, it has been found necessary to coat the SERS active NP with a protective shell (usually silica), which also provides a convenient surface for deposition or attachment of various biomolecules which could be used for bio-specific interactions with the analyte. These SERS barcoded NP's could be used for biochip-type detection scenarios or even inside living cells for biological function/imaging studies.

The requirement for metallic surface plasmons for SERS has also resulted in development of so-called nanopatterned metallic platforms, which are surfaces with nanostructured features (usually constructed from nanospheres, nanorods, etc) overlaid with a thin metallic film. These platforms have the great advantage of stability against aggregation-induced irreproducibility (a problem with metal colloids) in SERS spectra, and can be used either for direct or indirect biosensing of various analytes. Biosensing microarrays can be constructed using the nanopatterned metallic platform concept, and using variously labeled and functionalized probes. Thus, one approach used was to employ single-walled carbon nanotubes (SWNT), constructed with either C^{12} or C^{13} and attaching antibodies specific for various proteins onto the SWNT surface. The SWNT become SERS-based sensors which can bind to spatially localized protein targets on the nanopatterned metallic surface. Sensitivities of the order of fM were obtained with this system (Kho et al., 2011).

Some investigators have developed SERS analogs of the molecular beacon concept. In this case, target DNA (or RNA) sequences are detected with a stem-loop structure, which has a SERS-active dye on one end of the stem and a NP on the other end of the stem in close apposition. Thus the SERS signal is strong when the stem-loop structure is intact, but when the target DNA hybridizes to the complementary sequence on the loop, loss of the SERS signal occurs when the stem –loop structure unfolds Detection of biomolecules using SERS has encompassed a wide variety of targets including lipids, nucleic acids, proteins, metabolites, sugars, bacteria and eukaryotic cells. Detection limits usually attainable range from fM to nM, but, greater sensitivities are reported in certain experimental set-ups. One particularly attractive area for future research efforts in SERS might involve the well-known ability of SERS to respond sensitively to changes in chemical structure. A practical example could be placing a SERS-active tag at the proximity of a binding site on one member of an interacting pair, such as tagging the antigen-binding site of an antibody, and then using the SERS tag to report on changes associated with the binding event. This has potentially much wider applicability than just antigen-antibody interactions (Kho et al., 2011).

Intracellular biosensing using the SERS technique has great potential, but relatively few studies have so far been done. Since the SERS technique depends upon metallic surfaces and the resulting plasmon resonances, the usual procedure is to use NP's tagged with SERS-active dyes for intracellular sensing. Part of the problem is then the difficulty in directing NP's to the appropriate intracellular compartments. Another issue is the strong tendency for NP aggregation in the highly complex intracellular environment. It should be recognized that NP's are much larger than typical molecular fluorophores, so their intracellular behavior would be expected to be much different. Intracellular delivery of NP's by many common techniques results in most NP's residing in vesicles inside the cell. Thus various techniques, such as microinjection or cell penetration by a nanofiber, have been used to 'force' the SERS

sensor into desired intracellular locations. One approach to introduce NP's into the specific intracellular compartments is the incorporation of, for example, nuclear targeting peptides such as the HIV-1 TAT on the NP surface. Other procedures can also be used to overcome aggregation problems and improve targeting. Using a stabilizing agent (i.e., protein, like BSA, silica or polyethylene glycol) to coat the NP's using appropriate chemical procedures will protect the NP's, partially prevent aggregation, and these coatings have the further advantage that biomolecules such as antibodies can be appended to the surface coat, resulting in NP's targeted to specific intracellular sites (Yuan et al., 2013).

Optical Resonators: Whispering Gallery Mode Sensors, Photonic Crystals

Whispering Gallery Mode (WGM) biosensors and other optical resonant microcavity-based sensors are adding a whole new class of sensing devices to the armamentarium of analytical techniques with biological applications. Potential advantages of these devices are that they are able to operate without the need for labels, can sense in real-time, and can display extreme sensitivity, even down to single molecule or single virion detection. Excellent recent reviews of these sensors as applied to chemical and biomolecular analysis can be found in Luchansky and Bailey (2012) and Yoshie et al. (2011). The optical theory describing the functioning of WGM sensors can become rather involved, but the basics can be described in a qualitative manner. When light propagates through an optical fiber, and is totally confined (total internal reflectance), then an evanescent wave is induced in the medium surrounding the fiber, and this wave extends ~100-200 nm beyond the surface of the fiber. If molecules are now attached to the fiber's surface, the evanescent wave can interact with them, resulting in an increase in the path length and number of wavelengths of light traveling through the guide. Unfortunately, this effect is so weak that trying to resolve the resulting change in wavelength by interferometry is essentially futile. Now, however, if the photons, guided by total internal reflectance, travel around a silica (for example) sphere (say of radius 100 μm), then each photon is recirculated many times. If the wavelength of the light is tuned so that the light is in phase after each revolution around the sphere (i.e., integral number of waves in one revolution of the sphere's circumference), then the light drives itself in a resonant manner. Practically, a tapered fiber-optic waveguide is attached to a tunable laser and is referred to as the driving fiber. This fiber is arranged in close apposition (for evanescent coupling) to a silica sphere which is formed on the end of a tapered glass fiber. Tuning the laser to a wavelength such that the evanescent wave circles the sphere an integral wavelength number of times results in a dip in light intensity at the other end of the driving fiber, where a photodetector is located. This is the resonance wavelength. Now, if molecules are attached to the surface of the sphere (say, by reaction with receptors previously bound to the surface), then the photon path length increases, and the light is perturbed out of resonance. Tuning the laser to return the sphere to the resonant state will result in an increase in the wavelength of the light to achieve resonance. The shift in the wavelength of the resonant light is the event detected, but this shift cannot be observed unless the resonance linewidths are very narrow. The sensitivity of the WGM sensor is directly related to the spectral resonance linewidth. Resonance theory indicates that the sensitivity of this resonance shift is proportional to the number of times photons recirculate around the sphere, which is several thousand to hundreds of thousands for devices so-far used.

One can also think of the sensing aspect of WGM resonators as follows. If a resonance dip is detected when the wavelength of the orbiting light is such that integral multiples of this wavelength match the microsphere circumference, this means that power is being extracted from the fiber, which results in loss of light intensity at the detector end of the fiber. Even a very thin coating of molecules adhered to the surface of the microsphere will change the radius or orbital refractive index of the sphere, and so the resonant wavelength must change. Practically, achieving requisite sensitivity depends on the Quality (Q) factor of the resonator. For example, Vollmer and Arnold (2008) discuss a hypothetical WGM sensor with a Quality factor of 2×10^6, and estimate that a shift in wavelength of 6 fm (femtometer) can be detected. They indicate that only a very thin layer of bound material is necessary to achieve this shift. In fact, further calculations indicate that a wavelength shift of 6 fm is equivalent to a change in radius of the microsphere of ~0.5 pm and since the size of a typical protein is 1–10 nm, this suggests only a small fraction of a monolayer should be detectable.

The quality (Q) factor of a device using this sensing scheme can be thought of as a measure of the resonant photon lifetime within the optical structure, so Q is directly related to the number of times a photon is recirculated and reacts with the analyte. The evanescent field on the exterior of the optical waveguide, associated with light which is confined by total internal reflection, interacts with molecules in close proximity to the surface of the sensor. High Q optical sensors produce light resonances at very specific wavelengths, and these resonances change as the RI at the sensor surface changes upon changing surface properties (i.e., attachment of analyte to surface). Higher Q values are associated with narrower resonant peaks; narrower resonant peaks translate into sensors with greater sensitivity, because narrower resonance peaks can be resolved better, even if the resonance shift is very slight.

Vollmer et al. (2008) carried out studies of WGM sensors, first using polystyrene (PS) microspheres and then testing detection of Influenza A virus particles. Successful adsorption of individual PS microspheres of 250 nm radius to a silica microsphere of ~45 micron radius was detected by definite steps of resonance shift which could be clearly seen above background cavity noise. These experiments allowed studies of the dependence of the wavelength shift upon the microsphere radius. Detection of individual Influenza A virions was attempted using a laser wavelength of 763 nm and a microsphere of radius 39 microns. Adsorption of individual virus particles was observed as a step function of shifts of resonance wavelengths, above background noise. The radius of Influenza A virion particles as calculated from equations associated with the WGM studies was 47 nm, while the size from SEM micrographs was 45–55 nm.

A variety of optical configurations have been devised to take advantage of the WGM concept. For example, microrings microdiscs and microtoroids have been used, in which the driving fiber is closely apposed to either a ring, disc, or toroidal surface, while yet

another configuration is a tapered optical fiber which is apposed perpendicularly to a cylinder, so that the light circulates around the surface of the cylinder (and the interior of the cylinder could be a microfluidic channel, for example). Microring resonators and microdisk resonators have the advantage of being able to be fabricated on planar substrates, and so have the potential to be manufactured by standard chip fabrication techniques. Current microfabrication techniques are difficult to standardize in constructing these devices. Another significant factor is the requirement for precise alignment of the sensing device with the optical fiber to achieve WGM excitation. This is particularly of concern for the microtoroidal configuration, but is true for other configurations as well. In fact, fluidic flow may easily disrupt these critical alignment factors for WGM resonance, so liquid-based detection of analytes can present problems. Q factors ranging from $\sim 10^4$ to $\sim 10^8$ or 10^9 have been measured with various microresonators of shapes described above (Luchansky and Bailey, 2012).

Armani et al. (2007) used a silica-based microtoroidal resonator which uses the WGM sensing technology, and which had a remarkable Q factor of 10^8, as opposed to Q factors of $\sim 10^6$ for microsphere-based sensors. The higher Q factor of the microtoroidal WGM sensor as opposed to the microsphere WGM sensor is apparently due to the planar nature of the microtoroid. A typical diameter of the microtoroid fabricated was 80 microns. With a Q factor of 10^8, a single target molecule on the microtoroidal surface is sampled more than 100,000 times by the input light, thus producing incredible sensing amplification. Studies involving detection of Interleukin 2 (IL-2), using microtoroidal cavities modified with antibodies to IL-2, indicated a working range of an astonishing 10^{12} in concentrations able to be detected, although the response was not linear over this whole range. Further studies with very dilute solutions of IL-2 showed step function increases in resonance shift, indicating single molecule binding events such as was detected in the work of Vollmer et al. (2008). A variety of experiments were carried out to verify the single-molecule nature of these events.

One way in which to increase sensitivity and specificity using WGM sensors such as those described above, is to take advantage of so-called 'sandwich' assay formats. Luchansky and Bailey (2012) give several examples of such techniques in their review article, such as using a secondary antibody specific for a protein analyte. Thus one antibody captures the analyte on the sensor surface, while the second antibody provides another specific recognition element, while at the same time further increasing the RI change which the sensor can detect. Another concept to further enhance sensitivity is to use very large tags, such as microbeads or nanoparticles, to produce large changes in RI when reagents tagged with such particles binding to the sensor surface, thus providing greater shifts in resonance peaks. In fact, a variety of biological techniques are potentially applicable to such WGM sensors, like using various shapes of nanoparticles, or rolling circle amplification or enzymatic amplification.

Photonic crystals (PC) are essentially made up of spatially periodic constructs of dielectric materials with different refractive index properties, and so confine light due to this periodic structure. For PC sensors, light is back-reflected at only certain resonance wavelengths, which are not allowed to propagate through the periodic dielectric structure (the range of wavelengths which are not allowed to propogate are called the photonic bandgap). The photonic bandgap can be tuned by either varying the RI contrast of the dielectric materials making up the PC or by altering the periodicity of the structure. Frequently, the periodic structure consists of a series of nanoholes in the dielectric surface. Generally, a defect (acting as a resonance cavity) is introduced somewhere in the periodic structure. Certain frequencies of light become confined in the defect structures, thus resulting in high optical field densities around the defect. The RI at the defect site determines the resonant frequency coupled into the crystal. If biomolecules are immobilized in the defect microcavity, changes in the RI occur, with resulting changes in the resonance frequency. The fundamental sensing paradigm is similar to the sensors described above, where changes in RI are detected. Moderately high Q factors can be attained using these devices, so great sensitivity is possible. For instance, less than 10 fg of BSA have been detected using a PC. It has been reported, however, that the sensitivity of detection for PC's is at least 1 order of magnitude less than for resonant microcavity or interferometric sensors (Washburn and Bailey, 2011).

Waveguide Mode Sensors

There are other approaches which make use of evanescent waves detecting interaction of analytes and biorecognition elements at surfaces, by changes in RI. In one approach, denoted as optical waveguide light-mode spectroscopy (OWLS), a thin-film, single mode planar waveguide (denoted as optical grating coupler sensor chip) is fabricated, with a grating etched into the waveguide at the point of optical input. The waveguide layer is sandwiched between two other layers. Light is launched into the waveguide at an incident angle such that it propagates by total internal reflection. An evanescent wave is generated which penetrates into the upper layer (assuming light is launched from the bottom) which has ligands for binding the analyte of interest. Binding of analyte on the surface of the sensor produces a change in the RI, which affects the evanescent wave. This, in turn, generates a change in the angle of incidence at which light is maximally coupled into the waveguide. This change in incident light angle is what is monitored. Other, similar sensors are called wavelength-interrogated optical sensors. In these sensors, wavelength changes are measured as the output signal, rather than incidence angle. Gopinath et al. (2012) discuss using aptamers as biorecognition elements on waveguide mode sensors. Of particular interest are two experimental procedures they use to increase sensitivity. One involves the perforation of the waveguide surface with nanoholes (e.g., 50 nm diameter) to increase molecular accumulation of ligands on the waveguide surface. Size of the nanoperforations is critical however, as too large a size increases scattering of incident light and interference, decreasing sensitivity. Another technique to increase sensitivity is to attach a chromophore to, say, the target molecules. If proteins are targeted, using aptamers which bind a specific protein, then one easy technique is to add Coomassie Brilliant Blue (a non-specific protein stain) to the analyte solution. Proteins captured by the aptamer will also have the dye attached to them. This dye -coupled protein produces a much larger detection signal (i.e., resonant peak) than in the absence of dye.

Applications of Biosensors

Bacterial Biosensors for Environmental Monitoring

Whole cell bioreporters which respond to certain pollutants/toxicological conditions are beginning to find applications in environmental sensing. Applications range from detection of contaminants, measurement of pollutant toxicity, monitoring of genetically engineered bacteria released into the environment, and even uses for detection of stresses such as ionizing radiation and oxidative damage. In general, such whole cell sensors should not be called biosensors unless the cells are integrated somehow into a stand-alone sensing/transduction/readout system. Some whole cell sensors use genetically engineered bacteria into which a *lux* gene construct (coding for an active luciferase) under the control of an inducible promoter is introduced. The general sensing strategy is to put the luciferase construct under control of a promoter that recognizes the analyte of interest although in some cases, the bioreporter organism can be engineered to respond to many analytes (broad specificity) by using a heat shock promoter or other general stress response mechanism, fused to the lux cassette.

For example, the seminal work in this arena describes a naphthalene biosensor organism (genetically engineered *Pseudomonas fluorescens*) that detected the bioavailability of naphthalene (serving as a surrogate for polyaromatic hydrocarbons) and salicylate in contaminated soil by induction of the lux reporter, and subsequent bioluminescence as a result of expression of the reporter gene. The naphthalene biosensor described above is an example of a catabolic bioreporter. Catabolic bioreporters produce luminescence only in the presence of a specific contaminant, since the *lux* genes are linked to a degradation pathway promoter. Metabolic bioreporters, on the other hand, have reporter genes downstream of a strong constitutive promoter, and thus luminescence is directly affected by toxic agents that interfere with metabolism. These concepts have been subsequently used to develop a variety of bioreporter organisms. Other investigators have used genetically engineered bacteria in which the green fluorescent protein (GFP), (or derivatives thereof), initially derived from the jellyfish *Aequorea Victoria* functions as a bioreporter in the same manner as the *lux* gene. Some arguments have been advanced that the expression of bioluminescence is a more direct bioreporter than GFP expression, since the expression of GFP is only detected upon excitation with the correct wavelength of light. However, in practical terms, bioluminescence must be quantified by some sort of photodetector/luminometer, so the difference between that and a fluorescence excitation/emission detection system seems minimal. Beta-galactosidase enzymes have also been used as biomarkers. Others have used naturally luminescent bacteria (i.e., *Vibrio fischeri*) as bioreporter organisms, since toxic compounds can disturb metabolic processes, thus causing a reduction in their bioluminescence. Microbial biosensors have been developed to assay biological oxygen demand (BOD), a value related to total content of organic materials in wastewater. These sensors often measure BOD by evaluating the rate at which microbial cells deplete oxygen. It is not possible to provide a comprehensive list of whole cell sensor applications in this article. Very seldom are these bioreporter organisms integrated into a complete sensing package where a microculture environment, integrated luminometer or flurometer, and light-tight enclosure can produce a true biosensor device.

Food Biosensing

Some of the factors of interest in regard to analysis of foods include monitoring nutritional parameters, food additives, contaminants, microbial contamination, shelf life assessment and factors such as smell and odor. Many of the aforementioned factors have to do with quality of the food, while others reflect directly on food safety. There are obvious overlaps between food quality and food safety. The assessment of food quality can be subjective, as factors such as appearance, taste, smell, and texture may enter into the overall evaluation. Sensor technologies that measure specific parameters such as sugar content and composition, total titratable acidity (e.g., for fruit), specific chemicals such as glucose, sucrose, lactate, alcohol, glutamate, and ascorbic acid provide more objective means of evaluating food quality and freshness or spoilage. Also, quantitative detection of contaminants of food such as growth hormones fed to animals, antibiotics and pesticides is important. Finally, there is the issue of pathogen and/or pathogen toxin contamination of food. This last is of great concern because of the potential for outbreaks of foodborne illnesses, such as are seen periodically. Fatalities can result from such exposures, and recent outbreaks have generated much media interest.

There is a clear role for biosensors in many areas relating to food quality and food safety. The space limitations of this article will not permit consideration of all aspects, and so the authors have chosen to focus on pathogen detection in food products. Recent reviews of statistics regarding pathogen testing (all types of tests including biosensors) in the food industry prove interesting in regard to future biosensor development. For example, for 1999, *c.* 16% of all microbial tests in the industry were for specific pathogens, *c.* 16% were for yeast and mold, *c.* 31% were for coliform and *E. coli*, and *c.* 37% were for total viable organisms. The same statistical survey found that microbial testing in each of the food sectors was as follows: 36% for the processed food sector, 10% for fruits and vegetables, 22% for meat, and 32% for dairy. The US food industry performed *c.* 144.3 million microbiological tests in 1999. Unfortunately, heavy reliance is still placed upon conventional culturing techniques, so there are significant time gaps between sampling for microbial contamination and detection of microbial contamination, and these techniques are often labor-intensive and require expertise in interpreting results. Other investigators also point out the importance of pathogen detection in the food industry, that is, that the food industry accounts for *c.* 38% of total research in the field of pathogen detection. The other major areas of research interest for biosensors are water and environment quality control (16%) and clinical applications (18%). In terms of research articles describing biosensors applied to pathogen detection, a review article, 'Pathogen detection: A perspective of traditional methods and biosensors', summarizes a number of interesting points. Thus, of research articles concerning pathogen detection, techniques for detection of Salmonellae species are the most abundant (33%), followed by *E. coli* (27%), with *Listeria*

(14%) and *Campylobacter* (11%) species the other major pathogens. *Legionella* account for 7% of research articles discussing detection techniques with all other bacterial species accounting for 8%. This relative abundance of research articles published with regard to detection schemes clearly indicates the emphasis upon pathogen detection for food safety. This same review also discusses some relevant statistics regarding detection technologies for pathogens, gleaned from a review of the relevant literature over the past 20 years. The most popular methods by far relate to culture/colony counting with PCR following closely behind. ELISA assays are in third place in terms of abundance, with biosensors following in fourth place. In the biosensor category, the most used techniques are optical (35%), followed by electrochemical (32%), piezoelectric (16%), and all other categories (16%).

The tried and true methods of microbiology involve concentration of microbes from food samples. Plating, culture, growth in selective media, and colony counting are still the gold standard with regard to identification and quantification. Nevertheless, as pointed out above, these techniques are time- and labor-intensive and do not provide answers in a timely manner. Even PCR often requires a time investment of several hours (at minimum) before analysis is complete, and also relies on technical expertise. There is clearly a need for rapid, low cost techniques that provide automated or semi-automated pathogen analysis. Even better would be systems which integrate all aspects of the analysis from sampling to final quantitative result. Biosensors seem well-suited to fill at least some of this niche in the food safety testing market. Yet, a main problem facing all such attempts to move pathogen testing from classical microbiological procedures to biosensor-based analysis is the issue of sensitivity. Recent literature indicates that the infectious dose of *Salmonella* or *E. coli* 0157:H7 is ten organisms, while the existing coliform standard for *E. coli* in water is 4 cells/100 ml. Culture methods can and do attain this sensitivity. From a review of recent biosensor literature the present authors carried out, this level of sensitivity is not achievable by most of the biosensor research devices currently proposed. It seems clear that biosensors will only see their full potential realized with regard to pathogen detection for food safety, when specificity and sensitivity can compare to established methods and such biosensors can also be cost-competitive (or cost-saving) with current techniques. It should also be pointed out that testing for genetically modified organisms (GMOs) will probably become a more frequent analysis with regard to food and some authorities expect this area to see the fastest growth of any testing market in the food industry. Biosensor research for GMO detection seems to be mainly focused on DNA-based detection technologies at present.

Biodefense Biosensing Applications

Events of recent years have indicated the need for sensors of pathogens/toxins which could be used by military enemies or for terrorist purposes. Much research effort is currently focused on analytical strategies to detect these agents. A major difficulty with biological attacks is actually determining whether the attack has occurred, in a timely manner, to enable early response, and minimize casualties. The ideal sensor for biological agents which could be used for attack (hereafter called biowarfare agents (BWAs)) would provide highly sensitive and selective identification of threat organisms in virtually real time. Also, since the potential release of BWAs could be on the battlefield or in urban settings, the surrounding environment/atmospheric milieu may be highly complex (smokes, dust, and particulate matter), and the analytical technique must be able to detect organisms of interest without interference from background material. To date, these requirements have provided a daunting analytical challenge.

Attempts to meet this challenge have employed molecular techniques that can identify chemical markers of BWAs. In general, detection of BWAs can follow one of two paths. In one, positive identification of a BWA must be obtained in a few hours, the so-called detect-to-treat option. Such detection would give medical personnel the means to successfully treat individuals that have been exposed to BWAs. The time frame of several hours makes the analytical task easier then is the case with the other detection scenario. In this latter case, a detect-to-warn sensor must be able to provide a warning within a few minutes that a BWA release has occurred. If such sensors could be developed, then perhaps therapeutic treatment of all members of an exposed population might be an option. The variety of BWA that potentially could be used, that is, bacteria (vegetative or spores, Gram-negative or Gram-positive), viruses, and toxins, adds difficulty to the sensing task. Specificity and sensitivity are both critical for useful BWA sensors. False positives are unacceptable due to the consequences attendant upon an assumed BWA incident. False negatives are also unacceptable for obvious reasons. It is true that nucleic acid-based biosensing technologies have been demonstrated to have the sensitivity to detect one organism or one spore. Also, in general, nucleic acid-based sensing systems are more sensitive than antibody-based detection systems. Such analyses, however, depend on amplification of the target nucleic acid, a process that takes time (although intensive efforts have been and are being made to shorten this step). Further, nucleic acids from nonrelevant sources may interfere in PCR amplifications, and nucleic acid-based detection will not work for toxins. Detection of BWA with extreme sensitivity is important since some of the organisms manifest infectivity at extremely low levels. Nucleic acid-based detection also offers the best chance for detection of novel, engineered organisms or so-called stealth organisms, where virulence genes are inserted into an otherwise innocuous microbe. In these cases, the nucleic acid sequence of the virulence elements still provides a diagnostic signature. Integration of all steps (lysing cells/spores to prepare nucleic acids, amplification of target sequences, and detection of the same) required for nucleic acid sensing is crucial for a true biosensor, and so far, reducing the entire assay time to a few minutes has been unreachable. Thus, there has been interest in nucleic acid-based techniques that provide gene-based specificity, but do not require amplification steps to attain detection sensitivity to the required levels. Such exquisitely sensitive detection almost precludes techniques like SPR and recommends procedures like fluorescence detection.

This difficulty has led others to suggest that detectors based on surface features (e.g., immunogenic molecular species) of the threat organisms may be a more tractable route for development of detect-to-protect devices. Such devices must have a rapid response, must not give false positives, must have high sensitivity and the ability to detect the target in aerosol samples, when the natural environment for most detecting biomolecules is aqueous. The major factors for successful immunosensing of BWA are the

efficiency of the antigen–antibody complex formation and the ability to sensitively detect these complexes. Nonspecific binding can be a problem for such techniques, particularly as sensitivity is pushed. There is also an urgent need for multiplexed detection in BWA sensors, since the list of potential BWAs, while not overly long, still numbers more than a handful.

New Biosensing Directions

In this section, we wish to highlight several areas of biosensor research and development which, in our opinion, either already have demonstrated, or have the potential to demonstrate novel and powerful sensing capabilities. We will briefly discuss each of the technologies we wish to highlight in the following text. Some areas which we see as having unusual potential for application to a variety of sensing needs are: (1) virus-based biosensors; (2) genetically encoded biosensors based on fluorescent proteins; and (3) nanomaterials, and their applications as components of biosensors.

Virus-Based Biosensors

Viruses continue to intrigue researchers because of their varied structures and range of capabilities, all contained in packages of such small size. As structural entities, viruses can be thought of as highly structured nanosystems with a well-defined 3-dimensional structure which can occur in a wide variety of shapes and sizes. Of particular importance for sensing applications, viruses are characterized by multiple identical repeating units of coat proteins (thus providing numerous identical sites for scaffolding of various ligands and tags), water solubility, stability in aqueous buffers and perhaps most importantly, particles of a single type of virus are essentially identical and thus mono-disperse in size and shape. Because of the repeating nature of the coat proteins, attachment of modifying molecules at precisely defined positions is possible. Thus, fluorescent dyes can be bound at defined positions to make a 'super-bright' viral particle in which the spacing between dye molecules is such as to mostly eliminate fluorescence quenching, while multivalent display of ligands on the viral surface can improve binding affinity for cellular targets. By using site-directed mutagenesis it is also possible to express new reactive residues on the viral coat proteins. It is also possible to modify the viral coat protein to display not only fluorescent tags, but also targeting ligands, such as antibodies. The use of phage display to genetically engineer the capsid proteins of filamentous bacteriophages to produce recognition peptides of almost unlimited specificity for any target analyte has already been mentioned in this article and will not be discussed further here. Bacteriophages (afterward shortened to phage) are not the only viruses of interest as sensing moieties, however, as a variety of plant viruses such as cowpea mosaic virus, tobacco mosaic virus, and turnip yellow mosaic virus have also been employed. It is also possible to use the interior compartment of viruses, once the nucleic acid is removed to entrap entities such as nanoparticles, polymers, enzymes, fluorophore tags, etc.

Only in recent times has the potential usefulness of viruses as sensing entities been exploited. Not surprisingly, phage-based sensors have been applied in a variety of ways: (1) as target-recognizing probes (think antibody substitute); (2) peptides isolated from phage display techniques and used directly as probes; (3) lytic phages used directly as sensors by means of lysing their target bacteria and detection of components of lysed contents; and (4) phages conjugated to other nanomaterials to form a composite sensor. Phages have been immobilized on the surface of a QCM and used for the detection of pathogenic bacteria. Phages displaying specific peptides on their major coat protein have also proven to be effective replacements for antibodies in ELISA-type assays. In a similar manner, phages have also been used as sensing elements in immunofluorescence assays, in SPR detection and in electrochemical sensors. As already mentioned, removing the interior contents of the virus and using the interior cavity for encapsulation of various reagents is a novel modification which is just now being explored. Such constructs are referred to as virus-like particles (VLP). A natural property of intact virus is binding between viral coat proteins and specific receptors on cell surfaces. Thus, VLP can retain this binding specificity, while carrying labeling reagents on the interior surface. For example, canine parvovirus, which has a natural affinity for transferrin receptors, could be modified into VLP containing a fluorescent label. These labeled VLP became a biosensor for certain tumor cells which express transferrin on their surface.

Genetically Encoded Biosensors using Engineered Fluorescent Proteins

This remarkable group of naturally fluorescent proteins, occurring in the biological realm in certain sea creatures, have proved to be powerful tools for elucidating information regarding complex biological interactions. Perhaps some measure of their significance to biological research can be gleaned from the fact that the 2008 Nobel Prize in Chemistry was awarded to three investigators (Shimomura, Chalfie and Tsien) who played pivotal roles in isolation, study and application of these proteins to biological systems. Isolation of the first green fluorescent protein (GFP) from the *Aequorea victoria* jellyfish was followed by eventual sequencing of the gene coding for the fluorescent protein, and the discovery that the fluorescent protein (FP) could be expressed in virtually any cell, not just in species of origin. Clever site-directed mutagenesis has produced variants of GFP which have enhanced fluorescent properties relative to the native protein, and which have different excitation/emission maxima, e.g., CFP (cyan) and YFP (yellow) being among the most useful. Further studies of various Anthozoa species (reef corals) have permitted the color palette of FP's to be greatly extended into the orange and red regions of the visible spectrum. These studies have spawned a wide variety of modified fluorescent proteins with enhanced fluorescence emissions and with fluorescence properties spanning essentially the whole visible spectrum. Recently, expansion of the FP spectrum into the near IR has also been reported, using a bacteriophytochrome from a microorganism.

To understand the applications of these FP's to biological science, a few words need to be said about the FP structure. The tripeptide responsible for the fluorescence of FP's is in a central alpha helix which is enclosed in an 11-stranded beta barrel structure. The size of the FP varies between 20–30 kDa. Mutations within the chromophore site, as already alluded to, can result in changes in fluorescence properties. Once the gene for the GFP was identified and sequenced, it was found possible to place it under control of promoters and express the protein in, for instance, animal cells. This application, however, was by no means the most significant use of FP's. It was found possible to fuse the FP gene to other protein genes, whilst still retaining the fluorescent properties of the FP. This immediately led to tremendously diverse applications, as the FP could be fused to protein scaffolds which could act as molecular recognition elements or subcellular localization signals. Thus the FP's can become biosensors, reporting on molecular interactions in addition to protein localization with heretofore unachievable spatial and temporal resolution.

A powerful advantage of these fluorescent proteins (FP's), as compared to sensor molecules such as Quantum dots or fluorescent probes, which by definition are exogenous to the cell, and must be introduced by various techniques, is that FP's are introduced into cells as a genetic construct, and therefore are manufactured directly by the cellular machinery. Thus these genetically encoded fluorescent probes, by definition, are noninvasive. A further advantage of these FP's is that they can be directed to certain subcellular compartments by incorporation of signal sequences which control subcellular localization, while exogenous fluorophores may be constrained to only certain subcellular compartments. Fusion constructs of FP genes and other protein genes are easy to construct by standard molecular biology techniques. The potential to resolve molecular events on the time scale of 100's of milliseconds exists using these FP probes. Further, the use of FP's coupled with FRET-based techniques (see below) allows one to capture the intricacies of spatial interactions on a distance scale which has been difficult or impossible to achieve by other techniques. Also, using FP's allows one to gain information about biorecognition processes in the natural habitat of the proteins of interest, thus providing information about processes occurring within the complex environment of living cells, as opposed to the test tube.

Applications of FP's span an enormous spectrum of investigations. Here we can only provide a generalized overview with emphasis on common thematic elements. Fusion chimeras of FP's and a molecular recognition element (MRE) can either involve an endogenous or exogenous MRE, with the changes in FP properties being the sensor endpoint. On the other hand, FRET-based sensors, by analogy, may involve either intramolecular or intermolecular FRET interactions occurring as MRE's on the same protein chain or separate protein chains interact, with alterations in FRET properties as the endpoint. Also, the FP chromophore may be fragmented into two parts, with the biorecognition event reconstituting the intact chromophore. Some further explanation of these possibilities with examples will now be provided.

Perhaps the simplest application of an FP/single polypeptide chain fusion is in studying colocalization of separate proteins to subcellular compartments. Here, the use of two FP's with quite different fluorescent properties, each fused to one of a pair of proteins under study, permits the imaging of the chimeras in various subcellular compartments and evidence either for or against colocalization. In terms of FP's with endogenous MRE's, a variety of biosensors have been developed where changes in pH or ion concentration, etc, can produce changes in fluorescence properties of the FP. In these cases, the FP itself serves as the sensor, i.e., the analyte directly influences the FP's fluorescence properties. Fusion to other polypeptides may or may not be necessary for its intended use. Due to the chromophore's environment within the protein, GFP demonstrates alterations in fluorescence properties as pH changes. Thus, with FP fused to organelle-specific targeting peptides, a subcellular pH sensor has been constructed, providing information about pH of various cellular compartments. FP constructs have been made to serve as halide sensors, in which fluorescent properties of the FP change upon halide interaction, although here the additional requirement of engineering an ion channel into the chromophore part of the FP structure is needed to make the sensor effective. Also, interestingly, a redox sensor has been constructed in which site-specific mutagenesis has produced two cysteines in close enough apposition to the chromophore element of the GFP so as to alter the fluorescent properties depending on the redox state of the cysteines. If the cysteines are oxidized to a disulfide pair, there is a ratiometric change in the excitation spectrum of the GFP which can be monitored. This particular GFP has been fused to a human glutaredoxin, which increases the specificity for the reduced glutathione/oxidized glutathione pair and abolishes the dependence on endogenous glutaredoxin. Using this sensing fusion FP, it has been possible to examine oxidation states in mitochondria in real time and in vivo.

In the case of exogenous MRE's fused to FP's in which the total construct is still one polypeptide chain, an ingenious technique is used to generate the sensing structure (these constructs are sometimes referred to as circularly permuted GFP's). Here, the original amino and carboxyl terminal amino acids of the FP are connected with a flexible peptide linker, while in some other location within the FP structure, a peptide bond is broken and an exogenous MRE is inserted at this point. The FP structure is not so perturbed as to lose all fluorescence properties, but when analyte binds to the MRE, inducing a conformational change, this change is relayed to the chromophore, resulting in altered fluorescence properties. Thus, for example, a Ca sensor was devised where calmodulin was inserted in the fusion construct in such a location, that upon Ca binding, a large change in fluorescence emission was seen. A sensor giving information about relative ATP:ADP concentrations was developed by inserting an adenylate-binding protein into the YFP structure. Shifts in excitation spectra resulted when different ratios of the two ligands bound to the adenylate-binding protein. Other sensors in this class include hydrogen peroxide and Zn sensors.

We now discuss the FRET-based biosensors which employ various FP pairs. These sensors provide potential for incredible spatial resolution of molecular interactions. This is by virtue of the fact that FRET is an energy transfer process which is critically dependent upon both distance and orientation between donor and acceptor groups. In fact, effective distances where FRET interaction occurs are usually considered to be 10 nm or less. This is well below the standard fluorescence imaging resolution of 100's of nm. Thus FRET provides an insight into molecular interactions which is hardly to be obtained by other means. In intramolecular FRET configurations of FP sensors, two different FP's (most often CFP and YFP) are fused in tandem to a polypeptide sequence which is an

MRE. Upon binding of the analyte to the MRE, there is a change of structure of the peptide chain, which results in an alteration of the distance between the donor and acceptor fluorophores of the FRET pair. This in turn causes a change in the efficiency of FRET, which is the signal change that is detected. For example, if two FP's are within FRET interaction distance and separated by a peptide sequence which is sensitive to a particular proteolytic enzyme, presence of that enzyme will result in cleavage at the peptide site, thus separating the two FP's and abolishing FRET interaction, a result easily distinguished by loss of acceptor emission. Intramolecular FRET is also a powerful tool for studying the action of kinases, enzymes which phosphorylate various sites in proteins and have critical roles in regulatory cascades. In this sensor format, a peptide substrate for the kinase of interest and the binding domain for phorphorylated substrate are both fused to two FP's which form a FRET pair. When the kinase encounters this substrate, and phosphorylation occurs, the binding domain binds to the substrate, there is a rather large change in conformation, and the FRET interaction between the FP's is altered, which is detected by spectroscopy. This same general paradigm has been applied to other types of sensors. Bacterial periplasmic binding proteins, for instance, can have binding pockets specific for certain sugars. When the specific sugar binds, the more open configuration of the protein becomes more condensed, and FP's on either side of the binding pocket can come into FRET apposition. Calcium sensors called chameleons have been developed in which calmodulin, and M13 peptide are fused to CFP and YFP. In the absence of Ca^{++}, the fusion protein assumes a dumbbell shape and distances between the FP's are too great for FRET, but upon Ca^{++} binding, configuration changes to a compact shape and FRET is now detectable.

Analogous to the above FRET analysis system, it is possible to use intermolecular FRET-based assays, in which the two FP's are fused to two distinct polypeptide chains. Here again, if protein/protein interaction occurs upon presence of the analyte, FRET interaction can occur. These assays become somewhat more complicated because two separate fusion constructs must be co-transfected. A biosensor for cAMP has been constructed using this principle, in which two FP's of a FRET pair were fused respectively to the regulatory RII and catalytic subunits of protein kinase A, the main effector of cAMP. Protein kinase A consists of a holotetramer composed of two regulatory and two catalytic subunits. Activation of protein kinase A by cAMP causes dissociation of the catalytic from the regulatory subunits. In the FP –labeled intact protein kinase A, FRET interaction occurs. Upon binding of cAMP, dissociation of the two subunits is induced, resulting in loss of FRET energy exchange. The same type of intermolecular FRET can be used to examine other protein-protein interactions.

Yet another type of sensor strategy is available for FP-based sensors. This is referred to as bimolecular fluorescence complementation. The principle here is that a biorecognition event (analyte binding) brings together two fragments of a split FP into close enough apposition so as to effectively constitute an active chromophore (i.e., by refolding into the beta barrel structure). Another way to conceptualize this is to think of a MRE fused to one FP fragment and an analyte protein fused to another FP fragment. Upon interaction of analyte portion with MRE, a 'reconstituted' active chromophore is generated. This type of sensing strategy has been used to study the interaction between three protein subunits that constitute the influenza A polymerase complex, and to examine oligomerization between adenosine A_{2A} and dopamine D_2 receptors.

It is not possible in this brief review to give more than a taste of what can be done with these very versatile FP reporters. The subject should not be left, however, without mentioning some potential difficulties associated with use of FP fusion constructs, as well as very recent developments. Unfortunately, because the FP's themselves are quite large proteins, the size of fusion constructs can cause deterioration of the fused construct. Also, the cell may mislocate the fusion protein, again probably because of the hybrid structure of the construct. The FRET-based applications, while potentially very powerful, are very dependent not only on distance but *orientation* of the adjoining fluorophores, and orientation may be critically changed in complex protein constructs. Also, FRET, while seemingly conceptually straightforward, is, in practice, somewhat more complex, and ratios of fluorescence changes between donor and acceptor molecules must commonly be examined, when attempting to assess conformational changes as monitored by FRET.

In spite of these caveats, the use of genetically encoded fluorescent biosensors bids fair to provide us with ever greater insights. One can envision a complete repertoire of these biosensors, each with specific properties that will allow interrogation of the status of the event under examination. Furthermore, other more advanced spectroscopic techniques like fluorescence lifetime imaging and fluorescence recovery after photobleaching, will permit more detailed analysis of these various FP fusion hybrids as they interact in sensor configurations. Finally, recent developments herald the applications of so-called photoactivatable or photoswitchable FP, which have been termed optical highlighters, in which illumination of the FP at certain wavelengths can produce photochemical reactions that change the properties of the chromophore. These optical highlighters have enabled imaging of fast protein dynamics and sub-diffraction limit imaging.

Nanomaterials and Applications to Biosensors

Introduction

The recent development of nanomaterials science has produced an incredibly rich array of novel materials which are likely to find multiple applications, among them being as components of biosensors. Various inorganic nanomaterials have been produced and characterized, including nanoparticles (metals, for example, gold, silver, iron), nanocrystals of semi-conductors (e.g., Quantum Dots [QD's]), nanorods, nanowires, and various carbon nanostructures (both single walled and multi-walled carbon nanotubes, nanofibers, etc). This list is not by any means all-inclusive, as more complex geometric shapes and various hybrid structures are being continuously developed. The field is so vast that it is not possible to cover every recent development in a short review.

Rather, in this article, we hope to focus on general principles which are being utilized in numerous applications of these nanomaterials to biosensing, and to provide a few examples illustrating such applications.

What then are the potential advantages which nanomaterials bring to biosensing applications? Due to their extremely small size, these materials have unique physical properties which recommend them as scaffolds for biosensing. Included among these properties must be items such as electrical conductivity, unique optical properties, unique structural characteristics (i.e., very high surface area-to-volume ratio), and certain biological properties predicated upon their nano-size. Indeed, the nanometer-size scale of these materials allows relatively facile entry into cells, providing potential for intracellular sensing, although the innocuousness of these materials within the cell is still somewhat of an open question. The large surface area of these materials allows derivitization with many biologically active ligands, and the resultant high density of such bioreceptors may increase affinity toward target molecules by a multivalent binding effect, while also shortening the response time for sensing. In the ideal case, nanomaterial-based sensors would selectively bind to the intended target, without interfering with the underlying biological process, and produce a strongly amplified signal which could be detected with commercially available detection technology. An inherent advantage of nano-sized materials as sensors, in general, is that the devices based upon such materials probe a much smaller volume or surface area than macro- or even micro-sensors. Thus, the total amount of analyte required to produce a measurable transduction signal is significantly lower, and so the limit of detection is (sometimes) greatly lowered.

How are nanomaterials utilized for biosensing applications? This is a complex question to answer, as innovative paradigm shifts are being constantly developed, but some general principles can be elucidated. First, nanomaterials may be used as scaffolds/platforms upon which to immobilize various biomolecules which are the actual biosensing element. Thus biorecognition molecules such as antibodies, aptamers, nucleic acids, carbohydrates, proteins, lipids, and subunits of the preceding, may all be immobilized (absorbed, covalently linked, etc) onto nanomaterials. Second, nanomaterials may bring unique capabilities to transducer output (i.e., signal which is detected) compared to conventional transducer mechanisms. For example, carbon nanotubes (CNT) have unique electrical conducting activities, making them highly useful for electrical sensing applications such as field effect transistors, electrochemical sensors, etc. Third, homogeneous or solution phase sensing using nanoparticles which have been functionalized to both capture analyte and serve as a detection platform are also being developed. As opposed to detection on surfaces, solution sensing has the potential advantage of more rapid and efficient kinetics in terms of analyte binding. For such applications, quantum dots have received much attention recently. Also, gold nanoparticles show unique optical changes depending upon degree of aggregation, thus forming the basis for colorimetric sensing applications. Gold nanoparticles provide ready-made surfaces for SPR sensing, while gold nanorods can serve as interdigitating electrodes for electrical sensing applications. Nanoparticles can also serve as intermediaries to increase sensitivity of detection in such sensing devices as QCM's and micro-cantilevers. Here the nanoparticles, appropriately derivitized so as to bind to a specific analyte, add further mass to the microbalance or cantilever, thus hopefully increasing the sensitivity without sacrificing selectivity.

Third, nanomaterials, by virtue of their size, are potentially very attractive intracellular biosensing probes. Entry into the cell can be a problem, but not necessarily always is. For instance, cells may endocytose nanomaterials, particularly if they are decorated with biomolecules or bio-like-molecules. Once inside, the nanomaterials can provide unique advantages for sensing applications, for example, the outstanding fluorescence intensity of the quantum dots. Further, biomaterials (nucleic acids for example) show increased stability and protection from intracellular degradation when coupled to nanomaterials. Some studies have indicated that the nanomaterials have little or no toxicity upon intracellular introduction, but this issue still needs further investigation. Coating the nanoparticle with an inert or biocompatible matrix has been investigated as an approach to mitigate any toxicity issues. Finally, nanomaterials may serve as molecular barcodes, that is, as identification elements which can serve to uniquely tag specific analytes. As such, they can provide multiplexing capabilities for bioassays. We now provide some examples, by no means comprehensive, of how nanomaterials have been utilized in biosensing applications.

Nanomaterials as Biosensors: Examples of Nanofiber-Based Systems as Integrated Biosensor Devices

An example of a nanomaterial biosensor that realizes all three of these criteria, i.e., molecular scaffolding, integrated transduction mechanisms, and intracellular deployment, is the vertically-aligned carbon nanofiber or VACNF. The VACNF is a unique subclass of carbon-based nanomaterials whereby plasma enhanced chemical vapor deposition is used to synthesize vertically-aligned high aspect ratio carbon spikes on a variety of growth substrates. VACNFs are compatible with most microfabrication processes including chemical and physical deposition techniques, chemical and physical etch processes, lithographic patterning, chemical/mechanical polish, etc., and thereby can be incorporated into complex multifunctional devices featuring VACNFs as nanoscopic components of mesoscale systems. The physical, chemical, and morphological characteristics of VACNFs provides a nanostructured element well suited for biosensor applications.

VACNFs are formed of stacked cones of graphitic carbon 'cups' that can either be directly functionalized to provide scaffolding for biomolecular reagents, or modified using various chemical or microfabrication techniques to enable indirect functionalization strategies. McKnight et al. (2006) demonstrated a variety of direct functionalization strategies that could be spatially defined using photoresist masking laterally across the surface of VACNF-array populated samples, as well as vertically along the height of these vertically-aligned elements. EDAC chemistry (1-ethyl-3-(3-diaminopropyl)carbodiimide) was used to spatially modify exposed VACNFs and VACNF-tips emergent above a photoresist layer with a variety of biomolecular species including DNA, streptavidin, and biotin. The latter could be used to capture streptavidin conjugated quantum dots, FITC-streptavidin, and peroxidase avidin. VACNFs have also been surface modified for subsequent functionalization, acting as scaffolds for example for deposition of

electron-beam evaporated gold films, whereby only the extreme tips of nanofibers emergent above a photoresist layer were gold modified, and later used for thiol affinity capture of active enzymes, nucleic acid probes, and full length copies of linearized or circular plasmid DNAs.

The microfabrication compatibility of VACNFs have also made them attractive candidates as fully-integrated transduction elements in biosensor devices. Using lithographic techniques, individual VACNF elements, or arrays of multiple high aspect ratio VACNFs, can be integrated into electrically addressable electrode systems, whereby the high aspect ratio carbon spike or even just its extreme tip can be deployed as the active element of a much larger mesoscale device. Guillorn et al. (2002) demonstrated the first integration of an electrically addressable array of individual VACNFs in a microfabricated device, verifying the electrochemical performance of individual spike tips using cyclic voltammetry of a redox active couple, ruthenium hexamine trichloride. Subsequent efforts have demonstrated site specific functionalization of individually addressed VACNF elements, such as via the electrodeposition of polypyrrole (pPy) and enzyme doped pPy-films; site specific transduction of molecular events occurring at nanofiber tips, including the exocytotic bursts of neurotransmitters from nearby cultured cells (McKnight et al., 2006); and electrophysiological interrogation and stimulus of organotypically cultured hippocampal slices (Yu et al., 2007).

Perhaps the most exciting aspect of VACNF-based devices is the ability to incorporate the high-aspect ratio carbon spike, and spike arrays, as intracellular elements that can be assimilated into the working microenvironment of individual or multiple cells. McKnight et al. (2003) demonstrated that when functionalized with covalently bound plasmid DNA, VACNF spike arrays could be introduced into the intracellular domains of many cells in parallel, and that cellular survival was maintained, even over long time periods (22 days) with the residence of individual spikes being retained in some cells. Subsequent applications of this technique have been used for a variety of gene and macromolecular delivery applications, including introduction and expression of covalently tethered DNA, releasable DNA cargoes using disulfide linkages cleaved via intracellular esterase activity, and protein localization and mislocalization studies via delivery and expression of fluorescent protein labelled gene products. Intriguing aspects of these demonstrations include the potential for achieving a genetic modification that is non-inheritable and whose extent in time can be directly and precisely controlled, and the potential of VACNF arrays as an intracellular interface for monitoring and controlling subcellular and molecular phenomena within viable cells for applications including biosensors, in vivo diagnostics, and in vivo logic devices.

Nanowires and Nanocrystals as Biosensors

The configuration of semiconductor nanowires (NW) as field effect transistors (FET) has opened new horizons in highly sensitive biosensing. In this arrangement, a single NW (for example, a silicon nanowire or a carbon nanotube) is placed between two patterned microscale contact pads, one of which is the source and one of which is the drain, in analogy to FET's. Binding of analytes to NW surface (either directly or more commonly via bioreceptor) produces a change in the electric field or potential at the NW surface, which in turn, results in a change in conductivity. Silicon NW's can have diameters as small as 2–3 nm. These small structures, on the same order of size as macromolecules, have a tremendous advantage over macro sensors because a smaller number of analyte molecules are able to produce a measurable change in NW electrical properties, due to the wire's nano-dimensional size. The binding of macromolecules to the NW surface can lead to depletion or accumulation of charge carriers over the bulk of the NW (thus changing the conductance), while with a planar FET, only the surface region of the transistor is affected. Commonly, as mentioned previously, the NW's are modified with biorecognition molecules which can bind the analyte of interest. In the case of silicon NW, the oxide layer that forms on the surface facilitates derivatization. When the NW FET is exposed to a solution of the analyte, binding takes place, and depending upon the net charge of the analyte in aqueous solution, either an increase or decrease in net negative NW surface charge takes place, with a corresponding increase or decrease in conductance, which is detected by measuring current flow between the electrical contacts. A potentially very important advantage of NW FET's is the fact that no labeling of the analyte or bioreceptor is needed for successful detection to take place. In principle, the concept of NW FET's is straightforward. One important consideration for successful functioning is to insure the use of aqueous solutions of low ionic strength, since high ionic strength buffers or biological solutions of high ionic strength interfere with the conductance. Several examples of biosensing applications of NW FET's follow. Reviews of this area have been published by Wanekaya et al. (2006) and Patolsky et al. (2006). Silicon is not the only material of use for nanowire FET's. Metal oxide nanowires have been used with success, such as ZnO, IrO_2 and In_2O_3. Further, conducting polymers have also been used, as have carbon nanotubes. Reproducible integration of these other materials with the electrodes is one of the present difficulties to their more widespread use.

Both proteins and nucleic acids, being generally charged in aqueous solutions, are readily detected by NW FET's. If peptide nucleic acid (PNA) sequences which are complementary to the DNA sequence of interest are attached to the NW, then hybridization of the polyanionic analyte DNA to the PNA can be detected. Importantly, PNA's need to be used as the bioreceptor, since they are uncharged. Direct electrical detection of single stranded DNA was possible down to at least 10 fM levels. Also, the NW FET could be used to detect single base-pair mutations. Thus, NW's modified with the complement of the wild-type sequence for one mutation site in the cystic fibrosis transmembrane receptor gene showed conductance changes in the FET upon hybridization to the wild-type gene sequence, while the sequence bearing the delta F 508 mutation would not hybridize with the complementary probe for the wild-type sequence and thus did not show a change in conductance. (The above work was reported in Hahm and Lieber, 2004.) Proteins are equally amenable to detection. Thus, for example, detection of PSA (prostate-specific antigen) was carried out using NW elements which had antibody to PSA immobilized on their surfaces. Detection was possible even with PSA concentrations of $\sim$2 fM.

Experiments with a different protein such as BSA or PSA pre-reacted with antibody to PSA showed no change in conductivity. It is also possible to construct NW arrays, to allow multiplexed detection of marker proteins, such as would potentially be of great value in defining cancer diagnosis. Patolsky et al. (2006) demonstrate this with an array of NW's which detected PSA, carcinoembryonic antigen and mucin-1 simultaneously, in real-time, at high sensitivity (femtomolar) and with essentially 100% selectivity. Interestingly, this same research group showed (by extensive and careful experiments) that a single virus particle could be detected on a NW FET in which the NW was modified with a monoclonal antibody against the virus. It appears that the future of NW-based FET's is bright considering their advantages, including direct, real-time, label-free electrical detection, coupled with great sensitivity and selectivity, along with the potential for development of integrated arrays (Patolsky et al., 2006).

Carbon nanotubes (CNT) can also be used as NW elements in FET's. Advantages over semi-conductor (i.e., silicon) NW include the fact that single –walled nanotubes have higher electron mobilities and diameters in the sub-nm range, making it possible to detect lower charge densities. On the other hand, the synthesis of carbon nanotubes is more difficult to precisely control as compared to synthesis of nanowires. Wang (2005) provides a review of this area. A particular advantage of CNT for electrochemical sensing is that it has been found that CNT can promote electron transfer reactions, particularly for redox enzymes, and can enhance the electrochemical reactivity of a number of biomolecules. For example, multiwall CNT carbon nanofiber arrays vertically aligned on an underlying electrode, act as molecular wires, which allow electrical communication between the electrode and redox enzymes immobilized on the CNT's. A variety of electrochemical techniques have been employed to detect glucose using CNT's as part of the electrode. Beside enzymes such as glucose oxidase, CNT's have also been used as facilitators for dehydrogenase reactions, as well as peroxidase and catalase reactions. CNT have also been utilized for electrochemical detection of DNA, that is, hybridizations to capture DNA molecules immobilized on electrode surfaces. Here again, vertically aligned CNT arrays have been found to be of great utility and can produce extremely sensitive analytic results.

The above discussion does not exhaust the types of nanomaterials being employed for biosensor use with electrical detection. For example, ingenious detection schemes for nucleic acids have been developed wherein gold microelectrodes were fabricated on silicon wafers, capture DNA strands were immobilized in the electrode gaps, and target DNA was captured within the electrode gaps. Then gold nanoparticles with an oligonucleotide complementary to the unhybridized end of the target DNA were added, hybridization took place, and gold nanoparticles were now immobilized between the electrodes. Subsequent coating with a silver layer increased electrical conductivity and sensitivity of the assay and lower limits of detection in the fM region were attained.

Kar and Shankar (2013) review the application of aligned one-dimensional arrays of nanotubes and nanowires constructed of various semiconductors. They provide a table comparing detection sensitivity for some common bio-analytes using both these types of structures (nanotubes and nanowires) and other transducers. For each analyte listed, the semiconductor nano-arrays showed better sensitivity of detection compared to other devices. Sensing techniques used included fluorescence, electrochemical, and FET. The increased sensitivity was attributed to greater efficiency of binding analyte/biorecognition element to the nanoroughened surface, along with increased sensitivity of transduction. The authors also provide a discussion of how these nano-arrays can be implemented for fluorescence, electrochemical, FET, and nanomechanical biosensing.

Quantum Dots – Nanoparticle Fluorescence

The development of semiconductor nanocrystals, called quantum dots (QD's), has had great impact upon the applications of fluorescence sensing for biosenors. The inherent weaknesses of organic molecule fluorophores can be largely overcome by these fluorescent nanoparticles. Quantum dots (typically CdSe, CdTe, CdS) have broad excitation spectra and also feature very narrow emission bandwidths, which can be tuned to various wavelengths depending on the size of the nanoparticle. A further advantage is good chemical stability. The name quantum dot is somewhat of a misnomer, as the structures are actually light-emitting semiconductor nanocrystals. The usual size of QD's are 2–10 nm. As the QD size increases, the emitted luminescence shifts to longer wavelengths. Among the advantages of quantum dots are size-tuned fluorescence emission, photostability (minimal photobleaching), high quantum yield, and the narrow, symmetrical emission profiles, thus facilitating multiple analyte detection in a given sample. The narrow emission profiles of QD's also facilitate their use in multiplexed assays and in FRET-based sensors. The very broad excitation spectral profiles of QD's allows excitation at a wavelength far removed ($>$100 nm) from emission maxima, so that multiple QD's with different fluorescent emissions can be excited with just one excitation wavelength.

The most common QD composition is CdSe, which forms the core, and has a thin shell of some other composition, frequently ZnS. The ZnS outer shell can facilitate conjugation of various molecules to the QD surface. Another strategy to link molecules to QD surfaces is to use a polyhistidine (usually 6 histidine residues) which coordinates to the Zn ions in the shell. Because of continuing concerns about the cellular toxicity of Cd, however, other semiconductor elements are beginning to be investigated for QD usage. Thus, InP/ZnS (core/shell) have been found to induce less oxidative stress genes in cells in culture than the usual Cd Se/ZnS QD's.

Modification of the surfaces of quantum dots is usually necessary to produce sites for biomolecules to bind, as quantum dots, in general, are hydrophobic after synthesis. Modification of the QD surface with biorecognition elements can be problematic both as regards orientation of the biorecognition element for most efficient target binding, as well as density of decoration on the QD surface. Peptide modification of QD surfaces has attractive features, since peptides can, in many instances retain the key biological activity desired for biosensing purposes, as well as retaining a separation distance between donor and acceptor molecules which can be compatible with FRET or BRET, a constraint which can be a problem with full-sized proteins. Nagy et al. (2014) provide a review of various biosensing applications of QD's conjugated with peptides. One of the commonest uses is as protease sensors, where the QD is conjugated to a peptidyl sequence which is a target sequence for a specific protease, and the distal end of the peptide sequence

has an acceptor fluorophore. If the peptide sequence is intact, the fluorescence of the QD is quenched via FRET, but when protease action cleaves the peptide at the target site, the QD recovers its fluorescence emission due to the acceptor being now separated from the QD. The same principle operates if, instead of an acceptor fluorophore, a Gold Nanoparticle (GNP) is attached to the end of the peptide, causing quenching via FRET or an organic quencher is used. Examples of proteases detected by this technique include caspases, serine proteases, metalloproteinases, and collagenase. It has been noted that GNP/QD FRET-based systems seem to, at least in some systems, produce better sensitivities of detection compared to QD/organic dye pairs, perhaps due to nanosurface energy transfer. Multiplexed assays of several proteases have been demonstrated using QD's with different emissions. Also, QD's can serve as the acceptor molecule rather than the donor in a FRET pair, and so donors like *Renilla* luciferase and mCherry protein have been used as donors in protease-type assays.

Besides proteases, protein kinase activities have been adapted to QD-based biosensing schemes. In these type assays, a similar FRET scheme is used as described above for proteases, but one of the FRET pair is an anti-phosphotyrosine (or anti-phosphoserine or anti-phosphothreonine) antibody coupled to a fluorophore, while the other member of the pair is a QD conjugated to a peptidyl substrate of the kinase being assayed. If the kinase phosphorylated the target peptide, the anti-phospho-antibody bound to the phosphorylation site, and FRET occurred, thus reducing the emission fluorescence of the QD. Other more complex assays have also been developed using QD's in which, for example, FRET 'relays' are produced where energy is transferred in turn from QD to one acceptor fluorophore and subsequently to another acceptor fluorophore.

Delivery of QD's intracellularly is of great interest, as the outstanding optical properties of QD's recommend them for a variety of intracellular sensing applications. Thus, investigations have focused on modifying QD's with cell-penetrating peptides, to facilitate cellular uptake. Uptake into the endosomal pathway seems to be a common theme, so that although the QD's indeed become intracellularly localized, they do not, in general, leave the endolysosomal pathway so that targeting to other intracellular locations is still not readily realized. Some examples of intracellular sensing using QD's include intracellular pH measurements and intracellular protease activity,

A very interesting application of QD's is their use as a type of optical barcode, which can be used as a sort of microchemical lab. To implement this concept, QD's of different sizes (and thus different colors in regard to emitted light) are incorporated in precisely defined ratios into a polymer microbead. The polymeric microbead is easily derivitized with biorecognition molecules, such as antibodies or nucleic acids, while each different bead species has a unique ratio of QD's. Thus, for each bead type having a unique biorecognition element, that same bead type will have a unique color/intensity code for facile target identification. The fact that all the QD's can be excited with one excitation wavelength makes this assay possible. The investigators who developed this assay found that the QD's embedded in the polymeric sphere were separated enough spatially that FRET did not occur. Han et al. (2001) estimate that at least 10,000 recognizable QD-based codes could be developed. The authors provide some bioassay results using this scheme.

The use of QD in combination with electrodes for sensing of analytes is a topic of recent interest. Quantum dots may be immobilzed on electrode surfaces, and then illuminated with light, which thus generates charge carriers and produces a current. Analytes at the electrode surface which can act as electron donors or acceptors can modify this current. An important point to consider in such an analysis is that only at the point at which light is illuminating the electrode do the reactions occur. Thus, if different biochemical systems are immobilized at spatially different sites, there are possibilities for multiplexing, when different sites are illuminated sequentially. There is another possible analysis which can take place in a system where QD's and electrodes are coupled. Here, if the electrochemical reactions taking place at the electrode surface generate light (electrochemiluminescence), then this light can be used as an excitation source to induce QD luminescence. Lisdat et al. (2013) provide a review of this developing field.

Quantum dots are not the only nanomaterials with intrinsic fluorescence properties. Graphene oxide also has intrinsic and tunable fluorescence. Fluorescent grapheme-based materials are also being investigated as sensors. Long et al. (2013) provide a brief overview of this subject.

Other Nanoparticle-Based Biosensing Modalities

Optical nanoparticle sensors for intracellular detection of a variety of analytes, called felicitously PEBBLES, have been developed by Kopelman's group. Most of these sensors are not strictly biosenors by the definition of this article since they do not involve a biomolecule as a recognition element. Nevertheless, the principles involved lend themselves to broader applications. The nanomaterials involved in these sensors include quantum dots, polymeric nanoparticles (i.e., organic molecule polymer or silica-based) as well as metallic nanoparticles. The sensing element is generally an organic fluorophore, or in the case of quantum dots, the nanoparticle itself. A unique aspect of these sensor nanosystems is the inclusion of a reference fluorescent dye, along with the detecting fluorophore, which allows some measure of quantification. Another research group has developed a maltose biosensor using a maltose-binding protein as a receptor attached to a quantum dot. Maltose binding was detected by FRET changes, as a quencher molecule was displaced from the monosaccharide binding site.

Noble metal (Ag, Au) nanoparticles have also proven their utility for optics-based methods of detection. Gold nanoparticles (GNP) can be utilized as part of a FRET pair by virtue of their fluorescence quenching properties, and they can also participate in direct colorimetric assays, based upon color changes between the aggregated and non-aggregated states of GNP's of appropriate size. If gold nanoparticles are surface-modified with biorecognition elements such that presence of an analyte produces aggregation of the nanoparticles, the resulting color change can be a simple visual assay. Thus, by attaching complementary DNA oligomers to two

separate batches of nanoparticles, the hybridization event could be detected by a change in color of the solution. Interestingly, the melting point profile for the hybridized DNA oligomers attached to nanoparticles showed very sharp transitions, apparently due to the nanoparticle attachments. Thus, due to the sharp transitions in melting temperature, it was possible to adjust the sensitivity of this assay so that single base pair mismatches could be detected. To detect specific nucleic acid sequences (target sequences), an assay format was developed in which capture oligonucleotides complementary to part of the target sequence were immobilized to a solid substrate, and gold nanoparticles derivitized with oligonucleotides complementary to another portion of the target formed a sandwich, binding the target sequence between them. To increase sensitivity, a silver coating (so-called silver enhancement) was deposited upon the gold nanoparticles and the resulting surface was scanned in a flatbed scanner. This assay was found to be 100-fold more sensitive than an assay based upon conventional fluorophore techniques.

Gold nanoparticles have a variety of unique optical properties which recommend them for use in imaging and detection modalities. Investigators have developed striped metallic micron-length nanorod particles, termed nanobarcodes. Identification of the stripe pattern can be made by means of a microscope. Labeling these nanorods with various bioreceptors allows one to identify and quantify a number of biomolecule targets simultaneously. Mirkin's group have developed a technique, called nanoparticle-based bio-bar codes for ultrasensitive detection of proteins. Here two types of particles are used, one a magnetic particle which has a capture antibody attached to it, and another a gold nanoparticle with an antibody against another epitope of the antigen of interest attached to it. The antigen (protein) of interest is first captured by the magnetic particle, and subsequently the gold nanoparticle with second antibody also attaches to the antigen. The key to this assay is that the gold nanoparticle has also attached to it double-stranded (complementary) oligonucleotides, in which only one of the oligonucleotide strands is bound to the nanoparticle. Following separation by a magnetic field of the particle/particle complex from reactants and a dehybridization step, the released oligonucleotide becomes a 'bio-bar code' specific for the protein of interest and also the means by which that particular protein is detected. Since many oligonucleotides can be bound to a single nanoparticle, there is an enormous potential for amplification. The released oligonucleotide strand can be readily detected by a variety of techniques such as the silver enhancement assay discussed above or PCR. The sensitivity of this method for detection of a clinically relevant protein (PSA) was found to be approximately 6 orders of magnitude better than standard immunological (ELISA) techniques.

Applications of carbon nanotubes (CNT) and other carbon-based nanostructures to novel biosensing strategies continue to grow. A variety of properties of CNT's recommend them for sensing functions. Because of electron or energy transfer, single walled CNT's can serve as very effective quenchers for a variety of fluorophores. Thus, single walled CNT's have been substituted for organic molecules used as quenchers in molecular beacons, and have better quenching efficiency, low background and high signal-to-noise ratio. Other analytical arrangements in which single or double-stranded nucleic acids are linked to single walled CNT's have also been explored as platforms for optical sensing. Aptamer-based molecular beacons for protein sensing can also be linked to single walled CNT's. Single walled CNT's can also be functionalized with several different probes for multiplexed sensing. Single walled CNT's have also been used as transducing elements with a thrombin-specific aptamer, but in this case the CNT was employed as a field effect transistor. CNT's have also been used as electrochemical sensors with applications to a wide variety of analytes. The features of CNT's which make them most suitable for these applications include high electrical conductivity, rapid electrode kinetics, chemical stability and mechanical strength. Also, a biosensor based upon antibodies immobilized on CNT's for detection of specific antigens has been described in which electrochemical sensing is the detection endpoint.

Nanoparticles (NP's) of various descriptions continue to receive much attention as potential biosensing and imaging agents. This review is not, per se, directed to imaging agents and techniques. We highlight here two recent interesting applications of nanoparticles as examples of novel concepts, but emphasize there are many more techniques being developed. Von Maltzahn et al. (2011) developed a NP-based system which consisted of a so-called signaling module and a receiving module, with a natural amplifying step, which was the clot-forming (coagulation) cascade, ending with the formation of fibrin. The signaling module employed gold nanorods or an engineered human protein, designed to detect host vessels for tumor receptors. In the case of gold nanorods, there was passive targeting of tumor tissue, and the clotting mechanism could be activated at the tumor site by using near IR energy (laser light) which was transduced by the nanoparticles into heat. The localized heating disrupted the tumor vasculature, generating the clotting mechanism. Magnetic nanoworms were then derivatized with molecules which provided specific targeting to the elements of the fibrin clot previously generated. The presence of the nanoworms in the tumor tissue was detected by MRI. Using these strategies, it was found that there was up to a 40-fold enhancement in accumulation of the receiving module (magnetic nanoworms) using the coagulation cascade, as compared to accumulation in the absence of the cascade. This is a rather unique experimental system, using as it does, both natural (coagulation) and artificial (nanoparticles) components in which the artificial input (signaling module) and artificial output (i.e., magnetic nanoworms – 'receiving module') are joined to a natural biological pathway.

Ghosh et al. (2012) have described another unique system in which a bacteriophage (a natural biological entity) is used as a carrier of nanoparticles to deliver larger amounts of imaging agent, via specific targeting, to tumor targets. In this case, the key to success is the fact that the bacteriophage M13 can be genetically manipulated so that the coat proteins can be modified by genetic engineering for either foreign (that is, to the bacteriophage) peptide or protein display. In this case, one of the coat proteins (p3) is genetically modified so that it has a targeting ligand for a specific protein associated with various cancers. Another coat protein (p8) is genetically modified so that this protein has a net negative charge, and thus binds strongly magnetic iron NP's with net positive charge. Since the M13 bacteriophage is filamentous, the magnetic NP's, which are attached to the p8 coat in a longitudinal manner, align along the long axis of the phage with a length of ~800 nm, the approximate length of the phage. Approximately 26 MNP could be bound per phage. The p3 coat protein, on the other hand is located at one end of the phage, so that the tumor

recognition site and nanoparticle binding sites are spatially separated, potentially avoiding problems with either targeting molecule or imaging moiety functionality. Thus the engineered phage, carrying the MRI-imaging NP's can bind specifically to tumor sites by virtue of its targeting peptides on one end of the phage. This is a paradigm shift in terms of attempts to target NP's to tumor sites, since the usual approach is to derivatize NP's with multiple targeting ligands, for improved binding. Here, by combining the carrying capacity of the phage for imaging/detecting NP's it is possible to achieve multiple detector agents per every phage which binds to tumor-specific targets. Ghosh et al. (2012) further demonstrate the effectiveness of this system by demonstrating selective targeting in vivo, as well as improved targeting in vitro, compared to magnetic NP's which were derivatized with the same targeting peptide as was used for the ligand attached to coat protein p3 in the modified phage.

The list of applications in which nanomaterials are playing a crucial role could go on and on. This short summary will, perhaps, provide a flavor of the scope of the potential uses of nanomaterials. As more nanomaterials are developed, with more unique properties, the list of biosensor applications can only grow.

Concluding Remarks

The above discussion of biosensors shows that this is an emerging field that has not by any means reached its full potential. During preparation of this article, the authors reviewed many research papers with very ingenious ideas for biosensors. Unfortunately, the gap between biosensor prototypes that work in the laboratory and commercial biosensors which can see routine use in real-world environments is often still large. The many varied biorecognition elements, transducers, and detection strategies lead to a wealth of potential applications. The field of biosensor research/applications is still probably in the stage where it is not clear what biorecognition elements/transducers/detection schemes will be most productive, and indeed, this may well continue to evolve in different directions as techniques like MIP become more established. Also, the large number of potential applications may well demand a quite varied armamentarium of biosensor techniques. In general, robustness of the biorecognition element continues to be a concern for many applications. The use of transducers/detection mechanisms that involve sophisticated/expensive instrumentation also will preclude such devices from many routine applications. Simpler is not necessarily always better, but dipstick sensors for certain applications are attractive.

A few comments regarding two other areas which the authors of this article believe will have increasing importance in the field of biosensor applications in the future will close out this article. Array biosensors, biochips, labs-on-a-chip, that is, devices that permit multianalyte detection (multiplex analysis) and, ideally, that can integrate all processing steps into a microanalytical system, will certainly see increased interest in the future. Such integrated devices have a number of advantages, such as ability to assay large numbers of samples in minutes, savings of expensive reagents, and reduction in time for individual assay steps due to decreased assay volumes. Unfortunately, there are a number of obstacles still to be overcome with regard to these systems. Among these are such things as further development of microfluidics systems, sample preparation modules, sensitive detection modules, and robust assay methodologies. Another area receiving much recent interest is in the area of nanotechnology, more specifically the use of nanomaterials such as nanoparticles in biosensor development. Nanoparticles do not serve as biorecognition elements *per se*, but can be modified by attachment of appropriate molecular species (e.g., antibodies and DNA fragments) to become nanosized carriers of biorecognition elements which can also serve valuable transduction functions (i.e., magnetic properties and fluorescence). The unique properties of nanoparticles, that is, colloidal gold and quantum dots, also recommend them for novel sensing applications. A number of investigators have mentioned the potential use of nanoparticles, particularly quantum dots, as molecular bar codes. Metallic nanoshells and semiconductor quantum dots are highly promising markers for use in high throughput screening and barcoding, by virtue of spectral multiplexing. However, much work remains to be done before the full promise of these materials is applied to practical and useful biosensors.

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Bioterrorism

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Glossary

Adenopathy An enlargement involving lymph nodes.
Bioterrorism A deliberate use of biological agents (such as bacteria or viruses) or any other organisms as a weapon.
CDC Centers for Disease Control and Prevention.
Edema/oedema Synonym with swelling, it represents an abnormal accumulation of fluid in body cavities or beneath the skin.
Incubation period The period between exposure to a pathogen and the appearance of the first signs or symptoms.
Lethargy A lack of interest or energy to do something.
Necrosis A cell injury that can be caused by an infection, a trauma, or a toxin and results in cell death.
PCR Polymerase chain reaction.
Prophylaxis Protective or preventive treatment in the absence of a disease.
Septicemia An infection (often bacterial) of the blood.
Spore A single reproductive body, usually resistant to heat and desiccation, capable of giving rise to a new organism, characteristic of some bacteria and fungi.
WHO World Health Organization.
Zoonosis A disease that can be transmitted to humans from animals (directly or using a vector).

Introduction

Webster's dictionary defines bioterrorism as a deliberate attack using biological agents such as bacteria, viruses, or any other organisms as weapons of biological warfare. These agents are used to disrupt our way of living; spread fear; and destroy plant, animal, and human ecosystems. Biological agents can spread through the ambient air, potable water or food, and animals, and can be modified to increase their transmissibility into the environment or to make them more resistant to the antimicrobial agents. What are the different biological threats that clinicians and hospitals should be prepared to manage or should develop proactive emergency plans should a mass casualty event occur? What are the different published tools and guidelines to help the medical community to manage large numbers of sick and potentially contagious patients and the worried well visiting the emergency room at the same time? This article is intended to answer to those questions.

Bioterrorism is not an invention of the recent past. In the Middle Ages, *Yersinia pestis* was used as a biological weapon when soldiers threw plague-infected corpses over the walls of besieged cities, trying to infect and kill the enclosed population. Smallpox was used as a biological weapon in the sixteenth century. Cortez unintentionally imported smallpox to the New World and killed a large part of the Native American population. In 1754, the English intentionally used smallpox as a weapon of mass destruction, distributing infected blankets to Native Americans, decreasing the population by 50%. Additionally, in the last 50 years, five bioterrorism events have occurred, the latest episode being the 2001 release of anthrax spores through the US Postal Services. This event affected 22 people (11 pneumonia and 11 cutaneous infections – 7 confirmed and 4 suspected cases) in four states (Connecticut, Florida, New York, and New Jersey) and the District of Columbia (DC) resulting in five deaths but also in postexposure prophylaxis for more than 10 000 (Bush and Perez, 2012). Among the four other bioterrorism attacks that took place since the middle of the twentieth century, three were deliberately spread gastrointestinal (GI) pathogens in targeted populations (*Salmonella* Typhi; *Salmonella* Enteritidis; and *Shigella dysenteriae*). One involved four students exposed to an intentional massive dose of *Ascaris suum* ova leading to pulmonary symptoms and infiltrates (Ippolito et al., 2006). These examples remind us our vulnerability and the need to plan for appropriate responses to the possible agents that could be used by malicious terrorist groups or individuals.

The Classification of the Different Bioterrorism Agents

The Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) classifies bioterrorism agents into three categories (A, B, and C) according to the following characteristics: their ease of spread in the environment and the associated severity of the illness or mortality rate. Category A agents are the most threatening for public health and include organisms (or toxins) based on their ease of spread or person-to-person transmission, their high mortality rates, and potential for overwhelming impact to the public health

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(input of patients to the emergency department, social disruption, and public panic). The category A agents require robust public health preparedness to manage the resulting impact on public health and health-care infrastructure. Those agents considered category A are the highest priority for public health planning, implementation of surveillance, and diagnostic capacity development. Categories B and C follow with lower priorities. Category B agents are moderately easy to disseminate with moderate morbidity and lower associated mortality rates than those in category A. Finally, category C agents include emerging pathogens with the capacity of mass dissemination following laboratory modifications and are characterized by their availability, ease of production and dissemination, and potential for high morbidity or mortality.

Because specific guidelines have been especially developed mainly for the CDC category A agents, we will focus the attention of this article on these six diseases. Readers are invited to review the other articles of this encyclopedia to have more information about the diseases included in categories B and C.

CDC category A	Includes those agents that disseminate easily or where person-to-person transmission occurs and has high associated mortality rates with major public health impact. Public and social disruption may happen and require health authorities preparedness. ● Anthrax ● Botulism ● Plague ● Smallpox ● Tularemia ● Viral hemorrhagic fevers (e.g., Ebola, Marburg, Lassa, etc.)
CDC category B	Includes those agents that are moderately easy to disseminate and are associated with lower morbidity and mortality. ● Brucellosis ● Epsilon toxin of *Clostridium perfringens* ● Food safety threats ● Glanders ● Melioidosis ● Psittacosis ● Q fever ● Ricin toxin ● Staphylococcal enterotoxin B ● Typhus fever ● Viral encephalitis (alphavirus) ● Water safety threats (*Vibrio cholerae*, *Cryptosporidium parvum*)
CDC category C	Includes emerging pathogens with the capacity of mass dissemination following engineering and are characterized by their availability, ease of production and dissemination, and potential for high morbidity or mortality. ● Nipah virus ● Hantavirus

Adapted from the Centers for Disease Control and Prevention.

Category A Agents

Anthrax

Introduction and Microbiology

The Working Group on Civilian Biodefense identified Anthrax or the agent *Bacillus anthracis* as one of the most serious threats to a city or a country (Inglesby et al., 2002). In 1970, the World Health Organization (WHO) estimated that 50 kg of *B. anthracis* released over a city of 5 million people would probably infect 250 000 and kill 2% of the population.

Bacillus anthracis is a 4 × 1 μm Gram-positive, facultative anaerobic or aerobic spore-forming bacillus that stains as a dark blue or violet rod usually in chains when the endospore forms with a central or subterminal location. *Bacillus anthracis* grows well on sheep blood agar and colonies are typically white to gray-white, flat with a curly tail, or a 'Medusa's head' appearance at the edge. *Bacillus anthracis* is nonhemolytic, nonmotile, catalase-positive, and classically susceptible to penicillin. The main virulence factors of *B. anthracis* are its capsule and two binary toxins called the 'edema factor' and the 'lethal factor.' The release of these toxins leads to edema, hemorrhage, and necrosis of the tissues (Inglesby et al., 2002). Epidemiologically, the bacterium is naturally found in the environment and is primarily associated with diseases in animals. It is usually transmitted to humans following contacts with infected cattle, camels, goats, or other herbivores. Hence, it was commonly called the 'woolsorter's disease' or 'ragpicker's disease' reflecting the association described with the wool of infected animals. The spores can also survive for months in the environment. Of note, as exemplified by the events in the fall of 2001, anthrax can also be acquired following malicious intention if it is used as a bioterrorism weapon (Bush and Perez, 2012).

The WHO, in collaboration with the Louisiana State University, maintains detailed data cataloging endemic anthrax in animals by country (accessible at http://www.vetmed.lsu.edu/whocc/mp_world.htm). Most countries present sporadic or low endemic animal cases but hyperendemic areas are reported in Africa (including Guinea, Sierra Leone, Liberia, Cote-D'Ivoire, Ghana, Togo, Niger, Chad, Zambia, Zimbabwe, and Ethiopia), Turkey, Tajikistan, and Myanmar.

Clinical Manifestations and Diagnosis

There are four clinical forms of the disease: cutaneous, GI, inhalational, and meningoencephalitis. All these forms could be encountered following a bioterrorist attack.

Cutaneous Anthrax

Cutaneous anthrax is the most frequent form (95%) of the disease in humans and occurs following direct skin contact when handling any contaminated source, mostly of animal origin like wool, hair, hides, or fur; the bacteria or its spores then may enter the skin through an abrasion. The incubation period varies between 1 and 12 days. The initial lesion is characterized by a painless, but typically, pruritic sore with a central vesicular or bullous lesion and peripheral edema. The central lesion further develops into an ulcer with a hemorrhagic and necrotic center followed by formation of a black eschar. If untreated, 20% of patients will die of cutaneous anthrax (Inglesby et al., 2002). When treated, the mortality rate is reduced to less than 1%.

Gastrointestinal anthrax

Gastrointestinal anthrax is a rare form of the disease (<5%) and follows consumption of contaminated fluid or undercooked infected meat and has two clinical presentations: oropharyngeal and intestinal, the latter being more frequent. The first syndrome is characterized by the development of cervical adenopathy and soft tissue edema, fever, oral lesions followed by ulceration and central necrosis after the consumption of infected food (Inglesby et al., 2002). The second form has three phases: initially, patients develop low-grade fevers, malaise, and fainting followed by mild to severe abdominal pain, nausea, vomiting, mild diarrhea, ascites, intravascular depletion, and abdominal distension. During the third phase, patients show paroxysmal abdominal pain, increasing ascites, and often shock (Kanafani et al., 2003). Surgical interventions and antimicrobial treatment may benefit those with a severe clinical presentation.

Inhalational anthrax

Inhalational anthrax follows inhalation of airborne spores from air or spores found in the fur or wool of an infected animal. It should always raise the concerns of a bioterrorist attack. The incubation period is generally 9–10 days but ranges from 2 to 98 days. The typical picture without treatment includes hemorrhagic mediastinal adenopathy and/or pleural effusions and a rapid evolution to death. As it is the case with the GI form, the inhalational anthrax can present in a two-stage fashion with an initial flulike syndrome (low-grade fever, malaise, myalgia, headache) followed by respiratory, neurologic, GI, and cardiovascular compromise (Inglesby et al., 2002). Meningitis or meningoencephalitis forms have 95% mortality whether or not the patient is adequately treated. This form of the disease is also characterized by hemorrhagic changes of the meninges due to invasion of blood and the cerebrospinal fluid (CSF) by the bacteria.

Confirmatory testing should always be performed at a reference laboratory. Suspected cultures in a clinical lab should be transferred to a biosafety level (BL) 3 laboratory for further analysis. The CDC have issued guidelines for testing clinical samples when a clinical suspicion of anthrax is raised that included culture, immunohistochemistry, serology, and polymerase chain reaction (PCR) testing. For example, in case of a cutaneous disease, the CDC recommend that samples (e.g., vesicular fluid from an unopened vesicle, swab samples from underneath the eschar, or base of ulcer) are obtained for both culture and PCR. Blood should be cultured and acute and convalescent serology obtained. Biopsy specimens are also recommended for the papule or vesicle including adjacent skin. The recommended testing analyses for inhalational or GI anthrax are available on the CDC Web site (http://emergency.cdc.gov/agent/anthrax/lab-testing/).

Treatment

If anthrax is suspected, treatment should be promptly initiated using intravenous treatment initially. For adult patients including pregnant women, the treatment of choice for inhalational, GI, or oropharyngeal anthrax should combine either ciprofloxacin (400 mg IV every 12 h) or levofloxacin (500–750 mg IV every 24 h) or doxycycline (100 mg IV every 12 h) with at least one more agent including clindamycin (900 mg IV every 8 h) or rifampin (300 mg IV every 12 h). Clindamycin is thought to block toxin production and rifampin penetrates well different tissues including the CSF. Other possible combination regimens include imipenem, clarithromycin, vancomycin, or chloramphenicol (Inglesby et al., 2002). Furthermore, if the strain is reported susceptible to penicillin, this agent may also be used. Cephalosporins should not be prescribed. According to some laboratory data on 53 B. anthracis strains isolated in Turkey, all were susceptible to ciprofloxacin, levofloxacin, tigecycline, linezolid, and vancomycin (Ortatatli et al., 2012).

Children should also be prescribed the same regimen with adjusted doses (see http://emergency.cdc.gov/agent/anthrax/). A switch to oral route, once the clinical presentation improves is indicated with a total duration of antimicrobials of 60 days. Steroids may be considered as adjunct therapy for those with severe edema or meningitis.

For cutaneous disease, treatment is usually 7–10 days but in a bioterrorism situation, where the risk of inhalation is greater, 60 days are also recommended with either ciprofloxacin or levofloxacin for both adults (including pregnant women) or children and doxycycline as an alternative treatment.

Infection Prevention Strategies

Inhalational anthrax is not spread from person to person and does not necessitate specific infection control practices other than the standard precautions. There is a theoretical risk of transmission to health-care personnel in case of cutaneous anthrax and gloves are

then required. In the case of an attack exposure, any person in direct physical contact with the agent containing the potential spores should readily and profusely wash the exposed skin and pieces of clothing with soap and water. Also, only those deemed directly exposed to the contaminated source would require postexposure prophylaxis. Decontamination of the environment should follow the usual hospital protocol. In some cases additional decontamination of the environment may be needed and should include an agent with activity against spores. Cases must be reported to public health authorities immediately and the laboratory should be notified when a suspected case is admitted to the hospital in order to put in place the adequate laboratory practices.

Vaccination

AVA vaccine (BioThrax) is available in the United States following a 5 dose-preexposure regimen with annual booster or a 3 dose-postexposure regimen. This vaccine is not available for the general public and is intended to at-risk people only. Preand postexposure protocols exist and the vaccine was shown effective against inhalational anthrax when combined to the right postexposure antimicrobial prophylaxis. Readers are referred to the Advisory Committee for Immunization Practices (http://www.cdc.gov/vaccines/pubs/ACIP-list.htm) for more details about the specific AVA vaccine recommendations.

Postexposure Prophylaxis

Given the current data on inhalational anthrax, three doses of AVA vaccine administered at time 0, 2, and 4 weeks along with 60 days of oral antimicrobial prophylaxis (ciprofloxacin or doxycycline) for those exposed to *B. anthracis* is the treatment of choice. Levofloxacin is also FDA-approved for chemoprophylaxis but safety data for therapies longer than 28 days are lacking and should be considered as a second-line choice. Penicillin G (FDA-approved) or amoxicillin (not approved by FDA) are effective but cannot be used if beta-lactamase production by the *Bacillus* strains is reported (Inglesby et al., 2002). Penicillin derivatives should be used only when susceptibility data confirm their efficacy. Other drugs including clindamycin, chloramphenicol, rifampin, and vancomycin may be considered in those patients who cannot tolerate the first- and second-line therapies. Regimens have also been developed for special population including pregnant women and children where the risks associated with anthrax must be balanced against the side effects of the drug regimen.

Botulism

Introduction and Microbiology

Botulism is a disease caused by neurotoxins produced by the bacteria *Clostridium botulinum*, and rarely by *Clostridium butyricum* and *Clostridium baratii*. *Clostridium botulinum* is a Grampositive, spore-forming, obligate anaerobe bacillus that can be found in soil and marine sediments worldwide (CDC, 1998). Botulinum toxin is one of the most lethal substances, with an LD_{50} of 0.0001 μg kg^{-1}; as a result, 1 g of the crystallized toxin, evenly dispersed and inhaled, can kill more than 1 million people (Darling et al., 2002).

Seven different types of Botulinum toxins exist and are referred to as types A–G. Different intracellular protein targets occur for each serotype. The toxin causes flaccid paralysis of the muscles. Although spores of *C. botulinum* can survive for several hours at 100 °C, their toxins are heat labile, and can be inactivated after 5 min at 85 °C (Darling et al., 2002).

Before 1949, the case-fatality rate was approximately 60%. Mortality is lower when the disease is recognized and treated early, proper supportive and respiratory intensive care is provided, and there is an effective public health response (CDC, 1998).

Clinical Manifestations

Botulinum toxin can be introduced to the human body via the GI route (foodborne and infant botulism), the pulmonary route (inhalation botulism), and absorption through the bloodstream from infected wounds (wound botulism) (Arnon et al., 2001).

Clinical manifestations of all types of botulism are characterized by a classic triad: (1) lack of fever, (2) symmetric and descending flaccid paralysis with prominent bulbar palsies, and (3) intact sensorium. The prominent bulbar palsies may be summarized as the '4Ds': diplopia, dysarthria, dysphonia, and dysphagia (Arnon et al., 2001). Foodborne botulism may have GI symptoms such as nausea, vomiting, abdominal cramps, or diarrhea.

Foodborne Botulism

Botulism caused by the ingestion of botulinum toxincontaminated food and it is the most common type of botulism worldwide. Sources of contaminated food include improperly canned or preserved food. Among those foods, vegetables with low acid (pH > 4.6) such as beans, garlic, pepper, carrots, and corn are considered as those with the highest risk of contamination. Meat has been reported as another source of foodborne botulism including fermented foods, seal oil, salmon, and dried foods. In the United States, the toxin types represented in the 444 foodborne outbreaks from 1950 to 1996 were type A (37.6%), E (15.2%), B (13.7%), and F (0.7%). Approximately 33% were unidentified with respect to toxin type. Signs and symptoms of foodborne botulism can occur after 2–8 days following the exposure, depending on the rate and amount of toxin absorption. Average incubation period is 12–72 h after the implicated meal (CDC, 1998).

Inhalation Botulism

Aerosol dissemination of botulinum toxin can also cause the disease and may not be difficult to recognize because of a common temporal and geographical exposure among cases. Incubation period of inhalation botulism is not established. Onset of symptoms

was approximately 72 h after exposure in a few reported cases. Animal data show signs of the disease after 12–80 h of exposure (Dembek et al., 2007).

Infant Botulism

Infant botulism, while distinct from foodborne botulism, is one of the most common forms of botulism, yet it is unlikely to be linked to bioterrorism. Infant botulism develops after the ingestion of *C. botulinum* spores, not toxin, leading to *in vivo* toxin production in infants' intestines. In general, cases occurred after honey or corn syrup was fed to infants. Clinically, infant botulism starts with constipation, and is followed by neuromuscular paralysis. Disease severity is broad, and ranges from only lethargy to respiratory failure (CDC, 1998).

Diagnosis and Differential Diagnosis

Early recognition of botulism using clinical criteria is essential because a confirmatory test requires days to complete. Differential diagnosis includes polyradicular neuropathy (Guillain–Barré or Miller Fisher syndrome), myasthenia gravis, or intoxication (e.g., depressants, organophosphates, carbon monoxide, or nerve gas). Botulism can be distinguished from these diseases by a clear sensorium and its prominent cranial nerve palsies disproportionate to milder weakness and hypotonia below the neck. Epidemiological clues that include a careful travel and dietary history should be obtained from suspected patients. Contact the local public health authorities and hospital epidemiologist promptly to coordinate treatment, diagnostic needs, and the epidemiological investigation (Arnon et al., 2001).

Diagnostic Laboratory

Obtain clinical samples including preantitoxin treated serum ($\geq$30 ml in adult, less in children), stool, gastric aspirate, vomitus, and suspected food (if available). All clinical specimens have to be kept refrigerated after collection. In general, these tests are performed in a public health laboratory. The standard mouse bioassay is very sensitive, and detects more than 0.03 ng of botulinum toxin but the turnaround time is 1–2 days. The bioassay can also demonstrate a specific toxin (CDC, 1998).

An electromyogram with repetitive nerve stimulation at 20–50 Hz may differentiate botulism from other causes of acute flaccid paralysis. CSF analysis is usually normal in botulism (Dembek et al., 2007).

Treatment

Treatment for botulism requires supportive care and passive immunization with antitoxin. Prompt administration of antitoxin using recommended doses can neutralize circulatory botulinum toxin and minimize subsequent nerve damage (Dembek et al., 2007). Unfortunately, the antitoxin cannot reverse existent paralysis (Table 1). Skin tests should be performed prior to administrating these products as there is risk of hypersensitivity reactions to the equine components.

Infection Prevention Strategies

Botulism is not transmitted by person-to-person contacts. Isolation is not necessary and standard precautions are used. After aerosolized toxin exposure, decontaminate clothing and skin with soap and water. Contaminated objects should be cleaned with 0.1% hypochlorite solution (Arnon et al., 2001).

Prevention

Preexposure Prophylaxis

The pentavalent botulinum toxoid (PBT) against botulinum toxin types A–E has been used since 1959 but is not FDA approved. Because of its reactogenicity and declining of immunogenicity, PBT administration should only be considered in people who are at risk of botulism such as laboratory workers and military personnel. The CDC's current recommendation of PBT protocol requires a

Table 1 Antitoxin therapy

Product	*Serotype*	*FDA approved*	*Availability*
Monovalent equine antitoxin	A, B	–	WHO[a]
Bivalent equine antitoxin	A/B	Yes	CDC[b]
Monovalent equine antitoxin	E	No	CDC[b]
Heptavalent despeciated equine antitoxin	A–G	No	CDC[b], UDAMRIID[c]
Human botulism immune globulin (Baby-BIG)	A/B	Yes	CDHS[d]
Human botulinum immune globulin	E	No	CDHS[d]

[a]WHO indicates World Health Organization.

[b]CDC indicates Centers of Disease Control and Prevention.

[c]USAMRIID, US Army Medical Research Institute of Infectious Diseases.

[d]CDHS, California Department of Health Services.

Adapted from Jones, R.G., M.J., Corbel, et al., 2006. A review of WHO International Standards for botulinum antitoxins. Biologicals 34 (3), 223–226; Dembek, Z.F., Smith, L.A., et al., 2007. Botulism: cause, effects, diagnosis, clinical and laboratory identification, and treatment modalities. Disaster Med. Public Health Prep. 1 (2), 122–134.

primary series of four subcutaneous injections of 0.5 ml at 0, 2, 12, and 24 weeks, followed by a booster dose at 12 months and booster doses annually, thereafter. Antitoxin titers are not needed. Research to develop newer toxoids and recombinant vaccines is ongoing (Rusnak and Smith, 2009).

Postexposure Prophylaxis

PBT is not recommended for postexposure prophylaxis. Because a study in nonhuman primates demonstrated that exposed individuals may not develop disease, asymptomatic individuals who are exposed to botulinum toxin should not receive passive immunoprophylaxis (Darling et al., 2002). Data also demonstrate that botulinum immune globulin administered within the first 24 h after a high-dose exposure to the toxin is beneficial. The risk of potential adverse effects from equine antitoxin and the expected morbidity and mortality from the disease should be weighed (Arnon et al., 2001).

Plague

Introduction and Microbiology

Bioterrorism attacks using *Y. pestis* should be considered if many cases of severe pneumonia in critically ill previously healthy adults come to medical attention. *Yersinia pestis*, the causative agent of plague, is a member of the family *Enterobacteriaceae* and is another agent of great concern given the capacity for its mass production and dissemination, its high fatality rate, and the possibility of person-to-person transmission (Inglesby et al., 2000). The Black Death is one of three historically important outbreaks caused by plague. It decimated one-fourth of the European population in the Middle Ages. Plague is a zoonotic disease that is transmitted from infected animals to humans via either the bite of an infected flea, *Xenopsylla cheopis*, direct contact with or inhalation of infected particles or rarely following ingestion of contaminated material.

Plague remains endemic among small animals in many countries in Africa, Asia, the Americas, and the former Soviet Union, even today. In 2003, WHO reported 2118 cases and 182 deaths in nine countries. Among these reports, 98.7% of cases and 98.9% of deaths occurred in Africa. Most recently, Peru reported 17 cases (4 pneumonic plague, 12 bubonic plague, and 1 septicemic plague). Infected rodents and domestic cats were the source of the outbreak (http://www.who.int/csr/don/2010_08_10/en/index.html). Hence, plague remains a credible threat and a potential agent of bioterrorism.

Clinical Manifestations

Different plague syndromes have been described: bubonic plague, septicemic plague, pneumonic plague, and meningeal plague.

Bubonic Plague

The incubation period of bubonic plague is 2–8 days after the flea bite infected with *Y. pestis*. The organism initially proliferates in the regional lymph nodes. Clinical manifestations include sudden onset of fever, chills, malaise, headache, prostration, lethargy or agitation, seizures (in children), and shortly after an enlarged painful lymph node (bubo) develops in the affected region. Typically, the mass is firm, tender, and nonfluctuant and measures between 1 and 10 cm and the skin is usually warm and erythematous. The most common sites for painful lymphadenopathy include the groin, axillary, and cervical regions (Inglesby et al., 2000). In addition, GI symptoms including abdominal pain, nausea, vomiting, and diarrhea may precede the bubo formation. Of note, bubonic plague can rapidly evolve without appropriate treatment leading to shock and death within 2 or 3 days of symptom onset.

Septicemic Plague

Following the initial proliferation of organisms in the local lymph nodes, the organism can spread hematogenously. Primary septicemic plague may develop without a bubo and in this form, patients develop high fever without lymphadenopathy and can die rapidly. An inflammatory response can lead to disseminated intravascular coagulation and organ failure that precipitate death. Purpuric lesions on the trunk and limbs, associated with necrosis of acral parts of the body (finger tips, nose, and toes) are characteristic (Inglesby et al., 2000). As with bubonic plague, patients may initially present with digestive complaints.

Pneumonic Plague

Pneumonic plague develops following either hematogenous spread of the bacillus from the bubo (10% of cases) or inhalation of infectious droplets (primary inhalation pneumonia) following close contact with a case of symptomatic pneumonic plague. This disease form evolves rapidly and patients often develop septic shock and organ failure. Classic symptoms include dyspnea, tachypnea, chest pain with cough, and hemoptysis or watery sputum. Untreated, the disease is always fatal and mortality increases as the treatment is delayed (Inglesby et al., 2000). Recent cases of primary pneumonic plague can follow laboratory incidents or exposure to domestic cats with respiratory symptoms.

Meningeal Plague

Meningeal plague is usually a complication of untreated plague (bubonic) and manifests with symptoms of acute nervous system infections including headache, nuchal rigidity, fever, and meningismus (Inglesby et al., 2000). Cultures generally grow organisms and pleocytosis of the CSF is common. Rare GI manifestations of plague including pharyngitis can follow the ingestion of the bacillus.

Suspected clinical cases of plague should be reported diligently to the Public Health Authorities to initiate an investigation.

Diagnosis

Physicians should have a high level of suspicion when patients returning from a plague endemic zone report exposures (contact with fleas, animals, or infected people) and present with an acute febrile illness to the hospital. Elevated white blood cells and decreased platelet counts are frequent findings in the various disease forms. Blood should be cultured before antimicrobial treatment is initiated and the laboratory notified of the clinical suspicion as specimens are processed with BL2 protocols. Other specimens including bubo aspirates, sputum, broncho-alveolar lavage, pharyngeal swab, CSF, or swabs of skin lesions should be obtained if symptoms are present. *Yersinia* spp. grows well on different media used in the microbiology laboratory including brain–heart infusion broths, sheep blood agar, MacConkey agars, etc. Smears from bubo, sputum, blood, or central nervous fluid can be stained (Gram) to look for the characteristic bipolar-staining (safety pins) Gram-negative organisms. Other diagnostic methods include serologic testing (hemagglutination test, enzymelinked immunosorbent assays (ELISAs)), and PCR techniques are available in specialized laboratories.

Treatment

When the disease is suspected, cultures should be obtained immediately and antimicrobials started. Streptomycin is the treatment of choice (Inglesby et al., 2000) (7.5 mg kg^{-1} of body weight each 12 h) for 7 days. The dose should be adjusted in case of renal insufficiency and it should be used cautiously in pregnant women. Most of the patients become asymptomatic within a few days. While streptomycin is not commonly used now, gentamicin (5 mg kg^{-1} for loading dose then 1.7 mg kg^{-1} every 8 h for 10 days) or a combination of gentamicin (same doses) and doxycycline (100 mg IV or PO every 12 h) for 10 days are as effective as streptomycin. Doxycycline or tetracycline can be given orally following an initial intravenous regimen using an aminoglycoside. Ciprofloxacin 400 mg IV every 12 h (adjusted in case of renal insufficiency) or chloramphenicol 1 g IV four times a day for 10 days are alternative agents. For pregnant women risk and benefits must be balanced but gentamicin 5 mg kg^{-1} every 24 h for 10 days has been recommended with alternative treatments using ciprofloxacin or doxycycline for 10 days.

Infection Prevention Strategies

Pneumonic plague is spread through respiratory droplets. Hence, household members and health-care workers are at increased risk of acquisition if appropriate measures are not observed. Symptomatic patients should be placed in droplet precautions (medical mask, gown, gloves, eye protection). Usually, droplet precautions can be stopped following 48 h of appropriate therapy and with clinical improvement (Inglesby et al., 2000). Contact precautions are also used for patients with bubo.

Prophylaxis is indicated for those exposed and in close contact (2 m, not using barrier precautions) as in the household, work place, or at the hospital to a symptomatic contagious patient. The preferred prophylaxis regimen is doxycycline 100 mg PO BID for 7 days but some experts recommend ciprofloxacin 500 mg orally twice everyday or levofloxacin 750 mg orally twice everyday for 7 days. The same regimens apply for pregnant women although the risk benefit to the mother child must be weighed.

Smallpox

Introduction and Microbiology

Smallpox (*Poxvirus variolae*) is a serious, contagious, lifethreatening disease caused by a brick-shaped, double-strand DNA virus in the genus *Orthopoxvirus*. It is a credible biological weapon because of its high case-fatality rate, no established definitive treatment, and its possibility of transmission via aerosols (Cleri et al., 2006). WHO declared smallpox eradicated in 1980, which lead to abandonment of smallpox vaccination worldwide. With declining immunity in human populations and the lack of natural exposure to the disease, large populations are vulnerable to infection with this virus.

Clinical Manifestations

The average incubation period is 10–12 days. After the virus penetrates the respiratory epithelium, within 72 h, it is transported to regional lymph nodes by infected macrophages. Once it has replicated for 96 h in the lymphoid tissues, a second viremia develops and patients present with symptoms. Initial symptoms of the disease are nonspecific and include fever, malaise, head and body ache, and sometimes, vomiting. After 2–4 days of the symptoms, patients develop maculopapular rash that will become pustular lesions and finally scab. In the normal host, this maculopapular and vesiculopustular rash develops centrifugally (Henderson et al., 1999).

There are two major forms of smallpox: variola major and minor. Variola major is more virulent and contributes to higher case-fatality rate. This type is divided into four disease entities: ordinary (most frequent type); modified (vaccine-modified and relatively mild form); flat (malignant); and hemorrhagic (fulminate) (Cleri et al., 2006). The latter two forms are rare and more severe. Overall, the mortality of variola major is 30%. Variola minor is less common and much less severe disease. Its mortality is less than 1% (Veenema, 2003).

Smallpox needs to be distinguished from chicken pox (varicella). The clinical syndrome and febrile syndromes caused by both viruses overlap; symptoms, incubation periods, rash evolution, and rash presence on the palms and soles are similar (Cleri et al., 2006). Hence, confirmatory laboratory testing is crucial. Clinical specimens include the vesicular fluid for electron microscopic examination and viral culture that must be processed using BL4 protocols (Moran et al., 2008). Antibody testing

in patients can be measured in the convalescence period for confirmation but may not be helpful in the initial disease management.

Treatment

Treatment for smallpox is limited. No promising antiviral or immunotherapy has been proven to improve outcomes in patients with smallpox. Parenteral cidofovir is licensed for treatment of cytomegalovirus, and is effective against other viruses in the genus *Orthopoxvirus* (Moran et al., 2008). Its utility in smallpox is not known and the severity of the disease should be balanced with the drug's side effects. Supportive treatment and antibiotic therapy for superimposed bacterial infections are standard of care (Henderson et al., 1999).

Infection Prevention Strategies

The contagious period of smallpox occurs after symptoms are present. Data from observational studies have not demonstrated disease transmission during the incubation period. Viral shedding occurs in the first 10 days after patients develop the rash and is present until all scabs and crusts fall off. Smallpox can spread from person to person, primarily from infected droplet nuclei, or aerosol. Contact with the contaminated crusts, contaminated clothes, and bed linens of the patients can transmit the disease as well. It is widely acceptable that only a few virions can cause the disease (Henderson et al., 1999).

Because of infectious properties of the virus, infection control strategies include airborne and contact precautions. Patients should be isolated for both confirmed and suspected cases. Patients must be placed in negative pressure rooms and equipped with high-efficiency particulate air filtration if available. In settings with limited resources a room with large windows and extensive air movement is best. Contact precautions include the use of gloves, gowns; and fitted respirators (airborne) are also recommended. All household and face-to-face contacts must be vaccinated as soon as possible to prevent disease. Postexposure prophylaxis with vaccine is best administered within the first 4 days after exposure. In an epidemic, an emergency vaccination program is indicated for all front-line staff with a high risk of exposure including healthcare personnel working in health-care facilities that might receive patients, firemen who may handle infected bodies, or emergency management staff. An expert working group also recommended smallpox vaccination in all hospitalized patients during an outbreak setting. Those at risk of exposure with a contraindication to live vaccine may require vaccinia immune globulin (VIG) instead (Henderson et al., 1999).

Preexposure Prophylaxis: Vaccine and Its Adverse Events

Edward Jenner first developed smallpox vaccine in 1796 by inoculating the cowpox virus to a human. Since this time, the smallpox vaccine has evolved into a live attenuated virus that uses vaccinia virus, which is closely related to variola virus (Metzger and Mordmueller, 2007). Because of the eradication of smallpox and the risks of the vaccine that outweigh the benefits, the CDC do not recommend preexposure prophylaxis for general population. High-risk groups, which include laboratory workers directly exposed to smallpox virus or other virus in the genus, are still offered the vaccine (Wiser et al., 2007).

Preexposure prophylaxis is contraindicated in those with immunosuppression, HIV infection, eczema, and household or sexual contact with an individual with known contraindications. The vaccine is also contraindicated in pregnancy. The vaccine complications vary from self-limited to life-threatening conditions, such as postvaccination encephalitis, vaccinia necroforum or progressive vaccinia, and eczema vaccinatum, that occur most commonly in immunocompromised hosts and household contacts (Cono et al., 2003; Table 2).

Postexposure Prophylaxis

There are no absolute contraindications for postexposure prophylaxis and the risks must be carefully weighed with the benefits. For individuals with the contraindications mentioned above who have been in close contact with smallpox patients, VIG may be given simultaneously (0.3 ml kg^{-1} body weight) to prevent complications. In case that VIG is not available, vaccination is still useful because of higher risk of smallpox disease itself than from vaccine complications (Henderson et al., 1999).

Table 2 Complications associated with the smallpox (vaccinia) vaccine

Vaccination status	No. vaccinated	Postvaccinal encephalitis		Vaccinia necrosum		Eczema vaccinatum		Generalized vaccinia	Accidental infection	Other complications[a]
		Cases	Fatalities	Cases	Fatalities	Cases	Fatalities			
Primary vaccination	5.6 million	16	4	5	2	58	0	131	142	66
Revaccination	8.6 million	0	0	6	2	8	0	10	7	9
Contacts	Unknown	0	0	0	0	60	1	2	44	8

[a]Erythema multiforme, painful primary take, bacterial superinfection, miscellaneous rashes, and burns.

Adapted from Lane, J.M., Ruben, F.L., et al., 1969. Complications of smallpox vaccination, 1968. N. Engl. J. Med. 281 (22), 1201–1208.

Decontamination

Vaccinia virus has been used as a model with smallpox properties in certain circumstances. The virus can be destroyed relatively easily in the settings of high temperatures and humidity. For example, the virus is killed when exposed to temperatures of 31.5–33.5 °C, and 80–83% humidity for at least 6 h. Contaminated laundry used by patients should be autoclaved or laundered in hot water with bleach (0.05–1% sodium hypochlorite) (Kumar et al., 2010). Disinfectants currently used in hospitals, such as hypochlorite and quaternary ammonium compounds, are optimal for decontamination (Henderson et al., 1999).

New Vaccine Development

The search for new smallpox vaccines and formulation is being investigated actively because of the limitations in the existing vaccine and possible use of smallpox as bioweapon (Metzger and Mordmueller, 2007).

Tularemia

Introduction and Microbiology

The fifth member of CDC category A bioterrorism organisms is *Francisella tularensis*, the causative agent of tularemia. Tularemia is a zoonosis and transmission to humans occurs when a person is exposed to diseased animals or intentional spread. Tularemia has been included as a potential agent for years with modern beginnings in the United States biological warfare program in the 1950s and 1960s. Many clinical manifestations and infection routes are described (see below) but according to The Working Group on Civilian Biodefense, aerosolization would be the most likely method of spread in case of an intentional release (Dennis et al., 2001). The attack rates of aerosolized *F. tularensis* (82.5%) and its case mortality rate (6.2%) render the organism suitable for a large-scale attack.

The organism is a small, pleomorphic aerobic Gram-negative coccobacillus that is catalase and oxidase positive. Importantly, in the laboratory, most of the strains do not grow on blood sheep or MacConkey agar because the organism requires cystine or cysteine. Chocolate agars, thioglycolate broth, or Thayer-Martin medium are more suitable in order to isolate the bacteria. Two species of *Francisella* have been described (*F. tularensis* and *F. philomiragia*) and *F. tularensis* includes four subspecies (*F. tularensis* subsp. *tularensis*; *F. tularensis* subsp. *holartica*; *F. tularensis* subsp. *Novicida*; and *F. tularensis* subsp. *mediaasiatica*). The most common are the subspecies *tularensis* (mainly seen in North America) and *holartica*.

Clinical Manifestations and Diagnosis

Sporadic cases of tularemia are encountered in the Northern Hemisphere following contact with infected animals (rabbits, hares, muskrats, beavers, squirrels) or infected ticks but transmissions through aerosols, contaminated water, and animal bites are reported. Increased risk of infection has been reported among those in close contact with potentially infected animals including farmers, veterinarians, hunters and trappers, landscapers, laboratory workers, and meat handlers (Dennis et al., 2001).

There are six classical clinical presentations. The different forms are glandular (enlarged loco-regional lymph nodes without cutaneous lesions), ulceroglandular (adenopathy with a necrotic ulcer), oculoglandular (painful conjunctivitis following direct inoculation in the eye or aerosolization associated with painful adenopathy in the preauricular, submandibular, or cervical regions), oropharyngeal (exudative pharyngitis or tonsillitis following swallowing of contaminated food, water, or droplets), typhoidal (a febrile illness not fitting in the other categories), and pneumonic (pneumonia) (Dennis et al., 2001). The syndromes may overlap in symptoms on presentation and manifestations.

The incubation period ranges from 1 to 21 days with most of the cases occurring 3–5 days after exposure. Usually the onset of the first symptoms is an influenza-like initial presentation (fever, headache, chills, malaise, and fatigue) with cough, sore throat, and myalgias. Abdominal pain, vomiting, and diarrhea may also occur. Disease can progress and acute renal failure, rhabdomyolysis, hepatitis, disseminated intravascular coagulation can develop.

Laboratory diagnosis relies on culturing the organism from blood, sputum, wound, lymph nodes, or pleural fluid. Suspected cultures should be manipulated in a BL3 cabinet to avoid transmission to laboratory personnel. If the appropriate facilities are not available, the suspected strain should be sent to a reference laboratory for further analysis. The final identification can be confirmed using slide agglutination, antisera, direct fluorescent antibodies, or cell wall fatty acid composition analysis. PCR has also been developed for use on human specimens (including blood and skin lesions biopsies). These rapid identification systems are likely important in an event of mass terrorism. Serologic studies are also available but take longer for results (2 weeks) and are not suitable in this context.

Treatment

Treatment relies on the use of aminoglycosides: streptomycin (1 g IM every 12 h) or gentamicin (5 mg kg^{-1} IV every 24 h) are the preferred agents and should be administered for 10 days. Ciprofloxacin, doxycycline, or chloramphenicol are alternative agents but should be administered for 14–21 days (Dennis et al., 2001). Pregnant women should receive the same regimens as other adults and aminoglycosides, ciprofloxacin, and doxycycline are also recommended for children following mass exposure.

Infection Prevention Strategies

Standard precautions are indicated. There is no human-tohuman transmission of the disease although outbreaks may occur following exposure to a common contaminated source or intentional large-scale release (Dennis et al., 2001). Mainly, infection

prevention interventions should target assuring that specimens are appropriately handled (safety cabinets) and prophylaxis administered if needed. Autopsy personnel are also at increased risk of exposure to aerosolized infected tissues and should use contact and droplet precautions when a case is suspected. Contaminated surfaces in the laboratory can be cleaned using a fresh solution of 10% chlorine (bleach solution).

Prophylaxis

Following exposure to tularemia, patients should be offered a prophylaxis with either doxycycline (100 mg PO every 12 h) or ciprofloxacin (500 mg PO every 12 h) for 14 days (Dennis et al., 2001). Attempts to develop a reliable vaccine against *F. tularensis* infection began in the early 1930s. A live vaccine was developed in the 1950s but is no longer available and regimens based on antimicrobial prophylaxis are the only available at this point.

Viral Hemorrhagic Fevers

Introduction and Microbiology

Viral hemorrhagic fever (VHF) generally refers to a syndrome characterized by acute febrile illness with bleeding diathesis and is caused by several families of small RNA viruses with lipid envelops. In 2000, these viruses were classified as category A based on their ability to pose a significant threat to public health because of their ease of dissemination or transmission, high mortality and morbidity, the lack of an effective vaccine, feasibility of large-scale production, and significant impact on public health including panic, social, and economic disruption (Borio et al., 2002). In this section, we focus on hemorrhagic fever viruses in four families including Filoviridae (Ebola, Marburg), Arenaviridae (Lassa, New World Arenaviridae), Bunyaviridae (Rift Valley fever), and Flaviviridae (yellow fever, Omsk hemorrhagic fever, Kyasanur forest disease). Crimean-Congo hemorrhagic fever and dengue virus may produce high morbidity and mortality but they are not mentioned in this article because of technically difficult to produce in large-scale amount that could be widely distributed.

Clinical Manifestations

VHF presents with nonspecific symptoms including an abrupt onset of fever, headache, myalgia, nausea, vomiting, diarrhea, and followed by maculopapular rash, particularly involving the trunk. Findings may include hypotension, relative bradycardia, conjunctivitis, and pharyngitis. A bleeding diathesis with petechiae, ecchymosis, hemoptysis, melena, conjunctival hemorrhage, and disseminated intravascular coagulopathy may develop later (Borio et al., 2002; Moran et al., 2008). The causative agent depends on the geographic distribution of the virus and its host. The average incubation period is 2–21 days (Table 3). In areas where these diseases are not endemic, a strong index of suspicion is needed and a bioterrorism attack should be suspected. Other organ involvement may suggest a particular viral etiology. For example, jaundice is typical in Rift Valley fever and yellow fever; (Moran et al., 2008) moreover, Lassa fever may present with leukocytosis instead of the leukopenia associated with other viruses (Pigott, 2005). No pathognomonic laboratory findings distinguish the causes of the disease.

VHF agents should be manipulated in BSL-4 containment facilities. Testing methods include antigen detection by antigen-capture ELISA, IgM antigen detection by antibodycapture ELISA, RT-PCR, and viral isolation (Pigott, 2005).

Treatment

Supportive treatment with careful maintenance of fluid and electrolyte balance, blood pressure, along with early recognition and treatment of disease complications (e.g., DIC, acute renal failure) are the foundation of VHF treatment. No antiviral agent is

Table 3 Hemorrhagic fever viruses and their distribution and disease characteristics

Virus	*Vector in nature*	*Natural distribution*	*Incubation period (days)*	*Person-to-person transmission*	*Mortality (%)*	*Treatment*
Ebola	Unknown	Africa	2–21	Yes	50–90	Supportive
Marburg	Unknown	Africa	2–14	Yes	23–70	Supportive
Lass Fever	Rodent	West Africa	5–16	Yes	15–20	Ribavirin, supportive
New World Arenaviruses	Rodent	Americas	7–14	Yes	15–30	Ribavirin, supportive
Rift Valley Fever	Mosquito	Africa, Saudi Arabia, Yemen	2–6	No	<1	Ribavirin, supportive
Yellow Fever	Mosquito	Africa, Tropical Americas	3–6	No	20	Supportive
Omsk Hemorrhagic Fever	Tick	Central Asia	2–9	No	0.5–10	Supportive
Kyasanur Forest disease	Tick	India	2–9	No	3–10	Supportive

Adapted from Borio, L., Inglesby, T., et al., 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. J. Am. Med. Assoc. 287 (18), 2391–2405.

available for the treatment of the disease (Borio et al., 2002). Oral ribavirin has demonstrated some antiviral activity against Arenaviridae and Bunyaviridae in limited human studies. Ribavirin is in a category class X that is contraindicated in pregnant patients. Hence, the risk of fetal toxicities and maternal mortality must be weighed (Bausch et al., 2010). There is currently no indication for corticosteroids. Aspirin and nonsteroidal anti-inflammatory drugs, and intramuscular administration of drugs are contraindicated because of potential bleeding complications (Borio et al., 2002).

Infection Prevention Strategies

Health-care-associated infections have been associated with reuse of blood-contaminated medical items and among healthcare workers who have provided patient care without appropriate barriers to prevent exposure to blood and other body fluids. Person-to-person transmission has been recognized in filoviruses and arenaviruses. Airborne transmission does not readily occur except in Lassa fever where the index patient had severe pulmonary involvement (Monath, 1975).

Standard precautions with contact and droplet isolation are recommended for suspected VHF cases. Hospitalized patients should be placed in private rooms. Airborne precautions can be used in suspected cases with severe pulmonary symptoms, or who undergo aerosol-generating procedures (such as aerosolized or nebulized medication administration). Barrier methods include gloves, impermeable gown, face shields, and goggles for eye protection. Additional barriers (e.g., plastic aprons, leg and shoe coverings) may be needed depending on the likelihood and magnitude of contact with body fluids. The Working Group on Civilian Biodefense also recommended using of double gloves in all suspected VHF patients. Nonessential health-care personnel and visitors should be restricted from entering the room of the patients (Borio et al., 2002).

Environmental Decontamination

Environmental surfaces or inanimate objects contaminated with blood, and other body fluids, secretions, or excretions (including vomitus) should always be decontaminated. In general, household bleach in a dilution of 1:100 should be used, whereas 1:10 dilutions should be used only in grossly soiled surfaces. Medical wastes (e.g., feces) can be disposed in the sanitary sewer following local sewage disposal requirement, while contaminated solid medical waste (e.g., needles, syringes) should be managed according to existing local and state regulations. Alternatively, on-site decontamination in an incinerator or a gravity-displacement autoclave can be used (CDC, 2005).

Postmortem Practices

Disease transmission in Ebola outbreaks has occurred after contact with cadavers hence standard precautions, and isolation as described above should be used in postmortem situations Prompt burial or cremation with minimal handling of bodily secretions is recommended (Borio et al., 2002).

Postexposure Prophylaxis

Use soap and water and wash aggressively when percutaneous or mucocutaneous exposures to contaminated blood or body fluids of suspected VHF patients occur. There are no data supporting the use of ribavirin preemptively. Exposed individuals should be placed under medical surveillance. Early recognition of any symptoms such as fever (101 °F, or 38.3 °C), and prompt administration of ribavirin are recommended (Borio et al., 2002). No effective vaccines for these viruses are currently available (Pigott, 2005; Hartman et al., 2010).

Hospital Preparedness

Health-care practitioners are the primary source of reporting potential cases of bioterrorism to local health departments. Health departments must scrutinize disease patterns and diagnostic clues that suggest an unusual infectious diseases outbreak (CDC, 2001). Indications suggesting a possible intentional release of a biologic agent include: (1) an unusual temporal or geographic clustering of illness or a large number of patients presenting with clinical signs and symptoms that suggest an infectious disease outbreak, (2) an unusual age distribution for a disease, or (3) a large number of cases of acute flaccid paralysis with prominent bulbar palsies. Because bioengineered pathogens may have unusual or unexpected characteristics, and may not be detected by existing assays, outbreaks with unexpected or unusual clinical or epidemiologic characteristics should be pursued with added urgency (Ippolito et al., 2006). They may be considered the source of previously unidentified pathogens.

Syndromic surveillance activities for health-care providers can include using International Classification of Diseases that may facilitate implementing disease prevention and control strategies (CDC, 2003). Of note, because coding must occur, this strategy alone may delay the identification of a bioterrorism event. These syndromes are listed as follows:

1. *Botulism-like syndrome* defined as acute condition, mainly neurological involvement that may represent exposure to botulinum toxin (e.g., lateral rectus palsy, diplopia);
2. *Hemorrhagic illness* defined as specific diagnosis, acute condition with multiple organ involvement and acute blood abnormalities that may be consistent with exposure to any virus that causes VHF (e.g., yellow fever, Ebola, dengue, Crimean-Congo);
3. *Lymphadenitis* defined as acute regional lymph node swelling and/or infection consistent with a bubo associated with Y. pestis infection;
4. *Localized cutaneous lesion* (resembling cutaneous anthrax, or tularemia) includes acute localized edema and/or cutaneous lesion/vesicle, ulcer, eschar, or specific diagnosis of localized cutaneous lesion/ulcer consistent with cutaneous anthrax or tularemia;

5. *Gastrointestinal syndrome* defined as acute infection of the upper and/or lower GI tract, both specific (e.g., *Salmonella*) and nonspecific symptoms;
6. *Respiratory syndrome* defined as acute infection of the upper and/or lower respiratory tract, including acute specific diagnosis (e.g., pneumonia due to influenza), acute nonspecific diagnosis (e.g., sinusitis), and acute nonspecific symptoms (e.g., shortness of breath, cough);
7. *Neurological syndrome* defined as acute neurological infection of the central nervous system (CNS) that includes specific diagnosis of acute CNS infection (e.g., pneumococcal meningitis), acute nonspecific diagnosis (e.g., meningitis not otherwise specified), and acute nonspecific symptoms (e.g., meningismus);
8. *Rash* (resembles smallpox) defined as acute condition that may present as consistent with smallpox, specific diagnosis of acute rash (e.g., smallpox), and acute nonspecific diagnosis of rash compatible with infectious diseases (e.g., viral exanthem);
9. *Specific infection* defined as acute infection of known cause not covered in syndromes listed above, includes septicemia of known bacteria, other febrile illnesses (e.g., scarlet fever);
10. *Fever* defined as acute potentially febrile illness of origin not specified;
11. *Severe illness or death potentially due to an infectious disease* defined as acute onset of shock or coma from potentially infectious etiology.

Challenges in Bioterrorism and Mass Casualty Preparedness

Emergency departments would be among the first responsible health-care entities affected after a biological attack. Most hospitals have emergency response plans for responding to chemical, biological, nuclear, and explosive incidents. Written protocols to encounter rapid, mass influx of patients who may have been exposed to biological hazard have to be addressed, and drilled with other local health-care facilities and their communities (Vinson, 2007). Health-care personnel have to be educated and trained for their roles in such events (Ippolito et al., 2006). Moreover, those who have no clinical responsibilities, or patient contacts may be needed for support and should be assigned positions in the situation. Resources for mass casualty to avoid spreading of harmful pathogens, such as personal protective equipment, have to be available (Grow and Rubinson, 2003). Hospital preparedness for bioterrorism, along with other mass casualty emergency plans, should be encouraged in all health-care facilities, both in metropolitan and nonmetropolitan areas for proper management in biological attacks (Beam et al., 2010).

Conclusion

Political conflicts and changing approaches to these conflicts globally make biological attacks still be possible. Bioterrorism preparedness is essential and requires that health-care professionals be engaged. New challenges in preparedness include the need for using resources efficiently and effectively and should be balanced by economic constraints. Restrictions in funding from research budgets may affect drug or vaccine development for the potential agents associated with bioterrorism. Although some difficulties exist, fundamental disease preparedness can be applied following international, national, and local health authority recommendations. Awareness of health-care personnel for changing disease patterns or syndromes, and preparedness of mass casualty for all hazardous materials are imperative. Hospital and healthcare facility roles are a priority for bioterrorism emergency response planning.

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Brochothrix thermosphacta

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Glossary

Bacteriocin A peptide produced by bacteria, with an antimicrobial activity toward bacterial species closely-related or non-related to the producer bacterium.
Biopreservation The most commonly used definition has been proposed by Michael E. Stiles in 1996 as "Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and (or) their antibacterial products".
Coding sequence Portion of a gene that is transcribed into a messenger RNA and translated into a protein.
Contig A set of overlapping DNA sequences (reads) that together represent a consensus region of DNA.
Genome The collection of an organism's entire genetic information, organized in genes. In bacteria the genome is most of the time constituted by a single chromosome and sometimes plasmids, the number of which varies depending on strains.
Genomics Science that studies all genes in an organism for analyzing their physical structure and organization (structural genomics) as well as the function of genes, their regulation mode, and their interactions (functional genomics or post-genomics).
Microbial spoilage Defect of the organoleptic properties of a food product including aspect, texture, color, odor, and flavor resulting from microbial growth and metabolism with the production of molecules (volatile compounds, pigments, gas, exopolysaccharides. . .).
Microbiota All microorganisms that typically inhabit a particular environment (here the food environment) considered as a group.
Operon A functional unit of DNA containing a cluster of genes controlled by a single promoter. The genes are transcribed together into a messenger RNA.
Peptidoglycan Also called murein, is an essential and specific component of the bacterial cell wall preserving cell integrity and located on the outside of the cytoplasmic membrane of bacteria.
Psychrotrophic Ability of microorganisms to grow at 0°C. Their maximum growth temperature is above 20°C. They are widespread in natural environments and in foods.
Scaffold A set of non-contiguous series of oriented genomic sequences or contigs separated by gaps of known length but of unknown sequence.
Taxonomy Classification and description of living organisms. It includes naming and defining of species, and collecting data about their biology (phenotypic, genotypic characteristics), biogeography, and ecology).

Abbreviations

BA	biogenic amines
CDS	coding sequence
CFU	colony forming unit
MAP	modified atmosphere packaging
STAA	streptomycin-thallous acetate-actidione
VOC	volatile organic compound
VP	vacuum packaging

Introduction

The genus *Brochothrix* belongs to the *Listeriaceae* family which encompasses only two genera: *Listeria* and *Brochothrix*. To date, in the *Brochothrix* genus only two closely related species have been described. *Brochothrix thermosphacta* has been first isolated from pork sausages (McLean and Sulzbacher, 1953) and *Brochothrix campestris* from grass and soil (Talon et al., 1988). Although, these two species are non-pathogenic, *B. thermosphacta* gained a scientific interest because of its implication in the spoilage of meat and seafood products. Indeed, *B. thermosphacta* is a psychrotrophic and ubiquitous bacterium that has been reported as present in various meat and seafood products stored at cold temperatures, and as responsible for the production of undesired off-odors such as

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cheesy or buttery odors (Rattanasomboon et al., 1999; Ercolini et al., 2006; Russo et al., 2006; Casaburi et al., 2015). In the case of meat, *B. thermosphacta* was reported as the dominant spoiler of products stored under vacuum packaging (VP) or modified atmosphere packaging (MAP) (Gill and Newton, 1977; Gill, 1983).

Food spoilage consists in any change of a food product leading to its rejection based on sensory characteristics (Gram et al., 2002). Food spoilage can result from physical damages or chemical and enzymatic reactions, but microbial growth and the resulting microbial metabolic activities are the major causes of food spoilage. Although spoiled foods may be not dangerous for human consumption, they are rejected by the consumer or earlier in the food production chain because they do not fit with quality standards. Therefore spoilage has important economic and ecological consequences. Indeed, one third of food produced for the human consumption is lost or wasted every year (Gustavsson et al., 2011) and part of losses are the consequence of microbial spoilage. All types of food can be concerned by microbial spoilage, but meat and seafood products are recognized as the most perishable ones due to their near neutral pH, water content, and abundance of nutrients essential for the growth of various microorganisms (Gram et al., 2002). Several studies showed that bacteria are the dominant spoilage microorganisms of meat and seafood (Gram et al., 2002). Microbial spoilage may appear as a visible bacterial growth, as changes in the texture, aspect, or as off-odor and off-flavor development (Gram et al., 2002). It is a complex process to which many bacteria can contribute. The food microbiota and its dynamics during the shelf life is affected by the nature of the food matrix (pH, water activity, and chemical composition), the stabilization process (cooking, smoking, drying, salting...), the hygienic conditions during the processing, and the packaging and storage conditions from the distribution of the food product until its consumption (Borch et al., 1996; Samelis et al., 1998, 2000a). Although many preservation methods have been developed to enhance the storage stability and maintain quality of foods for extended periods, excessive amounts of foods are lost every year due to microbial spoilage (Gustavsson et al., 2011).

Thus understanding the behavior of *B. thermosphacta* during food production and storage and the mechanisms responsible for its spoilage activity should contribute to find solutions for avoiding its presence or development in perishable meat and seafood products. This review focuses on the characteristics of this species, its role in food spoilage, and some strategies for fighting it in the food industry.

Taxonomy History

B. thermosphacta first isolated from pork sausage (Sulzbacher and McLean, 1951) was initially named *Microbacterium thermosphactum* (McLean and Sulzbacher, 1953). However, the same authors already mentioned notable discrepancies in the cell morphology between *M. thermosphactum* and the type species of the genus *Microbacterium lacumin*. Later studies confirmed the differences in the morphology and noted other dissimilarities especially in enzymology and protein profiles, in the DNA base composition and in peptidoglycan structure (Stackebrandt and Jones, 2006). Moreover, *M. thermosphactum* isolates formed a distinct cluster sufficiently distant from other species of the genus *Microbacterium* and from the closest Gram-positive genera (Stackebrandt and Jones, 2006). Consequently, this species was reclassified to a new genus *Brochothrix* and renamed as *B. thermosphacta* and first placed in the *Lactobacillaceae* family (Sneath and Jones, 1976). Thereafter, bacteria of the genus *Brochothrix* were found to be phenotypically closer to those of the genus *Listeria* than to those of *Lactobacillus* and were therefore moved to the *Listeriaceae* family (Sneath and Jones, 1986). The phylogenetic relationship of *B. thermosphacta* with *Listeria monocytogenes* was later reinforced by the results of a study analyzing the 16S rRNA sequence of the two species (Ludwig et al., 1984). In 1988, the isolation and description of a new species belonging to *Brochothrix*, *B. campestris* (Talon et al., 1988) confirmed the genus and its classification in the *Listeriaceae* family. To date it encompasses two species: *B. thermosphacta* (type strain ATCC 11509/DSM 20171) and *B. campestris* (type strain ATCC 43754/DSM 4712), which are highly related as their 16S rDNA sequences share 99.3% identity (Stackebrandt and Jones, 2006). Recently, the *rpoB* gene sequence, encoding the beta subunit of the RNA polymerase, appeared as a powerful tool for bacterial identification and phylogenetic analyses, especially for studying closely related isolates. Alignments of *rpoB* gene sequences have been reported to enable resolution at the species and subspecies levels (Adékambi et al., 2009). Additionally, it has been reported that *rpoB* gene sequencing provided higher discriminating power than 16S rRNA gene sequencing (Adékambi et al., 2009). We have drawn a phylogenetic tree based on the alignment of *rpoB* gene sequences from 12 *B. thermosphacta* genomes and from the unique *B. campestris* genome described to date. *Listeria* species were used as an outgroup (Fig. 1). The resulting tree confirms that *B. campestris* is located on a separate branch.

Characteristics and Ecology of *B. thermosphacta*

Habitat

The natural habitat of *B. thermosphacta* is not fully known. Indeed, initially isolated from pork sausage and considered as a psychrotrophic bacterium responsible for the spoilage of meat products, it did not attract the attention of microbiologists from other fields. In addition, its successive reclassifications mentioned above may have contributed to misidentification of some isolates. Nevertheless other possible sources of *B. thermosphacta* have been reported such as soil and feces (Gardner, 1966). More recently, it has been also isolated from a wide variety of food of animal origin *i.e.* meat from mammals and birds, seafood, milk and dairy products (Stackebrandt and Jones, 2006). Moreover, *B. thermosphacta* was described omnipresent along the production chain of beef meat, from the animal to the final product. It was isolated from beef carcasses, animal skin and rumen, walls, equipment of slaughterhouses, processing plants, hands of manipulators, and ambient air of chilling rooms (Nychas et al., 2008). It is therefore

Fig. 1 Phylogenetic tree of the *rpoB* gene from *B. thermosphacta* and *B. campestris*. The *rpoB* gene sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov); MAFFT alignment was performed using Geneious software R9; The phylogenetic tree was drawn with FastTree 2.1.5 then visualized with FigTree (v1.4.3); *rpoB* gene sequences from different species of the genus *Listeria* were used as outgroup species.

possible that this species lives in a wide range of habitats but has been found only in some environments where it was systematically searched, or in which it can be selected as growth conditions are optimal, such as cold temperatures and nutritious meat matrixes.

B. thermosphacta has been isolated from food, even in the absence of detectable spoilage (Nychas et al., 2007; Liang et al., 2012). Because of its ubiquitous and psychrotolerant nature, *B. thermosphacta* is frequently found as a dominant spoiler bacterium of a wide range of foodstuffs stored at low temperature. It has been identified as the emblematic bacterial species among the microbiota of 8 spoiled meat and seafood products stored at low temperature (Chaillou et al., 2015). Indeed, it has been associated to beef, veal, pork, and poultry spoiled meat (Rattanasomboon et al., 1999; Samelis et al., 2000b; Sakala et al., 2002; Bohaychuk and Greer, 2003; Vasilopoulos et al., 2008; Ercolini et al., 2011; Liang et al., 2012; Nieminen et al., 2012; Dias et al., 2013; Delhalle et al., 2016; Piotrowska-Cyplik et al., 2017) and seafood products (Drosinos and Nychas, 1996; Drosinos et al., 1997; Mejlholm et al., 2005; Jaffrès et al., 2011; Noseda et al., 2012; Parlapani et al., 2014). In most of these reported cases, *B. thermosphacta* numbers in the spoiled food had reached levels above 7 log CFU g^{-1}.

B. thermosphacta commonly belongs to aerobic microbiota of chilled meats and is a specific spoiler of meat stored under MAP and VP (Dainty and Mackey, 1992; Nychas et al., 2007, 2008; Vasilopoulos et al., 2008; Ercolini et al., 2011; Remenant et al., 2015; Piotrowska-Cyplik et al., 2017).

General Phenotypic Description of the Species

Strains belonging to the genus *Brochothrix* are Gram positive, non-spore forming, non-motile regular unbranched rods (0.6–0.8 μm diameter and 1-2 μm long) that occur singly or in pairs. Unlike *B. campestris*, *B. thermosphacta* can arise in short chains or in long filamentous chains. In old cultures, the cell morphology of this bacterium changes from rod to coccoid but cells resume the rod form when subcultured in a suitable culture medium (Stackebrandt and Jones, 2006). *Brochothrix* sp. are aerobe and facultative anaerobe, catalase positive, and oxidase negative. These bacteria can grow at temperatures ranging from 0 to 30°C with an optimum growth between 20 and 25°C (Stackebrandt and Jones, 2006). The optimum pH for growth is pH 7.0, but *B. thermosphacta* is able to grow within the pH range from 5 to 9.

Glucose is the preferred carbon source used by *B. thermosphacta* in meat (Gill and Newton, 1977). In the IMViC tests (indole, methyl-red, Voges-Proskauer, and citrate) used for differentiating bacterial isolates, the species is considered as methyl-red and

Voges-Proskauer positive meaning ability to mixed acid fermentation and production of 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol. Its metabolism is influenced by dioxygen and carbon dioxide contents in the atmosphere. Under aerobic conditions, 3-hydroxy-2-butanone (acetoin) and 2,3-butanedione (diacetyl) are the major metabolites produced from the consumption of glucose, and also free fatty acids as isobutyric and isovaleric acids which result from valine and leucine consumption at limiting concentrations of glucose (Dainty and Hibbard, 1980). In the absence of O_2, *B. thermosphacta* produces mainly L-(+)-lactic acid and ethanol (Dainty et al., 1985; Pin et al., 2002). When glucose is depleted, glycerol appears to be an alternative carbon source for growth (Macaskie et al., 1984). Nitrate is not reduced to nitrite by *B. thermosphacta* and this species does not appear to have a nitrite reductase system (Collins-Thompson and Rodriguez-Lopez, 1980; Holley, 2014). Indole and H_2S are not produced by *B. thermosphacta* (Stackebrandt and Jones, 2006).

Information about lipolytic and proteolytic activities of *B. thermosphacta* is scarce and remains ambiguous. Several studies reported that the optimal temperature for production of lipases by this species is 20°C (Papon and Talon, 1988; Braun and Sutherland, 2004; Nowak and Piotrowska, 2012). Nevertheless (Nowak et al., 2012) found that most *B. thermosphacta* strains showed a lipolytic activity at 25°C and only some at 4°C, while (Casaburi et al., 2014) did not observe any lipolytic activity from strains neither *in vitro* nor in meat. These contradictory results may be explained by the different *B. thermosphacta* strains used by these authors and suggest important intra-species diversity.

Factors Affecting Growth of *B. thermosphacta*

Various parameters are used to prolong the shelf life of food by diminishing microbial growth. These include the use of various MAP conditions (varying the O_2, CO_2 and N_2 ratios), storage at low temperature, addition of ingredients such as salts (especially in fermented and cured meat or in salted fish products), but also preservatives such as nitrite and sodium lactate. The growth of *B. thermosphacta* on meat and seafood products is highly affected by such abiotic factors (Hitchener et al., 1979; Blickstad, 1983; Labadie, 1999; Pin et al., 2002; Nychas et al., 2008). Table 1 summarizes the main effects of various factors on the growth of *B. thermosphacta* recorded from the literature.

Isolation and Identification Tools

Methods developed for isolating *B. thermosphacta* have been mainly devoted to its recovery from meat products. A selective medium has been designed and is still routinely used (Gardner, 1966, 1985). This so-called STAA medium contains streptomycin, thallium acetate, and actidione. *B. thermosphacta* is resistant to high concentrations of streptomycin-sulfate, which inhibits other Gram-positive and most Gram-negative bacteria. Thallium acetate inhibits most yeast species as well as many facultative aerobic and anaerobic microorganisms. The incorporation of actidione (cycloheximide) inhibits yeast and filamentous fungi (Gardner, 1966). Standard ISO norm 13722:1996 reports a colony-count technique for enumerating *B. thermosphacta* from meat products by plating on the selective medium STAA followed by incubation of the plates at temperatures ranging from 22°C to 25°C during 48 hours. Further tests may be necessary to check the colonies selected on STAA plates, such as an oxidase test for differentiating *B. thermosphacta* from some Pseudomonads that can also grow on this medium. Indeed, although the medium preferentially selects *B. thermosphacta*, a number of other bacterial species can also grow on it (Gardner, 1966).

Table 1 Abiotic factors encountered in meat products and their influence on growth of *B. thermosphacta*

Factor	*Effect on B. thermosphacta growth*	*Reference*
Gas composition of the atmosphere	Presence of O_2 improves growth	Samelis et al. (2000a) Argyri et al. (2011)
	High level of CO_2 is not inhibitory as long as O_2 is present	Samelis et al. (2000a) Koutsoumanis et al. (2006) Asensio et al. (1988) Lopez-Galvez et al. (1995)
Temperature	Growth is favored at low temperature (0 and 5°C)	Argyri et al. (2011) Koutsoumanis et al. (2006)
pH	Growth is inhibited at low pH	Blickstad (1983)
Glucose	Increasing concentrations improve growth	Macaskie et al. (1984) Nychas et al. (2008)
NaCl	Growth still possible with 8–10%	Collins-Thompson and Rodriguez-Lopez (1980)
Nitrate	Growth inhibition by nitrate, more important inhibition in combination with low pH and low temperature	Talon et al. (1988)
Nitrite	Combination of low pH+low temperature+nitrite, increase the growth inhibition	Collins-Thompson and Rodriguez-Lopez (1980)
Sodium lactate	Levels reduced by sodium lactate in acidified chicken meat model (pH=5.0) stored at 22°C	Lemay et al. (2002)

Table 2 Main characteristics that differentiate *Brochothrix* spp. from phylogenetically close genera

Characteristics	*Brochothrix*	*Listeria*	*Carnobacterium*	*Lactobacillus*
Growth at 35°C	−	+	+	+
Growth on STAA agar	+	−	−	−
Motility	−	+	−	−
Catalase activity	+	+	−	−
GC content (%)	36–38	36–42	33–37	32–53

Both phenotypic and genotypic methods can contribute to a further fast screening of colonies isolated on STAA medium. Some key tests for the phenotypic identification of new isolates of *Brochothrix* spp. have been traditionally used such as Gram staining, detection of catalase activity, and assessment of growth at different temperatures. Table 2 summarizes the main characteristics that differentiate *Brochothrix* from other non-sporulating Gram-positive bacilli that can contaminate the same food matrices (Stackebrandt and Jones, 2006).

However, the above mentioned tests do not enable the discrimination of *B. thermosphacta* from *B. campestris*. Those can be differentiated by their ability to grow in media containing 8%–10% NaCl or 0.05% of potassium tellurite, as *B. thermosphacta* can grow on these media whereas *B. campestris* cannot. Moreover, unlike *B. thermosphacta*, *B. campestris* is able to hydrolyze hippurate and produces acids from rhamnose (Stackebrandt and Jones, 2006).

Recently, molecular techniques have been widely applied for detecting and typing foodborne pathogens and spoilers (Van Der Vossen and Hofstra, 1996; Scheu et al., 1998; Böhme et al., 2014; Ceuppens et al. 2014). For example, 16S rRNA gene sequencing is the most commonly used method for studying bacterial phylogeny and taxonomy. However, although it is a powerful tool for many genera and species, it has poor discriminatory power in some cases (Bosshard et al., 2006; Mignard and Flandrois, 2006). As mentioned above, the two *B. thermosphacta* and *B. campestris* type strains share >99.3% sequence identity with regard to their 16S rRNA genes (Stackebrandt and Jones, 2006). Real-time PCR assays have been developed for detecting and quantifying *B. thermosphacta* from beef, seafood, and pork sausage (Mamlouk et al., 2012; Papadopoulou et al., 2012; Fougy et al., 2016). Some were based on primers designed from the 16S rRNA gene sequence (Mamlouk et al., 2012; Papadopoulou et al., 2012) and may not differentiate the two species. On the contrary, in a most recent report, the primers designed from the *rpoC* gene were specific for *B. thermosphacta* only (Fougy et al., 2016). Two PCR-based tests for specific detection and differentiation of *B. thermosphacta* and *B. campestris* were developed (Gribble and Brightwell, 2013). The first is a real-time TaqMan PCR based on a single nucleotide polymorphism (T/A) and targeting 16S rRNA gene sequences. This test allows the specific differentiation of *Brochothrix* species from the closely related *Listeria* and *Lactobacillus* genera. The second is a standard PCR assay for the specific identification of *B. campestris* based on the amplification of the brochocin-C encoding operon. This bacteriocin is produced by the *brcABIDT* operon (McCormick et al., 1998) which is absent from *B. thermosphacta* (Siragusa and Cutter-Nettles, 1993; Gribble and Brightwell, 2013). However, this reportedly species specific PCR based test may not be reliable as the studies on brochocin-C have all been performed with the type strain, *B. campestris* ATCC 43754. Thus, there is no information about the presence of the genes for brochocin-C production in other *B. campestris* strains that could validate this PCR test as accurate on more strains.

Other DNA based methods such as PFGE (Pulsed Field Gel Electrophoresis), REP-PCR (Repetitive Extragenic Palindromic PCR) were also applied on *B. thermosphacta* strains with the aim to characterize the intra-species diversity (Xu et al., 2010; Papadopoulou et al., 2012). The results obtained by these authors confirmed a genetic diversity between the strains. MALDI-TOF MS (Matrix assisted laser desorption/ionization time of flight Mass spectrometry) based on the analysis of protein profiles enabled the identification of *B. thermosphacta* colonies isolated from poultry cuts but no indication about the possible identification of *B. campestris* with this method was mentioned (Höll et al., 2016).

The use of culture-independent methods, performed with no need of prior bacterial cultivation, has greatly improved the knowledge about the diversity and the structure of microbial communities of food. By such methods the presence of *B. thermosphacta* among the dominant species has been highlighted in many food matrixes including meat and seafood products (Drosinos and Nychas, 1996; Jaffrès et al., 2009; Pennacchia et al., 2009, 2011; Ercolini et al., 2011; Nieminen et al., 2012; Chaillou et al., 2015; Delhalle et al., 2016; Piotrowska-Cyplik et al., 2017).

Spoilage Potential of *B. thermosphacta*

Production of Malodorous Molecules

The spoilage of food can be evaluated by sensory analyses which reveal color, aspect, odor, taste with attributes (such as buttery odor or wet dog as examples) and sometimes notation. Chemical analyses can also be performed to identify and sometimes quantify the molecules that are produced and considered as responsible for spoilage. In the case of off-odor production, methods aiming at detecting volatile molecules have been largely used. As *B. thermosphacta* has been reported to be one of the most common species associated with spoilage, many studies have analyzed volatile organic compounds (VOCs) in food contaminated by this species, to establish a link with its presence or activity in spoiled products. Table 3 summarizes the different molecules that have been identified as putatively linked to the presence of *B. thermosphacta* and its involvement in spoilage of various food matrices.

Table 3 Volatile Organic Compounds produced by *B. thermosphacta* during storage of meat or seafood under air, vacuum packaging (VP) and modified atmosphere packaging (MAP)

Volatile organic compounds (VOC)	*Matrix*	*Storage conditions*	*Odor description*	*Other spoilage bacteria*	*References*
Hydrocarbons					
Dimethylbenzene	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Strong butter, buttermilk, sour, nauseous sweet	/	Laursen et al. (2006)
2-Octene	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Strong butter, buttermilk, sour, nauseous sweet	/	
Alcohols					
Ethanol	Inoculated beef meat	VP, 5°C, 15 days		/	Stanley et al. (1981)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)
1-Propanol	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
2-Methyl-1-propanol	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	
2-Methyl-1-butanol	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50% N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	
3-Methyl-1-butanol	Spoiled chicken carcasses	Air		*Shewanella, Serratia liquefaciens*	Nychas et al. (1998)
	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Wet dog	*Carnobacterium maltaromaticum, Carnobacterium divergens Carnobacterium mobile*	Laursen et al. (2006)
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50% N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
2-Methylpropanol	Spoiled chicken carcasses	Air		*Pseudomonas* spp. *S. liquefaciens,*	Nychas et al. (1998)
	Inoculated beef meat	VP, 5°C, 15 days		/	Stanley et al. (1981)
2-Methylbutanol	Inoculated beef meat	VP, 5°C, 15 days		/	
1-Octanol	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
1-Octen-3-ol	Inoculated beef meat	Air, 4°C, 7 days		/	
2-Ethyl-1-hexanol	Inoculated beef meat	Air, 4°C, 7 days		/	
	Beef meat chops	Air, 4°C		/	Ercolini et al. (2011)
2-Ethyl-1-decanol	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
2,3-Butanediol	Inoculated beef meat	Air, 4°C, 7 days		/	
	Beef meat chops	Air, 4°C		*Pseudomonas* spp., *Enterobacteriaceae*	Ercolini et al. (2011)
	Inoculated beef meat	VP, 5°C, 15 days		/	Stanley et al. (1981)
Butanediol	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Strong butter, buttermilk, sour, nauseous sweet	/	Laursen et al. (2006)
2-Butoxy-ethanol	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
Hepanol	Inoculated beef meat	Air, 4°C, 7 days		/	
Phenylethyl alcohol	Beef meat chops	Air, 4°C		*Pseudomonas* spp., *Enterobacteriaceae*	Ercolini et al. (2011)
2-Hexanol	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)

(*Continued*)

Table 3 (Continued)

Volatile organic compounds (VOC)	*Matrix*	*Storage conditions*	*Odor description*	*Other spoilage bacteria*	*References*
Aldehydes					
Acetaldehyde	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
2-Methyl-1-propanal	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	
2-Methylpropanal	Spoiled chicken carcasses	Air		*Pseudomonas* spp., *S. liquefaciens,*	Nychas et al. (1998)
2-Methyl-1-butanal	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
2-Methylbutanal	Spoiled chicken carcasses	Air		*Pseudomonas* spp. *S. liquefaciens*	Nychas et al. (1998)
3-Methyl-1-butanal	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Strong butter, buttermilk, sour, nauseous sweet	/	Laursen et al. (2006)
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Wet dog	*C. maltaromaticum, C. divergens, C. mobile*	
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
3-Methylbutanal	Inoculated beef meat	Air, 5°C, 6–8 days		*Hafnia. alvei, Enterobacter agglomerans, S. liquefaciens, Alteromonas putrefaciens, Aeomonas hydrophila, Pseudomonas fragi*	Dainty et al. (1989)
	Inoculated beef meat	VP, 5°C, 15 days		/	Stanley et al. (1981)
Pentanal	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
Hexanal	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
Heptanal	Inoculated beef meat	Air, 4°C, 7 days		/	
Nonanal	Inoculated beef meat	Air, 4°C, 7 days		/	
	Inoculated beef meat	VP, 5°C, 15 days		/	Stanley et al. (1981)
Decanal	Inoculated beef meat	VP, 5°C, 15 days		/	
Ketones					
3-Hydroxybutanone (acetoin)	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
	Inoculated beef meat	VP, 5°C, 15 days		/	Stanley et al. (1981)
	Inoculated beef meat	Air, 5°C, 6–8 days		*H. alvei, E. agglomerans, S. liquefaciens, A. putrefaciens, A. hydrophila, P. fragi*	Dainty et al. (1989)
	Beef meat chops	Air/MAP (60% O_2, 40% CO_2), 4°C		*Pseudomonas* spp., *Enterobacteriaceae, C. maltaromaticum*	Ercolini et al. (2011)
	Spontaneously spoiled meat	Air		*Enterobacteriaceae*	Dainty and Mackey (1992)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)
	Raw atlantic salmon	Air; 4°C, 4 days		*Pseudomonas* spp., LAB, H_2S producing bacteria	Mikš-Krajnik et al. (2016)

2,3-Butanedione (diacetyl)	Inoculated beef meat	Air, 5°C, 6–8 days		*H. alvei, E. agglomerans, S. liquefaciens, A. putrefaciens, A. hydrophila, P. fragi*	Dainty et al. (1989)
	Spontaneously spoiled meat	Air,		*Enterobacteriaceae*	Dainty and Mackey (1992)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Strong butter, buttermilk, sour, nauseous sweet	/	Laursen et al. (2006)
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Wet dog	*C. maltaromaticum, C. divergens, C. mobile*	
2-Propanone	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)
2-Hexanone	Inoculated cold-smoked salmon	VP, 6°C, 40 days (Spoiled)	Blue cheese odor	/	Joffraud et al. (2001)
2-Heptanone	Inoculated cold-smoked salmon	VP, 6°C, 40 days (Spoiled)	Blue cheese odor	/	
2,3-Heptanedione	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50%N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
3-Octanone	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
Esters					
Ethyl acetate	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 50% N_2), 8°C, several weeks (spoiled)	Cheese/feet and sour/fermented	/	Jaffrès et al. (2011)
Ethyl butanoate	Inoculated sterile and none sterile meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
Ethyl 3-methylbutanoate	Inoculated beef meat	Air, 4°C, 7 days		/	
Ehylhexanoate	Beef meat chops	Air, 4°C		/	
	Inoculated beef meat	Air, 4°C, 7 days		/	
Ethyloctanoate	Inoculated beef meat	Air/MAP (60% O_2,40% CO_2), 4°C		/	Ercolini et al. (2011)
	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
Ethyldecanoate	Inoculated beef meat	Air, 4°C, 7 days		/	
Acids					
Acetic acid	Inoculated meat	Air		/	Dainty and Hibbard (1983)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)

(*Continued*)

Table 3 (Continued)

Volatile organic compounds (VOC)	*Matrix*	*Storage conditions*	*Odor description*	*Other spoilage bacteria*	*References*
	Inoculated peeled and cooked shrimp	MAP (50% CO_2, 30% N_2, 20% O_2), 5°C, 10 days	Wet dog	*C. maltaromaticum, C. divergens, C. mobile*	Laursen et al. (2006)
	Sea bream filet	Air/ MAP (60%CO_2, 10% O_2, 30% N_2), 5°C		*Pseudomonas* spp., *Enterobacteriaceae,* LAB, H_2S producin bacteria	Parlapani et al. (2014)
Formic acid	Model system and spoiled meat	Air		*Lactobacillus* spp.	Nychas et al. (2007)
				Leuconostoc spp., *Carnobacterium* ssp.	Nychas et al. (2008)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)
Hexanoic acid	Beef meat chops	Air, 4°C		*Pseudomonas* spp., *Enterobacteriaceae*	Ercolini et al. (2011)
2-Methyl butanoic acid	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
					Dainty and Hibbard (1983)
	Model system and spoiled meat	Air		/	Nychas et al. (2007)
					Nychas et al. (2008)
	Spoiled meat	Air, 5°C, 3 days		/	Dainty et al. (1985)
	Beef meat chops	Air, 4°C		*Pseudomonas* spp., *Enterobacteriaceae*	Ercolini et al. (2011)
3-Methyl butanoic acid	Inoculated beef meat	Air, 4°C, 7 days		/	Casaburi et al. (2014)
					Dainty and Hibbard (1983)
	Model system and spoiled meat	Air		/	Nychas et al. (2007)
					Nychas et al. (2008)
	Spoiled meat	Air, 5°C		/	Dainty et al. (1985)
Iso-butyric acid	Model system and spoiled meat	Air		/	Nychas et al. (2007)
					Nychas et al. (2008)
	Spoiled meat	Air, 5°C, 3 days		/	Dainty et al. (1985)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)
	Inoculated meat	Air		/	Dainty and Hibbard (1983)
Sulphur compounds					
Dimethyl sulphide	Spoiled meat	VP, 4°C		*Enterobacteriaceae, Clostridium algidicarnis, Clostridium putrefaciens, H. alvei, Lactobacillus curvatus, Lactobacillus sakei, S. liquefaciens, Shewanella Baltica*	Ercolini et al. (2011)
	Inoculated chicken breast fillets	MAP (30% CO_2, 70% O_2), 4°C, 7 days		/	Franke and Beauchamp (2017)

The spoilage effect of *B. thermosphacta* has been studied in different food matrices either from naturally contaminated products (Dainty et al., 1985, 1989; Dainty and Mackey, 1992; Ercolini et al., 2011; Noseda et al., 2012) or in challenge test experiments performed after inoculating sterile or low contaminated food matrices (Stanley et al., 1981; Joffraud et al., 2001; Jaffrès et al., 2011; Casaburi et al., 2014; Mikš-Krajnik et al., 2016; Franke and Beauchamp, 2017). As shown in Table 3, these studies reported that *B. thermosphacta* brings about spoilage by producing various undesirable VOCs comprising aldehydes, ketones, esters, alcohols, and small amounts of short chain fatty acids causing off-odors and off-flavors (Ordonez et al., 1991; Casaburi et al., 2014, 2015). This species also produces organic acids (lactic and acetic acids) and ethanol (Hitchener et al., 1979; Pin et al., 2002).

Some of the spoilage-related molecules produced by *B. thermosphacta* may have a very intense off-odor or off-flavor, and therefore affect the sensory quality of the product even at low levels (Refsgaard et al., 1999). For example, acetic acid has an undesirable pungent, acidic, and vinegar flavor even at low concentrations (Vermeiren et al., 2005). The amounts of the spoilage related molecules may vary depending on strains and on the nature and concentration of the substrates available (glucose, glycerol, ribose, branched-chain amino acid, and fatty acids among others) (Macaskie et al., 1984; Borch and Molin, 1989; Vermeiren et al., 2005).

The apparition of creamy/dairy and buttery odors are associated with the production of high amounts of acetoin and diacetyl, respectively (Stanley et al., 1981; Dainty and Hibbard, 1983; Dainty and Mackey, 1992; Nychas et al., 2008; Casaburi et al., 2014). Both compounds are major end products of the aerobic catabolism of glucose by *B. thermosphacta* (Blickstad, 1983; Pin et al., 2002).

In relation to seafood products, *B. thermosphacta* has been reported to have an important role in the spoilage of cooked and peeled shrimps stored under MAP (Mejlholm et al., 2005; Jaffrès et al., 2011), and of fresh and VP smoked salmon filets (Mikš-Krajnik et al., 2016); (Joffraud et al., 2001; Stohr et al., 2001). For example, in cold-smoked salmon *B. thermosphacta* produced mainly 2-hexanone and 2-heptanone responsible for the formation "blue-cheese" off-odor (Joffraud et al., 2001; Mejlholm et al., 2005; Laursen et al., 2006). In cooked and peeled shrimp, *B. thermosphacta* was related to the strong butter, buttermilk-like, nauseous, sour/fermented and cheese/feet off-odors associated with the production of 2,3-butanedione (diacetyl), 3-methyl-1-butanal, and 3-methyl-1-butanol (Mejlholm et al., 2005; Laursen et al., 2006; Jaffrès et al., 2011). It has been shown that factors affecting the biochemical composition of food products may determine the nature of the VOC and therefore affect the type and the intensity of spoilage (Koutsoumanis and Nychas, 1999; Skandamis and Nychas, 2002; Nychas et al., 2008). Other factors can influence spoilage by *B. thermosphacta*, as notably the presence of diverse bacterial genus and species in the food microbiota, and the possible interactions between them. Indeed, it has been reported that when co-inoculated on cooked and peeled shrimps, *B. thermosphacta* and *C. maltaromaticum* were able to form a typical wet dog off-flavor, whereas neither produced this off-flavor when inoculated separately (Laursen et al., 2006). No new metabolite was detected in *B. thermosphacta*–*C. maltaromaticum* co-cultures by comparison with those produced by both species inoculated alone. It can therefore be hypothesized that the wet dog off-flavor is most probably resulting from an interaction between metabolites formed by both species.

Obviously, a more in depth investigation is necessary to clearly understand the metabolic pathways which lead to the production of VOCs by *B. thermosphacta*.

Production of Biogenic Amines (BAs)

B. thermosphacta has been also associated with the production of biogenic amines (BAs) in some studies (Paleologos et al., 2004; Casaburi et al., 2014). These compounds, such as histamine, tyramine, tryptamine, putrescine, and cadaverine, often result from decarboxylation of free amino acids. Some BAs are responsible for strong putrid off-odors and some, as histamine, for foodborne poisoning. Histidine decarboxylase and tyrosine decarboxylase, responsible for histamine and tyramine synthesis, respectively, have been reported for several bacterial species. Although histidine decarboxylase activity has been reported in *B. thermosphacta* (Casaburi et al., 2014), no histidine decarboxylase gene was found in the genome (Stanborough et al. (2017). This may suggest that BAs production is strain dependent in *B. thermosphacta*, as already described in lactic acid bacteria (Coton and Coton, 2009). Furthermore, during co-cultures, *B. thermosphacta* has been reported to enhance the cadaverine production of *Escherichia coli* and can also promote the production of histamine by *Lactobacillus sakei* (Nowak and Czyzowska, 2011). This shows the difficulty to clearly demonstrate the spoilage activity of bacteria in food products.

Strategies for Fighting *B. thermosphacta* Food Spoilage

Biopreservation

Biopreservation has been described in 1996 by M.E. Stiles as "extended storage life and enhanced safety of foods using the natural microflora and (or) their antibacterial products" (Stiles, 1996). The addition of bacteria as protective cultures, of their metabolites like bacteriocins or other antagonistic compounds, or the use of bacteriophages having antagonistic effects reflect examples of biopreservation. The inhibition of *B. thermosphacta* using bacteriocins has primarily focused on nisin. This bacteriocin, produced by *Lactococcus lactis*, is applied as a food preservative (E234) in the food industry, particularly in some dairy products but not in meat or fish where *B. thermosphacta* commonly occurs. However, several studies focused on the addition of nisin in beef carcasses and meat products to inhibit the growth of *B. thermosphacta* (Cutter-Nettles and Siragusa, 1996a,b, 1997; Tu and Mustapha, 2002). Cutter-Nettles and Siragusa (1994) demonstrated that nisin spray treatment of beef carcasses can reduce the level of *B. thermosphacta* by up to 3.6 log·CFU cm^{-2} after aerobic incubation during 24 h at 4°C. The same authors showed later on that nisin spray treatment combined by VP was more effective as resulting in reducing *B. thermosphacta* by up 4.5 log CFU cm^{-2}

(Cutter-Nettles and Siragusa, 1996b). Additionally, nisin immobilized in a calcium alginate gel limited the growth *B. thermosphacta* on ground beef and beef carcasses to undetectable levels (<1.30 log CFU g^{-1}) (Cutter-Nettles and Siragusa, 1996a, 1997). Other authors demonstrated that nisin and nisin combined with EDTA treatments followed by VP could increase the shelf life of fresh beef stored at 4°C up to 25 days by inhibiting *B. thermosphacta* (Tu and Mustapha, 2002).

Brochocin-C, the two peptide bacteriocin produced by *B. campestris* ATCC 43754, inhibits *B. thermosphacta* and other closely related Gram-positive species associated with food and meat such as *Listeria* ssp., *Lactobacillus* spp. and *Carnobacterium* spp. (Siragusa and Cutter-Nettles, 1993; Greer and Dilts, 2006). Brochocin-C has been described as a potential food preservative since this bacteriocin was shown to be heat stable at 100°C and its activity preserved at pH ranging from 2 to 9 (McCormick et al., 1998). However *B. campestris* can also grow and cause spoilage on vacuum packed lamb making the use of this bacteriocin producing bacterium not suitable for meat biopreservation (Gribble and Brightwell, 2013).

Protective cultures active against *B. thermosphacta* in laboratory medium and also in meat and seafood products have been reported (Fall et al., 2010, 2012; Leroi et al., 2012, 2015; Olaoye et al., 2015; Hwanhlem et al., 2017). Inoculation of cooked and peeled shrimps with *Lactococcus piscium* CNCM I-4031 inhibited the growth of *B. thermosphacta* by 3–4 log CFU g^{-1} (Fall et al., 2012) and totally stopped its growth in cooked-smoked salmon (Leroi et al., 2015). Additionally, *L. lactis* subsp. *lactis* I23, a nisin producer, and the multi-bacteriocin producer strain *Lactobacillus curvatus* CRL705 and other lactic acid bacteria prevented the growth of *B. thermosphacta* in meat (Castellano and Vignolo, 2006; Russo et al., 2006; Olaoye et al., 2015).

Bacteriophages have also been proposed for biopreservation as an alternative for extending the storage quality of refrigerated meats (García et al., 2008). Sensory analysis of pork tissue showed a better acceptability of samples inoculated with *B. thermosphacta* and a phage solution than those inoculated only with *B. thermosphacta*, which produced undesirable odors within 4 days making the product rejected by the panel (Greer and Dilts, 2002).

Organic acids, often naturally produced by microorganisms, are used in food industry as food preservatives (Theron and Lues, 2007). The effect of lactic acid on *B. thermosphacta* has been evaluated (Grau, 1980; Niemand et al., 1983; Greer and Dilts, 1995). According to Grau (1980), lactic acid is more effective for inhibiting the growth of *B. thermosphacta* under anaerobic condition. Moreover, the product characteristics influence the inhibitory effect of lactic acid on *B. thermosphacta*. For example, lactic acid causes a stronger inhibition in fat tissue than in lean tissue (Greer and Dilts, 1995).

Plant-Derived and Other Antimicrobials

Many plants and their derived essential oils are known to delay or inhibit the growth of bacteria, yeast, and molds. These compounds have been therefore investigated for their potential and applicability to preserve food (Tiwari et al., 2009). Some studies have been published on the inhibitory effects of plant-derived antimicrobials on *B. thermosphacta* (Nowak et al., 2012; Casaburi et al., 2015; D'Amato et al., 2016). For example in beef stored under oxygen-enriched MAP, thyme and rosemary essential oils have been shown to prevent the growth of *B. thermosphacta.* However, the level of their bactericidal effect varied depending on the strain they were applied to Nowak et al. (2012). Thyme essential oil was also found to strongly inhibit the growth of *B. thermosphacta* in pork meat (D'Amato et al., 2016). Thus, these antimicrobial molecules could be good alternative methods for extending the shelf life of meat. However to be suitable for commercial application, the amount of essential oils to be added is determinant for the acceptance, since their strong aromas might be transmitted to food products (Chouliara et al., 2007).

Physical Treatments

The effect of physical treatments used in food processing, as heating, gamma irradiation of high hydrostatic pressure, has been evaluated. *B. thermosphacta* is heat sensitive bacterium that is destroyed by a heating at 63°C during 5 min (Stackebrandt and Jones, 2006). *B. thermosphacta* has been reported more resistant to irradiation than other meat spoilage organisms such as *Pseudomonas* (Giroux et al., 2001). Although it has been frequently isolated from irradiated meat and poultry, this species is affected by irradiation doses of 0.5 and 2.0 kGy (Stackebrandt and Jones, 2006). Various studies have shown that high hydrostatic pressure is of great interest for food industry to extend the shelf-life of solid food products as it can inactivate most micro-organisms. Inactivation of *B. thermosphacta* has been evaluated in high pressure treated ground beef (Ouattara et al., 2002), ready-to-eat meats (Hayman et al., 2004), cooked ham (Han et al., 2010), and Indian white prawn (Ginson et al., 2015). In the case ready-to-eat meat stored at 4°C, high pressure was shown to reduce *B. thermosphacta* counts to undetectable levels until 95 days after the treatment (Hayman et al., 2004). In vacuum-packed cooked ham similar results were observed with *B. thermosphacta* counts maintained at 2 log CFU g^{-1} 90 days after the treatment (Han et al., 2010).

Genomic Characteristics of *B. thermosphacta*

To date, 12 *B. thermosphacta* and one *B. campestris* draft genome sequences are publicly available. Genome sequences of the type strains *B. thermosphacta* ATCC 11509 and *B. campestris* ATCC 43754 were sequenced by DOE Joint Genome Institute (JGI) in 2014 in the framework of the project: Genomic Encyclopedia of Bacteria and Archaea (GEBA). More recently, eleven draft genome sequences of *B. thermosphacta* strains were determined (Stanborough et al., 2017). These strains were isolated from various meat products stored aerobically or under MAP.

Table 4 Genome overview of *B. thermosphacta* strains and *B. campestris*

Strain name	*Source*	*Genbank accession Number*	*Genome size (Mb)*	*GC content (%)*	*CDS*	*Contigs/scafolds*
B. thermosphacta						
DSM 20171[a]	Pork sausage	MDLK00000000	2.49	36.4	2290	33/33
7803	Beef[b]	MDLL00000000	2.57	36.3	2348	61/61
7804	Beef[b]	MDLU00000000	2.61	36.3	2415	56/56
7806	Beef[c]	MDLM00000000	2.54	36.3	2328	41/41
7807	Lamb[c]	MDLN00000000	2.48	36.4	2277	31/31
7808	Lamb[c]	MDLO00000000	2.56	36.3	2328	68/68
7809	Beef[c]	MDLV00000000	2.49	36.3	2273	22/22
7810	Beef[c]	MDLP00000000	2.47	36.4	2244	22/22
7811	Beef[c]	MDLT00000000	2.55	36.3	2292	186/186
7813	Minced veal[b]	MDLQ00000000	2.64	36.2	2417	166/166
7816	Lamb[c]	MDLR00000000	2.58	36.3	2342	154/154
7818	Beef[c]	MDLS00000000	2.59	36.3	2364	130/130
B. campestris						
DSM 4712[a]	Soil	NZ_AODH01000000	2.37	40.2	2424	128/128

[a]Type strain
[b]Stored under MAP
[c]Stored under aerobic conditions

Table 4 summarizes the characteristics of these genomes. All are draft genome sequences. Their size is quite similar, ranging from 2.43 to 2.64 Mb with a G+C content from 36.2% to 36.4%. Genome-based analysis performed by Stanborough et al. (2017) showed a high degree of genomic sequence similarity between the *B. thermosphacta* strains. The genome analysis highlighted diverse repertoire of substrate-specific genes from the phosphotransferase system (PTS). This includes the genes for glucose, maltose, sucrose, fructose, mannose, trehalose, cellobiose, β-glucoside, mannitol, and N-acetylglucosamine transport and phosphorylation systems. Ribose, glycerol-3-phosphate, maltose/maltodextrin and inositol transporter genes were also identified. All genes required for glycolysis were present. All genes required for the pentose phosphate pathway were also found, while coding sequences (CDS) for only four of the eight enzymes of the citrate cycle (TCA cycle) were detected. This analysis revealed also the presence of genes involved in the production of malodorous compounds as acetoin and 2,3-butanediol, with the presence of genes coding for α-acetolactate synthase, acetolactate decarboxylase and an (S,S)-butanediol dehydrogenase. As it has been suggested that free fatty acid production in *B. thermosphacta* results from amino acid catabolism rather than from lipolysis (Holley, 2014). Stanborough et al. (2017) have also searched for genes involved in the degradation of the branched-chain amino acids. They found all enzymes of these pathways leading to the production by *B. thermosphacta* of the branched-chain fatty acids isovaleric, isobutyric, and 2-methylbutyric acids from the degradation of leucine, valine, and isoleucine, respectively. These fatty acids are also responsible for off-odors production on food (Casaburi et al., 2014, 2015). As mentioned above no gene for histidine decarboxylase and histamine production was detected. Stress response regulatory genes were also identified, as for example the alternative sigma factor σ^B, which most probably plays important roles in the survival and growth under high-salt conditions and adaptation to refrigeration temperatures, and in the spoilage process. This suggests that despite the high degree of genomic sequence similarity between the *B. thermosphacta* strains, the spoilage potential for each strain is most probably related to the regulation of spoilage-specific genes expression.

Despite the temporal and the geographic diversities of the strains used for genome comparison, and also the difference in term of the meat source and their packaging conditions for strain isolation, the strain panel used does not represent an exhaustive diversity of *B. thermosphacta*. Indeed, this species has been isolated from various other ecological niches including seafood products and the environment (Stackebrandt and Jones, 2006). Therefore, sequencing other strains isolated from wider origin may help to better estimate and evaluate the diversity of *B. thermosphacta*.

Conclusion

Fig. 2 summarizes the information available on the spoilage potential of this bacterium depending on the food matrix and the storage conditions. *B. thermosphacta* is gaining the interest of researchers because it is increasingly isolated from various meats and seafood. It has been even recognized as a specific spoilage microorganism of meat products. Several studies aimed at understanding its metabolism as a function of the environmental parameters. Recently, analyzing the genome content of several *B. thermosphacta* isolates identified several genes involved in the metabolic pathways related to the development of spoilage reactions already described in *B. thermosphacta*. However, most of the genes responsible for the high number of molecules involved in the spoilage caused by *B. thermosphacta* remain yet unknown (Fig. 2). The limited genetic diversity among *B. thermosphacta* genomes may result

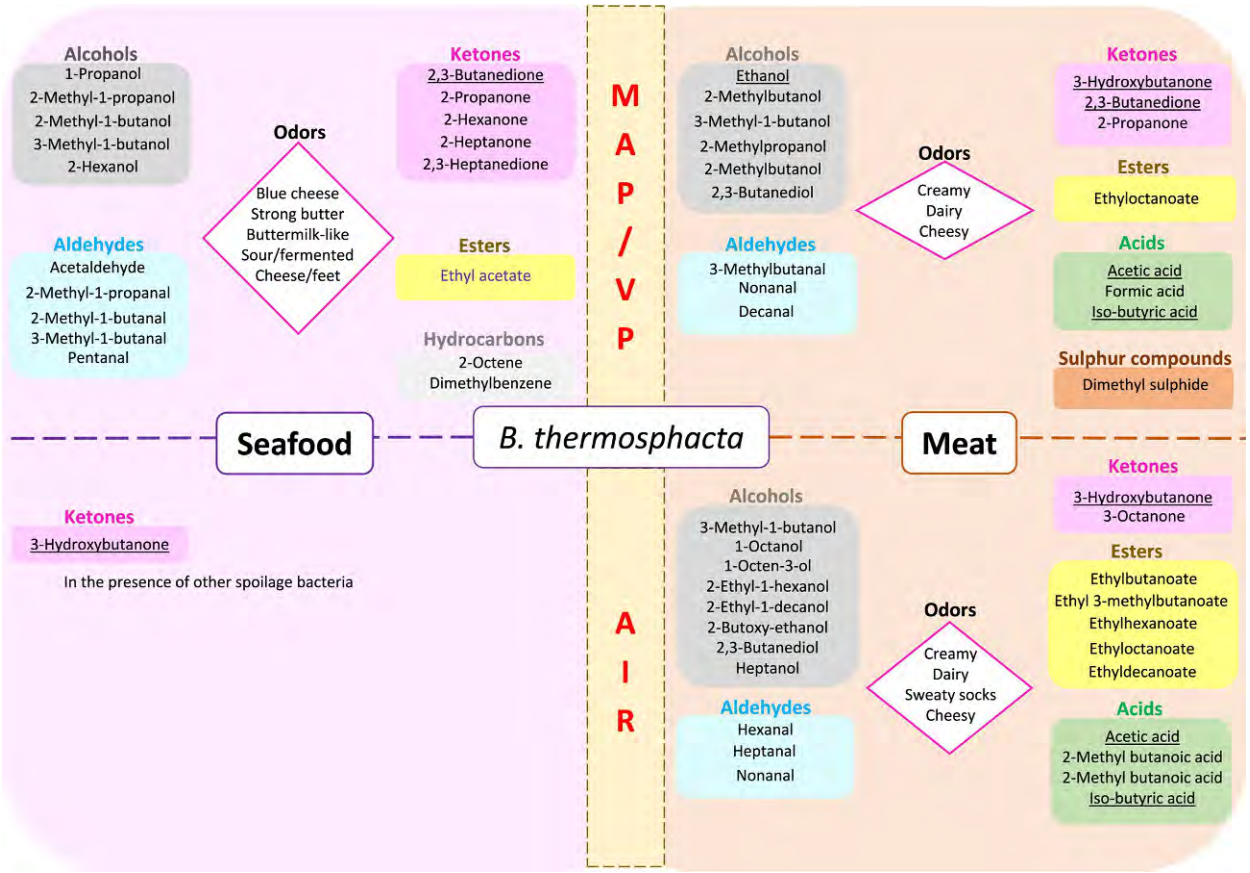

Fig. 2 Spoilage potential of *B. thermosphacta*. VOCs and off-odors produced in meat and seafood stored under air or MAP/VP are indicated. VOCs, which biosynthetic pathways are known, are indicated in underlined.

from the narrow ecological niche (red meats) for selecting strains to be sequenced. Therefore, sequencing the genome of other strains isolated from wider sources may help to better estimate and evaluate the diversity of *B. thermosphacta*.

In order to better understand *B. thermosphacta* spoilage, there is still a need for revealing the role of specific genes that contribute significantly to food spoilage. Discovering the influence of abiotic factors like food composition and storage conditions and of biotic factor (composition of the microbiota contaminating food) should help identifying genes responsible for spoilage activity and proposing strategies for fighting *B. thermosphacta*. A link between spoilage molecules and gene expression should be established for assessing the metabolic pathways involved in food spoilage. This should enable to deduce pertinent spoilage biomarkers (genes or molecules) for developing technologies that can prevent meat and seafood spoilage by this bacterium.

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Capsules and Secreted Extracellular Polysaccharides

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CPS and EPS Structures

Capsular polysaccharides (CPSs) maintain an association with the cell surface that is retained on centrifugation and this typically involves some form of covalent linkage to the surface. This results in a protective surface layer known as the capsule, which is clearly distinguished in micrographs (Fig. 1). In contrast, secreted exopolysaccharides (EPSs) have limited association with the cell surface post-export. Nevertheless, encapsulated bacteria inevitably shed surface material during growth, so CPS and EPS distinctions can be complicated.

It is also impossible to distinguish CPS from EPS based on the glycan components alone and these encompass a large and highly diverse range of structures. A few examples relevant to this review are presented in Figs. 2 and 3. The glycans can be composed of a wide variety of sugars and non-carbohydrate substituents, with a range of glycosidic linkages, creating polymers with repeat-unit structures of varying complexity. Most of the known structures are catalogued in a searchable database (http://csdb.glycoscience.ru/index.html). Structural variability can occur within a single species resulting in different serotypes. For example, there are ~80 recognized CPS serotypes each in *Escherichia coli* and *Klebsiella pneumoniae* (where they are called K antigens) and ~100 in *Streptococcus pneumoniae*, although genomics data from isolate collections suggest the actual numbers for some are potentially higher. While anionic properties are most common, some of the glycans carry no net charge. In a few rare cases, some CPSs carry both positive and negative charges due to free amines and carboxylates; the polysaccharide A (PSA) CPS of *Bacteroides fragilis* provides a high-profile example of such zwitterionic polymers and their unusual biological properties are described below. The polysialic acid CPS shared by *E. coli* K1 and group B meningococci, and the glycosaminoglycan-related *E. coli* K5 CPS, are examples of CPS structures resembling mammalian glycans and they have important implications for pathogenesis. Cellulose is an important structural component in plants and is often described as the most abundant biopolymer on earth; it is also produced as an EPS by a wide range of bacteria.

Decoration of peptidoglycan with additional glycans is a recurring theme in Gram-positive bacteria. Wall teichoic acids provide the best studied examples and are attached through a phosphodiester linkage and a linker oligosaccharide to the C6 position of muramic acid residues in the peptidoglycan backbone. A similar strategy links mycolylarabinogalactan to the peptidoglycan of mycobacteria. Covalent linkage to peptidoglycan is also employed to secure CPS in streptococci and *Staphylococcus aureus*. Although the precise structural arrangement is uncertain, serotype III CPS in group B streptococci is also linked to peptidoglycan via a phosphodiester bond but the site of linkage is the C6 of GlcNAc residues, rather than muramic acid. However, this linkage mode is not universal for streptococcal capsules because CPS from pneumococcus serotype 2 (and several other serotypes) are linked instead via a glycosidic linkage to the C6 position of peptidoglycan GlcNAc residues.

Despite extensive research, the mechanism(s) by which most Gram-negative CPS are linked to the cell surface have not been elucidated. Non-covalent charge-based interactions with other components on the cell surface may be important but these do not adequately explain the stable association with the cell surface and the inability to easily remove CPS with salt washes. Where the linkage of CPS to Gram-negative cell surfaces is known, the anchor involves a glycolipid. In the most prevalent mechanism, several CPS serotypes of *E. coli* employ a conserved glycolipid composed of several β-linked 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues, attached to (lyso)phosphatidylglycerol (Fig. 2). These capsules are collectively called "group 2" in *E. coli* due (in part) to a conserved assembly process. Meningococcal capsules are anchored by the same glycolipid and, based on conserved biosynthetic enzymes, this strategy is also used by a variety of other pathogens including *Haemophilus influenzae* and *Campylobacter jejuni*. The involvement of a Kdo linker between the glycan and lipid moities is reminiscent of lipopolysaccharide (LPS) structures. However, they are distinguished by the use of α-linked Kdo residues in LPS and β-linked Kdo in CPS. Human-adapted Salmonellae (including isolates of serovar Typhi) and some soil bacteria belonging to the Burkholderiales produce a CPS called Vi antigen, which is anchored by a different method. Vi antigen is composed of α-linked poly-*N*-acetylgalactosaminuronic acid modified with 3-*O*-acetyl residues, resembling a modified pectin. The glycolipid anchor for Vi antigen consists of an unidentified *N*-acetylhexosamine residue modified with 2 acyl chains (Fig. 2), although the exact structure has not been determined yet.

The relationships between LPS-linked polysaccharides (O antigens) and capsular polysaccharides are sometimes difficult to resolve. In *E. coli*, group 4 capsules (also called O antigen capsules) possess the same repeat-unit structures as LPS-linked O antigens and LPS-free CPS; serotype K40 provides an example. In contrast, assembly and attachment of high-molecular-weight group 1 CPS

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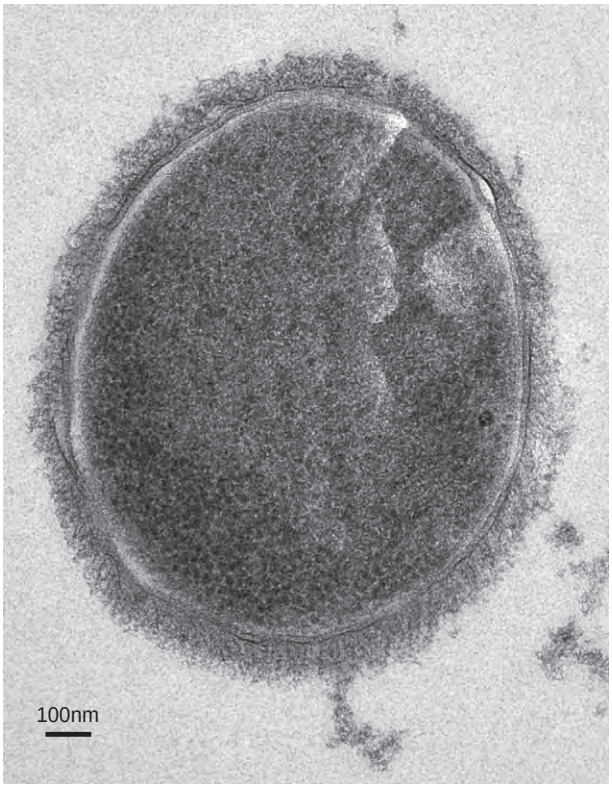

Fig. 1 Electron micrograph of encapsulated *E. coli* possessing a K1 capsule. Courtesy of Dr. Elyse Roaches, University of Guelph.

in *E. coli* (such as K30) is independent of LPS, but short K30 oligosaccharides can be linked to LPS. No covalent anchoring mechanism has been identified to explain how group 1 and 4 capsules are attached to the cell surface but Kdo-containing glycolipids are certainly not involved.

Production of CPS and EPS is not mutually exclusive. As an example, the envelope stress-regulated EPS, colanic acid, is produced by many isolates of *E. coli* and *Salmonella*. *E. coli* isolates with group 2 capsules also produce colanic acid under appropriate conditions. In contrast, those with group 1 capsules do not because the CPS biosynthesis gene locus has replaced the colanic acid locus. Like group 1 CPS, short oligosaccharides of colanic acid can be linked to LPS but the polymeric form is LPS-free. The ability of bacteria to produce and secrete multiple glycans derived from different assembly pathways is highlighted by Mycobacteria, which possess capsules composed of long chain α-glucans and release complex glycans including arabinomannan and mannan into the surrounding environment.

Functions of CPS and EPS

In 1928, F. Griffiths published a classic study that demonstrated the dependence of pneumococci on CPS production for the ability to cause infection in mice. These experiments have been particularly influential because they also demonstrated the transfer of genetic material and the concept of natural transformation, more than a decade before DNA was identified as the molecule carrying genetic information. CPS is now known to be a pivotal virulence factor for pneumococci and for many other pathogens of humans and livestock.

The complement system plays a critical role in host innate immunity. Bacteria possess a range of cell-surface molecules that activate the complement pathway, and the presence of a highly hydrated capsule structure can prevent the interaction of surface-bound complement with receptors on phagocytic cells. This protection is potentially overcome by deposition of C3b on CPS itself, so bacteria have developed different strategies to avoid this. For example, serotype III group B streptococci produce large amounts of CPS and the presence of Neu5Ac (sialic acid) residues mediates crucial interference with regulatory proteins that activate the alternative pathway. A comparable strategy (with different regulatory targets) occurs with polysialic acid CPS produced by *E. coli* K1 and serotype B meningococci (Fig. 2).

Opsonophagocytosis of encapsulated bacteria can be achieved via the classical pathway in the presence of CPS-specific antibodies. This provides the essential foundation for several CPS-based vaccines that have had major impact in reducing the burden of infectious diseases on a global scale. Examples include successful vaccines against *H. influenzae*, meningococci,

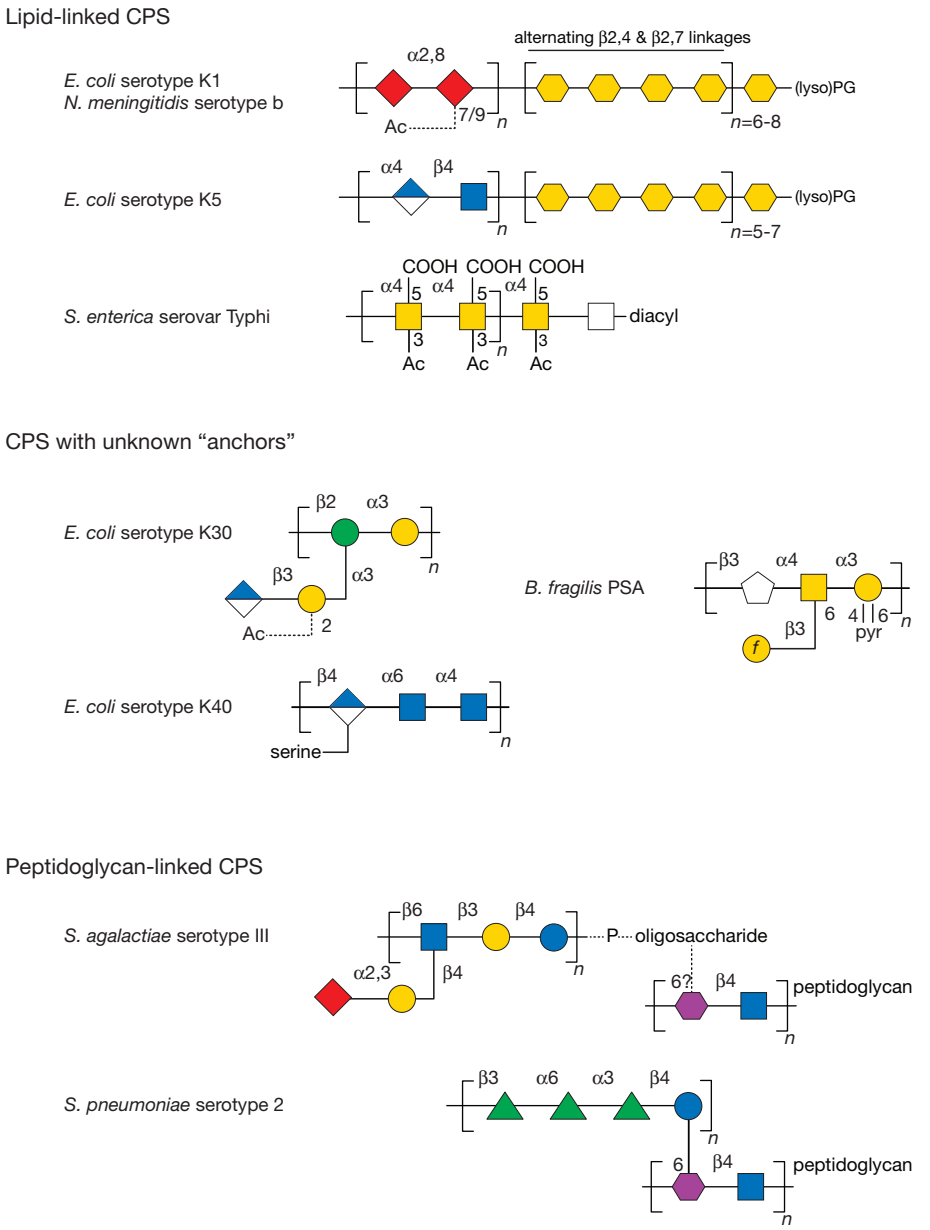

Fig. 2 Structures of examples of capsular polysaccharides. Shown are the repeating unit structures and the modes of attachment to the cell surface (where known). The carbohydrate structures are given using the symbol convention for the sugars. Fructose ⬟, galactose ●; galactosamine ■; glucose ●; glucuronic acid ◆; mannose ●; rhamnose ▲; glucuronic acid; *N*-acetylgalactosamine ■; *N*-acetylglucosamine ■; *N*-acetylmuramic acid ⬢; *N*-acetylneuraminic acid ◆; 2-acetamido-4-amino-2,4,6-trideoxygalactose ⬠; 3-deoxy-D-manno-oct-2-ulosonic acid ⬢; unidentified *N*-acetylhexosamine □.

and pneumococci. The majority of polysaccharides are T cell-independent antigens and the T cell-dependent (memory) response in effective vaccines is achieved by conjugating CPS (or fragments of CPS) to appropriate carrier proteins. The challenge of serotype diversity can be addressed with multivalent vaccines that encompass prevalent clinical serotypes. For example, Prevnar-13 targets 13 serotypes of pneumococcus and achieves sales in excess of a billion dollars annually. The effectiveness of vaccines that target defined serotypes can diminish over time due to the emergence of serotypes not covered by the vaccine, requiring new formulations. As successful as these approaches have been, they cannot overcome the problem of CPS that are poorly immunogenic due to structures that mimic "self" host cell glycans. Classic examples include a variety of CPS structures resembling host glycosaminoglycans and polysialic acids. Polysialic acid CPS mimics the glycans found on neural cell adhesion molecule (N-CAM) and certain other host components and helps neuropathogens cross the blood-brain barrier, increasing their invasiveness and leading to the high morbidity and mortality associated with meningitis. A relatively recent vaccine effective against group B meningococci (e.g., Bexsero) therefore relies on targeting multiple protein epitopes, rather than CPS.

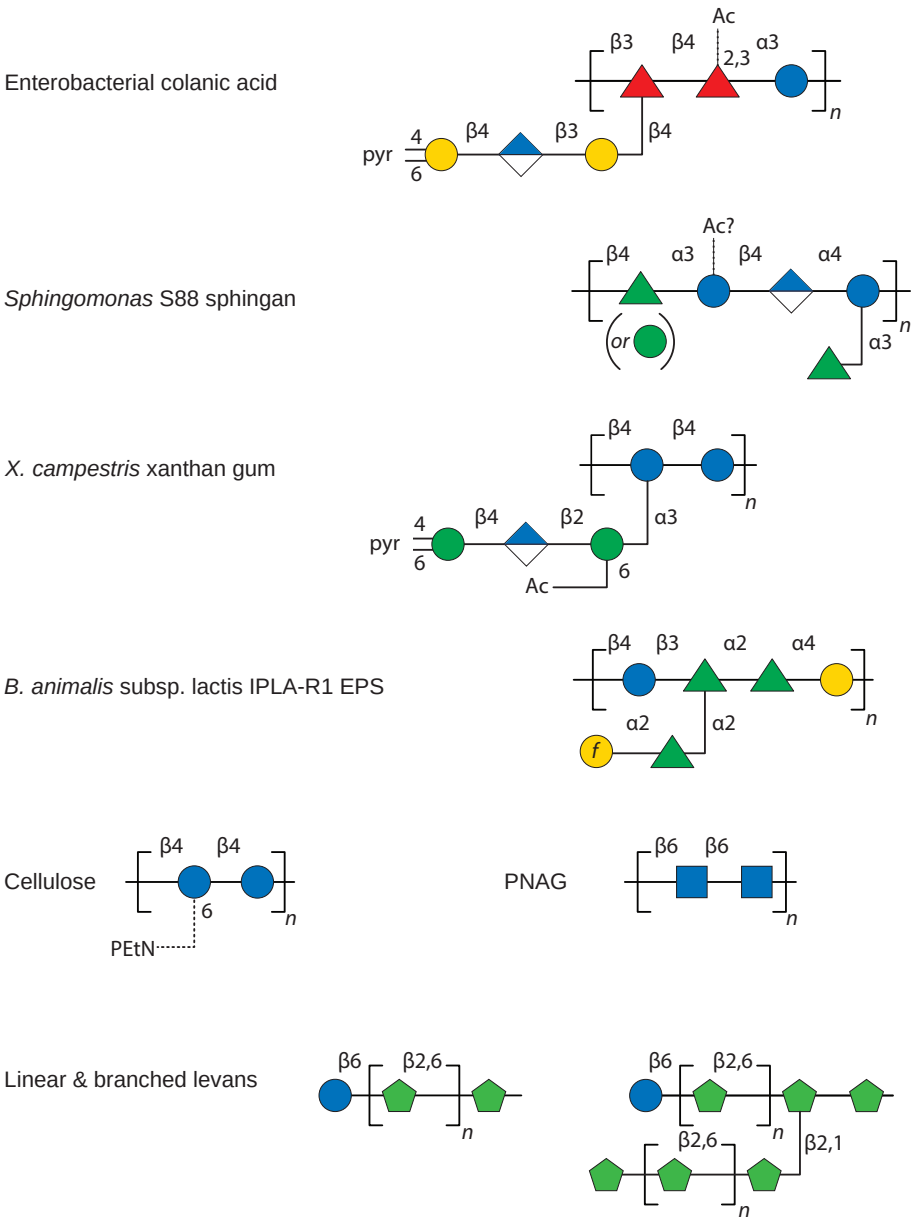

Fig. 3 Structures of examples of extracellular polysaccharides. Shown are the repeating unit structures. The carbohydrate structures are given using the symbol convention for the sugars. Fucose ▲, galactose ●; galactosamine ■; glucose ●; glucuronic acid ◆; mannose ●; rhamnose ▲; *N*-acetylgalactosamine ■; *N*-acetylglucosamine ■. The modification of bacterial cellulose with phosphoethanolamine (PEtN) is a recent discovery in γ- and β-proteobacteria.

The interaction between polysaccharides and the immune system is more complicated with zwitterionic structures. These are sometimes produced by commensal bacteria, such as *B. fragilis*, a low abundance anaerobic commensal in the gut microbiota. This organism is capable of producing at least eight CPS structures but only PSA and PSB are zwitterionic and PSA has been studied in most detail. Unlike other typical glycan structures, PSA is presented in a manner resembling protein antigens, and it induces a potent T cell-dependent response. Experiments in germ-free mice revealed that PSA influences the maturation and development of the innate immune system, as well as the maintenance of its homeostasis. In addition, PSA makes critical contributions to reducing potentially harmful inflammatory responses caused by other members of the gut microbiota. Collectively, these effects provide a major benefit in human health.

Lactic acid bacteria in the intestinal microbiota are also implicated in homeostasis of innate and adaptive immunity and their EPS may also possess immunostimulatory properties. Their ability to promote proliferation of macrophages and modulate levels of pro- and anti-inflammatory cytokines has been known for some time and several studies have also suggested certain lactobacillus EPS possess the ability to suppress the proliferation of tumor cell lines. Levans have been ascribed a wide variety of properties, including provision of dietary fiber, as well as offering antiobesity, anticholesterolemic, hyoplipidemic, antioxidant, and

immunstimulatory activities. The underlying mechanisms for these effects are mostly uncertain but they are presumably important elements in the probiotic capabilities of bacteria such as lactobacilli and *Bifidobacterium* species.

While the prior discussion focuses on interactions with mammalian (primarily human) hosts, CPS and EPS structures also play important roles in plant-associated bacteria. Plant symbionts, such as members of the Rhizobiaceae, produce complex branched EPSs (e.g., succinoglycan) with frequent non-sugar substitutents, as well as CPS structures which bear structural and biosynthetic relationships to the group 2 capsules of *E. coli*. These glycans are essential for success of the symbiotic relationship with legumes, although their precise involvement is not fully understood. Some functional overlap seems to exist between various bacterial surface glycoconjugates in these bacteria, reflecting the complexity of host-symbiont interactions. EPS and CPS may be involved in the later stages of infection thread formation and nodule development. However, they are also implicated in suppressing the plant host defense response. In plant pathogens, EPS (such as xanthan gum in *Xanthomonas campestris*) causes wilting by blocking xylem vessels and this can lead to the production of lesions that promote invasion and survival in plant tissues.

The biofilm mode of growth is a key part of the lifestyle of many bacteria, occupying a wide range of environmental niches. In biofilm communities, bacteria are embedded in a matrix of proteins, polysaccharides and extracellular DNA. As a result, CPS and EPS contribute (directly or indirectly) to the wide range of properties ascribed to biofilms. CPS is implicated in the formation of intracellular biofilm-like communities (IBCs) in uropathogenic *E. coli* and O antigen capsules are important for gallstone biofilms formed by *Salmonella*. However, such examples are limited and in some cases CPS actually impair biofilm formation. In contrast, EPS are central architectural components in biofilms. The importance of poly-*N*-acetylglucosamine (PNAG) and cellulose (Fig. 3) is well documented in this context. Cellulose production is predominantly a Gram-negative trait and the capacity of cellulose to assemble into filaments, as well as higher-order structures with proteins, is an important element in its function. PNAG was initially reported as polysaccharide intercellular adhesin in *S. aureus* but is prevalent in a remarkably wide range of Gram-positive and Gram-negative bacteria. Other bacteria make EPS that share structural features (and presumably physical properties) with PNAG; PEL EPS from *Pseudomonas aeruginosa* provides a good example. Growth in biofilms is often associated with elevated intrinsic resistance to antibiotics and several studies with bacteria including *P. aeruginosa* and *Acinetobacter baumannii* have described the ability of purified EPS to bind or adsorb antibiotics. Such associations depend on the glycan structure and the antibiotic examined, so structural principles are not understood, nor is the overall importance of this process relative to the additional cellular defenses against antibiotics. Some negatively-charged EPS are known to bind metals and recent work with colanic acid in *E. coli* suggests that this may contribute to preserving transmembrane potential during envelope stress in biofilms. Finally, EPS has been implicated in the ability of several bacteria to survive dessication.

While the majority of functional studies have focused on microbial pathogenesis and host interactions, CPS also play a pivotal role in protecting against infection and possible lysis by bacteriophages. The CPS layer prevents the bacteriophage from interacting with (more conserved) receptors at the cell surface. To counter capsular layers, some bacteriophages possess highly specific glycosidases in the tail assembly that cause local CPS depolymerization and allow access to underlying receptors. The host range of these bacteriophages is typically restricted to cells bearing a particular capsule structure since engagement of the glycanase seems to be required for infection. However, possession of glycanases with multiple specificities is possible, with one recent example encoding genes for nine CPS depolymerases active against different *K. pneumoniae* CPS types. Nevertheless, resistance to bacteriophage infection may be a leading driver for the diversification of CPS structures within many species. Given the current interest in bacteriophage therapy, it is perhaps not surprising that capsule-specific bacteriophages have been used to clear model infections by encapsulated bacteria. The depolymerases themselves are considered to have potential as therapeutic agents for acute infections, since their activity removes a cellular layer which is deployed to protect the pathogen against host defenses.

Biosynthesis of CPS and EPS

The basic principles of the biosynthesis of CPS and EPS are shared with those of LPS O antigens. Both require activated sugar nucleotide precursors in the cytoplasm and end with a polymerized glycan at the external face of the cytoplasmic membrane. Three different strategies have been described for CPS/EPS biosynthesis and these are distinguished by the type of acceptor used in polymerization, the membrane topology of the polymerization step, and the process for export across the cytoplasmic membrane (Fig. 4). Post-polymerization the cell envelope of Gram-negative bacteria dictates additional complexity for surface translocation of the product. The main assembly approaches are described here but some emerging systems have features that cross the boundaries.

Wzy Polymerase-Dependent Systems

Wzy-dependent polysaccharide biosynthesis was first described in the late 1960s in O antigen-biosynthesis. This process involves undecaprenol phosphate as an acceptor and appears to be the predominant mechanism when the glycan product has a complex branched structure. Examples include the group 1 CPS of *E. coli*, most streptococcal CPS, as well as colanic acid, xanthan gum and EPS from lactobacilli (Figs. 2 and 3). Their biosynthesis pathways are characterized by generation of undecaprenyl diphosphate-linked repeat units in the cytosol. The process (Fig. 4) is initiated by a phosphoglycosyltransferase (PGT) enzyme, with the pyrophosphoryl linkage in the lipid intermediate created by transfer of a sugar-1-phosphate from a nucleotide diphosphosugar to the undecaprenol phosphate acceptor. Additional glycosyltransferases then complete the carbohydrate structure in the repeat

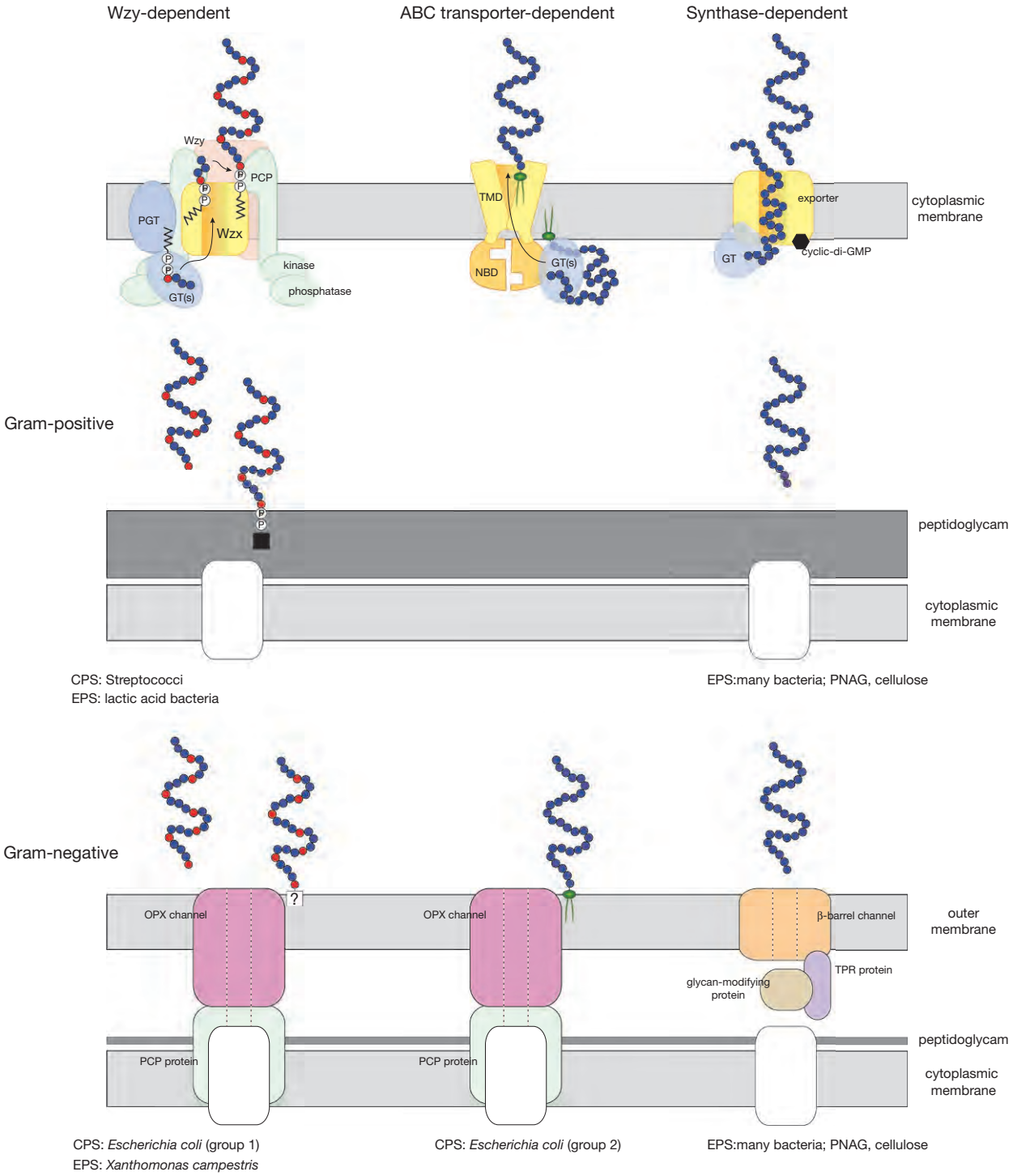

Fig. 4 Cartoon showing the three main assembly strategies for production of CPS and EPS from sugar nucleotide donors. The upper panel shows the typical components of membrane-embedded assembly complexes and these are discussed in detail in the text. The products of the assembly processes can follow different paths in Gram-positive and Gram-negative bacteria. The lower panel shows the typical components for glycan translocation in Gram-negative bacteria. The manner in which the translocation complexes penetrate the peptidoglycan is unknown. Generalized working models are described here but some emerging systems have features that cross the boundaries, so exceptions do exist. The unfilled shapes in the cytoplasmic membrane in the lower two panels represents the relevant biosynthesis complex for each system.

unit, which can be further modified by non-carbohydrate substituents (usually in the cytosol). This is followed by export across the cytoplasmic membrane by a Wzx protein homolog; these are membrane transport proteins belonging to the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) family. The MurJ flippase involved in peptidoglycan biosynthesis is another MOP family member. Following export, the lipid-linked repeat units serve as substrates for the strategy-defining Wzy polymerase, whose

catalytic-site is located at the periplasmic side of the membrane. The structure and mechanism of the integral membrane Wzy protein remains a key open question in this field. The polymerization reaction is regulated in an unknown manner by polysaccharide copolymerase (PCP) family 2 proteins, which possess cytosolic bacterial tyrosine (BY) kinase activity. Most Gram-negative members are PCP-2a proteins and contain transmembrane and cytosolic kinase domains in a single polypeptide, while the Gram-positive PCP-2b versions frequently separate these domains in a two-polypeptide format. The polymerization activity is dictated by cycling the phosphorylation state of C-terminal tyrosine residues, through the opposing activities of the PCP-2a/b kinase and a cognate phosphatase. Oligomers of PCP-2 proteins facilitate transphosphoryation between monomers in the complex and, in the streptococcal example, phosphorylation also coordinates capsule production with cell division.

In Gram-positive bacteria, the finished product is ligated to oligosaccharides of peptidoglycan. This reaction has clear parallels with the late steps in wall teichoic acid assembly. The phosphodiester linkages attaching teichoic acids to muramic acid residues are catalyzed by proteins belonging to the LytR-Cps2A-Psr family. The inclusion of Cps2A in this family suggests a common linkage mechanism for attachment of capsules but the actual situation is still unclear given the linkage of CPS to GlcNAc, the apparent use of phosphodiester and glycosidic bonds in different systems, and the retained CPS attachment in some *cpsA* mutants.

In Gram-negative bacteria, undecaprenyl diphosphate-linked oligosaccharides provide substrates for LPS O-antigen ligase and, after attachment of O antigen to LPS, the finished molecules then enter the LPS translocation pathway. In contrast, high-molecular-weight glycans follow a specific CPS/EPS translocation pathway composed of a tetramer of PCP-2a proteins interacting with an oligomer of outer membrane polysaccharide export (OPX) proteins. This creates a contiguous channel spanning the periplasm and outer membrane for CPS. Unlike porin proteins, which use β-strands to form channels across outer membranes, the prototype OPX protein (Wza from *E. coli*) uses amphipathic α-helices. Wza forms a stable octameric structure that contains the nascent CPS/EPS during its passage to the cell surface but details of the entry of the glycan from the polymerization center into the channel are unknown. Working models have involved comparison with tripartite drug efflux pumps in Gram-negative bacteria, with the PCP component fulfilling the role of the adapter proteins that provide an interface between the membrane pump and the outer membrane channel.

The features that distinguish CPS and EPS in this late step are also still unanswered. EPS could potentially be released by an incomplete Wzy reaction that would normally transfer the nascent glycan from its undecaprenol diphosphate carrier to the next repeat unit. However, this does explain retention of capsules, where no additional anchor molecule has yet been identified. While it is possible that the nascent glycans are never released from the final undecaprenol diphosphate carrier in the capsular form, this would limit each assembly complex to the production of a single CPS chain. Such questions offer intriguing avenues for future research.

Export of CPS Glycolipids by ABC Transporters

CPSs with defined glycolipid anchors are assembled without undecaprenol phosphate involvement. The widespread lyso(PG)-Kdo anchor from *E. coli* group 2 (and related) capsules offers the best studied example and involves the catalytic activity of two conserved CMP-Kdo-dependent β-Kdo glycosyltransferases (called KpsS and KpsC in *E. coli*). The β-Kdo oligosaccharide is then extended by addition of the CPS repeat-unit domain in a polymerization process involving sequential sugar transfer and is completed in the cytoplasm. In the *E. coli* K1 and *S.* Typhi Vi CPS, a single catalytic site is responsible for polymerizing the CPS domain. In other representatives, multiple glycosyltransferase catalytic sites are often accommodated in single "polymerase" polypeptides. The biochemical pathway for Vi antigen is less certain as the current data cannot confidently distinguish between models where the terminal diacyl *N*-acetylhexosamine residue is an acceptor, from one where its addition serves as a terminating reaction.

The details of the export of the completed CPS glycolipids via the ABC transporter are unknown but, given the size constraints imposed by the potential lumen of ABC transporters, a processive strategy is favored in working models, resembling that recently proposed for an ABC transporter that exports undecaprenyl diphosphate-linked O antigen. In this context, it is perhaps significant that most documented CPSs relying on these transporters have linear backbones, or possess single residue substituents. Structures for ABC transporters that export undecaprenyl diphosphate linked oligo- and polysaccharides for protein *N*-glycosylation and O antigen synthesis, respectively, invoke a model where the lipid is excluded from the transporter lumen and the same may be true for CPS. The transporter itself likely recognizes structural motifs in the shared lyso(PG)-Kdo glycolipid acceptors since transporters can be exchanged between systems with diverse repeat-unit structures.

Currently, for CPS assembly, ABC transporters are confined to Gram-negative bacteria and are therefore coupled to additional translocation machinery to complete the transfer to the cell surface. This consists of a PCP-3 protein and an OPX representative. PCP-3 proteins form multimers but lack BY-kinase domains. Genetic data supports PCP3 recognition of cognate OPX proteins but the OPX proteins are unable to form stable complexes, and their structural features have yet to be described. As with the Wzy-dependent pathways, comparisons have been made with tripartite drug efflux pumps in Gram-negative bacteria, but recent studies concerning Vi antigen export indicate some exposure of periplasmic intermediates, rather than a protected translocation pathway from the cytosol to the outside of the cell. The glycolipids on the CPS chain termini provide a means to attach the nascent product to the cell surface and create a capsule, and this may explain why ABC transporters have not been implicated, to date, in EPS export.

Synthase-Dependent Mechanisms for EPS Production

Bacterial cellulose and poly-*N*-acetylglucosamine (PNAG) are examples of EPSs secreted by a wide variety of bacteria. The characteristic biosynthetic feature of these systems is that polymerization occurs without the involvement of a lipid acceptor, and

synthesis and export across the cytoplasmic membrane are catalyzed by the same protein(s). Cellulose synthase provides the best example from the perspective of biochemical and structural insight, with two proteins (BcsA and BcsB) forming the active complex. BcsA is an integral membrane protein with a cytosolic processive glycosyltransferase. Most of BcsB is located in the periplasm but it also contributes a transmembrane helix to the integral membrane structure formed primarily by BcsA. BcsA and BcsB are both required for synthesis and they create a channel leading from the active site of the glycosyltransferase domain across the cytoplasmic membrane, so synthesis and export are obligatorily coupled. This two-protein architectural principle seems to be conserved in other EPS-producing synthases, as is the possession of a PliZ regulatory domain to facilitate regulation of the synthase by cyclic di-GMP. Binding of the effector to the PilZ domain in BcsA results in a conformational change that allows access of the UDP-glucose substrate into the catalytic site. In some members of the α- and β-proteobacter (including E. coli and Salmonella), additional enzymes modify the cellulose backbone with phosphoethanolamine (PEEN).

In Gram-negative bacteria, translocation of synthase products to the cell surface requires a β-barrel outer membrane channel. It is thought that a multiprotein complex spans the periplasm linking the channel and the synthase. A periplasmic scaffold is contributed by a protein containing an extended region composed of tetra-tricopeptide repeats (TPR). TPR motifs are protein interaction domains and synthase-dependent EPS production machinery often possess associated periplasmic enzymes that depolymerize or modify the glycan product. In PNAG, for example partial de-*N*-acetylation can have a significant effect on PNAG rheological properties. Such modifications can influence the assembly and export processes.

Levansucrases

The systems described above build glycans or their repeat units in the cytoplasm. Several Gram-negative and Gram-positive bacteria produce polysaccharides called levans from sucrose-based substrates, using fructosyltransferase or levansucrase enzymes that are attached to the outer cell surface or secreted into the growth medium. These enzymes can produce both oligo- and polysaccharides, catalyzing different reactions depending on the nature of the acceptor: hydrolysis using water as the acceptor, transfructosylation of monosaccharides-oligosaccharides, or polymerization of levan EPS using growing fructan chains.

Inhibition of CPS and EPS Biosynthesis

Given the important functions of CPS and EPS in pathogenic bacteria, there is significant interest in identifying inhibitory compounds that block production and potentially render the bacterium susceptible to host defenses. The similarities in folds and mechanisms shared by glycosyltransferases and sugar nucleotide synthases used to generate glycans in both host and pathogen create some challenges in these approaches, as does the wide range of activities for the repertoire of glycan structures. However, there are sufficient novel elements that are shared within a given assembly-export strategy to offer some avenues. Small molecule inhibitors targeting regulatory proteins and outer membrane channels for CPS translocation have been reported. In addition, some attempts to eradicate biofim-forming bacteria have focused on interfering with the synthesis and signaling mediated by cyclic-di-GMP, which would have a direct impact on the function of key synthase enzymes. However, a very promising different strategy for eradicating biofilms has been developed by using glycosylhydrolases that depolymerize the EPS within the biofilm. In an unusual twist, some of the effective candidate matrix-degrading enzymes tested to date actually originate from the periplasmic modification machinery involved in EPS production.

Biotechnological Applications

Since their varied structures confer distinct physical properties, CPS and EPS have received considerable commercial attention. Bacterial cellulose is produced in large quantities by organisms like *Komagataeibacter* (formerly *Acetobacter*) *xylinum*, where microbifrils intertwine to create impressive sheets or "pellicles" at the air-liquid interface of broth cultures. The genes required for modification of cellulose with PEEN residues are absent in the larger (commercial) producers such as *K. xylinum*. Bacterial cellulose has a smaller filament diameter and higher purity than cellulose sourced from plants, making it the preferred material in pharmaceutical applications such as wound dressings, artificial skin, and tissue grafts. The product can be also impregnated with a variety of compounds to promote tissue regeneration or prevent infection.

The gelling properties of EPS have been exploited in a range of applications. Xanthan gum has a large market presence but products belonging to the sphinghan family of EPSs (Fig. 3) also have potential. Xanthan is frequently found as a food additive where a stable colloidal emulsion is required. It can be produced in optimized fermentation conditions in yields approaching 30 g/L and forms high molecular weight chains (exceeding 10^6 Da), and is an excellent thickener, even at low concentrations. This EPS possesses unusual and valuable rheological properties, where its viscosity is reduced at high temperature or low ionic strength, which offers advantages in handling and application. As a result, it has seen a variety of other applications as a stabilizer in other commercial settings, including as cosmetics, textiles and oil recovery. Xanthan has also been used (alone or combined with other polymers) in tablets and hydrogels for drug release or as supports for cell proliferation approaches. Lactobacillus EPSs provide another important additive in the food industry because these bacteria are generally regarded as safe and are found in a variety of

fermented diary and vegetable products. The EPS, and its interactions with other components, contributes to the texture of the product. However, unlike an additive like xanthan, the EPS is produced in situ.

In a different biomedical application, polysialic acid is of interest for improving the effectiveness of therapeutic proteins and drugs. The objective is to enhance the (often) short half-lives of therapeutics in circulation after administration. Chemical modification with polyethylene glycol (PEG) is often used in this role, to reduce susceptibility to proteolysis, antibody response and clearance that hampers therapeutic protein efficacy. However, PEGylation has some drawbacks and modification with polysialic acid offers an alternative strategy, where the lack of immunogenicity of polysialic acid presents a significant advantage.

There is considerable interest in glycoengineering strategies that seek to use the enzymes from biosynthesis pathways (CPS, EPS and others) to create tailored glycan structures or entirely new glycoconjugates for biomedical and industrial purposes. This is already founded in natural glycan diversity, where lateral gene transfer and recombination within a biosynthesis gene locus leads to structural and antigenic diversification. This same principle can be used in targeted fashion, where glycosyltransferases with different specificities can be used to create new backbone or side-chain structures. This approach is limited by the acceptor specificities of known enzymes and must preserve processes that initiate on a required lipid carrier, as well as structural motifs that are essential for export and surface presentation. The efficiency of the combinatorial system is paramount, since yield determines commercial potential. However, one area where glycoengineering has been already seen success is the production of neoglycoconjugate vaccines using the protein glycosylation machinery. Periplasmic *O*- and *N*-glycosylation transfers oligosaccharides and polysaccharides to a protein. This provides an alternative to the chemical conjugation approach and allows precise determination of the site and frequency of glycosylation of a carrier protein of choice. The mechanism of the oligosaccharyltransfase requires the glycan be assembled on undecaprenol phosphate and the periplasmic location creates a need for export of the glycosylation donor to the periplasm. The power and flexibility of this strategy is evident in its successful application in the generation of glycoconjugates bearing LPS O antigens and CPS from pneumococci and *S. aureus*, which possess Wzy-dependent assembly mechanisms.

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Relevant Website

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Caulobacter☆

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Glossary

Chemotaxis Motility directed by a sensory/motor system along a gradient of a solute.
Cytoskeleton Protoplasmic proteins that participate in the development and/or maintenance of cell shape.
Oligotrophy Of the habitat: Condition of the habitat characterized by low organic productivity and low density of microbial populations. Of microbes: Nutritional class of microbes whose competitiveness in their natural habitat depends on their ability to exploit a scarcity of nutrients.
Periplasm Region immediately external to the cell membrane; in Gram-negative bacteria, the region is separated from the environment by a second superficial, outer membrane.
Phosphorelay Systematic transfer of phosphoryl groups from a nucleotide triphosphate through a series of proteins, thereby altering the functional activity of each protein; important in many signal-transduction systems.
Prostheca An outgrowth of the Gram-negative bacterial cell surface that includes the outer membrane, the cell membrane, and the peptidoglycan layer; may or may not include cytoplasmic elements.
Sacculus The rigid peptidoglycan layer of a bacterial cell envelope; in Gram-negative bacteria, the sacculus lies within the periplasm.
Stalked sibling The product of asymmetric fission in caulobacter that bears a prostheca and is not motile.
Swarmer sibling The product of asymmetric fission in caulobacter that bears a flagellum and is motile.
TonB-dependent receptors Periplasmic proteins that assist in providing metabolic energy for active transport of solutes across the outer membrane of a Gram-negative bacterium into its periplasm.
Transcription cascade System of sequential transcription of genes within which transcription of each 'later' gene requires activation by the product of an 'early' gene.

Abbreviation

GS/GOGAT Glutamine synthetase/glutamate-oxoglutarate amino transferase

Defining Statement

Caulobacters are aquatic, oligotrophic bacteria with a distinctive reproductive cycle that produces flagellated swarmer and non-motile stalked cells as siblings. Correlations between their oligotrophy and their asymmetrical division are drawn against the background of description of the molecular details of development now available from extensive research with *Caulobacter crescentus*.

Caulobacter and Caulobacters

Prologue: It was not a dark and stormy night; it was an overcast day, and the sky was a smooth gray. Nevertheless, the water of the lake was blue; it was unquestionably clean and oligotrophic. A caulobacteriologist scooped samples from the lake's surface with great expectations. Within 2 weeks, the collector was rewarded by finding that 75% of the 3177 aerobic, chemoorganoheterotrophic bacteria per milliliter that formed visible colonies on a dilute peptone medium were caulobacters.

Recognizing Caulobacters

The Genus *Caulobacter*

Species assigned to the genus *Caulobacter* are aquatic, oligotrophic, aerobic, freshwater, Gram-negative Alphaproteobacteria. All the distinguishing morphologic features of the genus *Caulobacter* are evident in the dividing cell (see Figs. 1–4):

☆*Change History*: June 2017. Emanuele G. Biondi replaced Figs. 9 and 10; modified section "Control of Development: Protein Synthesis, Modification, Localization, and Destruction"; and updated "References" and text throughout the article.

This article is an update of J.S. Poindexter, Caulobacter, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 57–73.

- An elongated, unicellular form – straight (rods or fusiform cells), curved (curved rods or subvibrios), or twisted (vibrios) – with poles that are blunt (rods) or tapered (vibrios, subvibrios, and fusiform cells);
- A different appendage at each pole: a single flagellum at the younger pole, and at the older pole a prostheca composed of the cell envelope (outer membrane, peptidoglycan layer, and inner membrane);
- A discrete blob of adhesive material, the 'holdfast', at two sites: at the flagellated pole at the base of the flagellum, and at the outer tip of the prostheca (hence its designation as a cellular 'stalk');
- A constriction at approximately the midcell that, when completed, will separate a flagellated 'swarmer' cell from its nonmotile stalked sibling;

Fig. 1 Vibrioid cells of *Caulobacter crescentus* strain CB2 grown in dilute peptone yeast extract medium. See also Fig. 8(a). EM, Pt-shadowed.

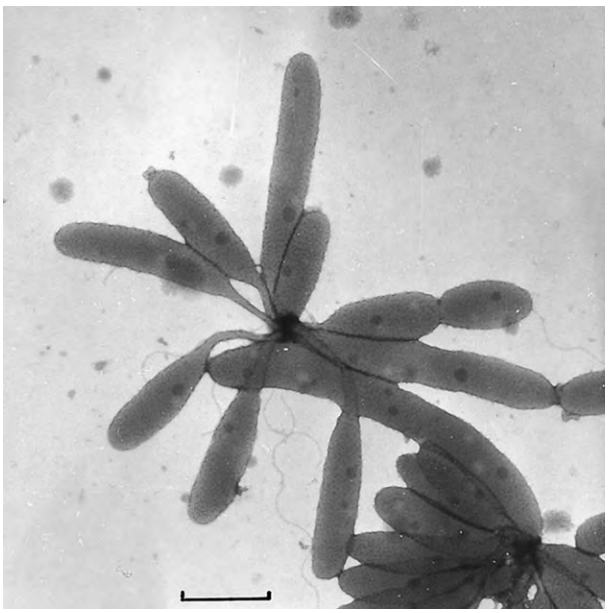

Fig. 2 Bacteroid cells of *Caulobacter* sp. strain CB417 grown in dilute peptone yeast extract medium. EM, negatively stained with AmMoO4. Marker=1 μm.

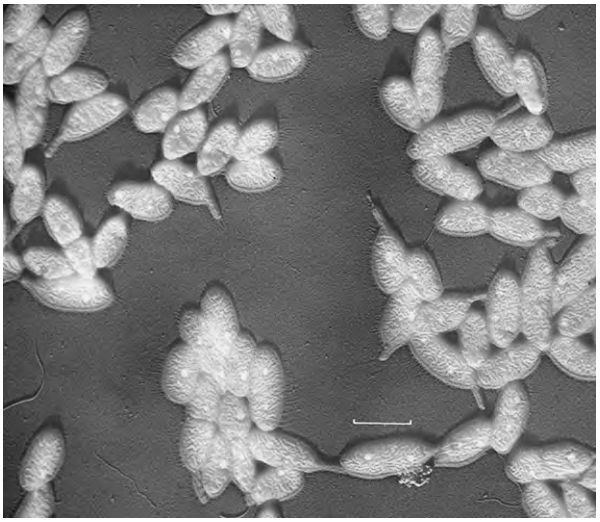

Fig. 3 Fusiform cells of *Caulobacter leidyi* strain CB37 grown in dilute peptone yeast extract medium. See also Fig. 8(b). EM, Pt-shadowed. Marker=1 μm.

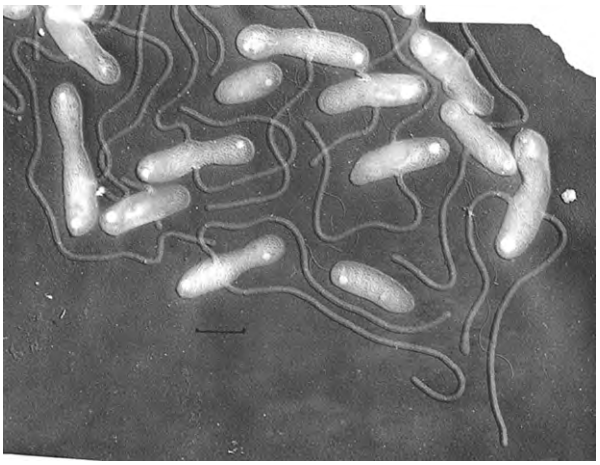

Fig. 4 Biprosthecate cells of *Asticcacaulis biprosthecum* strain C-19 grown in dilute peptone yeast extract medium. EM, Pt-shadowed. Marker=1 μm.

- Ringed disks of peptidoglycan and proteins, called stalk bands, at intervals along and within the stalk; one band is installed during each cell cycle and records the number of fissions completed by the cell since it began to develop its stalk. The rings look very much like the flagellar base plates first described in spirilla.

Other Caulobacters

Variations on this morphology are exhibited by closely related Alphaproteobacteria. The closest relative is the genus *Asticcacaulis* (literally, nonadhesive stalk). Although not adhesive, *Asticcacaulis* stalks are banded. The genus is distinguished from caulobacter by several morphologic features.

- Cells of all isolates are blunt rods.
- Locations of the holdfast, flagellum, and prostheca are not coincidental; the holdfast is located excentrically on the pole, the flagellum excentrically at the same pole, and the prostheca may be excentral, subpolar, or lateral. Isolates with lateral prosthecae may bear one or two prosthecae, although only one flagellum.
- Fission occurs by septation rather than constriction.
- A difference in size of the siblings, with the swarmer regularly much shorter than its stalked sibling during and immediately after fission, and a greater difference in cell cycle times between the siblings (see Fig. 9).

Both *Caulobacter* and *Asticcacaulis* fall into the 16S rRNA family '*Caulobacteraceae*', as do *Brevundimonas* (a composite taxon of phenotypically diverse bacteria) and *Phenylobacterium* (a genus of non-prosthecate bacteria). Some of the species currently assigned

to *Brevundimonas* were originally named as *Caulobacter* species because they possess all the ecologic, morphologic, and physiologic features described for that genus.

Two other 16S rRNA families of the class *Alphaproteobacteria* include genera with caulobacterial morphologies. One species (*C. leidyi*) is placed in the family *Sphingomonadaceae* on the basis of its lipid content as well as its 16S rRNA. Isolates similar to *C. leidyi* are known, but not yet named or classified. The family *Rhodobacteraceae* includes the marine caulobacters of the genus *Maricaulis*. Dividing cells of these alpha-proteobacteria are identical to *C. crescentus* in microscopical appearance of their dividing cells, and their stalks are adhesive; however, stalk bands are typically absent.

The Caulobacterial Stalk

The term 'prostheca' was introduced by J.T. Staley to designate an outgrowth of the bacterial cell surface that includes at least the peptidoglycan sacculus (Fig. 5); this definition distinguishes prosthecae from proteinaceous structures such as flagella and pili/fimbriae, and from extracellular polysaccharidic capsules and slime stalks. Known prosthecate bacteria are Gram negative, but not all are Alphaproteobacteria. Among dimorphic prosthecate bacteria that produce swarmer cells, the features of the dividing cell described above for caulobacters are – as far as known – unique to the three families of Alphaproteobacteria already mentioned. The caulobacterial prostheca lacks cytoplasm and DNA. It does not generate progeny, a reproductive process that occurs in the dimorphic bacteria of the genera *Hyphomicrobium* and *Hyphomonas*, where the nonadhesive prostheca produces swarmers by budding from its distal tip.

The value of motility in an aquatic organism is never debated. Adhesion, likewise, is generally regarded as adaptive because it can anchor the organism in a favorable locale. Although adhesion of a bacterium typically does not require a prostheca, adhering bacteria are very often perpendicular to the substratum. While this allows most of the cell surface to continue in contact with the liquid phase of the aquatic environment, an adhesive prostheca increases that advantage by preventing crowding of the cell body against other bacteria attached to the same substratum.

Only among the caulobacters is the prostheca typically adhesive, and adhesiveness is not a universal property even within this group. It is therefore an unavoidable challenge to infer possible functions for the stalk other than a role in adhesion. To date, three further functions have been proposed: (1) increased resistance to settling in an aquatic environment ('buoyancy'); (2) increase of the nutrient uptake-mediating area of the cell surface; and (3) boosting the incipient swarmer above the crowd to free it from entrapment in a biofilm. (1) The difference in buoyancy of non-stalked and stalked siblings is the mechanical basis for segregation of swarmers from their stalked siblings by differential centrifugation, which is the most widely used method for the establishment of synchronously developing populations used in cell cycle research with caulobacters (see Section 'Bacterial Filaments'). (2) Besides straightforward geometric reasoning, there is biochemical, physiological, developmental, and molecular (genetic and proteomic) evidence that supports the proposed role of the stalk in nutrient scavenging. Even stalks severed from the cell body mechanically or as a consequence of genetic mutation are demonstrably capable of accumulating solutes, and to consume metabolic energy doing so. (3) Observations of caulobacters attached to natural materials reveal only dispersed and not crowded caulobacters; swarmers tend to be well away from their stalked siblings before settling down. However, on both animate and inanimate surfaces exposed to

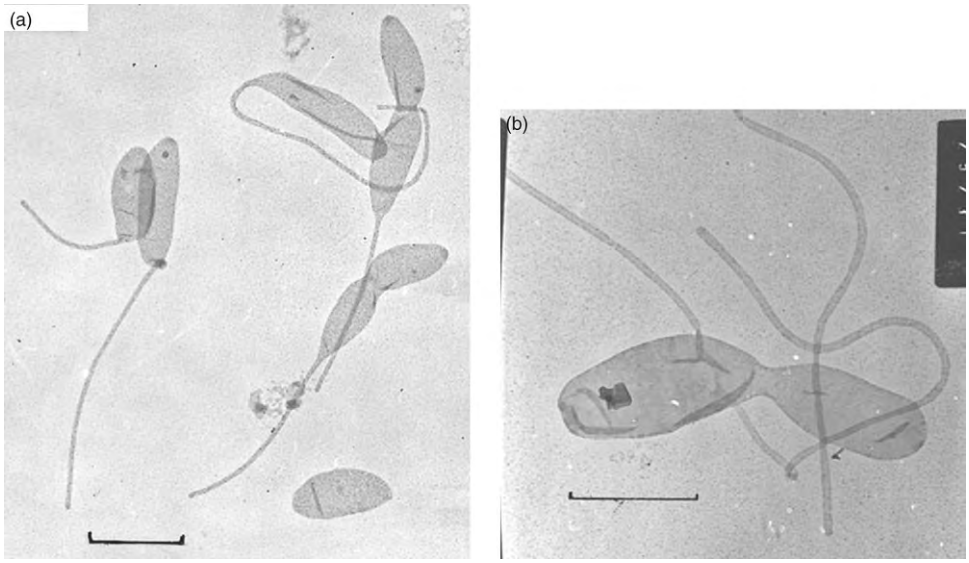

Fig. 5 Peptidoglycan sacculi of caulobacters. EM, positively stained with uranyl and rotary shadowed with Pt. Marker=1 μm. (a) *Caulobacter crescentus* strain CB2. (b) *Asticcacaulis biprosthecum* strain C-19. Marker=1 μm.

caulobacter populations at 1000-fold or greater than their usual density in natural waters, and in the absence of predators such as protozoa and microfauna, caulobacters can be forced into crowds – a technique that can be useful in technological applications that employ immobilized caulobacters.

Accordingly, the caulobacter stalk is demonstrably capable of providing at least four functions. It mediates adhesion in a fashion that simultaneously exposes most of the cell surface to the liquid surroundings, the source of the cell's nutrients and lifts the cell body above a biofilm, promoting free dispersal of the motile sibling. Because the stalk engages in little biosynthetic activity except at the cell-stalk junction (see Section 'Placement of Cytoskeletal Proteins and Cell Wall Synthesis Complexes'), it could serve (like microvilli in a mammalian intestine) to increase the ratio of nutrient-absorptive surface to nutrient-consuming protoplasm. Finally, it provides the capacity for the third ecologic habitat available in an aquatic environment: floating, as well as swimming in the water column and settling on submerged surfaces. Stalked caulobacters not attached to submerged surfaces accumulate in the neuston, or air–water interface, the shallow vertical zone in an aquatic habitat where organic substances accumulate because they diffuse less freely there than in the water column; where O_2 enters from the atmosphere; and where the local phototrophs, the primary producers, tend to gather during the day and catch the sun's energy that will support the local chemo-heterotrophs such as caulobacters. Thus, bacteria with this peculiar morphology and developmental cycle are well suited to living competitively in low-nutrient-flux aquatic environments as aerobic, metabolically efficient, nutrient-scavenging chemo-organo-heterotrophs. As mentioned in the Prologue, it is precisely where they are most dependably found as the predominant (but not crowded) aerobic, non-photosynthetic bacteria.

Capture and Cultivation of Caulobacters

The natural environment in which caulobacters thrive and can sometimes even predominate is low-nutrient-flux water, both fresh and marine. Even when predominant, caulobacters do not accumulate as dense populations; they are rarely as crowded as 10^5–10^6 cells per milliliter. Natural waters are almost never constant in nutrient flux; even the most oligotrophic water will experience transient surges of nutrients that will support a responding increase in the density of the microbial populations and of the organisms that feed on the microbes. During such periods, caulobacters do not necessarily disappear, but they do not respond by multiplying rapidly. Consequently, they can be overwhelmed numerically by faster-growing bacteria such as pseudomonads and become difficult to detect – microscopically or by cultivation.

The most dependable way to encourage caulobacters to accumulate in a culture and to enable their isolation as colonies on solidified media is to depend on their development as a dimorphic population. Let the water sample stand in a tall bottle (such as a milk dilution bottle) at room temperature in the dark. In 4–14 days, a thin, delicate surface film of caulobacters will accumulate as the swarmers rise toward the air–water interface and then develop their 'water wings' – their stalks. Their accumulation can be accelerated by adding peptone to no more 0.01% (w/v); higher concentrations will encourage the multiplication of bacteria that can grow faster, but are less capable of scavenging nutrients from low concentrations. Similarly, incubation in the dark reduces primary production by phototrophs in the sample. Their activities interfere with the enrichment of caulobacters in two ways: by producing and exuding organic compounds, often amino acids, thereby enriching the water; and by serving as attachment surfaces for the caulobacters and reducing their buoyancy. The stalked bacteria probably do quite well attached to phototrophs, but they are harder to find because they sink out of the surface film.

A sample of surface film can be examined in a wet mount to determine whether the relative abundance of stalked cells is increasing, and when it is, a sample can be streaked on an unbuffered medium containing 0.05% peptone and not more than 1% agar, and incubated at room temperature. As colonies begin to appear, it is instructive to mark those that are macroscopic by the third day of incubation; in the author's experience, such colonies are never generated by caulobacters from enrichment samples. After 1–2 weeks, new colonies no longer appear and the preparation for screening can begin.

There is a fast, frustrating way to screen, and a tedious, fruitful way. The fast way is to examine samples of colonies in wet mounts. Because the medium is dilute, the colonies are very small (rarely more than 0.5–0.8 mm), and a colony (especially if cohesive) is easily consumed in the preparation of the mount; consequently, finding them is not capturing them. The tedious way is to patch a sample of each colony onto a gridded plate of medium, incubate for about 2 days, and then prepare wet mounts from the growth in the patches. This actually saves considerable time because the patches can be sampled in a pattern and marked off if they contain caulobacters, whereas using primary colonies requires that the colonies be mapped. Better yet, the patch is not consumed in the preparation of a wet mount.

Water can be surveyed for the presence of caulobacters by molecular tools such as FISH probing. There are, however, practical problems with fluorescence microscopical techniques. For example, natural populations of caulobacters are sparse, whether they are predominant or vastly outnumbered by non-caulobacters. It is often more difficult to detect them as fluorescent dots than as bacteria that wave about on their stalks in a focal plane above other bacteria settled on the slide. Their motion reveals their presence and their stalks identify them. Fluorescence intensity of a probe-binding caulobacter cell may be low and difficult to discern, for more than one reason: (1) Caulobacter cells in wild samples are relatively small, often not much wider than 0.5 μm, binding too little probe to be discerned readily; (2) Ribosomal content is often very low in cells from oligotrophic conditions, and relatively little probe is bound; (3) Most of the 'universal' probes used in community surveys are intended to bind to 16S rRNA. However, the caulobacters most frequently encountered in freshwater are members of the rRNA family *Caulobacteraceae*, the only family in the order *Caulobacterales*, which is a fairly discrete and congruous taxon as currently defined. Again, reaction with a fluorescent probe

may be weak, and even competent application of probes can fail to detect a caulobacter signal in a sample that will yield a dozen or more different kinds of isolates – from which more can be inferred than just natural distribution.

To identify the colonies or patches containing stalked bacteria, the wet mount should be examined with phase-contrast illumination; the hydrated stalk is about 0.2 μm in diameter, just at the limit of resolution of a good phase-contrast microscope. If only ordinary light is available, preparing the mount in a droplet of dilute methylene blue will provide some contrast and help reveal some stalks. When crowded (as when growing on an agar surface), caulobacters adhere to each other in rosettes (see Figs. 6 and 7), and as their stalks elongate they will appear in one or another arrangement. In *Caulobacter*-like organisms, the stalks will be oriented like the spokes of a wheel, with the cell bodies sticking outward toward the rim of the wheel or like pins in a pincushion (Fig. 6). In *Asticcacaulis*-like organisms, the cells will appear to be in contact with each other at the poles, with the nonadhesive prosthecae extending from the cell cluster. *Asticcacaulis biprosthecum* rosettes (Fig. 7) are especially distinctive as 'hairy' rosettes. The remainder of the procedure is a routine bacteriological restreaking until only one type of colony can be found on plates streaked from successive colony samples.

Colonies of caulobacters may be translucent or opaque. They occur in various shades of yellow/gold/orange/red-orange due to cell-associated carotenoid pigments; pale pink due to an unidentified non-heme, non-carotenoid pigment; or colorless (unpigmented). As they age, colorless colonies often become dark tan-pink or pink-red due to heme accumulation. The colony margins are entire, the outline is circular, and the elevation is convex. Texture may be soft and watery, butyrous, compact and slightly sticky, or very cohesive and smooth or granular. Under each strain's optimal conditions of cultivation, colonies of most isolates become visible macroscopically between 60 h and 4 days of incubation – 3–4 days being most common. In liquid culture, doubling times of

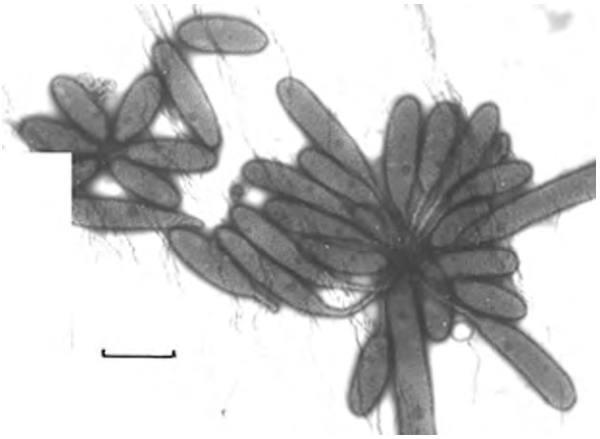

Fig. 6 Newer and older rosettes of *Caulobacter* sp. strain CB417. The newer rosette includes six cells with almost no stalk elongation yet; the older rosette includes 21 cells, most of which have developed stalks. EM, negatively stained with AmMo04. Marker=1 μm.

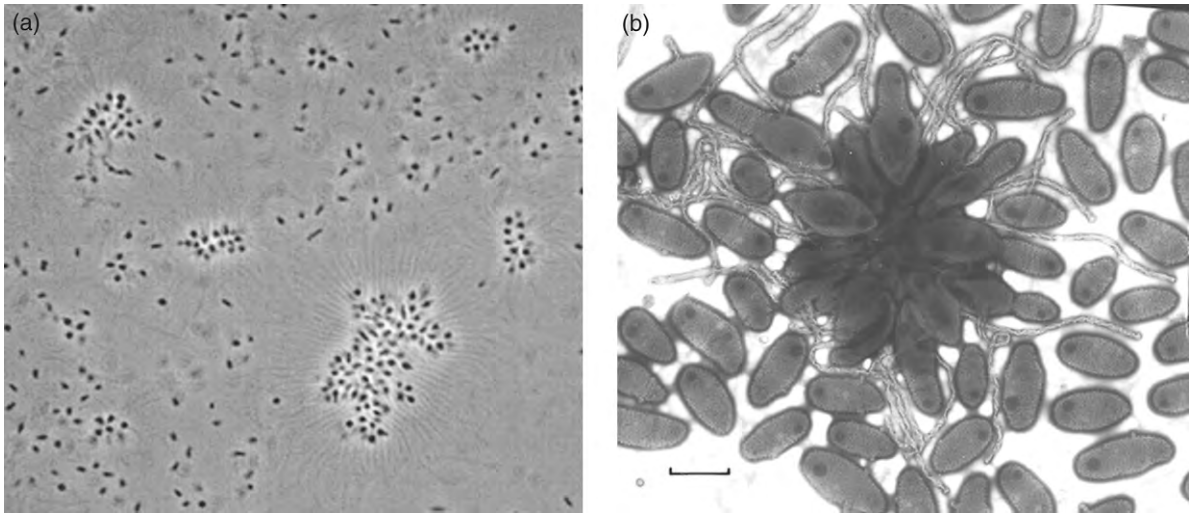

Fig. 7 Rosettes of *Asticcacaulis biprosthecum* strain AC402 appearing 'hairy' due to the extension of the nonadhesive prosthecae away from the point of mutual adhesion of the cells. (a) Phase-contrast photomicrograph. (b) EM, negatively stained with AmMo04. Marker=1 μm.

recent isolates range from 2.5 to 5–6 h, which is somewhat faster than they seem to manage in nature. As determined by the rate of accumulation of bands in the stalks of caulobacters captured by their adhesion to EM grids submerged in a mesotrophic lake, the reproductive rate in situ in early summer is three generations per day or 8 h per generation.

Faster-growing clones with doubling times close to only 2 h have been generated by laboratory cultivation from at least two species (*C. crescentus* and *C. leidyi*). These two species are the only caulobacters that can be grown in chemically defined media; vitamins (riboflavin, biotin, or cyanocobalamin) are required by some isolates, but are not sufficient for growth without complex supplementation with low concentrations of peptone, casamino acids, or yeast extract. Amino acids – commonly exuded by algae – are generally their preferred sources of both carbon and nitrogen; besides amino acids, many organic acids and several sugars can be consumed as carbon sources. Cellobiose, the disaccharide produced by the digestion of cellulose, is used by almost all isolates in the author's collection; this might explain in part the frequent occurrence of cytophagas in successful caulobacter enrichments.

Morphogenesis and Oligotrophy

It has long been suspected that one of the most important functions of the stalk is to increase the capacity of the caulobacter cell to scavenge nutrients from the oligotrophic environments where caulobacters are competitive. Very early in the studies with pure cultures of caulobacters, it was discovered that the nutrient with the most dramatic influence on morphogenesis of the stalk was phosphate: phosphate inhibits stalk elongation, and phosphate limitation results in a marked acceleration of stalk outgrowth (see Fig. 8 and Section 'Phosphate and Stalk Elongation'). These observations were a stimulus to the notion that stalk development improves specific nutrient scavenging by the caulobacter cell, at least for phosphate. The inhibitory effect of phosphate can be mitigated by calcium, and the accelerating effect of phosphate scarcity requires available calcium. Although an abundance of the molecular details of development has been elucidated in *C. crescentus*, the interaction of phosphate and calcium in stalk elongation is yet to be explained in comparable detail. Nevertheless, in the natural habitat of caulobacters, phosphate and calcium are not typically available in excess together because of the insolubility of calcium phosphate; in caulobacters, calcium could serve as an indicator of phosphate scarcity.

Nitrogen-source availability has little demonstrable effect on stalk elongation, but starvation or limitation for nitrogen has a marked effect on stalk initiation (see Section 'Nitrogen Source and Stalk Initiation'). In nitrogen-adequate media in the laboratory, the swarmer cell sheds its flagellum and begins its maturation (initiating stalk development and DNA synthesis) after a motile stage that lasts about 25–33% of the cell cycle time. In nitrogen-inadequate media, the transition from motile, juvenile swarmer to non-motile, reproductively capable stalked cell is postponed indefinitely and may not occur until nitrogen becomes available. The only nutrient-specific chemotactic response so far demonstrated in *C. crescentus* by the Adler capillary assay is the attraction of nitrogen-limited swarmers toward nitrogen sources (ammonium and amino acids); in contrast, even swarmers produced under nutrient-limited conditions (whether for carbon or nitrogen or phosphorus) do not clearly respond to carbon sources or phosphate. Thus, two types of observation reveal that nitrogen limitation is the only nutritional signal that results in perpetuation of motility, in specific chemotaxis, and in delay of maturation of the swarmer into the reproductive stage. This phenomenon depends on glutamine deprivation triggering ppGpp production, which in turn extends the swarmer stage. Together, these observations imply that the motility of the swarmer stage serves within the cell cycle to lead the cell toward a nitrogen-adequate site.

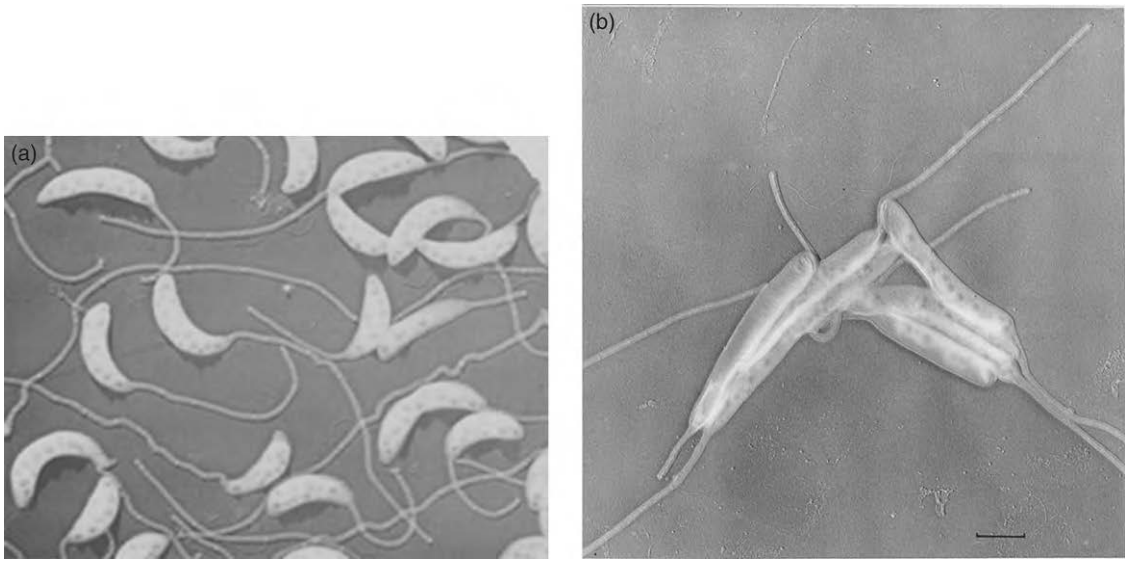

Fig. 8 Cell and stalk elongation in phosphate-exhausted cultures of caulobacters. EM, Pt-shadowed. Marker=1 μm. (a) *C. crescentus* strain CB2; See also Fig. 1. (b) *C. leidyi* strain CB37; see also Fig. 3.

Caulobacters generally prefer amino acids as their nitrogen source, but they can consume inorganic nitrogen. However, *C. crescentus*, at least, lacks glutamic dehydrogenase and depends on the high-affinity GS/GOGAT (glutamine synthetase/glutamate-oxoglutarate amino transferase) pathway for the assimilation of ammonium. As in enteric bacteria, the activity of the *C. crescentus* system is inhibited and its synthesis is repressed by ammonium. As a consequence, *C. crescentus* growth is ammonium sensitive in minimal media, and the bacteria can starve for nitrogen in the midst of abundance when ammonium is the only nitrogen source available. In laboratory cultures, relief of that interference is achieved by the supplementation of defined media with glutamate to enable ammonium assimilation via GS/GOGAT or by the substitution of nitrate for ammonium.

Limitation for any one of the three macronutrients carbon, nitrogen, and phosphorus dramatically increases the uptake rate and affinity specifically for the limiting nutrient. Again, as in the case of stalk hypertrophy, phosphate limitation has the most striking effect: the relative increase in uptake rate (calculated as per unit area of cell surface) is about 80-fold for phosphate, but only about eightfold for carbon or nitrogen sources. Uptake of non-limiting nutrients does not change with the stalk length. The long-stalked cells of phosphate-limited populations take up phosphate rapidly and with high affinity through both the cell body and the stalk, and their swarmer siblings exhibit a comparable enhancement of phosphate uptake. Nevertheless, while the stalk surface seems no more active in nutrient uptake than the rest of the cell, it lacks cytoplasmic components such as ribosomes and DNA. Thus, the hypertrophied stalk should provide the cell with an extension of the supply route for nutrients without increasing the nutrient demand by the most expensive metabolic activity, which is protein synthesis, or by the demand for phosphorus by nucleic acid synthesis.

A further aspect of caulobacterial nutrient-scavenging capacity was revealed in the fully sequenced genome of *C. crescentus*. There are in that genome loci that are interpreted as genes for TonB-dependent receptors, which are components of the systems for transducing metabolic energy to the outer membrane of Gram-negative bacteria that enables active uptake of solutes into the periplasm. Enteric bacteria have a few (not more than ten) such genes, and there are 34 in the highly versatile and competitive bacterium *Pseudomonas aeruginosa*. *C. crescentus* possesses 65. At least 19 such receptors appear in the proteome of isolated stalks, providing the strongest molecular evidence that the stalk is capable of energized, high-affinity uptake of solutes. Translocation to the cell body cytoplasm of solutes taken into the periplasm of the stalk probably involves periplasmic ATP-binding proteins, but the mechanism of translocation is still hypothetical.

In the oligotrophic waters that are the natural habitat of caulobacters, primary production of organic carbon is typically limited by the availability of phosphate or – less often – of nitrogen compounds, particularly nitrate. The dimorphy of caulobacters presents itself as a composite of adaptations that fits their ecologic role as nutrient scavengers, with the motile stage sensing nitrogen adequacy for stalk development and stalk elongation proceeding as needed for phosphate acquisition. The remaining major nutrient is organic carbon, which they presumably take up and transport into the cytoplasm with their exceptional battery of high-affinity TonB-dependent receptors. Those systems may enable caulobacters to collect the by-products of photosynthetic CO_2 fixation as those substances are released by the phototrophs to which caulobacters so commonly adhere and with whom they must compete for phosphorus and nitrogen.

Nutrient gradient-directed motility, extension of nutrient uptake surface relative to cytoplasm, nutrient uptake assisted by high-affinity TonB-receptor systems, and adhesion to phototrophic microbes all combine to make typical caulobacters highly competent for oligotrophy. Their unique reproductive cycle, which produces one offspring that will move away from its immobile sibling, also largely ensures that siblings will not crowd each other and therefore will not compete for sparse nutrients. Some of the details of the mechanisms that confer on caulobacters' unique approach to the problem of resource acquisition are described in the next section.

Development in *Caulobacter crescentus*

In 1935, on the basis of microscopical observations of mixed, wild (uncultivated) populations of caulobacters, Henrici and Johnson correctly inferred that a swarmer cell would develop a stalk and then proceed to asymmetric fission of a cell with a flagellum at one pole and a stalk at the opposite pole. They observed that such fission generated two siblings: a flagellated swarmer cell and a non-motile sibling bearing a stalk. However, because they lacked pure cultures, they could not determine whether a swarmer cell could divide without first attaching to a substratum and/or developing a stalk. This double question was answered in the first extensive studies with pure cultures of a variety of caulobacters, during which it was found that mechanical segregation of non-stalked (swarmer) cells from stalked cells allowed separate cultivation of each cell type. Early studies with such populations revealed that newborn swarmer cell populations were synchronous with respect to flagellum shedding, stalk development, and completion of fission, and that fission was invariably preceded by the other two events. Synchrony in a stalked cell population was less stringent; nevertheless, a full round of cell division was completed in about 75% of the time required for a full round of cell division in a population that began as swarmers.

Continuous direct observation of mixed populations in microcultures revealed behavior that was entirely consistent with the behavior of the synchronous cultures: the cell cycle was significantly shorter for a cell that entered the cycle with its stalk than for a cell that was observed from its motile stage, and the latter type of cell became non-motile and began to develop a stalk before it elongated, constricted, and divided. Fission of non-stalked cells was not detected. The absence of any difference in sequence or timing of morphogenetic and cell cycle events in populations attached to glass cover slips or suspended in agitated liquid cultures implied that adhesion was not required as a signal for flagellum shedding or the initiation of stalk outgrowth.

Within a few years after these initial studies, one species, *C. crescentus*, attracted the attention of microbial cell biologists who began to explore its dimorphic cell cycle. In the hands and minds of these diligent scientists, *C. crescentus* has proved a fertile experimental system that has yielded details of the mechanisms that enable a unicellular bacterium to order and integrate its morphogenesis, reproduction, and physiologic properties. The studies have also established that this 'simple' organism is a complex cell in which functional compartments can be established by the localization of proteins, without the elaboration of intracellular barrier membranes so familiar in eukaryotic cells.

Although there are some variations (rigidly enforced within a species), the cyclical sequence of developmental events in the lives of all caulobacter cells is largely a story of poles. The entire tale can be perceived at every *C. crescentus* pole from the birth of the pole as a product of fission to its maturation as an adhesive, banded stalk. It begins with the formation of the pole when fission is completed in generation I (see Fig. 9). During generation II, begun by that fission, the sequence of developmental events at each new pole will be the same, but the time course of those events will depend on the age of the opposite pole; developmental events will occur sooner at the new pole of the sibling with a stalk at its older pole (in the 'stalked sequence' in Fig. 9) than at the flagellated cell's new pole (in the 'swarmer sequence' in Fig. 9). Maturation of each pole formed in generation I will occur in generation III. In short, the pole's story has three chapters:

1. The pole is formed by cell fission.
2. Extrusion events occur that confer motility, adhesiveness, and phage reception.
3. The flagellum and motility and phage receptors are lost and the pole elongates as a stalk.

The long version of this story is now remarkably detailed as the result of research that has employed mutations, inhibitors (particularly, inhibitors of cell wall synthesis), genomic and proteomic analyzes, physiological studies, environmental manipulations, and studies of cellular composition and protein localizations. The research has yielded an elaborate description of the development of cell shape and of functional appendages in a unicellular bacterium whose unique morphology and cell cycle have been creatively exploited as a model system for the elucidation of bacterial cell biology (For the reader interested in full-length versions, several reviews are listed in the Further Reading section).

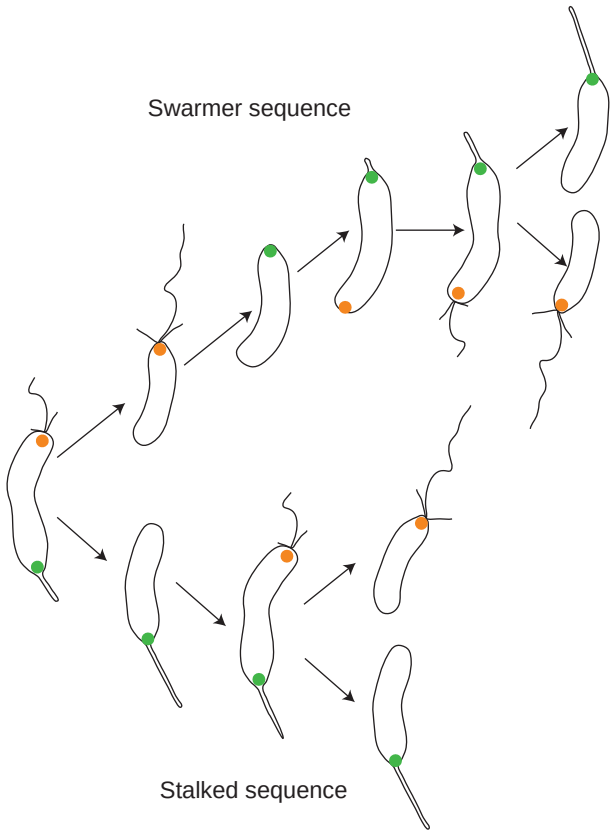

Fig. 9 Sequence of pole development events in several successive generations. The sequences are displayed here in parallel to emphasize that the sequences are identical at the sibling poles (green is the stalked pole, while orange is the swarmer pole) formed by any one division process. However, the swarmer sequence in the second generation is typically about 1.33 times as long as the sequence occurring in its stalked sibling.

Division and the Development of Cell Shape

Cell Wall Synthesis

The shape of a bacterial cell requires maintenance by a rigid element, which is a sacculus (see Fig. 5) of peptidoglycan, sometimes called 'murein'. The best-known pathway of synthesis in a Gram-negative bacterium occurs at three locations in a Gram-negative bacterial cell:

1. In the cytosol: small-molecule precursors are synthesized by intermediary metabolism, then combined into precursors of the repeating unit of the cell wall polymer.
2. At and within the cell membrane: the saccharide pentapeptide is delivered to the inner, cytoplasmic surface of the cell membrane, transferred to a lipid carrier, and moved into the membrane, where a second saccharide unit is added to the lipid-carried precursor. This disaccharide-pentapeptide-lipid carrier is then conveyed through the membrane to its outer periplasmic surface.
3. On the outer surface of the cell membrane and in the periplasm: the disaccharide pentapeptide is transferred from the lipid carrier to an acceptor site (either an end recently synthesized or a position opened by a lytic enzyme) on the preexisting peptidoglycan of the sacculus in the periplasm. This process elongates the glycan backbone of the peptidoglycan as the lipid carrier is recycled within the membrane. Transpeptidases then strengthen the sacculus by crosslinking between the outer D-ala-D-ala pair of one pentapeptide and the free amino group on the diamino amino acid (most commonly diamino pimelic acid) of another glycan chain.

The details of synthesis vary among bacteria; nevertheless, the overall process is general for Eubacteria, and similar systems occur in some Archaea.

Peptidoglycan synthesis complexes can affect the shape of an elongated cell when they are active at three regions:

1. Along the length of the cell to support elongation along the cell's long axis. So far as known at present, the complexes are arranged as a helix in rods, vibrios, spirilla, and fusiform cells.
2. At midcell to provide new peptidoglycan for constriction or septation. So far as known at present, the complexes are arranged as a ring in this location.
3. At a cell pole to support polar outgrowth such as occurs in budding and/or prosthecate bacteria. A specific geometric pattern of installation has not yet been discerned.

Research on bacterial cell shape development is, in large measure, research on peptidoglycan synthesis, and is aided by the availability of natural and artificial products that specifically inhibit one or another synthetic step. Example of inhibitors used in studies of *C. crescentus* morphogenesis include cycloserine, which inhibits the formation of D-ala-D-ala from L-ala in the cytoplasm; vancomycin, which inhibits trans-glycosylation at the outer surface of the cell membrane; and β-lactam compounds such as amdinocillin that inhibit crosslinking in the periplasm. Van-FL, a derivative of the cell wall synthesis inhibitor vancomycin, binds to and detects the D-ala-D-ala moiety of uncross-linked peptidoglycan. It can be used to tag nascent peptidoglycan in bacteria with highly crosslinked peptidoglycan, particularly Gram-positive species. However, the peptidoglycan of *C. crescentus* is not highly crosslinked, and Van-FL tagging probably does not adequately distinguish sites of particularly active peptidoglycan synthesis from less active or even inactive sites.

Bacterial Filaments

Three bacterial proteins homologous to cytoskeletal proteins of eukaryotic cells appear to guide the placement of cell wall synthetic complexes in *C. crescentus*:

1. FtsZ, a GTPase, is a tubulin homolog that participates somehow in cytokinesis (pole formation); in *C. crescentus*, it also participates in stalk initiation (pole outgrowth).
2. MreB is an actin homolog whose principal role appears to be to guide cell elongation.
3. Crescentin may be homologous to intermediate filaments of animal cells; it accumulates within the cytoplasm on the concave side of vibrioid cells and appears to generate cell curvature and possibly also twisting. Discovered in *C. crescentus*, its role in bacteria other than caulobacters has not yet been established, but similar proteins are anticipated.

The properties and functions of these cytoskeletal proteins have been investigated in rods such as *Escherichia coli* and *Bacillus subtilis*, and in staphylococci, and the research with *C. crescentus* has been particularly fruitful in elucidating the dynamic nature of their localization during the course of a cell cycle. In such research, *C. crescentus* cells offer two marked advantages over other bacteria: the relative age of each cell's two poles can be inferred in microscopical preparations, and one synchronous developmental sequence can be followed by the procedurally simple means of differential centrifugation that segregates the non-stalked cells (swarmers) from their distinctly more buoyant stalked siblings.

Placement of Cytoskeletal Proteins and Cell Wall Synthesis Complexes

In *C. crescentus*, microscopical studies have revealed a hierarchy of proteins' subcellular localizations in which the accumulation of one protein at a site facilitates or is necessary to the subsequent accumulation of other proteins.

These studies have employed a molecular biological toolkit of chemicals, mutants, and highly specific genetic manipulations, the details of which are beyond the scope of this article. They have enabled investigators to follow events in deletion mutants that cannot produce specific proteins, in strains that overproduce specific proteins, and others that can be depleted (by inducible promoters or temperature) of specific proteins due to the cessation of synthesis or when a specific protein accumulation is dispersed by being depolymerized. Sequence, duration, and location of specific events are followed by the fluorescence of fusion proteins or by protein-specific fluorescent antibodies. The studies have depended heavily on observations of the events in the swarmer sequence (see Fig. 9), where synchrony is far tighter than in populations of stalked cells.

In *C. crescentus*, as in most bacteria so far examined, FtsZ is present at the site of constriction during cytokinesis. Associated with this ring are peptidoglycan-synthesizing complexes that contain MurG, the trans-glycosylase that adds the second sugar to the monosaccharide-pentapeptide-lipid carrier. The completed precursor is then flipped from the cytoplasmic to the periplasmic surface of the cell membrane, an activity currently attributed to RodA, an integral cell membrane protein. As constriction is completed, FtsZ is visible within each incipient pole. When the siblings separate, the polar FtsZ disappears due to proteolytic destruction that seems faster in the stalked than in the swarmer sibling.

As the swarmer sequence progresses, FtsZ synthesis resumes when the flagellum is shed and DNA replication begins (see Section 'The Core Regulatory Cascade of Transcription Regulation'). The new FtsZ appears first as an ensemble of short arches that extend the entire length of the cell within the cytoplasm. It is accompanied by MreB, also able to form arches, and by a third arch-forming protein, MreC, in the periplasm. A fourth protein (this is not a full list) known to become part of this system is PBP2, a periplasmic transpeptidase. As the complexes mature, peptidoglycan synthesis can occur along these arches, and the cell elongates.

Early during DNA replication, the three membrane-associated proteins FtsZ, MurG, and MreB (at least) are directed to begin accumulation as a ring at midcell. As this region matures, the synthesis of peptidoglycan causes elongation from midcell toward the poles. At about the same time, a third site of synthesis is established at the older pole and initiates elongation of that pole as a stalk. The proteins apparently responsible for stalk elongation, which occurs only at the cell-stalk junction, include MreB and RodA. After the midpoint of DNA replication, constriction becomes apparent as the midcell site of peptidoglycan synthesis develops into two new poles. Meanwhile, because the swarmer's sibling lacked a motility period during which DNA synthesis and peptidoglycan synthesis at the pole were suspended, these events have already occurred in the stalked-cell sibling. In both siblings, cell wall synthesis at the cell-stalk junction appears to pause during cell separation, then resume, probably as soon as DNA synthesis resumes in the next cycle.

Two mechanisms could explain how a protein recognizes and positions itself at a particular location. Its structure could confer on it an affinity for another macromolecule, the target; such molecular recognition is one of the most important properties of proteins. Alternatively, it could encounter a barrier at some sites that prevents its close approach or binding; by default, it would then bind only at barrier-free sites. Both mechanisms are reasonable and already described; both beg the question of what places the target or the barrier. So far, in *C. crescentus* studies of these peptidoglycan-shaping proteins, only FtsZ seems capable of finding its own way within the cell relatively independent of the other pole- and surface-localizing proteins involved in shaping the cell. Nevertheless, others in this set of proteins (particularly MreB) are required to lead the cell wall synthetic complexes to the FtsZ. In some bacteria, FtsZ rings can form only in areas within the protoplast that do not contain proteins that inhibit polymerization of FtsZ by stimulating its GTPase activity. These proteins also tend to be DNA-binding proteins that associate with specific genome regions so that their location is determined by the location, position, and state of replication of the DNA. Such proteins so far identified in *C. crescentus* include MipZ and ParB, which may provide a direct molecular connection between cell shape development during the cell cycle (most importantly, in this instance, constriction) and the DNA replication cycle.

What happens to a cell in which these events are deranged by chemical abuse, genetic modification, or malnutrition? Cell shape becomes bizarre – lemons rather than vibrios; abnormally wide stalks; stubby or branched or multiple or misplaced (ectopic) stalks; filamentous cells that divide infrequently; and so on. Specific aberrations help investigators interpret the function of a missing participant in shape determination or protein placement, as does recovery toward normal shape following relief of an inhibitory condition. Without or before relief, many cells disintegrate and die.

Extrusion Events at the Younger Cell Pole

'Extrusion' is used here to refer to the assembly of cell components, other than the typical outer membrane of a Gram-negative bacterium, that are located superficially, external to the cell membrane, and installed only at cell poles (except, of course, in *Asticcacaulis*; see Section 'Other Caulobacters'). Freshwater caulobacters such as *C. crescentus* also produce and assemble a surface array, or S-layer, on the exterior of the outer membrane. However, that layer is made constantly through the cell cycle, covers the entire cell body and stalk surface evenly, and consists of a single major exported protein. Although it is presumably of great value to the cell and has been exploited in technological applications of *C. crescentus*, it is not described here because its production, assembly, and possibly its function are neither unique to caulobacters nor an aspect of the asymmetry of development in this bacterium.

Motility: Flagellum and Chemotaxis Proteins

Assembly and activation of a flagellum at the younger pole of a dividing cell can be observed as the appendage, its activity, or its component proteins. Preparation for the development of a new flagellum begins in the swarmer cycle as the swarmer's original

flagellum is shed, motility ceases, and stalk outgrowth and DNA replication begin. Within the cell, a transcription cascade of three sets of genes begins. The products of some of these genes regulate expression of other genes in the cascade, while others' products are functional components of the flagellum apparatus. The first set ('class II genes') produce the motor switch and its ring; the second set ('class III') produce the basal body and hook; and the third set ('class IV genes') produce the flagellin subunits that are assembled into the rigid external helical filament whose rotation will propel the cell through its liquid environment. In the cascade, transcription of each successive class is required for the expression of the next class, and each set of proteins is assembled onto products of the previous set.

During the swarmer cycle, this regulatory system results in temporal (because of transcription dependence) and spatial (because of assembly dependence) ordering of the development of the next flagellum. Meanwhile, protein components needed for chemotactic sensors and response regulators are produced and accumulated in the incipient swarmer progeny as the cell begins to divide. The flagellum begins to rotate as constriction progresses at midcell, and the newborn swarmer is freely motile immediately upon separation from its stalked sibling. Within the constriction site, two proteins, TipN and TipF, accumulate that seem to mark each of the new poles as sites of flagellum assembly in the next cell cycle.

The final step in the life of a flagellum occurs in the next swarmer cycle at about the time that DNA replication begins: release of the flagellum from the older cell pole. This process is accompanied by proteolytic destruction of the motor switch protein FliF by a specific protease, ClpXP. While the mechanism of release is not yet certain, the appearance of the shed flagella that accumulate like litter in the liquid phase of laboratory cultures is consistent with a cleavage-effected separation. The filament, the hook, and the rod are still an intact unit, but the other membrane-associated components are absent.

Thus, the overall organization of the development of flagellar structure and function follow mechanistic principles similar to those of sacculus development, although regulation of transcription may play a somewhat larger role here. An immediate role for cytoskeletal proteins has not been demonstrated for flagellar morphogenesis, but it is clear that the positioning of the flagellar apparatus derives from the ultimate guidance of the constriction site placement by the cytoskeleton.

Adhesiveness: Holdfast and Pili

Many kinds of bacteria – aquatic, terrestrial, plant and animal associates – adhere to bulky (bulkier than bacterial cells) substrata by means of some type of adhesive substance, most commonly composed of or containing a significant amount of polysaccharide. For mechanical and/or electrostatic reasons, initiation of adhesion is often facilitated by motility of the bacterium. In many bacteria, it is also aided by sticky protein filaments (pili or fimbriae) that extend from the cell surface and enlarge the effective diameter of the cell, increasing its frequency of contact with other particles.

The younger pole of a *C. crescentus* cell is provided with all three devices for establishing persistent, stable adhesion to substrata. At about the same time that the new flagellum becomes visible and active on a dividing cell, extrusion of adhesive material (the 'holdfast') occurs at the same pole. The glue is composed of poly-N-acetylglucosamine and other components. It is the strongest adhesive known among biotic glues, stronger than the adhesives of marine mollusks. It works under water and allows caulobacters to adhere to a wide diversity of organic and inorganic inanimate materials and to other microorganisms, most commonly algae and cyanobacteria in natural samples, without damaging the substrate cells. Once sessile, a *C. crescentus* cell remains fixed in place; there seems to be no mechanism for the cell to reverse adhesion, and the holdfast seems to retain its adhesiveness indefinitely.

In several species of caulobacters, pili are also extruded by assembly at the same pole as the flagellum and holdfast, but unlike those structures, pili are not universal among isolates. In *C. crescentus*, the pili accompany the other polar structures in position and in time during the cell cycle.

Caulobacters are fun to watch. Prior to sibling separation, if the stalked pole is not attached to the slide or cover slip, the single flagellum drives the dividing cell stalk foremost. The flagellated pole can attach without causing cessation of flagellar rotation, swinging its stalked sib around with the stalk waving in the liquid in a direction that reveals that, most of the time, flagellar rotation is counterclockwise. When the siblings separate with neither of them attached to a substratum, the swarmer pauses briefly and then resumes swimming at a speed noticeably greater than it achieved while it carried the additional mass of its stalked sibling.

One seemingly awkward aspect of adhesion initiated by the swarmer is direction: the flagellum drives the cell predominantly with the adhesive, flagellated pole as posterior and the newborn, non-adhesive pole in the lead. While this should prevent adhesion to most of the particles the cell approaches, motility should assist adhesion only when the cell backs up.

In most caulobacters, the three structures important to adhesion arise at the site on the younger pole of the dividing cell that will grow outward as the stalk. In the closely related genus *Asticcacaulis*, stalk outgrowth occurs at a separate site, in one species not even at the pole (see Section 'Other Caulobacters' and Figs. 4 and 7). As the flagellum is shed and stalk outgrowth begins, the pili are retracted by depolymerization within the periplasm, but the holdfast remains on the tip of the growing stalk. In contrast to stalk outgrowth, which probably pauses during cytokinesis and certainly continues (or resumes) in each cell cycle, synthesis and extrusion of the holdfast, pili, and flagellum occur only once in the life of a pole. If the cell does not adhere to a substratum while motile, it is much less likely to do so after it sheds its flagellum and begins to develop a stalk, even though the holdfast persists. It can migrate to a new locale only through its swarmer progeny. Given such a long-term commitment to a substratum, is there any mechanism that promotes or guides adhesion of the cell to a favorable environmental site?

The answer presumably lies in the ability of the cell to move to a favorable location chemotactically. Like other motile bacteria, caulobacters are provided with a chemical gradient sensory system that influences the activity of the flagellar motor. Unlike peritrichously flagellated bacteria, these mono-flagellate swarmers do not tumble, but they do occasionally stall. Brownian motion

is sufficient to reorient the cell during a stall, and this passive tumbling results in a redirection of the cell's swimming when rotation resumes. The components of the chemotaxis system, synthesized before midcell constriction occurs, are placed at the cell poles. However, even before constriction is completed, they are swiftly destroyed by proteolysis at the stalked pole and so are inherited almost exclusively by the swarmer sibling. Proteolysis eventually eliminates the system in the swarmer sibling as it begins the transition from swarmer to stalked cell, in concert with the shedding of the flagellum.

In the limited studies of specific stimuli of chemotaxis in caulobacters, the strongest influences on this response have proved to be potential nitrogen sources. This is consistent with the striking effect of nitrogen limitation as an inhibitor of the events that begin the maturation of a swarmer into a stalked cell. It implies that a major purpose of motility in these oligotrophic bacteria is to reposition the young swarmer not only away from its sibling and into an aerated region, but toward a substratum fixed in a nitrogen-adequate location.

Bacteriophage Receptors

There is a final set of functions that are transiently present at the younger pole of a dividing cell: reception of bacteriophage. With few exceptions, the attachment of bacteriophage produced by caulobacters is pole-specific, and the pole is the same for all such viruses – the flagellated, sticky pole. All the RNA phages of caulobacters attach to the pili; at least one type of DNA phage attaches to the flagellum; and the majority of DNA phages attach directly to the pole, either to unidentified components of the outer membrane or to protein(s) situated at the site through which pili are extruded. None is known to be holdfast-specific. The unusual consequence of this pole specificity is that bacteriophage infection is a 'childhood' disease of caulobacters, in the sense that susceptibility to infection depends on traits of the juvenile stage, the swarmer. As a consequence, virus infection spreads through a caulobacter population only when the environmental conditions are suitable for completion of at least one reproductive cycle – and for the production of virions.

Influence of Macronutrient Availability on Stalk Initiation and Elongation

Almost all of the nutritional experiments have been conducted with the species *C. crescentus*, which is amenable to the studies of nutritional influences because it can grow in chemically defined media. Nevertheless, one principle regarding development in caulobacters is observable with every isolate tested: development responds significantly to changes in nutrient concentration, even in complex media. Phosphate and nitrogen-source effects were recognized early; effects of carbon source, calcium, and magnesium are also known, but have received relatively little attention to date.

Nitrogen Source and Stalk Initiation

In general, caulobacter-like isolates can be cultivated in complex media containing peptones, which provide nitrogen as amino acids and peptides. *C. crescentus*, *C. leidyi*, and some *Asticcacaulis* isolates can be cultivated in media that provide inorganic nitrogen as ammonium or nitrate salts. However, because *C. crescentus* lacks the low-affinity ammonium uptake system of glutamate dehydrogenase and depends on the high-affinity GS/GOGAT system for ammonium assimilation, it starves for nitrogen when ammonium is in excess. Such starvation is relieved by supplementation of the medium with glutamate or by substitution of nitrate for ammonium. Nitrogen limitation has one long-known, demonstrable effect on the cell cycle: nitrogen unavailability causes cell cycle arrest that postpones release of the flagellum, onset of DNA replication, and initiation of stalk outgrowth. This swarmer stage arrest is linked to ppGpp production and it depends on SpoT activity.

Phosphate and Stalk Elongation

In considering stalk length in caulobacters, the age of the stalk, which grows during each cell cycle, needs to be accommodated. First-generation stalks have had only one growth period, third-generation stalks have had three growth periods, and so on. To compare two differently manipulated populations with each other with respect to stalk length, results will be informative only if stalk length is measured only for the stalks in the same generation. The first generation is by far the most variable, probably due to composite influences on two stages of stalk development – stalk initiation and stalk elongation. After the first stalk band has been installed, stalk outgrowth occurs by stalk elongation only, and that process can be evaluated separately from stalk initiation.

Phosphate concentration in finite cultures and phosphate flux in perpetual cultures (chemostats) exert striking effects on stalk development. At high phosphate concentrations or fluxes, stalk length by the end of the second cell cycle does not significantly exceed average length of siblings at the time of completion of cell constriction. During phosphate exhaustion in a phosphate-limited finite culture and most of the time in a phosphate-limited chemostat culture, second-generation stalks (with one or two bands) are several times longer than newborn cells. It is readily inferred that abundant phosphate retards stalk elongation. It probably also inhibits stalk initiation, at least in mutants that appear 'stalkless' until grown in low-phosphate conditions. Stalk initiation is, however, difficult to recognize until it is followed by stalk elongation, and so cannot be studied independently of elongation.

Like many other bacteria, *C. crescentus* possesses a Pho regulon of genes whose expression is governed by the availability of phosphate. Most of these genes occur in an operon containing at least three genes for high-affinity phosphate transport proteins and

at least three for sensing and responding to the availability of phosphate; another transport gene, *pstS*, is at a separate locus. If these genes work together in a manner similar to such genes in E. coli (which seems probable), the relationship between phosphate abundance and rate of stalk elongation would be as follows.

1. The proteins PstC, PstA, PstB, and PstS form a cell membrane complex capable of high-affinity phosphate transport. The complex is associated with the peripheral cytoplasmic protein PhoU, and through PhoU with the integral membrane protein PhoR. PhoU is a sensor protein and PhoR is a response modulator. The response regulator, PhoB, is cytoplasmic, not membrane-associated.

2. When phosphate is abundant, PhoR remains with the complex, but when phosphate is scarce, it is transported via the Pst complex. The passage of phosphate through the complex releases PhoR from the complex, but not from the cell membrane. PhoR then autophosphorylates (see Fig. 10(c)) and transfers its phosphate group to PhoB.

3. PhoB-P activates genes of the Pho regulon, increasing the abundance of the Pst proteins and providing other features for dealing with phosphate limitation, such as synthesis of phosphatases that enable the cell to consume phosphorus from organic phosphate compounds. In *C. crescentus*, these events are accompanied by a marked acceleration of stalk elongation.

Studies with cell wall synthesis inhibitors indicate that some of the immediate effectors of stalk outgrowth (initiation and elongation) catalyze peptidoglycan synthesis and/or modification, but no such enzyme has yet been identified as unique to stalk sacculus development. Studies of mutants that seem able to divide without stalk outgrowth when cultivated in phosphate-rich media have consistently been found able to initiate at least some outgrowth when subjected to phosphate limitation. Decades of searching for *C. crescentus* mutants that are viable yet lack at least one piece of genetic information essential to stalk development has not yet yielded such a mutant. Every *C. crescentus* laboratory has recognized the implication that because genetic stalklessness appears to be lethal, it is reasonable to regard stalk development as a process of cell surface morphogenesis that is common to pole outgrowth and pole formation. The latter process cannot be lost without eliminating division. Nevertheless, it is not unreasonable to expect that some step late in the hierarchy of regulation of stalk development could be eliminated without preventing fission, and a viable unconditionally stalkless mutant may yet be found.

Proteomic characterization of the *C. crescentus* stalk has identified several proteins that are known to participate in nutrient acquisition – for example, TonB-dependent receptors, certain outer membrane pore/channel proteins, and phosphatases and other hydrolytic enzymes that assist in increasing the transportability of charged substrates. Altogether, the stalk proteins so far identified appear to be a subset of the cell body periplasmic and outer membrane proteins; none appears to be unique to the stalk. Whether any of the stalk proteins are installed in the stalk only during phosphate limitation has not yet been systematically explored.

Control of Development: Protein Synthesis, Modification, Localization, and Destruction

The Core Regulatory Cascade of Transcription Regulation

For the first three decades or so of developmental research in *C. crescentus*, attention focused intensely on determining the cause of the postponement of developmental events in the swarmer sibling – events that occur in the stalked sibling in the same order and at the same rate, but suspended during motility in the swarmer sibling. By the early 1990s, a lengthy description of regulation through transcription control, protein phosphorylation that alter protein activities, and installation of specific proteins at specific cellular locations had been elucidated. Advances since then in genome manipulation, sequencing, and interpretation, as well as in subcellular microscopy of living cells and in physicochemical analysis of protein structure, have greatly accelerated the elucidation of further details. The core of the cell cycle's direction as described by about 1990 has proved adequate as new information has been fit into that core. Following is a brief description of the major regulatory features that govern development in both siblings: swarmer and stalked cell.

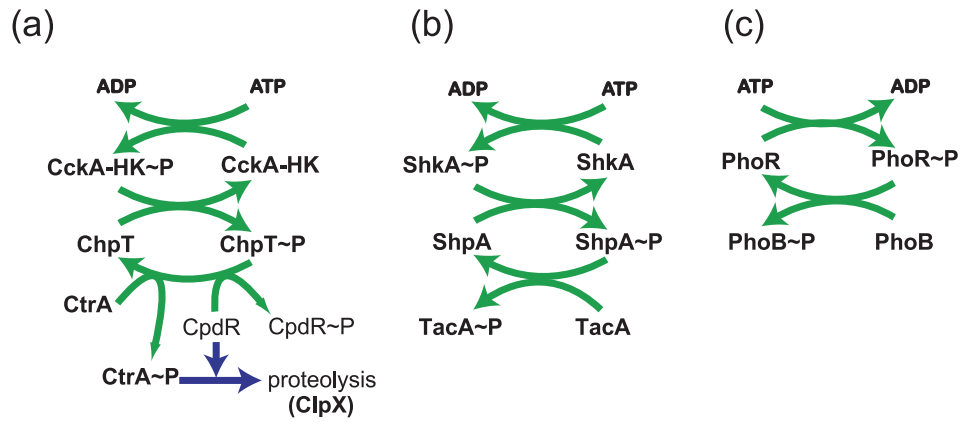

Fig. 10 Example phosphorelays (*p*-relays) known to influence pole development and progress through the cell cycle in *C. crescentus*. (a) CckA-initiated, ChpT-mediated, to the gene activator CtrA-P and CpdR (CpdR-P is unable to activate CtrA proteolysis). (b) ShkA-initiated, ShpA-mediated, to the gene activator TacA. (c) PhoR-initiated under conditions of phosphate limitation, to the gene activator PhoB-P.

Four regulatory proteins constitute the core of the transcriptional regulatory mechanism: CcrM, DnaA, GcrA, and CtrA. The last three of them are transcription regulators that interact with each other's genes and ccrM; as a group, they also control hundreds of genes whose products mediate the cell changes and assist in regulation. Most of the principles of cell cycle regulation in *C. crescentus* can be laid out in terms of these four proteins – their regulated expression, their placement relative to the cell poles, and their cyclical destruction by proteolysis.

Description of a cycle can begin at any point; the point used here is the epigenetic step: methylation of specific nucleotides close to a gene's promoter that affects the gene's transcription. Each of the other regulatory proteins activates or inhibits transcription.

1. CcrM is the DNA methylase: its methylation of DNA inhibits expression of *ctrA* and of its own gene, *ccrM*.
2. DnaA activates *gcrA*: through a multitude of other genes in its regulon, DnaA is essential for initiation of DNA replication.
3. GcrA (modulated by CcrM methylation) activates *ctrA* transcription from the first of *ctrA*'s two promoters, P1 and P2.
4. CtrA is phosphorylated by the CckA-ChpT P-relay (see Fig. 10(a)). CtrA-P has multiple regulatory effects as it accumulates:
   a. Activation of *ctrA* through its second strong promoter, P2, providing positive feedback regulation of its own transcription;
   b. Inhibition of *ctrA* transcription through P1, providing negative feedback of control of its transcription by GcrA;
   c. Inhibition of transcription of *gcrA*; a, b, and c together shift responsibility for regulation of *ctrA* from GcrA to CtrA-P;
   d. Prevention of initiation of DNA synthesis by binding near the origin of replication (ori), blocking the binding of the replication proteins. Initiation of DNA replication happens only once in each cell cycle in *C. crescentus*, necessarily occurring before CtrA-P accumulates;
   e. Activation of *ccrM*, reinitiating this regulatory cascade by activating DNA replication by inhibiting transcription of *ctrA*.

The operation of this cascade results in the sequential transcription of the four genes in order: *dnaA*, *gcrA*, *ctrA*, *ccrM*. Each of the gene products DnaA, GcrA, and CcrM is effective as an unmodified protein and begins to function as it accumulates, although GcrA is further modulated by CcrM methylation of promoters. CtrA, in contrast, must be converted into CtrA-P by phosphorylation, which is the role of a phosphorelay (*p*-relay) initiated by an auto-phosphorylating kinase, CckA. CckA itself has kinase and phosphatase activity being modulated by c-di-GMP and stress conditions, both triggering the dephosphorylation of CtrA and CpdR.

Roles of Phosphorelays

Protein phosphorylation is a mechanism for controlling the activity of catalytic proteins, which may be activated or inactivated by the addition of one or more phosphate groups to target sites, typically serine, threonine, or histidine residues. Cells also possess phosphatases that remove the phosphate groups and reverse the direction of control. P-relays provide control of metabolism by making the phosphate groups available to the families of proteins whose activation-inactivation depends on the presence and activity of the relay components; the relays thereby serve as a buffer to balance activation among pathways and their branches and so to direct metabolic traffic. Operation of a P-relay requires, at a minimum, a kinase that transfers the phosphate group ('∼P') from a nucleotide triphosphate to a protein (including itself, if capable of autophosphorylation), and a phosphotransferase to move the ∼phosphorus to at least one other protein. In a P-relay involved in regulation of transcription rather than of enzyme activities, one of the proteins along the relay is converted from an inactive form into a gene-activating protein that will target promoters that contain a unique, shared sequence. Activation of a set of such genes (a regulon) provides the cell with new, usually related properties simultaneously.

The genome of *C. crescentus* contains 106 genes – an exceptionally high number – for two-component signal-transduction proteins that function via P-relays. The activity of one-third of these genes rises and falls as the cell progresses through the cell cycle, implying that they participate in the regulation or they are under the control of developmental processes in *C. crescentus*. Such roles are known for two of the three examples of *C. crescentus* P-relays illustrated in Fig. 10. The CckA-ChpT P-relay (Fig. 10(a)) passes phosphate groups from ATP to ChpT, a branch point protein in the relay from which the ∼phosphorus can be passed to more than one other protein. One of those proteins is CtrA; another is CpdR. The CckA-initiated relay is, therefore, integral to the core of cell cycle regulation in *C. crescentus*.

The second relay illustrated (Fig. 10(b)) transfers ∼phosphorus from ATP through ShkA (the kinase) to ShpA (the phosphotransferase) and then to TacA (the response regulator). TacA-P is an enhancer-binding protein that can also be phosphorylated through CtrA-P. One of the genes activated by TacA-P, staR, coding an essential transcription factor for stalk elongation in nutrient-rich medium; the requirement for StaR may account in part for the known need for intact TacA for that process. The third, two-component system (Fig. 10(c)) illustrates the role of a P-relay in the response of Gram-negative bacteria, presumably including caulobacters, to environmental limitation for phosphate as a nutrient, which was discussed above (see Section 'Phosphate and Stalk Elongation').

Chromosome and Protein Localization

In the dividing cell, one copy of the origin of replication (ori) of the *C. crescentus* chromosome is situated at each pole. Completion of constriction separates the siblings, each with a copy of ori at its now-older pole. In the next cycle, as soon as replication begins (sooner, in the stalked sibling; later, in the swarmer cycle), one copy of ori migrates to the younger pole. Thus, for most of the cell cycle, begun either by a swarmer or by a stalked cell, the two copies of ori are at opposite poles.

Also prior to the completion of cell fission, the protein CpdR has somehow guided the installation of the protease ClpXP at the stalked pole. Several other proteins become localized to the poles late in the cell cycle. Motility-associated proteins of the entire

flagellar structure and chemotaxis system (e.g., McpA) are found only in the swarmer sibling, some installed in the cell envelope, others dispersed in the cytoplasm. Proteins associated with constriction (including FtsZ, MurG, and RodA; see 'Placement of cytoskeletal proteins and cell wall synthesis complexes') have gathered at about midcell. By the time the cell divides into two morphologically distinguishable siblings, they are also distinguishable by the presence of and arrangement of internal proteins. Probably most significantly, the core regulatory protein CtrA remains at ori within the stalked pole, but disperses into the cytoplasm in the swarmer sibling.

Starting Over: Proteolysis

Following the separation of the two copies of the origin of replication (ori), CtrA-P accumulates and blocks initiation of another round of replication during the remainder of the cell cycle, in both siblings. However, during constriction, while newly synthesized CtrA remains dispersed in the swarmer compartment, CtrA becomes localized at the stalked pole in the other compartment. Also localized at the stalked, but not the flagellated, pole is a complex of proteins that includes the protease ClpXP. Within that complex, ClpXP destroys the local CtrA and frees ori for the initiation of DNA replication.

ClpXP is positioned at the stalked pole of the dividing cell and does not diffuse into the incipient swarmer. Adding to the asymmetry of the next events is a role of the CckA-initiated P-relay, which is a phosphatase mode in the stalked sibling. As phosphorylation of CtrA fails, the protein becomes more susceptible to proteolysis and is destroyed, allowing DnaA to initiate DNA replication.

CtrA-P persists for a while in the newborn swarmer, but proteolysis of CtrA will occur in the swarmer sibling later – after the period of motility – and involves proteins and events that are not yet as certain as those identified in the stalked sibling. There are roles for the CckA-ChpT P-relay, a phosphatase (PleC) localized in the swarmer cytoplasm, DivK/DivK-P, and once again for ClpXP. In addition to the effects of CtrA-P noted above, CtrA-P activates *divK*, whose product inhibits the P-relay from CckA-ChpT to CpdR. Inhibition of CtrA proteolysis, an effect of CpdR-P, probably declines, allowing CtrA proteolysis in the newly-differentiated stalked cell, finally resetting the core regulatory cascade. This seems to coincide with the release of the flagellum, retraction of the pili (if present), initiation of DNA synthesis, and the onset of stalk outgrowth.

Besides CtrA, ClpXP also attacks TacA (see above), FliF, the flagellar motor switch, and McpA, a chemotaxis protein, both located at the flagellated pole. Proteolysis of FliF is a candidate as a crucial step in the release of the flagellum, which occurs close in time to the onset of DNA replication. The McpA will be replaced by renewed synthesis as the transcription cascade of flagellar genes begins again later in the cell cycle.

In summary, although the story of development in this dimorphic bacterium is a 3-chapter tale of poles, the cast of participants includes scores of proteins. The plot involves regulation of transcription; covalent modification of proteins that alters their activities; and localization of cytoskeleton-like proteins, cell wall synthesis complexes, DNA-binding proteins, and structural proteins to target sites or away from barriers. Unraveling the plot has revealed that these bacteria achieve functional compartmentalization of the protoplast by localization of proteins rather than by the familiar elaboration of internal membrane barriers seen in eukaryotic cells. Ultimately, degradation of certain participants resets the sequence and renews the cycle of events. The greater the detail in which this story is related, the stronger the implication that the unique dimorphy of caulobacters expresses a complex and sophisticated genetic heritage, and that dimorphy is of great significance to the success of caulobacters as they eke out their existence by competing for nutrients that are perpetually scarce.

In today's biology, we accept the notion that cells do not create genes; they copy them. The remarkably detailed molecular studies of *C. crescentus* development should help us recognize the parallel throughout the cell: some molecules in addition to DNA must be present for still other molecules to be synthesized and ordered. Like peptidoglycan and DNA, which can be polymerized only in a cell that has some of each, the cascades, feedback loops, protein phosphorylation pathways, and other controlling events, which occur during *C. crescentus* development, occur normally only in cells in which certain key events are already in progress and where certain key proteins are already in place. *C. crescentus* research, with an abundance of molecular detail, is contributing to cell biology an elegant lesson in the meaning of the biotic continuum.

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Chlamydia☆

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Glossary

Chlamydia muridarum A mouse-adapted *Chlamydia* species.
Chlamydia pneumoniae The bacterium that causes community-acquired pneumoniae.
Chlamydia trachomatis The bacterium that is the most common cause of sexually transmitted disease and preventable blindness worldwide.
Elementary body The infectious but non-metabolically active form of *Chlamydia*.
Inclusion The intracellular vacuole in which all *Chlamydia* species differentiate and replicate within.
Lipid droplets Endoplasmic reticulum-derived lipid storage organelles that are a source of neutral lipids.
Reticulate body The noninfectious but metabolically active form of *Chlamydia*.
Sphingomyelin A Golgi-derived sphingolipid that usually consists of ceramide and phosphorylcholine.
Type III secretion system A secretion system present in Gram-negative bacteria that functions to deliver effectors directly from the bacteria into the host cytoplasm.

Abbreviations

CADD	*Chlamydia* protein associating with death domains;
CPAF	Chlamydial protease-like or proteosome-like activity factor;
EAE	Enzootic abortion of ewes;
EB	Elementary body;
EEA1	Early endosomal antigen;
EUO	Early upstream open reading frame;
GrgA	General regulator of gene transcription A;
HGT	Horizontal gene transfer;
IDO	Indolameanine 2,3-dioxygenase;
Ig	Immununoglobulin;
IL	Interleukin;
Inc	Inclusion Membrane Protein;
INF-γ	Gamma interferon;
LCTs	Large cytotoxins;
LD	Lipid droplets;
LGV	Lymphogranuloma venereum;
MIF	Microimmunofluorescence assays;
MTOC	Microtubule organizing center;
MVBs	Multivesicular bodies;
OEA	Ovine enzootic abortion;
ORFs	Open reading frames;
p.i.	Postinfection;
Pgp3	Chlamydial plasmid-encoded protein 3;
PI3K	Phosphatidylinositol-3-kinase;
PZ	Plasticity zone;
RB	Reticulate body;
SNAREs	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors;
T3SS	Type III secretion system;
Tarp	Translocated actin recruiting protein;
TepP	Translocated early phosphoprotein;
TNF	Tumor necrosis factor

☆*Change History:* January 2015. HF Rutgers and MA Scidmore changed Sections entitled 'Abstract', 'Introduction', 'Taxonomy', 'Intracellular Survival', '*C. muridarum*', 'Conclusions' and 'Further Readings'; three sections entitled 'The plasmid', '*C. avium*' and '*C. gallinacean*' added; Figures 2 and 3 deleted.

This article is an update of M.A. Scidmore, Chlamydia, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 74–86.

Defining Statement

Chlamydiae are obligate intracellular bacteria that replicate within a nonacidified vacuole, termed the inclusion, which is actively modified by chlamydiae to promote their intracellular survival. To mediate their pathogenesis, chlamydiae secrete effector proteins into the host cytoplasm and inclusion membrane that directly target host signaling and trafficking pathways.

Introduction

Chlamydia is a Gram-negative obligate intracellular bacterium that is pathogenic for humans and a large variety of veterinary animal species. Chlamydiae replicate within a nonacidified vacuole, termed the inclusion, which is actively modified by chlamydiae to create a replication-competent intracellular compartment. Significant progresses in the understanding of the molecular mechanisms that mediate the pathogenesis of chlamydiae have been elucidated in the post-genomic era. It has become clear that chlamydiae secrete both type III secretion system (T3SS)-dependent and -independent effectors into the host cytoplasm to directly target and exploit basic host cellular signaling and trafficking pathways. The understanding of *Chlamydia* biology is being rapidly accelerated as a result of novel genetic tools, particularly newly developed transformation methodologies, and various expression and silencing vectors.

Taxonomy

Chlamydiae belong to the Chlamydiaceae family (order Chlamydiales) that contains a single genus *Chlamydia*. Since 1999, three new families have been classified within the order Chlamydiales. These include the families Parachlamydiaeceae, Simkaniaeceae, and Waddliaceae, all of which contain *Chlamydia*-like or environmental *Chlamydia* species. Currently, the genus *Chlamydia* contains 11 species: *Chlamydia trachomatis*, *Chlamydia muridarum* (formerly *trachomatis* mouse pneumonitis agent or MoPn), *Chlamydia suis*, *C. pneumoniae*, *C. psittaci* (formerly *psittaci* avian), *C. pecorum*, *C. abortus*, *C. felis*, and *C. caviae* (formerly *psittaci* GPIC), *Chlamydia avium* and *Chlamydia gallinacean* (*Sachse et al., 2014*). It should be pointed out that, near the end of the last century, two genera were proposed for Chlamydiaceae: *Chlamydia* and *Chlamydophila*. However, that taxonomy received little support from chlamydial researchers.

Chlamydiae have a diverse range of hosts. Man is the only known host for *C. trachomatis*. In comparison, the host range of *C. pneumoniae*, once thought to be an exclusively human pathogen, has been extended to other mammals, amphibians, and reptiles. All other *Chlamydia* species primarily infect nonhuman animals and birds, although occasional zoonotic infections do occur. *C. suis* infects swine; *C. abortus* infects ruminants; *C. caviae* infects guinea pigs; *C. felis* infects cats; *C. pecorum* infects mammals such as cattle, koalas, sheep, and swine; *C. psittaci*, *C. avium* and *C. gallinacea* infect birds.

Within the species *C. trachomatis*, there are two biovariants (trachoma and lymphogranuloma venereum, LGV) that are distinguished based upon their anatomical distribution and severity of disease. The trachoma biovars are further classified serologically into at least 20 distinct serovars. Chlamydiae of serovars A–C primarily infect ocular epithelial surfaces and are associated with endemic blinding trachoma, a leading cause of preventable blindness worldwide. Chlamydiae of serovars D–K infect the urogenital epithelial mucosa and are the most frequent cause of bacteria-caused sexually transmitted disease, but they can also cause inclusion conjunctivitis in adults, presumably by autoinoculation from a genital site of infection. The LGV biovars (serovars L1, L2, and L3) are also sexually transmitted and infect urogenital epithelial mucosal surfaces. However, unlike the trachoma biovars, which remain localized to mucosal surfaces, the LGV serovars are more invasive. The LGV serovars penetrate the submucosa, resulting in spread to the lymphatic system.

In humans, *C. pneumoniae* is primarily a respiratory pathogen that is a leading cause of community-acquired pneumonia. Additionally, *C. pneumoniae* has been linked with several chronic conditions such as coronary heart disease and is thought to be an added risk factor for the development of atherosclerosis. Finally, *C. pneumoniae* has been isolated from the brain of Alzheimer disease patients, but it is not clear what (if any) role this organism plays in the pathogenesis of the disease.

Developmental Cycle

Despite extreme diversity in tissue tropism, disease expression, and *in vitro* growth properties, all chlamydiae are characterized by a unique biphasic developmental cycle that alternates between two morphological and functionally distinct cell types: the elementary body (EB) and the reticulate body (RB) (Figure 1). The EB is the smaller developmental form measuring 0.3–5 μm in diameter. It is metabolically inert and is the infectious form of the bacterium. The physical features that define the EB include a highly condensed nucleoid structure as well as an extensively disulfide cross-linked outer membrane. In the absence of measurable amounts of peptidoglycan, a cell wall component that provides structural support to most other bacterial species, the cross-linking between cysteine-rich outer membrane proteins imparts structural rigidity to the EB. The properties of the EB facilitate the extracellular survival of the organism at the initial infection or transmission stages and mediate internalization, and hence infection, of the organism into host eukaryotic cells.

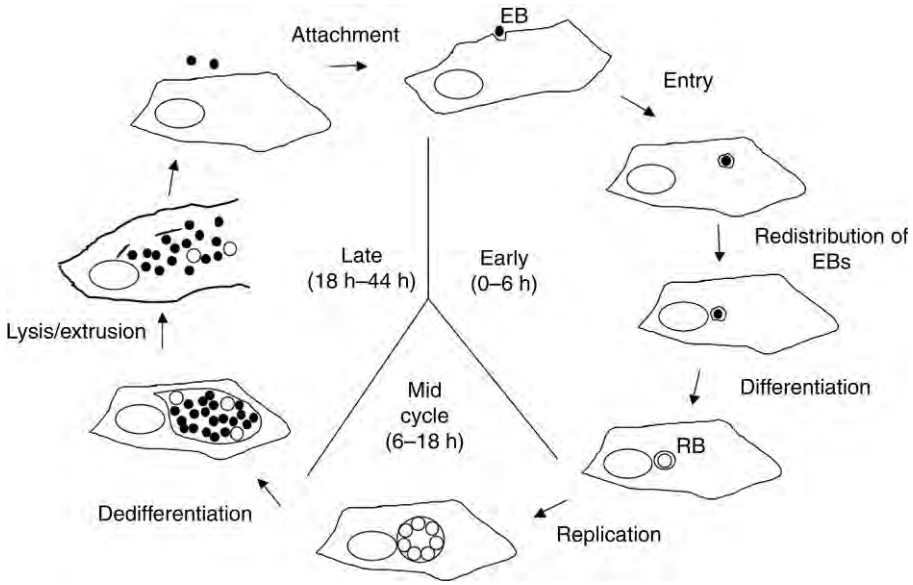

Figure 1 Chlamydial developmental cycle. The developmental cycle begins with the attachment and parasite-mediated endocytosis of the elementary body (EB). After internalization the EB is found within a membrane-bound vacuole, termed the inclusion. By 2 h p.i., the EB differentiates into the reticulate body (RB) and is trafficked to the peri-Golgi region of the host cell. Between 8 and 10 h p.i. the RBs begin to replicate and between 18 and 24 h p.i. some of the RBs begin to dedifferentiate back into infectious EBs. The cycle ends with either host cell lysis or extrusion of the inclusion from the infected cell, resulting in release of infectious EBs. All *Chlamydia* species undergo a similar developmental cycle. However, the time it takes to complete the cycle may vary from 36 to 96 h depending on the particular species.

In contrast to the EB, the RB is larger at approximately 1 μm in diameter; it is also metabolically active and noninfectious. In addition, the nucleoid is more diffuse within the bacteria, making the DNA accessible to the transcriptional and replication machineries. Furthermore, the outer membrane is less cross-linked, which makes the RB much more fragile than the EB and enables the replication of the organism. Whereas the EB functions in extracellular survival and internalization, the RB is the metabolic and replicative form and thus functions in intracellular survival and replication.

The developmental cycle is initiated by the attachment and internalization of the EB into the host eukaryotic cell, resulting in the envelopment of the EB in a membrane-bound vacuole, which has been termed the inclusion (Figure 1). The bacteria remain confined within the inclusion during the entire developmental cycle. Within the first several hours postinfection (p.i.), the internalized EBs differentiate into RBs. Depending on the specific strain or species, the newly generated RBs replicate by binary fission starting between 6 and 18 h p.i. The developmental cycle is asynchronous. While some RBs continue to divide, others begin to differentiate back into EBs, resulting in a mixture of EBs and RBs. Since RBs are localized adjacent to the inclusion membrane, while EBs are localized to the lumen of the inclusion, it has been proposed that detachment from the inclusion membrane and inactivation of the T3SS apparatus may signal the dedifferentiation process. As the organisms replicate, the original inclusion membrane increases in size to accommodate the increasing numbers of organisms. By the end of the cycle, the inclusion occupies the majority of the host cytoplasm. The increase in size of the inclusion is likely due to incorporation of both *Chlamydia*- and host-derived proteins into the expanding inclusion membrane. The cycle ends with either activation of cysteine proteases and host cell lysis or extrusion of the inclusion out of the cell by an actin- and myosin-dependent mechanism. Release of newly generated infectious EBs that can infect neighboring cells ensures continuation of the chlamydial developmental cycle.

All *Chlamydia* species undergo a similar developmental cycle that differs primarily in the time it takes to complete the cycle, which can range from 36 h for the fast-growing *C. muridarum* strains to 96 h for the slower-growing *C. pneumoniae* strains. The *C. trachomatis* strains fall in the middle of this spectrum with cycle lengths of 44–72 h depending on the specific biovar or serovar, and culture condition.

Whole genome microarray and RT-PCR analyses of specific subsets of chlamydial genes revealed that chlamydiae regulate the transcription of their genome in a temporal fashion. Three classes of gene transcription were identified that correlate with morphological aspects of the developmental cycle. Expression of 'early' genes is detected from 1 to 6 h p.i. 'Early' genes are likely to include genes involved in early metabolic processes, nutrient uptake, inclusion biogenesis, and EB to RB differentiation. Expression of 'mid cycle' genes is detected between 6 and 18 h p.i., and these genes are likely to be involved in replication and metabolic processes. Finally, expression of 'late' genes is detected 18 h and 24 h p.i. 'Late' genes include those involved in the dedifferentiation of RBs to EBs. Although the chlamydial genome encodes two alternative sigma factors, temporal regulation of chlamydial gene transcription is not regulated by these alternative sigma factors. Other types of regulation, such as response to stress, heat, nutrients, and host inflammatory cytokines, are superimposed upon the global temporal regulation.

Intracellular Survival

As obligate intracellular bacteria, chlamydiae have evolved highly successful strategies to promote and maintain their intracellular lifestyle including specific mechanisms to (1) gain entry into host cells, (2) avoid or prevent degradation by degradative lysosomal enzymes, (3) obtain nutrients and biosynthetic precursors while sequestered within a protected niche, and (4) evade host immune detection and destruction. Importantly, chlamydiae accomplish these tasks while sequestered within the protected environment of the inclusion through the exploitation of host signaling and trafficking pathways. Because *Chlamydia* species display such differences in tissue tropism and *in vitro* growth properties, and because of differences arising from experimental protocols, observations made about one species or serovar may or may not apply to all species.

Entry

For most *Chlamydia* species, columnar epithelial cells, which are nonprofessional phagocytes, are the primary sites of infection. However, other cell types can be naturally infected by some species. For example, the LGV biovars of *C. trachomatis* can replicate in monocytes/macrophages, while *C. pneumoniae* can replicate in many different cell types including epithelial, endothelial, and smooth muscle cells, and monocytes/macrophage. Chlamydiae enter nonprofessional phagocytes by a process that has been defined as 'parasite-mediated' endocytosis since it is 10–100 times more efficient than the uptake of inert particles such as latex beads or opsonized yeast. *In vitro*, multiple modes of entry have been documented including both clathrin- and non-clathrin-mediated endocytosis, microfilament-mediated phagocytosis, microfilament-independent pinocytosis as well as lipid raft-mediated endocytosis. Although different modes of entry have been established for *Chlamydia* species, a novel host cell actin-dependent entry mechanism has recently been reported. In this model, attachment of the EBs to the host cell surface is followed by the translocation of the T3SS effectors, translocated actin recruiting protein (Tarp) and translocated early phosphoprotein (TepP), into the host cell. Upon translocation, Tarp and TepP are sequentially phosphorylated by host cell kinases and concentrate on the cytoplasmic side of the eukaryotic plasma membrane at sites of EB attachment in *C. trachomatis*-infected cells. Tarp is an actin-binding protein that facilitates localized actin recruitment and polymerization at sites of EB entry. TepP likely serves as a downstream effector in this process. Subsequent to the phosphorylation of these secreted proteins, there is activation of host Rho GTPases, which are small ras-like GTPases that regulate actin dynamics. In *C. trachomatis*-infected cells, Rac1 is activated, while in *C. caviae*-infected cells, both Rac1 and Cdc42 are activated. The activation of Rho GTPases leads to the recruitment of several host proteins, including WAVE2, Abi-1, and Arp2/3, which are critical molecules in actin signaling and polymerization, to sites of EB attachment. Collectively, the activation and recruitment of these host signaling molecules, and others including another small GTPase, Arf6, leads to localized actin polymerization and cytoskeletal rearrangements that mediate the internalization of EBs. For other species, such as *C. pneumoniae*, the attachment of EBs induces the activation of other host signaling pathways including phosphatidylinositol-3-kinase (PI3K)-dependent and ERK1/2-dependent signaling pathways that are important for the entry of *C. pneumoniae* EBs.

Early Intracellular Events

During the first 2–3 h following internalization several critical events occur that are dependent on early *de novo* chlamydial gene expression. One of these critical events is EB to RB differentiation. Morphologically, differentiation is manifested by the reduction of the disulfide-linked outer membrane, the decondensation of the nucleoid, and an increase in size of the bacterium. The decondensation of the chlamydial chromatin plays an important role in silencing gene transcription in EBs. Two basic histone-like proteins Hc1 and Hc2 are thought to play critical roles in this process as overexpression of chlamydial Hc1 in *Escherichia coli* leads to condensation of the *E. coli* chromosome, gene silencing, and death. However, since Hc1 is expressed throughout the developmental cycle, degradation of Hc1 cannot be a mechanism that triggers DNA unwinding. Insights into this intriguing dilemma were revealed when researchers found that the death induced by overexpression of Hc1 in *E. coli* could be rescued by the overexpression of a second chlamydial gene product, CT804. CT804 is predicted to encode a kinase that shares homology to *E. coli IspE*, an enzyme that functions in a nonmevalonate pathway of isoprenoid biosynthesis. Currently, researchers believe that a metabolite of the pathway may act as a competitive inhibitor that blocks the DNA binding activity of Hc1, thereby allowing the DNA to unwind and enabling the initiation of the developmental transcriptional program.

EUO is one of few genes that are transcribed almost immediately (<1 h p.i.) after EB entry into the cell. By binding selectively to later gene promoters, EUO allows the transcription of early genes. Expression of early genes may also be facilitated by the newly identified transcription factor GrgA synthesized during the mid-late developmental stages.

Together with the physical changes in the organism, there are also critical interactions between the bacteria and the host that are established during this time period including the avoidance of lysosomal fusion, the intracellular redistribution of EBs, and the interception of host-derived secretory vesicles.

Avoidance of lysosomal fusion

One of the obstacles that all intracellular bacteria encounter is the harsh degradative environment of host cell lysosomes, which a bacterial pathogen would normally encounter upon endocytosis and trafficking along the default endocytic/lysosomal pathway. Bacterial pathogens have evolved unique mechanisms to circumvent their destruction in lysosomes. Some alter their phagosomal

maturation at an early prelysosomal stage such that they avoid fusion with lysosomes, while others like *Chlamydia* escape the default endocytic/lysosomal pathway and replicate in vacuoles that share properties with other host organelles, such as secretory organelles. Chlamydiae exit the classical endocytic/lysosomal pathway early during the developmental cycle as evidenced by absence of classical endocytic markers by processes dependent on early chlamydial protein expression. Incs, chlamydial proteins targeted to the inclusion membrane, likely play important roles in the inhibition of lysosomal fusion. Structurally, Incs mimic SNARE proteins essential for membrane fusion in the host cell.

Translocation of EBs

By 2–3 h p.i, EB-containing vacuoles are transported from the periphery of the cell and aggregate at the peri-Golgi region or the region of the cell that corresponds to the microtubule organizing center (MTOC) by processes that are dependent on early chlamydial gene expression. During this initial infection period, at least 60 'early genes' are expressed. Several of these early expressed genes are localized to the inclusion membrane and thus are likely candidates to facilitate the redistribution of EBs.

Host factors are also required for the intracellular trafficking of the EB-containing early inclusion. Some chlamydiae exploit microtubule-dependent host trafficking pathways to mediate their intracellular redistribution. Consistent with the trafficking of EBs toward the minus-ends of microtubules, the redistribution of EBs has been shown to be dependent on dynein, a minus-ended microtubule-dependent motor protein. Most dynein-dependent trafficking pathways in the cell require the assistance of dynactin, a multiprotein complex that facilitates cargo binding to dynein. Interestingly, although the transport of EBs requires dynein and at least one component of dynactin, $p150^{glued}$, localizes to inclusions, trafficking does not require dynactin. These data suggest that EBs exploit a novel dynein-dependent but dynactin-independent transport pathway to facilitate their redistribution within the eukaryotic cell. Protein tyrosine kinases Fyn and Src are also required for the trafficking of chlamydial inclusion. Fyn and Src are recruited to the inclusion where they directly interact with Incs.

Chlamydia-mediated remodeling of the inclusion is absolutely essential for the intracellular survival of chlamydiae. In the absence of early chlamydial gene expression all of these early events fail to occur and eventually the chlamydiae will be trafficked to host lysosomes and destroyed.

Nutrient and Biosynthetic Precursor Acquisition

Chlamydiae are auxotrophic organisms that rely on their eukaryotic host for essential nutrients such as nucleotides (including ATP, GTP, UTP but not CTP or any of the deoxynucleotides), amino acids, and a variety of host-derived lipid species including glycerophospholipids, cholesterol, sphingomyelin, and neutral lipids. Chlamydiae acquire host-derived lipids by targeting both vesicular- and non-vesicular-mediated host trafficking pathways. Beginning by 2 h p.i., coincident with the redistribution of EBs to the peri-Golgi region, the inclusion becomes competent to fuse with a subset of Golgi-derived exocytic vesicles containing endogenously synthesized sphingomyelin and cholesterol. Subsequent to this discovery, it was revealed that chlamydiae also acquire sphingomyelin from the delivery of multivesicular bodies (MVBs) that originate from late endosomes to the inclusion. Chlamydiae also recruit lipid droplets (LD) to the inclusion. LDs are ER-derived lipid storage organelles that are a source of neutral lipids. Recent work has shown that IncD recruits host lipid-binding proteins GBF1 and CERT to acquire host sphingomyelin. This process likely also involves Fyn and Src, and the Raf/MEK/ERK signal transduction pathway.

Exiting

Two alternative exiting mechanisms have been demonstrated for *Chlamydia*: lysis and extrusion. The lytic pathway is initiated with a loss of the inclusion integrity, which is followed by rupture of nucleus and plasma membrane. Lysis requires proteases and a rise of intracellular calcium concentration. The extrusion pathway causes the release of intact inclusions, and requires actin polymerization; neuronal Wiskott–Aldrich syndrome protein, myosin II, and Rho GTPase are all required for extrusion.

Virulence Factors

Intracellular pathogens exploit basic host cellular processes to mediate their entry and intracellular survival. Since chlamydiae remain sequestered within the inclusion, proteins that function to subvert host cellular processes need to directly access the host cytoplasm. Bioinformatics and heterologous expression systems have been used to demonstrate that chlamydiae express two categories of proteins that directly access the host cytoplasm: soluble, secreted proteins that are translocated directly into the host cytoplasm and membrane-anchored proteins that localize to the inclusion membrane. In addition, like other bacteria, chlamydiae carry a plasmid that encodes virulence factors. At least one plasmid-encoded protein (Pgp3) is targeted to the host cell cytoplasm.

The plasmid

All *Chlamydia* species carry a plasmid. Loss of the so called 'cryptic' plasmid in both *C. trachomatis* and *C. muridarum* results in attenuation of pathogenicity, as demonstrated in a nonhuman primate model and a mouse model, respectively. The plasmid is highly conserved in these two bacteria, and carries 8 open reading frames (ORFs), pgp1-8. Rapid revelation of the roles for these

ORFs has been made possible by the recently developed chlamydial transformation technique. Pgp1, 2 and 6 and the coding sequence of pgp8 serve as plasmid maintenance factors, Pgp4 as a transcriptional regulator for some 20 chromosome-encoded virulence determinants, and Pgp3, 5, 7 and 8 as dispensable for chlamydial growth in vitro.

Pgp3 is secreted into the host cell cytoplasm likely through a T3SS-independent mechanism. Several lines of evidence support a role of Pgp3 plays a critical role in promoting chlamydial pathogenicity. First, recombinant, LPS-free Pgp3 strongly induces IL-8 and TNF-α production from macrophages. Second, X-ray crystallography reveals that Pgp3 is a trimeric protein with the C-termini of the subunits forming a structure resembling that of TNF-α, providing a structural basis for Pgp3 to activate the TNF receptor-mediated inflammatory pathways. Third, *C. muridarum* carrying a plasmid with inactivated *pgp*3 gene show normal growth but cannot induce hydrosalpinx in mice.

Chromosome-encoded Virulence Factors

T3SS

Many Gram-negative bacterial pathogens, including *Chlamydia* species, share a unique virulence trait, a T3SS. T3SS is a specialized needle-like secretion system designed to translocate proteins from the bacteria directly into the host cell cytoplasm. T3SS effectors have been shown to modulate a large variety of host functions and facilitate many aspects of bacterial pathogenesis including entry, vacuolar biogenesis, intracellular trafficking, and inhibition or induction of host apoptotic pathways. Although electron micrographs obtained in the early 1970s depicted structures that resembled T3SS needle structures originating from the bacteria and extending through the inclusion membrane, genetic evidence that *Chlamydia* species encoded a T3SS was not obtained until 1997, when four genes with significant homology to known T3SS genes were identified in *C. caviae*. The following year, genomic sequence analysis revealed that *C. trachomatis* encoded within their genome a complete, albeit abbreviated, T3SS.

T3SS consist of a conserved core secretory apparatus, T3SS-specific chaperones, mobile translocation proteins, and a highly diverse set of secreted effectors. Because the core secretory apparatus and associated accessory molecules have retained DNA sequence conservation, bioinformatics approaches have successfully identified genes homologous to these conserved components in all *Chlamydia* species. Based upon comparison to the DNA sequences of T3SS in other bacteria, chlamydiae do not appear to encode the entire complement of T3SS genes, although they do encode enough of the components to produce a functioning T3SS. Chlamydiae likely encode T3SS structural components that have yet to be identified by conventional approaches due to divergence in DNA sequences. In support of this hypothesis, CdsF, which shares no sequence homology with other T3SS proteins, was identified as a possible functional homologue of the needle subunit protein SctF. Interestingly, there is no evidence that *Chlamydia* have acquired these genes through horizontal gene transfer (HGT), as is thought to be the case for T3SS in other facultative pathogenic bacteria. Although loosely organized into four genomic locations, genes encoding the T3SS are scattered throughout the genome. In addition, there are no differences in GC content of the T3SS-encoded genes as compared to the rest of the genome, nor is there evidence of associated insertion or transposon-like elements.

T3SS effectors

In contrast to proteins that make up the secretion and translocation apparatus, effector proteins that are secreted into the host cytoplasm generally share no sequence homology as they normally execute functions specific to the bacterial pathogen and host cell type. Therefore, it has been more difficult to identify chlamydial effectors by DNA sequence homology alone. However, progress has been made in the identification and characterization of several important chlamydial type III effectors. By taking advantage of heterologous T3SS systems of *Yersinia*, *Salmonella*, and *Shigella*, several researchers have confirmed T3SS-dependent secretion of specific chlamydial proteins or have identified novel effectors among the numerous 'hypothetical ORFs' present in the various chlamydial genomes. To date, there are about 80 predicted T3SS effectors including a number of Incs, Tarp, TepP, components of the T3SS apparatus (CopN, CopB, CopB2), CT847, Pkn5 (a serine/threonine protein kinase), *Chlamydia* protein associating with death domains (CADD), and numerous hypothetical ORFs.

Incs were originally identified in the pregenomic era through the isolation of proteins recognized by convalescent sera from guinea pigs infected with *C. caviae*. Upon completion of the genomic sequences from several *Chlamydia* species, based upon the presence of the signature hydrophobic motif, it was determined that *Chlamydia* species are predicted to encode 60–80 different Incs. Except for the conserved hydrophobic motif, Incs share no homologies with any other proteins in the databases nor do they share extensive homologies between themselves. In fact, *Chlamydia* species appear to express different complements of Incs. Furthermore, microinjection studies using domain-specific antibodies have demonstrated that many of the Incs directly access the host cytoplasm, making them likely candidates to exploit host cellular signaling and trafficking pathways through direct interaction with host proteins. Finally, Incs are expressed at all stages of the developmental cycle, suggesting that they may function at multiple times throughout infection. Biological roles for several inclusion-localized proteins have been proposed. For example, IncA is thought to promote homotypic vesicle fusion between *Chlamydia*-containing vesicles via either IncA–IncA interactions or the recruitment of host soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) to the inclusion. Consistent with a role in homotypic vesicle fusion, clinical *C. trachomatis* isolates that do not express IncA replicate in multiple nonfused inclusions. *C. trachomatis* IncD interacts with lipid-binding proteins of the host to acquire lipids. IncG has been shown to recruit mammalian 14-3-3β to the inclusion. 14-3-3β is a phosphoserine binding protein that plays multiple roles in cell signaling and cell survival. The recruitment of 14-3-3β to the inclusion contributes to the ability of chlamydiae to inhibit cellular apoptosis by sequestering and inhibiting the pro-apoptotic protein BAD at the inclusion. Finally, two Incs have been shown to recruit Rab

GTPases to the inclusion. Rab GTPases are small ras-like GTPases that play critical roles in vesicular-mediated trafficking pathways. *C. trachomatis* Inc CT229 recruits Rab4 to the inclusion, while *C. pneumoniae* Inc Cpn0585 interacts with multiple Rab GTPases, suggesting that chlamydiae may exploit Rab-dependent trafficking pathways to facilitate their intracellular trafficking or to modulate the identity of the inclusion.

CopN, a homologue of *Yersinia* YopN and component of the T3SS apparatus, CT147, and Cap1, a $CD8^+$ T cell epitope, are also localized to the inclusion membrane but lack the signature hydrophobic profile of Incs. CT147 is an early expressed inclusion localized protein that is homologous to the mammalian early endosomal antigen (EEA1). EEA1 contains a 'FYVE' domain that interacts with phosphatidylinositol (Ptd)-3-P, regulating tethering of early endosomes, and a separate domain that interacts with Rab5, regulating fusion of early endosomes. Interestingly, CT147 contains only the 'FYVE' domain that interacts with Ptd-3-P, suggesting that CT147 may function to tether early endosomes to the inclusion but, since it lacks the Rab5 interacting domain that promotes fusion, fusion with early endosomes is prohibited. However, the specific function of CT147 remains to be determined.

CADD was identified through a bioinformatics approach due to its homology with mammalian death domain receptors of the tumor necrosis factor (TNF) family. It has been shown to interact with several human death receptor domain family proteins including TNFR1, Fas, DR4, and DR5, and it partially co-localizes with Fas adjacent to the inclusion membrane in infected cells. Although ectopic expression of CADD induces host cell apoptosis, neither endogenous expression of CADD nor localization of Fas to the inclusion is sufficient to induce apoptosis in *Chlamydia*-infected cells. These conflicting results suggest that the ability to influence the host cell apoptotic pathways may be due to the specific localization of CADD, and therefore the exact role that endogenous CADD plays has not been elucidated.

Secretion of CADD into host cells can be inhibited by a compound designated C1. However, whether CADD is a T3SS effector is not entirely certain since C1 and other salicylidene acylhydrazide-based T3SS inhibits also inhibit iron metabolism. Likewise, other T3SS effectors proposed solely based on the use of salicylidene acylhydrazides have yet to be affirmed by additional experimentation.

Other secreted effectors

Several *Chlamydia* species encode several partial and complete ORFs that share significant homology to the catalytic domains of large cytotoxins (LCTs) produced by *Clostridium difficile*. LCTs inhibit the activity of Rho family GTPases, key regulators of the actin cytoskeleton. Some chlamydial strains are cytopathic to the host when infected at high multiplicities of infection in a phenomenon that has been termed 'multiplication independent immediate cytotoxicity'. Interestingly, there is a direct correlation between the ability to induce cytotoxicity and the number of cytotoxin-like genes that a strain encodes. More importantly, the immediate toxicity induced by chlamydiae is phenotypically indistinguishable from cells that have been exposed to LCTs. In both cases, there is a dramatic reorganization of the cytoskeleton that is manifested with the disassembly of actin stress fibers, which results in rounding up of the cells. Although the cytotoxins are expressed during later parts of the developmental cycle, it is thought that they are present in EBs as preformed effectors and they function early during the developmental cycle. One possible model suggests that the cytotoxins act to inhibit host Rho GTPases at sites of chlamydial entry. Alternatively, the cytotoxins also share homologies to *E. coli* lymphostatin protein (LifA) and therefore may play a role in immunosupression.

Chlamydial protease-like factor (CPAF) is a *Chlamydia*-encoded serine protease found in the cytoplasm of infected cells. Cleavage of numerous host proteins by CPAF has been reported in the literature. However, it is now clear that those reports were results of experimental artifacts arising during sample preparation. Although the physiological substrates of CPAF remain at large, it likely functions as an important virulence factor since antibody against CPAF can neutralize the infectivity of EBs, and immunization of mice with CPAF has been shown to elicit a protective immune response.

Host Tropism

Surprisingly, comparative genomic analysis revealed that the human pathogen *C. trachomatis* and the mouse pathogen *C. muridarum* share near genomic synteny. Even more strikingly, *C. trachomatis* serovar A, an oculotropic serovar, and *C. trachomatis* serovar D, a genitotropic serovar, share 99.6% DNA sequence identity and yet display different tissue tropisms *in vivo*. Most of the strain-specific genes are localized to a single region of the chromosome, which has been termed the hypervariable plasticity zone (PZ). Several of the genes that are localized to the PZ encode tryptophan synthase and the cytotoxins, both of which are thought to play crucial roles in host and tissue tropism. First, genes encoding functional tryptophan synthase are present in the PZ of only *C. trachomatis* genital tract and not ocular species. The genes are completely absent from *C. muridarum*. None of the *C. trachomatis* strains are able to produce tryptophan, an essential amino acid, from most tryptophan metabolic precursors. However, the genital tract *C. trachomatis* strains that express tryptophan synthase are capable of generating tryptophan, from exogenously added indole, whereas strains deficient for this enzyme cannot. The important host proinflammatory molecule INF-γ acts in part to inhibit chlamydial growth by the induction of indolameanine 2,3-dioxygenase (IDO), which functions to degrade tryptophan. Therefore, genital tract *C. trachomatis* strains that can synthesize tryptophan from indole, perhaps derived from the vaginal flora, may use this as a mechanism to overcome INF-γ-induced IDO activity. As such, tryptophan synthase is considered a virulence factor that differentiates the ocular and the genital tract serovars of *C. trachomatis* as well as differentiates the human genital tract *C. trachomatis* strains from murine *C. muridarum*.

Differences in susceptibility to INF-γ also distinguish *C. trachomatis* strains from *C. muridarum*. Susceptibility to INF-γ correlates with the presence or absence of cytotoxins in each of the respective genomes. The growth of *C. trachomatis*, which lacks or contains truncated forms of the cytotoxins, is inhibited by INF-γ, whereas the growth of *C. muridarum*, which contains three complete copies of the cytotoxins, is not. Recent evidence suggests that differential susceptibility to INF-γ may be due to INF-γ-inducible GTPases such as the p47 GTPase, Iigp1, a mouse-specific immune regulator that inhibits the growth of intracellular pathogens. Iigp1 contains motifs similar to YopT targeting sequences. Since the chlamydial cytotoxins share similarities with YopT, the cytotoxins present in *C. muridarum* may target and inhibit Iigp1, thus providing a model for how *C. muridarum* may resist the inhibitory effects of INF-γ. Consistent with this hypothesis, knockdown of Iigp1 makes *C. trachomatis* strains, which do not express the cytotoxins, less susceptible to INF-γ-mediated growth inhibition. Based upon these observations, the prevailing model suggests that the diversity in the PZ arose to counteract species-specific host immune effectors such as INF-γ.

Clinical Disease, Diagnosis, and Treatment

Although chlamydiae cause a wide range of diseases in many different host species, most sequelae resulting from chlamydial infections are primarily due to the fibrosis and tissue damage that results from chlamydial-mediated induction of host proinflammatory cytokine responses that can occur during repeated or persistent infections.

C. trachomatis: Trachoma

C. trachomatis servars A, Ba, Bb, and C infect ocular mucosal surfaces, causing trachoma, the leading cause of preventable blindness worldwide. Although virtually absent from industrialized countries, trachoma is still endemic in tropical and subtropical areas with poor hygienic conditions, including North Africa, the Middle East, and Northern India. The disease is transmitted from human to human through fomites. It may also be potentiated by the transfer of human secretions by flies. Infection begins as conjunctivitis, which is followed by follicle formation. Scarring and distortion of the eyelids may be caused by subsequent follicle rupture, which in turn causes in-turning of the eyelids. In-turning of the eyelid causes the eyelashes to scrape the cornea, which causes physical damage to the cornea. It is the physical damage to the cornea that ultimately leads to blindness. Therefore, blindness does not result from an acute infection, but rather results after repeated or persistent infections.

Diagnosis of trachoma is made by isolation of the chlamydial organism from conjunctival scrapings or identification of inclusions in epithelial cells derived from conjunctival scrapings. Trachoma is still a disease associated with poverty and poor hygienic conditions. An eradication program known as the SAFE strategy has been in place in endemic countries to try to eliminate the disease. The SAFE strategy includes **s**urgery to repair damaged eyelids, **a**ntibiotic treatment to eliminate the bacteria, **f**ace cleanliness to improve personal hygiene, and **e**nvironmental improvements to reduce the fly population to reduce bacterial spread. The disease is currently treated with antibiotics such as doxycycline and azithromycin. The latter is more expensive but more convenient, as a single dose is all that is required.

C. trachomatis: Urogenital Tract Infections

Serovars D, E, F, G, H, I, J, and K of *C. trachomatis* infect the columnar epithelial cells of the urogenital tract. The epithelium of the respiratory tract of newborns can be infected after passing the birth canal of infected mothers. Each year, close to 90 million new cases are reported, making *C. trachomatis* infections the leading bacterial cause of sexually transmitted disease. There is evidence that the gastrointestinal tract is another naturally infected site. *C. trachomatis* causes a wide variety of disease manifestations in both men and women. These include adult and infant inclusion conjunctivitis (which rarely, if ever, leads to blindness), pneumonia, salpingitis, cervicitis, female urethral syndrome, postpartum endometris, male urethritis, epididymititis, and arthritis. Fibrosis and scarring in the urogenital tract are the hallmarks of chlamydial infections due to the elicitation of a strong proinflammatory host cell response to the invading bacteria. Scarring causes the devastating sequelae that develop upon repeated or persistent chlamydial infections. Due to the asymptomatic nature of most chlamydial infections, chlamydial infections often go undiagnosed and untreated. In women, untreated chlamydial infections can lead to pelvic inflammatory disease, ectoptic pregnancy, and infertility. In addition, chlamydial infections may lead to increased susceptibility to HIV infection.

Laboratory diagnosis can be made by isolation of the chlamydial organisms from cervical or urethral swabs or by visualizing infected cells with direct fluorescence antibody tests, enzyme-linked immunoassays for antigen, or nucleic acid detection. Nucleic acid amplification tests using either self-collected vaginal swabs or voided urine are much more sensitive than culture for detecting genital *Chlamydia* infections in women. Similar to ocular infections, genital tract infections are treated with tetracyclines or macrolides.

Lymphogranuloma Venereum

The LGV serovars (L1, L2, and L3) of *C. trachomatis* also infect mucosal surfaces of the urogenital tract. However, in contrast to infections by the trachoma, which remain localized to the epithelium, the LGV serovars infiltrate the submucosa, resulting in

infection of the lymphatic drainage system and a more systemic disease. Common in tropical areas of Asia, Africa, and Central America, LGV has been virtually eliminated in the United States, but an epidemic has been taking place among men who have sex with men in Europe for more than a decade. In the initial stages of infection, asymptomatic primary lesions develop on the labia, vagina, cervix, or penis and may result in mild proctitis. If the infection is not diagnosed and treated, lymphoadenopathy in the nodes draining the site of primarily infection (usually inguinal and femoral) may form. The nodes may rupture, resulting in chronic draining fistulas. At this stage, the disease can take two courses, the abscesses will either resolve or the disease will become systemic, which can result in fever, mylagia, and headache. Chronic inflammation and scarring may occur in some cases. Diagnosis is made upon isolation of the bacteria from either a primary lesion or aspirated pus, or by microimmunofluorescence assays (MIF).

C.pneumoniae

C. pneumonaie is a newly recognized species that is primarily a respiratory pathogen. Although the species was first isolated in 1965 from a child's conjunctiva during a trachoma vaccine trial study in Taiwan, it was not considered a respiratory pathogen until 1983, when it was isolated from the pharynx of a pharyngitis patient. The original isolate, TW-183, and the respiratory isolate, AR-39, were subsequently discovered to be the same organism, and thus have become known as the TWAR isolates. Infections by *C. pneumoniae* cause 10% of all community-acquired pneumonia infections and 5% of all bronchitis cases. Although most clinical manifestations are not easily distinguishable from other atypical pneumonias (nonproductive cough, headache, and malaise), there are several clinical signs that are more typical of infections caused by *C. pneumoniae*. These include a subacute onset and the presence of pharyngitis during the initial stages of infection. There may also be a biphasic pattern with resolution of the pharyngitis prior to the development of bronchitis. Finally, the presence of a prolonged cough and the absence of fever are also typical of *C. pneumoniae* infections. Most infections are mild or asymptomatic and do not require hospitalization. Despite antibiotic treatment, recovery is often slow with persistence of a cough for 2–6 weeks. All age groups are at risk; however, respiratory disease is most common among children 5–14 years old. The majority of adults are seropositive for *C. pneumoniae* antibody titers. These data suggest that by the time most people reach adulthood, they have been infected with *C. pneumoniae* at least one time during their life.

Laboratory diagnosis for respiratory infection is often unreliable due to the absence of well-standardized and commercially available diagnostic tests. The most common diagnostic test used involves serologic detection of *C. pneumoniae*-specific antibodies by MIF. *C. pneumoniae* infection is positively diagnosed with a single immununoglobulin (Ig)M titer greater than or equal to 1:16 or a fourfold increase in the IgG titer. In primary infections, *C. pneumoniae*-specific IgM and IgG titers do not appear until 2–3 weeks and 6–8 weeks after infection, respectively. Therefore, most physicians will prescribe antibiotic treatment that is effective against most bacteria-caused atypical pneumonias prior to a definitive diagnosis. Typically, antibiotic therapy consists of a macrolide such as azithromycin or clarithromycin. If a definitive diagnosis of *C. pneumoniae* is made, antibiotic therapy is usually switched to a 10–14 day course of doxycycline. *C. pneumoniae* infection may be linked to a variety of chronic inflammatory conditions such as asthma, atherosclerosis, myocardial infarctions, and multiple sclerosis.

C.muridarum

C. muridarum was isolated from a normal mouse. The organism causes pneumonia in intranasally inoculated mice, and had been classified as a separate biovar of *C. trachomatis* until just prior to the new millennium, and continued to be frequently referred as *C. trachomatis* mouse pneumonitis agent in the literature for a few more years into the new millennium. *C. muridarum* has been a commonly and a very useful organism for modeling human urogenital *C. trachomatis* infection in mice.

C.psittaci

C. psittaci is a common avian pathogen and causes 'chlamydiosis'. In the past, infections of psittacine birds were termed 'psittacosis' and infections of wild or domestic fowl were called 'ornothosis'. Because the diseases caused are so similar, the term 'chlamydiosis' is used for infections in all birds. More than 130 species of birds are known to be infected with *C. psittaci*. Clinical signs vary between the different bird species. However, early signs may include dyspnoea, sinusitis, unilateral conjunctivitis, and diarrhea. During an acute infection, egg production may be reduced. In addition, weight loss, reduced body temperatures, dehydration, and loss of appetite may also be present. Morbidity and mortality rates may be as high as 50–80% and 30%, respectively. Presumed diagnosis may be made on physical examination and history.

Definitive diagnosis of avian infections can be made by isolation of the organism and identification of inclusions in tissue culture cells or through the direct identification of the organism in infected tissue samples. Serological tests are not widely used for diagnosis. Chlortetracycline, delivered in the bird's food supply, is the antibiotic of choice. Oxytetracycline, doxycycline, and enrofloxacin are also used to treat infected populations. Quarantine and antibiotic treatment of imported birds are common control measures used to prevent spread of *C. psittaci* infections.

C. psittaci is a zoonotic pathogen. Manifestations of human infection can range from asymptomatic to a systemic toxic syndrome without respiratory infection. Common clinical signs include fever, chills, muscular aches and pains, and severe headaches. Due to the introduction of bird feed containing antibiotics and a 30 day quarantine period for imported birds, the cases of human *C. psittaci*

infections have dramatically decreased, with less than 50 confirmed cases being reported per year. People most at risk include pet shop and poultry industry workers.

Diagnosis *C. psittaci* infection is made by either isolation of the organism by culture, which is often not done due to the infectious nature of the organism, or a fourfold increase in *C. psittaci*-specific antibody titers. The treatment of choice is a 10–21 day regimen of tetracycline or doxycycline.

C.abortus

C. abortus infects the genital tracts of ruminants (sheep, cattle, and goats) and mostly causes abortions. Infections by *C. abortus* can also cause stillbirths and the delivery of weak full-term newborns. *C. abortus* infections are the most common cause of abortions in sheep, known as ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE). The organism is usually transmitted during lambing seasons, as infected animals undergoing abortions secrete large amounts of organisms in placental tissues, uterine discharges, and in feces. In the initial stages of infection, the organism is found in the main organs of the ewe. Infection remains subclinical until the last 4 weeks of pregnancy, when abortions occur. At this time, the placenta becomes infected, while the organism is cleared from the other parts of the animal. The fetus becomes infected, leading to death and subsequent abortion. Infected ewes generally recover with their subsequent fertility unaffected.

Diagnosis is made by identifying the bacteria in impression smears of placental cotlyedons, by isolation of the organism by passage in tissue culture cells, and by detection of *C. abortus*-specific antibodies. Infections can be treated with oral chlortetracycline if administered prior to the appearance of the second chlamydemia. However, a more effective treatment plan includes intramuscular injections of oxytetracycline. *C. abortus* infections are controlled by management procedures and by vaccination.

Human infection due to exposure to aborting sheep has been documented and can lead to abortion or severe respiratory disease in nonpregnant individuals.

C.felis

Infection by *C. felis* causes conjunctivitis, rhinitis, or pneumonia in cats. Although cats of all ages are susceptible to infection by *C. felis*, infection is more common in young kittens (5–12 weeks old). Clinical signs include fever, depression, sneezing, anorexia, coughing, occasional pneumonia, and serous discharge from the eyes and nose. *C. felis* infection of humans has been documented but it is rare. If left untreated, infections often persist for 6–8 weeks. Treatment usually involves both topical antibiotics in the form of eyedrops or ointment and oral antibiotics for 6–8 weeks. Feline vaccines exist that help reduce the severity of clinical disease.

C.pecorum

C. pecorum infections occur in some species of mammals including cattle, sheep, goats, koalas, and swine. Depending on the specific biotype, clinical symptoms may include conjunctivitis, polyarthritis, pneumonia, abortion, encephalitis, and enteritis. In cattle, clinical signs also include metritis, salpingitis, and infertility. Laboratory diagnosis is difficult because of difficulties in growing the organisms in culture and cross-reactivity of antibodies with *C. abortus*.

C.caviae

The natural host of *C. caviae* is the guinea pig. Clinical signs include conjunctivitis. *C. caviae* infection of the guinea pig is routinely used as an animal model system to study human chlamydial genital tract infections.

C.suis

C. suis is a swine pathogen and is believed to be endemic in pigs. Clinical syndromes include conjunctivitis, pneumonia, genital tract disease, and enteritis. Due to its similarity to human isolates, recent concern has arisen due to the identification of tetracycline-resistant isolates of *C. suis*.

C.avium

C. avium has been isolated from sick pigeons and psittacine birds. It is not known whether or not their natural host range is limited to these animals.

C.gallinacean

C. gallinacean can be isolated from domesticated poultry including chicken, guinea fowl and turkey. Although there is currently no evidence that this organism is pathogenic in these birds, a link between this organism and atypical pneumonia among slaughterhouse workers has been suspected.

Conclusion

Exciting advances over the last several years have revealed that *Chlamydia* species target multiple host trafficking and signaling pathways to mediate their intracellular survival and pathogenesis. Chromosome- and plasmid-encoded effector molecules are secreted into the host cell cytoplasm or the inclusion membrane through T3SS-independent and -dependent mechanisms. While some of the effectors have been shown to specifically interact with host proteins, targets of other effectors have yet to be identified. Exciting new research tools, which include transformation, inducible expression, gene targeting, mutagenesis coupled with high throughput genome sequencing, and *in vivo* imaging system for monitoring chlamydial dissemination in live animals, will help clarify the functional role of these chlamydial effectors. These tools will also aid in identification of new virulence factors and therapeutic targets, and vaccinology research.

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Chlamydophila pneumoniae, A Pathogen Causing More Than Pneumonia

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Recognition of *Chlamydophila pneumoniae* as a Human Pathogen

Chlamydophila (*Chlamydia*) *pneumoniae* was first recognized as a cause of human respiratory tract infections in 1986 (Grayston et al., 1986, 1990; Grayston, 1992a). In the following decade, *C. pneumoniae* became well established as a significant cause of both upper and lower respiratory tract infections, including pharyngitis, laryngitis, sinusitis, bronchitis, and pneumonia (Grayston, 1992b; Blasi et al., 2000; Burillo and Bouza, 2010). Seroepidemiology studies in most industrialized countries (Fig. 1) have demonstrated that approximately 80% of adults are infected with *C. pneumoniae* at least once in their lifetime (Grayston, 1992b; Villegas et al., 2010; Miyashita et al., 2002; Puolakkainen, 2013). Both reinfections and recurrent infections are thought to be common; pulmonary infections can become chronic and have been associated with acute exacerbations of COPD (Grayston, 1992b; Blasi et al., 1993). Importantly, the ability of *C. pneumoniae* to disseminate from the lung to distant tissue sites and then to persist in these distant host tissues for extended periods of time is a critical aspect of the pathogenesis of *C. pneumoniae* infection (Moazed et al., 1998; Gieffers et al., 2004a; Witte et al., 2011). This pathogenesis must be clearly understood in order to appreciate the potential role of *C. pneumoniae* in human disease. In particular, this ability to cause persistent infection in distant tissues is the reason that *C. pneumoniae* is a pathogen that causes more than pneumonia (Roulis et al., 2013).

Molecular Pathogenesis of Chlamydial Infections

C. pneumoniae, like other members of the *Chlamydiae* family, is an obligate intracellular human pathogen with a unique life cycle during which the microorganism exists in different phenotypes (Fig. 2). This unique life cycle is largely responsible for the molecular pathogenesis of chronic chlamydial infections in human disease (Moulder, 1991; Beatty et al., 1994; Stephens, 2003; Hogan et al., 2004; Kern et al., 2009; Elwell et al., 2016). In brief, the life cycle for *Chlamydophila*/*Chlamydia* species begins with the elementary body (EB), which is an infectious "spore-like" form with minimal metabolic activity (Omsland et al., 2014) that is able to attach to a target host cell and stimulate uptake by this host cell. For *C. pneumoniae*, airborne infectious EBs produced through coughing by infected persons appears to be the primary manner in which this infection is transmitted from one person to another (Falsey and Walsh, 1993; Theunissen et al., 1993). After attachment to and internalization within the host cell, the EB transforms into a non-infective, metabolically active form called a reticulate body (RB) (Moulder, 1991; Beatty et al., 1994; Stephens, 2003; Hogan et al., 2004; Kern et al., 2009; Elwell et al., 2016). RBs replicate by binary fission within a host-derived vacuole called an inclusion body. Energy for chlamydial replication appears primarily to be obtained from host cell ATP via ATP/ADP translocases (Schmitz-Esser et al., 2004; Trentmann et al., 2007). During the replication process, some of the RBs transform back to EBs in an asynchronous manner. Lysis of the host cell and/or exocytosis of the RBs allows the extracellular release of these infectious forms for another cycle. This replicative life cycle is characteristic of acute chlamydial infection. This biphasic nature of the acute infection with an extracellular metabolically inactive infectious form and an intracellular noninfectious metabolically active form has been recognized for some time (Roulis et al., 2013; Moulder, 1991). More recently a shift in this biphasic paradigm (Beatty et al., 1994; Stephens, 2003; Hogan et al., 2004; Kern et al., 2009; Elwell et al., 2016) has occurred with the identification of a non-replicative persistent intracellular form of *Chlamydiae*; this cryptic form is now thought to be the predominant form in chronic chlamydial infections. Moreover, the cryptic form of *C. pneumoniae* in chronic human infections appears to be adapted to the availability of iron and tryptophan as well as to the presence of interferon gamma (Falsey and Walsh, 1993; Theunissen et al., 1993; Schmitz-Esser et al., 2004).

The persistent form of *Chlamydiae* is called a cryptic body (CB) and is characterized by an altered gene expression profile (Mathews et al., 2001) as well as ultrastructural changes (Kutlin et al., 2001). Cryptic bodies do not replicate, but are metabolically active using ATP obtained from the host cell (Schmitz-Esser et al., 2004; Trentmann et al., 2007). Persistent *C. pneumoniae* organisms retain a fully functional type III secretion apparatus (Subtil et al., 2001; Mueller et al., 2014; Engel et al., 2016), which allows the continuous release of effector proteins into the host cell cytoplasm and thus ensures a *C. pneumoniae*-adjusted environment. One important aspect of a *C. pneumoniae*-adjusted environment is that *Chlamydiae* are able to gain control over the host cell apoptotic regulation, which allows a favorable environment for replication or persistence (Fisher et al., 2004; Itoh et al., 2014; Sarkar et al., 2015). Persistent *C. pneumoniae* organisms demonstrate enhanced expression of the genes required for DNA replication, but not of the genes required for cell division (Byrne et al., 2001). In particular, there is a marked upregulation of major outer membrane protein (MOMP), heat shock protein 60 (Hsp-60) and proteins with functions in DNA replication (GyrA), transcription (RpoA, PnP), translation (Rrf), glycolysis (PgK, GlgP), and type III secretion (SctN) (Molestina et al., 2002). MOMP and Hsp-60 are thought to play an important role in persistent chlamydial infections. MOMP has been shown to bias the T cell-mediated immune response towards a type 2 response rather than a

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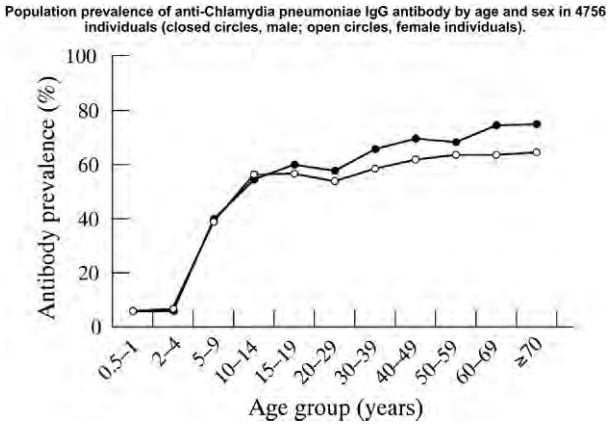

Fig. 1 Shows the age related incidence of anti-*C. pneumoniae* antibodies during the entire period. The antibody detection rate was 57%, broken down by age as follows: 6 months to 1 year old, 4%; 2–4 years old, 5%; 5–9 years old, 40%; 10–14 years old, 56%; 15–19 years old, 59%; 20–29 years old, 57%; 30–39 years old, 63%; 40–49 years old, 67%; 50–59 years old, 67%; 60–69 years old, 70%; and $\geq$70 years old, 71%. In subjects over 15 years old, anti-*C. pneumoniae* antibodies were present in 1331 (67.5%) of 1971 males and 1078 (60.6%) of 1777 females. There were 14 cases (0.3%) of recent *C. pneumoniae* infection among these healthy individuals, as determined by IgM.

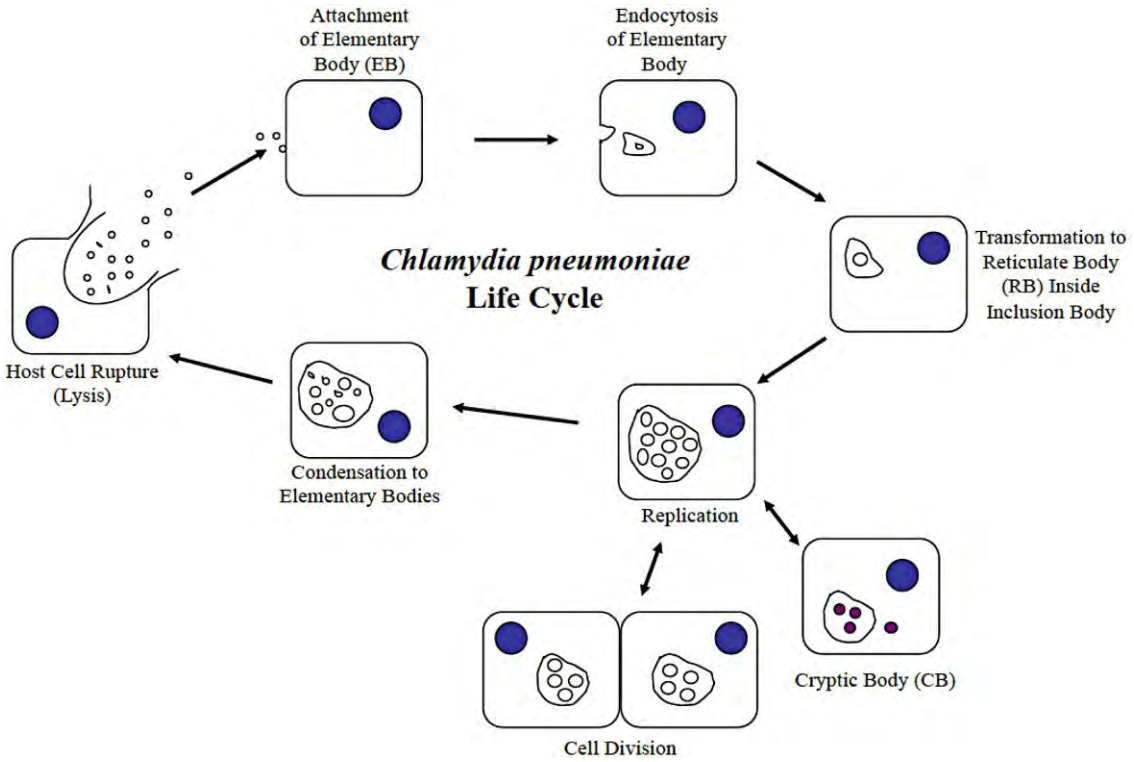

Fig. 2 Intracellular life cycle of *Chlamydia pneumoniae.*

type 1 response; the type 2 response is less protective (Shaw et al., 2002). Heat shock proteins are important antigens expressed during chronic infection and inflammation and markedly influence and sustain anti-infectious and autoimmune responses (Zugel and Kaufmann, 1999). Chlamydial Hsp-60 is pro-inflammatory (Da Costa et al., 2004) and is known to activate macrophages (Bulut et al., 2002); *C. pneumoniae* Hsp-60 also has been shown to induce pulmonary inflammation in mice (Bulut et al., 2009) as well as inflammation in human atheroma (Kol et al., 1998). Cells persistently infected with *Chlamydiae* secrete other pro-inflammatory substances (Mannonen et al., 2011). For example, it has been noted that epithelial cells infected in vitro with *Chalmydiae* secrete large amounts of pro-inflammatory cytokines, including IL-8, GROα, granulocyte-macrophage colony-stimulating factor, IL-1α, and IL-6 (Rasmussen et al., 1997). Finally, persistent *C. pneumoniae* infection of vascular cells such as endothelial cells and smooth muscle cells leads to proliferative and pro-inflammatory phenotypes with endothelial dysfunction within the infected vasculature in vitro and in vivo (Gaydos, 2000; Fryer et al., 1997; Dechend et al., 1999; Coombes and Mahony, 1999; Rupp et al., 2005a).

Systemic Dissemination of *C. pneumoniae* From the Respiratory Tract

The ability of *C. pneumoniae* to cause upper and lower respiratory tract infections is well known (Grayston et al., 1986, 1990; Grayston, 1992a,b; Blasi et al., 2000; Burillo and Bouza, 2010; Blasi et al., 1993). The inflammatory response to these acute infections has been shown to be the polymorphonuclear cell (Yang et al., 1994; Pizzichini et al., 1997). The polymorphonuclear cells appear to be capable of internalizing *C. pneumoniae*, but are unable to kill this organism (Sarkar et al., 2015; Van Zandergen et al., 2004). In fact, *C. pneumoniae* are able to extend the life span of infected polymorphonuclear cells and can replicate in these cells prior to a delayed apoptosis (Rupp et al., 2009). Moreover, these infected polymorphonuclear cells after apoptosis are taken up by macrophages; *C. pneumoniae* is then able to silently infect and propagate in these macrophages (Laskay et al., 2008). The apoptotic neutrophil as a "Trojan horse" for intracellular pathogens such as *Chlamydiae* has recently been recognized as an infection-promoting factor (Laskay et al., 2008). In a rabbit model, polymorphonuclear cells have been shown to act as a reservoir for *C. pneumoniae* (Gieffers et al., 2004a). Subsequent infection of alveolar macrophages by *C. pneumoniae* resulted in transmigration of these infected macrophages through the mucosal barrier to the lymphatic system and the systemic circulation of the rabbit. In addition, infected peripheral blood monocytes in the rabbit were shown to transmit chlamydial infection to the vascular wall. Systemic dissemination of *C. pneumoniae* by macrophages in the murine model also has been described (Moazed et al., 1998; Yang et al., 1995). Evidence for systemic dissemination in the human is provided by a number of studies investigating the prevalence of *C. pneumoniae* in white blood cells collected from healthy blood donors (Boman et al., 1998; Kaul et al., 2000; Bodetti and Timms, 2000; Haranaga et al., 2001; Rassu et al., 2001; Cirino et al., 2006). In particular, one study has demonstrated a *Chlamydiae* carriage rate of 24.6% within white blood cells (WBCs) from the normal blood donor population (Cirino et al., 2006). *Chlamydia* organisms were not only present within the mononuclear/macrophage cells as previously described (Boman et al., 1998; Kaul et al., 2000; Bodetti and Timms, 2000; Haranaga et al., 2001; Rassu et al., 2001; Cirino et al., 2006), but were also present within neutrophils and eosinophil/basophil cells (Cirino et al., 2006). Moreover, this study also demonstrated that the *Chlamydia* organisms in lysates of these peripheral WBCs were infectious and could be cultured (Cirino et al., 2006). This strongly suggests that such carriage of *Chlamydia* organisms in peripheral WBCs could lead to infection of other tissues. Such infection by *C. pneumoniae* has been described in the bone marrow (Nebe et al., 2005) as well as on a heart valve (Gdoura et al., 2002). Clearly, *Chlamydiae* can disseminate and cause infection in distant tissues of humans. Phylogenetic analysis of *C. pneumoniae* isolates from cardiovascular tissues and from brain have been compared to published whole genomes from respiratory isolates (Roulis et al., 2015); fine-detailed analysis of these new *C. pneumoniae* sequences have revealed several genetically variable loci suggesting that minor genetic differences may contribute to adaptation of particular strains in human disease.

Evolving Role of *C. pneumoniae* in Human Disease

The role of *C. pneumoniae* in human disease is evolving. *C. pneumoniae* is known to infect a large variety of human cells including alveolar epithelial cells, macrophages, monocytes, dendritic cells, B-cell, T-cells, polymorphonuclear cells, vascular endothelial cells, smooth muscle cells, fibroblasts, neuronal glia cells, and neuronal ependymal cells (Moulder, 1991; Beatty et al., 1994; Stephens, 2003; Hogan et al., 2004; Kern et al., 2009; Elwell et al., 2016). *C. pneumoniae* is known to be present in an infectious elementary body form within peripheral WBCs in up to 50% of humans (Boman et al., 1998; Kaul et al., 2000; Bodetti and Timms, 2000; Haranaga et al., 2001; Rassu et al., 2001; Cirino et al., 2006). *C. pneumoniae* also is able to cause chronic intracellular infections for which the hallmark is a chronic inflammatory response due to secretion of heat shock proteins and proinflammatory cytokines/chemokines (Moulder, 1991; Beatty et al., 1994; Stephens, 2003; Hogan et al., 2004; Kern et al., 2009; Elwell et al., 2016). Therefore, an important aspect for ongoing investigations of the pathogenesis of *C. pneumoniae* is which tissues are involved/infected, what are the specific roles of the various chlamydial forms (EB, RB, and CB), what are the clinical manifestations of such a chronic infection, and what is the evidence for causation?

To date, there have been a number of important associations of *C. pneumoniae* with chronic human diseases for which the exact role of *Chlamydiae* is still being elucidated (Choroszy-Krol et al., 2014). Four examples of such associations will be briefly reviewed in the following sections. The potential current and future roles of antimicrobial therapy for chronic chlamydial infections also will be discussed.

Association of *C. pneumoniae* With Asthma

An important site-specific pathogenesis association for chlamydial respiratory tract infections is asthma (Hahn et al., 1991; Hahn, 1998). It has been estimated that *C. pneumoniae* is responsible for approximately 20% of lower respiratory tract infections (Grayston, 1992a,b; Blasi et al., 2000; Burillo and Bouza, 2010; Villegas et al., 2010; Puolakkainen, 2013; Kauppinen and Saikku, 1995). *C. pneumoniae* respiratory tract infection may be an important factor in the pathogenesis and course of bronchial asthma in both adults (Hahn et al., 1991; Endo et al., 2017) and children (Emre et al., 1994; Webley et al., 2005; Asner et al., 2014; Iramain et al., 2016). Bronchial smooth muscle cells are potential host cells for *C. pneumoniae* during acute or chronic chlamydial respiratory tract infections; chlamydial infection of human smooth muscle cells has been shown to elicit basic fibroblast growth factor and interleukin 6 (Rodel et al., 2000). This type of bronchial response is thought to contribute to airway wall remodeling in a subset of

patients with *Chlamydia*-associated chronic asthma (Rodel et al., 2000; Hahn and Peeling, 2008; Metz and Kraft, 2010). Moreover, asthma and reactive airway disease is almost always associated with some kind of IgE-related reaction (Burrows et al., 1989). Therefore, *C. pneumoniae*-specific IgE has been investigated as a potential factor in the pathogenesis of reactive airway disease associated with chlamydial infections of the respiratory tract. Anti-*C. pneumoniae* IgE has been found in both children (Emre et al., 1995; Patel et al., 2012) and adults (Hahn et al., 2012) with asthma with *C. pneumoniae*-specific IgE being associated with the severity of asthma in both groups (Patel et al., 2012; Hahn et al., 2012). Finally, a number of studies have noted that antimicrobial agents such as macrolides and doxycycline are beneficial as adjunct therapy for asthma (Johnston et al., 2006; Dzhindzhikhashvili et al., 2013; Gibson et al., 2017), raising the valid question as to the role of such antimicrobial therapy on asthma caused by chronic *C. pneumoniae* lung infection (Hahn and Webley, 2016; Webley and Hahn, 2017).

Association of *C. pneumoniae* With Cardiovascular Disease

Another important association is that first described by Saikku involving the serologic association of *C. pneumoniae* with atherosclerosis and cardiovascular disease (Saikku, 1993, 1999, 2000). In the subsequent two decades, there have been a large number of studies and reports in the medical literature suggesting that chlamydial infection of vascular tissues may contribute to the pathogenesis of atherosclerosis (Saikku, 1993, 1999, 2000; Shor and Phillips, 2000; Palikhe et al., 2009; Fazio et al., 2009; Sessa et al., 2009; Player et al., 2014; Pigarevskii et al., 2015; Assar et al., 2015; Filardo et al., 2015; Yazouli et al., 2017); these include seroepidemiological and pathological studies as well as in vivo and in vitro studies. There are a number of ways that *C. pneumoniae* might play a role in atherosclerosis. Infectious elementary bodies circulating in the blood stream (Cirino et al., 2006) might directly infect endothelial cells and thus initiate the process of atherosclerosis (Kaukoranta-Tolvanen et al., 1994; Kreutmayer et al., 2013; Sun et al., 2014). Infection of endothelial cells could lead to infection of smooth muscle cells (Rodel et al., 2000; Gaydos et al., 1996). *C. pneumoniae* could also infect monocytes/macrophages before (Rupp et al., 2009) or after (Kalayoglu and Byrne, 1998; Mei et al., 2009) the monocytes/macrophages migrate to the infected endothelial cells in vascular tissue; *C. pneumoniae*-infected monocytes/macrophages in vascular tissue would result in foam cells (Kalayoglu and Byrne, 1998; Mei et al., 2009). Macrophage foam cell formation is the hallmark of early atherosclerosis (Kalayoglu and Byrne, 1998; Mei et al., 2009; Yu et al., 2013). Moreover, foam cells have been histologically implicated in the pathogenesis of acute myocardial infarction (Horie et al., 1978). *C. pneumoniae* has been detected in 32 of 51 (63%) coronary primary lesions of symptomatic patients; a highly significant prevalence of these lesions were associated with acute coronary syndrome suggesting a pathological role of *C. pneumoniae* in coronary plaque rupture (Baauriedel et al., 1999). Although these epidemiologic, laboratory, animal, and clinical studies have clearly established an association of *C. pneumoniae* with atheroscerosis, the question of whether this organism plays a contributory role versus a causal role or is a simple symbiotic passenger remains controversial (Mahoney and Coombes, 2001; Baker et al., 2009; Honarmand, 2013). This controversy, in part, is due to the lack of response with antimicrobial intervention in large randomized controlled clinical trials (Muhlestein, 2003; O'Conner et al., 2003; Grayston et al., 2005). Interestingly, in one large trial (WIZARD), stable post-myocardial infarct patients were randomized to receive a 3-month course of azithromycin or placebo; those receiving the single antibiotic demonstrated a significant reduction in death and myocardial infarction by 6 months, but this effect was not sustained (O'Conner et al., 2003). This lack of efficacy may be related to the fact that *C. pneumoniae* causes persistent infection that is refractory to antimicrobial therapy (Campbell and Rosenfeld, 2014). Additional studies are needed (Campbell and Rosenfeld, 2014, 2015).

Association of *C. pneumoniae* With Neurodegenerative Diseases

C. pneumoniae also has been associated with neurodegenerative diseases (Boelen et al., 2009; Contini et al., 2010) such as Alzheimer's disease (Stallings, 2008) and multiple sclerosis (Fainardi et al., 2008). Each of these two associations will be reviewed. The demonstration of *C. pneumoniae* by histopathological, molecular, and culture techniques in late-onset Alzheimer's disease dementia was first reported in 1998 by Balin and colleagues. These investigators then utilized electron and immunoelectron microscopic techniques (EM, IEM respectively) in order to characterize the ultrastructure of *C. pneumoniae* in Alzheimer's disease (AD) brain tissue (Arking et al., 1999). The AD brain tissue examined demonstrated both intracellular reticulate bodies and extracellular elementary bodies, with some of the intracellular organisms undergoing binary fission. Moreover, some AD brain tissue demonstrated atypical forms exhibiting a less distinct cell membrane and a granular center containing possible nucleic acid dense bodies. These atypical forms may represent the cryptic phase of *C. pneumoniae* (Hogan et al., 2004; Kern et al., 2009; Elwell et al., 2016; Arking et al., 1999). Because other investigators were unable to confirm the presence of *C. pneumoniae* in AD brain tissue using either PCR or immunohistochemistry methods (Gieffers et al., 2000; Ring and Lyons, 2000), Balin et al. utilized immunohistochemistry with a battery of commercially available anti-*C. pneumoniae* antibodies and confirmed that *C. pneumoniae* was present in areas typically associated with AD neuropathy (Hammond et al., 2010). Gerard et al. utilized PCR targeting the Cpn1046 and Cpn0695 genes and confirmed the presence of *C. pneumoniae* in an addition 20/27 cohort of patients with late-onset Alzheimer's disease (Gerard et al., 2006). In addition, *C. pneumoniae* has been cultured from AD brain samples from different geographic regions of North America (Dreses-Werringloer et al., 2009). Finally, Balin et al. have used a murine model to demonstrate that intranasal infection with *C. pneumoniae* induces Alzheimer-like amyloid plaques in the brains of BALB/c mice (Little et al., 2004, 2014). Another possibility for other investigators not being able to confirm the presence of *C. pneumoniae* in AD

brain tissue is that other pathogens might also be triggers for Alzheimer's disease (Maheshwari and Eslick, 2015). In particular, spirochetes also are associated with Alzheimer's disease (Maheshwari and Eslick, 2015; Miklossy et al., 2004; Miklossy, 2011, 2015; Allen, 2016). In summary, the evidence strongly suggests that *C. pneumoniae* is one of several bacteria that play an important role in the neuropathogenesis of Alzheimer's disease (Maheshwari and Eslick, 2015; Balin et al., 2008). Moreover, unlike single agent antimicrobial therapy for atherosclerosis, combination antimicrobial therapy with doxycycline and rifampin for 3 months in a randomized controlled trial demonstrated significantly less decline as measured by the Standardized Alzheimer's Disease Assessment Scale cognitive subscale (Loeb et al., 2004).

Another neurodegenerative disease in which *C. pneumoniae* may play a role is multiple sclerosis (Contini et al., 2010; Stratton and Wheldon, 2006; Stratton, 2016). *C. pneumoniae* is known to infect neutrophils and inhibit apoptosis of these cells (Sarkar et al., 2015; Van Zandergen et al., 2004). This allows *Chlamdyia*-infected neutrophils to serve as transport vehicles that can serve as "Trojan horses" and spread to macrophages as well as to distant sites such as the brain (Rupp et al., 2009; Laskay et al., 2008; Gieffers et al., 2004a). *Chlamydia*-infected macrophages/monocytes are able infect vascular cells, including those in the brain (Rupp et al., 2005b). In order to understand how *Chlamdyia*-infected neutrophils and/or macrophages/monocytes might pass through the blood brain barrier and directly infect the brain, a review of the choroid plexus and the circumventricular organs is useful (Wolburgh and Paulus, 2010; Duvernoy and Risold, 2007). The choroid plexus is a complex epithelial–endothelial vascular interface within the ventricular system of the vertebrate brain (Wolburgh and Paulus, 2010). It is comprised of epithelial cells, fenestrated blood vessels, and stoma; the choroid plexus is primarily involved in the production of cerebrospinal fluid (CSF). The circumventricular organs are structures lining the cavity of the third and fourth ventricle and possess a vascular architecture in which their capillaries have a wall devoid of blood-brain barrier (Duvernoy and Risold, 2007). Indeed, the hallmarks of the circumventricular organs are fenestrated capillaries with high permeability that are located in the third and fourth ventricles. This unique arrangement permits direct exchange between the blood and the nervous tissue located near these organs. The circumventricular organs are thought to be a possible entry site for circulating white blood cells into the CSF (Schulz and Engelhardt, 2005). The fenestrated capillaries of the circumventricular organs are regulated by angiogenesis, which involves the active proliferation of endothelial cells (Miyata, 2015). Such endothelial cells could be easily infected by transendothelial migration of *C. pneumoniae* from infected peripheral blood mononuclear cells as has been demonstrated in an experimental transendothelial migration model (Rupp et al., 2005b). Evidence that this can occur is seen in a report of chlamydial infection of ependymal cells in the ventricular tissues of some patients with multiple sclerosis (Sriram et al., 2005a). These investigators suggest that the circumventricular organs may allow entry of *C. pneumoniae*-infected mononuclear cells into the CNS. Infection of ventricular ependymal cells by *C. pneumoniae* could then lead to cytokine-mediated damage to brain tissue surrounding these ventricles. Moreover, infection of ventricular ependymal cells by *C. pneumoniae* would explain the visualization by electron microscopy of chlamydial elementary bodies in the CSF of MS patients (Sriram et al., 2005a). Such ventricular infection by *Chlamydia* would also explain the observations that affinity-driven immunoblot studies have demonstrated reactivity of oligoclonal bands in CSF from MS patients against elementary body antigens of *C. pneumoniae* (Yao et al., 2001; Fainardi et al., 2009). Finally, ventricular infection by *C. pneumoniae* would explain the presence of chlamydial DNA and mRNA transcripts in CSF from some patients with MS (Dong-Si et al., 2004; Contini et al., 2008a).

C. pneumoniae is known to produce large amounts of heat shock protein 60 (HSP 60) during chronic, persistent infections (Zugel and Kaufmann, 1999; Da Costa et al., 2004; Bulut et al., 2002, 2009; Kol et al., 1998); this HSP 60 is thought to play an important role in the pathogenesis of chronic chlamydial infections (Stephens, 2003). Heat shock protein 60 is an evolutionarily conserved protein in both eukaryotes and prokaryotes that is recognized as potent activator of the innate immune system and is capable of inducing the production of pro-inflammatory cytokines by the monocyte–macrophage system (Tsan and Gao, 2004b,b; Habich and Burkart, 2007; Henderson et al., 2013). Importantly, human mitochondrial HSP 60 has been proposed as a danger signal of stressed or damaged cells (Miyata, 2015). Both chlamydial HSP 60 and human HSP 60 contribute to inflammation in human atheroma and are able induce inflammation to levels induced by *Escherichia coli* lipopolysaccharide (Kol et al., 1998). Infection of ventricular ependymal cells by *C. pneumoniae* or other pathogens could serve as a focus of HSP 60, which in turn would stress and/or damage neurons and oligodendrocytes resulting in neuronal cell death, axonal injury, loss of oligodendrocytes, and demyelination (Rosenberger et al., 2015). Such HSP 60 mediated neurodegeneration and demyelination in the CNS has been described in a murine model in which intrathecal HSP 60 is injected (Lehnardt et al., 2008). The fact that neurodegeneration and demyelination in the CNS involves TLR4 and MyD88 confirms that this molecular pathway is able to the release of endogenous TLR ligands from injured CNS cells and is a common process in many different types of brain injury. Indeed, once there a brain injury involving release of HSP 60 has occurred, a vicious cycle can occur due to the activation of microglia, the resident CNS monocyte. Such a cycle involving release of HSP 60 from injured CNS tissue with activation of toll-like receptor 4 has been shown to medicate neurodegeneration in the CNS of mice (Prabhakar et al., 1994) and has been implicated in multiple sclerosis and myalgic encephalomyelitis (Elfaitouri et al., 2013; Amor et al., 2014).

There is clearly a subset of patients with multiple sclerosis in which an association between culture and/or PCR positivity for *C. pneumoniae* in CSF and disease activity has been noted (Sriram et al., 1999; Layh-Schmitt et al., 2000; Contini et al., 2004; Tang et al., 2009). Moreover, intrathecal production of anti-*C. pneumoniae* high-affinity IgG predominated in progressive forms of multiple sclerosis (Yao et al., 2001), and oligoclonal bands include antibodies that react with antigens from *C. pneumoniae* (Fainardi et al., 2009). Metabolically active *C. pneumoniae* has been identified in the CSF of patients with progressive forms of multiple sclerosis (Dong-Si et al., 2004; Contini et al., 2008a). Finally, *C. pneumoniae* has been identified in CSF and/or brain tissue of multiple sclerosis patients using immunohistochemical, molecular, and ultrastructural methods (Sriram et al., 2005a). Finally, the presence of *Chlamydia*-like organism DNA has been reported in several patients with relapsing-remitting MS

(Contini et al., 2008b). Although antimicrobial treatment for chlamydial infection in patients with multiple sclerosis has not been well studied, long-term treatment with a single agent, roxithromycin, was not effective (Woessner et al., 2008) whereas combination therapy with azithromycin and rifampin for 6 months showed a significant reduction in brain parenchymal fraction loss despite the lack of statistical power due to the small number of subjects (Sriram et al., 2005b). A prolonged antimicrobial regimen consisting of combination therapy has also been shown to improve the extra-cranial circulation in patients with MS (Thibault et al., 2017). This beneficial effect was statistically significant in patients with positive serology for *C. pneumoniae*.

Association of *C. pneumoniae* With Inflammatory Arthritis

Chlamydia species, including both *Chlamydia trachomatis* and *C. pneumoniae*, have been associated with a chronic inflammatory arthritis following acute infections at distant sites (Carter and Hudson, 2017). In reactive arthritis induced by either of these two *Chlamydia* species, organisms are viable and metabolically active in the synovium where they exist in a persistent state of infection (Gerard et al., 2000, 2013). Of note is that mRNA from the chlamydial hsp60 gene has been detected in synovial fluid in patients with reactive arthritis. HSP 60 is a well-recognized pro-inflammatory factor secreted by both *C. pneumoniae* (Zugel and Kaufmann, 1999; Da Costa et al., 2004; Bulut et al., 2002, 2009; Kol et al., 1998) and *C. trachomatis* (Cohen et al., 2005). Moreover, HSP 60 is typically secreted by the cryptic forms of *Chlamydia* species (Beatty et al., 1994; Stephens, 2003; Hogan et al., 2004; Kern et al., 2009; Elwell et al., 2016). The term "reactive arthritis" has been used to describe the association of *Chlamydia* species with this inflammatory process involving joints (Carter and Hudson, 2017; Gerard et al., 2000, 2009, 2013; Cohen et al., 2005; Villareal et al., 2002; Carter and Hudson, 2010) because micro-organisms have never been cultured from synovial effusions in early disease (Taylor-Robinson and Keat, 2015). However, reactive arthritis is generally a form of seronegative spondyloarthritis associated with migratory oligoarthritis and extra-articular symptoms that typically follow a gastrointestinal infection or urogenitial infection (Selmi and Gershwin, 2014). The fact that there are metabolically active *Chlamydiae* in the joints strongly suggests that the role of these *Chlamydiae* is causal; the causal nature is further strengthened by the response to prolonged antimicrobial therapy (Zeidler and Hudson, 2016). Prolonged antimicrobial therapy with a single antichlamydial agent such as azithromycin or doxycycline has been shown to be ineffective in reactive arthritis (Kvien et al., 2004; Carter et al., 2004) in contrast to combination therapy with either azithromycin or doxycyline combined with rifampin, which have demonstrated a therapeutic benefit for the treatment of reactive arthritis (Carter et al., 2004, 2010; Rihl et al., 2010).

Clearly, the role of *C. pneumoniae* in chronic human diseases is evolving. The four examples described above may be only the first of many instances where persistent infection with *C. pneumoniae* plays a role in chronic human diseases. Antichlamydial therapy may ultimately be beneficial in such chronic human diseases.

The Future of Antimicrobial Therapy for Chronic Chlamydial Infections

Persistent pulmonary infections with *C. pneumoniae* are a recognized complication of acute respiratory illness with this pathogen (Grayston et al., 1990; Grayston, 1992a,b; Blasi et al., 1993, 2000; Burillo and Bouza, 2010), and this microorganism has proven difficult to eradicate from the respiratory tract with a single agent using currently available antibiotics (Hammerschlag et al., 1992; Branden et al., 2004). Initial attempts to eradicate *C. pneumoniae* using a single agent in patients with cardiovascular disease have all failed (Muhlestein, 2003; O'Conner et al., 2003; Grayston et al., 2005). These observations can be explained, in part, by the fact that the persistent phase of chlamydial infection is relatively refractory to antimicrobial therapy (Gieffers et al., 2001; Yamaguchi et al., 2003). Moreover, first-line antichlamydial agents in subinhibitory concentrations have been shown to induce chlamydial persistence (Gieffers et al., 2004b).

Although clinical data is limited at this time, combination therapy with a regimen that includes a rifampin (Siewert et al., 2005) for a prolonged period of time does appear to be more effective against chronic chlamydial infections than does a single agent. Treating chronic chlamydial infections seems to be similar to treating tuberculosis in that prolonged therapy (i.e., months) with a combination of antichlamydial agents appears to be effective whereas shorter courses (i.e., weeks) with a single antichlamydial agent are not. Optimal therapy for chronic chlamydial infections is apt to required combinations of antimicrobial agents that ideally should be selected on their activity against in vitro persistent chlamydial infections. However, susceptibility testing of *Chlamydia* species is difficult since this pathogen is an obligate intracellular microorganism and not amenable to standard antimicrobial susceptibility testing methods.

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Chloroflexi

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Introduction

The phylum *Chloroflexi* is a deeply branching phylum of the *Bacteria*, which harbors extensive intraphylum diversity and is found in a wide range of aquatic and terrestrial environments. Before the name *Chloroflexi* was first proposed in 2001, members of this phylum were referred to as 'Green non-sulfur bacteria'. The phylum was named after the genus *Chloroflexus*, and its type species, *Chloroflexus aurantiacus*, a filamentous anoxygenic phototrophic bacterium that was first found in hot springs and described by Pierson and Castenholtz in 1974. As a 'green' phototrophic bacterium that lived in 'non-sulphidic' environments, it represented the group of 'green non-sulfur bacteria' (GNSB), which now is termed 'filamentous anoxygenic phototrophs' (FAP), a term used specifically for phototrophic members of the *Chloroflexi*. Members of this phylum have first been described in microbial mats associated with extreme conditions such as thermal and hypersaline environments. Subsequently, *Chloroflexi* have further been detected in a variety of mesophilic freshwater and marine environments, including wastewater treatment plants and even are part of the human microbiome.

Taxonomy and Phylogeny

Formerly, the phylum *Chloroflexi* had been divided into four major subphylum (class)-level taxa, i.e., 'subphyla I, II, III, and IV', which have been re-classified into at present (2018) eight classes containing isolated and described representatives: the *Anaerolineae*, *Ardenticatenia*, *Caldilineae*, *Chloroflexia*, *Dehalococcoidia*, *Ktedonobacteria*, *Thermoflexia* and *Thermomicrobia*. In addition, ribosomal sequences recovered from a variety of environments indicate the presence of many more, still uncultured, class-level groups within the *Chloroflexi*; approx. 25–30 total classes are estimated to exist in this phylum (Fig. 1). In this article we will give an overview of the known members of the phylum *Chloroflexi*, regardless of their official standing in nomenclature. Published taxonomic names are included also for not 'validly' described species and uncultured '*Candidatus*' taxa. (Suppl. Table 1).

The class *Anaerolineae* (Supp. Fig. 1(A)), together with the *Caldilineae*, used to constitute the '*Chloroflexi* subphylum I'. The class contains a single family, *Anaerolineaceae*, which is composed of ten genera with isolated representatives, *Anaerolinea, Bellilinea, Flexilinea, Leptolinea, Levilinea, Longilinea, Ornatilinea, Pelolinea, Thermanaerothrix and Thermomarinilinea;* as well as many additional uncultured clades, including "*Candidatus* Brevefilum" and the freshwater clade CL500–11. All isolated members belonging to these families possess multicellular filamentous morphology, and are strictly anaerobic chemotrophic organisms isolated from anaerobic digesters, hot springs or submarine sediment. In contrast to the members of the *Chloroflexia* (see below) they do not possess gliding motility.

The class *Ardenticatenia* was proposed based on a single species, the ferric ion- and nitrate-reducer, *Ardenticatena maritime*, isolated from a costal hydrothermal field. The species forms thin multicellular filaments and can grow at temperatures of up to 75°C. At the moment, the class contains only one family, *Ardenticatenaceae*, and one order, *Ardenticatenales*. However, ribosomal genes obtained in cultivation-independent studies from a variety of different environments indicate the presence of at least two more family level groups, one including the proposed taxon "*Candidatus* Promineofilum breve", detected in activated sludge flocs and formerly known as "Eikelboom morphotype 0092".

The class *Caldilineae* contains a single described family, *Caldilineaceae*, which consist of two genera, *Caldilinea* and *Litorilinea*. Species belonging to these genera are facultative aerobic or strictly anaerobic filamentous organisms found in hot springs or hot aquifers. Additional uncultured clades, possibly representing several genera in a second family, one including "*Candidatus* Defluviifilum", are indicated by ribosomal gene sequences.

The class *Chloroflexia* (Suppl. Fig. 1(B)) (sometimes also called *Chloroflexi*), formerly known as 'subphylum III', contains the first known members of this phylum, and three orders, *Chloroflexales, Herpetosiphonales* and *Kallotenuales*. Members of this class mostly possess filamentous growth morphology, and show photoheterotrophic and/or chemoheterotrophic growth under mesophilic or moderately thermophilic conditions. All isolated and described filamentous, anoxygenic phototrophic bacteria (FAP) are found in this class. Similar to the phylum, the class was named after the first isolated representative, *Chloroflexus aurantiacus*. All isolated and described phototrophic members are found in the order *Chloroflexales* which can be divided into three families, *Chloroflexaceae*, *Roseiflexaceae* and *Oscillochloridaceae*. The former two families are composed of thermophilic FAPs isolated from terrestrial hot springs, while the *Oscillochloridaceae* contains phototrophic mesophilic freshwater species. Although phototrophy in *Chloroflexi* isolates is limited to this class, metagenomic studies indicate the existence of phototrophic members also in other classes (see below).

The family *Chloroflexaceae* contains *Chloroflexus* (*Cfl.*) as its only validly described genus, which presently consists of three validly described species, i.e., *Cfl. aurantiacus* as the type species, *Cfl. aggregans* and *Cfl. islandicus*. The existence of additional undescribed species in this genus has been indicated by isolated strains and ribosomal gene sequences from uncultured members obtained from

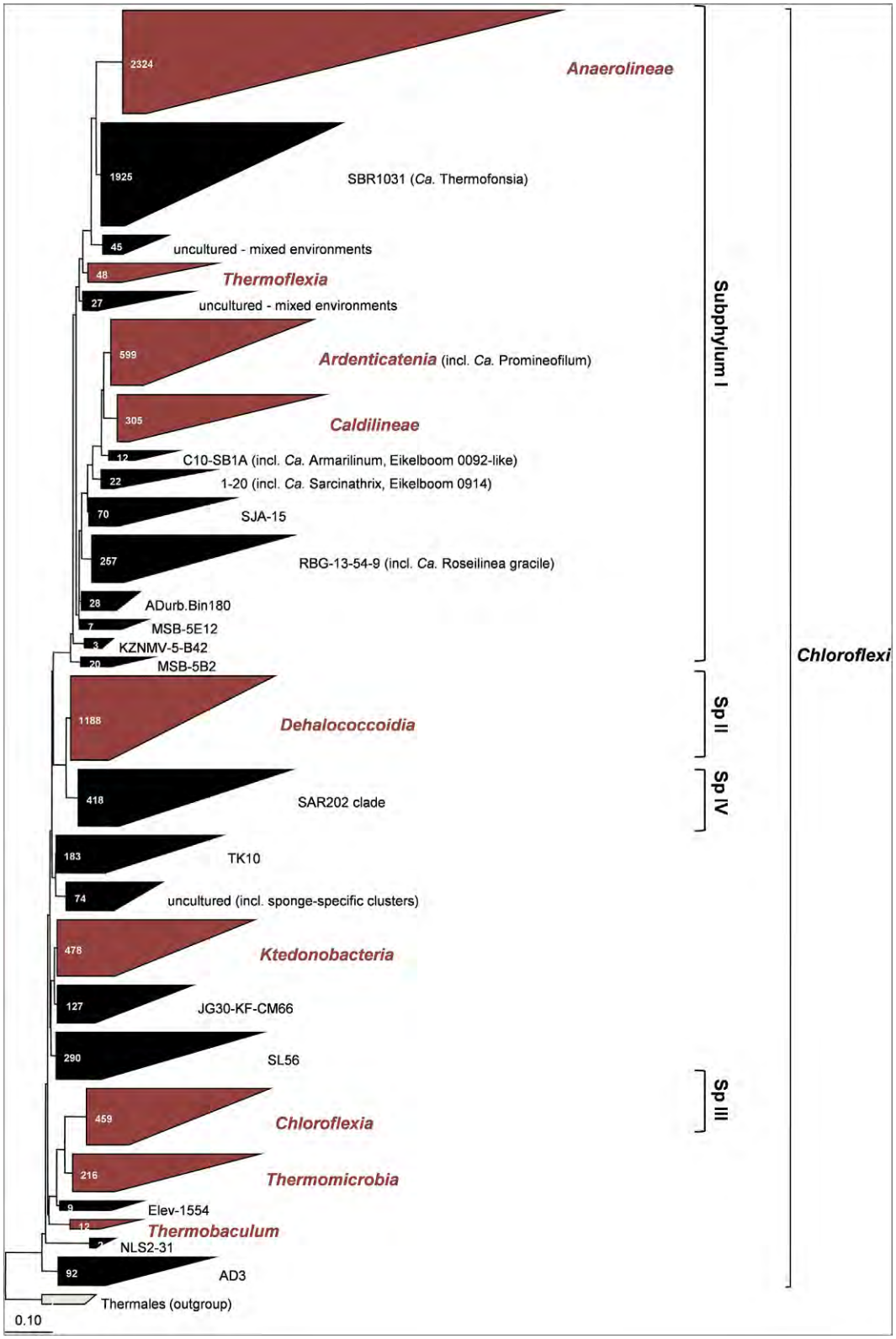

Fig. 1 Phylogenetic tree showing cultured and uncultured classes in the *Chloroflexi.* The tree displays the 16S rRNA gene sequence based SILVA SSU Ref_NR_99 backbone tree (SILVA Release 132, released on 13.12.2017) with some additional sequences and names added. Clades displayed in red contain isolated representatives, while black clades contain only sequences from uncultured microbes Ca. = Candidatus; Sp = Subphylum. Available at: https://www.arb-silva.de/download/arb-files/.

different hot spring environments. All isolates from this genus are 'green' thermophilic anoxygenic phototrophic organisms able to grow at 55°C, which contain the green bacteriochlorophyll (BChl) *c* as their major photosynthetic pigment, specifically found in light harvesting units called "chlorosomes", as well as BChl *a* in their photosynthetic reaction centers. Further, mesophilic FAP isolated from mesophilic alkaline environments are included in this family as members of the genus "*Candidatus* Chloroploca". "*Candidatus* Chloroploca asiatica" members are characterized by immotile, short trichomes which are surrounded by a mucous sheath, and a high sulfide tolerance (up to 8 mM). They resemble green FAP; contain BChls *a* and *c*, and are capable of photoautotrophic growth using sulfide as electron donor.

The family *Oscillochloridaceae*, containing the two genera *Chloronema* and *Oscillochloris*, harbors filamentous anoxygenic phototrophic bacteria from non-thermophilic environments. *Chloronema giganteum*, the only described species of the genus *Chloronema*, is a mesophilic FAP which contains BChl *d* as well as *c* and was isolated from a freshwater lake. *Oscillochloris chrysea* and *O. trichoides* were found by Russian researchers in mesophilic fresh- and brackish-water environments. The families *Chloroflexaceae* and *Oscillochloridaceae* are combined in the suborder *Chloroflexineae*.

Other green FAP related to *Chloroflexus* spp. have been detected in marine and hypersaline environments, and termed 'Marine *Chloroflexus*-like organisms', or MCLOs. One representative of this group, "*Candidatus* Chlorothrix halophila", has been grown in the laboratory in a mixed enrichment culture. These bacteria share the characteristic cell morphology of all FAP i.e., an unbranched multicellular filamentous shape, and show gliding motility, a type of flagella-independent motility described in more detail below.

The family *Roseiflexaceae*, the only family of the suborder *Roseiflexinaea*, contains the so called 'red FAP', in two genera with described representatives with a single described species each, *Roseiflexus* and *Heliothrix*, as well as the so far not validly described "*Kouleothrix aurantiaca*". Red FAP owe their name to their orange-red color, which is due to the lack of BChl *c*-containing chlorosomes which give the green FAP of the *Chloroflexineae* their characteristic green color. *Heliothrix oregonensis* and *Roseiflexus castenholzii* were isolated from hot springs in USA and Japan, respectively. A strain of a second but so far undescribed species of *Roseiflexus* has been isolated from hot springs in Yellowstone National Park (USA). "*Kouleothrix aurantiaca*" is a member of the 'Eikelboom morphotype 1851' and was isolated from activated sludge in an industrial wastewater treatment facility. Phototrophy has not been observed in the isolate, but has been suggested by the presence of all genes required for anoxygenic phototrophy in its genome. The presence of several more so far uncultured genera is indicated by ribosomal gene sequences obtained from a variety of habitats.

The other two orders of the class *Chloroflexia*, the *Herpetosiphonales* and *Kallotenuales* include one family each, the *Herpetosiphonaceae* and *Kallotenuaceae*. Unlike the members of the *Chloroflexales*, the members in these families are aerobic chemotrophic bacteria, which use organic compounds for growth but lack the ability to use light energy. However, similarly to the FAP they show filamentous morphology and gliding motility allowing them to move over solid surfaces. The four described species of the *Herpetosiphonales* were isolated from soil, freshwater and hot spring environments, while *Kallotenue papyrolyticum* represents a thermophilic and cellulolytic bacterium isolated from Great Boiling Spring, NV (USA). The species of the genus *Herpetosiphon* are specifically interesting as they have been described to feed on other bacteria and destroy whole colonies of several species of microorganisms (see below).

The class *Dehalococcoidia*, previously known as '*Dehalococcoides*' or '*Chloroflexi* subphylum II' has only recently (in 2013) been proposed as a class. It possesses only one family, *Dehalococcoidaceae*, containing two described genera, *Dehalococcoides* and *Dehalogenimonas*. Unlike many other members in this phylum, their cells are not filamentous but disc-shaped coccoid. Dehalogenation of chlorinated alkanes is a remarkable feature of all isolated members of the class.

The uncultured 'SAR202 clade', formerly known as '*Chloroflexi* subphylum IV', is most closely related to the isolated members of the class *Dehalocccoidia*; in some phylogenetic trees, such as the SILVA Tree Viewer (see Section 'Relevant Websites'), the clade is considered to be included in this class. It was first obtained from the Sargasso Sea and prevalent in deep oceans environments, especially in the marine subsurface (see Section Ecology).

The class *Ktedonobacteria* consists of three families, *Ktedonobacteriaceae*, *Thermosporotrichaceae* and *Thermogemmatisporaceae*, containing the genera, *Ktedobacter*, *Thermosporothrix*, and *Thermogemmatispora*, respectively. Members of the families *Thermosporotrichaceae* and *Thermogemmatisporaceae* are thermophilic bacteria, while *Kredobacter racemifer* represents a mesophilic aerobic heterotroph. All members were isolated from soil samples, *Thermogemmatispora* spp. from fallen leafs within such. The species in this class share the unusual characteristic of forming branched vegetative or aerial mycelia as well as spores similar to members of the phylum *Actinobacteria*.

The class *Thermoflexia* containing the single family *Thermoflexaceae* was named after a filamentous chemotroph, *Thermoflexus hugenholtzii*, isolated from a hot spring. The species is a thermophilic, chemotrophic, microaerobe able to grow at up to 8% O_2 with the optimal growth at 1%.

The class *Thermomicrobia* is composed of thermophilic chemoheterotrophic aerobes with irregular rod morphology. The class involves two families, *Thermomicrobiaceae* and *Sphaerobacteraceae* with the genera *Thermomicrobium*, *Sphaerobacter* and *Nitrolancetus*. *N. hollandicus*, the type species of the genus *Nitrolancetus*, is a chemolithoautotrophic nitrite oxidizer, growing on nitrite and CO_2; and is the first (and to date only) nitrifying member of the phylum *Chloroflexi*.

Thermobaculum terrenum is a thermophilic, strictly aerobic, non-phototrophic member of the *Chloroflexi*, most closely related to the class *Thermomicrobia*. It was isolated from thermal soils, and displays rod-shaped cell morphology. It stains gram-positive, which is unusual for this phylum. Its taxonomic affiliation has not been specified, but based on ribosomal genes it can be suggested to represent a novel class in this phylum.

Metagenome based analysis of hot spring microbial mats indicate the presence of novel phototrophs in this phylum, which are not affiliated with the *Chloroflexia*. The uncultured class "*Candidatus* Thermofonsia" has been proposed to contain some of these phototrophs. Also the novel phototroph, "*Candidatus* Roseilinea gracile", which was detected in a hot spring microbial mats in Yellowstone National Park (USA), was affiliated with this class based on RpoB genes. However, based on its 16S rRNA gene sequences and the SILVA backbone tree, it most likely represents the uncultured class 'RBG-13-54-9'. "*Candidatus* Roseilinea gracile" has been suggested to grow photoheterotrophically, contains thin short filamentous cell morphology, and the ability to produce photosynthetic pigments under aerobic conditions. They do not contain the characteristic green BChl *c* or chlorosomes, and thus resemble the red FAP such as *Roseiflexus castenholzii*. These findings point at a greater phototrophic diversity within the phylum *Chloroflexi* than previously recognized, and indicates horizontal gene transfer of photosynthesis genes within the phylum.

Physiology, Metabolism and Motility

A diverse range of metabolisms and physiologies are found in the phylum *Chloroflexi* (Table 1, Fig. 2). Anoxygenic phototrophy, obligate aerobic/anaerobic heterotrophy, organohalide-respiration and CO-oxidation have been observed. The phylum contains filamentous, coccoid and rod-shaped cell morphologies, thermophilic as well as mesophilic members, halophilic organisms, and even predatory microbes.

Most isolated members in the majority of the classes of this phylum share a multicellular filamentous morphology: all described species belonging to the classes *Anaerolineae*, *Ardenticatenia*, *Caldilineae*, *Chloroflexia*, *Ktedonobacteria* and *Thermoflexia* show multicellular filamentous morphology without exception (Table 1, Fig. 2(A),(B),(F) and (G)). The filamentous members of the *Chloroflexia* are capable of gliding over solid surfaces (see below). Only members of the classes *Dehalococcoidia* and *Thermomicrobia* do not show filamentous growth, but are represented by disc-shaped, coccoid, or rod-shaped cells (Table 1, Fig. 2(C–E)). Members of the class *Ktedonobacteria* are further characterized by a special type of filamentous growth, forming branched mycelia similar to *Actinomycetes*.

The first metabolism known to occur in this phylum was anoxygenic phototrophic growth as found in the members of the *Chloroflexaceae* (Table 1). The filamentous phototrophs use light energy in a chlorophyll-dependent photochemical process to generate chemical energy in form of ATP and reduction equivalents in form of NAD(P)H. As they cannot split water, they do not produce oxygen (hence the name 'anoxygenic' photosynthesis). Electron sources used are reduced sulfur compounds, such as (poly) sulfide, thiosulfate, small organic molecules, or molecular hydrogen. Phototrophy has been indicated to be present also in other *Chloroflexi* classes based on cultivation-independent genomic studies. However, as of now, all other isolated members of this phylum only grow chemotrophically. Almost all phototrophic members of the *Chloroflexi* have been shown to grow photoheterotrophically assimilating organic carbon compounds. Although many contain genes for known carbon fixation pathways, photoautotrophic growth has been demonstrated only in a few isolates (see below).

Almost all phototrophic members have also been shown to grow chemotrophically under aerobic dark conditions. Further, anaerobic chemoheterotrophic growth is found in the *Anaerolineae*, *Ardenticatenia*, and *Dehalococcoidia*, while members of the

Table 1 Differential characteristics of the eight described classes in the phylum *Chloroflexi*

Characteristics	Chloroflexia	Anaerolineae	Ardenticatenia	Caldilineae	Dehalococcoidia	Ktedonobacteria	Thermoflexia	Thermomicrobia
Morphology	Filamentous	Filamentous	Filamentous	Filamentous	Disc-shaped coccoid	Filamentous (forming mycelia)	Filamentous	Irregular rod-shaped
Habitat	Hot springs, Ponds, Brackish waters	Anaerobic digesters, Hot springs, Submarine sediment	Costal hydrothermal field	Hot springs, Hot aquifer	Contaminated environments (digester sludge, sediments, or aquifers)	Soil, Fallen leaves on geothermal soil	Hot springs	Hot springs, Sewage sludge, Nitrifying reactor
Gram stain	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Positive/ Negative
Thermophily	Thermophilic or mesophilic	Thermohilic	Thermohilic	Thermohilic	Mesophilic	Mesophilic	Thermohilic	Thermohilic or Moderately thrmophilic
Phototrophy	±	±[a]	–	–	–	–	–	–
O_2 requirement	Facultative or anaerobic	Anaerobic	Facultative	Facultative	Anaerobic	Aerobic	Facultative (microaerophilic)	Aerobic

[a]No phototrophic isolate has been obtained, but culture-independent analyses revealed that an uncultured phototroph putatively affiliated with the class *Anaerolineae* thrived in a hot spring microbial mat.

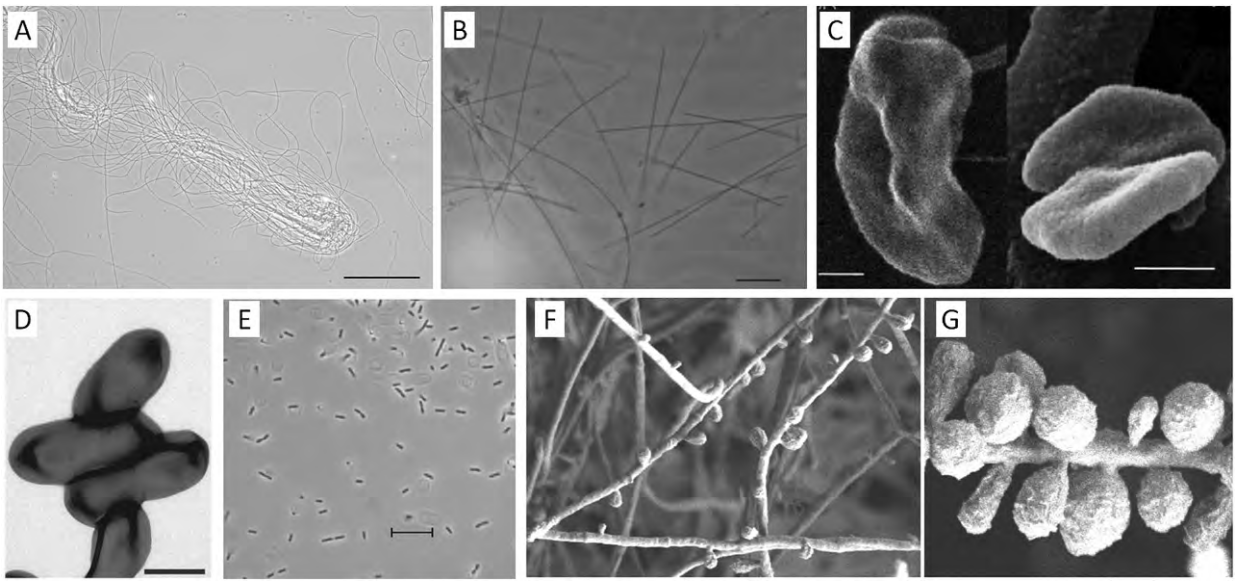

Fig. 2 Micrographs of different cell morphologies found in the phylum *Chloroflexi*. (A, B) Filamentous growth found in the classes *Chloroflexia*, *Anaerolineae*, *Ardenticatenia*, *Caldilineae* and *Thermoflexia* (here: (A) *Chloroflexus aggregans* and (B) *Ornatilinea apprima*). (C) Disc-shaped coccoid cells of the genus *Dehalococcoides*. (D, E) Rod-shaped cells in the class *Thermomicrobium* (here: (D) *Thermorudis pharmacolihpila* and (E) *Thermomicrobium carboxidum*); (F, G) Branched mycelia containing spores as found in the *Ktedonobacteria* (here: *Thermogemmatispora onikobensis*). Image (A) S. Fukushima; (B) Podosokorskaya, O.A., Bonch-Osmolovskaya, E.A., Novikov, A.A., Kolganova, T.V., Kublanov, I.V., 2013. *Ornatilinea apprima* gen. nov., sp. nov., a cellulolytic representative of the class Anaerolineae. International Journal of Systematic and Evolutionary Microbiology 63 (1), 86–92.https://doi.org/10.1099/ijs.0.041012-0. (C) Löffler, F.E., Yan, J., Ritalahti, K.M., *et al.*, 2013. *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and famil. International Journal of Systematic and Evolutionary Microbiology, 63 (PART2), 625–635. https://doi.org/10.1099/ijs.0.034926-0. (D) Houghton, K.M., Morgan, X.C., Lagutin, K., *et al.*, 2015. *Thermorudis pharmacophila* sp. nov., a novel member of the class *Thermomicrobia* isolated from geothermal soil, and emended descriptions of *Thermomicrobium roseum, Thermomicrobium carboxidum*, *Thermorudis peleae* and *Sphaerobacter thermophilus*. International Journal of Systematic and Evolutionary Microbiology 65 (12), 4479–4487. https://doi.org/10.1099/ijsem.0.000598**.** (E) Houghton, K.M., Morgan, X.C., Lagutin, K., *et al.*, 2015. *Thermorudis pharmacophila* sp. nov., a novel member of the class *Thermomicrobia* isolated from geothermal soil, and emended descriptions of *Thermomicrobium roseum, Thermomicrobium carboxidum*, *Thermorudis peleae* and *Sphaerobacter thermophilus*. International Journal of Systematic and Evolutionary Microbiology 65 (12), 4479–4487. https://doi.org/10.1099/ijsem.0.000598**.** (F–G) Yokota, A., 2012. Cultivation of uncultured bacteria of the class *Ktedonobacteria* in the phylum *Chloroflexi*. Makara Journal of Science 16 (161), 1–8. https://doi.org/10.7454/mss.v16i1.1273/Makara.

Caldilineae as facultative anaerobes, can switch between aerobic and anaerobic pathways (Table 1). The difference between the strictly anaerobic metabolisms of *Anaerolineae* and *Dehalococcoidia* is that the former contain a fermentative metabolism, while the latter perform anaerobic respiration of halogenated organic compounds (organohalide-respiration). No other metabolism has been observed in any of the *Dehalococcoides* isolates. However, recent genome analyses of uncultured *Dehalococcoidia* members have indicated more metabolic diversity, such as the potential to oxidize complex organics, ability to fix CO_2, conduct anaerobic respiration or to conserve energy as ATP via substrate-level phosphorylation.

Strictly aerobic chemoheterotrophic metabolism is found in the classes *Ktedonobacteria*, the order *Herpetosiphonales* within the class *Chloroflexia*, the *Thermoflexia* and *Thermomicrobia*, and the unaffiliated isolate *Thermobaculum terrenum* (Table 1). Members of the class *Ktedonobacteria* are further characterized by their filamentous cell morphology forming branched mycelia with spores. They grow chemoheterotrophically under obligatory aerobic conditions at either mesophilic or thermophilic conditions. Some have been shown to oxidize carbon monoxide.

Members of the *Thermomicrobia* differ from the majority of the *Chloroflexi*, as they display non-filamentous cell morphology and unusual chemotrophic metabolisms (Table 1). Some members of the *Thermomicrobiales* have been shown to oxidize carbon monoxide, and although autotrophic growth has not been observed in the laboratory, the capability for CO_2 fixation was suggested by genome analysis of *Thermomicrobium roseum*. The most unusual member, however, is *Nitrolancea hollandica,* the first nitrifying (nitrite-oxidizing) member of the *Chloroflexi*. It grows obligately aerobically under chemolithoautotrophic conditions, using nitrite as electron donor and CO_2 as carbon source.

Members of the *Herpetosiphonales* are exceptional as they are known as 'predatory microbes'. Grown on agar plates in vicinity of other bacteria, *Herpetosiphon* species have been shown to destroy whole colonies of several species of microorganisms. For killing other microbes, they appear to utilize a 'wolf pack' strategy, in which a large number of predatory *Herpetosiphon* cells congregate in order to lyse the prey by the secretion of hydrolytic enzymes. Members of this genus also have been suggested to be competent producers of bioactive secondary metabolites with potential use in medical or biotechnical applications.

Autotrophic Growth in *Chloroflexi* Members

In the laboratory, all isolated FAP strains have shown preferences for growing heterotrophically in media containing organic compounds. All *Chloroflexaceae*, *Roseiflexaceae* and *Oscillochloridaceae* isolates studied are able to grow photoheterotrophically under anaerobic conditions in the light and chemoheterotrophically under aerobic conditions in the dark.

While all isolated strains of the genus *Chloroflexus* grow well under photoheterotrophic conditions, photoautotrophic growth has been reported only for a few strains. For example, *Cfl. aurantiacus* OK-70-fl can use sulfide or hydrogen as electron donor for photoautotrophic growth, and *Chloroflexus* sp. strain MS-G showed autotrophic growth in anaerobic agar-deep cultures containing sulfide and inorganic carbon. Members of the *Chloroflexaceae* fix inorganic carbon via the 3-hydroxypropionate bi-cycle, which has been first described in *Cfl. aurantiacus* (Fig. 3). All available genomes of *Chloroflexus* and *Roseiflexus* species contain genes encoding for this pathway. This suggests that all members of these genera – even those that have not been observed to grow autotrophically in the laboratory – have the potential to perform carbon fixation. This hypothesis is currently being tested and autotrophic growth has been observed for various *Chloroflexus* spp. strains in ongoing cultivation studies.

Also mesophilic FAP, e.g., *Oscillochloris* species show growth under photoautotrophic, anaerobic conditions. They can use either sulfide or hydrogen as electron donor for autotrophic growth. In contrast to the thermophilic members, CO_2 fixation in *Oscillochloris trichoides* DG-6^T is performed through the Calvin-Benson-Bassham cycle.

Further, the MCLO representative '*Candidatus* Chlorothrix halophila' has been demonstrated to grow photoautotrophically, which indicates an ecological role as primary producer in their natural environments. Similarly to *O. trichoides* and in contrast to the thermophilic mat members *Chloroflexus* spp. and *Roseiflexus* spp., this hypersaline microbial mat member uses the Calvin-Benson-Bassham cycle for autotrophic growth.

The *Thermomicrobia* member *Nitrolancea hollandica* grows autotrophically under nitrite-oxidizing, aerobic conditions (see above). Further, capability for autotrophic growth has also been suggested for *Thermomicrobium* spp., as well as members of the *Dehalococcoidia* (see above).

Gliding Motility

Motility in the *Chloroflexi* has been reported only in filamentous bacteria belonging to the class *Chloroflexia*. These possess the so-called 'gliding motility', i.e., a smooth movement on solid or semi-solid surface without flagella. The filamentous cells typically glide straightforward along the long axis and occasionally reverse the travelling direction. The gliding speed differs between species and surface materials. On agar plates, gliding speeds of 0.01–0.04 μm/s (*Chloroflexus aurantiacus*); 0.1–0.4 μm/s (*Heliothrix oregonensis*); ~0.2 μm/s (*Oscillochloris trichoides*); and 10 μm/s in *Chloronema* species as the fastest gliders, have been observed.

The gliding motility enables the microbes to form dense microbial mats and migrate tactically within the mats and sediments, as has been observed for FAP in hot spring and hypersaline mats. The thermophilic phototroph *Chloroflexus aggregans* forms dense cell aggregates in liquid culture (Fig. 4, Video 1). The filaments glide at speeds of 1–3 μm/s on glass surface under conditions supporting optimum growing, whereas the speed of aggregation in liquid culture was up to 20 μm/s. The rapid aggregation is thought to occur through a process in which a filament glides on the surfaces of other filaments, and the filaments entangle with each other. The

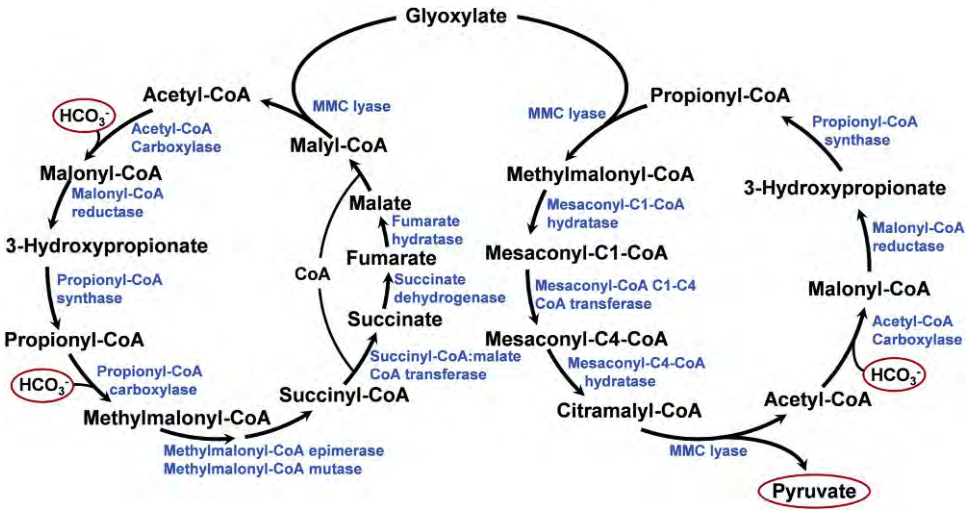

Fig. 3 Schematic map of metabolic reactions of the 3-hydroxypropionate bi-cycle and enzymes involved. Reproduced from Thiel, V., Hügler, M., Ward, D.M., Bryant, D.A., 2017. The dark side of the Mushroom Spring microbial mat: Life in the shadow of chlorophototrophs. II. Metabolic functions of abundant community members predicted from metagenomic analyses. Frontiers in Microbiology 8 (June), 1–23. https://doi.org/10.3389/fmicb.2017.00943.

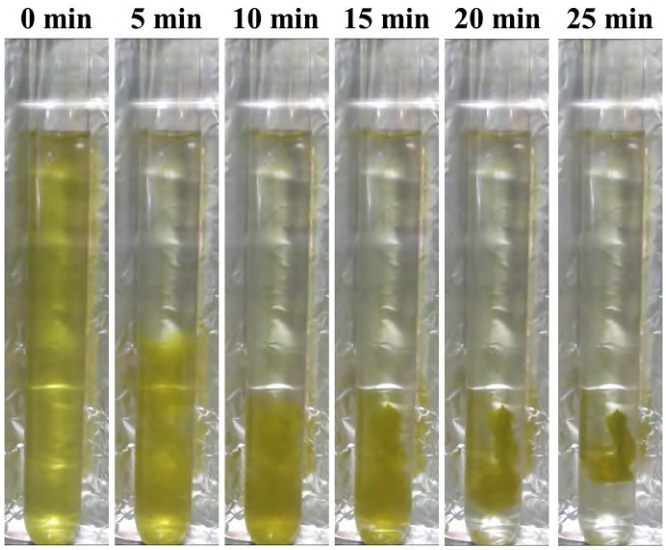

Fig. 4 Sequential photographs of aggregate formation in *Cfl. aggregans*. The cells which had been uniformly suspended within a liquid medium at 0 min were quickly aggregated under phototrophic growing condition, and a dense aggregate was formed. Image: S. Fukushima.

aggregation speed is accelerated by exogenous protease which is produced by other bacteria coexisting with *Cfl. aggregans* in microbial mats, suggesting that the aggregation should be a response for protecting the population of the cells from predation by proteases. Cyclic AMP is likely to play a key role in control of the tactic movement, because the aggregation is also promoted by increased intracellular concentration of cyclic AMP. Mesophilic non-phototroph *Herpetosiphon* species are known as predators utilizing a 'wolf pack' strategy as described above, which may be supported by gliding motility.

Supplementary material related to this article can be found online at doi:10.1016/B978-0-12-811736-1.20771-X.

Although the mechanical and molecular system of gliding motility in this phylum has not been completely elucidated, recent and ongoing studies indicate a possible involvement of pili observed on the cell surface of different *Chloroflexus* spp. Once the extended pili grip the ground surface, the cells are pulled forward by the retraction of the pili (Fig. 5, Video 2). A similar mechanism has been proposed for gliding motility in multicellular *Cyanobacteria*; and is possibly shared with *Chloroflexus*.

Supplementary material related to this article can be found online at doi:10.1016/B978-0-12-811736-1.20771-X.

Ecology

The phylum *Chloroflexi* comprises taxonomically and physiologically highly diverse bacteria which inhabit a wide range of natural and engineered environments including microbial mats, the deep oceans, freshwater aquifers and lakes, soils, as well as bioreactors and wastewater treatment plants; they have even been recognized as constituents of the human microbiome (Fig. 6). The phylum has been recognized as a typical ubiquitous bacterial taxon, which contains a number of diverse uncultured 16S rRNA gene sequences in a wide range of temperature (0–65°C) and salinity environments, where they most likely play significant ecological roles.

Microbial Mat Environments

Microbial mats are laminated biofilms, and a wonderful opportunity to see and observe the 'unseen majority', as they harbor purely microbial communities of *Bacteria* and *Archaea*, and sometimes also eukaryotic microorganisms. Microbial mats develop under conditions which are too extreme for most eukaryotes to thrive, such as thermal springs, sulfidic and/or high salinity environments. These structures provide a three-dimensional habitat in which metabolically diverse microbial populations closely coexist and interact. The microenvironment in these habitats is characterized by steep biogeochemical (e.g., O_2, pH, redox) gradients with a number of microhabitats in which different species and populations can live. *Chloroflexi*, and especially FAPs, are common members of phototrophic microbial mat communities, especially those associated with hot spring waters between 40–70°C. In these mats, oxygen tolerant phototrophic *Chloroflexaceae* can be found co-existing with *Cyanobacteria*, but also as dominant phototrophs in *Cyanobacteria*-free mats. Phototrophic microbial mats associated with neutral and alkaline hot springs can be observed all over the world and have been extensively studied in the USA and Japan since the 1960s. Those were the first environments in which phototrophic *Chloroflexi*, back then termed 'green non-sulfur bacteria' (GNSB), were detected and isolated from. The ecological role

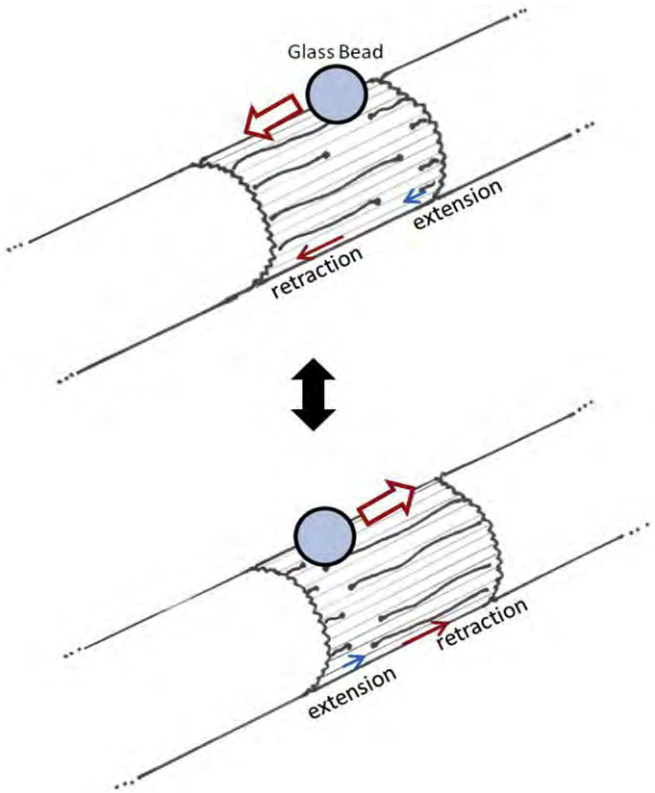

Fig. 5 Schematic drawing of the proposed mechanism for gliding motility in *Cfl. aggregans*. A glass bead attached to the cell-surface is moved on the cell surface by bi-directional pili retraction. If the extended pili is to grab a solid surface instead of the glass bead, the filament would be pulled over the surface by the retraction of the pili. Image: S. Fukushima.

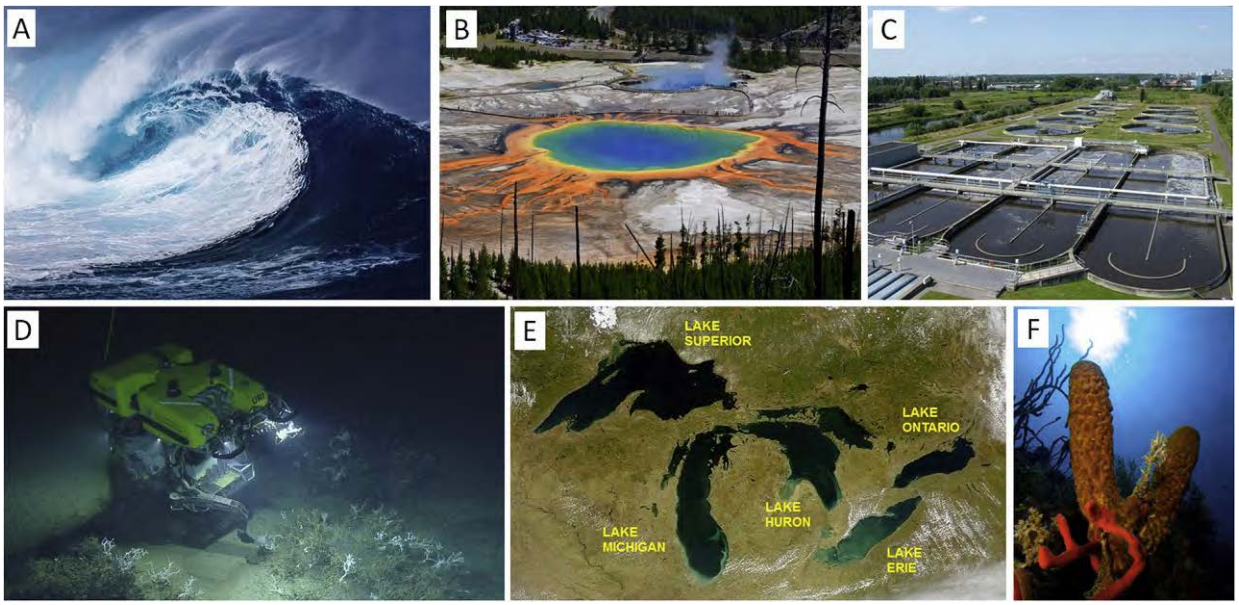

Fig. 6 Examples for natural environments for *Chloroflexi*. Members of this phylum have been found in marine habitats, such as seawater, sponges, and deep sea sediments, microbial mats associated with extreme environments, e.g., hot springs such as Grand Prismatic Spring in Yellowstone National Park, as well as freshwater habitats such as the Great Lakes and wastewater treatment plants. (A) Available at: https://pixabay.com/en/wave-atlantic-pacific-ocean-huge-1913559/, (B) Available at: https://commons.wikimedia.org/wiki/File:Grand_Prismatic_Spring,_Yellowstone.jpg, (C) Available at: https://commons.wikimedia.org/wiki/File:WWTP_Antwerpen-Zuid.jpg, (D) Available at: http://www.nautiluslive.org/sites/default/files/styles/photoswipe_display/public/originals/photos/2013-06-26/cam2_20130625235339.jpg?itok=Q_ciqSFH, (E) Available at: https://en.wikipedia.org/wiki/Great_Lakes#/media/File:Great_Lakes_from_space_crop_labeled.jpg; http://visibleearth.nasa.gov/view_rec.php?id=793. (F) Available at: https://commons.wikimedia.org/wiki/File:Aplysina_fistularis_(Yellow_Tube_Sponge)_behind_Amphimedon_compressa_(Erect_Rope_Sponge-_red).jpg.

for phototrophic *Chloroflexi* in these mats is primary and secondary production. Despite the fact that most phototrophic *Chloroflexi* in the laboratory don't show autotrophic growth, active CO_2 fixation by those mat members has been shown to occur in the mats. The most likely growth mode in their natural environments when growing with *Cyanobacteria* is a so-called 'mixotrophic' growth, in which small organic molecules and CO_2 are taken up and utilized as carbon sources simultaneously. In purely anoxygenic mats which develop in elevated temperatures and under more sulfidic conditions, under which the growth of *Cyanobacteria* is inhibited, phototrophic *Chloroflexi* are predicted to act as the main primary producers and thus to supply the mat with organic carbon. Up to now, there has been no evidence that *Chloroflexus* spp. or other phototrophic *Chloroflexi* are able to grow in acidic (< pH 6) or brackish-saline hot springs.

In addition to neutral to alkaline thermal environments, *Chloroflexi* have been observed also in marine and hypersaline microbial mats, in which they often represent the most abundant community members. The best studied hypersaline microbial mats are located in Baja California Sur, Mexico. In contrast to hot spring microbial mats, hypersaline microbial mats are highly diverse ecosystems, which also may contain a range of protistan, metazoan and invertebrate consumers. The phototrophic *Chloroflexi* inhabiting these mats resemble green FAPs, but were shown to be phylogenetically different from the thermophilic *Chloroflexus* species. They have been observed in a variety of marine and hypersaline environments since the 1980s and are referred to as 'Marine *Chloroflexus*-like Organisms' (MCLOs) in the literature. In their natural environments they co-occur with other salt-loving phototrophic bacteria of the phyla *Proteobacteria*, *Chlorobi* and *Cyanobacteria*, but also with e.g., non-phototrophic sulfur-oxidizing bacteria, such as *Beggiatoa*. One representative of this MCLO group, "*Candidatus* Chlorothrix halophilia", has been cultured in the laboratory in a stable co-culture and studied in detail. It resembles other green FAP, such as *Chloroflexus* spp. in its phototrophic properties as well as gliding motility. In intertidal sandy sediments migration of MCLOs has been observed most likely following the light. Unlike *Chloroflexus* spp. MCLOs require between 5%–12% NaCl for growth, which occurred best at mesophilic temperatures of about 35–38°C and only at a narrow pH range of 7.5–8.0. Photoautotrophic growth in the laboratory implies an ecological role as primary producer in their natural environments. This carbon fixation activity depended on sulfide, for which '*Candidatus* Chlorothrix halophila' showed an exceptionally high tolerance (up to 100mM). In their natural environment MCLOs live closely together with sulfate-reducing bacteria which produce sulfide as end-product of their anaerobic sulfate-respiration. High concentrations of sulfate as are typically found in marine and hypersaline environments also lead to high sulfide concentrations, which MCLOs seem to be suitably adapted to.

In addition to phototrophic *Chloroflexi*, also non-phototrophic members, e.g., of the *Anaerolineae* inhabit microbial mats. Their ecological role in these communities, however, has not been revealed yet. But their predicted chemotrophic metabolism indicates involvement in the degradation of organic matter and a role in carbon and other nutrient cycling.

Marine Environments

Aside from marine and hypersaline microbial mats, *Chloroflexi* have also been detected in a number of other marine environments, such as associated with marine sponges, sediments and deep water habitats. In these habitats, *Chloroflexi* do not necessarily dominate the microbial communities, but constitute only one of many phyla present.

Marine Sponges

Chloroflexi have been shown to be regular inhabitants in marine sponges, especially so-called HMA (high microbial abundance) sponges, sometimes also called 'bacteriosponges', where they co-dominate the microbial community together with *Acidobacteria* and members of the "*Candidatus* Poribacteria" phylum, as well as a variety of other phyla present. An enormous diversity of estimated 14–25 genera and 17–31 species of *Chloroflexi* seem to be specifically associated with these marine animals. *Chloroflexi* living in sponges belong to the classes *Caldilineae* and *Anaerolineae*, as well as to the uncultured environmental SAR202 clone group related with the *Dehalococcoidia*, but none is closely related to any isolated members of this phylum. Ongoing genomic analyses revealed aerobic chemoheterotrophic metabolisms for these sponge-associated *Chloroflexi* and specifically carbohydrate degradation in *Anaerolineae* and *Caldilineae* members, while the SAR202 members have the ability to produce vitamins and co-factors with potential benefit to the sponge host. These studies suggest that sponge symbionts collectively engage in the degradation of this dissolved organic matter, and thus contribute to DOM (dissolved organic matter) re-cycling and primary productivity in reef ecosystems via a pathway termed the 'sponge loop'.

Marine Sediments

Chloroflexi-related bacteria affiliated with the *Dehalococcoidia* have been repeatedly found in different marine sediments worldwide. High relative abundances of this group, sometimes up to 80% of all bacterial ribosomal gene sequences obtained, has been described in sediment samples all over the world. None of the widely distributed marine *Dehalococcoidia* members have been cultivated yet, and little is known about their physiological properties. The co-existence of a great variety of different *Dehalococcoidia* phylotypes within the same habitats indicates varying metabolic properties within this group. Further, differences between abundance and presence of specific clades with depth may relate to changes in biogeochemical factors and thus indicate specific adaptations to the corresponding environments, e.g., available electron donors and acceptors, and carbon sources. Genomic studies

have revealed some insights into possible metabolic functions. Unlike all isolated *Dehalococcoidia* members, the inhabitants of marine sediments do not seem to have the ability to perform reductive dehalogenation, contained a significantly larger genome size, and a greater metabolic versatility with metabolic potential. Genes for oxygen protection, which are all absent in isolated *Dehaloccoidia*, might represent adaptations to growth in shallow marine sediments where these organisms can be expected to periodically be exposed to oxygen. Additionally, the ability to oxidize reduce sulfur compounds has been proposed for members of this group, indicating a role in sulfur cycling. Overall, a wider metabolic and genomic diversity is now expected within this group, which also contributes to their widespread occurrence in the marine subsurfaces.

In addition to *Dehalococcoidia*, also *Anaerolineae* and *Caldilineae* members as well as the SAR202 clade have been detected in marine subsurface environments. *Anaerolineae* and *Caldilineae* sequences were found to be especially dominating in deeply buried sediments of more than approx. 1 m depth, where they often co-exist with members of the uncultured JS-1 group. Based on the chemoorganotrophic anaerobic metabolism of isolated *Anaerolineae*, contribution to terminal organic matter mineralization processes is also suggested for the uncultured *Anaerolineae* members in the deep biosphere. Further, indications for the metabolic ability for sulfate reduction and iron utilization have been found in an *Anaerolineae*-related partial genome.

Deep Ocean Environments

The environmental gene cluster SAR202, also '*Chloroflexi* subphylum IV', has first been detected in early investigations of seawater and since then been recovered from a variety of environments; they are consistently identified in the marine oceans where they constitute up to 10% of the bacterioplankton. It was the first microbial lineage discovered to specifically inhabit the dark, aphotic zones of the oceans, below the deep chlorophyll maximum where they are highly abundant and were found to persist down to 3600 m in the Atlantic Ocean and to 4000 m depth in the Pacific Ocean. Based on recent metagenome studies, the SAR202 cluster contains two distinct clades which members are suggested to be free-living chemoheterotrophic organisms with medium sized cells. They most likely metabolize multiple organosulfur and other organic compounds, possibly have the ability oxidize sulfite, and are predicted to play a major role in sulfur turnover in the dark water column.

Freshwater Environments

Chloroflexi have been detected in a wide range of natural and human-made freshwater environments, such as lakes, aquifers, sediments, lagoons and wastewater treatment plants. Most of the detected cultured and uncultured representatives in these habitats have been affiliated with the 'subphyla I', thus the *Anaerolineae and Caldilineae*, 'subphylum IV', i.e., the SAR202 clade, but also the 'subphylum III', the *Chloroflexia*.

Pelagic Freshwater

Freshwater bacterioplankton also often contains members of the *Chloroflexi* phylum, although often not as dominant taxa (average: 1%, range: 0%–4%), most of them affiliated with 'subphyla I and IV', i.e., the classes *Anaerolineae*/*Caldilineae* and the SAR202 clade. Overall, genomic diversity of pelagic freshwater taxa within the *Chloroflexi* is remarkably diverse; however communities living in the deeper layers of stratified lakes, the hypolimnion, have been shown to be less diverse than the upper layer, the epilimnion. Two clusters repeatedly detected in the he hypolimnion are affiliated with the uncultured CL500–11 cluster within the class *Anaerolineae*, and the unaffiliated TK10 cluster. In oxygenated hypolimnions of lakes such as the Laurentian Great Lakes and Crater Lake (USA), as well as Lake Biwa (Japan), *Chloroflexi* can dominate the bacterioplankton community. Members of the uncultured CL500–11 clade are among the largest prokaryotic cells in the water column of deep lakes. Based on genome reconstruction from metagenomic data, they are predicted to have an aerobic, motile, heterotrophic lifestyle with the additional capability to use light via bacteriorhodopsin and one-carbon compounds; and are believed to contribute significantly to deep lake carbon flow. In contrast to the CL500–11 clade, there is very little ecological information on the other *Chloroflexi* repeatedly found in freshwater lakes, the uncultured hypolimnion specific class-level TK10 cluster, and the epilimnion-specific uncharacterized class-level SL56 clade.

Freshwater Sediments

Chloroflexi have also been detected in freshwater sediments from lakes and rivers, where they constitute between 5%–25% of the bacterial communities. Most of those *Chloroflexi* have been affiliated with the 'subphylum I' (*Anaerolineae*/*Caldilineae*) and 'subphylum II' (*Dehalococcoidia*), but some also with undescribed new subphyla/classes. Based on the isolated members of the *Dehalococcoidia*, an environmental role involving the degradation of halogenated solvents and organics is predicted. However, metagenome studies conducted from terrestrial aquifer sediments which covered partial genomes of *Chloroflexi* members from 15 distinct lineages, found genes for organohalide respiration to be rather rare. Near complete genomes suggest aerobic chemoheterotrophic respiratory metabolism for an uncultured lineage in the *Anaerolineae*, and proposed roles in sugar and plant-derived-compound degradation for two uncultured class-level clades related to the *Dehalococcoidia*. Overall, *Chloroflexi* in those aquifer sediments were abundant and highly diverse, with possible roles in carbon cycling by respiration of sugars, fermentation, CO_2 fixation and acetate production via acetogenesis.

Wastewater Treatment Plants and Bioreactors

Filamentous *Chloroflexi* have long been observed in wastewater treatment plants. The interest on bacteria in these environments were mainly driven by the search for causes and control of activated sludge bulking, foaming and other solids separation problems. Many were described only based on microscopic studies and given different 'morphotype' numbers by Eikelboom (e.g., Eikelboom morphotype 0914, now termed '*Candidatus* Sarcinathrix'). Most belong to the classes *Anaerolineae* and *Caldilineae*, but also represent other, uncultured, class-level clades in 'subphylum I', which are being characterized *in-situ* by microscopy and genome analyses. *Chloroflexi* have been demonstrated to be constituents of mesophilic and thermophilic anaerobic (methanogenic) as well as activated sludge systems, and other bioreactors treating municipal wastewater; but also wastewaters containing compounds recalcitrant to biodegradation, such as phenol, phthalates, benzoate and others, as well as microbial fuel cell systems, e.g., fed with cellulose. Also nitrifying systems were shown to contain *Chloroflexi* in abundance, such as the nitrifying *Thermomicrobia* member *Nitrolancetus hollandicus*. The wide range of human-made environments, their high abundance and often dominance within these strongly suggest an indispensable role and ecological significance as well as physiological diversity of *Chloroflexi* in these habitats. Based on physiological properties of isolated strains from these habitats, it has been suggested that they may contribute, e.g., to the degradation of carbohydrates and other cellular components, such as amino acids, in the bioreactors. *Chloroflexi* of the 'subphylum I', and especially their filamentous morphology, are further considered important for the granulation of sludge in those bioreactors which is a major premise for the start-up and stable operation; while some have been suggested to be responsible for impaired sludge settleability known as 'bulking' which proposes an operational problem in the systems.

Soils

Although not the most dominant phylum, *Chloroflexi* can be considered as one of the dominant taxa in soils. On average it has been found to be the sixth out of nine abundant phyla, with an average of 3% (range 0%–16%). However, *Chloroflexi* in soil have not been thoroughly studied and data on physiology and ecological importance for these soil inhabitants are rare. One study found a correlation of *Dehalococcoides*-like *Chloroflexi* with the concentrations of organochlorine compounds in soils pointing towards an ecological role of organohalide-respiring *Chloroflexi* in the biogeochemical chlorine cycle in soils. Isolation studies showed that soil *Chloroflexi* affiliated with the uncharacterized E05 (within TK10) and G04 (within SL56) clades were rather slow-growing and mini-colony forming, pointing at the cultivability of these uncultured groups with sufficient patience by the scientist.

Human Microbiome

A distinct group of uncultured *Chloroflexi* related to free-living anaerobic *Anaerolineae* has been reported as a low abundance (<1%) but consistent component of human oral and skin microbiota, as well as the gut of other mammals. They are considered to represent a minor component of the overall healthy human microbiota. However, in some cases, *Chloroflexi* have been associated with diseases, such as periodontitis, an inflammation of the gums and supporting structures of the teeth, where they were found to proliferate in the subgingival pockets of the patients. The 'oral taxon 439' group is closely related to the recently described *Flexilinea flocculi* (class *Anaerolineae*). Similar to *F. flocculi* and other isolated members of the *Anaerolineae*, single-cell genome studies indicate the oral *Chloroflexi* to be anaerobic chemoheterotrophs. Their genomes encode abundant carbohydrate transport and metabolism functionalities. Other genes suggest an important ecological niche for the oral *Chloroflexi* in scavenging material from lysed bacterial cells and human tissue. Their ability to produce sialic acid, which is widely distributed in animal tissue, may enable them to hide from the host defense system and then colonize the subgingival space and to proliferate in periodontitis.

Genomics

With the invention of Next-Generation-Sequencing methods, prices for genome sequencing dropped significantly over the past two decades and the numbers of genomes available in public databases increase continuously. At the time of writing (July 2018), the databases of the "National Center for Biotechnology Information" (NCBI) listed 426 genomes for the phylum *Chloroflexi*, while the databases of the 'Joint Genome Institute' (JGI) contained 310 *Chloroflexi* genomes (Table 2). Most of the published genomes belong to uncultured and unclassified *Chloroflexi* and (often also uncultured) members of the class *Anaerolineae*.

The majority of these genomes are still in the process of being thoroughly analyzed and published. A quick search in literature databases, such as 'Web of Science' or 'PubMed', but also in 'Google Scholar' will reveal any early and most recent literature.

Early genome studies focused on pure culture isolates. Those studies allowed elucidation of pathways and metabolic capacities beyond the ones observed in the laboratory. Genome analysis of FAPs, for example, suggest their capability to fix CO_2 via the 3-hydroxy-propionate bi-cycle even for those strains that do not express this trait under laboratory culture conditions. The verification of this hypothesis is currently underway by isolation studies demonstrating autotrophic growth in several *Chloroflexus* spp. strains.

With the increasing power of sequencing techniques, the decreasing prices as well as the increase in available computing power environmental genomics have revealed valuable information also about uncultured members of this phylum. Of the approx. 25–30 or more *Chloroflexi* classes, only eight contain isolated members. Only thanks to environmental metagenomics we are learning about the metabolic potential of representatives of lineages previously characterized only by 16S rRNA gene sequences. In an aquifer

Table 2 Number of genomes listed for the different classes in the phylum *Chloroflexi* in the NCBI database

Taxon	*Genomes*
Phylum Chloroflexi	426
Class *Anaerolineae*	134
Class *Ardenticatenia*	3
Class *Caldilineae*	5
Class *Chloroflexia*	13
Class Dehalococcoidia	89
Class *Ktedonobacteria*	6
Class Thermoflexia	1
Class *Thermomicrobia*	5
unclassified *Chloroflexi*	170

sediment environment the analysis of uncultivated *Chloroflexi* members indicate a complex set of potential roles of these *Chloroflexi* in sediment carbon cycling, including organohalide and sugar respiration, as well as fermentation, CO_2 fixation and acetogenesis. Genome analyses also enable insights into the evolution of life, bacteria and functional traits. Comparative genomics, for example, suggests that horizontal transfer of genes encoding a nitrite oxidoreductase (NXR) was a major driver for the spread of nitrite oxidation during bacterial evolution.

Appendix A Supplementary Material

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/B978-0-12-811736-1.20771-X.

Further Reading

Bayer, K., Jah, M.T., Slaby, B.M., Moitinho-Silva, L., Hentschel, U., 2018. Marine sponges as *Chloroflexi* hot-spots: Genomic insights and high resolution visualization of an abundant an diverse symbiotic clade. mSystems 3:e00150-18. https://doi.org/10.1128/mSystems.00150-18.

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Cholera, Historical

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Glossary

anticontagionism The belief that disease originates not from the transmission of germs from person to person but rather from interaction with an unsuitable environment, contact with rotting or deteriorating organic material, inhalation of polluted or contaminated air, or immoral behaviors.

contagionism The belief that disease is transmitted by direct or indirect contact between living organisms, especially humans, in the form of microscopic living entities (e.g., germs).

miasma Literally, foul or bad air arising from decaying animal or vegetable matter, thought by anticontagionists to cause disease.

quarantine Originates from the Italian words *quarantina* and *quaranta giorno*, referring to the 40-day period ships entering the port of Venice were required to remain in isolation before any disembarkment. Defined by public health authorities in the nineteenth century as the process of inspecting all ships, cargos, and passengers for evidence of contagious diseases.

sanitarians Public health officials who worked to eradicate disease by disinfecting the environment and imposing sanitary restrictions on citizens.

Defining Statement

Cholera is an acute diarrheal disease in which *Vibrio cholerae*, serogroup 1, found only in humans, are present in large numbers in the small intestine. Marked by severe abdominal pains and rapid dehydration, cholera has brought horrific and untimely death to millions as it swept the world in as many as seven pandemics in the past two centuries. Cholera's modern history as a worldwide pandemic disease begins in 1817. Long endemic in India, cholera at this time began to make its way out of the subcontinent and spread as far as the United States by 1832. Cholera, described by one historian as 'the classic epidemic disease of the nineteenth century', has played a major role in medical history, particularly due to its widespread physical and emotional effects on individuals, societies, and cultures. The disease manifests with little warning, induces frightfully painful symptoms in its victims, and kills between 30 and 80% of those infected. Moreover, the cholera pandemics spanned generations, crossed political and geographic boundaries, and fueled fears of disease during a time of dramatic growth in the life sciences, public health, and international migration.

Etymological Considerations

The Greek word *kholera*, meaning 'bile' or 'to flow', is found in sources as early as the Hippocratic Corpus. Classical writers such as Celsus (first century AD) used the term 'cholera' to describe illnesses marked by sporadic diarrhea, vomiting, and gripping abdominal pain. Although this term has been in use for millennia, it was applied in various ways to describe a variety of conditions. For example, the term 'choleric', meaning 'easily moved to anger', is also derived from *kholera*, but was a concept employed by humoralists to describe the condition resulting from vitiated humors. It was not until 1669 that Thomas Sydenham first used the term 'cholera morbus' to distinguish sporadic and endemic diarrhea from the choleric concept. By the early nineteenth century, when cholera reached global epidemic proportions, the term 'cholera nostras' had come to designate diseases with symptoms similar to sporadic and endemic cholera (i.e., profuse diarrhea, dehydration, and cramps), whereas cholera morbus described the more virulent occurrences of epidemic cholera. Other terms synonymous with epidemic cholera are cholera asiatica, malignant cholera, cholera asphyxia, and cholera spasmodica.

Pathogenesis, Signs, and Symptoms

The disease called cholera today occurs when the etiological organism *Vibrio cholerae* is ingested and passes through the stomach into the small bowel, where it colonizes and multiplies. The *V. cholerae* is an acid-sensitive bacterium and a human must ingest a relatively large inoculum for sufficient numbers to evade the acidic environment of the stomach and reach the small intestine. The organism then releases a toxin that enters the epithelial cells, disrupting the absorption of electrolytes, and results in copious, watery discharges relatively free of fecal material accompanied by shredded mucous and epithelial cells giving it a characteristic 'rice water' appearance.

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The signs and symptoms of cholera are the result of a rapid loss of fluid and electrolytes. Symptoms begin with the victim's sense of generally not feeling well. This condition lasts only a few hours and is then followed by a violent bout of vomiting and diarrhea. The victim experiences painful spasms of the abdominal muscles as a result of forceful propulsions of the gut, and matters only worsen as hypovolemic shock ensues. The effects of dehydration are painfully apparent on the face of the victim; the skin becomes blue and tight, the eyes are sunken deeply in their sockets, and the lips and mucus membranes of the mouth are dry and cracked. As diarrhea and vomiting continue, patients become increasingly more disoriented and eventually lapse into convulsion and/or coma. From 30 to 80% of all cholera cases end in death.

Etiology

From antiquity until well into the nineteenth century, the medical theory of the cause of cholera was relatively static. For the most part, illness was perceived as an imbalance in the bodily humors due to poor diet, change in weather or seasons, geographic environment, or exposure to poisoned air or miasmas, such as the foul air caused by rotting organic material or animal and human waste. Although humoral theory waned during the nineteenth century, miasmatic theory and environmental influences continued to serve as explanations for disease. In a world still influenced largely by religious morality, people also often invoked metaphysical conceptions to explain cholera and other diseases. In America during the 1830s, for example, many ministers, moralists, and physicians agreed that those suffering from cholera had engaged in sinful activities and thereby predisposed themselves to sickness.

When the second pandemic reached Europe and the United States (1832), delegations of scientists and physicians were sent out to study the spread of cholera, and questions about its contagious nature slowly began to surface. Germ theory was far from well established at this time, but burgeoning interests in contagionism and increasing knowledge of microorganisms stirred the imagination of medical scientists to search beyond the traditional metaphysical and environmental explanations of disease.

Perhaps one of the greatest challenges to miasmatic theories on the cause of cholera came from John Snow, a British physician and epidemiologist. In the summer of 1849 he published a pamphlet "On the Mode of Communication of Cholera", in which he proposed that cholera was a waterborne disease. In 1854, his theory would be put to the test. He mapped incidences of cholera that occurred near Golden Square in London and noted that many cases were clustered around a particular water pump on Broad Street. After careful analysis, his cholera pamphlet was expanded and reprinted in 1855, reporting findings that sewage had seeped into the well that the recent victims of cholera used and concluding that contaminated water was the 'predisposing cause' of cholera. Although Snow was unaware of the causative agent, he posited that *materis morbi* (poisonous matter) must have caused the infection. Although many papers and pamphlets were published on studies similar to Snow's (such as the work of William Budd) and some measures were taken to avoid contaminated water, many medical and scientific elites who subscribed to anti-contagionist theories, such as the German public health expert Max Von Pettenkofer, remained resolute in their belief that cholera was caused by imbalances in the air, soil, and environment or exposure to human and animal filth in overcrowded cities. Shortly after Snow announced his findings, Italian microscopist Filippo Pacini reported his discovery of a microorganism with a terminal flagellum, isolated from the excreta of a cholera victim. This report, however, did not attract much interest among the medical community. Moralist ideas and claims about miasmatic theory were slow to die.

Anticontagionism, however, steadily lost scientific credibility as the nineteenth century progressed. For example, in 1868, the French chemist Louis Pasteur first articulated the germ theory when he elucidated the microbial cause of silkworm diseases. Fifteen years later, the famed microbiologist Robert Koch isolated the *V. cholerae* during the cholera epidemics in Calcutta and Alexandria. Indeed, Koch's studies confirmed that cholera is transmitted through drinking water and food contaminated with fecal matter from those infected with cholera.

The acceptance of germ theory and rapid growth in the science of bacteriology yielded more advances in the field of cholera research at the turn of the century. Recent investigation has elucidated the existence of 60 or more serogroups of *V. cholerae*, of which only a single group, serogroup 1, is responsible for the epidemic cholera; others are responsible for sporadic diarrhea and symptoms similar to epidemic cholera. Serogroup 1 of the *V. cholerae* exists as two serotypes, Ogawa and Inaba, each characteristic of different somatic antigen structures in the organism. *V. cholerae* also occur as two major biotypes: the classic (first described by Koch) and El Tor. The El Tor vibrio was discovered in 1905, during the sixth pandemic, in the El Tor quarantine camp in Egypt. Six unusual strains of vibrios were isolated from the dead bodies of pilgrims returned from Mecca. Gotschlich, who isolated the strains, performed tests that revealed the reactions of classic *V. cholerae*, though the victims did not show any signs of the cholera disease post mortem or while they were alive. Upon reexamination of the strains, Kraus and Pribram found that the El Tor vibrios produced a hemotoxin found to be lethal in experimental animals. They hypothesized that variability in the biotypes could be responsible for individual cholera epidemics. This was clearly demonstrated in the 1960s when El Tor was found to be the cause of many cholera outbreaks throughout the world.

The previous hypothesis was further supported in early 1993 when a *V. cholerae* strain, with an antigenic structure differing from that of El Tor, was found responsible for a large outbreak in India. The existence of the newly identified strain, named O139 Bengal, demonstrates that epidemic potential is not exclusive to the classic and El Tor vibrios bearing the O1 antigen. The existence of O139 Bengal is indicative of an emerging level of complexity in the relationship of genetic and epidemiological factors facing today's researchers and public health officials.

The Pandemics

First Pandemic (1817–1824)

In 1817, northeastern India experienced a particularly heavy rainfall that brought deluge to the state of Bengal and other regions between the Ganges and Brahmaputra Rivers. Floods resulted in failed crops and destroyed villages, and following the devastation cholera appeared with extraordinary virulence. In July of that year, cholera broke out in several districts in Bengal. Within a month, 25 000 people in Calcutta were being treated for the disease, 4000 of whom perished. This episode marks the beginning of cholera as a worldwide pandemic disease. Only 3 months later the province of Bengal was consumed by the disease, and by 1818 cholera extended in all directions to Nepal, Burma, and further into India: Delhi, Bombay, and as far as Ceylon. Although cholera affected these regions in the past, the rapidity with which it spread and the numbers mortally endangered in 1817 cast a shadow over any previous record of the disease. By 1820, the disease had spread by sea and land routes to Southeast Asia, moving from Burma to Siam's (Thailand) capital city of Bangkok and to Indonesia, where as many as 100 000 people succumbed to the cholera menace.

By 1822, incidences of cholera began to subside in India, although the disease was widely established outside the subcontinent. Ships traveling from Southeast Asia carried the epidemic to China, where it had been previously introduced by land travelers as early as 1817. Soon after, the British army landed infected troops from Bombay into Oman, Arabia, from where the disease spread and was established in Muscat by 1821. In the same year, 15 000–18 000 people were killed along the Persian Gulf in port cities such as Basra. From this region cholera continued on, moving across the Mediterranean region, carried by caravans into countries such as Syria, where it raged until the end of 1823. The spread of cholera was greatly facilitated by Persian troops victoriously returning home to present-day Iran after warring with Turks; they served as vectors for the deadly vibrio, disseminating cholera in areas between the Tigris and Euphrates Rivers.

After raging across the Far and Middle East for as many as three or four seasons, cholera subsided but remained active in Lower Bengal, India. Some have posited that control measures may have been responsible for cholera's retreat in the early 1820s, although others have ascribed its decline to a severe winter during 1823 and 1824.

Second Pandemic (1829–37)

By 1824 cholera settled back in its home, the endemic area of the Ganges Delta in Bengal, India. There the disease remained active until 1827, when it was reported to have moved west to the Punjab. Cholera continued to exist in isolated regions outside of India during the interpandemic period; therefore, when the disease spread to the north and west of the Caspian Sea, into Russia and neighboring countries, it was thought that the pandemic's origin came from Astrakhan, Azerbaijan, where cholera had persisted throughout the 1820s. However, cholera did not disperse from Astrakhan until 1830, although it was observed to be a problem in Orenburg, Russia, as early as 1829. Reports from that period in China demonstrated that the second pandemic made its way back into areas of China, particularly in and around Peking, via India, and from there gradually extended into Russia. Similarly, cholera was creeping across Persia from Afghanistan into Russia by way of caravans. Although these scenarios might not explain cholera's appearance in Orenburg, they demonstrate the vast space through which cholera steadily advanced early in the second pandemic.

Well established in Russia by 1830 in cities such as Moscow and Kharkov, cholera was then introduced into Poland by the Russian army. A ship transported the disease from Riga to the Prussian city of Danzig, resulting in a wave of infection that soon spread to Berlin, Vienna, and Hamburg by late 1831. At this time, some of the first cases of cholera were appearing in England in the port city of Sunderland, which had shipping connections with Germany. Cholera effectively made its way through England in 1832, infecting as many as 15 000 people and leaving more than one-third of them dead. The same year cholera visited Ireland and France. All of Paris' districts were reportedly overcome with the disease and as many as 7000 people died in 18 days. By early 1832, much of Europe was suffering from the devastating effects of cholera. Having traveled from Asia through the Near East and into Europe, it crossed the Atlantic and released its fury in North America. On 8 June, a ship from Dublin carried 173 passengers to Quebec City and Montreal, of which 42 died before reaching port. On 23 June, cholera appeared in New York, and 2 weeks later it surfaced in Philadelphia. From the north the epidemic spread to Mexico, Latin America, and the Caribbean. It was not until 1837 that the second cholera pandemic finally subsided.

Third Pandemic (1846–55)

Scholars have long debated the dates of the second and third pandemics. Authorities such as Pollitzer considered the second pandemic to have run until 1851, marking the commencement of the third in 1852. Others, however, noting the significant decrease in cases of cholera and its withdrawal in some areas during the period of the mid-1830s to the mid-1840s, have concluded that the resurgence of the disease in 1846 marks the beginning of a third pandemic.

In 1846 cholera again erupted in and around the endemic regions of South Asia. Having already extended into Persia and central Asia during 1844 and 1845, Europe was again invaded by cholera by 1848 in much the same fashion as the disease progressed during the second pandemic. In the same year, New York and New Orleans were miserably reacquainted with cholera. From these cities cholera spread rapidly across North America, reaching as far as California in 1850. Present throughout Europe since 1849, cholera continued its course, eventually surging in 1854, making this year one of the worst on record.

Fourth Pandemic (1863–79)

In the fourth pandemic, cholera flourished in the same regions as it had in past pandemics, but it infiltrated Europe via new routes. Instead of moving from Asia, across Persia and the Caspian Sea, and into the heart of Europe, cholera traveled across Arabia. In Mecca, for example, 30 000 of 90 000 pilgrims died in 1865. The scourge progressed throughout the Mediterranean regions from Alexandria, Egypt, and this time entered Europe through Italy and southern France. From Egypt, Africa's countries along the eastern coast were ravaged as far south as Mozambique. When cholera traveled north in 1866, some of the worst episodes occurred in Germany; where up to 115 000 deaths were reported. In Berlin, approximately 5500 deaths occurred out of 8000 cases. It has been suggested that Austria's wars with Germany and Italy may have contributed to cholera's dissemination.

In 1866, the United States was just recovering from the Civil War when cholera returned for a third American epidemic. New Orleans was hit by several waves of cholera through 1868 and the disease spread through the South and the Midwest via the extension of the railway system. However, the outbreaks were mild in comparison with previous epidemics. In New York, measures such as the cleaning of city streets and the isolation of the ill were successful in preventing the further transmission of disease. Cholera resurfaced again in New York and New Orleans in 1873–75, though casualties were minimal. Russia, Persia, Arabia, Africa, India, and much of East Asia, however, suffered repeated serious attacks of cholera during this period. Central Europe also had its share of cases, but epidemics were less widespread due to the increasing awareness of public health.

Fifth Pandemic (1881–96)

Many of the early epidemics of this period occurred in Asia, particularly China, and around the Mediterranean. However, in 1892 Hamburg suffered a huge outbreak of cholera due to the contamination of unfiltered water from the Elbe River; approximately 7500 deaths occurred. In North America, potential epidemics were again prevented due to advanced notice and modern sanitary measures, although isolated cases were recorded, particularly in New York City.

Sixth Pandemic (1899–1923)

Cholera recrudesced in India in 1899, marking the start of the sixth pandemic. Cholera never disappeared completely from China and other regions of Asia after the previous pandemic, as was also the case for Egypt. From these regions, cholera spread to Persia, Arabia, and Afghanistan. During this period, America and most of Europe were largely unaffected, although sporadic cases occurred in southern Europe and Hungary.

Seventh Pandemic (1961–Present)

Many of the epidemics occurring in the present era have been traced to the El Tor biotype. El Tor was isolated in Indonesia and China in 1961 and in 1962 the World Health Organization recommended the inclusion of diseases caused by El Tor to appear in the definition of cholera. Both the classic and El Tor biotypes were detected in Afghanistan, from where the El Tor spread to neighboring Iran. An upsurge in cholera infection was also documented to have spread into Europe and Africa in 1970 and 1971. Since 1972, the number of countries affected in any 1 year has declined. However, in South America, where nearly a century had passed since the last reported cholera cases, the disease broke out violently in Peru in early 1991. El Tor was found responsible for this episode and for the recurrent Latin American epidemics that followed in the early 1990s. In late 1992, cholera-like disease appeared in Madras, India. The outbreak spread north into Bangladesh, where 100 000 cases were reported in the next 6 months. The new strain of *V. cholerae*, O139 Bengal, was the source of this epidemic that subsequently spread to Pakistan, Nepal, and regions of Southeast Asia. Cholera remains endemic in areas of Asia, Africa, and many countries of South and Central America, and rarely isolated cases of cholera have been imported to America and Europe. One relatively recent occurrence was reported in Los Angeles in 1993 after a woman returned from a 6-week visit to Hyderabad, India.

Geographical Considerations

Cholera owes much of its geographical distribution to pilgrimage, the industrial revolution, international shipping, traveling armies, and immigration. For example, large religious festivals in India convene near sacred sites, usually along the banks of sacred rivers. Upon people's return home from such festivals, where thousands camped and used the same water supplies, cholera often followed. A similar process has been observed during the Islamic pilgrimages to Mecca. As transportation, such as with the steamship, became more efficient and continental and intercontinental railways linked city and village, the dissemination of the disease accelerated. The need for large workforces during the industrial revolution and the desire of many to emigrate because of either political or religious persecution contributed to the crowding of cities and the contamination of water supplies.

Control, Prevention, and Treatment

Some of the most significant topics explored in the history of medicine, such as the modern-day understanding of contagious diseases, the rise of sanitation and public health institutions, and developments in medical therapy, find context in the history of

cholera. For much of the nineteenth century, doctors found that they could do very little to prevent or cure cholera. Revolutions in industry and transportation brought about the rapid growth of urban populations. It was in this setting, including the living conditions of crowded and dirty cities, contaminated water supplies, and large-scale migration of peoples, that cholera proliferated before traveling into bucolic areas. Before the 1830s, city officials had little interest in public health. With news of cholera moving from East or West, however, both European and American newspapers and popular magazines reported the spread of the disease, raising the attention of civic leaders and the public. Physicians and public health officials published pamphlets and articles and posted signs encouraging personal hygiene. Anticontagionists such as Von Pettenkofer warned of bad air and soil and encouraged proper nourishment and sanitary measures. John Snow, on the contrary, encouraged attention to maintaining clean water and Koch's discovery of the etiology of cholera, *V. cholerae*, led to the development of modern public health policies and sanitation procedures and techniques that continue to maintain health in much of the industrialized world.

One of the initial responses to cholera's appearance or impending crossing of borders was the use of quarantine. For example, soon after it was reported that cholera broke out in Canada in 1832, both Canadian and US ports issued quarantine measures on all shipping and goods. In 1892, an epidemic of cholera was effectively prevented by the quarantining of ships in New York Harbor, where infected passengers were found. Entire vessels were quarantined for days until it was certain that no cholera would be introduced to the population on land. As a result of bacteriologists' increasing abilities to recognize organisms and epidemiologist's facility to track disease, scientists, physicians, and public health officials have been successful in controlling the spread of cholera.

Treatment of cholera before and during the early nineteenth century employed the use of emetics, purgatives, and even bloodletting. Such practices probably only worsened the conditions of the ill. As early as 1830 research in fluid balance led physicians to attempt injecting water into terminally ill cholera patients. Many of these patients demonstrated the return of a strong pulse, but soon died. Injections of saltwater proved slightly more helpful, although determining a proper and effective solution was not always possible. Hence, such a treatment did not become successful until the early decades of the twentieth century when physicians had a clearer understanding of water and electrolyte balance. After the discovery of antibiotics in the 1940s, their use and a carefully planned regimen of fluid replacement greatly improved the therapeutic resources of physicians.

Cholera and Society

Epidemics are commonly viewed as crises and engender massive reactions by physicians, public health workers, and laypeople. One unfortunate theme in the history of cholera (as with other epidemic diseases) is the scapegoating of those people and social groups perceived to be the vectors of the epidemic in question. The history of immigration illustrates such examples, where massive movements of peoples resulted in the transmission of disease across borders. In large cities that attracted great numbers of immigrants, such as New York City, the meeting of various ethnic groups and cultures set the stage for dramatic shifts in civic and social organization. When dealing with the cholera menace, societies, governments, and groups such as public health organizations at times did not hesitate to point the finger at marginalized peoples, labeling them as the bearers of disease. Immigrants were perceived to be predisposed to disease based on the association with the relatively few immigrants who did arrive in US ports ill with cholera and hence stigmatized. In reality, cholera was a social leveler that attacked people of all social classes and backgrounds. Nevertheless, the fear of cholera often inspired certain majority social groups to scapegoat and blame minority groups, such as immigrants and the urban poor.

On the contrary, the cholera pandemics in the nineteenth century helped solidify ideas and sentiment toward concerns for public health in a time of scientific, economic, and societal growth. During this period, the pandemics also inspired the formation of the first international health agency (the International Sanitary Conferences), demonstrating that epidemics also have the power to bring nations together. Hence, the history of cholera illustrates that epidemic disease is an intimate part of social organization and change.

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Chromosome Replication and Segregation☆

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Glossary

Anaphase-promoting complex (APC) A ubiquitin ligase activated in mitosis that triggers degradation of cyclin-dependent kinases and cohesins.
Autonomously replicating sequences (ARS) Yeast chromosomal sequences that provide a replicator function to plasmids and produce high-frequency transformation. ARSs are the most thoroughly studied replication origins in yeast.
Cell cycle The life span of the cell, from cell division to cell division.
Centromere *Cis*-acting sequences that control movement and segregation of chromosomes. In eukaryotic microbes, centromeres are attached to microtubules of the mitotic spindle apparatus. In prokaryotic microbes, the centromere and its attachment structure are less well defined.
CMG complex The active eukaryotic DNA replication fork helicase comprising Cdc45, Mcm2-7 and GINS.
Cohesins Proteins holding newly replicated yeast chromosomes together until the sister chromatids become attached to spindle microtubules.
Cyclin-dependent kinase Serine/threonine kinases involved in cell cycle regulation when complexed with cyclins.
DNA unwinding element (DUE) Regions of double-stranded DNA, where the helix is easily destabilized to achieve a single-stranded state. The DUE is associated with replication origins and is rich in A–T base pairings.
DnaA-Trio element DUE-associated region in bacteria containing arrays of the nucleotide triads 3′-GAT-5′, 3′-AAT-5′, and 3′-GAA-5′. Following DUE unwinding, DnaA oligomers assembled along the trios assist in loading the replicative DNA helicase.
Equipartition Allocation of one from each pair of newly replicated chromosomes into each progeny cell.
Fluorescence in situ hybridization (FISH) Microscopy technique used to locate specific genetic sites within an intact cell. DNA probes tagged with a fluorescent protein are hybridized to fixed cells and the position of the glowing spot is determined relative to intracellular structures or other genetic sites.
Fluorescent repressor operator system (FROS) Microscopy technique used to locate specific genetic sites within an intact cell. Fluorescent-tagged proteins are bound to their cognate binding sites after inserting multiple copies of these binding sites at specific genomic locations.
Kinetochore A multicomponent DNA-protein structure that forms on centromeres to link spindle microtubules and motor proteins with centromeres on the newly replicated chromosomes.
Origin of replication Chromosomal sites that interact with specific proteins to produce unwound DNA regions required to start polymerization of new DNA from existing template.
Origin recognition complex (ORC) Multiprotein complexes associated with origins of replication throughout the cell cycle, used to recruit pre-RC.
Prereplication complexes (pre-RC) Assemblage of proteins associated with ORC that is necessary to load DNA helicase and establish bidirectional DNA replication.
Sister chromatids The two linked products of chromosome duplication in eukaryotes, each containing a newly polymerized DNA strand and a template strand formed during an earlier round of DNA replication.
Spindle Microtubules emanating from a microtubule organizing center called the spindle pole body (SPB) on each side of the yeast nucleus. The microtubules grow toward the newly replicated paired chromosomes.

Abbreviations

ABF1	ARS-binding factor
ACS	ARS core sequence
APC	Anaphase-promoting complex
ARS	Autonomously replicating sequence

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CDK	Cyclin-dependent kinase
DUE	DNA unwinding element
FISH	Fluorescence in situ hybridization
FROS	Fluorescent repressor operator system
GFP	Green fluorescent protein
IHF	Integration host factor
MCM	Minichromosome-maintenance
ORBS	Origin recognition boxes
ORC	Origin recognition complex
pre-RC	Prereplicative complexes
RIDA	Regulatory inactivation of DnaA
SMC	Structural maintenance of chromosomes
SPB	Spindle pole body

Defining Statement

Chromosome replication and segregation are essential events during the microbial cell cycle. Diverse regulatory mechanisms exist to initiate new rounds of DNA synthesis at origins of replication with exquisite timing, limit initiation to only once per generation, and ensure correct partitioning of newly synthesized chromosomes into newly dividing daughter cells.

Introduction

Chromosome replication and segregation are key events during the microbial cell cycle that must be completed before a cell divides. To reproduce successfully, every cell must replicate its chromosome(s) and distinguish nascent sister chromosomes from one another. Each sister chromosome must then be physically segregated into one of two new cells prior to completion of cell division. These are not simple tasks considering the large size of microbial genomes (0.2–5 mm) and the extremely limited space these genomes occupy (0.1–10 μm^3). Yet, errors (production of chromosome-less cells) are rare ($<10^{-5}$/cell division in *Escherichia coli* and *Saccharomyces cerevisiae*). To ensure a low error rate, microbial cells require complex and precise regulatory mechanisms to both properly time new rounds of replicative DNA synthesis and orient newly synthesized DNA with respect to fixed intracellular locations. This article is focused on the nature of molecular mechanisms that regulate chromosome replication and segregation in prokaryotic and eukaryotic microorganisms. Most examples will be taken from the bacterium *E. coli* and the budding yeast *S. cerevisiae*. Emphasis will be on assembly of DNA–protein complexes at replication origins and localization of specific chromosomal sites during segregation.

Prokaryotic and Eukaryotic Chromosomes: Replication Origin Placement and Initiation Timing

Chromosomal DNA replication initiates from specialized sites termed origins of replication. In the Replicon model presented in 1963, Jacob, Brenner, and Cuzin formalized the premise of a specialized replicator site that binds regulatory initiator proteins. This model remains essentially correct, although many details of the initiation triggering process in bacteria and yeast are not yet completely understood. It is instructive to compare and contrast current knowledge about the location, arrangement, and number of replication origins on bacterial chromosomes and yeast chromosomes. This simple comparison reveals fundamental similarities and differences between the control mechanisms in these cell types.

Bacteria

Bacterial genomes, with very few exceptions, are double-stranded, circular, supercoiled DNA molecules. Each genome contains at least one fixed site, termed *oriC*, from which DNA replication initiates. In every cell cycle, two newly initiated DNA replication forks proceed from *oriC*, moving bidirectionally until the entire chromosome is duplicated. The majority of gene transcription proceeds away from the replication origin, in the same direction as replication forks. In most species of bacteria, the fixed position of *oriC* is closely associated with genes whose products play a direct role in regulation of DNA replication (e.g., see *dnaA* below). Some bacterial replication origins, such as *Bacillus subtilis* and *Helicobacter pylori*, are bipartite due to positioning of the initiator protein gene. *E. coli*'s *oriC* and those of its close relatives remain exceptional, positioned between the genes encoding ATP synthetase and asparagine synthetase, which are not directly involved in DNA synthesis.

Since there is only one *oriC* locus on most bacterial chromosomes, the number of intracellular copies of *oriC* might be expected to fluctuate from one to two during the cell cycle. This is indeed the case for slow-growing bacteria. However, during rapid growth

under nutrient-rich conditions, bacterial doubling times are shorter than the time needed to complete chromosome replication and septation. For example, *E. coli* doubling times approach 20 min in broth containing beef extract, but approximately 60 min is required after initiation of DNA replication to produce a complete copy of the 4600 kb genome and to complete cell division. Therefore, to produce two chromosomal copies for segregation to daughter cells prior to division, rapidly growing bacteria must trigger new rounds of chromosome replication from *oriC*s before previously initiated rounds are completed. As a result, these bacteria contain dichotomously forked chromosomes that contain more copies of *oriC* per cell (as many as eight in *E. coli* at cell division) than slower growing cells. Despite the unusual appearance of chromosomes in fast-growing *E. coli* (Fig. 1), each copy of *oriC* is identical and functionally equivalent to all others. In all prokaryotes examined thus far, replication initiates synchronously from all available copies of *oriC* at the start of the DNA synthesis phase of the cell cycle.

In *E. coli*, it is possible to increase further the number of functional copies of *oriC* per cell by introducing minichromosomes, plasmids whose sole origin of replication is a cloned copy of *oriC*. Minichromosomes are governed by the same regulatory mechanisms as chromosomal copies of *oriC*, that is, all *oriC* copies replicate coincidentally with cell cycle specificity. Although it might be expected that the copy number of minichromosomes would be equivalent to the number of chromosomal *oriC*s, minichromosomes are defective for equipartition at division (see segregation, below) and are inherited as though they are clumped together. The result is that, at cell division, half the cells receive the majority of minichromosomes, and half receive few or none. After sufficient numbers of generations under selective growth conditions to remove minichromosomes-less cells, minichromosome copy number increases. Regardless of number or location, all *oriC* copies initiate coincidentally, and the cell can support more than 20 copies of *oriC* without perturbing cell growth. Clearly, based on these observations, the mechanism that regulates bacterial chromosome replication is capable of triggering DNA replication without counting the numbers of origins in the cell. In addition, it is clear that this regulatory mechanism, unlike those in eukaryotes, senses neither completion of previously initiated rounds nor cell division. Initiation of DNA replication is coupled to cellular growth rate, and mechanistically must depend on the accumulation of sufficient numbers of at least one *oriC*-binding protein required for initiation (DnaA protein, see below).

Yeast

Bidirectional replication also ensues from origins on eukaryotic chromosomes, although characterization of these sites is a far more difficult task due to the large size of most eukaryotic genomes. *S. cerevisiae* contains 16 linear chromosomes, ranging from 250 to 2000 kb with a total genomic content that is three times larger than that of *E. coli*. Despite the larger amount of DNA, the time required to replicate the yeast genome in rich media is nearly identical to the time needed to duplicate the *E. coli* chromosome. This is because the yeast genome has between 250 and 400 different origins placed 40–90 kb apart (Fig. 1). A number of yeast replication origins are available for study as autonomously replicating sequences (ARSs), but genomic origins are difficult to determine by sequence features alone, and ARS-containing DNA fragments are sufficiently different from one another in that they do not cross hybridize. Despite this fact, common sequence modules are reported (see below).

Unlike the synchronous initiations seen in eubacteria, yeast origins do not all fire at the same time (Fig. 1). Using a combination of density labeling and microarray analysis in *S. cerevisiae*, the majority of origins (332 origins spanning the genome) were observed to fire near mid-S phase, but origins in centromeric regions initiated earlier and telomeric origins initiated later than the majority of mid-S phase initiators. Although adjacent origins tend to fire at the same time, origin-firing times may be separated by as much as 20 min. There also appears to be a preferred range of origin-firing times that is distinctive for each chromosome. This is a remarkable burden to place on the initiation-triggering mechanism because origins that fire late must be identified amidst an ever-increasing background of origins that have already initiated DNA replication. Obviously, reinitiations in any single S phase are not permitted and stringent regulatory mechanisms are required to ensure once-only origin firing each cell cycle.

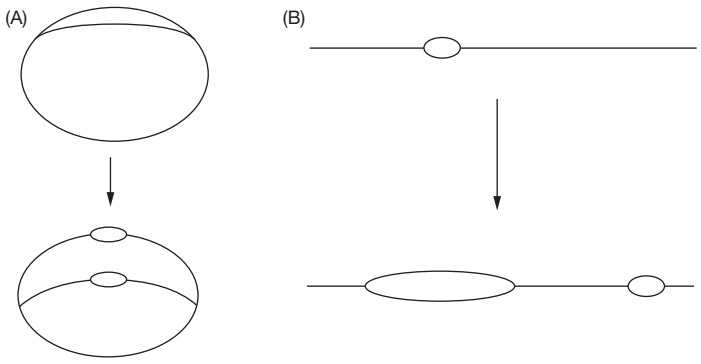

Fig. 1 Multiple origins of replication in bacteria and yeast. (A) In rapidly growing bacteria, multiple origins initiate chromosome replication synchronously. All origins fire before the previous round of replication is completed, resulting in dichotomous replication forks on a single chromosome. (B) In eukaryotic microbes, not all origins initiate synchronously. A single origin functions only once during S phase, and no reinitiations are possible until the cell has gone through mitosis.

It is not yet clear what specific qualities distinguish early firing origins from late firing ones. Clearly, differences in nucleotide sequence could affect the timing of one origin relative to another given the fact that key sequences bind the same regulatory proteins. Alternatively, the relative position of an origin to local chromatin structure and overall nuclear architecture might constrain initiation to mid- or late-S phase.

Archaea

Archaea, the third domain of life, can now be added as a model system for the study of regulatory mechanisms controlling DNA replication. Early studies indicate that although prokaryotic, the archaeal DNA replication machinery contains many attributes similar to those observed in eukaryotic cells. For example, although archaeal replication initiator proteins are not well conserved compared to other domains, they more closely resemble the origin recognition proteins (ORC) of eukaryotes rather than DnaA. Of great interest is the finding that some archaeal genomes, in *Sulfolobus* and haloarchaea, have circular chromosomes that contain multiple replication origins. Although these origins appear to fire synchronously, an initiator protein encoded by an origin-proximal gene regulates each origin independently. The regulatory mechanisms that control the timing of DNA synthesis from these origins are not yet known, although similarities to both bacterial and eukaryotic systems are reported. As more information becomes available, it will be interesting to examine the relationships among the different cell systems and determine the roles played by multiple origins on archaeal chromosomes.

Summary

As is the case for eukaryotes, many prokaryotes (eubacteria and archaea) are capable of triggering DNA synthesis from multiple replication origins, with each origin being restricted to firing only once per cell cycle. In this sense, the regulatory mechanisms governing initiation are fundamentally similar. The mechanisms are different in that yeast origins, unlike eubacterial origins, are not triggered synchronously. This finding suggests that eukaryotic origins exist in both easily triggered and delayed states. Thus, the initiation-triggering system in eukaryotic microbes must remain active for a considerable fraction of S phase. Under these circumstances, it is reasonable to suggest that the number of functional origins on eukaryotic chromosomes is dynamic, increasing or decreasing depending on developmental or physiological conditions that modify origin activity. These modifications may include changes in transcription activity and/or chromatin structure nearby or within origin regions.

Features of Microbial Replication Origins and Origin DNA–Protein Complexes

The primary function of replication origins is to provide regions where double-stranded DNA is easily unwound in response to cell growth-regulated signals. In microbes, these signals are mediated by multiple interactions of one or more specialized proteins with specific recognition sites within the replication origin. Ultimately, protein–DNA interactions produce an unwound DNA substrate suitable for the loading of DNA helicases followed by recruitment of a DNA polymerization machine to copy template strands. Once new replication forks are assembled, the initiation complex must be inactivated/disassembled and the origin reset for the next cell cycle. In all microbes studied thus far, persistent origin recognition protein complexes (ORC) serve as a scaffold for the cell cycle-specific assembly of pre-replicative complexes (pre-RC) that load DNA helicase.

Nucleotide Sequence Features of Replication Origins

What are the features of replication origins that distinguish them from other sites found on microbial genomes? This question is difficult to answer since nucleotide sequence alone does not always designate sites where DNA replication initiates. In *E. coli*, 245 bases are required (Fig. 2), but budding yeast (*S. cerevisiae*) origins vary in length from 100 to 200 bases, and up to 500–1500 bases are required for fission yeast (*Schizosaccharomyces pombe*) origins. However, there are some features that are common among all origins. For example, all microbial origins contain discrete modules that are highly A–T rich and can be easily unwound. These regions are termed DNA unwinding elements (DUEs). In *E. coli*, the DUE, located in the left half of the replication origin (Fig. 2), contains three tandem repeats of a 13 base sequence whose consensus is GATCTNTTNTTTT. Unwinding of *oriC* is restricted to the 13-mer region, with primary unwinding in the rightmost 13-mer. Similar (A–T)-rich regions are found in other eubacteria and archaea, but there are no consistent rules regarding placement of the DUE within prokaryotic replication origins. In budding yeast, analysis of single-strand nuclease hypersensitivity and thermodynamic helical stability indicates the presence of DUEs in the B domain of ARSs (see below). However, there is no identified consensus sequence for yeast DUEs, and information from budding yeast does not extend to other yeast genera.

In addition to DUEs, all microbial origins contain recognition sites for origin-binding proteins. Prior to assembly of replication forks, specific origin-binding protein recognition sites must become occupied and oligomeric complexes must assemble at the correct time during the cell cycle (see below). All bacterial origins contain multiple 9-mer sequences with consensus 5′-TTATC/ACAC/AA-3′. These recognition sites, termed R boxes, have strong affinity for the initiator protein DnaA (see below). In *E. coli oriC*, three R boxes (R1, R2, and R4) are mapped (Fig. 2), but the arrangement and number of R boxes varies among bacterial types (up to 20 DnaA-binding sites are found in some bacterial genera). In many diverse bacterial types, including *E. coli*, *Caulobacter crescentus*,

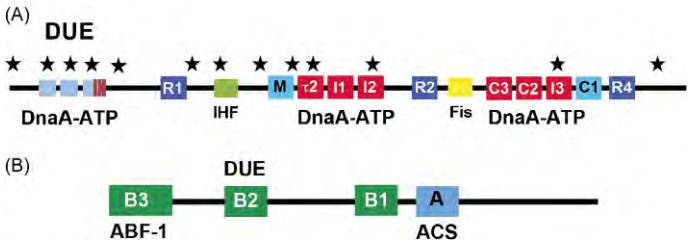

Fig. 2 Structure of replication origins. (A) Schematic map of *oriC*, the *E. coli* replication origin. Relative locations of the consensus sequences for DnaA are shown. The high and low affinity sites (R1–R4, R5(M), C1), which bind DnaA–ATP and DnaA–ADP equivalently, are shown in dark and light blue, respectively. The sites that preferentially bind DnaA-ATP (I1–I3; C2–C3; τ2), are shown in red. The DnaA–ATP sites in the 13-mer repeats, in the region where the origin unwinds (DUE), are shown by *gray* boxes. Fis and IHF binding sites are also indicated in yellow and green, respectively. The positions of the 13 GATC sites are marked by stars. The position of DNA-Trio sites are indicated by dark red rectangles to the left of R1. (B) Schematic map of *S. cerevisiae* ARS1. Relative locations of the A domain (*blue*) containing the ACS sequence, the B domains (*red*), and the DUE and ABF1-binding sites.

and *Mycobacterium tuberculosis*, DnaA does not bind all its recognition sites with equal affinity (K_d varies from ~1 to >50 nmol l^{-1} in *E. coli*) and this feature promotes the ordered assembly of initiation complexes.

Direct observation reveals that, in *E. coli*, DnaA occupies R1, R2, and R4 throughout the cell cycle and any further assembly of pre-RC must be due to low affinity DnaA interactions (see below). However, the occupation of all three sites is required to constrain *oriC* DNA and prevent spontaneous unwinding of the DUE. The ability to constrain is likely to require DNA looping to allow each of the bound DnaA molecules to interact with one another, presumably by Domain 1 interactions (see below). In recent studies, symmetrical arrays of four low affinity DnaA recognition sites were identified in each half of *E. coli* (Fig. 2). These 9-mer recognition sites (I boxes, Tau boxes and C boxes) differ from R box consensus sequence by three or more nucleotides and guide the assembly of DnaA oligomers along *oriC* DNA. Within each array, sites are separated from one another by two base-pairs, placing all the DnaA molecules on the same face of the helix. Each DnaA oligomer is nucleated from a peripheral high affinity R box (R4 and R1), but assembly is regulated so that the right oligomer precedes the left (see below). Unlike R boxes, which bind both the active form of DnaA (DnaA–ATP) and inactive DnaA–ADP with equal affinity, three of the low affinity sites in each array preferentially (fourfold) interact with DnaA-ATP. This binding preference is determined by the nucleotide sequence of these recognition sites and these sites couple pre-RC assembly to the availability of DnaA-ATP. However, mutant versions can be constructed which bind DnaA-ADP at all low affinity sites with equivalent affinity to DnaA-ATP. The DUEs of these versions can be unwound by DnaA-ADP and on chromosomes these origins are functional, but initiation of DNA synthesis is improperly regulated during the cell cycle. It appears that both forms of DnaA are functionally equivalent, but the predominant role of DnaA-ATP is in origin recognition and timing regulation.

Although less extensively studied than *E. coli*, other bacterial replication origins are also known to contain noncanonical DnaA binding sites. While the arrangement of the weak DnaA binding sites differs among bacterial types, low affinity sites tend to be located in the gap regions between high affinity DnaA sites. The number and spacing of weak sites also differs among bacteria, and may be tailored to organism-specific requirements for DnaA oligomerization. Since DnaA-ATP and DnaA-ADP are functionally equivalent in *E. coli* if both forms are permitted equal access to recognition sites, it is possible that DnaA-ADP makes a greater contribution to pre-RC assembly in some bacterial types and these differences are likely to contribute to *oriC* sequence diversity. Interestingly, all DnaA binding sites in origins of slow growing bacteria, such as *C. crescentus* and *H. pylori*, tend to have a lower affinity for DnaA than sites in origins of bacteria capable of rapid growth, such as *E. coli* or *B. subtilis*, suggesting either form of DnaA can be involved in pre-RC assembly.

Oligomers of DnaA-ATP also assemble along the single-stranded DNA of the unwound DUE, and these interactions are guided by distinctive DnaA-Trio motifs (3′-GAT-5′, 3′-AAT-5′, and 3′-GAA-5′, Fig. 2) recently identified in a wide range of bacterial types. DnaA–ATP oligomers within the DUE are proposed to stabilize localized DNA unwinding and assist with helicase loading.

In budding yeast, an 11-bp ARS core sequence (ACS), 5′-A/TTTTATA/GTTTA/T-3′, lies within a highly conserved and essential region called domain A (Fig. 2). The ACS binds ORC, a heterohexamer of Orc1–6 proteins, conserved in all eukaryotes (see below). In ARS1, ACS is not sufficient for origin function, and approximately 100 bp of 3′ DNA is also required. This region is termed domain B (Fig. 2), and there is a great deal of sequence variability in this region among different ARSs isolated to date. The B domain is reported to carry the replication origin's DUE, and is further divided into essential subdomains (B1, B2, and B3), based on linker substitution mutagenesis. Domain B1, nearest to the ACS, is the most highly conserved. ORC binding extends over B1, based on in vivo footprinting experiments. B2 mutants are impaired for loading minichromosome-maintenance (MCM) proteins (see below), the presumptive replicative helicase in yeast. In addition, mutations in the B2 element reduce the frequency of origin firing. The B3 element binds the accessory protein ARS-binding factor (ABF1) (see below).

Although mapped in less detail, archaeal origins contain multiple conserved inverted repeat elements (about 36 bp) termed origin recognition boxes (ORBS) that are required for binding of the initiator Orc1/Cdc6. Smaller versions of ORB repeats termed mini-ORBS, perhaps analogous to the low-affinity initiator recognition sites in bacteria, were also mapped. Although the archaeal initiator proteins appear more related to the eukaryotic ORC proteins than to DnaA, assembly of multimeric complexes of Orc1/Cdc6 on archaeal origins is similar to the formation of DnaA complexes on bacterial *oriC*, mediating origin recognition, origin unwinding, and helicase loading. The presence of Orc1/Cdc6 binding sites of differing affinities (ORBS vs. mini-ORBS) suggests that staged initiator complex assembly is also required in at least some archaeal types to properly initiate DNA replication.

Common Properties of Origin Recognition Proteins

Origin recognition proteins from all domains of life are members of the AAA+ superfamily of ATPases. These proteins carry a nucleotide-binding domain that interacts with ATP and are able to hydrolyze the bound ATP to ADP. Conformational changes that accompany ATP hydrolysis produce a switch-like mechanism to activate and inactivate origin DNA–protein complexes (see below).

DnaA is the initiator in all known bacterial species, with DnaA from *E. coli* being the most extensively studied. DnaA is essential, and only conditional DnaA mutations have been isolated in any bacterial type. *E. coli* DnaA contains 467 amino acids, which are separated into four domains. Domain I is required for loading of DnaB, the replicative helicase, and also plays a role in DnaA oligomerization (a requirement for the assembly of functional pre-RC, see below). Domain II is a poorly conserved linker region. Domain III contains the motifs involved in ATP binding and hydrolysis, and is also required for DnaA oligomerization. DnaA associates with ATP immediately after synthesis and, in *E. coli*, levels of DnaA–ATP increase during the cell cycle prior to the onset of DNA synthesis. Following initiation in *E. coli*, DnaA–ATP is inactivated by a DNA replication-coupled mechanism termed regulatory inactivation of DnaA (RIDA, see below), in which the Hda protein stimulates the hydrolysis of ATP to ADP. Not all bacterial types contain an Hda homolog, and in some, intrinsic hydrolysis of DnaA-ATP is reported to be much faster than observed in *E. coli*, although the significance of this remains to be determined. Domain IV is responsible for DNA binding and possible interaction of DnaA with the cell membrane. In all known bacterial genera, Domains III and IV are highly conserved, and form what is known as the DnaA core. Structural analyses revealed that DnaA-ATP formed right-handed helical filaments capable of binding to, and stretching, single-stranded DNA. The *E. coli* helicase loader protein, DnaC, also binds to the end of the filament. Other forms of DnaA oligomers are also likely to exist, however, since Domain IV in the helical DnaA-ATP filament is folded back to make contact with residues in Domain III, and is not available for double-stranded DNA binding. The ability to assemble different complexes on single and double-stranded DNA could allow DnaA to promote origin unwinding as well as stabilize the unwound DUE as directed by the DnaA-Trio sequences.

In budding yeast, functional ORC is a heterohexamer of Orc1–6 proteins, with Orc1–5 being essential for site-specific DNA binding. Although five ORC subunits are reported to be AAA+ proteins capable of interacting with ATP, only the association of ATP with Orc1 is required to bind DNA, and binding to DNA inhibits the intrinsic ATPase activity of Orc1. Orc1, Orc4, and Cdc6 (see below) also carry a conserved winged-helix domain that is necessary for DNA binding, and perhaps is also used for protein–protein interactions within the ORC.

Although yeast ORC resides at origins throughout the cell cycle, some ORC subunits are specifically phosphorylated during the cell cycle by S-phase cyclin-dependent kinases. In addition, one ORC subunit may play a role in chromosome segregation. Orc6 associates with nuclear chromosomes during the cell cycle, but is reported to bind kinetochores during mitosis and promote cytokinesis (see below). ORC is reported to be limited in living *S. cerevisiae* (about one ORC complex per origin).

The AAA+ domains of eukaryotic Orc proteins and archaeal Orc1/Cdc6 are reported to contain conserved structural elements similar to those found in the DnaA core that may promote helical structure formation. In fact, a helical pentamer of DnaA can be docked within ORC at a position dominated by Orc5. This result suggests that helical structures may be a feature of all origin-recognition proteins.

Additional Proteins Associating With Bacterial and Yeast Origins

DnaA is not the only protein that associates with bacterial *oriC*. While the catalog of associated proteins is large and not conserved among all bacterial types, the associated proteins seem to use only a few common strategies to regulate formation of initiation complexes: modulation of low affinity DnaA binding, promoting or inhibiting DnaA oligomerization, and/or changing the conformation of *oriC*. In both *E. coli* and *H. pylori*, as well as other proteobacteria, DiaA/HobA promotes DnaA oligomerization and cooperative binding during initiation complex assembly. Interestingly, while loss of DiaA/HobA is tolerated in *E. coli* cells, it is required in *H. pylori*. The reason for this is not clear, but may be related to the generally reduced affinity of DnaA binding in *H. pylori*. Factors that associate with DnaA to reduce cooperative DnaA binding, such as YabA, DnaD, SirA, and Soj have also been identified in *B. subtilis*. Associated proteins that block DnaA's access to low affinity DnaA binding sites have been identified in *E. coli*, *C. crescentus*, *M. tuberculosis*, and *H. pylori*; these include SeqA (see below), CtrA, MtrA, and HP1021, respectively. Additionally, DNA bending proteins bind to replication origins of several bacterial types, changing the DNA conformation and modulating DnaA binding. For bacteria with bipartite origins, bending proteins may be needed to bring the two halves of the origin together, although further study is required to determine if this is the case. In *E. coli*, two histone-like bending proteins, integration host factor (IHF) and factor for inversion stimulation (Fis), specifically bind *oriC* (Fig. 2). IHF has also been shown to bind to the origins of other bacteria, including *C. crescentus*. In *E. coli*, Fis and IHF are not required for *oriC* function but appear to facilitate efficient synchronous assembly of the nucleoprotein complex that triggers initiation. IHF stimulates the binding of DnaA to low affinity recognition sites, while Fis inhibits such binding. It appears that conversion from a Fis-bound ORC to an IHF-bound pre-RC serves as a molecular switch to limit the time window required to trigger new rounds of DNA replication. Since Fis is also a growth rate-regulated protein with highest levels measured during rapid growth conditions, it remains to be determined whether this switch is required for initiation synchrony during rapid growth or whether the absence of Fis under slow growth conditions alters the assembly of the pre-RC.

For yeast ARS1, an accessory protein, ABF1, binds to the B3 subdomain. ABF1 is a multifunctional protein analogous to Fis or IHF in that it is involved in transcription regulation and is not absolutely required for either ORC binding or ARS function. Like IHF, ABF1 acts as an enhancer of origin function by producing changes in DNA conformation.

Prereplicative Complex (Pre-RC) Assembly in *E. coli* and Budding Yeast

The assembly of pre-RC is a dynamic, multistep process that is critically timed during the cell cycle. The process has been most extensively studied in *E. coli* and *S. cerevisiae*. In both of these organisms, ORCs, persisting throughout most of the cell cycle, are converted into pre-RC to license origins for DNA replication, and then reset to ORC after initiation (see below and Fig. 3).

In *E. coli*, *oriC* is bound to DnaA at only R1, R2, and R4 throughout the majority of the cell cycle, in a complex that is functionally and temporally analogous to yeast ORC. During rapid growth conditions, Fis is associated with the *E. coli* ORC and inhibits pre-RC assembly (Fig. 3). However, as new DnaA–ATP is synthesized during the cell cycle, it accumulates at *oriC* and three events, which result in pre-RC formation, can be measured: (1) Fis is displaced from its site; (2) IHF binds to its site between R1 and R2; and (3) IHF binding stimulates DnaA binding to the remaining lower affinity sites (Fig. 3). IHF binding and filling of the weaker DnaA sites forms the pre-RC, and results in origin unwinding in the DUE.

During unwinding, DnaA protein also interacts directly with the single-stranded 13-mer region in the DUE, and recruits DnaB–DnaC complex to the origin. The AAA+ protein DnaC is required to load the DnaB helicase hexamer (Fig. 3). DNA polymerase is then recruited to form the replication fork.

In budding yeast, a stable complex of Orc1–6 proteins and ABF1 binds to origins immediately after initiation, and remains bound throughout the cell cycle (Fig. 3). Formation of the pre-RC is accomplished during late mitosis/G1 and assembly of this complex licenses origins for helicase loading and new DNA synthesis. Licensing is possible only when cyclin-dependent kinase (CDK) activity is low, but high CDK activity is subsequently required to trigger movement of replication forks (see below).

A key step in converting ORC into pre-RC is the association of Cdc6, apparently via interaction with Orc1. Cdc6 is a highly conserved AAA+ protein with strong amino acid homology to Orc1. ORC and Cdc6 bind cooperatively to origin DNA and this binding is dependent on Orc1's ATPase activity. Cdc6 expression is cell cycle-regulated, with mRNA levels peaking at mitosis. Phosphorylation by CDKs causes rapid degradation of Cdc6 protein at G1/S transition. Temperature-sensitive *cdc6* mutant strains cannot progress to S phase at nonpermissive temperature, and overexpression of Cdc6 causes origin rereplication within a single cell cycle.

ATP hydrolysis by both ORC and Cdc6 is required to proceed with loading of an oligomeric ring-like complex of six different MCM proteins (Mcm2-7). MCMs are conserved among eukaryotes and each carries a 240 amino acid region similar to DNA-dependent ATPases. The MCM complex is the best candidate for the replicative helicase. A loading factor, Cdt1, forms a complex with Mcm2-7, and then this complex associates with ORC/Cdc6 (Fig. 3). Intranuclear levels of both MCM proteins and Cdt1 are

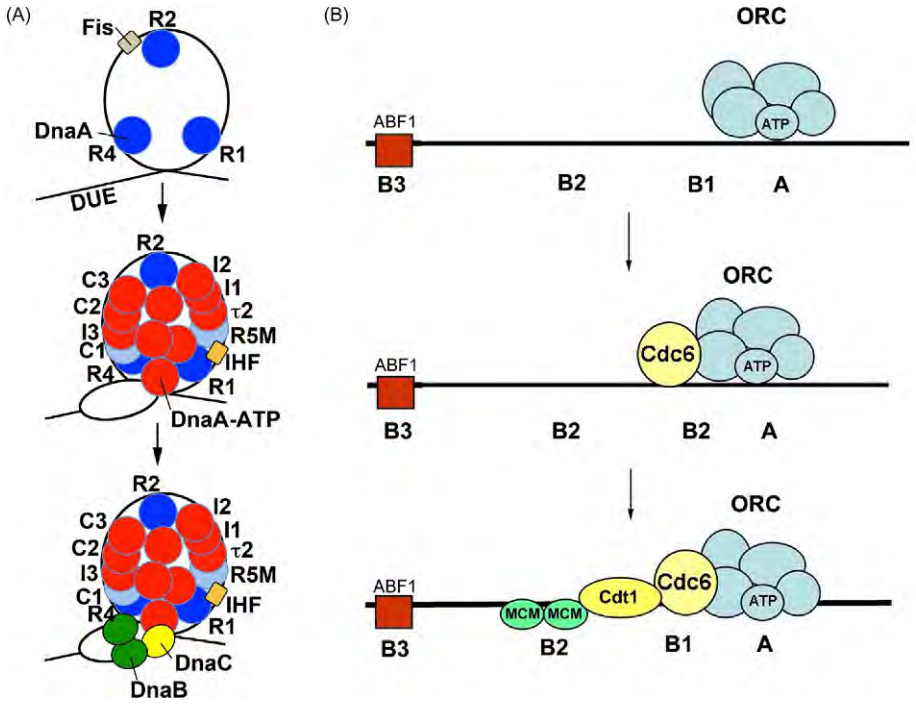

Fig. 3 Multistep assembly of nucleoprotein complexes at replication origins. (A) Formation of pre-RC in *E. coli*. *E. coli oriC* is maintained throughout most of the cell cycle with DnaA (either nucleotide form; *blue*) bound to only R1, R2, and R4, with Fis binding adjacent to R2 in rapidly growing cells. Immediately before DNA replication initiates, the remaining, lower affinity sites are filled; this step requires DnaA–ATP (*red*) although R5M and C1 bind equally well to DnaA-ADP or DnaA-ATP. Additionally, Fis is displaced, and IHF binds between R1 and R5M. The DNA strands in the DUE then separate, and are stabilized by binding of DnaA–ATP in the DnaA-Trio region (shown in Fig. 2). Finally, DnaC loads DnaB onto the single-stranded DNA. (B) Formation of pre-RC in budding yeast. ARS1 is bound throughout the cell cycle to ORC in the A domain, overlapping the B1 domain. ABF1 may bind the B3 domain as the pre-RC assembles. When CDK levels are low, Cdc6 is recruited to ORC. After Cdc6 binds, Cdt1 and MCM proteins are recruited, and MCM proteins are loaded onto the B2 domain, forming the pre-RC.

regulated so that these factors are only available in the nucleus during G1; they are retained in the cytoplasm during the remainder of the cell cycle. Surprisingly, multiple copies of Mcm2-7 are loaded at individual origins. The reason for this is unclear, but MCMs may be required at considerable distance from the origin, and the MCM complex moves with replication forks.

In yeast, loading of the MCMs completes pre-RC assembly, but several additional steps are required to convert Mcm2-7 in the pre-RC into an active DNA unwinding complex that allows cells to exit G1 and initiate DNA replication in S phase. These steps require high levels of CDK activity. First, a cell cycle-specific protein kinase, termed Cdc7-Dbf4, activates MCMs via phosphorylation. Second, additional factors must interact with phosphorylated MCMs; these include Cdc45, the GINS complex comprising four proteins (Sld5, Psf1, Psf2, and Psf3), and a complex termed 11-3-2 comprising Dpb11, Sld2, and Sld3. An S-specific CDK directly phosphorylates members of 11-3-2 prior to their association with MCMs and constitutes a key switch mechanism for the G1- to S-phase transition. The ultimate large complex of Cdc45-Mcm2-7-GINS, referred to as CMG, is the DNA-unwinding proficient version of the eukaryotic replicative helicase.

Limiting Initiation to Once per Cell Cycle and Replication Origin Resetting

Replication origins may be utilized once during each cell cycle, but never more than once. All cell systems studied thus far use multiple and redundant mechanisms to ensure the stringency of this rule. Most of these mechanisms focus on preventing reformation of the pre-RC. One way to do this is to regulate levels of proteins necessary to assemble pre-RCs; an example of this is degradation of Cdc6 at the G1/S transition. Another option is to modulate the activity of pre-RC proteins in a cell cycle-specific fashion. For example, once utilized, pre-RC proteins of the AAA+ family may be directly inactivated by ATP hydrolysis. Spurious reassembly of initiator complexes may also be prohibited by a variety of blocking agents. Finally, initiator proteins may be titrated away from origin sites by directing them to alternative DNA sites in prokaryotes, or exporting them from the nucleus in eukaryotes.

E. coli

To properly regulate timing of DNA synthesis, DnaA must be synthesized de novo each cell cycle. DNA replication begins when enough DnaA–ATP has accumulated to unwind the origin DNA and recruit the replication machinery. After replication forks begin their journey, rebinding of any remaining free DnaA–ATP to *oriC* must be prohibited to prevent reforming the pre-RC. At least three different mechanisms have been identified in *E. coli* to accomplish this goal (Fig. 4). First, hydrolysis of active DnaA–ATP to inactive DnaA–ADP is stimulated by a DNA replication-dependent mechanism termed RIDA. RIDA requires the β-subunit of DNA polymerase III holoenzyme complexed with a cofactor termed Hda. Presumably, any DnaA–ATP bound to DNA that contacts the replication fork during ongoing DNA replication will be inactivated. In particular, sites that bind large amount of DnaA (e.g. the *datA* locus) may be "hot-spots" for DnaA-ATP inactivation. Hda is not widely conserved among bacterial genera, and in some bacteria, such as *B. subtilis*, the rate of spontaneous DnaA-ATP hydrolysis may remove the requirement for this protein. *B. subtilis* cells do contain factors that limit DnaA's activity through means other than hydrolysis; one of these, YabA, associates with the β-clamp and limits cooperative DnaA binding by means that are not fully understood.

Although fork movement is probably responsible for the bulk of DnaA inactivation, it is unlikely that sufficient DnaA-ATP can be inactivated immediately after initiation to prevent reformation of the pre-RC. A second mechanism of downregulation involves blocking initiator protein access to *oriC*. In *E. coli*, *oriC* is blocked by a DNA-binding protein, SeqA, which recognizes and binds to hemimethylated GATC sequences, which are produced by replication of *oriC*. *E. coli*'s *oriC* contains 13 GATC sites (more than would be expected in a 245 bp region; see Fig. 2), and the placement of eight of these sites is conserved among enterobacterial origins. Normally, deoxyadenosine methyltransferase (Dam methylase) would fully methylate the palindromic hemimethylated GATC sequences shortly after they are formed. However, SeqA appears to "sequester" *oriC* from Dam methylase and other proteins,

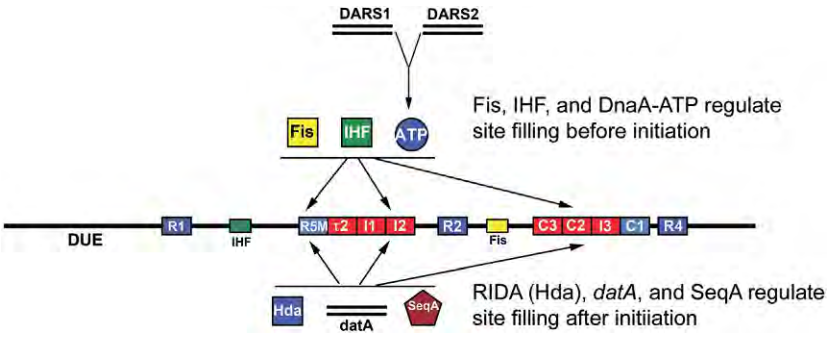

Fig. 4 Regulation of pre-RC formation in *E. coli*. In *E. coli*, multiple mechanisms target low-affinity DnaA-binding sites to regulate formation of pre-RC before and after initiation. Before initiation, Fis, IHF, and DnaA–ATP regulate binding to low affinity sites to ensure proper initiation timing. After initiation, inactivation of DnaA–ATP by RIDA, blocking of weak sites by SeqA, and titration of DnaA by the *datA* locus prevent reinitiation by inhibiting reformation of the pre-RC. Inactive DnaA-ADP is recharged to DnaA-ATP by interacting with DARS.

including DnaA, for approximately one-third of a generation. In this sequestered state, a new round of replication cannot be triggered. Mutant strains deficient in either SeqA or Dam methylase initiate more than one round of DNA replication per cell cycle. The GATC binding sites for SeqA overlap several lower affinity DnaA binding sites (Fig. 4). By targeting only the weak sites, SeqA specifically blocks pre-RC assembly, but permits DnaA loading at strong sites R1, R2, and R4 to reset the origin immediately after initiation. While SeqA is not a conserved protein, other bacterial types use a similar blocking strategy to inhibit initiations. In *Caulobacter* species, the global regulator CtrA binds to *oriC* and blocks at least one key DnaA binding site. In *M. tuberculosis*, the inhibitory protein MtrA also binds to *oriC* in regions that overlap DnaA recognition sites.

The third mechanism to downregulate pre-RC reformation depends on the duplication of a high-capacity DnaA titration site downstream from *oriC*. This site, termed *datA*, is reported to bind as many as 370 molecules of DnaA. Consequently, duplication of *datA*, approximately 8 min after DNA synthesis initiation, provides a cell cycle-specific mechanism to reduce the availability of DnaA during the sequestration period, so that there is not enough free DnaA to reassemble pre-RC when *oriC* loses its SeqA blocker (Fig. 4). *DatA* also likely provides a reservoir of DnaA for inactivation by RIDA.

Upregulation also exists. Under fast growth conditions where sufficient DnaA-ATP may not be synthesized de novo, *E. coli* is able to reactivate DnaA-ADP to active DnaA-ATP. The reactivation mechanism is based on specific nucleotide sequence motifs termed DnaA Reactivating Sequences (DARS) located at two different sites on the genome (DARS1 and DARS2 (Fig. 4). Although the exact mechanism for reactivation remains obscure, DARS both carry three DnaA recognition sites. The activity of DARS2 requires the interaction of DNA bending proteins Fis and IHF, suggesting that a specific DNA conformation is required for the reactivation of DARS-bound DnaA.

Yeast

In yeast, pre-RC formation must also be prohibited following the onset of S phase during each cell cycle. Since the eukaryotic cell cycle is driven by fluctuations in the levels of cyclins, it is not surprising that CDKs and their regulator, anaphase-promoting complex (APC), play key roles in restricting origin-firing to once and only once per cycle. Multiple CDK-regulated mechanisms act on the components of pre-RC, analogous to multiple mechanisms reducing pre-RC formation in prokaryotes. For example, Orc2 is directly phosphorylated by CDKs in late G1, S, G2, and M phases to inhibit its activity. Additionally, association of Cdc6 with Orc1 absolutely depends on low CDK activity, since phosphorylation, triggered by a rise in CDK levels at the end of G1, targets Cdc6 for degradation by ubiquitin-mediated proteolysis. Transcription of new Cdc6 is also reduced by CDK activity. The combination of reduced transcription and proteolysis results in Cdc6 levels that are insufficient to form the pre-RC. Other components of pre-RC are also targeted after initiation to prevent origin licensing. Mcm2-7, associated with Cdt1, normally enters the nucleus at the end of mitosis and remains in this location during G1. At the G1/S interface, rising CDK-dependent phosphorylation targets MCMs and Cdt1 for nuclear export, making them unavailable to reform active pre-RC.

All CDK-dependent inhibitions of origin licensing are eliminated by the increase in APC from late mitosis through early G1. APC is a ubiquitin ligase that begins the process of CDK proteolysis. After the formation of pre-RC, APC is inactivated to allow CDK levels to again rise, so that a new round of DNA synthesis can begin.

Summary

Both prokaryotic and eukaryotic microbes control initiation of chromosome replication by multistep, ordered assembly of a nucleoprotein complex at an origin of replication. *Re*initiation is prevented by mechanisms that inhibit reformation of the pre-RC. In bacteria, the rate at which the initiator protein DnaA accumulates at the origin determines the frequency of initiation, and cell cycle-specific expression of *oriC*-binding proteins is not necessary for proper initiation timing. Inactivation of DnaA, blocking of DnaA binding sites to prevent pre-RC reassembly, and titration of DnaA away from *oriC*, prohibit reinitiation events in the critical time following origin firing. In contrast, yeast cells contain cell cycle-specific kinases that regulate all aspects of origin function, such that key components are present and/or functional during a discrete time period of the cell cycle. The processes of initiation, chromosome replication, and segregation are separated temporally, and this separation is governed by a series of molecular checkpoints. With such a sophisticated regulatory network in place, it is not surprising that the yeast cell rarely commits an error. In fast-growing *E. coli*, DNA replication and segregation are ongoing when new rounds of replication initiate from *oriC*. If bacteria are to maintain the survival advantages resulting from rapid growth, they apparently cannot afford the luxury of restricting times that DNA may replicate during the cell cycle. Instead, bacterial cells have evolved regulatory mechanisms that are coupled to DNA replication but not to any other specific events during the cell cycle including cell division. Although this form of regulation is more streamlined than that found in eukaryotic microbes, it is no less precise.

Chromosome Segregation Systems in Prokaryotic and Eukaryotic Microbes

After successfully replicating their genomes, microbes must ensure that each new daughter cell receives the correct complement of chromosomes after cell division. Mechanistically, this is not a trivial process and there are obviously many steps where errors could lead to the production of nonviable cells. A successful segregation system requires several components: (1) a mechanism to identify the location of each nascent daughter chromosome, (2) a means for physical attachment to chromosome copies, and (3) a polarized

motor activity to pull chromosome copies into separate physical spaces destined to become new cells. During mitosis in eukaryotic microbes, structures for physical separation of chromosomes, the spindle pole bodies (SPB), are visible with the aid of a microscope. The vehicle for segregation is not so obvious in bacteria. Bacteria contain few visible intracellular structures and, until recently, there has been no evidence of centromeres (see below); little information on intracellular organization of segregating chromosomes is available. In addition, the segregation mechanism in bacteria must, under rapid growth conditions, be functional while DNA replication is ongoing. Because of these clear differences, it was expected that bacteria and yeast would segregate their chromosomes via markedly different mechanisms. However, there is now evidence that active mechanisms exist to precisely localize replication origins and distribute newly replicated genetic loci in bacteria. Current data suggest that in some ways, eukaryotic and bacterial microbes use similar strategies to segregate chromosomes. Furthermore, studies on bacterial chromosome segregation clearly show that profound spatial organization of the genome and mechanisms must exist to maintain this organization during a progressive segregation process.

Segregating Chromosomes in Budding Yeast

Prior to the onset of chromosome replication, specialized proteins, called cohesins, are loaded onto yeast chromosomes. After S phase, these cohesins pair and hold together the newly replicated sister chromatids (Fig. 5). Then, as cells enter mitosis, the yeast chromosomes are condensed by the action of a variety of proteins, including the condensin complexes, which makes the chromosomes more rigid and compact. The condensation process requires factors associated with the centromere. In mitosis, the kinetochores of the paired, compact chromosomes attach to the spindle, comprising interdigitated microtubules emanating from the SPB. This process is regulated to ensure that there is bipolar (amphitelic) attachment of microtubules to each sister chromatid's kinetochore. This amphitelic attachments cause tension between the sister chromatids, as kinetochore motor proteins try to separate the attached chromosomes. As the cells enter anaphase, the cohesins holding sister chromatids together are synchronously dissolved, allowing the motor proteins to segregate the chromosomes to opposite poles. Although this scenario seems simple, the molecular machinery of segregation in yeast is complex. It must include protein–DNA interactions that control cohesion and condensation of sister chromatids, as well as the assembly of a multicomponent DNA–protein structure, termed the kinetochore, that associates microtubules and motor proteins with centromere DNA. Failure to assemble or disassemble these components of the segregation system results in cell cycle arrest.

The linkage of sister chromatids by cohesins is beneficial, since it obviates the need to localize each sister chromatid individually before bipolar attachment to spindle microtubules. Initially, the replication process generates intertwining of DNA and produces physical linkage of sister chromatids. These DNA catenanes are removed by DNA topoisomerase II, which is part of the condensed

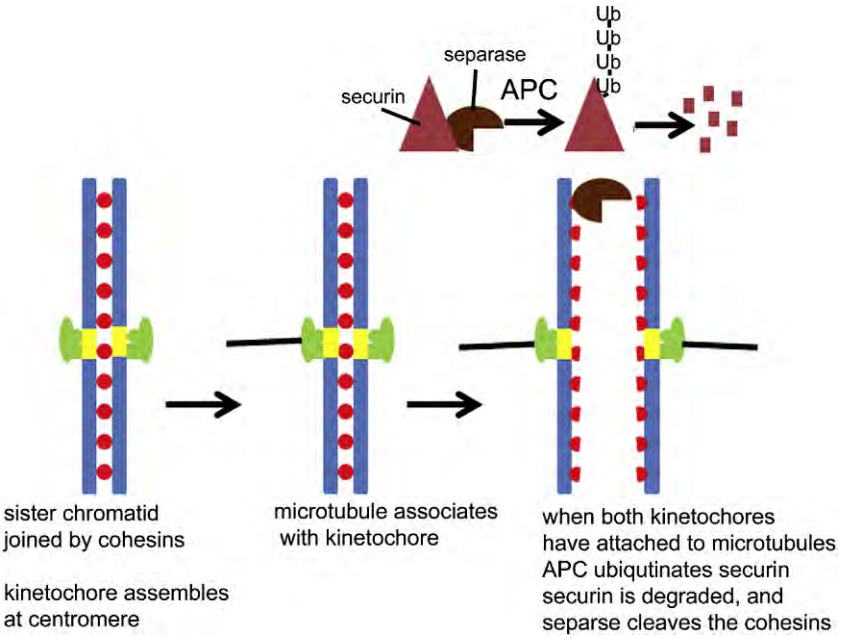

Fig. 5 Separation of sister chromatids in yeast. After chromosomes (*blue*) are replicated, they are paired by cohesion complexes (*red*). Kinetochores (*green*) assemble at the centromeres (*yellow*) of the sister chromatids. Microtubules emanating from the SPB bind to the kinetochore. Cohesion is maintained until both the kinetochores of sister chromatids are attached to separate microtubules. Then, APC ubiquitinates securin (rust *triangle*), triggering it for degradation and releasing separase (*brown*). Separase cleaves the cohesion complex, and the sister chromosomes are separated by motor protein activity associated with the kinetochores and microtubules.

chromosome scaffold. However, the cohesins, loaded prior to chromosome replication, ensure that sister chromatids do not separate until anaphase.

In *S. cerevisiae*, a cohesion complex comprises four proteins: Smc1, Smc3, Scc1/Mcd1, and Scc3. SMC (structural maintenance of chromosomes) proteins are conserved among some bacteria and most eukaryotes, including humans. Smc1 and Smc3 carry hinge and long-coiled coil regions that assemble into a V-shaped heterodimer with an ATP-binding motif. The V-shaped heterodimer is closed into a ring by the specific binding of Scc proteins to the globular heads of the Smc proteins. Once assembled, the cohesion complexes are loaded onto chromosomes with the assistance of Scc2 and Scc4 and the passage of the replication fork. Loading requires an ATPase activity associated with Smcs. The nucleosome remodeling mechanism in yeast is also involved since RSC (Remodels the Structure of Chromatin complex) recruits Scc2-Scc4 to nucleosome-free regions of the chromosome. Cohesion is maintained until anaphase begins, and this allows sufficient time for the kinetochore to be attached to the spindle (Fig. 5).

Kinetochores assemble via specific protein–DNA interactions at centromeres. The centromeres of *S. cerevisiae* chromosomes are quite small (120 bp) relative to other eukaryotic centromeres (which are contained in megabase domains of repetitive DNA). The *S. cerevisiae* centromere consists of three nucleotide sequence elements, termed CDEI, CDEII, and CDEIII. CDEI is an 8 bp sequence conserved among all yeast chromosomes. CDEII is a 78–86 bp, >90% (A–T)-rich sequence that separates CDEI and CDEIII. CDEIII is a conserved 24 bp sequence. CDEI and CDEIII are essential for chromosome segregation, since deletion of CDEI increases chromosome loss 10-fold and deletion or point mutations of CDEIII destroy centromere function.

In contrast to the centromere sequence, centromere-binding proteins of *S. cerevisiae* show little similarity to analogous proteins in other yeasts and higher eukaryotes. However, in all cases, kinetochores are large protein assemblies. In *S. cerevisiae*, kinetochores comprise 60 different subunits arranged in layers as 14 discernable complexes. A four-protein inner complex, termed Cbf3, binds specifically to CDEIII and is key for initiating kinetochore assembly. After binding to CDEIII, one of the Cbf3 proteins, p58, is activated by phosphorylation and later degraded by ubiquitin-mediated proteolysis. By this mechanism, assembly of the kinetochore can be limited to one per chromatid and assembly can be restricted to a limited time during the cell cycle.

The kinetochore links spindle microtubules to sister chromatids (Fig. 5). The spindle microtubules emanate from the SPB, which is embedded in the nuclear membrane throughout the cell cycle. Duplication of the SPB takes place prior to the onset of S phase in *S. cerevisiae*, and is facilitated by RSC-dependent distribution of nuclear membrane insertion factors Nbp1 and Nde1 to each new SPB. Surprisingly, the spindle (SPB with microtubules) first appears during S phase in budding yeast, suggesting that chromosome replication and spindle assembly comprise two separate regulatory pathways. Spindle elongation and subsequent chromatid separation requires kinesin-related proteins Kip1 and Cin8 and a dynein-related protein Dyn1 to provide both pushing and pulling forces. There is also evidence for mechanochemical motor activity associated with the kinetochore.

In budding yeast, each kinetochore captures only one independent microtubule. A mechanism to detect improperly oriented attachments exists, and is dependent on the ability of proteins in the kinetochore to sense tension generated by the action of motor proteins trying to separate chromosomes held together by cohesins. Attempts to reattach are continued until the correct orientation is achieved. It is likely that only one of the sister chromatids initially becomes attached to a spindle microtubule. The attachment, however, causes the movement of both chromatids, which oscillate as the microtubule grows and shrinks. This oscillation may prevent the remaining kinetochore from attaching to other microtubules from the same SPB. Only binding of the remaining kinetochore to a microtubule from the opposite SPB can produce the bidirectional force required to trigger dissolution of cohesions holding the chromatids together (Fig. 5). Since the release of cohesion during the cell cycle is such a dramatic event, there are several checkpoint proteins complexed with the kinetochore to monitor the events in early mitosis and inhibit chromosome segregation until all chromatids are properly attached to microtubules.

The loss of cohesion is synchronous and very tightly regulated. Scc1 is cleaved by a cysteine protease termed separase (Esp1), whose activity is held in check by a chaperone protein called securin. Securin is degraded by APC to release active separase only in anaphase (Fig. 5). When Scc1 is cleaved, the linkage holding the sister chromatids together is broken, and the chromosomes are segregated by the motor forces in the microtubules and kinetochore.

Origin Localization and Chromosome Segregation in Prokaryotes

Little obvious structural organization within the cytoplasm of prokaryotes has been observed in *E. coli* or any other eubacteria, and there is certainly no structure equivalent to the mitotic spindle segregation system. However, bacterial cells must have a highly effective equipartitioning system to segregate sister chromosomes, since anucleate cells are rarely produced. This perplexing situation resulted in models that included the cell surface as part of the segregation machinery. The 1963 Replicon hypothesis proposed by Jacob, Brenner, and Cuzin suggested that the replication origin could act as centromere, and the sites at which the origin attached to the membrane could function as an SPB (centrosome) in *E. coli*. Although the physical force separating bacterial chromosomes has yet to be identified, it is clear that bacteria contain mechanisms to accurately relocate newly replicated chromosomal origins to specific intracellular sites followed by ordered, progressive segregation of duplicated loci in a way that maintains the orientation of segregated chromosomes into newly divided daughters (Fig. 6).

Most information on the intracellular position and movement of genomic loci has come from *E. coli*, *B. subtilis*, and *C. crescentus*, using several elegant methods. One approach, termed fluorescent repressor operator system (FROS), involves inserting DNA cassettes containing multiple copies of the *lac* operator into the chromosome at the genetic location to be tracked. Time lapse microscopy then follows the intracellular location of green fluorescent protein (GFP) linked to Lac repressor, which binds to the cassettes. An alternate approach is fluorescence in situ hybridization (FISH), in which fluorescently labeled DNA probes for specific

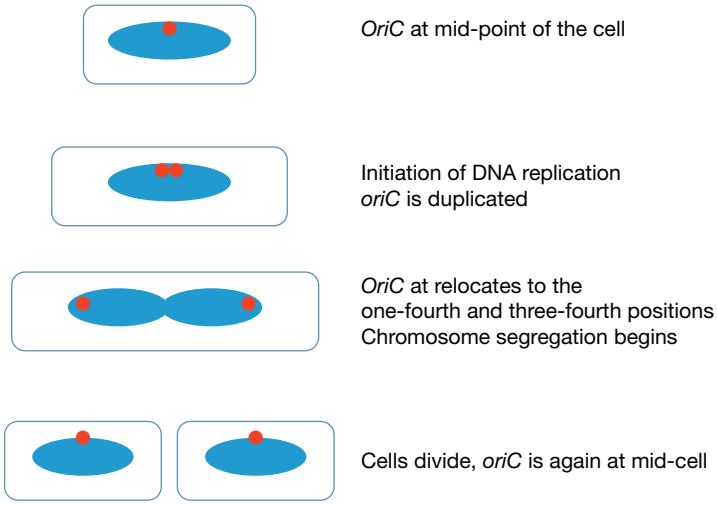

Fig. 6 Localization of *oriC* during the cell cycle. Schematic diagram of the localization of *oriC* in *E. coli* or *B. subtilis* during the cell cycle. *OriC* is at mid-cell in newly divided cells, and remains there until DNA replication initiation. After duplication of *oriC*, the two origins are actively relocated to positions that are one-fourth and three-fourth the length of the cell. As the chromosome is replicated, the sister chromosomes are also separated into each half of the cell. After cell division, each daughter cell has a complete chromosome, and *oriC* is again located at mid-cell.

loci are hybridized to fixed cells on microscope slides. Partition proteins have also been localized during the cell cycle using fluorescent antibodies as in situ probes.

Bacterial replication origins are positioned at specific cellular locations prior to the onset of DNA synthesis (polar in *C. crescentus* and near mid-cell in *E. coli* and *B. subtilis*; see Fig. 6). How the correct placement of *oriC* is achieved in most bacteria remains to be determined, but it is likely that the origin localization mechanisms will vary depending on cell shape and life style. After DNA synthesis initiates, duplicated origins are actively separated to the one-fourth and three-fourth cell position in *E. coli* and *B. subtilis* (Fig. 6) or to opposite poles in *C. crescentus*. There have been various proposals to explain how the force acting on replication origins is generated; it has been suggested to be derived from the replication process itself, by a mechanism related to transcription, to the synthesis of membrane components between newly replicated origins, or by a dynamic change in nucleoid structure (see below). While none of these mechanisms can be ruled out and perhaps several will act cooperatively, origin translocation across cellular space is unusually rapid, raising intriguing issues about the existence of kinetochore-like components that specifically act on origin regions to determine their position in cells and move them apart during the replication process.

Several candidate components of a bacterial kinetochore are described in the most widely studied bacterial systems. Two related systems were uncovered in sporulating *B. subtilis*. During sporulation, an origin-localizing protein, RacA, targets *oriC* to cell poles after binding preferentially to 25 regions spread over 612 kb across the origin portion of the chromosome. RacA has the ability to condense chromosomal DNA. *B. subtilis* also contains chromosomally encoded proteins that are similar to members of the Par family, partition proteins encoded by plasmids. Most plasmid systems encode two interacting Par proteins (ParA, B) and carry a *cis*-acting centromere-like element (*parS*). In most plasmid systems, ParA is an ATPase, and ParB binds directly to *parS*. In *B. subtilis*, Spo0J is a homolog of the plasmid-partitioning protein ParB, binding to at least eight sites spread over 800 kb in the *B. subtilis oriC* proximal region. Loss of Spo0J leads to 100-fold increase in anucleate cells. Spo0J also plays a role in origin positioning in the forespore, suggesting redundancy in *B. subtilis* kinetochore systems. Par family proteins also exist in *C. crescentus*, with a role in preventing anucleate cells, but an actin-like protein called MreB is thought to direct origin movement, without affecting other chromosomal loci. MreB appears to associate directly with origin proximal DNA and forms a spiral corkscrew on the cell surface that could serve as a track for origin movement. MreB is also present in *E. coli* and *B. subtilis* where it has been implicated in determining cell shape as well as playing a role in chromosome segregation. Additional candidate components include the bacterial members of the SMC family of proteins. Mutations in the *smc* genes of *B. subtilis* and the SMC-like Muk complex (encoded by *mukB*, *E*, and *F*) in *E. coli* produce less condensed nucleoids and anucleate cells. These complexes are expected to play a role in chromosome compaction, but it was recently shown that in both bacterial types the SMC proteins are also responsible for correctly separating and positioning new *oriC* copies.

It remains unclear whether the forces responsible for separating origins are also responsible for segregating the bulk of chromosomal DNA. Perhaps the moving force is found in the nucleoid itself. All recent studies show clearly that the nucleoid is well organized and exhibits a highly regulated spatial location in cells during replication and partitioning of daughter chromosomes. Based on studies in *C. crescentus* and *E. coli*, replication divides the circular genome into two distinct and equal arms termed replicores that occupy separate halves of the cells. This spatial orientation is maintained generation after generation by the sequential layering of newly replicated DNA to the inner and outer edges of developing nucleoids. Recent studies clearly show that the nucleoid is a remarkably dynamic structure undergoing rapid fluctuations in density, and several abrupt global reorganizations. These mechanical changes may be sufficient to drive nucleoids apart during the cell cycle without the need for any

additional motors. If this is correct, perhaps the segregation mechanism will only require guiding proteins, like those described above, to orient the DNA as it is replicated.

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Circadian Rhythmicity in Prokaryotes

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Glossary

Constant light (LL) A condition where organisms are held in continuous light, i.e. no dark period is given to the cells. After entrainment, cyanobacterial cells are released into LL to observe the free-running rhythm.
Diazotrophic Refers to organisms that are capable of fixing atmospheric nitrogen into ammonia.
Diel Cycle A 24-h period consisting of alternating light (day) and dark (night) conditions.
Entrainment A property of circadian rhythms in which the clock can be reset by external cues, thus establishing a particular phase relationship with the external environment. In typical experimental conditions we entrain the samples to an LD 12:12 cycle so that peak expression of reporter genes occurs at a particular time, dusk or dawn, relative to the provided LD cycle.
Free-running period (FRP) The periodicity of the rhythm in constant conditions. The typical FRP is approximately, but not exactly, 24-h.
Hourglass timer A timing system that once triggered can execute a temporal program. However, the program does not persist in constant conditions and cannot reinitiate a new cycle without environmental input.
Light-Dark (LD) Environmental cycle consisting of alternating periods of light and dark. LD 12:12, also referred to as a diel cycle, would indicate having the lights on for 12 h followed by 12 h in darkness.
Persistence A property of circadian rhythms meaning that the rhythm continues in the absence of environmental cues. Persistence is often demonstrated by placing organisms in either constant light or constant dark conditions and monitoring for sustained rhythmicity.
Temperature compensation A property of circadian rhythms in which the periodicity of the circadian clock remains ~24-h over a range of biological temperatures. In contrast to most enzymatic activities, for which reaction rates increase 2–3 fold with a 10°C increase in temperature, the circadian clock is buffered or compensated against changes in temperature that occur over the course of the day, and shows less than 10% change in period over that range.
Transcription-translation feedback loops (TTFL) A negative feedback loop in which genes code for proteins that, directly or indirectly, feed back to inhibit their own expression. Once protein levels have dropped through degradation, repression is relieved and the cycle begins again as the factor's gene can be transcribed and its message translated until the factor accumulates enough to repress its own expression. Post-translational modification and proteolysis contribute to these timing loops.
Ultradian rhythm A recurrent rhythm that has periodicity of less than a 24-h cycle. For example, rhythms that have a periodicity of 2 h, 4 h, 6 h, 8 h and 12 h would all be considered ultradian, as would very fast rhythms such as heartbeats in humans.

Fundamental Properties of Circadian Rhythms

Organisms growing in a changing environment use many methods to synchronize with their surroundings. One such mechanism that is very wide-spread in nature is a biological clock, driven by an oscillator. 24-h biological clocks that drive circadian rhythms, oscillations in biological activity that peak approximately once per day, are crucial for many organisms to coordinate biological activity over the course of the day. Evidence for circadian rhythmicity can be found everywhere; all one has to do is look. As early as 1729, Jean-Jacques d'Ortous de Marian reported that the daily opening and closing of the leaves of the mimosa plant persists in constant darkness, that is, in the absence of a *light-dark (LD)* cycle. This early experiment provided a hint that this process was not occurring in response to the rising and setting of the sun, but rather that the mimosa plant had an internal timing system independent of the external conditions. However, de Marian himself was hesitant to draw such conclusions, as he was not able to rule out other external factors such as temperature or gravity. Proving the existence of a circadian clock that could anticipate, rather than respond to, daily changes in our environment, was a challenging scientific problem that was settled only centuries later.

Circadian rhythms can be found ubiquitously throughout nature and are studied extensively in model systems that span the tree of life, including bacteria, fungi, plants, flies, mice, and humans. Although prokaryotic cells were originally thought not to be complex enough to support – or have need for – a circadian clock, we now know that circadian rhythms are indeed a property of some prokaryotes. While cyanobacteria are currently the only known prokaryotes to possess a *bona fide*, robust circadian clock that runs in continuous conditions, 24-h rhythmicity in a *diel* cyclic environment is pervasive throughout the prokaryotic world. New reports have suggested the existence of prokaryotic circadian clocks outside of cyanobacteria, as well as 24-h oscillations in

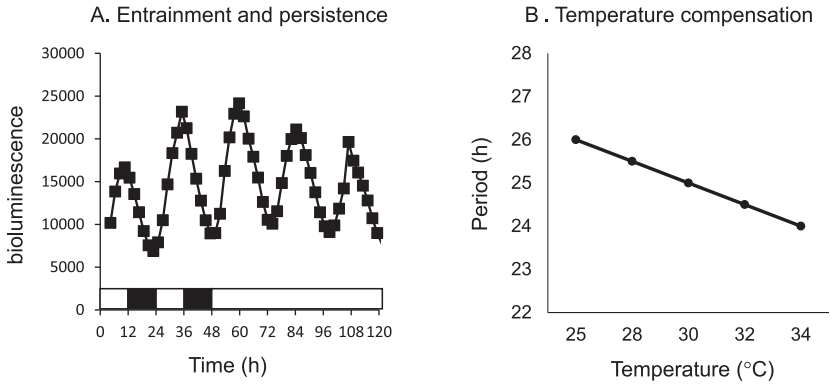

Fig. 1 Fundamental principles of entrainment, persistence and temperature compensation. (A) Bioluminescence (y-axis) measured from an *S. elongatus* reporter strain, P_{kaiB}-*luciferase*, for two days in LD 12:12 and three days after release into constant light conditions (LL). Rhythms of gene expression persist even in the absence of a light-dark cycle. Moreover, the phase of the oscillation remains the same in LL, demonstrating that the phase was entrained to the preceding LD cycle. (B) Graphical representation of temperature compensation. The period of the circadian rhythm as monitored from a luciferase reporter (y-axis) plotted as a function of temperature (x-axis) shows that the period varies no more than 10% over a 10-degree range of biologically relevant temperatures. Original data from Kondo, T., Strayer, C.A., Kulkarni, R.D., *et al.*, 1993. Circadian rhythms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria. Proc. Natl. Acad. Sci. USA 90 (12), 5672–5676.

biological activity from organisms that don't have a circadian clock but may instead respond to a cue(s) from circadian organisms in the same community.

While the mechanism of the prokaryotic oscillator is different from that in eukaryotic models, they share a remarkable number of properties, including the three fundamental hallmarks of circadian rhythms: *persistence, entrainment* and *temperature compensation* (Fig. 1). Circadian rhythms are defined by the ability to persist in constant conditions, meaning the ~24-h oscillation will continue in the absence of environmental cues, i.e., in *constant light (LL)* or constant dark conditions, demonstrating that circadian rhythms are not driven by the environment but rather are endogenous and genetically programmed. The periodicity of the rhythm in constant conditions is referred to as the *free-running period (FRP)*. The typical FRP is approximately, but not exactly, 24-h. The second criterion of circadian rhythmicity is the capacity for entrainment, meaning that the clock can be reset by external cues, thus establishing a particular phase in relationship to the external environment. In the natural world, circadian oscillators all need to adjust slightly in order to synchronize with the 24-h environmental cycle, as the FRP of the organism is not exactly 24-h. However, when the environment is changed more drastically, e.g., a time zone change, the circadian clock takes longer to synchronize with the surroundings, resulting in jet lag. Finally, circadian clocks are temperature compensated, meaning that they maintain a periodicity of ~24-h over a range of biologically relevant temperatures. In contrast to most enzymatic activities, for which reaction rates increase with temperature, the ticking of the circadian clock is buffered or compensated against changes in temperature that occur over the course of the day.

By definition, circadian clocks enable the anticipation of daily environmental changes, so that the organism is prepared for the rising and setting of the sun. This strategy is in contrast to the function of an *hourglass timer* that is set in motion by a change in the environment and would need a new stimulus every day. In addition to summarizing the current status of the prokaryotic circadian clock, we will provide examples of organisms with hourglass timers, which do not persist in constant conditions, and discuss how these two types of clocks may function to contribute to rhythms in complex environments.

Clocks in Prokaryotes

Even before the discovery of circadian clocks in prokaryotes, ~24-h rhythms of photosynthesis and nitrogen fixation that hinted at their existence were reported in several *diazotrophic* genera of cyanobacteria. At the time, prokaryotes were thought to be too simple to have a circadian clock; thus, these rhythms were always attributed to regulation dependent on the external environment or a consequence of the cell cycle. Experiments to test whether these daily rhythms fulfilled the criteria for circadian rhythmicity were not performed until 1986, when Huang and colleagues demonstrated that the rhythms of nitrogen fixation and amino acid uptake in *Synechococcus sp*. RF-1 satisfied the three criteria accepted by the scientific community to be requirements of a *bona fide* circadian rhythm: persistence, entrainment and temperature compensation. For further genetic analysis of the cyanobacterial circadian clock, however, two things were required: an organism amenable to genetic manipulation, and an easy and robust reporter system. In 1993, a joint effort by the laboratories of Takao Kondo, Carl Johnson and Susan Golden, reported the use of luciferase to monitor circadian rhythms of gene expression in *Synechococcus elongatus* PCC 7942. Today, the genetically tractable *S. elongatus* serves as the paradigm for prokaryotic circadian rhythms. Work in this elegant

system has led not only to an understanding of the genetics that underlie the prokaryotic circadian clock, but also to an atomic resolution of its structure and function.

S. elongatus as a Model for Prokaryotic Circadian Rhythms

In *S. elongatus* the KaiA, KaiB and KaiC proteins compose the core or central oscillator required to keep 24-h time. Unlike the *transcription-translation feedback loops (TTFL)* that typically from the basis of eukaryotic circadian oscillators, the Kai proteins function as a post-translational oscillator where associations with KaiA and KaiB drive 24-h rhythms of KaiC phosphorylation. KaiC is an auto kinase, auto phosphatase and ATPase, comprising two domains (CI and CII) that come together to form a hexameric structure that looks like two rings stacked on top of each other (Fig. 2). During the day KaiA promotes KaiC autophosphorylation by binding to the A-loops, flexible C-terminal extensions on KaiC, promoting a conformation of KaiC that favors the autokinase activity (Fig. 2 (B)). KaiC phosphorylates itself first on Threonine 432 (KaiC-SpT), followed by phosphorylation of Serine 431 (KaiC-pSpT), both present in the CII domain of KaiC. Phosphorylation make the KaiC domains rigid and increases interaction between the rings. When KaiC is in its fully phosphorylated form, the interactions between the CI and CII domains of KaiC become so rigid that the CII stacks tightly on the CI ring. Stacking exposes the B-loops, or the binding site for KaiB in the CI domain of KaiC. KaiB associates with KaiC via an interaction with the B-loops as well as with KaiA, sequestering KaiA away from the A-loops and forming the nighttime KaiABC complex. The form of KaiA that is captured by KaiB is an inactive state. By sequestering KaiA, away from the A-loops, KaiB promotes the auto-dephosphorylation of KaiC during the nighttime portion of the cycle, during which KaiC will first remove the phosphoryl group from the Threonine, followed by dephosphorylation of the Serine, returning KaiC to its original non-phosphorylated state (Fig. 2(B)). Rhythms of KaiC phosphorylation that are temperature compensated and have a periodicity of ~24-h can be reconstituted *in vitro*, confirming that the KaiABC proteins are sufficient to drive 24-h rhythms in cyanobacteria. More recent structural investigations of the KaiABC complex, discussed in more detail below, have revealed several fundamental mechanistic insights into the function of the oscillator.

While the KaiABC oscillator can run in isolation *in vitro*, the oscillator within the cell needs to be able to synchronize with the environment and relay time to clock-controlled activities. The core oscillator synchronizes with the environment both directly and indirectly via proteins that monitor cellular redox. CikA, Circadian Input Kinase A, is a critical component of the extended oscillator

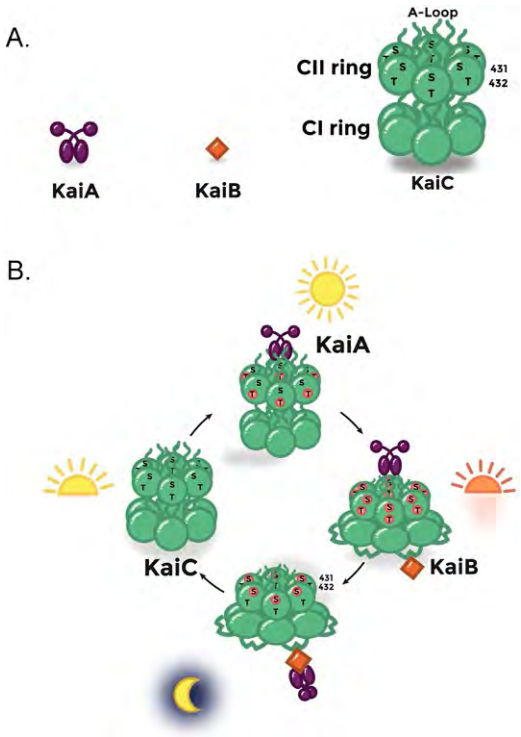

Fig. 2. Model of the *S. elongatus* core oscillator. (A) Cartoon representations of the KaiA dimer, KaiB in the fold-switched monomeric state, and the KaiC hexamer. Phosphorylation sites Serine 431 and Threonine 432 reside in the CII ring of KaiC. (B) At dawn KaiC starts off in a non-phosphorylated state, with CI and CII rings loosely stacked. KaiA binds to the A-loops of KaiC, promoting KaiC autophosphorylation during the day. Red circles represent phosphorylation events. Once KaiC is fully phosphorylated the CI and CII rings stack such that the B-loops on the CI ring become exposed. KaiB, once bound to KaiC, can sequester KaiA in an autoinhibited conformation, promoting KaiC autophosphatase activity during the night. Graphics courtesy of the UC San Diego Bio Clock Studio.

that plays roles both in input to and output from the clock, and affects circadian period. Strains that lack *cikA* display a variety of circadian phenotypes including short period, low amplitude rhythms of gene expression, and most strikingly, a failure to reset the phase of the oscillator by several hours in response to a dark pulse, a feat executed efficiently by WT. CikA plays critical roles in synchronizing and resetting the oscillator with the environment via its ability to interact with quinones, whose redox status varies as a function of photosynthetic activity. Specifically, CikA as well as core oscillator protein KaiA, directly bind to the oxidized form of quinone, an indicator of the onset of nighttime. Moreover, the addition of oxidized quinone can reset the oscillator both in *in vitro* and *in vivo*, suggesting that the redox status of the quinone pool is critical for relaying environmental signals to the oscillator. Even though a Δ*cikA* mutant does not reset the phase of the oscillator in response to a sudden dark pulse, the strain shows a coherent rhythm after entrainment to 2 LD cycles, suggesting that there is more to the story of environmental synchronization. Indeed, LdpA, an Fe-S binding protein, plays a role in aligning the clock with the environment by fine-tuning the circadian period in a light-intensity dependent manner, potentially via regulating CikA protein levels. Additionally, KaiC itself is sensitive to the [ATP/ADP] ratio in the cell, which declines slowly during the nighttime portion of the daily cycle; indeed, altering the [ATP/ADP] ratio can reset the oscillator *in vitro*. The current model to explain how the oscillator becomes entrained with the environment is that the oxidation state of the quinone pool, which becomes rapidly oxidized upon the change to dark, signals the onset of the nighttime period, while the slow change in the [ATP/ADP] ratio in the cell reports the duration of the night. Together, these cues allow the core oscillator to know when night has begun and how long the night persists, enabling the oscillator to synchronize with the external environment.

The oscillator requires a mechanism of circadian output to coordinate biological activity over the course of the day. CikA, SasA and RpaA are the key players that enable the oscillator to transmit temporal information to clock-controlled behaviors (Fig. 3(A)). Briefly, association with the Kai complex at different times during the cycle activates the kinase or phosphatase activity, respectively, of two regulators of a master transcription factor, RpaA (Fig. 3(A)). SasA is a histidine kinase that becomes activated via an association with KaiC to phosphorylate itself and transfer that phosphoryl group to its cognate response regulator, RpaA. SasA associates with KaiC during the day, thus increasing [RpaA-P/RpaA] (Fig. 3(B)). CikA, in addition to its known roles in relaying environmental information to the oscillator, dually functions in relaying information from the oscillator by acting as a phosphatase towards RpaA. This phosphatase activity is stimulated by CikA association with KaiB after formation of the KaiC-KaiB complex, with CikA likely competing with KaiA for KaiB binding (Fig. 3(B)). While SasA phosphorylates RpaA during the day, CikA dephosphorylates RpaA at night, resulting in a 24-h rhythm of RpaA-P, with RpaA-P levels peaking at dusk (Fig. 3(B)). The regulation of SasA and CikA functions are discussed in more detail below.

RpaA is a DNA-binding transcription factor that, in its active phosphorylated state, activates the expression of dusk-peaking genes and represses the expression of dawn-peaking genes. The deletion of *rpaA* eliminates genome-wide rhythms of gene

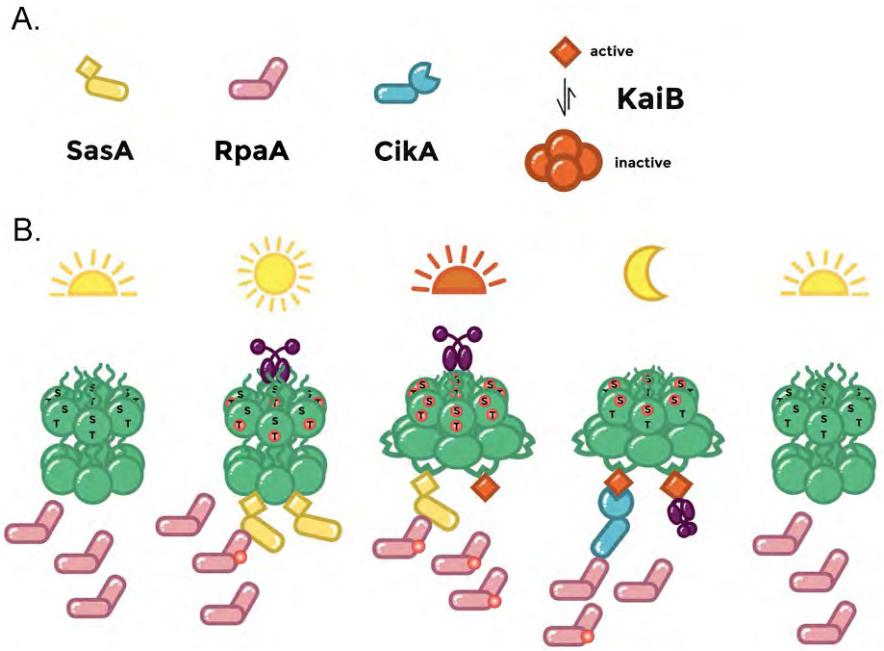

Fig. 3. Model of the *S. elongatus* output pathway. (A) Cartoon structures of output components SasA with N-terminal KaiC-interacting domain and C-terminal histidine kinase domain; the master circadian transcriptional regulator RpaA; CikA, which plays roles in both the input and output mechanisms. Additionally, KaiB morphs from an inactive tetramer in a ground-state fold to an active fold-switched monomer, capable of interacting with KaiC. (B) Over the course of the day SasA becomes activated as a kinase towards RpaA via an association with KaiC, with RpaA-P peaking at dusk. Red dots represent phosphorylation events. Once KaiC has reached the fully phosphorylated state rare fs-KaiB monomers are stabilized by binding to KaiC at a site that partially overlaps with SasA binding, likely dislodging SasA from KaiC. Additionally, fs-KaiB engages CikA, activating its phosphatase activity towards RpaA, causing RpaA-P levels to decrease at night. Graphics courtesy of the UC San Diego Bio Clock Studio.

expression. Surprisingly, RpaA-P binds directly to only ~100 positions in the genome; however, essentially the entire *S. elongatus* genome is expressed rhythmically. Included amongst the ~170 genes expressed as part of the RpaA regulon are those that encode 4 sigma factors, suggesting that RpaA-regulated global rhythms of gene expression are carried out via the activation of a transcriptional cascade. Additionally, RpaA-P directly regulates genes required for metabolism (glycolysis, glycogen and oxidative pentose phosphate pathways), proteolysis, and the bacterial tubulin homolog FtsZ, suggesting that processes such as energy production/storage, protein homeostasis and cell division are closely tied to the clock. However, this picture is not complete, as we know that RpaB, a paralog of RpaA that is regulated not by clock but by the environment, also plays a role in controlling diurnal gene expression. While roles for RpaB in rhythmic gene expression have yet to be determined, RpaB can inhibit the phosphorylation of RpaA, and binding of RpaB to some promoters is antagonized by RpaA, suggesting that RpaB is a hub that integrates both circadian and environmental information to regulate gene expression rhythms.

One of the primary reasons that circadian rhythms were originally not expected to be a property of bacteria is that many bacteria have a doubling time shorter than a day. Intuitively, an organism whose life cycle was less than 24-h did not seem to need a circadian clock. Work in *S. elongatus* demonstrated that the rhythmicity of the clock is independent of the cell division cycle. The clock maintains ~24-h periodicity whether the cells are dividing multiple times or not at all in a 24-h time period. Moreover, single-cell analysis has shown that the phase of the oscillator is robust at the single-cell level and is inherited from mother to daughter cell with negligible intercellular coupling. In populations of rapidly dividing cells a TTFL involving RpaA-activated transcription of *kaiABC* helps to maintain robustness of the clock, because enhanced variations in phase are observed in single cells when the TTFL is compromised. These results suggest that, while a TTFL is not the primary driver for rhythms of gene expression, such a loop is important for the sustained robustness of the post-translational oscillator under certain circumstances, perhaps via buffering protein levels in individual cells. Although cell division is not synchronous in *S. elongatus*, there is an ~6-h block of time in the early subjective night (i.e., circadian night, even in the absence of an environmental cycle) when cell division is specifically inhibited by the circadian clock. The mechanism by which this inhibition or gating occurs is unknown; however, the current model proposes that the clock regulates the expression of a factor or set of factors that inhibit the bacterial tubulin homolog FtsZ from forming the cytokinetic ring at mid-cell.

One virtue of the *S. elongatus* circadian clock, in comparison to those of eukaryotes, is that the core oscillator can be reconstituted *in vitro*. Moreover, the *in vitro* system mimics the *in vivo* system so well that it has led to many key insights into clock function. Nonetheless, there are aspects of the *in vivo* system that cannot be reproduced *in vitro*. An example is the natural variation in protein abundance: KaiB and KaiC exhibit robust oscillations in cellular protein levels throughout the day, whereas KaiA levels remain nearly constant. Another is that KaiA and KaiC change their subcellular localization patterns over the course of the day. Specifically, KaiA and KaiC are diffuse throughout the cell during the daytime portion of the cycle, but then become localized to a cell pole at night. The nighttime complex at the poles of cells also includes CikA, which is consistent with the known roles for CikA in the circadian mechanism. The observed changes in protein levels and subcellular localization patterns likely relay information to and from the input/output pathways and influence the robustness of the clock in the context of a cell, which is a much more complex system than a test tube.

Mutants with an altered clock or absent clock grow about as well as WT strains in constant light, which raises the question of whether the circadian clock is of any use to the cell. Key experiments performed by Johnson and colleagues showed that, while clock mutant strains do not exhibit a growth defect when grown in pure culture, a competitive fitness advantage is conferred to strains whose internal period matches the external environment. For example, when a WT strain with a FRP of ~25-h is co-cultured with a short-period mutant that has an ~22-h FRP, the WT strain will outcompete the short-period mutant if the mixed culture is grown in a 12-h light:12-h dark cycle (LD 12:12). However, in a shorter day cycle of LD 11:11, the short-period mutant will outcompete the WT. These experiments were the first to demonstrate experimentally, for any organism, the long-accepted assumption that circadian clocks confer a fitness advantage.

A Circadian Clock With Atomic Resolution

Recent structural investigations provided critical insights into clock function including the nature of KaiB's roles in timekeeping and time delay as well as relaying temporal information to clock-controlled activities. The structures of KaiA, KaiB and KaiC were solved more than a decade ago by X-ray crystallography and/or NMR spectroscopy. Structural analysis of each component has shown that KaiA is a homodimer, with each monomer comprising two independently folded domains: an N-terminal PsR domain, which binds oxidized quinone, and a C-terminal KaiC-interacting domain, connected by a linker. KaiC is a hexameric protein in which each monomer bears two homologous domains, CI and CII, such that oligomerization creates stacked hexameric rings. Although the CI and CII domains are duplications, the phosphorylation sites Ser 431 and Thr 432 are limited to the CII domain. However, the CI domain of KaiC possesses a weak ATPase activity, which hydrolyzes only ~15 molecules of ATP per day per KaiC monomer. The ATPase activity is temperature compensated and is important for the periodicity of the circadian clock. The structure of KaiB in isolation has been solved by several labs and was demonstrated to be a tetramer that took on what was referred to as a rare fold.

While the structure of each Kai protein had been solved individually, visualization of the KaiABC complex long remained elusive. Moreover, the mechanism by which KaiB inhibits KaiA stimulation of KaiC autophosphorylation had long been a mystery. The site of KaiB's association with KaiC was also contentious, as some groups presented evidence that it associated with the CII domain of KaiC while others proposed that it associated with the CI domain. The structure of KaiB in association with KaiC as well

as the KaiABC complex structure were conclusively resolved in 2017 (see Chang *et al.* and Tseng *et al.*), solving some long-standing mysteries and also leading to a few surprising discoveries. First, KaiB was shown to interact with the B-loops present in the CI domain. Although, CI and CII are homologous, the B-loops are present only on the CI domain of KaiC, where they remain hidden until KaiC becomes phosphorylated on S431. Only after S431 becomes phosphorylated are the B-loops get exposed, allowing KaiB to bind to the CI domain of KaiC. However, the real surprise came in the discovery that KaiB bound to KaiC not as a tetramer but rather as a monomer, capable of forming an additional hexameric ring on KaiC. Moreover, this monomeric form of KaiB had a completely different structure than the rare fold adopted in the tetrameric form. KaiB that associates with KaiC adopts a thioredoxin-like fold, which happens to be the same fold as the N-terminus of SasA. We now know that the KaiB is part of a class of metamorphic proteins capable of reversibly changing its structure. The rare fold in the tetrameric structure represents an inactive form of KaiB that is not capable of associating with KaiC, also referred to as ground-state KaiB (gs-KaiB). KaiB must morph, via unfolding and refolding, into an active, fold-switched (fs-KaiB) state in order to be competent to associate with KaiC (Fig. 2(A)). This switch from gs- to fs-KaiB is rare and unstable; the fs-KaiB fold is stabilized only in association with exposed B-loops on KaiC. The instability of KaiB's fold-switched form explains why it had not been discovered until its atomic structure was resolved in its KaiC-bound state. Moreover, the expectation that the structure that had been crystallized would associate with KaiC confused the analyses that pointed to binding on the CII domain. The switching from inactive gs-KaiB to active fs-KaiB, and the requirement for a specific status of KaiC to bind and stabilize it, accounts for a significant portion of the 24-h period of KaiC phosphorylation reaction cycle. Because thioredoxin is an ancient fold, perhaps fs-KaiB is the ancestral form of KaiB and evolution of the inactive, gs-KaiB, was an important evolutionary step in acquiring a clock mechanism.

This new understanding of how KaiB associates with KaiC also shed light on how KaiB inhibits KaiA and promotes the autophosphatase activity of KaiC, switching the oscillator from a daytime to a nighttime mode. Once in the fully phosphorylated state, the CII ring of KaiC stacks tightly onto the CI ring, causing the B-loops to become exposed. When fs-KaiB is present it can rapidly associate with the newly exposed B-loops, ultimately forming a ring consisting of 6 fs-KaiB monomers on the CI domain of KaiC. In addition to associating with KaiC, fs-KaiB can bind and sequester KaiA away from the A-loops of KaiC, promoting KaiC autophosphatase activity during the nighttime portion of the cycle. Recent structural work has shown that fs-KaiB associates with an auto-inhibited form of KaiA, which has undergone a large-scale conformational change such that its KaiC interaction domain is unavailable. Thus, KaiA can no longer stimulate KaiC kinase activity in CII because it has been sequestered by KaiB on CI, and cannot stimulate the activity of a neighboring KaiC from its KaiB-bound position because it is in an auto-inhibited conformation (Fig. 2(B)).

The identification of fs-KaiB also led to critical insight as to how the oscillator communicates information to the output pathway. SasA that is not in association with KaiC is not competent to phosphorylate RpaA. When the N-terminal domain of SasA associates with the CI domain of KaiC, at a site that partially overlaps with the B-loop, SasA becomes activated as a kinase towards RpaA. The binding of fs-KaiB to the B-loops, occurring after S431 phosphorylation of S431, likely displaces SasA from KaiC. Additionally, fs-KaiB in the KaiBC complex interacts with CikA, stimulating its phosphatase activity. Thus the binding of fs-KaiB to KaiC not only switches KaiC from a daytime autokinase mode to a nighttime autophosphatase mode, but also inactivates SasA as a kinase toward RpaA and activates CikA as a phosphatase towards RpaA, resulting the rhythms of RpaA-P and, thus links the oscillator to output function.

Variations on the KaiA-KaiB-KaiC Oscillator

The *S. elongatus* KaiABC oscillator is the only prokaryotic circadian clock whose molecular details have been elucidated. In *S. elongatus* KaiA, KaiB, and KaiC are all essential components of the clock. However, variations of the KaiABC system exist amongst cyanobacteria. Here, we discuss cyanobacteria that encode homologs of *kaiB* and *kaiC* but not *kaiA*, as well as species that encode multiple paralogs of *kaiB* and *kaiC*.

While circadian rhythmicity that persists in the absence of external cues is a property of many cyanobacterial species, it may not be present in all cyanobacteria. *Prochlorococcus marinus*, one of the predominant primary producers in the open ocean, likely has an hourglass timer that is not self-sustained in the absence of external cues. In the laboratory as well in natural populations, *P. marinus* growing in an LD cycle display a daily cell cycle rhythm, where the DNA is replicated in the afternoon and cell division occurs in late afternoon/early evening, as well as rhythms of gene expression. However, these rhythms quickly damp in constant light. These data suggest that the *P. marinus* clock functions as an hourglass timer, requiring the rising of the sun to initiate a 24-h program of temporal regulation. While the *P. marinus* genome has *kaiB* and *kaiC* homologs, it appears to have lost the full length *kaiA* sequence, with a remnant *kaiA* gene remaining. In contrast to the *S. elongatus* KaiC, whose autophosphatase activity dominates when the protein is in isolation, KaiC from *Prochlorococcus sp.* MED 4 exhibits ATPase activity and readily autophosphorylates on serine 427 and threonine 428. *Prochlorococcus* also lacks *cikA* homologs, consistent with the notion that *cikA* is evolutionarily the newest member of the circadian network. Moreover, an hourglass timer that responds directly to the environment may not require a protein such as CikA that functions to entrain the core oscillator, consistent with its absence in *Prochlorococcus*. The molecular details of how the *Prochlorococcus* clock functions are still unresolved and the lack of molecular genetics in this system has hindered progress in this area.

The loss of *kaiA* is not the only variation to the *kaiABC* theme among diverse cyanobacteria. Several cyanobacterial species including the model system *Synechocystis* sp. PCC 6803 have one *kaiA* gene and three paralogs of *kaiB* and *kaiC*. Using luciferase

reporters the *kaiA-kaiB1-kaiC1*locus of *Synechocystis* has been shown to be required for cyclic gene expression. As in *S. elongatus*, KaiC1 autophosphorylation is dependent on KaiA. However, KaiC2 and KaiC3 can autophosphorylate independently of KaiA. While it still remains to be determined how each of the three *kai* clusters may contribute to the circadian clock and rhythmic phenomena, it has been proposed that *kaiB3* and *kaiC3* are important for fine-tuning the period length and phase of the clock whereas *kaiB2* and *kaiC2* have no attributable clock functions. However, supporting data for these proposed functions of *kaiB2*, *kaiC2*, *kaiB3* and *kaiC3* have not been published.

Our understanding of how circadian clocks function is dependent on our ability to grow and manipulate the organisms in a laboratory setting. Their function in the organisms' native environments have not been studied as extensively. Time-resolved whole-genome transcriptome profiling of marine microbial populations over a 3-day period found robust 24-h diel oscillations of cyanobacterial transcripts; surprisingly, it also revealed diel cycling of transcripts in heterotrophic bacterioplankton that follow those of the photosynthetic organisms. One hypothesis is that the heterotrophic bacterioplankton are responding to metabolic cues from the cyanobacteria, thus resulting in staggered multispecies waves of diel gene expression in the ocean. The metabolic coupling between primary producers (cyanobacteria) and consumers (heterotrophic bacteria) results in the coordination of diverse biogeochemical activities in complex microbial communities, such as in the ocean.

Kai Genes Outside of Cyanobacteria

While the *kai* genes have been studied extensively only in cyanobacteria, potential homologs of *kaiB* and *kaiC* (but not *kaiA*) are found in eubacteria and Archaea. While evidence for 24-h timing systems is clear in many of these organisms, some appear to possess an hourglass timer whereas others may have a free-running circadian clock. Although the existence of circadian timekeeping in some of the organisms discussed below may seem obvious, the definitive experiments to demonstrate the existence of a robust mechanism that satisfies the three criteria accepted to comprise a *bona fide* circadian clock have not yet been performed, nor has the role for the *kai* homologs in these organisms been explored.

Phototrophic bacteria including purple bacteria and the green nonsulfur bacterium *Chloroflexus* have homologs of *kaiB* and *kaiC*, but not of *kaiA*. Extrapolating from what is known in *Procholorcoccus*, a KaiBC-based hourglass timer may be present in these organisms. However, the purple bacterium *Rhodobacter sphaeroides*, which grows photosynthetically under anaerobic conditions, exhibits sustained rhythms of gene expression, as monitored using a luciferase reporter system, suggesting the existence of a circadian oscillator. Under aerobic conditions, where the organism grows chemoheterotrophically, these rhythms exhibit a 20.5-h period and under anaerobic, photosynthetic conditions they have an *ultradian* period of 10.6–12.7 h. A related purple non-sulfur bacterium, *Rhodopseudomonas palustris*, which similarly exhibits different growth modes depending on the availability of oxygen, displays rhythms of nitrogen fixation in LD. However, these rhythms do not persist in constant light. A mutant that lacks *kaiC* also exhibits nitrogen fixation rhythms in LD, but with a peak time that varies from WT, suggesting a role for *kaiC* in the phasing of these rhythms. *R. palustris* KaiC is capable of autophosphorylation, but rhythms of KaiC phosphorylation were not observed in LL *in vivo*. Taken together, these data suggest that KaiB and KaiC proteins play roles in both circadian and hourglass timekeeping mechanisms. Additionally, these data may suggest roles for the cyanobacterial-specific KaiA in promoting sustained rhythmicity in the absence of external cues.

Potential *kaiB* and *kaiC* homologs have been found in eubacteria as well, but their functions have not been investigated. In the human pathogen *Legionella pneumophila kaiB* and *kaiC* are expressed in an operon under the control of the stress sigma factor RpoS, and have been proposed to function in stress-resistance pathways, but tests for circadian rhythmicity have not been performed. Intriguingly, the crystal structure of KaiB in isolation from *L. pneumophia* has been solved and was found to adopt the fold-switch or thioredoxin-like fold. These data support the notion that the thioredoxin-like fold is ancestral, and that the ground state in *S. elongatus* is an adaptation that enabled the evolution of the clock.

There is also evidence for circadian rhythmicity in archaea. In the halophilic archaeon *Halobacterium salinarum NRC-1* 12% of all genes show ~24-h rhythmicity in LL after 3-day entrainment to an LD cycle. While mechanistic studies in this organism have not been performed, a related haloarcheon, *Haloferax volcanii*, has for four cryptic *kaiC*-like genes, *cirA*, *cirB*, *cirC*, and *cirD*, that have been the subject of some investigation. While *kaiC*-like genes were identified, no homologs of *kaiA* or *kaiB* were found in this organism. CirA, CirB and CirD are single-domain proteins that share 48%–55% similarity to the CI domain of KaiC from *S. elongatus* and do not have a CII domain. The clock in *S. elongatus* requires both domains in order to function as the circadian timekeeper, as interdomain interactions figure largely in the timing mechanism. CirC, on the other hand, has a double-domain structure similar to KaiC of *S. elongatus*. Expression of the *cir* genes oscillates with daily periodicity in LD but not in LL. Intriguingly, rhythms of gene expression in LD are lost if any of the *cir* genes is disrupted, implicating all as participating in diel cycling. However, limitations of working with these archaeal species have hindered progress in this area.

Some reports have identified peroxiredoxins as a conserved marker of circadian rhythms across all domains of life. Peroxiredoxins are a ubiquitous type of antioxidant enzyme. Peroxidase activity is dependent on the oxidation state of a key cysteine residue; once this residue is hyperoxidized it is catalytically inactive and can be recycled. Peroxiredoxins that undergo ~24-h redox cycles that persist for several days in the absence of external cues, and are entrainable and temperature compensated, have been reported in species across all three domains of life including circadian systems *S. elongatus*, flies, fungi, plants and also in archaea and even red blood cells. While eukaryotic circadian oscillators are driven by TTFLs, red blood cells lack a nucleus and could not support such a mechanism, suggesting that peroxiredoxin redox rhythms are driven by a posttranslational mechanism. In *S. elongatus* peroxiredoxin redox rhythms persist even when the *kai*-based oscillator is compromised, albeit with altered phasing. This finding

suggests that the KaiABC complex do not drive peroxiredoxin redox rhythms, but the two oscillators are somehow coupled. Similarly, the peroxiredoxin-redox oscillator is not dependent on known clock components in eukaryotic systems, suggesting the existence of a novel timing mechanism that predates more recently evolved robust circadian oscillators. These results, with the observations from *Legionella* and archaea, suggest that Kai proteins may have functions beyond acting as a circadian oscillator and that Kai proteins may not be the only drivers of prokaryotic circadian clocks.

Microbiome Rhythms

Recently, there has been an explosion of work demonstrating the importance of the human gut microbiome (the ~100 trillion microbes that reside in our gut, also referred to as microbiota) to human health and disease. The composition and function of the microbiome exhibits 24-h diurnal oscillations that are important for metabolic homeostasis of the host. Functional profiles of the microbiome at different times of day reveal that energy harvest, DNA repair, and cell growth primarily occur during the active phase of the host, whereas detoxification and chemotaxis are more prevalent during the host resting period. These microbiome rhythms are not self-sustained and are dependent on the circadian clock of the host. Oscillations in neither abundance nor function of the microbiome are observed if the host circadian clock is perturbed. Intriguingly, changes in microbiota induced by jet-lag led to enhanced weight gain and glucose intolerance compared to control microbiota samples, highlighting the importance of microbiota rhythmicity to human health.

In addition to light perceived through the eyes, we now know that food intake is an important input to the mammalian circadian clock. Mice fed a high-fat diet exhibit attenuated clock function, and these adverse effects can be prevented with time-restricted feeding, suggesting that the cause of attenuation is not the high-caloric diet itself but rather the misalignment of nutrient availability with circadian metabolic activity. Not surprisingly, microbiota oscillations are controlled by feeding time of the host in addition to regulation by the host circadian clock.

While the host circadian clock regulates rhythms within the microbiome population, new evidence suggests that the reverse is also true. Oscillations in key microbial-derived metabolites can regulate or modify the host central and hepatic circadian rhythm and host metabolic function. Alteration to microbiota rhythms resulting in inappropriate timing of key metabolites can alter circadian rhythms of gene expression in the liver and promote diet-induced obesity. Additionally, the location of particular members of the microbiome changes over the course of the day. Oscillating biogeographical distribution of the microbiome, as well as oscillations in metabolism, determine rhythmic exposure of the intestinal epithelium to different bacterial species and their metabolites over the course of the day. Thus, the host circadian clock regulates rhythmicity in population, function, and location of the gut microbiome. In turn, metabolites produced by the microbiome provide critical feedback to the host circadian clock.

Because the observed oscillations in the gut microbiome are not self-sustained, but rather dependent on the host clock, it would suggest that these rhythms rely on an hourglass timer. However, it is possible that some members of the microbiome community possess a circadian clock themselves. In fact, one member of the microbiome community, *Enterobacter aerogenes*, exhibits circadian patterns of swarming and motility as well as gene expression in response to the neurohormone melatonin. These rhythms can be entrained to the presence of melatonin and are temperature compensated. However, *E. aerogenes* does not encode homologs of *kaiA*, *kaiB* or *kaiC*, suggesting that a novel mechanism is driving these rhythms. One possibility is that this oscillator is the same as the one that drives peroxiredoxin oscillations. The motility, and therefore localization, of bacterial species within the gut may be controlled by the microbial circadian clock. Further investigation is sure to lead to new insights into the interplay between the microbiome and the host.

The discovery of circadian clocks in cyanobacteria surprised a community that underestimated the sophistication of prokaryotic cells and organisms. Work in *S. elongatus* has led to an exquisite understanding of how bacterial circadian clocks function. However, circadian rhythms in prokaryotes are likely not limited to cyanobacteria, as examples of bacterial rhythms are now being discovered everywhere. Moreover, work from organisms outside of *S. elongatus* has led to the idea that Kai proteins may have functions outside of the circadian clock system and that there are prokaryotic clocks that are not composed of Kai proteins (Table 1). While circadian

Table 1 Summary table of different species, the genes that have been tied to clock behaviors, and whether the observed rhythmic behaviors are circadian, hourglass or unknown. Circadian? represents organisms for which the existing data are highly suggestive of a circadian clock; however, the definitive experiments have not yet been performed

Organism	*Clock genes*	*Clock*
S. elongatus PCC 7942	*kaiA, kaiB, kaiC*	Circadian
Procholorcoccus spp.	*kaiB, kaiC*	Hourglass
Synechocystis sp. PCC 6803	*kaiA, kaiB1, kaiC1, kaiB2, kaiC2, kaiB3, kaiC3*	Circadian
Rhodobacter sphaeroides	*kaiB, kaiC*	Circadian?
Rhodopseudomonas palustris	*kaiB, kaiC*	Hourglass
Legionella pneumophila	*kaiB, kaiC*	None known
Halobacterium salinarum NRC-1	*cir genes*	Circadian?
Enterobacter aerogenes	Not known	Circadian?

biological oscillators can be found ubiquitously throughout nature, hourglass timers, which require cues from the environment to be sustained, are also important for coordinating activity over the course of the day. Studies of environmental systems and bacterial-host interactions have led to the notion that circadian oscillators can work in conjunction with hourglass timers to orchestrate the timing of a complex community. Continued research in this area is required to determine their mechanisms, but the next few years are sure to be an exciting time for bacterial circadian rhythms.

Further Reading

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Relevant Website

https://ccb.ucsd.edu/the-bioclock-studio/about-bioclock-studio/–U.C. San Diego BioClock Studio-Video tutorials and animations.

Clostridia

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Glossary

Acetone–butanol fermentation A bacterial fermentation carried out by certain sugar-metabolizing species of *Clostridium*. The typical end products include acetone, *n*-butanol, and acetic acid (collectively referred to as 'solvents').
Anaerobe An organism that has the ability to grow in the absence of oxygen.
Antitoxin Protective factors, usually antibodies, produced in animal and human sera in response to injection with toxin or detoxified toxin (toxoid).
Bioremediation Conversion of pollutants to nontoxic compounds by microorganisms and enzymes.
Botulinum neurotoxin The most poisonous toxin known for humans and certain animals and the cause of botulism. Botulinum neurotoxin is also being increasingly used for the treatment of a diversity of disease syndromes and for cosmetic enhancement.
Botulism The rare paralytic disease of humans and other animals caused by the potent neurotoxin produced by the bacterium *Clostridium botulinum*.
Clostridium Obligately anaerobic, endospore-forming bacteria that occur widely in soils and in the intestines of animals and occasionally humans.
Endospores Resistant bodies formed by certain genera of bacteria, including *Bacillus* and *Clostridium*.
Enzymes Biochemical compounds (particularly enzymes) that catalyze chemical reactions.
Fermentation Energy-yielding metabolism in which an energy substrate is metabolized without the molecular involvement of an external electron acceptor, such as oxygen.
Gas gangrene A rapidly spreading disease that results from the infection of a wound by certain anaerobic bacteria, particularly by species in the genus *Clostridium*.
Tetanus The extremely painful and often deadly spastic disease (commonly called lockjaw), affecting humans and animals, produced by the bacterium *Clostridium tetani*.
Toxins Microbial products or components that cause injury or disease in multicellular organisms, including humans and animals. The clostridia produce more protein toxins than any other bacterial group.
Vaccine An antigenic preparation administered to humans that elicits protective immunity to a specific pathogen or toxin.

Defining Statement

Clostridium comprises a genus of spore-forming, strictly anaerobic bacteria that have important roles in human disease and in biotechnology. This article describes their biological properties and their importance to humankind.

Introduction

The clostridia are an extremely diverse group of bacteria, and several species have an illustrious history in medical microbiology and in biotechnology. Clostridia were probably first alluded to in history by the Greek physician Hippocrates from the island of Kos (460–370 BC), who reported on wound infections in his *Epidemics III*. The symptoms were a yellowish-red swelling in the wound and followed by the formation of copious pus. In severe cases, flesh was destroyed and limbs were lost. Tetanus, commonly referred to as lockjaw, was vividly illustrated in Sir Charles Bell's *Essays on the Anatomy and Philosophy of Expression* (1824), in which in a painting he depicted a soldier in agony from severe contractions. The famous original painting can be observed in the museum at the Royal College of Surgeons of Edinburgh. In World War I, wound infections caused by clostridia were common among soldiers and caused much suffering and mortality. Clostridia also have several positive characteristics and utilities in industry and medicine. They have been used as a source of fine chemicals, solvents, and enzymes used in the chemical industries, and clostridia are ecologically important in the degradation of various xenobiotics. One of the most remarkable attributes of clostridia is the development of botulinum neurotoxin for the treatment of a variety of neurological maladies by direct injection of small quantities of toxin into neuromuscular tissue. Botulinum toxin is also used in cosmesis, for the presumptuous beautification of the human body.

This article is an update of E.A. Johnson,Clostridia, *Encyclopedia of Microbiology (3rd Edn)*, edited by Moselio Schaechter, Academic Press, 2009, pp. 87–93.

Clostridia grow only in the absence of oxygen and are characterized as obligate anaerobes. Although life without oxygen was envisioned as early as 1680 by Antonie van Leeuwenhoek, the discovery of life without oxygen was by Pasteur, who showed in the mid-1800s that butyric acid formation by clostridia occurred in the absence of oxygen. Pasteur devised anaerobic culture conditions by boiling the medium to drive out absorbed gases from the culture medium and by using anaerobic culture conditions. He isolated an organism that grew and produced butyric acid under anaerobic conditions; he called this organism '*Vibrion butyrique*,' which probably corresponds to *C. butyricum*, the type species of the genus *Clostridium*. In 1877, Pasteur and Joubert also described the first pathogenic anaerobe of humans and animals, now known as *C. septicum*, which is a serious cause of wound infections and bacteremia or blood infections. *C. tetani*, the cause of lockjaw or tetanus in humans and animals, particularly horses, was discovered in 1889 by Nicolaier, who enriched the spores from pus from a lesion of an animal that had died. Kitasato isolated the organism in pure culture and showed that it produced a potent toxin, which when injected in an inactive form into animals gave rise to specific antitoxin in the serum that was protective against the disease when administered prophylactically. This groundbreaking research contributed to the development of vaccines, currently used for the prevention of disease in billions of humans and animals.

Interest in clostridia was also stimulated by their roles in food spoilage and foodborne disease. Several species of clostridia grow at refrigerator or high temperatures and spoil foods, which is noted by gas production from carbohydrate fermentation, putrid smells from protein degradation, and detriments in texture and organoleptic properties of the foods. Several clostridia are highly resistant to heat and survive canning and cooking processes by virtue of their ability to form spores and subsequent germination of the spores and growth leading to spoiled cans and packages that may explode due to gas formation. Several federal laws implemented for food preservation and food safety have been derived to prevent the survival and growth of *C. botulinum*, which has highly resistant endospores and produces its extremely potent neurotoxin that is the cause of the disease botulism.

Clostridia have had important historical and contemporary roles in various biotechnological processes. Reports indicate that clostridia were used by the Romans for the production of indigo for dyeing of the skin and later for linen, paper, and other materials. Pasteur found in the 1860s that clostridia produced different solvents and acids including butanol and butyric acid. Certain nonpathogenic clostridial species are important in the formation of solvents and organic acids by anaerobic fermentations and as sources of enzymes for biocatalysis. The importance of clostridia in industrial fermentations was demonstrated prior to World War I, when an industrial process was developed for butanol as a precursor of butadiene (for synthetic rubber) and of acetone, used in the manufacture of munitions. Solventogenic clostridia are currently being extensively studied for the production of acetone and butanol by fermentation from renewable agricultural materials. Another important biotechnological process of clostridia was their use in flax retting, resulting in the production of clothing and oil. In the twentieth century to the present, clostridia have been investigated as sources of novel enzymes for biotransformations, for biomass degradation, and for their ability to degrade pollutants and xenobiotics.

A remarkable discovery postulated in the eighteenth century that became a reality in 1970–80s and escalating in importance to the present is the use of botulinum neurotoxins as pharmacological agents for the treatment of a diversity of neurological diseases including movement disorders, pain syndromes, cerebral palsy, spasticity, depression, excessive sweating, and more than 100 syndromes. The basis for therapeutic use of botulinum neurotoxin is its absolute specificity and action on nerves in the peripheral system. It blocks the release of the neurotransmitter acetylcholine, thus quieting the activity of muscles adjacent to the affected nerves. The toxin has also been used for cosmetic purposes such as smoothing frown lines, crow's feet, and other appearances that come with age. Recent evidence indicates that botulinum toxin can enter the central nervous system and lead to relief of mood and affect disorders.

Owing to the extreme toxicity of botulinum neurotoxin, it has been considered as potential agents of bioterrorism. Historically, botulinum toxin was administered to prisoners in certain cultures and regimes. In 1990, during the first Gulf War, it was acknowledged that 20 000 l of botulinum toxin was produced and potentially weaponized on missiles. The Japanese cult sect Aum Shinrikyo attempted to dispense aerosolized botulinum neurotoxin in metropolitan Tokyo. Fortunately, the cult was unsuccessful in producing active aerosols of the toxin. These attempted incidences and evil plans have raised awareness regarding the potential use of botulinum neurotoxin as a bioterrorist agent.

The importance of clostridia in medical and industrial microbiology contributed significantly to the development of important microbiological techniques. The culturing of strict anaerobes from the environment and infected humans and animals, as well as the strictly anaerobic colon of mammals, presented a considerable challenge to scientists. It became necessary to develop oxygen-free culture media and equipment including anaerobic incubation systems to isolate and study the clostridia and other strictly anaerobic microorganisms. The availability of anaerobic culture techniques led to the discovery of many new groups of organisms and recognition of unique pathways of anaerobic metabolism.

Properties of Clostridia

Classification

The genus *Clostridium* was first proposed in the mid-1800s. Since then, most anaerobic endospore-forming bacteria have been included in the genus. The genus *Clostridium* belongs to the family Bacillaceae of the phylum Firmicutes of Bacteria. The genus *Clostridium* comprises a large and diverse group of at least 12 lineages of anaerobic and occasionally aerotolerant, Gram-positive, rod-shaped bacteria that form resistant endospores. The endospores are often wider than the vegetative organisms in which they arise, giving cells the characteristic spindle shapes or clostridium forms. The clostridia have certain common properties: (1) a Gram-positive cell wall structure, (2) formation of resistant endospores that often swell the cell, (3) an anaerobic and fermentative metabolism, and (4) the inability to reduce sulfate to sulfide. The genus *Clostridium* has a wide range of 21–54 mol% GC content of their DNA, supporting the heterogeneity of this group of organisms.

Traditionally, clostridia are classified on the basis of morphology, spore formation, and physiological properties including anaerobiosis and fermentative metabolism, disease association, toxin formation, DNA relatedness, and ribosomal RNA gene sequence homologies. The vast majority of clostridia have a rod-shaped morphology and are motile by peritrichous flagella. Several taxonomic groups of *Clostridium* have been described on the basis of nucleic acid homologies; sequencing of macromolecules, particularly 16S rRNA; mol% GC; cell wall structure; production of toxic and antigenic proteins; and type of anaerobic metabolism. Advances in whole genome sequencing are becoming more important in the classification of clostridia. Currently, more than 200 species have been proposed, and *Clostridium* represents one of the largest genera of prokaryotes. The genus needs to be better defined by traditional and advanced molecular methods to delineate natural lineages.

Habitat

The clostridia are widely distributed in nature by virtue of their ability to form environmentally resistant endospores and by their diverse metabolic properties. The clostridia appear to reside in two primary habitats: soils and water sediments throughout the world and within the gastrointestinal tracts of certain animals. Some species inhabit the human gastrointestinal tract, and ~40 species have been found in human feces, including several pathogenic species. Many species of pathogenic clostridia are free-living and generally do not have an obligate association with an animal host, in contrast to many other pathogenic bacteria. One species, *C. perfringens*, has been found in nearly every soil sample worldwide for which it has been sought. Since spores of clostridia are present in the soil, they are associated with many foods, including vegetables and milk, as well as in environmental sources such as dust and sewage. Although most species usually lead a strictly saprophytic nonpathogenic existence, several species are the cause of well-recognized diseases in humans and animals mainly through the production of potent toxins.

Physical Properties

The clostridia vary widely in their structure and in certain physical properties. Most clostridia are rod-shaped; however, some species are pleomorphic, and morphology of a strain differs markedly, both among and within species. The morphology of the strains and the presence of endospores vary depending on the stage of the growth cycle. Generally, clostridia are large, rod-shaped organisms that range approximately from 0.4 to 1.3 μM in breadth and from 3 to 8 μM in length. The vegetative forms often occur singly, in pairs, or as long chains. Most of the clostridia are actively motile by peritrichous flagella.

Clostridia produce specialized structures, referred as endospores, which are very resistant to heat, radiation, drying, and chemicals. All *Clostridium* species are able to form spores, but there is considerable variation in the formation in nature and in the laboratory. Spores can be visualized by phase-contrast microscopy. The spores of most species are wider than the vegetative cells, imparting a distinctive appearance depending on where they are formed within the cell. When they are formed at the equator, the cells are spindle-shaped; when present or terminally, they impart a marked club-shaped or tennis racket appearance (Figure 1). Spores of most species can be produced in chopped meat broth or in complex media such as trypticase–peptone–glucose–yeast extract, a clear medium from which they can be purified and studied.

The spores released from the mother cell are distinct in structure and resistance properties. They are low in water content, possess distinct layers and compartments, and resist inactivation by heat, other physical treatments, and exposure to chemical agents. They are dormant and exist in an inactive solid state. Certain clostridia, such as *C. botulinum*, produce spores of high heat resistance, and the spores will remain viable after boiling in aqueous media for more than 1 h. This is the reason that additional barriers are needed to prevent clostridial growth when minimal heat treatment is used in many foods. Inactivation of *C. botulinum* spores or control of spore germination and outgrowth provides the basis for many food processing technologies and food regulation laws. Survival of spores of nonpathogenic heat-resistant mesophilic and thermophilic spores during processing of foods can lead to huge economic losses.

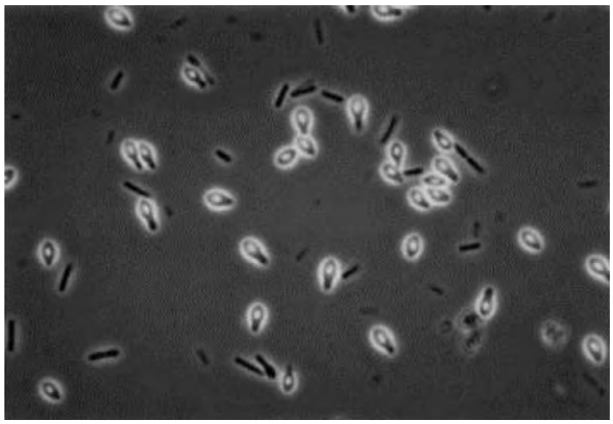

Figure 1 Characteristic morphology of *Clostridium botulinum* with the presence of endospores. The photograph shows a phase-contrast visual micrograph (×750) of a culture of *C. botulinum* type A.

Metabolic Properties

The clostridia are sensitive to oxygen and only grow under anaerobic conditions. They fail to thrive in aerobic environments, which differentiate them from aerobic spore formers including the anthrax bacillus (*Bacillus anthracis*) and the common hay bacillus (*B. subtilis*), which has been used extensively as a model organism for the study of sporulation and other properties. Under permissive environmental and nutritional conditions, spores germinate to form vegetative cells that divide by binary fission to reach high cell numbers in suitable nutritional media. When nutrients are limiting, vegetative cells form endospores in an intricate differentiation process. The spores exist as dormant bodies until they encounter a permissive growth environment, and the spores germinate, thus perpetuating the vegetative/spore cycle. In certain clostridia such as *C. perfringens*, extracellular toxin formation appears to coincide with sporulation, while in other species such as *C. botulinum* and *C. tetani*, the highest yields of toxin occur in populations of vegetative cells that do not sporulate.

Species differ widely in the substrates that they ferment. Some species ferment biomass including cellulose, starch, hemicellulose, and pectins by virtue of production of extracellular degradative enzymes, and they anaerobically convert the resulting sugars to ethanol, organic acids, and other products including various gases. Several species are putrefactive, degrading proteins with the formation of odorous nitrogenous products characteristic of rotting foods. Some clostridia can utilize nucleic acids, purines, and pyrimidines, and a few species such as *C. pasteurianum* utilize molecular gaseous nitrogen as their sole source of nitrogen and convert it to organic nitrogen cellular components (nitrogen fixation).

Some species of clostridia can grow over a wide range of pH and temperatures. Most species prefer growth within the pH range of 6–7, but some grow at pH below 4 or above 8. Clostridia favor temperatures of growth from 25 to 37 °C, but several species of psychrophilic clostridia grow at temperatures below 10 °C and several thermophilic species grow at temperature above 60 °C. The psychrophiles are important in food spoilage, while many of the thermophilic clostridia are valuable as sources of durable enzymes for industrial processes.

Special considerations are needed for isolation and cultivation of clostridia. First, since most species are strict anaerobes, specialized equipment and methodologies are required to provide an anaerobic environment for growth. Anaerobic jars, specialized culture tubes, glove boxes, and reduced media are needed to exclude oxygen and provide the needed redox environment. Second, clostridia tend to grow as mixed cultures with other anaerobes, and strict attention is needed to affirm the purity of the culture under study or the culture used in an industrial process. Many clostridia are subject to bacteriophage infection that will decrease solvent, enzyme, or toxin yields. Safe laboratory practices and immunization of personnel may be needed for the study of toxigenic species such as *C. botulinum* and *C. tetani*.

Genetics of Clostridium

The genetic study of the clostridia is in its infancy, but significant advances have been made in recent years. The application of molecular biology techniques and genomics has led to certain genetic tools including cloning and expression vectors and gene inactivation systems. Gene transfer technology using transformation (usually electroporation) or conjugation has been developed for *C. perfringens*, *C. acetobutylicum*, *C. difficile*, and *C. botulinum*, and genetic tools to understand virulence and to improve industrial processes are becoming increasingly available. The achievement of the complete genome sequences of a growing number of *Clostridium* spp. has provided a foundation for elucidating important genetic and phenotypic properties and for providing interesting comparative lifestyle and evolutionary analyses. Since the coding genomic DNA of most clostridial species is A–T-rich, it can be difficult to express cloned clostridial genes, such as in *Escherichia coli* or *Saccharomyces cerevisiae*, due to the limited availability of the required cognate tRNAs for protein synthesis. Much work is still needed to elucidate the modes of genetic regulation and the integration of pathways into the overall biology of the clostridia.

Plasmids and bacteriophages are commonly found in clostridia, particularly in pathogenic species. Although most plasmids encode unknown (cryptic) functions, some have been demonstrated to possess genes for toxins and antibiotic resistance. Bacteriophages are widespread in pathogenic and industrial strains of clostridia. In certain pathogens, phages carry genes for toxins that can be transferred to normally nonpathogenic clostridia by infection. Phage infection and culture lysis have been a continual problem of clostridial cultures used for solvent production. Strain degradation and the loss of capacity of clostridia to produce high yields of solvents have also been problematic for industrial processes.

Medical Importance and Industrial Applications of Clostridia

Several clostridial species are of medical and industrial importance (Table 1). Many species are pathogenic to humans and animals, mainly through the production of protein toxins, enzymes, and virulence factors during infection. Other species have beneficial aspects in biotechnology, such as production of valuable metabolites, formation of enzymes catalyzing bioconversions, secretion of enzymes and proteins that convert wastes to higher value products, and formation of enzyme systems that metabolize pollutants or detoxify xenobiotics under anaerobic conditions (Table 1). One of the most remarkable applications of the clostridia in industrial microbiology is the utilization of botulinum neurotoxin for the pharmacological treatment of a diversity of human disorders characterized by involuntary or hyperactive muscle movements. The practical significance of clostridia to humankind is summarized in Table 1.

Table 1 Significance of clostridia in human welfare

Property or role	*Important products and species*
Ancient dyeing	Indigo formation by *Clostridium isatidis*
Cause of disease in humans and animals, for example, tetanus, botulism, gas gangrene, wound infections, enteric infections, blackleg, malignant edema	Various toxins are virulence factors including neurotoxins, cytotoxins, collagenases, lipases, lecithinases, DNases, RNases, ADP-ribosyltransferases, hyaluronidases, hemolysins, and other protein toxins produced by toxigenic *Clostridium* spp. including *C. tetani*, *C. botulinum*, *C. perfringens*, *C. difficile*, *C. histolyticum*, and *C. septicum*
Production of solvents and organic acids	Production of various acids, alcohols, and gases by various clostridia, particularly *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum*, *C. pasteurianum*, *C. propionicum*, and *C. sporogenes*. Products include acetic acid, propionic acid, lactic acid, butyric acid, ethanol, acetone, 2-propanol, 1, 2-propanediol, 1,3-propanediol, and butanol
Food spoilage	Several clostridia cause various types of food spoilage. *C. tyrobutyricum* and *C. butyricum* cause gaseous spoilage of cheeses. Several species of psychrotrophic clostridia cause gas formation in vacuum-packaged chilled meats and certain other food products. Thermophilic clostridia including *C. bifermentans* and *C. nigrificans* cause thermophilic sulfide spoilage of canned foods or foods in tropical environments. Clostridia growing at acid pH conditions such as *C. pasteurianum*, *C. butyricum*, and *C. thermosaccharolyticum* can lead to gaseous spoilage of canned and pouched products
Source of industrial enzymes	Clostridia produce a variety of enzymes that are important industrially. These include polymer-degrading enzymes such as cellulases, amylases, pectinases, proteinases, and collagenases. Many of these enzymes have high thermal stability. Important clostridial species as sources of enzymes include *C. thermocellum*, *C. thermosulfurogenes*, *C. thermohydrosulfuricum*, and *C. thermosaccharolyticum*. Enzymes used in bioconversions and fine chemical syntheses as well as for the degradation of pesticides and xenobiotics are produced by *Clostridium* spp.
Source of pharmaceuticals and use in cancer therapy	Botulinum neurotoxin is produced by *C. botulinum* and has been used for the treatment of a variety of neurological disorders including dystonias, involuntary muscle disorders, pain, inflammatory syndromes and for cosmetic purposes. Certain clostridial species have been investigated for use in cancer therapy. The spores of clostridia target tumors, where they are able to proliferate in the malignant tissue. They produce native toxins or elicit compounds that are deleterious for tumor survival

Clostridial Diseases

Many clostridia cause devastating diseases of humans and animals. Known diseases produced by clostridia include botulism, tetanus, gas gangrene (clostridial myonecrosis), antibiotic-associated diarrhea and pseudomembranous colitis, foodborne diarrhea, necrotic enteritis of humans and domestic animals, and blackleg and malignant edema in cattle and sheep. Recently, wound infections and botulism have been detected in intravenous drug users from spore contamination in the illicit drugs.

One of the most fascinating features of the clostridia is their production of a wide array of biologically active antigens, many of which cause diseases in humans and animals. About 20 clostridial species produce protein toxins that are lethal for animals and/or humans. Genomic analyses have supported that clostridia produce a number of virulence factors, especially a large variety of enzymes and toxins. The clostridia produce more types of protein toxins than any other bacterial genus. Some of the toxins are notable for their extreme potency; for example, botulinum and tetanus toxins are the most lethal poisons known. Botulinum toxin can be preformed in foods and cause a true intoxication, whereas most of the other pathogenic clostridia produce toxins and inflict damage during infection of the host.

Food Spoilage and Food Poisoning

The spores of clostridia are widespread in the environment and present in many foods. Spores from several species resist minimal food processing procedures, and they can later germinate and cause spoilage or safety problems in foods. Two clostridial species, *C. botulinum* and *C. perfringens*, are the major species causing food poisoning. Several other clostridial species can spoil foods by forming gas from organic acids or carbohydrates or by carrying out putrefactive degradation of food proteins. Recently, clostridia that grow at cold temperatures (psychrotrophic clostridia) have been increasingly involved in gas formation and organoleptic spoilage of several classes of foods.

Solventogenic Clostridia

Clostridia produce fermentation end products of industrial importance, including acetone, butanol, ethanol, 1,2-and 1,3-propanediol, acetate, and butyrate. The acetone–butanol fermentation process employing *C. acetobutylicum* was used for about 30 years on a large industrial scale, but due to economic constraints, these solvents are now mostly produced by chemical synthesis. Ethanol synthesis from thermophilic clostridia growing on waste feedstocks, such as cellulose, has attracted much interest, but the process is limited by low ethanol yields and formation of side products. Industrial interest in clostridial metabolites currently is focused on products that are difficult to prepare in good yields by chemical synthesis, such as chiral compounds.

Industrial Enzymes from Clostridia

Several thermophilic clostridia produce remarkably stable, highly efficient enzymes that selectively degrade inexpensive substrates, including cellulose, xylan, pectin, pullulan, collagen, and starch. These enzymes are being developed for industrial uses in food processing and production, in detergents, and for other applications. Proteases from clostridia are used for chillroofing of beer and for other applications in food processing. Clostridia also produce enzymes of value in bioconversions and in degradation of pollutants and xenobiotics. Thus, the clostridia appear to have important roles in environmental bioremediation.

Use of Clostridial Toxins as Pharmaceuticals

Botulinum neurotoxin is the most poisonous substance known on Earth. Botulinum toxin has become an extremely important pharmaceutical for more than 100 neurological disorders. Selective injection of botulinum toxin into nerve–muscle regions produces a local weakening of proximal muscles, providing relief from excessive involuntary muscle contractions and pain or disfigurement. Botulinum neurotoxin is also used for cosmetic enhancement of humans. The outstanding properties of botulinum toxin as a pharmacological agent is its extreme specificity for nerves and its long duration of action. Clostridial toxins or fragments of the entire toxins are being developed for drug delivery to the nervous system, prevention of food poisoning, prevention of inflammatory responses, and stimulation of the immune system. It is remarkable that nature's most poisonous substances are being employed as therapeutics to benefit humans.

Clostridia in Cancer Therapy

It has long been recognized that spores administered to animals localize in necrotic tissues and in tumors. This is mainly due to the anaerobic environment present in these tissues. The first attempt to use clostridia for the treatment of cancer in animals was reported in 1935. Currently, there is interest in enhancement of clostridial oncolysis to suppress growth or to kill tumors in humans, as well as in cancer diagnosis.

Conclusions

The clostridia are ancient evolutionary ancestors within the Gram-positive bacteria. Outstanding biological properties of this important group of bacteria include their strict anaerobic metabolism, the formation of resistant endospores, and the synthesis of a wide array of distinctive toxins and enzymes. Although clostridia have been known for centuries to cause devastating diseases in humans, such as tetanus, botulism, and gas gangrene, they also are a source of metabolites and enzymes that have considerable utility in biotechnology. Advances in our knowledge of ecology, metabolism, genetics, and genomics of clostridia promise to increase the biological understanding and appreciation of the clostridia, prevent dreadful diseases characteristic of the clostridia, and enhance their industrial utility to humankind.

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Commensal to Pathogen Transition of *Candida albicans*

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Glossary

Attenuated (virulence) Reduced capacity to induce damage/disease.
Biofilm A structured community of microorganisms that adhere to each other and are commonly embedded within a self-produced extracellular matrix composed of polysaccharides, proteins, and DNA.
Candidiasis Infections caused by *Candida* species.
Colonization Growth (or prolonged survival) of microorganisms in a distinct (host) niche.
Commensal Microorganism that growths within a host without (negatively) affecting the host species.
Dysbiosis Disturbance of the microbiota leading to microbial imbalance and commonly reduced species diversity; often associated with increased abundance of a few taxa or species.
Filamentation The production of filamentous growth forms (hypha, pseudohypha).
Hypha Long filaments of connected fungal cells, characterized by directed growth at the tip, branching, parallel cell walls and the absence of constriction at the septa separating individual cells.
Hypha-associated Genes or proteins that are usually only expressed by hypha and not by yeast cells.
Inoculation Introduction of a microorganism into a host or growth medium.
Macronutrients Nutrients that provide energy, for example, carbohydrates and amino acids.
Microbiota Ecological community of commensal, symbiotic and pathogenic microorganisms found at a distinct site/within a distinct host niche.
Micronutrients Nutrients required by organisms throughout life in small quantities as necessary cofactors for metabolism, for example, trace minerals and vitamins.
Morphogenesis The process by which an organism develops its shape. In the context of *Candida* it usually refers to the formation of hypha from yeast cells, whereas the term "reverse morphogenesis" is sometimes used to describe the formation of yeast cells from filamentous growth forms.
Morphotype Morphological growth form; *C. albicans* has different morphotypes, for example, yeast, pseudohypha, and hypha.
Mycobiota Ecological community of fungi found at a distinct site/within a distinct host niche.
Nosocomial infection An infection contracted by a patient while under medical care.
Opportunistic infection An infection that usually does not occur in healthy hosts but only if predisposing factors (weakened immune system, breached epithelial barriers) are present that give the pathogen the opportunity to establish an infection.
Pathobiont A microorganism that under normal circumstances lives as a commensal but has the potential to cause disease.
Pathogen A microorganism that causes disease.
Pathogenesis The mechanisms leading to disease.
Pseudohypha Elongated fungal cells, characterized by constrictions between individual cells.
Pyroptosis A form of programmed cell death of immune cells that occurs most frequently upon infection with intracellular pathogens and leads to the release proinflammatory cytokines, thereby contributing to inflammation.
Virulence factor Gene or attribute that contributes to the virulence of a pathogen.
Virulence The ability of microorganisms to cause damage to a host.

Commensal to Pathogen Transition of *Candida albicans*

Introduction: *Candida albicans* as Commensal and Pathogen

Candida albicans is an archetypical opportunistic pathogen: While it is the most common cause of systemic fungal infections, it is also carried by two out of three humans on mucosal membranes without causing any damage (Kett et al., 2011; Odds, 1987; Pfaller and Diekema, 2007; Richardson and Rautemaa, 2009; Thewes and Hube, 2008). As a commensal, the fungus is commonly found within the oral cavity, gastrointestinal tract (GI tract), and on the urogenital mucosa. On all sites, it has to adapt to the local environment that differs significantly between various anatomical locations. Environmental conditions are on the one hand defined by properties of the local mucosa. On the other hand, *C. albicans* has to establish its niche within the complex microbiota that also

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varies depending on the anatomical site (Morgan et al., 2013). Adaptation to these different niches requires versatile nutrient acquisition, metabolic flexibility, and stress resistance.

C. albicans adaptability contributes to its success as an opportunistic pathogen. If the balance between host defense systems, microbiota, and *C. albicans* is disturbed, for example, due to immunosuppression, impairment of mucosal barrier integrity, or disturbance of the bacterial microbiota by antibiotics, *C. albicans* can cause both local and disseminated infections (Goncalves et al., 2016; Kullberg and Arendrup, 2015; Suleyman and Alangaden, 2016). Mucosal infections can occur at all sites that are colonized with *C. albicans* and are often associated with specific risk factors that do not predispose to infections at other sites: For example, oropharyngeal candidiasis is very common in AIDS patients but these patients do not have an increased risk for other forms of candidiasis (Fidel, 2011). Chronic mucocutaneous candidiasis is often associated with gene mutations that impair Th17 immunity. Even though these patients do suffer continuously from invasive local candidiasis, the exact kind of mutations in the IL-17 pathway determines whether they additionally have an increased risk for disseminated infection (Okada et al., 2016). These examples illustrate the complexity and niche specificity that applies to the host–pathogen interactions that control the commensal-to-pathogen shift in *C. albicans*.

In the following parts we will summarize the current knowledge on which features make *C. albicans* a successful commensal, focusing on the GI tract, and discuss some of the main attributes that contribute to its success as an opportunistic pathogen.

Candida albicans as a Commensal: Living With the Host and Microbial Neighbors

As mentioned above, both the environmental conditions and the present microbiota vary between different mucosal sites. Consequently, different aspects of *C. albicans* physiology contribute to varying extend to fungal survival in the different mucosal niches. In the following part we focus on the GI tract; for further information on *C. albicans* as a commensal and pathogen in the oral and vaginal niche we refer the reader to recent comprehensive reviews (Cassone, 2015; Donders and Sobel, 2017; Goncalves et al., 2016; Hebecker et al., 2014; Lalla et al., 2013; Peters et al., 2014; Singh et al., 2014).

Fungal Factors That Contribute to *Candida albicans* Survival in the Human Gut

In the human body, the GI tract is not only the major reservoir of *C. albicans*, but also the dominant source for candidiasis, a life-threatening systemic infection caused by this yeast (Mittal and Coopersmith, 2014). Research in fact provides evidence that the majority of systemic *Candida* infections are endogenous, so patients are infected with their very own *C. albicans* strains (Miranda et al., 2009). As fungi were long underrepresented in microbiome studies due to technical reasons, the exact densities of fungi in the GI tract and other body niches are still poorly understood. However, there is increasing interest toward the entity of fungi living on and in the human body, termed mycobiota, and its interaction with the human host (Underhill and Iliev, 2014). In approximation, recent studies estimate at least 0.1% of all microorganisms (so 10^{11} out of 10^{14}) in the human GI tract to be fungi (Ianiro et al., 2016; Qin et al., 2010). In this context, it is interesting that *C. albicans* is among the few microbial species enriched in patients upon intensive care, while the overall microbial diversity under these circumstances is decreased (Zaborin et al., 2014). *Candida* colonization is also more common among patients with GI tract pathologies, like inflammatory bowel disease (IBD) or Crohn's disease (Kumamoto, 2011). Furthermore, antibiotic treatment, reducing the number of bacteria, is associated with increased fungal colonization (Underhill and Iliev, 2014). This indicates that *C. albicans* is well adapted to the GI tract and can grow to high densities if bacterial competition is reduced. As increased colonization with *C. albicans* constitutes a risk factor for candidiasis (Muskett et al., 2011), recent research increasingly focuses on the properties that allow the fungus to colonize mucosal surfaces and especially the gut (recently reviewed in Prieto et al. (2016)).

Within the length of the GI tract and across the diameter of the intestine, several environmental parameters, including pH, oxygen and nutrient availability, and concentrations of hydrolytic enzymes and antimicrobial peptides (AMPs), vary significantly (Khutoryanskiy, 2015; Zheng et al., 2015a,b). Some of these factors are interconnected: the pH for instance ranges from highly acidic in the stomach to alkaline in the small intestine. It influences availability of micronutrients, such as iron, the proton gradient, that is important for nutrient uptake, and affects the efficacy of fungal enzymes (Davis, 2009). *C. albicans* can grow within a broad pH range, mediated by the sensing of environmental pH via the plasma membrane receptors Rim21 and Dfg16. The associated signaling cascade leads to activation of the transcription factor Rim101 at neutral to alkaline pH. Other factors involved in the response to changing pH are the cell wall β-glycosidases Phr1 and Phr2, Mds3 and calcineurin. Additionally, *C. albicans* is able to actively increase the local pH by metabolizing amino acids or polyamins, leading to the production and release of ammonia (Vylkova et al., 2011). To which extend pH sensing and adaptation exactly contribute to survival of *C. albicans* in the gut has yet to be experimentally addressed.

Even though the GI tract might appear rich in nutrients, specific types of carbon and nitrogen sources can be scarce in distinct microniches (recently reviewed in Fischbach and Sonnenburg (2011), Flint et al. (2012)). Furthermore, *C. albicans* has to compete with other members of the microbiota for available nutrients. The metabolism of *C. albicans* is very versatile and the fungus can use a wide range of both carbon and nitrogen sources (Brown et al., 2014; Ene et al., 2014). Peptides, amino acids, sugars, and organic acids can be taken up by specific transporters and used to produce energy or as building blocks. As they commonly need to be released or extracted from macromolecules, *C. albicans* possesses a number of proteases and lipases to support nutrient liberation (Polke et al., 2015). Typically, those enzymes include secreted aspartic proteases (Saps), lipases (Lips), and Plb phospholipases as well as several enzymes for the degradation of polysaccharides (Klis and Brul, 2015).

Overall, the gene expression of *C. albicans* colonizing the mouse gut appears to be distinct from other profiles. By comparing *C. albicans* cells invading host tissue during disease with *C. albicans* colonizing the mouse cecum, interesting similarities were

revealed, but also niche- and mode-specific differences were detected (Rosenbach et al., 2010; Thewes et al., 2007; Walker et al., 2009; Zakikhany et al., 2007). The commensal gene expression profiles shared similarities with both, logarithmically growing cells and cells in stationary phase under standard in vitro conditions (Rosenbach et al., 2010). The glucose metabolism was induced even though the gut is considered to be a low-glucose niche (Rosenbach et al., 2010). Remarkably, although *C. albicans* grows predominantly as yeast in the mouse cecum (see also below), several genes that encode certain adhesins were also expressed despite that they were previously thought to be exclusively hypha-associated (Herwald and Kumamoto, 2014; Rosenbach et al., 2010).

So far, studies aiming at experimentally testing the contribution of factors to *C. albicans* fitness as a commensal of the GI tract have focused mainly on transcription factors (TFs) due to their central role for controlling expression of physiological traits. One well studied TF is Efg1 that is involved in filament formation (Stoldt et al., 1997). *EFG1*-deficient *C. albicans* strains are unable to form filaments under many in vitro conditions (Stoldt et al., 1997). In colonization experiments using mice, Efg1 influences *C. albicans* survival in the GI tract as *EFG1*-deficient strains were found to be more efficient colonizers of the mouse gut (Pierce et al., 2013). The stress response in the cecum was also induced in an Efg1-dependent manner (Pierce et al., 2013). Further TFs required for *C. albicans* filamentation under certain laboratory conditions and important for GI tract colonization are Cph2 and Tec1 (Rosenbach et al., 2010). Another transcription factor involved in the intestinal commensal state is Efh1, a paralog of *EFG1* whose deletion does not affect filamentation under in vitro conditions (Doedt et al., 2004). Efh1 appears to actively promote *C. albicans* commensalism but is dispensable for systemic infections (White et al., 2007). Using a signature-tagged mutagenesis approach, Pérez *et al.* tested a library of 77 TFs that did not affect growth in vitro to assess their ability to colonize the mouse gut. Thereby they identified six factors that contributed to colonization: *TYE7*, *RTG1*, *RTG3*, *LYS144*, *HMS1*, and *orf19.3625* (Perez et al., 2013). Rtg1, Rtg3, Tye7, and Lys144 affect expression of genes involved in nutrient acquisition and metabolism (Perez and Johnson, 2013; Rosenbach et al., 2010). Furthermore, *C. albicans* mutants lacking the gene *PHO4*, encoding a transcription factor involved in phosphate metabolism, were outcompeted by the wild type during colonization (Urrialde et al., 2016). These results thus underline the role of metabolic adaptation and nutrient acquisition for fitness in the gut. Finally, the kinase Hog1, a central factor in the general stress response of *C. albicans*, was shown to contribute to intestinal fitness (Prieto et al., 2014), indicating that *C. albicans* is exposed to stressful conditions in this environment.

The current knowledge implies that morphogenesis, metabolism and stress response affect *C. albicans* survival in the GI tract. This picture is certainly still incomplete and to which extent and by which mechanisms distinct pathways or factors contribute to fitness remains to be determined. Currently ongoing research will likely provide further information on distinct factors influencing the life of *C. albicans* as a gut commensal in the near future.

Morphology of *Candida albicans* in the Gut

One of the most remarkable features of *C. albicans* is its morphological flexibility (Noble et al., 2016). This includes growth as typical round, budding yeasts and filamentous growth either as long true hyphae or pseudohyphae. Both morphotypes are essential for virulence: Yeasts are highly proliferative and thought to be important for dissemination, while hyphae are important for invasion and tissue destruction (Gow et al., 2002; Jacobsen et al., 2012; Whiteway and Oberholzer, 2004). While yeasts, pseudohyphae, and hyphae are isolated from sites of infection, filamentous forms of *C. albicans* are found only rarely during gut colonization (Kayser, 2010; Noble et al., 2016; White et al., 2007). As the intestinal environment provides strong triggers for filamentation (e.g., 37°C, high CO_2, and peptidoglycan shed by bacteria) (Noble et al., 2016; Sudbery, 2011; Wang and Xu, 2008), the absence of prominent filamentation is unlikely due to a lack of hypha induction but more likely mediated by other environmental factors or influenced by the bacterial microbiota.

In addition to normal yeasts, also known as white a/α cells, a gut-specific yeast-like morphotype termed gastrointestinally-induced transition (GUT) was recently identified in this particular niche (Pande et al., 2013). GUT cells are phenotypically and metabolically distinct from other morphotypes and can be produced by passaging *C. albicans* through the murine GI tract. GUT cells are characterized by overexpressing *WOR1*, encoding a transcription factor that is not expressed in white cells and controls the white-opaque switching important for mating (Huang et al., 2006). However, GUT cells are distinct from mating-competent opaque cells and remain heterozygous at the mating type locus. Compared to other yeast forms, GUT cells have an elongated shape with smooth surface leading to altered colony morphology. GUT cells outcompete white cells in murine colonization, possibly due to an adapted metabolism that facilitates highly efficient utilization of the nutrients available in the gut (Pande et al., 2013).

Animal Models Used to Investigate *Candida albicans* Commensalism in the Gut

Currently, our knowledge on specific factors required for commensalism in the gut is still limited and nearly exclusively deduced from studies in mice, and a few studies in rats (Koh, 2013). Still, there are significant differences between humans and mice that need to be kept in mind when transferring knowledge from one organism to the other. Differences that might influence pathogenesis of infections are, for example, the composition of blood leukocytes, the functionality of neutrophils, or the inflammatory response (Ermert et al., 2013; Mestas and Hughes, 2004; Seok et al., 2013; Warren, 2009). At the commensal state of *Candida*, there are also some factors to be considered when mimicking men in mice. Laboratory mice are usually not colonized with *C. albicans*, even though other *Candida* species such as *Candida krusei* can be present (Dollive et al., 2013). This might point to a distinct role of the murine gut microbiota mediating colonization resistance toward *C. albicans*. However, differences in exposure might also affect these findings. Laboratory mice kept under hygienic conditions are probably more likely to harbor

environmentally-derived yeasts (such as *C. krusei*) than *C. albicans* that is only found associated with warm-blooded hosts (Odds, 1987). Furthermore, timing of exposure might be relevant. Humans are believed to be colonized during birth or the first days of life when the gut microbiota is in its earliest stages. Indeed, also infant mice are more susceptible both to colonization and spontaneous dissemination of *C. albicans* (Guentzel and Herrera, 1982; Pope et al., 1979). Furthermore, food composition affects colonization resistance (Gunsalus et al., 2016; Kadosh et al., 2016).

In general, however, many studies have shown that the gut microbiota of adult mice and other rodents mediates colonization resistance toward *C. albicans* (Kennedy and Volz, 1985a,b; Koh, 2013). Therefore, depletion of bacteria is necessary to facilitate stable colonization of adult mice with *C. albicans* (see Fig. 1). Experimental procedures published in different studies vary in the choice of antibiotics and *C. albicans* inoculation regiment (selected recent examples in Table 1), but mutually a combination of antibiotics is used. The infectious dose appears to have less influence on long-term colonization as proliferation to high, stable colonization levels occurs within a few days, even after inoculation of a minimal dose of 100 *Candida* cells (Prieto and Pla, 2015). Importantly, colonization experiments with single *C. albicans* mutants might not always reveal the influence of genes on colonization efficiency and competition experiments might be required to detect fitness defects (Prieto et al., 2014).

According to our current understanding, *C. albicans* generally does not spontaneously disseminate in significant numbers from the gut to the blood stream of adult mice (Ekenna and Sherertz, 1987; Koh, 2013). In order to achieve translocation and disseminated infection, impairment of the epithelial barrier and suppression of the innate immune system are required

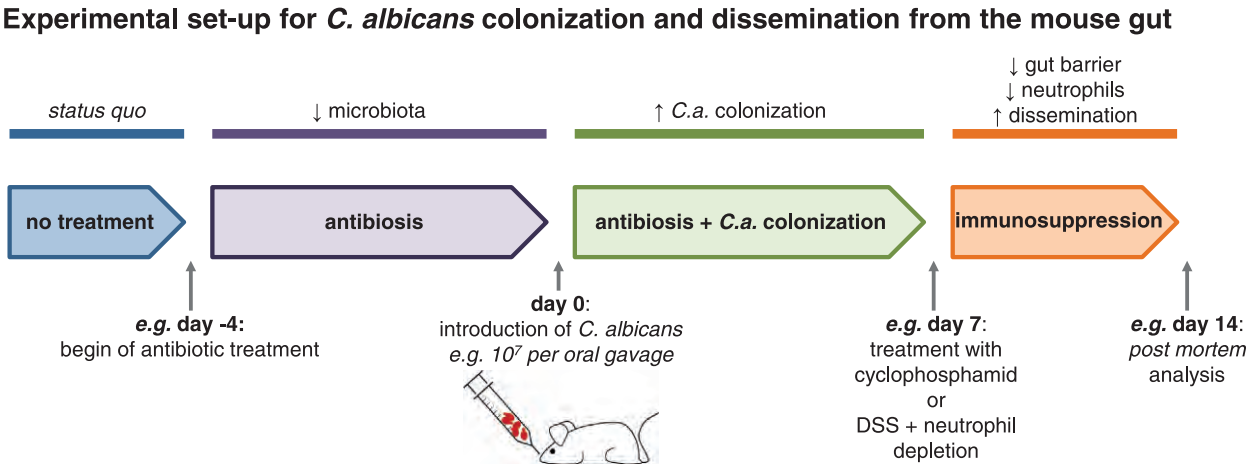

Fig. 1 Set-up for *Candida albicans* colonization and dissemination from the mouse gut. Widely-used experimental strategies to reduce the preexisting microbiota (blue), to colonize with *C. albicans* (*C.a.*, green), and to induce dissemination (orange) from the mouse gut. Illustration by I.D. Jacobsen.

Table 1 Overview of colonization strategies with antibiosis prior to *Candida albicans* inoculation of mice

Antibiosis	*Inoculum*	C. a. *strain*	*CFUs/g feces*	*Time point* p.i.	*References*
Bacitracin Streptomycin Gentamycin	Oral gavage, 10^7 cells	CAF2	10^6–10^7	3 d	Wiesner et al. (2001)
Tetracycline Streptomycin Gentamycin	Oral gavage, 5×10^7 cells	DAY185	10^5	18 d	White et al. (2007)
Streptomycin Penicillin G	Drinking water, 10^7 CFUs/mL	SC5314, CAF2-1	10^7	21 d	Koh et al. (2008)
Tetracycline Streptomycin Gentamycin	Oral gavage, 5×10^7 cells	CKY101	10^7	3 d	Pierce et al. (2013)
Streptomycin Bacitracin Gentamycin	Oral gavage, 10^7 cells	CAF2	10^6–10^7	40 d	Prieto et al. (2014)
Streptomycin Bacitracin Gentamycin	Oral gavage, 10^6 cells	CAF2	10^7	3 d	Prieto and Pla (2015)
Streptomycin Penicillin	Drinking water, 10^7 CFUs/mL	SC5314	10^6–10^7	10 d	Vautier et al. (2015)

Note: Colonization strategies presented in different publication varied in choice of antibiotics, application procedure, dosage of *C. albicans* (*C.a.*) inoculum, stability of colonization (by fungal load in feces) as finally the time point of analysis.

Source: Contributed by A. Elisabeth Greßler.

(Koh et al., 2008). This can be achieved, for example, by induction of colitis using the detergent dextran sulfate sodium and selective depletion of neutrophils. Alternatively, treatment with cyclophosphamide, a chemotherapeutic drug leading to the death of fast-proliferating cells, results in a combination of both, reduced barrier function of the enteric epithelium and immunosuppression via leukopenia (see Fig. 1). Consequently, mice treated with cyclophosphamide are more likely to develop disseminating candidiasis originating from the gut (Ekenna and Sherertz, 1987). Murine sepsis models based on mechanical disruption of the gut, such as cecal ligation and puncture, are not commonly used as a model for gut-derived candidiasis (Toscano et al., 2011; Warren, 2009), but might be an alternative approach. A typical experimental set-up for a colonization-dissemination experiment as currently used in the field is depicted in Fig. 1.

Candida albicans as Part of the Microbiota

In addition to immunosuppression and breach of mucosal barriers, the disturbance of the microbiota constitutes a major risk factor for disseminated candidiasis. Long-term antibiotic treatment and malnutrition during intensive medical care are prominent causes of dysbiosis (Zaborin et al., 2014). This indicates that the *C. albicans* population in the gut is usually controlled by commensal bacteria and possibly other fungi colonizing the respective niche in the body. This is mirrored in mouse models of *Candida* gut colonization: As mentioned above, extensive antibiosis prior to introduction of the fungus is necessary to achieve stable colonization with *C. albicans* (Fan et al., 2015; Koh, 2013). Like the bacterial microbiota, the fungal "mycobiota" is more and more appreciated for its immune-modulatory properties, and not only as a source of infection (Gouba and Drancourt, 2015; Suhr and Hallen-Adams, 2015; Underhill and Iliev, 2014). To date, little is known about the exact mechanisms of microbial competition and microbe–microbe interaction and microbe–host cross-talk and how this affects host health and disease development. Yet, recent research provides evidence for an intricate interplay of many actors that exceeds simple competition for resources.

Notably, interactions between microbes are numerous and occur naturally on all surfaces of the human body, i.e., the skin, the lung, the oral cavity, the urogenital tract and the GI tract (Peleg et al., 2010; Peters et al., 2012). The specific nature of these interactions varies depending on the species involved, but it can be expected that some general concepts are conserved. To date only few studies have specifically addressed the interactions of *C. albicans* with intestinal bacteria. Here, we will briefly summarize the current knowledge on *Candida*-bacteria interplay in general (for more detailed information we refer to Polke et al. (2015)).

Candida and bacteria can interact by (1) direct physical contact, (2) communication via released molecules, and (3) indirectly by modification of the environment and the host response. Given the high density of microorganisms on mucosal surfaces, direct contact is highly anticipated. Interestingly, different bacteria have been described to form aggregates with *C. albicans* and are often found to bind fungal hyphae better than yeast cells (Adam et al., 2002; Bagg and Silverwood, 1986; Bamford et al., 2009; Brand et al., 2008; Harriott and Noverr, 2010, 2011; Holmes et al., 1996; Jenkinson et al., 1990; Kong et al., 2015; Metwalli et al., 2013; Peters et al., 2010; Schlecht et al., 2015; Shirtliff et al., 2009). Binding can involve both *C. albicans* and bacterial adhesins as well as mannosylation of fungal surface proteins (Bamford et al., 2009; Brady et al., 2010; Dutton et al., 2014; El-Sabaeny et al., 2000; Holmes et al., 1996; Klotz et al., 2007; Kong et al., 2015; Schlecht et al., 2015; Silverman et al., 2010; Tati et al., 2016; Wright et al., 2013). In the oral cavity, these interactions can promote *C. albicans* colonization and persistence (Cannon and Chaffin, 2001; Shirtliff et al., 2009). Furthermore, adhesion to invading *C. albicans* hyphae can aid bacteria that otherwise would be unable or little efficient to penetrate host cells, as it has been shown for *Staphylococcus aureus* (Alves et al., 2014; Kong et al., 2015; Schlecht et al., 2015). Synergistic interactions of *C. albicans* and bacteria are also found in mixed-species biofilms, an important source of nosocomial infections (Hirota et al., 2017; Shirtliff et al., 2009; Taff et al., 2013). Bacteria, including species present in the gut such as *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, or *Salmonella typhimurium*, have been found to also affect filamentation of *C. albicans*, an effect often mediated by small, secreted signaling molecules (Brand et al., 2008; Cruz et al., 2013; Hogan et al., 2004; Kim and Mylonakis, 2011; Peleg et al., 2008; van Leeuwen et al., 2016). Most of these interactions negatively affect hypha formation and it is thus tempting to speculate that it is the microbiota that keeps *C. albicans* in the less invasive yeast form in the gut (Förster et al., 2016). Reducing bacterial microbiota by antibiotics might relief this restriction and thereby promote filamentation and invasion; however, this hypothesis has not been tested experimentally yet. These examples illustrate that *Candida*-bacteria interactions can be considered antagonistic, and therefore beneficial for the host, or mutualistic and thereby detrimental for the host (Förster et al., 2016; Polke et al., 2015).

Recent studies indicate that the human gut microbiota is very diverse with more than 1000 bacterial species and up to 75 fungal genera described (Mar Rodriguez et al., 2015; Qin et al., 2010). It furthermore becomes increasingly clear that a balanced composition of bacteria and fungi is associated with intestinal health, while dysbiosis is accompanied by a range of diseases (Lagunes and Rello, 2016; Mittal and Coopersmith, 2014; Sokol et al., 2016; Wheeler et al., 2016). The composition of the microbiota affects *C. albicans* survival in the gut and nonpathogenic gut-residing bacteria, i.e., Firmicutes and Bacteroidetes, that are involved in maintaining *C. albicans* colonization resistance in mice (Fan et al., 2015). One specific member of this group, *Bacteroides thetaiotamicron*, was demonstrated to mediate this effect by inducing the production of host antimicrobial effectors (Fan et al., 2015). Another group of bacteria with antagonistic relations to *C. albicans* are lactobacilli. Lactobacilli negatively affect *C. albicans* colonization by direct mechanisms, modification of the environment, and indirectly via cross-talk with host cells (Kelly et al., 2015; Noverr and Huffnagle, 2004; Romani et al., 2015; Wynne et al., 2004). While not being the only source, lactobacilli are known for their capacity to produce short-chain fatty acids (SCFAs) in the gut. SCFAs were demonstrated to increase barrier function of the gut epithelium and inhibit *C. albicans* filamentation (Kelly et al., 2015; Noverr and Huffnagle, 2004) and this mechanism might also contribute to colonization resistance and yeast growth.

Notably, while *C. albicans* is clearly affected by bacteria in the gut, the fungus likewise affects composition of the bacterial community and bacterial virulence. In mice, the introduction of *C. albicans* affects the composition and development of the microbiota after antibiotic depletion, leading to an enrichment of *Bacteroidetes* and *E. faecalis*, but slower regrowth of lactobacilli (Erb Downward et al., 2013). *C. albicans* furthermore dampens the virulence of *P. aeruginosa* in a colonization-dissemination model by reducing the expression of bacterial virulence factors (Lopez-Medina et al., 2015). In addition, *C. albicans* was also shown to promote growth of obligate anaerobic *Clostridium* ssp. under aerobic conditions, potentially boosting their ability to colonize the human gut (Fox et al., 2014; van Leeuwen et al., 2016). While we are just at the beginning of unraveling the triangle of *C. albicans*, gut-residing bacteria and the human host, these findings indicate that bacterial–fungal interactions affect not only the composition of the microbiota but might also significantly influence the development of either bacterial or fungal infections.

Features That Contribute to the Success of *Candida albicans* as Pathogen

Virulence is a multifaceted feature of microorganisms that includes the ability to thrive within host tissue, withstand rapid elimination by the immune system and the capability to cause tissue and/or organ damage. Keeping this in mind, it is not surprising that genes involved in various processes, from basic cell biology to specific interactions with host cells, have been demonstrated to affect the virulence potential of *C. albicans* (see (da Silva Dantas et al., 2016) for an overview). Here, we will focus on factors that influence the interaction of the fungus with host cells, nutrient acquisition and we will briefly discuss the role of the cell wall and morphogenesis for candidiasis.

Interaction of *Candida albicans* With Epithelial Cells

As a commensal *C. albicans* lives in close contact with – or depending on the presence of a mucus layer, in proximity to – epithelial cells without causing overt damage. This changes during infection characterized by increased fungal load, invasion into epithelial barriers (and underlying tissue), induction of an immune response and tissue damage. The factors that trigger the transition from commensalism to infection are only incompletely understood, but the disturbance of the microbiota clearly plays an important role (Fan et al., 2015; Lopez-Medina et al., 2015) and recently reviewed in Förster et al. (2016) and Höfs et al. (2016).

The first step toward infection is attachment of yeasts to host cells, in case of mucosal membranes epithelial cells (Fig. 2). This complex process is likely initiated by passive forces, for example, hydrophobic cell–cell interactions, followed by specific

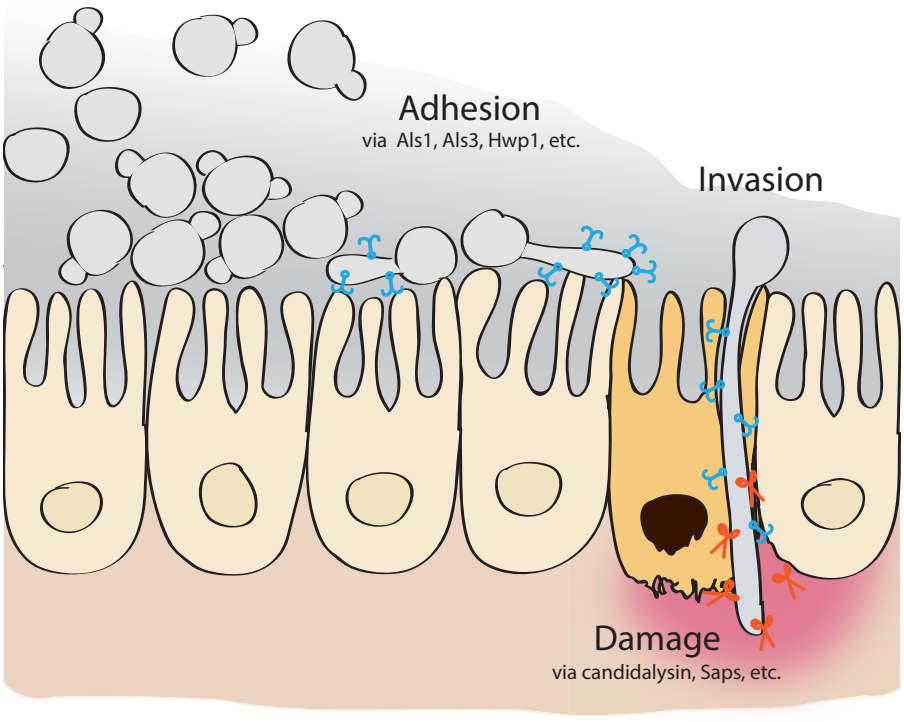

Fig. 2 Adhesion, invasion, and damage by *Candida albicans* in the human gut. Major steps of the *C. albicans* life cycle during the switch from commensal to pathogen. Hyphae-associated adhesins (blue anchors) mediate the attachment to enterocytes. Growing hyphae and subsequent release of candidalysin, proteases (orange scissors) and lipases penetrate the enterocyte barrier and destroy underlying cell layers. Illustration by M.J. Niemiec and M. Polke.

interactions between fungal and host cell surface molecules (a comprehensive overview has recently been published by Moyes et al. (2015). While some adhesins also occur on yeast cells, hyphae express adhesins, like Als3 and Hwp1, in larger number and more efficiently (Phan et al., 2007; Wächtler et al., 2011). Targeted host structures include cadherins, integrins and components of the extracellular matrix (Chaffin, 2008; Phan et al., 2007; Zhu et al., 2012). Of note, adhesion to host cells is a strong trigger of filamentation in vitro and hypha formation is important for the second step during infection: invasion (Fig. 2). Invasion into host cells occurs by two mechanisms, induced endocytosis and active penetration. Active penetration is a fungal-driven process that requires directed filamentous growth (Wächtler et al., 2012). The current knowledge suggests that in addition to physical forces provided by the turgor pressure, hydrolytic enzymes, especially Saps, support this process. Inhibition of aspartic proteases by pepstatin A significantly reduces invasion (Dalle et al., 2010); the contribution of individual Saps to this process is however still controversially discussed (Naglik et al., 2008, 2003). In contrast to active penetration, induced endocytosis requires the activity of host cells and occurs even with inactivated fungal cells. It is initiated by the recognition of distinct fungal surface proteins ("invasins") by host cell receptors, which triggers rearrangement of the cytoskeleton leading to uptake of the fungal cell. The first *C. albicans* invasin identified was Als3 that binds to cadherins on host cells (Phan et al., 2007). Another known *C. albicans* invasin is Ssa1 and further host receptors include the EGF receptor and the human epidermal growth factor receptor 2 (Sun et al., 2010; Zhu et al., 2012). Importantly, as Als3 is a hypha-specific protein, both active penetration and induced endocytosis depend largely on hypha formation. Therefore, hyphae are considered to be the invasive morphology in *C. albicans*.

Invasion is a prerequisite for damage but not necessarily linked to damage of host cells: As the host membrane remains intact following initial invasion, forming a glove-like structure surrounding growing hyphae (Wächtler et al., 2012; Zakikhany et al., 2007), the host cell is not immediately lyzed. Indeed, specific *C. albicans* mutants have been identified that show unaltered adhesion and invasion but significantly reduced damage in the interaction with epithelial cells. One of these mutants is *C. albicans eed1*Δ/Δ, lacking *EED1*, a factor that is essential for maintenance of hyphal growth and thus hyphal elongation. Following invasion, this mutant reverts back to yeast growth intracellularly, thereby reducing the strain on the host cell membrane afflicted by growing hyphae of wild type *C. albicans* (Martin et al., 2011; Zakikhany et al., 2007). Other examples are mutants lacking either *DUR31* or *HSP21*, that likewise show reduced hyphae formation following invasion (Mayer et al., 2012a,b). Most important, however, was the discovery that an *ECE1* deletion mutant is highly attenuated in epithelial damage, despite normal filamentation. *ECE1* was already identified in the 1990s as one of the genes most highly expressed by hyphae compared to yeast cells (Birse et al., 1993). Yet, its function was only elucidated in 2016 (Moyes et al., 2016): *ECE1* encodes a protein that is cleaved into eight short peptides by Kex2. Peptide 3 generated by this cleavage adopts an α-helical structure and integrates into host cell membranes, leading to permeabilization and lysis. Due to this effect, the peptide was named candidalysin (Moyes et al., 2016). Thus, candidalysin links hyphal morphology and damage beyond physical rupture by continuous filamentous growth and provides further explanation for the significant role of filamentation in pathogenesis (Wilson et al., 2016).

Interaction of *Candida albicans* With the Immune System

Immune cells are rapidly recruited during candidiasis and especially innate phagocytes are efficient and essential effector cells during fungal infections (Fig. 3; Duggan et al., 2015; Lionakis, 2014; Lionakis et al., 2011). In fact, impairment of the immune system represents a risk factor for candidiasis, but not all patients suffering from disseminated *C. albicans* infections are perceptibly immunocompromised (Guery et al., 2009; Nolla-Salas et al., 1997; Yapar, 2014). To be a successful pathogen, *C. albicans* needs to be able to either prevent recognition or survive antimicrobial mechanisms employed by immune cells to a certain extend. As the immune response to *C. albicans* is addressed in detail in another module of this series, we will focus here on some of the mechanisms by which the fungus withstands elimination of the immune system.

The immune system can be roughly divided into cellular and humoral effector mechanisms. Humoral factors include antibodies, the complement system, and AMPs. Antibodies are readily produced in humans and mice following exposure to *C. albicans* (Mochon et al., 2010; Pitarch et al., 2006, 2009, 2011) but naturally induced antibodies are considered to have only a limited protective effect (recently reviewed in Richardson and Moyes (2015)). The complement system and AMPs are evolutionary old and comprise two of the first lines of defense against invading microbes. Furthermore, AMPs are produced by mucosal epithelial cells even in the absence of a pathogenic threat and thereby control the commensal microbiota. Several AMPs, β-defensins, the cathelicidin LL-37, and histatin 5, have been shown to kill *C. albicans* in vitro (Chang et al., 2012; Vylkova et al., 2007a,b). It has furthermore been proposed that the reduced levels of histatin 5 observed in the saliva of HIV^+ patients contribute to the increased risk of these patients for oropharyngeal candidiasis (Khan et al., 2013). Although this and the hypothesis that AMPs control fungal growth on mucosal surfaces in vitro appear plausible, they still need to be experimentally verified. Nonetheless, *C. albicans* also seems to have evolved AMP resistance mechanisms, like inactivation through proteolytic cleavage of AMPs by Sap9 and Sap10 and inactivation by a secreted glycodomain of Msb2 (Meiller et al., 2009; Puri et al., 2012; Swidergall et al., 2013; Szafranski-Schneider et al., 2012). Tolerance to AMPs can furthermore be induced by induction of compensatory mechanisms, for example, Pbs2 via the Hog1 stress response pathway, or stabilization of mitochondrial integrity via Ssd1 and Bcr1 (Argimon et al., 2011; Jung et al., 2013). Similarly, *C. albicans* interferes with complement activation by various means (recently reviewed in Cheng et al. (2012), Luo et al. (2013)), including proteolytic cleavage of active complement components by Saps and binding of host factors that regulate complement activation. To which extend these immune evasion mechanisms contribute to pathogenesis is not fully clear; however, complement defects render mice more susceptible to systemic candidiasis (Ashman et al., 2003; Tsoni et al., 2009). Furthermore,

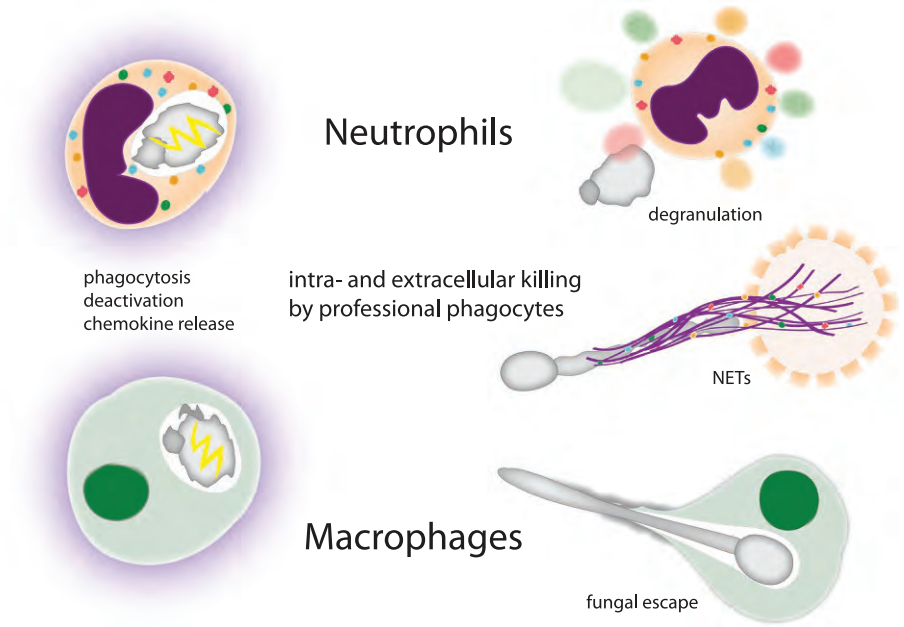

Fig. 3 Killing of *Candida albicans* by professional human phagocytes. Killing of *C. albicans* by neutrophils and macrophages occurs intra- and extracellularly (IC/EC). IC: internalization of *Candida* via phagocytosis leads to inactivation of fungus by ROS (yellow lightning) and host effector proteins. EC: chemokines are released (purple glow) by activated phagocytes to recruit more phagocytes. Antifungal effector proteins are released into cell environment during degranulation. Neutrophils release extracellular traps (NETs) to entangle and kill *Candida*. Notably, *C. albicans* can outgrow macrophages, not neutrophils. Ilustration by M.J. Niemiec.

opsonization by complement enhances phagocytosis and fungal killing (reviewed in Rambach and Speth (2009)), thereby linking complement evasion to other survival strategies of *C. albicans*.

Professional phagocytes, especially neutrophils and monocytes/macrophages, constitute cellular components of the first line of defense against *C. albicans* (Basu et al., 2008; Kullberg et al., 1999; Romani et al., 1996; van't Wout et al., 1988; van Enckevort et al., 1999). Following recognition of the fungus via interaction of pattern recognition receptors (PRRs) with fungal surface components acting as pathogen-associated molecular patterns (PAMPs) (recently reviewed in Erwig and Gow (2016), Miramon et al. (2013), Plato et al. (2015)), immune cells become activated and either phagocytose the pathogen or, in the case of neutrophils, can undergo degranulation or form neutrophil extracellular traps (NETs) via programmed cell death to release antifungal effector molecules (Gazendam et al., 2016; Stephenson et al., 2016; Fig. 3). The cell wall composition and, more importantly, exposure of distinct components on the surface significantly influences the efficacy of recognition and the subsequent response induced; thus, cell wall modifications are one way by which *C. albicans* can influence recognition by phagocytes and, additionally, phagosome maturation (Bain et al., 2014; Lewis et al., 2012). These mechanisms are discussed in detail below.

Phagocytosis requires physical uptake of the pathogen and is therefore influenced by size and shape. *C. albicans* hyphae can grow to a length that prevents complete phagocytosis (reviewed in Erwig and Gow (2016)). Therefore, the ability of *C. albicans* to form filaments affects the interaction with phagocytes. However, yeast cells can readily be phagocytosed and phagolysosome maturation in macrophages creates a hostile environment characterized by reduced pH, a high concentration of AMPs, reactive oxygen and nitrogen species (ROS and RNS, respectively), hydrolytic enzymes and nutrient starvation (Erwig and Gow, 2016; Miramon et al., 2013). *C. albicans* is able to escape from macrophages by either triggering pyroptosis, a programmed cell death pathway, or by affecting phagolysosome maturation, development of long hyphae, and physical piercing of macrophages. The exact mechanisms by which *C. albicans* triggers pyroptosis are only incompletely understood, but include neutralization of the phagosome by ammonia excretion, hypha induction, and remodeling of the cell wall associated with increased 1,3 β-glucan exposure (Uwamahoro et al., 2014; Vylkova and Lorenz, 2017; Wellington et al., 2014). The production of ammonia is intimately linked to amino acid metabolism and depends on the transcription factor Stp2, which controls expression of amino acid permease genes (Vylkova and Lorenz, 2014). Amino acids serve to generate ammonia, which is then excreted by Ato5 and neutralizes the phagosome (Danhof and Lorenz, 2015). As alkalinisation in turn promotes filamentation (Vylkova et al., 2011), this process might link induction of pyroptosis and active escape via hyphal growth.

Professional phagocytes typically produce significant amounts of ROS and RNS upon encountering *C. albicans* and these oxidative mechanisms are important for killing of *C. albicans* (Aratani et al., 2002; Brothers et al., 2013; Sasada et al., 1987; Vazquez-Torres et al., 1996). The fungus responds by activating a general stress response that involves the protein kinase Hog1 and the more specific oxidative and nitrosative stress response pathway involving the TFs Cap1 and Cta4, respectively (Alonso-Monge et al., 2003; Arana et al., 2007; Brown et al., 2009; Chiranand et al., 2008; Patterson et al., 2013; Smith et al., 2004;

Wang et al., 2006). The activated stress response results in increased expression of detoxification systems (reviewed in detail in Dantas Ada et al. (2015), Erwig and Gow (2016), Miramon et al. (2013)) that include superoxide dismutases (SODs) (Frohner et al., 2009; Hwang et al., 2002; Miramon et al., 2012), catalase 1 (Nakagawa et al., 2003; Wysong et al., 1998), a glutathione-based system including glutathione peroxidases and reductases (Chaves et al., 2007; Enjalbert et al., 2007; Fradin et al., 2005; Miramon et al., 2014; Rubin-Bejerano et al., 2003; Yadav et al., 2011), and flavodoxin-like proteins (Li et al., 2015), that are protective against oxidative stress. Resistance against nitrosative stress depends on the flavohemoglobin NO scavenger Yhb1 (Ullmann et al., 2004). Upon phagocytosis, *C. albicans* furthermore upregulates certain metabolic pathways, including gluconeogenesis, enzymes for the utilization of alternative carbon sources, β-oxidation of fatty acids and the glyoxylate cycle, whereas glycolysis and protein synthesis are downregulated (Fradin et al., 2005; Lorenz et al., 2004). As the phagosome is generally considered to be poor in nutrients, this metabolic adaptation contributes to survival of the fungus in this specific environment (Fernandez-Arenas et al., 2007; Miramon et al., 2012). Finally, the upregulation of uptake systems for trace metals (discussed in detail below, see Fig. 4) by phagocytosed *C. albicans* cells likely contributes to overcome restriction of these micronutrients within host cells (Lorenz et al., 2004).

The Cell Wall as a Dynamic Factor in *Candida*–Host Interactions

As the outer layer of the cell, the cell wall and associated proteins present the structures that mediate contact of *C. albicans* with the environment and host cells. While on the one hand providing the major outer physical structure for the cell and mediating resistance to adverse environmental conditions, the cell wall is also presenting PAMPs that are recognized by PRRs on professional and associate immune cells (recently reviewed in Barreto-Bergter and Figueiredo (2014), Erwig and Gow (2016), Miramon et al. (2013), Plato et al. (2015)). Thus, the cell wall is critical for host–pathogen interactions of *C. albicans*. The fungal cell wall consists of different β-glucans, chitin and chitosan, which are located in an inner layer, and mannans attached to proteins in an outer layer (for details see (Free, 2013)). Most of the cell wall molecules are carbohydrates, which all contribute to immune recognition (reviewed in detail by Becker et al. (2015), Erwig and Gow (2016), Gow and Hube (2012), Netea et al. (2008), Plato et al. (2015), Zheng et al. (2015a,b)). The receptors involved in recognition and the response of immune cells differs depending on the cell wall moiety that is recognized: β-1,3 glucan interacts with the C-type lectin Dectin-1, which triggers a strong inflammatory response (Brown, 2011; Cheng et al., 2011). Mannans can be recognized by Toll-like receptors (TLRs), the Mannose Receptor (MR), DC-SIGN, Mincle and Galectin-3 (Barreto-Bergter and Figueiredo, 2014; Netea et al., 2008, 2006), likewise inducing a proinflammatory response which is, in comparison to β-1,3 glucan, less pronounced (Gow and Hube, 2012). Chitin is sensed by NOD2, TLR9 and the MR, but in contrast to the other components can reduce inflammatory responses (Mora-Montes et al., 2011; Wagener et al., 2014). Thus, the relative exposure of the different carbohydrates determines the quality and quantity of subsequent immune responses. This is exemplified in studies of *C. albicans* mutants with defects in mannosylation which lead to increased exposure of inner cell wall components, resulting in altered interaction with immune cells (reviewed in Hall and Gow, (2013)).

Importantly, the cell wall composition and relative exposure of the different carbohydrate components is highly dynamic and influenced by the morphological state, environmental conditions and the interaction with immune cells (recently reviewed in Hall (2015)). Yeast and hypha differ in the types of glucan (Lowman et al., 2003, 2014) and the amount of chitin, with chitin being more abundant in hypha (Munro et al., 1998). Furthermore, the distribution of chitin differs; while it is found along the cell wall of hypha, it is concentrated at the bud scar in yeast (reviewed in Gow et al. (2012)). Furthermore, some mannoproteins are differentially expressed depending on the morphology, for example, hypha-specific proteins like Hwp1 and Als3 (reviewed in Sundstrom (1999), Trevijano-Contador et al. (2016)), and the mannan composition in yeast and hypha is different (Shibata et al., 2007). As a consequence of these differences, immune cells respond differently to yeast and hypha (reviewed in more detail in Erwig and Gow (2016), Gow et al. (2011), Jacobsen et al. (2012), Trevijano-Contador et al. (2016).

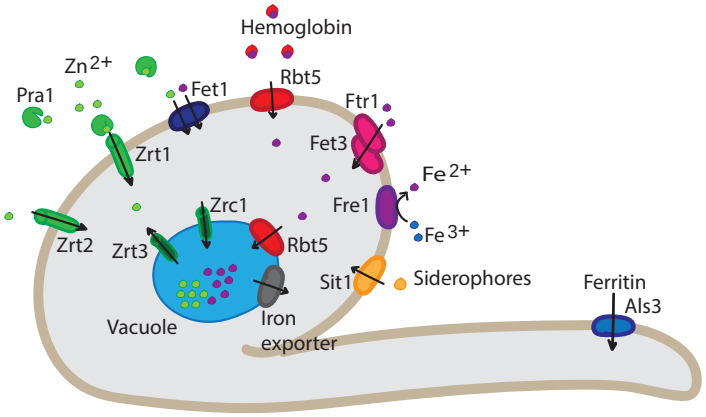

Fig. 4 Micronutrient acquisition by *Candida albicans*. *C. albicans* imports, stores, and exports trace elements during its life cycle. In the mammalian host, the availability of micronutrients is limited. Depicted are the transport systems for zinc and iron. Illustration by M. Kapitan.

The environment influences the cell wall composition and structure. Mannan structures (Ene et al., 2012; Kruppa et al., 2011; Lowman et al., 2011) and the levels of glucan and chitin (Ene et al., 2012), for example, vary depending on the culture conditions. Lactate induces active β-glucan masking while cell wall stress induced by the antifungal echinocandins leads to increased chitin and enhanced β-glucan exposure both in vitro and during in vivo treatment (Ballou et al., 2016; Lee et al., 2012; Walker et al., 2008; Wheeler et al., 2008). Importantly, in the absence of antifungals, unmasking of β-glucan also occurs during systemic candidiasis in mice as a result of active cell wall remodeling in response to damage by NETs (Hopke et al., 2016). Chitin can also be upregulated during infection, affecting recognition by Dectin-1, a phenomenon that varies between different *C. albicans* strains (Marakalala et al., 2013). Increased chitin in *C. albicans* is furthermore associated with reduced virulence in a systemic mouse model (Lee et al., 2012), highlighting the importance of the cell wall and its dynamic modifications for the interactions *C. albicans* with its host.

The Influence of Macronutrients on *Candida*–Host Interaction

As both a commensal and a pathogen, *C. albicans* can thrive at different anatomical sites. These differ not only in pH, oxygen levels, and host defense systems but also regarding the quality and quantity of available carbon and nitrogen sources. Its success at the different niches suggests that *C. albicans* is metabolically versatile and has evolved means to assimilate nutrients from a range of sources. Furthermore, the dissemination into various tissues during systemic infection requires fast adaptation to local nutrient availability. Indeed, *C. albicans* can utilize a vast variety of different sugars and amino acids (Brunke and Hube, 2013) and a range of secreted hydrolytic enzymes contributes to the liberation of nutrients from complex molecules (Brunke and Hube, 2013; Naglik et al., 2004; Schaller et al., 2005). Complex regulatory networks mediate the response of *C. albicans* to nutrient starvation or alterations in the available carbon or nitrogen sources, leading to altered expression of hydrolases, transporters, and metabolic pathways like gluconeogenesis, glycolysis, or fatty acid β-oxidation (Askew et al., 2009; Barelle et al., 2006; Lee et al., 2013; Morschhauser, 2011; Ramachandra et al., 2014; Sabina and Brown, 2009). In comparison to the related baker's yeast *Saccharomyces cerevisiae*, substantial transcriptional and posttranslational rewiring has occurred in *C. albicans*, contributing to the metabolic flexibility of *C. albicans* (Ramirez and Lorenz, 2007; Sandai et al., 2012; Whiteway et al., 2015).

In addition to providing the necessary energy for fungal survival, growth, and production of effector molecules, metabolic adaptation is directly linked via transcriptional networks to the expression of different fitness and virulence attributes such as morphogenesis, stress resistance, adhesions, and hydrolytic enzymes (Han et al., 2011). Cell wall composition is also influenced by nutrients, especially carbon metabolism, and thereby metabolism indirectly influences the interaction with immune cells (recently reviewed in Hall (2015)). One well studied example is the growth of *C. albicans* on lactate in comparison to glucose and the changes caused by this. While most growth media used in the laboratory are rich in glucose, glucose availability is limited within the host. Thus, *C. albicans* has to use alternative carbon sources such as lactate. Growth on lactate in vitro led to alterations in cell wall architecture and secretome, with consequences for adhesion, stress resistance, and interaction with immune cells: Lactate grown cells were phagocytosed less by macrophages while fungal escape was enhanced; at the same time lactate-grown *C. albicans* cells induced more IL-10 but less IL-17, thereby leading to a reduced proinflammatory response (Ene et al., 2012, 2013). As a consequence, fitness of lactate-grown *C. albicans* cells in vivo was enhanced (Ene et al., 2012).

Due to the link between metabolism and fitness/virulence traits, the adaptation of *C. albicans* to different environments and nutrient availability within the host simultaneously affects expression of virulence factors (recently reviewed in Brown et al. (2014)). This linkage of metabolic responses to environmental clues and virulence can be interpreted as "adaptive prediction" (Mitchell et al., 2009), by which the availability of distinct nutrients serves as a clue for further subsequent environmental changes, for example, attack by immune cells following translocation into the blood stream, leading to oxidative stress due to ROS production. From an evolutionary perspective, this linkage is likely to be highly advantageous to the fungus and likely contributes to its success in diverse anatomical niches both as a commensal and a pathogen. For more information and a summary of the open questions, we refer the readers to the review by Brown et al. (2014).

Acquisition of Micronutrients by *Candida albicans*

Micronutrients are as essential for microbial growth as carbon- and nitrogen sources. Trace metals such as iron, zinc, copper and manganese are essential cofactors in enzymes and stabilize three-dimensional protein structures (Palm-Espling et al., 2012; Waldron et al., 2009). However, their free availability in the host is limited by sequestration and binding to host molecules. Host-mediated nutrient depletion from invading pathogens is also termed "nutritional immunity," a mechanism that has gained increased attention in recent years, and is therefore described in some detail below.

Every aspect of nutritional immunity, from the host or pathogen perspective, reflects the coevolutionary nature of these interactions. Best studied is the competition for iron, but most concepts apply to all transition metals in eukaryotes (Sutak et al., 2008; Weinberg, 1975). In the host, iron is mainly kept intracellular – free iron is practically unavailable in blood or other extracellular liquids, with exception of the gut lumen. Iron is bound to transferrin in plasma and lactoferrin in external secretions. However, in order to grow within the host, microbial pathogens cope with these limitations using different strategies: In case of direct physical contact between the host iron source and the pathogen, host iron-retaining proteins might be manipulated to release their iron load into the extracellular environment for uptake. Secondly, many bacteria and fungi secrete siderophores: Small

molecules with exceptionally high iron affinity that free iron from host proteins followed by resorption into the pathogen cell. In response, mammals produce lipocalin-2 to intercept siderophores. This can in turn be counteracted by many pathogens which disguise their siderophores by glycosylation (Sutak et al., 2008). Iron is a common cofactor in enzymes and one of the most important direct functions of iron during host–pathogen interactions is likely the detoxification of H_2O_2 by the iron-dependent heme-enzyme catalase (Hamilton and Holdom, 1999). Iron uptake in *C. albicans* occurs by reductive and nonreductive mechanisms (Almeida et al., 2009; Waldron et al., 2009; Fig. 4). In a nutshell, the high affinity reductive uptake system functions via the interplay of three enzymes. A reductase (Fre) converts ferric Fe^{3+} to ferrous Fe^{2+}. The actual uptake is mediated by a permease (Ftr) and a multicopper ferroxidase (Fet). *C. albicans* relies on this pathway to acquire iron from ferritin and transferrin (Almeida et al., 2008; Knight et al., 2005). Probably due to functional redundancy due to gene expansion of the reductases and multicopper oxidases, only the permease Ftr1 has been shown to be crucial in blood stream infections by *C. albicans*. Nonreductive iron uptake is based on the import of iron-containing proteins, namely hemoglobin and siderophores. *C. albicans* has receptors for heme (e.g., Rbt5), but also for ferritin (reductive uptake). The ferritin receptor and adhesin Als3 is expressed only by hyphae (Almeida et al., 2008). Siderophores are not produced by *C. albicans*, but they can be utilized as an iron source even if produced by other microbes (Almeida et al., 2009).

In contrast to other sites in the human body, the gut lumen is probably the only site where microbes encounter high iron levels. Accordingly, as a colonizer of this niche, *C. albicans* is able to adapt not only to iron limitation but also to relatively high iron concentrations. To do so, an intricate system of three transcriptional regulators has evolved that controls iron uptake in *C. albicans* (Blankenship and Mitchell, 2011; Polke et al., 2015). The transcriptional repressor Sfu1 and the virulence regulator Hap43 dampen iron uptake and Sfu1 is required for persistence of *C. albicans* in the gut. In contrast, during blood stream infections, when iron is limited, iron uptake is upregulated by Sef1, a process essential for virulence in the systemic mouse model (Chen et al., 2011). Sfu1 represses Sef1, thereby interconnecting the response to low and high iron and adaptation to distinct niches in the host.

Uptake of iron and copper are very tightly connected. The ferroxidase (Fet) is actually a copper-dependent enzyme, and the iron reductases (Fre) are indeed ferric/cupric reductases that reduce Cu^{2+} to Cu^{1+} through the oxidation of Fe^{2+} to Fe^{3+}, and vice-a-versa (Niemiec, 2015). Reduced Cu^{1+} is taken up by Cu transporters (Ctr). Ctr1 and Ctr3 are plasma membrane-localized, Ctr2 is vacuolar – and all three transport toward the cytosol. Interestingly, the number of *CTR* copies varies between fungal species and might affect virulence due to an effect on, for example, hyphal growth (Ding et al., 2014). During infection, pathogens face copper starvation as well as copper intoxication. For example, excess copper has been described as a mode of action against intraphagosomal *Mycobacteria* in macrophages (Rowland and Niederweis, 2012). Therefore, copper export and copper detoxifying metallothioneins are important to maintain copper homeostasis. Metallothioneins are conserved in prokaryotes and eukaryotes. A potential role in virulence was demonstrated in *Cryptococcus neoformans*, a facultative intracellular fungal pathogen, but not yet in *C. albicans*. However, *C. albicans* is known to export excess copper, indicating that fungi are primed to resist potential copper poisoning in vivo (Ding et al., 2014). At the same time, copper is essential for pathogenic survival. Most fungal SODs, crucial to withstand superoxide stress, have copper in their active site. In *C. albicans*, Sod1, Sod4, and Sod6 are copper/zinc-dependent, Sod5 was recently described to be a copper only SOD (Gleason et al., 2014). *C. albicans* extracellular Sod4 and Sod5 were demonstrated to detoxify superoxides produced during interactions with macrophages (Frohner et al., 2009).

Of all metal-containing enzymes, 9% use zinc – so zinc is the second most abundant metal cofactor after magnesium (Waldron et al., 2009). Further, zinc stabilizes zinc-finger domains, a function that is especially important in eukaryotic organisms (Decaria et al., 2010). Several studies have demonstrated the importance of zinc in pathogenic and nonpathogenic fungi. *C. albicans* is very sensitive to zinc starvation. Zinc can actually be the limiting factor if all other nutrients are available (Bedell and Soll, 1979; Soll et al., 1981). Zinc acquisition in fungal pathogens is mediated by a small number of proteins and is conserved in *C. albicans* (Fig. 4): Zinc enters the cytoplasm via the Zrt plasma membrane transporters (Zrt1, Zrt2). From there, zinc can be transported further into the vacuole through ZnT transporters (in *C. albicans*: Zrc1). In return, zinc can be mobilized from the vacuolar pool by Zrt exporters (Zrt3). Interestingly, no cellular zinc exporter has been described in fungi, yet. The homeostasis of zinc is regulated on two levels: the expression of Zrt importers, controlled by Zap1, and zinc import and export from subcellular organelles (Wilson et al., 2012). Vacuolar storage might be especially important during infection, since it allows proliferation even under zinc-restricting conditions (Simm et al., 2007). In addition, *C. albicans* uses a zincophore system – a secreted protein that sequesters zinc from host cells and re-associates with Zrt1 to deliver zinc to the fungal cell: Pra1 (Citiulo et al., 2012). The expression of Pra1 is repressed under acidic conditions, but induced under zinc starvation or at neutral/alkaline pH (Citiulo et al., 2012). Remarkably, Pra1 is not only beneficial for the fungus, it is actually immunogenic and immune-modulatory (Wilson, 2015). Amongst many functions, Pra1 can serve as a ligand to neutrophil integrin receptor αMβ2 and enhance neutrophil killing (Soloviev et al., 2007). Probably due to this "side effect," Pra1 is in not conserved in all pathogenic fungi (Wilson, 2015). A role in virulence for zinc acquisition systems has not been directly demonstrated; however, zinc is important for biofilm formation, and involved in tolerance to intrinsic oxidative stress by the cytoplasmic Sod1 (Wu et al., 2009). Sod1 is a copper/zinc SOD. Notably, the cell surface Sods Sod4 and Sod6 that are crucial to survive host-derived ROS, have copper and zinc as their active site (Frohner et al., 2009).

In non-plant eukaryotes, manganese function is restricted to mitochondrial enzymes, including Sod2 and Sod3. The human host restricts access to manganese by releasing calprotectin that is capable of binding manganese and zinc (Kehl-Fie et al., 2011; Sohnle et al., 2000). Furthermore, manganese (and iron) are actively depleted from the macrophage phagosome (Kehl-Fie and Skaar, 2010). Manganese restriction is important during bacterial infections, but its role during fungal pathogenesis requires more investigation (Corbin et al., 2008; Kehl-Fie et al., 2011; Posey and Gherardini, 2000).

Candida albicans Morphology – Does It Determine the Difference Between Commensalism and Infection?

As mentioned throughout the previous parts, the formation of filamentous growth forms during morphogenesis is generally thought to be essential for *C. albicans* virulence. This concept is based on the observation that *C. albicans* mutant strains locked in either the yeast or hyphal form were attenuated in their virulence in murine infection models (Braun et al., 2000, 2001; Lo et al., 1997; Murad et al., 2001). Given that hyphae have the ability to physically penetrate host cell barriers and hypha formation is associated with production of candidalysin (Brand, 2012; Moyes et al., 2016), it appears obvious that hypha formation significantly affects the ability of *C. albicans* to penetrate tissue, damage host cells, and cause disease. However, hypha formation is co-regulated with several virulence-associated factors, leading to rearrangement of the cell wall and thereby affecting the response of immune cells, and is further associated with stress response and metabolism (Dantas Ada et al., 2015; Inglis and Sherlock, 2013; Monge et al., 2006; O'Connor et al., 2010). It is thus difficult to determine to which extend the formation of hyphae per se contributes to virulence (Kumamoto and Vinces, 2005). Most likely, both, yeast and hypha, play distinct roles during the different steps of *C. albicans* infections (Gow et al., 2002; Kumamoto and Vinces, 2005; Saville et al., 2003).

While invasive growth and host damage are intrinsically linked during infection, commensalism is characterized by a balance between host and fungus that does not cause host damage (Gow and Hube, 2012). Interestingly, genetic analyses revealed that colonization of the gut is associated with significant expression levels of hypha-associated gene expression, such as *EFH1*, *ECE1*, *RBT4*, and *RBT1* in yeast cells (d'Enfert, 2009; Doedt et al., 2004; Rosenbach et al., 2010; White et al., 2007). Thus, transcriptional programs that were previously considered to be morphology-associated based on in vitro observations might be uncoupled from the filamentation program under distinct environmental conditions. Furthermore, *ECE1*, the gene encoding for candidalysin and required for host cell damage, is upregulated in the gut. This raises the question whether growth as yeast in the gut truly reflects a benign, commensal transcriptional program and whether separate commensal and pathogenic transcriptional programs exist. Possibly, the yeast morphology in the gut might be the consequence of complex interactions between host, microbiota, and *C. albicans*. Factors that have so far been only associated with *C. albicans* infections may be required for fungal survival in the gut. Indeed, recent gene network analyses of TFs required for gut colonization and/or fitness during systemic infection revealed several connections between the regulons of TFs important for either commensalism or virulence (Perez and Johnson, 2013).

Further research directed at a better understanding the entire *C. albicans* biology, not only as a pathogen but also as a commensal on different mucosa, is required to decipher what it is exactly that facilitates this fungus to be a successful member of the human microbiota. Hopefully, this will contribute to eventually elucidate the specific factors that predispose patients to disseminated candidiasis.

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Complement

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Historical Aspects

Several studies described the bactericidal activity of normal serum as far back as the 1880s, which was lost when blood was heated to 55°C. Buchner called the bactericidal heat labile factor alexin. Pfeiffer and Issaef showed that whole blood from guinea pigs that survived *Vibrio cholerae* infection could protect naïve animals from developing disease. Subsequently, Jules Bordet showed that the bactericidal activity of heated immune serum was restored by fresh serum that itself lacked intrinsic bactericidal activity. Thus, the bactericidal activity of immune serum required a heat-stable factor ('amboceptor', which now called antibody) as well as a heat labile factor (alexin, which subsequently named 'complement' by Paul Ehrlich). The combination of amboceptor and alexin also caused hemolysis.

Euglobulin and pseudoglobulin fractions in serum were both required for complement activity, leading to the initial nomenclature of C′1 and C′2 (the "prime" was omitted following the WHO nomenclature proposed in 1968). Loss of complement activity following treatment of serum with cobra venom and absorption of this activity by yeast led to the discovery of the third component (C3). In the 1940s, Manfred Mayer and colleagues developed a standardized method of hemolytic titration. Mayer proposed the "one-hit" theory, whereby only one complete sequence of complement proteins was required to lyse an erythrocyte, which permitted titrating the hemolytic activity of sera. The identification of C3 as β1c-globulin – a major component of serum that was present at concentration of ∼1 mg/ml – by Müller-Eberhard and colleagues, paved the way to further characterize complement proteins.

Although not appreciated at the time, the existence of the alternative pathway (*i.e.*, not requiring antibody) was suggested by experiments where cobra venom factor and zymosan consumed complement activity without affecting the classical pathway. Pillemer and his coworkers first characterized the properdin pathway, but his critics ascribed the observations to small amounts of contaminating antibody. Following his untimely suicide in 1957, Pillemer's work was carried on by Irwin Lepow and several other groups, which led to characterization of the alternative pathway by the early 1970s. In Pillemer's memory, alternative pathway proteins were designated with letters of the alphabet, as he had originally proposed.

Evolution of Complement

The complement system developed long before the evolution of adaptive immunity. Acquired immunity appears to have arisen early in vertebrate evolution, between the divergence of cyclostomes (lampreys) and cartilaginous fish (sharks). Genes encoding molecules of the acquired immune system, including immunoglobulin (Ig), T-cell receptor (TCR), major histocompatibility complex (MHC) class I and II, and recombination-activating gene (RAG), have been identified only in sharks and higher vertebrates. The alternative and the lectin pathways are phylogenetically older than the classical pathway. C3, the central molecule of complement, C4 and C5 are all derived phylogenetically from a serum protease inhibitor called α2-macroglobulin. Only a single C3/C4/C5 gene that is distinct from the α2-macroglobulin gene was identified in the lamprey, the ascidian and the sea urchin, which suggested that the gene duplication that gave rise to distinct C3, C4 and C5 molecules occurred later in evolution, around the time of the appearance of jawed vertebrates. The classical and terminal complement pathways are believed to have developed around the same time as the adaptive immune system.

Synthesis, Distribution and Catabolism of Complement

The liver is a major site of synthesis of most complement proteins. Several other cell types, such as neutrophils, monocytes, macrophages, adipocytes, microglia, astrocytes, fibroblasts, cervical epithelial cells and endothelial cells also contribute to complement production locally. Complement proteins have higher fractional catabolic rates and are metabolized more rapidly than most serum proteins. Several complement proteins are acute-phase reactants and their synthesis is modulated by cytokines such as IFN-γ, IL-1, and TNF and by endotoxin. Monocytes and macrophages synthesize complement in amounts sufficient to opsonize microbes, therefore upregulation of complement production by proinflammatory signals enhances anti-microbial activity at sites of infection. Locally synthesized complement is important for several physiologic processes, such as the shaping of adaptive immune responses and neural remodeling.

Complement is found at almost every mucosal site and body fluid. Complement concentrations at mucosal sites approach 5%–10% that seen in serum. An increase in complement concentrations at sites such as the cerebrospinal fluid occurs during infections or inflammatory processes because of increased vascular permeability and because of increased local production by inflammatory cells. Complement levels in the female genital tract is profoundly affected by hormonal regulation.

Both preterm and full-term newborns have lower complement function compared to adults. Complement activity correlates inversely with the age of gestation, and approaches adult levels at about 6 months of age.

Activation of the Complement System

Complement activation has traditionally been considered to occur through one of three pathways – the classical, lectin or alternative pathways (Fig. 1). In most instances, complement is activated through more than one pathway simultaneously. As an example, C3b deposited through the classical or lectin pathways often recruits the alternative pathway. For ease of understanding, each pathway is considered separately. Characteristics of each of the components of complement in the fluid phase are listed in Table 1.

The Classical Pathway

The classical pathway is initiated by binding of antibodies to their target antigens, which is followed by specific noncovalent interactions between Fc regions of proximate IgG molecules to form ordered hexamers. These hexamers can engage each of the six globular heads of C1q (a member of the C1 complex that contains C1q, C1r and C1s), thereby converting low-affinity Fc-C1q interactions ($K_d \approx 10^{-4}$ M) to high-avidity interactions. C1r and C1s are arranged as a tetramer (C1s-C1r-C1r-C1s) and form a Ca^{2+}-dependent catalytic subunit. A conformational change in the C1 complex as a result of C1q binding auto-activates C1r, which in turn activates C1s. C1r and C1s are both activated through cleavage of an Arg-Ile bond.

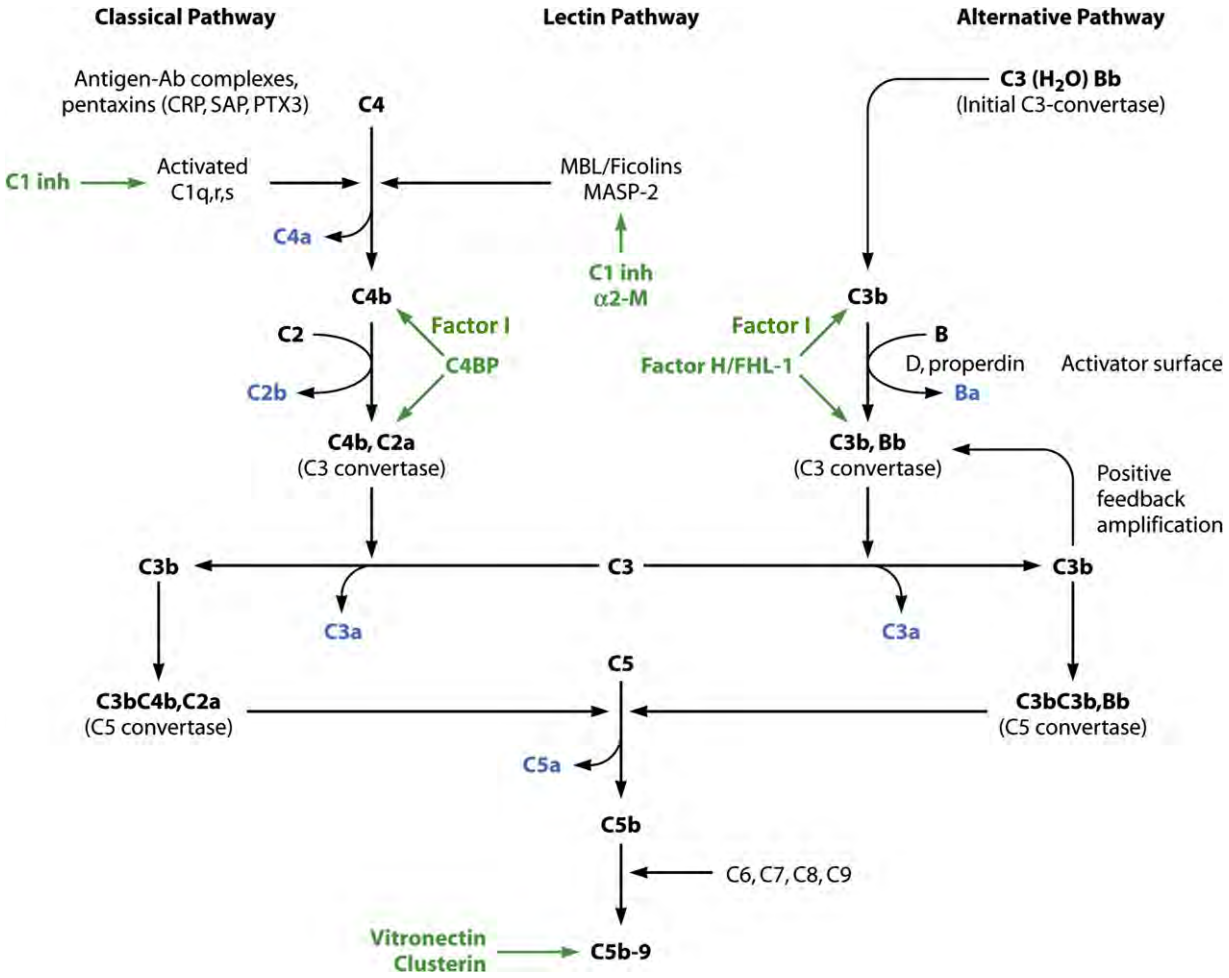

Fig. 1 Schematic representing the activation of the complement cascade. The fragments released into solution are indicated in blue font. The key fluid-phase regulators are indicated in green font. CRP, C-reactive protein; SAP, serum amyloid P component; PTX3, pentraxins 3; C1 inh, C1 inhibitor; α2-M, α2-macroglobulin; C4BP, C4b-binding protein; FHL-1, factor H like protein-1. From Ram, S., Lewis, L.A., Rice, P.A., 2010. Infections of people with complement deficiencies and patients who have undergone splenectomy. Clin. Microbiol. Rev. 23 (4), 740–780.

Table 1 Characteristics of soluble proteins of the complement cascade

Component[a]	*Plasma conc. (μg/ml)*	*Mol. mass (kD)*	*Structure*	*No. of genetic loci*	*Chromosomal assignment*
Proteins that activate complement					
Classical pathway proteins					
C1q	70	459	18 polypeptide chains; 6A, 6B, 6C; A-B and C-C linked by disulfides	3 (A, B, C)	1p34–1p36.3
C1r	34	173	Comprises a CUB (C1r/C1s, uEGF, bone morphogenetic protein) module, an epidermal growth factor (EGF)-like module, a second CUB module, two complement control protein (CCP) modules and a C-terminal chymotrypsin-like serine protease domain; dimer (A and B chain linked by disulfide bond)	1	12p13
C1s	31	80	Dimer (A and B chain linked by disulfide bond); modular structure s described for C1r	1	12p13
C4	600	206	β-α-γ; 1 β-α and 2 α-γ disulfide bonds	2 *(C4A, C4B)*	6p21.3
C2	11–35	100	1 chain	1	6p21.3
Alternative pathway proteins					
Factor B (FB)	200	90	1 chain	1	6p21.3
Factor D (FD; adipsin)	1–2	25	1 chain	1	19p13.3
Properdin	5–10	55	Cyclic polymers in head-to-tail orientation; dimers: trimers:tetramers in 26:54:20 ratio; each monomer comprises 6 thrombospondin-like repeats (TSRs) and has 14 sites of C-mannosylation	1	Xp11.4–p11.23
Lectin pathway					
Mannan-binding lectin (MBL)	1–5	~25 (subunit monomer)	Subunit – trimers of identical polypeptides; subunits organized into larger oligomers (n ~2 for variant (B, C, and D) alleles and 4–6 for wild-type (A) allele)	1	10q11.2–q21
Ficolin-1 (M-ficolin; ficolin/P35-related protein)	0.04–0.1 (monocytes and PMNs main source)	~32 (subunit monomer)	Subunit – trimers of identical polypeptides; subunits organized into larger oligomers	1	9q34
Ficolin-2 (L-ficolin; hucolin; EBP-37; ficolin/P35)	3–4	34 (subunit monomer)	As with Ficolin-1	1	9q34
Ficolin-3 (H-ficolin; Hakata antigen; thermolabile β-2 macroglycoprotein; thermolabile substance)	18	35 (subunit monomer)	As with Ficolin-1	1	Chr 1
Collectin-10 (Collectin Liver 1; CL-L1)	1.9	44 (subunit monomer)	N-terminal segment (19 aa), collagen-like region (72 aa), α-helical coiled-coil neck region (34 aa), carbohydrate-recognition domain (CRD; 125 aa). Each chain forms trimers, which combine to form higher order oligomers		8q23–q24.1
Collectin-11 (Collectin Kidney 1; CL-K1)	0.3	34 (subunit monomer)	Domain structure same as Collectin-10		2p25.3
MASP-1	6	97	Active form consists of heavy and light chains linked by disulfide bond	1	3q27–28
MASP-2	0.02–0.8	83	Active form consists of A and B chains linked by disulfide bond	1	1p36.3–p36.2
MASP-3	2–12.9	105	Activation splits 105 kD disulfide-linked dimer into A (58 kD) and B (42 kD); B chain is serine protease domain	1	3q27–28
MAp19	?	19	Alternatively spliced version of MASP-2 – contains first 2 domains and 4 additional C-terminal aa's; head-to-tail homodimer	1	1p36.3–p36.2
C3					
C3	1000–1500	190	β-α, linked by disulfide bond. Crystal structure shows organization into 13 domains; 8 macroglobulin	1	19p13.3–p13.2

Table 1 Continued

Component	Plasma conc. (μg/ml)	Mol. mass (kD)	Structure	No. of genetic loci	Chromosomal assignment
			domains, and 1 CUB, thioester (TED), anaphylatoxin, linker and C345c domain		
Terminal complement components					
C5	75	190	β-α, linked by disulfide bond	1	9q34.1
C6	45	100	1 chain	1	5p13
C7	90	95	1 chain	1	5p13
C8	55–80	151	α-γ dimer linked by disulfide, noncovalently associated with β	3	(α,β)1p32; (γ) 9q34.3
C9	60	71	1 chain	1	5p13
Complement inhibitory proteins					
C1 inhibitor (C1-INH)	150	104	1 chain; highly glycosylated	1	11q11–q13.1
C4b-binding protein (C4BP)	150–300	~550	7 disulfide-linked α-chains (8 SCRs) linked to β-chain (3 SCRs) via disulfide (major isoform; α7/β1); minor isoforms α7/β0 and α6/β1. β-chain associated with protein S of the anti-coagulation system (C4BP-Protein C4BP-Protein S)	2	(α,β) 1q32
Factor H (FH)[b]	500	155	1 chain (20 SCRs)	1	1q32
Factor I (FI)	34	90	1 chain; comprises FI-MAC domain, scavenger receptor cysteine-rich or CD5 domain, two low-density lipoprotein receptor (LDLr) domains and a serine protease domain	1	4q
FHL-1 (Factor H-like protein 1)	25	43	1 chain (7 SCRs; identical to 7 N-terminal SCRs of factor H, plus 4 unique C-terminal aa's)	1	1q32
Vitronectin (Vn; S-protein)	500	75 (65 kD proteolytic fragment also seen)	1 chain	1	17q11
Clusterin (Cn; SP-40,40; apolipoprotein J)	100–300	60 (predicted); 80 (observed)	Heterodimer linked by 5-disulfide bond motif	1	8p21–p12

[a]The FH family of proteins includes five FH-related molecules called FHR1, 2, 3 4 and 5. The function of these proteins remains to be fully elucidated. See text for details.
[b]The terminology proposed in 2014 jointly by the International Complement Society (ICS) and the European Complement Network (ECN) has been used.

Note: Modified from Ram, S., Lewis, L.A., Rice, P.A., 2010. Infections of people with complement deficiencies and patients who have undergone splenectomy. Clin. Microbiol. Rev. 23 (4), 740–780.

Human IgG subclasses differ in their ability to activate complement; in general, the hierarchy is IgG3>IgG1>IgG2, while IgG4 does not activate complement. In contrast to IgG, where cooperation between several molecules is required to activate complement, a single IgM molecule can activate complement. This is because IgM is a polymer (pentameric or hexameric in the presence or absence of the J chain, respectively) and each target-bound IgM can bind a C1 complex. Thus, on a molar basis, IgM is the most potent activator of the classical pathway.

The classical pathway can also be activated when members of the pentraxin family (includes C-reactive protein (CRP), serum amyloid P component (SAP) and pentraxin 3 (PTX3)) bind to surfaces and engage C1q. A novel mechanism of classical pathway activation described in mice is initiated by the binding of certain pneumococcal polysaccharides to Specific Intracellular Adhesion Molecule (ICAM)-Grabbing Nonintegrin R1 (SIGN-R1), a C-type lectin.

Activated C1s cleaves the C4a fragment (77 amino acids) from the N-terminus of the α-chain of C4 to form the metastable C4b molecule. This exposes the internal thioester bond of C4b that reacts rapidly with nucleophilic (*i.e.*, electron-donating groups) groups such as –OH or $-NH_2$ on surfaces to form covalent ester or amide bonds, respectively. If the nascent carbonyl group in the thioester moiety fails to interact with a surface, it reacts with water and remain in solution. Two isoforms of C4, called C4A and C4B, dictate the type of bond formed by C4b (note that C4a and C4b represent cleavage products of C4 that should not be confused with C4A and C4B, the isoforms of intact C4). A His residue at position 1106 in the α-chain of C4B imparts the ability to form ester linkages, while Asp at position 1106 results in 'C4A-like' functionality and forms amide bonds. The type of bond formed may have functional consequences. C4B is believed to possess greater hemolytic activity than C4A. On the other hand C4A binds to complement receptor 1 (CR1) more efficiently and may more effectively clear immune complexes from the bloodstream, which may explain the association between C4A deficiency and autoimmune diseases. In addition to the fact that C4 exists as distinct isoforms, the number of copies of the C4A and C4B genes varies across individuals. Deletions or duplications of C4 genes occur frequently and affects plasma levels of C4. The frequency of C4 gene dosages of 2, 3, 4, 5 and 6 in the Caucasian population is 2%, 25.3%, 52%, 17.3% and 3.3%, respectively. As a result, complete C4 deficiency is extremely rare. Conversely, heterozygous C4

deficiency is very common, and occurs in approximately 25% of the general population. Complete deficiency of either C4A or C4B is also relatively common and occurs in about 6% of the population.

The next step in classical pathway activation involves C2 binding to C4b. C2 is cleaved by activated C1s into the C2a fragment, which remains attached noncovalently to C4b, and C2b, which is released into solution. C4bC2a forms the C3 convertase (C3 cleaving enzyme) of the classical pathway. In this manner, a single C1 complex can cleave several substrate molecules and augment complement activation.

The Lectin Pathway

Activation of complement through the lectin pathway also generates C4bC2a. To date, six lectin molecules that can bind to a variety of terminal monosaccharides and initiate complement activation that have been described. These include the collectins (collagen-containing C-type [calcium-dependent] lectins) mannan binding lectin (MBL) and collectins 10 and 11, and ficolin-1, -2 and -3 (also called M-, L- and H-ficolin, respectively). Ficolins contain a fibrinogen-like domain combined with a collagen-like domain, thus are distinct from collectins.

The recognition molecules of the lectin pathway are organized as trimers; each identical polypeptide subunit in a trimer terminates in a calcium-dependent carbohydrate recognition domain. Trimers are organized into higher order oligomers that resemble a bouquet. MBL shares structural and functional homology with C1q. Similar to C1q, MBL is complexed with serine proteases, termed MBL-associated serum proteases (MASPs). Four such molecules – MASP-1, MASP-2, MASP-3, and MBL-associated plasma protein of 19 kD (MAp19) – are the products of two genes arising from a common ancestor shared with C1r and C1s. MASP-1 and MASP-3 are alternatively spliced products of *MASP1*, while MASP-2 and MAp19 are alternatively spliced products of *MASP2*. MASP-2 cleaves C4 and C2 to generate the classical pathway C3 convertase, as described earlier. An individual with a nonsense mutation in *MASP1* (and therefore lacking both MASP-1 and MASP-3) had a non-functional lectin pathway, which supports a role for MASP-1 in complement activation. Reconstitution of this individual's serum with MASP-1 resulted in MASP-2 cleavage and full restoration of lectin pathway activity. Further, MASP-1 and MASP-2 exist as a co-complex with MBL, supporting a model in which MASP-1 trans-activates MASP-2, analogous to C1r and C1s activation.

Plasma MBL levels are influenced by mutations in the first exon of MBL2 that encodes the signal peptide and the collagen-like region of the molecule. Three mutations associated with MBL deficiency are G→D at position 54, G→E at position 57 and R→C at position 57, which are termed the B, C and D alleles, respectively. These three mutant alleles are collectively referred to as the "O" alleles, while the wild-type protein is designated as the "A" allele. Each of these point mutations (O alleles) interferes with oligomerization of the three single chains that form the mature protein and are associated with low levels of MBL. In addition to the mutations in the coding region of the gene, three polymorphic sites are found in the 5′ untranslated promoter region of the *mbl2* gene:H/L, X/Y, and P/Q. The promoter alleles are found in linkage disequilibrium with the exon 1 SNPs (the O alleles), which results in a limited number of haplotypes. Seven haplotypes have been described: HYPA, LYPA, LYQA, LXPA, HYPD, LYPB and LYQC. When the A, or wild-type alleles are in *cis* with promoter −550/−221 haplotypes HY, LY and LX, the MBL concentrations are high, intermediate and low, respectively. Studies where only genotyping has been used to infer MBL levels should be interpreted cautiously because MBL levels may differ by as much as 10-fold across individuals with identical MBL genotypes.

MBL binds to a variety of terminal monosaccharides, including mannose, *N*-acetyl-mannosamine, *N*-acetyl-D-glucosamine, fucose, and glucose. Collectin-11 binds preferentially to L-fucose and D-mannose. The ficolins appear to bind preferentially to acetylated sugars such as N-acetyl-D-glucosamine. In addition, Ficolin-1 (M-ficolin) binds to N-Acetyl-D-galactosamine and select sialoglycans, such as those present in the capsule of *Streptococcus agalactiae*. Ligands reported for Ficolin-2 (L-ficolin) include β-(1→3)-D-glucan, N-acetylneuraminic acid, lipoteichoic acid, C-reactive protein, fibrinogen, DNA and certain corticosteroids, while H-ficolin binds to fucose. Collectin-10 binds mannose, fucose and galactose with high affinity, while collectin-11 binds preferentially to L-fucose and D-mannose. These sugars frequently decorate microbial surfaces but rarely appear as the terminal unit of oligosaccharides or glycoconjugates on human cells, which enables 'self-nonself' discrimination and targets complement activation to foreign surfaces. Renal ischemia in mice enhances local production of collectin-11, which binds to L-fucose expressed on the surface of stressed renal tubule cells and triggers complement-mediated damage. Lectins share several critical features with IgM ('natural antibodies'): both are polyreactive, bind to surface carbohydrates and binding of a single molecule activate complement.

The Alternative Pathway

Similar to the lectin pathway, the alternative pathway does not rely on initiation by antibodies and thus protects the host from pathogens prior to the development of specific immune responses. Alternative pathway activation is characterized by a unique positive feedback loop that permits self-amplification of the pathway. The principal component of this feedback loop is C3b, which may be generated by "tickover" of C3 (which generates a molecule functionally similar to C3b, as discussed below), or by alternative or classical pathway C3 convertases. Given its central role in complement activation, the structure of C3 is discussed next.

C3 – The central component of complement

All complement pathways converge at the level of C3, the most abundant complement component (plasma concentrations range from 1.0 to 1.5 mg/ml). C3 fragments serve a variety of functions: C3b and iC3b deposited on surfaces are opsonins for phagocytes, while the anaphylatoxin C3a modulates inflammation, lipid metabolism (C3a-desArg, which is generated by cleavage of the C-terminal Arg by carboxypeptidase N is also called acylation stimulating protein) and tissue regeneration.

C3 is composed of an α- and a β-chain linked by a disulfide bond (Fig. 2(A)). Similar to C4, the α-chain of C3 also possesses an internal thioester moiety that forms covalent bonds with target surfaces. The crystal structure of C3 revealed that it is organized into 13 domains (Fig. 2(C)). Cleavage of the C3a fragment from the α-chain activates C3, which is accompanied by marked structural

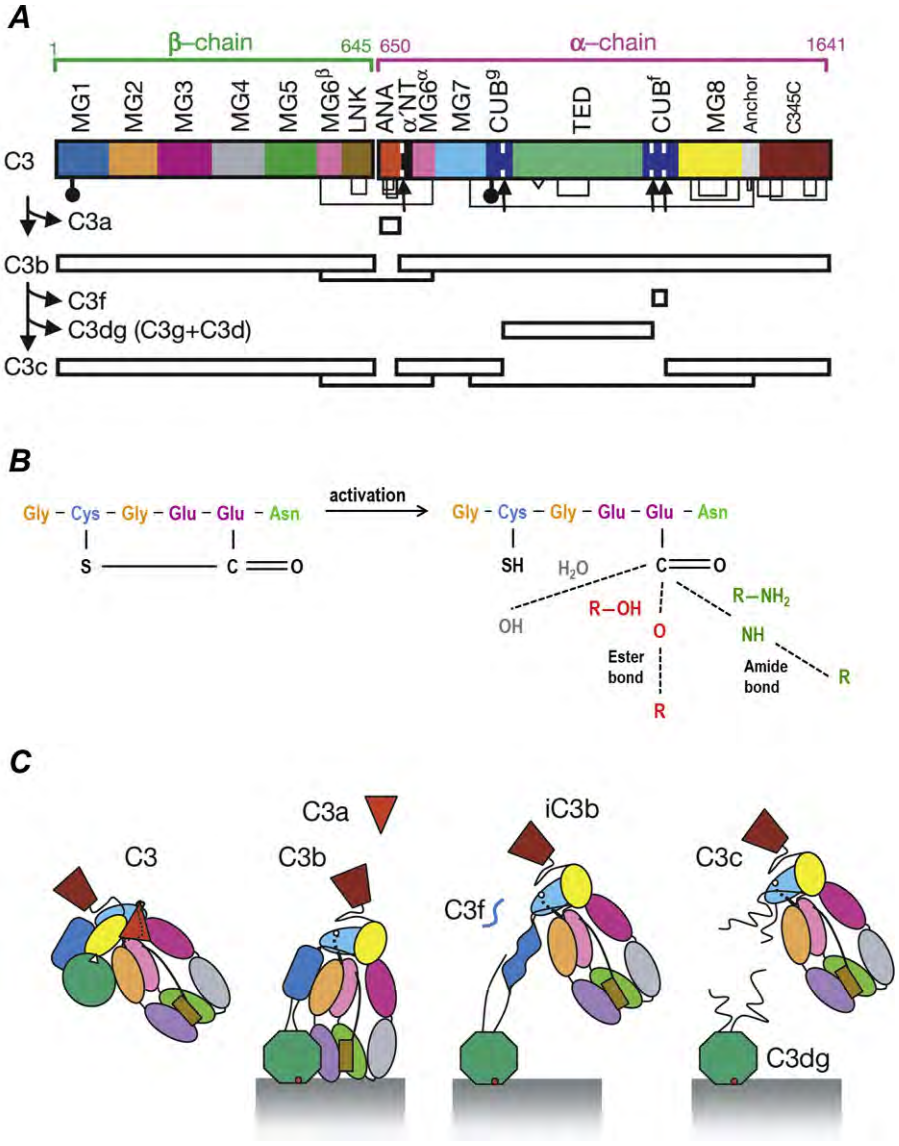

Fig. 2 C3 structure and activation. (A). The C3 molecule represented as its α and β chains and its degradation products. Arrows indicate physiologic cleavage sites. The location of the thioester bond is indicated by the inverted white triangle. Sites of N-linked glycosylation are shown by the 'inverted lollipop' symbol. Locations of disulfide bridges are also shown. Colors of the amino acid stretches of the α and β chains correspond to domain colors in panel C. (B). The internal thioester bond of C3. Asn, asparagine; Cys, cysteine; Glu, glutamic acid; Gly, glycine. R represents a surface target. (C). The domain organization of C3 and its cleavage products. C3 is organized into 13 domains. Upon activation, C3a is released from the amino terminus of the α-chain of C3. The internal thioester bond becomes exposed and accessible to nucleophilic attack and can react with water or available hydroxyl or amine groups on cell surfaces (B). Analogous reactions occur with C4. Together, these reactions involving C3 and C4 are responsible for covalently linking complement deposition to the cell surface. Activation of C3 is accompanied by an ~85 Å displacement of the thioester domain and the resulting C3b molecule can form covalent (either ester or amide) bonds with targets (C). Cleavage of C3b to iC3b also results in conformational changes that contribute to ligand specificity. MG, macroglobulin; LNK, linker; ANA, anaphylatoxin; CUB, complement C1r/C1s, Uegf, Bmp1; TED, thioester-containing domain; α′NT, α′ amino-terminal segment. From Janssen, B.J., Huizinga, E.G., Raaijmakers, H.C., et al., 2015. Structure of complement component C3 provide insights into the function and evolution of immunity. Nature 437, 505–511, Janssen, B.J., Christodoulidou, A., McCarthy, A., et al., 2006. Structure of C3b reveals conformational changes that underlie complement activity. Nature 444, 213–216.

rearrangements among its various domains (Fig. 2(C)). The thioester domain that is 'buried' in native C3 moves about ~85 Å, becomes exposed and can react with nucleophiles through formation of a highly reactive acyl-imidazole intermediate with a remarkably short calculated half-life of ~30 μs. If this unstable and highly reactive group does not bind to a surface –OH (or under some conditions, a $-NH_2$ group), it will react with a water molecule and remain in solution (Fig. 2(B)). The high reactivity and short life of the nascent thioester domain restricts C3 deposition to sites proximate to the site of C3 activation, while sparing more distant (and possibly normal) tissue from 'bystander' damage.

Activation of the alternative pathway

The "tickover" model

As discussed above, the internal thioester bond mediates covalent attachment of C3 to surfaces. Although well concealed in the native C3 molecule, the thioester undergoes spontaneous hydrolysis at a low rate of 0.2%–0.4%/h. The generated molecule, called $C3(H_2O)$, has C3b-like properties, but differs from C3b in that it still possesses the C3a fragment. Similar to C3b, $C3(H_2O)$ also can bind to factor B (FB), properdin, C5, can be further cleaved by the combined action of factor H (FH; cofactor) and factor I (FI; enzyme), and can bind to cellular receptors for C3. In the brief period before its degradation by FH and FI, $C3(H_2O)$ can bind to FB to form $C3(H_2O)B$, which in the presence of FD and Mg^{2+} forms $C3(H_2O)Bb$ (the Ba fragment is released from FB). Akin to C3bBb, $C3(H_2O)Bb$ has C3 convertase activity and generates more metastable C3b molecules capable of forming covalent bonds with surfaces. Each $C3(H_2O)Bb$ produces on average three to five metastable C3b molecules before being inactivated by FH. Surface-bound C3b then recruits FB and FD to generate surface-bound C3 convertase (C3bBb) and set into motion the positive feedback loop of the alternative pathway. The sequence of events that generate C3 convertases through the "tickover" of C3 are illustrated in Fig. 3(A). C3bBb is inherently unstable and dissociates into its components with a $t_{1/2}$ of about 90 s. Binding of properdin – the only known positive regulator of complement – to C3bBb stabilizes the complex and prolong its half-life 5- to 10-fold, thereby amplifying C3 activation. $C3(H_2O)$ may constitute an important source of intracellular C3 and play an important role in the homeostasis and differentiation of T cells, as discussed below.

The properdin-directed model

The properdin-directed model put forth by Hourcade et al. lent support to Pillemer's original proposal of alternative pathway activation. Each properdin subunit is highly positively charged and composed of six complete thrombospondin type 1 repeat (TSR) domains and a truncated N-terminal TSR domain. Each subunit oligomerizes in a head-to-tail manner to form dimers,

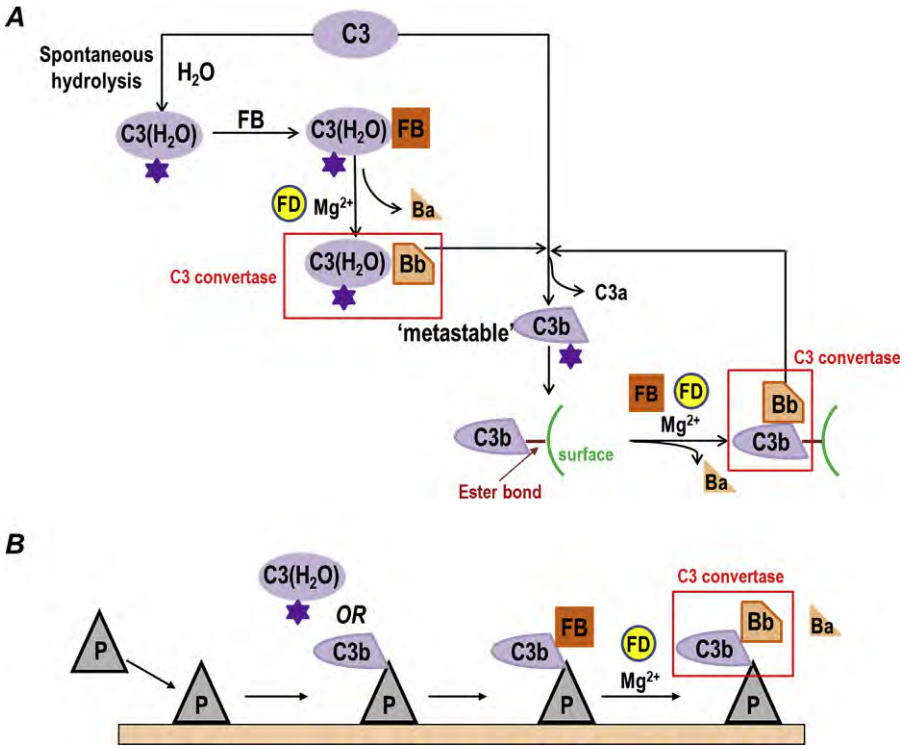

Fig. 3 Initiation of alternative pathway activation. (A). The 'tickover' model. C3 undergoes slow, spontaneous hydrolysis, where the internal thioester bond (indicated by a star) that is normally tucked away becomes exposed and reacts with a water molecule to form $C3(H_2O)$. Subsequent Mg^{2+}-dependent reactions with FB and FD results in formation of fluid-phase C3 convertase, $C3(H_2O)Bb$, which then activates more native C3 molecules and enables them to bind covalently to surfaces and further activate the alternative pathway. (B). The properdin-directed model. Properdin binding to activator surfaces can recruit fluid-phase C3b or $C3(H_2O)$ and serves as a platform for formation of C3 convertases on the surface.

trimers and tetramers at a ratio of 26:54:20, respectively. Phagocytes, and in particular neutrophils, are the main site of properdin synthesis and storage. Release of properdin from these cells may increase alternative pathway activation locally at sites of inflammation. Properdin multimers may bind to a select cell surface sulfated glycoconjugates and initiate the alternative pathway. Surface-bound properdin can then recruit C3b or C3(H_2O) that then binds FB and act as a platform for further alternative pathway activation (Fig. 3(B)).

Studies using purified properdin should be interpreted cautiously because properdin forms higher order oligomers and aggregates upon freeze-thawing or with prolonged storage, which may result in artefactual binding to surfaces. Increasing evidence suggests that properdin may bind to cell surfaces solely through C3b.

The Terminal Complement Pathway: Assembly of the Membrane Attack Complex

Binding of C3b to classical and alternative pathway C3 convertases generates C5 convertases (C4bC2aC3b and C3bBbC3b) that can cleave C5 and initiate the assembly of membrane attack complex (MAC). C5 bears structural homology with C3 and C4, but lacks a thioester domain. The addition of C3b to C3 convertases lowers the K_m for C5 >1000-fold, from far above the physiological concentration of C5 in plasma to far below it. Therefore, situations that favor complement activation and rapid C3b generation also facilitate the generation of MAC. Cleavage of C5 releases the 11 kDa C5a fragment, an anaphylatoxin with diverse functions (discussed below). Carboxypeptidase N removes the C-terminal Arg residue to yield C5a-desArg, which possesses only 1%–10% of the inflammatory activity of C5a.

Binding of C5b to hydrophobic sites on cell surfaces exposes binding sites for C6 and C7 to form the C5b-7 complex, which has amphiphilic properties and inserts into cell membranes. C8 then binds to the β-chain of C5b, followed by the addition of one or more C9 molecules. C6, C7, C8 and C9 all belong to the MACPF/CDC (MAC-perforin/cholesterol dependent cytolysin) superfamily of proteins. In its fully assembled state, MAC contains one molecule each of C5b, C6, C7 and C8 and up to 18 molecules of C9. Poly-C9 is responsible for the ring-like configuration of MAC. The external aspect of the tubule is hydrophobic and intercalates into membranes, while the hydrophilic inner aspect of the pore permits passage of water and ions. Cryo-electron microscopy analysis of the entire C5b-9 complex revealed a 'split-washer' configuration, as opposed to a symmetric closed-ring conformation seen with perforin and other cholesterol-dependent cytolysins. Disruption of the membrane proton motive force during pore formation and osmotic damage mediated by the channel may both contribute to the cytolytic and bactericidal actions of MAC.

The Intracellular Complement System

Recent studies have shown the presence of intracellular stores of C3 and C5, which are cleaved by intracellular proteases such as cathepsin L to release C3a and C5a, respectively. C3 may be expressed by T cells themselves, or C3(H_2O), the C3b-like hydrolyzed form of C3 may be taken up from the circulation by cells. Activation of intracellular complement plays an important role in the homeostasis, effector response and contraction of T cells. Intracellular C3a-C3aR interactions induce low levels of mammalian target of rapamycin (mTOR) activation to facilitate T cell survival (homeostasis). T cell activation is triggered by signaling through the T cell receptor (TCR), which results in translocation of C3a and C3b to the cell surface, where they engage C3aR and CD46, respectively. Activation of CD46 increases expression of the CD46 isoform with the Cyt-1 intracytoplasmic 'tail'. Cleavage of Cyt-1 by γ-secretase leads to translocation of Cyt-1 to the nucleus which drives the expression of glucose and amino acid transporters, increases nutrient influx and glycolysis and oxidative phosphorylation, resulting in Th1 expansion. Stimulation of CD46 and T cell receptor increases cleavage of intracellular C5 to release the C5a fragment. C5a-C5a receptor 1 (C5aR1) interactions generates reactive oxygen species (ROS), which in turn activates the NLRP3 inflammasome and IL-1β release. IL-1β expression is required for optimal production of IFN-γ and Th1 differentiation. Th1 contraction occurs when the CD46 isoform with the Cyt-2 tail predominates, which in conjunction with the IL-2R signaling, produces the Th1-inhibiting cytokine, IL-10.

Inhibition of the Complement System

Tight regulation of the complement cascade is essential to limit complement activation only to sites of tissue injury or infection, thereby minimizing collateral damage to normal host cells. Targeted activation is achieved by the specificity of antibodies and lectins. Once initiated, complement activation is amplified by the alternative pathway positive feedback loop. However, excessive and uncontrolled activation is limited by the short half-lives of the convertases and anaphylatoxins. Several fluid-phase inhibitory proteins act at various levels of complement activation, including C1, C4b, C3b and MAC. The high plasma concentrations of these inhibitory proteins highlight their importance in minimizing unnecessary complement activation. Several membrane-associated complement inhibitors also dampen complement activation on cell surfaces. Absence or dysfunction of complement inhibitors is associated with diseases such as atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy and age-related macular degeneration, further underscoring their physiological importance.

Fluid-Phase Complement Inhibitors

C1 inhibitor (C1-INH)

C1-INH belongs to the family of protease inhibitors called serpins (*ser*ine *p*rotease *in*hibitors) that function by a unique "trapping" mechanism, whereby the enzyme cleaves the inhibitor at its reactive center (between Arg 444 and Thr 445 in C1-INH), which is followed by formation of a covalent complex between the enzyme and the inhibitor (*i.e.*, the inhibitor is a "suicide substrate"). C1-INH is one of the most heavily glycosylated serum proteins. C1-INH directly inactivates both C1r and C1s. Binding of C1-INH to C1r also dissociates C1r and C1s from the activated C1 macromolecule, as well as the entire activated C1 complex ($C1q\text{-}C1r_2\text{-}C1s_2$) from immobilized human IgG. C1-INH also inhibits the alternative pathway; C1-INH binds to C3b and blocks FB-C3b interactions, thereby preventing the formation of alternative pathway C3 convertase.

Although its name suggests specificity for C1, C1-INH acts on a variety of substrates including the contact system proteases (factor XII, plasma kallikrein), an intrinsic coagulation protease (factor XI) and the fibrinolytic proteases (plasmin, tissue plasminogen activator). C1-INH deficiency results in hereditary angioneurotic edema, characterized by excessive bradykinin production and increased vascular permeability.

Factor I (FI)

FI is a serine protease that inhibits the classical and alternative pathways by cleaving the α' chains of C4b and C3b,respectively, in the presence of cofactors – C4b-binding protein (C4BP) for C4b, factor H (FH) for C3b and membrane cofactor protein (MCP) and complement receptor 1 (CR1) for both C3b and C4b – to their hemolytically inactive forms. Cleavage of C3b yields iC3b, while cleavage of C4b yields C4c and C4d. The primary site of FI synthesis is the liver, but FI also synthesized by fibroblasts, monocytes, keratinocytes, endothelial cells, myoblasts and primary cervical epithelial cells. FI is an acute-phase protein and is upregulated in endothelial cells, hepatocytes and fibroblasts by LPS and IFN-γ. Complete absence of FI is rare and results in uncontrolled complement activation and complement consumption. These individuals are functionally deficient in complement and predisposed to infections, in particular, invasive meningococcal disease. Loss of function mutations in FI lead to alternative pathway overactivity and results in aHUS or C3 glomerulopathy. FI polymorphisms, some of which result in low serum levels of FI, are associated with an increased risk of AMD.

C4b-Binding Protein (C4BP)

C4b-binding protein (C4BP) inhibits the classical pathway by acting as a cofactor in the factor I (FI)–mediated cleavage of C4b to C4c and C4d, and accelerating dissociation of C2a from the classical pathway C3 convertase (C4bC2a), a property called 'decay-accelerating' activity. C4BP is composed entirely of short consensus repeat (SCR) domains, also called complement control protein (CCP) domains. Each SCR domain consists of about 60 amino acids, with four invariant cysteine residues and several other conserved amino acids that are folded into a compact unit. Other complement proteins that contain SCR domains include FH, DAF, MCP, CR1, CR2, FB and C2. Interestingly, most SCR-containing complement proteins interact with C3b and/or C4b.

Factor H (FH) and the FH family of proteins

Analogous to inhibition of the classical pathway by C4BP, FH is a cofactor for FI–mediated cleavage of C3b to iC3b and accelerates the decay of C3bBb. FH also blocks binding FB to C3b to prevent formation of the alternative pathway C3 convertase. FH contains 20 SCR domains organized as a single chain; the first four N-terminal SCRs are necessary and sufficient for complement inhibition. In addition to inhibiting the alternative pathway in the fluid phase, FH also limits alternative pathway activation on host cells. This property stems from the ability of FH to interact simultaneously with C3 fragments deposited on host cells and specific host glycosaminoglycans (including sialic acid in certain configurations) through domains 19 and 20, respectively. The interaction between FH and "self-polyanions" on cells increases the affinity of FH for C3b, and simultaneously decreases FB-C3b interactions, thus preventing C3 convertase formation. Conversely, the FB binding to C3b is favored over FH-C3b interactions on "activator" surfaces. Similar to complete deficiency of FI, loss of FH is associated with complement consumption and predisposition to meningococcal infections, and with renal pathology (dense deposit disease). Mutation in FH that impair recognition of cell surfaces and C3d – most of which reside in domains 19 and 20 – result in aHUS. A homozygous polymorphism in domain 7 (His instead of Tyr at position 402) significantly increases the risk of age-related macular degeneration (AMD). Compared to FH containing Tyr402, FH with His402 shows impaired binding to malondialdehydes that accumulate in 'drusen' (the subretinal lesions of dry AMD). Reduced FH (His402) bound to drusen permits more alternative pathway activation and increased uptake of malondialdehyde-modified proteins by macrophages, with resulting inflammation and ocular damage.

An alternatively spliced variant of factor H called factor H-like protein 1 (FHL-1) contains the first seven N-terminal SCRs of FH and therefore possesses cofactor and decay accelerating activities. There are also five FH-related molecules (FHRs 1 through 5) that are the products of separate genes. FH and the related proteins are arranged in tandem within the Regulation of Complement Activation (*RCA*) gene cluster on human chromosome 1q32. The FHRs also composed entirely of SCR domains that share varying levels of homology with FH, but none of the FHRs have cofactor or decay accelerating activity. FHRs 1, 2 and 5 exist in circulation as homo- or heterodimers. FHRs have been postulated to block binding of FH to microbial surfaces. As an example, CFHR3, competes with FH for binding to meningococci and promotes complement activation. The homology and proximity of the FH family of genes results in gene deletions, duplications and rearrangements. Expression of FH-FHR hybrid proteins result in disordered regulation of the alternative pathway and are associated with renal disorders, such as aHUS, C3 glomerulonephritis, dense deposit disease and retinal damage in age-related macular degeneration.

Vitronectin (Vn)

Vitronectin (Vn; also known as S protein protein) inhibits the terminal complement complex at various stages. Vn can occupy the metastable membrane-binding site of the nascent C5b-7 complex to form sC5b-7. Although the sC5b-7 complex can take up further C8 and C9 molecules to form sC5b-8 and sC5b-9, respectively, these vitronectin-containing complexes lack hemolytic activity because the number of C9 molecules is limited to three, which is insufficient to form a pore. Vn also blocks pore formation by perforin, a product of cytotoxic T lymphocytes and natural killer (NK) cells. Vn is a multifunctional protein with several distinct ligands and modulates coagulation, fibrinolysis, pericellular proteolysis, vascular remodeling, cell attachment and spreading.

Clusterin (Cn)

Clusterin (Cn; apolipoprotein J; serum protein 40,40 (SP40–40)) is a heterodimer linked by five disulfide bonds. The highest levels of Cn occur in semen, which contains Cn levels 10-fold above the serum concentrations. Although structurally unrelated to Vn, Cn shares functional similarity with Vn and binds to several sites in MAC to prevent C9 polymerization.

Membrane-Associated Complement Inhibitors

Membrane-associated complement inhibitors are critical to prevent damage to host tissue. Diseases associated with their absence or dysfunction highlights their physiological importance – as examples, loss of CD46 function is associated with aHUS and loss of CD55 and C59 causes paroxysmal nocturnal hemoglobinuria (PNH). A schematic representing the five major membrane-associated complement inhibitors discussed below is shown in Fig. 4.

Complement receptor 1 (CR1; CD35)

CR1 is an integral membrane glycoprotein composed of a C-terminal transmembrane region and an extracellular region composed of a linear array of 30 SCR units. The N-terminal 28 SCRs are further organized as four tandem, long homologous repeats (LHRs) of 7 SCR units each. Four allelic variants of CR1 have been described in humans, which express three to six LHRs. CR1*1 (or CR1-A) is the most commonly encountered allotype and occurs in >80% of most populations studied. CR1 copy number on erythrocytes constitutes another polymorphism.CR1 binds C3b, C4b, C1q and MBL. CR1 possesses cofactor activity and can facilitate FI cleavage of C3b to iC3b, and is the only molecule that can facilitate further cleavage of iC3b to C3c and C3d. CR1 also mediates decay acceleration of C3 and C5 convertases of the classical and alternative pathways.

CR1 is found on erythrocytes, neutrophils, monocytes, glomerular podocytes and certain T cells. Human erythrocyte CR1 mediates binding of complement-opsonized immune complexes or microorganisms and forms the basis for the phenomenon of immune adherence. Clustering of CR1 clusters on cell surfaces allows multivalent binding of immune complexes to RBCs. RBC-bound complexes or organisms are removed as they transit the liver or spleen; in the process CR1 is also lost cell from the surface.

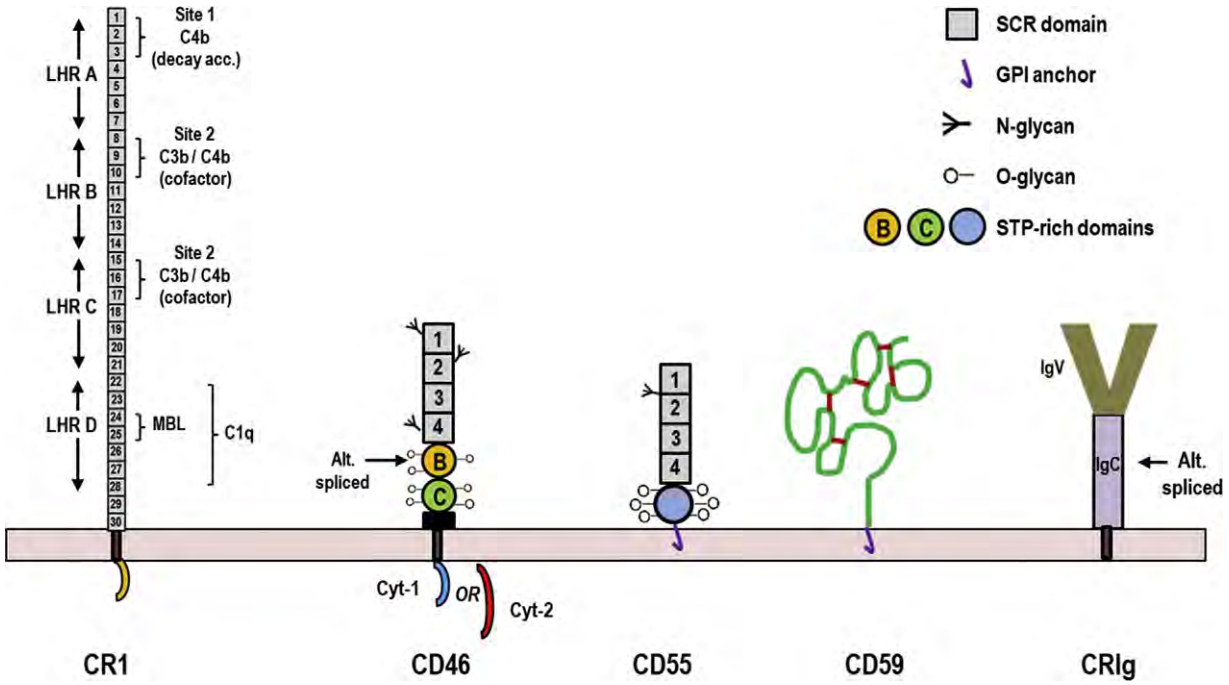

Fig. 4 Schematic representation of membrane-bound complement inhibitors. SCR, short consensus repeat; LHR, long homologous repeat; GPI, glycophosphatidylinositol; STP, serine threonine proline.

Given its importance in clearing immune complexes, CR1 expression levels on erythrocytes may also modulate the severity of systemic lupus erythematosus (SLE).

SCR1 of CR1 interacts with the *Plasmodium falciparum* protein PfRh4. In addition to facilitating the entry of *P. falciparum* into RBCs, CR1 promotes erythrocyte rosetting that may contribute to the pathogenesis of cerebral malaria. Several epidemiologic studies have attempted to correlate CR1 allotypes and expression levels with the severity of malaria, often with conflicting results.

CD46 (membrane cofactor protein [MCP])

CD46 is a cofactor for FI-mediated cleavage of C3b and C4b to iC3b and C4d, respectively. CD46 expressed on most nucleated cells. Accordingly, human erythrocytes lack CD46. CD46 contains four SCR domains followed by an O-glycosylated serine/threonine/proline-rich (STP) domain, 12 residues of unknown function and an intracytoplasmic tail. Alternative splicing of the STP region (encoded by the B and C exons; the A exon is rarely used) to the B exon and the cytoplasmic tail to either Cyt-1 or Cyt-2 gives rise to four major isoforms of CD46 called C1, C2, BC1 and BC2. N-glycans in SCRs 2 and 4 are required for cofactor activity. The STP region modulates CD46 function. For example, the BC isoform binds C4b more efficiently than the C isoform and better protects cells against the classical pathway.

CD55 (decay accelerating factor [DAF])

CD55 is a single chain glycoprotein that comprises four SCR domains and a heavily glycosylated STP-rich domain that is attached to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor. CD55 accelerates the decay of classical and alternative pathway C3 and C5 convertases and protects host cells against autologous complement-mediated injury. DAF is present on all blood cells and most other cell types. High levels of DAF are seen on cells that line extravascular compartments, such as the cornea, conjunctiva, oral and gastrointestinal mucosa, exocrine glands, renal tubules, ureter and bladder, cervical and uterine mucosa, and pleural, pericardial and synovial membrane.

CD59 (homologous restriction factor 20 [HRF-20]; Membrane attack complex inhibitory factor [MACIF]; Protectin)

CD59 is also GPI-anchored to the cell membrane. CD59 binds to C8 in MAC and prevents incorporation of pore-forming poly-C9. CD59 may also bind to C9 in partially assembled MAC complexes. The term 'homologous restriction factor' is a misnomer because CD59 can protect cells against 'non-host' complement, albeit to varying degrees. Thus, activity may be host-selective rather than host-restricted. Host-selective complement inhibition also applies to other membrane-bound complement inhibitors such as CD46 and CD55. The ability of non-human membrane complement inhibitors to protect against damage by human complement may have implications in xenotransplantation.

Complement receptor of the immunoglobulin superfamily (CRIg)

CRIg, a type 1 transmembrane IgG superfamily member, exists as two alternatively spliced forms: the longer form, huCRIg(L) encodes both V and C_2-type terminal Ig domains, while the short form, huCRIg(S) encodes only an IgV domain. CRIg is expressed by Kupffer cells, $CD14^+$ dendritic cells and non-inflammatory resident macrophages in various tissues, but not on infiltrating macrophages during inflammation or on peripheral blood $CD14^+$ monocytes, suggesting a role in maintenance of homeostasis rather than in inflammation. CRIg binds to C3b-opsonized particles and in a murine model accelerates the clearance of *Listeria monocytogenes* and *Staphylococcus aureus*. CRIg also binds to the C3b component of alternative pathway C3 and C5 convertases and prevents their interaction with C3 and C5, thereby inhibiting the alternative pathway.

Complement Receptors

Complement receptors may be classified into: (i) receptors that bind to soluble complement fragments (*e.g.*, C3a and C5a) or (ii) receptors for complement components deposited on surfaces (*e.g.*, C1q, C3b, iC3b, C3d and C4b). The characteristics of the membrane-associated complement receptors are summarized in Table 2.

Receptors for C5a and C3a

C5aR (CD88; C5aR1) is expressed on myeloid-derived cells and non-myeloid cells including vascular smooth muscle, endothelium, epithelium and glial cells and binds to C5a with high affinity (Kd≈1 nM), but with relatively low affinity (Kd≈660 nM) to C5a-desArg. C5aR1 is a G-protein coupled receptor, which interestingly is 'pre-coupled' to G-proteins even in the absence of its ligand. C5a-C5aR1 interactions lie below the level of detection when C5aR1 is uncoupled from G-proteins. Activation of C5aR1 results in Ca^{2+} fluxes from intra- and extracellular sources, which is followed by binding of β-arrestins 1 and 2 to C5aR1, resulting in internalization of C5aR1 via clathrin-coated pits. Subsequently, several downstream pathways are stimulated, including PI3K-γ kinase, phospholipase C β2, phospholipase D and Raf-1/B-Raf mediated activation of MEK1.

A second receptor for C5a, called C5a receptor-like protein (C5L2 or C5aR2), is coexpressed with C5aR1. In addition to C5a, C5L2 also binds to C3a and C3a-desArg. Unlike C5aR, C5L2 couples poorly to Gi-like G protein-mediated signaling pathways and is not internalized following ligand binding. The role of C5L2 remains controversial. While some reports suggest that C5L2 functions as a decoy receptor to antagonize C5aR, other studies show that C5L2 is required for optimal C5a signaling in phagocytes and fibroblasts.

Table 2 Complement receptors and membrane bound complement inhibitors

Protein	*Characteristics*
Membrane bound complement inhibitors	
CR1	Cofactor for factor I cleavage of C3b to iC3b and further to C3d, and C4b to C4d; binds to MBL and C1q; clearance of opsonized pathogens and C3b/C4b associated with immune complexes ("immune adherence")
CD46	Cofactor for factor I cleavage of C3b and C4b. Ligand for Notch family member Jagged1. Roles in T cell differentiation
CD55	Accelerates the decay of C3 convertase assembled on cells.
CD59	Inhibits the assembly of membrane attack complex (C9 polymerization)
CRIg	Ligand for the β-chain of C3b/iC3b; inhibits alternative pathway C3 and C5 convertases by binding to C3b and preventing interaction of the convertases with C3 and C5, respectively; role for pathogen clearance demonstrated in mouse model
Complement ligands	
CR2	Binds primarily to C3d and C3dg; part of the CR2/CD19/CD81 complex that mediates B cell responses to antigens linked to C3 fragments; receptor for Epstein-Barr virus
CR3	Ligand for iC3b; phagocytosis
CR4	Binds for C3d/C3dg; function not known
C1q receptors (cC1qR and gC1qR)	cC1qR (calreticulin) binds to the collagenous region of C1q; gC1qR binds to the globular domain of C1q; cC1qR – phagocytosis of apoptotic cells, chaperone, Ca^{2+} homeostasis; gC1qR – modulation of complement, kallikrein-kinin and coagulation systems.
SIGN-R1	Complement receptor identified as a murine homolog of DC-SIGN; binds select pneumococcal polysaccharides and C1q and can activate the classical pathway in Ab-independent manner
Receptors for anaphylatoxins	
C3aR	Binds C3a/C3a des-Arg; vasodilatation
C5aR	Binds C5a/C5a des-Arg; chemotaxis; modulates inflammation and sepsis, T cell differentiation

Note: Modified from Ram, S., Lewis, L.A., Rice, P.A., 2010. Infections of people with complement deficiencies and patients who have undergone splenectomy. Clin. Microbiol. Rev. 23 (4), 740–780.

Similar to C5aR, C3aR is also a G-protein coupled receptor. C3aR is present on B lymphocytes, guinea pig ileum, vascular endothelium, adipocytes, and mast cells. Unlike C5aR, stimulation of C3aR initiates Ca^{2+} flux only from the extracellular pool and therefore does not activate PI3K-γ. C3aR activation results in activation of protein kinase C by phospholipase C or mitogen activated protein (MAP) kinases. C3a-C3aR interactions induce cytokine expression through ERK and Akt phosphorylation.

A variety of functions have been ascribed to anaphylatoxins and their receptors. They increase chemotaxis and vascular permeability in sepsis. Higher levels of C3a and C5a are often associated with poorer outcomes. C3a and C5a are important in guiding T-cell responses and differentiation. Interactions between FcγRs and C5aR has also been postulated. Treatment of macrophages with C5a upregulates the activating FcγRIII (possess an immunoreceptor tyrosine-based activation motif (ITAM)) and downregulates the inhibiting FcγRIIb (possesses an immunoreceptor tyrosine-based inhibition motif (ITIM)), thereby reducing the threshold for FcγR-mediated stimulation of macrophages. Macrophage activation releases C5, which produces C5a through the activity of proteases, further upregulating activating FcγRs. Engagement of FcγRIIb on the other hand, inhibits signaling through C5aR. Immune complexes containing IgG with highly galactosylated Fc N-glycans link Dectin-1 with FcγRIIb, which blocks downstream responses mediated by C5aR.

Receptors for C1q

Cellular receptors for C1q include cC1qR (calreticulin (CR); cC1q/CR) that binds to the collagen domain of C1q and gC1qR (also known as hyaluronic acid-binding protein 1, or p33) that recognizes the globular domain of C1q. cC1qR also binds to the collectins surfactant protein A (SP-A) and MBL. cC1qR is expressed by most cell types, except erythrocytes. cC1qR facilitates clearance of C1q-coated apoptotic cells. Calreticulin also located in the storage compartments of the endoplasmic reticulum. Along with calnexin, intracellular calreticulin functions as a molecular chaperone that regulates glycoprotein folding. By virtue of being a high-affinity Ca^{2+} storage protein, calreticulin also regulates Ca^{2+} homeostatsis.

gC1qR is part of the receptor complex for high molecular weight kininogen (HK) on endothelial cells and also binds hepatitis C virus core protein. gC1qR modulates the activity of the kallikrein-kinin and coagulation systems. Soluble gC1qR released by endothelial cells acts as an autocrine signal to induce expression of the bradykinin receptor 1. Engagement of gC1qR on $CD4^+$ T cells by HIV-1 glycoprotein 41 (gp41) induces expression of NKp44L, which targets destruction of these $CD4^+$ cells by NK cells and contributes to $CD4^+$ T cell depletion in HIV infection. Expression of gC1qR and cC1qR are both upregulated in most malignant cells. They appear to have opposing roles in carcinogenesis; cC1qR enhances phagocytosis and increases tumor destruction, while gC1qR promotes tumor growth by enhancing angiogenesis and metastasis.

Complement Receptor 2 (CR2; CD21)

CR2 (or CD21) is a glycosylated transmembrane protein that is composed of a series of 15 or 16 SCRs. C-terminal to the SCRs is a 22–24 amino acid transmembrane domain followed by a 34-amino acid intracellular domain. CR2 is found on mature

B lymphocytes, follicular dendritic cells, thymocytes, a subpopulation of $CD4^+$ and $CD8^+$ T cells, basophils, keratinocytes, astrocytes and lacrimal and ocular epithelial cells. CR2 is the principal ligand for C3d and Epstein-Barr virus glycoprotein 350/220. CR2 helps couple innate recognition of microbial antigens to B cell activation. Activation of complement results in deposition of C3 fragments on foreign antigens or microbes; the interaction of C3-tagged antigens with CR2 (CD21) results in formation of the CD21/CD19/CD81 complex and activates B cells, as discussed below.

CR3 (Mac-1; CD11b/CD18; $\alpha_M\beta_2$-integrin)

CR3 is a β_2-integrin that mediates binding of neutrophils to endothelial cells and is critical for neutrophil recruitment to sites of infection. CR3 mediates phagocytosis of iC3b-coated microbes, enables extravasation of leukocytes from the circulation to sites of injury or infection and facilitates homing of lymphocytes to tissues. The arginine-glycine-aspartic acid sequence (Arg-Gly-Asp, or the 'RGD motif') present in C3 and other CR3 ligands is an important binding motif for CR3. The C-terminal domain of CD11b contains a lectin site that recognizes microbial polysaccharides such as β-glucan, β-oligomannan and GlcNAc. CR3-dependent cytotoxicity requires engagement of the lectin domain; iC3b-coated cells or pathogens that do not engage the lectin binding site on CR3 only adhere to cells but are not phagocytosed. The lectin site of CR3 also promotes formation of cell surface transmembrane signaling complexes between CR3 and membrane glycoproteins that are attached to cells through GPI anchors and therefore cannot signal on their own, such as CD16b (FcγRIIIB) and CD87 (urokinase plasminogen activator receptor, or uPAR).

CR4 (CD11c/CD18)

CR4 is also an integrin that binds to iC3b. In addition, CR4 binds to fibrinogen, ICAM-1, LPS and denatured peptides. CR4 is expressed on myeloid cells, tissue macrophages, dendritic cells, activated B cells and lymphoid cells.

Functions of Complement

Innate Immunity – Combating Infection

As discussed above, the complement system discriminates between self and 'non-self' structures such as invading pathogens to target them for elimination. The amplification of C3 and C5 convertases is favored over their decay on 'non-self' structures. Complement activation generates C3and C5a, which promote vascular dilatation, endothelial permeability and neutrophil chemotaxis. Pathogens marked, or opsonized with C3b and iC3b are targeted for removal by phagocytes through the interactions with CR1 and CR3, respectively. Opsonophagocytosis is mediated mostly through iC3b-CR3 interactions in conjunction with engagement of IgG by Fc receptors (FcγR). In contrast to gram-negative bacteria can be killed by MAC insertion, the thick cell walls of gram-positive bacteria and fungi render them resistant to killing by MAC and therefore phagocyte recruitment is required to kill these pathogens.

Most gram-negative bacteria isolated from the blood are resistant to killing by complement, whereas most isolates of the same species isolated from mucosal sites tend to be complement-sensitive. These data point to a central role for complement in host defenses. The increased incidence of infections in individuals with complement deficiencies is further evidence for the role of complement in innate immune defenses.

Complement is critical in combating meningococcal infections. The incidence of invasive meningococcal disease in individuals with defects in the alternative (FD and properdin) or terminal complement (C5 through C9) pathways is increased about 1000- to 2000-fold over rates in the general population. Pharmacologic blockade of C5 by the therapeutic monoclonal antibody eculizumab, an approved treatment for paroxysmal nocturnal hemoglobinuria (PNH) and aHUS, is also associated with a high rate of meningococcal disease.

Paradoxically, persons with terminal complement defects and meningococcal infection experience a lower mortality than complement-sufficient individuals. Severity of meningococcal disease correlates directly with the extent of complement activation and endotoxin levels. This is because an intact terminal pathway is necessary for lipopolysaccharide (LPS) release from the surface of gram-negative bacteria, which could explain the milder disease course observed in individuals with terminal complement defects.

Modulation of the Adaptive Immune Response

B Cells and Humoral Immunity

A role for complement in shaping adaptive immune responses was suggested about 45 years ago, when C3b and C3d were shown to bind to B lymphocytes and follicular dendritic cells (FDCs). Covalent binding of C3b to an antigen marks the antigen for uptake by phagocytic cells or retention by FDCs for recognition by cognate B cells. CD21 (CR2) forms a receptor complex with CD19 and CD81 on B cells and plays a central role in enhancing B cell immunity. Coligation of CD21 and B cell receptor with C3 fragments and antigen, respectively, in antigen-C3 complexes lowers the threshold for B cell activation. As an example, hen egg lysozyme (HEL) coupled to two and three copies of C3d were 1000- and 10,000-fold more immunogenic, respectively, than HEL alone. This 'adjuvant-like' role for C3d enhances responses to antigens that have a low affinity for the B cell receptor.

Complement also enhances B cell immunity by localizing antigen to FDCs within lymphoid follicles. High expression of CD21 and CD35 on FDCs facilitate efficient trapping of immune complexes bound to C3 fragments within the lymphoid compartment.

An intact classical pathway, CD21 and CD35 are necessary for efficient uptake of immune complexes by FDCs. Optimum activation of B cells requires an intact classical pathway and ligands for C3 and C4 fragments, evidenced by the observation that mice deficient in C1q, C4, C3, or CD21/CD35 have impaired humoral responses to thymus-dependent and thymus-independent antigens.

Studies using $C1q^{-/-}$, $C4^{-/-}$ or $C3^{-/-}$, or $CD21/CD35^{-/-}$ knockout mice have all demonstrated the importance of complement at several stages of B cell differentiation. B cells first express the CD19/CD21/CD81 complex as they migrate from the bone marrow to the periphery. Cross-linking of the BCR at this early stage in their development results in cell death or anergy rather than activation, thus eliminating self-reactive B cells. B1 cells, the main source of natural antibody, are positively selected during early development. Complement plays a role in the selection and maintenance of B1 cells, evidenced by the altered natural antibody repertoire of $CD21/CD35^{-/-}$ mice.

Regulation of T Cells

C3-deficient mice are highly susceptible to primary infection with influenza A virus. C3-knockout mice show delayed viral clearance and increased viral titers in the lung, attributable to reduced priming of T-helper cells and cytotoxic T lymphocytes in lymph nodes draining the lung and the impaired recruitment into the lung of virus-specific $CD4^+$ and $CD8^+$ effector T cells that produce IFN-γ. Accordingly, T-helper cell-dependent IgG responses are also reduced in $C3^{-/-}$ mice. C3-coated viral particles are taken up by antigen presenting cells (APCs) through receptors such as CR3 (CD11b/CD18) and CR4 (CD11c/CD18) and results in T cell priming. Lack of C3 reduces APC function and limits T cell priming. Independent of APC function, lack of C3 prevents C3a and C5a generation. Engagement of C3aR and C5aR by these anaphylatoxins may contribute to the pulmonary response to influenza virus.

Cross-linking of CD46 (which is also a receptor for the measles virus) with an anti-CD46 antibody or with C3b (a ligand for CD46) inhibits monocyte IL-12 production, which could contribute to the immunosuppression associated with measles infection. Co-engagement of CD3 and CD46 in the presence of IL-2 induces a T-regulatory 1 (Tr1)-specific cytokine phenotype in $CD4^+$ T cells, which produce IL-10 and thus inhibit the activation of bystander T cells. CD46 also binds to Jagged1, a member of the Notch family of proteins; loss of CD46-Notch crosstalk stunts T_H1 responses.

Local production and activation of complement and signaling through C3aR and C5aR determines the outcome of T cell responses. Engagement of Toll-like receptors on dendritic cells (DCs) results in secretion of alternative pathway components and upregulates C3aR and C5aR expression. C3a and C5a act on their cognate receptors on DCs to induce secretion of IL-6, IL-12 or IL-23. Stimulation of CD28 on T cells induces expression of C3aR and C5aR; engagement of the latter by C3a and C5a generated by DCs induces IL-12R expression and subsequently, IFN-γ and IL-2 production. Interleukins secreted by DCs then determine whether responses are skewed toward T_H1 or T_H17. In the absence of activation of DCs through pattern recognition receptors, local complement production ceases and the lack of C3aR and C5aR signaling is associated with increased production of TGF-β and induction of suppressive $Foxp3^+$ T_{reg} cells. During this process C5L2 is upregulated and sequesters C5a, further limiting C5aR activation.

Complement and Autoimmunity

Complement plays an important role in solubilizing and clearing immune complexes (ICs). Fc-Fc interactions leads to precipitation of ICs. C1q binding to Fc interferes with Fc-Fc interactions and prevents IC precipitation. Subsequent C3b deposition, aided by alternative pathway amplification, disrupts forces within the IC and prevents further lattice formation. Separation of smaller complexes from the lattice results in solubilization of immune complexes. Thus, the classical pathway prevents IC precipitation while C3b deposition facilitates IC solubilization. Complement is about ten times more efficient in preventing IC precipitation than solubilizing them. This function of complement explains the strong association between defects of the classical pathway and lupus.

ICs bearing C3b are targeted for removal by CR1. Erythrocytes express ~1000 CR1 molecules per cell, while neutrophils express ~60,000 CR1 molecules on their surface. Because erythrocytes outnumber neutrophils in circulation by a factor of about 1000, over 95% of the CR1 in the circulation is found on erythrocytes. IC removal from the circulation occurs as erythrocytes traverse the liver and spleen, where tissue macrophages lining the sinusoids of these organs remove both, CR1and the ICs adherent to it.

Complement also plays an important role in the disposal of apoptotic cells. The surface of apoptotic cells often contains molecules such as phosphatidylserine, annexin 2 and annexin 5 that bind C1q and activate complement. The 'complement-marked' cell is then eliminated through engagement of C1qR and CR3 on macrophages and dendritic cells. The R77H variant allele in CD11b that is associated with impaired phagocytosis is a strong risk factor for SLE, highlighting the importance of CR3 in clearing apoptotic cells. Factors that contribute to limited inflammation associated with apoptosis include binding of complement inhibitors such as FH and C4BP, which reduces complement activation and elimination of apoptotic cells via CR3 on phagocytes.

Metabolism

Metabolic syndrome is associated with increased systemic inflammation and complement activation. Insulin resistance and obesity are associated with increased concentrations of C3. Fat cells are the main source of FD (FD is therefore also called adipsin), and also secrete FB and C3. Local C3 activation generates C3a, which is rapidly converted to C3a-desArg by carboxypeptidase N. C3a-desArg, which is also known as acylation-stimulating protein, promotes triglyceride synthesis in fat cells by increasing the activity of diacylglycerol acyltransferase.

Complement and Cancer

Complement may play a role in immune surveillance against malignant cells by promoting antibody-dependent cellular cytotoxicity and also through lysis by MAC, but on the other hand may promote tumor growth and metastasis. Cancer cells evade complement by expressing high levels of membrane complement inhibitors such as CD46, CD59 and DAF, and can also recruit fluid-phase complement inhibitors such as FH and FHL-1. C3a and C5a induce the secretion of vascular endothelial growth factor (VEGF), which promotes neovascularization. Complement can degrade the extracellular matrix, which may facilitate tumor invasion and migration. C5a also attracts myeloid-derived suppressor cells (MDSCs) to tumors, which generate reactive oxygen and nitrogen species that interfere with the ability of T cells to respond to tumor antigens. Signaling through C5aR increases proliferation of endothelial and colon cancer cell lines. Activation of C3aR guides cell migration that may promote metastasis. Insertion of sublytic amounts of MAC in cell membranes promotes cell proliferation, inhibits apoptosis, and enhances their resistance to complement-mediated lysis.

Tissue Regeneration, Organogenesis and Synaptic Pruning

Accumulating experimental evidence supports a role for complement in tissue growth and regeneration. Locally produced C3 and C5 facilitates limb regeneration in newts. C3 and C5 knockout mice show impaired liver regeneration. Signaling through the canonical Wnt pathway contributes to age-associated decline in tissue repair and regeneration.

The classical pathway prunes and eliminates unwanted synapses in the developing brain. A recent study associated higher levels of C4A expression in the brain with schizophrenia; the mechanism postulated was excessive complement-mediated synaptic pruning during adolescence. Complement activation also contributes to synaptic loss in mouse models of Alzheimers disease.

C3a-C3aR interactions guide neural crest migration in *Xenopus* embryos. Impaired collectin 11 (CL-K1) and MASP-1 function results in misdirected migration of neural crest cells during development, which may contribute to development of the 3MC syndrome, a term that encompasses four rare autosomal recessive disorders with overlapping clinical features: Mingarelli, Malpuech, Michels and Carnevale syndromes. The 3MC syndrome is characterized by developmental abnormalities including facial dysmorphism, learning disability, and genital, limb and vesicorenal anomalies.

The context and the extent of complement activation may be important in determining the outcome to the host. Controlled complement activation is important in eliminating apoptotic cells, facilitating adaptive immune responses, aiding tissue regeneration and in organogenesis, while unregulated complement activation may contribute to the pathology of acute myocardial infarction, stroke, hyperacute rejection of organ transplants and to tissue injury in conditions such as hepatic, pulmonary and renal fibrosis, Alzheimer's disease, Parkinson's disease and multiple sclerosis.

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Conjugation, Bacterial☆

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Glossary

Coupling protein An ATPase responsible for the transport of DNA during conjugation. It is a hallmark of conjugative systems and homologues are widely distributed throughout nature.
ICE (integrating conjugative element) Chromosomally encoded elements, similar to conjugative transposons, capable of excision, conjugation, and reestablishment in a new host via integration. The excision and integration operations are formally similar to those of integrative phages.
Plasmid An extrachromosomal DNA segment, usually circular, which is capable of autonomous replication via a segment of the plasmid called the replicon.
Relaxase The protein responsible for site-specific nicking at the origin of transfer (*oriT*) in the DNA as well as recircularization after transfer. It covalently attaches to the 5′ end of the nicked DNA via a tyrosine. It is a key component of the relaxosome.
Transconjugant A general term for a recipient cell that has successfully been converted to donor cell by conjugation.
Transposon A segment of DNA that is replicated as part of a chromosome or plasmid. It encodes a mechanism, called transposition, for moving from one location to another, leaving a copy at both sites.
Type IV secretion system (T4SS) A widely distributed mechanism for the secretion and uptake of protein and nucleic acids via secretion, conjugation, and transformation.

Abbreviations

Cma	Chromosome mobilization ability
Eex	Entry exclusion
fi/Fin	Fertility inhibition
Hfr	High frequency of recombination
HFT	High frequency of transfer
HGT	Horizontal gene transfer
HSL	Homoserine lactone-like
ICEs	Integrating conjugative elements
IHF	Integration host factor
Inc	Incompatibility groups
kb	Kilobases
LPS	Lipopolysaccharide
Mpf	Mating pair formation
Mps	Mating pair stabilization
NLS	Nuclear localization signals
T4SS	Type IV secretion system
Tc	Tetracycline

Defining Statement

Bacterial conjugation is a widespread mechanism for the transfer of DNA between cells in close contact with one another. This entry summarizes past findings and discusses the better-studied systems in Gram-negative and -positive bacteria as well as the phenomena of mobilization and tumorigenesis in plants, which are related processes.

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Introduction

Bacterial conjugation was first described by Lederberg and Tatum in 1946 as a phenomenon involving the exchange of markers between closely related strains of *Escherichia coli*. The agent responsible for this process was later found to be a site on the chromosome called the F ('fertility') factor. This finding was the basis of bacterial genetics in the 1940s and 1950s and was used extensively in mapping the *E. coli* chromosome, making it the preeminent prokaryotic organism at that time. It was also shown that F could excise out of the chromosome and exist as an extrachromosomal element or plasmid. It was capable of self-transfer to other bacteria and could cotransfer the chromosome, a serendipitous function of F, and integrate randomly into its host's DNA. The F sex factor of *E. coli* also imparted sensitivity to bacteriophages that required the F pilus, which is encoded by the F transfer region, as an attachment site during infection. In the 1960s a number of other conjugative plasmids were isolated, many carrying multiple antibiotic resistance markers. These plasmids were termed R ('resistance') factors and were found in many instances to repress pilus expression and conjugation by F, a process termed fertility inhibition (fi^+). The number of conjugative plasmids discovered has grown tremendously in the last few decades and includes self-transmissible plasmids isolated from Gram-negative -positive bacteria, Archaea, as well as mobilizable plasmids. Conjugative transposons or integrating conjugative elements (ICEs), which move between cells using a conjugative mechanism, excise and integrate into the host chromosome via a process reminiscent of lysogenic phages; an example of a conjugative phage has been described for *Staphylococcus aureus*.

In general, the transfer and replication functions of these mobile elements are often physically linked and the type of transfer system is closely aligned with the nature of the replicon that is described by incompatibility groups (Inc). An excellent summary of the properties of many conjugative plasmids is given in Shapiro (1977).

Bacterial conjugation is now realized to be one of the principal conduits for horizontal gene transfer (HGT) among microorganisms. The process is extremely widespread and can occur intra- and intergenerically as well as between kingdoms (bacteria to yeast or to plants). DNA sequence analysis has revealed that conjugation, and in some cases transformation, two of the main conduits for HGT, are effected by a transenvelope protein complex that belongs to the type IV secretion system (T4SS). The effect of this process on evolution has been immense with bacteria rapidly acquiring traits both good (hydrocarbon utilization) and bad (antibiotic resistance, toxins). Once again, bacterial conjugation is at the forefront of microbiology but this time the emphasis is on the process itself rather than its utility as a geneticist's tool. Excellent reviews of the topic are provided in *The Horizontal Gene Pool, Bacterial Plasmids and Gene Spread* (C.M. Thomas, ed.) and *Plasmid Biology* (Phillips, G. and Funnell, B., eds.).

Conjugative Process

Unlike other processes like transformation and transduction that contribute to HGT, conjugation can be distinguished by two important criteria. There must be close cell-to-cell contact between the donor and recipient cells and DNA transfer must begin from a specific point on the transferred DNA molecule, be it a plasmid, transposon, or chromosome (Figure 1). This point is encoded

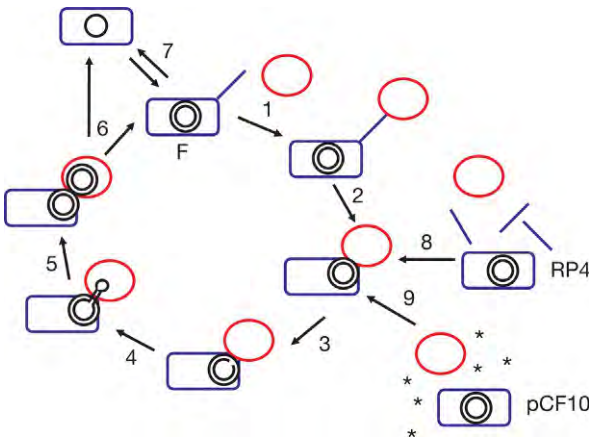

Figure 1 Summary of the mating process for universal (plasmid F) and surface-preferred (plasmid RP4) conjugation systems in Gram-negative bacteria and the pheromone-activated system of *Enterococcus faecalis* (plasmid pCF10). In universal systems, the pilus attaches to a receptor on the recipient cell surface (1) and retracts to form a stable mating pair or aggregate (2). DNA transfer is initiated (3), causing transport of a single strand in the 5′ → 3′ direction (4). Transfer is associated with synthesis of a replacement DNA strand in the donor cell and a complementary strand in the recipient (5). The process is terminated by disaggregation of the cells, each carrying a copy of the plasmid (6). The transfer systems of conjugative plasmids in Gram-negative bacteria can be repressed (7) or derepressed (constitutive; 8). Cells carrying RP4 and related plasmids express pili constitutively but the pili are not seen attached to the bacteria. Such cells form mating pairs by collision on a solid surface (8). In Gram-positive bacteria, such as the enterococci, the donor senses the presence of pheromone (*) released by the recipient cell, which triggers mating pair formation (Mpf) and DNA transfer (9). Donor cells are shown as oblongs (blue) and recipient (red) cells as ovals. Pili are blue.

within the origin of transfer (*oriT*) called *nic*. The proteins that act on this site are encoded by *tra* (transfer) or *mob* (mobilization) regions although other designations such as *vir* are now common. In general, each conjugative element encodes an array of proteins for mating pair formation (Mpf) while another set of proteins are involved in processing and transferring the DNA (Dtr). The Mpf genes can further be classified into the genes for pilus formation or mating pair stabilization (Mps) in Gram-negative bacteria or aggregate formation in Gram-positive cocci. A system to prevent close contact between equivalent donor cells is called surface exclusion. The gene products that process the DNA in preparation for transfer usually include a protein (relaxase) that cleaves the DNA in a sequence- and strand-specific manner at *nic* and remains covalently bound to the 5′ end in all cases that have been examined. This nucleoprotein complex plus other auxiliary proteins bound to the *oriT* region is called the relaxosome whereas the complex formed between the relaxosome and the transport machinery is known as the transferosome. A hallmark of conjugative systems is the coupling protein, within the cytoplasmic membrane, that connects the relaxosome to the transferosome. A process that prevents the transfer of DNA into the recipient cell after Mpf has occurred is called entry exclusion (Eex). Previously, the terms surface exclusion and entry exclusion were used interchangeably; however, as the details of the process have been refined, it is important to make this distinction.

In Gram-negative bacteria, the process of DNA transfer is triggered upon cell contact whereas in *Enterococcus faecalis* and T-DNA transport by *Agrobacterium tumefaciens*, among others, contact between cells induces a complex program of gene expression leading to DNA transport. Whereas the sequences for a number of conjugative elements have been completed and comparisons have revealed information on the evolution of conjugative elements, a study of the conjugative process has only been undertaken in some depth for IncF, IncI, IncP, IncW elements and the Ti plasmid of *A. tumefaciens* and for the pheromone-responsive system found in some plasmids in *Ec. faecalis*, although studies on other systems such as pIP501 are ongoing. Information is now available on the integration and excision processes of conjugative transposons and ICEs as well as the role of the *mob* genes in mobilizable plasmids. In addition, conjugation in *Streptomyces* has been studied in some detail but is quite different than that described and may use a DNA transport mechanism related to the process of DNA partition during septation in *Bacillus subtilis* (see '*Streptomyces*').

Physiological Factors

The level of transfer efficiency varies dramatically among the various systems. For derepressed or constitutively expressed systems such as F (IncFI) or RP4 (IncPα), maximal levels of mating (~100% conversion to plasmid-bearing status) are possible within 30 min. Plasmids undergoing fertility inhibition usually have a 100- to 1000-fold reduction in mating efficiency whereas other plasmids, especially the smaller plasmids of Gram-positive bacteria and conjugative transposons, mate at barely detectable levels even under the best of circumstances. Factors affecting mating efficiency include temperature with very precise optimums usually being the rule. For instance, F and RP4 mate optimally at 37–42 °C, and IncH plasmids and the Ti plasmid at about 20–30 °C. Other factors include oxygen levels, nutrient availability, and growth phase. Silencing by host-encoded factors such as H-NS is an important phenomenon that is thought to provide control of gene expression by newly acquired DNA through HGT, a process now termed 'xenogeneic silencing'. F^+ cells in late stationary phase are known as F^- phenocopies because they are able to accept incoming F DNA and are not subject to surface or entry exclusion. Available literature indicates conjugation to be maximal over a short temperature range, in nutrient-rich environments with good aeration for aerobic organisms.

Liquid versus Solid Support

The ability of some conjugative systems to mate equally well in liquid media or on a solid support is one of the hallmarks of conjugation. Whereas all conjugative elements can mate well on a solid support, usually a filter placed on the surface of a prewarmed nutrient agar plate, many transfer systems, including those of the IncF group and the pheromone-responsive plasmids of *Enterococcus*, mate very efficiently in liquid media. This difference can be attributed to the nature of the Mpf process as thick, flexible pili of Gram-negative bacteria are associated with systems that mate well in liquid media whereas rigid pili, not usually seen attached to the cells (e.g., IncPα), require a solid support for efficient mating. The aggregation substance of *Ec. faecalis* allows high levels of transfer in liquid media but other Gram-positive systems and conjugative transposons mate at low levels and absolutely require a solid support. In general, it appears that mating systems requiring a solid support depend on collision between donor and recipient cells whereas systems that mate well on either medium have a mechanism for initiating contact between freely swimming cells (thick, flexible pili, and aggregation substance). The description of media requirements for many Gram-negative plasmid transfer systems is given in Bradley et al. (1980).

Conjugative Elements

Naturally occurring conjugative elements including plasmids, conjugative transposons, or ICEs, which are incorporated into the host chromosome, can lead to chromosome mobilization ability (Cma), resulting in high frequency of recombination (Hfr). Free plasmids can be divided into self-transmissible (Mpf plus Dtr genes) or mobilizable (Dtr or Mob genes) plasmids and can vary in size from a few kilobases (kb) to large plasmids 100–500 kb in size.

Table 1 Selected conjugative/mobilizable plasmids and conjugative transposons.

Mobile element	*Size (kb)*	*Inc group/host/pheromone*[a]	*Copy number*	*Mating surface/pilus type*[b]	*Mating efficiency/host range*
Gram-negative bacteria					
F	100	IncFI	1–2	Liquid/flexible (II)	High (derepressed)/narrow
RP4	60	IncPα	4–6	Solid/rigid (II)	High (constitutive)/broad
ColIB-P9	93	IncI1	1–2	Liquid/rigid (II),thin (IV)	Low (repressed)/narrow
pTiC58	~200	*Agrobacterium tumefaciens*/HSL	1–2	Solid/rigid (II)	Low (repressed)/narrow
vir	25 (T-DNA)	Plant exudates	1–2	Plants/rigid (II)	
Gram-positive bacteria					
pAD1	60	*Ec. faecalis*/cAD1	1–4	Liquid	High (10^{-2} per donor)/narrow
pIP501	30.2	Inc18/*Streptococcus agalactiae*	3–5	Solid	High (10^{-4} per donor)/broad
pIJ101	8.8	*Streptomyces*	~300	Solid	High/broad (*Actinomycetes*)
Mobilizable plasmids					
ColE1	6.6	*Escherichia coli*	~10	Liquid	High (IncF, −P, −I)/narrow
RSF1010	8.9	IncQ	~10	Solid	High (IncP)/broad
pMV158	5.5	*Streptococcus* B	12–16	Solid	Low (pAMβ1/pIP501)/broad
Conjugative transposons					
Tn*916*	18.5	*Enterococcus faecalis*		Solid	Low (~10^{-8} per donor)/broad
CTnDOT	80	*Bacteroides*/Tc		Solid	Low (~10^{-5} per donor)/narrow
Integrating conjugative elements					
SXT	99.5	*Vibrio cholerae*		Solid	Low (~10^{-4} or 10^{-5} per donor)/narrow
R391	89	*Providencia rettgeri*		Liquid	Low (~10^{-4} or 10^{-5} per donor)/narrow

[a]Incompatibility groups (Inc) are listed for *E. coli* except pIP501, which uses the Inc group classification for *Streptococcus*. HSL is homoserine lactone. cAD1 is a pheromone specific for pAD1 of *Ec. faecalis*. CTnDOT transfers at 1000-fold higher frequencies in the presence of tetracycline (Tc)
[b]Pili can be classified as type IV or type II that are assembled by type II (T2SS) or type IV (T4SS) secretion systems, respectively

Plasmids

In general, Gram-negative transfer systems are approximately 20–35 kb and reside on plasmids from 60 to 500 kb whereas mobilizable plasmids are under 15 kb. The transfer or mobilization regions often represent half or more of the coding capability of the plasmid. Table 1 contains a list of selected plasmids and their characteristics including their pilus type and mating medium preference. In nonfilamentous Gram-positive plasmids, the smaller plasmids (<30 kb) usually have a requirement for a solid support during mating and mate at low levels whereas the larger plasmids mate efficiently in liquid media and express genes for aggregate formation (e.g., *Enterococcus*, *Staphylococcus*, *Lactobacillus*, *Bacillus thuringiensis*). *Streptomyces* is able to mate at high frequency and has the added property of Cma. Each large self-transmissible plasmid can supply the needed Mpf functions for a number of mobilizable plasmids. These mobilizable plasmids have been used to construct vectors that either are maintained in the recipient cell or deliver their cargo of DNA but are unable to replicate in the new host (suicide vectors). This has been a boon for the study of genetics of otherwise recalcitrant bacteria.

Chromosome Mobilization

F undergoes integration into the chromosome via four transposable elements (IS2, IS3a and b, and Tn1000), which either mediate cointegrate formation via a transposition event or more frequently undergo homologous recombination between these sequences and similar elements on the chromosome. Once incorporated into the chromosome, the F replicon is suppressed by the chromosomal replication machinery allowing stable maintenance of the Hfr strain. Like F, which was found incorporated into the host chromosome, other examples of naturally occurring Hfr strains have been reported in the literature including ICEs. Hfr strains have also been constructed using homologous gene segments shared by a plasmid and its host for mapping the host's genome. Since the advent of pulsed-field gel electrophoresis for mapping chromosomes and large-scale sequencing facilities, the utility of Hfr strains for mapping purposes is waning. The procedure for using Hfr strains for chromosome mapping requires that the plasmid integrate near a locus with a few genetically defined markers. The direction of transfer of the chromosome and the time of entry of the markers into the recipient cell is a function of the position and orientation of the plasmid's *oriT* in the chromosome

and its distance from each marker. By laboriously measuring the time of entry of each marker (e.g., antibiotic resistance, amino acid biosynthesis), which must be able to recombine into the recipient's chromosome in such a way as to announce its presence, a map of the chromosome can be generated. The process of mobilizing the entire *E. coli* chromosome takes about 90 min and markers that are distal from F *oriT* are transferred much less efficiently than those more proximally located, a process called the 'gradient of transmission'. The last portion of the chromosome to be transferred contains the F transfer region; consequently, the recipient cells in an Hfr mating are seldom, if ever, converted to F^+ (Hfr) status.

Another related property of F is imprecise excision out of the chromosome with adjoining chromosomal sequences being incorporated into the circular F element that are often large enough to encode complete operons. These elements are known as F′ (F prime) factors, and examples include F*lac*, F*gal*, and F*his*.

Conjugative Transposons and ICEs

The first conjugative transposon, Tn*916*, which expressed *tetM* (tetracycline (Tc)) resistance, was isolated from *Ec. faecalis* (Table 1) in Clewell's laboratory in 1981. This element excises from the chromosome, circularizes into a nonreplicative intermediate, and expresses functions for transfer to a wide range of recipient cell types at low frequency on solid surfaces. The enzymes responsible for excision and integration (Int, Xis) are related to the corresponding enzymes in lambda (λ) phage. The absence of a small repeated sequence flanking the conjugative transposon as well as a nonrandom site selection mechanism for integration suggests that these elements are evolutionarily more related to phages than true transposons. Excision of the element results in staggered ends that form a heteroduplex structure upon circularization. This heteroduplex structure is derived from the flanking sequences in the chromosome ('coupling sequences'). On the basis of mapping and sequencing the site of insertion and determining which end of the heteroduplex is inherited in the recipient, a model involving single-stranded DNA transfer has been proposed. A second important group of conjugative transposons has been found in the anaerobic Gram-negative genus *Bacteroides*. These elements are usually associated with antibiotic resistance (*tetX*, *erm*) and exhibit increased transfer proficiency in the presence of Tc. That IncJ conjugative plasmids could not be isolated from *E. coli* was puzzling. The realization that they were related to the chromosomally located, conjugative, integrative phage-like SXT element in *Vibrio cholerae* explained why they were not true plasmids. These ICEs appear to lack the property of random site selection during integration but instead integrate at *att*-like sites (e.g., *prfC*), similar to lysogenic phages.

Conjugative transposons demonstrate amazing versatility in mobilizing DNA. They can mobilize coresident plasmids directly or form cointegrates with plasmids or the chromosome. They are also able to harbor other mobile elements such as transposons and move them between cells. In the case of *Bacteroides*, the conjugative transposons are able to excise and mobilize small, nonconjugative, nonreplicative segments of DNA found in the chromosome called NBUs (nonreplicating *Bacteroides* units). While conjugative transposons have been identified in many genera of bacteria especially in Gram-positive bacteria, the details of the conjugation process remain obscure although orthologues of T4SS have been identified within the transfer regions, suggesting that Gram-negative and -positive conjugation are related.

Gram-Negative Conjugation

With the possible exception of *Bacteroides*, all Gram-negative transfer systems encode a conjugative pilus (type II), which is essential for Mpf and DNA transport (Figure 1). In thick, flexible pilus mating systems, the pili are attached to the donor cell and pili-mediated contact between donor and recipient cells is easily visualized with a electron microscope. In the case of rigid pilus mating systems, pili are rarely seen attached to cells but are seen as bundles of pili accumulating in the medium.

Considerable homology has been found among all the transfer systems examined to date within the Gram-negative group of organisms as well as detectable homology with systems in Gram-positive bacteria and Archaea. In Gram-negative bacteria, two broad classes can be identified as F-like or P-like Mpf, with I-like Mpf systems being a subclass of P. Relaxase, as well as the protein that energizes DNA transport, the coupling protein, is conserved throughout these systems as are key proteins in the T4SS, as described below. To date, the principal systems studied include IncF (F, R100-1, R1) and IncHI1, which have F-like Mpf genes, and IncP (RP4, R751), IncI1 (ColIb-P9, R64), IncN (pCU1, pKM101), IncW (R388), and the Ti plasmids of *A. tumefaciens*, which encode IncP-like Mpf genes.

Pilus

Structure

The conjugative pilus is a thin filament expressed in relatively low numbers (1–3 per cell), has no set length (usually ~1 μm), and is randomly distributed on the surface of the cell (Figure 2). The diameter of the pilus is approximately 6–11 nm, with most pili being around 9 nm. Pili isolated from cells usually contain a 'knob' at the base that represents unassembled pilin subunits derived from the inner membrane of the cell. They may also have a pointed tip suggesting another protein or unusual configuration of pilin subunits at the tip and can aggregate into large bundles that can be pelleted in an ultracentrifuge. The pilus is usually composed of a single repeating subunit of pilin arranged in a helical array with a hollow lumen clearly visible in negatively stained electron micrographs. F pili, which are the best studied, have a diameter of 9 nm with an inner lumen of 2 nm and a mass per unit length of 3000 Da $Å^{-1}$ (Arutyunov and Frost, 2013).

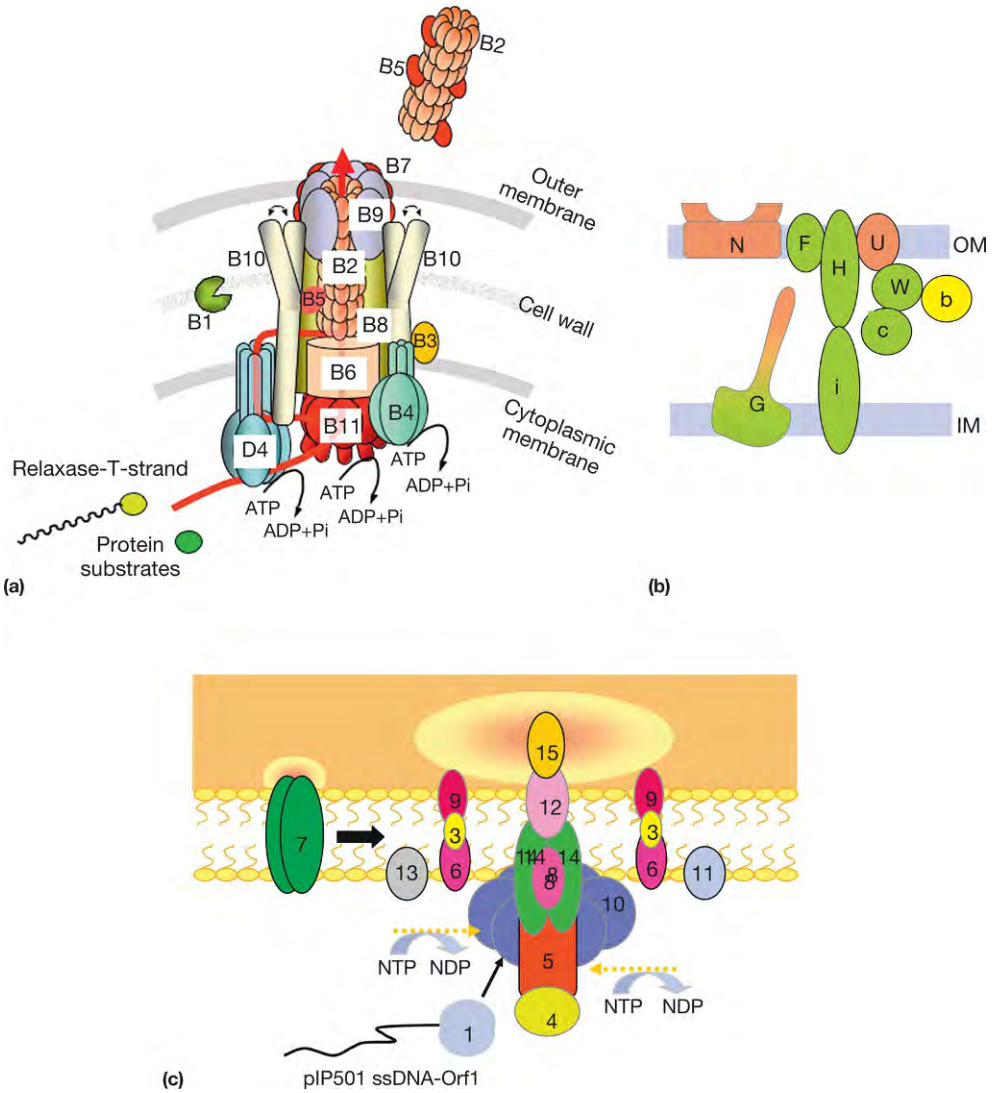

Figure 2 Transfer apparatuses of Gram-positive and -negative bacteria. The simplest transfer mechanism is exemplified by Tra in *Streptomyces*, which is homologous to the coupling protein of other systems (not shown). (a) The transfer apparatus of the Ti plasmid illustrates the complexity of the P-type Gram-negative transfer systems. The red arrow is the putative path of the DNA during transfer. Note the presence of three ATPases involved in pilus assembly and DNA transfer. Reprinted with permission from AAAS. (b) The F-type Gram-negative system is similar to that for the Ti plasmid except that it lacks homologues of VirB11 (ATPase) and VirB8. It contains proteins for mating pair stabilization (Mps) (TraG, −N, and -U in orange) as well as a cluster of proteins involved in pilus assembly that form an interaction network (shown in green) (based on Harris and Silverman (2004) *Journal of Bacteriology*. 186: 5480–5485). TrbB (yellow) has DsbC-like activity; TraN, −U, and -H are cysteine-rich. TraD, the coupling protein, is not required for pilus assembly. The relaxase, other members of the relaxosome, and inessential proteins are not shown. (c) The transfer apparatus of pIP501 illustrates the comparative simplicity of a system that spans only one membrane. Homologies among the various systems as well as functions are given in Table 2. Reproduced from Abajy et al. (2007), redrawn with permission

The F pilus is expressed as propilin of 121 amino acids (*traA*), which requires TraQ, a putative chaperone, for insertion in the inner membrane where it is stored in a pool of approximately 100 000 subunits. The 51-amino acid leader peptide is cleaved by the host leader peptidase (LepI) and the pilin subunit is acetylated at the N-terminus by TraX. Transposon insertion studies have revealed that mature pilin is oriented within the inner membrane as two α-helical transmembrane segments with the N- and C-termini facing the periplasm. Assembly by the TraL, −E, −K, −B, −V, −C, −W, −U, −F, −H, −G and TrbC proteins results in the subunits oriented within the fiber such that the acetylated N-terminus is buried within the structure and the C-terminus is exposed on the sides. The pilus appears to assemble at its base rather than the tip, based on evidence using the slowly assembled pili expressed by the IncHI1 plasmid R27.

The RP4 pilin subunit is expressed as a 15 kDa prepilin polypeptide (*trbC* in Tra2), which is processed three times to give a 7.5 kDa mature product that is circular with the N- and C-termini covalently linked. The cleavage reactions at the N- and C-termini are completed by LepI of the host as well as the cyclase, TraF, that removes four amino acids at the C-terminus and cyclizes the pilin. The RP4 transfer region is separated into two parts: Tra1 and −2. In addition to *traF* of Tra1, an essential gene, *trbD-L* are required for

pilus assembly with the exception of TrbK, which is involved in Eex. A homologue of F TraX is present in RP4 (TraP) although its substrate and function are unknown. Circular pilins have been identified in the IncHI1 plasmid, R27, and T-pili of the Ti plasmid of *A. tumefaciens*, that uses a host-encoded cyclase, and are suspected to be present in other mating systems that encode a P-like TraF orthologue.

Phage attachment

Conjugative pili act as the primary receptor for a wide range of bacteriophages. These phages can be divided broadly into those that bind to the pilus tip and those that bind to the sides of the pilus. The structure of the phages includes the single-stranded DNA filamentous phages, the small isometric RNA phages, and the complex double-stranded DNA tailed phages that usually attach near the pilus tip. The filamentous phages specific for F-like pili (Ff phages; M13, f1, fd) attach via a defined region of the pIII attachment protein to an unknown receptor at the pilus tip. The RNA phages such as R17 and Qβ, which belong to different phage groups, bind to specific residues in F pilin exposed on the pilus sides. For RP4 (IncPα), the filamentous phage Pf3 binds to the sides of the pilus as does the RNA phage PRR1. The tailed phages such as PRD1 and PR4 bind to the pilus tip, although it appears that infection requires binding to a tip exposed at the cell surface rather than at the end of an extended pilus. These phage have a broad host range including cells bearing IncP, −W, −N, and -I plasmids.

Whereas the pilus is required for initial attachment, the transfer region is not necessarily required for phage penetration or growth. The Ff phages are thought to contact the cell surface via the process of pilus retraction where they interact with the TolA protein and penetrate the cell via the TolQRA pathway. The RNA phages R17 and Qβ have differing requirements for the F TraD coupling protein, which energizes the transport of nucleic acid through the conjugation pore, suggesting that R17 is imported via the F transfer machinery whereas Qβ is taken up via another pathway. RNA phages have the property of phage eclipse, an extracellular event that involves detachment of the phage capsid from the attachment (A) protein–RNA complex, which is then susceptible to exogenous ribonuclease. This process requires that the pilus be extended from a living cell, suggesting that the cell supplies the energy for phage eclipse in some unknown manner. Mutations in F-pilin that block phage eclipse have been identified.

Role in conjugation

The role of the pilus in conjugation has been controversial but it is now generally believed that pilin is part of the conjugative pore. Mutations that affect pilus formation block both Mpf and DNA transfer. Mutations that affect Mps (e.g., F TraN and TraG) allow initial contacts to form between cells via the pilus and also allow the initiation of DNA synthesis in the donor cell but block DNA transport into the recipient cell. While indirect, this is the best evidence that the pilus is involved in the signaling process whereas the Mps genes are involved in forming the conjugation pore. Other experiments in which donor and recipient cells were not allowed to establish cell-to-cell contact suggested that mating was possible through the extended pilus. The pilus has also been implicated in DNA transport in both the F plasmid (mutations in pilin that block transfer) and the Ti plasmid (cross-linking T-DNA to pilin).

Mating Pair Formation

Conjugation involves the establishment of a mating pair via Mpf, which may be augmented by the process of Mps, a feature of F-like systems. The pilus is thought to identify a receptor on the recipient cell surface that triggers retraction of the pilus into the donor cell, although the route of the pilin subunits in this process is unknown. Pilus outgrowth requires energy whereas retraction occurs by default in the absence of assembly. Thus factors that negatively affect cell metabolism (temperature, poisons, and carbon source) promote retraction. Pilus outgrowth and retraction are ongoing processes whereas binding of recipient cells or phage probably trigger retraction.

Early studies identifying mutations in the recipient cell that affected conjugation (Con^{-}) revealed that various components of the heptose-containing inner core of the lipopolysaccharide (LPS) were generally important in Mpf whereas OmpA was required for efficient conjugation by the F transfer system. The F-like systems are each affected by different mutations in the *rfa* (now *wra*) locus in the recipient cell whereas the IncH plasmids seem to recognize a generalized negative charge on the recipient cell surface. The requirements for OmpA by F as well as for specific side chains in the LPS appear to be a function of the outer membrane protein F TraN, which is involved in Mps. The idea that the pilus recognizes negatively charged surfaces nonspecifically remains a possibility. A second protein identified in F that is involved in Mps is TraG. Mutations in *traG* fall into two classes: those in the first two-thirds of TraG that affect pilus formation and Mpf and those that only affect Mps.

Recent studies on the F-like *tra* and Ti *vir* gene products have revealed the presence of a complex transenvelope structure, the transferosome, composed of core T4SS proteins and auxiliary proteins involved in pilus retraction and Mps (Figures 2(a) and 2(b); Table 2). The scaffold for the transferosome consists of a TraB (VirB10)–TraK (VirB9)–TraV (VirB7) complex that spans the envelope. VirB10 has been shown to have TonB-like activity that transduces energy from the inner membrane to the outer membrane; TraK/VirB9 are related to secretins of other secretion systems and TraV/VirB7 are lipoproteins in the outer membrane. Other transfer proteins are also required for pilus assembly (Figure 2(b)) with a group of proteins (TraF, −G, −H, −N, −U, −W, TrbB, −C, and -I), which are characteristic of F-like transfer systems, additionally involved in pilus retraction and conjugative pore formation. Some of these proteins have a high cysteine content (TraH, −N, and -U) whereas others are homologous to thioredoxin with one protein (TrbB, a homologue of DsbC) shown to be involved in disulfide bond formation and protein stabilization.

Table 2 Orthologues in various conjugative systems.[a]

xfunction	*F*[b]	*P(RP4)*	*Ti(pTiC58)*	*I1(R64)*	*pIP501*	*pAD1*	*HI1(R27)*
SLT[c]	P19	TrbN	VirB1	TrbN	Orf7	Orf41/50	Orf169
Acetylase	TraX	TrbP					
Pilin cyclase		TraF	Unknown				TrhP
Pilin	TraA	TrbC	VirB2	TraX			TrhA
Pore, pilus assembly	TraL	TrbD	VirB3				TrhL
Pilus assembly, ATPase	TraC	TrbE	VirB4	TraU	Orf5		TrhC
Pore, pilus assembly	TraE	TrbF	VirB5				TrhE
Pilus assembly, Mps	trag^{N}[b]	TrbL	VirB6				TrhG
Lipoprotein, OM	TraV	TrbH	VirB7	TraI			TrhV
Pore		TrbF	VirB8				
Pore (secretin-like)	TraK	TrbG	VirB9	TraN			TrhK
Pore (TonB-like)	TraB	TrbI	VirB10	TraO			TrhB
Transport ATPase		TrbB	VirB11	TraJ			
Relaxase	TraI	TraI	VirD2	NikB	Orf1(TraA)	TraX	TrhI
Coupling protein ATPase	TraD	TraG	VirD4	TrbC	Orf10	TraW	TraG

[a]Based on Lawley et al., 2003 and Grohmann et al., 2003; Abajy et al., 2007.
[b]F-like systems contain a characteristic gene cluster encoding mating pair stabilization (Mps), disulfide bond isomerization, and pilus retraction proteins (TraF, −H, −G, −N, −U, −V, and -W; TrbB, −C, and -I). G^{N} refers to the N-terminal region of TraG that is required for both pilus assembly and Mps
[c]SLT refers to soluble lytic transglycosylase

An interesting variation of Mps for the IncI1 transfer systems (R64, ColIb-P9) that express two types of pili has been identified: thin, flexible pili, which are required for Mps, and thick, rigid pili, which are required for DNA transfer. Research on the thin pili of R64 by Komano's group has revealed that they are composed of type IV pilin (similar to the pili found in pathogens such as *Neisseria gonorrhoeae*) of 15 kDa (*pilS*). These pili have a protein at their tip (*pilV*) whose gene undergoes rearrangement via site-specific recombination by the *rci* gene product to form seven possible fusion proteins, each recognizing a specific LPS structure (e.g., *pilVA′*). Whether these pili retract in order to bring the donor and recipient cells together is unknown although retraction is a general feature of type IV pili. Besides these two cases (F and R64 thin pili), little is known about Mps in other Gram-negative systems.

Surface and entry exclusions

Surface or entry exclusion reduces redundant transfer between equivalent donor cells. Such transfer is thought to be deleterious to the donor cell and is exemplified by the phenomenon of lethal zygosis, which occurs when a high ratio of Hfr donor to recipient cells is used. Multiple matings with a single recipient cell result in its death because of severe membrane and peptidoglycan damage as well as induction of the SOS response resulting from the influx of a large amount of single-stranded DNA. The surface exclusion genes were first identified as the *ilz* locus (immunity to lethal zygosis) because of their role in protecting recipient cells during matings with a high ratio of Hfr donor to recipient cells.

The mechanism of entry or surface exclusion is unknown although an exclusion mechanism has usually been found associated with the transfer systems studied to date. One exception is the conjugative transposons that transfer at such low frequency that redundant transfer might not be an important factor. Surface exclusion in the F system involves TraT, a lipoprotein found in the outer membrane, which forms a pentameric structure and blocks Mps. Whether it interacts with the pilus or another component of the F transfer system is unknown. The TraS protein of F is an inner membrane protein that blocks the signal that DNA transfer should begin and is thus associated with the property of Eex. TraS interacts with TraG in the inner membrane of another donor cell as demonstrated by Eex specificity experiments using F and R100 plasmids and R391 and SXT ICEs. Thus, TraG appears to contact the inner membrane of the recipient cell, thereby stapling the cells together as part of the Mps process, which is blocked by TraS. TrbK, a lipoprotein found in the inner membrane of RP4-containing cells, is also thought to cause Eex.

DNA Metabolism

Organization of oriT

In Gram-negative transfer systems, the origin of transfer (*oriT*) is ~40–500 bp in length and contains intrinsic bends and direct and inverted repeats that bind the proteins involved in DNA transfer. The *nic* site itself, which is a strand- and sequence-specific cleavage site, is cleaved and religated by relaxase. In most cases, relaxase requires auxiliary proteins that direct relaxase to the *nic* site and ensure the specificity of the reaction. The sequence of the *nic* sites identified to date reveal four possible sequences represented by IncF, −P, and -Q and certain Gram-positive plasmids such as pMV158. In addition, there is usually a protein that binds to multiple sites within *oriT* forming a higher-order structure on the DNA, which is essential for the process. This protein also appears to have a function in anchoring the relaxosome to the transport machinery.

Mechanism of DNA transfer

After Mpf, a signal is generated that converts the relaxosome from the cleavage/religation mode to one where unwinding of the DNA is coupled to transport through the conjugation pore in an ATP-dependent manner. The transfer rate is ~750 nucleotides per second with the F plasmid (100 kb) transferred in a little over 2 min.

In IncF plasmids, TraI is the relaxase/helicase enzyme that binds to a site near *nic* and generates an equilibrium between cleavage and religation. This reaction requires supercoiled template DNA and Mg^{2+} as well as the auxiliary proteins F TraY and host integration host factor (IHF) *in vitro*. TraM is known to promote nicking although it is not absolutely required. The signal that triggers the helicase activity of F TraI, which is essential for DNA transfer, is unknown as is the function of TraI* produced by a translational restart in the *traI* mRNA. TraY binds near *nic* whereas TraM binds to multiple sites, in conjunction with IHF, within the nucleoprotein complex. TraM binds TraI as well as the coupling protein, TraD, an inner membrane protein that utilizes ATP via its two NTP-binding motifs. Thus TraM could mediate the initial interaction between TraI and TraD in F, whereas in other systems, relaxase and coupling protein interact directly. This step precedes transport of relaxase, covalently bound to the 5′ end of the DNA, into the recipient cell where religation is thought to occur. The transport of relaxase has been demonstrated for several systems other than F and is now thought to be a general feature of conjugation.

In RP4, a similar arrangement of proteins at *oriT* exists except that there is no role for the host protein, IHF. The relaxase protein, also called TraI, cleaves at *nic* and is part of a complex with TraJ whereas TraH stabilizes the TraI–TraJ complex. TraK binds and bends the DNA at *oriT* to form the nucleosome-like structure thought to be needed to initiate DNA replication. The DNA is delivered to the TraG coupling protein, possibly by another ATPase, specific to P-like systems, called TrbB in RP4 and VirB11 in the Ti plasmids, found in the inner membrane that is independent of the T4SS.

In all cases, the 5′ phosphate generated by the cleavage reaction remains covalently bound to the relaxase enzyme via a tyrosine residue using a mechanism that is similar to the initiation of rolling circle replication in some phage and plasmid replicons. The DNA is transferred in a 5′ → 3′ direction with the first genes to enter the recipient cell called the leading region. Transfer seems to be a precise process with termination of transfer after one copy of the plasmid has been delivered to the recipient cell. A sequence in *oriT*, near *nic*, is important for termination by relaxase in a religation reaction. Both strands of the DNA are replicated by the PolIII enzyme using discontinuous synthesis in the recipient and continuous synthesis either from the free 3′ end at *nic* or from an RNA primer in the donor. Although synthesis and transport are coupled in conjugation, DNA synthesis does not drive, nor is it required, for DNA transfer.

In RP4 and IncI plasmids, the transport of a primase protein, Pri (*traC* encoded in Tra1), or Sog in RP4 or IncI plasmids, respectively, has been demonstrated to occur simultaneously with the transport of the DNA, with hundreds of copies being transferred. This protein appears to initiate DNA synthesis in the recipient cell via primer formation although it is not essential for conjugation. In F, no primase is transferred and DNA synthesis is thought to begin via a mechanism utilizing *ssi* sites for single-stranded initiation.

Leading region expression

The first genes to enter the recipient cell in the leading region include genes for preventing the SOS response (*psi*) and for plasmid maintenance via poison–antidote systems such as CcdAB and Flm in F, Hok/Sok in R1, and Kil/Kor in RP4. Although homologues of a single-stranded DNA-binding protein, Ssb, are found on many conjugative plasmids, they are not essential for conjugation. Another interesting but inessential gene is *orf169* in F or TrbN in RP4 that is related to transglycosylases such as lysozyme. Perhaps this gene, which is the first to enter the recipient cell during F transfer, has a role in establishing a new transferosome in the recipient cell. Slowly growing bacteria in natural environments might require this transglycosylase to rearrange peptidoglycan in preparation for pilus assembly and DNA transfer. Homologues in Gram-positive conjugation systems are essential for conjugation, suggesting a role in penetrating the thick Gram-positive cell wall during erection of the transport apparatus.

Regulation

The regulation of genes involved in conjugation has been extensively studied in F, RP4, and Ti (see 'Transfer to plants') plasmids and in Gram-positive conjugation but there is little information on other systems. The regulation of F transfer gene expression depends on both host and plasmid-encoded factors whereas the regulation of RP4 appears to be independent of the host. Also, F is unusual in that there is no evidence for coregulation of transfer and replication, a salient feature of other conjugation systems.

In F there are three main transcripts encoding *traM*, *traJ* (the positive regulator of transfer operon expression), and *traY-I* (33 kb) (Frost and Koraimann, 2010). The P_{Y-X} promoter is controlled by a consortium of proteins, including the essential TraJ protein, TraY, the first gene in the operon, and IHF and SfrA (also known as ArcA) encoded by the host. TraY also controls *traM* expression from two promoters that are autoregulated by TraM. The translation of the *traJ* mRNA is controlled by an antisense RNA FinP, which requires the RNA-binding protein FinO for activity (fertility inhibition; see 'Fertility inhibition'). Extracytoplasmic stress, which upregulates the CpxAR regulon, decreases the levels of TraJ by upregulating the HslVU protease/chaperone pair that degrades TraJ. F transfer gene expression is also silenced by H-NS as it enters the stationary phase with TraJ involved in desilencing the transfer region. This silencing has also been described for other plasmids in response to temperature flux and some plasmids such as F-like R100 encode H-NS-like proteins that form heterodimers with host H-NS, thereby inactivating it and desilencing the transfer region. Other factors have been shown to affect F transfer gene expression including LRP and CRP, which sense the nutritional state of the host cell, and Dam methylation of sites within the *traJ* promoter region during conjugation, which monitors the methylation state of the incoming DNA.

In RP4, transfer is tied very closely to replication with the main replication promoter for TrfA divergently oriented and overlapping with the first of two promoters for the Tra2 transfer region, P_{trbA} and P_{trbB}. In this system, the *trfA* promoter is activated first in the transconjugant, promoting plasmid replication. The P_{trbB} promoter, as well as the promoters in Tra1 that express the genes for Dtr, is also activated in order to establish a new transferosome. Eventually, the main global regulators KorA and KorB repress expression from these promoters and allow transcription from P_{trbA} that maintains the level of Tra2 proteins in the donor cell. TrbA is a global regulator that represses P_{trbB} as well as the three promoters in Tra1 encoding the genes for Dtr. Thus conjugation leads to a burst in transcription that establishes the plasmid in the new donor cell followed by transcription from either the *trfA* or the *trbA* promoters during vegetative growth.

Fertility inhibition

Fertility inhibition (Fin) is a widespread phenomenon among related plasmids that limits the transfer of competing plasmids coresident in a single cell. The Fin systems of F-like plasmids (R factors) repress F and also autoregulate the expression of their own transfer regions. These systems have two components, the antisense RNA FinP and the RNA-binding protein, FinO, which together prevent translation of the *traJ* mRNA, TraJ being the positive regulator of the $P_{Y\text{-}I}$ promoter. FinO protects FinP antisense RNA from degradation by the host ribonuclease, RNase E, allowing FinP concentration to rise sufficiently to block *traJ* mRNA translation. Whereas *finP* is plasmid-specific, *finO* is not and can be supplied from a number of F-like plasmids. This is the basis of the fi^+ phenotype noted in the 1960s for various R factors. F lacks FinO since *finO* is interrupted by an IS3 element and consequently is constitutively derepressed for transfer.

In F-like plasmids ($FinOP^+$), 0.1–1% of a repressed cell population express pili. If conjugation is initiated, the transconjugant is capable of high frequency of transfer (HFT) for about six generations until fertility inhibition by FinOP sets in. This phenomenon, along with surface or entry exclusion, contributes to epidemic spread followed by stable maintenance of a plasmid in a natural population of bacteria.

Other Fin systems are specified by one plasmid and are directed against another. For instance, F encodes PifC that blocks RP4 transfer whereas RP4 encodes the Fiw system that blocks the transfer of coresident IncW plasmids. Each system has a unique mechanism that has made the study of Fin systems more difficult and has tended to downplay the importance of this phenomenon in the control of the dissemination of plasmids in natural populations.

Gram-Positive Conjugation

Conjugative elements in nonfilamentous Gram-positive bacteria can be subdivided into three groups: small plasmids (<30 kb), usually associated with MLS resistance, exhibiting moderate transfer efficiency on solid surfaces over a broad host range (pAMβ1, pIP501); large plasmids (>60 kb) that mate efficiently in liquid media over a narrow host range and undergo clumping or cell aggregate formation (pAD1, pCF10 in *Enterococcus*, pSK41, pGO1 in *S. aureus*); and conjugative transposons.

Studies on Gram-positive plasmids have revealed that their conjugative systems also encode homologues of T4SS proteins especially relaxase, the coupling protein (an ATPase associated with pilus assembly), and a lytic transglycosylase (Ti VirD2, −D4, −B4, −B1, respectively; Figure 2(c), Table 2). Although constructing the T4S apparatus within the Gram-positive cell envelope has different challenges, the basic mechanism of conjugation appears to be conserved throughout the phylogenetic tree.

In the few systems studied in detail, detection of a recipient cell results in expression of an aggregation substance (AS, Agg, or Clu) that covers the surface of the donor cell and results in the formation of mating aggregates that are visible to the naked eye. This 'fuzz' is the result of a complex pattern of gene expression that has been studied in detail in only a few instances, mostly in the large plasmids of *E. faecalis*. This group of plasmids responds to pheromones expressed by the recipient cell whereas the signal that triggers mating aggregate formation in other systems is poorly understood. Plasmids in *S. aureus* produce pheromones that trigger transfer by *E. faecalis*, suggesting a mechanism to broaden the host range for the plasmids of this latter organism.

Enterococcus faecalis

The first conjugative plasmid identified in Gram-positive bacteria was pAD1, which carried a hemolysin/bacteriocin determinant responsible for increased virulence and which caused clumping or aggregation 30 min after the addition of plasmid-free cells. Later it was shown that aggregation and subsequent plasmid transfer were induced by pheromones produced by the recipient cell and released into the medium. These pheromones are hydrophobic peptides of 7–8 amino acids with a single hydroxyamino acid derived from the signal sequences of surface lipoproteins. Each recipient cell releases several pheromones with plasmids from a particular incompatibility group recognizing a specific pheromone with specificity residing in the N-terminus of the peptide. Once the plasmid has become established in the transconjugant, expression of the pheromone is repressed by preventing its synthesis in, or release from, the donor cell. As little as picomolar amounts of pheromone added to donor cultures can induce the expression of the transfer genes. Usually the concentration hovers around 1–10 nmol l^{-1} and a slight increase in pheromone levels is sufficient to induce clumping. Pheromones are named after the transfer system that recognizes them; thus, pAD1 recognizes cAD1 and produces iAD1, an inhibitory peptide that blocks accidental induction of transfer.

The pheromone is recognized by a plasmid-encoded protein and is imported into the cell via the host Opp system (oligopeptide permease). In pAD1, it binds to a repressor, TraA, inactivating it and allowing transcription of TraE1, a positive regulator required for

induction of transfer gene expression. In pCF10, the pheromone cCF10 causes antitermination of transcription of the *prg* genes (pheromone-responsive gene), generating the 530-nucleotide RNA Q_L and the mRNA for aggregation substance and transfer proteins. The Q_L RNA, in conjunction with the pheromone, associates with the ribosome causing preferential translation of the transfer genes. In either case, transcription of the aggregation substance (AS or Asa1 for pAD1, Asc10 for pCF10) ensues, which is deposited asymmetrically on the surface of the cell until the cell surface is covered. This binds to the binding substance (BS, lipoteichoic acid) on the recipient cell to give the mating aggregates so characteristic of conjugation. Interestingly, both AS and BS have been associated with increased virulence in a rabbit endocarditis model with AS having RGD motifs (arginine-glycine-aspartic acid), which are known to promote binding to the integrin family of cell surface proteins.

Once the pCF10 transferosome is established in the transconjugant, a cytoplasmic membrane protein binds pheromone and prevents its release. The inhibitory peptide, iCF10, is synthesized from a shorter version of Q_L called Q_S. A surface exclusion protein, Sea1 or Sec10 (for pAD1 or pCF10, respectively), is also expressed to reduce aggregation between donor cells and provides a second level of control to prevent redundant mating between plasmid-bearing cells.

There also seems to be a close relationship between replication and transfer in these plasmids with the replication protein PrgW embedded within a region that negatively regulates transfer in pCF10. The replication protein also appears to have a requirement for pheromone that is not yet understood. There are two *oriT*s for pAD1: one within the *rep* gene for plasmid replication and a second dominant one near the genes for relaxase (TraX), and the coupling protein (TraW) on the opposite side of the plasmid. The *oriT* of pCF10 is located near *pcfG*, encoding the relaxase, which is interrupted by a functional group II iteron suggesting tight control of the conjugative process.

Streptomyces

The transfer systems found on conjugative plasmids in the large genus *Streptomyces* differ significantly from those of both nonfilamentous, Gram-positive bacteria and Gram-negative systems in that there is only a single essential transfer protein and no evidence for a relaxase or *nic* site has been found. *Streptomyces* are a medically important source of antibiotics and other therapeutic compounds and are thought to be a major reservoir for antibiotic resistance mechanisms that protect these bacteria from the arsenal of antibiotics they produce.

The conjugative plasmids of *Streptomyces* range in size and structure from the circular 9 kb plasmid pIJ101 to the large linear 350 kb plasmid SCP1. The phenomenon of conjugation in this genus was first identified by the ability of certain integrating plasmids to mobilize chromosomes (Cma). One such plasmid SLP1 (17 kb) excises and integrates at the 3′ end of an essential $tRNA^{Tyr}$ gene in *Streptomyces lividans* with tRNA genes providing the loci for integration of a number of phage and plasmids.

Streptomycetes are soil microorganisms that undergo a complex differentiation program whereby spores germinate and form substrate mycelia that penetrate into the support surface followed by the erection of aerial hyphae that are multinucleate mycelia. These mycelia eventually septate and form spores. As the cells enter the hyphal stage, they begin to produce an array of secondary metabolites characteristic of the organism and also enter a phase where they are competent for conjugation between mycelia or mycelia and other organisms such as *E. coli*.

The intermycelial transfer of a plasmid requires one essential protein, for example, Tra, in pIJ101, which has homology to the proteins FtsK and SpoIIIE. This protein is located at the asymmetric septum of sporulating *B. subtilis* cells and ensures that a copy of the chromosome enters the forespore. Since no relaxase protein has been found associated with pIJ101, this might be a conjugation system closely related to partitioning mechanisms involving double-stranded DNA.

Once the DNA has been transferred to one compartment of a long mycelium, the plasmid is distributed to all compartments by the *tra* and the *spd* gene products. This process slows the growth rate of the cell and areas on agar plates where plasmids are spread via inter- and intramycelial transfer form 'pocks' of more slowly growing cells, which resemble plaques on a phage titer plate. This phenomenon has proven useful in identifying cells containing conjugative plasmids.

Mobilization

Mobilization is a widespread phenomenon whereby a smaller plasmid encoding its own *nic* site and Dtr genes (*mob*) utilizes the transport machinery of a usually larger plasmid to effect its own transfer. Many plasmids can be mobilized by plasmids from a number of Inc groups. For instance, ColE1 can be mobilized by plasmids from IncF, −P and, −I groups and less effectively and with different requirements by IncW plasmids. The Mob proteins of ColE1 consist of MbeA (relaxase) and MbeB and C that aid in relaxosome formation. MbeD has an Eex function. In the vector pBR322, derived from ColE1, the *mob* genes are deleted and only an *oriT* region for IncP plasmid mobilization remains. While ColE1 requires TraD, the coupling protein, from F for mobilization, the closely related plasmid CloDF13 supplies its own TraD-like protein, a difference that is commonly seen among mobilizable plasmids.

The most remarkable mobilizable plasmid is RSF1010 and its relatives (~8.6 kb in size) from the IncQ group. These plasmids are mobilized very efficiently by plasmids from the IncP group into an extremely broad group of recipients including bacteria, yeast, and plants. This plasmid encodes three Mob proteins, with MobA being the relaxase. Like ColE1, it requires TraG of RP4, a coupling protein, for efficient conjugation. The *oriT* region is a mere 38 bp in size and is homologous to *oriT* regions in plasmids from Gram-positive bacteria, all of which use a rolling circle mechanism during transfer. RSF1010 can be mobilized into plants and between

agrobacteria by the *vir* region (not *tra*; see 'Transfer to plants') and between strains of *Legionella* using the virulence determinants encoded by the *dot* and *icm* loci involved in macrophage killing (see 'Evolutionary relationships').

Small Gram-positive plasmids are also mobilizable by self-transmissible plasmids but less is known about them although recent studies confirm that they encode T4SS homologues and require a transglycosylase (SLT) (Table 2). The utility of conjugative transposons as genetic tools in Gram-positive bacteria has overshadowed interest in these plasmids that tend to be mobilized at low frequencies over long periods. One plasmid that replicates and transfers via the rolling circle mechanism using different origins is pMV158. It encodes a relaxase (MobM) that cleaves at a *nic* sequence unique to a group of mobilizable plasmids found in Gram-positive bacteria representing the fourth class of *nic* sequences.

Transfer to Plants

The phenomenon of DNA transfer from *A. tumefaciens* to plant cells has features of both Gram-negative (pilus expression) and Gram-positive (induction of *tra* gene expression) bacteria and has been dealt with separately (Figure 3). *A. tumefaciens* carrying large conjugative plasmids such as Ti (tumor-inducing) or Ri (root-inducing) greater than 200 kb cause crown gall disease in plants whereby they induce the formation of tumors at the site of infection. The Ti plasmid encodes a sensor-response regulator system, VirA and VirG, which in conjunction with ChvE, a chromosomally encoded periplasmic sugar-binding protein, process signals from wounded plant tissue. The phosphotransfer reaction from VirA to VirG induces gene expression from the *virA*, *−B*, *−D*, *−E*, and *-G* operons on the Ti plasmid. In addition, the *virC*, *−F*, and *-H* operons are induced but these operons express inessential gene products that affect host range or the degree of virulence. The signals generated by the plant include phenolic compounds, simple sugars, and decreased pH or phosphate content among others.

The *virB* region encodes 11 proteins that are homologous to the gene products in the Tra2 region of RP4 and are distantly related to the gene products of F. They encode the gene for prepropilin (VirB2), which is processed to pilin via a mechanism similar to that for RP4 pilin. A potential peptidase, homologous to TraF in RP4, has been identified (VirF) but its role has not been proven. The assembly of the VirB pilus is highly temperature dependent with an optimum of 19 °C, which is also maximal for the transfer process. The pili along with the T4SS transfer apparatus are localized to the pole of the cell, where transfer occurs.

The specific segment of single-stranded DNA that is transferred to the plant nucleus is called the T-DNA and can be characterized by the right (RB) and left (LB) borders, which are direct repeats of 25 bp. The T-DNA of nopaline-producing Ti plasmids is about 23 kb in length and contains genes for plant hormone expression (13 kb), a central region of unknown function, and a third region for opine (nopaline) biosynthesis (~7 kb). The relaxase, VirD2, in conjunction with VirD1 that is similar to RP4 TraJ, cleaves at RB and subsequently LB in a TraI-like manner and remains attached to the 5′ end. VirC1, which binds to an 'overdrive' sequence near the RB, and VirC2 of certain Ti plasmids, enhance T-intermediate formation. Unlike other transfer systems, many copies of the T-DNA segment accumulate in the bacterial cytoplasm suggesting replication is important in this system. The accumulation of T-DNA strands has been puzzling but might represent a strategy by the bacterium to ensure infection of the larger, more complex plant cell.

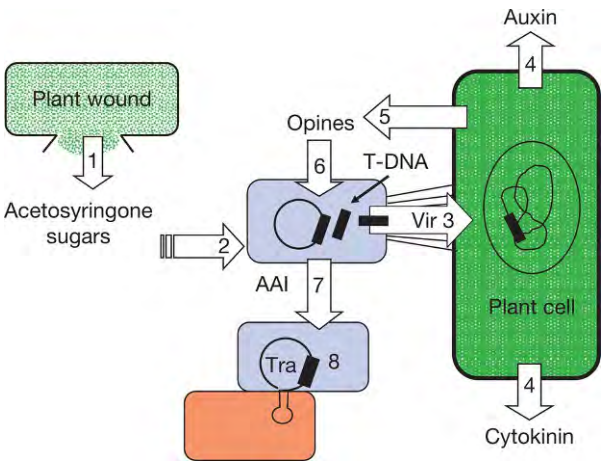

Figure 3 Signaling pathway used to stimulate T-DNA complex transfer to the plant nucleus and Ti conjugative transfer between Agrobacteria. Wounded plant tissue releases phenolics (acetosyringone) and sugars (1) that are detected by the two-component VirA and VirG regulatory system (2). This induces expression of the *vir* genes that encode the transfer apparatus at the pole of the cell that transports the T-DNA to the plant nucleus (3). The T-DNA is incorporated into the plant genome and produces the phytohormones auxin (indoleacetic acid) and cytokinin (4) that trigger tumorigenic growth of the plant tissue. The plant also produces opines (5) whose synthesis is encoded on the T-DNA. These unusual amino acids serve as a food source for *Agrobacterium* and also result in the induction of synthesis (6) of the conjugation factor, *N*-β-oxo-octanoyl-homoserine lactone (AAI; 7). AAI allows quorum sensing, which determines cell density with respect to Ti-plasmid-bearing cells resulting in conjugative transfer to other agrobacteria (8).

The DNA in the VirD2–T-DNA complex (T-complex) is thought to be coated with the single-stranded DNA-binding protein, VirE2, in preparation for transport through a conjugation pore composed of the VirB proteins (VirB2–VirB11). The VirD4 coupling protein (an ATPase) appears to work in conjunction with VirB11, another ATPase specific to P-like T4SS systems, to transport the T-complex (Figure 2(b)). Recent evidence suggests that VirE2 and VirD2–T-DNA transport are uncoupled and can occur using separate transfer pores. One of the Vir proteins, VirB1, which is inessential, resembles the transglycosylase of F (Orf169) and RP4 (TrbN) whereas a truncated version of VirB1 (VirB1*) is excreted into the rhizosphere and mediates adhesion between the bacterium at the site of transfer and the plant. Once the T-complex has entered the plant cytoplasm, the DNA is transported to the nucleus via nuclear localization signals (NLS) on the VirE2 and VirD2 proteins. The T-DNA is randomly integrated into the plant genome whereupon it begins to elicit signals for plant hormone production resulting in tumor formation. The T-DNA encodes for the synthesis of auxin (indoleacetic acid) and cytokinin (isopentenyl adenosine), plant hormones that elicit uncontrolled growth at the site of infection. The bacteria derive nutrients from the tumor by the devious method of opine (unusual amino acid) production encoded by the T-DNA. Opines can be classified into about nine different types of compounds including octopine, nopaline, and agrocinopine, with up to three different opines being encoded by a particular T-DNA. Thus Ti plasmids are often referred to as octopine- or nopaline-type plasmids, for instance, depending on the opine they specify. The opines are excreted from the plant and taken up by the *A. tumefaciens* bacteria encoding a region on the Ti plasmid involved in opine utilization. The genes for opine catabolism (e.g., Occ for octopine catabolism) match the genes for the synthesis of that class of opines on the T-DNA.

An interesting aspect of Ti plasmid biology is the induction of conjugative transfer between agrobacterial cells in response to the presence of opines. The genes for this process (*tra*) are distinct from the genes for T-DNA transfer (*vir*) and encode a transfer region with homology to RP4 as well as the *vir* region itself.

Conjugative transfer by Ti has a narrow host range, limited to the genus of *Agrobacterium*. However, the host range can be extended to *E. coli* if an appropriate replicon is supplied, suggesting that it is plasmid maintenance and not conjugative functions that affect host range.

The process of inducing conjugative transfer in these bacteria is unique and fascinating. Initially, there is a low-level uptake of opines, which activates the regulatory protein OccR in octopine-type plasmids and inactivates the repressor protein AccR in nopaline-type plasmids such as pTiC58. This leads to increased expression of the *tra* and opine utilization genes by activation of TraR. TraI (not to be confused with relaxase proteins of F and RP4 plasmids) is a LuxI homologue that synthesizes a signaling compound, *N*-β-oxo-octanoyl-homoserine lactone (*Agrobacterium* autoinducer or AAI), belonging to a diverse class of homoserine lactone-like (HSL) compounds involved in quorum sensing or gene activation in response to changes in cell density (Figure 3). TraR is a LuxR-like regulatory protein that detects increased levels of AAI and induces transfer gene expression to maximal levels. The result is the dissemination of the genes for opine utilization among the agrobacteria in the rhizosphere. Thus the system demonstrates a certain degree of chauvinistic behavior since the original colonizer of the plant cell shares its good fortune with its neighbors who then outcompete other bacteria in the rhizosphere.

Evolutionary Relationships

With the advent of high-throughput sequencing and easily available databases, comparison of gene sequences has become routine. Considering that the mechanism for conjugation varies surprisingly little among the systems described above, the high degree of relatedness of these systems with one another is expected (Table 2). However, the remarkable finding that there is homology between conjugative systems and the transport mechanisms for a number of toxins and virulence determinants has generated increased interest in these systems. There is almost gene-for-gene homology between the transport system for pertussis toxin of *Bordetella pertussis* (*ptl*) and the *virB* region of the Ti plasmid, which is, in turn, homologous to the genes for pilus synthesis in IncN, −P, and -W transfer systems and is distantly related to those of F-like plasmids. More recently, five *vir* homologues (VirB4, −9, −10, −11, and VirD4) have been found in the *cag* pathogenicity island of *Helicobacter pylori* and some of the Dot/Icm proteins involved in the pathogenesis of *Legionella pneumophila*, which can mobilize RSF1010, are homologous to genes in the Tra2 region of RP4 as well as the Trb region of the IncI1 plasmid R64. In fact, many bacteria carry more than one T4SS that are involved in conjugation, transformation, DNA release, and protein translocation. The number of orthologues is astounding and the versatility of T4SS is only now being appreciated.

Conjugation in Natural Environments

While the process of conjugation is thought to be relevant to the adaptation of organisms to environmental conditions such as the acquisition of antibiotic resistance under continuous pressure for selection, there is much to be learned about the process in nature. Conjugation can be demonstrated in a wide number of situations including the gut of animals, biofilms, soil, aquatic environments including wastewater, and on the surface of plants and animals. However, the level of transfer is usually very low. Most experiments have utilized common lab strains and plasmids, which are good model systems for study but might be irrelevant in nature. Considering the diversity of bacterial species, their vast numbers, and the timescale for their evolution, we have only scratched the surface of this phenomenon in the natural environment. However, studies on domesticated lab strains and plasmids have allowed predictions about the conditions that favor transfer. Most conjugative systems require actively growing cells in exponential phase

and have a fairly precise temperature optimum. The majority of systems studied to date mate more efficiently on solid media. Those systems that mate efficiently in liquid media seem to be found in enteric bacteria and might be associated with diseases transmitted via water. More information is required on the natural hosts for conjugative elements and their contribution to the evolution of these elements in an ecological niche. In addition, the most likely route of transmission, which appears to involve many intermediate organisms, is usually impossible to predict or detect because of the complexity of the system and the unknown role of nonculturable organisms in this process. Thus, we can isolate a plasmid from its environment and we can find evidence for its transfer to a new species but we cannot, at this time, follow the plasmid as it makes its way in the world.

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Continuous Cultures (Chemostats)☆

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Glossary

Auxostat A continuous culture system in which the rate of feeding is determined by controlling a single growth-dependent parameter while all other parameters, including the dilution rate (D) and the specific growth rate (μ), are adapted accordingly.
$\bar{C}_S$ Steady-state concentration of growth-limiting substrate (S) in fermentor (mol l^{-1} or g l^{-1}).
$\bar{C}_X$ Steady-state concentration of biomass in fermentor (g DW l^{-1}).
Chemostat Named from the phrase *chem*ically *stat*ic, the chemostat is a continuous culture system in which the dilution rate (D), and hence the specific growth rate (μ), is set externally and all other growth parameters will adapt accordingly.
Continuous culture An open culture system in which fresh (sterilized) medium is introduced at a steady flow rate (ϕ), from which the culture fluid is continuously removed at the same rate.
Critical dilution rate (D_C) Rate above which cells are washed-out faster than they can grow.
C_s Concentration of growth-limiting substrate (S) in fermentor (mol l^{-1} or g l^{-1}).
C_{Si} Concentration of substrate in medium supply (mol l^{-1} or g l^{-1}).
C_X Concentration of biomass in fermentor (g DW l^{-1}; DW = dry weight of cells).
Dilution rate (D) Flow rate of incoming medium divided by the volume of the culture in the continuous culture vessel (h^{-1}).
Fermentor Cultivation vessel with appropriate stirring device and control of temperature, aeration, gas supply, pH, pO_2, and ports for addition and removal of gas and liquids.
Flow rate (ϕ) Rate at which fresh medium is supplied to the fermentor (lh^{-1}).
Maintenance coefficient (q_m) Maintenance energy requirement of the biomass in culture (g or moles substrate (g DW h^{-1}).
Maximum specific growth rate (μ_{max}) Rate of increase in biomass (h^{-1}) relative to biomass present when all nutrients are present in excess and no growth inhibitors are present.
Monod saturation constant (K_S) Substrate concentration (g^{-1} or mol l^{-1}) at which the specific growth rate is equal to half of the maximum specific growth rate.
Recycling chemostat A chemostat in which a (major part) of the biomass is retained in the fermentor by using a membrane module. The ratio of the volume of culture effluent and the volume of the filtered spent medium determines the recycle ratio and the density of the retained biomass.
Retentostat A chemostat equipped with a membrane module to allow full retention of the biomass, only spent medium is pumped away.
Specific consumption rate (q_s) Substrate (g or moles) consumed per gram biomass per hour, also equal to μ/Y.
Specific growth rate (μ_s) Rate of increase of biomass relative to the biomass already present (h^{-1}), numerically equal to ln 2/t_d. The subscript s refers to the growth-limiting substrate, but is used optionally.
Steady state Condition of a continuous culture in which changes in cell density and physiological state of the cells are no longer detectable.
Turbidostat A continuous culture with a growth-dependent feedback system, in which the dilution rate is controlled by an internal sensor monitoring turbidity.
Y''_{SX} (*yield factor for biomass on substrate*) Quantity of cells produced per substrate consumed (g DW produced per g or moles substrate consumed).
Y_{SX}^{max} True yield factor for biomass on substrate, that is, the experimental yield factor Y''_{SX} if no maintenance exists (g DW produced per g or moles substrate consumed).

Abbreviations

GOGAT-GS	Glutamate oxoglutarate aminotransferase and glutamine synthetase
PMF	Proton motive force
PTS	Phosphotransferase system

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Defining Statement

Continuous culture techniques enable the cultivation of microorganisms at submaximal growth rates under highly controlled, nutrient-limited conditions and, if desired, at high cell density, thereby providing researchers with powerful and reproducible tools that have several advantages over batch cultivation. Continuous cultivation is invaluable for (eco)physiological investigations of microorganisms, including functional genomics studies.

Introduction

Continuous culture is a set of techniques used to reproducibly cultivate microorganisms at submaximal growth rates at different growth limitations in such a way that the culture conditions remain virtually constant (in "steady state") over extended periods of time. In the steady state, the growth of organisms can be studied in great detail under precisely controlled physiochemical states. Such conditions are amenable to a great deal of mathematical modeling that enables powerful quantitative analysis of microbial activities. Continuous culture principles first appeared in the literature near the middle of the 20th century, notably from work performed in the labs of Herbert, Monod, and Novick. Since that time, continuous culture techniques have become common tools in both research and industry. A large diversity of continuous culture applications exists, of which only a modest subset will be mentioned in the present work. Focus will be on a number of classic and a few up-to-date examples of the use of continuous cultivation in various applications. As will be described, the use of continuous culture has enabled studies into several ecological phenomena, including the relationship between growth rate and intracellular metabolic fluxes, the transcriptional responses of microorganisms to various nutrient limitations, the competitive strategies between microorganisms at low nutrient concentrations, as well as the selection and competition between spontaneous or designed mutants for biotechnological applications. As synergistic tools continue becoming more powerful and widely available, the number of uses and the value of the classic continuous culture techniques will likely continue growing at a comparable rate.

Theory of Continuous Culture

General

Microorganisms, inoculated into a suitable growth medium, will grow at a rate that is the maximum possible under the given conditions. During their growth, the environment will continuously change, but as long as the conditions remain favorable, growth will continue until at least one of the essential substrates in the medium becomes limiting. If all other nutrients are present in excess, this is called the growth-limiting substrate. The specific growth rate of a microorganism is dependent on the concentration of the growth-limiting substrate according to the empirical equation of Monod:

$$\mu = \mu_{\max} \frac{C_s}{K_s + C_s} \tag{1}$$

where μ is the specific growth rate, μ_{max} the maximum specific growth rate, C_s the concentration of the growth-limiting substrate, and K_s the Monod saturation constant, which is numerically equal to the substrate concentration at which $\mu = \frac{1}{2}\,\mu_{max}$. We will use this very simple mathematical model for growth to introduce the mathematics of the operation of the chemostat. Note that the doubling time (t_d) is related to μ as $t_d = \ln 2/\mu$.

To obtain sizeable population densities, the substrate concentrations employed in a batch culture are much higher than K_s (K_s values are usually in the nmol l^{-1} range), so that growth occurs at the maximum specific growth rate. Most microbial and biochemical research has been carried out on microorganisms grown under these conditions. An experimental drawback of the batch culture is that during growth the medium composition will change, excretion products may accumulate, and cell density will increase. In view of the adaptability of the physiology of the microbes, this means that the results obtained from such cultures will depend on the time of harvest. Another drawback of cultivation in batch is that questions concerning general physiology, such as cellular metabolic pathways, composition, and enzymology at submaximal growth rates under nutrient-limited conditions, cannot be answered.

In contrast, in continuous culture, it is possible to maintain steady-state concentrations of a growth-limiting nutrient in the culture, which permits growth of microorganisms at submaximal rates. In addition, in continuous culture, parameters such as pH, oxygen tension, concentration of excretion products, and population densities can easily be controlled. Several types of continuous culture methods exist (i.e., auxostat, turbidostat, and the chemostat), but by far the most common is the flow-controlled continuous culture, the chemostat, which will be discussed first.

As already mentioned, the chemostat is used for the cultivation of microorganisms at growth-limiting substrate concentrations. In the medium reservoir all the compounds necessary for growth are present in excess, with the exception of the growth-limiting substrate, C_S. Under these conditions, the microorganisms grow at a specific growth rate, μ, which is lower than the maximum specific growth rate, μ_{max}. The chemostat is an "open" culture system (often a lab-scale growth vessel or fermentor) in which fresh (sterilized) medium from a medium reservoir is introduced at a steady flow rate, ϕ, from which the culture fluid emerges at the same rate, often by a simple overflow system. With a constant volume, V, and an in-flow rate, ϕ, the dilution rate, D, is defined as

$$D = \frac{\phi}{V} \tag{2}$$

where the dilution rate is expressed in h^{-1}.

In the following a simple mathematical treatment of the operation of the chemostat is presented. To this goal the Monod model for growth as shown in Eq. (1) is used since it leads to simple mathematical formulae that can easily be explained and presented graphically. In this treatment it is also assumed that according to Monod a fixed relationship exists between the amount of substrate consumed and the amount of biomass produced over a large range of growth rates:

$$\frac{dC_X}{dt} = -Y''_{SX}\frac{dC_S}{dt} \tag{3}$$

where dC_X/dt is the change in biomass concentration over time. Y''_{SX} is the yield factor and is defined as the amount of cell material produced per amount of substrate consumed. However note that Y''_{SX} is only a constant as long as the substrate consumption for maintenance purposes is not taken into account as will be explained later in this article.

Biomass: If the medium in the fermentor is inoculated, for example with bacteria, the culture will grow at a given rate. At the same time, a quantity of bacteria will be washed out via the overflow, because the culture is continuously fed and diluted with fresh medium from the medium supply. For the culture it thus follows that the accumulation of biomass is equal to growth minus washout or

$$\frac{dC_X}{dt} = \mu C_X - DC_X = (\mu - D)C_X \tag{4}$$

Hence if $\mu > D$, C_X will increase, while if $\mu < D$, C_X will decrease. If $\mu = D$, an equilibrium will exist. While the formula given above accurately describes the general situation, it can easily be shown that, starting from nonsteady-state conditions, a steady state must inevitably be reached, provided that D does not exceed the critical value D_C:

$$D_C = \mu_{max}\frac{C_{Si}}{(K_S + C_{Si})} \tag{5}$$

where C_{Si} is the concentration of the growth-limiting substrate in the medium supply. If $C_{Si} \gg K_S$, which is usually the case, then $D_C \approx \mu_{max}$. However, if $C_{Si} \ll K_S$, the culture will be washed-out at $\mu \ll \mu_{max}$. If $D < D_C$, the establishment of a steady state may be considered as follows. At $\mu > D$, the biomass concentration, C_X, will increase. Owing to the resulting decrease in substrate concentration (C_S), the specific growth rate μ will then decrease. If μ becomes lower than the dilution rate, D, then C_X will decrease because of washout. Consequently, C_S will increase again. Therefore, it can be concluded that the dynamic equilibrium $\mu = D$ is a stable situation. Accordingly, a steady state will be established automatically. It is usually assumed that a steady state has been reached if C_X has not changed during two volume changes and at least five volume changes, in total, have occurred.

Growth-limiting substrate: Substrate enters the culture vessel at a concentration C_{Si}. Consumption of the substrate by the organisms results in a concentration C_S. The net rate of change in the culture vessel is obtained by a balance equation:

$$\frac{dC_S}{dt} = \text{in} - \text{out} - \text{consumption} \tag{6}$$

in which

$$\text{consumption} = \frac{\text{growth}}{\text{yield}} = \frac{\mu C_X}{Y''_{SX}}$$

It follows that

$$\frac{dC_S}{dt} = D(C_{Si} - C_S) - \frac{\mu C_X}{Y''_{SX}} \tag{7}$$

At steady state, dC_X/dt and dC_S/dt are both equal to zero. This, when combined with Eqs. (4) and (7), gives the equilibrium concentrations $\bar{C}_X$ and $\bar{C}_S$.

If

$$\frac{dC_X}{dt} = (\mu - D)C_X = 0$$

then

$$\mu = D \tag{8}$$

Hence

$$D = \mu_{max}\frac{\bar{C}_S}{(K_S + \bar{C}_S)} \tag{9}$$

or

$$\bar{C}_S = K_S\frac{D}{\mu_{max} - D} \tag{10}$$

Furthermore

$$D(C_{Si} - \bar{C}_S) = \frac{\mu \bar{C}_X}{Y''_{SX}} \tag{11}$$

Combining Eqs. (8) and (11), it follows that

$$\begin{aligned} \bar{C}_X &= Y''_{SX}(C_{Si} - C_S) \\ &= Y''_{SX}\left(C_{Si} - \frac{K_S D}{\mu_{max} - D}\right) \end{aligned} \tag{12}$$

where K_S, μ_{max}, and Y''_{SX} are constants for a microorganism under the specified condition of temperature, medium composition, and the nature of the growth-limiting substrate, respectively. For C_{Si} and D a constant value can be chosen. From Eq. (10) it appears that $\bar{C}_S$ solely depends on D. If K_S, μ_{max}, and Y''_{SX} are known for a given microorganism, the relationship between $\bar{C}_X$ or $\bar{C}_S$ and D can be predicted at a chosen C_{Si}. This is illustrated in Fig. 1.

The amount of cell material produced per unit of time is given by $D\,\bar{C}_X\,(g\,l^{-1}\,h^{-1})$. This term is also known as the productivity. Note that the theoretical lines often do not follow experimental values when the dilution rate is below ~10% of μ_{max}. This is because at lower μ, the assumption that Y''_{SX} is a constant (i.e., the Monod model for growth) does not hold (see below). Eq. (12) shows that $\bar{C}_X$ depends on D and C_{Si} and is proportional to C_{Si} if $\bar{C}_S \ll C_{Si}$, which is usually the case in the experimental lab situation. At varying C_{Si}, the relationship between D and $\bar{C}_X$ or $\bar{C}_S$ is illustrated in Fig. 2.

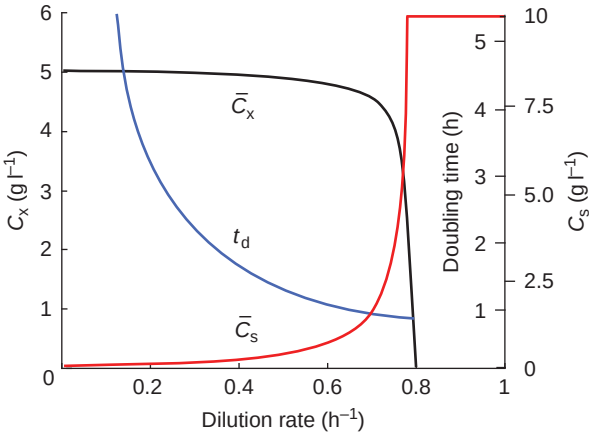

Fig. 1 Steady-state relationships in a continuous culture (theoretical). The steady-state values of substrate concentration, bacterial concentration, and doubling time at different dilution rates are calculated from Eqs. (10) and (12), for an organism with the following growth constants: $\mu_{max} = 0.8\ h^{-1}$, $Y'_{SX} = 0.5\ g\ g^{-1}$, $K_S = 0.15\ g\ L^{-1}$, and a substrate concentration in the medium supply of $C_{Si} = 10\ g\ L^{-1}$.

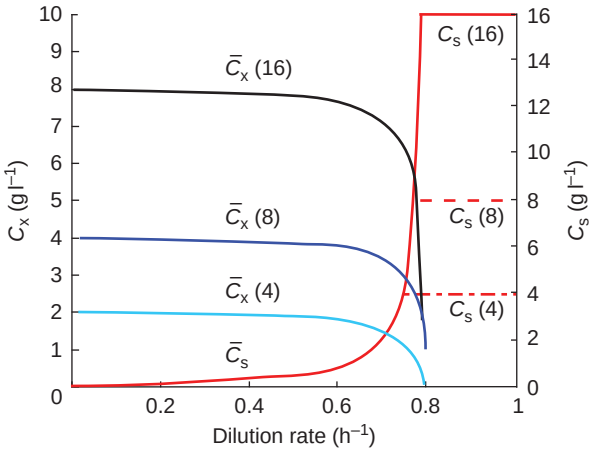

Fig. 2 Effect of varying the concentration of substrate in the medium supply (C_{Si}) on the steady-state relationships in a continuous culture (theoretical). The curves are calculated from Eqs. (10) and (12) for an organism with $\mu_{max} = 0.8\ h^{-1}$, $Y'_{SX} = 0.5\ g\ g^{-1}$, and $K_s = 0.15\ g\ L^{-1}$, for three media with different substrate concentrations of 4, 8 and 16 g L^{-1}.

It is important to note that $\bar{C}_S$ is independent of C_{Si}. At dilution rates well below D_C, relatively high cell concentrations can be obtained at very low, growth-limiting concentrations of the substrate. Hence, high biomass samples of cells that are growing at a submaximal rate and maintained in an active, controlled physiological state, are available for (eco)physiological studies. This is one of the great assets of chemostat cultivation.

In practice, the parameters of a culture such as μ_{max}, Y''_{SX}, and K_S can be determined in various ways. In the chemostat, μ_{max} can usually be determined more reliably than in a batch culture for the following three reasons: (1) prior to the determination of μ_{max} the culture can be grown at a rate close to μ_{max}, ensuring that all cells are optimally adapted to growth at their near-maximum rate; (2) lag phases will not interfere with the measurement; and (3) possible influences of changing the substrate and the product concentrations are minimized. For the actual measurement, the dilution rate is increased (in one step) from a value slightly below μmax to a value of 20–50% above the critical dilution rate. This results at once in alleviation of the substrate limitation and in gradual washout of the culture. The rate at which this washout proceeds can be expressed as given in Eq. (4), which after integration gives

$$\ln C_X = (\mu - D)t + \ln C_{Xo} \tag{13}$$

where C_{Xo} represents the cell density at the start of the washout period. Because the substrate is no longer limiting, the culture grows at μ_{max} and a plot of ln C_X versus t yields a line with a slope of $(\mu - D)$. Because D is fixed at a known value, μ_{max} can be determined.

K_s can be obtained from continuous culture experiments by selecting D so large that $\bar{C}_S$ can be measured. Usually $\bar{C}_S$ can only be measured over a small range of dilution rates, and hence the data may give inaccurate results. It is essential to minimize residual substrate consumption during the sampling. This can be done (1) by very rapid sampling in a tube with precooled stainless-steel beads, followed by immediate filtering and (2) by decreasing the C_{Si} so that the steady-state biomass concentration, C_X, and hence the rate of consumption, $\mu C_X/Y''_{SX}$, are lowered. A common way to obtain K_S is to perform a nonlinear fit (with least-squares regression), of the measured C_S values in the Monod equation ($\mu = D$) with a proper computer-fitting program. The most accurate method is to use the formula for the specific consumption rate (q_s), discussed below (Eq. (16)). An outdated method for the graphical determination of K_S is mentioned here because it is very commonly used to linearize Eq. (9), which can be rewritten to produce the so-called "Lineweaver–Burk plot":

$$\frac{1}{D} = \frac{K_S}{\mu_{max}\bar{C}_S} + \frac{1}{\mu_{max}} \tag{14}$$

At varying D values, a graphical plot of the reciprocal of $\bar{C}_S$ measured versus the reciprocal of D gives a straight line with an intercept with the y-axis equal to $1/\mu_{max}$ and an intercept with the x-axis equal to $-1/K_S$. The slope of the line is K_s/μ_{max}. Practical values of K_s can lie in a range between 10^{-8} and 10^{-3} mol l^{-1}, but are usually between 10^{-7} and 10^{-3} mol l^{-1}. However this method gives inaccurate results because those points obtained at the low end of the substrate concentration range (which usually are the most inaccurate) have the greatest effect on the position of the line in such a plot. This graphical method has been replaced by the direct linear plot.

The yield factor, Y''_{SX}, can be obtained from batch experiments using a series of cultures with increasing C_{Si} and further by a graphical plot of C_{Si} versus $\bar{C}_X$. However, this method often neither gives data as reproducible nor dependable as that observed from the chemostat. In continuous cultures, Y''_{SX} can be calculated from Eq. (12), according to the simple Monod model for growth, but now we must refine the mathematical treatment of the operation of the chemostat, because in practice it is observed that at low growth rate the yield is strongly influenced by the fact that an organism requires maintenance energy for a number of purposes. The resulting deviation is illustrated in Fig. 3.

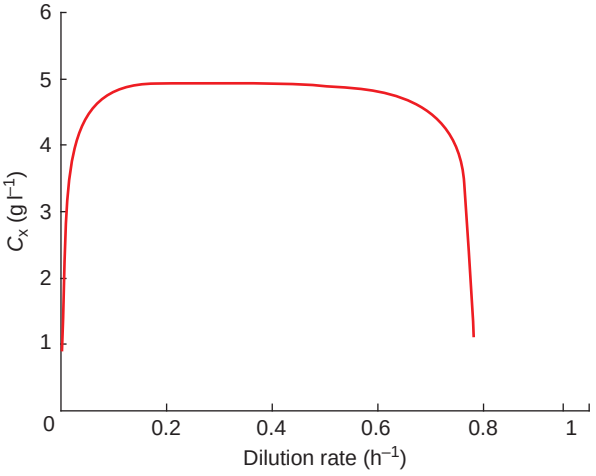

Fig. 3 Steady-state biomass concentration $\bar{C}_X$ as a function of the dilution rate D in a chemostat when there is a maintenance requirement for the growth-limiting substrate: $= Y_{SX}^{max} = 0.5$ g g^{-1}, $C_{Si} = 10$ g L^{-1}, $q_S^{max} = 1.6$ g g^{-1} h^{-1}, $q_m = 0.08$ g g^{-1} h^{-1}, and $K_S = 0.15$ g L^{-1}.

In other words, in Eq. (12), Y''_{SX} will not always be a constant. The percentage of the total consumed substrate used for maintenance will increase as μ decreases and, thus, Y''_{SX} will decrease as μ decreases. Marr, and later Pirt, have given an explanation based on the assumption that for its maintenance a cell requires a certain amount of energy per unit of time, independent of the specific growth rate. This means that the total consumption of substrate is equal to the consumption of substrate for maintenance plus consumption of substrate for growth:

$$\frac{\mu C_X}{Y''_{SX}} = q_m \cdot C_X + \frac{\mu C_X}{Y^{max}_{SX}} \tag{15}$$

If we divide by C_X, we obtain the specific consumption rate of the microorganism, q_s:

$$q_s = \frac{\mu}{Y''_{SX}} = q_m + \frac{\mu}{Y^{max}_{SX}} \tag{16}$$

where q_m is the specific maintenance energy requirement expressed as amount (moles or grams) of substrate consumed per unit of biomass per unit of time. Note that in the literature we often see the symbol m_s instead of q_m. Y^{max}_{SX} is the maximum yield (also known as the true yield), that is, the growth yield if no maintenance energy is required. The experimentally obtained Y^{max}_{SX} value should not be confused with the "theoretical" maximum yield that can be calculated from metabolic pathways, which is at least twice as high as the Y^{max}_{SX}.

Maintenance energy is necessary, in the first place, to maintain the proton motive force (PMF). This is a proton gradient across the cell membrane that is essential for various metabolic functions (e.g., maintaining ion gradients across the cell membrane). Furthermore, energy is used in the "turnover" of proteins and mRNA, for repair and motility, among other things. The existence of a maintenance energy requirement can be deduced from the fact that all microorganisms at rest (i.e., not growing) retain a certain respiratory level.

In the older literature, a graphical plot of $1/Y''_{SX}$ versus $1/D$ is used to derive the value of q_m (slope) and $1/Y_{SX}$ (intercept with the y-axis). However, in this type of reciprocal plot the same drawback is observed as pointed out above for the K_S determination. In this case, a plot of q_s against D gives much more dependable results. An example is shown in Fig. 4 (*lower line*), where the specific rate of oxygen consumption of a carbon- and energy-limited culture is plotted against D. The value of q_s can easily be calculated by monitoring the oxygen content of in- and out-flowing gas and by measuring the biomass concentration in the steady state. The specific maintenance coefficient in this case is expressed as $q_m(O_2)$, that is, the specific oxygen consumption for glucose respiration at $D = 0$. If the specific glucose consumption rate, $q_{s(glucose)}$, instead of qO_2, would be plotted as a function of D, the experimental yield is measured and the $q_{s(glucose)}$ is calculated from the equation $q_s = \mu/Y''_{SX}$.

Although q_m can directly be read from the graph at $D = 0$, the Y^{max}_{SX} must be calculated from the slope of the curve under carbon and energy limitation. The experimental data indeed often show a linear relationship. In such cases it is assumed that q_m is a constant. However, sometimes a straight line is not obtained. It is clear that in such a case Pirt's concept does not hold. An essential assumption, which was previously not formulated explicitly, is that Pirt's concept can only be applied when growth is energy-limited. This means that under other limitations the "apparent" q_m may have a variable value (see Fig. 4), and does not refer to the "minimum" energy required for maintenance of the integrity of the living cell. In practice, energy limitation often involves growth-limiting amounts of an organic compound that is simultaneously the energy and carbon source. With limitations other than energy

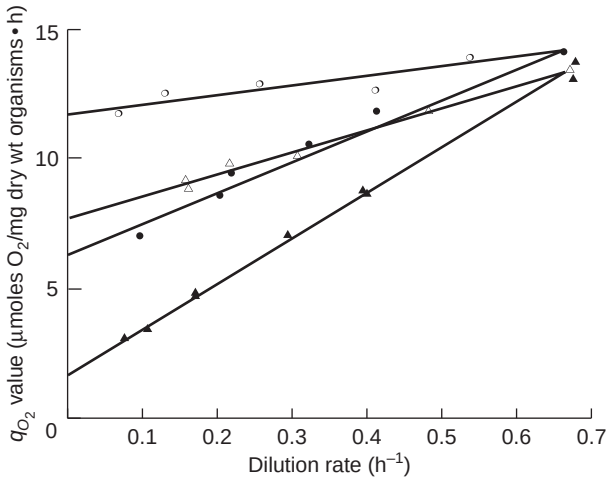

Fig. 4 Relationship between the specific growth rate and the specific rate of oxygen consumption in variously limited chemostat cultures of *Klebsiella aerogenes* growing in a glucose-containing medium. Cultures were, respectively, carbon-limited (▲), NH_3-limited (△), SO_4^{2-} -limited (●), and phosphate-limited (○). Reproduced from Neijssel, O. M. and Tempest, D. W. (1976). Bioenergetic aspects of aerobic growth of *Klebsiella aerogenes* NCTC-418 in carbon-limited and carbon-sufficient chemostat culture. *Archives of Microbiology*, 107, 215–221.

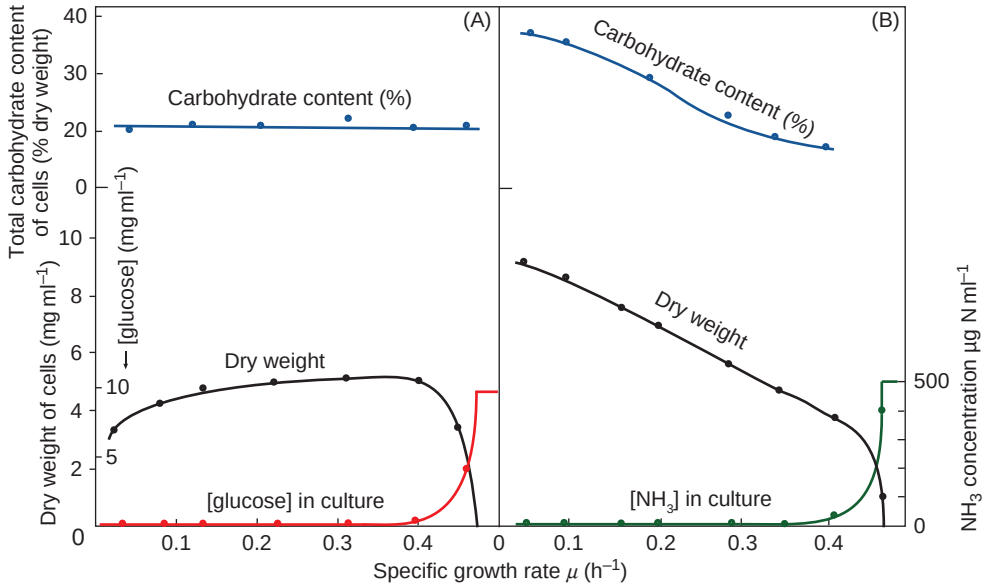

Fig. 5 Carbohydrate content of *Torula utilis* as a function of growth rate and limiting nutrient (unpublished data of Herbert and Tempest). The organism was grown in a continuous culture at a number of different growth rates in a glucose-NH_3-salts medium (A) with glucose as limiting nutrient, and (B) with NH_3 as limiting nutrient. Dry weight of cells in the culture and their total carbohydrate content (anthrone method), as well as steady-state levels of glucose and NH_3 in the culture, are plotted against growth rate. Reproduced from Herbert, D. (1961). The chemical composition of microorganism as a function of their environment. In: Meynell, G. G. and Gooder, H. (eds.) *Microbial reaction to environment: 11th symposium*, pp. 391–416. Reading: Society of General Microbiology: Cambridge University Press.

(and carbon), much higher consumptions may be observed as glucose may be consumed for purposes other than growth. This is very well described in the literature for microorganisms grown under nitrogen (ammonium) limitation, and is demonstrated in Fig. 5 from an experiment with *Torula* (*Candida*) *utilis*, grown under nitrogen limitation. At low growth rates, the cell will store reserve material that does not contain nitrogen (e.g., polyglucose or poly-β-hydroxybutyrate), because the cell has an excess of carbon and energy. This phenomenon is quite common and may have ecological implications, because if a shortage of energy and/or carbon sources occurs, the cell can then use the stored reserve material. This ability of a cell to consume more substrate than strictly necessary for the synthesis of "standard" cell material is based upon the surplus capacity of the respiratory system under substrate excess. This will be further discussed later.

The consequence of the introduction of the maintenance energy requirement is that the empirical Monod model for growth no longer holds. If maintenance energy is playing a role, then clearly a discrete rate of substrate supply and/or consumption can take place without growth, when the rate of supply is lower than required for maintenance purposes. This implies that the Monod type of saturation curve (hyperbola Eq. (1)) must be written for q_s rather than for μ:

$$q_s = q_{max}\frac{C_s}{K_s + C_s} \tag{17}$$

This is the formula commonly used in computer models to calculate K_S from (weighted) experimental data.

After rearrangements of Eq. (17) one obtains an alternative formulation for μ:

$$\mu = \mu_{max}\frac{C_s}{K_s + C_s} - \frac{K_s}{K_s + C_s}Y_{SX}^{max}q_m \tag{18}$$

This equation shows that at $C_s = 0$, $\mu = -q_m Y_{SX}^{max}$, while at $C_s = \infty$, $\mu = \mu_{max}$. This equation is the Monod equation in the first part, corrected with a term that becomes zero at very high C_S. Accounting for maintenance, the formulae for the steady state of C_X and C_S become

$$\bar{C}_X = \frac{\left[DY_{SX}^{max}(C_{Si} - C_S)\right]}{D + q_m Y_{SX}^{max}} \tag{19}$$

$$\bar{C}_S = \frac{\left(D + q_m Y_{SX}^{max}\right)K_S}{\left[Y_{SX}^{max}\left(q_s^{max} - q_m\right)\right] - D} \tag{20}$$

Fig. 3 has been constructed from Eqs. (19) and (20), using assumed kinetic parameters: Y_{SX}^{max}, C_{Si} q_S^{max}, q_m, and K_S. Further considerations concerning the maintenance energy requirement will be discussed under the physiological studies.

In continuous culture experiments, many other deviations from the theoretical behavior of chemostat cultures can be found. For example, if the cells present in the culture are not all viable (i.e., if a certain percentage of cells continuously die), a deviation will appear because the living cells must grow faster than D to maintain the value of C_X. Another problem might be the toxicity of the growth-limiting substrate, which could give deviation at high D (i.e., high $\bar{C}_s$). Mathematical models incorporating these extra variables can be found in the literature.

In the laboratory practice there are a number of potential problems related to the growth of microorganisms in the chemostat. In homogeneities may often occur because the culture is not well stirred, or because wall growth takes place.

Computer modeling of the chemostat operation. The Eq. (17) for the specific consumption rate of the (limiting) substrate and Eq. (16), combined with two balance equations for substrate and biomass, respectively, are commonly used in the engineering treatment and modeling of the chemostat operation. The two balance equations are similar to Eqs. (7) and (8), but in Eq. (7) the specific growth rate μ is replaced by the specific consumption rate qs. This allows the description of the growth and substrate consumption of a specific microorganism both in steady state and in nonsteady state when the culture is started up. Recently, Hakkaart et al. (2017) have published a paper describing a "Simulator-assisted workshop for teaching the chemostat." Using the four equations they developed a MATLAB based program ("Chemostatus") visualizing key parameters (i.e. biomass, substrate concentrations) of simulated chemostat cultures from start-up growth conditions to steady state. The workshop has successfully been used by these authors to teach quantitative microbial physiology. The files of the Chemostatus are freely available and the simulator can be requested at HYPERLINK "mailto:chemostatus@gmail.com" chemostatus@gmail.com.

Continuous Cultivation by Other Controls: The Turbidostat and pH-Auxostat

The flow-controlled chemostat is not suited for the growth of microorganisms at a value μ near D_C ($\approx \mu_{max}$). Small experimental errors in the pumping rate, and hence in the D, will have dramatic effects on $\bar{C}_X$ (see Fig. 1). In such a case, a turbidostat is preferred. In a turbidostat, the density (turbidity) of the culture is measured continuously and kept constant by a proportional adjustment of the pumping rate. A practical problem is that the measuring device, such as a flow-through cell in a spectrophotometer, used to measure the turbidity and generate the feedback to the pump can easily be fouled by wall growth.

Other types of continuous cultures are controlled by monitoring a variety of measurable chemical or physical parameters and appropriate feedback. Examples are carbon dioxide, sulfide, light, oxygen, and protons (i.e., pH). Such systems are called "auxostats." The pH-auxostat has frequently and successfully been used in practice. The theory is as follows. Assuming that during growth protons (H^+) are excreted into the culture liquid, the change in their concentration as a function of time can be expressed as follows:

$$\frac{dH^+}{dt} = \mu C_X h + D\left[H_R^+\right] - D\left[H_C^+\right] - DB_R \tag{21}$$

where h is the stoichiometry of proton formation per gram dry weight of cells (moles (g DW)$^{-1}$), $[H_R^+]$ the proton concentration in the medium reservoir (moles l^{-1}), $[H_C^+]$ the proton concentration in the culture (moles l^{-1}), and B_R the buffer capacity of the reservoir medium (moles l^{-1}). For the simplest situation, in which only a small difference exists between the pH of the medium and that of the culture, Eq. (21) reduces to

$$\frac{dH^+}{dt} = \mu C_X h - DB_R \tag{22}$$

and in steady state with $\mu = D$ the following expression for cell density is the result:

$$\bar{C}_X = \frac{B_R}{h} \tag{23}$$

This shows that the steady-state cell density is a linear function of the buffering capacity of the medium, assuming that h is independent of B_R. Combining Eq. (21) with the general nutrient balance for continuous culture Eq. (7) yields the following expression for C_S in steady-state cultures:

$$\bar{C}_S = C_{Si} - \frac{B_R}{hY''_{SX}} \tag{24}$$

Finally, solving the conventional Monod expression (Eq. (1)) for the obtained steady-state values of C_S allows a plot of the specific growth rate (μ), the steady-state substrate concentration ($\bar{C}_s$), and the steady-state cell density ($\bar{C}_X$) as a function of the buffering capacity (B_R) (Fig. 6). As can be seen from this illustration, the specific growth rate of the cells remain at a value close to μmax over a large range of buffering capacities but, of course, will decrease at high buffering capacities due to the decreasing concentration of remaining growth substrate. Obviously, this effect will be most prominent with cells possessing relatively high K_S values for the substrate used. In principle, this will provide the opportunity to choose the buffering capacity such that the substrate concentration becomes strongly growth rate-limiting, thus creating an overlap with the conventional mode of substrate-limited growth.

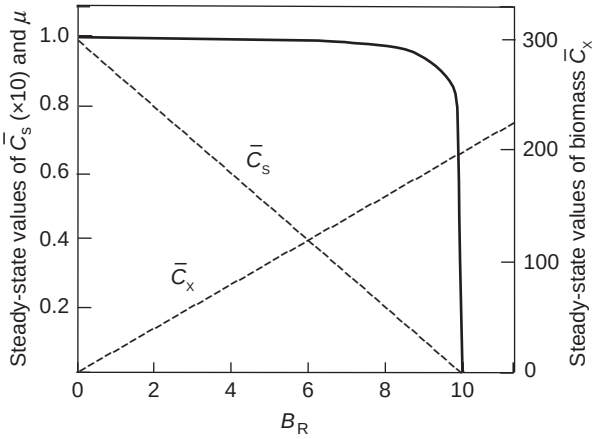

Fig. 6 Major growth parameters in a pH-auxostat. Residual substrate concentration C_s (moles L^{-1}), specific growth rate μ (h^{-1}) and cell density C_x (g DW L^{-1}) as a function of the buffering capacity B_R (moles L^{-1}), in the reservoir medium. Arbitrarily chosen values: $Y_{sx}'' = 22$ (g DW (mole)$^{-1}$), $h = 0.05$ (moles (g DW)$^{-1}$), $C_{si} = 10$ (mole L^{-1}) and $\mu_{max} = 1$ (h^{-1}). Adapted from Gottschall, J. C. (2000). Continuous culture. In: Lederberg, J. (ed.) *Encyclopedia of microbiology*, 2nd edn, vol. 1, pp. 873–886. New York: Academic Press Inc.

Continuous Cultivation in the Retentostat or Recycling Chemostat

The chemostat is recommended to cultivate microorganism at submaximal specific growth rates. The technique can be used to grow pure cultures of microorganisms, or to enrich for microorganisms at low growth rates, that is, low substrate concentrations. In practice dilutions rate between 0.005 and 1.0 h^{-1} have been used with chemostats of 1–2 L working volume. However the chemostat clearly has its technical limits when it comes to very low rates of growth and/or substrate supply, since the low flow of nutrients will cause fluctuations in the limiting substrate in the first place due to the drop size of the incoming medium and also to the potential effect of wall growth. An excellent tool to circumvent this problem is the use of a recycling- or retention-chemostat, in which part of the biomass is retained in the culture by using a membrane that keeps the microorganisms in the chemostat, but filters out the spent medium. Examples and suitable mathematical models will be cited in the following sections.

Competition of Microorganisms for a Growth-Limiting Substrate

Consider microorganisms A and B, which have μ–C_S curves of the types presented in Fig. 7A and B. In the example shown, if organisms A and B are grown in batch culture (with a maximum specific growth rate of μ_{max}), organism A will grow faster than organism B in both cases and therefore becomes dominant (assuming that both have started with equal numbers and neither has a lag phase). In the example shown in Fig. 7B, however, if A and B are grown together in a continuous culture with growth limitation by substrate C_S, then, at low D, organism B will dominate. This can be rationalized by assuming a steady state with organism B at

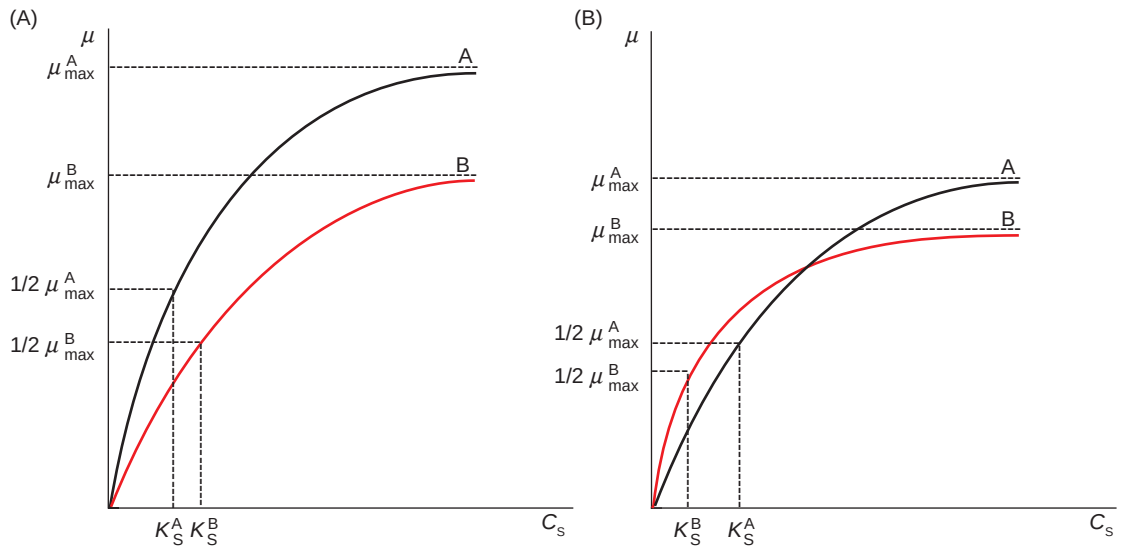

Fig. 7 The μ–C_S relationship of two organisms A and B (A) $K^A_S < K^B_S$ and $\mu^A_{max} > \mu^B_{max}$; (B) $K^A_S > K^B_S$ and $\mu^A_{max} > \mu^B_{max}$.

$D = {}^{1}/_{2}\mu^{B}_{max}$. The steady-state concentration of the growth-limiting substrate would be equal to K^{B}_{S}. At that nutrient concentration, organism A will grow at a $\mu < D$, and hence it will be washed out. Conversely, if we would have a steady state of organism A at that same D, organism B would be able to grow faster at the concentration of the growth-limiting nutrient than A, and hence would outcompete (replace) organism A. In other words, in spite of the lower μ_{max} of B shown in Fig. 7B, this organism will grow faster than organism A at low $\bar{C}_s$. In fact, it is the slope of each of the curves, that is μ_{max}/K_S, which determines the outcome of the competition. This slope is often referred to as the "affinity" for the substrate. A high affinity, in other words, will equip the organism with the ability to grow relatively fast at very low nutrient concentrations. This is most relevant to the (semi)natural environment. In the later examples of competition for growth-limiting nutrients, we will not only refer to mixtures of different organisms, but also to competition between mutants of one species, which is very important for the general study of selection. However, mutant selection is also a potential practical problem in the continuous cultivation of pure cultures in the laboratory (see below).

Physiological and Functional Genomic Studies with the Chemostat

Physiological Studies

As pointed out in the introduction, the chemostat is a unique tool for the study of the physiology of microorganisms at different growth rates and with different growth limitations under controlled, nutrient-limited conditions. For example, by means of this technique it has been found that the composition of the cell changes strongly, both qualitatively and quantitatively, with changing growth rates and with the type of growth limitation. At low growth rates, cells appear to possess fewer ribosomes (rRNA) than at higher growth rates. This can be directly related to the requirement of the cell to synthesize protein more rapidly when growing fast as the rate of protein synthesis per ribosome is about constant. Especially when growth is limited by the carbon and energy source, the amount of catabolic enzymes (for energy production) present in the cell may be observed to increase with increasing growth rate. Furthermore, the amount of cytochromes in a cell also often increases or decreases in response to environmental changes. However, a general rule for alterations in enzyme activity with changing growth rate cannot be given, because these changes are usually the result of very complex regulatory mechanisms.

Metabolic pathways and growth rates: As already stated, the chemostat is used for the study of all forms of limitation. Examples involving nitrogen and carbon/energy supplies as well as different limitations will be discussed here.

Most organisms grown in batch culture with ammonia as the nitrogen source possess glutamate dehydrogenase for ammonia assimilation. The same enzyme is used by chemostat cultures under limitation by the carbon and energy source. However, if growth is limited by ammonia, the combination of glutamate oxoglutarate aminotransferase and glutamine synthetase (the GOGAT-GS system) is induced. The latter enzyme has a considerably lower K_m value for ammonia than does glutamate dehydrogenase (about a factor of 5–10). The assimilation of ammonia through glutamine synthetase costs 1 ATP, but it is clear that under conditions of energy excess, this is not a problem for the cell.

While the biomass yield is, of course, dependent on the substrate (as is the case in batch culture), it is also dependent on the metabolic pathway used by the organism, which in turn may be dependent on the growth rate. The group of Stouthamer has observed a very interesting example. They found that at high growth rates *Lactobacillus casei* ferments glucose, producing lactate. Accordingly, when grown in the chemostat under glucose limitation at high D values, the bacterium produced only lactate. However, when D was lowered, the density of the culture increased and acetate appeared in the culture. The explanation of this phenomenon is that at low D values *L. casei* can make additional ATP by converting pyruvate into acetyl CoA rather than lactate. By means of a phosphoroclastic splitting of acetyl CoA, one additional ATP is formed. To keep a proper redox balance the organism produces formate and ethanol:

$$\text{High } \mu : \text{glucose} \rightarrow 2 \text{ pyruvate} + 2 \text{ ATP} + 2 \text{ NADH}$$
$$\text{pyruvate} + 2 \text{ NADH} \rightarrow 2 \text{ lactate}$$
$$\text{yield} : 2 \text{ ATP}$$

$$\text{Low } \mu : \text{glucose} \rightarrow 2 \text{ pyruvate} + 2 \text{ ATP} + 2 \text{ NADH}$$
$$2 \text{ pyruvate} + 2 \text{ NADH} \rightarrow 2 \text{ formate} + \text{ethanol} + \text{acetate} + 1 \text{ ATP}$$
$$\text{yield} : 3 \text{ ATP}$$

There is no definite answer to the question of why the organism at high growth rates does not also produce energy from glucose as economically as possible. It is possibly due to the fact that the reaction that yields 3 ATP is not fast enough for higher growth rates. It was found that the lactate dehydrogenase is activated by fructose-l,6-biphosphate. At low growth rates the concentration of this intermediate is low and the lactate dehydrogenase does not function. In this situation, the pyruvate can be converted into formate, ethanol, and acetate.

Neijssel and Tempest studied the response of *Klebsiella aerogenes* grown fully aerobically under different limitations in the chemostat with glucose as the only carbon and energy source as shown in Fig. 4, which is discussed in the theory section. The specific oxygen respiration rate qO_2 was recorded as a function of D and in all cases a linear relation existed. In the carbon-limited cultures glucose was completely consumed, but in all other (glucose-sufficient) cultures the sugar was partially respired and also partially fermented, as indicated by the appearance of different products such as gluconate and acetate (not shown in Fig. 4). In many

publications, it is also shown that under limitations other than by the carbon and energy source the cell-composition changed as mentioned for nitrogen limitation. For example, during phosphate or sulfate limitation the cell may synthesize alternative cell wall components not containing the limiting nutrient.

As mentioned earlier, according to the Pirt maintenance concept the extrapolated consumption rate under carbon and energy limitation at $D = 0$ represents the true maintenance energy requirement, q_m. Extrapolation of the other lines to $D = 0$ simply demonstrates that the organism respires more glucose than required for biosynthesis. The apparently wasteful use of glucose under the other limitations may in part be due to the need for more energy for transport of the limiting nutrients, but the authors also clearly showed that a certain uncoupling of energy metabolism and biosynthesis occurred. The interpretation of this phenomenon is that under the other limitations the organism is unable to match (excess) uptake of glucose with the requirement for growth, and hence must waste energy since without it, it would be unable to maintain the proper energy charge and redox balance in the cell. Neijssel and Tempest argue that the maintenance energy requirement is not necessarily limited to the Pirt-type μ-independent maintenance requirement, but may be in part growth rate dependent in a linear manner. The discussion of this point falls beyond the scope of this article. The physiology of maintenance requirement has been studied in "retentostats" with full biomass retention/recycling of pure cultures of organisms that have relatively high maximum specific growth rates (Van Verseveld et al., 1986; Boender et al., 2009).

Yields under fluctuating-nutrient supply: Another point that deserves attention is that energy-limited cultures, when exposed to sudden increases of their limiting energy source, often show lower yields. For example, when glucose-limited cultures of the yeast *Saccharomyces cerevisiae* were given the same amount of glucose per unit of time, but in discrete pulses, rather than continuously, the experimental biomass yield decreased. Directly after the pulse the yeast would excrete ethanol, which would be rapidly consumed again once all glucose had been taken up. The ethanol consumption was proceeding through acetate, which was transiently observed. Before the next pulse of glucose appeared all substrates had been converted into CO_2 or taken up into biomass. Apparently under these conditions, the (excess) uptake of glucose could not be met by biosynthesis, and a certain uncoupling of energy metabolism and biosynthesis would take place and some substrate was wasted as heat. This phenomenon was not observed in the yeast *Candida utilis*, which under these fluctuating conditions would control its glucose uptake to match the requirement for biosynthesis, and hence in the latter case the yields were the same.

Functional Genomic Applications of the Chemostat

With the booming interest in "functional genomics" and "systems biology" of microbes, which may well be considered as a modern version of microbial physiology, the chemostat has obtained renewed interest not only because it allows one to study the (controlled) response of an organism to different limitations, but also because of the reproducibility of the experiments. This was put to the test by the groups of Pronk and Nielsen, who did a transcriptome analysis of *S. cerevisiae* grown in the chemostat under both aerobic and anaerobic conditions in two independent laboratories. Using triplicate experiments in each laboratory and adequate statistical analysis, they were able to show 95% agreement for transcripts that changed by more than twofold. This is remarkably reproducible for these types of biological experiments and shows the great values of the use of the chemostat for genomic research. The Pronk group also used the chemostat to grow the same organism under carbon, nitrogen, phosphorous, and sulfur limitations at the same dilution rate. In doing so, they could rule out that major differences would be caused by differences in specific growth rate. Experimental design and medium composition were carefully checked to ensure that the chosen growth limitation was realized. They observed that 31% of the annotated genome transcripts changed significantly in these experiments with four separate limitations. Nearly 500 transcripts could specifically be linked to one of the four nutrient limitations. Fifty-one genes showed tenfold changes under one particular limitation. They concluded that the responsible genes may be targets for future diagnostic study and characterization of specific limitations for metabolic engineering strategies. For the cultivation of industrially relevant organisms the use of poorly defined, complex media (such as molasses) is common and hence this type of analysis would be most important. These two examples underline the power and usefulness of controlled cultivation in the chemostat for reproducible and quantitative functional genomic research.

Simultaneous Use of Mixed Substrates in Continuous Culture

Equivalent Substrates

Diauxic growth is a phenomenon that can be observed when, for example, *Escherichia coli* is grown in batch culture on a mixture of glucose and lactose. Growth of the culture clearly shows two phases: first the glucose is consumed and, after a lag phase, the lactose can then be used.

At high glucose concentrations the organism grows at the maximal rate and lactose metabolism is repressed (known as catabolite repression). This is because lactose transport is inhibited at high-energy status of the phosphotransferase (PTS) system. It should be remembered that the PTS system is in dynamic equilibrium with the PMF and the energy charge of the cell. Under simultaneous limitation of growth by glucose and lactose in a continuous culture, at relatively low dilution rate ($D = \mu$), the energy status of the cell is relatively low and the use of lactose does not appear to be repressed. Thus at low D values, the simultaneous use of both compounds occurs, whereas at high D values lactose does appear in the fermentation broth. In *E. coli*, $\mu_{max(glucose)}$ is approximately 1.0 h^{-1}, while the $\mu_{max(lactose)}$ is approximately 0.8 h^{-1}. In this case, the complete simultaneous use occurs below $D = 0.5$ h^{-1}, and

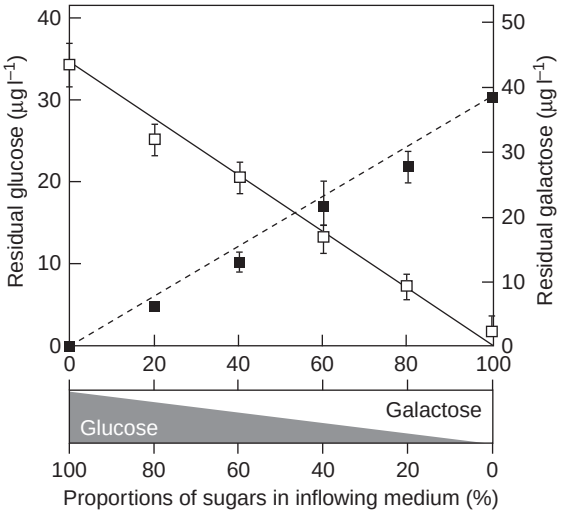

Fig. 8 Steady-state concentrations of glucose (□) and galactose (▪) during growth of *E. coli* at a constant dilution rate of 0.30 h^{-1} in carbon-limited chemostat culture with different mixtures of two sugars. The proportion of the sugars in the mixture fed to the culture is given as weight percentages. The total sugar concentration in the feed was held at 100 mg L^{-1}. Reproduced from Egli, T. (1995). The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. In: Jones, J. G. (ed.) *Advances in microbial ecology*, vol. 14, pp. 305–386. New York: Plenum Press.

glucose and lactose are detectable only at microgram per liter levels. Between $D = 0.5$ and 0.8 h^{-1} lactose gradually appears at increasing levels and a plot of C_S (lactose) against D shows a normal Monod relation as shown in Fig. 1. Above $D = 0.8$ h^{-1} lactose is no longer used and the organism shows typical diauxic behavior as in batch culture with excess glucose. The culture was washed out above the D_C for glucose, and hence also for the glucose the organism displayed normal Monod kinetics (Fig. 1).

The group of Egli has performed similar experiments with other (two or three) mixtures of sugars, for example glucose and galactose, which they were able to measure directly in the steady-state culture liquid at extremely low levels using a new sensitive assay. Fig. 8 shows the results of chemostat experiments run at $D = 0.3$ h^{-1} with different ratios of glucose (0–100%) and galactose (100–0%) in the in-flowing medium.

It can be seen that the residual, steady-state concentration of each of the two growth-limiting substrates was lower in the presence of the second substrate than in its absence. In a 50:50 mixture the residual sugar concentrations were around 20 µg L^{-1} (≈ 100 nmol L^{-1}), that is, almost half of that compared with the presence of one substrate only. This important observation, predicted by mathematical modeling, indicates that in the competition for substrates in Nature, the capability to use substrates simultaneously has a competitive advantage (also see the paragraph on competition for mixed substrates).

The simultaneous, that is mixotrophic, use of substrates with similar roles in metabolism is not limited to sugars but also applies to all kinds of mixtures of carbon and energy sources, mixtures of organic compounds and inorganic energy sources, as well as to mixtures of nitrogen sources, such as ammonium and nitrate. Likewise, the same mixotrophic behavior occurs with respect to electron acceptors such as limiting mixtures of oxygen and nitrate. An extensive study in this area has been done by Gottschal and Kuenen on mixotrophic growth of a facultative sulfur-oxidizing bacterium, *Thiobacillus versutus* (presently named *Paracoccus versutus*) on a mixture of acetate and thiosulfate as an additional energy source and/or CO_2 as an additional carbon source. The organism showed typical diauxic behavior in batch culture at high acetate (consumed first) and thiosulfate, but under simultaneous limitation (at $D = 0.05$ h^{-1}) by the two substrates both were used simultaneously. With high acetate to thiosulfate ratio the latter substrate served as supplementary energy source, while at low ratio acetate was primarily used as carbon source with CO_2 as supplementary carbon source for autotrophic metabolism. Thus a remarkably efficient metabolic control and use of resources were revealed, which made this organism very competitive under conditions of mixed-substrate supply and/or short-term fluctuation in the supply of the separate substrates.

Simultaneous Limitation by Nonequivalent Substrates

When in a carbon-limited culture the in-flowing nitrogen (originally in excess) is lowered, the residual nitrogen in the culture will go down. At a stage where, academically speaking, the nitrogen is still in slight excess the organism may, however, begin to induce its high-affinity GS-GOGAT system for ammonium assimilation and hence employ the ATP-requiring assimilation pathway, in spite of its carbon and energy limitation. This was experimentally verified by Egli, who showed that below suboptimal nitrogen content in the in-flowing medium the organism displays an actual physiological double limitation.

A recent study by Ihssen and Egli with glucose-limited *E. coli* cultures confirmed and extended the generally observed phenomenon of de-repression of pathways in the lower D-range. Under single limitation by glucose at $D = 0.3$ h^{-1} (i.e., at 40%

of μ_{max}) a large number of metabolic pathways were de-repressed, without the inducer being present, in contrast to what is observed in batch culture. These cultures can metabolize and grow instantaneously on the new substrates, such as sugars, alcohols, and organic acids. It is generally believed that this type of response offers an advantage for survival because it reduces the time required to react to a change in nutrient supply. Clearly under natural conditions, mixed substrates will be available and mixotrophic growth on a variety of substrates will be the rule rather than the exception. In these experiments, the authors ensured that the selection of particular mutants did not play a significant role. This was accomplished in practice by establishing each steady state from a fresh inoculum.

Ecological Studies in the Chemostat

General Considerations

In Nature and in man-made environments such as wastewater-treatment plants, concentrations of nutrients are generally very low. The order of magnitude is usually in the nanomolar (microgram per liter) level and KS values of organisms are adapted to these low concentrations. The physiological or competitive behavior of organisms at these low levels can be studied by using chemostat cultures grown under the appropriate limitation. It is true that in Nature actual steady states rarely occur but the chemostat is an excellent tool to look at the principles of (eco)physiological response and competition, under controlled conditions. In addition, continuous cultivation in chemostat equipment under dynamic condition can also be done for simulation of natural conditions, though the mathematics of such operations is much more complicated.

The groups of Jannasch and Veldkamp have extensively studied the competition of bacteria under nutrient limitation in the chemostat. It appears that in nature there are many organisms of type B (Fig. 7B), which possess a high affinity for a growth-limiting nutrient (i.e., low K_S combined with a relatively low μ_{max}) for the substrate. An example is that of a rod-shaped bacterium (R) and a spirillum (S) as shown in Fig. 9.

Both strains were originally isolated from phosphate-limited continuous cultures inoculated with ditch water. One chemostat was run at a high *D*, with a second one at a low *D*. In the culture with the low *D* the spirillum became dominant, while in the culture with the high *D* the rod dominated. When the two pure cultures were mixed, and cultivated again at the same *D* values, the cultures appeared to behave like the original chemostat enrichments. When experiments were performed with the same set of two organisms under other nutrient limitations, that is, succinate, ammonium, or potassium, very similar crossing curves were obtained showing that the spirillum had a generally higher affinity for substrates than the rod. This property, which points to a generally higher transport capability at low nutrient concentration, is linked to the much higher surface to volume ratio of the spirillum. This allows the accommodation of more membrane-transport proteins per unit of biomass.

Competition for Mixed Substrates

In nature many growth-limiting substrates are available simultaneously, and hence an understanding of the selection and competition for more than one substrate has also been studied in the chemostat. Unquestionably, other abiotic and biotic variables also play a role in the establishment of a community of organisms in any environment. Theoretical calculations by Fredrickson indicate that the maximum number of coexisting species is determined by the number of variables in a particular environment. As a simple example, a steady state with two growth-limiting substrates will allow a maximum of two existing species in the culture. When a variation of the pH would be admitted as a third variable, the number would increase to a maximum of three.

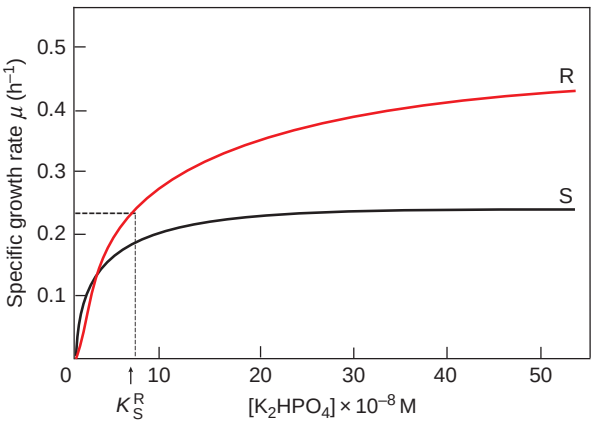

Fig. 9 Specific growth rate of fresh water rod-shaped bacterium (R) and a spirillum (S) as a function of phosphate concentration. The curves are schematic and based on two measurements each at the growth rates indicated by the arrows. K_s values: rod-shaped bacterium, 6.6×10^8 mol L^{-1}; spirillum, 2.7×10^{-8} mol L^{-1}. Reproduced from Kuenen, J. G., Boonstra, J., Schroder, H. G. J., and Veldkamp, H. (1977). Competition for inorganic substrates among chemoorganotrophic and chemolithotrophic bacteria. *Microbial Ecology*, 3, 119–130.

Table 1 Maximum specific growth rates of two specialists and one versatile bacterium.[a]

Organism	*Lifestyle*	*Relevant physiology*	μ_{max} *thiosulfate (T)*	μ_{max} *acetate (A)*	μ_{max} *(T + A)*
Thiobacillus neapolitanus	Specialist	Obligate chemolithoautotroph	0.35	0	0.35
Thiobacillus versutus	Versatile	Facultative	0.1	0.22	0.22
Heterotroph G7	Specialist	Obligate heterotroph	0	0.43	0.43

[a]The specialists can only grow on a single specific substrate. The versatile organism shows diauxic behavior towards thiosulfate, but simultaneously consumes acetate and thiosulfate in the chemostat at low $D = 0.05\ h^{-1}$.

Gottschal and colleagues published an interesting example of a set of three organisms competing for two substrates. The properties of the three organisms are listed in Table 1 and refer to *Thiobacillus neapolitanus*, *T. versutus*, strain A2, and Spirillum G7.

T. neapolitanus (presently known as *Halothiobacillus neapolitanus*) is a specialized, obligately chemolithoautotrophic sulfur oxidizer, which can grow only on an inorganic sulfur compound (thiosulfate) as the energy source and CO_2 as carbon source. Spirillum G7 is a specialized chemoorganoheterotroph, which can grow only on acetate as carbon and energy source. Both specialists have a high μ_{max} in batch (excess substrate) on their respective substrates. In contrast, *T. versutus*, presently known as *P. versutus*, is a facultative organism capable of growing in both modes, but at lower μ_{max} in batch. As mentioned above, when grown in a mixture of the two substrates in batch culture at high substrate concentrations, the organism shows diauxic behavior and consumes acetate first and then thiosulfate. Therefore, μ_{max} (T + A) does not increase. When a mixture of the three organisms is inoculated in excess acetate and thiosulfate in batch culture, we end up with a dominant mixture of the two specialists. However under limitation by a mixture of acetate and thiosulfate at a low dilution rate in the chemostat, the versatile organism can very effectively use the two substrates simultaneously. It was shown that, as a result of this capability at low D ($0.05\ h^{-1}$) it can coexist or even out-compete the two specialists in a mixed culture of the three organisms. This is due to the fact that in the mixture of the second substrate (thiosulfate or acetate) the versatile organism can lower the concentration of the other limiting nutrient, as was shown above for the mixture of glucose and galactose (Fig. 8) and mathematically modeled by Gottschal and Thingstad. In line with theoretical predictions concerning the maximum number of coexisting species in relation to the number of variables, it was shown that *T. versutus* was able to maintain itself at low number in the steady-state culture of *T. neapolitanus*, in spite of its lower affinity for thiosulfate, since the specialist excreted glycollate. The glycollate was mixotrophically consumed by the versatile organism. This demonstrates the important principle that metabolic (excretion) products can not only lead to simple cometabolic consumption of this product by a second population, but that by excreting a consumable product the excreting organism may generate more competition for its main substrate.

Continuous cultivation not only provides a tool to create reproducible steady-state cultures, but also offers controlled alternations of environmental and nutritional conditions, such as feast and famine cycles, changes of substrates, or temperature. In such experiments, a true steady state is not established but nevertheless highly reproducible cycles will allow the precise monitoring of the response of the organism(s). Indeed in the case of the two specialists and the versatile organism, it was shown that the latter could also out-compete the two specialists at low D when acetate and thiosulfate were alternately supplied. As long as the period of the cycle was below 4 h the versatile organism would continue growing, but when longer cycle times were introduced the versatile organism would repress its autotrophic potential too far down to be able to compete with the specialists in the next cycle. This example serves to emphasize the usefulness of continuous cultivation for ecophysiological research.

Industrial Applications of Continuous Culture

In the industrial production of (secondary) metabolites, such as antibiotics, the production organism (i.e., a fungus like a *Penicillium* or a *Streptomyces* sp.) is cultivated at submaximal μ, because only under these conditions will the organism produce at a sufficiently high rate. Clearly for optimization reasons, it is essential to study the performance of the organism in the chemostat at different dilution rates. The production process is, however, rarely performed in continuous culture. The main reasons for this are: (1) the cell density and hence the product concentration can never be very high because the aeration capacity of the fermentors have limited oxygen transfer and cooling capacities. This leads to insufficiently high product concentrations for economical down-stream processing. (2) The inevitable selection of less productive spontaneous mutants under the imposed carbon and energy limitation (see below). Therefore, in industry, the cultivation method of choice is usually fed-batch cultivation. After an initial stage of growth in excess substrate the organism is fed (in batch) with a concentrated feed of the growth-limiting nutrient (i.e., glucose or ammonium) in such a way that the instantaneous consumption of the added nutrient limits the growth of the organism. Using this method, high product concentrations may be reached and in the short-term cultivation period mutants will not become dominant.

In the practice of wastewater treatment many (semi) continuous operations are used, be it that biomass retention and recycling of biomass are practiced. An example of a chemostat-type large-scale process is the production of a 50:50 ammonium/nitrite mixture from ammonium-containing reject water using selected, oxygen-limited mixed cultures of nitrifying bacteria. The effluent is used to feed an anaerobic anammox reactor, which converts the mixture into nitrogen gas as demonstrated by Van Dongen and

colleagues. The recycling chemostat with major biomass recycle has been used to grow (mixtures) of microorganisms at a constant very low growth rate, down to 0.05/day. This has been particularly successful for enrichment of very slow growing microorganisms from natural resources and from wastewater treatment plants (Van der Star et al., 2008).

Competition and Selection of Mutants in the Chemostat

Nutrient-Limited Wild Types and Mutants

A monoclonal bacterial culture will contain mutants. It is known that the average number of mutants for a particular gene is of the order of 1 out of every 10^6–10^9 replications. This means that mutants, which can perform better under nutrient limitation than any wild-type organism, may out-compete the parent strain. Therefore, the study of competition and selection does not require two different species. The technique is sometimes intentionally utilized to isolate mutants, which by spontaneous (or induced) mutation have obtained a competitive advantage. A classical example of this is the development of a constitutive mutant for β-galactosidase of *E. coli* in a lactose-limited culture as described by Novick. Once the constitutive mutant has been enriched, so-called super-constitutive mutants, which produce more β-galactosidase often arise. This is illustrated in Figs. 10 and 11.

These results can be understood by making the assumption that the uptake of lactose (and the linked conversion of lactose into galactose and glucose) is the rate-limiting step. By producing more permease and β-galactosidase (which is transcribed under the same promoter), the cell would be able to convert lactose somewhat faster at the same $\bar{C}_s$. For example, as long as the lactose permease is the real bottleneck in the rate of metabolism of lactose, a doubling of the permease concentration will allow the organism to maintain the same overall rate at half the concentration of the lactose. Consequently, it will be able to grow at the same rate at half the lactose concentration and hence out-compete the wild type. Consequently, the μ–C_S curve of the mutants changes. This is illustrated in Fig. 11, where Novick has given the wild-type μ–C_S curve an "S"-shape to accommodate the fact that, in contrast to the constitutive mutant, it requires a relatively high concentration of lactose to fully initiate transcription of the lactose operon.

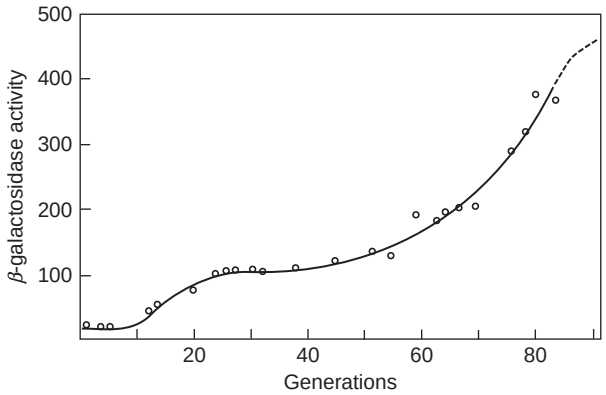

Fig. 10 Increase in β-galactosidase activity of *Escherichia coli* strain E-102 grown in a lactose-limited chemostat. Reproduced from Novick, A. (1961). Bacteria with high levels of specific enzymes. In: Zarrow, M. X. (ed.) *Growth in living systems, purdue growth symposium*, pp. 93–106. New York: Basic Books, Inc.

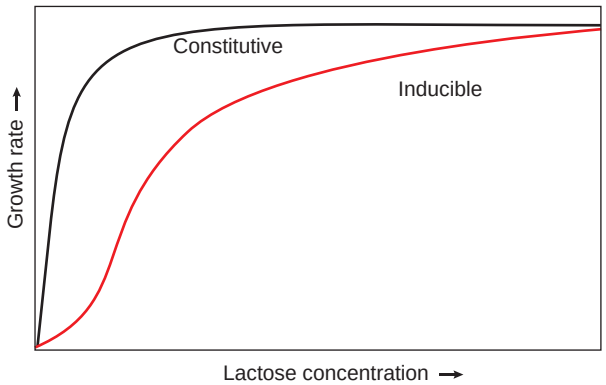

Fig. 11 Hypothetical relationship between growth rate and lactose concentration when the growth-limiting enzyme is constitutive and when it is inducible. Reproduced from Novick, A. (1961). Bacteria with high levels of specific enzymes. In: Zarrow, M. X. (ed.) *Growth in living systems, purdue growth symposium*, pp. 93–106. New York: Basic Books, Inc.

In the above case, 25–30% of the total protein of the "super-constitutive" mutant appeared to be β-galactosidase. This mutant is, in fact, extremely vulnerable, competitively, under any other form of growth limitation since then the overproduction of the enzyme would be a waste of energy. For this reason, if this culture is transferred to glucose limitation, other mutants, which cannot be distinguished from the original (wild type) parent strain, will rapidly appear. In summary, the striking changes shown in the final mutant demonstrate the extreme selective forces that can be exploited in the chemostat to obtain mutants that have taken advantage of the chosen limitation. For a representative set of other examples the reader is referred to the reviews by Kuenen and Harder and Sikyta.

Egli's team has studied the selection of mutants of *E. coli* in glucose-limited chemostats. With a combination of highly sensitive analytical tools for residual sugar concentrations as well as the availability of tools for physiological screening, a thorough analysis has been made of the events taking place during the selection of *E. coli* mutants under glucose limitation in the chemostat. It appeared that the selection events were consistent with Monod kinetics. Consecutive mutants, with higher affinity (i.e., a mutation leading to either higher μ_{max} or lower K_S) for glucose, gradually took over in the chemostat, as evidenced by the stepwise drop in the residual glucose concentration in the culture. Initially μ_{max} improved, but this stopped after the first 150 h and then further improvements were primarily due to the lowering of the K_S. The experiments were reproducible in cultures with high number of cells in the culture (10^{11}), that is, the stepwise improvement could be predicted. However at low numbers of total cells (10^7), the mutation frequency for advantageous mutations (estimated to be $1/10^7$ cell duplications) caused a stochastic behavior. Evidently, at 10^{11} cells per culture, in the order of 10^4 favorable mutants would have been present after the first generation and the system did not have to wait for favorable mutants to overgrow the existing population. Over the time span of 500 h in these experiments, no mutant types other than the high-affinity strains established themselves in the culture. They obtained no evidence for the establishment of secondary populations of mutants with a lower affinity for glucose, as was observed by Rosenzweig et al. (1994), after long-term selection at $D = 0.2\ h^{-1}$. In this experiment, a mixture of mutants had been selected with a distribution of tasks after 773 generations (2500 h). This mixed culture remained essentially the same for an additional 450 generations. One dominant mutant had the highest affinity for glucose but excreted glycerol and acetate, which were consumed by a second and third (satellite) mutant, respectively. Hence under the extreme and permanent selection conditions, the distribution of tasks in the breakdown of glucose ("resource partitioning") apparently was the most effective way to deal with the regulatory consequences of metabolic rearrangement in the respective mutants. Although the exact steady-state fluxes of glycerol and acetate in the chemostat were not known, it must be assumed that the second and third mutants, having the capacity for glucose metabolism, were both growing mixotrophically on glucose and glycerol or acetate, respectively.

The selection of mutants in nutrient-limited chemostat has extensively been studied by the group of Ferenci and colleagues, who showed that depending of the type of limitation a different spectrum of mutants is continuously produced and maintained in the chemostat which results in the selection ("evolution") of different types of mutants over time. They studied carbon, phosphate, nitrogen, oxygen or iron limited chemostat cultures of *E. coli* and observed a number of different types of mutation caused by base pair changes, transposition or indel changes that occurred at different frequencies and preferences under the influence of the specific limitation, demonstrating that the environment not only influences selection but also genetic variation.

Research by Stevenson and Schmidt on batch and chemostat grown *E. coli* has shown that multiple rRNA operons may confer a competitive advantage during fluctuations in nutrient supply.

Metabolic Engineering

The group of Pronk has recently constructed a xylose-fermenting *S. cerevisiae* strain for alcohol production from the pentose. They inserted a xylose isomerase from a fungus in this yeast, which was originally incapable of metabolizing xylose even under aerobic conditions. The xylose isomerase converts the sugar into xylulose, which can be metabolized by the yeast. Once this was accomplished, oxygen-limited and anaerobic batch enrichment in the presence of high-xylose concentrations led to the selection of mutants that grew faster on xylose and eventually they ended up with a mutant that grew anaerobically on xylose. Genetic engineering to add bottleneck enzymes of the pentose phosphate cycle yielded a mutant that could grow anaerobically on xylose at a rate of $0.09\ h^{-1}$. The organism showed, however, a strong diauxic behavior towards xylose in the presence of glucose, which was undesirable for application in the commonly available industrial sugar mixtures derived from processing of wood for paper production. Subsequent selection was then performed in an anaerobic chemostat culture under simultaneous limitation by a mixture of glucose and xylose. Progressive decrease of the residual xylose in the culture indicated the appearance of mutants. A strain was obtained with much improved xylose uptake kinetics and rate of metabolism of the pentose in the presence of glucose, even in batch culture. The authors concluded that the bottleneck in this case was primarily caused by uptake of xylose. This again shows how one can take advantage of the strong selective forces existing in nutrient-limited continuous cultures. Further improvement was accomplished by enrichment of mutants in anaerobic sequencing batch culture to select for faster growth. In the end, a mutant was selected with a $\mu_{max} = 0.22–0.25\ h^{-1}$ in a mixture of (10%, *w*/*v*) glucose and (2%) xylose, in which sequential but effective-anaerobic consumption of xylose at a rate of 0.9 g per gram biomass per hour occurred.

Practical Aspects Related to Inevitable Presence of Mutants in the Culture

It is important to note that due to the selective forces operating in the chemostat cultures the (chemostat) culture never is a genetically homogenous "pure" culture. This is augmented by the fact that in most cases the mutation only may give a slight advantage in terms of an increased specific growth rate in the order of $0.01\ h^{-1}$ or less. It is easy to calculate, using Eq. (4) that it would take such a mutant 100 days to make up 1% of the culture in a "steady state." One may therefore say that it is inevitable that a

number of mutants with slight growth rate advantages will be present in the culture, but that they may only become significant after many volume changes. In practice a steady state is reached in 4–5 volume changes (at $D = 0.01\ h^{-1}$ a volume change takes 100 h). Given the presence of these competitive mutants the maintenance of prolonged steady states it is not advisable in laboratory studies aimed at investigating the wild-type strain. It is advisable to switch dilution rates after each steady state and to start with a fresh culture (using an original new inoculum from stock) after not more than 3–5 consecutive steady states have been investigated. Reproducibility of any particular steady state must be checked with independent cultures.

Other Interactions in Continuous Culture

When we consider competition for single or mixed substrates, we assume that there are no interactions between the organisms other than competition for the growth-limiting substrate. Commensalism was briefly mentioned, and in its "pure" form it is defined as an interaction involving the excretion of a substrate that is used by a second organism, without any further interaction. Examples are the use of fermentation products or products from an electron acceptor, such as nitrite from nitrate or sulfide from sulfate. Some of these may be toxic and in such cases the first organism may need the second to perform optimally. Secondary products such as vitamins or antibiotics may play a crucial role as well. This may lead to stable coexistence if an organism with the highest affinity for the limiting substrate requires the vitamin from an organism with lower affinity. In the case of an antibiotic, the cell density may determine whether or not coexistence is possible.

Mixed cultures of organisms may also be stable if one organism consumes a substrate toxic to the second. In this way, mixtures of aerobic and anaerobic organisms have been studied in oxygen-limited continuous culture. Oxygen is removed by the first organism, before the second can carry out its obligate anaerobic metabolism as has been demonstrated for a mixture of aerobic heterotrophs and sulfate reducers and even methanogens. More complex interactions include the degradation of xenobiotics by consortia of organisms, where one organism initiates the degradation of the compound, but cannot grow on that product. It needs a second organism to metabolize the first product and excrete a second product that can be used by the first. Examples are known in which four or five organisms maintain themselves in continuous culture in a tightly closed interactive network.

Essential Equipment in Continuous Culturing

From the preceding theoretical consideration, it is clear that the only fundamental design requirement of any continuous culture system is that the culture be kept growing by a continuous input of fresh medium that is balanced by the removal of culture fluid at the same rate. It is essential that the culture is "ideally" mixed, because the theory assumes a totally homogeneous system. A great variety of continuous culture systems have been used over the years, with properly evaluated systems ranging in size from just a few milliliters in modified Hungate tubes that were used for radioactive tracer analysis of metabolic fluxes, up to a 1500 m^3 industrial system that is used for the nitrification step in wastewater treatment.

For all practical purposes, it is recommended to use a commercially available fermentor of 1–2 L working volume with a potentially high oxygen transfer capacity to ensure good aeration for aerobic cultivation. If the fermentor is built of glass and stainless steel, or from autoclavable oxygen-impermeable polymers, such a system can also be used for anaerobic continuous cultivation.

It is beyond the scope of this article to elaborate on the detailed design of these systems, but a summary of the important general considerations is included because they are important for the construction of most general-purpose research chemostats. Today many very well-designed chemostat systems are commercially available. In Fig. 12, an example of a small bench scale ($\approx$0.5 L working volume) continuous culture system is shown. Some of its design characteristics are as follows:

1. It can be used for both aerobic and anaerobic cultivation. For aerobic use, silicone seals and tubing are ideal. For anaerobic cultivation, seals and tubing are typically made from neoprene rubber or similarly impermeable materials, into which holes can easily be drilled to fit the desired assortment of tubing and probes.
2. Sterility is easily maintained because the entire unit can be autoclaved and mixing is done with a magnetic coupling between the motor and the stirring shaft, eliminating potential problems associated with the sealing of the stirring shaft through the lid of the chemostat. For simplicity Fig. 12 shows a stirring bar. However, in fermenters with larger working volumes ($\gg$0.5 L) much more elaborate stirrers with shaft and impellers are required to ensure homogeneity.
3. The medium is supplied by means of a peristaltic pump using a durable rubber such as ismaprene or norprene for anaerobic cultivation. Silicone or any other compatible rubber can be used with aerobic cultures. The culture volume is maintained at a constant level by the removal of fluid at the same rate that it enters. In the example, an overflow is shown for simplicity. However, the drawback is that such devices may not remove representative materials from the culture. Therefore the best device is a second pump removing liquid from below the liquid surface and which is activated by a sensor touching the surface of the culture.
4. In some cases, an appropriate low cost and/or simple continuous culture system may be used. It is essential, however, that such a system be designed appropriately for its intended purpose and thoroughly checked for adequate performance. For example, the use of potentially hazardous and/or expensive compounds, such as ^{14}C- or ^{13}C-labeled substrate, may require using very small working volumes. A simple test that is often overlooked in continuous culture studies and that can easily demonstrate whether the culture is actually limited by the expected substrate is to check the relation and the proportionality of $\bar{C}_X$ with C_{Si} at one chosen intermediate D, when it can be expected that $\bar{C}_s \ll C_{si}$ (Eq. (12)). A very good example is described by Sauer and

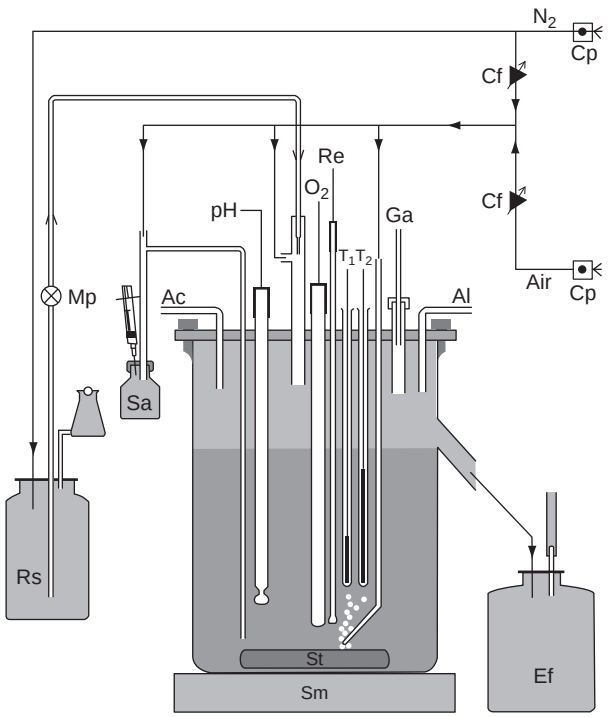

Fig. 12 Schematic drawing of a small-scale (500-mL working volume), low-cost glass chemostat. All gases pass through cotton wool filters (not shown) before entering the fermentor. For anaerobic cultivation the N_2 is freed of traces of oxygen by passage over heated copper turnings. *Rs*, reservoir medium; *Mp*, medium supply pump; *Sa*, sampling bottle; *Ac* and *Al*, acid and alkaline titration inlets, respectively; *pH*, autoclavable pH electrode; O_2, autoclavable polarographic oxygen electrode; *Re*, redox electrode; T_1 and T_2, temperature sensor and heating element; *Ga*, sampling outlet for head-space gas analysis; *Cf* and *Cp*, constant flow and pressure regulators, respectively, for maintaining a stable (mixed) flow of N_2 and air over and through the culture; *St* and *Sm*, magnetic stirring bar and motor unit, respectively; *Ef*, effluent from the culture.

colleagues, who used 17 mL Hungate tubes as chemostats to study the dependency of intracellular fluxes of metabolite as a function of specific growth rate. The validation concerned aeration sufficiency, and predicted linearity of the specific consumption rate with the dilution rate as well as biomass yield, residual glucose, and acetate as a function of *D*.

5. Other potential issues of chemostat design may arise from the use of highly volatile compounds in which an open gas phase may not be appropriate. The use of hydrophobic compounds can be problematic as they are often permeable in rubber tubing and rubber seals, and so proper attention toward chemical compatibility is essential. Most general-purpose chemostats are also insufficient for growth under pressure so the continuous cultivation of hyperthermophiles requiring elevated pressure typically must be done in chemostats whose exteriors are made exclusively from stainless steel. Insufficient stirring and aeration can easily become a problem in cultures grown at very high cell densities, especially in combination with high dilution rates. These can also become a problem at low cell densities if the volume of the culture is scaled up too high and these parameters should therefore be carefully considered when setting up any continuous culture system.
6. One detailed comment must be made on the design of the nutrient-medium inlet, which is of critical importance. The inlet must be able to deliver a regular flow of small droplets to the culture vessel (to minimize any effects of discontinuity in the supply of fresh medium) while simultaneously preventing contamination of the nutrient reservoir by the back growth of organisms in the culture vessel. This is typically accomplished by using a device through which medium droplets fall freely and directly into the culture fluid through a relatively wide glass tube that is kept dry on the inside by a continuous flow of sterile gas in the direction of the culture vessel. However if this device is used at low dilution rate and small culture volume, the drop-wise addition will cause dramatic fluctuations of the concentration of the limiting nutrient in the culture. As a result the coupling between catabolism and anabolism may no longer be optimal, which leads in turn to the low yield.

Important Aspects of Continuous Culture

1. Continuous culture in a chemostat enables the reproducible growth of bacteria and other microorganisms. Consequently, the chemostat is an appropriate tool for quantitative (eco)physiological research.
2. Microorganisms can be studied while growing at submaximal rates.
3. The effect of different growth limitations on the metabolism of the cells can be measured reproducibly.
4. The chemostat is also appropriate for studying the competition of microorganisms and mutants for growth-limiting substrates.

5. Continuous cultivation can also be performed under fluctuating environmental conditions.
6. Recent publications show the great value of chemostat cultivation for functional genomic research and for the selection of industrially relevant mutants.
7. The commercial availability of well-designed chemostat equipment greatly facilitates the introduction of continuous cultivation in the laboratory.

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Corrosion, Microbial[☆]

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Glossary

Cathodic polarization Consumption of hydrogen accumulated on the surface of metals by microorganisms attached on the surface.

Exopolymers Organic molecules, mostly carbohydrates and proteins, secreted by microorganisms.

Hydrogen embrittlement Permeation of molecular hydrogen into the metal matrix, resulting in crack and structure deformation.

Microbial-induced (influenced) corrosion An electrochemical process catalyzed by microorganisms through formation of electrochemical potential between the cathode and the anode, cathode depolarization, and attack on metals by generated corrosive products.

Defining Statement

Microorganisms are known for their role in the corrosion and passivation of a wide range of metals and alloys through generation and consumption of electron, but the mechanisms, including depolarization of materials, biomineral formation, complexation through exopolymeric materials, H_2 embrittlement, electron shuttling and nanowire, are still not well understood. The best-known model is the involvement of sulfate-reducing bacteria (SRBs) via sulfate reduction and production of H_2S, but further knowledge about the specific genes and enzymes involved is incomplete. Other proposed mechanisms need substantive experimental evidences to confirm the specific biological basis of those processes. Polymeric polymers are used as physical barrier to separate materials from water and microorganisms to achieve corrosion inhibition, but microorganisms are found to destruct the protective coating over long time of exposure. Prevention of microbial influence corrosion (MIC) is a major challenge because microbial biocides and toxic chemicals cannot kill and inhibit them over a long period of applications.

History and Significance

Corrosion is part of the natural processes of decomposition, providing the key driving force for the Earth's dynamic system. Unfortunately, it has a severe economic consequence to our society and this fact has been recognized at a very early stage of research and development. The estimated loss is ~4% of the gross national product (GNP) due to corrosion, of which 70% of the corrosion is by microorganisms in gas transmission pipelines. The American refinery industry loses $1.4 billion a year from microbial corrosion alone. Microorganisms are the first colonizer on earth and they are ubiquitous. A wide variety of microorganisms are capable of corrosion and degradation, and the causative microorganisms include both aerobic and anaerobic bacteria as well as fungi. Among them, the SRBs have been the long-known microorganisms in a large number of earlier studies on biocorrosion and they are also the culprits for control and elimination in utilities, and oil and gas industries. In addition to SRBs, exopolymer (slime)- and acid-producing microorganisms are also recognized for their active participation in corrosion processes through mechanisms by which metal ions are complexed and released from matrices of materials with functional groups of the not-well-defined exopolymers, resulting in release of metallic species in the solution. Similarly, fungi are involved in the corrosion of aluminum and its alloys by a process in which organic acids of fungal origin attack the underlying material through the acids produced. Fungi are also shown to be the causative organisms in degradation of concrete, stone and polymeric materials that are widely used for structure as well as protective purposes against metal corrosion.

The importance of microorganisms in the corrosion of metallic materials was first reported by Dutch scientist von Wolzogen Kuhr in 1922 when anaerobic SRBs were postulated to contribute to iron corrosion through removal of hydrogen accumulated at the cathodic site on substratum material surfaces. Since then, microbial influenced corrosion (MIC) has been reported for a much wide range of conditions, including oil fields, offshore, pipelines, pulp and paper industries, armaments, nuclear and fossil fuel power plants, chemical manufacturing facilities, wastewater treatment, drinking water distribution system, food industries, and facilities with membrane applications to ship, airplanes, space station and nuclear depositories. The terminology of microbiological

[☆]*Change History*: In addition to making corrections and improving the coherence of the article, text relating to the proposed role of electron shuttling in corrosion has been added.

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 https://doi.org/10.1016/B978-0-12-809633-8.13026-2

corrosion has frequently been used interchangeably with microbiological fouling, but the two are not synonymous on scientific basis. MIC is still not clearly defined on a scientific basis as the biochemical mechanisms involving the responsible microorganisms are still not well defined, and ambiguity and misuse are common in the literature.

Microbial processes contributing to corrosion of metals are several. In general, the presence of inorganic deposits and differential concentration cells of oxygen and chloride without microbial participation are important factors determining the corrosion mechanisms and the extent of corrosion. The presence of microflora and fauna on surfaces of materials alters the local environment further, providing conditions for initiation of metal dissolution or passivation of the material surface. Microbial involvement in corrosion of metals is a result of electron or proton movement at the interface between a substratum and the colonizers, in which adhesion on surfaces is a prerequisite for subsequent alteration of material stability through metabolic activity. Microorganisms form complex communities on surfaces of materials, and develop into a complex heterogeneous structure of biofilms under natural conditions (Fig. 1). Such microbial associations on surfaces can be corrosive or passive and are also responsible for destruction or protection of the underlying materials. Specific aerobic microorganisms obtain electrons from metal oxidation and, at the same time, reduce CO_2 for cellular synthesis and growth. Bacteria growing on these surfaces alter the microenvironments, resulting in depolarization and depletion of local oxygen to create concentration cells for electrochemical process to initiate, and also altering the diffusivity of metabolites and nutrients within the 3 dimensional biofilm structures of microorganisms. During growth and building up of the biofilm, the anaerobic microorganisms can establish near the surface of materials where oxygen is depleted so that SRBs produce H_2S, which reacts and corrodes metals. Corrosion is typically mediated by the hydrogenase activity of the SRBs, particularly in the genus *Desulfovibrio*.

A scanning electron micrograph showing a complex biofilm community developed on surfaces of synthetic materials in a polluted river. The sample was dehydrated and critical point-dried before being coated with palladium and gold for viewing under Scanning electron microscope (scale bar, 2 μm).

Microbial Biofilms and Corrosion

Microorganisms are normally associated with physical surfaces in the natural environment. This is especially true for those in aquatic and terrestrial environments of either oligotrophic or eutrophied conditions. Microorganisms adhere to both non-living and living (tissue) surfaces under submerged or moist conditions, in industrial environments exposed to moisture, and enclosed environments of submarine, space station and deep subsurface depositories. Physical surfaces concentrate nutrients under nutrient-poor conditions and the nutrients serve as chemotactically attractants for certain microorganisms. Microbial growth on such surfaces may result in well-defined colonies to further utilize the nutrients available more efficiently from the surfaces. Adhesion of microorganisms onto surfaces of metals alters the electrochemical characteristics of the material by establishing microelectrochemical concentration cells, in which electron flow can be initiated from anode to cathode. The resultant microbial biofilm can lead to cathodic depolarization due to oxygen depletion near the microbial colonies because of microbial activity and diffusion limitation of O_2 in aqueous phase coupling with an increasingly localized acidity around the microbial colonies.

The structure of a biofilm community on any surface is highly heterogeneous in composition and in structure as well as over time and space. The community composition reflects changes in the local environment, nutritional conditions, and selective competition pressure under the specific environments. Nearly a pure culture of *Pseudomonas aeruginosa* can be resulted in the lung of patients suffering from cystic fibrosis. Microbial attachment onto surfaces can have several effects: (1) enhance the initiation of electrochemical process, corrosion, (2) recruit invertebrates for settlement, and (3) passivate metallic surfaces. An understanding of microbial adhesion processes and characteristics of biofilms is essential for better knowledge of initiation and control of corrosion.

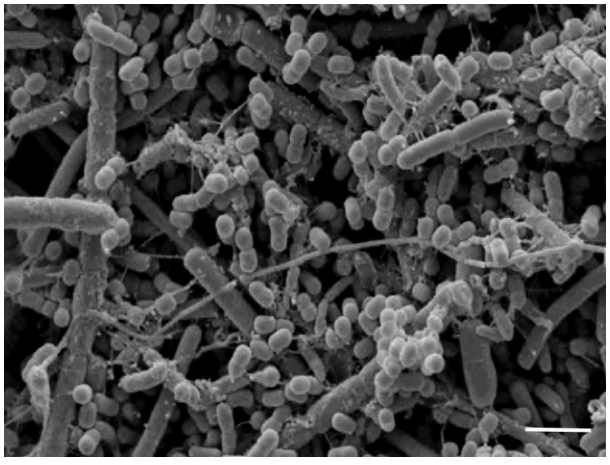

Fig. 1 A scanning electron micrograph showing a complex biofilm community developed on surfaces of synthetic materials in a polluted river. The sample was dehydrated and critical point-dried before being coated with palladium and gold for viewing under Scanning electron microscope (scale bar, 2 mm).

Biofilms can affect corrosion by means of any or a combination of the following factors: (1) direct effects on cathodic or anodic processes to affect electron movement between material and the ambient surrounding, (2) changes in surface film resistivity by microbial metabolites and exopolymeric materials, (3) generation of microenvironmental conditions promoting corrosion, including low oxygenation conditions and acidic microenvironments, (4) establishment of concentration cells facilitating electrochemical reaction to take place, and (5) microbial products in the forms of H_2S and other organic acids that promote the aggressive corrosion of underlying metals. Because microorganisms are ubiquitous and biofilms are present in various environments, their influence on materials covers a wide range of temperature, humidity, salinity, acidity, alkalinity, and barometric conditions. In some cases biofilms may also cause ennoblement of metal rather than corrosion, but the underlying biological mechanisms are still poorly understood. Obviously, there is an apparent lack of fundamental scientific information to reveal the basic biological mechanisms at biochemical and physiological levels. In addition, prediction of both corrosion and passivation of materials especially the rate is almost impossible, indicating the lack of large amounts of scientific data and the factors contributing to the phenomena observed under field conditions.

Adhesion of bacteria onto surface is by means of long-range and short-range forces operating between the bacterial cells and the surfaces from a physics point of view. The known Diffuse Double Layer Theory can be used to explain the process involved. At a distance, attractive forces dominate and allow the close movement between a bacterium and the surface. When moving to a critical distance near to the surface, the repulsion forces emerge and become dominant gradually, keeping bacteria away from the surface. Microorganisms form patches of colonies on any kinds of materials, inducing the formation of differential concentration cells on metals. After the initial adhesion by electrostatic attraction or random collision, organisms divide and form 3-dimensional structures of biofilms, which deplete oxygen and release hydrogen ions. Microorganisms have a specific tendency to selective sites for initial attachment and roughness of material surfaces is generally fundamentally important in the early establishment of biofilms. Such physical factor effects can be identified from investigation by using Atomic Force Microscopy, but the lack of a biological basis for such observations is a big obstacle for further substantiation of the results obtained. For example, surface treatment can modify the metal surfaces to be smoother at the microscopic level and attachment of bacteria on these surfaces is negatively correlated to surface smoothness, offering practical applications to achieve biofilm control by the choice of materials. In food processing and pharmaceutical industries, this approach can lead to significant savings from cleaning and disinfection to maintain high quality of products. The adhesion and biofilm forming process may be gene regulated to synthesis of exopolymeric materials to form a complex biofilm community. Under oligotrophic conditions, microorganisms synthesize large quantities of exopolysaccharides, serving as protectants from desiccation and energy reserves. When nutrients are further depleted, the cells can recycle these polymers as a reserved source of carbon and energy for survival.

The existence of biofilms on surfaces as a concept has gained wide acceptance and knowledge of this aspect has been advanced significantly in recent years. A lack of fundamental understanding of adhesion processes prevents us from formulating effective preventive strategies for the effective control of bacterial biofilms or predicting the potential for biofilm development and damage caused. Biomolecules of signaling have been implicated in these processes and significant advances were made in this line of research. Unfortunately, large quantities of biocides and antibiotics are used in industrial and medical areas, but control of biofilms has little success. Selective microorganisms develop mechanisms to resist toxic chemicals and utilize them as a source of carbon and energy. Microorganisms have ability to sense the chemicals in the environment through active movement closer or away from a specific chemical substance called chemotaxis. Selective chemicals can repel marine bacteria under experimental conditions, but the application challenge is to retain these potent chemicals in a formulated coating on surface for slow release over an extended long period of time.

Aerobic Corrosion Processes

Molecular oxygen (O_2) serves as an electron acceptor for microorganisms to achieve maximal energy for growth under aerobic conditions. When molecular oxygen becomes limited, bacteria have other alternative strategies in utilizing other electron acceptors, including NO_3^-, NO_2^-, Fe^{3+} and Mn^{4+} as examples. Since microorganisms living under natural conditions tend to adhere onto surfaces, cells within a biofilm face shortage in oxygen availability. During aerobic corrosion, the area of metal beneath these colonies acts as an anode, while the area further away from the colonies, where oxygen concentrations are relatively higher, serves as a cathodic site (Fig. 2). Electrons flow from anode to cathode and the corrosion process is initiated. Electrolytes affect the distance between the anode and cathode, being shorter at low and longer at high salt concentrations. An electrochemical potential is eventually developed across the two sites and corrosion reactions take place, resulting in the dissolution of metals. Dissociated metal ions can form ferrous hydroxides, ferric hydroxide, and a series of Fe-containing minerals in the solution phase for iron, depending on the species of microorganisms present and the chemical conditions. Electron flow via oxidation and reduction is fundamental and must occur to consume electrons produced for corrosion to proceed. However, the electrochemical reactions never proceed at theoretical rates because the rate of oxygen supply to cathodes and removal of products from the anodes limit the overall reaction. In addition, impurities and contaminants of the metal matrices also stimulate corrosion by initiating the formation of differential cells and accelerated electrochemical reactions.

When aerobic corrosion occurs, corrosion products usually form a structure consisting of three layers called tubercles (Fig. 3). The inner green layer is mostly ferrous hydroxide $Fe(OH)_2$. The outer one consists of orange ferric hydroxide $Fe(OH)_3$. In between

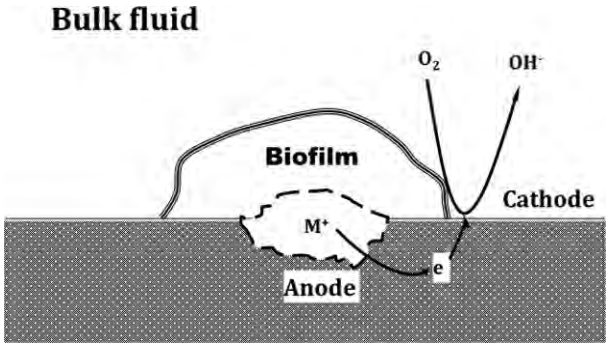

Fig. 2 A schematic diagram of a differential aeration cell created by biofilm under aerobic conditions on a metal surface leading to corrosion of metal.

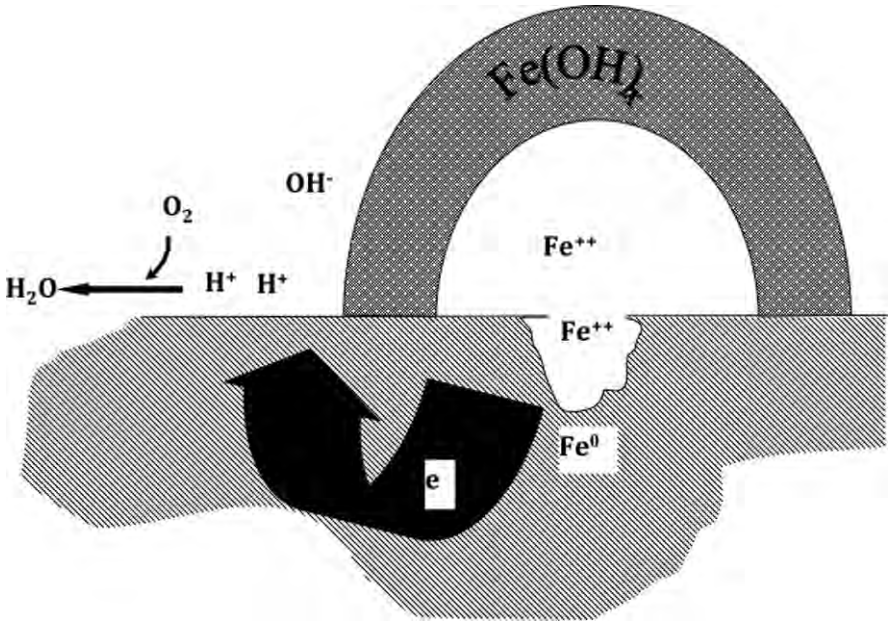

Fig. 3 A schematic diagram of a tubercle formed under pitting corrosion condition of metals.

these two, magnetite Fe_3O_4 forms a black layer. The most aggressive form of corrosion is tuberculation caused by the formation of differential oxygen concentration cells on metal surfaces. The overall reactions are expressed as follows:

$$Fe^0 \rightarrow Fe^{2+} + 2e^- \text{ (anode)} \quad (1)$$

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \text{ (cathode)} \quad (2)$$

$$2Fe^{2+} + 1/2O_2 + 5H_2O \rightarrow 2Fe(OH)_3 + 4H^+ \text{ (tubercle)} \quad (3)$$

Initial oxidation of Fe of mild steel at near-neutral pH is driven by dissolved O_2. Subsequent oxidation of Fe^{2+} to Fe^{3+} is an energy-producing process carried out by a few specialized species of microorganisms autotrophically. The amount of free energy gained from this reaction is small, approximately −31 kJ, so large quantities of Fe^{2+} are oxidized to support microbial growth. This is especially true in an intertidal environment where alternation of oxic and anoxic conditions is facilitated by tidal activity. Because the Fe^{2+} oxidative reaction is rapid under natural conditions, microorganisms compete with chemical processes for Fe^{2+}. As a result, biological involvement under aerobic conditions may be underestimated.

A number of aerobic microorganisms play an important role in corrosion, including the sulfur bacteria, iron- and manganese-depositing and slime-producing bacteria, fungi, and algae. At neutral pH, Fe^{2+} is not stable in the presence of O_2 and is rapidly oxidized to the insoluble Fe^{3+} state. In fully aerated freshwater at pH 7, the half-life of Fe^{2+} oxidation is less than 15 min. Because of this, the only neutral pH environments where Fe^{2+} is present are interfaces between anoxic and oxic conditions. Improved culturing techniques allowed the isolation of new Fe^{2+}-oxidizing bacteria under microaerophilic conditions at neutral pH. Ferric oxides may be enzymatically deposited by *Gallionella ferruginea* and non-enzymatically by *Leptothrix* sp., *Siderocapsa*, *Naumanniella*, *Ochrobium*,

Siderococcus, *Pedomicrobium*, *Herpetosyphon*, *Seliberia*, *Toxothrix*, *Acinetobacter*, and *Archangium*. Questions remain as to the extent of microbial involvement in specific processes of corrosion involving iron oxidation.

Other microorganisms in the genus of *Thiobacillus* are also responsible for oxidative corrosion. Because they oxidize sulfur compounds to sulfuric acid metabolically, the acid around the cells may attack metals and alloys as well as stone and concrete. *Thiobacillus* is the most common and *Thiobacillus ferrooxidans* oxidizes Fe^{2+} to Fe^{3+}, but the product limits growth of the organisms. SO_4^{2-} is required by the Fe-oxidizing system in *T. ferrooxidans* at the surface of the bacteria. The electrons removed from Fe^{2+} are passed onto periplasmic cytochrome *c*. The reduced cytochrome *c* binds to the outer plasmic membrane of the cell, allowing transport of electrons across the membrane to cytochrome oxidase located in the inner membrane. Recent findings also emphasize the importance of bacterial physiology and water chemistry as the most important determinants of microbial corrosion through generation of mutants defect of biofilm formation and measurement of electrochemical impedance spectroscopy (EIS).

Most microorganisms accumulate Fe^{3+} on their outer surface by reacting with acidic polymeric materials. Such mechanisms have important implications not only for corrosion of metals, but also for the accumulation of metals under natural conditions. *Aquaspirillum magnetotacticum* is capable of taking up complexed Fe^{3+} and transforms it into magnetite Fe_3O_4 by reduction and partial oxidation. The magnetite crystals are single-domain magnets and they play an important role in bacterial orientation to the two magnetic poles of the earth in natural environments. However, some non-magnetotactic bacteria can also form extracellular magnetites. The role of these bacteria has been articulated in carbon cycling in natural ecosystem but their role in metal corrosion is still not known. Recent studies indicated that microorganisms may also contribute to corrosion through electron shuttling process, but such mechanism must be confirmed to realize their importance and significance in the corrosion of metals.

Anaerobic Corrosion Processes

Oxygen is a relatively new invention by microorganisms and has altered the evolution history on Earth. Anaerobic microorganisms were the early colonizers and then survive the increasing concentration of oxygen by living in oxygen limited sediment and intestinal tracts of animals. The gelatinous matrix of a heterogeneous biofilm contains both oxic and anoxic zones, permitting both aerobic and anaerobic processes to take place simultaneously. In the absence of oxygen, anaerobic bacteria, including methanogens, SRBs, and acetogens, may actively participate in corrosion processes. Emphasis has traditionally been on SRBs and hydrogenases, in which the hydrogen on metal surfaces is consumed by microbial metabolism. Severe damage of metals by SRBs is common in oilfields and sewage systems. However, the involvement of SRBs and their hydrogenases in corrosion of mild steel is still controversial.

SRBs are among the most intensely investigated groups of microorganisms on their role in biological corrosion. Under anaerobic conditions, oxygen is not available to accept electrons produced. Instead, SO_4^{2-} or other compounds are used as electron acceptors under such conditions. Each type of electron acceptor is unique in the pathway of metabolism. When corrosion begins, the following reactions take place:

$$4Fe^0 \rightarrow 4Fe^{2+} + 8e^- \text{ (anodic reaction)} \quad (4)$$

$$8H_2O \rightarrow 8H^+ + 8OH^- \text{ (water dissociation)} \quad (5)$$

$$8H^+ + 8e^- \rightarrow 8H \text{ (adsorbed) (cathodic reaction)} \quad (6)$$

$$SO_4^{2-} + 8H \rightarrow S_2^- + 4H_2O \text{ (bacterial consumption)} \quad (7)$$

$$Fe^{2+} + S_2^- \rightarrow FeS\downarrow \text{ (corrosion products)} \quad (8)$$

$$4Fe + SO_4^{2-} + 4H_2O \rightarrow 3Fe(OH)_2\downarrow + FeS\downarrow + 2OH^- \quad (9)$$

von Wolzogen Kuhr and van der Vlugt suggested that the above set of reactions is caused by SRBs. This electrochemical generalization has been accepted and is still prevalent. During corrosion, the redox potential of the bacterial growth medium is −52 mV. After inoculation of SRBs into a corrosion testing cell, the overall internal resistance decreases from the initial value of 15 Ohms to approximately 1 Ohm, while the sterile cell actually shows an increase in resistance. Several phases of change in the electrical potential of steel can be observed after inoculation with SRBs. Before inoculation, the value is determined by the concentrations of hydrogen ions in the medium. A film of hydrogen molecules forms on surfaces of Fe and steel, inducing polarization. Immediately after inoculation, SRBs begin to grow to cause depolarization, resulting in a drop of 50 mV in the anodic direction. The SRBs by means of their hydrogenase system remove the adsorbed hydrogen, depolarizing the system. The overall process was described as depolarization, based on the theory that these bacteria remove hydrogen that accumulates on the surface of metals and alloys. The electron removal as a result of hydrogen utilization results in cathodic depolarization and forces more iron to be dissolved at the anode. A typical polarization curve is shown in Fig. 4 where both current density and polarization potential of the metal specimen are shown.

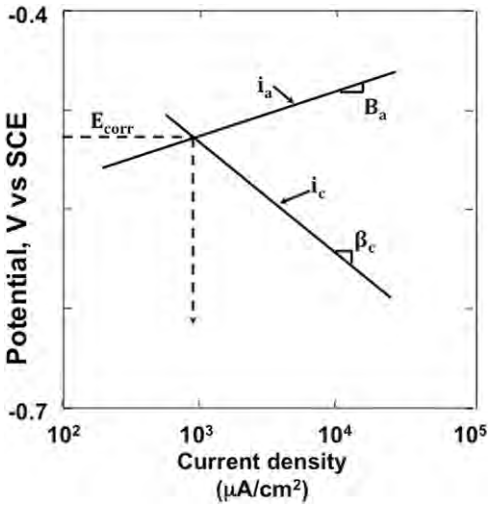

Fig. 4 A polarization curve showing the relationship between corrosion potential and current density during corrosion of metals.

Since direct removal of hydrogen from the surface is equivalent to lowering the activation energy for hydrogen removal by providing a depolarization reaction, the hydrogenase enzyme in many species of *Desulfovibrio* spp. is believed to be involved in this specific depolarization process. Under anaerobic conditions, particularly in the presence of SRB, SO_4^{2-} in the aqueous phase can be reduced to S^{2-} microbiologically. The biogenically produced S^{2-} reacts with released Fe^{2+} to form precipitate of FeS on metal surfaces. Controversy surrounding the mechanisms of corrosion includes more complex mechanisms involving both sulfide and phosphide, and processes related to hydrogenase activity. The addition of chemically prepared Fe_2S and fumarate as electron acceptors also depolarizes. However, higher rates are always observed in the presence of SRBs. As a result of the electrochemical reactions, the cathode always tends to be alkaline with an excess of OH^-. These hydroxyl groups also react with ferrous irons to form precipitates of hydroxy iron. Precipitated iron sulfites are frequently transformed into minerals, such as mackinawite, greigite, pyrrhotite, marcasite, and pyrite. Biogenic iron sulfides are identical to those produced by purely inorganic processes under identical conditions.

SRBs are divided into two physiological groups: one group utilizes lactate, pyruvate, or ethanol as carbon and energy sources, reducing sulfate to sulfide. Examples are *Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum*, and *Desulfobulbus*. The other group oxidizes fatty acids, particularly acetate, reducing sulfate to sulfide. This group includes *Desulfobacter*, *Desulfococcus*, *Desulfosarcina*, and *Desulfonema*. Some species of *Desulfovibrio* lack hydrogenase. For example, *Desulfovibrio desulfuricans* is hydrogenase negative and *Desulfovibrio salexigens* is positive. The rate of corrosion by these bacteria is correlated with their hydrogenase activity. Hydrogenase-negative SRBs were completely inactive in corrosion. Apparently, hydrogenase-positive organisms utilize cathodic hydrogen depolarizing the cathodic reaction, which controls the kinetics of corrosion. In contrast to this theory, ferrous sulfide FeS is suggested to be the primary catalyst for initiation of corrosion.

Corrosion by SRBs is mediated by the hydrogenase enzyme system. Available information suggests that a protective film of atomic hydrogen is formed on the surface of metals and this neutralizes any further electrochemical reaction, for example, initial exposure of metal to soil and water. However, SRBs disrupt this delicate balance by utilizing this hydrogen as an energy source, resulting in further oxidation of iron or metals to maintain the initial electrochemical steady state. In this process, biochemically, an iron hydrogenase takes an electron from the steel surface and the enzyme is thought to be located on the exterior surface of the outer membrane in order to utilize the surface-adsorbed hydrogen on metals. Hydrogenases have been found only in the periplasm, cytoplasmic membrane, and cytoplasm of the SRBs. *Desulfovibrio vulgaris* contains three hydrogenases, Fe, NiFe, and NiFeSe, in various quantities and in specific locations of the cells. Approximately 95% of the hydrogenase activity is found to be associated with periplasmic Fe hydrogenases and the remaining with cytoplasmic membrane-bound NiFe hydrogenase and a very small fraction by the NiFeSe cytoplasmic hydrogenase. To explain this, Fe^{2+} regulation may play an important role in controlling the synthesis of key proteins associated with out-membrane and periplasm for hydrogen generation. The depolarization theory suggests that bacteria, for example, SRBs, utilize the protective hydrogen layer on metal surfaces through the hydrogenase. This requires the hydrogenase to be localized on the outer membrane's exterior surface to access and utilize the hydrogen. Since only a very small fraction, that is, 1%, of periplasmic Fe hydrogenase activity is associated with the out-membrane, high molecular weight cytochrome in the out-membrane may be the initial electron transporter for hydrogen to be passed onto Fe hydrogenase, which is located next to the cytochrome but inward.

Other microorganisms should be noted for their potential role in anaerobic corrosion. They include methanogens, acetogens, thermophilic bacteria, archaea and obligate proton reducers. Among them, only methanogens have been implicated for their role in corrosion of metals. As all microorganisms have metabolic capability in utilizing hydrogen, it would not be a big surprise to confirm their role in corrosion of metals. More research work is needed to elucidate the role of these organisms in corrosion to further enrich our understanding of this subject.

Alternating Aerobic and Anaerobic Conditions

Corrosion under natural conditions may not be constantly anoxic or anaerobic over time, and most of the time alternation of aerobic and anaerobic conditions may take place. In this case, corrosion can be accelerated greatly when transformation of element sulfur is considered as sulfate is reduced to H_2S by SRBs under anaerobic conditions, but H_2S is oxidized to elemental S° and sulfate and other S species, which generates acidity. Acid attack is a significant process contributing to the corrosion of metals and also inorganic materials including stone and concrete. Since elemental S can be recycled further, such process is carried out continuously, resulting in dissolution of metals and significant deterioration of inorganic materials. Since constant oxic or anoxic conditions are rare in natural or industrial environments, alternating of the two conditions, depending on oxygen gradient and diffusivity in a specific environment and microbial activity, is common. Microbial corrosion under such conditions is quite complex, involving two generally different groups of microorganisms; an interface that serves as a transitional boundary of the two conditions can also be established. Such system is complicated for scientific investigation, and a recognized and generally agreed model system should be used for such research to obtain information on the relative contribution of each group of microorganisms. Resultant corrosion rates are often higher than those observed under either continuous oxic or anoxic conditions. Microbial activity reduces the oxygen level at the oxic–anoxic interface, facilitating anaerobic metabolism when nutrients are available. The corrosion products resulting from anaerobic processes, such as FeS, FeS_2, and SO, can be oxidized when free oxygen is available.

Stainless steel is more resistant to corrosion than mild steel because of the formation of a passivation film on the metal surfaces. Pitting corrosion of stainless steel is often initiated, in particular, in areas of welding and crevices or impurity as commonly observed in applications. Corrosion of stainless steel AISI 304 and AISI 316 is often associated with marine environments. During oxidation of reduced sulfur compounds more corrosive sulfides are produced under anoxic conditions through microbial physiological processes, causing cathodic reactions. The corrosion rate increases as the reduced and oxidized FeS concentrations increase. Corrosion rate of iron by H_2S is accelerated through formation of FeS, which decreases hydrogen concentration at the cathode site of the metal surface. In such a system, metal serves as an anode. During corrosion of iron and steel by SRB, a thin layer of $\sim$1 μm thickness is always observed as an adherent layer of tarnish and has been confirmed to be mackinawite (tetragonal FeS1−x). This layer becomes loosen when it grows. When Fe^{2+} concentration is low, the mackinawite changes to greigite. This transformation of minerals is only observed when microorganisms are involved. If Fe^{2+} concentration is high, mackinawite is accompanied by green rust, which is a ferrosoferric oxyhydroxide. Mackinawite under condition of SRB can be further transformed to greigite Fe_3O_4, smythite Fe9S11, and finally to pyrrhotite FeS1+x. Formation of these minerals also accelerates the equilibrium established between soluble fraction and metals.

Corrosion Via Microbial Exopolymeric Products

Bacteria are known to produce copious quantities of exopolymers, which are important because of their pathogenicity and survival in natural and artificial environments. Such organic materials, in addition to their ecosystem function in recruitment of larvae in aquatic environment and formation of mutualistic or pathogenic relationship with plants and animals, appear to be implicated in their role in corrosion process. These exopolymers consisting mostly of polysaccharides and proteins are acidic and contain reactive functional groups that bind metal ions tightly. These exopolymers play an important role in facilitating the adhesion of bacterial cells onto surfaces during their initial natural development. These materials have been found to be involved in severe corrosion of copper pipes of water supplies in buildings and hospitals after water stagnation for a period of time. Using surface analysis employing XPS, the organic fraction of the chemicals influences the electrochemical characteristics of metals through functionality-rich materials to complex metal ions from the surface of metal matrix, releasing them into aqueous solution in soluble and complexed forms. As a result, corrosion is initiated. Proteins in bacterial exopolymeric materials use their disulfide-rich bonds to induce corrosion very effectively through chemical reaction. Current understanding on the chemical constituents in the exopolymeric materials of any bacteria is still very rudimentary as the analysis has been on several different categories of chemicals, for example, polysaccharides, proteins, uronic acid, 2-keto-3-deoxyoctanoic acid, and deoxyribonucleic acid. Other chemicals, especially those at low concentration but significant role to corrosion, must be identified and evaluated.

Purified exopolymeric materials of *Deleya* (*Pseudomonas*) *marina* were used in an investigation. Proteins in the exopolymeric materials are responsible for reduction of molybdate MoO_4^{2-} under deaerated conditions through comparison with the same exopolymeric materials where proteins have been removed. The reduction process is thought to be due to the presence of proteins containing disulfide moieties. The resultant Mo^{5+} species can be reoxidized when molecular oxygen is available in the ambient environment. Such reduction process can also be observed on the surface of the MoO_4^{2-}-treated austenitic type 304 SS and formation of MoO_2 was detected. Though exposure to the exopolymer results in surface depletion of Fe and enrichment of Cr, rendering an increased hydration of Cr, corrosion resistance of the SS is not compromised by the exopolymer attachment on the surface. Since the duration of the experiment was short and the chemicals were purified, such factors must be taken into account when considering long-term exposure and life-span assessment. On the other hand, aluminum is very susceptible to corrosion by fungi and results have shown that fungal hyphae formed imprints into the aluminum alloys through acids produced during fungal metabolism. It is clear here that microorganisms play at least two roles in inducing corrosion, one by the exopolymeric materials synthesized and the other by direct attack through the acidic products released.

In addition to their genetic basis, production of bacterial exopolymers is affected by several environmental factors. Polysaccharide production by *Enterobacter aerogenes* is stimulated by the presence of Mg^{2+}, K^{+}, and Ca^{2+} ions in the culture media. Toxic metal ions, for example, Cr^{6+}, can also enhance the synthesis of polysaccharides and other exopolymeric materials in selective bacteria as a response to the chemical in the culture medium. In particular, synthesis of the exopolymeric materials is positively correlated with Cr^{6+} concentration in *Vogesella indigofera*. Specific chemical molecules involved in corrosion process need to be identified and the corrosion mechanisms by them shall be postulated and confirmed to further advance our understanding on the science of corrosion and then the controlling strategies against corrosion in various applications.

Microbial Hydrogen Embrittlement

Biologically produced hydrogen (H_2) has been more widely known in energy-related research and development in recent years. This process of release of hydrogen ion or molecular hydrogen by microorganisms is a common phenomenon associated with many different groups of microorganisms. However, the role of bacteria in embrittlement of metallic materials is not fully understood and the limited number of publications only postulates the potential importance of such biological process on material integrity. During the growth of bacteria, fermentation processes covert complex organic compounds to simpler organic acids and molecular hydrogen H_2. Such hydrogen, when generated and released on the surface of materials, can be absorbed into material matrix, causing polarization. Some bacteria, particularly the methanogens, sulfidogens, and acetogens, are also capable of hydrogen utilization.

Initially, a possible role for microbial hydrogen in hydrogen embrittlement of metal was proposed. Permeation of microbial hydrogen into metal was measured using a modified Devanathan cell with a mixed microbial community commonly found in nature, hydrogen production and consumption occur simultaneously. In the complex natural system, competition for hydrogen between microbial species determines the ability of hydrogen to permeate into metal matrices, causing crack initiation. In a pure culture of *Clostridium acetobutylicum* ATCC 824, hydrogen produced by bacteria can be absorbed by palladium foil was demonstrated through monitoring of current density during bacterial growth. Microbial hydrogen involved in material failure may be explained by two distinctively different hypotheses: pressure and surface energy changes. The kinetic nature of hydrogen embrittlement of cathodically charged mild steel is determined by the competition between diffusion and plasticity. The strength level and the susceptibility of the alloy are positively correlated. However, microstructures were also proposed to be the more critical determinant of material susceptibility. Hydrogen permeation increases the mobility of screw dislocations, but not the mobility of edge dislocations.

Corrosion by Other Microbial Metabolites

The microbial word is complex and majority of them are too small to be observed visually. Bacteria have a complex metabolic network to produce a diverse group of chemicals as their degradation intermediates. Some of the chemicals can accumulate and may be corrosive depending on the metabolic process. Fermentative microorganisms thriving under reduced availability of oxygen and in the presence of sufficient carbon source can produce simple organic acids, formic, acetic, butyric, and so on. In addition to these physiological and biochemical capabilities, microorganisms are also of producing extracellular electron shuttling molecules, which may have a significant role in the corrosion of metals.

Fungi produce highly corrosive metabolites including a wide range of organic acids, which corrode fuel tanks. They survive very well at the water–fuel interface and aqueous phase, metabolizing the fuel hydrocarbon as carbon and energy sources. Fungi are also capable of generating corrosive oxidants, including hydrogen peroxide. Degradation of protective coating is an issue because the underlying metals are dependent on the integrity of the polymer for corrosion control. Fungi cause deterioration of a wide array of polymeric materials, including electronic polyimides, packaging cellulose acetate, epoxy resins, and protective coatings.

Electron Shuttling in Corrosion

Electron transfer is the most fundamental and basic electrochemical process in the corrosion of metallic materials regardless of the participation of microorganisms. Such electron transfer process is facilitated by natural molecules, for example, humic substances, commonly found in the environment, both aquatic and terrestrial. More recently, results showed that bacterial quinone, an effective electron transferring molecule, can be excreted by the bacterium *Shewanella putrefaciens*, suggesting that extracellular process of electron transport may initiate and participate actively in the corrosion of metals. It would be interesting to further investigate the extent of such microbial electron transport process in corrosion and the mechanisms involved using candidate electron transfer chemical molecule.

Direct contact between bacterial cells and the metal oxide is required for the energy conservation process and such system may provide further insights in understanding the mechanisms involved in metal corrosion. For example, enzymatic reduction of Cr(VI) has been observed in a number of bacteria and the reduction of Cr(VI) is achieved by either soluble enzyme systems or the membrane-bound system. Membrane-associated chromate reductase activity was first observed in *Enterobacter cloacae* HO1 where

the insoluble form of reduced chromate precipitates was detected on the cell surfaces. In the presence of ascorbate reduced phenazine methosulfate (PMS) as electron donor, active chromate reduction has been shown in membrane vesicles of *E. cloacae* HO1. Membrane-associated constitutive enzyme that mediated the transfer of electrons from NADH to chromate was later elucidated. In the case of *Sh. putrefaciens* MR-1 chromate reductase activity is associated with the cytoplasmic membrane of anaerobically grown cells. Formate and NADH serve as electron donors for the reductase. No activity is observed when NADPH or L-lactate is provided as the electron donor. However, in *Pseudomonas putida*, unlike in *Sh. putrefaciens*, NADPH serves as an electron donor. The presence of soluble chromate reductase is possible in *Escherichia coli*. Cr(VI) reduction in another Gram-negative bacterium, *Pseudomonas* species CRB5, was mediated by a soluble enzyme in cytoplasm. In addition to Gram-negative bacteria, soluble chromate reductases have also been observed in Gram-positive strains. NADH is the preferred electron donor for the reduction of chromate by the soluble enzyme in *Bacillus coagulans*.

Enzymes capable of Cr(VI) reduction are often referred to as 'chromate reductases' in the literature. Several bacterial Cr(VI) reductases, some conferring resistance to chromate, have been characterized. These enzymes commonly show a NADH:flavin oxidoreductase activity and use Cr(VI) as electron acceptor. The ability to reduce chromate may be a secondary function for Cr(VI) reductases, which have a primary role other than Cr(VI) reduction. This is likely true as the gene sequences available from GenBank indicate that the *P. putida* chromate reductase is a quinone reductase (AF375641) that has a bound flavin and *Pyrus ambigua* ChrR is a flavin reductase (D83142). The nitroreductases NfsA/NfsB from *Vibrio harveyi* possess a nitrofurazone nitroreductase as primary activity and a Cr(VI) reductase activity as a secondary function. Similarly, ferric reductase FerB from *Paracoccus denitrificans* uses both Fe(III)-nitrilotriacetate and Cr(VI) as substrates. These secondary functions may be related to the bacterial enzymatic adaptation as a result of the relatively recent increase of Cr(VI) in the environment due to anthropogenic activities.

YieF is a flavoprotein containing the FMN cofactor and the enzyme is able to reduce chromate in vitro. YieF possesses quinone reductase activity, which appears to guard against oxidative stress by preventing redox cycling of quinones, which would otherwise generate ROS, and by maintaining a pool of reduced quinone in the cell that is able to quench ROS directly. The quinone reductase activity of YieF is likely the primary biological role of this enzyme. ChrR of *P. putida* is the currently best-studied Cr(VI) reductase. During Cr(VI) reduction, ChrR shows a quinone reductase activity that generates a flavin semiquinone. Cr(VI) is reduced to Cr(V) by ChrR, previously reduced by NADH; Cr(V) is next converted to Cr(III) by diverse biomolecules generating reactive oxygen species (ROS). ROS may be eliminated by alternative mechanisms (i.e., catalases or peroxidases) or by the additional function of ChrR. ChrR in a reduced status may reduce quinines (such as Vitamin K or Coenzyme Q), which may then detoxify previously formed ROS. The soluble bacterial quinone reductase has the ability to reduce a variety of quinone substrates. The above model may provide a unique system for verification of electron shuttling in corrosion of metals.

Prevention and Control

A common practice to eradicate the initiation and development of microbial biofilm on the surfaces of a wide range of materials in engineering systems of industries is through introduction of biocides. Chlorination and glutaldehyde are routinely used as a preventive measure in water cooling system and oil fields. Chlorination yields secondary halogenated by-products, resulting in environmentally unacceptable residues of chemicals. In addition, biofilm bacteria are distinctively different from the planktonic ones in their resistance to toxic chemicals and environmental shock. In addition, biocidal effect is compromised with biofilm formed on surfaces of materials. However, this has not been taken into account for testing the effectiveness of biocides in different industries against biofilm bacteria.

Biofilm structures are advantageous for the microorganisms because diffusion is limited to prevent penetration of disinfectants and to facilitate more effective exchange of genetic materials between members. Organic biocides, used to prevent bacterial growth in industrial systems for an extended period of time, enrich a population capable of biocide resistance by genetic modification in bacteria, for example, by recruitment of extracellular DNA. However, a new generation of environmentally acceptable biocides is also available, and their application and effectiveness have to be tested over time to provide further information on effectiveness. It is probable that some of these biocides are capable of either preventing biofilm formation or killing the microorganisms in formed biofilms, but it is unrealistic to expect a magic biocide for eradicating microbial biofilms over a long period of time.

Corrosion protective coatings also have wide applications because of the development of metallic materials and susceptibility to both environmental and microbiological corrosion. Polymeric coatings are designed to prevent contact of the underlying materials with corrosive media and microorganisms. However, microbial degradation of coatings may accelerate and severely damage the underlying metals. A typical example is the corrosion of underground storage tanks and oil tanks of airplanes and ship. Natural populations of bacteria form microbial biofilms on surfaces of coating materials, including epoxy and polyamide primers and aliphatic polyurethanes. Surprisingly, the addition of biocide diiodomethyl-*p*-tolylsulfone in polyurethane coatings did not inhibit bacterial attachment or growth effectively due to development of biofilm and bacterial resistance.

Metal protective coatings are physical barrier separating metal surface from water and microorganisms so that corrosion can be inhibited. Polyurethane coating is commonly used and polyurethane-degrading microorganisms including *Fusarium solani*, *Curvularia senegalensis*, *Aureobasidium pullulans*, and *Cladosporidium* sp. through esterase activity detected with *C. senegalensis* are also known. A number of bacteria, including four strains of *Acinetobacter calcoaceticus*, *Arthrobacter globiformis*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *P. putida*, and two other *Pseudomonas*-like species, are capable of degrading polyurethane. In addition, *Pseudomonas chlororaphis* encodes a lipase responsible for degradation. Using EIS, both primers and aliphatic polyurethane coatings

were shown for their biodegradation by bacteria and fungi, indicating that primers are more susceptible to degradation than polyurethane top coating. The degradation process is similar to mechanisms as shown for electronic insulation polyimides. Aliphatic polyurethane-degrading bacteria can be isolated and one of them is *Rhodococcus globerulus* P1 base on a 16S rRNA sequence.

Microbial growth and propagation on material surfaces can be controlled efficiently by physical and chemical manipulations of the material types and the artificial environments. Surface engineering so to prevent the attachment by and susceptibility to microorganisms can be used to limit occurrence of biodeterioration. As a control measure, lowering humidity is a very effective means to slow down the growth of microorganisms on surfaces in an enclosed environment and prevention against potential contamination can extend the life time of the materials.

Biocides are commonly applied in repairing, cleaning, and maintenance of artworks. Chlorine, iodine, and other organic biocidal compounds are used widely and routinely in controlling biofilms that cause corrosion and deterioration of a wide range of materials in industries and for conservation of art. These chemicals have been shown to be ineffective in killing biofilm bacteria. In addition to their environmental unacceptability, most of the time because of toxicity, biocides induce the development of biofilms that are highly resistant to the levels of chlorine normally utilized to prevent biocorrosion. Organic biocides, used to prevent bacterial growth in industrial systems, may selectively enrich populations of microorganisms capable of biocide resistance. No solution to these problems is currently available and alternative biocides have been screened from natural products. Current research by materials scientists focuses on the prevention of adhesion of corrosive microorganisms to surfaces through surface treatments and modification.

Since bacteria are capable of forming biofilms on surfaces of materials, future tests should focus on the dynamics of biofilm and quantification than descriptively show biofilms of scanning electron micrographs. In particular, tests for assaying efficacy of biocides should be conducted based on biofilm condition than for liquid culture efficacy. This major discrepancy has not been resolved fully. Because biofilm bacteria are more resistant to antibiotics and biocides, tests based on planktonic cells are not truly representative of their actual conditions on surfaces of materials. New initiative is needed for innovative methodology to assess biocidal effects and mechanisms using surface-oriented assays similar to the application conditions.

Conclusions

Under both aerobic and anaerobic conditions, microorganisms can directly or indirectly participate in the electrochemical process of metal corrosion. Under anaerobic conditions bacteria corrode metals by cathodic depolarization and the formation of FeS, or by consuming hydrogen produced by polarization. Other corrosion mechanisms, including microbial hydrogen embrittlement, complexation of metals from matrices by microbial exopolymeric materials, or extracellular electron respiration, have also been implicated in recent years. It is clear that a better understanding of the complexity of interactions between microflora and metals leading to corrosion can be facilitated by the elucidation of specific mechanisms involved. Molecular biology-based investigation coupled with electrochemistry and chemical biology should permit a better knowledge of the fundamental biochemical processes, genes and the role of microorganisms in corrosion of metals. Equipped with basic knowledge, it is feasible to formulate strategies for controlling biofilms and MIC in industrial and other environments. Use of biocides cannot solve the MIC problems, but spreading of antibiotic-resistant microorganisms is a much more serious to be addressed by the professionals and also the general public.

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CRISPR–Cas9

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Introduction

Forward and reverse genetics have provided a wealth of information about the organization and function of biological systems. For decades, genome modification has relied on transgenesis, spontaneous gene replacement by homologous recombination (HR), and chemically induced or spontaneous generation of mutations. Although these approaches greatly contribute to advancing our knowledge of biological systems, they have some limitations. For example, spontaneous gene replacement and chemically induced mutations occur at random, and, in some cases, their frequency of occurrence is not sufficient to conduct forward genetic studies in a timely manner. Transgenesis requires the generation of large and complex DNA constructs, and their integration within a genome occurs at random, which often affects transgene expression. Moreover, transgenesis cannot be used to modify a specific location within a genome. Although gene replacement by HR is more precise than transgenesis and allows the modification of endogenous genes, HR is an infrequent event in cells. Consequently, the process requires the generation of large and complex DNA constructs with sequence homology to the target region and selectable markers to facilitate clone identification through positive and negative selections. Further, this technique is amenable to only a subset of cells in which HR is effective and in model organisms for which stem cells are available. The process is not always reliable and several rounds of blastocyst injections and transfer of blastocysts are required to generate mice that can transmit the mutant allele to their offspring.

Early studies in yeast and mammalian cells demonstrated that insertion of a DNA double-strand break (DSB) at a specific locus in a genome increases the frequency of gene replacement at that locus by several orders of magnitude compared with spontaneous gene replacement (Choulika et al., 1995; Cohen-Tannoudji et al., 1998; Donoho et al., 1998; Smih et al., 1995). Building upon these findings, scientists have developed targetable nucleases for genome engineering. These enzymes can introduce DNA DSBs within genomes with good accuracy.

Meganucleases, the first generation of targetable nucleases, were mainly used for proof-of-concept studies on HR in eukaryotic cells (Choulika et al., 1995; Cohen-Tannoudji et al., 1998; Donoho et al., 1998; Smih et al., 1995). Meganucleases recognize and cleave particularly long DNA sequences, which gives them an exquisite degree of specificity. However, the long DNA sequences recognized by meganucleases are practically absent from genomes of most species, thus impeding the use of these enzymes as a genome-editing tool.

In search of a more versatile genome-editing tool, scientists turned to zinc-finger nucleases (ZFNs) (Bibikova et al., 2003) and transcription activator-like effector (TALE) nucleases (TALENs) (Christian et al., 2010). These enzymes use naturally occurring DNA-binding domains fused to the non-specific endonuclease *Fok*I (Li et al., 1992; Looney et al., 1989; Nwankwo and Wilson, 1987). Arrays of TALEs or zinc-finger proteins can recognize virtually any DNA sequence and can thus be programmed to target virtually any given DNA sequence. Although these protein domains provide the long-awaited versatility, programming and reprogramming of these enzymes to target other sites is difficult due to the repetitive nature of TALE DNA-binding domains and the complexity of ZFP engineering.

Adaptation of the microbial clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9) immunity into a genome-editing tool finally provided the much-sought versatility and simplicity of programming while retaining efficacy and specificity (Hsu et al., 2013; Jinek et al., 2012; Mali et al., 2013b). In the CRISPR–Cas9 system, a large DNA endonuclease guided by a small RNA transcript recognizes and cleaves double-stranded DNA. Unlike ZFNs and TALENs that rely on protein–DNA interactions for target recognition, the CRISPR–Cas9 system uses conventional RNA–DNA base pairing. Hence, in contrast to the complete reengineering of nucleases required for ZFNs and TALENs reprogramming, the simple modification of the RNA sequence is sufficient to redirect the endonuclease Cas9.

In this article, I describe adaptation and implementation of the CRISPR–Cas9 technology for genome editing and regulation in mammalian systems. I also summarize the applications of CRISPR–Cas9 modalities in agriculture, biotechnology, biology, biomedical research, and medicine.

CRISPR–Cas9 Genome Editing

Class 2 Type II CRISPR-Cas9 Adaptive Immunity

Several CRISPR–Cas9 systems have been adapted for editing and regulation of genomes, most being derived from class 2 type II or type V bacterial adaptive immune systems (Makarova and Koonin, 2015). These systems function by incorporating DNA sequences from invading bacteriophages for their reuse by large DNA endonucleases as targeting devices to direct the selective degradation of invading DNA upon subsequent infections.

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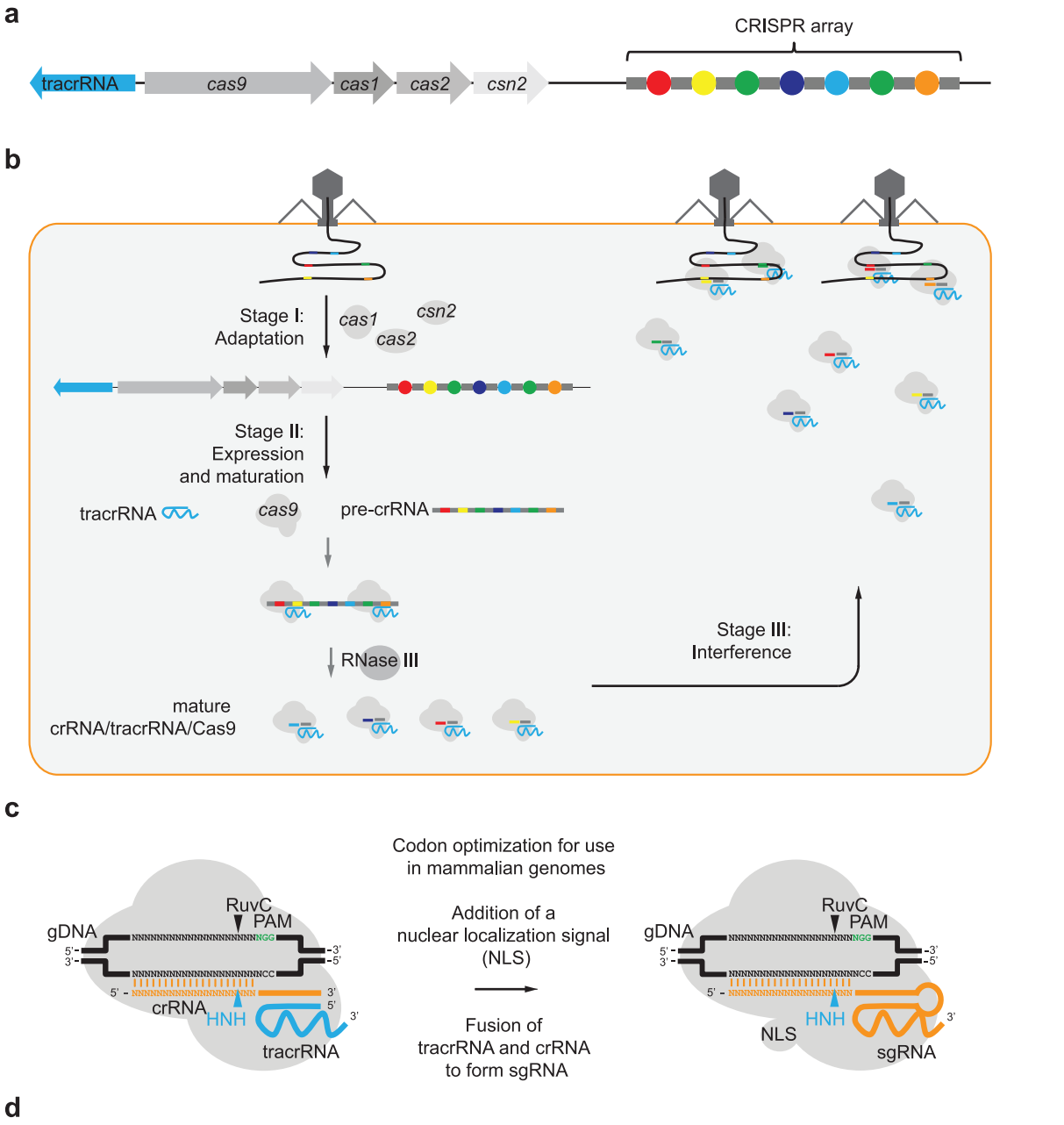

Fig. 1 Class 2 type II CRISPR–Cas9 from *Streptococcus pyogenes* and its adaption as a genome-editing tool. (a) The type II CRIPSR–Cas9 operon from *S. pyogenes* contains 4 genes (gray arrows), a CRISPR array containing repeat elements (gray rectangles) regularly interspaced with spacer elements (colored circles), and sequences encoding the transactivating crRNA transcript (tracrRNA, blue arrow). (b) Schematic representation of type II CRISPR–*Sp*Cas9 adaptive immunity. CRISPR–Cas9 immunity is divided into 3 stages: adaptation (stage I), expression and maturation (stage II), and interference (stage III). In the adaptation stage, Cas1, Cas2, and Csn2 coordinate the excision and integration of small DNA fragments from invading DNA molecules (bacteriophages or plasmids) into the CRISPR array. In the expression stage, the CRISPR array, tracrRNA, and Cas9 are expressed, form a ribonucleoprotein complex, and precrRNA is further processed into mature crRNAs by RNase III. In the interference stage, the mature tripartite ribonucleoprotein complex targets invading RNA molecules for degradation upon subsequent infection. (c) Adaptation of type II CRISPR–*Sp*Cas9 for genome editing. Some modifications have been made to *Sp*Cas9 and RNA transcripts for use as genome-editing tools. First, tracrRNA and crRNA have been linked by an artificial loop to form the single-guide RNA (sgRNA) molecule. Second, *Sp*Cas9 cDNA has been optimized for mammalian codon usage. Third, a nuclear localization signal has been added to *Sp*Cas9 to facilitate its translocation to the nucleus of eukaryotic cells. Cloud-like gray shape, Cas9; black, genomic DNA; orange crRNA (left) or sgRNA (right); blue, tracrRNA; green, PAM sequence; black arrowhead, RuvC-like endonuclease cleavage site; and blue arrowhead, HNH cleavage site. (d) Sequence of the sgRNA transcript used by *Sp*Cas9. Nucleotides shown in red, orange, and blue are complementary and forms hairpins. N represents any nucleotide. The 5′-most end of the sgRNA, represented by Ns, corresponds to the guide sequence.

Like most genetic regulatory systems found in bacteria, CRISPR–Cas9 systems are organized in operons (Makarova and Koonin, 2015). The type II CRISPR–Cas9 operon from *Streptococcus pyogenes*, which is the prototypical CRISPR–Cas9 system discussed in this article, comprises 4 genes *Cas1*, *Cas2*, *Csn2*, and *Cas9* (or *SpCas9* for *S. pyogenes Cas9*); a CRISPR array; and DNA sequences encoding a short RNA transcript called trans-activating CRISPR RNA (tracrRNA) (Fig. 1(a)). Cas1, Cas2, and Csn2 are involved in the adaptation stage (stage I), whereas Cas9, tracrRNA, and the CRISPR array are mainly involved in expression and maturation and interference stages (stages II and III, respectively) of adaptive immunity (Fig. 1(b)). In the adaptation stage, Cas1, Cas2, and Csn2 regulate the incorporation of small foreign DNA fragments derived from an invading bacteriophage or plasmid into the genome of host cells. These fragments, called spacers, are incorporated into the CRISPR array, which is intercalated by short repeat elements (Amitai and Sorek, 2016). In the expression stage, both tracrRNA and pre-crRNA are expressed and pre-crRNA is processed to mature crRNA by the joint action of Cas9, tracrRNA, and RNase III (a ribonuclease that processes double-stranded RNA molecules) (Deltcheva et al., 2011). At this stage, Cas9 is associated with both RNA molecules, and the tripartite ribonucleoprotein complex can recognize and degrade invading DNA molecules.

Target specificity for CRIPSR–Cas9 adaptive immunity is provided by the mature crRNA, more specifically via the 5′-most 20-nucleotide (nt) segment of the RNA transcript (Jinek et al., 2012). This sequence forms a RNA–DNA hybrid with its cognate genomic DNA sequence, called protospacer element, according to Watson–Crick base-pairing rules. The 3′ end of the crRNA contains the repeat element of the CRISPR array and associates with the 5′-most end segment of the tracrRNA molecule. The RNA–RNA duplex binds Cas9 via the scaffolding region of tracrRNA.

Cas9 possesses 2 distinct endonuclease domains: (1) a RuvC-like domain, which cleaves the DNA strand displaced by formation of the RNA–DNA hybrid, and (2) an HNH domain, which cleaves the annealed strand. Cleavage of target DNA also requires the presence of a specific DNA sequence located at the 3′ end of the protospacer element. This sequence, called protospacer adjacent motif (PAM), often differs among species. For example, Cas9 from *S. pyogenes* or *S. mutans* requires the trinucleotide 5′-NGG-3′ (where N represents any nucleotide) for maximal activity, whereas Cas9 from *C. jejuni* requires the heptanucleotide sequence 5′NNNNACA-3′. Table 1 summarizes PAM requirements for Cas9 endonucleases from different species. Cas9 from different species also differs in size and crRNA/tracrRNA-binding properties and cleaves DNA at various locations within the protospacer element.

Adaptation of CRISPR–Cas9 for Genome Editing

For the purpose of genome editing, the CRISPR–*Sp*Cas9 system has been simplified and requires only 2 (and not 3) components (Fig. 1(c) and (d)) (Jinek et al., 2012). The tracrRNA and crRNA have been fused to form a 96-nt single-guide RNA (sgRNA) molecule (Fig. 1(d)). The first 20 nt correspond to the spacer element, followed by 30 nt that correspond to the direct strand of the repeat element and the 5′ end of tracrRNA, which is complementary to the repeat element, linked by a 4-nt artificial hairpin. The last 46 nt correspond to the scaffolding portion of tracrRNA.

Modifications have also made to *Sp*Cas9 (Fig. 1(c)). It has been optimized for codon usage in mammalian cells and to include a nuclear localization signal (NLS) (Hsu et al., 2013; Mali et al., 2013b). The NLS of Cas9 facilitates transport of the endonuclease to the nucleus of the target cell. In this system, simple modification of the first 20 nt of the sgRNA is sufficient to reprogram Cas9 to target another locus within any given genome (Jinek et al., 2012).

Efficacy and Specificity

The efficacy of CRISPR–Cas9 systems to cleave DNA is equal or superior to that of other gene editing platforms. However, unlike ZFNs and TALENs that can target virtually any location within a genome, CRISPR–Cas9 systems require the presence of PAM

Table 1 PAM requirements for Cas9 endonucleases from various bacterial species

Endonuclease	*Species*	*PAM*	*References*
Naturally occurring Cas9	*Streptococcus pyogenes*	NGG	Hsu et al. (2013)
	Streptococcus mutans	NGG	van der Ploeg (2009)
	Staphylococcus aureus	NNGGGT	Ran et al. (2015)
		NNGAAT	
		NNGAGT	
	Streptococcus thermophilus (CRISPR3)	NGGNG	Fonfara et al. (2014)
	Streptococcus thermophilus (CRISPR1)	NNAAAAW	Fonfara et al. (2014)
	Campylobacter jejuni	NNNNACA	Fonfara et al. (2014)
	Neisseria meningitidis	NNNNGATT	
	Pasteurella multocida	GNNNCNNA	Fonfara et al. (2014)
	Francisella novicida	NG	Fonfara et al. (2014)
	Treponema denticola	NAAAAN	Esvelt et al. (2013)
Engineered Cas9	*Streptococcus pyogenes* (VQR)	NGAG	Kleinstiver et al. (2015)
	Streptococcus pyogenes (VRER)	NGCG	Kleinstiver et al. (2015)

Note: N represents any nucleotide.

sequences, which are specific nucleotide sequences at the 3′ end of target sequences. Although this was originally viewed as a major limitation for the use of CRISPR–Cas9 systems for genome editing, it was later shown *Sp*Cas9 PAM sequences (5′NGG-3′) occur at every 10 nt in the mouse genome and theoretically at every 8 nt in other genomes (including the human genome), supporting that *Sp*Cas9 can target virtually any location within genomes (Pelletier et al., 2015). Moreover, several other CRISPR–Cas9 systems, including engineered ones, have been adapted for genome editing (Table 1). These CRISPR–Cas9 systems have distinct PAM sequence requirements, which expand their targeting capabilities. In addition, sequence composition of the sgRNA can also influence Cas9 activity. Nucleotide composition, in particular the nature of the 3′-most nucleotide of the guide sequence, can have different effects on the cleavage efficacy of Cas9: the presence of a guanine can increase Cas9 activity whereas that of a thymidine can have the opposite effect (Chari et al., 2015).

The most important limitation to using CRIPR–Cas9 systems as genome-editing tools is that they can target sites other than those for which they were designed. Studies show that *Sp*Cas9 can tolerate the presence of several nucleotide mismatches between the sgRNA and its genomic target (Hsu et al., 2013; Jinek et al., 2012). Although bioinformatics tools have been developed to design and predict sgRNA on- and off-target sites, it is still difficult to identify all potential target sites of an sgRNA by using in silico models. To minimize off-targeting, we and others have developed single-guide selection procedures based on the singularity of guide sequences within a genome. Our procedure, which has been detailed previously (Pelletier et al., 2015), uses the algorithm developed by the Center for Genome Engineering, Institute for Basic Science, Korea (see Relevant Websites section). Our strategy provides a comprehensive list of all potential off-target sites associated with all possible guides for a given locus, which facilitates the selection. Approximately 70 mouse lines and several mouse and human cell lines have been designed and generated under my guidance by using this procedure (Gingras et al., 2017; Ippagunta et al., 2016; Karki et al., 2016; Lin et al., 2016; Man et al., 2017; Martinez et al., 2015; Pelletier et al., 2015; Van de Velde et al., 2016). In most cases, we have been able to avoid off-target cleavage.

In addition to the careful selection of guide sequences, several other approaches have been developed to limit Cas9 activity at undesired sites. These include the use of paired DNA nickases (Fig. 2(a)), shorter sgRNA molecules, inducible Cas9 systems, and self-inactivating Cas9 plasmids or engineered Cas9 with increased specificity (Fu et al., 2014; Guilinger et al., 2014; Mali et al., 2013a; Moore et al., 2015; Nihongaki et al., 2015a; Nihongaki et al., 2015b; Ran et al., 2015; Slaymaker et al., 2016). Paired nickases (*Sp*Cas9–D10A), in which the HNH domain has been inactivated, or the d*Sp*Cas9–Fok1 fusion, which employs a catalytically inactive Cas9 fused to the non-specific endonuclease Fok1, which function as obligate dimer (Fig. 2(b)), provide increased specificity by targeting 2 juxtaposed genomic sequences oriented in opposite directions. The chances of having the same 2 target sequences anywhere else in the genome are quasi inexistent, thereby reducing the risk of off-target cleavage. Shorter sgRNAs are thought to increase target specificity by reducing the affinity of sgRNAs for their cognate on- and off-target sites. Shortening the duration of Cas9 activity by using inducible systems or self-inactivating plasmids or through direct delivery of DNA by plasmid-free systems (e.g., RNA transcript or RNA–protein complexes; see below) can reduce off-target activity. Cas9 systems with improved specificity have also been engineered by substituting some amino acids that have nonspecific interactions with the backbone of the DNA target.

CRISPR–Cas9 Plasmids and Delivery Methods

Several plasmids encoding various components of CRISPR–Cas9 have been developed for genome editing and other applications. These include conventional and viral vectors encoding both Cas9, generally under the control of strong promoters such as CBh (a hybrid form of the chicken b-actin [CBA] promoter), or sgRNA under the control of the U6 promoter. These vectors also generally express selection markers in the form of genes conferring resistance to chemical drugs or fused with fluorescent proteins. CRISPR reagents can also be produced in vitro, without the need of a vector, for physical delivery of reagents. Cas9, in the form of mRNA transcripts or protein, and sgRNA can be produced in vitro and delivered by microinjection and other approaches (see below). Plasmids encoding individual components of these systems have been developed, and some are available at Addgene (see Relevant Websites section).

Viral or non-viral methods can be used to deliver CRISPR–Cas9 reagents. Viral delivery systems use viral vectors to package various components of the genome-editing system. Several viral vector platforms have been used to deliver CRISPR–Cas9 components, such as adenoviruses, adeno-associated viruses, retroviruses (in particular lentiviruses and integrase-deficient lentiviruses), and baculoviruses. In contrast, non-viral delivery systems use physical and/or chemical methods to deliver components. Non-viral delivery systems include electroporation, chemical transfection, mechanical stress transduction, or microinjection of ribonucleoprotein particles or plasmids encoding various components of systems. Cell-penetrating peptides genetically or chemically fused to Cas9 are also used to deliver Cas9 via endocytic pathways. Both viral and non-viral delivery methods have been successfully used in vivo, ex vivo, and in vitro.

Basic Principles of Genome Editing Using CRISPR–Cas9 Technology

It has long been recognized that insertion of DNA DSBs increases the gene replacement frequency in mammalian cells (Choulika et al., 1995; Cohen-Tannoudji et al., 1998; Donoho et al., 1998; Smih et al., 1995). Insertion of DNA DSBs triggers DNA repair mechanisms within cells, of which HR and nonhomologous end joining (NHEJ) are the most common (Fig. 3) (Chapman et al., 2012). DNA repair via the NHEJ pathway often results in the insertion or deletion of relatively small DNA segments or nucleotide substitutions. Repair via HR results in proper rearrangement of the DSB and uses the sister chromatid as the repair template. These pathways compete with each other within cells, and in most cells NHEJ is the more prevalent repair pathway.

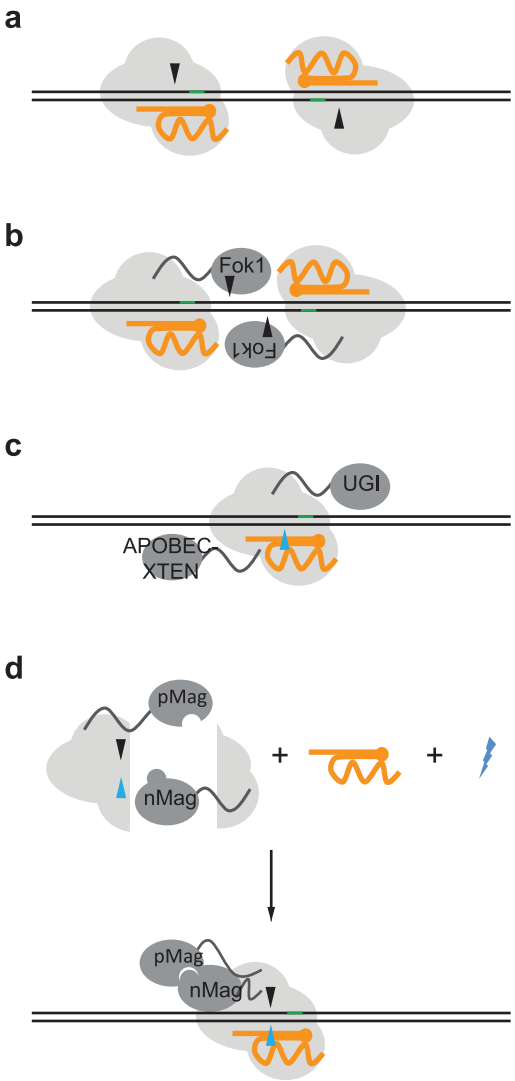

Fig. 2 CRISPR–*Sp*Cas9 modalities for genome editing. (a) Genome editing using a paired *Sp*Cas9–D10A. The D10A mutation inhibits the HNH endonuclease activity of *Sp*Cas9. A paired *Sp*Cas9–D10A recognizing the opposite strand of a locus can be used to introduce scattered DNA double-strand breaks (DSBs). The use paired nickases can reduce off-target cleavage. (b) Genome editing using a pair of catalytically inactive Cas9 fused to the endonuclease *FokI*. *FokI* functions as obligate dimer. Targeting d*Sp*Cas9–*FokI* enzymes on opposite strands (head to head) introduces scattered DNA DSBs at the locus, which can serve as a substrate for DNA repair pathways. (c) Base editing using the RuvC-like–deficient but HNH-competent APOBEC–XTEN–RuvC–*Sp*Cas9–UGI fusion. APOBEC–XTEN catalyzes the deamination of a uracil to a cytidine. The substitution is stabilized by the presence of a UGI. Genome editing employing base editing does not require the insertion of DNA DSBs. (d) Genome editing using inducible split *Sp*Cas9. A split Cas9 fused to the photoactivatable system Magnet (pMag and nMAg). Blue light triggers dimerization of the split Cas9. A similar system in which FK506-binding protein 12 (FKBP) and FKBP–rapamycin binding (FRB) domains have been fused to split Cas9 has also been developed. In this case, dimerization is triggered by rapamycin. Light-gray cloud-like shape, *Sp*Cas9; orange traces, sgRNAs; green lines, PAM sequences; dark gray shapes, functional entities (*FokI*, a non-specific endonuclease; UGI, uracil glycosylase inhibitor; APOBEC–XTEN, cytidine deaminase); black arrowhead, RuvC-like endonuclease cleavage site; blue arrowhead, HNH endonuclease domain; black lines, genomic DNA locus; pMag, positive magnet; nMAG, negative magnet.

Specific mutations within a genome are usually introduced by co-administration of exogenous DNA templates containing the desired mutations. This process, generally referred to as homology-directed repair (HDR), uses single-stranded DNA (ssDNA) or double-stranded DNA molecules (linear or circular) as a template for repair. Introducing mutations by using double-stranded DNA templates likely uses the HR pathway (Chapman et al., 2012), and recent evidence suggests that ssDNA editing may involve the Fanconi anemia DNA repair pathway (see Relevant Websites section), which involves components of the HR pathway, nucleotide excision repair pathway, and mutagenic translesion synthesis (Moldovan and D'Andrea, 2009).

Genome-Editing Strategies

Virtually any type of genomic alteration can be introduced through CRISPR–Cas9 technology. A gene can be inactivated by inserting a single DNA break within a critical exon. Repair of the break by the NHEJ pathway may result in insertion or deletion of

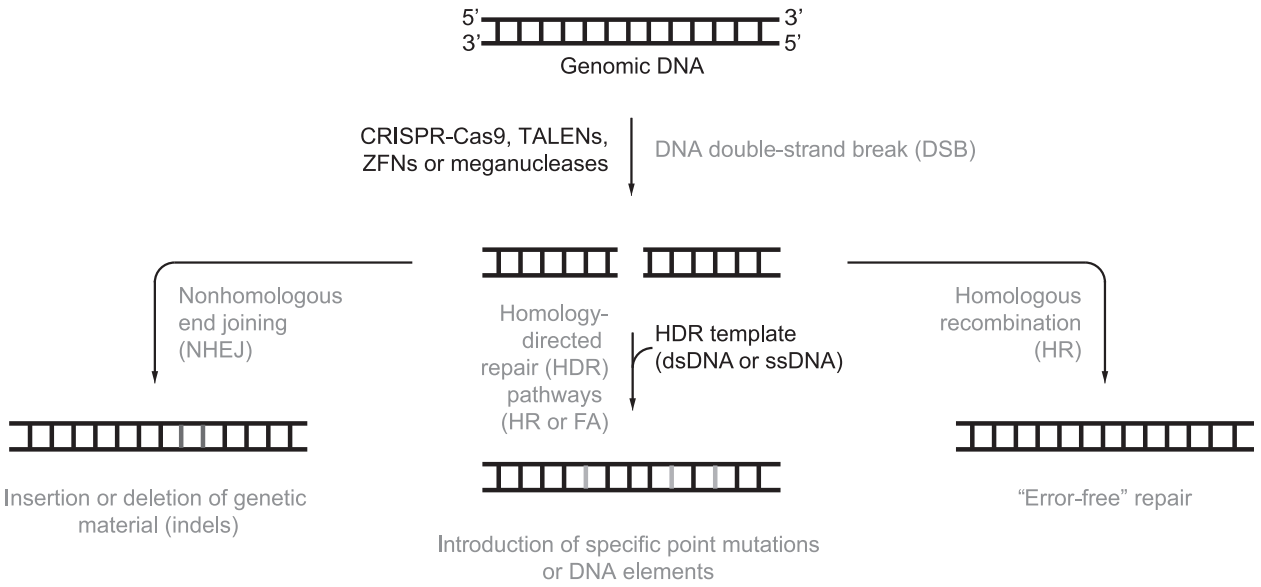

Fig. 3 DNA repair pathways engaged by targetable nucleases. Homologous recombination (HR) and nonhomologous end joining (NHEJ) are the most common DNA DSB repair pathways. Resolution of DNA DSBs by the error-prone NHEJ pathway usually results in insertion or deletion of genetic material caused by misalignment of resected strands during the repair process. Resolution of the DSB by HR uses the sister chromatid as a repair template and is thought to be error free. Specific mutations or large DNA elements can be inserted by co-administration of a homology directed repair (HDR) template. HDR templates are usually single-stranded (ss) or double-stranded (ds) DNA molecules that have sequence homology to the target locus. Repair pathways involved in this process depend on the type of repair templates used. ssDNA repair templates may be used by the Fanconi anemia repair pathway, whereas dsDNA templates may be used by the HR pathway.

nucleotides, which causes a nonsense or frameshift mutation, leading to the formation of a premature stop codon (Fig. 4(a)). mRNA transcripts with premature stop codons are often recognized as defective and are degraded via the nonsense mRNA decay pathway (Popp and Maquat, 2016). A shortcoming of this strategy is that break repair by the NHEJ pathway can also result in the insertion of missense or silent mutations that do not disrupt gene function or potentially provide adverse properties to the encoded protein. This limitation can be resolved by co-administering a repair template that contains one or more stop codons in one or all reading frames (Martinez et al., 2015).

Another strategy for gene inactivation is partial or complete removal of a gene, assuming that the targeted region does not contain other genes such as microRNAs or transfer RNAs (tRNAs). Large chromosomal deletions can be achieved by introducing 2 DNA DSBs in the coding or noncoding regions of a gene (Fig. 4(b)). Break repair can result in complete removal of a gene located between the 2 breaks. Importantly, introduction of 2 DNA DSBs can also result in inversion of the intervening region, which might have unpredictable consequences (Fig. 4(b)). This strategy has been used to not only inactivate genes in model organisms and cell lines (Gingras et al., 2017; Karki et al., 2016; Man et al., 2017; Van de Velde et al., 2016) but also remove regulatory elements termed microRNAs, including those located in introns of genes, tRNAs, long noncoding RNAs, and gene clusters of the same family (Fig. 4(c)) (Van de Velde et al., 2016). This strategy can also be used to inactivate splice variants of a gene without affecting other isoforms. We are currently using this strategy to remove specific domain(s) of proteins in vivo. HDR templates are co-administered with the RNA-guided nuclease to promote specific recombination events.

Conditional alleles, which allow the spatial and/or temporal removal of genes, can also be generated by introducing 2 DNA DSBs and co-administering DNA repair templates containing recombinase recognition sites (e.g., *LoxP*, *FRT*, *Lox511*) flanked by DNA sequences homologous to the target site (Fig. 4(b)). In addition to inserting *loxP* sites independently, other methods to insert loxP sites include the insertion of 2 DSBs flanking a single critical exon and repair of the break using a large DNA molecule (single stranded or double stranded) containing the targeted exon flanked by 2 loxP sites and sequences homologous to the target site (Ippagunta et al., 2016).

Genes can also be conditionally inactivated by regulated expression of Cas9 by either using plasmids encoding Cas9 under the control of an inducible promoter or by expression of a photoactivatable (or chemically induced) split Cas9 or nuclease-deficient Cas9 (dCas9) fused to transcriptional activators or repressors (Fig. 2(d)). Chromosomal translocations, a frequent rearrangement in cancer cells, can be generated by inserting 2 DNA DSBs located on 2 distinct chromosomes. Chromosomal translocations generated using this strategy can be facilitated by co-administering an HDR template with sequence homology to the targeted chromosomes (Fig. 4(d)). Such translocations can also be made conditional by inserting recombinase recognition sites at break sites (Fig. 4(d)).

In addition to inactivating genes, RNA-guided nucleases also facilitate the study of specific or disease-associated mutations in the context of the endogenous gene or the whole organism. This is of the utmost importance, especially for understanding disease mechanisms and performing structure–function studies. Overexpression of mutant proteins may have nonspecific and/or

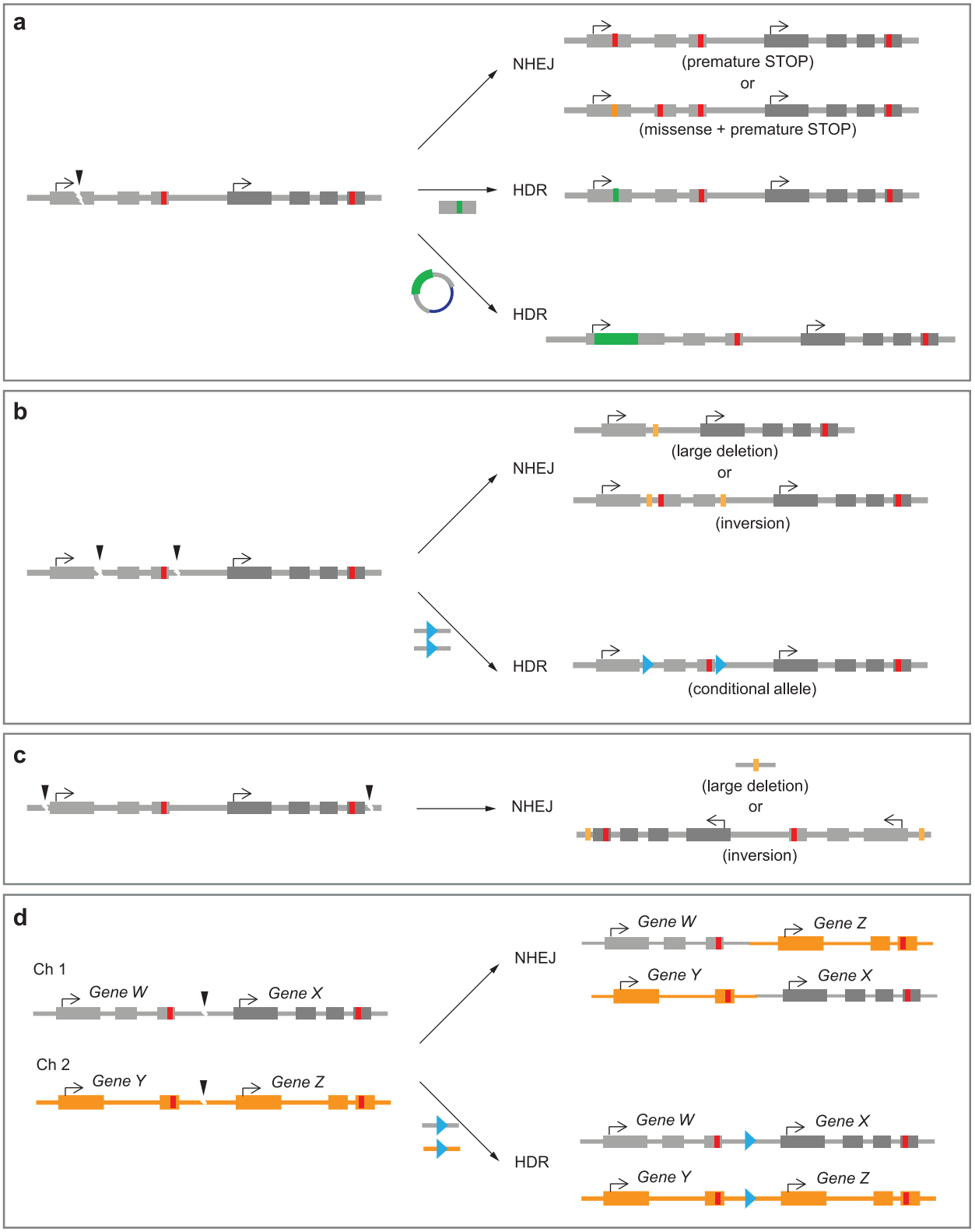

Fig. 4 Genome-editing strategies using CRISPR-Cas9. (a) A single DNA DSB can be inserted to generate various alleles. Null alleles can be generated by inserting a single DNA DSB. Break repair via the NHEJ pathway can result in insertion of a nonsense or frameshift mutation, leading to the formation of a premature STOP codon (red) and mRNA decay. Single DNA DSBs can also be inserted to introduce a STOP codon or other desired mutations (green) or insert large DNA elements such as DNA sequences encoding epitope tags, fluorescent proteins, or other functional entities. (b) Two DNA DSBs can be inserted within a gene to inactivate genes or generate conditional alleles. Break repair via the NHEJ pathway often results in deletion of the region flanked by DNA DSBs. Inversion of the intervening region is also possible. Co-administration of HDR templates containing recombinase recognition sequences can be used to generate conditional alleles. (c) Two DNA DSBs can be inserted to delete entire chromosomal regions. Break repair via the NHEJ pathway can result in removal or inversion of an entire chromosomal section.(d) Two DNA DSBs can be inserted in 2 distinct chromosomes to promote chromosomal translocation, an aberration observed in cancer cells. LoxP sites or other recombinase recognition sequences can be co-administered to make the translocation conditional. Boxes, exons; lines, non-coding region of a gene (introns, 5′ and 3′ untranslated regions); red boxes, STOP codons; yellow boxes, frameshift mutations or indels; green boxes, designed mutations or inserted DNA elements; blue triangles, DNA recombinase recognition sequences; HDR pathway, homology-directed repair pathway (HR or FA); NHEJ, nonhomologous end joining.

deleterious effects on cellular processes that would normally not be present in the context of the endogenous gene or organism. Specific mutations can be introduced by inserting a DNA DSB and co-administering a repair template containing the desired mutation flanked by sequences homologous to the target region (Fig. 4(a)). This strategy has been used to introduce mutations in cell lines as well as in model organisms (Lin et al., 2016; Martinez et al., 2015; Pelletier et al., 2015; Wang et al., 2013; Yang et al., 2013).

In addition to facilitating the insertion of point mutations and recombinase recognition sites, CRISPR–Cas9 systems are used to introduce small or large DNA elements such as epitope tags, Lox-STOP-Lox cassettes, transcriptional response elements, and cDNAs encoding fluorescent proteins. These elements are introduced by inserting 1 or 2 DNA DSBs and co-administering a DNA repair template containing the element to be inserted flanked by sequences homologous to the target site (Fig. 4(a)) (Yang et al., 2013).

Small edits can also be introduced within genomes without introducing DNA DSBs (Komor et al., 2016). For example, base editing uses an endonuclease-defective mutant of Cas9, in which one of the nuclease domains, the RuvC-like domain, is inactivated and various enzymatic activities are fused to it (Fig. 2(c)). These enzymes include the cytidine deaminase APOBEC-XTEN (catalyzes the conversion of cytosine to uridine) and a uracil glycosylase inhibitor (UGI; prevents reversion of uracil to cytosine). Nicking of the unmodified strand by the HNH domain of Cas9 RuvC− stabilizes nucleotide conversion. Like other CRISPR–Cas9 systems, the modified Cas protein is targeted to specific genomic loci through the binding of an sgRNA.

Genome-Wide Screens

Single-guide RNA libraries have also been developed for genome-wide screens studies and are available at Addgene. These libraries contain several guide RNAs (gRNAs) targeting each gene of the fly, mouse, or human genome. Some libraries also include gRNAs that target microRNAs and long-coding RNAs. Most libraries enable genome-wide screens that require the use of next-generation sequencing to measure enrichment or depletion of specific gRNAs. These genome-wide screens thus require robust assays for monitoring enrichment or depletion, such as cell death and survival or proliferation. GE Healthcare has also developed libraries that do not impose such limitations on assay designs and are available as lentiviral particles delivered in 96-well-plate formats.

CRISPR–Cas9 Genetic Regulation

Gene Expression or Repression (CRISPR Activation and CRISPR Interference)

Although primarily developed for genome editing, CRISPR–Cas9 systems have also been designed to upregulate or downregulate gene expression without modifying the target cell's genome (Cheng et al., 2013; Gilbert et al., 2014; Qi et al., 2013). Gene expression is modulated by the fusion of transcriptional activators such as VP64 (Fig. 5(a)) or transcriptional repressors such as KRAB to a catalytically inactive but gRNA-competent Cas9 (dCas9) (Fig. 5(b)). Transcriptional activation or repression is achieved by targeting one or more dCas9 fusion proteins to a gene promoter. Differential regulation can be obtained by increasing or reducing the number of transcriptional regulators to a specific promoter. Genome-wide libraries for activation and repression are available at Addgene. Screens using these libraries also require next-generation sequencing and robust functional enrichment or depletion assays.

Modulation of Epigenetic Markings

Epigenetic markings occur on both DNA and DNA-binding proteins. These markings, such as DNA methylation; histone acetylation and methylation; and ADP ribosylation, ubiquitination, sumoylation, and phosphorylation can affect gene expression. Selective modulation of these DNA markings at specific sites in the genome can provide information about the functional relevance of these posttranslational modifications on gene regulation. Epigenetic markings can be modulated by fusing the DNA or DNA-binding protein marking enzymes to catalytically inactive but gRNA-competent Cas9 (Fig. 5(c)) (Vojta et al., 2016). In this way, these functional entities can be targeted to various regions in an sgRNA-dependent manner to any genomic location and enable site-directed DNA or histone modifications.

CRISPR–Cas9 Tracking Devices

In addition to modulating gene expression and editing genomes, CRISPR–*Sp*Cas9 has been repurposed as tracking device to visualize specific DNA loci or mRNA transcripts (Chen et al., 2013; Nelles et al., 2016). The chromosomal region is visualized using dCas9 fused to a fluorescent protein marker such as green fluorescent protein (GFP). The tiling of Cas9–GFP fusions using several sgRNA molecules targeting a specific region of a chromosome can then promote the accumulation or fluorescently tagged dCas9 to that locus (Fig. 5(d)). This strategy was first used to visualize telomeres in cultured cells (Chen et al., 2013).

A similar strategy has also been devised to visualize endogenous mRNA transcripts within cultured cells (Nelles et al., 2016). In this system, a catalytically inactive Cas9 is fused to a fluorescent protein. The modified Cas9 is targeted to specific RNA transcripts, using both a sgRNA with sequence complementary to the RNA transcript and an oligonucleotide called PAMer. This oligonucleotide also possesses sequence complementarity to the mRNA transcript as well as a decoy PAM sequence that is recognized by Cas9 (Fig. 5(e)).

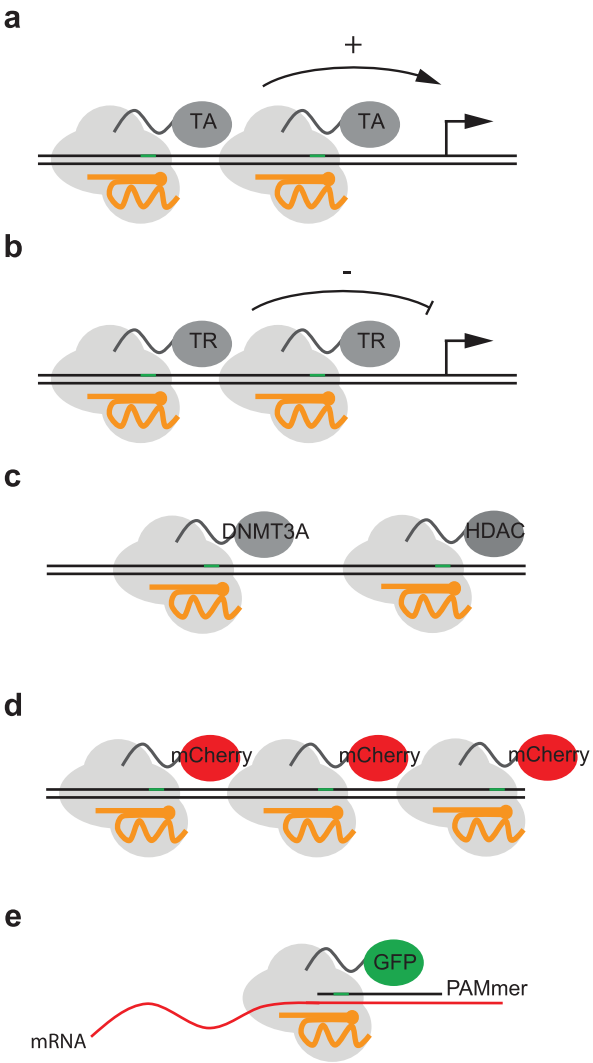

Fig. 5 CRISPR–Cas9 for genome regulation. (a) Transcriptional activator. *Sp*Cas9 has been converted into a transcriptional activator by fusion of a catalytically inactive *Sp*Cas9 to transcriptional activators (TAs) such as VP16/VP64 or p65 activation domains. Tiling of these site-specific transcriptional devices on gene promoters can modulate gene expression. (b) Transcriptional repressor. *Sp*Cas9 has been converted to site-specific transcriptional repressors by the fusion of a catalytically inactive *Sp*Cas9 to transcriptional repressors (TRs) such as KRAB or SID. Tiling of these site-specific transcriptional repressors on genes promoters can be used to modulate repression. (c) DNA and DNA-binding protein modifying devices. *Sp*Cas9 has been converted to locus-specific epigenetic marking enzymes. For example, the fusion of a catalytically inactive SpCas9 to DNA methylases such as DNMT3A or histone deacetylases can promote site-specific epigenetic modifications. (d) Genomic DNA tracking devices have been generated by fusing a catalytically inactive *Sp*Cas9 to fluorescent proteins such as mCherry or GFP. Tiling of these fusion proteins to a genomic locus can enable its visualization in real time. (e) Live imaging of the RNA transcript using a catalytically inactive *Sp*Cas9 fused to a fluorescent protein such as GFP can enable the visualization of mRNA transcripts. In this system, a small oligonucleotide PAMer is co-administered with the sgRNA and *Sp*Cas9. The PAMer serves as molecular decoy by providing a PAM sequence to the DNA–RNA hybrid.

Orthogonality of CRISPR–Cas9 Platforms

As mentioned earlier, several CRISPR–Cas9 and CRIPSR–Cpf1 systems have been adapted for genome editing and regulation, with each system having distinct PAM requirements and Cas9-binding properties. These orthogonal properties of CRISP–Cas9 systems allow the use of one system in combination with another to perform distinct functions within a single cell. For example, using these systems, it is possible to induce the expression of a specific gene while simultaneously inactivating another (Esvelt et al., 2013).

Applications of CRISPR–Cas9 Systems

CRISPR–Cas9 systems have found applications in a broad range of disciplines such as agriculture, biotechnology, biomedical research, drug development, and medicine. In crops and livestock, the CRISPR–Cas9 platform has been used to increase

productivity or provide resistance to pathogens. In biomedical research, they have been used to identify gene function, study structure–function relationships, and rapidly and efficiently generate cellular or animal models of human diseases. The ease with which cellular and animal models are generated also accelerates target validation for drug development. In medicine, CRIPSR–Cas9 technologies have been used to repair disease-causing mutations in model organisms. In the last 5 years, the platform has moved from proof-of-concept to clinical trials. In biotechnology, CRISPR–Cas9 platforms have been used to modify cells and microorganisms to produce more effective recombinant pharmaceuticals. Also, microbe or plant metabolism can be manipulated to increase the efficiency of biofuels production.

Concluding Remarks

We are currently redrawing the blueprint of life with the hope of finding cures to human diseases. Among all genome-editing tools, RNA-guided nucleases, in particular CRSIPR–Cas9 technologies, are the most promising. Not only are these genome editing systems significantly contributing to our understanding of biology in general, they also hold great promise to curing many genetic disorders through gene function identification and modeling human diseases. As we move forward with the development of these incredibly powerful genome-editing tools, it is also important to develop ethical and regulatory guidelines for this technology to ensure their safe use.

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Crystalline Cell Surface Layers (S-Layers)☆

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Introduction

The different cell wall structures observed in prokaryotic organisms, particularly the outermost envelope layers exposed to the environment, reflect evolutionary adaptations of the organisms to a broad spectrum of selection criteria. Crystalline cell surface layers (S-layers) are now recognized as common features of both bacteria and archaea.

Most of the presently known S-layers are composed of a single (glyco)protein species endowed with the ability to assemble into two-dimensional arrays on the supporting envelope layer. S-layers, as porous crystalline membranes completely covering the cell surface, can apparently provide the microorganisms with selective advantages by functioning as protective and anti-fouling coats, molecular sieves, molecule and ion traps, and as a structure involved in cell adhesion and surface recognition. In those archaea that possess an S-layer as exclusive envelope component outside the cytoplasmic membrane, the crystalline arrays act as a framework that determines and maintains cell shape and may also aid in cell division. In pathogenic organisms S-layers have been identified as virulence factors.

S-layers, as the most abundant of bacterial cellular proteins and thus on earth too, are important model systems for studies of structure, synthesis, assembly, and function of proteinaceous components, and evolutionary relationships within the prokaryotic world. S-layers also have considerable application potential in biotechnology, biomimetics, biomedicine, molecular nanotechnology, and synthetic biology (Table 1).

Location and Ultrastructure

Although considerable variation exists in the complexity and structure of bacterial cell walls, it is possible to classify cell envelope profiles into the following main groups on the basis of structure, biochemistry, and function (Fig. 1):

1. Cell envelopes formed exclusively of a crystalline S-layer composed of (glyco)protein subunits external to the cytoplasmic membrane (most halophilic, thermophilic and acidophilic, alkaliphilic, barophilic, and Gram-negative archaea (Fig. 1A). The inner part of the S-layer protein partially inserts into the hydrophobic domain of the cytoplasmic membrane or even penetrates it. Anchoring of the S-layer may also involve lipid modified glycoprotein subunits (Fig. 1B).
2. Gram-positive cell envelopes of bacteria with a rigid peptidoglycan-containing sacculus of variable thickness outside the cytoplasmic membrane (Fig. 1D), and Gram-positive cell envelopes of archaea with a rigid sacculus composed of pseudomurein or other polymers (Fig. 1C).
3. Gram-negative envelopes of bacteria with a thin peptidoglycan sacculus and an outer membrane (Fig. 1E).

Although not a universal feature as in archaea, crystalline arrays of (glyco)proteins have been detected as outermost envelope components in organisms of most major phylogenetic branches of Gram-positive and Gram-negative bacteria.

The most useful electron microscopy preparation procedure for detecting S-layers on intact cells is freeze-etching (Fig. 2).

S-layers completely cover the cell surface at all stages of cell growth and division in both archaea and bacteria. High-resolution electron microscopy and scanning force microscopy studies revealed that S-layer lattices can have oblique (p1, p2), square (p4), or hexagonal (p3, p6) lattice symmetry (Fig. 3) with a center-to-center spacing of the morphological units (composed of one, two, three, four, or six identical monomers) of approximately 5–35 nm. Among archaea, hexagonal lattices were shown to be predominant. S-layers are generally 5–25 nm thick and have a smooth outer and a more corrugated inner surface. Since S-layer lattices are monomolecular assemblies of identical subunits, they exhibit pores of identical size and morphology (Fig. 3). S-layers can display more than one type of pore. From high-resolution electron microscopy, scanning force microscopy, and permeability studies, pore sizes in the range of approximately 2–8 nm and a porosity of the protein meshwork between 30% and 70% were estimated.

Comparative studies on the distribution and uniformity of S-layers have revealed that in some species individual strains can show a remarkable diversity regarding lattice symmetry and lattice dimensions. In some organisms, two or even more superimposed S-layer lattices have been identified.

☆*Change History*: May 2018. Uwe B. Sleytr, A. Breitwieser and D. Pum are involved in preparing the update. The sections Abstract and Keywords, Isolation and chemical characterization, Assembly and morphogenesis, Genetics and biosynthesis, Further reading were updated. Figures 1, 3, and 4, Table 2, and relevant websites were updated. Selected milestones and historical data on S-layers research are provided in Sleytr et al. (2014) FEMS Microbiology Reviews 38: 823-864.

This article is an update of U.B. Sleytr, P. Messner, Crystalline Cell Surface Layers (S Layers), *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 89–98.

Table 1 General and specific functions of S-layers

General function	*Specific function*
Determination and maintenance of cell shape	Determination of cell shape and cell division in archaea that possess S-layers as exclusive wall component
Isoporous molecular sieve and antifouling coatings	Molecular sieves in the ultrafiltration range
	Delineating a compartment (periplasm) in Gram-positive bacteria
	Prevention of nonspecific adsorption of macromolecules
	Prevention of molecules reaching the cell wall proper (e.g., lytic enzymes)
Adhesion zone for exoenzymes	High-molecular-mass amylase of *Geobacillus stearothermophilus* wild-type strains
	Pullulanase and glycosyl hydrolases of *Thermoanaerobacter thermosulfurigenes*
Protective coat	Prevention of predation by *Bdellovibrio bacteriovorus* in Gram-negative bacteria Phage resistance by S-layer variation
	Prevention or promotion of phagocytosis
	Adaptation of *Bacillus pseudofirmus* to alkaline environment
Template for fine grain mineralization	Induction of precipitation of gypsum and calcite in *Synechococcus* and shedding of mineralized S-layers
Pathogenicity and cell adhesion	Virulence factor in pathogenic organisms
	Important role in invasion and survival within the host
	Specific binding of host molecules
	Protective coat against complement killing
	Ability to associate with macrophage and to resist the effect of proteases
	Production of immunologically non-crossreactive S-layers (S-layer variation)
Surface recognition and cell adhesion to substrates	Physicochemically and morphologically well-defined matrices
	Masking the net-negative charge of peptidoglycan-containing layer and antifouling surface in Bacillaceae

Adapted with permission from Sleytr et al. (1997). *FEMS Microbiology Reviews* **20**, 5–12.

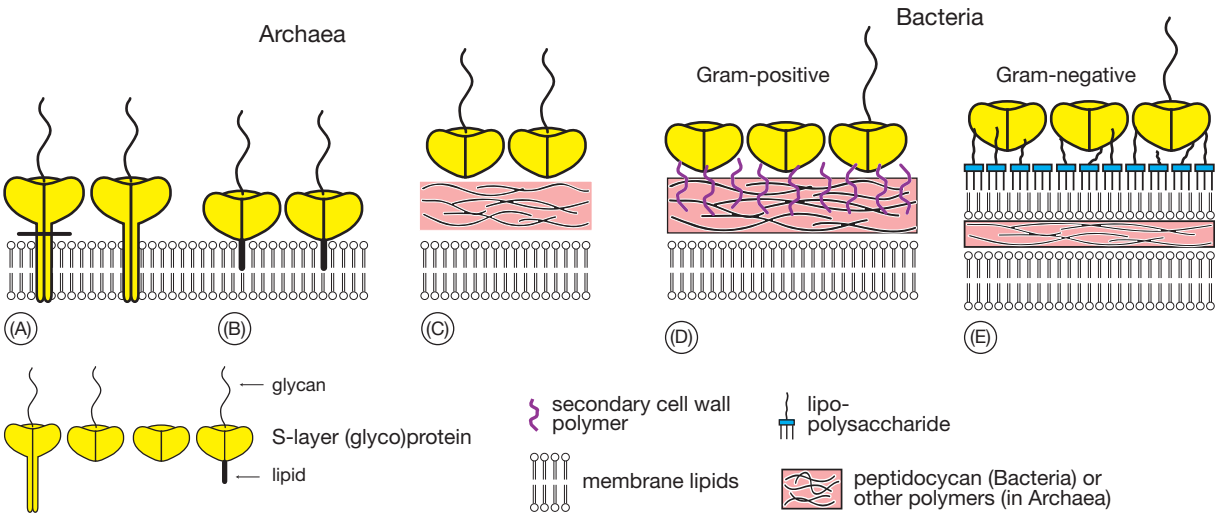

Fig. 1 Schematic illustration of the supramolecular architecture of the major classes of prokaryotic cell envelopes containing surface (S)-layers. S-layers in archaea with glycoprotein lattices as exclusive wall component are composed either of mushroom-like subunits with pillar-like, hydrophobic transmembrane domains (A), or lipid-modified glycoprotein subunits (B). Individual S-layers can be composed of glycoproteins possessing both types of membrane anchoring mechanisms. Few archaea possess a rigid wall layer (e.g., pseudomurein in methanogenic organisms) as intermediate layer between the plasma membrane and the S-layer (C). In Gram-positive bacteria, (D) the S-layer (glyco)proteins are bound to the rigid peptidoglycan-containing layer via secondary cell wall polymers. In Gram-negative bacteria, (E) the S-layer is closely associated with the lipopolysaccharide of the outer membrane. Reproduced with permission from Sleytr, U. B., Schuster, B., Egelseer, E. M. and Pum, D. (2014). S-layers: Principles and applications. *FEMS Microbiology Reviews* **38**, 823–864.

Isolation and Chemical Characterization

S-layers of different bacteria may vary considerably with respect to their resistance to disruption into their monomeric subunits, and a wide range of methods has been applied for their isolation and purification. The subunits of S-layers from the domain Bacteria interact with each other and with the supporting envelope layer through non-covalent forces.

In Gram-positive bacteria, a complete disintegration of S-layers into monomers can be obtained by treatment of intact cells or cell walls with high concentrations of hydrogen bond-breaking agents (e.g., urea or guanidine hydrochloride). Secondary cell wall

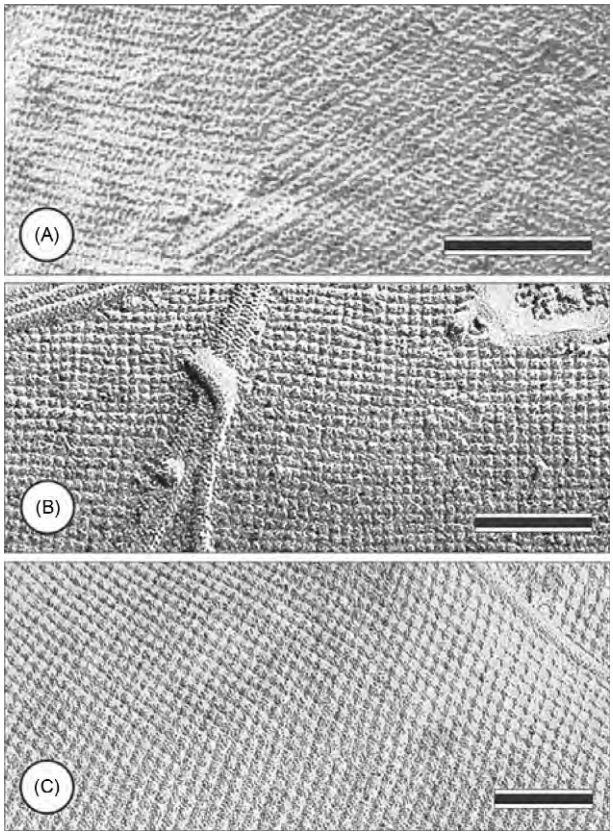

Fig. 2 Electron micrographs of freeze-etched preparations of intact bacteria. (A) *Lactobacillus buchneri* 41021/251; (B) *Desulfotomaculum nigrificans* NCIB 8706; (C) *Thermoanaerobacter thermohydrosulfuricus* L111-69. The oblique (A), square (B), and hexagonal (C) S-layer completely covers the cell surface. Bars = 100 nm.

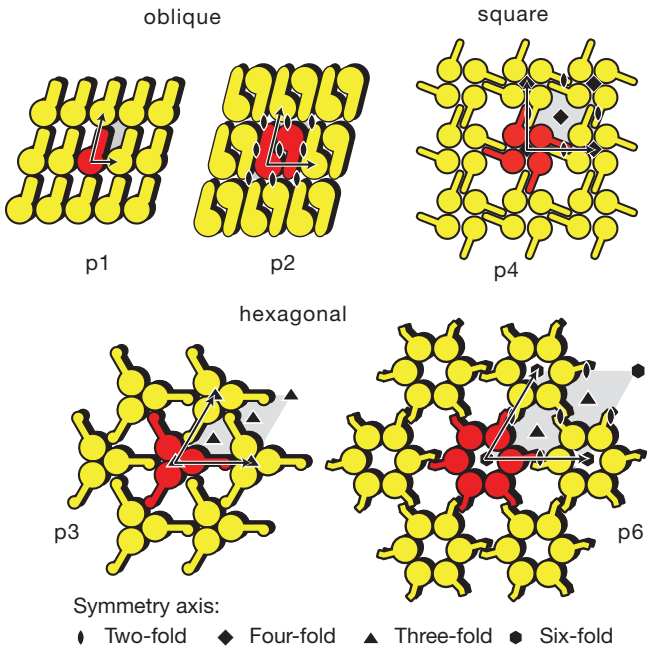

Fig. 3 Schematic drawing of different S-layer lattice types. The regular arrays exhibit oblique (p1, p2), square (p4), or hexagonal (p3, p6) lattice symmetry. The morphological units are composed of one, two, three, four, or six identical subunits. Reproduced with permission from Sleytr, U. B., Schuster, B., Egelseer, E. M. and Pum, D. (2014). S-layers: Principles and applications. *FEMS Microbiology Reviews* **38**, 823–864.

polymers (SCWPs; sugar polymers covalently linked to the peptidoglycan of the cell wall) have been recognized as components that facilitate a specific interaction between S-layer monomers and the peptidoglycan sacculus. In bacilli, the N-terminal region was found to be responsible for anchoring the S-layer subunits to the underlying rigid cell envelope layer whereas in many lactobacilli binding occurs via the C-terminal portion in a defined orientation to the SCWP. Structurally, the SCWPs resemble to some extent teichoic acids of Gram-positive organisms but beside having the common negative charge they can also be uncharged. Therefore, at least two types of binding mechanisms between S-layer proteins and SCWPs are now considered. The first one involves so-called SLH (S-layer homology) domains and pyruvylated SCWPs. The negative charge of pyruvic acid was demonstrated to participate in binding by the construction of knockout mutants in *Bacillus anthracis* and *Thermus thermophilus*. In the case of the S-layer protein SbsB of *Geobacillus stearothermophilus* PV72/p2, the SLH domain is part of the SCWP-binding domain. The C-terminal part of SbsB turned out to be highly sensitive against deletions and even removal of <15 amino acids led to water soluble S-layer protein forms. In contrast, in SbpA, the S-layer protein of *Lysinibacillus sphaericus* CCM 2177, up to 237 amino acids from the C-terminus of that S-layer protein could be deleted without interfering with the formation of the square lattice structure. From surface plasmon resonance (SPR) measurements it is known that the binding mechanism between SLH domains and pyruvylated SCWPs is highly specific and is therefore exploited for a biomimetic approach for an oriented recrystallization of S-layer (fusion) proteins on various SCWP-coated solid supports as required for many nanobiotechnological applications.

A second type of binding mechanism between S-layer proteins and SCWPs has been described for *G. stearothermophilus* wild-type strains and involves an SCWP that contains 2,3-dideoxy-diacetamidomannuronic acid as the negatively charged component. In *Geobacillus tepidamans* an SCWP with an identical backbone structure is present but instead of a free, negatively charged carboxyl group an amide carrying different substituent is present. Therefore, this SCWP is not charged, as are the SCWPs in several *Thermoanaerobacterium thermosaccharolyticum* strains. In those S-layer (glyco)proteins that are devoid of N-terminally located SLH domains, arginine and tyrosine, which typically occur in carbohydrate binding proteins such as lectins, are accumulated in the N-terminal part.

S-layers from Gram-negative bacteria frequently disrupt upon application of metal chelating agents (e.g., ethylenediaminetetraacetic acid (EDTA) and ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA)), cation substitution (e.g., Na^+ to replace Ca^{2+}), or pH changes (e.g., pH < 4.0). From extraction and disintegration experiments it can be concluded that the bonds holding the S-layer subunits together are stronger than those binding the crystalline array to the supporting envelope layer. There are some indications that S-layers of some archaea (e.g., *Thermoproteus* species and *Methanospirillum hungatei*) are stabilized by covalent bonds between adjacent subunits.

Most S-layers are composed of a single, homogeneous protein or glycoprotein species. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed apparent molecular masses for subunits in the range of approximately 40–220 kDa. In general, sequencing of the genes and mass spectrometry confirmed these results but for glycosylated S-layer proteins usually much lower molecular masses have been obtained. Amino acid analyses and genetic studies on S-layers from both archaea and bacteria have shown that the crystalline arrays are usually composed of weakly acidic proteins. Typically, they contain 40%–60% hydrophobic amino acids and possess a low portion, if any, of sulfur-containing amino acids. The isoelectric points pI are in the range of 4–6 but for *Methanothermus fervidus* and for lactobacilli, pI values in the range of 8–10 have been determined.

Information regarding the secondary structure of S-layer proteins is derived either from the amino acid sequence or from circular dichroism (CD) measurements, indicating that approximately 20% of the amino acids are organized as α-helices and about 40% occur as β-sheets. Aperiodic foldings and β-turn content may vary between 5% and 45%. More detailed studies have been performed with the S-layer proteins rSbsB from *G. stearothermophilus* PV72/p2 and rSbpA from *B. sphaericus* CCM 2177. Truncated forms of these S-layer proteins were constructed comprising either the N-terminal SLH domain, which is responsible for binding of the S-layer subunits to the rigid cell wall layer, or the middle and C-terminal self-assembly domain required for assembling into two-dimensional protein arrays. For both S-layer proteins, CD spectroscopy studies confirmed that most α-helical segments are arranged in the N-terminal SLH domain, whereas the middle and C-terminal parts exist as a β-sheet protein.

The fact that only a few structural models at atomic resolution of S-layer proteins are available may be explained by the molecular mass of the subunits being too large for nuclear magnetic resonance analysis, as well as by the intrinsic property of S-layer proteins to self-assemble into two- dimensional lattices, thereby hindering the formation of isotropic three-dimensional crystals, as required for X-ray crystallography. The X-ray structure of the SbsB S-layer protein of *G. stearothermophilus PV72/p2* was determined by the use of nanobody-aided crystallization down to a resolution of 2.4 Å. The SbsB protein consists of seven domains consisting of an amino-terminal cell-wall attachment domain and six consecutive immunoglobulin-like domains which are stabilized by inter-domain Ca^{2+} ion coordination. In the case of the bacterial S-layer protein SbsC from *G. stearothermophilus* ATCC 12980, water-soluble N- and C-terminally truncated forms were used for initial crystallization experiments. Native and heavy atom-derivative data confirmed results of the secondary structure prediction, indicating that the N-terminal region comprising the first 257 amino acids of the mature S-layer protein is mainly organized as α-helices, whereas the middle and C-terminal parts of SbsC consist of loops and β-sheets. While most of the high-resolution work was done with self-assembled S-layer lattices, most recently, the structure of the hexagonal surface layer on *Caulobacter crescentus* cells was obtained in situ at a resolution of 2.7 Å.

A few post-translational modifications are known to occur in S-layer proteins, including cleavage of carboxy- or amino terminal fragments, protein phosphorylation and protein glycosylation of amino acid residues. The latter is a remarkable characteristic of many archaeal and some bacterial S-layer proteins. In fact, S-layer proteins were the first glycoproteins detected in prokaryotes and still are among the best studied examples of glycosylated prokaryotic proteins. The glycan chain and linkages of bacterial and archaeal glycoproteins are significantly different from those of eukaryotes. The *Halobacterium salinarum* S-layer glycoprotein was the

first non-eukaryotic protein shown to be *N*-glycosylated. Most archaeal S-layer glycoprotein glycans consist of only short heteropolysaccharides, usually not built of repeating units. Moreover, the predominant linkage types are *N*-glycosidic bonds where the glycan moieties are covalently linked to select asparagine residues of the target protein. Although most S-layer proteins are either *N*- or *O*-glycosylated, in few cases both modifications can be present on one protein at the same time. An example constitutes the well characterized *Halobacterium volcanii* S-layer glycoprotein contain both *N*- and *O*-linked glycans.

As mentioned before, at the same time when glycosylation on haloarchaea was reported for the first time, glycosylation of S-layer proteins from the *Bacteria Thermoanaerobacter thermohydrosulfuricus* and *T. thermosaccharolyticum* was discovered. Since then, S-layer glycoproteins from several other bacteria have been extensively studied, leading to the awareness of the wide distribution of S-layer glycoproteins among bacteria.

In bacteria, *N*-glycosylation is considered to be a rare event and is represented mainly in *Campylobacter* spp., *Helicobacter* spp. and *Desulphovibrio* spp. Thus, *N*-glycosylation apparently is far more common in archaea than in bacteria. The degree of glycosylation of bacterial S-layer proteins, that is, the covalent *O*-glycosidic linkage of glycan moieties to select serine, threonine, and tyrosine residues, varies generally between 2% and 10% (*w*/w) and the S-layer proteins are typically multiple glycosylated.

Moreover, the lipid carrier molecules also differ between archaea and bacteria. In archaea isoprene-based lipids like dolichol phosphate and dolichol pyrophosphate play essential roles in the *N*-glycosylation process by delivering their bound glycan cargo to selected protein targets. In bacteria, however, undecaprenol pyrophosphate is recognized to play an essential role in S-layer protein *O*-glycosylation. To sum up, S-layer glycoproteins are among the best-studied examples of glycosylated prokaryotic proteins. Hence, a more detailed and comprehensive summary cannot be given and is out of the scope of this review. Nevertheless, at this point we would like to refer to several recent reviews providing deeper information on the glycosylation process in general.

Recently, yet another post-translational modification of S-layer glycoproteins was reported. It could be demonstrated that a subset of secreted euryarchaeal proteins, including the S-layer glycoprotein, is processed and covalently linked to membrane-embedded lipids involving membrane-spanning enzymes referred to as archaeosortases.

Assembly and Morphogenesis

Isolated S-layer subunits of Gram-positive and Gram-negative bacteria have shown the ability to recrystallize on the cell envelope fragments from which they had been removed, or on those of other organisms, or on untextured charged or uncharged inanimate surfaces. To date, the most detailed self-assembly and reattachment experiments have been performed with S-layers from Bacillaceae. S-layers of these organisms reveal a high anisotropic charge distribution. The inner surface is net negatively or net positively charged, whereas the outer face is charge-neutral, approximately with pH 7. This characteristic of the S-layer subunits appears to be essential for proper orientation during local insertion in the course of lattice growth.

Detailed studies have been performed to elucidate the dynamic process of assembly of S-layers during cell growth. For maintenance of a good long-range order during cell growth, S-layer protomers must have the ability to recrystallize on the supporting envelope layer. Labeling experiments with fluorescent antibodies and colloidal gold/antibody marker methods indicated that different patterns of S-layer growth exist for Gram-positive and Gram-negative bacteria. In Gram-positives, growth of the S-layer lattice primarily occurs on the cylindrical part of the cell by insertion at multiple bands or helically arranged bands; in Gram-negatives, incorporation of new subunits occurs at random. In both types of organisms, entirely new S-layer material also appears at regions of incipient cell division and the newly formed cell poles.

Reassembly of isolated S-layer subunits at the air/water interface, on Langmuir–Blodgett (LB) films, liposomes, emulsomes, or a great variety of solid supports (e.g., polymers, silicon wafers, metals, graphene) has proven to be an easy and reproducible way for generating extended coherent S-layer lattices (Fig. 4). Moreover, S-layer proteins are ideal model system to study nonclassical nucleation and growth of protein crystals at surfaces. The self-assembly process may be divided into several stages: (i) formation of amorphous clusters that are (ii) transformed into crystalline patches. (iii) Subsequently crystal growth into extended lattices continues by the incorporation of S-layer protein subunits at the edge sites of the crystalline domains. It has to be stressed that the final conformational transformation of the monomer to the oligomeric form is an inherent part of the assembly process. In accordance with S-layer proteins recrystallized on solid surfaces, the orientation of the protein arrays at liquid interfaces was determined by the anisotropy in the physicochemical surface properties of the protein lattice. Electron microscopy and atomic force microscopy examination revealed that recrystallized S-layer proteins from Bacillaceae were oriented with their outer charge-neutral, less hydrophilic surface against the air/water interface and with their negatively charged, more hydrophilic inner surface against the positively charged or zwitterionic head groups of phospholipid or tetraether lipid films.

Genetics and Biosynthesis

The fact that S-layers fully cover the bacterial cell at all stages of growth underlines the importance of these outermost two-dimensional (2D) crystalline highly ordered arrays for the bacteria. In order to form such a closed proteinaceous layer by self-assembly on a growing cell surface, high rates of subunits must be synthesized. At a generation time of about 20 min (e.g., for bacilli), at least 500 copies of a single polypeptide species with a molecular mass of approximately 100,000 Da have to be synthesized per second.

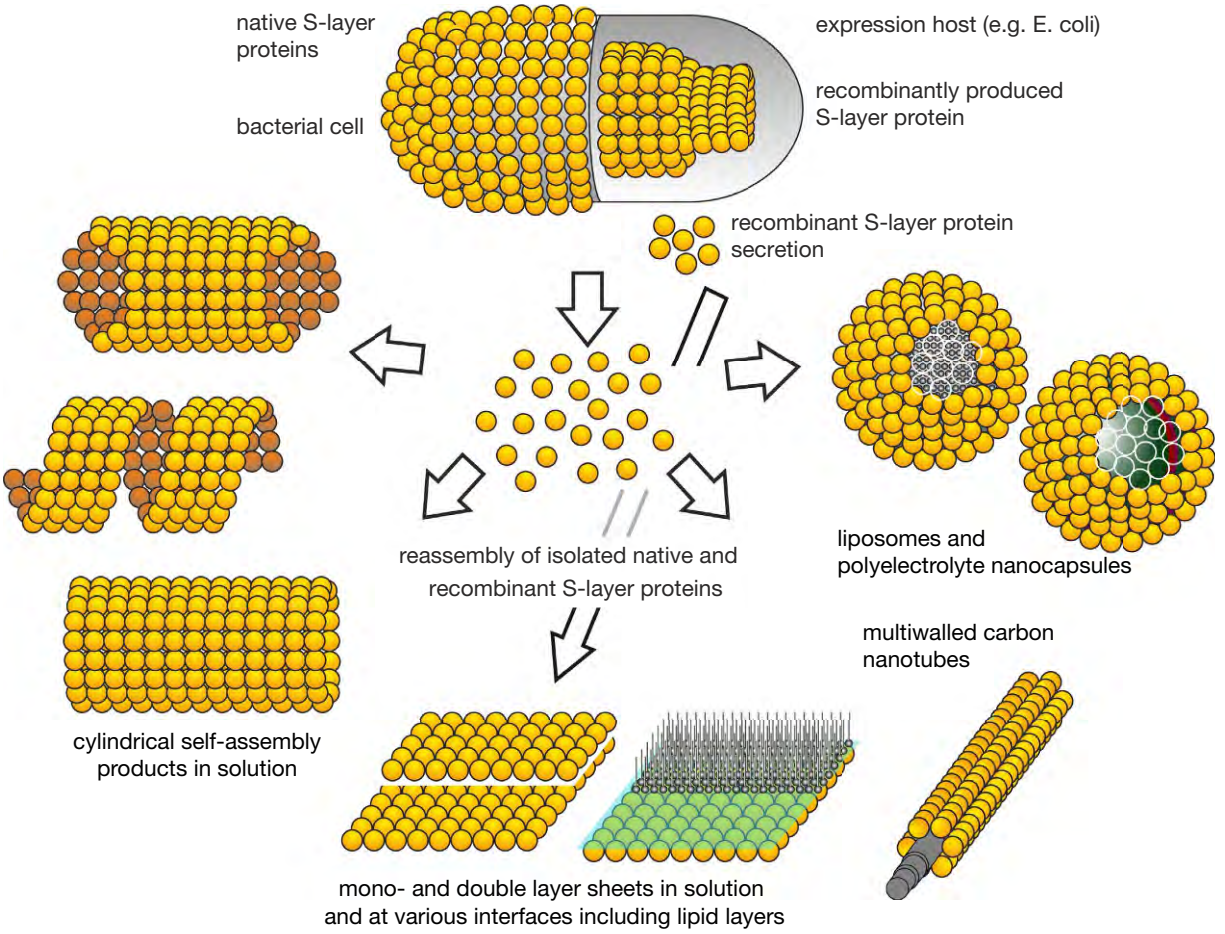

Fig. 4 Schematic illustration of the recrystallization of isolated S-layer subunits into crystalline arrays. The self-assembly process can occur in solution, at the air–liquid interface, at the solid–liquid interface, at lipid films, on liposomes, nanocapsules, emulsomes, and on carbon nanotubes. Modified with permission (CC BY 3.0) from Pum, D., Toca-Herrera, J. L. and Sleytr, U. B. (2013). S-Layer protein self-assembly. *International Journal of Molecular Sciences* **14**, 2484–2501.

Since S-layers are ubiquitous and represent one of the most abundant cellular proteins, numerous S-layer genes from bacterial and archaeal organisms of quite different taxonomical affiliations have been sequenced and cloned in the past. The studies revealed that homologies between nonrelated organisms are low, although their amino acid compositions show no significant differences. High homology scores are usually explained by evolutionary relationships but other factors, such as growth conditions and environmental stress may also be determinative for structural homologies of S-layer genes. A homology search among the *G. stearothermophilus* S-layer protein precursors SbsA–SbsD, SgsE, and SgsF revealed that the amino acid sequences showed overall identities between 21.6% and 93.9%. Less but distinct identities were found with several S-layer proteins from related organisms, such as *Bacillus thuringiensis*, *Bacillus firmus*, *Brevibacillus brevis*, or *Clostridium thermocellum*. By comparing the amino acid sequences of the *G. stearothermophilus* S-layer proteins it became evident that they share similarities with respect to the following features: (i) they are synthesized with a typical N-terminal signal sequence consisting of 30/31 amino acids; (ii) the signal peptide cleavage site is between the Ala–Ala motif and; (iii) all S-layer proteins exhibit a weakly acidic isoelectric point.

Interestingly, within the first 270 N-terminal amino acids of these S-layer proteins, sequence identities were in the range of 82.5%–98.1%. The only exception was SbsB, which did not reveal significant sequence homology in the N-terminal region; instead, it is the only one among the compared *Geobacillus* S-layer proteins to possess SLH domains between amino acids 31 and 168.

Within the domain Archaea overall sequence comparisons have only been performed for selected organisms. Studies of methanococcal S-layers have shown that the S-layer protein of *Methanotorris igneus* shared 43% identity with *Methanothermococcus thermolithotrophicus*, 39% with *Methanocaldococcus jannaschii*, 35% with *Methanococcus vannielii*, and 34% with *Methanococcus voltae*. The S-layer protein of *Mt. igneus* possesses a 28-amino acid signal peptide with typical characteristics of signal sequences such as a positively charged N-terminal region, a hydrophobic core, a polar C-terminal region, and an alanine residue at the peptide cleavage site. Further, it is highly conserved (approximately 68%) in all S-layer proteins of *Methanococcales*.

Although the low homology for S-layer proteins on the sequence level, common structural organization principles have been identified. Two separated morphological regions could be addressed to distinct properties; one responsible for binding to the cell

wall and one required for the self-assembly. Bacillaceae possess specific binding domains on the N-terminus for SCWP. These N-terminal binding domains (SLH motifs) anchor S-layer proteins to distinct SCWPs carrying pyruvic acids.

For the S-layer of *G. stearothermophilus* PV72/p2 SbsB, recognition of SCWP by three SLH motifs could be confirmed. In *L. sphaericus* CCM2177 an additional 58-amino acid long SLH-like motif is required for attaching the S-layer to the underlying cell wall components. In contrast to the SLH motifs of most S-layer proteins, which reveal a positive net charge, those of *L. sphaericus* strains are net negatively charged, which explains the importance of bivalent cations for binding the S-layer subunits to the rigid cell envelope layer. S-layers lacking the SLH motifs are anchored to different types of SCWP via their N or C-terminal regions. Studies performed for *G. stearothermophilus* wild type strains revealed a highly conserved N-terminal region lacking a SLH domain and nonpyruvylated SCWP containing 2,3-diacetamido-2,3-dideoxymannuronic acid as the negatively charged binding site. Although no SLH motifs were found in S-layer proteins from lactobacilli, which display a variety of regions responsible for cell wall binding or self-assembly, the SCWP was found to act as counterpart anchoring the S-layers.

For understanding gene regulation of S-layer, the observation that single bacterial strains can express different genes was important. In the pathogen *Campylobacter fetus* all studies have demonstrated that only a single promoter exists and that antigenic variation is due to recombination events. This allows expression of different S-layer gene cassettes. The variation enables the organism to overcome the host's immune response.

The presence of multiple S-layer protein genes is common for lactobacilli; *Lactobacillus brevis* ATCC 14869 possess three S-layer protein genes, while for *Lactobacillus crispatus* JCM5810 and *Lactobacillus acidophilus* ATCC 4356 encoding genes for two S-layer protein genes has been described. A multiple-promoter structure has been described for several S-layer protein genes of lactobacilli. for example, two promoter regions for the S-layer protein genes of *Lactobacillus brevis* ATCC 8287 and *Lactobacillus acidophilus* ATCC 4356 are required for efficient transcription. The S-layer protein gene of *Lactobacillus brevis* ATCC 14869 was shown to be expressed differently dependent upon growth conditions. Under aerated conditions, both S-layer specific transcripts were detected, whereas under anaerobic conditions only one specific transcripts could be observed.

The genome of *B. anthracis* encompasses 24 open reading frames whose predicted translation products each contain a secretion signal and three tandem SLH domains. The *B. anthracis* gene cluster encodes the two S-layer proteins, surface array protein and extractable antigen 1, as well as a pyruvyl transferase responsible for decorating SCWP with ketal-pyruvate.

An intact, "closed" S-layer on an average-sized, rod-shaped cell consists of approximately 5×10^5 monomers, the biogenesis of which requires high amounts of metabolic resources. S-layer monomeric proteins must be generated, hindered to self assemble within the cells during translocation to the cell surface and there rapidly incorporated into the existing S-layer lattice. Bacteria exhibit distinct secretory pathways for the secretion and anchoring of the S-layer to the cell wall. In many Gram-positive bacteria (described in detail for *Clostridium difficile* and *B. anthracis*) secretion of the S-layer precursors is dependent on the accessory Sec secretion system, which appears to involve one or two major S-layer proteins as well as S-layer-associated proteins. Precursors can travel via the canonical secretory pathway involving SecA or a SecA2 pathway responsible for the secretion of S-layer proteins.

Here the amino terminal signal peptide of the S-layer protein directs the polypeptide to the secretion apparatus and is cleaved upon membrane translocation. The accessory ATPase SecA2 is required to guide the S-layer proteins across the cytoplasmic membrane through distinct pores. The *C. difficile* S-layer is composed of two proteins, which are produced by proteolytic cleavage of the precursor S-layer protein SlpA. The low molecular weight S-layer protein, which probably faces the environment, shows considerable sequence variability among strains generating antigenic diversity. Another *C. difficile* S-layer protein, cell wall protein V, undergoes phase-variable expression, leading to phenotypic heterogeneity in the population.

In several Gram-negative bacteria S-layer secretion is based upon a type I secretion system. for example, in *C. fetus* and *C. crescentus* secretion is performed with an inner membrane ATP-binding cassette (ABC transporter) in combination with an outer membrane pore. Secretion of the S-layer proteins in the fish pathogens *Aeromonas salmonicida* and *Aeromonas hydrophila* relies on a specific two-step type II secretion system. Here, the unfolded precursor is translocated across the cytoplasmic membrane by the canonical Sec secretion system followed by folding of the protein and transportation across the outer membrane under the support of a multi-protein secretion apparatus.

An interesting feature is that many bacterial and most archaeal S-layer proteins are glycosylated. Although the function of bacterial S-layer glycoprotein glycans is still unclear so far, the enormous biosynthesis effort by various bacteria from different phylogenetic branches to glycosylate their S-layer proteins can lead to the assumption that glycosylation adds significantly to the potential functional spectrum of S-layer proteins.

The remarkable diversity in glycan structures of S-layer glycoproteins raised interesting questions about the biosynthesis of prokaryotic glycoproteins. In the domain Bacteria, four complete and one incomplete *slg* (surface-layer glycosylation) gene clusters have been identified. Slg gene clusters have been originally found based on the presence of L-rhamnose in the respective S-layer glycans and the knowledge that the *rmlABCD* genes involved in biosynthesis of L-rhamnose are highly conserved among Gram-positive and Gram-negative bacteria.

A polycistronic *slg* gene cluster encodes the S-layer glycan biosynthesis in *G. stearothermophilus* NRS 2004/3a. Three rhamnosyltransferases are responsible for the transfer of L-rhamnose from a nucleotide diphosphate-activated precursor across the membrane by a process involving an ABC transporter to the growing S-layer glycan chain. It seems that the variable part in the center of the *slg* gene clusters is responsible for the biosynthesis of the individual repeating units and terminating elements, whereas the region with higher homology codes for proteins involved in assembling the core region, transport of the glycan to the cell surface, and its ligation to the S-layer protein.

A bacterial protein tyrosine *O*-glycosylation system was described for the S-layer glycoproteins of *T. thermohydrosulfuricus* strains S102–70 and L111–69, *Th. thermosaccharolyticum* strains D120-70 and E207-71, and *Pa. alvei* CCM 2051^T in which glycan chain might be synthesized in a process similar to the ABC transporter-dependent pathway of the LPS O-PS biosynthesis.

In contrast to the previously described long-glycan chain S-layer glycoproteins of thermophilic and mesophilic bacilli, short-glycan chain S-layer glycoproteins have also been described for the Gram-positive organism *La. buchneri* CD034 or the Gram-negative periodontal pathogen *Tannerella forsythia* ATCC 43037.

In the following study, for the first time a glycosylated S-layer protein has been described for a Gram-negative organism. The newly emerged periodontopathic pathogen *T. forsythia* possesses an S-layer protein that is involved in hemagglutination, adhesion/invasion of epithelial cell, and murine subcutaneous abscess formation. The structure of the glycan partially imitates that of host glycoproteins and the glycobiology of this pathogen, including its repertoire of glycosidases, seems to be key to its physiology and, potentially, pathogenicity. Biochemical and molecular genetic characterization revealed that the S-layer is composed of two different glycosylated proteins *TfsA* and *TfsB*. Amino acid sequence comparison exhibited a 24% similarity between the two, 200 and 210 kDa proteins. Further sequence analyses showed that both possess putative signal peptide sequences with cleavage sites at alanine residues, and transmembrane domains on the C-terminal region. Comparison with other known S-layer proteins of prokaryotic organisms shows that the *T. forsythia* S-layer is very unique, since it appears to be composed of two large glycoproteins, and it does not reveal any homology to other known S-layer proteins or glycoproteins of prokaryotic organisms.

Very recently, a glycosylated S-layer was shown to be present also on the anamox bacterium candidatus *Kuenenia stuttgartiensis*. This S-layer showed hexagonal symmetry, which has no homology to any known protein and is present in glycosylated form.

Work on the identification of genes involved in the biosynthesis and attachment of *Methanococcus voltae* glycans provided the first detailed insights into N-linked glycosylation pathways in archaea at the genetic level. In addition to the previous work on halophilic archaea, a novel trisaccharide N-linked to asparagine residues in *Methanococcus voltae* flagellin and S-layer proteins could be characterized. Several candidate genes within the *Methanococcus voltae* genome were inactivated by insertional mutagenesis and have effects on flagellin and S-layer protein molecular mass, the N-linked glycan structure and are further involved in the transfer of the complete glycan to the flagellin and S-layer proteins.

Functional Aspects and Application Potential

Since S-layers represent one of the few examples in nature where proteins reveal the intrinsic capability to self-assemble into closed monomolecular lattices, a considerable potential in biological and nonbiological applications is evident. Data obtained in fundamental studies demonstrated that S-layers represent an ideal patterning element for nanobiotechnological, biomimetic, and nanotechnological applications. Particularly, the repetitive physicochemical properties and isoporosity of S-layer protein lattices down to the subnanometer scale make them unique matrices and building blocks for generating complex and supramolecular assemblies. The prime attractiveness of such "bottom-up" strategies lies in their capability to generate uniform nanostructures, and the possibility to exploit such structures at the meso- and macroscopic scale. Most importantly, it has been shown that S-layers can be combined with all major species of biological or synthesized (macro)molecules. Particularly, cloning and characterization of genes encoding S-layer proteins opened new areas of applied S-layer research as the incorporation of single or multifunctional domains has been possible without loss of their self-assembly capabilities (Table 2). As S-layers are highly anisotropic structures with regard to their topography and the physicochemical properties of their inner and outer surface, it was essential to ensure the recrystallization of S-layer fusion proteins in defined orientation on solid supports (e.g., polymers, metals, semiconductors), lipid membranes, and liposomes. Biomimetic approaches copying the physicochemical properties of the cell envelope structures supporting native S-layer proteins (e.g., SCWP, peptidoglycan) provided a remarkably easy solution for this problem. S-layer lattices are also used as support and stabilizing structures for lipid membranes and liposomes, mimicking the supramolecular construction principle of archaea dwelling under extreme environmental conditions (Fig. 1A and B) or virus envelopes. This technology allows exploitation of specific membrane functions with different formats while considerably increasing the life-time of the membranes as required for biosensors, lipid chips, drug screening, diagnostics, targeting, and drug delivery systems. S-layer self-assembly products have also been demonstrated to be well suited for a geometrically defined attachment of haptens and immunogens or immunostimulating and immunomodulating substances. Moreover, it is expected that highly specific immunogenic components with intrinsic targeting and delivery functionalities can be developed by combining recombinant S-layer proteins with the supramolecular construction principle of virus envelopes.

Chemical analyses of different S-layer proteins have demonstrated the presence of covalently linked carbohydrate residues. Since cell surface glycoconjugates play critical roles in many biological processes, in organisms from bacteria to man, glycosylated S-layers are a promising means for the development of functionalized nanoscaled materials. The detailed knowledge of glycoprotein biosynthetic pathways and cloning of the involved key enzymes are a prerequisite for the exploitation of glycobiology within the fields of nanobiotechnology (e.g., glycotherapeuthics with controllable, nanometer-scaled display of epitopes). Engineering of tailor-made bacterial glycoproteins will decisively change our capabilities in influencing and controlling complex biological systems (e.g., control of serum half-life of glycosylated metabolites and interference with glycosylated receptors).

Table 2 Functional recombinant S-layer fusion proteins and their applications

Recombinant S-layer protein	*Functionality*	*Length of function*	*Application*
SbpA SbsB	Core streptavidin	118 aa	Binding of biotinylated ligands (DNA, protein), Biochip development
SbpA, SbsC	Major birch pollen allergen (Bet v1)	116 aa	Vaccine development, treatment of type 1 allergy
SbpA	*Strep*-tag II, Affinity tag for streptavidin	9 aa	Biochip development
SbpA	ZZ, IgG-binding domain of Protein A	116 aa	Extracorporeal blood purification
SbpA	Enhanced green fluorescent protein (EGFP)	238 aa	Coating of liposomes, development of drug and delivery systems
SbpA	cAb, heavy chain camel antibody	117 aa	Diagnostic systems and sensing layer for label-free detection systems
SbpA	Hyperthermophilic enzyme laminarinase (LamA)	263 aa	Immobilized biocatalysts
SbpA	Cysteine mutants	3 aa	Building of nanoparticle arrays
SbpA, SbsB	Mimotope of an Epstein-Barr virus (EBV) epitope (F1)	20 aa	Vaccine development
SbpA, SbsB	*Mycoplasma tuberculosis* antigen (mpt64)	204 aa	Vaccine development
SbpA	IgG-binding domain of Protein G	110 aa	Downstream processing
SgsE	Glucose-1-phosphate thymidylyltransferase (RmlA)	299 aa	Immobilized biocatalysts
SgsE	Enhanced cyan fluorescent protein (ECFP)	240 aa	pH biosensors in vivo or in vitro, fluorescent markers for drug delivery systems
	Enhanced green fluorescent protein (EGFP)	240 aa	
	Yellow fluorescent protein (YFP)	240 aa	
	Monomeric red fluorescent protein (RFP1)	225 aa	
SbsA	*Haemophilus influenzae* antigen, (*Omp*26)	200 aa	Vaccine development
SlpA	Antigenic poliovirus epitope (VP1)	11 aa	Development of mucosal vaccines
	Human c-myc proto-oncogene	10 aa	
SLH-EA1, SLH-Sap	Levansucrase of *B. subtilis*	473 aa	Vaccine development
SLH-EA1	Tetanus toxin fragment C of *Clostridium tetani* (ToxC)	451 aa	Development of live veterinary vaccines
RsaA	*Pseudomonas aeruginosa* strain K pilin	12 aa	Vaccine development
RsaA	IHNV glycoprotein	184 aa	Development of vaccines against hematopoietic virus infection
RsaA	Beta-1,4-glycanase (Cex)	485 aa	Immobilized biocatalysts
RsaA	IgG-binding domain of Protein G	$GB1_{xs}$	Development of immunoactive reagent
RsaA	Domain 1 of HIV receptor CD4	81 aa	Anti-HIV microbicide development
	MIP1α ligand for HIV coreceptor CCR5	70 aa	
RsaA	His-tag, Affinity tag	6 aa	Bioremediation of heavy metals (Cd) from aqueous systems, bioreactor
RsaA	Protective coat	6 aa	Protection against antimicrobial peptide in *Caulobacter crescentus*

S-layer proteins: SbsB of *Geobacillus stearothermophilus* PV72/p2, SbpA of *Lysinibacillus sphaericus* CCM 2177, SbsC of *Geobacillus stearothermophilus* ATCC 12980, SgsE of *Geobacillus stearothermophilus* NRS 2004/3a, SbsA of *Bacillus stearothermophilus* PV72/p6, SlpA of *Lactobacillus brevis* ATCC 8287, SLH (S-layer homology domain of EA1 or Sap) of *Bacillus anthracis*, RsaA of *Caulobacter crescentus* CB15A, * personal communication.

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Another line of development is directed to the use of S-layer lattices for non-life science applications. It has already been shown that S-layer protein meshworks can be exploited for biological templating, biomineralization, and the formation of arrays of metal clusters and nanoparticles as required in molecular electronics, biocatalysis, and nonlinear optics. Recently, it was demonstrated that fabrication of spatially well-defined ordered S-layer arrays on silicon support can be achieved by applying micromolding in capillaries (a soft lithography technique).

Finally, S-layers as isoporous structures can be used for the production of ultrafiltration membranes with very sharp molecular exclusion limits. These membranes are manufactured by depositing and cross-linking S-layer fragments on microfiltration membranes.

Although a remarkably broad spectrum of nanobiotechnological applications for S-layers has already been developed, many other areas will eventually emerge. Particularly, data on specific biological functions and biophysical properties of S-layers of organisms growing and surviving under extreme environmental conditions will stimulate applied S-layer research and broaden the application potential.

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Cutaneous Fungal Infections☆

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Cutaneous Host Defenses

Structure of the Skin

The physical and chemical structure of the skin represents a form of defense against fungal pathogens. The skin surface is relatively inhospitable to fungal growth because of exposure to ultraviolet light, low moisture conditions, and competition from the normal bacterial flora of this site. Therefore, this surface acts as a barrier to the entry of fungi. The stratum corneum is made up of keratin, which most microorganisms cannot use for nutrition. However, *Candida albicans* and the dermatophytic fungi produce keratinases, which hydrolyze this substance and facilitate the growth of these organisms in the stratum corneum itself. This very superficial site of infection may protect the infecting organisms from direct contact with at least some of the effector cells of the immune system. Although neutrophils and small numbers of lymphocytes may enter the epidermis, the major infiltrates of cell-mediated immune responses are generally confined to the dermis.

Keratinization and Epidermal Proliferation

The process by which the stratum corneum is continually renewed through keratinization of the epidermal cells may also present a form of defense against organisms infecting the skin. The basal epidermal cells cause continued growth of the epidermis as they undergo repeated cell divisions that move the resulting daughter cells (keratinocytes) outward, toward the surface. As they mature and differentiate, these cells lose their nuclei and become flattened to form the keratinized cells. This process results in continuous shedding of the stratum corneum, which may remove infecting fungal microorganisms residing there. Inflammation, including that produced by cell-mediated immune reactions, appears to enhance epidermal proliferation so that rates of transit of epidermal cells toward the stratum corneum are increased. A number of studies have demonstrated that epidermal proliferation is important in the defense against superficial mycosis.

Antifungal Substances

Sebaceous gland secretions and other epidermal lipids, including the sphingosines, fatty acids, polar lipids, and glycosphingolipids, have been shown to be fungistatic against both dermatophytes and *C. albicans*. Unsaturated transferrin is another well-known antimicrobial substance that appears to be active in inflamed skin and inhibits microbial growth by competing for iron. Keratinocytes of the epidermis produce several other antimicrobial proteins, including cathelicidins, human β-defensin 2, and calprotectin. These proteins appear to be generated in greater quantities when the keratinocytes are stimulated by contact with microorganisms or cytokines. A variety of other antimicrobial proteins are also found in the skin, including granzyme B, adrenomedullin, antileukoprotease, and melanins. Others are undoubtedly present also, and the presence of all these antimicrobial compounds adds to the difficulty that potential pathogens face when trying to invade into the skin.

The Innate Immune System

The innate immune system is composed of cells and mechanisms that defend an organism against nonself entities in a nonspecific manner; it represents a first line of defense against invading pathogens and does not require a preliminary exposure to microbial antigens to generate an effective response. Unlike adaptive immunity, the innate immune system does not confer long-lasting memory or protective immunity against the foreign entity. Rather, innate immunity provides immediate defense against pathogens. This system is especially prominent in those body surfaces that contact the external environment and are colonized by potential pathogens; these areas include the skin, digestive and urinary tracts, and airways. Therefore, cutaneous fungal pathogens must somehow evade these processes when they first try to invade the skin.

All known plants and animals have innate immunity, suggesting that this system appeared early during evolution. The major function of this system seems to be the initial recruitment of leucocytes, such as macrophage and neutrophils, to areas of infection. These cells then phagocytose and kill pathogens while also eliciting continued inflammatory responses. After microbial killing occurs in the phagosomes of macrophage, the organism's components may also be presented to T lymphocytes to activate the adaptive immune system. Thus, there is interplay between these two arms of the immune system.

A critical part of this process is the ability of the host cells to discriminate between themselves and a large number of potential pathogens using a restricted number of germ-line-encoded pattern recognition receptors. The latter recognize particular chemical

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characteristics of microorganisms, referred to as pathogen-associated molecular patterns (PAMPs). The best-described host receptors involved in this process are the toll-like receptors (TLRs), whose genes are present in all eukaryotes. At present there are ten known TLRs in humans, with different PAMPs being recognized by different ones. Other similar receptors include Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs). Once the pattern recognition receptor identifies a PAMP, it begins a cell-signaling cascade that initiates a defensive response directed at eliminating the pathogen. This cascade involves multiple cytokines that promote inflammation, stimulate adaptive immunity, and cause an acute phase response. Factors such as age, nutritional status, and hormone levels may alter innate responses.

The Inflammatory Response

With various kinds of superficial fungal infections, there appears to be an inverse relationship between the degree of inflammation produced by a particular fungal pathogen and the chronicity of that infection. *Malassezia sp.* and the anthropophilic dermatophytes, *Trichophyton rubrum* and *Epidermophyton floccosum*, generally produce little inflammation in their cutaneous lesions and frequently cause infections that persist for long periods. On the other hand, many of the geophilic or zoophilic dermatophytes, for example, *Trichophyton verrucosum*, produce highly inflammatory infections that are usually self-limited. Thus, the local inflammatory process may indeed be involved in the defense against this group of pathogens.

Several mechanisms have been described by which inflammatory cells are attracted into sites of cutaneous fungal infections, in addition to the innate immunity processes described above. Fungal organisms are generally capable of activating complement by the alternative pathway to produce chemotactic activity for neutrophils and can also produce low molecular weight chemotactic factors analogous to the ones made by growing bacteria. Keratinocytes themselves can generate chemotactic cytokines that could also be responsible for some of the inflammation in the lesions of cutaneous fungal infections.

Neutrophils and monocytes/macrophage appear to be important in the defense against fungi, including those involved in the cutaneous mycoses. Neutrophils can directly attack pathogens using a variety of microbicidal processes. These processes depend upon either microbicidal oxidants or nonoxidative granule antimicrobial peptides. The latter include defensins, bactericidal/permeability-increasing protein, lactoferrin, lysozyme, and cathelicidin. Most of these compounds have been studied primarily for their ability to kill organisms, although lactoferrin may have both microbistatic and microbicidal effects. Macrophages have an additional antimicrobial mechanism whereby they can produce nitric oxide to inhibit growth of ingested fungal pathogens. Neutrophils also appear to have significant growth inhibitory activity in their cytoplasm. These cells, as do the keratinocytes themselves, produce large amounts of calprotectin, a calcium- and zinc- binding protein that has potent microbistatic activity against *C. albicans*, the dermatophytes, and other fungi.

The Cutaneous Immune System

Since cutaneous fungal infections are more frequent and more severe in patients with immunologic defects, immune responses to fungal antigens would seem to play an important role in the host defense against these infections. Immunologic host defense mechanisms in normal hosts seem to be effective even when the infections are limited to superficial locations, such as the stratum corneum. A number of studies suggest that the epidermis not only represents a passive barrier against the entry of infecting organisms, but also acts as an immunologic organ with some unique elements. The skin-associated lymphoid tissue (SALT) appears to act as immune surveillance unit. A variety of cell types are believed to be involved in this cutaneous immune system, including Langerhans cells, dermal dendritic cells, epidermal T lymphocytes, keratinocytes, and microvascular endothelial cells. The mechanisms employed are complex, involving a network of fixed or mobile cells interacting either by the trafficking of the cells themselves from one site to another or by the production of cytokines that influence the function of other cells. Such skin-initiated immune responses act against a broad spectrum of foreign antigens including contact allergens, tumors, and transplants, and it is likely that they are also active against the fungal pathogens of interest here. Therefore, this system is probably responsible for initiating immune responses that work to eliminate the infecting organisms in the immune host. In addition, such responses may also produce some of the inflammation that results in much of the symptomatology of these infections.

Description of the Diseases

Superficial Fungal Infections

These infections are limited to the most superficial layers of the epidermis and/or its keratinized appendages, such as the hair and nails. The most common pathogens causing superficial mycoses are listed Table 1.

Cutaneous candidiasis

Cutaneous candidiasis is an infection of the skin that is generally caused by the yeast-like fungus *C. albicans* and that can be either acute or chronic in nature. *C. albicans* is part of the normal flora of the gastrointestinal tract, rather than that of the skin, although it can be found on the skin on occasion. This organism can grow as either yeast cells or filamentous forms, with mixtures of the two phases generally seen in tissue infections. Cutaneous candidiasis on occasion may be caused by other species of this genus, including *Candida parapsilosis*, *Candida tropicalis*, or *Candida glabrata*, but these are unusual.

Table 1 Superficial cutaneous mycoses and the most common responsible pathogens

Type of infection	*Pathogens*
A. Cutaneous candidiasis	Candida albicans
Dermatophytosis	Trichophyton, Microsporum, Epidermophyton
1. Tinea pedis	T. rubrum, T. mentagrophytes, E. floccosum
2. Tinea cruris	E. floccosum, T. rubrum
3. Tinea barbae	T. rubrum, T. verrucosum, T. mentagrophytes
4. Tinea unguium (onychomycosis)	T. rubrum, T. mentagrophytes
5. Tinea capitus	T. tonsurans, T. schoenleinii, T. rubrum, M. canis, M. audounii
6. Tinea corporis	T. rubrum, T. mentagrophytes, M. canis, T. verrucosum, M. gypseum
Skin and nail infections by nondermatophytes	Scytalidium dimidiatum, Scopularis brevicaulis, Fusarium oxysporum
Tinea versicolor	Malassezia globosa, M. furfur, M. sympodialis
Malassezia folliculitis	Malassezia sp.
Tinea nigra	Hortaea werneckii
White piedra	Trichosporon ovoides, T. inkin, T. asahii
Black piedra	Piedraia hortae

Acute cutaneous candidiasis may present as intertrigo, producing intense erythema, edema, creamy exudates, and satellite pustules within folds of the skin. Other infections may be more chronic, as in the feet where there can be a thick white layer of infected stratum corneum overlaying the epidermis of the interdigital spaces. Candida paronychia is marked by infection of the periungual skin and the nail itself, resulting in the typical swelling and redness of this type of candidal infection.

Superficial candidiasis is common in otherwise healthy neonates and young infants, and mainly manifests as either oropharyngeal candidiasis (oral thrush) or candidal diaper dermatitis. In the latter case, moist macerated skin in the diaper area appears to be particularly susceptible to candida invasion. The infection usually starts in the perianal region and may spread to involve the perineum and perhaps the lower abdomen and upper thighs. The lesions are typically scaly papules that progress to weeping eroded lesions that often have satellite pustules. Congenital cutaneous candidiasis is a more severe infection of neonates wherein the disease presents within 6 days of birth; there is generally widespread desquamating and/or erosive dermatitis and a significant risk of systemic and sometimes fatal candida infection. The affected neonates require systemic antifungal therapy.

In some cases, superficial *C. albicans* infections may be particularly severe, persistent, and recalcitrant to treatment, producing the uncommon disorder known as chronic mucocutaneous candidiasis. This condition consists of persistent and recurrent infections of the mucous membranes, skin, and nails, along with a variety of other manifestations. The superficial infections last for years in affected patients unless they are properly treated, although deep candida infections are very rare in this situation. Oral thrush and candida vaginitis are fairly common in patients with chronic mucocutaneous candidiasis. There is often infection of the esophagus, although further extension into the viscera is unusual. Epidermal neutrophilic microabscesses, which are common in acute cutaneous candidiasis, are rare in the lesions of chronic mucocutaneous candidiasis. The oral lesions are generally tender and painful.

A number of other disorders are associated with the syndrome of chronic mucocutaneous candidiasis, including endocrine dysfunction, vitiligo, dysplasia of the dental enamel, congenital thymic dysplasia, thymomas, and certain other infections. The term autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) has been used to describe some of these patients, and mutations in a particular gene, called the autoimmune regulator (AIRE), appear to be responsible. More recently, some patients with chronic mucocutaneous candidiasis have been demonstrated to have a deficiency in IL17 cytokine production, which is the function of a recently described subdivision of T cells (Th17 cells) with important proinflammatory and host defense functions. In addition, a family with mucocutaneous candidiasis was found to have a mutation in the CARD9 (caspase recruitment domain-containing protein 9) gene that was associated with low numbers of Th17 cells in the affected family members. The Th17 pathway appears to be important in protecting against chronic superficial candida infections. Otherwise, chronic mucocutaneous candidiasis no doubt represents a group of related syndromes with a variety of predisposing or secondary abnormalities in host defense function, most commonly deficient cell-mediated immune responses against candida antigens.

The diagnosis of superficial candidiasis is usually suspected on clinical grounds and can be confirmed in skin scrapings by demonstrating the organisms using potassium hydroxide preparations and/or culture on appropriate antifungal media. Long-term (3–9 months) treatment with azole antifungal drugs can produce good results in chronic mucocutaneous candidiasis, although occasional failures have occurred due to the development of resistant strains of *C. albicans*. Patients who present with chronic mucocutaneous candidiasis should be evaluated for the presence of infection with the human immunodeficiency virus and, if presenting as adults, for the possibility of thymoma.

Dermatophytosis

Dermatophytosis is the infection of keratinized structures, such as the nails, hair shafts, and stratum corneum of the skin, by organisms of three genera of fungi termed dermatophytes. Although they are not part of the normal human skin flora, these organisms are particularly well adapted to infecting this location because they can use keratin as a source of nutrients – unlike most

other fungal pathogens. The different types of dermatophytes are often classified according to body site, using the word 'tinea', followed by a term for the particular body site. The major types of dermatophytosis and the most frequent organisms associated with them are listed in Table 1. The degree of inflammation produced in the lesions appears to depend primarily on the particular organism and perhaps also to some extent on the immunological competence of the patient.

Tinea pedis (athlete's foot) is probably the most common form of dermatophytosis. This condition is a chronic toe web infection that can be scaly, vesicular, or ulcerative in form and can sometimes produce hyperkeratosis of the sole of the foot. Tinea cruris is an expanding dermatophye infection in the flexural areas of the groin and occurs much more frequently in males than in females. Dermatophyte infection of the major surface areas of the body is termed tinea corporis. These infections frequently take the classical annular, or 'ringworm', shape. Involvement of the beard area in men, a condition known as tinea barbae, is often caused by zoophilic organisms such as *T. verrucosum*. Infection of the hair and skin on the scalp is called tinea capitis and is more common in children than adults. Hair infection by dermatophytic fungi can be classified as either endothrix (with organisms present within the hair shaft) or ectothrix (with most of the organisms outside the hair shaft, forming a sheath of spores around it).

Tinea unguium is a form of onychomycosis, or fungal infection of the nails, that is caused by dermatophytes such as *T. rubrum*. This type of infection may be classified into three types by the way the organisms enter the nails: (1) distal subungual onychomycosis, in which the organisms initially enter the distal nail plate and nail bed; (2) proximal subungual onychomycosis, in which the organisms invade proximally in the cuticle area and then migrate distally; and (3) white superficial onychomycosis, in which the fungi invade the superficial layers of the nail plate directly. Nail infections, particularly of the toenails, are among the most difficult types of dermatophytosis to treat.

Diagnosis of dermatophytosis is made in a similar manner to that of cutaneous candidiasis, with examination of skin scrapings by potassium hydroxide preparations and culture on appropriate fungal media. The treatment of this condition has improved markedly in recent years with the development of new antifungal agents for topical application or oral administration. However, certain kinds of dermatophytosis, including widespread infections and those of hair and nails, will often respond poorly to topical therapy and will require prolonged courses of an oral antifungal agent, such as griseofulvin, ketoconazole, itraconazole, fluconazole, and terbinafine.

There is an opportunistic fungal organism, *Scytalidium dimidiatum* (formerly known as *Hendersonula toruloidea*), that can produce conditions clinically mimicking those caused by the usual dermatophyte species, but which does not respond well to conventional antifungal therapy. In addition, some nondermatophytic fungi such as *Scopulariopsis brevicaulis* and *Fusarium oxysporum* can invade the nails to produce the white superficial onychonmycosis pattern of infection. Whereas dermatophytes rarely invade the deep tissues or produce systemic infections, even in severely immunocompromised patients, *Fusarium* spp. can disseminate to produce severe and often fatal infections in patients with inadequate host defenses. On the other hand, deep and severe dermatophytosis caused by the usual dermatophytes has been found in some families with inherited CARD9 deficiency.

Tinea (pityriasis) versicolor and malassezia folliculitis

Tinea versicolor is a chronic superficial fungal infection of the skin generally affecting the trunk or the proximal parts of the extremities and caused by various species of *Malassezia*, primarily *M. globosa*, *M. furfur*, and *M. sympodialis*. The organism is lipid requiring and will not grow on most laboratory media. The lesions resulting from infection with *Malassezia sp.* are macules that may coalesce into large, irregular patches characterized by fine scaling, along with hypopigmentation or hyperpigmentation. These infections can persist for years unless treated appropriately. *Malassezia sp.* have also been postulated to play a role in certain other diseases, including atopic dermatitis, seborrheic dermatitis, psoriasis, and reticulate papillomatosis. Malassezia folliculitis is a condition that resembles several other cutaneous infections, including acne vulgaris, the macronodular lesions of disseminated candidiasis in immunosuppressed patients, the candidal papular folliculitis of heroin addicts, and graft-versus-host disease in bone marrow transplant patients. The papules of this condition begin as inflammation of the hair follicles, instead of macules typical of tinea versicolor, and may progress to frank pustules.

In tinea versicolor, potassium hydroxide preparations of skin scrapings reveal the typical grape-like clusters of yeast and tangled hyphae of the causative fungus, confirming the diagnosis of this condition. The organism is not usually cultured because of the requirement for specialized media. Tinea versicolor can be treated topically with lotions or creams containing selenium or sodium thiosulfate, specific antifungal agents, or sulfur–salicylic acid shampoo. Oral azole antifungal drugs can also be used for more difficult cases. Malassezia folliculitis can be treated using topical antifungal agents or an oral azole antifungal agent.

Miscellaneous superficial fungal infections

Tinea nigra is a superficial mycosis of the palms that is most often caused by *Hortaea werneckii* (formerly called *Phaeoannellomyces werneckii* or *Exophiala werneckii*). The lesions are generally dark-colored, nonscaling macules that are asymptomatic, but can be confused with melanomas and can perhaps result in unnecessary surgery. Tinea nigra is most often seen in tropical or semitropical areas of Central and South America, Africa, and Asia, although some cases do occur in North America. This condition can be treated effectively with either keratinolytic agents or topical azoles. White piedra is an asymptomatic fungal infection of the hair shafts that is caused by *Trichosporon beigelii* (also known as *Trichosporon cutaneum*). This infection produces light-colored, soft nodules on the hair shafts and may cause the involved hairs to break. Otherwise, this condition appears to be asymptomatic, although the causative fungus can produce serious infections in immunocompromised patients. Black piedra is similar to white piedra in that it is a nodular, generally asymptomatic, fungal infection of the hair shafts. It is caused by *Piedraia hortae* and most commonly affects the scalp hair. Black and white piedra are generally treated by clipping off the affected hairs.

Cutaneous and Subcutaneous Mycoses

The dermis and subcutaneous tissues can be infected by a variety of fungal agents that are directly implanted into the skin by punctures with sharp objects contaminated by the organisms. Organisms causing the deep cutaneous and subcutaneous mycoses are listed in Table 2.

Sporotrichosis

This condition is generally caused by accidental implantation of the causative fungus *Sporothrix schenckii* into the skin. The lesions most often consist of cutaneous and subcutaneous nodules extending up the limb from the site of inoculation. However, spread may occur through the lymphatics or blood vessels, causing infections of the bones, joints, or other organs. It is also possible to develop lesions in the lungs by inhalation of the fungal elements. The causative organism is a dimorphic fungus that exists as either hyphae or elongated yeast cells.

The most common reservoir of the fungus in nature is on vegetation, although it may also be found in the soil. Rose bushes often have the organism, and inoculation by rose thorn punctures is common. The site of implantation may develop into a papule or pustule and cutaneous nodules may then develop proximally in a linear fashion. If the fungus is inhaled, it may cause a granulomatous pneumonitis that can cavitate and produce a clinical picture similar to tuberculosis. Immunosuppressed patients are more likely to develop disseminated disease.

Demonstration of the characteristic small, cigar-shaped yeast cells is diagnostic but often difficult. Multiple sections may have to be examined. Asteroid bodies are stellate, periodic acid–Schiff (PAS)-positive eosinophilic material that surround the organisms. The diagnosis of sporotrichosis is best made by culture of material from the lesions on appropriate fungal media. Isolation of this organism is usually indicative of sporotrichosis in that the fungus is not part of the normal flora of humans. Iodides may be given for cutaneous sporotrichosis, with oral intraconazole or fluconazole being used if these measures fail. For disseminated disease, either amphotericin B or itraconazole is generally effective, although relapse is common.

Chromoblastomycosis

Certain species of the dematiaceous (darkly pigmented) fungi can cause chronic cutaneous and subcutaneous infections. A number of genera can be involved, but *Fonsecaea*, *Phialophora*, and *Cladophialophora* are most common. The dark pigment of these organisms is dihydroxyphenylanine melanin also associated with *Cryptococcus neoformans*. Dematiaceous fungi can also cause mycetoma and phaeohyphomycosis (see below). Chromoblastomycosis is characterized by the presence of sclerotic (muriform) bodies in the tissues. Chromoblastomycosis usually results from implantation of the organisms during local trauma, generally to the feet or legs. Usually, the first lesion is an erythematous papule, followed by scaling and crusting, with eventual development into a warty structure. The pathology is characteristic of a suppurative granuloma, often with overlying pseudoepitheliomatous hyperplasia. The distribution of cases is worldwide, although most come from Central and South America.

The finding of the characteristic cross-walled, pigmented sclerotic bodies is pathognomonic of chromoblastomycosis. However, since those formed by all the relevant dematiaceous fungal species are similar, culture of the infecting organism on fungal media containing cycloheximide and antibiotics is necessary to identify it. Treatment may be difficult in that the organisms may not be sensitive to antifungal agents. Surgery or local heat may be other options in the early stages of the disease.

Phaeohyphomycosis

Dematiaceous fungi can also cause this closely related condition, with *Exophiala jeanselmei*, *Wangiella dermatitidis*, and *Bipolaris sp*. being most common. These infections usually begin as papules with indolent and painless spread into the subcutaneous tissue, sometimes with cyst formation. However, especially in immunosuppressed patients, the organisms can spread to sinuses, lungs, and brain; it should be noted that brain abscesses have occurred in immunocompetent patients. Surgical debridement may be necessary for cure of these infections. In deep infections such as brain abscess, amphotericin B has been used with some success. Otherwise, the newer azoles such as voriconazole or posaconazole may have a role also.

Table 2 Deep cutaneous and subcutaneous mycoses and the most common responsible pathogens

Type of infection	*Pathogens*
A. Sporotrichosis	Sporothrix schenckii
B. Chromoblastomycosis	Fonsecaea pedrosoi, F. compacta, Cladophialophora carrionii Phialophora verrucosa
C. Phaeohyphomycosis	Exophiala jeanselmei, Wangiella dermatitidis, Bipolaris sp.
D. Eumycotic mycetoma	Pseudoallescheria boydii, Madurella mycetomatis, M. grisea, Acremonium spp.Leptosphaeria senegalensis, Aspergillus nidulans
E. Endemic fungal infections	Blastomyces dermatitidis, Coccidioides immitus, Histoplasma capsulatum, Paracoccidioides braziliensis
F. Infections with immunosuppression	Cryptococcus neoformans, Trichosporon beigelii, Aspergillus spp., Zygomycetes (Rhizopus spp. and Mucor spp.), Fusarium spp., Blastoschizomyces capitatus, Penicillium marneffei

Mycotic mycetoma

Mycetomas are swellings with draining sinuses and grains. They usually affect the feet, legs, or hands and begin with direct implantation of the causative organisms. The latter are either actinomycetes (actinomycotic mycetoma) or the true fungi (eumycotic mycetoma). About half of the cases of mycetoma are caused by true fungi, including the genera *Madurella*, *Leptosphaeria*, *Pseudoallescheria*, *Acremonium*, plus several others. Initially, pain and discomfort develop at the implantation site, followed weeks or months later by induration, abscess development, granulomas, and draining sinuses. The lesions may extend to bone and cause severe bony destruction. Eumycotic mycetomas are rare in the United States, although those caused by *Pseudallescheria boydii* do occur there.

Specimens of exudates or biopsy material should be examined by the naked eye for the presence of grains. The latter can be Gram-stained and examined microscopically to differentiate actinomyctic from eumycotic mycetoma. The grains should be washed with saline containing antibacterial compounds and then cultured on Sabouraud's dextrose agar containing chloramphenicol and cycloheximide, as well as on media for bacterial and actinomycotic organisms. Identification of the organisms is based on gross colonial morphology, pigmentation, and mechanism of conidiogenesis. Treatment of eumycotic mycetoma is often unsatisfactory because the causative organisms generally show poor sensitivity to available antifungal agents. Amputation of an infected limb or surgical debridement of infected tissue may be necessary. Amphotericin B or azole antifungal drugs can be used if the particular fungal strain is sensitive. If not treated effectively, mycetomas may progress for years and produce marked tissue damage, deformity, and even death.

Systemic Mycoses with Cutaneous Manifestations

A number of deep fungal infections may produce cutaneous lesions as part of a disseminated disease process. In these infections, the portal of entry is usually the lung, with development of a pneumonia and spread to other organs. Dissemination is most likely to occur in patients with compromised host defenses. This process is different from that discussed in the last section wherein direct implantation into the skin or subcutaneous tissues is the usual mode of entry. The organisms causing systemic infections with spread to the skin are listed in Table 2; they include both the endemic fungi and opportunistic fungal organisms that infect immunosuppressed patients.

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Cyanobacteria[☆]

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Glossary

Benthos Collection of organisms living on or within the sediments of water bodies.
Monophyletic A taxon or group of organisms that contains all extant descendants of a common ancestor.
Oxygenic photosynthesis Type of metabolism based on the coordinated action of two photosystems by means of which radiant energy is converted into chemical energy in the form of ATP, and reduction equivalents are obtained in the form of NADPH from the (photo)oxidation of water to free molecular oxygen.
Phycobilisome Macromolecular aggregates serving as antenna systems for the capture of light in photosynthesis, typical of cyanobacteria. They are composed of multimers of different phycobiliproteins and of linker polypeptides. Phycobiliproteins are polypeptides containing covalently bound open tetrapyrroles chromophores: the Phycobilins.
Plankton Collection of organisms living suspended in open waters. According to their function, oxygenic phototrophic organisms in the plankton are termed phytoplankton. By an unorthodox convention, planktonic organisms smaller than 2 μm in size are called picoplankton.
Sheath Structurally well defined, usually laminated, extracellular polysaccharide investment. The term is usually, but not exclusively, applied to those of filamentous cyanobacteria.
Trichome Row of vegetative cells in filamentous cyanobacteria, excluding the extracellular polysaccharide structures (sheaths). The term is complemented by 'filament', which includes both the trichome and the sheaths.

Defining Statement

Cyanobacteria are a monophyletic, ancient, morphologically diverse, and ecologically very important group of bacteria, most of which carry out water-oxidizing, oxygen-evolving, plant-like photosynthesis. With few exceptions, they synthesize chlorophyll *a* as the major photosynthetic pigment and phycobiliproteins as light-harvesting pigments. They typically fix CO_2 as the sole source of carbon using primarily the reductive pentose phosphate pathway.

Introduction

Cyanobacteria constitute a phylogenetically coherent group of evolutionarily ancient, morphologically diverse, and ecologically important bacteria. The majority of strains carry out oxygenic photosynthesis (water-oxidizing, oxygen-evolving, plant-like photosynthesis), although some intracellular symbionts have lost this ability. With few exceptions, they synthesize chlorophyll *a* as the major photosynthetic pigment and phycobiliproteins as light-harvesting pigments. They are able to grow using CO_2 as the sole source of carbon, which they fix using primarily the reductive pentose phosphate pathway. Their chemoorganotrophic potential is restricted to the mobilization of reserve polymers (mainly glycogen) during dark periods, although some strains are known to grow chemoorganotrophically in the dark at the expense of external sugars. As a group, they display some of the most sophisticated morphological differentiation among the bacteria, and many species are truly multicellular organisms. Two recently recognized bacterial groups, the *Melainabacteria* and *Sericytochromatia*, are their closest non-phototrophic relatives, which some consider members of a single phylum, rather than sister groups to the cyanobacteria. Cyanobacteria have left fossil remains as old as 2000–3500 million years, and they are believed to be ultimately responsible for the oxygenation of Earth's atmosphere. During their evolution, through an early symbiotic partnership, they gave rise to the plastids of algae and higher plants. Today cyanobacteria make a significant contribution to the global primary production of the oceans and are locally dominant primary producers in many extreme environments, such as hot and cold deserts, hot springs, and hypersaline environments. Their global biomass has been estimated to exceed 10^{15} g of wet biomass, most of which is accounted for by

[☆]*Change History*: March 2019. Tanya Soule and Ferran Garcia-Pichel updated the text and Further Reading. This is an update of Garcia-Pichel, F., 2009. Cyanobacteria. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology, third ed. Academic Press. pp. 107–124. ISBN 9780123739445. doi:10.1016/B978-012373944-5.00250-9.

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the marine unicellular genera *Prochlorococcus* and *Synechococcus*, the filamentous genus *Trichodesmium* (a circumtropical marine form), as well as the terrestrial *Microcoleus vaginatus* and *Chroococcidiopsis* sp. of barren lands. Blooms of cyanobacteria are important features in the ecology and management of many eutrophic fresh and brackish water bodies. The aerobic nitrogen-fixing capacity of some cyanobacteria makes them important players in the biogeochemical nitrogen cycle of tropical oceans, terrestrial environments, and in some agricultural lands. Because of their sometimes large size, their metabolism, and their ecological role, the cyanobacteria were long considered algae; even today it is not uncommon to refer to them as blue-green algae, especially in ecological studies.

With the possible exceptions of their capacity for facultative anoxygenic photosynthesis, and a group of symbionts of marine unicellular eukaryotes which lack crucial photosynthetic machinery, cyanobacteria in nature are known for being oxygenic photoautotrophs. It can be logically argued that after the evolutionary advent of oxygenic photosynthesis, the evolutionary history of cyanobacteria has been one geared toward optimizing and extending this metabolic capacity to an increasingly large number of habitats. This article provides an overview of the characteristics of their central metabolism and a necessarily limited impression of their diversity. Generalizations might, in the face of such diversity, easily become simplifications. Whenever they are made, the reader is reminded to bear this in mind.

Biodiversity: Taxonomy and Phylogeny

Taxonomy

For the newcomer, the taxonomy of cyanobacteria can easily become a nightmare; for the initiated, it is a persistent headache. Since in many cases cyanobacteria are indistinguishable ecologically from eukaryotic microalgae, they had been studied mostly by botanists. The epithets blue-green algae, Cyanophyceae, Cyanophyta, Myxophyceae, and Schizophyceae all apply to cyanobacteria. The botanical taxonomy was built using overwhelmingly morphological criteria based on observations from natural samples. There are actually two parallel botanical taxonomic treatments that exist. The 'Geitlerian' system, recognizing approximately 1300 species grouped in 145 genera and three orders, has been the most widely accepted, and has been the subject of more modern updates and modifications in the 'Anagnostidis/Komarek' system. The Drouet system represented an attempt to arbitrarily simplify the previous one, recognizing only 62 species in 24 genera. It was never judged appropriate by most taxonomists, but it was welcome by many biochemists and physiologists because of its ease of use. Many names of laboratory strains stem from Drouet's system, such as *Anacystis nidulans* (also known as *Synechococcus*), the *Escherichia* coli of cyanobacteria. The prokaryotic nature of cyanobacteria began to be fully recognized in the 1970s, when a taxonomic system based on the International Code of Nomenclature of Bacteria was initiated. This system relies on the study of cultured axenic strains, and it draws heavily on morphological and cytological information, but integrates some genetic and physiological traits as well. The maximal taxonomic resolution so far achieved in this system is at the genus level. The current state of the bacteriological taxonomy of the cyanobacteria is described in the second edition of the *Bergey*'s *Manual of Determinative Bacteriology*. Agreement exists that eventually both systems should converge, but to date they coexist with their own advantages and shortcomings. One is left with the choice of either using a system that allows identification of species but is largely unreliable or using a more reliable system in which species are yet to be defined. To complicate the matter further, recently described nonphototrophic *Melainabacteria* and *Sericytochromatia* are placed within the phylum by some authors. Until the issue is resolved, and for the sake of clarity, we use the term cyanobacteria as a synonym of oxyphotobacteria. The diagnostic key to the cyanobacterial subsections (a taxon akin to Order), as given in the *Bergey's Manual*, is reproduced in Table 1. Most of the so-called form genera recognized within each subsection can be found in Fig. 1, in which they have been gathered according to some simple, mostly visual keys. This unavoidably abridged overview is not intended as a substitute for more thorough generic descriptions. In this article, for consistency, taxa not recognized in the *Bergey's Manual* appear in quotation marks, without intended detriment to their validity.

Table 1 Diagnostic key to the subsections of the cyanobacteria, according to the *Bergey*'s Manual of Determinative *Bacteriology*

Subsection (traditional order)	*Definitory criteria*
Subsection I (Chroococcales)	Unicellular, nonfilamentous. Cells occurring singly or in aggregates. Cell division by binary fission in 1, 2, or 3 planes, symmetric or asymmetric, or by budding
Subsection II (Pleurocapsales)	Unicellular, nonfilamentous. Cells occurring singly or in aggregates. Reproduction by multiple fission without growth, yielding beaocytes (cells smaller than the parent cell), or by binary and multiple fission
Subsection III (Oscillatoriales)	Filamentous, binary fission in one plane, yielding uniseriate trichomes without true branching. No heterocysts or akinetes formed
Subsection IV (Nostocales)	Filamentous, division occurring only in one plane to yield uniseriate trichomes without true branching. Heterocysts formed when combined nitrogen is low
Subsection V (Stigonematales)	Filamentous, division occurring periodically or commonly in more than one plane, yielding multiseriate trichomes, truly branched trichomes, or both. Heterocysts formed when combined nitrogen is low

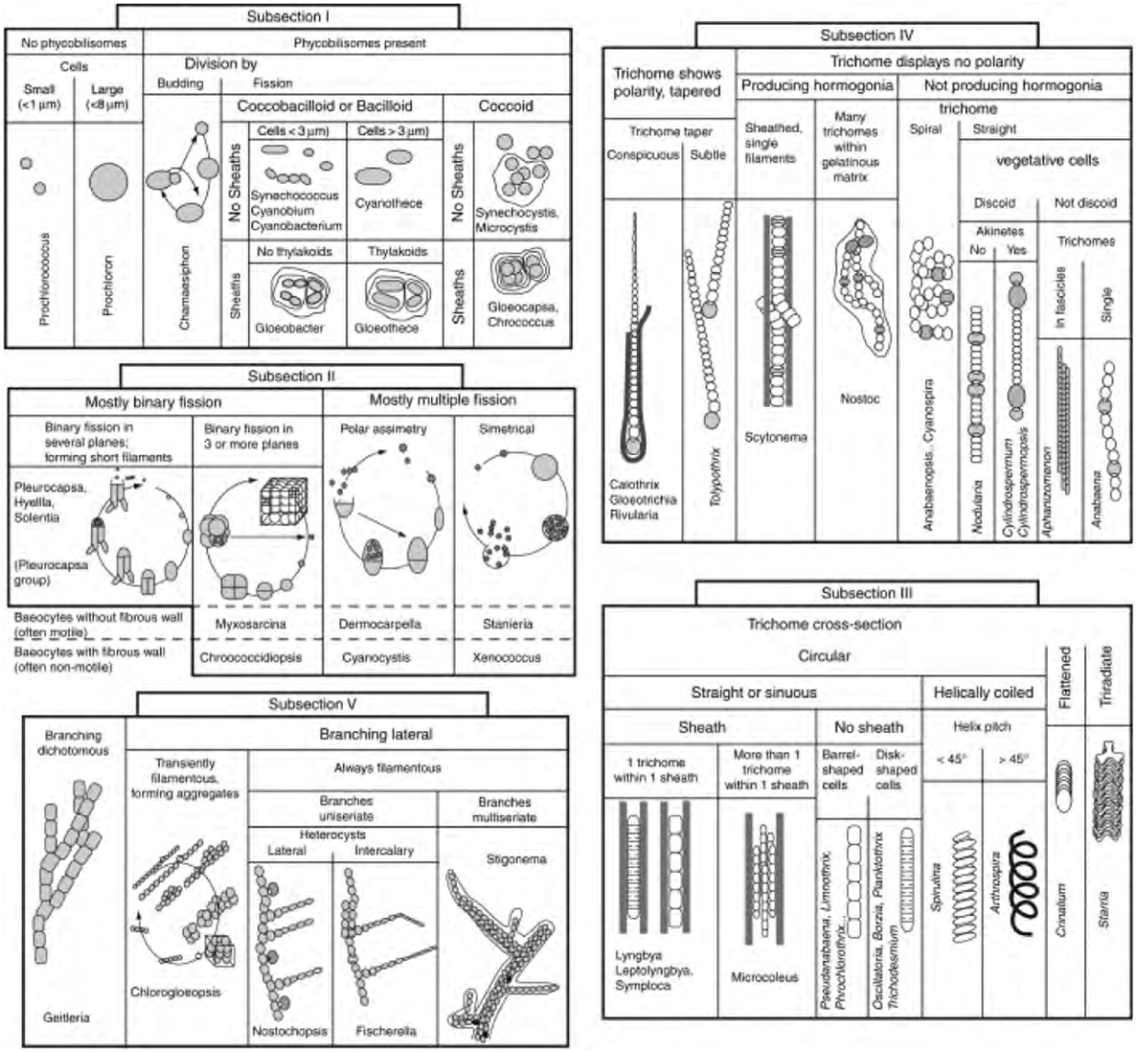

Fig. 1 Genera (form genera) recognized in the *Bergey*'s Manual of Determinative *Bacteriology* within each of the five subsections of the cyanobacteria. Genera have been arranged according to the most important (abridged) definitory criteria, and drawings are provided depicting their simplified morphological appearance and/or their life cycles.

Phylogeny

The most widely accepted reconstructions of cyanobacterial phylogeny are those based on comparisons of the 16S ribosomal RNA gene sequences. The salient traits of cyanobacterial ribosomal phylogeny have found support when other genes or multilocus analyses have been carried out, even with large sets of genes. Currently, several characteristics of cyanobacterial evolution can be inferred, and some taxonomic controversies have been settled. However, several apparent paradoxes have also been unveiled. Extreme caution should be exercised in the interpretations of phylogenetic trees of cyanobacteria because the nomenclatural chaos has already pervaded the molecular databases. In Fig. 2, a phylogenetic tree for the cyanobacterial radiation based on 16S ribosomal RNA gene sequences is presented, color coded to indicate the subsections of Fig. 1. The cyanobacteria are a diverse phylum, and studies support clearly the endosymbiotic theory for the origin of plant chloroplasts because they place plastids (from all eukaryotic algae and higher plants investigated) in a very diverse but monophyletic deep-branching cluster within the cyanobacterial radiation. However, the gross structure in the evolutionary history of extant cyanobacteria cannot be easily resolved; most of the cyanobacterial diversity probed seems to be due to an explosive radiation that took place early during their evolution. It seems clear that the current taxonomic treatment of the cyanobacteria diverges considerably from a natural system that reflects their evolutionary relationships. For example, subsections I, III, and II (orders Chroococcales, Oscillatoriales, and Pleurocapsales, respectively) do not find support in the trees as coherent units. The heterocystous cyanobacteria (subsections IV and V; Nostocales and Stigonematales,

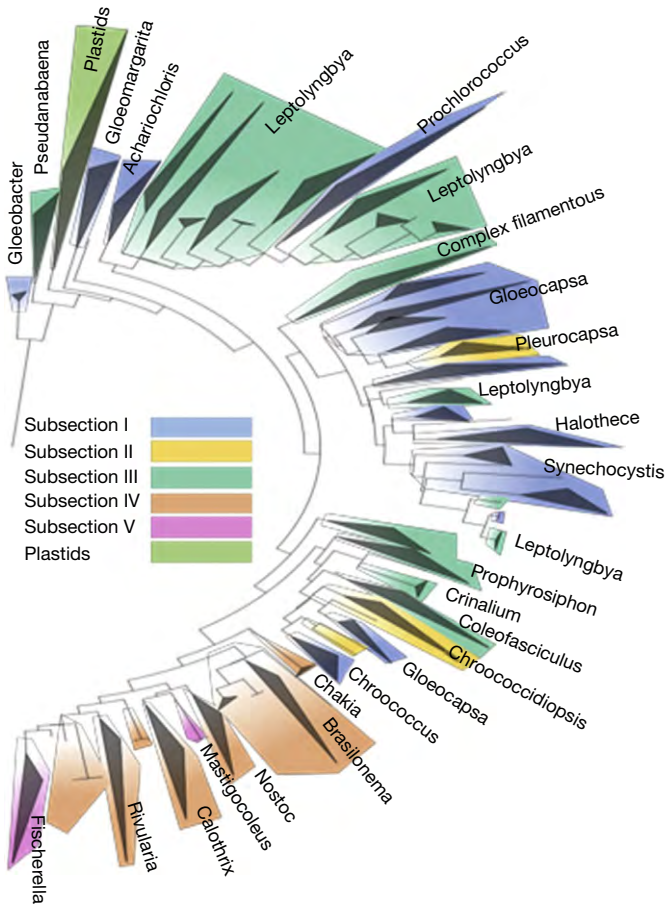

Fig. 2 Phylogenetic tree of the cyanobacteria based on (complete) 16S ribosomal RNA sequences obtained from the Cydrasil open resource (https://github.com/FGPLab/cydrasil). Representative generic names have been added to many of the branches, but the list is not complete. Many morphogenera (i.e., *Leptolyngbya*) encompass a large diversity of phylogenetically unrelated clades, and the classification based on traditional subsections finds no support in phylogeny.

respectively) together form a monophyletic group with relatively low sequence divergence, but they are not monophyletic in their own right. Several other statistically well-supported groups of strains can be distinguished that may or may not correspond to currently defined taxa. With the advent of high-throughput sequencing, phylogenetic analyses using hundreds of protein-encoding genes are now possible. These phylogenies are largely congruent with those formed using 16S ribosomal RNA gene sequences. The picture that seems to emerge from these studies is that ecology and physiology are extremely important parameters to understanding the phylogenetic relationships of cyanobacteria and to achieving an evolutionarily coherent taxonomic system. Reaching this goal will necessitate continued efforts in the future.

Cytology and Morphogenesis

Cytology and Ultrastructure

Cyanobacterial cells range in width between 0.5 μm (e.g., *Prochlorococcus*) and 50–100 μm (e.g., some '*Chroococcus*' and some *Oscillatoria*); the modal size of the described species is significantly larger than that of most other bacteria and archaea (~4 μm). In unicellular and colonial forms, cells may be spherical, bacilloid, or fusiform, and some strains present considerable pleiomorphism. Cells of filamentous cyanobacteria may range from discoid to barrel-shaped, and the trichomes often attain lengths on the order of millimeters. The filamentous genus *Starria* has triradiate cells. Several types of cells may be present in morphologically complex cyanobacteria. The cells of most cyanobacteria are surrounded by a more or less-defined exopolysaccharide investment. In some species this may form a distinct, structured capsule or sheath, where the steric constrictions to cell growth imposed by the presence of mechanically strong capsules or sheaths may even dictate cellular shape (Fig. 3(p)). Several ultrastructural features are typical for cyanobacteria. The cell envelope is of a Gram-negative type but may attain a considerable thickness in the peptidoglycan layer (from several to 200 nm). Pores of different sizes, whether or not orderly arranged, perforate the cyanobacterial cell wall. Pore pits may allow close contact of the cytoplasmatic membrane with the lipopolysaccharide outer membrane. The photosynthetic machinery of cyanobacteria resides on intracellular membranes (thylakoids). Each thylakoid consists of a double-unit membrane enclosing an

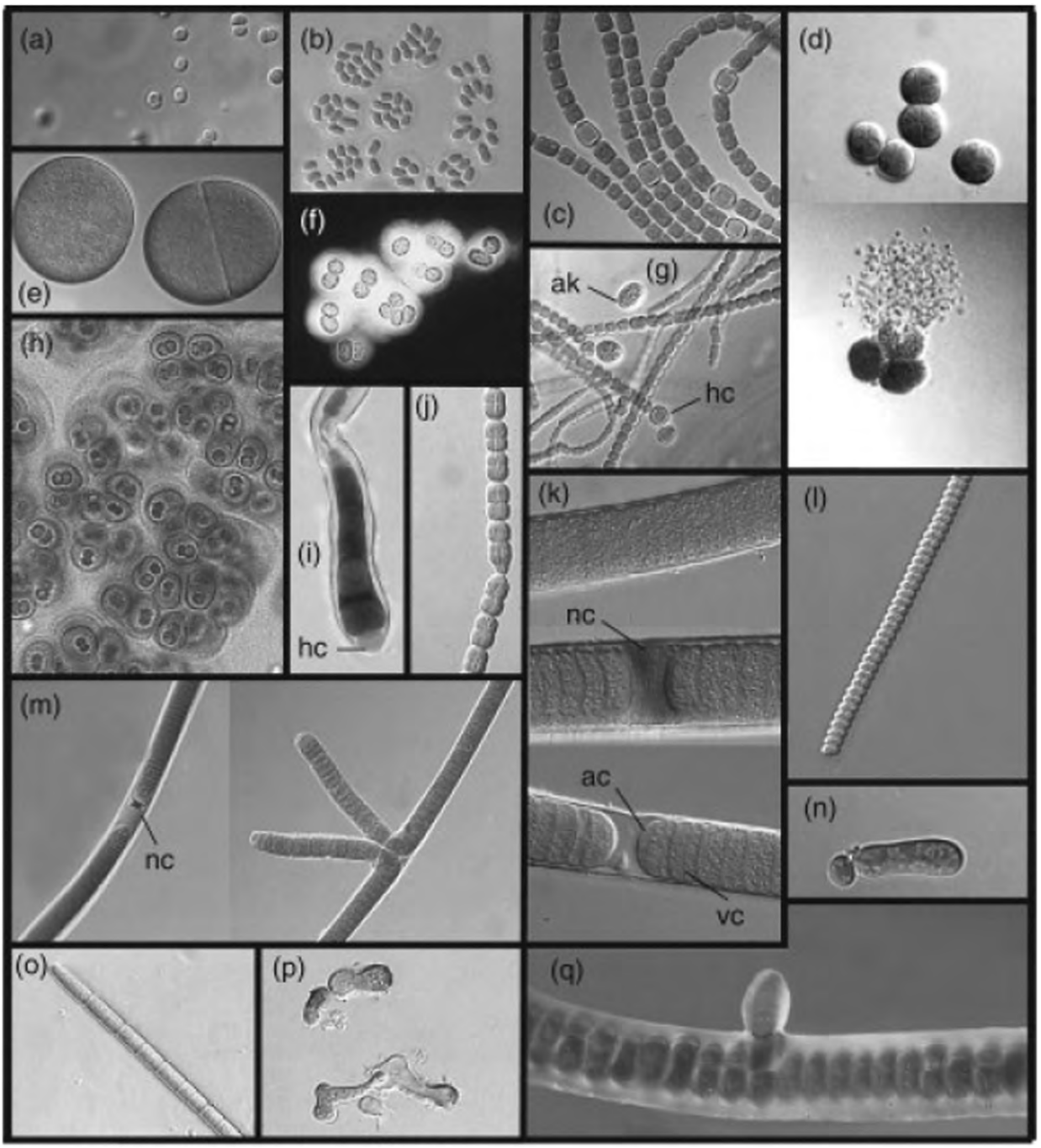

Fig. 3 Aspects of morphological diversity and morphogenetic processes in cyanobacteria. (a) Coccoid cells of *Synechocystis* sp. strain PCC 6803, one of the most widely studied cyanobacteria. (b) Loosely aggregated colonies of a unicellular, extremely halotolerant cyanobacterium belonging to the Halothece cluster. (c) Filamentous *Anabaena* with intercalary heterocysts (hc). (d) Formation and extrusion of baeocytes in unicellular colonial cyanobacteria after repeated rounds of division without growth (sequence from top to bottom). (e) *Chroococcus turgidus*, the largest unicellular cyanobacterium known with right cell undergoing fission. (f) Negative stain (India ink) of a colony of *Gloeothece* sp., rendering the diffuse extracellular polysaccharide investments visible. (g) Cell differentiation in *Nostoc* showing vegetative barrel-shaped cells, heterocysts (hc), and akinetes (ak). (h) Field sample of *Gloeocapsa sanguinea*, with red-stained, well-structured extracellular polysaccharide sheaths. (i) A young filament of *Calothrix*, displaying a terminal heterocyst (hc) and a tapering trichome. (j) Filament of a *Pseudanabaena*-like, *Konvophoron*, with deep constrictions at cross walls and displaying trichome division by direct differentiation of mamillated (nipple-bearing) apical cells without mediation of necridia. (k) Trichome division in a *Lyngbya*-like (*Porphyrosiphon*) filamentous strain. The top-to-bottom sequence displays the formation of necridial cells (nc), their degradation, and the formation of two rounded apical cells (ac), different from the discoid intercalary cells and a leftover vestigial cell. (l) Helically coiled filament of *Spirulina*. (m) Heterocystous *Scytonema* showing the formation of false branches, as recently divided trichomes (see vestigial necridium, nc) break free from the sheath and continue to grow (not glide). (n) Asymmetric division (budding) in unicellular cyanobacteria of the genus *Chamaesiphon*. (o) Single trichome of *Microcoleus chthonoplastes*, with slight constrictions at the cross walls and bullet-shaped apical cell. (p) Pleiomorphy in a unicellular cyanobacterium, *Cyanophanon*, morphology being a result of steric constrictions to growth by their tough sheath. (q) Biseriate filament of *Stigonema*, with a true lateral branch.

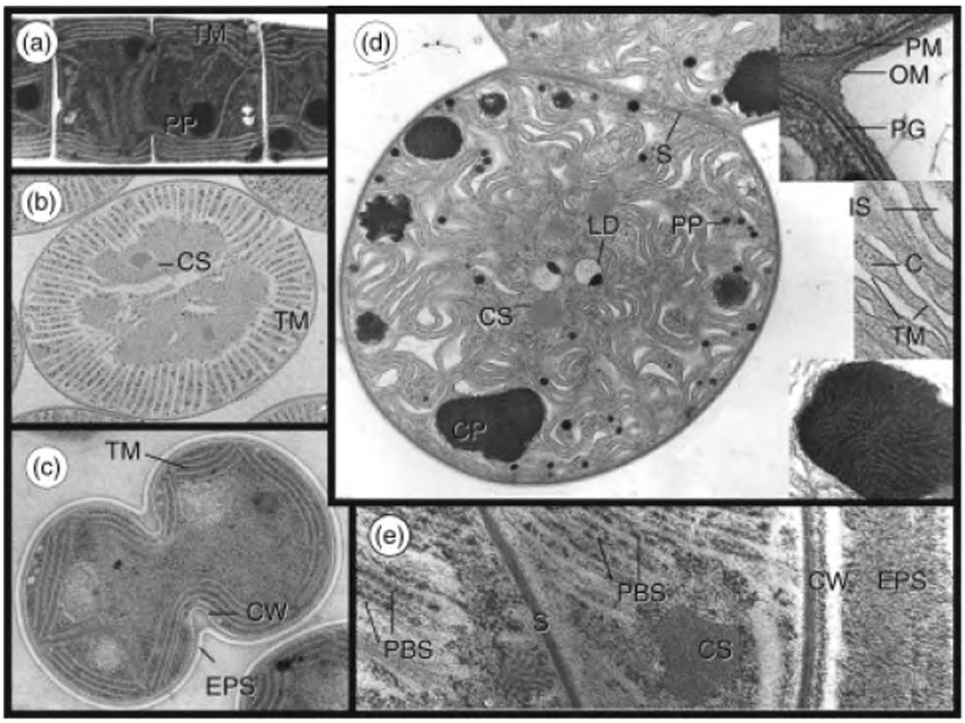

Fig. 4 Cyanobacterial ultrastructure as seen by transmission electron microscopy. (a) Longitudinal section through a trichome of *Oscillatoria* '*lacus-solaris*', showing thylakoid membranes parallel to the cell wall (TM), and large polyphosphate (PP) granules, as well as incipient septa (cross-walls). (b) Traversal section through a trichome of *Microcoleus chthonoplastes* showing radial, peripheral arrangement of thylakoids, a clear centrosome containing carboxysomes (polyhedral bodies, CS). (c) Dividing *Synechocystis* strain PCC6803, showing clearly equatorial invagination of all tegumentary layers including exopolysaccharide (EPS) and cell wall (CW), and parallel sets of thylakoids membranes. (d) End cell in a *Nostoc pruniforme* trichome, with characteristic cyanophycin granules (CP), carboxysomes (CS), polyphosphate granules (PP), unordered arrangement of thylakoids, lipidic droplets (LD), and septum or cross wall (S). The top insets show a detail of the tegumentary layers around the cross wall constrictions, with the plasma membrane (PM), and the peptidoglycan wall (PG) entering to form the septum, to the exclusion of the outer membrane (OM). The middle inset shows a thylakoid-rich region with thylakoid membranes (TM), intrathylakoidal space (IS), and the cytoplasm (C). The bottom inset shows a cyanophycin granule. (e) Outer regions in a *Oscillatoria* filament with, from right to left, exopolysaccharide (EPS) layer, cell wall (CW), a carboxysome (or polyhedral body, CS), rows of phycobilisomes (PBS) sitting on the cytoplasmatic side of the thylakoid membranes, and a septum (S). Unpublished photomicrographs are from Mariona Hernández-Mariné (University of Barcelona, all but (c)) and Robert Roberson (Arizona State University, (c)), with permission.

intrathylakoidal space (Fig. 4(d)). Thylakoids may be arranged parallel to the cell membrane, radially, or in small disorderly stacks, depending on species (Fig. 4). They may be single or stacked, usually depending on illumination conditions. *Gloeobacter*, a representative of the most ancient cyanobacterial lineage, has no thylakoids and the photosynthetic apparatus resides in the plasma membrane. The phycobilisomes, used by most cyanobacteria as light-harvesting structures, can be distinguished in electron microscopic preparations as rows of particles ~50 nm in diameter on the cytoplasmatic side of the thylakoids (Fig. 4(e)). These are obviously absent from chlorophyll *b*-containing, phycobiliprotein-lacking species (*Prochlorococcus*, *Prochloron*, and *Prochlorothrix*). A central electron-clear region in the cell, the nucleoplasm, or the centroplasm, hosts the cellular DNA. Several intracellular nonmembrane-bound granules typically correspond to polymeric reserve materials, such as glycogen (polyglucose, usually present in the intrathylakoidal space), polyphosphate, poly-β-hydroxyalkanoates, and lipid droplets found in the cytoplasm proper, which are also common in other bacteria (Fig. 4). Cyanophycin (multi-L-arginyl-poly(L-aspartic acid)) is a cytoplasmatic, exclusively cyanobacterial nitrogen reserve polymer. Carboxysomes (or polyhedral bodies) are commonly seen as membrane-bound intracellular inclusions, and they consist of accumulations of the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), responsible for the initial carboxylation step in the Calvin cycle (see 'Dark reactions of photosynthesis: carbon fixation and uptake'), bound by a proteinaceous, viral capsid-like case (Fig. 4(e)). Gas vesicles are air-filled, cylindrical proteinaceous structures present in many planktonic species and in the dispersal stages of benthic forms; they provide buoyancy to the organisms. A large variety of ultrastructural inclusions of restricted occurrence are also known from particular species or strains.

Growth and Cell Division

Some strains cultured under optimal conditions display doubling times as short as several hours. In nature, one or more doublings per day is common in blooming planktonic populations, and cell divisions may be strongly governed by daily periodicity. Under

extreme conditions in the natural environment, net growth is sometimes best measured in units of percentage increase in biomass per year. Many cyanobacteria owe their ecological dominance and success not so much to the fast growth as to their ability to grow slowly and steadily in the face of environmental adversity. Slow growth rates may be an integral, genetically fixed part of the biology of many cyanobacteria. Cyanobacteria cells divide basically by fission, but the patterns of cell division, which vary, form the base of a morphological diversity unparalleled among prokaryotic organisms (Fig. 1). It is surprising that, because morphological complexity is such a unique cyanobacterial trademark in the bacterial world, our knowledge of the regulation of cell division patterns and morphogenetic implications at the biochemical and molecular level is virtually nonexistent. The extracellular slime layers or sheaths may contribute considerably to morphogenesis in some cyanobacteria by simply holding cells together after division in disorderly colonies (as in *Microcystis*), by holding filaments together into bundles (as in *Microcoleus*), or by allowing a localized extrusion of trichomes that result in 'false branching' (as in *Scytonema*; Fig. 3(m)).

Unicellular and Colonial Types

Cell division occurs here by inward growth of all tegumentary structures (cytoplasmatic membrane, cell wall, outer membrane, and slime sheath), usually at an equatorial position (Fig. 4(c)). Asymmetrical division in one pole of unicells may result in small-sized daughter cells (budding; Fig. 3(n) and (p)), as in *Chamaesiphon*. A genetic capacity to alternate between two or three orthogonal division planes yields spatially ordered colonial forms, as in *Merismopedia* (planar colonies – two alternating division planes) or in *Myxosarcina* (cubical colonies – three alternating division planes). Multiple divisions occurring in the absence of cell growth result in the formation of a multiplicity of minute daughter cells (baeocytes) that eventually break free from the cell wall remains of the parental cell, as in all 'pleurocapsalean' (subsection III) cyanobacteria (Fig. 3(d)).

Filamentous Types

Repetitive division in a single plane without complete cell separation yields simple filamentous forms. The filamentous nature of some cyanobacteria may simply be due to this fact and may not necessarily imply a functional integration of the cells into a truly multicellular organism. In morphologically complex filamentous forms, however, the outer tegumentary layers may be continuous along the trichome. The formation of cross walls or septa may involve the invagination of the plasma membrane and the cell wall only, as has been shown in *Nostoc* (Fig. 4(d)), resulting in a continuous periplasm along the trichome. Septal junctions, analogous to metazoan gap junctions, then mediate intercellular molecular transfer by traversing the peptidoglycan of adjacent cells. In filamentous oscillatorian cyanobacteria, one or more tegumentary invaginations (future cross walls) may be initiated before cell division is completed. A widespread change of division plane in the cells along the trichome results in biseriate or multiseriate trichomes (two or more rows of cells). A change in the plane of division occurring in a single cell and maintained for several rounds of fission results in true branching, as in *Stigonema* (Fig. 3(q)). In morphologically complex filamentous forms, cell division may be meristematic, occurring only in certain portions of the trichome (e.g., *Calothrix* and allied forms).

Multicellularity and Cell Differentiation

The magnitude of the morphological complexity evolved within the cyanobacterial radiation is exemplified by the achievement of multicellularity. The heterocystous group (subsections IV and V) and some oscillatorians are clearly not mere linear arrays of cells but truly multicellular organisms, possessing all the attributes required for such a distinction: supracellular structural elements, integrated behavioral responses to environmental stimuli, and distribution of labor through cell differentiation into distinct cellular types. The most common and important examples of cellular differentiation in cyanobacteria are reviewed below in detail, even though adaptation to particular environments have resulted in unique cell-types, such as the calcicytes of endolithic *Mastigocoleus testarum* or the diazocytes of *Trichodesmium*. Additionally, in many cases cyanobacteria form colonies or structurally organized macroscopic bodies (thalli in botanical terminology) that are quite large in size. Spherical colonies of *Nostoc* '*pruniforme*' exceeding 15 cm in diameter are known from the benthos of oligotrophic cold springs, but somewhat smaller, ordered arrangements of cells and filaments are common in other *Nostoc* and '*Aphanothece*' species as well as in *Calothrix* and allied genera (e.g., '*Rivularia*'). This metadifferentiation is seldom achieved in culture.

Hormogonia

Hormogonia (s. hormogonium) are short chains of ~5–25 cells formed and released from the parental, larger trichome. They serve a function in the dispersal of the organism, often in response to hormogonium-inducing factor secreted by plant host cells initiating symbiotic relationships with nitrogen-fixing cyanobacteria. Hormogonial cells may or may not be different in size and shape from vegetative cells. Detachment may involve the differentiation of a necridic cell separating the vegetative trichome from the hormogonium. Dispersal is aided by the expression of phenotypic traits, which may vary according to strains, such as gliding motility, development of gas vesicles, or change in surface hydrophobicity. Hormogonia eventually settle and dedifferentiate into a typical vegetative organism, which may subsequently differentiate into a heterocyst for a symbiotic relationship with a plant host. Transcriptomic analysis of cells differentiating into hormogonia in *Nostoc punctiforme* PCC 73102 resulted in differential expression of about 25% of the genes in the genome, many of which are of unknown function.

Heterocysts

Heterocysts (Fig. 3(c), (g), and (i)) are morphologically distinct cells that develop in response to a lack of combined nitrogen sources in the environment. The ability to develop heterocysts occurs without exception within a monophyletic group of filamentous cyanobacteria (heterocystous; subsections IV and V). They are usually larger than vegetative cells, develop thick tegumentary layers and intracellular hyaline buttons at the points of attachment to the vegetative cells, and display a pale coloration and reduced autofluorescence. As such, heterocysts are easy to recognize under the microscope. They may differentiate from end cells (terminal heterocysts, as in *Calothrix*) or from cells within the trichome (regularly spaced intercalary heterocysts, as in *Anabaena*, or lateral as in '*Mastigocoleus*'). Heterocysts are highly specialized in the fixation of dinitrogen under aerobic conditions. They represent a successful solution to the nontrivial problem of avoiding nitrogenase inactivation by free oxygen in oxygen-evolving organisms. Heterocyst biology has been relatively well studied at the biochemical and molecular levels. Heterocysts are the only cells that express *nif* (nitrogen fixation) genes and synthesize nitrogenase in heterocyst-forming cyanobacteria. Heterocysts do not evolve oxygen themselves (photosystem II (PSII) activity is absent or restricted) but a functional photosystem provides ATP. The source of reductant for nitrogen fixation is provided (as organic carbon) by the adjacent vegetative cells, which in turn obtain fixed nitrogen from the heterocyst in the form of amino acids (mostly glutamine). The heterocysts protect their nitrogenase from oxygen inactivation by maintaining reduced internal partial pressures of oxygen, a situation that is attained by means of increased rates of cellular respiration and, apparently, by restricting diffusive entry of oxygen from the environment as a result of their thick envelope. The developmental regulation of heterocysts is beginning to be understood at the genetic level. The autoregulated gene *hetR*, which is activated by a deficiency in combined nitrogen, plays a crucial role in the initiation of heterocyst development. On the other hand, the small peptide PatS inhibits heterocyst formation for the purpose of pattern formation to ensure that intercalary heterocysts are regularly spaced.

Akinetes

These nonmotile cells (Fig. 3(g)) are characterized by their enlarged size with respect to vegetative cells, their thick cell wall and additional tegumentary layers, and their high content of nitrogen reserves in the form of cyanophycin granules. They are formed exclusively by heterocystous filamentous cyanobacteria (but not by all) and they may differentiate *en masse* or at special locations within the filaments (usually close to or next to a heterocyst). Because akinetes are resistant to desiccation, low temperatures (including freeze–thaw cycling), and digestion in animal guts, they are considered resting stages in the life cycle, but they are fundamentally different from typical bacterial endospores in that they are not heat-resistant. Transcriptomic analysis of *Nostoc punctiforme* PCC 73102 cells differentiating into akinetes revealed that many of the down-regulated genes were associated with central metabolism, supporting their role as resting stages of the cell. In natural planktonic populations, massive akinete formation occurs at the end of the growth season. Germination of akinetes occurs when the environmental conditions become favorable for the growth of a vegetative filament. Genetic evidence suggest that the early regulatory process of akinete development and that of heterocysts has a common basis.

Terminal Hairs

These are multicellular differentiations occurring at the tips of trichomes in some members of the genus *Calothrix* and allied cyanobacteria (botanical family Rivularaceae). In response to nutrient limitation (e.g., phosphorous), the terminal parts of the trichome differentiate irreversibly into thin and long rows of narrow, almost colorless, vacuolated cells (hence the term hair). The hair is a site of preferential expression of cell surface-bound phosphatase activity.

Necridic Cells (Necridia)

Necridic cells occur in truly multicellular cyanobacteria (Fig. 3(n) and (k)). Necridic cells undergo a suicidal process (apoptosis), which begins with the loss of turgor and leakage of some cellular contents and continues with shrinkage and the separation of the cross walls (septa) from the adjoining cells. Eventually, the necridic cells will either rupture and disintegrate or remain as small, isolated vestigial cells. Cells adjacent to the necridium will usually develop morphologies typical for terminal (apical) cells. The formation of necridia may lead to the separation of one trichome into two (proliferation) or in the detachment of hormogonia from the vegetative filament. Most of the information about necridia is observational, and no studies have been performed to investigate the regulation of this morphogenetic mechanism.

Physiology and Metabolism

Cyanobacteria are photoautotrophic organisms *par excellence* and their metabolism is typically geared toward anabolic reactions. The basis of their metabolism is the conversion of radiant energy into chemically usable energy and the reduction of CO_2 into organic matter. The electron donor for the reduction of CO_2 is water, which is oxidized to molecular oxygen. The name of this type of metabolism is derived by the release of oxygen: oxygenic photosynthesis. It is exclusively carried out by organisms in the cyanobacterial radiation (cyanobacteria proper and the plastids of algae and plants).

Photosynthesis

In the light reactions of photosynthesis, radiant energy is captured and used to generate energy in the form of ATP and reduction equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH). The light reactions of oxygenic photosynthesis are based on the coordinated action of (1) light-harvesting systems; (2) two chlorophyll *a*-containing, membrane-bound, multisubunit enzymes known as photosystems; and (3) a series of soluble and membrane-bound electron-carrier proteins linking both photosystems (Fig. 5).

Light Harvesting

Light harvesting in most cyanobacteria (and in red algal plastids) is accomplished by highly ordered and structurally versatile supramolecular complexes known as phycobilisomes, which are primarily composed of phycobiliproteins. Phycobiliproteins are water-soluble proteinaceous pigments containing covalently bound, open-chain tetrapyrroles (phycobilines) as chromophores. Universal cyanobacterial phycobilines are the blue-colored allophycocyanin (absorbing maximally at a wavelength of 650 nm) and phycocyanin (maximum at 620 nm). Phycoerythrocyanin (maximum at 575 nm) and the red-colored phycoerythrin(s) (maxima vary from 495 to 560 nm) are also common. Multimeric disc-shaped phycobiliprotein complexes are stacked into either central cores or radially protruding rods (Fig. 5) to form a functional phycobilisome. The radiant energy absorbed by the phycobiliproteins along the rods is channeled vectorially (as excitation energy) toward the core region and from the core onto the reaction center of PSII or (partially) onto that of photosystem I. Phycobiliproteins are arranged orderly within the phycobilisome: the shorter the wavelength of maximum absorbance, the more peripheral their location. This allows for the centripetal channeling of excitation energy down a thermodynamically allowed sequence. In such a light-harvesting system, 300–800 phycobilin chromophores capture additional energy for ~50 Chl *a* molecules associated with PSII. In some strains, the phycobilin composition of phycobilisomes can be regulated to optimize the capture of photons according to the color of the available light, a phenomenon known as complementary chromatic adaptation. Excess light, however, can result in the production of toxic oxidative intermediates. The soluble orange carotenoid protein has been shown to play a critical role in photoprotection by initiating nonphotochemical quenching in the phycobilisome protein complex.

In addition to Chl *a*, some cyanobacteria use far-red (long-wavelength) chlorophylls. Chl *d* is the only other known cyanobacterial chlorophyll to participate in the reaction center. Chl *a* and Chl *d* both contain a similar tetrapyrrole structure with Mg^{2+} but differ in that Chl *a* absorbs maximally in the short-wavelength (visible) spectrum while Chl *d* absorbs in the long-wavelength spectrum (>700 nm). Chl *d* has been largely characterized in *Acaryochloris* where it is serves as the predominant chlorophyll and facilitates light absorption at depths under phytoplankton surface layers which absorb most of the visible light energy. Chl *b*, Chl *c*, and Chl *f* serve as accessory antennae pigments that do not participate in the photosynthetic electron transport chain.

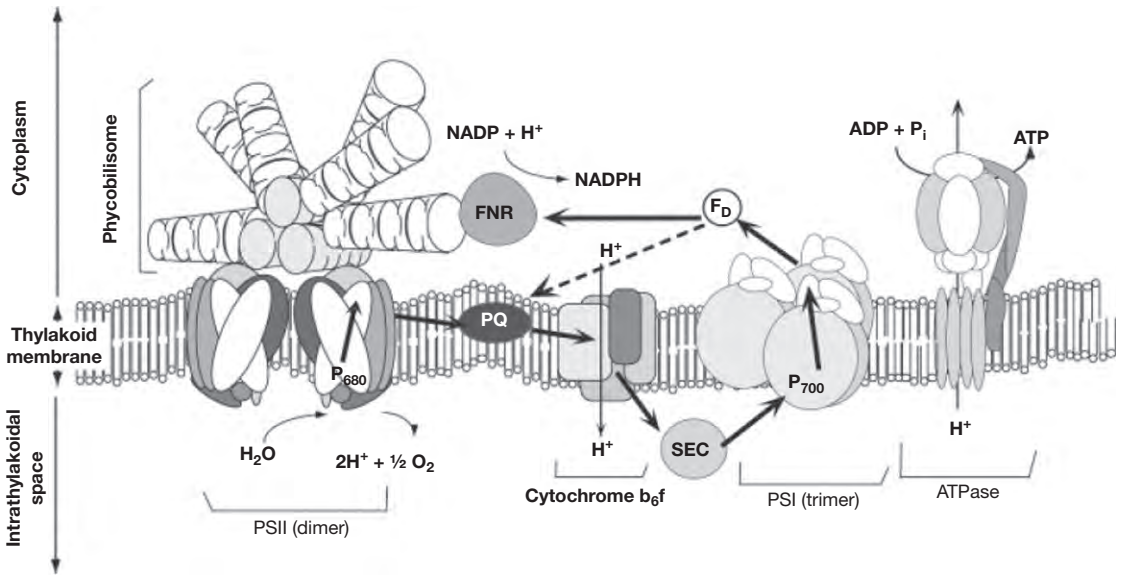

Fig. 5 Idealized organization of the photosynthetic components in and around cyanobacterial thylakoids and their associated activities. Multimeric complexes are indicated by labeled brackets. Abbreviations not explained in text are bound quinone (Q), plastoquinone (PQ), soluble electron carrier (SEC), ferredoxin (FD), ferredoxin/NADP oxidoreductase (FNR). Thick black arrows indicate the direction of electron flow from water to NADPH; dashed arrow depicts the shortcut under cyclic phosphorylation conditions. Thin arrows depict either transformation of chemical reactants or the traffic of protons across the membrane. Modified from Bryant, D.A. (Ed.), 1996. The Molecular Biology of Cyanobacteria. Dordrecht: Kluwer, as cited, with permission.

Light Reactions of Photosynthesis

PSII, which contains a reaction center, known as P680, of very high basal reduction potential (+1 V), catalyzes the transfer of electrons from water to a bound quinone, with the production of O_2. The electrons then enter an electron transport chain involving successive redox reactions of a membrane-bound protein (plastoquinone), a membrane-bound protein complex (cytochrome b_6f), and one of the two intrathylakoidal soluble electron carrier proteins (cytochrome c_{533} or plastocyanin). An electrochemical gradient of protons is created across the thylakoid membrane in the process of electron transport. This is used by the thylakoidal f-type ATPase complex to generate ATP, the cell's energy currency. When excited, PSI, with a reaction center (known as P700) of intermediate reduction potential, catalyzes the reoxidation of reduced plastocyanin (or cytochrome c_{533}) with the concomitant reduction of ferredoxin (a soluble iron–sulfur protein) against a steep thermodynamic gradient. Reduced ferredoxin is used by ferredoxin: NADP+ oxidoreductase (an enzyme physically tethered to phycobilisomes, if present) to generate the NADPH necessary for the dark reactions. In short, the light-driven formation of ATP and NADPH has been achieved. Additionally, electron flow around PSI alone may also occur (cyclic electron transport). In this case, electrons flow from reduced ferredoxin directly to plastoquinone, through the cytochrome b_6f complex and plastocyanin, back to PSI and, with light, to oxidized ferredoxin, closing the cycle. The net effect of the cycle is the generation of energy but no reductant.

Dark Reactions of Photosynthesis: Carbon Fixation and Uptake

The reduction of CO_2 to organic matter (carbon fixation) occurs in all cyanobacteria, mainly through the reductive pentose phosphate (Calvin) cycle, in which the net formation of a triose from $3CO_2$ is powered by ATP and NADPH formed in the light reactions. This cycle supplies important intermediates for anabolic reactions (triose, pentose, and hexosephosphate). Additional CO_2 may be fixed by phosphoenolpyruvate carboxylase, yielding C_4 acids, and by carbamyl phosphate synthetase/carbamylphosphateornithine carbamyl transferase, yielding citrulline and glutamate from glutamine, ornithine, and CO_2. The Calvin cycle is related to the catabolic (oxidative) pentose phosphate pathway, differing in two key enzymes that allow it to function anabolically. These are PRK (phosphoribulosekinase) and RubisCO, a very interesting enzyme and the most abundant protein on Earth. RubisCO is characterized by a low affinity for CO_2 and by possessing internal monooxygenase activity. This results in a competitive inhibition of carboxylation by free oxygen, a fact of obvious importance for oxygen-producing phototrophs. Under conditions of low CO_2 and high O_2 partial pressure, RubisCO catalyzes the oxidation of ribulose bisphosphate to phosphoglycerate and phosphoglycolate. After dephosphorylation, glycolate is excreted by the cells in what seems to be a wasteful loss of carbon. Probably, to prevent conditions leading to such losses, cyanobacteria possess a carbon concentrating mechanism by which inorganic carbon, either as bicarbonate or as CO_2, is active at the expense of energy transported into the cell. This can result in intracellular concentrations of CO_2 1000-fold higher than those outside the cells. A carbonic anhydrase-like enzyme keeps intracellular carbon in the form of bicarbonate to prevent leakage of CO_2. A carbonic anhydrase located within the carboxysomes (or polyhedral bodies, the site of RubisCO accumulation; Fig. 4) generates CO_2. With this system, a high CO_2 partial pressure is maintained locally in close proximity to the carboxylation sites of RubisCO, and the carboxylating activity of the enzyme is promoted.

Dark Metabolism

Energy generation in the dark occurs through aerobic respiration at the expense of glycogen accumulated during the light phase. Monomeric sugars are degraded using the oxidative pentose phosphate cycle. Most cyanobacteria are unique in that they have a non-traditional tricarboxylic acid cycle, in which 2-oxoglutarate is converted to succinate by 2-oxoglutarate decarboxylase and succinate semialdehyde dehydrogenase in the absence of α-ketoglutarate dehydrogenase, which has never been detected in any cyanobacterium. NADPH formed in sugar catabolism is fed to the membrane-bound electron transport chain at the level of plastoquinone. Terminal oxidases are cytochrome oxidases of the aa_3 type. The respiratory electron transport chain of cyanobacteria is housed in both the plasma and the thylakoidal membrane and it shares many functional components with photosynthetic electron transport. Approximately half of all cyanobacterial strains tested are obligate phototrophs, unable to use exogenous carbon sources aerobically. Some are photoheterotrophs, able to use some sugars as a carbon source, and some are facultative heterotrophs, able to grow, albeit slowly, at the expense of externally supplied sugars (usually only one) in the dark. All strains retain pigmentation and all components are necessary for photosynthesis under dark growth conditions. The lack of sugar transport systems has been heralded as one of the main reasons for the inability of many strains to use exogenous sugars while being able to respire endogenous glucose.

Cyanobacteria may also be subject to periods of anoxia, particularly in the dark (e.g., benthic forms thriving in sulfidogenic environments and biofilm or colony formers under diffusion limitations of O_2 supply). The only known electron acceptors alternative to oxygen for cyanobacterial chemoorganotrophy are internal organic compounds and elemental sulfur. Fermentation is not universal but is a relatively widespread ability in benthic and bloom-forming cyanobacteria. As in aerobic heterotrophy, fermentation occurs at the expense of endogenous sugars (usually glycogen but also sugar osmolytes such as trehalose or glucosylglycerol) accumulated in the light period. Some strains ferment, or even grow on, exogenous substrates anaerobically. Homolactic, heterolactic, homoacetate, and mixed-acid fermentation have all been described. There is evidence that the

Embden–Meyerhof–Parnas glycolytic pathway, unoperative for aerobic respiration, is used in the fermentative degradation of sugars by cyanobacteria. Several cyanobacteria, notably *Lynbgya aestuarii*, produce copious amounts of hydrogen gas during the fermentation of photosynthate, a fact that makes them interesting for biotechnology. An *Oscillatoria* strain oxidizes endogenous carbohydrates largely to CO_2 in the presence of elemental sulfur with the concomitant production of sulfide. In other cyanobacteria, sulfur may be used as a sink for electrons, otherwise released as H_2, with or without concomitant modification of the fermentative products. A thermophilic *Synechococcus* reduces sulfate and thiosulfate to sulfide anaerobically in the dark. It is yet to be demonstrated that the reduction of sulfur is coupled to electron transport or energy generation.

Close Relatives of Cyanobacteria

The classes *Melainabacteria* and *Sericytochromatia* currently include only nonphototrophic members, and may in fact constitute a single phylum with cyanobacteria proper. *Melainabacteria* and *Sericytochromatia* sequences have been retrieved from anoxic environments such as the human gut, wastewater treatment facilities, subsurface groundwater, and a methane coal bed. Genomic data of these groups reveal that they lack the capacity for photosynthesis, but otherwise contain genes for fermentation, while some also appear capable of aerobic respiration. Also, unlike oxyphotobacteria, some of their genomes encode for flagellar proteins and some even exhibit a predatory lifestyle. Further study of these bacteria may provide interesting insight into the evolutionary history of cyanobacteria.

Secondary Metabolism

Cyanobacteria synthesize a variety of compounds that are not components of universal biochemical pathways, but have restricted distribution among taxa (secondary metabolites). These are thought to serve particular functions relevant to the survival of the strains in question, but their specific role has been deduced only in a few cases. Several important cyanobacterial metabolites are peptides synthesized in a nonribosomal setting by specific peptide synthetases. Compounds such as cyanobacterin (Fig. 6(c)), an herbicide produced by some *Scytonema* strains, potently inhibit PSII of algae and cyanobacteria other than the producing strain, thus wiping out the competition. Scytonemin, a widespread indole alkaloid, is synthesized, excreted, and accumulated in large quantities in extracellular sheaths in response to ultraviolet (UV) radiation exposure, serving a sunscreen role (Fig. 6(h)). A similar sunscreen role has been proposed for a large variety of colorless mycosporine-like compounds (Fig. 6 (f) and (g)). Triterpenoids of the hopane series found in thermophilic strains may stabilize the cell membranes under high temperatures. Other compounds display antibiotic activity, such as the antibacterial brominated biphenyls from *Oscillatoria* '*chalybea*' (Fig. 6(k)) or the methoxytetradecanoic acid of *Lyngbya*'*majuscula*' (Fig. 6(d)). However, for most cyanobacterial secondary metabolites identified, their biological function remains elusive. Such is the case for the volative compounds 2-methylisoborneol and geosmin (Fig. 6(a) and (b), respectively), which are of common occurrence and responsible for the earthy smell and off-flavors in lakes harboring cyanobacterial blooms. A biological role for the notoriously famous cyanotoxins (Fig. 6(i) and (j)) is also difficult to define but some have suggested it provides them with a competitive advantage in response to grazing or resource competition. Another possibility for toxin synthesis is that production of complex secondary metabolites improves cell physiology by benefitting homeostasis, improving photosynthetic efficiencies, and accelerating growth rates. One study found that the toxin microcystin enhances the response to oxidative stress conditions in *Microcystis* by binding to proteins and decreasing their susceptibility to proteases. Among the bioactive compounds of unknown natural function, some have antioxidant, antineoplastic, antiviral, antiinflammatory, antimitotic, ichtiotoxic, and dermatitic activities. Efforts to study the largely untapped cyanobacterial inventory of secondary metabolites and their biology has increased substantially due to their relevance to pharmaceutical research and public health. To this end, advances in genome sequencing efforts have accelerated the ability to use genome mining to discover novel natural products from cyanobacteria. In particular, *Lyngbya*, *Nostoc*, *Microcystis*, and *Haplosiphon* strains serve as rich repositories for discovery, as their genomes encode for hundreds of gene clusters related to natural product biosynthesis.

Metabolic Engineering

Metabolic engineering is the use of recombinant DNA technology to improve cellular activities. Most often metabolic engineering focuses on chemical manufacturing, biofuel production, or bioremediation. Cyanobacteria are ideal photosynthetic hosts for this purpose because there are many strains that are amenable to genetic manipulations. Classical genetic approaches to knock-in or knock-out genes have been used in a variety of cyanobacteria and are extensively reviewed elsewhere. More recent advances have taken advantage of clustered regularly interspaced short palindromic repeats (CRISPR) gene editing technology. Originally identified in bacteria as a defense mechanism against foreign DNA (e.g., viruses and mobile genetic elements), the Cas proteins associated with CRISPR sequences are RNA-guided DNA endonucleases capable of introducing blunt-ended double-strand breaks in specific target sequences. The CRISPR/Cas9 system was used successfully in *Synechococcus elongatus* PCC 7942 to improve yields of succinate by deleting the glucose-1-phosphate adenylyltransferase (*glgC*) gene in an effort to divert carbon away from glycogen and towards succinate. An alternative to CRISPR/Cas9 and Cas12a gene editing systems is CRISPR interference (CRISPRi) which utilizes an enzymatically inactive dead Cas9 (dCas9) to reduce the expression of genes that cannot be deleted. CRISPRi was first used in *Synechocystis* sp. PCC 6803 to demonstrate Green Fluorescent Protein (GFP) repression and has since been used successfully in

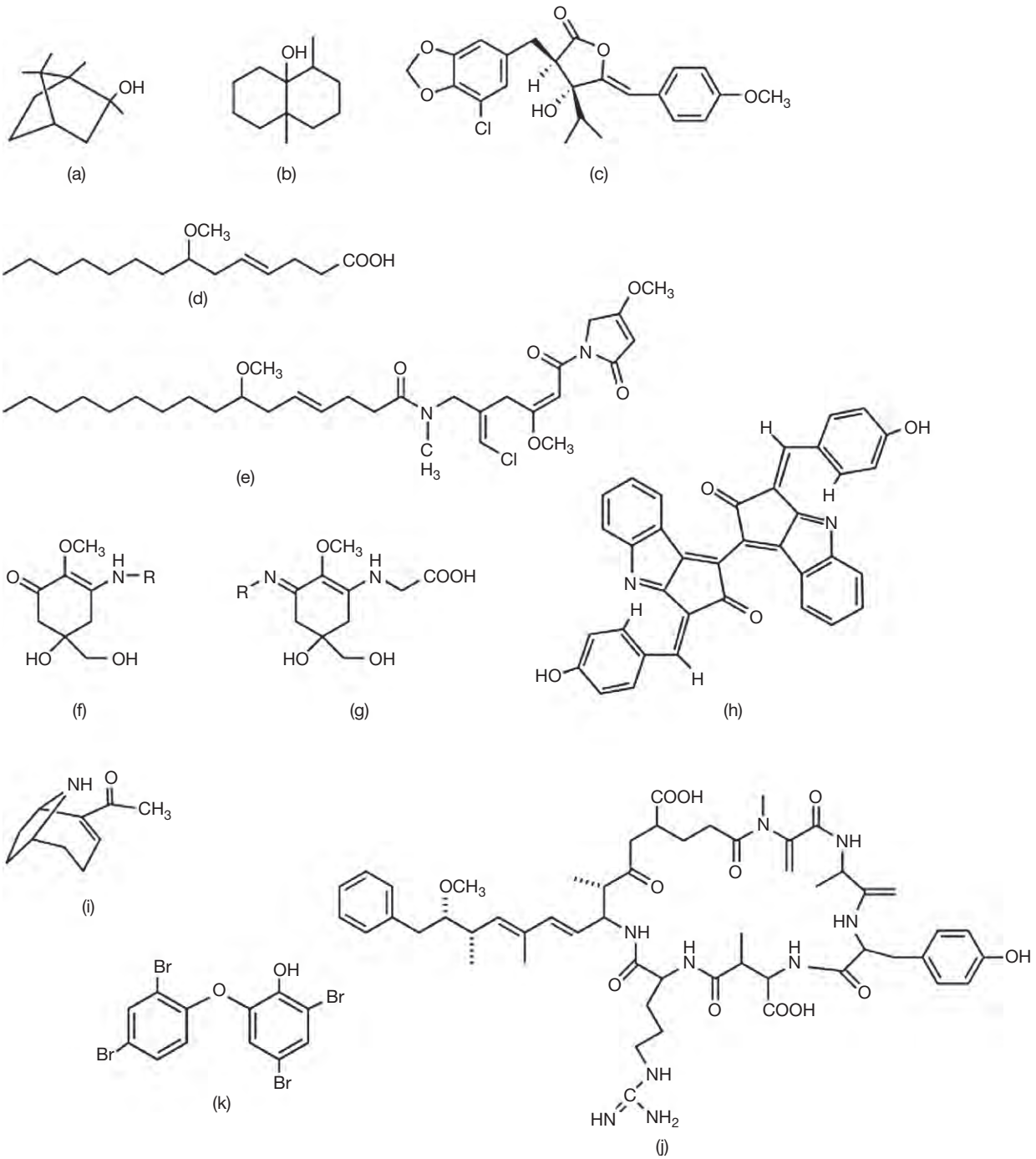

Fig. 6 Diversity of cyanobacterial secondary metabolites. (a) 2-methylisoborneol; (b) geosmin; (c) cyanobacterin; (d) 7-methoxy-4-tetradecanoic acid; (e) malyngamide A; (f) and (g) mono- and bisubstituted mycosporines, respectively, where R stands for (amino) acidic moiety; (h) scytonemin; (i) anatoxin-a; (j) microcystin-YR; (k) a brominated phenyldiphenol.

Synechococcus sp. PCC 7002 to repress the phycobiliprotein complex and in the heterocyst-forming strain *Anabaena* PCC 7120 to regulate ammonium production.

Nutrition

Apart from liquid water, light, and inorganic nutrients, few additional requirements for growth are known in most cultivated strains. A requirement for vitamin B_{12} has been demonstrated in some strains. Metabolic processes devoted to the provision of nutrients

may account for a significant part of the energy and reduction equivalents obtained in the light reactions of photosynthesis. Cyanobacteria possess specific uptake systems for nutrient assimilation. Orthophosphate can be taken up and stored intracellularly as polyphosphate (Fig. 4(a) and (d)), and the uptake may be aided by the action of surface-bound phosphatases, which release phosphate bound to organic molecules. The availability of phosphorous may often be the growth-limiting factor in natural freshwater populations. The production of siderophores (iron-chelating organic compounds) seems to be important in the assimilation of iron because Fe^{3+} ions, required for many of the enzymes involved in redox reactions, are very insoluble in water. The availability of iron may be growth limiting in oceanic planktonic species. Nitrogen-fixing cyanobacteria have complex sets of amino acid uptake systems, probably geared toward the recovery of leaked fixed nitrogen. In addition to uptake mechanisms, sulfur and nitrogen assimilation require additional reduction steps and are discussed separately in the following sections.

Nitrogen Assimilation

Among inorganic nutrients, nitrogen is of paramount importance as it accounts for ~10% of the dry weight of cyanobacterial cells. Nitrate (NO_3^-) and ammonium (NH_4^+) are virtually universal sources of nitrogen for cyanobacteria, but urea or other organic nitrogenous compounds can be used by some strains. In addition, many strains can fix gaseous dinitrogen (N_2). Plasma membrane-bound transport systems exist for both NO_3^- and NH_4^+, whereas N_2 enters the cells by diffusion. Intracellular NO_3^- must be reduced to NH_4^+. This is accomplished by the stepwise reduction to nitrite (catalyzed by nitrate reductase) and NH_4^+ (catalyzed by nitrite reductase), the reduction equivalents for both processes stemming from reduced ferredoxin. Ammonium (either taken up or endogenously generated) is assimilated by the glutamine synthetase/glutamate synthase enzyme system. The net action of this system is the formation of glutamate from α-ketoglutarate and NH_4^+, with the expenditure of ATP and the oxidation of ferredoxin. Glutamate can donate its amino moiety to various precursors of central metabolism by the action of specific transaminases. Many, but not all, cyanobacteria are able to fix N_2; this is of great ecological significance because N_2 is ubiquitous in the environment. The process is carried out by the enzyme nitrogenase and is a costly one, involving the consumption of both ATP and reduction equivalents (supplied by ferredoxin). In addition, nitrogenase will also inevitably reduce protons to H_2 in what represents a wasteful decrease in efficiency.

Nitrogenase is inherently and irreversibly inactivated by O_2. Several strategies have evolved in cyanobacteria to circumvent this problem. Some strains will only carry out N_2 fixation under anoxic conditions, but some will also do it in the presence of oxygen. Several strains have been shown to temporally restrict N_2 fixation to the dark period, thus decreasing the exposure of nitrogenase to photosynthetic oxygen. Strains belonging to the Nostocales and Stigonematales have evolved a specialized cell type (the heterocysts; see 'Heterocysts') in which nitrogen fixation is spatially separated from photosynthesis and protected from O_2 inactivation. Heterocystous strains display the highest specific rates of N_2 fixation among all cyanobacteria. However, some nonheterocystous cyanobacteria, such as *Trichodesmium*, are able to fix substantial, biogeochemically significant amounts of N_2 in the light. It is now known that nitrogen-fixation in *Trichodesmium* is spatially separated in specialized diazocyte cells and that oxygen concentrations are lowered by the Mehler reaction, in which the oxygen produced by PSII is reduced again after PSI. Some cyanobacteria that lack nitrogen fixation genes, like *Microcoleus vaginatus* of desert soils, enter into loose symbiotic relationships with heterotrophic diazotrophs in exchange for organics. The various mechanisms for nitrogen assimilation are tightly regulated so that the presence of less costly sources (NH_4^+) immediately inhibits NO_3^- (and NO_2^-) uptake, or N_2 fixation activity, and represses the expression of the enzymes involved in the reduction of alternative N_2 sources. In the same way, the presence of abundant NO_3^- represses the expression of nitrogenase genes and results in the halting of new heterocyst differentiation.

Sulfur Assimilation

Sulfate ($SO4_2^-$) is seemingly the universal source of sulfur for cyanobacterial cells, and it is only rarely growth limiting in the environment. Other sources of sulfur may be taken up alternatively, such as sulfate esters, sulfonate, hydrogen sulfide, and organic thiols. Sulfate is taken up by a $SO4_2^-$ permease in an energy-dependent process, reduced to sulfide, and incorporated into cysteine. The cyanobacterial assimilatory sulfate reduction pathway is similar to that of other bacteria, involving the activation of sulfate by binding to ADP and the reduction of the sulfonucleotide to free sulfite using thioredoxin as a reducing agent. Sulfite is further reduced to sulfide by sulfite reductase using NADPH as an electron donor, and free sulfide is incorporated into cysteine by specific synthases. An oxidized sulfur source may also be a requirement for growth because, unlike other bacteria, cyanobacteria possess important structural components containing oxidized sulfur moieties: the sulfolipids of the photosynthetic membranes and, in some strains, the sulfate esters constituent of the extracellular polysaccharide sheaths.

Regulation

The regulation of cellular activities in cyanobacteria is similar in nature to that found in other prokaryotes, but photobiology plays a particularly important role. The presence and nature of the cyanobacterial photoreceptor systems (a cell's light meter) are well documented. Although specific photoreceptor molecules, some structurally similar to plant phytochromes, do exist, and some have been postulated as sensors of UV radiation, in many cases it is the indirect effect of light supply on the overall redox state of the cell that determines cellular responses. Small redox-sensitive proteins such as thioredoxin may act as general modulators of enzyme activity in carbon and nitrogen metabolism. The balance between carbon and nitrogen metabolism is typically sensed through the

levels of 2-oxoglutarate. Short-term (photo)responses can also be based on protein phosphorylation mechanisms, as seems to be the case for the process leading to the redistribution of captured energy between photosystems I and II (the so-called state transitions) or for the direct regulation of phosphoenolpyruvate carboxylase activities. By means of its multiple targeting, phosphorylation of a serine residue of P_{II}, a small regulatory protein, is thought to provide coordinated regulation of carbon and nitrogen metabolism. There is abundant evidence for light-mediated regulation of gene expression, leading to long-term responses, either to light intensity or to spectral composition. This is particularly true for genes encoding components of the photosynthetic apparatus, such as phycobiliproteins and PSII polypeptides. Some strains grown under light–dark cycles are capable of incorporating specific metabolic tasks into the swing of the cycle, relegating, for example, protein synthesis or nitrogen fixation to the dark periods. At least some of these daily patterns are maintained by an internal clock system, because the periodicity remains even in the absence of environmental stimuli. The core of this central clock, virtually universal among cyanobacteria, is an autophosphorylating enzyme, KaiC, that oscillates between nonphosphorylated and phosphorylated forms, and can modulate the expression of various central genes. Two-component (histidine kinase/response regulator) regulators seem to be common for signal transduction, particularly in responses to environmental stress or shock (cold, heat, salt, UV, and light), as well as for adaptation to some forms of nutritional limitation. Several histidine kinases with GAF-domains that function as photosensors or in phototaxis have been described in *Synechocystis*. Quorum sensing, using chemical signal molecules such as acyl-homoserine lactones, has been observed in some cyanobacteria to sense cell-population densities as part of biofilm formation. Quorum-sensing inhibition, through the production of inhibitory metabolites, has also been documented from cyanobacteria in a hypersaline system.

Noncoding RNAs (ncRNAs), also known as small RNAs (sRNAs), are known to play a role in post-transcriptional regulation by binding to complementary target mRNAs to affect their translation or stability. Cyanobacteria display a broad percentage in the amount of noncoding transcriptional start sites in a genome, from ~5% in *Procholorococcus* to 26% in *Trichodesmium erythraeum*. ncRNAs are involved in a wide variety of functions, including regulation of photosynthesis and stress responses. In *Synechocystis* most of them have been shown to function as antisense transcripts which can have activating or repressive effects on gene expression.

Motility and Taxes

Flagellar proteins have only been identified in the nonphototrophic cyanobacteria while many unicellular and filamentous oxyphotobacteria display gliding motility. In some strains of oceanic marine *Synechococcus*, slow-swimming motility has also been described. Gliding is a movement across a solid or semisolid material in the absence of flagella or other conspicuous propulsion mechanisms and without apparent change in cellular (or trichome) shape. Gliding is typically accompanied by the secretion of slime and in filamentous cyanobacteria this polysaccharide layer may provide a suitable surface for motility. In *Nostoc* hormogonia and *Synechocystis*, gliding motility is facilitated by type IV pili in an ATP-dependent manner. In filamentous forms, rotation of the trichomes along their main axes often occurs while gliding. The structural involvement of a Ca^{+}-binding glycoprotein, oscillin, in cyanobacterial gliding has been determined; it forms supracellular helical fibrils in the outermost surface of the trichomes. Gliding motility may be displayed only transiently (i.e., in hormogonia or in baeocytes but not in vegetative cells). Photosensory and chemosensory systems, allowing the organisms to respond to temporal or spatial environmental gradients, are tightly coupled to motility, resulting in the so-called tactic behavior. Positive tactic responses to chemical species (chemotaxis) such as bicarbonate, nitrate, and plant exudates in symbiotic species have been shown in cyanobacteria (i.e., they move up chemical gradients of concentration of those substances). Terrestrial cyanobacteria are the only microorganisms shown to have tactic responses to water. Even halotaxis, movement towards optimal salinity, has been reported in cyanobacteria from an intertidal hypersaline microbial mat. All motile cyanobacteria display phototactic behavior so that the populations are able to seek optimally illuminated areas. Both positive and negative phototaxis systems exist, to achieve both optimal illumination as well as avoid damaging UV radiation. Like other bacteria, cyanobacteria usually respond by stopping and changing the direction of movement (reversing) upon crossing a sharp boundary in light intensity (photophobic response); however, some are also capable of perceiving the angular direction of the light and respond by steering toward or away from the direction of the incoming light. Spherical cells of *Synechocystis* act as microlenses to sense light in what has been described as the "world's smallest and oldest example of a camera eye". In this analogy the cell acts as a spherical microlens and the cell membrane, which contains a variety of photoreceptor proteins, acts as the retina, providing vision for the phototactic response. This capacity (known as true phototaxis) has no parallel in any other prokaryote.

Molecular Genetics

Genomes

Cyanobacterial genomes are typically prokaryotic in nature and located in the centroplasm. Most genomes are circular but linear chromosomes have been observed in *Cyanothece*. Many cyanobacteria are polyploid or oligoploid, with 53 chromosome copies per cell in *Synechocystis* sp. PCC 6803 and as few as two copies in *Synechococcus elongatus* PCC 7942. The genomes of free-living cyanobacteria vary widely in GC base composition from 32% to 71%, a range comparable to that spanned by all bacteria. They also vary in size, approximately correlating with morphological complexity, from 1.6 to 14 $\times$ 10^6 bp (base pairs) (~1700–10,000 genes). The smallest cyanobacterial genomes are thus similar in size to those of most bacteria, whereas the

largest ones are in the range of eukaryotic fungal genomes. Symbiotic cyanelles and plastids have retained only 0.13×10^6 bp in their genomes. To date, more than 300 cyanobacterial genomes have been fully sequenced, including many strains of *Synechococcus* and *Prochlorococcus*. Based on an analysis of 58 cyanobacteria, a core cyanobacterial genome of 559 genes has been suggested, along with three signature genes for thermophilic and 57 signature genes for heterocystous cyanobacteria. The DNA of cyanobacteria is subject to very extensive modification, and DNA methylation patterns in nitrogen starvation and nitrogen recovery over twelve generations in *Synechocystis* suggest that transgenerational epigenetic processes exist in cyanobacteria. The presence of widespread, highly iterated short palindromic sequences is a trait shared by many, but not all, cyanobacterial genomes and CRISPR sites are also common. Genomic rearrangements involving deletion, operon fusion, and translocation events are known to occur during heterocyst differentiation. Plasmids or extrachromosomal replicons are commonly encountered (some as large as 1.5×10^5 bp), but they are usually cryptic and do not appear to be responsible for antibiotic resistance phenotypes, as in other bacteria. Some are known to bear genes encoding for isozymes involved in assimilatory sulfate reduction.

Gene Transfer

There is evidence from phylogenetic comparisons that horizontal genetic exchange among related cyanobacteria has played a significant role in their evolution. Nevertheless, the mechanisms leading to genetic exchange are difficult to pinpoint. Despite the abundance and spread of cyanobacterial plasmids, natural conjugation among cyanobacteria has not been reported. The same is true for viral transduction, despite the wealth of cyanophages described in the laboratory and from natural populations, some clearly carrying cyanobacterial genes in their genomes. Some strains are naturally highly competent for taking up foreign DNA, but unaided transformation seems to be restricted to some unicellular strains of the genera *Synechococcus* and *Synechocystis*. The latter of which has been shown to use type IV pili and DNA-binding proteins to enhance transformation efficiency. In the laboratory only transformation (or electroporation) and conjugation are used to transfer DNA into cyanobacterial cells. Even though transformation has been used on some filamentous strains, most gene transfer of these strains in the laboratory is done by conjugation.

Gene Expression

Control of gene expression at the level of transcription seems to play a significant role in the adaptation to changing environmental conditions. Cyanobacteria possess a transcriptional apparatus of unique characteristics among bacteria. The cyanobacterial DNA-dependent RNA polymerase is structurally different from that of the common bacterial type, possessing an additional subunit in its core, and several sigma factors (polypeptides, whose association with the core of the polymerase is needed for effective initiation of transcription) have been identified. It has been shown that a 'principal' sigma factor is commonly present under normal growth conditions, *sigA* in *Synechocystis*, whereas alternative factors are temporarily expressed upon, for example, a change to nitrogen-limiting conditions. Because cyanobacterial promoters lack some of the distal consensus sequences of other bacteria, it has been hypothesized that regulation of transcription may often be activated by accessory factors other than sigma factors. Small noncoding RNA transcriptional regulatory elements (see 'Regulation') appear to be widespread among the cyanobacteria, with documented studies in unicellular, filamentous, and heterocystous types. Examples of sRNAs/ncRNAs involved in regulation include IsaR1 and NsiR4, which are involved in iron and nitrogen depletion, respectively, and PsrR1 which is involved in the high light stress response.

Systems Biology

Systems biology approaches to studying cyanobacteria have generated a variety of 'omics' datasets, most of which are focused on genomics, transcriptomics, proteomics, and metabolomics. The sequencing of over 300 cyanobacterial genomes has expanded the capabilities of applying these approaches to better understand these complex biological systems. Early studies relied on the development of DNA microarrays which were limited to only a handful of cyanobacteria. More recent approaches utilize RNA-Seq (RNA Sequencing), which provides whole-transcriptomic analysis using next-generation sequencing. RNA-Seq also offers annotation of transcriptional start sites, promoter regions, and identification of new genes, particularly noncoding RNAs. Furthermore, RNA-Seq can be applied to any sequenced organism, although current datasets are mainly from *Synechocystis* sp. PCC 6803. Proteomics began with the use of two-dimensional gel electrophoresis with peptide identification through mass spectrometry and has now advanced into gel-free approaches such as selected reaction monitoring (SRM), which allows precise quantification of targeted proteins. SRM has been applied to the *Synechocystis* sp. PCC 6803 proteome to quantify proteins controlled by the transcriptional factor SufR and the noncoding RNA IsaR1. Advanced proteomic methods also offer the advantage of identification of protein modifications such as phosphorylation and glutathionylation. Metabolomics uses gas or liquid chromatography in combination with mass spectrometry to quantify cellular metabolites. This method is currently capable of providing quantitative analysis of about 100 cyanobacterial metabolites. An extension of metabolomics is the measurement of flux (fluxomics) using stable isotopes such as ^{13}C. This type of analysis can reveal candidate enzymes and pathways for targeting in order to optimize growth or output under specific growth conditions. For example, ^{13}C-flux experiments using *Synechococcus* sp. PCC 7002 cells revealed changes in cell composition in response to different light regimes.

Ecology and Adaptations

The range of environmental conditions under which cyanobacteria can develop is impressively wide, and equally wide is the variety of ecological adaptations they display. One can find cyanobacteria as an important part of the primary producer community in almost any habitat in which light penetrates. Thermophilic cyanobacteria mainly of the genera *Synechococcus* and *Thermosynechococcus* can grow up to temperatures of 73°C in hot springs, which is the upper temperature limit for photosynthesis. Cyanobacteria can also develop stable populations in polar soils, rocks, and ponds in which temperatures rarely exceed a few degrees Celsius. In fact, low-temperature ecotypes can be differentiated from warmer temperature strains by comparing ribosomal sequences. Some forms thrive in rain or snow-melt puddles of extremely low inorganic solute concentrations, and some halotolerant types grow in NaCl-saturated brines. Halophilic cyanobacteria, which are often nonheterocystous strains, can be found in saltern evaporation ponds at salinities above 200 g L^{-1}. One of the major strategies for coping with salt stress is to accumulate compatible solutes such as sucrose, trehalose, glucosylglycerol, and glycine betaine. It was even shown that the salt tolerance of cyanobacteria could be predicted by which compatible solutes were encoded for in their genomes, with sucrose and trehalose more common in freshwater strains and glycine betaine common in hypersaline strains. Some cyanobacteria thrive in caves, deep in lakes, and in coastal areas, where light is extremely dim, while some terrestrial forms develop permanent populations in mountainous tropical areas exposed to the highest levels of solar radiation found on Earth. Many terrestrial cyanobacteria are desiccation resistant, and they withstand freeze–thaw cycles. Benthic marine cyanobacteria flourish under supersaturated oxygen, often exceeding 1 atm in partial pressure during daytime, but they are exposed to anoxia at night. It is common that more than one of these extreme conditions coincide in one particular habitat. One of the most conspicuous limitations to the development of cyanobacteria seems to be acidity: although many are known from alkali lakes, no *bona fide* reports of growth below pH 4.5 exist. The ecological success of cyanobacteria in many of these extreme habitats is often a result of their metabolic resilience in the face of environmental insults rather than a consequence of sustained growth. A few environmentally relevant cyanobacterial habitats are discussed in the following sections.

Marine Plankton

With the possible exception of polar areas, morphologically simple cyanobacteria of small size (0.5–2 μm) inhabit in large numbers in the upper zone of the oceans where light penetrates. These are referred to as picoplankton, and consist of two phenotypically distinct but phylogenetically related groups (Fig. 2): the open-ocean marine *Synechococcus* and *Prochlorococcus*. Population sizes typically range between 10^4 and 10^5 cells per ml for both types. The global biomass of picoplankton must be on the order of 1–2 billion metric tons (1600×10^{12} g). This shear size indicates their ecological importance. It has been calculated that as little as 11% and as much as 50% of the primary production of nonpolar open ocean regions is due to their activity. This group has developed interesting adaptations to the light field of clear oligotrophic (nutrient-poor) waters: their light-harvesting complexes have differentiated to match the predominantly blue light available. *Synechococcus* cells synthesize a special kind of bilin chromophore, phycourobilin, absorbing maximally at 490–500 nm, thus increasing the ability of cells to use blue light. Evolutionary pressure of a similar nature has probably resulted in the virtual loss of phycobiliprotein-based light harvesting in *Prochlorococcus* and the evolution of antenna mechanisms based on (divinyl) chlorophylls (*a* and *b*), which are optimally suited to capture blue light. The life strategy of picoplankton populations is based on fast growth, with cells often displaying several doublings per day. Grazing pressure and viral infection seem to be the major factors controlling population sizes. The comparatively small size of picoplankton genomes, the absence of nitrogen-fixing capacity and of some reserve polymers such as phycocyanin, and the lack of mechanisms to withstand small concentrations of toxic metals such as copper may be the result of reductionist evolutionary pressures favoring fast growth. Their small size (large surface to volume ratio) may provide selective advantage in nutrient-poor environments. Furthermore, *Prochlorococcus* is capable of utilizing non-phosphorous lipids such as phosphonates (organic carbon-phosphorous compounds) in the oceans. Phenotypic and genetic variation exists within the picoplanktonic cyanobacteria, resulting in strains that diverge in light and temperature optima for growth: high- and low-light-loving strains of *Prochlorococcus* have been described as well as mesophilic and moderately psychrophilic strains of *Synechococcus*. Double-stranded DNA cyanobacterial myoviruses abundant in marine environments have been studied in both *Synechococcus* and *Procholorococcus*, for which there appears to be a 'cyanophage core genome' that exists across at least 16 cyanophage genomes. Some of these cyanophage genomes carry genes for the light reactions of photosynthesis, the pentose phosphate pathway, and phosphorous acquisition, enhancing these processes in marine cyanobacteria.

The intensely red (phycoerythrin-containing) oscillatorian cyanobacteria of the genus *Trichodesmium*, which typically occur as bundles of filaments in the wild, constitute the second most important group of marine planktonic cyanobacteria, with a global biomass estimated at 100×10^{12} g. They are inhabitants of oligotrophic tropical open ocean regions worldwide, in which they may form blooms that can be detected as surface accumulations with the naked eye. They are responsible for much of the global oceanic nitrogen fixation, and this nitrogen-fixing capacity is a key factor of their ecological success. The particular adaptations that allow nonheterocystous *Trichodesmium* filaments to fix nitrogen in the light involve specialized diazocyte cells and reduction of oxygen after PSI (see 'Nitrogen assimilation'). They also contain large amounts of gas vesicles that provide positive buoyancy to the filaments so that they remain in the upper wind-mixed layers of the ocean. *Trichodesmium* gas vesicles are among the sturdiest in prokaryotes, apparently so that they can withstand the large hydrostatic pressures experienced upon mixing of the deep mixed layers of open-ocean waters. Large, unicellular populations of '*Crocosphaera*' fix nitrogen at night and also contribute significantly to the inputs of new nitrogen in the oceans along with *Trichodesmium*.

Freshwater Plankton

Although not a major component of the global biomass (with only some 30×10^{12} g), a large variety of cyanobacteria are found as components of the phytoplankton of fresh waters, and are particularly prominent or dominant under conditions of nutrient eutrophication. In eutrophic lakes and man-made reservoirs (and in enclosed brackish water basins such as the Baltic), the formation of cyanobacterial blooms results in serious water quality problems regarding not only the degradation of the recreation potential, musty odors, and off-flavors that are associated with bloom development, but also the likelihood of fish kills due to anoxic events after bloom decay and the production and release of cyanobacterial toxins. These are known to have caused animal and, in extreme cases, human deaths. Bloom-associated health effects reach beyond local environmental agencies and are being considered in rulings of the World Health Organization. Gas-vacuolated species in the heterocystous genera *Anabaena*, *Nodularia*, '*Gloeotrichia*', and *Aphanizomenon*, as well as in the nonheterocystous genera *Oscillatoria* and particularly *Microcystis*, are notoriously responsible for bloom formation and for reported cases of intoxication.

Terrestrial Environments

Desiccation-resistant terrestrial cyanobacteria have widespread occurrence. They may be found growing on bare surfaces (rocks, trees, buildings, and soils) or several millimeters within more or less soft diaphanous substrates (soils, sandstone, and limestone). Some species actively bore into the rock substrate. The availability of liquid water, in the form of rain or dew, determines the potential spurts of growth of cyanobacteria in the terrestrial environment. Growth of terrestrial cyanobacteria can be fast and luxurious in tropical humid climates, but in most other regions it is usually only intermittent. Their life strategy is usually one of slow growth and enhanced resilience. Adaptations to this environment are directed to withstand both desiccation (e.g., by abundant exopolysaccharide production) and exposure to solar radiation under inactive conditions (by the synthesis of sunscreen pigments). The negatively charged polysaccharides surrounding the cells also aids in sequestering metal cations necessary for growth. The exclusion of higher plant vegetation by climatic rigors determines the relative importance of cyanobacteria in terrestrial habitats. Thus, extensive endolithic cyanobacterial communities (accounting for as much as 140×10^{12} g of biomass globally), usually dominated by members of the genus *Chroococcidiopsis*, have been described from tropical, desert, and polar environments. These communities play a significant role in rock erosion processes, and their actions have become a concern for the preservation of stone monuments. Edaphic (soil dwelling) cyanobacteria are also distributed worldwide, and represent one of the largest global reservoirs of biomass, with some 540×10^{12} g. Sheathed oscillatorian forms such as *M.*'*vaginatus*', possibly the most common and widespread, along with heterocystous ones (*Nostoc* and *Scytonema*), are major ecological players in arid and semiarid regions, both hot and cold. Edaphic cyanobacteria in the so-called biological soil crusts contribute significantly to the physical stability and fertility of arid soils worldwide.

Sulfidogenic Environments

Hydrogen sulfide interferes with PSII and acts as a potent inhibitor of oxygenic photosynthesis. Many marine and freshwater habitats, such as hot springs, marine littoral sediments, and the deep water of lakes, may contain significant amounts of free sulfide. Cyanobacteria develop the most conspicuous populations of oxygenic phototrophs in such environments when sufficient light is available. Specific adaptations to these habitats include the ability to express sulfide-resistant forms of PSII so that oxygenic photosynthesis can proceed even in the presence of sulfide (e.g., in the marine benthic *Microcoleus* '*chthonoplastes*' and in some hot spring and freshwater oscillatorians) and also an ability to perform anoxygenic photosynthesis using hydrogen sulfide as a source of electrons instead of water (e.g., in *Oscillatoria* '*limnetica*' and members of the 'Halothece' cluster from hypersaline waters, *Oscillatoria* '*amphigranulata*' from hot springs, or *Pseudanabaena* sp. from hardwater lakes). Many strains display both adaptations simultaneously. The ability to use sulfide as an electron donor has been traced to the inducible expression of a soluble enzyme, sulfide:quinone oxidoreductase, which can transfer electrons from sulfide to plastoquinone, thus allowing the noncycling function of PSI (Fig. 5) with the formation of both ATP and NADPH. Although some strains in culture show continued growth using anoxygenic photosynthesis alone, they cannot compete successfully for sulfide with phototrophic sulfur bacteria in the environment. It is thought that cyanobacteria use anoxygenic photosynthesis as a means for sulfide detoxification. Indeed, many use anoxygenic photosynthesis only temporarily, until local concentrations of sulfide are sufficiently low and (sulfide-resistant) oxygenic photosynthesis can begin.

Symbioses

Although they show an apparent lack of taste for sexual matters, cyanobacteria have displayed a considerable evolutionary promiscuity, entering into intimate symbiotic associations with various unrelated organisms. The list of cyanobacterial symbioses is large. There is also a large variation in the degree of independence maintained by the cyanobacterial partners. In some cases, the distinction of two organisms may no longer be possible because cyanobacteria lose their typical appearance and a large portion of their genomes to their hosts. This is obviously the case in higher plant and eukaryotic algal plastids, in which massive loss of genes to the eukaryotic nucleus has occurred. Cyanelles (plastid-like endosymbionts of eukaryotic protists) have retained the peptidoglycan and phycobilines, but their identity loss is substantial as well. These cases are no longer considered symbioses. At the other end of the spectrum, loose but mutualistic relationships between cyanobacteria and other bacteria or fungi (the so-called consortia) have been

described, but these are not considered here. Well-known cyanobacterial symbioses can be functionally divided into those formed by heterocystous cyanobacteria, in which the main contribution of the cyanobacterial partner is the supply of fixed nitrogen, and those formed by nonheterocystous types, in which their contribution is often the supply of fixed carbon. According to the degree of intimacy attained, they can be classified into intracellular (in which cyanobacterial cells are found within cells of other organisms) and extracellular (in which cyanobacterial cells are located within the tissues but outside the cells of other organisms). The most common extracellular symbioses of nonheterocystous cyanobacteria (involving the unicellular genera *Chroococcidiopsis*, *Gloeocapsa*, '*Chroococcus*', and *Gloeothece*) are in the form of cyanolichens. *Prochloron*, *Acaryochloris*, and large-celled *Synechocystis* are known from extracellular symbioses with ascidians in tropical or subtropical marine waters. Extracellular symbioses of Pseudanabaena-like '*Konvophoron*' occur in Mediterranean sponges. Filamentous *Phormidium* has been reported in symbioses with some green algae. Intracellular symbioses of nonheterocystous cyanobacteria are known from tropical sponges ('*Aphanocapsa*', *Oscillatoria*, *Synechocystis*, and *Prochloron*), from green algae (*Phormidium*), and from dinoflagellates (*Synechocystis* and *Synechococcus*). Heterocystous cyanobacteria in the genus *Nostoc* are known to form extracellular symbioses with liverworts and higher plants (Cycads and duckweed). *Anabaena* enters into symbiosis with water ferns of the genus *Azolla*. Cyanolichens are known to contain members of the heterocystous genera *Nostoc*, *Calothrix*, *Scytonema*, *Stigonema*, and *Fischerella* as photobionts. Intracellular symbioses of heterocystous cyanobacteria occur in oceanic diatoms of the genera *Hemiaulus* and *Rhizosolenia* (cyanobacterial genus '*Richelia*') and in *Trifolium* (clover) with *Nostoc*. *Nostoc* also enters into intracellular symbioses with the terrestrial nonlichenic fungus *Geosiphon* pyriforme.

Fossil Record and Evolutionary History

The fossil record of cyanobacteria contains the oldest entries that can be confidently assigned to any extant group of organisms. Excellently preserved microfossils, 1000 million years old, bear virtually indisputable cyanobacterial morphologies. Fossil cyanobacteria showing considerable morphological diversification have been described dating back at least 2500 million years, which is also corroborated by phylogenetic analyses of scytonemin genes, dating heterocyst-producing clades as early as 2450 million years. Additionally, filamentous bacteria of putative cyanobacteria identity are known from as far back as 3500 million years. In fact, it has been suggested that cyanobacteria have evolved only very slowly in the intervening time because present and past morphologies are very similar. In view of the biochemical and physiological diversity of adaptations that particular cyanobacteria display, some doubts may be cast on such a perception. Lipid biomarkers, such as 2-methylhopanoids preserved in rocks as old as 2700 million years, have been used to identify cyanobacteria in the rock record. However, the use of 2-methylhopanoids to date cyanobacteria must be used with caution since genes involved with 2-methylhopanoid biosynthesis are found in other prokaryotes. The fossil record of the Archaean and Proterozoic Eons (before 500 million years ago) offers strong evidence not only for the presence of cyanobacteria but also for a type of environment they must have inhabited: the sedimentary environment of shallow coastal waters. This is recorded in the abundant organosedimentary laminated macrofossils known as stromatolites or microbialites, which became geographically restricted in the Phanerozoic. Stromatolites are analogous to present-day cyanobacterial mats, benthic compact assemblages built by cyanobacteria in extreme environments, and have provided evidence for the sustained importance of photoresponses in the ecology of cyanobacteria in the form of 'heliotropic' accretions. Precambrian fossil microboring on marine carbonaceous substrates reveals the sustained role of cyanobacteria in small-scale geomorphological processes. Fossil evidence for the presence of eukaryotic algae is also quite old, perhaps as much as 2000 million years, which is in agreement with the early offshoot of the plastidic line of evolution suggested by phylogenetic reconstructions. The oldest fossil evidence for terrestrial cyanobacteria, in the form of *Gloeocapsa*-like cells symbiont in lichens, is comparatively young (400 million years). Thus, cyanobacteria have inhabited Earth for a long time and survived through geological periods of environmental conditions very different from those reigning today. In the early days of cyanobacterial evolution, high fluxes of short-wavelength UV radiation penetrated the oxygen- and ozone-free, carbon dioxide-rich atmosphere. Oceans were shallow and rich in reduced iron and poor in sulfate and nitrate. In fact, it is thought that oxygenic photosynthesis was the ultimate cause, regulated by geological events of carbon burial, for the change in most of these parameters, including the late Proterozoic oxygenation of the atmosphere.

Phylogenetic reconstructions suggest that the genes for photosynthesis were acquired late in cyanobacterial evolution and that the nonphototrophic *Melainabacteria* and *Sericytochromatia* represent the ancestral state of this phylum. The hypothesis has been presented that genetic fusions between different oxygenic phototrophs may have led to the evolution of a two-photosystem photosynthetic apparatus in the predecessor of cyanobacteria, perhaps using iron as an electron donor. Comparative biochemistry of the proteins in the different photosystems suggests homologies between PSII of cyanobacteria and the photosystems of purple sulfur bacteria, as well as between PSI and the photosystems of green sulfur bacteria. The evolutionary lowering of the basal potential of the type II photosystem, allowing the retrieval of electrons directly from water, would have supposed the tapping of a virtually unlimited and ubiquitous source of electrons for CO_2 reduction. This would have provided the first oxyphotobacterium with a wide range of potential niches and possibly enabling an early explosive radiation of particular adaptations.

Commercial Use and Applications

The commercial use of cyanobacteria has long been sought for but in most cases has not reached the production stage. Procedures and modified strains have been devised, for example, for the industrial production of amino acids, ammonia, and for the control of

mosquito larvae using genetically engineered strains that produce *Bacillus* toxins. The main commercial use of cyanobacteria is for the production of bulk biomass for human consumption, a practice that has a long history in traditional cultures. Natural blooms of *Arthrospira* (previously assigned to *Spirulina*) were collected, sun-dried, and cut into cakes for human consumption in preHispanic Mexico; this 'tecuitlatl' of the Aztecs was highly regarded and commercialized at that time. A very similar procedure is used today to manufacture 'Dihé' cakes by the Kanembu tribeswomen from the shores of Lake Chad. Indeed, dried *Arthrospira* contains 60%–70% protein. Today, it is also produced commercially in outdoor man-made facilities and commercialized under the trade name 'Spirulina' for the health-food market as a protein-rich, low-calorie, cholesterol-free, vitamin-loaded food supplement. Due to the cult that has developed regarding this form of food supplement, blooms of other species ('Aphanizomenon', traditionally not consumed and strains of which are known to contain toxins) are also being commercially sold. *Nostoc commune*, a terrestrial cyanobacterium, is considered a delicacy and has been collected and marketed for centuries in China. Given the central role that natural populations of cyanobacteria play in maintaining the long-term fertility of paddy soils for rice cultivation, inoculating rice fields with cyanobacterial mixtures is currently a standard agricultural practice in some Asian countries. The symbiotic association *Anabaena*/*Azolla* (see 'symbioses') is intensively cultivated in the Far East for its use as green manure and as fodder for poultry and swine; written instructions for this practice date to 500 BCE.

There are many cyanobacterial metabolites considered as "high-value products". Carotenoids such as beta-carotene and astaxanthin, as well as the phycobilin phycocyanin are commercialized as food colorants. Chlorophyll *a*, radiolabeled nucleotides, amino acids, and some restriction endonucleases of cyanobacterial origin are sold for research purposes. There are many examples of cyanobacteria bioactive compounds with antibacterial, antifungal, and antiviral properties. Furthermore, the production of biopolymers such polyhydroxyalkanoates (PHAs) by cyanobacteria are used as a biodegradable alternative to petroleum-based plastics, especially in the biomedical industry.

The possibility of using cyanobacteria to produce biofuels is currently being pursued by a variety of academic and industrial ventures worldwide. Cyanobacteria are especially attractive for this purpose since they require only sunlight and water and can incorporate atmospheric or water-dissolved CO_2 into hydrocarbons for biofuels. Biofuel products produced by cyanobacteria include biodiesel, alcohols (e.g., ethanol, butanol), aldehydes, terpenoids (e.g., isoprene), alkanes, free fatty acids, hydrogen, and ethylene. For example, high levels of hydrogen were produced by a wild-type strain of *Cyanothece* sp. ATCC 51142 under aerobic conditions in the presence of glycerol, and *Lyngbya aestuarii* produces naturally copious hydrogen from the fermentation of photosynthate. However, an added benefit of using cyanobacteria to produce these biofuels is that many strains can be genetically manipulated to increase yields. Approaches to optimize the metabolic capability of cyanobacteria to synthesize these products include improving rates of CO_2 fixation, optimizing pathway flux, increasing tolerance to toxic products, and elimination of competing pathways. Modification of the glycolytic pathway of *Synechococcus elongatus* PCC 7942 resulted in increased shuttling of CO_2 to the Calvin-Benson cycle and production of 2,3 butanediol in the presence of light. Furthermore, alkane production was accomplished by heterologous expression of a two-gene operon from *Synechococcus elongatus* PCC 7942 in *E. coli* to produce a mixture of C13 to C17 alkanes and alkenes. Another approach to increasing the efficiency of using cyanobacteria for fuel production is to improve downstream processes, such as biomass extraction. In *Synechocystis* sp. PCC 6803 a strain capable of secreting free fatty acids was engineered to improve the secretion of some fatty acids up to 30% compared amounts secreted by the wild type.

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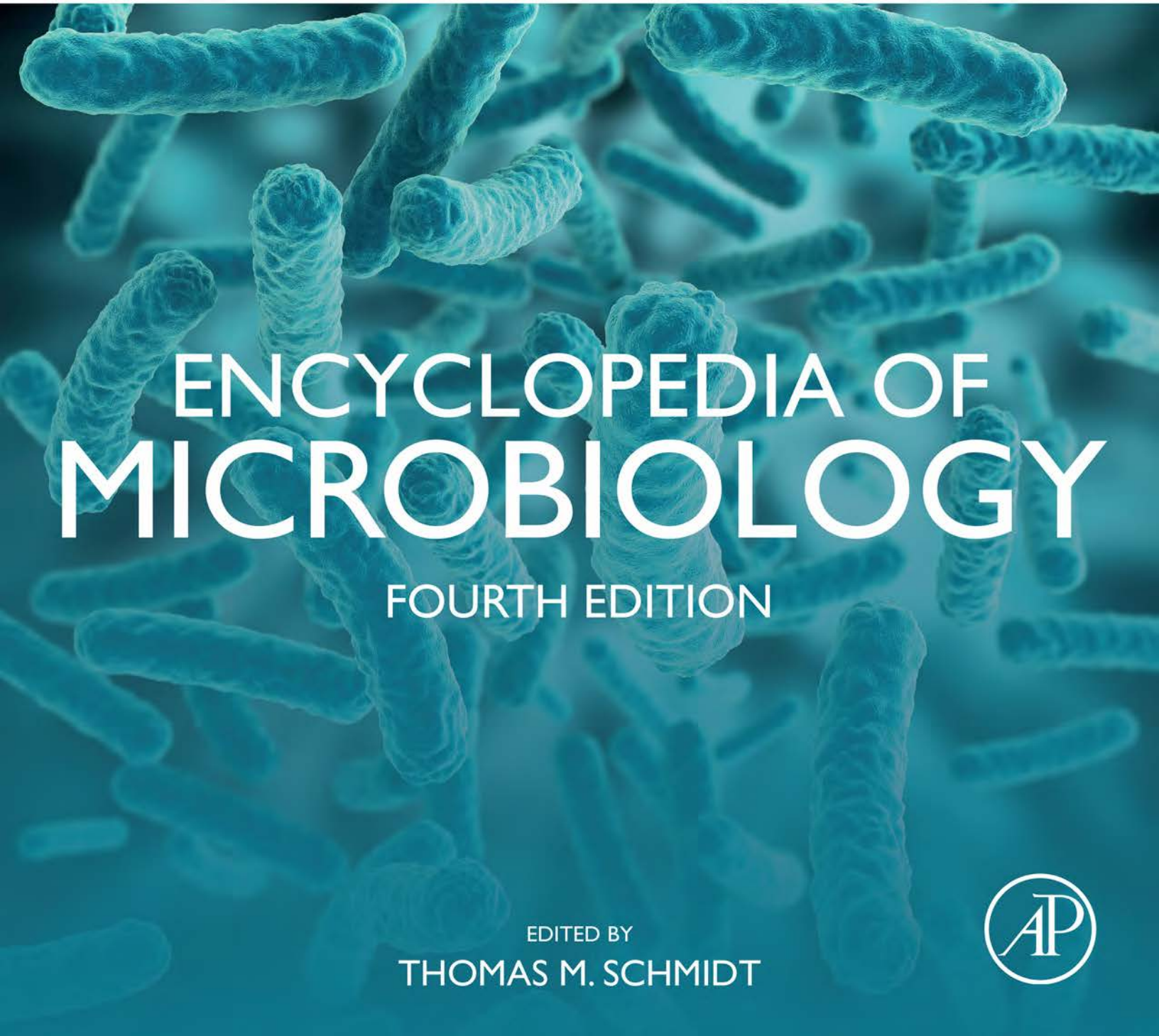

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EDITOR-IN-CHIEF

Thomas (Tom) Schmidt is a microbial physiologist and ecologist who studies diverse microbes and microbial communities. Tom received a PhD from The Ohio State University and conducted postdoctoral research at Scripps Institute of Oceanography and Indiana University. He spent much of his career studying the ecology of microbes in soil that are responsible for the exchange of greenhouse gases with the atmosphere. More recently, he joined the University of Michigan and focused his research on the human microbiome. His joint appointment in the Departments of Internal Medicine and Ecology & Evolutionary Biology reflects his expertise in applying ecological and evolutionary principles to understand the functioning of complex microbial communities.

Tom is a fellow of the American Academy for Microbiology and was director of the Marine Biological Laboratory's Microbial Diversity Course in Woods Hole. Through that course and in his laboratory, he has helped numerous scientists incorporate molecular approaches into traditional strategies for studying the microbial world. He currently teaches a university course that merges his research and teaching goals by engaging students in a coordinated study of the effects of diet on the gut microbiome, and he directs a graduate program that combines laboratory sciences and modeling. Tom was a section editor for previous editions of the *Encyclopedia of Microbiology* and was delighted to assume the role as editor-in-chief to help bring the microbial world to an expanding audience.

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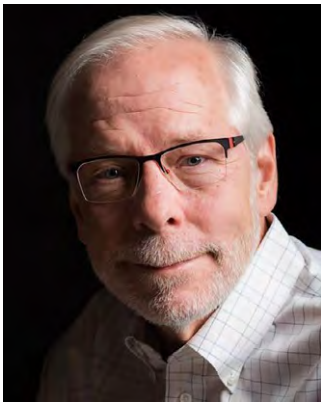
Bianca Brahamsha is a research microbiologist at Scripps Institution of Oceanography at the University of California, San Diego. She was a biology and history major at Goucher College, earned her PhD in microbiology at Cornell University, and carried out postdoctoral work in molecular genetics as an American Cancer Society fellow at the University of Chicago. Her research interests have centered on bacterial motility, the genetics and physiology of marine cyanobacteria, and on the interactions between cyanobacteria and their eukaryotic predators.

James (Jim) Brown was born in Atlanta, Georgia, and lived in Dade City (Florida), Bloomington (Indiana), Lima (Peru), and Muncie (Indiana) while growing up. From the beginning, he had an intense interest in nature, including anything found in the woods, rivers, beach or ocean that were always nearby.

Jim attended Ball State University beginning in 1976. A single lecture on microbial diversity in a general microbiology class, followed by the announcement of the discovery of the Archaea (George Fox and Carl Woese, 1977), sparked his lasting interest in microbiology. This led to undergraduate research examining *Beggiatoa* in a sulfur spring in French Lick, Indiana. After receiving his BS in biology in 1980, he joined the graduate program in microbiology at Miami University, where he worked on plant tissue culture mRNAs with Prof. Ronald Treick. After obtaining an MS degree in 1982, he moved to the MCD Biology Program at The Ohio State University to work on the molecular biology of methanogenic Archaea in the Department of Microbiology with Prof. John Reeve. While there, Jim worked on polyadenylation of mRNAs, RNA polymerase, and promoters in Archaea, and received his PhD in 1988. Jim then went to Indiana University for 5 years of postdoctoral work in Prof. Norman Pace's lab on the comparative analysis of the structure of a bacterial ribozyme, RNase P.

In January of 1994, Jim started as an assistant professor in the Department of Microbiology and moved to North Carolina State University. Research in Jim's lab focused on the comparative sequence and biochemical analysis of RNA, and in particular RNase P in Archaea. Students in the Brown lab developed a high-resolution model for the structure of this RNA, how it changed over the diversification of the Archaea, the protein subunits associated with the RNA, and how they contribute to the function of the holoenzyme. Jim developed, teaches, and wrote the textbook for a senior-level undergraduate lab course in microbial diversity. Jim is now Professor Emeritus in the Department of Biological Sciences at NC State University.

Jim has a son and two daughters, and is married to Melanie Lee-Brown, professor of biology and director of Research and Creative Endeavors at Guilford College. For fun, Jim enjoys skin and scuba diving, driving (i.e., working on) his 1968 Lotus Super Seven, and restoring vintage racing bicycles. Jim and Melanie have been renovating an abandoned fishing lodge on North Caicos Island (Turks and Caicos Islands, BWI), which will be reopened soon as a bed and breakfast.



Larry Forney is a university distinguished professor and a member of the American Academy of Microbiology with academic appointments in the Department of Biological Sciences and Bioinformatics and Computational Biology at the University of Idaho. Dr. Forney is an evolutionary ecologist who conducts research on the complex array of factors that influence the function, composition, structure, and temporal dynamics of bacterial communities in a wide array of habitats. In recent years he has largely focused on the community ecology of the human vagina across a woman's lifetime with an eye on understanding how changes in community structure and function affect a woman's risk to bacterial vaginosis, sexually transmitted infections, and pre-term birth, and how host factors shape these communities. His research extends to understanding the mutation-selection processes that govern the occurrence and persistence of genetic diversity of bacterial populations in spatially structured environments such as microbial biofilms and porous media. These studies have shown that spatial structure creates conditions in which mutation frequencies are high and selective sweeps are protracted, which leads to extraordinary within species diversity that increases the resilience of populations to environmental changes.

Robert Haselkorn was the F. L. Pritzker distinguished service professor of molecular genetics and cell biology at the University of Chicago, retiring several years ago. He was an undergraduate at Princeton, a graduate student at Harvard, and a postdoctorate in Cambridge, England. He started his teaching career at Chicago in 1961 in biophysics, extending later to microbiology, biochemistry, and chemistry. His research interests have centered on heterocyst differentiation in nitrogen-fixing cyanobacteria, in bacterial genomics, and in the enzyme acetyl-CoA carboxylase in plants, parasites, and people. He is a member of the National Academy of Sciences, a fellow of the American Academy of Arts and Sciences, and a member of the American Philosophical Society. Among other external activities he was a founder and adviser for 20 years to the International Center for Genetic Engineering and Biotechnology, located in Italy and India. For the past 15 years Haselkorn and his wife Margot have been working on selection and supporting a speaker for the Haselkorn Lecture at the University of Chicago, an award they have endowed. Many of the Haselkorn Lecturers have been Nobel Prize winners or will be soon.

Jennie C. Hunter-Cevera received her PhD from Rutgers University in 1978 (microbial physiology and biochemistry), an MS in microbial ecology in 1972, and a BS in biology from West Virginia University in 1970. She is the founder of Hunter and Associates, a consulting firm focusing on finding integrative solutions to complex problems involving sustainability in the life sciences arena. From July of 2009 to August 2012, she was the executive vice president of Discovery and Analytical Sciences (DAS), Corporate Development and Government Relations. She has 22 years of experience in the pharmaceutical and biotechnology industries (E. R. Squibb and Sons, Cetus Corporation, GeoBiotics, and Universal Foods). She was the co-founder of The Biotic Network and BlueSky Laboratory that contracted with biotechnology and pharmaceutical companies on basic and applied natural product research. Dr. Hunter-Cevera was an

employee for 5 years with the Department of Energy as the head of the Lawrence Berkeley National Laboratory's Center for Environmental Biotechnology. She worked in academia for 10 years as the president of the University of Maryland Biotechnology Institute and 2 years as the Interim Provost of Mount St. Mary's University. She also served as project manager for UC Davis's CIFAR (Center for Industrial Food and Agricultural Research). She has published many papers and chapters, and served as senior editor of the *Journal of Industrial Microbiology and Biotechnology* for 10 years. She is a co-section head of microbiology for Faculty of 1000. She holds 15 patents and specializes in areas of screen design for the discovery of natural compounds in the area of human therapeutics, biodefense, sustainable agriculture, bioremediation, and biocatalysis for industrial processes in the food and clothing industries.

While in Maryland, she served as a member of the Executive Committee for Governor Erhlich's transition team, his Committee on science, technology, engineering, and maths, and was the Technology Representative for Governor Glendenning on the Southern Governor's Association, and she served on the Technology Economic Development Corporation, TEDCO (Board of Directors for 4 years and was Chair for 2 years). Dr. Hunter-Cevera also served on the Advisory Board for the Maryland Industrial Partnerships, the MDBio's Board of Directors, and the MDBio Foundation for 10 years and on the BioIT Coalition for 5 years. She served as an Entremed Board Member for 10 years and served on the Executive Committee for 2 years. She was a board member for Patients with Power, a software company that designs programs for cancer patients to make the best decisions for their treatments. Jennie also served as acting secretary for Maryland's Higher Education Commission in 2015.

She is a member of several professional societies and has served as president of the Society for Industrial Microbiology, the International Marine Biotechnology Association, and the United States Federation of Culture Collections. Dr. Hunter has served on many national committees and commissions and was Chair of the National Research Council's Committee on Large Scale Production of Biofuels from Algae. She also chaired two other NRC Committees: Standing Committee on DOD's Translational Medicine and the DOE's Genome to Life for Biofuels.

She is the recipient of several awards and honors including Maryland's Top 50 Influential People (2007, 2009) and Maryland's Top 100 Women (2003, 2006, 2009), American Society for Microbiology's Porter Award for distinguished research in microbial systematics and taxonomy, elected as a SIM fellow, a member of the ASM Academy of Microbiology, and an AAAS fellow. She is also a WVU Distinguished Alumni Awardee and Nath lecturer and served as an entrepreneurial coach for the UNC Executive MBA program. She was awarded an Honorary Doctorate from West Virginia University, May 2013, and the Rutgers Cook College Dennis M. Fenton Distinguished Graduate Alumni Award in 2014.

She currently serves on the International Advisory Council for Brazil's Fundação Dom Cabral which is a world-class Brazilian business school that develops strategic thinking skills of executives, entrepreneurs, and public-sector managers. In addition, Jennie served 6 years on the Edison Awards Steering Committee and currently serves on the Edison Awards Advisory Board.

Stanley Maloy is a professor of microbiology and associate vice president for research and innovation at San Diego State University. Stanley's research has focused on bacterial and phage genetics and physiology, evolution of infectious diseases, and development of antimicrobials and vaccines. In addition, he has consulted with large and small companies in multiple sectors, played a role in starting several Biotech companies, and served leadership roles in multiple start-up companies.

Dr. McCormick earned her PhD in microbiology in the topic area of intestinal ecology, and completed postdoctoral training at Harvard Medical School. She remained on the faculty of Harvard Medical School where she was an associate professor of pediatric gastroenterology, and director of research for the Mucosal Immunology and Biology Research Center at Massachusetts General Hospital. In 2008, she joined the University of Massachusetts Medical School where she is professor and vice chair of the Department of Microbiology and Physiological Systems. Dr. McCormick is also the founding executive director of the University of Massachusetts Center for Microbiome Research, which she established in 2014. Dr. McCormick is one of the original pioneers in the field now known as cellular microbiology. Her work provided the first evidence that epithelial cells in response to pathogen contact orchestrate a pro-inflammatory program, which recruits inflammatory cells. Dr. McCormick has since identified new, previously unidentified and unexpected virulence mechanisms that are key to the inflammatory response, leading to both novel biological principles of host–microbe interactions and therapeutic intervention strategies for the treatment inflammatory bowel disease, and cancer. Her work continues to identify novel ways in which microbes interact with the intestinal epithelium, publishing over 100 original research papers and opinion pieces in this area. Dr. McCormick is an elected fellow of the American Academy of Microbiology, is on the Board of Editors for *Gastroenterology*, and *Gut Microbes*, and serves as a member of four Editorial Review Boards.

Dr. Mobley received his BS degree in biology from Emory University in 1975 and his PhD degree in microbiology and immunology from University of Louisville in 1981. He conducted postdoctoral training in biological chemistry and then bacterial genetics in the Center for Vaccine Development at the University of Maryland School of Medicine. He served on the faculty at the University of Maryland School of Medicine from 1984 until 2004 in the Division of Infectious Diseases (1984–97) and then the Department of Microbiology and Immunology (1997–2004) where he led the graduate program. During that time, he held a joint appointment in the Department of Biochemistry and Molecular Biology and trained graduate students in that program. In 2004, Mobley moved to the University of Michigan to chair the Department of Microbiology and Immunology and was installed as the inaugural Frederick G. Novy Collegiate Professor of Microbiology and Immunology.

Dr. Mobley's research interests focus on the molecular mechanisms of bacterial pathogenesis and on the fundamental basic research that will lay the groundwork for future therapeutics and vaccines. His lab studies virulence mechanisms of uropathogenic *Escherichia coli* and *Proteus mirabilis* that cause uncomplicated and complicated urinary tract infections, respectively, and, in the recent past, *Helicobacter pylori* that causes gastritis and peptic ulcer disease. For *E. coli* and *Proteus mirabilis*, his lab is focused on identifying surface-exposed proteins that are both synthesized by the bacteria during a urinary tract infection and conserved among uropathogenic *E. coli and Proteus* strains. Using these conserved antigens, his lab is determining the efficacy of candidate proteins as components of a multivalent subunit vaccine to protect against urinary tract infection.

Dr. Mobley is a fellow of the American Academy of Microbiology and a fellow of the American Association for the Advancement of Science and a member and past president of the Association of Medical School Microbiology & Immunology Chairs. He was the recipient of the inaugural University of Michigan Postdoctoral Association Excellence in Mentorship Award in 2012. He is a member of the editorial review boards of *Infection and Immunity and Microbiology Spectrum* and has served as a study section member for the National Institutes of Health. Dr. Mobley was awarded and named a distinguished university professor in 2015. This is the University's most prestigious professorship established to recognize senior faculty with exceptional scholarly achievements, national and international reputations for academic excellence, and superior records of teaching, mentoring, and service.

Dr. Mobley has published 255 peer-reviewed articles which have been cited in the literature over 18,000 times as of June 2019, as well as 49 book chapters and 5 books. He has trained 29 PhD students and 38 postdoctoral fellows, and has delivered 232 invited lectures in 21 countries.

Carlos Pedrós-Alió graduated in biology at the Autonomous University of Barcelona and got his PhD in bacteriology at the University of Wisconsin–Madison. After a postdoctoral stay at the Autonomous University he became an assistant professor of microbiology. He moved to the Marine Sciences Institute (CSIC, Barcelona) in 1989 where he was a research professor since 2000. In 2016 he moved to the National Center for Biotechnology (CSIC, Madrid). Dr. Pedrós-Alió's interest is to understand the ecology of aquatic microorganisms. Around 2005 he started to use genomics as a tool to generate hypotheses that could later be tested experimentally. He also likes to study extreme environments such as hypersaline systems, thermal springs, or polar waters. Another interest is in finding the mechanisms maintaining a large number of rare bacteria in aquatic ecosystems. He is also interested in outreach, relationships between art and science, biology of spirituality, fiction writing, and birding.

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SUBJECT CLASSIFICATION

HISTORICAL

AIDS, Historical
Cholera, Historical
Historical Plague
Historical Smallpox
History of Microbiology
History of Virology
Methods, Philosophy of
Spontaneous Generation
Syphilis, Historical
Typhoid, Historical
Typhus Fevers and Other Rickettsial Diseases, Historical

MICROBIAL DIVERSITY

Acidobacteria
Amitochondriate Protists (Diplomonads, Parabasalids and Oxymonads)
Amoebozoa
Aquificae
Archaea – An Introduction
Aspergillus: A Multifaceted Genus
Bacillus Thuringiensis: Mechanisms and Use
Bacteriophage: Overview
Brochothrix thermosphacta
Caulobacter
Chlamydia
Chloroflexi
Clostridia
Cyanobacteria
Dictyostelium
Dinoflagellates
Escherichia coli
Green Algae: Chlorophyta and Streptophyta
Haemophilus Influenzae
Halophilic Archaea
Helicobacter pylori
Legionella and *Bartonella*
Leishmania
Listeria monocytogenes
Mollicutes
Nanoarchaeota
Phylum *Verrucomicrobia*
Picoeukaryotes
Planctomycetes
Plant Pathogens, Minor (Phytoplasmas)
Protozoa
Rhizobia
Spirochetes
Staphylococcus

Streptococcus Pneumoniae Evolving – Impact of Antibiotics and Vaccines
Streptomyces
Trypanosomes
Viroids/Virusoids

PHYSIOLOGY AND GENOMICS

Archaellum
Autotrophic CO2 Metabolism
Bacterial and Archaeal Cell Membranes
Bacterial and Archaeal Cell Structure
Bacterial Bioluminescence
Bacterial Cell Cycles and Division
Bacterial Chemotaxis: Conservation and Variation on a Theme
Bacterial Development
Bacterial Flagella
Bioluminescence in Eukaryotic Microbes
Chromosome Replication and Segregation
Circadian Rhythmicity in Prokaryotes
Conjugation, Bacterial
CRISPR–Cas9
Crystalline Cell Surface Layers (S-Layers)
Energy Transduction Processes
Fundamentals of Metabolic Systems Biology
Gene Transfer Agents
Genetically Modified Organisms: Guidelines and Regulations for Research
Glycogen Biosynthesis
Horizontal Gene Transfer: Uptake of Extracellular DNA by Bacteria
Intracellular Structures of Prokaryotes: Inclusions, Compartments, and Assemblages
Iron Metabolism
Lipid Biosynthesis
Magnetotaxis
Methanogenesis
Methylation and other Modifications of Nucleic Acids and Proteins
Microbial Solute Transporters
Nitrogen Assimilation in Bacteria
No Bones About It: The Bacterial Cytoskeleton
Nonflagellar Bacterial Motility
Outer Membrane, Gram-Negative Bacteria
Peptidoglycan (Murein)
Phototaxis in Archaea and Bacteria
Phototrophy and Phototrophs
Pili, Fimbriae
Posttranscriptional Regulation
Regulation of Carbon Assimilation in Bacteria
Regulation of Replication Origin Firing
Regulatory RNAs
Restriction-Modification Systems
RNA Processing
Sensory Transduction in Bacteria
Single-Particle Cryo-Electron Microscopy
Stress, Bacterial: General and Specific
Stress Responses: Heat
Swimming and Swarming Motility
The Bacterial Glycome: From Monomers to Complex Carbohydrate Polymers
The Social Evolution of Bacterial Quorum Sensing
Transcription Regulation in Bacteria
Transduction: The Transfer of Host DNA by Bacteriophages
Translational Control and Fidelity

ECOLOGY AND EVOLUTION

Adaptations of Microorganisms to Low Nutrient Environments: Managing Life in the Oligotrophic Ocean
Adaptive Radiation in Microbes
Algal Blooms
Bacteriophage Ecology
Ecology of Rare Microorganisms
Ecology, Microbial
Endophytic Microbes
Evolutionary Theory and Experiments With Microorganisms
Extremophiles and Acidic Environments
Extremophiles: Cold Environments
Extremophiles: Dry Environments (Including Cryptoendoliths)
Extremophiles: Hot Environments
Extremophiles: Hypersaline Environments
Freshwater Habitats
Genomes From Uncultivated Microorganisms
Intracellular Symbionts and Parasites
Marine Deep Biosphere
Microbial Biofilms
Microbial Cycling of Methane
Microbial Ecology of the Rumen
Microbial Mats: Impact on Geology
Mixotrophy Among Freshwater and Marine Protists
Models in Microbial Ecology
Nitrogen Cycle
Origin of Life, Theories of
Overview of Plant Diseases
Palaeontology, Microbial
Paramecium Molecular Evolution
Phosphorus Dynamics in the Environment
Plastics: Colonization and Degradation
Quorum-Sensing in Bacteria
Rhizosphere
Secondary and Tertiary Endosymbiosis
Sediment Habitats, Including Watery
The Evolutionary Ecology of Microbes
Virus Evolution

PATHOGENESIS AND IMMUNOLOGY

Adhesins During Infection
Airborne Infectious Microorganisms
Antibiotic Resistance
Antifungal Agents
Antigenic Variation in Bacterial Pathogens
Antiviral Agents
Arboviruses
Bacterial Iron Acquisition Strategies
Biofilms and Disease: A Persistent Threat
Capsules and Secreted Extracellular Polysaccharides
Chlamydophila pneumoniae, A Pathogen Causing More Than Pneumonia
Commensal to Pathogen Transition of *Candida albicans*
Complement
Cutaneous Fungal Infections
Diagnostic Microbiology
Emerging and Reemerging Infectious Diseases
Entamoeba Histolytica: Biology and Host Immunity
Enteric Viruses
Epidemiological Concepts and Historical Examples

Exotoxins
First Principles of Clinical Microbiology: Collection, Handling, and Diagnostics
Fungal Biofilms
Fungal Infections, Systemic
Gastrointestinal Microbiology in the Normal Host
Gut Microbiota in Human Health and Diseases
Hemorrhagic Fever Viruses
Hepatitis Viruses
Herpesviruses
HIV and Retroviruses
How Microbial Pathogens Subvert Host Innate Immune Defenses
Human Fungal Infections
Lipopolysaccharides (Endotoxins)
Medically Relevant *Mycoplasmas and Ureaplasma*
Microbial Agents to Treat Cancer
Microbiology of the Cystic Fibrosis Airway
Mycotoxins
Oral Microbiology
Pathogen Sensing: Toll-Like Receptors and NODs (Innate Immunity)
Phage Therapy
Phagocytes (Innate Immunity)
Polyomaviruses and Papillomaviruses
Quinolones
Rabies
Respiratory Viruses
Retroviruses
RNA Viruses: Plant Pathogenic
Role of B Cells and Antibodies in Controlling Bacterial Pathogens
Sexually Transmitted Diseases
Toxoplasmosis
Unusual Infectious Agents

TECHNOLOGICAL ADVANCES AND APPLIED MICROBIOLOGY

Amino Acid Production
Amylases
Antimicrobial Susceptibility Testing
Bacterial Targeting of Tumors
Bacteriophages and Rapid Detection of Bacterial Pathogens: A Novel Approach
Beer/Brewing
Biocides
Biodeterioration – Including Cultural Heritage
Biological Warfare
Bioreactors
Biosensors
Bioterrorism
Continuous Cultures (Chemostats)
Corrosion, Microbial
Directed Evolution
DNA Cloning Strategies
Drinking Water
Drinking Water Microbiology
Foodborne Pathogen Detection, Using Rapid Technologies
Fungal Extracellular Vesicles
Genome Sequence Databases: Annotation
Genome Sequence Databases: Sequencing and Assembly
Genome Sequence Databases: Types of Data and Bioinformatic Tools
Genome Sequencing of Microbes
Heavy Metal Pollutants: Environmental and Biotechnological Aspects
Industrial Biotechnology (Overview)

Industrial Production of Glycosaminoglycans
Infectious Waste Management
Insecticidal Toxins from *Photorhabdus and Xenorhabdus*
Metal Extraction and Biomining
Microbial Adhesion
Microbial Forensics
Microbiology of Fermented Dairy Products
Municipal Water Treatment
Organic and Fatty Acid Production, Microbial
Patenting of Microorganisms
Pesticides, Microbial
Phylogenetic Methods
Pigments, Microbial
Polysaccharides, Microbial
Solvent (Acetone–Butanol: AB) Production
Teaching Resources, Microbiology
Technology Advances in Medical Microbiology
Trehalose: A Crucial Molecule in the Physiology of Fungi
Type Culture Collections their Databases
Vitamins and Vitamin-Like Compounds: Microbial Production
Water Treatment, Industrial
Wine
Xylanases

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PREFACE

As with previous editions of the *Encyclopedia of Microbiology*, the 4th edition takes on the challenge of providing a contemporary overview of microbes—the most abundant and diverse forms of life on Earth. These single-celled organisms were the first life forms to inhabit Earth and they transformed the planet and its atmosphere. They continue to maintain Earth's atmosphere, drive essential processes in terrestrial and aquatic ecosystems, and form intimate relationships with all plants and animals. Microbes can reproduce by doubling every 20 minutes or can remain dormant for many thousands of years. They colonize every known habitat on Earth, accounting for approximately half of the living biomass on our planet. Microbes also cause the most devastating diseases known to humans and are winning the antibiotic war we wage against them. Yet, we cannot live without microbes as symbionts of humans and drivers of Earth's biosphere.

Despite the central roles of microbes, we are only just becoming aware of our limited knowledge of interactions among microbes, other organisms, and the environment. Molecular techniques continue to lead a revolution in our understanding of microbial diversity and function, with the new "big data" sciences of genomics, transcriptomics, proteomics, and metabolomics now being applied to communities of microbes. These techniques provide the ability to identify and study complex communities of microbes in diverse environments including the human gut, forest soil, water-treatment biofilters, and the open ocean.

Given the remarkable physiological and phylogenetic diversity of microbes, their capacity for rapid evolution, and the pivotal role of host-associated and environmental microbiomes, it is difficult, if not impossible, to identify contemporary questions in biology that are not influenced by microbes. It is also increasingly difficult to assemble an encyclopedia that provides comprehensive coverage of the vast universe of microbes. Despite these challenges, we have engaged the expertise of scientists around the globe to provide an exceptional overview of and perspective on the microbial world. This edition of the *Encyclopedia of Microbiology* will help readers develop a framework for understanding microbes and will provide references directing readers to the primary literature that is needed for a more thorough appreciation of specific topics.

We have included a number of articles which provide a historical perspective of microbiology that is focused on disease—one of humanity's earliest acknowledgment of microbes. These help frame more contemporary issues in microbiology that have moved far beyond the relatively small collection of microbes that cause disease. Rather than simply alphabetize topics as in traditional encyclopedias, articles in the 4th edition are also listed under the following themes in a Subject Classification: Historical, Microbial Diversity, Physiology and Genomics, Ecology and Evolution, Pathogenesis and Immunology, and Technological Advances and Applied Microbiology. We hope this helps readers navigate the work more effectively.

On behalf of an insightful and collegial editorial team, I hope that you emerge from your forays into the *Encyclopedia of Microbiology* with some of the awe that we share for the elegance and power of the microbial world.

Thomas M. Schmidt
Editor-in-Chief

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INTRODUCTION: MICROBIAL DIVERSITY

It is what we think we know already that often prevents us from learning.

—Claude Bernard

One of the difficult lessons for any professional scientist is that, as much as we know, as good as our tools and questions are, our knowledge is crude and far smaller than we imagined when we were students. Particularly troublesome is that most fundamental aspects of various scientific fields are simply not very well understood. The physicist wonders what, exactly, "time" is, how space is structured, and why the Planck Constant is $6.62607004 \times 10^{-14}$ m^2 kg/s instead of any other number you might care to choose. Chemists argue about the nature of a covalent bond, or whether or not transition states exist in reality. Biologists are at a loss to describe how life originated, or even what exactly life (in a general sense) actually is. In the most important book of modern times, "On the Origin of Species," Charles Darwin clearly (but not concisely) deconstructs the idea of the biological "species," and despite what many of us learned in school, our view of the "species" even for multicellular sexually reproducing creatures remains murky. The modern reader of "On the Origin of Species" might be forgiven for concluding that the reason for this is that it is the actual *biology* of species, and by extension "life," our origin, etc., rather than our concepts of them, that are fundamentally disordered.

The subject of this section of the Encyclopedia is Microbial Diversity. What does this mean? What *is* "microbial diversity?"

Let us start with "microbial." Literally speaking, the term "microbial" means small living things, usually understood to mean life too small to see with the unaided eye. The scientific field of microbiology, however, mostly emerged from our need to solve crucial health dangers created by a tiny fraction of microbes. As a result, "microbiology," and so "microbes," refer to Bacteria (and in modern times the Archaea), fungi, a scattering of problematic single-celled or small multicellular eukaryotes, and the physiological systems of the human body that manages our interactions with these creatures. This sells the microbial world very short. For 85% of the history of our planet, life was *entirely* microbial, and it remains *predominantly* microbial. In the words of Stephen J. Gould, "*We live now in the 'Age of Bacteria.' Our planet has always been in the 'Age of Bacteria,' ever since the first fossils—bacteria, of course—were entombed in rocks more than 3 billion years ago. On any possible, reasonable or fair criterion, bacteria are—and always have been—the dominant forms of life on Earth.*" Another view of this comes from the phylogenetic perspective. If you scrutinize any objective/quantitative "Tree of Life," based on molecular sequence analysis or any other criterion of choice, you will quickly discover that nonmicrobial life is limited to the tips of a small number of otherwise un-noteworthy branches. Only from our self-centered anthropocentric viewpoint is microbiology distinguishable from biology as a whole.

And what of "diversity?" Living things and their morphology, structure, physiology, ecology, internal mechanisms, behavior, interactions, etc., are far more diverse than most of us have been led to believe. For example, if you think you know how mitosis works, look it up in *Giardia* and be amazed. The notion of what constitutes a "gene" in the kinetoplast of *Trypanosoma* requires you to throw almost everything you think you know about molecular genetics away. Then have a look through the genome of the archaeon *Nanoarchaeum.* These are just the tips of icebergs; these and many more can be found within the chapters that follow. And yet . . . all of this diversity rests over an almost entirely uniform biochemistry that speaks to the shared ancestry of all living things on Earth. How, then, do we assess the "diversity" of living things? Historically, this meant comparing the morphology of different living things; this was the origin of Linnaean taxonomy and, to be fair, is very useful for organisms on the human size scale. More recently molecular phylogenetics has allowed us to more clearly understand how creatures are related, and draw an objective and quantitative graph of these

relationships; the molecular "Tree of Life" is a more useful roadmap of biological diversity. But it is clear that an even more sophisticated view of biological diversity is required, for example to describe how horizontal gene transfer impacts "diversity," and many of these questions emerge from microbiology. Perhaps the most important fundamental question in microbiology today is "What is a microbial species?" In other words, how do we conceive of a "species" in organisms that do not reproduce sexually? This is not a trivial or parochial question; remember that the concept of biological evolution, and so the foundation of the science of biology, emerged directly from exploration of what a "species" is and how it originates, in plants and animals. But this was over 150 years ago. Broadening our view of microbial diversity, how it is structured and its history and origins, is the key to new and deeper biological insight.

And so, as is usual in science, attempt to answer a simple question that raises many more questions and provides few answers. What *is* microbial diversity? I dunno, but let us have a look. . ..

James Brown

INTRODUCTION: PHYSIOLOGY AND GENOMICS

The first two editions of EOM were edited entirely by Dr. Joshua Lederberg. The third edition was also planned largely by Dr. Lederberg, but he died during that phase, in 2008, and was replaced by Dr. Moselio Schaechter, promoted from the ranks of associate editors. Dr. Thomas Schmidt was also an associate editor for that edition and moved up to editor-in-chief for the fourth edition.

The time that has passed from the first to the fourth edition has seen remarkable advances in understanding the variety of microbes. Complete genome sequences were determined for 18 microbes by October 1998 and published in the second edition in 2000. By 2019, a single company had determined the complete annotated genome sequences of several thousands of microbes. Comparative genomics, including the ancillary "omics," as well as the application of systems biology to reconstruct metabolic networks, have provided new insights into many aspects of microbial physiology and genetics. The advent of cryo EM and of single particle cryo EM has revolutionized how we understand the structure of cells and macromolecules. For this fourth edition, we were guided by the splendid organization of the third and additional areas in which considerable advances have been made. The fourth edition now includes articles describing additional features of microbiology: cryo-electron microscopy, CRISPR/*cas*, and metabolic systems biology, as examples of completely new methods to study microbes, circadian rhythms as an important aspect of the physiology of at least one group of microbes, and new developments in our understanding of the many ways in which bacteria and archaea move. We have also made use of updates of chapters that were included in the third edition.

Bianca Brahamsha
Robert Haselkorn

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INTRODUCTION: ECOLOGY AND EVOLUTION

Evolution

Seven articles deal with different aspects of evolution. Two articles analyze the remains of the past evolution of microbes in the fossil record. Three other look at theories, mechanisms, and experimental procedures. Two more examine specific aspects of the evolution of viruses and of a ciliate. Finally, one article bridges ecology and evolution looking at the emerging field of evolutionary ecology of microbes.

Ecology

The ecology section contains a few general articles about what microbial ecology is about, and the particularities of the ecology of viruses and rare microorganisms.

The remaining articles can be placed in two complementary approaches: habitat oriented or process oriented. In the first approach there is a lot of attention devoted to extreme environments. These environments are usually exclusively microbial and show the capacity of microbes to deal with really difficult conditions. Besides, the relative simplicity of such ecosystems allows somewhat easier manipulations than in "normal" ecosystems. Several of the latter are also examined in articles ranging from the deep ocean to plastics. Special attention is given to animal and plant habitats and how microbes interact with their hosts.

The process-oriented approach shows, on the one hand, adaptive traits of microorganisms such as the ability to live in diluted environments or to use chemotaxis to find the optimal conditions. One promising and novel avenue to study adaptations is the analysis of the genomes of uncultured microorganisms. On the other hand, several articles review processes relevant at the ecosystem level, from feeding strategies such as myxotrophy to element cycles such as methane formation and oxidation and, finally, to the use of models in microbial ecology.

We hope this collection of articles provides an overview of the exciting times that microbial ecology is going through, with many new discoveries appearing every few years, and a fascinating and invisible world to be explored.

Carlos Pedrós-Alió
Larry Forney

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INTRODUCTION: PATHOGENESIS AND IMMUNOLOGY

This section of the encyclopedia focuses on the relatively narrow slice of all microbes that infect living hosts and how the hosts combat these microbes using innate and adaptive immunity. In addition, we examine how antimicrobial agents including unique metabolites and even bacteriophages have been enlisted to combat these infections. This section takes a broad look at infectious agents ranging from prions, viroids, and satellites to the more traditional etiologic agents like bacteria, viruses, fungi, and selected parasites. Also addressed are epidemiologic and diagnostic techniques used to identify and track the spread of infectious microbes including the most advanced diagnostic methods used by the clinical microbiology laboratory. A closer look at pathogenic mechanisms includes groups of virulence factors such as adhesins, iron acquisition, exotoxins of bacteria and fungi, and bacterial endotoxin (lipopolysaccharide). Strategies adopted by bacteria and fungi to colonize implanted foreign bodies such as catheters are also discussed. Plus, infections of the gastrointestinal tract, lung, oral cavity, and the skin are examined. In addition to pathogenic microbes, some articles examine the protective effect of the microbiome and commensal organisms. With respect to immunity this section also focuses on the interplay of the host immune system with pathogenic microbes covering evasion immune strategies in humoral and cellular immunity as well as innate immunology and the complement immune system.

Beth McCormick
Harry Mobley

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INTRODUCTION: TECHNOLOGICAL ADVANCES AND APPLIED MICROBIOLOGY

This section focuses on the development and refinement of the tools commonly used for both basic and applied microbiology. The articles in this section are written by authors who personally have worked on developing and fine-tuning technology for research in microbiology. Many of the products and processes described have multiple uses in a variety of disciplines such as health, the environment, food and agriculture, and industrial biocatalysis. The advances made in sequencing, protein analysis, and bioinformatics have given microbiologists new insights into old problems. These advances have also enabled the rapid identification of microorganisms in studies of biowarfare, infectious disease, and pollution of our water sources. At the same time the integration of mathematics and physics with biology and chemistry has provided instrumentation that enables a greater depth of analysis than ever before. Never in the history of microbiology have so many technological advances been developed and commercialized for use by scientists. Microbiologists today are equipped with improved "tools" within the toolbox to push the boundaries of not only elucidating the relationship of microbes to humans, plants and animals but also moving basic research much faster from the bench to development of new products and processes that benefit society and the planet. The renaissance of applied microbiology powered by technological advances has been both exciting and promising in all fields of study.

Jennie C Hunter-Cevera
Stanley Maloy

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Diagnostic Microbiology☆

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Abbreviations

CIA	Chromatographic immunoassay
CMV	Cytomegalovirus
CPE	Cytopathic effect
CRE	Carbapenem-resistant *Enterobacteriaceae*
EIA	Enzyme immunoassay
FDA	Food and Drug Administration
HAD	Helicase-dependent amplification
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
NASBA	Nucleic acid sequence-based amplification
NGS	Next generation sequencing
PCR	Polymerase chain reaction
PNA	Peptide nucleic acid
SDA	Strand displacement amplification
TMA	Transcription-mediated amplification
VRE	Vancomycin-resistant enterococci

Defining Statement

Diagnostic microbiology determines if microorganisms present in specimens collected from human beings, animals, and environment, are pathogens. Additionally, it defines infectious processes and aids in the management and treatment of patients based on the detection, quantification, and characterization of specific pathogens. This module provides an overview of the fundamental principles and techniques of diagnostic microbiology.

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Development of Diagnostic Microbiology

Diagnostic microbiology dates back to over three centuries when van Leeuwenhoek first observed bacteria and protozoa with his primitive microscope. However, it was not until the late 1800s that the work of Pasteur, Koch, and others ushered in the modern era of germ theory as well as the use of isolation techniques on artificial growth media. Since then, the capabilities of modern diagnostic microbiology have expanded and rapidly improved due to technological revolutions in microbiology, immunology, and molecular biology. A microorganism in a sample can be detected and identified by: (1) cultivation using artificial media or living hosts, (2) direct microscopic examination, (3) measurement of microorganism-specific immune responses, and (4) detection of microorganism-specific macromolecules, especially nucleic acids and antigen detection. The contrast of these techniques is summarized in Table 1, the following sections will discuss them separately.

During the last 30 years, diagnostic microbiology has significantly evolved. Until the early 1970s, definitive laboratory diagnoses of infectious diseases had been largely accomplished through the use of cumbersome, costly, time-consuming, and often subjective techniques. However, in the 1980s and 1990s, advances in molecular techniques were routinely incorporated within the laboratory. The development of radioimmunoassay and immunofluorescence, enzyme immunoassay (EIA) and immunoblotting, became routine diagnostic microbiology applications. These techniques, especially EIA, have supplanted many labor-intensive and relatively insensitive and nonspecific procedures such as complement fixation and hemagglutination inhibition assays for viral serology. Commercially available direct immunoassay kits make detection of certain important groups of viruses, such as respiratory syncytial virus and rotavirus, practical and economical. Application of monoclonal antibodies and antigen-recognizing probes, such as peptides, has significantly increased the sensitivity and specificity of antigen detection.

Direct microorganism identification based on antigen or nucleic acid detection through immunologic and molecular techniques has significantly shortened the test turnaround time. Oligonucleotide probes were used for culture confirmation and differentiation of slow-growing microorganisms, such as mycobacteria. Specialized nucleic acid hybridization probes, such as peptide nucleic acid (PNA) probes, are used for in-situ identification of organisms to the species level under a fluorescent microscope. Automated or semiautomated computerized commercial systems are now available for microorganism identification and antimicrobial susceptibility determination. Bacteremia and fungemia are now often detected by automated, instrument-assisted blood culture systems , which have significantly improved the sensitivity and specificity for detecting most such pathogens and, by virtue of automation, have eliminated much of the hands-on time for detection of positive cultures.

One of the greatest advances in the field of diagnostic microbiology has been in nucleic acid detection. The polymerase chain reaction (PCR) and other amplification techniques have simplified and accelerated the *in vitro* process of nucleic acid amplification. Rapid techniques of nucleic acid amplification and characterization, along with the increased use of automation and user-friendly software, have significantly broadened microbiologists' diagnostic arsenal. New real-time PCR, multiplex PCR, and microarray techniques provide relevant and accurate microbial quantification and typing, which play important roles in the selection and monitoring of antimicrobial therapy.

Advances in omics-based approaches (genomics, proteomics, and metabolomics) have led to their use in diagnostic microbiology and epidemiology. The development of robust next-generation sequencing platforms has dramatically increased sequencing capacity and reduced cost per base. These changes have made sequencing more accessible for the diagnostic laboratory such that sequencing is becoming a routine diagnostic tool in many laboratories. One of the most revolutionary technologies within the diagnostic laboratory has been the implementation of mass spectrometry (MS)-based microbial identification and characterization. Besides providing accurate and rapid identification of microorganisms, it has also significantly impacted laboratory costs (Patel et al., 2017; Perez et al., 2013). Transcriptomic and metabolomic profiles have also been shown to have clinical utility. Changes in transcriptomic and metabolite profiles can be used to diagnose and monitor severe microbial infections and differentiate between bacterial and viral infections (Barral-Arca et al., 2018; Herberg et al., 2016; Warsinske et al., 2018).

Table 1 Methods used for microorganism diagnosis.

Test	*Ease of performance*	*Turnaround time*	*Result interpretation*	*Advantages*	*Disadvantages*
Direct examination	Can be performed in routine clinical laboratory and in nurse station	1–3 h	Direct if correlated with symptoms	Rapid	Poor sensitivity and specificity; special skills are needed for interpretation
Culture	Can be performed in sophisticated clinical laboratory and in research laboratory	2–14 days	Definite; gold standard for most infection diagnosis	For phenotypic drug susceptibility testing	Time-consuming; poor sensitivity; limited microorganisms are culturable
Serology	Can be performed in larger and sophisticated clinical laboratory	4–6 h	Indirect	Automation	Results are generally retrospective; immunosuppressed host may be unable to mount a response
Molecular diagnostics	Can be performed in sophisticated clinical laboratory and in research laboratory	0.5–8 h	Direct without knowing microbial viability	High sensitivity and specificity	Quantification results awaits standardization; results might not be used as a test of cure

In summary, the implementation of these technologies within the diagnostic laboratory has had a positive impact in patient care. The old diagnostic microbiology model could only service inpatients because the methods used were labor intensive and time consuming. More so, these test results took days to months to be available. Because of the significant reduction in turnaround time, the modern diagnostic microbiology laboratory has gradually begun servicing an increasing number of emergency room patients and outpatient populations.

Direct Identification of Microorganisms

Microscopic Examination

Many test specimens may be examined in their native state under a microscope. A wet mount can be prepared by applying specimens directly onto the surface of a slide, which is often used to detect motile trophozoites of parasites such as *Giardia lamblia* in stools and *Trichomonas vaginalis* in vaginal discharges. Certain bacteria are so thin that they cannot be resolved in direct preparations. Their characteristic motility, however, is an important feature of presumptive identification. Darkfield microscope examination, a method of allowing light to be reflected or refracted off the surface of the object, is used to identify these bacteria; spirochetes, such as *Borrelia burgdorferi*, the microorganism causing Lyme disease, are primarily visualized with this method. This method is also used for the demonstration of motile treponemes in exudates collected from a primary chancre of syphilis.

Direct examination also allows the identification of certain characteristics that represent specific infections. The presence of cytoplasmic morulae in peripheral blood leukocytes is a timely way of confirming *Ehrlichia* infection. Detection of viral inclusions in smears or tissues has been the traditional means of directly demonstrating virus infections. Although not used routinely within the diagnostic laboratory, electron microscopy (EM) has been used to directly detect viral causes of gastroenteritis such as rotaviruses (Brandt et al., 1981).

The most common method of direct examination involves staining either the specimen itself or cultures. Microorganisms may be visualized and assigned to morphologic and functional groups using special stains. Gram stain is the most popular stain used within the diagnostic laboratory. It allows the staining of bacteria and yeast present in test samples. It is most commonly used to classify bacteria based on their forms, sizes, cellular morphologies, and color reactions. Nevertheless, there are other organisms for which Gram stain is not ideal. For example, mycoplasma lack a cell wall, therefore they will not be stained by Gram stain and their presence is visualized using acridine orange. Whereas for bacteria that possess unique cell wall components (e.g., mycobacteria), acid-fast stain is preferred. A modified acid-fast stain is useful for detecting certain parasites present in stool samples (e.g., *Cyclospora*). For systemic protozoa (e.g., malaria), Giemsa stain is the stain of choice. In the case of fungi, Calcofluor white binds to fungal cell walls in the presence of 10% potassium hydroxide, and fungal elements can be observed under a microscope. This allows sensitive direct fungus detection, including *Pneumocystis carinii*, a common opportunistic infection in AIDS and other immunocompromised hosts. Table 2 summarizes common staining methods used in diagnostic microbiology.

Table 2 Stains commonly used for detection of microorganisms.

Stain method	*Organisms detected*	*Advantages*	*Disadvantages*
Gram stain	Bacteria, yeast	Rapid; direct differentiation; assess specimen for culture	Cell wall-deficient bacteria stain unpredictably; pink background often masks Gram-negative organisms
Acridine orange stain	Bacteria, mycoplasma, plasmodia, Babesia	Good for organisms with damaged cell walls; background levels are low for structures that lack nucleic acid	Specific light source is needed; cannot differentiate bacterial Gram status reaction
Acid-fasting stain (Kinyoun or Ziehl-Neelsen)	Mycobacteria	Direct diagnosis of infection in untreated host	Cannot speciate mycobacteria; high background in tissue slide
Auramine-rhodamine stain	Mycobacteria	Lower power can be used for examining the slide	Fluorescence microscope is needed; artifact staining in tissue slide
Modified acid-fast stain	Nocardia, Cryptosporidia, Cystoisospora, Cyclospora	Rapid and specific diagnosis	Tissue homogenates often mask presence of the organism
Calcofluor white stain with potassium hydroxide	Pneumocystis, fungi	Rapid stain for fungi detection	Fluorescence microscope with specific filter is needed; species differentiation requires skills
India ink stain	*Cryptococcus neoformans*	Diagnosis of meningitis when positive in spinal fluid	Low sensitivity; a messy technique with false positives from lymphocytes in hypotonic solution
Giemsa stain	Plasmodia, trypanosomes, Leishmania, Toxoplasma, Histoplasma, Pneumocystis	Detection of multiple organisms; shows the relationship between organisms and host cells	Not specific for viral inclusions; cannot determine bacterial Gram status

Microbial Antigen Testing

Immunological approaches to the detection of microorganisms' protein antigens remain important tools for the diagnosis and management of infectious diseases. Great technical advances have been made since the introduction of the early precipitation and agglutination assays. For immunologic detection of microbial antigens, latex particle agglutination, immunofluorescence assay, and EIA are the techniques most frequently used. Antibodies to a specific antigen are bound to latex particles to produce agglutination. This technique has been extremely useful in the detection of *Cryptococcus neoformans* in bodily fluid specimens, such as plasma and cerebrospinal fluid. Direct immunofluorescence assay has been included in the shell vial technique for rapid virus culture detection (see below) and the detection of *Legionella pneumophila* in sputum.

There are several approaches to enzyme-linked antigen detection assays; the one most frequently designed for the detection of microbial antigens uses an antigen-specific antibody that is fixed to a solid-phase well in a plastic tray. Antigen present in the specimen binds to the antibody. The test is then completed by adding a second antigen-specific antibody bound to an enzyme that can react with a substrate to produce a colored product. Lateral flow chromatographic immunoassays (CIA) have been introduced to rapidly detect several respiratory virus-specific antigens and cryptococcal antigen. CIAs consist of a test strip which contain immobilized antigen specific antibodies conjugated to a chromogenic label. If the antigen is present in the patient's sample, it binds to the antibodies and the antigen–antibody complex continues to migrate laterally up the test strip via capillary action until it reaches the test line. The test strip contains a secondary antibody that captures the antigen–antibody complex. The accumulation of antibody–antigen complexes at the test line produces a band indicating a positive reaction. In comparison to regular EIA procedures, these lateral flow CIA-based procedures require very little hands-on time and often are capable of a fast test turnaround time. Some of them require a hands-on time of only 1–2 min, have only one incubation period, and are very easy to interpret and can be semiquantitative (Theel et al., 2015).

The use of microorganism-specific monoclonal and recombinant antibodies has improved reagent availability and reproducibility and enhanced test specificity. Examples include rapid viral antigen detection kits, which have been used widely in the clinical virology laboratory for the rapid detection of influenza virus, respiratory syncytial virus, and rotavirus in clinical specimens, though these techniques are steadily being replaced by PCR-based methods (see below). To overcome the time-consuming and costly procedure for natural antibody production, phage display has been used to discover antigenic peptides, which are either chemically synthesized or produced in *Escherichia coli*, and used to produce antibodies that can be probes for microbial antigen detection. They have been used for the detection of antigens of *E. coli* O157:H7, *Borrelia*, Epstein–Barr virus, and panels of biological threat agents.

Genetic Probe Hybridization

The ribosomal RNA (rRNA) possesses distinct features that make it a good universal marker for bacterial identification. Molecular biology has exploited this characteristic to design methods to target these "signature" sequences. DNA or RNA probes complimentary to these unique nucleotide sequences within the rRNA of a microorganism serve as a way to detect the presence of the organism itself. These probes are linked with reporter molecules that allow visualization of hybridization reactions without the need to amplify the target sequence. In general, nonamplified-probe based techniques are used in diagnostic microbiology primarily for culture confirmation of organisms after a brief period of *in vitro* cultivation. Because they provide a fast and reliable result, probe assays are routinely used within the laboratory. They are useful in identifying organisms that can take a long time to grow in culture (e.g., *Mycobacterium tuberculosis*). Gen-Probe, Inc. (San Diego, CA, USA) and Becton, Dickinson and Company (Sparks, MD, USA) make several culture identification nucleic acid probes that have been approved by the FDA. These can be used for mycobacterial, fungal, and bacterial identification.

Other companies have developed other assays that are currently not FDA-approved yet offer the ability to quickly identify microorganisms with suspected pathogenicity present in clinical samples. As an example, AdvanDx Inc. (Woburn, MA, USA) offers a panel of PNA fluorescence in situ hybridization (FISH) tests including QuickFISH that allow the identification of common bloodstream infection pathogens directly from positive blood cultures in approximately 30 min (http://www.opgen.com/pathogenid/pna-fish-products/). Although these commercial products are more expensive than conventional approaches, the decrease in time-to-identification has the potential to improve patient outcomes and reduce overall health-care costs (Seo et al., 2018).

Microorganism Culture and Identification

Isolation and cultivation of a microorganism, either in an artificial medium or in a living host, is definitive evidence for the presence of a microbe. In many cases, culture techniques remain the "gold standards" for diagnostic microbiology even though lengthy incubation periods preclude the use of the test results as useful diagnostic procedures. Culture is usually the most specific method for establishing the presence of a particular pathogen in a specimen. In addition, a pure culture of either a virus, fungus or a bacterium is essential for performing *in vitro* phenotypic antimicrobial susceptibility tests.

Culture Using Artificial Media

Bacteria, mycobacteria, mycoplasma, and fungi are cultured either in liquid or solid artificial media. Liquid media provide greater sensitivity for the isolation of small numbers of microorganisms since larger volumes of a specimen can be inoculated into broth than onto agar plates. However, liquid media cannot be used for diagnosis of mixed infections and organism quantification cannot be performed unless real-time growth indicators are measured. On the other hand, though somewhat less sensitive than liquid

media, agar media provide the ability to isolate colonies that can be identified, sometimes based on their colony morphologies, and quantified by calculating the number of colony-forming units. The quantification of microorganisms is important when determining if the presence of an organisms represents colonization or not.

Some organisms require specific conditions to grow. For example, anaerobic pathogen recovery is achieved by incubating media in an anaerobic environment. An anaerobic container or chamber can be used. Additionally, culture media can be made selective by incorporating compounds such as antimicrobial agents that inhibit endogenous flora, while permitting the growth of specific microorganisms resistant to these inhibitors. This is extremely important in the isolation and identification of pathogenic microorganisms in sputum and stool specimens. For instance, several selective and differential media such as Hektoen enteric, xylose-lysine desoxycholate, and salmonella–shigella agars are available for the isolation and differentiation of enteric pathogens from stool samples. Bile esculin agar plus vancomycin has been widely used as a selective and differential medium for screening vancomycin-resistant enterococci. In some instances, a growth indicator medium can be developed by incorporating one or more carbohydrates in the medium along with a suitable pH indicator. These differential media can be used to isolate and screen for certain microorganisms present in different clinical samples. As an example, many different chromogenic media have been developed to differentiate between bacterial genus and species and between yeast species (Fig. 1). In addition, chromogenic media have been developed to screen for antibiotic—resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and carbapenem-resistant *Enterobacteriaceae* (CRE) (Perry, 2017). Isolation of microorganisms from blood specimens is a major task of clinical microbiology laboratories.

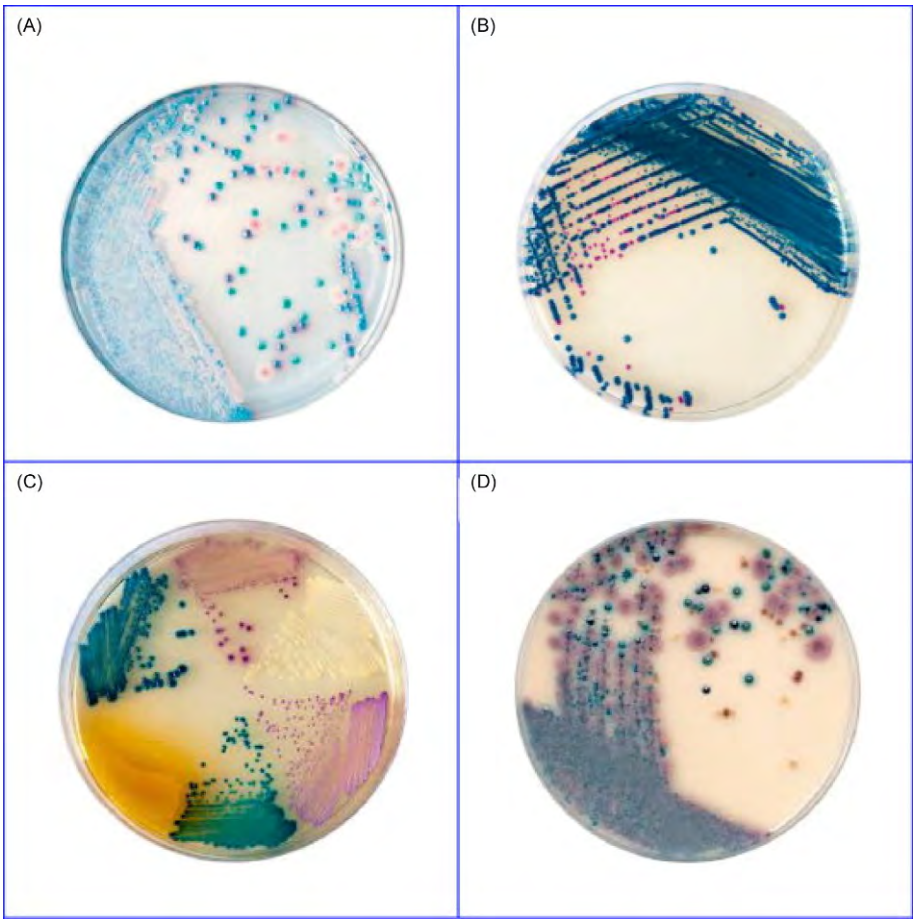

Fig. 1 Chromogenic medium used to (A) Screen for MRSA, mauve colonies (CHROMagar MRSA from Becton, Dickinson and Company) (https://www.bd.com/resource.aspx?IDX=32508). (B) CHROMagarTM StrepB plate to differentiate group B streptococci from other streptococci (http://www.chromagar.com/clinical-microbiology-chromagar-strepb-focus-on-streptococcus-agalactiae-30.html#.U-plm_PD9Qs), (C) CHROMagar ESBL is used to select for and differentiate between Gram negative organisms producing extended-spectrum beta lactamase (http://www.chromagar.com/clinical-microbiology-chromagar-esbl-focus-on-esbl-resistance-31.html#.U-pl7_PD9Qs), and (D) Brilliance Candida Agar (Oxoid) differentiates between *Candida albicans* and *Candida tropicalis* from other species of Candida within 48 h (http://www.oxoid.com/pdf/24085_oxoid_brilliance_candida.pdf).

Culture Using Living Cells

In the 1960s, it was shown that *Chlamydia trachomatis* could be recovered in eukaryotic cell culture using McCoy cells (Gordon and Quan, 1965; McNabb et al., 2004). Besides *Chlamydiae*, many viruses can be recovered and identified using eukaryotic cell culture. The virologic component of diagnostic microbiology has become increasingly more common with the advent of effective antiviral treatments as well as the need to identify and treat viral infections in the human immunodeficiency virus (HIV)-infected and other immunocompromised hosts. Viral isolation is very sensitive and in theory a positive result can be obtained with a single infectious virion. However, not all viruses can be recovered *in vitro*. Several culture systems have been used for virus isolation, and their characteristics are listed in Table 3. After inoculation, a virus can initially be identified and differentiated by (1) the pattern of the cytopathic effect (CPE), (2) the specific cells in which CPE is induced, and (3) the rapidity of the appearance of CPE. For example, the majority of enteroviruses cause CPE in rhesus monkey kidney cells within 48–72 h (Klespies et al., 1996; Lipson et al., 2001). However, poliovirus-induced CPE appears within 1 day after inoculation, whereas it takes 4 days for echovirus to induce the same amount of CPE. Typically, specimens are inoculated into different cell cultures to provide a susceptible host for each virus that may be present. Some of the most common cell culture cell lines are shown in Table 3.

CPE can be visualized under a light microscope without the need of a stain. Because CPE can be a result of cellular deterioration, direct fluorescent antibody (DFA) tests have been developed to confirm the presence of the virus. Some organisms may take a long time to grow in cell culture or to cause CPE, therefore shell vial isolation has been widely used within the diagnostic laboratory. This technique combines cell culture and antigen detection. It can be used for the detection of several viruses as well as *Chlamydiae*. One of the advantages of this method is that it is a relatively rapid at identifying agents whose identifications may ordinarily take as long as 3 weeks in a traditional tube culture. Vials with coverslips are typically seeded with a monolayer of cells in growth medium. When the cells are nearly confluent, the growth medium is aspirated and replaced by an aliquot of the clinical sample. The vials are centrifuged and then growth medium is added. The vials are incubated for specific times and temperatures (depending on the agent being sought), and the cells on the coverslip are then incubated with fluorescein-conjugated virus-specific antibody directly in the shell vial. The stained coverslip is removed and placed on a slide for examination under the fluorescence microscope. This technique is advantageous specially for the detection of cytomegalovirus (CMV) infections, since CMV-induced CPE is relatively slow to develop. Quidel (San Diego, CA, USA) offers multiple DFA kits that allow the detection of respiratory viruses, enteroviruses, herpes viruses as well as *Chlamydiae* (http://www.quidel.com/cultures-fluorescent-tests). They also offer a genetically manipulated cell line that accumulates an enzyme when infected by HSV-1 or HSV-2. This system is known as the enzyme-linked virus inducible system (ELVIS®). This method allows rapid (<1 day) detection of HSV without the need to detect CPE. Additionally, transgenic and cocultured cell lines have been used to enhance the sensitivity of recovery of enteroviruses and eliminate the need for multiple shell vials. Mixed cell lines stably transfected with host cofactors involved in the binding and entry of some enteroviruses (Super *E*-Mix ReadyCells) are commercially available (Quidel). These mixed cell cultures have been shown to be more sensitive for virus recovery from clinical samples compared to conventional tube or shell vial cultures with single cell lines.

Culture Using Other Living Hosts

Microorganism isolation, especially for some viruses, occasionally requires the inoculation of test specimens into animals and embryonated eggs. Viruses require intracellular replication, therefore inoculating a clinical specimen into cell lines (discussed previously), animal models or embryonated eggs, can aid in their isolation. As an example, several arthropod-borne viruses (arboviruses) that cause encephalitis can be isolated by inoculating the implicated specimen into suckling mice (Reynolds et al., 2017). Susceptible animal inoculation, such as animal models like the severe combined immunodeficiency mice, as well other rodents, have aided in the elucidation of unknown pathogens. Whereas, embryonated eggs are still the best culture medium for influenza viruses.

The ability to grow pathogens in animal models also provides scientists the opportunity to study the mechanisms of virulence and thus aid in the development of new diagnostic techniques, vaccines and treatments. A classic example of the use of animals as a vector to elucidate the etiology of infectious diseases is the discovery of *L. pneumophila* as the etiology of Legionnaires' disease. *L. pneumophila* was first identified by inoculating the clinical materials into susceptible guinea pigs (McDade et al., 1977). Guinea pigs have served as important animal models throughout microbiology's history. In fact, these aided in the development of Dr. Robert Koch's postulates of infectious disease etiology.

Table 3 Common cell cultures used in diagnostic microbiology.

Culture type	*Characteristic*	*Cell line examples*	*Primary use*	*Comments*
Primary	Diploid	Primary rhesus monkey kidney (rhMK)	Influenza, parainfluenza, enteroviruses	Endogenous virus infection, for example, SV-40; 1–2 passages only
Diploid cell lines	Diploid	Human diploid fibroblast MRC-5	Herpes simplex virus, cytomegalovirus, rhinovirus	Limited passage (50–70)
Established cell lines	Heteroploid	HEp-2, HeLa	Respiratory syncytial virus, adenovirus	Continuous passage; mycoplasma contamination

Measurement of Host Responses to Infection

Instead of detecting the presence of the microorganism, serologic tests measure the host's humoral immune response to a microorganism infection. Serologic tests are, in certain circumstances, the mainstay for diagnosis of certain infections whenever recovery of those organisms in culture is difficult or impossible. However, there is usually a lag between the onset of microorganism infection and the development of antibody to the organism. The presence of IgM specific antibodies is considered evidence of an active or recent infection, whereas the presence of IgG specific antibodies is evidence of a past exposure or chronic infection. Although IgM antibody appears relatively rapidly, IgM testing may be subject to interference by rheumatoid factors. For IgG testing, it is often necessary to obtain paired serum specimens, one taken during the acute phase of the disease and one taken during convalescence, to look for a rising titer of IgG antibody. A limitation on the use of serology as a diagnostic tool is that immunosuppressed hosts may be unable to mount an antibody response. In general, however, detection of specific antibody responses can be a very useful diagnostic approach in the postacute setting where causative organisms may be rare or completely absent. For example, serological testing for Lyme disease remains the mainstay of diagnostic evaluations because at the time many patients present, organisms have disseminated from the site of infection and are difficult or impossible to detect by direct methods based on culture or PCR (Theel et al., 2015).

Immunofluorescence Methods

Immunofluorescence techniques have been widely used in the past for detection of microorganism-specific antibodies and still have many applications. The common procedure used in a clinical serology laboratory is an indirect one. If present, the antibody in the serum specimen reacts with the microorganism-specific antigen, which was prefixed on a glass slide. After a wash step, fluorescein-conjugated immunoglobulin directed against the test serum species is overlaid on the slide. After washing, the slide is examined for specific fluorescence under a fluorescence microscope. This technique is still the primary choice for diagnosis of several virus infections, including Epstein–Barr virus and measles virus, in the majority of clinical virology or serology laboratories. However, fluorescence techniques are quite labor intensive and interpretation of the results is subjective; these techniques have, therefore, been gradually replaced by automatable and more objective serologic techniques such as EIAs.

Enzyme Immunoassays

EIAs have replaced radioimmunoassays in most diagnostic applications, and have become among the most widely used diagnostic methods in a clinical serology laboratory. In its most used format, the solid-phase EIA technique uses plastic microtiter plates or beads to which antigens are passively adsorbed. In a noncompetitive procedure, a test serum specimen is added. If present, the microorganism-specific antibody binds to the antigen. After washing, an enzyme-labeled immunoglobulin specific for the test serum species is added. After another wash step, a chromogenic enzyme substrate is added, and the color that develops is proportional to the amount of specific antibody present. The application of EIA serologic techniques for diagnosis of viral and rickettsia infections has expanded significantly. This assay is most frequently used in screening for immune status, such as in rubella testing, but they are also used as primary screening tests for infections due to HIV, HCV, HBV, and human T-cell lymphotropic virus.

Immunoblotting

The specificity of a serologic test is largely determined by the antigen used to capture the antibody in the direct test formats. An immunoblot (or Western blot) is one of the most specific serologic methods available. The general components of immunoblotting as used for immunodiagnosis with human sera include (1) electrophoretic separation of protein antigen on sodium dodecyl sulfate polyacrylamide gels, (2) electrophoretic transfer of the protein bands to a nitrocellulose or other support membrane, (3) blocking of free protein-binding sites on the membrane, (4) addition of the test serum, and (5) detection of the specifically bound serum antibodies. It is then possible to determine whether the patient's antibodies are directed against pathogen-specific or cross-reactive antigens. This additional level of analysis allows the discrimination of specific and nonspecific reactions. Immunoblotting has become a mainstay for confirming infection with HIV, HCV, and *B. burgdorferi* after initial positive EIA results.

Other Serologic Techniques

Other methods to detect immune humoral response to microorganism infections include agglutination and complement fixation assays. Agglutination reactions can be defined as the specific immunochemical aggregation of polystyrene (latex) particles coated with microorganism antigens that can be used to detect antigen-specific antibodies. Agglutination assays are simple to perform, and require only 15–30 min for completion, offering a useful alternative to EIA. This test has been used in the clinical serology laboratory for detection of rubella virus antibodies and quantification of rapid plasma reagin, which can be used as an indication of the therapeutic efficacy of syphilis treatment. Although the complement fixation test is still used in the serodiagnosis of several fungal infections, the system is extremely labor intensive, relatively insensitive, and will be gradually replaced with other formats. New techniques and formats in antibody testing have been emerging. A multianalyte profile (xMAP) allows simultaneous detection and differentiation of a panel of antibodies in a single reaction by using xMAP multiplexed technology (Luminex, Austin, TX, USA), which is highly desirable in clinical laboratories to provide a differential diagnosis of nonspecific conditions (Reslova et al., 2017).

Other Host-Response Determining Techniques

In addition to humoral immune responses, other host responses have been used in diagnostic microbiology field. For many years the tuberculin skin test (TST) has been used for screening *M. tuberculosis* infection. However, this test fails to discriminate between actual *M. tuberculosis* infection or vaccination with the Bacillus Calmette-Guérin (BCG) strain. Additionally, other nontuberculosis mycobacteria could also cause false-positive responses. Recently, interferon (IFN)-γ release assays (IGRA) have been developed to detect *M. tuberculosis* infection without cross-reactivity of the BCG vaccination. These tests measure the IFN-γ release following stimulation by specific *M. tuberculosis* antigens. There are three FDA-approved IGRAs manufactured by two companies, the T-SPOT. TB (Oxford Immunotec, Oxford, UK), the QuantiFERON-TB Gold (Qiagen, Venlo, Netherlands) and a newer version of the latter, the QuantiFERON-TB Gold Plus.

Sepsis is a life-threatening condition that requires early diagnosis and antimicrobial intervention. Hence, host response markers such as C-reactive protein (CRP) and PCT have been used to screen for sepsis. Nevertheless, blood culture remains the gold standard for isolation of bloodstream infection etiologies. C-reactive protein (CRP) is pentameric protein that aids in nonspecific host defense against inflammatory processes and cellular breakdown products by activating the classical complement cascade. CRP is one of the most sensitive acute-phase measures of inflammation, with concentrations rising to 1000-fold over baseline levels after such events as infection, trauma, surgery, burns, tissue infarction, and advanced cancer. Concentrations of CRP are generally higher in patients with bacterial infections relative to those with viral infections. However, high CRP levels may also be observed in individuals with influenza or mononucleosis. Overall, changes in CRP concentrations provide useful diagnostic information about the acuity and severity of a disease as the increase in CRP with inflammation occurs within 6–12 h and peaks at 48 h. However, caution must be taken when interpreting CRP results, as CRP elevations are nonspecific and the magnitude of CRP levels may be less pronounced in patients with liver disease (Gabay and Kushner, 1999). The diagnostic utility of CRP has also been evaluated for meningitis, but procalcitonin (PCT) has been shown to be more useful in discriminating between viral and bacterial meningitis (Alkholi et al., 2011). Recently, three host protein biomarkers, tumor necrosis factor-related apoptosis-inducing ligand, interferon gamma induced protein-10, and CRP, provided the diagnostic value to differentiate between bacterial and viral infections in children with lower respiratory tract infection or fever without source (van Houten et al., 2017).

Molecular Techniques in Diagnostic Microbiology

Identification methods based on biochemical phenotypic parameters can sometimes result in confusing or misleading results, especially when the number of features that can be analyzed is limited. Nucleic acid amplification technology has opened new avenues for the detection, identification, and characterization of pathogenic organisms in diagnostic microbiology. Molecular applications enhance the speed, sensitivity, and sometimes the specificity of an etiologic diagnosis. One good example is the detection and identification of pathogenic organisms directly from positive blood cultures. The promise of these techniques is the replacement of biological amplification—growth in culture—by enzymatic amplification of specific nucleic acid sequences, which is extremely useful to identify microorganisms that cannot be cultivated. The disadvantage, on the other hand, is the lack of recovered isolates for antimicrobial resistance determining. A full molecular procedure consists of specimen processing, nucleic acid amplification, and amplification product identification. Nucleic acid amplification techniques fall into three general amplification categories, which all share certain advantages over traditional methods, particularly for the detection of fastidious, unculturable, and/or highly contagious organisms (Table 4).

Nucleic Acid Extraction

A good specimen preparation is comprised of efficient target recovery, establishment of the integrity of nucleic acid targets, optimal removal of amplification inhibitors, elimination of components that affect other enzymatic substrates, and sterilization of potentially hazardous organisms (Thatcher, 2015). Numerous commercial methods and kits that are manual or automatic, with low or high throughput, are available for the extraction of microbial nucleic acids from a variety of clinical specimens. QIAamp MinElute Virus Kits (Qiagen, Inc., Valencia, CA, USA) have been demonstrated to effectively extract viral total nucleic acids from cerebrospinal fluid and respiratory specimens. Automated platforms such as the NucliSens easyMAG platform (bioMérieux, Durham, NC, USA) allow for high throughput processing, and high yield of quality total nucleic acids for clinical specimens and reduces the risk of contamination.

In Vitro Nucleic Acid Amplification Systems

According to the theoretical basis, *in vitro* nucleic acid amplification techniques can be placed into one of three broad categories, which all share certain advantages over traditional methods, particularly for the detection of fastidious, unculturable, and/or highly contagious organisms (Table 4). The first category is a target amplification system, which includes PCR, transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), and helicase-dependent amplification (HDA), where many copies of the nucleic acid target are made. Among these amplification systems, PCR and PCR-derived techniques are the best developed and most widely used methods of nucleic acid amplification in the clinical setting. Semiautomated and automated systems have now been manufactured by Roche Molecular System, Inc. (Indianapolis, IN,

Table 4 Nucleic acid amplification methods.

Amplification method	*Amplification category*	*Manufacturer (trade mark name)*	*Enzymes used*	*Temperature requirement*	*Nucleic acid target*
Polymerase chain reaction	Target	Roche Molecular System, Inc. (Amplicor, Cobas®); Abbott Molecular (m2000); Cepheid (GeneXpert); Qiagen (Rotor-Gene Q MDx); Quidel (QuantStudio™ Dx), etc.	Taq DNA polymerase	Thermal cycler	DNA or RNA
Transcription-mediated amplification	Target	Bio-rad (Droplet Digital™); Thermo Fisher Scientific (QuantStudio™ 3D) Gen-Probe, Inc. (Aptima®)	Reverse transcriptase, RNA polymerase, RNase H	Isothermal	RNA or DNA
Nucleic acid sequence-based amplification	Target	bioMérieux (NucliSens®, EasyQ®)	Reverse transcriptase, RNA polymerase, RNase H	Isothermal	RNA or DNA
Strand displacement amplification	Target	Becton, Dickinson and Company (ProbeTec™)	Restrictive endonuclease, DNA polymerase	Isothermal	DNA
Helicase-dependent amplification	Target	Quidel/BioHelix Corp. (IsoAmp®)	Helicase, Taq DNA polymerase	Isothermal	DNA or RNA
Cycling probe technology	Probe	ID Biomedical, Vancouver, BC, Canada (CPT-strip)	RNase H	Isothermal	DNA or RNA
Ligase chain reaction	Probe	Abbott Laboratories, Abbott Park, IL, USA (LCx)	DNA ligase	Thermal cycler	DNA
Hybrid capture system	Signal	Qiagen/Digene (Hybrid Capture)	None	Isothermal	DNA
Branched DNA	Signal	Siemens/Bayer Diagnostics, Emeryville, CA, USA (Versant®)	None	Isothermal	DNA or RNA
Invader technology	Signal	Hologic/Third Wave Technologies (Invader®)	Cleavase enzyme	Isothermal	DNA

USA), Abbott, Cepheid, Becton Dickinson and other companies for detection and/or quantification of several organisms including HIV, HCV, HBV, CMV, HSV, HPV, MRSA/*S. aureus*, *Clostridium difficile*, enterovirus, influenza, respiratory syncytial virus, group B *Streptococcus*, vancomycin-resistant enterococci, norovirus, *C. trachomatis*/*Neisseria gonorrhoeae*, *M. tuberculosis* and a variety of other organisms. In addition, numerous user-developed PCR-based DNA amplification techniques have been developed and applied to the detection of microbial pathogens, identification of clinical isolates, determination of antibiotic resistance, and strain subtyping. Commercially available products based on TMA, NASBA, HDA, and SDA are available from Gen-Probe, Inc., bioMérieux, BioHelix Corp. (Beverly, MA, USA), and Becton Dickinson and Company to detect and quantitate several microorganisms including *M. tuberculosis*, *C. trachomatis*, *N. gonorrhoeae*, MRSA, *C. difficile*, HIV, HCV, and enteroviruses (Table 4).

A second approach is probe amplification in which many copies of the probe that detects the target nucleic acid amplified after target-specific hybridization occurs. The ligase chain reaction (LCR) was the most widespread application of probe amplification in diagnostic microbiology until its withdrawal from the market (Table 4). When used following a target amplification method, such as PCR, LCR can be sensitive and useful for the detection of point mutations. Although convenient and readily automated, the main drawback of LCR was the difficult inactivation of postamplification products. The nature of the technique does not allow for the most widely used contamination control methods to be applied. Another technique in the system, cycling probe technology (CPT), which incorporates a unique chimeric DNA–RNA–DNA probe, has been designed to detect a specific sequence with *mecA* and *vanAB* genes for diagnostic use as a culture confirmation assay for MRSA and vancomycin-resistant enterococci.

The last amplification system is signal amplification, which is designed to strengthen a signal by increasing the concentration of label attached to the target nucleic acid. Unlike procedures which increase the concentration of the probe or target, signal amplification increases the signal generated by a fixed amount of probe hybridized to a fixed amount of specific target. The fact that signal amplification procedures do not involve a nucleic acid target or probe amplification is a theoretical advantage, because of lower susceptibility to contamination problems inherent in enzyme-catalyzed nucleic acid amplification. Sensitivity, however, compared to target nucleic acid amplification techniques, may be a limiting factor. Another limitation of signal amplification is background noise due to the nonspecific binding of reporter probes. Currently, three diagnostic companies have their signal amplification products available for molecular diagnostic purposes. The Digene Hybrid Capture System (Digene Diagnostics, Inc., Gaithersburg, MD, USA) is widely used to determine HPV infection in cervical swabs. Homogeneous Invader Technology (Hologic/Third Wave Technologies, Madison, WI, USA) is another signal amplification system, which is a powerful tool for genetic analysis of single nucleotide polymorphisms in both microorganisms and hosts.

Detection and Identification of Amplification Products

After amplification, the millions of target DNA copies that accumulate are invisible to the naked eye; therefore, an appropriate detection method is needed to "visualize" amplified DNA molecules. On the other hand, an additional identification system is critical to enhance both sensitivity and specificity of the PCR amplification procedure. Detection and identification of amplification products, or amplicons, has become a routine procedure in the molecular diagnostic laboratory. The classical means of detection and characterization of the PCR product was agarose gel electrophoresis, where sensitivity can be enhanced by Southern blot hybridization using DNA probes (Procop, 2007). Several user-friendly and rapid techniques have been adopted by molecular diagnostic laboratories, including direct sequencing, nucleotide microarray, mass spectrometry, and a "real-time" procedure. Due to the advancement of gene sequencing techniques, gene sequencing has become a powerful tool for molecular diagnostic laboratories. High throughput sequencing has reduced the cost and increased output for laboratories to identify and differentiate between closely-related bacteria and viruses. For example, gene sequencing is used to monitor HIV-1 responses to therapy and to identify genomic antiviral drug resistance mutations. In addition, gene sequencing has been applied to Mycobacterial spp. identification. The 16S rRNA gene has traditionally been the most frequently used target for differentiation of *Mycobacterium species*. However, the 65-kDa heat-shock protein (hsp65) has been shown to have more inter-species variability than the 16S rRNA gene and is, therefore, more useful for differentiating between closely-related species (Kapur et al., 1995; McNabb et al., 2004). Microarray technology is becoming more accessible for pathogen detection in the clinical setting. New devices are being developed that automate DNA extraction from patient specimens. In one example, the preliminary array technology included 11 different gram-negative, gram-positive, and yeast pathogens. These arrays successfully identified organisms collected from infected wound swab samples (Palka-Santini et al., 2009).

Arguably the biggest advance in PCR technology has been the development of closed system detection of amplification products which obviates the need for contamination-prone removal of amplification products. Early applications of PCR comprised PCR amplification followed by a separate identification of PCR products. With the introduction of a fluorescence resonance energy transfer, nucleic acid amplification, detection, and probe-specific confirmation of PCR products, no transfers of PCR products out of the reaction tube are required (Mackay, 2004). RT-PCR is closed and, therefore, avoids the important concerns of carryover contamination with previous amplification products. As a result of shorter thermocycling and simultaneous product detection, the turnaround time for results has also been significantly shortened. This technique is widely used to detect and quantify microorganisms and determine mutations in both microorganism and host genes. Real-time detection and characterization is also available in other *in vitro* amplification systems such as the Invader and NASBA systems.

Nongenomic Molecular Testing

Detection of Metabolic Products

The causal relationship between *Helicobacter pylori* colonization of the gastric mucosa and gastritis was first proposed in 1983 by Warren and Marshall, who received the Nobel Prize in physiology or medicine in 2005. *H. pylori* produces large amounts of urease which hydrolyzes urea to form ammonia and soluble carbon dioxide. Based on this, the noninvasive urea breath test (UBT) was developed. UBT allows screening for *H. pylori* infections without the need of an invasive procedure such as endoscopy or tissue biopsy. It also helps assessing the efficacy of eradication therapy (Wang et al., 2015). Either ^{13}C- or ^{14}C-labeled urea are used, but ^{13}C-labeled urea is preferred given that is not radioactive as is ^{14}C-labeled urea. In brief, ^{13}C- or ^{14}C-labeled urea ingested by the patient is hydrolyzed to labeled CO_2 in the stomach. The labeled CO_2 is absorbed in the blood and exhaled. Patients breath into a collection bag and the levels of labeled CO_2 are measured by mass spectrometry. Overall, UBT is a highly accurate and reproducible test with a sensitivity and specificity of 95% (Wang et al., 2015). One drawback of the test is the possibility of false positive results based on the presence of other urease producing pathogens in the stomach.

The application of volatile organic compounds (VOC) as biomarkers for the diagnosis of infectious diseases has been of great research interest. The use of VOCs for the detection of pulmonary infections caused by *M. tuberculosis*, *Pseudomonas aeruginosa*, and *Aspergillus fumigatus* have been evaluated. Several nicotinic acid metabolites produced by *Mycobacterium* species have been evaluated but shown unremarkable sensitivities and specificities (Sethi et al., 2013). This is also the case of other VOCs evaluated for detection of *P. aeruginosa* and *A. fumigatus*, since the same VOCs could be found in certain foods and thus cause false positive results (Sethi et al., 2013). Similarly, breath fungal secondary metabolite signature determined by thermal desorption-gas chromatography/mass spectrometry was used to diagnose invasive aspergillosis (Koo et al., 2014). When these metabolomic biomarkers are used in the clinical practice, we must remember the extreme heterogeneity of clinical illness, and strive for generalizable disease-defining diagnostics (Sweeney and Khatri, 2017). While detection of exogenous microbial volatile metabolites in the breath has opened up a new and exciting dimension of diagnostic research and development in infectious diseases, the daunting challenges to volatile diagnostic biomarker discovery and clinical development have to be kept in mind (Acharige et al., 2018).

Microbial Identification by Mass Profiling

Few developments in microbiological diagnostics have revolutionized the routine identification of microorganisms in clinical microbiology laboratories as matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Due to

its reliable capability to identify microbial pathogens in a rapid, high-throughput, accurate, and cost-effective manner, MALDI-TOF MS has become a gold standard for microbial identification. There are two commercially available MALDI-TOFs, the MALDI Biotyper® (Bruker Daltonics, Germany) and the VITEK MS® (BioMérieux, France). Both systems allow the phenotypic characterization of Gram-positive and Gram-negative bacteria, yeast, mycobacteria and mold. MALDI-TOF MS identification of microorganisms has an accuracy comparable with nucleic acid sequencing (Bizzini and Greub, 2010; Dhiman et al., 2011; Marko et al., 2012; Rychert et al., 2013). However, certain clinically relevant organisms cannot be distinguished by this method and require further testing for identification, as is the case of *Shigella* spp. and *E. coli* (Khot et al., 2012; Lan and Reeves, 2002). Another limitation of MALDI-TOF MS is the inability to discriminate among the members of certain bacterial groups/complexes (e.g., *Citrobacter freundii* complex and *Enterobacter cloacae* complex) (Richter et al., 2013).

Due to the high mortality risk of sepsis, it is imperative to identify microorganisms growing in blood cultures. Thus, both MALDI-TOF MS manufacturers have developed research only protocols to identify bacteria directly from positive blood cultures. Recently, the performance of these protocols in correct species level identification of monomicrobial blood cultures was shown to be >90% accurate (Chen et al., 2013). Nevertheless, these protocols are not useful to identify organisms in polymicrobial cultures. Research laboratories have also evaluated direct microorganism detection from urine samples. In brief, the urine sample is centrifuged to pellet the bacteria and then analyzed by the instrument (Demarco and Burnham, 2014; Ferreira et al., 2010; Inigo et al., 2016; Wang et al., 2013).

Besides microbial identification, research laboratories have explored the application of MALDI-TOF MS for microbial subspecies typing and antibiotic resistance marker detection (e.g., β-lactamases and carbapenemases) (Burckhardt and Zimmermann, 2011; Camara and Hays, 2007; Ghebremedhin et al., 2016; Hrabak et al., 2012; Idelevich et al., 2018). The recently developed direct-on-target microdroplet growth assay (DOT-MGA) allows rapid universal antimicrobial susceptibility testing using MALDI-TOF MS (Correa-Martinez et al., 2019; Idelevich et al., 2018). The ability of MALDI-TOF MS to type *Streptococcus pneumoniae* has also been explored, but serotyping of *Salmonella* by this method has been unsuccessful up to date (Kang et al., 2017; Nakano et al., 2015; Pinto et al., 2017). A recent study used quantitative proteomics to evaluate the host proteome response in septic patients secondary to community-acquired pneumonia. Bioinformatic analyses of differentially expressed proteins showed alteration in the cytoskeleton, cellular assembly, movement, lipid metabolism and immune responses in septic patients (Sharma et al., 2017).

Transcriptomics

Expression levels of specific RNAs have been associated with disease and infection. Utilizing transcriptomics, these RNAs can serve as biomarkers to monitor disease progression or aid in diagnosis when an organism cannot be recovered. Next generation sequencing and microarray technology has enabled the identification of these specific RNAs associated with infections and disease such as sepsis, HIV and tuberculosis (Correia et al., 2017). Using a series of RNA arrays on RNA extracted from pediatric patients with sepsis, Herberg et al. identified two host transcript RNA signatures which can distinguishing bacterial from viral infection (Herberg et al., 2016). A further study demonstrated that the 2-transcript host cell signature can distinguish viral from bacterial diarrhea and it was influenced by the severity of symptoms (Barral-Arca et al., 2018).

Efforts to develop strategies for eradication of HIV-1 have intensified in recent years. However, while $CD4^+$ T cell count and plasma viral load have been important markers of disease progression, their reliability has recently come into question as these markers do not reliably identify individuals on anti-retroviral medication with virological failure (Moore et al., 2008) and patients treated using immunotherapies that utilize recombinant viral proteins such as CAR T-cell therapy may produce false positive results (Ariza-Heredia et al., 2017; Laetsch et al., 2018). Recent reports have identified levels of miR-150 and miR-146b-5p in peripheral blood mononuclear cell and plasma as predictive of HIV/AIDS disease progression and therapy (Munshi et al., 2014).

In the case of *M. tuberculosis* infections, effective treatment regimens rapidly decrease the number of viable *M. tuberculosis* organisms in sputum, with the number of cultivatable bacilli typically reduced by approximately 10-fold within the first 1–2 weeks. However, because of the slow growth rate of *M. tuberculosis*, results of cultures for samples obtained early in treatment to monitor treatment efficacy will not be available in a timely manner (Mdivani et al., 2009). Bacterial clearance in TB patients receiving chemotherapy is important for a number of reasons, including early diagnosis of treatment efficacy, prevention of relapse due to incomplete cure, and as endpoints in clinical trials evaluating new tuberculosis medications (Bertholet et al., 2011). As a consequence, an mRNA RT-PCR assay analyzing antigen 85B mRNA from sputum samples has been developed as a test-of-cure for *M. tuberculosis* infections. This assay allows real-time, rapid monitoring of the response to anti-TB therapy and has a sensitivity and specificity of 85.2% and 88.6%, respectively. The antigen 85B mRNA RT-PCR assay data correlated clinically with resistance to anti-TB drugs, whereas the DNA PCR assay showed a high false-positive rate (Bertholet et al., 2011).

Molecular Diagnosis and Monitoring

Detection and Identification of Microorganisms

Over the past century, the identification of microorganisms has relied principally upon phenotypic characteristics such as morphology and biochemical characterization. Genotypic identification has emerged as an alternative or complement to established phenotypic methods. Broad-range PCR primers are designed to recognize conserved sequences in the phylogenetically informative gene of a variety of bacteria, and highly variable regions between the primer binding sites are amplified by PCR. The

amplified segment is sequenced and compared with known databases to identify a close relative. The most successful example is the rRNA gene sequence-based microbial identification system, which includes the extraction of nucleic acids, PCR-mediated gene amplification, sequence determination, and computer-aided analysis.

The growing availability of next-generation sequencing has allowed for whole-genome sequencing of bacteria, which has identified additional PCR targets to identify organisms, detect virulence factors, antibiotic resistance markers, and for molecular genotyping. The time, effort, and cost associated with sequencing data analysis were previously major limitations of using sequencing technologies in a clinical setting. However, next generation platforms such as Ion PGM (Life Technologies) and the MiSeq (Illumina) have decreased sequencing costs while simultaneously increasing efficiency and improving turnaround times (Didelot et al., 2012).

Microorganism Quantification

Quantification of nucleic acid targets has become desirable in the clinical practice to monitor therapeutic responses and provide prognostic information for infectious diseases. The task of quantitative amplification has been problematic since the amplification techniques yield products in an exponential manner until a plateau is reached; any factor interfering with the exponential nature of the amplification process would thereby affect the result of the quantitative assay. To overcome this problem, a competitive RT-PCR assay was developed based on coamplification of an internal competitor with the target sequence. Currently, quantification of microorganism load is based primarily on (1) target amplification, which includes both PCR- and NASBA-based techniques, and (2) branched DNA-based signal amplification. Commercially available kits are available to make it possible for many clinical laboratories to carry out quantitative analysis. RT-PCR-based systems are widely being used for microorganism quantification in clinical microbiology services. The unique characteristic, simultaneous, cycle-by-cycle detection of PCR products has made possible a rapid, high-throughput quantification of nucleic acid sequences (Mackay, 2004). Several RT- PCR systems have been successfully used to manage infections caused by HBV, HCV, HIV, CMV, Epstein–Barr virus, and BK virus.

Digital PCR (dPCR) represents a relatively new method of nucleic acid quantification in microbiology. Like conventional PCR and RT-PCR, dPCR involves amplification of target nucleic acids using fluorescent probes, however the reaction is randomly distributed into thousands of partitions in such a way that ideally, each partition only contains one target molecule to be amplified. Depending in the platform, partitions can be in the form of microwell plates, capillaries, oil emulsions, or chip arrays. After amplification, absolute quantification can be achieved by counting the number of positive (fluorescent) and negative partitions (no fluorescent) and calculating Poisson's distribution. Digital PCR provides an advantage over qPCR in that it allows for absolute quantification without calculation of CT values and standard curves. In addition, dPCR also allows for detection of rare alleles and clinical samples in that contain low amounts of nucleic acids body fluids or tissues (Kuypers and Jerome, 2017). Currently, dPCR has been applied to detection of HPV in tumors of patients with head and neck cancers (Jeannot et al., 2016), detection and quantification of HIV viral load, CMV and surveillance for antibiotic and antiviral resistance (Luo et al., 2017; Taylor et al., 2015).

Syndromic, Multiplex Testing

Certain infectious diseases present themselves with similar or nonspecific clinical manifestations, thus making an accurate diagnosis challenging. Traditionally, different methods of detection are employed for detecting different pathogens responsible for these similar syndromes. This requires special media, equipment, and sophisticated facilities. Much effort has gone into developing syndromic, multiplex molecular techniques that are more effective and easier to perform within the diagnostic laboratory. By utilizing numerous primers within a single reaction tube, nucleic acid fragments from multiple targets can be amplified at the same time, thus allowing screening of a clinical specimens for several probable pathogens. Nevertheless, the ability to screen for large numbers of potential etiologies limit detection efficiency. Several syndromic panels commercially available for detection and simultaneous identification of common pathogens involved in respiratory, gastrointestinal and blood infections, respectively. Additionally, some panels also detect antimicrobial resistance markers which can aid in optimal selection of antimicrobial treatment.

There are several FDA-cleared gastrointestinal panels available in the United States. The xTAG Gastrointestinal Pathogen Panel (Luminex, Austin, TX, USA) was the first FDA-cleared multiplex PCR for detecting gastrointestinal pathogens (Dunbar et al., 2013). This panel constitutes of 15 gastrointestinal pathogens, including bacteria, viruses and parasites. It has a turnaround time of <5 h, whereas the FilmArray® (BioFire Diagnostic, Salt Lake City, UT, USA) can detect 22 targets (bacteria, viruses and parasites) in a total run time of about 1 h. The Verigene® Enteric Pathogens Nucleic Acid Test, which was recently acquired by Luminex, targets nine analytes (bacteria and viruses). BioCode Gastrointestinal Pathogen Panel (Applied BioCode, Santa Fe Springs, CA, USA) is another recently FDA-cleared gastrointestinal panel testing for 17 most common bacteria, viruses, and parasites in stool with high throughput BioCode MDx-3000 molecular system. The FDA-cleared respiratory panels include the FilmArray® respiratory panel (20 bacterial and viral targets), Verigene® Pathogens Flex test (16 targets, bacteria and viruses) and the xTAG® Respiratory Viral Panel (viral only). As for the meningitis/encephalitis panels, these are offered by the FilmArray® (14 targets including bacteria, fungi and viruses) and the AllPlex™ Meningitis (Seegene, Rockville, MD, USA) (18 bacteria and viruses). One limitation of these panels is that since they target microbial nucleic acid (DNA or RNA), they cannot distinguish between viable, replicating organisms responsible for disease from traces of nucleic acid (Binnicker, 2015).

Table 5 Food and Drug Administration-cleared bloodstream infection rapid diagnostic methods.

Test manufacturer	*Detection method*	*Analytical time*	*Specimen*	*Comments*
FilmArray® Blood Culture Identification (BCID) Panel (BioFire Diagnostics)	Nucleic acid amplification	1 h	Positive blood culture	Identifies Gram-positive and Gram-negative bacteria, yeast and detects 3 resistance markers
VERIGENE® (Luminex Corporation)	Microarray	2.5 h	Positive blood culture	Identifies Gram-positive and Gram-negative bacteria, and detects 6 resistance markers
Accelerate PhenoTest™ BC Kit (Accelerate Diagnostics)	FISH	1.5 h (ID) 7 h (AST)	Positive blood culture	Identifies bacteria and 2 *Candida* spp. Performs phenotypic antimicrobial susceptibility testing (AST) for bacterial targets
ePlex® (GenMark)	Nucleic acid amplification	1.5 h	Positive blood culture	Gram-positive panel: 20 bacteria, 4 resistance markers Gram-negative panel: 21 bacteria, 6 markers Fungal panel: 15 targets

Due to the urgency in determining the etiology of a bloodstream infection, these companies have also developed multiplex PCR assays that allow this. Besides PCR, other molecular techniques that enable multiplex testing of positive cultures have been developed and are summarized in Table 5.

The Accelerate PhenoTest system (Accelerate Diagnostics) utilizes gel electrofiltration and fluorescence in situ hybridization FISH for identification but besides identification, it also performs antimicrobial susceptibility testing (AST) (Ramanan et al., 2018). The T2Dx instrument (T2Biosystems, Inc., Wilmington, MA, USA) is FDA-approved for bacteremia and candidemia. Their assay combines nuclear magnetic resonance and PCR to directly detect and define the organisms from whole blood samples with a limit of detection as low as 1 cfu/mL of whole blood (Clancy and Nguyen, 2018).

Rapid, On-Demand, and Point-of-Care Testing

Rapid provision of results can facilitate better clinical decision-making, improved patient adherence, and greater patient satisfaction, all of which lead to improved clinical outcomes with wider patient, operational, economic, and societal benefits. For example, early and accurate diagnosis of the central nervous system infections has been critical to clinical intervention in terms of choosing chemotherapy and predicting prognosis. Early diagnoses of meningitis improves patient care and in return decrease the costs of care by reducing hospital stay length and antibiotic use. Similarly, direct detection of *Streptococcus pyogenes* antigens in throat swabs can improve the treatment of pharyngitis by detecting the majority of streptococcal infections while the patient is still in the office. Detection of specific nucleic acids is more sensitive but also less rapid and more expensive than antigen detection.

Technology advances in molecular microbiology now enable a wide range of diagnostic tests to be provided at the point of care. The GeneXpert® Dx diagnostic system (Cepheid) is an important new development in the field of molecular diagnostics. It automates all of nucleic acid diagnostic steps in a disposable, microfluidic cartridge and is the first real-time PCR system that can be used to provide "real-time service" (Boehme et al., 2010; Xie et al., 2017). Although it was approved by the FDA as a moderately complex test, it is simple enough to be performed reliably by individuals without a background in nucleic acid diagnostics. Sample preparation reagents and samples are all that are required to add to the cartridge by the user. It has independently controlled and operated analysis modules that facilitate testing of individual samples in a random-access mode (Fig. 2A). The complete automation and rapid result capability of the GeneXpert make it uniquely suited for on-demand and point-of-care testing for testing respiratory specimens for Influenza, MRSA surveillance, virus and stool samples of suspected *C. difficile* patients.

The Alere™ i (Fig. 2B) is a small isothermal nucleic acid amplification platform that allows for the qualitative point-of-care detection of infectious diseases. For example, the Alere™ i Influenza A & B panel produces flu results in <15 min making it the first FDA-cleared, CLIA-waived molecular device used in point-of-care setting (Wang et al., 2018). The Cobas® Liat™ Analyzer (Fig. 2C) automates all nucleic acid testing processes, including reagent preparation, target enrichment, inhibitor removal, nucleic acid extraction, amplification and real-time detection. The machine has a very small foot print and is built with a touch screen, eliminating the need for an additional neighboring computer, and reports results in 20–60 min depending on the test (Ling et al., 2018). Quidel/Biohelix has developed several Helicase-dependent isothermal amplification diagnostic assays (AmpliVue® series) (Fig. 2D). These assays do not require any instrument and can be performed at room temperature. Quidel AmpliVue assays available include *C. difficile*, group A and B streptococci, and HSV 1 and 2.

Laboratory Automation

Historically, microbiology laboratories have been less automated than other clinical laboratories however, technological advances have brought on availability of more automated devices for the busy clinical microbiology laboratory. Different levels of

Fig. 2 Rapid, on-demand, and point-of-care testing. (A) The Xpert® EV assay (Cepheid) comes as a unit dose, self-contained kit (Xpert enterovirus self-contained cartridge) run on the GeneXpert® Dx diagnostic system (http://www.cepheid.com/us/cepheid-solutions/systems/genexpert-systems/genexpert-xvi). The only extra equipment required is a pipette that can deliver 140 mL of Lysis Reagent and sample into the cartridge. The whole test takes <5 min of hands-on time, and results are available in about 2.5 h. (B) The Alere™ i Influenza A & B panel is a point-of-care tests that offers results in as few as 15 min (https://www.alere.com/en/home/product-details/alere-i-influenza-ab.html). (C) The Cobas® Liat Analyzer™ automates molecular testing for influenza A and B (https://diagnostics.roche.com/us/en/products/systems/cobas-liat-system.html). The machine has a small footprint and 20–60 min turn-around time enabling its use for point-of-care diagnostics. (D) The Quidel AmpliVue® kits are available for instrument-independent, room-temperature molecular diagnostics (http://www.quidel.com/molecular-diagnostics/amplivue-products).

automation can be incorporated each phase of testing, from sample processing to total lab automation (TLA). In the preanalytical phase, the Previ® Isola (bioMérieux; Fig. 3A) and the Walk-Away Specimen Processor (WASP®; Fig. 3B) are examples of automatic specimen processing and plate inoculation platforms currently on the market. The Previ® Isola plates, streaks, and labels as many as 180 plates per hour. The Wasp is a comprehensive system which encompasses all aspects of liquid specimen processing including plate streaking, slide preparation, and enrichment broth inoculation. In the analytical phase, automated antibiotic susceptibility testing platforms including Vitek® (bioMérieux), MicroScan® (Siemens), Phoenix™ (BD) systems and The Sirscan2000automatic by i2a incubate, read and publish results (Fig. 3C). TLA systems such as the BD Kiestra™ (BD; Fig. 3D) and The Wasp Lab (Copan) include specimen processing, incubation in aerobic and CO_2 atmospheres, and digital imaging of plates. Automation has allowed for standardization, more rapid results, reduction in labor costs and improved safety (Croxatto et al., 2016; Dauwalder et al., 2016; Novak and Marlowe, 2013).

Concluding Remarks

Diagnostic microbiology has been developing new methods to rapidly detect and accurately identify implicated microorganisms in test specimens through a variety of techniques. Technologic changes have made significant progress in the various areas including bacteriology, mycology, mycobacteriology, parasitology, and virology during the last two decades in the field of diagnostic microbiology. The physical structure of laboratories, staffing patterns, work flow, and turnaround time have all been influenced

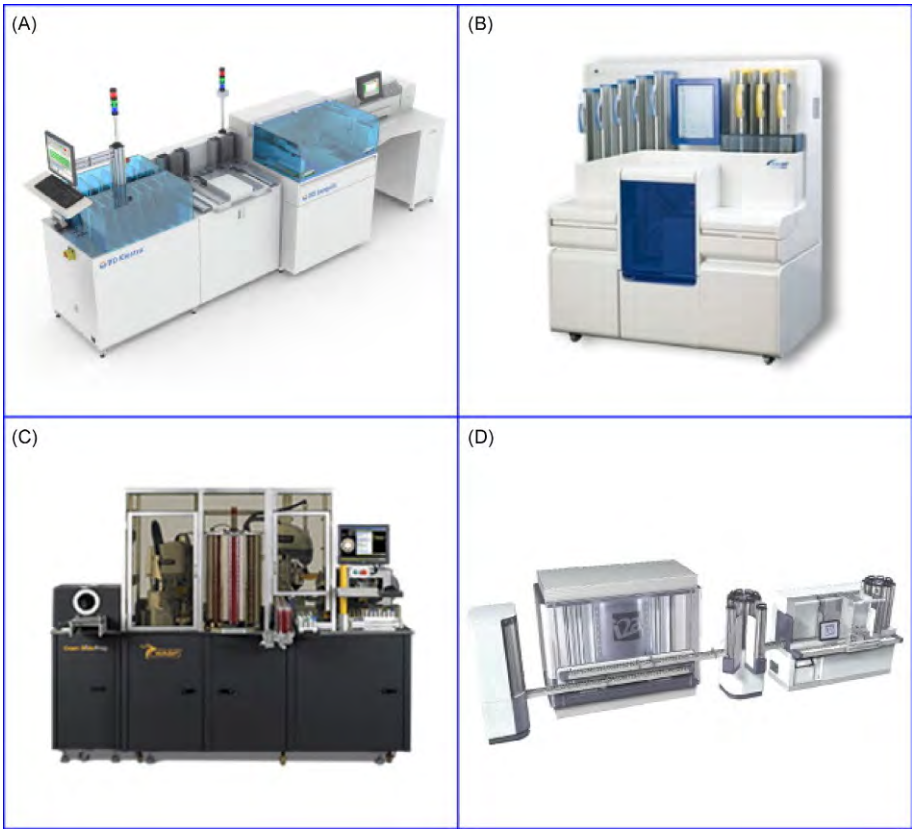

Fig. 3 (A) The Beckton Dickenson Kiestra™ InoqulA™ (D) is a fully automated, high throughput machine that processes both liquid and solid samples (photo courtesy of Paul Bourbeau). The InoqulA™ system fully integrates with additional products from the BD Kiestra™ line which automates incubation, reading, identification, and susceptibility of samples https://www.bd.com/en-us/offerings/capabilities/lab-automation/bd-kiestra-inoqula-plus-specimen-processor). (B) The bioMérieux Previ® Isola provides clinical laboratories with an automated means to quickly and accurately identify and perform antimicrobial susceptibility testing (http://www.biomerieux-diagnostics.com/previ-isola). (C) The Walk-Away Specimen Processor (WASP®) by Copan encompasses all aspects of liquid specimen processing including plate streaking, gram slide preparation, and enrichment broth inoculation (http://www.copanusa.com/products/automation/wasplab/). (D) The Sirscan2000automatic by i2a incubates samples and reads samples (photo courtesy of Christian Curel) (https://www.i2a-diagnostics.com).

profoundly by these technical advances. The implementation of nucleic acid amplification-based molecular techniques provides complementary, rapid, and on-demand diagnosis services. These changes will continue, and lead diagnostic microbiology inevitably to be a modern discipline, which will be equipped to face inevitable challenges in the future.

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Dictyostelium[☆]

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Glossary

Amoebas Protozoa that move using pseudopods.
cAMP, cyclic AMP, adenosine 3′5′-monophosphate An ATP derivative that is most commonly used as an intracellular intermediate for cellular responses to hormones and other extracellular signals.
Chemoattractant Organic or inorganic substance that induces directional cell movement in motile cells.
Chemotaxis Directional cell movement toward or away from chemicals in the environment.
Culmination The process of forming a fruiting body.
Cytokinesis Contraction and division of cytoplasm to form two identical daughter cells each containing one nucleus.
Dictyostelids The order of cellular slime molds, also known as social amoeba.
differentiation The process by which a single cell becomes more specialized, or a group of cells start to differ from each other.
Diploid The state of cells containing two copies of each chromosome.
Fruiting body A structure formed to carry spores aloft for improved preservation and dispersal.
Homologous recombination Genetic recombination event involving physical rearrangement between two strands of DNA.
Homothallic Refers to a species whose members can mate with any other individual, as opposed to heterothallic where only members of opposite sex or mating type can mate.
Parasexuality A phenomenon where two cell nuclei merge without any sexual process and the chromosome count is doubled.
Phagocytosis The cellular process of engulfing solid particles in membrane-bound compartments to form a phagosome.
Phototaxis Directional cell movement toward or away from a light source.
Thermotaxis Cell movement toward or away from a heat source.
Transformation Altering the genetics of a cell by introducing foreign DNA.

Abbreviations

ALC	anterior-like cell
asp	aspartate
cAMP	cyclic AMP
cAR	cAMP receptor
PdsA	phosphodiesterase
PIP2	phosphatidyl inositolbisphosphate
PIP3	phosphatidyl inositoltrisphosphate
PKA	cAMP-dependent protein kinase
REMI	restriction enzyme-mediated integration
SDF	spore differentiation factor

Defining Statement

Dictyostelid social amoebae present a useful model for the study of cell biology in the context of free-living single cells and during multicellular development.

Taxonomy, Evolution, and Ecology

All members of the Dictyostelid taxonomic group grow as free-living amoebae that feed on bacteria in decaying vegetation in the soil. When the food source is depleted they enter one of three different development programs, the most spectacular of which is the formation of the multicellular fruiting body.

[☆]*Change History*: (1) New sections on social evolution and "farming". (2) Revised section on the genome and genomics. (3) Revised and new sections on cell biology. (4) Revised section on chemotaxis. (5) Revised section for prestalk cell and stalk formation. (6) Revision of section on intracellular mechanisms of cAMP in culmination.

This article is an update of C. Scott, P. Schaap, Dictyostelium, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 606–616.

 https://doi.org/10.1016/B978-0-12-809633-8.13033-X

Of the 100 or more known species in the group, *Dictyostelium discoideum* is the most extensively studied. When *D. discoideum* cells starve, cells start to secrete pulses of cyclic AMP (cAMP), a molecule that regulates events inside the cell in most other organisms. When receiving a cAMP pulse, neighboring cells migrate toward the signal source in a process called chemotaxis. In turn these cells secrete their own cAMP pulse. The pulse propagates as a wave through the cell population, coordinating the movement of up to 1 million cells to come together to form a packed mound of cells. Subsequent signaling between cells directs their differentiation into different cell types and directs cell movement to form a fruiting body. This consists of thickly walled spores within a spore head lifted up by a stalk that is formed from cellulose-encased stalk cells. Additional ancillary structures support the spore head and form a disc of stalk cells at the base, from which the species gets its name, to anchor the stalk to the substratum. An intermediate, motile "slug" stage allows the whole multicellular structure to relocate before fruiting body formation and spore dispersal to a more nutrient-rich habitat (Fig. 1).

In addition to fruiting body formation, amoebae have two alternative responses to starvation. Some species can encyst as individual cells to form dormant microcysts (Fig. 2). Microcysts are less dehydrated than spores and have a thinner two-layered cell wall instead of the thick three-layered spore coat. Alternatively, cells can form sexual macrocysts. This requires the presence of cells with opposite mating types, of which there are three, although homothallic mating is also common. Macrocyst formation begins when two cells fuse to form a zygote. The zygote then attracts other starving cells and cannibalizes them. Eventually, the zygote synthesizes a highly resistant thick cell wall and enters a long period of dormancy. Macrocysts are more commonly formed under dark and wet conditions that are not favorable for fruiting body formation.

Molecular taxonomy based on gene or protein sequence comparisons place Dictyostelid within a supergroup known as Amoebozoa. This group is separate from, but closely related to, the opisthokonts, the group containing the animals and fungi. Further phylogenic studies subdivide the Dictyostelids into four major divisions. Species in groups 1–3 generally form small clustered and branched fruiting structures from a single aggregate (Fig. 3). Group 4, species usually form a single robust unbranched structure. Most species in groups 1–3 can form microcysts, whereas group 4 species have lost this survival strategy. The sexual cycle,

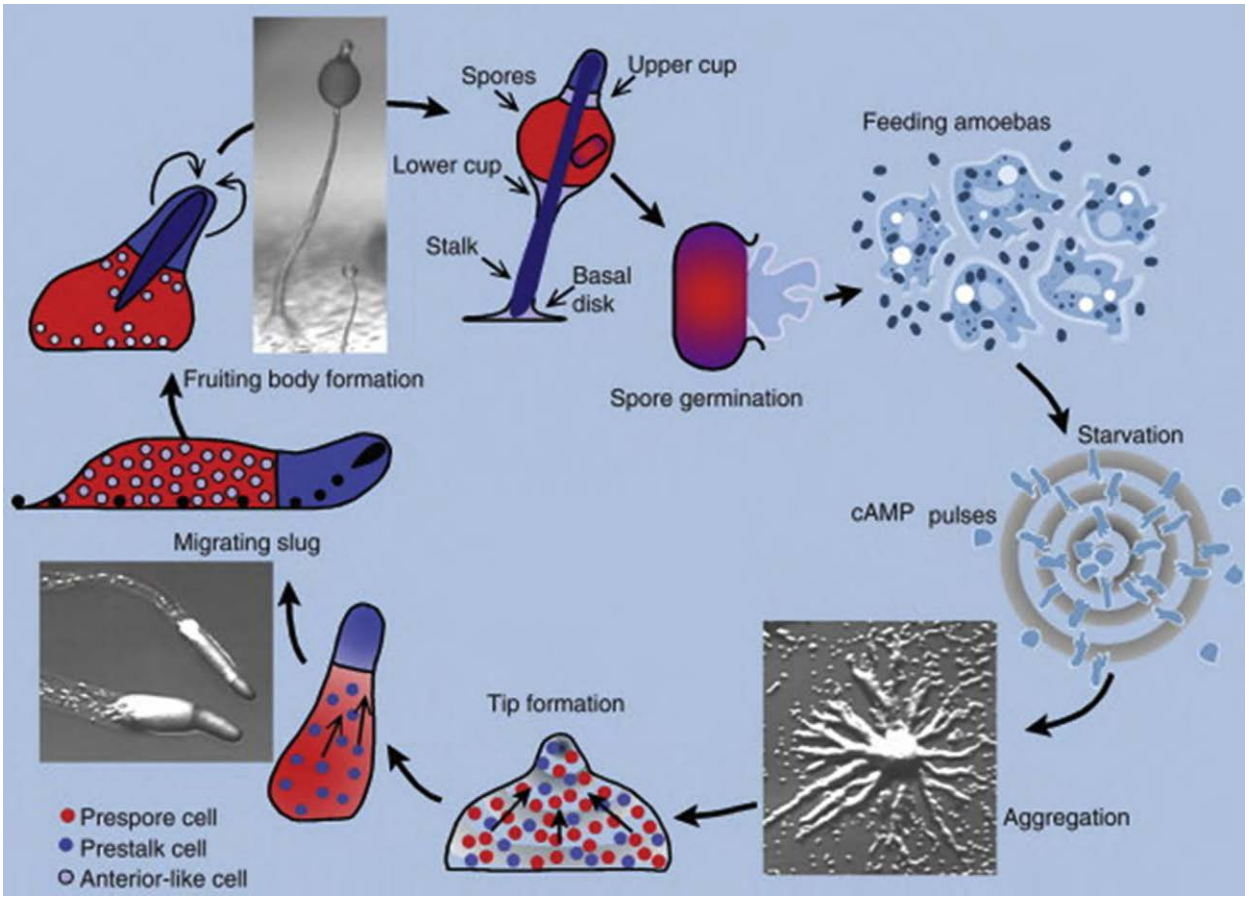

Fig. 1 The life cycle of *Dictyostelium discoideum*. Unicellular amoeba aggregate to form mounds in response to starvation. Within the mounds the cells begin to differentiate into prestalk and prespore cells and the mound morphs into a migratory slug. When suitable conditions for spore dispersal are found, the slug sits down and fruiting body formation commences. Reproduced from Scott, C., Schaap, P., 2009. Dictyostelium. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology. Oxford: Elsevier, pp. 606–616.

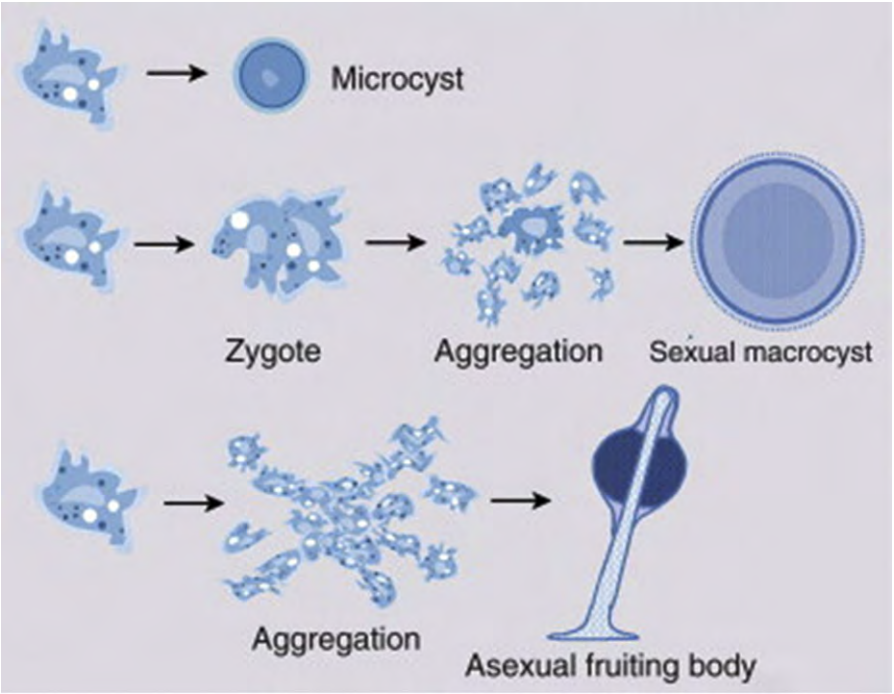

Fig. 2 Surviving starvation. Amoebas can adopt one of three survival strategies when faced with starvation. Solitary amoeba can encapsulate individually to form microcysts, two amoebas can fuse to form a zygote, which then attracts other cells to form a sexual macrocyst, or amoebas can aggregate to form fruiting bodies, where part of the cells die to form a stalk and the others survive as highly resistant spores. Reproduced from Scott, C., Schaap, P., 2009. Dictyostelium. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology. Oxford: Elsevier, pp. 606–616.

resulting in macrocyst formation, occurs in all groups. Group 4 species also stand out by their use of cAMP as the chemoattractant for aggregation, with a variety of other compounds being used by the other groups (Fig. 3).

Dictyostelids have been found all over the world and adapted to many environmental conditions. Forest soils contain the largest variety of species, but also cultivated soils and even deserts harbor at least some species. Some species seem happy to grow under almost any conditions, coping with a wide variety of changes in humidity and altitude. Cosmopolitan species grow almost anywhere and include the species *Dictyostelium mucoroides* and the exquisite *Polysphondylium pallidum*. However, others are far more particular about their choice of residence, with disjunct species, including *D. discoideum*, being widely geographically distributed, but found in similar habitats and climates. Generally speaking, the tropics contain the highest number of species and species variety decreases as latitude and altitude increase.

The cooperative behavior seen in the dictyostelid social amoebae offers a powerful molecular cell model to study the mechanisms of social evolution. At the heart of its social interaction is the process of determining whether each individual amoeba differentiates into a viable spore or a dead stalk cell. While benefiting the population by forming the fruiting body, stalk formation is detrimental to the survival of the individual cell. So how does this altruistic behavior become evolutionary stable, and why do cells not "cheat" by always forming into spore cells? Such "cheater" mutants can be isolated in the laboratory, however strains isolated from the wild always develop fruiting bodies with stalks, although they can exhibit genetic variation in the ratio of spore to stalk cells. Such studies have shown that this altruistic behavior combines elements of an individual competitive drive for efficient spore formation, coercion of other cells to form stalk and the mechanism of kin selection, in which loss of individual cells is balanced by increased survival of a genetically closely related population. This latter effect requires an ability to distinguish relatedness, and an allo-recognition system has been discovered that requires the *TgrB-C* genetic locus that encodes a family of cell-cell adhesion proteins capable of sorting cells of different strains during aggregation.

In addition to interactions between *Dictyostelium* cells, the interaction with soil bacteria is more complex than simple predation of a food source. Some *Dictyostelium* strains have been found to be "farmers" with an ability to accumulate bacteria in the fruiting body to disperse both amoebae and bacterial cells. This is an endosymbotic relationship, with bacteria present within spore cells. One interesting interaction is seen with strains of Burkholderia bacteria, which are not a food source themselves, but induce farming of other bacteria species. Consequently, Burkholderia confer advantages when spores are dispersed into environments that are low in bacteria, and hence provide benefit for survival. In the wild only about a third of *Dictyostelium* isolates naturally farm, however it would appear that this is dependent on the propensity for the cells to secrete the protein Discoidin, which protects bacteria and allows entry into the cell. However, not all bacteria associations are beneficial, for instance the bacterium *Mycobacterium marinum* will infect *Dictyostelium* by hijacking the cells machinery for autophagy, a mechanism that normally surrounds cell debris for degradation. This is a good model for infection of human lung cells with *Mycobacterium tuberculosis*, the cause of TB.

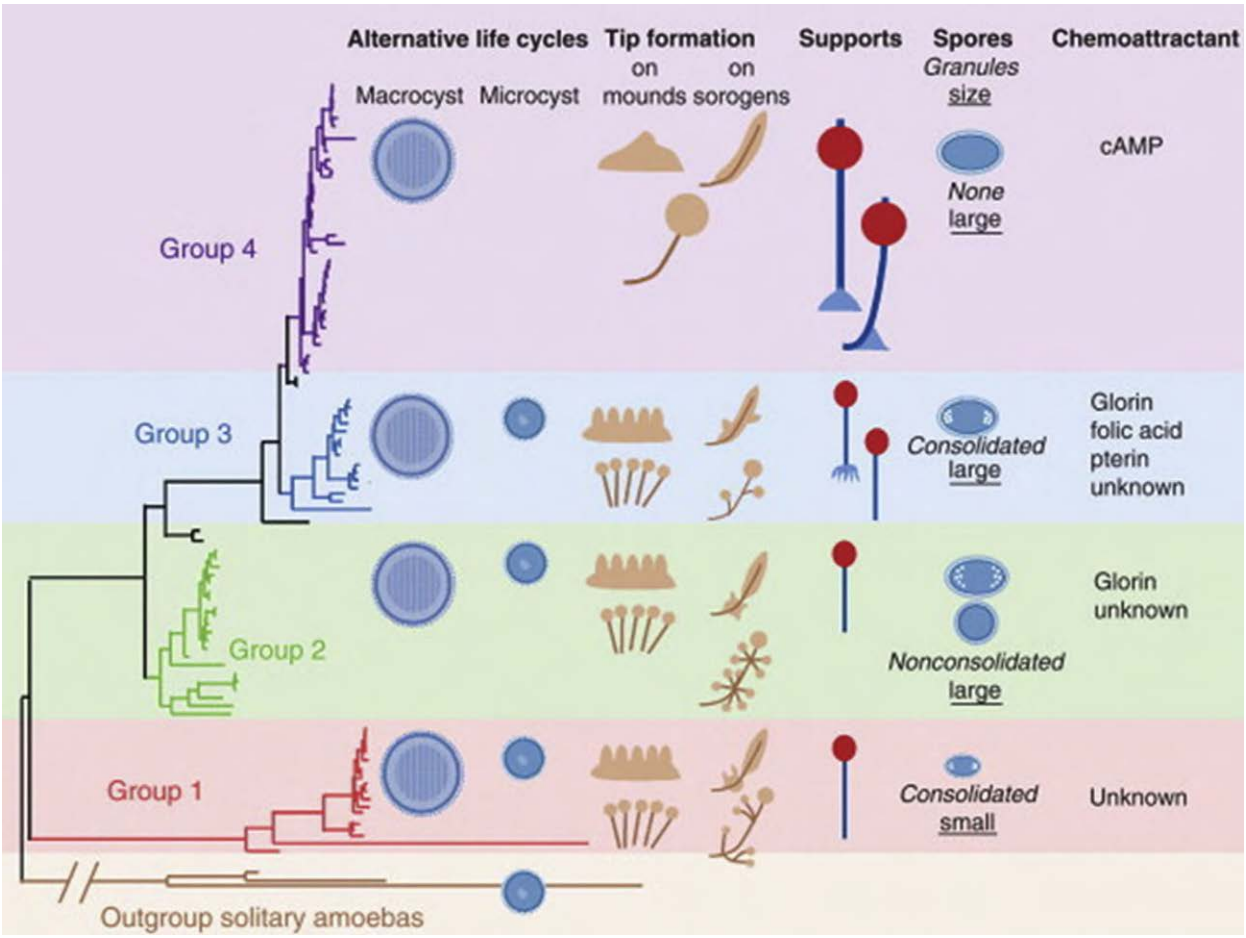

Fig. 3 Trends in the evolution of morphology. Cartoon representation of the distribution of morphological characters across the molecular phylogeny. Spore size and the position of polar granules in the spores shows strong group-specific trends. Sexual macrocysts are formed by multiple species in each group, but microcysts are no longer formed in group 4. Species in groups 1–3 usually form multiple fruiting bodies from a single aggregate that often carry side branches, while species in group 4 tend to form large solitary and unbranched structures. They also form cellular supports or basal discs to hold up the stalk. Only a subset of species in group 3 forms crampon-like supports. In contrast to Group 4 species, none of the species in groups 1–3 use cAMP as a chemoattractant. Reproduced from Scott, C., Schaap, P., 2009. Dictyostelium. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology. Oxford: Elsevier, pp. 606–616.

Genomics

The D. Discoideum Genome

The whole *D. discoideum* genome sequence was published in 2005. It is haploid, 34 Mb in size and arranged over six chromosomes. This makes it about 95 times smaller than the human genome. However, it contains approx. 12,750 protein coding genes, giving it a much greater gene density than the human genome, with ~60% of the number of human genes in 1% of the space. Due to its compact genome, genes have relatively small intergenic regions and introns, although still 11% of sequence in the *Dictyostelium* genome is of low complexity, much of which is present as nucleotide repeats. Such repeats can be found in protein coding regions, such as the long sequence stretches of glutamine (polyQ) and asparagine (polyN). The genome complement is completed by 490 non-coding RNA genes, clusters of transposable elements, the array of rRNA genes with the rDNA element and its palindromic extrachromosomal 88-kb derivatives present at ~100 copies per nucleus; and the 56 kb mitochondrial genome. The genomes of three other Dictyostelid species have also been completely sequenced covering all 4 phylogenetic groups: *D. fasciculatum* (group 1); *Polysphondylium pallidum* (group 2), and *D. lacteum* (group 3). dictyBase is an online genomic resource that links the genomic sequences of the 4 dictyostelid species to descriptions of each gene, associated mutant phenotypes, current bibliography and RNA-seq data.

The genome nucleotide composition of *D discoideum* is very biased with an A/T enrichment of approx. 70% within coding sequences and approaches 90% in non-coding regions. Each chromosome has a telocentric centromere (i.e., located near one end of the chromosome), which is characterized by a single cluster of repeats rich in DIRS (Dicty intermediate repeat sequence) elements. *Dictyostelium* does not possess conventional telomeres, but instead the ends of chromosomes are associated with partial copies of

the rRNA element. Recombination between DIRS and rDNA elements on Chromosome 2 are thought to be responsible for the inverted duplication of 608 genes seen in AX4 laboratory strains.

The *Dictyostelium* genome possesses nearly all of the gene families found in the human genome. These include 60 G-protein coupled receptors (GPCRs), 285 protein kinases and 152 transcription factors. Some protein families have more members in *Dictyostelium* than humans, such as the Frizzled receptors and polyketide synthase genes, which at greater than 40 is the largest number known in any organism. Interestingly, it also contains a number of xenologues, genes horizontally transferred from bacteria. These include histidine kinases, 2-component signaling proteins and genes for synthesis of cyclic nucleotides, including cAMP and dicyclic-GMP.

Classical genetic procedures and linkage analysis in *D. discoideum* remains problematic due to the very slow and infrequent germination of sexual macrocysts. However, *D. discoideum* can also undergo a parasexual cycle whereby two haploid cells can fuse together to form a diploid cell, which contains the chromosomes of both parents contained within a single nucleus. This occurs with a frequency of about 1 per 30,000 amoebae. The resultant diploid cells are relatively stable and divide as normal, producing diploid progeny. However, occasionally the diploids loose a copy of each chromosome and revert to a haploid condition with a random mixture of chromosomes from the parental strains. Diploid cells can be selected by using parental strains with complementary markers. Thus, the parasexual cycle of *Dictyostelium* can be used to generate multiple knockout mutants by recombining several existing mutations.

Protocols for genetic transformation of *D. discoideum* were established in the mid-1980s and have now been expanded into a broad repertoire of techniques. Genes can be expressed from constitutive and inducible promoters with a variety of tags for visualization of proteins in living cells, or purification of proteins and protein complexes. *Dictyostelium* multicellular structures are transparent and are therefore fully amenable to all microscopic investigation. By using these approaches, *Dictyostelium* has become a paradigm for studying cytoskeletal remodeling in living cells during chemotaxis, cell division, and vesicle trafficking.

Genes can be disrupted or replaced with mutant alleles using homologous recombination, which works very efficiently in *Dictyostelium*. Because development is separated from growth and cell division, most developmentally regulated genes are not essential and their mutations can be investigated for their effect on the developmental programme. This has opened up *Dictyostelium* as a powerful molecular genetic tool for studying gene function. Multiple gene deletions can be achieved by consecutive transformations using different selectable markers or by reiterated excision of the same selectable marker by cre-lox recombination.

Creation of random insertion mutants was more problematic until the application in *Dictyostelium* of the restriction enzyme-mediated integration (REMI) method. This enables efficient screens for genes that confer specific phenotypes. Briefly, a restriction enzyme digested plasmid carrying a resistance cassette is introduced into *Dictyostelium* cells along with a restriction enzyme that generates cuts in the genome with sticky ends that are compatible with those of the digested plasmid. The cell DNA ligases repair the cuts, occasionally inserting the plasmids. Resistant cells are recovered, cloned and inspected for mutant phenotypes. Plasmid rescue or inverse PCR methods can be used to identify the genome insertion site. REMI can be adapted for population screens for drug sensitivity or cell competition. A high throughput REMI method (REMI-seq) that incorporates next-generation sequencing to simultaneously screen 1000 s of REMI clones is making a large impact on the number of *Dictyostelium* mutants available for study. The current Genome-wide Dictyostelium insertional mutant (GWDI) bank contains over 5000 gene insertions.

Cell Biology

The combination of being a haploid, free-living unicellular organism and its advanced genomics makes *Dictyostelium* a powerful model for many areas of cell biology, and a useful workhorse for the study of human diseases. Much of this cell biology hinges on control of the actin cytoskeleton, which is utilized in a plethora of functions from including cell motility, membrane trafficking and cytokinesis, often using the same proteins in different ways. There is an extensive body of work in isolating, cloning and study of the regulators of Actin polymerization and its reverse.

Feeding on bacteria, *Dictyostelium* cells have very active phagocytosis, and can engulf relatively large particles, providing a good basis for genetic screens for regulation of phagocytosis. A key decision that the cell must make is the balance between deploying actin polymerization for phagocytosis or cell motility, as these processes appear to be mutually exclusive. Interestingly, factors from bacteria such as folate can both act as a chemoattractant to direct cell movement towards bacteria, but when the cells make contact with the bacteria, they switch into phagocytosis.

Most wild-type cells can only grow by phagocytosis of bacteria, however the commonly used laboratory strains have been selected for the useful property of growing in bacteria free liquid medium. This process is known as macropinocytosis as the cells have gained the ability to engulf large volumes of liquid. The gene responsible for this unusual ability has been cloned, and is the *Dictyostelium* orthologue of Neurofibromatosis type I, NF1, a gene responsible for one of the most common human genetic disorders, and causes tumours of the nervous system. NF1 is a negative regulator of the oncogene ras, and in *Dictyostelium* leads to misregulation of actin polymerization during phagocytosis.

Dictyostelium also offers opportunities for modeling other aspects of human disease. The DNA repair mechanisms of *Dictyostelium* have close similarities to those seen in mammalian cells, and offers a good opportunity to study the processes that monitor and fix damaged DNA. Mutant screens have been used successfully to screen for resistance to commonly used drugs, such as the anti-cancer drug cisplatin, and the mood stabilizers lithium and valproic acid. This has provided new evidence for the mechanism of action of these drugs.

The Developmental Programme of *D. Discoideum*

Upon starvation, *Dictyostelium* enters it developmental programme. For simplicity this can be divided into three phases: aggregation, multicellular development and culmination (Fig. 4).

Aggregation

Although starvation is the primary trigger for commencing development, quorum-sensing factors ensure that the developmental program only commences if there are sufficient cells. These factors down-regulate genes needed for cell growth and induce low expression of aggregation genes, such as cAMP receptor cAR1 and adenylyl cyclase ACA. During aggregation cAMP is synthesized by ACA, which is regulated by cAR1 that is coupled to heterotrimeric G-proteins. Activation of cAMP receptors initially stimulates cAMP synthesis, but as the receptor saturates, it desensitizes and ACA activity drops. Extracellular cAMP is rapidly broken down by the cAMP phosphodiesterase PdsA, allowing the receptor to reactivate and become sensitive to further cAMP stimulation. This cycle results in the production of cAMP pulses in the nanomolar concentration range (Fig. 5). In addition to triggering chemotaxis and cell aggregation, the cAMP pulses strongly accelerate the expression of aggregation genes, causing cells to aggregate to form the multicellular mound after 8–10 h. cAMP pulses also induce competence, that is, components of novel signal transduction pathways for the gene expression events that occur after aggregation.

A key function of extracellular cAMP pulses is to act as a chemoattractant to bring cells together to form the mound, initiating multicellular development. At its simplest level, chemotaxis involves detection of a gradient of chemoattractant and translation of this external gradient into an internal gradient that causes directional movement. However, the molecular mechanisms that underlie chemotaxis are extremely complex and an area of intense study both in *Dictyostelium* and for cell biology in general. *Dictyostelium* cells move by protruding pseudopods in the direction of movement in a process that requires cARs and the G-protein Gα2. Pseudopods are enriched in filamentous actin (F-actin), which provides rigidity to the cell cortex of the extending pseudopod. Pseudopods tend to form close to the base of the previous pseudopod, and regions of the F-actin cytoskeleton are enriched in a number of major signaling proteins, including ras proteins and the downstream effector PI3-kinase, the enzyme that generates phosphatidyl inositol trisphosphate (PIP_3). These in turn promote actin polymerization (Fig. 6(a)). Away from the extending pseudopod, the back of the cell is enriched in both the PIP_3 phosphatase, PTEN, and myosin. Myosin both cross-links F-actin and squeezes the back of the cell (Fig. 6(b)). Time-lapse video-microscopy often detects the rapid protrusion of membrane, blebbing, in a mechanism that requires myosin, but occurs in advance of actin polymerization, suggesting that actin polymerization occurs relatively late in the pathway controlling chemotaxis. Indeed, cells that lack the ability to generate PIP_3 also undergo chemotaxis. Current thinking views PIP_3 synthesis as one component of local excitable network that both drives cell movement towards cAMP and couples to a global inhibitory signal that suppresses pseudopod in the back of the cell – a local excitation global inhibition

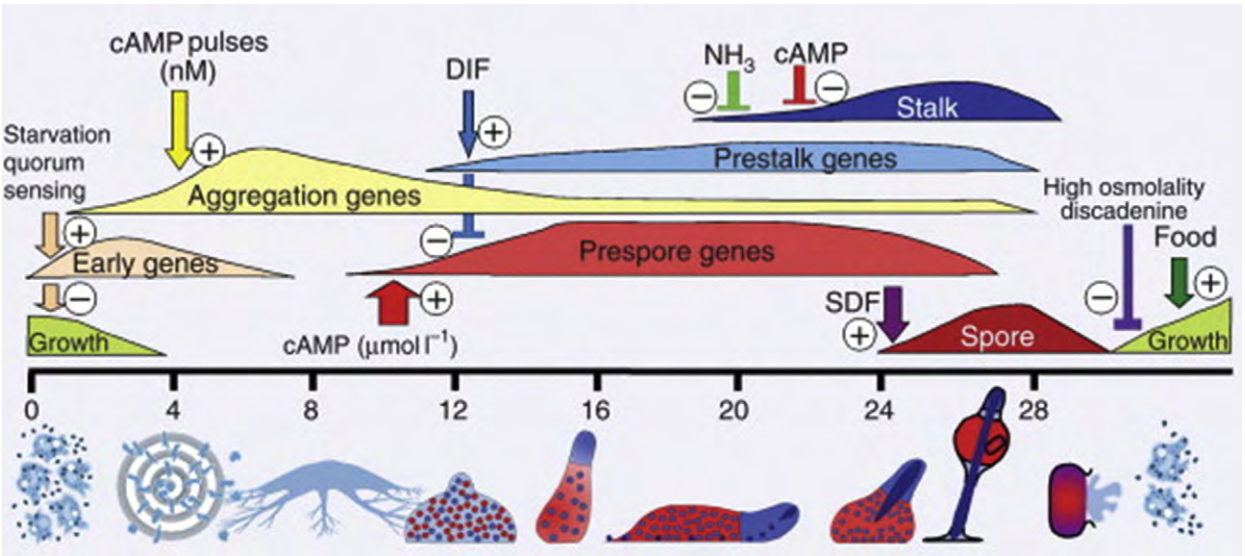

Fig. 4 Gene regulation by cell–cell signaling during *Dictyostelium discoideum* development. Starvation and the quorum sensing factors CMF and PSF trigger expression of early development genes and low levels of aggregation genes. cAMP pulses further upregulate aggregation genes. Once aggregated higher cAMP levels induce prespore gene expression and DIF production. DIF in turn induces expression of some prestalk genes. Ammonia, which accumulates through protein degradation, prevents terminal stalk and spore maturation in migrating slugs. Rising culminants lose ammonia by gaseous diffusion, stalk cells mature and produce a peptide, SDF, which triggers spore maturation. High osmolality and discadenine prevent spore germination in the spore head, but, once dispersed, spores germinate when exposed to food. Reproduced from Scott, C., Schaap, P., 2009. Dictyostelium. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology. Oxford: Elsevier, pp. 606–616.

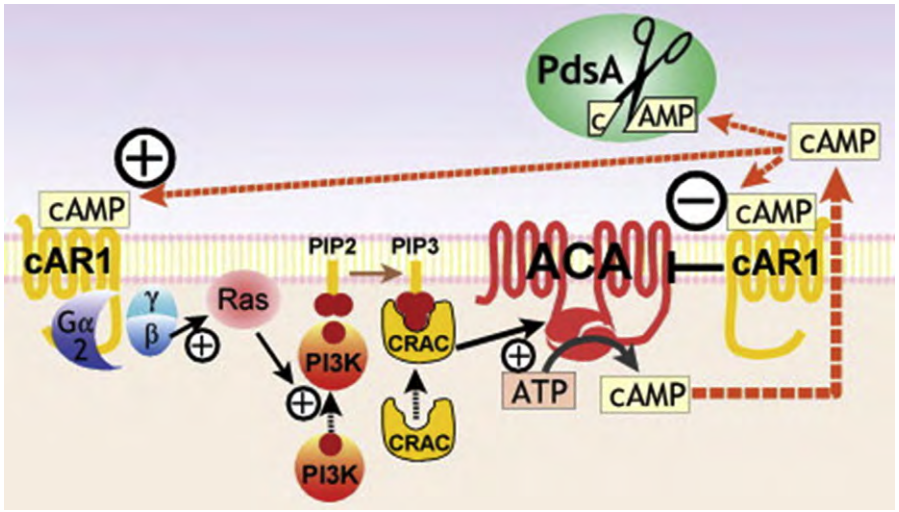

Fig. 5 The network for production of cAMP pulses. Pulsatile cAMP secretion results from positive and negative feedback loops acting on cAMP production. Amoebas first secrete a little cAMP, which binds to the cAMP receptor cAR1 to activate the adenylyl cyclase ACA in an indirect manner that involves phosphorylation of the membrane phospholipid PIP2 to form PIP3. Adenylyl cyclase activation terminates because cAMP also causes desensitization of cAR1, which blocks further cAMP accumulation. Hydrolysis of extracellular cAMP by the cAMP PdsA resets the system for the next pulse. Reproduced from Scott, C., Schaap, P., 2009. Dictyostelium. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology. Oxford: Elsevier, pp. 606–616.

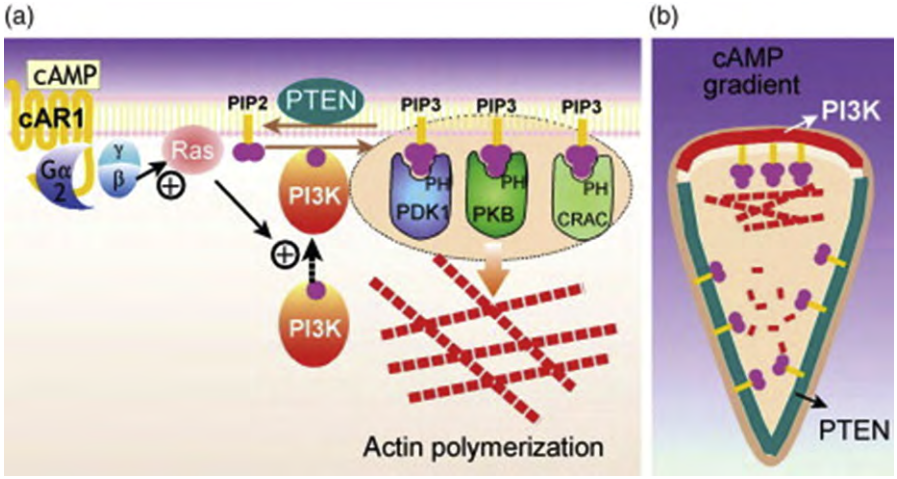

Fig. 6 How do cells detect a cAMP gradient and move forward? (a) Amoebae move up a cAMP gradient by extending a pseudopod in the direction of the highest cAMP concentration and then pulling in their rear end. Pseudopod extension requires polymerization of the major cytoskeletal protein actin at the front of the cell, and is associated with a cAMP increase of the membrane phospholipid PIP_3. PIP3 is formed via ras activation of PI3 kinase, which phosphorylates PIP_2. (b) PI3 kinase activity increases PIP3 in the F-Actin leading edge of the cell, whereas the phosphatase PTEN degrades PIP_3 in the rear of the cell. Reproduced from Scott, C., Schaap, P., 2009. Dictyostelium. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology. Oxford: Elsevier, pp. 606–616.

(LEGI) mechanism. However, the mechanisms initiating cell orientation towards the cAMP source and maintaining the "memory" of that direction as the cAMP pulse passes the cell remain to be established, but may involve the protein kinase, ERK1, and the protein complex assemble around Scar, also known as WAVE.

Multicellular Development

After cells have formed the mound (Fig. 4), cAMP pulses continue to be emitted by the aggregation centre. The source of the pulses now moves to the top of the aggregate. The cells on top of the mound start to secrete cellulose and matrix proteins, which form a sheath around the structure that eventually extends over the entire aggregate. The cells continue to chemotax toward cAMP pulses emitted by the tip, but due to the pressure imposed by the sheath are forced to move upward, forming a finger-shaped slug that due to gravity eventually falls over. At this point the slug begins to migrate toward warmth and light, which in nature would be the soil

surface. These so-called thermotactic and phototactic responses are important, since localization of the fruiting body at the soil surface facilitates the dispersal of the spores.

While the mound forms, cells begin to differentiate into prespore and prestalk cells, the precursors of the spore and stalk cells that form during culmination. They are initially randomly distributed, but sort so that a group of the prestalk cells, the pstA cells, are present in the front 20% of the slug, and the prespore cells are in the rear 80%. This rear portion also contains other group of prestalk cells, the so-called anterior-like cells (ALC). Progression through the developmental program is due to regulated expression of genes at different stages, controlled by cell–cell communication. cAMP pulses continue after aggregation, but ACA is lost from most cells in the rear of the slug. These cells start to express a different adenylyl cyclase, ACG, which produces a constant output of cAMP that soon reaches micromolar concentrations. This is the signal that triggers the expression of prespore genes.

Prespore cells also secrete a dichlorinated alkyl phenone, known as DIF. This low molecular weight molecule inhibits prespore differentiation. DIF addition to isolated cells induces both prestalk cells and formation of stalk cells. The DIF biosynthesis genes have been identified and DIF-less mutants created. Remarkably, when developed these mutants still form pstA cells and a stalk. However, they lack the ALC cells that eventually form the upper cup cells during culmination. In contrast to the pstA cells, a second population of prestalk cells, the pstB form the basal disc, but are suppressed by cAMP. Loss of the *Dictyostelium* orthologue of GSK-3, a regulator of developmental cell fate in animal cells, de-represses the effect of cAMP on pstB cells and changes the proportions of basal disc cells to spores in the fruiting body. During later stages of *Dictyostelium* development GSK-3 is also required to prevent premature expression of stalk genes during culmination. This leaves the question of what controls the formation of pstA and stalk cells. This is not fully understood but a series of transcription factors, have been show to control pstA cell formation. These include the STAT orthologues, DstA and DstC, the bZip proteins, DimA and DimB, and an myb family gene, MybE. Interestingly, the conversion of pstA cell to stalk cells requires dicyclic-GMP, a signal molecule normally only found in bacteria.

Culmination

At the onset of fruiting body formation, culmination, the slug points its tip upward and the prestalk cells start to construct a cellulose tube. This occurs within the tip region, with cells joining the stalk tube at its top, where they differentiate into stalk cells. This process involves the construction of a cellulose cell wall, the formation of a central vacuole, and uptake of water to provide rigidity, and ultimately cell death. The outer surface of the stalk tube is laid down by a collar of cells, held together by adherens junctions in a similar configuration to the polarized epithelia of animals. The prespore cells are raised up the growing stalk, where they mature into spores. The process of spore maturation involves fusion of prespore vesicles with the plasma membrane forming the first layer of the three-layered spore coat. The ALCs move both up and down through the spore mass to form the ancillary supporting structures of basal disc and upper and lower cups.

Ammonia (NH_3) is an important regulator of culmination. During aggregation and particularly in the prestalk cell population during multicellular development, cells degrade their own proteins for energy and metabolites. This causes production of large amounts of ammonia, which has several roles. First, ammonia suppresses the prestalk and prespore cells from maturing into stalk cells and spores. Second, it prevents the ALCs from moving into the prestalk region. If prestalk cells in the tip are lost during migration and no longer produce ammonia, the inhibitory effect is relieved and the ALCs move to the front of the slug to take the place of the lost prestalk cells. All this changes during culmination. Incident light causes the slug to point its tip upward and initiate the culmination process. The tip is now exposed to air and loses ammonia by gaseous diffusion. The loss of ammonia allows the prestalk cells to mature into stalk cells. Ammonia binds to the sensor domain of a histidine kinase, DhkC. to activate RegA via its response regulator domain. RegA contains a phosphodiesterase catalytic domain, and suppresses cAMP-dependent protein kinase (PKA) activity by lowering intracellular cAMP levels. So when ammonia is lost during culmination, RegA loses its phosphoryl group and becomes inactive, allowing PKA activation.

PKA is an important intracellular regulator in eukaryotes. Its cAMP binding domain is also conserved in prokaryotes, where it is part of the catabolite repressor protein, a cAMP-activated transcription factor. *Dictyostelium* PKAs consist of a single catalytic subunit (PKA-C) and a single regulatory subunit (PKA-R). Metazoan PKAs have two of each. PKA is activated when cAMP binds to the regulatory subunit, which then dissociates from the catalytic subunit, thus activating the enzyme. PKA-C and PKA-R levels are low in growing cells and PKA activity is not required for growth. PKA-C is translationally upregulated at the onset of starvation in a process that is most likely activated by the quorum sensor, PSF. This up-regulation is essential for initiation of development, and PKA activity is required for cAMP secretion during aggregation. However high levels of PKA activity are required for both spore cell and stalk maturation.

Three adenylyl cyclases are active during development, ACA during aggregation, ACB in stalk cells and ACG in prespore and spore cells. They all generate the cAMP to activate PKA. However, during culmination it is cAMP degradation by RegA that holds the balance for PKA activation. In addition to ammonia acting via DhkC, the peptide SDF controls RegA via a second histidine kinase DhkA. This peptide is generated by cleavage of the protein AcbA, secreted by prespore cells and cleaved by a protease TagC present on the surface of prestalk cells. In contrast to DhkC, SDF de-activates DhkA causing a reversal of its associated phosphorelay pathway to remove the phosphoryl group from RegA, an increase of cAMP and PKA activation in prespore cells to cause their maturation into spores. Together, ammonia and SDF make sure that stalk cells and spores are formed at the right time and position in the fruiting body (Fig. 7).

It is remarkable that many of the signals that control *Dictyostelium* development, such as ammonia, high osmolarity, and discadenine, do so by regulating the activation status of PKA. In *Dictyostelium*, PKA plays a central role in timing and coordination of

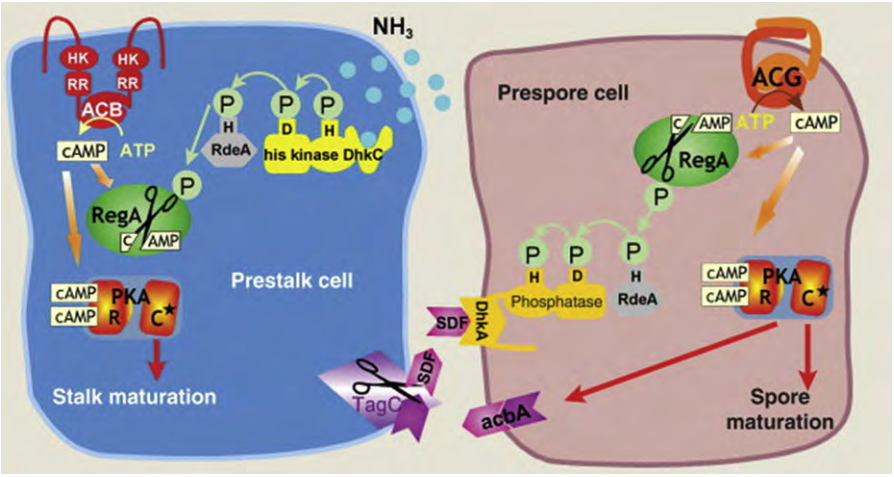

Fig. 7 External signals regulate spore and stalk maturation by altering intracellular cAMP levels. Terminal spore and stalk maturation require high levels of cAMP-dependent protein kinase (PKA) activation by accumulation of intracellular cAMP. In prestalk cells cAMP is produced by ACB and in prespore cells by ACG. The cAMP phosphodiesterase RegA degrades intracellular cAMP but requires phosphorylation to be active. The phosphoryl group is provided by a sensor histidine kinase that can act as either a kinase or a phosphatase when a ligand binds to the sensor domain. The kinase autophosphorylates a histidine residue and via a series of histidine to aspartate to histidine transfers the phosphoryl group is carried over to RegA. Ammonia activates the histidine kinase activity of DhkC, thereby causing activation of RegA and preventing cAMP accumulation. Reproduced from Scott, C., Schaap, P., 2009. Dictyostelium. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology. Oxford: Elsevier, pp. 606–616.

development. It does not stop there, because although stalk cells die in the fruiting body, spore cells remain viable for long periods until dispersal. It is vitally important that spores only germinate when they are dispersed and the emerging amoebas are surrounded by food. Two factors, ammonium phosphate and a modified adenine called discadenine, are present in the spore head to prevent spore germination *in situ*. Ammonium phosphate acts by raising the osmolality in the spore head to high levels. Again this involves a PKA mediated process, as high osmolality activates the osmosensor of the adenylyl cyclase ACG, causing intracellular cAMP production and PKA activity that inhibits spore germination.

Conclusions

The Dictyostelid social amoebas have fascinated biologists for almost 150 years by their alternation between a single state during growth, and multicellular development into fruiting bodies. Combined with their ease of culture and excellent experimental and genetic tractability this versatility makes them an outstanding tool to resolve a broad range of questions in molecular, cellular, and developmental biology. With the discovery that many human disease genes are conserved in Dictyostelids, investigation of the molecular basis of human afflictions has recently been added to this repertoire.

This article provides a generalized overview of current knowledge of the biology of the Dictyostelids, summarizing studies of many of our colleagues. More detailed information about their work can be found in the Further Reading section.

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Dinoflagellates[☆]

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Glossary

Antioxidant enzymes SOD (superoxide dismutase) Metalloenzyme that degrades superoxide radicals into hydrogen peroxide and molecular oxygen.
Basic nuclear proteins DNA-binding proteins of low molecular weight present in the chromatin of dinoflagellates.
Dikaryon Cell with two nuclei.
Dinoflagellates (phylum Dinoflagellata) A group of unicellular eukaryotes that are essentially biflagellated and possess a dinokaryon.
Dinokaryon The nucleus of dinoflagellates, characterized by the presence of a permanent nuclear envelope and chromosomes in a quasi permanently compacted state.
Dinomitosis Mitosis of the dinokaryon characterized by the presence of an extranuclear mitotic spindle crossing the nucleus *via* cytoplasmic channels.
Ecdysis Act of molting or shedding of the theca.
Endosymbiosis The condition of one organism living inside the cells of a different species where neither organism is harmed.
Nucleosomes Protein complexes consisting of histone protein octamers, which are present in most eukaryotic chromatin except that of dinoflagellates.
Ocellus The most complex algal eyespot, present only in several dinoflagellate species.
Pelagic Describing or referring to animals living in the marine domain, either swimming or passive; opposite to benthic (living on the bottom).
Peridinin A carotenoid pigment specific to most autotrophic dinoflagellate.
Theca Total cell wall, consisting of cellulose plates in dinoflagellates.

Abbreviations

FeSODs Iron SODs
SOD Superoxide dismutase

Defining Statement

The diversity of dinoflagellate morphology and lifestyles is presented with emphasis on model organisms. These include heterotrophic (*Crypthecodinium cohnii*, *Noctiluca scintillans*), autotrophic (*Prorocentrum micans*), mixotrophic (*Blastodinium* sp.), and parasitic (*Syndinium* sp.) species. Cellular and molecular analyses including some puzzling questions regarding dinomitosis, evolution, cell architecture, eyespots and an antioxidant enzyme are discussed.

Introduction

With their large distribution throughout all the seas and freshwater bodies of the world, dinoflagellates play a prominent role in the trophic chain and in water ecology. They constitute a major group of unicellular eukaryotic microbes and can be autotrophic (about half are photosynthetic), heterotrophic, parasitic and/or mixotrophic, or symbiotic. Current classification recognizes approximately 161 genera, 48 families, and 17 orders or a total of ~6000 described species. Cells are typically biflagellated and generally the two flagella are oriented perpendicular to one another. This results in a characteristic spiral locomotion from which the group derives its name (*dino*, in Greek means 'to turn'). Most autotrophic dinoflagellates possess a characteristic carotenoid pigment, peridinin, in association with chlorophylls *a* and *c* and other pigments such as β-carotene, diadinoxanthin, and dinoxanthin.

[☆]*Change History*: October 2018. Marie-Odile Soyer-Gobillard updated the text (souligned in yellow) and add three figures (Figs. 11, 17, and 21). Fig. 11: From Soyer-Gobillard, M.-O., et al., 2002. By copyright permission from Vie Milieu Life and Environment. Fig. 17: Reproduction of a draw from an original plate (Pl. XIV) of the doctoral Chatton's thesis (1920) Archives de Zoologie Expérimentale et Générale 1920, 59, 1–475. Journal not yet published. Chatton del. Fig. 21: From Moon, E., et al., 2015. With permission of Elsevier.

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One of the most striking features of dinoflagellates is their nucleus (dinokaryon). Although the pattern of dinoflagellate DNA synthesis is typically eukaryotic, they are the only eukaryotes in which the chromatin is totally devoid of histones and nucleosomes. Instead, it contains specific DNA-binding basic proteins. The only known exception to this is the plasmodial genus *Syndinium* in which basic nuclear proteins (histone-like) have been detected using alkaline fast green staining.

Dinoflagellate diversity has inspired not only numerous scientific studies but also artistic representations. These include the wonderful drawings of Ernest Haeckel (1834–1919) and Edouard Chatton. For example, Fig. 1 shows a drawing of several free-living dinoflagellate of the genus *Pyrocystis*, which is still actively studied for its bioluminescent properties (see "Relevant Website section").

Dinoflagellate Evolution

The great protistologist and cell biologist Edouard Chatton (1883–1947) was the first to distinguish between prokaryotic and eukaryotic microbes and to propose a tentative protist classification. However, it was Haeckel who coined the term 'protist' in the nineteenth century, although he also included bacteria in that group. Dinoflagellates are now recognized as one of the major division of the eukaryotic supergroup Alveolata, which is further included in the supergroup Chromalveolata. Like other members of the chromalveolates, phototrophic dinoflagellates have acquired their plastids by secondary endosymbiosis. This appears to have occurred multiple times during dinoflagellate evolution and at least two cases of tertiary endosymbiosis have now been documented in this group.

The first and undisputable fossil dinoflagellate cyst is from the Triassic (*c*.200 million years ago). However, biogeochemical evidence suggests the presence of dinoflagellate ancestors as far back as the Early Cambrian. This is based on the discovery of triaromatic dinosteroid, sedimentary biomarkers characteristic of dinoflagellates. This suggests that these protists are much older than their fossils suggest. The major diversification of modern dinoflagellates occurred during the Jurassic radiation based on the presence of numerous cysts and thecal plates, which were first formed by photosynthetic dinoflagellates. However, these dates based on morphological and cell biology data are not yet fully supported by all the molecular phylogenetic analyses.

Dinoflagellate Diversity

Three species of free-living dinoflagellates and two mixotrophic species belonging to the genera *Blastodinium* and *Syndinium* have been extensively studied as model organisms, and much of what is known about dinoflagellate cell structure is based on this work. These organisms are the heterotrophic species *Crypthecodinium cohnii* and *Noctiluca scintillans*, the photosynthetic species *Prorocentrum micans*, and the mixotrophic species of the genera *Blastodinium* and *Syndinium*.

Crypthecodinium cohnii Biecheler

C. cohnii Biecheler is a marine dinoflagellate whose vegetative cells are devoid of plastids but rich in starch. Two orthogonally disposed flagella are present (Fig. 2), and these disappear just before cell division (dinomitosis), which precedes a complex reproductive cycle. Since 1887, two forms of *C. cohnii* swimming cells and cysts that lack flagella have been recognized. Detailed *in vivo* studies published in the 1990s described the complex cell cycle of this dinoflagellate, which lasts 26 h and results in four daughter cells (Fig. 3). The organism has been used as a model system to study the cytoskeleton and its role in mitosis, the superoxide dismutase (SOD) evolution.

The cytoskeleton of *C. cohnii* has been intensively studied in our lab, particularly the role of F- and G-actin (see further article) and β-tubulin in the maintenance of cell shape (Fig. 4) and their importance in dinomitosis. It was also shown in this species that cortical microtubules do not depolymerize during mitosis, and now this has also been shown in the species *Oxyrrhis marina*. Microtubules of cytoskeleton are accompanied by heat shock protein P72. This may play a protective role for the cortical microtubules, for example, by preventing microtubule depolymerization during mitosis. This well-conserved protein could also play an important role in the regulation of microtubule formation.

SODs, which catalyze the degradation of toxic superoxide radicals, have been studied only recently in dinoflagellates. A multicopy gene family (about 20 genes) encoding iron SODs (FeSODs) was discovered and characterized in a heterotrophic dinoflagellate, while MnSOD and FeSOD activities were detected in *Symbiodinium* sp., a dinoflagellate living in symbiosis with the sea anemone *Anemonia viridis*, and FeSOD, MnSOD, Cu/ZnSOD were found in the photosynthetic dinoflagellate *Lingulodinium polyedrum* (formerly *Gonyaulax polyedra*). In another photosynthetic dinoflagellate, *Karenia brevis*, both MnSOD and FeSOD have been identified but only by Western blotting. It is thought that these enzymes might play an important role in the survival strategy of this Florida red tide microorganism. Enzymatic assays of SOD activity indicate the presence of two different FeSOD activities in *C. cohnii* lysates, whereas MnSOD and Cu/ZnSOD were not detected in this species. This contrasts with the situation previously characterized in other photosynthetic dinoflagellates.

Phylogenetic analyses including 110 other dimeric FeSODs from a wide range of bacteria and eukaryotes showed that the *C. cohnii* SODs form a monophyletic group (Fig. 5). These results (2008) suggest that all dinoflagellates primitively had a cytosolic FeSOD of bacterial origin, while the MnSOD of mitochondrial ancestry was lost and replaced by a second copy of FeSOD.

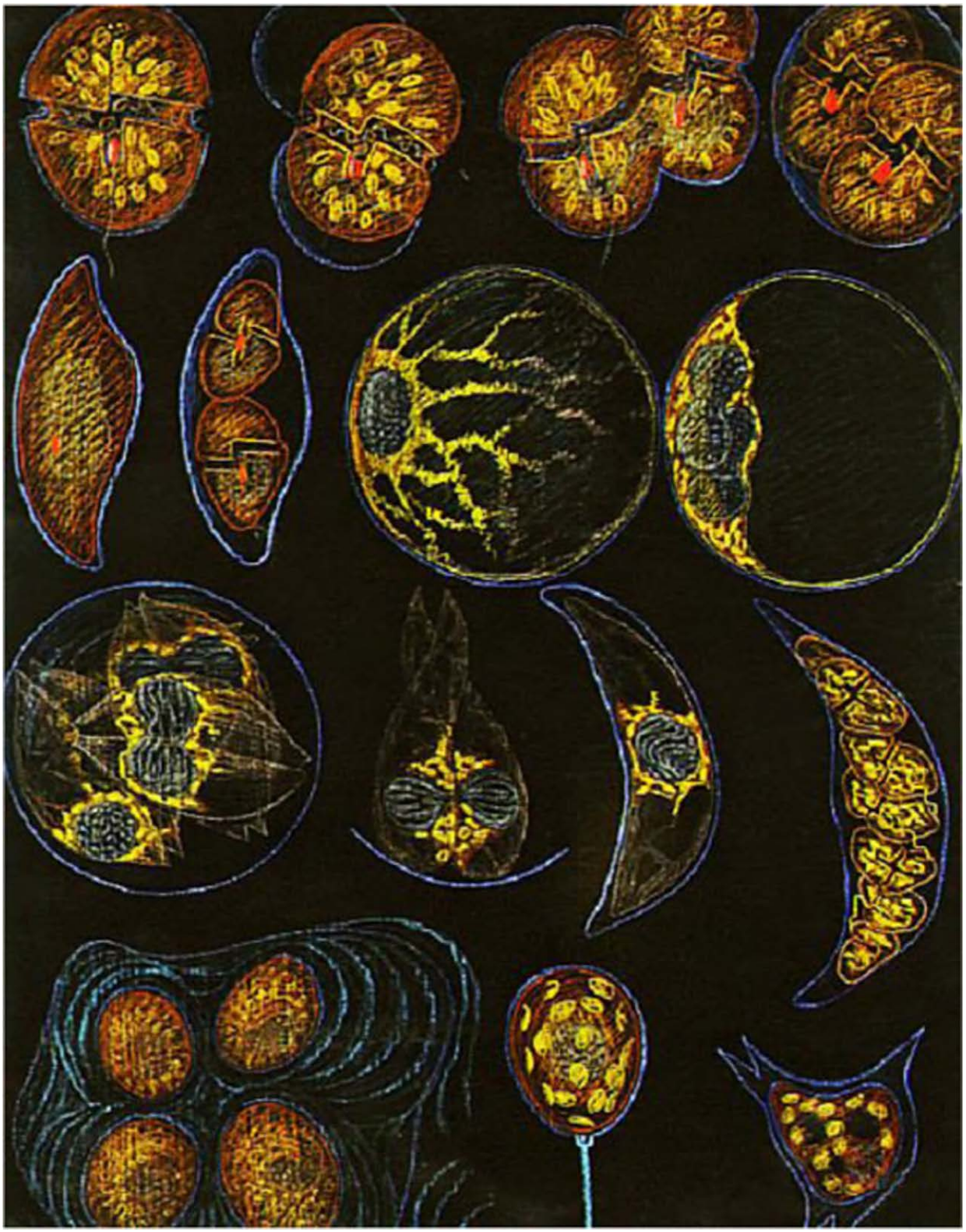


Fig. 1 Several free-living bioluminescent dinoflagellates of the genus *Pyrocystis* Murray ex Haeckel, 1890. The species *Pyrocystis pseudonoctiluca* is shown in the second row at the right-hand side; *Pyrocystis lunula* in the third row at the right-hand side. The drawing is from a course board for students. Drawn by Chatton, E., 1930. Copyright is courtesy of "Les Archives du Laboratoire Arago", bequest LWOFF, F-Banyuls-sur-mer, and all rights are reserved for potential further use.

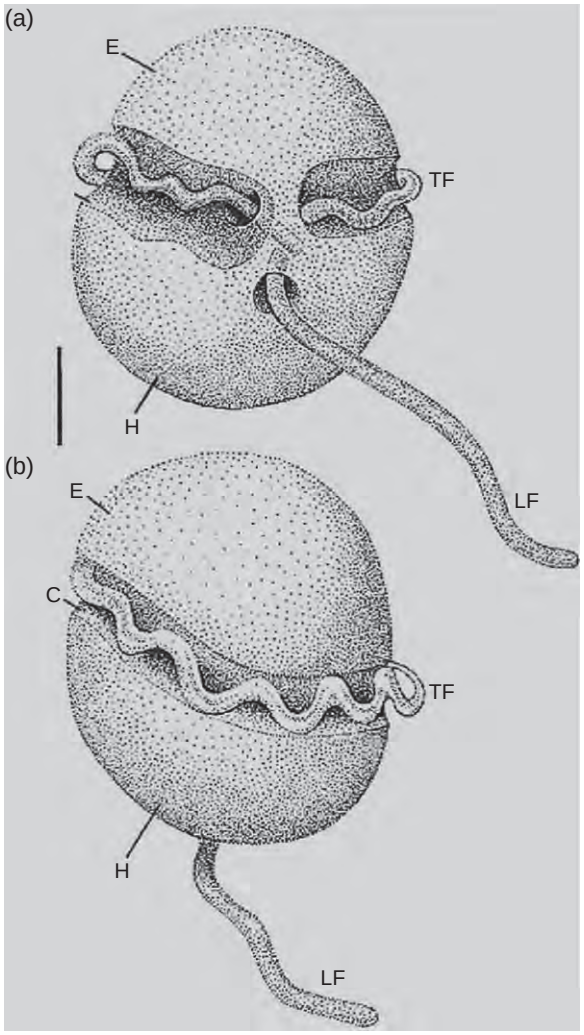


Fig. 2 Schematic representation of the *Crypthecodinium cohnii* cell. The diagram is drawn from previously published SEM views. (a) Ventral view and (b) dorsal view. Also indicated are the episome (E), hyposome (H), longitudinal flagellum (LF), and cingulum (C). Reproduced from Perret, E., Albert, M., Bordes, N., et al., 1991. Microtubular spindle and centrosome structures during the cell cycle in a dinoflagellate *Crypthecodinium cohnii* B.: An immunocytochemical study. BioSystems 25, 53–65. Perret, E., Davoust, J., Albert, M., et al., 1993. Microtubule organization during the cell cycle of the primitive eukaryote dinoflagellate *Crypthecodinium cohnii.* Journal of Cell Science 104, 639–651. With permission of the Company of Biologists Limited.

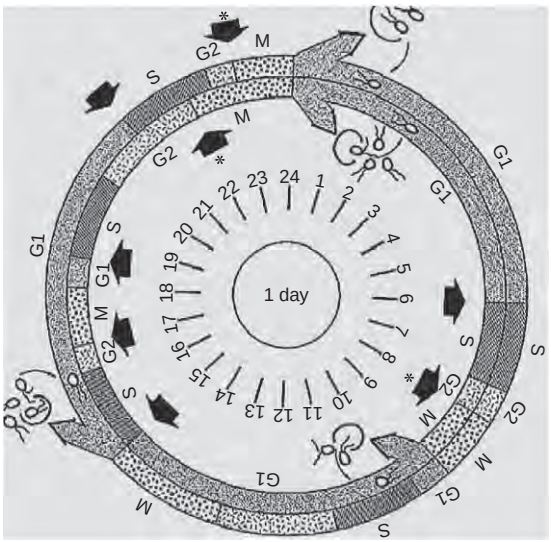


Fig. 3 Diagram of some successive *Crypthecodinium cohnii* cell cycles over 24 h. In this particular example, one vegetative cell performed two successive complete cell cycles (16 h) and released four daughter cells. One of these new swimming cells released two daughter cells 10 h later (external circle of the diagram). During this time, other swimming cells gave an inverse alternation (in alternance with the other cycle) (internal circle). Different diagrams could be possible with other alternations. Transition points G1S ('start' point) are represented by arrows and G2M by arrows plus star. Reproduced from Bhaud, Y., Barbier, M., Soyer-Gobillard, M.O., 1994. A detailed study of the complex cell cycle of the dinoflagellate *Crypthecodinium cohnii* Biecheler and evidence for variation in Histone H1 kinase activity. Journal of Eukaryotic Microbiology 41, 519–526. With permission of the Company of Biologists Limited.

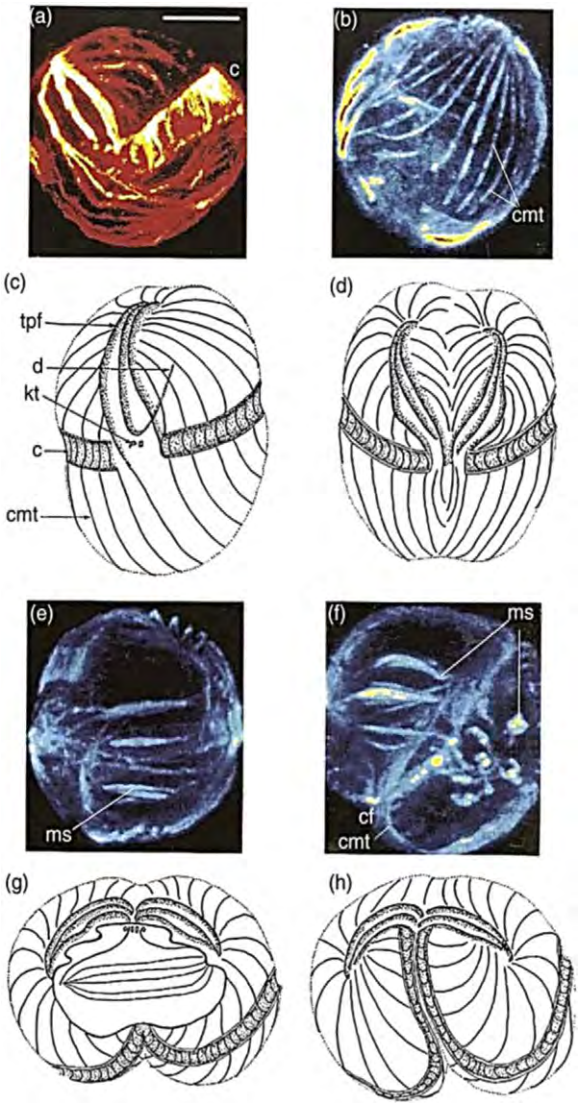


Fig. 4 Reconstructions (each of 16 confocal laser scanning sections) of the free-living heterotrophic dinoflagellate *Crypthecodinium cohnii* Biecheler. Cells are labelled with anti-β-tubulin antibody. (a) In the cortical region of *C. cohnii*, the cingulum (c) in which the transverse flagellum lies (data not shown) and microtubular bundles are visible (in blue color) (Scale bar = 10 μm). (b) Organization of the cortical microtubular rows (cmt) of *C. cohnii.* (c) Cytoskeleton of a nondividing *C. cohnii* cell in G1 phase. Close to the two kinetosomes (kt), three important cortical microtubular bundles are merging at the apical pole. (d) At the beginning of the division, duplicated microtubular bundles migrate. (e) In this dividing (anaphase) cell, microtubules of mitotic spindle (ms) and of the cortex are both visible. (f) Two dividing daughter cells in a cyst, showing two orthogonally oriented mitotic spindle (ms) and cleavage furrow (cf). (g) and (h) Peeled back views of the same dividing cell are shown. The diameter of a dividing cell is about 20 μm. Drawn by M.J. Bodiou. Reproduced from Soyer-Gobillard, M.O., Besseau, L., Géraud, M.L., et al., 2002. Cytoskeleton and mitosis in the dinoflagellate *Crypthecodinium cohnii:* Immunolocalization of P72, an HSP70-related protein. European Journal of Protistology 38, 155–170. With copyright permission from Elsevier publisher.

Eyespot

The ocellus of dinoflagellates, an unusual photosensitive organelle, is the most complex algal eyespot known. It is present in several heterotrophic dinoflagellates such as *Nematodinium*, *Warnowia*, *Erythropsis* and several Woloszynskioids. Eyespot is considered as an important phylogenetic marker in dinoflagellates. More often located toward the posterior end of the cell, the most sophisticated structure measures about 25 μm long by 15 μm wide. It is composed of a pigment cup, retinoid, and lens and it functions as a photoreceptor. As suggested by W. J. Gehring in 2004, this photosensitivity probably arose first in cyanobacteria, and was later acquired by the first photosynthetic eukaryotes (archaeplastida) *via* the cyanobacterial endosymbiosis that gave rise to the plastid. Dinoflagellates would then have acquired this photosensitivity from a red alga *via* secondary chloroplasts. In some dinoflagellate species this plastid evolved into an elaborate and sophisticated photoreceptor organelle, which is found in some dinoflagellate species that lack chloroplasts, such as those cited above. Because some dinoflagellates such as zooxanthellae are commonly found as symbionts in cnidarians, for example, these organisms could then have transferred their photoreceptor to cnidarians.

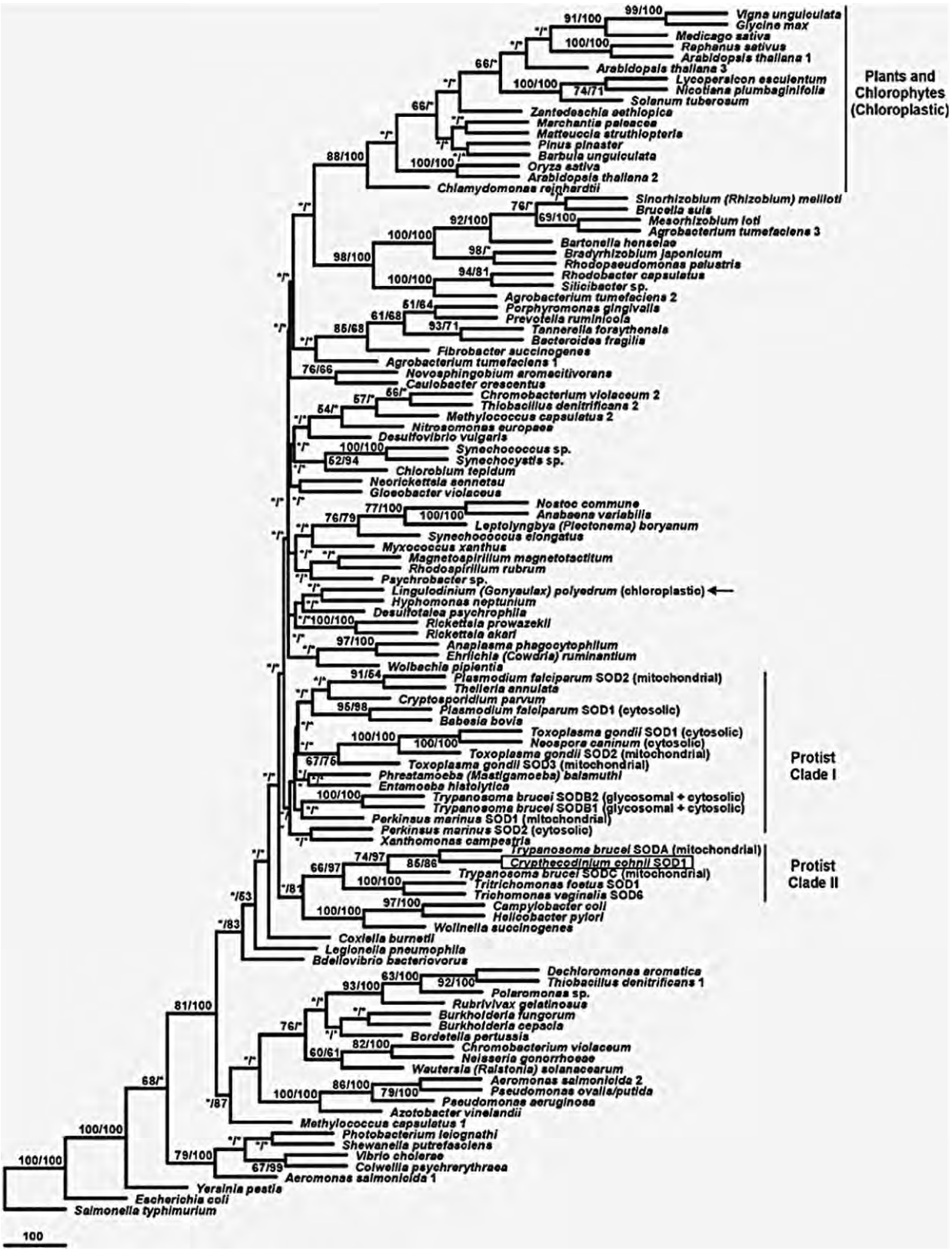


Fig. 5 Neighbor-joining tree based on protein sequences of dimeric FeSODs and closely related SODs. Accession numbers or references of the sequences retrieved from genome sequencing projects are given in Dufernez, F., Yernaux, C., Gerbod, D., et al., 2006. The presence of four iron containing superoxide dismutase isozymes in Trypanosomatidae: Characterization, subcellular localization, and phylogenetic origin in *Trypanosoma brucei.* Free Radical Biological Medicine 40, 210–225. The full-length *Crypthecodinium cohnii* SOD1 sequence obtained in this study is boxed, whereas the chloroplast sequence from *Lingulodinium (Gonyaulax) polyedrum* is indicated by an arrow. If experimentally determined, the subcellular localization of protist SODs is indicated in parentheses. The scale bar corresponds to 0.1 substitution (corrected) per site. Reproduced from Dufernez, F., Derelle, E., Noël, C., et al., 2008. Molecular characterization of iron-containing superoxide dismutases in the heterotrophic dinoflagellate *Crypthecodinium cohnii.* Protist 159, 223–238. By copyright permission from Elsevier GmbH.

Noctiluca scintillans McCartney

N. scintillans McCartney is a model for studying the complex heterotrophy found in some dinoflagellates. Cytoskeletal elements that participate in the motility of the *N. scintillans* trophozoite are also involved in related nutritional functions. These elements are located at the level of the tentacle (Fig. 6) and of the cytostome where filaments are found organized into myonems and cytoplasmic fibrils organized into striated and contractile strip. As shown in Fig. 8, microtubules are located just under the peripheral ectosarc, which is responsible for calcium exchange by means of epiplasmic channels, and are oriented parallel to the major axis of the tentacle. In the schematic drawing of transverse section (Fig. 7), the convex part is equipped with a large number of microtubule rows that are crosslinked with each other (Fig. 8, arrows). Striated myonems are distributed along the tentacle or linked to one another by a knot (Fig. 7). The tentacle also contains numerous mitochondria. This complex system together with extracellular calcium ions sequestered in mitochondria is responsible for this dinoflagellate's motility and feeding. Tentacle contraction involves ectosarc deformation, myonem contractility, and microtubule modifications. The major contractile protein of this system that plays an important role in determining cell shape is the epiplasmic actin protein, P45. Cytochemical investigations about the presence of histone-like proteins in the trophocyte nucleus, using in particular alkaline fast green, were negative. At the molecular level, the unusual nucleotide containing the base 5-hydroxymethyluracil has been found in the nuclear DNA of *N.scintillans*. In the up to now investigated dinoflagellate species this unusual base replaces 12%–68% of the thymines that constitutes a relatively important level of replacement amount.

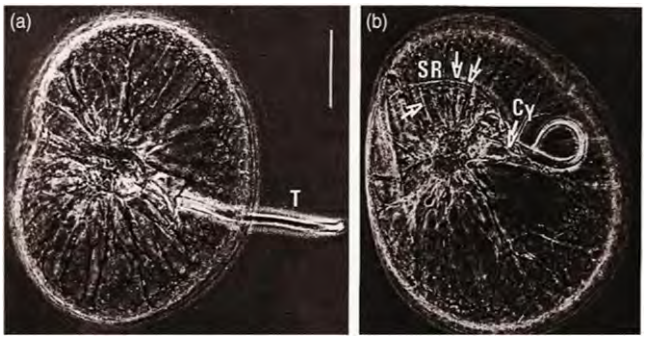


Fig. 6 *Noctiluca scintillans* McCartney. (a) and (b) Interference phase-contrast views. (a) Trophozoite with its tentacle (T) seen relaxed and (b) contracted. Tracts of myonems (arrows) are anchored between the cytostome (Cy) and the supporting rod (SR). Scale bar = 200 μm. (Photos J. Lecomte). Magnification ×200. Reproduced from Métivier, Ch., Soyer-Gobillard, M., 1988. Organization of cytoskeleton during the tentacle contraction and cytostome movement in the dinoflagellate *Noctiluca scintillans* McCartney. Cell and Tissue Research 251, 359–370. By copyright permission from Springer-Verlag.

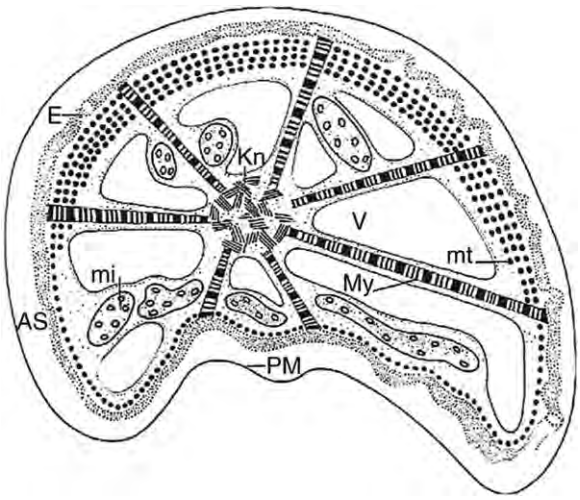


Fig. 7 Schematic drawing of a transverse section of the *Noctiluca scintillans* tentacle. Note the distribution of the three characteristic elements of the cytoskeleton: the ectosarc (E), microtubules (mt), and myonems (My). Also indicated are the knot (Kn), plasma membrane (PM), vacuoles (V), mitochondria (mi), and alveolar space (AS). Reproduced from Métivier, Ch., Soyer-Gobillard, M.O., 1988. Organization of cytoskeleton during the tentacle contraction and cytostome movement in the dinoflagellate *Noctiluca scintillans* McCartney. Cell and Tissue Research 251, 359–370. With copyright permission from Springer-Verlag.

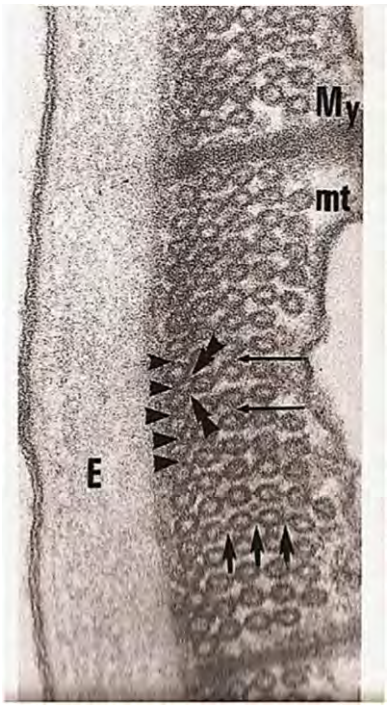


Fig. 8 Ultrastructural organization of the tentacle of *Noctiluca scintillans*. High magnification of part of the tentacle on its convex side. Myonems (My) between the microtuhules (mt) on the ectosarc (E) of the first row are crosslinked with each other by bridges (single arrowhead), links with the second row result in a Y bond (double arrowheads). In the other rows, links are always observed between each row (thick black arrows); they are sometimes visible between microtubules of the same row (thin black arrows). Scale bar = 0.1 μm. Magnification ×140,000. Reproduced from Métivier, Ch., Soyer-Gobillard, M.O., 1988. Organization of cytoskeleton during the tentacle contraction and cytostome movement in the dinoflagellate *Noctiluca scintillans* McCartney. Cell and Tissue Research 251, 359–370. By copyright permission from Springer-Verlag.

Prorocentrum micans Ehrenberg

P. micans is a model for autotrophic dinoflagellates. In this species, the pellicle covering the whole surface of the theca (the epitheca) is a kind of polysaccharidic 'skin'. This fragile structure surrounding the entire cell was observed only under SEM with careful preservation (Fig. 9). The cell cycle of *P. micans* is typically eukaryotic despite the unusual structure of the dinoflagellate chromatin when compared with that of most eukaryotes. The population doubles every 6 days and the S phase of DNA synthesis lasts 4 h. From the end of replication to the end of mitosis (G2 + M phases), the time taken on average is 8 h. Phases S + G2 + M take less than 12 h and the G1 phase lasts 120 h (Fig. 10). This is despite the fact that *P. micans* has more than 100 chromosomes of 1 μm in diameter and a large DNA content of 42 pg cell^{-1}.

In *P. micans*, vegetative cells are haploid and reproduce by binary division. Sexuality can be experimentally induced by changing the culture medium (switching from Erd Schreiber's to Provasoli's medium) or by subjecting cultures of this photosynthetic species to unfavorable conditions such as darkness and low temperature (4°C).

Mating in dinoflagellates occurs by conjugation (Figs. 11 and 12). This begins with the formation of a conjugation tube between the two conjugants, followed by passage of one nucleus *via* the tube. Then, this tube enlarges and the sexual cells fuse, leading to the formation of a dikaryon (cell with two nuclei). There is commonly an absence of fundamental morphological differences between vegetative cells and gametes. The only visible differences are a small variation in pigmentation and cell size. Many different sexual processes occur in dinoflagellates (Table 1), but *P. micans* is unique in having a fertilization tube that permits the transfer of all genetic material (male gametic nucleus) and in the formation of a partition between the empty sexual cells and the dinokaryon. Dinoflagellates appear to be highly specific in terms of environmental factors that induce sexuality. This is probably related to the extreme diversity in their life cycles and way of life.

Mixotrophic Dinoflagellates

Mixotrophic (parasitic) Dinoflagellates were first described by Chatton in his magisterial doctorate thesis, published in 1920. He was particularly interested in *Blastodinids*, which he first discovered in the digestive tract of pelagic copepods. As shown in Figs. 13 and 14, Blastodinids are binucleated cells. Two kinds of cells are present: the trophocyte, which remains temporarily undivided while it increases in size; and the gonocyte, which produces sporocytes in which the chromatin is progressively more and more

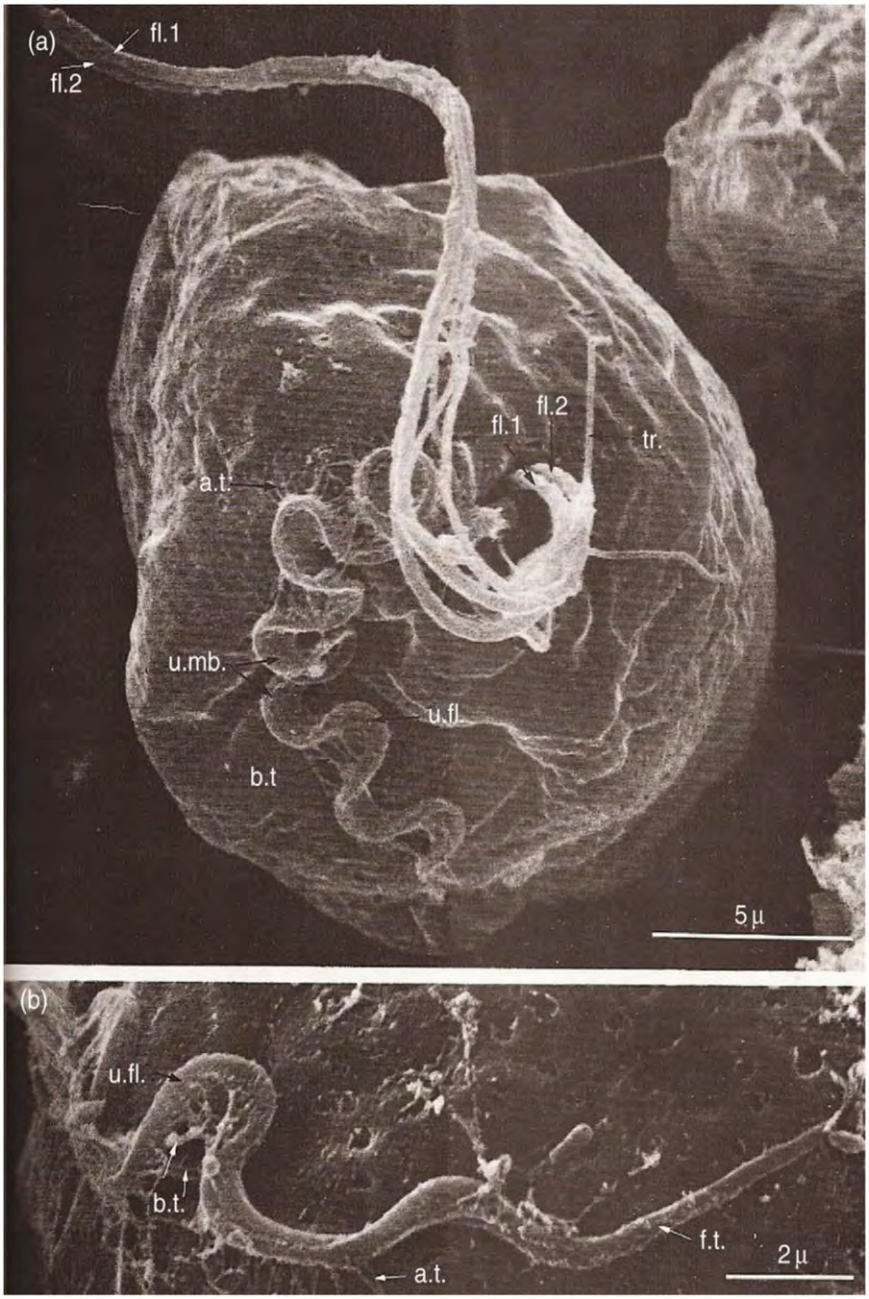


Fig. 9 (a) *Prorocentrum micans* autotrophic cell apical view observed with scanning electron microscope. Observe the peripheral polysaccaridic 'skin'. Two longitudinal flagella of this predividing cell run more or less parallel to each other. One undulating flagellum arises from the same opening of the epitheca as the longitudinal ones, but adheres to its outer layer; anchoring threads strengthen this adhesion, and bridging threads connect every second wave of the undulating membrane. On the epitheca also lie adsorbed extruded fibrous trichocysts and undefined filaments, probably of bacterial origin. (b) Terminal portion of the undulating flagellum. There are no anchoring or bridging threads on its free tip. Magnification ×11,500. a.t., anchoring thread; b.t., bridging thread; fl1, fl2, longitudinal flagella; f.t., free tip; tr. trichocyst; u.fl., undulating flagellum; u.mb., undulating membrane. Reproduced from Soyer-Gobillard, M.O., Prévot, P., De Billy, F., et al., 1982. *Prorocentrum micans* E., one of the most primitive dinoflagellates. I. The complex flagellar apparatus as seen in scanning and transmission electron microscopy. Protistologica XVIII, 289–298. Journal no longer published.

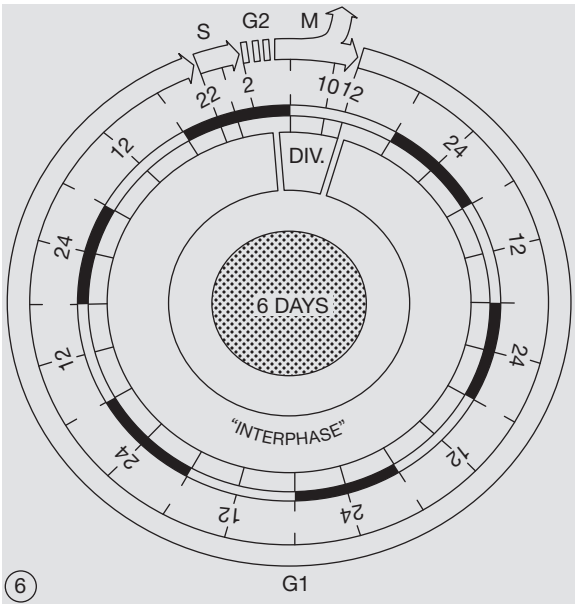


Fig. 10 Cell cycle and mitotic clock of *Prorocentrum micans* cell. S = 4 h; G2 is short or nonexistent, duration undetermined; G2 + M = 8 h; S + G2 + M = 12 h; G1 = 120 h. Reproduced from Bhaud, Y., Soyer-Gobillard, M.O., 1986. DNA synthesis and cell cycle of a primitive dinoflagellate *Prorocentrum micans* Ehr. Protistologica XXII, 23–30. Journal no longer published.

condensed after successive divisions. Layers of sporocytes lead to the formation of biflagellate swarming dinospores, which are later released in seawater. Trophocytes can also divide longitudinally and produce other parasites into the same digestive tract. In Fig. 14, the two nuclei are seen clotted in anaphase with 'plasmodendrites' (cytoplasm) passing through the nuclei. In 1971, Soyer (Gobillard) showed with transmission electron microscopy that the *Blastodinium* mitotic spindle is located in these channels and linked to the spindle poles, which lack centrioles. During mitosis, the chromosomes of the sporocytes become increasingly compacted and are attached to the nuclear envelope. The latter has differentiated cytoplasmic channels that allow the microtubules of the mitotic spindle to pass through the nuclear membrane (Fig. 15).

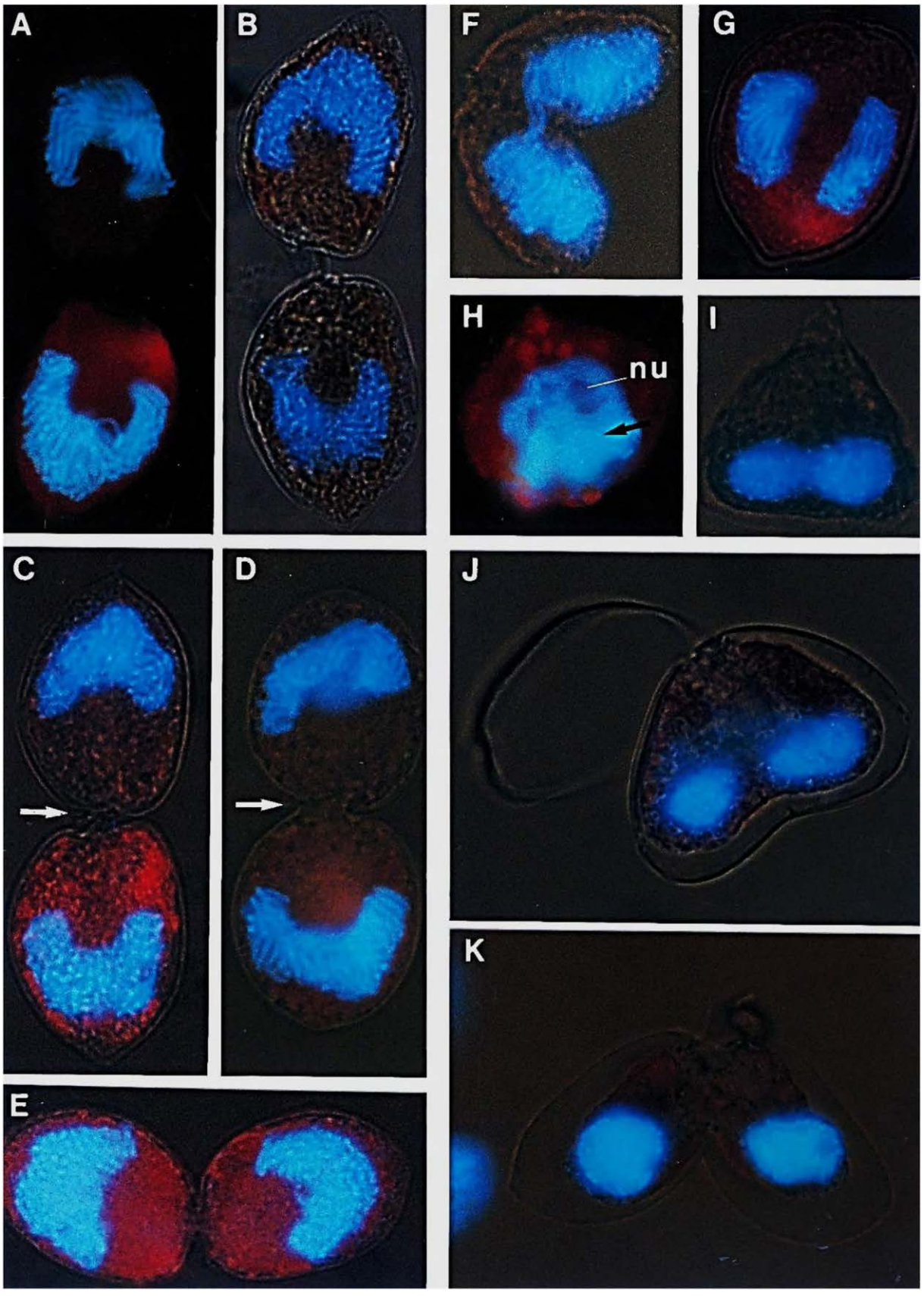
Chatton also described the genus *Syndinium*, whose various species parasitize marine copepods or various radiolarians. On the basis of the morphology of the spores released by their free-living swarmers, Chatton considered *Syndinium* a 'specialized' dinoflagellate. This was also due to the apparent simplicity of *Syndinium* cell division, and the fact that the species had only five chromosomes (Fig. 16). On this basis, Chatton proposed it as a model for dinoflagellate mitosis, specifically referring to it as 'syndinian mitosis' (Fig. 17).

Later, ultrastructural studies showed this to be very particular peridinian mitosis. The mechanism consists of a permanent closed nuclear membrane outside of which lies an extranuclear mitotic spindle with centrioles and kinetochores that attach to the inner surface of the nuclear membrane (Figs. 18 and 19). This is in contrast to most other dinoflagellates, which have neither centriole nor kinetochore. Moreover, most typical dinoflagellate chromosomes lack basic proteins such as histones, but instead have specific basic nuclear protein associated with their DNA. Two exceptions to this are *Syndinium* sp. and the symbiotic zooxantellae, which have appreciable amounts of basic proteins of the histone family associated with their DNA. Further biochemical and molecular studies are needed to analyze more thoroughly and to characterize these basic nuclear proteins.

Detailed description of a new planctonic free living mixotrophic Dinoflagellate, *Paragymnodinium shiwhaense*, was published: its morphology, pigments and ribosomal DNA gene sequence analysis suggested that it is a new species into a new genus. The distinctive feature of this species is the presence of both chloroplasts and nematocysts, organelles used for the prey capture as also present in the polynucleated *Polykrikos* dinoflagellate, in addition to fibrous trichocysts, which are detoxication organelles. In addition to the photosynthesis, *P. shiwhaense* sucks the preys through its peduncle. Several photosynthetic species of Dinophyceae as *Dinophysis acuminata or D.norvegica* were the first (1994) to be described as mixotrophic with the presence of food vacuoles into the cell. They are predators for other dinoflagellates or other unicellular planctonic organisms and can have an important grazing impact on algal populations.

Evolution of the Mitotic Apparatus

Most dinoflagellates have a closed mitosis in which the nucleus is surrounded by a persistent nuclear envelope. Within this nucleus the chromosomes are maintained in a quasipermanently compacted state. Chromosome separation is accomplished by an extranuclear microtubular mitotic spindle located in cytoplasmic channels that pass through the nuclear membrane during mitosis.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Two centrosome regions without centriole are linked to the kinetosomes by two microtubular structures called 'desmoses' as shown on the diagrammatic representation of a dinoflagellate in Fig. 20: in this diagram, is represented the mitotic apparatus schematized in anaphase with listed and framed the up-to-now detected associated proteins. Exceptions among these species-models include rare species such as the coelomic parasite *Syndinium* sp. (see below), *Oxyrrhis marina* and *N. scintillans*, which show considerable variations when compared with the well-known *C. cohnii* model of mitosis.

Syndinium and related species that parasitize invertebrates were classified as dinoflagellates by Chatton and others on the basis of the morphology of their zoospores. However, the biochemistry of their chromosomes (presence of specific basic proteins) and electron microscopic observations demonstrated fundamental differences in their mitotic apparatus as compared with the rest of the dinoflagellates. In *Syndinium* and relatives, the four V-shaped chromosomes are permanently attached at their apex to a specific area of the nuclear membrane through a kinetochore-like trilaminar disk inserted into an opening in the membrane (Fig. 19). Microtubules connect the dense layer of each kinetochore to the base of the two centrioles located in an invagination of the nuclear envelope (Fig. 18). In these species, centrioles or basal bodies are intimately involved in chromosome segregation. This is not the case in *Crypthecodinium*, where the basal bodies are indirectly involved with the microtubular spindle, or in *Blastodinium*, where the nuclear envelope functions as the motor for chromosome segregation.

As soon as 1996, for the first time, by means of monoclonal and polyclonal anti-actin antibodies, Soyer-Gobillard et al. were able to demonstrate the presence of F- and/or G-actin in two species of dinoflagellates *Prorocentrum micans* and *Crypthecodinium cohnii*, either by biochemistry, immunofluorescence and in TEM. SDS-PAGE electrophoresis and immunoblottings made either from total or nuclear protein extracts revealed the presence of a 44-kDa band reacting with monoclonal anti-actin antibody in two species, and thus demonstrated the presence of actin in nuclear and cytoplasmic fractions. After squash preparation of *P. micans* cells, actin was identified within the nucleus and in some regions of the cytoplasm by immunofluorescence microscopy. Labelling of both nucleolus and centrosome region was evident together with amorphous nucleoplasmic material surrounding the chromosomes. The use of cryosections of intact *P. micans* and *C. cohnii* cells for immunofluorescence along with staining with DAPI to delineate the chromosomes themselves, yielded finer resolution of the intranuclear network labelling pattern. ln *P. micans*, in addition to the centrosome region, the cytoplasmic channels passing through the nucleus in dividing cells are labelled. ln *C. cohnii*, the cortex, the centrosome region, the cytoplasmic channels, the region surrounding the nucleus, the filaments linking it to the cortex and the cleavage furrow are also labelled. ln the nucleus of the two species, there is a prominent 'weft' of fine actin filaments in the nucleoplasm forming a matrix of varying density around the persistent chromosomes. This actin matrix, of unknown function, is most conspicuous at the end of the S-phase of the cell cycle. Fluorescent derivatives of phalloidin, used as diagnostic cytochemical probes for polymeric actin (P-actin), gave similar results. Positive TEM immunolabelling of intranuclear actin confirms its presence in the nucleoplasm and in the nucleolus where the preribosomal region is labelled. ln the cytoplasm, lips of the cleavage furrow and kinetosome regions are labelled as weIl as the centrosome region. The possible functions of this protein located in several compartments of dinoflagellate cells were recently lightened and all these observations confirmed by Berdieva et al. (2018), in another Prorocentrale, *Prorocentrum minimum* (Pavillard) Schiller 1933: *Prorocentrum minimum* is an armoured, marine, planktonic, bloom-forming dinoflagellate. It is a toxic cosmopolitan species common in cold temperate brackish waters to tropical regions. In this species, actin is also involved in fundamental nuclear processes and participates in cell shape determination, cell cover rearrangement and development during ecdysis.

Fig. 11 Course of pairing (A)–(C), mating (D)–(F) and beginning of sexual reproduction (G)–(K) of *Prorocentrum micans* as observed *in vivo* after staining of the nuclei with DAPI and fluorescence coupled with Nomarski interference light microscope observations. During pairing and conjugation, several cell pairs presented a bright (red) autofluorescence (A), (C), (E), (G), (H). (A)–(C). During pairing the cells are disposed with the anterior part of one cell in front of the other anterior part, apical spine against apical spine. The two nuclei are distally disposed. (D) Note the fixation of one of the apical spines anchored into the partner cell (arrow) at the beginning of mating. (E)–(G) After the formation of a fertilization canal, and injection of one nucleus of the donor cell into the receiver (F), the two nuclei are located in the same cell (G). H, After conjugation of the nuclei, replication occurs. The nucleus containing 4q DNA presents very thin and punctuated chromosomes (arrow). An intense rotation movement (cyclosis) is also observable in the nucleus. The outline of the nucleus is wavy and three nucleoli (nu) are visible. (l)–(K) The first nuclear meiotic division takes place but cytoplasmic segregation does not occur. Meiosis is incomplete. Reproduced from Bhaud, Y., Soyer-Gobillard, M.O., Salmon, J.M., 1988. Transmission of gametic nuclei through a fertilization tube during mating in a primitive dinoflagellate, *Prorocentrum micans* Ehr. Journal of Cell Science 89, 197–206. Soyer-Gobillard, M.O., Bhaud, Y., Saint-Hilaire, D., 2002. New data on mating in an autotrophic dinoflagellate *Prorocentrum micans* Ehrenberg. Vie Milieu 52, 167–175. By copyright permission from Vie Milieu Life and Environment.

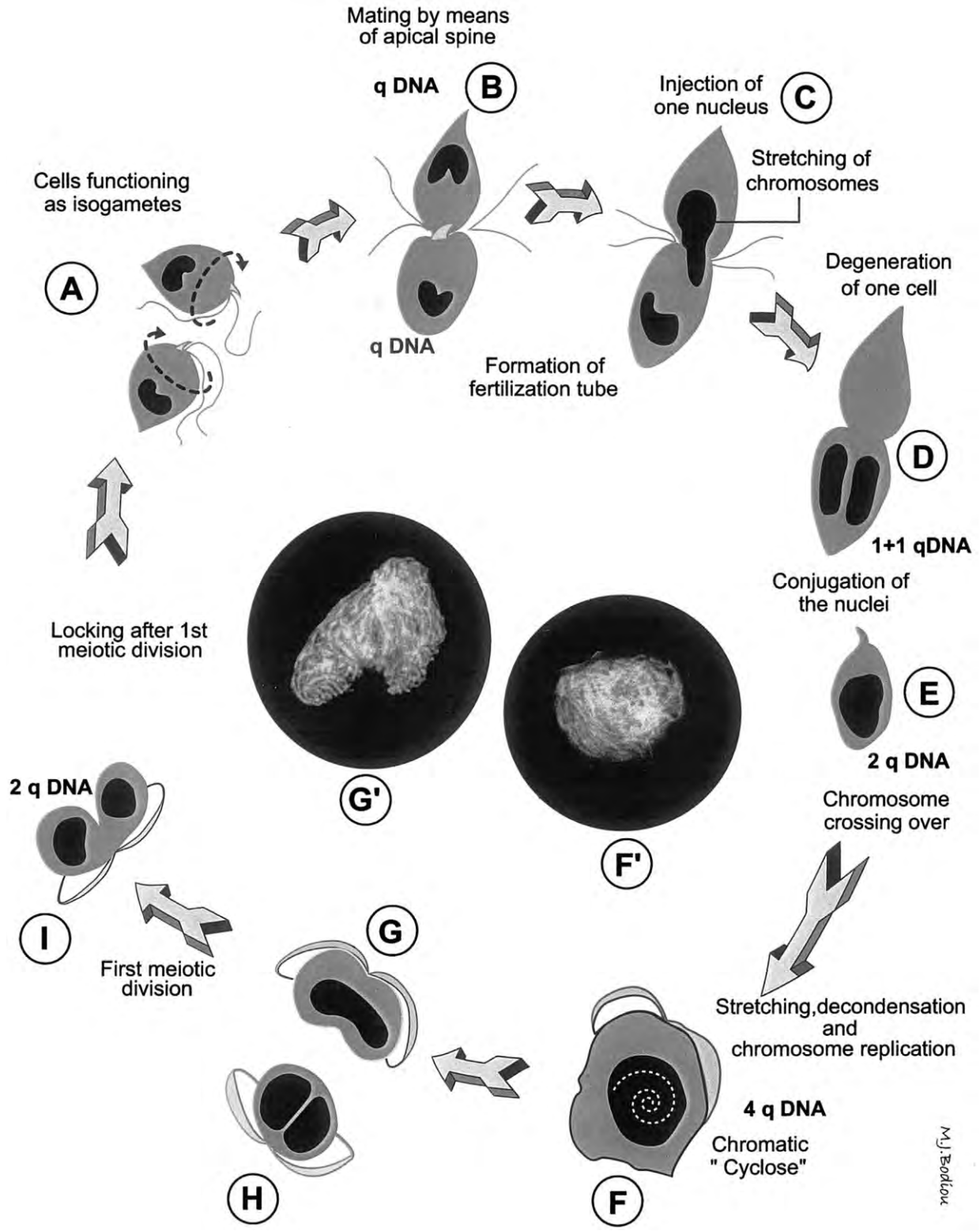

Fig. 12 (A)–(I) Diagram of the partial course of sexual reproduction phases of the dinoflagellate *Prorocentrum micans* from *in vivo* and TEM observations of the nuclei after exposure of the cultured cells to low temperature (4°C) and darkness. Vegetative cells functioning as isogametes (A) and containing *q* DNA become paired by means of their respective apical spines (B), then the donor cell injects its nucleus with stretched chromosomes into the receiver cell (C). After the two nuclei conjugate (D) in the zygote containing 2*q* DNA (E), crossing over of the chromosomes occurs. Chromosomes become completely unwound, stretched, and replicated. In the nucleus containing therefore 4*q* DNA (F), chromatin begins to spin round (chromatic cyclose) giving a round shape to the nucleus as shown in the photograph (F′). Only one meiotic division occurred (G, G′, H), leading to an incomplete separation of the 2*q* DNA-containing cells (I). Reproduced from Bhaud, Y., Soyer-Gobillard, M.O., Salmon, J.M., 1988. Transmission of gametic nuclei through a fertilization tube during mating in a primitive dinoflagellate, *Prorocentrum micans* Ehr. Journal of Cell Science 89, 197–206. Soyer-Gobillard, M.O., Bhaud, Y., Saint-Hilaire, D., 2002. New data on mating in an autotrophic dinoflagellate *Prorocentrum micans* Ehrenberg. Vie Milieu 52, 167–175. By copyright permission from Vie Milieu Life and Environment.

Table 1 Some properties of sexual reproduction in 25 dinoflagellate species

Species	F or M[a]	Different from vegetativ cells[b]	Iso (+) aniso(−) gamy	Naked (−) or thecated (+)	Fertilizat tube[c]	Protoplas fusion[d]	Planozyg[e] (nb. flagellae)	Hypnozy[f]	Authors
Peridinium cinctum	F	+	−	−	?	+	+(2)	+	Pfiester (1975)
idem	F	+	−	−+	+	+	+(4)	+	Spector et al. (1981)
Peridinium willei	F	+	−	−	−	+	+(2)	+	Pfiester (1976)
Peridinium gatunense	F	+	−	+	+	+	+(?)	+	Pfiester (1977)
Peridinium volzii	F	+	−	−+	+	+	+(?)	+	Pfiester and Skvarla (1979)
Peridinium limbatum	F	+	−	+	−	+	+(?)	+	Pfiester and Skvarla (1980)
Peridinium inconspicuum	F	+	−	+	?	+	+(?)	+−	Pfiester et al. (1984)
Peridinium cunningtonii	F	+	−	−+	−	+	+(?)	+	Sako et al. (1984)
Gymnodinium excavatum	F	+	−	?	?	?	+(?)		Von Stosch (1972)
Gymnodinium paradoxum	F	+	−	?	?	?	+(?)	+	Von Stosch (1972)
Gy. pseudo palustre	F	+	−	?	+	+	+(?)	+	Von Stosch (1973)
Amphidinium carteri	M	−	−	?	+	+	+(?)	+	Cao Vien (1967, 1968)
Ceratium cornutum	F	+	+	+	+[g]	+[h]	+(?)	+	Von Stosch (1972)
Ceratium horridum	M	+	+	+	+[g]	+[h]	+(?)	+	Von Stosch (1979)
Oxyrrhis marina	M	+	−	?	?	?	+(?)	?	Von Stosch (1972)
Crypthecodinium cohnii	M	+	+−	?	?	+	+(?)	+	Tuttle and Loeblich (1975)
idem	M	?	+−	+	+	+	+(?)	+	Pfiester (1984)
Gonyaulax tamarensis	M	+	+	?	−	+	+(4)	+	Turpin et al. (1978)
idem	M	+	?	?	?	?	+(?)	+	Anderson and Lindquist (1985)
Go. excavata	M	?	?	?	?	?	?	+	Anderson and Wall (1978)
Go. monilata	M	+	−	+	−	+	+(4)	+	Walker and Steidinger (1979)
Woloszynskia apiculata	F	+	−	+	+	+	+(4)	+	Von Stosch (1973)
Protogonyaulax catenella	M	+	−	+	−	+	+(?)	+	Yoshima (1981)
Gymnodinium breve	M	+	−	+	−	+	+(4)	−[i]	Walker (1982)
Gyrodiniumun catenatum	M	+	+	+	+	+	+(4)	+	Coats et al. (1984)
Helgolandicum subglobosum	M	+	+	?	?	?	+(?)	−	Von Stosch (1972)
Noctiluca miliaris	M	+	−	−	−	+	+(0)	?	Zingmark (1970)
Prorocentrum micans	M	−	−	+	+	−	+(2)	−	Bhaud and Soyer-Gobillard (1988)

Note: Bhaud, Y., Soyer-Gobillard, M.O., Salmon, J.M., 1988. Transmission of gametic nuclei through a fertilization tube during mating in a primitive dinoflagellate, *Prorocentrum micans* Ehr. Journal of Cell Science 89, 197–206. With permission of the Company of Biologists Limited.

[a]F, freshwater; M, marine.

[b]Differences observed between vegetative cells and gametes are often morphological variations such as variations in cell volume and color; gametes are generally smaller and less pigmented than vegetative cells; +, different; −, the same.

[c]Fertilization tube or similar structure: +, present; −, absent.

[d]Protoplasmic fusion of the two gametes: +, fusion; −, no fusion.

[e]Planozygote: +, present; −, absent.

[f]Hypnozygote: +, present; −, absent.

[g]The female engulfs the male.

[h]Protoplasm from female and male is misled.

[i]No hypnozygote in the culture.

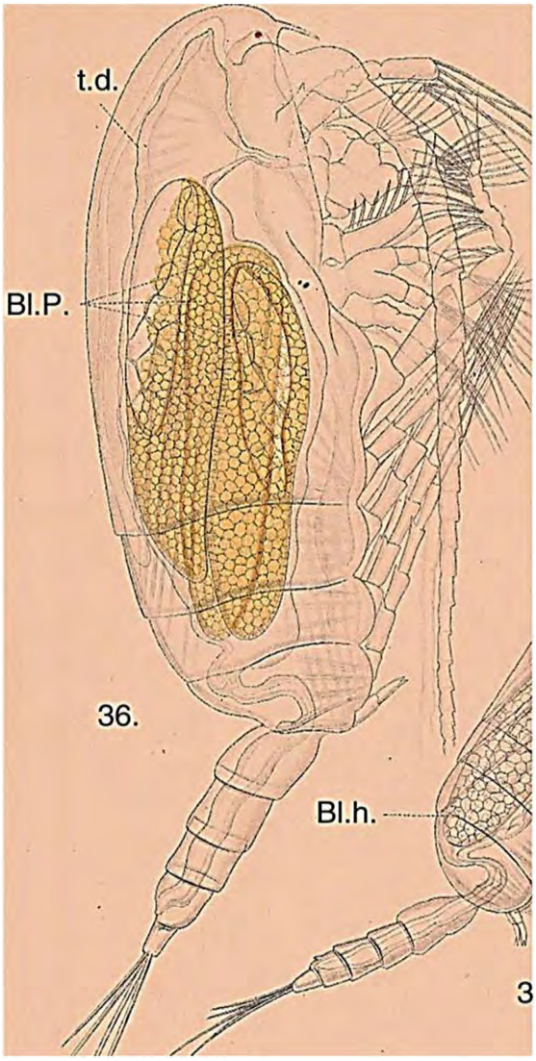

Fig. 13 A pelagic copepod (*Clausocalanus arcuicornis*) in which stomach is distended by three *Blastodinium pruvoti* Chatton. Total length is 1.3 mm. t.d., digestive tract. From an original plate (Pl. IV) of the doctoral Chatton's thesis (1920) Archives de Zoologie Expérimentale et Générale, 1920, 59, 1–475. Journal not yet published. *E. Chatton del.*

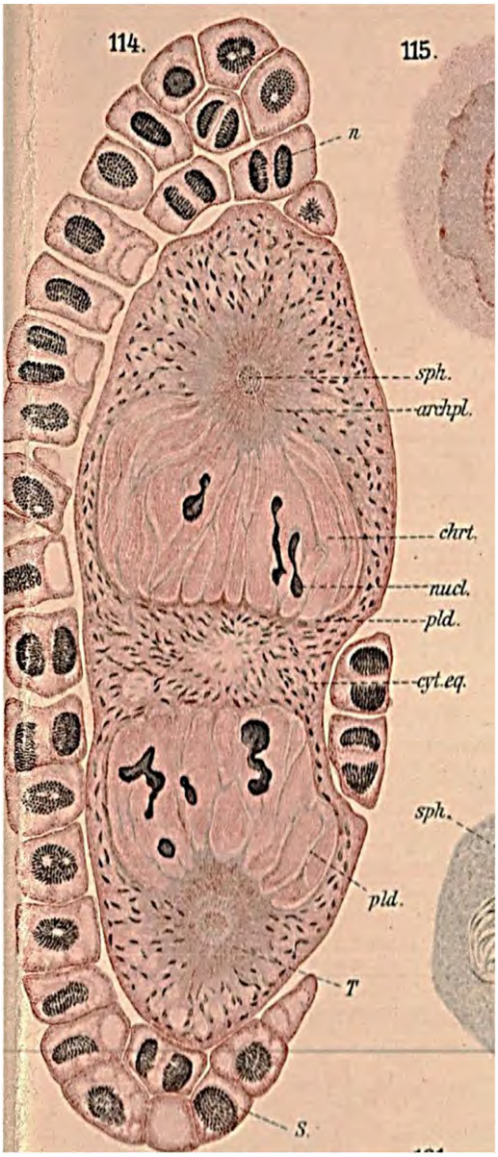

Fig. 14 Cytology of *Blastodinium crassum* Chatton, a parasitic and mixotrophic dinoflagellate living in the digestive tract of pelagic copepods (*Clausocalanus arcuicornis*). Longitudinal section of the vegetative binucleated cell, the depicted 'trophocyte' (T) containing no functional etioplastids, surrounded by several layers of numerous dividing sporocytes (s) containing functional plastids. The centrosome region-like or 'centro spheres' (sph) is surrounded by archoplasm, specialized cytoplasm containing Golgi bodies. chrt.,chromatin; chrs, chromosomes; nucl., nucleolus; pld, plasmodendrites, cytoplasmic channels. Length of the trophocyte is 300 μm. From an original plate (Pl.X) of the doctoral Chatton's thesis (1920) Archives de Zoologie Expérimentale et Générale 1920, 59, 1–475. Journal not yet published. *Chatton del.*

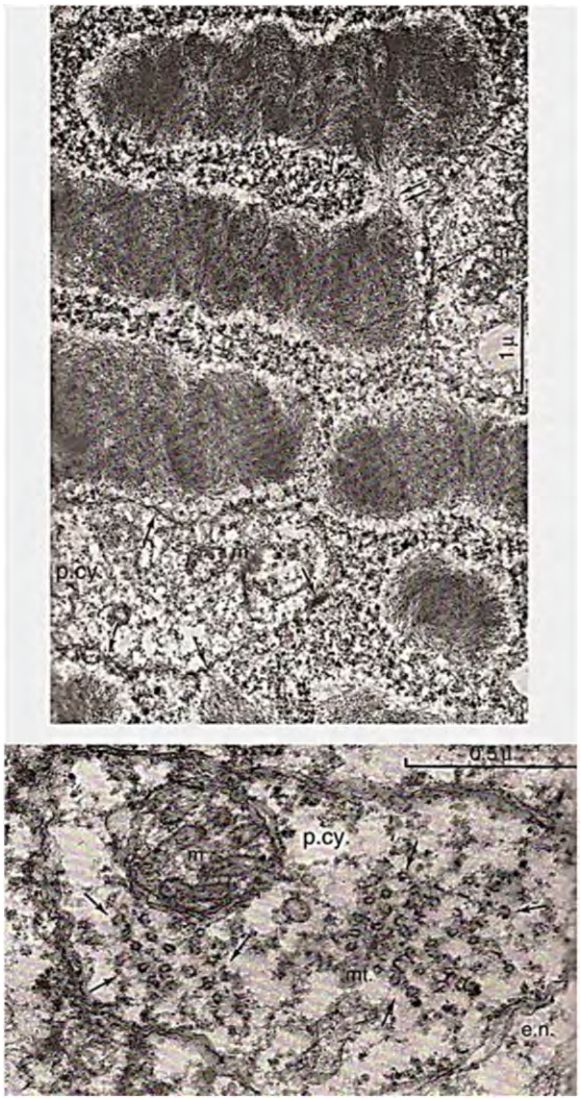

Fig. 15 Part of a *Blastodinium contortum* Chatton dividing nucleus. Several chromosomes are attached to the nuclear envelope (arrows) and two dividing chromosomes are yet linked by DNA fibers (double arrow; upper panel). Detail of a transversally sectioned cytoplasmic channel (p.cy.) passing through the nucleus. Numerous microtubules (mt.) of the mitotic spindle and mitochondriae (m) are visible (lower panel). Reproduced from Soyer-Gobillard, M.O., 1971. Structure du noyau des *Blastodinium* (dinoflagellés parasites). Division et condensation chromatique. Chromosoma 33, 70–114. By copyright permission from Springer-Verlag.

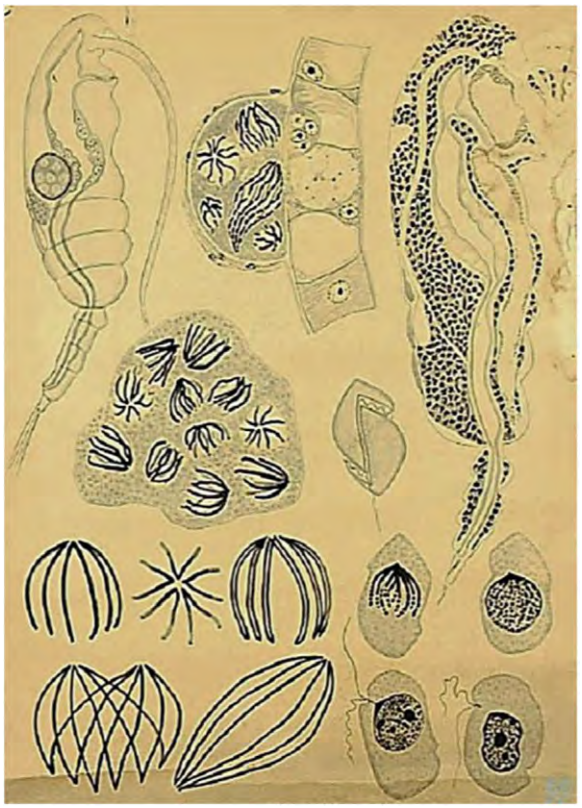

Fig. 16 Course board (1.60 m by 1 m) drawn by Edouard Chatton (1930). Chatton discovered the genus *Syndinium* Chatton in 1910 in the sea, close to Banyuls-sur-mer (France). Detail of the syndinian mitosis: *Syndinium turbo* is a plasmodial coelomic and parasitic dinoflagellate living in the general cavity of pelagic copepods (*Paracalanus parvus, Clausocalanus arcuicornis*, or *Corycoeus venustus*). During metaphase, the five chromosomes of *S. turbo* congress to an equatorial plate before anaphase segregation. The motile bi-undulipodiated zoospores, later released, are of gymnodinian (free-living dinoflagellate) type. Note at the lower right the release from the coelomic mass of the newly formed karyomastigont during zoospore maturation. At the lower right, the characteristic signature of Edouard Chatton (E and C interlaced). © Archives of the National Museum of Natural History, Paris (Donation of the Dr Marie-Odile SOYER-GOBILLARD, Bequest LWOFF).

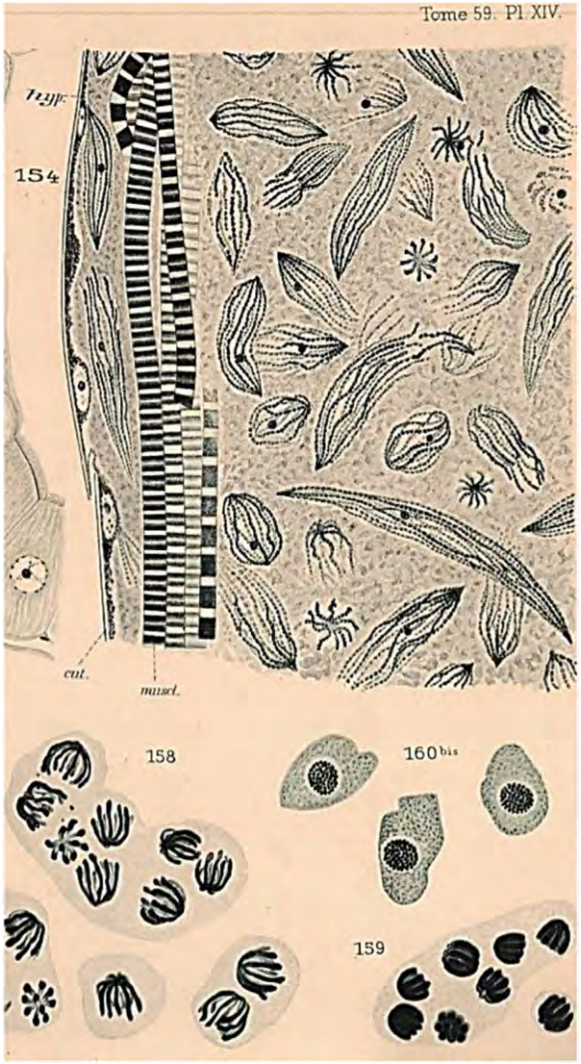

Fig. 17 Detail of *Syndinium turbo* Chatton cytology showing at the top of the draw many nuclei with 5 chromosomes in mitosis. The striated bands are the abdominal muscles of the Copepod host as seen in longitudinal section. At the bottom of the draw, nuclei with condensed chromosomes are represented and prespores before their release in the marine environment. From an original plate (Pl.XIV) of the doctoral Chatton's thesis (1920) Archives de Zoologie Expérimentale et Générale 1920, 59, 1–475. Journal not yet published. *Chatton del.*

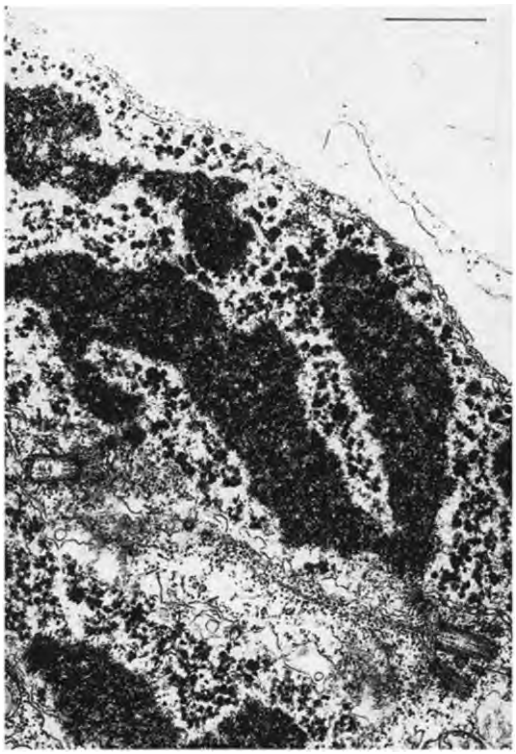

Fig. 18 Ultrathin section through a nucleus of *Syndinium* sp. in an intermediate stage of division. The central microtubular spindle lies in a central channel and is linked with two pairs of centrioles. The 'V' shape of one of the chromosomes is clearly visible in this micrograph (as previously shown by Chatton, see Fig. 15). Scale bar = 1 μm. Reproduced from Ris, H., Kubai, D.F., 1974. An unusual mitotic mechanism in the parasitic protozoan *Syndinium* sp. Journal of Cell Biology 60, 702–720. By copyright permission from The Rockefeller University Press.

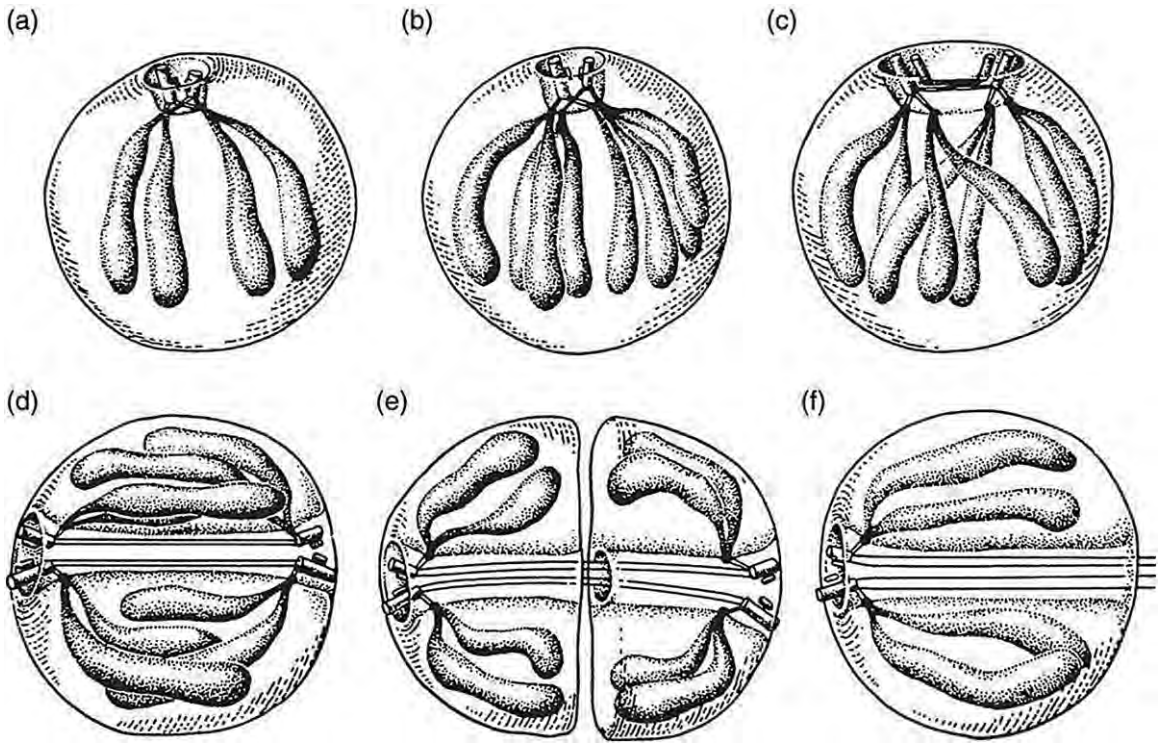

Fig. 19 Diagrammatic representation of nuclear division in *Syndinium* sp. (a) Interphase. (b) Early division, kinetochores and chromosomes have duplicated. (c) Early stage of chromosome segregation. Central spindle between separating centrioles. (d) Late stage of chromosome segregation. Central spindle in cytoplasmic channel throughout the nucleus. (e) Division of nucleus. (f) Early daughter nucleus with persisting channel and microtubules. Reproduced from Ris, H., Kubai, D.F., 1974. An unusual mitotic mechanism in the parasitic protozoan *Syndinium* sp. Journal of Cell Biology 60, 702–720. By copyright permission from The Rockefeller University Press.

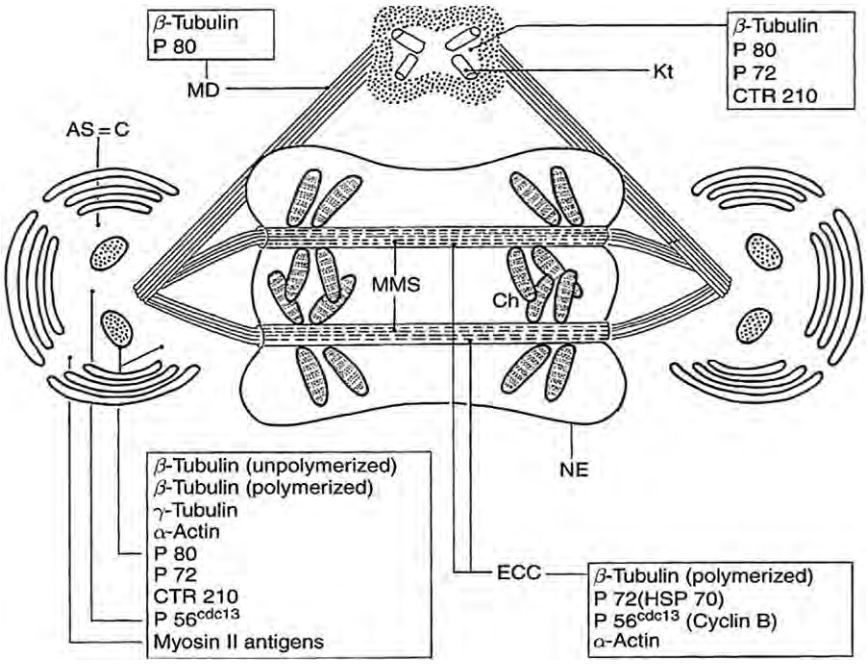

Fig. 20 Diagrammatic representation of a dinoflagellate (except *Syndinium*) mitotic apparatus schematized in anaphase with listed and framed the up-to-now detected associated proteins. Microtubular mitotic spindles lie throughout the nucleus, pass into the archoplasmic spheres, and are linked to the two pairs of kinetosomes. AS, archoplasmic sphere (containing Golgi bodies); C, centrosome (without any centriole); ECC, extranuclear cytoplasmic channel; MMS, microtubular mitotic spindle; MD, microtubular desmose; Kt, kinetosomes; N, nucleus; NE, nuclear envelope (permanent); Ch, chromosomes. Reproduced from Ausseil, J., Soyer-Gobillard, M.O., Géraud, M.L., 2000. Dinoflagellate centrosome: Associated proteins old and new. European Journal of Protistology 36, 1–19. By copyright permission from Elsevier publisher.

Conclusions

The first evidence of dinoflagellates in the fossil record is the biogeochemical analyses of early Cambrian sediments (*c.*520 million years ago), which detect the presence of dinoflagellate-specific dinosterols. Despite the great diversity of dinoflagellates in terms of their physiology, lifestyle, and cell cycle, they are remarkably similar in their mitosis except for *Syndinium* spp. and *Oxyrrhis marina.* The system of cytoplasmic channels passing through the intact nucleus indicates that microtubules are never in direct contact with the chromosomes but are always separated from them by the persistent nuclear envelope. Despite the absence of histones and nucleosomes in the chromatin of some species, dinoflagellates still have a typical eukaryotic cell cycle. According to Marinov and Lynch (2016), which have applied large-scale mRNA sequencing to 11 species of dinoflagellates, including strains of the syndinean genera *Hematodinium* and *Amoebophrya*, parasites of crustaceans and dinoflagellates, respectively, to optimize and update the dinoflagellate tree, we now know that dinoflagellates belong to the alveolates, together with apicomplexans and ciliates, and that it is highly probable that the loss of nucleosomes is a derived feature. Although an enormous amount of work have yet began about dinoflagellate chromatin, transcription, transcriptional and post-transcriptional regulation as see in Soyer-Gobillard and Dolan (2015), the mystery of dinoflagellate chromatin remains largely unresolved and there is still too little molecular data to sufficiently resolve relationships among the diverse dinoflagellates. Thus, morphological and cell biological analyses will continue to constitute a vital tool for dinoflagellate phylogeny (Fig. 21).

In addition to their fundamental interest for cell and evolutionary biologists, autotrophic dinoflagellates constitute part of the enormous energy potentially available from the phytoplankton. The heterotrophic *C. cohnii* is also intensively cultivated as a source of maternal milk due to the presence of long chain fatty acids in these organisms. Dinoflagellates are also important as symbionts of corals, where they play important role(s) in the health and survival of their hosts. The impact of toxic dinoflagellates such as species of *Pfisteria*, *Alexandrium*, *Gymnodinium*, *Dinophysis*, *Karenia*, and others on fish and shellfish industries is substantial and possibly is still significantly underestimated. Indeed, toxic incidents resulting from these different dinoflagellate species are being reported frequently throughout the world, particularly near the coasts. This increase of toxic blooms seems linked to the eutrophization of coasts and the spreading of species due to an increase in commercial exchanges. Thus, the importance of these species, which produce some of the most potent neurotoxins known to man, should not be underestimated.

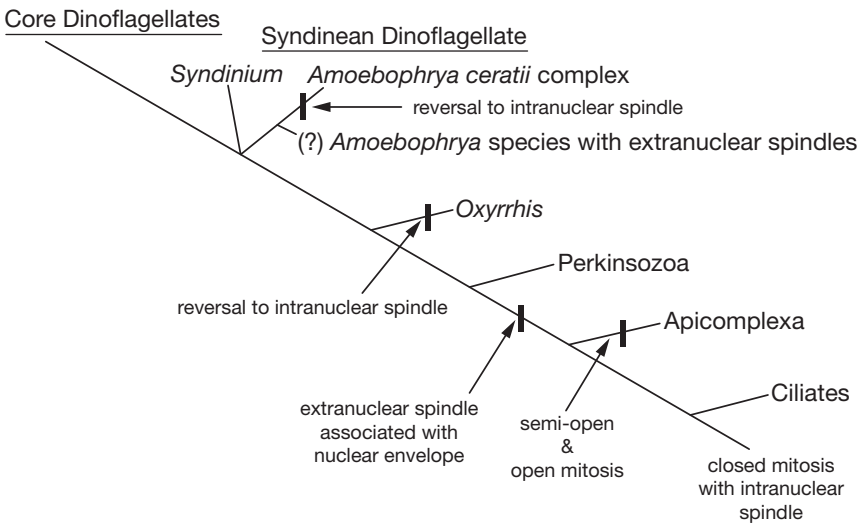

Fig. 21 Evolution of mitosis in Alveolates according to the recent phylogeny of Bachvaroff et al., 2014. From Moon, E., Nam, S.W., Shin, W., Park, M.G., Coats, D.W., 2015. Do all dinoflagellates have an extranuclear spindle? Protist 166, 569–584. With permission of Elsevier.

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Relevant Website

www.mcb.harvard.edu/—Department of Molecular and Cellular Biology, Harvard University.

Directed Evolution[☆]

L Gold and J Walker, SomaLogic Inc., Boulder, CO, United States; The University of Colorado at Boulder, Boulder, CO, United States

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Glossary

Aptamer From the Latin *aptus* meaning "to fit" and the Greek *mer* meaning "repeating unit." Aptamers are nucleic acid molecules (RNA or DNA) selected to bind to target molecules. The name was born in Jack Szostak's lab, namely Andy Ellington, who attributes it to friend Nina Tovish.

Chemically modified nucleotide A nucleotide that has been modified chemically to create a new chemical structure that is distinct from standard RNA and DNA nucleotides used by life. For example, 5-benzylaminocarbonyl2′-deoxyuridine or 2′-deoxyuridine modified at its 5-position with a benzyl group.

Directed evolution A synthetic process that harnesses the selection to evolve molecules, typically proteins or nucleic acids, from large, stochastically permuted pools or combinatorial libraries, to have desired properties.

In vitro evolution A general term that refers to a process or method of directed evolution, such as SELEX (Systematic Evolution of Ligands by EXponential amplification), that is carried out in test tubes as opposed to in vivo, or carried out in living systems like cells or organisms.

Selection In directed evolution, a method or process used to select or pick out molecules that have desired properties, such as binding to a target analyte.

SELEX Systematic Evolution of Ligands by EXponential amplification. A process of directed evolution by which molecules are evolved from stochastically permuted pools or combinatorial libraries using exponential amplification, such as polymerase chain reaction (PCR), to have desired properties.

SOMAmer reagents Slow Off-rate Modified Aptamer reagent. A single-stranded nucleic acid molecule that contains chemically modified nucleotides that is selected to have desired properties such as binding to a specific ligand, and has slow dissociation rates.

Evolution and the Central Dogma – DNA, RNA, Proteins

Francis Crick articulated the Central Dogma, and thus directed the next 50 years of research in molecular biology and genetics. While the edges of the Central Dogma have been discussed vigorously, one achieves substantial understanding of biology by keeping the Central Dogma clearly in mind.

The Central Dogma described information flow within the central macromolecules of life – DNA, RNA, and proteins. Single-stranded DNA was easily converted to its complementary strand, and thus DNA could exist as a double-stranded helix or as either single strand without (except for mutations during the replication events) losses or changes in the information encoded by the sequence of nucleotides in DNA. Similarly, transcription (from DNA to RNA) is an accurate process that requires nothing more than accurate reading of DNA by the RNA polymerases to make RNA molecules whose nucleotide sequences reflect the sequences of nucleotides in DNA. The process of translation is also an accurate process, using a complex machine called the ribosome, through which RNA sequences are read, three nucleotides at a time, into the amino acids that comprise a protein. Famously, Crick understood that nucleotide complementarity made all interconversions between DNA and RNA rather simple, while protein sequences could not (in biochemical terms) be read "backward" into nucleic acid sequences. The Central Dogma is discussed fully in the encyclopedia.

The Central Dogma and "Undirected" Evolution

Evolution operates at the level of the Central Dogma, over long time periods. Creatures speciate and evolve (ie, restrict their mating events to others of the same kind) through a slow process related to the multiplication time for that creature (an hour for many bacteria, years for many mammals) and the mutation rate for that creature (ie, the number of nucleotide changes – of any kind – that occurs stochastically to that creature's genome during each multiplication event). Because of the Central Dogma, changes in the genome (most frequently DNA, although some viruses use RNA, is their genomic substance) lead to changes in RNA sequences and protein sequences. The process of natural evolution occurs over long time periods and is undirected, most of the time. That is, mutations arise in the genomes of creatures and they can be harmful, beneficial, or neutral, but the underlying chemical process (changes in the genome) are almost always random.

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A classic study of undirected evolution was the extraordinary work on the bacteriophage T4 rII cistrons by Seymour Benzer and by Francis Crick and Sydney Brenner (and, of course, their many colleagues). In many ways, the apogee of modern classic genetics were the studies of the rII cistrons, which led to understanding of the mechanisms of mutagenesis as well as an understanding that the genetic code was read during translation at three nucleotides (a codon) per amino acid.

Breeders of all kinds have tried to enhance the rates of evolution toward desired phenotypes. Tulips, corn, chickens, dogs, cats, race horses, and laboratory strains of mice have been improved through natural evolution; breeders identify a trait of interest and select (historically, by choosing from a group of progeny the ones that seem to have the desired trait) those variants for further mating, and thus screen and isolate natural changes in the genomes of those creatures under study. Because genomes tend to "breed true," this process has been successful for a long time. ("Breed true" is of course a quantitative term – some creatures, like humans, have low intrinsic mutation rates (perhaps one mutation per generation per 10^9 nucleotides), while viruses often replicate with high error rates (perhaps one mutation per generation per 10^3 nucleotides).) Breeding is thus similar to natural (undirected) evolution, except that it is guided by a predetermined human desire to achieve a particular evolved quality. The essential element of natural undirected evolution is time, largely due to three facts: "breeders" are historically operated from visual clues; the intrinsic mutations rates – at the genomic nucleotide level – are low; and, often, the "growing season" for the creature of interest is brief.

Molecular biologists made possible faster undirected evolution by enhancing the mutation rate at the sites on genomes which were thought to be involved in a process of interest. This work, which was started in the mid-1970s (because DNA synthesis in the lab became widely available), was enabled by the ability to both prepare nucleic acids and replace genes in organisms. With those tools available, scientists were able to "hammer" a region of a genome with heavy mutagenesis to allow a large pool of interesting genomic variants to be screened for a desired trait. In some cases, depending on the creature under study, heavy mutagenesis of a region of a genome was followed by selection of a desired phenotype rather than merely looking for that phenotype. This kind of work was common and was most frequently done in bacteria because gene replacement protocols were powerful for many species of bacteria. The metaphor for such an experiment is simple to imagine: if a bacterial species is unable to grow on some sugar (but is able to grow on many different sugars), one might do heavy mutagenesis of the genes responsible for using sugar #1 as a carbon source and transform the bacteria with copies of the mutated DNA, and then selecting for bacteria that can now grow on the previously unacceptable sugar. This simple paradigm works because of the Central Dogma – hammered genes are transcribed and translated into proteins whose sugar preferences might have been changed by sequence changes in the "sugar utilization" proteins.

One sees in this simple example that "faster undirected evolution with selection" approaches "directed evolution" – the differences in the end are quantitative rather than qualitative. Natural undirected evolution in bacteria, while slow, is rather extensive. We estimate that "every letter of DNA" in every bacterial species on Earth has been altered some 100,000,000 times (kind of like a metronome – first a G, then an A, then a G, then a C, then a T, and so on, 100,000,000 times) over the evolutionary history of those bacterial species (some three billion years), and thus natural undirected evolution in bacteria is complete for short stretches of their genomes. "Hammered" is an appropriate word for what has happened to bacterial genomes over time, and it is likely that the bacteria have been optimized for many physiological functions. Scientists cannot plan and execute experiments over billions of years, and thus directed evolution has emerged as the preferred way to do exhaustive genetics for a problem of interest.

The Central Dogma and "Directed" Evolution Through Selections In Vitro

High mutation rates through chemistry of DNA synthesis (above) led inexorably to directed evolution. The deep mutagenesis of DNA is a first requirement. Imagine chemical synthesis of a DNA molecule, from one end to the other, using the modern chemistry that makes such synthesis possible – one starts from one end and places into the reaction vessel first one monomer (or letter), then the second, then the third, and so on. Modern machines can easily make single-stranded DNA or RNA molecules of length 100 or more. One can put in as substrates at each step of the synthesis one activated nucleotide (ie, one letter of DNA – an A, C, G, or T) or one could, at any position, put in all four activated nucleotides and allow stochastic additions to the growing chain of any of the four nucleotides. This idea – deep mutagenesis – was powerful.

The first example of a large in vitro evolution experiment was published in 1989 by Arnold Oliphant and Kevin Struhl. The experiment they did was elegant and simple. They knew that a yeast protein (named GCN4) bound (as a transcription factor) to double-stranded DNA in a sequence-specific manner (the biology does not matter for this example). Oliphant and Struhl synthesized double-stranded DNA (how they did this is also unimportant for this discussion) containing random sequences 25 nucleotides long. That is, since they were studying 25 positions in double-stranded DNA, each containing only four letters, the total number of sequence possibilities was 4^{25}, or approximately 10^{15}. Oliphant and Struhl then asked which members of the huge combinatorial library would bind to the protein GCN4, and found a repeated sequence that allowed that binding. In principle, because of the scale of the initial synthesis, every sequence of 25 nucleotides was in their starting library many times. This first experiment was aimed at understanding a natural double-stranded DNA-binding sequence, and the experiment worked powerfully.

Directed evolution has also been aimed at proteins. Again, because the Central Dogma allows combinatorial libraries of DNA sequences to be transcribed and translated into proteins, one has access to large libraries of protein sequences. Libraries of around 10^9 protein sequences can be made and studied using in vivo methods, while larger libraries (up to 10^{15} protein sequences) can be studied in vitro. Phage display is one common way to prepare and select protein and peptide variants.

Directed evolution has been aimed at single-stranded nucleic acids through the in vitro methodologies described as SELEX (Systematic Evolution of Ligands by EXponential enrichment). Tuerk and Gold understood in 1990 that single-stranded

combinatorial oligonucleotide libraries contained large numbers of molecules with properties that went beyond linear sequence space – a "tapes versus shapes" or "strings versus things" discussion. It was of course known that many single-stranded RNAs in biology have "globular" structures – the transfer RNAs were the first to be studied by X-ray crystallography (in 1974) and shown to have an extraordinary shape. The power of SELEX lays in the vast numbers of molecules from an initial library that could be screened for a particular property. Similar selection methods also led to novel ribozymes – single-stranded RNA molecules with catalytic properties – and even novel DNAzymes. Both phage display for proteins and SELEX for oligonucleotides moved toward chemistry – that is, the vast potential of directed evolution made the resulting molecules of interest (for new drugs, diagnostics, and nanomaterials) outside of the basic science questions first studied by these technologies. The molecules that were identified through SELEX were named "aptamers" by Ellington and Szostak.

In the past few decades, much emphasis has been aimed at the use of modified nucleotides to make the resulting aptamers more potent and useful for the biotech and pharma industries. The SELEX protocols include in vitro synthesis and selection of a desired oligonucleotide, and thus, one is not limited to the nucleotides found in biology. Novel aptamer reagents, called SOMAmer (Slow Off-rate Modified Aptamer) reagents, have been identified as binding reagents for more than 5000 human proteins – with more to come (there are more than 20,000 proteins in a human). The special chemistries of modified nucleotides in a short single-stranded molecule provide unique binding qualities, elucidated now in a series of four published co-crystal structures of SOMAmer reagents binding their target proteins.

The Future of Directed Evolution

The scientific, research, and medical communities have been surprised by the emergence of ribozymes/aptamers/SOMAmers as molecules with diverse capabilities. Like monoclonal antibodies, such molecules have the potential to be therapeutics or key reagents for diagnostic purposes (either in vivo, for imaging, or in vitro, for protein measurements). Directed evolution today is limited only by one's imagination; vast combinatorial libraries can be made with unusual chemistries and special molecules can be selected from those libraries. That is, directed evolution can be directed toward many molecular properties that have not yet been sought. Novel successful selections will encourage more thinking about chemical evolution on the early earth, and widen our appreciation for the transition from a chemical earth to a self-replicating chemical earth to a biological earth, the transition that allowed undirected evolution to create numerous creatures with special adoptions to the niches in which they live. Directed and undirected evolution will inform each other in the decades ahead.

Further Reading

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DNA Cloning Strategies☆

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Glossary

cDNA Complementary DNA is a DNA copy of mRNA made by the enzyme reverse transcriptase.
Cloning The process of inserting foreign DNA into a vector. Recombinant DNA vectors are typically amplified in an *E. coli* host.
Ligation The enzymatic formation of a covalent phosphodiester bond between two nucleic acid molecules by DNA ligase.
multiple cloning sites (MCS) Short sequences engineered in vectors that contain a number of unique restriction sites for cloning DNA.
polymerase chain reaction (PCR) The enzymatic replication and amplification of DNA *in vitro*. Reactions are done in a thermal cycler with a thermoresistant DNA polymerase.
restriction enzymes Proteins or endonucleases that cleave phosphodiester bonds in DNA at specific sequences.
Screen The process of visually inspecting mutant or transformed organism for altered phenotypes. In cloning, blue/white screens are commonly employed to identify clones with insert DNA.
Selection The process of forcing a cell to contain a desired phenotype and genotype. For example, plasmid uptake by bacteria can be selected for by antibiotic resistance.
synthetic DNA The ability to chemically make DNA *de nova* in the absence of a template or a polymerase.
Transformation The process of introducing new or recombinant DNA into an organism. Some bacteria are naturally transformable. Most bacteria require artificial methods, chemical or high–voltage (electroporation), to compromise cell permeability for DNA uptake.
Vectors DNA vehicles that allow foreign DNA to be combined in new ways and amplified. Vectors encode *cis* elements to initiate DNA replication (*ori*), site to insert foreign DNA (MCS) and contain a selectable marker, such as antibiotic resistance. Vector examples include plasmids, cosmids, BACs and YACs.

Cloning Strategies

At the heart of recombinant DNA technology is how different molecules are joined or ligated together. Specifically, the nature of the 5′ and 3′ ends of each DNA molecule is important for covalent bond formation. DNA ends can be mechanically created by shearing or sonication forces. Such techniques create random breaks in DNA that are useful for cloning genomic libraries. Physical forces break different chemical bonds and produce heterogeneous or damaged ends. To allow efficient cloning these ends are enzymatically repaired with DNA polymerase or exonuclease treatment to produce 'polished' blunt ends. Alternatively linear DNA molecules are generated with precise ends by PCR or restriction digestion. In most cases the underling cloning strategy is relatively simple, but in practice there are numerous steps and the process can be difficult. Thus when designing and conducting a cloning experiment attention to detail is essential for success.

PCR Based Cloning

There are many ways to clone DNA. Over the past two decades, great strides have been made in sequencing whole genomes. As a result investigators frequently know the sequence of their target DNA for cloning. This information is invaluable and allows one to easily amplify target DNA by PCR (Figure 1). A power of PCR cloning is that the DNA or gene of interest (GOI) can be directly and precisely amplified from biological material or cell cultures. One does not necessarily need a pre-existing clone or even purified genomic DNA material, though such preparations do facilitate PCR amplification. As shown, to amplify the GOI two primers are designed that specifically hybridize with template DNA. Primers are routinely synthesized at research institutions or are readily available through commercial venders at an economical price. An additional advantage of PCR cloning is that desired DNA sequences can be engineered into primer sequences. For example, to facilitate cloning primers are designed with unique restriction sites in their 5′ tails that do not interfere with priming at the 3′ end (Figure 1). After amplification the PCR products contain incorporated restriction sites. To clone the PCR products they are digested with restriction enzymes. Plasmid DNA is digested with the same enzymes producing complementary sticky ends that can anneal by forming hydrogen bonds with insert DNA. These two DNA fragments are covalently bonded with DNA ligase. The ligated plasmids are transformed into an *E. coli* host by selecting resistance to the antibiotic kanamycin. Transformant clones are screened to identify clones with the correct insert DNA.

☆*Change History:* August 2014. D Wall introduced small edits in the text of the article including citations, added the sections "Applications" and "References"and added Figure 6

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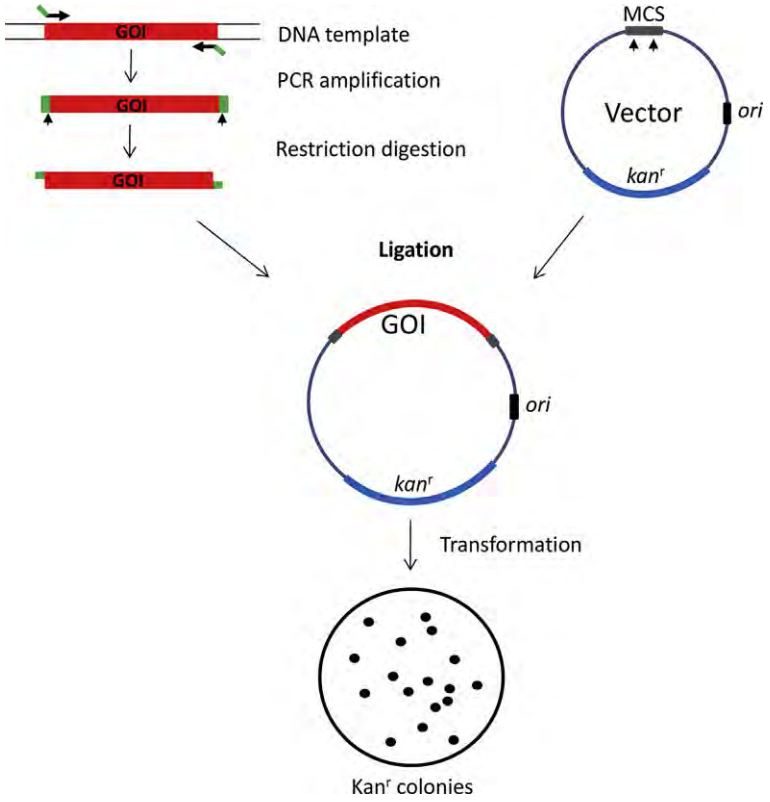

Figure 1 PCR cloning strategy. Two primers are designed to specifically amplify the 5' and 3' end of a gene of interest (GOI). These primers include restriction sites (green) engineered in the 5' tails. The vector DNA is digested with the same enzymes within the MCS (arrowhead). The DNA is ligated generating a recombinant plasmid that is transformed into *E. coli* selecting Kan^r.

A number of technologies have simplified and reduced the time it takes to construct recombinant clones. Central to this development is PCR and the rapid pace of genome sequencing. Consequently a key part on cloning involves primer design. Primer design essentially has two parts. First, primers are optimized for robust and specific amplification of the target DNA. Key parameters to consider are primer length, melting temperature, avoiding secondary structure within and between primers and ensuring the 3' end is specific toward target DNA. Primers can be manually design, but commercial or web-based computer algorithms work better and faster. In some cases there are few choices in design because a primer needs to start at specific sequence, e.g. beginning or end of a gene. The second factor to consider is engineered sequences. For example, a particular mutation can be incorporated into a primer. Alternatively, restriction sites or epitope tags are included. In other cases degenerative primers might be sought to amplify gene homologs. In sum, the ability to engineer any sequence into a primer provides tremendous latitude to make unique recombinant DNA molecules and to simplify complex cloning steps.

Investigators that rarely employ or want to shorten cloning steps may consider a number of products that simplify the process. One technology is called TOPO cloning (Life Technologies). The advantage of TOPO cloning is that PCR products are efficiently and directly cloned into commercially prepared vector reaction that is ready for ligation. Here, the linearized vector has been prepared with Vaccinia virus topoisomerase I covalently attached to each 3' end. Addition of insert DNA with free 5' ends allows rapid ligation to the vector catalyzed by topoisomerase I. This technology has also exploited the unique activity that thermophilic *Taq* DNA polymerase adds a single deoxyadenosine (A) to the 3' ends of PCR products. Thus some of the TOPO cloning kits include linearized plasmid DNA that has complementary 3' deoxythymidine (T) overhangs covalently bonded to topoisomerase I. Other TOPO vectors are prepared for blunt end cloning. Significantly, neither the PCR product nor the plasmid needs to be restricted or purified for ligation. Instead material directly from the PCR reaction is mixed with prepared TOPO vector. The ligation is completed in 5 minutes and is ready for transformation. TOPO vectors are available in a variety of configurations. Thus in many cases an ideal vector is found and eliminates subsequent cloning to make the desire construct.

Restriction Endonucleases

Restriction endonucleases are enzymes that recognize specific DNA sequences for cleavage. These enzymes have formed the backbone of recombinant DNA technology. Stanley Cohen, Stanford University, and Herbert Boyer, University of California, San Francisco, and colleagues first published a cloning experiment in 1973. Here they used *Eco*RI to digest two plasmids, pSC101 and

pSC102, to form a new recombinant plasmid that contained the tetracycline and kanamycin resistant markers from each parent plasmid. Today investigators no longer need to purify their own restrictions enzymes, but instead chose from around 300 commercially available endonucleases. Most restriction enzymes used for molecular cloning are Type II that cleave within or adjacent to the recognition sequence. Type I and Type III restriction enzymes, which also contain DNA modification activity, are rarely used for cloning. The names of restriction enzymes begin with the first letter of the genus followed by two letters from the species name from which they were isolated. This abbreviation is followed by numbers or letters that typically refer to more specific strain information or order of isolation. Some restriction enzymes also have a corresponding commercially available DNA methylases. These enzymes recognize the same DNA sequences as their cognate restriction enzyme and block DNA cleavage by methylation.

The length of DNA that restriction enzymes recognize varies, though most enzymes recognize 4 or 6 base pairs (bp). The frequency of tetranucleotide sites appear once every 256 bp (4^4), while a hexanucleotide site appears once every 4096 bp (4^6). The frequency of sites within the DNA is also influenced by G + C content. Most restriction sites have dyad symmetry: the double stranded sequence is identical after 180° rotation. The decision on what particular restriction enzymes to use partly depends on the size of DNA sought. To prepare a plasmid for cloning one typically uses a 6-cutter, since a 4-cutter would generate too many fragments. Along with generating smaller DNA fragments, 4-cutters are useful to generate a relatively random pool of larger DNA fragments by a partial digestion. The size of the DNA fragment pool is controlled by simply changing incubation times. For this reason genomic libraries are often constructed with 4-cutter enzymes. Other restriction enzymes recognize longer sequences, such as 8 bp, and consequently rarely cut DNA.

Another important distinction among restriction enzymes is the type of ends they produce. Some enzymes cleave each strand at similar locations of the opposite sides of the axis of symmetry, creating ends that carry protruding single-stranded cohesive or "sticky" termini. Depending on the restriction enzyme either 5′ or 3′ protruding ends are produced (Figure 2). Other enzymes cleave both strands exactly at the axis of symmetry, generating blunt ends, e.g. *Sma*I (Figure 3). All cleavage reactions produce 5′ phosphate and 3′ hydroxyl ends, both of which are required for ligase to produce a recombinant DNA molecule.

Figure 2 illustrates the details of how two DNA molecules are cut by the *Sal*I and *Xho*I restriction enzymes. Both enzymes recognize specific 6 bp sequences that have dyad symmetry. The digested DNA fragments contain 5′ protruding sticky ends. Although these restriction enzymes recognize different DNA sequences the core 4 bp are identical. Consequently the 4 bp 5′ protruding ends are the same and are annealed and ligated. Figure 2 shows the resulting 6 bp sequence that neither *Sal*I nor *Xho*I recognize, though the core 4 bp sequence remains and contains a *Taq*I 4-cutter recognition site. This is one on many examples of how different restriction enzymes can produces ends that are compatible for ligation. Such ligated ends may also produce new restriction sites for use in a subsequent cloning step.

Figure 3 outlines a more complex cloning scheme. Here, insert DNA was cleaved with *Eco*RI to generate 5′ protruding sticky ends, while vector DNA was cut with *Sma*I to generate blunt ends. In order to clone the insert DNA into the vector's *Sma*I site it must be blunted. 5′ overhangs can be blunted by a T4 DNA polymerase fill-in reaction (Figure 3) or by removal of overhangs with mung bean exonuclease. If the foreign DNA was instead digested with an enzyme that produced 3′ overhangs it would have to be blunted by an exonuclease (mung bean or T4 polymerase which contains 3′ to 5′ exonuclease activity). Since blunt end ligations do not

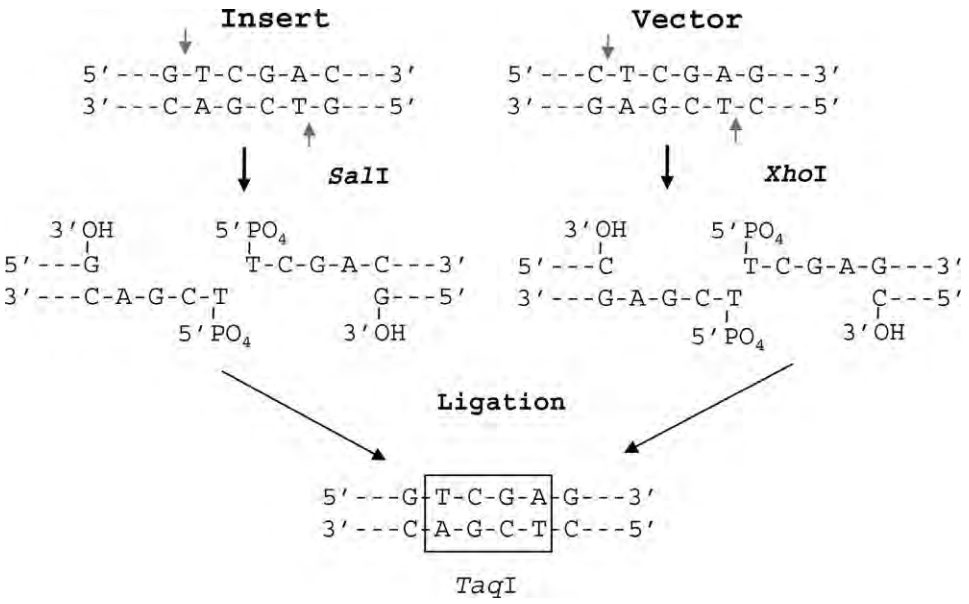

Figure 2 Restriction digestion and ligation. The restriction sites have dyad symmetry. Both digestions produce 5′ protruding ends that are identical and complementary for sticky end ligation. One end of the ligated product is shown and generates a new site that neither *Sal*I nor *Xho*I recognize, but does contain the site for the *Taq*I 4-cutter restriction enzyme (box).

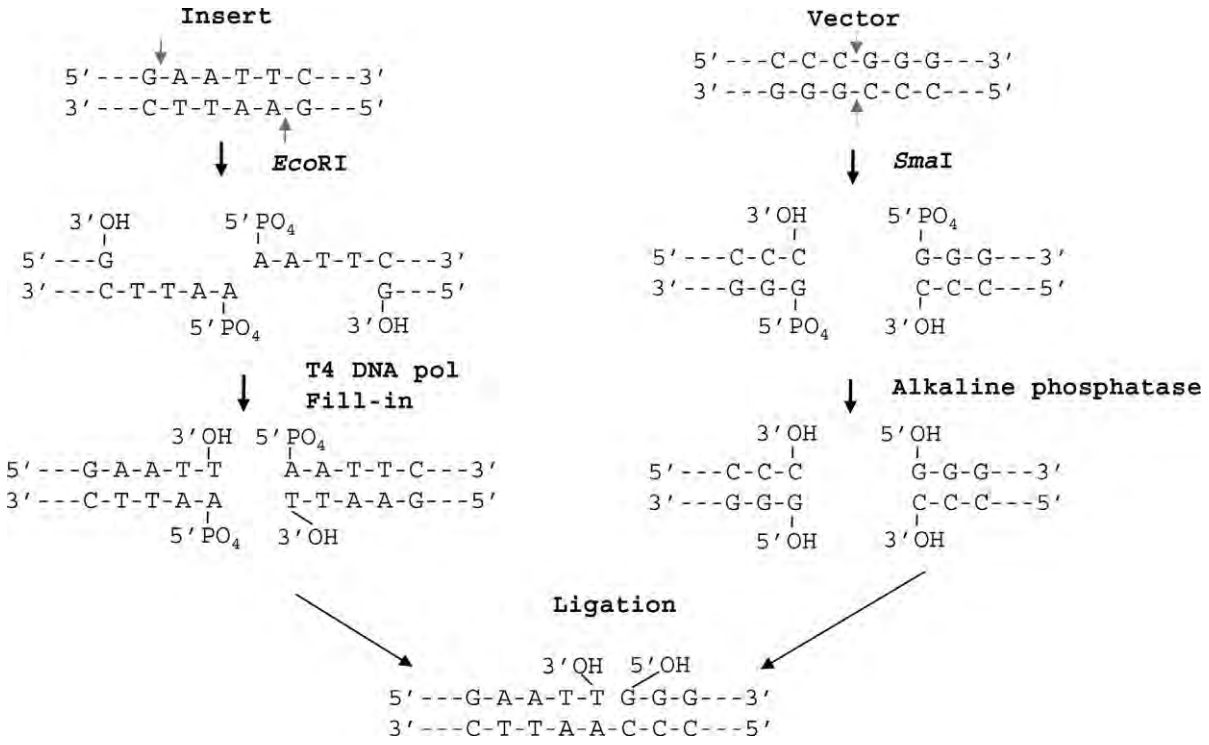

Figure 3 Outline of a blunt end cloning strategy. Insert DNA is digested with *Eco*RI which produces 5′ overhangs. T4 DNA polymerase treatment results in filled-in blunt ends. Vector DNA is cleaved with *Sma*I that produces blunt ends. To prevent vector self-ligation it is treated with alkaline phosphatase to remove 5′ phosphate groups. Only ends with 5′ phosphate and 3′ hydroxyl groups can ligate, resulting in a nicked ligation product.

contain annealed sticky ends they are less efficient. To improve the frequency of recovering ligation products with inserts the vector DNA is treated with alkaline phosphates to remove the 5′ end phosphates (Figure 3). Consequently the vector cannot self-ligate, however the vector can ligate to blunt end inserts that contain phosphorylated 5′ ends. Thus at each ligated end the DNA contains one ligated strand and one nicked strand (Figure 3). Once transformed into *E. coli* the host machinery will repair the DNA nicks. Though phosphatase treatment involves an extra step and reduces ligation efficiencies, the procedure helps to ensure the desired is obtained. Another strategy to prevent vector self-ligation is to cut vector and insert DNA with two different restriction enzymes that produce ends that are not compatible for annealing. This highly efficient cloning method forces the insert DNA to be directionally cloned, since the insert is only annealed in one orientation. This method is frequently employed as most vectors contain MCS and clones are often sought in a particular orientation so, for example, they can be expressed from a vector based promoter.

Homology based and Site-Specific Recombination

Restriction enzymes and DNA ligase have formed the foundation of recombinant DNA. However today there are other strategies to recombine DNA into new purposeful configurations. TOPO cloning described above is one such example. Another recently developed strategy is called Gibson cloning. This method is named after one of the inventors, Daniel Gibson, and is powerful because multiple DNA fragments can be simultaneously assembled into a plasmid. This is possible by the use of a cocktail of enzymes, which includes an exonuclease to create single-stranded 3′ overhangs that facilitates annealing of ends that are complementary. The cocktail also includes DNA polymerase to fill in the gaps within each annealed fragment and DNA ligase to seal the nicks and assemble a recombinant clone. For the reaction to work the enzymes must be present at appropriate concentrations. Toward this end commercial cocktails are available (New England Biolabs Inc.). A further utility of Gibson cloning is that different DNA fragments can be seamlessly assembled. To assemble the fragments complementary ends can be made by primer design and PCR.

Another cloning strategy is called GATEWAY cloning (Life Technologies), which relies on bacteriophage λ site-specific recombination system that integrates the phage genome into the *E. coli* chromosome. In this system neither restriction enzymes nor DNA ligase is used to form recombinant molecules. Instead engineered vectors act as donor (entry) and recipient (destination) substrates for subcloning between plasmids. The donor plasmid contains a GOI flanked on both sides by the *attL* sequence, while the recipient plasmid contains a counter-selectable marker (*ccdB*) flanked on both sides by attR sequences. These plasmids are mixed in vitro with the Int, Xis and IHF enzymes that catalyze site-specific recombination between the *attR* and *attL* sequences. The recombination product contains the GOI in the recipient plasmids flanked by the recombined *attR*/*attL* sequence (*attB1*), while the *ccdB* gene now resides in the donor plasmid flanked by *attP* sequences ('by-product' plasmid). The reaction mixture is transformed into *E. coli* selecting the desired antibiotic resistance, while the original donor and by-product plasmids are selected against because of the toxic

ccdB gene product. This reaction is analogous to phage λ excision from the *E. coli* chromosome. The reverse (integration) reaction is performed by assembling the same reaction, but excluding the Xis protein. The utility of the GATEWAY system is that a GOI can quickly and efficiently be subcloned into a wide range of vectors for genetic or protein expression studies.

In an alternative strategy, plasmid DNA can be recombined *in vivo*. One system relies on homologous recombination techniques in *E. coli* to swap pieces of DNA between different plasmids ('MAGIC'). In short a donor plasmid is transferred by conjugation, bacterial mating, into a recipient strain that harbors a recipient plasmid. Both plasmids are engineered with homology regions and I-*Sce*I sites that flank the regions to be recombined. Upon induction in *E. coli* of the I-*Sce*I restriction endonucleases, which recognizes an 18-bp sequence, and the bacteriophage λ-*red gam* gene products (recombinase), the target DNAs are excised and the linear products are homologously recombined *in vivo*. The desired chimeric plasmid is then selected for *in vivo* (antibiotic resistance and counter selection techniques).

DNA Synthesis

DNA can be chemically synthesized *de nova* in the laboratory. A key advantage for using DNA synthesis is that any sequence can be easily and quickly made. For this core technology molecular biologists typically rely on commercial venders or core nucleic acid facilities at larger research institutes. In the case of oligonucleotide synthesis, which is typically used as primers for DNA synthesis or as probes, this technology has been available for several decades at a reasonable cost. Here oligonucleotides, typically 15 to 100 bases long, can be embellished with desired restriction sites or with tag sequences. A tag can encode a motif to facilitate protein purification or an epitopes to assist with protein detection. Oligonucleotide synthesis also allows primers to be easily made for use in site directed mutagenesis. In summary, oligonucleotide synthesis has great flexibility in the construction of recombinant DNA molecules.

In the past few years the technology to synthesize large DNA molecules has significantly improved, while the costs have also dropped. At the time of writing of this review commercial vendors can synthesize DNA from 100 to 10 000 bp. At a price of about \$0.30 bp^{-1} a typical sized gene of 1000 bp can be synthesized and cloned at a reasonable cost. These developments have several important implications. First, in many cases it is more time and cost effective to have a desired gene or DNA molecule synthesize than for a researcher to make it themselves by standard recombinant DNA methods. For example, if one is interested in a particular gene found in a database, but the genomic DNA is not in the laboratory, they can simply have it synthesized, instead of requesting or ordering the template DNA for PCR based cloning. Second, for investigators that are not familiar with cloning approaches they can circumvent this problem by simply ordering the desired clone. Third, the ability to synthesize DNA *de nova* allows one to create or optimize any sequence they want. For example, to maximize recombinant protein expression in a heterologous host, codon usage can be optimized for that host. Alternatively, if one wants to introduce restriction sites, tags, mutations, create chimeras or any combination thereof; they can easily do so by having their optimized gene synthesized. Additionally, these changes can be seamlessly made without leaving scares in the DNA, which sometimes occurs using standard cloning methods. Because of these advantages, even highly skilled molecular biologists will sometimes prefer a synthetic DNA cloning strategy over multi-step cloning approaches.

Clone Isolation and Characterization

After recombinant DNA molecules are made *in vitro* and transformed the correct clone must be isolated. Two general methods for clone identification are genetic selections and screens. Selection is preferred because it demands an essential activity for cell survival and allows rapid and rare clone isolation. For example, the cloning of the tetracycline resistant marker is easily selected for by growing transformed cells on tetracycline plates. This strategy allows a rare Tet^R marker to be readily cloned from genomic DNA. Such techniques are commonly used to isolate genomic DNA linked to a transposon marker. Similarly, if a strain contained a temperature sensitive mutation in an essential gene, such as DNA gyrase, the corresponding wild type gene can be readily cloned from a genomic library by selecting growth at a non-permissive temperature. Although cloning by selection is powerful it is not infallible. In this latter scenario, complementing candidate clones may include DNA gyrase as well as other genes that function as multicopy suppressors of the temperature sensitive mutation. Frequently, selection strategies are not available and one resorts to screening. α-complementation allows a quick blue/white screen for insert clones and is described below. If selections or phenotypic screens are not available then molecular techniques are employed. Common techniques include diagnostic colony PCR or restriction analysis. These methods are more labor intensive, but are always available for clone validation. If a very rare clone is sought, for example one gene out of a genomic library, then methods such as colony hybridization may be employed. Here colony libraries are transferred to membranes and screened with labeled (radiolabeled, bioluminescent, etc.) probes (e.g. nucleic acid or antibody) to identify a specific DNA, RNA or protein clone.

As mentioned, part of the cloning process includes clone characterization. To do this there are a number of available methods to extract and purify vector DNA. Two common protocols are extraction by alkali or boiling lysis of *E. coli* cells. These steps selectively release plasmid DNA from large chromosomal DNA that is trapped and precipitates with lipids and cell wall debris. Vector DNA is further purified by selective precipitation of proteins or organic extraction with phenol and chloroform. RNA is removed by RNAse treatment. Ethanol precipitation allows quick concentration of DNA from solution. Plasmid DNA can be further purified by ultracentrifugation in a cesium chloride (CsCl) density gradient with ethidium bromide. This procedure results in a highly purified band of supercoiled plasmid DNA in the CsCl gradient. In lieu of these time-consuming procedures commercial plasmid purification kits, which selectively bind DNA to membranes, are available from a variety of vendors. Once purified, vector DNA is characterized by restriction digestion or PCR, followed by agarose gel electrophoresis separation and visualization with ethidium bromide staining.

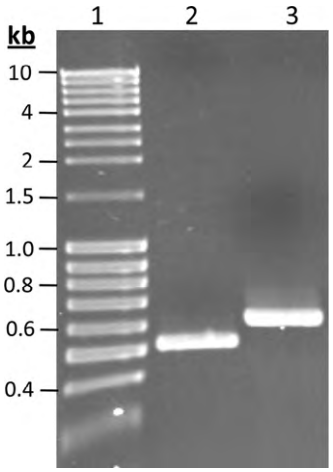

Figure 4 Agarose gel electrophoresis and ethidium bromide straining of DNA samples. Lane 1 is a commercial DNA ladder used as molecular weight standards. Selected sizes are shown at the left. Lane 2 and 3 are PCR products with predicted sizes of 522 and 623 bp, respectively. Image kindly provided by Yao Xiao.

Electrophoresis of DNA through an agarose gel is a standard and rapid method to separate DNA fragments based on size. Commercially available agarose is derived from seaweed and composed of polysaccharide and galactose sugars. The dynamic range for size separation is easily adjusted by changing the agarose concentration. DNA migrates through pores in the gel in response to an applied electric field. Migration occurs from negative to positive potential due to the net negative charge of the phosphate backbone. A representative agarose gel is shown in Figure 4. Following electrophoresis the bands were stained with ethidium bromide which intercalates in the DNA helix. The bound ethidium bromide is fluorescently visualized by ultraviolet light exposure. Cloned DNA can be further characterized by Southern blot analysis. In this method DNA is transferred or blotted from a gel onto a nitrocellulose or nylon membrane which is then hybridized with a labeled nucleic acid probe. For high resolution DNA separation, for example to resolve single base differences, agarose is substituted with polyacrylamide. The exact base composition of cloned DNA can be determined by sequencing reactions and resolution on a polyacrylamide gel. Obtaining the DNA sequence is import to conduct homology searches and to predict protein functions and study evolutionary relationships between sequences. The DNA sequence also facilitates clone construction to identify restriction or primer sites. Due to low costs, today most labs use core facilities or commercial venders for DNA sequencing.

Subcloning

Sometimes the original GOI clone will be derived from a vector used to clone large DNA fragments or cDNAs and is not suitable for subsequent studies. For example, a researcher might want to study gene expression and protein localization in the original host organism and might separately want an expression vector for protein purification. Each of these experiments will likely require different promoters, replicons and selectable markers. Consequently the GOI needs to be subcloned into different vectors to address specific experimental needs.

Protein expression

A common recombinant DNA objective is the overexpression of a target protein. Overexpression is achieved in a wide variety of expression vectors. Once validated clones are constructed they are assessed for overexpression. In some cases overexpression is phenotypically observed, such as the ability of a cell to produce the green fluorescent protein. However, typically expression is evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). If overexpression is significant the protein product is directly visualized upon coomassie blue staining. If expression is modest and not readily detected by SDS-PAGE then other more sensitive methods are employed.

The T7 RNA polymerase/promoter system is both a powerful and sensitive system for expression and detecting recombinant proteins. The two key factors of the system are: (i) T7 RNA polymerase is highly selective and consequently does not recognize any *E. coli* promoter. Only the recombinant gene fused to the vector T7 promoter is expressed. (ii) The antibiotic rifampicin selectively inhibits host RNA polymerase and does not inhibit T7 RNA polymerase. Thus when a host cell is induced to express T7 RNA polymerase and then treated with rifampicin the only promoter that is transcribed is vector borne. If cells are grown in minimal media and given radiolabeled [^{35}S] methionine shortly after rifampicin treatment the only protein labeled in the cell is from the T7 promoter. Selective protein labeling is the result of rapid mRNA degradation in *E. coli*. Subsequently, proteins are separated by SDS-PAGE and visualized by autoradiography. Alternatively, commercial coupled *in vitro* transcription-translation systems allow selective expression and labeling of genes contained in a purified plasmid.

Western blot analysis is yet another sensitive method to detect recombinant proteins. In many cases commercial antibodies are available that selectively bind target proteins or are cross-reactive. If antibodies to the native protein are not available then an epitope tag can be fused in-frame to the recombinant protein at the amino- or carboxyl-terminus. A variety of commercially available antibodies detect these epitopes by Western blot or ELISA (Enzyme-Linked ImmunoSorbent Assay).

In some cases *E. coli* is not a suitable host for the expression of recombinant proteins. Complicating factors include: unstable or unfolded or aggregated proteins, low expression levels or lack of proper post-translational modification such as glycosylation. Some of these problems are addressed by changing strain background or growth conditions. In other cases the gene needs to be subcloned into a shuttle vector for expression in a different bacterial or eukaryotic host. Another factor that affects expression levels is codon use. Since each species has its own preferred codon set, a foreign gene will likely not contain optimal codon preference for high expression. To circumvent this problem a gene can be synthetically made with optimal codon use for the intended host. Depending on gene size this can be an expensive endeavor, but is done for highly sought proteins, such as those with commercial applications.

Cloning Vectors

Cloning vectors have three common properties: (1) a selectable marker, which is almost always antibiotic resistance; (ii) an origin of DNA replication to allow vector propagation; and (iii) MCS or polylinker that contains a number of restriction sites to clone foreign DNA. Vectors also frequently contain other elements to assist cloning. For instance, a facile way to screen clones for insert DNA is important. Typically this can be screened by lose of an antibiotic resistance marker, insertional inactivation, or α-complementation with β-galactosidase (below). Vectors may also contain regulatable promoters to express a GOI. Expression vectors typically also contain a transcriptional terminator immediately following the MCS to prevent transcription of downstream DNA. Some plasmids contain an origin of replication for filamentous phage such as M13. When a helper phage is added the cloned DNA is packaged as single-stranded DNA in bacteriophage heads. This DNA serves as template for sequencing.

Plasmids

The preferred cloning vehicles are plasmids, because high quantities of vector DNA are rapidly purified and they are typically small. Both of these factors are important because they allow easy manipulation and characterization. Small plasmids, 3 to 4 kilobase pairs (kb), are ideal because they contain relatively few restriction sites and are efficiently transformed into host cells. Small plasmids are less likely to contain multiple restriction sites and thus are more readily interpreted on agarose gels. In contrast, large vectors may require extra steps that involve autoradiography methods to characterize clones. Practically, the efficiency of transformation significantly decreases as plasmid size exceeds 10–15 kb. This is especially critical when ligations are transformed.

Plasmid copy number varies greatly depending on replicon use. Low copy or stringent replicons such as the F plasmid have 1–2 copies per cells. Low copy replicons are ideal to clone toxic DNA to ensure tight gene repression and maintain plasmid stability. Medium copy number plasmids exemplified by the ColE1 and pMB1 (pBR322 and derivatives) replicons are maintained at about 15–20 copies per cell. High copy number plasmids, typified by the pUC vectors, are maintained around 500 copies per cell and are said to replicate in a "relaxed" (not stringent) fashion. Initiation of replication in the widely used pMB1 and ColE1 replicons is primed by an RNA primer. This primer hybridizes to the template DNA near the *ori* and is processed by RNAase H into a mature form (RNA II) to prime plasmid replication. The activity of RNA II is in turn regulated by RNA I, which is plasmid encoded on the opposite strand of the *rna II* gene. The binding of RNA I to RNA II prevents RNA II priming. The pairing of these RNAs is enhanced by a plasmid encoded protein called Rop, which reinforces the negative regulation of RNA I and reduce plasmid copy number. The copy number of pMB1 or ColE1 plasmids can consequently be increased by mutations in *rop* or *rna I*. Thus, for example, the popular pUC derived plasmids are high copy pMB1 replicons that contain a *rna I* mutation that impairs plasmid copy number control resulting in elevated levels of plasmid.

In addition to controlling copy number, RNA I, II and Rop determines the plasmid compatibility group. Plasmid compatibility groups are important when two different plasmids are sought in the same host cell. When two different plasmids belong to the same incompatibility group, such as the pMB1 and ColE1 replicons, they become unstable in the same cell. In this case the same copy control mechanism regulates both plasmids; that is copy number of both plasmids is the same as a single plasmid. Since the plasmid partitioning is not regulated in these vectors, there is a stochastic distribution of plasmids into daughter cells. Over time and cell divisions the ratio of plasmids varies and one is eventually lost. In contrast, the pMB1 and ColE1 replicons are compatible with p15A (pACYC and derivatives) and pSC101 replicons and are stably maintained together.

Most plasmids do not require synthesis of plasmid encoded proteins for replication. They instead rely on long-lived host enzymes for replication, while the host requires continued protein synthesis for chromosomal replication. Plasmid DNA yields are accordingly increased by up to 50-fold by treating late log cultures with translation inhibitors such as chloramphenicol. This treatment efficiently blocks chromosomal replication and enriches plasmid recovery.

Recombinant DNA tools and methods have primarily been developed and optimized for *E. coli*. Thus regardless of the organism that one is studying the initial cloning experiments are usually done in this host. However, investigators frequently study biological process in other species, whether eukaryotic or prokaryotic, and as a result need to move their vector into those cells. Since many of the *E. coli* plasmid genes or genetic elements do not function in distantly related hosts, the use of shuttle vectors is employed. Shuttle vectors contain two replicons and replicate in two different hosts. A mammalian shuttle vector, for example, may contain an adenovirus

replicon to allow mammalian cell replication. In other cases a vector might not replicate in the host cell but instead recombine into the genome by homologous or site specific recombination. Still other vectors are not stably maintained in their host, e.g. transient transfections. Aside from the replicon, other genetic elements, such as selectable markers and promoters, need to be suited for the intended host. For all of these reasons and more there are literally thousands of different vectors used by molecular biologists.

Bacteriophage, Cosmid, BAC and YAC Vectors

When large pieces of DNA are cloned other vectors beside plasmids are typically used. One alternative vector that was developed early in recombinant DNA evolution was bacteriophage λ. In phage λ it was recognized that much of the nearly 50 kb genome was dispensable for lytic growth and thus suited for replacement with foreign DNA. To construct a λ clone or libraries, insert fragments of approximately 20 kb are ligated to the left and right arms of λ genomic DNA that had been restricted and purified. Ligated DNA is then packaged with two complementary *E. coli* strain extracts infected with different λ mutants that cannot replicate or package their own DNA. The physical requirement that DNA molecules with λ *cos* sites, 12 bp single stranded complementary ends of the linear genome, be separated by approximately 50 kb for packaging provides a strong selection for inserts of a given size. A variety of λ vectors are available with different cloning sites and accommodate different insertion sizes or allow expression of cloned DNA. Advantages of λ vectors include large insert cloning and phage amplification. Because the host cell is killed during lytic growth toxic or unstable DNA fragments may be better suited in λ vectors.

Cosmid vectors are plasmids containing the λ *cos* site. Cosmids allow relatively large DNA fragments, 30–45 kb, to be cloned. Ligated recombinant cosmids are packaged in λ phage heads by *in vitro* packaging with cell extracts. A phage particle is produced which is transduced into *E. coli*. Transductants are selected by antibiotic resistance encoded on the autonomously replicating cosmid. Still larger DNA fragments, 100 to 300 kb, are cloned into bacterial artificial chromosomes (BACs). The BAC is based on the F plasmid replicon and partitioning system which maintains 1–2 copies per cell. Thus foreign DNA is simply ligated into the BAC vector and optimized electroporation conditions transform the BAC into *E. coli* by selecting antibiotic resistance. Because of the low copy number BACs are stably maintained and the supercoiled DNA it is relatively easy to purify and manipulate. Yeast artificial chromosome (YAC) vectors can accommodate 100 to 1000 kb of foreign DNA. These linear vectors contain a centromere, replicon, telomers and selectable marker which are ligated *in vitro* DNA of interest and transformed into yeast. Although very large fragments are cloned, YAC clones are unstable and difficult to manipulate.

E. Coli Host

E. coli generally serves as the host cell when constructing recombinant clones. However, there are exceptions to this rule, for example YACs are directly transformed into yeast. There are similar examples in other bacteria. In most cases, however, *E. coli* serves as the host for constructing recombinant clones because of its: (i) high electrocompetent transformation efficiencies ($>10^{10}$ transformants per μg DNA) or rapid chemical competent protocols, (ii) fast high density growth, (iii) availability of advance genetic tools, such as regulatable promoters, (iv) there are a plethora of established academic and commercial vectors available for cloning, and (iv) in-depth understanding of *E. coli* genes and biology important for cloning.

When selecting an *E. coli* host strain for cloning there are a number of genetic variables to consider. First, like many bacteria, *E. coli* contains host restriction modification systems to destroy foreign DNA. The *Eco*K I restriction system, encoded by *hsdRMS*, cleaves DNA that is not protected by adenine methylation at the ACC[N6]GTGC or GCAC[N6]GTT sites. A *hsdR* mutation abolish restriction but not methylation (r^-m^+), while *hsdS* mutations abolish both (r^-m^-). In contrast, the *mcrA*, *mcrBC* and *mrr* genes encode methylation requiring systems to attack DNA only when it is methylated at specific residues. All three of these endonucleases restrict DNA modified with CpG methylation. Thus when cloning DNA from mammals, plants and some bacteria it is wise to use strains that lack the Mcr and Mrr systems.

DNA methylation systems in *E. coli* can affect the susceptibility of cloned DNA to subsequent restriction analysis. Strains that encode a functional *dam* gene methylate DNA at GATC sites, while the endogenous *dcm* gene product methylates CC(A/T)GG sequences. In some cases restriction enzyme cleavage is blocked by Dam or Dcm methylation, e.g. *Cla*I and *Stu*I, while in other cases restriction site methylation does not block cleavage, e.g. *Bam*HI and *Kpn*I.

There are a number of other factors to consider when selecting a host *E. coli* strain for cloning. If the cloned DNA has high homology to the host chromosomal DNA, e.g. an *E. coli* gene, or to sequences within the clone, such as repeat elements, it might recombine is undesirable ways. To avoid this problem select a strain with a *recA* mutation that abolishes homologous recombination. Mutations in *endA*, which encodes endonuclease I, blocks nonspecific degradation and yields high quality cloned DNA that is not nicked or degraded. If a high copy plasmid is unstable, presumably by expression of a toxic product, it can be stabilized by a *pcnB* mutation, which reduces the copy number of the popular ColE1-type plasmids. If expression of a recombinant protein is unstable it may be stabilized by mutations in *lon*, *clpP* or *rpoH*, all of which reduce cellular protease activity.

Certain strains of *E. coli*, e.g. DH5α, have been constructed to optimize transformation efficiencies and clone identification. A common tool to rapidly identify clones that contain insert DNA is the use of α-complementation. Vectors that allow α-complementation contain a short 146 amino acid fragment of the *E. coli* β-galactosidase gene (*lacZ*). This DNA fragment is typically embedded in the polycloning site. When expressed this amino-terminal α-complementation fragment can associate in appropriate strains that contain a ω fragment derived from the carboxyl-terminal portion of β-galactosidase. These Lac^+ bacteria are

easily recognized as blue colonies in the presence of chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). However, when foreign DNA is cloned into the MCS the α-complementation fragment is destroyed and the recombinant clones appear as white colonies. This simple screen allows quick identification of candidate recombinants.

There are a number of other factors that one should be mindful of when selecting an *E. coli* host for cloning. For example, the construction of many strains of *E. coli* has resulted in the placement of drug resistance markers in the chromosome. In some cases these are drug resistant cassettes, such as tetracycline or kanamycin resistance. In other cases mutations in host genes, for example *rpsL* mutations, confer antibiotic (streptomycin) resistance. To select transformants the host must not contain the same antibiotic resistant marker as the vector. Another consideration is whether the host contains the appropriate transcription factors to regulate expression of cloned DNA. For example many vectors contain the *lac* promoter/operator and thus to repress gene expression the host needs to encode the Lac repressor (*lacI*). The Lac repressor is allosterically inactivate by isopropyl β-D-1-thiogalactopyranoside (IPTG) and triggers expression of the *lac* operon. The popular pET vectors contain the bacteriophage T7 promoter and in order to drive transcription the T7 RNA polymerase gene needs to be encoded in the host.

Applications

Recombinant DNA has revolutionized biological research and has had huge impact on the pharmaceutical and biotechnology industries. These techniques have provided the foundation for sequencing the human genome and hundreds of other genomes by providing genomic libraries. This information is invaluable for the molecular understanding of how cells and organisms function. Genomic sequence information allows a clear understanding of evolutionary relationships between species and genes. Genomic information also allows an understanding of how the environment impacts cellular physiology and genetic composition. This is particularly true with microorganisms as they are found in a plethora of environments; e.g. soils, oceans, streams and animal and plant hosts. These environmental conditions can vary greatly with respect to temperature, pH, osmolarity, oxygen tension, radiation levels or nutrient availability. Knowing the genomic sequence of bacteria found in different environments provides a molecular understanding how genes and organisms adapt to their environment.

In the pharmaceutical industry recombinant DNA methods are common tools used in drug discovery and development. These techniques help to find and elucidate interactions between therapeutic compounds and target macromolecules such as protein or RNA. Potential effects on cellular physiology, toxicologically along with mechanism of compound action and resistant profiles can be tested with molecular genetic or biochemical methodologies. Natural product therapeutics, derived from microbial secondary metobolites, are optimized by recombinant methods to improve yields or alter chemical structures.

The biotechnology industry uses recombinant DNA to isolate, improve and produce protein therapeutics such as insulin, human growth factor, interferon, erythropoietin (Epogen) and filgrastim (Neupogen). Many other therapeutic recombinant proteins are clinically used or under development. Recombinant DNA technology has also resulted in improved vaccines that simply contain isolated antigen and no other bacterial or viral component that often leads to adverse side effects. In agriculture recombinant DNA has improve plant growth by increasing nitrogen fixation efficiencies, by cloning bacterial genes and inserting them into plant cells. Other plants have been engineered to be resistant to caterpillar, pests and viruses by inserting resistant genes into plant genomes. In humans gene therapy may one day improve health. Here cells are removed and transformed with altered genes and replaced back into the patient to provide missing functions. Alternatively, recombinant viruses or other methods could be used for gene therapy delivery in live hosts. Gene therapy has the potential to replace drug therapy, which takes many years to develop safe and efficacious molecules for treating human genetic disorders. Lastly, recombinant DNA methods are used to isolate, develop and purify catalytic enzymes used in industrial processes. Enzymes have practical implications in many chemical synthesis processes by improving the rate and specificity of reactions. Such enzymes are used for pulp bleaching in paper making, oil processing, house hold detergent ingredients and conversion of biomass or oils into ethanol or biofuels.

In forensic science, recombinant DNA techniques help law enforcement agencies test relationships between biological crime samples and individuals to prove guilt or innocence. These methods are also used to understand family heredity and to screen individuals for the presence or absent of genetic diseases. One technique is DNA fingerprinting by restriction fragment length polymorphisms (RFLP). Here unique repeating DNA sequences in individuals are characterized by restriction digestion analysis in which specific bands on blots are probed with labeled nucleic acids. Alternatively, polymorphisms can be identified by high throughput DNA sequencing methods.

In biological research recombinant DNA methods are omnipresent. They are used to study gene function by constructing mutant alleles to test phenotypes. Site-specific mutations are readily engineered into primers used in PCR amplification. This approach is particularly attractive when structural information is available or evolutionarily conserved sequences/motifs are found within proteins. Thus site-specific changes in gene sequences directly test structure-function relationships. Alternatively random mutagenesis of a cloned gene is used in an equally informative and unbiased approach to study gene function. DNA regulatory elements, such as promoters, can be studied *in vivo* by fusing them to reporter proteins, such as β-galactosidase. These reporters are used to test gene expression responses to environmental or cellular changes. Reporters can be conveniently vector encoded or integrated into the chromosome to allow accurate physiological measurements. Subcellular localization of proteins is visualized by fusing a gene product to a reporter, such as the green fluorescent protein, and observed by fluorescent microscopy. Regulatable expression vectors allow a gene product or alleles thereof to be conditionally expressed in cells. Such systems test biological responses to gene products or provide a means protein overexpression.

When biochemical methods are used to study biological processes the investigator may identify protein product before the gene is known. In these cases reverse genetics is used to isolate the corresponding gene and to construct mutants to study protein function *in vivo*. Here the partial protein amino acid sequence is determined. With this information a degenerative DNA sequence is deduced for every combination of codons to each amino acid. If the genome sequence is available the corresponding gene is found with bioinformatic tools. If the genome sequence is not available then degenerative oligos are synthesized to the peptides and a gene fragment is PCR amplified. Alternatively, the oligos (or PCR product) is labeled as a hybridization probe to identify the corresponding clone from a genomic library. Recombinant DNA and genetic techniques provide the means to insert mutant alleles into the host to study mutant phenotypes. Depending on the genetic tools available for a host a variety of mutants can be constructed such as a null, insertion, truncations, point or promoter mutations. Recombinant DNA methods continue to improve and will inevitably serve as a foundation to approach biological and commercial problems in the foreseeable future.

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Drinking Water☆

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Abbreviations

AOC	Assimilable organic carbon
ATP	Adenosine triphosphate
CCL	Contaminant Candidate List
CFU	Colony-forming units
DE	Diatomaceous earth
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon ED electrodialysis
EPA	Environmental Protection Agency
EU	European Union
FR	Federal Register
GAC	Granular activated carbon
GWR	Groundwater Rule
HACCP	Hazard Analysis and Critical Control Point
HPC	Heterotrophic plate count
LT2ESWTR	Long-Term 2 Enhanced SWTR
MCL	Maximum contaminant level
MCLG	Maximum contaminant level goal
MF	Microfiltration
MST	Microbial source tracking
NF	Nanofiltration
NTU	Nephelometric turbidity units
POE	Point-of-entry
POU	Point-of-use
PVC	Polyvinyl chloride
PWS	Public water system
RNA	Ribonucleic acid
RO	Reverse osmosis
RTCR	Revised Total Coliform Rule
SWTR	Surface Water Treatment Rule
TCR	Total Coliform Rule
UF	Ultrafiltration
WHO	World Health Organization

Defining Statement

The primary objective of undertaking drinking water microbiology is to prevent waterborne disease. This objective can be achieved through proper assessment of source water quality, matching it against sufficient water treatment and control practices including drinking water distribution, and by monitoring their effectiveness. This article addresses characteristics of drinking water microbiota, water treatment practices in use, and monitoring and control strategies.

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Introduction

Safe drinking water is one of the most successful public health interventions ever developed. Ancient civilizations practiced water treatment, as evidenced by Egyptian inscriptions and Sanskrit writings. Babylonians had piped water since 4000 BC, and sand filtration was in use in some cities as early as the sixteenth century. Chlorination of drinking water, so widely used today, was not introduced until the first decade of the twentieth century. Sand filtration and chlorination practices dramatically decreased the incidence of waterborne disease, although waterborne disease is still a problem in the United States and elsewhere when systems are inappropriately managed or unusually challenged.

As with many developed regions, the United States takes a multiple-barrier approach in preventing pathogen entry into drinking water. This means that a drinking water distribution system is required to use several barriers/treatment steps to counter enteric pathogens, throughout the entire process from source water management (e.g., intake) to drinking water exposure (e.g., the tap). Systems using surface water may control human activities on the watershed supplying the system. In groundwater systems natural barriers such as soil, aquitards and bedrock function in preventing passage of pathogens into well water. As part of this approach, a system should use the cleanest source water available (including use of watershed controls), provide appropriate water treatment for the water source used, and minimize pathogen entry into the distribution system via cross-connections and back-flow, pipe breaks, and other distribution system deficiencies. In addition, the multiple-barrier approach includes a good management plan, minimum operator standards and training, a state laboratory accreditation program, periodic on-site sanitary surveys, and a host of other quality assurance programs that collectively reduce the potential for drinking water contamination. An integral part of this approach is for a system to understand the potential sources of contamination in the watershed, including human activity and livestock practices to minimize human-infectious enteric pathogens. Implementing strong plumbing system codes provides additional protection to drinking water quality.

This article discusses how water is treated and monitored to insure its microbiological safety for human consumption. The primary focus is on water treatment practices as used in developed regions like the United States. The article also includes a brief description of the drinking water regulations for microbiology in the United States and discusses emerging concerns from water-based pathogens and antibiotic-resistant genes in the environment.

Indicator Organisms in Microbial Monitoring

Drinking water can be a vector of waterborne and water-based pathogens and therefore exposure to contaminated drinking water can result in a number of illnesses such as gastroenteritis (when ingested) and respiratory diseases (when such organisms enter via the respiratory tract). Waterborne pathogens are enteric agents that pass in feces and are ingested via water, and include numerous viral, bacterial and parasitic protozoa pathogens (Ashbolt et al., 2001). Water-based pathogens are non-enteric microorganisms that spend part of their life cycle in water environments, some of which are pathogens of vulnerable groups, such as the immunocompromised and elderly. Ideally, specific detection of the various waterborne and water-based pathogens would be the most direct approach in determining their presence and the need for control. Unfortunately, in actual practice, this is impractical for several reasons. The variety of potential pathogens is so broad, that a search for all, -especially on a routine basis, would be extremely difficult, time-consuming, and expensive. Moreover, the efficiency of current techniques for recovering and detecting known drinking water pathogens is often poor, primarily due to the low levels of these organisms in drinking water. In addition, detection does not always translate into risks as some strains of the same species are more pathogenic than others, and current detection methods cannot easily discriminate between pathogenic and nonpathogenic strains.

Although culture techniques for isolating many bacterial pathogens are available, some are nonselective, thus allowing nontarget organisms to proliferate in numbers that overgrow the pathogen. Viral pathogens are fastidious in their growth requirements and grow only in special tissue cultures that are expensive and often difficult to maintain. Some viruses cannot yet be cultivated in the laboratory, such as human noroviruses. Currently, methods for pathogenic protozoa are inefficient due to the poor recovery yields of methods used for detection and the need to filter large volumes of water to detect protozoan in situ levels. Extended delays can be involved in carrying out specific identification procedures for pathogens, and only certain laboratories with specially trained technologists may have the expertise to carry out some of these procedures. Moreover, if pathogens were present in drinking water, their concentrations would usually be sufficiently low to require analysis of large-volume samples.

Because of the problems associated with trying to detect specific enteric pathogens, indicator organisms are used as fecal indicators, as treatment surrogates to assess the microbiological quality of drinking water, and to monitor the integrity of distribution systems. The ideal drinking water indicator has the following attributes.

- It is suitable for all types of drinking water.
- It is present in sewage and fecally-polluted waters at much higher densities than enteric pathogens.
- Its environmental persistence is at least that of waterborne pathogens.
- It is at least as resistant to disinfection as the hardiest waterborne pathogens.
- It is easily detected by simple, inexpensive, laboratory tests in the shortest time with accurate results.
- It is stable and nonpathogenic.
- It is generally not present in waters not contaminated by feces from warm blooded animals.

The indicators generally employed worldwide are total coliforms (e.g., as a measure of disinfection efficacy), thermotolerant coliforms, and/or *Escherichia coli* (as fecal indicators). Total coliforms constitute a broad group of bacterial species, all phylogenetically affiliated to the family *Enterobacteriaceae*. They are usually not pathogenic and are widespread in ambient water, where some grow unassociated with fecal contamination. Commonly, they are more numerous than *E. coli* in sewage and animal excreta, and hence are used to provide a flag for potential fecal contamination. While total coliforms are encompassed by multiple genera with a variety of physiological traits, they are defined by their ability to produce acid and gas from lactose on selective culture media designed to recover enteric bacteria. The preferred culture media used to detect and enumerate vary by country (e.g., in the US and European countries). This bacterial group include most species of the genera *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Escherichia*, although some species of *Serratia*, and other genera may also be included, depending on the medium used. Total coliforms are used to determine the effectiveness of water treatment processes, to monitor the integrity of the underground pipe network called the drinking water distribution system, and as a screening group for fresh fecal contamination. Treatment and other water system practices that provide coliform-free water should also reduce pathogens to minimal levels. However, as with *E. coli*, total coliforms are inadequate for predicting the potential presence of parasitic protozoan cysts and enteric viruses, since coliforms are more susceptible to disinfection than these other organisms. Furthermore, for microfiltration processes, bacteria and cysts maybe removed, but limited levels of the smaller enteric viruses may remain. Therefore it is important to match the appropriate treatment indicator with the class of pathogen and treatment process to be monitored.

Thermotolerant coliforms are a subset of the total coliform group, primarily including *E. coli* and a few thermotolerant strains of *Klebsiella*. Thermotolerant coliforms and *E. coli* are the preferred indicators of fresh fecal contamination, not total coliforms, as reflected in recent changes to the total coliform rule (EPA, 2009) and by WHO (2008). However, total coliforms are a more suitable indicator than thermotolerant coliforms or *E. coli* for determining the vulnerability of a distribution system to surface water contamination, especially in the absence of fecally contaminated samples at the times and locations of sample collection. Total coliforms are usually present at much higher concentrations in source waters than these other indicators, and they are relatively more resistant to chlorine and other environmental stresses. Most countries that have formal drinking water rules, including the United States, monitor for total coliforms to flag potential contamination, loss of disinfection treatment efficacy and breaches of distribution system integrity. From a regulatory perspective, *E. coli* (or enterococci and coliphages in groundwater) are used to flag fecal contamination and their presence would normally result in follow-up action to identify and manage the source of contamination.

In addition to the indicators just described, other drinking water indicators also have been used or suggested, usually as a supplement to total coliforms or *E. coli*. For example, coliphages are used as surrogates of human enteric virus behavior (particularly for groundwater sources), while spores of *Clostridium perfringens* are used as surrogates for parasitic protozoa oo/cysts removal. As with total coliforms, heterotrophic bacteria (as measured by the heterotrophic plate count [HPC] method) are used to assess treatment performance. Specific molecular methods targeting genes associated to members of the *Bacteroidales* and to *Methanobrevibacter smithii* are also used to aid in identifying the source(s) of fecal contamination. Also for source tracking several chemicals have been used, such as, fecal sterols, caffeine, and disinfectant residuals. For monitoring ambient freshwaters, the US Environmental Protection Agency (EPA) suggests the use of enterococci or E. coli as indicators, with enterococci providing the strongest link to health outcome for both fresh and marine waters (EPA, 2012).

EPA Drinking Water Regulations

General

The EPA publishes enforceable regulations under the Safe Drinking Water Act, which was passed by the US Congress in 1974 and subsequently amended. Regulations under this Act apply to every public water system (PWS), that is, to those systems that regularly serve 25 or more people at least 60 days out of the year, or that have 15 or more service connections. There are approximately 160000 PWSs in the United States, of which close to 15000 are surface water systems and the rest are groundwater systems. About 9000 systems serve 3300 people or more, and these 9000 systems serve a total of approximately 258 million people. Based on EPA's definition, PWSs can be classified as a Community Water System (a public water system that supplies water to the same population year-round), a Non-Transient Non-Community Water System (a public water system that regularly supplies water to at least 25 of the same people at least six months per year, but not year-round. Some examples are schools, factories, office buildings, and hospitals which have their own water systems), or a Transient Non-Community Water System (a public water system that provides water in a place such as a gas station or campground where people do not remain for long periods of time. The systems can also be classified according to the number of people they serve, that is, from very small (serving 25–500 people) to very large systems (serving >100000 people) (www.water.epa.gov/infrastructure/drinkingwater/pws/factoids.cfm).

The EPA has four classes of regulations to protect the public against waterborne pathogens: the Total Coliform Rule and followon Revised Total Coliform Rule, the Surface Water Treatment Rule and supplemental Long Term 2 Enhanced Surface Water Treatment Rule, the Groundwater Rule, and the Aircraft Drinking Water Rule. These rules are summarized here. The Code of Federal Regulations (40 CFR 141 and 142) provides detailed requirements about the existing rules. The *Federal Register* (FR) also provides detailed requirements, along with the rationale for these requirements. (The general website for the Code of Federal Regulations and the Federal Register is http://www.gpoaccess.gov).

Total Coliform Rule and Revised Total Coliform Rule

The Total Coliform Rule (TCR) (54 FR 27544; 29 June 1989) sets a maximum contaminant level (MCL) for total coliforms, so that only *E. coli* now has a MCL, which is 0 detects in a 100 ml sample of drinking water. Total coliforms are used in the Revised Total Coliform Rule to indicate the need for follow-up sampling and for system assessments. Provisions of the Revised Total Coliform Rule become effective April 1, 2016. If a system exceeds the MCL for repeat samples according to the conditions described in CFR 141.63 c(1) through c(4), it must notify the public using mandatory language developed by the EPA. The required monitoring frequency for a system depends on the size of the population served and whether the system operates full time or not. This frequency ranges from 480 samples per month for the largest systems to once annually for certain of the smallest systems. The regulation also requires all systems to have a written plan identifying where samples are to be collected.

If a system has a total coliform-positive sample, it must analyze for the presence of *E. coli* on three different samples (for small systems, four) within 24 h of being notified of the positive sample. In addition, systems that collect fewer than five samples per month must, with some exceptions, collect at least five routine samples the next month of operation. Both routine and repeat samples count toward calculating compliance with the MCL. If two consecutive samples at a site are total coliform-positive and one is also thermotolerant coliforms- or *E. coli*-positive, the system is in violation of the MCL and must notify the public using more urgent mandatory language than used for the presence of total coliforms alone. The TCR also requires each system that collects fewer than five samples per month to have the system inspected every 5 years (10 years for certain types of systems using only protected and disinfected groundwater). Some of the regulations implemented in the TCR will change in the forthcoming Revised Total Coliform Rule (RTCR).

Surface Water Treatment Requirements

The Surface Water Treatment Rule (SWTR) (54 FR 27486; 29 June 1989) was designed to protect all PWSs that use surface water, or groundwater under the direct influence of surface water, against waterborne pathogens, specifically including *Giardia lamblia*, viruses, and *Legionella*. The SWTR requires systems to use sufficient water treatment (removal and/or inactivation) to reduce *G. lamblia* cysts by at least 99.9% (3 logs) and enteric viruses by at least 99.99% (4 logs). The SWTR specifies criteria for meeting these standards, and criteria for avoiding filtration. More specifically, the SWTR requires all systems covered by the rule to disinfect. It also requires all such systems to filter their water, unless (1) the system has an effective watershed control program; (2) it complies with the TCR and MCL for trihalomethanes; (3) it uses a good-quality source water, as defined as having no more than 20 thermotolerant coliforms per 100 ml or no more than 100 total coliforms per 100 ml, and a turbidity (opaqueness) no greater than 5 nephelometric turbidity units (NTU), and (4) it meets stringent disinfection conditions.

The regulation and associated EPA guidance assist the system in meeting the specified pathogen reduction levels by identifying pertinent *CT* values for disinfection inactivation (*C*, disinfectant concentration in mg l^{-1}; *T*, time of disinfectant contact with the water in min). *CT* values are provided for *Giardia* and enteric viruses by disinfectant type (e.g., chlorine, chloramines, ozone, or chlorine dioxide), water pH, and temperature. The regulation also requires a system using surface water to have a 0.2 mg l^{-1} disinfectant residual continually entering the distribution system and at least a detectable disinfectant residual in at least 95% of the sample sites throughout the distribution system. The rule specifies the monitoring frequency and locations for determining these disinfection residuals and (for unfiltered systems) testing source water quality.

The SWTR has been strengthened by three subsequent rules, the Interim Enhanced SWTR (63 FR 69478, 16 December 1998), Long-Term 1 Enhanced SWTR (67 FR 1812; 14 January 2002), and Long-Term 2 Enhanced SWTR (LT2ESWTR) (71 FR 6135; 5 January 2006). These three rules collectively tighten the turbidity standards, require systems to monitor the turbidity levels leaving each individual filter, require periodic on-site sanitary surveys by the state or primary authority, require additional treatment for systems with elevated source water *Cryptosporidium* concentrations, and require the system either to cover all finished drinking water storage facilities (e.g., reservoirs) or treat the water in those facilities, as well as other new requirements. The primary purpose of these new requirements is to control *Cryptosporidium* oocysts. Another purpose is to ensure that utilities do not degrade pathogen control while complying with other regulations that control for disinfection by-products (formed by the reaction of disinfectants with organic material in the source water). Under these three SWTRs, a system must provide sufficient water treatment to reduce Cryptosporidium by at least 99% (2 logs). However, because of a concern that systems drawing water from a poor-quality source might be exposing the public to a greater pathogen risk than is reasonable, the LT2ESWTR requires systems to provide additional *Cryptosporidium* treatment if required source water monitoring (initially screened by *E. coli* levels for small systems) indicates the average density of *Cryptosporidium* oocysts is 0.075 l^{-1} or greater (for unfiltered systems, > 0.01 l^{-1}). The actual required minimum level of additional treatment depends upon how much greater than 0.075 l^{-1} is the average *Cryptosporidium* density. In this manner, in theory, the consumer would have approximately the same risk of infection, regardless of the system used.

Groundwater Rule

The Groundwater Rule (GWR) (71 FR 65574; 8 November 2006) applies to all PWSs that use groundwater (about 142000 systems). However, systems that use groundwater that are under the direct influence of surface water must instead comply with the SWTR. Under the GWR, the state will determine whether a system is fecally contaminated or is vulnerable to fecal contamination. This determination may be made by a variety of means, including direct monitoring of the source water (usually the well), periodic

on-site sanitary surveys by a trained inspector, and an examination of the site's hydrology. A fecally-contaminated site is required to take corrective action such as providing an alternate water source, eliminating the contamination source, correcting all significant deficiencies found during a sanitary survey, and/or providing treatment (removal, inactivation, or both) that reliably achieves at least a 4-log (99.99%) virus reduction. Under the rule, a system may use *E. coli*, enterococci, or coliphage for source water monitoring; the rule approves specific analytical methods for these three indicators. The rule specifies when, where, and how often a system must be monitored; the frequency of required on-site sanitary surveys, minimum disinfectant requirements, and other provisions.

Aircraft Drinking Water Rule

The Aircraft Drinking Water Rule (74 FR 53590; October 19, 2009) applies to aircraft PWSs. It applies to scheduled and non-scheduled charter air transportation. Airlines which do not regularly serve water to passengers, or serve at least 25 passengers for at least 60 days per year are exempt from this regulation. Sampling should be at least one sample from a galley and one from a lavatory for total coliform and disinfectant residual. For airlines of >20 aircraft, 25% of the fleet should be sampled quarterly, and for airlines of <20 aircraft, all should be sampled quarterly. Each aircraft should have its water system disinfected and flushed no less than quarterly and watering points (trucks, carts, hoses etc.). Coliform sampling is required once per year for aircraft that are flushed and disinfected quarterly, once per six months for aircraft that are flushed and disinfected one to three times per year, once per quarter for aircraft that are flushed and disinfected twice per year, and monthly for aircraft that are flushed and disinfected once per year or less. The sample volume for coliform testing is 100 ml. A total coliform sample should be followed up by *E. coli* testing. Four repeat samples are required for a total coliform and E. coli positive samples. In the event of a positive *E. coli* sample, public access to the water should be restricted, and the system should be disinfected and flushed. If a sample is total coliform positive only, corrective actions should be taken until repeat samples are total coliform negative.

Other US Rules

EPA has published a list of pathogens and chemical contaminants that are not specifically regulated but occur, or are likely to occur, in water systems, pose a potential health risk, and may need to be regulated (63 FR 10274; 2 March 1998). The Contaminant Candidate List (CCL), most recently published as CCL-3 in 2012 includes adenoviruses, caliciviruses, *Campylobacter jejuni*, enteroviruses, *E. coli* O157, *Helicobacter pylori*, hepatitis A virus, *Legionella pneumophila*, *Mycobacterium avium* complex, *Naegleria fowleri*, *Salmonela entericia*, and *Shigella sonnei*. The EPA is conducting research on each of these pathogens to determine whether new regulations are needed to control them. Some of these organisms are opportunistic pathogens; hence the EPA is required to pay special attention to consumers at higher risk (i.e., immunocompromised).

In developing future regulations, EPA will assess the risk of waterborne and water-based pathogens (i.e. *L. pneumophila* & non-tuberculous mycobacteria). This assessment will be facilitated by the development of risk models that would specify, for example, the level of pathogen inactivation/removal needed by a ground or surface water system and, thus, whether that system would need further monitoring or other requirements such as disinfection. A satisfactory assessment of risk depends on the evaluation of a number of factors, including infectious dose, virulence, ratio of infection to disease, identification of susceptible groups, the extent to which a pathogen is likely to be present in drinking water and the percentage that are viable. Despite the development and evaluation of microbial risk assessment and modeling tools for water-associated pathogens, this approach has not been widely adopted.

Rules and Guidelines of Other Countries

Several multi-country organizations have strong drinking water directives or guidance. These include the World Health Organization (WHO) and the European Union (EU). WHO's *Guidelines for Drinking-water Quality*, third edition (2006), contains a comprehensive strategy for ensuring safe drinking water, from the water source though treatment and distribution to the consumer – in what it calls a Water Safety Plan that is risk-based management and follows the hazard analysis critical control point (HACCP) approach used in the food industry. The guidelines do not set specific numerical goals for removal/inactivation of pathogens or indicators of water quality. Rather WHO takes the position that a community should set its health-based goal, such as one Disability Adjusted Life Year (DALY) per million people in a year, then based on reference pathogens (addressing relevant viral, bacterial and parasitic protozoa) determine overall log reductions to stay below the risk-based targets for each pathogen group. Therefore, there is no single list of drinking water standards, but individual regions set these according to local needs and capacities. Guidelines are provided for a large variety of drinking water systems and circumstances, including large metropolitan systems, small community piped systems, non-piped systems in communities and in individual dwellings, large buildings, food production, emergencies and disasters, and travelers and commercial transport. The document describes the approaches used in deriving the guidelines, including guideline values, and includes fact sheets on significant microbial and chemical hazards.

A key component of the WHO guidelines is the Water Safety Plan (WSP). The WSP has several elements, for example, the development of an individual water safety plan that includes an assessment of risk from source to tap; the identification of control measures and, for each measure, appropriate monitoring that, collectively, will control the identified risks in a timely manner; and

the development of management plans describing actions to be taken during normal operation or incident conditions, as well as supporting programs (http://www.who.int).

EU directive 98/83/EC (3 November 1998) specifies that no *E. coli*, enterococci, or coliform bacteria should be detected in 100 ml of drinking water. It also states that no abnormal change in heterotrophic colony counts (22 °C) should occur. The directive also specifies that if water originates from or is influenced by surface water, it must also monitor *C. perfringens* (including spores). If the density of *C. perfringens* exceeds 1/100 ml, the utility must investigate to ensure that water has not been contaminated by enteric pathogens. In contrast to US standards, the monitoring frequency for bacterial indicators is based upon the volume of water produced or distributed each day, rather than by the number of people served by the utility (http://europa.eu). Among individual countries with an effective drinking water management plan approach are Canada (http://www.hc-sc.gc.ca), Australia (http://www.waterquality.crc.org.au), and New Zealand (http://www.mfe.govt.nz). These countries focus more on the whole of system HACCP approach, knowing that to provide water at an annual risk threshold below one infection per 10000 people or <1 disability adjusted life year (DALY) per million it is not feasible to monitor at the drinking water product end alone. For example, drinking water would need to be sampled every 15 min to be sure 95% of the time to have <1 virus/10^6 l, the risk-based target to achieve <1 infection per 10000 people (Smeets et al., 2010). Hence, there is a need to provide validation of likely human-infectious pathogen numbers upstream and relevant surrogates of treatment removal to estimate these risk-based targets.

Sources, Treatment, and Distribution

Sources of Water Supply

Drinking water sources can be divided into two categories: surface water and groundwaters; treatment requirements vary with the source. Microbiologically, groundwaters tend to be of better quality than surface water sources and, consequently, require less intensive treatment. Most groundwaters supplies used for drinking water are pumped wells (dug, drilled, or driven), artesian wells, or springs.

Surface water sources include streams, rivers, ponds, lakes, and manmade impoundments and reservoirs. These sources include precipitation and runoff that is not lost to the atmosphere by evaporation and does not enter the ground via infiltration and percolation. Surface waters become contaminated by human pathogens through direct and indirect inputs of municipal sewage and other sources of human and animal excreta. Removal or inactivation of these microorganisms to provide safe drinking water involves a multiple-barrier concept, using a variety of treatment processes, in addition to measures aimed at controlling or reducing source water pollution (source protection).

Bacterial concentrations in groundwater, as measured by the HPC, are only a very small fraction of the total active cells and often <100 colony-forming units (CFU) ml^{-1}; coliforms are normally absent in that volume. Bacterial concentrations in surface waters depend on the degree of contamination with human and animal fecal material. Pristine and relatively uncontaminated surface waters commonly contain total coliform bacteria that range from <1/100 to >10^6/100 ml, and thermotolerant coliforms/*E. coli* range from <1/100 to >10^5/100 ml. Raw water containing more than 2000 thermotolerant coliforms/100 ml should not be used as a drinking water source unless there is considerable pre-treatment, such as by slow deep-sand filtration or riverbank infiltration.

Water Treatment

The major water treatment processes for improving the microbiological quality of surface waters for human consumption include coagulation and flocculation, sedimentation, filtration, and disinfection. Most groundwater used as drinking water sources are is only disinfected, if they are treated at all, hence the importance to identify the likelihood of surface water intrusion for groundwater systems. Figure 1 is a schematic of the unit processes in a conventional drinking water treatment system used for surface waters. Several additional treatment processes are widely used, for example, softening, fluoridation, and iron removal, but these are for aesthetic or non-microbiological reasons and not discussed here.

Another process called 'bank filtration' is used in various countries (e.g., Finland, Germany and the Netherlands) as a replacement for coagulation/flocculation and filtration treatment steps to provide water for drinking water supply purposes. Bank filtration wells are installed adjacent to a river or other source of water. The wells capture ground water from the landward side and surface water that is induced by well pumping to infiltrate through the subsurface and into the well. The produced water is processed through other treatment steps for drinking water. The use of 'bank filtration' in the United States has seen limited application.

Coagulation, flocculation, and sedimentation

In the first step, raw water is pumped into a rapid mix unit, where chemical coagulants are added to destabilize particles in the water electrostatically (i.e., make them 'sticky'). This step is referred to as coagulation. Then water enters a flocculation basin, which is often a series of chambers with slow moving paddles. During flocculation, the destabilized particles are brought into contact with each other so they aggregate into larger particles, or flocs. During this process microorganisms become trapped by, or attached to, the flocs. The optimal coagulation practice varies with water pH and temperature, raw water turbidity, and type of coagulant(s) used. Commonly used coagulants include aluminum sulfate (alum), calcium oxide (lime), ferrous sulfate, and ferric

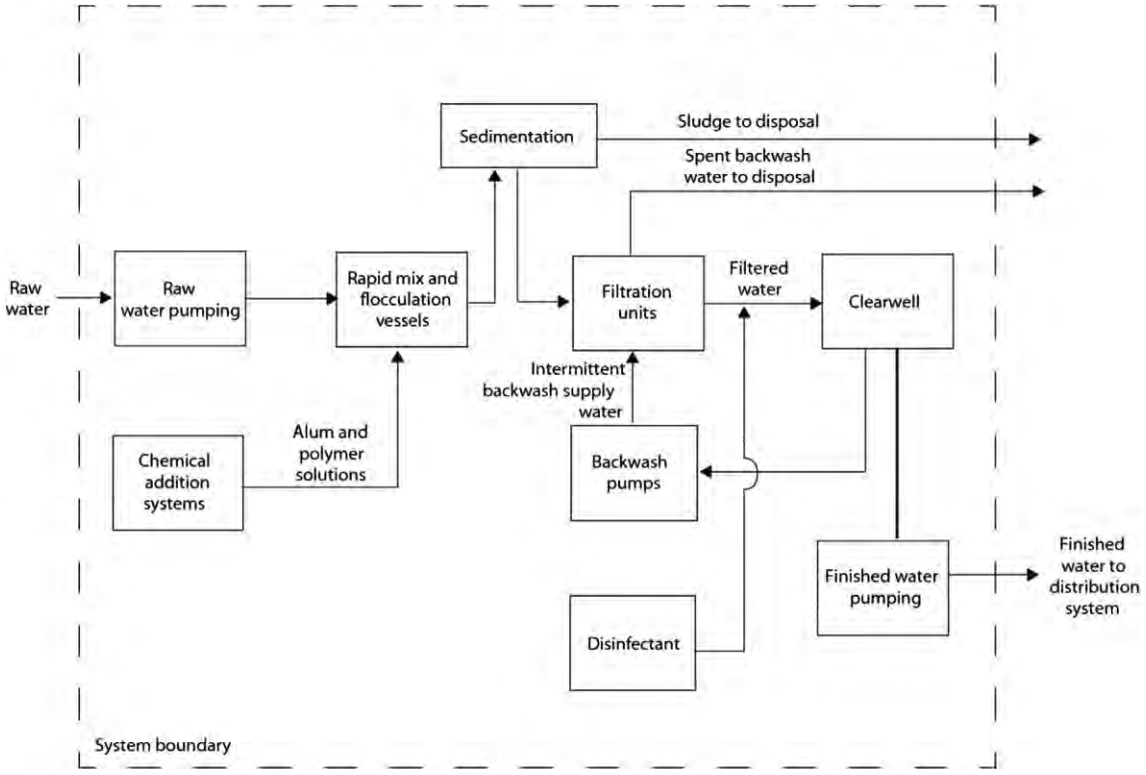

Figure 1 Conventional filtration system for drinking water treatment.

chloride. Often, coagulant aids such as activated silica, bentonite clay, and polyelectrolytes (synthetic polymers of varying charge) are also used to reduce the concentration of primary coagulants employed.

After flocculation, water enters the sedimentation basin(s), where flocs are given time to settle by gravity. Some systems omit this step and feed the flocculated water directly to filters (direct filtration). If the source waters are highly turbid, systems may include an additional sedimentation step prior to coagulation (presedimentation). Sludge in the sedimentation basins is removed and discharged to a municipal sewer, lagooned, or dewatered and hauled to a landfill. The sedimentation basins are generally equipped with sludge removal mechanisms; if not, they usually have a sloping bottom so that most of the sludge flows out with the water when the basin is drained for cleaning.

Filtration

The next treatment step is filtration, which removes suspended and colloidal material that has not settled. The filtration unit consists of steel or concrete vessels containing granular materials such as graded sand, anthracite, and/or gravel. Three types of filters are commonly used: rapid granular filters, slow sand filters, and diatomaceous earth (DE) filters.

Rapid granular filters

The most commonly used filter in the United States is the rapid granular filter. These filters may consist of silica sand, anthracite coal, and/or other materials. Filters are usually set up to provide gradation in filter media, with the coarsest particles on top and the smallest on the bottom. In a rapid granular filter, the rate at which water is applied is at least 2 gal min^{-1} per square foot of surface area.

Rapid granular filters gradually accumulate a large amount of particles that impede water flow; thus, the filters must be periodically cleaned. In this process, water flow through the filter is reversed in a process termed 'backwashing.' Backwashing expands the filter media, thereby releasing trapped particles into the water. Jets of water, air injection, or mechanical agitation at the surface may improve the process. The filter back-wash water should be disposed of in an environmentally acceptable manner.

Slow sand filters

Slow sand filters are similar to the rapid granular filters, except that water flows through the filter at a much slower rate, 0.05–0.15 gal min^{-1} per square foot of surface area, and pretreatment (coagulation, flocculation, and sedimentation) is often omitted. Removal occurs primarily in the upper portion of the sand, by straining, sedimentation, adsorption, and chemical and microbiological action. As contaminants are removed, a layer of deposited material called a 'schmutzdecke' forms. Schmutzdecke contains a large number of bacteria and associated extracellular matrix (biofilm) that break down organic material in the water and acts as an excellent microbial pathogen trap. However, periodically the schmutzdecke clogs the filter, and thus it must be removed by scraping

off the top layer of sand so as to restore sufficient water flow through the sand filter. Normally, slow sand filters are used by systems with relatively clean source water (turbidity of 10 NTU or less and no undesirable chemical contaminants). They require far less maintenance than rapid granular filters, but must be much larger in surface area to produce a given volume of drinking water.

Diatomaceous earth (DE) filtration

In DE filtration, solids are removed by passing water through a thin filter consisting of a layer of DE supported on a rigid base (septum). DE is composed of crushed siliceous shells of diatoms (microscopic algae). To maintain adequate water flow, additional DE called body feed is continually added to the raw water during operation. Like slow sand filters, systems that use DE are usually small with relatively clean source water, and often pretreatment is omitted.

Granular activated carbon (GAC) filters

GAC filters are sometimes used in conjunction with rapid granular filters to control taste and odor problems as well as to remove cyanobacterial toxins and other undesirable chemical contaminants. In Europe, many systems treat the water with ozone (ozonation) before applying it to the GAC filters. Ozonation not only disinfects the water but partly degrades organic contaminants so they are more readily utilized by heterotrophic bacteria in the filter. The high density of bacteria that grow on the surface of the filter media significantly reduces the level of organic substances in the water as it passes through the filter. However, where there is biofilm growth, there are also amoebae and other protozoa/metazoan feeding on bacterial cells. Some of these protozoa may host the amplification of water-based pathogens, such as *Legionella* that may then seed the downstream drinking water distribution system.

Membrane processes

Membrane technology has become increasingly popular in potable water treatment. It has been used for desalination, removal of dissolved inorganic chemicals and various pesticides, water softening, and removal of solids. The simplest way to describe membrane technology is that water is forced through a porous membrane under pressure while suspended solids, larger molecules, or ions are held back or rejected by the membrane.

Membrane processes may be pressure- or electricity-driven. The pressure-driven processes are microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO). During MF water is forced through a porous membrane with nominal pore sizes of 0.2–45 μm, which is relatively large compared with other membrane processes. Hence, MF is suitable for the removal of small particles, including bacteria and protozoa. For UF a membrane with a pore size less than 0.1 mm is use to remove colloids, and when used with <60 k Dalton molecular weight cut-off (MWCO) membrane viruses and high-molecular-weight organic compounds are removed. Indeed, UF is a good way to sample and concentrate enteric viruses from water for further testing. MF or UF will not remove dissolved organic and/or inorganic species without advanced pretreatment to transform the dissolved species into particulate form. NF treatment rejects even smaller molecules than UF, and has been used for water softening and removal of total dissolved solids and viruses. RO systems have the highest rejection rate of all of the membrane processes. It has been used primarily for desalination of seawater. The electricity driven processes are electrodialysis and electrodialysis reversal. Electrodialysis (ED) transfers ions though a membrane as a result of direct electric current, and ED reversal is similar to ED except that the polarity of the direct current process is periodically reversed. These types of systems are used primarily for the treatment of brackish water. Of these processes, NF seems to have the greatest potential for increased use in water treatment.

Disinfection

Disinfection is the primary means for inactivating pathogenic microorganisms in water. It should be noted that disinfection treatments do not sterilize water as viable non-pathogenic bacteria and other microorganisms may still be present. Also, depending on the disinfectant used, some pathogens may persist. For systems using groundwater, disinfection is generally the only treatment practiced. Several disinfectants are available, including chlorine, chloramines (chlorine combined with ammonia or organic amines), ozone, chlorine dioxide, and ultraviolet light. All are oxidizing agents.

Chlorination is, by far, the most common disinfection technique practiced in the United States. The dose of chlorine usually applied is sufficiently high to meet chlorine demand (i.e., the tendency of organic substances and ammonia to react with chlorine) and still leave a sufficient concentration (chlorine residual) to inactivate microorganisms throughout the distribution system. A major shortcoming is that chlorine combines with organic substances in the water to produce disinfection by-products (DBP), some of which are toxic (e.g., trihalomethanes such as chloroform). Unlike chlorine, monochloramine (NH_2Cl) (the only effective chloramine disinfectant) does not result in any known significant trihalomethane formation, but may produce low levels of other more toxic DBPs, such as nitrosamines. The microbial inactivation rates of monochloramine are substantially less than that of chlorine. Chlorine dioxide produces few DBP, but its intermediates (chlorite and chlorate) are toxic. Like chlorine, monochloramine and chlorine dioxide are useful because they provide disinfectant residuals in the water throughout the distribution system.

Ozone is the most effective disinfectant generally available, and is widely used in water treatment in Europe. Fewer toxic by-products have been identified for ozone than for chlorine, but ozone is more expensive and does not leave a significant residual for distribution systems. There is increased interest in the United States in using ozone as a pre-disinfectant, followed by chlorine or monochloramine. This combined approach would allow a system to control the formation of toxic by-products, yet maintain a disinfectant residual in the distribution system.

Mostly used by smaller systems and individual domestic systems (especially those using groundwater), ultraviolet light is also being considered by some larger drinking water treatment facilities to comply with the LT2ESWTR. The optimum wavelength for biocidal effectiveness is 254 nm. Dosage is expressed as the product of radiation intensity (mJ) and the time (s) per unit area (cm^2). Ultraviolet light does not produce disinfectant by-products in water and leaves no disinfectant residual. Its effectiveness is reduced by high turbidity, air bubbles, some dissolved chemicals that absorb UV, the lack of reliable methods or meters to measure dosage, and equipment maintenance and reliability considerations.

It has been demonstrated that UV irradiation is very effective against (oo)cysts of *Cryptosporidium* and *Giardia*. These two pathogenic organisms are of major importance for the safety of drinking water as they exhibit more resistance to chlorination. Results of inactivation studies have shown that UV is effective against all waterborne pathogens; however enteric viruses, specifically adenoviruses, and bacterial spores show some resistance to 254 nm UV inactivation and so-called medium pressure, polychromatic UV treatment is more effective against these microorganisms.

Microbiology of Treatment Processes

The removal of microorganisms by the treatment processes through the filtration step is variable due to a variety of factors, including fluctuating source water quality, coagulant dose, pH, water temperature, depth of filter, filter medium particle size, and filtration rate. Drinking water treatment processes and the approximate percentage removal/inactivation of microorganisms achieved by each of the processes are shown in Table 1. Removal percentages represent only removal percentages for that stage, not the cumulative percentage. It is difficult to establish the cumulative removal percentages from one step to the next because they vary depending on what treatments are used and due to the considerable variation in the data reported from different studies. These differences reflect different source waters and qualities, different treatment processes, and different operating conditions. Thus, direct comparison of study results is difficult, but sufficient studies have been conducted to permit general characterization of the effectiveness of water treatment processes for removing microorganisms.

Slow sand filters

The efficiency of microorganism removal by slow sand filters is influenced by several factors, including the particle size of the filter medium and the extent to which the schmutzdecke has developed. A filter with a smaller particle size is more efficient in microbe removal, but results in shortened filter runs, that is, more frequent filter cleaning is necessary to maintain a suitable water flow rate. In addition, new or newly cleaned slow sand filters require some conditioning (ripening) before they are effective in removing microorganisms. The ripening period allows a biologically active schmutzdecke to build up on the particles and in the filter bed. This layer then assists in the filtration of other particles and colloids from the water. The schmutzdecke is important in microbe removal, particularly for bacteria and viruses. Virus removal by sterile sand is negligible, and removal by clean nonsterile sand is variable but poor.

Removal of coliform bacteria by slow sand filters ranges from about 83% with new filter sand to nearly 100% for sand with an established schmutzdecke. Removal of poliovirus type 1 by slow sand filters ranges from 22% to 96% for clean sand to >99.9% for

Table 1 Microorganism removal efficiency (%)[a]

Unit process	*Bacteria*	*Viruses*	*Protozoa*	*Helminths*
Storage reservoirs	80–90	80–90		
Aeration				
Pretreatment[b]	90–99	90–99	>90	>90
Hardness reduction				
High lime	90–99.9	99–99.9		
Low lime	90–99	90–99		
Slow sand filtration Without pretreatment[b]				
	35–99.5	10–99.9	59–94	
With pretreatment[b]	90–99.9	90–99.9	59–99.98	
Rapid granular filtration				
Without pretreatment[b]	0–90	0–50	0–90	
With pretreatment[c]	90–99	90–99	90–99	
With pretreatment[b]	90–99.9	90–99	90–99.9	
Diatomaceous earth filtration[d]	90–99.9	99–99.96	99–99.999	
Activated carbon		10–99		
Microfiltration	<99.999	<99	<99.9999	

Pretreatment, filtration, and disinfection. (Reprinted in part from Amirtharajah A (1986)

[a]Removal can also expressed using log units. 1 log = 90% removal, 2 log = 99% removal, 3 log = 99.9% removal, and so on.

[b]Pretreatment includes coagulation, flocculation, and sedimentation.

[c]Pretreatment, except no sedimentation.

[d]With pretreatment and precoating of filter.

sand with an established biological population. Removal of *Giardia* cysts (protozoan) by slow sand filtration ranges from 59% to 99.98%.

Cold water temperatures normally decrease microorganism removals by slow sand filtration. At water temperatures <5 °C, removal of heterotrophic bacteria (measured by HPC) and coliforms decreases by about 2 and 2–10%, respectively, compared with removals at temperatures greater than 15 °C. Virus removals decrease by about 0.5% and protozoan cyst removals decline by 0–6.2%. Increased water flow through the filter (filtration rate) also results in decreased microorganism removals.

Rapid granular filters

Systems that use rapid granular filtration must first pretreat the raw source water (coagulation, flocculation, and sedimentation) for effective microbial removal, unless the water is clear (turbidity < 10 NTU). Effective pretreatment should reduce the turbidity of muddy surface water to a level well below 1 NTU. Effective pretreatment and filtration collectively should reduce the turbidity to 0.1 NTU or less.

Microorganism removals achieved by pretreatment and rapid granular filtration are high. Bacterial removals (heterotrophic bacteria and coliforms) range from 86% to 98.8%, and virus removals range from 90% to >99.99%. The coagulation and filtration processes generally can achieve removals of protozoan cysts ranging from 83% to 99.99%. Efficient removal of Giardia and other protozoan cysts by filtration is dependent on an adequate coagulant dose. A change in alum dose from 5 to 10 mg l^{-1} can increase the removal of *Giardia* cysts from 96% to >99%.

Factors that adversely affect removal efficiency include interruption of chemical feed (coagulants and polymers), poor filter efficiency at the beginning of a filter run, sudden increases in water flow, and turbidity breakthrough that can occur with higher filter head loss (resistance to water passage through the filter) at the end of a filter run. Any of these factors can seriously degrade the microbiological quality of the filtered water. In addition, some source waters are so highly polluted that full treatment (pretreatment, filtration, and disinfection) may not achieve a suitable level of microbiological reduction.

Diatomaceous earth

Bacterial removals by DE filtration are affected by the grade of DE used. When fine DE is used that yields a median pore size of 1.5 mm in the filter cake, bacterial removals of nearly 100% can be achieved. Lower percentage removals occur when coarser DE is used that provides increased median pore size. However, by chemical conditioning of coarser grades of DE, good bacterial removals can be obtained.

DE filtration can satisfactorily remove viruses from water, but to be most effective, the raw water must be pretreated with a coagulant aid (polymer) or the DE filter cake must be chemically conditioned to enhance virus attachment to the filter material during filtration. Overall, DE filtration is most effective for removal of microorganisms in the size range of *Giardia* or *Entamoeba histolytica* cysts. *Giardia* cyst removals of 99% or greater can be achieved by proper DE filter operation. The use of DE to remove smaller organisms (bacteria and viruses) can be improved by coating it with aluminum hydroxide precipitate. This coating gives the DE a positive surface electrical charge. Bacteria and viruses with a negative electrical charge are then removed by surface attachment.

Membranes

Membrane processes can be effective at removing various microbial contaminants. For example, MF and UF are generally capable of removing 6–7 logs of protozoan cysts and greater than 5.5 logs of bacteria. Lower removal rates are achieved for viruses: less than 2.5 logs removal.

Disinfection

General considerations

The final treatment process for drinking water to address microbial contamination is chemical or physical disinfection intended to inactivate pathogens that enter the distribution system. The effectiveness of disinfection is a function of the types of organisms to be inactivated, the quality of the water, the type and concentration of the disinfectant, the exposure or contact time, and the temperature of the water.

As stated previously, *CT* values are used to identify the level of inactivation provided by a given disinfectant for an organism under a specific environmental condition. These values are useful for comparing biocidal efficiency. Table 2 provides *CT* values for several microorganisms. Most of the available *CT* data for microorganisms of health concern were developed from laboratory studies that might not be indicative of actual *CT* values achieved in operating water treatment plants.

Water temperature can affect disinfection rates (and thus *CT* values). Microorganism inactivation rates decrease as water temperature decreases. Water pH can also affect disinfection rates. In most water systems, the pH is kept in the range of 7–9. Water pH, for example, determines the proportions of the most important chlorine species, hypo-chlorous acid (HOCl) and hypochlorite (OCl^-). Lower pH values (pH 6–7) result in the formation of HOCl, which is favorable for rapid inactivation, whereas higher pH values (pH 8–10) result in formation of OCl^-, which results in slower inactivation. For chlorine dioxide (ClO_2), which does not dissociate, inactivation is more rapid at higher pH values (pH 9) than at lower pH values (pH 7). Ozone disinfection efficacy does not appear to be affected by pH.

An important factor in bacterial inactivation is the phenomenon of cell injury. Disinfection and other environmental stresses may cause nonlethal physiological injury to waterborne bacteria. This phenomenon causes problems for monitoring water quality and calculating *CT* values, because injured bacteria may not grow on selective media normally used to detect or enumerate the

Table 2 *CT* value ranges for inactivation of microorganism by disinfectants

Microorganism	*Free chlorine (pH 6–7)*	*Preformed chloramine (pH 8–9)*	*Chlorine dioxide (pH 6–7)*	*Ozone (pH 6–7)*
E. coli	0.034–0.05	95–180	0.4–0.75	0.02
Poliovirus-1	1.1-2.5	768–3740	0.2–6.7	0.1–0.2
Rotavirus	0.01–0.05	3806–6476	0.2–2.1	0.006–0.06
Phage f2	0.08–0.18	ND	ND	ND
G. lamblia cysts	47–>150	2200[a]	26[a]	0.5–0.6
G. muris cysts	30–630	1400	7.2–18.5	1.8–2.0
Cryptosporidium parvum	7200[b]	7200[c]	78[c]	5–10[b]

ND – No data.
All *CT* values are for 99% inactivation at 5 °C except where noted.
[a]Values for 99.9% inactivation at pH 6–9.
[b]99% inactivation at pH 7 and 25 °C.
[c]90% inactivation at pH 7 and 25 °C.

bacteria. Thus, the actual number of viable cells may be underestimated. In some cases, injured pathogens remain infective. Problems with detecting injured cells can be mitigated by the use of media and procedures that remain selective, yet permit the injured cells to repair metabolic damage.

Microorganism inactivation

Table 2 shows the effect of disinfectant concentration, contact time, pH, and temperature on microbial inactivation.

Chlorine Table 2 shows that enteric viruses (represented by poliovirus type 1) are more resistant to inactivation by chlorine than are bacteria (represented by *E. coli*), and protozoan cysts are nearly two orders of magnitude more resistant than the enteric viruses. *Cryptosporidium* oocysts are virtually unaffected.

Monochloramine Comparison of monochloramine with chlorine for disinfection of microorganisms (Table 2) shows that, in general, for all types of microorganisms, *CT* values for monochloramine are higher than *CT* values for free chlorine species.

Chlorine dioxide Chlorine dioxide *CT* values in Table 2 show that at pH 7.0, ClO_2 is not as strong a biocide as HOCl. However, as the pH is increased, the efficiency of ClO_2 for inactivation of viruses increases.

Ozone Overall, comparison of *CT* values for ozone with those for chlorine and ClO_2 indicates that ozone is a much more effective biocide than the other disinfectants. *E. coli* is about tenfold (1 $\log_{10}$) more sensitive to ozone (Table 2) than poliovirus type 1. *Giardia muris* cysts are about tenfold more resistant to ozone than poliovirus type 1. Since ozone is a powerful oxidant, it reacts rapidly with both microorganisms and organic solutes and is very useful as a primary disinfectant.

The order of microbial disinfectant efficiency is $O_3 > ClO_2 > HOCl > OCl^- > NH_2Cl > NHCl_2 > RNHCl$ (organic chloramines). However, for technical reasons, practical handling considerations, cost and effectiveness, the frequency of use of disinfectants by utilities in the United States is generally chlorine > chloramines > O_3 > ClO_2.

Ultraviolet Light Sensitivity of the various microbial groups to ultraviolet light is similar to that for chemical disinfectants. For example, enteric bacteria are known to be more sensitive than enteric viruses and protozoan cysts. Organisms that are sub-lethally injured by UV light exposure may, under appropriate conditions, be able to repair the damage (i.e., photoreactivation or dark repair). Ranges of UV doses required for 99.9% inactivation of microorganisms of concern in drinking water are: bacteria, 7–167 mJ cm^{-2}; viruses, 16–167 mJ cm^{-2}; protozoan oo(cysts), 11–119 mJ cm^{-2}.

Distribution Systems

Description

Water transmission and distribution systems are needed to deliver water to the consumers. In 2006, the US National Academy of Sciences published a report, "Drinking Water Distribution Systems: Assessing and Reducing Risks" that recommended the following measures to control waterborne pathogens entering the distribution system.

1. Storage facilities should be inspected on a regular basis.
2. Better sanitary practices are needed during installation, repair, replacement, and rehabilitation of distribution system infrastructure.
3. Water residence times in pipes, storage facilities, and premise plumbing should be minimized.
4. Positive water pressure should be maintained.
5. Distribution system monitoring and modeling are critical to maintaining hydraulic integrity.
6. Microbial growth and biofilm development in distribution systems should be minimized.
7. Residual disinfectant choices should be balanced to meet the overall goal of protecting public health.
8. Standards for materials used in distribution systems should be updated to address their impact on water quality, and research is needed to develop new materials that will have minimal impacts.

9. Although it is difficult and costly to perform, condition assessment of buried infrastructure should be a top priority for utilities.
10. Cross-connection control should be in place for all water utilities.
11. Where feasible, surge protection devices should be installed.
12. Prior to distribution, the quality of treated water should be adjusted to minimize deterioration of water quality.

Distribution systems represent the major investment of a municipal water works and consist of large mains that carry water from the source or treatment plant, service lines that carry water from the mains to the buildings or properties being served, and storage reservoirs that provide water storage to meet demand fluctuations, for firefighting use, and to stabilize water pressure. The branch and loop (or grid) are the two basic configurations for most water distribution systems.

The layout of a branch system is similar to that of a tree branch, with smaller pipes branching off from larger pipes throughout the area served. This system, or a derivative of it, is normally used to supply rural areas where water demand is relatively low and long distances must be covered. Disadvantages of this configuration are the possibility that a large number of customers will be without service should a main break occur, and the potential water quality problems in parts of the system resulting from the presence of stagnant water. System flushing should be accomplished at regular intervals to reduce the possibility of water quality problems.

The loop configuration currently is the most widely used distribution system design. Good design practices for smaller systems call for feeder mains to form a loop approximately 1 mi (1600 m) in radius around the center of the town with additional feeder loops according to the particular layout and geography of the area to be served. The area inside and immediately surrounding the feeder loops should be gridded with connecting water mains on every street.

The most commonly used pipes for water mains are ductile iron, pre-stressed concrete cylinders, polyvinyl chloride (PVC), reinforced plastic, steel, and asbestos cement.

Microbiology

Microbiologically, water distribution systems are interesting microbial ecosystems that present a real challenge to the water utilities in terms of maintaining good-quality water with low microbial densities. The construction characteristics, operation, and maintenance of a water distribution system provide ample opportunities for microbial recontamination of the treated water during distribution. Pipe joints, valves, elbows, tees, and other fittings as well as the vast amount of pipe surface provide both changing water movement and stagnant areas where bacteria can attach and colonize. Water distribution systems are susceptible to intrusion, backflows and mains repairs that may allow entry of pathogens into the system. A cross-connection is any direct or indirect connection between the drinking water distribution system and any non-potable fluid or substance, when the water mains pressure is reduced by back siphoning or back pressure.

Biofilms in water distribution systems

Microbes found in water distribution systems can be classified into indigenous (autochthonous) and exogenous (allochthonous) populations. The indigenous organisms are well-adapted biofilm-forming bacteria that represent a stable ecosystem that is difficult to eradicate. The exogenous bacteria are contaminants that are transported into the system by a variety of mechanisms.

The development of a permanent biofilm in the distribution system occurs because several microbial groups find physical and chemical conditions conducive to colonization and growth at the solid surface/water interface. These conditions include an ample supply of nutrients (such as measured by assimilable organic carbon, AOC) for growth, a relatively stable temperature, and some degree of protection from exposure to harmful chemicals such as water disinfectant(s).

When an adequate disinfectant residual is maintained throughout the distribution system, microbial growth is usually well controlled and the density of bacteria in the bulk water traveling through the pipes will remain low – in the range of <10 to several hundred CFU ml^{-1} (HPC). The density of bacteria in the biofilm, however, may be several orders of magnitude higher, ranging upward of 1×10^5 CFUcm^{-2} (HPC). The disinfectant residual concentration needed to control the growth of the bacteria varies from one water system to another.

The choice of the medium and method used to determine the bacterial density may result in low or high counts of heterotrophic bacteria. In general, rich culture media and incubation at 35 °C will yield lower counts than dilute nutrient media and incubation at 20–28 °C. Factors that appear to be critical are pH, temperature, dissolved organic carbon (DOC) concentration, AOC concentration, and type of disinfectant used. The disinfectant residual will also reduce or suppress extensive biofilm growth if the residual is maintained throughout the system. Nonetheless, only a small fraction of bacteria are culturable. Methods that measure adenosine triphosphate (ATP) can provide an idea of the total viable microbial biomass in a drinking water sample. Recently, RNA-based methods have been developed to measure and identify the active bacteria present in drinking water.

Disinfectant residuals should be at least 0.2 mg l^{-1} for chlorine and 0.4 mg l^{-1} for monochloramine (NH_2Cl). Higher concentrations of disinfectant residual may be applied and maintained, but if the water contains high levels of DOC, it may be difficult to maintain an adequate disinfectant residual to control bacterial growth and still have water that is aesthetically acceptable to the consumers. Bacterial concentrations in distribution water vary from <1 CFU ml^{-1} in the water leaving the treatment plant to as high as 10^5–10^6 CFU ml^{-1} in water from slow flow or stagnant areas of the distribution system. The concentrations of bacteria in the water and on the pipe surfaces vary spatially and temporally in the distribution system. Bacterial densities in the pipe wall biofilm and in sediments may reach 10^7 CFU cm^{-2}. The biofilm contributes bacteria to the flowing water through shear loss (erosion) and by migration of actively motile bacterial cells into the water.

Some of the bacteria commonly found in drinking water, sediments, and biofilms are listed in Table 3. Many of these bacteria are found in both the water and the biofilm, indicating the influence of the biofilm on the bacterial quality of distribution system water. Indeed, biofilms are where the most significant bacterial growth occurs in the distribution system. Moreover, such multispecies biofilms form relatively protected microniches where pathogens can seek refuge. Bacteria in drinking water are detected and enumerated using traditional culture media and methods (coliforms, thermotolerant coliforms, heterotrophic plate count, and so on). The recent development of culture-independent molecular methods (DNA- and RNA-based) has begun to reveal the great diversity of bacteria in water, biofilms, and sediments. Genes of many bacteria that cannot yet be cultured have been detected using the DNA- and RNA-based methods.

Iron and sulfur bacteria are 'nuisance' organisms that cause taste and odor problems and are often associated with groundwater sources. Nitrifying bacteria and fungi cause problems in chloraminated drinking water systems, including depletion of total chlorine residual, conversion of reduced nitrogen compounds such as ammonia to nitrite and nitrate, and high levels of heterotrophic bacteria. Invertebrates and protozoa are nuisance organisms associated with aesthetic complaints about water quality. Invertebrates and protozoa may also harbor pathogenic and nonpathogenic bacteria, either internally or attached to their surfaces, and this association provides the bacteria with some degree of protection from the inactivation by disinfectant residuals. http://www.epa.gov/ogwdw/disinfection/tcr/pdfs/whitepaper_tcr_biofilms.pdf

Information about the development and control of biofilms in drinking water is sparse (EPA, 1992). In addition, there is meager data on other issues related to biofilms, including the role of iron and sulfur bacteria in biofilm development, the role of biofilms in the corrosion of pipe material, the role of biofilms in nitrification for systems that use chloramines as a secondary disinfectant, and the effect of added corrosion inhibitors on bacterial populations in biofilms.

The presence of chlorine or chloramine retards the development and affects the spatial distribution of biofilm. The type of disinfectant residual provided selects for bacteria that are more tolerant to the specific disinfectant used. The biofilm environment provides protection for the cells by diffusional resistance and neutralization of the disinfectant. Therefore, biofilm organisms are less inhibited by the disinfectant residual than are planktonic cells. Differential effectiveness of chlorine and monochloramine for control of biofilm growth has been shown. Monochloramine, because it is less reactive and therefore more persistent, apparently can penetrate the biofilm and is more effective than chlorine in controlling biofilm growth.

In some cases, coliforms that gain entry into the distribution system may attach to pipes or pipe sediments and proliferate, thus becoming a biofilm constituent. The intermittent, sporadic, or persistent sloughing of coliform bacteria from biofilms into the water of the distribution system may cause systems to repeatedly violate standards for total coliforms. This problem is most frequently associated with utilities that use a surface water supply where the water temperature is $\geq$ 15 °C. Public safety dictates that all coliforms detected by testing be regarded as representing system vulnerability, unless strong evidence suggests otherwise.

The utility should review its treatment operations and increase monitoring of the quality of water entering the distribution system to be sure that inadequate or failed treatment is not responsible for the total coliform occurrences and that E. coli is not

Table 3 Examples of microorganisms found in treated distribution water, sediment, and distribution system biofilm as reported in the peer-reviewed scientific literature

Microorganism	*Distribution water*	*Sediment*	*Biofilm*
Pseudomonas spp.	X	X	X
Alcaligenes spp.	X		X
Acinetobacter spp.	X		X
Moraxella spp.	X	X	X
Arthrobacter spp.	X	X	X
Corynebacterium spp.			X
Bacillus spp.	X	X	X
Enterobacter spp.	X	X	X
Micrococcus spp.	X		
Flavobacterium spp.	X	X	X
Klebsiella spp.	X	X	X
Mycobacterium spp.	X	X	X
Porphyrobacter	X		
Blastomonas	X		
Sphingomonas	X		
Bosea	X		
Phenylobacterium	X		
Delftia	X		
Iron/sulfur bacteria	X	X	X
Nitrifying bacteria	X	X	X
Yeasts/fungi	X	X	X
Invertebrates/protozoa	X	X	X
Enteric viruses	X	X	X

present in the water. The water utility should also review the operation and management of the water distribution system to ensure the absence of *E. coli*, a disinfectant residual is present, an adequate cross connection control program is in effect, and maintaining adequate pressure to prevent intrusion of microbial contaminants. Finally, the water utility should insure that large volumes of water held in storage in reservoirs, standpipes, or above ground tanks are not the source of the total coliform problem.

During warm-water periods, it is more difficult to maintain a disinfectant residual in the water in all parts of the distribution system. With increasing temperature, the disinfectant reacts more rapidly with dissolved organic chemicals in the water and in the biofilm, and bacteria grow faster. Increased reaction rates and bacterial growth are often compounded by a lack of knowledge of system hydraulics and of the actual water movement throughout the system. In many cases, there are large areas where minimal movement of the water occurs. Many water utilities may create microbial or chemical contaminant problems by their failure to understand how their systems work; better system management can reduce those problems.

Biofilms in household and building plumbing systems

Biofilms develop in household and building plumbing systems and can degrade water quality more rapidly and to a greater extent than in the distribution mains. In such systems, pipe materials, long residence times, stagnation/ low-flow conditions, residual disinfectant concentration and water temperature may differ considerably from those found in the main distribution piping. Increased disinfectant consumption in household and building networks may be related to the smaller diameters of service line piping, which results in a greater surface-to-volume ratio than in the pipes of the main distribution system. Depletion of the disinfectant residual due to water stagnation, organic deposits on the pipe surfaces and increased water temperature result in increased biofilm bacterial growth.

In general, there is little evidence that high levels of heterotrophic bacteria in drinking water from plumbing systems have adverse health effects. However, for specific organisms such as *Legionella pneumophila* and nontuberculous *Mycobacterium* spp. that have been found as part of the biofilm in such systems, there is evidence of adverse health effects. Plumbing system components such as hot water storage tanks and shower heads may permit significant bacterial growth. For example, outbreaks of Legionnaire's disease in healthcare facilities have been related to amplification of *L. pneumophila* in hot-water storage tanks, shower heads and drinking waters used in dentistry.

In the case of nontuberculous mycobacteria, some evidence suggests that the type of residual disinfectant used in the drinking water treatment process may select for colonization of biofilms by these organisms, and species found in treated drinking water have been linked to human infections in immunocompromised patients.

Alternative Water Sources

In areas where both surface and ground-water sources may not be suitable as potable source water, other sources of drinking water may be necessary. Bottled water is one alternative source, but is very expensive and generates additional waste (plastic bottles); therefore this source of drinking water is not viewed as a sustainable long-term solution.

Bottled Water

The bacterial quality of noncarbonated bottled water varies both among brands and from lot to lot within a brand. Chemical quality varies as well. Bottled water in the United States is usually treated by RO, ozone, and/or ultraviolet light. Bacterial densities in noncarbonated bottled water, determined by plate count methods, vary from 0 to $>1.0 \times 10^5$ CFU ml^{-1}. Fresh noncarbonated bottled waters often have low bacterial densities, but during storage the bacteria can multiply, often by several orders of magnitude. Carbonated (sparkling) bottled waters generally have low or no detectable bacteria because of the low pH. In most bottled waters examined, coliform bacteria have been absent. Bottled water quality is regulated by the US Food and Drug Administration, but must comply with EPA drinking water regulations to the extent it is possible to do so. It should be noted that bottled water regulations include many of the same maximum levels as the drinking water regulations, although they do not include maximum levels for all regulated contaminants. For instance, FDA does not regulate contaminants that are considered primarily distribution system contaminants because bottled water is not subject to distribution system contamination potential (e.g., bottle water is not expected to contain *Cryptosporidium*).

Emergency Drinking Water

Treatment of water for drinking use in an emergency generally involves either boiling the water or chemically disinfecting the water using liquid chlorine laundry bleach, tincture of iodine, or iodine or chlorine tablets. Water to be disinfected should be strained through clean cloth to remove particles and floating matter.

Boiling is the most effective means of inactivating pathogens. For heat disinfection, the strained water should be boiled vigorously for at least one minute and allowed to cool before use. For chemical disinfection, add liquid chlorine bleach (6–8.25% chlorine) at a rate of two drops (clean clear water) or four drops (cloudy water) per quart, mix thoroughly, and allow to stand for 30 min. A slight chlorine odor should be detectable in the water; if not, repeat the dosage and let stand for an additional 15 min before using. If tincture of iodine is used, add five drops (clean clear water) or ten drops (cloudy water) of 2% tincture of

iodine solution per quart of water. Allow the water to stand for 30 min before use. For chlorine or iodine tablets (obtained from drug or sporting goods stores), follow the instructions on the package. Keep all purified water in clean closed containers. Chlorine and iodine may not inactivate the protozoan pathogens, especially *Cryptosporidium*.

Point-of-Use/Point-of-Entry Treatment

Point-of-use/point-of-entry treatment (POU/POE) devices (or filters) are small package units designed to treat water at a designated tap in a household or to treat all of the water entering the household. These devices are becoming increasingly sophisticated and are growing in popularity. POU devices are generally placed under a sink and POE devices might be located in a basement or a garage. Currently POU/POE treatment is used to reduce the levels of a wide variety of contaminants in drinking water, including organic material, turbidity, fluoride, iron, radium, chlorine, arsenic, nitrate, ammonia, and pathogens (including cysts/oocysts). Taste, odor, and color can also be improved with POU/POE treatment.

The types of technology that may be used in POU/ POE treatment devices include adsorption, ion exchange, RO filtration, chemical oxidation, distillation, aeration, and disinfection such as chlorination, iodination, ozonation, and ultraviolet light. Activated carbon filtration, a type of adsorption device, is the most widely used POU system for home treatment of water. The performance of an individual unit depends on a combination of factors, such as unit design, type and amount of activated carbon, and contact time of the water with the carbon. Most units use GAC in their design, although other types of carbon such as pressed block, briquettes of powdered carbon, and powdered carbon are available.

Bacterial growth in activated carbon units may be a problem. The organic material adsorbed onto the carbon provides nutrients for bacterial growth. Stagnation periods between operations allow time for bacteria to grow and high densities will develop in newly installed filters within 2 weeks. To help minimize this problem, some units contain GAC impregnated with silver or some other bacteriostatic agent. Silver, although useful against some bacteria (e.g., coliforms and related enteric bacteria) as a bacteriostatic agent, does not effectively inhibit the growth of all bacteria in GAC, POU, or POE filters. Bacterial levels in water leaving GAC filters, with or without silver, range from 10^2 to 10^5 CFU ml^{-1}, but it may take longer for bacterial levels in a GAC filter with silver to reach the higher concentrations. When a GAC filter is used as the key treatment technique and the incoming water is not microbiologically safe, water from the unit should be disinfected either chemically or by UV irradiation to reduce bacterial levels in the water and to inactivate opportunistic pathogens that may colonize the carbon particles. Silver resistant nontuberculous mycobacteria have been shown to colonize point of use water filters containing silver, suggesting a potential risk to immunocompromised persons who consume water from such devices.

Some carbon block units do not contain silver but claim bactericidal effects, probably due to straining through small carbon pore size (0.5 μm). Some POU/ POE systems use a disinfectant such as ozone or UV irradiation to control bacteria.

Household Water Treatment in Developing Countries

Developing countries often lack the resources for the types of water treatment systems used in developed countries. The WHO, US Centers for Disease Control and Prevention, and other organizations are developing and testing simple and inexpensive alternatives that households in such countries can use to control waterborne disease. Among the more promising alternative disinfection methods being tested and used are boiling, solar disinfection using clear plastic containers (combined UV radiation and thermal effects), solar disinfection using dark, or partially dark, opaque containers (thermal effects alone), UV lamps, chlorine tablets, and liquid solutions of sodium hypochlorite.

Sometimes, a filtration process is needed, given that high organic matter levels in the water affect chlorine efficiency and high water turbidity levels affect UV treatment efficiency. Simple filtration processes used by households in the developing regions include adsorption by clay, activated carbon, charcoal, porous ceramic filters, fibers, cloth, paper, and crushed vegetative matter such as seeds. To minimize post-treatment contamination, the WHO recommends household water storage in narrow-mouth containers with a spigot or spout.

The candidate treatment alternatives mentioned above are by no means exhaustive. The alternative selected depends upon such issues as raw water quality, treatment effectiveness and reliability, and availability of resources such as electricity (e.g., for UV lamps), wood or other fuel (for boiling water), and hours and days of sunlight per year (solar heating). It also depends upon broader issues such as affordability, technical simplicity, education level, cultural acceptance of candidate alternatives, and the prospect for continuing the use of candidate alternatives over time. More information on household treatment systems in developing countries is available at http://www.who.int and http://www.cdc.gov.

Other Issues Associated with Water Treatment

Drinking Water Microbial Diversity

Traditionally, the microbial water quality of distribution systems has relied on culture-based methods that target fecal indicator bacteria and heterotrophic bacteria. Disinfectants can kill most of the pathogenic microorganisms and the surrogate indicators but a fraction may persist and not accounted for as they lose their ability to grow on selective media. Some drinking water bacteria may enter a viable but nonculturable (VBNC) status, although the health risks impacts of VBNC bacteria is poorly understood.

Additionally, the bacterial composition of drinking water has been based solely upon bacteria that can form colonies on agar media under a narrow set of incubation conditions. Only a small fraction of the bacterial community can grow under such conditions, resulting in a biased picture of the *in situ* microbial diversity. A similar situation holds for the viruses and fungi. Moreover, there are no culture-based methods to detect or enumerate some microbial groups relevant to public health (e.g., noroviruses). Detection of protozoan parasites normally relies on the use of cumbersome and time-consuming microscopic techniques.

Recently developed molecular tools have circumvented some of the aforementioned limitations and have deepened our understanding of the microbial community dynamics in water distribution systems. For example, DNA-based sequence analyses of the 16S rRNA gene have revealed a rather complex microbial community composed of different bacterial groups, mostly dominated by alpha-, beta-, and gamma-Proteobacteria and Actinobacteria (primarily mycobacteria) species (Figures 2 and 3). These methods have shown that the bacterial community structure can vary within a distribution system. Moreover, the bacterial community is dynamic as it can change from season to season and in response to changes in disinfectant. Eukaryotic microorganisms have also been identified using similar approaches. Among some of the eukaryotes identified are different amoebae, ciliate, metazoan, fungi, and cercozoan species. Next generation sequencing methods are now used in conjunction with barcoded primers to simultaneously describe the composition of hundreds to thousands of drinking water samples simultaneously. This facilitates addressing complex questions via robust experimental design. Sequence analyses and databases are not only important to confirm the identity of the residing microbiota but also to develop novel methods that specifically detect the different microbial groups that reside in distribution systems.

Several methods have been used to detect microbes in drinking water, often aided by the amplification of specific genetic markers via polymerase chain reaction (PCR). Such methods have detected bacteria closely related to public health-relevant genera such as *Legionella* and *Mycobacterium* in drinking water (Figure 2). While species of the latter genera have been associated with waterborne illness, their detection does not necessarily imply a health risk or a deficiency in water treatment, as most species within these genera are not pathogenic to humans. Nucleic acid-based methods have also detected fastidious organisms in drinking water such as nitrifying bacteria, whose presence may degrade drinking water quality and thus may reflect inadequate water treatment practices.

Studies using methods targeting phospholipid fatty acids and RNA have demonstrated that different disinfection treatments (i.e., chlorination vs. chloramination) modify the characteristics of the active and dynamic microbial community in drinking water biofilms. Unlike DNA, microbial phospholipid fatty acids and RNA are thought to degrade fairly rapidly in environmental settings and thus their detection can be used as evidence for the presence of metabolically active cells and hence disinfection efficiency in drinking water. For most bacterial groups phospholipid analyses however do not provide the resolution needed to identify bacteria at the species level. Thus the use of RNA based methods to identify active bacterial species promises to be an important tool in the future of drinking water microbial water quality (Revetta et al 2010). RNA has also been shown to increase the detection limits of fecal bacteria in treated source water (Pitkänen et al., 2013).

The advent of next generation sequencing methods is providing other unique opportunities such as the possibility of sequencing the entire genome of different microbial species autochtonous to distribution systems. Understanding the genetic makeup of autochtonous bacteria can lead to a better understanding of their survival capabilities, discovery of factors that can be used to promote their growth in artificial media, how they might interact with other members of the community, and their potential link to health risks. Novel sequencing methods are now also used to get an idea of all the different genes associated with a microbial sample (metagenomics) as well as the genes that are transcribed (metatranscriptomics). Metagenomic studies have already provided a significant amount of information on the genetic functional network in a sample, including the presence of antibiotic resistance mechanisms that would have been difficult to unveil (Gomez-Alvarez et al., 2012)). Metagenomic sequencing can also help us construct entire microbial genomes without the need of relying on the culturability of the organism or availability of culturable methods. In the future, metatranscriptomics will be of great value at assessing which metabolic pathways are responding to the different drinking water conditions and to further confirm the identity of the active microbial members within drinking water samples. For those bacterial pathogens that interact with amoeba, metagenomic sequencing will help better understand the genes responsible for infectious pathways and to survival mechanisms with host cells.

Source Water Protection

Preventing the pollution of waterbodies that serve as sources of drinking water (e.g., groundwater, lakes, and rivers) is becoming an important challenge to both developed and developing countries. Source water pollution has a direct impact on treatment plants, particularly as the level of pollution can have an effect on the removal and disinfection efficiency of enteric pathogens and their eventual presence in the distribution system. In the United States, under the Clean Water Act, each state sets water quality standards that include limits on the concentration of specific pollutants that can be present in an ambient water body (usually a lake or a river) and this limit depends on its designated use (e.g., swimming, boating, fishing, drinking water source, and shellfish harvesting). To limit pathogens, fecal indicator bacteria such as *E. coli* or enterococci are normally used. Discharges of the fecal indicator into that water body are set at levels that meet water quality standards. One goal of this requirement is to limit the level of fecal matter that reaches a water system intake. This limit facilitates treatment and reduces the health risk in the event of a treatment failure. In spite of these standards, a significant portion of US surface waters exceeds regulatory limits.

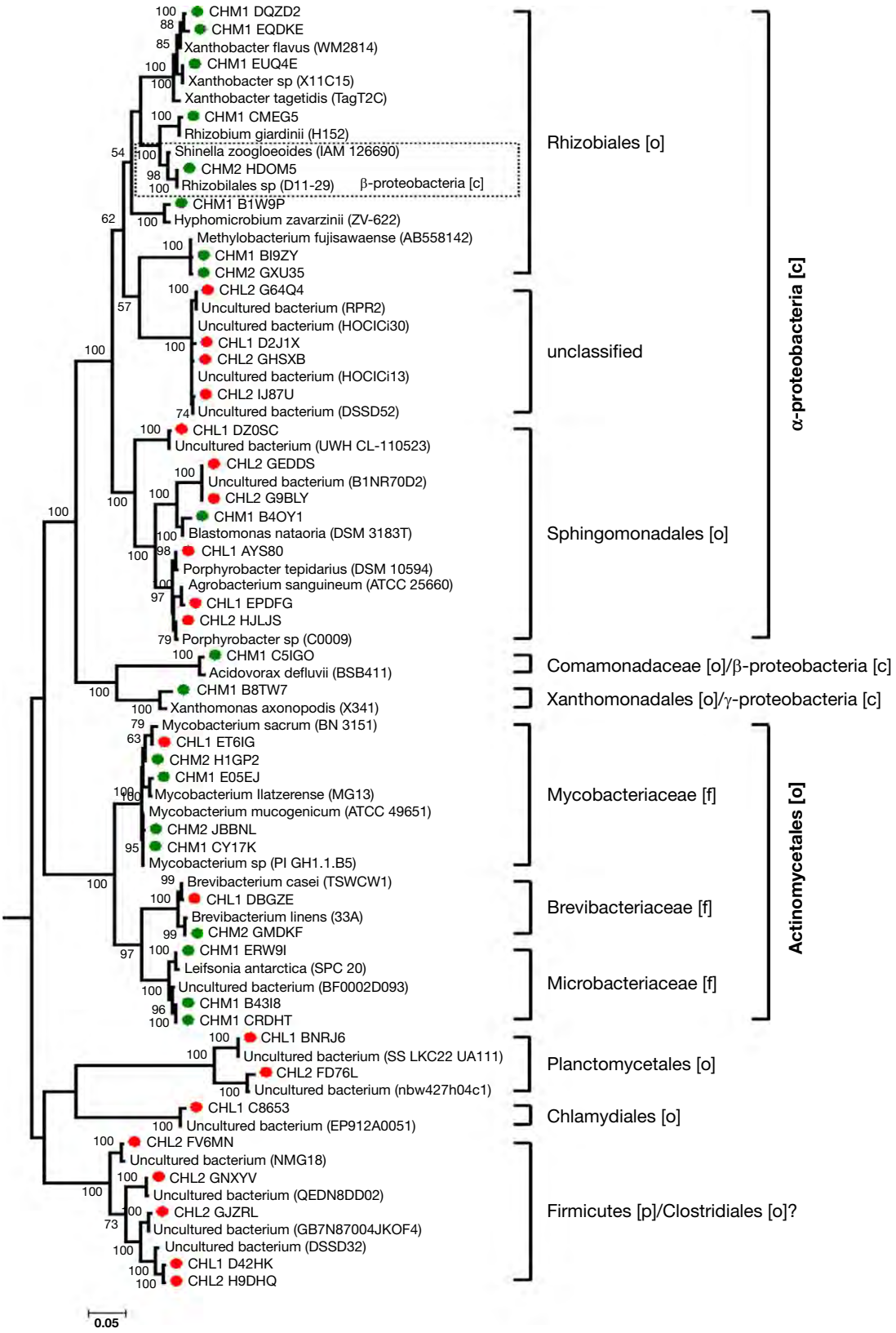

Figure 2 Phylogenetic affiliation of 16S rRNA sequences recovered from bulk water metagenomes: chloraminated (CHM, green) and chlorinated (CHL, red). Reads were identified by phylum [p], class [c], order [o], and family [f]. The tree was inferred using maximum likelihood analysis of aligned 16S rRNA gene sequences.

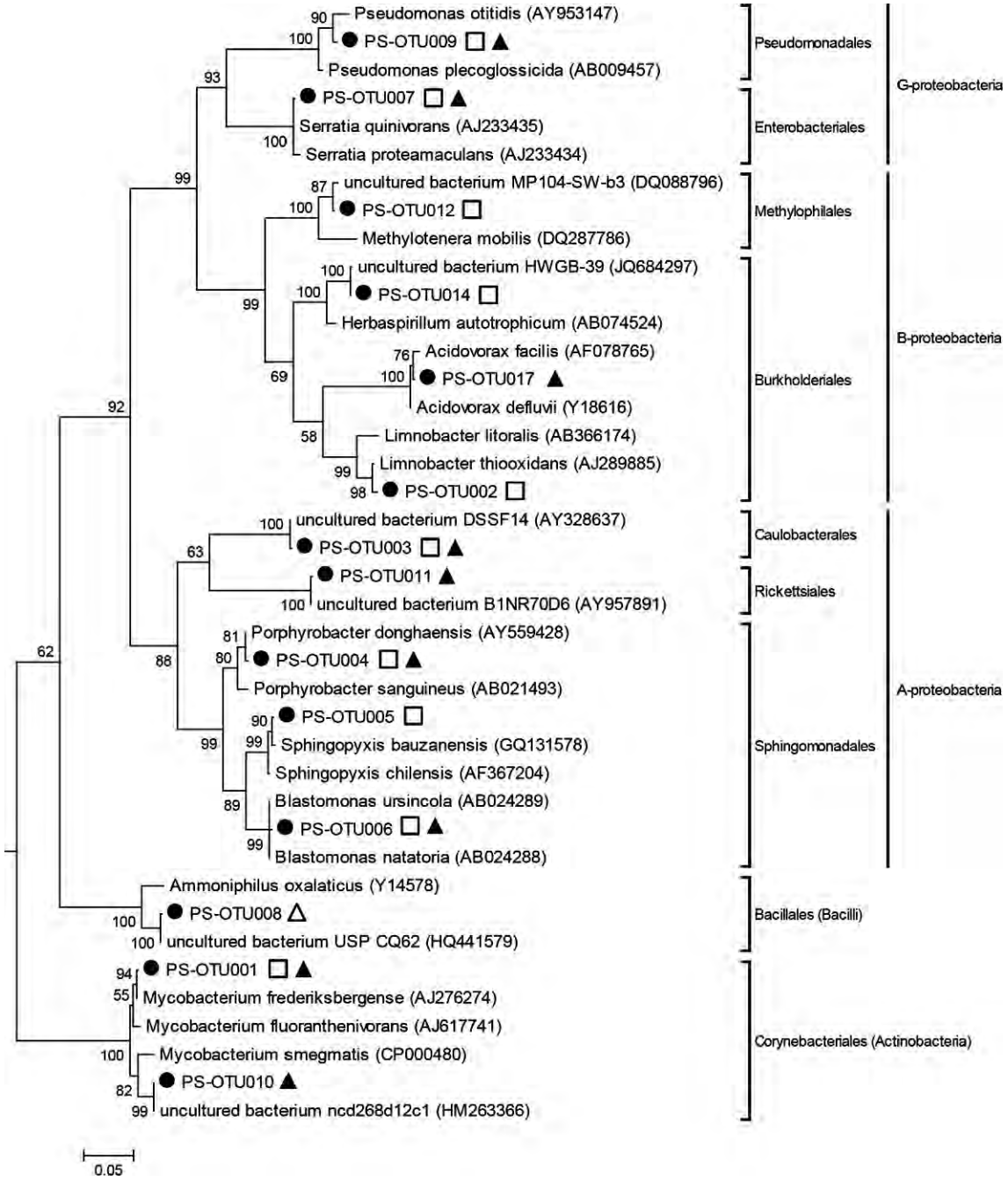

Figure 3 Phylogenetic relationships among the ten most abundant operational taxonomic unit (OTU) groups from each groundwater (GW, □) and surface water (SW, ⊠) communities that explained ≈68% (SIMPER analysis) of the dissimilarity within all samples (one-way ANOSIM, R = 0.134, p = 0.034). The tree was inferred from a maximum likelihood analysis of aligned 16S rRNA gene sequence (≈800 bp); nodes with a bootstrap value ≥50% of 500 replicates are identified.

Several microbial source tracking (MST) methods have been developed to identify the primary sources of pollution and to establish adequate pollution control practices (Santo Domingo et al., 2007). The use of MST data could lead to measures that decrease the level of enteric pathogens entering water bodies used by drinking water systems. Many MST methods have been proposed in the last 15 years, but those that detect host-specific markers directly from water DNA extracts have gained the most acceptance. Such MST methods are based on quantitative PCR (qPCR) and usually target the 16S rRNA gene of fecal bacteria within the Bacteroidales. A reliable MST assay must target populations that do not grow in the environmental waters, exhibit high levels of host specificity and host distribution, target markers that are present in high abundance within the specific fecal source, cross-react with markers that are present in low abundance within non-specific sources, detect targets present at low levels within a water sample, exhibit geographic stability and be seasonal. Ideally, an MST method should also correlate with the presence of the

indicator that is used as the water quality standard for the drinking water source. Unfortunately, there is no perfect MST assay and therefore, whenever possible, multiple assays should be used to determine the primary sources and to quantify their relative fecal bacteria loading contributions.

Homeland Security

Concerns regarding the vulnerability of municipal water supplies to biological attacks have prompted renewed interest in treatment issues, particularly as they relate to the survival of select agents in potable water (e.g., *Bacillus anthracis*, *Francisella tularensis*, and smallpox virus). Data on the inactivation of bacterial bioterrorism agents by chlorination are available. The detection of bioterrorism agents in drinking water requires methods with low detection limits as these agents need to be detected at very low densities, given that they have low infectious doses. As a result, future early warning detection systems will rely on the development of efficient concentration methods as well as rapid molecular procedures with low detection limits (e.g., polymerase chain reaction assays).

Conclusions

Overall, the treatment technology available today is capable of providing microbiologically safe drinking water to all consumers. In practice, however, many systems are still vulnerable to waterborne disease. Reasons include (1) inability to fund the installation and use of adequate technology, (2) ignorance of the need for adequate technology, (3) inadequate or improper monitoring to insure that barriers against waterborne disease remain intact, (4) lack of real-time monitoring data, and (5) lack of adequate data on the effectiveness of various treatment practices against certain pathogens. Degrading infrastructure is another significant issue which will become more critical in coming years. In addition, with increased population pressure, source water quality may decline over time, especially in areas where water is scarce; thus the technology used becomes inadequate. These problems are common on a global basis. The challenge globally is to highlight the imperative of safe drinking water and to find solutions to these problems.

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Drinking Water Microbiology

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Introduction

Access to clean drinking water is required for healthy, thriving communities. It is estimated that more than 5 billion people (71% of the global population) used what the WHO has defined as a safely managed drinking water service (Fig. 1); that is, one located on premises, available when needed and free from contamination. Unfortunately, in 2015, 29% of the global population (2.1 billion people) lacked safely managed drinking water services—defined by the World Health Organization (WHO) as water at home, available, and safe. There were 844 million people in the world who lacked a basic drinking water service, which is defined as drinking water from an improved source, provided that the collection time is not more than 30 min for a round trip, including the time standing in line waiting. Fig. 2 shows the regional differences in access to different types of water across the world.

Concern over the lack of access to safe drinking water led the WHO/UNICEF Joint Monitoring Program to establish the goal to "Ensure Availability and Sustainable Management of Water and Sanitation for All" as its Sustainable Development Goal (SDG) 6, with a target of achieving "universal and equitable access to safe and affordable drinking water for all" by 2030. Further, they have defined safe drinking water as water that "has a risk level equal to or less than a 1 in 10,000 annual probability of infection or 1 in 1 million disability-adjusted life years" (DALY).

Significance of Waterborne Disease

On a global basis, every year, 1.5 million people die from waterborne disease. The majority of these individuals are young children who die of dehydration caused by diarrhea. According to the World Health Organization, there are almost 1.7 billion cases of diarrhea in young children annually, and diarrhea is the second leading cause of death for children under 5 year of age.

In the United States and other countries in which drinking water treatment is routinely practiced, people typically assume that all drinking water is treated, however, that is not the case. For example, in the United States, the Environmental Protection Agency (EPA) estimates that 15% of the population obtains their drinking water from untreated private wells or other untreated sources. Based on statistics compiled by the Centers for Disease Control and Prevention, most of the drinking water outbreaks in the United States are associated with untreated or inadequately treated ground water, and distribution system deficiencies. For example, in the latest update on waterborne outbreaks in the United States (2013–14), the CDC reported 1006 cases of illness, 130 of which were caused by *Legionella*, and 86% of which originated from outbreaks in which acute gastrointestinal illness was the predominant illness. Epidemiological investigations have estimated that between 12 million and 19.5 million waterborne illnesses occur annually in the United States, only a small fraction of which are recognized and/or reported.

Waterborne Pathogens

Worldwide, it is believed that the most common route of infection with a waterborne pathogen is the "fecal-oral" route: infection occurs as a result of exposure to water or food improperly treated sewage or directly contaminated with untreated human or animal feces. Such microorganisms are referred to as enteric pathogens.

Although other microorganisms (e.g., *Legionella*, *Naegleria*) can cause waterborne disease, most of the concern and focus on a global basis has been on microorganisms of enteric origin. Much of the reason for this is that many of these microorganisms can be readily handled through the implementation of relatively simple interventions such as handwashing and other sanitation measures.

The fecal material of infected individuals is the major source of pathogenic microorganisms in domestic wastewater: hundreds of different types of microorganisms may be present in the fecal material of infected humans and animals. In addition, urine may be a source of certain pathogenic microorganisms, such as *Leptospira* and polyomaviruses. Numerous factors affect the concentrations and types of pathogens found in domestic wastewater at any given over time; these include the disease incidence in the population producing the wastewater, season of the year, climate factors (such as rainfall, temperature), the amount of water being used, the economic status of the population, and quality of the drinking water. If fecal material or wastewater contaminated with fecal material enters a water body that is used as a source of potable water, there is a potential for disease transmission. The major groups of pathogenic microorganisms transmitted by water and food contaminated with feces include bacteria, viruses, and parasites.

As stated previously, other, nonfecal microorganisms also cause waterborne disease from the consumption or use of potable water; these are typically microorganisms indigenous to the water or sediment. For example, *Legionella*, a bacterium that is transmitted via aerosols, is a frequent cause of drinking-water associated disease. Indeed, in 2001, the CDC began including reports of *Legionella* outbreaks with the drinking water surveillance data, and *Legionella* was the most frequently-identified causative agent of waterborne disease in reported outbreaks in the United States in 2013–14. Notably, it was responsible for all 13 of the deaths

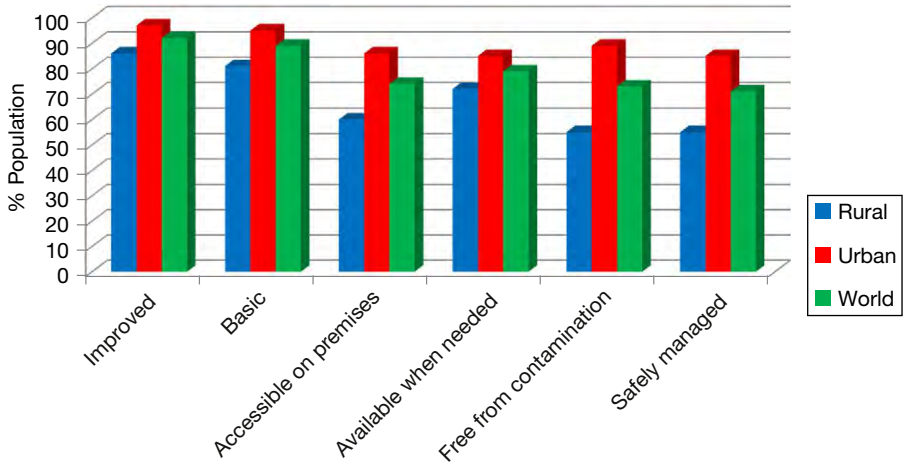

Fig. 1 Population using drinking water sources meeting SDG criteria for safely managed services, 2015 (WHO/UNICEF, 2017).

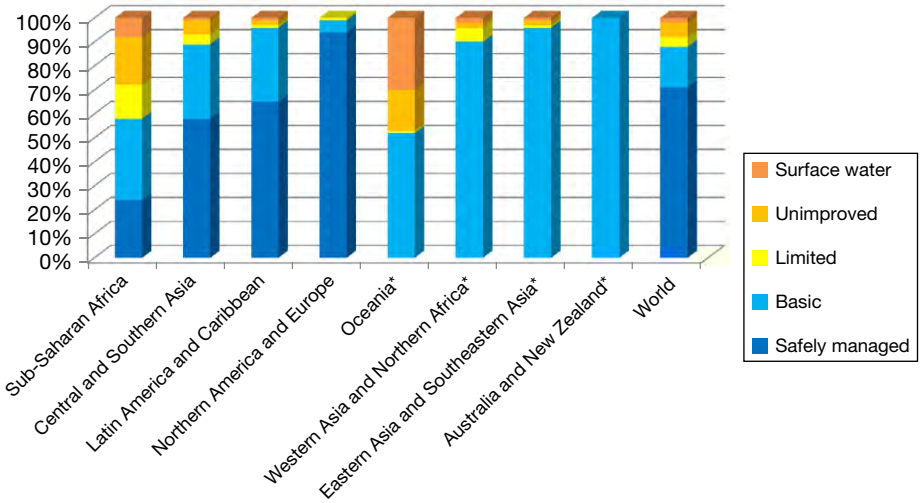

Fig. 2 Regional drinking water coverage, 2015 (WHO/UNICEF, 2017).

associated with waterborne disease in that time period. *Naegleria fowleri*, an amoeba that naturally occurs in the sediments of certain warm water bodies, has also been the reported as the cause of several waterborne disease outbreaks. Infection by this microorganism typically results in death. A series of fact sheets that describe the human health effects, source and occurrence, routes of exposure, and significance in drinking water for many waterborne pathogens has been compiled by the World Health Organization.

Bacteria

Bacteria are naturally present in the gastrointestinal tracts of humans and animals; in fact, they are necessary for the proper functioning of the digestive system. The vast majority of these bacteria are harmless. However, when an individual is infected by an enteric bacterial pathogen, these microorganisms grow and reproduce in the gastrointestinal tract, and they are shed in the fecal material. Because the enteric bacteria are well-adapted to the conditions present in the gastrointestinal tract, which include high organic carbon concentrations, low pH, and a relatively high temperature (~37°C), they tend to have a relatively difficult time growing in the environment. This is because the conditions in soil, water, etc. are generally very different from those in the gastrointestinal tract: nutrients are generally present in low concentrations and/or in difficult-to-metabolize forms, the temperature is not optimal for growth, the moisture content is lower, etc. As a result, the enteric bacteria typically do not compete well with the indigenous soil or water bacteria for the available nutrients. This adversely affects their ability to reproduce, or even survive without reproducing, in the environment. Thus, their longevity in the external environment is typically limited to a few days to weeks, depending on the environmental conditions.

Historically, the bacterial pathogens have been the focus of waterborne disease, mainly because they were among the first microorganisms recognized. Bacteria cause many diseases, such as cholera, typhoid, and dysentery that have been responsible for

large epidemics (and, in the case of cholera, pandemics) all over the world. In general, many of the bacteria pathogens are relatively easy to remove through wastewater and drinking water treatment processes, and, therefore, are of less concern than the other groups of pathogens in many industrialized countries. However, these pathogens are still of great concern in many of the less-industrialized countries, due to the lack of adequate sanitary treatment processes in those countries. According to the WHO/UNICEF, in 2015 only 39% of the global population (2.9 billion people) used a safely managed sanitation service; that is, excreta safely disposed of in situ or treated off-site. More than 2 billion people lack basic sanitation, and almost 1 billion people still practice open defecation.

Some of the most common bacterial pathogens found in human and animal waste that are transmitted by contaminated water and food are listed in Table 1. Some relevant characteristics, such as infectivity, survival in water, resistance to chlorine, and health impacts are also provided.

Viruses

Although fecal material always contains high concentrations of nonpathogenic bacteria because they are part of the normal flora of the gastrointestinal tract, like pathogenic bacteria pathogenic viruses are typically only present in fecal material if the individual is infected. Enteric viruses are very different from enteric bacteria in a number of ways. Some of the most important differences in this context are:

- Viruses are nonliving. They can only reproduce inside of susceptible host cells, as they use the host's biochemical mechanisms to reproduce themselves.
- Viruses are relatively more host-specific than bacteria. Thus, many viruses are only able to infect a single species or a few species. This can make it easier to identify the source of contamination. For example, if a human-specific virus is found in water, it is clear that the water has been contaminated by human fecal material.
- Because viruses are metabolically inert when outside of a host cell, they are not affected as much as bacteria by the presence of nutrients in the external environment. As they cannot reproduce in the environment, their numbers can only decrease over time.
- Viruses are typically able to survive for longer periods in the environment, compared to bacteria. Typically, viruses can survive for weeks to months; survival has even been reported for 2 years in a cold groundwater.
- Viruses are much smaller than bacteria. The typical enteric virus is 25–80 nm in size, while the typical size of an enteric bacterium is 1–2 mm. This affects their ability to be transported through the soil and into the groundwater.

More than 100 types of enteric viruses can be present in domestic wastewater. The types of symptoms that result from enteric virus infections range widely—from asymptomatic infections to relatively mild, self-limiting diarrhea, to more severe illnesses, including respiratory illness, infectious hepatitis, paralysis, encephalitis, and myocarditis. Several of the pathogenic human enteric viruses, along with some relevant characteristics, such as infectivity, survival in water, resistance to chlorine, and health impacts, are listed in Table 1.

Parasites

Another group of pathogenic microorganisms that can be transmitted through contaminated drinking water is the parasites. This is an extremely diverse group of organisms, the common feature of which is that they live on or in a host organism and obtain their food from or at the expense of the host. The enteric parasites that are pathogenic to humans can be classified into two groups: the protozoa (sometimes referred to as the protists) and the helminths, which include the intestinal worms. Protozoa are single-celled microorganisms whose life cycles include at least one vegetative as well as at least one resting stage. The infective stage of the protozoa is different for different parasites. In the case of *Cryptosporidium*, the resting oocyst form must be ingested to initiate infection; for others, the vegetative form must be ingested for infection to occur. The resting stage of the organism (cyst, ovum) is generally relatively resistant to inactivation from environmental stresses and during conventional drinking and wastewater treatment processes. Thus, those parasites whose resting stage is the infective stage may be of greater concern than those whose infective form is the vegetative stage, even for consumers of (inadequately) treated water.

Many of the intestinal protozoa are transmitted by fecally-contaminated water, food, or other materials. Some of the most common protozoan pathogens found in human and animal waste that are transmitted by contaminated water and food are listed in Table 1. Some relevant characteristics, such as infectivity, survival in water, resistance to chlorine, and health impacts are also listed.

Indicators of Fecal Contamination of Water

As stated previously, bacteria are always present in the gastrointestinal tract of humans and other animals, and therefore are present in fecal material. On the other hand, pathogenic enteric microorganisms are typically only present when an individual is infected. Therefore, if one wants to assess the safety of water for human consumption, the water would have to be continuously monitored for the presence of pathogens in order to be certain of their presence or absence. Additionally, there are hundreds of different pathogens that may be present in feces, so the water would need to be monitored for all of these microorganisms to assure the safety for consumption.

Table 1 Selected pathogens that can be transmitted by water

Organism	*Disease*	*Incubation Period*	*Duration of Illness*	*Health Significance*[a]	*Resistance to Chlorine*[b]
Bacteria					
Burkholderia pseudomallei	Pneumonia			High	Low
Campylobacter	Gastroenteritis	3–5 days	1–4 days	High	Low
Legionella	Pontiac fever	5–66 h	2–7 days	High	Low
	Pneumonia	2–14 days	Weeks to months	High	Low
Leptospira	Weil's disease (headache, chillls, fever, nausea, neck or joint pain)	2–20 days	3 days to 3 weeks	High	Low
Shiga-toxin producing *E. coli*	Gastroenteritis, hemolytic uremic syndrome, kidney failure	12 h - 8 days	1 day to 3 weeks	High	Low
Salmonellca enterica serovar typhi	Typhoid fever	7–28 days	Weeks to months	High	Low
Salmonella	Salmonellosis	8–48 h	3–5 days	High	Low
Shigella	Bacillary dysentery	1–7 days	4–7 days	High	Low
Vibrio cholerae 01	Profuse, watery diarrhea, vomiting, rapid dehydration	9–72 h	3–4 days	High	Low
Vibrio cholerae non-01	Watery diarrhea	1–5 days	3–4 days	High	Low
Yersinia enterocolitica	Gastroenteritis	2–7 days	1–21 days	Moderate	Low
Protozoa					
Cryptosporidium parvum	Diarrhea	1–2 weeks	4–21 days	High	High
Cyclospora cayatanensis	Watery diarrhea alternating with constipation	2–11 days	days	High	High
Entamoeba histolytica	Amoebic dysentery	2–4 weeks	weeks to months	High	High
Giardia intestinalis	Diarrhea, malabsorption	5–25 days	weeks to months	High	High
Microsporidium	Chronic diarrhea, weight loss				
Naegleria fowleri	Primary amoebic meningoencephalitis	Minutes to hours		High	Low
Toxoplasma gondii	Toxoplasmosis			High	High
Viruses					
Adenovirus	Respiratory illness, conjunctivitis, vomiting, diarrhea	1–4 days	2–3 days	Moderate	Moderate
Astrovirus	Vomiting, diarrhea	3–4 days	2–7 days	Moderate	Moderate
Calicivirus	Vomiting, diarrhea			Moderate	Moderate
Coronavirus	Vomiting, diarrhea			Moderate	Moderate
Enterovirus:		3–14 days	variable		
Poliovirus	Paralysis, meningitis, fever			High	Moderate
Coxsackie A	Meningitis, fever, herpangina, respiratory illness, paralysis			High	Moderate
Coxsackie B	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory illness, pleurodynia			High	Moderate
Echovirus	Meningitis, encephalitis, respiratory illness, rash, diarrhea, fever, myocarditis, endocarditis			High	Moderate
Enterovirus 68–71	Meningitis, encephalitis, respiratory illness, acute hemorrhagic conjunctivitis, fever			High	Moderate
Hepatitis A virus	Infectious hepatitis	15–50 days	1 week to several months	High	Moderate
Hepatitis E virus	Hepatitis	15–65 days	1 week to several months	High	Moderate
Norovirus	Epidemic vomiting and diarrhea	1–3 days	1–3 days	High	Moderate
Reovirus	Respiratory illness			Moderate	Moderate
Rotavirus	Diarrhea, vomiting	1–3 days	3–7 days	High	Moderate
Sapoviruses	Gastroenteritis			High	Moderate

[a]Health significance relates to the severity of impact, including association with outbreaks.

[b]When the infective stage is freely suspended in water treated at conventional doses and contact times and pH between 7 and 8. Low means 99% inactivation at 20°C generally in <1 min, moderate 1–30 min and high >30 min. It should be noted that organisms that survive and grow in biofilms, such as Legionella and mycobacteria, will be protected from chlorination.

Modified from Yates, M. V. (editor in chief) (2016). Manual of Environmental Microbiology, 4th ed. American Society for Microbiology, Washington, DC. 1088 pp.

However, it is not technically or economically feasible to test wastewater for the presence of all different types of pathogens, nor is it feasible to continuously monitor water for pathogenic microorganisms. As a consequence, the microbiological quality of water historically has been assessed by monitoring for the presence of alternative organisms—those enteric organisms that are always present in fecal material—the so-called indicator microorganisms. The rationale for using fecal indicator microorganisms is that, if they are found in water, the water contains fecal material, and therefore may also contain pathogenic microorganisms. In contrast, if fecal indicators are absent, the water is considered not to be contaminated by fecal material, and therefore, no fecal pathogens are present. Common indicators or groups of indicator organisms used are the total coliform bacteria, the thermotolerant coliform bacteria (also called fecal coliform bacteria), the enterococci (also called fecal streptococci); examples include *Escherichia coli*, *Clostridium perfringens*, and *Bifidobacterium*.

While the initial focus of monitoring water for safety was on the potential presence of pathogenic bacteria, in industrialized countries, many of the bacterial diseases have been controlled through the use of drinking water and wastewater treatment, including disinfection. As this has occurred, more of the focus in drinking water and wastewater treatment has been on controlling the concentrations of parasitic and viral pathogens. Unfortunately, the bacterial indicators mentioned above do not always correlate well with the presence or behavior of the nonbacterial pathogens. Efforts are underway to identify more appropriate indicators, such as bacteriophages (viruses that infect fecal indicator bacteria) for these pathogens.

Characteristics of Microbial Indicators

In 1966, Bonde developed the first systematic delineation of the attributes of an ideal indicator organism for the assessment of health risk or treatment efficiency. These criteria state that an ideal indicator should:

- Be present whenever the pathogens are present;
- Be present only when the presence of pathogens is an imminent danger; that is, they must not proliferate to any greater extent in the aqueous environment;
- Occur in much greater numbers than the pathogens;
- Be more resistant to disinfectants and to the aqueous environment than the pathogens;
- Grow readily on simple media;
- Yield characteristic and simple reactions enabling as far as possible an unambiguous identification of the group;
- Be randomly distributed in the sample to be examined, or it should be possible to obtain a uniform distribution by simple homogenization procedures; and
- Grow widely independent of other organisms present, when inoculated in artificial media; that is, the indicator bacteria should not be seriously inhibited in their growth by the presence of other bacteria.

Unfortunately, no indicator organism or group of organisms was found to have all of these attributes. The criteria that have been most difficult to meet are the first and fourth: many waterborne disease outbreaks have occurred in the absence of indicator microorganisms, which, at least in some cases is due to the fact that the pathogens are less resistant to disinfectants and environmental stressors than the indicator microorganisms. This is especially true for nonbacterial pathogens.

Over time, as new pathogens are identified, more/different applications for indicators have been found, and new microbial sampling and detection methods have been developed, Bonde's criteria have been reevaluated. For example, a National Academies of Science committee developed a set of criteria recommended for use when assessing the appropriateness of any indicator or group of indicators. These separate the biological attributes of the indicators themselves from the attributes of the methods used to detect the indicators. The desirable biological attributes of indicators, as developed by the NRC are:

- Correlated to health risk
- Similar (or greater) survival to pathogens under environmental conditions
- Similar (or greater) transport to pathogens
- Present in greater numbers than pathogens
- Specific to a fecal source or identifiable as to source of origin

The desirable attributes of the indicator detection methods, according to the NRC, are:

- Specificity (independent of matrix effects)
- Broad applicability
- Precision
- Adequate sensitivity
- Rapidity of results
- Quantifiable
- Measures viability or infectivity
- Logistical feasibility

For most situations, there is agreement that the most important characteristic of any indicator or indicator system is its ability to indicate possible health risk. This characteristic can be shown directly through conducting epidemiological studies in which the association between the indicators and health outcomes are assessed. A different, more common, and generally less expensive way

to demonstrate the correlation between indicators and health risk is to monitor water for the presence and/or concentration of pathogen and compare those data to data on potential indicator microorganisms. While the latter approach might be simpler and less expensive, it may be more problematic. This is because most pathogen detection methods detect a single pathogen or closely related group of pathogens, and a correlation (or lack thereof) between an indicator and a single pathogen or pathogen group does not infer that a correlation exists (or does not exist) with other waterborne pathogens. To obtain complete and accurate information regarding the potential for an indicator to provide information on the microbiological safety of the water, correlations for each pathogenic microorganism or related group of microorganism would need to be examined.

Common Indicators

It is technologically, economically, and practically not possible to test water for all possible pathogenic microorganisms. As a result, the typical practice has been, and continues to be, to monitor water for one or more indicator microorganisms (or groups of indicator microorganisms) to indicate whether or not there is a health risk associated with the consumption of water. Different groups have been used for many years as indicators of the microbiological quality of drinking water and of recreational waters. For example, total coliform bacteria have been used for almost a century to assess the microbiological quality of drinking water.

Coliform bacteria

The group of indicator microorganisms in longest use for assessing the microbiological quality of drinking water and wastewater is the total coliform bacteria. More recently, other coliform bacteria, including the thermotolerant coliforms (previously termed fecal coliforms and a subgroup of the total coliforms), and *E. coli*, a specific thermotolerant coliform bacterium, considered specifically of fecal origin, have been added to the commonly used indicator organisms. The US Environmental Protection Agency requires the monitoring of all public sources of drinking water for total coliform bacteria. The use of the total coliform standard to assess the microbiological safety of water has had a dramatic effect on reducing waterborne disease outbreaks in countries that have implemented one.

Numerous studies, however, have documented that many pathogenic microorganisms, especially enteric viruses and parasites, are not as easily inactivated by water and wastewater treatment processes as are coliforms. The fact that waterborne disease outbreaks continue to occur every year in countries that have implemented coliform monitoring standards is clear evidence that better ways to assess the microbiological quality of water must be found. Although there is no perfect indicator organism, research is ongoing to find a rapid, relatively inexpensive, and accurate way to assess the microbiological quality of water.

Fecal enterococci

Like the coliform bacteria, the fecal enterococci or fecal streptococci, which include some members of the genera *Streptococcus* and *Enterococcus*, are found in the intestinal tract of humans and many animals. Use of this group of organisms has some advantages over the coliform group for assessing the microbiological safety of water. These include the fact that they rarely multiply in environmental waters, they are more resistant to treatment processes and environmental stresses, and they generally persist for longer in the environment than do the coliform bacteria. The most common use for these organisms is to indicate the microbiological quality of recreational bathing waters. In addition, enterococci are recommended as one of the groups of fecal indicator organisms that can be used to assess the microbiological quality of groundwater by the US Environmental Protection Agency.

Clostridium perfringens spores

As indicated previously, the resting stages of many protozoan parasites are especially resistant to inactivation during water and wastewater treatment. Therefore, the use of bacterial indicator organisms may not provide accurate information regarding the presence of pathogenic parasites in water. The search for a surrogate organism for parasite removal during treatment processes has led to investigations into the potential use of *Clostridium perfringens* spores. These bacterial spores are present, typically in very low numbers, in human and animal feces. However, like the protozoans, they are relatively resistant to many forms of drinking water and wastewater treatment processes, so may be useful indicators for parasite and virus removal by treatment. *Clostridium* spores are used in conjunction with enterococci as secondary indicators of water quality in Hawai'i, due to problems with coliform regrowth in the warm temperatures of the tropical environment.

Heterotrophic plate count bacteria

Another group of bacteria that can be used to assess the microbiological quality of water, especially with respect to the efficacy of treatment processes, is the heterotrophic plate count (HPC) bacteria. The HPC is defined as "the number of aerobic and facultatively anaerobic bacteria that obtain their carbon and energy from organic sources." This is a very general test of the bacteriological quality of water, and both pathogenic and nonpathogenic microorganisms are enumerated, but they are not and cannot be differentiated from one another using this method. Studies to assess the health effects of consumption of water containing high HPC have not documented adverse health effects. In addition, some studies have found that the presence of HPC bacteria in water do not indicate potential adverse human health effects.

The numbers of HPC bacteria in a specific water are typically less useful than changes in the number at a particular location, as the change may indicate a problem with the water/wastewater treatment processes, a lack of integrity of the water distribution system, or the potential for bacterial regrowth in the system.

Bacteriophages

The large differences between the physical properties, especially size, of bacterial indicators and waterborne viruses has caused concern over the use of bacterial indicators to evaluate the virological quality of water, especially groundwaters. This has led to investigations of the use of bacteriophages, viruses that infect bacteria, as indicators for groundwaters used as drinking water sources. The US Environmental Protection Agency allows the use of bacteriophages as an indicator of fecal contamination of groundwater in the Ground Water Rule.

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Ecology of Rare Microorganisms

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The topic of the diversity of life on the planet has always fascinated scientist (Erwin, 1991), and the diversity of microbes has become even more fascinating with the discovery of their ubiquity and the important role that they play in a variety of ecosystems. It is, however, difficult to delineate microbial species and to count them globally. Therefore, estimating their diversity and providing an inventory is very challenging. Recent estimations based on species abundance distribution suggest that there are 10^{12} microbial species on Earth (Locey and Lennon, 2016), while other estimates put the number as low as a few millions (Amann and Rosselló-Móra, 2016a,b). The findings are strongly debated (Amann and Rosselló-Móra, 2016a,b; Lennon and Locey, 2016; Willis, 2016) and a true estimate is yet to come. Microbial communities in most natural environments are composed of numerous individuals that are spread across a very large number of different species. For example, each drop of seawater (1 mL) can contain 500,000–1 million prokaryotic microbes (bacteria and archaea) that are distributed across hundreds of microbial species (Lozupone and Knight, 2007). Soils show similar patterns, with often higher number of microbes (5.5×10^7 cells/g of soil, Elshahed et al., 2008) and thousands of species (Lozupone and Knight, 2007). The fact that microbes are numerous and diverse is long known, but the extent of their diversity really started to be understood with the development of new sequencing technologies during the mid-2000s. With these new sequencing technologies, it became possible to probe the diversity of communities in a variety of environments, with data always revealing a tremendous number of taxa. Interestingly, the distribution of microbes within samples normally showed communities dominated by a few abundant taxa followed by numerous rare ones (Fig. 1). The extent of that diversity and the presence of numerous rare taxa naturally begs the question of the ecological role that so many different microbes can have within one environment.

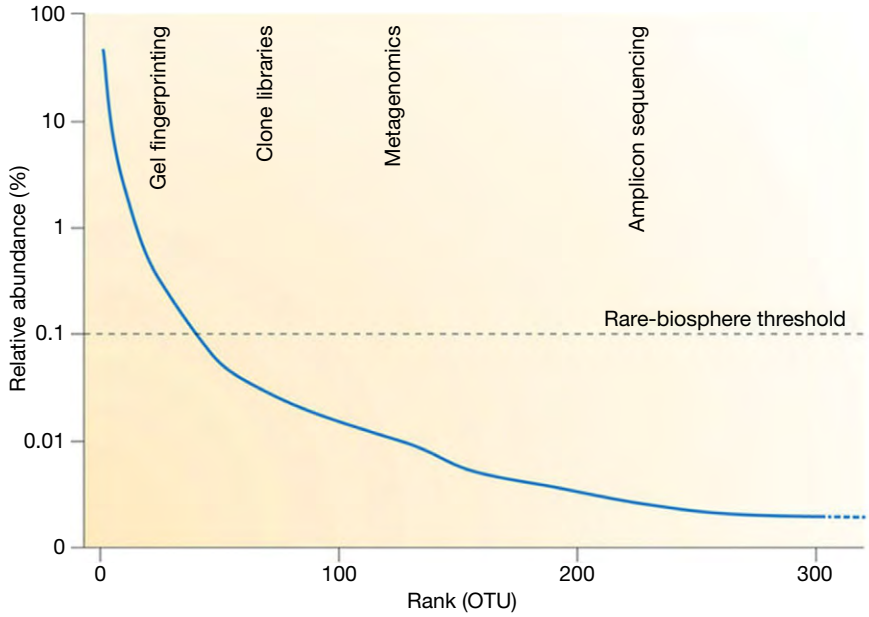

Fig. 1 Schematic rank–abundance curve. The coverage depth of different methods used for studying the diversity of microbial communities is indicated: gel fingerprinting, clone libraries/sanger sequencing, metagenomics and high-throughput amplicon sequencing. The arbitrary threshold of 0.1% for the detection of rare-biosphere organisms is indicated. Reproduced with permission from Lynch, M.D., Neufeld, J.D., 2015. Ecology and exploration of the rare biosphere. Nature Reviews Microbiology 13, 217–229.

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The term "rare biosphere" was coined in 2006 by Sogin et al. (2006). At the time, a new high throughput sequencing technology was just starting to be used and they were the first to apply the "454" pyrosequencing technology to study microbes in environmental samples. They sequenced deep sea samples and observed thousands of low-abundance taxa that made most of the observed community diversity. They suggested that members of the rare biosphere may eventually become dominant in response to environmental change. The same year Pedrós-Alió (2006a,b), published a review paper on the topic of microbial diversity in which he explored and discussed the concept of core versus rare species, which he named the "seed bank". Members of the seed bank were hypothesised to have extremely low loss rates because their low abundances made them less prone to viral infection or death by predation. Rare taxa could be promoted from the seed bank to the core population if conditions became suitable for the growth of the particular taxon. Pedrós-Alió concluded with a very fascinating statement that said: "If it was true that all microbes are easily dispersed globally, the long tail of any ecosystem would include all of the microorganisms on Earth. In effect, the biodiversity of any ecosystem would be identical to the biodiversity on the planet." Even though that assumption may be difficult to test, recent works do suggest that the global ocean contains a persistent microbial seed bank (Caporaso et al., 2012; Gibbons et al., 2013). However, the structure of the rare biosphere would appear to be much more complex than originally thought as we will now see.

The two founding papers on the rare biosphere (Pedrós-Alió, 2006a; Sogin et al., 2006) rapidly attracted the interest of microbial ecologists. The exciting definition of a new category of microorganisms, combined with the rapid development of relatively cheap sequencing technologies, paved the way for research on the rare biosphere that would cover both the taxonomical and functional aspects of the topic over a large range of ecosystems.

What is Rare?

The first thing to think when studying rare microorganisms is to find a way to define what is rare. Microbial communities are made of thousands of individuals that can be described through a range of tools that are evolving and changing fast with technological advances. Before 2006, microbial communities were studied with molecular tools that included fingerprinting, cloning and Sanger sequencing. All methods relied on amplification of DNA by PCR and often targeted the 16S or 18S rRNA gene as taxonomical marker. This approach first originated in the 70's with the work of Norman Pace and colleagues on the ribosomal gene (Pace, 1973). The power of that approach was revealed when Carl R. Woese revolutionised the field by using the ribosomal gene as universal marker for the classification of organisms. He demonstrated the strength of the method in 1977 when he first published a new classification of life that was separated in 3 domains (Woese and Fox, 1977) later defined as Eucarya, Bacteria and Archaea (Woese et al., 1990). The separation of life in 3 major domains is still used today. The 16S and 18S rRNA became gold standards in the field to describe microbial diversity (Lane et al., 1985), and were applied through two main approaches: fingerprinting and cloning/sequencing. Fingerprinting technics such as RFLP or DGGE gave a rapid overview of the composition of communities, but they did not provide the identity of microbes. Cloning and sequencing were needed to identify their taxonomy but the approach was time and cost consuming. The taxonomic resolution of the fingerprinting method was good, but it only allowed detection of the most common members of the communities (Fig. 1). It was said that only bacteria with occurrence >1% could be seen (Muyzer et al., 1993). As for cloning, the costs of the technique limited the number of sequences that could be obtained and often communities were described with less than 100 sequences. Sequences were grouped by similarity into operational taxonomical units (OTUs). Since uncultured bacteria cannot be defined as species, OTUs serve as species proxies when studying community composition. The methodological limitation of PCR-dependent techniques at the time led Pedrós-Alió to set the cut-off for separating abundant and rare taxa at 1% (Pedrós-Alió, 2006a). Bacteria that could at the time be retrieved by PCR approaches would be considered as the abundant members of the communities. They were the bacteria that were important and active in the ecosystem. The diversity and identity of rare taxa would then not or only seldom be accessed by molecular approaches. Only rare microorganisms that could be grown in enrichment or pure cultures could be identified. Thus, only very few rare microbes were described at the time and the composition of the rare biosphere remained a mystery.

The discovery of high throughput sequencing transformed the field of microbial ecology (Logares et al., 2012). Instead of a few tens or hundreds of sequences, microbial communities could now be described by thousands of sequences (6505 to nearly 23,000 sequences in the first study of Sogin et al. (2006). The new sequencing techniques gave birth to the term "rare biosphere", but, in that first publication, this community component was not defined by any threshold; instead, it was just described as low abundance taxa. Low abundance taxa were the ones that constitute the long tail seen in rank abundance curves (Fig. 1). The development of a new sequencing technology and the subsequent discovery of the extent of the diversity of natural microbial communities raised a debate about the role of PCR and sequencing bias in artificially creating microbial OTUs. Could the rare biosphere be actually the "fake biosphere"? (Reeder and Knight, 2009; Kunin et al., 2010). Following the advance in sequencing technologies, tools were developed to clean the sequences and ensure that the data represented true diversity (Huse et al., 2010; Quince et al., 2011). With increasing sequencing depth, the cut-off for defining rare microbes was pushed down to 0.01% (Fuhrman, 2009; Galand et al,., 2009; Logares et al., 2014a), but varied according to studies and sequencing depth up to 0.1% (Quero and Luna, 2014) or 1% (Campbell et al,., 2011; Alonso-Sáez et al., 2015). The cut-off, and thus the definition of the rare biosphere, has been until now set arbitrarily and is dependent on the tool used for the study. In addition, it should be noted that defining abundance should be done within a common spatial or temporal scale. Microbes can be classified as rare within one sample, which is the most common case, but they can also be defined across a number of samples within a common environment, but at different time points, or within a

common time frame but at different locations. The scale used to define rarity will strongly impact the result of the analysis. An interesting thought is that even though a bacteria can be rare in one marine sample, e.g., 1 cell per liter, at the whole ocean level, it would not be that rare since 93,300,000,000,000,000,000 cells would be expected in the whole ocean roughly. With that in mind, one could think of a theoretical number for the whole ocean below which a species is rare.

Crespo et al. (2016) attempted to exhaustively map the rare biosphere in two marine samples by conducting very deep sequencing (~500,000 final reads per sample). Even with that sequencing depth, they were not able to cover the full diversity of the water samples and they calculated that ~2 million final reads would be adequate to sequence 90% of the total identifiable bacterial species richness. In an attempt to move forward and away from the sequencing depth-dependent definition of rarity, Kalenitchenko et al. (2018b) recently set up an experiment that use bacterial cell numbers to define rarity. The principle of the experiment was to let large organic substrates, in this case wood, be colonised by specialised microbes originating from sea water. The idea came from the observation that wood found on the seafloor was often colonized by mats made of sulfur oxidizing microorganisms that were not detected in the surrounding seawater (Kalenitchenko et al., 2015, 2016). These microorganisms belong to the planktonic rare biosphere and become abundant when they encounter a suitable substrate. The study design consisted in a dilution approach. Wood pieces were inoculated with different dilutions of sea water and the presence of key microorganisms involved in the sulfur cycle was analysed after 4 weeks of incubation. Through the classical approach in microbiology called the most probable number (MPN), the cell number of the colonizing organisms was estimated in the original seawater inoculum. Seawater communities were simultaneously described by deep Illumina sequencing. Interestingly, the taxa growing on the wood were identified as marine microorganisms, but they could not be detected in sea water by sequencing. Their abundance was so low that even deep sequencing could not reveal their presence. The MPN approach showed that they had occurrences as low as 1 cell in 10 L of seawater or as little as 0.00000002 % of the cells in the aquaria. It means that theoretically, if PCR or sequencing bias were not considered, one would thus need at least 8,038,282,890 sequences (the number of bacteria present in average in 10 L of seawater) to be able to detect these rare bacterial cells. This estimation exceeds by orders of magnitude earlier numbers. The authors thus proposed an additional category of rare microbes that they named the ultra-rare. The ultra-rare microbes would be defined as microbes that are typically not detected by high throughput sequencing approaches, but that are nevertheless viable and able to colonize new ecosystems with the potential to fulfil an important function in the marine ecosystem (Fig. 2).

The Ecology of the Rare Biosphere

New sequencing technologies have revealed the true extent of microbial diversity in a number of ecosystems. Despite the early debate on the reality of the observed richness, it is now acknowledged that microbial communities in nature are composed mostly of rare taxa. The logical question is therefore why are there so many rare taxa and what do they do? When Pedrós-Alió first reviewed the concept of the rare microbes, he defined them as a seed bank of species that could become abundant when conditions become favourable (Pedrós-Alió, 2006a). The majority of rare microbes that compose the diversity of communities would thus be dormant or slow growing (Jones and Lennon, 2010a), just waiting for an opportunity to wake up and multiply. The first studies dedicated to

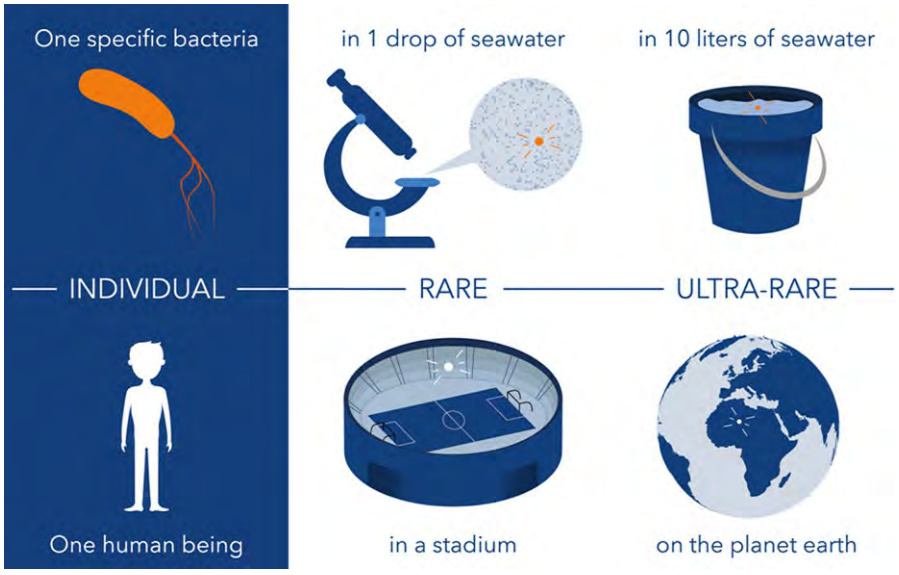

Fig. 2 Infographic illustrating the concept of rare and ultra-rare microbes (upper panel) put into human perspective (lower panel). Rare microbes have a frequency of occurrence lower than 1 cell among 10,000 (<0.01%). Ultra-rare microbes may represent only 1 cell among 8 billion. Drawing by Carole Petetin.

the rare biosphere then gave a more detailed picture of what rare microbes are, showing a more complex picture. A study on the biogeography of rare species, i.e., their large scale spatiotemporal distribution, first showed that rare species were not stochastically distributed, but that they rather show distribution patterns similar to those observed in abundant species (Galand et al., 2009). This finding implied that rare species were most likely subjected to ecological processes such as selection, speciation, and extinction. Then, a study used RNA to target the active fraction of the community, and compare it to the entire community described with DNA. Interestingly, this work on marine microbes showed that a significant portion of the rare community was active (Campbell et al., 2011), thus contradicting the idea that rare microbes represented a seed bank of dormant organisms.

A first proposition to categorise rare microorganisms was published in a study on soil microbes that, at the time, was still based on cloning/sequencing methods. The authors were able to produce a large number of clones and thus describe rare species. Due to technological limitation at the time, the scope of the study was restricted to a single sample but it allowed a first classification of rare species into 2 groups (Elshahed et al., 2008). Group I contained non-unique and non-novel members of the rare biosphere that act as a backup system, which is equivalent to the seed bank, and group II comprised rare bacterial taxa with no close relatives within the dominant species. A study on marine Archaea that focused on the seasonal dynamics of communities year after year then went further in separating the rare biosphere into 3 distinct groups (Hugoni et al., 2013). One group contained the rare microorganisms that became abundant at different times within the same ecosystem. The second group contained cells that were uncommon in public databases and not active. The third fraction contained microbes that were always rare but actively growing. Following that later publication and the latest review by Pedrós-Alió (2012), we can thus propose the following groups of rare microbes.

The Local Seed Bank

The local seed bank is composed of cells that are dormant or little active and that are specific to an ecosystem and essential for its functioning during cycling seasonal environmental conditions. They have also been named as the "conditionally rare taxa" (Shade et al., 2014; Fuentes et al., 2016) and are defined as taxa that are typically found in low abundances in one locality, but occasionally become prevalent. For example, in marine ecosystems with strong seasonality, the local seed bank seen in summer contains microorganisms that become abundant in winter. The local seed bank is adapted to certain environmental conditions, blooms when these conditions are optimal, and then moves back to the rare biosphere until next season. This has been observed for bacteria in the Atlantic Ocean (Bay of Biscay) over a 3 years study in which the authors classified microbes as transient or intermittent depending of their occurrence across seasonal samples (Alonso-Sáez et al., 2015). The role of rare populations as seed banks was also highlighted in the Sargasso Sea, where sequences were partitioned based on frequency of detection as frequent and infrequent (Vergin et al., 2013). In the Chesapeake Bay, over half of the bacterial taxa cycled between being abundant and rare over a 3 year period (Campbell et al., 2011). Archaea have also been targeted in rare biosphere studies. In the coastal Mediterranean Sea, the presence of a local archaeal seed bank contributing to the seasonality of communities was also observed (Hugoni et al., 2013). In lakes, the conditionally rare taxa have been shown to become abundant when the epilimnion and hypolimnion thermal layers were forced to mix to mimic the weakening of the stratification observed in autumn and recreate the following winter mixing (Shade et al., 2014). In soils, studies have tested the response of communities to rewetting events that can imitate the seasonal dry and rainy periods found under various climates. The experiment demonstrated that seed bank microbes "resuscitated" and became abundant following precipitation (Aanderud et al., 2015).

Potential New Colonizers

The potential new colonizers consist of aliens to the studied ecosystem and represent a nonlocal seed bank of microbes that are not found under normal conditions but that have a potential for colonisation. They can be identified as a distinct group because they are usually dormant and composed of organisms that are taxonomically and functionally distinct from the ones found in the studied ecosystem (Elshahed et al., 2008; Hugoni et al., 2013). These rare microbes are not the ones that cycle from abundant to rare between seasons. They lay waiting in the ecosystems, or pass through them, until drastic changes in the environment promote their growth, or they simply die. They can respond to disturbances that occur only exceptionally. For example, in aquatic microbial communities, exposure to salinity stress, either increasing or decreasing salinity, transformed community composition. The disturbed communities had changed due to the recruitment of phylotypes present in the rare biosphere of the original community (Sjöstedt et al., 2012). Another example of severe disturbance is pollution. Rare species have been shown to respond to organic pollutant in freshwater mesocosms (Wang et al., 2017), and rare soil bacteria were shown to bloom in response to hydrocarbon pollution (Fuentes et al., 2016). The group of potential colonizers can also represent microbes waiting for a substrate on which to grow, and this is particularly true in aquatic ecosystems. It has been observed on the sea floor, where specialized chemosynthetic communities become extremely abundant on large organic falls (Kalenitchenko et al., 2018a) or at specific features such as hydrothermal vents (Anderson et al., 2015). These microbes originate from the surrounding seawater, but are so rare that they often go undetected (Kalenitchenko et al., 2018b). They can also represent microbes that are found associated in symbiosis with animals such as corals (Ainsworth et al., 2015) or sponges (Taylor et al., 2013). Again, they will be part of the rare marine-water biosphere before they come into contact with the host.

These potential new colonizers may also be on their way to local extinction (Pedrós-Alió, 2012). If they do not encounter a suitable niche or substrate for growth, they could, with time, disappear. If they do not get extinct, they can also become remnants of microbial evolution that, although currently out-competed in all global ecosystems, possess an exceptional ability to survive and escape extinction (Elshahed et al., 2008).

Always Rare but Active

This last group of rare microbes cannot be considered a seed bank because it is composed of active microbes that, in some cases, can show similar taxonomic affiliation and seasonal dynamics compared to the abundant microbes (Hugoni et al., 2013). Several studies have used RNA as a marker for activity in microbial communities. By calculating a ratio of RNA to DNA after amplifying the 16S rRNA gene, one can estimate the level of metabolic activity of an OTU in the environment. There are strong limitations to the approach and the interpretation of the results obtained by targeting RNA are very much discussed (Blazewicz et al., 2013). Nevertheless, the method is used and a study has showed that at least half of the rare bacteria found in coastal waters of the Delaware coast were metabolically active (Campbell et al., 2011), and that many rare archaea were active in the coastal Mediterranean Sea (Hugoni et al., 2013). Similarly, for freshwater ecosystems, a high proportion of rare bacteria were found to be active in lakes (Jones and Lennon, 2010a). Some of these rare active bacteria could carry out major ecological roles. For example, a specific group of rare methane oxidizing bacteria regulated methane emissions from floodplains (Bodelier et al., 2013). In peatland ecosystems, sulfate reduction can be conducted by single genus of bacteria that comprised less than 0.006% of the total microbial community (Pester et al., 2010) or by a consortium of low-abundance bacteria (Hausmann et al., 2016). Analysis of bacterial activity at the single cell scale also showed that rare microbes could play a significant role in the nitrogen and carbon cycles in the environment (Musat et al., 2008).

The intriguing question is why would active microbes that are key players in the ecosystem remain rare. One hypothesis is that by remaining at low abundance, microbes could minimise predation or viral lysis. Both processes depend on prey encounter probabilities, which are lower for rare microbes. This would fit the killing the winner hypothesis that states that the most abundant microbes, the winners, will be exposed to strong predatory pressure (Winter et al., 2010).

What About Protists?

Protists are key components of microbial communities (Caron et al., 2009; Worden et al., 2015), yet, for historical reasons, they have been overlooked in community ecology studies as well as in studies focusing on rare taxa. Protists feature fundamental differences with prokaryotes in terms of cellular structure, feeding habits, diversity of metabolisms, growth rates and behaviour (Massana and Logares, 2013; Keeling and Del Campo, 2017). Therefore, understanding rare prokaryotes does not imply that we understand rare protists. The available studies thus far indicate that protistan communities feature a large number of rare taxa that constitute a "rare biosphere" (Caron and Countway, 2009; Logares et al., 2015). Even though there are no figures as in prokaryotes, we suspect that with the current amount of sequencing we are only sampling the tip of the iceberg of the protistan rare biosphere. Interestingly, Massana and Logares (2013) proposed that the size of the rare protistan biosphere could be smaller than in its prokaryotic counterparts, given that survival mechanisms in protists could be less developed than in prokaryotes. Another reason is that the "delights of a diluted life" (Pedrós-Alió, 2006b), may not be so obvious for protists. Some picoeukaryotes may not be able to escape predation even after reducing their cell size to the minimum (Massana and Logares, 2013). Furthermore, most small protists are naked, the lack of a rigid cell-wall may limit their opportunities for dormancy. In addition, several protist species may engage in sex, which represents a process that helps maintaining the cohesiveness of eukaryotic species. If the chances of finding a sexual partner become extremely low, as it could happen in the rare biosphere, then the advantages of rarity would be reduced among protists. One important difference between the latter groups is that while prokaryotes seem to enter into a dormant state easily and then establish a seed-bank, this behaviour does not seem to be that common in aquatic protists (Massana and Logares, 2013; Logares et al., 2015). Thus, it follows that rare protists are likely metabolically active (Logares et al., 2015). This has been supported by a study in lakes that found a strong coupling between the total number of protists detected (rDNA fraction) and the active protists (rRNA fraction) (Jones and Lennon, 2010b). In contrast, the total and active bacterial communities analysed in the latter study were decoupled. For protists, similar results to those obtained by Jones and Lennon (2010b) were obtained by Logares et al. (2014b) in a study of marine-coastal communities from Europe. There, researchers did not find large deviations in the total and active communities, pointing to metabolic activity in abundant as well as in rare taxa. In sum, aquatic protistan communities also feature a rare biosphere, yet, whether its size is equal or smaller than that of prokaryotes is a matter of debate. What seems to be clear is that protists do not form extensive seed-banks as prokaryotes, and therefore, communities experiencing repeatable changes (e.g., seasonal) probably reconstitute previous states by recruiting metabolically active, yet low-abundance, taxa.

Perspectives

The development of sequencing technologies has revealed the true extent of microbial diversity in all ecosystems on earth, pointing to a never ending long tail of rare microbes. But, as more and more sequences are obtained for each single studied sample, it appears

that we are still not able to describe all the microorganisms found in the environment. Recent publications gave estimates ranging from 2 millions (Crespo et al., 2016) to 8 billions (Kalenitchenko et al., 2018b) sequences needed to fully describe communities in a ca. 10 L of seawater. The wide range of numbers proposed (3 orders of magnitudes) illustrates the difficulty of estimating the true diversity of microbial communities in nature. It also shows that with the sequencing effort routinely used today, from 20,000 to 100,000 sequences per sample, studies only depict the most common members of the rare biosphere.

Two kinds of approaches would be useful in the future to better understand the ecology of the rare biosphere. First, a massive sequencing effort should target samples from different ecosystems to probe the extent of the diversity of communities. For a reliable result, the approach should combine different sets of universal 16S rRNA primers so that microbial diversity would theoretically be covered extensively, and taxonomic blind spots avoided. The DNA extraction method should also be carefully chosen, so that the DNA of most of the microbes present in a sample is released. Finally, the size of the sample, for example volume of water or grams of soils, should be large enough so that it can represent the diversity of an ecosystem. Metagenomic approaches would also be useful as they are thought to be less biased than PCR-based methods. The number of metagenomic sequences required to get a good coverage of the diversity of environmental DNA is, however, very large and probably beyond reach with the existing sequencing technology.

A second approach would rely on culturing. It is well known that strain isolation from environmental samples only recover a small proportion of the organisms. Long referred as the great plate count anomaly (Staley and Konopka, 1985), culturing tends to be biased toward microorganisms that are not common in the environment. This bias can be exploited to get a better insight into the metabolisms of rare microbes (Lynch and Neufeld, 2015). Culturing approaches have already successfully allowed the detection of rare members of the communities in different ecosystems, such as soils (Shade et al., 2012) or deep sea corals (Jensen et al., 2015).

Finally, experimental approaches have proven to be useful tools to promote the growth of rare species. Experiments designed to provoke drastic changes in the environment have always showed members of the rare biosphere becoming abundant and thus being potentially characterized (Sjöstedt et al,., 2012; Shade et al., 2014; Kalenitchenko et al., 2018b; Wang et al., 2017). These were often so rare in the original community that they were not detected at all. Such approaches will allow the indirect quantification of rare species and a better understanding of their role in the environment.

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Ecology, Microbial

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Glossary

Biogeochemistry The study of processes and reactions that govern the composition of the natural environment and the cycles of matter and energy that transport the earth's chemical components in time and space.
Eutrophic Enriched with inorganic nutrient resources, such as N or P, to the level that excessive amounts of phototrophic biomass is produced. The term is primarily applied to aquatic ecosystems.
Functional redundancy A property of a microbial community in which different species have the potential to perform the same functional role in an ecosystem. A consequence is that reductions in species diversity may not affect ecosystem functionality.
In situ Located in the natural environmental habitat.
Limiting nutrient A nutrient resource whose concentration or flux rate limits the amount of biomass in the habitat, the growth rate of organisms in the habitat, or both.
Microenvironment The chemical, physical, and biological factors that are located within a few micrometers of the exterior surface of a microbial cell.
Oligotrophic An aquatic ecosystem that has low nutrient resource levels, and as a result, low biomass levels.
Planktonic Free-floating microbiological organisms dispersed in solution within an aquatic habitat.
Subsurface The zone below the surface of the earth. For ocean systems, depths >200 m may be considered the subsurface. In terrestrial systems, sediment at depths below the rooting zone of the plant community may be considered the subsurface.
Thermodynamically favorable reaction A reaction in which the Gibbs free energy in the products is less than in the reactants. That is, the reaction releases energy. These reactions can occur spontaneously, although the reaction rate may be greatly enhanced by a catalyst such as an enzyme.

Abbreviation

DOC dissolved organic carbon
HGT horizontal gene transfer
HPK histidine protein kinase
PCR polymerase chain reaction
RTFs resistance transfer factors
SIMS secondary ion mass spectrometry

Defining Statement

This article provides an overview of the field of microbial ecology by delineating its principles, historical development, areas of study, and experimental approaches.

What is Microbial Ecology?

The term ecology was devised by Ernst Haeckel from the Greek *oekologie* meaning 'the study of the household of nature' in 1866. Hence, microbial ecology concerns the interactions of microbes with both their abiotic and biotic environments. Microorganisms are ubiquitous – they grow not only in soil, freshwater and marine habitats, and on or within plants or animals, but also in so-called extreme environments with chemical and physical conditions that exclude most if not all other forms of life. Thus, the field is extraordinarily broad; a number of other articles in this encyclopedia discuss specific topics within this broad field. This article represents a synthesis of developments that may have arisen from the study of different environments or different organisms, and puts them into a historical context of how microbial ecology has developed and (more importantly) where it may be heading in the future.

This article is an update of A. Konopka,Ecology, *Encyclopedia of Microbiology (3rd Edn)*, edited by Moselio Schaechter, Academic Press, 2009,pp. 91-106.

The definition of microbial ecology is very important, because it leads to some important points regarding the discipline. Microorganisms (by definition) are too small to be seen with the naked eye; bacterial, archaeal fungal, and protistan cells typically have diameters between 1 and 100 μm, with bacterial sizes heavily skewed to the smaller end of this distribution and protists at the larger end. Consequently, the 'environment' that these organisms are sensing must also be at a micron scale (the microenvironment). This scale is far too small for humans to sense directly. We can analyze larger, macroscopic samples and infer what occurs at the microscale, although there is a great risk that the properties we measure in the large sample merely represent the average of a rich heterogeneity at the microscale. Because of this, technological innovations are important to microbial ecology when they allow authentic analysis of the *in situ* environment. The definition of microbial ecology also indicates that the intellectual interests and approaches of the microbial ecologist are quite distinct from those of the laboratory-oriented microbiologist who focuses on analysis of pure cultures of microbes. Microbes in nature interact not only with their abiotic environment but also within biological communities of other microbes and perhaps plants and animals. These complex systems may exhibit behaviors that are not predictable from study of the individual organisms in isolation.

That being said, most microbial ecologists have been trained as laboratory-oriented microbiologists rather than as ecologists; as a result, their understanding of ecological theory is often rudimentary. This can lead to conceptual problems, either because problems in microbial ecology do not become well integrated into ecological theory or because there is no clear recognition where the ecology of microbes has elements that are unique from those of plants or animals.

Historical Development of Microbial Ecology

The realization that there was a microbial world depended upon technological innovation – Anton van Leeuwenhoek improved techniques in lens grinding that led to his visualization of 'animalacules' in the late 1600s. However, the discipline of microbiology did not really develop until the late nineteenth and early twentieth centuries when Louis Pasteur and Robert Koch took on practical problems that arose from the interaction of microbes with their environments (such as the germ theory of disease). Their studies were not ecological, but they did develop technologies for the fundamental manipulation of microbes that are still used today. Also in the early twentieth century, Martinus Beijerinck and Sergei Windogradsky became interested in the activities of microbes in natural habitats. They took into consideration the chemical conditions in the natural environment and devised selective conditions (enrichment cultures) by which they could isolate organisms that carried out specific biogeochemical activities. However, their focus was not on the habitat *per se* but on the physiology of the microorganism, and their studies did not focus on ecological interactions.

Modern microbial ecology had its origins in the 1950s, in the study of the rumen ecosystem by Robert Hungate and his colleagues. Hungate is well known for his development of techniques for the cultivation of strict anaerobes. In addition, he had an interest in quantitative analysis of microbial ecosystems. This followed from his studies with C.B. van Niel on cellulose hydrolysis by gut microbiota first in beetles and termites and subsequently in the bovine rumen. He pioneered studies in which the analysis did not end at the isolation and characterization of pure cultures, but necessarily included the enumeration of organisms in the habitat and quantification of their activities. This quantitative analysis distinguished major from minor microbial species and catabolic pathways in the rumen ecosystem.

As in other areas of science, progress in microbial ecology has been intimately tied to the development of new technologies. The availability of radioisotopes beginning in the 1950s was a major impetus for the measurement of biogeochemical process rates in nature. A major problem is that samples must usually be removed from the habitat and incubated under artificial conditions. As incubation time lengthens, the risk of bottle effects that produce results different from *in situ* rates increases. Thus, there is a premium on sensitive detection methods in which incubation time is minimized. The use of $^{14}CO_2$ allowed analysis of rates of primary production by phototrophs and chemoautotrophs, and both ^{14}C- and ^{3}H-isotopic forms of a broad variety of organic compounds have been applied to analyze fundamental processes such as nutrient uptake and assimilation, mineralization to CO_2, and microbial community growth rates. $^{32}PO_4$ and ^{35}S isotopes have been applied to measure biogeochemical process rates in the phosphorus and sulfur cycles. Unfortunately, no practically useful N radioisotope is available, although advances in the sensitivity of stable isotope analyses may make ^{15}N analyses at short incubation times feasible. Another significant breakthrough in analysis of microbial process rates came in the 1980s, when microelectrodes sensitive to certain chemical species were developed. These electrodes have a spatial resolution of 50–100 μm, and have produced great insights into the spatial and temporal dynamics of microbial processes in habitats such as microbial mats and sediments.

Quantitative analyses of biogeochemical process rates blossomed in the period after radioisotopes became widely available, but these studies were incomplete because the specific microbial catalysts were not identified. The techniques of pure culture microbiology were broadly recognized to be ineffective in cultivating numerically abundant organisms from nature. However, over the past 25 years, a set of cultivation-independent techniques have evolved to determine the relative abundance of microbes in a habitat. Carl Woese demonstrated that the sequence of the small subunit ribosomal RNA molecule could be used as a molecular chronometer to analyze the phylogenetic relationships among all forms of life. Norm Pace took this seminal idea and applied it to the analysis of 'who's there' in natural environments. That is, by isolation of nucleic acids directly from a natural environment, amplification of small subunit rRNA gene sequences by polymerase chain reaction (PCR), DNA sequencing of the amplicons, and comparison of the retrieved sequences to the ever-growing database of sequences from pure cultures and environmental DNA, it was unnecessary to cultivate the organism to determine their presence. This has become the major activity in the field of microbial ecology over the past 15 years, and has provided tremendous insights into the phylogenetic diversity present within microbial habitats. However, the determination of what microbes are present in a habitat does not directly resolve the problem of specifically

linking specific microbes to their quantitative biogeochemical role in habitats. Metagenomics, the sequencing of total DNA recovered from an environment, now provides insights into biogeochemical potential, but measuring actual expression of that potential remains a difficult technical problem in the discipline.

The Scope of Microbial Ecology

Microbes are present in almost all aquatic and terrestrial environments, and associated with all plant and animal species. Although each of these habitats may have unique aspects that select for particular strategies among microbes, in this general discussion of microbial ecology, the focus will be on elements that are common to different physical habitats.

Microbes' impact at different spatial scales

The biosphere can be construed as a series of interlinked systems that operate at different spatial scales. Microbes physically operate at a micrometer scale, but both impact and are impacted by processes that operate at larger scales. At the largest scales (global and landscape) are aquatic and terrestrial biomes. Biomes are large geographical areas defined by macroecologists on the basis of their distinctive flora and fauna, which presumably reflect selection and adaptation to climate and geography. For example, terrestrial biomes include temperate deciduous forests, grasslands, rainforests, deserts, taiga, and tundra. Marine aquatic biomes would include polar, temperate, and tropical oceans, and freshwater biomes comprise flowing water (rivers and streams), lakes, and ponds, or wetlands. Whereas biogeography (the study of the distribution of organisms over space and time) is well established for plants and animals, the concept has been less intensively studied for microbes until very recently. There are studies in which there is a correlation between geographic distance and genetic distance for specific bacterial groups. In addition, an increase in the number of taxa detected with increases in sampling area has been found for microbes, as has been found many times for plants and animals. However, the underlying mechanisms responsible for the species-area relationship may be quite different in microbes, due to unique mechanisms for phylogenetically broad genetic transfer and (in some cases) high rates of recombination.

At the global and landscape scale, biotic and abiotic planetary processes can impact each other across biomes. For example, oceanic photosynthesis may be limited by the amount of iron; iron is a cofactor for electron transport proteins essential for conversion of solar energy to a biologically useful form of energy. Arid conditions in deserts produce iron-rich dust particles that can be transported thousands of kilometers through the atmosphere and deposited in the ocean. As another example, the global dispersal of animal viruses such as West Nile virus and influenza virus H5N1 can be mediated by migratory birds.

At the ecosystem scale, there are rich, complementary interactions between physical, chemical, and biological components. For example, photosynthetic sulfur bacteria may occur in freshwater lakes; they require light energy, sulfide as an electron donor that will be used in large part to reduce CO_2 to the oxidation level of cell organic matter, and anaerobic conditions. This combination of factors only occurs in lakes where morphometry, nutrient inputs, and biology are constrained within certain limits (Fig. 1).

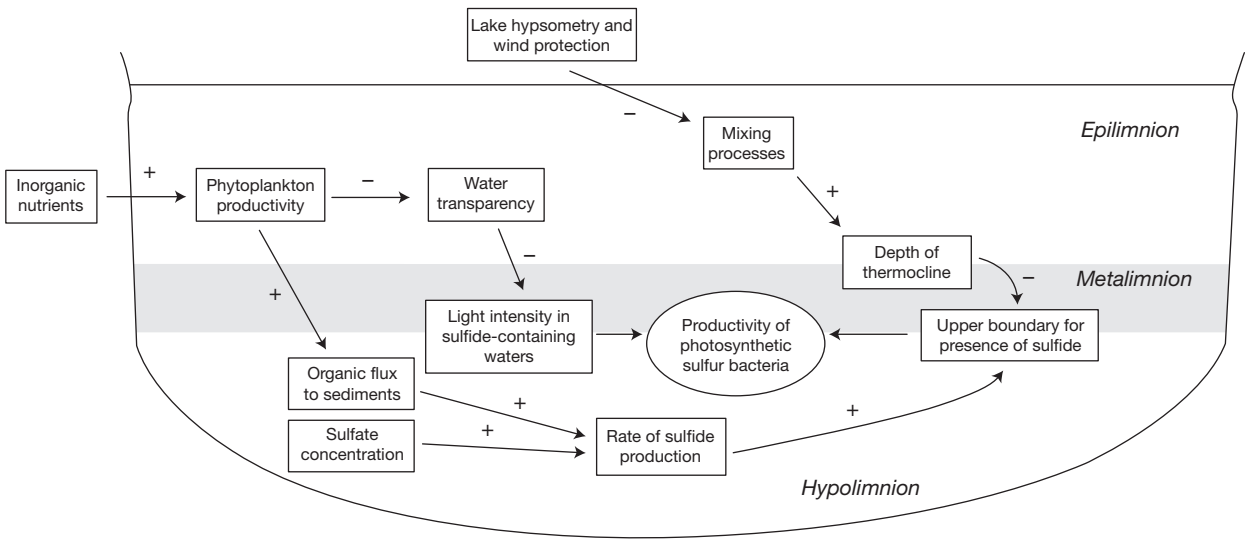

Fig. 1 System interactions affect the occurrence and intensity of photosynthetic bacteria in lakes. These microbes will only be active at strata where light, anaerobic conditions, and reduced S cooccur. The diagram depicts important physical, chemical, and biological factors, and whether an increase in intensity of one factor produces an increase (+) or decrease (−) in a related factor. For example, increases in inorganic nutrient concentrations stimulate phytoplankton growth. This has a negative affect on water transparency at depth (necessary for photosynthetic bacteria) but also results in a larger flux of organic matter to sediments. This stimulates sulfide production, which is also required for photosynthetic bacterial production. The morphometry of the lake basin and its degree of wind protection drive the intensity of lake mixing, which affects the depth from the sediment to which anaerobiosis can extend. Reproduced from Parkin, T.B., Brock, T.D., 1980. Photosynthetic bacterial production in lakes – the effects of light intensity. Limnology and Oceanography 25, 711–718.

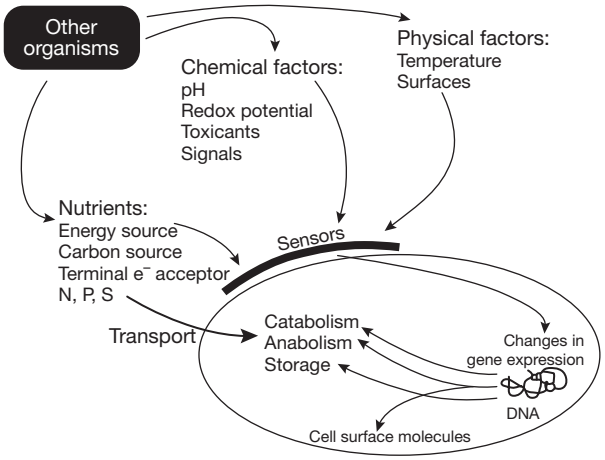

Fig. 2 The microenvironment. The volume within a few micrometers from the microbial cell surface has the greatest impact upon it. External resources are transported across the cell membrane via membrane-bound permeases. Microbial cells contain sensors at their cell surfaces that can detect chemical signals in the environment. This can lead to changes in gene expression and alterations in microbial cell activity. Reproduced from Konopka, A., 2006. Microbial ecology – searching for principles. Microbe 1, 175–179.

However, to understand the mechanistic basis for interactions of a microbe with its environment, one must consider the conditions within a few microns of the cell surface, the microenvironment (Fig. 2). The cell's surface interacts with the external physical environment – this includes physical factors such as temperature or light but may also involve interactions with the surface of minerals or microbial or metazoan cells. A broad suite of chemicals are present – some are required nutrient resources, but others may be toxic or represent signals arising from other cells. The chemicals may arrive at the cell surface by diffusion from the bulk phase liquid, or via intimate contact in dense biofilms. For nutrient resources, there are membrane-bound permeases that transport molecules into the cell. As single-celled organisms, microbes have evolved a series of sensors on cell surfaces to detect and respond to chemical cues. One such system results in a molecular memory of past chemical concentrations and controls the rotation of bacterial flagella in a way that effects chemotaxis. In N_2-fixing root nodule symbioses, chemical cues are passed between *Rhizobium* species and specific plant legumes; flavonoids excreted from plant roots are the key signals in initiation of nodule formation in the nitrogen-fixing symbiosis. They bind to NodD proteins in the rhizobial cytoplasmic membrane and these sensors also activate transcription of nodulation genes critical for the initiation of root nodule formation. Some of these *nod* genes specify enzymes that synthesize lipochitooligosaccharides, molecules that are sensed by root cell sensors and lead to morphological changes in them. Environmental sensors may also be important in animal pathogenesis. The low pH of the human stomach is a defense mechanism against bacterial infection of the gastrointestinal tract. In the human pathogen *Vibrio cholerae*, however, transcription of genes required for colonization and pathogenesis is induced by exposure to acid and higher temperatures as would occur after a host ingested the organism from the environment.

A phylogenetically widespread regulatory system that can detect environmental signals and produce changes in bacterial gene expression is two-component regulatory systems, in which one component is a histidine protein kinase (HPK). Approximately 5000 have been identified by bioinformatic analysis of more than 200 bacterial genomes. The proportion of the genome that encoded HPKs increased as genome size increased, and many of the organisms with very high proportions of HPK exist in anaerobic sedimentary habitats in which there are steep chemical gradients over short scales (such as δ- and ε-proteobacteria) or have relatively complex life cycles (the cyanobacterium *Nostoc* and aquatic chemoheterotroph *Caulobacter crescentus*). On the other hand, bacteria that are intracellular parasites of eukaryotes might be expected to encounter a more constant external environment and they were found to have few HPK genes.

Categorizing microbial ecosystems

A focus on the microscopic scale suggests a set of microbial ecosystems (Table 1), based upon the relevant physical and chemical characteristics at that scale. The quintessential characteristic of aquatic habitats is the planktonic (free-floating) lifestyle. Growth of planktonic chemoheterotrophic bacteria is generally limited by labile, dissolved organic carbon (DOC) present as sugars, amino acids, and organic acids that arise from larger organisms via excretion, lysis, or hydrolysis of macromolecules. The total DOC concentration in ocean waters may approach 1 mg l^{-1}, but this represents a pool of several hundred different organic molecules, each present at very low (nmol l^{-1} to μmol l^{-1}) concentrations. The concentrations are low because the bacteria have evolved high-affinity transport systems, and competitive forces select for microbes that can exploit multiple substrates at low concentrations.

Other microbes must deal with strong concentration gradients of chemical substances, because they are relatively fixed in a solid matrix (such as an aquatic sediment, subsurface aquifer, microscopic biofilm, or macroscopic microbial mat). Strong spatial gradients form in these systems because the chemical sources and sinks differ in location. If microbes are sinks or sources for chemical species, their numbers and activity will affect the steepness of the gradients. These gradient systems may be stable in time

Table 1 Microbial-scale ecosystems

Ecosystem type	*Examples*	*Characteristics*
Planktonic	Open ocean, lakes	'Oligotroph lifestyle' High affinity for uptake of multiple nutrients
Surface-associated saturated water	Freshwater and ocean sediments, subsurface sediments, microbial mats, biofilms	'Gradient lifestyle' Hydrodynamic processes and fluid flow determine nutrient fluxes Biomass density affects gradient steepness
Surface-associated unsaturated water	Surface and vadose zone soils	Water availability as limiting factor for activity and dispersal Patchy nutrient distribution Dormancy
Macroorganism associated	Gastrointestinal tract, rhizosphere, epiphytes	Coevolution, specific molecular interactions with surface-associated molecules

Reproduced from Konopka, A., 2006. Microbial ecology – searching for principles. Microbe 1, 175–179.

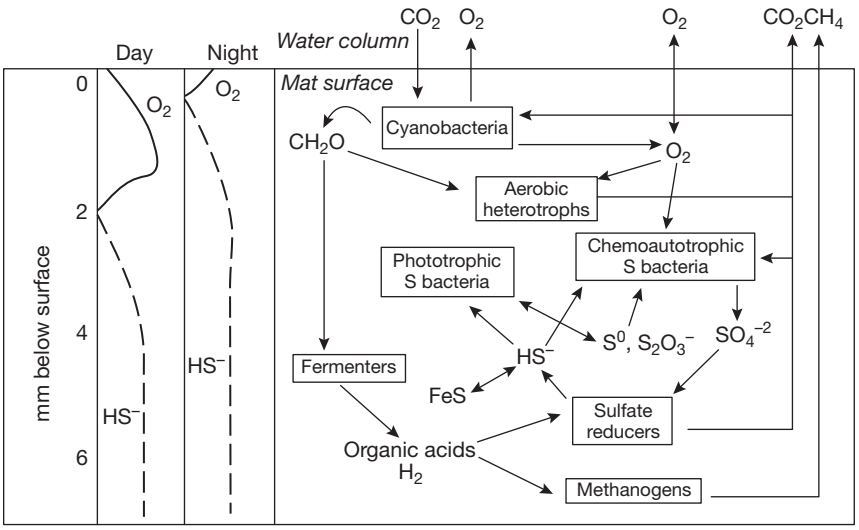

Fig. 3 Microbial interactions and dynamics in a microbial mat. The flow diagram illustrates major functional groups of microbes and transformations in the biogeochemical C and S cycles. The depth profiles on the left present idealized gradients of O_2 and H_2S during the day (when photosynthetic oxygen evolution occurs) and at night (when respiratory processes lead to anoxic conditions through much of the mat). Reproduced from DesMarais, D.J., 2003. Biogeochemistry of hypersaline microbial mats illustrates the dynamics of modern microbial ecosystems and the early evolution of the biosphere. The Biological Bulletin 204, 160–167.

or show significant temporal fluctuations. For example, microbial mats contain photoautotrophic cyanobacteria in the top few millimeters of the mat and a variety of chemoheterotrophic bacteria are located deeper. Experiments with microelectrodes have shown that there are large and rapid changes in chemical species such as O_2, H_2S, and H^+ just after the sun rises and after it sets (Fig. 3).

Terrestrial soil habitats that are not saturated with water may be likened to vast deserts in which there is an occasional convergence of water, nutrients, and microbial cells on a particle that results in a burst of microbial activity. Soils may seem to be rich with microbes (10^9 bacterial cells and thousands of different bacterial genomes per gram), but these numbers occupy $<0.01\%$ of the soil volume. The spatial location of microbial activities in soil can be conceptualized as a series of three-dimensional maps that overlay each other within a matrix of pores. These maps describe the spatial location of (1) different microbes (which have distinct biochemical functionalities), (2) chemical resources (organic and inorganic), and (3) water and gases. Each is necessary but none by themselves is sufficient – activity will proceed only where appropriate levels of all necessary factors intersect. The result is a complex three-dimensional landscape in which the physical structure, determined by not only the mineral matrix but also by plant roots, microbial cells, and extrapolymeric substances, sets the conditions for the activity of microbes and the fate of organic and inorganic C, N, and other elements.

In the case of plants or animals as microbial ecosystems, there is an additional dimension to the chemical gradients and physical structure of the ecosystems described above. That is the capacity for very specific molecular interactions with biomolecules on cells of the plant or animal, and the capacity for (co)evolution by both the microbe and its 'environment'.

Thinking like a microbe

Microbial activity at the microscopic system level produces effects at the macroscopic and landscape level. These effects have relevance for scientific disciplines such as macroecology, biogeochemistry, and geology. However, there are principles unique to the

ecology of microbes, and they only emerge from 'thinking like a microbe'; that is, by considering interactions with the environment at the microscopic scale. It is important to keep in mind some characteristics that distinguish microbes from macroorganisms:

- Small size – Optical amplification (microscopy) is an invaluable tool to assess the distribution of microbes in natural habitats.
- Fast rates of growth and metabolism – Per unit biomass, microbes grow and metabolize much more rapidly than macroorganisms. Generation times of 1–2 days in natural environments are not unusual.
- Exponential growth of populations – The growth of individual microbial cells is autocatalytic; a cell grows to form two cells, each of which can catalyze further growth. When coupled with the relatively fast growth rates of microbes, favorable environmental conditions can produce explosive increases in microbial populations over short period of times.
- Rapid dispersal can occur over long distances – Due to their small size, microbes can be carried for long distances by air or water turbulence. In addition, animals may transport bacteria via mechanisms such as fecal transmission.
- Genetic flexibility – Although Bacteria, Archaea, and many fungi lack sexual recombination mechanisms, many microbes possess genetic elements that mediate vectorial gene transfer from donors to recipients. Analysis of microbial genomes shows that gene transfer is not limited to within a species, but may occur across broad phylogenetic boundaries. In the cases of resistance to newly introduced antibiotics and metabolism of novel xenobiotic organic compounds, evolution has occurred over a period of decades rather than millennia.
- Adaptability to environmental extremes – Most microbial species tolerate and grow within what we consider the normal range of environmental factors such as temperature, pH and chemical concentrations. However, species that are optimally adapted to these environmental extremes have evolved.

Principles of Microbial Ecology

A statement of general principles is difficult, because the attention of microbial ecologists is diffused over many different habitats. Thus, there is a tendency to focus on the idiosyncratic rather than the general. In this section, an attempt is made to synthesize phenomena and search for fundamental principles among the unique features of diverse ecosystems.

Overarching Principles

Principle 1. The primary role of microbes in the biosphere is as catalysts of biogeochemical cycles. That is, they mediate kinetically inhibited but thermodynamically favorable reactions. Some microbes may also have strong effects on the ecology of specific micro- or macroorganisms, via their roles as pathogens or symbionts.

Solar energy is the external energy source that drives the biosynthesis of organic matter from inorganic C, N, P, S, and trace elements by photosynthetic primary producers. These inorganic nutrient sources are present in finite amounts; microbes are often largely responsible for catabolism of organic compounds by which the mineral forms used by primary producers are regenerated.

In terrestrial ecosystems, plants are responsible for the great bulk of primary production. Plant organic matter becomes available for microbial decomposition in forms that vary in molecular structure, susceptibility to degradation, and temporal availability. These include root exudates, the fine meshwork of root tissue in soil, and the seasonal fall of plant litter onto the soil surface. Each source may contain complex mixtures of organic substrates that range from low molecular weight sugars, organic acids, and amino acids to macromolecules. Macromolecule catabolism involves initial hydrolysis to monomers by microbial extracellular hydrolases and subsequent intracellular metabolism. Not only is organic carbon oxidized back to CO_2, but catabolism converts organic N, P, and S to NH_4^+, PO_4^{-3}, and SO_4^{-2}. In this process, energy is released by breaking C–C covalent bonds; some of the released energy is conserved by microbes and used to drive their growth.

The cycling of organic C in soil intersects with biogeochemical reactions in which inorganic chemical species serve either as terminal electron acceptors or as electron donors. These reactions are particularly important with respect to N, because these transformations affect the bioavailability of inorganic N for plants. For example, NH_4^+ is a reduced form of N that is used as an energy source by nitrifying bacteria, and NO_3^- is the end product of nitrification. However, NO_3^- is more mobile than NH_4^+ in soil solution, and hence more susceptible to loss. Furthermore, if the soil becomes anoxic, NO_3^- can substitute for O_2 as a terminal electron acceptor for chemoheterotrophic microbial metabolism. The oxidized end products, N_2O and N_2, are gaseous and thus represent a loss of N for plants.

In addition to their biogeochemical function in nature, the specific interaction that microbes have with plants or animals can have major consequences, not only on the affected organisms but also on the ecosystem. Frank pathogens of plants and animals can generate selection pressures to evolve resistance determinants upon their hosts. For example, humans with the sickle cell trait (one allele of an altered form of hemoglobin along with one normal allele) are more likely to survive disease caused by the malaria protozoan parasite than individuals homozygous for normal hemoglobin. The incidence of sickle cell trait in humans is highly correlated with areas in which malaria is endemic.

Microbes can also positively affect macroorganisms via mutualisms. A well-studied example relates to another aspect of the nitrogen biogeochemical cycle. Bacteria such as *Rhizobium* and *Frankia* can form specific associations within nodules on plant roots. These associations make photosynthetically produced organic C available to the microbes. When in association with plants, these microbes express nitrogenase, an enzyme that fixes N_2 into NH_4^+; microbial metabolism is regulated such that >90% of fixed N is translocated to the plant.

Principle 2. Although generally unseen to the naked eye, microbes comprise more than 25% of all biomass on Earth. All habitats suitable for plants and animals also harbor microbial populations. In addition, some microbes are adapted to grow under physical and chemical conditions that are too extreme for plant and animal growth.

Determining the amount of microbial biomass on a global scale is difficult to do with precision, but estimates suggest that the amount of organic carbon in Bacteria and Archaea (ca 200 Pg) is approximately one-third of that found in land plants. Estimates of eukaryotic microbial biomass (e.g., fungi in terrestrial habitats) are difficult to obtain, but would certainly augment this estimate significantly. more than half of the biomass on Earth is likely to be microbial. Near-surface terrestrial and aquatic habitats make larger contributions to overall microbial growth and activity. There are few measurements of growth rate in the deep subsurface, but existing measures suggest a generation time for microbes measured in tens to thousands of years; this may reflect occasional periods or locations of microbial growth with most of the microbes surviving in a metabolically quiescent state.

Microbes associated with plants or animals can exert strong ecological effects via parasitism or mutualism. However, in terms of global biomass, these organisms are minor players; they account for less than 1/100,000th of microbial biomass on Earth. Bacteria that function in digestion and nutrition of animal herbivores (ruminants and termites) comprise the largest proportion, due to the large biomass of these animals on Earth.

Humans characterize habitats in which the physical or chemical conditions preclude the existence of plants or animals as 'extreme'. However, some microbes have evolved adaptations in molecular structure such that they are optimally adapted to growth in a particular extreme environment such as very acidic or basic pH, very cold or hot temperatures, or high salt concentrations. These adaptations usually mean that the organisms cannot grow under the conditions we humans consider normal. In the case of thermophiles and psychrophiles, evolution in cellular macromolecules has occurred. For example, cellular enzymes in thermophiles retain structure and activity at higher temperatures; this may be a consequence of more extensive secondary structure and reduced content of certain labile amino acids. A significant problem at low temperature is fluidity of membranes, and the lipids in psychrophiles have increased content of fatty acids that will remain fluid at lower temperatures.

Principle 3. The effects of microbial catalysis (the rates of chemical and energy flow through an ecosystem) depend upon the population size of microbes (the number of catalysts) and their physiological activity (as determined by extant physical and chemical conditions). In stable ecosystems, small numbers of microbes can still produce significant effects over geological time spans.

Microbial population levels and cell-specific activities can range over many orders of magnitude in nature. In aquatic ecosystems such as lakes and coastal marine areas, excessive nutrient enrichment leads to undesirable ecological effects (eutrophication). The amount of P_i loaded into an aquatic ecosystem will often determine the amount of photoautotrophic biomass (Fig. 4) in the water column, and systems with higher levels of photoautotrophic biomass exhibit increased levels of primary productivity. However, physiological studies of phototrophs have shown that the cell-specific rate of photosynthesis will vary significantly as a function of irradiance, and that even at saturating irradiances the photosynthetic rate may vary 5- to 20-fold, depending upon the nature and stringency of nutrient limitation.

The effects of temperature upon activity may be even more striking, when one realizes that much of the biosphere (the deeper waters of oceans and lakes, and many subsurface terrestrial systems) has ambient temperatures of $<10\,^{\circ}C$. As microbes are exposed to temperatures near their minimum, not only do the catalytic rate of enzymes decline, but membrane lipids stiffen, thereby decreasing the effectiveness of membrane-bound transport proteins. As a result, substrate affinity declines and cells are less effective in concentrating substrates from dilute environments. The physiological consequences are that limiting resources can have even more acute affects on activity in cold habitats.

Some processes may occur very slowly, yet have significant impacts on biogeochemical cycles. For example, the anaerobic oxidation of methane in deep-sea sediments is biogeochemically important, given the vast expanse of seabed floor. However, the

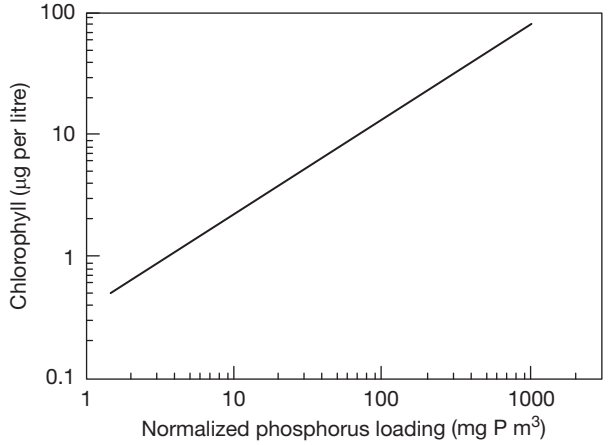

Fig. 4 The Vollenweider model of the relationship between the amount of P that enters a lake (P loading in mg P per m^3 of lake volume) and the standing crop of phytoplankton biomass (measured as chlorophyll *a*).

process occurs at rates of nmol cm^{-3} $year^{-1}$, and enrichment cultures of the consortium of methanogen and sulfate-reducing bacteria have a doubling time of 7 months, whereas when grown axenically, these organisms double in < 40 hours.

Principle 4. The results of microbial catalysis can have profound effects on the physical and chemical characteristics of the macroenvironment. However, microbial activity is determined by the physical and chemical characteristics of the microenvironment.

There are many habitats in which spatial heterogeneities at the submillimeter scale produce a richer diversity of habitats and niches than is apparent from measurement of bulk average concentrations of chemicals. In the open ocean, there is a spatial mosaic of nanometer-sized organic colloids as well as sheets of organic compounds that may be up to 100 μm in size. Aggregation of organic matter and organisms into millimeter-sized particles create a habitat for a rich and diverse community of phytoplankton, protists, and bacteria. In well-aerated soil, local deposition of labile organic matter will create a site of intense activity by aerobic bacteria in which the rate of oxygen consumption exceeds its diffusion and an anoxic microsite in a soil particle can arise.

Strong concentration gradients in environments that result from physico-chemical processes, high densities of active microbes, or (most commonly) the interaction between abiotic and biotic forces also produce strong effects at the microscale. The gastrointestinal tract of insects is a fermentation chamber with a high concentration of microbes; the fermentation products (organic acids) are absorbed through the wall of the gut to fuel the nutrition of the animal. However, O_2 can also diffuse from the trachea across the gut wall, so that much of the gut volume is microoxic rather than fully anaerobic. Furthermore, H_2 is a fermentation product that arises in the anoxic lumen of the gut and is not used by the insect. It is an energy source for aerobic chemoautotrophic bacteria, which have a niche where overlapping gradients of O_2 and H_2 occur in the lumen. In dense microbial mat systems, diurnal changes in light irradiance drive changes in the rate of photosynthetic oxygen evolution that result in rapid temporal changes over millimeter distances in the profiles of O_2, H_2S, and pH in the mats.

Principle 5. The microbial world exhibits much greater metabolic versatility than is found among macroorganisms. This versatility is exhibited in several different ways:

- Essentially all reduced chemicals found on Earth (both organic and inorganic) can be used as an energy source by some microbe if its oxidation can be coupled to reduction of a terminal electron acceptor in a thermodynamically favorable reaction.

  Many biogeochemical cycles operate because some microbe can extract biologically useful energy from the reduced form of an element (e.g., H_2S) and transfer the electrons to a molecule (such as O_2) that has a greater tendency to become reduced (the terminal electron acceptor). The oxidized product (SO_4^{2-}) can then be reduced to H_2S when it serves as a terminal electron acceptor for a different physiological group of microbes.

  For organic compounds, all naturally occurring organic compounds and most synthetic organic chemicals can be used as energy sources by microbes. It is easier to list the recalcitrant exceptions – lignin (under anaerobic conditions), highly halogenated organic compounds (under aerobic conditions), branched alkanes, and insoluble synthetic polymers (e.g., polyethylene).
- Individual organisms may be capable of metabolizing a broad range of different substrates.

  From an ecological perspective, much of the organic matter that becomes available to chemoheterotrophic bacteria arises via cell lysis from the death of other organisms (plants, animals, or other microbes). The enzymatic hydrolysis of cell macromolecules will produce a rich mixture of various sugars, amino acids, and organic acids. Thus, it is not surprising that individual microbes may possess transporters and enzymes that provide the potential for utilization of many components of these mixtures.

  The study of biodegradative enzymes in bacteria has provided insights into some mechanisms whereby a broad substrate utilization range may arise. In some cases, a key enzyme with a broad substrate range has evolved. A halidohydrolase from an *Arthrobacter* sp. is active against more than 50 different halogenated organic compounds that differed in their halogen substituent and ranged from aromatic to saturated or unsaturated alkanes. The evolution of biodegradative metabolic pathways has also made it facile for an organism to utilize multiple organic substrates. There are a large diversity of substituted aromatic compounds that can be aerobically catabolized by microbes. Catabolic pathways entail the action of enzymes that funnel different substrates to one of a few common intermediates (usually dihydroxy benzene derivatives), which are then further catabolized by a common set of enzymes. Thus, the growth substrate range can be augmented by acquisition of enzymes to process specific substrates, once it possesses a core aromatic catabolism pathway.
- Complex microbial communities may be metabolically redundant and contain a number of different populations that can metabolize the same substrate. This metabolic diversity increases resiliency to environmental perturbations, as extreme conditions are less likely to kill all species performing a particular ecological function.

  A critical issue in all areas of ecology is to understand the relationship between biodiversity and the functioning and stability of ecosystems. Imagine an ecosystem that is devoid of microbes, and that different species are introduced one by one. Initially, each new species is likely to add a new function to the ecosystem, because it can utilize substrates that the previous species could not. At some point, however, new species are likely to be redundant – an existing species can already carry out that function. The ecosystem possesses its maximum microbial functionality. However, higher biodiversity (with its associated functional redundancy) is valuable if the ecosystem is exposed to changes in environmental conditions such as a change in pH or addition of a toxic metal. If functional redundancy were low, then all species that can carry out a particular function might be eliminated. At higher functional redundancy the probability that at least one species is tolerant of the new environmental condition is higher and the ecosystem then retains this function and is perceived as functionally stable.
- Microbial populations are capable of rapid evolutionary responses to environmental perturbations; this is due to mechanisms that generate relatively high mutation frequencies and a capacity for horizontal gene transfer (HGT) across large phylogenetic boundaries.

Evolution via differential reproduction under environmental selection pressures was the central premise of Darwin's work. In the case of plant and animal species, genetic variation (the raw material upon which selection acts) is generated within one species via the shuffling of genes during sexual reproduction. In microbes, however, there are mechanisms of HGT that can cross broad phylogenetic boundaries and generate completely novel gene combinations in much shorter time frames.

Humans have performed two great natural experiments that illustrate this point – the large-scale introductions of antibiotics and xenobiotic organic compounds. Antibiotic-resistant forms of a pathogen have often arisen within 10–30 years after introduction of a new antibiotic. The acquisition of multiple antibiotic resistance by clinical isolates is conferred by resistance transfer factors (RTFs), broad host range plasmids that contain a set of transposons, each of which has a gene for resistance to a different antibiotic. From where did these plasmids arise? A set of clinical isolates of Enterobacteriaceae that had been archived in the preantibiotic era was found to contain plasmids, and their core genes for replication, maintenance, and conjugal transfer were similar to those found in modern RTFs. However, they did not contain transposons with antibiotic resistance determinants. The implication of these findings is that the use of antibiotics in clinical practice and agriculture results in a strong selection pressure for cells with RTFs in which the appropriate transposon has integrated.

Why can microbial evolution proceed more quickly than in the case of macroorganisms? Microbial generation times can be comparatively short, and each replication of genetic material will result in a small number of mutations. When one considers the total population size of microbial cells in habitats (millions to billions per gram), there are ample opportunities for genetic variation to arise.

The 'evolution' of specific taxa is particularly enhanced by bacterial mechanisms of gene transfer (the uptake of naked DNA by a cell, or transfer of DNA into a recipient bacterium by direct contact with a donor bacterium or infection with a bacterial virus) that may permit horizontal transfer of genetic material across broad phylogenetic barriers. Gene transfer frequencies are directly proportional to cell density – crowded habitats such as animal gastrointestinal tracts, soil microcolonies, the plant rhizosphere, biofilms, and marine snow probably represent important foci. In some instances, the acquisition of a single gene (e.g., an antibiotic resistance gene or exotoxin genes within prophage of *Corynebacterium diphtheriae* or *Clostrdium botulinum*) can substantively alter the organism's ecological properties. In other instances, there are functional modules of multiple genes that confer novel properties. Some examples include sets of genes for organic catabolism or pathogenicity islands that contain virulence genes.

Principles in Population Ecology

Principle 6. The types and numbers of different microbes present in a habitat are a function of the diversity and amounts of nutrient resources available; in ecology, this is termed bottom-up control. However, the abundance of a microbe will also be modulated by its ability to withstand forces that eliminate it from the habitat. These loss forces may be biotic (due to the activity of predators and parasites and termed top-down control), physical (flushing or scouring action), or physiological (nutrient starvation).

The effect of nutrient resources upon microbial activity and growth will be discussed below in the context of physiological ecology. Although much of the historical effort in microbial ecology has been directed to the study of bottom-up control, there have been significant studies since the 1980s on the effects that biotic loss factors may have on structuring microbial communities. The recognition of the role played by eukaryotic protists in biogeochemical cycling in the ocean was important in this regard. Bacteria have been found to utilize up to 50% of the carbon fixed by photosynthesis in the ocean. The 'microbial loop' (Fig. 5) provides a means for these resources to be reintroduced into the traditional food chain (via zooplankton to fish). Bacterial numbers are controlled by nanoflagellates and to a lesser degree small ciliated protists, which in turn are preyed upon by microzooplankton. As these predators are of similar size to phytoplankton, they can be consumed by zooplankton and the resources reenter the planktonic food chain. Detailed investigation of other habitats has shown that bacteriovorous protists are important in a range of ecosystems, including soil.

There is a vigorous debate between those who contend that bottom-up mechanisms control microbial biomass levels and those who emphasize top-down control. The truth likely lies in the middle, and may differ depending upon season and ecosystem

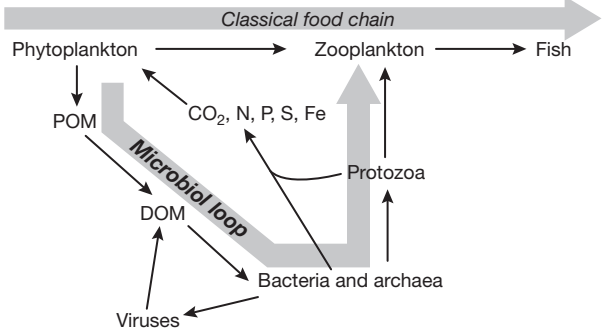

Fig. 5 The microbial loop in aquatic food chains. Filter feeding by protists upon bacteria can result in the return of primary production back to the classical food chain, as the protists are preyed upon by zooplankton. POM, particulate organic matter; DOM, dissolved organic matter.

productivity. Both empirical and theoretical evidence suggest that bacterial numbers may be more tightly controlled by protistan predation in nutrient-poor (oligotrophic) ecosystems, whereas in nutrient-rich habitats competition for nutrients has primacy. Intuition might lead one to the opposite inference, but eutrophic habitats will have higher population levels of higher-level predators (zooplankton and fish) that keep tighter control on the abundance of bacteriovorous protists. As a result, predation pressure on bacteria is reduced and higher bacterial numbers result in more stringent nutrient limitation.

Protistan grazing not only can control bacterial numbers, it can also shape bacterial community composition. Protists employ either filter or interception feeding. As a result, bacterial cells with diameters of 1–3 μm are most susceptible to predation. And, therefore, aquatic protists are very effective predators on rapidly growing single-celled bacteria. Thus, there will be strong selective pressure for antipredator strategies in bacteria that result in smaller or larger particles. Strategies that yield larger particles include morphological changes such as filament formation or production of exopolymers to produce cell aggregates. Ultramicrobacteria (those with a cell diameter of 0.3 μm or less) can be isolated from natural marine samples; these organisms are sized below the optimal feeding range of protists. Chemical defenses have also been found – the purple pigment of *Chromobacterium violaceum* has been found to induce apoptosis in flagellates when a bacterial cell is ingested.

Over the past 25 years, aquatic ecologists have come to recognize that viruses are also significant causes of microbial mortality. There are approximately 10^8 virus particles per milliliter in productive, coastal marine waters. There is significant genetic diversity among these viral particles, and within that diversity are viruses that can infect algae, bacteria, or protists. It has been technically challenging to accurately measure the proportion of microbes that are lysed by viruses; current estimates range from 10% to 50% of the bacterial population per day and the impact of viruses as a loss factor for bacterial production may equal that of bacteriovorous protists in marine systems.

Principle 7. Microbial populations and communities often exhibit much larger dynamics in biomass, composition, and activity than do plant and animal populations.

These complex, nonequilibrium dynamics are thought to be primarily driven by the temporal frequency of changes in important environmental factors (and hence, changes in selective environmental forces). An obvious example is a tidal salt marsh, but there are periodicities that exist at shorter and longer frequencies to which microbes can respond. Temporal changes can occur over very different spatial scales, from kilometers in coastal marine upwellings to millimeters in laminated microbial mats. In addition to these environmentally driven changes, there are also internal biological cycles in ecosystems that alter selective forces. Predator–prey cycles are perhaps the best known, but the identification of cross-species molecular signaling in the laboratory suggests that population dynamics may have a larger biological component than we have appreciated.

Microbes respond more dynamically to these changes than macroorganisms as a consequence of their biological properties: a rapid pace of physiological adaptation to changing environmental conditions, a broad variety of positive and negative interactions between different microbes and the capacity for rapid genetic change. An important consequence of principles 6 and 7 is that the observed temporal changes in microbial populations (whether termed 'community dynamics', 'successions', or 'oscillations') are not solely determined by environmental or biological factors, but rather by an intricate interaction among these factors.

Principles in Physiological Ecology

Microbial physiological ecology is a field in which the molecular details of gene regulation can be evaluated in the context of ecological effects under the selective pressures experienced by microbes in nature.

Principle 8. The amount of one essential limiting resource is most likely to determine the amount of microbial biomass in an ecosystem.

A nutrient resource that is taken up may be (1) conserved in functional or structural molecules (i.e., become biomass), (2) utilized and excreted for bioenergetic purposes (energy source and terminal electron acceptor), or (3) function both as a component of biomass and in bioenergetics. All organisms must acquire an energy source (light of the appropriate wavelengths for phototrophs and a reduced chemical for chemotrophs) and the appropriate molecular form of atoms that comprise biomass. The most important atoms are C, N, P, S, and several metals (Fe is often the most quantitatively important) that serve as enzyme cofactors. Chemotrophic bacteria also require a terminal electron acceptor, a molecule to which electrons are ultimately donated after oxidation of the energy source. Autotrophs require reducing power to reduce CO_2; in photoautotrophs an external electron donor is used for this purpose via photosynthesis whereas chemoautotrophs may use their reduced energy source directly or generate reductant via reverse electron transport.

The molecules that comprise this elementary set of requirements can be called 'complementary substrates' in that all are necessary, but none by themselves are sufficient for growth. However, within each requirement, there may be substitutable forms. All microbes use $NH4^+$ as an N source, a number can use $NO3^-$, and some fix N_2. There are hundreds of different organic substrates in the environment; if a specific microbe has uptake systems and enzymes to catabolize them, these represent substitutable substrates.

The limiting nutrient is not necessarily the one present in the lowest absolute concentration in the habitat. Rather, it is the resource in scarcest supply relative to its requirement for biomass synthesis (Liebig's law of the minimum). It is important to recognize that cellular composition is not immutable. Despite the general biochemical similarity of all organisms, some taxa may have unique requirements. For example, diatoms require silica for their extracellular frustules whereas other groups of phytoplankton do not have this requirement. In addition, physiological conditions may substantially affect the elemental stoichiometry of biomass. Iron is required in a variety of electron transfer proteins such as cytochromes and nonheme iron proteins; it usually

comprises 0.2% or less of a bacterium's dry weight. However, in N-limited environments where N_2-fixing bacteria would have a selective advantage, the synthesis of Fe-rich nitrogenase and ferredoxin would increase cellular iron content by at least fivefold.

Principle 9. Growth of microbes on the limiting nutrient (or a set of substitutable nutrients) will depress its concentration to a value that limits microbial growth rate. The nutrient flux rate in an ecosystem may become physically limited by hydrodynamics or mass transport.

The 'limiting nutrient' in a habitat has two distinguishable effects on microbial growth. It limits the total amount of biomass. If microbes are inoculated into a sterile environment, they will grow at their maximum rate and at some point the total demand of biomass for nutrient uptake exceeds its supply rate, and the concentration of limiting nutrient decreases. At some low concentration (in the nmol l^{-1} to low μmol l^{-1} range), transport rate into the cell is determined by this low extracellular substrate concentration and a reduced intracellular flux produces a reduced growth rate.

Principle 10. Competition among microbes for low concentrations of limiting substrate and temporal variability in nutrient availability is a strong selective force in natural habitats. A variety of physiological and morphological adaptations can have adaptive value under specific conditions.

1. The number, diversity, and affinity of transporters in the cell membrane: Nutrient concentrations in the nmol to low μmol l^{-1} range are likely to constrain the growth rate. The organisms that can maximize nutrient flux into the cytoplasm will have the highest growth rate and therefore a competitive advantage. In the case of chemoheterotrophic bacteria in the open ocean, organic substrates are likely to be the limiting nutrient. The dissolved organic C level in seawater totals ~1 mg C l^{-1}, but this represents nmol l^{-1} concentrations of a wide variety of specific substrates derived from the lysis of phytoplankton cells and the hydrolysis of their macromolecules. One physiological strategy would be to express genes for high-affinity transporters, because at these low external substrate concentrations, the specific affinity (the initial slope of the uptake rate vs. substrate concentration function) provides a competitive advantage. Furthermore, derepression of gene expression to increase the number of transporters inserted in the membrane will enhance substrate flux via the law of mass action. The maximum rate of transport (V_{max}) normalized to a per cell basis is a function of the number of transporters per cell. Increases in V_{max} not only provide a higher transport rates at saturating substrate concentrations, but also increase flux rates at rate-limiting concentrations (Fig. 6).

   When there are approximately 1500 transporters of one specific type in the membrane, they compete with each other for substrate molecules, and further investment in additional transporters of this type is not productive. However, only 2% of the membrane surface is covered with transporters; synthesizing transporters with many different specificities will maximize the total flux of limiting organic C into the cell, if other organic substrates are available. Thus, organic C-limited chemoheterotrophs are predicted to express the capacity for the transport and catabolism of multiple organic substrates. They need only generate a fraction of their total growth rate from the transport flux of each individual organic compound.

2. Regulation of the capacity for substrate uptake versus substrate assimilation: The capacity for nutrient uptake is maximized by increasing the number, diversity, and specific affinity of transporters. However, in a competitive environment this will push ambient substrate concentrations to values where the growth rate is far below maximal. The optimal use of resources under these conditions will regulate decreased amounts of the macromolecular machinery for biosynthesis and assimilation. The most obvious example of this is the direct relationship between growth rate and cellular ribosome content. Ribosomes are energetically expensive to produce; a cellular content that exceeds the required rate of protein synthesis is energetically inefficient.
3. Cell size: When bacteria are grown in a chemostat at a variety of growth rates, slow-growing cells have a smaller size and volume than fast-growing populations. The direct physical reason for this may be the differences in ribosome content mentioned above. A tenfold increase in cellular ribosome content can only be accommodated by increasing the volume of a cell. Direct microscopic

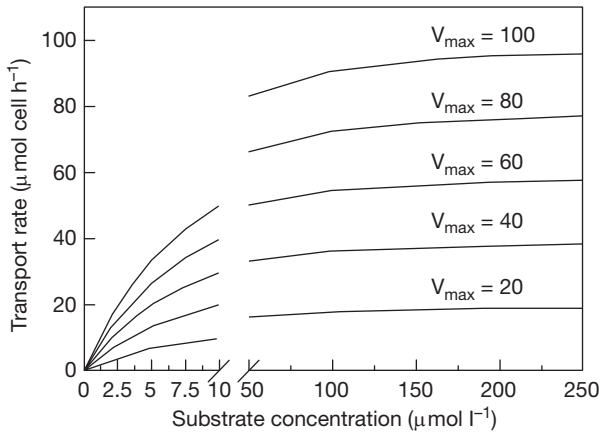

Fig. 6 The effect of increasing the number of membrane-bound permeases per cell, at both low, transport-limiting external substrate concentrations and saturating concentrations. Calculations were made employing the Michaelis–Menten equation at K_s of 10 μmol l^{-1}, and different V_{max} values. V_{max} is proportional to the number of permeases per cell.

examination of bacteria in aquatic environments indicates that many cells are quite small, with diameters of 0.5 μm. Size-selective predation by protists may play a role here, but the low growth rates typically found in planktonic aquatic ecosystems (doubling times are often measured in the range of 16–50 h) also lead to small cells. The physical nature of small cells has another beneficial effect for nutrient-limited habitats – the surface-to-volume ratio increases as cell diameter decreases. Membrane-bound surface area is the location of substrate transporters, whereas the cytoplasmic volume contains soluble biosynthetic machinery. Thus, decreases in cell size found when nutrient-limited growth is slow have the consequence of increasing the ratio of transporters-to-assimilation machinery.

4. Storage polymers: The physiological responses to nutrient limitation in nature include derepression of nutrient transport systems and downregulation of biosynthetic pathways. However, a microbe may be exposed to pulses of higher nutrient concentration either on a regular diurnal cycle or at irregular intervals (e.g., by encountering particulate organic matter in an aquatic ecosystem). At this physiological state, transport of the limiting nutrient occurs at a much faster rate than can be assimilated into proteins and nucleic acids. The solution to this mismatch is intracellular storage as a polymer that can be assimilated later when external substrate concentration is again limiting.

Storage polymers of C and energy include glycogen, starch, and polyhydroxyalkanoates. P is stored as polyphosphate by a number of microbes. N is the macronutrient for which there is no storage polymer, with the exception of cyanophycin produced in many cyanobacteria and a few heterotrophic bacteria. The polymer consists of an aspartic acid backbone to which arginine residues are linked.

The synthesis and degradation of glycogen by the cyanobacterium *Oscillatoria aghardii* during a diurnal light–dark cycle illustrates the interplay between resource acquisition, storage, and cell growth. During the light period, fixed CO_2 is used for immediate growth (synthesis of proteins and nucleic acids) and stored as glycogen. Of particular note is that growth rate (measured as rate of protein synthesis) during the light period is lower than in cells cultured in continuous light. However, during the dark period the intracellularly stored glycogen is used as both a carbon and an energy source – the rate of protein synthesis in the dark is very similar to that measured in the light. That is, the microbes grow chemoheterotrophically in the dark on stored glycogen. This physiological strategy produces a higher daily growth rate than if the cyanobacteria had a growth rate of μ_{max} in the light and 0 in the dark.

Strategic Approaches to the Study of Microbial Ecology

An examination of articles published in microbial ecology journals will illustrate not only the breadth of habitats that are studied by microbial ecologists, but also a variety of technical approaches. Some of the major categories are described below.

Descriptive Studies of the Distribution and Activity of Microbes in a Natural Habitat

'Descriptive studies' has become a pejorative term in science, but this type of analysis is a necessary first step when initiating a study on a new habitat. In addition, field studies have an overhead not found in well-controlled laboratory studies – the collection of routine data on physical, chemical, and biological characteristics of the system. It is essential to monitor the natural variation that lies behind any hypothesis-driven field experiments. These routine data can be mined using statistical techniques to uncover relationships between environmental factors and biological activities. These relationships suggest hypotheses that can be experimentally tested.

The most common types of descriptive data published in ecological studies are lists of microbial community members identified from sequences of 16S rRNA gene amplicons or metagenomic analyses. About 23% of the 22,000 papers published on microbial ecology over the past 10 years have included at least one of these data types. Although knowledge of community composition on its own is useful, it is far more impactful to conduct deeper analysis of datasets comprising hundreds of samples. By employing statistical frameworks, changes in microbial community composition in space or time can be used to infer the relative importance of ecological forces (dispersal, selection, and drift) that shape microbial composition. Furthermore, when related to field-scale measurements of physical and chemical variations, the effects of environmental variable variation upon the intensity of those ecological forces can be mapped.

Analysis of *In Situ* Rates of Growth or Biogeochemical Activity

These analyses can rarely be accomplished under true *in situ* conditions, as they entail addition of chemical tracers or measurement of changes in biomass. Thus, natural samples are collected and confined in containers, but incubated under the physical conditions found *in situ*. The removal of a sample from the open conditions of the natural system to a confined bottle will lead to changes with time. Therefore, it is advantageous to keep the incubation time as short as possible. The use of radiotracers (i.e., the addition of a radioactive molecule in an amount that is small enough to have a negligible impact on *in situ* chemical concentration) has been invaluable in rate measurements of biogeochemical processes. Bacterial growth rates have been estimated by measuring the rate at which radioisotopic forms of biosynthetic precursors (such as thymidine or leucine) are incorporated into cellular macromolecules. These calculations require several assumptions, and give an 'average' growth rate for a heterogeneous population of microbes.

These results generally suggest that the doubling times of aquatic bacterioplankton are in the range of 1–2 days, much longer than what is achievable with pure cultures in the laboratory. *In situ* growth rate is a fundamental parameter of interest in ecological studies, but it remains a difficult one to accurately and conveniently measure for microbial populations in nature.

Experimental Manipulation of Natural Samples Under Simulated Conditions (Microcosms)

The intent of microcosm experiments is generally not to accurately measure *in situ* activity rates, but rather to perform experimental manipulations of natural microbial communities to test hypotheses regarding the effects of environmental perturbations upon microbial community activity or composition. Incubation times are likely to be much longer than when measuring *in situ* activity rates; therefore, caution in interpretation is warranted as confinement of the sample can introduce major effects.

An example study examined the population structure and activity of a microbial community in soils that were cocontaminated with lead, chromium, and aromatic hydrocarbons. It was difficult to separate the effects of different contaminants by direct analysis of spatial differences in community composition and environmental contaminants. Therefore, microcosm studies were set up in which soil samples from both contaminated and uncontaminated sites were challenged with different terminal electron acceptors and a graded series of heavy metal concentrations, to analyze the effects upon microbial activity (the mineralization of organic carbon) and community composition. The experiments illustrated that if one wanted to detect changes in community composition, it was important to add an energy source (organic substrate) that could drive significant changes in composition. Of course, this energy source will itself represent a strong selective force in structuring the microbial community. However, by comparison of sample treatments, it was possible to demonstrate that whereas resource factors such as the organic energy source and terminal electron acceptor were a major force in structuring species richness, the effects were modulated by toxic factors such as heavy metals. In addition, the factorial design of the experiments (in which a series of combinations of energy sources, terminal electron acceptors, and heavy metal concentrations were tested) showed that microbial activities carried out by a diverse group of functionally redundant community members were more resistant to high toxic metal inputs than activities for which a more restricted diversity of microbes was likely to be present.

Model Laboratory Systems

A distinction between microcosm experiments and model laboratory systems is that the former are initiated with many of the physical and chemical characteristics as well as the taxon richness of the original habitat whereas the latter is often a highly simplified version of nature. In many cases, it may be the study of a pure culture in the laboratory. This can be controversial in microbial ecology, because the laboratory system will lack a large number of features found in nature. However, ecophysiological studies in the laboratory provide the means to rigorously ascertain the physiological responses made by a microbe to environmental changes. These can be approached with a degree of experimental control and replication that is not possible in the habitat. If the study remains housed entirely in the laboratory, it probably ceases to be an ecological one. However, there are advantages to integration of field and laboratory studies, and recursion between real-world complexity and the experimental control that can be applied in the laboratory.

Ecological studies on planktonic cyanobacteria have provided an example of the value of this type of recursion. In eutrophic lakes, cyanobacteria proliferate to high cell densities (blooms) in which >90% of the biomass in the sample consists of one morphological type of cyanobacterium. Under these unique conditions, one can measure physiological properties in whole samples (photosynthetic rate, allocation of fixed C to different macromolecules, rates of macronutrient uptake) and reasonably ascribe these properties to the dominant biomass component. From these measurements on field populations, hypotheses arise regarding the regulatory responses made by the organism to limiting environmental factors. These hypotheses are testable in the laboratory by experiments with cyanobacterial isolates cultivated in chemostats under control of growth rate and stringency of nutrient limitation. As deeper insights are gained into the details of ecophysiological responses from laboratory experiments, it is important to revisit the field and ascertain that those details actually operate under field conditions. This iteration between laboratory and field is straightforward when the natural population has a high relative abundance of the organism of interest. A remaining challenge for microbial ecology is to develop technologies that allow application of this iterative approach to community members present at low relative abundance. This requires the capacity to rapidly detect specific taxa and make physiological measurements on single cells.

Future of Microbial Ecology

The Microbiome

There has been an explosion in the past ten years of work on the 'microbiome,' the microbial community in a specific habitat. As often occurs in science, explosions are driven by technology developments – in this case, fast and cheap high-throughput DNA sequencing coupled with bioinformatics algorithms, databases, and methods of data analysis. Currently, the most common approaches after isolating DNA directly from environmental samples are PCR amplicon sequencing of a portion of the 16S rRNA gene and shotgun metagenomic sequencing. These methods have produced tremendous insights into the diversity of microbes and their functional genes, and will continue to be a major component of microbial ecology research in the future. However, these

molecular approaches have not yet reached the aspirations held for them to unlock deeper insights into microbial ecology. The issues include:

1. DNA sequence information provides insight into metabolic potential, but not actual activity. For example, a high proportion of *nif* genes in a metagenome does not indicate that nitrogen fixation actually occurs in the habitat. The dominant biota may have been selected for other traits, but happen to also possess *nif* genes. Advances in the practicality of metatranscriptome and metaproteome analyses would address this gap.
2. Most studies do not conduct adequate sample replication in site, space, or time to derive statistically rigorous conclusions.
3. Hypothesis testing typically involves multivariate statistical analysis of molecular sequence and environmental data. However, these approaches can only indicate correlation but never prove causation.
4. The technical choices for DNA isolation method, primers, and sequencing technology all introduce biases. Hence, comparisons across laboratories and historical studies is difficult. The Earth Microbiome Project is attempting to introduce standards for these issues.
5. Reference databases are inadequate. There is a bias in databases (both for taxa and genes) towards certain relatively well-understood phyla (for example, the Proteobacteria). As a result:
   a. The commonly used 97% sequence similarity threshold for delineating "taxa" from 16S rRNA sequences probably blurs or does not capture the breadth of microbial ecological heterogeneity.
   b. In terms of inferring potential microbial physiology from metagenomes, the inadequacies of reference databases mean (a) some annotations are wrong, (b) gene calls are conservative, hence 30–50% of genes found are of undetermined function, and many others identify a protein family rather than a specific enzymatic activity, (c) only environmental genes with >40% identity to a homologous database sequence are very likely to share functional similarity.

Microbiology at the Microscale

It is a central principle that the direct interaction of microbes with their environment occurs at a spatial scale of micrometers. Thus, analysis of the authentic ecology of microbes is enhanced by technologies that increase the spatial resolution of experimental measurements. This applies not only to analysis of the physical and chemical environment around a cell, but also to measuring the physiological and genetic properties of a set of individual microbial cells. In addition, analysis of a set of individual microbial cells can provide the basis to understand not only physiological responses at the microscale, but also the distribution of properties among a population of cells within a natural habitat.

Future possibilities include the continued development of microfluidic devices that can be designed to impose gradients of environmental factors or simulate natural microhabitats (e.g., soil pore network or gastrointestinal villi). Optical sensors for important metabolic reactant (such as O_2 and CO_2) can be incorporated into such devices.

To achieve microscale microbiology, the development and application of high-resolution imaging approaches that couple biochemical analyses to micron scale interactions of microbes with each other and their environment is critical. There have been impressive advances using a variety of approaches in analytical chemistry. These include label-free techniques such as Raman and FTIR microspectroscopy which have been applied to the analysis of microbial cells, and nuclear magnetic resonance that can be used to image biofilms and also provide information on chemical gradients within them. New fluorescence probes and optical imaging systems provide the capacity to interrogate physiological state, gene expression, and phylogenetic identity of individual cells in a natural sample.

Radioisotopes have been a powerful technology in advancing microbial ecology; however, they also have practical limitations for performing *in situ* experiments and handling waste. Stable isotope methodologies have the potential to revolutionize the field of microbial ecology. Sophisticated improvements in nuclear magnetic resonance, mass spectrometry, and Raman spectroscopy are approaching the point that allow the kinetics of metabolic function in microbial communities to be determined with great sensitivity. In contrast to radioisotope-based techniques, stable isotopes provide a means to specifically isolate cellular macromolecules that have incorporated the label and therefore have a unique signature. There remains great scope for technical refinements and advancements in the use of stable isotopes for metabolic and molecular analyses of microbial communities; these future methodological innovations will provide the means to mechanistically understand *in situ* function of microbial communities in a quantitative way. With technology developments regarding sensitivity and spatial resolution, this has the potential to provide a direct link between measurements of community function (metabolism) and expression of functional genes (via labeling and analysis of either mRNA or protein). An example of this has been the application of nanoSIMS (secondary ion mass spectrometry) in microbial ecology, which makes possible the determination of chemical and stable isotope composition at the submicron level.

Single-cell microbial genomics is now a reality, but remains tedious due to current sequencing technologies. If accurate single molecule DNA sequencing comes to fruition, imagine that 300 individual microbes from a habitat are physically separated, and each are subjected to single-molecule genome sequencing. One has in hand a community metagenome in which environmental sequences are inherently placed in their genomic context, and information on the relative abundance of different genomes (and genetic microdiversity within a specific clade) is directly available.

Theory in Microbial Ecology

Technology is not enough. New technologies will generate a blizzard of data at an ever-increasing rate, but understanding can only come from a synthesis of that information. Data-rich technologies carry the risk that they drive science to idiosyncratic pathways

that do not yield deep understanding of fundamental principles. The antidote is to ground studies to sound theory, a synthesis of how we imagine microbial populations and communities operate. In comparison to the ecology of plants and animals, microbial systems are much more experimentally tractable, which provides the opportunity to test theoretical syntheses that are either unique to microbial ecology or derived from the rich body of macroorganismal ecological theory.

There are several means to articulate a theoretical synthesis; a simulation model is one of them. This approach has the virtue of forcing a qualitative, descriptive discipline to become a quantitative, predictive one. The challenges are great, particularly at the community level, because functional redundancy will make precise, unambiguous prediction of community composition uncertain. However, robust model predictions may hold at the cellular, population, and ecosystem levels and lead to deeper insights into principles of microbial ecology than presented here. The greatest value of a simulation modeling approach is an iterative interaction with experimentation. The test of a simple model against ecological reality illuminates its weaknesses, and aspects in which greater depth of detail is necessary. Sensitivity analyses of a refined model suggest the most insightful experiments to carry out in the field, which lead to another round of model refinement.

Does Cultivation Have a Place?

For more than 30 years, the conventional wisdom in microbial ecology is that less than 1% of bacteria in nature can be cultivated. That observation in fact was a driving force for the development of cultivation-independent analyses of nucleic acids that are now the driving force in the field. Does there remain a role for cultivation?

There have been important innovations that can in many cases raise the proportion of cultivatable microbes from a habitat to 10%–25%. Culture chambers that mimic natural environments (for example, by using microfluidic devices) have been successful. Co-culturing has also been effective. Techniques that employ substrate concentrations at *in situ* levels or the addition of substances such as signaling molecules or resuscitation factors have been successful in cultivating relatively high proportions of the population. However, it is true that cultivation remains a time-consuming undertaking that is resists automation.

But cultivation still plays two important roles in microbial ecology, that complement molecular analyses of the microbiome. First, it provides the raw material to improve the genome reference databases that metagenomics relies upon. Secondly, the future entails understanding the functional potential of individual microbial ecotypes (metabolic versatility) as well as functional redundancy in microbial communities, and here molecular approaches have fundamental weaknesses. In particular, metagenomic approaches can uncover novel gene sequences or identify familiar ones, but in the end only allow the formulation of hypotheses that still must be subjected to biochemical analysis for rigorous proof. In contrast, propagation of an isolate under controlled conditions permits application of a broad array of tools to investigate its specific ecophysiology and genetics.

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Emerging and Reemerging Infectious Diseases

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Glossary

Emerging infectious disease(s) Infections that are newly recognized in a population or have existed previously but are rapidly increasing in incidence or geographic range.
One health An interdisciplinary approach that brings together human, animal, and environmental health professionals to address complex global health problems.
Urban sprawl The uncontrolled movement of urban development away from a city central.
Vector Any organism, though usually an arthropod, that can transmit an infectious agent to a host.
Zoonoses Diseases that can be transmitted from an animal to human or a human to animal.

Introduction

Throughout history, infectious diseases have vastly impacted human civilization. This impact has been demonstrated by the relentless appearance of various infectious disease outbreaks, including plague that scourged Europe during the Middle Ages, yellow fever that decimated Napoleon's forces in Haiti during the early nineteenth century, and influenza that claimed the lives of as many as 50 million people in 1918 (Zietz and Dunkelberg, 2004; Patterson, 1992; Johnson and Mueller, 2002). In the twentieth century, public health knowledge and interventions increased, particularly in more developed countries, reducing the burden of infectious diseases (Armstrong et al., 1999). Industrialization and urbanization influenced improvements in sanitation, structural development (e.g., window screening), and vector control that collectively lowered transmission rates by reducing the population's contact with infectious agents (Armstrong et al., 1999). In addition, the discovery of penicillin in 1928 and the continual development of vaccines ushered in an age of treatment and prevention strategies that many believed could eradicate infectious diseases from the globe (Clardy et al., 2009; 1999).

This idea was personified by organizations that made pronouncements to 'take up arms' against the most burdensome diseases. A well-known example comes from the Rockefeller Foundation, an organization that allocated substantial resources in the early twentieth century to combat yellow fever in the United States and other highly impacted territories, efforts that established precedence for future work (Fosdick, 1989). After World War II, new health organizations were established, including the US Centers for Disease Control and the World Health Organization (WHO) that led multiple campaigns to completely eradicate specific infectious diseases. One of the most notable efforts was the vaccination campaign against smallpox, a highly infectious viral disease that was completely eradicated by 1980 (Henderson, 2009). These campaigns established a great confidence and optimism in the ability to combat and control infectious diseases worldwide.

Despite these achievements, infectious diseases still pose a considerable threat today (Jones et al., 2008). Currently, it is estimated that at least 25% of total global mortality is attributable to infectious diseases, translating into over 15 million deaths per year (Mathers et al., 2008). The majority of deaths occur among children less than 5 years of age, living in countries with low to middle incomes (Mathers et al., 2008). Certain diseases have greater mortality, such as acute respiratory infections, tuberculosis, diarrheal diseases, malaria, measles, and HIV/AIDS, which account for 9 out of every 10 infectious disease deaths (2001).

Data from the past 30 years reflect various degrees of resurgence or reemergence of different infectious diseases worldwide. Two important examples of this phenomenon include the increased incidence of wild-type poliomyelitis across geographic pockets of Northern Africa, as well as an increase in the number of individuals being infected by the mosquito-borne disease caused by dengue viruses. Additionally, novel infectious diseases continue to emerge in virtually every region of the world. Examples include Hendra virus, discovered in 1994 in Australia (Murray et al., 1995); Nipah virus, identified in 1999 as the causative agent of outbreaks among pig farmers in Malaysia (Chua et al., 2000); severe acute respiratory syndrome (SARS) responsible for an outbreak of respiratory disease in multiple countries in 2003 (Marra et al., 2003; Peiris et al., 2003); and the 2009 emergence of a new influenza strain, originating in North America, responsible for the first pandemic of the twenty-first century (2009b). In public health, these types of events are referred to as emerging or reemerging infectious diseases, or collectively known as emerging infectious diseases (EIDs). EIDs have been defined as infections that are newly recognized in a population or have existed previously but are rapidly increasing in incidence or geographic range (Morse, 2004; Morens et al., 2004; Fauci, 2005, 2001; Institute of Medicine (US). Committee on Emerging Microbial Threats to Health et al., 1992). EIDs can be considered emerging, recently reemerging/resurging, or deliberately emerging, depending upon the pathway of emergence (Morse, 2004; Fauci, 2005; Morens et al., 2008).

EIDs are influenced by a wide variety of often complex factors, including ecology, human behavior, globalization, microbial adaptation, and public health infrastructure (Morse, 1995, 2004; Lashley, 2003; Morens et al., 2004, 2008; Fauci, 2005; Jones et al.,

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2008). More recently, the threat of bioterrorism, or the deliberate release of viruses, bacteria, or other agents used to cause illness or death in people, animals, or plants (Inglesby et al., 2002; Jernigan et al., 2002; Traeger et al., 2002), has added its own complexities to how EIDs affect global health. A number of recent bioterrorism events have had a dramatic impact on public health policies and resource allocations. New research and technology have provided better detection and response capabilities, as well as basic general knowledge of the various factors and determinants affecting the emergence and spread of EIDs; however, much is still unknown. This article offers a review of the most relevant literature associated with EIDs, as well as perspectives on how to address the most critical future public health questions.

Global Distribution

EIDs exist in most regions of the world, often with distinct and identifiable transmission patterns that are driven by various predictors or risk factors. These include the globalization of travel and trade, country and individual social–economic status, as well as population dynamics. Risk factors are used to identify trends in the movement of EIDs throughout various populations. When coupled with Geographic Information Systems mapping software, transmission patterns can be modeled, offering a pictorial view of EID distribution. These models are very useful, allowing for predictions to be made as to where future EID events are likely to occur and estimating the impact of public health interventions.

Globalization

Modern globalization has created ubiquity in world travel and trade. For example, a piece of fruit that is grown in Chile today can be purchased in a market on the other side of the world in 1–2 days. EIDs travel via these rapid global transport systems as made evident by the frequent international foodborne epidemics and zoonotic disease outbreaks that have occurred over the past few decades. SARS in 2003 demonstrated how quickly a highly transmissible respiratory pathogen can be spread, originating in China, and making its way to more than 15 countries in just a few months (Shen et al., 2004). Another example is the cholera outbreak that began in Haiti following a devastating earthquake in 2010, with genomic evidence suggesting that the epidemic *Vibrio* strain that ignited the outbreak was likely carried into the country by foreign security forces (Keim et al., 2011; Chin et al., 2011; Pun, 2011; Piarroux et al., 2011; Ali et al., 2011). These types of events make control extremely difficult, reflecting how quickly EIDs can be transmitted and established in new populations before public health officials can intervene.

EIDs and Economic Status

Geographically, EIDs are often more highly prevalent among underdeveloped and economically disadvantaged populations. Along with a high incidence of EIDs these populations also experience a greater severity from infections, often translated into higher rates of mortality (Farmer, 1996). This trend is attributed to various social determinants of health that elevate the susceptibility of an individual or population to infection (Marmot, 2005). Poverty both promotes and results from social determinants including access to health care, clean water, food, and other important environmental factors that influence disease transmission (Marmot, 2005). The cyclic nature of this relationship makes public health interventions complicated, since most, if not all, determinants must be addressed in order to have a lasting positive impact.

The socioeconomic status of a country can be an important determinant for the transmission of EIDs, influenced by both the availability of an appropriate public health infrastructure and the necessary resources to carry out prevention and control strategies. This could include instituting effective surveillance systems, as well as providing adequate health-care services to individuals affected by EIDs. Without this necessary capacity, a disease can quickly become well established or endemic in a population before a public health response can be initiated, if one is initiated at all.

Historically, tuberculosis, chiefly caused by *Mycobacterium tuberculosis*, is an EID associated with economics, often being coined a 'disease of poverty' due to the increased impact it has on economically poor individuals. Today, data indicate 95% of all tuberculosis cases and deaths occur in the developing world (2012b). In the past 30 years, tuberculosis has begun to reemerge across the globe, partly due to the emergence and spread of HIV, which is now a primary risk factor for tuberculosis transmission. Nearly half of all individuals in the developing world who have HIV also are coinfected with *M. tuberculosis* (2012b). This emphasizes the importance economics play as a driver for disease emergence and reemergence, as well as the treatment strategies available for those infected.

Despite a disproportional distribution of EIDs in low- and middle-income countries, high-income countries experience their own burden of EIDs. In the United States, West Nile virus continues to cause disease in man. Lyme disease is also well established with an annual incidence that has increased in over the past 10 years, reaching over 30 000 and 60 000 reported annual cases in the United States and Europe, respectively (Radolf et al., 2012). In 2003, the global pet trade contributed to an outbreak of monkeypox virus in the United States that originally infected individuals who had close contact with prairie dogs purchased as pets through a common supplier. Epidemiological studies traced the origin of this outbreak back to an exotic African rodent species that transmitted the virus to the prairie dogs during transport to the United States (Reed et al., 2004).

Higher socioeconomic countries also experience a substantial burden of foodborne related EIDs, the more common pathogens being bacteria, including *Salmonella* spp., *L. monocytogenes*, *Brucella* spp., *Campylobacter* spp., and pathogenic strains of *E. coli* (Newell et al., 2010). Even with strict regulations on food production, outbreaks frequently occur due to the centralization and mass production by the food industry, which is capable of distributing large quantities of food over vast geographic areas. In this

type of system, if food becomes contaminated, then rapid dissemination of the pathogen before detection is much more likely. This is especially dangerous when the pathogen being disseminated is very pathogenic and the population is more susceptible. This was the case when a rare subtype of enterohemorrhagic *E. coli* (O104:H21) was responsible for a foodborne outbreak throughout Europe in 2011 that caused over 3800 cases and 54 deaths (Frank et al., 2011). Upon investigation, the source of the outbreak was found to be sprouts that were grown with contaminated seed attained from a supplier in another country (Buchholz et al., 2011).

Population Density and Expansion

Since 1960, the global population has more than doubled, from 3 billion to nearly 7 billion people (2012a). As the population has grown, population density has also increased, creating in some countries what are called megacities (e.g., Tokyo, Mexico City, New York City, etc.). These cities may have upward of 20 million people or more, sometimes residing in extremely confined geographic areas. These conditions promote the proliferation of new diseases, especially when the pathogen is highly transmissible, forming emerging disease 'hot spots' (Jones et al., 2008; Heymann and Rodier, 2001). In addition, some of these countries operate wet markets where livestock, including poultry and swine, are slaughtered in poor hygienic conditions and sold directly to the public (Webster, 2004). These practices further promote favorable environments for disease emergence, with SARS and highly pathogenic avian influenza (HPAI) virus being recent examples.

In response to rising population densities, many cities have seen an increase in urbanization, particularly away from a city central. This practice is often referred to developmentally as urban sprawl. While the practice has helped to reduce the rate of growing population density inside cities, it has concurrently moved more groups of people into previously undeveloped areas. This rapid expansion in land use and development has combined what was once fairly separated animal and human habitats, allowing for more frequent exposure of humans to potential disease reservoirs, increasing the risk of EID proliferation.

Pathways of Emergence

It has been proposed that EID events occur in two steps (Morse, 2004). A pathogen must first be introduced into a new population and then disseminated within that population. With this construct in mind, understanding the origin of novel microbes becomes critical. Microbes often exist in the environment in a nonpathogenic state, with limited contact with a viable host. However, when appropriate conditions are met, opportunistic microbes can exploit new niches, including human hosts, resulting in a successful introduction. Once successful, this type of event is sometimes referred to as a microbial 'jump' or 'crossover'. This crossing of the species barrier is often necessary before widespread dissemination can occur.

Dissemination is then dependent upon the transmissibility of the pathogen in the new population. Dissemination can occur directly from one host to another, or can establish an intermediate host in its transmission cycle, such as a vector. If a pathogen is unable to be transmitted beyond an immediate or intermediate host, then further dissemination is not possible. These interactions can often be complex, involving pathogen, environment, and host, making it challenging to understand and identify what contributes to the most optimal conditions promoting disease emergence.

Zoonoses

The transmission of a pathogen between animals and humans is known as zoonotic transmission or zoonosis. Zoonosis is perhaps one of the most important pathways of emergence, with an estimated 75% of all known EIDs originating from some type of animal reservoir (Taylor et al., 2001). Disease examples include HIV/AIDS, Lyme disease, plague, SARS, several hemorrhagic fevers, and zoonotic influenza. Each of these possesses a unique etiology, and though it is not an exhaustive list, it highlights the diversity of disease emergence related to zoonosis. Additionally, effectively controlling zoonotic diseases is particularly difficult, since recognizing an emerging zoonotic disease often does not occur until a major outbreak is already underway. If the responsible zoonotic pathogen is highly transmissible, then future epidemic spread occurs even after recognition.

As previously discussed, population growth has had a drastic impact on society. Greater zoonotic disease potential can result from this growth primarily due to an increased interaction between humans and animal habitats, Figure 1. Population growth has also resulted in a higher demand for food commodities. To meet this growing demand, industrialization of food production is increasing, especially in developing countries. This is true of all types of food, including meat production that has moved away from small farms toward large-scale cattle, poultry, and swine operations, Figure 2. These sites have been able to streamline meat production; however, it comes at the cost of creating favorable environmental conditions for the zoonotic transmission of opportunistic microbes (Drew, 2011).

Other food acquisition practices pose a greater risk of acquiring an EID. This includes the long-standing practice in Africa, Asia, and the Americas of hunting and consuming wild animals, also called bushmeat. Eating bushmeat has been associated with a number of new diseases, including two highly fatal hemorrhagic diseases caused by Ebola and Marburg viruses (Peters, 2005; Wolfe et al., 2005). Additionally, the practice of consuming raw or unpasteurized milk products (e.g., milk, cheese) can increase the transmission risk of certain zoonotic bacteria, such as *L. monocytogenes*, *Brucella* spp., and *Camylobacter* spp. This is particularly common in areas where pasteurization is not an accepted practice.

Figure 1 The risk of zoonotic transmission of a pathogen is increased with man's close and frequent contact with other species.

Figure 2 Large dense populations of animals, particularly in herds, can promote zoonotic pathogen transmission to man.

Vectors

Some EIDs are vector-borne, caused by the transmission of a pathogen through the feeding activity of a vector, usually an arthropod. Some of the most common and effective vectors are insects including flies and flees, as well as arachnids such as ticks and mites. These vectors are often attracted to humans and animals because they are obligate blood feeders, using biting or piercing mouthparts to obtain a blood meal from their host. Pathogens have coevolved with vectors to exploit this behavior, often relying on transmission to allow for the propagation of new progeny. An example of a model vector that does this is the mosquito, which is the most widely distributed and abundant vector in the world. Mosquitoes are of particular concern due to their sometimes aggressive feeding behavior and ability to effectively transmit a broad range of pathogens, causing diseases such as malaria, dengue fever, and many others.

In terms of importance to emergence, it has been suggested that vector-borne EIDs constitute nearly a quarter of all EID events occurring in the last decade (Jones et al., 2008). Also, there has been an increase in the total number of vector-borne EIDs during this same time period, while other types of EIDs have slightly decreased (Jones et al., 2008). Increased human population density and changing demographics seem to be associated with this rising trend of vector-borne EIDs, as well as climate change that some have suggested is promoting an expansion in vector distribution and range (Jones et al., 2008; Epstein, 2001).

Animals are also important in the life cycles of vector-borne diseases, since many can be competent hosts and serve as reservoirs for different pathogens. In fact, some EIDs only occur when humans become an incidental or dead-end host in an already established transmission cycle between an animal and vector. This is true for Japanese Encephalitis (JE), a flavivirus transmitted by certain mosquitoes, causing viral encephalitis cases primarily in Asia. For JE, humans are dead-end hosts, capable of infection if bitten by an infected mosquito, but unable to amplify and transmit the virus further, whereas, swine and other wild birds are able to propagate the virus and maintain a complete transmission cycle. Hence, if humans are removed from the environment, the virus would still be sustained as long as the vector, pathogen, and reservoir were to remain.

Factors Associated with EIDs

Since the early 1990s, discussion has been centered on the factors that are most associated or attributable to EIDs. A report published by the Institute of Medicine in 1992 originally identified six contributing factors (Institute of Medicine (U.S.). Committee on Emerging Microbial Threats to Health et al., 1992). Since the list was published, new emerging threats have resulted in additional factors being added, all thought to be important contributors by the global health community, Table 1.

This list provides guidance as to where research efforts and interventions should be targeted. It is important to note that many of these factors are often interrelated with each other. For instance, a lack of political measures will often result in the breakdown of public health measures. Economic development and land use can be associated with technology and industry, as well as changing ecosystems, and shaping conditions of poverty. Hence, effective interventions must be multidimensional for sustained change.

Ecological Changes

Perhaps one of the most apparent and pervasive factors affecting the incidence of EIDs is environmental alterations that result in a drastic change in ecology. One common ecological model works from a premise that disease occurs at the intersection of environment, pathogen, and host, Figure 3. A change to any one of these entities could impact the disease outcome. It has been suggested that classical ecological tools are limited in their ability to effectively assess the multifaceted complexities of EIDs. This is clearly indicated by the limited number of published studies that have effectively evaluated EIDs from an ecological perspective (Meentemeyer et al., 2012). An ecological relationship could be as simple as the symbiosis of certain types of fungus and plants where no other factors play a role in the survival of everyone. Others can be much more complicated, such as the multistage life cycle of the guinea worm (cause of the disease dracunculiasis), a parasitic nematode that uses two different species, a copepod and humans, to complete its life cycle. These parasites are impacted by human movement, water availability, copepod abundance, as well as climate, all of which can impact the nematode life cycle.

However, the research that does exists suggests that the most important ecological changes encouraging EIDs are those that put humans in closer or more frequent contact with current or potential pathogen reservoirs. For instance, the incidence of Lyme disease in the United States and Europe seems to be associated with the number of available hosts in a given area. Deer and small mammals play an important role in the life cycle of ticks that vector the spirochete causing Lyme disease. Studies have shown that host populations directly correspond to tick abundance, often influenced by ecological factors that promote population growth (Levi et al., 2012). When this is coupled with urbanization and reforestation, transmission can drastically increase, since both the host and environment serve as the drivers for disease emergence.

Climate Change

Evidence shows that the earth's average surface temperatures have increased at least 0.6°C over the past century (Houghton and Intergovernmental Panel on Climate Change. Working Group I., 2001). While many agree with the empirical evidence of rising temperatures, there is still controversy as to why the earth is warming. Some argue that the earth is experiencing a natural temperature fluctuation similar to what has occurred throughout history, citing previous ice ages and warming periods. Others have suggested that the rising temperatures are attributed to increased atmospheric concentrations of carbon dioxide related to the

Table 1 Factors of emerging and reemerging infectious diseases

- Microbial adaptation and change
- Human susceptibility to infection
- Climate and weather
- Changing ecosystems
- Human demographics and behavior
- Economic development and land use
- International travel and commerce
- Technology and industry
- Breakdown of public health measures
- Poverty and social inequality
- War and famine
- Lack of political will
- Intent to harm

Reproduced from Smolinski, M.S., Hamburg, M.A., Lederberg, J., Institute of Medicine (U.S.) Committee on Emerging Microbial Threats to Health in the 21st Century, 2003. Microbial Threats to Health: Emergence, Detection, and Response. National Academies Press, Washington, DC; Morse, S.S., 1995. Factors in the emergence of infectious diseases. Emerg. Infect. Dis. 1, 7–15; Lashley, F.R., 2003. Factors contributing to the occurrence of emerging infectious diseases. Biol. Res. Nurs. 4, 258–267; and Morse, S.S., 2004. Factors and determinants of disease emergence. Rev. Sci. Tech. 23, 443–451.

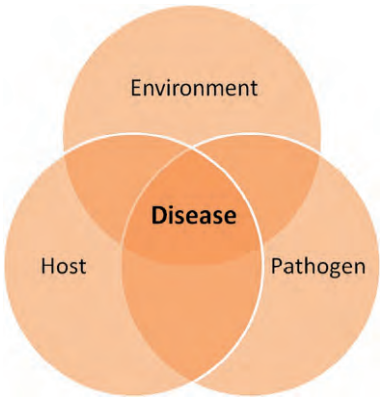

Figure 3 A basic ecological model illustrating the interaction of host, pathogen, and environment in disease.

burning of fossil fuels and deforestation. Despite the discourse, the reality of climate change in the context of EIDs remains an important topic, with research showing associations between disease indicators and climate factors (Patz et al., 1996; Epstein, 2001).

Climate change primarily impacts the range of infectious diseases, particularly those that are transmitted by vectors. Warmer temperatures allow vectors to more readily survive conditions at higher altitudes and latitudes, as well as having shorter periods of overwintering. Weather patterns are also influenced, as the hydrolytic cycle can be disrupted by warmer ocean temperatures, causing severe weather at greater frequency. Particularly concerning are weather events that release high volumes of rain, as they may result in temporary explosions in vector populations, especially mosquitoes.

Microbial Adaptation and Change

From a pathogen perspective, microbial adaptation and change significantly contribute to the likelihood that a microbe will become pathogenic in a population. As mentioned, when certain environmental conditions are met, a microbe can experience alterations in genetic makeup that can affect its pathogenicity or virulence. This type of adaptation can either occur gradually or rapidly by means of random mutations, reassortments, or adaptive pressures brought on by stressors such as antimicrobial agents.

A good example of microbial adaptation is demonstrated by the influenza A virus, an RNA virus that has been shown to undergo both gradual and rapid genetic changes. Just in the last 20 years, new variants of influenza have caused major human outbreaks, including HPAI subtype H5N1, first detected in 1997 (1997) and the pandemic influenza subtype H1N1, first detected in 2009 (2009a). Influenza A contains two surface proteins (glycoproteins), called hemaglutinin (HA) and neuraminidase (NA): HA proteins are associated with viral attachment and cell entry using a fusion pathway, while NA proteins regulate the release of progeny virus from an infected cell. Antigenic drift and shift are two mechanisms that create variations in the antigenic properties of these two proteins that allow influenza A to bypass the acquired immunity of a population, Figure 4. Antigenic drift occurs when small point mutations accumulate gradually, altering the antigenic properties of the two surface proteins. This can cause population immunity to partially decrease, resulting in seasonal epidemics. Antigenic shift involves major changes in proteins, sometimes through the swapping of entire gene segments. The genetic reassortment occurs when two or more unique viruses infect the same cell and generate mixed progeny viruses (Chen and Deng, 2009; Kaye and Pringle, 2005). In particular, genetic reassortments of the HA antigens may result in large worldwide epidemics or pandemics. Waterfowl are thought to be the largest source of diverse viruses from which gene reassortment may occur, as they are known to carry viruses with 16 different HA proteins. When a population has no or little immunity to a new subtype and the new subtype is highly transmissible, pandemics may result.

Like influenza A virus, other pathogens undergo similar mechanistic adaptive changes and sometimes variation results from external pressures that promote microbial evolution. Antibiotic resistance is an example of this kind of adaptation. Since their discovery, the use and variety of antibiotics have greatly increased over time and many pathogens have adapted to their presence. These antimicrobial resistant pathogens are having an increased impact upon clinical care and medical costs.

Bacteria, such as methicillin-resistant *Staphylococcus aureus* and multidrug resistant *M. tuberculosis*, are good examples of the severe impact that this type of microbial adaptation can have on individual health outcomes. Individuals infected with these bacteria are often prescribed newer and more expensive, 'last defense' antibiotics. These diseases may be life-threatening and require specialized care. Hospitalized patients with these resistant pathogens may seed the hospital staff and environment and cause nosocomial transmission among immunocompromised patients.

Important EID Examples

EIDs result from a large variety of causative agents, each one possessing a unique etiology and ecology. Agents may be organized according to transmission pathways, genetic markers, or the pathology a pathogen might exhibit in a population. Most often,

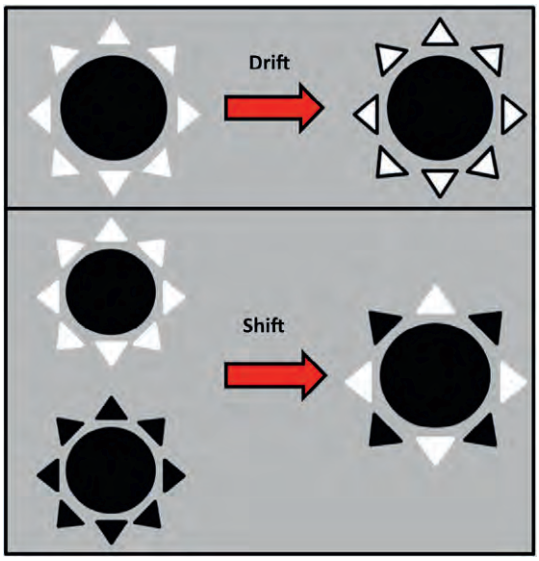

Figure 4 Graphical representation of antigenic drift and shift among influenza A viruses. Top panel: Antigenic drift – minor genetic changes (e.g., through mutation) lead to slight changes in surface glycoproteins or season variation in strains. Bottom panel: Antigenic shift – exchanges of entire segments of genome lead to major changes in the surface glycoproteins, which may lead to pandemics.

however, EIDs are categorized into bacteria, virus, fungus, parasites, and more recently prions. Table 2 presents key examples of each of these disease categories.

Conclusions

As the world's population rises and public health problems increase in complexity, it will be critical to establish innovative and dynamic strategies to counter EID threats. These strategies should include the reinforcement of public health systems, better diagnostics for EIDs, stronger surveillance systems, and better interdisciplinary and international collaborations. A novel interdisciplinary strategy, called One Health, is gaining popularity as an approach that attempts to focus efforts on complex public health issues such as EIDs.

Public Health Infrastructure

Public health infrastructure is critical to the current mitigation and future prevention of EID events. An effective infrastructure should include the following: a legal framework that allows for enforcement of public health measures and a system to monitor outcomes; dissemination and utilization of health knowledge, including the training of health workers; and physical environments or services conducive to targeting important health threats, like sanitation infrastructure (e.g., sewers). Infrastructure can subsist on a local level, but most often relies upon the coordination and leadership of nationallevel governments, providing guidance for task prioritization and implementation. However, national and international organizations can also provide assistance by supporting government-led efforts with technical and financial resources, or filling in the necessary gaps of a fragmented infrastructure.

EID Diagnostics

Diagnostics are an important tool for identifying and characterizing infectious disease agents from both clinical and environmental samples. Common techniques include molecular assays, such as multiplexing, microarrays, deep sequencing, and traditional and real-time polymerase chain reaction, as well as immunoassays, including variations of the enzymelinked immunosorbent assay and other techniques exploiting antigen and antibody binding activity. The variety and availability of these techniques have greatly improved over the last two decades, with significant increases in sensitivity and specificity, as well as marked decreases in costs. For instance, the cost of full genome sequencing of a moderately large virus has seen substantial reductions in the past decade, dropping from tens of thousands of dollars per run to a couple thousand dollars, with much more accurate analysis. Much of this improvement can be attributed to new technological developments, such as third and fourth generation sequencing that is greatly improving novel pathogen detection capabilities.

Table 2 Examples of recent human emerging infectious disease threats

Emerging infectious disease threat	*Pathogen*	*Emerging/reemerging*	*Primary transmission*	*Key geographic area(s)*
Bacteria				
Bartonella infections	*Bartonella spp.*	Emerging	Zoonotic	Australia, Europe, United States
Vancomycin-resistant *Staphylococcus aureus* infections	*Staphylococcus aureus*	Reemerging	Person-to-person	Worldwide
Pathogenic *Escherichia coli* infections	*Escherichia coli*	Emerging	Foodborne	Europe, United States
Cholera	*Vibrio cholerae*	Reemerging	Waterborne	Africa, Asia, South America, Haiti
Plague	*Yersinia pestis*	Reemerging	Vector-borne	Africa, Asia, South America, United States
Typhoid fever	*Salmonella typhi*	Reemerging	Foodborne, waterborne	Africa, Asia, Latin America, Carribean
Diphtheria	*Corynebacterium diphtheriae*	Reemerging	Respiratory (person-to-person)	Eastern Europe, India
Multidrug-resistant tuberculosis infections	*Mycobacterium tuberculosis*	Reemerging	Respiratory (person-to-person)	Worldwide
Lyme disease	*Borrelia spp.*	Emerging	Vector-borne	Eastern Asia, Europe, United States
Fungal				
Cryptococcus gattii infections	*Cryptococcus gattii*	Emerging	Environmental exposure	Australia, Canada
Parasite				
Cyclosporiasis infections	*Cyclospora cayetanensis*	Emerging	Foodborne, waterborne	Worldwide
Drug-resistant malaria	*Plasmodium spp.*	Reemerging	Vector-borne	Africa, Asia, South America
Protein				
Variant Creutzfeldt–Jakob disease	Prion	Emerging	Zoonotic, foodborne	Europe
Virus				
West Nile fever	West Nile virus	Reemerging	Vector-borne	Africa, Asia, Europe, North America
Hantavirus pulmonary syndrome	Hantavirus	Emerging	Zoonotic	Canada, East Asia, Europe, South America, United States
Dengue fever	Dengue virus	Reemerging	Vector-borne	Central Africa, Central America, Latin America, Southern Asia
Yellow fever	Yellow fever virus	Reemerging	Vector-borne	Central Africa, South America
Lassa fever	Lassa fever virus	Emerging	Zoonotic	Central and Western Africa
Marburg hemorrhagic fever	Marburg virus	Reemerging	Zoonotic	Central Africa
Ebola hemorrhagic fever	Ebola virus	Reemerging	Zoonotic	Central Africa
Rift Valley fever	Rift Valley fever virus	Reemerging	Vector-borne	Africa
Hendra virus infection	Hendra virus	Emerging	Zoonotic	North-eastern Australia
Nipah virus infection	Nipah virus	Emerging	Zoonotic	Asia
Highly pathogenic avian influenza	H5N1 influenza virus	Emerging	Zoonotic	Asia
Severe acute respiratory syndrome	SARS coronavirus	Emerging	Respiratory (person-to-person)	Asia[a]
2009 pandemic influenza	2009 H1N1 influenza virus	Emerging	Respiratory (person-to-person)	Worldwide
Japanese encephalitis	Japanese encephalitis virus	Reemerging	Vector-borne	Asia

[a]Originated in Asia, but had rapid global transmission.

Reproduced from Armstrong, G.L., Hollingsworth, J., Morris, J.G., Jr., 1996. Emerging foodborne pathogens: Escherichia coli O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiol. Rev. 18, 29–51; Rey, M., 1996. Resurgence of diphtheria in Europe. Clin. Microbiol. Infect. 2, 71–72; Collinge, J., 1999. Variant Creutzfeldt-Jakob disease. Lancet 354, 317–323; Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug-resistant malaria. Lancet Infect. Dis. 2, 209–218; Crump, J.A., Luby, S.P., Mintz, E.D., 2004. The global burden of typhoid fever. Bull. World Health Organ. 82, 346–353; Mackenzie, J.S., Gubler, D.J., Petersen, L.R., 2004. Emerging flavi viruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat. Med. 10, S98–S109; Shen, Z., Ning, F., Zhou, W., He, X., Lin, C., Chin, D.P., Zhu, Z., Schuchat, A., 2004. Superspreading SARS events, Beijing, 2003. Emerg. 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A major need is the better recognition of EIDs, especially in low-resource areas where EIDs are common. This can be accomplished by prioritizing laboratory infrastructure development, continuing to direct efforts in targeting disease 'hot spots' where EID outbreaks are most likely to occur. Emphasis should be placed on making new diagnostic assays timely, affordable, and feasible for laboratories with limited resources. In addition, employing assays that are able to detect multiple agents in a single specimen is important in EID management. This kind of technique allows for more rapid identification of causative agents, an important component in how public health responses are planned and delivered.

Global Surveillance and Communication

Disease surveillance is defined as the ongoing systematic collection, consolidation, and analysis of outcome specific data for the purpose of planning, implementation, and evaluation of various health-related events (Thacker, 1988). Many information and surveillance networks that are currently in place can be characterized as fragmented or unrepresentative of actual disease circulation in a population, making it difficult to estimate actual incidences. There have been some successes in improving surveillance capabilities, particularly passive surveillance that uses techniques such as monitoring healthcare clinics and emergency rooms for various health outcomes indicative of an EID.

For instance, global influenza surveillance, following the 2009 H1N1 pandemic, received substantially more prioritization and resource allocations from countries that were lacking such capabilities beforehand. This has resulted in a rather comprehensive collection and dissemination of data to stakeholders worldwide, through a network called FluNet. This network is overseen and coordinated by WHO, and helps provide a more comprehensive resource regarding the incidence and circulation of different strains of influenza. Information is used not only to enhance outbreak response, but is also the source of prototype strains that are used in annual seasonal vaccination manufacturing. This is an example of how successful surveillance can greatly improve the public health response capabilities.

For EIDs this may be more difficult, only since it takes a large amount of coordination and resources to create global surveillance for any specific pathogen. Furthermore, loweconomic countries often do not have the necessary resources to carry out such endeavors. The Internet may be useful in supporting countries that lack traditional surveillance capabilities, as has been demonstrated by the international e-service called ProMED-mail. This service is a consortium of public health professionals that disseminate disease outbreak updates, often in real time, through a global e-mail distribution list. Since telecommunications are becoming well established, even in low-economic countries, this has proven to be a very practical approach, providing the international community with reliable information and even sometimes serving as a first report of an index case. Further developing these types of approaches will be useful in communicating EIDs, pre- and postevent.

International Collaboration

Today, EIDs impact our entire world. It will be important that governments and agencies concur with the need for strong international communication and collaboration regarding EID events. This will include the sharing of resources and knowledge, particularly regarding EIDs that are considered a global threat. It is also ideal that low-economic countries receive priority in the enhancement of their public health systems, since a large proportion of EID risk exists in these areas. Collaborations offer unique diversity in expertise, improve cultural competencies, and enhance response efforts in the event of an EID outbreak.

'One Health'

It is clear that EID ecology is often complex, requiring a sophisticated, interdisciplinary response to mitigate disease impact. One attempt to address these complexities has been coined 'One Health'. One Health is a moniker for the interdisciplinary approach, bringing together human, animal, and environmental health professionals, to address complex global health problems. While the terminology may be relatively new, the foundations of the approach are not. Throughout history, individuals from different disciplines have often worked together to create solutions for some of the most important health threats. One Health attempts to convert those cooperative successes into a practical methodology to be used in future public health problems.

One of the strongest arguments for One Health is the role zoonoses play in the emergence of new infectious diseases, and the recognized long-standing gap of cooperation that has existed between the fields of animal and human health. As previously described, the global impact of HIV, H5N1 avian influenza, SARS, and 2009 H1N1 pandemic influenza, all diseases with origins from animal reservoirs, have led to a consortium of doctors, veterinarians, and public health officials beginning to work together to identify effective solutions that bridge the gap between each discipline. In addition, experts from fields of geography have begun to pursue new models to help predict the movement of different EIDs. Engineers are developing new technologies, based upon the recommendations of field workers that may mitigate contamination of food and water supplies. Economists are calculating the cost effectiveness of vaccination campaigns to help guide policy makers in better using resources.

Overall, One Health is an approach that can improve effectiveness of public health response and interventions, as it allows for a more targeted application of multiple areas of expertise, not relying on a single discipline approach that may not address all of the minute facets that accompany most public health problems of today. To be successful, it will require an intentional effort of current public health professionals reaching out to one another, to strengthen collaborations, communication, and the ability to be open to novel ideas. Since this approach promotes flexibility, it should be able to adapt to the rapid changes demonstrated by emerging

diseases in the last two decades. It is this characteristic that makes One Health not just a temporary solution, but a philosophy that can have serious long-term impact on EID morbidity and mortality.

Acknowledgments

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Endophytic Microbes☆

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Glossary

Ascospore The meiospore of Ascomycota produced inside an ascus.
Ascus (pl. asci) A sac-like structure characteristic of Ascomycota. An ascus usually contains eight ascospores.
Axenic (of cultures) A pure culture of microorganism that is completely free of the presence of other organisms, uncontaminated, consisting of a single organism.
Conidium (pl. conidia) A specialized, nonmotile, asexual spore of mitosporic fungi.
Defensive mutualism A form of symbiosis where the host is defended from predation or disease by other symbiont.
Endophyte A microbe growing within tissues of a plant.
Endophytic Living within tissues of a living organism.
Epibiotic Living on the surface of a living organism.
Epiphyllous A microbe growing on the surface of a leaf.
Epiphyte A microbe or plant that lives on the surface of a plant.
Hypha (pl. hyphae) One of the filaments of a mycelium. A basic, threadlike element that forms the thallus of most fungi.
L-form A wall-less protoplast form of a bacterium.
Loco An unusual behavior and neurological syndrome of grazing animals poisoned by certain legumes.
Mutualistic symbiosis A form of symbiosis where both organisms benefit from the association, with an overall increase of fitness and survival.
Mycelium (pl. mycelia) A mass of hyphae; the thallus of a fungus.
Mycosome A wall-less protoplast form of a fungus.
Mycotoxin A compound produced by a fungus that is toxic to humans and other organisms.
Rhizophagy cycle A nutritional process where symbiotic microbes cycle between a root intracellular phase and a free-living soil phase. Microbes (bacteria or fungi) acquire soil nutrients in the free-living soil phase; nutrients are extracted from wall-less protoplast forms of microbes oxidatively in the intracellular endophytic phase.
Stroma (pl. stromata) A mass or matrix of mycelium, with or without tissue of the host or substrate, in or on which spores or fruit bodies bearing spores are produced.
Volatile organic compounds (VOCs) Are organic chemical compounds that have high enough vapor pressures under normal conditions to significantly vaporize and enter the atmosphere.

Defining Statement

Endophytic associations between microbial and autotrophic organisms in nature are ubiquitous. Because of the complexity of the endophytic associations with their hosts we are only starting to understand these interactions. In this article, we compare the biology and ecology of different groups of endophytes associated with hosts. The functional roles of endophytic microbes are also discussed. We also evaluate the potential of endophytic microorganisms as sources of bioactive metabolites with potential use in agriculture and medicine.

Introduction

Microorganisms are found virtually in every biotic and abiotic niche on earth. This includes extremophiles living in deserts, rocks, thermal springs, freshwater, marine, and arctic environments, and associated with terrestrial and aquatic animals. A wide diversity of microorganisms may be isolated from most terrestrial and marine plants. Microorganisms are present on the surface of and within tissues of most parts of plants, especially the leaves. When microbial organisms colonize a plant and the plant tissue is healthy, the relationship between the microorganism and its host plant may range from latent pathogenesis to mutualistic symbiosis. Therefore, the microbial organisms may be latent pathogens, epiphytes (epibionts), or endophytes (endobionts).

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Endophytes are generally any organisms that under normal circumstances are contained within tissues of living plants (usually autotrophs) without causing noticeable symptoms of disease, and the host tissues remain intact and functional. However, the same organisms may also be described as saprobic or pathogenic at another time; for example, when the host is stressed, some endophytes may become pathogenic. The delicate balance between host and endophytic organisms seems to be controlled in part by chemical factors, for example, herbicidal natural products produced by the fungus versus antifungal metabolites biosynthesized by the host plant.

The term 'endophyte' is derived from the Greek *endon* (within) and *phyte* (plant), and was introduced by Heinrich Anton de Bary in 1866 and applied to any organism found within a plant. Traditionally, the term had been broadly applied to fungi in plant tissue, including the mycorrhizal fungi in plant roots. However, some authors do not include mycorrhizal fungi in this group. The term endophyte has also been adapted to other microorganisms, such as endophytic bacteria. The associations of endophytic organisms with their host plants are varied and complex and we are only starting to understand these interactions. Endophytic microbial organisms often contribute to the normal health and development of their hosts in exchange for a relatively privileged niche. Microbial endophytes have been isolated from tissues of algae, mosses and hepatics, ferns and fern allies, grasses, other herbaceous plants, and trees growing in tropical, temperate, boreal, and arctic environments.

Endophytes represent a large reservoir of undiscovered genetic diversity. Species composition of endophyte assemblages and infection frequencies vary according to (1) host species, (2) growth stage of the host, (3) tissue type, (4) the age of host organs and/or tissues, (5) position in the canopy and associated vegetation, and (6) site characteristics, such as elevation, exposure, and latitude. Usually, one to a few species dominate the endophyte community, while the majority of the species are rare. Distribution of rare and incidental species is influenced more by site than by host, and the number of rare and incidental species isolated is proportional to the intensity of sampling.

Many plant fungal endophytes are transmitted horizontally by production of conidia or other spores on plants that may spread to adjacent uninfected plants, and are not directly inherited through germplasm from the previous generation of the host. Therefore, with each host generation, environmental fungi must compete to colonize the empty niches within new host individuals. Alternatively, some endophytes, including Epichloë species, grow intercellularly throughout aboveground parts of plants and are transmitted vertically via seeds. These endophytic fungi are spread by hyphae growing into the developing seeds or vegetative parts of infected maternal host plants.

Epibiosis is another type of association of two organisms, the epibiont and the basibiont. The majority of organisms that exist on the surfaces of plants are referred to as epibionts or epiphytes. The epibiotic organism may be sustained entirely by nutrients and water received nonparasitically from within the host (basibiont) on which it resides. However, the interaction of epibiont with basibiont is usually unknown. In some epibionts water and nutrients are taken up entirely from suspended soils and other aerial sources such as dead host tissues, airborne dust, mist, and rain. Any negative effect on the host, if it occurs, is indirect. In general usage the terms epiphyte and epibiont are used interchangeably.

The assemblage of epiphyllic flora on the surface of healthy plant leaves is usually composed of bacteria, yeasts, and filamentous fungi that belong to different systematic categories. The density and the number of epiphyllic microorganisms can rapidly change and it strongly depends on environmental conditions, host species, habitat of the host, and the age and the surface structure of the organ on which the epiphyllic organism resides.

While some fungi are adapted to the plant surface, the community also includes propagules of airborne species, fungi that would not otherwise be considered epibionts. The surface of the plant, especially the leaf is an extreme environment. Though plants may leak nutrients through the cuticle, the surface is dry, waxy, and affected by UV radiation. Epibionts have many obvious characteristics that enable continuation through the stressful conditions of the leaf surface. For example, some epibionts can digest lipidic substrates, and thus may utilize the waxy layer covering the leaf. Epibionts are likely to be melanized, and thus able to resist UV radiation. Epibiotic yeasts are able to multiply during the short periods of appropriate environmental conditions. Although epibiotic organisms differ markedly from endophytes, it has been shown that some endophytic fungi are also able to produce epiphytic stages with fungal reproductive structures on the surface of the host plant.

Bacterial Endophytes

For approximately 60 years endophytic bacteria have been observed to exist within plants. Bacterial endophytes similar to endophytic fungi are microorganisms that live inside plant tissues and can be isolated from surface-disinfected plant tissues or extracted from within a plant. Although the bacterial endophytes do not visibly harm the host plant, traditionally they were assumed to be latent pathogens or contaminants.

Endophytic bacteria have been isolated from several tissue types of numerous plant species including both mono- and dicotyledonous plants. It is assumed that the root zone is the primary site where endophytic bacteria enter plant tissues. However, aerial portions of plants may also be used for entry. Inside a plant the endophytic bacteria can spread from point of entry throughout the entire plant. Various studies indicate that endophytic bacteria inhabit mainly intercellular spaces of plant tissues, but they can also live in intracellular spaces and inside the vascular system. Variations in the populations of endophytic bacteria have been reported, and they depend on plant species, plant age, tissue type, and time of sampling and environmental conditions. Generally, bacterial endophyte populations are larger in roots and gradually decrease in the stems and leaves. Active exchange can also occur between epiphytic and endophytic populations. Although leaf surfaces and interior spaces of a plant are two different

environments to which the foliar bacteria have to adopt to survive and multiply, some of the bacteria can colonize both the surfaces of the leaf and the interior of a plant. Because of the dual colonization of interior and exterior spaces of the plants, distinction between epiphytic and endophytic bacteria may be difficult in practice. The endophytic colonization of plants is probably the standard for plant-associated bacteria to develop sustainable epiphytic populations.

Endophytic bacteria in native and agronomic plants that have been reported most commonly are represented by a broad range of taxa, including α Proteobacteria, β Proteobacteria, γ Proteobacteria, Firmicutes, Bacteroides, Actinobacteria. For example, *Pseudomonas syringae* is an opportunistic plant pathogen capable of colonizing aerial plant surfaces and leaf tissue. Pseudomonas species are sometimes reported as a plant endophyte, and some species of the genus are known for their beneficial associations with host plants and for their antagonistic activity against plant pathogens.

The diversity of bacterial endophytes has been mainly studied using cultivation-dependent techniques. Due to insufficient knowledge about growth requirements of many microorganisms as well as the presence of viable but nonculturable bacterial cells within plant tissues, our knowledge of actual diversity of bacterial endophytes is limited. Recent reports have confirmed detection of a broad spectrum of endophytic bacteria from plant tissues by using culture-independent molecular biological methods. For example, the 16S ribosomal RNA gene is a phylogenetic marker frequently employed to describe the microbial community in natural environments without a need for cultivation. It has been found that isolation from internal tissue based on cultivation-dependent methods captured less than 50% of the bacterial community retrieved by cultivation-independent techniques.

Intracellular Root Endophytes: Rhizophagy Symbiosis

In the last several years it has been shown that plant roots internalize bacteria and yeasts into roots cells where microbes appear to be degraded in time – in a process denominated as 'rhizophagy' to denote that roots appear to be 'eating' microbes. It has become clear that certain microbes are not completely degraded, and instead cycle between a naked-protoplast phase inside root cells and a free-living walled-cell phase in soil in a process that has been denominated 'rhizophagy symbiosis' or 'rhizophagy cycle'. Microbes (bacteria and yeasts) acquire soil nutrients in the free-living soil phase; nutrients are extracted from microbes oxidatively in the intracellular endophytic phase. Plants have a limited capacity to sequester micronutrients in soils, while microbes are particularly good at acquiring soil micronutrients using siderophores and hemophores. Because of this it has been proposed that extraction of micronutrients (such as iron) from rhizophagy microbes is the probable function of the rhizophagy symbiosis. In the rhizophagy cycle symbiotic microbes (bacteria or yeasts) grow on the rhizoplane in the exudate zone behind the root meristem. Microbes enter root tip meristem cells before cell walls are fully hardened – locating within the periplasmic spaces between cell wall and plasma membrane. In the periplasmic spaces of root cells, microbes convert to wall-less protoplasts (called 'L-forms' in bacteria, and 'mycosomes' in fungi). As root cells mature, microbes are exposed to reactive oxygen (superoxide) produced by NADPH oxidases on the root cell plasma membranes. Reactive oxygen degrades some of the intracellular microbes – and likely causes membrane porosity and electrolyte leakage in other microbes, effectively extracting nutrients from them. Surviving microbes in root epidermal cells trigger root hair elongation, and as hairs elongate microbe protoplasts are forced out of root hairs at hair tips, reforming cell walls and cell shapes as they emerge into the rhizosphere where microbes may obtain additional nutrients. Later attraction of microbes to the root exudate zone behind the root tip meristem again places bacteria in position to enter root meristem cells. It is currently thought that most or all plants engage in rhizophagy symbiosis, although very few details are currently known with regard to how the rhizophagy cycle works or how important it is for plant growth and development.

Fungal Endophytes

Many groups of fungi exist as plant endophytes. The fungal endophytes have commonly been divided into two major groups. The first group comprises a large number of fungal species with a broad range of host plants, and the second group includes a smaller number of specialized fungal species that colonize some monocotyledonous hosts. Every plant species examined to date maintains endophytic associations with fungi and this association is a ubiquitous and cryptic phenomenon in nature. Although most of the endophytic fungi belong to the phylum Ascomycota and its anamorphs, Glomeromycota and some Basidiomycota and Zygomycota are also known.

Endophytes associated with healthy organs of nongrass plants are still poorly known. However, fungal surveys conducted during the last 30 years have demonstrated that tissues of the vast majority of nongrass plants are colonized by endophytic microfungi. Plants from over 100 plant families have been reported to host endophytic fungi. Most fungal surveys of angiosperms show that fungi from ascomycetes orders Diaporthales, Dothideales, Helotiales, Hypocreales, Leotiales, Pezizales, Phyllachorales, Pleosporales, Sordariales, Xylariales, and fungal species belonging to basidiomycetous orders Agaricales and Polyporales are frequently encountered as endophytes. Particularly, fungi from the genera *Acremonium*, *Alternaria*, *Chaetomium*, *Cladosporium*, *Cryptocline*, *Cryptosporiopsis*, *Leptostroma*, *Phoma*, *Phomopsis*, *Phyllosticta*, and *Trichoderma* are well represented in endophyte assemblages. Fungal endophytes within the host may inhabit many different tissues of roots, stems, branches, twigs, bark, leaves, petioles, flowers, fruits, and seeds, including xylem of all available plant organs. Endophytic microfungi in tissues of nongrass hosts are usually highly diverse and occur as numerous localized infections that increase in number, density, and species diversity with organ age. It may be because the fungal endophytes associated with nongrass plants generally appear to be transmitted horizontally (i.e., from plant to

plant in populations). The profile of fungal endophyte assemblages in specific organs can be completely different from those in other plant organs or tissues. In general, the main tissues that have been analyzed for the presence of endophytes in woody perennials are the leaves, twigs, branches, and roots; a few studies have looked for endophytes in meristems, flowers, or fruits of woody perennials. However, the profile of fungal endophyte assemblages in flowers and fruits could be radically different than that in other types of plant structures because these organs are young and rapid in development.

The host range, ecology, and geographical distribution of most groups of endophytic fungi are still poorly known. A study by Betsy Arnold (University of Arizona) and François Lutzoni (Duke University) indicated that endophyte infection of host plants increases along latitudinal gradient from the Arctic to the Tropics, with less than 1% to more than 99% of tissue segments (2 mm^2) with endophytes, respectively. This study also showed that foliar endophyte assemblages, at both an individual host species and the community level, increases in diversity with decreasing latitude.

Diversity and composition of endophytic fungal communities of almost all plant species examined to date have been assessed mostly by culture-based approaches and have been identified using morphological characteristics with support of molecular analysis for identification of some endophytes that remain sterile in culture. Abundance and diversity of unculturable endophytes is still mostly unknown, limiting our understanding of endophyte infection frequencies, taxonomic composition, and diversity. Many researchers suggest that culture-based methods alone underestimate diversity and misrepresent the taxonomic composition of endophyte communities. Therefore, recently cultivation-independent approaches, such as RFLP analysis and sequencing of rDNA, have gained popularity as a means to assess the diversity and composition of uncovering endophytes with obligate host association, that is, fungal species that do not grow on standard media or fungi that grow slowly and are lost during the culturing process in competitive interactions.

Endophytes of Grasses

Fungal endophytes of grasses are widely studied; with most work concentrating on fungi of the family Clavicipitaceae (Hypocreales; Ascomycota). The following genera of this family have been identified as grass endophytes or epiphytes: *Atkinsonella*, *Balansia*, *Balansiopsis*, *Echinodothis*, *Epichloë*, *Myriogenospora*, and *Parepichloë*. Clavicipitaceous endophytes, also known as e-endophytes, are widespread in grasses; approximately over 10% of all grass species are estimated to harbor clavicipitaceous fungal endophytes. The genus *Epichloë*, with anamorphs previously in *Neotyphodium*, is the most studied group of grass endophytes. These endophytes are found growing systemically in the aboveground tissues of some temperate grass species of the cool-season grasses. Based on the relative costs and benefits to the hosts, these grass–fungal endophyte symbioses have been showed to range from pathogenic to mutualistic. Some endophytic species from the genus *Epichloë* are often considered to be pathogenic and may cause partial or complete sterilization of hosts due to the production of a fungal stroma on the flowering culms ('choke disease') of the host. However, the degree of effect on host reproduction varies with species. In mutualistic associations, that is, in some *Epichloë* spp. that do not cause choke disease, the endophyte grows systematically within its host (Fig. 1), including the developing seeds (Fig. 2), and is entirely dependent on the survival and growth of the grass host plant for its own growth.

It is believed that fungal endophytes of grasses have two modes of reproduction. The sexual species of genera *Epichloë* and *Balansia* can be horizontally transmitted through development of ascospores. Because *Epichloë* species are obligately outcrossing ascomycetes, development of the sexual spores is dependent upon transfer of spermatia of one mating type to an unfertilized stroma of the opposite mating type occurring on different individuals of the host plants. Transfer of spermatia of *Epichloë typhina* (Pers.) Tul. & C. Tul. is accomplished by flies of genus *Botanophila* (Anthomyiidae; Diptera), which visit stromata for feeding and oviposition. Immediately after cross-fertilization of the fungus, perithecia begin to develop on the stroma. During flowering of the host plant, the ascospores produced within the perithecia of infected individuals in the population are forcibly ejected. The ascospores, possibly dispersed by air currents, may land on another healthy grass plant and may initiate infection. In contrast,

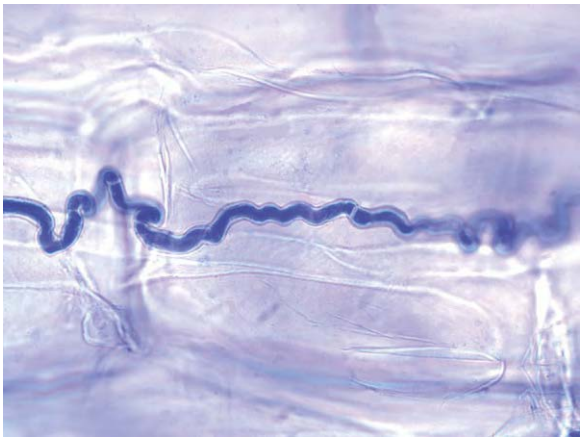

Fig. 1 Endophytic convoluted hypha of *Epichloë coenophiala* (Morgan-Jones & Gams) C.W. Bacon & Schardl. in a culm-scraping preparation from *Festuca arundinacea* Schreb. (× 1600).

Fig. 2 Four-week-old culture of *Epichloë typhina* subsp. *poae* (Tadych, K.V. Ambrose, F.C. Belanger and J.F. White) Tadych from seeds of *Poa secunda* subsp. *juncifolia* (Scribn.) Soreng grown on potato dextrose agar at room temperature (× 0.7).

many sexual Epichloë species and all of their asexual mutualistic species are typically transmitted vertically from maternal plants to their offspring. For most of their life cycle the endophytes inhabit, asymptomatically and systemically, the apoplasts of the aboveground organs of infected host plants, including the embryos of viable seeds, and can be disseminated vertically to successive generations of the host plant. Infected seeds and vegetative tillers of infected host plants are the only known modes of propagation of these endophytes. Additionally, several *Epichloë* spp., such as *Epichloë festucae* Leuchtm., Schardl & M.R. Siegel, are represented by species that have a remarkable mixed-transmission strategy, that is, horizontal and vertical transmission modes. In these cases, some tillers produce stromata while other tillers on the same plant are asymptomatic and produce normal, vigorous, endophyte-infected seeds.

During the last decade some *Epichloë* endophytes have been also shown to emerge from plants and produce a network of mycelium, conidiophores, and conidia on the surfaces of plants (Fig. 3). This epiphyllous mycelium is most evident on leaf blades of certain species of the Pooideae, including the bent grasses, fescues, forest hedgehog grass, ryegrasses, wild barley, and some blue grasses. The epiphyllous conidia are viable and spread via water currents, rain splash, or drip splash to adjacent plants. It is likely that epiphyllous conidia are responsible for some of the parasexual recombination 'hybridization' that may occur in the *Epichloë* endophytes.

In addition to the e-endophytes a number of other seed-transmitted grass fungal endophytes have been described. Certain cool-season grasses harbor nonclavicipitaceous fungal endophytes that are less frequent than e-endophytes. These fungi belong to two different groups, that is, p-endophytes and a-endophytes. The p-endophytes include *Gliocladium*- and *Phialophora*-like endophytes ('p' for penicillate disposition of conidiophores, common to *Gliocladium*-like and *Phialophora*-like fungal species) that can be isolated from culms and seeds of numerous festucoid grasses, for example, from perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis* Huds.), Arizona fescue (*Festuca arizonica* Vasey), and giant fescue (*Festuca gigantea* (L.) Vill.). These endophytes are represented by species of the order Eurotiales (Ascomycota). The a-endophytes is a group of grass endophytes found in Italian ryegrass (*Lolium multiforum* Lam.), other annual species of the *Lolium* genus, and *Festuca paniculata* (L.) Schinz & Thell. The a-endophytes belong to parasitic species of *Acremonium* similar to *Acremonium chilense* Morgan-Jones, J.F. White & Piont. (Hypocreales; Ascomycota), an endophyte of orchard grass (*Dactylis glomerata* L.). At present, the ecological and physiological importance of p- and a-endophytes of grasses are not determined or understood.

Fig. 3 Epiphyllous growth of *Epichloë typhina* subsp. *poae* on a two-week-old *Poa secunda* subsp. *juncifolia* seedling (× 3).

The warm-season grass, *Trichachne insularis* (L.) Nees, was found to harbor a nonclavicipitaceous, seed-transmitted endophyte identified as *Ramulispora trichachnicola* (J.F. White & Morgan-Jones) U. Braun, an anamorphic species of genus *Mycosphaerella* (Mycosphaerellaceae; Ascomycota). Another seed-transmitted fungus *Gibberella fujikuroi* (Sawada) Wollenw. (Nectriaceae; Ascomycota) has been isolated from maize (*Zea mays* L.), sorghum (*Sorghum* spp.), and other plants in the Poaceae family as a symptomless endophyte. The horizontally transmitted entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Clavicipitaceae; Ascomycota) is also known to form an endophytic association with maize.

Nonsystemic grass endophytes present another group of fungal endophytes. At present, little is known about nonsystemic endophytic fungi that inhabit grass species. Most studies of grass endophytes have focused on systemic endophytes. However, some studies indicate that in addition to systemic infections, endophytes that form localized infections in grasses may be also diverse and widespread. Generally, dominant nonsystemic grass endophytes are represented by the genera *Alternaria*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Phoma*, and pathogens typical of grass hosts. Because of their mode of dissemination, diversity and dispersion of this group of grass endophytes is probably more variable than that of systemic grass endophytes and depends on availability and viability of spores of the fungus.

Some common endophyte–grass associations

Tall fescue – *Epichloë coenophiala* association

Tall fescue (*F. arundinacea* = *Lolium arundinaceum* (Schreb.) Darbysh.) is one of the best-known examples of a grass with an endophyte that causes toxicity. The grass was brought to the United States from Europe in the late 1800s. It was officially discovered in Kentucky in 1931, tested at the University of Kentucky, and released in 1943 as 'Kentucky 31'. From the mid-1940s it became popular with farmers, spreading quickly throughout the midwestern and southern United States. Today it accounts for well over 16 million hectares of pasture and forage land in the United States. The problem of livestock neurotoxicosis ('fescue toxicosis' also known as 'fescue foot' or 'fescue lameness') became a major concern in the United States. Several studies show that consumption of endophyte-infected tall fescue decreases the feed intake of cattle and therefore lowers animal weight gains. Affected cattle also produce less milk, have higher internal body temperatures and respiration rates, develop a rough hair coat and demonstrate an unthrifty appearance, salivate excessively, have poor reproductive performance, and maintain reduced serum prolactin levels. By the end of the 1970s, the association of this toxicity with the endophyte had been discovered. The endophyte was originally identified as a strain of *E. typhina*, but was later described as *E. coenophiala*. *E. coenophiala* produces several alkaloids, particularly, the alkaloid ergovaline that is structurally related to ergotamine, a major factor in ergot poisoning due to ingestion of *Claviceps purpurea* (Fr.) Tul. 'ergots' contaminating rye flour.

Perennial ryegrass – *Epichloë festucae* var. *lolii* association

Perennial ryegrass (*L. perenne* L. subsp. *perenne*) is a valuable forage and soil stabilization plant. In New Zealand the neurotoxic disease 'ryegrass staggers' (also known as 'perennial ryegrass staggers') of sheep and cattle has long been reported and attributed to the consumption of perennial ryegrass. In 1898 for the first time the presence of a fungal endophyte in the seeds of *L. perenne* was observed, and 40 years later the endophytic fungus from *L. perenne* was isolated and grown in agar culture. The association between the *L. perenne* endophyte and 'ryegrass staggers' was finally established in 1981. *Epichloë festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C.W. Bacon & Schardl that infects perennial and hybrid ryegrasses was shown to synthesize several alkaloids. Three of these are known to be particularly important to pasture management, specifically (1) lolitrem B, a tremorgenic molecule responsible for livestock 'staggers', (2) ergovaline, an ergopeptine that has vasoconstrictive effects and causes heat stress in grazing animals, also responsible for 'fescue toxicosis', and (3) peramine, a tripeptide that deters some insects, particularly the Argentine stem weevil, from feeding on ryegrass but is not toxic to mammals. Perennial ryegrass staggers is a very serious problem for grazing livestock (sheep, cattle, horses, deer) in New Zealand; however, mortality rates are generally low. Ryegrass staggers occur sporadically in North and South America, Europe, and Australia.

Darnel ryegrass – *Epichloë* occultans association

Darnel ryegrass = darnel (*Lolium temulentum* L.) is another common endophyte-infected grass species. This species has a long-recorded history as a plant poisonous to humans and animals. Darnel was known as a weed and as a poisonous plant in earlier times. The earliest written references to darnel indicating it to be a noxious and toxic weed that caused problems for humans and animals may be found in the Gospel of Matthew of the New Testament and in authors such as Plautus, Virgil, Ovid, Dioscorides, and Shakespeare. Darnel seed from 4000-year-old archeological materials in ancient Egypt contained endophyte mycelium. It was generally understood that human beings would be poisoned by ingestion of flour or baked products containing seeds of darnel as a contaminant. Contamination was also undesirable because of the strong taste of darnel seeds, which, for example, resulted in inferior bread. Darnel seeds were sometimes added to beer as a flavoring. By the end of the 1800s it was discovered that a fungus infects seeds of darnel. Currently we know that darnel seeds contain an endophytic fungus, and this symbiotic fungus is known as *E. occultans* (C.D. Moon, B. Scott & M.J. Chr.) Schardl.

The sleepygrass – *Epichloë* chisosa association

Sleepygrass (*Achnatherum eminens* (Cav.) Barkworth) is a perennial grass forming stout, erected clumps in dry plains, hills, and open woods. The grass is native to North America, and is abundant in southwestern United States. It was found to be toxic and narcotic to grazing animals, that is, to horses, cattle, and sheep. Animals, after consumption of relatively small quantities of the

grass, go to sleep for 2–3 days, and then gradually recover. Ergot alkaloids, that is, ergonovine, ergonovinine, lysergic, and isolysergic acid amides, have been identified as the sleep-inducing agents in sleepygrass. These alkaloids are produced by *E. chisosa* (J.F. White & Morgan-Jones) Schardl, the endophyte isolated from this plant and related species of the genus. However, recent studies suggest that the level of ergot alkaloids in native sleepygrass populations may be highly variable within and among populations despite the level of endophyte infection. Animal toxicity is localized in particular areas where particular strains of the endophyte may predominate.

The 'drunken horse grass' – *Epichloë* association

Drunken horse grass (*Achnatherum inebrians* (Hance) Keng) is a perennial bunchgrass, distributed on alpine and subalpine grasslands of northwestern China and Mongolia. Horses that have grazed *A. inebrians* develop a stagger; the animals walk as if drunk, with some being unable to stand after falling. The symptoms persist for 6–24 h, and usually they appear to be completely recovered after 3 days. Severely affected animals die within 24 h of consumption of the grass. Toxicity of *A. inebrians* was reported by Marco Polo and the Russian explorer Nikolai Mikhaylovich Przhevalsky. In the last decade the presence of two endophytes, *E. gansuensis* (C.J. Li & Nan) Schardl and *E. inebrians* (C.D. Moon & Schardl) L. Chen & C.J. Li, in this grass species was confirmed. In the study of alkaloid content, it was found that infected *A. inebrians* had two major toxic alkaloids: ergonovine and lysergic acid amide.

The 'dronkgras' – *Epichloë* melicicola association

Staggers grass = 'dronkgras' (*Melica decumbens* (L.) Weber) is a tufted perennial, very coarse grass with usually rolled and very rough leaves. *M. decumbens* is endemic to Africa and has a limited distribution in South Africa, being found only in the arid areas of central South Africa. It grows amongst rocks, under trees, and shrubs on hill- and mountainsides, occasionally in areas along roadsides. The name 'staggers grass' comes from the fact that this grass has narcotic effects on cattle, horses, donkeys, and sheep. The tremorgenic neurotoxins produced by the endophyte *E. melicicola* (C.D. Moon & Schardl) Schardl were found to be responsible for the narcotic effect of *M. decumbens* on grazing livestock. Usually, consumption of grass is not lethal and the animal recovers. This is probably because the leaves of the grass are very coarse, and animals do not graze it frequently, except when the grass is very young.

Forest hedgehog grass – *Epichloë* association

Forest hedgehog grass (*Echinopogon ovatus* (G. Forst.) P. Beauv.) is a tufted perennial, *meso*phytic grass often found in moist forested areas (in wet sclerophyllic woodlands and by creeks). The grass is endemic to Australia, New Guinea, New Zealand, and Tasmania. Young plants of *E. ovatus* cause 'staggers', sometimes known as 'wobbles' in stock. The grass was found to harbor endophytes *Epichloë aotearoae* (C.D. Moon, C.O. Miles & Schardl) Leuchtm. & Schardl and *Epichloë australiense* (Moon & Schardl) Leuchtm. The presence of indole-diterpenoid and loline alkaloids in endophyte-infected *E. ovatus* was confirmed.

'Huecú' grasses – *Epichloë* tembladerae association

Poa huecu Parodi and several other grasses of South America (e.g., *Festuca argentina* (Speg.) Parodi, *Festuca hieronymi* Hack., *Festuca magellanica* Lam., *Festuca superba* Parodi ex Türpe, and some other *Bromus* spp., *Melica* sp., *Phleum* spp. and *Poa* spp.) are colonized by the endophytic fungus *E. tembladerae* (Cabral & J.F. White) Iannone & Schardl. The association of the endophyte with *F. hieronymi* and *P. huecu* is probably responsible for toxicosis syndromes, called 'tembladera' or 'huecú' in grazing animals. 'Tembladera' is from the Spanish word that means 'tremble', and word 'huecú' is from the indigenous Araucanian language of the tribes that lived in the region and means 'intoxicator'. Huecú toxicosis results from consumption of *P. huecu* and is frequently lethal to animals. Studies on *E. tembladerae* suggest that toxicity of the infected grasses to mammals is associated with the ergot alkaloids, lolitrems, and some glycoproteins, produced by the fungus.

Locoweed and Poison Pea Endophytes

The name locoweed is derived from Spanish 'loco', which means insane or crazy. In English, the term loco has been used by the people of North America for over a century to describe an unusual behavior and neurological syndrome of grazing animals poisoned by certain legumes (Fabaceae). These certain legume species in the related genera of *Astragalus*, *Oxytropis*, commonly known as 'locoweeds' or 'milkvetch', and *Swainsona*, known as 'poison peas', may be found in semiarid regions of Asia, the Americas, Europe, North Africa and Australia. Both wildlife and livestock animals that feed on these plants exhibit similar neurological syndromes, called 'locoism' in North America or 'pea struck' in Australia, characterized by weight loss, altered behavior, depression, decreased libido, infertility, abortion, birth defects, and death.

The mechanism of toxicity caused by these plants has been explained decades ago by isolation of the indolizidine alkaloid, swainsonine (Fig. 4) from *Swainsona canescens* (Benth.) F.Muell. (Darling pea), a legume native to Australia, and isolation of swainsonine N-oxide from *Astragalus lentiginosus* Douglas (spotted locoweed or freckled milkvetch). For years, it was thought that swainsonine was a plant-derived secondary metabolite, however, in fact, it was first isolated from a fungus, not a plant. Recently, it was shown that endophytic fungi associated with the *Astragalus*, *Oxytropis*, and *Swainsona* plants produce swainsonine that intoxicates animals, and deters many herbivores from consuming the plants. Although numerous species within each of the three genera are colonized by fungal endophytes and reported to contain swainsonine, many other species are endophyte-free, thus nontoxic to the animals.

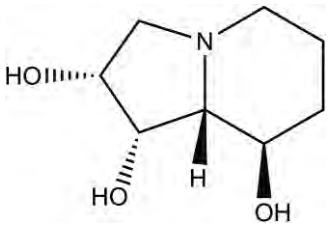

Fig. 4 Swainsonine.

All swainsonine-containing locoweeds/milkvetches and poison peas investigated thus far are associated with endophytes belonging to *Alternaria* spp. section *Undifilumi* (Pleosporales; Ascomycota). Mycelia of the endophytic fungi systematically colonize the parts of the host, which are aboveground, i.e., stem, leaf, inflorescence, and seed, but cannot be found in underground parts. In the seed the mycelia are primarily found in the seed coat, but are not present in the embryo. The endophytes are vertically transmitted from seed to progeny. Until now the following endophytic species, i.e., *Alternaria oxytropis* (Q. Wang, Nagao & Kakish.) Woudenb. & Crous, *Alternaria cinereal* (Baucom & Creamer) Woudenb. & Crous, and *Alternaria fulva* (Baucom & Creamer) Woudenb. & Crous have been isolated from the locoweed/milkvetch and poison pea plants, and identified to produce swainsonine.

Marine Endosymbionts

The marine environment may offer a variety of unexplored endosymbiotic microorganisms. Due to the complicated nature of marine symbiotic associations and experimental limitations, the ecology and chemistry of these interactions has not been well studied. Only in a few cases a specific microbe–host relationship, for example, symbiosis or permanent association, has been proven. Most of the literature reports describe the sporadic occurrence of variable microorganisms for certain hosts. Mostly bacteria but also fungi are to be found in marine animals and algae as epi- and endobionts. However, marine fungi are not taxonomically well defined. In general, the marine environment includes obligate marine fungal species, which are considered to be fungi that grow and sporulate exclusively in a marine habitat. It also includes facultative marine fungi, that is, those that can be isolated from both terrestrial and marine environments, and are adapted to and isolated from various marine habitats, like near-shore or estuarine environments. Marine fungi that do not germinate in the natural marine habitat are not included in these groups. One of the better-documented relationships between marine derived fungi and other marine organisms is the fungal–algal symbiotic association 'mycophycobiosis'. It was estimated that one-third of all known marine fungi are associated with marine algae and these fungi reside inside the algal tissues. Studies of marine-derived fungi indicate the enormous diversity of the fungal community in the world's oceans and their biochemical uniqueness.

Ecological Impacts of Endophytes

Abiotic and biotic stresses due to noninfectious and pathogenic plant diseases, pests, and unfavorable growing conditions are major causes for plant productivity losses. Productivity of cultivated plants relies heavily on high chemical inputs. Recently, natural and biological control of diseases and pests affecting cultivated plants has gained much attention as a way of reducing the use of chemical products in agriculture. Biological control offers an alternative or supplement to chemical pesticides in plant protection. This approach incorporates one or more organisms to maintain another pathogenic organism below a level at which it is no longer an economical problem. Use of endophytic organisms could enhance plant growth and productivity on a worldwide basis.

Some groups of endophytic microorganisms have been described as 'defensive mutualists' that protect host plants against biotic and abiotic stresses. In return, the endophytic symbionts acquire nutrients from their host plants. Endophytic arbuscular mycorrhizal fungi, which live in mutualistic symbiosis with at least 80% of plants, effectively protect host plant from many root diseases, reduce infection by nematodes, increase stress tolerance like drought resistance, tolerance to heavy metals and salinity, and increase phosphorous uptake in phosphorous-deficient soils. Endophytes of upper parts of grasses and other plants also benefit their hosts. The benefits frequently reported include systemic resistance against pathogens, reduced herbivory, increased drought resistance, improved tolerance to heavy metals, and generally enhanced growth and an increase in the plant's fitness to environmental extremes. The fungal endophytes may also influence the plant pathogen assemblages, and reduce their diversity and abundance. For example, in *F. arundinacea*, endophyte infection reduced seedling blight caused by *Waitea circinata* Warcup & P.H.B. Talbot and crown rust caused by *Puccinia coronata* Corda relative to endophyte-free plants, but colonization by *E. coenophiala* did not affect rust caused by *Puccinia graminis* Pers. Also, Alternaria leaf spot caused by *Alternaria triticina* Prasada & Prabhu was significantly more common on endophyte-free *Panicum agrostoides* Spreng. than on plants with endophyte. Endophyte-infected grasses may also experience a lower incidence of disease when insects, vectors of plant pathogens, are deterred by endophytes. Although the ecological roles played by endophytic fungi are more diverse and varied, these benefits arise in part from the

production of fungal metabolites (usually alkaloids) by the endophyte or endophyte–plant complex. However, in some cases the mechanisms of defense are not fully understood and require further study.

In contrast, in horizontally transmitted endophytes associated with phytosynthetic tissues such as leaves of nongrass plants, the benefits are less clear. Generally, these endophytes are believed to also function as defensive mutualists of host plants; however, in most cases their ecological roles have not been assessed experimentally. Recently, several studies have provided evidence for important roles of endophytes in enhancing plant defenses against biotic stresses, such as protection from herbivores, pathogenic fungi, nematodes, and neighboring plants' competition, and abiotic stresses. Recent studies have shown that the endophyte *Trichoderma ovalisporum* Samuels & Schroers that colonized stems and fruits of *Theobroma gileri* Cuatrec. has the ability to parasitize and antagonize the necrotrophic mycelium of pathogenic *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora, and *Moniliophthora roreri* (Cif.) H.C. Evans, Stalpers, Samson & Benny. Another study showed that seedlings of *Theobroma cacao* L. inoculated with seven endophytic fungal species and exposed to *Phytophthora* spp., species that are pathogens of the plant, were more resistant than endophyte-free plants. Leaves of the control endophyte-free plants died in greater numbers and suffered much more pathogen damage than endophyte-infected plants. Also, the relative benefit of endophyte inoculation was higher in older than in younger leaves.

Endophytic fungi contribute to and may also be responsible for the adaptation of host plants to environmental stresses. For example, the heat-tolerant perennial panicgrass (*Dichanthelium lanuginosum* (Elliott) Gould) is symbiotic with a fungal species of the genus *Curvularia*. The endophytic fungus was isolated from the grass collected from geothermally heated soils of Yellowstone National Park (Wyoming, Montana, and Idaho, USA) and Lassen Volcanic National Park (California, USA). Geothermal soils of Yellowstone and Lassen Volcanic National Parks may reach temperatures as high as 57°C, and on an annual basis the grass is exposed to high temperatures as well as periods of drought conditions. Laboratory and field studies conducted by Rusty Rodriguez (US Geological Survey/University of Washington), Regina Redman (US Geological Survey/University of Washington), and Joan Henson (Montana State University) have shown that the endophytic fungus confers thermotolerance to the host plant and that this fungal–plant association is responsible for survival of both species in geothermal soils. When these organisms were grown asymbiotically under controlled conditions, the maximum growth temperatures of *D. lanuginosum* and *Curvularia* sp. were 40 and 38°C, respectively; while with the endophyte plants grew at considerably higher temperatures. Another example is the fungus *Piriformospora indica* Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken originally isolated from a spore of mycorrhizal fungus *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler. Some studies showed that *P. indica* is able to endophytically colonize roots of various crop plants. It has been shown that isolates of *P. indica* in roots of barley plants enhanced development of the host plant and increased grain yield. It was speculated that this endophytic fungus elevated antioxidative status of the infested roots and therefore protected roots from root pathogens like *Fusarium culmorum* (W.G. Sm.) Sacc. and *Bipolaris sorokiniana* (Sacc.) Shoemaker. Also, the systemic plant response, as a result of induced resistance by the endophyte, causes a reduction of powdery mildew (*Blumeria graminis* (DC.) Speer) infection of barley leaves. The fungus also protects the barley plants from salt stress.

Production of Nontoxic Endophytes

It is also well documented that endophytic fungi have been implicated in toxicity of some poisonous plant species. Grass fungal endophytes are a particularly important group of fungi that have been found to naturally produce a range of mycotoxins, alkaloids, and physiologically active chemical compounds. Some of these substances cause problems for livestock and are recognized as, for example, the causative agents of economically important livestock toxicoses, such as 'fescue toxicosis' and 'ryegrass staggers'. It has been found that some endophytes of ryegrasses and some other related grass species have reduced toxicity to grazing livestock while at the same time they enhance tolerance to pests and/or abiotic stresses. It has also been discovered that some toxic and beneficial alkaloids have separate biosynthetic pathways, allowing the selection or development of endophyte strains that show low animal toxicity but still possess anti-insect qualities. Axenic cultures of selected grass fungal endophytes may be produced and inoculated into seedlings of grasses. The discovery and commercialization of low-toxicity *Epichloë* endophytes along with the technology for their reinoculation resulted in the development of elite grass cultivars that are persistent, productive, and enhance animal performance, for example, Greenstone™ tetraploid hybrid ryegrass with Endosafe™ endophyte, and tall fescue cultivars with MaxQ™ endophyte.

Endophytes as Sources of Bioactive Metabolites

Microorganisms demonstrate many unique characteristics. Among these characteristics is the production of a vast range of biologically active substances and secondary metabolites. Microorganisms are one of the richest resources of biologically active substances and secondary metabolites with novel structures and potential activities. Secondary metabolites, also known as idiolites, have been defined as low-molecular weight and naturally produced substances that often possess chemical structures quite different from primary metabolites, such as amino acids, organic acids, and sugars from which they are produced. Their functions in the producing organisms are not obvious. They are usually hypothesized to function as chemical defenses for the hosts, but may be potential viable weapons against other organisms. In addition, secondary metabolites may be agents of symbiosis and agents of metal transport. Finally, these metabolites may act as sex hormones, plant growth stimulants, and as effectors of differentiation. For

many reasons these compounds may have tremendous economic importance to humankind and an extraordinary impact on the quality of human life. Some fungal secondary metabolites are beneficial (antibiotics such as cephalosporin and penicillin) while others are harmful (carcinogens such as aflatoxin and ochratoxin). The secondary metabolites produced by microbes are generally large and complex chemicals that are not readily synthesized using recombinatorial synthetic approaches. However, a number of drugs derived from fungal metabolites have been developed as their modified analogs. Antibiotics, antifungal, anticancer, immunosuppressive agents, and hypocholesterolemic agents that are derived from fungal compounds have been used for over 50 years.

During the last three decades the fungi living internally in living tissues of plants have been targeted as valuable sources of new bioactive compounds and they have become a mainstay of natural product screening programs. Growth of endophytic fungi within hosts without causing apparent disease symptoms and metabolic interaction of endophytes with hosts may favor the synthesis of biologically active secondary metabolites. According to a study performed by Barbara Schulz (Technical University of Braunschweig) and her colleagues, the proportion of novel structures produced by endophytic isolates (51%) was considerably higher than that produced by soil isolates (38%). Among the fungal genera, endophytic fungi from *Acremonium*, *Chaetomium*, *Colletotrichum*, *Fusarium*, *Pestalotiopsis*, *Phoma*, and *Phomopsis* are known to produce bioactive compounds of medical importance. In addition, endophytic fungi growing in axenic culture can also produce biologically active compounds, including several alkaloids, antibiotics, and plant growth-promoting substances. However, the amount and kind of compounds that are produced by a fungus will be affected by factors like temperature, degree of aeration, and the composition of the medium used for culturing. Some endophytic fungi have been identified as sources of anticancer, antidiabetic, and immunosuppressive compounds. For example, anticancer molecule paclitaxel (Taxol, Fig. 5), originally discovered in the bark of the Pacific yew tree (*Taxus brevifolia* Nutt.), has also been found to be produced by the endophytic fungus *Taxomyces andreanae* Strobel, Stierle, Stierle & Hess. Another endophytic fungus *Muscodor albus* Worapong, Strobel & Hess has an ability to produce a mixture of volatile organic compounds (VOCs) that were fatal to a wide variety of human- and plant-pathogenic fungi and bacteria.

Endophytic fungi are also more often recognized as a group of organisms capable of providing a source of novel bioactive compounds and secondary metabolites for biological control in agriculture as well as for biotechnology and industrial applications. It was shown that production of herbicidally active substances by endophytic fungi is 2 and 3 times higher than phytopathogenic fungi and soil fungi, respectively.

Endophytic fungi play an increasingly important role in the integrated pest management programs in agriculture. Many of the secondary metabolites produced by *Epichloë* grass endophytes significantly increase deterrence of vertebrate herbivores, insects, and nematode pests. It has been demonstrated that the secondary metabolites produced by the fine fescue endophyte *E. festucae* are inhibitory to other fungi. It has also been shown that endophyte-infected grasses contain a range of biologically active compounds

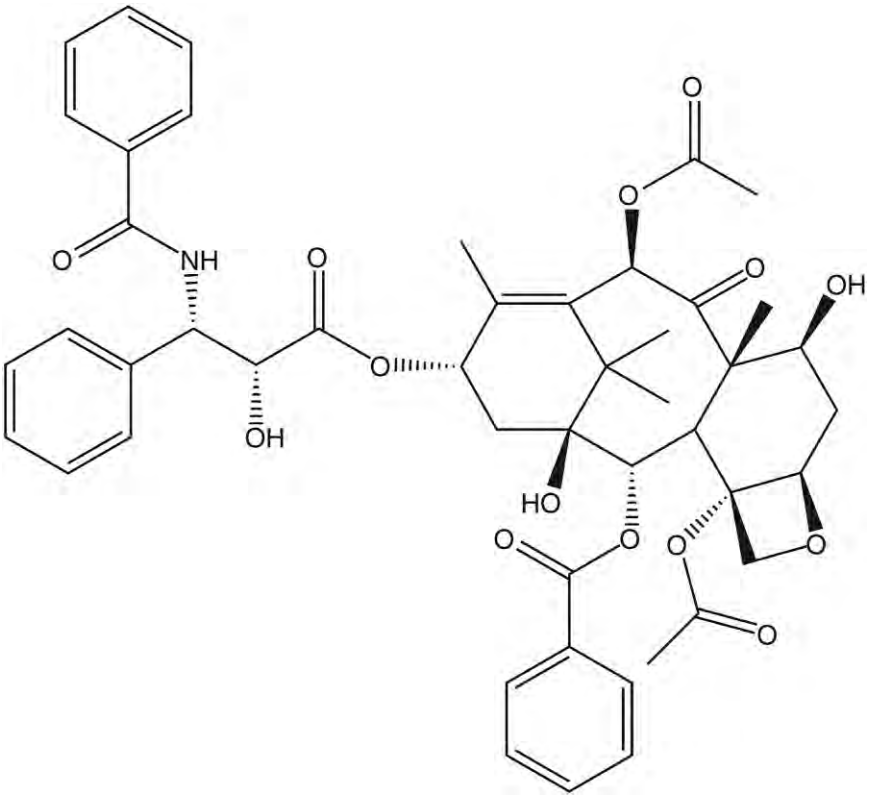

Fig. 5 Paclitaxel (Taxol).

that either are derived from endophyte or are produced as a result of the association. Four main classes of defensive compounds have been associated with endophyte presence in grasses and the following effects have been described.

Ergot Alkaloids

Ergot alkaloids cause toxicoses in grazing mammals with a range of symptoms from stupor and appetite suppression through reproductive problems and dry gangrene to death. The ergot alkaloids (Fig. 6) are produced by a wide range of filamentous fungi, primarily by members of the Clavicipitaceae family, including the above-mentioned grass endophytes of the genera *Epichloë* and the genera *Claviceps* and *Balansia*.

Indole-diterpene metabolites

Indole-diterpene metabolites produce a number of biological effects, including anti-insect activity (feeding deterrence, modulation of insect receptors functions, toxicity) and mammalian tremorgenic activity (staggers). Indole-diterpenoides (Fig. 7) have been reported from *Epichloë* as well as from some fungi of the genera *Claviceps*, *Aspergillus*, and *Penicillium*.

Peramine

Peramine is an unusual pyrrolopyrazine, and is produced by most *Epichloë* endophyte species symbiotic with grasses (Fig. 8). It is a metabolite that has specific biological activity as an insect feeding deterrent. Peramine is deterrent to invertebrate herbivores, especially the Argentine stem weevil, and is also active in preventing feeding activity of aphids, but is not toxic to mammalian herbivores.

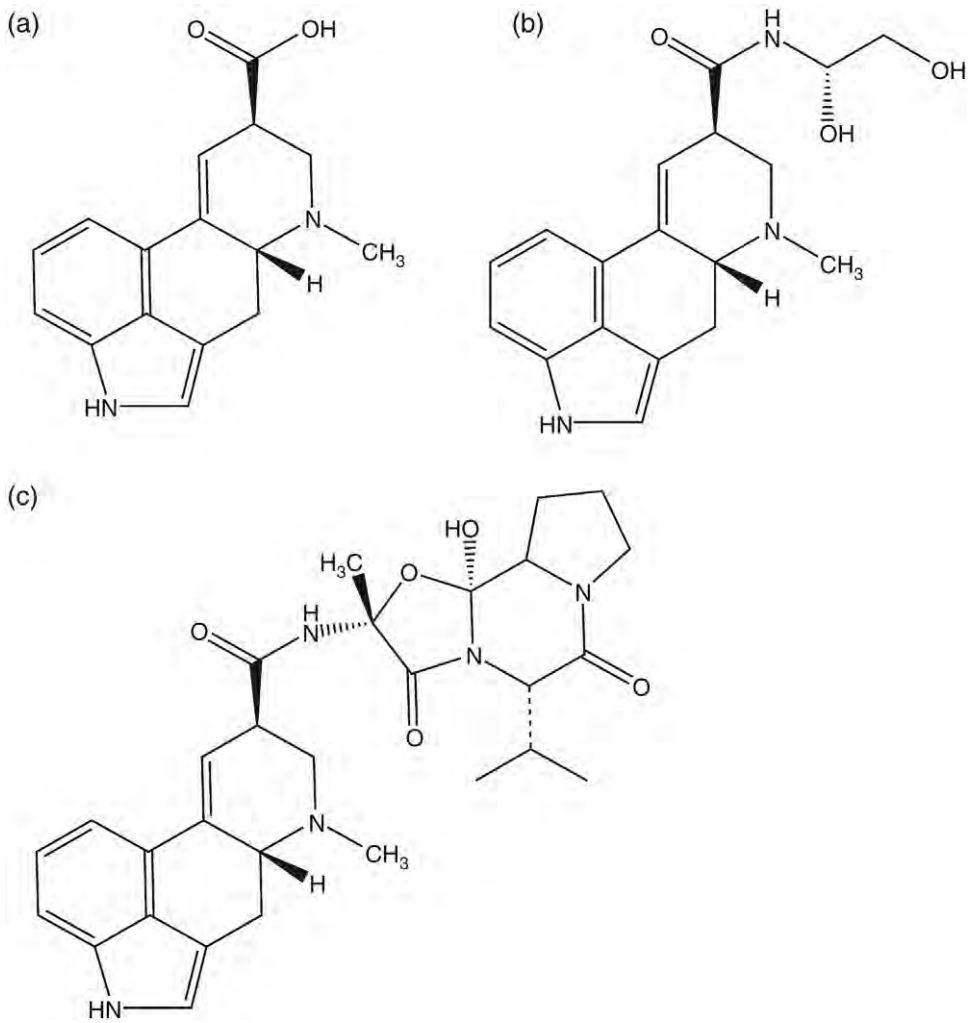

Fig. 6 Ergot alkaloids: (a) lysergic acid, (b) ergonovine, and (c) ergovaline.

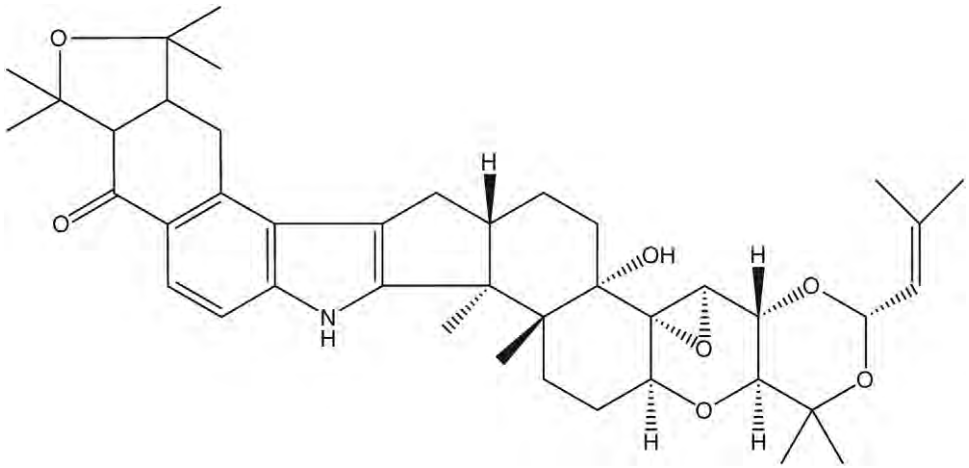

Fig. 7 Indole-diterpene: Lolitrem B.

Fig. 8 Peramine.

Lolines

Lolines are classified as pyrrolizidines, a class that also includes plant alkaloids known for their insecticidal activity (Fig. 9). These substances are insecticidal alkaloids with insect-deterrent activities, possessing little or no activity against large mammals. Lolines are neurotoxic to a broad range of insects, and when produced by endophytes in plants they have been shown to defend the plants from aphids. Lolines are only known from endophyte-infected grasses, and plants of the genus *Adenocarpus* (Fabaceae) and *Argyreia*

Fig. 9 Loline.

Fig. 10 Auxin – 3-indoleacetic acid (IAA).

mollis (Burm.f.) Choisy (Convolvulaceae). It is possible that undiscovered fungal symbionts might be responsible for loline production in *Adenocarpus* and *Argyreia* species.

Many, but not all, *Epichloë* species produce up to three classes of these alkaloids. In addition to the above, several simple indole alkaloids have been isolated from cultures of endophytic fungi; these include the simple auxins, for example, 3-indoleacetic acid (IAA, Fig. 10) and the indole glycerols, for example, 3-indolybutanetriol.

Reports of the secondary metabolites from true marine endophytes are relatively rare. This may be due to the relative difficulty in collecting and culturing of marine endosymbionts and their usually slow growth in laboratory conditions rather than lack of ability to produce secondary metabolites. In recent years, an increasing number of natural products from marine-derived fungal endophytes have been reported. Described below are some examples of secondary metabolites from marine-derived endophytic fungi. Isolated from the green alga *Enteromorpha* sp., the endophytic fungus *Wardomyces anomalus* F.T. Brooks & Hansf. (Microascaceae; Ascomycota), collected around Fehmarn island in the Baltic Sea, showed (1) antimicrobial effects of the crude extract toward *Microbotryum violaceum* (Pers.) G. Deml & Oberw. and *Aspergillus repens* (Corda) Sacc. and (2) inhibition of HIV-1 reverse transcriptase (HIV-1-RT). Investigation of the extract yielded several xanthone derivatives. Xanthones are a unique class of biologically active compounds possessing numerous bioactive capabilities, such as antimicrobial, antitubercular, antitumor, antiviral, and antioxidant properties. Xanthone derivatives occur in a number of higher plant families and fungi. Some fungal species are well known as sources of xanthone derivatives, for example, *Penicillium raistrickii* G. Sm., *Phomopsis* sp., *Actinoplanes* sp., *Ascodesmis sphaerospora* W. Obrist, and *Humicola* sp. The cultivation of the marine fungus *Arthrinium arundinis* (Corda) Dyko & B. Sutton isolated from inner tissue of the North Sea alga *Polysiphonia violacea* Grev. led to the isolation of several new secondary metabolites, where some of them exhibited significant cytotoxicity against human cancer cell lines. From the green alga *Ulva* sp., the endophytic and obligate marine fungus *Stagonosporopsis salicorniae* (Magnus) Died. was isolated. This fungus was found to produce, among others, the unusual tetramic acid-containing metabolites ascosalipyrrolidinones A and B. Ascosalipyrrolidinone A has antiplasmodial activity toward (1) strains K1 and NF 54 of *Plasmodium falciparum* Welch (causing malaria in humans) and (2) general antimicrobial activity. Penostatins, new cytotoxic agents toward leukemic cell lines, were reported from a *Penicillium* sp., inhabiting the marine environment and originally isolated from the marine alga *Enteromorpha intestinalis* (L.) Nees. Antimicroalgal substances, halymecins, were isolated from *Fusarium* and *Acremonium* spp. isolated from a marine alga *Halymenia dilatata* Zanardini.

Conclusion

Endophytic microbes are relatively common in all families of plants from polar to tropical regions. The endosymbionts have in several cases proven to play defensive roles in the ecology of host plants. Relatively little is known about the relationships between microbial endophytes and their hosts, especially with respect to chemical ecology. Nonetheless, some observations suggest the importance of the host as well as the ecosystem in influencing the general metabolism of endophytic microbes. Although endophytes are still a poorly investigated group of organisms, with the exception of the clavicipitaceous endophytes of grasses, many studies have proven that they are relatively rich and promising sources of bioactive and chemically novel compounds with a wide variety of potential uses in medicine, agriculture, and industry.

Acknowledgments

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Energy Transduction Processes☆

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Glossary

Aerobic respiration The energetically downhill electron transfer from a donor molecule or ion to oxygen, which is reduced to water, with concomitant coupled ion, usually proton, translocation and thus, generation of an electrochemical gradient.

Anaerobic respiration The energetically downhill electron transfer from a donor molecule or ion to a molecule other than oxygen, or to an ionic species, with concomitant coupled ion (proton) translocation and, thus, generation of an electrochemical gradient. The reduction products of the acceptors can either be released from the cell or, sometimes, used as further electron acceptors.

Antiport The transport of a molecule or ion up its chemical or electrochemical gradient with the concomitant movement in the opposite direction, but down its electrochemical gradient, of one or more protons or other e.g. sodium ions.

Bacteriorhodopsin A protein of the cytoplasmic membrane of the halophilic archaebacterium *Halobacterium salinarum* (formerly *halobium*) that has a covalently attached retinal molecule. Absorption of light by the latter pigment results in proton translocation across the membrane.

Chemiosmotic mechanism The transduction of energy between two forms via an ion electrochemical gradient (usually of protons, but sometimes of sodium) across a membrane. Examples of such membranes are the cytoplasmic membranes of bacteria, the inner mitochondrial membranes of eukaryotes, and the thylakoid membranes of plants.

Cytochrome Hemoprotein in which one or more hemes is alternately oxidized and reduced in an electron transfer process.

Electrochemical gradient The sum of the electrical gradient or membrane potential ($\Delta\psi$), and the ion concentration gradient across a membrane (the latter is often defined as ΔpH for protons).

Electron acceptor A low-molecular-weight inorganic or organic compound or an ion – which is reduced in the final step of an electron transfer process.

Electron donor A low-molecular-weight inorganic or organic compound or an ion – which is oxidized in the first step of an electron-transfer process.

Electron transport The transfer of electrons from a donor molecule (or ion) to an acceptor molecule (or ion) via a series of components (a respiratory chain), each capable of undergoing alternate oxidation and reduction. The electron transfer can either be energetically downhill, in which case it is often called respiration, or energetically uphill when it is called reversed electron transfer.

F_oF_1 ATP synthase The enzyme that converts the proton-motive force into the synthesis of ATP. Protons (or more rarely sodium ions) flow through the membrane sector of the enzyme, known as F_o, and thereby cause conformational changes, with concomitant ATP synthesis, in the globular F_1 part of the molecule. Under some circumstances, the enzyme can generate the proton-motive force at the expense of ATP hydrolysis.

Oxidase The hemoprotein that binds and reduces oxygen, generally to water. The name oxygen reductase would better reflect the function.

Oxidative phosphorylation ATP synthesis coupled to a proton or sodium electrochemical gradient, generated by electron transport, across an energy-transducing membrane.

P/O (P/2e) ratio The number of molecules of ATP synthesized per pair of electrons reaching oxygen, or more generally any electron acceptor.

Photophosphorylation ATP synthesis coupled to a proton or sodium electrochemical gradient generated by light-driven electron transport, a process that is often cyclic in bacteria.

Proton-motive force The proton electrochemical gradient (*q.v.*) across an energy-transducing membrane in units of volts or millivolts.

Quinone A lipid-soluble hydrogen (i.e., proton plus electron) carrier that mediates electron transfer between respiratory chain components. The principal types are ubiquinone (under aerobic or anaerobic conditions), menaquinone (usually under anaerobic conditions), and plastoquinone (in plants, but not photosynthetic bacteria). In each case, the reduced form is a quinol.

Redox potential A measure of the thermodynamic tendency of an ion or molecule to accept or donate one or more electrons. By convention, the more negative the redox potential the greater is the propensity for donating electrons and vice versa.

☆*Change History:* November 2014. SJ Ferguson updated the text and further readings to this entire article, added new section on Electron bifurcation, and updated figure legends.

This article is an update of S.J. Ferguson,Energy Transduction Processes, *Encyclopedia of Microbiology (3rd Edn)*, edited by Moselio Schaechter, Academic Press, 2009, pp. 123-131.

Respiration The sum of electron transfer reactions resulting in reduction of oxygen (aerobically) or other electron acceptor (anaerobically) and generation of proton-motive force.
Respiratory chain Set of electron-transfer components, which may be arranged in a linear or branched fashion, that mediate electron transfer from a donor to an acceptor in aerobic or anaerobic respiration.
Reversed electron transport The transfer of electrons energetically uphill toward the components of an electron-transfer chain that have more negative redox potentials. Such electron transfer can be regarded as the opposite of respiration, and is driven by the proton-motive force.
Symport The transport of a molecule up its chemical or electrochemical gradient with the concomitant movement, in the same direction but down its electrochemical gradient, of one or more protons or sodium ions.
Uniport The transport of an ionic species in direct response to the membrane potential across a membrane.

Defining Statement

Many microbes obtain energy for growth from either capture of light or oxidation of suitable reductants. This process involves transfer of electrons between proteins of the cytoplasmic membrane (bacteria and archaea) or the inner mitochondrial membrane (eukaryotes). The unifying feature is that the energy is conserved as a proton (sometimes sodium) electrochemical gradient across the membrane. This gradient then drives uphill reactions, notably synthesis of ATP. There are examples of electron transport-independent generation of the gradient. There are also examples of a process known as electron bifurcation in which some electrons flow energetically downhill so as to drive other electrons uphill, in effect a disproportionation reaction.

Introduction

The inner mitochondrial membrane of the eukaryote, the plant thylakoid and the cytoplasmic membrane of the prokaryote (bacteria and archaea) are the key sites where energy available from processes such as the oxidation of nutrients, or from light, is converted into other forms of stored energy that the cell needs. Most prominent among these other forms is ATP and thus these membranes are concerned with oxidative phosphorylation (or photophosphorylation). Energy-transducing membranes share many common components, but most importantly they operate according to the same fundamental chemiosmotic principle. This states that energetically downhill reactions that are catalyzed by the components of these membranes are coupled to the translocation of protons (or more rarely sodium ions) across the membranes. The direction of the movement is outward from the matrix of the mitochondria or the cytoplasm of the bacteria or archaea. The consequence of this translocation is the establishment of a proton electrochemical gradient. This means that the matrix of the mitochondria, or the cytoplasm of the bacteria, tends to become both relatively negatively charged (thus, called the N side) and alkaline relative to the other side of the membranes, the intermembrane space in the mitochondria and the periplasm in Gram-negative bacteria (and equivalent zone in Gram-positive bacteria and archaea), which is thus called the P side (Figure 1). This electrochemical gradient is under most circumstances dominated by the charge term, which means that there is often a substantial membrane potential across the membranes, frequently

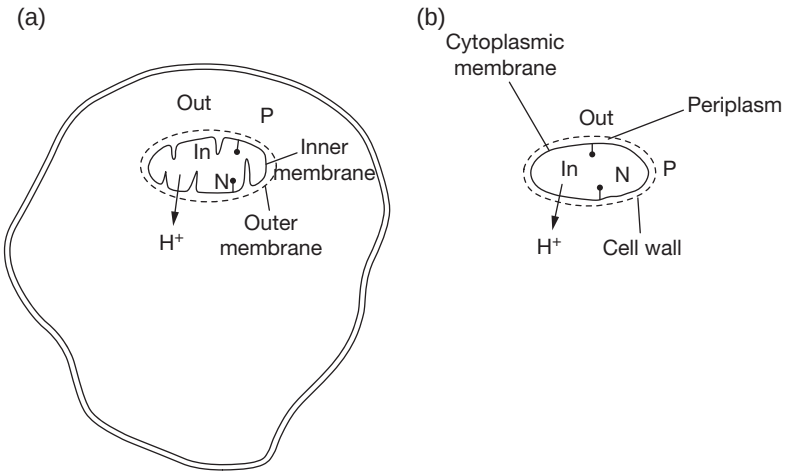

Figure 1 (a) Idealized mitochondrion in a eukaryotic cell and (b) Gram-negative bacterium showing the direction of proton translocation linked to an exergonic (energetically downhill) reaction. P represents a relatively positive aqueous phase (i.e., outside the inner mitochondrial membrane or the bacterial cytoplasmic membrane) and N a relatively negative aqueous phase (i.e., inside the mitochondrion (the matrix) or the cytoplasm of a bacterium). represents the ATP synthase enzyme.

estimated to be on the order of 150–200 mV. Under most circumstances, the pH gradient generated by the proton translocation is small, 0.5 unit is an approximate average value. The membrane potential is added to the pH gradient to give the total gradient, which is usually called the proton-motive force if given in millivolts. The conversion factor is such that 0.5 pH unit is approximately equivalent to 30 mV. Strictly speaking, the expression of the gradient as an electrochemical potential requires units of kJ mol^{-1} to be used; in practice, this is rarely done, sometimes causing confusion. I use the term proton-motive force in this article.

Mitochondrial Energetics

The best-known machinery for generating the proton-motive force is the mitochondrial respiratory chain. The standard mitochondrial respiratory chain is found, at least under some growth conditions, in many, but not all, eukaryotic microbes. Diversity is illustrated by yeasts with some species having the classic Complex I (Figure 2) whereas other species have an alternative form of NADH dehydrogenase. The key point is that as a pair of electrons traverses the chain from NADH to oxygen, there are three segments (formerly called sites, but the term is inappropriate because it implies equivalence, and relates to a very old idea that ATP is made at three sites within the electron-transport chain) where protons can be translocated across the membrane. The first and last of these segments effectively move four protons per two electrons, while the middle segment effectively moves only two (Figure 2), but note that this description is an oversimplification. For more details on protein translocation, consult the book by Nicholls and Ferguson mentioned in the 'Further Reading.' Overall, ten protons are moved per two electrons moving along the chain from NADH to O_2. Electrons may enter the chain such that they miss the first proton-translocating segment of the chain. Succinate and the fatty-acyl CoA intermediates generated during fatty-acid oxidation are the most prominent examples of such electron sources which do not involve the NADH-ubiquinone oxidoreductase (Complex I). Each of these sources delivers electrons to ubiquinone by different enzymes, succinate dehydrogenase or the electron transfer flavoprotein (ETF) in combination with the electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-UQ), respectively. In these cases, six protons are translocated per two electrons. The entry of electrons at the third segment, rare in mitochondria, gives a translocation stoichiometry of four.

The proton-motive force generated can then be used to drive various uphill reactions. Most prominent is ATP synthesis. This is achieved by protons flowing back across the membranes and through the ATP synthase enzyme, often called FoF_1 ATP synthase. Note that in archaea a related enzyme but with some quite distinct features is present and called by analogy the AoA_1 enzyme. There is increasing insight into the mechanism of the ATP synthase enzyme; it functions as a rotary motor in which the flow of protons through the F_o is coupled to rotation and structural changes in the F_1 part of the molecule, events that are linked to ATP synthesis. The number of protons that must pass through the ATP synthase to make one ATP molecule is widely accepted to depend on the number of a certain type of subunit, the c subunit, in the enzyme. An approximate value (see later for further discussion of this point) adopted here for illustration, even though it is not the reality, is three. On the basis of "what goes one way across the membrane must come back the other," it might therefore be thought that the stoichiometry of ATP production per pair of electrons (called the P/O or P/2e ratio) flowing from NADH to oxygen would be 10/3 (i.e., 3.3) for NADH and 6/3 (i.e., 2) for succinate. However, matters are a little more complicated. The combined process of entry of ADP and P_i (phosphate) into mitochondria and the export of ATP to the cytoplasm involves the movement of one proton into the matrix (Figure 3). Thus for each ATP made, the predicted stoichiometry is $10/(3+1)=2.5$ for NADH and $6/(3+1)=1.5$ for succinate. These values differ from the classic textbook values of 3 and 2, respectively, but they are now generally accepted. It is intuitive to expect that a cell should seek to maximize the yield of ATP, that is, the P/O ratio. However, such an intuition does not always conform to reality. Thus, many eukaryotic microorganisms possess the 'alternative oxidase,' which is best known in plant mitochondria and allows ubiquinol to be oxidized by oxygen with no associated proton translocation. The energy is instead dissipated as heat. Similarly, *Escherichia coli* lacks Complex

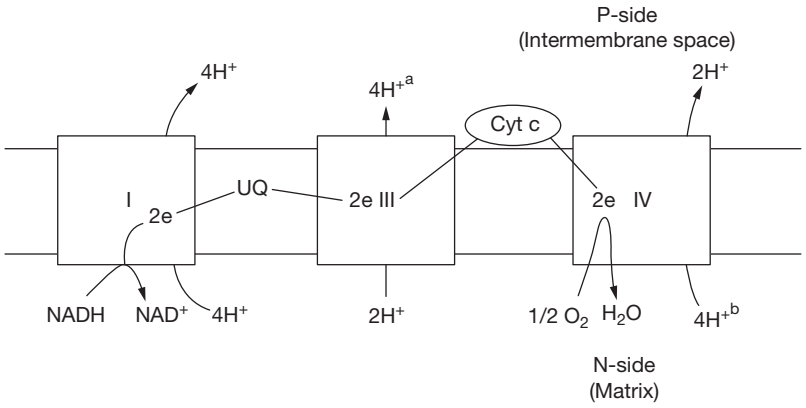

Figure 2 Considerably simplified representation of the proton-translocation stoichiometry, per two electrons, of the mitochondrial respiratory chain. I indicates Complex I (otherwise known as NADH dehydrogenase); III, Complex III (otherwise known as the ubiquinol-cytochrome *c* oxidoreductase or cytochrome bc_1 complex); IV, Complex IV (otherwise known as cytochrome *c* oxidase or cytochrome aa_3 oxidase); P, positive aqueous phase; and N, negative aqueous phase. [a]Simplifying has caused some unavoidable inaccuracies (see Nicholls and Ferguson (2013) for accurate detailed description). [b]Note that 2 of the $4H^+$ are used to give H_2O.

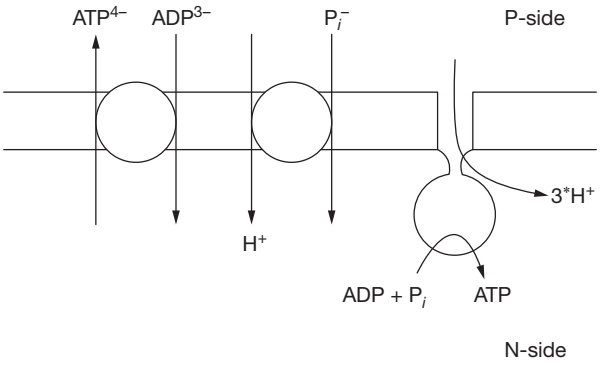

Figure 3 Charge movement, associated with ATP synthesis and translocation of adenine nucleotides and phosphate across the inner mitochondrial membrane. The stoichiometry of proton translocation through the ATP synthase has in recently commonly been taken to be 3, but this is rarely if ever the actual value which can be non-integral (see text for more information on this point). The translocated protons are not believed to pass through the active site of the ATP synthase enzyme. Note that the adenine nucleotide exchange moves one positive charge into the matrix per nucleotide exchanged and the operation of the phosphate transporter effectively moves the chemical part of the proton (but not the charge) into the matrix. Thus, in combination, the two transporters move one positive charge into the mitochondrion per ATP synthesized and returned to the P phase. Note that these transporters do not operate in bacterial ATP synthesis. This number would be 3 if there are 12c subunits. In practice current knowledge indicates 8 or 10c subunits in mitochondrial ATP synthases.

III and thus fewer protons are translocated as a pair of electrons is transferred from quinol to oxygen compared with an organism in which Complex III is present. There is no general explanation as to why energy is dissipated as heat rather than conserved as a proton-motive force, but the phenomenon implies that under certain circumstances organisms are not 'energy limited' and thus have another priority, for example oxidizing substrates so that the carbon skeletons can be converted into molecules required for the growth of cells. For example, butyrate is more reduced than the average carbon molecule in the cell; thus growth on butyrate often requires dissipation of excess reductant as heat.

The number of protons that must pass through the ATP synthase for a molecule of ATP to be made is uncertain, but is currently thought to vary with the structure of the ATP synthase enzyme in a given organism. It is generally accepted that the mechanism of this enzyme involves the rotation of a circular assembly of one type of subunit (the c subunit), belonging to the F_o part of the enzyme. A 360° rotation of this assembly drives a similar rotation of a subunit of the F_1 part of the enzyme, which thus interacts sequentially with the three catalytic subunits driving synthesis of three molecules of ATP. The 360° rotation of the assembly in the F_o part of the enzyme is believed to be driven by the sequential passage of the number of protons that equals the number of c subunits in the assembly. Thus ten subunits, as currently thought to be present in the microbial mitochondrial F_o, imply a proton to ATP ratio of $10/3 = 3.3$ but 8 in higher eukaryotes and thus $H^+/ATP = 2.66$. In prokaryotes that the number of these subunits varies from 10 to 15, depending on the organism, with variation between 13 and 15 even being seen in different species of cyanobacteria; the proton per ATP ration can thus be as high as 5. In some species such as *Propionegenium modestum* (see later) analogous movement of Na^+ via the circular assembly of c subunits occurs to drive ATP synthesis.

It has been generally thought that eukaryotes are only capable of aerobic respiration. However, there is now evidence of a form of mitochondrial anaerobic respiration in which nitrate is reduced to nitrous oxide (more typically a prokaryotic characteristic, see the following), and of a novel type of mitochondrion in the ciliate protist *Nyctotherus ovalis* that reduces protons to hydrogen. In both these examples, electrons are derived from NADH.

Bacterial Energetics

Many species of bacteria and archaea generate a proton-motive force by a respiratory chain similar to that found in mitochondria. However, there are many types of electron donors and acceptors that can be used by bacteria (eukaryotes are restricted to aerobic oxidative breakdown of reduced carbon compounds), and various forms of anaerobic respiration are widespread. A further general difference between bacteria and microbial eukaryotes is that in the former, the proton-motive force can drive a wider range of functions and be generated in more diverse ways than in the latter. Thus, functions alongside ATP synthesis (for which the enzyme is very similar to that found in mitochondria), such as driving of many active transport processes and the motion of the flagella, are important processes that depend on the proton-motive force in many organisms (Figure 4).

A common mode of active transport is known as the symport; the classic example of this is the lactose-proton symport, coded for by the *lacY gene* of the *lac* operon of *E. coli*, and for which a molecular structure is now available. In this case, a transmembrane protein translocates together a proton down its electrochemical gradient and a lactose molecule up its concentration gradient (Figure 5), but the exact mechanism is presently unknown. There are cases of analogous transporters known where Na^+ is the translocated ion. A second type of transport system is the antiport (Figure 5). Here the movement via a protein of the proton down its electrochemical gradient is obligatorily linked to the movement of another species, typically an ion, in the opposite direction and

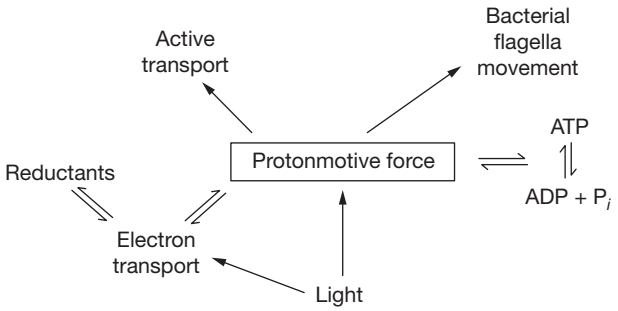

Figure 4 The central role of the proton-motive force in linking diverse reactions. Note that the direct generation of proton-motive force from light is unusual, but is exemplified by the proton-pumping bacteriorhodospin protein found in some archaea (halobacteria); a related protein, proteorhodopsin, is found in some bacteria . Normally, light drives electron-transport processes, which in turn generate the proton-motive force.

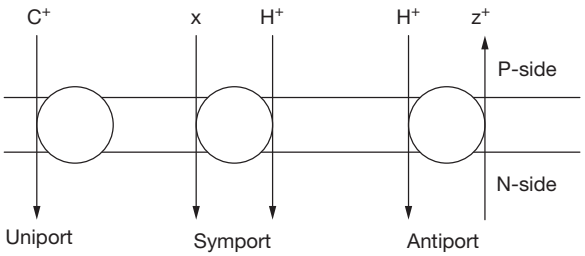

Figure 5 The three common modes of substrate transport across the bacterial cytoplasmic membrane.

up its electrochemical gradient. Sodium/proton exchange provides an example. The third type, uniport, is the case where an ion moves in direct response to the membrane potential and is rarer than the other two examples in the prokaryotic world.

It is important to appreciate that not all transport processes across the bacterial cytoplasmic membrane are directly driven by the proton-motive force. Some transport reactions are driven directly by ATP. Notable among such systems are ABC (ATP-binding cassette) transporters.

A more subtle aspect of prokaryotic energetics is that in some species of bacteria the proton-motive force must drive reversed electron transport under some circumstances (see later).

A common misconception is that the proton-motive force can be divided between different functions (e.g., 100 mV for ATP synthesis and 50 mV for flagella motion). This notion is incorrect because the proton-motive force across a membrane has a single value at any one time and the magnitude of this force acts simultaneously on all energy-transducing units, be they ATP synthases, active transporters, or flagella.

Principles of Respiratory Electron-Transport Linked ATP Synthesis in Bacteria

In principle, energy transduction on the cytoplasmic membrane is possible if any downhill reaction is coupled to proton translocation. The most familiar examples are probably those that also occur in mitochondria, for example, electron transfer from NADH to oxygen or from succinate to oxygen. In these cases, the electrons pass through a sizeable redox drop (Table 1). In contrast to mitochondria, various species of bacteria can use a wide variety of electron donors and acceptors. The fundamental principle is that the redox drop should be sufficient for the electron transfer to be coupled to the translocation of protons across the cytoplasmic membrane. Table 1 shows that such sizeable drops are associated with the aerobic oxidation of hydrogen, sulfide, carbon monoxide, and methanol, to cite just a few electron donors. Anaerobic respiration is also common with many suitable pairings of reductants and oxidants (e.g., Table 1). Thus, NADH can be oxidized by nitrate, nitrite, nitric oxide, or nitrous oxide. The flow of electrons to these acceptors, each of which (other than nitrate) is generated by the reduction of the preceding ion or molecule, is the process known as denitrification. In *E. coli* under anaerobic conditions, formate is frequently an electron donor, and nitrate and nitrite are the acceptors, with the latter being reduced to ammonia (Table 1) rather than to nitric oxide, as occurs in denitrifying bacteria. A wide variety of electron-transport components, including many different types of cytochrome, are involved in catalyzing these reactions. The mechanisms whereby electron transport is linked to the generation of the proton-motive force are frequently complex. However, nitrate respiration (Figure 6) provides an example of one of the simplest mechanisms that corresponds to Peter Mitchell's original redox loop mechanism feature of his chemiosmotic mechanism.

An important point is that the consideration of the energy drop between the donor and the acceptor (Table 1) is only a guide as to whether proton translocation, and thus ATP synthesis, can occur and, if so, with what stoichiometry. Thus while many bacterial

Table 1 Approximate standard redox potentials[a] of some electron-donor and electron-acceptor couples used in respiratory processes

Couple	*E°' (mV)*
N_2O/N_2	+1360
NO/N_2O	+1180
O_2/H_2O	+820
NO_3^-/NO_2^-	+430
NO_2^-/NH_4^+	+360
NO_2^-/NO	+350
Fumarate/succinate	−30
Methanol/formaldehyde	−180
$NAD^{+}/NADH$	−320
CO_2/formate	−430
CO_2/CO	−540

Conditions experienced by cells may vary significantly from these and thus the actual redox potentials of the couples should be calculated from the Nernst equation and may differ substantially from those in this table.

[a]Redox potential refers to the standard state (1 mol l^{-1} concentrations for solutes and 1 atm pressure for gasses).

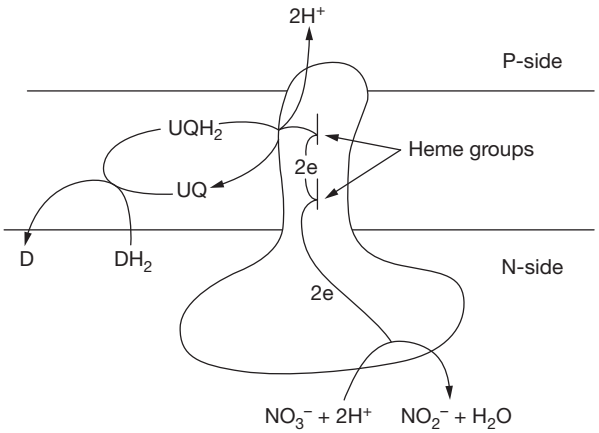

Figure 6 A simple mechanism for generating the proton-motive force, the bacterial nitrate reductase system. Oxidation of ubiquniol (UQH_2) to ubiquinone (UQ) on one side of the membrane is accompanied by release of protons to the P side and translocation of the electrons toward the N side, where they combine with protons and nitrate to produce nitrite. Overall, the process effectively translocates two protons per two electrons across the membrane. The mitochondrial electron-transport chain (see Figure 2) involves more complex mechanisms for proton translocation. DH_2 is an unspecified donor to the ubiquinone, and D is the product of oxidizing DH_2. The crystal structure of nitrate reductase fully confirms the scheme shown here and, in combination with the structure of formate dehydrogenase, shows how a complete redox loop mechanism links oxidation of formate by nitrite to the generation of a proton-motive force (see Further Reading).

species can form a respiratory chain with considerable similarity to that found in mitochondria, others vary from this pattern. Notably all strains of *E. coli* always lack the cytochrome bc_1 complex. Under some growth conditions, it uses cytochrome *bo* as the terminal oxidase and under others, cytochrome *bd*. The consequence is that when the former proton-pumping oxidase is operating, eight protons are translocated per pair of electrons flowing from NADH to oxygen, while with the latter oxidase, which does not pump protons but in common with the *bo* oxidase does effectively move two protons per two electrons as part of the mechanism of reducing oxygen to water, the stoichiometry would be only six. As explained earlier, the corresponding number for mitochondria is ten. This example illustrates the important point that it is not just the energy drop between a donor and an acceptor that is important, but also the details of the components (or molecular machinery) in between. Another example is methanol to oxygen. Periplasmic oxidation of methanol feeds electrons into the electron-transport chain close to the terminal oxidase, yet energetic considerations alone would indicate that electrons could span more proton-translocating sites just as they do when succinate is the electron donor (compare the redox potentials for fumarate-succinate and methanol-formaldehyde, Table 1). A final example to consider is the case in which both the electron donor and the acceptor are in the periplasm and they are connected purely by periplasmic components. In such a case, which applies to methanol (as donor) and nitrous oxide (N_2O as acceptor), the electrons do not pass through any proton-translocating complex. Thus, no proton translocation would occur no matter what the redox drop between the two components.

The electron transport systems can be completely distinct from those in *E. coli*. For example, the cytoplasmic membranes of sulfate-reducing bacteria catalyze electron transfer from hydrogen to APS reductase and sulfite reductase using a quite distinct set of cytochromes.

It is not necessary for electrons to flow over such a large energy drop as they do when they pass from NADH to oxygen (Table 1) to generate a proton-motive force. Thus, if the driving force associated with a reaction is very small, it might still be energetically possible for the passage of two electrons from a donor to an acceptor to cause the translocation of just one proton. If three protons are required for the synthesis of ATP, then the ATP yield would be 0.166 per electron flowing from the electron donor to acceptor. This seemingly bizarre stoichiometry is not only energetically possible but also mechanistically possible because the chemiosmotic principle involves the delocalized proton-motive force that is generated by all the enzymes of the membrane and also consumed by all of them. There is no case known that matches this extreme; nevertheless, there may well be organisms yet to be discovered that have such low stoichiometries of ATP synthesis.

One example of the lowest known stoichiometries of ATP synthesis per pair of electrons reaching the terminal electron acceptor (oxygen) occurs in *Nitrobacter*. Table 1 shows that the redox drop is small between nitrite and oxygen. This organism also illustrates the versatility and subtlety of the chemiosmotic mode of energy transduction. *Nitrobacter* species oxidize nitrite to nitrate at the expense of the reduction of oxygen to water in order to sustain growth. The energy available as a pair of electrons flows from nitrite to oxygen is sufficient to translocate two protons (a more detailed consideration of how this is done is outside the scope of this article). This means, recalling the current consensus that three protons are needed for the synthesis of one ATP molecule, that the ATP yield stoichiometry would be 0.66/2e. Nitrobacter also illustrates another important facet of energy transduction in the bacterial world. The organism is chemolithotrophic, which means that oxidation of nitrite provides not only ATP but also reductant (NADPH), which is required for reducing CO_2 into cellular material. Energetic considerations show that nitrite cannot reduce NADP directly. What happens in the cell is that a minority of the electrons originating from nitrite are driven backward up the electron-transfer system to reduce NAD(P) to NAD(P)H. This is achieved by the inward movement of protons reversing the usual direction of proton movement (Figure 7). This reversed electron-transport process is an important phenomenon in a variety of bacteria, especially those growing in the chemolithotrophic mode.

Most studies of electron transport-linked ion translocation have been carried out with species of bacteria. However, the same fundamental process also occurs in archaea, although with some novel features that reflect some of the extreme growth modes tolerated by these organisms. For example, a key step in methane formation by methanogenic bacteria is electron transfer from hydrogen or the other reductant to a small molecule that contains a disulfide bond. The latter is reduced to two sulfides and the overall process is coupled to the translocation of protons across the cytoplasmic membrane. The proton-motive force thus set up can be used to drive ATP synthesis.

Generation of the Ion Electrochemical Gradient Other than by Electron Transport

ATP Hydrolysis

Organisms that are incapable of any form of respiration still require an ion electrochemical gradient across the cytoplasmic membrane for purposes such as nutrient uptake. One way in which this requirement can be met is by using some of the ATP synthesized by fermentation for ATP hydrolysis by the F_oF_1 ATPase. This means that this enzyme works in the reverse of its usual direction and pumps protons out of the cell. Thus there are many organisms that can prosper in the absence of any electron-transport process, either as an option or as an obligatory aspect of their growth physiology.

Bacteriorhodopsin and Proteorhodopsin

A specialized form of light-driven generation of proton-motive force, and hence of ATP, occurs in halobacteria; these organisms are archaea. The key protein is bacteriorhodopsin, which is a transmembrane protein with seven α-helices, that has a covalently bound

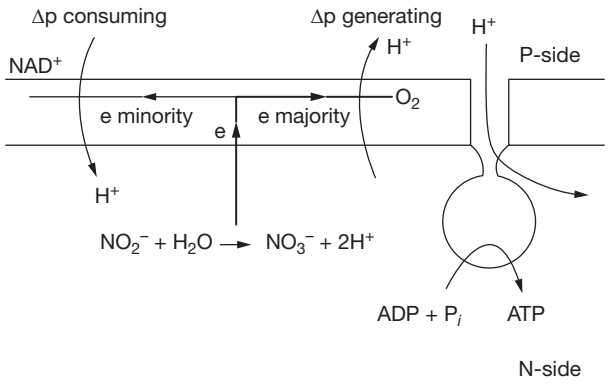

Figure 7 Reversed electron transport illustrated by the example of nitrobacter. The majority of eletrons is derived from nitrite flow energetically downhill to oxygen via a cytochrome oxidase, which generates a proton-motive force. A minority of electrons is driven energetically uphill by the proton-motive force so as to reduce NAD^+ to NADH. Note that in this diagram no proton stoichiometry values are implied.

retinal. The absorption of light by this pigment initiates a complex photocycle that is linked to the translocation of one proton across the cytoplasmic membrane for each quantum absorbed. Bacteriorhodopsin is one of a family of related molecules. Another, halorhodospin, is structurally very similar and yet catalyzes the inward movement of chloride ions driven by light. In certain groups of bacteria a protein called proteorhodopsin performs an analogous function to bacteriorhodopsin.

Methyl Transferase

One step of energy transduction in methanogenic bacteria involves an electron-transfer process (see earlier). Another important process in methanogens is the transfer of a methyl group from a pterin to a thiol compound. This exergonic (energetically downhill) reaction is coupled to ion, in this case sodium, translocation across the cytoplasmic membrane.

Decarboxylation Linked to Ion Translocation

In the bacterial world, the electrochemical gradients can be generated by diverse processes other than electron transport or ATP hydrolysis. For example, *Propionegenium modestum* grows by converting succinate into propionate and carbon dioxide. One of the steps in this conversion is decarboxylation of methyl-malonyl coenzyme A (CoA) into propionyl CoA. This reaction is catalyzed by a membrane-bound enzyme that pumps sodium out of the cells, thus setting up a sodium electrochemical gradient (or sodium-motive force). This gradient in turn drives the synthesis of ATP as a consequence of sodium ions reentering the cells through a sodium-translocating ATP synthase enzyme. Apart from illustrating that sodium, instead of proton circuits, can be used for energy transduction in association with the bacterial cytoplasmic membrane, this organism also illustrates that the stoichiometry of ATP synthesis can be less than one per CO_2 formed. It is believed that each decarboxylation event is associated with the translocation of two sodium ions and the synthesis of ATP with three. Thus, nonintegral stoichiometry is consistent with the energetics of decarboxylation and ATP synthesis. This is an important paradigm to appreciate; the underpinning growth reaction for an organism does not have to be capable of supporting the synthesis of one or more integral numbers of ATP molecules.

Metabolite Ion-Exchange Mechanisms

Another example of the generation of a proton-motive force is ion exchange across the membrane. For example, in fermenting bacteria there is evidence that under some conditions an end-product of metabolism, lactic acid, leaves the cell together (i.e., in symport) with more than one proton, resulting in the generation of a proton-motive force (Figure 8). A second example is provided by *Oxalobacter formigenes*, in which the entry of the bivalent anion oxalate is in exchange for the exit of the monovalent formate ion generated by decarboxylation of the oxalate, leading to the net generation of membrane potential (Figure 8). This seems to be the principal mode of generating membrane potential in this organism.

Photosynthetic Electron Transport

Prokaryotic photosynthesis involves a cyclic electron-transport process in which a single photosystem captures light energy and uses it to drive electrons around the cycle (Figure 9). The consequence of this cyclic electron flow is the generation of the proton-motive force. There are two types of photosystem found in prokaryotes. One is related to the water-splitting photosystem that is found is plants; typically this bacterial photosystem is found in organisms such as *Rhodobacter sphaeroides*. The second type of photosystem is closely related to the second photosystem of plants, the one that is concerned with the generation of NADPH. *Heliobacter* is an example of an organism carrying this type of center. Plants have both these photosystems, arranged to operate in series. The same is true for the prokaryotic cyanobacteria and the eukaryotic algae.

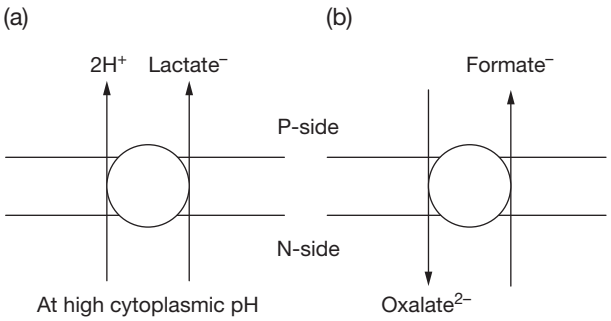

Figure 8 Two examples of generation of proton-motive force by end-product extrusion from fermenting bacteria.

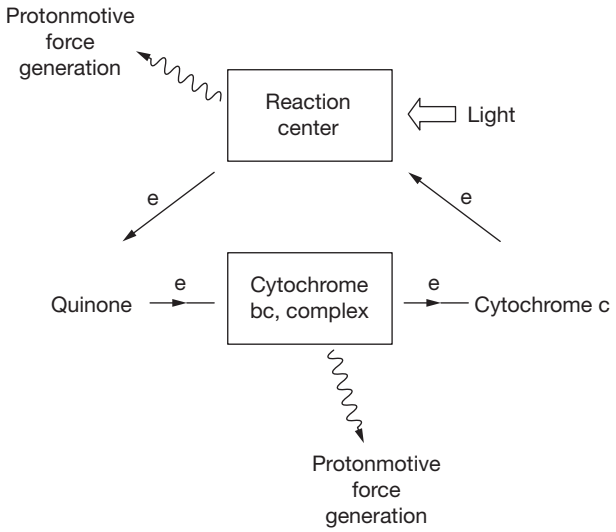

Figure 9 An oversimplified outline of the cyclic electron-transport process of photosynthetic bacteria. There are two types of reaction centers, depending on the organism. The molecular composition of the system depends on the organism. The two components contribute to the generation of the proton-motive force.

Alkaliphiles

An interesting unresolved problem relates to energy transduction in the alkaliphilic bacteria. The problem is straightforward. These organisms can grow in an environment with a pH as high as 11 or 12. A cytoplasmic pH even as high as 9 means that the pH gradient could be as much as 3 units (equivalent to 180 mV), the wrong way around in the context of the chemiosmotic mechanism. The membrane potential always seems to be larger than 180 mV, but the total protonmotive force can be very low (e.g., around 50 mV). For some organisms that use a conventional proton-translocating respiratory chain and ATP synthase, it is not understood how they survive energetically. In other organisms, there is evidence for the role of a sodium-motive force. This would sidestep the problem of the adverse proton concentration gradient.

Electron Bifurcation

Not all energetically 'uphill' reactions in bacteria are driven by the ion electrochemical gradient, important as this is. A process known as electron bifurcation has been recognized in recent years and may prove to be more important in microbial energetics than realized at the time of writing. An example is that of driving electrons uphill in *Rhizobia* from NADH to ferredoxin which in turn can act as the reductant for nitrogen fixation. There is a membrane bound system that can take four electrons from two molecules of NADH and send two on an energetically downhill path to an electron acceptor, in this case oxygen at low concentration. The energy so released can be used to drive a second pair of electrons energetically uphill to reduce two molecules of ferredoxin. The exact enzymology of this process is not fully understood but in energetic terms in it can be thought of as similar to a disproportionation reaction in chemistry. Another key role for electron bifurcation is in methanogenesis in organisms lacking cytochromes, whereas in organisms with cytochromes the required uphill reaction is driven by the ion motive gradient. Similarly transfer of electrons from NADH to ferredoxin in some photosynthetic bacteria is driven not by electron bifurcation but by the ion electrochemical gradient using a membrane protein known as Rnf.

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Entamoeba Histolytica: Biology and Host Immunity

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Abbreviations

ALA	Amebic liver abscesses
CLDN	Claudins
CLS	Cyst-like structures
COX	Cyclooxygenase
CP	Cysteine proteinase
CRD	Carbohydrate recognition domain
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
Eh	*E. histolytica*
EhC2PK	*E. histolytica* C2-domain-containing protein kinase
EP4	E-proastanoid-4
HSP	Heat shock protein
IL	Interleukin
LPPG	Lipophosphopeptidoglycan
MAC	Membrane attack complex
MCP	Monocyte chemotactic protein
MIF	Macrophage migration inhibitory factor
MLIF	Monocyte locomotion inhibitory factor
OCLN	Occludins
PGE_2	prostaglandin E_2
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
ROM1	Rhomboid protease
ROS	Reactive oxygen species
SAP	Soluble amebic protein
SREHP	Serine rich *Eh* protein
STIRP	Serine, threonine and isoleucine rich protein
TMK	Trans-membrane kinases
TNF-α	Tumor necrosis factor-α
ZO	Zona occludins

Introduction

Entamoeba histolytica is the causative agent of amebiasis, a disease common in developing countries around the world. It causes 100 million cases of disease per year, and was responsible for over 11,000 deaths in 2013 alone, making *E. histolytica* the fourth highest infectious parasite, and the only pathogenic intestinal amoebae. Interestingly, many people come in contact with *E. histolytica* but never develop any symptoms; only 10% of people carrying the parasite develop amebiasis or other symptoms, while the other 90% remain asymptomatic carriers. In both cases, an infectious cyst is ingested from stool with an infectious dose of over 1000 cysts. The nucleus of the cysts then divides through binary fission into eight uninucleated trophozoites in the terminal ileum. The trophozoites then migrate to and colonize the colon where undigested host nutrients and the host microbiota serve as a food source (Fig. 1). In asymptomatic cases, *E. histolytica* remains in the lumen and does not contact the host epithelial cells. The trophozoites re-encyst in the descending colon, recreating the infectious cyst that is excreted in stool and ready to infect a new host. A carrier of the parasite may pass up to 45 million cysts in stool daily. In case of infection, the multitude of parasite virulence factors are able to bypass the host mucosal layer (Table 1), leading to *E. histolytica* contacting the host epithelial cells and causing serious infection such as diarrhea and dysentery, and in some cases extra-intestinal symptoms such as liver granulomas. With such a high rate of infection worldwide, much research has gone into the pathogenesis of *E. histolytica* in the past few decades.

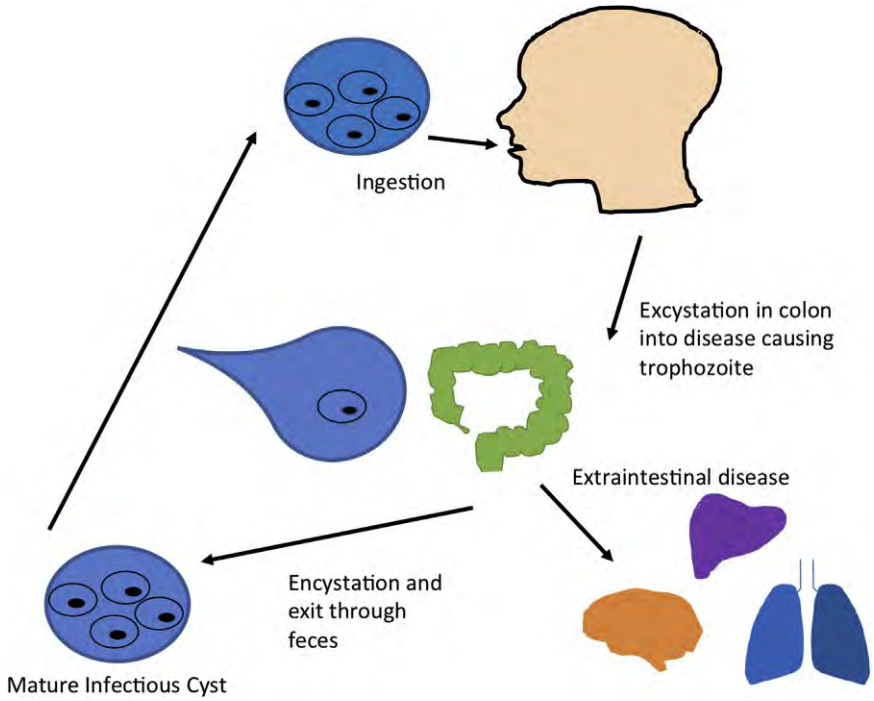

Fig. 1 Life cycle of *E. histolytica*. Mature cysts are ingested by the host, and travel through the gastrointestinal system. In the terminal ileum, the cyst undergoes excystation and gives rise to 4 daughter trophozoites, that migrate to the colon and now capable of causing disease symptoms. The trophozoites may remain in the colon, or may cause extraintestinal disease in the liver, lungs or brain. The trophozoites in the colon eventually undergo encystation and the infectious cyst excreted in stool is now able to infect a new host as the cycle repeats itself.

Table 1 *E. histolytica* virulence factors

Virulence factor	*Function*
Gal/GalNAc Lectins	binds to Gal/GalNAc of mucus layer to allow adherence and colonization
	binds to immune cells, facilitating phagocytosis
	activate macrophage
	induces host pro-inflammatory response
	antigenic similarity to host CD59 inhibits complement MAC attack
	stimulate macrophages to produce TNF-α
	binds to and activates NLRP3 inflammasome in macrophages
	binds to complement components C8 and C9 to block the MAC formation
Cysteine proteinases	degrade C-terminal of MUC2 to degrade mucus layer
	bind to $\alpha v\beta 3$ integrin on goblet cells to induce mucus hypersecretion
	cleave IgA and IgG
	degrade cathelicidins
	cleave C3 activating alternative complement pathway
	degrade anaphylatoxins C3a and C5a
	degrade tight junction structures to increase paracellular permeability
	convert pro-IL-1β from damaged intestinal cells to active IL-1β
	role in liver abscess development
Amoebapores	creates membrane pores in host cell leading to necrosis and apoptosis
	role in liver abscess development
PGE_2	disrupt tight junctions by altering claudin-4
	disrupt tight junctions by binding to host EP_4 receptors, inducing a pro-inflammatory response
	induce neutrophil chemokine IL-8
	inhibits Th1 cytokine release, oxidative burst and NO synthesis

History

Since bloody and mucus filled diarrhea are symptoms not unique to amebiasis, early reports of amebic infection are hard to decipher with complete certainty. Those being said, as early as 1000 BCE reports of bloody, mucose diarrhea in the Brigu-samhita, a Sanskrit document, have been located. Later, in 600 BCE, both Assyrian and Babylonian texts describe counts of bloody stool. Similarly, liver and perianal abscesses consistent with amebiasis were described in *Epidemics* and *Aphorisms* in the *Corpus Hippocratorum*. Counts of bloody diarrhea and liver abscesses continued until 16th century when amebiasis was recorded in Europe and Asia among other places, until Europeans colonized the New World and seemingly spread the disease. Intestinal and hepatic amebiasis became even more spread as world trade became more prevalent, making spread of the parasite even easier. Even though these accounts are recognized as amebiasis, it was the book "*Researches into the Causes, Nature and Treatment of the More Prevalent Diseases of India and of Warm Climates Generally*" by James Annersley in 1855 that first distinctly described both intestinal and hepatic amebic infection. It was not until 1857 that the English physician William Budd described the connection between the intestinal disease and liver abscesses. Shortly after in Russia in 1873, as the field of microscopy and germ theory advanced, Friedrich Lösch discovered not only *E. histolytica* itself, but also established the relationship between the parasite and disease from working with a farmer with ongoing dysentery and periods of bloody stool. Descriptions of dysentery and amebic cysts continued to surface over the coming years, solidifying the presence of an infectious dysentery-causing ameba. At the end of the 19th century, William Thomas Councilman and Henry Lafleur definitively described the pathology of amebiasis and first used the terminology "amebic dysentery" and "amebic abscess", terminology that is still used today in describing amebiasis. In 1925, Emile Brunt suggested that there must be two distinct yet morphologically identical *Entamoeba* species, as not every patient with *Entamoeba* displayed symptoms of amebiasis. In the 1990s, this was proven to be true; pathogenic *E. histolytica* was distinguished from the non-infectious but morphologically identical *E. dispar*.

Morphology and Characteristics

E. histolytica exists as either the infectious cyst or the disease causing trophozoite. The cyst is round in shape and 10–15 μm in diameter. Each cyst contains 4 nuclei, chromatoid bodies and glycogen, and is surrounded by a refractile chitinous wall. The glycogen forms a characteristic vacuole that becomes more diffuse as the cyst matures. The trophozoite is typically pleomorphic in shape and larger in size, ranging from 10 to 50 μm in length and contain usually one nucleus, but up to four nuclei in one trophozoite has been reported. Unlike the cysts, the trophozoites are motile through pseudopodium. TNF-α, fibronectin, the anaphylatoxin C5a and lysed erythrocytes act a chemoattractants of *E. histolytica*. When the parasite chemoreceptors sense one of these stimuli, the cell polarizes and pseudopodia form, allowing the parasite to move. The trophozoites contain chromatoid bodies, glycogen and ingested food including bacteria and red blood cells. *E. histolytica* trophozoites lack classical mitochondria, rough endoplasmic reticulum and a Golgi apparatus, however nuclear bodies including a nucleolus and speckles are present. In both forms, the nuclei are spherical and approximately 4–7 μm in diameter. The nuclei membrane is lined by a single layer of chromatin granules, and contains a 0.5 μm spherical karyosome centrally located in a colorless capsule-like structure. DNA is contained in chromatin, and *E. histolytica* has acetylation, deacetylation and methylation proteins in order to modify chromatin structures and alter gene expression. *E. histolytica* also has a variety of miRNAs, siRNA and sRNA, as well as a functional RNA interference machinery, strongly suggesting iRNA as another method of gene regulation that requires further research.

Although the cyst and trophozoite stages receive the most attention, *E. histolytica* Cyst-Like Structures (CLS) have also been reported. This stage is named for its structural similarities with the cyst, small, rounded, multinucleated structures with a chitin wall. Interestingly, although the CLS resembles the cyst, it shares significantly more proteins with the trophozoite stage. Proteomic analysis has shown that proteins related to stress response were highly abundant in the CLS, whereas metabolic proteins were far less abundant. Based on these characteristics, it is suggested that CLS stages are a rapid trophozoite response to stressful conditions; the chitin wall protects the parasite from perceived threats in the area. Once the danger has passed, the CLS is able to revert back into the trophozoite form.

Amebiasis

The main disease caused by *E. histolytica* infection is termed amebiasis and includes amebic colitis and amebic liver abscesses (ALA). Amebiasis is contracted by ingesting fecally contaminated substances including food and water. After excystation in the terminal ileum or colon, the trophozoites bind to and colonize the host mucus layer. In disease situations, *E. histolytica* then disrupts the protective mucosal barrier, allowing access to the underlying epithelial tissue and invasion occurs. Amebiasis presents as abdominal cramping and pain, watery or bloody diarrhea, and potentially weight loss. Amebic colitis is a rare manifestation, and includes more serious symptoms including gastrointestinal lesions, abdominal pain, and loose mucoid bloody stool. Furthermore, the colonic muscoa may develop flask-shaped ulcers, and potentially fatal necrotizing colitis. Amebomas, or amebic granulomas, can also occur in rare circumstances and present as white nodular masses, similar to colon carcinomas. Amebomas can become problematic if they

become so large in size that they obstruct the bowel. Toxic megacolon may also occur, typically when the patients also used corticosteroids, and may require surgery. Other life-threatening complications include peritonitis, perforation, shock or in some cases death.

In extreme cases, the parasite is able to leave the colon and can travel through the bloodstream to other locations including the liver, brain, lungs or urinary tract. This even can happen years after initial contact with *E. histolytica* making diagnosis difficult. The most common dissemination occurs via the portal vein, where *E. histolytica* travels to the right lobe of the liver and leads to amebic liver abscesses. ALA presents with symptoms including fever, cough, rigor, chills and dull aching abdominal pain, as well as nausea, vomiting, diarrhea, constipation and abdominal distention, and can be fatal. Anemia and leukocytomia is also common, as is increased levels of alkaline phosphatase and alanine amino-transferase.

There are a number of risk factors that increase this risk of infection with *E. histolytica* and the chance of mortality. Since the parasite is transferred through feces, areas with poor sanitation and unhygienic drinking water are at high risk for *E. histolytica* infection. As with many diseases, malnutrition is linked with *E. histolytica* susceptibility; malnourished individuals are prone to immune deficiency and therefore at risk for *E. histolytica* infection. Living in developed countries does not exempt individuals from amebiasis; travelers and immigrants coming from less industrialized countries may come in contact with *E. histolytica* while away. There is substantial evidence supporting the notion that the composition of the commensal microbiota largely affects the outcome of amebiasis. For example, one study demonstrated that the presence of *Closteridia* species is protective against amebiasis in murine models. Conversely, an increase in *Prevotella copri* has been observed in *E. histolytica* infections, potentially since *P. corpi* is associated with inflammation in autoimmune diseases. Certain strains of *E. coli* and *S. dysenteriae* have been found to increase *E. histolytica* virulence, while other *E. coli* strains can actually decrease virulence. Furthermore, it is necessary for commensals to be present for *E. histolytica* to cause a pathogenic disease state. Lastly, there is a link between men having oral-anal or oral-genital sexual contact with other men.

Diagnosis and Recognition

Amebiasis is often suspected when the patient presents with amebiasis-like symptoms and has a history of being exposed to the pathogen. Amebiasis is diagnosed by analyzing stool samples for cysts or trophozoites. It is recommended that a minimum of 3 stool samples be taken during a 10-day period from each patient as *E. histolytica* may appear intermittently. Even with this precaution, it is sometimes impossible to detect *E. histolytica* in the stool of an infected patient. In the case of ulceration, biopsy samples can also be examined to look for the presence of *Entamoeba* species. Intestinal scrapings or aspirates may also be obtained as a sample. Microscopic detection is a standard clinical diagnosis method, however poses a number of challenges. Due to the morphological similarity *of E. histolytica* to non-pathogenic strains *E. dispar* and *E. moshkovskii*, it is almost impossible to microscopically distinguish the species. The exception is when a host erythrocyte is ingested in the ameba; this signifies the presence of *E. histolytica* as the other two species do not phagocytose red blood cells. There is also a risk of mistaking host macrophages or leukocytes for an amebic cyst, leading to a false positive diagnosis. For these reasons, further tests need to be done to confirm the ameba present is in fact *E. histolytica*. A number serological tests such as complement fixation, indirect immunofluorescence assay, the amebic gel diffusion test and immunodiffusion can successfully identify *E. histolytica,* however, they can cannot determine if the parasite is currently present or if the patient has been infected in the past. Currently, PCR or ELISA based assays using fecal samples, abscess pus or serum are available to detect *E. histolytica* DNA or antigens and can successfully distinguish between pathogenic *E. histolytica* and non-pathogenic *E. dispar* and *E. moshkovskii*. Since samples are taken at the time of the test, these samples also ensure that the patient is currently infected with the parasite. It is also important to exclude other bacterial and parasitic infections including *Salmonella*, *Shigella*, *E. coli* or *B. coli,* as well as non-infectious causes of dysentery such as inflammatory bowel disease or carcinoma.

Treatment

When determining proper treatment for a patient, it is critical to determine if a pathogenic or non-pathogenic species of *Entamoeba* is present; only *E. histolytica* requires medical treatment. Even if a patient is not presenting with any adverse symptoms, if *E. histolytica* is confirmed it is advised to treat the infection to prevent the parasite from invading the host and causing infection, as well as to prevent the spread of the parasite. Usually in asymptomatic infections, a 7-day course of the amebicidal luminal agent paromomycin is given. In the case of symptomatic amebiasis, nitromidazoles are prescribed. Numerous nitroimidazoles exist, however, metronidazole is the standard antibiotic given due to its availability in most countries. Although nitromidazoles, including metronidazole, have unpleasant side effects including diarrhea, nausea, vomiting and abdominal discomfort, it continues to be used as 90% of patients respond to it if followed with luminal agents such as paromomycin or diloxanide to ensure the parasite is removed from the intestinal lumen. Recently, Resveratrol, a plant-based natural phenol, has had success in attenuating *E. histolytica* infection in animal with fewer side effects than nitroimidazoles. This is an exciting development as previous plant-based treatments that were successful in killing *E. histolytica* also killed human cell lines, making them unsuitable for treatment. Another alternative candidate has been found in auranofin, a drug currently used to treat rheumatoid arthritis, as it can decrease *E. histolytica* numbers in various models of amebic colitis and is already approved for use in humans. Furthermore, it has proven to be successful in treating other parasitic infections.

In recent years, metronidazole resistant strains of *E. histolytica* have been created in the laboratory. Although the parasite can survive, multiple important virulence and survival factors are altered. Metronidazole resistant *E. histolytica* show decreased adhesion, a crucial factor in both colonization and invasion for the parasite. Furthermore, the resistant trophozoites are less phagocytic and cytopathogenic, suggesting that they have a severely reduced ability to cause disease. ALA are usually treated the same as intestinal amebiasis; a course of metronidazole followed by either paromomycin or diloxanide. Occasionally, ALA require further treatment than antibiotics. This is the case when antibiotic treatment does not seem to be clearing the infection, or when the patient is at risk of the abscess rupturing due to its size or location. The abscess may be drained either by needle aspiration or catheter drainage; surgical interventions are less likely to be used. Because the abscess may because coinfected with bacteria either before or as a result of the drainage, antibiotics may also be prescribed to inhibit a secondary infection. Surgical interventions may be necessary if the infection leads to abdominal or gastrointestinal bleeding, or if toxic megacolon develops.

As with many diseases, the ideal treatment would be a preventative vaccine to eradicate the disease and eliminate need for treatment entirely. To date, no successful vaccine has been identified, but there have been some potential vaccine candidates that have been investigated. Only animal models of vaccination candidates have been tested so far, and although some have had success in protecting against ALA and amebic colitis, no potential vaccine has been able to induce long-term immunological memory against the pathogen. Of these potential vaccines, only 1 has been tested in non-human primates. Although more testing will be needed before it can be tested in humans, a primate study holds promise for a successful candidate against *E. histolytica*.

Virulence Factors

Gal/GalNAc Lectin: Gal/GalNAc lectin is one of the first parasite proteins to encounter host proteins. The Gal/GalNAc lectin protein binds to abundant amounts of galactose and N-acetly-galactosamine on mucus, allowing the trophozoites to colonize the mucus layer where it can inhabit indefinitely. *E. histolytica* has also been shown to bind to the colonic epithelium, neutrophils and erythrocytes, as well as some types of bacteria. This is a large 260-kDa protein composed of 3 chains; a heavy 170-kDa chain, a light 35 kDa chain, and an intermediate 150 kDa chain. The heavy and light chains are covalently linked via disulfide bonding, whereas the intermediate chain is non-covalently linked to the other 2 subunits. The heavy chain contains a carbohydrate recognition domain (CRD) within a cysteine rich extracellular domain. The heavy chain is also composed of a transmembrane region and a cytoplasmic tail. The light chain is extracellular and contains a glycosylphosphatidylinositol-anchored tail. The light chain has been attributed to amebic virulence; antisense inhibition of the light chain causes less cytopathic and cytotoxic activity, but does not interfere with adhere to target cells. Gal/GalNAc lectin can bind to various host cell receptors and leads to a strong host pro-inflammatory response and the production of various pro-inflammatory cytokines. The heavy chain of Gal/GalNAc lectin is a common antigen targeted by the host's humoral immunity; 90% of patients with a previous *E. histolytica* infection have antibodies against the heavy chain. The Gal/GalNAc lectin adherence to a host cell is a critical prerequisite step for *E. histolytica* to kill host cells. This has been demonstrated by blocking adherence with excess galactose or N-acetyl-galactosamine; when the lectins do not bind to the host cell, there is a decrease in host cell death.

Proteinases: E. histolytica has over 50 known cysteine proteinases (CPs), many of which it uses to invade and infect the host while evading host immunity and enhancing survival. These proteins are both secreted and membrane bound. One of the most studied CPs expressed by *E. histolytica* is CP5 as it is present in *E. histolytica* but not in the non-pathogenic *E. dispar*, and has been described in multiple roles of pathogenesis. One of the primary and important roles of CPs is their ability to degrade the protective mucus barrier that overlies the host epithelial layer. The mucus layer is composed primarily of the glycoprotein MUC2, and *Eh*CPs are able to cleave the C-terminal of MUC2 at the IRTT and GKTT sites, destroying the protective barrier and allowing direct access to the epithelial cells. This cleavage is assisted by *E. histolytica* produced glycosidases that can cleave the sugar residues of the MUC2 polymer, exposing it to further degradation by the CPs. Interestingly, *E. histolytica* can also act as a mucus secretagogue. *Eh*CP5 binds to αvβ3 integrin on goblet cells and stimulates goblet cells hypersecretion of mucus, which depletes the goblet cell storage of mucus through a protein kinase C (PKC) and Ca^{2+}-dependent pathways. CPs have the ability to cleave both subtypes of IgA in order to avoid antibody recognition and expulsion in the colon, as well as IgG. Purified CPs can also cleave elastin, fibrinogen, collagen and laminin of the extracellular matrix. *E. histolytica* CPs also have a role in complement, an integral part of host humoral immunity. CPs can degrade components of the complement pathway, inhibiting the cytotoxic and pro-inflammatory effects of the complement pathway. Although many of the roles of *Eh*CPs revolve around immune evasion and host invasion, it has also been suggested that CPs play an important role in nutrient acquisition and proper encystation. E-64 (L-*trans*-epoxysuccinyl-leucylamido(4-guanidino) butane) from the fungi *Aspergillus japonicas* has been shown to be a broad cysteine proteinase inhibitor, and indeed E-64 does inhibit the proteinase activity of *E. histolytica* and therefore prevents the destruction of host epithelial cells. Antisense inhibition of CP5 greatly reduces the parasites ability to degrade mucus, and also prevents the formation of liver abscesses, demonstrating the importance of CP5 for *E. histolytica*.

Amoebapore: Another virulence factor *E. histolytica* uses to infect and invade host cells is its use of a pore-forming peptide termed the amoebapore. Like other pore-forming toxin, the amoebapore kills host cells by forming a transmembrane pore in the host cell wall. The amoebapore is a 5kDa protein made of 77 amino acids including 6 cysteines that forms a α-helical structure held together by disulfide bonds. The amoebapores are stored in granules in the cytoplasm of *E. histolytica* until released. Granules also contain lysozyme, phospholipase A2, calcium-binding protein and cysteine proteinase. To date, 3 pore-forming proteins have been identified and are entitled amoebapore A, B and C, with the latter being the most potent. The amoebapore functions optimally

in acidic conditions of approximately pH of 5.2, and has no activity above pH 6. The virulent peptide is able to kill eukaryotic cells as well as both gram positive and gram-negative bacteria. Once in contact with a human host cell, the host cell swells and loses its membrane integrity, consistent with necrosis, which then leads to inflammation due to an influx of leukocytes. The proposed mechanism of action for the amoebapore is the barrel-stave mechanism. Briefly, the amoebapore polypeptides form helices and insert into the cell membrane of the target cell, forming a channel that allows the cytoplasmic contents of the target cell to pass through, resulting in a drastic change of cytoplasmic content and eventually cell lysis and death. Hydrophobic regions of the helices interact with the phospholipids of the membrane, and the hydrophilic regions create the inner side of the channel where water, ions and other cytosolic components pass through. Interestingly, amoebae are immune from their own amoebapores as the peptide is unable to bind to the lipid membrane of *E. histolytica* and therefore the amoebapore cannot insert and damage the parasite itself. Removing the amoebapore through either transcriptional silencing or antisense inhibition drastically affects the pathogenicity of *E. histolytica* and removes the ability of the parasite to form liver abscesses.

Apoptosis and Phagocytosis: As previously mentioned, Gal/GalNAc adherence to the target cell is a necessity for killing host cells. In addition to Gal/GalNAc *E. histolytica* has a STIRP (serine, threonine and isoleucine rich protein) protein and surface LPPG (lipophosphopeptidoglycan), both of which are associated with adherence and cell cytotoxicity. Adherence to a target host cell induces the first step in amebic caused apoptosis: an increase in calcium. Up to a 20-fold increase in host cell calcium has been reported. As the calcium increases intracellularly, the host cell becomes dephosphorylated. This dephosphorylating starts with calcium activating the protein calpain, a calcium dependent protease. Once active, calpain proteolyically cleaves PTP1b, a PTPase that is known to cause cell dephosphorylation. Both of these events appear to be mandatory; blocking calcium influx or the dephosphorization inhibits *E. histolytica* killing of the host cell. These events lead to a change in the host cell morphology including chromatin condensation, blebbing and DNA fragmentation. This inevitably leads to caspase-3 dependent apoptosis. There have also been observed host cells dying in a fashion more consistent with necrosis following contact with *E. histolytica*, so it is important to note that cytotoxicity may not be strictly an apoptotic response. Host cell death is observed within minutes.

After apoptosis, *E. histolytica* phagocytoses the cells. This is beneficial as it removes dead cell components that could alert host immune cells of a problem from the area, avoiding detection and inflammation. This can also explain how *E. histolytica* can cause high degrees of tissue damage without causing inflammation. Interestingly, apoptosis appears to be a pre-requisite for phagocytic uptake in *E. histolytica*; healthy cells nor necrotic cells are not phagocytosed by the parasite, but apoptotic cells readily are engulfed. Phosphatidylserine is exposed on apoptotic cells and is used by *E. histolytica* to recognize apoptotic cells. There is also evidence of *E. histolytica* trans-membrane kinases (TMK) and serine rich *Eh* protein (SREHP) facilitating phagocytosis in addition to phospatidlyserine. Additionally, F-actin and myosin 1B are necessary to form a phagocytic cup and ingest the apoptotic host cell. Phagocytosis-deficient *E. histolytica* are less virulent and cannot cause liver abscesses. The avirulent *E. dispar* does not phagocytose host cells. Accordingly, the presence of visible phagocytosed erythrocytes is used to differentiate *E. histolytica* from *E. dispar*.

Trogocytosis: A unique method *E. histolytica* uses to kill host cells is amebic trogocytosis, a form of cell killing based off the Greek word "to nibble". Within as little as 1 minute of contact with a human host cell through the Gal/GalNAc, *E. histolytica* has been shown to internalize small fragments of the host cell's membrane, which eventually leads to cell death due to an increase in intracellular calcium and eventually the loss of membrane integrity. The phenomenon of trogocytosis only occurs with live host cells; once the cell dies, the ameba detaches from the host cell and trogocytosis ceases. Similarly, when *E. histolytica* comes in contact with host cells that are already dead, trogocytosis does not occur. Instead, the parasite ingests pre-killed cells via phagocytosis. Killing host cells through trogocytosis, especially epithelial cells, may be another method *E. histolytica* uses to invade the epithelium. Trogocytosis occurs at physiological pH, and requires actin polymerization at the site of host cell contact, as well as *E. histolytica* C2-domain-containing protein kinase (EhC2PK) and phosphoinositide-3-kinase (PI3K) signaling.

Alteration of Host Tight Junctions: Removing the mucus layer allows direct contact with the host epithelial cells, but does not allow access to the basolateral side, as epithelial cells are tightly bound together creating tight junctions. This protective barrier is designed to keep unwanted pathogens and toxins out of the basolateral surface. The families of proteins claudins (CLDN) and occludins (OCLN) are transmembrane proteins that create the backbone of the barrier between neighboring cells. These proteins bind with intracellular proteins such as zona occludins (ZO) to anchor and stabilize the tight junction structure. When the tight junction complex is altered, pathogens and their secreted protects can invade the underlying basolateral surface and cause further disease and damage. *E. histolytica* has a number of ways to overcome this host mechanism and further its invasiveness.

When in contact with the epithelial cells, *E. histolytica* proteases have been shown to remove portions of the tight junction structure leading to an increase in paracellular permeability and a decrease in TER. Live *E. histolytica* contact with epithelial cells lead to ZO-1 and ZO-2 releasing from each other, ZO-2 dephosporylation, and decreased presence of ZO-1, leading to decreased integrity of the tight junction complex. *E. histolytica* also induces a very strong pro-inflammatory response through activation of the transcriptional regulator NF-κB. Multiple cytokines are upregulated including IL-1β, IFN-γ, TNF-α. Intriguingly, upregulation of these pro-inflammatory cytokines has been shown to alter the tight junction proteins leading to increased paracellular permeability. Therefore, *E. histolytica* can disrupt the tight junctions of host epithelial cells directly by proteinases interacting with tight junction proteins, or indirectly by inducing the host immune system to up regulate tight junction-altering cytokines. *E. histolytica* also has an occludin-like protein that can disrupt tight junction barrier integrity possibly by displacing and replacing the host occludin protein.

Another method is based on the inflammatory molecule prostaglandin E_2 (PGE_2), a prostanoid found throughout the GI tract that regulates physiological functions such as mucosal protection, intestinal secretions including increased mucus secretions, vasodilation and peristaltic movement. An increase in PGE_2 has been shown in inflammatory disease such as colitis, Crohn's disease and intestinal inflammation. *E. histolytica* has been shown to stimulate host macrophages to up regulate PGE_2. Furthermore,

the parasite can synthesize and secrete PGE_2. PGE_2 is formed when arachidonic acid, a fatty acid found commonly in the gastrointestinal tract, is transformed to prostaglandins by cyclooxygenase (COX) enzyme. Interestingly, *E. histolytica* has a COX-like enzyme, and is therefore able to synthesize and secrete PGE_2, and can disrupt tight junctions by altering claudin-4 and induce an inflammatory response through PGE_2 binding to host E-proastanoid-4 (EP4) receptors. PGE_2 also creates a NaCl gradient that may cause water flow and eventually diarrhea, a frequent event in intestinal diseases that can also increase the spread of infection. *E. histolytica* can also alter the tight junctions through its *Eh*CPADH112 complex. Composed of the cysteine proteinase *Eh*CP112 and an adhesin *Eh*ADH112, this virulent complex can bind to tight junction components including occludin, claudin-1 and ZO-1 and ZO-2. Once bound to the complex, these proteins are targeted for proteasome degradation or dissociation, resulting in a decrease in tight junction function.

Host Immune Evasion

Innate Host Immunity: The human body is filled with complex innate immune strategies that are constantly present and ready to defend against any potential pathogens (Table 2). When *E. histolytica* is initially ingested as an infectious cyst, it enters the stomach, which is rich in acidic secretions meant to destroy pathogens. However, the cyst's chitinous cell wall is resistant to acid in the stomach, and therefore is able to pass through and enter the intestine. The human mucus layer, composed primarily of MUC2, is the next major innate defense against *Eh*. The mucus layer is designed to keep the microbiota and potential pathogens away from intestinal epithelial cells, however, *E. histolytica* can easily overcome this barrier using its cysteine proteinases to cleave the MUC2 monomers, destroying the mucus layer and allowing contact with the epithelial cells and inducing the expression of antimicrobial peptides. Human β-defensin 2 and cathelicidins are some of the main antimicrobial peptides released upon contact with *E. histolytica*, but the parasite has been shown to be less susceptible to both defensin and cathelicidin killing, overcoming yet another innate defense.

Pro-inflammatory response: Since *E. histolytica* is able to overcome various innate immunity strategies employed by the host, further immune responses are perpetuated in order to clear infection. When an epithelial cell comes in contact with *E. histolytica* through its Gal/GalNAc lectin, the host cells elicits a potent pro-inflammatory response including a variety of cytokines to alert immune cells and recruit them to the site of infection, including interleukin-1 (IL-1β), IL-8 and tumor necrosis factor-α (TNF-α) through NF-κB. Similarly, CP5 can bind αVβ3 integrin via the RDG motif on colonic epithelial cells, stimulating a pro-inflammatory response through NF-κB.

Neutrophils: The first immune cells to respond to an *E. histolytica* infection are neutrophils. These host cells try to remove the parasite from the area by releasing reactive oxygen species (ROS). The parasite is not harmed by the oxidative stress as it contains a number of mechanisms to overcome neutrophil attack. It can inhibit the release of ROS from neutrophils all together, or it can use its superoxide dismutase and NADPH:Flavin oxidoreducatae enzymes to turn ROS species into H_2O_2. The parasite then detoxifies H_2O_2 with a surface protein, peroxiredoxin, a potent antioxidant and can degrade H_2O_2, rendering it undamaging. *E. histolytica* also secretes *EhSERP*, a serine protease inhibitor that binds and inactivates the neutrophil serine protease cathepsin G, rendering it useless. Furthermore, a single *E. histolytica* trophozoite can kill as many as 3000 neutrophils through inducing NADPH oxidase and ROS-mediated apoptosis. Not only does the neutrophil death help *E. histolytica* spread and survive, but it also contributes to the extensive tissue destruction associated with amebiasis. As neutrophils die, their intracellular contents such as lysozymes, hydrolases and proteases are released and can damage the nearby intestinal epithelial cells. As intestinal cells are damaged, they release

Table 2 Host immunity against *E. histolytica*

Immune component	*Function*
Mucus layer	Prevents pathogens from reaching underlying epithelial cells
Neutrophil	release ROS to kill pathogens
	contains serine protease cathepsin G that degrades pathogens
	contain various lysozymes, hydrolases and proteases
Macrophage	PRRs detect pathogens
	release NO
	produce a pro-inflammatory response to stimulate apoptosis, cell proliferation and attract other cells
	activate inflammasome response to secrete pro-inflammatory cytokines
Complement	cascade leads to the formation of the membrane attack complex which lyses pathogens
	components of the complement pathway are potent anaphylaxatoxins
Immunoglobulins	produce memory antibodies against pathogens
	neutralize pathogen surface molecules
	activate complement
Tight junctions	prevents pathogens from reaching the basolateral surface of the epithelial cells
Stomach acid	low pH kills many pathogens as they enter the stomach

pro-IL-1β, which CPs can convert into active IL-1β. Conversely, neutrophils activated by both IFN-γ and TNF-α are successful in killing 67% of *E. histolytica* trophozoites in 6 h. Interestingly, PGE_2 from *E. histolytica* can induce the neutrophil chemokine IL-8.

Macrophages: Another type of immune cell that is quick to infiltrate an amebic infection is macrophages. They are recruited by monocyte chemotactic protein (MCP) from intestinal epithelial cells that have come in contact with *E. histolytica* soluble proteins. Gal/GalNAc recognition by PRRs TLR-2 and TLR-4 can stimulate naïve macrophages to produce the pro-inflammatory cytokine TNF-α which can not only upregulate other pro-inflammatory cytokines but can also induce apoptosis, stimulate cell proliferation and act as a chemoattractant. This pro-inflammatory response can assist macrophages in killing trophozoites, or it can limit the growth of the parasite. Macrophages can also be activated by IFN-γ; higher levels of IFN-γ correlate with lower frequency of *E. histolytica* infecting a host and appear to have a protective role in amebic infection. Macrophages release NO in order to harm parasites, and can effectively kill *E. histolytica* trophozoites by inhibiting cysteine proteinases and alcohol dehydrogenase. However, *E. histolytica* has developed multiple strategies to evade the macrophage immune response. The *E. histolytica* arginase enzyme limits the amount of NO produced by converting L-arginine, the substrate for NO, into L-ornithine, stopping the production of NO. The parasite also produces monocyte locomotion inhibitory factor (MLIF) that inhibits NO production. In addition to MLIF, *E. histolytica* also produces macrophage migration inhibitory factor (MIF), which can increase macrophage TNF-α secretion, leading to a more pronounced pro-inflammatory response, furthering tissue damage. *E. histolytica* can synthesize PGE_2, which can inhibit Th1 cytokine release as well as oxidative bursts and NO synthesis. PGE_2 can also inhibit class II MHC genes, blocking the antigen presenting capabilities of macrophages. An interesting interaction between *E. histolytica* and host macrophages is the activation of the NLRP3 inflammasome in macrophages. When live *E. histolytica* binds to a macrophage via the Gal/GalNAc lectin, the inflammasome becomes activated and secretes pro-inflammatory IL-1β and IL-18. This cytokine response is beneficial for the host as it can enhance the pro-inflammatory response against the parasite, but it can also be detrimental as it can lead to further tissue damage. Macrophages must be stimulated with IFN-γ, TNF-α and macrophage colony-stimulating factor-1 in order to mount a successful response against *E. histolytica*.

Eosinophils: Recently, a role for eosinophils has been discovered. IL-25 from eosinophils is down regulated in cases of amebic colitis, however replenishing IL-25 can reduce the severity of *E. histolytica* infection and protect the intestinal epithelial cells by suppressing pro-inflammatory cytokines, including TNF-α, which has been shown to have a harmful effect in *E. histolytica* infection. Furthermore, induction of eosinophils may reduce the severity and size of ALA.

Complement Cascade: Complement is another immune strategy employed by the host in case of *E. histolytica* infection. When activated successfully, complement creates the membrane attack complex (MAC) that can lyse invading pathogens, including *E. histolytica*. The Gal/GalNAc lectin of *E. histolytica* antigenically resembles host CD59, a MAC-inhibitory protein, and therefore inhibits and avoids MAC-activated lysis. The parasite cysteine proteinases can activate the alternative complement pathway by cleaving the α-chain of C3. Furthermore, *Eh*CPs cleave complement anaphylaxatoxins C3a and C5a, inhibiting the complement pathway and stopping the toxins from causing mast cell histamine release, macrophage IL-1β release, chemotaxis of neutrophils and increase vascular permeability. Lastly, *E. histolytica* Gal/GalNAc lectin can bind complement components C8 and C9, blocking them from forming the MAC.

Humoral Immune Response: *E. histolytica* induces a strong humoral immune response. Over 80% of amebiasis patients develop circulatory antibodies within the first week of infection. The Gal/GalNAc lectin is immunogenic; plasma cells in the lamina propria produce mucosal IgA against this portion of *E. histolytica*. The presence of sIgA correlates with a decrease in future *E. histolytica* infections. Anti-ameba IgG is also produced during amebic invasion; however, it correlates with more frequent and more severe future infections. Regardless of the consequence of the immunoglobulin, cysteine proteinases cleave both sIgA and circulatory IgG. *E. histolytica* has also been shown to utilize an immune evasion strategy known as receptor capping, a method where the parasite redistributes surface receptors that have been recognized by host immune cells. Recognized surface-bound molecules are translocate to the posterior end of the cells where they are cluster to form a cap and are then released as membrane bound vesicles, eliminating the host immune system's ability to recognize the parasite. The serine proteinase rhomboid protease (ROM1) is partially responsible for the translocation and release of antigenic materials. Interestingly, ROM1 has been shown to cleave the Gal/GalNAc lectin, a protein that is often recognized by host immune cells. It is suggested that ROM1 may continually cleave Gal/GalNAc lectin from the surface of the parasite, hindering the immune system from targeting the parasite.

Immune Evasion: Since 90% of *E. histolytica* infections are asymptomatic, there must be some methods by which the parasite can avoid the potent pro-inflammatory and invasive response. Soluble amebic proteins (SAPs) have been found to suppress NF-κB signaling; thereby inhibiting NF-κB mediated inflammation. SAPs unregulated heat shock proteins 27 and 72 (Hsp27 and Hsp72), associated with stress response and protective effects. Hsp27 and Hsp72 have been shown to inhibit NF-κB signaling in response to SAPs, lessening the pro-inflammatory and apoptotic events, alleviating epithelial damage. This could explain why the vast majority of people carrying *E. histolytica* do not display symptoms; however, it is unknown why or how the parasite turns from harmless to invasive.

Concluding Remarks

Dating back for centuries, *E. histolytica* has become a well characterized human pathogen. In most circumstances, *E. histolytica* lives as a harmless member of the hosts microbiota. However, in a small portion of the population, the organism invades tissues causing a range of symptoms causing disease both in and outside of the intestinal tract including diarrhea, fever, intestinal ulcers and liver

abscesses. Although diagnostic technologies have improved with advances in science, the similarities of pathogenic *E. histolytica* with non-pathogenic *E. dispar* and *E. moshkovskii* can make diagnosis challenging. Furthermore, although largely successful, the current effective treatment options often are accompanied with a variety of unpleasant side effects. The success of *E. histolytica* as a pathogen is due to its numerous virulence factors such as Gal/GalNAc lectin, cysteine proteinases and the amoebapore. Although the host is able to create a robust immune response in the presence of infectious *E. histolytica*, the parasite has a number of ways to evade and overcome host immunity, and often the host immune response is responsible for some of the intestinal damage associated with *E. histolytica* infection. Clearly, more studies are needed to understand how to prevent *E. histolytica* infection globally with vaccines and how to treat infection without creating more symptoms and host damage.

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Enteric Viruses☆

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Infectious gastroenteritis or diarrhea remains a significant health burden worldwide. Although global diarrheal deaths have declined in recent years, morbidity remains unchanged. Many pathogens, including viruses, bacteria and parasites, can cause diarrhea. These pathogens target the gastrointestinal tract for replication. Transmission to new hosts occurs via the fecal-oral route, when the pathogens are ingested via contaminated food or water or fomites, and are shed in the feces following replication. Thus, the pathogens that infect via the intestine have evolved numerous strategies to overcome host barriers at the intestinal surface to establish a successful infection. Most viruses that infect the intestine are non-enveloped. This allows these viruses to survive in the harsh environment of the stomach *en route* to the intestine. It also makes these enteric viruses relatively resistant in the environment and to disinfection, making processes like water treatment more difficult. Enteric viruses are shed from days to weeks in very large quantities (up to 10^{11} viruses/g stool), and they are infectious at low doses, promoting their spread. Many different viruses cause food- and waterborne infections. These include viruses with DNA genomes (e.g., adenovirus serotypes 40 and 41, bocavirus, and Torque teno virus), viruses with positive-sense, single-stranded RNA genomes (e.g., norovirus, sapovirus, astrovirus, aichivirus, the picornaviruses, cosavirus, salivirus, enterovirus, hepatitis A virus, and hepatitis E virus), and viruses with double-stranded RNA genomes (rotaviruses). While many of these infections are asymptomatic or mild and not diagnosed, others lead to morbidity and mortality. Given their prevalence, the focus of this review will be on noroviruses, sapoviruses, rotaviruses, adenoviruses and astroviruses.

Caliciviridae

The name "Caliciviridae" is derived from the ancient Greek word *calyx*, meaning "cup" or "goblet", because of the chalice-like structures of the viral capsid that can also appear as six-pointed stars in electron micrographs. Their icosahedral capsids are ~32 nm in diameter, non-enveloped and encapsidate a non-segmented, positive-sense, single-stranded RNA genome. Viruses in this family infect a range of vertebrate animals, including dogs, cats, dolphins, rabbits, mice, pigs, cows and humans. They are classified into genera, subdivided into genogroups, and further separated into genotypes based on phylogenetic analysis. Currently, five genera are officially recognized: Vesivirus, Nebovirus, Lagovirus, Norovirus, and Sapovirus. These viruses cause a wide range of symptoms. Vesiviruses, like feline calicivirus (FCV), cause respiratory disease among cats; neboviruses, like the Newbury-1 virus, cause gastrointestinal disease in cows; and lagoviruses, like rabbit hemorrhagic disease virus (RHDV), cause hepatitis in rabbits; while noroviruses and sapoviruses cause sporadic and epidemic outbreaks of gastroenteritis in humans and thus will be discussed below in more detail.

History and Epidemiology

The first norovirus, Norwalk virus, was visualized by electron microscopy in the stool of an elementary school student from Norwalk, OH, in 1972. A few years later in 1977, the first sapovirus, Sapporo virus, was discovered in a stool sample from a child in Sapporo, Japan. Noroviruses and sapoviruses cause primarily vomiting and/or diarrhea. While either disease is rarely lethal, norovirus infects all age groups and more severe disease is observed in the very young, the elderly and immunocompromised individuals. The incubation period for norovirus is between 24 and 48 hours, the duration of illness for otherwise healthy adults is 1–3 days, and the mean duration of shedding is 10 days. Compared to noroviruses, sapovirus infections occur more often in children and typically result in less severe disease. Of all enteric viruses, noroviruses are the most prevalent and responsible for almost one fifth (18%) or 685 million cases of acute gastroenteritis worldwide with an estimated price tag of about $60 billion per year. Approximately 200,000 children under the age of 5 die every year in developing countries from norovirus infections. Asymptomatic infections are common and approximately 7% of healthy individuals worldwide unknowingly shed virus. Norovirus is spread most commonly via person-to-person contact but also by contaminated food and water, or in vomitus. Thus, outbreaks occur most often where large populations interact in close quarters, such as schools, military bases, cruise ships, and nursing homes, but frequent transmission also occurs in restaurants or via catered meals. Outbreaks occur all year long and propagate easily due to the low infectious dose of noroviruses (estimated to be as low as 18 particles). Furthermore, norovirus particles are highly stable in the environment, *e.g.*, particles remain infectious in ground water for several months and are resistant to standard concentrations of chlorine in drinking water.

Noroviruses are highly genetically diverse. While genogroup I (GI) strains are more often transmitted via water, most outbreaks are caused by genogroup II (GII) viruses, in particular the genogroup II genotype 4 (GII.4) viruses. This cluster is unique as it is undergoing continuous genetic and antigenic drift resulting in new antigenic variants that can cause global epidemics. Other GI and

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GII genomes have differed only slightly over several decades, they remain "static". The genetic diversity of noroviruses is further increased by frequent mixed infections resulting in recombination between strains.

Structure

The norovirus genome is approximately 7.5 kb long and consists of three or four open reading frames (ORFs). Sapovirus genomes are of similar length and encode two or three ORFs. The 5′ end of the norovirus genome is linked to a viral protein (VPg), while the genomic 3′ end of both viruses has a poly(A) tail. The capsid structure of sapovirus is more similar to animal caliciviruses in the Vesivirus genus than to norovirus.

The norovirus ORF-1 encodes a large polyprotein that is cleaved by the viral protease into 7 non-structural proteins, which are required to replicate the viral genome, including the protease and RNA-dependent RNA polymerase. ORF-2 and ORF-3 encode the major capsid protein VP1 and minor capsid protein VP2, respectively. 180 copies of VP1 form the viral capsid, while a few copies of VP2 are located inside the capsid to facilitate stability and genome packaging. VP1 is divided into two domains. The S domain on VP1 is highly conserved and forms the shell (S) around the genome. It is used by the virion as a scaffolding for the P domain (protruding domain), which contains the receptor binding and antigenic sites and is more variable. The neutralizing antibody response is mainly directed against the P domain.

Binding and Replication

Human noroviruses have remained refractory to cell culture for over 40 years. Currently, two cell culture systems have been described that enable replication of some isolates in either primary human intestinal epithelial organoid cultures or the human B cell line BJAB. However, human sapoviruses remain unculturable to date. Knowledge about the cellular life cycle of these viruses thus comes largely from the related murine norovirus and porcine sapovirus, which can be cultured. Following attachment of murine norovirus to carbohydrates on the cell surface, the virus binds its proteinaceous receptor CD300lf, which also binds ceramide on the cell surface. Expression of the proteinaceous receptor is required for infection and determines host cell tropism. Following receptor binding, norovirus virus particles enter the cell via endocytosis. Porcine sapovirus binds to cell surface glycans and requires bile acids for infectivity *in vitro* but proteinaceous receptor(s) and entry mechanisms remain unknown. Like all positive-strand RNA viruses, once the genome has reached the cytosol, it functions as mRNA in protein translation. The 5′-linked VPg recruits the cellular translation apparatus. After translation of the ORF-1 polyprotein, it is cleaved by the viral protease to release the non-structural proteins, which recruit intracellular membranes and form the replication complex. Genome replication is performed by the viral polymerase, which generates genomic and subgenomic length RNAs via a negative-sense intermediate. The subgenomic RNA is translated to make capsid proteins, which then encapsidate the viral genome. Packaged virions are released from the cell by lytic and non-lytic mechanisms.

Pathogenesis

Noroviruses and sapoviruses cause species-specific infections. Thus, information on viral pathogenesis comes from a combination of epidemiological studies, human volunteer studies, and studies in animal models. Typically, these animal models either investigate mechanisms of human viruses in non-human hosts or related, non-human viruses (also referred to as "surrogate" viruses) in their native hosts. Each of these models has strengths and limitations but none of these models recapitulates all aspects of human noro- or sapovirus infections.

Noroviruses and sapoviruses cause villus blunting and atrophy in the small intestine. While the cell types infected by human sapovirus *in vivo* remain unknown, porcine sapovirus antigen has been detected in villous enterocytes in the proximal small intestine. Human, murine and bovine noroviruses were shown to replicate in epithelial and immune cells in the small intestine. More specifically, human and murine noroviruses replicate in macrophages, dendritic cells, B and T cells, in addition to intestinal epithelial cells. Extraintestinal spread of noroviruses can also occur with some strains. The cause of norovirus diarrhea is still under investigation but one study suggested epithelial barrier and secretory pathway dysfunction. This dysfunction may be due to a cytokine storm as another study demonstrated increased serum cytokine levels in symptomatic versus asymptomatic patients despite similar viral loads. On the other hand, nausea and vomiting may be caused by abnormal gastric motor function and delayed gastric emptying, which has been observed in some human volunteers.

Immunity to human noroviruses is poorly understood and resistance to infection is both genetic and immunologic. Histo-blood group antigens (HBGAs) are a genetic susceptibility factor for human noroviruses. Human noroviruses bind to these carbohydrates, which are present on intestinal epithelial surfaces, red blood cells, and in saliva. Related structures are also found on intestinal bacteria and even plant leaves. These molecules are thought to serve as attachment factors on the host cell surface, but the precise function during pathogenesis remains unclear. No susceptibility factor has been identified for human sapoviruses to date. While human sapoviruses have not been shown to bind HBGAs, other cell attachment factors like sialic acid might be used by both virus families. Noroviruses and sapoviruses are sensitive to interferons, and innate immune responses are required to control levels of norovirus titers *in vivo*. Furthermore, adaptive and cellular immune responses develop following norovirus infection. However, immunity to noroviruses is short-lived and volunteer studies indicate that people can be reinfected by the same strain after only one year. In mice, antibody and CD4 and CD8 T cell responses are required for murine norovirus clearance

from immune cells, while interferon lambda clears infection from specialized intestinal epithelial cells called tuft cells during persistent infection.

Vaccination and Treatment

No specific vaccine or antiviral is approved for use against norovirus or sapovirus infections. Supportive treatment, rest and rehydration for the infected individual are currently used for standard care. Strategies to limit spread, such as enhanced hygiene, disinfection, and isolation techniques, are imperative, for example, in hospitals, food service establishments, and schools to reduce outbreaks.

Virus-like particles and the P domain subunit are under development for a norovirus vaccine. However, biological and logistical/programmatic challenges need to be overcome in the future to have a global impact on disease burden. For example, noroviruses are highly antigenically and genetically diverse and major gaps exist in our knowledge about anti-norovirus immunity, presenting a challenge for generating broadly protective immune responses. At the same time, approaches for both virus-targeted and host-based antivirals are being investigated, which ultimately may complement the arsenal available to combat these highly prevalent viruses.

Astroviridae

Astroviruses resemble stars when viewed by electron microscopy, so these viruses were given the name "Astrovirus" from "astron" meaning "star" in Greek. Like caliciviruses, astroviruses have icosahedral, non-enveloped capsids of ~32 nm containing a positive-sense, single-stranded RNA genome. They infect vertebrates and typically cause gastroenteritis in the young, including humans, pigs and birds, but fatal hepatitis and growth-stunting nephritis have also been observed in birds. Several new astroviruses have been isolated recently, increasing genetic diversity within this virus family. Based on the capsid sequences, astroviruses are classified into two genera *Mamastrovirus* and *Avastrovirus*, which are currently further subdivided into 19 and 3 genotypes, respectively. Astroviruses infecting humans are found in 3 clades: classical human astrovirus (HAstV), MLB-like and VA-like. Although astroviruses were thought to cause species-specific infections, recent phylogenetic evidence suggests that cross-species infection events have occurred.

History and Epidemiology

Human Astrovirus (HAstV) was first observed in 1975 using electron microscopy in the stool of children with acute gastroenteritis. In the same year, astrovirus was also found in hospitalized infants suffering from acute gastroenteritis. In 1981, HAstV was first grown and passaged in cell culture, leading to the characterization of 8 different serotypes for classical HAstV. Typical HAstV infections are mild or asymptomatic, and disease is found in children, the elderly, and the immunocompromised. The incubation period is approximately 4.5 days long before the onset of watery diarrhea, which lasts 1–4 days and is sometimes accompanied by vomiting, fever, and abdominal pain. Symptoms usually resolve spontaneously but in immunocompromised patients the virus can spread systemically, and on rare occasions can be fatal. Infections occur worldwide, are very common, and most children are found to be seropositive but their immunity wanes over time. The classical HAstVs cause ~2%–9% of acute gastroenteritis cases in children worldwide but studies including the recently identified MLB- and VA-like astroviruses are urgently needed to assess the full prevalence of these viruses. Infections predominantly occur in the winter months or during the rainy season and are common in locations where crowds gather. Food, water and fomites are transmission vehicles, hence the exacerbation of outbreaks can be due to poor hygiene practices.

Structure

Astroviruses encapsidate their non-segmented, ~7 kb RNA genome into non-enveloped particles of icosahedral symmetry. Their genome has a viral protein (VPg) linked to the 5′ end and a 3′ poly(A) tail. It encodes three ORFs: ORF1a, ORF1b, and ORF2. ORF1a and ORF1b encode two nonstructural polyproteins, which are further proteolytically processed and are responsible for transcription and replication. ORF2 encodes the capsid protein VP90. The protein is divided into 3 domains: the conserved N-terminus, a hypervariable domain, and a genetically variable C-terminus. Capsid assembly first yields an immature capsid following cleavage of the capsid protein VP90 to the VP70 form. Further proteolytic cleavages then result in a fully mature capsid with the C terminal domain (VP27 and VP25) forming the spikes on the virion surface and the N-terminus (VP34) forming the core surrounding the genome.

Binding and Replication

Astrovirus replication is poorly understood. The identity of any cellular receptors for these viruses is unkown. Treatment with trypsin has been found to enhance the virus' infectivity 3- to 5-fold by cleaving the viral polyproteins resulting in dramatic structural changes. Treatment with dextran sulfate, heparin sulfate, and heparin prevented infection of cells with HuAstV while treatment with sialic acid has no effect suggesting that a carbohydrate molecule acts as a receptor for the virus, similarly to caliciviruses. Binding of the virus to differentiated intestinal epithelial cells results in changes in tight junction proteins and

increased barrier permeability. The replication cycle for astroviruses is similar to that of viruses from the *Caliciviridae* family. The virus is endocytosed into cells, the genome is released into the cytoplasm and the two nonstructural polyproteins are translated from the incoming genomic RNA. The proteins then form viral replication complexes that associate with intracellular membranes and replicate the genome. New virions are formed and mature until their release from the cell in a largely non-lytic manner.

Pathogenesis

Little is known about astrovirus pathogenesis. These viruses are thought to infect mainly intestinal epithelial cells in the small intestine, but some mamastroviruses have also been observed to infect sub-epithelial macrophages and M cells of the small intestine. How astroviruses cause disease is still under investigation. In general, infections are not associated with an inflammatory response or cell death in the intestine.

Turkey astrovirus-2 (TAstV-2) in young turkeys is the most common animal model to study astrovirus pathogenesis. Turkeys suffer from diarrhea within 1–3 days of infection and symptoms persist for an additional four days. After infection, virus can be found in the blood and many other tissues of young turkeys, indicating viremia. However, the gut is the only place where replication occurs. Infection causes only mild histopathologic changes and is not associated with inflammation in the intestine. One study demonstrated that oral administration of the TAstV-2 capsid protein caused diarrhea in turkeys by increasing intestinal epithelial barrier permeability and internalization of the sodium hydrogen exchanger 3. Based on these data, the astrovirus capsid is thought to function as an enterotoxin. More recently, murine astrovirus was discovered in biomedical mouse research colonies. No obvious symptoms are observed in mice, likely resulting in the endemic spread of this virus. In the mouse model, both innate and adaptive immune responses are required to clear the infection and type I interferons protect from virus-induced barrier permeability.

Vaccine and Treatment

No specific antiviral treatments or vaccines are available. Supportive treatment is used for those who require hospitalization. HuAstV spread is best controlled by limiting its transmission. Hygienic practices like disinfection of fomites, cleaning and cooking of food, and decontamination of drinking water can prevent the virus from causing an outbreak. However, little information is available regarding effective decontamination procedures. Limited studies indicate that different strains exhibit different levels of resistance to environmental decontamination by chlorine and alcohol.

Rotavirus (Reoviridae)

Rotavirus was named after the Latin word “rota”, meaning “wheel” because the structure is such that the particle appears to have spokes and a smooth rim. Rotaviruses (RV) are a genus in the *Reoviridae* family, which cause significant morbidity and mortality worldwide. They are the second largest cause of non-bacterial acute gastroenteritis in the world in all age groups, behind noroviruses. In children, rotaviruses are the leading cause of life-threatening diarrhea. In addition, 14 other genera are classified within the *Reoviridae* family. Members of the *Orthoreovirus* genus can also infect humans via the intestine but infections are rarely symptomatic. When the virus does cause symptoms, they can include a cough, pharyngitis and gastroenteritis. Orthoreovirus infections can also be associated with neonatal biliary atresia which entails inflammation of intrahepatic bile ducts, infection of the central nervous system, myocarditis, and pneumonia.

History and Epidemiology

Rotavirus (RV) was first discovered in the 1970s alongside norovirus. In 1973, viral particles were visualized by electron microscopy in feces. Although this was the first rotavirus infection recorded in humans, it was later discovered that gastroenteritis infections in other mammals in years previous had also been due to rotavirus. To date, at least eight rotavirus species, *Rotavirus A* (RVA) to *Rotavirus H (RVH)*, have been classified. These viruses are further classified into genotypes based on nucleotide sequences of the VP7 (G) and VP4 (P) capsid genes. Most recently, the classification is being expanded to contain sequence information of the other nine proteins. Most cases of rotavirus gastroenteritis are caused by 5 genotypes but regional and temporal differences in the distribution of genotypes have been noted. However, new strains evolve continuously due to point mutations by the viral RNA-dependent RNA polymerase and reassortment of the gene segments during co-infections. Although largely species-specific, zoonotic transmission events have been detected, particularly for *RVA*, which infects birds and mammals.

Symptomatic RV infections mostly occur in children <5 years of age, at which point almost all children have been infected. Prior to universal vaccination, half a million children died from excessive fluid loss due to RV gastroenteritis each year, mostly in developing countries. All age groups can become infected, but mild or asymptomatic infections are typically seen in older children and adults. While infections can occur throughout the year, most cases show seasonality during the winter months or the dry season. The incubation period is ~2 days and symptoms last 3–5 days. Spread occurs via person-to-person contact and often occurs in settings with small children, e.g., daycares, schools, and households.

Structure

The icosahedral particles are approximately 100 nm in diameter, which is large for a non-enveloped enteric virus. Particles are triple-layered with VP7 and VP4 (spike) forming the outer layer and mediating attachment and cell entry. VP6 forms the middle layer, which functions as an adaptor between the outer and inner layer. The inner layer is formed by VP2, which encapsidates the RNA genome. Upon cell entry, double-layered particles are observed, which are transcriptionally active and needed for proliferation. The triple- and double-layered particles have been clearly resolved via x-ray crystallography and cryo-electron microscopy, which has helped in determining the geometry of the particles. Rotaviruses have an icosahedral symmetry with different surface lattices on different layers of the particle. Channels run from the outside of the particle through the second layer and have different functions based on their size, for example the export of messenger RNA (mRNA).

The *RVA* genome is about 18 kb long, composed of 11 dsRNA segments and contains conserved non-coding sequences at the 5′ and 3′ ends required for packaging, enhancing translation, gene regulation, and replication. It encodes six structural and six non-structural proteins. Each gene segment encodes only one protein except for genome segment 11, which encodes two. In homologous strains of the virus, non-coding regions within the genes are highly conserved.

Binding and Replication

Most of what is known about the rotavirus infection cycle was learned through infection studies of monkey kidney cells or polarized intestinal epithelial cells. Infection requires the presence of exogenous proteases to cleave the outer capsid protein VP4, which initiates attachment to the host cell membrane. RV enters the host cytosol by receptor-mediated endocytosis, uncoats and viral RNA is transcribed by the viral RNA-dependent RNA polymerase (RdRp) inside the subviral particle. The positive-sense RNA serves as messenger RNA for protein synthesis and packaging into newly made double-layered particles. Within a special area of the cell, called the viroplasm, negative-sense RNA and double-stranded RNA is synthesized and the virus is packaged into these particles. Assembly is aided by intracellular calcium and particles mature *en route* to the plasma membrane. Virions are released from the host cell via cell lysis or non-lytic means. In polarized cell lines, entry and release occur primarily via the apical surface.

Pathogenesis

Rotavirus exhibits a predominant tropism for differentiated enterocytes, particularly those at the top of the villi in the small intestine, but can also infect mesenchymal cells and enterochromaffin cells. However, extraintestinal spread is also observed in humans and animal models. Infection of enterocytes can cause vacuolization, epithelial cell loss, villous blunting and crypt hyperplasia. Inflammation in the intestine is mild. In immunocompromised hosts, RV may also replicate in the liver, biliary system, and pancreas. Histological findings, severity, and exact location of infection within the intestine can vary greatly between animal species. Symptoms in animal hosts also vary greatly depending on the age of the host and the infecting virus strain. Rotavirus diarrhea is thought to occur by a combination of malabsorption as a result of enterocytes destruction and via activity of the virus-encoded enterotoxin NSP4. Release of NSP4 from an infected cell initiates a signal transduction pathway that releases intracellular calcium and chloride from neighbouring cells. In addition, virus infection of enterochromaffin cells results in secretion of serotonin, which stimulates the enteric nervous system and is thought to trigger rotavirus-induced vomiting.

Adaptive immunity is critical for protection from infection. Neutralizing antibodies against the surface antigens VP7 and VP4 are elicited by wildtype infections and a high titer of serum anti-RV IgA antibody correlates with protection from infections by the same serotype. The inner core protein VP6 is also a strong antigen but it elicits antibodies, which can neutralize the infection intracellularly, and is thought to be responsible for protection from disease, but not infection. However, since people can be re-infected with rotavirus, sterilizing immunity does not develop after a single infection and immunity wanes with age. Studies in mouse models of rotavirus infection point to a role for not only B cells but also T cells in clearing the primary infection and mediating short-term protection from re-infection. Other studies have identified VP7 neutralizing antibodies titers, intestinal, salivary or serum IgA levels as correlates of rotavirus immunity in humans. Although not fully understood, heterotypic immune responses develop during infection and are the basis for the monovalent rotavirus vaccine.

Innate immunity is critical to shape early responses to rotavirus infection. Rotaviruses are susceptible to anti-viral interferon responses and cells or calves pre-treated with interferons show reduced infection. Yet, at the same time the rotavirus non-structural protein NSP1 antagonizes the interferon response, in a species- and strain-specific manner. In general, homotypic virus strains are more effective in blocking innate immune responses than heterotypic ones. Thus, while cells sense the infection and initiate antiviral responses, the virus has developed strategies to counteract those to enable its replication.

Vaccine and Treatment

Prior to the development of a vaccine, supportive oral rehydration therapy was the preferred treatment of choice. Today, live, attenuated vaccines are available. The first rotavirus vaccine was quadrivalent and consisted of a mixture of attenuated reassortant viruses. It was developed after evidence of protection against RV was observed in response to antibodies and T cells transferred into animal models. Studies of young children showed that primary RV infection was often symptomatic and occurred very early in life, reinfections were frequent, and protection against the virus developed in association with the presence of RV antibodies. The

original vaccine was withdrawn due to adverse effects, but two new vaccines are currently in clinical use, a monovalent vaccine and a pentavalent vaccine. Both vaccines are effective in reducing the burden of RV gastroenteritis in developed countries but do not block infection and shedding. Reduced effectiveness is seen in developing countries. The reasons for this discrepancy are under investigation, as is the effect of the vaccines on genotype distribution.

Adenoviridae

Adenoviruses are double-stranded DNA viruses that cause a range of infections in humans, including respiratory disease, gastrointestinal disease and eye infections, particularly in younger hosts. While most infections are mild infections can be severe in immunocompromised patients and neonates and result in fatal pneumonia, hepatitis, or encephalitis. The *Adenoviridae* family consists of four genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus*. Human adenoviruses (HAdV) can be found within the *Mastadenovirus* genus, while members of the other genera typically infect birds and/or reptiles. Infections are generally species-specific. At least 57 human adenovirus serotypes have been isolated and categorized into seven species or subgroups (A-G). Recombination within species is common, increasing genetic diversity. Serotypes 40 and 41 (subgroup F) are specifically associated with childhood gastroenteritis, although serotype B, C, D, and G viruses can also infect via the gastrointestinal tract, replicate there and be shed in the stool. The subgroup F adenoviruses have been less studied compared to the other HAdV due to the difficulties growing them in cell culture.

History and Epidemiology

Adenovirus was first characterized in 1953 while looking for infectious agents of respiratory disease. The incubation period of HAdV varies based on the serotype, but can range from 48 hours to two weeks. Shedding after enteric adenovirus infection lasts weeks to months. Shedding can be prolonged in immunocompromised patients, similarly to other enteric viruses. The virus can remain latent in tissues including the pharynx, intestine, urinary tract, and lymphocytes for years.

Adenoviruses are relatively resistant to UV light and thus are found widely in the aquatic environment, especially in sewage. However, chlorine disinfection is effective in eliminating infectious virus. Infections do not typically vary between seasons, unlike other enteric viruses, but do cause similar gastrointestinal symptoms. Transmission can occur after sneezing if the potential host inhales aerosolized droplets, via the fecal-oral route, or by direct exposure to mucosal membranes, blood, or tissue. Outbreaks are typically sporadic, but epidemics do occur occasionally. HAdV infects mostly infants and children and is common in places like schools, military bases, and hospitals where hosts are in close quarters. Immunocompromised patients who are infected have a higher chance of fatality and are more likely to acquire an infection after a hematopoietic stem cell or organ transplant.

Adenovirus-associated diarrhea typically occurs in children <4 years of age. Prevalence numbers vary widely depending on the location. Rates are typically between 2%–10% but as many as 38% of diarrheal cases have been attributed to Subgroup F Ads in some studies. Although these infections do not occur as frequently as rotavirus and norovirus infections, the symptoms from each virus infection are often not clinically distinguishable, and asymptomatic infections are common.

Structure

HAdV is a non-enveloped, icosahedral, double-stranded DNA virus. The capsid is ~100 nm in diameter. Fibers extending from the viral capsid engage the cellular receptor and contribute to neutralization and immune responses. The genome is approximately 36 kb long, making it one of the largest enteric viruses. More than 30 structural and non-structural proteins are encoded by the HAdV genome.

Binding and Replication

HAdV binds to target cells using the fibers that protrude from the viral capsid. Most human adenovirus types recognize the coxsackie-adenovirus receptor (CAR) protein *in vitro*, a transmembrane protein found in cells in the heart, pancreas, central and peripheral nervous system, lung, liver, and intestine. More recently, factor X has been identified *in vivo* as another receptor that bridges the interaction of the hexon with cell surface-associated heparan sulfate proteoglycans. The penton base then interacts with integrins to promote intracellular transport to the nucleus following endocytosis. Inside the nucleus, ~20 "early" viral genes are expressed first, whose gene products mediate viral DNA replication. Following replication, ~15 "late" viral genes are expressed prior to virus assembly in the nucleus. Virions are then packaged and ultimately released by cell lysis.

Pathogenesis

HAdV exhibits a tropism for many cells, including epithelial cells during an enteric infection. However, the specific tropism for Ad 40 or 41 is unknown.

Induction of type I interferons during infection is part of the host response to adenovirus. The proteins encoded by the early gene E1 and E3 encode activities to counteract host cell innate immune responses. To date, multiple mechanisms have been revealed.

They include counteracting the protein kinase R, prevention of MHC class I transport to the cell surface to evade cytotoxic T cell-mediated killing of infected cells, inhibition of tumor necrosis factor (TNF)-mediated cytolysis, and internalization and lysosomal degradation of multiple cell surface molecules, such as epidermal growth factor receptor, Fas, TNF receptor, and TRAIL receptor. While most findings were made in cell culture, limited experiments in animal models support those functions *in vivo*.

Infection with adenovirus confers life-long immunity to the infecting serotype. Adaptive immune responses are critical for this protection. Antibodies to the virus can be detected in the serum as early as 7 days post-infection, and still effectively neutralize the infection at least 10 years later. Neutralizing antibodies are directed against the three capsid proteins; the hypervariable fiber, hexon and penton base. They function extracellularly via virus aggregation, virus destabilization, blockade of virus-receptor interaction, and intracellularly by mediating proteasomal degradation or blocking microtubule spread. In addition, CD4+ and/or CD8+ T cell responses against viral capsid proteins and the DNA polymerase have also been identified and shown to be protective in humans.

Vaccine and Treatments

No specific antivirals or vaccines are available to treat adenovirus 40 and 41 infections. However, an effective oral, live adenovirus vaccine has been developed by the military against Ad 4 and Ad 7. It is given orally since the enteric infection is asymptomatic and protects well against the more severe respiratory infection typically caused by these strains. Adoptive transfer of T cells is also effective in treating Ad infections in allogenic stem cell transplant patients. In addition, acyclic nucleosides (*e.g.*, cidofovir) are effective against many DNA viruses, including adenoviruses, since these compounds have a higher affinity for viral DNA polymerases than cellular polymerases. Multiple other antiviral compounds are effective against adenovirus infections in cell culture and animal models and may be developed into effective treatments in the future.

Adenoviruses are also being used as gene therapy vectors and account for ~25% of all gene therapy clinical trials. The beneficial aspects of these vectors include the ability to grow high titer virus stocks, the ability to infect dividing and non-dividing cells of many different cell types, and the maintenance of the vector DNA as episomes instead of chromosomal integration. While Ad 40 and 41 are not in clinical use as vectors to date, they are being developed for future use in applications that target the intestine.

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Epidemiological Concepts and Historical Examples

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The Epidemiologic Approach

Epidemiology is generally defined as the study of the distribution of disease or some other physiologic characteristic and its determinants in a population. Epidemiologists are sometimes called "disease detectives" because they identify the causes of disease or "doctors for the population" because they monitor population health, and design, implement and evaluate interventions to protect the public's health. Even before the cause of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) was identified, epidemiologists demonstrated that HIV/AIDS could be transmitted by sexual contact enabling the implementation of effective strategies to prevent HIV/AIDS transmission. Epidemiologic studies identified the associations of hepatitis B and human papillomavirus with cancer which led to the development of vaccines, and the efficacy of those vaccines was evaluated in epidemiologic studies, with their eventual inclusion as recommended immunizations. On a day to day basis, epidemiologists in health departments and hospitals conduct surveillance of diseases that significantly impact public health. By monitoring disease trends epidemiologists detect disease outbreaks, and evaluate the effectiveness of treatment and prevention strategies (see Box 1 Epidemiologic Surveillance). Epidemiologists also investigate outbreaks, and design, implement and evaluate interventions to reduce disease occurrence. A cluster of salmonella cases? A measles outbreak? A case of meningococcus? An epidemiologist is the one leading the outbreak investigation and guiding interventions to prevent additional cases.

Epidemiologic studies characterize the natural course of a disease from onset to resolution, generate or test hypotheses about disease etiology, and identify disease outbreaks and their sources. They also describe the extent of disease in a population, and patterns and trends in disease occurrence—essential data for the development and evaluation of interventions aimed at preventing disease. Epidemiologic studies are designed to evaluate: (1) whether an individual with disease has characteristics that distinguish them from those without disease—generating or testing hypotheses about disease etiology; (2) if disease occurrence varies by time—suggesting that one or more factors might be present that enhance or decrease disease occurrence; and (3) if disease occurrence varies by geographic area—which can generate hypotheses about disease reservoirs (see Table 1 for definitions of common epidemiologic terms). While the methods of epidemiology, that include study design, demography, and statistics, can be broadly applied to any feature occurring in a population, this article will focus on the application to the study of diseases caused by microbes (infectious diseases), and to microbial populations.

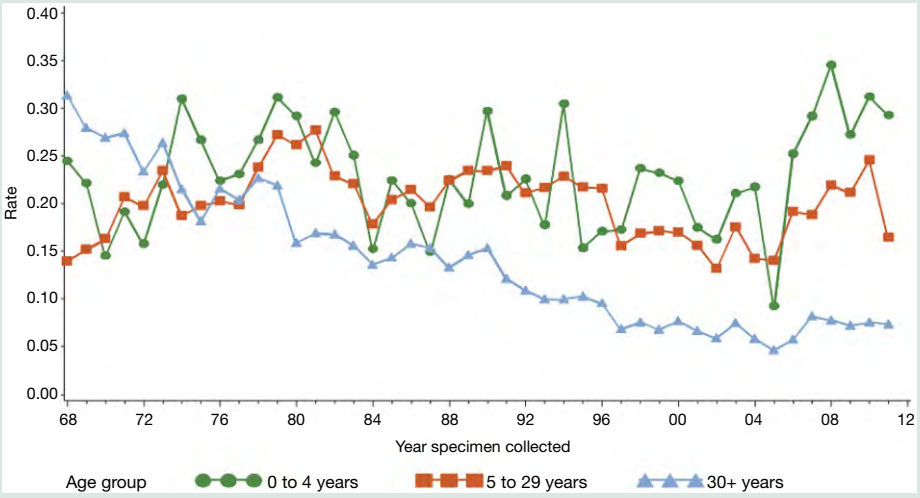
Understanding the natural history of infection is essential for diagnosis, treatment, and prevention of disease spread. The first step of infection is colonization: the time from colonization to disease can vary wildly, depending on the infecting organism, host characteristics, and presence of other factors. Some infections can be transmitted before the appearance of symptoms, others primarily only when symptoms are present, and others long after symptoms disappear. For example, chickenpox (varicella) is infectious 1–2 days before appearance of symptoms and lasts until the pox lesions are crusted. Following symptom resolution, varicella-zoster virus remains in the body. The latent virus can re-emerge with a different clinical presentation: shingles, which are also infectious. Many microbes that can cause infectious disease ("infectious agents") only cause disease under certain conditions. How many individuals become ill following exposure depends on the infectious dose, the individual's characteristics, and other factors—including the characteristics of the infectious agent. Up to 15% of the general population carries *Clostridium difficile* in their gut, however, *C. difficile* diarrhea generally only emerges following treatment with broad spectrum antibiotics (Caroff et al., 2017).

Characterizing a disease by person, place and time is a powerful method for generating hypotheses regarding the cause of disease, how it is spread, and how it might be prevented even if the cause is unknown. Dr. John Snow conducted investigations of cholera epidemics occurring in London during the 19th century. Caused by *Vibrio cholera*, cholera causes profuse watery diarrhea and vomiting leading to dehydration and shock in 5%–10% of those infected (CDC.gov). During the 19th century several waves of cholera swept Great Britain causing over 23,000 deaths in 1831–32, and at least 250,000 deaths in 1848–49. At the time cholera was defined solely by the clinical presentation. There were several hypotheses regarding the cause of cholera, but most believed that the miasma ("bad air") theory was correct. Snow conducted a series of studies that characterized cholera cases by person, place and time; the studies' results suggested that cholera might be transmitted by water. In London, water companies drew water from different parts of the river Thames, some upstream and others downstream of where the sewage was dumped. Water companies competed for customers - so individuals in the same city block or even the same house would buy water from different companies. Snow took advantage of this and carefully documented deaths by water company. In houses receiving water from the Southwark and Vauxhall Companies that took water downstream of the sewage there were 315 deaths/10,000 households, compared to 37/10,000 households whose water source was Lambeth Company that took water upstream. In the rest of London the rate of cholera deaths was 59/10,000 households. Most famously, in a later cholera outbreak, Snow tracked the outbreak to water obtained from a single water pump, leading to the removal of the pump handle. In this Broadstreet pump outbreak there were 127 cholera deaths over a 3 day period, and 616 deaths before the end of the outbreak. Fig. 1 shows Dr. Snow's map of the outbreak. Each bar represents a death occurring in a specific household. Note the clustering of deaths near the Broad Street pump, and that there are few or no

Box 1 Epidemiologic surveillance

In most countries, there is ongoing monitoring for diseases considered of importance. Some surveillance systems are a global effort; for example, there is surveillance worldwide for influenza (WHO.int influenza). Influenza surveillance monitors which influenza types are circulating, and the emergence of antigenic drifts and shifts. As influenza types vary from year to year, surveillance information is critical for vaccine design, and planning for pandemics.

Components of a surveillance system include data collection, analysis and feedback, so the results can be used to describe disease trends (including identifying outbreaks), plan and target interventions, and evaluate their effectiveness. Depending on the disease there may be one or more ongoing surveillance systems. For example, the United States Centers for Disease Control and Prevention (CDC) lists seven different surveillance systems for *Salmonella* (CDC.gov salmonella). The rate of disease from *Salmonella* serotype Typhi varies by year and age; the disease is most often detected in those aged 0–4 years (the *green line* in figure below). The CDC also monitors antibiotic resistance: in 1999 ~20% of *S. Typi* was resistant to ciprofloxacin; in 2011 it was greater than 70%.



(Figure used with permission from Centers for Disease Control and Prevention (CDC) (2013). An atlas of *Salmonella* in the United States, 1968–2011: Laboratory-based enteric disease surveillance. Atlanta: US Department of Health and Human Services, CDC.)

deaths located near other water pumps (UCLA.edu). This example highlights the power of population studies to identify how a disease is transmitted—even if the transmitting agent is not known—leading to effective interventions.

When the transmitting agent is known it is easier to demonstrate transmission—especially using modern molecular techniques for strain typing. The combination of epidemiologic methods with modern molecular techniques facilitates outbreak investigations, and also provides a powerful strategy for discovering new infectious agents. During the early part of the HIV epidemic, it was observed that many individuals with AIDS had Kaposi sarcoma, a rare type of cancer. The epidemiology and natural history of the sarcoma was different from cases of Kaposi sarcoma found in individuals without HIV/AIDS. The epidemiology suggested that the sarcoma was caused by an infectious agent—but no agent could be grown using standard culturing techniques. However, using a case-control design (see Section Epidemiologic Study Designs) and molecular tools—which did not require growing the infectious agent—a virus was identified, now known as herpes simplex virus 8 or Kaposi sarcoma virus (Chang et al., 1994). Similarly, in 1977 an explosive outbreak of pneumonia occurred in Pennsylvania; as the disease occurred mainly among attendees at a convention of the American Legion, the disease was dubbed Legionnaires' disease (Fraser et al., 1977). There was no evidence of person to person spread, but the pattern of disease suggested a common-source airborne exposure. Cases of disease occurred over a relatively short period and infected individuals tended to have stayed in or visited a single hotel. This outbreak led to a successful search for the causal agent, that was named *Legionella pneumophila* in recognition of the original outbreak (Kirby et al., 1980).

Epidemiologic approaches also can provide important insights to studies of populations of microbes. While in the laboratory the microbes studied may be clonal, in vivo they are not. HIV, for example, exists in vivo as a heterogeneous, complex mixture of quasispecies with different antigenic and phenotypic properties (Santoro and Perno, 2013). Bacteria are more likely to spread clonally within an individual, but they are similarly heterogeneous at the population level. In the United States a clone of *Staphylococcus aureus*, USA300, has been a major driver of community-associated methicillin-resistant *S. aureus* (MRSA). However, whole genome sequencing of USA300 from members of two households with persistent USA300 colonization showed notable changes in the DNA of the isolates over 15 months demonstrating adaptation to individual hosts (Uhlemann et al., 2012). Further, analysis of four individuals with recurrent USA300 MRSA infection showed variability in diversity, accumulation of mutations and mobile genetic elements—and even replacement with genetically distinct USA300 MRSA strains over a 5 year period

Table 1 Definitions of common epidemiologic terms

Term	*Definition*
Attributable risk	Difference between the risk of disease among those exposed and risk of disease among those unexposed. A measure of the excess number of disease cases associated with exposure; or if there is a causal relationship, the excess number of disease cases attributed to exposure. Also known as the risk difference.
Case-fatality rate	Number of deaths due to a specific disease divided by the number of people with that disease. Usually expressed as a percent.
Colonization	Persistent growth of a microorganism in or on a body site that does not cause disease.
Communicable period	The amount of time when a person (or animal) can transmit the disease to another person, animal or arthropod.
Endemic	The usual frequency of a disease in a community or defined geographic area.
Epidemic	A higher than expected number of cases of a disease for a given time period and geographic area as compared with previous experience with that disease in that geographic area.
Exposure	A characteristic of a person, animal or microorganism of interest that is used to categorize a population for comparison. E.g., risk of disease among persons exposed to tobacco compared to those not exposed.
Host	The person, animal or arthropod where a microorganism usually lives or can infect under natural conditions.
Incidence	The number of new infections or disease cases in a given population per unit time; can be expressed as a probability or rate.
Incubation period	The time between exposure to an infectious microorganism and appearance of signs or symptoms of illness.
Index case	The first identified case of illness in a defined population that may infect others. Term is usually used in context of an outbreak investigation.
Morbidity rate	The number of ill persons in a defined population per unit time.
Mortality rate	The number of persons dying in a defined population per unit time.
Odds ratio	The ratio of the odds of disease among exposed divided by the odds of disease among the unexposed. Is algebraically equivalent to odds of exposure among those with disease divided by the odds of exposure among those without disease. A measure of the strength of the association between exposure and disease. The odds ratio approximates the risk ratio when disease is rare.
Pathogenicity	The proportion of colonizations that result in clinically apparent infection (disease).
Prevalence	The number of existing cases of disease divided by the number in the total population for a defined time period.
Reservoir	A person, animal or media in which a microbe normally lives and grows that can be a source of infection to another host.
Risk or rate ratio	Incidence among the exposed divided by the incidence among the unexposed. A measure of the strength of the association between exposure and disease. Also known as relative risk.
Surveillance	Ongoing, systematic collection, analysis and interpretation of health data used in the planning, implementation, and evaluation of public health programs.
Transmission	Any mechanism which can spread a microbe to another host: person-to-person direct contact, indirect contact, from mother to child during pregnancy or labor and delivery (vertical), via air, a fomite or food, or via a vector (e.g., mosquito).
Virulence	The proportion of clinically apparent infections (disease cases) that result in serious disease. The case-fatality rate is one measure of virulence.

Definitions modified from (1) Evans, A. S. and Brachman, P. S. (eds.) (1991). *Bacterial infections of humans: epidemiology and control* (2nd edn.). New York: Plenum Book Company. (2) Thomas, J. C. and Weber, D. J. (eds.) (2001). *Epidemiologic methods for the study of infectious diseases*. New York: Oxford University Press. (3) Aschengrau, A., Seage, G. R. (2014). *Essentials of epidemiology in public health*. Burlington: Jones & Bartlett Learning.

(Azarian et al., 2016). This underscores the necessity of evaluating claims about the contribution of particular clones or genes to disease pathogenesis by using population-based samples, proper study design and examination of the role of cofactors and confounders. An illustrative example of this phenomena is the putative association of Panton-Valentine leucocidin (PVL) toxin with poorer clinical outcomes from invasive disease caused by MRSA. This putative association seems to have resulted from the strong correlation between PVL and MRSA in the United States. Analyses of strains from Europe where most PVL are methicillin sensitive, and from Australia where half of community MRSA clones are PVL-positive but much of PVL-positive disease is caused by methicillin sensitive *S. aureus* do not support an association of PVL with poorer clinical outcomes (Shallcross et al., 2013). This is not to say that PVL is not a virulence factor (see, for example, Bamberger, 2017; Prince et al., 2017), but the amount of severe disease caused by MSRA that is attributable to PVL alone—and thus the importance of developing specific therapies against PVL, is likely not as extensive as previously believed.

In summary, the epidemiologic approach is characterized by examining variations in population distributions. Epidemiologic surveillance is a cornerstone of public health, used to monitor population health for outbreaks and evaluate the effectiveness of interventions. Epidemiologic studies are conducted to identify causes of disease outbreaks, generate and test hypotheses about disease etiology and transmission, and evaluate the effectiveness of interventions.

Epidemiologic Study Designs

Studies in human populations are very challenging: humans vary in genetic background, behavior and environment. Large sample sizes are needed for study so variations that might modify or obscure an association can be taken into account in the analysis. Further, humans may choose whether to participate in a study, and whether to complete the entire study protocol. These choices

Fig. 1 Dr. John Snow's map of the cholera outbreak. Each black bar represents a death occurring in a specific household. Note the clustering of deaths near the Broad Street pump (*gold star*), and that there are few or no deaths located near other pumps (UCLA.edu).

may bias any observed associations and limit the generalizability of study results. Even in animal models using clonal lineages, it can be challenging to design experiments to eliminate all extraneous factors, so that the signal of an association between an exposure and outcome of interest (if it exists) can be identified, for example, cage effects. Clinical trials, experiments conducted in human populations, are usually much larger than similar experiments in animal systems because of the inherent variability in study participants. Further, it is not considered ethical to test a treatment in humans unless there is strong evidence that it might be more effective than standard of care. Therefore, because of these and other challenges (e.g., cost) a majority of epidemiologic studies are observational. Although the investigator does not assign individuals to exposure or treatments in an observational study, a carefully designed epidemiologic study can approximate an experiment (see Table 2 for a description of epidemiologic study designs). The design closest to an experiment is a cohort study where the investigator follows individuals that vary in the exposure(s) of interest until the outcome of interest occurs.

Cohort studies vary in size and purpose. Cohorts are designed to describe the natural history of disease, and to estimate the strength of association between one or more exposures and one or more outcomes of interest, for example, as a follow-up to outbreak investigation (see Box 3 in Section Outbreak Investigations). Because participants are sampled by their exposure status, cohort studies are ideal for evaluating the effects of rare exposures. Mounting and maintaining a study cohort is very expensive and therefore often are used to explore a variety of exposures and outcomes. There are several, ongoing, large, long-term cohort studies.

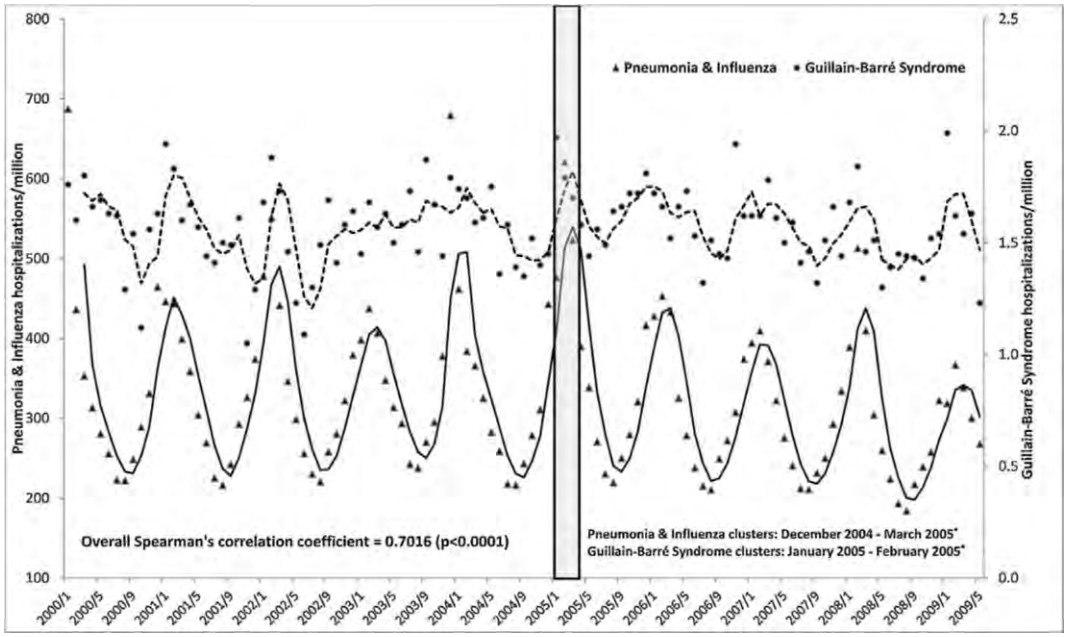


Fig. 2 Monthly rates per million population of Guillain-Barre syndrome and Pneumonia and Influenza hospitalizations in the United States, Jan. 2000–May 2009. *$P = .001$; Trend lines are 3-month moving averages. Used with permission from Iqbal, S., Li, R., Gargiullo, P. and Vellozzi, C. (2015). *Relationship between Guillain–Barré syndrome, influenza-related hospitalizations, and influenza vaccine coverage.* Vaccine **33**, 2045–2049.

Table 2 Characteristics of epidemiologic study designs, from most to least definitive

Design	*Sampling for inclusion*	*Timing of measurement*	*Parameters that can be estimated*	*Purpose*
Experiment	By population	Individual: multiple time points	● Incidence ● Risk or rate ratio, odds ratio	● Hypothesis testing
Cohort	By exposure or population	Individual: multiple time points	● Incidence rate ● Prevalence of disease or exposure ● Risk or rate ratio, odds ratio ● Attributable risk	● Hypothesis testing ● Hypothesis generating
Case Control	By disease (outcome)	Individual: single time point	● Odds ratio	● Hypothesis testing ● Hypothesis generating
Cross-sectional	By exposure, disease (outcome) or population	Individual: single time point	● Prevalence of disease or exposure, prevalence odds ratio ● Point prevalence ratio	● Descriptive ● Hypothesis generating
Ecological	By exposure, disease (outcome) or population	Groups: (e.g., neighborhood, factories, cities, counties, countries): single or multiple time points	● Correlation coefficient	● Hypothesis generating of population-level effects

Two of the best known are the Framingham Heart Study [www.framinghamheartstudy.org], begun in 1948 to study risk factors for heart disease, and the Nurses' Health Study [www.nurseshealthstudy.org], begun in 1976 to investigate the potential long-term effects of using oral contraceptives. Framingham originally enrolled 5209 men and women aged 30–62 living in Framingham Massachusetts; subsequently the original participants' children and grandchildren were enrolled. Participants complete a physical examination, questionnaires on medical history and life style, and laboratory tests every 2 years. The study has yielded over 3500 papers on the risk factors for and genetics of heart disease and related conditions. The Nurses' Health Study has enrolled over 280,000 nurses in three waves. Cohort members complete a questionnaire every 2 years about medical history and lifestyle variables, including diet. Biological specimens have been collected from a subset of the study participants. Hundreds of publications on topics ranging from effects of diet and exercise on cancer risk to rotating night shift work and heart disease have resulted.

In a case-control study, participants are sampled by the presence and absence of the outcome of interest and the investigator compares the frequency of exposure among those with and without the outcome. Cheaper to conduct than a cohort study, case-control studies often are used as part of outbreak investigations (see Box 3 in Section Outbreak Investigations). Other applications are studies of rare outcomes, as it is more efficient to compare cases with a sample of controls than to follow a cohort of 10,000 for something that occurs only in one out of a thousand individuals.

Cross-sectional studies describe the distribution of outcome and exposure at a single point in time. Cross-sectional studies are used to allocate resources, plan interventions and future cohort studies, and to generate hypotheses. Ecologic studies compare frequency of exposure and outcome among populations, rather than individuals (see Box 2).

Identifying naturally occurring events that facilitate answering research questions of interest and designing appropriate studies to address those questions is a major component of epidemiologic research. Outbreak investigations, for example, are excellent opportunities to gain new knowledge regarding the factors that influence transmission of infectious agents. The investigation of an outbreak starts with description and usually ends with conducting a case-control or cohort study or both.

Outbreak Investigations

The first step in an outbreak investigation is verifying that there is an outbreak. An outbreak or epidemic is defined as a significant increase in the number of cases of a particular disease occurring in a given geographic area for a defined time period. This entails describing the incidence rate, the number of new cases that occur over a defined time period, and comparing it to historical rates in the same population (see Table 1 for definitions of common epidemiologic terms). If the incidence rate significantly exceeds that expected for a given time period an outbreak is suspected. As measles has been eradicated in North America, a single case of measles is considered an outbreak; introduction of a new organism, for example, an antibiotic resistant organism with resistance caused by a mechanism infrequently reported in a geographic region, is also an outbreak. Assuming that the increase in cases is not due to increased testing or a change in diagnostic procedures, the next step is determining the extent of the outbreak by conducting a field investigation. A case definition is established, and then the investigator uses various methods to identify any additional cases. Methods of case finding range from reviewing medical records, calling attendees at the event associated with the outbreak, to advertising in the media. Identified cases are described by person, place and time characteristics. For outbreaks caused by microbes, strain typing information can be very important, as the increase in cases might be associated with a more virulent strain, but also might be associated with a breakdown in sterile hygiene or some other factor. If the excess of cases are all of a single strain the strain type might be used in the case definition, which increases the power of the study to identify the factor(s) that led to the outbreak. Descriptive analysis usually leads to working hypotheses about the outbreak cause which are used in the next steps of the investigation.

Following description, the investigator has a choice of study design (see Table 2 for a comparison of study designs). A cohort study is optimal if feasible. The cohort for an outbreak investigation consists of all those who were at risk of the outcome of interest. For an outbreak investigation of foodborne illness, say at a picnic, this would entail talking to everyone who attended the picnic and asking what foods they ate. The incidence of disease by exposure to various foods is calculated, and the attack rates by food are compared. As it can be challenging (and costly) to identify and contact everyone attending an event, an alternative strategy is to conduct a case-control study. The investigator identifies those meeting the case definition and compares them to a sample of those without disease but who were at risk (controls); for a picnic, controls would be picnic attendees who did not get sick. One or more controls can be selected per case. A key criteria for control selection is that they stem from the same underlying population from which the cases arose. Ideally, they would be a sample of the entire population at risk of outcome (see Further Readings for a more detailed discussion). Proper control selection is essential to minimize bias. Cohort and case-control designs provide complementary information, and both might be applied in an outbreak.

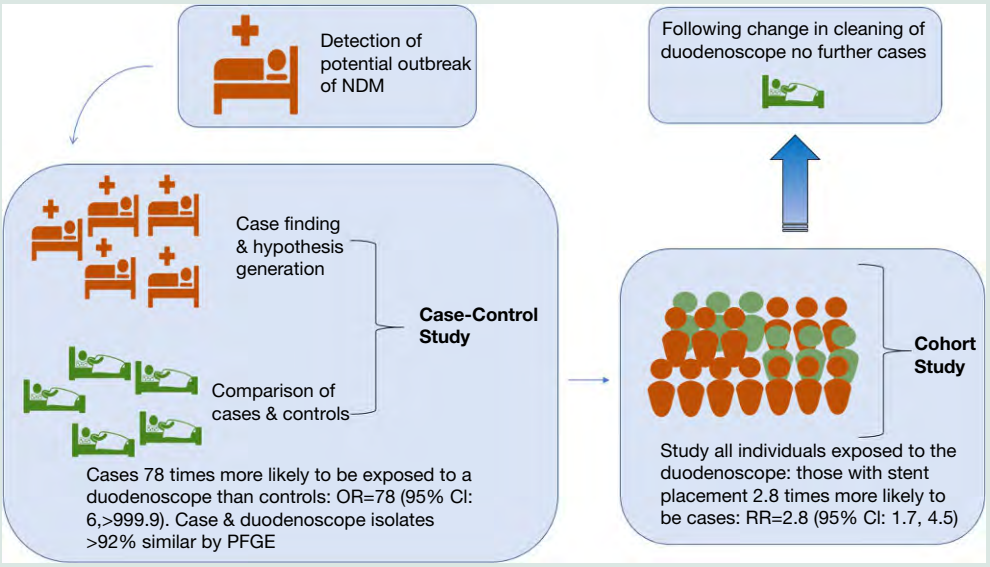
As an illustration of outbreak investigation and subsequent studies see the diagram in Box 3. After observing the occurrence of 6 cases of Delhi Metallo-β-Lactamase-Producing Carbapenem-Resistant *Escherichia coli* (NDM) in a tertiary facility several investigations were conducted (Epstein et al., 2014). At the time, a single occurrence of NDM was considered worthy of investigation to prevent transmission. After confirming the presence of NDM, investigators identified cases from clinical cultures, and performed rectal screening of cases' roommates, and patients admitted to the ward where the first patient was treated. They reviewed medical records to generate hypotheses. History of procedures using a duodenoscope was common among initial cases. Investigators then randomly selected controls from patients who had negative rectal screening cultures. After finding that cases were 78 times more

Box 2 Ecologic studies

In an ecologic study the unit of analysis is a population, e.g., a town, city, county or country. These studies generally use existing data (often collected for other purposes) to generate or test hypotheses. Correlations between population trends in disease and exposure should be interpreted with caution, but can be very useful. For example, in the 1950s, a ground breaking study by Doll and Hill showed a strong correlations between increasing tobacco use since the early 1900s and incidence of lung cancer (Doll and Hill, 1950). For some hypotheses, such as changes in disease rates by weather patterns or population-level interventions (e.g., imposing taxes to decrease consumption), an ecologic study is the only feasible method of assessing an effect. For example, Iqbal et al. demonstrated a relationship between hospitalization for pneumonia and influenza and hospitalizations for Guillain-Barre syndrome, the most common cause of acute flaccid paralysis (Iqbal et al., 2015) (Fig. 2).

Box 3 Outbreak investigation

Diagram of investigation of an outbreak of New Delhi Metallo-β-Lactamase-Producing Carbapenem-Resistant *Escherichia coli* (NDM) in a tertiary care facility (Epstein et al., 2014).



likely than controls to have undergone a procedure using a duodenoscope, the hospital notified all patients who had undergone a procedure using a duodenoscope and offered rectal screening. Duondenoscopes were cultured and compared to the isolates from cases using pulsed-field gel electrophoresis (PFGE). A single duodenoscope was implicated. All patients exposed to this duodenoscope were included in a cohort study, to identify what specific procedures were associated with increased risk of NDM transmission. After implementing a change in cleaning procedures, no further cases were identified.

Summary

The epidemiologic approach provides a powerful strategy for solving complex problems and evaluating potential solutions. Ongoing epidemiologic surveillance provides essential information for making health decisions for public health practitioners, clinicians and the public. Epidemiologic training includes study design, statistical methods, the biological basis of disease and the contribution of social factors to disease etiology. The methods are inherently interdisciplinary, integrating multiple perspectives to understand disease patterns and their determinants. Epidemiology is a key component of training for public health practitioners, infection preventionists, directors of clinical microbiology laboratories, and physicians specializing in preventive medicine, infectious diseases or antibiotic stewardship.

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Escherichia coli ☆

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Introduction

First described by Theodor Escherich from stool samples of healthy infants in 1885, *Escherichia coli* (formerly *Bacterium coli* and *Bacillus coli*), a Gram-negative facultative anaerobic nonspore-forming motile rod, has undergone considerable changes in its profiles during the subsequent decades. From an innocuous intestinal commensal to an important pathogen of both humans and animals, pathogenic *E. coli* is now known to cause a spectrum of clinical diseases in human and animal hosts, including urinary tract infection (UTI, which may sometimes evolve to haemolytic-uremic syndrome or HUS), blood and central nervous system infections (sepsis/meningitis), and gastroenteric infection.

Taxonomy

Escherichia coli is classified taxonomically in the genus *Escherichia* (named after its discoverer Theodor Escherich), family Enterobacteriaceae, order Enterobacteriales, class Gammaproteobacteria, phylum Proteobacteria. Currently, the genus *Escherichia* comprises five recognised species: *E. albetii, E. coli* (the type species), *E. fergusonii, E. hermanii* and *E. vulneris*. Two former *Escherichia* species, *E. adecarboxylata,* and *E. blattae,* have been reclassified as *Leclercia adecarboxylata* and *Shimwellia blattae,* respectively.

On the basis of its surface antigens (i.e., somatic O antigen and flagellar H antigen), *E. coli* can be separated into >190 serogroups (serotypes), with the serotype of an *E. coli* isolate being defined by a specific combination of O-and H-antigens (e.g., O157:H7). In many cases, the O-antigen (which forms part of lipopolysaccharide layer) is 'masked' by a heat-labile, acidic polysaccharide capsule (K-antigen). Specific serogroups are often associated characteristically with certain clinical syndromes, but some may be implicated in more than one category of *E. coli* pathotypes.

Using genotyping techniques, *E. coli* may be distinguished into six phylogenetic groups (A, B1, B2, D, E, and Shigella). Despite its close association with other members of *E. coli,* and possibly representing the clones of *E. coli,* the phylogenetic group Shigella has continued to be treated as a separate genus *Shigella* (which includes the species of *S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*) due to historic considerations.

In accordance with its pathogenic potential, *E. coli* is divided into non-pathogenic and pathogenic groups. Although the non-pathogenic *E. coli* exists as a commensal in the intestine of warm-blooded animals, it may be involved in opportunistic infections at times. The pathogenic *E. coli* is responsible for three main types of disease in humans: urinary tract infection (UTI, which may sometimes evolve to haemolytic-uremic syndrome or HUS), blood and central nervous system infections (sepsis/meningitis), and gastroenteric disease.

Through their unique interactions with eukaryotic cells, adhesion/colonization mechanism, toxin/virulence factor production and clinical disease profiles, the pathogenic *E. coli* strains are further distinguished into ten pathotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), Shiga-toxin-producing enteroaggregative *E. coli* (STEAEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), adherent invasive *E. coli* (AIEC), uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MAEC) (Table 1). Collectively, ETEC, EPEC, EHEC, EAEC, STEAEC, EIEC, and DAEC are known as diarrhoeagenic *E. coli*, while AIEC is enteric pathogen causing Crohn disease in which diarrhoea may present as one of the symptoms.

Interestingly, while some diarrhoeagenic *E. coli* prefer the sparsely colonised small bowel as their preferred site of infection (ETEC, EPEC), others execute their pathogenic strategies at the level of the colonic mucosa (EIEC, EHEC and probably EAEC), in more intimate-proximity to the enterocyte than non-pathogenic flora. Furthermore, whereas EHEC, EPEC and EIEC rely on type 3 secretion system (T3SS) to translocate bacterial proteins (known as effectors) directly into the eukaryotic host cell in order to subvert host cell processes, ETEC, EAEC, STEAEC, DAEC and AIEC are non-T3SS dependent pathotypes, which have comparatively simple and efficient molecular mechanisms of virulence requiring effective colonization factors followed by secretion of toxins that subsequently enter the host cell.

Structural and Genomic Characteristics

E. coli possesses a number of structural and genomic features that are important for maintaining its life cycle, and for invading and surviving inside host cells.

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Table 1 Classification and characteristics of *E. coli* pathotypes

Category	*Serogroups*[a]	*Toxin*	*Adhesin*	*Clinical disease*
Enterotoxigenic (ETEC)	Chiefly O6, O8, O15, O20, O25, O27, O63, O78, O80, O85, O115, O128ac, O139, O148, O153, O159, O167 among 117 serogroups	Heat-labile enterotoxin (LT), Heat-stable enterotoxin (ST), Cytolysin A (ClyA)	Colonization factors (CF), Porcine A/E associated adhesin (Paa)	Acute watery diarrhoea (<5 yo) Travelers' diarrhoea
Enteropathogenic (EPEC)	O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, O158	-	Intimin, Bundle forming pili (BFP) Paa, LPF, Iha, EhaA	Infant diarrhoea
Enterohemorrhagic (EHEC)	O26, O91, O111, O157	Stx	Intimin, Paa, Toxin B (ToxB), *E. coli* factor for adherence (Efa)-1, LPF, STEC autoagglutinating adhesin (Saa), *E. coli* immunoglobulin-binding protein (EibG), EHEC autotransporter encoding gene A (EhaA), Outer membrane protein A (OmpA), Iha	Food poisoning (Haemorrhagic colitis or bloody diarrhoea, HUS)
Enteroaggregative (EAEC)	O3, O15, O44, O77, O126	EAEC heat-stable enterotoxin 1 (EAST1), Shigella enterotoxin (ShET)1, Haemolysin E (HlyE)	Aggregative adherence fimbriae (AAF) (I, II, III, Hda), Toxigenic invasion loci A (Tia)	Travelers' diarrhoea, Infant diarrhoea
Shiga-toxin-producing enteroaggregative (STEAEC)	O114	Stx	AAF, IrgA homolog adhesin (Iha)	Food poisoning
Enteroinvasive (EIEC)	O28ac, O29, O112ac, O124, O136, O143, O144, O152, O164, O167	ShET1/2	-	Shigellosis
Diffusely adherent (DAEC)	O86, O127, O142, O158 (isolated from children and containing Afa/Dr genes)	-	afimbrial (Afa) or fimbrial (Dr) adhesins	Acute diarrhoea (<5 yo)
Adherent invasive (AIEC)	O6, O25, O83	-	Type 1 pili, long polar fimbriae (LPF)	Crohn's disease, with a chronic transmural, segmental, and typically granulomatous inflammation of the intestine
Uropathogenic (UPEC)	O1, O2, O4, O6, O7, O8, O16, O18, O25, O75, O78, O114, O142, O164, O157	α-Haemolysin, Cytotoxic necrotizing factor 1	P fimbriae (coded by *pap*), S fimbriae (coded by *sfa*), Afa adhesions (coded by *afa*)	Asymptomatic bacteriuria, cystitis, pyelonephritis, septic shock
Meningitis-associated (MAEC)	O1, O2, O18, O45, O83	-	P fimbrial adhesin, S fimbrial adhesin	Fever, irritability, seizures

[a]ETEC, EPEC, EHEC, EAEC, STEAEC, EIEC, and DAEC are collectively known as diarrhoeagenic *E. coli*. Some serogroups are associated with more than one diarrhoeagenic disease category. For example, although serogroup O86 is mainly considered as EPEC, it has also been implicated in ETEC, EAEC, and DAEC infections.

Sources: Clements A, Young JC, Constantinou N, Frankel G. Infection strategies of enteric pathogenic *Escherichia coli*. Gut Microbes. 2012;3:71–87; Totsika M, et al. Uropathogenic *Escherichia coli* mediated urinary tract infection. Current Drug Targets 2012;13:1386–99; Smith EJ, Thompson AP, O'Driscoll A, Clarke DJ. Pathogenesis of adherent-invasive *Escherichia coli*. Future Microbiology 2013;8:1289–300.

Fimbriae (Pili)

E. coli strains have one or two kinds of fimbriae, common and conjugative (sex pili). Common fimbriae (numbering 100–1000 per cell) are mainly composed of an acidic hydrophobic protein called fimbrin. According to the amino acid sequence of their major fimbrin, common fimbriae are separated into seven groups. Fimbriae are highly antigenic, comprising many F antigens. It is notable that *E. coli* strain K12 contains only type 1 common fimbriae, and it may alternate between the fimbriated and nonfimbriated condition, a phenomenon known as phase variation. This may be due to the possibility that the presence of common fimbriae allows organisms in their first efforts to colonize their host by attaching to epithelial cells. Inside the body, turning off the synthesis of common fimbriae may lessen the chances that the organisms will be phagocytized by white blood cells.

The sex fimbriae (usually called pili, with one or a few copies per cell) are encoded by plasmids such as F or R. These structures cause the donor and recipient bacteria to make contact, allowing the transfer of DNA during conjugation.

Flagella

E. coli possesses 5–10 flagella per cell, which are responsible for its motility. The flagella are typically 5–10 μm-long and are arranged randomly around the cell surface, a pattern called peritrichous flagellation. Structurally, *E. coli* flagella are composed of a long filament, a hook, and a basal body, with an *N*-methyl-lysine-rich protein (55 kDa in size) known as flagellin being the principal component. Around 20 000 subunits of this protein assemble to make the flagellar filament. *In vitro*, flagellin self-assembles into flagella-like filamentous cylindrical lattices with hexagonal packing.

The *E. coli* flagellar genetic system consists of about 40 genes arranged in five regions. These genes are involved in structure, function, assembly, and regulation. Flagella are highly antigenic, comprising a large number of H antigens, with the N- and C-termini of various H antigens being highly conserved.

Capsule and Outer Membrane

The outer membrane of some *E. coli* strains is covered by a polysaccharide capsule composed of K antigens. Other polysaccharides, the M antigens (colanic acids, which are polymers of glucose, galactose, fucose, and galacturonic acid), are synthesized under conditions of high osmolarity, low temperature, and low humidity. Additionally, *E. coli* has a glycolipid anchored in the outer leaflet of its outer membrane, called the enterobacterial common antigen (ECA). The outer membrane of *E. coli* consists of a lipid bilayer whose inner leaflet is made up largely of phospholipids and whose outer leaflet is made of lipopolysaccharide (LPS). Interspersed are several kinds of membrane proteins.

One membrane protein, the murein lipoprotein, is small (7.2 kDa), with 7×10^5 copies per cell. This protein contains three fatty acid residues that help anchor it into the inner leaflet of the outer membrane, whereas the rest of the molecule is located in the periplasm. About one-third of the molecules are covalently linked to the cell-wall peptidoglycan. The major outer-membrane proteins include the pore-forming proteins, called porins Omp C, Omp F, and Pho E. Together, these porins are present in about 10^5 copies per cell. Their sizes vary from 36.7–38.3 kDa. The diameters of the pores are 1.16 nm for Omp F and Pho E and 1.08 nm for Omp C. Synthesis of Omp F is repressed by high osmotic conditions or high temperature, that of Omp C is depressed by high osmotic conditions, and Pho E is synthesized when cells are starved for phosphate.

Certain compounds that are too large to diffuse through *E. coli* porin channels are carried across the outer membrane by special transport proteins. These compounds include maltose oligosaccharides, nucleosides, various iron chelates, and vitamin B12. The proteins involved, as well as the porins, also act as receptors for the attachment of bacteriophage and colicins.

Periplasm and Cell Wall

The periplasm (the space between the inner and outer membranes) of *E. coli* makes up 20–40% of the cell volume. This compartment is osmotically active, in part because it contains large amounts of membrane-derived oligosaccharides (molecules of 8–10 linked glucose residues substituted with 1-phosphoglycerol and O-succinyl esters). The *E. coli* periplasm contains over 60 known proteins, including binding proteins for amino acids, sugars, vitamins, and ions; degradative enzymes (phosphatases, proteases, and endonucleases); and antibiotic detoxifying enzymes (β-lactamases, alkyl sulfodehydrases, and aminoglycoside phosphorylating enzymes). The periplasmic environment is oxidizing, whereas that of the cytoplasm is reducing. This explains why certain secretory proteins that require disulfide bonds for activity are inactive in the cytoplasm. The cell wall of *E. coli* consists of a peptidoglycan layer responsible for cell shape and rigidity. The peptidoglycan layer (of one or a few molecules thick) is anchored to the outer membrane at some 400 000 sites via covalent links to the major membrane lipoprotein and noncovalent links to porins. The periplasm is spanned by 200–400 adhesion zones between the outer membrane and the cytoplasmic membrane. These appear to be the sites of attachment of certain bacteriophage and of export of outer-membrane proteins and lipopolysaccharide. In addition to these apparently scattered junctions, the two membranes appear joined at defined periseptal annuli, ring-shaped adhesion zones that are formed near the cytoplasmic membrane.

The cytoplasmic membrane of *E. coli* is made up of about 200 distinct proteins and four kinds of phospholipids. Proteins make up about 70% of the weight of the structure. Under aerobic conditions, the *E. coli* cytoplasmic membrane contains a number of dehydrogenases (e.g., NADH-, D- and L-lactate, and succinate dehydrogenases), pyruvate oxidase, cytochromes (of the o and d complexes), and quinones (mainly 8-ubiquinone). Anaerobically grown *E. coli* may contain other dehydrogenases (e.g., formate and glycerol- 3-phosphate dehydrogenases) and enzymes involved in anaerobic respiration (nitrate and fumarate reductases). The cytoplasmic membrane is the site of adenosine triphosphate (ATP) synthesis. The cytoplasmic membrane systems involved in the transport of solutes are highly efficient and enable its growth in relatively dilute nutrient solutions. The cytoplasmic membrane of *E. coli* contains over 20 proteins involved in various aspects of peptidoglycan biosynthesis, cell wall elongation, and cell division. About 10 of these proteins have been identified by their ability to covalently bind β-lactamantibiotics. They are known as the penicillin-binding proteins, and some have been shown to be involved directly in cell-wall synthesis.

Cytoplasm

Biochemical reactions necessary for the growth of *E. coli* mostly take place in the cytoplasm. These activities are divided into those concerned with metabolic fueling (production of energy, reducing power, and precursor metabolites), biosynthesis of building

blocks, polymerization into macromolecules, and assembly of cell structures. In fast-growing *E. coli*, much of the cytoplasmic space is taken up by ribosomes. The number of ribosomes per cell is proportional to the growth rate, ranging from about 2000 in cells growing at 37 °C peptidoglycan layer peptidoglycan layer at doubling rates of 0.2 per hour to >70 000 at a doubling rate of 2.5 h per hour, where they make up about 40% of the cell mass. In *E. coli*, the genes for the four ribosomal RNAs (16S, 23S, 5S, transfer) are arranged in seven operons located at different sites on the chromosome.

Most of these operons are found near the origin of chromosome replication and, thus, are replicated early. This arrangement ensures that ribosomal RNAs are made in large amounts during rapid growth. Each operon encodes one, two, or three transfer RNAs at sites between the 16S and 23S RNA genes or at the end of the operon. The 52 ribosomal proteins are encoded by 21 transcriptional units. Ribosomal RNAs and proteins assemble into particles via a precise sequence of reactions that has been well studied *in vitro*.

Genome and Nucleoid

The *E. coli* strain MG1655 (a laboratory strain K-12 derivative) genome consists of a circular DNA molecule of 4.64 Mb in length, with 4288 protein-coding genes (organized into 2584 operons), seven ribosomal RNA (rRNA) operons, and 86 transfer RNA (tRNA) genes. It also includes a significant number of transposable genetic elements, repeat elements, cryptic prophages, and bacteriophage remnants. The genomes of some pathogenic *E. coli* are about 1 Mb larger than that of the commensal K-12 strain, as exemplified by enterohaemorrhagic *E. coli* strains O157:H7 Sakai (5.50 Mb); enteroaggregative *E. coli* strain O42 (5.36 Mb) and UPEC isolates CFT073 (5.23 Mb), 536 (4.94 Mb), UTI89 (5.07 Mb). The extra genetic segments were probably acquired by horizontal gene transfer.

Comparative analyses of *E. coli* and *Shigella* genomes indicate that the predicted pan-genome consists of 15 741 gene families, and only 993 (6%) of the families are found in every genome, comprising the core genome. The variable or 'accessory' genes account for >90% of the pan-genome and about 80% of a typical genome. The plasticity of the *E. coli* genome is conferred mainly by two genetic configurations, virulence-related plasmids and chromosomal pathogenicity islands. Although many strains (e.g., strain MG1655) do not contain plasmid, some may possess 1–5 plasmids in their genome. All seven categories of diarrhoeagenic *E. coli* carry at least one virulence-related property on a plasmid. In the case of EIEC, EHEC, EAEC and EPEC, a member of a highly conserved plasmid family is often present. Encoding multiple virulence factors, the plasmid is typically large (>60 megadalton or MDa), of low copy number, and either conjugative or of transmissible incompatibility group. While clusters of virulence traits are encoded plasmids and pathogenicity islands, individual traits (e.g., shiga toxin) may be encoded by transposons.

Similar to other bacteria, the *E. coli* genome is condensed into a compact structure called the nucleoid. Nucleoid activities such as transcription, replication, recombination, and repair are influenced by the structural properties and the special conformations of nucleoid. The nucleoid of *E. coli* is a highly lobular intracytoplasmic region, generally located toward the center of the cell. In this region, the DNA is found at a local concentration of 2–5% (w/v). *In vivo* the DNA is negatively supercoiled into some 50 individual domains. Transcription takes place at the nucleoid–cytoplasm interface, as the nucleoid forms a significant barrier to the diffusion of many macromolecules. The highly irregular shape of the nucleoid may contribute to the availability of genes for transcription. At least four small-molecular-weight proteins that bind to DNA are known to play a role in transcription, recombination, and replication. These nucleoid-associated proteins range in molecular weight from 9.2 to 15.4 kDa. Two, HU and IHF, are among the abundant *E. coli* proteins and are present in 20–50 000 monomers per cell.

The initiation of DNA replication takes place at a specific origin site, *oriC*, and is under the influence of a protein that is highly conserved among many bacteria, DnaA. Once initiated, DNA replication takes place at a nearly constant rate in moderately fast and fast-growing *E. coli*, until it reaches a terminus. Segregation of the nucleoids takes place with considerable fidelity and, thus, cannot result from partitioning into progeny cells by chance alone. *in vitro*, recently replicated (hemimethylated) origin DNA binds to the membrane with great specificity.

Biology and Epidemiology

Biology

The central metabolism of *E. coli* is conducted via the Embden–Meyerhof–Parnas pathway, the pentose pathway, the tricarboxylic acid cycle, and the Entner–Doudoroff pathway (for the metabolism of gluconate). As a facultative anaerobe, *E. coli* fulfils its energy needs by either respiratory or fermentative pathways. Under anaerobic conditions, the main products are formate, acetate, lactate, succinate, ethanol, 2,3- butanediol, CO_2, and H_2. Its need for biosynthetic building blocks is met by the production of 12 precursor metabolites common to all bacteria. *E. coli* has a complete system of transporting and using organic phosphates, including an inducible alkaline phosphatase in its periplasm.

E. coli is a chemoheterotroph with the capability to grow on any of a large number of sugars or amino acids provided individually or in mixtures. Some strains found in nature have single auxotrophic requirement such as thiamin. The growth of many strains is inhibited by the presence of single amino acids, such as serine, valine, or cysteine. *E. coli* grows faster with glucose than with any other single carbon and energy source and reaches a doubling time of 50 min under well-oxygenated conditions at 37 °C. Slow rates of growth can be achieved by using an externally controlled continuous culture device or by adding a metabolic analogue and its antagonist to the culture at proper ratios. *E. coli* grows more rapidly in rich-nutrient broths (containing amino acids, nucleosides, sugars, and vitamin precursors, etc.), reaching doubling times of 20 min at 37 °C. *E. coli* can grow at temperatures between 8 and

48 °C, depending on the strain and the nutrient medium. Its optimum growth temperature is 39 °C. *E. coli* does not grow in media containing a NaCl concentration greater than about 0.65 M. In response to changes in the osmotic pressure of the medium, *E. coli* increases its concentration of ions, especially K+ and glutamate. The pH range for growth is between pH 6.0 and 8.0, although some growth is possible at values approximately 1 pH unit above and below this range.

Epidemiology

E. coli is well adapted to colonizing the mammalian intestine and seldom causes disease. It colonizes not only the large intestine of vertebrates but also the ileum, the distal segment of the small intestine. In the ileum, *E. coli* may have been selected for rapid growth under oxygenated conditions; in the colon, in competition for limited nutrients and in the presence of noxious chemicals under anoxic conditions. When human strains are cultivated in the laboratory, they tend to lose the ability to colonize. Included among these is K12, the most widely used laboratory strain in the molecular microbiology.

Periodically deposited from their intestinal residence into soils and waters, *E. coli* bacteria do not survive for extended periods of time in such environments and could be cultured only for a few days (seldom weeks) after their introduction. For this reason, their presence has been taken as a measure of recent fecal contamination, and the coliform count of the drinking water supply or of swimming facilities is still a common measure of microbiological water purity.

Mammals become colonized with *E. coli* within a few days of birth, possibly during passage through the birth canal or, shortly after birth, via the fecal–oral route from the mother or other attendants.

Primary reservoir of UPEC isolates is within the human intestinal tract. Two important routes by which UPEC bacteria can invade and spread within the urinary tract are the ascending and haematogenous pathways. Ascending route is a widely accepted paradigm of infection, which involves the ascension of bacteria from the gut microbiota to the vagina and then the bladder i.e., the faecal-perineal-urethral pathway and rectal flora serving as reservoir for the strains infecting the urinary tract. Uropathogens gain entry into the bladder, by means of the urethral massage that may accompany sexual intercourse. Once the bacteria ascend into the bladder, they may multiply and pass up the ureter, particularly if vesicoureteral reflux is present, and then to the renal parenchyma. The subsequent development of infection depends upon the particular organism, the inoculum size and the adequacy of host defences. Haematogenous route facilitates infection of the renal parenchyma by blood-borne organisms.

Clinical Features

E. coli strains are responsible for a large number of clinical diseases in humans, with the most common infections involving the intestinal and urinary tracts, leading to watery diarrhoea or locally invasive forms of infection (e.g., dysentery). *E. coli* infects deeper tissues, including the blood (septicemia) as a complication of focal extraintestinal infections such as pyelonephritis and, meningitis in newborns.

ETEC

Usually acquired from food or water contaminated with human or animal feces, enterotoxigenic *E. coli* (ETEC) induces watery diarrhoea of varying severity (ranging from mild, self-limiting disease to severe cholera-like, life threatening illness). Beginning with a sudden onset of watery stool (without blood or inflammatory cells), and vomiting, the disease may present with dry mouth, rapid pulse, lethargy, decreased skin turgor, decreased blood pressure, muscle cramps, and shock as a result of progressive loss of fluids (dehydration) and electrolytes (sodium, potassium, chloride, and bicarbonate). Lasting for only 3 to 4 days, the diarrhoea is self-limited; if hydration is maintained, the patients survive, without any sequelae. Besides diarrhoea in infants younger under 2 years of age in the developing world, ETEC is also associated with travellers' diarrhoea.

EPEC

Enteropathogenic *E. coli* (EPEC) is a common cause of watery diarrhoea among infants in the developing world, and is also responsible for sporadic diarrhoeal outbreaks among infants in the developed world. In addition to watery diarrhoea containing mucus but not blood, other symptoms such as vomiting, fever, malaise and dehydration may be present. These symptoms may persist for several days but chronic EPEC has been also observed. EPEC may sometimes cause bloody diarrhoea after the colonization of the mid-distal small intestine (ileum). This pathotype induces a characteristic attaching/effacing (A/E) lesion on intestinal epithelium, without the production of Shiga toxins. Two groups of EPEC have been recognised: typical EPEC (tEPEC) strains (including classical O26, O55, O86, O111, O119, O125, O126, O127, O128ac, O142, O158), and atypical EPEC (aEPEC) strains, which lack the EPEC adherence factor plasmid (or pEAF) but contain other virulence factors.

EHEC

Enterohaemorrhagic *E. coli* (EHEC) typically causes an afebrile bloody colitis (bloody stools with ulcerations of the bowel) known as hemorrhagic colitis. In about 10% of patients (e.g., children and the elderly), infection with EHEC O157 may result in haemolytic

uraemic syndrome (HUS), which is defined by acute renal failure, haemolytic anaemia and thrombocytopenia. Like EPEC, EHEC elicits an attaching and effacing lesion of the intestinal mucosa, this time of the colon. The elaboration of an oligomeric Shiga toxin (Stx1 or Stx2) contributes to the haemorrhagic colitis and to the development of the systemic sequelae of disease. Because its cytotoxins closely resemble the Shiga toxin produced by *Shigella dysenteriae* 1 (Stx1 and Stx2), EHEC is also known as Shiga toxin-producing *E. coli* (STEC).

EAEC

Enteroaggregative *E. coli* (EAEC) infection manifests clinically by a watery diarrhoea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever. EAEC can cause both an acute and a chronic (>14 days) diarrhoeal illness. Malnourished hosts may be unable to repair mucosal damage, and thus prone to persistent or chronic diarrhoea. Without enterotoxins LT or ST, EAEC adheres to HEp-2 cells in an aggregative adherence (AA) pattern, which is characterized by prominent, 'stacked brick' auto-agglutination of the bacterial cells to each other. EAEC is associated with persistent diarrhoea in the developing world and is also implicated in the developed world as causes of both outbreaks and sporadic diarrhoea among AIDS patients.

STEAEC

Shiga toxin-producing enteroaggregative *E. coli* (STEAEC) is an EAEC strain (O104:H4) that has acquired typical EHEC phenotypes, most notably Stx production. STEAEC O104:H4 is responsible for a high percentage of patients developing hemolytic uremic syndrome (HUS) and a mortality rate of 1%. The high morbidity and mortality associated with this strain may reflect the stronger adherence of EAEC compared with EHEC allowing more Stx to be transferred and more resultant pathology.

EIEC

Enteroinvasive *E. coli* (EIEC), which is biochemically, genetically and pathogenetically closely related to *Shigella* spp., induces an invasive inflammatory colitis with a watery diarrhoea syndrome indistinguishable from that caused by other *E. coli* pathotypes and *Shigella* spp. In severe cases, scanty dysenteric stools may contain blood and mucus. Fever and severe cramps may be also present. Like *Shigella*, it may invade intestinal epithelium, principally in the large intestine.

DAEC

Diffusely adherent *E. coli* (DAEC) cause a watery diarrhoea in adults and children that can become persistent. The relative risk of diarrhoea associated with DAEC increases with age of children from 18 months to 5 years. DAEC are differentiated from other diarrhoeic *E. coli* by generating a distinct adhesion phenotype on Hep-2 cells.

AIEC

Adherent-invasive *E. coli* (AIEC) has the capacity to colonize the intestinal mucosa by adhering to intestinal epithelial cells, invade intestinal epithelial cells and replicate in macrophages, contributing to the development of Crohn's disease (CD). The symptoms may range from fever, fatigue, abdominal pain, cramping, nausea, vomiting, bloody stool, mouth sores, reduced appetite, weight loss, perianal disease, to diarrhoea.

UPEC

Uropathogenic *E. coli* (UPEC) is the primary cause of urinary tract infections, including both cystitis and pyelonephritis. This pathotype has evolved a multitude of virulence factors and strategies to facilitate its growth and persistence within the adverse settings of the host urinary tract.

Pathogenesis

ETEC

ETEC produces a heterogeneous group of pertinacious surface structures known as colonization factors (Cuffs), colonization factor antigen I (CFA/I), or coli surface (CS) antigen to facilitate its adhesion and colonization to small-bowel enterocytes. The Cuffs can be fibril, non-fibril or febrile, and to date at least 23 Cuffs have been identified. ETEC febrile confer the species-specificity of the pathogen.

Following the initial adhesion and colonisation in the terminal small intestine, ETEC strains elaborate the heat-labile (LT) and/or heat-stable (ST) enterotoxins that induce diarrhoea. ETEC strains may express an LT only, an ST only, or both.

The heat-labile enterotoxins (LTs) produced by ETEC are an oligomeric class of toxins closely related in structure and function to the cholera enterotoxin (CT) expressed by *Vibrio cholerae*. The LT from human isolates (called LT-I) is ~75% identical at the amino

acid level with CT and shares several phenotypes, including its primary receptor, and mechanism of action. Found primarily in animal *E. coli* isolates and rarely in human isolates, the LT-II toxin has not been associated with disease.

LT-I (or LT) is an oligomeric toxin of ~86 kDa composed of one 28 kDa A subunit and five identical 11.5 kDa B subunits. The B subunits are arranged in a ring or 'doughnut' and bind strongly to the ganglioside GM_I and weakly to GD_{1b} and some intestinal glycoproteins. Two closely related variants of LT-I have been described, called LTp for porcine and LTh for human, which exhibit partial antigenic cross-reactivity. The A subunit is responsible for the enzymatic activity of the toxin and is cleaved by proteolysis to yield A_1 and A_2 peptides joined by a disulphide bond. The A_1 domain constitutes the active toxin and the A_2 fragment is the helical portion of the molecule and anchors the A subunit to the B pentamer which binds irreversibly to GM1 ganglioside as receptors on cell surface.

Both toxins act by changing the net fluid transport activity in the gut from absorption to secretion. LT is structurally similar to cholera toxin and activates the adenylate cyclase–cyclic adenosine monophosphate system, whereas ST works on guanylate cyclase. The intestinal mucosa is not visibly damaged, the watery stool does not contain white or red blood cells, and no inflammatory process occurs in the gut wall. Gut cells activated by LT or cholera toxin remain in that state until they die, whereas the effects of ST on guanylate cyclase are turned off when the toxin is washed away from the cell.

Genes that encode LT, called *elt* or *etx*, reside on plasmids, which may also contain genes encoding ST or CFAs. The genes are arranged as an A-B cluster in which the B ribosomal binding site and the A coding region overlap. The A and B subunits are synthesised individually and secreted through the inner membrane coupled with processing of their typical signal sequences. In the periplasm, the subunits assemble into the mature A_1B_5 configuration, which is held together by non- covalent bonds. The last four residues of the A subunit are required for stability of the holotoxin structure.

After the LT holotoxin binds to the host cell membranes, it is endocytosed and translocated through the cell in a process involving trans-Golgi retrograde transport. Through a series of subsequent steps, the major chloride channel, CFTR, of epithelial cells is activated. This leads to increased secretion of chloride ions by secretory crypt cells. In addition, LT serves to inhibit absorption of sodium chloride by villus tip cells. One additional mechanism by which these toxins may act involves prostaglandins of the E series (PGE_1 and PGE_2) and platelet-activating factor (PAF). Another alternative mechanism involves the enteric nervous system (ENS), which regulates intestinal motility and ion secretion. Serotonin (5-HT) and vasoactive intestinal peptide (VIP), both of which can stimulate intestinal epithelial cell secretion via the ENS, are released into the human small bowel after treatment with CT. A third potential mechanism could involve a mild intestinal inflammatory response due to CT and LT.

The heat-stable enterotoxins (STs) of ETEC are small, monomeric toxins that contain several cysteine residues whose disulphide bonds account for their heat stability. ST is a 71 or 72-amino acid preprotoxin which is processed into an 18–19 amino acid active toxin called STa and a 42 amino acid toxin called STb. STa is produced by both human and animal strains, whereas STb is mainly detected in strains of animal origins.

Genes for both STa and STb classes are predominantly found on plasmids and some ST-encoding genes have been found on transposons. STa (also referred to as ST-I) toxins are produced by ETEC and several other Gram-negative bacteria, including *Yersinia enteroeolitiea* and *V. cholerae* non-OI.

STa is synthesised as a 72 amino acid precursor ('pre-pro form') that is cleaved by signal peptidase 1 to a 53 amino acid peptide. The disulphide bonds are formed in this 53 amino acid 'pro-form' in the periplasm by the DsbA protein. An undefined periplasmic protease then processes pro-STa to the final 18- or 19-residue mature toxin with a molecular mass of ~2 kDa, which is released by diffusion across the outer membrane.

Two STa variants are known, designated STp (porcine) or STh (human). Both of these can be found in human ETEC isolates and are presumed to be equally pathogenic. STh and STp arc nearly identical in the 13 residues necessary and sufficient for enterotoxic activity; of these 13 residues, 6 are cysteines which form three intramolecular disulphide bridges.

The major receptor for STa is a membrane-spanning guanylate cyclase (GC-C). This belongs to a family of receptor cyclases that includes the atrial natriuretic peptide receptors GC-A and GC-B. Binding of STa to GC-C stimulates guanylate cyclase activity, leading to increased intracellular cyclic GMP levels, which in turn produces stimulation of chloride secretion and inhibition of sodium chloride absorption. Ultimately, STa is believed to stimulate the CFTR chloride channel, as does LT. The mechanism of activation by ST is apparently different, however, and may involve cGMP-dependent kinases instead of or in addition to A kinase. The secretory response to STa may also involve phospha-tidylinositol and diacylglycerol release, activation of protein kinase C, elevation of intracellular calcium, and microfilament (F-actin) rearrangement.

STb is synthesised as a 71 amino acid precursor protein, which is processed to a mature 48 amino acid protein with a molecular weight of 5.1 kDa. Unlike STa, STb induces histological damage to the intestinal epithelium, which consists of loss of villus epithelial cells and partial villus atrophy. STb also stimulates the release of PGE_2 and serotonin, suggesting that the ENS may also be involved in the secretory response to this toxin.

Apart from CF, two other proteins, the outer membrane protein Tia and the glycosylated autotransporter TibA, are shown to mediate intimate cell attachment and to induce ETEC invasion into epithelial cells. While ETEC binds to leaf surfaces through the flagellum shaft, a novel adhesin, EtpA, located on the tip of ETEC flagella mediates attachment to mammalian host cells. EtpA is degraded by the serine protease autotransporter of Enterobacteriaceae (SPATE), EatA, thereby modulating bacterial adhesion and accelerating delivery of heat labile (LT) toxin into host cells. It is thought that the long-range flagella-EtpA first anchors the bacterium to the host cell and allows shorter CFs to interact. EatA then degrades EtpA and finally intimate attachment is mediated by Tia and TibB.

EPEC

Enteropathogenic *E. coli* (EPEC) strains cause watery and sometimes bloody diarrhoea after the colonization of the mid-distal small intestine (ileum). They recognize their preferred hosts and tissues by means of plasmid-encoded surface adhesins specific for receptors on the intestinal brush-border membranes, called the bundle-forming pili (or fimbriae). Characteristic of EPEC infection is an attachment–effacement (A/E) lesion on the surface of enteric epithelial cells. The affected cells form a broad flat pedestal (effacement) beneath the attached microorganism, which, by damaging the absorptive surface (villi), contributes to the diarrhoea. The genes required for the formation of A/E lesions are located on a 35-kb pathogenicity island called the locus of enterocyte effacement (LEE). Once bound to the epithelial cells, EPEC export critical virulence factors by a type III secretion apparatus, causing several host signals to be activated.

The distinctive histopathology induced by EPEC is the attaching and effacing (A/E) lesion, which results from the intimate attachment of bacteria to the intestinal epithelial cells and subsequent effacement of enterocyte microvilli. Structurally, marked cytoskeletal changes are seen directly beneath the adherent bacteria, which sometimes sit on a pedestal-like structure, with the accumulation of polymerised actin, which can be stained using fluorescein isothiocyanate (FITC)-labeled phalloidin (so called fluorescent actin staining or the FAS test).

The pathogenesis of EPEC infection involves three steps: (i) localised adherence; (ii) signal transduction; and (iii) intimate adherence.

EPEC typically possesses a plasmid which encodes a fimbrial antigen with the ability to aggregate and form bundles (so called 'bundle-forming pilus' or BFP). Production of BFP protein induces the localized adherence (LA) pattern. Most EPEC carry a highly conserved IncF adherence plasmid (i.e., pEAF), and adherence requires at least two large and highly conserved plasmid regions. A 1-kb fragment from one of these regions has been shown to be a useful diagnostic DNA probe (the EAF probe). The N-terminal sequence of the purified fimbriae demonstrates similarity to the TCP pilus of *V. cholerae* and other members of the type IV fimbrial family. A cluster of 13 genes including *per* (encoding plasmid-encoded regulator, Per or BfpTWV) on the EAF plasmid is required for the expression and assembly of BFP. Further, the chromosomal *dsbA* gene encoding a periplasmic enzyme is also required to mediate disulphide bond formation.

After colonisation of the small bowel via BFP, EPEC initiate a more complex cascade of events leading to the A/E lesion and secretory diarrhoea. Adherence of EPEC to epithelial cells activates a variety of signal transduction pathways in the eukaryotic cell. The bacterial genes responsible for this signal transduction activity are encoded on a 35 kb pathogenicity island called the locus of enterocyte effacement (LEE), which encodes a type III secretion system, multiple secreted effector proteins, and outer membrane protein intimin. Mutation of the genes (*espA*, *espB* and *espD*) encoding the secreted proteins or the gene (*sep*) encoding the type III secretion system abolishes these multiple signaling events. The product of the *espA* gene forms a short pilus-like structure that serves both as an adhesin in the early stages of EPEC pathogenesis and as a conduit through which the other Esp proteins pass on their way to the eukaryotic cell cytoplasm.

The binding of EPEC to cultured epithelial cells triggers the release of inositol phosphates including IP_3, and IP_4 in infected cells, which results in release of calcium from intracellular stores. The increase in inositol phosphates is due probably to activation of phospholipase C in the host cell. Buffering of intracellular calcium greatly reduces the polymerisation of actin and formation of the A/E lesion. Moreover, increases in intracellular calcium can inhibit absorption or sodium and chloride ions, and stimulate chloride secretion in enterocytes. In addition, adherence of EPEC to epithelial cells results in phosphorylation of several epithelial cell proteins on serine and threonine residues, the most prominent of which is myosin light chain (MLC). Activation of protein kinase C (PKC) induces rapid changes in intestinal water and electrolyte secretion and phosphorylation of MLC together contributes to increased permeability of tight junctions.

Binding of EPEC to HeLa cells also induces protein phosphorylation on tyrosine residues. The major tyrosine-phosphorylated protein is a 90-kDa epithelial cell membrane protein called Hp90. The tyrosine-phosphorylated proteins are part of the A/E lesion and the distribution of the phosphorylated proteins is restricted to an area immediately beneath the adherent bacteria at the tip of the pedestals. The tyrosine phosphorylated Hp90 serves as a receptor for the intimin adhesin. Indeed, the signal transduction induced in epithelial cells by EPEC may induce formation of the mature receptor as well as cause subsequent cytoskeletal re-arrangements.

Intimate adherence of EPEC to epithelial cells is mediated by a 94–97-kDa outer-membrane protein called intimin which is encoded by *eae,* a gene located on the locus of enterocyte effacement (LEE). The *eae* gene is present in all EPEC, EHEC, *Citrobacter rodentium,* and *Hafnia alvei* strains capable of producing the A/E histopathology but is absent from *E. coli* of the normal flora, ETEC and other bacterial species that do not produce the A/E lesion. Intimin is essential for full virulence of EPEC strain E2348/69, but that additional virulence factors are required for disease. Intimin is immunogenic and may be useful as an antigen in EPEC vaccine development.

EHEC

Several EHEC serogroups (e.g., O26:H11, O91:H21, O111:H8, O157:NM, and O157:H7) are associated with EHEC infection in humans. Of these, O157:H7 is the most common in the United States is, whereas O26 is found with greater frequency elsewhere in the world. EHEC strains have two special characteristics of pathogenic importance. First, they produce high levels of two related cytotoxins that resemble toxins of *Shigella*, with the same protein synthesis-inhibitory action and binding specificity. These toxins are therefore called Shiga-like toxins (SLT) I and II. The SLT are cytotoxic for endothelial cells in culture. Second, they possess a gene highly homologous to the EPEC attaching and effacing pathogenicity island. In combination, the proteins encoded by this gene and

the SLT presumably damage the gut mucosa in a manner characteristic of hemorrhagic colitis. EHEC strains cause systemic manifestations (hemolytic–uremic syndrome or thrombotic thrombocytopenic purpura in adults) that are believed to be related to systemic absorption of SLT, possibly in combination with endotoxin, which up-regulates the expression of the SLT receptor on host cells. These syndromes represent the clinical response to endothelial damage of glomeruli and the central nervous system. Organ damage is sometimes permanent.

It is evident that O157:H7 may have emerged through four sequential events: (i) acquisition of an stx2-containing bacteriophage, (ii) acquisition of pO157 and the *rfb* region, (iii) acquisition of the stx1-containing bacteriophage, and (iv) loss of the ability to ferment D-sorbitol and loss of beta-glucuronidase (GUD) activity.

The shiga toxin family contains two major, immunologically non-cross reactive groups of toxins, Stx1 and Stx2. The genes for Stx1 and Stx2 are encoded on lysogenic lambdoid bacteriophage. A single EHEC strain may express either or both toxins, or even multiple forms of Stx2. As Stx2 is more potent in inducing cytotoxicity and more important in the development of HUS than Stx1, O157:H7 strains expressing Stx2 alone are more likely to be associated with progression to HUS than strains producing Stx1 alone or, curiously, both Stx1 and Stx2. Stx1 from EHEC is identical to shiga toxin from *Shigella dysenteriae* I, but for a single amino acid difference. Production of Stx1 by *E. coli* and *S. dysenteriae* is repressed by iron and reduced temperature, but expression of Stx2 is unaffected by these factors.

Structurally, Stx is composed of one enzymatically active A subunit (A1) and five identical receptor-binding B subunits (B5). The prototypic Stx1 and Stx2 toxins respectively share 55 and 57% sequence identity in the A and B subunits. After the B5 subunit binds to the specific host receptors globotriaosylceramide (Gb3) or globotetraosylceramide (Gb4), Stx is endocytosed through coated pits and is transported to the Golgi apparatus and then to the endoplasmic reticulum. The A subunit is translocated to the cytoplasm, where as an *N*-glycosidase it inhibits protein synthesis by the specific removal of a single adenine residue from the 28S rRNA of the 60S ribosomal subunit. The resulting disruption of protein synthesis leads to death of renal endothelial cells, intestinal epithelial cells, Vero or HeLa cells, or any cell which possesses the Gb3 receptor, or for Stx2 the Gb4 receptor [10].

The locus of enterocyte effacement (LEE) pathogenicity island, which confers the A/E phenotype on EPEC, is of 43 kb in *E. coli* O157:H7, which contains an additional 7.5 kb prophage sequence compared with EPEC strains (whose LEE is of 35 kb). The LEE comprises at least 41 different genes organized into three major regions: (i) a type III secretion system (TTSS) that exports effecter molecules; (ii) an adhesion called intimin and its translocated receptor, Tir, which is translocated into the host cell membrane by the TTSS; and (iii) several secreted proteins (Esp) as a part of TTSS, which are important in modification of host cell signal transduction during the formation of A/E lesions.

E. coli O157:H7 has is a highly conserved nonconjugative F-like plasmid (pO157) with a size of 92–104 kb. The pO157 shows a dynamic structure and includes different mobile genetic elements such as transposons, prophages, insertion sequences (IS), and parts of other plasmids. IS or remnants of IS are frequently associated with the virulence-related segments, which are similar to compositions of the large virulence plasmid in Shigella spp. Of the 100 ORFs in pO157, 43 show sufficient similarities to known proteins, including a haemolysin (ehxA), a catalase-peroxidase (katP), a type II secretion system apparatus (etp), a serine protease (espP), a putative adhesin (toxB), a zinc metalloprotease (stcE) and an eae conserved fragment (ecf).

The classical intestinal histopathology of haemorrhagic colitis due to *E. coli* O157:H7 includes haemorrhage and oedema in the lamina propria. Biopsies of the colon from many patients also show focal necrosis and neutrophil infiltration. The overall pattern resembles a combination of ischaemic and infection injuries similar to those described in toxin-mediated *Clostridium difficile*-associated colitis, and pseudomembranes are seen in many patients.

Similarly to EPEC, *E. coli* O157.H7 induces a host inflammatory response that is apparently linked to the attaching and effacing (A/E) histopathology. The ability of EHEC to cause the A/E lesions is probably sufficient to cause non-bloody diarrhoea, but shiga toxin (Stx) is essential for the development of bloody diarrhoea and haemorrhagic colitis. Stx induced damage to the microvasculature endothelium is the underlying mechanism of the syndrome.

The typical human renal histopathology includes swollen glomerular endothelial cells and deposition of platelets and fibrin in the glomeruli. Stx is believed to damage the glomerular endothelial cells leading to narrowing of capillary lumina and occlusion of the glomerular microvasculature with platelets and fibrin. The decreased glomerular filtration rate is presumably responsible for the acute renal failure that is typical of HUS. The fragmented erythrocytes characteristic of HUS may be the result of red cell injury caused by passage across the occluded microvasculature.

Although the simplest mechanism for HUS involves a direct cytotoxic action of Stx on renal endothelial cells, several studies also support a role for cytokines in this process. Purified Stx induces the expression of proinflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin 6 (IL-6) by murine peritoneal macrophages, as well as specific synthesis of TNFα in the kidney. TNFα and IL-1β can enhance the cytotoxic effect of Stx on human vascular endothelial cells *in vitro* and these two cytokines, as well as TNFβ and bacterial LPS, induce expression of globotriaosylceramides (Gb3) and increase binding of Stx to human endothelial cells. In clinical studies, elevated levels of IL-6 are found in serum and urine of HUS patients and the levels of IL-6 correlate with severity and outcome of disease.

EAEC

Infection with EAEC induces cytotoxic effects on the intestinal mucosa. The lesion is characterised by shortening of the villi, haemorrhagic necrosis of the villus tips and a mild inflammatory response with oedema and mononuclear infiltration of the submucosa. The bacteria adhere to enterocytes without formation of the attaching and effacing lesion [12].

The mode of action of EAEC comprises three stages. Stage I involves initial adherence to the intestinal mucosa and/or the mucus layer, and aggregative adherence fimbriae (AAF)/I and /II are the leading candidates for factors which may facilitate initial colonisation. Stage II includes enhanced mucus production, leading to deposition of a thick mucous-containing biofilm encrusted with EAEC. The mucous blanket may promote persistent colonisation and perhaps nutrient malabsorption. Stage III includes the elaboration of an EAEC cytotoxin(s) and enterotoxin which result in mucosal damage and fluid secretion.

Aggregative adherence fimbriae (AAF) are encoded by a 55–65 MDa plasmid named pAA. A transcriptional activator known as 'AggR,' encoded by pAAs, is the major EAEC virulence regulator controlling diverse virulence genes encoded by pAAs (including biogenesis of AAF) as well as by chromosomes. Adherence of EAEC to the mucosa is characterized by the formation of a thick, aggregating mucus layer inside which they survive and this biofilm production has been attributed to the activity of *fis* and *yafK* genes. A secreted protein of 10 kDa encoded by pAA and called antiaggregation protein (Aap) or dispersin, facilitates the movement of bacteria across the surface of the cells for subsequent aggregation and adherence.

EAEC produce a variety of SPATEs of either class I (cytotoxic) or class II (non-cytotoxic). Pic (protease involved in colonization, also found in Shigella flexneri and UPEC) is a class II SPATE with haemagglutinin and mucinolytic activity. It may help penetrate the mucus layer in which EAEC resides on enterocytes. Pet is a class I SPATE that is endocytosed by host cells, undergoes retrograde trafficking and utilizes the ER-associated degradation (ERAD) pathway to be released into the cytosol. Pet then cleaves the actin binding protein spectrin in the host cytosol, disrupting the actin cytoskeleton and causing cell rounding and detachment. As Pet is only present in a small minority of strains, alternative class I SPATES (Sat, SigA, EspP) may have similar roles. Sat in particular has 52% amino acid identity with Pet.

In addition, EAEC elaborate an EAEC ST-like toxin (EAST1), which is a non-SPATE toxin showing 50% homology to the enterotoxic domain of STa. EAST1 is a 38 amino acid protein of 4.1 kDa with 4 cysteine residues, unlike the 6 residues characteristic of *E. coli* STa. Similar to STa, EAST-1 activates guanylate cyclase leading to increased cGMP, although the toxigenic effect is milder than for Sta. EAST1 is detected in ~40% of EAEC strains, as well as in other *E. coli* categories, notably the EHEC. EAEC also produce cytotoxin, which is s a 108 kDa protein with enterotoxic activity. Other toxins include ShET1 (an AB_5 toxin in *Shigella flexneri*) and HlyE (a pore-forming toxin) may also contribute to EAEC virulence.

STEAEC

STEAEC outbreak strain O104:H4 carries Pic on the chromosome and a pAA-like virulence plasmid encoding AAF, AggR, Pet, ShET1 and dispersin. A second virulence plasmid encodes multiple antibiotic resistances. Besides these standard EAEC virulence determinants, STEAEC O104:H4 has a stx2-harbouring prophage integrated into the wrbA locus, allowing production of Stx, a defining characteristic of the EHEC pathotype. The outbreak strain has also acquired the IrgA homolog adhesin (Iha) and a tellurite resistance cluster, which are common features of EHEC strains. Therefore, STEAEC O104:H4 is a combination of known virulence determinants from two pathotypes.

EIEC

The current model of *Shigella* and EIEC pathogenesis involves: (i) epithelial cell penetration, followed by; (ii) lysis of the endocytic vacuole; (iii) intracellular multiplication; (iv) directional movement through the cytoplasm and (v) extension into adjacent epithelial cells. When severe, this sequence of events elicits a strong inflammatory reaction cluster of secreted effector proteins.

Both EIEC and *Shigella* invade the colonic epithelium, a process that requires specific chromosomal and plasmid-borne virulence genes.

Invasion-related genes are carried on a 120-MDa plasmid (pInv) in *S. sonnei* and a 140-MDa plasmid in other *Shigella* serotypes and in EIEC. Acquisition of the invasive plasmid (pInv) encoding the ability to invade host tissues has contributed to the evolution of both *Shigella* and EIEC from non-pathogenic *E. coli*. The *mxi* and *spa* gene clusters, which encode a plasmid-borne type III secretion system (T3SS), are required for the secretion of multiple virulence The movement of *Shigella* and EIEC in the cytoplasm is mediated by nucleation of cellular actin into a 'tail' which extends from one pole of the bacterium. As additional actin is added to this structure, the bacterium is propelled through the cytoplasm, generally in a lateral direction. A surface protein, VirG (IcsA), is essential for the nucleation of actin filaments and movement through the cytoplasm and into adjacent cells. In addition, many chromosomal genes (e.g., *virR* which encodes a histone-like protein) are carried on the chromosome with key roles in pathogenesis. VirR apparently acts in concert with VirF, a transcriptional activator encoded on pInv. VirF exerts pleiotropic effects, some of which are through the intermediate transcriptional activator VirB. EIEC strains may also produce a 63 kDa toxin designated Sen with enterotoxic activity, and mutation of the *sen* gene causes a significant diminution of the enterotoxic activity of the parent strain.

Interestingly, of the three T3SS-dependent pathotypes, EHEC and EPEC primarily remain extracellular during infection while EIEC are found intracellular. Despite these very different lifestyles and the different T3SS origins (LEE encoded or pINV encoded, respectably) EPEC/EHEC and EIEC/Shigella actually share a number of T3SS translocated proteins, e.g., EspG/VirA, EspO/OspE, NleE/OspZ reflecting similar infection strategies.

DAEC

The typical class of DAEC includes *E. coli* strains that harbour AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, and NFA-I adhesins (Afa/Dr DAEC); these strains (i) have an identical genetic organization and (ii) allow binding to human decay-accelerating factor

(DAF) (Afa/Dr(DAF) subclass) or carcinoembryonic antigen (CEA) (Afa/Dr(CEA) subclass). The atypical class of DAEC includes two subclasses of strains; the atypical subclass 1 includes *E. coli* strains that express AfaE-VII, AfaE-VIII, AAF-I, AAF-II, and AAF-III adhesins, which (i) have an identical genetic organization and (ii) do not bind to human DAF; and the atypical subclass 2 includes *E. coli* strains that harbour Afa/Dr adhesins or others adhesins promoting diffuse adhesion, together with pathogenicity islands such as the LEE pathogenicity island.

The diffusely adherent phenotype of DAEC on HEp2 cells is due largely to the action of afimbrial (Afa) or fimbrial (Dr) adhesins (Afa-Dr adhesins). While all Afa/Dr adhesins bind the DAF, a subfamily of Afa/Dr adhesins (AfaE-III, Dr and F1845 adhesins) also bind carcinoembryonic antigen-related molecules (CEACAMs) and the Dr adhesin also bind type IV collagen. A small number of DAEC strains may also express the CS20 colonization factor from ETEC.

After the Afa/Dr adhesins bind their cellular target on enterocytes (DAF or CEACAMs) they relocalize the target around the site of bacterial attachment. For the Dr adhesin this relocalization is dependent on Src kinase activation. Following target mobilization, enterocyte signaling pathways (e.g., MAPK and PI3K) are activated and IL-8 is synthesized (for the F1845 adhesin this requires HIF-1α) inducing transepithelial migration of human polymorphonuclear neutrophils (PMN). This stimulates the enterocytes to synthesize TNFα and IL-1β and upregulate DAF to strengthen bacterial adhesion. DAEC can interact with the transmigrating PMNs and induce type I pili-dependent IL-8 release. Transmigrated PMNs are also induced to undergo apoptosis after interaction with DAEC and have a diminished phagocytic capacity, prolonging bacterial persistence in the gut.

The secreted factor associated with DAEC infection is the SPATE Sat, which can induce rearrangement of the tight junction proteins ZO-1, ZO-3 and occludin increasing paracellular permeability but not transepithelial resistance and can also bind spectrin, rearrange focal adhesion associated proteins vinculin and paxillin, and cause cell detachment and caspase-independent cell death.

AIEC

AIEC has the ability to (i) adhere to and invade intestinal epithelial cells, and (ii) survive and replicate expansively within macrophages without triggering host cell death and inducing the release of tumor necrosis factor alpha. AIEC strains are clonally diverse and belong to distinct serotypes. AIEC strains carry many virulence-associated genes characteristic of extraintestinal pathogenic *E. coli* (ExPEC) strains.

UPEC

UPEC typically expresses an array of fimbrial and afimbrial adhesins, secreted toxins, haemolysin, iron acquisition systems, capsular polysaccharide etc. to facilitate entry and survival in urinary tract. Specifically, UPEC utilises type 1 and P pili to bind and invade host cells and tissues within the urinary tract, and employs iron-chelating factors (siderophores) to pilfer host iron stores. In addition, UPEC makes use of an array of toxins, such as haemolysin and cytotoxic necrotizing factor 1, to inflict extensive tissue damage, which contributes to bacterial dissemination, releasing host nutrients and disabling immune effector cells. However, unlike many diarrheogenic *E. coli* isolates, UPEC lacks the type III secretion system to inject VFs into target host cells.

Diagnosis

E. coli is a Gram-negative, non-spore forming, facultative anaerobic, rod-shaped bacterium of about 0.6×2 μm in size. On EMB agar, the bacterium often forms non-spreading colonies with a characteristic green sheen.

Biochemically, *E. coli* is capable of reducing nitrates to nitrites and producing lactate, succinate, ethanol, acetate and carbon dioxide. Most *E. coli* strains are positive for catalase, but negative for oxidase, citrate, urease and hydrogen sulfide. In addition, it is positive for indol production and the methyl red test. *E. coli* can be discriminated from the closely related *Shigella* and *Salmonella* by its ability to ferment lactose. For this reason, lactose is included as the sole added sugar together with a pH indicator in agar media. Lactose fermenting colonies (presumptively those of *E. coli*) turn a distinctive color due to the production of acid. Nevertheless, it should be noted that only about 90% of *E. coli* are lactose-positive. The indol test, positive in about 98% of *E. coli*, is a better test for biochemical differentiation from other Enterobacteriaccac. EHEC strains of the serotype O157:H7 are nearly unique in their inability to ferment sorbitol. Nonfermenting colonies are detected on sorbitol containing MacConkey agar and confirmed with a sensitive and specific latex agglutination test.

A more specific approach to define *E. coli* strains and pathotypes is through examination of their antigenic compositions, such as O (lipopolysaccharide), H (flagellar), K (capsular), and F (fimbrial) antigens (proteins), and their phage and colicin sensitivity. This is assisted with the use of polyclonal or monoclonal antibodies in various serological procedures, ranging from mannose-resistant agglutination of erythrocytes, enzyme-linked immunosorbant assay (ELISA), passive latex agglutination, to immunoprecipitation in agar and Biken test.

Use of cultured cells such as epithelial HEp-2 and HeLa cells provides a valuable means to characterise *E. coli* pathotypes. Indeed, HEp-2 cell-adherence assay allows differentiation among EPEC, EAEC, and DAEC isolates, while HeLa cell monolayers provide a reliable method to detect plaque-forming EIEC isolates. Given that LT produces morphological changes on Y1 adrenal and Chinese hamster ovarian cell lines that are neutralizable by antitoxin, tissue culture tests may be employed in preference to the rabbit ileal loop for LT detection. In addition, infant mouse physiological assay may be applied for ST identification, and guinea pig may be useful to detect keratoconjunctivitis caused by EIEC isolates.

The availability of detailed knowledge of the *E. coli* genetic structures enabled the development of rapid, sensitive and specific laboratory techniques for identification and typing of *E. coli* strains and pathotypes. Molecular and fluorogenic typing methods useful for diagnostic purposes have been developed to detect toxins, pili, and other virulence factors. For example, PCR procedures are useful for detection of *pap*, *afa*, and *sfa* genes in UPEC. In addition, multiplex PCR and real time PCR permit simultaneous detection of one or more diarrhoeagenic *E. coli* pathotypes.

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Evolutionary Theory and Experiments With Microorganisms☆

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Glossary

Adaptation A feature of an organism that enhances its reproductive success and that evolved by natural selection.
Epistasis An interaction among genes, such that the effect of a mutation depends on the genetic background in which it occurs.
Evolution Change in the genetic properties of populations and species over generations, which requires the origin of variation (by mutation or mixis) as well as the subsequent spread or extinction of variants (by natural selection and genetic drift).
Fitness Average reproductive success of a genotype in a particular environment, usually expressed relative to another, standard genotype.
Genetic drift Changes in gene frequency caused by the random sampling of genes during transmission across generations (rather than by natural selection).
Mixis Production of a new genotype by recombination of genes from two sources.
Natural selection Changes in gene frequency caused by specific detrimental or beneficial effects of those genes.
Population Group of individuals belonging to the same species and living in close proximity, so that individuals may potentially recombine their genes, compete for limiting resources, or otherwise interact.

Abbreviations

GASP Growth Advantage in Stationary Phase
MOI Multiplicity of infection

Defining Statement

Evolution in action can be studied by experiments in the laboratory using bacteria and other microorganisms with suitably rapid generation. These experiments have confirmed the main principles of modern evolutionary theory, while also providing new insights into the genetics, physiology, and ecology of microorganisms.

Review of Evolutionary Theory

Evolutionary theory seeks to explain observable patterns of biological diversity in terms of a few fundamental evolutionary processes. These processes are presumed not only to have operated in the past, but also to continue to operate today. Thus, they can be studied experimentally in the laboratory. Before discussing a broad range of experiments that have used microorganisms to examine evolutionary processes, the major elements of evolutionary theory will be reviewed.

Evolutionary Patterns

The three most conspicuous products of organic evolution are (1) the wealth of genetic variation that exists within almost every species; (2) the divergence of populations and species from one another and from their common ancestors; and (3) the manifest adaptation, or fit, of organisms to the environments in which they live.

Genetic variation

The existence of extensive genetic variation within species has been demonstrated by a variety of means. Variation in certain traits, such as seed shape in pea plants and blood type in humans, can be shown to have a genetic basis by careful examination of pedigrees. For many other traits, such as milk production in cows or body weight in humans, quantitative genetic analyses are required to partition the phenotypic variation that is due to genetic versus environmental influences. Biochemical and molecular techniques have also revealed extensive variation in DNA sequences and the proteins they encode.

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Divergence and speciation

All biological species differ from one another in some respects. It is generally possible to arrange species hierarchically, depending on the extent and nature of their similarities and differences. This hierarchy is reflected in the classification scheme of Linnaeus (species, genus, family, and so on). This hierarchical arrangement also suggests a sort of "tree of life" in which the degree of taxonomic relatedness between any two species reflects descent with modification from some common ancestor in the more or less distant past. Investigating the origins of groups and their relationships requires an historical approach, which is not amenable to direct experimentation. Even so, historically based hypotheses can be tested by phylogenetic and comparative methods, which utilize data on the distribution of traits across various groups and environments, sometimes supplemented with information from the fossil record.

The extent of evolutionary divergence that is necessary for two groups of organisms to be regarded as distinct species is embodied in the biological species concept, according to which "species are groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups" (E. Mayr, in 1942). Speciation thus refers to the historical process by which groups of organisms become so different from one another that they no longer can interbreed. However, many organisms (including most microorganisms) reproduce primarily or exclusively asexually, and the preceding definition is not applicable. For such organisms, the extent of evolutionary divergence that corresponds to distinct species is somewhat arbitrary and often more a matter of convenience than of scientific principle.

Adaptation

Many phenotypic features of organisms often exhibit an exquisite match to their environments. For example, the bacteria that live in hot springs have special physiological and biochemical properties that allow them to survive and grow at very high temperatures, which would kill most other bacteria; often these thermophiles cannot grow at all under the lower temperatures where most other bacteria thrive. However, organisms are also generally not perfectly adapted to their environments. Evidence for the imperfection of organisms can be seen when species go extinct, usually as a consequence of some change in the environment to which they cannot quickly adapt.

Evolutionary Processes

Biological evolution occurs whenever the genetic composition of a population or species changes over a period of generations. Four basic processes contribute to such change: mutation, mixis, natural selection, and genetic drift. Selection and drift will not produce evolutionary changes, however, unless there exists genetic variation among individual organisms.

Sources of genetic variation

Genetic variation among individuals is generated by two distinct processes, mutation and mixis. In terms of evolutionary theory, these processes are usually distinguished as follows: mutation refers to a change at a single gene locus from one allelic state to another (eg, *abcd*→ *Abcd*, where each letter indicates a locus), whereas mixis refers to the production of some new multilocus genotype by the recombination of two different genotypes (eg, *abcd*+*BCD*→ *aBcD*).

There are many different types of mutations, including point mutations, rearrangements, and transposition of mobile elements from one site in the genome to another. Some mutations cause major changes in an organism's phenotype; for example, a bacterium may become resistant to attack by a virus (bacteriophage) as a result of a mutation that alters a receptor on the cell surface. Other mutations have little or even no effect on an organism's phenotype: many point mutations have absolutely no effect on amino acid sequence (and hence protein structure and function) because of the redundancy in the genetic code.

Any number of factors may affect mutation rates, including both environmental agents (eg, UV irradiation) and the organism's own genetic constitution (eg, defective DNA repair genes). Evolutionary theory makes no assumptions about the rates of mutations or their biophysical bases, with one exception: mutations are assumed to occur randomly, that is, irrespective of their beneficial or harmful effects on the organism.

Recombination among genomes can occur by a number of different mechanisms. The most familiar one is eukaryotic sex, which occurs by meiosis and fertilization. Many eukaryotic microorganisms, including fungi and protozoa, engage in sexual mixis. Bacteria generally reproduce asexually, but may undergo mixis via conjugation (plasmid-mediated), transduction (virus-mediated), or transformation. Even viruses may recombine when two or more coinfect a single cell.

Unlike mutation, these various mechanisms do not necessarily produce organisms with new genes; instead, they may produce organisms that possess new combinations of genes. This can have very important evolutionary consequences. In the absence of mixis, two or more beneficial mutations can be incorporated into an evolving population only if they occur sequentially in a single lineage. Mixis allows beneficial mutations that occur in separate lineages to be combined and thereby incorporated simultaneously by an evolving population. Thus, mixis may accelerate the rate of adaptive evolution by bringing together favorable combinations of alleles.

Natural selection

One of the most conspicuous features of biological evolution is the evident "fit" (adaptation) of organisms to the environments in which they live. For centuries, this match between organism and environment was taken as evidence for the design of a Creator. But in 1859, Charles Darwin published "*The Origin of Species*", in which he set forth the principle of adaptation by natural selection.

This principle follows logically from three simple premises. First, variation among individuals exists for many phenotypic traits. Second, these phenotypic traits influence survival and reproductive success. Third, phenotypic variation in those characters that affect survival and reproductive success is heritable, at least in part. (Many phenotypic traits are subject to both genetic and environmental influences.) Hence, individuals in later generations will tend to be better adapted to their environment than were individuals in earlier generations, provided that there is heritable phenotypic variation and the environment has not changed too much in the intervening time. (Environments do sometimes change, of course, and when this happens a population or species may go extinct if it cannot adapt to these changes.)

Darwin himself did not know about the material basis of heredity (DNA and chromosomes), nor did he even understand the precise causes of heritable variation among individuals (mutation and mixis). What he clearly understood, however, was that this heritable variation did exist and its causes could be logically separated from its consequences for the reproductive success of individuals and the resulting adaptation of species to their environments.

Darwin's theories were influenced, in part, by his observations on the practices of breeders of domesticated animals and plants. These practices are now commonly referred to as artificial selection. It is useful to distinguish between artificial and natural selection, and to relate this distinction to experimental evolution in the laboratory. Under artificial selection, organisms are chosen by a breeder, who allows some but not all individuals to survive and reproduce. Individuals are thus selected on the basis of particular traits that are deemed desirable to the breeder. By contrast, under natural selection, no one consciously chooses which individuals within a population will survive and reproduce and which will not. Instead, the match between organismal traits and environmental factors determines whether or not any given individual will survive and reproduce.

At first glance, one might regard laboratory studies as examples of artificial selection. Such usage, however, would not reflect the critical distinction between artificial and natural selection, that is, whether a breeder or the environment determines which individuals survive and reproduce. In experimental evolution, an investigator typically manipulates environmental factors, such as temperature and resource concentration, but he or she does not directly choose which individuals within an experimental population will survive and reproduce. Instead, natural selection in the laboratory, like natural selection in the wild, depends on the match between organismal traits and environmental factors.

Genetic drift

The frequency of genes within populations, and hence also the distribution of phenotypic traits, may change not only as a consequence of natural selection, but also as a consequence of the random sampling of genes during transmission across generations. This random sampling is called genetic drift. In practice, it can be difficult to distinguish between natural selection and genetic drift, although statistical methods have been developed that may allow one to distinguish between these forces by comparing DNA sequences among related strains. Alternatively, by using microorganisms to study evolution experimentally, it is possible to compare the survival and reproductive success of different genotypes that are placed in direct competition with one another. With proper replication of such experiments, one can distinguish systematic differences in survival and reproductive success from chance deviations due to drift.

Experimental Tests of Fundamental Principles

Two key principles of evolutionary theory are the randomness of mutation and adaptation by natural selection. According to the former, mutations occur irrespective of any beneficial or harmful effects they have on the individual organism. According to the latter, organisms in later generations tend to be better adapted to their environment than were those in earlier generations, provided that the necessary genetic variation exists and the environment itself does not change.

Random Mutation

For many years, it was known that bacteria could adapt to various environmental challenges. For example, the introduction of bacteriophage into a population of susceptible bacteria often caused the bacterial population to become resistant to viral infection. It was unclear, however, whether mutations responsible for bacterial adaptation were caused directly by exposure to the selective agent, or whether this adaptation was the result of random mutation and subsequent natural selection. Two elegant experiments were performed in the 1940s and 1950s, which demonstrated that mutations existed prior to exposure to the selective agent, so that these mutations could not logically have been caused by that exposure.

Fluctuation test

The first of these experiments was published by Salvador Luria and Max Delbrück in 1943, and it relies on subtle mathematical reasoning. Imagine a set of bacterial populations, each of which grows from a single cell to some large number of cells (N); the founding cells are identical in all of the populations. If exposure to the selective agent causes a bacterial cell to mutate with some low probability (p), then the number of mutants in a population is expected to be, on average, pN. Although this probability is the same for each of the replicate populations, the exact number of mutants in each population may vary somewhat due to chance (just as the number of heads and tails in 20 flips of a fair coin will not always equal exactly 10). Mathematical theory shows that the expected

variance in the number of mutants among the set of replicate populations is equal to the average number of mutants under this hypothesis.

Now imagine this same set of populations, but assume that mutations occur randomly, that is, independent of exposure to the selective agent. During each cell generation, there is a certain probability that one of the daughter cells is a mutant. A mutant cell's progeny are also mutants, and so on. According to mathematical theory for this hypothesis, the variance in the number of mutants among the replicate populations should be much greater than the average number of mutants. This large variance comes about because mutations will, by chance, occur earlier in some replicate populations than in others, and each of the early mutations will leave many descendant mutants as a consequence of the subsequent population growth.

Luria and Delbrück designed experiments that allowed them to measure both the average and the variance of the number of mutants in a set of populations of the bacterium *Escherichia coli*. The observed variance was much greater than expected if exposure to the selective agent had caused the mutations. Hence, their results supported the hypothesis of random mutation.

Replica-plating experiment

Joshua and Esther Lederberg devised a more direct demonstration of the random origin of bacterial mutations, which they published in 1952. In their experiment, thousands of cells are spread on an agar plate that does not contain the selective agent; each cell grows until it makes a small colony, and the many colonies together form a lawn of bacteria (master plate). By making an impression of this plate using a pad of velvet, cells from all of the colonies are then transferred to several other agar plates (replica plates) that contain the selective agent, which prevents the growth of colonies except from those cells with the appropriate mutation. If mutations are caused by exposure to the selective agent, then there should be no tendency for mutant colonies found on the replica plates to be derived from the same subset of colonies on the master plate. However, if mutations occur during the growth of the colony on the master plate (ie, prior to exposure to the selective agent), then those master colonies that give rise to mutant colonies on one replica plate should also give rise to mutant colonies on the other replica plates. Indeed, Lederberg and Lederberg observed that master colonies giving rise to mutants on one replica plate gave rise to mutants on the other replica plates. Moreover, they could isolate resistant mutants from those master colonies, without the cells having ever been exposed to the selective agent. This experiment thus demonstrates that the mutations had occurred randomly during the growth of the colony on the master plate.

Adaptation by Natural Selection

In addition to demonstrating the random occurrence of mutations, the fluctuation test and the replica plating experiment both demonstrate adaptation by natural selection. Two other types of experiments also demonstrate adaptation by natural selection. One type is very complicated and involves monitoring the dynamics of mutation accumulation at one genetic locus, which is not under selection, in order to study indirectly what is happening at some other loci, which are under selection.

The other type of experiment that demonstrates adaptation by natural selection involves direct estimation of an evolving population's fitness relative to its ancestor, and it is conceptually quite simple. A population is founded by an ancestral clone, which is also stored in a dormant state (usually at very low temperature). The population is then propagated in a particular environment, and one or more samples are obtained after many generations have elapsed. These derived organisms are placed in direct competition with the ancestral clone under the same defined environmental conditions (after both types have acclimated physiologically to these conditions). If the derived organisms increase their number relative to the ancestral clone, in a systematic and reproducible fashion, then the evolving population has evidently become better adapted to the environment as the result of mutation and natural selection.

To distinguish the derived and ancestral types from one another in a competition experiment, it is usually necessary to introduce a genetic marker that can be scored into one of them. This genetic manipulation necessitates an appropriate control experiment to estimate the effect of the genetic marker on fitness.

Genetic and Physiological Bases of Fitness

The fact that one strain may be more fit than another in a particular environment usually says little or nothing about the causes of that difference. It is interesting to know why one strain is more fit than another in terms of their genotypes and their physiological properties. By using both classical and molecular genetic methods, one can construct genotypes of interest and then determine the effects of their differences on physiological performance and fitness.

Effects of Single and Multiple Mutations

A study by Santiago Elena and Richard Lenski examined systematically the fitness effects of a large set of random mutations. Using transposon mutagenesis, they made 225 genotypes of *E. coli* that each carried one, two, or three insertion mutations. Each genotype was then placed in competition with the unmutated progenitor strain in order to measure the fitness of the mutant relative to the progenitor. Single insertion mutations reduced fitness by about 2%, on average, and there was tremendous variability in the

mutational effects, which ranged from approximately neutral to effectively lethal under the conditions where the competitions were performed. The relationship between average fitness and mutation number was approximately log-linear. That is, subsequent mutations were neither more nor less harmful, on average, than was the first mutation. However, many pairs of mutations interacted strongly with one another, so that their combined effect on fitness was different from what was expected given their separate effects. (The overall relationship between average fitness and mutation number was approximately log-linear because some interactions were synergistic whereas others were antagonistic.) These data imply that a full understanding of the genetic basis of any organism's functional capacity cannot depend entirely on the step-by-step analysis of individual genes and pathways; instead, a more integrative approach is necessary.

Another approach is to investigate both the actual and possible alternative evolutionary paths that lead from an ancestor to a derived state that has accumulated multiple mutations. A study by Daniel Weinreich and colleagues examined a set of five point mutations in *E. coli* that, in combination, increase resistance to the β-lactam cefotaxime by a factor of ~100,000. With five mutations, there are 120 possible paths to obtain the full complement sequentially, but only 18 of them result in strictly increasing levels of resistance to this antibiotic. The large fraction of mutational paths along which antibiotic resistance either decreased or remained the same across one or more steps indicates pervasive epistasis in this system. In contrast, a study by Aisha Khan and colleagues examined the first five mutations that arose in a population of *E. coli* as it evolved in a nutrient-limited environment; the combination of these mutations increased fitness by ~30% relative to the ancestor. In this study, 86 of the 120 paths from the ancestor to the genotype with all five mutations were monotonically increasing in fitness, indicating that epistasis was not a substantial barrier to adaptation. Which of these two situations is more typical remains an open question in the field.

The study by Khan *et al*. also provided evidence of diminishing returns as populations adapt to a constant environment. That is, when they moved beneficial mutations into different genetic backgrounds, they found that the resulting fitness gains were smaller on the more-fit backgrounds than on the less-fit backgrounds. A similar tendency has been seen in other experiments, including one in which Hsin-Hung Chou and colleagues engineered a strain of *Methylobacterium extorquens* to be deficient in growth on methanol and then allowed it to evolve. They then identified four mutations that led to almost a doubling of fitness, constructed all of the possible combinations of those mutations, and measured the fitnesses of the constructed genotypes. Here, too, the fitness gains were usually smaller on the more-fit backgrounds, indicating a pattern of diminishing returns.

Beyond these detailed gene-by-gene analyses of adaptive trajectories, other studies have used the dynamics of adaptation in evolving populations to make inferences about the tempo and sustainability of evolution. In a recent study, Michael Wiser, Noah Ribeck, and Richard Lenski examined fitness changes across a set of 12 populations of *E. coli* as they evolved in and adapted to a nutrient-limited laboratory environment for 50,000 generations. Consistent with the earlier work by Kahn and colleagues, this study supported the role of diminishing-returns epistasis in adaptive evolution. However, the diminishing returns were not sufficient to stop fitness from continuing to increase. Instead, the dynamics of adaptation in this system were well described by a mathematical function called a power law, according to which improvements scale with the logarithm of time. As a consequence, fitness can increase indefinitely, but an ever-declining rate of improvement.

Fitness Effects Due to Possession of Unused Functions

A number of studies have used well-characterized bacterial genotypes to examine the effects on fitness caused by the carriage and expression of superfluous gene functions. These studies have measured the relative fitnesses of (1) bacteria with constitutive (high level) and repressed (low level) expression of enzymes for catabolism of carbon sources in media where those resources are not available; (2) prototrophic bacteria (which produce an amino acid or other required nutrient) and auxotrophic mutants (which cannot produce that nutrient) in media where the required nutrients are supplied; (3) phage-sensitive bacteria and resistant mutants when the phage are absent; and (4) antibiotic-sensitive bacteria and resistant genotypes in media that contain no antibiotics.

These studies have often, but not always, demonstrated substantial fitness disadvantages due to possession of unnecessary gene functions. In some cases where such disadvantages have been detected, they are mu greater than can be explained by the energetic costs of synthesizing the extra proteins and other metabolites. For example, a study by Daniel Dykhuizen found that the fitness disadvantage associated with synthesis of the amino acid tryptophan, when it was supplied in the medium, was much greater than could be explained on the basis of energetic costs alone. Evidently, the expression of superfluous functions can sometimes have strong indirect effects, which presumably arise through the disruption of other physiological processes.

The idea that microorganisms may have reduced fitness owing to possession of unnecessary functions has two important practical implications. First, in many bioengineering applications, microorganisms are constructed that constitutively express high levels of some compound (eg, a pharmaceutical) that can be harvested for its commercial value. However, the microorganisms themselves do not benefit from producing that compound, and so any mutant that no longer produces the compound may have a selective advantage. Such a mutation would thus spread through the population and thereby reduce the efficiency of the production process. Second, the spread of antibiotic-resistant bacteria has become a serious concern in public health. It has been proposed that the prudent use of antibiotics, including even the elimination of their use in certain environments (eg, animal feeds), might favor antibiotic-sensitive bacteria, thereby restoring the efficacy of antibiotics. This proposal rests, in part, on the presumption that resistant bacteria are less fit than their sensitive counterparts, in the absence of antibiotic, due to their possession of the superfluous resistance function. This tradeoff often appears to be the case, but sometimes antibiotic-resistant bacteria evolve solutions that reduce or even eliminate the cost of resistance, thus complicating efforts to contain their spread.

Effects Due to Variation in Essential Metabolic Activities

It is clear that the expression of unnecessary metabolic functions is often disadvantageous to a microorganism. An equally important issue concerns the relationship between fitness and the level of expression of functions that are required for growth in a particular environment. This latter issue is more difficult to address experimentally, because it demands careful analyses of subtle differences between strains in their biochemical activities. Daniel Dykhuizen, Antony Dean, and Daniel Hartl performed a pioneering study to examine the relationships between genotype, biochemical activities in a required metabolic pathway, and fitness. Their study examined growth on lactose by genotypes of *E. coli* that varied in levels of expression of the permease used for active uptake of lactose and the β-galactosidase required for hydrolysis of the lactose. (The genotypes were otherwise essentially identical.) Given that both enzymes are necessary for growth on lactose, how do the activities at each step affect the net flux through this metabolic pathway? And how does net flux affect fitness?

Metabolic control theory consists of mathematical models that describe the dynamics across multiple steps in a biochemical pathway. Using this theory, Dykhuizen and colleagues predicted how the net flux through this pathway would depend on the activities of the permease and p′-galactosidase enzymes, and they measured these activities for several different genotypes using biochemical methods. They then predicted that the relative fitness of any two genotypes would be directly proportional to their relative fluxes if lactose was provided as the sole energy source. In order to test the theory and its predictions, they estimated the relative fitnesses of the various genotypes in a medium in which lactose was the sole source of energy for growth. The observed fitnesses were very close to the predicted values.

Dykhuizen and Dean have also successfully extended this mechanistic approach to predicting fitness in competition for mixtures of lactose and glucose. However, the results to date (for both single and mixed sugars) were obtained with genotypes in which gene regulation was eliminated in order to simplify the analysis. An important challenge for the future is to include the complex dynamical effects of gene regulation in the models and experiments.

Effects of Genetic Background

It is obvious that the fitness effects caused by particular genetic differences strongly depend on the environment. For example, an antibiotic resistance gene function that is essential for survival and replication of a bacterium in the presence of antibiotic may hinder growth in an antibiotic-free environment. Similarly, the fitness effect that is due to a particular gene function may often depend on the genetic background in which that gene is found.

For example, one study found that different alleles at the 6-phosphogluconate dehydrogenase (6-PGD) locus in *E. coli* had similar fitnesses in a gluconate-limited medium, provided that these alleles were present in a genetic background that also encoded an alternative metabolic pathway for 6-phosphogluconate utilization. In a genetic background where this alternative pathway was defective, however, these alleles had quite variable fitnesses in that same medium.

In another study with *E. coli*, it was observed that the selective disadvantage associated with bacteriophage resistance mutations in a virus-free environment was substantially reduced during several hundred generations of experimental evolution. This fitness improvement resulted from secondary mutations in the genetic background that compensated for the maladaptive side effects of the resistance mutations, but which did not diminish the expression of resistance.

Other studies, including one by Stephanie Schrag, Veronique Perrot, and Bruce Levin, have demonstrated similar compensatory evolution among antibiotic-resistant bacteria. When bacteria resistant to an antibiotic first arise, they are typically less fit in the absence of the antibiotic, thus helping to control their proliferation. Over time, however, the evolving bacteria become very good competitors in the absence of antibiotic while retaining their resistance to antibiotic. That is, the bacteria find ways to "have their cake and eat it, too." Such compensatory evolution unfortunately makes it more difficult to devise strategies to manage the spread of antibiotic-resistant pathogens.

Speciation and Genetic Exchange

As populations diverge from one another over time, the potential for gene flow between them lessens. The increased barriers to gene flow between more distantly related organisms reflect both molecular processes and selective factors. At the molecular level, highly diverged DNA sequences recombine less efficiently than similar sequences. From the standpoint of selection, genes that evolved in one lineage may not function as well when they are moved into a different lineage, where they must function within a different physiological context. Therefore, when two lineages diverge and adapt to different environments for a sufficiently long time, they become separate species. An experiment by Jeremy Dettman and colleagues examined the effects of adaptation to different environments on the fitness of progeny in the yeast *Saccharomyces cerevisiae*. The researchers generated hybrid progeny by sexually crossing yeast strains, using three different types of strain pairings: (1) two strains that had independently evolved in the same environment; (2) two strains that evolved in different environments; and (3) one evolved strain and its ancestor. The hybrid progeny of strains that had both evolved in the same environment were more fit than the other types of hybrids, while the least fit progeny resulted from crosses between strains that evolved in different environments. These results demonstrate that incipient speciation can occur rapidly as populations adapt to different environments and diverge from one another, so that their hybrid progeny are not well adapted to the environment of either parent.

Over time, another process, called reinforcement, may occur that further separates and isolates two incipient species. Reinforcement occurs when organisms evolve mating preferences so that they become less likely to mate with individuals that are ecologically and genetically distinct, thereby avoiding the production of mal-adapted hybrid offspring. In another experiment with yeast, Jun-Yi Leu and Andrew Murray showed the rapid evolution of mate discrimination. After only a few dozen rounds of selection for assortative mating, yeast cells had evolved that were five times more likely to mate with their own type than with the ancestor.

Genomic Analyses of Experimental Evolution

As technologies change, so do the questions that biologists can address. Our understanding of the extent and patterns of genetic diversity has grown with each new advance. For many years, geneticists had to rely on differences that were visible to the naked eye – for example, the round and wrinkled seeds, and white and purple flowers, of the pea plant that Gregor Mendel studied to discover the basic laws of inheritance in plants and animals. Several decades ago, biochemists discovered they could discern subtle differences in proteins, caused by mutations in the genes that encode them, by examining how the variant proteins moved through gels that were subject to electrical fields. This approach revolutionized biology by demonstrating tremendous levels of genetic diversity in almost every species that was investigated, from bacteria to humans. More recently, the ability to sequence DNA molecules now allows scientists to examine and compare the hereditary information of organisms at its most fundamental level. It has become possible to sequence genes isolated directly from the environment, allowing ecologists to examine patterns of diversity even among microbes that have never been directly observed or cultured. It has also become possible to sequence the entire genome of any organism. With improving technologies and declining costs, it is becoming feasible to pursue whole-genome sequencing to discover and investigate all of the genetic changes that occur during laboratory-based evolution experiments.

A fascinating question in evolution – and one that has benefited from the improving genomic technologies – concerns the reproducibility of evolutionary outcomes. The late paleontologist Stephen Jay Gould imagined the thought experiment of "replaying life's tape" to address this question. Of course, it is impossible to rerun evolution for billions of years on the scale of an entire planet, but with microbes one can perform careful experiments to ask whether replicated populations that start with the same ancestor and evolve in the same environment will arrive at the same or different solutions to the challenges they face. A long-term study by Richard Lenski and colleagues has monitored the evolution of 12 initially identical populations of *E. coli* as they evolved in and adapted to the same laboratory environment for over 60,000 generations. They identified many parallel, or repeatable, changes in the phenotypes of the evolving lineages, including improved competitiveness in the glucose-based medium where they evolved, reduced performance on certain other sugars including ribose and maltose, larger cell volumes, altered supercoiling of their chromosomes, and so on. Based on the tremendous advances in DNA sequencing technology, a recent paper by Olivier Tenaillon, Jeffrey Barrick, and colleagues analyzed the complete genomes of 264 isolates from the first 50,000 generations across the replicate populations, allowing them to characterize the long-term dynamics of genome evolution. The authors found that many genes had acquired mutations in several or even all of the evolved lines, even though most genes acquired no substitutions in any of the lines. These data indicate a high degree of evolutionary reproducibility even at the genetic level.

Holly Wichman, James Bull and colleagues previously documented even more striking evolutionary reproducibility in the bacteriophage (φ)X174. In this case, the reproducibility, or parallelism, often extended to the nucleotide level. Christopher Herring, Bernhard Palsson and colleagues have used new methods for rapidly resequencing entire bacterial genomes to identify several genes that changed repeatedly in short-term experiments in which *E. coli* evolved in and adapted to a glycerol-based medium. Gregory Lang and colleagues also used this approach with the yeast *S. cerevisiae*, finding many cases in which multiple mutations arise at similar times and compete to become fixed within a given population. They also found a subset of genes that showed striking parallelism as mutational targets across dozens of replicate populations. Outside the laboratory, these new sequencing approaches have been used to study evolutionary dynamics in clinical settings, which sometimes provide a so-called "natural experiment" in which multiple infected hosts are analogous to replicate culture vessels. For example, Tami Lieberman and colleagues sequenced over a hundred genomes of pathogenic *Burkholderia dolorosa* that were isolated over many years from the lungs of patients with cystic fibrosis, finding a number of genes that underwent parallel evolution during the adaptation to different individual hosts.

Genome-level resequencing was also used by Gregory Velicer and colleagues to discover mutations that arose during a two-stage evolution experiment with the bacterium *Myxococcus xanthus*. This species is fascinating because cells, when they are starving, form multicellular aggregations. Some of the cells in these aggregations differentiate into stress-tolerant spores that can be dispersed and germinated in more favorable environments, but the majority of cells die while forming a mound-like structure that elevates the spores and helps them disperse. In the first stage of their experiment, Velicer and colleagues evolved *M. xanthus* strains that "cheated" on their progenitors during aggregation and differentiation. By themselves, these cheaters were poor at aggregating and producing spores, but when they were mixed with their cooperative progenitors, the cheaters were disproportionately likely to end up among the surviving spores. In the second stage of this evolution experiment, Velicer and colleagues found one cheater-derived strain that had recovered the ability to cooperate, although its mechanism of cooperation was somewhat different from the ancestral cooperator. By resequencing the genomes of both the evolved cheater and the restored cooperator, and comparing them to the ancestral genome, they found 14 mutations that led to the cheater, but only a single mutation – although obviously an important one – was responsible for the evolved restoration of the cooperative behavior.

Genetic Variation Within Populations

In nature, there exists abundant genetic variation in most species, including microorganisms. Some of this variation exists within local populations, while other variation may distinguish one population from another. This section describes some of the dynamic processes that influence genetic variation within populations.

Transient Polymorphisms

A population is polymorphic whenever two or more genotypes are present above some defined frequency (eg, 1%). A polymorphism arises whenever an advantageous mutation is increasing in frequency relative to the ancestral allele. This type of polymorphism is called transient, because eventually the favored allele will exclude the ancestral allele by natural selection.

Selective Neutrality

At the other extreme, some polymorphisms may exist almost indefinitely precisely because the alleles that are involved have little or no differential effect on fitness. Such selectively neutral alleles are subject only to genetic drift. Daniel Dykhuizen and Daniel Hartl sought to determine whether some polymorphic loci in natural populations of *E. coli* might exist because of selective neutrality, or whether other explanations are needed. To that end, naturally occurring alleles at certain loci were moved into a common genetic background, and the fitness effects associated with the various alleles were determined. Even when the bacteria were grown under conditions where growth was directly dependent on the particular enzymes encoded by these loci, there were often no discernible effects on fitness due to the different alleles. These results therefore support the hypothesis that random genetic drift is responsible for some of the genetic variation that is present in natural populations.

Frequency-Dependent Selection

In the course of growth and competition in a particular environment, microorganisms modify their environment through the depletion of resources, the secretion of metabolites, and so on. When this happens, the relative fitness of genotypes may depend on the frequency with which they are represented in a population, and selection is said to be frequency-dependent. Frequency-dependent selection can give rise to several different patterns of genetic variation.

Stable equilibria

Two (or more) genotypes can coexist indefinitely when each has some competitive advantage that disappears as that genotype becomes more common. In that case, each genotype can invade a population consisting largely of the other genotype but cannot exclude that other genotype, so that a stable equilibrium results.

Several different ecological interactions can promote a stable equilibrium. For example, an environment may contain two different resources. If one genotype is better at exploiting one resource and another genotype is superior in competition for the second resource, then whichever genotype is rarer will tend to have more resource available to it, thereby promoting their stable coexistence. In some cases, a resource that is essential for one genotype may be produced as a metabolic by-product of growth by another genotype; such interactions are often called cross-feeding. Stable coexistence of genotypes in one population can also occur when the environment contains a population of predators (or parasites); predator-mediated coexistence requires that one of the prey genotypes be better at exploiting the limiting resource while the other prey genotype is more resistant to the predator. The evolution of two or more stably coexisting bacterial types from a single ancestral type has been demonstrated in several experiments involving both cross-feeding and predator–prey interactions.

A striking example of the rapid evolution of several stably coexisting genotypes comes from an experiment on *Pseudomonas fluorescens* performed by Paul Rainey and Michael Travisano. The experiment started with a single clone that was placed in a static (unshaken) flask containing a nutritionally rich liquid medium. Within a few days, the bacteria evolved into three distinct genotypes that could be distinguished by the appearance of their colonies, and these types then coexisted with one another. By reconstituting the various combinations of these three types, the authors showed that each type had a selective advantage when it was rare relative to one or both of the other types. As a consequence, each genotype could invade and coexist with the others, so that a stable community was formed. However, if the medium and cells were thoroughly mixed by physically shaking the flask, then this coexistence was disrupted and one genotype prevailed. The three genotypes coexisted in the static flask because they had evolved different abilities to exploit gradients, such as in oxygen concentration, which were generated by the organisms' metabolic activities in concert with the physical environment. When these gradients were eliminated by continually shaking the flask, stable coexistence of the three genotypes was impossible.

Unstable equilibria

Those ecological interactions that promote the stable coexistence of two or more genotypes contribute to the maintenance of genetic variation in populations. However, certain ecological interactions give rise to unstable equilibria. An unstable equilibrium exists when each of two genotypes prevents the other from increasing in number. Such interactions do not promote polymorphisms

within a local population. However, they may contribute to the maintenance of genetic differences between populations, because neither type can invade a resident population of the other type.

One form of ecological interaction that can give rise to an unstable equilibrium is interference competition. This interaction occurs when one genotype produces a toxic substance that inhibits the growth of competing genotypes; it is distinguished from exploitative (scramble) competition, which occurs by the depletion of resources. Many microorganisms secrete such toxins, including fungi that produce antibiotics. Similarly, some strains of *E. coli* produce colicins that kill some competing strains of *E. coli*, but to which the producing genotypes are immune. The producing types, when common, make so much toxins that they can eliminate a sensitive strain that is more efficient in exploitative competition. When the producing cells are rare, however, the cost of their colicin production is greater than the benefit of the resource that becomes available by the killing of sensitive cells, and the producing type loses out to the more efficient colicin-sensitive competitor. The outcome of competition between colicin-producing and sensitive strains also depends on the physical structure of the environment, as shown in experiments by Lin Chao and Bruce Levin. In particular, the advantage shifts to the colicin-producing cells on agar surfaces, even when they are rare, because the resources made available by the killing action of colicins accrue locally to the producers, rather than being dispersed evenly as in a well-stirred liquid medium.

Nontransitive interactions

In some cases, the frequency-dependent interactions among three or more genotypes are so complex that they become nontransitive. For example, genotype *A* out-competes genotype *B*, and genotype *B* out-competes genotype *C*, but genotype *C* out-competes genotype *A*. A familiar example is the game of rock-paper-scissors, in which rock beats scissors, scissors beat paper, and paper beats rock. Benjamin Kerr, Brendan Bohannan and colleagues studied an example of this game played by three strains of *E. coli* on the surface of agar plates. One strain produced a toxic colicin, while the second strain was sensitive to the colicin. The third strain was resistant to the colicin, although it did not produce any toxin. In pairwise interactions, the colicin-producing strain out-competes the sensitive strain by poisoning it. The resistant strain out-competes the producing strain by not wasting resources to produce the colicin, as both strains are resistant to it. The sensitive strain, in turn, out-competes the resistant strain because there is no colicin around, and the resistant strain pays a fitness cost for its resistance. This research team further showed, both by experiments and in computer simulations, that the three strains could coexist with one another only in a spatially structured environment, such as on the surface of an agar plate. By contrast, in a well-mixed liquid medium, one strain dominates, although the identity of the winner depends on the initial abundances.

As another example, Charlotte Paquin and Julian Adams found nontransitive interactions in populations of the yeast, *S. cerevisiae*, that were evolving in chemostats fed with glucose as a sole carbon source. Nontransitive interactions can lead to situations in which the average fitness of an evolving population declines relative to some distant ancestor, even though each successive dominant genotype has increased fitness relative to its immediate predecessor. Indeed, Paquin and Adams observed precisely this phenomenon.

Evolution in a Changing Environment

Most evolution experiments operate within a controlled, defined environment, which facilitates the analysis and interpretation of these experiments. However, many environments change over time owing to either external processes, such as a change in temperature, or internal processes, such as resource depletion or coevolution of interacting species. This section examines research on the evolution of bacteria in response to prolonged resource deprivation, while the next section will address the coevolution of interacting species.

When bacteria are inoculated into fresh medium, they grow exponentially until they exhaust the available nutrients, at which point the cells typically enter a "stationary" phase during which they neither grow nor die, at least for many hours or even days. However, if the bacteria are left in the nutrient-depleted medium indefinitely, they eventually enter a death phase, although not all the cells necessarily die even after a very long time. Roberto Kolter and Steven Finkel studied the ecological and evolutionary dynamics in long-term cultures of *E. coli* that were grown in a nutrient-rich medium and then left for over 5 years without additional nutrients, except that sterile water was added occasionally to compensate for evaporation. Some 99% of the cells died over the first few weeks of the death phase. If that rate of decline had continued throughout the experiment, then there would soon have been no survivors at all. However, Kolter and Finkel found that the surviving cells entered a sort of second stationary phase, in which the population declined only very slowly. During this period, the rates of cell division and death were nearly equal, so that the population was dynamic rather than static. Moreover, the environment itself changed continually owing to the buildup and breakdown of metabolic by-products released by both living and dying cells. This changing environment, in turn, favored mutants that were better able to survive and grow under these challenging conditions than was the strain used to begin this experiment. The authors called these winners "GASP" mutants because they had a growth advantage in stationary phase (GASP). Further analyses of this system found multiple waves of GASP mutants that arose and swept to high frequency in the population, only to be displaced later by other, even tougher, mutants. Thus, many different mutations can produce GASP phenotypes, and the various mutants differ in the details of their physiological and ecological advantages.

Coevolution of Interacting Genomes and Species

Microorganisms in nature rarely, if ever, exist as single species, as they are usually studied in the laboratory. Rather, they exist in complex communities of many interacting populations. Some interactions are exploitative, such that one population makes its living by parasitizing or preying upon another population. Other interactions are mutualistic, such that each population obtains some benefit from its association with the other. In many cases, these interactions are plastic both genetically and ecologically. For example, a single mutation in a bacterium may render it resistant to lethal infection by a bacteriophage. And a plasmid that confers antibiotic resistance may be beneficial to its bacterial host in an antibiotic-containing environment but detrimental in an antibiotic-free environment.

As a consequence of this variability, microorganisms have proven useful for investigating questions about the coevolution of interacting populations. Are there evolutionary "arms races" between host defenses and parasite counter defenses? Why are some parasites so virulent to their hosts, whereas others are relatively benign? How can mutualistic interactions evolve, if natural selection favors "selfish" genes that replicate themselves even at the expense of others?

Exploitative Interactions

A number of studies have demonstrated the stable coexistence of virulent bacteriophage (lytic viruses) and bacteria in continuous culture. In these studies, the virus population may hold the bacterial population in check at a density that is several orders of magnitude below the density that would be permitted by the available resource if viruses were not present. In most cases, however, bacterial mutants eventually appear that are resistant to the virus, and these mutants have a pronounced selective advantage over their virus-sensitive progenitors. The proliferation of bacteria that are resistant to infection by the original virus provides a selective advantage to host-range viral mutants, which are capable of infecting the resistant bacteria. Thus, one can imagine, in principle, an endless "arms race" between resistant bacteria and extended host-range viruses.

In fact, there are constraints that often preclude this outcome. Experiments performed by Lin Chao, Richard Lenski, and Bruce Levin using *E. coli* and several lytic viruses found that bacterial mutants eventually evolved for which it was very difficult or impossible to isolate corresponding host-range viral mutants. This asymmetry may arise because bacterial resistance can occur via mutations that cause either the structural alteration or the complete loss of certain receptors on the bacterial surface, whereas viral host-range mutations can counter only the former. Despite this asymmetry, these experiments also demonstrated that the virus population often persisted because the virus-resistant bacterial mutants were less efficient than their sensitive progenitors in competing for limiting resources. In such cases, the result was a dynamic equilibrium, in which the growth-rate advantage of the sensitive bacteria relative to the resistant mutants was offset by death due to viral infection. Such tradeoffs between competitiveness and resistance commonly occur, because the same receptors used by viruses to adsorb to the cell envelope often serve to transport nutrients into the cell or to maintain its structural integrity.

A widely held belief is that a predator or parasite that is too efficient or virulent will drive its prey or host population extinct, thereby causing its own demise. However, virulent phage often coexists with bacteria, even though successful reproduction of the virus is lethal to the infected bacterium. Moreover, the process of natural selection does not involve foresight, so the mere prospect of extinction cannot deter the evolution of more efficient predators or more virulent parasites. Nevertheless, there exist many viruses (lysogenic and filamentous bacteriophage) that are replicated alongside the host genome, and whose infections, although deleterious, are not necessarily lethal. These viruses, as well as conjugative plasmids, have life cycles that include both horizontal (infectious) and vertical (intergenerational) transmission.

At present, the evolutionary forces that favor these alternative modes of transmission are not fully understood. One factor that is thought to be important is the density of hosts. If susceptible hosts are abundant, then there are many opportunities for horizontal transmission. In that case, selection favors those parasites that replicate and infectiously transmit themselves most rapidly, regardless of the consequences of these activities for the host's fitness. On the other hand, if susceptible hosts are scarce, then horizontal transmission becomes infrequent. Vertical transmission, by contrast, does not depend upon the parasite or its progeny finding another host. Instead, the success of a vertically transmitted parasite is determined by the success of its infected host. The greater the burden that such a parasite imposes on its host, the slower the host can reproduce its own genome and that of the parasite. Hence, when the density of susceptible hosts is low, selection may favor those parasites that minimize their replicative and infectious activities, and thereby minimize their deleterious effects on the host.

Two studies sought to test this hypothesis by manipulating the supply of susceptible hosts. One experiment, by Jim Bull and colleagues, with a filamentous bacteriophage supported the hypothesis. The other study, by Paul Turner and Richard Lenski, used a conjugative plasmid, and their experiment did not support that hypothesis, for reasons that are unclear. However, both studies demonstrated genetically encoded tradeoffs between the parasites' rates of horizontal and vertical transmission. That is, parasites that were transmitted between individual hosts at higher rates reduced the host's growth rate – and hence their own vertical transmission – more severely than did parasites that were infectiously transmitted at lower rates.

In addition to subtle changes in the interaction between a virus and its current host, evolution can occur when a virus evolves the ability to infect a different host species. Most viruses are severely constrained in the organisms that they can infect because they must enter their host via specific proteins or other receptors on the host's cell surface, and these receptors typically differ between host species. Two viral genomes can only recombine genetically when they both infect the same host. Therefore, if a virus evolves the new

ability to infect a different host species and, moreover, loses its ability to infect its previous host, then this host shift may lead to speciation, whereby a single virus population eventually evolves to become two distinct types of virus.

A study by Wayne Crill, Holly Wichman, and Jim Bull examined the adaptation of a virus to two different host species. They alternated a bacteriophage population between two bacterial hosts, *E. coli* and *Salmonella enterica*. The experiment was designed so that the virus saw only "naive" bacteria that had not previously been exposed to the virus; hence, the bacteria could not evolve resistance that might complicate their analysis of the evolving virus. Because the viral genome is small, the authors could completely sequence several isolates of the virus after each round of their adaptation to the alternating hosts. The authors found a single base pair in the virus genome that alternated in perfect concordance with the host species to which the virus had most recently adapted. Owing to this and other mutations, viral growth rate was always higher on the current host than on the alternate host. However, there was a strong asymmetry in this host-specific adaptation. Specifically, adaptation to *S. enterica* led to reduce viral growth on *E. coli*, but adaptation to *E. coli* did not cause a comparable reduction in growth on *S. enterica*. This finding has potential relevance for the production of vaccines, because it is common practice to produce weakened versions of infectious viruses by adapting them to new cell types, and then using these attenuated viruses in vaccines. If viral adaptation to a new host type does not always impair viral growth on its usual host, then it becomes all the more necessary to test carefully the safety of such putatively attenuated viruses. More generally, these results show that even single mutations can sometimes allow viruses to infect new host species, a finding that is of interest not only for understanding evolution but also to public health.

Mutualistic Interactions

It has been proposed that many mutualisms evolved from formerly antagonistic interactions. In fact, mathematical models predict that, at low host densities, genetic elements such as plasmids and phage can persist only if they are beneficial to their host. Many plasmids encode functions useful to their bacterial hosts, including resistance to antibiotics, catabolic pathways, production of bacteriocins, and so on. Also, some plasmids are unable to promote conjugation, thus relying exclusively on vertical transmission. This limitation requires that the plasmid does not harm its host, because any plasmid-free derivative would otherwise out-compete those cells that carry a costly plasmid. Indeed, several studies have found unexpected competitive advantages for bacteria that are infected by plasmids, transposons, and even temperate phage, relative to cells that are not infected but otherwise genetically identical.

Two studies have even demonstrated the evolution of mutualistic interactions from formerly antagonistic associations. Kwang Jeon showed that the growth of the protist *Ameba proteus* was initially greatly reduced by a virulent bacterial infection. The harmful effects of the bacteria were diminished, however, by propagating the infected amoebae for several years. In fact, the amoebae eventually became dependent on the bacterial infection for their viability. In another study, Judith Bouma and Richard Lenski found that a certain plasmid initially reduced the fitness of its *E. coli* host in antibiotic-free medium. However, the plasmid enhanced the fitness of its host in this same medium after 500 generations of experimental evolution. Interestingly, the mutation responsible for the newly evolved mutualistic interaction was in the host, not in the plasmid. Both of these experiments show that hosts can become dependent on, or otherwise benefit from, formerly parasitic genomes, thus giving rise to mutualistic interactions.

Evolution of New Metabolic Functions

Microbes exhibit a tremendous diversity of metabolic activities, some of which function in degradative pathways (catabolism) while others work in synthetic pathways (anabolism). How has this diversity evolved? One area of research in the field of experimental evolution seeks to elucidate the various processes by which microorganisms can acquire new metabolic functions. This research is timely as humans seek to employ certain microbes that degrade toxic pollutants in the environment, and to harness others that may be useful in the production of biofuels.

Acquisition by Gene Transfer

Perhaps the simplest way in which a microorganism can acquire some new metabolic function is by gene transfer from another microorganism that already encodes that function. For example, antibiotic resistance functions are often encoded by plasmids, which are transmitted from donors to recipients by conjugation. However, acquiring new functions by gene transfer is not always so simple. Biodegradation of certain recalcitrant compounds may require the complex coordination of several steps in a biochemical pathway, which are encoded by complementary genes from two (or more) different microorganisms. The acquisition of activities that depend on such pathways may require not only genetic exchange, but also subsequent refinement of the new function by mutation and natural selection.

Changes in regulatory and structural genes

In a number of studies, microorganisms have been shown to evolve new metabolic functions without any horizontal gene exchange. The evolution of these new functions often occurs by selection for mutations in existing regulatory or structural genes that previously encoded some other function. For example, the bacterium *Klebsiella aerogenes* cannot normally grow on the sugar D-arabinose, although it does possess an enzyme, isomerase, that is able to catalyze the conversion of D-arabinose into an

intermediate, D-ribulose, which can be further degraded to provide energy to the cell. This isomerase is normally expressed at a very low level that does not permit growth on D-arabinose. Robert Mortlock and colleagues demonstrated that mutations in regulatory genes that increase the level of expression of this isomerase are sufficient to enable *K. aerogenes* to grow on D-arabinose. They also showed that the ability to grow on D-arabinose could be further improved by certain mutations in the structural gene that change the amino acid sequence of the isomerase in ways that improve its efficiency in converting D-arabinose into D-ribulose.

Mutations that place existing genes in a new regulatory context can also have profound effects on an organism's phenotype. After more than 30,000 generations into the long-term evolution experiment with *E. coli* discussed elsewhere, one of the 12 populations grew visibly turbid, as bacteria began to grow on citrate that is present in the medium as a chelating agent. At first this growth was thought to indicate contamination, because *E. coli* cannot normally grow on citrate in an oxygen-containing environment. However, work led by Zachary Blount showed that a subpopulation had evolved the ability to consume the citrate in the medium. Blount's work further showed that a genetic rearrangement – specifically, tandem duplication – had placed a previously unexpressed gene encoding a citrate transporter protein under the control of a promoter that was expressed in the presence of oxygen as the cells exhausted the glucose on which they fed. Even with this mutation, efficient growth on citrate was contingent on one or more mutations that had evolved earlier in this lineage, and subsequent mutations were also important for the bacteria to effectively exploit its new ecological niche.

These studies, as well as other experiments and comparative analyses, show that the evolution of new metabolic functions often involves "borrowing" gene products that were previously used for other functions. It is not surprising that this process may sometimes also encroach upon, and disrupt, the previous function. Such encroachment could, in turn, favor gene duplications, whereby a single copy of an ancestral gene gives rise to two copies, each of which may then evolve toward different metabolic capabilities.

Evolution of Genetic Systems

The process of adaptation by natural selection requires genetic variation in traits that influence the survival and reproduction of organisms. As discussed earlier, the two sources of genetic variation are mutation and mixis. Rates of mutation and mixis depend not only on environmental factors (eg, ultraviolet irradiation), but also on properties of the "genetic system" intrinsic to the organism itself. Here, genetic system refers to all those aspects of the physiology, biochemistry, and reproductive biology of an organism that affect rates of mutation and mixis. For example, organisms have mechanisms of varying efficacy to promote the accurate replication and repair of their DNA. And while sex is an integral part of reproduction for some organisms, many others reproduce asexually, so that the progeny are usually identical to their parent and siblings.

Some of the most interesting questions in evolutionary biology concern the significance and evolutionary consequences of alternative genetic systems. Why do some organisms reproduce sexually, whereas others reproduce asexually? If mutation generates variation that is necessary for adaptation by a population, but most mutations have deleterious effects on the individual, then what mutation rate is optimal? Might organisms somehow be able to choose only those mutations that are beneficial to them, given their present circumstances?

Sexuality and Mixis

Sexual reproduction imposes several costs relative to asexual reproduction. These costs include finding a mate, the risk of disease transmission, and, in higher organisms, the genetic dilution that occurs because a female produces an offspring that carries only half of her genes. Therefore, biologists have long sought to understand the advantages for sexual reproduction that could overcome these disadvantages. Numerous hypotheses have been proposed, and all of them depend, in one way or another, on the genetic variation that results from mixis. Most efforts to test these hypotheses have relied on comparing distributions of sexual and asexual organisms to find variables that correlate with reproductive mode. However, several experiments have tested the evolutionary consequences of mixis using microbes in which one can manipulate the extent of intergenomic recombination.

For example, mixis in viruses can be experimentally manipulated by varying the multiplicity of infection (MOI) of host cells, since recombination of viral genotypes can occur only if two or more viruses infect the same host cell. An experimental study by Russell Malmberg compared the rate of adaptive evolution in bacteriophage populations propagated at high and low MOI; the total number of viruses per population was standardized for both treatments. The average fitness increased more rapidly under the high-MOI (equal to high recombination) treatment than under the low-MOI (equal to low recombination) treatment. This result is consistent with the hypothesis that sexual populations can adapt more rapidly than asexual ones because two or more beneficial mutations can be incorporated simultaneously in the former, but only sequentially in the latter.

Another experiment indicates that the benefit of mixis in accelerating adaptive evolution may depend on the environment. Matthew Goddard, Charles Godfray, and Austin Burt compared the rate of fitness improvement in evolving sexual and asexual populations of the yeast *S. cerevisiae*. By deleting two genes in the yeast, they were able to construct an asexual version that could not undergo meiosis. They then established separate populations of sexual and asexual yeast in both harsh and benign environments, and allowed the yeast to evolve for several hundred generations. Neither sexual nor asexual strains showed significant improvement in the benign environment, implying that they were already well adapted to those conditions. However, both strains adapted to the

harsh environment, with the rate of improvement consistently greater for the sexual than the asexual populations. This experiment suggests, therefore, that genetic recombination can be especially important for adaptation to stressful and changing environments.

Some experiments have suggested that another advantage of mixis may occur when the rate of deleterious mutation is high and the effective population size is very small. Such conditions may apply especially to microorganisms with high error rates during replication (eg, RNA viruses) or those with large genomes (eg, protozoa), if their populations experience periodic "bottlenecks." In these cases, deleterious mutations tend to accumulate in asexual lineages, a process called "Muller's ratchet" (after the geneticist, H. J. Muller, who first described this phenomenon). However, even occasional mixis can purge lineages of their accumulated load of deleterious mutations, as demonstrated by Lin Chao and colleagues using a segmented RNA virus that infects bacteria. This effect occurs because two recombining genomes may each complement the deleterious mutations that are present in the other, thereby generating some progeny that have a reduced load of deleterious mutations (as well as other progeny with an increased load, which will be removed by natural selection).

In some cases, mixis might not be an adaptation to recombine genes but rather a coincidental consequence of the movement between cells of parasitic entities. In many bacteria, for example, mixis occurs only when cells are infected by viruses (transduction) or plasmids (conjugation). The new combinations of chromosomal genes that sometimes result from such infections may occasionally be advantageous. However, one cannot view phage and plasmids as benevolent agents of bacterial carnal pleasure, because these infectious agents often harm or even kill their hosts.

Evolutionary Effects of Mutator Genes

"Mutator" genes increase the mutation rate throughout an organism's genome by disrupting DNA repair functions. Experiments performed by Lin Chao and others have investigated the effect of mutator genes on bacterial evolution. These studies have revealed a pattern that seems, at first glance, rather curious. When a mutator gene is introduced in a population above a certain initial frequency (eg, 0.1%), it tends to increase in frequency over the long term. However, if that same gene is introduced at a frequency below that threshold, then it typically goes extinct.

What causes this curious effect? In a sense, there is an evolutionary race between two clones, with and without the mutator gene, to see which one gets the next beneficial mutation. The rate of appearance of new mutations for each clone depends on the product of its population size, N, and its mutation rate, u. When the ratio of mutation rates for the mutator and nonmutator clones, u'/u, is greater than the inverse ratio of their population sizes, N/N', then the mutator clone is more likely to have the next beneficial mutation and thus prevail over the long term. When u'/u is less than N/N', the nonmutator clone, by virtue of its greater numbers, is likely to produce the next beneficial mutation and thereby exclude the mutator clone.

However, this explanation presents a problem for understanding the evolution of mutators in nature, where they are moderately prevalent in some circumstances. If mutator genes are useful only when they are common, then how do they become common in the first place? Mathematical models and evolution experiments with bacteria indicate that a process called "hitchhiking" can resolve this paradox. In hitchhiking, a deleterious mutation (such as the one that disrupts DNA repair) gets carried along to high frequency if it is genetically linked to a beneficial mutation. In bacteria, which usually have a single chromosome and reproduce asexually, the entire genome is effectively linked. Moreover, a mutator gene is more likely than any other deleterious mutation to be associated with a new beneficial mutation because of the high mutation rate it causes. It is unlikely that any particular mutation that produces a mutator will yield a beneficial mutation that allows the mutator to hitchhike. Given enough time, however, one "lucky" mutator may do so, and the mutator gene can then increase in frequency by hitchhiking with the beneficial mutation that it caused.

It has also been proposed that mutator genes may be more common in pathogenic bacteria than in their nonpathogenic counterparts. The idea is that pathogenic bacteria face especially rapidly changing selective conditions owing to immunological and other host defenses. By having a high mutation rate, pathogens would have a better chance of evolving a counter defense. This explanation also fits well with the hitchhiking hypothesis, because every change in the host that favors a new mutation in the pathogen population creates an opportunity for a mutator allele to hitchhike to high frequency. By contrast, for an organism living in a constant environment to which it is already adapted, beneficial mutations would be much rarer and hence there would be fewer opportunities for a mutator gene to hitchhike. Antonio Oliver, Fernando Baquero, Jesús Blázquez, and colleagues examined the frequency of mutator genes in samples of *Pseudomonas aeruginosa*, a bacterial species that often causes chronic (long-term) lung infections in people with cystic fibrosis. This same species is common in the environment, and it can cause acute (short-term) infections in patients with severe burns or otherwise weakened immune systems. The authors documented a much higher frequency of mutator clones in samples from patients with chronic infections, which supports the hypothesis that frequent changes in host immunity and antibiotic regimens can promote the evolution of high mutation rates.

An experimental study by Csaba Pal, Angus Buckling, and colleagues examined the effect of viral parasites on the evolution of mutation rates in bacteria. They found that *P. fluorescens* that were coevolving in environments with bacteriophage present were much more likely to become mutators than were bacteria that were evolving in the absence of the bacteriophage. This study also supports the general hypothesis that rapidly changing environments – including coevolving parasites as well as hosts – can favor the evolution of elevated mutation rates.

Thus, aspects of genetic systems that increase variation – whether by mutation or mixis – may accelerate adaptive evolution. On the other hand, mutation and mixis can also break down genotypes that are already well adapted to particular environments. The evolution of genetic systems may often reflect the balance between these opposing pressures.

Directed Mutations?

The experiments of Luria and Delbrück and the Lederbergs demonstrated that mutations arose before bacteria were exposed to a selective agent, and thus the mutations were not a response by the bacteria to that agent. However, in 1988, John Cairns and colleagues called into question the generality of random mutation in bacteria. Their paper and some other studies seemed to show that certain mutations occurred only (or more often) when the mutants were favored, and such mutations were called "directed." However, several subsequent experiments indicated that some of the evidence for directed mutation was flawed or misinterpreted, and this subject became very controversial. However, there is now widespread agreement that the most radical hypotheses put forward to explain this phenomenon – for example, a reverse flow of information from the environment through proteins and RNA back to the DNA – are incorrect.

Nonetheless, this controversy generated renewed interest in bacterial mutation, especially the mechanisms by which a cell might exercise some control over the mutational process. Attention became focused on understanding the effects of stress, such as due to starvation, on DNA repair and mutation, as well as the extent of variation among local DNA sequences in their mutability. For example, numerous studies have documented unusually high mutation rates in short repeated sequences (eg, TTTT); the mutations are typically frameshift events involving the loss or gain of a repeated element, and they occur via the slippage of DNA strands during replication. These hypermutable sequences are not distributed randomly throughout bacterial genomes. Rather, they are found more often in genes encoding products (such as fimbriae and lipopolysaccharides on the cell surface) that are involved in pathogenicity and evasion of host immune surveillance. This distribution suggests that bacteria have evolved a simple but effective strategy to increase the mutation rate in genes that help them cope with unpredictable aspects of the environment, without inflating the load of deleterious mutations in "housekeeping" genes whose products interact predictably with the environment. These mutations are apparently random insofar as a particular mutation does not occur as a direct response to an immediate and specific need, but they are nonrandom in their genomic distribution and may thereby promote a more favorable balance between evolutionary flexibility and conservatism.

Evolving Multicellularity

Experimental evolution is also being used to investigate certain major transitions in evolution. In a pioneering study, William Ratcliffe, Michael Travisano, and colleagues demonstrated that a simple selection procedure that favored faster settling in the yeast *S. cerevisiae* led to multicellular yeast. That is, those individuals that reached the bottom of the culture vessel the fastest were used to seed the next generation. In principle, this procedure might have favored sticky cells that simply adhered to one another. However, subsequent analyses revealed that the clusters – shaped almost like snowflakes – were formed by cells that did not completely separate after division. Moreover, these clusters continue to replicate as multicellular configurations, with daughter clusters breaking off from an existing cluster, and then growing in size before releasing their own multicellular propagules. The ability to observe such a major transition in the laboratory demonstrates the power of experimental approaches to addressing evolutionary questions.

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Exotoxins☆

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Glossary

AB structure–function Structure–function organization of most bacterial exotoxins; the **A** domain comprises a catalytic activity and the **B** domain comprises the binding and translocation domains.
Exotoxin A soluble protein produced by a microorganism that can enter a host cell and catalyze the covalent modification of a cellular component to alter host-cell physiology.
Heat-stable enterotoxins Soluble peptides that are secreted by bacteria, which bind to host cells and stimulate a signal transduction pathway within that cell.
Mechanism of action The specific reaction by which each exotoxin modifies a host-cell component, such as the ADP-ribosylation of elongation factor-2 by diphtheria toxin.
N-terminal and C-terminal Bacterial toxins are often organized into distinct domains termed the amino- terminal (N-terminal) and carboxy-terminal (C-terminal) domain based upon their location within the primary amino acid sequence of the toxin.
Pore-forming toxins Soluble monomeric proteins that are secreted by bacteria, which bind to the surface of the host cell and oligomerize to form a pore to release soluble components from the host cell.
Posttranslational modification A covalent modification to a cellular component that occurs subsequent to its synthesis.
Proenzyme The form in which exotoxins are secreted by bacteria; these are processed to exhibit catalytic activity.
Superantigens Soluble proteins that are secreted by bacteria and bind to the major histocompatibility complex of antigen presenting cells outside the peptide-binding grove. This complex binds to the T cell receptor of other T lymphocytes, which stimulates antigen-independent poly-clonal proliferation of T lymphocytes and cytokine secretion.
Toxoid The detoxified form of an exotoxin that is used for immunization. Conventional toxoiding is achieved with chemicals such as formalin; genetic engineering approaches can also produce toxoids.
Type III secreted cytotoxins Soluble proteins that are translocated into the host-cell cytoplasm via a type III secretion apparatus by host-cell surface-bound bacteria. Subsequent studies identified several analogous secretion systems termed the type IV, V, and VI secretion systems which also deliver cytotoxins from the bacterium into the host cell.
Vaccination The administration of an immunogen (toxoid) to stimulate an immune response that protects the host from infection by the microorganism that produces the immunogen.

Exotoxins are a group of soluble proteins which are secreted by bacteria that enter host cells, and catalyze the covalent modification of a host-cell component(s) to alter the host-cell physiology. Both Gram-negative and Gram-positive bacteria produce exotoxins. A specific bacterial pathogen may produce a single exotoxin or multiple exotoxins. Each exotoxin possesses a unique mechanism of action, which is responsible for the elicitation of a unique pathology. Thus, the role of exotoxins in bacterial pathogenesis is unique to each exotoxin. For example, *Corynebacterium diphtheriae* produces diphtheria toxin, which is responsible for the systemic pathology associated with diphtheria, whereas *Vibrio cholerae* produces cholera toxin, which is responsible for the diarrheal pathology associated with cholera. Exotoxins vary in their cytotoxic potency, with the clostridial neurotoxins being the most potent exotoxins for humans. Exotoxins also vary with respect to the host that can be intoxicated. Exotoxin A of *Pseudomonas aeruginosa* can intoxicate cells from numerous species, whereas other toxins, such as diphtheria toxin, are more restricted in the species that can be intoxicated. Some bacterial toxins, such as pertussis toxin, can intoxicate numerous cell types, whereas other exotoxins, such as the clostridial neurotoxins, show a specific affinity and intoxicate only cells of neuronal origin. Bacterial exotoxins catalyze specific post-translational modifications of host cell components, such as the ADP-ribosylation reaction catalyzed by diphtheria toxin or the deamidation reaction catalyzed by the cytotoxic necrotizing factor of *Escherichia coli*. These chemical modifications may either inhibit or stimulate the normal action of the target molecule to yield a clinical pathology. Bacterial exotoxins possess an **AB** structure - function organization, in which the **A** domain comprises the catalytic domain and the **B** domain comprises the receptor-binding domain and the translocation domain. The translocation domain is responsible for the delivery of the catalytic **A** domain into an intracellular compartment of the host cell.

Many bacterial exotoxins can be chemically modified to toxoids that no longer express cytotoxicity, but retain immunogenicity. Bacterial toxins can also be genetically engineered to toxoids, which may lead to a wider range of vaccine products. Exotoxins have also been used as therapeutic agents to correct various human disorders, including the treatment of muscle spasms by botulinum toxin. Nontoxic forms of exotoxins have been used as carriers for the delivery of heterologous antigens to elicit an immune response

☆*Change History:* FC Blum and JT Barbieri updated text throughout. Figures 1 and 3 and the figure legends were updated. References and Websites were updated.

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and as agents in the development of cell-specific chemotherapy. In addition, bacterial toxins have been used as research tools to assist in defining various eukaryotic metabolic pathways, such as G-protein-mediated signal transduction.

Classification of Exotoxins

Exotoxins are soluble proteins produced by microorganisms which can enter a host cell and catalyze the covalent modification of a cellular component(s) to alter host-cell physiology. The term 'host cell' refers to either vertebrate cells or cells of lower eukaryotes, such as protozoa because some bacterial exotoxins intoxicate a broad range of host cells. The recognition that some pathogenic bacteria produced soluble components capable of producing the pathology associated with a particular disease was determined in the late nineteenth century. Roux and Yersin observed that culture filtrates of *Corynebacterium diphtheriae* were lethal in animal models and that the pathology elicited by the culture filtrate was similar to that observed during the infection by the bacterium. Subsequent studies isolated a protein, diphtheria toxin, from the toxic culture filtrates and showed that the administration of purified diphtheria toxin into animals was sufficient to elicit the pathology ascribed to diphtheria. Diphtheria toxin is a prototype exotoxin and has been used to identify many of the biochemical and molecular properties of bacterial exotoxins.

The ability of a bacterial pathogen to cause disease frequently requires the production of exotoxins, but the mere ability to produce a toxin is not sufficient to cause disease. Cholera toxin is the principal virulence factor of *Vibrio cholerae*. Administration of micrograms of purified cholera toxin to human volunteers elicits a diarrheal disease that mimics the magnitude of the natural infection. Nonetheless, nonvirulent, toxin-producing strains of *V. cholerae* have been isolated and shown to lack specific biological properties, such as motility or chemotaxis. Similarly, although anthrax toxin is the principal toxic component of *Bacillus anthracis*, nonvirulent toxin-producing strains of *B. anthracis* have been isolated and shown to lack the production a poly-glutamic acid capsule. In contrast, the botulinum neurotoxins can be ingested preformed in contaminated food and elicit the pathology of botulism; food poisoning by the botulinum neurotoxins is an intoxication rather than an infection.

Bacterial exotoxins are classified according to their mechanisms of action. The covalent modifications of host-cell components that are catalyzed by bacterial exotoxins include ADP-ribosylation, deamidation, depurination, proteolysis, and glucosylation (Table 1). Most cellular targets of bacterial exotoxins are proteins, although there are exceptions such as shiga toxin, which catalyzes the deadenylation of ribosomal RNA. In addition to exotoxins, there are several other classes of toxins that are produced by pathogenic bacteria, such as pore-forming toxins, type III-VI secreted cytotoxins, heat-stable enterotoxins, and superantigens. Each of these toxins fails to perform one of the properties associated with exotoxins. The pore-forming toxins are not catalytic in their action, but instead disrupt cell physiology through the formation of pores in the host-cell plasma membrane. The type III-secreted cytotoxins cannot enter host cells as soluble proteins, but instead are injected directly into the host cell by the type III secretion apparatus of a host-cell bound bacterium. The heat-stable enterotoxins and superantigens do not enter the intracellular compartment of the host cell, but elicit host-cell responses by triggering signal-transduction pathways upon binding to the host-cell membrane or by complexing host cells, respectively. In this article, initial emphasis will be placed on the molecular properties of bacterial exotoxins, with a subsequent description of the general properties of pore-forming toxins, type III-secreted cytotoxins, heat-stable enterotoxins, and superantigens.

The pathology elicited by a specific exotoxin results from the catalytic covalent modification of a specific host-cell component. Although diphtheria toxin and cholera toxin are both bacterial ADP-ribosylating exotoxins, the pathogenesis elicited by each exotoxin is unique. This is due to the fact that diphtheria toxin ADP-ribosylates elongation factor-2, resulting in the inhibition of protein synthesis and subsequent cell death, whereas cholera toxin ADP-ribosylates the G_s component of the heterotrimeric protein, which stimulates the activity of host adenylate cyclase. This stimulation of adenylate cyclase elevates intracellular cAMP, which triggers the secretion of electrolytes and H_2O from the cell, resulting in the clinical manifestations of cholera.

General Properties of Exotoxins

Genetic Organization of Exotoxins

The genes encoding bacterial exotoxins may be located on the chromosome or located on an extrachromosomal element, such as a plasmid or a bacteriophage. Elegant experiments characterizing diphtheria toxin showed that the gene encoding this exotoxin was located within the genome of the lysogenic-phage. Although both nonlysogenic and lysogenic strains of *C. diphtheriae* could establish local upper-respiratory-tract infection, only strains of *C. diphtheriae* lysogenized with the lysogenic beta-phage that encoded diphtheria toxin were capable of eliciting systemic disease. This established a fundamental property for the pathology elicited by bacteria that produce exotoxins; bacteria establish a localized infection and subsequently produce an exotoxin, which is responsible for pathology distanced to the site of infection.

Most exotoxins are produced only during specific stages of growth with the molecular basis for the regulation of toxin expression varying with each bacterium. This differential expression often reflects a complex regulation of transcription, including responses to environmental conditions, such as iron. Multi-subunit toxins are often organized in operons to allow the coordinate expression of their subunit components.

Table 1 Properties of Representative Bacterial Exotoxins.

Modification	*Exotoxin*	*Bacterium*	*AB*	*Target*	*Contribution to pathogenesis*
ADP-ribosylation	Diphtheria toxin	*C. diphtheriae*	**AB**	Elongation factor-2	Inhibition of protein synthesis
	Exotoxin A	*P. aeruginosa*	**AB**	Elongation factor-2	Inhibition of protein synthesis
	Cholera toxin	*V. cholerae*	**AB5**	Gsα	Inhibition of GTPase activity
	Heat-labile enterotoxin	*E. coli*	**AB5**	Gsα	Inhibition of GTPase activity
	Pertussis toxin	*B. pertussis*	**AB5**	Giα	Uncouple signal transduction
	C2 toxin	*C. botulinum*	**A-B**	actin	Actin depolymerization
	ExoS	*P. aeruginosa*	**A (type III delivered)**	Multiple targets including Ras	Uncoupling of Ras signal transdcution
	ExoT	*P. aeruginosa*	**A (type III delivered)**	Crk	Uncoupling of Rac signal transdcution
	SpvB	*Salmonella*	**A (type III delivered)**	Actin	Disruption of the Actin cytoskeleton
Glucosylation	Lethal toxin	*C. sordelli*	**AB**	Ras	Inhibition of effector interactions
	Toxin A and B	*C. difficile*	**AB**	RhoA	Inhibition of Rho signaling
Endoprotease	Lethal toxin (Anthrax toxin)	*B. anthracis*	**A-B**	MAP kinase	Cell death
	Botulinum toxin (A–G)	*C. botulinum*	**AB**	SNARE proteins	Inhibition of vesicle fusion in neurons (flaccid paralysis)
	Tetanus toxin	*C. tetani*	**AB**	Vesicle Associated Membrane Protein (VAMP)	Inhibition of vesicle fusion in neurons (spastic paralysis)
Deamidation	Cytotoxic Necrotizing Factor	*E. coli*	**AB**	RhoA	Stimulation of RhoA
Deadenylation	Shiga toxin	*Shigella* spp.	**AB5**	28S ribosome	Inhibition of protein synthesis
	Verotoxin	*E. coli*	**AB5**	28*S* RNA	Inhibition of protein synthesis
Adenylase cyclase	Adenylate cyclase toxin	*B. pertussis*	**AB**	cAMP prodcution	Uncouple cell signaling
	Edema Toxin (Anthrax toxin)	*B. anthracis*	**A-B**	cAMP prodcution	Uncouple cell signaling
	ExoY	*P. aeruginosa*	**A (type III delivered)**	cAMP prodcution	Uncouple cell signaling
RhoGAP	SptP	*Salmonella spp.*	**A (type III delivered)**	Inhibition of RhoGTPase function	Inhibition of phagocytosis
	YopE	*Yersinia*	**A (type III delivered)**	Inhibition of RhoGTPase function	Inhibition of phagocytosis
	ExoS	*P. aeruginosa*	**A (type III delivered)**	Inhibition of RhoGTPase function	Inhibition of phagocytosis

Secretion of Exotoxins from the Bacterium

Most bacteria secrete exotoxins across the cell membrane by the type II secretion pathway. The secretion of exotoxins by the type II secretion pathway was predicted by the determination that the amino terminus of mature exotoxins had undergone proteolysis relative to the predicted amino acid sequence derived from the gene sequence encoding the exotoxin. Type II secretion is also called the general secretion pathway. Type II secretion involves the coordinate translation and secretion of a nascent polypeptide across the cell membrane. During the translation of the mRNA that encodes a type II-secreted protein, the amino-terminal leader sequence of the nascent polypeptide targets to and is secreted across the cell membrane. After secretion across the cell membrane, the nascent protein folds into its native conformation and the leader sequence is cleaved by a periplasmic peptidase to yield a mature exotoxin.

Some Gram-negative bacteria export the assembled exotoxin from the periplasm into the external environment via a complex export apparatus. While the heat-labile enterotoxin of *Escherichia coli* remains localized within the periplasmic space, *V. cholerae* and *Bordetella pertussis* assemble their respective exotoxins, cholera toxin and pertussis toxin, within the periplasm and then transport the mature exotoxin into the external environment. Although the multiple protein components of the export apparatus have been identified, the exact mechanism for export across the outer membrane remains to be resolved.

Bacteria Produce and Secrete Exotoxins as Proenzymes

Although one property of a bacterial exotoxin is the ability to intoxicate sensitive cells, early biochemical studies observed that, *in vitro*, many bacterial exotoxins possessed little intrinsic catalytic activity. These perplexing observations were resolved with the determination that bacteria produce and secrete exotoxins as proenzymes, which must be activated (processed) to express catalytic activity *in vitro*. The observed requirements for *in vitro* activation reflect the activation steps of the exotoxin *in vivo*. For example, an exotoxin may require specific conditions for activation, including proteolysis, disulfide-bond reduction, or association with a nucleotide, cofactor, or a eukaryotic accessory protein. Some activation processes result in the release of the catalytic **A** domain from

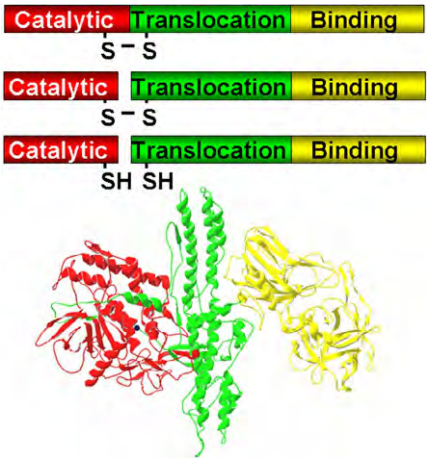

Figure 1 Bacterial exotoxins are produced as proenzymes. (Upper panel) Most bacterial exotoxins are produced as proenzymes that undergo processing to express catalytic activity. The sequential processing of botulinum toxin involves protein cleavage between the catalytic **A** domain (Red) and the Translocation (Green) and Receptor Binding (Yellow) **B** domain. The **A** and **B** domains are connected by a disulfide bond, which is reduced as the **A** domain is translocated into the host cytoplasm by agents such as reduced glutathione. (Lower panel) A ribbon diagram of botulinum neurotoxin is shown (Protein Data Bank # 3BTA). The catalytic **A** domain (Red) is linked to **B** domain, which includes the Translocation domain (Green) and the Receptor Binding domain (Yellow).

the **B** domain, whereas other activation processes appear to result in a conformational change rendering the catalytic **A** domain active. Some exotoxins require sequential activation steps. Diphtheria toxin is activated by limited proteolysis, followed by disulfide-bond reduction (Figure 1).

The determination of the activation mechanism of exotoxins often provides insight into several physiological pathways of host cells. The eukaryotic protein ARF (*A*DP-*r*ibosylation *f*actor), which activates cholera toxin *in vitro*, was subsequently shown to play a central role in vesicle formation within the eukaryotic cell. The ability of a host-cell extract to activate cholera toxin is often used as a sign of the presence of ARF. Cholera toxin is activated through a series of steps where the catalytic domain of the toxin is cleaved into an A1 and A2 fragment. The A1 fragment binds ARF which activates the protein to ADP-ribosylate the alpha subunit of the heterotrimeric G_s protein to uncouple cAMP regulation within the host cell. Characterization of the mechanisms that pertussis toxin and cholera toxin use to intoxicate eukaryotic cells provided insight into the pathways for eukaryotic G protein-mediated signal transduction. The ability of pertusis toxin to inhibit the action of a ligand in a signal-transduction pathway is often used to implicate a role for G proteins in that signaling pathway.

AB Structure–Function Properties of Exotoxins

Most bacterial exotoxins possess **AB** structure-function properties (Figure 2). The **A** domain is the catalytic domain, whereas the **B** domain includes the translocation and binding domains of the exotoxin. Exotoxins are organized into one of several general types of **AB** organization. The simplest **AB** organization is represented by diphtheria toxin, where the **A** domain and **B** domain are contained in a single protein. Diphtheria toxin is the prototype for this class of **AB** exotoxin. Diphtheria toxin is a 535-amino-acid

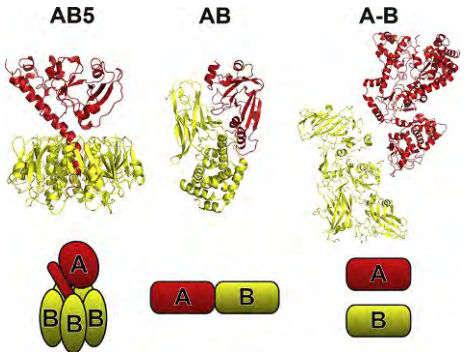

Figure 2 Bacterial exotoxins possess **AB** structure–function organization. There are three general **AB** organizations of bacterial exotoxins. The **A** domain (Red) represents the catalytic domain whereas the **B** domain comprises the translocation and receptor-binding domains (Yellow). **AB5** is represented by cholera toxin of *V. cholerae*, which is composed of six noncovalently associated proteins (PDB: 1XTC). **AB** is represented by diphtheria toxin of *Corynebacterium diphtheriae*, in which the **A** and **B** domain are included in a single protein (PDB: 1MDT). **A-B** is represented by protective antigen and lethal factor from anthrax toxin of *Bacilus anthracis*, which is composed of two non-associated proteins; the two proteins associate after the binding and processing of the **B** component on the host-cell membrane (PDB: 1ACC and 1JKY).

protein in which the amino terminus constitutes the ADP-ribosyltransferase domain and the carboxyl terminus comprises the translocation domain and receptor-binding domain. The **AB5** exotoxins are composed of six proteins that are noncovalently associated as an oligomer. Cholera toxin is the prototype for the **AB5** exotoxin. The **A** domain of cholera toxin constitutes the ADP-ribosyltransferase domain, whereas the **B5** domain is composed of five identical proteins, forming a pentamer, which is organized into a ring structure on which the **A** domain is positioned. The five proteins that make up the **B** domain may be identical, as is the case for cholera toxin and the heat-labile enterotoxin of *E. coli*, or may be an oligomer of different proteins that form a non-symmetrical ring structure, as observed with the **B** domain of pertussis toxin.

The third class of **AB** exotoxin is composed of proteins that are not associated in solution, but where the **A** and **B** domains associate following the binding and oligomerization of the **B** domain on the host cell. C2 toxin is an example of this class of **A-B** exotoxin. C2 toxin is a bipartite exotoxin composed of a protein that encodes the catalytic **A** domain and a separate protein that encodes the **B** domain. C2 toxin **A** domain ADP-ribosylates actin. The **B** domain of C2 toxin binds to sensitive cells, is nicked by a eukaryotic protease, and the nicked **B** domain oligomerizes and is then capable of binding the **A** domain protein. Anthrax toxin is a major virulence factor of *Bacillus anthracis*. Anthrax toxin is organized as an **AB** exotoxin that is composed of two unique **A** domains (edema factor, an adenylate cyclase, and lethal factor, a zinc protease) and a **B** domain (termed the orotective antigen) which are not associated in solution. The **A** domains associate with the **B** domain subsequent to the binding and oligomerization of the **B** domain on the surface of sensitive host cells. Recent studies observed that three **A** domains can bind to each heptameric **B** domain on the host cell surface.

The **B** domain possesses two specific functions, receptor binding and translocation capacity. Each exotoxin uses a unique host-cell surface component as a receptor. The host cell surface receptor for an exotoxin may be a specific molecule or a non-specific group of molecules. For example, the cell surface receptor for cholera toxin is the ganglioside GM1, and the cell surface receptor for diphtheria toxin is the epidermal growth factor precursor. In contrast, the binding of pertussis toxin is less specific, as pertussis toxin binds numerous cell surface proteins. The ability to bind a cell surface receptor is an absolute requirement for an exotoxin to intoxicate a host cell, as the deletion of the receptor-binding domain renders the exotoxin noncytotoxic. After binding to the cell surface, some exotoxins are proteolytically processed or are processed during intracellular endosome transport.

The second function of the **B** domain includes translocation capacity, which is responsible for the delivery of the **A** domain across the cell membrane. The presence of a translocation domain was predicted from early structure-function studies of diphtheria toxin, which showed that in addition to the catalytic domain and receptor-binding domain, a third function was required for the efficient expression of cytotoxicity. This third function was subsequently shown to correspond to a region of diphtheria toxin that had the propensity to interact with membranes. The crystal structure of diphtheria toxin revealed the presence of three distinct domains, representing the catalytic, translocation, and receptor-binding domains.

Exotoxins Enter Host Cells via Distinct Pathways

Although **A** domain translocation is one of the least understood aspects of the intoxication process of exotoxins, there are several general themes that are involved in translocation of the **A** domain across the cell membrane. One translocation mechanism uses a pH gradient within the endosome to stimulate protein conformational changes in the **B** domain, making the **B** domain competent to interact with the membrane of the endosome. After insertion into the endosome membrane, the **B** domain generates a pore that is involved in the translocation of the **A** domain across the vesicle membrane in an unfolded form. After the translocation across the endosome membrane, the **A** domain refolds to its native conformation. Upon translocation of the **A** domain across the endosome membrane, reduced glutathione may reduce the disulfide that connects the **A** domain with the **B** domain, and release the **A** domain into the cytoplasm. The potency and catalytic potential of exotoxins was demonstrated by the observation that the introduction of one molecule of the catalytic domain of diphtheria toxin into the intracellular cytoplasm was sufficient to inhibit host-cell protein synthesis, resulting in cell death. Recent studies on the translocation process of anthrax toxin support the direct translocation of the **A** domain through a stable, gated channel formed by the heptameric **B** domain.

Other toxins, such as cholera toxin and exotoxin **A** of *Pseudomonas aeruginosa*, use retrograde transport to enter the interior regions of the cell. Movement occurs through retrograde transport of the endosome to the Golgi apparatus and ultimately to the endoplasmic reticulum. Many exotoxins that are ultimately delivered to the endoplasmic reticulum possess a KDEL (Lys-Asp-Glu-Leu)-like retention signal sequence on their carboxyl terminus. Studies with chimeric proteins have shown that the introduction of a KDEL retention sequence is sufficient to retrograde transport a protein from the endosome to the endoplasmic reticulum. Thus, there is physiological precedence for the use of the KDEL sequence to retrograde transport exotoxins toward the endoplasmic reticulum. The host mechanism that recognizes unfolded host proteins also translocates the **A** domain from the lumen of the endoplasmic reticulum into the host cell cytosol.

Covalent Modification of Host-Cell Components by Exotoxins

Exotoxins use several unique mechanisms to covalently modify host-cell components. The major classes of reactions are the covalent addition of a chemical group to the target protein, the cleavage of a chemical group from a target protein, or the endoproteolytic cleavage of a peptide bond of the target protein.

The ADP-ribosylation of host proteins is the prototypical mechanism of action of bacterial exotoxins. Numerous bacterial exotoxins catalyze the ADP-ribosylation of specific host proteins and elicit physiological changes. In the ADP-ribosylation reaction,

exotoxins use the oxidized form of nicotinamide adenine dinucleotide (NAD) as the substrate, and transfer the ADP-ribose portion of NAD to a specific amino acid via an *N*-glycosidic linkage of ADP-ribose onto the host target protein. The specific type of amino acid that is ADP-ribosylated within the target protein varies with the specific exotoxin. Diphtheria toxin ADP-ribosylates elongation factor-2 on a post-translationally modified histidine residue called diphthamide. ADP-ribosylated elongation factor-2 is unable to perform its translocation of nascent polypeptides in the ribosome, which results in the inhibition of protein synthesis and subsequent cell death. In contrast cholera toxin ADP-ribosylates the G_s component of a heterotrimeric G protein. ADP-ribosylated G_s is locked in an active conformation, which stimulates host adenylate cyclase and the subsequent elevation of intracellular cAMP. Thus, ADP-ribosylation may either activate or inhibit the activity of the target protein. Deamidation of Gln63 on RhoA by *E. coli* cytotoxic necrotizing factor (CNF) results in a constitutively active RhoA protein. Note that although most host targets for exotoxins are proteins, Shiga toxin catalyzes the deadenylation of a specific adenine on 28*S* RNA.

Recall that each exotoxin modifies a specific host-cell component, which is responsible for the specific pathology elicited by that exotoxin. Although there are no absolute rules for the types of proteins targeted for covalent modification, the most frequent targets are nucleotide-binding proteins that are involved in signal-transduction pathways, including both the heterotrimeric G-proteins and the small-molecular-weight GTP-binding proteins of the Ras superfamily.

Molecular and Structural Properties of Bacterial Exotoxins

Early biochemical studies provided significant advances in defining the structure-function properties of exotoxins, resolving many of the exotoxin mechanisms of action and developing the concept that exotoxins have **AB** organization. Molecular genetics and structural biology have extended these earlier studies and provide a more detailed understanding of the biochemical and molecular relationships among the exotoxins. The biochemical characterization of diphtheria toxin and exotoxin A (ETA) of *Pseudomonas aeruginosa* showed that these two exotoxins catalyzed kinetically identical reactions during the ADP-ribosylation of elongation factor-2. In addition, both diphtheria toxin and ETA possess an active site glutamic acid, which was subsequently shown to be a signature property of exotoxins that catalyze the ADP-ribosyltransferase reaction. These observations predicted that ADP-ribosylating exotoxins would possess considerable primary amino acid homology. Thus, the determination that the genes encoding diphtheria toxin and ETA shared little primary amino acid homology was unexpected. This paradox was resolved after the analysis of the three-dimensional structures of ETA and the heat-labile enterotoxin (LT) of *E. coli*. The three-dimensional structures of ETA and LT showed little similarity in their respective receptor-binding domains and translocation domains; however, the catalytic domains of ETA and LT, which are composed of seven discontinuous regions of each protein, could be superimposed on each other despite possessing homology at only three of the 43 amino acids. One of the homologous amino acids in ETA and LT was the signature active site glutamic acid. This was a remarkable finding because ETA and LT ADP-ribosylated different host target proteins and possessed different **AB** organization. A common theme has evolved for describing the structure-function properties of this family of bacterial exotoxins in which the ADP-ribosylating exotoxins possess a conserved three-dimensional structure in their active sites, despite the lack of primary amino acid homology. These findings provided a framework for the study of other classes of exotoxins produced by divergent groups of bacteria.

Conversion of Exotoxins into Toxoids

Chemical Detoxification of Bacterial Exotoxins

Shortly after the determination that toxic components were associated with bacterial pathogens, cell extracts or cell cultures of a pathogen were shown to be treated with chemical denaturants, such as formalin, to produce nontoxic-immunogens as vaccines to prevent disease upon subsequent exposure by that pathogen. In the case of diphtheria toxin and tetanus toxin, chemical modification with formalin produced toxoids that were used as subunit vaccines in large-scale immunizations. This resulted in a remarkable decrease in the incidence of both diphtheria and tetanus within the immunized population. In areas where these toxoids are not administered, diphtheria and tetanus remain clinically important diseases. In addition to formalin, other chemicals have been used to detoxify bacterial exotoxins, including glutaraldehyde and hydrogen peroxide. In contrast, the production of a chemical toxoid of other exotoxins, such as cholera toxin and pertussis toxin, has been more difficult because the treatment of these toxins with denaturants and reduced immunogenicity. Thus, there is a need to develop alternative strategies, such as genetically engineered and subunit vaccines, to eliminate the cytotoxicity of certain exotoxins without compromising their immunogenicity.

Genetic Detoxification and Subunit Vaccines of Bacterial Exotoxins

Developments in genetic engineering provide an opportunity to produce recombinant forms of bacterial exotoxins that possess greatly reduced toxicity, but retain immunogenicity. The use of genetic engineering to develop a toxoid of pertussis toxin has been successful. The whole-cell pertussis vaccine is composed of a chemically treated preparation of *Bordetella pertussis*, which is effective in the elicitation of a protective immune response after mass immunization. However, the whole-cell pertussis vaccine is acutely reactive when administered to children due to the crude nature of the vaccine that includes endotoxin. Recently a component pertussis vaccine has been developed that is administered with the diphtheria toxoid and tetanus toxoid (termed the DTaP vaccine)

which produces fewer adverse reactions in children than the whole cell pertussis vaccine. The reactivity of the acellular pertussis vaccine is so low that administration of this vaccine in adolescents and adults has been approved, an important development as adults are considered a primary carrier of *Bordetella pertussis*. Pertussis toxin, a primary virulence determinant of *B. pertussis*, is an exotoxin that ADP-ribosylates the G_i component of heterotrimeric G proteins and effectively uncouples signal transduction between the G protein-coupled receptor and the G protein. Genetically engineered forms of pertussis toxin have been produced that possess essentially no catalytic activity or cytotoxicity, but that maintain native conformation and elicit a protective immune response when used as an immunogen. These recombinant noncytotoxic forms of pertussis toxin have been engineered with multiple mutations in their active site, virtually eliminating the risk of reversion to a cytotoxic form. Similar strategies are being applied to other bacterial exotoxins with the goal of engineering acellular vaccine candidates.

Subunit vaccines represent another approach to developing safe and efficient vaccines. This strategy implies that a domain of the exotoxin can be identified that elicits a protective immune response against exotoxin challenge in the host. One example of a subunit vaccine is the development of a subunit vaccine against botulism. Botulism is a toxin-mediated disease elicited by botulinum toxin. Botulinum toxin is the most toxin protein for humans and is an **AB** toxin (Figure 2). Recent studies have shown that immunization with the C-terminal portion of the **B** domain stimulates a protective immune response against challenge by botulinum toxin. Recombinant forms of this C-terminal **B** domain have been produced in yeast and *Escherichia coli* and represent the next generation of botulism vaccines.

Therapeutic Applications of Exotoxins

One of the most exciting areas of bacterial exotoxin research has been the development of strategies to use exotoxins in therapeutic disciplines. Some therapies use the native cytotoxic form of the exotoxin. Other therapies use the **A** domain, which is conjugated to a heterologous binding component or to effector elements, respectively, to produce a chimeric molecule with directed properties.

Botulinum toxin and tetanus toxin (BT/TT) are single-chain proteins organized as **AB** exotoxins. The amino-terminal **A** domain of BT/TT express protease activity, cleaving SNARE proteins, which inhibits fusion of neurotransmitter vesicles to the plasma membrane of neurons. The carboxy-terminal **B** domain of BT/TT possesses motor neuron-specific receptor-binding activity that is unique for each exotoxin. BT enters synaptic vesicles of motor neurons and inhibits synaptic vesicle fusion at the neuromuscular junction, causing flaccid paralysis, while TT enters endosomes of motor neurons and retrograde traffics into the central nervous system to inhibit of fusion of neurotransmitter vesicles to the plasma membrane of inhibitory neurons, yielding spastic paralysis. Thus, the specific intracellular trafficking of BT/TT is responsible for the clinical manifestation of each neurotoxin. BT is also a potent therapy of human diseases, and reduces muscle spasms associated with several clinical disorders, such as blepharospasm, an involuntary contraction of eye muscles. This application has expanded and BT is one of the most widely used therapeutic proteins in clinical medicine. The extreme potency of BT to humans has also made this a potential agent for malicious application.

Diphtheria toxin is a carrier of antigens to stimulate an immune response against several polysaccharide epitopes. One epitope is polyribitolphosphate, a component of the polysaccharide capsule of *Haemophilus influenzae* type b (Hib). Early attempts to elicit an effective immune response to purified Hib antigen resulted in a T-cell-independent immune response that did not yield an effective immune memory. A non-catalytic mutated diphtheria toxin, CRM197, is one of the protein carriers for the Hib epitope. Immunization with the CRM197-Hib conjugate yields a strong T-dependent immune response. Mass immunization with conjugate-Hib vaccines has dramatically reduced the number of cases of Hib in the immunized population.

Due to their potency, the catalytic **A** domain of exotoxins have been used in the construction of chimeric immunotoxins that target cancer cells. Early studies used conjugates that were composed of the **A** domain of the diphtheria toxin coupled to an antibody that recognized a cell surface-specific antigen. The **A** chain of the diphtheria toxin was used in the first generation of immunotoxins due to the **A** domains potency. Similar chimeras continue in development to treat specific human cancers.

Other Classes of Bacterial Toxins

Pore-Forming Toxins

The lack of a catalytic **A** domain differentiates the pore-forming toxins from exotoxins. Thus, the pathology associated with pore-forming toxins is due solely to the generation of a pore within the membrane of the host cell. Several bacterial pathogens produce pore-forming toxins, some of which are secreted by a type I secretion pathway. Unlike type II-secreted proteins, the amino terminus of type I-secreted proteins is not processed. Type I-secreted proteins possess a polyglycine signal sequence in the carboxyl terminus of the mature toxin to facilitate translocation. There are several classes of pore-forming toxins, including members of the hemolysin family of pore-forming toxins, the aerolysin family of pore-forming toxins, and the alpha-toxin of *Staphylococcus aureus*. Host-cell specificity differs among pore-forming toxins. The crystal structures of several of the pore-forming toxins have been determined. The molecular events generating a pore in the membrane of a host cell have been proposed for the aerolysin family of pore-forming toxins, where aerolysin is exported by *Aeromonas hydrophilia* as a monomeric molecule, which binds to the host cell. A host protease nicks the monomer, which is then processed and subsequently oligomerizes. The oligomerized complex inserts into the membrane and generates a pore in the center of the complex, causing the release of the cytoplasmic components of the host cell.

Type III-Secreted Cytotoxins

The lack of a **B** domain differentiates the type III-secreted cytotoxins from exotoxins. Thus, the organization of the type III-secreted cytotoxins may be described as **A** domains that are specific effector proteins. Cell surface-bound bacteria transport type III-secreted cytotoxins directly into host cells. Type III-secreted proteins are unique from proteins secreted by either the type I or II secretion pathways, where the amino terminus of type III-secreted cytotoxins is not processed and the carboxy terminus does not possess a poly-glycine motif. Although progress has been made in establishing the components of the type III secretion apparatus, the mechanism for the delivery of type III-secreted proteins across the host-cell membrane remains to be resolved. Numerous bacteria possess type III secretion pathways, including members of the genera *Escherichia*, *Pseudomonas*, *Shigella*, *Salmonella*, and *Yersinia*. Cytotoxicity elicited by type III-secreted cytotoxins has an absolute requirement for the type III secretion apparatus of the bacterium, as purified forms of the cytotoxins are not toxic to host cells.

The **A** domains of type III-secreted cytotoxins possess several unique mechanisms of action, including the depolymerization of the actin cytoskeleton, phosphatase activity, ADP-ribosyltransferase activity, and the stimulation of apoptosis. Each type III secretion apparatus appears capable of delivering numerous type III-secreted proteins into the host cell. Recent studies have identified additional secretion systems that bacteria use to translocate proteins across the bacterial cell envelope. The type IV secretion system utilizes an apparatus related to the apparatus for DNA transfer between bacteria. The type IV secretion apparatus of *B. pertussis* transfers pertussis toxin from the periplasm across the cell envelope and into the extracellular environment, while the type IV secretion apparatus of *Agrobacterium tumefaciens* injects effector molecules directly into the host cells. The type V secretion system is an autotransporter system where a protein is composed of a catalytic domain that is transported across the bacterial cell envelope by another component of the protein termed the autotransporter domain. There are numerous type V secreted proteins, with VacA, a virulence factor of *Helicobacter pylori*, one of the most noted examples of a type V secreted protein. The type VI secretion system has been described best in *P. aeruginosa* and involves two protein families, Hcp, which forms nanotubes on the bacterial surface, and VgrG, which form trimers on the bacterial cell surface. Both proteins pierce membranes and transport proteins from the bacterial cell.

Heat-Stable Enterotoxins

The inability of the heat-stable enterotoxins to enter the host cell or possess catalytic activity differentiates the heat-stable enterotoxins from exotoxins. Several genera of bacteria produce heat-stable enterotoxins, including *Escherichia* and *Yersinia*. The heat-stable enterotoxin a (STa) of *E. coli* is the prototype toxin. *E. coli* secretes STa into the periplasm as a 72 amino-acid precursor, where three intramolecular disulfide bonds form and help process STa into a 53 amino-acid peptide. The 53 amino-acid peptide is exported into the environment, where a second proteolytic cleavage results in the production of an 18 or 19 amino-acid mature STa molecule. The mature STa binds a protein receptor on the surface of epithelial cells, which stimulates an increase in the intracellular concentrations of cGMP. The intracellular increase in cGMP stimulates chloride and H_20 secretion, resulting in diarrhea.

Superantigens

The inability of superantigens to enter the host cell or possess catalytic activity differentiates the superantigens from exotoxins. Superantigens are soluble proteins of approximately 30-kDa secreted by both *Streptococcus* and *Staphylococcus*. The superantigens bind to a component of the major histocompatibility complex of T lymphocytes through an antigen-independent mechanism, which stimulates binding of the T-cell receptor via an antigen-independent mechanism leading to the proliferation of a large subset of T lymphocytes, and the production of a pro-inflammatory immune response. The superantigens are especially potent toxins.

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Extremophiles and Acidic Environments

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Glossary

Autotroph An organism that obtains its carbon by fixing carbon dioxide, bicarbonate or other C1 compound.
Chemolithotroph A prokaryote that uses an inorganic energy source and fixes carbon dioxide.
Heterotroph An organism that obtains both its carbon and energy from an organic source.
Phototroph An organism that uses solar energy.
Snotites Small gelatinous growths of micro-organisms that grow suspended from the roofs of underground mines and caves.

Abbreviations

AMD	Acid mine drainage
ARD	Acid rock drainage
C1 compounds	Organic compounds containing only one carbon atom
Ci	Inorganic carbon
EPS	Extracellular polymeric substances
LTSEM	Low temperature scanning electron microscopy
Pi	Inorganic phosphorus
PCS	Phytochelatin synthase
SEM	Scanning electron microscopy
SEM-BSE	Scanning electron microscopy combined with analysis of back scattered electrons
16S rRNA	Small sub-unit fraction of ribosomal ribonucleic acid

Defining Statement

This article gives an overview of the nature of extremely acidic environments and of the biodiversity of prokaryotic and eukaryotic micro-organisms found within them. How acidophiles interact with each other in both positive and negative fashions are described and the microbial ecology of some of the most widely studied extremely acidic environments on our planet is discussed. The importance of acidophilic micro-organisms in existing and emerging biotechnologies is highlighted throughout the article.

Nature and Origin of Extremely Acidic Environments

Marine waters constitute the largest biome on planet earth. The dominant bicarbonate buffer dictates that sea water is mostly moderately alkaline (pH 8.2–8.4), though more alkaline (e.g., at the Lost City hydrothermal field in the mid-Atlantic ridge) and acidic waters (e.g., in the vicinities of black smoker hydrothermal vents) can be found in small scale and micro-niches. Terrestrial environments (soils, fresh waters and brackish waters) have greater potential for extremes of pH to develop and become established for protracted periods. Acidic mineral soils and peatlands are widespread where bedrocks are non-basic (granitic etc.) and where precipitation exceeds evapo-transpiration potentials. However, with the notable exception of acid sulfate soils, these rarely develop pH values <4.2 (mineral soils) or <3.0 (acidic peats). The dominant acids here are aliphatic and aromatic organic acids, and they tend to have pK_a values that are considerably larger than "strong" mineral acids. For example, the pK_a values of acetic and benzoic acids are 4.75 and 4.2, respectively, while those of hydrochloric and nitric acids are both <0. It follows that biological (or abiotic) processes that generate strong mineral acids have far greater potential to result in the formation of very low pH environments than those, such as fermentation, that produce organic acids (which themselves are susceptible to being degraded). Biological generation of mineral acids other than sulfuric acid are, however, rare. Gastric juices produced by mammalian stomachs have pH values of 1.5–3.5, due to their content of hydrochloric acid. Nitrification (dissimilatory oxidation of ammonium and nitrite) generates nitrate, rather than nitric acid, since the vast majority of nitrifying microorganisms are pH sensitive and the process can therefore become self-limiting. In contrast, while many bacteria and archaea that oxidize elemental sulfur and reduced sulfur oxy-anions are also acid-sensitive (e.g., lower limits of ~pH 5 for some *Thiobacillus* spp. and ~pH 3.0 for *Thiomonas* spp.) others are far more tolerant of low pH, and are among the most extremely acidophilic of all known life-forms.

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Microbiological generation of sulfuric acid by coupling the oxidation of elemental sulfur (S°) to the reduction of oxygen (aerobic oxidation) is represented by Eq. (1):

$$S^0 + 1.5O_2 + 3H_2O \rightarrow 2H_3O^+ + SO_4^{2-} \quad (1)$$

Sulfuric acid has two pK_a values (−2.8 and +1.9) and as pH falls below ~2.0 the bisulfate (HSO_4^-) anion becomes increasingly relatively abundant and one, rather than two, hydronium ions are generated for each sulfur oxidized, which in turn slows down environment acidification, at least if quantified solely by pH measurements. These reactions can be seen in solfatara fields which are widely distributed in volcanic and geothermal areas (Fig. 1). Sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) are important components of volcanic gases and can react together to generate elemental sulfur (Eq. 2),

$$SO_2 + 2H_2S \rightarrow 3S^0 + 2H_2O \quad (2)$$

or dissolve in water (both are fairly soluble gases) and be subjected again to microbiological oxidation, generating sulfuric acid. Some acidophilic bacteria are also able to oxidize zero-valent (S°) and reduced sulfur in the absence of oxygen using iron (III) (ferric iron) as an alternative electron acceptor. As shown in Eq. (3) this actually generates considerably more acidity than does aerobic oxidation of sulfur:

$$S^0 + 6Fe^{3+} + 12H_2O \rightarrow 8H_3O^+ + SO_4^{2-} + 6Fe^{2+} \quad (3)$$

Sulfur is one of the more abundant elements present in the earth's crust, accounting for 0.05% of the lithosphere on a weight basis, and can exist in any of nine oxidation states, varying from −2 to +6. It occurs in oxidized minerals such as gypsum

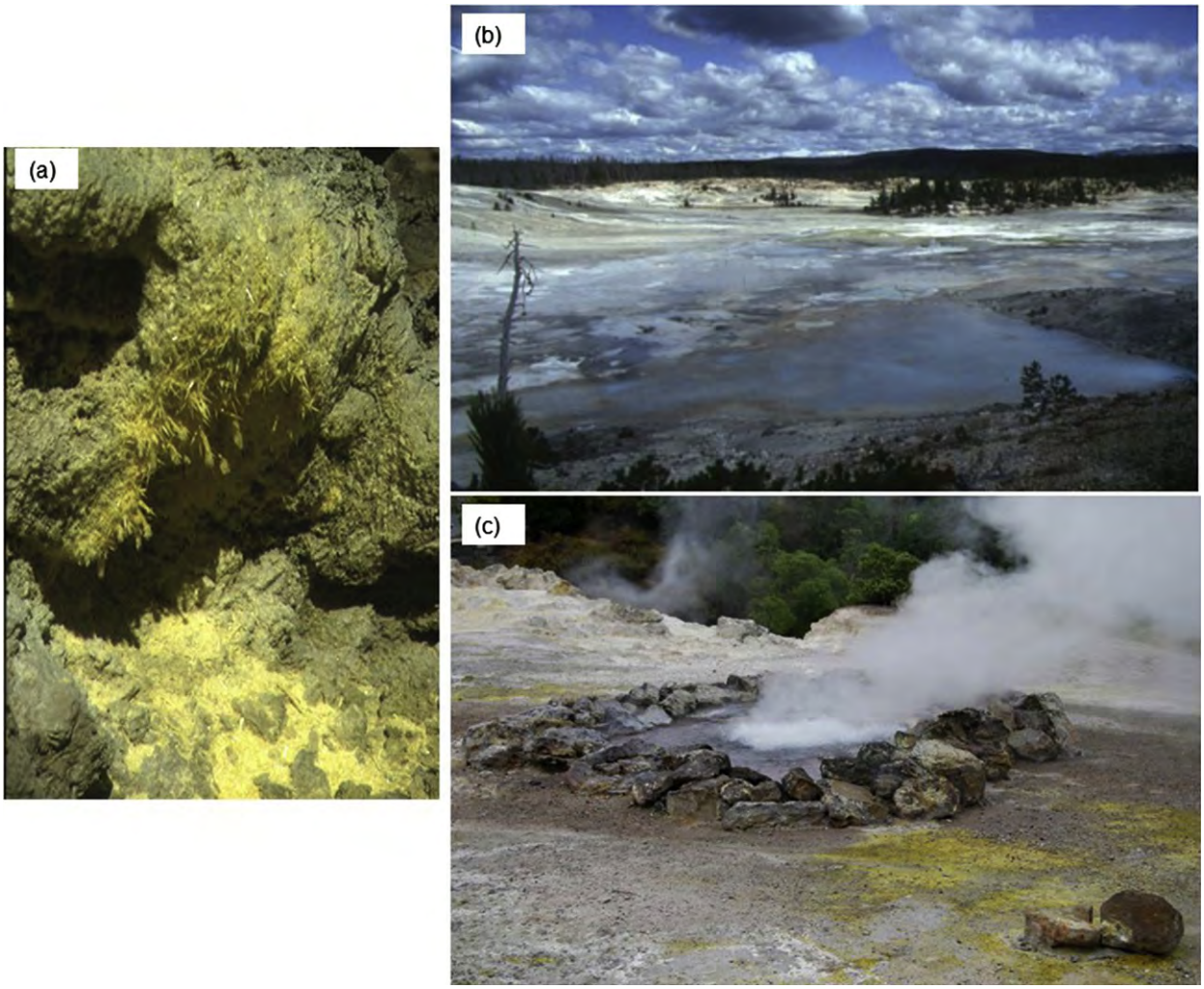

Fig. 1 (a) Elemental sulfur forming from gases venting the Soufriere Hills volcano on Montserrat, West Indies; (b) an acidic geothermal area (Norris geyser basin) in Yellowstone National Park, Wyoming; (c) a sulfur-rich hot spring in São Miguel, Azores.

($CaSO_4{\cdot}2H_2O$) and baryte ($BaSO_4$) in both of which sulfur has an oxidation state of +6. However, from the perspective of extremely acidic environments, the most important are reduced sulfide minerals where sulfur occurs in oxidation states of 0, −1 or −2. The most abundant, and in environmental terms, significant sulfide mineral is pyrite (FeS_2; "fools' gold"). This and other sulfide minerals can be formed in high or low temperature environments, provided that they are anoxic. They are among the hardest and most dense minerals known, but are susceptible to being destroyed when exposed to both water and an oxidizing agent. The latter may be (as with sulfur oxidation) either molecular oxygen or soluble ferric iron, and the process can be entirely abiotic. However, some specialized acidophilic prokaryotes have evolved to essentially tap into the free energy available from sulfide mineral oxidation and use this to fuel carbon (and in some cases nitrogen) fixation and other metabolic processes. To mediate this, they not only have genetic information that codes for proteins involved in the oxidation of iron (II) and/or reduced sulfur but also to allow them to withstand the extremely acidic external liquors in which they exist.

How extremely acidic environments may form as a consequence of microbiological sulfide mineral oxidation can be understood by considering pyrite. This mineral degrades relatively slowly even in highly acidic solutions. It is, however, readily oxidized by soluble ferric iron which (in the absence of chelating agents such as oxalic acid) exists in significant concentrations only in very acidic liquors (pH values below ~2.5). Six ferric iron ions are required to oxidize one "FeS_2" and are reduced to ferrous iron in the process, with the sulfur moiety being released initially as thiosulfate (equation 4):

$$FeS_2 + 6Fe^{3+} + 9H_2O \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H_3O^+ \quad (4)$$

This is an abiotic reaction that generates acid but does not require oxygen. However, unless the oxidant (ferric iron) is continuously inputted from an external source, the process comes to a halt except where, as in the vast majority of both natural and anthropogenic extremely acidic environment, species of acidophilic bacteria and archaea that are able to regenerate ferric iron are present, and oxygen is available for them to catalyze the oxidation reaction. Since spontaneous chemical oxidation of ferrous iron proceeds very slowly at low pH, these micro-organisms play a critical role in pyrite oxidation, and are regarded as the primary agents involved in the oxidative dissolution of sulfidic minerals at low pH. The thiosulfate generated in Eq. (4) is unstable in low pH liquors and transforms to elemental sulfur and a variety of sulfur oxy-anions, all of which can be oxidized by "secondary" microbial members of the consortium present, forming sulfuric acid, as described previously. Some or all of the ferric iron generated from pyrite oxidation can undergo mineralization reactions to form secondary oxidized minerals such as jarosites, schwertmannite or ferrihydrite. Eq. (5) depicts a situation where ferric iron is partially mineralized to form schwertmannite:

$$16FeS_2 + 60O_2 + 60H_2O \rightarrow Fe_8O_8(SO_4)(OH)_6 + 8Fe^{3+} + 38H_3O^+ + 31SO_4^{2-} \quad (5)$$

As can be seen, this reaction is strongly acid-generating, which limits the extent to which secondary mineralization to this particular ferric iron mineral can occur. The system also provides a strong pH buffer to acidic iron-rich water bodies, helping to maintain their pH often to within the range 2.2–2.8. This is seen in the most well-known and researched extremely acidic environment on planet Earth, the Rio Tinto in south-west Spain, which remains within this pH range for most of its 60 km length. Another important attribute that this pH buffer/mineralization system affords is the strong coloration of acidic iron-rich environments: soluble ferric iron (complexed by hydroxyl and sulfate ligands) imbues an amber to ruby-red color to waters while underlying ferric iron-rich sediments have pigmentation ranging from straw to orange to deep brown (referred to as "ochre" or "yellow boy" Fig. 2(a)).

In contrast to extremely acidic environments that derive from sulfur oxidation, most low pH environments associated with sulfide mineral oxidation are anthropogenic and not located in volcanic or geothermal areas. In some pristine areas, such as Greenland and Antarctica, natural exposures of iron-rich deposits overlying sulfidic bedrocks (gossans) can generate acidic run-off waters, though these are generally limited in the amount of acid (and soluble metals) they generate by the physical nature of the bedrock which limits its exposure to oxygen and water. In contrast, when deep-buried sulfidic rock strata are mined as ore bodies, haulage to the land surface followed by crushing and grinding (comminution) of the metal-rich fractions greatly increases the exposed surface areas of minerals and therefore their susceptibility to oxidative dissolution. Many of the most important base metals used by modern society occur either predominantly (e.g., copper and zinc) or extensively (e.g., nickel) as sulfide minerals in the lithosphere. While most of these target minerals are recovered from crushed ore by froth floatation, other sulfide minerals, such as pyrite, end up in fine-grain mineral wastes known as "tailings". These are generally stored underwater in vast lagoons in order to limit oxygen ingress and the microbially-accelerated oxidative dissolution of reactive minerals, except in arid areas such as the Atacama dessert. Even so, oxidation of pyrite and other sulfide minerals (which may contain highly noxious metals and metalloids such as cadmium and arsenic) in an on-going process, even in storage lagoons, particularly where water levels fluctuate, causing the generation of acidic, metal-rich waters which may accumulate within and eventually drain the site (Fig. 2(b)), and being the cause of more widespread environmental pollution. Waters of similar chemistries may also form within abandoned deep mines, in waste rock and spoilage dumps, at and around coal mines as well as metal mines and in the voids of closed and abandoned opencast mines (Fig. 2(c)). The latter become infilled, at different rates, with groundwater, forming pit lakes that typically are stratified with surface layers often being moderately or severely acidic. Waste waters associated with metal and coal mining (often referred to generically as "acid mine drainage") have long been recognized as a major environmental threat to areas adjacent to, and sometimes distant from, abandoned mines. The causative agents of the formation of these metal-rich, acidic water bodies are again the specialized, mostly acidophilic, microbial consortia described above (and in more detail below) that exploit the opportunities given

Fig. 2 (a) The Rio Tintillo, an iron-rich extremely acidic river that flows through south-east Spain, showing the accumulation of secondary ferric iron minerals and terraces and the orange-red coloration typical of extremely acidic iron-rich water bodies; (b) a pit lake formed by the ingress of ground water into an abandoned opencast metal mine (San Telmo, Spain); (c) an acidic metal rich stream draining an abandoned copper mine in Avoca, Ireland.

to them in human activities inadvertently making accessible sulfide minerals from which they can indirectly obtain the energy they need to sustain their somewhat unusual life-styles.

Extremely Acidophilic Micro-Organisms

Although highly acidic and often metal-rich environments are toxic to most life-forms, a surprisingly large number of diverse organisms can thrive in some situations, though *bona fide* (metabolically-active) indigenous life-forms are microbial and mostly single-celled. Although some angiosperms have been observed to grow in highly acidic lakes, their root systems grow in sediments in which the pH is usually much higher than the water body itself. The axiom that as an environmental parameter (in this case, acidity) becomes more extreme, biodiversity declines holds true for both simple and complex life-forms. Research over the past 30 or so years has unveiled a large diversity of acidophilic microorganisms, which occur randomly throughout the "tree of life" in all three domains – Bacteria, Archaea and Eukarya – indicating that acidophily is a trait that has developed on many occasions during the evolution of life on planet Earth, rather than from a single adaptation event. As with other extremophiles, acidophiles tend to be specialized life-forms, and most extreme acidophiles are unable to grow in neutral pH environments. Another trait they have in common with most other extremophilic life-forms is that, while acidophiles that tolerate other physico-chemical extremes (e.g., very high temperatures or hyper-salinity) as well as extreme acidity do exist, these tend to be less acid-tolerant than those species that can tolerate extreme acidity alone. This is particularly noticeable among eukaryotic acidophiles. The biodiversities of extremely acidophilic prokaryotic and eukaryotic is described in the following sections.

Prokaryotic Extreme Acidophiles

Prokaryotes comprise Bacteria and Archaea. Although these are the most simple life-forms in terms of their morphologies (single celled organisms, occurring mostly as rods, spheres, filaments or vibrios), in terms of the physiological and metabolic potential they

(as a group) are vastly superior to eukaryotes. One reason for this is that prokaryotes have been resident on planet Earth for an estimated 3.8 billion years, while the first (single celled) ancestors of eukaryotes are thought to have emerged about 2.7 billion years ago.

The first extremely acidophilic micro-organism to be isolated and characterized (by Waksman and Joffe in 1921) was the sulfur-oxidizing bacterium, *Acidithiobacillus (At.) thiooxidans*, though it was referred to at the time as *Thiobacillus thiooxidans*. Some years later, another sulfur-oxidizing bacterium was isolated from water draining a coal mine that had the unique trait (at the time) of also being able to oxidize ferrous iron to ferric at very low pH. *(Acidi)thiobacillus ferrooxidans* has subsequently become the most well-studied of all acidophilic micro-organisms. It is now known, however, that many acidophilic bacteria that were previously considered to be strains of *At. ferrooxidans*, on the basis that they were aerobic straight rods that could oxidize both iron and sulfur, were probably other species which were unrecognized until relatively recently. Currently, the genus *Acidithiobacillus* comprises seven species, all of which (by definition) oxidize reduce sulfur and four of which (*At. ferrooxidans*, *At. ferridurans*, *At. ferrivorans* and *At. ferriphilus*) can also oxidize ferrous iron, and the genus will undoubtedly undergo further revision and expansion in future years. Both of the early *Acidithiobacillus* isolates (and all since) are autotrophic chemolithotrophs, i.e. they use inorganic electron donors and fix carbon dioxide. Such a metabolic life-style is highly appropriate in extremely acidic environments which tend to contain elevated concentrations of potential inorganic "energy sources" (electron donors, such as ferrous iron and reduced sulfur) but often low concentrations of dissolved organic carbon.

The major method by which single-celled prokaryotes generate ATP is by generating proton (hydronium ion) concentration gradients across their cell membranes, and then allowing controlled influx of protons through specific channels (ATPases). This is also the case for mitochondria in eukaryotic cells. On the face of it, therefore, acidophilic prokaryotes have an immediately available energy source as their cytoplasms have a typical pH values of around 6.5 while their external solutions are often in the pH range of 1.0–3.0 (i.e., differential proton concentrations of 4–6 orders of magnitude). The immediate energy source for acidophilic prokaryotes is this pre-existing proton gradient, though a major challenge to them is to prevent ingress of protons into their cytoplasms other than through defined channels. Obviously even tightly controlled influx of protons will ultimately lead to severe internal acidification and death, and with almost all electron donors used by these prokaryotes, they are faced with the challenge of pumping out protons against concentrations gradients that are immensely different to their neutrophilic counterparts. The single exception to this is ferrous iron, which is used as an electron donor by many species of chemolitho-autotrophic and chemolitho-heterotrophic acidophiles. In Gram-negative acidophiles, ferrous iron is oxidized within the periplasm (the space between the inner and outer membranes). This is necessary, as the ferric iron produced is highly insoluble in all but acidic liquors and would precipitate and accumulate inside bacteria and archaea if this was an intracellular reaction. The periplasm, however, has a similar pH to the bathing (external) acidic liquor, which facilitates the diffusion of the ferric iron waste product away from the cells. The electron captured from ferrous iron is transferred through the inner membrane, eventually being used to "neutralize" inflowing protons, forming, in combination with molecular oxygen, water (reaction (6)):

$$4Fe^{2+} + 4H_3O^+ + O_2 \rightarrow 4Fe^{3+} + 6H_2O \quad (6)$$

It should be noted that oxidation of ferrous iron consumes acidity, which can be considered as "de-energizing" these extreme environments, in contrast to reactions involving zero-valent sulfur (reaction (1)) or reduced sulfur oxy-anions which generate acidity and can therefore be considered to "re-energize" these environments.

There are various ways in which acidophilic prokaryotes can be, and have been, sub-categorized. Traditionally this has been on the basis of physiological traits, and this can be very useful, for example when considering their applications in biotechnologies. However, in more recent times categorizing acidophiles on the basis of their phylogenetic affiliations has become more commonplace.

Categorization Based on pH and Temperature Responses

The ability to tolerate elevated concentrations of protons (hydronium ions; H_3O^+) is obviously what defines an acidophile. While there is no official cut-off pH value that delineates whether an organism is or is not an acidophile, the generally accepted view is that, as a group, these can be divided into extreme acidophiles that have pH optima for growth at pH <3, and moderate acidophiles that have pH optima of between 3 and 5. The term "hyper-acidophiles" has been proposed for the relatively few species that grow optimally below pH 1.0. Acid-tolerant organisms have pH growth optima above 5, though can also grow at lower pH values. As would be anticipated, the most extremely acidic environments have less potential biodiversity than those that are moderately acidic. The number of prokaryotes that are known to grow at pH <1 is relatively small, and includes some Gram-positive bacteria (e.g., *Sulfobacillus* spp.), Gram-negative bacteria (e.g., *Leptospirillum* spp. and *At. thiooxidans*) and Archaea (e.g., *Ferroplasma* spp.) that oxidize iron and/or sulfur. The most acidophilic of all currently-known life forms is, however, a heterotrophic archaeon, *Picrophilus*. Two species are known, *P. oshimae* and *P. torridus*, both of which have optima pH for growth of ~0.7, and grow in synthetic media poised and pH ~0. These hyper-acidophiles are also thermophilic, with optimum temperatures for growth at ~60°C.

One of the most widely used methods to categorize acidophilic prokaryotes is on the basis of their temperature characteristics, i.e. their optimum temperatures for growth and range of temperatures within which they are active (Table 1). Three groups of acidophiles have often been recognized in this way: (i) Mesophiles, with temperature optima of 20–40°C; (ii) moderate

Table 1 Categorization of validated species of extremely acidophilic prokaryotic micro-organisms, based on growth temperature optima

		Electron donors			
Acidophile	*Carbon assimilation*	*Fe^{2+}*	*ZVS/S oxy-anions*	*H_2*	*Fe^{3+} reduction**
(a) Mesophiles (temperature optima 20–40°C) & psychro-tolerant species					
At. ferrooxidans	OA	+	+	+	+
At. ferridurans	OA	+	+	+	+
At. ferrivorans	OA	+	+	(−)**	+
At. ferriphilus	OA	+	+	−	+
At. thiooxidans	OA	−	+		−
L. ferrooxidans	OA	+	−	−	−
Fv. myxofaciens	OA	+	−	−	−
Fm. acidiphilum	OH	+	−	−	+
Atx. ferrooxidans	OH	+	−	−	+
Acidiphilium spp.	OH	−	+	−	+
A. acidophilum	FA	−	+	−	+
Acidocella spp.	OH	−	−	−	+
(b) Moderate thermophiles (temperature optima 40–60°C) & thermo-tolerant species					
At. caldus	OA	−	+	+	−
L. ferriphilum	OA	+	−	−	−
Af. thiooxydans	OA	+	+	−	+
Sulfobacillus spp.	FA	+	+	+	+
Alicyclobacillus spp.	OH/FA	+/−	+/−	nr	+/−
Am. ferrooxidans	FA	+	−		+
Fx. thermotolerans	OH	+	−	nr	+
Acd. organivorans	OH	−	+	nr	−
Ferroplasma spp.	OH	+	−	−	+
Acidiplasma spp.	OH	+	−	−	??
C. divulgatum	OH	−	−	nr	−
Thermoplasma spp.	OH	−	−	nr	−
Picrophilus spp.	OH	−	−	nr	−
(c) Extreme thermophiles (temperature optima >60°C)					
H. acidophilum	OA	−	−	+	−
S. acidocaldarius	OH	−	−	−	−
S. solfataricus	OH	−	−	−	−
S. metallicus	OA	+	−	+	−
S. tokodaii	OH	+	−	+	−
Metallosphaera spp.	FA	−−	−	+	−
Sulfurococcus spp.	FA	−	−	+	−
Ac. infernus	OA	−	−	+	+
Ac. ambivalens	OA	−	−	+	+
Ac. brierleyi	FA	+	−	+	+
Sg. azoricus	OA	−	−	−	+
Ss. ohwakuensis	FA	−	−	−	+

Source: Key: OA, obligate autotroph; FA, facultatative autotroph; OH, obligate heterotroph. Genera abbreviations: *A., Acidiphilum; Ac., Acidianus*; *Acd., Acidicaldus; Af., Acidiferrobacter; Am., Acidimicrobium; At., Acidithiobacillus; Atx., Acidithrix*; *Fm., Ferrimicrobium; Fx., Ferrithrix; C., Cuniculiplasma; H., Hydrogenobaculum; L., Leptospirillum; S., Sulfolobus; Sb., Sulfobacillus; Sg., Stygiolobus; Ss., Sulfurisphaera.*

thermophiles, with temperature optima of 40–60°C; (iii) extreme thermophiles, with temperature optima of 60–80°C. The term "thermo-tolerant" has also been used to describe those acidophiles, such as *L. ferriphilum*, that can grow above 40°C but not above 50°C, and also for those that are very active over a wide temperature range (e.g., 25 to >45°C in the case of *At., caldus*). While some acidophiles (*At. ferrivorans, Ferrovum myxofaciens* and some strains of *Acidiphilium*) have been demonstrated to be active at very low (<5°C) temperatures, all of these have temperature optima well above 20°C, and are therefore psychro-tolerant rather than psychrophilic micro-organisms. At the other end of the temperature spectrum, the most thermophilic extreme acidophile known is the facultatively anaerobic sulfur-metabolizing archaeon *Acidianus infernus*, which has a growth temperature optimum of 90°C and a maximum of about 96°C. However, relatively few hyper-thermophilic acidophiles are known, and the fact that the maximum temperature for growth of an acidophile is about 25°C lower than that of the most thermophilic life-forms known (neutrophilic *Pyrolobus*-like archaea) is possibly a reflection of the difficulty that living organisms have when challenged by the dual stresses of extreme temperature and acidity.

As with neutrophilic prokaryotes, extremely thermophilic acidophiles are mostly Archaea while mesophiles are predominantly Bacteria. The majority of moderate thermo-acidophiles and thermo-tolerant acidophiles are also Bacteria, and mostly Gram-positives, while most known Gram-negative acidophilic bacteria grow best at below 40°C. There are exceptions to this general trend. Indeed, the most thermophilic acidophilic bacteria known - the sulfur-oxidizing autotroph *Hydrogenobaculum acidophilum*, which grows at up to 70°C, and the heterotroph *Acidicaldus organivorans*, which grows at up to 65°C – are both Gram-negative.

Categorization Based on Requirement for Molecular Oxygen

The majority of known acidophilic prokaryotes have been described, when first characterized, as obligate aerobes. More detailed examination has revealed that, in many cases, they can also grow in the absence of oxygen and are therefore facultative anaerobes. Of the various options that micro-organisms use for living in the absence of oxygen, by far the most widespread among acidophiles appears to be ferric iron respiration. This is understandable since iron, as both ferrous and ferric, is usually abundant in extremely acidic environments, particularly those originating from the oxidative dissolution of sulfide minerals. There is also a thermodynamic advantage to be gained from using ferric iron, in that the redox potential (E_h value) of the ferrous/ferric couple in acidic sulfate-rich waters is ca. +680–700 mV, a value which is not much below that of the oxygen/water couple (+840 mV at pH 7) and considerably more positive than alternative inorganic electron acceptors such nitrate and sulfate. Most Bacteria (and the euryarchaeotes, *Ferroplasma* and *Acidiplasma*) that can oxidize ferrous iron in the presence of molecular oxygen can also reduce ferric iron when oxygen is absent. Notable exceptions are species of *Leptospirillum*, though this is explained by the fact that these highly specialized bacteria have not been demonstrated to use an electron donor other than ferrous iron. Other acidophilic bacteria that also reduce ferric iron to ferrous are obligate heterotrophs, one of which (*Ferrimicrobium acidiphilum*) is also an iron-oxidizer, while others (*Acidiphilium*, *Acidocella* and *Acidobacterium* spp.) are not. Recently it has been shown that sulfur-oxidizing *At. caldus* and *At. thiooxidans* can reduce ferric iron when grown under aerobic conditions. The mechanism for this is indirect and not well understood, though it has been demonstrated that neither acidophile can grow via ferric iron respiration.

Sulfur respiration (the use of elemental sulfur as electron acceptor) is a widespread characteristic of acidophilic archaea: *Acidianus*, *Stygiolobus*, *Sulfurisphaera* (all thermo-acidophilic crenarchaeotes) and *Thermoplasma* (a moderately thermo-acidophilic euryarchaeote) can all grow anaerobically by reducing sulfur to hydrogen sulfide. *Acidianus* spp. and *Sulfurisphaera ohwakuensis* are both facultative anaerobes that couple the oxidation of hydrogen to the reduction of sulfur in anoxic environments, while *Stygiolobus azoricus* is an obligately anaerobic thermo-acidophile that can do the same. In contrast, both classified species of *Thermoplasma* (*Tp. acidophilum* and *Tp. volcanium*) are facultative anaerobes that couple the oxidation of organic carbon to the reduction of zero-valent sulfur. No extremely acidophilic sulfur- or sulfate-reducing bacteria (SRB), or sulfate-reducing archaea, have yet been isolated and characterized, though moderate acidophiles and acid-tolerant species, such as *Desulfosporosinus acididurans*, some of which are metabolically active at pH <3, have recently been described.

Clones of methanogenic archaea have also been identified in gene-libraries constructed from DNA extracted from some extremely acidic environments, but no extremely acidophilic methanogens are known. Likewise, no acetogenic acidophiles have been characterized, though this may be explained on the biotoxicity of acetic acid in low pH liquors. The general toxicity of aliphatic acids may also help account for the apparent absence of fermentative metabolism among extreme acidophiles, with the exception of the thermophilic archaeon *Acidilobus aceticus*, which can grow by fermenting starch to acetic acid. The scarceness of nitrate in most acidic environments, apart from those in the vicinity of rock blasting, and the fact that acidophiles are more sensitive to nitrate and nitrite than most other bacteria, are probably why nitrate respiration is apparently absent in these micro-organisms.

Categorization Based on Carbon Assimilation

Some extremely acidophilic prokaryotes fix CO_2, like green plants (obligate autotrophs), others have an absolute requirement for pre-fixed (organic) carbon (obligate heterotrophs), while a third group fix CO_2 but switch to assimilating organic carbon if and when this becomes available. The metabolic logic for the latter is obvious; CO_2−fixation is a highly energy-consuming process (e.g., the iron-oxidizing acidithiobacilli have been estimated to utilize most of the energy they obtain from oxidizing iron on this single process) and using pre-fixed carbon, assuming that it is readily incorporated and metabolized, avoids this expenditure of energy. Various terms have been used to describe such micro-organisms, which include some eukaryotic algae as well as some prokaryotic acidophiles, though the most appropriate (and least ambiguous) is to refer to them as facultative autotrophs.

Acidophilic autotrophic prokaryotes appear, either predominantly or exclusively, to use inorganic electron donors (iron, reduced sulfur and hydrogen) rather than solar energy, to sustain their growth. Cyanobacteria and green- and purple sulfur-bacteria that are found in higher pH environments appear not to occur (or at least be active) in extremely acidic environments. In some situations, chemolithoautotrophs are the only primary producers present (e.g., in abandoned underground mines and acidic caves) yet, as evidenced by the scale of life that has been reported in some of these sites, their activities can underpin large accumulations of acidophilic biomass. Where solar energy is accessible however, they mostly take a more minor role in net primary production than acidophilic micro-algae.

Bacteria, in particular, are renowned as a collective group of micro-organisms for their abilities to degrade a multitude of small and large molecular weight organic compounds, including many synthetic materials. Acidophilic prokaryotes, on the other hand, appear to use a far more restricted range of monomeric organic substrates, and few polymeric materials. Simple sugars and alcohols

are utilized by many heterotrophic acidophiles, but small molecular weight aliphatic acids tend to be lethal to acidophiles when present in only micro-molar concentrations. The reason for this relates to the fact that these organic acids exist primarily as undissociated, lipophilic molecules in low pH liquors. These can freely permeate microbial membranes and accumulate in the circum-neutral pH cell interiors where they dissociate and cause intracellular acidification of the cytoplasm. Di- and tricarboxylic organic acids, such as citric acid, are not so toxic and are actually used as substrates by many heterotrophic acidophiles. Some organic acids, most notably glutamic acid, also serve as appropriate substrates for many acidophiles, though others (e.g., glycine) do not. Complex, nitrogen-rich organic substrates, such as yeast extract and tryptone, are also suitable substrates for isolating and cultivating many heterotrophic acidophiles, and supplementing defined organic growth media with, for example, yeast extract, often promotes growth of heterotrophic acidophilic bacteria and archaea. One of the few known examples of an acidophile being able to grow on an organic polymer is the archaeon *Acidilobus aceticus*, which grows anaerobically on starch, forming acetate as the main metabolic product.

Specialized and Generalist Acidophilic Prokaryotes

Acidophiles as a group are highly versatile, and are able to utilize a wide variety of energy sources (solar, and inorganic and organic chemicals), grow in the presence or complete absence of oxygen, and at temperatures of between 4°C and 96°C. However, individual species display very different degrees of metabolic versatility. Among the most specialized acidophiles are *Leptospirillum* spp. and *Ferrovum myxofaciens*, all of which are known only to use a single electron donor (ferrous iron) to support their growth and, because of the high redox potential of the ferrous/ferric couple these bacteria have, by necessity, to use molecular oxygen as electron acceptor, restricting them to being metabolically active only in aerobic environments. *Leptospirillum* spp. and *Ferrovum myxofaciens* are obligate autotrophs, and can also fix dinitrogen. Their metabolic limitations appear, however, to be compensated for by other physiological advantages that they have over other iron-oxidizing acidophiles. In the case of *Leptospirillum* spp., their greater affinity for ferrous iron and tolerance of extremely high concentrations of ferric iron and for growth in temperatures >40°C than, for example, the iron-oxidizing acidithiobacilli, enables them to out-compete the latter bacteria in many natural and anthropogenic environments, such as stirred tank bioreactors used to bioleach or bio-oxidize sulfide ores.

Species of the genus *Acidithiobacillus* are, in contrast, more generalist bacteria. Some can use a variety of inorganic electron donors (iron, sulfur and hydrogen) while others use a more limited range. Even within species, there may be differences in this regard. For example, only one strain of *At. ferrivorans* and one of *At. thiooxidans* have been found to use hydrogen as electron donor. While all iron-oxidizing acidithiobacilli have been confirmed to be facultative anaerobes, species, such as *At. thiooxidans*, that cannot oxidize iron do not grow under anoxic conditions.

The most generalist of all acidophiles are spore-forming Firmicutes of the genus *Sulfobacillus*, and to a lesser extent *Alicyclobacillus* and *Acidibacillus* spp.. *Sulfobacillus* are Gram-positive bacteria that can grow in aerobic or anaerobic environments, and are facultative autotrophs. They use a wide range of electron donors, including ferrous iron, hydrogen, zero-valent sulfur and reduced sulfur oxyanions, and a variety of organic compounds (such as glucose and glycerol) as carbon sources and electron donors, though their capacities for heterotrophic growth are more limited than the more heterotrophically-inclined *Alicyclobacillus* spp..

Categorization of Acidophilic Prokaryotes on the Basis of Their Phylogenetic Affiliations

As elsewhere in (micro)biology, classification based on biomolecular signatures (mostly on comparative sequences of 16S/18S rRNA genes) has become the *prime facie* way of categorizing extremophilic prokaryotes. Acidophilic micro-organisms can be found apparently randomly distributed throughout the tree of life. This is particularly apparent for prokaryotic acidophiles within the domains Bacteria and Archaea.

Domain Bacteria. Many acidophilic bacteria are Gram-negative Proteobacteria, the largest of the bacterial phyla. The Alphaproteobacteria includes three genera of extreme acidophiles: *Acidiphilium*, *Acidocella* and *Acidimonas*. All three are obligate heterotrophs, except (at present) for one species of *Acidiphilium* (*A. acidophilum*) which is a facultative autotroph which, like *Acidithiobacillus* spp., can use oxidation of zero-valent sulfur to fuel the fixation of carbon dioxide. Several other *Acidiphilium* spp. can also oxidize sulfur, though they require organic carbon. *Acidiphilium* spp. are also frequently found in tight association to iron-oxidizing *Acidithiobacillus*, possibly because they are the most acidophilic of the three genera. *Acidocella aromatica* is unusual (for an acidophile) in being able to grow on a range of aromatic compounds and aliphatic acids. *Acidimonas methanolica* (currently the only species of this genus) was named as such to reflect its growth on C_1 compounds, though this is something that is also characteristic of *Acidiphilium* spp.. In contrast, only one extreme acidophile is currently affiliated to the Betaproteobacteria, the iron-oxidizing autotroph *Ferrovum myxofaciens*, which forms macroscopic "streamer-like" growths in flowing, acidic (pH >2) ferruginous waters, and also to the Gammaproteobacteria (the iron/sulfur-oxidizing autotroph *Acidiferrobacter thiooxydans*). Previous phylogenetic uncertainty regarding acidophiles of genus *Acidithiobacillus* (Table 1) was resolved by allocating these (and the closely related neutrophilic genus, *Thermithiobacillus*) to a new class within the Proteobacteria phylum, the Acidithiobacillia. Other Gram-negative extreme acidophiles are found within the deeply-branching bacterial phyla Nitrospirae, Aquificae and Verrucomicrobia. The most widely studied of these, primarily because of their importance in mineral processing biotechnologies, are *Leptospirillum* spp. (Nitrospirae), described above. *Hydrogenobaculum acidophilus* (Aquificae) grows autotrophically on hydrogen

at an optimum temperature of 65°C, while *Methylacidiphilium infernorum* (Verrucomicrobia) is a methylotrophic acidophile that grows optimally at pH 2.0–2.5 and at 60°C.

Gram-positive extreme acidophiles currently fall within two phyla: Actinobacteria and Firmicutes. The Actinobacteria houses six described (though not all validated) species, most of which can both oxidize and reduce iron, though sulfur-oxidation is a relatively rare trait within this group. Species vary in both their temperature characteristics and morphologies, growing as mesophilic and moderately thermophilic cells that form single rods and long entwined filaments. Spore-forming Gram-positive extreme acidophiles (Firmicutes) comprise three genera: *Sulfobacillus*, *Alicyclobacillus* and *Acidibacillus*. *Sulfobacillus* spp. are described above. The first *Alicyclobacillus* spp. were moderate acidophiles that were isolated from pasteurized fruit juices, while more recent species and strains were isolated from mineral leaching environments, and catalyze the dissimilatory oxidation of ferrous iron and/or sulfur. Most recently, three species of the novel genus *Acidibacillus* have been described. These are obligately heterotrophic extreme acidophiles that oxidize iron (and some also oxidize sulfur) and which vary in their temperature characteristics. *Acidibacillus* have global distribution in a wide range of environments (e.g., geothermal sites, mine waste and sulfur deposits) and have been isolated from neutral pH as well as acidic sites.

Domain Archaea. Currently, there are six recognized genera of extreme acidophiles located within the archaeal phylum Euryarchaeota, all within the order *Thermoplasmatales*: *Ferroplasma*, *Acidiplasma*, *Thermoplasma Picrophilus*, *Thermogymnomonas* and *Cuniculiplasma*, All of these are heterotrophic, though it has also been claimed that one (*Fp. acidiphilum*) can also fix inorganic carbon. Another notable feature is that some species lack cell walls, causing cells to have irregular and often variable morphologies. *Thermoplasma acidophilum* was the first extremely acidophilic heterotrophic prokaryote to be described, while *Picrophilus* spp. have the distinction of being the most acidophilic of all currently known life forms, with pH optima of ~0.7 and minima of <pH 0. *Ferroplasma* and *Acidiplasma* frequently occur in commercial biomining operations, though their precise roles here have yet to be elucidated. While both can oxidize ferrous iron (and thereby contribute, in theory, to accelerating the oxidative dissolution of metal-containing minerals) they are both scavengers of organic carbon (such as dead bacteria), and their relative numbers in bioleaching operations increase in response to the mortality of other bioleaching micro-organisms.

Extremely acidophilic crenarchaeotes are all thermophiles and members of the order *Sulfolobales*, and include the first extremely thermo-acidophilic archaeon to be characterized, *Sulfolobus acidocaldarius*. Some species, though not *S. acidocaldarius*, can oxidize reduced sulfur and one (*Sulfolobus metallicus*) can also grow chemolitho-autotrophically on ferrous iron. Other *Sulfolobales* (e.g., *Metallosphaera*, *Sulfurococcus* and *Acidianus* spp.) also oxidize sulfur and/or iron, and are thought to be important in high temperature (>60°C) mineral bio-processing operations. Some *Acidianus* spp. are able to use sulfur either as an electron donor under aerobic conditions, or as an electron acceptor (coupled to the reduction of hydrogen) under anaerobic conditions. Two other species of thermo-acidophilic archaea, *Sulfurisphaera ohwakuensis* and *Stygiolobus azoricus*, can also grow via sulfur respiration, generating H_2S.

Eukaryotic Extreme Acidophiles

The pioneering studies conducted by Thomas Brock on extremophiles in the hydrothermal vents of Yellowstone National Park focused on life in extreme environments of prokaryotes and their metabolisms. However, eukaryotic microbial life has also been observed in almost all extreme settings where there is a source of energy to sustain it, the only exceptions being high temperature environments (>70°C) and the deep subsurface biosphere. Eukaryotic organisms have been found to be highly adaptable, and molecular analysis of different extreme environments, including extremely low pH sites, has unveiled new protist genetic diversity. As is the case with prokaryotic acidophiles, representatives from multiple evolutionary eukaryotic lineages occur in low pH environments, suggesting that the ability to adapt to this environmental extreme is widepread. Which environmental parameters are most influential in determining eukaryotic microbial diversity patterns at pH extremes remains under-explored.

Diversity of Eukaryotic Acidophiles

Species belonging to the four main eukaryotic superclades, *SAR* supergroup *(Stramenopiles, Alveolates and Rhizaria)*, *Excavata*, *Archaeplastida* and *Unikonta* have been described in extremely pH environments. Within *Stramenopiles*, diatoms from the genera *Pinnularia* are the most widespread, as well as *Alveolates* such as *Oxytricha* ciliate. Species related to the order *Cercomonas* are the main micro-organisms within the *Rhizaria*. Members of *Excavata* (heterotrophic flagellates) include species belonging to *Bodo* and *Ochromonas* genus as well as Euglenozoans such *Euglena mutabilis*. AMD-related *Archaeplastida* include phototrophic red (*Rhodophyceae*) and green algae (mainly *Chlorophyta*); these organisms depend on sunlight and are present in open-air systems. Additionally, *Heterolobosea* (amoebas) genera *Naegleria* and *Vahlkampfia*; *Rotifera (i.e. Cephalodella acidophila)*; heliozoan (*Actinophyrs*) and *Fungi* (*Opisthokonta*) complete the general set of eukaryotes found in acidic emplacements. The *Ascomycota* and *Basidiomycota* fungi are the primary eukaryotic members in sub-surface low-pH biofilms.

In acidic geothermal springs, the unicellular rhodophyte *Galdieria sulphuraria* (formerly *Cyanidium caldarium*), is often the dominant eukaryote worldwide. This moderate thermophile (35–56°C) may grow as a heterotroph in the absence of light (as may *Euglena*) and has been reported to grow at pH values around zero. Related *Galdieria* species can be found also in acidic AMD and ARDs since they are extremely tolerant to heavy metals being able to absorb high concentrations of them from the acidic water.

These eukaryotic microbial communities are distributed in extensive biofilms, formed mainly by photosynthetic species surrounded by an extracellular matrix. Microbial biofilms are organized multicellular systems with a structural and functional architecture which influences metabolic processes, response to nutrients, predation and other factors of the ecosystem. Thus, it has been suggested that biofilm structures generate micro-niches in which the environmental conditions are less stressful, thereby facilitating physiological activities that would not be feasible in the water column. As in other habitats, most acidic biofilms are composed of mixtures of different micro-organisms. The macroscopic shape and species composition of the biofilms vary greatly throughout these ecosystems. Some of them adopt filamentous morphologies in flowing water (Fig. 3(a–c)) while others form thick colourful patches firmly attached to the mineral substrates (Fig. 3(d–e)).

Some of the most extensively studied photosynthetic acidic biofilms are located in the Río Tinto (Spain). These biofilms are usually distributed on the riverbed as independent patches easily differentiated by their texture and color, although after microscopic observations some areas indicated mixing of communities mostly at the interface with the substrate. Most of the naturally grown biofilms are composed of different species organized in different layers separated by extracellular polymeric substances (Fig. 4).

Biodiversity studies show the presence of sequences related to uncultured organisms present in acidic or contaminated soil habitats, such as *Betaproteobacteria* and *Acidobacteria*, suggesting that biofilms appear to facilitate the development of less extreme adapted organisms coming from soil habitats. Besides, prokaryotic communities present in thinnest photosynthetic biofilms (<100 μm) resembled the biodiversity present in the water column, while thicker biofilms (>200 μm) show higher degree of prokaryotic diversity than in the water column. Thus, the presence of different environmental conditions inside the biofilms might explain why the greatest degree of prokaryotic diversity is associated with localities in which extensive photosynthetic biofilms are developed. The development sequence of the biofilms starts with the accumulation of unstructured material mainly formed by bacteria and inorganic particles of minerals. Within the next two months, fungi and some heterotrophs such as amoeba and flagellates (i.e., *Bodo* and cermonads) appear. After the mycelial matrix is formed, diatoms begin colonization, closely followed by green algae (*Dunaliella* and *Chlamydomonas*) as well as *Euglena*. Sessile species of algae such *Chlorella* or *Cyanidium* are the last colonizing microorgamisms followed by the filamentous algae, *Zygnemopsis* and *Klebsormidium*.

Acidophilic micro-algae: Micro-algae are the dominant eukaryotic micro-organisms found in extreme acidic environments that are exposed to light. Chlorophyta such as *Chlamydomonas*, *Dunaliella* and *Chlorella* are frequently found (Fig. 5(a–b)). *Eunotia* and *Pinnularia* diatoms are also widely distributed in acidic environments (Fig. 5(c)). Filamentous algae from the genera *Klebsormidium* and *Zygnemopsis* are extremely abundant in summer months, producing large biomass areas on the water surface (Fig. 5(d)). The photosynthetic protist *Euglena mutabilis* is probably the most widespread species reported in acidic environments, being considered as a biomarker of low pH waters (Fig. 5(f)). The reasons for the dominance of these particular algal groups in these ecosystems are presumed to lie in their adaptive capabilities, their ecophysiological specialisation and the ecological advantages that they possess.

Apart from *Dunaliella acidophila*, the only other algal species reported to grow at pH 0 belongs to the order Cyanidiales. This order contains a group of asexual, unicellular red algae, which thrive in acidic (pH 0.5–3.0) and high temperature (50–55°C) conditions around hot springs and/or acidic sulfur fumaroles as well as in some acidic mining areas. Three genera (*Cyanidium*, *Cyanidioschyzon* and *Galdieria*) distributed in six species are identified in this order. Cyanidiales show a spherical morphology, one plastid, up to three mitochondria, a vacuole for products storage, the nucleus and are surrounded by a thick cell wall. The Cyanidiales is one of the most ancient groups of algae, located at the base of the Rhodophyta, and showing characteristics of Cyanobacteria. Within them, *Galdieria sulphuraria* is a remarkable species in terms of tolerance to high concentrations of metals and salt, exhibiting also a wide metabolic versatility, being able to grow photoautotrophically or heterotrophically using more than 50 carbon sources. *G. sulphuraria* is frequently found in volcanic hot sulfur springs, solfatara soils, and some contaminated anthropogenic environments. In habitats with high concentrations of toxic metals, easily represents up to 90% of total eukaryotic biomass.

Compared with prokaryotes, acidophilic micro-algae have received little attention, despite their abundance and remarkable presence in these environments. This might be due to them not being directly involved in geochemical transformations of iron and sulfur, though their indirect impact on these transformations can be considerable. For example, acidophilic micro-algae produce and secrete organic substrates (i.e., glycolic acid, fructose, glucose and mannitol) that stimulate the growth of acidophilic heterotrophic bacteria that can reduce both iron and sulfur/sulfate.

Acidophilic euglenoids and protozoa: *Euglena mutabilis* is one of the most abundant and conspicuous acidophilic micro-organisms in acidic environments. It has been described in acidic volcanic lakes, acidic geothermal areas and acidic mine waters, as is often considered a bio-indicator of the latter. *E. mutabilis* secretes metabolites, such as amino acids, polyamine compounds, urea and some sugars, into the external media that influences the composition and the metabolism of the whole microbial community.

Other groups of protozoa are frequently also found in acidic environments, and play key roles in the pelagic food web of acidic mining lakes. These include several species of ciliates (e.g., *Urotricha*, *Oxytricha* and *Frontonia* spp.), flagellates (e.g., *Ochromonas* and *Bodo* spp.), amoeba (e.g., *Vahlkampfia* and *Naegleria* spp.) and the heliozoan, *Actinophyrs* sp. Many of these species are acid-tolerant rather than obligate acidophilic, and are able to grow over a broad pH range. In acidic pit lakes, the phytoplankton is often dominated by the mixotrophic flagellate *Ochromonas* spp. Ciliates seem to be of minor importance in these environments, relative to mesotrophic circum-neutral pH lakes.

Acidophilic fungi and yeasts: Acidophilic fungi and yeasts have received far less attention than algae or protozoa, although among the filamentous fungi isolated from acidic sites are some of the most acidophilic of all known micro-organisms: three species of filamentous fungi (*Acontium cylatium*, *Trichosporon cerebriae* and a *Cephalosporium* sp.) are able to grow at *ca*. pH 0. Most fungi found

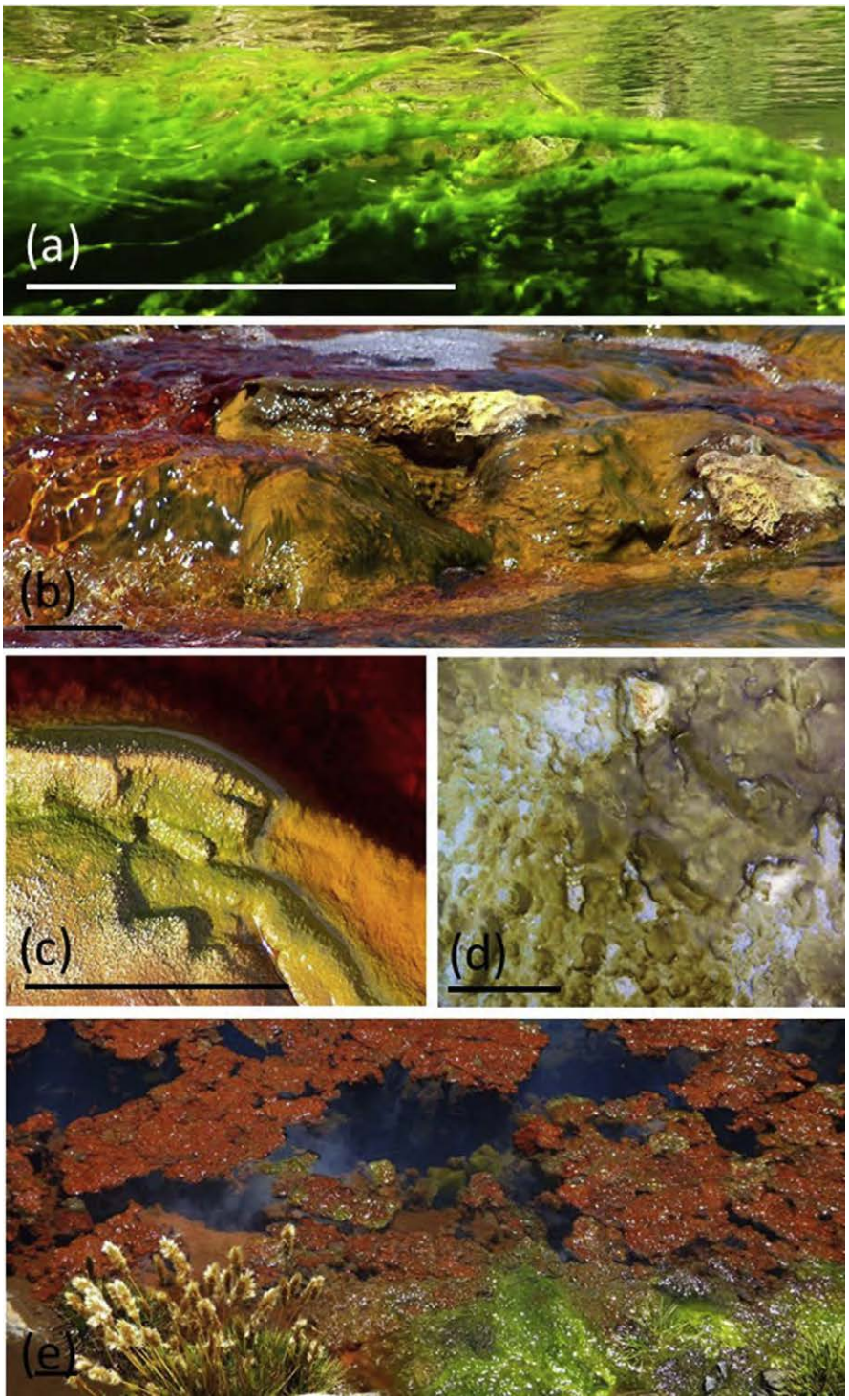

Fig. 3 Microbial eukaryotic biofilms found in different acidic environments: (a) Green filaments formed mainly by *Klebsormidium* spp. in the Pachacoto River (Huaraz National Park, Peru); (b) filamentous *Zygnemopsis* spp. in the Río Tinto (Huelva, Spain); (c) floating mats of filamentous zygnemas covered by iron oxides at Pastoruri Glacier (Huaraz National Park, Peru); (d) *Chlorella* spp. forming a thin layer over Rio Tinto sediments; (e) biofilms of the diatom *Pinnularia* spp. in the Seltun geothermal area (Reykjanes Peninsula, Iceland). The scale bar represents 10 μm.

in acidic habitats are, however, acid-tolerant rather than strictly acidophilic. Fungi play important roles in these environments because, together with other micro-organisms, they form biofilms on the surfaces of rocks. These biofilms are the site of metal and mineral precipitation and provide physical support for other microbial populations. Fungi can display metal resistance and can sequester specific metals allowing less tolerant species to exist. Recent studies have shown that biogenic formation of jarosite in

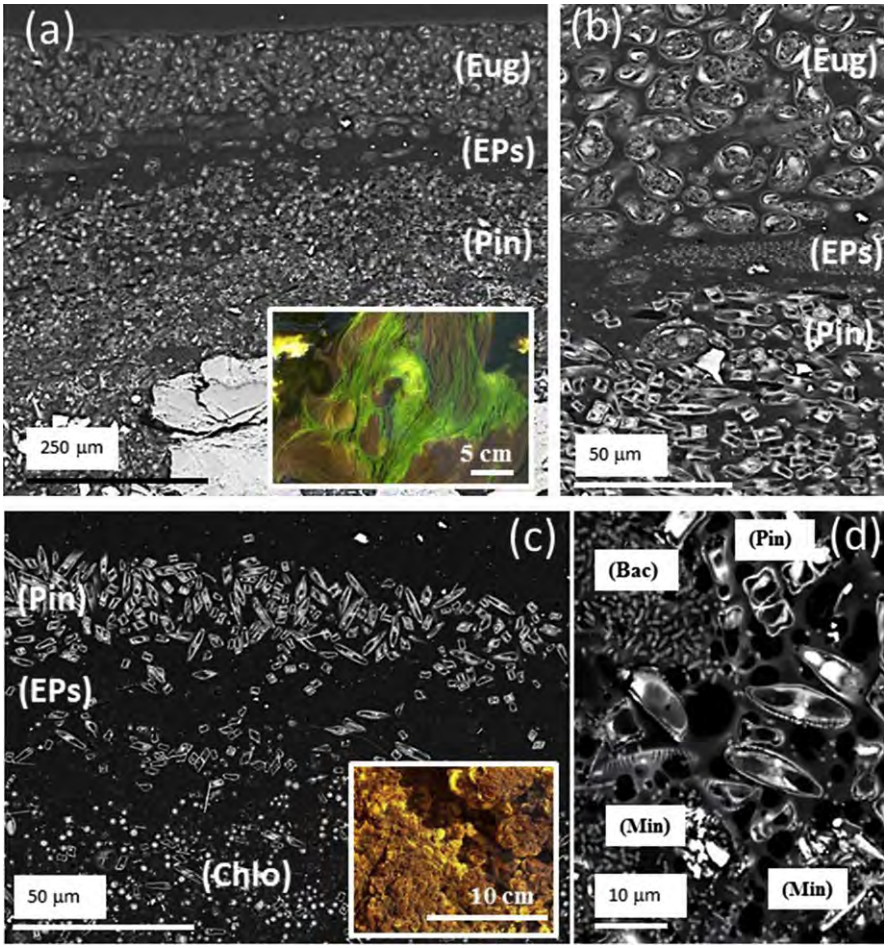

Fig. 4 SEM-BSE images of photosynthetic biofilms from Río Tinto: (a) Transverse section shows *Euglena* (Eug) and *Pinnularia* (Pin) species separated by an EPS layer (EPs), which dominate this biofilm above the rock fragments on the riverbed; (b) detailed image of same biofilm, showing a clear separation of the species; (c) transverse section showing *Pinnularia* (Pin) diatoms and *Chlorella* (Chlo), separated also by an EPS layer; (d) detailed image of the same biofilm showing bacterial communities (Bac) between *Pinnularia* (Pin) cells and some minerals (Min).

natural acidic environments, which is usually attributed solely to bacterial activity, is also enhanced by acidophilic fungi. *Purpureocillium lilacinum*, a fungal strain isolated from the Río Tinto, specifically precipitates hydronium-jarosite. The mineral starts to nucleate on the fungal wall of both living and dead cells as well as on extracellular polymeric substances (EPS).

Studies on yeasts in acidic environments are even scarcer and are usually related with *in situ* biodiversity, rather than adaptive mechanisms to acid stress. *Rhodotorula* spp., forming pink colonies, is usually found in acid mine drainage waters.

Adaptation Mechanisms in Eukaryotic Acidophiles

pH: As with prokaryotic acidophiles, the cytoplasmic pH of acidophilic eukaryotes is close to neutral, so they have to contend with extremely trans-membrane pH gradients. Acidophilic *Chlamydomonas* spp. have been reported to be able to survive a broad range of extracellular pH, by maintaining cytosolic pH values relatively neutral and faster rates of ATP consumption when living in acidic conditions than at neutral pH. Active proton extrusion into a cytosolic vacuole is suspected to be the pervasive mechanism that provides protection. Near neutral cytosolic pH may be maintained with the help of the contractile vacuoles in these micro-algae, without proton exchangers or transporters being exposed to the severe extracellular environment. In contrast, *Dunaliella acidophila*, one of the most extreme acidophiles known, shows a different adaptive mechanism for living at low pH. Several mechanisms have been described for its maintenance of trans-membrane pH gradients: (a) the existence of a potent plasma membrane H^+-ATPase, (b) a positive surface charge and (c) a positive inside trans-membrane potential. In addition, the possession of positive surface charges is probably another factor that reduces the permeability of *D. acidophila* cells to protons, and also to cationic metals.

Heavy metals resistance: Since cation transition metals are often much more soluble in acidic liquors, acidophilic eukaryotes, like their prokaryotic neighbors, must be tolerant to elevated concentrations of metals top survive in many low pH environments. Their

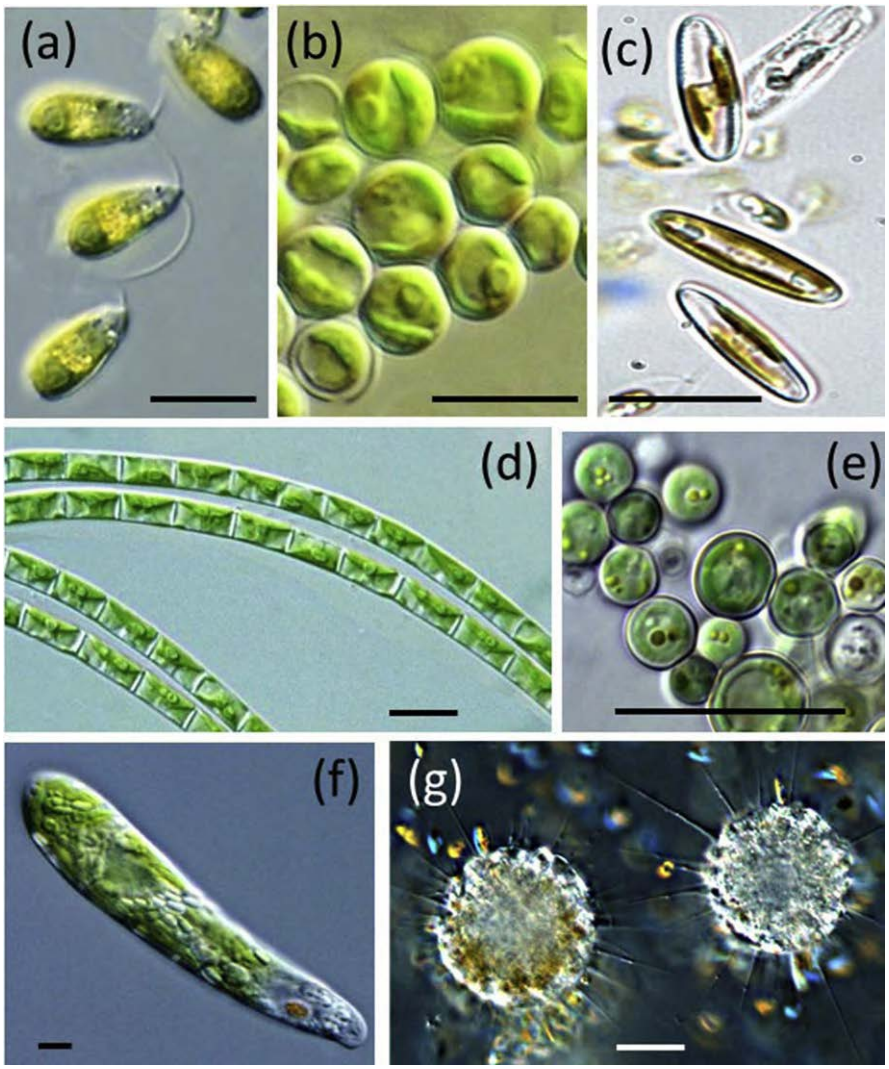

Fig. 5 Light microscopy photographs of different eukaryotic species isolated from Río Tinto (Huelva, Spain): (a) Green algae *Dunaliella acidophila* cells; (b) Green algae *Chlorella* spp. cells; (c) *Pinnularia* sp. diatoms; (d) Filamentous of *Klebsormidium* spp; (e) Red algae *Galdieria* sp.; (f) Photosynthetic protist *Euglena mutabilis*; (g) Heliozoa *Actinophrys* sp. Scale bar=10 m.

ability to survive high metal concentrations in acidic environments is due to a combination of extracellular passive and intracellular active systems. Within the passive resistance mechanisms, eukaryotic acidophiles usually form biofilms including extracellular polymeric substances (EPS) that can sorb metals to provide a further degree of metal tolerance. (Fig. 6).

The most frequent defence mechanism against heavy metal stress found in eukaryotic cells is the production of peptides capable of binding heavy metals, such as phytochelatines and metallothioneins. These molecules, as organometallic complexes, are further partitioned inside vacuoles to facilitate appropriate control of the cytoplasmic concentration of heavy metal ions, thus preventing or neutralizing their potential toxic effect. A recent study regarding transcriptomic response to cadmium, identified novel phytochelatin synthase (PCS) genes from two acidophilic green algae, *Dunaliella acidophila* and *Chlamydomonas acidophila* strains isolated from the Río Tinto. These novel PCS genes appeared to have a bacterial origin and were subsequently inherited by different groups, in some cases multiple times.

Acidophilic organisms are also able to transform metal species to less toxic or bioavailable forms. For example, *Cyanidium caldarium* is able to oxidize, reduce, and methylate arsenical compounds, the latter process leading to the generation of volatile arsenic species such as trimethylarsine oxide. In addition, *E. mutabilis* has been shown to be particularly tolerant to arsenic, which could depend on a novel response to the presence of this toxic metalloid. Transcriptomic analysis performed on both *Euglena* species, revealed that *E. mutabilis* avoids the accumulation of arsenic in the cell through the expression of several transporters, and can induce the expression of active DNA repair and protein turnover mechanisms, which allow *E. mutabilis* to maintain functional integrity of the cell under challenging conditions. Taken together, all these results suggest the existence of a specific arsenical response of *E. mutabilis* that may play a role in its hyper-tolerance to this toxic metalloid. Transcriptomic data and metabolomics approaches carried out in *Coccomyxa* sp., a micro-algae isolated from the AMD of Carnoulès (France), highlighted also the induction

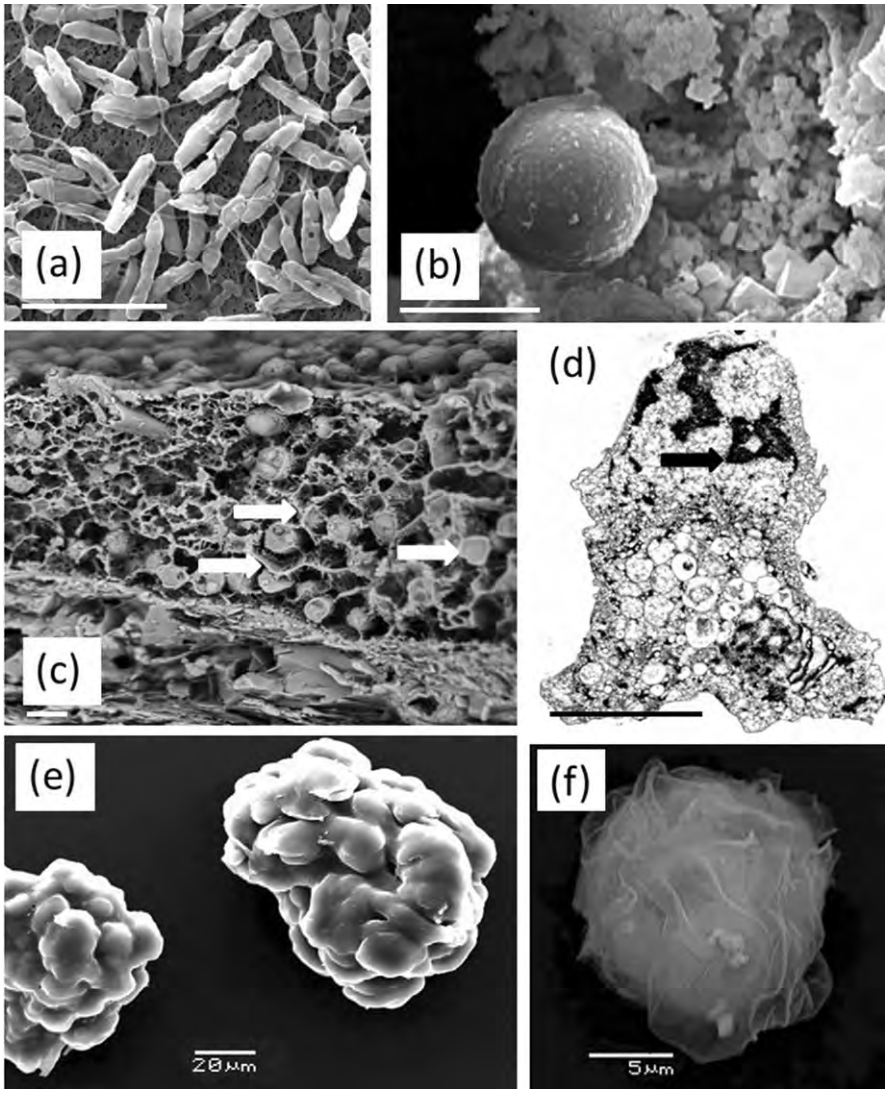

Fig. 6 Electron micrographs of acidophilic eukaryotic micro-organisms and biofilms from the Río Tinto (Huelva, Spain): (a) SEM of *Dunaliella acidophila*; (b) SEM micrograph of *Chlorella acidophila*; (c) LTSEM image of a natural *Chlorella*-dominated biofilm, arrows point to the mineralized EPS around the cells; (d) transmission electron micrograph of an amoeba isolated from the river, with electron-dense vacuoles containing transition metals arrowed; (e) SEM of *Chlamydomonas acidophila* cells surrounded by EPS; (f) SEM of *Chlorellla* spp. encased by EPS. The scale bar represents 10 μm, unless indicated otherwise.

of a set of transporters that could potentially prevent internal accumulation of arsenic. Electron microscope observations have indicated the presence of electron-dense bodies in vacuoles in *Chlamydomonas acidophila* and *Cyanidium caldarium*, as well as in other micro-algae species, treated with heavy metals. These dense bodies contain heavy metals, suggesting that metal deposition in vacuoles is also a mechanism that may contribute to heavy metal tolerance. Metal accumulation in vacuoles seems to be the most effective system for maintaining low cytoplasmic metal concentrations.

Nutrient limitations: Most extremely acidic environments contain relatively low concentrations of inorganic carbon (Ci) and phosphorus (Pi), and may therefore be considered oligotrophic, limiting the growth of autotrophic organisms. The low pH of acidic waters dictates that negligible concentrations of bicarbonate are present, and dissolved CO_2 is the sole inorganic carbon source. *Chlamydomonas acidophila* can accumulate Ci and shows high affinity CO_2 utilization under low-CO_2 conditions, similar to organisms with an active CO_2 uptake mechanism, although both processes are down-regulated under high CO_2 conditions. Thus, Ci availability is improved in *C. acidophila* under ecologically relevant conditions, which may enable optimal survival in its extreme Ci- and Pi-limited habitat. A further metabolic advantage is the ability of acidophilic micro-algae to produce phosphatases. This is an apparent adaption to the poor nutrient situation in these pit lakes and other acidic mine waters, and may be induced by aluminum. *C. acidophila* responds to phosphate deficiency not only by enhancing total phosphatase activities but also by producing another phosphatase with an optimum at pH 2. This is thought to be one reason why *C. acidophila* is one of the most abundant eukaryotic species in both extreme acidic environments, mining-impacted areas and volcanic habitats.

Photosynthesis: It is well known that photosynthesis is highly sensitive to stressful environmental conditions, such as pH and temperature extremes, and the presence of toxins. However, it appears that metal stress or low phosphate concentrations rather than elevated hydronium ion concentrations limit photosynthetic activity in extremely acidic environments. Light is often a limiting factor for primary productivity in acidic lakes, due to the presence of particulate materials and soluble ferric iron. Adaptation to low light intensities has been reported for benthic biofilms of diatoms in acidic lakes, where photosynthesis at low light intensities has been described, suggesting an efficient absorption of red light, the dominant wavelength available in these iron-rich acidic waters. All of the photosynthetic biofilms analyzed at Río Tinto (euglenas, chlorellas and diatoms biofilms) are acclimatized to low light intensities, and are mostly located at the bottom of the river bed, covered by several centimeters of a highly colored red water, while in contrast filamentous *Zygnemopsis* spp. float on the water surface, where the sun irradiance is extremely high during the summer. This difference in their ecological niches may explain their different photosynthetic behavior. Intense solar radiation can induce large-scale transcriptomic reprogramming during midday periods, though this is due to UV radiation rather than to light intensity. In order to minimize the negative effects of solar radiation, the transcriptomic results support the presence of a circadian rhythm in this euglenophyte that increases their opportunity to survive, with photosynthetic related processes enhanced in the morning, DNA synthesis occurring in the evening, and cell division taking place at night. The transcription pattern during the day is mainly altered in genes involved in Photosystem II stability and repair, UV damaged DNA repair, non-photochemical quenching and oxidative stress. Adaptation to low light intensities has also been reported in *Galdieria and Cyanidium*, which often grow endolithically (within rocks), and therefore, autotrophic cell growth is restricted to upper rock layers and/or to periods of high photosynthetically-active irradiation.

Biotechnological Applications of Acidophilic Eukaryotes

Cultivation and exploitation of extremophile micro-organisms has attracted interest due to their ability to accumulate high-value compounds, including various metabolites, enzymes and surfactants. Recent genetic studies on acidophilic algae may lead to novel biotechnological applications. During the last decade, several biotechnological approaches using acidophilic micro-algae have been reported with the food industry (carotenoids production, lipid bioaccumulation) and bioremediation of metal-contaminated environments as the main areas of interest.

Acidophilic micro-algae as a food supply: The use of micro-algae as a food source for humans and animals has been increasing since the early 1950s. Micro-algae can represent a valuable source of both vitamins and fatty acids and, because of the high protein content of some species, they could also have potential for manufacturing food-stocks both for humans and animals. Although micro-algae are usually photosynthetic organisms, a few species are able to grow under heterotrophic conditions. This possibility opens a promising field of research, through the use of cheap fermenters instead of expensive photo-bioreactors, allowing high densities of micro-algal cells and therefore high yields. In this respect, *Galdieria* species are of particular interest as they grow between pH 1.5 and 2.0, thus preventing contamination with pathogenic bacteria, which is one of the key problems of large scale micro-algae cultures. Heterotrophic cultures of *Galdieria sulphuraria* contain high levels of phycocianin, a pigment used as a dye in food and cosmetics as well as a fluorescent marker in medical diagnostics. In addition, *Galdieria* species also have significant potential as a source of protein and other macronutrients due to the high protein contents of their cell walls. The difficulties in introducing micro-algal-based ingredients in many foods are due to the strong green color and the susceptibilities of lipid moieties to oxidation, but the peculiar features of *G. sulphuraria* have the potential to overcome these obstacles. Nutritional analysis of 43 strains of *Galdieria* showed that they were rich in proteins (26%–32%) and polysaccharides (63%–69%) but contained relatively small amounts of lipids. Under heterotrophic cultivation conditions, the lipid moiety mainly contained monounsaturated fatty acids, suggesting that *G. sulphuraria* biomass has a potential use as food ingredients both for protein-rich or insoluble dietary fibre-rich applications.

Some issues related with the use of micro-algal biomass as a direct food source for animals have been reported, including their high nucleic acid content and possible contamination, and concern has been raised regarding potential toxicity and long-term effects on human health. Due to this, the future use of micro-algae biomass in the food industry is likely to be primarily as a source of nutraceuticals for functional foods rather than the direct use of such biomass. However, studies carried out with the acidophilic micro-algae *Coccomyxa onubensis* as dietary supplementation in laboratory rats have shown no adverse effects on rat health. In addition, *C. onubensis*-supplemented diets exhibited a potent hypocholesterolemic and hypotriglyceridemic effect in experimental animals. These results support the idea that acidophilic species might be better model systems for investigating the potential use of microalgae in animal feed.

Acidophilic micro-algae as source of industrially interesting compounds; anti-oxidant carotenoids and lipids: The extreme oxidative conditions of acidic environments suggest that acidophilic micro-organisms should express anti-oxidant mechanisms to defend themselves from oxidative stress. For this reason, the biotechnological value of several species of acidophilic micro-algae has been analyzed. *Chlamydomonas acidophila* accumulates high concentrations of lutein, a well-known antioxidant also known to be accumulated by other micro-algae; this compound has recently attracted interest for use in treatment of oxidative pathologies such as macular degeneration. Mixotrophic growth of *C. acidophila*, in terms of carotenoid production, resulted in even higher productivity of carotenoids (mainly lutein) than obtained with photo-autotrophic cultures. The accumulated lutein concentrations of *C. acidophila* under these conditions (about 10 g/kg dry weight) are among the highest reported for a microalga. Similar results were obtained with *Coccomyxa onubensis* (about 6 g lutein/kg dry weight).

The use of micro-algae as a carbon neutral source of biofuel has also been considered in recent years. Current micro-algae biofuel methodologies usually depend on growing high-lipid producing laboratory strains of micro-algae in open or closed photo-bioreactors. Unfortunately, these micro-algae species are usually highly sensitive to competition by indigenous strains or to environmental stresses. Contamination by invasive species can decrease productivity of commercial processes, increasing the price of production. The use of high-lipid acidophilic algae which thrive in restrictive culture conditions that reduce the risk of contamination could help to solve the contamination problems. Cultures of acidophilic micro-algae *Scenedesmus* spp. and a *Pseudochlorella* sp., have shown to accumulate high amount of storage lipids (30% of dry weight) by cultivating them in sulfuric acid-containing open ponds (generated from mine drainage waters), acidic hot springs or industrial wastes, demonstrating their potential use in next-generation biofuel industry.

Although current knowledge of oil accumulation mechanisms in micro-algae is relatively sparse, recent studies have yielded information on how to increase the cellular oil by genetic manipulation in tractable model algae such as *Chlamydomonas reinhardtii*, and also procedures for transformation in several other algal species. These developments and a further screening of algal strains from acidic environments could enhance oil productivity and biomass in acidic open ponds in the future.

Acidophilic eukaryotes and bioremediation: Typically, high water consumption in mining operations results in large amounts of waste water contaminated with heavy metals, salts and various other inorganic compounds. Traditional water treatment techniques such as reverse osmosis and membrane filtration are energy intensive and costly, and the use of micro-organisms to remove heavy metals and other potentially harmful inorganic materials in waste waters is a promising alternative. Extracellular polymeric substances (EPS) have attracted attention because of their biotechnological potential in removing contaminants, especially heavy metals. Acidophilic micro-organisms could play an important role in this area, since their EPS production is several orders of magnitude higher than in neutrophilic species. The potential for heavy metal remediation growing endemic acidophilic micro-algae biofilms was illustrated with phosphorus-enriched water from a nickel refinery. Indigenous *Chlorella*-like micro-algae and biofilms were shown to remove up to 25% from the total metals present. In addition, the use of *Galdieria sulphuraria* for the recovery of metals, and particularly rare earth metals, has also been explored. *G. sulphuraria* was able to recover lanthanoids ions present at only 0.5 mg/L with greater than 90% and, at pH between 1.0 and 1.5, the lanthanoids were sequestered much more efficiently into cell biomass.

Bioprospecting for new species and strains could well identify new may identify novel acidophilic micro-algae that have even more useful characteristics for emerging biotechnologies. Acidophilic eukaryotes are currently an under-exploited resource that requires to be further investigated.

Ecosystems and Interactions

Extremely acidic environments can develop in a range of ecological niches. Within them, microbial communities of varying complexities occur within which individuals interact with each other in a wide variety of ways. These interactions, in turn, can have major impact of the geochemical dynamics of these extreme environments.

Diversity of Extremely Acidic Environments

(1) Geothermal Areas. Geothermal areas occur is discreet zones in various parts of the world. The most well-studied land-based geothermal area is Yellowstone National Park, Wyoming, U.S.A., where the acidity is derived chiefly from the oxidation of sulfur. Much of the early pioneering microbiological work in Yellowstone was carried out by Thomas Brock and co-workers in the 1970s and formed the basis of later research on thermophilic micro-organisms (including thermo-acidophiles). One of the major breakthroughs around the time was the isolation, by James Brierley, of the first thermophilic acidophile (a *Sulfolobus*-like archaeon, though it was not recognized as such at the time) from an acid hot spring in Yellowstone. Two of the more important solfatara fields in Yellowstone are located in and around the Norris Geyser Basin, and at Sylvan Springs, though there are numerous other smaller scale, often ephemeral sites (such as around the Gibbon River) where acidic ponds and streams may be found. Moderately thermophilic and acidophilic phototrophs (*Galderia*/*Cyanidium*-like rhodophytes), Gram-positive (*Sulfobacillus*, *Alicyclobacillus*, *Acidibacillus*, *Acidimicrobium* and *Ferrithrix* spp.) and Gram-negative (*Acidicaldus* and *Hydrogenobaculum* spp.) bacteria have been isolated from these and other sites. Mesophilic bacteria, such as *Acidiphilium* spp., are more abundant in cooler, extremely acidic sites. In contrast, sulfur-oxidizing crenarchaeotes (species including *Sulfolobus*, *Sulfosphaera*, *Metallosphaera* and *Stygiolobus*) dominate hotter low pH environments in Yellowstone.

Other geothermal areas where the distribution of acidophilic micro-organisms has been studied include New Zealand, the Caribbean island of Montserrat, and northern California. A site in New Zealand, an acidic (pH 2.5) stream water on White Island, contained significant concentrations (2–4.4 g/L) of chloride in addition to soluble iron and sulfate. Among the clones identified from DNA extracted directly from the acid stream were those closely related to a green sulfur bacterium (*Chlorobium vibrioforme*), the marine, purple non-sulfur bacterium *Rhodovulum*, and the heterotrophic bacterium *Ralstonia solanacearum*, while those obtained from enrichment cultures also included a bacterium that was closely related to *Acidicaldus. organivorans*. Pure cultures of *Acidiphilium* and *Cyanidium caldarium* were also obtained from the site. Results of a large-scale survey of

geothermal sites (many of which were also extremely acidic) on Montserrat, carried our shortly prior to the major eruption of the Soufriere Hills volcano in 1996, showed that temperatures of pools and streams in the volcanic southern region of the island ranged from 30 to 99°C, and pH from 1.0 to 7.4. Most of the acidophilic bacteria that were isolated were similar to known strains, though some *Sulfobacillus*-like isolates had novel traits in being able to grow either as mesophiles, or at higher maximum growth temperatures (up to 65°C) than classified species. Clone libraries constructed from DNA extracted from acidic sites with different temperatures indicated the presence of: (i) *Acidiphilium*-like bacteria and *Acidithiobacillus caldus* (33°C site); (ii) *At. caldus*, and a putative moderately thermophilic sulfate-reducing bacterium of the *Desulfurella* group (48°C site); (iii) novel *Ferroplasma*-like and *Sulfolobus*-like archaea (78°C site); (iv) an archaeon distantly related to *Acidianus infernus* (98°C site). Elsewhere, a microbiological survey of high temperature (82°–93.5°C) acidic (pH 1.2–2.2) hot springs located in the Lassen Volcanic National Park in northern California failed to detect any bacteria, though archaea distantly related to the crenarchaeotes *Stygiolobus azoricus* and *Sulfolobus solfataricus* (both extreme thermo-acidophiles thermophiles) were identified in clone libraries. Extremely acidic pools and lakes may develop naturally in volcanic areas, for example, Lake Kawah Idjen in Indonesia, which has a pH of ~0.7. The Copahue volcano is the source of geothermal activity in the Neuquén province of Argentina, where acidic ponds, pools and hot springs input into the acidic Rio Agrio, which in turn discharges into a large low pH water body, Lake Caviahue. Microbial diversity and interactions in the acidic environments in this region are dictated primarily by pH and temperature, and a wide range of known species, as well as novel species (e.g., the thermo-acidophilic archaeon *Acidianus copahuensis*) have been isolated from and identified within Copahue-Caviahue.

(2) Acid mine drainage waters and pit lakes. Waters draining abandoned mines, mine spoils and mineral tailings deposits (often referred to as acid mine/rock drainage) are often characterized by low pH and elevated concentrations of soluble metals (particularly iron) and sulfate (Table 2). Mineral acidity, due to the presence of dissolved aluminum, manganese and iron can contribute greatly to the net acidity of these water bodies. Pit lakes are relics of open-cast coal and metal mining, where worked out voids have not been back-filled and become progressively filled with rising ground water or river water. Where the surrounding bedrocks are rich in sulfide minerals (normally chiefly pyrite and marcasite) and contain small amounts of carbonates, the oxidative dissolution of the former can lead to the formation of extremely acidic mine lakes. Acidic pit lakes are particularly abundant in central Europe (Germany, Poland and the Czech Republic) where extensive reserves of lignite have been extracted (and still are on a more limited scale) by open-cast mining on a massive scale, leaving a legacy of a very large number of man-made lakes of varying sizes and chemistries. In the Lusatia district of eastern Germany alone there are an estimated 200 mining lakes of > 1 ha that have pH values of <3. Pit lakes resulting from opencast metal mining have more complex chemistries and can develop more extreme acidity, particularly in their upper (oxidized) zones. The often complex microbial geochemistries of these water bodies are described in a later section of this article.

Knowledge of the biodiversity of mine drainage has expanded considerably since *Acidithiobacillus ferrooxidans* was first isolated from a stream draining a bituminous coal mine in the U.S.A. in 1947. The most important factors in determining which microbial species are present in mine waters appear to be pH, temperature and concentrations of dissolved metals and other solutes. The Richmond mine at Iron Mountain, California, which can have negative pH values, represents the most extreme end of this spectrum, and the microbiology of water bodies within the mine has been studied extensively. At this site, pyrite is undergoing oxidative dissolution at a rate that is sufficient to maintain air temperatures of between 30 and 46°C, and produce mine waters containing ~200 g/L of dissolved metals. A novel iron-oxidizing archaeon, *Ferroplasma acidarmanus*, was found to be dominant in waters within the mine that had the lowest pH and highest ionic strengths, while *Leptospirillum ferriphilum* and *L. ferrodiazotrophum* were also associated with exposed pyrite. *Sulfobacillus* spp. were more important in some of the warmer (~43°C) waters. Iron-oxidizing acidithiobacilli were rarely found in sites that were in contact with the ore body, though they were found in greater abundance in the cooler, higher pH waters that were peripheral to the ore body. In contrast, a

Table 2 Examples of mine water chemistries (all units are mg L^{-1}, except pH)

	pH	*[Fe_{total}]*	*[Fe^{2+}]*	*[Al]*	*[Cu]*	*[Zn]*	*[SO_4]*
Coal mines:							
Bullhouse (U.K.)	5.9	61	45	1.2	<1	<1	–
Ynysarwed (U.K.)	6.2	160	140	20	–	–	460
Oatlands (U.K.)	5.5	287	–	0.97	<0.007	0.05	146
Sverdrupbyen (Norway)	2.7	179	–	27.5	0.168	1.3	1077
Metal mines:							
Mynydd Parys (U.K.)	2.5	650	650	70	60	40	3100
Roeros (Norway)	3.7	6.7	–	4.3	11	3.76	–
Wheal Jane (U.K.)	3.6	130	130	50	2	130	350
Cwm Rheidol (U.K.)	2.6–2.7	–	–	104–128	1.2–9.35	577–978	250
Sao Domingos (Portugal)	1.7	31,000	10,000	–	–	–	14,850
Iron Mountain (California)	1.5	2670	2470	–	293	58	14,000

Data are from Johnson, D.B., 2006. Biohydrometallurgy and the environment: Intimate and important interplay. Hydrometallurgy 83,153–166. And Nordstrom, D.K., Alpers, C.N., Ptacek, C.J., Blowes, D.W., 2000. Negative pH and extremely acidic minewaters from Iron Mountain, California. Environmental Science and Technology 34, 254–258.

microbiological survey of much cooler and higher pH mine waters at an abandoned sub-arctic copper mine in Norway found that the psychro-tolerant acidophile *Acidithiobacilllus ferrivorans* was the dominant iron-oxidizer isolated. This *Acidithiobacillus* sp. has been found in many cold, acidic and iron-rich surface and sub-surface environments, ranging from Siberia to Antarctica, where its ability to remain metabolically active where other species are inactive clearly presents it with a strategic advantage. Heterotrophic extreme acidophiles (*Acidiphilium* and *Acidocella* spp.) have also been encountered in permanently and transiently low temperature acidic environments. *Ferrovum myxofaciens* (which is also psychro-tolerant) is also frequently encountered in such environments, particularly in flowing waters > pH 2.

(3) *Acid streamers, mats and slimes in surface and sub-surface low pH environments*: The most obvious and dramatic manifestations of microbial life in extremely acidic environments are macroscopic growths, referred to as acid streamers, slimes and mats (Fig. 7). Streamers may occur in flowing AMD streams within and outside of abandoned mines; these have distinct filamentous morphologies and each filament may be more than a meter in length. Acid mats are more dense in texture, and are often found below growths of acid streamers. Acid slimes are thick, macroscopic biofilms that grow on moist surfaces of exposed rock faces. In addition, macroscopic filaments (or pipes) composed of acidophilic micro-organisms may attach to, and suspend from, mine roofs and pit props. Where these are small-scale, they have been referred to as snotites, though larger structures have been described as microbial stalactites (Fig. 7(a)). Because of the macroscopic nature of acid streamer growths, they were among the first life-forms to be reported in extremely acidic environments (in 1938). Most of the early reports described acid steamers as being composed of bacteria embedded in a gelatinous matrix. The first attempts, in the 1970s, to characterize their component micro-organisms used classical microbiological (cultivation-based) techniques. In the main, neutrophilic (chiefly spore-forming *Bacillus* spp.) were isolated from the acid streamers examined, leading to the erroneous conclusion that acid streamers were composed of neutrophilic heterotrophic bacteria that maintained circum-neutral pH within the macroscopic growths. In contrast, other researchers noted that streamers found in an iron/sulfur mine in Japan were able to catalyze the oxidation of both ferrous iron and sulfur, though some sub-samples of streamers have very limited capacity to oxidize iron. *Acidithiobacilus ferrooxidans*-like bacteria were isolated from these growths, leading researchers to conclude that acid streamers were a mass of iron-oxidizing acidithiobacilli embedded in a gelatinous matrix. However, it is only since biomolecular tools

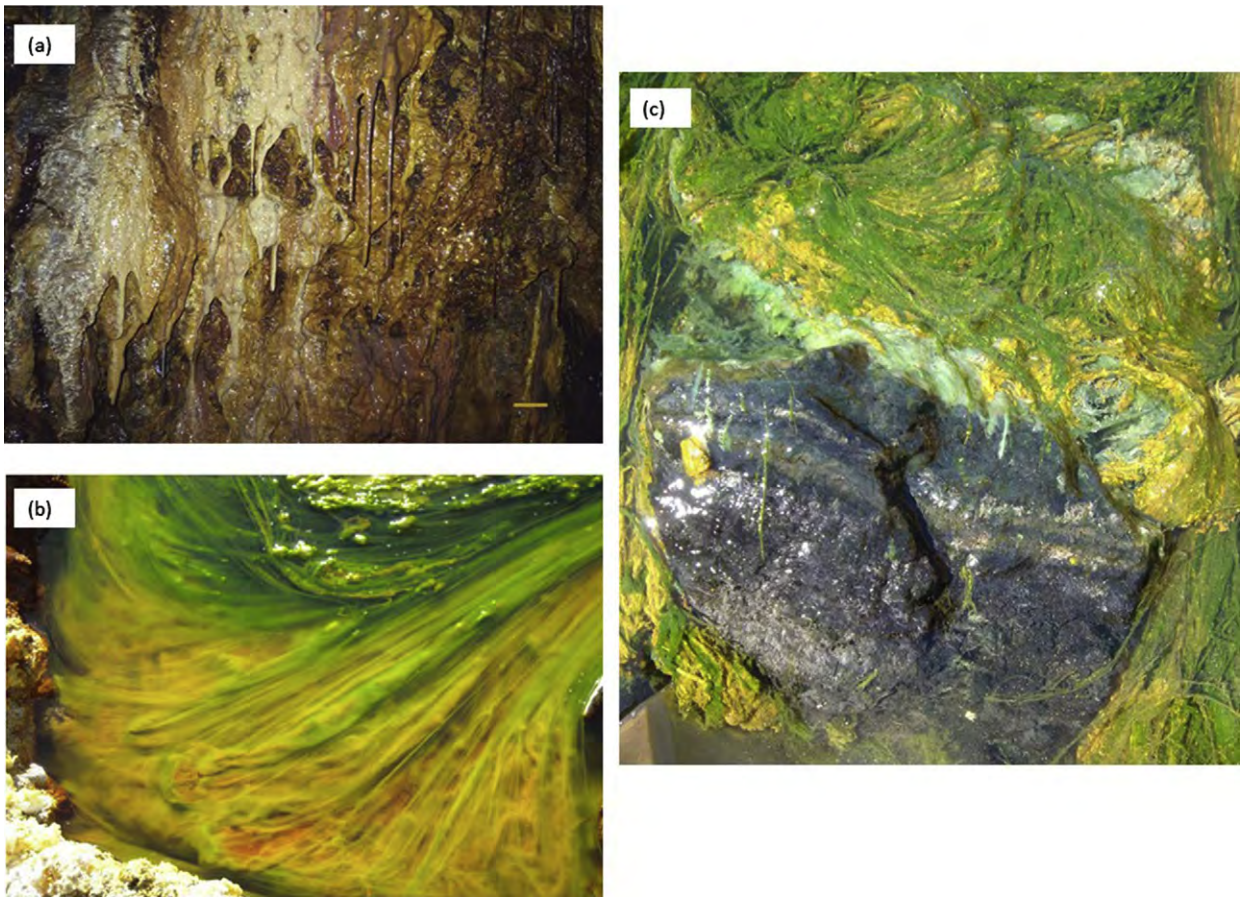

Fig. 7 (a) Microbial slime and stalactite growth deep underground in an abandoned copper mine (Mynydd Parys, Wales); (b) growths of micro-algae on bacterial acid streamers in the Rio Tintillo, Spain; (c) stratified acid streamer/mat growths in a drainage channel at the abandoned Cantareras copper mine, Spain.

have been used to analyze acid streamers and related macroscopic growths that their true nature has been elucidated. This approach, coupled with major advances in techniques for isolating and cultivating acidophiles made over the past 30 years, has shown that streamer communities are both highly complex and vary from site to site. Superficial similarities in their gross morphologies may mask completely contrasting microbial diversities.

One of the first intensive biomolecular examinations of acid slime (~1 cm thick) and snotite growths was carried out with materials collected from the Richmond mine at Iron Mountain. Microscopic examination showed that both growth forms were composed mostly of spirillum-shaped cells embedded in an extracellular polymeric matrix. Phylogenetic analysis based on 16S rRNA genes showed that most of the recovered sequences were novel, but related to known iron-oxidizing acidophiles. The single most dominant sequence recovered from the slime growths were of a novel strain of *Leptospirillum* (subsequently named *L. ferrodiazotrophum*). *L. ferriphilum*-related clone sequences were also identified. Other iron-oxidizing Bacteria identified were related to the Gram-positive actinobacteria, *Acidimicrobium* and *Ferrimicrobium*, while sequences that affiliated with deltaproteobacteria (which includes anaerobic sulfate- and iron-reducers) were also detected, suggesting that micro-zones of low redox potential existed within the macroscopic growths. Archaeal genes were also amplified, and sequences related to *Ferroplasma acidarmanus* identified. Other archaeal sequences were, however, only distantly related to known Archaea. In contrast to the Richmond mine slimes, acid streamer growths in less extremely acidic and cooler sites in north Wales were both found to be composed predominantly of Betaproteobacteria, occasionally with large numbers of Acidithiobacillaciae. *Ferrovum myxofaciens* was identified as the dominant betaproteobacterium, and *Acidithiobacillus ferrivorans* as the other dominant Proteobacterium, which correlated with their known traits of psychro-tolerance and tolerance to acidity above pH ~2.

Other subterranean extremely acidic sites that have been subjected to detailed investigation include abandoned pyrite mines in Wales and Germany, a copper mine (again in Wales) and a sulfur-rich cave system in Italy. The abandoned Cae Coch pyrite mine in north-west Wales is home to the most extensive and diverse macroscopic acidophile growths yet reported, with an estimated acid streamer biovolume of >100 m^3 alone, in addition to extensive slime biofilms and mats, microbial stalactites and snotites. Although the temperature in the mine shows very little seasonal fluctuation (8.5+/−1°C), other physico-chemical factors, including pH, dissolved oxygen and concentrations of dissolved metals and other solutes, vary from site to site within the mine. This, together with the fact that the underground mine has been largely undisturbed for 90 years, has facilitated the colonization of different niches by different streamer/slime acidophilic communities. *Ferrovum myxofaciens* and *Acidithiobacillus ferrivorans* are again highly abundant in this underground mine, together with smaller numbers of other extreme acidophiles (e.g., *Leptospirillum*, *Ferrimicrobium*, *Acidiphilium* and *Acidocella*) and moderate acidophiles. The abandoned Drei Kronen und Ehrt pyrite mine in the Harz Mountain area of central Germany also contains small microbial stalactite-like structures attached to the mine ceiling. Molecular analysis of the microbial growths showed that they were dominated by *Leptospirillum*- and *Ferrovum*-like bacteria with smaller numbers of heterotrophic (*Acidiphilium*-like) bacteria. Archaeal sequences closely related to those detected in Iron Mountain, and also to uncultivated members of the Thermoplasmatales have been detected in the biofilm communities in this site.

Mynydd Parys in north Wales was, in the 18th century, the world's most productive copper mine, worked initially as a deep mine and later as an opencast operation. The underground workings were drained in the early 21st century, allowing previously inaccessible areas to be explored. Large, two-dimensional gelatinous materials, referred to by the early cavers as "drapes" hanging from previously inundated pit props were discovered, together with microbial stalactites and streamer-like materials were found to be growing from the adit roofs and in newly-formed pools. Bacteria in the "drapes" were related to heterotrophic, rather than autotrophic, acidophiles, and included two species (*Ferrimicrobium* and *Acidimicrobium*) that are known to both oxidize and reduce iron and another (*Acidobacterium* spp.; moderate acidophiles) known to catalyze the dissimilatory reduction of iron (III). Archaea were also detected by molecular methods, most of which were related to methanogenic euryarchaeotes.

The Grotta Grande del Vento-Grotta del Flume (Frasissi) cave complex, Italy, is an example of a sulfidic cave formed when sulfide-rich groundwaters contact oxygen within subterranean carbonate rock strata. Sulfuric acid formed by (microbiological) oxidation of the sulfide results in the dissolution of the carbonate minerals, a process that can lead to the development of extensive cave systems, also found elsewhere.

In the Frasissi cave, viscous biofilms occur in extremely low pH (0−1) waters. The pendulous biofilms ("snotites") have very limited biodiversity, and are dominated by bacteria related to the mesophilic sulfur-oxidizing autotroph *Acidithiobacillus thiooxidans*. Other bacteria identified include bacteria related to Gram-positive *Sulfobacillus* and *Acidimicrobium* spp., and archaea related to *Ferroplasma*. Interestingly, although *Sulfobacillus* spp., like *Acidithiobacillus* spp., can use reduced sulfur species as electron donors, those *Acidimicrobium* and *Ferroplasma* spp. that have been characterized are known to oxidize ferrous iron but not sulfur, raising the probability that the latter two species in Frasissi (where ferrous iron is not a significant source of energy) represent new phenotypes. Other microorganisms detected within the slime growths include protists and filamentous fungi, as well as uncharacterized bacteria.

Streamer-like growths are also commonly found in flowing extremely acidic waters outside of mines, but here access to solar energy promotes the growth of micro-algae as well of chemolitho-autotrophic prokaryotes, and streamer growth often have surface green colorations (Fig. 7(b)). The addition of phototrophic biomass adds to the biodiversity of these ecosystems both directly and indirectly. One such site that has been studied in detail is a small abandoned copper mine (Cantareras) in south-west Spain. Surface growths grow as a dense microbial mat; that fills the ~100 m long drain channel and show distinct stratification (Fig. 7(c)). The surface layer is green due to the presence *Zygnema*, *Chlamydomonas* and other phototrophic eukaryotes that both aerate the anoxic mine water and provide organic carbon which supports the growth of bacteria within the mat. In contrast to most other acid streamer communities that have been described, heterotrophic (mostly iron-reducing) acidophiles dominate sub-surface layers at

Cantereras. Complex biogeochemical cycling of iron (acting as both electron donor and electron acceptor) and sulfur in the Cantareras streamer community helps to sustain the highly diverse population of acidophiles that occurs there.

Microbial Interactions at Extremely Low pH

Extremely acidophilic bacteria, archaea and eukaryotic microorganisms live, sometimes in very close proximity, with others of their own species and with different species, and within these communities they can exhibit a variety of interactions which may have positive, negative or nil effects on their neighbors. Most of the types of interactions that occur between microorganisms in environments considered to be non-extreme have also been observed in those that are extremely acidic. These include: (i) *Neutral symbioses*, in which neither partner derives any positive or negative effect from sharing an environmental niche (these tend to be relatively rare); (ii) *positive symbioses*, where one or more partners derive some benefit from sharing an environmental niche. These include: (a) mutualistic interactions, where both partners derive benefit from their association; (b) commensalism (or facilitation), where only one of the partners is perceived to derive benefit; (c) synergistic interactions that result in the complimentary activities of both (or all) participants being more efficient in, for example, degrading a substrate, than the individual species working alone; (d) syntrophy, where the degradation of a substrate by one species is only made thermodynamically possible through the removal of an end product by another species; (iii) *negative symbioses* (also referred to antagonism), in which the activities of one or both partners sharing an environmental niche are impaired or suffer more serious negative impact. These include: (a) competition, where populations of co-habiting species are limited by their communal requirement of an electron donor, electron acceptor or inorganic nutrient (nitrogen, phosphorus, carbon dioxide etc.) which is present in limited quantities; (b) amensalism, in which one species can repress another, e.g., via the production of toxin(s); (c) predation, where one organism is ingested by another.

One of the first mutualistic interactions amongst acidophilic bacteria to be recognized was the flow of organic carbon from chemo-autotrophic acidithiobacilli to obligately heterotrophic *Acidiphilium* spp.. The latter have longed been known to grow, sometimes cryptically, in "inorganic" liquid media used to cultivate iron- and sulfur-oxidizing chemolitho-autotrophs. Some of the carbon dioxide fixed by the latter and incorporated into organic molecules is leaked into the surrounding media during their growth, and also arises from lysed cells. Some of these organic materials are oxidized back to CO_2 by the heterotrophs, and this in turn benefits the autotrophs in two ways. Firstly, certain organic exudates and lysates are toxic to the carbon-fixers, and can inhibit their growth, especially in closed systems, and their removal is therefore advantageous. Secondly, it has been demonstrated that some of the CO_2 generated is actually re-incorporated into biomass by the acidithiobacilli. This is potentially an important source of inorganic carbon for these bacteria, as the availability of CO_2 is often limited (due to its poor solubility in acidic liquors) in low pH environments.

Symbioses involving microbial redox transformations of iron and sulfur are also important in low pH environments. Ferrous iron acts as an electron donor for many acidophilic prokaryotes, which oxidize it to ferric iron, which in turn has widespread use as an electron acceptor in oxygen-limited (or depleted) environments. Since both ionic forms of iron are soluble at pH <2.5, they can freely diffuse between, for example, aerobic surface waters and underlying micro-aerobic or anoxic sediments. Dynamic oxdo-reduction of iron is a feature of many extremely acidic environments. This is true also, to some extent, for sulfur. Many acidophilic prokaryotes, as noted previously, can oxidize reduced forms of sulfur, ultimately to sulfate. Sulfate-reducing bacteria (which catalyze the dissimilatory reduction of sulfate to hydrogen sulfide) are widely distributed in anoxic sediments etc., including those in low pH stream and lake sediments. Several species of acid-tolerant, though not extremely acidophilic, sulfate reducing bacteria have been isolated from these environments and shown to be involved in sulfur cycling *in situ*.

The importance of microbial consortia and interactions in commercial bio-processing of sulfide minerals is well established. Three "groups" of acidophiles are ubiquitous in these operations. The first, and most important, are iron-oxidizing (mostly autotrophic) acidophiles which generate ferric iron, the oxidant that solubilizes the minerals. Iron-oxidation is, as noted, a proton-consuming reaction, which is ultimately counter-productive to the process, which requires low pH to maintain ferric iron in solution. The second players in the consortia oxidize reduced sulfur released from the partly-oxidized minerals to sulfuric acid, thereby maintaining suitable pH for both the process and the prokaryotes involved. Again these are mostly autotrophs, and organic carbon from these (and the iron oxidizers) is degraded by the "janitor" members of the consortium (heterotrophic acidophiles), which both de-toxify the liquors and release CO_2 for the autotrophs. A schematic of this system is shown in Fig. 8.

A more rarified consortium, comprising just two acidophiles that can successfully thrive by degrading pyrite together which neither has the potential to do this alone, has also been described. *Ferrimicrobium acidiphilum* is an obligately heterotrophic acidophile that oxidizes ferrous iron and can degrade sulfide minerals such as pyrite when provided with a source of organic carbon, such as yeast extract. *Acidithiobacillus thiooxidans* is an autotrophic acidophile that oxidizes reduced sulfur, but not iron, and can therefore not oxidize pyrite. Pure cultures of either organism (in inorganic media) show no potential to grow on and degrade pyrite, but mixed cultures of the two can thrive on this mineral. Interactions involve inter-related flows and transformations of iron, sulfur and carbon, as depicted in Fig. 9.

Although biogeochemical cycles in extremely acidic environments usually center on transformations of iron, sulfur and carbon, they can involve other elements which in turn can have overriding influences on the nature and dynamics of these environments. This is clearly seen in the case of acidic pit lakes that form in abandoned metal mines. Studies carried out in several sites in Spain have revealed an intriguing level of geomicrobiological complexity within them. These pit lakes can be deep (>50 m) and have characteristic permanent stratification, with the water column composed of an upper oxic layer which displays seasonal variation in temperature, a bottom anaerobic layer which has a more limited temperature range, and a transitional zone (the chemocline)

Fig. 8 Microbial consortia and interactions involved in the oxidative dissolution of sulfidic minerals, such as pyrite (FeS_2; illustrated) at low pH. Iron-oxidizers are primarily responsible for degrading the minerals, while sulfuric acid-generating and organic carbon-degrading acidophiles maintain conditions that are conducive for the process to continue.

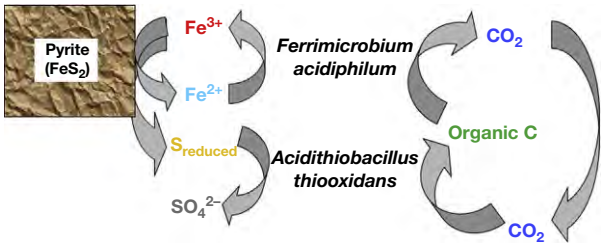

Fig. 9 Dissolution of pyrite by a mixed culture of *Ferrimicrobium acidiphilum* and *Acidithiobacillus thiooxidans*. *Fm. acidiphilum* can oxidize ferrous iron to ferric iron but requires organic carbon to grow. *At. thiooxidans* can fix CO_2 and releases some of this as organic carbon, but it cannot access its energy source (reduced sulfur) directly from pyrite. Neither bacterium can therefore grow in organic carbon-free pure cultures using pyrite as an energy source, though together they form a successful synergistic consortium.

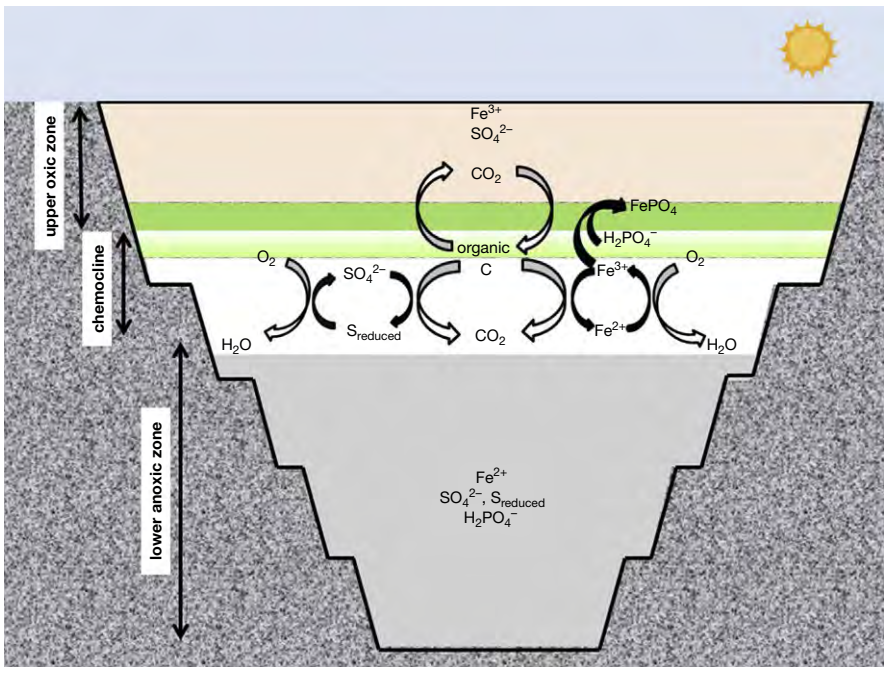

Fig. 10 Geochemical stratification of a typical pit lake formed by the flooding of an abandoned opencast metal mine in the Iberian Pyrite Belt. The green-shaded area indicates the zone where algal numbers and activity (and primary production) are greatest.

separating them. Dynamic cycling of iron and sulfur, and fixation and catabolism of organic carbon, occur in these lakes. An apparent major anomaly identified in pit lakes in the Iberian Pyrite Belt was that maximum primary production and accumulation of algal biomass occurred at depths where photosynthetically-active radiation was <2% of that close to the water surface. While concentrations of inorganic nitrogen were found to be fairly uniform throughout the water columns in these lakes, phosphate was present in far greater concentrations in the anoxic bottom waters than in the aerobic surface waters. Further investigations showed that this was due to the formation of insoluble ferric phosphate in the upper waters, corresponding to the microbial oxidation of ferrous iron in and above the chemocline, leading to the formation of insoluble ferric phosphates. Algal growth and activity was therefore dictated primarily by the availability of phosphate in these iron-rich lake waters, rather than by the intensity of sunlight. The chemoclines (which were about 2 m in depth in these pit lakes) were demonstrated to be the zones within the water columns that housed the greatest biodiversity and numbers of micro-organisms, and where the geochemical cycling of carbon, iron and sulfur were most dynamic (Fig. 10).

Further Reading

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Extremophiles: Cold Environments☆

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Glossary

Barophilic Descriptor for cultured members of the domain Bacteria or Archaea that grow more rapidly under elevated hydrostatic pressures of the deep sea than at atmospheric pressure (also piezophilic).
Barotolerant Descriptor for cultured members of the domain Bacteria or Archaea that grow under elevated hydrostatic pressures but more rapidly at atmospheric pressure.
Cold-active General descriptor for enzymes that are catalytic or viruses that are infective at low temperature.
Cold-adapted General descriptor for microorganisms, cultured or uncultured, that express traits enabling activity at low temperature.
Cryopegs Ancient (>100,000 years) lenses of liquid brine of marine origin, found in deep layers of Arctic permafrost at temperatures of −6 to −12°C.
Enzymes Proteins that catalyze chemical reactions which otherwise would occur only slowly if at all; extracellular enzymes are held near the surface of the cell or released into the environment.
Eutectic temperature Lowest temperature at which a mixture of salt and water can contain any liquid (about −55°C for seawater); below it, liquid water converts to solid (ice) and dissolved salts precipitate; eutectophile refers to a microbe living near the eutectic temperature in a natural (saline) ice formation.
Freezing point Temperature at which liquid water begins to convert to solid phase (ice); the freezing point of pure water (0°C) is lowered by the presence of impurities (salt, organics).
Lipid bilayer Critical component of the semipermeable membrane enclosing a microbial cell; when composed of unsaturated fatty acid-rich complexes, the lipid bilayer imparts greater flexibility to the membrane at low temperature.
Mesophilic Thermal descriptor for cultured members of the domain Bacteria or Archaea that reproduce at a minimal temperature of ∼10°C, optimal temperature near 37°C, and maximal temperature of ∼45°C.
Oligotrophic Descriptor for nutrient-poor environments, where nutrients refer to organic substrates utilizable by heterotrophic microbes.
Permafrost Perennially frozen soil or rock material, not subject to seasonal warming, found in polar regions.
Psychrophilic Thermal descriptor for cultured members of the domain Bacteria or Archaea that grow at a minimal temperature of 0°C or lower, optimal temperature of 15°C or lower, and maximal temperature of ∼20°C (also stenopsychrophilic).
Psychrotolerant Thermal descriptor for cultured members of the domain Bacteria or Archaea that grow at a minimal temperature of 0°C or lower and maximal temperature above 20°C (also eurypsychrophilic).

Earth has not always been cold. Clear signs of the first glaciation period date to the middle of its history, at about 2.2 billion years ago. Since then, the planet has undergone epochs of warming and cooling, implying that cold-adapted microbes periodically faced extinction as a thermal class or else found refugia, most likely in a cool (≤15°C) deep sea. Current global warming is again reducing the extent of cold, and especially frozen, habitats that arguably provided conditions for the evolution of today's cold-adapted microbes. More than at any time in the history of modern science, research on the extremophiles of cold environments is warranted. Motivations for such research have always included the desire to understand fundamental cellular and molecular aspects of cold adaptation, often centering on how or when a microbe produces lipid membranes flexible enough to transport and exchange solutes and ions with its environment or enzymes capable of catalyzing essential biochemical reactions in the rigidifying cold. There is also now a heightened sense of urgency to understand how microbes currently driving biogeochemical cycles in the cold may respond to environmental warming and thus impact existing food webs and carbon sequestration from the atmosphere. The further expectation that cold-adapted microbes and their products can be harnessed for societal needs, from bioremediation of cold environments to development of alternative energy sources through cold hydrolysis, drives applied research on the extremophiles of the cold. How microbes function or simply survive in frozen environments and unusual ice formations that approach the eutectic has become a central issue in the emerging field of Astrobiology. Most of the current targets for potential (or past) life in our solar system present us only with surfaces at extremely low temperatures, from a planetary-scale average on Mars of −55 (the eutectic for Earth's seawater) to −160°C on Europa, the ice-covered moon to Jupiter believed to harbor an ocean larger than ours.

☆*Change History*: September 2016. Jody W. Deming made changes in Sections "Text revisions to Earliest Observations and Terminology", "Exploration of the Cold Deep Sea", "Exploration of other Low Temperature Environments", "Influence of Astrobiology", "Molecular Basis for Cold Adaptation" and "Membrane Fluidity"; added new Figures 1 and 4 and updated "Further Reading" section.

This article is an update of J.W. Deming, Extremophiles: Cold Environments, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 147–158.

Discovery of Cold-Adapted Microbes

Earliest Observations and Terminology

The earliest known report of microbial life in a cold environment dates back to the fourth century BC and the writings of Greek philosopher Aristotle who made observations of what later proved to be photosynthetic Eukarya ("red algae") that turned snow to a reddish color. More than two millennia later in the 19th century (1887), the German scientist Forster described the ability of a bioluminescent bacterium, derived from fish preserved in the cold, to reproduce at 0°C. In the 20th century (1902), the term "psychrophile" for cold loving was introduced by Schmidt–Nielsen to describe such microorganisms.

Over the next 60 years, the term psychrophile continued to be in use to describe cold-adapted microbes according to the ability to reproduce in the cold, regardless of the upper temperature limit for growth. That limit, with few exceptions, fell above room temperature, thus overlapping with the thermal category of mesophiles. These microbes today are called psychrotolerant (or psychrotrophic), with the descriptor psychrophilic reserved for organisms that fit the more precise definition provided by the American marine microbiologist Morita (in 1975), based on cardinal growth temperatures: minimal temperature (T_{min}) of 0°C or lower, optimal temperature (T_{opt}) of 15°C or lower, and maximal temperature (T_{max}) of about 20°C (Fig. 1). By this definition, which provided much needed clarity at the time and remains in wide use today, the first true psychrophile was discovered by Tsiklinsky as part of a French expedition to Antarctica (1903–05). Another half-century would pass before the abundance and functional roles of true psychrophiles in cold environments would be appreciated. First, microbiologists had to learn about the sensitivity of cold-adapted microbes to room temperature (even to unchilled pipets) during isolation procedures. Eventually, after many enrichment studies using 4–6°C (convenient refrigerator temperature) and reports of predominantly psychrotolerant isolates, came the understanding that the temperature of initial enrichment influences the thermal nature of the resulting isolates: enrichments near the freezing point (e.g., at −1°C for marine samples) are more likely to yield psychrophilic than psychrotolerant bacteria.

Morita expected that future adjustments to his definition of psychrophily would be in the direction of lower cardinal temperatures, based on new isolates obtained by paying attention to detrimental (and influential) temperatures during isolation procedures. Such microbes with considerably lower cardinal growth temperatures, called extremely psychrophilic, have been cultured from the marine environment; for example, the Gammaproteobacteria *Psychromonas ingrahamii* (T_{min}=−12°C, T_{opt}=5°C, T_{max}=10°C) and *Colwellia psychrerythraea* strain 34H (T_{min}=−12°C, T_{opt}=8°C, T_{max}=18°C) from subzero Arctic sea ice and sediments, respectively. The current record holder for low-temperature growth, however, is *Planococcus halocryophilus* Or1 (T_{min}=−15°C, T_{opt}=25°C, T_{max}=37°C), a Gram-positive bacterium derived from permafrost at ambient temperature of –16°C. This organism categorizes as psychrotolerant, growing across an unusually wide temperature range of 52°C (from –15 to 37°C), consistent with its thermally dynamic environment. Measurements of microbial metabolic activity at extremely cold temperatures in natural ice formations (down to at least −20°C), where measuring an ambient reproductive rate is methodologically challenging, has led to introduction of the term eutectophile for the most cold-adapted microbes, living in ice near the eutectic temperature with only nanometer-scale films of liquid water available.

Because cardinal growth temperatures cannot fully capture the adaptability of a microorganism to its environment and are unavailable for the vast majority of microbes in nature that evade cultivation, the term psychrophilic (and psychrotolerant) remains most useful for categorizing cultured members of the domains of Bacteria and Archaea. Cold-adapted is used very generally to

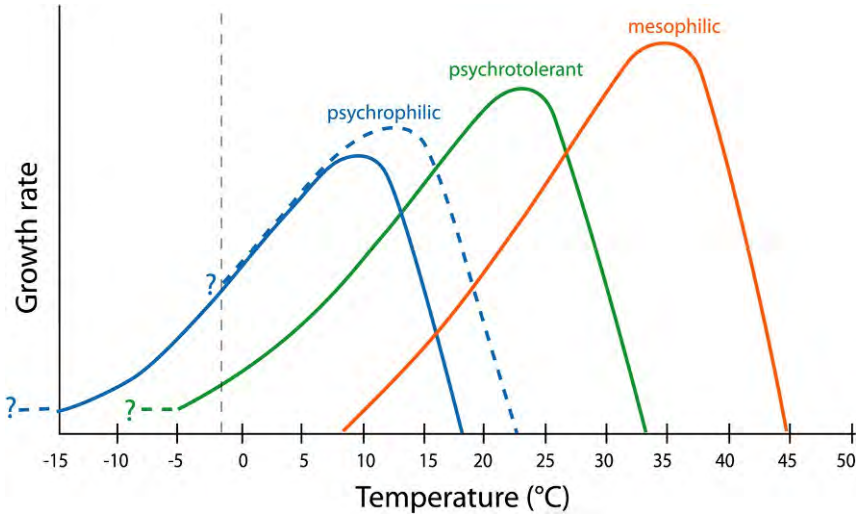

Fig. 1 Idealized depiction of bacterial growth rate as a function of temperature (at atmospheric pressure) for a psychrophilic, psychrotolerant, and mesophilic microbe. The dashed line depicts the shift in response of a deep-sea psychrophile when grown under elevated hydrostatic pressure. The dotted vertical line indicates the freezing point of seawater.

describe microorganisms, cultured or uncultured, that express a recognizable adaptation to low temperature, while cold-active is often reserved for enzymes and viruses with catalytic or infective activity at low temperature. Other terms based on cardinal growth temperatures, particularly stenopsychrophilic and eurypsychrophilic (comparable in operational meaning to psychrophilic and psychrotolerant, respectively), attempt to recognize that psychrotolerant microbes are not simply tolerant of the cold (some mesophiles and thermophiles can tolerate the cold) but also adapted to it, in spite of higher T_{opt} and T_{max} for growth than psychrophiles.

What constitutes a cold temperature is also subject to perspective. Combining cardinal growth temperatures for psychrophiles and some key environmental temperatures yields the following set of descriptors. Moderately cold is 15 to 5°C; that is, from the upper T_{opt} for psychrophilic growth, which is also the average temperature of the surface of the Earth, down to the (upper) temperature of 80% of the biosphere. Cold is 5 to −2°C, the approximate freezing point of seawater, while very cold is below −2°C. Extremely cold is below −15°C, the current lowest T_{min} for microbial growth in culture and representative of the temperature of the Earth's near-million year-old permafrost in polar regions. Cold in the term cold adaptation remains broadly defined by any temperature 15°C or lower.

Exploration of the Cold Deep Sea

The cold deep sea, as the volumetrically dominant and most persistently cold environment on Earth (over geologic time), has provided an important natural laboratory for studying and advancing understanding of cold adaptation in the microbial realm. Although its temperature is always above the freezing point of seawater and thus not as thermally extreme as most frozen environments, the cold deep sea is considered extreme for other reasons: its elevated hydrostatic pressure, which increases linearly at 10 atm (=1 MPa) per 100 m increase in water depth, and its generally oligotrophic state. The search for psychrophiles in the cold deep sea has often been coupled with the search for barophiles (also known as piezophiles), pressure-adapted microbes that grow more rapidly under elevated hydrostatic pressures than at atmospheric pressure when adequate nutrients in the form of organic substrates are available. The focus of these searches has usually been the heterotrophic bacteria.

Scientific exploration of oceanic life, in general, began with a series of deep-sea expeditions at the end of the nineteenth century (study of the productive surface layers and sea ice would come later). Until the discovery of deep-sea hydrothermal vents in the latter half of the twentieth century (vent-supported life was first reported in 1977), the deep ocean was understood to be uniformly cold, the temperature not exceeding about 5°C (except in the deep Mediterranean and Sulu seas where temperatures reach about 15°C). Yet, early expeditions had no facilities for incubating samples shipboard at such low temperature. The first opportunities to discover cold-adapted microbes from the vast, cold deep sea were thus thwarted by lack of refrigeration. Remarkably, the early French explorer Certes was able to test for pressure-adapted microbes from the deep sea in the 1880s, even if cold adaptation was beyond reach.

More than a half-century later, American marine microbiologist ZoBell began the study of deep-sea microbes under both in situ pressure and temperature, documenting with Morita in 1957 the first psychrophilic barophiles. Although these cultures were lost to future study, similar efforts by several groups beginning from the 1970s eventually yielded sizeable culture collections of psychrophilic barophiles; indeed, all barophiles cultured from the cold deep sea are also psychrophilic. The synergistic effects of temperature and pressure on microbial growth (or other activities) have not been fully explored, but for some psychrophilic bacteria their cardinal growth temperatures can be shifted upward by incubating under higher pressure (Fig. 1). When the growth responses of deep-sea bacteria have been examined according to a matrix of three parameters – temperature, pressure, and salinity – salt concentration is also observed to shift cardinal growth temperatures, though the direction of the shift is variable and strain-dependent. Microbiological exploration of the cold deep sea has thus raised general awareness that cold adaptation must be understood in relation to other parameters and not exclusively to temperature.

Not least of other parameters is the concentration of available energy sources or organic substrates in the case of the heterotroph. For heterotrophic psychrophilic barophiles the barophilic trait depends upon available substrate concentration: at low substrate concentrations, growth rate is similar under both atmospheric and elevated pressures (barotolerant, within the pressure range for growth), while at high substrate concentrations, growth rate is higher under elevated pressures than at atmospheric pressure (barophilic). When substrates are in adequate supply in the cold deep sea, for example, from the hydrolysis of freshly deposited organic detritus, psychrophilic barophiles will outcompete other cold-adapted microbes that may be present. Genetic work with pressure-adapted psychrophiles indicates that membrane proteins involved in substrate uptake are upregulated under elevated pressures when provided with sufficient substrate.

Somewhat analogous work with marine psychrophiles from shallow cold waters, considering only temperature and substrate concentration, indicates that the lower the temperature, the higher the required substrate concentration to achieve a comparable growth (or oxygen respiration) rate. Low temperature or viscosity-driven reduction of the diffusive flux of various solutes to the cell contributes to but is insufficient to account for this increase in the required substrate concentration threshold. Resolving the issue is important to understanding why, at least at times, the microbial role in biogeochemical cycles of surface polar waters appears limited until sufficient organic solutes accumulate. Although gene expression work that may help to explain this phenomenon remains to be conducted, the available whole-genome analyses of heterotrophic psychrophiles reveal a capacity to store substrate reserves intracellularly, a potential means around the problem for the individual cell. Also awaiting a gene expression-based explanation is the repeated observation that cold temperature favors higher bacterial growth efficiency: a greater fraction of the organic carbon consumed in the cold goes to new biomass (gain to the food chain) than to respiration (loss as carbon dioxide).

Warming temperatures in polar waters are thus expected to shift the role of cold-adapted microbes in the cycling of organic carbon to increased remineralization to carbon dioxide.

Exploration of Other Low-Temperature Environments

Many other cold environments have been explored over the past several decades for cold-adapted microbes. Moderately cold to cold environments include a wide range of aquatic and sedimentary environments, both marine and freshwater (rivers, lakes, and subsurface aquifers), terrestrial soil environments of varying degrees of desiccation, and numerous surfaces that support microbial biofilms, from moist rocks in caves and mines to fluid-bathed tissues of vertebrate and invertebrate animals that live in the cold. Unlike the cold deep sea, many of these environments experience fluctuating temperatures (and other parameters) on a seasonal and diurnal basis. Exceptions with stably cold temperatures include animal-associated microbial environments, where the animal host spends its life history in cold water, as well as the many deep subglacial lakes of Antarctica (close to 400 have been discovered) with stable temperatures just above the freezing point, given separation from the lower atmosphere and solar radiation by kilometers of glacial ice. With the possible exception of subglacial lakes, where the temperature requirements of microbial inhabitants have not been addressed in live culture, all of these cold environments have in common their successful colonization by cold-adapted microbes, which then play active if not dominant roles in cycling the inorganic and organic materials within them. Based on classical chemostat studies pitting cold-adapted microbes with different cardinal growth temperatures against each other at constant cold temperature, the more psychrophilic organisms are the dominant players.

In contrast are the environments that experience very cold to extremely cold temperatures, including the upper atmosphere, where viable microbes have been found associated with microscopic particles, and the major types of ice formations on Earth – freshwater snow and glacial ice (formed from long-term compaction of snow), lake and river ice, polar and subpolar sea ice, and frozen soils. Except for sea ice (see below) and possibly lake ice (less well studied), frozen environments in general can be viewed as preserving an often cosmopolitan suite of microbes, largely inactive in the cold, rather than as actively colonized by cold-adapted microbes with all the attendant successional and adaptive responses. At extremely cold temperatures, the primary limitation to the latter scenario is the absence of sufficient water in the liquid phase. All ice formations on Earth derive from source waters containing impurities of one kind or another that depress the freezing point (especially inorganic salts), so they retain at least some liquid water. Only those that may drop below their eutectic temperature, for example, high-altitude glacial ice on Antarctica, become completely desiccated.

Some of these frozen environments experience seasonal and/or diurnal temperature swings, which intermittently relieve the limitation of insufficient liquid water. They can then support highly productive microbial ecosystems. On the surface of glacial ice, for example, wind-deposited dust called cryoconite absorbs solar radiation and melts small holes into the ice, where liquid and nutritional resources can be sufficient to allow cold-adapted microbes to flourish. Another example is sea ice, which during spring and summer seasons, with near-continuous sunlight and seawater flushing its base with nutrients, develops algal and microbial communities visible to the naked eye as strongly discolored ice (Fig. 2). These communities contribute 25% or more of total primary production in the Arctic with consequent effects on secondary production (the transformation and consumption of this biomass) and higher trophic levels. Sea ice (like lake and river ice), however, is not a stable environment, melting by late summer before reforming in fall. An exception is multiyear sea ice in the Arctic Ocean that until recently could survive 8–10 melting seasons before circulating out of the Arctic into warmer (ice-melting) Atlantic waters. Climate-driven declines in multiyear (and first-year) sea ice, which had been averaging about 10% per decade since satellite coverage began in the 1970s, recently accelerated beyond all model predictions. This particular frozen environment may soon represent a lost opportunity for the study of cold adaptation.

The upper layers of soil in alpine and polar regions also experience regular and wide fluctuations in temperature seasonally, from warm ($>20^{\circ}C$) to extremely cold, as well as climate-driven warming. Even during moderately cold periods, microbial activity increases such that the environment becomes a source of greenhouse gases, like carbon dioxide and methane, rather than a sink. In alpine soils, an insulating snow cover promotes substantial microbial activity through the winter. The deeper frozen layers (>50 m) of polar soils removed from atmospheric and solar influences, however, have been permanently frozen (permafrost) in the temperature range of –10 to $-12^{\circ}C$ for close to a million years in some Siberian locations. The typical diversity of soil microbes that have been recovered in culture from permafrost, including aerobes, anaerobes, heterotrophs, sulfate reducers, and methanogens, may represent some of the oldest viable forms available to study. Wedged between deep layers of permafrost are cryopegs, recently discovered multimeter-scale lenses of very old unfrozen water kept liquid by high salt concentration. Available isotopic and other evidence indicates an ancient marine source for these brines and active microbial and viral communities within them, despite total darkness and anoxia. The physical separation of these communities from contemporary ecosystems over geologic time makes them promising sites for the study of microbial evolution under extreme conditions that include very cold temperatures.

In the upper atmosphere, in high-altitude glacial ice and snow that covers Greenland and Antarctica, and in Arctic winter sea ice and its overlying snow cover, microbes experience extremely cold temperatures, sometimes approaching or reaching the eutectic of $-55^{\circ}C$ (for seawater). The thermal gradients inherent to glacial and sea-ice environments (Fig. 2) provide natural laboratories to examine the question of the lower temperature limits for microbial growth, activity, and survival. To date, studies of such environments and of artificially produced ices at extreme temperatures suggest that cellular reproduction may be limited to about $-20^{\circ}C$, metabolic activity to about $-40^{\circ}C$, and survival to the lowest temperatures yet to be tested ($-196^{\circ}C$ in liquid nitrogen). These general guidelines, however, are subject to change, especially as the field of Astrobiology stimulates increased experimentation under extremely cold conditions.

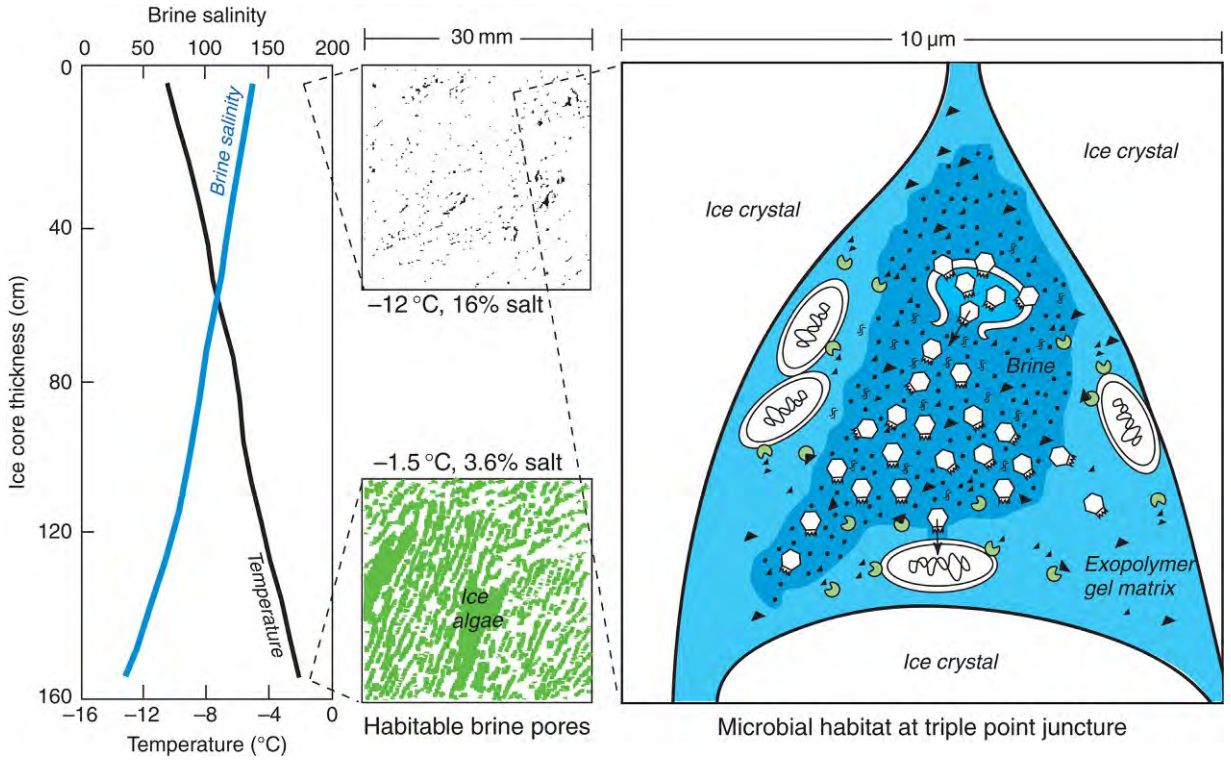

Fig. 2 Schematic depiction of some characteristics of sea ice. At left are vertical gradients, in temperature and in salinity of liquid inclusions, that develop in winter as temperature of the overlying atmosphere drops but underlying seawater remains near freezing point. Middle panels depict relative size of brine pores in a very cold section of ice vs. bottom ice, where larger channels flushed by seawater will support an ice-algal bloom in spring. At right is an enlarged schematic of very cold brine at the juncture of three ice crystals, depicting microbes embedded in a gelatinous matrix of exopolymers, brine, and organic substrates concentrated in the interior, and extracellular enzymes hydrolyzing the substrates. Also shown are proposed cold-active viral enzymes at work, enabling successful infection and viral reproduction, host lysis, and release of free DNA and new viruses, potential agents of horizontal gene transfer.

Early in the study of this varied array of low-temperature environments, a general paradigm emerged: stably cold environments tend to support a greater (culturable) community of psychrophilic microbes, while those with temperatures that fluctuate, especially above the T_{max} of psychrophiles, tend to support a greater community of psychrotolerant (eurypsychrophilic) microbes. The implication was that psychrophily requires a stably cold environment to evolve. Although this paradigm appeals to common sense, much of the early data supporting it relied upon enrichment temperatures that would have favored psychrotolerant microbes. When more stringent enrichment conditions are used (e.g., −1°C for saline environments), temperature-fluctuating environments that previously yielded mainly psychrotolerant isolates yield a predominance of psychrophiles instead. Psychrophiles have also been observed to reestablish dominance in an environment in a relatively short period of time (days), once fluctuating temperatures have stabilized at a cold temperature. Furthermore, the sea-ice environment, which has always consistently yielded greater numbers of psychrophiles, is an ephemeral one, with inhabitants released during the summer melt period to seawater that then warms under 24 h solar radiation. From initial encasement during ice formation in fall through the winter, spring and summer seasons, sea-ice microbes can experience a temperature fluctuation of more than 40°C, from −35 in winter to 6°C (and higher) in melt water. The corresponding fluctuations in other parameters, especially salt concentration (Fig. 2), add osmotic and other stresses to the thermal swing. Although psychrophiles have long been considered the more sensitive of cold-adapted microbes, in large part because some express narrow temperature ranges for growth (unlike those shown in Fig. 1), environmental robustness is a matter of perspective influenced by considering other parameters. What constitutes an ideal habitat for cold adaptation needs to be reconsidered.

Influence of Astrobiology

The ongoing and planned exploration of other bodies in our solar system for evidence of past or present life has opened a new chapter in the history of studying cold-adapted extremophiles. Even the extremely cold environments of Earth are thermally moderate relative to the extraterrestrial sites targeted for exploration. For example, the average surface temperature of the desiccated soils on Mars is about −55°C, coincidentally the eutectic temperature for Earth's seawater. Where the surface temperature is warmer, not even nanometer-scale films of liquid water are available and radiation intensity (in the absence of a protective atmosphere as on Earth) would be prohibitive. The average temperature of the deeply frozen surface of Europa, the Jovian moon that harbors beneath its ice cover a global ocean larger than Earth's, is about −160°C. On Enceladus, the Saturnian moon recently shown to release

plumes of water containing carbon and nitrogen compounds provocative of life, the average surface temperature is –201°C. Such extremely cold ice formations are not found on Earth, invoking the need to study forms of ice generated in the laboratory. Initial studies of the reactions of a known psychrophile to flash freezing, as might be experienced when oceanic waters on Europan and Enceladus rise through cracks of an extremely cold ice cover, have pointed to the importance of organic (sugar-rich) exopolymers in buffering cells against damage from the freezing process. Because the presence of exopolymers in saline ice formations are also known to alter the microstructure of the ice in readily detectable ways, similar effects in extraterrestrial ices may constitute a biosignature for life.

Although space missions have accessed only extremely cold surfaces (as on Mars), the subsurface environments of Mars, Europa and Enceladus, hidden from damaging radiation, hold greater promise for life. They are expected to be more moderate in temperature, favoring liquid water (perhaps in analogy to the brine layers of deeply buried permafrost), and to offer potential energy sources for chemolithotrophic life forms, for example, via the exothermic water-rock reaction known as serpentinization. In Earth's deep ocean at a mid-Atlantic site called Lost City, serpentinization is known to yield hydrogen and methane in support of luxurious archaeal biofilms and mats. The study of cold-adapted Archaea and chemolithotrophs in general remains in its infancy, relative to the heterotrophic bacteria.

Evolution of Cold Adaptation

Glaciation Periods on Earth

The temperature of the early Earth and its ocean is actively debated, but marine geological evidence points to a very hydrothermally active and thus warm ocean. The first hypothesized period of planetary-scale chilling or glaciation does not occur until about halfway through Earth's history at 2.2 billion years ago during the Proterozoic era. Less than a billion years ago, Earth is believed to have experienced a severe freezing episode resulting in what has been called "Snowball Earth". Since then, the planet has experienced a series of glaciation events, not always global in scale, eventually leading to the glacial/interglacial periods of recent Earth history. Their periodicity is estimated in tens of thousands of years. In between each major ice age, the planet is believed to have been completely free of ice with an average surface temperature above that permissive of a psychrophilic lifestyle.

Unless the deep sea remained sufficiently cold to provide refuge to a stock of psychrophiles, a difficult hypothesis to test, psychrophilic microbes likely evolved more than once during Earth's history. The implication is that the evolutionary steps between psychrotolerance and psychrophily must be accommodated by the time available between glaciation periods. In addition to vertical gene transfer from an ancestor (inherited beneficial gene mutations), horizontal gene transfer (e.g., mediated by viruses or free DNA) may have played important roles in achieving these steps. Leading the way to tests of this hypothesis are phylogenetic analyses of extant microbes, cultured and uncultured, comparative genomic evaluations of psychrophiles and other thermal classes of microbes, and experimentation with horizontal gene transfer in the cold.

Phylogeny of Cold Adaptation

The now classic 16S rRNA gene-sequencing approach to deducing relationships among organisms yields a universal tree of life on which known psychrophilic and psychrotolerant microbes can be located. Use of a phylogenetic tree originally designed to highlight hyperthermophilic genera of Bacteria and Archaea (those that grow at 90°C or higher) for this purpose emphasizes the late arrival of cold adaptation among extant organisms (Fig. 3). It also indicates the slightly deeper branching of groups containing only psychrotolerant members, reinforcing the expectation that psychrophiles evolved from psychrotolerant strains.

Most of the major branches within the domain of Bacteria, except those unique to thermophiles, contain psychrophilic members, including all five groups of the *Proteobacteria*, aerobes and anaerobes alike, the *Cytophaga-Flavobacteria*, the *Cyanobacteria*, and the Gram-positive bacteria. Some genera of the gammaproteobacteria, in particular *Moritella* and *Colwellia*, are comprised mainly or exclusively of psychrophiles. The Green nonsulfur bacteria, however, contain only psychrotolerant isolates so far.

In the domain of Archaea, only a single culture-authenticated psychrophile is known, the methanogen *Methanogenium frigidum*, isolated from an Antarctic lake. Its position within the archaeal domain of the tree also indicates a later evolutionary arrival (Fig. 3). The marine Crenarchaeota have a somewhat earlier branching position. Members of this archaeal group often dominate numerically in the cold deep sea and occur in many of the polar waters and sediments that have been examined, but only one of their members has been brought into culture (from an aquarium sample enriched at room temperature). It is not cold-adapted. A well-studied crenarchaeal symbiosis with a sponge host that dwells in cold waters clearly suggests cold adaptation, but whether psychrophilic or psychrotolerant awaits a cultured isolate. Because the marine Crenarchaeota that inhabit the cold deep sea are believed to be involved in the nitrogen cycle, especially the process of nitrification (microbial oxidation of ammonia to nitrite and nitrate) which generates the inorganic nitrogen required by primary producers in surface waters, they are targets of intensive study.

Given that the study of cold adaptation in the microbial realm has historically centered on heterotrophic bacteria, in large part because these organisms are more readily brought into culture than chemolithotrophs or Archaea in general, conclusions from the depicted phylogenetic tree (Fig. 3) should be drawn with caution. As more microbes are brought into culture and shown to be cold-adapted, the branching patterns evident today may change. Stable isotope (and other) probing techniques that allow recognition of microbial activity under different temperatures in the absence of cultivation, yet coupled to a sequencing identification, may also bring new information to the tree, as may targeted studies using transcriptomics, proteomics, and metabolomics.

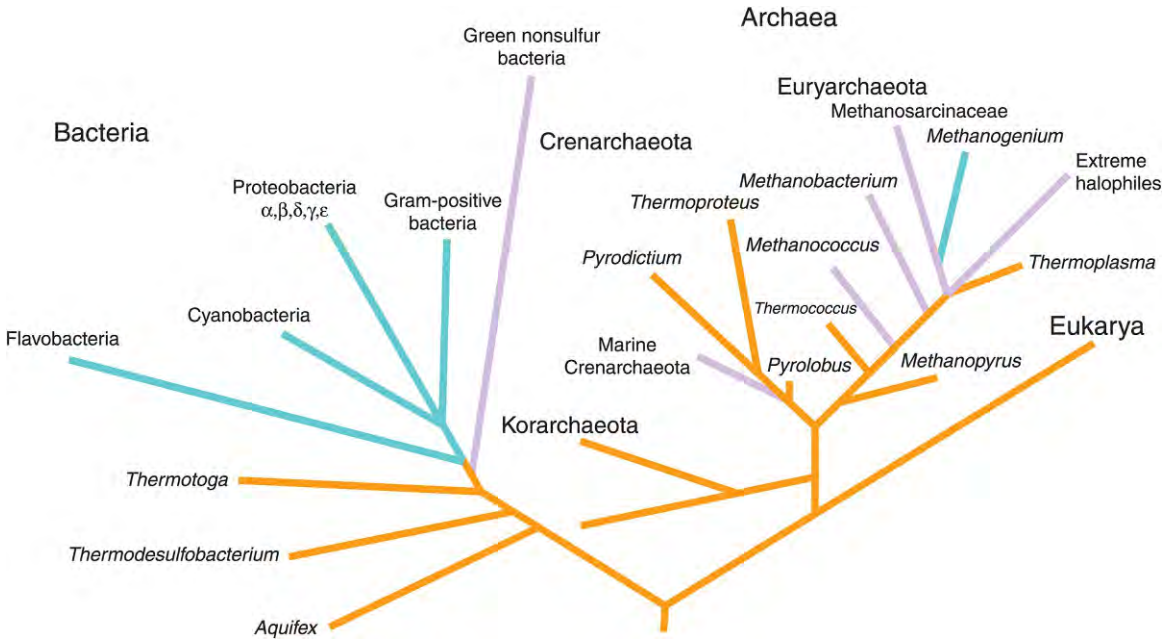

Fig. 3 Universal phylogenetic tree of life based on 16S rRNA sequences, emphasizing the domains of Bacteria and Archaea. Orange branches indicate hyperthermophiles that grow at $\geq$90°C; purple branches, groups that contain known (cultured) psychrotolerant strains; and blue branches, groups that contain known (cultured) psychrophiles. Note that the (uncultured) marine Crenarchaeota are colored purple because degree of cold adaptation is not known.

Genetic Mechanisms

Genetic mutation as a means to cold adaptation is evident from studies of the molecular interactions inferring enzymes with catalytic ability in the cold and in comparative analyses of whole-genome sequences from related organisms with different cardinal growth temperatures. In the former case, site-directed mutagenesis and related approaches indicate that, depending on the enzyme and often its size, anywhere from a single amino acid change to numerous amino acid substitutions or chemical alterations can explain the gain (or loss) of cold activity. In the latter case, and despite an oft-cited idea that only a critical subset of an organism's enzymes need be cold-active for it to function as a psychrophile, results of comparative genome studies for both Archaea and Bacteria suggest otherwise. Significant amino acid replacements were observed in over 1000 genome-deduced proteins from the psychrophilic methanogen, *M. frigidum*, relative to proteins from other methanogens covering a range of temperature growth optima, from 15 to 98°C. When the whole proteome of *C. psychrerythraea* 34H and three-dimensional architectures of its proteins were compared to nearest mesophilic neighbors with available genome sequences, the same general observation was made. In both cases, the interactions and locations of polar and charged amino acids (in particular serine, histidine, glutamine, and threonine) in protein tertiary structures appeared influential in imparting psychrophily.

In other analyses of the whole genomes from the several psychrophiles that have been sequenced so far, specific proteins and other features known from previous culture work to impart cold adaptation (discussed below) have been documented. A new genomic finding, not widely evident from prior culture work, is the prevalence of virally delivered genes in psychrophiles along with the presence of prophage (viruses residing benignly in the bacterial genome and thus implicated in gene transfer). That virally mediated horizontal gene transfer has played an important role in the evolution of psychrophily appears inescapable, although other forms of gene transfer also need to be considered (transformation by direct uptake of free DNA and plasmid exchange by conjugation between cells; Fig. 4), particularly since the transfer of plasmid DNA via conjugation between mesophilic and psychrophilic bacteria has been demonstrated.

In the same time frame that genomic analyses of psychrophiles have become available, the study of cold-active virus–host systems in culture has been renewed. While work over a half-century ago had documented infectivity at 0°C, the environmental and evolutionary implications had only rarely been pursued. Recently, several promising cold-active virus–host systems have been obtained by a number of researchers and the lower (known) temperature limit for infectivity has been pushed to −12°C (and 16% salt) in simulated sea-ice brines. The goal of demonstrating active horizontal gene transfer under realistic environmental conditions, whether or not mediated by viruses, as means to evolve cold adaptation remains for the future.

Molecular Basis for Cold Adaptation

As the temperature of an aqueous solution decreases, for example, from 37 to 0°C, its viscosity more than doubles, slowing solute diffusion rates, while chemical reaction rates (also influenced by viscosity) decrease exponentially. The cytoplasmic membranes and

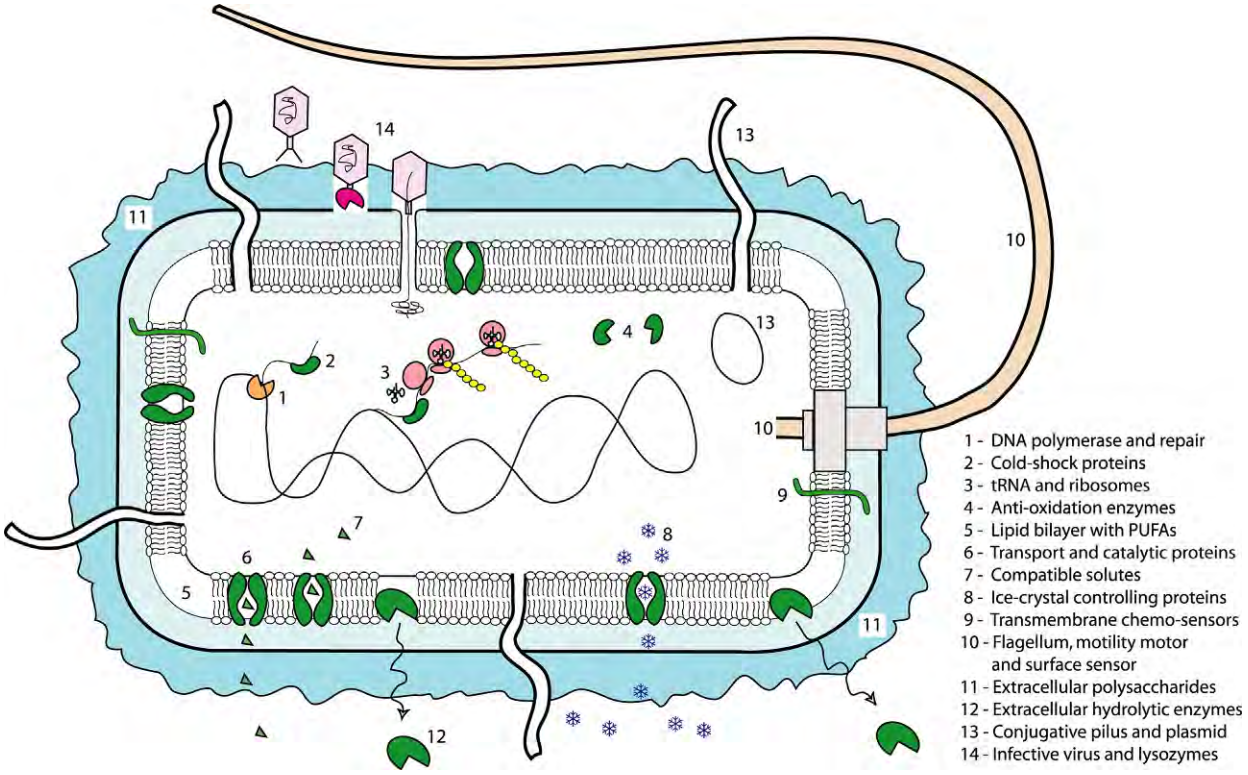

Fig. 4 Simplified schematic of a bacterial cell depicting some of the known components and processes linked to cold adaptation (see text).

enzymes of mesophilic microbes tend to rigidify under these conditions and lose function. Protein folding is impaired and nucleic acids assume secondary or super-coiled structures that interfere with their proscribed activities. A hallmark of microbial cold adaptation at the molecular level is thus to retain sufficient flexibility in its macromolecules such that essential functions can go forward in spite of the challenging effects of low temperatures. When arguably nonessential functions, for example, motility, can also go forward, the microbe becomes even more competitive in the cold. The lower temperature limit for bacterial motility, −13°C (in a glycerol solution), is held by an extreme psychrophile, *C. psychrerythraea* 34H, while recent work with a digital holographic microscope has revealed motility in natural sea-ice brine communities at –4°C, the lowest temperature tested. Enough direct research on psychrophiles and comparative studies of psychrotolerant and mesophilic bacteria has been accomplished in recent decades to identify key components and aspects of a cell that impart cold adaptation (Fig. 4).

Membrane Fluidity

The cellular membranes of cold-adapted microbes must remain flexible enough under rigidifying temperatures to facilitate their essential functions in the transport of nutrients and metabolic byproducts and the exchange of ions and solutes critical to maintaining intracellular integrity. The cold-adapted cell accomplishes this feat by fine-tuning the composition of its membrane lipid bilayer (Fig. 4), introducing steric hindrances that change the packing order of the lipids or reduce interactions between them and other membrane components. The genome must encode the ability to produce a flexible membrane in the first place (adaptation), as well as to adjust membrane components on the short-term (acclimation). The list of specific alterations known to enhance flexibility in the cold is extensive, if sometimes strain-specific, but typically includes producing a higher content of unsaturated (fewer double bonds between carbon atoms), polyunsaturated, and branched and/or cyclic fatty acids in response to cold. In some cases, shortening the length of the fatty acid can enhance flexibility, while in others, changing the content or size of the lipid head groups helps. Genes for enzymes involved in polyunsaturated fatty acid (PUFA) synthesis are clearly present in psychrophilic genomes, although without tests of their cold-active nature, such findings are not definitive for cold adaptation (some mesophiles also contain them).

Other components of the lipid bilayer include membrane proteins and carotenoid pigments. How membrane proteins interact with lipids affects both the active and passive permeability of the membrane, important for controlling the exchange of ions and organic solutes (Fig. 4). The genomes of *C. psychrerythraea* 34H and *P. halocryophilus* Or1, for example, contain numerous gene families involved in the transport of solutes, low-molecular-weight organic compounds (often sugars or amino acids and their derivatives) that accumulate to high intracellular levels under osmotic stress, and are compatible with the metabolism of the cell. They help to maintain cellular volume and turgor pressure and protect intracellular macromolecules in the face of changing salt

concentrations exterior to the cell, as occurs in sea-ice brines when the temperature drops (Fig. 3). Recent work with both psychrophilic and psychrotolerant isolates in culture, and with natural sea-ice brine communities, indicates widespread reliance on compatible solutes by cold-adapted bacteria. The cold-adapted membrane thus needs to be flexible, especially in very cold environments, to permit the uptake (and release) of compatible solutes as well as energy-yielding substrates, yet impermeable enough to prevent excessive passive exchange; adjusting the protein components of membranes can help. The sensitivity of some membrane proteins to temperature-driven conformational changes appears to provide a thermal sensor that results in the upregulation of genes involved in subsequent membrane adjustments, making life in a temperature gradient imminently feasible. As temperatures approach T_{max} for growth, cold-adapted microbes must also be able to keep their membranes sufficiently stable (inflexible) to avoid cell leakage and death by lysis. In some cases, adjusting pigment content appears to accomplish this goal.

Cold-Active Enzymes

The exponential drop in chemical reaction rates brought on by decreasing temperature highlights the impressive evolutionary development of all manner of enzymes with high catalytic activity in the cold. Complementing the known membrane adjustments to achieve flexibility in the cold are those expressed by cold-active enzymes, including essential intracellular enzymes required for nucleic acid and protein synthesis in the cell, membrane permeases for active solute transport and alteration, and extracellular enzymes released to perform hydrolytic functions in the environment (Fig. 4). For cold environments that experience strong UV radiation, the microbial production of enzymes that scavenge reactive oxidative (chemical) species may also be considered essential. As with achieving a more flexible membrane against the cold, achieving high enzymatic activity at low temperature involves creating greater molecular flexibility than that observed in enzymes active at warm temperatures. It also involves trade-offs; although not a firm rule, for many cold-active enzymes the very traits that impart cold activity also make them unstable at higher temperature. Unlike membrane adjustments, enzyme adaptations over an evolutionary time scale appear more relevant to cold activity than means for short-term acclimation. The strategies for increased flexibility of an enzyme leading to cold activity are numerous but not uniform. In some cases, increased flexibility is linked to a shift in primary structure (amino acid composition) of the entire protein, while in others only direct adjustments to the catalytic site of the three-dimensional macromolecule are involved. For some enzymes, including those released from the cell to perform hydrolytic functions in the environment, adjustments to flexibility are detected in the regions of the protein exposed to the solvent. Keeping an exterior shape firm as temperature drops can translate to keeping a more protected catalytic site flexible.

Considering the interface between the exterior shape of an enzyme and the solvent raises the need to consider enzyme interactions with other components in the environment, independently of the cell. For example, the extracellular polysaccharides released by *C. psychrerythraea* 34H have been observed to stabilize an extracellular protease (that it also produces) against thermal denaturation. The effective work of extracellular enzymes in the cold, for example to hydrolyze organic substrates to a size that can be transported into the cell, may be an important trait of heterotrophic psychrophiles. Over half of the enzymes assigned to the degradation of proteins and peptides in the *C. psychrerythraea* genome are predicted to be localized external to the cytoplasm, among the highest percentage in any completed genome (from all thermal classes). The successful infection (and reproduction) of a virus in this same psychrophile at very cold temperatures (–10 to −13°C) suggests that at least some viruses in cold environments may carry highly cold-active lysozymes for penetrating the cell membrane (Figs. 3 and 4).

In spite of multiple strategies to achieve enough flexibility for catalytic activity in the cold, intracellularly or extracellularly, some common trends have emerged from enzyme studies regarding specific chemical modifications required to reduce the strength or number of otherwise stabilizing factors for a protein. These include reducing ion pairs, hydrogen bonds and hydrophobic interactions, inter-subunit interactions, cofactor binding, and proline and arginine content. Increasing exposure of apolar residues to the solvent, accessibility to the active site, and the clustering of glycine residues also pertain. Such trends provide means to search available (and future) genomes for signs of cold adaptation. They also provide blueprints to identify or engineer proteins for applied uses in the cold, many of which have been identified by the food, detergent, and biotechnology industries, by those seeking means to remediate the contamination of cold environments, and by start-up companies interested in the possible production of cost-effective to fossil fuels that take advantage of enzymatic hydrolysis in the cold. A promising recent example is the discovery of potentially novel hydroxylases for alkane, a major component of crude oil, which could be scored as cold-active based on amino acid preferences and regions of increased flexibility typical of psychrophiles.

Cold-Shock Proteins

For the cold-adapted microbe, all nucleic acids and proteins involved in maintaining (if not synthesizing), transcribing, and translating genetic information intracellularly must be able to function in the cold. In some cases, this feat is thought to be accomplished not by primary alteration of the macromolecule itself, as already described, but by production of specific proteins that bind to them, presumably enabling proper conformation and flexibility, including required periods of destabilization. The production of cold-shock proteins to serve a similar function when mesophiles are subjected to a temperature downshift is well studied, but less is known about related responses of cold-adapted microbes to downshifts in temperature or to continuous life in the cold. Available information on psychrotolerant bacteria indicates that large numbers of cold-shock proteins (related to those in mesophiles) are always present and that production of cold-acclimation proteins is continuous in the cold, as is the expression of housekeeping genes (for basic cellular functions). By contrast, the mesophile *Escherichia coli* carries few cold-shock proteins prior to

cold shock, but a temperature downshift immediately results in repression of critical housekeeping genes and induction of cold-shock (but not cold-acclimation) proteins, which is transient.

The continuous production of a variety of binding proteins to maintain proper conformation, flexibility, and function of major macromolecules thus appears to be an important trait of cold adaptation (Fig. 4). Although work with live psychrophiles is needed, genomic sequence data support this idea; for example, the genome of *C. psychrerythraea* 34H encodes for multiple common cold-shock proteins. Furthermore, the genetic acquisition of cold-shock proteins may not require the longer term evolutionary process of vertical inheritance but may be facilitated by horizontal gene transfer. Genes for cold-shock proteins known only from the domain of Bacteria have been observed upon genomic sequencing of an uncultured population of marine Crenarchaeota from cold Antarctic waters.

Cryoprotectants and Exopolymers

In very cold environments, the cellular membranes of resident microbes are subject not only to rigidity but also to physical damage from ice-crystal formation during the freezing process. Some cold-adapted microbes are known to produce and release specific proteins that help to control the formation of ice crystals (Fig. 4), including ice-nucleating proteins (that provide a template for crystal formation away from the cell) and antifreeze proteins that inhibit ice nucleation by dropping the freezing point or repressing the recrystallization of ice. The rate of freezing experienced by the cell also influences the degree of damage, with faster rates limiting the damage. Natural ice formations on Earth freeze slowly (producing ice crystals) relative to the vitrification process, whereby the liquid phase converts directly to solid without ice-crystal formation. When microbial cultures are vitrified in the laboratory (using liquid nitrogen at $-196^{\circ}C$), their cell membranes remain intact with no morphological sign of damage. When vitrified in the presence of sugars, the likelihood of recovering them in culture after thawing increases.

Small molecular weight sugars (like glycerol) have long been used as cryoprotectants in the deep-freeze ($-80^{\circ}C$) storage of microbes, presumably providing a buffer between cells and ice crystals. Newly discovered, however, is the overproduction of complex extracellular polysaccharides by cold-adapted bacteria, both psychrophilic and psychrotolerant, when subjected to increasingly cold temperatures, especially below the freezing point. Sea ice through its seasonal lifetime is also recently known to harbor high concentrations of sugar-based exopolymers in its liquid brine inclusions (Fig. 3). These exopolymers, produced copiously not only by sea-ice algae but also by ice-encased bacteria, are understood to serve as natural cryoprotectants not only against potential ice-crystal damage but also by further depressing the freezing point such that more liquid water remains available within the ice matrix. In this regard, cellular coatings of exopolymers (Fig. 4), or exopolymers available in the environment, are believed to provide a hydrated shell that helps to buffer the cell against the osmotic stress of high salt concentrations in winter sea-ice brines (Fig. 3). Along with the possible stabilization of extracellular enzymes, this myriad of functions makes exopolymers a cold-adaptive trait worth examining in more detail. The organization of genes for exopolymers on the genome of the psychrophilic bacterium *Psychroflexus torques*, for example, suggests that they may be the result of a series of lateral gene transfer events.

Conclusion: A Model Habitat for Cold Adaptation

Considering that the ocean represents the bulk of Earth's cold biosphere, today and in the past, the annual freezing of its surface waters in polar regions takes on special significance as an important planetary driver of cold adaptation. Astronomical numbers of bacteria (10^5 in a single milliliter of seawater) pass through this frozen gauntlet annually, and over extended periods in geological time. Microbes that experience a winter in sea ice are subjected to the linked stressors of increasingly cold temperature and high brine salinity as shrinking pore space further concentrates all impurities in the source seawater, including microbes (Fig. 2). This concentrating factor brings microbes into close proximity to each other, as verified by microscopic observations of DNA-stained cells in unmelted ice, in a liquid environment of abundant low- and high-molecular-weight organic compounds, including complex exopolymers that serve many positive functions for the trapped cells. Model calculations and observed concentrations in melted sea ice indicate that agents of lateral gene transfer (free DNA and viruses) also surround the encased cells (Fig. 2). The virus–bacteria contact rate in a winter sea-ice brine may be as much as 600 times higher than in underlying seawater. Active virally mediated gene transfer has not yet been demonstrated, but the sea-ice environment would appear to favor it.

Even if horizontal gene transfer is not operative in sea ice, the vertical inheritance of genes for cold adaptation must be a regular occurrence. The habitat of *P. ingrahamii*, one of the most extremely psychrophilic bacteria on record (shown to reproduce at $-12^{\circ}C$), is sea ice. Isolates of *C. psychrerythraea* (strain 34H also grows at $-12^{\circ}C$) are readily cultured from sea ice. Virtually all of the canonical molecular traits of cold adaptation, along with some new ones, have been documented in the test tube or by genome analyses of such sea-ice psychrophiles. Rather than a stably cold environment, the key to the evolution of cold adaptation may be repeated exposure to the extreme cold and brine of sea ice, selecting for robustness in the face of multiple insults, including future ones like hydrostatic pressure. That salt-heavy water masses form in polar oceans actively growing sea ice and then sink to fill the cold deep ocean over time points to the concept of a cold refuge for cold-adapted bacteria during interglacial times when ice as an evolutionary driver was nonexistent, as we may witness again in this century.

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Extremophiles: Dry Environments (Including Cryptoendoliths)☆

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Glossary

Arid region Region on the earth's surface where the ratio of the mean annual precipitation to the mean annual potential evapotranspiration is less than 1; divided into the hyperarid, arid, semiarid, and subhumid zones.
Arid zone Region of the earth where the ratio of the annual precipitation to the annual potential evapotranspiration is between 0.03 and 0.20.
Biogeography The study of the distribution of organisms across geographical regions and the factors leading to observed distributions.
Chasmoendolithic habitat Lithic habitat consisting of the cracks and fissures in the rock, with more or less direct connection to the surface.
Compatible solute Low-molecular-weight organic compounds that can serve to lower the osmotic potential of a cell while maintaining cellular and molecular integrity.
Cryptoendolithic habitat Lithic habitat consisting of the naturally occurring pore spaces within the rock, usually only indirectly connected to the surface.
Deliquescence Process by which a substance absorbs water vapor from the air in sufficient quantities that it dissolves in the absorbed water.
Epilithic habitat Lithic habitat consisting of the upper exposed surface of a rock or stone.
Euendolithic habitat Lithic habitat consisting of holes and pits created by the metabolic activity of the microbial community.
Hyperarid zone Region of the earth where the ratio of precipitation to potential evapotranspiration is less than 0.03.
Hypolithic habitat Lithic habitat consisting of the lower surface of a stone in association with adherent soil particles.
Lithopanspermia Hypothesis suggesting that life can be transferred from planet to planet by means of rocks expelled from planetary surfaces through the force of meteorite impacts.
Perilithic habitat Lithic habitat consisting of the sides of a stone below the soil surface; distinguished from the hypolithic habitat on the lower surface of the stone.
Water activity (a_w) A measure of the concentration of water in a solution, as modified by the type of solutes present in the solution.
Water potential (ψ) A measure of the potential energy in a unit mass of water.

Nomenclature

ETP annual potential evapotranspiration
P annual precipitation

Defining Statement

This article summarizes the current state of knowledge concerning the microbial communities inhabiting arid regions, with an emphasis on lithic environments. The constraints put on these communities by their dry environment and their adaptations to survive the desiccated state are also addressed.

Introduction

It can be argued that dry environments present one of the most extreme set of conditions faced by microorganisms. The cell must be able to withstand the specific biochemical stresses created by the lack of water; also, it may be called upon to withstand a variety of other environmental insults – exposure to high levels of UV radiation, for example, or elevated temperatures during periods when its

☆*Change History*: July 2016. James A. Nienow added sections on biogeography of desert microorganisms and the relevance of desert organisms to astrobiology; made small changes to the rest of the text; added a new Figure, the terms biogeography and lithopanspermia to the glossary, citations, including citations to investigations published since 2009; and updated reference section.

This article is an update of J.A. Nienow, Extremophiles: Dry Environments (including Cryptoendoliths), *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 159–173.

metabolic activity has been reduced to minimal levels by the same lack of water. On one level, then, it is surprising that any cells possess the required set of adaptations. Yet, when one considers the obvious selective advantages conferred by these adaptations in the 30% of the world not covered by liquid water, perhaps it is not so surprising after all. As will be seen, there are a variety of ways to cope with dry environments and a diversity of microorganisms employing them.

Before proceeding, it may be worthwhile to spend some time discussing what constitutes a dry environment. Clearly, the concept includes some mechanism leading to a reduction in the liquid water content of the cell. In general, the reduction of liquid water can be achieved either by freezing the cell or by the net movement of water out of the cell. Freezing conditions, then, in the broadest sense, constitute a form of dry environment. However, the freezing process involves its own set of biochemical stresses, which are not necessarily related to the lack of liquid water, and, therefore, will not be treated further; interested readers are directed to the review by Crowe and Crowe (2002). Instead, the discussion will revolve around environments leading to the removal of cellular water. Technically, these include any environment with a reduced water activity or a low water potential, including syrups, brines, and even some hypersaline lakes and salinas. While peripheral to the main treatment, the microorganisms occurring in these rather wet "dry" environments provide some insight into some of the mechanisms available to microorganisms living in the truly dry environments – dry soils and all aerial and subaerial environments, especially those in arid regions, the exterior of spacecraft, and dried grains and other foodstuffs – and so will not be explicitly excluded. The discussion will focus, however, on the microorganisms of arid regions, because they experience the widest range of extreme conditions, and, in consequence, display the broadest set of adaptations. Readers interested in a more thorough understanding of hypersaline environments should consult the Further Reading section.

Microbial Associations in Arid Regions

Traditionally, deserts have been defined as regions with barren landscapes and limited water, with the degree of water limitation usually defined on the basis of annual precipitation (see Office of Arid Land Studies, University of Arizona, 1968; Rainey et al., 1999). Thornwaite (1948) pointed out that a definition based strictly on precipitation is incomplete, in that it ignores the effects of temperature and plant cover on the fate of the precipitation. Instead, he championed a comparison of the actual annual precipitation with the potential evapotranspiration in the delineation of climatic regions. This approach was followed in the development of some of the first precise global maps of arid regions in the early 1950s (Meigs, 1953). Currently, arid regions sensu lato are defined as those parts of the world where the ratio of the mean annual precipitation (P) to the mean annual potential evapotranspiration (ETP) is less than 1. This definition emphasizes the water deficit of the region, which is greater for hotter deserts where the rate of evaporation is greater. Four intergrading zones have been delineated: the hyperarid zone with P/ETP less than 0.03; the arid zone with P/ETP between 0.03 and 0.20; the semiarid zone with P/ETP between 0.20 and 0.50; and the subhumid zone with P/ETP between 0.50 and 0.75 (see UNESCO, 1979; Parsons and Abrahams, 1994). In this system, regions previously classified as deserts on the basis of a mean annual precipitation of less than 200 mm per year correspond roughly to the hyperarid and arid climate zones. Together, these comprise in excess of 20% of the Earth's terrestrial surface. Semiarid regions, including steppes, savannahs, and similar habitats, cover another 16% (Walton, 1969). Such a large area encompasses a diversity of habitats and associations. In this article, we touch upon three broad clusters of associations: the soil microbiota, biological soil crusts, and rock-inhabiting microorganisms (Fig. 1; Table 1).

The Microbiota of Desert Soils

Numerous studies over the past several decades have indicated the presence of a remarkably diverse assemblage of microorganisms in soils from both hot and cold deserts (see Vishniac, 1993, 2002; Kieft, 2002; Cary et al., 2010). Included in most assemblages are a number of cosmopolitan species, which gives the overall assemblage an appearance similar to what would be expected from milder climates. This raises questions concerning (1) how harsh the desert soil climate is; (2) what role the cosmopolitan species play in the species assemblage; and (3) whether there exists a microbiota indigenous to desert regions. It is possible that a number of the bacteria isolated from desert soils are recent additions, carried in by wind, dust, or human activity, and not truly active members of the ecosystem. This is especially true for the cold McMurdo Dry Valleys region of Antarctica (also known in the literature as the Ross

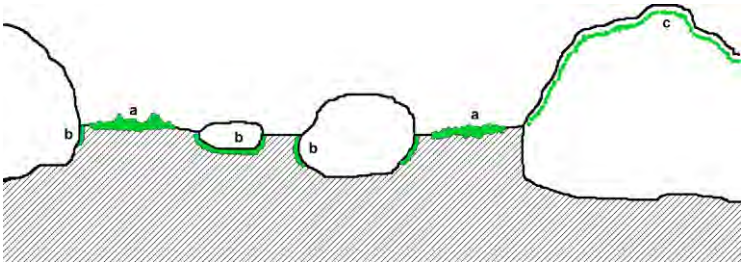

Fig. 1 Diagrammatic representation of the major microbial associations in dry environments. (a) Biological soil crust. (b) Hypolithic associations. (c) An endolithic association.

Table 1 Characteristics of the more common habitats

Habitats/ associations	*Ecological features*
Edaphic habitats	On or in bare soils; surface fully exposed to the environment; exposure decreasing with depth; subject to disturbance (burial and erosion) from wind and flowing water, animal movement
Biological soil crusts	Fully exposed to the environment; insolation, desiccation, and wind erosion may be ameliorated by the structure of the association and the degree of connectivity with the underlying soil
Lithic habitats	
Epilithic	Fully exposed to the environment, organisms experience extremes of insolation, desiccation, long- and short-period temperature variation, wind erosion; generally restricted to relatively moist regions
Endolithic	With varying degrees of protection from environmental conditions: light filtered by the rock substratum; water retained by the physical structure of the substratum; wind erosion reduced by the overlying rock material; short-period temperature variations reduced, long-periods variations similar to those of the epilithic habitat; limited space for growth of the association
Chasmoendolithic	Protection from environmental conditions depending, in part, on the nature of the habitat – macroscopic cracks lead to more exposure to desiccation, but may be more efficient in collecting water and wind-blown nutrients; habitats with microscopic cracks similar to cryptoendolithic habitats
Cryptoendolithic	Generally more protected from environmental conditions than chasmolithic habitats because of the absence of a direct connection to the surface
Euendolithic	Restricted to the upper millimeter of the rock surface, therefore with a high degree of exposure to environmental conditions, especially insolation and desiccation
Hypolithic	Highest degree of protection from environmental conditions: light reduced by transmission through the overlying stone; long- and short-term temperature variations reduced; possible transport of moisture and nutrients to the habitat from the underlying soil
Perilithic	Subset of the hypolithic habitat relying on the condensation of water on the relatively large upper surface of the stone

Desert), where the conditions would tend to preserve nonindigenous cells in a nonmetabolic state for extended periods of time. Even with the possible inclusion of a number of nonindigenous forms, in hot deserts there does seem to be a skew in favor of desiccation-tolerant forms. This manifests itself in the proportionally larger numbers of spore-forming Gram-positive bacteria, especially spore-forming actinobacteria, and in the relative proportion of filamentous fungi (Fuller, 1974; Rainey et al., 1999; Kieft, 2002). There is a shift toward radiation-resistant forms in arid soils, especially members of the genus *Deinococcus*, with up to nine species present in a single soil sample (Rainey et al., 2005). Such a shift is consistent with the suggestion that radiation resistance in bacteria is actually a side effect of adaptations to water stress (Mattimore and Battista, 1996). In the interior of the cold McMurdo Dry Valleys region, in contrast, actinobacteria may only comprise about 3% of the cultured soil microbiota (Vishniac, 1993). The numbers of yeasts and filamentous fungi are also very low (Vishniac, 1996; Connell et al., 2006). *Deinococcus*, including several newly described species, however, is present (Hirsh et al., 2004). The low numbers of yeasts in McMurdo Dry Valleys soils are somewhat surprising in light of the fact that the dominant yeast in the region, *Cryptococcus vishniacii*, is considered to be both endemic- and desiccation-resistant (Vishniac, 2002).

Early studies of desert microorganisms relied on standard culture-based techniques. It is now recognized that many of the bacteria in environmental samples cannot be cultured on standard media, or, for that matter, on any media yet devised. Therefore, in recent years a number of molecular techniques have been developed, primarily based on direct or indirect analysis of gene sequences, but including fatty acid analysis and community-level physiological profiling (Torsvik et al., 1998; Kirk et al., 2004). Application of some of these techniques to desert soils has indicated the presence of a number of heterotrophic organisms not previously seen in culture-based studies, including members of the domain Archaea (Dunbar et al., 2000; Rutz and Kieft, 2004; Adams et al., 2006; Chanal et al., 2006; Smith et al., 2006; Lester et al., 2007; Yergeau et al., 2007; Niederberger et al., 2008). Even bacteriophage, either as prophage (viral genetic material incorporated into the bacterial genome) or as virus particles in bacteria in a pseudolysogenic state (infecting the cell, but maintained separately from the genome), but not as free particles, have been detected in the subsurface desert sands (Prigent et al., 2005), and in Antarctic soils and lithic environments (Zablocki et al., 2014). The full significance of these results is yet to be elucidated. At a minimum they suggest that the diversity of desert soil bacteria is much greater than was previously realized (Niederberger et al., 2008; Cary et al., 2010).

Traditionally, the phototrophic assemblage in desert soils has been evaluated using direct microscopic observation of field samples supplemented with cultures derived from the samples (see, e.g., Friedmann et al., 1967). Based on these types of studies a picture arose of deserts populated by a relatively simple assemblage dominated by coccoid green algae (*Chlorococcum*, *Chlorella*, *Stichococcus* and others) and filamentous cyanobacteria (*Lyngbya*, *Schizothrix*, *Microcoleus*, *Scytonema*, *Nostoc* and others), with coccoid cyanobacteria and other groups of eukaryotic algae present in smaller numbers (Friedmann and Galun, 1974). As was recognized early on, the simple and variable morphology of many of the desert phototrophs makes identification from field material difficult, while culture techniques tend to favor cosmopolitan generalists instead of the ecological specialized, and perhaps more significant, members of the association (Friedmann et al., 1967); this is the same problem encountered in culture-based studies of heterotrophic bacteria. Improved culture methods and the application of molecular techniques are beginning to provide us with better understanding of the true diversity of the desert phototrophic assemblage (Cardon et al., 2008; Osorio-Santos et al., 2014; Patzelt et al., 2014; Pietrasiak et al., 2014; Fig. 2).

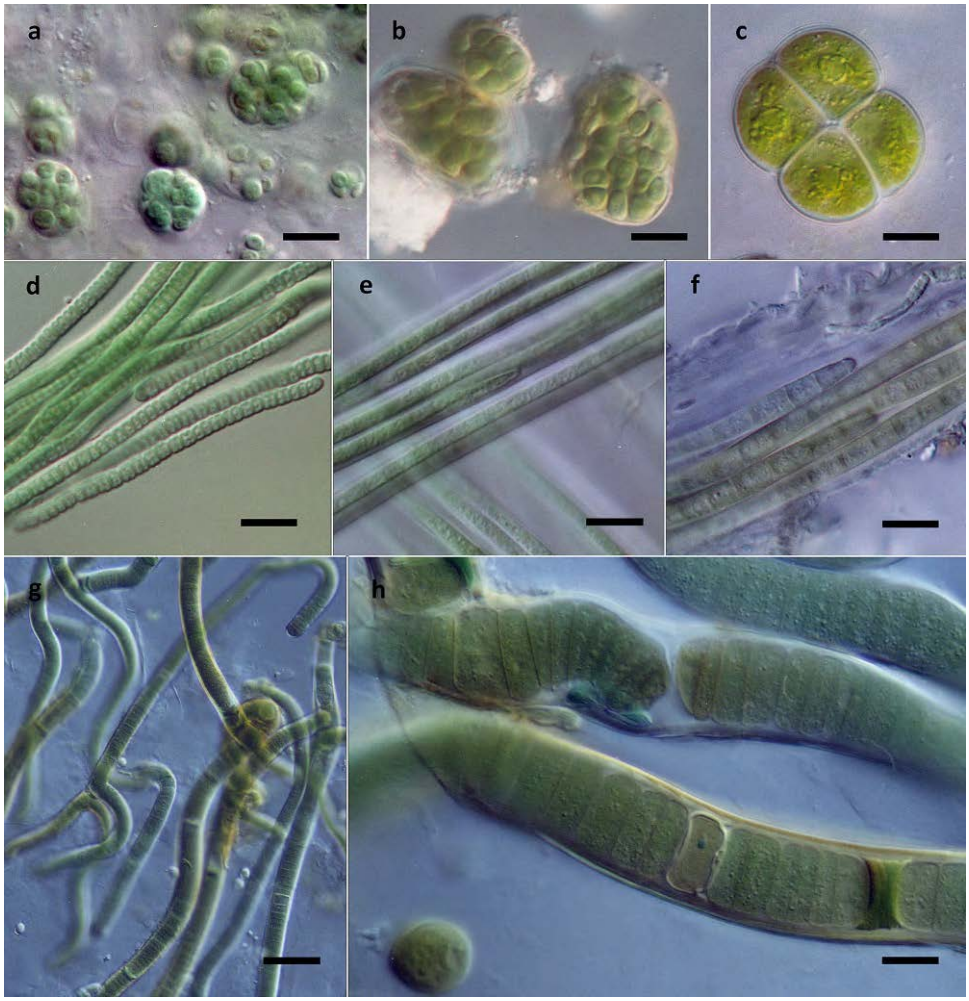

Fig. 2 Examples of desert cyanobacteria and algae. (a) Cyanobacterium *Chroococcidiopsis* sp. from the Negev Desert, Israel. (b) Cyanobacterium *Chroococcidiopsis*-like from a halite in the Atacama Desert, Chile. (c) Chlorophyte *Tetracystis* sp. from the Mojave Desert, United States. (d) Cyanobacterium *Leptolyngbya* sp. from the Mojave Desert, United States. (e) Cyanobacterium *Schizosthrix* sp. from the Mojave Desert, United States. (f) Cyanobacterium *Microcoleus* sp. from the Mojave Desert, United States. (g) Cyanobacterium *Scytonema* sp. from the Mojave Desert, United States. (h) Cyanobacterium *Scytonema* sp. Detail of material illustrated in figure (g). Scale bars represent 10 μm in figures (a)–(f), (h) and 50 μm in figure (g).

As expected, the numbers of soil bacteria in deserts tend to be lower than in comparable soils from more temperate climates. Reported plate counts from a variety of hot desert soils range from a low of less than 10 cfu g^{-1} to 1.6×10^{7} cfu g^{-1}, numbers at least 2 orders of magnitude lower than is typical for agricultural or forest soils (Kieft, 2002). The lowest recorded numbers are from the vicinity of Yungay in the hyperarid core of the Atacama Desert, Chile (Navarro-González et al., 2003). This region has generated considerable interest among microbiologists and astrobiologists because it is considered to be one of the oldest and most extreme of the hot deserts, and can be considered a terrestrial analogue of Mars (McKay et al., 2003). Some microbiologists, because of the low numbers of bacteria recovered, suggest that the dry conditions in the hyperarid core of the Atacama may be near the boundary for life in desert environments (Navarro-González et al., 2003). Others dispute this view on the basis of their own higher counts; they suggest that the boundary for life has yet to be found on Earth (Drees et al., 2006; Lester et al., 2007). No matter which group is right, it is clear that large-scale shifts in the moisture regime have a significant impact on the total number of desert soil bacteria.

The cold deserts of Antarctica have also been cited as being at or beyond the limits of life; there are several examples of studies where the number of colony-forming units (cfu) in soil from the McMurdo Dry Valleys region of the Antarctica was below detectable limits (Horowitz et al., 1972). However, the apparent sterility of at least some of the samples may have been an artifact of the medium used (standard nutrient agar); true oligotrophs and some nutrient-starved bacteria are unable to grow on this relatively rich medium. Later sampling of similar soils using oligotrophic media containing 0.1% peptone and 0.02% yeast extract enriched with soil extracts from the site yielded significantly higher numbers of bacteria, suggesting that viable cells can be presumed to be present in all soils not containing toxic substances (Parker et al., 1982; Vishniac, 1993, 2002). The absolute number and diversity of soil bacteria varies widely, depending on the type (e.g., ornithogenic, enriched with bird wastes vs. mineral, with limited organic material), the local climate, and the technique used (Vishniac, 1993; Aislabie et al., 2006; Yergeau et al., 2007;

Niederberger et al., 2008; Lee et al., 2012). Away from the coast, the numbers are similar to those encountered in the Atacama Desert, on the order of 10^3–10^5 cfu g^{-1} soil, when procedures similar to those used in the Atacama studies, plate counts on R2A agar, a low-nutrient medium designed to count slow-growing heterotrophic bacteria in stressed environments, are used. The more arid Antarctic soils generally harbor relatively small numbers of cyanobacteria and microalgae (Aislabie et al., 2006; Wood et al., 2008; Pointing et al., 2009; Khan et al., 2011).

The source of the organic carbon supporting the low numbers of heterotrophic bacteria in the Antarctic Dry Valleys, and other extreme deserts, is not completely clear. In the milder deserts, shrubs and animal mounds provide obvious sources of carbon and other nutrients, creating "islands of fertility" with bacterial populations up to two orders of magnitude larger than those found just a few decimeters away (Herman et al., 1995; Schlesinger et al., 1996; Kieft, 2002; Schade and Hobbie, 2005). These sources of nutrients are completely lacking in the more extreme deserts. Therefore, the heterotrophic microbial community must rely on in situ production by cyanobacteria and microalgae, importation of carbon from more productive regions, or relic carbon remaining in the soils from periods when the climate was more benign (Fountain et al., 1999; Virginia and Wall, 1999; Burkins et al., 2000, 2001; Elberling et al., 2006; Hopkins et al., 2006; Cary et al., 2010). There is evidence suggesting that, at least for the lower elevations of Taylor Valley, Antarctica, relic organics deposited either as entrained material in glacial tills or as primary production in ancient glacial lakes are the primary source of organic material in the soil and that this legacy material may sustain the microbial soil community in the absence of any other inputs to the system (Burkins et al., 2000). However, field measurements of short-term respiration rates, coupled with known meteorological parameters, indicate a carbon release rate of 6.5 g C m^{-2} $year^{-1}$; at this rate the mean residence time for carbon would be 23 years, not the thousands of years expected (Burkins et al., 2001). This suggests the presence of significant in situ production since the carbon signature of the organic material rules out inputs from the surrounding habitats. The legacy carbon may exist in a recalcitrant form unavailable to the community under normal conditions (Burkins et al., 2001; Barrett et al., 2006; Hopkins et al., 2006). This two-compartment model of soil carbon, one compartment labile, one compartment recalcitrant, may prove to be of value in the analysis of other desert systems.

Biological Soil Crusts

Biological soil crusts are created by surface-bound assemblages of microorganisms that consolidate the soil into crusts up to a centimeter thick (Garcia-Pichel, 2002; Fig. 3). Much of our current knowledge of this system has been summarized previously; those wishing a thorough review are directed to the Further Reading section (Belnap and Lange, 2003). Here we will just touch upon a few highlights.

Biological soil crusts are widespread, occurring on all of the continents and in most biomes, essentially wherever a shortage of water limits the growth of vascular plants, thereby allowing light to reach bare soil, but there is still sufficient water to allow the growth of phototrophic microorganisms (Garcia-Pichel, 2002; Büdel, 2003c; Belnap and Lange, 2003; Pócs, 2009). They reach their greatest development in semiarid and arid environments, where they can cover more than 90% of the ground surface (Johansen et al., 2001; Belnap et al., 2003; Thomas and Dougill, 2007; Zaady et al., 2007; Zhang et al., 2007). In such cases biological soil crusts may provide a significant fraction of the photosynthetically fixed carbon (Lange, 2003; Evans and Lange, 2003; Burgheimer et al., 2006; Thomas and Dougill, 2007; Pointing and Belnap, 2012), serve as sources of fixed nitrogen (Belnap, 2003a; Evans and Lange, 2003; Bhatnagar and Bhatnagar, 2005; Pointing and Belnap, 2012), reduce soil erosion (Belnap, 2003b; Eldridge, 2003; Warren, 2003a,b; Williams et al., 2012), and influence local and regional hydrologic cycles (Warren, 2003a,b; Eldridge, 2003; Yair, 2003; Belnap, 2006; Thomas and Dougill, 2007; Williams et al., 2012). Extensive growths of biological crusts can be mapped using aerial photography and satellite-based imagery (Karnieli et al., 2003; Zhang et al., 2007; see also Warren-Rhodes et al., 2007a,b,c).

The distribution, composition, and appearance of biological soil crusts depend primarily on moisture and climatic conditions, secondarily on soil properties (Johansen et al., 2001; Belnap et al., 2003; Büdel, 2003c; Ullmann and Büdel, 2003b; Bowker et al., 2006). On a broad scale, four distinct types of crusts can be recognized macroscopically (Belnap, 2003c, 2006). Smooth crusts, as

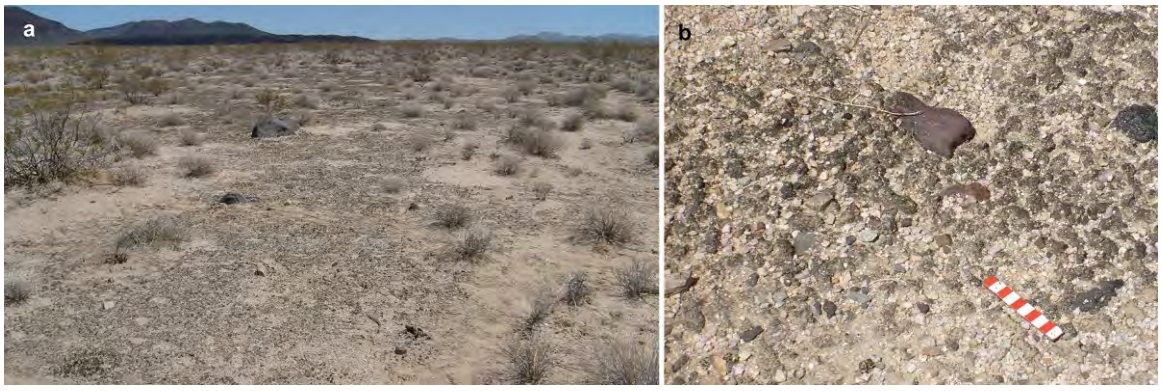

Fig. 3 Biological soil crusts in the Mojave Desert. (a) The desert landscape. Soil crusts appear as darker areas of the soil surface. (b) A closer view of the crust. In this instance the crust form a pattern of darker bands. The total length of the scale bar in the foreground is 10 cm.

suggested by the name, are recognized by the smoother appearance of the soil surface resulting from activity of cyanobacteria, algae, and fungi. The biological components spend much of the time just below the surface, only appearing when the soil is wet. This type of crust is most prevalent in hyperarid and arid regions with very low precipitation and very high temperatures (Belnap, 2003c). Rugose crusts, which give the soil surface a rough appearance, are common in arid regions with a slightly moister climate. They are also dominated by cyanobacteria, green algae, and fungi, but may include varying numbers of lichens and desiccation-resistant mosses (Belnap, 2003c). Pinnacled crusts are characterized by the presence of a patchwork of mounds up to 15 cm in height but only a few millimeters in width, caused by a combination of frost heaving during the winter and differential erosion during the rest of the year. Cyanobacteria are the dominant microorganisms, but lichens and mosses may contribute up to 40% of the cover. Pinnacled crusts are found in some of the cooler deserts, where freezing temperatures are possible (Belnap, 2003c). Rolling crusts are also associated with frost heaving. However, in this case, a thicker growth of mosses, lichens, and/or cyanobacteria reduces the amount of erosion, leading to a generally smoother appearance than that of the pinnacled crusts. Rolling crusts are characteristic of colder climates with a lower potential evaporation rate (Belnap, 2003c). Within these groupings, there is a tendency for sandy soils of low nutritional content to be dominated by cyanobacteria, with the proportion of lichens increasing with the carbonate, gypsum, and silt content of the soil (Büdel, 2003c; Ullmann and Büdel, 2003b).

From the limited data available, it appears that the abundance of soil crusts decreases as the climate becomes more arid. For example, in Central Asia, biological crusts are described as abundant in the eastern semideserts of Mongolia, present in the central Gobi Desert (Büdel, 2003a), and covering about 29% of the land in the relatively drier Gurbantunggut Desert farther west (Zhang et al., 2007). Within the Gurbantunggut Desert there is a marked decrease in the abundance of crusts in the northern regions of the desert, which corresponds to a decrease in the amount of precipitation (Zhang et al., 2007). Similarly, in southern Africa, the development of biological soil crusts appears to be linked to the frequency of rains and the duration of the dry periods (Büdel et al., 2009). The development of crusts in the Sahara Desert may be restricted by climate and surface instability to the marginal regions (Ullmann and Büdel, 2003a). However, there has only been a single study of soil crusts from the region (Ullmann and Büdel, 2003a). True soil crusts are rare in the Atacama Desert, usually restricted to moister sites in the south (Patzelt et al., 2014) and coastal regions near the port cities of Antofagasta and Caldera (Büdel, 2003b); *Nostoc* crusts are present in southern Peru (Rauh, 1985). In the drier parts of Antarctica, away from the coasts, small patches of cyanobacterial crusts occur sporadically in widely scattered locations (Green and Broady, 2003).

Common microbial constituents of biological soil crusts include members of the filamentous cyanobacterial genera *Microcoleus*, *Nostoc*, and *Scytonema* (Friedmann and Galun, 1974; Dor and Danin, 2001; Belnap et al., 2003; Büdel, 2003c). Filaments of *Microcoleus* are formed from numerous trichomes within a common sheath. The individual trichomes are motile within the sheath and may even, when conditions warrant, leave the sheath. In the latter case, trichomes will group and secrete a new sheath later. The buildup of used and new sheath material contributes to the stability of the soil crust (Dor and Danin, 2001; Belnap et al., 2003; Pointing and Belnap, 2012). Members of *Nostoc*, especially *Nostoc commune*, frequently form large masses of coiled trichomes within a communal polysaccharide sheath. Masses of *N. commune* can become large enough to harvest and are apparently used as a supplemental food source for humans in some regions (Belnap et al., 2003). Heterocysts, specialized nitrogen-fixing cells, are common within the colonies. Filaments of *Scytonema* are formed from a single trichome with heterocysts and false branching within a colored sheath. Trichomes reproduce through the formation of small motile filaments called hormogonia. Additional common phototrophic microorganisms include the cyanobacterium *Gloeocapsa* and members of the green algal genera *Chlorococcum*, *Macrochloris*, and *Stichococcus* (Belnap et al., 2003). It should be kept in mind, however, that the actual diversity of photosynthetic microorganisms in the desert crusts is much greater than indicated here. Molecular analysis of isolates and natural samples from North America (Garcia-Pichel et al., 2001; Lewis and Flechtner, 2002; Lewis and Lewis, 2005) suggests that biological soil crusts can almost be considered biodiversity hot spots. Somewhat surprisingly, soil crust fungi and heterotrophic bacteria have so far been virtually ignored (States et al., 2003).

Rock-Inhabiting Microorganisms

Most deserts contain extensive regions covered by rock outcrops or by bare soils with embedded or loose-lying cobbles, pebbles, and stones. The importance of rock, or lithic, habitats in these regions is suggested by the variety of terms derived from indigenous languages used to describe them. In the Middle East and northern Africa, regions characterized by rock outcrops, large boulders, and limited soil are called hamadas. Regions characterized by smaller stones and pebbles accompanied by sandy or gravelly soil are referred to variously as reg (western Sahara, the Iranian desert), serir (the Libyan and Egyptian Sahara), gobi (Mongolia), or gibber plains (Australia). Desert pavement is used specifically for those parts of the desert covered by embedded stones in a relatively compact arrangement (Dixon, 1994).

Lithic habitats can be divided into three distinct habitats corresponding loosely to the extremes of environmental conditions encountered by the microbial inhabitants. The *epilithic* habitat corresponds to the upper surface of the rock or stone, the part most directly exposed to atmospheric and climatic conditions and, therefore, more directly exposed to environmental extremes. The *endolithic* habitat corresponds to the interior of the rock or stone. Three distinct subdivisions of this habitat can be identified (Golubic et al., 1981): the *cryptoendolithic* habitat, consisting of naturally occurring pore spaces within the rock; the *chasmoendolithic* habitat, consisting of cracks and fissures in the rock leading to the surface; and the *euendolithic* habitat, consisting of holes and pits created by the metabolic activity of the microbial community (Fig. 4). The *hypolithic* habitat consists of the lower surface of a stone in association with adherent soil particles (The term "sublithic" is sometimes used, erroneously, as a synonym for hypolithic.).

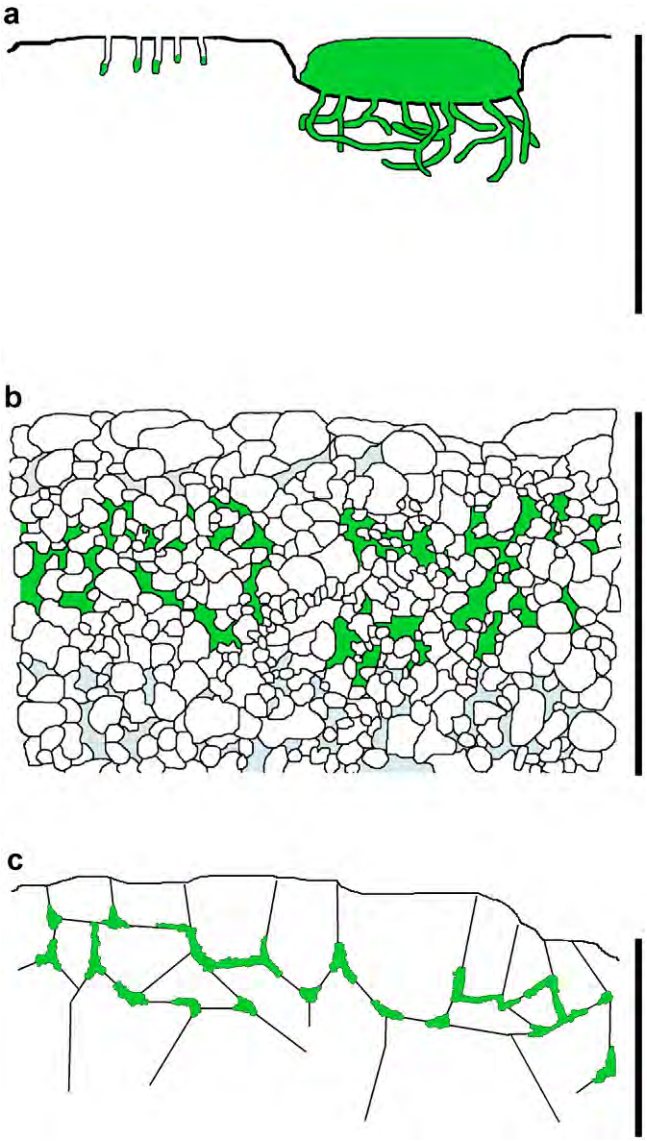

Fig. 4 Diagrammatic representations of endolithic habitats and associations. (a) Euendoliths, boring into the rock either individually (left) or as part of a lichen association (right). (b) A cryptoendolithic association. (c) A chasmoendolithic association inhabiting microfractures in a weathered substratum. The drawings are not to scale. However, scale bars have been included to provide an indication of the relative dimensions of the habitat – in figure (a) the bar represents 1 mm, in figures (b) and (c) it represents 1 cm.

Recently, the term perilithic has been introduced to distinguish the subsurface sides of large stones from the true hypolithic habitat below the stone (Warren-Rhodes et al., 2007a,b,c). The hypolithic and perilithic habitats are similar, but differ slightly in their moisture and light regimes.

Epilithic Associations

In relatively moist regions epilithic cyanobacteria and cyanophilous lichens form extensive growths, capable of completely covering the surface of large inselbergs, rock hills arising abruptly from the surrounding plains (Büdel, 1999). In hot arid regions, such growths are essentially absent (Büdel, 1991). Instead, the dominant epilithic microbial community is that associated with rock varnishes. Rock varnishes are thin, dark layered veneers of clay minerals held together by oxides and hydroxides of manganese and iron (Kuhlman et al., 2006). They are found in all weathering environments, but are most common in arid lands, where they are referred to as desert varnishes. There is a long-standing debate concerning the origins of rock varnishes with some advocating the predominance of physical–chemical mechanisms and others advocating biological mechanisms (see Nagy et al., 1991; Krinsley, 1998; Perry and Kolb, 2004; Perry et al., 2006 for brief reviews). It is also possible that the mechanism may change with changing climatic conditions (Liu and Dorn, 1996). Resolving this debate is beyond the scope of the present article. Our interest is in the

presence of a ubiquitous and relatively diverse microbial community in what is considered to be a dry environment (Kuhlman et al., 2006). Included are actinobacteria, *Bacillus*, *Micrococcus*, *Metallogenium*-like strains, cyanobacteria, and microcolonial fungi (see Krumbein and Jens, 1981; Kuhlman et al., 2006). Nearly all of the heterotrophic strains of bacteria isolated are Gram-positive and are capable of precipitating manganese. Populations of bacteria in rock varnishes from the eastern border of the Mojave Desert are on the order of 10^7–10^8 cells per gram of varnish, estimated on the basis of direct microscopic observation and phospholipid fatty acid analysis (Kuhlman et al., 2006), somewhat remarkable given their exposed position. Raman spectroscopy of varnishes from Colorado and Nevada indicates the presence of chlorophyll, scytonemin, and β-carotene (Edwards et al., 2004); the latter two are considered to be bioprotective molecules (see below).

Endolithic Associations

Euendolithic associations

Euendolithic microorganisms, which use chemical reactions to bore into the surfaces of rocks, are common in aquatic systems (see, e.g., Pentecost and Whitton, 2000; Priess et al., 2000; Radke and Golubic, 2005; McLoughlin et al., 2007), less so in terrestrial systems (Fig. 4). The decrease in abundance in terrestrial systems is caused, presumably, by the need for suitable substrates, generally carbonates although volcanic glasses are possible (McLoughlin et al., 2007), and for sufficient external moisture to support the chemical reaction. In fact, moisture is more of a problem in euendolithic systems than in other terrestrial endolithic systems because there is a direct connection between the colony and the external environment. One way around the moisture problem is the formation of a lichen association – the added mass and the structure of the lichen thallus could serve to maintain a moist environment long enough for both significant metabolic activity and the dissolution of the rock material to take place. In fact, euendolithic lichens are widespread, at least in Europe and the eastern Mediterranean, on calcareous rocks in microclimates where air humidity is high and light intensity is low (Tretiach and Pecchari, 1995; Tretiach and Geletti, 1997). Their activity may contribute significantly to the deterioration of marble buildings and monuments (Danin and Caneva, 1990).

Extensive growths of euendolithic lichens have even been reported from the moister regions of the Negev Desert (Danin et al., 1982; Danin and Garty, 1983; Danin, 1986; Garty, 1999). Here, the distribution is linked to the local microclimate through the imbibition time, an indicator of how many hours a year a lichen thallus is moist. If the conditions result in more than 450 h of daylight imbibition time epilithic lichens dominate, between 300 and 450 h of imbibition euendolithic lichens dominate, and at lower levels the free-living euendolithic fungus *Lichenothelia* dominate in loose association with cryptoendolithic and chasmoendolithic cyanobacteria and eukaryotic algae (Danin and Garty, 1983). Because the different associations form distinctive patterns on the surface of the rock, which can persist long after the association has disappeared, they can be used as indicators of the climate at the time the community was destroyed by fire or burial (Danin et al., 1982; Danin, 1986). The small pits and other scars in the rock surface may also facilitate the recolonization of the surface by desert microorganisms (Garty, 1992).

Free-living colonies of euendolithic coccoid cyanobacteria have also been reported from several locations in Israel. If true, these would represent the first such reports from a terrestrial setting (Danin and Garty, 1983; Danin et al., 1983; Danin, 1986). However, it is possible that the colonies were actually lichenized, but with a small proportion of fungi (Nienow et al., 2002). More recently a species of the filamentous heterocystous cyanobacterial genus *Matteia* has been isolated from Negev limestones. This species is capable of boring into limestone rocks both in culture and in the Negev, thus demonstrating unequivocally the existence of free-living terrestrial euendoliths (Friedmann et al., 1993a,b).

Outside of the Negev, there are almost no reports of euendolithic associations of any sort from arid regions. The euendolithic lichen *Verrucaria calciseda* is described as widely scattered in temperate and boreal regions rich in limestones, including northern Arizona (Brodo et al., 2001). The euendolithic lichen *Verrucaria rubrocincta* is present in caliche deposits in the Sonoran Desert (Bungartz et al., 2001). Filamentous and coccoid cyanobacteria coat the insides of small (0.1–0.5 mm) pits, presumably made by the action of the cyanobacteria, in the surface of limestone pinnacles in Western Australia (Pócs, 2009).

Chasmoendolithic associations

Like euendolithic systems, chasmolithic systems have a direct connection with the surface, allowing light, moisture, and nutrients to reach the association relatively unimpeded. As a result, a wide variety of rock types, including darker basalts and granites, can sustain chasmolithic growth at some depth. In spite of their widespread occurrence in a variety of climate zones, including desert regions, there have been relatively few detailed investigations of either the habitat or the organisms within (Friedmann and Galun, 1974; Garty, 1999; Nienow et al., 2002). The most extensive studies are from the relatively mild Vestfold Hills and Mawson Rock regions of Antarctica and from the hyper-arid portions of the Atacama Desert in Chile. In the Antarctic, the chasmoendolithic association contains a diverse group of microalgae, with members of cyanobacterial genera *Chroococcidiopsis* and *Plectonema* and the green algal genera *Desmococcus* and *Prasiococcus* the most widespread in the habitat. These can be grouped into associations dominated by *Desmococcus*, common in the drier regions, or by *Chroococcidiopsis*, common in the wetter regions of the Mawson Rock site (Broady, 1981a). The domination of *Chroococcidiopsis* in the wetter and not the drier sites was unexpected given the frequency of reports of *Chroococcidiopsis* from arid deserts. A few of the associations displayed a distinct zonation pattern, with either *Prasiococcus* or *Desmococcus* forming an outer green band and *Chroococcidiopsis* forming an inner bluish-green band. At the Vestfold Hills site, extensive development of chasmolithic associations is restricted to the slopes of hills and boulders sheltered from the wind (Broady, 1981a).

Chasmolithic lichen associations and cyanobacterial biofilms from the Granite Harbor region of Antarctica have been shown to develop acidic microenvironments within the association that may be involved in weathering of the substratum (de los Rios et al., 2003) – chasmoendolithic associations are often implicated in the weathering of marble and limestone monuments in the semiarid Mediterranean region (Garty, 1999). Molecular analysis indicated the presence of what may be a relative of the chlorophyll d-containing cyanobacterium *Acaryochloris marina* in the association; this is the first record of this group of cyanobacteria in a terrestrial endolithic environment (de los Rios et al., 2007).

As might be expected, the chasmoendolithic association in the drier Atacama Desert is less diverse, with no eukaryotic phototrophs detected either visually (microscopically) or through molecular methods (DiRuggiero et al., 2013). Instead, the system is dominated by members of the genus *Chroococcidiopsis*, with the presence of small numbers of filamentous cyanobacteria and heterotrophic bacteria indicated by molecular techniques (DiRuggiero et al., 2013).

Cryptoendolithic associations

Cryptoendolithic organisms inhabit the interstitial spaces of porous rocks. Because the organisms lie below the surface of the rock, without a direct light path to the surface, only rocks composed of light-colored or translucent grains are colonized by cryptoendolithic phototrophs. Cryptoendolithic associations are easily recognized macroscopically as a pigmented layer running more or less parallel to the surface of the rock at a depth of one to a few millimeters. They can develop in any environment, wherever suitable rocks are exposed to sufficient light (van Thielen and Garbary, 1999; Nienow et al., 2002). Examples have been reported from weathered granites, gneisses, limestones, and marbles, including some used as building materials (see Nienow and Friedmann, 1993; van Thielen and Garbary, 1999; Nienow et al., 2002; Wong et al., 2010 and the references therein), beachrock (Krumbein, 1979; Schreiber et al., 2002), gypsum crusts (Hughes and Lawley, 2003; Boison et al., 2004; Dong et al., 2007; Wierzchos et al., 2010), halites and evaporites (Rothschild et al., 1994; Wierzchos et al., 2006), sinters associated with geothermal environments (Walker et al., 2005; Phoenix et al., 2006), dark volcanic basalts (Jorge Villar et al., 2006), and gneisses altered by asteroid or comet impacts (Cockell et al., 2002). However, the most commonly colonized substrata appear to be porous quartz sandstones (Friedmann and Ocampo-Friedmann, 1984; Bell, 1993; Nienow et al., 2002; Omelon et al., 2006; Pócs, 2009; Fig. 5).

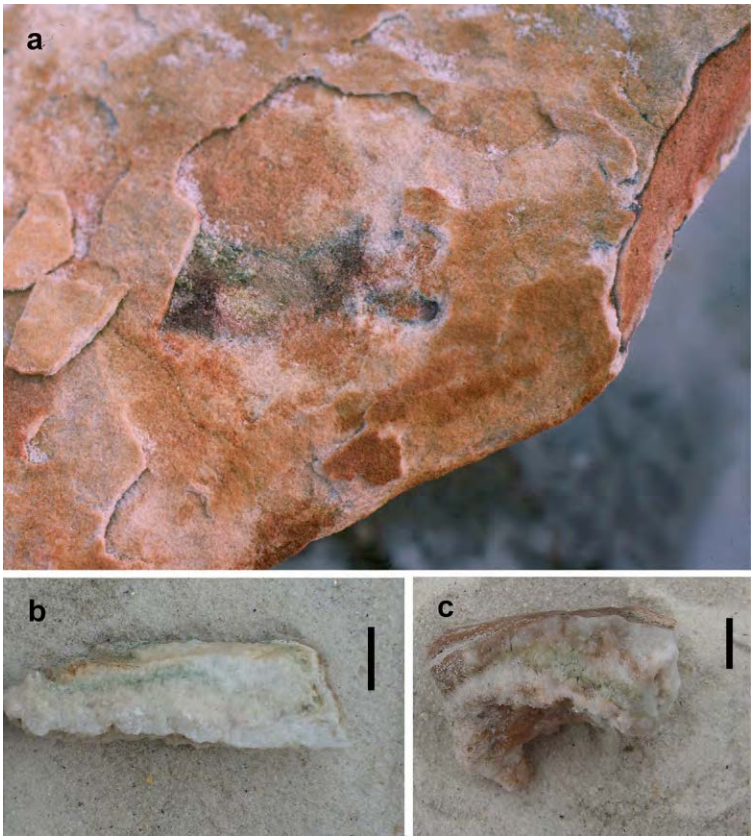

Fig. 5 Examples of endolithic associations. (a) Cryptoendoliths inhabiting a sandstone in the dry valley region of Antarctica. In this instance the association causes a characteristic weathering pattern visible on the surface of the rock that leads to flaking of the crust surface (seen in the center of the image). The association is visible to the right of the image, where the rock has been broken off. (b) An endolithic association from the Mojave Desert. (c) An endolithic association inhabiting halite in the Atacama Desert. The scale bars in figures b and c represent 1 cm.

The most extensively studied cryptoendolithic associations from desert regions are those found in porous sandstones in the McMurdo Dry Valleys regions of Antarctica (Nienow and Friedmann, 1993; Nienow et al., 2002; Sun et al., 2010). One of the most striking features of these associations is the complexity of the system. At least five distinct associations, three of which are widespread, have been described. The most abundant is generally referred to as the lichen-dominated community or the cryptoendolithic lichen community. It can be recognized macroscopically by the presence of parallel black, white, and green bands a few millimeters below the rock surface. The black layer consists of dark-pigmented filamentous fungi and lichenized cells of the green algal genus *Trebouxia* (or, possibly, *Pseudotrebouxia*). The white layer is populated by green algae and hyaline filamentous fungi, possibly a morphological variant of some of the black fungi from the upper layer; it appears white because the iron oxides that normally give the rocks their characteristic brown to orange-red color have been leached from this layer (Nienow and Friedmann, 1993; Nienow et al., 2002). Included among the dark-pigmented fungi are a number of nonlichenized meristematic black fungi, typically members of *Friedmanniomyces* and *Cryomyces* in the Dothideomycetidae, a taxonomic order known to have many stress-tolerant members (Onofri et al., 1999; Selbmann et al., 2005). A number of culture-based and molecular studies indicate the presence of a small but diverse heterotrophic bacterial community dominated by actinobacteria, but including members of *Deinococcus* (Nienow et al., 2002; de la Torre et al., 2003).

The *Hemichloris* community can be recognized as a green band several millimeters below the surface of the rock. Typically, it develops below the white layer of the lichen-dominated community, but can also be found as the sole community inhabiting the lower surface of overhanging sandstone ledges (Nienow et al., 1988, 2002). The community is named after its dominant member, the green alga *Hemichloris antarctica* (Tschermak-Woess and Friedmann, 1984). Included in the community is a diverse assemblage of cyanobacteria and eukaryotic algae, including a second species of *Hemichloris* (Tschermak-Woess et al., 2006), the endemic species *Heterococcus endolithicus* (Darling et al., 1987), and members of the green algal genus *Stichococcus* and the cyanobacterial genera *Gloeocapsa* and *Chroococcidiopsis*. Members of *Stichococcus*, *Gloeocapsa*, and *Chroococcidiopsis* are all common constituents of terrestrial systems; *Stichococcus bacillaris* is generally considered to be a cosmopolitan inhabitant of subaerial environments, including lithic environments in Antarctica (Nienow and Friedmann, 1993; Broady, 1996; Hughes, 2006). The species of *Chroococcidiopsis* in this community is similar to that described in other endolithic and hypolithic communities.

The *Hormathonema–Gloeocapsa* community is a complex cyanobacteria-dominated community only found on Battleship Promontory, where it colonizes white sandstone boulders permanently wetted by snowmelt percolating through the dolerite rubble at the base of the rocks; the drier upper surfaces of large outcrops in the region are colonized by the lichen-dominated community. It can be recognized macroscopically by the presence of two parallel color bands below the rock surface, without a leached zone in between. The upper layer is a complex assemblage of cyanobacteria dominated by members of the genera *Hormathonema* and/or a dark gray species of *Gloeocapsa*. The lower green band is dominated by a species of *Aphanocapsa*, occasionally accompanied by several species of *Gloeocapsa*, *Anabaena*, and *Lyngbya* (Friedmann et al., 1988). The heterotrophic component of the community includes flagellated protozoans, filamentous fungi, and yeasts and up to 39 morphotypes of heterotrophic bacteria (Hirsch et al., 1988). Molecular analysis indicates the presence, among others, of actinobacteria and members of the *Thermus–Deinococcus* group (de la Torre et al., 2003).

The remaining associations are referred to as the *Chroococcidiopsis* community and the red *Gloeocapsa* community (Friedmann et al., 1988). Both are dominated by unicellular cyanobacteria of similar morphology. The *Chroococcidiopsis* community was the first endolithic community discovered in Antarctica (Friedmann and Ocampo, 1976 (*Chroococcidiopsis* is referred to as *Gloeocapsa* in this paper)). It appears as a single green or brownish band just below the surface crust, and *Chroococcidiopsis* sp. is the sole phototrophic organism. When discovered, it seemed to confirm a pattern just then beginning to be recognized: in the most extreme deserts where environmental conditions preclude eukaryotes and filamentous cyanobacteria, the unicellular *Chroococcidiopsis* becomes the dominant or sole photoautotroph (Friedmann and Galun, 1974 (again *Chroococcidiopsis* is referred to as a probable member of the genus *Gloeocapsa*), Friedmann and Ocampo-Friedmann, 1995). The relatively limited distribution of the *Chroococcidiopsis*-dominated community in the Antarctic desert suggests that this polar desert may not be the most extreme on Earth, although it remains the closest terrestrial analogue to Mars.

It has been estimated that cryptoendolithic associations colonize 20–30% of the exposed surfaces of Beacon sandstones in the McMurdo Dry Valleys region, equivalent to a total colonized area of between 200 and 300 km^2 (Friedmann et al., 1993a,b). Given the abundance of material in an association, 1.4–7.4 g N (Kjeldahl)m^{-2}, or 35–190 g C m^{-2} (Friedmann et al., 1980; Sun and Friedmann, 1999), this represents a significant pool of organic matter in a desert region and suggests that endolithic organisms play an important role in the ecosystem. How significant is a matter of debate. Short-term laboratory measurements of CO_2 gas exchange under different temperature conditions coupled with multiyear records of environmental conditions with colonized surfaces suggest net and gross primary productivity rates on the order of 600 and 1200 mg C m^{-2} $year^{-1}$, respectively (Friedmann et al., 1993a,b). At this rate of incorporation, a fully developed community could replace itself within 300 years. Multiple lines of evidence suggest that the endolithic communities grow much more slowly than this. Radiocarbon dating of endolithic communities (McKay et al., 1986; Bonani et al., 1988), carbon turnover times (Johnston and Vestal, 1991), comparisons of the rates of biogenic and abiogenic weathering (Friedmann and Weed, 1987; Sun and Friedmann, 1999), and estimates of the number of metabolically active hours per year (Friedmann et al., 1987; McKay et al., 1993) and the number of divisions per year in the lower levels of the community (Nienow et al., 1988) all indicate that the age of a well-developed community is on the order of 1000–10,000 years. Some of the material, especially low-molecular-weight compounds lost during rewetting, may be leached from the community, ending up, eventually, in the surrounding soils (Friedmann et al., 1993a,b). It is not clear how significant this leaching is. Dr. H. Sun (Desert Research Institute, Las Vegas, personal communication) found that most of the free amino acids were concentrated in the

upper few centimeters of the lichen-dominated community. Chemical signatures indicative of the cryptoendolithic community are present in soils from the higher elevations in Taylor Valley, but not in soils from the lower elevations (Burkins et al., 2000).

Cryptoendolithic associations are widespread outside of Antarctica, although not nearly as intensely investigated (Nienow et al., 2002). In most cases, they occur in some sort of sandstone substratum. Here, they take on the same macroscopic appearance. There is a colored band beginning just below the surface of the rock, extending inward just a few millimeters at most (Friedmann and Galun, 1974; Bell, 1993). The composition of the community depends, at least to a degree, on the aridity of the environment. For example, in the vicinity of the Colorado Plateau in North America, the relative abundance of eukaryotic green algae increases in comparison with cyanobacteria in the milder upper elevations – at the milder sites, members of the green algal orders Chlorococcales and Chlorosarcinales comprise 50–75% of the phototrophic associations, while at the lower, more xeric sites, nearly 100% of the phototrophs are cyanobacteria (Bell et al., 1988). Generally speaking, however, most cryptoendolithic associations in hot deserts are dominated by members of the cyanobacterial genus *Chroococcidiopsis* (Friedmann and Galun, 1974; Bell, 1993; Nienow et al., 2002); in many cases *Chroococcidiopsis* appears to be the sole phototrophic taxon present (Friedmann and Galun, 1974; Bell, 1993; Nienow et al., 2002). The reduced importance of eukaryotic algae in hot desert endolithic associations is usually ascribed to temperature, rather than moisture concerns, although this has not been tested in detail; in fact, there are few data concerning the temperature ranges of hot desert forms.

Endolithic associations have also been reported from gypsum rocks in Antarctica, and in the hot Atacama, Jordanian, and Mojave deserts (Hughes and Lawley, 2003; Dong et al., 2007; Wierzchos et al., 2010). Preliminary investigations of the Antarctic community indicated the presence of a member of the cyanobacterial genus *Chloroglea* as the principal phototrophic member, with the fungal genus *Verticillium* and the bacterial genus *Sphingomonas* also represented (Hughes and Lawley, 2003). Overall, the biodiversity appears to be low (Hughes and Lawley, 2003). The hot desert gypsums contained a much more diverse assemblage (Dong et al., 2007; Wierzchos et al., 2010). The dominant phototrophs in most cases were morphologically similar to *Chroococcidiopsis*, with a small number of filamentous cyanobacteria in samples from the Mojave Desert (Dong et al., 2007). Somewhat surprisingly, Wierchos and his colleagues found calcium sulfate crusts from the drier regions of the Atacama Desert were dominated by lichenized eukaryotic algae (Wierzchos et al., 2010); they attribute the dominance of eukaryotes to the lack of liquid water and the reliance of the association on water vapor.

A unique endolithic community exists in halite evaporites of nonmarine origin in the hyperarid core of the Atacama Desert (Wierzchos et al., 2006). The evaporites form extensive nodular crusts at several sites in the Atacama, including Yungay, Salar de Llamara, and Salar Grande (Pueyo et al., 2001; Wierzchos et al., 2006). The community forms a macroscopically visible grayish layer beginning a few millimeters below the surface and extending another 2–5 mm into the rock (Wierzchos et al., 2006). The dominant phototrophic organism in the community is morphologically similar to *Chroococcidiopsis* with a brown sheath (Wierzchos et al., 2006); phylogenetic analysis, however, indicates that the dominant phototroph in the system is more closely related to *Halothece*, a cyanobacterium known from hypersaline environments (de los Ríos et al., 2010; Robinson et al., 2015). Heterotrophic bacteria and archaea are also present (Wierzchos et al., 2006; de los Ríos et al., 2010; Robinson et al., 2015).

Hypolithic Associations

Scientific study of hypolithic associations began with the pioneering work of Vogel (1955) in South Africa and Cameron and Blank (1966) in the American Southwest. Since then there have been additional reports from the Namib Desert in southern Africa (Rumrich et al., 1989; Büdel and Wessels, 1991; Warren-Rhodes et al., 2013); the Negev Desert in the Middle East (Friedmann et al., 1967; Friedmann and Galun, 1974; Berner and Evenari, 1978); the southwestern United States (Friedmann and Galun, 1974; Büdel and Wessels, 1991; Schlesinger et al., 2003); Australia (Tchan and Beadle, 1955; Tracy et al., 2010); the Atacama Desert in South America (Warren-Rhodes et al., 2006, 2007a,b,c); the arid northwestern region of China (Pointing et al., 2007; Warren-Rhodes et al., 2007a,b,c); the Dry Valleys region of Antarctica (Broady, 1981b; Nienow and Friedmann, 1993; Broady, 1996; Smith et al., 2000; Cockell and Stokes, 2006; Cowan et al., 2011; Khan et al., 2011); and the high Arctic (Cockell and Stokes, 2004, 2006). In most cases, colonized stones are composed of quartz, flint, or some other translucent material. These allow small but measurable amounts of photosynthetically active radiation to reach the phototrophic portion of the association, estimated to be on the order of 0.01–0.1% of the incident light at the lowest colonized levels (Vogel, 1955; Berner and Evenari, 1978; Schlesinger et al., 2003; see also Tracy et al., 2010; Cowan et al., 2011; Warren-Rhodes et al., 2013); similar numbers have been obtained for cryptoendolithic associations (Nienow et al., 1988; Matthes et al., 2001; Cockell et al., 2002). In one case, colonized dolomites from the Arctic, no light transmission was detected through 1-mm-thick sections of the substratum, suggesting that light reaches the community through the surrounding coarse-grained soil (Cockell and Stokes, 2006).

Phototrophic hypolithic associations form macroscopically visible green to bluish-green films on the lower surfaces of colonized stones (Fig. 6). In most of the instances, the reported community contains a remarkably diverse assemblage of species including a mixture of unicellular and filamentous cyanobacteria, green algae, and, in some cases, diatoms. For example, more than 23 species of hypolithic phototrophs have been recorded from Antarctica on the basis of light microscopy, including representatives of eight genera of cyanobacteria, three genera of green algae, two genera of yellow-green algae, and two species of diatoms (Broady, 1981b, 1996). In the Namib Desert, representatives of over 50 species of just diatoms have been reported from hypolithic associations (Rumrich et al., 1989). More recent investigations using molecular techniques have confirmed this diversity in Antarctic associations (Smith et al., 2000; Pointing et al., 2009; Khan et al., 2011). The hypolithic association in a semi-arid region of northern Australia

Fig. 6 Examples of hypolithic associations from the Mojave Desert. (a)–(c) Views of a single stone. (a) The upper surface. (b) The lower surface. (c) View from one of the sides. Note the limited distribution of the photosynthetic association (greenish band). (d)–(e) Two views of a smaller stone from the same region. (d) The upper surface. (e) The lower surface. In this case the photosynthetic association nearly covers the lower surface – the rock was less than 1 cm thick, allowing light to reach the entire lower surface. (f) and (g) Two views of a third stone. (f) The upper surface. (g) The lower surface, again with an almost complete cover by the hypolithic association. This example is somewhat unusual because of the dark color of the stone. Upon closer examination the coloration was found to be superficial, the interior of the stone is white. Scale bars in all images represent 1 cm.

was also diverse, including representatives of several genera of filamentous cyanobacteria in addition to *Chroococcidiopsis* (Tracy et al., 2010).

In more arid regions, however, the diversity decreases sharply. In both the Atacama (Warren-Rhodes et al., 2006), and the arid regions of China (Pointing et al., 2007), only cyanobacteria with a *Chroococcidiopsis*-like morphology have been observed, although molecular techniques indicate the possible presence of filamentous *Phormidium*-like cyanobacteria at colder or wetter sites. The percent abundance of hypolithic communities on suitable rocks is also moisture-dependent. In the relatively mild Mojave Desert, nearly 100% of quartz pebbles are colonized by hypoliths (Schlesinger et al., 2003). In the nearby hyperarid desert of Baja California, in contrast, only between 26 and 38% of quartz stones were colonized (Heckman et al., 2006). This shift is in keeping with extensive studies along a moisture gradient in the Atacama Desert (Warren-Rhodes et al., 2006). Here, 27.6% of quartz pebbles were colonized at the "moist" Copiapo end of the transect, while at the dry end of the transect, near Yungay, only 3 out of 3723 quartz pebbles were colonized. Thus, the hyperarid core of the Atacama appears to be near the dry limit of metabolic activity for both soil and lithic microorganisms. This makes the presence of what appears to be an active and extensive cryptoendolithic association in evaporites near Yungay even more intriguing (Wierzchos et al., 2006).

Recently, the source of inocula for hypolithic associations has become a topic of special interest in the context of the biogeography of desert microorganisms. According to one school of thought, microorganisms do not exhibit a true biogeography since, because of their small size and assumed ease of dispersal, they do not face geographic barriers of the same magnitude as those encountered by macroscopic organisms. As succinctly stated by Baas Becking (1934), "alles is overall: *maar* het milieu selecteert" ("everything is everywhere, *but* the environment selects"). If this hypothesis is true, we should expect to see (1) in regions where not all suitable stones are colonized, the distribution of colonized stones should reflect local differences in the environment and not sources of inocula; (2) the hypolithic to contain a subset of the types of microorganisms present in the surrounding soils, the subset selected by the unique environment found on the undersurface of the stone; and (3) a degree of global consistency in the structure of association in similar types of deserts.

The first of these was tested in the Atacama Desert in Chile (Warren-Rhodes et al., 2007a) and in the Taklimakan and Qaidam Basin deserts in western China (Warren-Rhodes et al., 2007b,c). In both cases, clustering of colonized stones was observed, not the random pattern expected from Bass Becking's hypothesis. In the Atacama Desert, clustering correlated well with rock size and

microtopography, both related to small-scale differences in moisture availability. Large rocks, as discussed previously, capture and retain more moisture, and are, therefore, more suitable for colonization. Slight depressions and elevations can also increase moisture availability; at one site 90% of colonized stones were located on a microtopographic high, where moisture from fog was presumed to be higher (Warren-Rhodes et al., 2007a). In this case the observed clustering actually mapped the location of favorable microclimates, where the habitat could, indeed, select. In western China, microtopographic data was not available, so small scale changes in microclimate could not be correlated with clustering. Instead, small scale dispersal from an initial colonized stone to surrounding stones was invoked to explain the existence of clusters of colonized stones (Warren-Rhodes et al., 2007b,c).

Surprisingly few studies have compared the hypolithic association with the microbiota in the surrounding soils. The diversity of cyanobacteria associated with hypolithic environments in semi-arid northern Australia does suggests some may be part of the larger biological soil crust association in the same area (Tracy et al., 2010). When direct comparisons have been made, there are consistent differences between the two associations. For example, in Namibia hypolithic associations were dominated by cyanobacteria (*Chroococcidiosis*) while soil systems were dominated by actinobacteria (Makhalanyane et al., 2013a,b). Similar results were found in Antarctica in Beacon Valley (Wood et al., 2008), McKelvey Valley (Pointing et al., 2009), and Meirs Valley (Khan et al., 2011; Makhalanyane et al., 2013a,b; Wood et al., 2008). These findings support the idea that the hypolithic environment provides a selective filter of the microbial community. Somewhat surprising was the fact that cyanobacteria were not detected in the soils of the drier Beacon and McKelvey valleys (Pointing et al., 2009; Wood et al., 2008); presumably, the organisms were present, but at such low numbers that they were not detected by the techniques used. Some of the heterotrophic bacteria from lithic habitats were also not detected in soils. In Meirs Valley, there is some evidence for successional stages in the hypolithic association (Makhalanyane et al., 2013a,b).

The presence of hypolithic associations dominated by members of the genus *Chroococcidiopsis* in hot and cold deserts from around the world permitted Bahl and his colleagues to analyze distribution global distribution patterns and relationships (Bahl et al., 2011). Their findings indicate the presence of three distinct clades within the genus, one adapted to cold deserts, the other two adapted to hot deserts, and that the three groups have been evolutionarily separated for a long period of time. Within each clade there is some evidence for a degree of regional grouping of phylotypes, especially within the hot desert clades. This would suggest that dispersal between regions may be limited, at least over short periods of time. However, genetic differences between variants of *Chroococcidiopsis* were not significantly related to geographic distance on a global scale (Bahl et al., 2011), in keeping with the basic ideas of Baas Becking.

Adaptations to the Environment

All desert microorganisms are subjected to extended periods in a desiccated, metabolically inactive state. However, in this inactive state, individual cells are subject to a variety of chemical and physical insults, resulting in damage that cannot be repaired until metabolism restarts. If the damage incurred while in the inactive state is too great, the cell is unable to make repairs fast enough, and dies (Walters et al., 2005). At the same time, the association as a whole is subject to losses by erosion and predation that cannot be replaced until cellular reproduction restarts. Therefore, any feature tending to prolong the periods of activity or to help the cell survive the periods of inactivity in a desiccated state would be adaptive. A number of such features have been identified (Brown, 1990; Potts, 1994; Bewley, 1995; Wynn-Williams, 2000; Hohmann, 2002; Grant, 2004; Cox and Battista, 2005). The principal adaptations are summarized below, following a brief review of the nature of the problem.

The Concepts of Water Activity and Water Potential

The net movement of water across a cell boundary is governed by the related concepts of water activity and water potential. Water activity (a_w) is a measure of the concentration of water in a solution, as modified by the type of solutes present in the solution. It is derived from a modification of Raoult's law relating the vapor pressure of a solution (p) to the vapor pressure of the pure solvent (p_0). In the ideal case, both a_w and p/p_0 would equal the mole fraction of water (N_w) defined as $n_w/(n_w+n_s)$, where n_w is the number of moles of water and n_s is the number of moles of all solutes in the system (the treatment here follows that of Brown (1990) and Potts (1994)). In reality, unless the solutions are very dilute, the solutes interact with water, necessitating the inclusion of an empirical coefficient (γ_s). The water activity is then defined as

$$a_w = p/p_0 = \gamma_s n_w/(n_w + n_s) = \gamma_s N_w$$

The chemical potential of the water (μ_w) is given by

$$\mu_w = \mu_w^\circ + RT \cdot \ln(a_w) + V_w P + z_w FE + m_w gh$$

where μ_w° is the chemical energy of water under standard conditions (J mol^{-1}), R is the universal gas constant (~8.31447 J mol^{-1} K^{-1}), T is the temperature in Kelvin, V_w is the change in volume associated with a change in the amount of water (~1.8×10^{-5} m^3 mol^{-1}), P is the hydrostatic pressure (N m^{-2}), z_w is the charge on a molecule of water, F is the Faraday constant (~9.6485×10^4 C mol^{-1}), E is electrical potential (J C^{-1}), m_w is the molar mass of water (~1.8×10^{-2} kg mol^{-1}), g is the acceleration due to gravity

($\sim$9.8 m s^{-2}), and h is the height of the cell (m). The electrical term can be ignored because the charge on a molecule of water (z_w) is negligible. The gravitational term can also be ignored because of the small size of the cells. The chemical potential then reduces to

$$\mu_w = \mu_w^\circ + RT \cdot \ln(a_w) + V_w P$$

which can be rewritten as

$$(\mu_w - \mu_w^\circ)/V_w = (RT \cdot \ln(a_w))/V_w + P$$

This equation leads to the definition of the water potential (ψ) of the system as

$$\psi = \frac{(\mu_w - \mu_w{}^\circ)}{V_w}$$

with units in terms of pressure (N m^{-2} or pascals). In this derivation, the water potential is the sum of two separate potentials, one based on the hydrostatic pressure (P), the other based on the chemical activity of water ($RT \cdot \ln(a_w))/V_w$. Conceptually, the water potential can be broken into additional terms, as appropriate. A typical formulation gives the total water potential (ψ) as

$$\Psi = \psi_\pi + \psi_p + \psi_m + \psi_g$$

where ψ_π is the osmotic potential and refers to part of the water potential associated with dissolved substances in the system, ψ_p is the pressure potential and refers to part of the water potential associated with hydrostatic pressure, ψ_m is the matric potential and refers to part of the water potential associated with the interaction of water molecules with surfaces and interfaces, and ψ_g is the gravitational potential and refers to part of the water potential associated with gravitational forces.

A simple example may be instructive. Suppose a cell is suspended in seawater so that the external matric and gravitational potentials are negligible, and that the external hydrostatic pressure (ψ_p) is 1 atm (1.01325 bars or 1.01325×10^5 Pa) directed inward. Because the activity of seawater at 25°C is 0.9806, the external osmotic potential (ψ_π) is -2.70×10^6 Pa (-2.70 MPa); the negative sign indicates that the "pressure" is directed outward. The total water potential experienced by the cell ($\Psi_{environment}$) is given by $\psi_\pi + \psi_p = -2.6$ MPa. At equilibrium, the total internal water potential of the cell (Ψ_{cell}), which equals the sum of the internal osmotic, hydrostatic, and matric potentials, would be the same, although none of the individual internal potentials would necessarily match any of the individual external potentials. If that same cell were now plunged into pure water ($a_w = 1.0$), $\Psi_{environment}$ would increase to $+0.1$ MPa, while Ψ_{cell} would start at -2.6 MPa. Such an energy differential cannot be maintained. Water would begin to flow down the energy gradient into the cell. The additional water would raise the mole fraction and water activity within the cell, thereby bringing ψ_π closer to 0; it is also possible that some of the solutes may be lost. At the same time, the increased volume of water would correspond to an increased hydrostatic pressure directed outward. If the cell membrane and/or cell wall can withstand the increased hydrostatic pressure without rupturing, the cell will eventually come to a new equilibrium, with Ψ_{cell} again equal to $\Psi_{environment}$. Of course, if the cell were moved to more concentrated seawater, with a salinity of 70‰ instead of 35‰ and a water activity of $\sim$0.96, $\Psi_{environment}$ would decrease to -5.82 MPa, and water would flow out of the cell until the new equilibrium is reached.

Seawater would represent an environment with relatively constant, even if reduced, water activity. Of course, seawater, even double-strength seawater, does not constitute a dry environment and a wide range of microorganisms can survive there without major modification. More relevant environments would be found in hypersaline lakes and salinas, seawater-based evaporites (a_w $\sim$0.75) or in cereals, candy and other confectionaries, and dried fruits (a_w $\sim$0.70). Organisms living in these habitats would be subjected to chronic conditions of low water potential and may even be considered to be under chronic water stress. The constancy of the stress allows for some degree of predictability and biological accommodation. Even so, there are only a limited number of microorganisms capable of growing at water activities below 0.85; only two, the yeast *Zygosaccharomyces rouxii* and the filamentous ascomycete *Xeromyces bisporus*, are capable of growth at water activities below 0.70 (Grant, 2004). Terrestrial microorganisms, especially subaerial and desert forms, face a much more difficult moisture environment. The water activity of the external environment, roughly equivalent to the relative humidity of the air divided by 100 (see Dose, 2002), is not constant, but can vary between lows near 0.10 and highs near 1.00, all within a single day. Upshifts in the external water activity due to changes in the relatively humidity are ameliorated somewhat by the slow pace of the diffusion of the water molecules in the air. When rains appear, however, the cells are suddenly plunged into a liquid medium with a much higher water potential. This sudden shift creates an acute stress not experienced by the food- or evaporite-inhabiting microorganisms.

Impacts of Low Water Activity on Unicellular Organisms

The effects of low water activity on cellular and macromolecular structure have been reviewed extensively by Potts (1994), Alpert (2005), Crowe and Crowe (2002) and Dose (2002) provide additional insights in their more recent reviews. Here we provide a brief summary of their major findings.

The irrefutable feature of exposure to low water activities and low water potentials is the removal of water from the cell. This process was first closely observed by Koga et al. (1966) in the desiccation tolerant yeast *Saccharomyces cerevisiae*. They identified four distinct states for intracellular water based on the total water content. In the initial state, the "solution domain," water is free-flowing

and metabolic reactions are still possible, albeit at possibly reduced rates. Once acertain water content is reached, ~20% in *S. cerevisiae*, the cell enters what is called a "gel domain," where water molecules no longer form a continuous solvent and metabolic activity ceases. Further reductions in water content lead to two additional states distinguished by how tightly the remaining water is bound.

These decreases in water content are accompanied by changes in the structure of the phospholipid bilayer (Crowe and Crowe, 1992, 2002; Dose, 2002). As the number of water molecules decreases, the hydration state of the individual molecules forming the bilayer changes, leading to an increase in the temperature at which the membrane changes from the biologically important liquid crystalline state to the gel state (Crowe and Crowe, 2002). Because of these changes, in the dehydrated state the membrane components are in the gel state at physiological temperatures (Crowe and Crowe, 2002). As water is added back into the system, and the gel–liquid crystalline transition temperature drops back to physiological levels, there is a transient state when the membrane is part liquid crystalline and part gel. Such two-part systems are highly permeable to small molecules (Potts, 1994; Crowe and Crowe, 2002). For non-desiccation-resistant microorganisms, these changes in the cell membrane may be the primary cause for cell death during freeze-drying (Crowe and Crowe, 2002).

Presumably, changes in the hydration states of individual proteins, similar to those experienced by molecules of the bilayer, will occur as the amount of water decreases. Such changes could impact the three-dimensional structure of the proteins, especially where hydrophobic interactions are involved, causing potentially irreversible denaturation (Dose, 2002). How much damage is caused by this mechanism is not clear (Potts, 1994). For the desiccation-tolerant cyanobacteria *N. commune*, proteins involved in lipid biosynthesis and in glycerol, sulfate, and phosphate uptake appear to be undamaged, while phycobiliproteins involved in light harvesting are rapidly degraded (Potts, 1985, 1994).

In contrast with the case for proteins, there is clear evidence that DNA is a major target of desiccation-induced damage (Potts, 1994). Conformational changes accompanying the loss of hydration shells allow the formation of crosslinks between the phosphodiester group of DNA and a protein-bound OH group (Dose, 2002). These crosslinks result in single-strand and double-strand breaks in the DNA, as many as ten breaks per single-strand genome after 12 min of drying in the desiccation-sensitive strain *Escherichia coli* K-12 AB 1157 (Dose et al., 1992; Dose, 2002). Even desiccation-resistant *Bacillus subtilis* spores and *Aspergillus ochraceus* conidia display increasing amounts of DNA–protein crosslinking during exposure to desiccation (Bieger-Dose et al., 1992). Depurination of individual nucleotides may also be a significant source of DNA lesions (Dose, 2002), with the rate for desiccated samples of *N. commune* estimated to be sufficient to depurinate 1% of the genome in 10 years (Potts, 1994).

The presence of reducing sugars and O_2 can increase the amount of damage sustained by all components. Maillard reactions, a series of browning reactions between reducing sugars and amino acids, may occur, thereby damaging proteins, although the rate would be expected to be low in the desiccated state (Dose, 2002). There is evidence that reducing sugars can also interact with DNA and membranes (Dose, 2002). These reactions would also occur in hydrated cells, but in the hydrated case repair mechanisms not available in the desiccated state would be in operation. It is also expected that active species of oxygen (O_2^-, H_2O_2, and OH^-) will form in drying cells under the influence of light (Potts, 1994). The presence of appreciable amounts of these species would lead to increased rates of single-stranded breaks in DNA, oxidation of proteins, and peroxidation of lipids, all of which cannot be repaired in the inactive state (Potts, 1994).

Adaptations to Arid Environments

As mentioned previously, a variety of adaptations to arid environments can be identified. In the following discussion, these are grouped according to which feature of the environment the adaptations appears to address. Note, however, that this is only a loose grouping and that many of these adaptations serve multiple purposes. For example, the presence of a sheath can serve to retard water loss, maintain cell membrane integrity, and protect against photodamage (Potts, 1994; Table 2).

Adaptations to Prolong the Period of Activity

Higher plants and terrestrial animals remain active in desiccating environments through the formation of complex multicellular structures with essentially watertight surfaces. This option is not open to unicellular forms. However, they can use extracellular structures to accomplish the same end, albeit not as efficiently. For example, nearly all terrestrial cyanobacteria, many eukaryotic algae, and the cryptoendolithic black fungi from Antarctica form extensive mucilaginous sheaths, as much as 98% of which may be water (Friedmann and Galun, 1974; Grilli Caiola et al., 1996; Potts, 1994; Wynn-Williams, 2000; Selbmann et al., 2005). This water reserve can prolong the period of activity under desiccating conditions from several hours to a few days (Shephard, 1987; Nienow and McKay, 2000). The sheaths may also control the rate at which water enters the cell after dehydration, preventing the resumption of metabolic activity until there is sufficient moisture in the environment to sustain activity for a prolonged period of time (Williams et al., 2014). In soil crusts and mats, the polysaccharide sheaths may form a physical barrier, which serves to retard evaporation and retain moisture in the lower levels (Davey and Clarke, 1992; Belnap, 2006). Soils and the porous rocks and stones of the cryptoendolithic habitat may also serve as water reservoirs for the community (Friedmann et al., 1987, 1993a,b; McKay et al., 2003; Omelon et al., 2006; Warren-Rhodes et al., 2007a,b,c), and help to capture and retain water at low water activities either through their physical or through their chemical structure (Palmer and Friedmann, 1990; Rothschild et al., 1994; Wierzchos et al.,

Table 2 A partial list of the major innate adaptations to the dry environment. Examples are for illustrative purposes only; the specific adaptations of most microorganisms found in dry environments have not been investigated

Adaptation	*Function(s)*	*Examples*
Mucilaginous sheaths (extracellular polysaccharides)	Water storage; cell membrane stabilization	Nearly all terrestrial cyanobacteria, including *Chroococcidiopsis*, *Gloeocapsa*, *Microcoleus*, *Scytonema*, and *Nostoc*; many chlorophytes including *Hemichloris*; black fungi *Friedmanniomyces*, *Cryomyces*
Production of compatible solutes	Cell membrane stabilization; stabilization of the tertiary structure of proteins and DNA	*Chroococcidiopsis*, *Nostoc*; the fungi *Zygosaccharomyces*, *Xeromyces*
Photoprotective pigments in sheaths and walls	Absorption of UV and high-energy visible light	Many cyanobacteria including *Chroococcidiopsis*, *Gloeocapsa* and *Scytonema* (scytonemin and MAA's); black fungi (melanin)
Carotenoid production	Quenching of free radicals	*Deinococcus*, cyanobacteria such as *Nostoc*; many chlorophyte algae including *Trentepohlia*, *Haematococcus*, *Chlorella* spp.
Efficient DNA repair mechanisms	Repair the damage incurred during desiccation or exposure to radiation	*Deinococcus*, possibly the cyanobacterium *Chroococcidiopsis*
Multiple copies of the genome	Provide multiple copies of essential genes, serve as template for DNA repair	Possibly *Chroococcidiopsis*, *Nostoc*
Spore formation; encystment	Specialized cells able to resist dry conditions	Endospore-forming bacteria, members of the Actinobacteria; most fungi
Motility	Avoidance of excessive insolation, possible movement toward higher concentrations of water	*Microcoleus*, *Oscillatoria*, and some other filamentous cyanobacteria; some flagellated eukaryotic algae

2010, 2012; Warren-Rhodes et al., 2013). Of special interest in this respect is the role mineral deliquescence plays in controlling the metabolism of the endolithic association living in halites in the Atacama Desert (Davila et al., 2008, 2013).

The hypolithic association enjoys a particularly favorable habitat in this regard. The dominant microorganisms, usually a member of *Chroococcidiopsis* or *Gloeocapsa* (Friedmann and Galun, 1974; Büdel and Wessels, 1991), form thick sheaths extending some distance from the surface of the stone. In addition, the nonporous stones overlying the association serve as efficient moisture barriers. Therefore, it is not surprising that the moisture content in the soil beneath the association is often much higher than in the surrounding soil (Cameron and Blank, 1966; Broady, 1981b, Smith et al., 2000; McKay et al., 2003). This effect can be enhanced by the thermal properties of the stone. Large stones can generate thermal gradients within the soil that result in the net movement of water vapor through the soil to the hypolithic environment; in effect, the lower surface of the stone acts as a water collection system (Jury and Bellantuoni, 1976a,b). However, for the water collection system to be effective, there must be significant quantities of moisture available in the soil. Therefore, it is not too surprising that attempts to measure this effect in the core of the Atacama have been unsuccessful (McKay et al., 2003). The upper surfaces of stones are also more efficient dew, fog, and mist collectors than soil surfaces. This may account for the presence of desert varnish communities in semiarid and arid regions. It is also possible for the collected water to pool on the surface and then trickle off the surface to moisten the soil around the edges and just below the stone, thereby supporting the hypolithic association. The effect is enhanced with large, nonporous stones, explaining the preference for colonization of larger quartz stones, especially in the perilithic habitat, in hyperarid deserts (Warren-Rhodes et al., 2007a,b,c; compare McKay et al., 2003; Warren-Rhodes et al., 2013).

A number of eukaryotic algae are capable of absorbing sufficient water vapor when the relative humidity is high to maintain some level of metabolic activity (see Lange et al., 1990; Nienow, 2002). Included in these are a number of forms commonly found in desert environments, including strains of the green alga *Stichococcus* (Nienow and McKay, 2000). It is generally believed that this ability is not present in cyanobacteria or other prokaryotic organisms (see Nienow, 2002). However, the desert crust cyanobacterium *Microcoleus sociatum* may be able to use water vapor at relative humidity in excess of 95% (Büdel, 1994). The mechanism behind this ability is unknown. In the case of *Microcoleus*, it may result from the structure of the filament – several trichomes within a communal sheath. The compact arrangement could lead to the formation of liquid water within small capillaries. In the case of unicellular forms such as *Stichococcus* or the subaerial taxon *Apatococcus*, it is presumed to be related to the buildup of compatible solutes in the cell (see below), although this is yet to be tested.

Some of the filamentous cyanobacteria present in desert crusts are known to be mobile during wet conditions, rising to the surface of the soil and causing a brief "greening" of the desert and then retreating back into the soil to take up residence a few millimeters below the surface (Brock, 1975; Dor and Danin, 2001; Belnap, 2006). In *Microcoleus*, the movement is apparently caused by a combination of physical exclusion of the living trichomes from the polysaccharide sheath as it swells with water and typical cyanobacterial gliding motility (Dor and Danin, 2001). In other cases, gliding motility alone is sufficient. Much of the movement appears to be directed upward toward the surface of the soil, possibly in a phototactic response, possibly as part of a random process (Pringault and Garcia-Pichel, 2003). As the soil dries, at least some members of the association track the moisture gradient back into the soil, in a hydrotactic response (Garcia-Pichel and Pringault, 2001; Pringault and Garcia-Pichel, 2003).

Adaptations to Survive Desiccation

Even with the most efficient means of prolonging water availability, desert microorganisms will become desiccated. At present, little is known of the mechanisms by which desert microorganisms survive the drying process. The first step seems to be the formation of some sort of compatible solute. Compatible solutes are low-molecular-weight organic compounds that can serve to lower the osmotic potential and, therefore, the total water potential within the cell while maintaining cellular and molecular integrity. A large number of different types of molecules have been implicated as compatible solutes, including polyols, sugars and sugar derivatives, betaines, some amino acids and amino acid derivatives, and ectoines. Trehalose and sucrose seem to be particularly important in organisms subjected to extreme water stress; thus, it is not surprising that desert *Chroococcidiopsis* sp. and *N. commune* are both known to accumulate trehalose and sucrose under conditions of water stress (Hershkovitz et al., 1991; Hill et al., 1997; Sakamoto et al., 2009). Trehalose and sucrose both seem to work by stabilizing cell membranes by replacing the water bound to phosphate groups, and thereby depressing the phase transition temperature of desiccated membranes to physiological temperatures, and by preventing the fusion of membrane vesicles (Crowe and Crowe, 1992; Potts, 1994, 1999); they may also stabilize protein and DNA structure (Potts, 1994). The extracellular polysaccharides may work in the same way to stabilize the outer membranes of bacteria or the outer layers of the cell membrane of other organisms. It is interesting, in this respect, to note that cyanobacteria with intact, lamellated sheaths have greater viability than those without (Grilli Caiola et al., 1996); similar sheaths have been noted in endolithic biofilms (de los Rios et al., 2007).

Adaptations to Reduce the Damage While in the Desiccated State

Phototrophic microorganisms are all subject to the accumulation of photooxidative damage resulting from the reduction of oxygen and other molecules by photosystems only partially active in the desiccated state (Smirnoff, 1993; Lao and Glazer, 1996; Gray et al., 2007). In addition, all desert microorganisms are subject to the accumulation of damage caused by UV light. Therefore, the existence of a variety of mechanisms to reduce overall exposure to light is not surprising. Simplest among these is the growth in low-light environments. As discussed previously, both hypolithic and cryptoendolithic microorganisms inhabit environments where the maximum photon flux is several orders of magnitude lower than that of the soil or rock surface. Motile crust-forming cyanobacteria, for example, *Microcoleus* and *Oscillatoria*, form layers several millimeters below the surface of the soil during dry conditions (Dor and Danin, 2001; Pringault and Garcia-Pichel, 2003); here, again, the photon flux is much reduced. Nonmotile members of desert crusts, for example, *Scytonema*, and cryptoendolithic lichens and cyanobacteria inhabiting the upper layers of the association usually form some sort of photoprotective pigment. Many desert and subaerial cyanobacteria produce the brown sheath pigment scytonemin and/or water-soluble mycosporine-like amino acids, both of which are known to provide some degree of protection against UV and high-energy visible light (Garcia-Pichel and Castenholz, 1991, 1993; Ehling-Schulz and Scherer, 1999; Oren and Gunde-Cimerman, 2007). Many lithophytic and lichen-forming fungi produce melanin, mycosporine and mycosporine-like amino acids, and/or carotenoids (Gorbushina et al., 2003; Onofri et al., 2004; Selbmann et al., 2005; Oren and Gunde-Cimerman, 2007). Terrestrial eukaryotic algae generally lack colored sheaths and walls. However, some species, notably members of the genera *Chlamydomonas*, *Chlorella*, *Chlorococcum*, *Haematococcus*, and *Trentepohlia*, have the potential to accumulate oil droplets colored by the carotenoids astaxanthin and/or zeaxanthin under high light, low water, or low nutrient conditions (Ho et al., 1983; Adams et al., 1993; Bidigare et al., 1993; Remias et al., 2005). These may serve to shield the cells from excess light, in a manner similar to the colored sheaths of cyanobacteria (Demmig-Adams and Adams, 1992; Gorton et al., 2001; Remias et al., 2005). They may also serve to scavenge the reactive oxygen species associated with photooxidative damage (Demmig-Adams and Adams, 1992; Trebst, 2003; Remias et al., 2005; Holzinger and Karsten, 2013). Ascorbic acid, glutathione and α-tocopherol in the plastid serve similar purposes in higher plants and at least some eukaryotic algae (Demmig-Adams and Adams, 1992; Trebst, 2003; Remias et al., 2005; Holzinger and Karsten, 2013). Changes in the pigment complement within chloroplasts may enhance the operation of the xanthophyll cycle, a mechanism for the thermal dissipation of light energy captured by the light harvesting complexes, thereby reducing the number of reactive oxygen species created (Demmig-Adams and Adams, 1992). Intracellular pools of manganese ions may serve to block the formation of iron-dependent reactive oxygen species (Daly, 2009).

Adaptations Leading to a Quick Recovery

The final set of adaptations seen in desert organisms are those leading to a quick recovery from damage associated with desiccation, especially damage to the DNA. These have been best studied in *Deinococcus radiodurans*, primarily in regard to damage from ionizing radiation (see Cox and Battista, 2005; Daly, 2009). Several mechanisms have been implicated, including the presence of multiple copies of the genome within individual cells, delays in DNA replication until damage has been repaired, physiologic responses including the induction of a suite of genes following exposure to desiccation and ionizing radiation, protein-based protection of the broken ends of the DNA molecule, RecA-independent double-strand break repair, and novel forms of RecA repair mechanisms. The multiple copies of the genome reduce the risk of inactivating all copies of individual genes at the same time. They also provide reserve of information that can be used to repair damaged sequences. Not all of the stress-induced proteins have been assigned functions, but at least five have been implicated in genome repair. One of these, DdrA, binds to the 3′ end of single-stranded DNA, protecting it from degradation by nucleases in vitro. Presumably, within desiccated or irradiated cells it would bind to the broken ends of the DNA, protecting them until repair can be accomplished. It is also worth noting that *D. radiodurans* is known to

accumulate manganese ions, which can protect the repair proteins (and others) during desiccation or exposure to ionizing radiation enhance recovery after the stress has been removed (Daly, 2009).

How far the mechanisms used by *Deinococcus* can be applied to other members of the desert community is not clear. Desert strains of *Chroococcidiopsis*, also known to be radiation-resistant, are postulated to rely on multiple genome copies, but this has not been investigated in detail (Billi et al., 2000). *N. commune* is thought to rely more on protective mechanisms, high trehalose concentrations, in particular, to reduce the amount of damage occurring during desiccation. Clearly, more research is necessary in this area.

Relevance to Astrobiology

The Search for Life on Mars

According to the currently accepted theory, about 3.8 billion years ago conditions on Mars included flowing liquid water and surface temperatures above zero (McKay, 1993). While these conditions did not last long, it is possible that they were of sufficient duration that life could have arisen independently on Mars at about the same time that it arose on Earth. In addition, much of the surface of Mars, unlike the surface of Earth, remains relatively unchanged since that period. Therefore, Mars may have the best record of the origin of life on Earth-like planets (McKay, 1993). One focus of astrobiolological research is to develop strategies and techniques for the in situ search for indications of the remnants of these early stages of life. Dry environments on Earth are useful in this context for two reasons. First, as the environment on Mars changed, the surface would have become increasingly colder and drier, approximating the conditions in arid and hyper-arid regions currently in existence on Earth. These regions, therefore, serve as models of the types of ecosystems that may have been present during the final stages of life on Mars. The continued development tools and techniques for the morphological and chemical analysis of the microbial associations found in the arid regions Earth is clearly central to the development of similar tools and techniques for the search for the remnants of life on Mars (see, e.g., Perry and Kolb, 2004; Warren-Rhodes et al., 2007d).

Interplanetary Exchange of Microorganisms – Testing Panspermia

Allen Hills 84001 (ALH 84001), a meteorite thought to have originated on Mars, gained notoriety when it was claimed that it contained evidence of extra-terrestrial life (McKay et al., 1996). While the initial claims remain, at best, controversial, the discussions that ensued led to a renewed interest in panspermia, the theory that organisms can be transferred between planets by natural processes. The current form of the theory, lithopanspermia, suggests that rocks containing microorganisms are ejected from a planetary body, such as Mars, by the impact of a large asteroid or comet. The rocks, with their microbiota relatively intact, eventually reach another planet where the microorganisms take up residence. Because the organisms would have to be present in the interior of the rock before ejection from the planetary body, endolithic communities, especially those from arid regions with a presumed level of pre-adaptation for withstanding the conditions associated with space travel, are considered candidates for testing this theory.

For lithopanspermia to be successful, the microbiota must survive the initial ejection from the planetary body, the subsequent journey through space, and re-entry on a second planetary body (Horneck et al., 2010). All three of these have been tested directly using cryptoendolithic cyanobacteria and cryptoendolithic associations, with, at best, mixed results. In a test of the initial ejection phase, dried cells of *Chroococcidiopsis* were placed between two gabbro plates and then exposed to shocks mimicking the impact of a meteorite (Horneck et al., 2008). The cells were able to survive shocks of up to 10 GPa; higher shocks resulted in mechanical damage to the cells. While encouraging, this is at the low end of estimates of the shocks experienced by examples of Martian meteorites recovered on Earth (Horneck et al., 2008). It should be noted, however, that other microorganisms, including at least one species of lichens and bacterial spores, could withstand much greater shocks, including shocks sufficient for ejection (Horneck et al., 2008). In a separate set of experiments, survival during transit was tested by exposing desiccated pieces of Antarctic sandstones colonized by cryptoendolithic associations to space conditions and to simulated Mars conditions aboard the International Space Station (Scalzi et al., 2012). After one and a half years, the samples were returned to Earth and cultured. The investigators were able to recover only one colony of the green alga *Stichococcus* from the simulated Mars conditions and three colonies of fungus *Acaropora* from the space conditions. This is a much lower diversity than what was recovered from the control samples and much lower than present in the original samples, and suggests that presence in the cryptoendolithic habitat does not guarantee survival over years in the space environment (Scalzi et al., 2012). In a third set of experiments, the ability of *Chroococcidiopsis* to survive re-entry was tested using the European Space Agency's STONE facility. In this system, small pieces of material can be affixed to the exterior of spacecraft, near the heat shield of a FOTON re-entry vehicle near the ablation point, and exposed directly to the heat of re-entry. In an initial experiment samples of impact-shocked gneiss were inoculated with cultures of *Chroococcidiopsis* then attached to the vehicle (Cockell et al., 2007). During re-entry, the vehicle entered the atmosphere at speeds close to 8 km per hour, somewhat slower than meteorites. However, even at these slow speeds, the surface of the sample melted and all biological material was destroyed (Cockell et al., 2007). In a follow-up experiment, *Chroococcidiopsis*, piece of lichen, and bacterial endospores attached to the carrier behind a 2-cm piece of shocked gneiss instead of on the exposed surface, in order to provide some degree of protection. Again, none of the biological material survived the high-temperatures associated with re-entry, although the remains of the cells and biochemical markers were detected. This places a severe constraint on the types of endolithic associations are suitable for consideration in the context of lithopanspermia (Cockell et al., 2007).

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Extremophiles: Hot Environments☆

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Introduction

The first report of life in a hot environment, herein defined as being above 70°C, came from the observation of high concentrations of microbes (or 'chlorophylless filamentous Schizomycetes') in an 89°C hot spring in Yellowstone National Park sampled in 1898. The hottest known microbes are 'hyperthermophiles', which are those organisms that grow optimally above 80°C. In hot environments, they are commonly joined by the hottest thermophiles, which are those organisms that grow optimally above 55°C. After 1980, many microbes that grow above 70°C were cultured and characterized from terrestrial geothermal environments and shallow marine vents. At the same time, microbes from deep-sea hydrothermal vents were isolated from mid-ocean spreading centers and volcanic seamounts. The advent of metagenomics and microbial community analysis has shown that hyperthermophiles and hot thermophiles are also found in other hot environments such as in petroleum reservoirs and deep marine sediments.

In general, thermophiles that grow below 70°C are found in a wide range of environments outside of the hot environments discussed herein. These include coal refuse, hot water tanks, and compost piles. Photosynthetic bacteria can grow up to 70°C and include cyanobacteria and green- and purple-sulfur bacteria. There are many spore-forming thermophiles such as *Bacillus*, *Clostridium*, and *Moorella* species; and there are thermophilic *Actinomycetes*, sulfur oxidizers, sulfate reducers, and Gram-negative aerobes such as *Thermus aquaticus*, the source of the Taq DNA polymerase. Even some *Alvinellid* polychaete worms from hydrothermal vents are known to live at temperatures of 55–60°C. However, this review focuses on those microbes that grow without sunlight and optimally above 70°C. Their growth characteristics will be described based on their metabolic function. This includes specialists such as methanogens, other autotrophs, hydrogen-producing heterotrophs, and thermoacidophiles as well as metabolic generalists from freshwater and marine environments that grow either autotrophically or heterotrophically and use numerous inorganic compounds to support their growth. The review will then discuss characteristics of hot environments, such as hydrothermal vents, hot springs, and hot sedimentary environments including petroleum reservoirs, and the organisms and geochemical processes within them.

Metabolic Functional Groups

Methanogens

Microbial methanogenesis is the largest source of methane globally with an annual production rate of 10^9 tons. All microbial methanogenesis is catalysed by archaea and mediated by obligately anaerobic methanogens in the *Euryarchaeota*, although there is recent metagenomic evidence for possible methanogenesis in the *Bathyarchaeota* and *Verstraetearchaeota*. Methanogens are most abundant in environments where alternative terminal electron acceptors such as sulfate, nitrate, and metals are depleted. They generate methane by using hydrogen and CO_2, formate, methylated compounds, and acetate as carbon and energy sources. The *Methanococcales*, *Methanobacteriales*, and *Methanopyrales* are the Orders that have representatives with optimal growth above 70°C (Table 1). Their growth is almost entirely hydrogenotrophic (i.e., $4H_2+CO_2 \rightarrow CH_4+2H_2O$), although a few species are capable of growth on formate. Like all methanogens, they couple methanogenesis to the Wood-Ljungdahl pathway for CO_2 fixation and energy conservation. The key enzyme in methanogenesis is methyl-CoM reductase, which serves as a genetic marker for methanogenesis in the environment (e.g., *mcrA*).

Methanocaldococcus (Fig. 1(A)) and *Methanotorris* are marine genera in the *Methanococcales* Order that grow best at temperatures of 80–88°C. *Methanocaldococcus jannaschii* is the most studied hyperthermophilic methanogen and was among the first microbes to have its complete genome sequenced. While nitrogen fixation in high-temperature methanogens is unusual, *Methanocaldococcus* strain FS406–22 is the hottest known organism capable of this process. The Order *Methanobacteriales* is divided into two Families: the *Methanobacteriaceae* and the *Methanothermaceae*. The *Methanobacteriaceae* do not grow optimally above 70°C, but does include *Methanothermobacter* species that grow best around 65°C and are common in many mildly hot environments. In contrast, all *Methanothermaceae* grow optimally at 83–88°C. Its sole genus is *Methanothermus*, whose species are the only hyperthermophilic methanogens found in terrestrial hot environments and grow up to 97°C. The Order *Methanopyrales* has the highest optimal growth temperatures of any methanogen. *Methanopyrus kandleri* is marine and the only species in the Order. It is the hottest life form known anywhere with growth under hydrostatic pressure at temperatures up to 122°C.

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Table 1 Taxonomy of microbes that grow above 70°C and arranged by functional group. The genera, optimal growth temperatures, carbon sources, and electron acceptors are provided for each listed Order

Functional groups by Order	*Genera*	T_{opt} (°C)	*Carbon sources*	*Electron acceptors*
Methanogens				
Methanobacteriales	*Methanothermus*	83–88	A	CO_2
Methanococcales	*Methanocaldococcus*, *Methanotorris*	80–88	A	CO_2
Methanopyrales	*Methanopyrus*	95–100	A	CO_2
Other obligate autotrophs				
Aquificales	*Aquifex*, *Balnearium*, *Caldimicrobium*[a], *Desulfurobacterium*, *Hydrogenivirga*, *Hydrogenobacter*, *Persephonella*, *Phorcysia*, *Sulfurihydrogenibium*, *Thermocrinis*[a], *Thermosulfidibacter*, *Thermovibrio*, *Venenivibrio*	70–85	A	S°, $S_2O_3^{2-}$, O_2, NO_3^-, Fe(III), AsO_4^{3-}, SeO_4^{2-}
Thermodesulfobacteriales	*Geothermobacterium*, *Thermodesulfatator*, *Thermodesulfobacterium*[a], *Thermosulfurimonas*	70–90	A	S°, $S_2O_3^{2-}$, SO_4^{2-}, Fe(III)
H_2 producers				
Thermoanaerobacteriales	*Caldicellulosiruptor*, *Thermoanaerobacter*	70–75	H	H^+, S°, $S_2O_3^{2-}$
Thermococcales	*Pyrococcus*, *Thermococcus*	80–100	H	H^+, S°
Thermotogales	*Thermotoga*, *Fervidobacterium*	70–80	H	H^+, S°
Marine generalists				
Archaeoglobales	*Archaeoglobus*, *Ferroglobus*, *Geoglobus*	70–88	A, H, FA	SO_4^{2-}, $S_2O_3^{2-}$, NO_3^-, Fe(III)
Desulfurococcales	*Aeropyrum*, *Desulfurococcus*, *Hyperthermus*, *Ignicoccus*, *Ignisphaera*, *Pyrodictium*, *Pyrolobus*, *Staphylothermus*, *Stetteria*, *Sulfophobococcus*, *Thermodiscus*, *Thermogladius*, *Thermosphaera*	85–106	A, H, FA	H^+, S°, $S_2O_3^{2-}$, SO_3^{2-} Fe(III), NO_3^-, O_2
Freshwater generalists				
Thermoproteales	*Caldivirga*, *Pyrobaculum*, *Thermocladium*, *Thermofilum*, *Thermoproteus*, *Vulcanisaeta*	75–100	H, FA	O_2, S°, SO_4^{2-} $S_2O_3^{2-}$, NO_3^-, Fe(III), AsO_4^{3-}, SeO_4^{2-}
Thermoacidophiles				
Acidilobales	*Acidilobus*, *Caldisphaera*	70–85	H	S°
Sulfolobales	*Acidianus*, *Metallosphaera*, *Stygiolobus*, *Sulfolobus*, *Sulfurisphaera*	70–85	A, FA	S°, O_2, Fe(III)

[a]Contains some species that are facultative autotrophs.

Other Obligate Autotrophs

Other obligate autotrophs in hot environments include the *Aquificales* and the *Thermodesulfurobacteriales*, which are both bacteria (Table 1). They primarily use hydrogen and sulfur compounds as their electron donor and acceptor, although there are a few facultative autotroph exceptions. The *Aquificales* grow optimally at 70–85°C and contains three Families: the *Aquificaceae*, the *Desulfurobacteriaceae*, and the *Hydrogenothermaceae*. With a few exceptions, the *Aquificaceae* are strict chemolithoautotrophs that fix CO_2 using the reductive Tricarboxylic Acid Cycle and use hydrogen, thiosulfate, and elemental sulfur as electron donors. Most are microaerophiles that use a low concentration of oxygen as a terminal electron acceptor, although sulfur compounds and nitrate can also be used as an electron acceptors. *Aquifex* uses oxygen as an electron acceptor at very low concentrations, oxidizes hydrogen, and makes water as the final product (i.e., Knallgas reaction) at temperatures up to 95°C. *Hydrogenivirga* and *Hydrogenobacter* use hydrogen, thiosulfate, and elemental sulfur as electron donors and nitrate and oxygen as electron acceptors. *Thermocrinis* forms grey and pink filamentous masses in streams that are visible to the eye. *Thermocrinis albus* produces individual filaments up to 60 μm long and can also grow heterotrophically on formate and formamide under microaerophilic conditions.

The *Desulfurobacteriaceae* are strict anaerobes and chemolithoautotrophs that couple hydrogen oxidation to the reduction of elemental sulfur, thiosulfate, sulphite, and nitrate. *Desulfurobacterium* (Fig. 1(B)) cells form filamentous masses that can be 8 cm in length. *Balnearium* only reduces elemental sulfur while *Thermovibrio* and *Phorcysia* reduce elemental sulfur as well as nitrate to ammonium.

The *Hydrogenothermaceae* are also strict chemolithoautotrophs, except for some *Sulfurihydrogenibium* that are facultative autotrophs. *Persephonella* use hydrogen, thiosulfate, and elemental sulfur as electron donors and elemental sulfur, nitrate, and oxygen as terminal electron acceptors. *Sulfurihydrogenibium* are also metabolically versatile using these compounds plus ferrous iron, arsenite, and selenite as electron donors and ferric iron, arsenate, and selenate as electron acceptors. They are important in sulfur cycling and iron mineralization. In contrast, *Venenivibrio* species grow solely using hydrogen and oxygen to make water.

The *Thermodesulfurobacteriales* are mostly obligate autotrophs that grow best at 70–90°C, but a few are facultative autotrophs. *Caldimicrobium* use hydrogen or ethanol as electron donors and thiosulfate or elemental sulfur as terminal electron acceptors. Freshwater *Caldimicrobium thiodismutans* and marine *Thermosulfurimonas dismutans* grow autotrophically by disproportionation of

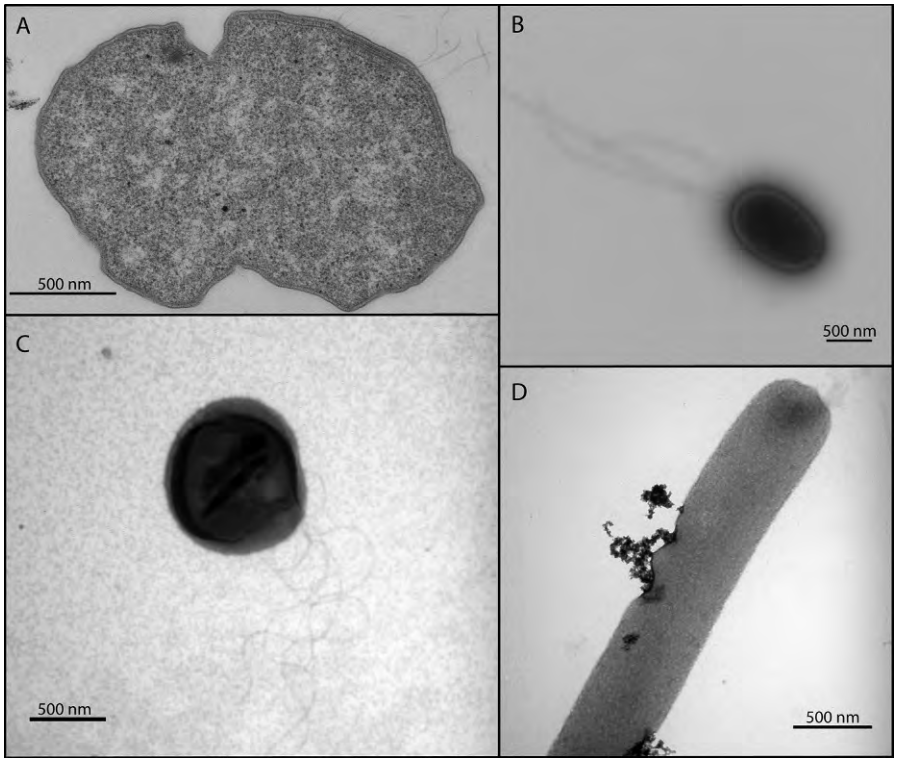

Fig. 1 Electron micrographs of various thermophiles and hyperthermophiles. (A) Thin section of the hyperthermophilic methanogen *Methanocaldococcus bathoardescens* while undergoing cell replication. (B) The thermophilic, autotrophic sulfur reducer *Desulfurobacterium* sp strain HR11 with its flagella. (C) The hyperthermophilic marine generalist *Pyrodictium delaneyi* with its flagella. (D) The hyperthermophilic freshwater generalist *Pyrobaculum islandicum* shown with iron oxide mineral particles as part of its growth on iron. Reproduced from Ver Eecke, H.C., Akerman, N.H., Huber, J.A., Butterfield, D.A., Holden, J.F., 2013. Growth kinetics and energetics of a deep-sea hyperthermophilic methanogen under varying environmental conditions. Environmental Microbiology Reports 5, 665–671. With Permission Stewart, L.C., Llewellyn, J.G., Butterfield, D.A., Lilley, M.D., Holden, J.F., 2016. Hydrogen and thiosulfate limits for growth of a thermophilic, autotrophic *Desulfurobacterium* species from a deep-sea hydrothermal vent. Environmental Microbiology Reports 8, 196–200. With Permission Lin, T.J., El Sebae, G., Jung, J.-H., *et al.*, 2016. *Pyrodictium delaneyi* sp. nov., a hyperthermophilic autotrophic archaeon that reduces Fe(III) oxide and nitrate. International Journal of Systematic and Evolutionary Microbiology 66 (9), 3372–3376. With Permission.

elemental sulfur, thiosulfate, and sulphite. *Thermosulfurimonas* fixes CO_2 using the Wood-Ljungdahl pathway. *Thermodesulfatator* are marine autotrophs that grow solely on sulfate as an electron acceptor, while *Thermodesulfobacterium* are autotrophs that reduce sulfate, sulphite, thiosulfate, or elemental sulfur. Some *Thermodesulfobacterium* species can also use organic compounds as electron donors. *Geothermobacterium* are freshwater obligate iron reducers that couple hydrogen oxidation with ferrihydrite reduction forming magnetite at temperatures up to 100°C, making them the hottest known bacteria.

Hydrogen Producers

High-temperature hydrogen producers (Table 1) use a variety of organic substrates for growth, contain an unusually high number of glycosyl hydrolases and transferases, and in some cases use energetically favorable electron carriers such as ferredoxin that help the organisms overcome hydrogen inhibition. Many of them also use sulfur compounds for growth, and produce hydrogen primarily in the absence of these electron acceptors. They can grow syntrophically with hydrogenotrophs through interspecies hydrogen transfer.

Within the *Thermoanaerobacteriales*, *Caldicellulosiruptor* and *Thermoanaerobacter* are bacteria that grow optimally at 70–75°C with similar fermentation and hydrogen generation pathways. *Caldicellulosiruptor* species degrade many organic compounds including lignocellulosic substrates and have high hydrogen yields. They are studied extensively for potential biofuel production. *Thermoanaerobacter* is saccharolytic, but not cellulolytic, and also reduces thiosulfate and elemental sulfur. Both genera use the Embden-Meyerhof pathway for sugar catabolism and generate hydrogen using NADH-dependent hydrogenases. Hydrogen production is only possible at very low hydrogen concentrations. When hydrogen concentrations increase, there is a shift to lactate production in *Caldicellulosiruptor* and ethanol in *Thermoanaerobacter*. In the *Thermotogales*, *Thermotoga* are bacteria that grow best at 70–80°C and catabolize sugars using the Embden-Meyerhof pathway to produce acetate, lactate, ethanol, CO_2, and hydrogen. They use a bifurcating enzyme, ferredoxin:NADH oxidoreductase, for hydrogen generation and generate lactate when inhibited by hydrogen. *Thermotoga* will preferentially use elemental sulfur over protons as its terminal electron acceptor when it is available.

The *Thermococcales* are archaea in the *Euryarchaeota* that grow optimally at 80–100°C and either require or are stimulated by elemental sulfur. They are the most studied hyperthermophiles due to their industrial applications and ease of growth. They are often the source of DNA polymerases used for the polymerase chain reaction (PCR) and in vitro DNA replication. They all use

a modified archaeal Embden-Meyerhof pathway for sugar catabolism that uses ADP-dependent rather than ATP-dependent hexokinases and phosphofructokinases as well as a ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase in place of NADH-dependent glyceraldehyde-3-phosphate dehydrogenase and ATP-producing 3-phosphoglycerate kinase. This results in no net ATP production in this pathway. They also use ferredoxin-dependent oxidoreductases to catabolize peptides that are linked with NADH-producing glutamate dehydrogenase. *Pyrococcus furiosus* grows optimally at 100°C and is one of the most thoroughly studied hyperthermophiles. It preferentially reduces elemental sulfur to hydrogen sulphide using a soluble heterodisulfide reductase but also makes hydrogen in the absence of sulfur. It uses a ferredoxin-dependent hydrogenase on its membrane to produce hydrogen and generate a H^+/Na^+ gradient that is used for oxidative phosphorylation and ATP generation on the membrane. It also has a soluble NADH-dependent hydrogenase that is used for biosynthesis reactions. *Thermococcus* are widespread in hot marine environments with over 30 species in culture and more than 20 of their genomes sequenced. They also preferentially reduce elemental sulfur to hydrogen sulphide and in some cases protons to hydrogen gas. In addition to the ferredoxin- and NADH-dependent hydrogenases found in *Pyrococcus*, some *Thermococcus* also have membrane-bound formate- and CO-dependent hydrogenases that expand the hydrogen production and H^+/Na^+ translocation capabilities of these organisms.

Marine Generalists

There are two Orders of marine generalist that are both archaea: the *Archaeoglobales* in the *Euryarchaeota* and the *Desulfurococcales* in the *Crenarchaeota* (Table 1). They are obligate heterotrophs or facultative autotrophs that use a wide range of electron donors and acceptors. The *Archaeoglobales* reduce sulfate, thiosulfate, iron, and nitrate; although elemental sulfur inhibits their growth. *Archaeoglobus* are facultative autotrophs or heterotrophs that reduce sulfate and thiosulfate to hydrogen sulphide. In contrast, neither *Ferroglobus* nor *Geoglobus* can reduce sulfate. *Ferroglobus* species can oxidize Fe(II), hydrogen, sulphide, acetate, and aromatic compounds and reduce nitrate, thiosulfate, Fe^{3+}-citrate, and ferrihydrite. *Geoglobus* species grow autotrophically by hydrogen oxidation and are obligately dependent on either Fe^{3+}-citrate or ferrihydrite as terminal electron acceptors.

The *Desulfurococcales* are comprised of two Families: the *Pyrodicteaceae* and the *Desulfurococcaceae*. The *Pyrodictiaceae* grow best at 90–106°C and often have unique cell structures that vary from coccoid to disk-shaped and the cells can form a network of cannulae that are tubular structures composed of glycoprotein subunits that connect the cells. They are autotrophs, facultative autotrophs, and heterotrophs that sometimes require hydrogen for growth and use a variety of terminal electron acceptors such as elemental sulfur, thiosulfate, nitrate, oxygen, and ferrihydrite. *Pyrolobus fumarii* is a strict H_2 oxidizing autotroph that reduces thiosulfate, nitrate, oxygen and can grow at temperatures up to 113°C. *Pyrodictium delaneyi* (Fig. 1(C)) reduces nitrate as well as ferrihydrite to the mineral magnetite. The *Desulfurococcaceae* have more genera than the *Pyrodicteaceae* and generally have cooler optimal growth temperatures of 85–95°C. They grow mixotrophically or heterotrophically. *Desulfurococcus*, *Hyperthermus*, *Sulfophobococcus*, *Staphylothermus*, and *Thermosphaera* ferment sugars and peptides. *Stetteria*, *Thermodiscus* and some *Desulfurococcus* species use organic compounds to reduce elemental sulfur and generate hydrogen sulphide, organic acids, and alcohols. They are obligate anaerobes with the exception of *Aeropyrum pernix*, which is a microaerophile that respires oxygen. *Ignicoccus* species are strictly autotrophic elemental sulfur reducers that produce hydrogen sulphide. *Ignicoccus hospitalis* forms a stable co-culture with the archaeon *Nanoarchaeum equitans* that lives on the outer surface of *I. hospitalis*. *N. equitans*, in the *Nanoarchaeota*, has a genome size of 490 kb and a cell diameter of 350–500 nm. It lacks almost all known genes for lipid, cofactor, amino acid, and nucleotide biosynthesis and is completely dependent on *I. hospitalis* for survival.

Freshwater Generalists

Similar to the marine generalists, freshwater generalists are all archaea that belong to the *Thermoproteales* in the *Crenarchaeota* (Table 1). The *Thermoproteales* grow best at 75–100°C and consists of two families: the *Thermoproteaceae* and the *Thermofilaceae*. They can be distinguished from each other morphologically based on cell diameter since *Thermofilum*, the only genus in the *Thermofilaceae*, form very thin (0.15–0.35 μm diameter) filaments. The *Thermoproteales* differ from other (hyper)thermophilic archaea in that they are rod-shaped instead of coccoids. They also form a unique 'golfclub' morphology where a spherical structure forms at the terminal end of the rod during certain growth conditions. *Pyrobaculum* (Fig. 1(D)) are the only members of the *Thermoproteaceae* that grow above 100°C. They are facultative autotrophs capable of growth on many terminal electron acceptors such as elemental sulfur, thiosulfate, oxygen, nitrate, nitrite, selenate, selenite, arsenate, glutathione, Fe^{3+}-citrate, and ferrihydrite. In contrast, *Thermoproteus* are facultative autotrophs that only respire elemental sulfur. *Vulcanisaeta*, *Caldivirga*, and *Thermocladium* are obligate heterotrophs that reduce elemental sulfur, sulfate, and thiosulfate. *Caldivirga maquilingensis* and *Thermocladium modestius* are the only species in their genera, and both are also microaerophiles. Archaeal cell wall extract was shown to stimulate the growth of some *Thermofilum*, *T. modestius*, and *C. maquilingensis*, but the largest effect was observed on the growth of *Thermofilum pendens*. This latter organism is depenent upon the polar lipid extract of *Thermoproteus tenax* for growth.

Thermoacidophiles

Thermoacidophiles live in hot, acidic (pH <4) terrestrial environments. They are *Crenarchaeota* belonging to the *Acidilobales* and the *Sulfolobales* (Table 1). The *Acidilobales* consist of the genera *Acidilobus* and *Caldisphaera*. Both are obligate anaerobes and heterotrophs

that use sugars and peptides for growth, and secrete short-chain fatty acids. Their growth is stimulated by elemental sulfur that they use to produce hydrogen sulphide. *Acidilobus* live optimally at 80–85°C and pH 3.5–4.0, while *Caldisphaera* live optimally at 70–78°C and pH 3.5–4.5. The *Sulfolobales* are more metabolically versatile than the *Acidilobales* and have an optimum pH near 2.0. They are facultative autotrophs that oxidize elemental sulfur, thiosulfate, dithionite, Fe(II), metal sulphides, and hydrogen while reducing oxygen or sulfur when growing autotrophically. Most *Sulfolobus* and all *Metallosphaera* species are aerobic, *Acidianus* and *Sulfurisphaera* are facultative anaerobes, and *Stygiolobus* are anaerobic. *Stygiolobus* is the only obligate chemolithoautotroph and grows by hydrogen oxidation coupled with elemental sulfur reduction. *Acidianus* and *Sulfurisphaera* can both oxidize and reduce elemental sulfur. *Sulfolobus metallicus*, some *Acidianus* species, and *Metallosphaera sedula* can remove metals from metal sulphide minerals and have high resistance to metal toxicity.

Habitats

Marine Hydrothermal Vents

Marine hydrothermal vents are sites where seawater percolates through cracks and pores in the Earth's crust and reacts with hot subsurface rocks. The seawater is transformed into hydrothermal fluid as the seawater exchanges ions with the rock. Fluid-mineral reactions result in the loss of some dissolved chemical and volatile components (e.g., oxygen, sulfate) and gain in others (e.g., hydrogen, methane, carbon dioxide, sulphide, and reduced metals) at progressively higher temperatures as modified seawater migrates deeper into the crust. Hydrothermal venting is associated with mid-ocean spreading centers along tectonic plate boundaries, intra-plate volcanic hot spots, and back-arc basins and island-arc volcanoes in tectonic subduction zones. They are found in the deep-sea as well as in shallow coastal marine environments.

Deep-sea hydrothermal vents were first discovered in 1977 near the Galápagos Islands, and since then dozens of deep-sea hydrothermal vent fields in all the oceans have been discovered. The hydrothermal fluid emanating from these vents can reach temperatures over 400°C, which are kept in a liquid state due to the high hydrostatic pressure exerted on the fluids. These temperatures are too hot to support life, but hydrothermal fluids mix with cool seawater inside the crust and within metal sulphide mineral deposits on the seafloor and create cooler habitats that support thermophilic and hyperthermophilic life. These organisms use chemicals from both the hydrothermal fluid and the seawater for growth. However, the chemical composition of the fluids vary depending on the kinds of rocks that the seawater reacts with. If seawater reacts with ultramafic rock such as olivine from deep in the Earth's crust or with newly erupted magma in the shallow crust, then large concentrations of hydrogen are produced in the fluids. The hydrogen then supports the growth of thermophilic and hyperthermophilic autotrophs, particularly methanogens such as the *Methanococcales*. In contrast, along subduction zones where highly weathered seafloor that is millions of years old is melted and reacts with seawater, the hydrothermal fluids have low hydrogen concentrations but are rich in metals and highly acidic. In these cases as well as non-eruptive mid-ocean spreading centers, the thermophiles and hyperthermophiles found are autotrophs such as the *Aquificales* and the *Thermodesulfobacteriales*. All deep-sea hydrothermal environments generally support the growth of heterotrophs such as the *Thermococcales* and marine generalists like the *Desulfurococcales* and the *Archaeoglobales*.

The discovery of life in environments lacking sunlight and oxygen profoundly changed the way in which scientists view the history of life on Earth and the likelihood of life beyond Earth. It was suggested that deep-sea hydrothermal vents may be where life first began on Earth. Indeed, there is evidence of ancient microbial life in hydrothermal vents from more than 3×10^9 years ago. This life may have been thermophilic and hyperthermophilic living inside the crust and existing solely on geothermally derived gases, sulfur compounds, and minerals. Furthermore, there is evidence for past hydrothermal activity on Mars and for currently volcanic activity in ice-covered oceans on some of the moons of Jupiter and Saturn. If life can exist in hydrothermal vents on Earth, then it is possible that extraterrestrial hydrothermal vents could support life beyond Earth in the same manner. Therefore, extraterrestrial hydrothermal vent sites are targets for space exploration and the search for life beyond our own planet.

Shallow marine hydrothermal vents are found at water depths of less than 200 m. These include the summits of seamounts and the flanks of volcanic islands. Because of the lack of hydrostatic pressure, the hydrothermal fluid temperatures are much lower. The highest measured temperature at shallow marine vent is 135°C just 22 m below sea level. Shallow hydrothermal vents are in the photic zone and thus are influenced by photosynthetic processes that occur near the surface and are often iron or sulfur dominated. Hydrothermal processes create geochemically distinct features such as metal sulphide-poor mineral deposits and focused brines. Directly above hydrothermal discharges, there is an increase in richness of heterotrophs belonging to the *Thermococcales* and the *Thermoproteales* and autotrophs belonging to the *Aquificales*.

Terrestrial Hot Springs

Hot springs are heated, low salinity (0.1%–0.5%) ecosystems associated with active volcanoes. They have pH values ranging from pH 0.5 to pH 9. Steam vents (i.e., fumaroles) at hot springs are features where water boils away before reaching the surface and have steam temperatures that range 150–500°C. Solfataras are steam vents that are rich in hydrogen sulphide gases and transport these gases to the surface. Hot springs provide many electron donors to the hyperthermophilic microbial communities such as hydrogen, sulphide, and ferrous iron and electron acceptors such as oxygen and elemental sulfur. Hot springs are found globally, but are particularly well known in Yellowstone National Park in the US; Hokkaido Island in Japan; Krýsuvík, Hveragerthi, and Kerlingarfjöll in Iceland; the White Island in New Zealand; the Kamchatka Peninsula in Russia; and Pisciarelli Solfatara in Italy.

Research on thermophilic life in Yellowstone National park resulted in characterization of many new *Crenarchaeota* in the 1990s. Hydrogen metabolism is the major energy source in these springs, especially at higher temperatures. The hydrogen in the fluids is geothermally produced by the interaction of meteoric water with Fe(II). Oxygen and oxidized sulfur species are the major electron acceptors. Extensive studies at Yellowstone National Park have shown that hot springs have a range of geochemical gradients due to variations in pH, temperatures of diffusing fluids, and trace element and ammonia concentrations that provide a large biotope for different hyperthermophilic microbes as observed with the *Aquificales*-rich "filamentous-streamer" communities and *Sulfolobales*, *Desulfurococcales*, and *Thermoproteales*-rich "archaeal-dominated sediments". Similar to hydrothermal vents, some of the earliest signs of life have been found in ancient hot spring deposits that are more than 3×10^9 years old.

Hot springs have a range of geochemical characteristics. Some fluids are oxygen-rich and have a pH $\leq$ 2 and some fluids are anoxic, metal sulphide-rich and have a pH 4–6. Transition zones between them are often rich in elemental sulfur. The thermoacidophiles *Sulfolobus*, *Metallosphaera*, *Stygiolobus* and *Acidianus* are found in acidic, ferric iron- and oxygen-rich fluids whereas *Desulfurococcus*, *Thermoproteus*, *Pyrobaculum*, and *Thermofilum* are found in neutral pH fluids. In the Pisciarelli solfatara arsenate was shown to be an electron acceptor for *Pyrobaculum arsenaticum* in presence of thiosulfate and organic compounds. The Japanese coastal solfataric vents in Kodakara-Jima Island is where one of the first aerobic hyperthermophiles, *Aeropyrum pernix*, was isolated.

Marine Sediments and Hydrocarbon Reservoirs

It was estimated that 35% of all marine sediments are above 60°C suggesting that they provide a large habitat for thermophiles and hyperthermophiles. In these environments, buried organic material from the surface are likely to be the carbon and energy sources for the high temperature microbes that are present. This environment represents a new and largely unexplored habitat that may have an impact on the production and quality of natural gas and petroleum. Hyperthermophilic heterotrophs such as *Thermococcus* and *Pyrococcus* were the predominant microorganisms found at some depths in sediment cores collected offshore of New Zealand and Newfoundland, Canada. *Thermococcus* were also found in an uncontaminated oil reservoir in the Norwegian Sea, in a 90°C water-flooded oil well in China, and in Marcellus and Utica shale bed produced waters in western Pennsylvania. These organisms may be participating in the degradation of buried organics with concomitant hydrogen production. The organics and hydrogen present also support the growth of other thermophiles and hyperthermophiles such as hydrogenotrophic methanogens like *Methanothermococcus* and sulfate reducers like *Archaeoglobus*. The methanogens may enrich hydrocarbon reservoirs with biogenic methane making them more economically profitable while sulfate reducers can spoil a petroleum reservoir if too much sulphide is generated in the fluids. Preventing microbial oil spoilage can be accomplished by preventing the introduction of sulfate into the well or by the addition of inhibitory compounds such as nitrate. The upper temperature limit for life in hydrocarbon reservoirs is near 85°C.

Conclusion

The discovery and study of life that grows above 70°C have changed our perspective on life. We now have insight into how biological molecules such as proteins, DNA, and lipids can be thermostable at temperatures that normally denature these molecules. These organisms provide new enzymes and processes for industrial applications such as in vitro DNA replication using DNA polymerases from thermophiles and hyperthermophiles. They have also shown us unique adaptations to metabolic pathways such as the use of tungsten-containing enzymes and ADP-dependent kinases for glycolysis. High-temperature environments have shown us that life can exist entirely without sunlight and use chemicals provided from hot water-rock reactions. This has provided new ideas about life on the early Earth and the possibility of life on other planets and moons that once had or currently have hydrothermal fluid circulation below their surfaces. Future research will continue to identify and characterize novel thermophiles and hyperthermophiles, and will model and predict the impact of these organisms on the biogeochemistry of hot environments and their surroundings. These are becoming more relevant as we learn that hot environments are more widespread on the Earth than originally imagined. Hot environments can also be artificially created for industrial applications and the production of commercial enzymes and biofuels.

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Extremophiles: Hypersaline Environments

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Introduction

According to the definition of Oren (2015), hypersaline environments are those with salt concentrations more than twice that of seawater, and include solar salterns, salt lakes, saline soils and deep-sea brines, among others. Hypersaline systems are extreme environments and are thus inhabited by extremophilic organisms, namely halophiles or extreme halophiles. In a wide sense, halophilic organisms (or halophiles, "salt-loving" in Greek) are those that grow best in media containing from 1 to above 30% dissolved salts. Although there are no sharp limits between the different categories, halophiles can be classified as slightly halophilic (optimum growth between 1 and 4% salt; this category includes most marine microbes which cannot be considered as extremophiles), moderately halophilic (4%–15% salt), and extremely halophilic or hyperhalophilic (above 15% salt). Since most halophiles have an absolute requirement for sodium, some authors instead of "salt" refer to "NaCl" concentrations, using the same values.

The existence of extreme halophiles, among other extremophiles, was already acknowledged by Darwin in "The Voyage of the Beagle" in which he wrote "... Whether lakes of brine, or those subterranean ones hidden beneath volcanic mountains – warm mineral springs – the wide expanse and depths of the ocean – the upper regions of the atmosphere, and even the surface of perpetual snow – all support organic beings".

Types of Hypersaline Environments

Hypersaline environments are widely distributed in the planet. In fact, around 50% of continental waters are hypersaline. In addition, there are terrestrial hypersaline environments such as soils (like that of the hyperarid Atacama Desert), salt crusts or sub-surface salt deposits. Despite their harsh conditions, most of these systems are inhabited by an active specialized microbiota. Hypersaline systems can be classified according to the origin of the salts (marine or thalassohaline and non-marine or athalassolhaline) or to whether they are natural or artificial (for instance, salt lakes versus solar salterns). While coastal hypersaline systems are normally thalassohaline, inland salt lakes can either be thalassohaline or athalassohaline.

Salt lakes are hypersaline natural systems that, depending on the origin of their waters, their dynamics and the local climate conditions, may be exposed to sharp seasonal salinity changes. The Great Salt Lake in Utah and the Dead Sea are probable the first natural salt lakes ever studied and represent two very different systems: the first is thalassohaline and the second athalassohaline, with Mg^{2+} concentration so high as to inhibit the growth of even the most hyperhalophiles known. Soda lakes, with high salt and high pH (i.e., poly-extreme), are also among the first hypersaline systems studied and have been sources of a wealth of haloalkaliphilic microbes. During the last 10 years, the microbiota of more natural salt lakes has been characterized in detail. This is the case, for instance, of Lake Tyrrel in Australia (whose study allowed the formal discovery of a new archaeal phylum), the Tuz Lake in Turkey, Rumanian lakes such as Ursu and Ocnei, the $MgSO_4$ rich Hot Lake in Washington, the Aran-Bidgol lake in Iran, or the Siberian lake Novosibirsk.

Other high salt natural systems which are among the most extreme environments in the planet but still harbor microbial communities are the hypersaline Antarctic lakes, such as the Deep Lake, and the deep-sea hypersaline anoxic lakes (DHALs). Deep Lake constitutes a unique system in which water remains liquid at −20°C due to its high salt concentration (around 27%). DHALs are hypersaline basins located thousands of meters below the sea level, have salt concentrations 5–10 times higher than seawater, lack oxygen and light, and are exposed to high pressure. Since DHALs are denser than the overlaying seawater, there is an overlaying normally very stable pycnocline which can support a unique microbial community.

Finally, solar salterns used to concentrate salt by evaporation of seawater (or from hypersaline springs) represent the paradigmatic example of artificial solar hypersaline environments. Multi pond solar salterns consist of a series of interconnected shallow ponds with increasing salinity, from sea water to NaCl saturation. As the water gets pumped through the ponds, there is serial precipitation of salts and an increase of the total salinity. These changes in salinity are accompanied by a succession of different groups of microorganisms, from marine to extremely halophilic microbiota, each adapted to a salinity range. Thus, solar salterns can be used as a model to study how microbiota changes with salinity (Fig. 1), as discussed below.

Why is Salt Limiting Life and How do Microbes Cope With it?

Why are halophiles extremophiles or, in other words, why does salt limit growth? The first obvious answer is that salts "sequester" water and thus reduce the water available to biomolecules (water activity). In the case of cells, hyperosmotic media force the water to exit the cell which may collapse and suffer what is known as plasmolysis (the cell shrinks). In addition, although all solutes reduce

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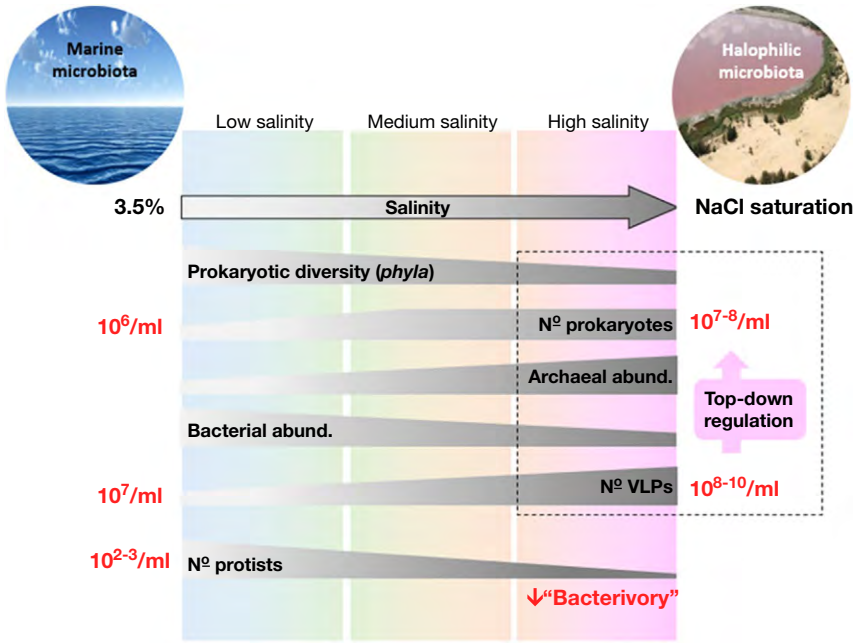

Fig. 1 Main changes in numbers and diversity of the microbiota along the salinity gradient from marine to NaCl saturation.

water activity, some, like $MgCl_2$, can act as chaotropes, weakening electrostatic interactions and destabilizing biological molecules. Other solutes (like NaCl) are in fact kosmotropes that strengthen these interactions and stabilize macromolecules. Thus, life in some hypersaline environments is limited not only by water availability but also by the chaotropicity of their salts. Such is the case, for instance, of the Dead Sea or deep sea hypersaline anoxic brines, mentioned above.

To cope with osmotic stress, microorganisms in hypersaline environments have developed two types of strategies to retain the water inside the cells and avoid plasmolysis, namely the "salt-in" strategy and the accumulation of compatible solutes. Salt-in strategy consists of the accumulation of high concentrations of inorganic ions in the cytoplasm, mainly potassium. This strategy implies that all the intracellular machinery is adapted to function in the presence of molar concentrations of salt, which implies having acidic proteomes. On the other hand, compatible solutes are organic compounds compatible with the cell metabolisms that accumulate in the cytoplasm and counterbalance external osmotic pressure. These two strategies allow the maintenance of turgor pressure, cell volume, and the cytoplasmic ionic concentration and are therefore essential for survival and growth in hypersaline environments. As a general rule, most hyperhalophilic microbes (like extremely halophilic *Archaea* and some *Bacteria* belonging to the *Haloanaerobiales* and *Bacteroidetes* such as *Salinibacter*) use mainly the salt-in strategy while other extreme halophiles and most moderate halophiles accumulate compatible solutes. Examples of such substances are glycerol, derivatives of aminoacids such as ectoine, hydroxyectoine, and the universal compatible solute glycine betaine.

Since the solubility of oxygen in water decreases with salinity, aerobic respiration can be compromised at high salt concentrations. In addition, very often hypersaline environments are exposed to high solar radiation. Likely these are the reasons why many extremely halophilic microbes harbor retinal binding proteins (RBPs or rhodopsins) that can transform solar radiation into metabolically active ionic gradients. In fact, microbial retinal-based proton pumps (that can be considered as the simplest photosynthetic apparatus that transform light into a H^+ gradient which is coupled with ATP synthesis) were first discovered in extremely halophilic *Archaea*, although at that time had not been considered as such and thus the proton pump was named as "bacteriorhodopsin" ('bacterio' being a holdover from older, "pre-archaeal" taxonomy). Another adaptation for protection of the high solar radiation that many hypersaline systems experience is the high presence of carotenoids in its inhabitants from the three domains of life. These pigments are responsible for the pink-orange colors that hypersaline waters offer display. In the case of the bacterium *Salinibacter ruber*, a frequent inhabitant of these systems, the pigments can act as antenna of the retinal-bindind proton pump xanthorhodopsin. This is highly unusual since antenna pigments are typical of other types of photosynthesis.

Microbiota of Hypersaline Environments: Solar Salterns as Models

Hypersaline environments are inhabited by organisms belonging to the three domains of life, although at very high salt concentrations (see Fig. 1) the system is dominated by extremely halophilic *Archaea* and *Bacteria* and a few (in numbers) representatives of the *Eukarya*, such as the phototrophic *Dunaliella* spp., some heterotrophic protists and fungi. Overall, the studies on microbiota

from different hypersaline environments show that salinity is one of the main drivers of microbial diversity in these systems, for the three domains of life. In this regard, it is useful to use the above-mentioned model of the solar salterns to classify the halophilic microbiota with respect to their salt requirements.

As a rule, as salinity increases from sea water to saturation (Fig. 1), overall prokaryotic diversity (at least at the phylum level, see Fig. 2(A)) decreases, *Archaea* numbers increase, and *Bacteria* and *Eukarya* numbers decrease. At salt concentrations from 20% to saturation, archaeal numbers are fairly stable while bacteria experience a sharp decrease (Fig. 3). In most close to saturation systems, mainly those from warm regions, total cell counts are in the order of 10^7–10^8 per milliliter (at least 10-fold over marine numbers) although there are examples of salt lakes and salterns with lower concentrations.

Overall, there is a transition along the salinity gradient from marine microbiota to a hyperhalophilic microbiota dominated by *Euryarchaeota* of the order *Halobacteriales* and frequently also members of de *Nanohaloarchaeota*. Very often, *Bacteria* of the *Bacteroidetes* group are also present as well as the green alga *Dunaliella*. In a wide sense, the range of different prokaryotic communities thriving at different salt concentrations can be divided into two groups: those living at salinities from seawater to 15% and the extreme halophiles inhabiting the 22%–37% salt range, with a mixed community in the intermediate range.

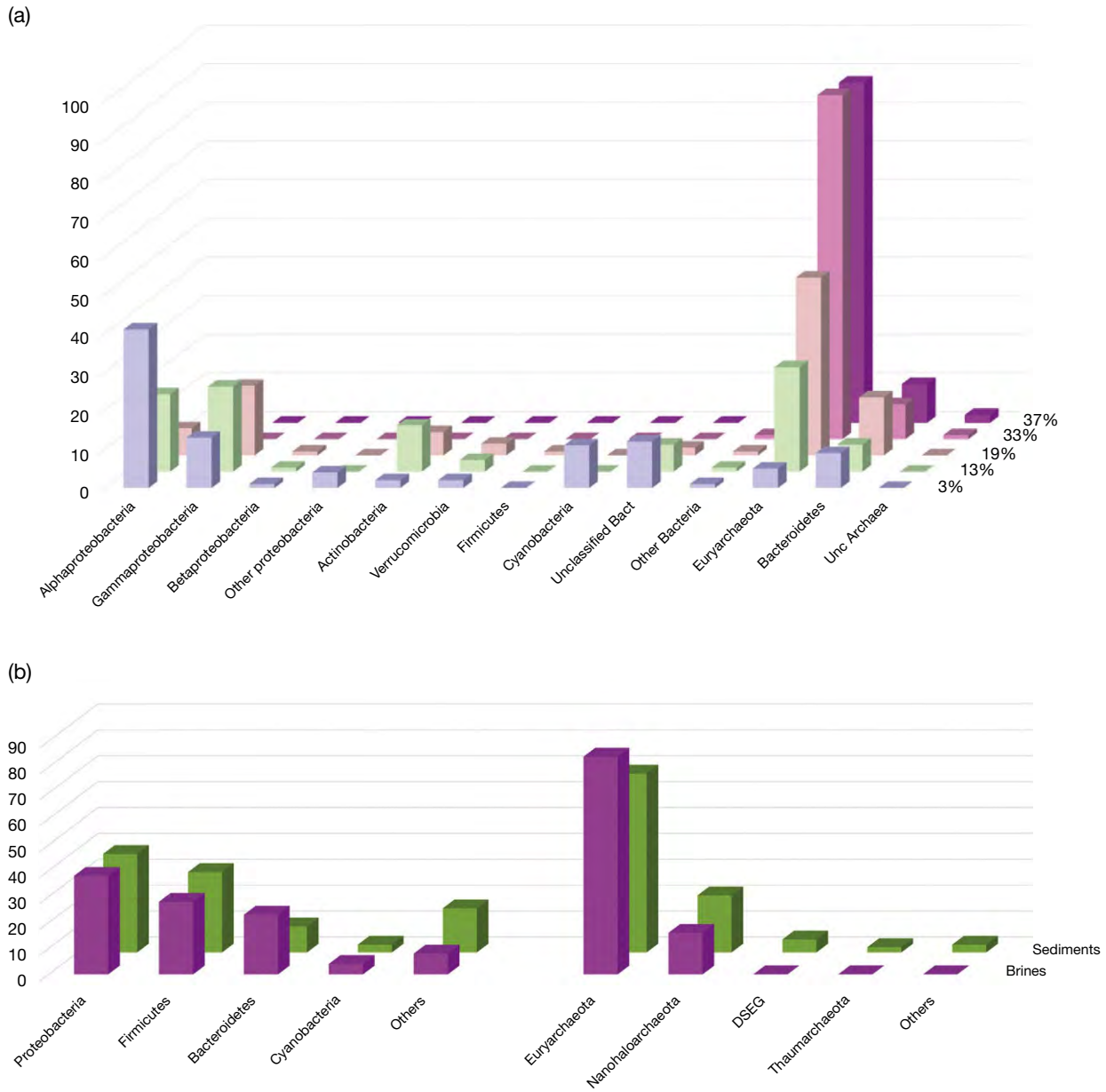

Fig. 2 Main prokaryotic groups present in (A) different ponds of Bras del Port solar salterns; (B) a collection of 50 brines and the overlying sediments from salterns around the world (in this panel, archaeal and bacterial values are referred to 100% independently.

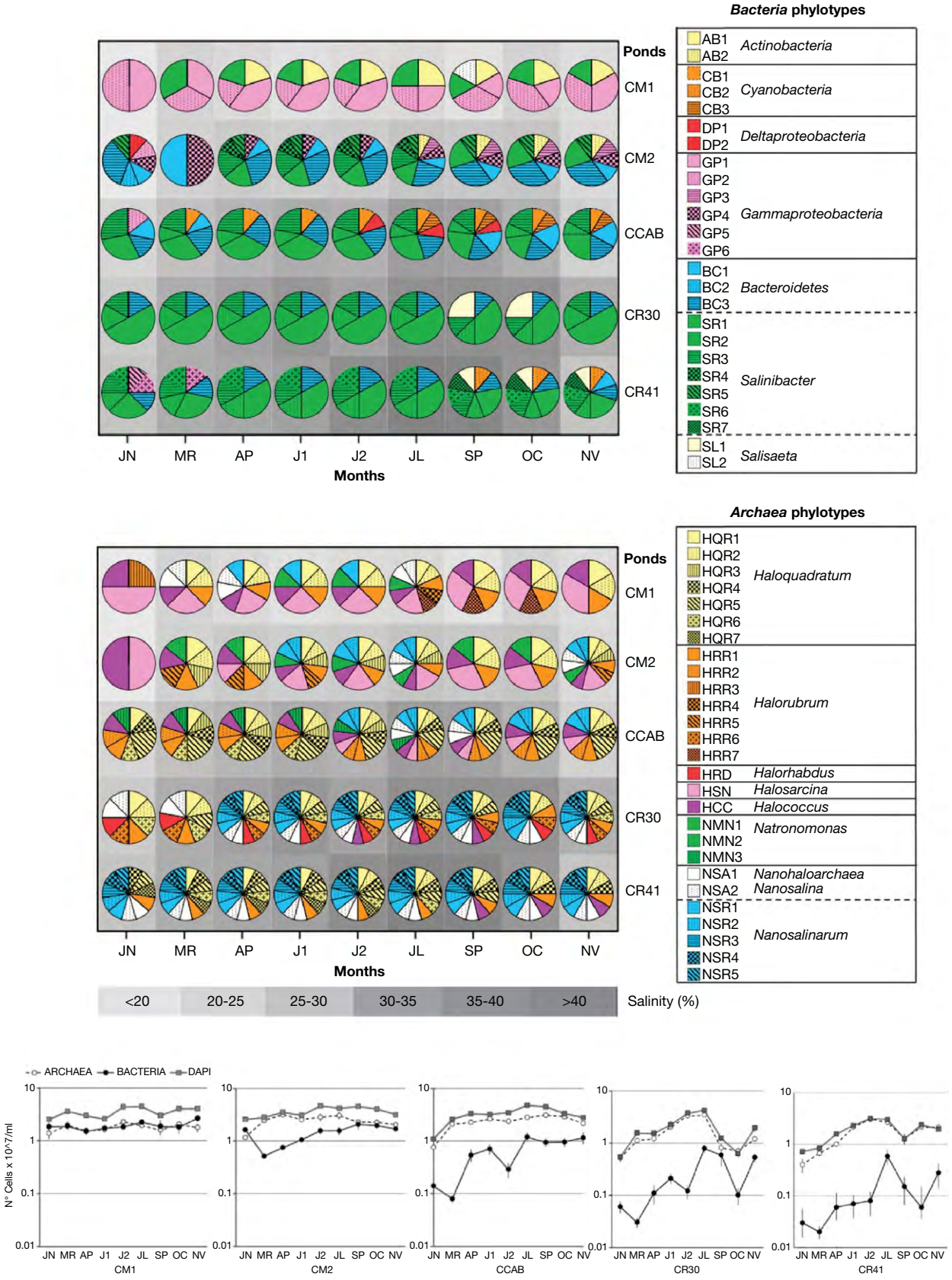

Fig. 3 Changes in the Bacteria and Archaea in five ponds of increasing salinity (CM1, CM2, CCAB, CR30 and CR41) from Bras del Port solar salterns, Upper panel show changes in diversity and lower panel, changes in numbers.

The oxygenic phototrophic assemblage also changes along the gradient: diatoms and green algae, present at lower salinities, are substituted by cyanobacteria and, at highest salinities, the green alga *Dunaliella* is the only phototrophic primary producer. However, as mentioned above, a large part of the prokaryotic community is able to use solar light to obtain metabolic energy by means of RBP.

In the lower part of the salt range, Alpha-, Beta-, Gamma- and Epsilonproteobacteria, together with *Bacteroidetes*, *Actinobacteria* and *Cyanobacteria* are the main *Bacteria* while *Archaea* are represented by *Euryarchaeota*. As the salinity increases, the presence of all the bacterial groups except *Bacteroidetes* decreases. At very high salt, *Bacteroidetes* (frequently from the *Salinibacter* group) dominate. Interestingly, in medium-intermediate salt ponds, high GC *Actinobacteria* are substituted by low GC, from which there are no currently isolated representatives. Conversely, the archaeal assemblage is being progressively dominated by *Euryarchaeota* of the order *Halobacteriales* and *Nanohaloarchaeota*.

The microbiota of the sediments underlying crystallizer ponds, although with some specific characteristics (see Fig. 2(B)), presents some overlap with that of the overlying brines. Furthermore, members of the archaeal MBSl-1 candidate division have been detected in anoxic hypersaline sediments. This euryarchaeotal group, for which a methanogenic metabolism was hypothesized, had been only previously detected in DHALs of the Western Mediterranean, providing an example of how different types of hypersaline environments can harbor similar microorganisms.

Until recently, all extremely halophilic archaea were included within the phylum *Euryarchaeota*. However, in 2011, two groups almost simultaneously described the presence in solar salterns of what was lately named as *Nanohaloarchaeota*. In fact, sequences of this group had been detected years ago in the African lake Magadi although they remained unclassified. This again provides an example of database bias. This phylum is included within the archaeal superphylum DPANN, formed by phyla belonging to the so-called "microbial dark matter", since no culture representative is available so far. Recently, many new groups have been included in the *Archaea* and *Bacteria* domains, such as the *Candidatus* Parcubacteria or the Woesearchaeota, which have also been detected in hypersaline environments. Their physiology, abundance and ecological relevance remain a mystery.

Very high salt systems, such as crystallizer ponds from solar salterns, are often dominated by the low GC euryarchaeon *Haloquadratum walsbyi* (the "square archaeon"), often comprising more than 60% of the archaeal assemblage. This organism has also been found in very high numbers in some solar lakes but there are other systems in which it is not relevant and can be outnumbered by other high GC euryarchaea like *Halorubrum* and *Halonotius* or other organisms.

One of the most intensely studied hypersaline systems are the multi pond coastal solar salterns Bras del Port, in Santa Pola (Spain), a widely documented example of how the description of natural microbial communities relies on the available techniques. In addition, these systems provide examples of how culture-independent techniques can guide the isolation of ecologically relevant microbes. The case of the gammaproteobacterium *Spiribacter* can be considered paradigmatic. This previously unknown bacterium was first detected as an abundant component of the community in a metagenomic dataset from a 13% pond. The genome assembled from the environment provided clues on how to culture this bacterium that was isolated shortly after. Before the description of this genus, bacteria readily isolated but present only at very low numbers in low-medium salinity ponds were taken as models for moderate halophiles.

Although the eukaryote assemblage along hypersaline systems has been way less studied than prokaryotes, high throughput 18S rRNA gene analysis has identified four salinity classes selecting for four different protistan communities: 4%–9%, 14%–24%, 27%–36% and 38%–44%. Overall, protistan diversity is remarkable, especially in the 14%–24% range, although the information regarding abundance is still scarce. In fact, some studies based solely on microscopy failed to detect protists at salinities above 30%. In the same way, the role that protistan bacterivory plays in controlling prokaryotic communities at very high salt concentrations is still an open question. It seems clear, however, that bacterivory decreases at very high salt, leaving viruses as the main biological factor controlling prokaryotic numbers.

Although close to saturation hypersaline systems where once considered steady-state systems and the same types of hyperhalophilic microbes were expected to be found all over the world, recent studies indicate that there are dynamic microbial successions in these systems, both for prokaryotes (Fig. 3) and eukaryotes. Furthermore, not all the high salt systems around the world are inhabited by the same microbial groups. Therefore, caution must be exerted when drawing general conclusions even for these "simple" environments.

Viral Communities in Hypersaline Environments

One of the most striking characteristics of close-to-saturation hypersaline environments is that they harbor the highest concentrations of virus-like particles (VLPs) reported so far for aquatic systems. These values range from 10^7 to 10^{10} VLPs/ml (Fig. 4). In multi-pond solar salterns there is a clear correlation between the salinity of each pond and its viral concentration. One could think that this increase in VLPs is due to the concentration of viruses present in previous ponds although metagenomic studies indicated that this is not the case, since each pond harbors a specific viral community. As with the host microbiota, viruses in the most hypersaline systems share some traits that differentiate them from the communities inhabiting at medium and high salt. In other words, there is a "hypersaline-ness" of the viral communities in very high salt systems, as there is a "marine-ness" of marine viral communities.

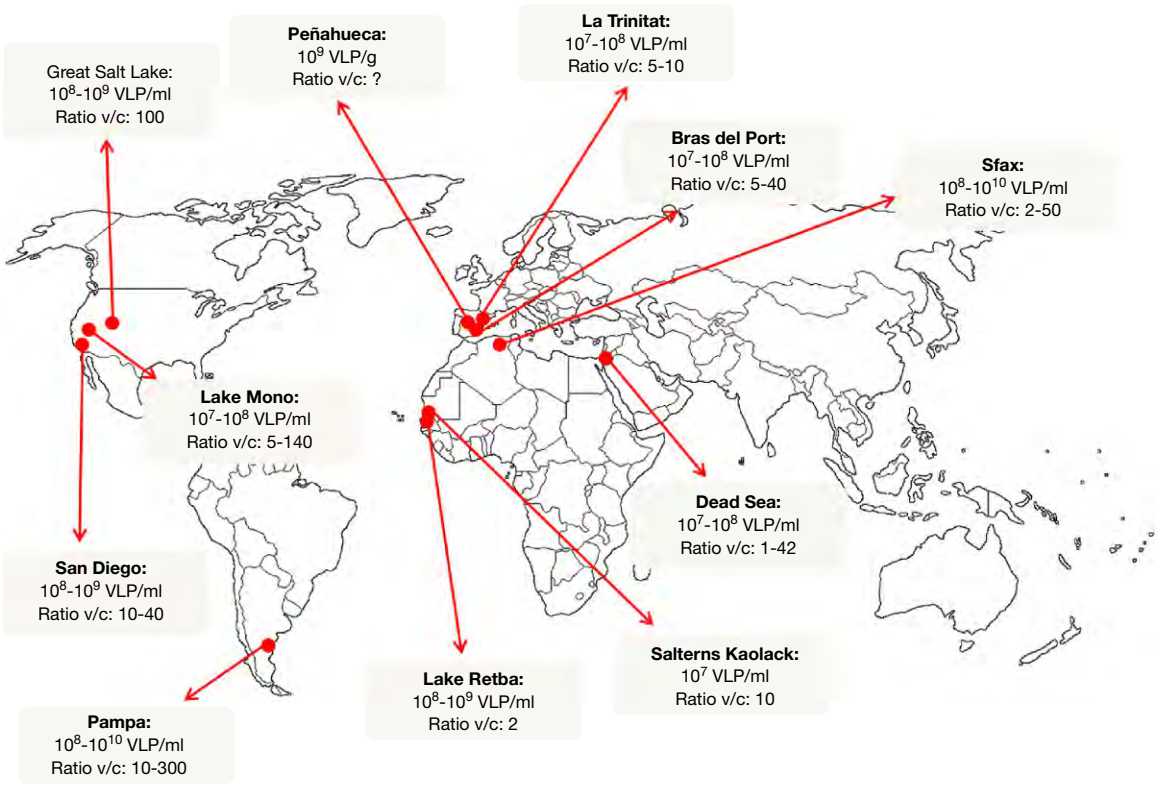

Fig. 4 Virus like particles (VLPs) concentrations and virus to cell rations measured in different solar salterns and salt lakes around the world.

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First Principles of Clinical Microbiology: Collection, Handling, and Diagnostics

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Introduction to Clinical Microbiology

Clinical microbiology focuses on the isolation and characterization of infectious organisms so they can be managed and treated in patients. Infections can be caused by bacteria, fungi, viruses, and parasites. To diagnose an infection, a sample must be collected from a patient at a body site where the detection of a pathogen or its associated biomarkers is likely to signify disease. The specimen must be transported to the laboratory in a manner that preserves the specimen for the intended testing. Then the specimen must be tested in a way that is sensitive and specific for the suspected organism causing the disease. Finally, these results must be communicated back to a clinician in a way that he or she can interpret and act on appropriately.

Clinical microbiology is arguably the first discipline of personalized medicine. As an example, a patient has the signs and symptoms of a urinary tract infection, including increased urgency, frequency, and pain with urination. A urine sample is collected and cultured quantitatively. Within 24 h, the clinical microbiology laboratory reports the species and quantity of bacteria found in the urine. 1–2 days later, the laboratory reports the susceptibility of that patient's bacterial isolate to a panel of antibiotics approved to treat urinary tract infections. The patient's clinician can then choose an antibiotic that is predicted to be effective against that patient's infection.

A pioneering microbiologist, Robert Koch, established the paradigm of clinical microbiology that is still in practice today. In proving that the bacterium *Bacillus anthracis* caused the disease anthrax, he developed what have become known as Koch's postulates which are: (1) the microorganism must be observed in all cases of the disease, (2) the microorganism must be isolated and grown in pure culture, (3) microorganisms from the pure culture, when inoculated into a susceptible animal, must reproduce the disease, (4) the microorganism must be observed in and recovered from the experimentally diseased animal. Many pathogens have been linked to disease by fulfilling these postulates. For these pathogens, clinical microbiology labs can diagnose infections in patients with compatible signs and symptoms by isolating the microorganism in pure culture, or detecting it by an alternative method. When Koch's postulates have not been met, interpretation of detection of the microorganism in a patient can be challenging or impossible.

Colonization and Infection

The primary goal of the clinical microbiology laboratory is to aid in the diagnosis of infections. However, many pathogens can live on or in a body site without causing signs and symptoms of disease. For bacteria, fungi, and parasites this is termed colonization, and establishes a normal flora of microbes in certain body sites. For viruses, an analogous process is latent infection, where the virus or viral genes persist within cells for long periods of time. Detecting colonization or latent viral infection can mislead a physician by incorrectly implicating the wrong microbe to explain a patient's medical condition. To accurately diagnose infections, the clinical microbiologist must have knowledge of the normal flora of various body sites. For example, *Streptococcus pneumoniae* is a frequent colonizer of the nasopharynx, particularly in young children. However, it can also cause meningitis and bloodstream infections. Isolation of *S. pneumoniae* from either the CSF or blood is compelling evidence that it is causing an infection whereas isolation from the throat is not. *S. pneumoniae* also causes pneumonia, but diagnosis can be challenging since the site of colonization is near the site of infection and may be sampled inadvertently. Therefore, diagnosis of pneumonia requires careful processes to ensure that the lungs, and not the upper respiratory tract, have been cultured.

Viral infections require integration of time and body location into diagnosis. Cytomegalovirus infects 40%–50% of people in the United States and approaches 100% in some countries (Specter, 2009). When it first infects someone, it may produce signs and symptoms of an infection. Once it establishes a latent infection within cells, it can persist for years or a lifetime without causing further symptoms. CMV can also reactivate from a latent infection to cause disease. Finally, some viruses cause an acute infection but are eventually cleared by the immune system. But the infection may trigger an immune response that can be measured as antibodies against the virus. If detection of antibodies (serology) is used as part of the diagnosis, then past infection must be distinguished from current infection, and latent infection must be distinguished from primary infection or reactivation.

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For many infections, colonization of one body site is a prerequisite for infection of another. This is particularly important for antibiotic-resistant bacteria, for which infections are difficult to treat. For example, colonization of the intestinal tract with vancomycin-resistant enterococci (VRE) is concerning because that patient may subsequently become infected or could spread that bacteria to another patient in the hospital. Therefore, clinical microbiology laboratories now also seek to identify patients colonized with microbes that could later cause significant infections.

Detection, Quantification and Characterization

At a minimum, diagnosis of infection requires detection of the pathogen or a pathogen-specific host response. Detection can be through targeted or non-targeted approaches. The enduring non-targeted approach, as pioneered by Koch, is culture. A specimen is introduced onto nutrient-containing media in either agar or a broth. The breadth of organisms that can grow on the media is limited only by the nutrients supplied and the time, temperature and atmospheric conditions of incubation.

Different combinations of media can be used to aid in detection and identification of suspected pathogens from a particular body site. Sheep blood agar supports growth of many, but not all, types of bacteria. Sheep blood agar can also differentiate bacteria for preliminary identification, as groups including Staplylococci and Streptococci hemolyze the blood creating clearings around their colonies. Media additives, such as antibiotics or other chemicals, can be used to select for certain organisms and aid in their rapid identification. MacConkey agar is selective for enteric bacteria such as *Escherichia coli* and *Salmonella* because it contains crystal violet and bile salts that are inhibitory to many other bacteria and differential because it contains lactose (Atlas, 2015). Most *E. coli* ferment lactose, reducing the pH of the media and causing the neutral red indicator to turn pink. *Salmonella* can grow in bile salts but doesn't ferment lactose and has clear colonies. The combination of sheep blood agar and MacConkey allows growth of many bacteria and can also aid in rapid diagnosis once a colony grows.

Targeted approaches to diagnosis include detection of molecules produced by the pathogen (antigens), pathogen-specific immune responses (antibodies or cell-mediated immunity), and RNA or DNA specific to the pathogen (molecular diagnostics). With these approaches, the timing of infection must be accounted for because they do not necessarily distinguish between viable and nonviable organisms. Antigens and nucleic acid may persist in the body for surprisingly long periods after clearance of viable pathogen. Pathogen-specific antibodies may last for the lifetime of the patient. In combination with direct detection of the organisms, these approaches can distinguish current from past infection, and primary, latent, and reactivation infections.

In addition to detection, diagnosis may also require quantification. Most diagnostic approaches in clinical microbiology focus on the detection of a pathogen that, when found in that body site indicates infection. But as mentioned above, colonization and latent infections can confound interpretation of simply detecting a bacteria or virus. Quantification is one method to improve the specificity of testing. For bacteria, quantification can aid in diagnosis of infections near sites of colonization, such as urinary tract infections. For viral infections, quantification is valuable in distinguishing latent infection from primary infection or reactivation. Latent infection by CMV can cause shedding of virus from mucosal surfaces and into the blood. Detection of a low level of CMV in the blood, along with the presence of CMV-specific antibodies would be consistent with latent infection. But higher levels in the blood, and an increase over time, indicate reactivation of the infection.

Characterization of a detected microbe is needed to determine its significance. Typically, identification to the species level is required to assess the likelihood of causing the infection and the prognosis. However, not all strains within a species may be pathogenic. Some strains have genes that enable virulence, the ability to cause disease, whereas other strains are avirulent. This variation is striking for *E. coli*, where some isolates colonize our intestines harmlessly, while others cause urinary tract infections, diarrhea, meningitis, and bloodstream infections. In recognition of these differences, Stanley Falkow created Molecular Koch's postulates to identify the genes that enabled virulence of certain isolates within a species (Falkow, 1988). This concept has begun to enter the clinical microbiology laboratory in the form of directed molecular tests. For example, diarrhea-causing strains of *E. coli* can be detected based on their gene content among the billions of bacteria and potential bystander *E. coli* in a stool sample from a patient. In other cases, antibiotic resistance is a primary concern in diagnosing both infection and colonization. From the intestinal tract, detection of VRE can be used to implement precautions to prevent spread to other patients, whereas detection of susceptible *Enterococcus* has little medical significance. Detection of VRE in the blood typically requires a rapid change in treatment to make sure an effective antibiotic is used.

Pre-Analytic, Analytic, and Post-Analytic Phases of Testing

Clinical microbiology testing, like all clinical laboratory testing, has three phases: pre-analytic, analytic, and post-analytic (Table 1). All phases are critical to making the correct diagnosis, and errors in any of them can lead to incorrect results and poor outcomes for the patient. The pre-analytic phase is all steps that take place before the samples are analyzed (Lifshitz, 2017). This includes assessing the need for the test, placing an order, determining the site for collection and collecting the sample, identifying the sample properly, transporting the specimen to the lab, and preparing the sample for analysis (Table 2). The analytic phase of testing includes inoculation and incubation of the specimen, detection, quantification, and characterization of pathogens, and recording test results (Table 3). The post-analytic phase includes the reporting of the test result, the clinician accessing the report and then interpreting the result, integrating it with other clinical data and acting on the result in the care of the patient (Table 4).

Table 1 Laboratory considerations for patients with suspected infection

Pre-analytic considerations	*Analytic considerations*	*Post-analytic considerations*
Differential diagnosis	Processing triage	Result interpretation
Test ordering	Assessment of specimen quality	Action taken
Specimen collection	Rejection criteria	
Specimen transport	Media selection	
Selection of transport device	Appropriate incubation	
	Examination/enumeration	
	Result reporting	

Table 2 Variables in pre-analytical phase of microbiology testing

Differential diagnosis	*Test ordering*	*Specimen type*	*Specimen collection*	*Specimen transport*
Bacteria	Culture	Body fluid	Timing relative to infection	Selection of transport device
Fungi	Molecular	Tissue	Cleaning of site	Temperature
Virus	Antigen	Swab	Method and equipment	Preserved vs. unpreserved
Parasite	Antibody	Foreign object		

Clinical microbiology laboratory acts as a consultant.

Table 3 Variables in analytic phase of microbiology testing

Processing triage	*Assessment of specimen quality*	*Rejection criteria*	*Media selection*	*Appropriate incubation*	*Examination/enumeration*	*Result reporting*
Centrifugation	Microscopy	Inadequate volume	Adequate nutrients	Temperature	Detection	Detected/not detected
Decontamination	Visual inspection	Improper temperature	Selective for suspected pathogen	Percent carbon dioxide	Quantification (culture, molecular)	Quantification
Homogenization	Measurement of volume	Contamination or incorrect body site	Differentiates pathogens	Oxygen/ reducing capacity (anaerobic organisms)	Characterization (species identification, antibiotic resistance, virulence genes)	Characterization of pathogen, including interpretation of antimicrobial susceptibility testing (if applicable)
Separation	Indicators of oxygen exposure				Flora, contamination, or pathogen	Comments on flora or contamination

Clinical laboratory assumes responsibility for testing.

Table 4 Variables in post-analytic phase of microbiology testing

Result interpretation	*Action taken*
Is this result consistent with the signs, symptoms, and other clinical data from the patient? Is there an alternative explanation?	Supportive care
Does this represent colonization, active infection, latent infection, past infection, or contamination?	Addition or alteration of prescribed antimicrobials
Does the infection need to be treated?	Additional diagnostic testing

Clinical laboratory provides education on result interpretation and acts as a consultant.

Although errors can occur at any stage of testing, the majority of testing errors occur in the pre-analytic phase. In this phase, the microbiology laboratory consults on which tests to order to diagnose a suspected infection, what type of specimen to collect, and how to transport the specimen. The clinical microbiology lab assumes direct responsibility for the testing towards the end of the pre-analytical phase when the specimen is received in the lab and is responsible throughout the analytic phase until the result is reported in the patient's medical record. In the post-analytic phase, the laboratory can educate the clinicians on how to interpret the result.

Specimen Collection

Proper specimen collection in the pre-analytic phase is essential to diagnose infections. This responsibility rests with the ordering clinician, who makes the choice of specimen site and collection technique. Based on the patient presentation, the clinician must make a list of pathogens that may be causing the patient's disease. Next, the clinician needs to decide on the best specimen to make the diagnosis. In general, there are four types of specimens for microbiology: tissue, body fluids, swabs, and foreign objects. Often the decision of specimen type is straightforward. If suspecting a bloodstream infection, the clinician should collect a sample of the patient's blood. However, sometimes the choice of specimen is more complex. If suspecting pneumonia caused by *Legionella pneumophila* or *S. pneumoniae*, the first specimen collected may be urine to detect a pathogen-specific antigen since this approach is rapid, relatively inexpensive, and usually non-invasive for the patient. Collecting multiple specimens consecutively or concurrently can increase the chances of making the diagnosis. For either of these organisms, the clinician may also collect a lower respiratory specimen for detection by culture or molecular methods, and a blood culture may detect *S. pneumoniae* in a patient with pneumonia.

In addition to collecting from the most informative site, the amount of specimen collected determines the chances of detecting the pathogen. With few exceptions, more specimen is better. In the case of diagnosing a bloodstream infection, the standard of care is to collect multiple specimens over time and with relatively large volumes (16–20 mL for an adult). This is because, despite the dramatic symptoms and deadly outcomes of bacteremia, the numbers of circulating bacteria (colony forming units, or cfu) can be less than 5 per mL. For other body fluids, again more is better and the laboratory can concentrate the specimen if needed. If the pathogen is suspected to be infecting body tissue, then as large a specimen as is safe and feasible should be collected. A saying in clinical microbiology is "Give me a liter or a lobe" (Paxton, 2004).

The method of specimen collection significantly impacts both the likelihood of detecting the pathogen. Swabs are a common method of collecting specimens for microbiology testing, but are only useful to diagnose certain infections. They are ideal for diagnosis of upper respiratory and urogenital infections, where vigorous sampling can collect infected epithelial cells and exudate. Swabs are less useful for diagnosing infections of tissue since they are only sampling the surface of the infected site. A biopsy of the specimen is almost always superior (Baron et al., 2013).

Collection technique is critical to avoid contamination with members of the body's colonizing bacteria, or microbiota. For bloodstream infections, the skin overlying the sampled must be cleaned rigorously, and the rates of contamination by skin bacteria are a standard measure of quality of care in a hospital. Similarly, urine must be collected in a way that minimizes contamination by bacteria colonizing the urethra and surrounding skin. Sampling the lung either requires protecting the sampling instrument on its way through the throat, or demonstrating that the sample is from the lower respiratory tract based on the type of human cells present in the specimen.

Timing of specimen collection is also important for both correct interpretation of results and maximizing chances of recovering the pathogen. The *Plasmodium* parasites that cause malaria periodically lyse out of red blood cells causing the symptom of fever. Therefore, collecting a specimen before a fever increases the chance that they will be detected within red blood cells by microscopy. Culture-based methods rely on a specimen with viable organisms, so at least one specimen should be collected prior to giving antibiotics. The timing of specimen collection relative to the suspected onset of infection is also important. If collected early, the pathogen may not have replicated sufficiently to be detectable in the specimen. If collected late, its density may be waning. Pathogen-specific host responses develop over time and the timing of specimen collection can determine if a response is detected, and if that response indicates past or current infection.

Specimen Transport

Once a specimen is collected, it must be transported to the laboratory in a manner that preserves the viability of the pathogen or the analyte that will be detected. For culture-based testing, preserving viability is paramount. Transport is usually at room temperature, but can differ with the type of microbe suspected (Leber, 2016). Swabs are often inserted into a tube with liquid media for transport, and separate transport devices are available for bacteria and viruses to preserve their viability. Bacteria with anaerobic metabolism may be killed by exposure to oxygen. Large pieces of tissue and volumes of fluid preserve viability and anaerobic conditions for short periods, reinforcing the hyperbole of "Give me a liter or a lobe." Smaller samples should be placed in specialized transport tubes that maintain anaerobic conditions and have color (E_h) indicators to determine if oxygen has contaminated the specimen. Swabs are generally a poor choice for anaerobic cultures because they sample the surface of the infection and not the infection itself, but swab transport media are effective at maintaining viability of any anaerobes recovered. Blood culture bottles serve as both the transport and culture vessel. For molecular diagnostic tests, transport tubes may have additives that preserve DNA and RNA but not the pathogen itself. For each test that can be ordered, clinical microbiology laboratories publish handbooks stating what body site of specimen to sample, what volume is suggested, the minimum required volume, the type of tube or container to be used, the temperature for transport, and how quickly the sample must reach the laboratory.

Specimen Processing

Once the specimen reaches the laboratory, usually it must be processed before it can be tested. Specimen processing involves concentration, decontamination, homogenization, separation, or a combination of these processes. These choices are made based

on a combination of what the clinician ordered and established policies in the laboratory, shifting responsibility for the specimen from the clinician to the laboratory.

Large volumes of fluid can be concentrated by centrifugation, pelleting the pathogen and human cells at the bottom of the tube. Although receiving more specimen is better, there are limits on the volume that than be added to a culture plate or tested in an instrument. The supernatant is removed and this pellet, potentially enriched in pathogens, can be tested further.

Decontamination is important to detect slow growing pathogens like *Mycobacteria* and *Legionella*, where small numbers of contaminating bacteria can overgrow the pathogen of interest. This can obscure the detection of these organisms in broth and agar cultures. Decontamination techniques use harsh chemical treatments, such as sodium hydroxide, that *Mycobacteria* can withstand more readily than other types of bacteria and acid shock that enriches for *Legionella*. This is a balancing act, and laboratories aim for low but detectable rates of contamination to indicate that the process was not so harsh as to also kill the suspected bacterium.

In a specimen with multiple anatomic parts or fluid phases, the choice is to combine through homogenization or separate. Tissues can be homogenized by sterile, mechanical processes such as tissue grinders and Stomachers, combining specimen with saline or culture media to release pathogens for detection. In contrast, blood can be tested whole, or separated into serum or plasma components. The type of blood transport tube determines the blood compartment that is tested, so the clinician must again decide where to look for the pathogen to maximize chances of detection.

To process a specimen properly, the technician must predict if the specimen contains one or multiple bacteria. This can determine if testing should be done in a liquid form or on agar. In specimens such as blood and sterile body fluids, there is usually only one pathogen, so the specimen can be tested as a homogeneous liquid by culture or other means. Other specimens will have multiple microbes present, either because they are part of a polymicrobial infection, or the pathogen and some colonizing microbes may be present. These specimens should be processed in a way that enables detection of each microbe separately. Through culture, this means inoculating the sample on selective and differential agar so colonies of each bacterium can be visualized and analyzed separately. For molecular methods, the test must detect multiple microbes as well as it detects single pathogens. Often if one pathogen is predominant, it can consume reagents in a molecular test, analogous to contaminating bacteria overgrowing mycobacteria in culture. To interpret mixed cultures, quantification can be helpful, but this must be anticipated at the time of specimen processing. Quantitative cultures on solid media, using a defined inoculum, can distinguish infection from colonization as discussed above for urine cultures. Even if the volume of sample cultured was not precisely measured, relative quantitation can be done to indicate that one pathogen is predominant in the sample.

Specimen Testing

Testing methodologies each have their own benefits and drawbacks relative to the pathogen being sought. Direct microscopy of a specimen provides a rapid and inexpensive way to detect pathogens. In addition to the Gram stain for bacteria, specific stains for fungi, mycobacteria, and blood parasites are routinely performed. The Gram stain also allows evaluation of human somatic cell types present in the specimen as a measure of specimen adequacy and risk of contamination from colonized body sites. The sensitivity of direct microscopy depends on the volume of sample used and the ability of the stain to highlight the organism. Fluorescent stains can increase the sensitivity of microscopy, and are used routinely to detect fungi and mycobacteria.

Antigen detection is of primary utility in non-invasive investigation of infections that infect deep tissues or fluids. The specimens tested are typically blood or urine that can be readily obtained from a patient. These approaches are particularly useful for fungal infections, where culture is slow and may require invasive procedures to sample the lungs or other sites of infection. Antigen detection may also be helpful in stool to diagnose parasitic infections. The main challenge with these approaches is cross-reactivity with other antigens found in these body fluids or other pathogens that cause a similar disease presentation.

The utility of antibody detection (serology) varies widely and is specific for each pathogen. In general, serology is used when other direct approaches of detection do not perform well. For fastidious bacteria and fungi, serology may be the only reliable method to determine if a patient has been infected. In other infections, the pathogen is present at very low concentrations or only transiently, making direct detection difficult. Furthermore, the patient may present with symptoms as the pathogen burden wanes. For example, encephalitis from arboviruses (e.g., west Nile virus) is most effectively diagnosed by serology from cerebrospinal fluid as opposed to viral detection. Serology is one of the few diagnostic methods that can provide information about time of infection, but the interpretation is specific to each pathogen. The clinician must have a basic understanding of the immune response to the suspected infection to know how long it takes to mount an initial IgM antibody response, when an IgG response may emerge, and how long each of them persist.

The strengths and drawbacks of culture are implicit in the fact that it requires growth of the organism of interest. The strengths are that it is a largely unbiased approach that may identify rare organisms causing a disease. It is also inexpensive. The main drawback is that culture is only as fast as the organism's growth rate and requires hours to days. It is also limited by which organisms the culture media can support for growth. Culture is a largely manual method, but the development of automated systems for specimen processing, incubation, and monitoring of growth is likely to speed up time to diagnosis and further reduce costs through decreased personnel time.

Current molecular detection methods are targeted to one or a set of pathogens of interest. These can be performed directly from a specimen or used for pathogen identification after culture. Molecular assays can either give a qualitative result of present or absent, or quantify the pathogen relative to the volume of sample. The benefits of molecular methods are exquisite sensitivity and specificity and precise quantification of pathogens. The main drawbacks are their directed nature and high cost. Unbiased sequencing

techniques, that identify nucleic acids directly from samples, are an intriguing approach to rapidly identify and perhaps quantify specimens in patient samples. Although not currently in wide use, these approaches have the potential to disrupt and revolutionize clinical microbiology.

Antimicrobial Susceptibility Testing

Once a pathogen has been detected, antimicrobial susceptibility testing is a key part of clinical microbiology testing. A few pathogens have predictable susceptibility or resistance to certain antibiotics, so further testing is unnecessary. But for pathogens where isolates vary in their ability to resist an antibiotic, antimicrobial susceptibility testing is critical to guide effective treatment of the infection. Bacteria, yeast, and mycobacteria are routinely tested for susceptibility in clinical laboratories. Anaerobic bacteria, molds, and viruses are more difficult to test, so susceptibility testing is usually performed in specialized reference laboratories or in response to treatment failures.

The goal of antimicrobial susceptibility testing is to predict if an infection with the detected pathogen can be treated with normally achievable antibiotic concentrations at the site of the infection. In most cases this is done through phenotypic testing. Varying concentrations of an antibiotic are added to a culture of the pathogen isolated from the patient, and the minimum concentration of antibiotic that inhibits growth is recorded (Minimum Inhibitory Concentration, MIC). This result is interpreted as the isolate being susceptible or resistant to the antibiotic based on what is known about patterns of resistance within the microbial species and pharmacokinetics and pharmacodynamics of the antibiotic in patients.

Genetics of the microbe often determines if it is susceptible or resistant to an antibiotic, so genotypic testing can also be useful. When the mechanistic relationship between a gene and resistance to an antibiotic is robust and predictable, molecular tests with their exquisite sensitivity and specificity can produce rapid and reliable results. However, the genetic diversity of microbes is such that many mutations or genes could lead to resistance against the same antibiotic. In these cases, phenotypic testing is more useful than genotypic testing. Again, as molecular techniques move towards unbiased approaches, rapid detection of multiple genotypes that yield the same phenotype may be possible.

Concluding Remarks

Clinical microbiology is focused on the detection, characterization, and quantification of pathogens from patient samples to enable the diagnosis, treatment, and management of infections. Clinical microbiologists must be experts in all aspects of pre-analytic, analytic, and post-analytic phases of microbiology testing. We must educate clinicians on the critical aspects of test selection, specimen collection and transport, and result interpretation that occurs outside the laboratory. We must also ensure excellent quality of testing within the laboratory, from the time when the laboratory receives the sample until the result is posted. This requires a combination of biological knowledge of how and where microbes colonize, remain latent, and cause symptomatic disease as well as an understanding of the analytical principles, strengths and drawbacks of each testing modality. As new technologies are introduced to the field, clinical microbiologists will be able to diagnose infections more rapidly and precisely than ever before.

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Foodborne Pathogen Detection, Using Rapid Technologies

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Glossary

Amplicon A piece of DNA resulting in an amplification process.
Antibody Glycoprotein (immunoglobulin) molecule developed by the body in response to, and interacting specifically with, an antigen, as part of the body's immune response.
Antigen A foreign substance that induces the formation of antibodies in the body that react to that substance, specifically.
Bacteriophage A virus that infects bacteria; often called a phage.
Bacterium A prokaryotic, microscopic, one-cell microorganism found as a free-living organism/parasite, which multiplies by binary fission.
Base pairs (bp) Formed by hydrogen bonds between purine and pyrimidine nitrogenous bases on opposite strands of DNA or RNA. Abbreviated bp, the length of a particular genome is usually measured and reported in bp.
DNA polymerase The enzyme that synthesizes or builds DNA from a DNA template using nucleic acids. The intact enzyme purified from bacteria has the ability to synthesize and edit DNA sequence. The editing function comes from nuclease activity.
Endotoxin A heat-stable lipopolysaccharide that is found in the outer membrane of Gram-negative bacterial cells. The toxin is released from the cells when lysis occurs.
Enterotoxin A toxin released from several types of bacteria in the intestines that specifically affects the host's intestinal mucosal cells, which causes vomiting and diarrhea.
Exonuclease An enzyme that digests or degrades nucleic acids starting from the 5′ or 3′ terminus and extending inward.
Expression To 'express' a gene (DNA or RNA sequence) is to cause it to work. A gene that encodes a protein will be transcribed and translated, when expressed, to produce a specific protein.
Gene library A genomic library is a mixture of thousands of different clones containing a vector (e.g., derived from bacterial plasmid, or phage) carrying an insert, the cloned DNA.
Immunoassays It is a serological technique that measures the presence or concentration of analyte (antigen) with the help of homologous antibody.
Internal control Nontarget analyte added to the test reaction to judge performance and validity of a particular test. This material should generate a positive result when testing conditions are optimal.
Klenow fragment A genetically modified version of bacterial DNA polymerase that has the polymerase function still active, but the polymerase 5′ → 3′ exonuclease activity has been removed.
Midivariant RNA sequence A 220-nucleotide-long sequence that is recognized by Q-beta replicase enzyme. The enzyme binds to the sequence and starts amplification.
Multiplex PCR The amplification of multiple targets by different PCR primers within the same reaction tube.
Neurotoxin A toxin released from bacteria that damages, destroys, or impairs the functioning of nerve tissues.
Polymerase chain reaction A molecular technique for synthesizing a specific sequence of DNA *in vitro*, even in the presence of excess nonspecific DNA. Primers (short DNA oligonucleotides) are added in the presence of nucleotides and Taq polymerase. When the temperature is changed, the target DNA sequence is repetitively denatured and copied. PCR can amplify sequences after converting the RNA to DNA using reverse transcriptase. This is called 'RT-PCR'.
Primer A small oligonucleotide (anywhere from 6 to 50 nucleotides long, sometimes longer) used to bind and prime DNA synthesis. A primer that binds to the DNA template is used to start the replication. Primers are important for DNA sequencing and PCR.
Restriction endonucleases These enzymes are among the most useful in recombinant DNA technology, capable of introducing a single cleavage site into a nucleic acid. The site of cleavage depends on the sequence; recognition sites contain from 4 to 10 specific nucleotides. The produced digested ends of the nucleic acid chain may either be blunt or contain a 5′ or 3′ overhang ranging from one to eight nucleotides.
Sensitivity The ability (probability) that a particular test will give a positive result when the test sample is positive for the targeted analyte (i.e., no false negatives).
Shotgun cloning It is a method used to amplify genomic DNA by cloning small DNA pieces generated by a cut using a restriction enzyme or a physical method. These DNA fragments are cloned into a vector (plasmid).
Single nucleotide polymorphism (SNP) A genetic variation resulting from a single nucleotide difference in the genome of a species. These differences can be exploited, for example, to discriminate strains of bacteria. This discrimination may help with determining a pathogenic from nonpathogenic strain or aid in tracing the source of an outbreak of illness.
Specificity The likelihood (probability) that a particular test will produce a negative result when only nontarget analytes are present (i.e., no false positives).

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Worldwide there are at least 200 different diseases known to be associated with bacterial or viral contamination of food related to bacterial and viral infections that are known to be transmitted by food. A recent study by the US Centers for Disease Control and Prevention found that 31 pathogens caused 9.4 million foodborne illnesses, 55 961 hospitalizations, and 1351 deaths. However, an additional 38.4 million illnesses, 71 878 hospitalizations, 1686 deaths were found to be related to unspecified food-related agents, suggesting that we have only begun to understand the scope of the problem (Scallan et al., 2011). We must not only use all the research tools at our disposal but also develop new technologies for identifying bacteria and adapt those tools for efficient use in clinical microbiology laboratories, farms, and food production areas. Molecular methods can play an important role in facilitating rapid and specific detection of pathogens on the farm before the food enters a global distribution network and in the clinical microbiology laboratory after occurrences of foodborne infection or poisoning in patients. Molecular methods can work as screening and qualitative (absence/presence) tools as well as quantitative measurements.

Although this article focuses on newer molecular and immunological methods, classical microbiology methods (not addressed in this article) are still important. Regulatory agencies such as the US Food and Drug Administration and the US Department of Agriculture require traditional isolation and culturing of foodborne pathogenic bacteria to verify contamination. Many of those methods rely upon the ability to first obtain an isolated organism.

Conventional Polymerase Chain Reactions

As with most technological advancements, there was a beginning that laid much of the necessary groundwork. In the case of modern molecular biology, the starting point is described in the 1953 paper published by James Watson and Francis Crick in the journal *Nature* (Watson and Crick, 1953a,b). In the article presenting a proposed structure for deoxyribonucleic acid (DNA), they state, "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material."

About 20 years after the publication of the Watson and Crick paper, the conceptualization of a DNA copying process was initiated (Kleppe et al., 1971) and later demonstrated (Saiki et al., 1985), leading to the invention of polymerase chain reaction (PCR), largely attributed to Dr Kary Mullis in 1983 while employed by the Cetus Corporation (Mullis et al., 1986). Six components are essential to the performance of PCR: the target DNA template; the deoxyribonucleotides (dNTPs) consisting of a nitrogenous base (adenine, guanine, thymine, or cytosine – usually abbreviated A, G, T, or C, respectively), a deoxyribose sugar, and a phosphate group; a DNA polymerase, the enzymatic driver that catalyzes copying of genetic material; a buffer to maintain a desired pH level; divalent cations (usually added as $MgCl_2$) needed for DNA polymerase function; and a primer(s) or a short piece of single-stranded DNA that will help 'prime' the copying of the target DNA by providing the empty hydroxyl group on 3′ end for sequence-specific incorporation of the dNTPs. The specificity of PCR is driven by the primer design. Most often, primers are carefully designed based upon knowledge of the nucleic acid sequence of target DNA so that it will bind in a specific region of the genome.

Briefly, the steps of PCR include heat *denaturation* to insure that the native double-stranded DNA (dsDNA) is now in a single strand form and ready to be copied, *annealing* of the primer(s), followed by the *extension* of the primer by the incorporation of the purine and pyrimidine, bases which occurs in a sequence complementary fashion (Figure 1). Before the discovery of a heat-stable DNA polymerase from *Thermus aquaticus*, a thermophilic bacterium (Chien et al., 1976), DNA polymerase had to be added after every denaturation step. After one cycle of PCR, the starting amount of DNA has doubled. Given this exponential growth, a theoretical 10^{12} copies are possible from a single target molecule after 40 PCR cycles (Figure 2). PCR automation is possible by the many commercially available thermocyclers that are now commonplace in even the most remote and resource-poor laboratories.

Conventional PCR utilizes end-point detection and sizebased discrimination by gel electrophoresis of any amplified DNA (Figure 3). In an agarose gel, the DNA is exposed to an intercalating agent like ethidium bromide and visualized under ultraviolet light. While conventional PCR offers advantages like increased sensitivity and specificity over traditional methods (e.g., biochemical profiling) for pathogen detection and characterization, there are some substantial limitations. Smearing can occur when too much DNA is loaded on the agarose gel and the resolution between similar-sized DNA amplicons is nearly impossible. Finally, to be semiquantitative, this technique requires to stop the amplification process on the exponential phase of the PCR (when initial DNA quantity but not dNTP, enzyme or primers is the limiting parameter of PCR product formation). Thus, the results with conventional PCR are more qualitative (i.e., absence/presence) than quantitative.

Quantitative Real-Time PCR

The technique of quantitative real-time PCR (qPCR) has existed for over a decade and was largely built upon the fundamentals used in conventional PCR (Saunders, 2009). DNA is still copied but master mix components that produce fluorescence are needed and a more sophisticated thermocycler with fluorescence detectors allow for increases in amplification to be measured as the procedure is happening (in 'real time'). This means that initial concentrations of pathogen DNA (or copy number) and small differences in quantities can be determined because amplification is monitored and data captured during each round of cycling. Figure 4 shows a typical qPCR amplification plot. After 30 or more cycles, DNA amplification reaches a plateau as the master mix components are depleted. Thus, by waiting until PCR completion to view amplification, as is done typically in conventional PCR, only the final plateau is observed and any differences in initial abundance are obscured. Also, eliminating the need to open PCR tubes, containing

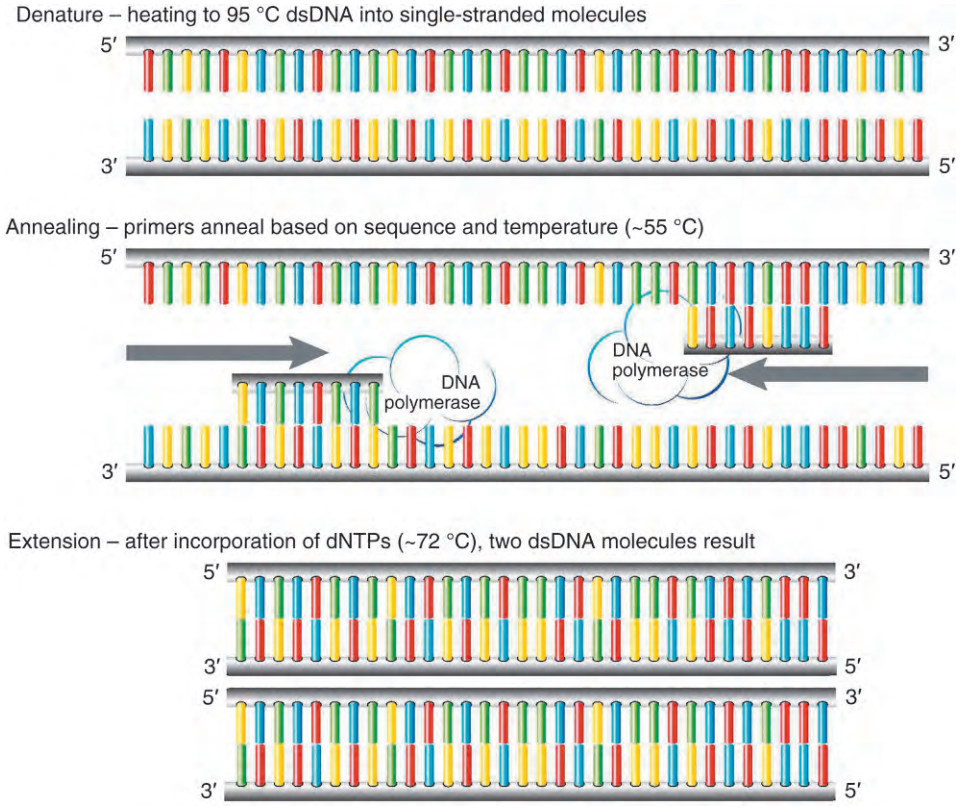

Figure 1 The three steps of PCR. The initial double-stranded DNA (dsDNA) piece is denatured to form two single-stranded molecules that can be copied to result in two dsDNA pieces after one round of amplification.

amplified product, means reducing the chance of contamination and time to result by at least 1 h. The differences between conventional (end-point) PCR and qPCR are summarized in Table 1.

With qPCR, fluorescence can be generated in a sequence-specific or non-sequence-specific manner; however, in both cases the fluorescent intensity is proportional to the amount of PCR products produced. The simplest, or nonspecific, approach employs the use of DNA intercalating chemicals like SYBR® Green that will emit a strong fluorescent signal when interwoven in dsDNA. Since all dsDNA will be detected, careful examination of the results is necessary to avoid misinterpretation of a positive result when target DNA was not present or to avoid an overstatement of the starting amounts of pathogen DNA. This method requires the analysis of the *melting curve* performed on each PCR reaction after the amplification program has finished. Any double-stranded PCR amplicon will dissociate in a temperature-dependent fashion mostly contingent on the guanine/cytosine content. Postamplification and within the thermocycler tubes are slowly heated to 95 °C while fluorescence is measured. As the doublestranded PCR amplicon dissociates or 'melts,' fluorescence will rapidly decrease since any DNA intercalation by chemicals that fluoresce will be lost. The point at which 50% of the DNA dissociates is referred to as the temperature of melting or T_m and is graphically represented as a peak when temperature is plotted against the negative first derivative of the fluorescence signal.

A more specific way of generating fluorescence most often consists of a donor and acceptor molecule linked to a primerlike short piece of DNA, or oligonucleotide probe. When the donor fluorophore or dye is excited by the thermocycler light source, fluorescence is produced and possibly transferred to an acceptor dye. The transfer occurs by fluorescence (or Förster) resonance energy transfer (FRET), a highly distant-dependent interaction where the reporter and quencher molecules must be in close proximity (10–100 Å). With FRET, a photon is not emitted by the donor dye, but rather the energy is transferred to the acceptor molecule. Depending on the strategy, the fluorescence emitted by the donor (e.g., hydrolysis probe) or the acceptor (e.g., hybridization probes) is monitored during the course of the experiment by instrument software.

Many qPCR chemistries exist and still more are under development every day. Some popular ones include hydrolysis probes (e.g., TaqMan® probes), molecular beacons, hybridization or FRET probes, and Scorpions® probes. Conceptually, hydrolysis probes are easy to understand and provide an excellent example of FRET. In this case, the acceptor molecule is a quencher, a fluorescent dye, or any other molecule that will absorb emitted light energy from the reporter molecule, effectively eliminating or quenching reporter molecule emission fluorescence from being detected by the thermocycler. This requires the wavelength emitted by the reporter to be near the absorbance spectrum of the quencher. Figure 5 provides a schematic illustration. Simply, a hydrolysis probe is constructed to have a reporter molecule on the 5′ end and a quencher molecule on the 3′ end, which if chosen properly will

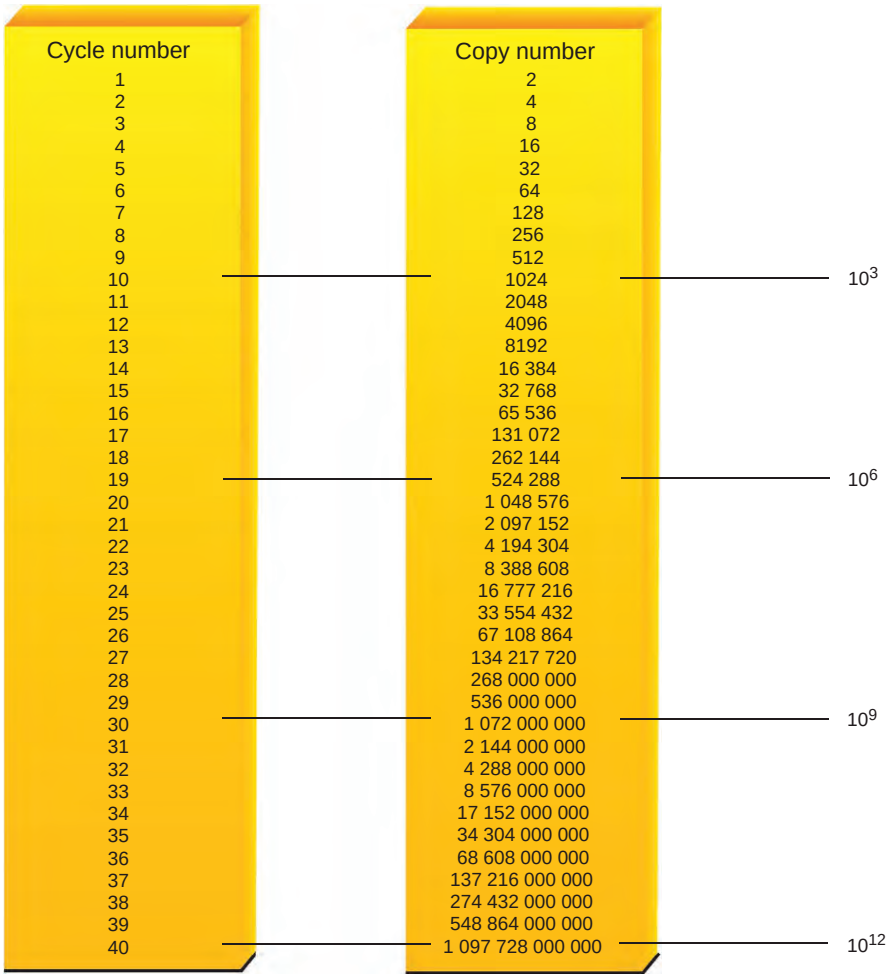

Figure 2 Illustration of the power of PCR due to exponential accumulation of PCR product. After 40 cycles of amplification by PCR, accumulation of 10^{12} gene copies is possible when starting with a single copy.

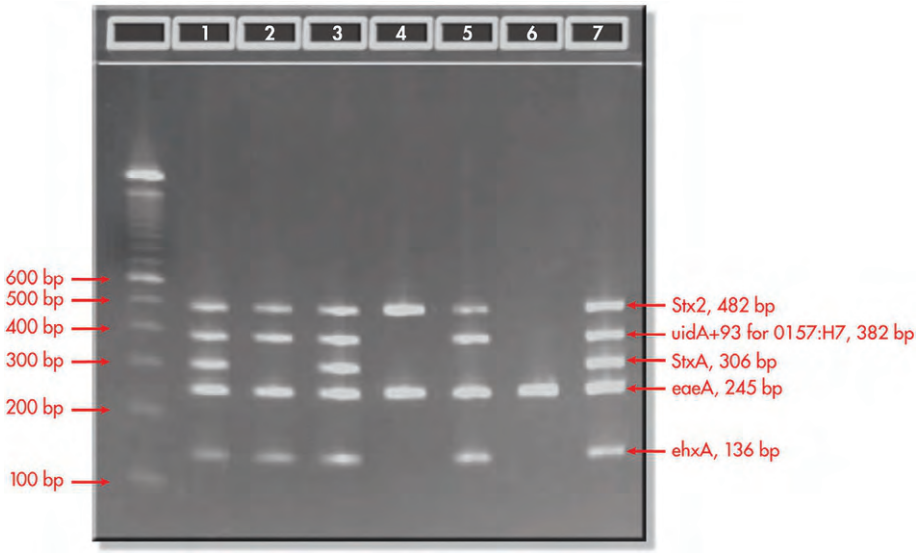

Figure 3 Example of PCR products observed after electrophoresis on 2% agarose gel stained with ethidium bromide. The 5-plex PCR results are shown for seven unknown samples (columns 1–7). Amplicon sizes range from 482 to 136 bp. The unmarked column is a DNA standard or 'ladder' used to confirm amplicon sizes.

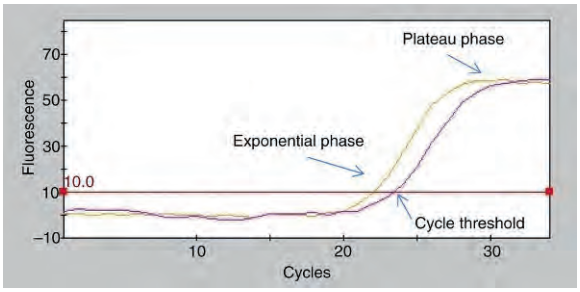

Figure 4 Example of a qPCR amplification plot (fluorescence (relative fluorescence units) vs cycles). Since measurements of fluorescence are collected during the exponential phase in qPCR, difference in starting concentration between two samples can be observed. Alternatively, in conventional PCR, samples are often analyzed after the run has finished (plateau phase) resulting in a loss of discriminatory power. Ct (cycle threshold) refers to the point where the fluorescence within a sample crosses the software- or user-defined threshold.

Table 1 Comparison of the attributes of conventional PCR and real-time PCR

Conventional PCR vs real-time PCR		
	Conventional PCR	Real-time PCR
Time to result	~4 h–2 days	~45 min
Analysis	Requires postrun manipulations	Closed tube system
Specificity	From primer only	From primers and probe(s), if used
Sensitivity	Higher detection limit	Lower detection limit
Dynamic range	Short; <2 logs	Increased; >6 logs
Resolution range	>10-fold	As low as 2-fold
Quantitative	No, results not expressed as numbers; size-based discrimination; ethidium bromide staining not very quantitative	Yes, Ct values produced (relative or absolute); increase in fluorescence signal is directly proportional to the number of amplicons generated
Multiplexing	Yes, multiple primers	Yes, multiple primers and fluorophores
Data collection	End point (typically)	During exponential growth phase

prevent any reporter fluorescence being detected until after binding to the DNA strand being amplified. Here, the probe is subjected to the $5' \rightarrow 3'$ exonuclease activity of the DNA polymerase enzyme as it encounters the probe. The nuclease activity hydrolyzes the probe separating the reporter and quencher molecules such that FRET can no longer occur, allowing for a photon to be emitted by the reporter molecule and detected by the thermocycler. Hydrolysis probes are a very popular qPCR chemistry that can be applied to single nucleotide polymorphism (SNP) detection for pathogen detection and discrimination.

Multiplex PCR

While the simple presence of a specific microorganism or microbial toxin is often enough for etiologic agent identification, for the linking of human, food, and environmental isolates, and for regulatory agencies to take action to protect public health, sometimes it is desirable to obtain additional information from a single PCR run. Case in point, a patient with flulike symptoms might be suffering from illness due to several different bacterial, viral, or parasitic agents, necessitating the screening for several pathogens. Multiplex PCR can provide a useful tool in this situation. In other cases, pathogen presence might be better determined by targeting multiple areas of its genome. For example, in biodefense work many assays are designed for the simultaneous detection of multiple genes in a microorganism due to concern over the manipulation of an agent's genetic makeup to evade detection by published PCR methods. Multiplex PCR could also be applied to subtyping an organism or genetically closely related organisms at the species or genotype level. Virulence profiling a particular organism might also be desired (i.e., what public health risk is posed by the presence of an organism with particular virulence genes).

The maximum number of targets that can be multiplexed has not been absolutely defined but software is available for 30-plex PCR assays (e.g., PrimerPlex, Premier Biosoft) and commercial kits are available for 20-plex conventional PCR reactions (e.g., Life Technologies, Grand Island, NY, USA). Multiplexing is also available in qPCR assays since nearly all instrumentation provides multiple optical channels that can distinguish different emission wavelengths of light in a single sample. For most qPCR instrumentation, filters embedded in the system provide light tuned to the excitation spectra of specific fluorophores and detect

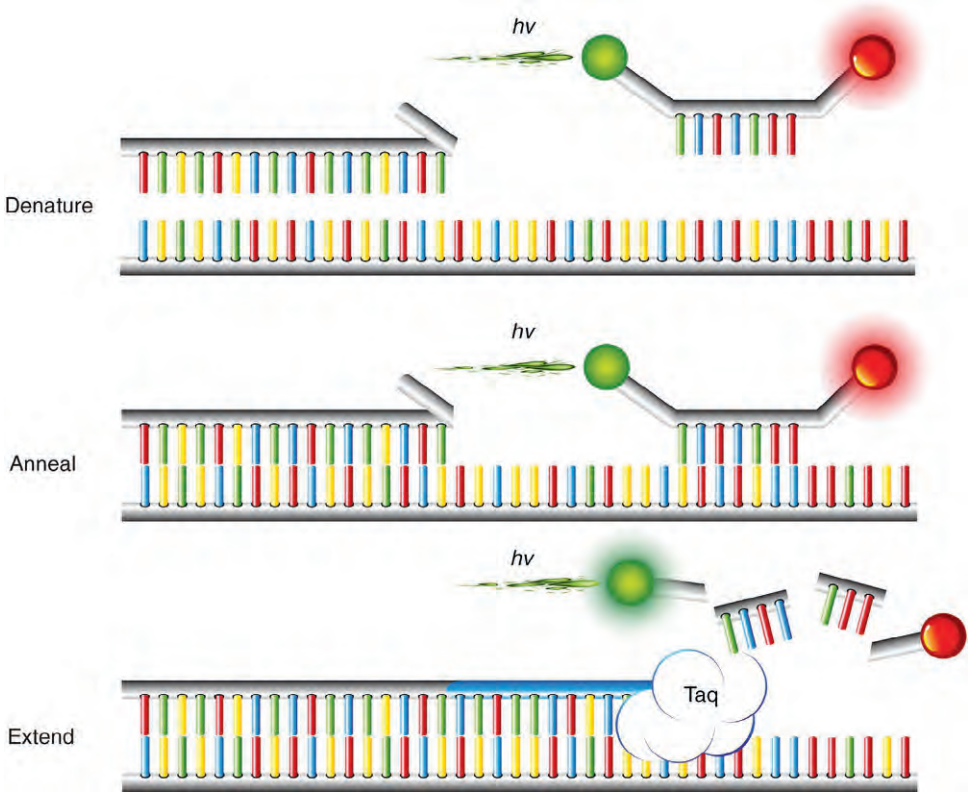

Figure 5 Concept of hydrolysis probes in qPCR. Hydrolysis probes have a reporter molecule on the 5′ end and a quencher molecule on the 3′ end. The nuclease activity of the DNA polymerase hydrolyzes the probe separating the reporter and quencher molecules such that FRET can no longer occur allowing for a photon to be emitted by the reporter molecule.

the corresponding emission spectra. One significant limitation of multiplexing in qPCR is the number of channels available with a particular platform (most often four or six). With many assays employing an internal control to judge the validity of a particular PCR run, this means one less channel is available.

Reverse Transcriptase Real-Time PCR

When the beginning target is ribonucleic acid (RNA) and not DNA, an additional step is necessary to convert the RNA to its DNA complement (complementary DNA (cDNA)) using the enzyme, reverse transcriptase. The conversion from RNA to cDNA is necessary since thermostable DNA polymerases are DNA-dependent enzymes. While this is a two-step process, it is possible to accomplish all in a single tube. In terms of pathogen detection, reverse transcriptase PCR is commonly applied to viruses like hepatitis A, *Norovirus*, or *Rotavirus* since their genome is a single-stranded RNA molecule. For viruses like hepatitis E and *Norovirus* that cannot be cultured *in vitro*, real-time PCR (RT-PCR) is a critical tool in detection and diagnosis. An additional advantage of RT-PCR is the ability to assess the viability of bacterial or parasitic pathogens, by detecting the presence of short-lived messenger RNA sequences which are only generated by living organisms. In contrast, standard PCR assays (conventional or real time), which can only detect DNA, offer no direct way to ascertain the viability of any pathogen found in a sample.

Microsphere-Based Suspension Arrays

The presence of pathogens can also be detected using microsphere-based suspension array technology, such as the Luminex®x-MAP™ (Luminex Corporation, Austin, TX, USA). Luminex has licensed this xMAP technology to other companies, including Bio-Rad (Hercules, CA, USA), which offers a popular suspension array system, the Bio-Plex 200 (Figure 6). Suspension arrays offer specific, rapid, and high-throughput screening of multiple targets within a given sample. The data output is median fluorescence intensity (MFI), which indicates the presence and amount of analyte in a given sample.

The backbone of the xMAP system is the polystyrene or magnetic microspheres, also referred to as beads, that can be differentiated by unique spectral signatures due to ratios of two (e.g., Luminex 100/200, MAGPIX system) or three (e.g., FLEXMAP

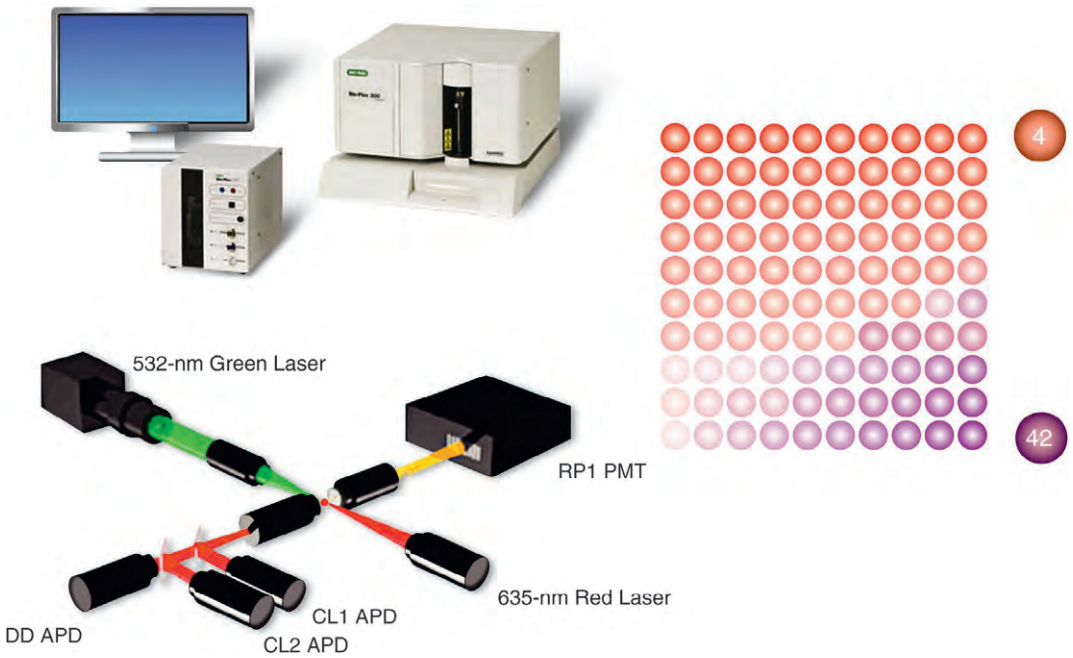

Figure 6 Example of a microsphere-based suspension arrays technology: Bio-Rad Bio-Plex 200 instrument, color-coded microspheres, and laser system used in suspension array work. The unique microsphere color comes from different ratios of two or three distinct fluorophores (ratios signify a particular microshpere region; regions 4 and 42 are shown as examples). The laser system housed within the body of the instrument consists of a red laser that determines the microsphere region and a green laser that generates fluorescence in a positive test sample. APD, avalanche photodiode detector; DD, doublet discriminator channel, discriminates single microsphere from aggregated microspheres; CL1, classify channel, allows multiplexing and detects dye inside microsphere; RP1, reporter channel, quantitates assay in this channel, photomultiplier tube.

3D system) different internally embedded dyes (Figure 6). Each spectral signature defines a microsphere's 'region' and up to 100 (Luminex 100/200, MAGPIX system) or 500 (FLEXMAP 3D system) different regions exist. This means that up to 500 different analytes can be measured within a single sample depending on the instrument model and the combination of different sets of microspheres, providing great flexibility in assay design. Consequently, microsphere-based suspension arrays have superior multiplexing capability over conventional PCR or qPCR since those assays are limited by resolution of band sizes on a gel or the number of fluorescent channels available, respectively.

To detect these analytes, a broad range of customizable bioassay formats is available utilizing capture antibodies, oligonucleotide probe sequences, peptides, or cell receptors covalently attached to the microsphere surface. When antibodies are used, the assay resembles a standard capture sandwich immunoassay (e.g., see section 'Enzyme-Linked Immunosorbent Assay (ELISA)'); however, xMAP assays provide better sensitivity as the traditional colorimetric detection has been replaced by fluorescence detection. Biotinylated detection antibodies can quantify analytes by detecting the fluorescence of biotin-bound streptavidin-R-phycoerythrin (SAPE) conjugates. An immunological approach, in which specific antibodies bind a target protein with high affinity, is particularly applicable to the detection of bacterial toxins. Detection of bacterial nucleic acid is not adequate since toxins may be present in the absence of the source microbe. Pauly and collaborators (Pauly et al., 2009) provide a practical example of xMAP technology applied to immunological detection of bacterial and plant-based toxins, botulinum neurotoxin type A and B, staphylococcal enterotoxin B (SEB), ricin, and abrin in food matrices.

When pathogen DNA is the analyte, PCR amplification is needed before direct hybridization can occur between the oligonucleotide probe attached to the microsphere and a sequence complementary area of the pathogen genome. Typically, this is done through an asymmetric conventional multiplex PCR assay. Asymmetric PCR is performed by using a master mix containing a higher concentration of a biotin-labeled primer than unlabeled primer. If the two strands are amplified nonpreferentially, the reannealing of these strands may outcompete binding to the oligonucleotide probe, producing lower MFI signals. Fluorescence generation is made possible by the attachment of the reporter molecule, SAPE, to the microsphere–probe–PCR amplicon complex (Figure 7).

Using the Luminex 100/200 or FLEXMAP 3D system, a flow cytometer ensures that the microsphere complexes are analyzed individually, with one laser (red) determining the microsphere region and a second laser (green) exciting the SAPE complex to generate fluorescence (Figure 6). The MAGPIX performs a similar analysis, less expensively, by using light-emitting diodes instead of lasers and spreading the microspheres on a monolayer as a charge-coupled device camera records an image requiring fewer microspheres per region. All platforms allow for high-throughput screening in a 96-well plate format (or 384-well plates using FLEXMAP 3D) and all assays, including those requiring PCR, can be completed in less than 6 h.

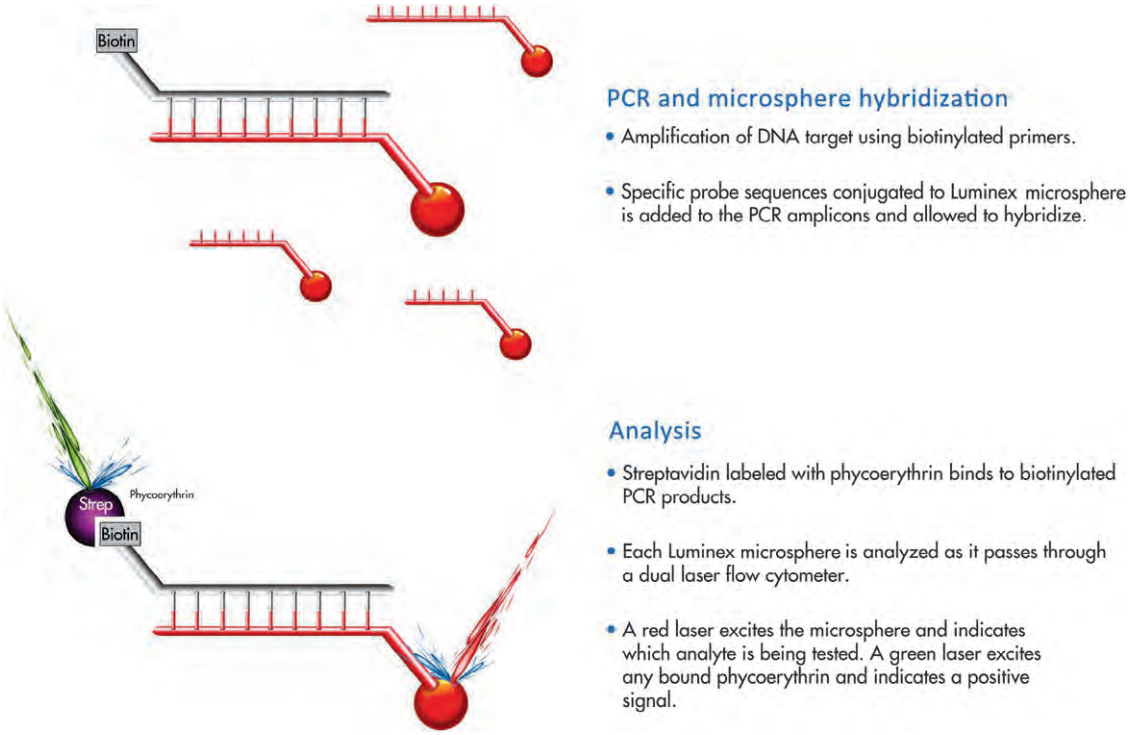

Figure 7 Experimental setup of the microsphere-based suspension array. The steps of PCR, microsphere hybridization, and analysis are presented.

Microsphere-based suspension arrays have been used to identify the Osero group of Shiga toxin–producing *Escherichia coli* in a single multiplex reaction (Lin et al., 2011), the molecular serotyping of *Salmonella* O-group has been reported (Fitzgerald et al., 2007), along with H antigen typing (McQuiston et al., 2011), the speciation of the fungal pathogen *Aspergillus* (Etienne et al., 2009), and the detection of four different animal pestiviruses (Deregt et al., 2006).

Ligase Chain Reaction

Ligase chain reactions (LCRs) can help researchers detect single base pair differences between target sequences. This is possible because mismatches between two oligonucleotide sequence complementary strands will not allow the thermostable DNA ligase to function, resulting in the absence of that ligated fragment (Figure 8). The LCR process involves adding two adjacent forward primers to the PCR reaction in the presence of DNA template. These two primers are at least one to three base pair(s) away from each other. If the complementary strands (primers added) differ due to an SNP, the ligation cannot occur, and the ligated amplified fragment will not appear in gel electrophoresis. This technology is useful for discriminating among DNA sequences differing in an SNP that is used to cluster (group) bacterial strains.

LCR has been used to detect the quantity of intestinal bacterial components (Tang et al., 2012) by identifying 16S rDNA from several bacteria (*Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Enterobacter*, and *Eubacterium*) living in the intestine. The researchers compared LCR results with those from with comparative PCR and ligase detection reaction. Another study used LCR as a reproducible molecular identification test of *Brucella* species (Wattiau et al., 2011). The strategy was used to identify multiple genetic markers at the single nucleotide level, resulting in a characteristic capillary electrophoresis profile for 10 *Brucella* species. Because LCR is insensitive to primer interactions in one single reaction, the method can be updated with new future primers without the fear of losing its ability of discrimination due to the addition of more species.

Isothermal Strand Displacement Amplification

Strand displacement amplification is a powerful *in vitro* DNA amplification method that provides an accurate compatible diagnostic nucleic acid assay. In this reaction, four to six primers and DNA polymerase are used to amplify DNA using strand displacement, loop formation, and synthesis of the singletemperature amplification of a specific fragment of a DNA template at a much faster rate than normal PCR (Figure 9). The reaction is based on the ability of a restriction enzyme (example BsoBI) to nick the unmodified strand at its recognition site and the ability of exonuclease-deficient Klenow (Exo$^-$Klenow) to extend the 3′ end at the nick and displace the downstream DNA strand (Walker et al., 1992a,b).

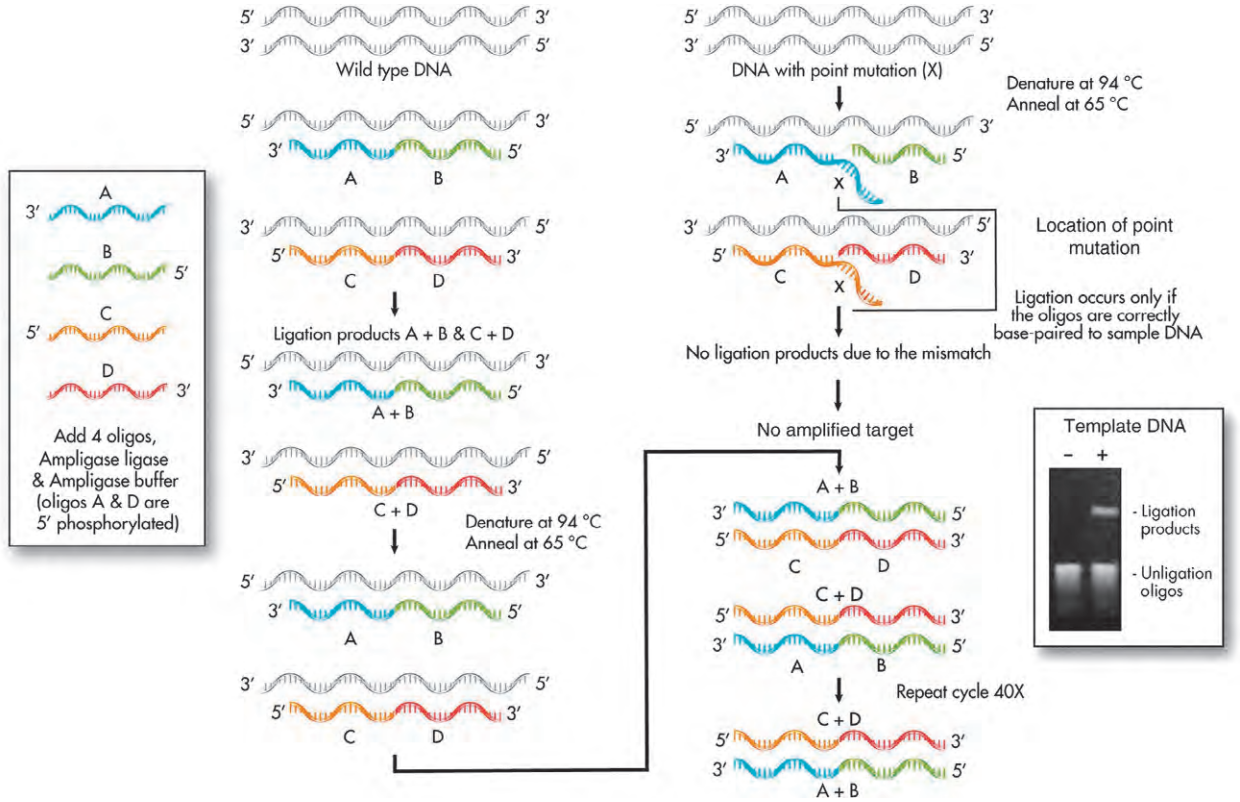

Figure 8 LCR. Two forward primers are added to the reaction in the presence of DNA template. The two primers are far from each other by 1–3 base pair(s). If one of the primers does not bind to the template due to mismatch – SNP – the ligation would not occur, and the ligated amplified fragment would not appear in the gel electrophoresis.

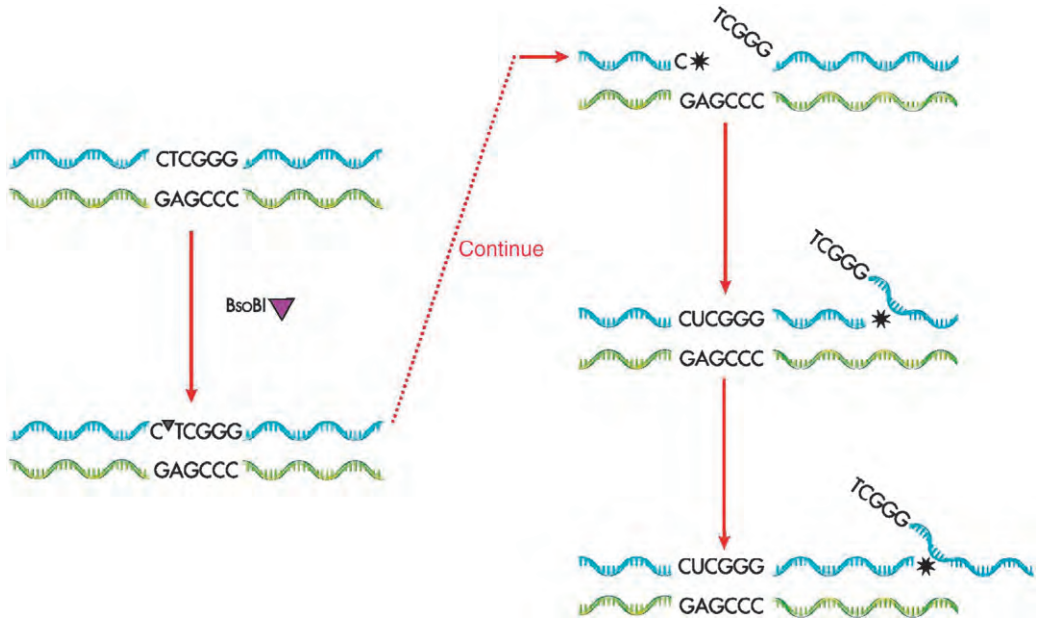

Figure 9 Isothermal strand displacement amplification (SDA). During the target generation phase (not illustrated), a double-stranded DNA presenting one modified strand of DNA (green strand) is generated. The addition of restriction enzyme (example *Bso*BI) to the reaction will create a nick in the unmodified strand. The nick will be used by exonuclease-deficient Klenow (Exo$^-$ Klenow fragment, represented by a star) to extend (amplify) the 3′-end and displace the downstream DNA strand. The incorporation of dUTP during amplification, which does not inhibit nick formation, is commonly used to prevent carryover contamination. As a result, the iterative nicking and displacement process create many new fragments.

Q-Beta Replicase Amplification

Q-beta replicase is an enzyme originally isolated from bacteriophage Q-beta, which can amplify only a specific oligonucleotide sequence in the presence of midivariant RNA sequence (around 220 nucleotides long, see Glossary). The target oligonucleotide sequence is usually inserted into the 3′ end of the probe to be amplified. The sequence acts as a reporter molecule in a hybridization assay: because the probe that hybridizes to the target is DNA, the probe is resistant to RNAse III activity and, as a result, it is amplified by Q-beta replicase. This can result in 10 000-fold amplification of 4200-nucleotide singlestranded RNA in 10–15 min at a constant temperature of 37 °C. Unlike traditional PCR, Q-beta replicase does not need changes in temperature for denaturing, annealing, and polymerizing. Each round of replication only takes approximately 20 s and proceeds exponentially, amplifying the target sequence until the number of replicons exceeds the number of the enzyme molecules. Figure 10 shows a general strategy where Q-beta replicase amplification can be used in hybridization assay.

Researchers (Smith et al., 1997a,b) used target capture technology and Q-beta replicase amplification to develop a rapid method for detecting the presence of *Mycobacterium tuberculosis*. Their method was very specific in capturing the target from clinical samples and in amplifying both capture and detector probe for several organism groups. In fact, a prototype instrument platform has been developed to automate the amplification steps inside a closed disposable test pack without the need for a highly skilled technician to operate the reaction (Smith et al., 1997a,b). Using this method also reduces contamination risks while increasing reproducibility and controlling biohazardous waste products.

Q-beta replicase amplification has also been used to identify *Plasmodium falciparum*, human immunodeficiency virus-1, *Cytomegalovirus*, *Chlamydia trachomatis*, and *Neisseria gonorrheae* (Cahill et al., 1991). Other molecular biology technologies have been developed to refine Q-beta replicase amplification, decreasing background noise by using reversible target capture (Morrissey and Collins, 1989).

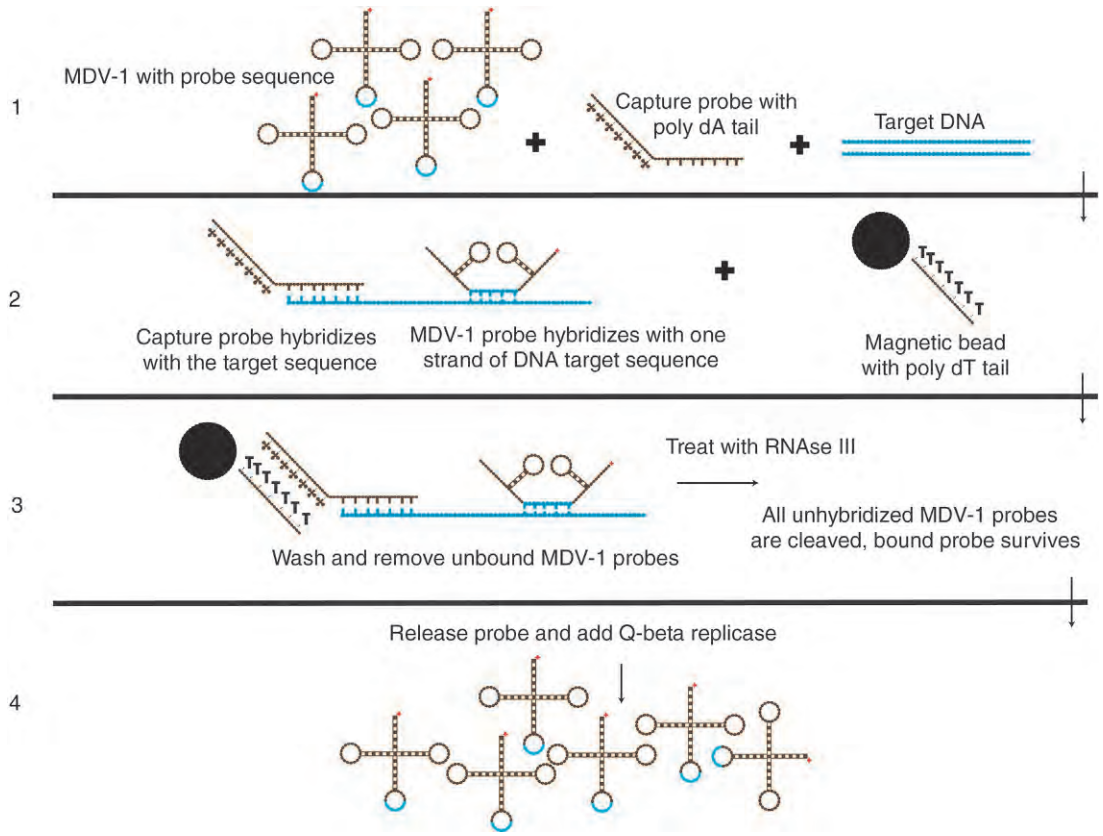

Figure 10 General strategy where Q-beta replicase amplification can be used in hybridization assay. Q-beta replicase enzyme amplifies only a specific sequence in the presence of midivariant RNA sequence (around 220 nucleotides long, which is recognized by the enzyme). When the sequence is inserted into 3′ end, the sequence works as a reporter molecule in a hybridization assay. The enzyme produces thousands of copies of the single-stranded sequence in 10–15 min at constant temperature 37 °C. MDV, midivariant RNA.

Gene Identification Using Microarray Chip Hybridization

Another method for gene identification is the chemically modified slide microarray (biochip), which has the ability to bind or capture DNA, RNA, antibodies, or peptides, which can then be detected optically using fluorescent or biotin labels for microarray hybridization. The biochip can screen large numbers of targets and use small amount of reagents with a high level of specificity in a wide range of complex matrices. Several chips capitalize on this sensitive and robust technology.

In Figure 11, the synthesis of DNA sequences or amplification of small fragments of DNA is followed by using a robotic arm to transfer the DNA from a microtiter plate to chemically modified glass slide (printing). The DNA sequence (oligoprobe) usually represents a fingerprint of a gene that has 20–1000 bp. After the printing process, the DNA covalently bonds to the surface of the chip through several chemical reactions. Depending on the technology used, the oligoprobe can also be synthesized *in situ* directly on the chip. The newly developed chip is then taken through hybridization steps with DNA amplified using multiplex PCR (or single PCR), producing fluorescent spots if it is positive (indicating hybridization). The DNA (isolated from tested sample) is labeled with a fluorescent dye such as Cy3 or Cy5 before hybridization. After mixing the labeled probe with printed chip in 50–60 °C for 1 h, the hybridized chip is then washed with several buffers before final readout. The microarray chip is then scanned using laser scanner, and the data analysis is conducted. Genes found in the lysed labeled tested samples light up (give fluorescence) on the chip surface because of hybridization, indicating the presence of the gene; genes absent in the tested samples will not light up.

Gene Expression Using Microarray Chip Hybridization

A second approach is to use DNA microarray hybridization in a high-density array to study gene expression. The printed DNA is usually synthesized from a 60 to 5000 oligonucleotide sequence representing a gene. The chip goes through the same previous steps of allowing the DNA to covalently bind to the chemically modified slide creating a chip. In a different strategy, the DNA oligoprobes can also be synthesized *in situ* directly on the chip (Affymetrix technology, Santa Clara, CA, USA).

Biochip arrays can also be used to analyze the RNA of bacterial strains isolated using two tested different conditions and labeled before hybridization with two different fluorescent dyes such as Cy3 and Cy5. Chip hybridization is conducted, and chips are washed several times with buffer before scanning. The signal intensity of each spot is measured using laser scanners (Figure 12). This process can be efficient in measuring the expression of thousands of genes in just a short time.

Because the chip has the ability to hold thousands of spots (genes, oligoprobes), the amount of gene expression data increases dramatically. The microarray gene expression profile is usually confirmed with other technologies like RT-PCR or microsphere-based suspension arrays. For example, it was possible to quantify the DNA of gastric epithelial cells infected with two different strains of *Helicobacter pylori*: wild type and arginase deficient (Kim et al., 2012). This established that arginase plays an important

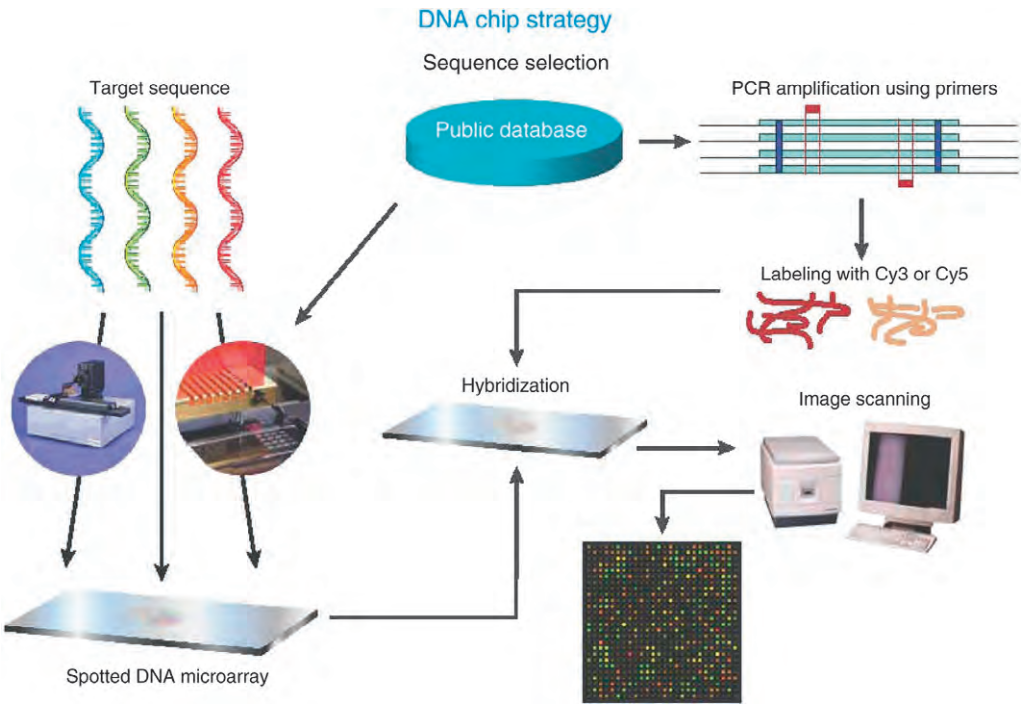

Figure 11 Steps involved in preparing a gene chip by using DNA microarray (spotted array) technology.

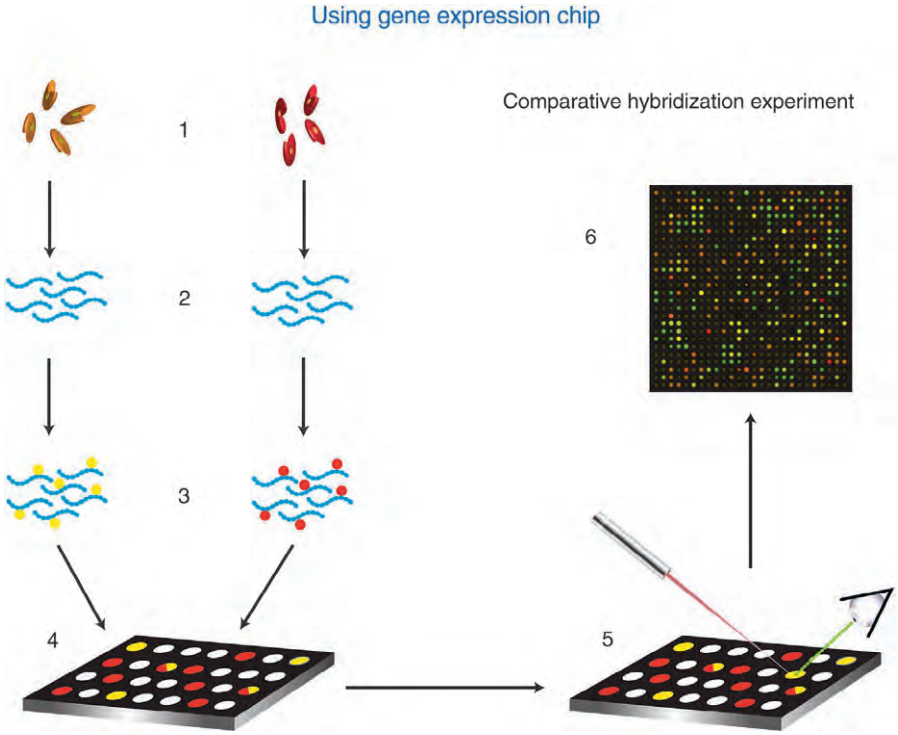

Figure 12 Steps involved in conducting gene expression analysis using microarrays: (1) tested cell sample will be compared with normal of wildtype cell; (2) RNA isolation; (3) RNA labeling or cDNA amplification and labeling; (4) RNA or cDNA hybridization; (5) chip scanning; (6) hybridization analysis and data mining.

role in helping to establish *H. pylori* infections. Later, the gene expression microarray data were confirmed by qPCR, microsphere-based suspension arrays, and ELISA (Kim et al., 2012). *Overall, DNA microarray gene expression is a great tool for screening and understanding the function of networking genes.*

Metagenomic Sequencing for Uncultured Bacteria

It is estimated that bacteria isolated using classical microbiology culture methods may represent only 1% of the total living cells in environmental samples (Amann et al., 1995). Therefore, we need other methods for understanding the 99% microbial cells and their communities. Culture-independent genomic analysis (metagenomic) can advance our understanding of these unculturable microorganisms, allowing us to discover new gene functions that are associated with natural populations.

In the past 5 years, DNA sequencing technologies have become cost effective for constructing libraries of DNA sequences representing several natural microbial communities. Metagenomic sequencing originally started in viral communities of the ocean (Breitbart et al., 2002; Venter et al., 2004) and human feces (Breitbart et al., 2003; Venter et al., 2004).

Metagenomic research can not only discover new genes and new natural products, but also study dynamic community interactions among microbes in extreme environments and discover new bacteriophages. Recent work by many research laboratories has showed the effectiveness of metagenomic approaches for understanding microbial habitats, discovering new DNA sequences, and identifying gene functions from unknown microorganisms. Metagenomic research requires cloning and sequencing DNA fragments using the following strategies:

- Screening DNA sequences for the phenotype gene expression using phylogenetic origins of DNA sequences (Brady et al., 2002).
- Screening DNA sequences for 16S RNA, followed by sequence DNA walking to find other novel genes residing near the 16S RNA genes (Beja et al., 2000a).
- Random DNA sequencing of certain genomic parts to reconstruct a DNA sequence map (Beja et al., 2000a,b; Breitbart et al., 2002, 2003).
- Using microarray technology to understand gene expression profiles of unknown microorganisms (Sebat et al., 2003).

The first large-scale metagenomic sequencing (Tyson et al., 2004; Venter et al., 2004) was started using shotgun cloning of environmental genomes inserted into cosmid libraries (Figure 13). This sequencing study revealed the presence of at least 1800 genomic species including 148 previously unknown bacterial phylotypes. New sequences (1.2 million representing 782 new rhodopsin-like photoreceptors) were found. Furthermore, 100-kb plasmids carrying arsenate, mercury, copper, and cadmium resistance sequences were identified. Transfer-related genes were identified in the ocean, suggesting substantial oceanic microbial diversity.

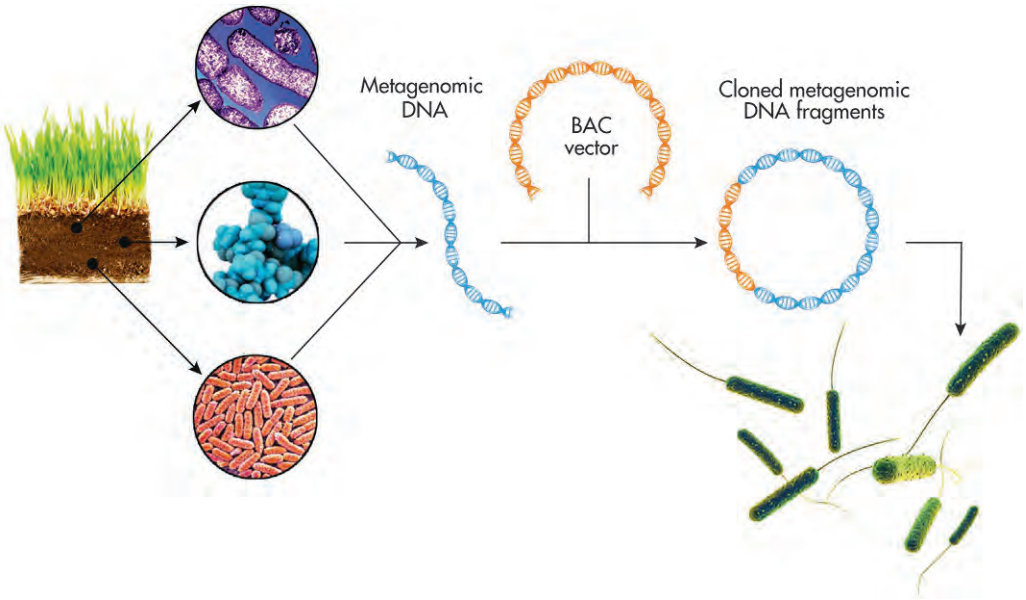

Figure 13 Metagenomic library in *Escherichia coli*. DNA is isolated from microbial communities of groundwater. The DNA is then inserted into the cosmic DNA library (bacterial artificial chromosome (BAC) vector) and sequenced. Inserts can be amplified by PCR, followed by DNA chip fabrication. These chips can be used to characterize microbial communities.

Similarly, a total of 76.2 million sequences representing 103–462 novel genes of natural acidophilic biofilm (Tyson et al., 2004) were found. Microarray platforms representing environmental DNA sequences used to screen 1-kb metagenomic libraries (Sebat et al., 2003) revealed the genetic profile of a metagenomic library.

The metagenomic research was also used to study viruses present in the human gut, which harbors approximately 500 human intestinal microbial species with around 40 predominant species in the total population (Moore et al., 1978; Moore and Holdeman, 1974). The occurrence of viral sequences was found in soil, the intestines of animals and humans, and even sea and drinking water. Metagenomic approaches helped to discover unknown genomic DNA sequences of an uncultured viral community (Breitbart et al., 2003). In fact, a total of 1200 viral genotypes were found, revealing the uniqueness of these microscopic lives. Examples of these viruses are siphophages.

It is important to realize that metagenomic DNA analyses helped to discover many bacterial species and viruses. Metagenomic DNA sequencing is now important in microbial investigation. Identification of new DNA sequences will enhance our lives.

Immunoassays

In vitro immunoassay methods are based on the binding of antibody to antigen. Antibodies are normally immune cells primed to detect and destroy foreign antigens or invading pathogens. The highly specific biorecognition property of antibodies to antigens have made antibodies one of the most indispensable molecules for broad research applications, not only in the diagnosis or detection of pathogens but also in the prevention and treatment of diseases. Immunoassays have been used to detect microorganisms (e.g., viruses and bacteria) and their products such as neurotoxins, which are produced by a variety of microbes, including *Clostridium botulinum*. The detection signals used can be radioactive, colorimetric, or fluorescent. The sensitivity and specificity of the immunoassays is highly dependent on the choice of antibody. Immunoassays are simpler, faster, and easier to perform than other *in vitro* assays. ELISAs, immuno-PCR, immunochromatography assays (ICAs), and flow cytometry are examples of immunoassays.

Enzyme-Linked Immunosorbent Assay

In the past 30 years, ELISA is a commonly applied *in vitro* method for reliable and quantitative detection of bacteria (e.g., *E. coli, Listeria, Staphylococcus aureus, Campylobacter, Vibrio cholerae, Bacillus cereus, Salmonella spp., and C. botulinum*) and their toxins in food, water, and environmental and clinical samples (Singh et al., 2013; Banada and Bhunia, 2008). ELISA is a rapid, high-throughput, quantitative immunoassay that is based on an antigen–antibody interaction and detected by an enzyme producing a chromogenic or fluorescent signal. ELISAs are performed in different formats depending on the location of antigen or the antibody on the solid surface. In general, ELISAs are performed in three formats: competitive inhibition, indirect, and sandwich ELISAs (Figure 14).

Competitive ELISA: the primary antibody is mixed in a separate tube with various dilutions of bacteria (or antigen/toxin) and added to the wells containing immobilized antigen. This allows only the free unbound antibody to bind to the immobilized

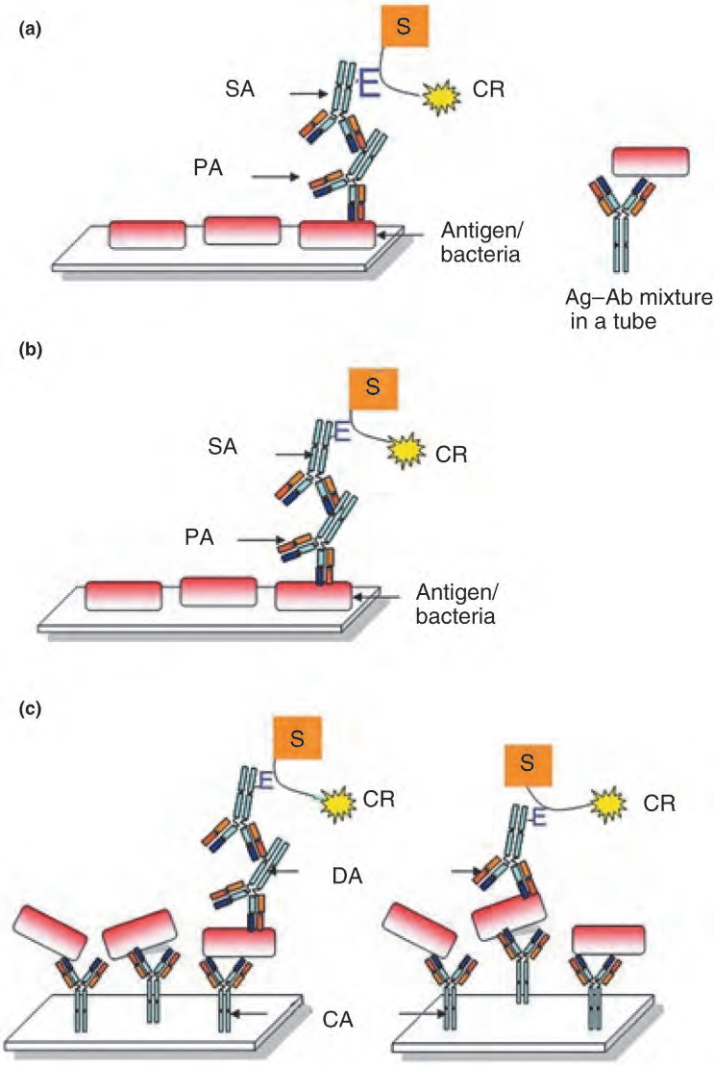

Figure 14 Diagrammatic representation of ELISA formats: (a) competitive inhibition ELISA, (b) indirect ELISA, and (c) sandwich ELISA. S, substrate; CR, color reaction; E, enzyme; PA, primary antibody; SA, secondary antibody; CA, capture antibody; DA, detection antibody; Ag–Ab, antigen–antibody.

antigen. A secondary antibody–enzyme conjugate and substrate system is added for color development. The highest dilution of cells showing the minimum reaction or equivalent to a background control is considered positive.

Indirect ELISA: the antigen is first immobilized in the wells of a microtiter plate and then incubated with the primary antibody solution. A secondary enzyme-tagged antibody is added to bind to the primary antibody and then the reaction is developed with an enzyme-specific substrate to produce color for the measurement. For a horseradish peroxidase (HRP)-tagged antibody, tetramethylbenzidine (TMB) is used to develop color. If a fluorescent molecule (e.g., fluorescein isothiocyanate, Cy5, Alexa-Fluor) is used, the reaction is quantified by the amount of fluorescence emitted.

Sandwich ELISA: this works similarly to indirect ELISA except that the microtiter plate is first coated with a capture antibody and then the antigen is added into the wells. The reaction is developed using the labeled detection or tracer antibody in either single step or in two steps with the help of an additional secondary conjugated antibody.

Immuno-PCR Assay

The immuno-PCR assay is a powerful tool for toxin detection. This method is similar to the ELISA in that the analyte (toxin) detection is based on formation of an antigen–antibody complex. However, the detected antibody in an immuno-PCR uses a reporter DNA-tagged antibody instead of an enzymetagged antibody. Normal or real-time PCR amplification of antibody-tagged reporter DNA is used for the detection of toxin with increased sensitivity of 10^2- to 10^5-fold. Furthermore, sensitivity of immune-PCR is enhanced by using a biotinylated DNA tag linked to a biotinylated antibody via streptavidin as shown in Figure 15 (Chao et al., 2004).

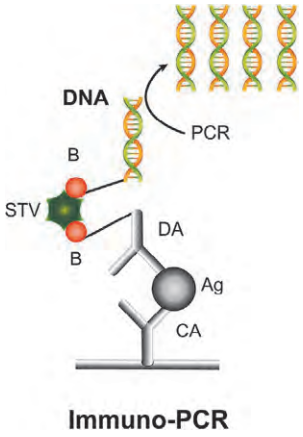

Figure 15 Schematic representation of the indirect sandwich immuno-PCR assays. Ag, antigen; B, biotin; CA, capture antibody; DA, detection antibody; STV, streptavidin.

Immunochromatography Assays

ICA methods are less sensitive than ELISAs because they use handheld devices and assessment of results is based on quick color development. The intensity of color is directly proportional to the presence of toxin concentration in test samples. ICAs have more field applications due to simplicity of devices and ease of operation, and do not require expensive instruments to perform the test. Lateral flow assays (LFAs) and affinity immunochromatography (AIC) are examples of ICAs.

LFAs: LFAs are handheld devices or a dip-stick method performed on a nitrocellulose strip. The LFA tests rely on a capture antibody–toxin–detector antibody interaction. In the lateral flow test, a line of a capture antibody is printed onto the nitrocellulose support, and the other reagents (e.g., detector antibody, buffers, and control antibodies) are dried onto a reagent pad. A drop of the toxin sample is applied to the reagent pad (sample zone) resulting in hydration of the reagents and allowed binding of toxin with detector antibody. Thus, the bound toxin with detector antibody migrates from the sample zone to the detection zone where it is trapped by the capture antibody. This trapping step results in concentration of the toxin bound to a labeled secondary antibody as a complex in a zone visible as a colored line (Figure 16) (Ching et al., 2012). Common labels include colloidal gold, resulting in a red

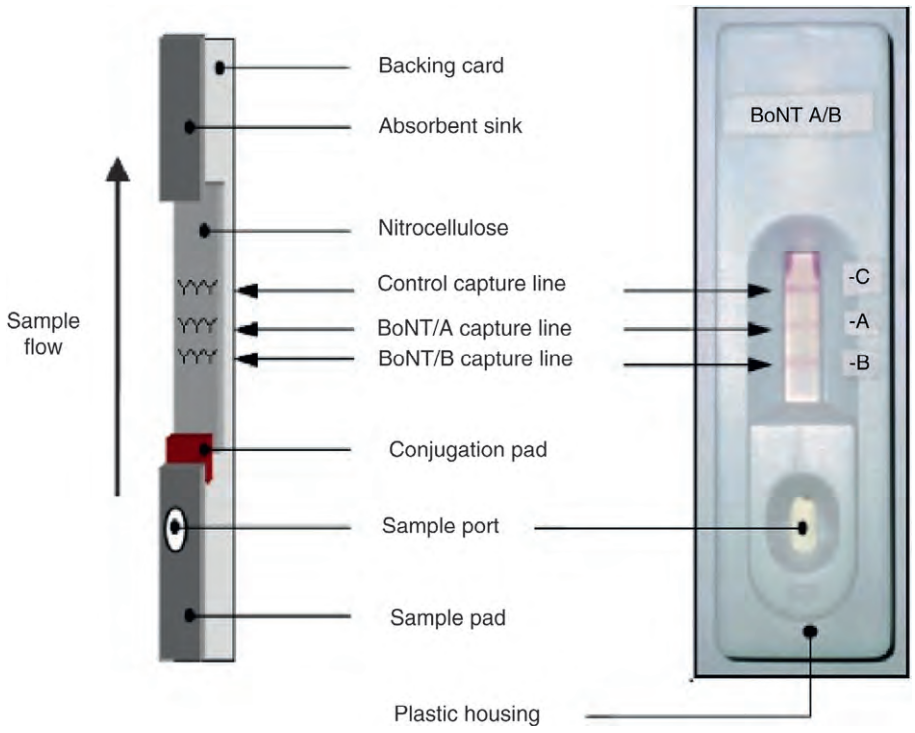

Figure 16 Schematic representation of a lateral flow device (LFD). The actual LFD (right) shows an example of positive detection of BoNT/A and B serotypes with the control line. Reprinted from Ching, K.H., Lin, A., McGarvey, J.A., Stanker, L.H., Hnasko, R., 2012. Rapid and selective detection of botulinum neurotoxin serotype-A and -B with a single immunochromatographic test strip. J. Immunol. Methods 380, 23–29. Copyright © 2012 with permission from Elsevier.

line, or various colored latex spheres conjugated to the detector (second) antibody. LFAs have significant advantages (e.g., self-contained, quicker, and do not require sophisticated equipment and an expert analyst) compared to other detection methods. Thus, they are ideal for field situations. LFA kits have been introduced in recent years for the detection of E. coli O157, *Salmonella* spp., *Listeria* spp., and *C. botulinum* from enriched food samples.

AIC assay: in this type of assay, a column is preloaded with matrix. The biotin-tagged antibody is mixed with a toxin sample, incubated for 30 min, and then loaded on to the column. The column was washed and passed through a solution of avidin-tagged HRP detection antibody. Then an insoluble solution of HRP-specific TMB substrate is added for color development (Figure 17) (Attree et al., 2007). The intensity of the color is directly proportional to the toxin concentration in the sample.

Flow Cytometry

The fluorescence sandwich immunoassay is a solid-phase immunoassay in which the capture antibodies are immobilized on Sepharose beads and used to detect and quantify toxin with the help of flow cytometry. This assay measures fluorescent intensity of individual particles in a flowing stream and can collect information in multiplexed format by using different color-coded Sepharose beads. A pentaplex flow cytometry assay was developed to test ricin, BoNT serotype A and B, abrin, and SEB in a variety of food matrices by applying fluorescent magnetic beads (Pauly et al., 2009). The sensitivity of flow cytometry assays can be further improved by using single domain antibody (sdAB) (Goldman et al., 2008), three heavy chain-specific antibodies, and introducing

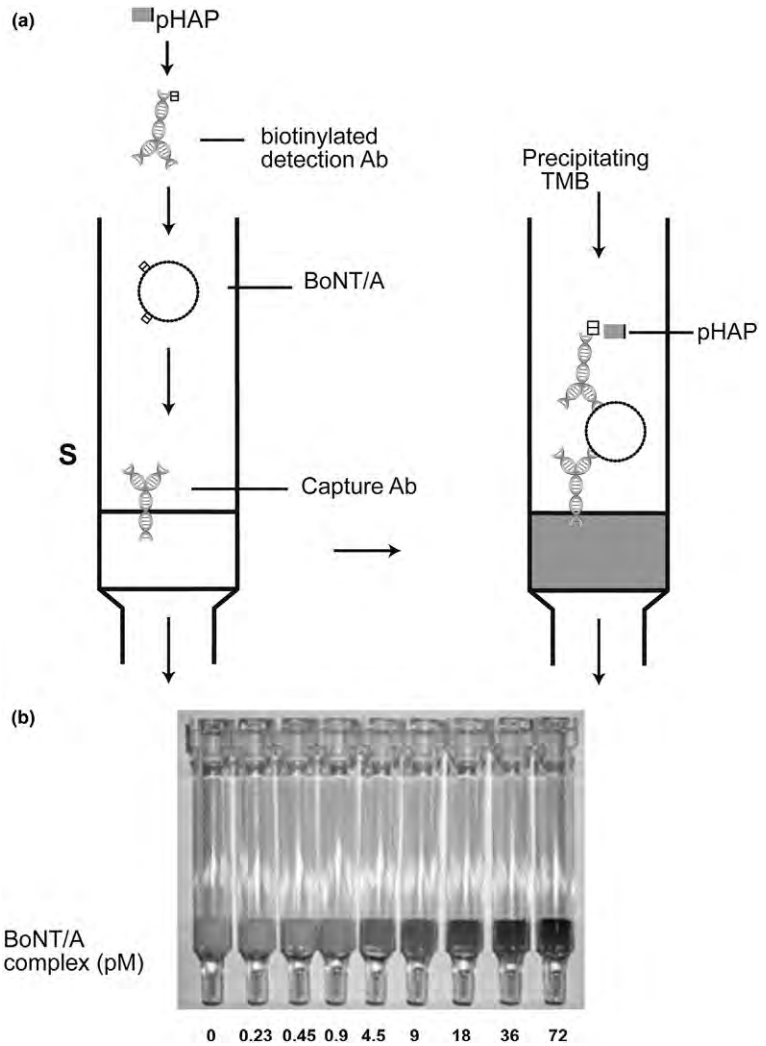

Figure 17 Schematic representation of AIC assay. (a) Prepare a column matrix by preincubation of toxin with biotinylated antibody for 10–30 min and pass antigen–antibody complex through AIC columns. Add solution of HRP-labeled antibody tagged with avidin that binds to the biotin. Finally pass through an insoluble substrate (TMB) for the HRP, which produces a visible band. (b) The intensity of color in AIC columns depends on toxin concentration. Streptavidin poly Horse Radish Peroxidase (pHAP). Reprinted from Attree et. al., 2007. J. Immunolo. Method. 325, 78–87. Copyright 2012 with permission from Elsevier.

an automated fluidic platform to process the sample, washing a way the sample matrix to get a higher sensitivity for BoNT/A heavy chain (Ozanich et al., 2009).

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Freshwater Habitats☆

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Overview

Freshwater environments are ubiquitous and critical to human success as sources of drinking water, conduits for waste materials, aesthetical and recreational purposes, among other uses. The great diversity in form and function of freshwater systems results in significant differences in microbial community structure and processes. Water flow, water depth, light level, nutrient inputs, pH, plant communities, and numerous other factors all influence the microbial ecology of freshwater (aquatic) ecosystems.

The presence of water dictates several key common properties of these environments, as do the interactions with the surrounding terrestrial environment within the watershed. Water provides a means for dispersal of organisms, transport of materials, and structural support while at the same time limiting light availability as depth increases, altering temperature regimes, and impacting oxygen concentrations. Some freshwater environments are fueled predominantly by allochthonous fixation of C while others are dominated by autochthonous C fixation. At the same time, microbial cells may enter aquatic ecosystems from outside sources and inter-mingle with autochthonous organisms.

Freshwater habitats are profoundly impacted by anthropogenic disturbance such as alterations in hydrology, land use, disposal of waste materials, contamination with fertilizers, heavy metals, xenobiotic organic compounds, introduction of exotic species, etc. Global climate change also has great potential impacts on freshwater resources through increases in carbon dioxide, elevated temperatures, an increase in sea level, and alterations in precipitation and snow melt seasonal patterns. Some of these are predicted to impact hydrology of freshwater systems while others may directly impact biota. The combination of these effects is predicted to alter freshwater habitats and thus, there is the potential for the microbial ecology of the systems to also change.

The central importance of bacteria, fungi and other microorganisms to biogeochemical cycles in freshwater systems is clear. There is also tremendous variability among freshwater systems and the diversity of microorganisms in these systems is still relatively understudied compared to soil and marine environments. Temporal changes in microbial community composition and diversity can be large and freshwater environments play host to communities of bacteria that are, in many ways, distinct. The number and diversity of freshwater systems that have been investigated continues to grow. Utilization of new methodological approaches is one driver of the expanding knowledge base.

As in other fields of microbial ecology, methodological advances, particularly those that facilitate linkage of community structure and function continue to have an impact on our understanding of freshwater systems. Particular advances have been made in the linkage in structure and function as it relates to key aspects of the nitrogen cycle. In addition, many microbial ecology concepts (such as those related to community assembly) and topics (like metagenomics) that have previously been only occasionally applied to freshwater systems are becoming more established although are still dramatically understudied compare to marine systems. Yet, freshwater studies may achieve more mechanistic insights because of their amenability to experimental manipulation and intensive spatiotemporal sampling. Functional genes, such as those involved in nitrogen fixation, denitrification and nitrification, have been studied with increasing frequency both in terms of diversity and quantity. Some studies of microbial gene expression (such as expression of *nif* genes for nitrogen fixation) in freshwater systems have also been conducted.

Although much focus is placed on microbial ecology in "natural" freshwater systems, be they pristine or impacted to a degree by humans, there is a much needed growing exploration of other systems. Most notable are those in urban areas as well as freshwater systems that are being restored or remediated. Studies of agricultural and wastewater impacts on microbial ecology are generally more common than those related to urban systems. Human impacts on freshwater systems are profound and part of the process of restoring natural functions involves creation or alteration of such systems. Such modifications include creation of artificial wetlands as a response to wetland loss or restoration of streams (for example, morphological modifications). In streams, management strategies may focus on achieving specific biological metrics (as related to fish communities, for example) or physical characteristics of the stream channel. The effects of such management on microbial community function and structure is largely unknown. Generally, study of the structure/function of the microbial community and biogeochemistry in created or restored freshwater systems is still in its infancy.

The three main types of freshwater habitats are discussed below and include wetlands, lakes, and lotic ecosystems (streams and rivers). Wetlands are arguably the least studied of these systems relative to structure of the microbial community although the functional role of microorganisms in wetlands and other freshwater habitats is well established. For each freshwater habitat type, the nature of the hydrology, geographic location and climate, and origin contribute to the large variations seen in ecosystem properties. The role of allochthonous versus autochthonous sources of organic compounds, nutrients, and microbial cells also varies among ecosystem type along with the contribution of plant detritus to the food web.

☆*Change History*: April 2018. New content was added to the overview section (3 paragraphs), new content was added to section III (lakes), Figure 4 was modified, new content was added to section IV (streams and rivers), IV Emerging Directions and Approaches is new content, references were added, and text was edited for clarity and accuracy.

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Freshwater Wetlands

Wetland Types and Properties

The nature of freshwater wetlands varies based on hydrology, origin of the wetland, plant community composition, and other features; these variations in turn affect the microbial communities of the environment. This complexity also limits the number of generalities than can be drawn about the microbial ecology of these ecosystems.

As landscape features, wetlands help to ameliorate environmental contaminants and manage flooding. At the same time, wetlands are highly threatened environments. For example, the percentage of wetlands in the continental United States is estimated to have declined by about 50% since pre-settlement times. Wetland loss in Europe as well as parts of Asia is thought to be much greater. The importance and vulnerability of freshwater wetlands leads to regulations in many countries regarding the use and destruction of wetlands. This, in turn, contributes to the issues associated with the definition of a wetland.

From a basic point of view, wetlands are shallow aquatic communities dominated by plants in which detritus plays a central role in the food web. From a stand point of delineation, the definition of wetlands is more complex and has been the subject of great debate. Overall, wetlands, for purposes of this article, can be defined as systems with hydrophytes (wetland-type plants), hydric soils, and, during the growing season, periods of standing surface water or water-saturated soils. The duration and depth of standing water varies greatly among wetlands and, within in a wetland, seasonally and from year to year.

The hydrology, origin, and geographic location of the wetland are a major determinant of plant community structure which in turn influences the microbial communities. Wetlands include those on the fringe of larger bodies of water, such as around the Great Lakes of the USA, prairie potholes, marshes (including freshwater tidal marshes), swamps, riparian zones and floodplains along rivers, bogs, playas, billabongs, etc. Each wetland is connected to the surrounding terrestrial environments in a variety of ways and they also can be connected to marine or other freshwater systems. For example, nutrients from a fringing wetland can be transported into lakes linked seasonally by water.

Wetland environments can be stressful and exhibit large seasonal changes in hydrology including periodic drying. This effect, coupled with the development of areas of low oxygen concentration, low redox potential, and wide array of different types of organic compounds, which in some cases, accumulate in the system, defines the microbial ecology of the wetland.

Human use of wetlands is also an important determinant of wetland function and biogeochemistry. Wetlands serve as sources of flood abatement, carbon sequestration, and phytoremediation and support a rich and unique flora and fauna. Wetlands are also created and used by humans for production of food, such as rice, fish and crayfish.

Overall, critical features of wetlands that affect the ecology of microorganisms are:

- Important role of plants and detritus in the food web,
- Critical role of hydrology which varies greatly among wetlands of different types,
- Seasonal variation in the extent of hydration of soils,
- Profound human impact on wetland extent, nature and hydrology,
- Creation of new wetlands, perhaps with different ecological properties, to replace wetlands lost through human use or to serve as sites of food production.

Microbial Processes

The importance of microbial processes in wetlands manifests itself in the biogeochemistry of these systems and the central role that detritus decomposition plays in the food web. Dissolved organic matter (DOM) is a major component of the organic matter pool and is made available to higher trophic levels by microorganisms. In some wetlands, often referred to as dystrophic, dissolved organic carbon (DOC) concentrations can be substantial and "stain" the water brown to black because of large amounts of humic substances. These wetlands, such as bogs, are often characterized by unique plant communities, including, in some cases, the occurrence of *Sphagnum*, formation of peat, and low pH.

Inundation with water creates anaerobic conditions in wetland soils providing the opportunity for alternative metabolic processes. The strong seasonality in the hydrology of many types of wetlands creates circumstances where there is flooding and corresponding chances for anoxia in lower layers of the soil at some times of the year, but not at other times (Fig. 1). This creates seasonal patterns in key microbial processes, such as methanogenesis and denitrification.

Plants play a seminal role in determination of microbial processes in wetlands including their impact on soil conditions, the detrital pool, and the oxygen status of the soil. Plant roots lose oxygen (from air-filled tissue in wetland plants called aerenchyma) to the surrounding soil altering the otherwise anaerobic status of the soils. The occurrence of oxygen is variable both spatially and temporally and, thus, the optimal conditions for growth of a particular microorganism may not always be present.

The C cycle of wetlands is characterized by a mixture of anaerobic and aerobic processes occurring at different locations or at different times. Often these processes can occur in spatially close proximity to each other in different portions of the soil. Fermentation is a common process in wetlands and appears to play a critical role in providing suitable organic compounds for other anaerobes. Methanogenesis in wetlands tends to be better studied than fermentation and can result in release of bubbles of methane gas (referred to as swamp gas). Methane emission from wetlands globally is quite large and is of particular interest because of the role of methane in global climate change and the possibility of management of methane entrance into the atmosphere. Quantification of methane production by wetlands reveals that there is substantial variation among wetland types and geographic locations.

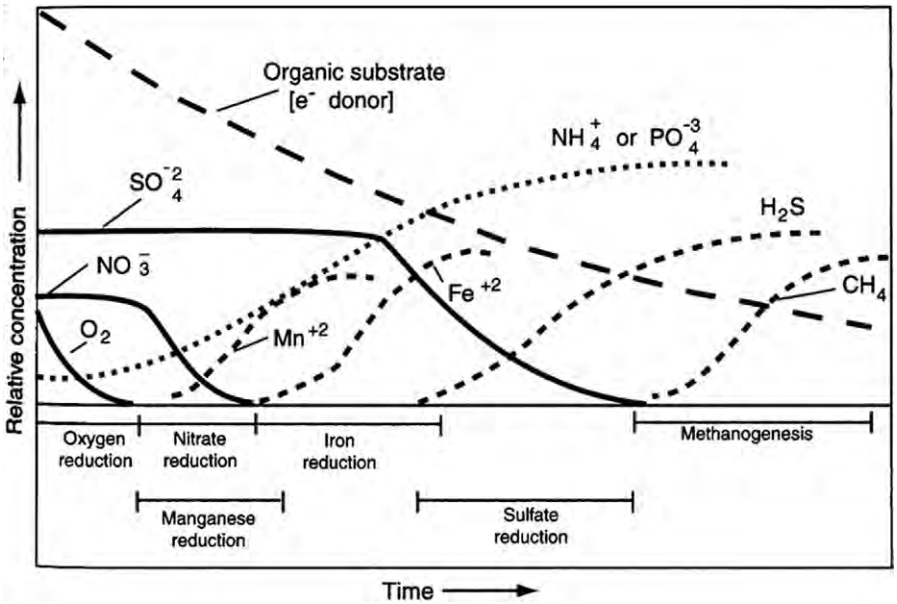

Fig. 1 Temporal changes in availability of alternative electron acceptors in wetland soil.

Properties of the N cycle in wetlands are highly variable depending on plant community type and hydrology. The sediments of wetlands play a critical role in the N cycle. N can be a limiting nutrient in wetland soil and depending on the N pool, N fixation will be more or less important. For example, the floating fern, *Azolla*, has a symbiotic nitrogen fixing cyanobacterium (*Anaebaena*). In wetlands created for rice cultivation the *Azolla: Anaebaena* may help rice production. In addition, in some cases, wetland fringing plants, such as alder (*Alnus*), may host nitrogen fixers that can impact the wetland N cycle. It appears that nitrogen fixation also does occur in association with wetland plants (such as some grasses), however, the organisms responsible of this activity defy cultivation and thus the amount of information on them is stilled relatively limited.

Denitrification is a potent source of N loss (as N_2) because of the extensive amounts of organic matter and anaerobic zones. The large amounts of N applied to landscapes can work their way into wetlands and perhaps these ecosystems can serve as N sinks in the global N cycle. Denitrification may be limited by low pH and thus loss of N in this fashion, in some wetlands, is more restricted than others. Hydrology also includes the potential for denitrification and the availability of nitrate. However, generally denitrification results in a major loss of N from wetlands that exceeds inputs from nitrogen fixation.

Nitrification plays a critical role in removal of ammonium from the soil which tends to accumulate because of ammonification. Aerobic zones supportive of nitrification occur in the top most layer of soil as well as in aerobic patches causes by plants in the rhizosphere. In some wetlands, ammonium is converted into ammonia which can be lost to the atmosphere and generally ammonium diffusion rates through wetland soil are a limiting process. In acidic wetlands, nitrification may be slower relative to other wetland types. Bacteria that oxidize ammonium anaerobically (anammox) are present in freshwater wetlands although their role in the N cycle of these systems is largely unexplored.

Sulfur transformations also occur in wetlands, although S is generally not a limiting nutrient and thus it is less frequently studied in freshwater wetlands. One manifestation of these transformations is the distinctive rotten egg smell from release of hydrogen sulfide when anaerobic wetland sediments are disturbed. Compared to marine wetlands, sulfide emissions from freshwater wetlands are much lower because S concentrations in marine systems greatly exceed those of freshwater systems. Sulfides produced by sulfate reduction can subsequently be oxidized by autotrophs. Sulfides that are not oxidized are toxic to plants and can precipitate with metals.

Phosphorus lacks the alternate oxidation states of N and S but is often a limiting nutrient in freshwater ecosystems. Some wetlands, such as bogs, are exceptionally nutrient limited while others receive P input from fertilizer and other sources. Both organic and inorganic P contribute to the P pool and insoluble P complexes can be formed that affect bioavailablity. For example, P can complex with iron or calcium or attach to clay or peat. In particular the sorption of P to clay can form complexes which may be carried into wetlands. When this material sediments, it helps make P available to the wetland plant community and allows the wetland to retain P.

Microbial Communities

Relative to other freshwater systems, the amount of information on microbial community structure in freshwater wetlands is relatively limited. Although microbial mediated processes are widely studied, as described above, the underlying communities

responsible for a particular function are unknown to varying degrees with more information available on methanogens, methanotrophs, and denitrifying bacteria, for example, relative to other functional groups, such as nitrogen fixers.

Although the role of fungi as decomposers of plant tissue is widely known, there is much less information available on the mycorrhizae associated with wetland plants. However, the potential importance of mycorrhizae in the success of plants may be particularly relevant in wetlands that are nutrient limited, such as bogs and fens, and can also be important to phytoremediation.

Although the number of investigations of bacterial diversity and community structure in freshwater wetlands is relatively limited, studies have revealed, that for the wetlands examined, *Proteobacteria* (Alpha, Beta and Gamma as well as the Delta *Proteobacteria* which are perhaps not as well represented in lakes and streams), the *Acidobacteria*, and the *Verrucomicrobia* are major constituents. The *Acidobacteria* are a widespread but largely understudied group because of limitations in our ability to culture these organisms. Likewise, there are few cultures from the group *Verrucomicrobia* with some of the major subgroups in this taxon having virtually no cultured representatives. *Verrucomicrobium* and other prosthecate bacteria are among the most well known members of this widespread and understudied group.

Amongst the Archaea, the occurrence of methanogens in wetland is the most often studied especially in light of the role of methane as a gas contributing to global climate change. In anoxic wetland soils, methanogenesis is a critical biogeochemical process and varies seasonally; both the availability of suitable substrates and nutrients impact methanogen function. The nearby occurrence of methanotrophs in oxic areas and their oxidation of methane greatly impacts methane release.

The role and diversity of protozoa in wetlands is largely unknown. Generally, the impact of protozoa as bacterial consumers is great in aquatic environments and less so in soil. There is at least some evidence that protozoan community composition can be very different than that of other freshwater habitats. The number of protozoa and consequently their impact on bacterial numbers fluctuates seasonally in temperate wetlands.

Anthropogenic Disturbance

Human impacts on wetlands are varied and, among these, perhaps the most important is the vast global destruction of wetlands. This leads to the phenomenon of wetland construction whereby new wetlands are created to replace those lost due to conversion into other uses (agriculture, construction, etc.). How well these created wetlands duplicate natural conditions and function as desired is variable and debatable. Hydrological modifications, to reduce flooding for example, are also common and considering the key role of hydrology in wetland microbial ecology undoubtedly greatly impact microbial processes.

Wetlands are also used for various agricultural and horticultural activities, such as peat mining and production of plants, such as rice. Like other freshwater habitats, impact of humans on water quality can translate into these environments. One manner in which some environmental issues may be addressed is through phytoremediation. Phytoremediation may be accomplished in either natural or constructed wetlands and potentially can remove xenobiotic compounds from water systems. Critical features for the success of phytoremediation in wetland include the redox potential, extent of above and below ground plant biomass, and hydrology.

Invasive species represent one area in which the effects on microbial communities in wetlands remains largely unknown. Of particular importance, are changes in native plant communities associated with the invasion of exotics, such as reed canary grass (*Phalaris arundinacea*), purple loosestrife (*Lythrum salicaria*), and alligator weed (*Alternanthera philoxeroides*). The role of plant communities in wetlands is a critical defining feature and thus alteration of the plant communities towards potentially nearly monospecific stands of exotic species is of great concern. Native plants are often out competed by exotic species of plant, perhaps resulting in a loss of the microbiota associated with the native plants (such as mycorrhizae), as well as an alteration in many other factors, such as soil moisture, quality and nature of the detrital pool, etc. In general, invasive plants are known to alter C, N, and water in soil, including altering N availability and fixation, and have differences from native species in productivity, growth form, chemistry, etc. Although the role of these differences in wetlands is less well studied than in terrestrial environments, the potential for a substantial change in wetland microorganisms is clear.

Lakes

Properties and Types

Like wetlands, lakes originate in various ways and the nature of their origin, hydrology, and morphometry impact environmental conditions and biotic functions. Basic morphometry is of particular importance because of the limitations that depth imposes on macrophyte success, the extent of the littoral zone, and because of impacts on the relative contributions of allochthonous and autochthonous production. Lake processes are very much dependent on the properties of the surrounding watershed, as those of other freshwater ecosystems. Lake age is also highly variable and depends on the lake type. In many ways, human impacts are poorly understood and not always recognized because so many key processes are hidden beneath the water column and thus unseen by the lay person.

One of the most critical determinants of the microbial ecology of a lake is it trophic status which ranges from eutrophic to oligotrophic (with various modifications of the terms, such as hypereutrophic). The trophic status of the lake profoundly influences the food web, biogeochemistry and other processes. Oligotrophic lakes are characterized by low nutrient concentrations and low primary production (as reflected in the low abundance of phytoplankton in the lake). Eutrophic lakes, in contrast, are characterized

by high nutrient concentrations and correspondingly high primary production. There is a gradient of trophic status between these two conditions with lakes in the middle of the gradient referred to as mesotrophic. The trophic status is the main determinant of the number, biomass, production, and diversity of microorganisms because of the differences explained above and their corresponding impacts on oxygen concentration.

Lakes, or lentic ecosystems, are also characterized by their stratification and mixing properties. In temperate zones, many lakes are dimictic with thermal stratification in winter and summer and turnover events in spring and fall. In some regions, there are lakes with permanent stratification called meromictic lakes, such as those where salt and fresh water combine, or those that are particularly deep, while other lakes never stratify. Amictic lakes are permanently ice covered, such as lakes found in Antarctica. Other lakes are monomictic with stratification once per year or polymictic with mixing and stratification multiple times throughout the annual cycle. The pattern of stratification and mixing depends on latitude and lake properties.

Seasonal stratification in temperate areas occurs because inputs and outputs of heat occur primarily at the surface of the lake. In such temperate zones, the heating of surface waters in summer creates a warm, circulating surface water pool (the epilimnion) with colder, undisturbed water below the thermocline in the hypolimnion (Fig. 2). As fall begins, the surface water cools and at some point reaches the same temperature as the underlying water; this results in a loss of density differences and as this surface water becomes cooler and denser it sinks causing the layers to mix or turn over.

In a dimictic lake that is productive (eutrophic) during times of thermal stratification (Fig. 2), the hypolimnion can be depleted of oxygen through biological activity. This happens when this layer is separated from and not mixing with the overlying water; biological activity in this confined layer resulting from decomposition can be great enough to deplete oxygen. This creates microbiologically important gradients with depth that can result in some interesting juxtapositions of biogeochemical processes as described below.

Another main way of categorizing lakes is their vegetation; with macrophyte dominated systems versus algal dominated systems representing alternative stable states. The dominance of macrophytes versus algae greatly influences the trophic interactions and paths of C transfer. The two alternative states which can occur in shallow eutrophic lakes are 1) clear water with abundant macrophytes or 2) less clear water because of phytoplankton abundance without abundant macrophytes. The clear water state is often associated with desirable fish and invertebrate communities, while the turbid water state tends to have lower diversity and undesirable algal blooms. These alternate states represent a shift in the lake community and may result from competition from nutrients, light limitation and turbidity, allelopathy or other factors. If the food web of a lake (such as by removal of macrophytes) or P concentrations are altered, it is possible to shift the state of a lake.

Overall, important lake properties that influence the microbial ecology of the ecosystem are:

- The trophic status of the lake (as determined by concentrations of N and P) and its point along the oligotrophic: eutrophic gradient.
- The morphometry and size of the lake which influences the size and relative contribution of the littoral zone and allochthonous inputs.
- The status of macrophytes in the lake; whether it is algal dominated or macrophyte dominated.
- The stratification and mixing patterns over the course of a year.

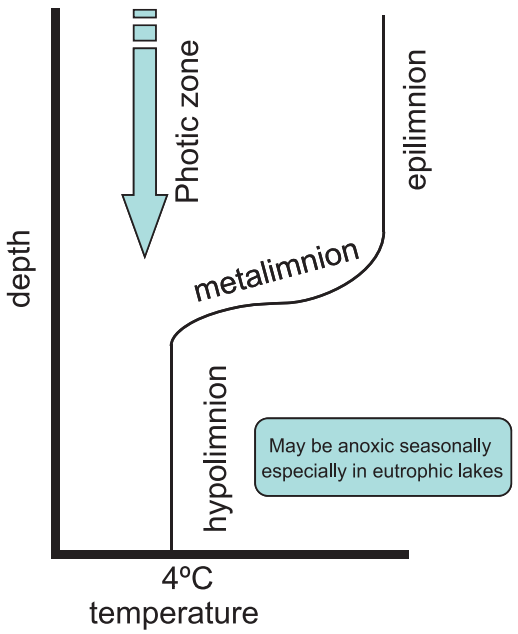

Fig. 2 The water layers and temperature profile in a typical seasonally stratified lake.

Microbial Processes

Both stratification and trophic status are major determinants of what microbial processes happen and where they happen. Stratification in a eutrophic lake, in particular, can ultimately lead to large, seasonal anoxic zones which greatly impact microbial processes as well as macrofauna. Occurrence of these so-called "dead zones" also significantly impacts lake quality as it may limit fish community success. The nature and extent of stratification as well as nutrient availability creates obvious differences in C and nutrient cycles amongst different lakes.

One emerging area of lake microbial ecology is the insight that can be obtained by comparison of different lakes. In some cases, a time can be exchanged for space to look at temporal succession while in other cases lakes with different levels of anthropogenic disturbance can be compared. Lakes can also serve as sentinels for global climate change; this work is facilitated by comparative studies, use of sensors and global networks, like the Global Lakes Observatory Network (see "Relevant Website section").

C cycle

Taken from a simplistic point of view, lake food webs include both a grazer "food chain" and a microbial "food chain" or microbial loop (Fig. 3). The relative amount of C transferred through these two components varies from lake to lake and within a lake (for example, near shore versus off shore). The dichotomy between these two paths is also reflective of the role of detritus in the system with the microbial loop reliant on the extensive dissolved portion of the detrital pool.

The occurrence of anoxic areas in the hypolimnion and benthos creates opportunities for the use of alternative electron acceptors at the appropriate depths within a lake. These processes can effect the C cycle; for example, methane is generated in these anoxic areas by anaerobic archaea and then can be utilized by methanotrophs in the overlying oxic area (the epiliminion). Likewise, fermentation occurs in the anaerobic zones resulting in a variety of small organic compounds for use by other microbes.

On macrophytes, extensive biofilms (often in limnology, such biofilms are referred to as periphyton) can form which may serve as competitors for nutrients with phytoplankton. These biofilms are complex structurally and feature diatoms interconnected by the extracellular matrix as well as cyanobacteria and heterotrophic bacteria. Some components of this assemblage penetrate into the macrophyte tissue while others are more loosely associated. Within these biofilms, release of extracellular organic compounds by algae supports bacterial growth and bacteria, in turn, release carbon dioxide and degradative enzymes. The surface area of macrophytes in a lake can be extensive depending on its state, depth, and relative littoral zone size. Thus, the role of these macrophyte-associated biofilms in nutrient acquisition may also be great.

N cycle

The N pool in lakes includes both inorganic and organic components that are utilized and transformed by microorganisms. Cyanobacteria, such as *Anabaena* and *Aphanizomenon*, are important nitrogen fixers especially, but not always, those that produce heterocysts. For example, members of the Oscillatoriaceae are also common and important in lakes but lack the ability to produce heterocysts. N fixing cyanobacteria are a valuable surface for attachment of bacteria and the heterocysts may create microzones of low oxygen concentration creating specialized bacterial niches. There is limited information on the importance of heterotrophs, such as *Azotobacter*, in nitrogen fixation in lakes. This is perhaps a result of the assumption that this process might be limited by the availability of labile organic compounds.

Nitrification in lakes occurs in aerobic zones and thus may be limited in the deeper sediments. In addition, specific dissolved organic compounds (such as tannins) may inhibit nitrification. Denitrification is important in areas with oxygen depletion (although it can also occur aerobically) such as the sediments of littoral zones of lakes and the hypolimnion of stratified eutrophic lakes. The ability to denitrify is widespread among different types of bacteria, including *Pseudomonas*, *Bacillus*, and *Achromobacter*.

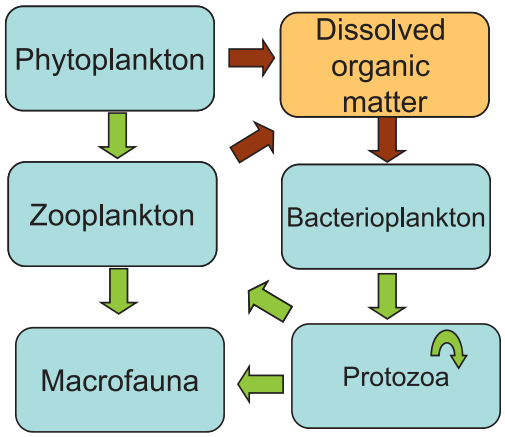

Fig. 3 Stylized and simplified food web in the water column of a lake illustrating paths of C transfer in grazer and microbial food webs; green arrows indicate grazing while brown arrows indicate production and use of detritus in the form of dissolved organic matter. Note within the protozoa box, multiple trophic transfers may occur.

P cycle

Each elemental cycle in the lake is intertwined with other cycles; this is illustrated, in particular, for the P cycle. Lake sediments are a major source of P in lentic ecosystems and P is often a limiting nutrient; additions of N and P lead to eutrophication which greatly impacts biotic processes. Most of the P in a lake is contained in the biomass and in organic compounds and total P concentrations vary considerably from hypereutrophic to ultra-oligotrophic lakes. Because P is often a limiting nutrient much attention has focused on internal loading from the sediments; the process by which sediment P is released into the water column. Mobilization of P is facilitated by bacteria such as *Pseudomonas* and *Chromobacterium* but chemical processes are dominant. Specifically, complexation with metals plays a major role in the lake P cycle; for example, phosphate can be released from ferric phosphate under anoxic conditions. Formation of iron sulfide associated with sulfate reduction can reduce the complexation of phosphate by iron.

Microbial Communities

The amount of information on lake microbial community structure has increased dramatically over the years. Prokaryotic communities have been studied in a variety of lakes of different sizes and trophic status. These studies have revealed information about the dominant bacterial taxa as well as differences between lake and marine communities.

The water column microbial community is distinctive in function and also structure from the benthic community. Depending on the water depth, the benthos may be a viable habitat for phototrophs. Often the benthos may be anaerobic (especially below the sediment surface) even if the overlying water is oxygenated providing opportunities for methanogens and dentrifiers.

Phototrophic prokaryotes fill a particular series of niches in the lake environment based on their physiological needs and pigments used. Cyanobacteria are abundant and can grow to nuisance levels especially in eutrophic lakes with abundant P. The gas vesicles produced by cyanobacteria contribute to their buoyancy and ability to maintain position in the water column which ultimately enhances their success. Many of these cyanobacteria can fix nitrogen resulting in undesirable blooms which have negative impacts on water quality. For example, *Microcystis*, which is abundant worldwide, produces microcystins which are of great concern for water quality from both a drinking water stand point and the success of aquatic animals. The hepatotoxins produced by *Microcystis aeruginosa* have damaging effects on the livers of mammals and control of this organism can be an important goal of lake management.

In many countries around the world, occurrence of toxin producing cyanobacteria in lakes is of concern. Cyanotoxins produced include hepatotoxins, neurotoxins, dermatoxins, and endotoxins which negatively impact water quality. Arguably the toxin of most concern in cyanobacterial harmful algal blooms are microcystins and much effort has focused on *Microcystis aeruginosa*. Efforts are made to control the blooms of cyanobacteria by biomanipulation (increasing grazing on the bacteria, top down control) and by decreasing nutrient availability (bottom up control). Bioremediation via microcystin degrading bacteria is also a potential tool in removal of toxins from water for consumption. The introduction of exotic species of cyanobacteria that produced toxins, such as *Cylindrospermopsis raciborskii*, can compound the problems associated with these organisms by introducing new types of undesirable algae into lakes.

Beyond the cyanobacteria, green sulfur, purple sulfur and purple non-sulfur bacteria can occupy niches in lakes. Their niches occur in stratified lakes in which the anoxic hypolimnion has adequate levels of light to support their growth and, for the sulfur bacteria, hydrogen sulfide in the concentrations needed. The occurrence of these ideal conditions creates relatively narrow bands in the water column where these organisms are abundant certain times of the year in temperate dimictic lakes. Factors including oxygen concentrations, temperature and light penetration are determinants of the vertical stratification of different types of photoautotrophs in lakes. The importance and perhaps diversity of non-oxygenic phototrophs is greater in lakes with permanent stratification; for example, in permanently frozen lakes of Antarctica or in meromictic lakes in other regions.

For the bacteria, overall, Beta-*Proteobacteria* appears to be the dominant group in freshwater making the community of freshwater systems distinct in some ways from marine systems. The Beta-*Proteobacteria* includes the freshwater ammonia-oxidizing bacteria and are common in high nutrient conditions and biofilms. In large oligotrophic lakes there is increasing evidence for the importance of the *Actinobacteria*. The Alpha-*Proteobacteria* are found in peak numbers, in many cases, under more oligotrophic conditions, and can be correlated with chlorophyll concentration and have a preference for labile organic matter. In addition, the gamma *Proteobacteria* are abundant as are the *Cytophaga-Flavobacterium-Bacteriodes* cluster and *Verrucomicrobia*. The *Cytophaga-Flavobacterium* are specialized for utilization of high molecular weight organic compounds and thus may exhibit spatio-temporal patterns of abundance related to the occurrence of molecules of this type.

The composition of the bacterial community and relative importance of each of these major groups can vary considerably among lakes as related to variations in trophic status, pH, temperature, and nutrient concentrations. Likewise, there are often differences in the relative abundances of major groups between the hypolimnion and the epilimnion in stratified lakes as well as between different portions of lakes (for example, areas that are shallower and close to riverine inputs might have a different community composition than off shore sites).

Fungi perhaps play a less important role in many lakes than other microorganisms because the role of plant detritus in some lakes is less significant. Aquatic fungi are important in littoral zones and in the degradation of macrophyte tissue. Many fungi that are encountered on plant material may be of allochthonous origin having colonized the tissue prior to submergence. However, the overall contribution of C from littoral zones and macrophytes is highly variable among lakes depending on size and morphometry. In many lakes, pelagic processes and in particular algal-bacterial coupling may be the central component of the microbial food web.

Algae in lakes have been studied for many years and are responsible typically for most of the C fixed in many lakes especially those that are large and too deep for growth of rooted macrophytes. Algal communities are diverse and different species fill varying ecological roles based on differences in motility, habitat preferences, and palatability to aquatic animals. Algae can be directly consumed by grazers and support bacterial growth via release of exudates (Fig. 3). In addition to prokaryotic algae, diatoms, green algae, chrysophytes, and dinoflagellates are common lake microbiota while some important marine groups (red and brown algae) are not as common in freshwater lakes. The relative balance between the prokaryotic algae (cyanobacteria) and eukaryotic algae depends on many factors including light and nutrient availability. The stoichiometric ratio between N and P is a particularly critical factor.

Protozoa play an important role in the microbial loop of lakes and are major consumers of bacteria. There is tremendous diversity of freshwater protozoa including organisms that are mixotrophs; protozoa that are photosynthetic and also consume prey items, such as bacteria. There are large spatio-temporal changes in the distribution and abundance of different species which is facilitated by the ability of some species to form cysts to persist undesirable conditions. There is vast morphological and function diversity amongst freshwater protozoa. Overall, from an ecological stand point, the smaller heterotrophic flagellates may be the most important bacterial grazers.

Anthropogenic Disturbance

Human impacts on lakes include those that are direct and those that are indirect. Indirectly, humans impact hydrology and nutrient loading into lakes while anthropogenic disturbance also has direct effects through invasive species, for example. Other anthropogenic disturbances of concern that may impact microbial ecology include changes in lake water level, decreasing pH from acid precipitation, input of gasoline additives from boating, heavy metal pollution (such as mercury), dredging, etc. An area of emerging concern in many aquatic ecosystems is the occurrence of plastic debris; microplastics are well studied in marine ecosystems and also are abundant in the Great Lakes, particular Lake Erie. Few studies have examined the occurrence of microplastics in other lakes.

Invasive species in lakes range from fish to invertebrates to plants to zooplankton. Examples include the zebra mussel (*Dreissena polymorpha*), water hyacinth (*Eichhornia crassipes*), and Eurasian watermilfoil (*Myriophyllum spicatum*). There are numerous other examples of invasive species including the widespread practice stocking of fish into lakes for fishing purposes. The role of exotic species in altering the food web and microbial processes in some cases is largely unknown while in other cases the impacts are at least partially explored. For example, the zebra mussel has invaded areas such as the Great Lakes in North America and greatly alters the environment by clearing the water while filter feeding and depositing materials in sediments through feces and pseudofeces. This acts to increase water clarity and decrease pelagic phytoplankton biomass. This alteration can correspondingly impact the role of the microbial loop in the food web; for example, they may alter the diversity and abundance of protozoa. In addition, these mussels have negative impacts on native bivalves and alter the community of benthic invertebrates.

Lakes are managed to enhance fish productivity, alter the type of vegetation present, reduce undesirable algae, make water bodies more suitable for boating and/or swimming, and improve water clarity. Many lake management strategies are focused on controlling loading of nutrients into the system. Lake management practices include aeration, biological control, treatment with alum, and treatment with algaecides. Management of undesirable biological features may take top down or bottom up approaches. Top down control includes grazer removal by addition of planktivorous fish, while bottom up includes inactivation of P and decreasing external or internal nutrient loading. Species-specific lake management, such as control of a specific undesirable cyanobacterium via cyanophages, is an emerging area.

Streams and Rivers

Properties of Lotic Ecosystems

Lotic systems (streams and rivers) are characterized by water with a unidirectional flow and are classified based on "size" as represented by stream order. Essentially, as illustrated in Fig. 4, the joining of two first order streams creates a second order stream,

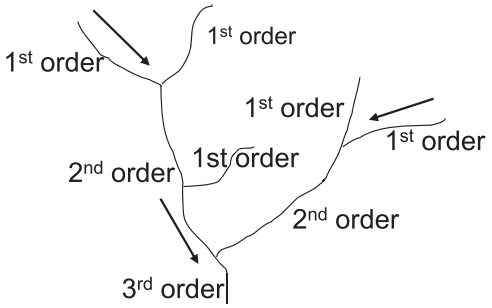

Fig. 4 Schematic illustrating manner in which stream order is determined. Arrows represent direction of water flow.

the joining of two second order streams creates a third order and so on. The processes at any site along the system are greatly influenced by upstream processes, as described, for example, in the River Continuum Concept (RCC), as well as by processes in the watershed. Water movement imposes a persistent and critical force on the biota increasing the importance of the benthos of the ecosystem and limiting stratification in contrast to what is typically the case in lakes.

The RCC illustrates the upstream-downstream connection and the role that riparian vegetation structure can play. Although streams in nature may not follow the RCC model, because of human alterations, like reservoirs, and other factors, the notion of this profound connectivity among parts, longitudinal changes, and gradients of biological processes are conveyed by the model.

In addition to the longitudinal and lateral connections, there are vertical connections with streams and the underlying water in a zone called the hyporheos. The extent of the hyporheos varies based on geomorphology and other features. A unique fauna can develop in these areas, in additional to communities of organisms that live in both the surface and the sub-surface. Biofilms formed in the interstitial spaces can be a potent site for microbial activity and create additional niches for microorganisms. Hyporheic processes depend on the redox potential of the surface and underlying waters and the occurrence of anaerobic hyporheic zones in proximity to aerobic surface substrates can create conditions in a relatively narrow spatial scale for a variety of oxidation and reduction reactions. The occurrence of upwelling and downwelling areas as well as whether the hyporheic water is oxic or anoxic determine the biogeochemistry of the system.

In temperate streams, autumnal leaf inputs are a major source of organic matter for the food web. The quality of this leaf material as a biological resource varies among species based on structural properties and chemical constituents. These leaf materials are conditioned by microorganisms, especially bacteria and aquatic hyphomycetes, and can then be consumed by macroinvertebrates, functionally defined as shredders (Fig. 5). The fecal production and fragmentation resulting from the feeding of these invertebrates results in the generation of fine particulate organic matter (FPOM). This FPOM is then available to another functional group of invertebrates, the collectors.

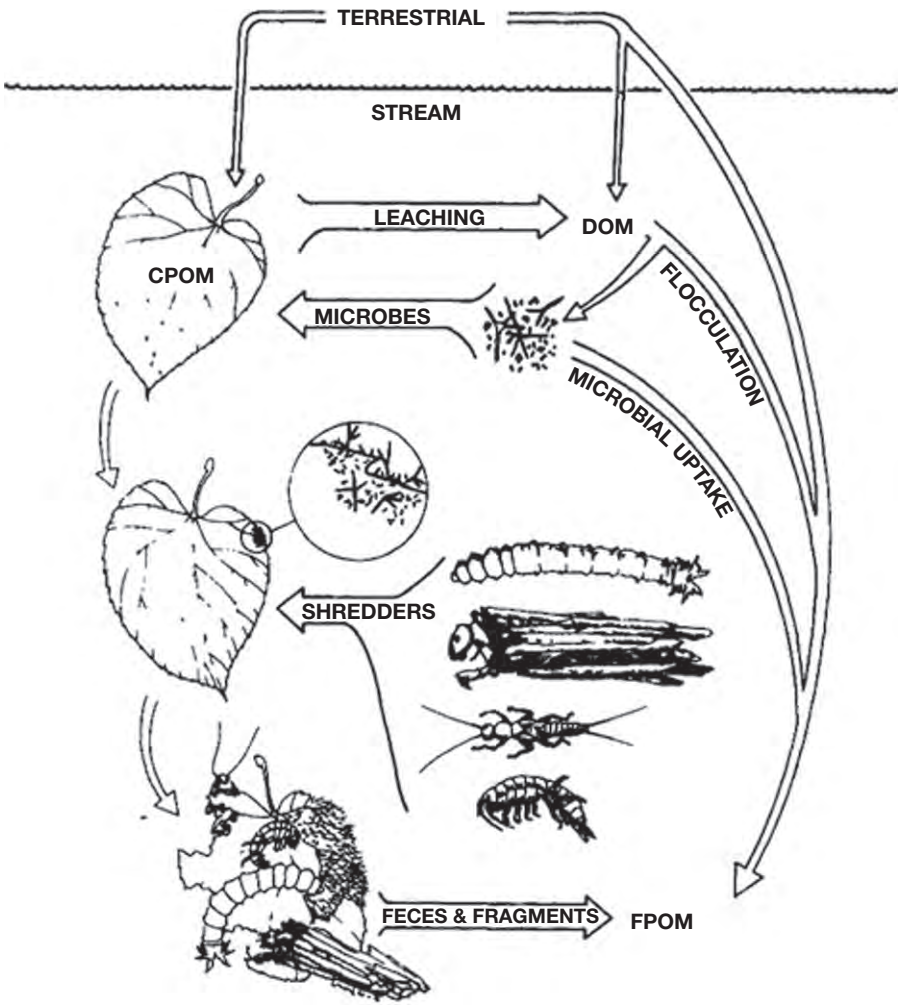

Fig. 5 Decomposition of leaf material in a stream depicting microbial colonization, consumption by macroinvertebrates shredders, and generation of fine particulate organic matter. Reproduced from Nikaido, H., 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiology and Molecular Biology Reviews 67, 593–656.

Disturbance via flooding is another major feature of streams that can have varying impacts on the biota depending on the seasonality, magnitude and duration of the event. Stream life is adapted to the flowing water conditions, but water velocity and depth drastically increase during flooding causing organisms to be dislodged. In addition, benthic materials can be entrained in the water column and be transported resulting in scouring of surfaces and loss of biofilm. Flooding also connects the stream proper to its floodplain and the input of allochthonous materials jumps (including microorganisms, nutrients and organic compounds).

Overall, lotic ecosystems features that are critical to microbial ecology are:

- The unidirectional flow of water.
- The high degree of mixing resulting in a lack of stratification and high aeration.
- Strong interconnection to the surrounding watershed and terrestrial environments.
- Important role of the benthos as a habitat for microorganisms and site of nutrient uptake.
- The significance of allochthonous inputs of microorganisms, organic compounds, nutrients, and particles.
- Importance to humans as water sources and transporters of waste water.
- Impact of humans on the environment through sewage, industrial pollutants, agricultural run-off, reservoir construction, removal of woody debris, channelization, etc.

Microbial Processes

Food webs and the C cycle

As in other freshwater environments, DOM is the dominant fraction of the detrital pool. DOM is made available to higher trophic levels through the microbial loop (Fig. 6); bacteria utilize the DOM and are in turn consumed by protozoa. Although rates of consumption per individual are much higher for ciliates then flagellates, small flagellates are the predominant protozoan predator of bacteria. Protozoa can then be eaten by meiofauna, such as nematodes or benthic copepods.

Another critical process in the C cycle is degradation of coarse particulate organic matter (CPOM) like leaves and wood. Aquatic hyphomycetes play a central role in degradation of CPOM and can penetrate into the tissue of decomposing leaves facilitating colonization by bacteria. There is evidence of competition as well as of facilitation among bacteria and fungi on leaves decomposing in streams. As noted above, these conditioned leaves are then palatable to leaf shredding invertebrates. There are clear patterns of temporal succession within the microbial community of leaves with some fungal and bacterial species serving as early colonizers and others appearing later in the process.

Conditioning of leaf material by microorganisms facilitates consumption by macroinvertebrates who assimilate various fractions of the microbial: leaf assemblage (Fig. 6). In addition, leaf-eating invertebrates play host to a variety of microorganisms; for example, the caecum of the crane fly larva, *Tipula*, boasts large numbers of bacteria.

The importance of algal production, and thus autochthonous C sources, varies greatly and light limitation is crucial. Both shading from riparian vegetation and turbidity can limit light and restrict algal production. Many low order streams with well developed canopies are considered heterotrophic because the P/R ratios are less than 1. In this case, the food web is dependent on C fixed in the terrestrial environment representing allochthonous sources of organic compounds. In contrast, the RCC predicts at

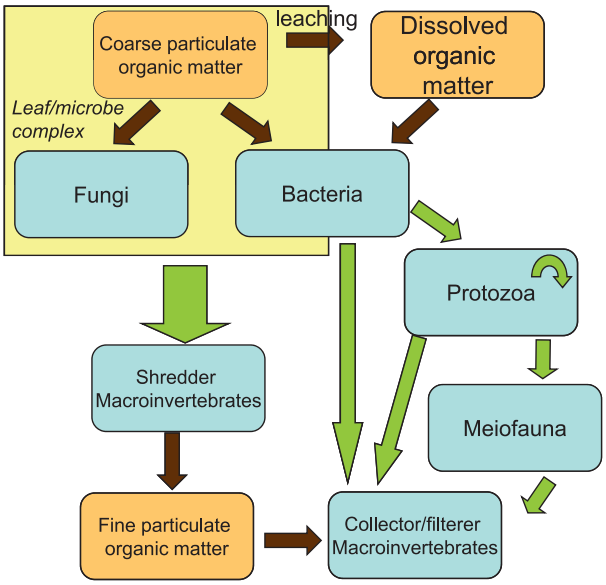

Fig. 6 Basic illustration of C transfers in a stream where the coarse particulate organic matter is leaves. Reproduced from Nikaido, H., 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiology and Molecular Biology Reviews 67, 593–656.

middle orders the P/R ratio will reach its maximum. The P/R ratio is also expected potentially to be greater than 1 in open canopy streams especially those with abundant nutrients and suitable substrates for algal attachment.

Algal: bacterial coupling, which is widely observed in marine and lentic ecosystems, can also be evident in streams. Although the absolute contribution of autochthonous organic C to the overall DOC pool may be limited, this fraction generally has higher lability than the allochthonous fraction. The contribution of breakdown products of plant structural materials (lignocellulose) to the allochthonous fraction is high and these products can be highly recalcitrant. The basis for the observed algal:bacterial coupling has two components: growth of bacteria on algal released organic compounds and use of the algal cells as a physical substrate for attachment.

Although the role of viruses in streams is largely unexplored, they potentially can alter C flow through the microbial loop by attacking bacteria and other organisms. Clearly, in other aquatic environments bacteriophages can be an important component of the microbial loop, and the abundances of viruses observed in lotic ecosystems, suggests that this is also the case in flowing waters.

N cycle

One characteristic of nutrient relations in streams is the spiraling manner with which nutrients as well as bacterial cells are transported (Fig. 7). The nutrient spiraling concept describes the process by which an atom that is released into the water column travels some distance downstream before being taken up again. The spiraling length varies depending on many stream properties such as retentiveness of the channel and water velocity. Transport of nutrients in this manner is experimentally evaluated by measuring the uptake length of material released into the water column.

In addition to biological processes, geochemical processes impact N dynamics in lotic ecosystems. Reactive sites in the benthos can result in chemical transformation and sorption of nutrients. This impacts nutrient availability to the biota, as well as transport downstream.

Nitrogen, in particular, has been the subject of numerous studies in stream ecology because of its potential to serve as a limiting nutrient, influxes of N from fertilizer, and role in water quality. The quantity of N is highly variable among streams based on influxes from outside sources and biological processes in the riparian zone. Both assimilatory and dissimilatory reactions occur in the N cycle in streams and the role of particular N transformations is variable. Both inorganic and organic N are important N cycle participants, although most emphasis has been placed on inorganic N.

Sorption of ammonium to sediments can be high and, correspondingly, ammonium concentrations in water of rivers and streams tends to be low. The benthos creates circumstances in which ammonification, denitrification and nitrification can take place within a comparatively narrow fraction of the benthos and there can be steep redox potential gradients (Fig. 8). Depending on the redox potential, the hyporheos can be potent sites of denitrification. Denitrification in the soil of the riparian zone can also contribute to the stream N cycle.

N enters streams from both upstream, terrestrial and groundwater sources where it is subjected to various microbial transformations (Fig. 9). Processes are dominated by benthic components and are highly dependent on the oxygen gradient within the sediment.

P cycle

Phosphorous in aquatic ecosystems is often a limiting nutrient and the form of P greatly impacts biological activity. Often soluble reactive phosphate is measured and represents a readily assimilated form; dissolved organic P is also present but is used more slowly. Sorption and desorption of phosphate to sediments (particularly fine particles acts to control the concentration of inorganic P in the water in streams and rivers. In addition, P concentrations can increase greatly after flooding especially with input from runoff or sewage.

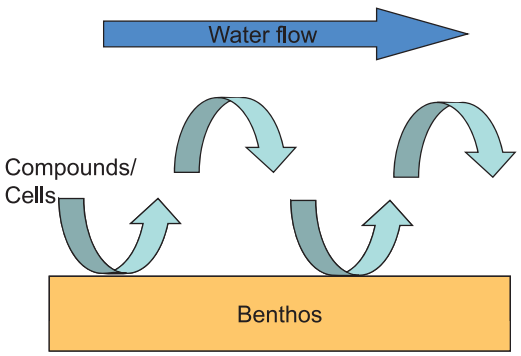

Fig. 7 Schematic illustrating the manner in which cells and chemical compounds are transported some distance downstream before uptake into the benthos (via attachment, sorption, biological assimilation, etc.). Materials are subsequently released creating a spiral-like pattern. Reproduced from Nikaido, H., 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiology and Molecular Biology Reviews 67, 593–656.

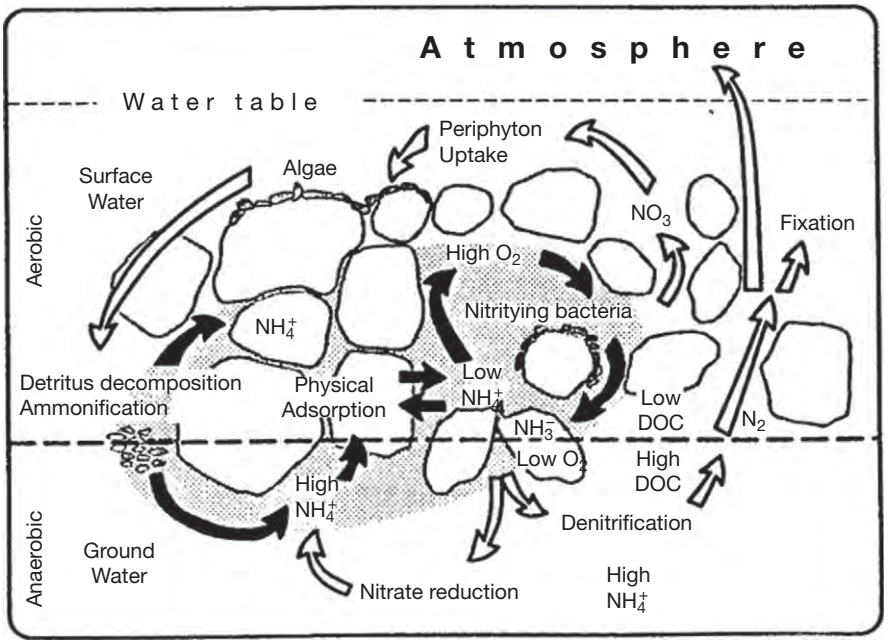

Fig. 8 Interplay between aerobic and anaerobic zones and N transformations in the benthos of a stream.

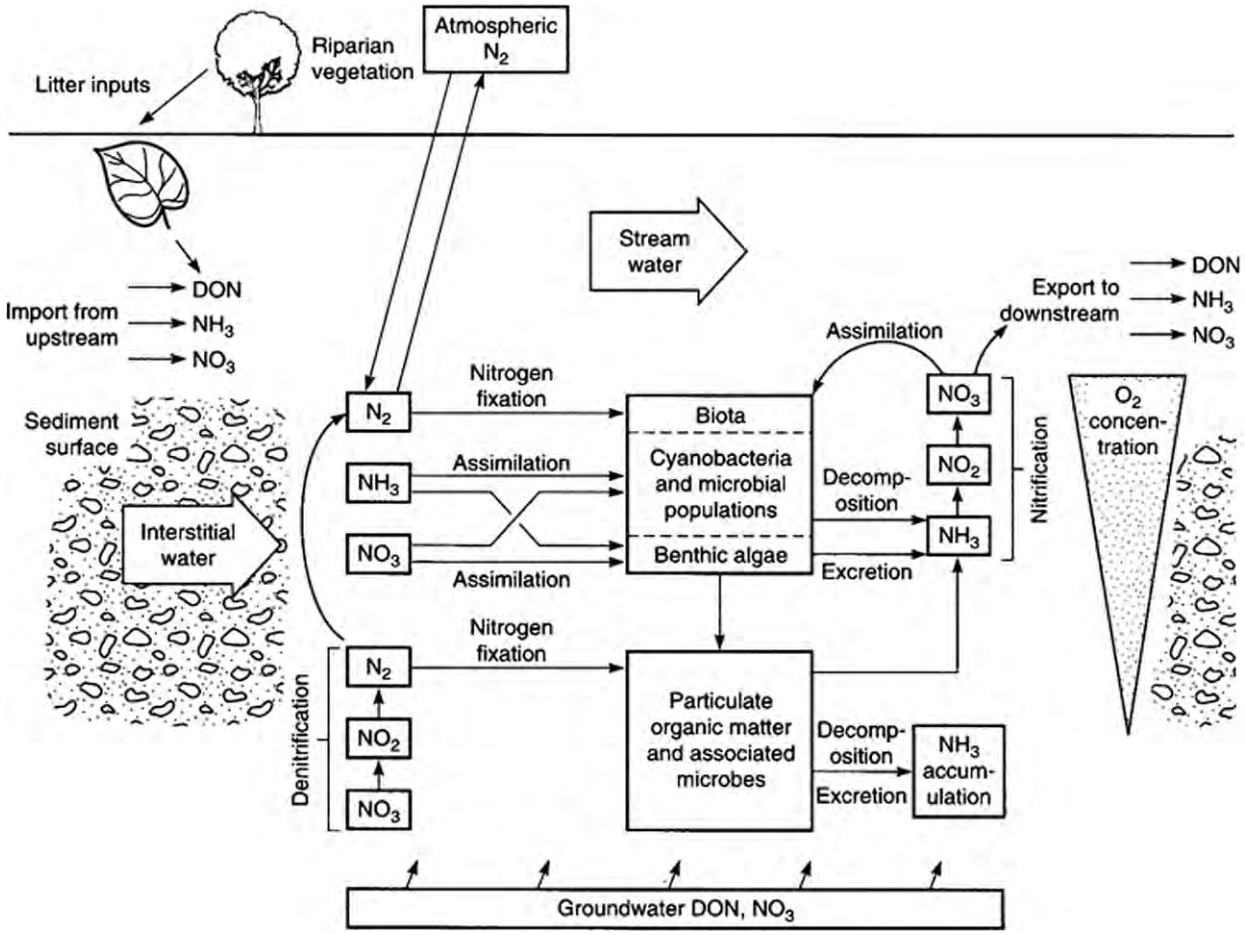

Fig. 9 Schematic of the nitrogen cycle in a stream.

Microbial Communities

Microbial communities in streams are a complex mixture of cells from allochthonous locations and those produced within the stream proper. Distinguishing amongst these components is quite problematic and the relative contribution of the fractions depends greatly on local events, such as rainfall, flooding, etc.

Biofilms with complex 3-dimensional structures form on stream surfaces including wood, leaves, rocks, and smaller particles. These biofilms are critical habitats for microbial production in lotic ecosystems and consist of complex mixtures of cells of different types embedded in a matrix of extracellular polysaccharide. The matrix is interspersed with channels serving as transport venues and plays host to a mixture of autotrophic and heterotrophic microorganisms. Water velocity is a major determinant of biofilm thickness in streams and physical disruption can "reset" the community by clearing the surface of many of biofilm residents.

The nature and activity of the biofilm may vary depending on substrate type and various terms are applied to biofilms on different surfaces, such as epilithon for biofilms on rocks, epiphyton for biofilms on macrophytes, and epixylon for biofilms on wood. Within the biofilm, various niches occur and there are opportunities for many types of biotic interactions, such as predation and competition. The biofilm creates an opportunity for cells to maintain position in the flowing water and to accumulate resources in transport. Of particular importance is the ability of the biofilm to aide in retention of enzymes released from the cells to degrade polymers.

Activity of enzymes in biofilms and other habitats, including phenol oxidase beta -D-glucosidase phosphatases, endocellulase, cellobiohydrolase and aminopeptidase, has often been measured to assess the potential for enzymatic degradation. Typically, assays rely on chromogenic or fluorogenic substrates. Although these methods do not allow the researcher to determine the organism that produced the enzyme, they do provide measures of specific activity and degradative potentials.

Like nutrients, microbial cells are also transported along the length of the stream; this process is described in the information spiraling concept. A variety of processes influence release of cells from surfaces, including the activities of animals, physical disruption, sloughing, and natural dispersal properties of the species. The uptake length of bacterial cells has been measured in streams through experimental addition of bacteria.

Bacterial communities in streams, as in lakes, are numerically dominated by *Proteobacteria*, particularly the Beta-*Proteobacteria*. Although the diversity of bacteria in streams and rivers is less studied than marine or lake environments, we do know that the *Proteobacteria*, *Actinobacteria*, and *Cytophaga-Flavobacterium* cluster are common in lotic ecosystems. Some bacterial groups may perform specialized roles in nutrient cycling or in degradation of particular organic compounds. For example, the actinomycetes participate in the decomposition of leaf material in streams along with other types of bacteria and the hyphomycetes. Based on meta-analysis of several studies, water temperature, hydrology, organic matter and nutrients are seasonally predictable and correlated with microbial diversity.

Although there is increasingly good data that describes the bacterial community composition in streams, there are still limitations in our ability to connect structure and function. In streams and rivers (as well as other freshwater habitats), the number of bacterial cells in the community that are active (such as those that are undergoing respiration) is often a small percentage of the total number of cells. This may be a particularly important component in lotic ecosystems because of the inputs of large numbers of allochthonous cells. Although these cells are not contributing necessarily to ecological functions, they are still detected via many of the widely used methods.

The contribution of archaea to lotic ecosystems is largely unknown beyond the role of methanogens. Relative to bacteria and other systems, there is very limited information about the abundance, diversity and activity of Archaea in streams and rivers.

Fungal communities in streams, as well as other freshwater environments, are dominated by aquatic hyphomycetes which are identified based on the structure of their conidia. Hyphomycetes are not a specific taxonomic group; rather they are a heterogeneous group of fungi. Hyphomycetes are one type of microsporic fungi and produce conidia directly from hyphae or via conidiophores. The group can be further subdivided and, in streams, much focus is on the so-called aquatic hyphomycetes, a polyphyletic group, that play an established role in decomposition of leaf material. Common genera of hyphomycetes found include: *Anguillospora*, *Tetrachaeum*, *Tetracladium*, *Clavatospora*, *Goniopila*, *Helicromyces*, *Lemonniera*, and *Heliscella*.

Algal communities in streams can be light limited (either by shading of riparian vegetation or turbidity) and nutrient limited and their contribution to the C in streams varies based on these limitations. Attachment to surfaces is another critical need as phytoplankton generally develops only in large rivers. Diatoms are both diverse and abundant in streams particularly attached to rocks and cobbles and are generally the most abundant algae in stream biofilms. The distribution of diatom species varies predictably among locations with different water velocities. In addition, the growth form of filamentous green algae (i.e., *Cladophora*) varies with velocity.

Algal stream communities are greatly impacted by light, temperature, herbivory, water velocity, substrate type and by nutrient availability. This latter includes silica which is essential for diatoms, however, in lotic ecosystems silica is generally found in adequate supplies. Diatoms, which include genera such as *Achnanthes*, *Gomphonema*, and *Navicula*, can be more successful under low light conditions than other algae; this is a likely contributor to their success in streams. In terms of nutrient limitations, autotrophs in streams are generally thought to be P limited not N (although exceptions to this trend occur).

Protozoa in streams are in many ways less well studied than in lakes. It is known that protozoa play a major role in the microbial loop in streams. Small flagellates are considered to be the most important consumers amongst the protozoa in streams and can greatly alter bacterial abundance.

Anthropogenic Disturbance

Human impact on streams is profound and has greatly altered the structure and function of streams and rivers globally. These impacts in turn influence? the role of the microbial community and essential biogeochemical processes.

Introduction of sewage effluent and fecal waste from human/animal activities into lotic systems is widespread and serves as a source of microorganisms, nutrients, organic compounds, as well as various pollutants used by humans and disposed of in sewage. The input of allochthonous microorganisms in this manner has critical impacts on water quality, human health, and the spread of water borne diseases. Inputs of organic compounds and nutrients from sewage alter the resource availability to the biota and can, in some cases, result in reduction of oxygen concentrations in the water. Often these inputs are monitored using biological indicators of sewage contamination such as enumerating fecal coliforms. In addition, other fecal-associated organisms and viruses may be monitored to assess different types of risks (like *Cryptosporidium* from cattle feces) or fecal streptococci (Gram positive cocci) from human sewage effluent. To reduce the risk of contamination of water by pathogens, treatment of effluent is often done, in particular using chlorination. Chlorination does have associated health risks through the production of trichloromethanes from natural occurring dissolved organic compounds and alternative strategies and dechlorination can be used to minimize these risks.

An area of emerging concern is the presence of personal care products, such as pharmaceuticals, in streams arising from wastewater effluent. Such compounds have been detected in a variety of streams and have an unknown impact on the microbial community. For example, the presence of antibiotics may promote occurrence of antibiotic resistance genes.

Humans also alter the physical structure of streams through channelization for navigation purposes, removal of woody debris, straightening of channels and removing of meanders, dam and reservoir construction, etc. Such impacts have gone on for many years and thus have had long term consequences on stream and river function. In particular, water residence time is altered by these activities that, in turn, alters transport of materials and uptake lengths. For some alterations, like addition of reservoirs, residence time is greatly increased creating unexpected lentic regions in the middle of otherwise "normal" river continua. At these discontinuities, material in transport can drop out of the water column and pelagic processes can replace benthic processes in relative importance.

Acid mine drainage represents one specific impact on streams and has huge impacts on the microbial ecology of the system. The problems start with the mining of coal that is mixed with pyrite. Iron and sulfur oxidation leads to a decrease in pH and favors the growth of specific types of microorganisms which maintain the low pH. This greatly lower pH has profound negative impacts on all other forms of stream life.

Comparison Among Freshwater Habitats

Hydrology, the potential for oxygen depletion, whether organic C is primarily of allochthonous or autochthonous origin, and the role of plant detritus are among the major determinants of variations among freshwater ecosystems in microbial processes. These variations couple with the relatively small number of studies on some aspects of ecology of microorganisms in freshwater habitats limits the number of generalities that have emerged.

A critical limitation on our ability to draw generalities about microbial ecology of freshwater habitats is the relatively limited number of studies performed on certain aspects of the topic. This fact coupled with the limited coverage of the broad range of diversity of ecosystems suggests that we have yet to reveal many of the underlying tendencies that may unite freshwater habitats. It is not known if specific locations that have been studied in some detail are representative of the greater array of locations that have never been studied.

Overall, major determinants of the structure and function of the microbial community in freshwater habitats are the occurrence and importance of plant detritus, the nature of the hydrology of the system, and the occurrence of oxic and anoxic zones. The potential for drastic spatial and temporal changes in critical factors, such as redox potential, creates opportunities for a diversity of different microbial niches and interconnectivities. This creates interesting spatio-temporal juxtapositions of complementary microbial processes over comparatively small scales. Each microbe-mediated process can be altered by anthropogenic disturbances, which are all too common in freshwater systems because of the proximity to human populations, strong connections to the surrounding landscape, and vital importance for human success.

Emerging Directions and Approaches

Molecular methods are key to the future of freshwater microbial ecology. However, there are limiting factors that will be overcome with time including the need for more sequence data from a wide range of freshwater habitats, meta analysis of these data sets, new pipelines for sequencing, etc. In addition, knowledge of understudied and uncultured organisms is needed to make sure that all taxa are well covered. Perhaps the biggest challenge is to attribute specific functional abilities to specific taxa.

Advances in freshwater ecology also have the potential to enhance our understanding of their role as habitats for microorganisms. More specifically the use of embedded wireless sensors has the ability to measure and transmit critical environmental data, like temperature and dissolved oxygen, in real time. Sensor platforms or small single sensors placed in freshwater systems are becoming an increasingly important tool for monitoring of environmental conditions; real time monitoring has become increasingly prevalent in marine systems over the past several years.

Beyond this capacity to measure physical and chemical conditions of the environment, sensor technology can also be used to detect specific microorganisms or their products. For example, there are sensors for fluorometric detection of specific pigments of cyanobacteria, such as phycocyanin, which have application in monitoring of harmful algal blooms as well as those for more general compounds, like chlorophyll. There is also technology now that allows detection of specific bacteria (such as *Escherichia coli*) and may rely on antibody based detection, for example. The next generation of biosensors which is just now being developed for and applied to freshwater systems are nucleic acid-based systems which can be targeted to detect a variety of bacteria and viruses.

Ultimately, the integration and analysis of many large data sets whether they be 'omics' or environmental data from sensors will continue to be important in the future. In this way, discrete findings from individual studies can be compared and built upon in search of generalities.

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Relevant Website

www.gleon.org—GLEON.

Fundamentals of Metabolic Systems Biology

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Topic 1: Systems Biology as Genome-Scale Science

Some 60 years ago, the promise of molecular biology held that if we knew and understood the function of the molecules that comprise cells, then we could understand cells and their functions. Although this was true in principle, the sheer number of molecules made it very difficult to comprehend so many simultaneous functions. Thus, to fulfill this promise, systems analysis is needed.

The simultaneous measurement of members of whole classes of biomolecules became possible over the past two decades (metabolomics, lipidomics, proteomics, etc.). In addition, methods (ChIP-Exo, Ribo-Seq, PPI, etc.) have been developed to measure interactions between large numbers of biomolecules. Consequently, there are a growing number of datasets available that give us the molecular composition of cells under a certain condition. Many of the chemical interactions (i.e., mechanisms) between many of these components are now known and this knowledge gives rise to reconstructed biochemical reaction networks on a genome-scale that underlie various cellular functions. Structured representations of this information can be converted into a mathematical form enabling computation and model building. Thus the formulation of '*in silico*' cells became possible that represent their *in vivo* counterpart based on our current state of knowledge. *In silico* cells became a foundation of the bottom-up approach to systems biology.

Systems biology is not focused so much on the biomolecules themselves, but rather on the activity of the links (i.e., their chemical and physical interactions) that connect them, and the computation of 'functional states' of reconstructed networks. Functional states of networks correspond to observable physiological or homeostatic states. Completing quantitative relationships between the chemical components of a cell (with their genetic bases) and their physiological functions is the promise of (molecular) systems biology. This undertaking represents the *de facto* construction of a high-dimensional, mechanistic genotype–phenotype relationship.

As an example of the systems biology approach, we illustrate computational prediction of essential genes, and synthetically lethal gene pairs, based on genome-scale metabolic models. Predictions of gene essentiality are made by computing the growth capabilities (*i.e.* the synthesis of all biomass components in the right ratios) of an *in silico* cell by removing the activities of a single gene. Synthetic lethals can be predicted by simultaneously removing two genes from an *in silico* cell and computing its growth capabilities. These predictions represent perhaps the largest scale and most intricate computational predictions of phenotypes performed to date, reaching hundreds of thousands of predicted experimental outcomes. The comparison of computed lethality and experimental measurement in a number of studies is shown in Fig. 1. The remainder of this article illustrates the concept of a network reconstruction, the formation of an *in silico* cell (i.e., a computable knowledge base), and its uses to understand biological functions.

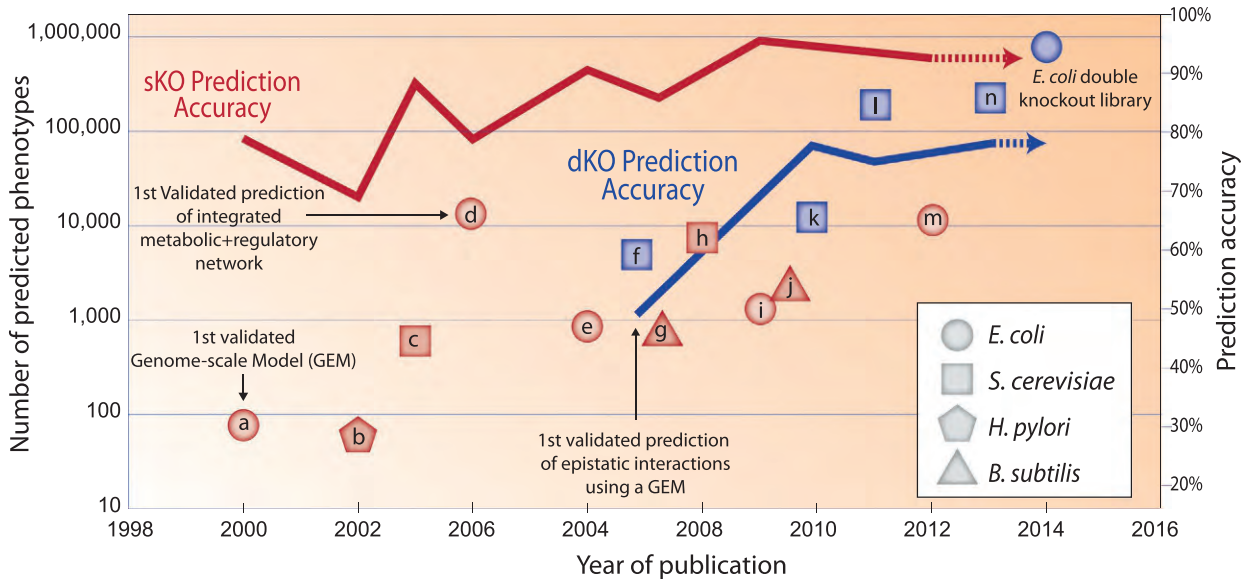


Fig. 1 Predicting experimental outcomes of cellular growth screens. The number of predictions made on growth screens that cross environmental conditions with gene knockouts has grown steadily over the past 15 years. Over this time, both single-gene knockout (SKO) predictions (red line) and double-gene knockout (DKO) predictions (blue line) have become increasingly accurate. Figure is taken from Monk, J., Palsson, B.O., 2014. Predicting microbial growth. Science 344, 1448–1449.

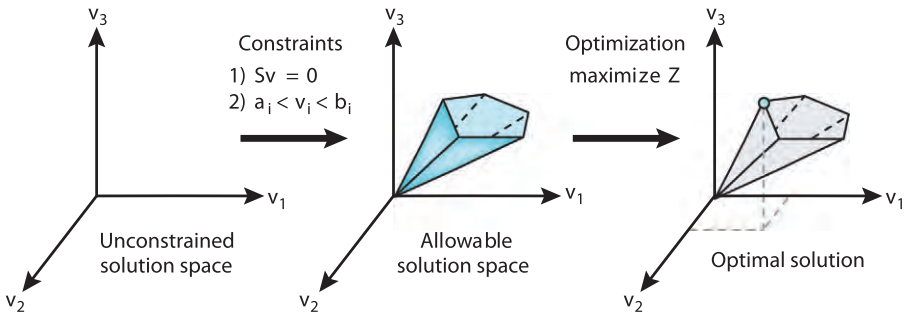


Fig. 2 The conceptual basis of constraint-based modeling. With no constraints, the flux distribution of a biological network may lie at any point in a solution space. When mass balance constraints (**Sv**=**0**) imposed by the stoichiometric matrix S (labeled 1) and capacity constraints imposed by the lower and upper bounds ($\mathbf{a_i}$ and $\mathbf{b_i}$) (labeled 2) are applied to a network, it defines an allowable solution space. The network may acquire any flux distribution within this space, but points outside this space are denied by the constraints. Through optimization of an objective function, one can identify an optimal flux distribution that lies on the edge of the allowable solution space. Figure is taken from Orth, J.D., Thiele, I., Palsson, B.Ø., 2010. What is flux balance analysis? Nat. Biotechnol. 28, 245–248.

Topic 2: Introduction to COBRA Methods and Reconstruction

Two fundamental steps: The underlying COnstraint-Based Reconstruction and Analysis (COBRA) methods are based on relatively simple concepts, but their application can result in non-intuitive, novel predictions. COBRA entails two fundamental steps (Fig. 2):

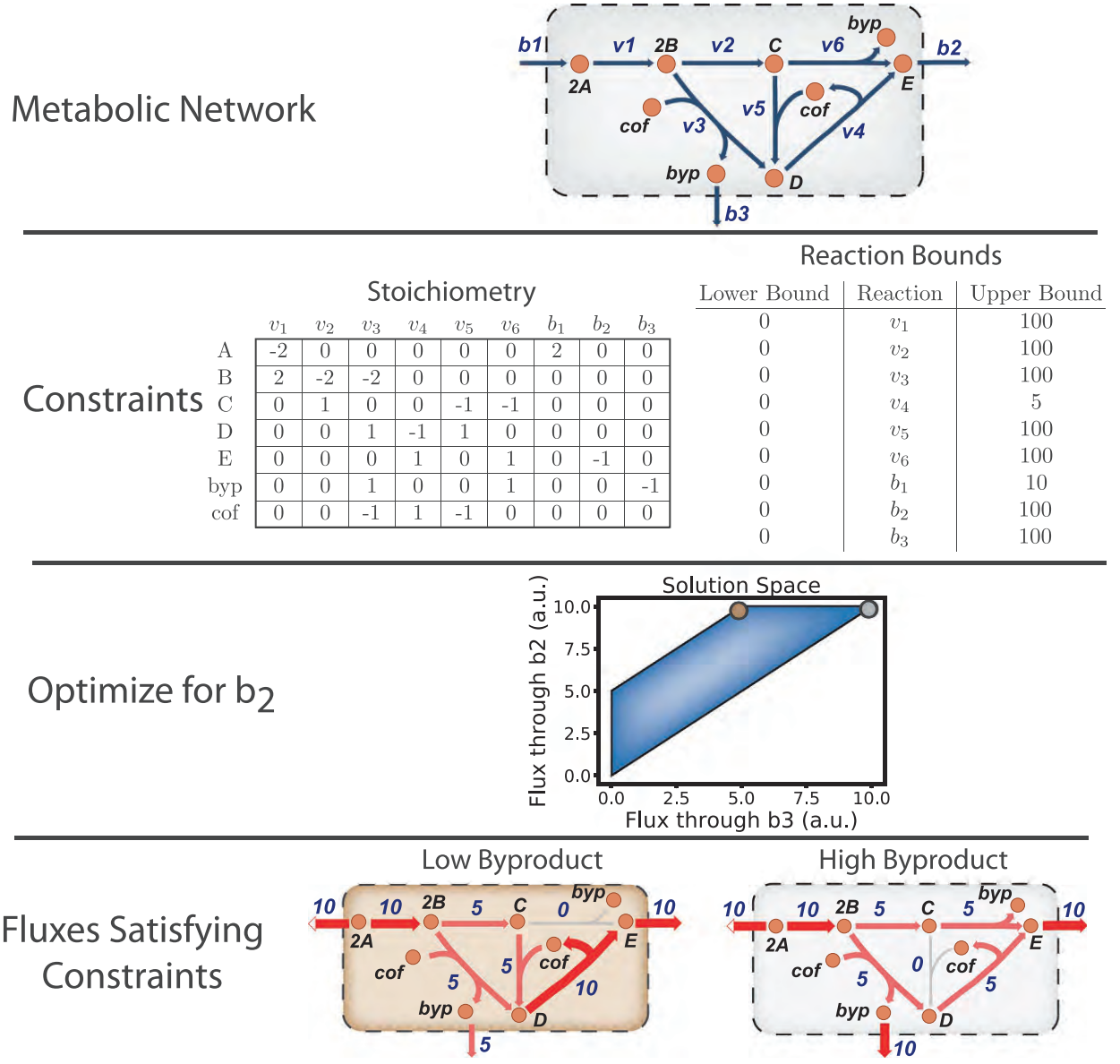
- The first step is the network reconstruction process described above. A reconstruction, once mathematically represented, can distinguish between the possible and impossible phenotypic states. Basically, one can find the fundamental constraints that come with all the interactions between the biomolecular components of a cell and form what is called a 'solution space.' This space contains all the allowable network states. It is analogous to the term 'reaction norm' used in ecology and genetics.
- The second step is to find the states within the solution space that are likely to represent the homeostatic state of an organism. These states are found by using constraint-based optimization methods. Optimization requires an objective function that describes the desirable properties of the homeostatic state. Constraint-based optimization finds the best points within the solution space based on the stated objective function. The most commonly used objective function is growth rate, although many others have been studied. Ultimately, the objective function describes distal causation, thus representing fundamental biological causation.

Illustrative example: An overview of the COBRA method is shown in Fig. 3 for a simple toy network, though the same procedure is used to produce COBRA models at the genome scale. The key differences being the number of reactions in the model and a few additional considerations, which will be discussed below in Topic 3.

A COBRA model for the simple toy network shown in Fig. 3 can be constructed using the following steps. First, the stoichiometries for all of the reactions in the toy network must be defined including all possible inputs (b_1) and all outputs (b_2 and b_3) of the system. This definition is trivial for the toy network shown, but when executing this process on a genome scale, there are numerous additional considerations that must be addressed. Next, the catalog of reactions must be transformed into a mathematical format that lends itself to computation. This transformation is accomplished by constructing what is called a stoichiometric matrix, shown in Fig. 3. In this format, each column represents a unique reaction and each row a unique metabolite. The numbers in the matrix thus indicate the stoichiometry of the metabolite in each reaction, with negative numbers representing reactants, and positive numbers representing products. The matrix entry is 0 if the metabolite does not participate in the reaction.

Constructing the stoichiometric matrix alone does not produce a useful COBRA model. Bounds must be placed on the activity of each individual reaction as well. Bounds provide the limits on the amount of flux each reaction can carry. In the toy example the units of the reaction flux is ambiguous, but at the genome scale reaction fluxes have units of mmol per gram dry weight of cell per hour (mmol gDW^{-1} hr^{-1}). In the toy example, all reactions are irreversible and operate only in the forward direction, indicated by reaction bounds being greater than or equal to 0 for each reaction. If reactions operate only in the reverse direction or are reversible, the lower reaction bounds should be set below zero. Reaction b_1 defines the input of species into the system, which is metabolite A in the toy network, and is limited to 10 units of uptake flux (Fig. 3). This flux limitation is analogous to the substrate uptake rate for an organism's genome-scale model.

After defining the stoichiometric matrix and reaction bound constraints, we can define a particular reaction to optimize (i.e., maximize or minimize its reaction rate, or flux) subject to the constraints inherent in the model. One of the several freely available software tools can then be used to compute with the model using a linear programming solver. Solutions to a COBRA metabolic model are non-unique, meaning there are an infinite number of flux states that can produce an optimal result. The full extent of fluxes that satisfy constraints can be represented as a solution space, depicted as the polygon for the toy network in Fig. 3. This example shows all of the fluxes possible through reactions b_2 and b_3 given the stoichiometric and reaction bound constraints of the toy network. Two extremes of the solution space are shown. These are equally optimal solutions that produce a maximum amount of flux through the b_2 reaction.



	v_1	v_2	v_3	v_4	v_5	v_6	b_1	b_2	b_3
A	-2	0	0	0	0	0	2	0	0
B	2	-2	-2	0	0	0	0	0	0
C	0	1	0	0	-1	-1	0	0	0
D	0	0	1	-1	1	0	0	0	0
E	0	0	0	1	0	1	0	-1	0
byp	0	0	1	0	0	1	0	0	-1
cof	0	0	-1	1	-1	0	0	0	0

Lower Bound	Reaction	Upper Bound
0	v_1	100
0	v_2	100
0	v_3	100
0	v_4	5
0	v_5	100
0	v_6	100
0	b_1	10
0	b_2	100
0	b_3	100

Fig. 3 Overview of the constraint-based modeling method. The toy metabolic network displayed in the top panel can be simulated by converting it into a mathematical format, as shown in second panel. Doing so involves first converting the stoichiometry of all of the reactions in the network into a matrix representation where the columns correspond to reactions, the rows correspond to metabolites, and the matrix values correspond to the stoichiometry of the metabolite in the reaction. Further, the upper and lower flux limits on each reaction must be defined as reaction bounds, as shown. This representation can be converted into a linear programming problem using available software and solved. The solution to this problem however is not unique, and the full range of possible solutions can be represented as a solution space, shown in the third panel. Two of the labeled vertices of the solution space are shown overlaid on the network in the bottom panel which are equally optimal solutions that give the most reaction flux through b2. These can be considered low byproduct and high byproduct solutions based on the amount of metabolite byp secreted.

Escherichia coli as a model organism for systems biology: The organism with the most extensive, validated, and widely-used COBRA model is *E. coli*. As a model organism, many metabolic processes were first discovered in *E. coli* and are best characterized in this organism. This rich *E. coli* bibliome has culminated in the recent, most comprehensive COBRA model of *E. coli* K-12 MG1655, *i*ML1515. Information regarding the pathways and processes modeled in *i*ML1515 and other high-quality models can be found at bigg.ucsd.edu.

Scalability: From a strain to a species to infections on a national scale: Beyond predicted metabolic capabilities of the lab strain, the *E. coli* K-12 MG1655 model can help us understand the *E. coli* species as a whole. An initial study of 55 sequenced *E. coli* strains demonstrated the ability to predict auxotrophies and niche-specific nutritional requirements (Monk et al., 2013), that were subsequently repeated with over 1200 strains (Monk et al., 2017).

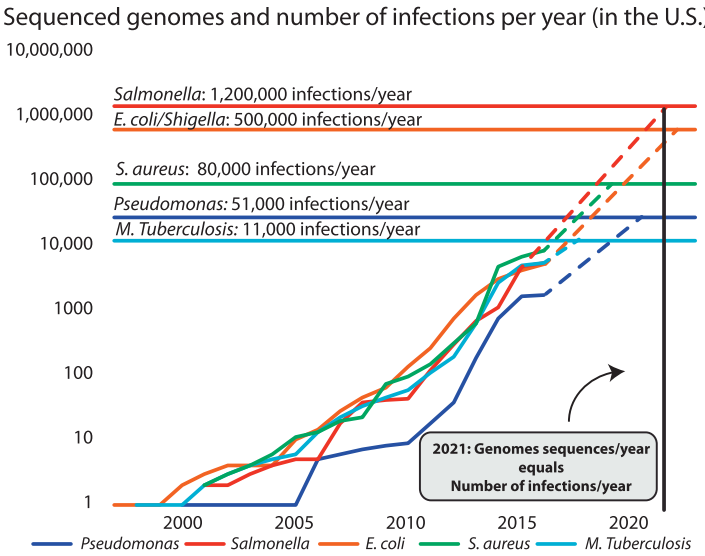


Fig. 4 Increase in sequenced genomes for five common clinical pathogens. If current trends are maintained, the number of sequenced genomes for all five pathogens will equal the number of infections per year by 2021. The number of sequenced genomes per organism were obtained from the Patric database (www.patricbrc.org). The infections per year were obtained from the Center for Disease Control (www.cdc.gov) with the infections per year obtained for S. aureus from the Pew Charitable Trusts (www.pewtrusts.org). Figure prepared by Jonathan Monk.

Prediction from sequence alone opens up new possibilities. For example, using a genome sequence of a clinical isolate, *i*ML1515 can be used to construct COBRA models tailored to that specific pathogen. With the number of sequenced clinical *E. coli* isolates, and those of other major pathogens, increasing at a staggering pace (Fig. 4), genome-scale models offer a means to extract essential information that might lead to guidelines on how to treat a specific unique infection. If such predictions materialize, we can move the field of infectious disease closer to truly personalized medicine. If the current rate of sequencing continues, it becomes feasible by ca. 2021 to sequence all clinical isolates in the United States (Fig. 4). A truly amazing prospect.

Topic 3: The Metabolic Requirements for the Production of Biosynthetic Precursors

Pathway versus genome-scale networks viewpoints: Traditionally, microbiology is taught from a pathway or biochemical reaction perspective. In reality, however, cellular survival relies on the activity of many such interconnecting pathways working in conjunction to produce the metabolites needed for growth in the proper amounts. Beyond this, these individual pathways vary among species and even to some extent within strains of the *E. coli* species itself. Obtaining actionable knowledge by mapping out the metabolic pathways within an organism is difficult. To this end, systems biology methods provide the tools that enable the use of this information to compute phenotypic and metabolic characteristics of an organism despite the complex reality of cellular metabolism.

The power of systems biology can be illustrated by analyzing a 11 reaction linear pathway to produce L-histidine from D-ribose. It is shown in Fig. 5 as is it might be discussed in a traditional microbiology book or class. What is typically glossed over, however, is the role that cofactor or metabolic precursor availability may play in shaping the functioning of this pathway or how the activity of any byproducts that are synthesized may affect other metabolic activities in the cell. When using flux balance analysis to simulate the production of L-histidine from D-ribose aerobically (oxygen uptake limit of 10 mmol gDW^{-1} hr^{-1}), 72 total reactions must be active in order to enable the function of the 11 reaction pathway in a fully mass balanced way.

Why are these 61 extra reactions necessary? L-histidine synthesis is an anabolic process relying on chemical or energetic contributions from various metabolites in the cell. For three reactions in the pathway energy from ATP is required to fuel metabolic conversions or to transfer high energy phosphate bonds to L-histidine precursors to fuel future metabolic conversions. Synthesizing ATP thus requires that some of the substrate (D-ribose) is diverted from reactions in the L-histidine synthesis pathway to the pentose phosphate pathway and, eventually, to glycolysis. Flux through glycolysis enables ATP synthesis directly as a product of glycolytic reactions and indirectly by reducing NAD^+ to NADH, which can be used for oxidative phosphorylation. Some additional ATP in this example is produced by fermentation and acetate secretion due to the limit on oxygen uptake being set to 10 mmol gDW^{-1} hr^{-1}. This value is below the amount needed for oxygen excess and fully aerobic metabolism in the simulation. Further, L-histidine contains three nitrogen atoms that it must acquire given that D-ribose does not contain nitrogen. One nitrogen is incorporated from adenosine and the other two are added from L-glutamine and L-glutamate. The pathways that incorporate inorganic nitrogen into these metabolites must therefore be active. Side products of the L-histidine synthesis pathway must also be degraded. One such byproduct is 5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamide (AICAR) which is

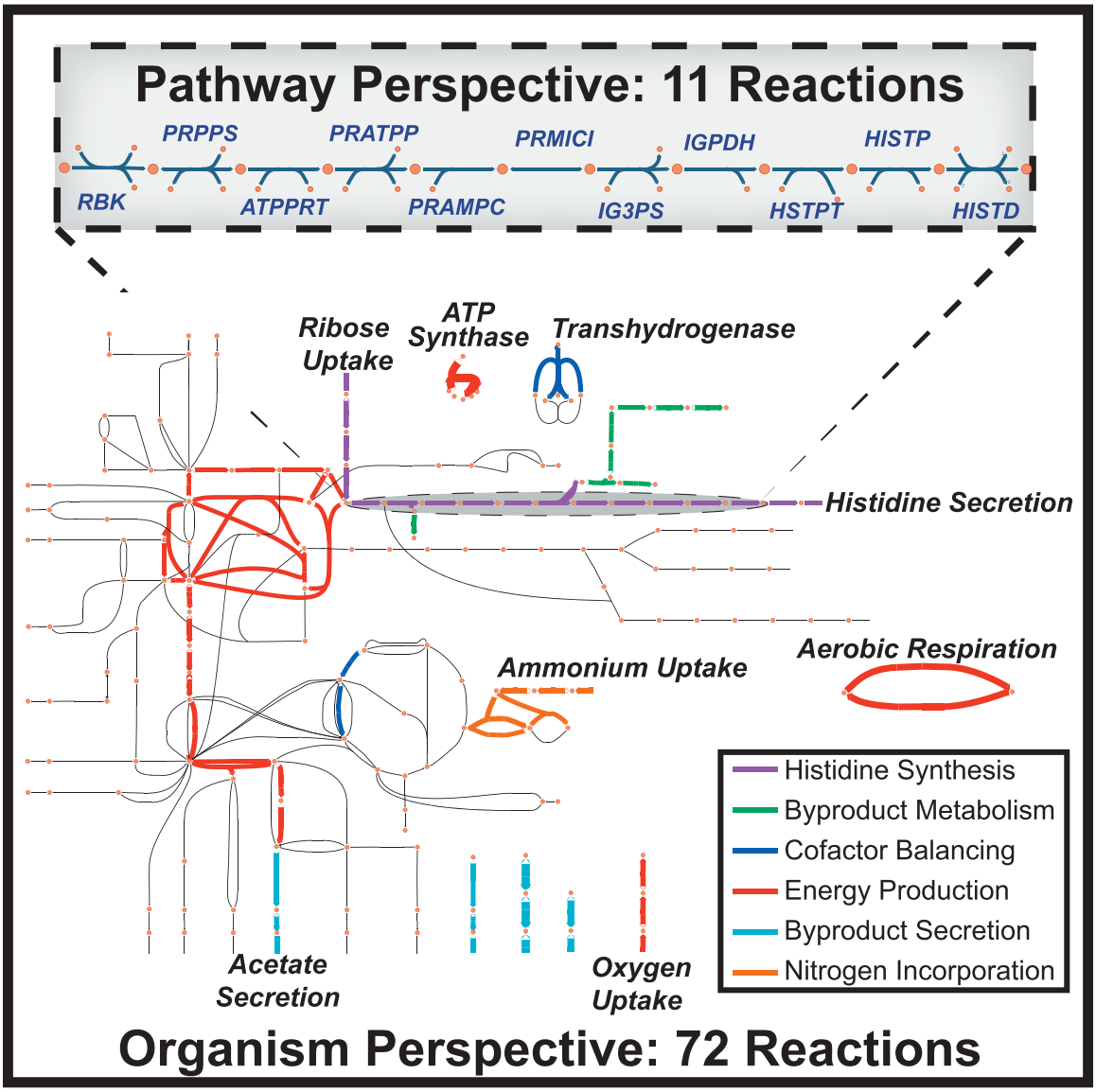


Fig. 5 Contrasting the traditional pathway view (i.e., textbook) and the network view (i.e., systems biology) of the biochemical processes needed to produce a single biosynthetic precursor. The L-histidine biosynthetic pathway is considered 'long', being composed of 9 enzyme-catalyzed reactions on 1 operon. When considering L-histidine synthesis from D-ribose, 2 additional reactions are required to phophorylate D-ribose to form 5-Phospho-alpha-D-ribose 1-diphosphate (PRPP) so that it can enter the L-histidine biosynthetic pathway. This brings the total number of reactions in this example pathway to 11. In order to synthesize L-histidine from D-ribose in a way that is fully mass-balanced, however, it can be seen that a total of 72 reactions (61 of which are enzymatic) are required. Below we discuss the full metabolic requirement for such biosynthesis.

degraded to ADP in this example, though *in vivo* AICAR is used for inosine biosynthesis. Lastly, all NAD^+/NADH cofactor usage must be balanced, meaning that NADH must be produced and consumed at equal levels (Fig. 5). Synchronizing all these functions requires a total of 61 additional active biochemical reactions.

Networks are highly interconnected, and thus their characteristics can be non-intuitive: Many important properties of the network cannot be determined based on simple characteristics or intuition. For instance, there is little relationship between the predicted carbon yield (i.e. the fraction of carbons in a substrate that end up in the product metabolite) and the number of reactions separating the substrate and product metabolite (Fig. 6). D-ribose, D-glucose, and other metabolites with few enzymatic reactions separating them from L-histidine are actually capable of synthesizing L-histidine with a carbon yield similar to substrates separated from L-histidine by many more intermediate reactions, like acetate.

Acetate, however, is a poor-quality substrate for *E. coli*, meaning that cells grows slowly when fed this metabolite as a carbon source. This is due to the fact that growth is dependent on the organism's ability to produce all biosynthetic precursors and energy

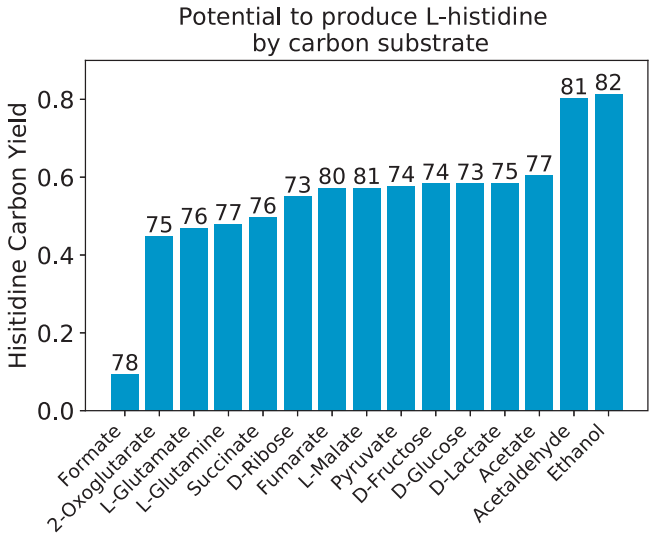


Fig. 6 Maximal aerobic (oxygen uptake limit of 10 mmol gDW^{-1} hr^{-1}) carbon yield of L-histidine from 15 *E. coli* carbon substrates. The number of active reactions required for each substrate to produce L-histidine is shown above each bar.

necessary to grow from the *in silico* growth media. Therefore, while acetate may be able to produce L-histidine efficiently, it is a highly oxidized carbon source meaning that it cannot produce ATP as efficiently as substrates like glucose, limiting its growth capabilities.

From a single amino acid to synthesis of all biomass constituents: This systems-level analysis has shown that even the activity of a short, linear pathway relies on the interconnectivity of many different metabolic subsystems in order to operate. Beyond this, all of the considerations discussed for the L-histidine biosynthesis pathway hold true for all other essential biosynthetic precursors that need to be synthesized in a growing cell. For an organism to successfully synthesize the dozens of macromolecular precursors and produce the amount of energy needed to grow, all of these pathways must be active and working in harmony.

In order to take the biosynthesis of these biomass constituents into account, COBRA models include a reaction called the biomass objective function. This function includes all of the essential metabolites (i.e., structural components, biomass building blocks, cofactors and currency metabolites, etc.) with empirically derived coefficients corresponding to their concentrations in a growing cell. The flux through this reaction corresponds to the *in silico* growth rate of the cell under the environmental conditions imposed. Since the production of each metabolite in the biomass objective function must be produced in the proper amounts and in a fully mass balanced way, the interconnectivity of the pathways producing each essential metabolite can be fully accessed in a model simulation. Remarkably, the power of systems science allows us to perform these computations.

Topic 4: The Proteomic Requirements for the Production of Biosynthetic Precursors

Proteome size is limited: The availability of protein within a growing cell is limited to about 2–3 million protein molecules per cell. Cells have therefore evolved to utilize the available proteome as efficiently as possible. This optimization of proteome investment has led to certain unintuitive and sometimes seemingly "wasteful" growth phenotypes (e.g., acetate overflow in *E. coli* or the Warburg effect in cancer cells) that require the consideration of protein cost to be accurately described and understood. While the COBRA methods described in *Topics 2 and 3* have proven effective in computing the metabolic potential and characteristics of an organism, they do not account for protein costs. In other words, metabolic network models do not consider the proteome investment required to synthesize the enzymes that catalyze flux through a metabolic network.

This lack of consideration of proteome cost means that any two pathways converting metabolite ***A*** to metabolite ***D*** are treated equally by the model regardless of the efficiency of the enzymes catalyzing the reactions, the length of the pathways, or the metabolic investment required to synthesize the enzymes making the metabolic conversions. Using an economic analogy, the 'operating expense' of a metabolic reaction is accounted for in a metabolic model but not the 'capital expense' required to build the protein that catalyzes the reaction. Ignoring proteome costs can lead to metabolic model predictions of incorrect or unrealistic pathway activity and the presence of alternative optimal reaction flux states (Fig. 3).

Genome-scale models can compute proteome composition: COBRA models have been developed that explicitly describe the processes involved in gene expression and proteome synthesis. These genome-scale models of proteome synthesis impose a biosynthetic cost of catalyzing a metabolic reaction in the model by requiring the synthesis of the catalyzing enzyme in order for a reaction to carry flux. They are called ME-models, for Metabolism and Expression.

The proteome cost is imposed by considering the dilution of macromolecules to daughter cells as the organism grows and divides (Fig. 7(A)). The faster the cell is growing, the more the macromolecules are passed on and therefore must be replaced through model synthesis. This relationship provides the basis for genome-scale models of proteome synthesis (ME-models) and

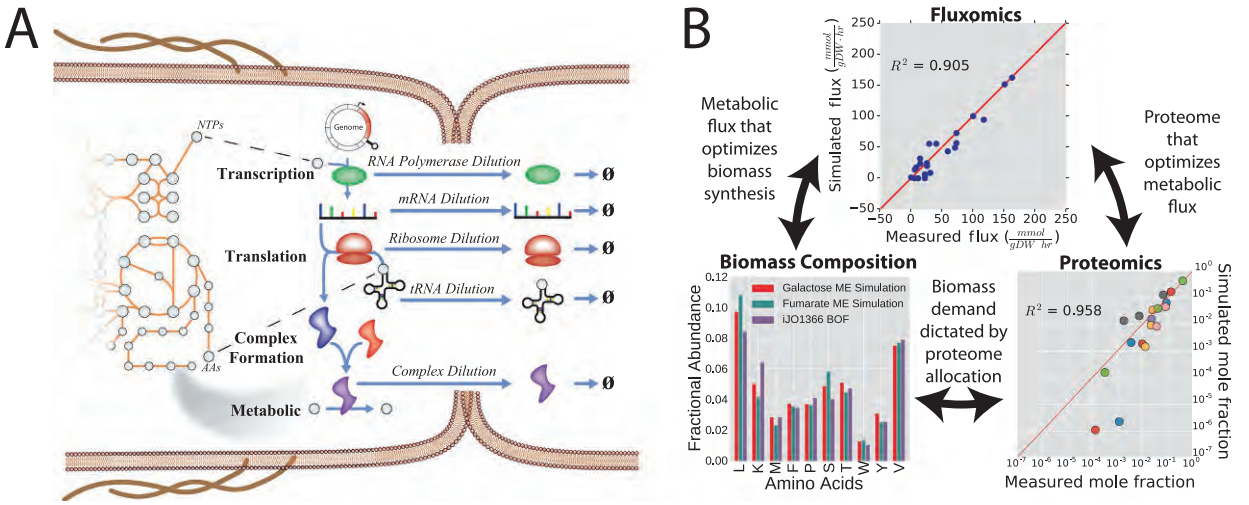


Fig. 7 (A). A depiction of each of the major cellular processes involved in gene expression, all of which are modeled in gene expression models. (B). In order for a single genome-scale model of proteome synthesis (ME-model) to solve, multiple processes involved in cellular growth must be reconciled. For example, the cellular reaction flux state must be supported by the proteome catalyzing all of these processes. The proteome, in turn, is itself supported by the synthesis of biomass precursors. The biomass precursor demand for the proteome then informs the optimal metabolic flux state. Solutions of ME-models can then be validated against disparate OMICs data types (i.e., ^{13}C fluxomics, proteomics, etc.). Panel (A) is from Lloyd, C.J., Ebrahim, A., Yang, L., et al., 2017. COBRAme: A computational framework for building and manipulating models of metabolism and gene expression. bioRxiv.

their ability to link the synthesis of a macromolecule to the reaction which it catalyzes. For a more thorough explanation on this computational method, refer to Lloyd et al. (2017) and O'Brien et al. (2013).

Proteome limitations lead to optimal proteome allocation that can be a governing constraint: How do ME-models improve the framework we introduced in *Topic 2*? First, the nutrient uptake and metabolic reaction fluxes are determined by optimal protein utilization as a consequence of limited proteome availability. As opposed to COBRA models of metabolism which require measured substrate uptake rates in order to make feasible predictions, ME-models are capable of determining the optimal nutrient uptake rates with reasonable accuracy. In addition, ME-model solutions more accurately estimate the metabolic flux distribution by eliminating biologically infeasible, alternative optimal flux distributions (described in *Topic 2*) that do not satisfy the constraint imposed from limited proteome availability. As a result, the optimal solution of a ME-model is effectively unique. Second, the relative abundance of proteins and other macromolecules required to support the metabolic phenotype is predicted by a ME-model. These macromolecule abundances provide novel predictions of macromolecular expression that can be compared against proteomics data or other numerous OMICs data types (Fig. 7(B)). The unique predictions enabled by ME-models open entirely new avenues of biological questions and discovery.

Topic 5: The Condition-Dependent Proteome

The genome-scale computation of the composition of the proteome is just the first step in computationally representing a functioning proteome. To become functional, many proteins require post-translational modification and engraftment of prosthetic groups. Additionally, there are constant degradative forces operating on a proteome. Remarkably, with the chemical basis for these functions, they can be explicitly reconstructed and added to ME-models that compute the composition of the proteome. Here we briefly describe how such *in silico* models of whole cells deal with, (1) the requirement of iron in the functional center of certain enzymes, and (2) the requirement of chaperones to keep the proteome correctly folded in the face of thermally caused protein misfolding.

(1) *Functionalizing the proteome: metalloproteins.* The catalytic activity of inorganic iron has played a central role in facilitating important metabolic processes in organisms dating back to the beginning of life on earth. As a result, many important processes in living cells still depend on the availability of iron in order to function (Fig. 8(A)). Using ME-models, we can predict how reductions in the availability of iron cause the metabolic phenotype of *E. coli* to change. In agreement with experimental observations, decreasing iron availability causes the cell to grow much slower and shift to secreting D-lactate instead of acetate (Fig. 8(B, C)). The predicted causes of this shift is the reduced activity of the metabolic enzymes that have iron in their catalytic site.

Beyond studying growth and metabolism under iron-limited conditions, ME-models have been developed that incorporate damage and repair processes of iron-containing proteins in the presence of oxidative stress. In doing so, these models have the capability to, for instance, correctly predict amino acid auxotrophies in *E. coli* when exposed to elevated oxidative stress in superoxide dismutase mutants (Yang et al., 2017).

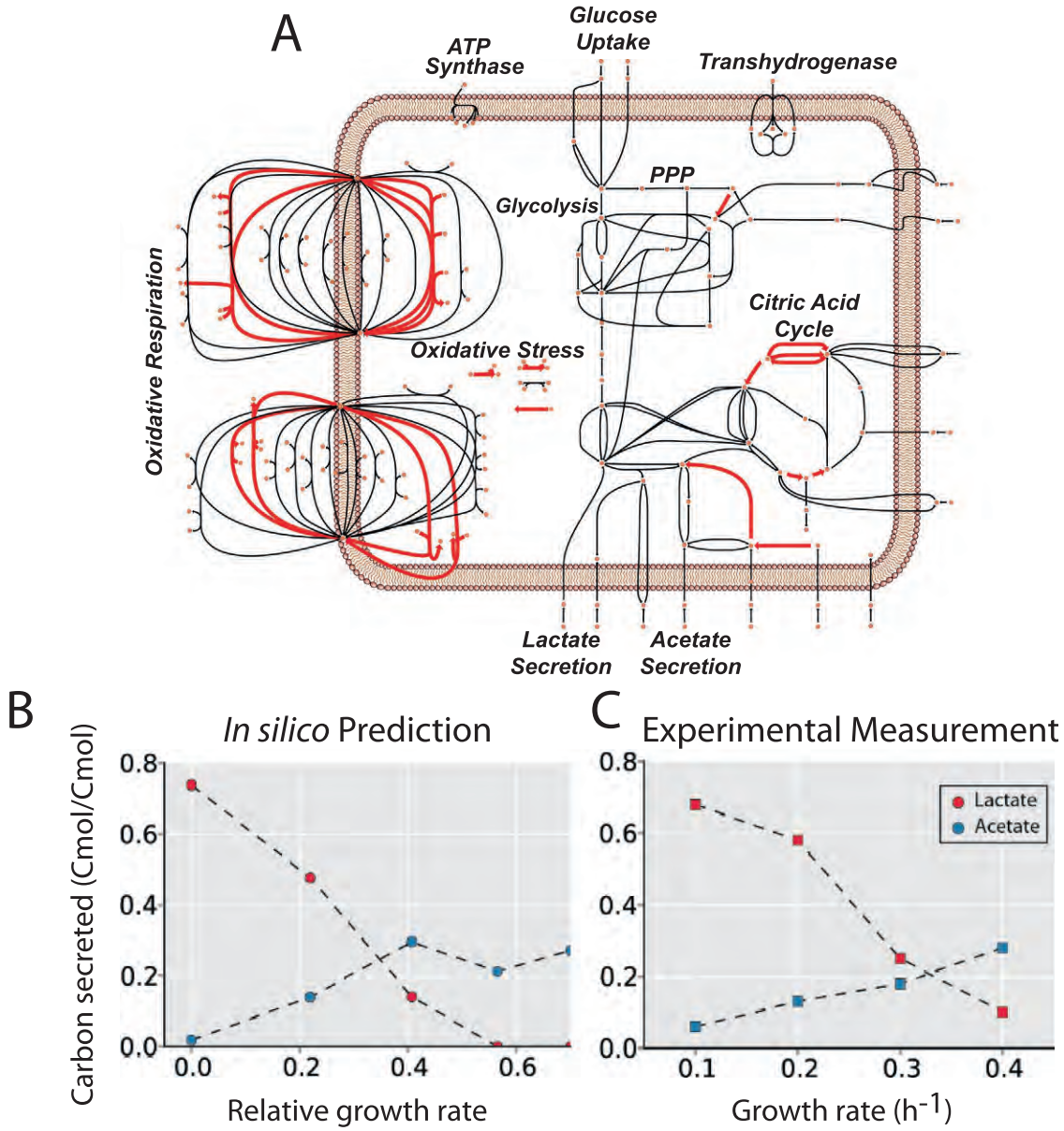


Fig. 8 (A) Central carbon metabolism with the reactions catalyzed by iron-containing enzymes in red. (B) *In silico* predictions of relative growth rate and metabolic byproduct secretion when under varying levels of iron limitation. (C) These *in silico* predictions agree with experimental measurements of *E. coli* growing in iron limitation. Parts (B) and (C) are adapted from O'Brien, E.J., Utrilla, J., Palsson, B.O., 2016. Quantification and classification of *E. coli* proteome utilization and unused protein costs across environments. PLoS Comput. Biol. 12, e1004998.

These two examples highlight the importance of accounting for the metallo-proteome when fully modeling cell metabolism. Both the availability of iron and the oxidation load on iron can be described by elementary processes and thus included in an *in silico* model of a cell.

(2) *The physical integrity of the proteome: proteostasis.* Beyond incorporating vital coenzymes, prosthetic groups or metal ions, proteins must also be properly folded to enable their proper catalytic function. By incorporating chaperone activity into these models we can now compute the systems level consequences of protein unfolding and refolding (proteostasis). To begin to model the protein unfolding response, we must first understand that proteins themselves have intrinsic capabilities to keep their shape. In a recent study, we described how computed properties of individual proteins can determine when they are targeted by the chaperones of *E. coli* (Fig. 9).

How are these protein properties incorporated into genome-scale models? The recently introduced GEM-PRO pipeline (genome-scale models with protein structures) enables the curation and computation of protein sequences and structures within genome-scale models (Fig. 9(A)). Thousands of 3D protein structures are solved experimentally every year, thanks to efforts of structural biologists

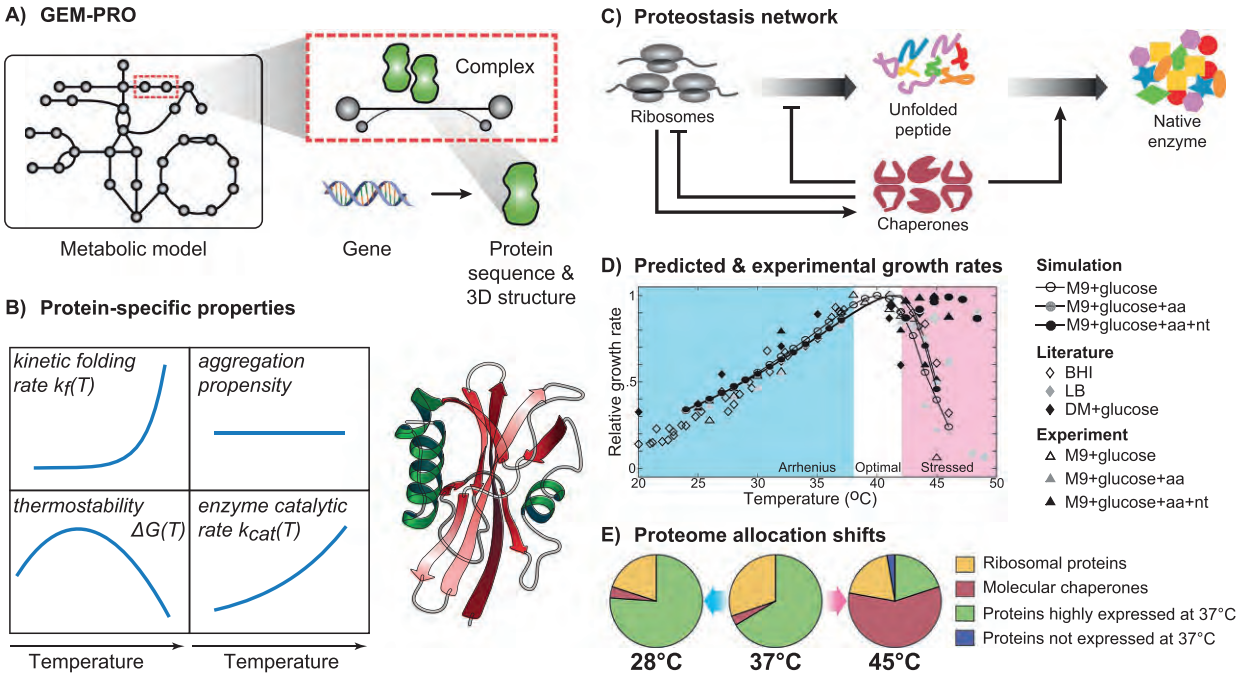


Fig. 9 Utilization of protein structural properties within a genome-scale model for the purpose of modeling thermal stress adaptations. (A) The formal integration is termed a "GEM-PRO", or genome-scale model with protein structures. Enzymatic reactions are mapped to their corresponding protein sequences and structures. (B) Protein-specific properties can be predicted from sequence and structure, and further computed to reflect protein property changes under stress. For example, four parameters are chosen to represent the influence of temperature on the status of the structural proteome. (C) Incorporation of chaperone folding networks adds the proteostasis network response to the model. (D) Simulated growth rates of an integrated model match very closely to measured experimental rates at different temperatures. (E) Proteome allocation of the cell under thermal stress can be inspected to find a dramatic shift towards chaperone production at high temperatures. Figure is adapted from Mih, N., Brunk, E., Chen, K., et al., 2018. ssbio: A python framework for structural systems biology. Bioinformatics 34, 2155–2157. And Chen, K., Gao, Y., Mih, N., et al., 2017. Thermosensitivity of growth is determined by chaperone-mediated proteome reallocation. Proc. Natl. Acad. Sci. USA 114, 11548–11553.

worldwide and programs such as the Protein Structure Initiative. However, a large gap remains between known structures and the total number of annotated open reading frames within sequenced genomes. Due to this gap, structural information remains incomplete for a number of reasons, such as the protein (membrane proteins are difficult to crystallize) or organism of interest (some organisms are not as well studied). Fortunately, for model organisms such as *E. coli*, we have now reached a point where structural information is available or can reliably be predicted for a large portion of the expressed proteome. This enables what is broadly known as a structural systems biology for genome-scale models.

Once a GEM-PRO has been generated for *E. coli*, we can then predict or compute protein properties as a function of temperature from first principles (Fig. 9(B)). Incorporating two major chaperone systems and their folding networks (Fig. 9(C)) to the ME-model thus allows us to understand how changes in these protein properties affect the systems-level response to changes in temperature. This model, named foldME, thus enables a surprisingly accurate prediction of relative growth rates under different temperatures (Fig. 9(D)). Furthermore, foldME allows us to inspect changes in the production levels of certain proteins (i.e., the cell's proteome allocation). Under high temperatures, there is a dramatic shift toward the production of chaperones while shifting away from the production of cytoplasmic proteins (Fig. 9(E)).

Thermostability has often been a property studied in the context of individual proteins, or as a global phenotypic response. FoldME exemplifies how incorporating atomic-level information can expand the systems biology paradigm, and it provides an understanding of how a cell balances its protein production and maintenance (folding) machinery under thermal stress.

Topic 6: Multi-Strain Reconstructions Enable Assessment of Strain Variation

Genomic sequences from multiple strains allow us to ponder the definition of a species. As shown in Fig. 4, there has been a staggering increase in the number of sequenced strains of *E. coli*. These sequenced genomes have allowed us to adapt reconstructed COBRA models to inspect variation at both the gene-level (i.e., absent metabolic reactions, which may cause auxotrophies) and at the base-pair level (i.e., changes to a protein sequence and their impact on structure). This sequence data availability brings into focus the question: "what is a strain and what is a species?" A comprehensive comparison of the metabolic capabilities of 55 strains of *E. coli* and *Shigella* revealed that these strains can adapt to catabolize nutrients with alternate pathways (Monk et al., 2013). This result exemplifies how COBRA models provide utility by predicting a strain's environmental niche. This approach has been applied to

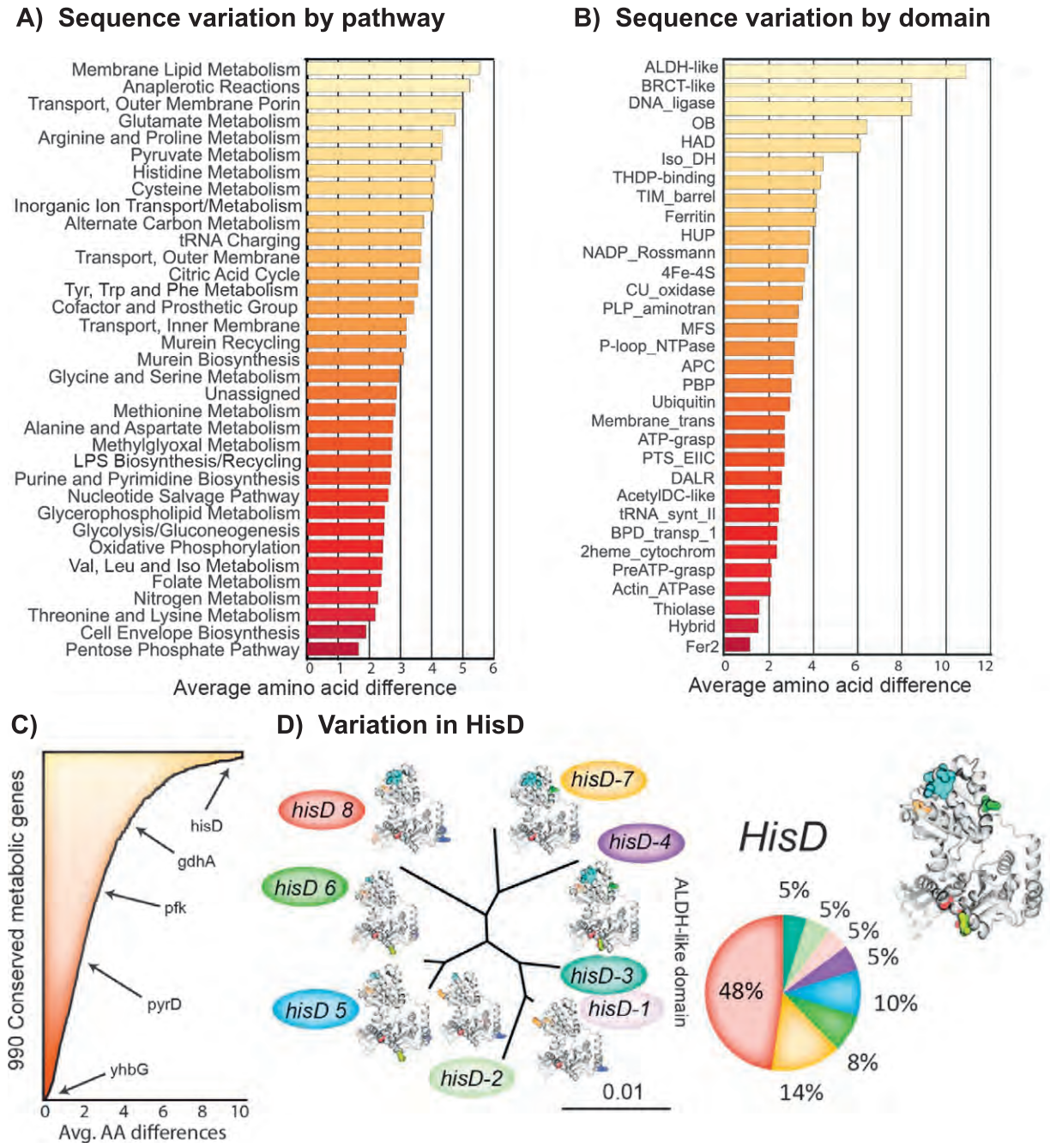


Fig. 10 Analyzing strain variation in *E. coli* at multiple levels. (A) Inspecting variation at the pathway level shows that membrane lipid metabolism and a number of amino acid synthesis pathways top the list of average number of changes in their catalyzing enzymes. (B) Incorporating protein domains into the analysis highlights specific domains that display this variation. (C) 976 genes with metabolic functions are conserved across 99% of *E. coli* strains, and this core set also has mutations. The bar chart shows the average number of amino acid mutations in these core genes for 1122 strains of *E. coli*. (D) L-histidine pathways showed high levels of amino acid differences among genes involved. The pie chart represents the percentage of strains that contain unique *hisD* alleles. The *hisD* allele in *E. coli* K-12 MG1655 is present in only 19 (1.7%) of the sequenced strains. Figure is adapted from Monk, J.M., Lloyd, C.J., Brunk, E., et al., 2017. iML1515, a knowledgebase that computes *Escherichia coli* traits. Nat. Biotechnol. 35, 904–908.

characterize differences in industrially relevant strains of *E. coli*, the aquatic bacterium *Shewanella*, *Staphylococcus aureus*, *Leptospira*, and *Pseudomonas putida*.

Structures can provide a template for analyzing sequence variation. However, some pathways are often quite conserved and essential in many organisms. The L-histidine biosynthesis pathway, for instance, is known to be essential across bacteria, fungi, plants, and archaea, and is present in most strains of *E. coli*. As such, differences in this pathway among strains must now be probed at a deeper level to begin to understand what enables the broad diversity seen within a single species. An initial analysis of sequence-level

variation in over 1000 clinical isolates of *E. coli* revealed a large number of alleles (unique variant forms of the protein) for enzymes in L-histidine biosynthesis and a number of other pathways related to amino acid synthesis (Fig. 10(A)). This analysis was then coarse-grained to consider protein domains, which can be thought of as the functional units of proteins. This domain-level analysis indicated that the aldehyde dehydrogenase (ALDH-like) domain has, on average, more variation across all studied strains compared to other domains (Fig. 10(B)). The last enzyme in L-histidine biosynthesis, *hisD*, contains this ALDH-like domain and had one of the highest counts of alleles, as well as the highest number of mutations conferring an allele (Fig. 10(C, D)). The potential for further characterization of these enzymes due to these variations remains, as these are but clues to begin to understand the true relationship between genotype and phenotype.

Closing

The whole genome sequences that appeared in the mid to late 1990s ushered in the genome era for microbiology. The availability of all the genetic elements on a genome sequence, in turn, ushered in the era of microbial systems biology where the simultaneous function of all the gene products is considered. The basic paradigms of microbial systems biology that have solidified over the past 20 years are manifested in two fundamental steps: first in the reconstruction of all the biochemical, genetic, genomic, and structural information available about a target strain in a formalized fashion, called a reconstruction, and second, in the development of a mathematical framework that allows computational interrogation reconstruction properties, with special emphasis on phenotypic traits. These two steps together comprise *de facto* a multivariate mechanistic genotype-phenotype relationship.

To date, metabolic and proteome synthetic networks have been reconstructed and characterized. Currently, protein structures, proteostasis, and stress responses are being reconstructed at the genome scale. We are thus approaching the point in time were we can explicitly compute over 90% of the proteome by mass and directly relate the outcome of such computations to phenotypic functions. These genome-scale models have invaluable utility to predict and guide hypothesis-driven experimental designs from a genome-scale standpoint and to compare properties of different strains.

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Fungal Biofilms

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Glossary

Antifungal drug An antibiotic drug used to prevent or treat fungal infections.
Biofilm A highly organized microbial community typically attached to a substrate and enveloped within a protective matrix of self-produced expolymeric material.
Biofilm dispersion The process by which some cells detach from the biofilm and become free-floating. Normally it is considered that these cells will disseminate and seed new infectious sites.
Opportunistic pathogen An infectious microorganism that under normal conditions is a commensal or does not normally harm its host, but can cause active infection when in a compromised host.
Quorum sensing A mechanism to detect population density through the use of signal molecules. This allows microorganisms to communicate and coordinate group behavior.
Virulence factor A molecule or property produced by or associated with a pathogen that contributes to the pathogenicity of the organism and its ability to cause infection.

Clinical Significance and Spectrum of Fungal Biofilms

Fungal infections represent an escalating problem affecting an expanding spectrum of immune- and medically-compromised patients (Brown et al., 2012). These infections carry unacceptably high morbidity and mortality rates, due in part to the limited arsenal of antifungal agents, their inherent toxicity, and the emergence of resistance (Ostrosky-Zeichner et al., 2010). One factor that further complicates treatment of fungal infections is the fact that fungi are capable of forming biofilms, which are attached and highly structured communities of cells surrounded by a self-produced exopolymeric matrix (Ramage et al., 2009). It is now well established that biofilms represent an important virulence factor associated with the pathogenesis of different fungal infections (Sardi Jde et al., 2014). Once formed, biofilms provide a safe haven in which fungal cells can resist treatment due to their increase resistance to antifungal therapy; and cells can also disperse from these biofilms to cause disseminated infections in target organs (Ramage et al., 2009; Uppuluri et al., 2010a). As such, biofilm formation results in increased mortality rates and associated economic costs.

Candidiasis is now the third most common nosocomial infection and a large proportion of these infections in different clinical settings involves biofilm formation (Kojic and Darouiche, 2004; Ramage et al., 2006). *Candida albicans* remains the most frequent causative agent, but infections caused by other non-albicans *Candida* spp., including among others *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis* and *Candida tropicalis*, are also associated with the formation of biofilms (Sardi et al., 2013). Different *Candida* strains vary in their biofilm-forming ability, and this correlates with their pathogenicity potential in patients (Tumbarello et al., 2012). Of note, *Candida* spp. represent the third leading cause of central catheter-related infections associated with the formation of biofilms, with the second highest colonization-to-infection rate and the overall highest crude mortality (Crump and Collignon, 2000). Other clinical manifestations such as vaginal and oral candidiasis, as well as denture stomatitis, are also associated with a biofilm etiology (Harriott et al., 2010; Ramage et al., 2006, 2004).

Other yeasts and filamentous fungi are also capable of forming biofilms on biological and/or artificial surfaces. These include the opportunistic yeasts *Cryptococcus neoformans* (the main causative agent of fungal meningoencephalitis in HIV/AIDS patients), as well as different *Trichosporon*, *Malassezia*, *Blastoschizomyces* and *Saccharomyces* species (Ramage et al., 2009; Sardi Jde et al., 2014). Even the growth of *Pneumocystis* spp. within the lung alveoli has been postulated to constitute a biofilm (Cushion et al., 2009). Dimorphic fungi, the etiological agents of endemic mycoses, such as *Coccidioides immitis*, *Histoplasma capsulatum*, *Penicillium marneffei* and *Paracoccidioides brasiliensis* can also form biofilms (Sardi Jde et al., 2014). Among filamentous fungi, perhaps the most important species capable of biofilm formation is *Aspergillus fumigatus*, the main causative organism responsible for invasive aspergillosis in cancer and transplant patients (Mowat et al., 2009). The biofilm-forming ability of other filamentous fungi, such as the Mucorales and *Fusarium* spp., has also been described (Sardi Jde et al., 2014). Unlike *Candida*, several of these opportunistic pathogenic fungi are also ubiquitous in nature, and it is conceivable that biofilm formation can play a role in their persistence and survival in the environment (Martinez and Casadevall, 2007; Pini et al., 2005; Ravi et al., 2009). In this case, it is possible that the extracellular matrix increases resistance to UV light, de-hydration, predation and other environmental threats.

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Development of Fungal Biofilms

Architectural Features of Fungal Biofilms

Fungal biofilms are defined as highly structured and organized consortia of cells attached to a substrate, either biological or artificial, and encased within a self-produced matrix of exopolymeric material Fig. 1. Although their structural properties can vary depending on the fungal species, the type of substrate and the growing conditions, overall fungal biofilms typically display a complex three-dimensional architecture with extensive spatial heterogeneity, including microcolonies and ramifying water channels (Blankenship and Mitchell, 2006; Chandra et al., 2001; Nett and Andes, 2006; Ramage et al., 2001b, 2005). This constitutes an almost optimal spatial arrangement for the uptake of nutrients, excretion of metabolic products, and for cell-to-cell communication, and may include some degree of specialization of cells depending on the location that they occupy within the biofilm. Also, the close proximity of cells within biofilms facilitates mating (Soll and Daniels, 2016).

The Different Phases of Fungal Biofilm Development

There are four major stages of fungal biofilm development. These are: (1) initial attachment and colonization, (2) proliferation, (3) maturation and (4) dispersion (Chandra et al., 2001; Ramage et al., 2009; Uppuluri et al., 2010a; Fig. 2). During the dispersion phase daughter cells, mostly in the yeast form and with increased infectivity potential, are released from the biofilm and attach at distal sites so that the "biofilm life-cycle" can start all over again (Uppuluri and Lopez-Ribot, 2016). The whole developmental process is quick and fully mature biofilms can be observed 24–48 h after the first *Candida* cell adheres to the surface or 48–72 h after the swollen *Aspergillus* spores touches the epithelial cells in the alveolar lung. In the case of *C. albicans* and also filamentous fungi, the process of biofilm formation is intimately linked to filamentation, as hyphae represent key elements for providing structural integrity and are responsible for the distinctive multi-layered architecture associated with fully developed biofilms. (Lopez-Ribot, 2005; Nobile et al., 2006a; Nobile and Mitchell, 2006; Ramage et al., 2002d; Uppuluri et al., 2010b). Quorum-sensing mechanisms (ie, cell-to-cell communication via small hormone-like molecules released by biofilm cells) as well as adhesive interactions, both cell-to-cell and cell-to-substrate, are also important during biofilm development (Nobile et al., 2006a,b, 2008; Nobile and Mitchell, 2005, 2006; Ramage et al., 2002b).

The Fungal Biofilm Matrix

As mentioned before, one of the main characteristics of fungal biofilms is the presence of a hydrated exopolymeric material that envelops the cells within the biofilms, commonly referred to as the "biofilm matrix," which is produced by cells during biofilm maturation (Mitchell et al., 2016). From a structural point of view, the cohesive and adhesive forces of the matrix contribute to the

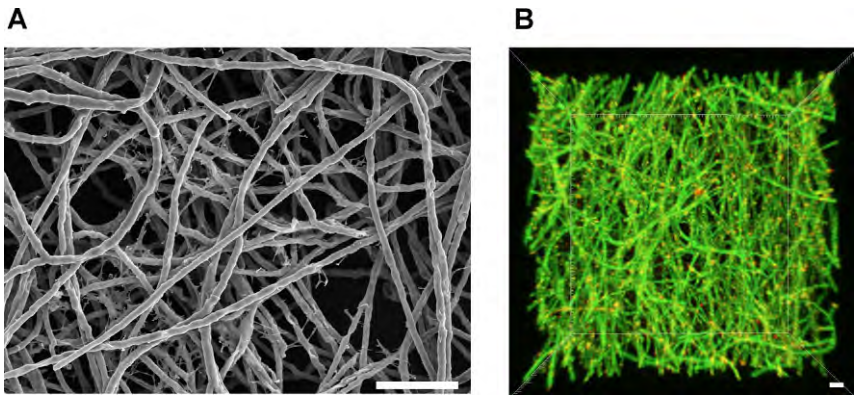

Fig. 1 (A) A scanning electron microphotograph showing an *Aspergillus fumigatus* biofilm (Courtesy of Dr. Gordon Ramage). (B) Three-dimensional reconstruction of a *Candida albicans* biofilm using confocal scanner laser microscopy and associated software. Bars are 20 μm.

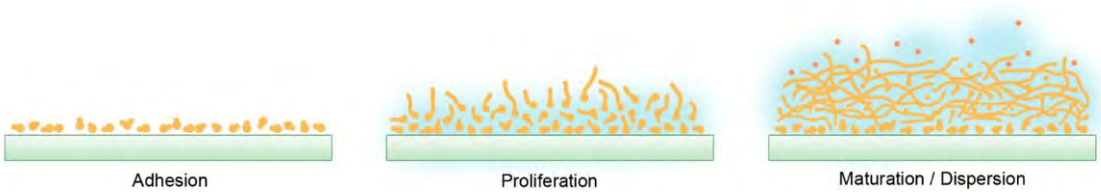

Fig. 2 A cartoon depicting the different phases of fungal biofilm development.

architectural and mechanical stability of the biofilm by mediating stable cell–cell and cell–surface interactions, essentially acting as a glue that holds the cells together and attached to the surface (Mitchell et al., 2016). The composition of the biofilm matrix changes according to physical, biological and environmental conditions and varies among different fungal species, but it has been demonstrated that carbohydrates and proteins constitute the most common and abundant matrix components (Mitchell et al., 2016). However, other macromolecules such as lipids and nucleic acids seem to be almost universally present in fungal biofilm matrices (Martins et al., 2010, 2012; Mitchell et al., 2016; Rajendran et al., 2013; Zarnowski et al., 2014). Moreover, the different components in the *C. albicans* biofilm matrix interact physically with each other resulting in the overall matrix displaying characteristics of an amalgam and emergent properties (Zarnowski et al., 2014). In general, biofilms formed under physiological conditions of flow display increased matrix production compared to those formed statically (Uppuluri et al., 2009a). From a physiological perspective the biofilm matrix acts as a shielding barrier, protecting against host defenses as well as preventing drugs from penetrating the biofilms, and as such contributes to biofilm resistance (see below) and to the recalcitrance of biofilms-associated infections (Mitchell et al., 2016). In polymicrobial biofilms, most commonly formed by fungal and bacteria that grows together in a mutualistic association, the fungal biofilm matrix contributes to protection of the bacterial cells against antibiotics and may even provide a hypoxic microenvironment that supports the growth of anaerobic bacteria (Fox et al., 2014; Harriott and Noverr, 2009), while matrix components secreted by the bacteria may also contribute impact resistance to antifungal agents (Montelongo-Jauregui et al., 2016).

Molecular Mechanisms Governing Fungal Biofilm Formation

Without any doubt, *C. albicans* has been the favorite fungal system used in studies by a growing number of groups trying to elucidate the molecular mechanisms underlying fungal biofilm development. Early studies established that morphogenesis plays a pivotal role during biofilm formation, with hyphae representing essential elements for providing structural integrity to fully developed biofilms (Baillie and Douglas, 1999), and already demonstrated a key role for *EFG1* as a key transcriptional regulator of biofilm development (Ramage et al., 2002d). Up-regulation of many hyphal-induced genes in biofilms detected in gene expression profiling studies corroborated these findings, and also pointed to a different metabolic signature of biofilm cells as compared to their planktonic counterparts (Garcia-Sanchez et al., 2004). *BCR1* codes for a key transcriptional regulator that activates cell-surface protein and adhesion genes required for biofilm formation, most notably cell wall mannoproteins encoded by the *ALS* gene family, and in particular Als1p and Als3p. These proteins mediate attachment and facilitate coaggregation with other fungal elements via complementary adhesins such as Hwp1 (Nobile et al., 2006a, 2008), and they also mediate binding to bacterial cells in polymicrobial biofilms (Silverman et al., 2010; Fig. 3). The zinc-responsive transcription factor Zap1 is the major regulator of the biofilm extracellular matrix production (Nobile et al., 2009). In addition, several key biofilm genes are involved in the quorum-sensing response to farnesol, a natural occurring inhibitor of the *Candida* yeast-to-hypha transition and important regulator of the biofilm growth. More recently, by screening a collection of *C. albicans* mutant strains, Nobile *et al.*, demonstrated that a network of six "master" transcription regulators (Bcr1, Efg1, Ndt80, Rob1, Tec1 and Brg1), now expanded to also include Flo8, Gal4 and Rfx2, orchestrates biofilm formation in this opportunistic pathogen (Fox et al., 2015; Nobile et al., 2012). The overall biofilm network is large, with approximately one thousand genes and twice that many connections, pointing to a high level of complexity associated with biofilm development. Interestingly, from an evolutionary point of view, this biofilm network seems to have evolved relatively recently in *C. albicans*, with a preponderance of "young" as compared to "old" genes, and this may have important repercussions in its adaptation to the mammalian host and pathogenic potential (Nobile et al., 2012). Not all biofilms are created equal, and in particular the configuration of the mating type locus (*MTL*) has a profound influence on biofilm thickness and function, which can translate in species- and strain-specific functional characteristics and further complicate biofilm elimination/treatment (Soll and Daniels, 2016). As discussed previously, dispersion of yeast cells from the biofilm is an important developmental phase of

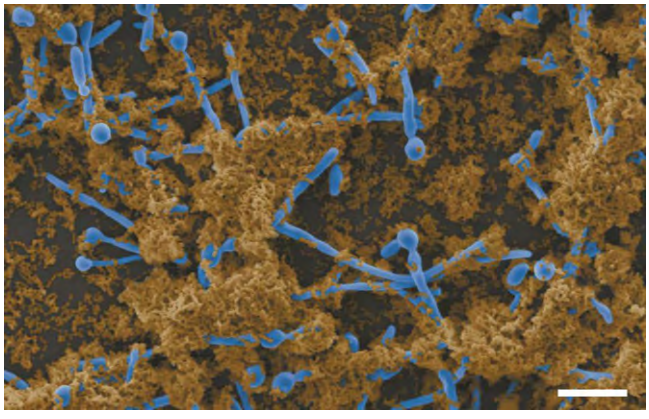

Fig. 3 Scanning electron microphotograph showing a polymicrobial biofilm of *Candida albicans* and the bacterium *Streptococcus gordonii*. The fungal and bacterial elements have been pseudocolored. Bar is 20 μm.

C. albicans biofilms as it allows the dissemination of infectious particles, favoring invasive life-threatening infections (Uppuluri et al., 2010a; Uppuluri and Lopez-Ribot, 2016). Ume6, Pes1, Nrg1 and Hsp90 have been identified as key regulators of the release of cells from a biofilm (Robbins et al., 2011; Uppuluri et al., 2010a,b) and understanding the main pathways that control the release of cells from the biofilm may help elucidate ways to curtail dispersion and control the related infection (Uppuluri and Lopez-Ribot, 2016).

Antifungal Drug Resistance of Fungal Biofilms

As mentioned before, fungal biofilm formation carries important negative consequences since cells within the biofilms tend to display high levels of resistance to a majority of clinically used antifungal agents (Pierce et al., 2013; Uppuluri et al., 2009b). As such the formation of biofilms by many fungal species further complicates antifungal therapy in an expanding population of at-risk patients and represents a major contributor to the high mortality rates associated with these infections.

Fungi are eukaryotic and there is paucity of targets which can be exploited for antifungal drug development. As a result, the antifungal arsenal is exceedingly short, and in particular for the treatment of invasive fungal infections is restricted to three classes of antifungal agents, polyenes (ie, amphotericin B), azoles (ie, fluconazole) and echinocandins (ie, caspofungin) (Ostrosky-Zeichner, et al., 2010; Pierce et al., 2013). Polyenes bind to ergosterol and compromise the integrity of the fungal membrane, azoles inhibit synthesis of ergosterol which is the main sterol in the fungal membrane, whereas echinocandins inhibit synthesis of glucan, a key structural component of the fungal cell wall. Unfortunately, it is well documented that fungal biofilms are intrinsically resistant to azoles; for example, preformed biofilms of *Candida* spp. are up to 1000 times more resistant to fluconazole compared to planktonic populations (Ramage et al., 2001a); however, voriconazole (and other azoles derivatives) can inhibit biofilm formation in different *Candida* species (Valentin et al., 2012). Fungal biofilms also showed decreased susceptibility to polyenes; although amphotericin B exhibits biofilm activity, it does so at high concentrations which are usually considered toxic and unsafe (Ramage et al., 2002c). Subsequent reports indicated excellent activity of liposomal formulations of Amphotericin B against *Candida* biofilms (Kuhn et al., 2002). Of note, all echinocandin derivatives seem to display excellent anti-biofilm activity even at relatively low therapeutic concentrations. Initial reports demonstrated the potent activity of caspofungin (the first echinocandin to reach the market) against *C. albicans* biofilms in vitro (Bachmann et al., 2002; Kuhn et al., 2002), and subsequent experiments expanded these observations to micafungin and anidulafungin, also in the case of different *Candida* spp. (Kucharikova et al., 2011). These findings were further corroborated in in vivo models and in clinical practice (Kucharikova et al., 2013, 2010; Tumbarello et al., 2012). Therefore, one could argue that the potent anti-biofilm activity of echinocandins has been one of the main factors responsible for this class of antifungals becoming first line therapy against candidiasis, particularly when biofilm formation is suspected (ie, catheter-related candidemia) (Pappas et al., 2009; Tumbarello et al., 2012). However it is important to note that the anti-biofilm activity of echinocandins is not universal. A more recent report indicated drug and species specific differences in their anti-biofilm activity, with *Candida lusitaniae* and *Candida guilliermondii* biofilms exhibiting reduced susceptibility to this new class of antifungals (Simitsopoulou et al., 2013). Furthermore *A. fumigatus* biofilms are relatively resistant to echinocandins (Ramage et al., 2009, 2012), and this class of drugs is not active against other opportunistic fungi frequently associated with biofilms, like *Cryptococcus*, Mucorales and *Fusarium*.

Mechanisms of Antifungal Drug Resistance

The investigation of the mechanisms responsible for the antifungal drug resistance of fungal biofilms at the molecular level has been an area of intensive study. Overall, there is a general agreement that fungal biofilm resistance is a complex multifactorial phenomenon involving distinct molecular mechanisms of resistance as compared to planktonic cells Fig. 4. Different groups of investigators have demonstrated the contribution of different mechanisms to fungal biofilm resistance including: (1) the increased cellular density of fungal cells within the biofilm, basically a simple concept of "safety in numbers," as elegantly demonstrated by the Chaffin's group (Perumal et al., 2007); (2) the existence of subpopulations of "persister" cells, dormant variants that are highly tolerant to the action of antifungals (LaFleur et al., 2006, 2010); (3) distinct physiological and metabolic signatures of biofilm cells (Baillie and Douglas, 1998a,b); (4) changes in sterol composition of the cell membrane of biofilm cells, most notably decreased levels of ergosterol; (5) an important protective role for the biofilm matrix, predominantly due to binding of azoles and polyenes to matrix glucans (Al-Fattani, and Douglas, 2006; Nett et al., 2007; Nobile et al., 2009) and extracellular DNA (Martins et al., 2012); (6) the differential expression of genes linked to resistance, particularly those encoding drug transporter resulting in increased efflux of antifungal molecules (Mukherjee et al., 2003; Ramage et al., 2002a); (7) a complex and highly orchestrated network of cellular responses mostly mediated by the hsp90 molecular chaperone (Robbins et al., 2011).

Conclusions and Future Outlook

It is now widely recognized that a number of fungal infections are associated with biofilm formation. Formation of fungal biofilms complicates therapy and contributes to mortality, also with soaring economic consequences. Although much information has been accumulated concerning *Candida* and *Aspergillus* biofilms, little is known regarding less frequent but highly threatening agents, such

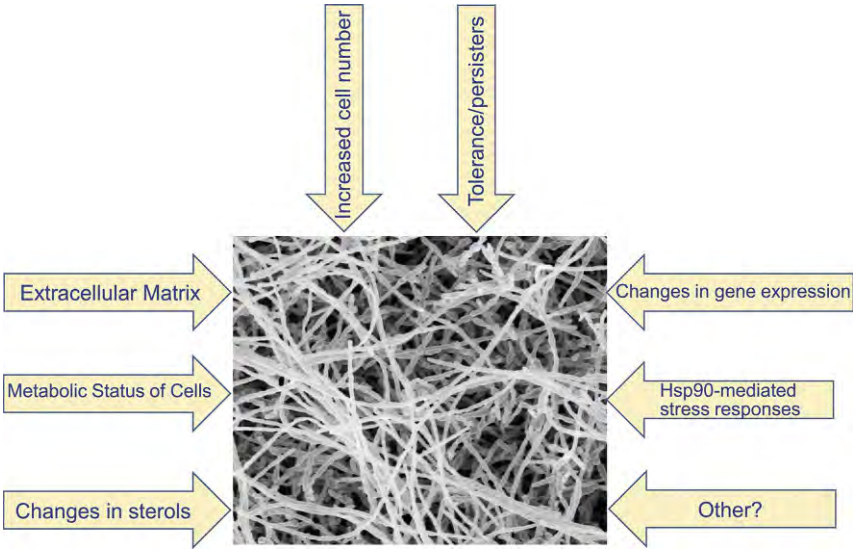

Fig. 4 Multiple factors contributing to fungal biofilm resistance.

as the Mucorales and *Fusarium* spp. It is our hope that the increasing understanding of mechanisms involved in fungal biofilm development and resistance at both the cellular and molecular levels, together with the increased recognition and awareness of the importance of these infections by the medical communities, will eventually lead to novel strategies for better clinical management and will offer new hope for a growing number of patients suffering from these devastating infections.

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Appendix A Supplementary Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/B978-0-12-809633-8.12092-8.

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Fungal Extracellular Vesicles

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Glossary

Cell wall Structural network that surrounds the lipidic cell membrane in bacteria, fungi and plants, and that confers structural maintenance (rigidity) and osmotic stability to the cells.
Chitin Polysaccharide obtained after the polymerization of N-acetyl glucosamine.
Dendritic cells Phagocytic cells whose main function is the presentation of antigens from internalized pathogens to other immune cells (mainly CD4, CD8 and B cells).
Endocytosis Process that results in the internalization of exogenous components by cells. When the cells internalize solid particles, it is referred as phagocytosis.
Exosomes Small intraluminal vesicles (30–150 nm) released when multivesicular bodies (MVBs) fuse with the plasma membrane.
Extracellular vesicles Any bilayered compartment released by living cells.
Galleria mellonella A lepidopteran species widely used as an alternative model of host in infectious disease research.
Glucan Polysaccharide composed of glucose monomers bound through glycosidic bonds.
Laccase Enzyme also known as diphenol oxidase, which is involved in some fungi in melanin synthesis from specific substrates, such as L-DOPA.
Lipid raft Membrane micro-compartment enriched in sterols, sphingolipids, glycolipids and GPI-anchored proteins that regulate functions such as endocytosis and cellular signaling.
Macrophages Specific type of white blood cells (leukocyte) specialized in the uptake and degradation of exogenous particles. There are two types of macrophages: M1 (also known as classical or proinflammatory macrophages), which have a strong antimicrobial activity, and M2 (also known as alternative or anti-inflammatory macrophages), which have an immunoregulatory function.
Mannan Polysaccharide formed by polymerization of mannose monomers. Can be divided in **N-linked mannan** (polymer attached to an asparagine through a nitrogen atom) or **O-linked mannans** (attached to a serine or threonine through an oxygen atom).
Microvesicles Extracellular vesicles that are formed by direct plasma membrane budding.
Th1 and Th2 immune response Defense mechanism elicited by hosts that protect from external challenges, mainly microbes and toxic compounds. Th1 and Th2 are different types of immune response, being Th1 those known as inflammatory, and Th2 as anti-inflammatory.

Abbreviations

AE	Atopic eczema
APT1	Aminophospholipid translocase 1
CD	Cluster of differentiation
Chitin	β-1,4-poly-N-acetyl-D-glucosamine
CHS	Chitin synthase
Cln1	G1/S cyclin 1
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DLS	Dynamic light scattering
EVs	Extracellular vesicles
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlcCer	Glucosylceramide
GRASP	Golgi reassembly and stacking protein
GSL	Glycosphingolipids
GXM	Glucuronoxylomannan
HBMEC	Human brain microvascular endothelial cells

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HSP60	Heat shock protein 60
IL-10	Interleukin-10
IL-12	Interleukin-12
mAb	Monoclonal antibody
MHCII	Major histocompatibility complex class II
MP65	Mannoprotein 65
PBMC	Peripheral blood mononuclear cells
PCA3	Prostate cancer antigen 3
PSA	Prostate–specific antigen
RNA	Ribonucleic acid
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
WT	Wild type

Biogenesis of Fungal EVs

EVs are produced virtually by all living organisms, including bacteria, fungi, plantae, protozoa and mammals (Marcilla et al., 2014; Brown et al., 2015; Rodrigues et al., 2015; Yanez-Mo et al., 2015). The term "EVs" is used to designate any bilayered compartment released by alive cells. In fungal organisms it includes exosome-like particles, microvesicles (also named ectosomal or shedding vesicles) and compartments generated by cytoplasmic subtractions (Gyorgy et al., 2011; Rodrigues et al., 2013) (Fig. 1). Exosomes are small intraluminal vesicles (30–150 nm) released when multivesicular bodies (MVBs) fuse with the plasma membrane (Fig. 1(A)) (Yanez-Mo et al., 2015; Rashed et al., 2017) (Fig. 1(A)). Microvesicles are usually larger (100–1000 nm) and assembled by direct plasma membrane budding (Fig. 1(B)). Cytoplasmic subtractions due to plasma membrane reshaping were recently described in *Saccharomyces cerevisiae* (Rodrigues et al., 2013). They are formed through an invagination process followed by a reorientation of the invaginating membrane to the plasma membrane, with subsequent membrane fusion and release of vesicles into the periplasmic space (Fig. 1(C)). Fungal EVs usually consist of one or two populations ranging between 50–250 and 300–800 nm (Albuquerque et al., 2008; Rodrigues et al., 2008; Oliveira et al., 2010; Wolf et al., 2014, 2015; Brown et al., 2015; Vargas et al., 2015). Differences in electron density between fungal EVs have been demonstrated, supporting the idea that EVs could have

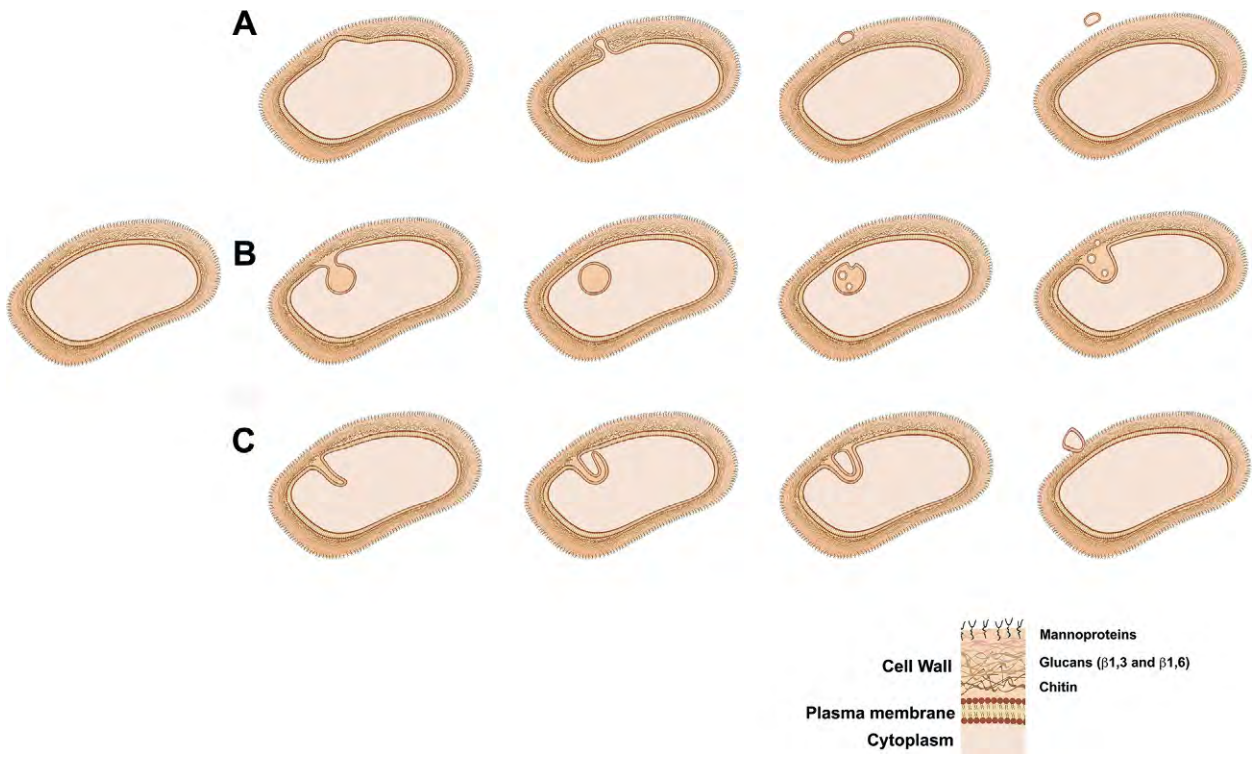

Fig. 1 Fungal EVs biogenesis. EVs are formed through at least three distinct mechanisms: (A) multivesicular bodies fusion with plasma membrane releasing exosomes, (B) plasma membrane budding and (C) cytoplasmic subtraction. In all cases the EVs could reach the extracellular environment intact.

Table 1 Related functions for proteins characterized in fungal extracellular vesicles

Related functions for proteins characterized in fungal extracellular vesicles:
Pathogenesis
Lipid metabolism
Carbohydrate metabolism
Cell well architecture
Amino acid and Protein metabolism
Ribosomal proteins
Cellular organization and biogenesis
Cell signaling
Response to stress
Endocytic route

multiple biogenesis pathways resulting in distinct cargo (Rodrigues et al., 2008). Remarkably, even strains of the same species can produce EVs with distinct sizes, confirming the heterogeneity of these compartments (Vargas et al., 2015).

Major components of the cell wall include chitin, β-1,3 and β-1,6 glucans and mannoproteins (regularly found as an outer cell wall layer of *C. albicans* and *S. cerevisiae*).

Fungal EVs Cargo

The mechanisms mentioned above are compatible with distinct vesicle sizes and high diversity of structures carried by fungal EVs, including proteins lacking secretory signals, metabolic enzymes, RNA, pigment and polysaccharides (reviewed in Nimrichter et al., 2016). A number of EV components are conserved among different fungal species and strains, but some are species-specific (Vallejo et al., 2012a, b). The major lipids characterized in fungal EVs are glucosylceramide (GlcCer), sterols and phospholipids (Rodrigues et al., 2007; Albuquerque et al., 2008; Vallejo et al., 2012a, b; Vargas et al., 2015). The variety of proteins carried by fungal EVs is enormous (Table 1) and includes proteins involved with multiple metabolic pathways (Rodrigues et al., 2008; Vallejo et al., 2012a, b; Rodrigues et al., 2014; Wolf et al., 2014; Gil-Bona et al., 2015; Vargas et al., 2015; Wolf et al., 2015; Matos Baltazar et al., 2016; Nimrichter et al., 2016).

Unexpectedly, major hits found in EVs from distinct fungal species included enzymes involved with glycolysis, fermentation, gluconeogenesis, pentose phosphate, tricarboxylic acid, and glyoxylate cycles (reviewed in Nimrichter et al., 2016). At least some of these enzymes are involved in fungal adhesion to host cells, such as enolase, GAPDH and phosphoglycerate mutase (Nimrichter et al., 2016). However, their participation during EV recognition by host cells has never been investigated. It is plausible that release of fungal EVs loaded with metabolic enzymes can be a mechanism for a rapid change in cell metabolism. Polymers such as polysaccharides and melanin are also carried by EVs (Rodrigues et al., 2007; Eisenman et al., 2009). Recently, the presence of RNA was characterized in fungal EVs from several species (da Silva et al., 2015). RNA-containing vesicles could be responsible for biological processes triggered in host cells during fungal infection or participate during communication among fungi or even with other organisms.

Traversing the Cell Wall

The plasma membrane is the interface between mammalian cells and the extracellular environment. In contrast, fungal organisms are encaged in a thick cell wall, which demands EVs to cross this barrier to be released into the extracellular environment (Rodrigues et al., 2013, 2015). In encapsulated fungal cells, such as those belonging to the *Cryptococcus* complex, EVs must also traverse the capsule (Rodrigues et al., 2007). The mechanisms by which fungal EVs traverse fungal outer layers and reach the extracellular environment is still unclear.

Cell wall composition can change considerably according to the fungal species and/or morphological stage (reviewed by Erwig and Gow, 2016). However, some common compounds are widely known. These compounds form a network that is basically composed by a matrix of polysaccharides where different classes of proteins and lipids are embedded (Nimrichter et al., 2005). In most species the presence of melanin has been reported (Gomez and Nosanchuk, 2003). The major structural polysaccharides include chitin and glucans and N or O-linked mannans (review in Latge, 2010). The most common prototypes for study of fungal cell walls are *S. cerevisiae* and *C. albicans*, where a layer of mannoproteins is depicted at the edge of the cell wall (Erwig and Gow, 2016). However, other species can have polysaccharides or hydrophobins, such as *Histoplasma capsulatum* and *Aspergillus* conidia, respectively, as outer layers (Erwig and Gow, 2016). In order to support turgor pressure and also permit budding and morphological changes the cell wall must be robust, elastic and very dynamic.

After their release at the periplasmic space, fungal EVs presumably interact with cell wall components. The exact number of EVs produced by each fungal cell is not known. Although mechanical pressure could facilitate EV passage through the wall (Brown et al., 2015), other mechanisms were proposed. For instance, Anderson and colleagues demonstrated the presence of channels at the cell wall of *C. albicans* (Anderson et al., 1990). Cell wall protuberances were associated with the edge of these channels where small

vesicles were depicted. In addition, flask-shaped vesicles were observed underneath the base of these protuberances (Anderson et al., 1990). In *C. neoformans*, Wolf and colleagues (Wolf et al., 2014) demonstrated that EVs interact directly with the cell wall. Notably, they also observed multiple or single leaving events between the plasma membrane and the cell wall. Thus the release of EVs in *C. neoformans* does not appear to be linked to channels.

EV-mediated cell wall hydrolysis was also proposed as a mechanism facilitating their passage through the cell wall. In fact, EVs isolated from cultures of *C. neoformans*, *Paracoccidioideis brasiliensis*, *H. capsulatum* and *C. albicans* carry a number of glycosidases including, chitinases, glucosidases and mannosidases (Nimrichter et al., 2016). Lipases and proteases are also transported by EVs. Although the activity of cell wall-related enzymes was never reported in *C. neoformans* EVs, active laccase and urease were detected biochemically, supporting the notion that other enzymes can be also functional (Rodrigues et al., 2008). However, direct EV-mediated hydrolysis has never been demonstrated.

In fungi, production of chitin and glucans occurs at the plasma membrane level (Latge, 2007). Once the newly synthesized polysaccharides are extruded to the cell wall they can be modified by cell wall enzymes. Polysaccharides are then cross-linked to each other or to GPI-remnant proteins (De Groot et al., 2005; Latge, 2007). Proteomic analysis suggested that fungal EVs also participate in cell wall synthesis (Albuquerque et al., 2008; Vallejo et al., 2012a, b; Rodrigues et al., 2014; Gil-Bona et al., 2015; Vargas et al., 2015). Chitin synthases were reported in EVs released by *H. capsulatum*, *C. neoformans* and *P. brasiliensis* (Nimrichter et al., 2016). Some chitin synthases are regulated by protease activity (Sburlati and Cabib, 1986). For instance, two chitin synthases (CHS1 and CHS2) from *S. cerevisiae* are more active after trypsin or chymotrypsin treatment (Duran and Cabib, 1978; Sburlati and Cabib, 1986) while the third one (CHS3) is inactivated (Orlean, 1987; Valdivieso et al., 1991). Thus, fungal EVs could potentially contribute to chitin synthesis directly, by their chitin synthases, or indirectly, through their proteolytic activity. Furthermore, EVs from *C. neoformans* carry chitin-deacetylase, an enzyme that converts chitin into chitosan. In this pathogen, chitosan is required for cell wall integrity (Baker et al., 2007). The absence of chitosan affects capsular size and promotes a "leaky" melanin phenotype (Baker et al., 2007).

Host Cell Modulation and Impact on Disease Development

Since fungal EVs carry a combination of virulence factors, it has been speculated that they could impact host cell responses (Oliveira et al., 2010; Huang et al., 2012; Vargas et al., 2015; Wolf et al., 2015; da Silva et al., 2016). EVs from *C. neoformans* and *C. albicans* co-localized with the lipid raft marker GM1 at the surface of macrophages and dendritic cells (Oliveira et al., 2010; Vargas et al., 2015). Fusion between fungal EVs and the host plasma membrane was not detected. EVs were internalized after few minutes of interaction, with a complete internalization after 1 h (Vargas et al., 2015). Exposure for longer periods demonstrated that EVs from *C. neoformans* and *C. albicans* are not toxic to primary mammalian cells (Oliveira et al., 2010; Vargas et al., 2015). Together these findings indicate that cell surface receptors are required for EV association with and internalization by phagocytes. Accordingly, Peres da Silva and colleagues reported that EVs from *P. brasiliensis* display mannose and N-acetylglucosamine residues that could be recognized by DC-SIGN (Peres da Silva et al., 2015). Internalization of fungal EVs by phagocytes culminated with production of nitric oxide and cytokines such as IL-12, IL-10, TNFα and TGFβ, confirming their immunomodulatory activity (Oliveira et al., 2010; Vargas et al., 2015). In addition, the fungicidal activity of macrophages was increased after exposure to EVs from *C. neoformans* or *P. brasiliensis* (Oliveira et al., 2010; da Silva et al., 2016). Furthermore, *P. brasiliensis* EVs promoted M1-polarization in macrophages and reversed the M2-polarization stimulated by IL-4/IL-10 (da Silva et al., 2016). The M1 phenotype is important for host defense during phagocytosis and killing by macrophages (Murray and Wynn, 2011). During chronic paracoccidioidomycosis the majority of macrophages are M2, favoring a Th2 response and the susceptibility to the infection (de Carli et al., 2016). Thus, EVs could contribute to paracoccidioidomycosis control.

The ability of fungal EVs to stimulate human cells was also observed. Gehrmann and colleagues showed that *Malassezia sympodialis*, a commensal yeast linked to atopic eczema (AE), releases EVs loaded with allergens (Gehrmann et al., 2011). *M. sympodialis* EVs were able to stimulate the production of TNFα and IL-4 by CD14- and CD34-depleted PBMC from healthy and AE patients. Remarkably, the response was significantly higher in *M. sympodialis* sensitized patients (Gehrmann et al., 2011). Collectively these results suggested that fungal EVs could activate cells of the innate immune system. Indeed, the capacity to stimulate the immune system was confirmed using *Galleria mellonella* larvae. *G. mellonella* immune mechanisms share a high degree of structural and functional homology to those used by the innate immune system of vertebrates (Browne et al., 2013). Treatment of *G. mellonella* larvae with EVs from *C. albicans* protected the insect against a subsequent challenge with the pathogen (Vargas et al., 2015). The number of viable yeasts recovered from EV-treated larvae was significantly reduced when compared to PBS-treated larvae. These results were accompanied by a higher percentage of larvae survival in the presence of fungal EVs.

Besides impacting the innate immune response, host cell activation mediated by fungal EVs could also change the outcome of the adaptive immune response as treatment of dendritic cells with EVs from *C. albicans* enhanced the surface expression of CD86 and MHC-II (Vargas et al., 2015). At least in part, this property could be linked to the high levels of MP65, a major protein in *C. albicans* EVs (Gil-Bona et al., 2015; Vargas et al., 2015). MP65 is a major mannoprotein at *C. albicans* cell surface (Gil-Bona et al., 2015). *In vitro*, MP65 stimulates dendritic cells inducing cytokine production, such as TNF-α and IL-6, and increases the expression of costimulatory molecules. These results indicate that fungal EVs can be potential antigen carriers to be explored in vaccine formulations.

The ability of fungal EVs to interact with host cells was also investigated in human brain microvascular endothelial cells (HBMEC) (Huang et al., 2012). Remarkably, treatment of HBMEC with EVs from *C. neoformans* induced fusion of the host cells and stimulated redistribution of membrane proteins in lipid rafts. The proteins CD44, a known receptor for *C. neoformans* in HBMEC (Jong et al., 2008), and caveolin-1 were significantly enhanced in lipid rafts fractions after exposure to EVs (Huang et al., 2012). In contrast, a significant reduction in raft-associated β-actin was observed. Thus, EVs from *C. neoformans* are able to activate HBMEC and promote changes that facilitate fungal adhesion and their ability to cross the brain blood barrier *in vitro.* Experiments *in vivo* confirmed that *C. neoformans* EVs could potentially impact disease development. Intravenous injection of EVs in a murine model of cryptococcosis significantly increased the fungal burden in the brain (Huang et al., 2012).

Genetically Modified Strains as Tools to Investigate Fungal EVs

As mentioned previously, fungal EVs were isolated for the first time from *C. neoformans* cultures (Rodrigues et al., 2007). To this date, most of the information based on genetic alterations and EV release in fungal pathogens comes from this model. The attempts to impair fungal EV release in genetically modified strains generally failed, although important differences in EV properties were observed. The first gene investigated was *SEC6*, which encodes a protein that participates in the fusion of exocytic vesicles with the plasma membrane in yeasts (TerBush et al., 1996). Panepinto and colleagues investigated the role of Sec6 during EV-mediated release of virulence factors by *C. neoformans* (Panepinto et al., 2009), including the enzymes phospholipase, urease and laccase, and the major capsular polysaccharide glucuronoxylomannan (GXM) (Rodrigues et al., 2008). Notably, laccase and urease activities were significantly reduced in i*SEC6* cells. In addition, the amount of secreted GXM was also decreased (Panepinto et al., 2009). Sec6 suppression in *C. neoformans* led to vesicle accumulation in the cytoplasm and abrogated EV release. In *iSEC6* cells laccase was restricted to the cytoplasm within vesicular compartments. Although phospholipase activity and capsular size were not affected in the *SEC6* knockout, the *iSEC6* strain was less virulent than WT cells. Fungal EVs appeared to be responsible for the correct transport of virulence factors to the cell surface, contributing to the disease development.

The influence of proteins that regulate membrane asymmetry in EV properties was investigated in *C. neoformans* (Rizzo et al., 2014). The gene *APT1* encodes a putative aminophospholipid translocase, a class of proteins named as flippases. Their role in membrane asymmetry helps to control trafficking events that precede secretion. Flippases were linked to EV production in *C. elegans* (Tuck, 2011; Wehman et al., 2011). In *C. neoformans* Apt1 is required for protein secretion and pathogenicity (Hu and Kronstad, 2010). Deletion of *APT1* affected Golgi morphology and GXM secretion, but not capsule size and GXM composition (Rizzo et al., 2014). The amount of GXM found in EVs from yeasts lacking Apt1, however, was considerably reduced when compared to vesicles obtained from WT cells (Rizzo et al., 2014).

The connections between proteins involved with unconventional secretion mechanisms and export of fungal molecules were explored in a few studies. In eukaryotes, Golgi *r*eassembly and *s*tacking *p*rotein (Grasp) participates in unconventional mechanisms of secretion (Kinseth et al., 2007). Deletion of *GRASP* in *C. neoformans* affected Golgi morphology (Kmetzsch et al., 2011) and reduced the dimensions of extracellular polysaccharides, as determined by dynamic light scattering (DLS). These results were reverted by addition of Ca^{2+} to the system, allowing GXM aggregation through calcium bridges (Nimrichter et al., 2007; Kmetzsch et al., 2011). In agreement with the reduction in extracellularly released GXM the mutants also displayed a hypocapsular phenotype. Recent data from our laboratory suggested that *GRASP* deletion affects EV populations of lower dimensions, suggesting that Grasp participate in vesicle biogenesis (Luna S. Joffe, unpublished data). EVs are affected by other cryptococcal genes. A considerable increase in EV release was observed in a *C. neoformans* strain lacking Cln1, a protein involved with cell cycle progression (Garcia-Rodas et al., 2014). In addition, deletion of *CAP10*, a putative xylosyltransferase, resulted in EVs with significantly reduced dimensions (Tefsen et al., 2014). Notably, increased laccase and acid phosphatase activities were observed at the fungal cell surface.

The relationship between secretory genes and EV formation was more deeply explored in *S. cerevisiae*. Vesicle dimensions and protein composition were analyzed in mutants lacking either conventional or unconventional secretory regulators. All mutants produced EVs, but amounts, composition and dimensions varied depending on the gene deleted. No direct correlation was established between secretory mechanisms (conventional or unconventional) and EV formation, since all genes were apparently required for normal vesicle release. These results are summarized in Table 2.

Table 2 Effects of gene deletion on the properties of *S. cerevisiae* EVs

Gene product	*Associated secretory pathway*	*Average EV dimensions*	*Protein composition*	*EV amount*
Sec4	Conventional	Increased	Slightly affected	Reduced
Bos1	Conventional	Not determined	Slightly affected	Not determined
Sec1	Conventional	Not determined	Slightly affected	Not affected
Snf7	Unconventional	Not affected	Heavily affected	Not affected
Vps23	Unconventional	Not determined	Slightly affected	Not determined
Grasp	Unconventional	Not determined	Not determined	Reduced

EVs as Targets of New Antifungal Drugs and Protective Antibodies

The potential participation of fungal EVs in physiological events and disease development suggests that they can become interesting targets for antifungal drugs and antibodies. As mentioned above GlcCer is a glycosphingolipid (GSL) component of fungal EVs (Rodrigues et al., 2007; Vallejo et al., 2012a,b; Vargas et al., 2015). Besides its structural function, GlcCer is also a virulence regulator in *C. neoformans* and *C. albicans* (Rittershaus et al., 2006; Noble et al., 2010). Recently, Mor and colleagues identified a new class of antifungal drugs that indirectly impact the synthesis of GlcCer. One of the effects was the accumulation of intracellular vesicles in yeasts of *C. neoformans* (Mor et al., 2015). Other drugs that impact lipid biosynthesis or proteins involved with EVs biogenesis could impair release of EVs and change the fungal physiology. For instance, Knechtle and colleagues identified a plant derived diyne-furan fatty acid named as EV-086 as a potent antifungal drug (Knechtle et al., 2014). This compound targets delta-9-fatty acid desaturation increasing the ratio of saturated/unsaturated fatty acids. Accumulation of saturated fatty acid would impact directly the membrane composition and fluidity (Ballweg and Ernst, 2017). Consequently, the function of several proteins could be modified as well as the biogenesis, cargo and release of EVs. These possibilities require experimental confirmation.

Administration of monoclonal antibodies (mAbs) that recognize structures carried by fungal EVs could be another strategy to impact EV activity during disease development. Three probable mechanisms are expected for mAb to interfere with fungal EVs *in vivo*, including (i) removal of released EVs, reducing their potential deleterious effect in host cells, (ii) preventing initial passage through the cell wall or (iii) impacting gene regulation in fungal metabolism. All these three mechanisms were previously described in models of fungal infections involving protective mAbs (Eisenman et al., 2005; McClelland and Casadevall, 2012). Indeed, it could explain why mAb to GlcCer promotes a protective effect in a cryptococcosis murine model (Rodrigues et al., 2007). Recently, Baltazar and colleagues investigated whether mAbs to HSP60 impact release and cargo of EVs in *H. capsulatum* yeast cells (Matos Baltazar et al., 2016). Noteworthy, HSP60 is a major EV compound in the *H. capsulatum* model (Albuquerque et al., 2008). Incubation of yeasts with mAb-HSP60 significantly changed the size of EVs, as well as their protein content and enzymatic activities. Remarkably, changes in protein composition varied depending on the epitope recognized by mAbs to HSP60 (Matos Baltazar et al., 2016). This observation confirms that antibody binding can modify the EV release to the extracellular environment.

Challenges in the Field of Fungal EVs

Although the number of publications and findings regarding fungal EVs increased significantly over the last decade (Joffe et al., 2016), there are unsolved questions that require further investigation. One major concern is whether EVs are released by fungal cells *in vivo*, as well as their amounts, and whether they contribute or not to infection development. Isolation of fungal EVs from infected tissues will likely represent a breakpoint in the field. On the basis of the current literature, it is plausible that EVs isolated *in vivo* could show considerable changes in composition when compared with EVs from culture supernatants. Furthermore, one must consider that EVs released during infection could express a mixture of fungal and host molecules, which makes these analyses even more complex. Although a number of methods can be used for EV preparation, successful protocols require a high recovery of EVs using small volumes of samples. The standard methods are differential and density gradient centrifugation, but the viscosity of some biological fluids and the similar densities between some EVs limit the use of centrifugation methods. EV markers are now known in mammalian samples (reviewed in Xu et al., 2016). For instance, survivin, PSA and PCA3 are exosome markers for prostate cancer in urine samples (Nilsson et al., 2009). Monoclonal antibodies have been used as tools to isolate EVs, including distinct subtypes, using immunological methods and commercial kits (Taylor et al., 2011; Lasser et al., 2012; Vlassov et al., 2012; Ghosh et al., 2014; Hudson et al., 2014; Enderle et al., 2015; Lobb et al., 2015; Brett et al., 2017). The absence of a fungal EV marker is challenging to the field. GlcCer seems to be an interesting fungal EV-marker candidate, although some species including *C. glabrata* and *S. cerevisiae* are unable to produce this GSL (Tavares et al., 2008). Protein markers would also facilitate separation of EVs originated from distinct biogenesis pathways. Finally, the participation of fungal EVs during cell-to-cell communication has never been demonstrated, although indirect evidence suggested that it could occur *in vitro* during fungal differentiation (Leone et al., 2017).

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Further Reading

Brown L, Wolf JM, Prados-Rosales R, and Casadevall A (2015) Through the wall: Extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat. Rev. Microbiol.* 13(10): 620–630.
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Relevant Website

https://www.isev.org—International Society For Extracellular Vesicles (ISEV).

Fungal Infections, Systemic☆

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Glossary

Adaptive immunity An arm of the immune system that responds to microorganisms in a specific manner. The adaptive immune response consists of antibodies or T lymphocytes generated to fungal antigens and is based on immune memory.
Dimorphism The ability of fungi to grow as two morphologies in response to temperature. Usually these fungi grow as filamentous forms at ambient temperatures and as yeasts at 37 °C.
Hyphae Filamentous cells characterized by parallel cell walls.
Immunosuppression An impaired immune system as a result of chemotherapy for cancer, conditioning prior to transplantation, treatment with anti-inflammatory drugs such as steroids, or diseases such as advanced HIV infection.
Innate immunity An arm of the immune system that responds to microorganisms in a generalized manner by phagocytic cells. This is usually the first line of defense against infection. The innate immune system confers protection against fungi in the absence of specific antibodies or T lymphocytes previously generated prior to infection.
Mold Fungi that grow exclusively in the hyphal morphology.
Opportunistic pathogen A microorganism that tends to cause disease in individuals with impaired host defenses. Many fungi cause disease in patients with cellular immunity defects or suppression.
Phylogenetics The science that utilizes genetic relatedness to classify organisms.
Transformation A system to introduce DNA into microorganisms. This tool allows for the study of the effects of gene function on virulence or infectivity.

Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
AIDS	Acquired immunodeficiency syndrome
CF	Complement fixation
CNS	Central nervous system
CSF	Cerebrospinal fluid
EIA	Enzyme immunoassay
HIV	Human immunodeficiency virus
ID	Immunodiffusion
MTL	*MAT*-type-like loci
PAMPs	Pathogen-associated molecular patterns
PEG	Polyethylene glycol
TLRs	Toll-like receptors

Defining Statement

Systemic fungal infections are infections of organs or tissues other than the skin or mucosal surfaces. These infections have increased in frequency and importance in recent years. This article reviews the fungi that cause most systemic infections in people and the key features of the diseases these fungi cause.

Introduction

Before the 1980s, serious fungal infections were relatively uncommon, occurring mostly in patients with cancer or other underlying conditions that cause severe immunodeficiency. Since then, several developments have greatly expanded the population at risk for

☆*Change History:* July 2014. Janet F Staab extensively updated section 'Molecular Approaches for Studying Fungal Pathogenesis' and Table 5 to reflect newer nucleic acid sequencing methods and completed genomes data. Wong updated epidemiology of coccidiomycoses, and *C. gattii*; treatment of *Candida* fungemia and diagnosis of *Pneumocystis*. Staab and Wong updated section 'Further Reading' to add recent journal publications.

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developing these fungal infections. These include advances in the treatment of many forms of cancer, advances in solid organ and stem cell transplantation, the ongoing human immunodeficiency virus (HIV) epidemic, and the widespread availability and use of antibiotics that are effective against a broad range of bacteria. For these reasons, the incidence and importance of serious fungal infections have increased markedly in the past 20 years. For example, data collected from community and teaching hospitals throughout the United States showed that the frequency of fungal bloodstream infections increased by more than threefold during the 1990s, when the frequency of Gram-positive and Gram-negative bacterial bloodstream infections changed little. Similarly, deaths from fungal infections in the United States increased more than threefold between the early 1980s and the mid-1990s. Thus, it is now clear that fungi are very important human pathogens that warrant attention and study.

Classification of Pathogenic Fungi

Fungi are among the most abundant and diverse of all organisms, but only a few species are responsible for a large majority of serious fungal infections in humans. The most important of these fungal pathogens of humans are listed according to traditional phylogenetic categories in Table 1. These classifications are based on the traditional growth and morphology of vegetative and reproductive fungal forms and also on genetic relatedness as determined by genome sequencing, especially of highly conserved elements like ribosomal DNA sequences. Most of the fungi that cause serious infections in humans are members of the phylum Ascomycota, although a few are members of Basidiomycota or Zygomycota.

Traditional phylogenetic classification is very useful for analyzing evolutionary relationships and for guiding studies of properties such as sexuality and genetic recombination within populations, but alternative approaches to classification are often more useful when one considers these fungi as human pathogens. As serious fungal infections are often first recognized when fungi are observed in infected tissues or isolated in culture, it is generally useful to divide the pathogenic fungi into groups that grow in tissues or in culture as yeast-like fungi (sometimes with associated elongated forms known as pseudohyphae), as filamentous fungi (or molds), or as dimorphic fungi (which grow as one morphologic form when they are observed in infected tissues or are cultured at 37 °C and as a completely different morphologic form in the environment or when they are cultured at ambient temperatures). Table 2 divides the major human pathogenic fungi into yeast-like fungi (which include both ascomycetes and basidiomycetes), filamentous fungi or molds (which include ascomycetes and zygomycetes), and dimorphic fungi (all of which are ascomycetes).

Some pathogenic fungi cause disease in people with normal host defenses, whereas others cause disease almost exclusively in people whose defenses against infection are seriously compromised. As the adequacy of an individual patient's host defenses is often known well before the etiology of any specific infection is known, it is also useful to divide the human pathogenic fungi into

Table 1 Phylogeny of the common invasive fungi

Phylum	*Subphylum*	*Class*	*Order*	*Genus*
Ascomycota				
	Pezizomycotina			
		Eurotiomycetes		
			Onygenales	*Blastomyces*
				Coccidioides
				Histoplasma
				Paracoccidioides
			Eurotiales	*Aspergillus*
				Penicillium
		Chaetothyriomycetes		*Exophiliala* (*Wangiella*)
		Sordariomycetes		
			Ophisostomatales	*Sporothrix*
			Microascales	*Pseudallescheria*
				Scedosporium
			Hypocreales	*Fusarium*
	Saccharomycotina			*Candida*
	Taphrinomycotina			*Pneumocystis*
Zygomycetes			Mucorales	*Rhizopus*
				Absidia
				Mucor
			Endomopthorales	*Basidiobolus*
				Conibiobolus
Basidiomycota		Hymenomycetes		*Cryptococcus*
				Trichosporon
		Ustilaginomycetes		*Malassezia*

Source: Adapted from Taylor, J. W. (2006). Evolution of human-pathogenic fungi: Phylogenies and species. In: Heitman, J., Filler, S. G., Edwards, J. E. Jr., & Mitchell, A. P. (eds.) *Molecular principles of fungal pathogenesis*, ch. 8, pp. 113–133. Washington, DC: ASM Press.

Table 2 Morphology of the common invasive fungi

	Genus	*Usual morphology in tissues*
Yeasts	*Candida*	Budding yeast, pseudohyphae, hyphae
	Cryptococcus	Encapsulated budding yeast
	Exophiala (*Wangiella*)	Black yeast, pseudohyphae
	Trichosporon	Pleomorphic yeast, pseudohyphae or hyphae
Molds	*Aspergillus*, *Fusarium*, *Pseudallescheria*, *Scedosporium*	Narrow septate hyphae, branching at 45°
	Rhizopus and other zygomycetes	Wide aseptate hyphae, branching at 90°
Dimorphic fungi	*Blastomyces*	Yeast with broad-based buds
	Coccidioides	Large endosporulating spherules
	Histoplasma	Small budding yeast
	Paracoccidioides	Yeast with multiple buds per cell
	Penicillium	Intracellular yeast
	Sporothrix	Elongated budding yeast
Other	*Pneumocystis*	Trophic forms, and cysts that contain up to 8 internal spores

true pathogens that can infect normal hosts and opportunistic pathogens that tend to infect only seriously compromised hosts (Table 3). It should be noted that most of the fungi listed as true pathogens can also act as opportunists because they tend to cause more extensive and severe disease in people with compromised host defenses.

Host Defenses Against Fungal Infections

Humans are continuously exposed to fungi. The majority of fungi are recognized and inactivated within hours by cellular and noncellular innate defense mechanisms. In addition, an important protective mechanism is provided by physical barriers such as intact skin and healthy mucosa. Many fungal infections occur in individuals with predisposing factors that affect or impair the function of skin, mucosa, or airways. A major predisposing factor for *Candida albicans* bloodstream infections, for example, is the placement of an indwelling catheter that provides the fungus with direct access into the bloodstream and internal organs, bypassing the protection provided by intact skin. Another example of a fungus that can take advantage of interruptions in the skin to invade deeper structures is *Malassezia furfur*, a basidiomycete that can contaminate and then proliferate within lipid-containing parenteral nutrition fluids. Filamentous fungi such as the *Rhizopus* can colonize skin that is interrupted by full-thickness burn injuries or by surgical incision, after which the fungi can invade deeper structures and disseminate widely.

Other factors that disturb the integrity of the intestinal mucosa, such as chemotherapy treatments for hematological or neoplastic malignancies, can also allow *C. albicans* to translocate from the gastrointestinal tract into the blood. Besides the mechanical barrier provided by intact skin, secretions that bathe the mucosal surfaces can also influence susceptibility to infection. A group of soluble peptides, defensins, and histatins, found in mucosal secretions and other tissue fluids, have antimicrobial activities, and play an important role in innate immunity. Most of these peptides exert their antifungal activities by interacting directly with the microbial membrane, destabilizing it and leading to cell death.

Protection against fungal infections is afforded by both innate and adaptive immunity. Cellular innate immunity relies upon professional phagocytic cells (tissue macrophages/monocytes and dendritic cells) that are able to ingest and kill certain fungal

Table 3 Pathogenic potential of the common invasive fungi

True pathogens	*Blastomyces*
	Coccidioides
	Histoplasma
	Paracoccidioides
	Sporothrix
Opportunists	*Aspergillus*
	Candida
	Cryptococcus
	Exophiala (*Wangiella*)
	Fusarium
	Penicillium
	Pneumocystis
	Pseudallescheria and *Scedosporium*
	Rhizopus and other zygomycetes

spores or yeasts, and have an important role in containing fungal growth and dissemination. Another aspect of this first line of defense is the recognition of conserved molecular patterns found on the surface or as part of microorganisms that signal infection to the host. These conserved molecular structures are known as pathogen-associated molecular patterns (PAMPs) that are usually units of cell wall or cell membrane structural components found in large groups of pathogenic microorganisms. PAMPs are 'seen' by host cells through a set of surface proteins called Toll-like receptors (TLRs) and by other membrane-bound proteins (such as Dectin-1, the receptor for 1,3-β-glucan) found on professional and nonprofessional phagocytic cells. Binding of PAMPs by TLRs and other non-TLR proteins signals the recruitment and activation of neutrophils to the site of infection in a manner that is tailored for the killing or containment of a particular pathogen. Recruitment and activation of neutrophils occur by signaling cascades generated by the secretion of chemokines and cytokines by macrophages to produce a beneficial inflammatory response. Inappropriate inflammatory responses have been implicated in allergic types of reactions that do not offer protection against the offending organism.

Adaptive immune responses are also important for protection against fungal infections. This can occur through a cell-mediated mechanism involving T lymphocytes that become activated by binding fungal antigens processed by macrophages, dendritic and other antigen-presenting cells. Dendritic cells are capable of bridging innate and adaptive immunity pathways by virtue of having some TLRs on their surfaces and being able to process fungal antigens to present to T cells in lymphoid tissues. The T cell implicated in cellular immunity to fungal infections is the CD4+ helper T cell. CD4+ T cells dictate the immune response by managing the activity of other lymphocytes. These T cells are biased (Th1 or Th2) to secrete specific cytokines that produce a protective type of inflammatory response or a maladaptive response that may lead to chronic infections or uncontrolled allergic responses. How these cells are programmed to produce an effective immune response is not entirely clear and is a topic of significant research interest.

Antibodies are also involved in immunity to fungi although their protective role is not as well defined as in the case of cell-mediated adaptive immunity. Most adults and children have naturally occurring antibodies to *C. albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Pneumocystis jirovecii*. Interestingly, populations at risk for *Cryptococcus* infections often have impaired B cell or immunoglobulin repertoires. Animal models have established a role for antibodies in the protection against certain fungal infections, implying that antibodies may also have important roles in fighting these infections in man. Several fungal antigens have been used experimentally to establish their usefulness as vaccines.

The host can also defend itself against microbial invasion by sequestering essential nutrients such as iron. Although this metabolic defense mechanism has been studied in more detail in bacteria, there is good evidence that patients with iron overload syndromes caused either by underlying metabolic diseases or by frequent blood transfusion are unusually susceptible to infection by zygomycetes such as *Rhizopus*. In fact, there have been outbreaks of life-threatening or fatal zygomycosis infections among iron-overloaded patients who have received the microbial iron-chelating siderophore desferrioxamine in an effort to reduce heavy metal levels in tissues. Whereas iron sequestered in mammalian tissues by proteins such as transferrin and lactoferrin is not usable by *Rhizopus*, iron chelated to desferrioxamine is readily usable by these fungi. Thus, these outbreaks highlight the importance of nutrient sequestration as a host defense against at least some fungi.

It should be clear from the discussion above that host defenses against fungal pathogen are complex and incompletely understood. Nevertheless, it is generally true that particular opportunistic fungi tend to take advantage of particular host defense abnormalities. Thus, Table 4 summarizes the fungi that tend to take advantage of interruptions in the skin or mucosal barriers, abnormalities in or deficient numbers of phagocytic cells, abnormalities in cell-mediated immunity, and inadequate iron sequestration.

The recognition of fungi by the innate immune system utilizes fungal molecules that are similar or are shared across species. Many fungi share characteristics such as cell wall composition or their ability to change their morphology in response to temperature, but not all fungi are able to cause disease. This implies that individual characteristics that give some fungi but not others enhanced infectivity exist. As discussed further, even closely related species are not equally pathogenic. How closely related fungi differ at the genomic level has been used to determine what characteristics are important for causing disease. Molecular tools to analyze genomes, inactivate genes, and introduce heterologous genes have tremendously advanced our knowledge of fungal

Table 4 Association of selected pathogenic fungi with host defense abnormalities

Disrupted skin or mucosa	*Candida* *Mallasezia* *Rhizopus* and other zygomycetes
Neutrophil deficiency or dysfunction	*Aspergillus* *Candida* *Rhizopus* and other zygomycetes
Cellular immunodeficiency	*Candida* *Cryptococcus* *Histoplasma* *Coccidioides* *Penicillium* *Pneumocystis*
Diabetes/acidosis/iron overload	*Rhizopus* and other zygomycetes

cellular processes and how these can influence infection of the human host. The section below discusses current molecular approaches to better understand fungal biology and how this information has helped us study how fungi cause disease.

Molecular Approaches for Studying Fungal Pathogenesis

The sequencing of multiple fungal genomes has significantly advanced the study of comparative genomics. Cellular processes that are common or unique among species can reveal information about shared structural and metabolic components, and individual characteristics that help a particular fungus evade the immune system or actively participate in the establishment of infection. Not only do genome sequences allow for the comparison of paralogous and orthologous proteins, cross-species genetic information allows for the comparison of the linear order of genes (or synteny), information that may yield clues as to how genetic information has been maintained or lost in different fungi. Newer high-throughput DNA sequencing technologies, called next-generation sequencing (or next-gen), are capable of producing more accurate and complete data sets of whole genomes at a much faster and cheaper rate relative to a few years ago. These advances have prompted the sequencing of many fungi and of multiple isolates of a given fungal species, sometimes revealing that two isolates may differ in genome size. One of the advantages of next-gen sequencing is the ability to determine the whole genome sequence data of fungi recovered from clinical samples. This is especially important for microorganisms that are considered 'unculturable' in the laboratory such as *Pneumocystis jirovecii*. The genomes of opportunistic fungi appear to differ widely in size and in complexity as seen by the number of chromosomes, genome lengths, and abundance of introns (Table 5). Although genome sequencing has revealed invaluable information, the data have yielded few obvious pan-fungal clues as to why relatively few fungi out of thousands tend to cause most of the systemic infections. This is also true of fungi of the same genus: one or two species tend to cause disease whereas the remaining dozen or more are rarely isolated from patients (*Aspergillus fumigatus* and *C. neoformans* being examples). One shared characteristic is the ability to grow well at the host temperature

Table 5 Genome organization of selected fungal pathogens

Classification by morphology	*Organism*	*Genome size (Mb)*[a]	*Introns?*	*Number of chromosomes*	*Ploidy*	*Mating*
Yeasts	*Candida albicans*	14.3	Few (8% of genes)	8	Diploid	Has *MTL***a**/α[b] loci; mating documented *in vitro*
	C. glabrata	12.3	Very few	11	Haploid	Has *MTL***a**/α loci; no mating *in vitro*; mating unknown
	Cryptococcus neoformans (serotype A)	18.9	Intron-rich	14	Haploid/diploid	Has *MAT***a**/α loci; mating documented *in vitro*
	C. gattii	17.5–18.3	Intron-rich	14	Haploid/diploid	Has *MAT***a**/α loci; mating documented by population genetic studies
Molds	*A. fumigatus*	29.3	Most of the genes have short 1–3 introns	8	Haploid	Has *MAT1-1*/*1–2* loci; mating documented *in vitro*
Dimorphics	*Blastomyces dermatitidis*	61–75.3	Yes[c]	ND	Haploid/diploid	Has *MAT1-1*/*1–2* loci; mating documented *in vitro*
	C. immitis	28.9	Most of the genes have short 1–3 introns	5	Haploid	Has *MAT1-1*/*1–2* loci; mating documented by population genetic studies
	Histoplasma capsulatum	30.4–39.1	Most of the genes have 1–3 introns	6	Haploid/diploid	Two mating types (+/−)[d]
	P. brasiliensis	30–32.9	Yes[c]	5	Haploid	*Has MAT1-1*/*MAT1-2 loci*; *mating documented in vitro*
	P. marneffei	28.4	Intron-rich	3–6[e]	Haploid	*Has MAT1-1*/*MAT1-2 loci*; *mating inferred from population studies although isolates are highly clonal*
Other	*Pneumocystis jirovecii*	8.1	Majority of genes have multiple short introns	ND	Haploid/diploid	Unknown

ND, not determined.

[a]Mega base pairs.

[b]*MTL*: mating type-like.

[c]Genes with introns have been cloned but the overall intron/exon organization of the genome has not been reported to date.

[d]+, strains are *MAT1-1*; −, strains are *MAT1-2*.

[e]The number of chromosomes is in question.

of 37 °C. How this and other growth attributes coded in the genome affect infectivity and virulence is the subject of much interest, and the ready availability of genome sequencing and molecular tools are important resources for furthering such studies.

Next-gen sequencing technologies have also been used to sequence the entire repertoire of RNA molecules expressed in a cell (or transcriptome). This technology or RNA-sequencing (RNA-seq) is capable of cataloging and quantifying any and all RNA species in a given cell. RNA-seq has virtually replaced microarray chips as a way to study gene expression of cells subjected to different stressors or during given growth stages or morphologies. RNA-seq has revealed regions of the *C. albicans* genome (and in the genomes many other eukaryotes) previously thought to be transcriptionally silent to express non-translated RNAs. An exciting and promising use of RNA-seq will be the dual analysis fungus and host transcriptomes. The latter experiment may provide clues as to how both organisms remodel their transcriptomes in response to each other and help researchers understand the biological processes of infection. A limiting factor thus far has been the handling and analysis of the vast amount of data that is generated from these experiments. In spite of this bioinformatics hurdle, the ability to quickly determine, assemble and examine entire genomes and transcriptomes of fungi is revolutionizing how we approach the study of fungal infections.

Comparative genomics has been helpful in many areas such as for the elucidation of signaling pathways, in the discovery of candidate genes involved in host survival or in virulence traits, for the identification of cell surface proteins perhaps associated with host adhesion, for uncovering mating gene loci, and, in general, for establishing genetic relatedness among fungi. An area of heightened interest has been the demonstration of mating in fungi that appear to exist as asexual organisms. Genetic diversity, as provided by mating, is thought as necessary for maintaining species robustness and fitness. Population genetic studies sometimes suggest that sexual reproduction does occur in fungi in the absence of laboratory demonstration of mating. Genomics has been instrumental in uncovering mating loci by homology searches of fungal genomes. Such has been the case for *A. fumigatus* whose genome harbors loci with good homology to *MAT* genes and was subsequently documented to undergo mating in the laboratory, altought it appears to propagate in a clonal fashion in nature. In *C. albicans*, the discovery of *MAT*-type-like loci (*MTL*) has led to the description of a parasexual modality of mating that does not produce haploid progeny. Instead, the tetraploid (4n) progeny undergo a concerted chromosome loss to return to the diploid state. The very low recovery of *MTL* homozygous clinical strains (**a**/**a** or α/α) appears to diminish the importance of mating in infectivity of the human host, and the description of five major distinct genetically similar groups (clades) suggests that *C. albicans* tends to propagate clonally and mating may be a rare event. In *C. neoformans*, mating type has been associated with virulence as α strains are more virulent that **a** strains in certain genetic backgrounds. It has long been observed that the α-mating type predominates in clinical and environmental samples, perhaps due to monokaryotic fruiting (a form of sexual reproduction by same mating-type strains) that has been mostly observed in α strains. Whether and how often 'successful' occurs and how this affects pathogenesis or tissue tropism in medically important fungi is unknown. As more genome sequences are completed and analyzed, together with empiric genetic data, similarities may begin to emerge that point toward characteristics that make certain fungi more apt to infect humans.

An invaluable molecular tool for the study of gene function in association with pathogenesis has been the ability to introduce DNA (transformation) into fungi and to specifically target the disruption of genes of interest (Table 6). Transformation systems can be divided into two steps: (1) DNA delivery into the fungus, and (2) integration of the gene disruption DNA cassette into the

Table 6 Molecular genetic tools used to study selected pathogenic fungi

Classification by morphology	*Organism*	*Transformation methods*	*Selectable markers*
Yeasts	*Candida albicans*	Spheroplasting, Li acetate/PEG, eletroporation, *Agrobacterium*	*URA3. ARG4*, *HIS1*, *LEU2*, *ADE2*, *SAT1*[a], *MPA*[b], *hph*[c]
	C. glabrata	Li acetate/PEG, electroporation, *Agrobacterium*	*URA3*, *TRP1*, *HIS3*, *neo*[d], *ble*[e], *hph*
	Cryptococcus neoformans	Biolistic, electroporation, *Agrobacterium*	*ADE2*, *URA5*, *NAT*[a], Neo, Hph
	C. gattii	Biolistic	*URA5*, *NAT*, Neo
Molds	*Aspergillus fumigatus*	Electroporation, protoplasting, *Agrobacterium*	*hgh*, *pyrG*, *argB*, *lysB*
Dimorphics	*Blastomyces dermatitidis*	Electroporation,*Agrobacterium*	*hph*, *ura5*, *sur*[f]
	C. immitis	*Agrobacterium*, protoplasting	*hph*, *ble*
	Histoplasma capsulatum	Li acetate/PEG, electroporation, *Agrobacterium*	*URA5*, *hph*
	P. brasiliensis	*Agrobacterium*	*hph*
	P. marneffei	protoplasting	*pyrG*
Other	*Pneumocystis jirovecii*	NA	NA

NA, not available.

[a]Nourseothricin marker.

[b]Mycophenolic acid marker.

[c]Hygromycin B (hygromycin phosphotransferase) marker.

[d]G418/Geneticin marker.

[e]Zeocin marker.

[f]Chlorimuron ethyl resistance (sulfonyl urea resistance). NA not available.

chromosome by homologous recombination, preferably at a rate that exceeds the inherent mutational rate. A gene disruption cassette consists of a DNA fragment with homologous DNA of your chosen gene flanking a selectable marker gene. This gene disruption DNA cassette is introduced into the fungal cell by one of various methods, after which homologous recombination at the chromosomal gene locus results in the replacement of the functional gene with a nonfunctional one carried on the disruption DNA fragment. Once a gene knockout strain is identified by molecular methods, it can be used to determine how the loss of function of the gene of interest affects virulence attributes. Many of the selectable marker genes in use restore a nutritional requirement (e.g., *URA3* for uracil or *ADE2* for adenine), which requires the use of a mutated strain that lacks the functional corresponding marker gene. A disadvantage of this methodology is the inability to create gene knockouts in clinical strains that normally are not mutated in your chosen gene marker. To overcome this problem, several dominant selectable markers such as genes coding for antimicrobials (e.g., hygromycin B resistance) have been engineered for use in transformation systems of several fungi. This obviates the need of an auxotrophic strain for performing genetic studies and also allows for the transformation of clinical strains. A very useful modification has been the development of gene disruption cassettes that are recycled such that one can generate multiple gene disruptions within one strain using the same selectable marker gene.

The ability to specifically target the disruption of genes to study their function has also undergone a methodology revolution in the last few years. A method that utilizes a bacterial enzyme called Cas9 is combined with short 'guide' RNA molecules to target the modification of DNA in the genome. This method termed CRISPR/Cas system for clustered regularly interspaced palindromic repeats/Cas9, has been very successful in generating gene deletions or other modifications (e.g. insertion of DNA sequences at specific sites within the genome) in genetically intractable systems such as in mammalian cells. The two major advantages of this system are the lack of selectable marker genes and the use of short homologous RNA molecules circumventing the often-laborious need to clone DNA disruption cassettes. The CRISPR/Cas system is still in early stages of use and optimization but promises to be an important innovation once applied to pathogenic fungi. The current methods to disrupt or modify genes in fungi are cumbersome thus ways that obviate the use of selectable markers or multiple rounds of transformations will make the generation of mutants faster and easier.

Common Fungal Diseases

In the following section, the characteristics of several of the most important fungal pathogens of humans are summarized, along with information about the epidemiology, clinical and pathologic manifestations, diagnosis, and treatment of the infections that these fungi cause.

Diseases Caused by True Pathogens

Blastomycosis

Causative organism

The causative agent of blastomycosis is *B dermatitidis*, the asexual form of the ascomycete *Ajellomyces dermatitidis*. *B. dermatitidis* is dimorphic in that it grows as a mold and produces conidia in the environment and in culture at $\leq$30 °C (Figure 1(a)), whereas it grows as yeast-like cells in infected mammalian tissues and when cultured at 37 °C. The yeast cells have thick walls; they are multinucleate and reproduce by budding from a broad base (Figure 1(b)). This fungus grows well in media rich in organic content and therefore it is assumed that they subsist in soil, perhaps in decaying wood or near bodies of water. The exact ecology of *B. dermatitidis* is not well understood because this fungus has only rarely been isolated from soil.

B. dermatitidis exists in two mating types (+and −), both of which are pathogenic. Mating type + and − strains are found in approximately equal proportions in infected people.

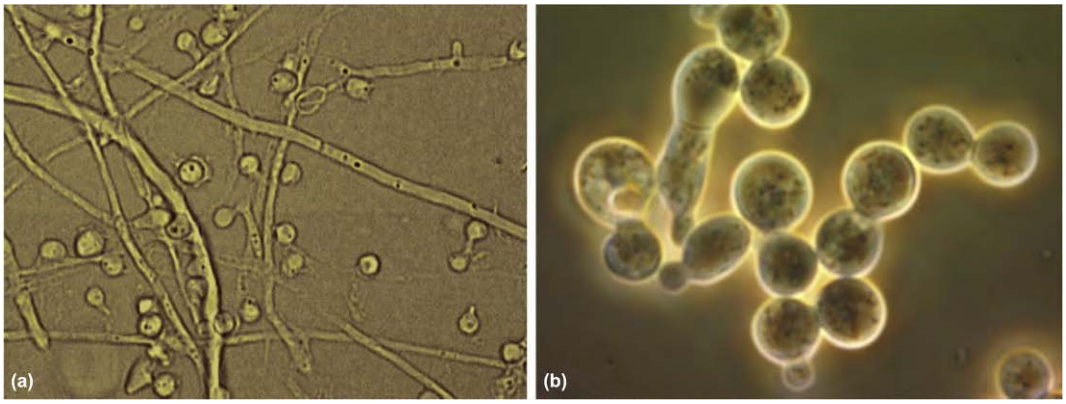

Figure 1 *Blastomyces dermatitidis* grows as a mold at 30 °C or below (a) and as thick-walled yeast cells that divide by budding on a broad base (b) in infected mammalian tissues or at 37 °C. Images courtesy of Bruce Klein, University of Wisconsin.

Epidemiology

Most cases of blastomycosis occur in the Central and Eastern United States. Areas of highest prevalence include the lower Mississippi River Valley, the Ohio River Valley, the Carolinas, and regions including the Great Lakes and the St. Lawrence River Valley. There are also microfoci in Central and South America and parts of Africa. Conidia are thought to be the infectious forms of *B dermatitidis*.

Analysis of sporadic cases indicated that persons at greatest risk in endemic areas include farmers, hunters, forestry workers, and campers. More males than females have historically been diagnosed with blastomycosis, but analysis of several distinct outbreaks has not established any gender or racial differences in susceptibility. Blastomycosis is also common in dogs and other mammals.

Pathogenesis and clinical features

Infectious particles (presumably conidia) are inhaled into the lungs of humans or animals, where they convert into the pathogenic yeast forms. Infection in the lungs elicits an inflammatory reaction with features of both acute inflammation and granuloma formation. Most infections resolve spontaneously and are thus asymptomatic and cause minimal symptoms, but some infections progress to cause progressive pulmonary infection and/or disseminated extrapulmonary disease. Several virulence-associated properties and genes have been studied. One example is a protein on the surface of *B. dermatitidis* (WI-1) that mediates adhesion to host cells.

The ability of an infected host to mount an acute inflammatory reaction and to acquire specific cell-mediated immunity is important for defense against *B. dermatitidis*. Although severe blastomycosis can occur in patients with abnormal immune defenses, *B. dermatitidis* is associated with infection of compromised hosts less often than are most other pathogenic fungi.

Because most pulmonary *Blastomyces* infections are asymptomatic or associated with only mild symptoms, blastomycosis is usually not recognized unless there is persistent or progressive pneumonia or a dissemination to extrapulmonary sites. Symptoms of pulmonary blastomycosis include fever, chills, productive cough, and chest pain. Individuals who do not recover from the early manifestations of infection develop a progressive granulomatous and suppurative pulmonary infection, or the infection may disseminate to other organs. The most common sites of dissemination are the skin and bones, although other tissues can also be involved. The typical skin lesion is a chronic progressive ulcer that is often misdiagnosed as neoplasia.

Diagnosis and treatment

The diagnosis of blastomycosis is based on demonstrating the causative organism in infected tissues or body fluids (e.g., sputum and bronchial washings, other body fluids, skin scrapings, and tissue biopsies), either by microscopy or by culture. The finding of thick-walled, broad-based, unipolar budding yeast-like cells, 8–15 μm in diameter in the collected material or within tissue, is highly suggestive of blastomycosis, and the diagnosis is proven by isolating *B. dermatitidis* in culture. Because the fungi isolated in culture are often molds without distinctive morphologic features, definitive identification of these fungi as *B. dermatitidis* can be accomplished with commercially available DNA probes. Serological tests for antibodies are of limited use because of problems with sensitivity and specificity. An immunologic test for *B. dermatitidis* antigens in serum or urine is commercially available.

Blastomycosis tends to be chronic and progressive, and mortality rates as high as 90% have been reported. Thus, the current consensus is that all people with proven pulmonary or extrapulmonary blastomycosis should receive antifungal therapy. *B. dermatitidis* is susceptible *in vitro* to the polyene antifungal amphotericin B and to the azole antifungals ketoconazole, itraconazole, and fluconazole. Case series and/or formal treatment trials indicate that all of these treatments are effective. With treatment, most people with blastomycoses recover.

Histoplasmosis

Causative organism

The causative agent of histoplasmosis is *H. capsulatum*, the asexual form of the ascomycete *Ajellomyces capsulatum*. *H. capsulatum* is dimorphic in that it grows as a filamentous mold in the environment and in culture at $\leq$30 °C (Figure 2(a)), and it grows as small uninucleate budding yeast cells (diameter 3–5 μm) in infected tissues and in culture at 37 °C (Figure 2(b)). The mold form of *H. capsulatum* produces both macroconidia (diameter 8–15 μm) and microconia (diameter 2–5 μm). Microconia are presumed to be the most important infectious particles because their small size allows them to reach the alveoli of the lung. *H. capsulatum* exists in two mating types (+and −). Approximately equal proportions of the + and − mating types have been isolated from environmental sources such as soil, but most strains isolated from infected people are of the – mating type.

H. capsulatum var. *duboisii* causes histoplasmosis in central Africa. The mold form of this fungus is indistinguishable morphologically from North American *H. capsulatum* strains, but yeast-phase cells of *H. capsulatum* var. *duboisii* are much larger (diameter 10–15 μm).

Epidemiology

Histoplasmosis is endemic in the Eastern and Central United States, specifically in the Ohio River Valley and the lower Mississippi River Valley. Skin test surveys conducted in US military recruits in the 1940s established that as many as 80% of people who live in highly endemic areas have acquired immunity to *H. capsulatum*. The fungus can be found in high concentrations in soil contaminated with bird or bat droppings, caves, areas harboring bats, poultry housing litter, and bird roosts. Outbreaks in endemic areas have been traced to the disturbance of contaminated sites during large-scale excavations or construction projects. Sporadic cases have been diagnosed in people who have neither lived in nor visited endemic areas. *H. capsulatum* var. *duboisii* is endemic in central Africa.

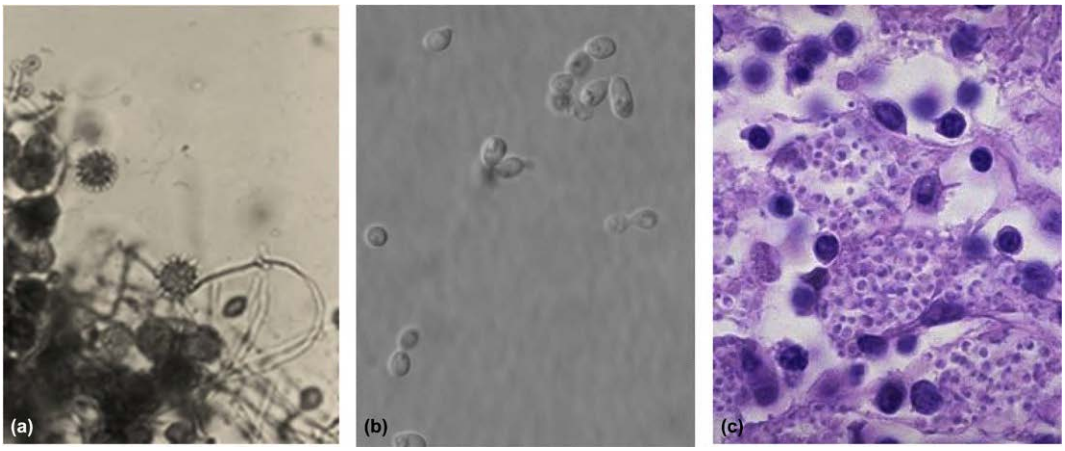

Figure 2 *Histoplasma capsulatum* grows as a mold with hyphae, microconidia, and macroconidia at 25 °C (a), and it grows as small budding yeast cells at 37 °C (b). Large numbers of *H. capsulatum* yeast cells can often be demonstrated within mononuclear phagocytic cells of people with disseminated histoplasmosis, as in this specimen of bone marrow (c). Images courtesy of George Deepe, University of Cincinnati.

Pathogenesis and clinical features

Inhaled microconidia are deposited in the lungs and are ingested by resident phagocytic cells via receptor-mediated endocytosis. Conversion of microconidia to the yeast-phase form of growth is presumed to occur intracellularly. Once viable fungi are ingested by phagocytes, some are killed, whereas others survive and can replicate within the phagolysosomes of monocytic phagocytes. When the *H. capsulatum* yeast cells proliferate within infected host phagocytes, they eventually kill the cells and go on to infect adjacent phagocytic cells. Also, the yeast cells can disseminate to distant sites within infected host phagocytes. Fungal replication in the lungs or in other organs is eventually controlled by cellular immune mechanisms, which eventually results in the formation of dense granulomata. Acquired T-cell immunity to *H. capsulatum* develops and can be demonstrated by skin testing with crude *H. capsulatum* antigen mixtures and by *in vitro* lymphocyte stimulation studies. Adoptive transfer studies in mice have established that acquired immunity to *H. capsulatum* is protective.

A large majority of people who acquire histoplasmosis by inhalation are asymptomatic or they develop only minimal respiratory symptoms. Acute pulmonary histoplasmosis develops in a small minority of individuals following initial infection, especially when the initial fungal inoculum is large. This form of histoplasmosis is characterized by fever, malaise, chest pain, and nonproductive cough. Chest radiographs demonstrate progressive pneumonia (which is often diffuse and bilateral) and sometimes also regional lymphadenopathy. Most people with pulmonary histoplasmosis recover completely without therapy, so the only evidence of past infection is skin test reactivity and sometimes the presence in radiographs of multiple calcified granulomas in the lungs, spleen, or other organs.

Some people who recover from their initial pulmonary infections develop chronic pulmonary histoplasmosis. As this can occur many years after an individual has left the endemic area, this form of histoplasmosis (which closely resembles pulmonary tuberculosis) presumably results from reactivation of latent infection. Symptoms generally include cough, fever, and weight loss, and these symptoms can progress over the course of months to years. Chest radiographs usually show upper lobe pulmonary disease, often with cavity formation and scarring. Differentiating histoplasmosis from tuberculosis in patients such as these can be very challenging.

H. capsulatum can also cause disease in extrapulmonary sites. In people with profound defects with regard to cell-mediated immunity (e.g., patients with advanced acquired immunodeficiency syndrome (AIDS) or organ transplant recipients) or whose immune systems are immature (e.g., infants and young children), widely disseminated histoplasmosis can develop. This form of histoplasmosis can follow primary infection, or it can develop in previously infected people who develop immune deficiencies. This severe form of disseminated histoplasmosis is characterized by extensive proliferation of *H. capsulatum* yeast cells within phagocytic cells in the blood and in organs such as the spleen, liver, and bone marrow. People with this form of histoplasmosis are usually severely ill with a clinical syndrome that resembles generalized sepsis. Key clinical and laboratory features include fever, chills, enlargement of the liver and/or spleen, hematologic abnormalities including anemia, leukopenia, thrombocytopenia, and disseminated intravascular coagulation. This form of disseminated histoplasmosis is fatal if untreated.

A second form of disseminated histoplasmosis tends to occur in older individuals without known immunodeficiency states. This type of disseminated histoplasmosis varies widely in severity and pace; in some people the only symptoms are gradual weight loss and fatigue and in others there is clear evidence of the involvement of many different extrapulmonary organs (e.g., liver, spleen, meninges, adrenals, and bone marrow). One clue that the problem may be disseminated histoplasmosis is the presence of painful oropharyngeal ulcerations. These lesions usually have raised borders, and they are often mistaken for neoplasms. When biopsied, these lesions show a mix of acute inflammatory reactions and granuloma formation, and special stains generally reveal abundant small budding yeast.

H. capsulatum can also cause disease by eliciting inadequately attenuated host immune responses. In normal individuals, tissues respond to *H. capsulatum* cells or antigens by forming granulomata, which are often dense and fibrotic. In addition to limiting fungal replication and containing infection, this process can lead to the development of pulmonary nodules (histoplasmomas) that

sometimes enlarge to several centimeters in diameter. These nodules are often removed because they can be difficult to differentiate from neoplasms. Histopathological examination shows that these lesions are well-organized granulomas with prominent fibrosis; budding yeast cells are generally sparse or absent and cultures are usually negative. Another clinical and pathological entity associated with host responses to *H. capsulatum* antigens is mediastinal granulomatosis and fibrosis. This late complication of histoplasmosis of the mediastinal lymph nodes and adjacent structures is characterized by granuloma formation and dense fibrosis that results in the obliteration and obstruction of key structures, including the airways, esophagus, lymphatics, and blood vessels. Lymphocytic infiltrates and scars in the choroid and retina of the eye occur in people who live in areas in which histoplasmosis is endemic and is presumed to result from cellular immune responses to *H. capsulatum* antigens.

Infection by *H. capsulatum* var. *duboisii* (which causes African histoplasmosis) is presumably also via the lungs, but acute and chronic pulmonary disease is rare. Rather, this organism tends to cause skin, subcutaneous, and bone infections.

Diagnosis and treatment

Histoplasmosis is diagnosed definitively by isolating *H. capsulatum* in culture. Because the filamentous form of *H. capsulatum* cannot readily be distinguished from several other fungi, definitive identification of *H. capsulatum* formerly required its conversion of hyphal-phase organisms to the yeast phase by incubating the cultures at 37 °C or by animal inoculation. However, DNA probes that can definitively identify fungi as *H. capsulatum* are now available commercially. *H. capsulatum* grows slowly; so cultures generally may require several weeks of incubation before growth is detected. Moreover, cultures from some patients are often negative even after prolonged incubation. Thus, it is sometimes necessary to make a presumptive diagnosis of histoplasmosis by microscopy. Histological sections or appropriately stained smears of peripheral blood or other body fluids are examined for the presence of small yeast-like cells that bud from narrow bases. These fungi are generally sparse or absent in pulmonary nodules or in material obtained from patients with mediastinal fibrosis or with ocular histoplasmosis, but they are often easily demonstrated in tissues or body fluids from patients with the acute or chronic forms of disseminated histoplasmosis (Figure 2(c)).

As histoplasmosis can be difficult to diagnose by culture or direct microscopy, serologic testing is an important adjunctive method. Antibodies to yeast and mycelial antigens are generally detected by complement fixation (CF) and immunodiffusion (ID) tests. High or rising antibody titers can provide evidence of active or recent infection, but interpretation of serologic tests is confounded by delayed or absent responses in some people (especially immunocompromised hosts) and by the persistence of detectable antibodies for many years after active infection in some others. Immunologic tests for *H. capsulatum* antigens in serum, urine, bronchial lavage fluid, and cerebrospinal fluid (CSF) have been used to diagnose histoplasmosis in people with acute pulmonary, acute disseminated, and meningeal histoplasmosis. These tests are available in commercial reference laboratories and can provide rapid and specific diagnoses, although some cross-reactions have been described. In AIDS patients or others with disseminated histoplasmosis, serial serum or urine antigen determinations are useful in assessing responses to treatment.

H. capsulatum is susceptible *in vitro* to amphotericin B and to the azole antifungals ketoconazole, itraconazole, and fluconazole, and all of these drugs have been used with success in people with histoplasmosis. Antifungal chemotherapy is required only in forms of histoplasmosis in which fungal proliferation is a prominent feature, such as severe acute pulmonary histoplasmosis, chronic pulmonary histoplasmosis, and the acute or chronic forms of disseminated histoplasmosis. Antifungal therapy is of no value in patients with stable or enlarging pulmonary nodules, mediastinal granulomatosis and fibrosis, or ocular histoplasmosis.

Coccidioidomycosis

Causative organisms

Coccidioides immitis and *Coccidioides posadasii* are the causative agents of coccidioidomycosis. These are dimorphic ascomycetes that grow as filamentous molds and produce barrel-shaped arthroconidia in the environment and in culture. In infected tissues and in specialized liquid growth media, *Coccidioides* produces thick-walled structures called spherules, which reproduce by endosporulation, a process by which the interior of the spherule is divided into as many as several hundred individual cells (endospores) (Figure 3). When the spherule ruptures, the individual endospores are released into the surrounding environment, and each spore is capable of generating a new spherule.

Sexual reproduction in *C. immitis* and *C. posadasii* has not been observed directly, but molecular genetic analyses of isolates collected from infected people and the environment support the view that meiotic recombination probably occurs in the environment.

Epidemiology

Coccidioidomycosis is endemic in arid regions in the southwestern United States, Mexico, and Central and South America. *C. immitis* is limited to the San Joaquin Valley in California and in parts of Arizona, whereas *C. posadasii* is more widespread in geographic distribution. The importance of environmental exposure to airborne spores is illustrated by new infections occurring most frequently in the dry seasons (late summer and early fall) and by large outbreaks of coccidioidomycosis occurring after dust storms, earthquakes, and other events that disturb and aerosolize the soil.

The importance of coccidioidomycosis has increased in recent decades as the population in arid regions of the southwestern United States has grown, especially with the influx of older retirees and immunocompromised individuals. In fact, new cases of coccidioidomycosis increased eightfold in several high-prevalence states between 1998 and 2011.

Pathogenesis and clinical features

The barrel-shaped arthroconidia of *C. immitis* and *C. posadasii* are highly infectious when they are inhaled into the lungs. Once they are deposited in the lung of a susceptible person, the arthroconidia swell and produce spherules that fill with endospores. The

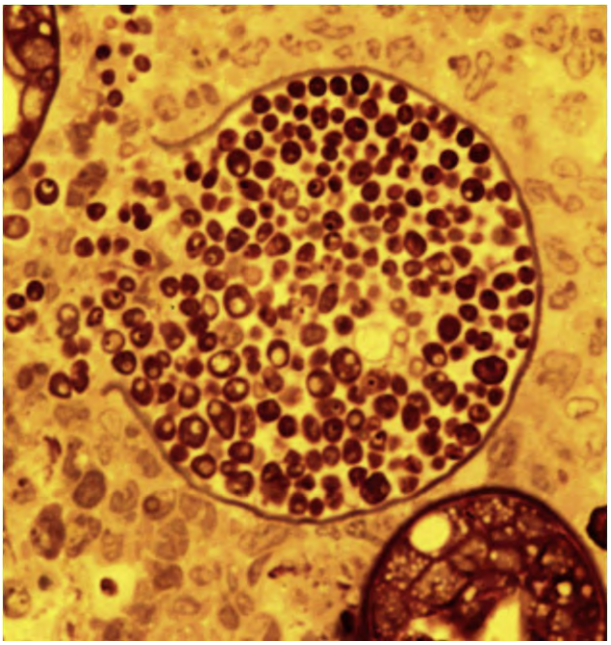

Figure 3 *Coccidioides posadasii* spherules filled with endospores are typically seen in infected tissues. The fungus can be induced to form spherules *in vitro* in special synthetic medium at 37 °C in the presence of CO_2. Image courtesy of Garry Cole, University of Texas at San Antonio.

endosporulating spherules rupture and release their endospores, each of which can generate a new spherule. This process results in primary pneumonia, which is the most common clinical syndrome associated with coccidioidomycosis.

Approximately 60% of primary *Coccidioides* infections cause no or only minor symptoms and thus are recognizable only later by a positive skin test. Symptoms in the remaining patients with pulmonary infections range from mild to severe. In general, the symptoms are nonspecific and include cough, chest pain, fever, chills, headache, and night sweats. Chest radiographs usually show pneumonia or pleural effusions, with or without hilar or mediastinal lymphadenopathy. Immune-mediated skin lesions such as erythema nodosum or erythema multiforme can also occur in association with pulmonary coccidioidomycosis. Most cases of pulmonary coccidioidomycosis resolve without treatment, although a minority of patients who recover have residual abnormalities such as single or multiple pulmonary nodules or thin-walled cavities on their chest radiographs. That active coccidioidomycosis sometimes develops in people who left the endemic area many years earlier implies that some of these residual lesions contain dormant but still viable fungi that are capable of reactivating when immune surveillance fails.

In a minority of patients, pulmonary coccidioidomycosis progresses to a chronic form of pneumonia that can persist for months or even years. These infections usually resemble pulmonary tuberculosis in that the upper lobes are often involved, and there is often prominent granuloma formation, fibrosis, and cavity formation. Large pulmonary masses and military disease also occur. Histopathological examination of these chronic or persisting lung lesions often shows a combination of suppurative and granulomatous features.

In a small minority of individuals, disseminated coccidioidomycosis develops, either soon after the primary pulmonary infection or much later when latent infection reactivates. Risk factors for the development of disseminated coccidioidomycosis include traditional causes of immunodeficiency such as AIDS, organ transplantation, or therapy with corticosteroids or other immunosuppressive drugs. Disseminated coccidioidomycosis is also more common in people of African American and Filipino ancestry, in young children and the elderly, and in pregnant women. The most common sites of extrapulmonary involvement are the skin, subcutaneous tissues, bones and joints, and meninges, but almost any organ can be involved. Meningeal coccidioidomycosis commonly causes hydrocephalus that requires surgical shunting, and this form of coccidioidomycosis is very difficult to cure.

Diagnosis and treatment

The diagnosis of coccidioidomycosis is established by demonstrating spherules by microscopy in sputum, pleural fluid, or tissues or by isolating *Coccidioides* in cultures of clinical samples. Clinicians should warn laboratory staff when coccidioidomycosis is suspected because cultures of *Coccidioides* are hazardous. A DNA probe that can identify a fungal isolate as *Coccidioides* is available commercially, so it is no longer necessary to convert the hyphal form to spherules in culture or by animal inoculation.

Serologic tests for antibodies to *Coccidioides* antigens are often useful. Enzyme immunoassays (EIA) for IgM antibodies are very sensitive, but also give many false-positive results. EIA tests for IgG and ID tests for IgM and IgG antibodies to *Coccidioides* antigens are less sensitive, but more specific. The CF test is usually positive at titers of 1:16 or higher in patients with disseminated or severe infections and declines with successful therapy. As is the case with most other infectious diseases, patients with profound immunodeficiency (e.g., those with advanced AIDS) are less likely to have measurable humoral immune responses to *Coccidioides* than are patients who are immunologically intact.

Skin testing for delayed-type hypersensitivity to *Coccidioides* is useful mostly as an epidemiologic tool, and the reagents are no longer available commercially.

C. immitis and *C. posadasii* are susceptible *in vitro* to amphotericin B and to the azole antifungals fluconazole, itraconazole, and voriconazole. Patients with pulmonary nodules, asymptomatic patients with a pulmonary cavity, and patients with acute pneumonia who are not seriously ill can often be observed because many improve without therapy. Patients with severe or progressive pulmonary disease or with extrapulmonary disease are treated with an azole or with amphotericin B. Meningeal coccidioidomycosis is usually treated with high-dose fluconazole or intravenous plus intrathecal amphotericin B; some patients require surgical shunting because of obstructive hydrocephalus.

The prognosis in normal hosts with pulmonary coccidioidomycosis is generally good. The outcome is less favorable in patients with disseminated infection, those who have underlying immunodeficiency states, and those with meningeal coccidioidomycosis.

Paracoccidiodomycosis

Causative organism

The causative agent of paracoccidioidomycosis is *Paracoccidioides brasiliensis*, a dimorphic ascomycete that grows as a mold in the environment and in culture at $\leq$25 °C (Figure 4(a)) and as budding yeast in infected tissues and in culture at $\geq$35 °C. One unique feature of yeast-phase *P. brasiliensis* is that single large mother cells can generate multiple small daughter cells (Figure 4(b)).

Epidemiology

Paracoccidioidomycosis is restricted to Central and South America, between latitudes 23° N and 34° S. Within this region, the disease is common in some areas and almost never observed in others. Favorable environments are characterized by the presence of tropical or subtropical forests, abundant rainfall and water, and moderate temperatures.

Skin test surveys show that approximately equal number of males and females have been exposed to *P. brasiliensis*, but many more males than females develop clinical disease. Paracoccidioidomycosis is unusual in children; so this is primarily a disease of adult men, most of whom are agricultural workers.

Pathogenesis and clinical features

Infection is presumed to follow the inhalation of conidia produced in the environment by the filamentous form of *P. brasiliensis*. Once the conidia are deposited in the lung, they transform into yeast cells, they begin to proliferate, and they can disseminate to distant sites via the bloodstream and lymphatic system. The fungi initially elicit an intense inflammatory reaction. With time, granulomas form and fungal replication ceases. As some patients develop active paracoccidioidomycosis years after they have left the endemic areas, it is likely that at least some fungi within granulomas or other lesions are dormant but still viable and can thus reactivate when host immunity wanes or becomes insufficient.

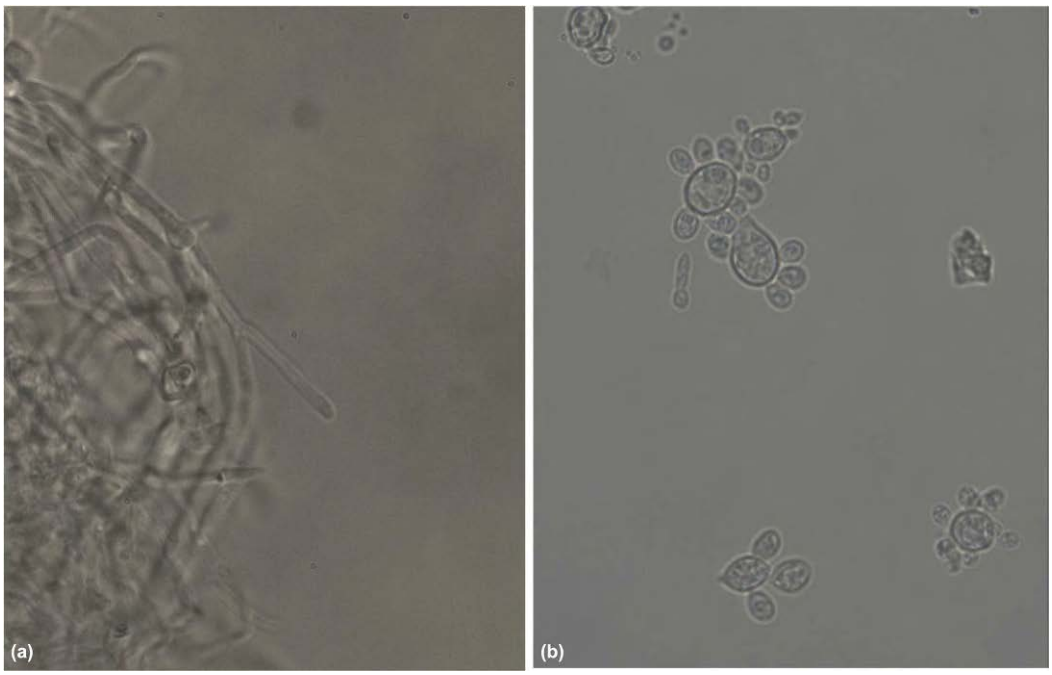

Figure 4 *Paracoccidioides brasiliensis* grows as hyphae at 25 °C (a) and as large yeast cells that produce multiple buds per cell at 37 °C (b). Images courtesy of Paulo Coelho, University of Sao Paulo, Brazil.

Most primary pulmonary infections are asymptomatic or cause only minimal symptoms and are recognized only in retrospect because of positive skin test reactivity. The most common clinical syndrome caused by *P. brasiliensis* is a chronic infection of the lungs and one or more extrapulmonary sites. This form of paracoccidioidomycosis tends to occur in adult males and is characterized by a chronic, progressive pneumonia that usually involves the central and lower portions of both lungs. The most common extrapulmonary sites involved are the skin and oropharyngeal or laryngeal mucosa, both of which develop ulcers characterized by granuloma formation and presence of large numbers of yeast-phase *P. brasiliensis* cells. Lymph nodes, the adrenal glands and the central nervous system (CNS) are also often involved in this form of disease.

A second form of disseminated paracoccidioidomycosis occurs primarily in children and in immunocompromised hosts (e.g., patients with advanced AIDS). This form of paracoccidioidomycosis resembles severe disseminated histoplasmosis in that large numbers of fungi are present in multiple organs such as the liver, spleen, and lymph nodes. Lung disease is much less prominent in patients with this type of paraccocidioidomycosis than in immunologically normal adults with more chronic paraccocidioidomycosis.

Diagnosis and treatment

Yeast-phase cells of *P. brasiliensis* are usually abundant in infected body fluids and tissues; so the diagnosis is usually established by demonstrating typical multiply budding yeast cells in potassium hydroxide preparations of appropriate clinical specimens or in histological sections. *P. brasiliensis* can also be identified in culture, but it generally takes several weeks to obtain hyphal-phase growth and then to convert the mold to yeast-phase cells by shifting to 36–37 °C.

Serologic tests for antibodies are useful in paracoccidioidomycosis. Antibodies can be demonstrated by ID in as many as 95% of patients with active disease. The CF test is somewhat less sensitive, but the ability to quantify the antibody response by generating titers is useful for monitoring responses to treatment. Immunologic tests for *P. brasiliensis* antigens in serum and CSF have been described, but these tests are not widely available.

P. brasiliensis is susceptible to sulfonamides, amphotericin B, and the azoles ketoconazole and itraconazole, and all of these drugs have been effective in clinical studies. Therapy with sulfonamides for 3–5 years is safe, inexpensive, and effective in approximately 70% of cases. Amphotericin B and ketoconazole are also effective in approximately 70% of cases, and itraconazole for 6 months is effective in as many as 95% of cases.

Sporotrichosis

Causative organism

The causative agent of sporotrichosis is *Sporothrix schenckii*, a dimorphic fungus that grows as a filamentous mold in the environment and in culture at 25–27 °C and as small, elongated 'cigar-shaped' yeast cells (3–5 μm) in infected tissues and in culture at 37 °C.

Epidemiology

S. schenckii is found throughout the world, especially in soil and in decaying or live plant matter. Most infections result from inoculation of hyphal-phase fungi through the skin at the sites of minor injury; so infections are most common in people who handle plants or plant products (e.g., gardeners and agricultural workers). *S. schenckii* can also be transferred from soil to people by infected or contaminated animals (e.g., cats). Several outbreaks have been described, including a large outbreak in South African mine workers who handled contaminated timber and outbreaks associated with contaminated plant materials used for gardening.

Pathogenesis and clinical features

The most common form of sporotrichosis is lymphocutaneous disease. This form of sporotrichosis follows inoculation of hyphal material through minor injuries in the skin. The fungus converts to yeast-phase cells, and the host responds by mounting an inflammatory reaction that includes neutrophils and also mononuclear phagocytes. Several days to a few weeks later, a papule or nodule usually develops, and then similar nodules develop over several weeks along the course of the lymphatics that drain the site of initial injury. Some of these nodules ulcerate and drain. The lesions are usually not painful.

Less common forms of sporotrichosis include pulmonary infection that closely resembles pulmonary tuberculosis, arthritis or osteomyelitis, and meningitis. Disseminated sporotrichosis has been described in patients with advanced AIDS and in patients who have received tumor necrosis factor antagonists.

Diagnosis and treatment

Definitive diagnosis of sporotrichosis requires that the causative fungus be isolated in culture and then converted from its hyphal phase to the characteristic yeast-phase cells. Because this generally takes several weeks, a presumptive diagnosis can be made if microscopic examination of appropriate fluids or tissues reveals typical elongated budding yeast cells. Unfortunately, these cells can be sparse and difficult to find. Serologic tests for sporotrichosis are generally not available, and there are no tests for *S. schenckii* antigens.

Most patients with lymphocutaneous, pulmonary, or bone and joint sporotrichosis can be treated effectively with itraconazole for at least several months. Potassium iodide by mouth is also effective in lymphocutaneous sporotrichosis; the mechanisms by which this treatment works are unknown. Amphotericin B can be used as initial therapy in people with *S. schenckii* meningitis or in immunocompromised patients with widely disseminated disease.

Diseases Caused by Opportunistic Fungi

Candidiasis

Causative organisms

Candidiasis refers to infections caused by fungi in the genus *Candida*. Together, these organisms are the most common causes of serious fungal infections in people. The most important pathogenic *Candida* species are *C. albicans*, *Candida glabrata*, *Candida tropicalis*, and *Candida parapsilosis*. All of these organisms grow in infected tissues and in culture as budding yeast-like fungi, and all except for *C. glabrata* can also form elongated buds (pseudohyphae). *C. albicans* and some strains of *C. tropicalis* can also form hyphae (Figure 5). The pathogenic *Candida* species are generally differentiated from each other based on (1) the abilities of *C. albicans* (and its close relative *Candida dubliniensis*) to form hyphae (also called germ tubes) when incubated in serum or other protein solutions, (2) the production of distinctive colors on indicator media (e.g., ChromAgar), (3) carbohydrate assimilation and fermentation patterns, and (4) morphologic features when grown in nutrient-limiting media.

C. albicans was once responsible for a substantial majority of serious *Candida* infections, but the importance of other *Candida* species has increased in recent decades. This trend was first noted in the 1970s and 1980s in cancer patients and bone marrow transplant recipients, and it has recently been documented in unselected populations. For example, a recent study of all *Candida* bloodstream infections in Baltimore County, Maryland, and in Connecticut between 1998 and 2000 showed that *C. albicans* was the causal agent in 45% of cases, *C. glabrata* in 24%, *C. parapsilosis* in 13%, *C. tropicalis* in 12%, and other *Candida* species in 6%.

One insight that emerged from sequencing of the genomes of several *Candida* species is that these fungi have mating loci that resemble the mating loci in ascomycetes with known sexual reproductive cycles. This led to the recognition that diploid *C. albicans* strains that were homozygous for the *MTL* **a** or the *MTL* α mating locus can mate with homozygous strains of opposite mating type to form tetraploid cells, which then return to diploidy by chromosome loss. Whether and how often events like this occur in natural populations of *Candida* cells is not known, but these observations raise the possibility that pathogenic eukaryotes that have lost the ability to grow as haploids or to undergo meiosis can acquire alternative mechanisms for genetic recombination.

Epidemiology

The epidemiology of candidiasis differs from the epidemiology of most other fungal diseases because normal people are colonized by *Candida*. Thus, candidiasis is acquired from endogenous sources rather than by exposure to fungi in the environment. Among the major *Candida* species, *C. albicans*, *C. tropicalis*, and *C. glabrata* populate the gastrointestinal tract and vagina of normal individuals, and *C. parapsilosis* is often found on the skin. Consequently, symptomatic infections generally develop when these fungi overgrow at sites of colonization (often as a consequence of suppression of the competing bacterial flora because of antimicrobial therapy) and when the host's defenses against infection are diminished.

Pathogenesis and clinical features

There are two general patterns of candidiasis. In people in whom overgrowth of *Candida* occurs because of suppression of competing bacterial flora by antibiotics or defects in cell-mediated immunity (e.g., corticosteroid recipients and patients with advanced HIV infection), the predominant form of disease is mucosal or cutaneous candidiasis. Typical mucosal lesions consist of white plaques that overlie shallow ulcers on an erythematous base. The ulcers are often filled with inflammatory cells and a mixture of *Candida* yeast cells and pseudohyphae, and the base may bleed if the white plaque is scraped off. Mucosal candidiasis of the mouth and pharynx is referred to as thrush, and similar lesions can occur anywhere in the gastrointestinal tract. Similar lesions also occur in the vagina. Finally, *Candida* can also infect the skin (especially in moist areas where skin maceration has occurred) and the fingernails and toenails (especially in patients with cell-mediated immune deficiency).

The second form of candidiasis is characterized by the fungal invasion of deeper organs and is generally referred to as disseminated or systemic candidiasis. In this form of candidiasis, the fungus usually enters the bloodstream through the gastrointestinal tract or vagina and the major risk factors are loss of mucosal integrity and lack of functional neutrophils. In recent years

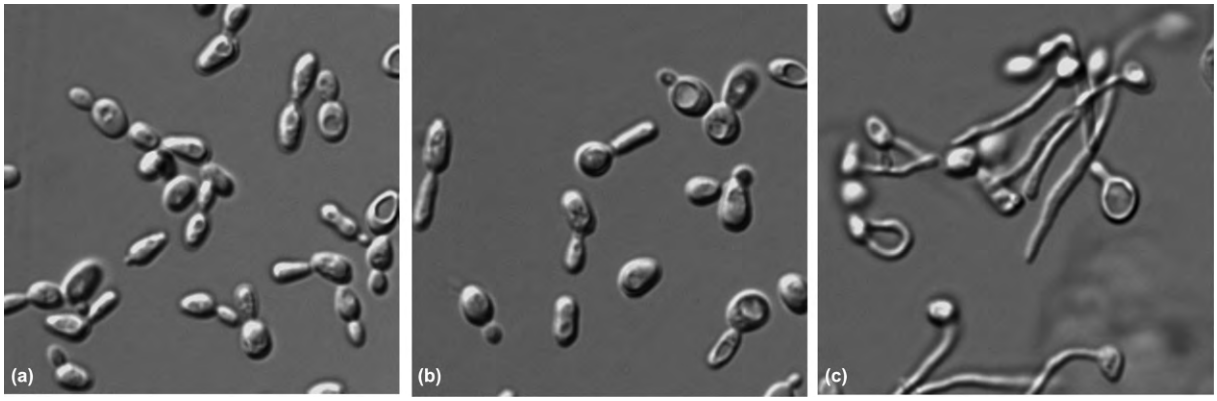

Figure 5 *Candida albicans* can grow as budding yeast (a), yeast cells with elongated buds called pseudohyphae (b), or as true hyphae (c). Short hyphal cells shown in (c) are also known as germ tubes.

bloodstream infections occur more commonly via an intravascular device such as an intravenous catheter. *Candida* invades distant organs by (1) adhering to the vascular endothelium, (2) exiting from the vascular space into surrounding tissues, (3) proliferating within those tissues, and (4) eliciting an intense inflammatory reaction. The cardinal feature of this form of candidiasis is the presence of multiple microabscesses within many different tissues. Most patients with disseminated candidiasis are febrile and have signs of generalized infection, but specific clues that the problem is caused by *Candida* are usually lacking.

Hepatosplenic candidiasis is characterized by the onset of fever, abdominal pain, and biochemical evidence of hepatic dysfunction when patients whose bone marrow function is depressed by cytotoxic drugs and/or radiation therapy recover the ability to mount an inflammatory response and thereby respond to *Candida* cells that previously seeded the liver and spleen. In addition to these more generalized syndromes, *Candida* species can also cause deep infections in individual organs, such as bones, meninges, retina and adjacent structures in the eye, and heart valves. Involvement of organs such as these generally develops as a complication of *Candida* fungemia, although the fungemic event may be unapparent or unrecognized.

Diagnosis and treatment

Candida infections can be diagnosed by demonstrating fungal cells with characteristic morphology by microscopy or by culturing the organisms from appropriate sites. Microscopic or microbiologic demonstration of *Candida* species from superficial body surfaces (e.g., skin, mucosal surfaces, and sputum) does not imply infection because *Candida* species are part of the normal commensal flora. Whether *Candida* is causing or contributing to mucosal or cutaneous disease generally must be determined by considering the number of fungi observed, the type of lesions, and relevant host factors. On the other hand, isolation of *Candida* species from body fluids such as blood or CSF or from biopsies of deep tissues constitutes strong evidence of invasive infection. However, at least 50% of patients with disseminated candidiasis have repeatedly negative blood cultures, and there are no reliable nonculture diagnostic methods. Consequently, the diagnosis of disseminated candidiasis is often delayed or missed.

Serologic tests for antibodies to *Candida* species are not useful because normal people have detectable antibodies to this commensal organism. There have been extensive efforts to develop tests for distinctive *Candida* antigens and nucleic acids, but none of these is generally available or used. Several pathogenic *Candida* species produce sufficient amounts of the 5-carbon sugar alcohol D-arabitol in infected tissues and serum and urinary levels of this compound are elevated in a majority of patients with disseminated candidiasis. D-arabitol is used as a diagnostic marker in Japan and Scandinavia, but these tests are generally not available in North America.

Most strains of the medically important *Candida* species are susceptible *in vitro* to amphotericin B, the azole antifungals (e.g., fluconazole, itraconazole, and voriconazole), and the echinocandins (caspofungin, micafungin, and anidulafungin). Clinical trials conducted mostly in AIDS patients with esophageal candidiasis and in patients with *C.* fungemia have established that antifungals in all of these classes are effective. Most trials in patients with *Candida* fungemia or other forms of invasive *Candida* infections have shown that these classes of drugs have similar efficacies, but a recent pooled analysis of multiple studies suggest that echinocandins may be more effective than fluconazole. Resistance to azole antifungals can develop in people who are heavily treated for prolonged periods, and some *Candida* species are inherently more resistant to some classes of antifungals than most others (e.g., *C. glabrata* and azoles, *C. parapsilosis* and echinocandins).

Most patients with mucosal or cutaneous candidiasis can be treated effectively with topical antifungals or systemic azoles. Despite the availability of multiple effective antifungals, disseminated candidiasis remains a serious infection. Attributable mortality rates in the range of 35–50% have been reported, and these have not changed substantially in recent decades.

Cryptococcosis

Causative organisms

Cryptococcosis refers to infections caused by fungi in the genus *Cryptococcus*, which are the asexual forms of basidiomycetes in the genus *Filobasidiella*. A unique feature of *Cryptococcus* is that these are the only medically important fungi that have thick polysaccharide capsules, which consist of chains of α 1,3-linked mannose units with xylosyl and β-glucuronlyl substitutions. Human cryptococcosis is mostly caused by *C. neoformans*, of which there are two varieties (var. *neoformans* (serotype D) and var. *grubii* (serotype A)). *Cryptococcus gattii* (formerly called *C. neoformans* serotypes B and C) is responsible for approximately 5% of all cases of cryptococcosis, especially in several geographically restricted areas (see below).

All pathogenic *Cryptococcus* species grow as encapsulated budding yeast in infected people and in culture. When haploid cells of opposite mating types (**a** or α) are mixed on nutrient-limiting media, mating generates diploid hyphal forms that produce clamp connections and basidia, and haploid basidiospores are produced as a result of meiosis. Filamentation and formation of basidia and basidiospore formation can also be induced by nutrient deprivation in haploid cells of a single mating type. Although mating and basidiospore formation have not been observed in nature, molecular genetic analyses of environmental and pathogenic strains provide strong indirect evidence that sexual recombination occurs.

One surprising observation is that almost all strains isolated from infected people and also a large majority of environmental isolates are of mating type α. Moreover, direct comparison of the virulence in animals showed that a *C. neoformans* mat α strain was much more virulent than a congenic **a** strain. Classical and molecular genetic studies have also established that the ability to grow at 37 °C to form melanin and capsules is required for wild-type virulence.

Epidemiology

C. neoformans is found throughout the world, especially in soil and other material that are heavily contaminated with bird (especially pigeon) feces. Some areas have much higher incidence and prevalence rates than others, which presumably reflects differences in environmental conditions and factors such as bird populations. *C. neoformans* infections were once quite uncommon,

tending to occur almost exclusively in people with cellular immunodeficiency states caused by neoplasia, organ transplantation, and administration of immunosuppressive drugs. However, the HIV epidemic has greatly expanded the population at risk for serious *Cryptococcus* infections. Cryptococcosis is now a leading cause of death among people with AIDS in Africa and elsewhere in the developing world.

C. gattii is primarily found in tropical and subtropical areas, often in association with trees (especially eucalyptus) and their surrounding microenvironments. However, a large cluster of cases of human and animal *C. gattii* infections was recently recognized on Vancouver Island in Western Canada, and this epidemic has since spread to the mainland of British Columbia, Washington, and Oregon. One unexpected aspect of this ongoing epidemic is that there is molecular genetic evidence that a hypervirulent epidemic strain may be the product of a sexual cross between a nonvirulent strain endemic and another strain that was recently introduced into this area. *C. gattii* causes serious pulmonary and extrapulmonary disease in immunologically normal hosts; so the epidemiology of *C. gattii* and *C. neoformans* diseases differs substantially. Since this epidemic of *C. gattii* infections in Western Canada and the Pacific Northwestern USA was recognized, additional cases have been documented in many additional geographic areas around the world.

Pathogenesis and clinical features

Cryptococcus is acquired by inhaling viable fungi in the environment into the lungs. Whether the infectious particles are desiccated yeast cells or basidiospores is not known. Once the fungi are deposited in the lungs, they proliferate as budding yeast cells, and the infection is usually controlled by the combined activities of natural and acquired host defenses. Neutrophils, mononuclear phagocytes, and lymphocytes all participate in cellular defenses against *Cryptococcus*, and phagocytosis and killing are also enhanced by humoral immune molecules such as antibodies to the capsular polysaccharide and its complements.

Most cases of pulmonary cryptococcosis are asymptomatic or result in only minor symptoms, and they resolve spontaneously without sequelae. In a minority of normal people and more often in people with cell-mediated immune deficiency, primary infection with *Cryptococcus* can lead to significant pulmonary disease. Symptoms can include cough, fever, chest pain and dyspnea, and chest radiographs can reveal pneumonitis, pulmonary nodules that may cavitate, pleural effusions, and regional lymphadenopathy.

Dissemination to extrapulmonary sites is common, especially in immunodeficient hosts. Extrapulmonary disease can develop at the same time or shortly after a patient has pulmonary cryptococcosis, but most cases occur without concomitant pulmonary disease. The most important extrapulmonary manifestation of cryptococcosis is meningoencephalitis, which is usually subacute or chronic. Patients with CNS cryptococcosis usually have headache, fever, and signs of meningeal irritation. Mental function abnormalities are common and can range in severity from subtle memory loss or personality changes to profound obtundation and coma. Cranial nerve dysfunction is common and often results from increased intracranial pressure. The pace and severity of CNS cryptococcosis can be highly variable. Some patients progress to coma and death within a few days, whereas others have had untreated chronic cryptococcal meningitis for >10 years.

Other extrapulmonary manifestations of cryptococcosis include mass lesions in the CNS (cryptococcomas), nodular skin lesions, chronic prostatitis, and bone lesions. Diffuse bilateral pulmonary disease that resembles miliary tuberculosis or *Pneumocystis* pneumonia can also occur in profoundly immunodeficient people.

Diagnosis and treatment

Cryptococcosis can be diagnosed by demonstrating encapsulated budding yeast cells in clinical specimens or in histological sections, by isolating the causative fungus in culture, or by demonstrating capsular polysaccharide antigens by immunoassay. As *Cryptococcus* is the only pathogenic fungus with a capsule, microscopic demonstration of encapsulating budding yeast cells in body fluids or tissues is diagnostic of cryptococcosis. The capsule of *Cryptococcus* is easily demonstrated in specimens such as CSF by its ability to exclude india ink (Figure 6). The capsule can appear as a clear halo around yeast cells in histological sections, and it is stained by mucicarmine. *C. neoformans* and *C. gattii* are usually isolated in culture within 2–3 days from CSF, blood, respiratory specimens, or tissue biopsies. Positive cultures can be identified as *Cryptococcus* by demonstrating the polysaccharide capsule, by India ink staining, or by immunoassay. Type-specific sera for differentiating *C. neoformans* from *C. gattii* are generally not available; however, *C. gattii* produces blue pigment on canavanine–glycine–bromothymol blue agar, and *C. neoformans* does not.

Several immunoassays are available for demonstrating the capsular polysaccharide of *C. neoformans* and *C. gattii* in body fluids. These tests are now highly sensitive and specific, and they can be used with CSF, blood, and bronchial lavage fluids. Among all the immunoassays for fungal antigens, the test for cryptococcal polysaccharide is the most reliable and widely used.

C. neoformans is susceptible *in vitro* to amphotericin B, the combination of amphotericin B and flucytosine, and the azole antifungals fluconazole and itraconazole. Clinical trials have established that all of these therapies are effective in patients with cryptococcal meningoencephalitis. In contrast, the echinocandins have no activity against *Cryptococcus* and should not be used. Combination therapy with amphotericin B and flucytosine sterilizes the CSF faster, and is associated with fewer late relapses and lower mortality than is therapy with amphotericin B or fluconazole alone. Relapses following initial course of therapy are common in AIDS patients and others whose underlying immunodeficiency cannot be corrected; so prolonged maintenance therapy with fluconazole is often required. Marked worsening of intracranial hypertension often occurs shortly after therapy is begun; so careful monitoring for this complication and prompt intervention are mandatory.

No formal treatment trials have compared different therapies in patients with nonmeningeal cryptococcosis. Also, *C. gattii* appears to be less susceptible to azole antifungals than *C. neoformans*, but whether patients with serious *C. gattii* infections should be treated with amphotericin B, amphotericin B plus flucytosine, or an azole antifungal has not been studied.

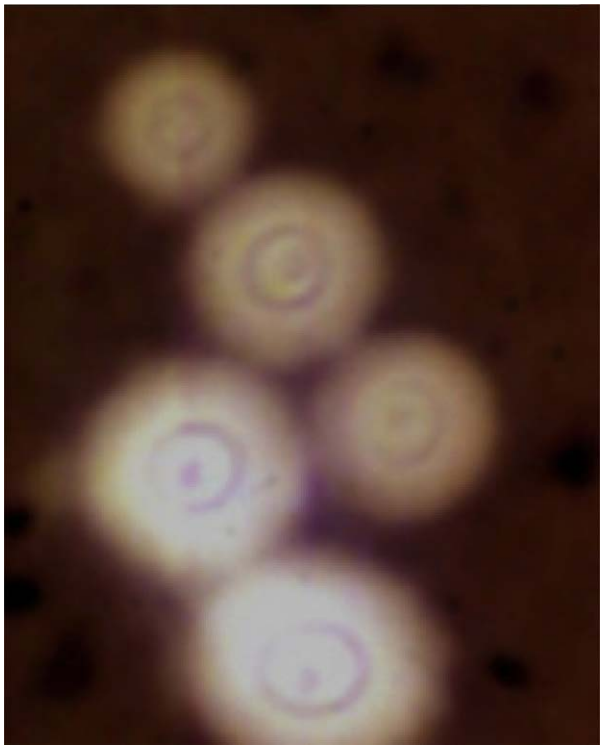

Figure 6 *Cryptococcus neoformans* produces a polysaccharide capsule, which is demonstrable as a clear halo surrounding cells in this India ink preparation. Image courtesy of Kausik Datta, Oregon Health & Science University.

Penicillosis

Causative organism

Penicillosis refers to diseases caused by fungi in the genus *Penicillium*. The most important *Penicillium* species is *Penicillium marneffei*, which causes serious infections in immunocompromised individuals in Southeast Asia. *P. marneffei* is a dimorphic fungus that grows as hyphae at 25–30 °C and as a yeast at 37 °C. Hyphal-phase *P. marneffei* produces a red pigment that diffuses into agar culture medium. Yeast-phase *P. marneffei* cells divide by fission (i.e., schizogony, as does the model fungus *Schizosaccharomyces pombe*) rather than by budding.

Epidemiology

P. marneffei is endemic in Southeast Asia. Cases of human disease have been reported in Thailand, Laos, Cambodia, Vietnam, Malaysia, Singapore, Burma, Taiwan, and Southern China. The organism has been isolated from healthy bamboo rats and from soil in and around these animals' burrows. Retrospective case–control studies have associated *P. marneffei* infection with exposure to soil and not with exposure to bamboo rats.

P. marneffei causes disease almost exclusively in people with profound abnormalities of cell-mediated immunity. Advanced HIV infection is the most common underlying condition, but cases are also observed in people with hematologic neoplasia, in organ transplant recipients, and in people receiving immunosuppressive drugs. *P. marneffei* infection is the third most frequent serious opportunistic infection among AIDS patients in Northern Thailand, following only tuberculosis and cryptococcosis.

Pathogenesis and clinical features

It is likely that infection is by inhalation of environmental spores, but this has not been proven. Most susceptible people with *P. marneffei* infections develop a subacute to chronic disseminated disease characterized by several weeks of fever, weight loss, and malaise. Many patients also have cough and other symptoms of lung infection, and a majority have skin lesions. These skin lesions are generally located on the face and upper body, and they can include papules (sometimes umbilicated), pustules, ulcers, or abscesses. At autopsy, there is usually widespread involvement of many organs, including the lungs, lymph nodes, bone marrow, liver, spleen, kidney, bowel, adrenals, bone, and meninges. Tissue reactions can include granulomatous and acute inflammatory reactions, often with necrosis or suppuration. Yeast-phase forms of *P. marneffei* are usually abundant in infected tissues.

Diagnosis and treatment

In endemic geographic locales, disseminated *P. marneffei* infection should be considered whenever an immunocompromised patient has a subacute to chronic progressive illness consistent with penicillosis. In nonendemic areas, a history of prior residence or travel to an endemic area should be sought, especially since recrudescence years after departure from an endemic area has been documented.

Once the diagnosis of penicillosis is considered, it is verified by demonstrating the causative organism in body fluids or tissues by microscopy or by culture. The diagnosis can often be established by demonstrating typical fungal forms in smears of skin biopsies, lymph node biopsies, or bone marrow aspirates or biopsies. If there is pulmonary involvement, the fungus can often be demonstrated by microscopic examination of respiratory secretions or bronchoalveolar lavage. *P. marneffei* can be isolated in culture from infected body fluids or tissues, including the blood, bone marrow, skin lesions, lymph nodes, sputum, or bronchoalveolar lavage, or other tissues. If hyphal-phase fungi with morphologic features typical of *Penicillium* species are isolated, identification of the organism as *P. marneffei* is established by conversion to yeast-phase growth by incubating at 37 °C.

Tests for distinctive antibodies to *P. marneffei* or for *P. marneffei* antigens or nucleic acid sequences are under investigation, but they are not generally available. *P. marneffei* has been reported to cross-react with tests for *Aspergillus* galactomannan.

P. marneffei is susceptible *in vitro* to amphotericin B, itraconazole, ketoconazole, and voriconazole, but not to fluconazole. Seriously ill patients are generally treated with intravenous amphotericin B initially (e.g., for 2 weeks) and then with itraconazole for at least 10 more weeks. Therapy with itraconazole alone is generally reserved for people with less severe disease. Relapses among AIDS patients with penicillosis are common; so long-term suppression with itraconazole or fluconazole is often given. Whether treatment can be stopped if immune reconstitution is achieved with antiretroviral therapy is not known.

Aspergillosis

Causative organisms

The ascomycetous fungi in the genus *Aspergillus* are the causative agents of aspergillosis. These environmental molds are present in abundant amounts in many outdoor and indoor environments. *A. fumigatus* is by far the most important pathogen of humans. *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus lentulus* also cause serious infections. All of these fungi grow in the environment and in culture as branching hyphae, and they also produce airborne conidia (Figure 7).

Epidemiology

People are exposed to spores of *A. fumigatus* and other *Aspergillus* species on a regular basis; so whether exposure to these fungal spores results in disease is determined primarily by host susceptibility. Cases of invasive aspergillosis have increased markedly in recent decades, which is no doubt due to an increase in the number of people with markedly abnormal host defenses. In one study, cases of aspergillosis per 100 000 people in the United States increased more than fourfold between 1981 and 1997.

Although host susceptibility determines in large measure who acquires aspergillosis, environmental factors are also important. Several studies have documented outbreaks of cases among immunocompromised people who were exposed to aerosols generated by construction projects either within hospital buildings or outdoors. It has long been known that removal of airborne particles by high-efficiency air filtration can substantially reduce the incidence of aspergillosis among highly susceptible hosts.

Pathogenesis and clinical features

Aspergillus causes several different types of human disease. Allergic bronchopulmonary aspergillosis (ABPA) is characterized by chronically recurring episodes of bronchospasm associated with sputum production, mucus plugging of airways with resulting atelectasis, sometimes resulting in pulmonary to fibrosis and/or bronchiectasis. This syndrome is caused by hypersensitivity to antigens expressed by fungi colonizing the airways. Most patients with ABPA have high circulating levels of total IgE, demonstrable IgE-mediated responses to *Aspergillus* antigens, and high circulating levels of IgG antibodies to *Aspergillus* antigens. The course is generally chronic and progressive. Patients generally do not improve without therapy.

Aspergillus can also colonize anatomically abnormal airways, thereby initiating a local inflammatory reaction. When this occurs in residual cavities in patients with healed tuberculosis, a mass of fungal hyphae and necrotic debris that eventually fills the cavity is

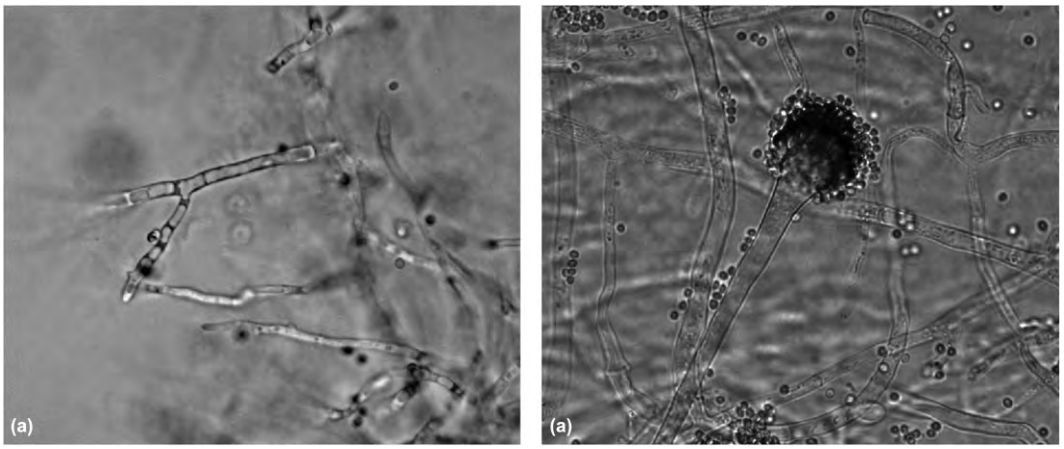

Figure 7 *Aspergillus fumigatus* and other *Aspergillus* species grow in infected tissues and in culture as narrow septate hyphae that branch at angles of approximately 45° (a) *A. fumigatus* produces airborne asexual spores (conidia) from columnar fruiting bodies when cultured on agar plates (b) but these structures are seldom seen in infected mammalian tissues.

referred to as an aspergilloma or fungus ball. Most aspergillomas cause no symptoms, but they erode the surface of the cavity, which can result in bleeding (hemoptysis) that can be severe. *Aspergillus* can similarly colonize bronchiectatic airways of patients with underlying chronic lung diseases such as cystic fibrosis.

Invasive pulmonary aspergillosis is the form of aspergillosis that has increased markedly in frequency and importance in recent decades. Patients at highest risk include people with prolonged neutropenia due to stem cell transplantation or cytotoxic chemotherapy for neoplasia. Transplant recipients and patients receiving high doses of corticosteroids or other immunosuppressive drugs are also at increased risk. When an immunodeficient host inhales viable *Aspergillus* conidia, the fungi evade ingestion and killing by host phagocytes, they swell and germinate to produce filamentous hyphae, and they invade the surrounding tissues. *Aspergillus* tends to invade and proliferate within blood vessels, thereby causing ischemic injury and widespread necrosis in affected tissues. Patients with invasive pulmonary aspergillosis usually have fever and cough, and pleuritic chest pain is often present. Chest radiographs show pulmonary infiltrates that are often peripheral and wedge-shaped, as would be expected in a lesion in which vascular ischemia is an important contributor. Cavity formation is common and can be a valuable clue to the diagnosis. Disease progression is often very rapid in profoundly immunocompromised individuals, often leading to respiratory failure and death in a few days. In others (especially those with less severe immune deficiencies), the pace of the disease is much slower; chronic progression with formation of multiple cavities can develop over several months.

Aspergillus can also cause necrotizing sinusitis in some profoundly immunocompromised individuals. The initial site of infection is the mucosal surface of a facial sinus or the nasopharynx, which is directly invaded by germinating fungal hyphae. Because *Aspergillus* tends to invade blood vessels and thereby causes extensive tissue necrosis, this process can rapidly extend into the orbit and into the cranium. For unknown reasons, *A. flavus* is especially likely to cause necrotizing sinusitis.

Aspergillus can also disseminate to distant organs via the bloodstream in patients who have primary aspergillosis of the lung or a facial sinuses. Once the fungus reaches these distant organs, it tends again to invade along blood vessels, thereby causing ischemia and tissue necrosis. Virtually any organ can be involved, including the brain, kidneys, liver, spleen, and skin.

Diagnosis and treatment

Aspergillus grows in infected tissues as narrow septate hyphae that branch at 30–45° angles, thus demonstrating fungi with these morphologic features in body fluids of tissues from a person with a compatible clinical syndrome can provide presumptive evidence of aspergillosis. However, the hyphae of several other pathogenic fungi (e.g., *Fusarium*) are similar in morphology; so a definitive diagnosis of aspergillosis requires both demonstration of the fungus in infected body fluids and/or tissues and isolation and identification of the fungus in culture. Interpreting the results of fungal cultures (especially from respiratory sites) can be difficult because (1) *Aspergillus* can colonize the respiratory tract without causing invasive disease and (2) some patients with proven invasive infections have repeatedly negative cultures. It should also be noted that *Aspergillus* is almost never isolated from blood, even in patients with widely disseminated disease.

Patients with ABPA usually have high total IgE levels and high levels of *Aspergillus*-specific IgE and IgG antibodies. Patients with aspergillomas or fungal colonization of bronchiectatic airways often have high levels of IgG antibodies to *Aspergillus* antigens. Serologic testing for antibodies to *Aspergillus* is not useful in invasive pulmonary or extrapulmonary aspergillosis. However, galactomannan derived from the cell wall of *Aspergillus* can be detected by immunoassay in a substantial portion of patients with invasive pulmonary or extrapulmonary aspergillosis, and galactomannan can be detected by immunoassay in bronchoalveolar lavage fluids from a substantial majority of patients with invasive pulmonary aspergillosis.

Growth of *Aspergillus* is inhibited *in vitro* by amphotericin B, the azoles itraconazole, voriconazole, and posaconazole (but not fluconazole or ketoconazole), and the echinocandins caspofungin, micafungin, and anidulafungin. Prospective clinical trials have established that itraconazole is an effective therapy for ABPA and that voriconazole is superior to amphotericin B for invasive aspergillosis. Uncontrolled studies suggest that caspofungin may be useful in patients who do not respond to or cannot tolerate alternative therapies. Controlled trials showed that posaconazole can prevent invasive aspergillosis in selected high-risk patients. Despite the availability of effective therapies, mortality in severely immunocompromised hosts with invasive aspergillosis remains very high. For this reason, there is considerable interest in combination chemotherapy, adjuvant immunotherapy, and other measures. Also, some patients with extensive necrotizing aspergillosis lesions may benefit from surgical debridement or resection, either to control the infection initially or to reduce the likelihood of relapse.

Zygomycosis

Causative organisms

The term zygomycosis refers to diseases caused by filamentous fungi in the class Zygomycota, the most important of which are in the genera *Rhizopus*, *Mucor*, and *Absidia*. As these fungi are in the order Mucorales, an alternative name for zygomycosis is mucormycosis. These fungi are widespread in nature. They can commonly be found in the soil and on decaying plant and animal matter; for example, zygomycetes include common bread molds. These fungi grow as molds consisting of broad hyphae that usually lack nonseptae. In the environment (but not in infected tissues), fruiting structures (sporangia) develop, which generate large numbers of airborne spores (sporangiospores). Consequently, most people regularly come in contact with airborne zygomycete sporangiospores.

Pathogenesis and clinical features

Most patients with zygomycosis have abnormal host defenses, although these often differ from those that underlie most other opportunistic mycoses. For example, the most important factor associated with zygomycosis is diabetes, especially with

ketoacidosis. Other forms of metabolic acidosis (e.g., uremia) also predispose to zygomycosis, as do states associated with availability in tissues of excess amounts of free iron (e.g., hemochromatosis or administration of iron-chelating agents). Corticosteroids, neutropenia, and interrupted skin (e.g., in burns or surgical incisions) are also important predisposing factors.

The most common form of zygomycosis is rhinocerebral zygomycosis, which begins when fungal spores deposit on the mucosal surfaces of the nasopharynx or palate. The spores then germinate into hyphae, which invade and then grow within blood vessels, thereby causing extensive thrombosis, ischemia, and necrosis. Zygomycosis typically breaches anatomical barriers and promptly involves the facial sinuses, the orbit and its contents, and then the cranial contents. Pain, swelling and cranial nerve palsies are often seen early, followed by frank skin necrosis, blindness, stupor, and coma.

In patients such as neutropenic stem cell transplant recipients and leukemia patients, inhalation of zygomyces spores into the lungs can lead to pulmonary zygomycosis, which is very similar in its clinical and pathologic features to pulmonary aspergillosis. As is also observed in rhinocerebral disease, the zygomycetes invade and then grow along blood vessels in the lungs, causing extensive thrombosis, ischemia, and tissue infarction. Most patients have fever, cough, and/or chest pain, and chest radiographs usually show diffuse pneumonia, more focal disease that may cavitate, and/or pleural effusions.

Dissemination via the bloodstream to distant organs occurs in patients with either the rhinocerebral or pulmonary forms of zygomycosis, especially in patients with prolonged neutropenia or in stem cell transplant recipients. The zygomycetes also cause necrotizing skin infections, either as a secondary manifestation of widespread hematogenous dissemination or when viable spores are introduced into burn wounds or surgical incisions via contaminated wound dressing materials. The zygomycetes rarely cause invasive disease of the gastrointestinal tract, especially in malnourished children in the developing world.

Diagnosis and treatment

Most cases of zygomycosis are diagnosed by demonstrating in infected tissues or fluids or in stained histological sections broad hyphae that branch at 90° angles that usually lack cross-septations (Figure 8). The causative fungi can often be demonstrated in potassium hydroxide preparations of material from necrotic eschars in the nasopharynx or palate or from infected sinuses of people with rhinocerebral zygomycosis.

The various zygomycete genera and species are differentiated by morphologic features of fruiting and 'rootlet' structures that develop only in culture. However, it is sometimes very difficult to isolate these fungi in culture, even from specimens that are known to contain abundant hyphae by direct microscopy. Like *Aspergillus*, the fungi that cause zygomycosis cannot be isolated from the blood of infected people. There are no serologic tests for antibodies to the zygomycetes or immunoassays for zygomycete antigens.

Because zygomycosis tends to progress rapidly and is associated with high mortality rates, multiple therapeutic measures are generally required. First, every effort should be made to correct underlying host defense abnormalities such as diabetic ketoacidosis or uremic acidosis as quickly as possible. Similarly, immunosuppressive drugs should be stopped or their dosages reduced, if possible. Second, necrotic tissues should be surgically debrided and removed, even if extensive and disfiguring procedures are required. Next, antifungal therapy should be administered. Amphotericin B inhibits the growth of most of the zygomycetes and has been used with success in many patients with zygomycosis. The newer azole antifungal posaconazole has *in vitro* activity against many zygomycetes, whereas most other azoles do not, and at least one uncontrolled case series suggested that posaconazole may be effective in patients with zygomycosis who have not responded to or cannot tolerate amphotericin B.

Pneumocystis infections

Causative organism

Organisms in the genus *Pneumocystis* were once thought to be protozoans because of morphology and other characteristics, but genome sequence analysis has established that these are fungi in the phylum Ascomycota. *Pneumocystis* has been found in the lungs of many mammalian species, but it has not been cultivated *in vitro*. Thus, much of what is known about these fungi has been gleaned

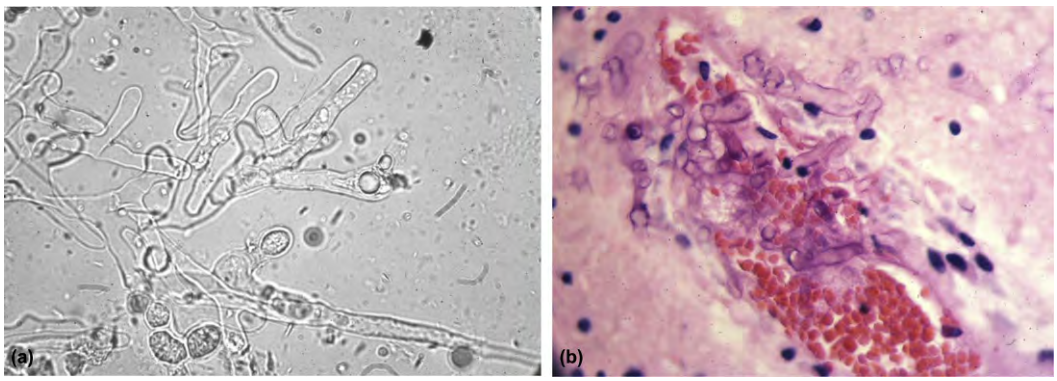

Figure 8 *Rhizopus* and other zygomycetes grow as broad nonseptate hyphae that branch at angles of approximately 90°. Typical hyphae can often be demonstrated in potassium hydroxide preparations of infected tissues. (a) Zygomycete hyphae tend to invade within and along blood vessels, as in this section from the brain of a patient with fatal rhinocerebral disease (b).

from its morphology in infected hosts and from inferences from analyzing their genome sequences. Two morphologic forms are observed in infected lung tissues. Trophic forms are small (diameter 1–5 μm), uninucleate cells that appear to reproduce asexually by fission. Cystic forms are larger (diameter 5–10 μm) spherical structures with thick walls and as many as eight internal spores. As these structures resemble asci, they are presumed to develop as a consequence of mating between trophic cells of opposite mating type.

As different species of *Pneumocystis* appear to infect different mammalian species, the human pathogen that was once called *Pneumocystis carinii* (now the rat pathogen) is now called *P. jirovecii*. Environmental forms of *Pneumocystis* have not been described.

Epidemiology

Serologic surveys have shown that most healthy people develop antibodies to *P. jirovecii* during childhood. Animal studies indicate that *Pneumocystis* is acquired by inhalation, but it is not known if the infectious particles are spores or trophic cells acquired from infected individuals or if they are produced by an unknown environmental form of this fungus. Clinically apparent disease develops almost exclusively in people with cell-mediated immunodeficiency states associated with conditions such as advanced AIDS, neoplasia, malnutrition, or immunosuppressive drugs.

Pathogenesis and clinical features

The most common clinical manifestation of Pneumocystis infection is bilateral diffuse pneumonia. Most patients have fever, dry cough, and dyspnea. In some patients, the pneumonia can progress to severe disease and death over a few days; in others, the course is indolent over the course of several weeks. Histopathological examination of infected lung tissue generally reveals foamy eosinophilic exudates within affected alveoli. These exudates typically contain abundant *Pneumocystis* trophic cells and cysts. The host inflammatory response is usually mild. Extrapulmonary involvement of many different organs has been documented in patients with advanced AIDS, but is seldom of major importance.

Diagnosis and treatment

Pneumocystis pneumonia is usually diagnosed by demonstrating morphologically characteristic trophic cells and cysts in respiratory specimens or lung biopsies. Fluorescein-labeled monoclonal antibodies are available and are more sensitive for identifying *Pneumocystis* organisms than conventional staining methods. Immunoassays for *Pneumocystis* antigens in respiratory fluids and polymerase chain reaction tests for *Pneumocystis* DNA sequences have been developed, but these tests are not widely available.

The combination of a sulfonamide and trimethoprim is highly effective for the treatment of *Pneumocystis* pneumonia. Alternative therapeutic include pentamidine (either parenterally or by inhalation), atovaquone, dapsone plus trimethoprim, primaquine plus clindamycin, and trimetrexate. Adjunctive therapy with corticosteroids improves the outcome in AIDS patients with severe *Pneumocystis* pneumonia, presumably by attenuating host inflammatory responses to dead or dying fungi. Chemoprophylaxis against *Pneumocystis* pneumonia is effective in high-risk individuals who have not previously been infected (e.g., AIDS patients with low CD4+ lymphocyte counts, children with acute leukemia) and also in patients who have recovered from an initial episode. Effective chemoprophylactic agents include sulfamethoxazole/trimethorpim, dapsone, atovaquone, and pentamidine administered by inhalation.

Summary and Conclusion

The burden of disease caused by pathogenic fungi has increased markedly in recent decades, primarily because there are now many more people with significant deficiencies in host defenses against infections than there were as few as 20 years ago. Along with the recent increase in the frequency and importance of these diseases, our understanding of the basic biology of the pathogenic fungi and of their interactions with mammalian hosts has also expanded greatly. These advances have resulted in large measure from the sequencing of the genomes of several pathogenic fungi, the application of molecular genetic methods to fungal pathogenesis, and advances in understanding both innate and adoptive immune responses to infection by fungi and other pathogens. There have also been significant advances in recent decades in our ability to diagnose, prevent, and treat several serious fungal infections.

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Gastrointestinal Microbiology in the Normal Host☆

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Defining Statement

The microflora of the human gastrointestinal tract has a profound influence on health and disease. The majority of the bacteria reside in the colon that they make up about one-half of the colon content and their total mass is 0.2 kg (Sender et al., 2016). The number of colon bacterial cells was most recently estimated as 3.8×10^{13}, the same order as that of human cells, which is 3.0×10^{13}. In 2014, an integrated human gut microbiome catalog of reference genes was established with 1267 sequenced metagenomics samples from 1070 people worldwide and it comprises 9,879,896 non-redundant genes (Li et al., 2014).

Problems Associated With the Study of Gastrointestinal Flora

The composition of the gastrointestinal (GI) microflora is complex and many different methodologies have been used to study it. Fecal matter is usually used as a proxy of gut microbiota. However, there is still no universally accepted protocol for fecal sample collection and the downstream analysis. Cultural studies were primarily used in the past but many of gut microorganisms are quite fastidious and are difficult (or impossible presently) to recover in culture, particularly if they are present in relatively small numbers. For cultural studies, a variety of media should be used, along with a variety of atmospheric conditions. Organisms vary greatly in their growth requirements. Unless selective and differential media are used, certain organisms present in lower counts may be overlooked. It is important to remember that many or most "aerobes" are actually facultative and therefore can grow on anaerobically incubated plate media. Selective media, while very useful, always lead to suppression of certain organisms. One of the principal problems of culture-based techniques is the fact that the majority of the indigenous flora of the GI tract cannot be detected. Most of these so-called "uncultivable" organisms (a better term for them would be "not yet cultured" organisms) are anaerobes or other very fastidious microorganisms. Molecular techniques provide the possibility to detect the majority of such organisms. Molecular studies using microbial DNA may also be inaccurate if the stool or other specimen is not thoroughly homogenized before the DNA is extracted. In studying mixed populations, there may be insufficient or preferential cell lysis, inhibition of PCR, differential amplification or formation of chimeric or artefactual PCR products.

In the past few years, sequencing technology have been vastly developed whereas the cost dropped dramatically. This makes next-generation sequencing (NGS) techniques become mainstreams in the study of GI flora. NGS is culture-independent and can produce huge amount of sequence data in a relatively short time. Being a complex microbial ecosystem, a human fecal sample usually has hundreds of members with complex interactions. NGS allows sequencing all members simultaneously, thus can better reflect the true microbial composition structure inside a sample. However, lack of reproducibility in GI flora investigations is common and still demands solutions. This problem is due to various known and unknown variations, which are from sample collection, storage, DNA extraction, sequencing strategies and data analysis (Thomas et al., 2015; Sinha et al., 2015; Costea et al., 2017).

Variation in GI flora among population is large since gut microbiota are influenced by many factors, for instance, diet, sex, age and health states of the host. In a Dutch population-based cohort with 1135 participants, 126 host factors investigated could account for 18.7% of the inter-individual variation in microbial composition. Of these 126 factors, which contained 31 host intrinsic factors, 12 diseases, 19 drug groups, 4 smoking categories, and 60 dietary factors, 110 in total were associated with 125 species (Zhernakova et al., 2016). Many clinical studies try to survey key microorganism or factors related to diseases by comparing several subject groups. A well designed study that can maximally minimize irrelevant variation is crucial for this purpose.

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There is growing interest to define a healthy human gut microbiome. Large-scale projects, such as the Human Microbiome Project (HMP) and the Metagenomics of Human Intestinal Tract (MetHIT), have sequenced microorganisms from normal humans and generated a microbiome baseline catalog. Besides phylogenic and taxonomic information, host biogeography, ecology, metabolism, and function are also included (Human Microbiome Project Consortium, 2012b; Qin et al., 2010). However, the definition of "healthy" or "normal" gut microbiome is still under controversy. Large degree of diversity among individuals even in the population without apparent disease is one of the major reasons. There may be a certain common "core microbiota," which in healthy adults appears to be dominated by Firmicutes and Bacteroidetes, followed by Actinobacteria, Proteobacteria, and Verrucomicrobia (Hollister et al., 2014). Some researchers have attempted to discover a species-level core in healthy adults, which including *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Bacteroides uniformis* (Qin et al., 2010). Unfortunately, the total abundance of these species was less than 0.5% in some other individuals (Turnbaugh et al., 2009). Given the complexity and diversity of gut microbiota in healthy adults, it may be difficult to define a "core microbiome" set at the species-level. An alternative conceptualization, "functional core microbiome" set is proposed in healthy individuals, collecting functional genes and pathways rather than species or genera (Nicholson et al., 2012; Zhang et al., 2015a,b). This is because healthy individuals, even with varied gut microbiota structure, all contain pathways for central carbohydrate and amino-acid metabolism. Some functions are variable among the population, such as vitamin and drug catabolism, pathogenicity islands, motility and nutrient transporters (Bäckhed et al., 2012; Lozupone et al., 2012). In 2011, based on unconstrained canonical correspondence analysis of the genus-level compositional profiles, the term "enterotypes" was proposed. Individuals were classified into three groups (enterotypes) according to the abundance pattern of three taxa *Bacteroides*, *Prevotella*, and *Ruminococcaceae*, which had largest variation among the population (Arumugam et al., 2011). The obtained three enterotypes appeared to be independent of BMI, age, gender or nationality, but were correlated with diet. Individuals on an animal-based diet had been proved to have a *Bacteroides* dominated enterotype, whereas those on plant-based diet have a *Prevotella* dominated one (Wu et al., 2011). However, debates on calculation that result in "enterotypes" still continue (Knights et al., 2014; Gorvitovskaia et al., 2016). Further research is necessary to determine whether discrete enterotypes or fluid "enterogradients" is a better to describe the human microbiota composition, and to reveal the further biological significance under enterotypes (Bäckhed et al., 2012; Costea et al., 2018).

Techniques for Study of Gastrointestinal Flora

There are many protocols for cultural studies of GI flora. It is important that good anaerobic conditions are provided during transport of the specimens and for the culture and examination processes. With GI flora, many organisms are quite fastidious and may not survive if they are cultured in an anaerobic jar and then removed to an aerobic atmosphere while the plates are examined before sub-culturing.

Various molecular approaches may be used and will provide a remarkable amount of additional information. Clone libraries, while tedious and time-consuming, have been used with excellent results. Terminal restriction fragment length polymorphism (T-RFLP) has been considered to be promising for analysis of communities such as GI flora but is somewhat lacking in precision. Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE) and fluorescent in situ hybridization (FISH) have also been used effectively for community flora analyses. Probe grids or DNA checkerboard hybridization may be used and DNA microarrays are available, but not with a comprehensive array for GI flora. A preferred technique is real-time PCR. It requires that one have primer-probes for target sequences of groups of organisms or for each individual organism that may be present (which immediately places limitations since we do not yet know the total makeup of GI microflora communities). For studies of known organisms for which there are primer-probes high-throughput methods, such as pyrosequencing techniques provide quantitation as well as accurate identification, and detection of organisms that are present in small numbers.

NGS technologies have revolutionized the studies of GI flora through a culture-independent, relatively high precision and high-throughput strategy to explore the microbial community in a complex ecosystem. The fast-evolving sequencing platforms include 454 pyrosequencing, Illumina, Ion Torrent and PacBio etc. Measurements of microbiota may be based on 16S rRNA and other marker genes. In addition of sequencing the near-full-length clones, much higher throughput methods are generally performed to sequence the PCR amplicons of targeted hyper-variable regions of 16S rRNA genes. Assigning such 16S rRNA gene amplicon sequences into operational taxonomic units (OTU) based on the similarity thresholds becomes a basic protocol in microbial community studies. The similarity thresholds of sequences can vary to represent different levels of taxonomic composition, e.g., 97% similarity stands for species and 95% similarity for genera (Goodrich et al., 2014). Several pipelines such as QIIME (Caporaso et al., 2012), mothur (Schloss et al., 2009), and UPARSE (Edgar, 2013) have been developed to combine the quality filtration, chimera detection, OTU picking, taxonomy classification and downstream analyses altogether. Other efforts have also been introduced to bypass the similarity threshold based OTU picking strategy to achieve more detailed resolution (Tikhonov et al., 2015; Shokralla et al., 2015; Burke and Darling, 2014). More recent methods include Swarm, DADA2 and Deblur. Swarm clusters OTUs by connecting nearly identical sequences (Mahe et al., 2015). DADA2 and Deblur provide denoised error-free sequences for the inference of single nucleotide differences-amplicon sequence variants (ASVs) (Callahan et al., 2016; Amir et al., 2017).

Whole metagenomics, which target on the whole DNA of the microbiota in the GI flora, are used to provide both structural and functional information. The first human gut microbial gene catalog (~3.3 million genes) was established in 2010 (Qin et al., 2010) and the integrated catalog of reference genes (~9.9 million genes) in the human gut microbiome was published in 2014 (Li et al., 2014).

Information from >1800 independent human gastrointestinal metagenomic samples had been included in Human Pan-Microbe Communities Database (HPMCD) since 2015 and could provide standardized phylogenetic classification at species level (Forster et al., 2016). High-quality microbial genomes can be assembled from metagenomics directly with the emerging methods (Nielsen et al., 2014; Kang et al., 2015; Sangwan et al., 2016). For instance, Parks et al. reconstructed ~8000 bacterial and archaeal genomes from >1500 metagenomic datasets, which increased phylogentic diversity and expanded our understanding of the tree of life (Parks et al., 2017). In addition, metatranscriptomics, which identifies RNA-based regulation and expression level in ecosystem, and metaproteomics, which is for large-scale characterization of the whole protein in the microbial community, have been applied in the study of GI flora. For instance, by combining the metatranscriptome and metagenome of the human gut, Franzosa et al. found the subject-specific whole-community regulation (Franzosa et al., 2014). In a review about human gut microbiome metaproteomics, Xiong et al. listed considerations for metaproteome measurement and subsequent information analysis (Xiong et al., 2015). They also highlighted the distinctness and complement of functional information between metaproteome and metagenome. Cheng et al. recently developed MetaLab, an integrated software platform for metaproteomic analysis, to facilitate microbiome studies (Cheng et al., 2017).

Normal Flora of Esophagus

It has generally been considered that the esophagus has no resident microflora that bacteria occur there only transiently and represent swallowed organisms from the oral cavity. However, a paper in 1983, using older techniques, did find aerobic organisms in all subjects studied and anaerobes in 80% of subjects. The most common aerobes might have represented oral flora—*Haemophilus*, *Neisseria*, and streptococci and yeasts, but coliforms were found in half the subjects. The most common anaerobes encountered were anaerobic cocci and *Bacteroides*, including *Bacteroides fragilis*. Recently, diverse techniques have been used to reveal the resident bacteria in the esophagus. A study based on 16S rDNA clone libraries of esophageal microbiota was performed on four persons and detected up to 6 phyla and 41 genera, among which *Streptococcus*, *Prevotella*, and *Veillonella* were prevalent (Pei et al., 2004). The cultivation based bacteria identification of both brush and biopsy samples in a cohort of 40 persons also validated that the esophageal bacterial community is predominated by viridans streptococci and fusiform rods, then N*eisseria*, *Haemophilus*, and *Prevotella* species. The similarity of oral and esophageal microbiota suggested that oral is the source of bacterial seeds transported to esophagus. The biopsy samples indicated that the bacteria are not just transient passing the surface but also residing in the epithelia, especially for Streptococci that have a strong capacity to adhere to mucosal linings (Norder Grusell et al., 2013). Further studies also revealed the correlation between the alteration of esophageal microbiome and the risk of gastroesophageal reflux disease, Barrett's esophagus, esophageal adenocarcinoma, esophageal squamous cell carcinoma, and eosinophilic esophagitis (Snider et al., 2016; May and Abrams, 2018). To satisfy the increasing requirements of sampling esophageal microbiota, a novel device using minimally invasive technology also been made to perform "Esophageal String Test" to accelerate the study of esophageal microbiome (Fillon et al., 2012).

Normal Flora of Stomach

Stomach is an interesting and unique niche for bacteria. Many bacteria enter the stomach through oral cavity and refluxing from the duodenum, but most of them are killed by the low pH of the stomach. For a long time, gastric mucosa was considered sterile. The discovery of *Helicobacter pylori* in 1983 changed this view and more bacteria have been identified in stomach due to the application of cultural independent technology. Bik et al. analyzed gastric mucosa of 23 healthy adults and found 128 phylotypes, of which most belonged to the Proteobacteria, Firmicutes, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* phyla (Bik et al., 2006). Based on cloning and 16S rRNA sequencing, Li et al. identified that 99.0% of gastric microbiota sequences from five healthy subjects belonged to five phyla (Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, and Proteobacteria) (Li et al., 2009). Engstrand et al. analyzed the gastric microbiota of 13 healthy subjects and identified similar results as mentioned before even though the DNA extraction methods, primer design and sequencing were different in each study (Engstrand and Lindberg, 2013). Delgado et al. investigated the microbiota of stomach mucosa biopsies and gastric juices from 12 healthy persons. They combined culture and culture-independent techniques and found four main genera: *Propionibacterium*, *Lactobacillus*, *Streptococcus*, and *Staphylococcus* (Delgado et al., 2013). The results of these studies based on the different populations were consistent with each other even though the large degree of inter-individual variability existed. The composition of gastric microbiota is dynamic. Researchers have identified several factors including diet, antibiotic medication, inflammation of gastric mucosa, pH and *H. pylori* colonization can affect it (Nardone and Compare, 2015).

Normal Flora of Small Intestine

The small intestine has three major regions: duodenum, jejunum and ileum. It is the main place where most nutrients are digested and absorbed. The small intestine is a relative harsh environment for microorganism due to the short transit time and the presence of digestive enzymes, bile, oxygen and pH limits, and there is a significant distinction exists in microbiota between the small

intestine and colon (Zoetendal et al., 2012; Donaldson et al., 2016). The knowledge of the microbial composition and metabolic pathways that are operating in the small intestine microbiota is limited due to poor physical accessibility. Some studies exploited a flexible silicone sampling probe, which was introduced by individual mouth until it reached the small intestine, being monitored by radioscopy (Zilberstein et al., 2007).

The small intestine exhibits lower bacterial diversity than the colon, and the microbiota composition varies between different locations in the intestine tract (Zoetendal et al., 2012, Donaldson et al., 2016). The five major phyla present in duodenal microbiome are Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Fusobacteria, and the dominant genera are *Streptococcus*, *Acinetobacter*, *Propionibacterium*, *Prevotella*, *Bacteroides*, *Neisseria*, *Haemophilus*, *Fusobacterium*, *Actinobacillus*, and *Porphyromonas* (Li et al., 2015, D'Argenio et al., 2016). The mainly enriched microorganisms in the jejunum are *Veillonella*, *Bacteroides*, *Clostridium*, *Proteus*, *Staphylococcus*, *Escherichia coli*, and *Klebsiella* (Zilberstein et al., 2007; Sullivan et al., 2003). Due to varied physiological environments, such as pH, phosphate buffer and bile salts, the floras in the upper ileum are similar to those of the jejunum. The upper ileum is enriched with *Veillonella*, *Bacteroides*, *Staphylococcus*, *Enterobacter*, *Corynebacterium*, *Escherichia coli*, and *Peptococcus*. Whereas in the distal ileum, the dominant microorganisms are *Veillonella, klebsiella, Clostridium*, *Corynebacterium*, *Propionibacterium*, *Escherichia coli*, and *Bacteroides* (Quintanilha et al., 2007; Zilberstein et al., 2007).

Normal Flora of Colon

Succession of Flora in Infants

The GI tract is sterile at birth and the colonization of GI microbiota undergoes dramatic changes in composition during the first year of life. Facultative aerobes (*Enterobacteriaceae*, *Enterococcus*, and *Streptococcus*) first colonize the neonatal gut at birth due to the presence of abundant oxygen in the gut, but once the oxygen is used up by these facultative aerobes, obligate anaerobic genera (*Bifidobacterium*, *Bacteroides* and *Clostridium*, etc.) take the place and predominate in the suckling period (Vael and Desager, 2009). The composition of gut microbes is less complex but more dissimilar among newborns (Backhed et al., 2015). During the weaning process, supplying solid foods causes a significant shift in the infant gut micro-ecology (Fanaro and Vigi, 2008), and finally a gut microbiota broadly similar to that of adults is established by the age of 1 year (Palmer et al., 2007).

Delivery mode, feeding type and mothers' gut microbiota affect the composition of the newborns' gut microbiota. Compared to babies delivered by C-section who receive the skin, mouth and environmental bacteria randomly as the seeds for their gut microbiota, the babies delivered vaginally get the first inoculum for their gut microbiota from the bacteria present in the birth canal of the mothers, so babies delivered naturally develop healthier gut microbiota with important microbes such as *Bacteroides* and *Bifidobacterium* (Backhed et al., 2015; Dominguez-Bello et al., 2010).

Breast milk is an ideal source for the babies' commensal microbiota and continuous promoter for beneficial gut bacteria since it contains both probiotics derived from the mothers' intestine and prebiotic oligosaccharides that promote the growth of beneficial probiotics (e.g., *Bifidobacterium*) (Martin et al., 2003, 2012; Coppa et al., 2006). The gut microbiota of breast-fed infants is dominated by *Bifidobacterium*, which is considered protective for infant health (Rivero-Urgell and Santamaria-Orleans, 2001). In contrast, the gut of formula-fed infants, who have a higher incidence of infections, is colonized by an adult-type gut microflora in which *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Lactobacillus*, Gram-positive cocci, coliforms, and other groups are all predominantly represented (Gad, 2007). The weaning process is the major driving force of the succession of gut microbiota in infants, resulting in the enrichment of *Roseburia, Clostridium, Anaerostipes* etc. However, such compositional and functional shift of gut microbiota does not happen even though solid food has been introduced to infants, until the breast-feeding is entirely stopped. The breast-feeding has been shown to be associated with the adult microbiota community type and may influence the life-long profiles of metabolic and immune systems (Ding and Schloss, 2014).

The gut microbiota established in first years of life plays a fundamental role in the host health not only in infancy but also in childhood and adulthood by affecting the digestion and nutrition, modulating the maturation of the immune system, resisting pathogens, influencing the energy storage and obesity, and even affecting the behavior of host through the metabolism of vitamins, iron, and amino acids that are essential for the development of neuro and brain (Vael and Desager, 2009; Fanaro and Vigi, 2008; Hsiao et al., 2013; Diaz Heijtz et al., 2011).

Cultural Studies of the Microflora of the Adult Colon

Cultivation of GI flora is time-consuming and laborious. Nevertheless, it is an indispensable procedure for providing detailed physiological and functional features of individual microorganisms. Rajilic-Stojanovic and de Vos systematically reviewed 1057 microbial species that can reside in the human gastrointestinal tract and have been identified, which consist of 92 *Eukarya*, 8 *Archaea*, and 957 *Bacteria* (Rajilic-Stojanovic and de Vos, 2014). More the remaining GI flora are expected to be cultured in the future.

Molecular Studies of the Microflora of the Colon

The rapid improvement of high-throughput sequencing technology greatly facilitates the molecular studies of colonic flora as a complex ecosystem. The composition of human gut microbiota encompasses over 1000 species (Hollister et al., 2014) and is

immensely variate between individuals and dynamic at different ages (Lozupone et al., 2012), though it is deemed to be essentially stable throughout adulthood (Nicholson et al., 2012). Host factors, including genetics, immune system, age, diet and drug administration, appear to affect the human gut microbiota in the normal state (Tuddenham and Sears, 2015). Dysbiosis of gut microbiota has been proved to be associated with many diseases, such as obesity, malnutrition, inflammatory bowel disease, neurological disorders and cancer (Dicksved et al., 2008; Fei and Zhao, 2013; Kau et al., 2011; Ley et al., 2006; Lupton, 2004).

The international initiatives of human microbiome research, like MetaHIT and HMP, have greatly accelerated the studies on gut microbiota. Financed by the European Commission under the 7th FP program, the main objective of MetaHIT is to study associations between the human intestinal microbiota and human health, in particular two diseases, inflammatory bowel disease and obesity (http://www.metahit.eu). MetaHIT had sequenced fecal samples of 124 Europeans using Illumina-based metagenomic sequencing platform and obtained 3.3 million non-redundant microbial genes from 576.7 gigabases of sequences. About 1000–1150 prevalent bacterial species in total were estimated and each individual harbors at least 160 species (Qin et al., 2010). Two phyla, Bacteroidetes and Firmicutes had the highest abundance.

Funded by the National Institutes of Health, HMP Consortium has established a framework to develop metagenomic protocols and to generate high-throughput metagenomic data. With 242 healthy adults, each sampled at 15 or 18 body sites up to three times, 5177 microbial taxonomic profiles from 16S ribosomal RNA genes and over 3.5 terabases of metagenomic sequence were obtained. About 800 reference strains isolated from the human body have been sequenced (Consortium, 2012b). Similar to the result from MetaHIT, the dominant phyla in the gut are Bacteroidetes and Firmicutes (Consortium, 2012a). In 2017, Lloyd-Price et al. reported an expanded dataset from 265 individuals of the HMP cohort, which contained 1631 new whole-metagenome sequencing samples at multiple time points (Lloyd-Price et al., 2017).

A deep 16S rRNA sequencing survey of fecal microbiota was conducted in China, which covered 314 healthy young adults from 7 ethnic groups living in 20 rural or urban regions of 9 provinces. The four most dominant bacterial phyla in this population were Firmicutes (73.47%), Bacteroidetes (14.13%), Proteobacteria (5.83%), and Actinobacteria (3.36%). In Archaea, Euryarchaeota (2.62%) was the only detected phylum (Zhang et al., 2015b). Inconsistent with previous study, *Phascolarctobacterium* was the most predominant genus, instead of *Bacteroides* (Arumugam et al., 2011) and *Faecalibacterium* (Nam et al., 2011). Among the 279 identified genera, nearly half of the total sequences were from nine predominant genera, *Phascolarctobacterium*, *Roseburia*, *Bacteroides*, *Blautia*, *Faecalibacterium*, *Clostridium*, *Subdoligranulum*, *Ruminococcus*, and *Coprococcus*. These genera co-existed in all samples and all of them are short-chain fatty acid producers. The cluster of 314 microbiota samples was mainly relevant to host's ethnicities/geography and less to lifestyles. The ratios of Firmicutes: Bacteroidetes among the individuals varied from 0.5 to 300.6.

Falony et al. investigated variation of fecal microbiome in two independent healthy populations: the Belgian Flemish Gut Flora Project with 1106 subjects and the Dutch LifeLines-DEEP study with 1135 subjects. They found medication had the largest effect size on the variability of fecal samples while influence from early-life events such as birth mode was minor (Falony et al., 2016). When the two datasets integrated with other U.K. and U.S. studies, 664 genera from 3948 subjects were identified. They further found a core microbiota containing 14 genera, including *Veillonellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Hyphomicrobiaceae*, *Erysipelotrichaceae*, *Clostridiales*, *Clostridiaceae*, *Roseburia*, *Faecalibacterium*, *Dorea*, *Coprococcus*, *Clostridium_XlVa*, *Blautia*, and *Bacteroides*. Through simulation, they estimated that the genera richness of total western gut microbiota was 784 and they need additional 40,739 individuals obtain the rest genera.

Studies of Individual or Special Groups

A recent clinical dietary intervention on 17 Prader-Willi syndrome and 21 simple obesity children showed that a diet rich in non-digestible carbohydrates could significantly reduce body weight, decrease serum antigen load and alleviated inflammation (Zhang et al., 2015a). Shifts of gut microbiota were observed after the intervention. Through 161 assembled bacterial draft genomes, increased functional genome groups were detected. These groups can produce acetate from carbohydrates fermentation. Meanwhile, trimethylamine N-oxide and indoxyl sulfate, detrimental host-bacteria co-metabolites, were significantly decreased in the urine after the intervention. These co-metabolites were correlated with some bacterial genomes, which encode enzyme genes for production of the co-metabolite precursors by fermentation of choline or tryptophan in the gut. Both pre- and post-intervention gut microbiota from a volunteer were transplanted into two groups of germ-free mice, respectively. Higher inflammation level and larger adipocytes were occurred in the pre-intervention group. Furthermore, Wu et al. found that the dietary intervention also significantly reduced the rinses and diversity of the gut resistome of the obese children, and especially reduced bacteria with multi-resistance potential (Wu et al., 2016). The concept "resistome" was introduced by D'Costa et al. in 2006, which refers to the collections of the enteric antibiotic resistance genes (ARGs). The human gut microbiota is a reservoir for ARGs. ARGs can appear in the gut microbiota in the early age of human beings. Even in the fecal samples of healthy infants and children, diverse and novel ARGs have been identified. As antibiotic resistance has been one of the greatest global threats to human health, there is considerable need to characterize the resistome of the human gut microbiota and to understand its diversity and richness, and the spreading mechanisms between different members, especially between commensals and opportunistic pathogens. A total of 1093 ARGs of gut microbiota were found from 162 individuals from China, Denmark and Spain. The Chinese individuals harbored the highest number of ARGs while the resistomes of the two European populations were more closely related (Hu et al., 2013). Forslund et al. found ARGs for 68 classes and subclasses of antibiotic in 252 fecal samples, at an average of 21 ARGs per sample (Forslund et al., 2013). They further derived and compared the resistomes of human gut microbes from 832 individuals located in 10 different countries. Significant differences were detected between samples and were associated with antibiotic usage and exposure in medical and food production

context (Forslund et al., 2014). Feng et al. compared the resistomes in fecal samples from 180 healthy individuals from 11 different countries and found 7 common multidrug ABC transporters in all individuals. In addition, via occurrence networks between ARGs and microbial taxa, they identified potential hosts for 58 ARG subtypes (Feng et al., 2018).

Addendum

GI microbiota affect human healthy and are also being influenced by various factors from host and environment, forming a complicated ecosystem. Many gastrointestinal and metabolic diseases have been associated with the dysbiosis of the GI microbiota. A large number of epidemiologic investigation and clinical intervention have provided helpful information in understanding the features of functional microbiota, which could be served as drug/diet targeted candidates in treating diseases associated with disrupted microbiota. For instance, Zhao et al. recently found that a diet rich in diverse fibers could selectively increase a group of short chain fatty acid producing gut bacteria and this promotion was associated with the alleviation of type 2 diabetes (Zhao et al., 2018). Such work provided promising solution to manage metabolic disease through gut microbiome targeted nutrition intervention. The application of emerging multi-omics technologies combined with statistical and bioinformatic tools facilitates the identification and elucidation of potential key microorganisms to human health. Advances in genomic techniques assisted with bioinformatics allow dissection of those microorganisms at strain-level. Though demonstration of causality is still not easy, functional strain candidates can be validated through gnotobiotic models.

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Gene Transfer Agents

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Introduction

Gene transfer agents (GTAs) are small bacteriophage-like particles produced by some bacteria and archaea (see Fig. 1). Each GTA particle contains a short fragment of cellular genomic DNA that it can inject into a closely related recipient cell. The transferred DNA can then recombine with the recipient's genome, possibly changing its genotype in a process similar to transduction. GTAs are typically encoded by bacteriophage-derived genes in the producing cell's chromosome, with the genes encoding the particle head and tail usually in a single operon or cluster; genes for other functions (regulation, cell lysis, etc.) may be dispersed at other locations. In the well-studied GTA systems the expression of GTA genes has been found to be under control of cellular regulatory systems. GTA genes have complex evolutionary histories and the evolutionary forces responsible for their maintenance are not yet well understood.

GTA particles resemble small DNA phages, but the relationship between GTAs and phages is complex. Phages and prophages are even more ubiquitous than bacteria, and their genes frequently recombine with each other. Since phage and bacteria have been coevolving for billions of years, and because of the coexistence within cells of the phage and host genomes, their intertwined histories can make it difficult to distinguish between phage and host genes.

Many phages are temperate and integrate their DNA into a host chromosome, where it may remain repressed as a prophage for many generations. Most bacterial genomes contain multiple prophage sequences; some of these appear intact and functional, but others are obviously defective, having undergone various forms of mutational and deletional degeneration. Several of the known GTAs are produced by sets of phage structural genes that have come under the control of cellular regulation machinery.

Phage evolve as infectious agents; introduction of phage DNA into a host cell leads to expression of phage genes and release of particles containing phage DNA, which can go on to infect new cells. In contrast, GTA particles are not infectious because they are typically too small to contain the gene clusters encoding them. However, both phage and GTAs can mediate gene transfer (Fig. 1(A)). Phage particles normally contain only phage DNA, but occasionally a particle will package a fragment of host DNA instead, and when the particle injects this DNA into another cell the DNA can recombine with the chromosome and change its genotype. This process is called transduction. Although transduction by phage appears to be an unselected consequence of accidental packaging errors, transduction-like gene transfer appears to be the primary function of GTAs because they consistently package chromosomal DNA, with no preference for GTA genes (Fig. 1(B)).

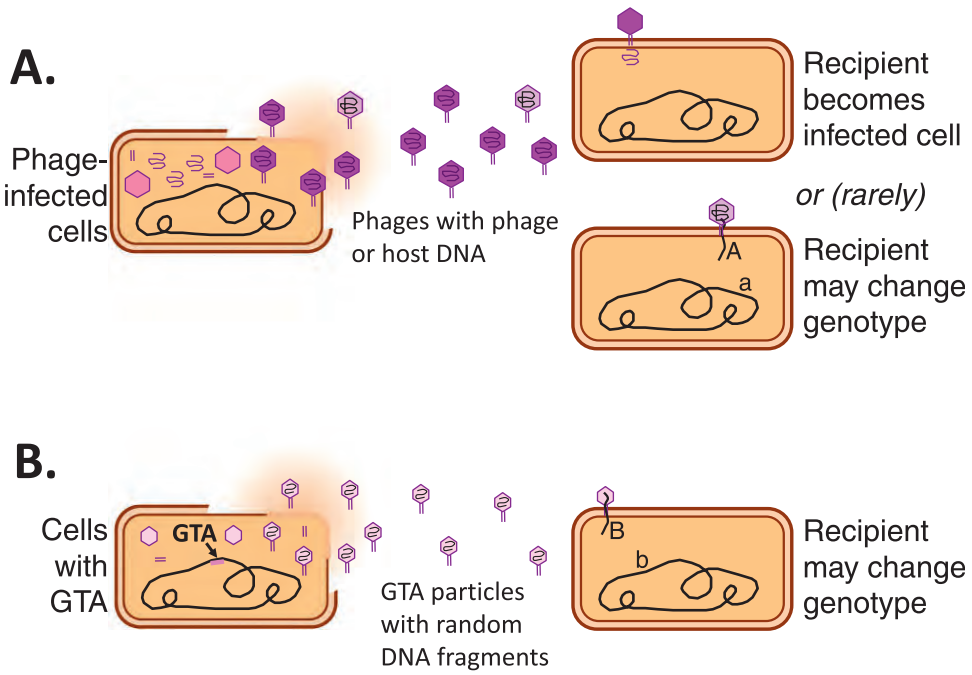

Fig. 1 Similarities and differences between typical DNA phage infection and induction of GTA. (A) Phage infection and transduction. (B) GTA production and gene transfer.

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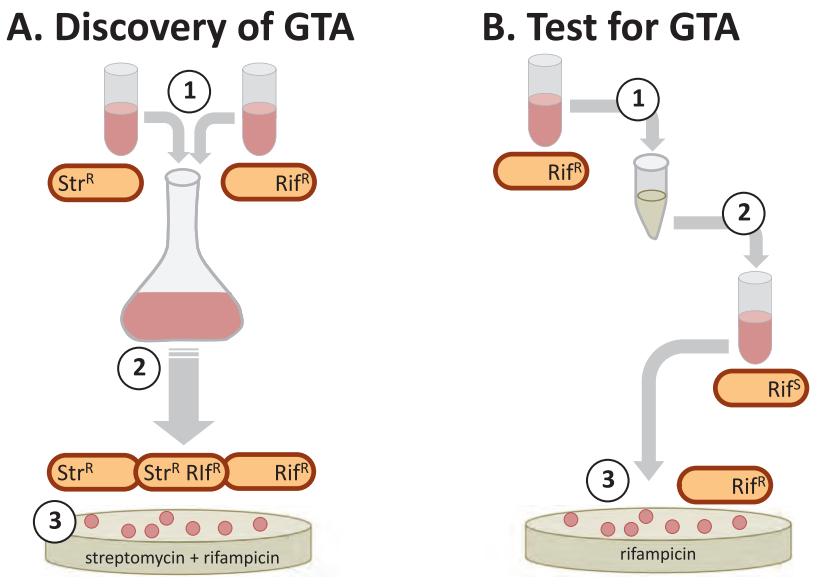

Fig. 2 Discovery and detection of GTAs. (A) Marrs' first experiment. Step 1, cultures with different antibiotic-resistance marker genes were mixed. Step 2, the mixed culture was grown for several generations. Step 3, recombinants were detected by plating on agar containing both antibiotics. (B) General assay for GTA-mediated gene transfer. Step 1, a culture of genetically marked donor cells (e.g., Rif^R) is filtered to remove the cells and treated with DNase to remove free DNA. Step 2, the filtrate is mixed with unmarked recipient cells. Step 3, the recipient cells are plated on selective agar to detect recombinants.

Table 1 Properties of different GTAs

GTA, producing organism(s)	*Size of DNA (kb)*	*Morphology type*	*Production in related organisms*
RcGTA, *Rhodobacter capsulatus*	4	Siphovirus	Yes
Dd1, *Desulfovibrio desulfuricans*	13.6	Podovirus	Not known
VSH-1, *Brachyspira hyodysenteriae*	7.5	Siphovirus	Unlikely
VTA, *Methanococcus voltae*	4.4	Siphovirus	Not known
BaGTA, *Bartonella henselae*	14	Non-tailed or podovirus	Yes

Discovery of GTAs

The first GTA was discovered in 1974 by Barry Marrs, using a coculture experiment designed to detect *Rhodobacter capsulatus* (then *Rhodopseudomonas capsulata*) recombinants arising by conjugation, transduction or transformation (see Fig. 2). Mixtures of some strains yielded many recombinant colonies, and the responsible process could not have been conjugation, because the transfer property remained in the culture medium after the cells were removed by filtration. Natural transformation by free DNA in the filtrate was also ruled out, because adding DNase to the filtrate did not reduce its activity. However, the process also differed from transduction, since inactivation assays suggested that the particles were small, and there were no signs of the cell lysis expected of a phage-mediated process. The entity responsible for genetic recombination was named the "Gene Transfer Agent".

This discovery was followed by electron microscopy analysis showing that the *R. capsulatus* GTA particles physically resemble unusually small tailed phages, and extraction of DNA from these particles, showing that they contain apparently random 4–5 kb segments of chromosomal DNA. Approximately 20 years then elapsed before the responsible genes were identified in the *R. capsulatus* chromosome, regulation by host proteins was characterized, and subsequently GTA-producing cells were shown to release their GTA particles by lysis.

Meanwhile, functionally similar GTAs were being discovered in other systems (Table 1), and to prevent confusion the *R. capsulatus* GTA was renamed RcGTA. As described in more detail below, RcGTA relatives are widespread in a large clade of the alpha-proteobacteria. A small subclade of these bacteria, the *Bartonella* species, contain an independently derived GTA (BaGTA). GTAs are now also known in the delta-proteobacterium *Desulfovibrio* (Dd1), in the spirochaete *Brachyspira* (VSH-1) and in the euryarchaeote *Methanococcus* (VTA).

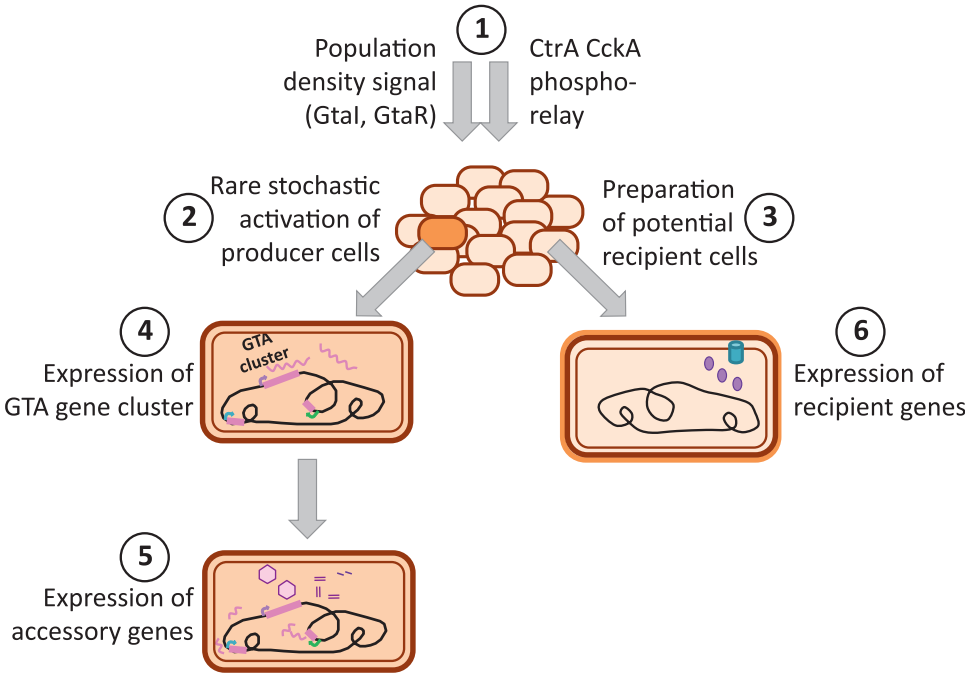

Fig. 3 Regulation of RcGTA production and recipient capability. Step 1, quorum-sensing and phosphorelay signals induce expression of GTA genes. Step 2, an uncharacterized stochastic process limits expression of the GTA cluster and lysis genes to a small fraction of the population. Steps 3 and 4 (only in the GTA-producer subpopulation), the GTA cluster is expressed and other genes needed for GTA production are expressed (lysis, head-spike and attachment functions) Step 5 (in the non-producer subpopulation), genes for capsule production and for DNA processing are expressed.

GTA Genes and Regulation

The GTA major head and tail proteins are typically encoded by a cluster of co-transcribed genes. Other proteins needed for GTA production (head spikes, tail fibers, lysis proteins) and DNA uptake (receptors and DNA-transport proteins) may be encoded elsewhere in the genome. Several GTA systems that have been investigated are regulated by cellular factors. Fig. 3 shows the regulatory processes for RcGTA, the best-studied system, which control both RcGTA production and the ability to be a recipient of genes carried by an RcGTA particle. In the best-studied systems, *R. capsulatus* and *Bartonella henselae*, regulation of GTA gene expression is under direct control of specific cellular regulators, with an uncharacterized stochastic process determining which cells will participate in production of particles.

In *R. capsulatus*, the activity of a cellular phosphorelay pathway culminating in the response regulator CtrA appears to differentially activate RcGTA structural genes early after the start of induction and, later in the inductive process, other genes needed for maturation and lytic release. This is analogous to early and late gene expression in the induction of prophages such as λ, but involves cellular genes instead of phage or phage-homologous genes. The production and release of RcGTA particles is activated in only a small subset of the population (<3%). Furthermore, the same regulatory processes (quorum sensing, the CtrA phosphorelay) act in the non-producing fraction of the population, enhancing the ability to bind RcGTA particles and import RcGTA-borne DNA. This recipient capability depends on the production of an extracellular polysaccharide that binds RcGTA particles, on proteins needed to translocate inserted DNA from the periplasm to the cytoplasm, and on the cytoplasmic DprA protein that, with RecA, promotes integration of DNA into the genome; all of these are induced by CtrA and quorum sensing. The related DsGTA, produced by *Dinoroseobacter shibae*, is also regulated by quorum sensing and the CtrA phosphorelay.

The above regulatory processes lead to maximal RcGTA production in post-growth cultures, but the *Bartonella* GTA BaGTA is maximally induced during exponential growth, with the ppGpp-dependent stringent response preventing expression at other times. In this system, the BaGTA genes are induced in approximately 6% of the population. The uptake of DNA from BaGTA particles is dependent upon cell division proteins, meaning that only actively dividing cells are able to act as recipients.

The *Brachyspira* GTA VSH-1 differs from other GTAs, and resembles many prophages, in being induced by DNA-damaging chemicals such as mitomycin C and peroxide. This could reflect control by a phage-derived damage-responsive repressor like the phage λ cI protein or regulation by a cellular SOS system. VSH-1 also differs from other GTAs in being induced throughout the cell population rather than in only a small subset of cells.

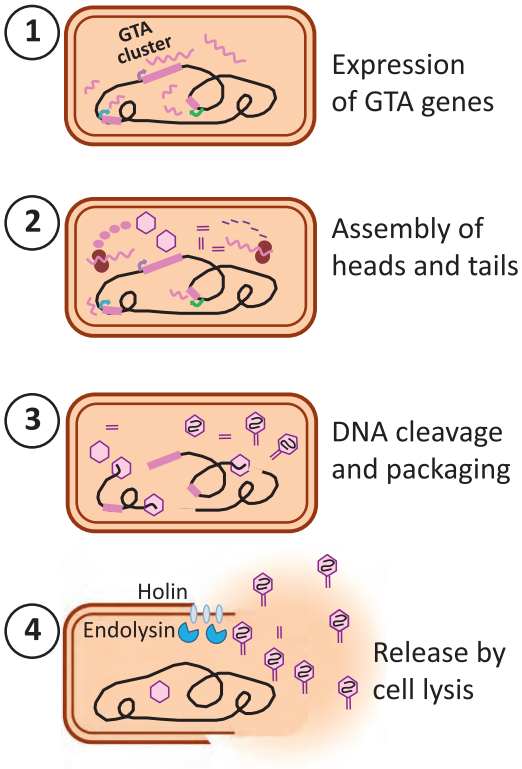

Fig. 4 Production of GTAs. (1) The genes encoding GTA structural components and necessary functions are transcribed. (2) Head and tail proteins are assembled into empty heads and detached tails. (3) Heads are filled with chromosomal DNA and tails are attached. (4) Holin and endolysin proteins cause cell lysis, releasing the particles.

GTA Production

Induction of GTA genes leads to production of GTA proteins but not to specific replication of the GTA-encoding DNA (see Fig. 4). The strong degree of sequence identity between phage and GTA proteins suggests that, as in phages, GTA head particles are initially assembled as empty shells that are then filled with uniform-length DNA segments by a terminase enzyme complex before the tail is attached to the head. Fragment lengths differ between systems from approximately 4 to 14 kb (Table 1) and this is thought to reflect 'headful' packaging rather than use of a specific packaging-recognition sequence like the λ *cos* sequence. Phage-homologous lysis proteins (holin and endolysin) then lyse the cell to release the GTA particles.

Lack of Specificity of DNA Packaging

Unlike phage, GTA particles do not preferentially package the DNA that encodes them. In RcGTA and BaGTA the DNA fragments are randomly derived from all of the DNA available in the cell (GTA-encoding genes are not preferentially packaged), although local DNA over-replication may enrich this DNA for certain regions. For RcGTA, analysis of DNA extracted from GTA particles shows even representation (±25%) across the *R. capsulatus* genome. A sharp dip in coverage at the site of the GTA gene cluster suggests that DNA packaging may be inhibited by the intense transcription of these genes in GTA-producing cells. In BaGTA particles, one region of the *Bartonella* genome is overrepresented about tenfold; this is not due to biased packaging but to over-replication, thought to be centered at an active phage-derived origin of DNA replication not associated with the BaGTA gene cluster.

A very different picture is seen for chromosomal DNA coverage in the GTA produced by *D. shibae*, with very distinct peaks and valleys not explained by differences in cellular DNA abundance. Different replicons within the *D. shibae* cells are also packaged at different frequencies, with the chromosome and chromosome-like plasmids packaged significantly more than the small plasmids. No clear correlation with GC-content or specific packaging motifs is seen, suggesting that differences in the DNA's physical properties may affect the initiation of packaging. The mechanism(s) underlying these biases is(are) currently unknown. As with RcGTA, the GTA genes are underrepresented in particles, again perhaps due to transcriptional interference with packaging.

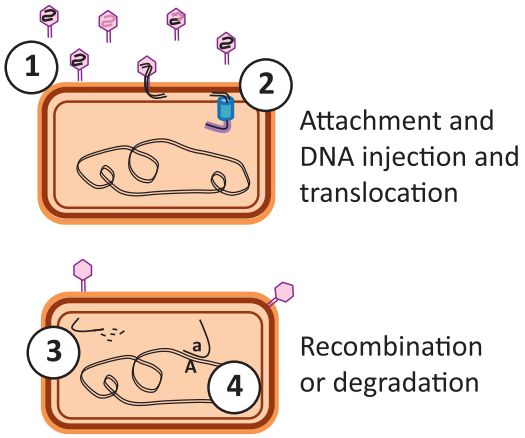

Fig. 5 GTA-mediated gene transfer. Step 1, GTAs attach to cell-surface polysaccharide capsule (outer layer shown in orange) and inject their DNA across the outer membrane. Step 2, competence-associated proteins transport one strand across the inner membrane. Step 3, non-homologous DNA is degraded. Step 4, homologous DNA replaces one chromosomal DNA strand by RecA-mediated recombination.

GTA DNA Injection and Recombination

Whether the GTA particles released by a cell go on to transduce other cells depends on their ability to survive in the environment and on the presence of suitably prepared recipients. Nothing is known about environmental effects on GTA survival, although the discoverers of RcGTA did report that the gene transfer properties of their GTA preparations were much less stable than similar phage lysates. The regulation of RcGTA production by quorum sensing presumably serves to ensure the availability of a dense population of closely related recipients.

As mentioned above, RcGTA attachment depends on the presence of a cell surface capsular polysaccharide (see Fig. 5). The next step, DNA injection, differs from that of phages because the RcGTA-packaged DNA is not injected directly into the cytoplasm. Instead the DNA is inserted into the periplasmic space between the inner and outer membranes. Movement of the DNA from the periplasm to the cytoplasm requires cellular homologs of the DNA-translocation proteins ComEC, ComF and ComM, previously known only for their roles in natural transformation by competent cells. In competent cells, and presumably in RcGTA recipients, these proteins transfer only a single strand of the initially double-stranded DNA across the inner membrane and coat it with the recombination-promoting DprA protein. Once in the cytoplasm recombination is thought to occur by the same RecA-dependent steps as in natural transformation, because the proteins DprA and RecA are essential.

Evolutionary History of GTAs

Prophage-derived gene clusters that might be GTAs are widespread in bacteria and archaea, often annotated as defective prophages. At present there is no good way to decide which of these might be genuinely functional GTAs and these organisms would need to be tested for high-frequency gene transfer, as illustrated in Fig. 1, or for production of small phage-like particles containing random segments of chromosomal DNA. In principle, sequence-based criteria could be developed for GTA genes, but these would need to be validated by experimental demonstration of GTA production.

Although the different GTAs are encoded by phage-derived genes with similar functions, they appear to have originated independently. The three genetically defined GTAs, RcGTA, VSH-1 and BaGTA, are not directly evolutionarily related. Furthermore, there are no obvious homologs of these GTAs' genes in the genomes of *Desulfovibrio desulfuricans* or *Methanococcus voltae*, indicating the GTAs produced by those organisms also have independent evolutionary origins.

The first step in the transition from prophage to GTA could be a simple deletion or rearrangement that replaces the left half of the prophage with a chromosomal promoter (Fig. 6). This would need to be followed by mutational changes to the terminase to facilitate packaging of all chromosomal regions and to the capsid to produce smaller heads relative to genuine phages. Genes for cell lysis appear to have undergone subsequent rearrangement and replacement events in *R. capsulatus*.

In addition to having different evolutionary origins, the different GTAs appear to have very different evolutionary histories within their organisms' chromosomes. Homologs of genes encoding VSH-1, the *B. hyodysenteriae* GTA, are present in the genomes of other species within the genus *Brachyspira*, but they are not syntenic, and homologs of the genes are not found outside of this genus. The BaGTA genes are widely conserved and usually syntenic within the genus *Bartonella*, with some BaGTA genes occasionally found outside the genus. Both of these GTA systems appear to be of relatively recent origin (Fig. 7). Conservation is much deeper for RcGTA. Complete sets of RcGTA gene homologs are well conserved across the order Rhodobacterales, and occasionally found in other alpha-proteobacterial orders. Additionally, partial sets of the genes are detected in the genomes of many other

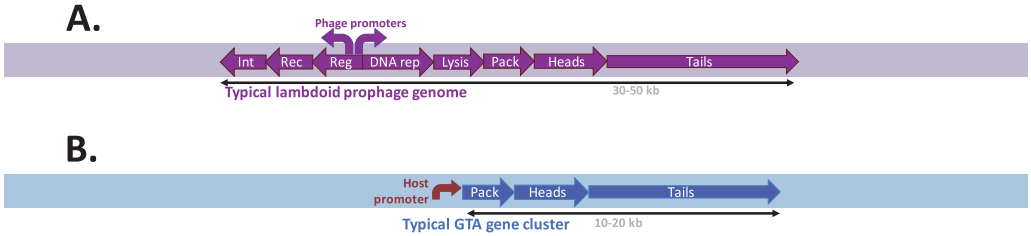

Fig. 6 Gene organization in typical DNA phage and GTA gene clusters. (A) Prophage genome integrated in bacterial chromosome. (B) GTA genes in bacterial chromosome. Gene function abbreviations: Int, prophage integration; Rec, recombination; Reg, regulation; DNA rep, Phage DNA replication; Lysis, lysis of host cell; Pack, packaging of DNA into capsid head; Heads, capsid head proteins; Tails, capsid tail proteins.

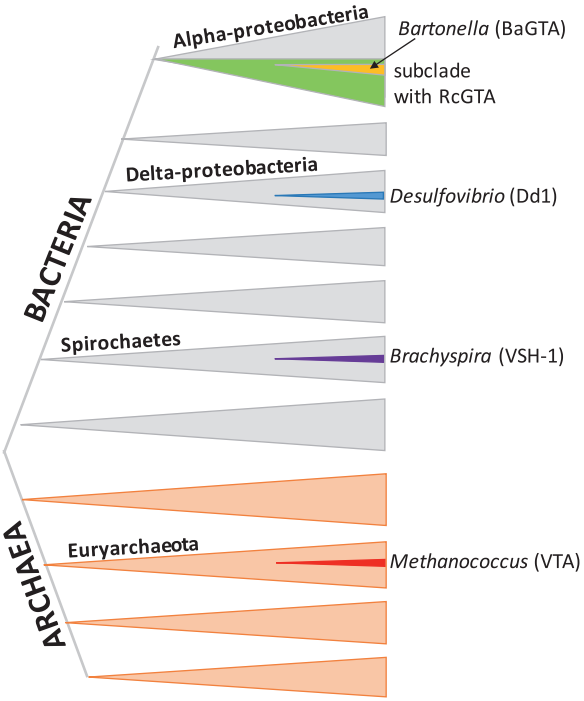

Fig. 7 Schematic GTA phylogeny. Gray wedges represent major bacterial and archaeal clades; many clades lacking GTAs are not shown. Colored wedges represent clades known to have GTAs (GTA names in parentheses).

alpha-proteobacteria, including members of the deep-branching order Rickettsiales. This suggests that the origin of RcGTA goes back to the origins of the class alpha-proteobacteria (at least 700 million years).

Although genuine gene transfer by BaGTA has been demonstrated for only one species, *B. henselae*, production of particles with the same properties in terms of DNA content (i.e., 14-kb random fragments) has been observed for additional *Bartonella* spp. The ability of these particles to perform gene transfer warrants further study to determine the level of conservation of GTA functionality within this group, which would provide further support for the idea that GTAs are maintained by cellular organisms due to their provision of benefit(s). For RcGTA, representatives of multiple genera have been documented to make similar particles, and some of these have been shown to mediate gene transfer. Therefore, production of functional GTA particles has been maintained by these organisms over long evolutionary time-frames.

Evolutionary Function(s) of GTAs

For GTAs, it has been assumed that the benefits of transferring random chromosomal genes overwhelms the costs of GTA production. However, this may not be consistent with the current understanding of how evolutionary forces act on gene transfer processes (Fig. 8). The perils of this notion are well illustrated by the mutually contradictory explanations given for regulation of RcGTA and BaGTA production. For RcGTA, production only when the culture growth stalls is said to help stressed cells acquire new genes, but BaGTA production by log-phase cells is said to help fit cells share their genes.

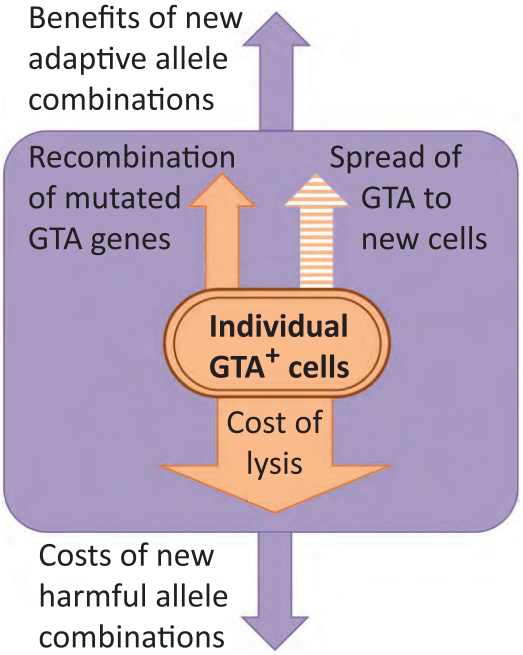

Fig. 8 Schematic representation of evolutionary forces acting on GTA genes and GTA$^+$ cells and populations. Upward-pointing arrows, forces causing an increase; downward-pointing arrows, forces causing a decrease; orange arrows, forces acting on individual GTA$^+$ cells; purple arrows, forces acting on GTA$^+$ populations.

The only known consequences of GTA production are lysis of the producer cell and transfer of fragments of its DNA to recipient cells. Because GTA production is lethal there is no direct benefit to the producing cell. Although regulation of GTA expression is thought to limit this lysis to a very small fraction of the population, it nevertheless represents a significant evolutionary cost of GTA production that must be opposed by evolutionary benefits if the GTA genes are to be maintained. Indirect evidence that such benefits must exist comes from analysis of mutation distributions in both BaGTA and RcGTA genes, which shows strong evidence of purifying selection; that is, natural selection is removing the non-synonymous mutations, which change amino acid sequences and are likely to interfere with the genes' functions. The maintenance of functional GTA systems across long evolutionary time-scales provides additional evidence that these systems must provide benefits of some sort.

The simplest explanation for persistence of GTA systems would be that they are "selfish" entities that exist only to spread the GTA genes to other cells. However, the lack of GTA-specific DNA replication and packaging, and the inability for GTAs to package all of their encoding genes, make this very unlikely. It remains possible that the GTA genes may contribute some other benefit to the producing organism that is not related to GTA production and gene transfer, but such benefits have not been identified.

GTAs as Part of the Virosphere

It is now recognized that viruses are the most abundant biological entities on the planet. In the oceans, for example, viruses typically outnumber cellular organisms by at least tenfold. This realization came from direct counting of virus particles through techniques such as fluorescence microscopy using DNA-binding stains that allow virions to be detected. However, these techniques have limitations and only particles with enough DNA content can be visualized and counted and these techniques would be unlikely to enumerate GTAs due to their packaging of smaller fragments of DNA. Now that we know that multiple organisms make GTAs, it seems likely that some fraction of the "viruses" in nature are in fact GTAs. This might be especially true for some portions of the oceans because of the high level of GTA gene conservation in members of the order Rhodobacterales, which are abundant in the ocean and with multiple representatives demonstrated to produce functional GTAs.

An observation frequently made during viral metagenomic studies is that a proportion of the genes contained within the viruses is always of bacterial origin. There are several possible, non-mutually exclusive, explanations for this. These include contamination of the viral DNA preparation by cellular DNA, the presence of transducing phages in the viral population, the presence of DNA-containing extracellular vesicles in the purified viruses, and the presence of GTAs in the viruses.

Future Outlook

For even the most well-studied GTAs there are many aspects of their biology that remain to be deciphered. For example, although some details are known about regulation of GTA production for some organisms, these are far from complete. Identifying any potential driving force(s) behind GTA evolution and maintenance is an important area for future work to help address the question of possible benefits to producing organisms. Identification of new GTAs in additional organisms will also be important.

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Genetically Modified Organisms: Guidelines and Regulations for Research☆

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Abbreviations

APHIS	Animal and Plant Health Inspection Service, United States
BIO	Biotechnology Innovation Organization, United States
CDC	Centers for Disease Control, United States
EPA	Environmental Protection Agency, United States
EU	European Union
FDA	Food and Drug Administration, United States
FFDCA	Federal Food, Drug, and Cosmetic Act, United States
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act, United States
GMO	Genetically Modified Organism
IBC	Institutional Biosafety Committee
NIH	National Institutes of Health, United States
OBA	Office of Biotechnology Activities, United States
OECD	Organization for Economic Cooperation and Development
RAC	Recombinant DNA Advisory Committee, United States
rDNA	Recombinant DNA
TSCA	Toxic Substances Control Act, United States
USDA	US Department of Agriculture

Defining Statement

Guidelines and regulations for research with genetically modified organisms (GMOs) have become standardized globally in principle but not yet in practice. Current regulated products from over 40 years of research are greater than 100 microbially derived products used in a variety of medical, industrial, and food applications. Only 12 microorganisms, of which nine are living and three are heat-killed, have been introduced into the environment.

Introduction

Oversight of GMOs continues to differ. Containment of microorganisms is dependent upon the location of the research with the organism, whether inside (contained) or outside (so-called field research), the type of organism (e.g., plant, animal, microorganism) or use (e.g., medical, agricultural, environmental), and the country in which one works. The oversight mechanism for contained research is principally through guidelines developed by scientists and endorsed by the private and public sectors. Outside research is currently overseen by a number of federal agencies. In some countries, such as the United States, there can be overlapping jurisdictions, differing interpretations of legal statutes, and different requirements or standards for compliance by scientists who do research with GMOs in the outside environment. Scientific issues deal with differences in perspective on the risks of introduction of GMOs into the environment, the types of data required before the introduction to conclude that the experiment is of low risk, and the types of monitoring and mitigation practices, if necessary. Nonscientific issues are also considered and include those dealing with legal and social concerns. These differences in interpretation along with considerations of efficacy and commercialization have resulted in few introductions into the environment of microorganisms. The potential is enormous, but progress is impeded by rules and regulations.

☆*Change History:* November 2018. S Tolin and A Vidaver have updated abbreviations, glossary and introduced small edits in the text of the article and also added further reading and relevant websites. Comments from C. Wozniak of EPA were incorporated.

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Concern Over GMOs

The new biology, dating to the 1970s and usually said to begin with the discovery of recombinant DNA (r-DNA) techniques, enabled scientists to perform modifications of organisms with great precision and to combine DNA of organisms that cannot, in evolutionary time, exchange DNA; yet these recombinants are derived from the DNA of naturally occurring organisms. The scientific community debated hypothetical scenarios about the safety of genetically engineered organisms, and the public questioned the potential adverse effects of organisms with the new combinations of genetic information on humans and the environment. It was argued that such organisms have not been subjected to evolutionary pressures, including survival, dissemination, and selection, and may pose unpredictable risks to humans or the environment. However, it was recognized that genetic modifications can arise by classical or molecular methods, ranging from selection of desirable combinations by farmers or bakers since antiquity to nucleotide insertion or deletion by molecular biologists or more recently by de novo synthesis of self-replicating agents from known viral sequences. Beneficial, neutral, and deleterious changes can occur, and will, whether or not microorganisms are manipulated.

Research with GMOs also generated concern that the new organisms may be unpredictable not only in survival and dissemination but also in the possibility that the gene for the modified trait may transfer to nontarget organisms where it might have a different fate and impact. However, gene transfer occurs whether or not humans intervene, and is quite common in certain bacteria harboring promiscuous plasmids. Such gene transfers are thought to have minimal consequences unless selection is imposed. Increasing evidence supports the conclusion that microorganisms, particularly bacteria, usually maintain their fundamental characteristics and their essential identities, and moderate the amount of change that can be absorbed by known and unknown mechanisms. Principles for assessing risk of GMOs, as developed in scientific and public forums that led to guidelines and regulations, include the premise that if one begins with an organism that normally exchanges genetic information with another organism, exchange by r-DNA techniques should pose no risks that do not occur naturally. A second principle is that if insertion of DNA into a benign or beneficial organism imparts a neutral or beneficial trait, the probability of harm from transfer of genetic information is minimal.

History of US Guidelines and Regulations Applicable to GMOs

Response to Concerns that Enabled Research

The concern over the potential risks of GMOs led to a call for the initiation of various mechanisms for the oversight of research conducted throughout the world. Although new laws were proposed at the time, the oversight was first in the form of guidelines, a set of principles and practices for scientists to follow, and physical facilities to minimize escape of organisms. Regulation by laws applicable to certain genetic modification processes or organisms used for certain purposes is explained later. The legal profession claims that this is the first case in which hypothetical or speculative risks have become the basis for regulation.

Codified guidelines date back to the 1970s, when the concerns described above were raised. The decision was made by the US government to give the lead to the National Institutes of Health (NIH) of the Department of Health and Human Services. After several meetings including the Asilomar Conference in 1975, NIH developed guidelines to assure minimization of risk through containment of research involving r-DNA molecules, and to allow research to proceed. The first guidelines were published in 1976, and established a public meeting body of peers and nonscientists. This group was assembled as the NIH Recombinant DNA Advisory Committee (RAC) by the Office of Recombinant DNA Activities (ORDA), and was given the task of reviewing all r-DNA experiments within the United States and to revise the guidelines as needed to incorporate their recommendations. The RAC has met periodically for over 40 years, and has revised, updated, and republished the guidelines numerous times, most recently in April 2016. With this revision, the scope of the guidelines was broadened to include research on synthetic nucleic acid molecules. All previous versions of the Guidelines since 1995, and minutes of RAC meetings, are publically available from the NIH website (https://osp.od.nih.gov/biotechnology/nih-guidelines/). The name of the office within NIH that is responsible for the RAC and the guidelines is now the Office of Biosafety and Recombinant DNA Policy in the Division of Biosafety, Biosecurity, and Emerging Biotechnology.

The RAC was to assess the risk of each proposed experiment and recommend containment conditions under which it thought the risk would be minimal for laboratory workers and the environment. Each time the RAC made a recommendation on an organism and containment level, the decision was added to the guidelines after it had been acted on by the director of NIH. The resulting guidelines spelled out the recommended facilities and procedures for safety to individuals and to the environment. They included such specifics as the type of pipetting procedures one should use, what clothing to wear, and proper sterilization, air filtration, and decontamination and mitigation procedures.

Within a short time, most of the microorganisms and experiments had been assigned a containment level, and the responsibility for overseeing such experiments was decentralized and delegated to local institutional biosafety committees (IBCs) and to other institutions in other countries.

Many experiments to modify common laboratory strains of bacteria and yeast were judged to pose no risk and were exempted from the guidelines or any containment requirements. Research with other microorganisms, including viruses, required containment no greater than one would use for research with the microorganisms that did not involve genetic modification experiments. These assignments were generally consistent with the classification of human-associated microbes into risk groups by biomedical authorities, such as the Centers for Disease Control (CDC) in the United States. Other countries soon followed suit, many adopting the US guidelines.

Approval Mechanisms for Previously Prohibited Experiments

The initial guidelines prohibited culture of GMOs in volumes of greater than 10 L, the rationale being that accidental spillage of smaller volumes was of lower risk. This restriction was challenged by the pharmaceutical manufacturer, Eli Lilly, whose scientists had discovered a method for producing human insulin in the common bacterium *Escherichia coli*. The argument made was that large-scale fermentation was used safely, without organisms escaping into the environment, for a number of human pharmaceutical products. The RAC recommended lifting this prohibition and added an appendix that provided guidance to large-scale fermentation activities.

The initial guidelines also prohibited deliberate release of r-DNA-modified organisms into the environment, whether for research purposes or other activities. Scientists conducting research with plants and with microorganisms associated with plants argued that field experiments were often conducted and were the equivalent of clinical trials with humans and animals to select for and demonstrate efficacy and responses to different environmental conditions. A working group of the RAC recommended elimination of the prohibition, and changed the activity to "planned introduction into the environment" that required RAC review and NIH approval. Requests for conducting research with plants and plant-associated bacteria were considered by the RAC and approved, but never conducted. After legal review, it was deemed that NIH could not approve such experiments as there was no program within the agency encompassing such research. The US Department of Agriculture (USDA) research agencies established a biotechnology research advisory committee whose members had greater expertise to review agricultural and environmental experiments, but authority was never given to this committee or to the USDA to issue approvals.

Only experiments with human gene therapy and the occasional new organism or special request for change in the recommended containment level now require review by the RAC and approval of the NIH. Most recently, the NIH will no longer accept new human gene transfer protocols for the protocol registration process under the *NIH Guidelines*, or convene the RAC to review individual human gene transfer protocols (<https://osp.od.nih.gov/biotechnology/nih-guidelines/>—August 2018).

Guidelines to Regulations

Federal government activities, spurred by the perceived benefits of GMOs in agricultural and environmental applications, reviewed all applicable laws and regulations governing potential products of biotechnology. A document was issued in the mid-1980s on the regulation of products, and thus the research leading to their development, to the agencies that regulated similar products that were not genetically modified by rDNA techniques. Thus approval for GMOs for introduction into the environment in the United States was transferred to regulatory agencies. The regulatory arm of the USDA used current laws governing veterinary medicine products and plants within the Animal and Plant Health Inspection Service (APHIS). The US Environmental Protection Agency (EPA) used the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) to regulate pesticides and the Toxic Substances Control Act (TSCA) to regulate toxic substances, including so-called 'new' organisms derived through transfer of intergeneric or synthetic DNA. GMOs considered as food or food-associated products became subject to the Federal Food, Drug, and Cosmetic Act (FFDCA) of the Food and Drug Administration (FDA). These changes to regulation were encouraged by industries as they provided a route by which products could be approved for commercialization. However, obtaining experimental use permits for field trials became an onerous activity for both academic and private researchers. The impact of regulations on GMO applications has been extensively debated in the last 40 or more years.

Thus the evolution of guidelines and regulations applicable to GMOs has led to a matrix of responsibilities for oversight depending on the stage of development, the intended application, and the location of the research as summarized in Table 1.

Compliance and Approvals for Contained Research Under the NIH Guidelines

Classification of Experiments

Since 1978, compliance with NIH guidelines has been mandatory for all research utilizing rDNA techniques at an institution receiving federal research funding. The guidelines are promulgated only for NIH-funded research, but all other federal agencies funding research also require compliance with these guidelines. Although not required, privately funded and industrial laboratories

Table 1 Types of oversight of genetically modified organisms[a]

Location and stage of development	*Oversight*
Laboratory, greenhouse, animal pen[b]	Decentralized oversight from federal agencies in the form of guidelines
Large scale fermentation	Guidelines from federal agency for oversight
Small-scale field research[c]	Guidelines and regulations: Combination of decentralized and federal oversight
Scale-up or large-scale testing	Federal and state regulations
Commercial products	Federal, state, international regulations

[a]Reflects current practices: The degree of oversight differs in each country, and among different funding and regulatory agencies.
[b]For unmodified organisms (naturally occurring, chemically altered, spontaneous or selected mutants), standards of practice apply in research, whether conducted by the public or private sector.
[c]Includes tests on land and in enclosed waters.

adhere to the guidelines, because some agencies approving commercial products of biotechnology research began to require compliance with the guidelines for product approval.

An investigator working with or constructing a GMO must first determine whether or not the research is exempt from or subject to the guidelines. If the research is exempt, no further action is needed but it is recommended that the containment conditions in biosafety level 1 (see below) be followed during the course of the research. If the particular research is not exempt from the guidelines, the biosafety level recommended for the research is specified in Section III of the guidelines, and certification of compliance is the responsibility of the local IBC before or at the time the research is begun (notification).

Classification of Organisms Based on Risk

Appendix A of the guidelines lists organisms exempt from oversight because they naturally exchange genetic information with each other. Only bacteria are currently listed. Sublist A is the largest and includes all species in the genera *Escherichia*, *Shigella*, *Salmonella*, *Enterobacter*, *Citrobacter*, *Erwinia*, and certain species in the genera *Pseudomonas*, *Serratia*, and *Yersinia*. Sublist B includes eight species of *Bacillus*. Sublists C and D each list three species of *Streptomyces*. Sublists E and F include certain species of *Streptococcus*. If the research is not exempt, the investigator determines the recommended containment depending on the type of experimentation and the risk level of the organism and vector system being used. The IBC certifies compliance for this level, in accordance with Section III of the guidelines.

Risk levels for many microorganisms can be found in Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, and are summarized in Table 2. Included are those biological agents known to infect humans as well as selected animal agents that may pose theoretical risks if inoculated into humans. The listing in Appendix B reflects the current state of knowledge and serves as a resource document for commonly encountered agents, but is not intended to be all inclusive. The classification is based on agent risk assessment information compiled by the CDC; further guidance on agents not listed in Appendix B may be obtained from CDC and the Belgian Biosafety Server websites. The US list is reviewed periodically by a special committee of the American Society for Microbiology, and its recommendations for changes are presented to the RAC for consideration as amendments to the NIH guidelines. Organisms that are not agents of human disease are not included in the listings in the guidelines. Those microbial agents associated with plant and animal diseases are subject to the regulations of the Plant Protection Act, the Animal Health Protection Act, and the Virus-Serum-Toxin Act, under the authorities of the USDA APHIS. Permits from the USDA APHIS are required for transporting interstate any plant pathogenic agent between laboratories, and specific containment conditions are specified on a case-by-case basis, often with on-site inspection by USDA personnel. Receipt of a regulated article (i.e., a microbial culture) by a laboratory is accompanied by terms of final disposal and use. Transfer to another laboratory will require amendment of the permit conditions.

In Appendix C, exemptions are explained and listed. Exempt research includes using, as host organisms for cloning DNA, *E. coli* K-12, *Saccharomyces cerevisiae* and *Saccharomyces uvarum*, asporogenic *Bacillus subtilis* and *Bacillus licheniformis*, and also certain extrachromosomal elements of Gram-positive organisms propagated and maintained in them. Using DNA from organisms that are risk group 3, risk group 4, or otherwise restricted is not exempt. Cloning potent vertebrate toxin genes is also not exempt, except under conditions explained in Appendix F. Appendix E lists certified cloning vectors, including plasmid, bacteriophage, and others, for each of the above host systems and for *Neurospora crassa*, *Streptomyces*, and *Pseudomonas putida*. The rationale for the safety of these host–vector systems, and the process for certification of new systems for a particular degree of biological containment based on the survival of the host and the potential for transfer of the vector to another organism, is explained in Appendix I—Biological Containment. Appendices P and Q also address biological containment for plants and animals and associated microorganisms.

Table 2 Classification of human etiologic agents by risk group[a]

Risk Group	*Definition*	*Examples*
Risk Group 1 (RG1)	Agents that are not associated with disease in healthy adult humans	*Bacillus subtilis*, *E. coli* K-12, Adeno Associated Virus-types 1–4
Risk Group 2 (RG2)	Agents that are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are often available	Forty bacterial genera, including *E.coli* 0157:H7; 12 fungal genera; 31 parasites; common viruses in 18 families
Risk Group 3 (RG3)	Agents that are associated with serious or lethal human disease for which preventive or therapeutic interventions may be available (high individual risk but low community risk)	Nine types of bacterial agents, including *Ricksettsia*; 2 fungi; severe viruses in 8 families including Hanta, HIV;CJD and other prions
Risk Group 4 (RG4)	Agents that are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are not usually available (high individual risk and high community risk)	Extremely severe and emerging viruses in six families, including Lassa, Ebola, undefined hemorrhagic fever agents

[a]From NIH Guidelines and CDC Classification of Etiologic Agents. See Table 3 for URLs.

New regulations after 11 September 2001 require biosecurity oversight for certain high-risk agents identified as potential agents that can be used in bioterrorist events. These regulations supersede the above, and apply to human, animal, and plant microbial agents designated select agents by CDC and USDA. The website should be consulted to access current information, as the list changes periodically (https://www.selectagents.gov/).

Physical Containment and Practices

Detailed descriptions of physical facilities and practices to be followed by researchers to ensure containment of organisms and safety to workers and the environment in standard laboratory research are given in Appendix G of the US, NIH Guidelines. Four biosafety levels, BL-1 through BL-4, represent increasing stringency and control of the environment. Their use generally corresponds to research with organisms in the risk group 1–4 classification of etiologic agents (Table 2), irrespective of rDNA technology being used.

Appendix K specifies physical containment facilities and practices for large-scale uses of rDNA molecules, and Appendices P and Q specify physical and biological containment facilities and practices for experiments involving plants in greenhouses and animals in animal pens, respectively. In practice, APHIS may assist the investigators and the IBCs in certifying containment for these facilities. Other appendices deal with shipment (H) and human gene therapy (M).

Compliance and Approvals for Research With Planned Introduction Into the Environment

Any research with GMOs that is to be conducted in facilities that are not specified in the NIH guidelines is considered a planned introduction into the environment. Experiments involving introduction of GMOs into the environment were first reviewed and approved by the RAC in the early 1980s. However, they were not conducted until 1986 after investigators received approval for their research from regulatory agencies. This action signaled the beginning of oversight for such experiments by centralized regulatory authorities rather than through guidelines that describe principles and practices for confinement of the GMO to minimize risk.

In the United States, regulatory authority over research involving genetically engineered microorganisms introduced into the environment falls primarily with the USDA-APHIS and/or the EPA. A GMO intended for field evaluation is regulated by both USDA-APHIS and EPA, under the Plant Protection Act and FIFRA, respectively, when it contains DNA sequences from a plant pest and the intent is to use the microorganism as a biopesticide.

If the purpose of the GMO is for bioremediation of waste or contaminated soil, production of biofuels, biosensors or manufacture of chemicals, EPA's Office of Pollution, Prevention and Toxics (OPPT) regulates the GMO under TSCA. Microbes arising from transfer of DNA derived from a species in a genus other than the genus of the recipient organism may be subject to TSCA oversight as "new chemicals." One critical aspect of oversight under TSCA is the commercial intent of the producer. Research performed for investigational purposes or proof of concept would not likely fall under this Section 5 TSCA authority. However, pesticidal intermediates (e.g., bacteria carrying marker transgenes with ultimate intent of developing a pesticide) may require a TERA (TSCA Experimental Release Application).

The FDA has oversight for GMO microorganisms which are intended for use in production of food and feed products, including enzymes, nutraceuticals and microbes used in fermentation (e.g., *Saccharomyces cerevisiae*). Laboratory research for these purposes is not directly regulated by FDA, but compliance with NIH Guidelines is required by FDA. However, if the microbially produced entity is going to enter into commerce (i.e., the food and feed supply), conditions used in the manufacturing process will be scrutinized as part of the review process under FFDCA. With the advent of genetic engineering of animals for food or pharmaceutical production, FDA has applied their new animal drug application and investigative new animal drug (INAD) authorities under FFDCA to GMO salmon for food and more recently to sterile mosquitoes developed for the intent of decreasing populations of disease incidence or virus titer from vectors such as *Aedes aegypti*, the vector of dengue fever. GE mosquitoes intended for population reduction are now the purview of EPA as pesticides. INAD status may be needed to transport a GMO across state lines if it qualifies as a "drug" under FFDCA. Details are given in an Animal and Veterinary section of the FDA website.

None of these agencies has a codification similar to that of NIH for contained research. Instead, each introduction is treated as a distinct case and an application must be submitted by the investigator to the appropriate federal agency for permission to conduct the research. Each of the agencies has published policy statements or has issued proposed or final rules describing how regulations they enforce are applicable to research with GMOs. More detailed descriptions of legal and jurisdictional issues can be found in the "Further reading" section. The current status of each agency and of approval processes can be obtained most readily by viewing materials online.

For example, the EPA Final Rule under the TSCA, published in the 11 April 1997 Federal Register (62 FR 17910–17,958), is accessible at the Office of Pollution Prevention and Toxics site. An 80-page document entitled "Points to Consider in the Preparation of TSCA Biotechnology Submissions for Microorganisms" is available from the EPA. In this regulation, research and development activities are subject to TSCA if funded, in whole or in part, by a commercial entity, or if the researcher intends to obtain an immediate or eventual commercial advantage from the research. Microorganisms that are covered are those new microorganisms that are intergeneric and have been formed by the deliberate combination of genetic material originally isolated from organisms of different taxonomic genera. Intergeneric microorganisms also include those containing a mobile genetic element

(including plasmids, transposons, viruses) first identified in a microorganism in a genus different from the recipient microorganism. Reporting to or obtaining approval from the EPA is required before manufacturing such organisms in containment or releasing them experimentally. However, an exemption is provided for researchers conducting small-scale field tests with the nitrogen-fixing bacteria *Bradyrhizobium japonicum* and *Rhizobium (Sinorhizobium) meliloti*, provided certain conditions are met. The field test must be less than 10 acres in size and containment measures must be employed to limit dissemination of the organism. Researchers complying with the NIH guidelines may also be granted an exemption from the EPA for contained research in closed systems for the following organisms: *Acetobacter aceti*, *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus licheniformis*, *Bacillus subtilis*, *Clostridium acetobutylicum*, *Escherichia coli* K-12, *Penicillium roqueforti*, *Saccharomyces cerevisiae* and *Saccharomyces uvarum*. This is based upon a history of safe use of the organism, safety of the DNA introduced, and documented conditions for containment and inactivation of the microbe to keep any releases from the production facility to a minimum. It should be noted that EPA is proposing to add two microorganisms (specific strains of the fungus *Trichoderma reesei* and specific subspecies of the bacterium *Bacillus amyloliquefaciens*) to the list of 10 recipient microorganisms (40 CFR 725.420) that are eligible for exemptions from full chemical notification requirements under the Toxic Substances Control Act.

Oversight Mechanisms

Standards of Practice

All trained professionals learn and adhere to certain accepted procedures and practices with respect to safety and to the appropriate scientific methods for the profession. Most persons trained in microbiology and related disciplines using microorganisms are exposed to the same standards of practice that are used in training medically oriented professionals. These include safe preparation, use, and disposal of inocula and inoculated organisms, including inoculated plants or animals, and appropriate decontamination and mitigation procedures such as sterilization in contained facilities or incineration of animals or burial of plant material.

Standards of practice for all scientists include procedures appropriate for conduct of experiments; the use of data, including statistical analysis techniques; publication ethics and standards; and sharing of biological materials after publication of results. Standards of practice in the open environment are particularly evident in agricultural and forestry research; the sites are evaluated, plots are designed to enable statistical analysis of results, criteria for evaluation of results are widely distributed and agreed upon through peer review, and results are disseminated through various publications. Practices to preclude significant risk to the environment and to mitigate possible untoward effects are routinely considered and used by researchers.

Guidelines and Directives

Guidelines may be considered a statement of policy by a group having authority over that policy. Sometimes such guidelines are also published as "points to consider." These guidelines offer assistance to investigators, and do not have legal authority, except if required by a funding or regulatory agency. Guidelines are generally considered far more flexible than are directives or regulations. Guidelines that have been adopted worldwide for contained research with GMOs are those originating from the US. The NIH guidelines have been considered de facto regulations, as commercial entities have also adopted them as standards of good manufacturing practice. Since NIH is not a regulatory agency it can require compliance only for its grantees. By policy, applicable research sponsored by other Federal agencies is to be conducted, de facto, in compliance.

European and international guidelines contain some questionable analyses. The International Codex Alimentarius Commission oversees food production. A key safety goal, number 60 (2003), is to determine "whether the food produced using a recombinant DNA microorganism is as safe as the conventional counterpart." It omits a major consideration dealing with safety of microorganisms, namely the assessment of potential benefits of rDNA. Thus, the possibility that food could be even safer, for example, lower or no allergens in peanuts, is not addressed.

Directives, particularly those issued by governments, are orders or instructions. Directives are currently an overarching method of oversight implemented by multinational groups such as the European Union (EU). Countries are being urged to implement the directives within the next few years that would ideally harmonize oversight within the EU. However, individual countries would still have the prerogative of overseeing the details of such directives or even making them more stringent. Groups such as the United Nations, the World Health Organization (WHO), the Organization for Economic Cooperation and Development (OECD), and others also make informed statements for governments to consider as they develop their own oversight policies and mechanisms.

Regulations

Regulations are laws or rules to control or govern procedures or acts. In the case of GMOs, some countries have implemented new laws for oversight of certain microorganisms and field tests, especially in Europe. In other countries, no laws are currently applicable. In the United States, legal interpretations of current statutes have resulted in extensive oversight of research involving field trials and even commodity imports. Details can be obtained from websites of agencies and other sources listed in Table 3. The laws have legal penalties for noncompliance, whether in the public or private sectors. This type of oversight in the United States has resulted in an elaborate permitting, evaluating, interagency coordination, and reporting system that may be viewed as time-consuming, cumbersome, and costly to some, and inadequate to others. In addition to federal and national laws, other governmental entities such as

Table 3 Information sources for policies, guidance documents, and regulations of microbial biotechnology[a]

Source	*URL*	*Comments*
Belgium Biosafety Server	www.biosafety.be	Managed by the Service Biosafety and Biotechnology (SSB) of Sciensano, a permanent center in the field of biosafety for the use of GMOs and microbes, both contained research and release into the environment. Includes links to historical background on risk assessment, OECD reports, national regulations and European directives
Biosafety Clearing House	https://bch.cbd.int	Set up by Cartagena Protocol on Biosafety to facilitate exchange of information on Living Modified Organisms
Biotechnology Innovation Organization (BIO), U.S.	www.bio.org	Areas of work include health care, food and agriculture, and environmental biotechnology
Center for Environmental Risk Assessment	www.cera-gmc.org	Focuses on application of sound science for risk assessment of GM plants and global knowledge dissemination
Codex Alimentarius Authority	www.codexalimentarius.net	International guidelines for food and feed safety
European Food Safety Authority	www.efsa.europa.eu	Guidance document (see Authority EFSA reference); excellent compilation of EU directives. Work concerns safety evaluation of GM plants and microorganisms as requested by EU
European Association of Bioindustries	www.europabio.org	Provides information and news on biotech products and regulations in healthcare, agricultural, and industrial sectors
Food and Agriculture Organization of the United Nations	http://www.fao.org/food/food-safety-quality/gm-foods-platform/en/	To share information on safety assessment of foods derived from rDNA plants under Codex
Organization for Economic Cooperation and Development (OECD)	www.oecd.org/biotrack https://biotrackproductdatabase.oecd.org	Public database of approved applications of biotech products, assigning a unique identifier for the Biosafety-Biotrack system
United States Centers for Disease Control (CDC)	www.cdc.gov www.emergy.cdc.gov/bioterrorism/	Classification of microbial disease agents based on risk in "Biosafety in Microbiological and Biomedical Laboratories (5th Edition)" Regulation of microbes of national security concern
U. S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS)	https://www.aphis.usda.gov/aphis/home	Regulation of animal disease agents Regulation of microbial plant pathogens by biocontrol; Biotechnology and GM plant approvals[a]
U.S. Environmental Protection Agency (EPA)	www.epa.gov/pesticides/biopesticides/ https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra	Genetically modified microbes used as pesticides and in production of industrial chemicals
U.S. National Institutes of Health (NIH)	http://osp.od.nih/office-biotechnology/nig-guidelines	Guidelines for research with recombinant DNA (rDNA) molecules

[a]Includes transgenic plants incorporating microbial genes.

states or cities have enacted their own regulations, compounding the difficulty of conducting field research. Confidential business information is protected in all cases, including oversight by guidelines or by competent authorities, unless such information relates to safety issues. There are also multilateral treaties such as the Cartagena Protocol on Biosafety that are legally binding for transboundary transfer of certain classes of Living Modified Organisms that may pose a threat to biodiversity. However, a review by Kinderlerer (2008) concluded that few countries have laws in place to deal with GMOs under the biosafety protocol, and view the risk as palpable.

Appropriateness of Guidelines and Regulations

Contained Research

The conditions described in the NIH guidelines have served as a codification of practices for conducting research with both unmodified and modified organisms within a traditional laboratory setting and for large-scale fermentations with many microorganisms to produce many products of biotechnology. There is no indication that exempting certain organisms from containment requirements, or conducting most other experiments at the lowest containment conditions, has caused any problem to individual workers or the environment. The guidelines have served both national and world interests well for nearly 45 years. Although mandatory only for federally funded research, private companies have embraced them and established their own IBCs.

Conditions for conducting research with other organisms that require other conditions for optimum growth, such as plants, animals, and microorganisms associated with them, have also been described in the guidelines. The practices are based on those

developed as standards of practice in research, and there is no evidence of adverse effects upon the environment when these practices have been followed.

Rapid advances in plant and animal biotechnology, however, have practically made the guidelines for contained research with certain genetically modified plants and animals obsolete. For example, engineered soybean and corn grow extensively in farmers' fields, yet when grown in a greenhouse for research purposes, such plants are not exempt from the NIH guidelines and may be treated by IBCs as requiring containment for research. The guidelines should be modified to give clear exemptions of those plants, as well as microorganisms and animals that have been approved by regulatory agencies for commercial use in the environment.

Rationale for Concerns

The concern over GMOs resides partly on a perceived increase in the ability of the organism to survive or persist. Thus, some scientists and consumers argue that oversight of GMOs should be as stringent as for toxic chemicals, physical disruptions such as water control projects, or exotic organism introductions. The strongest argument for these concerns is voiced by persons who compare the risks of GMOs with those of introducing exotic organisms. The appropriateness of this analogy can be questioned because there is generally a great familiarity with the organism being modified and the trait being introduced, and the fraction of the new genetic information is very small. Engineered organisms to date contain less than 10 introduced genes, versus many in standard and exotic hybridization classes, as well as 4000–20,000 for an exotic species, which can be familiar or unfamiliar, with entire genomes.

There is also a perception by some that, should there be a problem with survival or dissemination of a microorganism, nothing can be done. Essentially, the assumption is that once the gene is out, it cannot be recalled. However, both orderly and inadvertent movement and dissemination of microorganisms occur repeatedly because of their presence in humans, plants, animals, and inanimate objects that are moving throughout the world at increasing rates. There are also long-standing environmental practices that are in use to decontaminate or mitigate unwanted effects of microorganisms. Such practices are known for microorganisms associated with food production, plants, and animals as well as for free-living microorganisms. Immediate decontamination methods include, among others, burning, chemical control, and sanitation by various means. Short- and long-term methods are abundant for plant-, animal-, and human-associated microorganisms, since a great deal of research on developing mitigation methods is conducted by scientists in the disciplines of plant pathology, veterinary medicine, and human medicine. Many of these deal with management practices and the use of genetic resistance and application of biological control organisms. Immunization of humans and animals, including the use of genetically modified microorganisms for vaccines, is another type of long-term management practice to mitigate the effects of microorganisms.

Differences in microorganisms and their effects have long been recognized by consideration of risk and resulting designations into risk groups. In addition to previous risk groups applicable to humans, animals, and plants (see "Belgian classification for micro-organisms based on their biological risks—Definitions" in the website) the United States has classified microbes of public health and national security concern into select agent categories based on ease of transmission, severity of morbidity and mortality, and likelihood of use in bioterrorism. These were classed as select agents and toxins, which legally restricts the possession, use, and transfer of these agents. However, in 2007, the US CDC issued some exclusions depending on the genetic modification. Many parts of the world seem to be operating on the principle of paralysis by analysis with respect to commercial use of genetically modified microorganisms. Recent work on nitrogen fixing microbes may help to change this attitude as nitrogen is key to agriculture and the potential benefits of these novel microbes are immense.

Research Involving Planned Introduction Into the Environment

Scientists had many concerns about the safety of planned introductions and examined such introductions in multiple ways. In 1987, the US National Academy of Sciences made major conclusions regarding risk, one of which stated that there is no evidence that unique hazards exist, either from the use of rDNA techniques or from the movement of genes between unrelated organisms. Further, the risks of the introduction of GMOs carrying rDNA are similar to those associated with the introduction of unmodified organisms and organisms modified by other methods. The final conclusion was that assessment of risks of introducing GMOs carrying rDNA into the environment should be based on the phenotype of the organism and the environment into which it is introduced, not on the method by which it was produced—the product, not the process.

In going further with the assessment of risk, another study by the National Academy of Sciences in 1989 posed three fundamental questions to assess risk: (1) Are we familiar with the properties of the organism and the environment into which it may be introduced? (2) Can we confine or control the organism effectively? (3) What are the probable effects on the environment should the introduced organisms or genetic traits persist longer than intended or spread to nontarget environments? Similar questions for focusing on risk were elaborated by the Ecological Society of America in greater detail. Questions still remain on how to scale regulatory oversight on the basis of risk.

The principles espoused in the preceding types of publications have played a major role in the risk assessment policies developed by international bodies such as the EU, the OECD, and others. Various entities have provided for the responsible oversight of rDNA research and field trials in countries in which formal oversight mechanisms are lacking.

Concerns Over Genetically Modified Domesticated Organisms in Agriculture and the Environment

Virtually all domesticated organisms used in the production of food, feed, and fiber have been genetically modified over long periods, including certain live domesticated microorganisms used in making bread, beer, wine, various types of cheese, yoghurt, and other fermented foods. Selected microorganisms that have been shown to be beneficial are also widely used in the environment. These uses include, among others, microorganisms that fix nitrogen and provide nutrients for trees and other plants (mycorrhizae) as well as those used in sewage treatment plants and oil drilling. Also, naturally occurring pathogenic microorganisms are used in the testing of domesticated plants to ascertain their disease resistance. In such critical tests with known pathogenic organisms, there has been no documented case of untoward effects, such as a plant disease epidemic, arising from such standard field trials.

It is widely accepted that the first step in risk assessment, whether in containment or in confined field trials, is identifying the risk by determining how much is known about the parental organism. It is also recognized that the risk can be minimized by the preferential selection of parental organisms that are generally recognized as safe because of their long history of use. In the oversight of food, such foods are categorized as GRAS or generally recognized as safe (US FDA). A similar category might be considered for microorganisms that would be introduced into the environment: GRACE, or those microorganisms that are generally recognized as compatible with the environment (our proposal).

Examination of the food safety issues associated with genetic modifications has led to the conclusion that potential health risks are not expected to be any different in kind than those associated with traditional genetic modifications. All such evaluations rest on knowledge of the food, the genetic modification, the composition, and relevant toxicological data. A recent international body concluded that rarely, if ever, would it be necessary to pursue all such evaluations exhaustively. There is reasonable agreement that a threshold should exist for regulation, or even of concern below which further evaluations on a genetically modified food product or its individual components need not be conducted. The International Food Biotechnology Council recommends flexible, voluntary procedures between food producers and processors and a regulatory agency.

Regulation of Field Research

At the present time, there is no decentralized body in any nation for oversight of field research that is comparable to the IBC for contained research. The USDA published (Federal Register, 1 February 1991) a draft of guidelines for conducting research under confinement in the open environment prepared by the Agricultural Biotechnology Research Advisory Committee of scientists (ABRAC). However, these guidelines were never implemented even though they provided generalized principles for assessing and managing the risks of microorganisms, as well as plants and animals that have been genetically modified, particularly by r-DNA. The principles laid out in these guidelines were sufficiently generic that they were to be applicable throughout the world. Later in 1995, this USDA committee developed performance standards specifically for research with genetically modified fish and shellfish, since no statutory authority existed for oversight by a regulatory agency.

In many countries, the oversight of GMOs is essentially the same as for unmodified organisms, except for the contentious issue of planned introduction into the environment. In countries such as the United States and Canada, a sizable bureaucracy has been built up to oversee both research and product development. Even though the risks remain speculative, the fear of litigation and unknown hazards has served to minimize the actual number of introductions, particularly of microorganisms. Of the approximately 12,500 tests of plants, animals, and microorganisms introduced into the environment for research, less than 10% have been microorganisms. Most of these microorganisms were modified to have marker genes that enabled them to be monitored in the environment. The first functional genes added to microorganisms and field-tested in the early 1990s were those encoding an insecticidal toxin from *Bacillus thuringiensis* added both to a pseudomonad and to a coryneform bacterium. In the former case, the modified organism was killed before it was released. In the latter case, in field trials to test for insecticidal activity, plants inoculated with the GMO were successfully protected from the European corn borer. But commercialization of this gene succeeded only when it was introduced into the genomes of corn (maize) and cotton.

Commercialization

Some scientists are more concerned about the widespread adoption of a beneficial organism in commerce, rather than about small-scale field research, because greater exposure is likely to increase the probability of risk.

In over 40 years of research on microbial GMOs, thousands of papers have been published and patents issued. Some products of GMOs are widely known and used, yet most are not recognized, nor can most of them be easily determined. Selected products are described in Table 4 to show that virtually all of them are simply derived from microorganisms. We were able to confirm that only 12 microorganisms were commercialized for use in the environment at the time of this writing; some previous products have not been commercially successful and have been removed from the marketplace. Vaccines, however, have been commercialized either as whole viruses or as derived proteins and killed vaccines.

There are about 60 nonduplicative pharmaceutical products for human health (BIO), about 35 food and industrial applications (Europa Bio; information no longer available without membership charge), and two whole bacteria, one live (*A. radiobacter* K 1026) and one killed before use (*P. fluorescens*, with a gene for *Bacillus thuringiensis* δ endotoxin). Viruses are used as vaccines and as insecticidal biopesticides. Regulations on enzymes are not standardized in Europe and probably elsewhere. Not included: Bacteriophage-resistant mutants, bacteriocin producers, naturally selected mutant bacteria and fungi for bread, milk, cheese, and beer.

Table 4 Selected commercial GMO products, use and source

Product	*Use*	*Source*
Insulin	Human health	US Food and Drug Administration
Blood protein	Human health	US FDA
Human growth hormone	Human health	US FDA
Immunomodulators	Human health	Biotechnology Innovation Association (BIO) www.bio.org
Vaccines	Human health	BIO
Vaccines	Animal health	http://www.aphis.usda.gov/
Vitamin B2	Human health	www.europabio.org/
Preservatives	Foods	ibid.
Amino acids	Food additives	ibid.
Enzymes	Various food/feed additives	www.europabio.org/
Cheese, fruit juice		
Textiles, leather		
Detergents, waste water		
Confectionary, starch,		
Flavors		
Biopesticides in agriculture		US Environmental Protection Agency
Agrobacterium radiobacter K1026	Plant bacterial disease control	See URL in Table 3
Pseudomonas fluorescens, with *B.t.* Cry genes	Insect control	
Nucleopolyhedrosis viruses with insect toxin genes		

Needs, Challenges, and Options

Currently, there is no global database on regulations and oversight for genetically modified microorganisms, nor for commercialized products. While a minority of countries do not differentiate oversight on the basis of process, for example, the United States, the majority still do. Hence, the compilation of information would lend transparency to the public, regulators, and those in the commercial sector. The estimate of greater than 100 products (Table 4) is based on accessing a wide variety of sources; only 12 of these products are released into the environment as whole microbes, and three of them are heat-killed before release. Of the 194 recognized countries at the time of this writing, only about 15 have accessible information on regulations and products. However, the number of new websites and information sources on commercial biotechnology products is increasing, and our search is not exhaustive.

We have been unsuccessful in obtaining information on regulations and products from various segments of governments, industry, academic biotechnology institutes, and the International Union of Microbiological Societies (IUMS). The searchable database of worldwide use of genetically modified plants hosted by the Center for Environmental Risk Assessment (formerly hosted by Agbios) has been decommissioned and is no longer available for search and discovery. An annual summary of engineered crop plants in commercial agriculture assembled by ISAAA is available as well as a searchable database (http://www.isaaa.org/gmapprovaldatabase/default.asp). Many of the GM plants contain genes of microbial origin that impart viral, insect, or herbicide resistance traits. There is no database on microbial gene inserts into commercially grown animals.

The objectives of a sound oversight policy are to develop a sensible, scientifically based policy that is consistent with a reasonable and accepted degree of safety (rarely absolute or ensured safety). Regulatory agencies also recognize procedures that do not pose an unreasonable risk to humans or the environment, that is, one cannot be absolutely certain that no deleterious effects can occur. Oversight ideally should balance risks with expected benefits. Many issues that deal with GMOs, particularly introduction into the environment, are subject to change and remain unresolved. These include the following:

1. The scope of oversight: What organisms or parts of organisms (genetic elements and sequences) should be subject to oversight?
2. By whom and at what level (e.g., professional organization, standards of practice, IBCs, local regulatory bodies, federal agencies, or some combination) should oversight be conducted? Should all experiments be examined at a federal or country level or can some be decided upon at a local level? Decentralization of authority to make decisions on field releases has not yet occurred, although APHIS has expedited approvals for many plants.
3. Can consistent definitions be developed? Definitions differ among agencies and countries and can lead to problems in legal interpretations. The meaning of the phrase "release or planned introduction into the environment" and even "pathogen" or "pesticide" are yet to be agreed upon. For example, the EPA uses plant-incorporated protectant as a name for the class of pesticidal substances based on use of microbial components engineered into plants with the intent of making disease- or insect-resistant plants. This also affects whether different considerations apply to plant pathogens that are applied as beneficial biological control agents.
4. To what degree should the manner of open and peer review be part of policy-making? What appeal procedures, if any, should there be for scientists and others disagreeing with the oversight bodies?

5. Decision making: Who should decide whether approval of field-testing is needed? Is it the public, the scientists, or the courts? What role is there for common sense? Should the decision be based on risk alone, or include other factors such as socioeconomic considerations?
6. Should there be a "sunset" clause on termination of oversight of research with some organisms or certain types of experiments?

There has been general agreement that a centralized database for field trials would be desirable in order to compare information, including negative results that are not always published. The USDA through its Information Systems for Biotechnology program and the OECD through its Biotrack monitoring database have compiled a great deal of this information. As of September 15, 2017, on-line news reports from the ISB website have been archived, however, the data and information on permits and notifications issued by APHIS are exclusively available on USDA-APHIS websites (https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/brs-news-and-information/2017_brs_news/permit_data); (https://www.aphis.usda.gov/biotechnology/check-status). Whether or not these activities serve the purpose of the scientific and commercial community and allay the concerns of the public remains to be seen. Thousands of tests worldwide have been conducted up to the present time and no unpredictable effects have been detected. However, it can be argued that such effects may occur in later years, and hence monitoring will be necessary to assess any problems that might arise. Another question that remains unanswered is how long monitoring should occur, compared with wild-type organisms.

Given the different views of different countries and applicable laws, global agreements on oversight likely will not be forthcoming. However, there is reasonable general agreement on standards of practice through the scientific and professional societies of the world for conducting research. There are also areas of reasonable agreement in principle, acknowledging that the process by which a genetic modification is made is not as significant as the effects of that modification, that is, the phenotype. The same degree of oversight is not applied to unmodified organisms and organisms modified by traditional and newer (gene-editing) approaches. Hence the process of modification is still the trigger for oversight. A second area of agreement is that familiarity or knowledge of the organism and its modification are likely to be good predictors of the characteristics of the modified organism. A third is that knowledge of the ability to confine an organism or mitigate its effect, if need be, offers a reasonable indicator of expected risk and of the potential for its management.

Future Benefits and Concerns

Differing perspectives remain on the safety to humans and the environment of GMOs, particularly those that have been modified by rDNA techniques. The concerns are particularly high with respect to the use of microorganisms in the environment. Discussions are likely to continue for several more years. It is too early to predict whether or not such tests will go forward with reasonable ease, given the stringency of the requirements to conduct the tests. The scientific concerns may not warrant the expenditure of time, effort, and money to conduct field research, since risks unique to GMOs have not yet been identified. The same question could be asked about the oversight of contained research.

Several potential oversight mechanisms would be commensurate with risk assessment and risk management, and have been demonstrated as effective for laboratory research. These could include (1) categorical exclusions, (2) only notification requirements, (3) review and approval by a local organization (e.g., IBCs), (4) review and approval by a Federal agency with an advisory group consisting of members familiar with the relevant research area, and (5) review and approval by an international agency, in cooperation with a member country.

A reasonable policy of oversight will encourage research with GMOs, especially those modified by rDNA techniques. There is no perfect oversight mechanism for any human activity, including environmental releases for research, development, or commercial purposes. There is also the recognition that various viewpoints or perspectives cannot always be accommodated or reconciled. Thus, persons of reason and broad vision will be needed to resolve some of the contentious issues dealing with planned introduction of GMOs into the environment.

Fear overrides any benefits that live GMO microbes could provide, despite recognition that GMOs could be potentially less damaging than the continued application of chemicals, and we do not see this changing in the near future, even in contained environments such as fermentation tanks for alcoholic beverages or for bioremediation in select environments. The notion that mutants, natural gene transfer, and DNA shuffling are acceptable to the public and industry but not deliberately constructed GMOs remains a scientific paradox. In view of the billions of microbes transferred daily in global trade, commerce, and travel, such cautions should be reexamined. More derived products are expected in the coming years. An unresolved question is the de novo synthesis of new genomes or virus-like replicons and their oversight, whether for beneficial or biodefense purposes. There is also concern about activities or products that increase virulence or pathogenicity of known microorganisms. Most of these products are or would be constructed using rDNA techniques. Whether or not this is the case, the NIH Guidelines added synthetic nucleic acids in 2013.

With respect to accelerated mutagenesis or DNA shuffling (genome editing), at least in plants, only Canada at present has regulations governing their planned introduction into the environment and commercialization. Thus, Canada examines all novel, manipulated plant products, not only those produced by genetic engineering. Genome editing for microbes is still in the research phase and has not yet been used for commercial purposes. Recent technologies for modifying traits by CRSPR/Cas gene editing has increased interest on the oversight and regulation of resulting organisms.

Genetically engineered antimaterial microbial weapons are being pursued and perhaps other microbial arsenals, but research is not open to public scrutiny. Such agents may be of dual use, that is, for constructive or destructive purposes. Development of recent programs funding research into plant, insect and microbial agents at the Defense Advanced Research Projects Agency for countering accidental or intentional release of disease causing agents does, however, bode well for further field work on a variety of GMO organisms with practical value (https://www.darpa.mil/program/safe-genes; https://www.darpa.mil/program/insect-allies). EPA has also seen a significant increase in the number of applications for Biotech Notifications the last few years, which are specific for GMO microbes with pesticidal properties, including from academe and small and large companies developing products for disease and pest control.

The benefits of microbial GMOs remain to be more fully explored and managed for multiple purposes. To the extent that oversight occurs, it will continue to play a major role.

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http://www.fao.org/food/food-safety-quality/gm-foods-platform/en/—Food and Agriculture Organization of the United Nations.
www.icgeb.org/biosafety/—International Centre for Genetic Engineering and Biotechnology (ICGEB).
www.isaaa.org—International Service for the Acquisition of A⊠n-Biotech Applications.
www.oecd.org/biotrack—Organization for Economic Cooperation and Development (OECD).
www.cdc.gov—United States Centers for Disease Control (CDC).
www.aphis.usda.Gov—US Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS).
www.epa.gov/oppbppd1/biopesticides/—US Environmental Protection Agency (EPA).
www.epa.gov/oppt/biotech/—US Environmental Protection Agency (EPA).
http://osp.od.nih.gov/office-biotechnology-activities—US National Institutes of Health (NIH).

Genome Sequence Databases: Annotation☆

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Glossary

Annotation The process of identifying the locations of genes and coding regions in a genome and determining what those genes do.
Database It is a structured collection of records or information.
Genome The complete set of genetic information contained in the DNA of an organism.
In silico Characterization of biological experiments carried out entirely via computer systems.
Metagenome All the genetic material recovered from an environmental sample, comprising genomes of many individual organisms.
Single nucleotide polymorphisms (SNPs) Single-base variations in the genetic material between different individuals of the same species.

Abbreviations

BLAST	Basic local alignment search tool
BRENDA	BRaunschweig ENzyme Database
CAZy	Carbohydrate-active enZYmes database
CDD	Conserved Domain Database
COGs	Clusters of Orthologous Groups of Proteins
dbSNP	Database of single nucleotide polymorphism
DDBJ	DNA data bank of Japan
DoE	Department of Energy
EBI	European Bioinformatics Institute
EMBL	European Molecular Biology Laboratory
EST	Expressed sequence tag
HMM	Hidden Markov model
IMG	Integrated microbial genomes
INSDC	International Nucleotide Sequence Database Collaboration
JGI	Joint Genome Institute
KEGG	Kyoto Encyclopedia of Genes and Genomes
NCBI	National Center for Biotechnology Information
NIH	National Institutes of Health
ORF	Open reading frame
PIR	Protein Information Resource
TrEMBL	Translation from EMBL
UMBBD	University of Minnesota Biocatalysis/Biodegradation Database

Defining Statement

Genome sequence databases are portals to obtain valuable sequence information on microbial sequencing projects. With their rapid expansion and diversification, these resources are becoming virtual environments to explore microbial genomic space at the gene, protein, function, and pathway network level. The applications of these databases range widely from gene discovery to energy alternatives.

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Introduction

Over the last 20 years, there has been a meteoric rise in the volume of nucleic acid sequences available in the public domain. This trend has been driven to ever-increasing heights over the last decade primarily through improvements in the speed, accuracy, and availability of high-throughput DNA sequencing technologies. Since the sequencing of the first free-living microorganism, the bacterium *Haemophilus influenzae*, the use of high-throughput sequencing and genome assembly has seen the birth of the field of genomics, that is, the study of an organism at the whole-genome level. Moreover, identification of new applications of genome technology, for example, the sequencing of whole environmental microbial populations, referred to as metagenomics, continues to further accelerate the rate of genome sequence information availability. Due to the smaller size of microbial genomes (e.g., bacteria and archaea), a larger proportion of organisms from these domains have been sequenced in their entirety to date compared to eukaryotes. The main focus of this article is to highlight the content and diversity of genome sequence derived databases with particular emphasis on microbial space.

Genome Sequence Repositories

The presence of such large amounts of biological sequence information from genome sequencing projects has led inevitably to the creation of repositories to house these data. In this respect, it is important to discuss historically how such databases came into being. Prior to the recent advent of automated DNA sequencing, scientists had created repositories to accommodate biological sequence information released into the public domain. Thus, in the 1980s, two organizations launched their respective databases. First, the European Molecular Biology Laboratory (EMBL) produced their data library (EMBL-Bank) followed later in the United States by GenBank (Bethesda, the United States) administered by the National Center for Biotechnology Information (NCBI). The largest public repositories of nucleic acid sequences that exist now reside in the three members of the International Nucleotide Sequence Database Collaboration (INSDC; Table 1) that includes GenBank, EMBL-Bank, and the DNA Data Bank of Japan (DDBJ). As a consequence of a data exchange policy implemented by the INSDC member organizations, information is synchronized daily between each data repository, enabling scientists all over the world to have access to and query the latest sequence information.

GenBank and NCBI

Although all sequence repositories synchronize their data every day and thus possess similar content and volume of data, of the three major genome sequence repositories, GenBank receives by far the largest number of sequence depositions, followed by EMBL-Bank and then by DDBJ. Therefore, it is useful to review the types of information focused primarily on microbial organisms, which exists within the public databases maintained by the National Library of Medicine's NCBI. Although the major proportion of resources spent by NCBI remains with the maintenance, curation, and development of GenBank, NCBI provides an electronic web portal to support a variety of other tools and databases for public use. As with any database, information retrieval, that is, the handling of search queries to the database by the end user, is of primary importance. The major search engine for NCBI resources is Entrez. The NCBI Entrez portal provides a query tool to search a variety of other NCBI-related genomic-, proteomic-, and literature-based databases. NCBI Entrez supports text-based Boolean querying of the 42 databases that together contain 990 million records available at NCBI. As of September 2013, the example records include over 101 million DNA and RNA nucleotide sequences, over 94 protein sequences, over 1.1 million taxonomy terms, approximately 11 000 genomes, over 91 million gene expression records, over 1.2 million gene-based sequence clusters in UniGene (nonredundant eukaryotic gene data set), genetic variations in dbSNP (database of Single Nucleotide Polymorphism) (largely eukaryotic), over 95 000 protein structures from the Molecular Modeling Database (MMDB), 168 000 three-dimensional and 12 000 two-dimensional (alignment-based) protein domains, and the biomedical literature via PubMed, PubMed Central (PMC), and Online Mendelian Inheritance in Man (OMIM). A brief overview of some of the more prominent databases within the NCBI suite is given in the succeeding text:

- Trace Archive: The Trace Archive is a database of over 2.1 billion sequencing traces or chromatograms produced from the automated sequencing machines used for genome sequencing projects. The Assembly Archive links the raw DNA sequence information found in the Trace Archive with assembled genome information found in GenBank sequence depositions.

Table 1 International nucleotide sequence database collaboration

Data repository	*Organization*	*Location*	*URL*
GenBank	NCBI/NLM/NIH	Bethesda, the United States	www.ncbi.nlm.nih.gov
EMBL-Bank	EBI/EMBL	Hinxton, the United Kingdom	http://www.ebi.ac.uk
DDBJ	CIB–DDBJ	Mishima, Japan	http://www.ddbj.nig.ac.jp

NCBI/NLM/NIH, National Center for Biotechnology Information/National Library of Medicine/National Institutes of Health; EBI/EMBL, European Bioinformatics Institute/European Molecular Biology Laboratory; CIB–DDBJ, Center for Information Biology and DNA Data Bank of Japan.

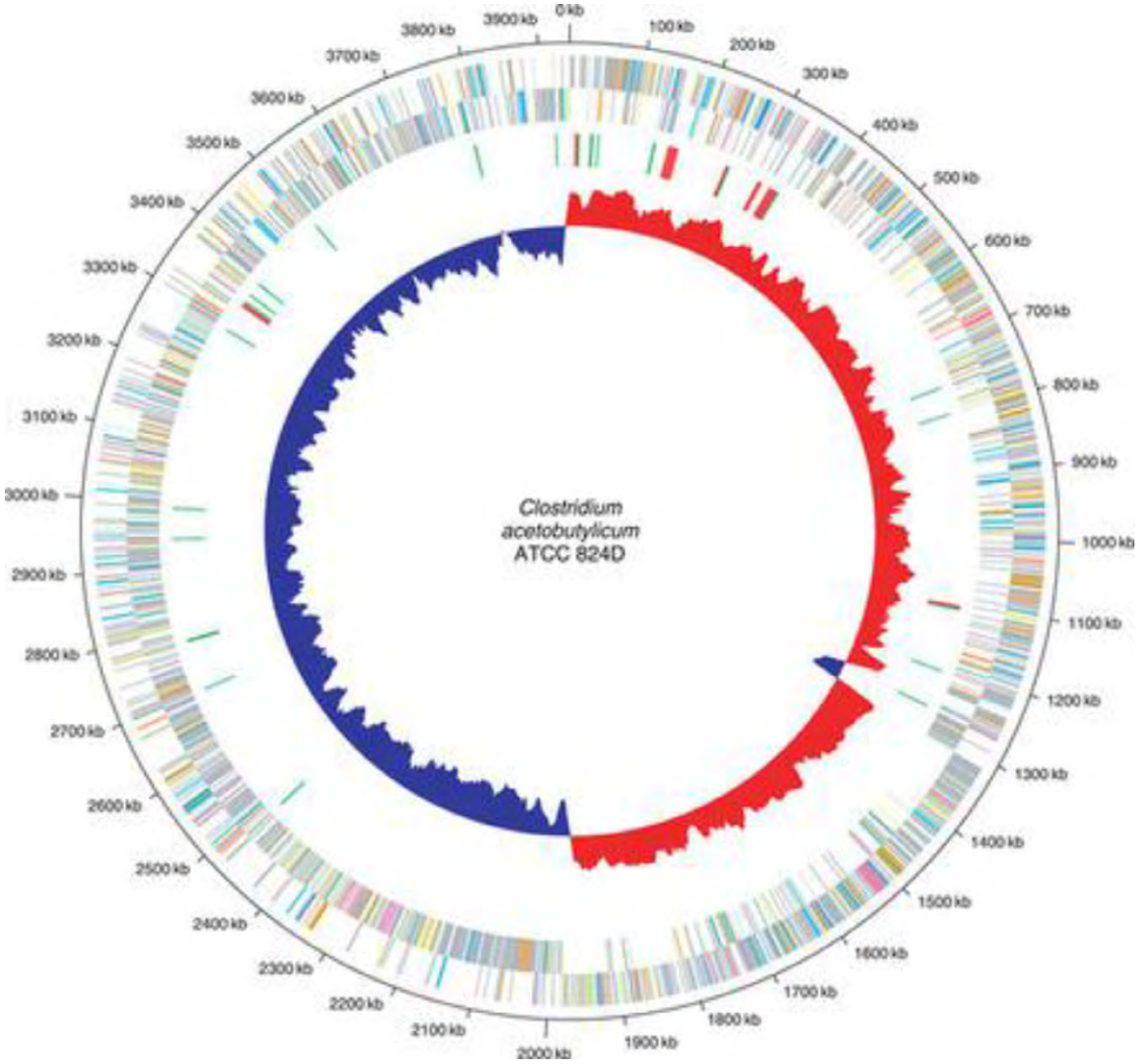

Figure 1 Genome of the industrial butanol-producing bacterium *Clostridium acetobutylicum* ATCC 824D main chromosome present in the Entrez Genome Project database. Courtesy of Integrated Genomics, Chicago, USA.

- Entrez Genome: This database contains sequence and map data from the whole genomes of over 1000 species or strains. The genomes represent both completely sequenced genomes and those with sequencing in progress. The database currently contains over 11 000 genomes, including genomes over 2000 eukaryotes, over 33 000 prokaryotes, 4630 viruses, 5700 plasmids, and 6500 eukaryotic organelles (e.g., mitochondria and chloroplasts). Each genome entry in this database is linked to a GenBank sequence file (identified by a unique GenBank accession number) containing the complete or draft DNA sequence information. The GenBank file is a specially formatted file comprising various fields including the organism name, taxonomy, published paper, the entire DNA sequence, all identified open reading frames (ORFs) with positional chromosome coordinates and gene assignments (also referred to as annotations), gene aliases (e.g., recA and mutS), and various other genome attributes and features. Details of this sort from a complete sequenced genome from this database can be used, with an appropriate tool set, to display a circular map diagram of an organism's genome (Figure 1).
- Entrez Genome Project: A supplemental database to Entrez Genome that provides information regarding status of incomplete sequencing projects as well as functional biological attributes of the organism that has or is being sequenced (e.g., taxonomy, why it is being sequenced, motility, and aerobic/anaerobic respiration).
- Taxonomy: The NCBI taxonomy houses approximately over 450 000 organisms with one or more nucleotide or protein sequence associated with each organism entry. The taxonomy browser visualizes the position of an organism in the hierarchical NCBI taxonomic ontology tree and is also able to retrieve entries in other NCBI databases (e.g., nucleotide and protein) linked via Entrez.

- Entrez Gene: The Entrez Gene database provides a portal to curated sequences and information about genes with links to other NCBI databases and viewers (e.g., Map Viewer, Evidence Viewer (EV), Basic local alignment search tool (BLAST) link (BLink), Conserved Domain Database (CDD), and other gene-related resources). The information within Entrez Gene is obtained and maintained through international collaborations as well as being subject to curation by NCBI scientists.
- Entrez Nucleotide: This is a nucleotide sequence database that comprises GenBank.
- Entrez Protein: This is a protein-oriented database assembled from a variety of data sources, including SwissProt, protein information resource (PIR), Protein Research Foundation (PRF), Protein Data Bank (PDB), and translations from annotated coding regions in GenBank and reference sequence (RefSeq) database.
- RefSeq: The RefSeq is a rapidly growing information set of over 61 million sequences (e.g., >52 million protein sequences from >51 600 organisms). RefSeq provides curated entries for gene transcripts, proteins, and genomic fragments, plus computationally derived nucleotide and protein sequences.
- PubMed: The PubMed database includes over 24 million citations from life science journals dating back to the 1950s. The PubMed citations are linked extensively to other Entrez databases including Nucleotide, Gene, Protein, Structure, and PubChem and provide the connection between sequence records and the primary scientific literature.
- Influenza genome resources: The Influenza Genome Project has generated over 12 000 viral sequences housed at NCBI, together with tools for the identification of genetic determinants of viral pathogenicity.

In addition to the databases discussed previously, there are numerous other databases that are part of the NCBI resources. Some of these rapidly growing databases are largely eukaryotic in relevance and content comprising, for example, the following:

- UniGene database: UniGene is a nonredundant database containing GenBank sequences and expressed sequence tags (ESTs) clustered into sets of related gene clusters or families. UniGene clusters are linked to the unique gene sequences and associated organism of origin. A set of UniGene clusters are assembly for any organism. In UniGene, human has over 7 million sequences in clusters, including 221 081 mRNAs and millions of EST sequences.
- Database of Single Nucleotide Polymorphism (dbSNP): dbSNP collects >299 million reference SNPs from nine organisms.
- ProtEST: The ProtEST database provides precomputed BLAST alignments between protein sequences from certain model organisms and the six-frame translations of nucleotide sequences in UniGene.

Sequence-Based Database Query: BLAST

By far, the most commonly utilized DNA sequence search tool prevalent in the armory of a bioinformatician is the BLAST suite of algorithms. BLAST programs perform sequence-similarity searches against a variety of databases, returning a set of gapped sequence alignments. Within the NCBI tool set, the output from BLAST is linked to full database records. An alignment produced by BLAST is scored and assigned a quotient of statistical significance referred to as the expectation value (E-value), which also takes into account the amino acid composition of the query sequence. This composition-based statistical analysis, used in conventional protein BLAST searches as well as PSI-BLAST searches, tends to reduce the number of false-positive database hits. The BLAST output includes the standard pairwise alignment between the query and the sequence in the GenBank archive, as well as a best-hit similarity table. More recently, new features including a dendrogram that clusters sequence output according to distance from the initial query sequence have been implemented. Further, more advanced versions of BLAST, for example, MegaBLAST, have been introduced that not only handle batch submission of nucleotide sequences for analysis but also run ten times faster.

Genomics and Proteomics Databases

It has been reported that there are over 719 databases of both prokaryotic and eukaryotic contents containing both DNA and protein sequence data. Although this is a conservative estimate, this number increased by 171 databases from the previous year and is rapidly increasing in both number and global geographic distribution. The presence of genome sequence records in a repository is important for public accessibility of the information. However, the derived data, for example, protein sequence and function information, are essential for the exploration of biological meaning encoded in a genome sequence. As a consequence in addition to the genome sequence databases per se, there have arisen a number of protein sequence databases that utilize the vast amounts of genomic sequence produced. Some of the more established protein databases currently available are summarized in Table 2.

Table 2 Public protein sequence databases

Protein sequence database	*Organization*	*URL*
SwissProt	Swiss Institute of Bioinformatics (SIB)	www.expasy.org
PIR	Georgetown University, the United States	http://pir.georgetown.edu
TrEMBL	European Bioinformatics Institute (EBI)	http://www.ebi.ac.uk
UniProt	UniProt Consortium (EBI/SIB/PIR)	http://www.uniprot.org

PIR, Protein Information Resource; TrEMBL, Translation from EMBL; UniProt, a combination of SwissProt, TrEMBL, and PIR-PSD.

When additional information is added to a protein sequence, this increases greatly the value and integrity of the resource for database users. Thus, curated databases provide, in addition to the mere protein sequence entry, information that validates the biological function associated with the gene product. This phase involves the manual curation by expert biologists who validate and ensure that information coupled to the protein sequence is reliable. An appraisal of some of the more commonly utilized protein databases is described briefly in the succeeding text:

- PIR: Established in 1984, PIR is a large nonredundant protein sequence database that organizes proteins into related protein families and superfamilies with additional annotations based on functional, structural, genetic, and bibliographic data. The resource is extensively cross-referenced with DDBJ/EMBL/GenBank gene identifiers, comprising over 273 000 classified entries organized into more than 100 000 protein families and 36 000 super families.
- SwissProt: This is one of the leading curated, nonredundant protein databases in existence. It comprises over 548 000 curated sequence entries from over 8300 different organism species. Each SwissProt sequence entry is annotated by a biologist to ensure database integrity using scientific literature-based curation methods. This information is augmented by other physical (e.g., protein secondary and quaternary structure and protein domain architecture) and biological properties (e.g., posttranslational modifications and diseases associated with deficiencies) of the protein.
- TrEMBL: Translation from EMBL (TrEMBL) consists of computer-annotated protein sequence entries obtained from translation of all coding sequences (CDDs) in the DDBJ/EMBL/GenBank nucleotide sequence repositories. It comprises over 46 714 000 entries from over 62 000 different species of organism.
- UniProt: Developed by the US National Institutes of Health (NIH), this database combines the SwissProt, TrEMBL, and PIR resources. Launched in 2003, it is composed of three components: the UniProt Knowledgebase (contains SwissProt, TrEMBL, and PIR curated entries), UniProt Archive (UniParc) of newly loaded protein sequences, and UniProt nonredundant reference database. Sequence entries are cross-referenced to its component databases as well as to DDBJ/EMBL/GenBank sequence entries.

Over the course of the last decade, the nature of genome sequence databases has seen a restructuring and consolidation of their content from being merely repositories of sequence information to portals to analyze in detail the functions of genes and the biochemical pathways and cellular subsystem to which they belong. In addition, with the advent of higher-capacity genome sequencing centers with more powerful technologies, this has allowed for not only generation of more complete genome sequences but tools for supporting comparative genomics efforts (comparative genomics: the comparison of multiple whole genomes and their encoded genes based on DNA–DNA alignments to visualize gene order or synteny and chromosomal rearrangements). The increase of more integrated one-stop genome sequence databases providing access to whole-genome sequence, entire gene annotation sets and reannotation tools, pathway collections, and comparative genomics tools has required a development of a genome processing pipeline to handle the workflow. For example, given the presence of a particular genome DNA sequence, various operations need to be performed to make sense of the encoded information. Thus, an overview of aspects of this pipeline is helpful in appreciating the overall process involved in preparing and submitting a genome to a sequence database and analyzing the information contained therein. While all genome sequence databases do not necessarily treat the data in the same way, genome sequencing organizations in particular, since they are responsible primarily for generating the raw sequence data and making sense of it, need to address these major issues. Some of these aspects are outlined and elaborated upon in more detail in the succeeding text and include the following:

1. Identification of the coding regions or genes in the genome
2. Functional gene assignment or annotation
3. Association of gene functions with cellular and biochemical pathway databases

Identification of Coding Regions

Once a microbial genome is completed, it generally consists of a number of DNA contigs that represent the main chromosome(s) and any accompanying extrachromosomal plasmid sequences. Most prokaryotes comprise at least a single covalently closed, double-stranded DNA chromosome (Figure 1). Although the actual DNA sequence is paramount to understanding the behavior of the organism, the raw DNA sequence needs to be processed further to be of use to microbiologists. One of the first steps in this processing, for example, is the identification of the CDSs or ORFs in the genome. It should be noted that the genome does not need to be entirely complete to identify ORFs. In fact, many incomplete or draft genomes have ORFs identified and can provide very useful biological information. The heart of this process of ORF finding requires in principle an accurate recognition of those genomic sequences that are most likely to be protein (or RNA) encoding as well as a choice of suitable translation start and end points. A variety of alternatives exist in order to do this ranging from performing BLAST analyses to identifying DNA regions homologous to existing proteins, to utilizing a plethora of gene-finding algorithms. Even within the context of ORF finding, there are bioinformatics tool sets to identify protein-coding regions as well as those designed to find various noncoding RNAs, such as tRNAs and rRNAs, as well as more esoteric RNA molecules.

The computational identification of genes can be categorized into two distinct classes of algorithms, specifically intrinsic and extrinsic. Intrinsic gene finders use heuristics that make no explicit use of sequence homology information about DNAs or proteins outside the sequence under investigation. In contrast, extrinsic gene finders utilize information derived from sequence-similarity search methods to identify the locations of protein-coding regions. It is commonplace for gene finders of both types to be used in

unison in a gene-finding project, owing to their complementary nature. The identification of the exact gene start positions remains a challenging problem in gene finding, as many genes have relatively weak patterns indicating sites of translation and transcription initiation. Examples of some of the commonly employed gene identification programs used in genome projects include GeneMark, Glimmer (both examples of intrinsic gene callers), and CRITICA, an extrinsic tool that uses a comparative approach to identify coding sequences based on other identified coding regions. Once the coding regions of the DNA chromosome are identified, the DNA sequence of the identified ORFs is translated *in silico* using a standard microbial translation table that associates a codon to an amino acid entity. Thus, a cognate set of translated proteins can be derived from the set of deduced ORFs in a genome sequence. The next phase involves conferring functions, enzymatic or otherwise, to this set of predicted proteins encoded in the genome, a process referred to as gene annotation.

Gene Annotation

The process of microbial genome annotation generally refers to the steps involved in assigning biological meaning to the raw sequence data by identifying gene regions or functional features and determining their biological functions. As discussed earlier, identification of the coding regions is the precursor to the subsequent phase of finding the functions for the set of translated proteins. Functional gene annotation, that is, the process of predicting *in silico* the function of a specific gene product, is a combination of automated and manual methods that generate a connection between a coding sequence and its biological function. The automated phase of this process generally involves comparing the query sequence (protein) with a nonredundant database of all other proteins available using comparison metrics based upon extent of sequence similarity of the query and protein in the database and the associated biological function(s) of these best-hit protein similarity matches. Thus, this is a process based on a system of standardized transfer of functional annotations from curated databases of well-characterized proteins. The manual phase involves using various bioinformatics tool sets to impart a function to gene products that were not conferred an annotation after the automated round.

Tools for microbial genome annotation

Numerous bioinformatics approaches and tools exist that allow annotators to deduce manually the putative protein functions *in silico*. The logic associated with assigning a function to a protein is based on a number of criteria including the presence of published literature documenting direct experimentation identifying the function, occurrence of diagnostic protein domains associated with a function, extent of protein similarity to a known protein with a specific function, and chromosomal clustering of the cognate gene with genes of related function. An example of the latter might be the clustering of a putative tryptophan biosynthetic gene with other tryptophan biosynthetic genes in the *trp* operon. Some examples of publicly available resources that are useful to bioinformaticians for discerning biological functions associated with proteins are summarized in Table 3 and outlined in the succeeding text:

- Clusters of Orthologous Groups (COGs) of proteins: The database of COGs, maintained at NCBI, is composed of protein families encoded in completely sequenced genomes, representing the major taxonomic lineages. Each COG category consists of individual proteins or of protein families from at least three lineages that correspond to conserved domains. The COG database consists of over 138 000 proteins that form 4873 COG families contained in 66 genomes comprising 38 taxonomic orders, 28 classes, and 14 distinct phyla. The COG protein classification scheme is based on orthologous (ortholog: the functional equivalent of a gene/protein function between two genomes) relationships between functionally related proteins from different organisms.
- Pfam: The Pfam database is a curated collection of over 10 000 protein families. Each family is represented by two multiple sequence alignments and two profile hidden Markov model (HMM: a statistical model that attempts to find patterns or hidden parameters from observable parameters. HMMs have applications in many fields of pattern recognition, for instance, modeling protein families of related sequence (e.g., Pfam)) (profile HMMs). These alignments denote functional features conserved in the protein or domain. The Pfam protein domain families are themselves classified into related protein family groups or clans.

Table 3 Tools for gene annotation

Annotation tool	*Functionality*	*URL*
COGs	Functional families or related proteins	www.ncbi.nlm.nih.gov
Pfam	Protein and domain families	http://pfam.Janelia.org
InterPro	Protein domains and functional motifs	http://www.ebi.ac.uk
ProDom		http://prodom.prabi.fr/
BLOCKS		http://blocks.fhcrc.org
ProSite	Collection of functional protein motifs	http://www.expasy.ch
SignalP	Identification of secreted proteins	http://cbs.dtu.dk/services
TmHMM	Prediction of transmembrane regions	
MEROPS	Protease/peptidase database	http://merops.sanger.ac.uk
CAZy	Carbohydrate/glycoconjugate domains	http://www.cazy.org

- InterPro: The Integrated Resource of Protein Domains and Functional Sites (InterPro) is, as the name suggests, a protein family and protein motif database. It is an integration of several databases (e.g., Pfam, ProSite, and ProDom) that can be used to discern functional properties of protein secondary structures. The public InterPro database, maintained by the European Bioinformatics Institute (EBI), has processed over 384 000 proteins derived from SwissProt and TrEMBL through its annotation pipeline.
- ProSite: The ProSite resource is a pattern database describing protein domains, families, and functional sites; it contains currently 1308 patterns and 1112 profiles.
- SignalP: The identification of proteins that are exported or secreted from a bacterium is important to determine since they can affect pathogenic properties of microbes, for example. The SignalP resource, maintained by the Technical University of Denmark, is a tool that predicts the location of signal peptide cleavage sites in protein sequences from Gram-positive, Gram-negative, and eukaryotic organisms. Predictions are derived using techniques such as neural networks and HMMs.
- Protein function-specific databases: In addition to the burgeoning collection of protein family, domain, and motif databases that exist in the public domain, there are also a variety of resources focused on specific functional groups of proteins. Thus, for instance, the MEROPS database is a structure-based, curated resource for proteases, proteinases, and proteolytic enzymes containing over 66 500 sequences associated with 346 discrete enzymatic specificities. MEROPS derives its sequence data primarily from publicly available complete microbial genome sequences. Similarly, the Carbohydrate-Active enZYmes database (CAZy) is a structure-based collection of catalytic and carbohydrate-binding protein domains of enzymes that degrade, modify, or create glycosidic bonds. The protein sequences present in these data are derived from the GenBank entries directly or from RefSeq. The information relating to carbohydrate and glycoconjugate breakdown and biosynthesis is gleaned from the genomes of 44 archaea, 501 bacteria, 25 eukaryotes, and 42 viruses.

Genome annotation databases

A variety of microbial whole-genome annotation databases, whether they are commercial, academic, or governmental in origin, have been developed over the recent years to handle the increasing volume of genomic data generated for public and commercial use. These databases provide resources to identify and annotate genes and their associated functions. This can be performed in an automated fashion, but also manual gene annotations can be conferred by users. Some of the more common genome annotation resources encountered are described briefly in the succeeding text:

- Bioinformatics Resource Centers (BRCs): BRCs are a group of five web-based research centers funded by US National Institute of Allergy and Infectious Diseases (NIAID). The five BRCs include Eukaryotic Pathogen Database Resources (EuPathDB), Influenza Research Database (IRD), PathoSystems Resource Integration Center (PATRIC) (for bacterial pathogens), Virus Pathogen Database and Analysis Resource (ViPR), and VectorBase (VB). These centers are intended to assist researchers to characterize various pathogens and generate drugs, vaccines, and diagnostic tools to combat them.
- Integrated Microbial Genomes (IMG): This data management system was created by the US Department of Energy (DoE) Joint Genome Institute (JGI) in 2005 to house and analyze the increasing volume of genomic data produced from DoE production sequencing facilities. The IMG platform provides public access to genome data from JGI sequencing projects. It contains genome, gene, function, and pathway information as well as visualization tools to compare genomes.
- SEED annotation environment: Developed through the US Department of Energy Argonne National Laboratory, the SEED annotation environment comprises 180 177 proteins with 213 distinct functional roles from 383 genomes.
- Proprietary databases: Genomics has been harnessed for the discovery of new genes, enzymes, and biochemical pathways. Consequently, the private sector has developed and exploited a variety of bioinformatics and genome analysis platforms to mine this wealth of genome data. An example of this is the subscription-based ERGO database created in 1998 to mine genomic information. This curated database comprises genomic data overlaid with gene, protein, and pathway information as well as metabolic reconstructions of entire genomes. Other commercial databases that exist are purely for corporate use and used for a variety of purposes ranging from data mining for novel enzymes to identification of drug targets in pathogenic microbes.

Integrating Genomics and Pathway Databases

As we have seen from earlier, the process of whole-genome sequencing and gene annotation can produce an extensive inventory of protein function assignments that can be propagated over a large set of sequenced genomes. With the improvement of both automated protein assignments and extensive manual tools to predict protein functions, an average bacterial genome can have between 50% and 65% of its protein complement assigned a discrete biological function. Some of these functions may participate in either metabolic (e.g., glucose dehydrogenase), nonmetabolic (e.g., DNA replication proteins), or regulatory (e.g., transcription factors) pathways. In turn, some of the protein functions, for instance, those with enzymatic roles, can be associated with their individual metabolic reactions and with each of its corresponding substrate and product metabolite compounds. Collections of enzymes, reactions, compounds, and pathways have been assembled and compiled from various sources through direct experimentation for over several decades. The fruits of this labor are available in published scientific journals. However, only recently as a direct consequence of genome sequence projects generating large quantities of predicted protein enzymatic functions have scientists connected the gene and protein information to pathway and reaction networks. This has led to the development of a more detailed understanding of the metabolic complexity inherent within a microorganism.

The integration of genome sequence with enzyme and pathway databases has attracted greater interest over recent years and led to a better characterization of microbial cell function. There are numerous applications of such integrated databases. This is of particular relevance to the biotechnology industry, for instance, where access to this information has a direct impact on the development of genetically engineered microbial strains that are capable of producing various compounds useful to society, including commodity and fine chemicals. Thus, for example, a detailed *in silico* metabolic network facilitates drug design for target enzymes in pathogenic microbes. Moreover, the availability of detailed reaction properties (e.g., thermodynamics and kinetics) aids the simulation of specific microbial pathway behavior to achieve a desired goal, exemplified by redirection of carbon flow to produce a useful metabolite.

Metabolic and cellular pathway databases are responsible for taking gene and protein sequences derived from genome sequencing projects and connecting them via their functional role(s) to a collection of individual compounds, reactions, pathways, and pathway networks (or subsystems). The ultimate goal of this information coupling is to generate a higher-level characterization of cellular metabolism and functionality than that obtained by looking merely at a gene inventory list. Examples of some of the enzyme and metabolic pathway databases under common use are summarized in Table 4. The strength of genome sequence databases leveraged with metabolic pathway content is a powerful scientific resource, and a description of some of these pathway databases and their content is merited.

- BRaunschweig ENzyme Database (BRENDA): The BRENDA database comprises information for metabolic enzymes and the molecules that bind them including potential enzyme inhibitors, as well as kinetic and thermodynamic data for the reactions. BRENDA comprises 3518 reactions with 47 630 associated compounds.
- Kyoto Encyclopedia of Genes and Genomes (KEGG): One of the most widely used public resources describing genes, functions, and metabolic reactions is KEGG, established in 1995 and maintained by the University of Kyoto, funded by the Japanese Ministry of Science. The database not only contains a large compound collection but also supports an extensive and ever-increasing collection of reactions and pathways, both metabolic and nonmetabolic. KEGG contains over 5473 reactions with 10 760 associated compounds in more than 200 pathways. The database is highly integrated with genomic data, compound, reaction, and pathway information. It is regularly updated with the latest publicly available complete genome sequences.
- MetaCyc: The goal of the curated MetaCyc database is to serve as an encyclopedia of metabolic enzymes and pathways. Maintained by SRI International (Menlo Park, California), it comprises a nonredundant collection of pathways that have been documented in the literature. MetaCyc contains 12 377 enzymatic reactions and over 11 900 compounds connected to 2310 pathways derived from over 500 reference genome sequences. Other derivatives of this database exist (e.g., EcoCyc) that comprise single organism (e.g., *Escherichia coli*) information repositories.
- University of Minnesota Database of Biocatalysis and Biodegradation Database (UMBBD): This resource is developed and maintained by the University of Minnesota (Minneapolis) and focuses on biodegradation pathways. It contains information regarding xenobiotic compound interconversions that are useful for industrial and bioremediation purposes.
- Commercial metabolic pathway databases: By far, the largest comprehensive commercial database, comprising both genomic and metabolic pathway data, is the ERGO database (Integrated Genomics, Chicago). It was developed from the Enzymes and Metabolic Pathways database (EMP) and Metabolic Pathways Database (MPW) but has expanded upon them to include 9506 reactions and 5978 pathways in >1000 complete and incomplete genomes. The resource integrates not only genomic data but also literature and high-throughput data (e.g., derived from whole-genome gene expression studies). Other databases are more specialized in nature, such as BioCarta that comprises pathways describing cellular signaling.

Many interaction pathway databases also exist (Table 5). Different from metabolic pathways, interaction pathways focus on the cellular signaling and regulatory reactions in cells. Besides KEGG, a brief introduction of these databases is discussed in the succeeding text:

- BioCyc: BioCyc is maintained by SRI International (Menlo Park, California). BioCyc collects 5500 pathway/genome databases and includes software tools to understand their data. BioCyc includes man derivatives, such as EcoCyc focused on *E. coli* and MetaCyc as described earlier.

Table 4 Enzyme and metabolic pathway databases

Metabolic databases	*URL*
MetaCyc	http://metacyc.org
BioCarta	http://www.biocarta.com
BRENDA	http://www.brenda-enzymes.org/
ENZYME	http://www.expasy.org
ERGO	http://ergo.integratedgenomics.com
KEGG	http://www.genome.jp/kegg/
SoyBase	http://soybase.org/
SEED	http://theseed.uchicago.edu
UMBBD	http://eawag-bbd.ethz.ch/

Table 5 Signaling and regulatory interaction pathway databases

Metabolic databases	*URL*
Advaita Bioinformatics	www.advaitabio.com/
BioCyc	www.biocyc.org
BioCarta	www.biocarta.com
Ingenuity	http://www.ingenuity.com/
KEGG	http://www.genome.jp/kegg/
PID	http://pid.nci.nih.gov
Reactome	http://www.reactome.org

- Pathway Interaction Database (PID): The PID is a freely available collection of manually curated and peer-reviewed pathways of human molecular signaling, regulatory, and cellular processes. This widely used database was created in a collaboration between the US National Cancer Institute (NCI) and Nature Publishing Group. PID contains 136 human pathways with 9248 interactions curated by NCI-Nature. It also imports 322 human pathways with 7575 interactions from BioCarta and Reactome. Although PID is widely used, the database has not been updated since September 2012.
- Reactome: This is a manually curated open data resource of human pathways and reactions. Currently, Reactome describes over 7000 human proteins, which participate in over 6700 reactions based on data extracted from over 15 000 research publications. The Reactome data resource links to other public resources such as the Gene Ontology (GO), UniProt, and ChEBI. User-friendly pathway analysis and visualization tools are also provided.
- Commercial interaction pathway databases and analysis tools: There are many commercial databases and tools to support interaction pathway studies. For example, BioCarta provides pathways describing cellular signaling, Ingenuity provides services and tools for variant and genomic data analysis, and Advaita Bioinformatics provides tools to support interaction pathway analysis.

Ontology-Based Gene and Omics Data Annotation

Biological/biomedical ontologies are sets of computer- and human-interpretable terms and relations that represent entities in the biological/biomedical world and how they relate to each other. Biomedical ontologies have emerged as a major tool for the integration and analysis of the large amounts of heterogeneous biological gene and omics data available in the postgenomics era. For example, the GO provides controlled and standardized terms for naming different types of biological processes, cellular components, and molecular functions. Creating such ontology-based annotations is highly valuable for both querying databases and analyzing high-throughput data. Since its first publication in 2000, GO has been cited by over 7000 peer-reviewed publications in PubMed and approximately 100 000 hits in Google Scholar. The Ontology of Genes and Genomes represents individual genes and genomes from different organisms. The Protein Ontology describes the relationships of proteins and protein evolutionary classes and delineates the multiple protein forms of a gene locus. The Ontology for Biomedical Investigations (OBI) has been used for representation of a wide range of investigations. The Vaccine Ontology has been used in different applications such as vaccine data integration and literature mining. Formatted based on the Web Ontology Language (OWL) technologies, these ontologies are being more and more widely used for a variety of biological and biomedical applications.

New Directions: Metagenomics

Genome sequencing of environmental samples or microbial communities, referred to as metagenomics, is the genomic analysis of organisms by direct extraction and cloning of DNA from an assemblage of microorganisms. This is in contrast to more conventional individual whole-genome projects where the genetic material from one organism is sequenced completely. The rationale underlying metagenomics arose from evidence (e.g., 16S rRNA gene sequence analysis) that as yet uncultured microorganisms represent the vast majority of organisms in most environmental niches. In many environments, approximately 99% of the microorganisms cannot be cultured by standard techniques. Although analysis of 16S ribosomal RNA genes provided only a phylogenetic description of community association, metagenomics facilitates study of the genetic diversity, physiology, and ecology of environmental microorganisms. The discovery of new genes and proteins identified through metagenomics approaches includes the first bacteriorhodopsin of bacterial origin, novel small molecules with antimicrobial activity, and antibiotic resistance determinants. The assembly of multiple genomes in an environmental sampling, for instance, has also provided insight into energy cycling within the community, genome structure, gene function, population genetics, and lateral gene transfer among members of an uncultured community.

The workflow for a metagenomics project is not dissimilar to conventional whole-genome projects, except that DNA is prepared from an assemblage of organisms rather than a single one. Libraries of genomic DNA fragments are then cloned into a standard vector (e.g., fosmids and bacterial artificial chromosomes (BACs)) and transformed in an *E. coli* bacterial host strain. Clones isolated from the bacterial plasmid library are then 'shotgun' sequenced on both strands of the insert DNA, that is, everything that has been cloned into the vector is sequenced in this way. The sequenced DNAs are then assembled in to larger DNA fragments, and this set of

DNA sequences can then be compared against databases of known sequences using BLAST algorithms or similar sequence analysis tools. The broad taxonomic category of the best BLAST hits can be utilized, for instance, using NCBI taxonomy, to provide some notion of which taxonomic clade the DNA sequence sample fragment may have originated from. Metagenomics sequencing projects have been performed on a number of environmental isolates including ocean water from the Sargasso Sea, human intestinal microflora, and heavy metal-contaminated effluent water from mining projects. The sampling of such genome sequence data sets is also beneficial from an industrial perspective and has been exploited to discover and clone new genes associated with novel enzymatic properties. The volume of large metagenome sequence submissions to GenBank and other public sequence repositories is beginning to increase rapidly as sequencing technologies improve and the tools to assemble and analyze these sequences become available.

Further Reading

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Genome Sequence Databases: Sequencing and Assembly☆

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Glossary

Assembler A computer program that pieces together overlapping reads to reconstruct the original sequence.
Captured gap (a sequence gap) Unsequenced area between two contigs spanned by at least one subclone or sequenced fragment
Contig 'A set of gel readings that are related to one another by overlap of their sequences. All gel readings belong to one and only one contig, and each contig contains at least one gel reading. The gel readings in a contig can be summed to form a contiguous consensus sequence and the length of this sequence is the length of the contig'.
Finishing The process of improving a draft assembly composed of shotgun sequencing reads, resolving misassembled regions, closing sequence gaps, and validating low-quality regions to produce a highly accurate finished DNA sequence (<1 error in 10 000 bp).
Gap Unsequenced area between two contigs.
Paired (sister) reads Sequences generated from both ends of a DNA fragment. Such reads are oriented toward each other and the distance between them is equal to the template length.
Quality score The probability of a wrong basecall. A Phrap quality score of X corresponds to an error probability of approximately $10–X/10$ (a score of 30 means that the error probability is 1/1000 or 99.9% accuracy for a base in the assembled sequence).
Read Gel reading generated during the DNA sequence process.
Repeats Sequences of varying lengths found in multiple copies in the genome.
Scaffold A group of ordered and oriented contigs.
Sequencing by Synthesis (SBS) A sequencing approach that involves multiple parallel microsequencing addition events occurring on a surface, where data from each round is detected by imaging.
SNP Single-nucleotide polymorphism.
Uncaptured (physical gap) Unsequenced area between two contigs with no subclones or sequenced fragment spanning it.

Abbreviation

AMOS	A Modular Open-Source
BAC	Bacterial artificial chromosome
EMBL/ENSEMBL	European Molecular Biology Laboratory
GAP	Genome Assembly Program
GEBA	Genomic Encyclopedia for the Bacteria and Archaea
LLR	Log-likelihood Ratio
NHGRI	National Human Genome Research Institute
NGS	next generation sequencing
PCAP	Parallel contig Assembly program
PCR	Polymerase Chain Reaction
SBS	sequencing by synthesis
SNP	single-nucleotide polymorphism
SPAdes	St. Petersburg genome assembler
SPS	Southwest Parallel Software
SSAKE	Short Sequence Assembly by progressive K-mer search and 3′ read Extension
TEM	transmission electron microscope
WGS	Whole-Genome Shotgun
YAC	yeast artificial chromosome

☆*Change History:* August 2014. A Lapidus introduced small changes into the Abstract to make it current, updated keywords and special terms, significantly updated sections 'DNA sequencing', 'Sequencing strategies', 'Libraries', 'Pre-Processing of raw Data', 'Assemblers', 'Algorithms for Second-generation Sequencing Data Assembly', 'Assembly of Metagenome Shotgun Sequences', Genome Assembly Improvement and Finishing', 'NCBI Trace and Assembly Archives' Updated sections 'Further Reading', 'Relevant Websites' Renamed and slightly updated section 'Microbial Genome project' (now it is 'Genome project'). Significantly updated Table 1. Added new Table 2. Added new sections 'Single-cell genome assembly', 'Third-Generation Sequencing and Genome Finishing', 'Assessing quality of draft genome assembly'. Created section 'References'.

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Defining Statement

Putting together data produced during the sequencing stage of a genomic project is comparable to solving a complicated puzzle made of several million pieces. Thanks to the combined efforts of enthusiastic software developers and biologists studying genome structure and functionality, a number of successful algorithms and approaches for genome assembly have been created. High-quality genomic assembly lays a solid foundation for functional genome analysis.

DNA Sequencing

The knowledge of the particular order of nucleotide bases of genomic DNA is widely used both in fundamental biological research and in many applications. This information plays a significant role in medical and forensic studies and is of great value for the pharmaceutical industry.

The era of DNA sequencing began about 30 years ago, when two methods of primary DNA structure determination appeared almost simultaneously. One of them was developed by A. Maxam and W. Gilbert at Harvard University and was based on the use of chemical modifications of DNA. The other method was published by F. Sanger and A. Coulson from Cambridge and is called the chain-termination method. Since then significant changes have been introduced both in the Sanger method (which has been completely automated and therefore has found wider application than Gilbert's method) and in a variety of other sequencing technologies.

Modern approaches are aimed at significantly reducing the costs associated with sequence determination. In 2004, the National Human Genome Research Institute (NHGRI) set a task for the global scientific community to lower the cost of sequencing of an individual human genome to $1000, with an intermediate goal being to reduce the cost of sequencing a mammalian-sized genome to $100 000. In January 2014 this goal –a whole human genome at a cost of $1000 –was achieved by launching the HiSeq XTen sequencing system]. This low price allows using DNA sequencing not only in research, but in clinical diagnostics, pharmaceutics, forensics, ecology etc. to simultaneously analyze the entire set of genes within an organism as well as between species and individuals.

The NHGRI initiative resulted in rapid proliferation of next generation sequencing technologies (NGS) including pyrosequencing, base-by-base sequencing by synthesis (SBS), sequencing by ligation, nanopore sequencing, and single-molecule sequencing by synthesis in real time. These newest technologies are currently at different stages of development and implementation.

As compared with the traditional Sanger sequencing, the greatest advantage of the first three NGS approaches is very high level of parallelization, as they are capable of performing up to 10^7 reactions in one experiment. In addition, the tiny volume of the individual wells (picoliters) in which sequencing reactions are running, and high data density (10 000 times higher, than in case of the latest microelectrophoresis-based capillary instruments) significantly decreases the volume of reagents necessary to perform the reactions. Another positive aspect of the new methods is the ability to circumvent the step of DNA cloning and propagation in *Escherichia coli* cells, thus avoiding the problems of biased genome coverage due to the presence of genomic areas, which are hard or even impossible to clone in *E. coli* (so called unclonable areas). The downside of the first NGS technologies, as compared with the Sanger approach, is the length of the reads produced. The 454 Genome Sequencer 20 System instrument (the first commercial NGS platform) produced reads of about 110 bp, although the read length has increased up to 1 kb with the latest GS FLX Titanium chemistry. Meanwhile, technologies using SBS and sequencing by ligation (Applied Biosystem SOLiD; polony-based approaches) produce reads of 25–50 bp long. Such short reads impose certain restrictions on the use of new technologies: whereas 454 sequencing has been successfully applied to *de novo* genome sequencing resulting in a gapped assembly with an error rate of about 1/3000–1/5000 bp, the ultrashort reads produced by Solexa technology however appear to be useful for resequencing purposes only, when a high-quality reference sequence is available for alignment. The use of 25–50 bp reads for *de novo* genome sequencing appears to be very problematic.

After being acquired by Illumina, Solexa became known as Illumina. This technology uses the SBS sequencing approach to provide massively parallel sequencing and detects single bases as they are incorporated into growing DNA strands. Read length varies from 100 to 250 bp depending on the sequencing instrument used (MiSeq, HiSeq 2000, HiSeq2500). Low error rate of Illumina reads ($\leq$0.1%) makes this sequencing technology very attractive for a number of applications including de novo genome assembly.

Ion Torrent is yet another NGS approach. It uses semiconductor sequencing technology of SBS. The Ion PGM sequencer detects changes in pH when a nucleotide is incorporated into the DNA molecules and a proton is released. This technology produces reads of 400 bp long with about 1% error rate allowing single nucleotide polymorphism (SNP) detection as well as whole genome assembly.

Among the numerous methods on the drawing board, special attention should be given to real-time sequencing of DNA molecules. Such methods use very small amounts of genomic DNA and require supersensitive detection methods. The success of these methods critically depends on the quality of genomic DNA used in the experiment, because any damage to DNA in the process of its isolation would result in corrupted sequence. Theoretically, the read length produced by this type of technology is equal to that of a full genome.

PacBio (SMRT) sequencing is one of the first real-time single-molecule sequencing techniques that generates long reads (10–15 Kb) without requiring an amplification steps and has the unique ability to distinguish methylated bases from normal nucleotides. This feature gives the possibility of studying the epigenetic potential of different organisms. Read error rate is high, but errors are random and thus can be corrected via increased coverage. Long reads play a crucial role in the gap closing step of the whole genome assembly and PacBio gave genome finishing, a step that for several years was set aside as time-consuming and not cost efficient, a much needed boost.

A number of development teams have used nanopores or an electron microscope for signal detection. When nanopore-based detection is used, DNA moves through 1.5-nm-wide pores in an electric field and a detector measures the change in conductance within the pore. ZS Genetics, Inc. was working on a single-molecule sequencing method, which uses a single-stranded DNA molecule to form a complementary strand via the incorporation of labeled nucleotides, thus making the DNA molecule visible under a transmission electron microscope (TEM). The company claims that their system is capable of sequencing DNA strands of 20 000 bp or longer.

The UK company Oxford Nanopore Technologies is developing a new generation of nanopore-based sequencing approach. The MinION system allows producing long reads up to 10 000 bases long. The current error rate is about 4% and it was observed that technology produces systematic sequencing errors, which is more challenging to correct bioinformatically than random ones.

The list of companies and university-based groups of researchers working on improving sequencing technology goes on. Some of them have already become commercially successful; others are still only approaching this goal, whereas some already failed or will fail (Table 1). In many cases a combination of different sequencing approaches allows to produce high-quality sequence fairly quickly and at very low cost.

Genome Project

The process of decoding a genome is a complex scientific task composed of complete genome sequence determination and functional analysis of the genes of the organism being sequenced. Thus each genomic project consists of sequencing, assembly, and annotation stages.

When starting a project, it is useful to estimate the size of the genome, its GC content, number of chromosomes (even some microbial cells contain more than one chromosome), and potential presence of plasmids (circular or linear extra chromosomal elements) in case of microbial projects. Choosing the most appropriate sequencing and assembly strategy is as important as deciding which tool to use for the subsequent functional analysis of the genome.

Table 1 Companies involved in DNA sequencing technology development

Company names	*Throughput*	*Read length (bp)*	*Approach*	*Application*	*Web site addresses*
Solexa/Illumina			Sequencing by synthesis (SBS)	Whole microbial genomes, Resequencing expression profiling, RNA-seq, teargeted sequencing	http://www.illumina.com
MiSeq	0.3–15 GB/5–6 h	2 × 300			
Hiseq 2500	10–1000 GB/ 7 h–6 days	2 × 125			
NextSeq 500	20–120 GB/ 12–30 h	2 × 150			
Thermo Fisher Scientific			Semiconductor sequencing	Human genome sequencing, exome sequencing, Targeted sequencing, de novo genome sequencing, gene sequencing, ChIP sequencing, methylation analysis, whole transcriptome, gene expression nalysis	http://www.appliedbiosystems.com
IonTorrent (PGM)	60 MB–2 GB/ 4–7.5 h	400			
Ion Proton	10 GB/ 2–4 h	200			
454 Life Sciences Corp, Roche			Pyrosequencing	Whole genome sequencing, targeted resequencing, metagenomics, transcriptome Sequencing	http://www.454.com
GS Junior System	35 MB/10 h	400			
Sequencer FLX System	700 MB/23 h	700–900			
Pacific Biosciences (PacBio RS II)	275–375 MB/ 30–180 min/ SMRT cell [1–16 SMRT cells/run]	5000–10 000	Single-molecule real-time sequencing (SMRT)	Targeted sequencing, *de novo* assembly, base modification detection	http://www.pacificbiosciences.com/
Oxford Nanopore Technologies Ltd	tens of Gb per day	5500–10 000	Nanopore-based single molecules sequencing	Scientific research, personalised medicine, crop science, security and defence, environmental applications.	https://www.nanoporetech.com/

Sequencing Strategies

Three sequencing strategies have been used in genomic projects sequenced using Sanger technology. The first one is based on the use of ordered collections of overlapping BAC (bacterial artificial chromosome) or YAC (yeast artificial chromosome) clones. To follow this approach, one should make a large-insert library (BAC, YAC), map clones to the genome using fingerprinting or hybridization, pick the minimal set of clones covering the entire genome, and sequence and assemble each clone from this set separately. This strategy appeared to be very labor-intensive and is not widely used today.

In 1995 the Whole-Genome Shotgun (WGS) sequencing approach was introduced and it quickly became the most popular strategy for microbial genome sequencing. According to this strategy, each sequencing project requires genomic DNA (gDNA) isolation, library construction, sequencing of the subclones, assembly of sequenced data, and genome finishing (Figure 1).

For the project to succeed, it is vital to begin with high molecular weight gDNA, which makes possible the creation of three genomic libraries with different insert length. Typically, the insert sizes are set at 3, 8, and 40 kb, thus creating 3-kb, 8-kb and fosmid DNA libraries. Library inserts are then sequenced from both ends, resulting in approximately 8–9*X* genome coverage with paired reads. Reads are aligned against each other by using various genome assemblers to produce a draft assembly, which consists of contigs linked into larger scaffolds based on the information about read pairs. The genome closure stage (finishing) is used to solve all possible misassemblies within contigs and to close gaps in (sequencing or captured gaps) and between (physical or uncaptured gaps) scaffolds to build a single high-quality contig spanning the entire genome (note that final assembly might contain more than one contig if the genome consists of a larger number of replicons).

Before moving on to a more detailed description of the WGS assembly and finishing stages, it is necessary to mention that new sequencing technologies gave birth to a third sequencing strategy. In many cases this is a combined approach that uses sequencing data produced by different sequencing platforms. The combined approach has its own advantages and drawbacks (which will be discussed later on), but seems to be promising considering it has the potential to speed up the completion of genome projects and lower their costs.

Libraries

WGS sequencing begins with random shearing of gDNA and used to continue with further cloning of small fragments of DNA into different cloning vectors. Depending on vector capacity to accept foreign DNA fragments of different length, small- or large-insert genomic libraries can be created. Libraries of different insert size play different roles in the genome assembly: whereas small-insert clones (3 kb on average) are needed to obtain the necessary genome coverage, larger insert libraries (8 kb, cosmids, fosmids, or BACs) serve to verify the accuracy of contig assembly and to order and orient them.

When dealing with small-insert libraries, it is important to be certain that each vector contains only one insertion. Simultaneous cloning of several random DNA fragments will lead to chimeric clones and thus will cause problems for the later stages of genome assembly. A number of approaches, such as vector/insertion ratio optimization in the cloning ligation reaction, efficient size selection, and the use of specially designed vectors allow minimization of the probability of multiple fragment co-cloning.

Large-insert libraries are usually created by cloning DNA fragments of 25–200 kb into cosmid, fosmid, or BAC vectors. In addition to the fact that such vectors can carry long fragments of foreign DNA, they are present in one or two copies in *E. coli* cells (low copy number vs. high copy number vectors used for 3- and 8-kb libraries). Low-copy-number vectors are very useful when so-called poisonous sequences (i.e., sequences whose presence or expression interferes with the biology of the host organism in some way resulting in host death) are cloned. Mechanisms of toxicity differ; they include cases of open reading frames that code for toxic proteins, operators that reduce effective concentration of an essential DNA-binding regulator, certain (AT)-rich DNA fragments, which can serve as strong promoters in *E. coli* and initiate transcription of genes encoded by the vector itself at a very high level. It has been shown, for example, that some membrane and ribosomal proteins are toxic for *E. coli* cells when highly expressed. This has been confirmed by numerous experimental observations that some areas of bacterial genomes are represented only by large-insert clones.

The next step is to sequence the inserts from the clone libraries. The WGS strategy does not mean that a complete sequence of the entire insert cloned into the vector will be produced. Typically, inserts are sequenced from both ends resulting in two sequencing reads of about 700 bp each. These pairs of reads, otherwise known as sister reads, form a mate pair. The sister reads are oriented toward each other. The distance between them is within the mean library insert size plus or minus three times the standard deviation of the insert size. When assembled (see below) sister reads may appear in the same or a different contig. Information about mate-paired reads is a very important input for assembly tools (assemblers).

If we assume that the library clones (subclones) cover the chromosome evenly (i.e., there is no cloning bias) and that the length of each read is approximately 700 bp, sequencing of 715 clones will be needed to cover (1 × read coverage) a 1-MB bacterial genome once. To obtain 1 × clone coverage of the same 1-MB genome one needs to have 333 clones from the 3-kb library or 125 clones from

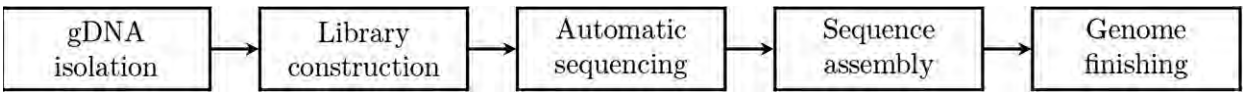

Fig. 1 Whole-Genome Shotgun sequencing steps.

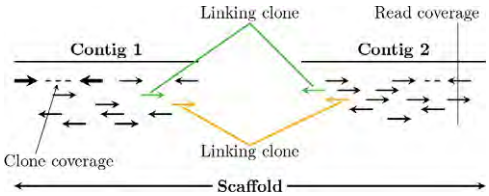

Fig. 2 Read and clone coverage.

the 8-kb library. In practice, clones are not randomly distributed and some areas remain unsequenced (gaps) even if $8\times$ read coverage is provided (Figure 2).

Most library preparation procedures for current NGS platforms consist of (i) a DNA fragmentation step performed by using physical, enzymatic or chemical methods, (ii) a DNA repair and end-polishing step followed by (iii) a platform-specific adaptor ligation. The PCR amplification step (iv) helps to produce enough DNA for further sequencing. As a result millions of small DNA fragments become available for sequencing in multiple parallel reactions. With minor differences these steps are used in library preparations protocols by major NGS platforms developed by 454/Roche, Solexa/Illumina, Thermo Fisher Scientific (SOLiD and Ion Torrent) and Pacific Biosciences (PCR amplification is not required).

High quality NGS libraries depend significantly on the amount and the quality of the DNA source material as well as the method used for DNA isolation. The term 'quality' is interpreted differently depending on the expected application (whole genome sequencing, genome re-sequencing, targeted sequencing, etc) (Chain et al., 2009).

Most of the existing NGS platforms produce paired-end or/and mate pair libraries allowing sequencing of DNA fragments from both ends. The distance between paired reads is called an insert size and is a critical parameter for subsequent genome assembly. A combination of several paired libraries with different insert sizes is needed to be able to resolve repeats of various lengths and nature. Requirements for the optimal insert size combination is dictated by genome features and do not differ from the ones used in Sanger libraries. While some NGS platforms do not support large insert size library creation, others like 454/Roche produced up to 20 kb libraries.

The recently emerged single-cell sequencing approach utilizes whole genome amplification with Multiple Displacement Amplification (MDA) (Lasken, 2009) to generate enough amplified gDNA to construct sequencing libraries. Single-cell libraries suffer from considerable bias, caused by nonlinear amplification and single-cell data demonstrates a highly non-uniform coverage, high rate of chimeric reads/read-pairs and a large number of sequencing errors. These properties of the sequencing data tend to further complicate genome assembly.

Assembly

Pre-Processing of Raw Data

The next step of the WGS project is to assemble the DNA reads produced during the sequencing stage to reconstruct the entire genome. The assembly of tens of thousands of reads into a few contigs (preferably into one for each replicon – the ultimate goal of any sequencing project) is a very complicated computational task. So, what makes the whole genome assembly so challenging?

Sequenced reads are never 100% error-free. Physical limitations of the gel electrophoresis technique, chemical artifacts, and human mistakes contribute significantly to assembly complications. The second equally or even more important factor further complicating the assembly is the presence of areas of identical or nearly identical sequence (repeats) in DNA molecules. Even the best assemblies contain gaps and imperfections due to genome complexity, sequencing biases, repeats and polymorphism, data and algorithm limitations.

A number of software tools were developed to address these issues with Sanger reads. Computer programs like ABI-basecaller, pregap4, and Phred analyze raw data produced by the automated DNA sequencing machines by converting them into a sequence of bases (basecalling) and assigning quality scores to each nucleotide. Because of its high accuracy, the Phred basecaller became the most widely used tool. Phred quality scores range from 4 to about 60 and define the probability that the base call is correct. A base with a quality score of 20 or higher is usually considered a high-quality base. 'Phred20' score means that the probability of the base being called incorrectly is 1 in 100. Quality at the beginning of the Sanger read and closer to its end is lower than in the middle of the read. This means that the error rate is higher (sometimes significantly) toward the end of the read, which makes assembly more difficult. (The same trend is observed for reads produced by Illumina sequencers).

The gold standard Sanger reads of about 1000 bp long have currently an error rate as low as 10^{-5} error per base (Shendure and Ji, 2008). The so called second generation sequencing (SGS) technologies (SOLiD, 454, Illumina, Ion torrent), while faster and cheaper, produce shorter reads of a lower accuracy compared to Sanger sequencing. Having a lot in common, these technologies use different reagents and signal detection systems, which leads to different types of errors made during sequencing. Some read errors can be corrected by increasing sequence coverage, but this will again raise the sequencing cost. More accurate base-calling allows decreasing the amount of coverage needed to reach the desired level of accuracy and therefore decreases the overall cost of sequencing.

Illumina provides its own base caller Bustard that creates a model from the raw intensities and performs base calling based on the produced model. Alta-Cyclic (Erlich et al., 2008) was developed to improve Illumina base calling. It uses machine learning to minimize noise factors, systematic biases and allows producing longer reads of a better quality. BayesCall, another model-based (a fully parametric model) base calling tool, 'improves Bustard's average per-base error rate by ~ 51%' (Kao et al., 2009) Ibis (Improved base identification system) (Kircher et al., 2009) is yet another base caller for the Illumina sequencing system. It incorporates both sequencing errors and neighboring signals into its statistical learning procedure. Further improvement of this tool called freeIbis (Renaud et al., 2013) represents an open source version of Ibis that requires less processing time and computational resources.

Errors in 454 reads are caused by mixed clusters, incomplete synthesis of the complementary DNA strand, possible incorporation of multiple different bases, and presence of consecutive runs of the same base (homopolymers). It was observed that the native 454 base caller underestimates the actual base accuracy assigning Q30 quality score to about 25% of bases produced. A base calling program, Pyrobayes (Quinlan et al., 2008), was developed to produce more accurate higher quality base calling. These and other base-callers for Illumina and 454 sequencing platforms are described in more detail in (Shendure and Ji, 2008).

New algorithms were also proposed and thus new programs were developed to convert Ion Torrent (pyrosequencing-like sequencing platform) flowgrams into reads. FlowgramFixer uses 'a linear-time method that combines a state machine with a Viterbi algorithm to find the nucleotide sequence that maximizes the likelihood of the observed flowgram' (Golan and Medvedev, 2013). The developed algorithm is applicable to any flowgram-based sequencing platform.

The error correction step for IonTorrent sequencing reads requires special attention. Existing approaches assume that most sequencing errors in the NGS data are mismatches, while in IonTorrent reads (similar to 454) homopolymer indels are most prevalent. Recently developed IonHammer introduces several novel algorithms based on homopolymer-space Hamming graph clustering and corrects both mismatches and indels. IonHammer has been shown to significantly reduce the average error rate (about eightfold), which is crucial for de novo assembly applications.

In order to be able to develop an error correcting tool one must be familiar with the different errors associated with the different sequencing platforms, which in turn makes it very important for downstream data analysis.

Another group of computer programs (available both as independent tools and as part of larger assembly packages) was developed to mark (pregap4 – part of the Staden package or trim out (Lucy, Arachne trimming module, MAGIC-SPP) low-quality tails of the reads and to mask or remove cloning vector sequences (Cross-Match provided by Phrap; vector_clip (Staden package-gap4), Lucy, and so on), mostly found in the beginning of the reads but also occasionally detected at the end of the read if the insert is very short.

Instead of vector sequence, NGS reads frequently contain adapter sequence in both the 5′ and 3′ ends of the read, which is also caused by the fact that the reads are longer than the insertions. If not trimmed, these sequences will create confusion during downstream assembly. This problem is very well known in relation to the Nextera library sequenced with 250 pb reads.

In contrast with the Sanger libraries, when the sequence of the cloning site is know and thus the exact start and/or end of the inserted gDNA fragment can be defined, it is not always easy to recognize the adapter especially if the sequence at the end of the read is of poor quality. To address this and other pre-processing problems new NGS tools were developed. Their goal is to recognize adapter sequences, perform read trimming and quality filtering (Trimmomatic, AdapterRemoval, Cutadapt, FASTX, Trim Galore!). Such preassembly data treatment significantly improves assemblers' performance and increases assembly quality.

Assemblers

Despite all the efforts high quality genome assembly is a complex task that so far remains unsolved. It is well known that majority of problems caused by repeats present in all genomes of any nature. The usage of multiple methods of genomic DNA isolation, different sequencing technologies and different types of genomic libraries for research projects introduces additional levels of complication to the genome assembly.

Generally speaking, most long reads Sanger assemblers follow a three-phase approach: overlap, layout, and consensus (OLC). During the first phase, the assembler determines which pairs of reads overlap; these reads may have been drawn from the same DNA region. During the layout phase, the assembler places the reads, thereby attempting to reconstruct contiguous sequences of DNA, or contigs, and to order and orient contigs to build scaffolds. During the final phase, the assembler uses the reads that have been placed in order to generate the consensus sequence – the assembler's best reconstruction of the original DNA sequence. Advanced algorithms employed by modern assemblers are also able to address such important problems as repeat locations and at least their partial resolution and identification of chimerical reads.

The first assembler was described by Roger Staden and has been significantly improved over the following few years. The current version of this assembler – GAP4 (Genome Assembly Program) – 'contains all the tools that would be expected from an assembly program plus many unique features and a very easily used interface'.

One of the most commonly used OLC assembly programs is Phrap. To detect overlaps, Phrap scans the set of reads to find pairs with perfectly matching subsequences and passes these sequences to a banded Smith–Waterman algorithm to find the optimal local alignment. Read couples that are near-duplicates are retained, while combined read couples that do not have good matching segments are considered to be not a match and are discarded. The Log-likelihood Ratio (LLR) score, a combined measure of match length and quality, is computed for each retained match. In the layout phase, Phrap constructs contig layouts using matches in decreasing order of LLR score. Version 3 of this assembler does not use paired read constraints and does not generate scaffold

information. A separate program, such as Bambus, may be used to order and orient contigs. Phrap 4 (also known as Southwest Parallel Software (SPS) Phrap) is an updated version of Phrap which, unlike Phrap 3, uses paired reads information and produces fewer misassemblies. Phrap 4 can create scaffold information using mate pair links, with an option to link contigs only if two mate pairs from the same library link the two contigs. Phrap 4 can also be instructed to ignore singleton mate pairs in the assembly. For consensus sequence generation Phrap analyses all individual reads and picks bases with the highest Phred quality score. Phrap is one of the best tools for working with low-quality reads and for assembling low-coverage nonrepetitive regions. The latest version of this assembler provides some support for next-generation sequencing reads.

Another widely used assembler is Arachne. To perform the overlapping stage, Arachne finds all reads that share a *k*-mer, an identical sequence of *k* bases (word), but excludes (masks) the reads that occur with extremely high frequency. In practice $k = 24$ is used. This exclusion improves the efficiency of overlap detection, and is thought to exclude high-fidelity repeat sequences. For layout, Arachne finds pairs of paired reads with sequence overlaps at both ends. Arachne iteratively identifies and extends 'paired pairs', forming minicontigs, and then assembles true contigs by avoiding assembling across repeat boundaries. Some of these contigs will represent repeat regions (they are identified by high depth of coverage, and by conflicting mate pair links to other contigs). The nonrepeat contigs are organized into scaffolds if they are linked by at least two mate pair links; priority is given to contigs that are separated by short distances and spanned by many mate pair links. Arachne then attempts to fill in the gaps between contigs by finding a path of repeat contigs across each gap, guided by mate pair links. The improved versions of Arachne first construct a set of contigs that are unlikely to include a misassembly, and then iteratively improving the initial assembly through read placement and contig breaking at suspicious points. Arachne is quite efficient at repeat resolution, with the exception of tandem repeats. To build the consensus sequence Arachne converts pairwise alignments of reads into multiple alignments and merges overlapping adjacent contigs in scaffolds. Quality scores are used to create and evaluate read alignments. This very powerful assembler was also successfully used for mammalian genome assembly.

Parallel Contig Assembly Program (PCAP) uses a parallelized local alignment technique and length of the word (*k*) of 12. For layout, pairs of reads are placed in order of decreasing local alignment score. The quality of the current layout is then determined by checking mate pair constraints. Corrections are made to the region with the largest number of constraint violations. The process of constraint assessment and correction is repeated until no areas with constraint problems are found. Finally, the assembler attempts to close gaps between linked contigs using the reads overlapping the ends of the contigs together with reads considered to lie in repetitive regions. PCAP computes a consensus sequence for contigs based on an alignment of reads in contigs and uses both quality values and coverage information for each base. The latest version of this assembler PCAP.REP does not use the constant word length to overlap reads. The developers implemented the idea of using superwords for more effective overlapping of repetitive reads. Superwords consist of more than one *k*-mer and can be of different length. It was demonstrated that the new method of overlap detection is very efficient in both unique and repetitive regions and that PCAP.REP produces more accurate and contiguous assemblies of whole-genome data sets.

The AMOS Comparative assembler (AMOS-Cmp, A modular, open-source whole genome assembler) skips the overlap step. It aligns reads of the target genome to the reference genome of a closely related organism by using a modified version of the MUMmer algorithm. Authors call this approach alignment–layout–consensus, since the assembler produces consensus from the alignment. However, even closely related organisms can significantly differ from each other due to insertions, deletions, rearrangements, repetitive regions and lateral gene transfer thus making comparative assembly challenging. The AMOS-Cmp is aimed to overcome these problems. Taking into consideration the increasing number of high-quality completed reference genomes in public depositories, the comparative (assisted) assembly approach has a bright future.

Algorithms for Second-Generation Sequencing Data Assembly

As was already mentioned above, second generation sequencing technologies produce short or ultra short reads (35–200 bp) and the number of reads for a given genomic project is significantly higher than what is obtained in the case of the Sanger method. This makes usage of the previously described assembly tools nearly impossible. Another problem with using traditional assemblers in connection with next-generation sequencing technologies is the fact that each of these platforms generates data in different formats (flowgrams, images, text format (fasta), and so on) and that these formats differ from the one generated by Sanger sequencers. As a result, there was an urgent need for new computational methods for analyzing massive amounts of very short reads and for managing this overwhelming volume of data. In response to this need new algorithms have been created to analyze short reads in a number of applications, including *de novo* genome assembly and visualization, polymorphism detection, gene expression, metagenomics and single-cell analysis.

The first short-read sequence assembler was described in a publication by EULER assembler developers (Pevzner et al., 2001). A modified version of EULER was used to assemble simulated reads of length 80–200 bp created to obtain $30\times$ coverage for several test cases (BACs and viral and bacterial genomes). The same main problems – repeats and sequencing errors – complicate short-read assembly, but shorter reads result in a larger number of repeats, which creates additional difficulties for assembly tools (repeats that are shorter than the read length). The EULER assembly approach, which performs fragment assembly by finding an Eulerian path through a de Bruijn graph representation of a genome, works well on error-free reads. To avoid or minimize the influence of the error rate, the modified version of EULER addresses the problem of errors by correcting them prior to assembly, thus eliminating alignment and basecalling steps. However, to adapt to variations of error rates and types in second generation sequencing methods, the corrective stage needs to be optimized. Experiments with the EULER assembler allowed assembly of short reads into relatively

long contigs and thus demonstrated the feasibility of such assembly for *de novo* draft sequencing. It was also predicted that significant finishing efforts would be required to resolve repeats and misassemblies to produce the finished genome.

454 Life Science company (Roche) has developed its own *de novo* flow-space assembler optimized for an increased number of 80–120 bp flow-based reads. The assembler, Newbler (Margulies et al., 2005), consists of three modules performing the overlap-layout-consensus operations and uses only high-quality flowgrams for better performance (it is stated that quality scores used by Newbler are in good correlation with phred scores). Errors most typical for the 454 platform are the ones related to homopolymers. They are caused by over- or underestimation of the intensity of the signal or by their combination.

To overlap reads the Overlapper module compares all flowgrams by using a hashing indexing method. The next module, Unitigger, groups the overlapped reads into 'unitigs'. As defined (Margulies et al., 2005) 'a unitig' is a collection of reads whose overlaps between each other are consistent and uncontested by reads external to the unitig. Unitigs are constructed from consistent chains of maximal depth overlaps'. Created unitigs then go through the all-against-all comparison to join the overlapping ones and to break the contigs at the repeat boundaries (Multialigner). For high-quality consensus generation the Newbler assembler averages the signals for each individual assembly position. This assembler was first tested on several microbial genome assemblies and is currently employed by all laboratories where 454 instruments are being used.

The latest versions Newbler is capable of assembling not only 454 data, but Ion Torrent PGM and Proton reads ((IonTorrent/Thermo Fisher Scientific) since these instruments produce a similar type of data (Table 2). This is one of the few programs that can handle data from multiple technologies to perform hybrid assembly and *de novo* assemble genomes up to 3 Gb in size using mate pair libraries with 3, 8, or 20 kb insert size in addition to paired end data. Newbler can also be used for assembling transcriptome data. To do so, it first collects reads into Isogroups, and then creates Isotigs out of those groups.

SSAKE (the Short Sequence Assembly by progressive *k*-mer search and 3′ read Extension) program was developed in an attempt to achieve the goal of *de novo* genome assembly using 25-bp reads. Simulated error-free data sets created for bacteriophage (PhiX174), SARS virus (SARS TOR2), bacterial (*Haemophilus influenzae*), and metagenomic (Sargasso sea) projects were aggressively assembled by using a prefix tree. Data sets are stored in hash tables and the assembly process goes through a number of iterations searching for progressively shorter 3′-most *k*-mers for every new read added into the assembly. This approach allowed a complete assembly of the PhiX174 genome and generation of reasonably long contigs covering the nonrepetitive regions of viral, microbial, and even metagenomic samples.

These were the first attempts to satisfy the growing need for advanced NGS assembly tools. In recent years, numerous assemblers have been created to assemble genomes sequenced with different sequencing technologies and approaches (Table 2). Majority of the newly developed assemblers use the de Bruijn graph approach to assemble genomic data. They aim to assemble genomes of different size (microbes, fungi, mammals, plants) and complexity (haploid, diploid).

One of the most notable microbial assemblers nowadays is SPAdes (St. Petersburg Academic University, Russia) (Bankevich et al., 2012; Nurk et al., 2013). This tool, while originally developed for the purpose of overcoming the complications associated with single-cell microbial data (uneven coverage and increased level of chimerical reads), was recognized by the scientific community (Magoc et al., 2013) as one of the best assemblers working with both isolates and single-cell data. The latest version of SPAdes allows working with different combinations of sequencing platforms including Illumina, Ion Torrent, PacBio and Sanger using both paired-end and mate-pair libraries of different insert sizes. Its error correcting modules (BaseHammer (Nikolenko et al., 2013) and IonHammer) ameliorate read quality prior to assembly and then later on quality of assembly is once again improved by running repeat resolution and scaffolding modules.

For greater details on algorithms used in different assemblers please read (Miller et al., 2010) and follow the links in Table 2.

Assembly of Metagenome Sequences

The interest in conducting genomic analysis of microbial communities that exist in a variety of natural habitats gave birth to Metagenomics and led to the appearance of numerous metagenomic sequencing projects. The Whole-Genome Shotgun sequencing approach was successfully used for a number of uncultivated microbial community projects. Despite this fact, assembly of shotgun-sequenced metagenomic DNA poses a serious challenge to traditional assembly methods that were developed to handle relatively uniform sequences derived from isolated microbes. In addition to the typically very large size of metagenomic sequencing projects, potential problems observed in metagenomic assemblies include chimeric contigs produced by co-assembly of sequencing reads originating from different species, and nonuniform sequence coverage resulting in significant under- and overrepresentation of certain community members. Communities with one or two highly abundant members represented by several strains can add to the complexity of metagenome assembly due to the presence of extensive strain-level heterogeneity. As a result, often times a nonuniform assembly of the genomes of these abundant members is produced. Depending on the assembler used and sequencing read depth, some fragments are resolved into strain-specific contigs corresponding to different haplotypes, while others are co-assembled into composite contigs with strain-specific variations appearing as single-nucleotide polymorphisms. Large-scale genome rearrangements and the presence of mobile genetic elements (phage, transposons) in the abundant community members result in assembly break points in the areas of synteny breakdown. Many parameters of metagenomic assemblies remain unknown, including the nature and extent of chimeric contigs, influence of the level of polymorphism on co-assembly of reads into composite contigs, overall quality of assemblies and binning (process of separation of scaffolds into species-specific groups), and so on. Assembly accuracy is essential for metagenomic projects since it has much more influence on subsequent analysis and interpretation of metagenomic data than in cases of individual microbes due to coverage-related increases in consensus error rate. Currently, there

Table 2 NGS assemblers

	Programming language	*Sequencing platform*	*Error correction*	*assembly approach*	*Output (contigs/ schaffolds)*	*Genome size*	*Libraries*	*Input file format*	*Output file format*	*Input datasets*	*Reads*	*Reference*
Celera	C++/C/Perl	Sanger, Illumina, 454, IonTorrent, PacBio CCS	Corrects reads, trims reads, removes poor quality reads and duplicates	OLC	Contigs and scaffolds	Prokaryotic/ mammalian	Unpaired, paired-end	.frg	.asm, .fasta .posmap, .qc	Microbes, mammals	Reads >75 bp	http://sourceforge.net/apps/mediawiki/wgs-assembler/index.php?title=Main_Page
CABOG	C++/C/Perl	Sanger, Illumina, 454, IonTorrent, PacBio CCS	Corrects reads, removes poor quality reads	OLC	Contigs and scaffolds	Prokaryotic/ mammalian	Unpaired, paired-end	.fastqa, .fastq, .sff, .frg	.asm, .fasta .posmap, .qc	Microbes, mammals	Reads >75 bp	http://wgs-assembler.sourceforge.net/
Newbler	C++	Sanger, Illumina, 454, IonTorrent, PacBio, hybrid data sets	n/a	OLC	Contigs	Prokaryotic	Unpaired, paired-end	.sff, fasta, .qual	.fna, .qual, .txt, .sff, .tsv, .ace	Microbes, mammals	Up to 2 kpb	http://454.com/contact-us/software-request.asp
MIRA	C++	Sanger, Illumina, 454, IonTorrent, PacBio CCS, hybrid data sets	Iteratively removes sequencing errors	OLC	Contigs and scaffolds with Bambus	Prokaryotic	Unpaired, paired-end	.scf, .fastq, .fasta, .qual, gff3, genebank	.caf, .ace, .fasta, .maf	Microbes (regular isolates), metagenomes	Short, medium, long (<32 kb)	http://www.chevreux.org/projects_mira.html
SGA	C++	Illumina	Corrects reads, removes poor quality reads	Overlap-based string graph	Contigs and scaffolds	Prokaryotic/ mammalian	Unpaired, paired-end	.fastq	.fasta	Microbes (regular isolates), mammals	Short, long	http://www.ncbi.nlm.nih.gov/pubmed/22156294
ABySS	C++	454, Illumina, SOLID	n/a	de Bruijn graph	Contigs and scaffolds	Prokaryotic/ mammalian	Unpaired, paired-end, mate pairs	.fastq, .fasta, .qseq, .export, .sam, .bam	.fasta, .hist, .dot, .adj, .dist, .path, coverage .hist	Microbes (regular isolates), mammals	Short	http://www.bcgsc.ca/platform/bioinfo/software/abyss
AllPaths-LG	C++	Illumina, PacBio	Corrects reads, removes poor quality reads	de Bruijn graph	Contigs and scaffolds	Prokaryotic/ mammalian	Unpaired, paired-end, mate pairs	.fastb, .qualb, .pairs	.fasta, .efasta	Microbes (regular isolates), mammals	Short	http://www.broadinstitute.org/software/allpaths-lg/blog
EULER-SR	C++/Perl*	454, Illumina	Corrects reads, removes poor quality reads	de Bruijn graph	Contigs and scaffolds	Prokaryotic	Unpaired, paired-end	.sff, .fastq, .eland	.fasta	Microbes (regular isolates)	Short	http://www.genome.cshlp.org/content/18/2/324.full
EULER +Velvet-SC	C++/Perl	Illumina	Corrects reads	de Bruijn graph	Contigs and scaffolds	Prokaryotic	Unpaired, paired-end	.sff, .fastq, .eland	.fasta	Microbes (single-cell)	Short	http://bix.ucsd.edu/projects/singlecell/
IDBA-UD	C++	Illumina, IonTorrent,	Corrects reads, removes poor quality reads	de Bruijn graph	Contigs and scaffolds	Prokaryotic/ metagenomes	Unpaired, paired-end	.fasta, .fastq	.fasta	Single-cell, regular isolates, metagenomes	Short (25–150 bp)	http://www.ncbi.nlm.nih.gov/pubmed/22495754
MaSuRCa	C++/Perl	454, Illumina, Ion Torrent, Sanger			Contigs and scaffolds		Unpaired, paired-end	.fastq, .frg	.fasta	Microbes (regular		

			Corrects reads, removes poor quality reads	combination of de Bruijn graph and OLC		Prokaryotic/ mammalian/ plant				isolates), mammals, plants	Short, medium, long	http://www.genome.umd.edu/masurca.html
Ray	C++	Illumina, 454	Corrects reads	de Bruijn graph	Contigs	Prokaryotic/ metagenomes	Unpaired, paired-end, mate pairs	TRACEINFO format, .fasta .fasta .gz .fasta .bz2 .fastq .fastq .gz .fastq .bz2 .sff	fasta	Microbes (regular isolates), metagenomes	Short (25–150 bp)	http://denovoassembler.sourceforge.net/
SOAPdenovo	C/C++	Illumina	Corrects reads	de Bruijn graph	Contigs and scaffolds	Prokaryotic/ fungal/ large plant/ mammalian	Unpaired, paired-end, mate pairs	.fasta, .fastq	.contig, .scafSeq	Microbes (regular isolates), mammals	Short reads	http://soap.genomics.org.cn/soapdenovo.html
SPAdes	C++/Python	Illumina, IonTorrent, PacBio, hybrid data sets	Corrects reads, removes poor quality reads	de Bruijn graph	Contigs and scaffolds	Prokaryotic/ fungal	Unpaired, paired-end, mate pairs	.fasta, .fastq, .fasta .gz, .fastq .gz	.fasta, .fastq	Microbes (single-cell, regular isolates), fungi	Medium + long	http://bioinf.spbau.ru/spades
Velvet	C	454, Illumina, SOLID, IonTorrent	Removes poor qulity reads	de Bruijn graph	Contigs and scaffolds	Prokaryotic/ mammalian	Unpaired, paired-end, mate pairs	.fasta, .fastq, .fasta .gz, fastq .gz, .sam, .bam, .eland, .gerald	.fasta, .afg, .txt	Microbes (regular isolates)	Short, medium	http://www.ebi.ac.uk/,zerbino/velvet
SHARCGS	Perl	Illumina	Removes poor quality reads	Gready	Contigs	Prokaryotic/ mammalian	Unpaired	.fasta, raw	.fasta	Microbes (regular isolates), mammals	Short	http://sharcgs.molgen.mpg.de/
SSAKE	Perl	Illumina, IonTorrent,	n/a	Gready	Contigs	Prokaryotic/ mammalian	Unpaired, paired-end	.fasta, raw	.fasta ??	microbes (regular isolates), mammals	Short	http://www.bcgsc.ca/bioinfo/software/ssake
VCAKE	Perl/C	Illumina	n/a	Gready	Contigs	Prokaryotic	Unpaired	.fasta, raw	.fasta	microbes (regular isolates)	Short	http://sourceforge.net/projects/vcake

is no single best pipeline to follow since each assembly program, each gene prediction algorithm, and each method of binning possesses its own set of benefits and problems. Moreover, each environment has its own particular complexities that are best dealt with using a combination of several tools for analysis. The high complexity and heterogeneity of metagenomic data call for an evaluation of the performance of different assembly tools on metagenomic sequences and require modifications to the assemblers and additional quality control throughout the entire process. To improve the quality of metagenomic assemblies, reads should be stringently quality- and vector- and adapter-trimmed prior to assembly (tools like Lucy, MAGIC-SPP, AdapterRemoval and Cutadapt, can be used for this purpose) and all possible attempts should be made to control quality of metagenomic assemblies produced. Assemblers containing modules to correct sequencing errors and to automatically detect and correct assembly problems (Arachne (Batzoglou et al., 2002), Phusion (Mullikin and Ning, 2003), EULER-AIR (Zhi et al., 2007; Table 2) can be used in metagenomic project assembly.

Depending on the complexity of the microbial community and the representativity of its members, assembly can contain a significant number of unassembled reads (up to 100%). As a result, some researchers choose to forgo assembly and instead focus analyses directly on the unassembled reads. While this method makes sense, genome assembly helps to diminish the size of the dataset for faster processing of further analysis. In addition to that, analytical tasks such as gene finding and taxonomic classification become much easier when applied to assembled contigs.

Several strategies can be used for Metagenomic samples analysis. Reference-based assembly can be done with software packages such as AMOS, or MIRA. These software packages include algorithms that are fast and memory-efficient. Reference based assembly works well, if the metagenomic dataset contains sequences where closely related reference genomes are available (Wu et al., 2009). Several tools are currently available for metagenome de novo short read assembly (MetaVelvet (Namiki et al., 2012), Meta-IDBA (Peng et al., 2012), Meta Ray (Boisvert et al., 2012)).

Directed sequencing is another approach used for metagenomic projects. This is based on the selective sequence of large-insert library clones of interest. The set of clones is screened for a desired function or the presence of phylogenetic markers. Shotgun and directed sequence approaches can be combined to help each other: random sequencing of large-insert libraries guides the selection of clones for complete sequencing using either traditional Sanger sequencing or one of the second generation DNA sequencing methods. This combination brings together the advantages of broader coverage provided by shotgun sequencing with the ability to sample specific genome areas in low-abundance organisms without oversequencing more abundant members of the microbiome. Despite the difference in sequencing strategies, processing of data generated by these approaches faces similar challenges due to inherent incompleteness and lower quality of the sequence data, and in many cases due to unknown origin of each sequence fragment.

A metagenome specific avenue of data analysis, called binning, assigns sequence fragments of a metagenome to particular species population or broader taxonomic groups based on information such as sequence similarity, sequence composition or read coverage. This is used for draft genome reconstruction, when sequencing coverage is insufficient for reconstruction based on assembly information alone.

Sequencing of paired-end and mate-pair libraries greatly improves genomic assembly by providing information about distances between two individual reads that can help with repeat resolution and contig scaffolding. The ordering and orientation of contigs within a scaffold can be used to check binning quality, which is why binning has been used to refine assembly in a feedback process. In recent studies, the combination of metagenome sequencing and taxonomic binning allowed to reconstruct several partial genomes.

Advances in metagenomic assembly will facilitate the linkage of function and phylogeny through the analysis of the assembly itself, but the assembly also helps with examining genetic variation and environmental distribution through fragment recruitment of different metagenomes to the assemblies.

Single-cell genome assembly

Single-cell genome assembly is a fairly new and challenging task. The main difference between single-cell and regular data sets becomes apparent when a whole genome amplification (WGA) method is used in order to amplify enough gDNA for the subsequent genomic library creation step.

As can be deduced from the term 'single-cell' this approach begins with a unique cell and therefore a very limited amount of gDNA starting material is available for the genomic project. Single cells can be isolated from the environment samples using such lab approaches as flow cytometry, micromanipulation, microfluidics, serial dilutions, laser capture microdissection, and fluorescence-activated cell sorting (FACS). The most popular WGA widely used for microbial single-cell gDNA preparation is called multiple displacement amplification (MDA) (Lasken, 2009). This method, while providing a sufficient amount of gDNA, complicates genome assembly by generating highly uneven coverage of the genome, as well as causing production of a significant amount of chimeric reads and reads with higher error rate. To overcome these problems new assembly algorithms and new single-cell assemblers (EULER+Velvet-SC (Table 2), SPAdes (Table 2), IDBA-UD (Table 2)) were developed. Another factor that affects assembly quality is the inconsistent outcome of MDA experiments ranging from a very low level of genome recovery to nearly complete reconstruction of the genome.

Nevertheless, single-cell genome sequencing is a powerful approach that demonstrates great promise for environmental biology since for the moment the majority of microbes cannot be grown in a lab environment and this is the only way to study them.

The recent development of a new amplification method called MALBAC (multiple annealing and looping-based amplification cycles) that 'introduces quasilinear pre-amplification to reduce the bias associated with nonlinear amplification' (Zong et al., 2012) was successfully applied during single human cell analysis. This opened the door for genomic studies of heterogeneous clinical samples, rare cell types, single-cell transcriptomic analysis and will undoubtedly have a significant impact on biology and medicine overall.

Genome Assembly Improvement and Finishing

None of the sophisticated computer algorithms described above is able to automatically reconstruct the entire genome from sequencing reads. They all produce so-called 'draft' assemblies, which are never perfect. Typically, there are problems such as misassembled areas usually caused by repetitive regions (repeats), sequence and physical gaps, areas of low coverage and/or poor quality. The better the assembler can handle such problems, the higher-quality draft will be created and the faster and easier the assembly improvement step (finishing) will be. Finishing is the process of transforming a draft assembly into a finished one. During this step all repeats are identified and assembled correctly, all misassemblies are resolved, all gaps are closed, and all bases are identified with high accuracy. Thus finishing is the process of incrementally improving an assembly by using computational tools, techniques and experimental protocols. Despite the fact that a solid suite of software tools and web applications have already been created, finishing genomes remains a labor-intensive process. It requires experienced personnel and laboratory experiments to support finishing strategies.

It is commonly believed that finishing begins by the ordering and orientation of contigs (scaffolding) for subsequent gap closing. However, the experience collected during the course of assembly and finishing of more than 500 microbes demonstrates that it is more efficient to begin the process of draft assembly improvement with misassembly resolution. It is rather logical, since incorrectly assembled contigs lead to the construction of erroneous scaffolds, created on the basis of false connections. In addition to real gaps, such assemblies will contain pseudo gaps. All this in turn will lead to further complications and the lengthening of the finishing process.

The areas of potential misassemblies can be recognized at different stages of genome assembly via the application of different methodologies. Some assemblers can identify and even correct repetitive areas during the assembly process (Figure 3). Some of them use paired reads information (detecting areas of clone mate inconsistency including reads placed too far apart, too close together or incorrectly oriented pairs), statistical information (areas of significantly higher coverage than the average) or mask out repeats (in this case additional efforts for assembly of repeats are required). Up to 80% of the repetitive areas can be addressed automatically, while the remaining ones (the most ambiguous) need additional laboratory experiments and manual efforts for their resolution. Additional tools have been developed for more effective detection of repeats. They can be used after the initial draft assembly has been created. For instance, RepeatMasker locates repetitive areas and excludes them (masks) from further searches for similarity regions. In addition to the exact repeats, a tool named REPuter recognizes degenerate repeats thus allowing for a certain rate of sequence errors. It is able to detect not just direct repeats, but also palindromic repeats and other closely related sequence features. Tools like equicktandem, mrep, and Tandem Repeat Finder find tandem repeats of certain size by using statistical (equicktandem) or heuristic (mrep, Tandem Repeat Finder) algorithms for their detection. Another software package, Vmatch, represents a collection of programs, for solving large-scale sequence matching tasks.

Repetitive elements can be of different size and may differ from each other by one or more bases. Most repeats of 2–3 kb are due to insertion elements. Multiple copies of ribosomal DNA sequences represent longer repeats of 5–8 kb. In some microbial genomes, repetitive elements of 70–100 kb were observed. They represent duplications of large areas of the genome, which could have occurred during the course of evolution of the particular microbe in question. Besides being differentiated by their length, repeats can be split into three categories by type: direct, inverted, or tandem repeats (Figure 4). While direct repeats and different copies of tandem repeats can collapse in one location or become rearranged during the assembly, copies of inverted repeats may be assembled in the wrong orientation thus creating pseudo physical gaps (Figure 4). By using for example the Miropeats program one can draw a graph that will help to distinguish tandem repeats, inverted repeats and palindromes; however, biological

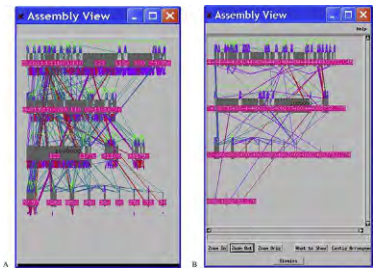

Fig. 3 Draft assembly views of burkholderia phytofirmans PsJN (GenBANK http://genbank:AAUH00000000): (a) Assembly produced by phrap; (b) Assembly produced by PGA. Grey boxes represent contigs. Purple lines above the contigs represent clones that span gaps between contigs and join them. Lines of different colors below or between contigs indicate misassembled paired reads.

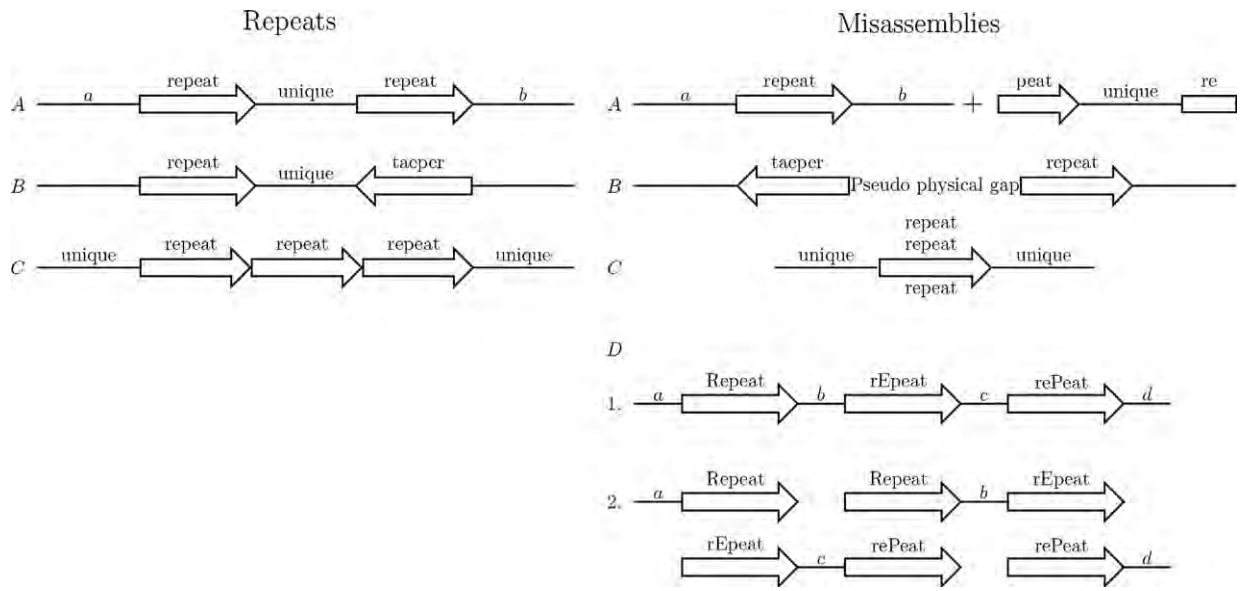

Fig. 4 Types of repeats and potential misassemblies. Repeats: A – direct repeat; B – inverted repeat; C – tandem repeat. Misassemblies: A – incorrectly linked non repetitive areas *a* and *b*, located up- and downstream of the direct repeat; B – pseudo physical gap caused by incorrect placement of copies of inverted repeat; C – collapsed copies of tandem repeat; D – rearrangement of identical or nearly identical copies of repeats (1 – correct order; 2 – misassembled area).

duplication events and assembly mistakes cannot be distinguished by this program. Another open source graph visualization software Graphviz represents structural information as diagrams of abstract graphs and networks and is widely used for genome assembly visualization and analysis.

One of the commonly used approaches to repeat resolution consists of the following steps: repeat localization, identification of the reads belonging to each repetitive element, pooling of (grouping) the defined reads, their assembly into separate subassemblies for each individual copy of a repeat and exporting the resulting consensus back to the main assembly as one 'long read' (fake read) for each subassembly. Tandem repeats represent the greatest problem in successful repeat resolution. They are especially troublesome if the length of one copy is longer than the insert sizes of the libraries available for the sequencing project. The reads belonging to different copies of a repeat and entirely lying within the repetitive region collapse into one pile during draft assembly. This means that assembler produces fewer copies of the repeat than the finished sequence contains. Because the library clones/fragments are not uniformly distributed over the genome, it is not sufficient just to estimate the extent of increased coverage in the area to discover the number of copies of a repeat gathered in such piles. An *in vitro* transposon insertion strategy involving a random insertion of a yeast transposable element into a repeat-covering, circular plasmid is one of the most powerful experimental approaches for tandem repeat resolution. Transposon 'bombing' allows random identification of new sequencing start points within the repeat and the generation of new sequence directed away from the insertion points.

Some modern assembly tools have repeat resolution and miss-assembly solving modules build in. For example the exSPAnder algorithm (Prjibelski et al., 2014) used in SPAdes assembler for repeat resolution, accurately resolves repeats when both single and multiple NGS libraries of read-pairs provided for standard and single cell assembly projects. In hybrid Illumina/PacBio or IonTorrent/PacBio assemblies SPAdes uses PacBio CLR reads for gap closure and repeat resolution. It can also use high quality contigs of the same genome generated by other assembler(s) and merge them into SPAdes assembly. Such contigs will be used for graph construction, gap closure and repeat resolution. If assembler is provided with preassembled contigs of a poor quality, it will use them for gap closure and repeat resolution only and will build a graph using Illumina or Iontorrent reads. ExSPAnder as well as Ray and Telescoper (Bresler et al., 2012) assemblers use a simple path extension approach for repeat resolution and combine it with some ideas from the Rectangle Graph approach.

After all the misassemblies have been resolved, it is time to fill in the gaps between the contigs, in order to produce contiguous sequence for each DNA. In practice, the process of closing gaps between the correctly assembled contigs can be started in parallel with repeat resolution.

aired reads information and the knowledge of the library insert size distribution allow ordering and orientation of correctly assembled contigs and organizing them into scaffolds (Figure 2). Software tools called scaffolders were created to assist finishers in this complex task. Consed and Bambus are among the most popular tools of this type. These and other similar programs (e.g., scaffolders within several assemblers – Table 2) provide a global overview of the interdependency between the contigs, and help with selection of particular clones that contribute to bridging gaps and planning of finishing experiments.

Small sequence (captured) gaps can be closed by custom primer walking on the existing clones (in case of Sanger libraries) that span gaps. Such clones can be distinguished by the presence of the sequence from each end of the insert in two separate contigs. During the primer walking procedure a custom primer designed for the end of the contig is used to perform sequencing reactions to

extend sequence information into the gap. This process is repeated until the entire gap-spanning plasmid is sequenced. It is faster and more cost efficient to close captured gaps of 5 kb or longer by transposon 'bombing' of gap-spanning clones (Epicenter Biotechnologies) or by producing a shatter library of appropriate plasmids or PCR fragment. In the shatter method, the chosen DNA template is sonicated into fragments of 100–300 bp, which are then subcloned into a vector and sequenced by using standard vector primers.

Many captured gaps are difficult to sequence by primer walking. Problems usually arise due to the presence of strong secondary structures (very common in genomes with GC content higher than 65%) and hairpin structures and/or long homopolymer stretches in the DNA. Sequencing very short inserts produced by shatter libraries usually helps with most DNA structures because only a small part of the secondary structure or hairpin is cloned. Short read NGS technologies also break down secondary structures or hairpins.

Linking information, usually provided by sister reads, is not available for physical gaps, existing between contigs or scaffolds. Sometimes the comparative analysis of closely related reference and target genomes can provide a clue for contig mapping. If the end sequences from the two contigs encode two different parts of the same protein, it may be assumed that these contigs should be linked. It has also been shown that the gene composition of some operons is very well conserved between genomes. This information can also be used to link the contigs containing parts of such operons at their ends. Several software tools have been developed to assist with this analysis by comparing the entire genomes or contigs from different assemblies against each other and against reference genome(s) (GMPTB, MUMmer, Projector2, SIBELIA and so on). Such assisted assembly strategies go beyond just helping with gap closing. They offer an opportunity to sequence new organisms to less than the typical sequence depth. For those cases in which none of the approaches described above is helpful, direct laboratory experiments need to be performed. For example, a technique called optical mapping compares the assembled contigs against the collection of restriction maps of a DNA molecule. Each map produced by a rare cutting restriction enzyme serves to order and orient contigs and scaffolds separated by uncaptured gaps. Selected restriction fragments can be used as templates to produce the sequence for gaps.

The recently developed BioNano Irys approach for generating optical maps attaches fluorescent labels to the specific sites of the DNA using single-stranded DNA nicks. Then the DNA fragments are linearized in nanochannels and images are taken with a CCD camera. The images are processed to obtain positions of the labels along each genomic fragment. The fragments are typically 100–200 kbp long and the labels are located approximately 5–10 kbp apart depending on the nick pattern selected for inserting the fluorescent labels. Combined with genome assembly this technique significantly improves repeat resolution and scaffolding steps.

When no other templates are available, polymerase chain reaction (PCR) products generated across the gaps can be used to map contigs or scaffolds and to produce sequence for the gaps. In order to build a PCR map, unique primers are designed for the end of each contig (it is necessary to make sure that primers correspond to regions outside of repeats) and used in a PCR experiment to test whether a particular pair of primers links the contigs. This approach is not feasible in the case of large numbers of contigs to be mapped, since it requires too many PCRs to be performed and analyzed.

An improved version of the combinatorial PCR method, Multiplex PCR, was developed to optimize this process. This approach is based on the simultaneous use of multiple primers (up to 32) in mapping experiments with further analysis of which two primers made the PCR fragment (Figure 5 schematically represents the use of Multiplex PCR approach for mapping of four contigs).

The advantage of the Multiplex PCR method versus the combinatorial PCR approach, where each end primer is verified against all others except the one that was designed for the second end of the same contig/scaffold, is that it requires fewer PCRs to map the contigs. Thus, to order and orient four contigs (Figure 5) one should perform nine multiplex reactions instead of 24 combinatorial ones. The difference becomes more prominent when larger numbers of contigs are involved in the experiment (only 17 multiplex PCRs versus 120 combinatorial PCR reactions are needed to map eighth contigs). The PCR products thus obtained are sequenced to close the gaps between mapped contigs or scaffolds.

Sequencing and mapping complications associated with strong secondary structures and unclonable areas present significantly fewer challenges for the second generation of sequencing technologies, as the majority of them rely on assembly of very short overlapping fragments and do not require fragment cloning in *E. coli*. As a result, 454 contigs, assembled by the Newbler assembler effectively cover physical gaps, produced by WGS. The highest success rate was observed for genomes with low GC% (<40%), for which a combination of Sanger and 454 sequencing was used. Such a combination allowed closing of up to 100% of uncaptured gaps (Figure 6) (unpublished data). The 454–Sanger combined approach also allowed the completion of the uncultured Gram-positive bacterium, *Candidatus Desulforudis audaxviator*, from a low-biodiversity water fraction collected at 2.8-km depth in South Africa.

In addition to the above-mentioned problems, a typical whole-genome draft assembly contains low-quality regions and regions poorly covered by clones or fragments. For the genome to be considered finished such areas should be improved by resequencing of the existing clones or of the appropriate PCR products (so called polishing step). Illumina and other new technologies, are very valuable for faster and cheaper polishing of microbial genomes. Results originally reported at the Advances in Genome Biology and Technology meeting (AGBT) in 2008 supported this notion: a Solexa/Illumina run aligned to a 454-assembled bacterial genome identified (and allowed correction of) 98% of frameshifting indels. More importantly, the remaining frameshifts were in fact true indels in the genome.

To completely wrap up a microbial project it is necessary to confirm the correctness of the final assembly and the accuracy of the finished sequence. The sequence quality standard used for Human Genome sequence data and named 'Bermuda' standard (1 error per 10 000 bp) has also been accepted in microbial sequencing. The final human inspection step supported by visualization tools (DNPTrapper, Hawkey, Orchid) aims to verify that all bases of the produced consensus are supported with enough coverage

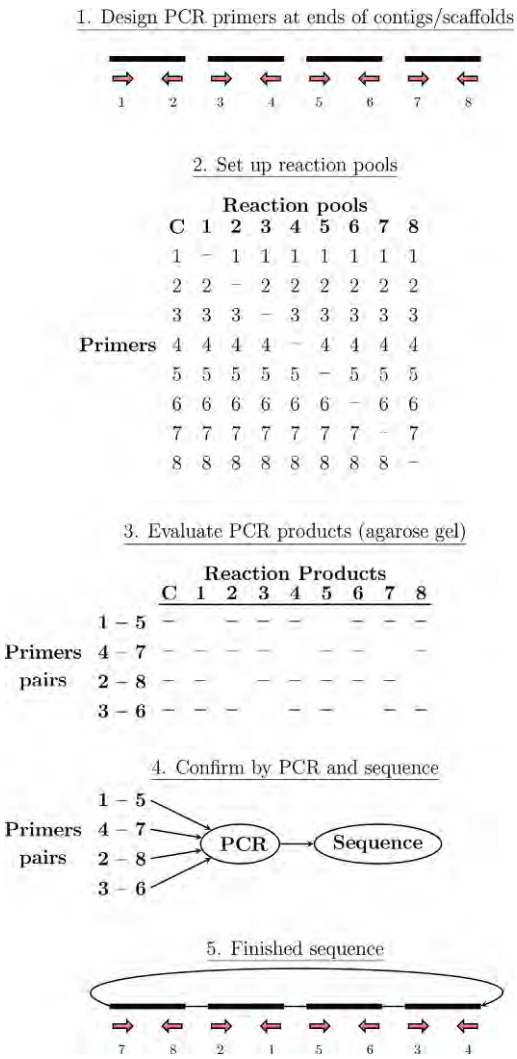

Fig. 5 Multiplex PCR approach: schematic representation. (1) Contigs are represented by black boxes; arrows indicate primers. (2) Primers mixed in each experimental reaction. C – control mix, containing all designed primers. (3) Scheme of electrophoretic analysis. (4) Confirmation by using identified primers pairs. (5) Final contig composition.

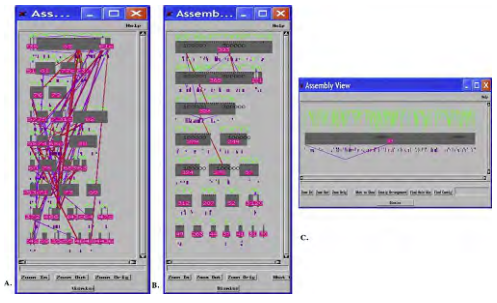

Fig. 6 Assembly views of desulforudis audaxviator: (a) draft assembly; (b) assembly after repeat resolution and two rounds of primer walk; (c) assembly after repeat resolution and two rounds of primer walk combined with 454 data (for details see legend for Figure 3).

(less frequently observed with new technologies), that consensus quality corresponds to the agreed standards (1 error per 100 000 bp for NGS assemblies), that all produced reads are correctly assembled (consistent), and that all repetitive areas are resolved (Chain et al., 2009).

Progress in DNA sequencing technology, improvements in finishing strategies and tools, as well as the availability of a number of assemblers and advanced methods for genome annotation have significantly reduced the time required for genome closure.

Despite this fact, the effort and time required for genome closure depends on the quality of the whole genome shotgun libraries created for the project, GC content of the genome, the size and frequency of identical or nearly identical repetitive structures, and the number of regions that cannot be cloned or are hard to clone in *E. coli* (if the cloning step is involved in a project). A finished genome represents the genome assembly of high accuracy and quality (with no gaps), verified and confirmed through a number of computer and lab experiments. The value of complete microbial genome sequences has been long established and appreciated by the scientific community. In addition to the genes that differ by a very few bases from each other and thus co-assemble in draft assembly, the genes coding for proteins toxic for *E. coli* remain undetected by the analysis of incomplete genomes. Furthermore, new sequencing technologies, developed for genome resequencing (Solexa/Illumina, SOLiD, IonTorrent) as well as the growing interest in metagenome sequencing projects, dictate a greater need for high-quality references. A significant number of microbial genomes still needs to be sequenced to full completion for successful use of new sequencing platforms in many areas of general microbiology, ecology, evolutionary studies, and so on.

The DOE Joint Genome Institute has established a project named GEBA (Genomic Encyclopedia for the Bacteria and Archaea), which is focused on filling gaps in the Tree of Life and has the long-term goal 'to generate reference genomes for every major and minor group of bacteria and archaea'. This is spearheading a major expansion of knowledge of bacterial phylogeny.

Third-Generation Sequencing and Genome Finishing

The best way to correctly assemble repeats, consequently not only closing, but possibly even avoiding gaps in assembly all together, is to generate reads that are significantly longer than the repeats themselves. This way they can unambiguously traverse repetitive areas. Pacific Biosciences (PacBio) is the first sequencing technology that allowed production of reads up to 15 Kbp long. Random error profile helps eliminate the problem associated with the high error rate (14–15%) of reads. Sufficient sequence coverage and/or error correction (Koren et al., 2012) significantly improve the quality of the consensus.

Looking at the PacBio production level one can ask why use PacBio when we get so much more data out of the Illumina sequencers? The answer is simple – single molecule approach enables unique opportunities for overcoming such short-falls of Illumina platform as GC bias, hard stops, need for multiple (short insert, mate pair, chemical methylation detection) library preps. Long reads, fast turnaround, flexibility to get high quality single molecule consensus (short insert CCS) or long reads for assembly/scaffolding/gap closure make this technology very advantageous.

This long read sequencing technology started the so-called third generation sequencing technology revolution that is currently being supported by Illumina (TruSeq synthetic long-reads), Oxford Nanopores, BioNano (Irys technology) and other companies. Such read length and consensus accuracy (up to 99.9%), as well as new assembly tools (Dazzler (the Dresden AZZembLER); SPAdes; AllPaths-LG; MIRA) and processes (HGAP (hierarchical genome-assembly process) (Chin et al., 2013)) will revive the production of completely assembled reference quality (Chain et al., 2009) microbial genomes that was slowed down for the last several years due to both high cost and labor intensiveness of finishing. An automated pipeline called PBJelly aligns long sequence reads to draft assemblies in order to close or improve captured gaps. It is currently applicable to PacBio RS reads, but can be generalized to any long read data.

The crucial role of completely finished microbial genomes in comparative genomics, metagenomics, diversity studies etc was already discussed above.

Assessing Quality of Draft Genome Assembly

Large amount of assemblers currently available for researches (not all of them are listed in this review) address many different aspects of genome assembly complications, but none of them are able to solve them all. This means that none of them are perfect and one has to decide which particular instrument to select based on the specifics of the project being worked on. Furthermore, assemblers can create different assemblies depending on the data sets provided and assembly parameters chosen.

Assembly evaluation tools can help answer the question of what assembly is better for further analysis by assessing the quality of an assembly and comparing different assemblies against each other. Developers of such tools need to choose what metrics to use to assess the assembly quality. Tools should work without relying on the high quality reference genome and be convenient for users with any level of experience (the latest requirement is true for most of other tools including genome assemblers).

QUAST (Gurevich et al., 2013) and GAGE (Salzberg et al., 2012) are the two most popular tools of this type used for microbial genome assembly QC. These tools compute different metrics to assess the assembly quality. The most common metrics used are: the total number of contigs in the assembly, the length of the largest contig, the total length of the assembled contigs and N50 (N50 length is the length of the shortest contig in the whole set such that the sum of contigs of equal length or longer contains at least 50% of the total length of all contigs). Parameter N50 becomes absolutely useless if the contigs are incorrectly assembled. So, additional information on missassemblies is very valuable. All of these and any other additional metrics should never be considered in isolation, since they begin to make sense only when being considered in combination with other assembly characteristics.

NCBI Trace and Assembly Archive

The Trace Archive was established in 2001 to collect raw data produced at sequencing centers around the world. This depository represents a collaborative effort between NCBI (http://www.ncbi.nlm.nih.gov/), European Molecular Biology Laboratory (EMBL/ENSEMBL) and DNA DataBank of Japan (DDBJ) and it currently (04/13/2014) contains 2 178 623 682 traces. The amount of data in the archive doubles approximately every 18 months and new sequencing technologies as well as large sequencing programs like the Microbial Earth project, Earth Microbiome Project, 100 K Pathogens, The Human Microbiome Project, Genome 10 K, 1000 genomes etc. will result in an even higher rate of data increase in the future. To accommodate massive flow of short sequencing reads generated by the next generation sequencing technologies NCBI has constructed (2008) a new repository named Short Read Archive (SRA).

NCBI's SRA, Trace and Assembly Archives (The Assembly Archive created at NCBI in 2004 (Salzberg et al., 2004) provide direct access to the raw traces and assemblies and give researchers a unique opportunity not only to reconstruct the assembly from raw shotgun and finishing reads obtained from the Trace Archive and/or SRA, but also to verify the quality of the assembled genome and its accuracy. Any potential or real frame shifts detected in the course of genome annotation can be thus double-checked and corrected on the basis of the details of assembly.

Because different sequencing centers and individual researchers use different tools to assemble sequences, the generated assemblies may come in a variety of formats. Regardless of the specific format, the data can still be submitted to The Assembly Archive, following detailed information provided in the NCBI Assembly Submission Guide.

Fig. 7 Assembly viewer display of *Salinispora tropica* CNB440 draft assembly (http://www.ncbi.nlm.nih.gov/). The overlapping traces comprising the assembly and detailed alignments are shown.

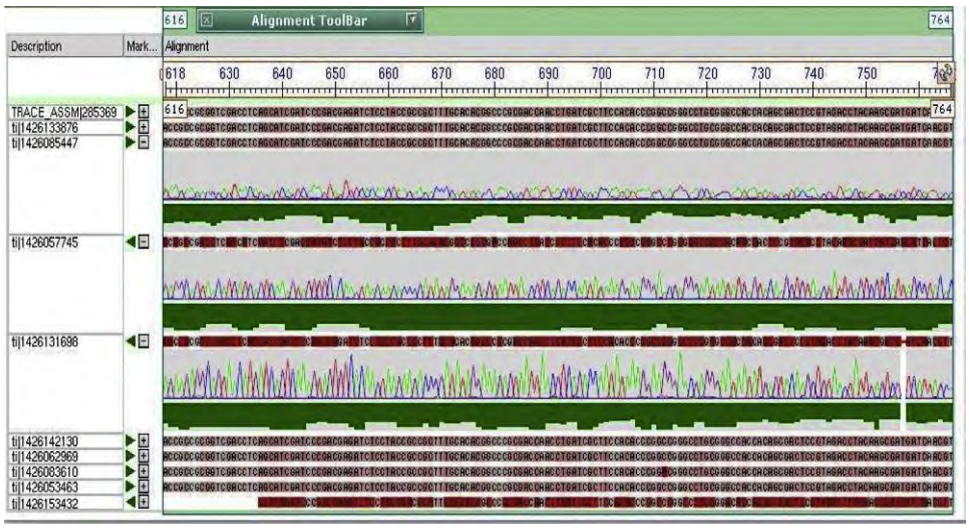

Fig. 8 The underlying sequences and traces from the draft assembly of *Salinispora tropica* CNB440.

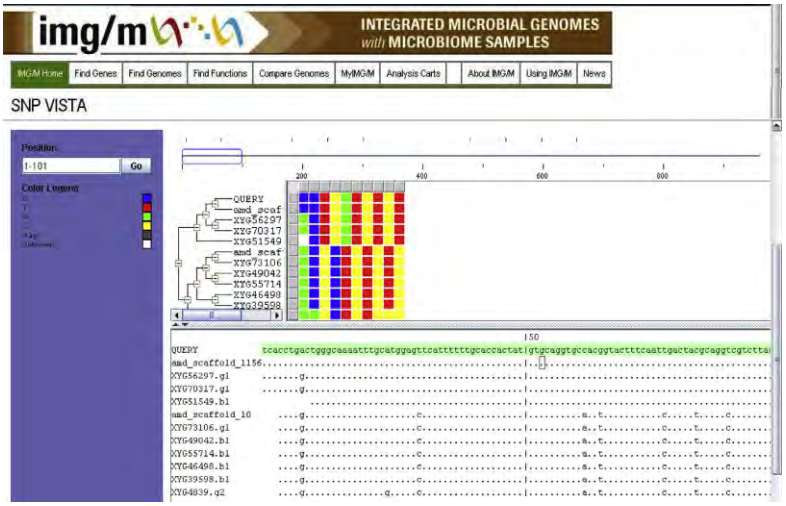

Fig. 9 Snapshot of the single-nucleotide polymorphism (SNP) VISTA viewer for the metagenomic project of acid mine drainage.

Figure 7 illustrates one small region of multiple sequence alignment of reads to the *Salinispora tropica* CNB440 genome sequenced at JGI. Another function made available by The Assembly Archive is the ability to see the individual bases in the DNA sequence by examining the aligned traces (Figure 8).

This is the feature that opened the door for analysis of single-nucleotide polymorphisms (SNPs) in a broad range of eukaryotes and for the haplotype analysis of metagenomic assemblies. The haplotype analysis of metagenomic data can also be performed in IMG/M (http://img.jgi.doe.gov/), a metagenome data management and analysis system developed by the DOE Joint Genome Institute (Figure 9).

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http://www.graphviz.org/—Graphviz.
http://www.hmpdacc.org/—The Human Microbiome Project.
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http://staden.sourceforge.net/—Staden package.
http://www.vmatch.de—Vmatch.
http://www.zsgenetics.com/—zs genetics.

Genome Sequence Databases: Types of Data and Bioinformatic Tools☆

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Glossary

α-Diversity Is the mean species diversity of a given sample or site, as opposed to β-diversity which takes into account the species diversity across different sites.
Average nucleotide identity (ANI) scores In the pregenomics era, a bacterial species was defined if 70% of the DNA from two bacteria hybridized in the molecular biology technique known as DNA–DNA hybridization (DDH). In 2007, the Kostas lab introduced the ANI scores by comparing DDH values to those of whole genome comparisons of those strains whose genomes were available at the time. Using both best hits (one-way ANI) and reciprocal best hits (two-way ANI) between two genomic datasets, they established a threshold of "same species" where their ANI values are above 95% (e.g., *Escherichia coli*). ANI values below 75% are not to be trusted. They keep an online tool, the ANI calculator that supports the comparison of any two genomes to be uploaded.
Barcode gene The term barcode, referring to the one you find on groceries, induces the idea that, ideally, any species could one day be identified simply by scanning a piece of genomic DNA and looking into the appropriate reference table to find the perfect match. Until that day comes, we'll have to settle for SSU genes (16S rRNA for prokaryotes and 18S rRNA for eukaryotes).
Clustering Clustering algorithms have the goal of grouping a set of objects, in this case, sequences, that are more similar based on certain criteria (usually, sequence identity). In the metabarcoding context, the process of iteratively assembling reads picked from a pool into OTU groups (or a taxon). It generally consists on aligning and measuring similarity/distance between read sequences.
Data Warehouse Is a collection of data from varied sources to provide meaningful insights blending components in order to allow the strategic use of data.
HMM A Markov model is a statistical model in which the probability of an event depends on the immediately previous event. In a HMM (for hidden Markov model) the parameters of the process are "hidden" and the challenge is to determine them from the observable data.
OTU Stands for Operational Taxonomic Unit.
Quality score Describes the quality associated to each base pair in a raw-read.
Raw-read Are those generated directly from the sequencing company, without any postprocess such as cleaning, trimming, adapter removal, etc.
Speciation Through accumulated mutations, specific niche adaptations, and isolation, organisms evolve to form new divergent species.
Taxon A monophyletic group of sequences or organisms that form a unit, which could be a species.

Introduction

When the previous edition of this encyclopedia was printed 10 years ago, bioinformatics had a different meaning and was applied differently. Although some techniques/approaches have not changed, we have clearly moved from studying single strains to studying microbial communities. Although we have learned a lot by studying model organisms, there is also a benefit in understanding how they interact with other members of their natural habitat, with the environment, and with their hosts. Focus may then be moved towards the particular physiology of that organism to understand its role in the ecosystem, and then back to the ecosystem to understand the metabolic functions of the global community. On top of studying individual species, we now aim to understand microbes and their communities. How do microbes behave in their respective ecosystem? Which metabolic functions do they perform? Do they do it by themselves or do they share the pathways with other commensals and interchange metabolites? Who are the primary producers in the community? Who fix carbon and who are the ones that ferment the byproducts made

☆*Change History*: January 2019. A. Gutiérrez-Preciado et al. have made major changes to the references and text.

This article is an update of A.G-Preciado, M. Peimbert, E. Merino, Genome Sequence Databases: Types of Data and Bioinformatic Tools, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 211–236.

available to the rest of the inhabitants? Once we know the identity of the major players and their genomes are available, more specific questions can be addressed. Which gene codes for a given transcript or protein that performs a particular function in a specific organism? How, and to what extent, is such a gene regulated? What is the three-dimensional structure of its corresponding protein? What other transcripts or proteins participate in the cellular pathway? Which scientific articles are related to this gene, protein, or pathway? If we change to a different ecosystem where the same genes and the same pathways are found, is the same organism the one that is performing such an action? Are there representative (core) organisms of certain environments? Or is it more that there are core metabolic functions for each ecosystem? Can the pattern of the present/absent species serve as a bioindicator of a healthy ecosystem?

This article will focus on the great advances that have changed bioinformatics in recent years, with a special spotlight on microbial communities, and an aim to review the tools and databases that may aid in answering these questions through either sequencing or accessing the enormous sequence collection the scientific community has gathered. In bioinformatics, there are no golden rules on how things should be done. The same problem can always be solved in several ways and, as such, for each problem, there are often multiple bioinformatics tools available to deal with it in different scenarios. We describe here a set of tools that we have found useful in our own experience but encourage the reader to go further and explore additional bioinformatics tools to gain a wider perspective on their microbial communities of interest.

Data Sources

The last 10 years have witnessed the democratization of high-throughput sequencing, to such extent that, in a single day, state-of-the-art sequencers can produce the equivalent of the entire nucleotide archive available when the previous edition of this encyclopedia was published (see Fig. 1). This massive amount of data has led to new challenges in bioinformatics, starting with storage issues, as nowadays it is cheaper to "read" a nucleotide than to permanently store its identity, position, and sequencing depth, all the way up to the dedicated analyses providing biological insights. In this section, we will cover how and where sequences are stored; and what kind of analyses can be performed in order to best utilize our sequence data.

Sequence Repositories

Sequencing machines do not really "read" biological polymers; instead they transform their read-out (i.e., the fluorescence associated to the release of a labeled pyrophosphate or the characteristic interference of the electrical current while a single biopolymer passes through a pore) into a string of characters. While the real "raw-reads" are the machine's read-outs, these are most commonly only available to whoever has paid for the sequencing, and seldom analyzed for other uses than quality control (although some can include biologically relevant information, such as base modifications that produce distinct read-outs, like methylation). For researchers outside of sequencing centers, the term *raw-read* is more often used to refer to the string of characters and an associated *quality score* that specify how confident the identity of a character is based on the sequencing reaction, itself depending on the sequencing chemistry and the local sequence properties. The character strings and their associated quality scores are stored in a human readable format called *fastq*. The sequence is stored in conventional fasta format and the quality scores in a legacy format called *phred*. Each read thus is stored in four consecutive lines, two normally containing the "read" identifier (header starting with "*@*" for the sequence and with "+" for the quality scores), one containing the sequence string in standard one letter code, and one containing the associated quality scores. Compressed fastq files are publicly stored in sequence repositories, the largest one being the Sequence Read Archive (*SRA*) from the International Nucleotide Sequence Database Collaboration (*INDSC*), consolidating data held by the National Center for Biomedical Information (*NCBI*), the European Bioinformatics Institute (*EBI*), and the DNA Data Bank of Japan (*DDBJ*).

About 21,078 of the entries in the SRA correspond to experiments of whole genome amplification, another 627,669 to RNAseq experiments (nearly half without reference genomes), 690,550 to metabarcoding and 111,155 to WGS metagenomes, these latter being particularly enriched with microbes lacking cultured representatives. Repositories of assembled genomes are presented in a dedicated chapter of this encyclopedia. How much of the total length is covered (completeness), how many times each position was identified (depth or coverage), what proportion of conserved genetic elements does it contain (representation) and the difference between assembled elements and chromosome-like structures (contiguity) are all are important metrics to assess the quality of a genome assembly. While most whole genome assemblies can be accessed through the sites of *INSDC* members, dedicated gateways, such as *EnsemblGenomes* and *GOLD* offer single entry points to finished and draft genomes. The *GOLD* repository also includes records from environmental samples, also known as metagenomes. The website *Integrated Microbial Genomes (IMG)* integrates viral, bacterial, archaeal, and selected eukaryotic genomic data collected from multiple data sources. It has an extended metagenomic *warehouse*: *IMG/M*, which is a data management and analysis system that provides tools and viewers for analyzing both metagenomes and isolated genomes individually or in a comparative context.

Besides being the underlying data for de novo genome or transcriptome assembling, sequence reads and their quality scores are also used for reference-based quantification of DNA modifications (Chip-Seq), differential expression of RNA molecules (RNAseq), population genomics (SNPs), and genome architecture and organization (Hi-C). Despite few model species, most of these techniques and analysis are seldom, if at all, used in the context of microbial genomics. Still, sequence reads are also the main source of data for modern metagenomics. Indeed, after years of surveying for species presence (more often listing of operative

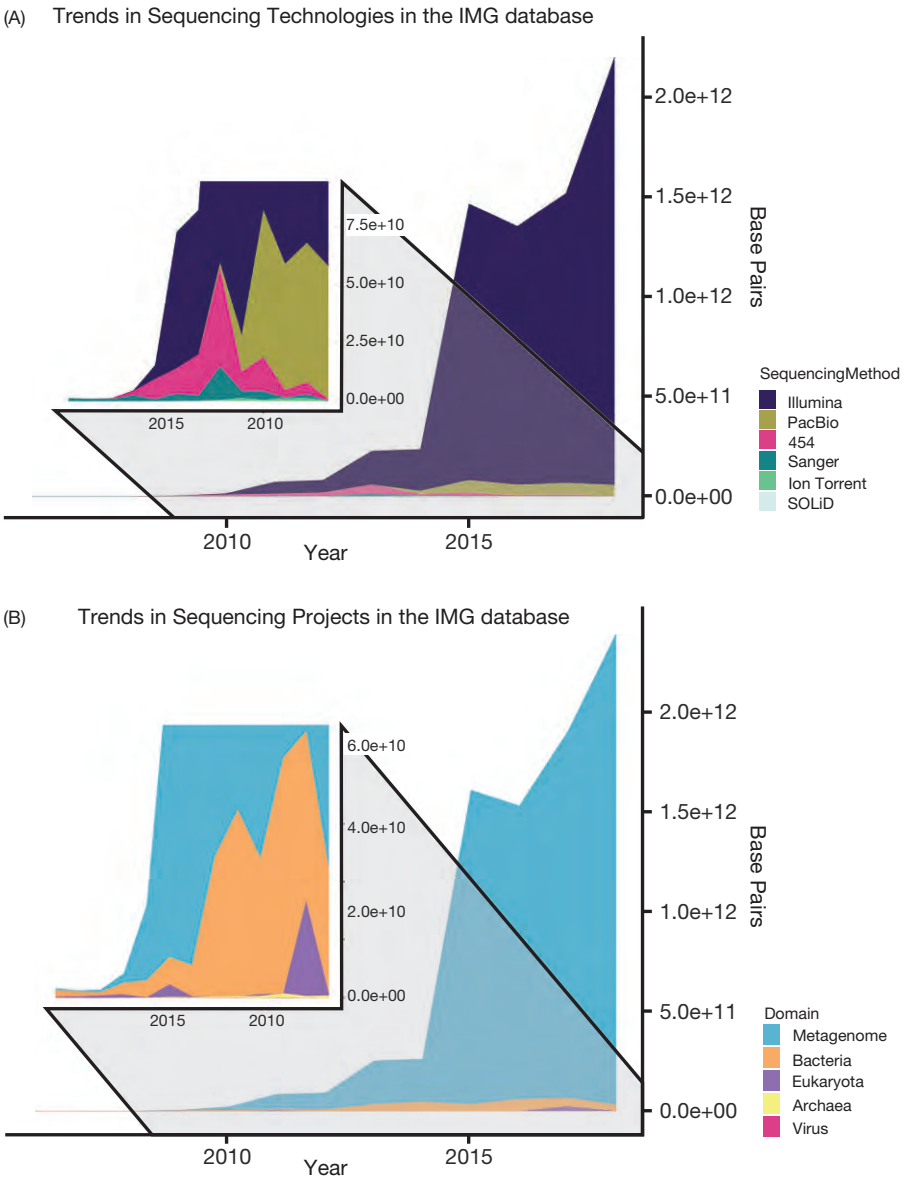

Fig. 1 Growth of sequencing archive as collected by the IMG database. (A) Trends in Sequencing Technologies in the IMG database. (B) Trends in Sequencing Projects in the IMG database.

taxonomic units (OTUs) based on hyper-conserved markers, i.e., the so-called meta-barcoding), the metagenomics field is now taking decisive steps into considering a more comprehensive representation of the biological diversity in all sorts of microbial communities.

Genome Analysis and Annotation Resources

Genome annotation is the process of overlaying other biological data onto the DNA sequence. While draft genomes might contain no annotation or include only basic information such as whether any particular base pair likely encodes a protein or not, genomes and metagenomes are most useful when they are close to completeness and rich in annotations. In the case of metagenomes, knowing the phylogenetic/taxonomic composition is also of paramount importance. To avoid duplication with other chapters of this Encyclopedia, we will limit this section to the introduction of some public resources that leverage already annotated genomes and metagenomes and we will close our article with an application note detailing *anvi'o*; a holistic bioinformatics platform to integrate and visualize all the data as different layers.

For many genome projects, the genome size is not known a priori and even when known, the coding density and redundancy is uncertain. So, how one can know if a draft assembly is already representative of the whole genome? Homologous genes conserved

across multiple taxonomic ranges tend not to appear in multiple copies (Single Copy Genes; SCGs) and the most closely related the taxonomic groups are, the larger will the group of conserved genes be. Identifying what proportion of such conserved, and conservative, genes are already covered by a given assembly, offers one of the quickest ways of answering this question. Implementations of the concept started back in the origins of phylogenomics in the form of Clusters of Orthologous Groups (the foundational, albeit outdated, *COGs*), later refined with more robust assignation of orthology (e.g., *phylomeDB*), and more recently benefited from the ever-increasing number of sequenced genomes (*BUSCO*).

Once annotations are available, genomes can be described and compared to each other. The underlying idea is that while similar genomic features (e.g., conserved protein families) would be the basis of common functions in the compared species or meta-communities, distinct genomic features should define what makes organisms or meta-communities unique. These analyses constitute the core of *comparative genomics*. The single-entry gateways presented above offer dedicated resources for microbial organisms (the *Integrated Microbial Genomes and Microbiomes, IMG/M,* and *EnsemblBacteria*, also including Archaea, *EnsemblFungi* and *EnsemblProtists*), including precomputed sets of orthologous genes. Another widely used resource is *OrthoDB*, a database of precomputed orthologous genes, which can be customized to display orthologous groups across up to 20 species. Conserved elements, such as single-copy orthologous groups, can then be used to resolve the phylogenetic relationships of the species under consideration.

Structure of Microbial Communities: Metabarcoding

Metabarcoding allows to quickly and easily screen the α-diversity of whole microbial communities without the need to culture organisms (Fig. 2). Barcodes are derived through sequencing a barcode gene that can be retrieved from genomic DNA extracted from homogenized biomass. Metabarcoding can describe the structure of very complex communities and has reshaped our understanding of the diversity of extant microbial life, exposing how much actual diversity was previously missed when relying only on cultures and microscopy.

Briefly, by massively sequencing the barcode gene of choice (usually the small subunit complex (SSU) of the ribosome, 16S rRNA for prokaryotes and 18S rRNA for eukaryotes), metabarcoding allows grouping and classification of living organisms into OTUs. OTUs are built upon the comparison of homologous DNA sequences from different organisms using a predefined and assumed difference metric.

Defining thresholds for OTU clustering is highly problematic. With SSU coding genes, there are two historical threshold values that have been widely used: 97% of similarity for 16S SSU rRNA sequences of prokaryotes and 98% of similarity for 18S SSU rRNA sequences of eukaryotes. How can we decide how much difference between sequences is not biologically discriminant? Since this will not always be true, the most recent algorithms dedicated to OTU inference allow "flexible" similarity threshold, or replace the fixed similarity value by local evaluations of informative variations for every read throughout a sample, taking into account the quality of the reads, the frequency of putative erroneous reads, and by measuring how informative are each position along reads.

Once OTUs are determined, the next step is to establish their biological identity. For this purpose, many dedicated reference databases have been built over the last two decades. Specialized databases provide the most reliable set of full-length barcode sequences in terms of sequence quality and taxonomic classification. The steps of defining the OTUs and assigning them a biological identity are key to unveiling the structure of the microbial community, and it is important to understand the algorithms and the resources available. It is worth mentioning that the common practice of inferring the taxonomy of OTUs using simple local similarity search tools like *blastn* can be misleading. More suitable strategies are available that involve a global pairwise alignment against the reference SSU databases (e.g., *SINA* or *NAST/pyNAST*), or phylogenetic placement (e.g., *pplacer*).

Finally, diversity analyses can be performed to understand the differential abundance of the inhabitants in the community and built co-occurrence networks of the OTUs with software such as *SparCC* to unveil possible interactions (e.g., symbiotic, commensalism, parasitism, detect key species, etc.; Fig. 2).

Application Note on Microbial Communities: Merging the "Omics"

Metagenomes aim to encapsulate the genetic pool present in the environment. Metagenomic datasets commonly comprise millions, if not billions, of short DNA sequencing reads and allow access to the entire genomic content of a wide range of abundant microorganisms, albeit in a fragmented form. In fact, sequencing reads are usually shorter than a gene. Nevertheless, metagenomics has proven itself a powerful approach to profile the taxonomy and functioning of microbial communities without relying on cultivation or metabarcoding.

In whole-genome sequencing projects, one might expect that the origin of the sequenced DNA is material anything but ambiguous. However, unintentional contamination and the throughput now standard in modern sequencing machines—in which multiplexed samples might come from very different or very similar organisms—have made necessary to develop sequence or composition based computational protocols to identify the likely origin of the sequenced DNA molecules. These methods can be and have been, used to resolve the taxonomic composition of metagenomes.

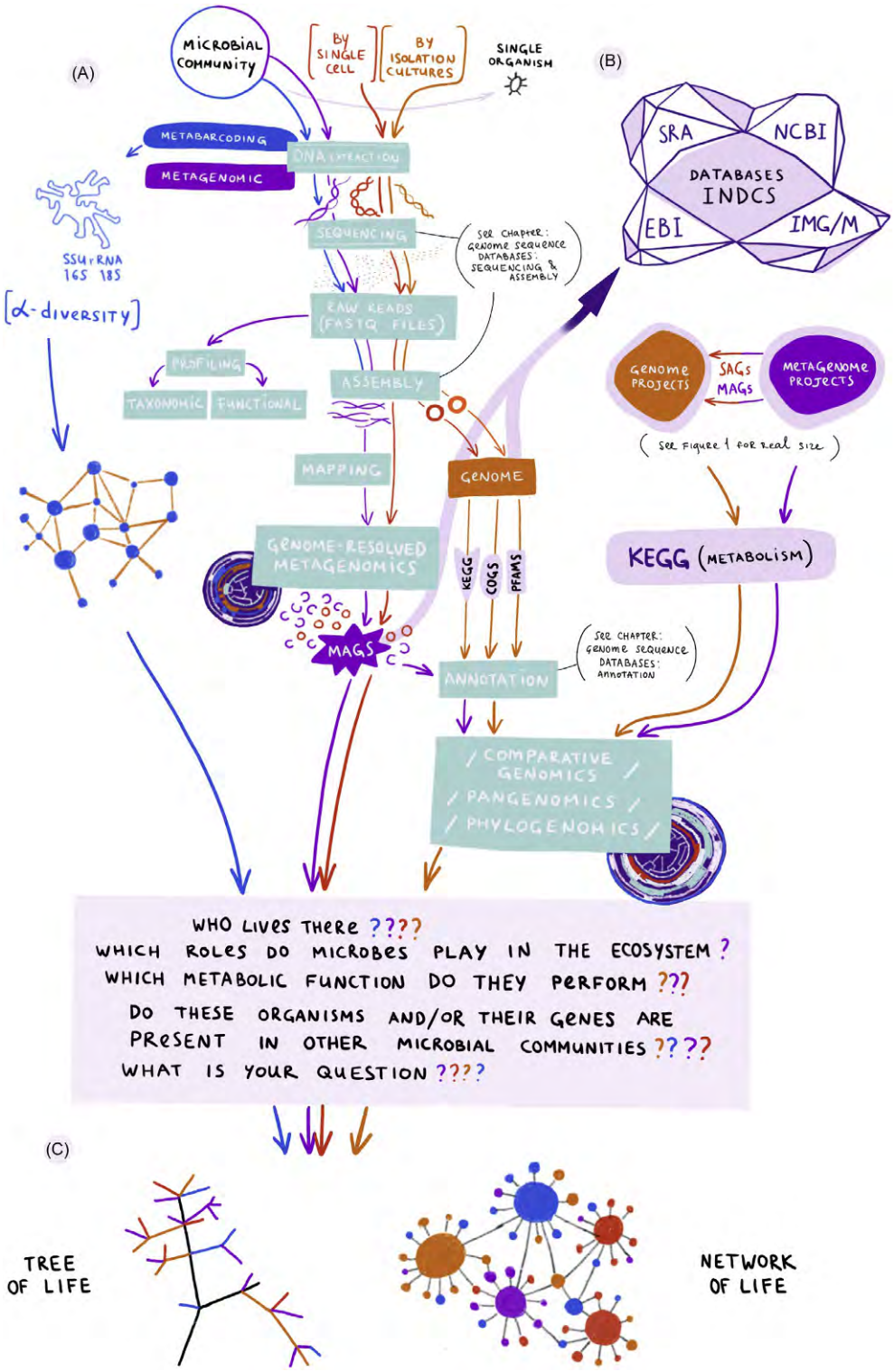

Fig. 2 A map through the Genome Sequence Databases, the Types of Data and Bioinformatic Tools. (A) The tools used to understand a microbial community through its genes, either by metabarcoding, metagenomics or genomics of an isolated microbe. The sequences generated from laboratories around the globe feed the (B) public databases, which are then used to contrast and compare new sequences generated from different microbial communities. All these tools and databases aid in answering biological questions which help the community to build an ever-evolving view of (C) the tree of life, and the network of life, through the evolution of the microorganisms and their interactions with each other. Artwork by Anna Ustrehova instagram: @ustrehova.

Profiling of a metagenome can be done twofolds: taxonomic profiling (who lives there) and functional profiling (what is the community capable of doing; Fig. 2). Profiling can be either performed at the read level, or at the contig level following an assembly process. Assembly of reads will not be covered in this chapter.

There is a large amount of software to assign taxonomy to reads. Some will focus on the *k*-mer composition of the reads (*Kraken*), others will perform a similarity search against a reference database (*MEGAN*) and others will exploit mapping algorithms (*Centrifuge*). Taxonomic assignment will depend on a combination of the selected algorithm and the database used, so it is important to keep in mind that taxonomic classification does not imply an evolutionary relationship.

Preliminary taxonomic and functional profiling of the reads is a useful guide for subsequent analyses and helps inform later decisions when adjusting parameters. It allows us to resolve taxa or functions in metagenomes but is strictly reference based. Sometimes we do not want to classify every read, so, for taxonomic profiling, *MetaPhlAn2* comes in handy. Through mapping, it relies on an impressive and growing dataset of unique clade-specific marker genes identified from reference genomes for unambiguous taxonomic assignments for bacteria, archaea, eukaryotes and viruses and an accurate estimation of organismal relative abundance. For functional profiling, *MEGAN* can generate *KEGG* profiles of the whole metagenome, and the user can explore the detail of individual pathways of interest.

The annotation of a metagenome gives important information on the functions the microbial community is performing (Fig. 2). In this chapter, we will not cover deeply on the how-tos of genome annotation, however, two points are worth mentioning. The first is the existence of an inclusive pipeline that comprises several annotating tools: *Prokka*. *Prokka*, as its name indicates, has been developed to annotate prokaryotic genomes and it is not recommended to annotate eukaryotes. *Prokka* will predict coding sequences (CDS), rRNAs, tRNAs, signal peptides, and ncRNAs. For the CDS, it will use curated models of protein families from *UniProt*, *Pfams*, and *TIGRFAMs* to provide a better functional annotation of the genes. Finally, it will generate several output files including a fasta file with the predicted proteins and those necessary to submit your sequences to NCBI (gbk and gff). Prokka an accurate way to annotate your sequences; however, there are several other databases available for more specific annotation, such as *KEGG* for metabolism.

The second regards working with very large datasets, such as metagenomes. When sequence similarity searches are needed, *BLAST* can take considerable computational time. An accelerated *BLAST* alternative exists but comes with a price in sensitivity and accuracy. The *DIAMOND* (Double Index AlignMent of Next-generation sequencing Data) software performs pairwise alignment of proteins and translated DNA at 500–20,000× speed of *BLAST* and is explicitly designed to make use of modern computer architectures that have large memory capacity and many cores. When it is impossible to *BLAST* a metagenome, *DIAMOND* is the next best option.

Genome Resolved Metagenomics: Metagenome Assembled Genomes

Metagenomes have provided new means to resolve genomes without relying on cultivation. These "genomes" called Metagenome-Assembled Genomes (MAGs) can be recovered from the set of contigs present in our assemblies and have transformed our understanding of the tree of life. This approach aims at transitioning from billions of short reads into millions of contigs and finally thousands of MAGs. Note that MAGs represent the consensus genome from nearly identical cells present in the microbial community. Since these cells maintain slight genomic variations, the exact sequences of MAGs recovered from metagenomic assemblies do not necessarily exist in nature.

Due to limitations at several steps in current technology (sequencing, assembly, and recovery of MAGs), it is challenging to recover complete genomes from metagenomes. In most cases, the technique will provide access to incomplete genomes. In addition, it is a tough task to recover them without contamination (i.e., contigs belonging to another taxonomic lineage). We will briefly describe tools one could use to optimize the outcome of genome-resolved metagenomics while minimizing risks of contaminations.

Binning

Binning is the process of classifying some data and group it in several more or less continuous values into a smaller number of "bins." We will refer to binning here, exclusively to classifying contigs out of the (co-)assemblies of metagenomes and bin them into MAGs. The goal is simple: solve a metagenomic puzzle by identifying contigs that belong to the same genome! In order to accurately classify our contigs, we rely on two independent properties: (i) sequence composition and (ii) differential coverage.

Genomes from different organisms possess different frequencies in tetranucleotide composition and GC-content. The tetranucleotide frequency (TNF) represents a genomic signature for each microbe, and genomic GC-content can range from 17% to 75%. These marked trends have enough power to classify contigs and bin them in broad categories. In fact, genomes were initially binned focusing on TNF, mainly with the *ESOM* (Emergent Self-Organizing Maps) software.

However, sequence composition alone lacks the resolution to distinguish between closely related microbes. These disadvantages can be overcome when we incorporate differential coverage to the binning process, taking advantage of the fact that abundance, and hence coverage, of each microbe differs in the microbial community. Comparing metagenomes from closely related sites, or from the same site at different time points allows us to exploit the property that different organisms exist in different abundances in the microbial community (differential coverage). Time series are ideal, as are sites close to each other. In addition, DNA extraction biases and the incubation of samples under different conditions can be used to take advantage of differential coverage.

Automatic binning software (e.g., *CONCOCT*, *MaxBin*, and *MetaBAT*) exploit these two properties to characterize bins from metagenomic assemblies and mapping results. Critically, automatic binning greatly vary in quality and the assessment of MAGs with SCGs is limited. Organizing and visualizing contigs in the context of biological (e.g., GC-content, TNF, taxonomical signal) and environmental information (mainly, the coverage of contigs across metagenomes and, if needed, individual nucleotides within a contig) can be used to manually curate MAGs and access their genome-wide quality. Such manual curation effort can be performed following the metagenomic binning workflow of *anvi'o*, which is described in the following section.

The anvi'o platform

Most bioinformatics processes we have described above for genome-resolved metagenomics have been integrated into the analysis and visualization platform *anvi'o. Anvi'o* allows either automatic or human-guided characterization of MAGs from metagenomic assemblies, with interactive interfaces that can link -omics data from multiple sources (e.g., DNA and RNA) into a single, intuitive display. Human-guided (a.k.a, manual) binning is time-consuming but recommended for quality purposes. Extensive web tutorials have been provided to describe the *anvi'o* programs (see http://merenlab.org/). In addition, international workshops are regularly organized so that the users can meet with the *anvi'o* developers and practice on reference datasets. Finally, users of the platform have formed an active slack community (https://slackin-ezbpfhwsmh.now.sh/) to help each other with technical and conceptual questions.

The *anvi'o* metagenomic workflow starts with (co-)assembly and mapping files, and the user will then be guided through the tutorials to annotate the metagenome and identify SCGs using HMMs. These SCGs provide the user with a rough estimation of how many bacterial, archaeal, and eukaryotic genomes are present in the assembly. Interestingly, this information can be used to determine how many clusters to automatically bin. *Anvi'o* incorporates all the information and properties of the assembly and combines it in a single display, allowing the user to explore the metagenomic assembly and its environmental signal interactively, and characterize and manually refine MAGs (a.k.a, the curation step).

Manual refinement of MAGs is an essential step to make sure we have a collection as flawless as possible for all our downstream genomic analyses, and for sharing them in the public databases. There is a guide on the Minimum Information of a Metagenome Assembled Genome (MIMAG) which classifies the MAGs into high-quality draft, medium-quality draft, and low-quality MAGs, based on the presence/absence of rRNAs, tRNAs, and on the levels of completion and redundancy of the MAGs. A perfect MAG would have every SCGs (100% completion) and only one copy of each of them (0% redundancy) or 100C/0R. While achieving this is some way off, MAGs with 90C/5R can be recovered using current techniques. Depending on our goals, we can afford low levels of completion up to 50%, but we don't want to reach the 10% redundancy cut-off due to contamination concerns.

Single-Cell Amplified Genomes

When Genome-Resolved Metagenomics is not enough to recover those genomes of the yet-unculturable and low abundant inhabitants of the community, single-cell approaches coupled with whole genome amplification and sequencing have already enabled the quantification of the heterogeneity in naturally occurring populations. The clearest example of this technological explosion is the recent accumulation of thousands of single-cell amplified genomes (SAGs) in databases like the *IMG/M* (3535 SAGs as of November 2018). Additionally, single-cell genomics is providing the opportunity to capture the total cellular DNA and thus associate chromosome and extrachromosomal elements, or cryptic endosymbiotic consortia as well as tight cell–cell interactions.

Single-cell experiments are a chain of challenging interdependent steps, from cell isolation to the recording of the downstream -omics or metabolic readouts. Main caveats include, but are not restricted to, maintaining the physiological conditions of unculturable/unknown microorganisms and reaching detectable levels of biological material while dealing with the omnipresent contamination.

Two flavors of single-cell isolation approaches are currently available: (i) free cells in continuous flow systems, which allow high throughput structural and/or metabolic differentiation of cell types based on their biochemical spectrum; (ii) confinement-based segregation, useful to study the effect of chemical/biological signals on cell growth, or when isolated cells need to be enriched for subsequent phenotypic measurements.

Further improvements in the field may lead to versatile technologies able to deliver sequence and metabolite measurements simultaneously for a large number of single-cells. This will help us to tease apart the complexity of microbiological networks and better predict their response to perturbations, undoubtedly triggering a new wave of targeted exploration of the metabolic and ecological networks mediated by microbial dark matter.

Pangenomics

In 2005, Tettelin et al. coined the term pangenome along with genome analyses of eight *Streptococcus agalactiae* strains. A pangenome is the union of the entire gene set known to exist in members of a group, typically in strains of the same species, or in members of a monophyletic clade. In order to define this "entire set" of genes, all genes in the selected genomes must be compared to each other to understand which genes are orthologous and group them together. There are several tools to cluster these orthologous genes in a given group of organisms. OrthoMCL has a web server, a database of orthologous protein sequences and command line-based software package that can be downloaded and used locally. Several online tutorials exist on how to use OrthoMCL. From a set of genomes, an all-against-all BLAST search of the proteins is performed. Putative orthologous relationships are identified between pairs of genomes by reciprocal best similarity pairs and a Markov Cluster (MCL) algorithm is employed to robustly cluster genes into orthologs, or "recent" paralogs from at least two species.

Once the grouping of the orthologous genes is performed, ad hoc scripts can be coded to extract the core genome of the pangenome, or alternatively, ITEP (Integrated Toolkit for Exploration of microbial Pan-genomes) can be used. The core genome usually refers to gene clusters identified in all (or most) genomes in the collection. These gene clusters commonly include ribosomal proteins and other housekeeping genes. It is also interesting to analyze the set of accessory genes, those genes that are specific to a subset of genomes. Genomes originating from organisms that share a particular trait (e.g., specific environment or stress) might possess accessory genes retained by selective pressure. Understanding the pangenome of a set of closely related organisms can also shed light on the evolutionary impact of HGT in the group.

The *anvi'o* platform includes a pipeline and tutorials to create pangenomes from reference genomes, newly characterized MAGs or a combination of the two sources. Notably, the platform allows visualization of the occurrence of thousands of gene clusters across the selected genomes, estimation of relationships between them based on gene clusters, computation and visualization of *average nucleotide identity scores*, and the linking of pangenomic traits with functional inferences.

The Universal Tree of Life

The amount and diversity of species with at least partial sequence information is rapidly increasing and the tree of life is constantly being redrawn. Phylogenetic trees represent a backbone of various biological studies and it is essential to have state-of-art tools for their display, customization, and interpretation. At your fingertips, an app available for Android and iPhone mobile phones, *Lifemap* developed by Damien de Vienne, allows you to locate (almost) any organism in a tree of life and calculate the distance to another one, drawing a route through possible common ancestors. Peer Bork's lab has an online tool, the *Interactive Tree of Life (iTOL)*, that allows you to explore precalculated phylogenies as well as displaying your own phylogenetic trees. Various types of data, such as genome size or protein domain repertoires, can be mapped onto the tree.

With the explosion of the metabarcoding techniques, an impressive collection of SSU genes has been made available. Now, metagenomics has unveiled a previously hidden biodiversity, obscured when viewed through the sole lens of metabarcoding. Some of these bacteria have introns in their rRNAs, making them invisible through amplification biased-techniques. Some other bacterial genomes lack some ribosomal proteins, which were thought to be part of the universal single-copy core collection of genes. All those bacteria and archaea that have long remained incognito, are now revealed through metagenomics and genome-resolved metagenomics. In 2016, Laura Hug et al. took advantage of MAGs to reconstruct a massive tree of life, changing of view of its topology with the incorporation of the Candidate Phyla Radiation (CPR) within the domain Bacteria.

It will take decades to fully appreciate the biological significance of MAGs affiliated to the CPR and other lineages with no cultured representatives. Without any doubt, all this unhidden diversity is revolutionizing the field once again and will outdate many aspects of this encyclopedia in its future editions. Indeed, now is an exciting time to explore the microbial world through bioinformatics!

Final Considerations

By routinely sequencing microbial communities and the genomes of those microbes that can be isolated (either by enrichments or by single-cell methodologies), scientists all over the world are contributing to an enormous collection of public genomic sequences. We can now compare microbial ecosystems from all over the world, from deserts to oceans, to skin microbiomes of frogs or humans to the New York subway. This huge volume of data is constantly challenging our concepts in Microbiology. In this past decade, our tree of life has expanded to unveil a large diversity of "Candidate Phyla," the metabolism and ecology of which we barely understand. We have also witnessed, thanks to genome-resolved metagenomics, the unmasking of an Archaeal lineage sister to the root of the Eukaryotes, shedding light on the possible last Eukaryotic common ancestor. The continuous flow of massive sequencing is continuously redefining our view of the world, we hope we have convinced the reader that computational tools and resources have been and will continue to be important for making such big contributions to the study of life.

Acknowledgments

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Relevant Websites

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http://enve-omics.ce.gatech.edu/ani/—The ANI calculator.

Genome Sequencing of Microbes

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Glossary

Core genome Represents the genes present in all strains of a species. It typically includes housekeeping genes for cell growth and maintenance.
***De novo* Sequencing** Sequencing a novel genome where there is no reference sequence available for alignment.
Draft genome An incomplete sequenced genome, with the possibility of missing nucleotides or segments.
Metagenome All the genetic material present in a sample consisting of all the genomes of the individual organisms in the environment.
Microbiome Collection of all the genomes of the microbes within a system or environment.
Microbiota Collection of microorganisms in a system or environment.
Next-generation DNA sequencing (NGS) DNA sequencing technologies that simultaneously determine the sequence of DNA bases from many thousands (or millions) of DNA templates in a single reaction.
Paired end sequencing Sequencing of both ends of the same fragment to generate alignable sequence data.
Pan genome The entire complement of genes representing all strains of a species.
Pseudogene A gene that has lost functionality due to a nucleotide point mutation, insertion or deletion.
Sequencing by synthesis The term used by Illumina to describe the base-by-base chemistry used in NGS machines.
SNP Single Nucleotide Polymorphism – a single base difference found when comparing the same DNA sequence from two different species.

Introduction

Genomic information has now become an almost essential component of microbiological and molecular biology research. Vast amounts of research and industrially-relevant genomic data is now publically available which undoubtedly increases our understanding of microbial genetics and host-microbe interactions. It is 22 years since the first microbial genome sequence of *Haemophilus influenzae* became available (Fleischmann et al., 1995) and 12 years since the advent of high throughput next generation sequencing (NGS) technologies which, in addition to sequencing bacterial genomes, has allowed the large accumulation of metagenomic data with many terabytes of sequencing data produced to date. With respect to DNA technologies, large progress has been made in terms of speed, read output, read length combined with a remarkable reduction in cost. This has allowed microbial genome sequencing to become a standard procedure and enables even the smallest of research groups to sequence their pathogen or organism of interest.

Genome sequencing, firstly can aid in the taxonomic classification in the study of an unknown organism. Additionally, access to even a basic draft genome with minimal annotation opens a wide range of opportunities and invaluable information on the genetic and metabolic capabilities of a microbe. A lack of accessible genomic data for a particular strain or species is now regarded as restrictive and the availability of such data is becoming a foundation for biological research and indeed increases overall research impact. In the instance where genomic information is existing for multiple strains of a species, extensive comparative genomics can be performed enabling core and even pan-genome analysis. Presently, the majority of bacterial phyla now have genome sequences available with many genera and species having multiple strains with full genomic data accessible. Additionally, there are over 20,000 metagenome projects accessible through repositories (Section 2) and although bacterial studies dominated initially, there is now also increasing interest in archaeal, viral and fungal communities (Section 4).

Recently, NGS has expanded past DNA genomics and is key to translating the research into practical applications such as in molecular diagnostics. In this article we focus on the availability and status of the many current sequencing technologies while providing a reference point to genomic repositories, databases and online resources. Additionally, we examine the impact of single genome sequencing on bacterial pathogenomics and probiotic research to date and finally we move beyond bacterial genomics and identify the key studies documented on viral, archaeal and fungal isolates.

Sequence Technologies and Online Resources

In the past 12 years, NGS technologies have been implemented in many applications, including whole genome sequencing, *de novo* assembly, metagenomics and transcriptome sequencing. NGS platforms perform massive parallel sequencing, during which millions of fragments of DNA are sequenced simultaneously. The technologies have advanced greatly since 454 Life technologies

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launched the first commercial next generation sequencer in 2005 which at that time could sequence a read length of 100–150 bp with an output of 20 Mb per run (Margulies et al., 2005). The 454 technology, developed by Roche, did improve from this and achieved approximately 1 million reads with read lengths of more than 700 bp in with the FLX+ system and data processing with outputs of up to 70 Mb with the bench top sequencer, the GS Junior in 2009. However, newer sequencing technologies emerged that could provide longer read lengths or a greater number of reads at a much lower cost which led to a move away from this technology and 454 sequencing was no longer available from mid-2016. NGS technologies past and present, including their sample preparations have described in many other reviews (Liu et al., 2012; Buermans and den Dunnen, 2014; Guinane et al., 2016; Mardis, 2017) so this article just briefly discusses the recently released technologies.

Current Sequencing Platforms

The choice of technology for a microbial sequencing project largely depends on the desired application; whether it is *de-novo* genomic sequencing by paired-end sequencing or resequencing by whole-genome shotgun strategies, compositional analysis from a large bacterial community or RNA-seq data from whole transcriptome data the strategy for sequencing will vary or perhaps require a combination of the technologies outlined below.

There are a number of different companies currently commercially active in the sequencing area. Qiagen (see Relevant Website section), a recent addition to the sequencing market, provides a complete NGS workflow on their recently launched GeneReader platform, however this is specifically designed for cancer biology rather than microbial genomes and therefore is outside the subject area of this article. Illumina, operating DNA sequencers since 2008, offers a range of sequencers adopting clonal amplification and the sequencing by synthesis (SBS) technology. Within it's sequencing platform repertoire, Illumina offers bench top sequencing systems (MiniSeq, MiSeq and NextSeq series) in addition to large production scale sequencers (HiSeq, HiSeqX and NovaSeq series). The MiSeq is the most established benchtop sequencer with a run time of just 4 h and an output of up to 25 million reads intended for targeted sequencing and for the sequencing of small genomes. The newest bench top sequencer launched in 2016, the MiniSeq allows for small scale targeted sequencing projects and is aimed at affordable sequencing for a smaller lab group. The MiniSeq can achieve read lengths of 100–150 bp and an output of ~7 Gb (see Relevant Website section). At a larger scale, Illumina supply the HiSeq 2500 and the HiSeq X 10, which together can each achieve an output of up to 600 Gb/day. The most recent addition to the sequencer repertoire is the NovaSeq series consisting of the NovaSeq5000 and the NovaSeq6000. These are cloud-integrated instruments and claim to provide access to powerful, high-throughput genomics at a lower price (see Relevant Website section).

Thermo Fisher Scientific (previously owned by Life Technologies) also provide a range of sequencers based on ion semiconductor sequencing which relies on the detection of hydrogen ions released during the sequencing process (Rothberg et al., 2011; see Relevant Website section). Bench top sequencers provided include the Ion Torrent Personal Genome Machine (PGM) and the Ion Torrent Proton released in 2010 and 2012 respectively. Ion technology has evolved very quickly which has allowed both machines to increase in read lengths and in data output (Buermans and den Dunnen, 2014). Many options are available for varying applications. The PGM, for example can provide reads up to 400 bp and therefore useful for targeted resequencing of small genomes. The Proton however generates shorter reads and therefore useful for applications such as RNA-seq transcriptomics. Due to the quick speeds of ion technology (2–8 h run times) these machines are potentially suitable for molecular diagnostics and other applications in a clinical setting (Mellmann et al., 2011).

Other sequencing technologies in, as yet, limited use include "third generation sequencers" named as such due to the fact that the technologies involve single molecule detection and the signal is captured in real time. Available platforms for these technologies include the Pacific Biosciences Single Molecule Real-Time (SMRT) system and the Oxford Nanopore system. Pacific Biosciences SMRT system is based on the properties of zero-mode waveguides and signals are generated in the form of light signals (reviewed in Buermans and den Dunnen (2014), Liu et al. (2012)). The PacBio RS instrument generates several thousands of up-to-several-kilobaselong reads ideal for *de novo* genome sequencing, however has been documented to have a high base error rate (Rhoads and Au, 2015). DNA sequencing using Nanopore technology was first introduced by Oxford Nanopore Technologies in 2014. The basic principle of Nanopore DNA sequencing involves the passing of an ionic current through a pore and measuring the current changes as the biological molecule passes through (Wang et al., 2014). The MinION was the first instrument launched by the company in 2015 in the form of a handheld portable device. This instrument also is low cost and can generate ultra-long reads (>100 kb) however high error rates have been documented for the MinION and although long read lengths are indeed achievable, low overall mean and median lengths have been reported on initial instruments (Laver et al., 2015). The company also recently launched the GridION X5 which can hold 5 MinION flow cells on a single bench top machine and a newly described PromethION which proposes sites for 48 flow cells promising industrial scale level of sequencing (see Relevant Website section). Other companies that provide promise in "third generation" sequencing include Roche which were one of the first companies active in next generation sequencing. Roche are also developing a nanopore-based platform for single molecule detection (see Relevant Website section). Quantum Biosystems, a relatively new company are developing a single-molecule electronic DNA sequencing technology that measures currents from DNA bases as the molecule passes by a sub-nanometer gap (see Relevant Website section).

Computational Challenges

The expansion in DNA sequencing technologies has indeed revolutionised biological research. However in creating vast amounts of raw sequence datasets, it has led to many bioinformatic challenges. Although the time taken for DNA sequencing has reduced

dramatically, there is still much time, difficulty and cost associated with the storage and analysis of the raw sequence reads. Scientists need to store, share and analyse vast amounts of datasets which require specialist computational skills. For single genome sequencing of a microbe, the raw read output from sequencers need to be quality checked and then assembled into contigs and scaffolds for feasible analysis of the genetic content. The software capable of performing many of these tasks are available on Linux-based operating systems demanding specific bioinformatics skills for competent use. Following genome assembly, it is then necessary to extract all the most valuable information from the sequence data, which involves gene prediction and functional annotation. Manual annotation can be a laborious and a time-consuming procedure. In fact the majority of genomes now available in Genbank are in draft format and not fully completed genomes due to the high volume of sequence data for microbial genomes currently being generated. There has, however, been many computational developments in this area in recent years and there are now many user-friendly software programs to assist in genome sequence analysis as outlined in the following section.

Genomic Repositories and Online Sequence Tools

In recent years, databases and online tools are playing a significant role in biology and are central to microbial genomic research. There are a number of data repositories for the storage and retrieval of biological data with the National Centre for Biotechnology Information (NCBI) and the European Molecular Biology Laboratory/European Bioinformatics Institute (EMBL-EBI) being the primary resources available that hold genomic collections. It is now mandatory to deposit all genome sequence data from any published genome project. Nucleotide sequence submissions can be made to any of the 3 databases; the DNA data bank of Japan (DDBJ), GenBank (USA) and the European Nucleotide Archive (Europe). These exchange data on a daily basis to ensure management between them and all nucleotide sequence data from all organisms is accessible from any of the databases. These are primary repositories, as they hold the original sequence data. These collaborate with the Sequence Read Archive (SRA), which stores raw reads from high-throughput instruments including metagenomic and compositional raw sequencing data.

In addition to the above tools there are also other resources that allow the integration of genomic and molecular biology data retrievable for further bioinformatic analysis. Examples of the main resources used by the research community include the Genomes on line database (GOLD) (see Relevant Website section) which integrates global genomic, metagenomic and associated metadata, the IMG/M: Integrated Microbial Genomes & Microbiomes (Markowitz et al., 2012a,b) the Microbial Genome Database (MBGD) (Uchiyama, 2003), the SEED viewer (Overbeek et al., 2014) which allows gene prediction and functional annotation and the MicrobesOnline (Dehal et al., 2010) resource that in addition to the availability of genomic data allows comparative genomic analysis and gene expression analysis. Databases specific to a particular pathogen are also available which allow in-depth analysis of an organism such as Fusobase (Ang et al., 2014) that provides genomic data for multiple strains and species of the emerging human pathogen *Fusobacterium* spp. and GenoList (Lechat et al., 2008) which allows multi-strain analysis of *Listeria* spp. There are also specialised databases that allow the detection of antibiotic resistance genes such as the comprehensive antibiotic resistance database (CARD) (McArthur et al., 2013) or to a particular strain trait such as antimicrobial peptide production including as Bagel 3 (van Heel et al., 2013) and antismash (Blin et al., 2017). Additionally, special purpose resources for specific functional gene analysis include STRING (see Relevant Website section) for protein-protein interactions, the CDD (see Relevant Website section) for conserved domain analysis, and PFam (see Relevant Website section) and SMART (see Relevant Website section) for protein family analysis and the Clusters of Orthologous Groups of Proteins (COGs) database (Tatusov et al., 2003) for the phylogenetic classification of proteins. Further information on available resources for microbial genomic analysis is documented by Zhulin (2015).

Catalogue of the Human Microbiome

Over the last 10 years, a number of research consortia have generated large amounts of sequence data of microbes with a vast array of accompanying online resources for data access and analysis. Data relating to microbial diversity, deposited metagenomic projects and reference genome collections from the human microbiome are now easily accessible. Large scale consortiums such as the EU-funded MetaHIT (Metagenomics of the Human Intestinal Tract) (Ehrlich et al., 2011) studied associations between the genes of the human intestinal microbiota and our health focusing on two specific disorders, Inflammatory Bowel Disease (IBD) and obesity (see Relevant Website section). The National Institutes of Health Common Fund Human Microbiome Project (HMP) was established in 2008, with the objective to characterise the human microbiome to understand its role in human health and disease (see Relevant Website section). The initial phase of the HMP resulted in the sequencing of 3055 reference microbial genomes from multiple body sites and included 1265 metagenomic projects. The second phase of the HMP aims to integrate datasets from the microbiome and the human host from specific studies of microbiome associated conditions such as IBD and type 2 diabetes (see Relevant Website section). An example of a database specific to one body site is the Human Oral Microbiome Database (HOMD) which provides sequence data and taxonomic information on ~700 prokaryote species that are present in the human oral cavity (Chen et al., 2010).

Overall these catalogues provide the biological research community access to the human microbiota furthering our understanding of these communities can impact on human health and disease. It is now possible to retrieve metagenomic, metatranscriptomic, human genetic, microbial culture, and many other data types from each project.

Understanding Bacterial Genomes

Our understanding of bacterial virulence and evolution has significantly improved with the sequencing of pathogen genomes. Since the first bacterial genome was sequenced over 20 years ago, there are now almost 2000 bacterial genomes fully sequenced including many strains of important clinical pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, Methicillin-resistant *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Clostridium difficile*.

The advent of DNA sequencing of microbes has provided significant understanding into the genetic mechanisms underpinning pathogenicity and evolution, the epidemiology of bacterial pathogens and provides a possible option of rapid detection and monitoring of outbreaks (Fig. 1). The improved resolution at nucleotide level provided by whole genome sequencing (WGS) has provided critical insight for determining the appropriate response to an infection. In disease progression, the ability to obtain detailed information on the pathogenic microbe from multiple patients or even multiple isolates from a single patient is extremely attractive. The reduction in sequencing cost has put WGS in scope of routine analysis in research and even clinical microbiological settings. There are comprehensive reviews (Bentley and Parkhill, 2015; Klemm and Dougan, 2016; Land et al., 2015) that have addressed this area, therefore here we outline just some examples of what we have learned from sequencing of pathogen genomes to date.

Architecture of the Pathogen Genome

The availability of complete genome sequence data from microbial pathogens has provided insights into key virulence genetic systems. Notably, studies have elucidated the evolution and function of bacterial protein secretion systems and protein-targeting mechanisms, such as sortases, which enable the attachment of substrate proteins including enzymes, pilins and adhesins to the bacterial cell surface (Loman and Pallen, 2015). The study of bacterial pathogens using traditional approaches identified many factors such as toxins, adhesins and invasins that are prerequisites to the development of pathogenicity and are often based on the acquisition of specific genetic elements. However, prior to WGS these genes and systems were studied largely independently of the complete genomic make-up of the microbe. Given the importance to human health, unsurprisingly the first microorganism whose complete genome was sequenced was a pathogen, *H. influenzae* (Fleischmann et al., 1995). In addition to colonizing the nasopharynges of healthy humans, *H. influenzae* causes acute respiratory infections in addition to invasive bloodstream infections such as meningitis and cellulitis. In common with many of the first complete genome sequences, a key finding was that 42% of the predicted coding regions did not have homologue or putative function assigned. In fact the results from the first completed

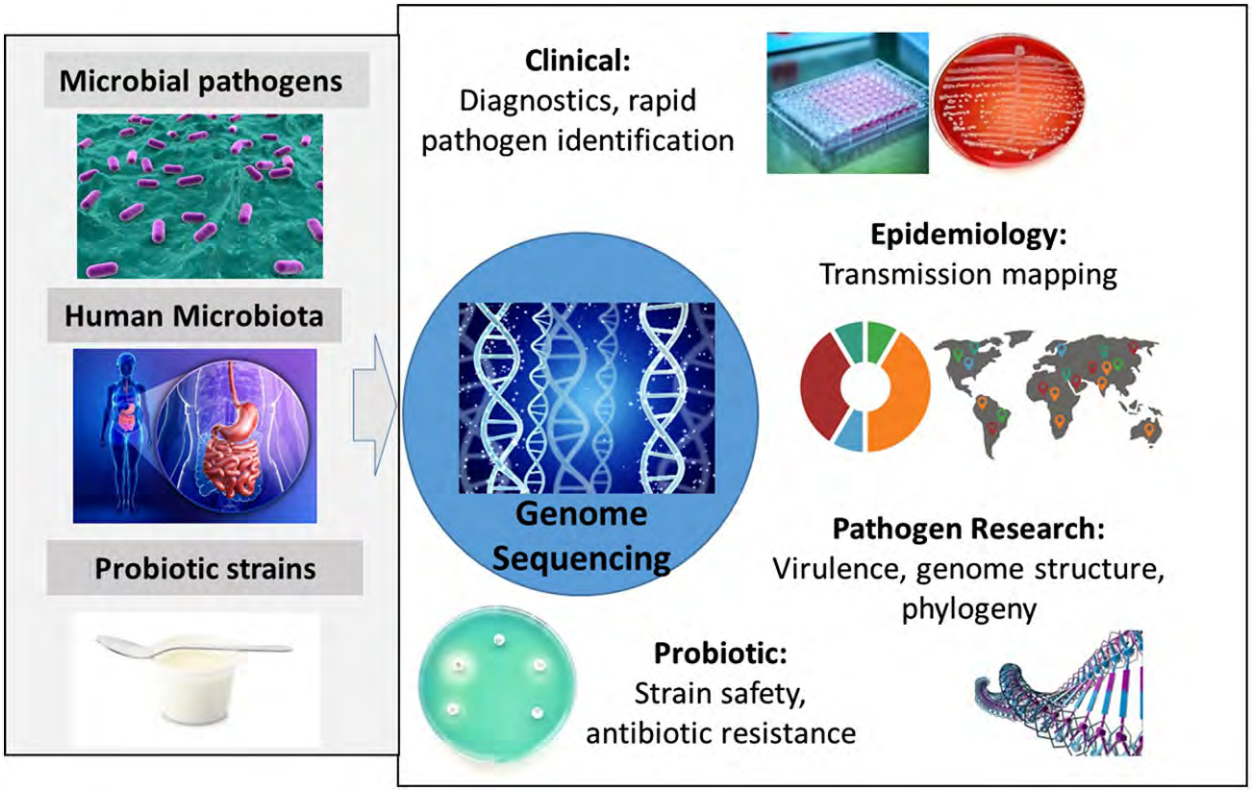

Fig. 1 The impact and possibilities of microbial genome sequencing on pathogen, probiotic and clinical research.

prokaryotic genome sequences showed that almost half of predicted coding regions identified were of unknown biological function (Fraser et al., 2000). Moreover, at the very early stage of genome sequencing approximately 25% of all predicted coding sequences in each species were found to be novel.

Early studies on strains of the same species centred on *Escherichia coli*. Comparisons between a highly pathogenic strain of *E. coli* O157:H7 and the *E. coli* K12 type strain revealed that over a quarter of the genes (26%) were unique to the pathogen compared with the type strain (Perna et al., 2001). It was known that the pathogenicity was largely associated with the gain or loss of genetic material through various mobile genetic element but the scale of the differentiation was unexpected. Further comparative genomic studies confirmed the earlier observations of substantial diversity within an individual bacterial species. For example, differences in 25% of the gene content was also observed amongst strains of *Neisseria meningitidis* and *Helicobacter pylori* (Boucher et al., 2001). Individual isolates contained very large numbers of unique genes and an increasingly small number of genes were observed in all members of any one species. The idea of the pan genome (representing all the genes in a species) subsequently emerged where species have a central core genome responsible for cell maintenance and regulation found in all strains and an accessory or more "expendable" set of genes that differ among the strains of a species (Medini et al., 2005). The *E. coli* species, as indicated from the initial studies, is an example of a genetically diverse organism with an extensive pan genome content. Recent comparisons of more than 2000 *E. coli* genomes revealed a core genome of more than 3000 gene families with a total of ~89,000 different gene families existing in the overall pan genome (Land et al., 2015). This level of diversity means in any one *E. coli* genome (~5 Mb), up to one third of each genome is part of the accessory genome and not present in all strains (Land et al., 2015).

In contrast to the diversity observed in *E. coli* genomes, comparative genomics has revealed that other human pathogens such as *Salmonella typhi*, *Yersinia pestis* and *Bordetella pertussis* demonstrate a high degree of 'celibacy' with little mobile DNA and limited genetic diversity (Parkhill et al., 2001a,b, 2003); reviewed by Bentley and Parkhill (2015). Genomic analysis of these pathogens identified many similar characteristics with a large amount of repetitive DNA and a high number of pseudogenes. Despite these organisms being successful pathogens many pseudogenes, surprisingly, appeared to be non-functional genes previously involved in virulence or host interactions. Also a common feature was that these organisms occupied an acquired restricted niche and are likely to have evolved to adapt to their environment. *Y. pestis* for example displayed 150 pseudogenes which were indicative of a redundant gastrointestinal organism but had gained the genetic determinants for life as a systemic invasive pathogen (Parkhill et al., 2001b).

It has become clear that we have learned that the genome structure varies greatly among species. Some organisms are extremely diverse and heterogeneous varying considerable in size and gene content whereas others show much conservation and limited genetic diversity. This knowledge is instrumental in developing strategies to deal with detection and control of these microbes.

Global Epidemiology and Transmission Mapping

The arrival of high-throughput DNA sequencing has also widely facilitated SNP-based phylogenetic analyses of bacterial pathogens enabling transmission mapping of pathogens and disease outbreaks (reviewed by Bentley and Parkhill (2015), Klemm and Dougan (2016)). The links between genomic diversity and geography have been studied for pathogens such as *Salmonella enterica* serovar Typhi (Typhi), the causative agent of Typhoid fever. As mentioned above, Typhi shows limited diversity among isolates with overall high conservation of genetic content. With such low levels of variation, the availability of whole genome sequences has impacted greatly on epidemiological studies. Holt *et al*., performed an early study on multiple pathogen isolates generating 19 Tyhpi whole genome sequences using a combination of NGS technologies (454 and Illumina). This intraspecies comparison study indicated large scale loss of gene function among isolates and limited evidence for recombination events indicating a small population was causing genetic drift of typhoid across the world (Holt et al., 2008).

An excellent example of where bacterial genomic analysis has been instrumental in deciphering the evolution and epidemiology is that of *Vibrio cholerae*. Cholera, an acute diarrheal infection, is a global threat to public health with previous epidemic dynamics poorly understood. Recent population genomic studies however have brought significant new understanding of the evolution and long-range spread of the causative agent, *V. cholerae*. The current ongoing seventh cholera pandemic began in Indonesia in 1961 and spread through Asia to Africa, Europe, and Latin America. This pandemic is caused by a new biotype of *V. cholerae* first isolated in 1905 in El Tor, Egypt (Sack et al., 2004). Genomic analysis by a number of authors showed that the El Tor lineage had emerged from the environmental reservoir of *V. cholerae* independent of lineage responsible for the first six pandemics (Robins and Mekalanos, 2014; Heidelberg et al., 2000). Further genomic analysis of isolates from the seventh pandemic allowed phylogenetic analysis of the El Tor lineage which demonstrated an evolutionary relationship between strains (Mutreja et al., 2011). Following genomic, SNP and phylogenetic analysis it enabled the authors to pinpoint the origin of the seventh pandemic clone concluding it most likely had originated from a single region in the Bay of Bengal and from there spread worldwide (Mutreja et al., 2011).

The availability of genome sequences was also pivotal to tracing the origins of the *E. coli* strain causing an outbreak of diarrhoea and Hemolytic–Uremic Syndrome in Germany. In the study by Rasko *et al*., third generation sequencing was used to determine the sequence of a number of *E. coli* strains including the outbreak strain (Rasko et al., 2011). Largely influential in transmission mapping and pathogen detection of the *E. coli* serotype involved was the rapid high throughput bench top sequencing performed, the open source availability of the sequence data and the out sourcing of data analysis to expert computational biologists (Rohde et al., 2011). In a logical extension of this application to a foodborne outbreak, the USDA have created the GenomeTrakr network (see Relevant Website section) which is the first distributed network of laboratories to utilize WGS for pathogen identification due to its superior resolution over conventional typing techniques. It has input from a number of public health and university

laboratories that collect and share all genomic and epidemiological data from foodborne pathogens which is then accessible by researchers and public health officials.

Clinical Applications

DNA sequencing technologies have much potential in clinical microbiology to detect microbes leading to the potential rapid diagnosis of infectious diseases. It could be extremely advantageous in the identification of certain bacterial and fungal organisms which grow slowly and require species-specific growth conditions. Some organisms can take days to culture possibly leading to a delay in the appropriate antimicrobial therapy being used. Within a clinical setting, initial bacterial population genomic studies focused on the pathogen *S. aureus*. *Harris et al.*, demonstrated WGS data was able to provide the resolution required to differentiate clonal isolates of *S. aureus* within a single hospital ward (Harris et al., 2010). Further work then showed that combining WGS with data from epidemiology studies enabled reconstruction of a hospital outbreak of Methicillin Resistance *S. aureus* (MRSA) from a special care baby unit identifying a persistent outbreak and transmission of infection into the community. WGS allowed the confirmation of MRSA carriage by a hospital staff member which assisted in maintaining the outbreak (Harris et al., 2013). A more recent study, again showed, genomics being used successfully to identify outbreak strains in a hospital-acquired infection of MRSA in China (Kong et al., 2016).

The availability of bacterial genomes has also given insight in to the pathogen evolution during patient infection and colonisation over time (reviewed by Didelot et al. (2016)). A critical area where WGS has provided important insights is the evolution of within-host antibiotic resistance. An example of this application was demonstrated through the genome sequencing from *Mycobacterium tuberculosis* isolates from a single patient revealing nucleotide substitutions responsible for rapid antibiotic resistance development. From a single patient over a period of just 3.5 years, the evolution of an extremely drug resistant *M. tuberculosis* isolate from a drug-sensitive strain was demonstrated (Eldholm et al., 2014).

WGS represents a molecular method that could be used to predict antibiotic resistance, assess virulence and genetically identify and type isolates. The possibilities of NGS as an effective diagnostic tool is an exciting prospect and would be particularly advantageous in the instance where an infecting microbe is not easy to culture. An increasing understanding of the capabilities of microbial genome sequencing is pivotal to its implementation into clinical medicine and in public health surveillance (Weinstock et al., 2016). For full application and routine use, however, many challenges have to be addressed. A regulatory framework for NGS technologies in clinical medicine needs to be implemented, in addition to a dedicated and standardised reference genomic database. Furthermore, operational hurdles need to be overcome such as user friendly bioinformatic software and computational expertise needs to match the technical ability of the sequencers to fully use and interpret the data outputs.

Genome Sequencing of Probiotics

As outlined above, initial efforts in bacterial genome sequencing concentrated on pathogen genomes as researchers sought to determine the genetic determinants for virulence and antibiotic resistance. A growing research area now however is the sequencing of microbes that are beneficial to human health to understand the genetic determinants for potential probiotic traits and subsequently for functional food strain selection (reviewed by Guinane et al. (2016)). To date, putative probiotic strains with an available genome sequence are those associated with the gut microbiome and confer a health benefit. Leading probiotics that are currently commercially available generally come from the 2 genera; *Bifidobacterium* and *Lactobacillus*. The first genome sequences for probiotic strains available were of *Lactobacillus plantarum* WCFS1 (Kleerebezem et al., 2003), *Lactobacillus johnsonii* NCC533 (Pridmore et al., 2004), *Lactobacillus acidophilus* NCFM (Altermann et al., 2005) and *Bifidobacterium longum* NCC2705 (Schell et al., 2002). Since these early genome sequencing projects, the probiogenomics area has grown rapidly with many species of *Bifidobacterium* (*B. longum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium animalis*) and *Lactobacillus* (*L. acidophilus*, *L. plantarum*, *L. johnsonii*, *Lactobacillus casei*, *Lactobacillus paracasei*) with multiple complete genomes available and numerous more in draft (see Relevant Website section). The genomics revolution and sequencing of the human microbiome has led to the discovery of many of other species that have probiotic potential now being called "next generation probiotics" expanding the likelihood of more live biotherapeutic products. There are many available complete genome sequences for strains of these proposed beneficial bacteria including *Faecalibacterium prausnitzii* (Heinken et al., 2014), *Akkermansia muciniphila* (van Passel et al., 2011), and draft genomes for strains of *Eubacterium hallii* considered a promising bacterium for maintaining intestinal metabolic balance due to its substrate utilisation spectrum (Duncan et al., 2004). High throughput sequencing technologies allows putative probiotics to be fully studied for potential health benefits and also for determining the safety of an organism at a genetic level to ensure the absence of transferable antibiotic resistance loci or virulent genes.

Current Developments in "Non-Bacterial" Sequencing

Although bacterial genome projects tend to dominate, high throughput sequencing has also revolutionised viral, fungal and archaeal research. The large diversity of the microbial world (ie small size of viruses compared to the large and complex genomes of a yeast) leads to the different sequencing technologies required depending on the required read length and depth of sequencing necessary. Each group has their own limitations which are very briefly discussed below.

Viruses, specifically have genomes much smaller than bacterial cells and therefore are obvious candidates for NGS technologies. Viruses are extremely genetically diverse and rapidly evolving with an explosion in the number of available viral genome sequences in recent years. NGS has become extremely important in overall viral research and in virus detection in a clinical setting. Difficulties in sequencing however can arise due to the very small size of some viruses or due to host contamination of viral nucleic acid (Marston et al., 2013) and as many virus are composed of RNA they are not detected by metagenomic DNA sequencing. Additionally, for clinical application, an enrichment step is often necessary to detect viral populations present in lower numbers (Datta et al., 2015). In recent years, many advances in genomic research has enabled analyses of the viral communities living within human body which are collectively called the human virome. The virome is particularly extensive and includes viruses that can invade and infect human cells, viral derived elements and bacteriophages that as yet remains relatively uncharacterised. However as sensitivities improve their impact within the human microbiome is likely to be determined.

Sequencing of archaeal genomes have also gained much attention and indeed the first Archeaon genome sequence, *Methanococcus jannaschii* was released in 1996 just one year after the first bacterial genome (Bult et al., 1996). The *M. jannaschii* genome enabled biologists to study prokaryotic evolution but also provided much insight in to archaeal biochemical and enzymatic pathways (Garrett, 1996). Subsequently there was much interest in archaeal genomes for evolutionary analysis and many formed part of the GEBA project: 'Genomic Encyclopedia of Bacteria and Archaea' (Wu et al., 2009). To date there are over 150 complete genomes available for Archaeal species (see Relevant Website section). Additionally, although receiving a lot less attention than their bacterial counterparts, a small number of archaeal genera have been identified in the healthy human microbiome, primarily in the gut with the species with *Methanobrevibacter smithii* being the best documented and most widely studied (Lurie-Weinberger and Gophna, 2015).

Fungi are a large group of eukaryotic organisms that ranges from yeast and moulds to mushrooms. As a large and diverse family, fungi are extremely important in human health and disease in addition to having a large role in many areas of biotechnology. The first fungal genome of the model yeast *Saccharomyces cerevisae* was sequenced in 1996 (Goffeau et al., 1996). This genome was just over 12 Mb, however the size of fungal genomes can vary from just under 9 Mb to 177.57 Mb. Until recently, fungal genomes did not dominate the genome repositories and due to the genetic diversity within this group a lack of genetic knowledge was apparent. However an initiative by the JGI is quickly changing this and bridging the gap with the 1000 fungal genome project. To date there have been more than 800 genomes made available by the JGI mycocosm resource (see Relevant Website section). The fungal components of our resident microbiota, collectively known as the human mycobiome, also remain somewhat undiscovered to date. However, due to their predicted involvement in a number of GI diseases, the gut mycobiome have begun to receive increasing research interest. The advances in HGS technologies has permitted in depth analysis of the mycobiome allowing a move away from the traditional culture based approaches. Optimisation of DNA extraction techniques and primer development are current priorities to sample the mycobiome efficiently. In recent years, there are more and more studies that have been performed which has surveyed the mycobiome in healthy patients and in disease states (for review see Huseyin et al. (2017)).

Future Perspectives

The development of high-throughput sequencing technologies has enabled microbial genomes to be sequenced in a matter of hours while metagenomic studies have transformed our ability to determine the composition of complex microbial communities. As outlined in this article, there are many sequencing platforms available to choose from a plethora online resources for bioinformatic analysis. Whole genome sequencing has permitted the full genetic information to be determined on all domains of microbial life including pathogenic and probiotic microorganisms allowing us to understand how they function in health and disease. With the advances in technologies and the reduction in sequencing costs, the use of WGS can potentially revolutionise clinical microbiology, biosurveillance and molecular diagnostics. The ability to replace time consuming and labour intensive techniques is very tempting. Rapid identification of pathogenic microbes without the need to wait for traditional culturing methods, determining the existence of antibiotic resistant clones, the ability to trace food-borne outbreaks are just some examples of uses of sequencing in food safety and biomedicine. There is no doubting the huge impact that NGS technologies have had on microbial and molecular research and demonstrates much future potential for routine application in commercial and clinical settings.

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Genomes From Uncultivated Microorganisms

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A Brief History of Microbial Genomics

In 1995, the first complete genome of a free-living microorganism, that of the bacterium *Haemophilus influenzae*, was sequenced by J. C. Venter and colleagues. This achievement proved the utility of shotgun genome sequencing and the discipline of microbial genomics was born. The years that followed were marked by sequencing genomes of bacterial and archaeal cultured isolates, and nearly 25 years later, well over 90,000 bacterial and nearly 900 archaeal isolate genome sequences are available in the public domain (Fig. 1). Due to our inability to cultivate the majority of microorganisms, cultivation-independent approaches to microbial genome discovery and identification, namely metagenomic sequencing, came to light in 2004 (Tyson et al., 2004; Venter et al., 2004) and have since been incredibly popular. Initially, cultivation-independent approaches were necessarily restricted to gene-centric analyses unless the microbial diversity of the sampled environment was very low; however, in recent years, genome-resolved metagenomics has become feasible through advances in sequencing technologies, metagenome assembly, and, importantly, computational binning algorithms (Wrighton et al., 2012; Albertsen et al., 2013). Genome-resolved metagenomics provides clear links between phylogeny and function, and offers population-level information on genome variability.

Complementary to genome-resolved metagenomics is microbial single-cell genomics, the sequencing of the genome from an individual cell directly isolated from the environment (for a review, see (Woyke et al., 2017)). Single-cell genomics emerged in 2005 when A. Raghunathan and colleagues demonstrated that sequence data from a single *Escherichia coli* cell could be obtained. Two years later the first genomes were recovered from the candidate phylum Saccharibacteria (formerly TM7) using single-cell sequencing. Since then, single-cell genomics methods have been widely adopted to complement metagenomics. To date, more than 5,000 bacterial and archaeal single amplified genomes (SAGs) and nearly 13,000 metagenome-assembled genomes (MAGs) from bacteria and archaea are in the public domain (Fig. 1). These genomes provide a rich resource for the phylogenetic and functional interrogation of the uncultivated majority within the tree of life.

Technical Aspects for Accessing Genomes From Uncultivated Microorganisms

Genome-Resolved Metagenomics

The term 'metagenomics' was coined in 1998 by J. Handelsman and colleagues to describe an approach for exploring phylogenetic and functional diversity by directly extracting and cloning environmental DNA. The basic experimental methodologies have remained stable, while the field has seen major advances in high-throughput sequencing technologies and downstream computational analyses. Mechanical or chemical lysis approaches are used to extract total nucleic acids from cells from a given environmental sample (Fig. 2). Extensive literature exists detailing impacts of sample preservation protocols, microbial biomass considerations, DNA extraction methodologies, and contamination considerations (for a review, see Quince et al., 2017). Based on sample quantification, subsequent library preparation approaches are predominantly geared towards sequencing on the Illumina platform. These library preparation methods include transposase-based 'tagmentation,' used in the Illumina Nextera and Nextera XT products, and PCR-free methods relying on physical fragmentation, used for Illumina TruSeq preparations. Known biases based on library preparation methods and the number of PCR cycles have been documented and are known to impact taxonomic abundance profile calculations. Increasing sequence output by leveraging the newly released Illumina NovaSeq platform promises upwards of 6 Tb in a dual flow cell run, enabling even deeper sequencing of environmental samples.

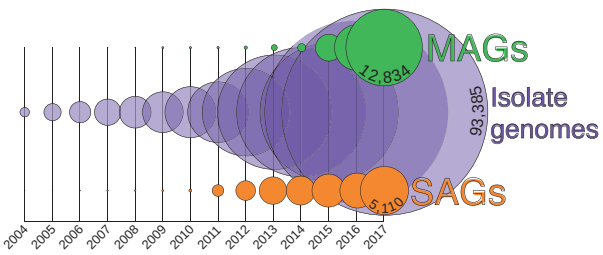

Fig. 1 The number of microbial isolate genomes, single amplified genomes (SAGs), and metagenome-assembled genomes (MAGs) sequenced over time. Data were extracted from the Genomes OnLine Database (GOLD; https://gold.jgi.doe.gov) on 11/21/2017.

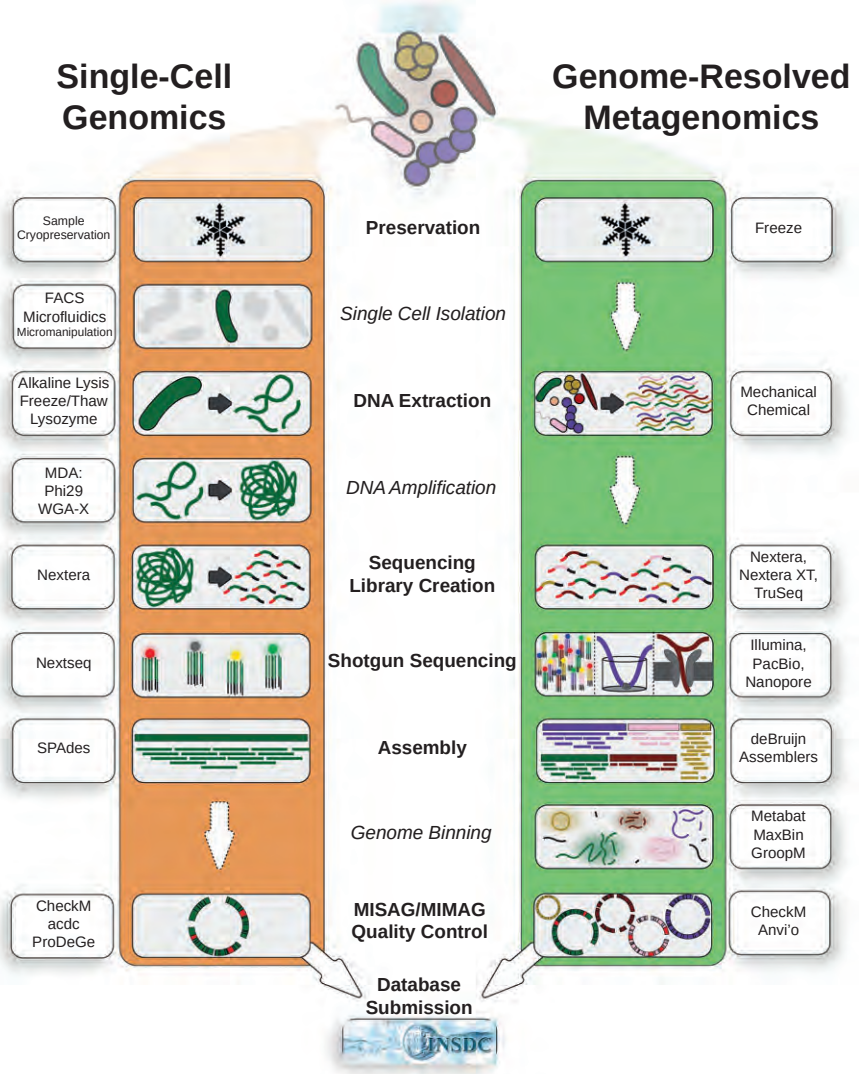

Fig. 2 Workflow for laboratory and analysis approaches of single-cell genomics and genome-resolved metagenomics.

Single-molecule long read sequencing technologies, including Oxford Nanopore and Pacific Biosciences, hold potential for metagenomic applications, yet are not widely utilized because it is challenging to extract high-quality, high molecular weight DNA. Alternatives to true single molecule sequencing generate 'synthetic long reads' from barcoded short reads or apply high-throughput chromosome conformation capture (Hi-C) technology. These and other technological developments on the horizon will undoubtedly shift the current paradigm of genome-resolved metagenomics away from computationally intense short-read assembly dependencies. For instance, a recently described novel approach from J. Beaulaurier and colleagues utilizes DNA methylation signatures derived from single-molecule real-time sequencing to resolve species- and strain-level bins, as well as link mobile genetic elements such as plasmids to their host.

Two avenues for computational analyses can be pursued with short-read shotgun metagenomics, namely *de novo* metagenome assembly and assembly-free metagenomic profiling to estimate taxonomic abundances. Here, we focus on *de novo* metagenome assembly as this approach enables subsequent reconstruction of population genomes to enable genome-resolved metagenomics. The de Bruijn graph approach underpins the majority of current metagenome assembly algorithms, although at present, there is no community consensus as to which assembly method is superior (Quince et al., 2017). While significant improvements in metagenome assembly workflows have been made, challenges remain for highly complex environments that consist of hundreds to thousands of strains.

To resolve metagenome-assembled genomes, termed MAGs, methods to link contigs to their respective genomes – termed binning – are required post-assembly. Binning methods can exploit sequence composition, species abundance, chromosome organization, or other inherent properties of the shotgun metagenomic data. A myriad of binning tools and approaches are available; however all tools are currently limited in their ability to distinguish closely related species and strains. Lastly, MAGs

are evaluated for estimated genome completeness and contamination using universal single copy genes. As can be observed in Fig. 1, the deposition of MAGs within public databases has proliferated in the last three years and has led to major scientific insights.

Single-Cell Genomics

Although the overall approach to single-cell genomics is rather simple (Fig. 2), several technical aspects should be considered when generating and analyzing genomes from individual environmental cells. On the experimental side, sample preparation, cell isolation, cell lysis and whole genome amplification are critical steps in the procedure (Rinke et al., 2014). Unless fresh samples are available for immediate processing, cryopreservation of the sample material using cryoprotectants such as glycerol, betaine, or DMSO is important to minimize cell damage and maximize maintenance of the integrity of the cellular structure and the genomic DNA. Further, samples should be prepared so that cells are well dispersed, facilitating efficient single-cell isolation. Although various methods are available for this next step (for a review see (Blainey, 2013)), including micromanipulation and microfluidics, fluorescence-activated cell sorting (FACS) has been used most prevalently by flow-sorting cells based on their size and level of fluorescence (stain-based or autofluorescence). A chief advantage of FACS is its accuracy and speed and thus high throughput. Following isolation, cells are lysed to make DNA accessible to whole genome amplification (WGA). To date, no universal lysis method exists and each sample may require custom adjustments based on the target taxa of interest. Most commonly used methods include alkaline lysis, though chemical lysis has more recently been combined with physical (i.e., freeze-thawing) and enzymatic (i.e., lysozyme treatment) lysis (Blainey, 2013; Rinke et al., 2014). Despite the continued development of a broad range of WGA methods (for a review, see (Blainey, 2013)), Phi29-mediated multiple displacement amplification (MDA) has remained the key technique used for microbial single-cell sequencing. Recently, a thermostable Phi29 polymerase mutant was shown by Stepanauskas and colleagues to yield improved genome recovery for single cells with high GC genomes. Both enzymes are strand-displacement polymerases with high processivity that rely on random hexamers to prime the reaction before amplifying long (>10 kb) DNA products to generate the SAG. Taxonomic identification of SAGs can be performed via direct PCR amplification and Sanger-based sequencing of the small subunit (SSU) rRNA gene. Illumina's Nextera protocol is recommended for generating the shotgun sequencing library because it minimizes sample handling, and thus cross-contamination, and reduces cost. For SAG sequencing, the Illumina NextSeq platform has proven most useful, as bleed-over between poolmates is minimal and short reads/ short insert libraries minimize the occurrence of chimeric reads or read pairs, which hamper the sequence assembly process (see following section for more details).

Genome amplification causes single-cell sequence data to have coverage biases and chimeric junctions occurring approximately every 20 kb. SAG-specific *de novo* genome assemblers, such as SPAdes are therefore recommended (Fig. 2), as they are optimized to correct for such data artifacts. Further, due to the amplification of low femtogram-range DNA via random hexamer primers, the single-cell genomics process is prone to contamination, and thus, thorough quality assessment and assurance (QA) of the data is advised (Bowers et al., 2017a). Several tools have been developed in recent years to facilitate single-cell data QA, such as ProDeGe (see "Relevant Websites section"), CheckM (see "Relevant Websites section"), acdc (see "Relevant Websites section"), and Anvio (see "Relevant Websites section").

Quality Considerations for Genomes From Uncultivated Microorganisms

MAGs and SAGs are often of draft quality, i.e., they are less complete and more fragmented than isolate genomes. The median completeness estimate of single-cell genomes from environmental cells is approximately 40%, though combined assembly of SAGs from the same species, as defined by average nucleotide identity (ANI), can yield more complete composite genomes. Only one complete, finished single-cell genome has been obtained to date. For MAGs, genomes are comparably incomplete, though several finished genomes have been reported (Albertsen et al., 2013; Brown et al., 2015). While most SAGs contain the SSU rRNA genes in the assembly, many MAGs do not due to the challenge of assembling and binning this highly conserved gene in a complex metagenome. When SSU rRNA genes are present, taxonomic inferences can be made based on the SSU rRNA gene based phylogeny, though phylogenetic placement of MAGs and SAGs is most often made through concatenated alignments of protein-coding single-copy marker genes.

Considering the variable quality of SAGs and MAGs, it is important to critically assess and report genomes quality. Recently, a set of community standards was put forward for reporting genome sequences of uncultivated microorganisms: the minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea (Bowers et al., 2017b). MISAG and MIMAG encompass a minimal set of mandatory genome quality criteria, such as the reporting of genome completeness and contamination estimates. Considering the various different downstream analyses for SAGs and MAGs (e.g., basic metabolic reconstructions for comparative genomics, fragment recruitment for biogeographic analyses, and phylogenetic inference for evolutionary analysis and population genomics), reporting completeness and contamination estimates is imperative (Fig. 2), as some analyses require finished or high-quality genomes, while others may be feasible with medium- or low-quality genomes. Additionally, the consistent reporting of basic environmental metadata is suggested (Bowers et al., 2017b), as downstream comparative genomics is only as good as the metadata of a genomic dataset. Adhering to these standards will facilitate more reproducible and robust data interpretation and comparative genomic analysis to make solid inferences about evolutionary relationships and ecosystem functions of uncultivated bacteria and archaea.

From Individual Cells to Ecosystems

The tremendous diversity of microbes that impact the environment, animal and plant health, and serve as major drivers of global biogeochemical cycles have remained largely uncatalogued and underexplored. Major scientific advances within the past decade have been achieved through applying single-cell genomics and genome-resolved metagenomics. In particular, the microbial tree of life has greatly expanded with genomic information from newly identified candidate phyla (those phyla for which no cultivated isolate exists). The Patescibacteria were first described using single-cell genomics (Rinke et al., 2013) and later expanded through genome-resolved metagenomics to the Candidate Phyla Radiation (CPR) (Brown et al., 2015). This group of bacteria has small reduced genomes and lacks certain ribosomal proteins, implying divergent and unusual ribosome structures and biogenesis mechanisms (Brown et al., 2015). Complementary to the Patescibacteria, C. J. Castelle and colleagues utilized genome-resolved metagenomics to characterize deep-branching lineages constituting a large radiation within the Archaea with similarly small genomes that encode metabolic capacity for carbon and hydrogen metabolism relevant to Earth's biogeochemical cycles. Further, metagenomic reconstructions of candidate phyla from the Archaea provided unique insight into the origin and evolution of the eukaryotes through the recovery of the Asgard superphylum (Zaremba-Niedzwiedzka et al., 2017). While the exact phylogenomic placement of this clade is currently hotly debated, these genomes are enriched for proteins of eukaryotic origin, including homologues of eukaryotic membrane-trafficking machinery components and vesicle biogenesis.

Beyond cataloguing phylogenomic diversity, single-cell genomics and genome-resolved metagenomics have enabled important insights into how microbial metabolisms impact elemental fluxes and how those transformations influence ecosystem processes. For example, novel modes of metabolic coupling in the candidate phylum Marinimicrobia (formerly SAR406 and Marine Group A) were recently revealed by A. K. Hawley and colleagues through single-cell genomics with important implications for sulfur and nitrogen cycling. Similarly, single-cell genomic approaches utilized by D. Tsementzi and colleagues uncovered genes for respiratory nitrate reductases (Nar) encoded within certain clades of the highly abundant marine bacterium SAR11. Heterologous expression of the putative SAR11 *nar* operons verified functionality for the first step of denitrification, linking this important marine microbe to nitrogen loss pathways within oxygen minimum zones and expanding its known ecological niche. Furthermore, genome-resolved metagenomic approaches have led to major advances in understanding carbon fluxes mediated by uncultivated lineages, including methane metabolism in the archaeal phylum Bathyarchaeota and carbon fixation in the bacterial phyla *Candidatus* Eremiobacteraeota and *Candidatus* Dormibacteraeota (formerly WPS-2 and AD3, respectively). Importantly, functional capabilities can be tracked through better genomic representation of uncultivated lineages to shed light on key evolutionary origins. An elegant example by Soo et al. (2017) demonstrated independent acquisition of aerobic respiratory complexes within the three classes of Cyanobacteria (*Oxyphotobacteria, Melainabacteria*, and *Sericytochromatia*), supporting the hypothesis that aerobic respiration evolved after oxygenic photosynthesis approximately 2.3 billion years ago.

Owing to their synergy, single-cell genomics and genome-resolved metagenomic approaches are increasingly being used in combination (Fig. 3). Single-cell genomics offers genome-level resolution from an individual cell and can provide better spatial

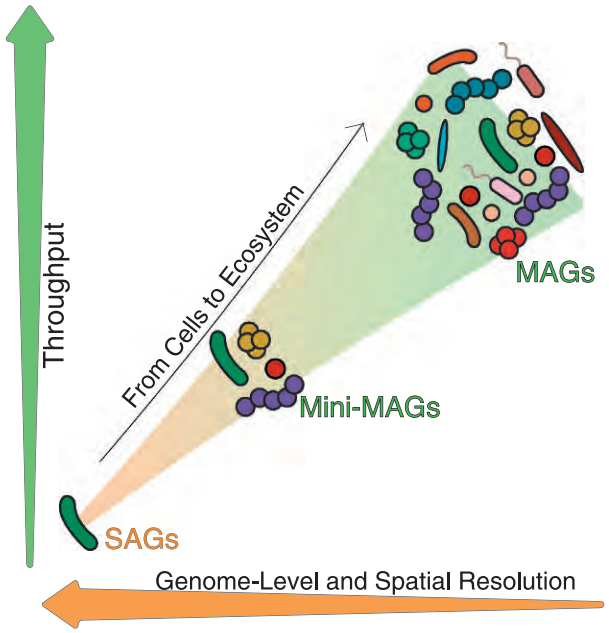

Fig. 3 From cells to ecosystems. Single cells provide genome-level and spatial resolution, which are critical to understand population structure in heterogeneous communities. MAGs from bulk metagenomes are produced at much higher throughput and without the bias of cell isolation and whole genome amplification. Mini-MAGs can be generated by subsampling an environmental sample.

resolution. However, partial genome recovery and the need for specialized instrumentation and whole genome amplification results in low throughput and biases. On the other hand, genome-resolved metagenomics provides a "consensus" genome which is typically more complete compared to SAGs (provided sufficient sequencing and a high-quality metagenome assembly), incorporating genetic information from genotypically heterogeneous populations. It is currently challenging to resolve strain-level variation from MAGs, although new approaches have recently been developed to address these challenges. Early studies applying both technologies predominantly utilized the partial SAGs to recruit metagenomic data, but more recent studies leverage both approaches to examine species diversification, adaptive properties and ecological patterns within microbial communities. Further, aspects of both single-cell and shotgun metagenome strategies have been combined through a newly described microfluidics-based mini-metagenomic method from F. B. Yu and colleagues, which allows single-cell resolution and improved genome recovery. We anticipate future studies leveraging both single-cell genomics and metagenomics will make significant strides towards addressing fundamental questions in microbial ecology, niche partitioning and microbial evolution.

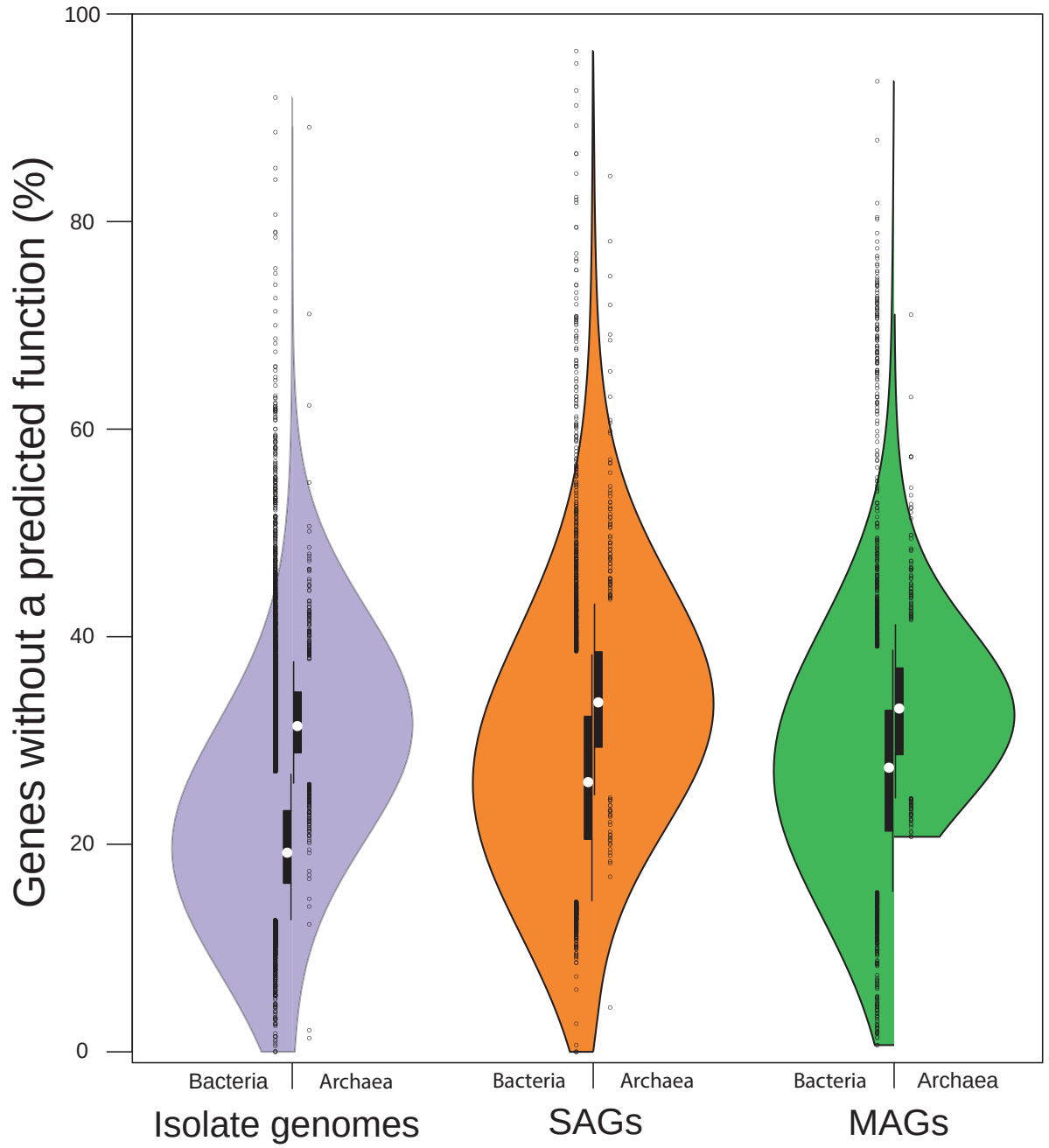

Fig. 4 Percentage of genes without a predicted function for bacterial and archaeal isolate genomes, SAGs and MAGs, illustrating a large knowledge gap in function assignment. Data were extracted from the Integrated Microbial Genomes and Microbiomes platform (https://img.jgi.doe.gov, IMG/M) on 1/16/2018.

Future Outlook

The tremendous increase in genomes from uncultivated microorganisms (Fig. 1), including from candidate phyla with no cultivated representatives, provides an exciting foundation for the functional interrogation of this microbial dark matter. The down side of the data deluge, however, is the increasing gap in our ability to assign function to many of the newly discovered genes, proteins, and pathways (Fig. 4). Steps to fill the function gap are most commonly achieved by moving from sequence to function via designing experiments to validate sequence-based predictions of function; these experiments rely on technologies such as DNA synthesis and protein expression followed by a functional assay. An alternate approach is "function-driven" (meta)genomics. Here moving from function to sequence, prior to sequencing, cells or DNA from organisms are selected and enriched based on a particular phenotype. Function-driven single-cell genomics (Doud and Woyke, 2017) may target cells of general metabolic activity or highly specific activities, as demonstrated in a recent study by M. Martinez-Garcia and colleagues, where populations degrading the substrate laminarin were captured and genome sequenced following the addition of a fluorescently labeled form of laminarin. Analogous to these approaches is the application of stable isotope probing (SIP) to link microbial activity (function) to taxonomic identity within an environmental sample. This function-driven metagenomic approach relies on the incorporation of heavy isotopes (for example, ^{13}C) into microbial DNA during growth on labeled substrates. Early successes of the technology include the identification of dimethyl sulphide (DMS) functional capacity within previously unknown bacterial groups by O. Eyice and colleagues, and most recently, the application of DNA-SIP for genome-resolved metagenomics by R. M. Ziels and colleagues. While a very promising approach, DNA-SIP is currently not widely utilized because it requires specialized laboratory equipment and technical expertise. However, we anticipate with advanced high-throughput protocols relying on robotic automation, along with improvements in depth of coverage for low-biomass samples, this technology will become more broadly accessible. While there is no magic bullet for validating function, and the aforementioned approaches are often rather custom, tedious and low throughput, it is important to continue making progress on the functional verification of genome sequence space. The integration of functional and phenotypic data with genomics will ultimately move us towards a better systems biology and ecosystem understanding of uncultivated bacterial and archaeal taxa.

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Relevant websites

https://github.com/mlux86/acdc. Acdc.
http://merenlab.org/software/anvio/. Anvio.
http://ecogenomics.github.io/CheckM/. CheckM.
https://gold.jgi.doe.gov. GOLD.
http://ecogenomics.github.io/GroopM/. GroopM.
https://prodege.jgi.doe.gov/. JGI.
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http://bioinf.spbau.ru/spades. SPAdes.

Glycogen Biosynthesis☆

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Abbreviations

ADP-Glc	Adenosine diphosphate-glucose
ADP-Glc PPase	Adenosine diphosphate glucose pyrophosphorylase
ADP-pyridoxal	Adenosine diphosphopyridoxal
BE	Branching enzyme
CRP	cAMP receptor protein
CSR	Carbon storage regulator
HCA	Hydrophobic cluster analysis
ORF	Open reading frame

Defining Statement

This article reviews the evidence indicating the energy-storage role of glycogen. Nutritional and physiological conditions enabling microorganisms to accumulate the polysaccharide as well as the reactions involved in their biosynthesis and subsequent utilization are described. In addition, the current state of knowledge of regulation of glycogen synthesis is discussed.

Introduction

Many bacteria accumulate glycogen, a polysaccharide that is considered to function as an energy reserve. Glycogen may accumulate during growth or at the end of the growth phase and may act as a source of energy no longer available from the environment. Glycogen usually accumulates when there is a carbon excess in the medium; that is, growth is limited by a nutrient other than the carbon source. The concentration of accumulated glycogen is dependent on the nutrient content of the medium as well as the growth phase of the organism and can at times be very high, at least 50% of the cell's dry weight. An advantage in using glycogen as a reserve storage compound is that because of its high molecular weight and physical properties, it has little effect on the internal osmotic pressure in the cell.

Various aspects of the enzymology and regulation of bacterial glycogen synthesis, including genetic regulation, have been extensively reviewed in the literature.

Criteria for Designating an Energy-Storage Function to a Compound

J.F. Wilkinson (1959) proposed three criteria for classification of compounds having energy-storage function. First, the compound should accumulate intracellularly under conditions in which the energy supply for growth of the organism is in excess. Second, the reserve polymer should be used when the components of energy or carbon in the media are no longer available for sustaining growth or other processes required for maintenance of viability. Wilkinson pointed out various cell functions that would require energy or carbon, for example, maintenance of a functioning semipermeable cytoplasmic membrane, replacement of proteins and nucleic acids during turnover, maintenance of intracellular pH, or other processes induced for bacterial survival, such as sporulation and encystment.

Third, the storage compound should be used by the cell for energy production that would enable it to survive in a nonsupportive environment. This energy required for survival is known as 'energy of maintenance'. This last criterion is perhaps the most important, for as Wilkinson notes, a number of other substances may be produced for other functions, such as, 'To detoxicate end products of metabolism which otherwise accumulate too rapidly and prove toxic.'

☆*Change History:* October 2014. J. Preiss introduced small edits in the text of the article. No edits in figures except figures 3 and 4 have been reversed in the order. Added new references to 'Further Reading' section.

This article is an update of J. Preiss, Glycogen Biosynthesis, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 145–158.

Occurrence of Glycogen in Bacteria

Many bacterial species accumulate glycogen either in stationary phase or under limited growth conditions with excess carbon in the medium. For *Escherichia coli* the rate of growth and the quantity of accumulated glycogen were inversely related when the cells were grown in media containing glucose as the carbon source and limited in nitrogen. Some bacteria, however, can optimally synthesize glycogen during exponential growth. Glycogen does not seem to be required for growth, since bacterial mutants having defective structural genes for the glycogen biosynthetic enzymes, and thereby unable to synthesize glycogen, can grow as well as their normal parent strains.

Glycogen or other similar α-1,4 glucans have been reported in more than 50 different bacterial species. The polysaccharide is not restricted to any class of bacteria as many Gram-negative and Gram-positive bacteria as well as archaebacteria have been reported to accumulate glycogen.

In many studies, it has been shown that glycogen accumulates in stationary phase, when growth either ceases because of the depletion of a nutrient, for example, nitrogen in the form of ammonia or amino acid, or diminishes because of unfavorable pH. When studied in detail, it is usually found that the largest accumulation of glycogen occurs under nitrogen-limited conditions.

Biological Functions of Bacterial Glycogen

Various strains of *E. coli*, *Enterobacter aerogenes*, and *Streptococcus mitis* not containing glycogen have been compared with strains containing glycogen, with respect to their viability in media having no exogenous carbon source. A more prolonged survival rate was observed for the strains containing glycogen. Moreover, it was shown that under starvation conditions, the glycogenless enteric cells degraded their RNA and protein constituents to NH_3. This either did not occur or occurred at a lower rate in cells containing glycogen. It was postulated that when glycogen was available, its use as an energy source minimized RNA and protein degradation for production of energy.

In various *Clostridium*, *Bacillus* and *Streptomyces viridochromogenes*, a glycogen-like molecule is accumulated up to 60% of the cell's dry weight before the onset of sporulation. The polysaccharide is then degraded during formation and maturation of the spore. Hence, it is believed that glycogen serves as an endogenous source of carbon and energy for spore formation. Although many experiments suggest that glycogen plays a role in the survival of bacteria, its precise function remains unclear. Further clarification on the role of bacterial glycogen is needed.

The synthesis and later degradation of glycogen by some oral bacteria may be an important factor in the development of dental caries. Consistent with this postulate are the findings that these organisms are capable of synthesizing glycogen, produce more acid when exogenous carbohydrate is present, and can produce acid in the absence of exogenous carbohydrate, when compared with oral bacteria that are unable to synthesize glycogen. The acid formed from polysaccharide catabolism may be of significance in the development of dental caries. It is formed over a considerable period of time and may consequently be responsible for the lower resting pH observed in dental plaque sampled from individuals with active caries.

Structural Studies of Glycogen

Microbial glycogen is very similar to mammalian glycogen in that it is composed mainly of α-1,4-glucosyl linkages and is a branched polysaccharide with about 8–12% of the glucosyl linkages being α-1,6.

Most bacterial glycogen structurally studied have chain lengths of ~10–13 glucose units and I_2 spectra with maximum absorption of ~410–480 nm. However, the bacterial α-glucans can differ to some extent in their structural properties, and the variation is attributable to a number of factors, including the relative amounts and types of branching and debranching activities.

Enzymatic Reactions Involved in Glycogen Synthesis

Sugar Nucleotide Pathway

Elaine Greenberg and Jack Preiss showed in 1964 that extracts of several bacteria contained both an ADP-Glc PPase synthetase (eqn [1]) and an ADP-Glc-specific glycogen synthase (eqn [2]):

$$\text{ATP} + \alpha\text{-glucose-1-P} \Leftrightarrow \text{ADP-Glc} + \text{PP}_\text{i} \quad [1]$$

$$\text{ADP} - \text{Glc} + \alpha\text{-glucan} \Rightarrow \alpha\text{-1, 4-glucosyl-glucan} + \text{ADP} \quad [2]$$

Subsequently, it was shown that branching enzyme activity (eqn [3]) was also present in many bacterial extracts and photosynthetic organisms.

$$\text{elongated } \alpha\text{-1, 4-glucan} \Rightarrow \text{branched } \alpha\text{-1, 4, } \alpha\text{-1, 6-glucosyl-glucan} \quad [3]$$

Synthesis of Glycogen Directly from Disaccharides

α-Glucans similar to glycogen have been shown to be formed from either sucrose or maltose. When *Neisseria* strains are grown on sucrose, they accumulate large amounts of a glycogen-type polysaccharide. The direct conversion of sucrose to an α-1,4 glucan (eqn [4]) is catalyzed by the enzyme amylosucrase. The glucosyl moiety of sucrose is transferred to a primer to form a new α-1,4-glucosidic linkage. The gene for the *Neisseria polysaccharea* amylosucrase has been cloned and can be expressed in *E. coli in vitro*-; the product formed is unbranched. Thus in *Neisseria* strains the α-1,6 linkages formed must be due to a branching enzyme activity:

$$\text{sucrose} + \alpha\text{-glucan} \Rightarrow \text{D-fructose} + \alpha\text{-1, 4-glucosyl-glucan} \quad [4]$$

Amylosucrase is limited to a few bacterial strains and is only induced in the presence of sucrose. Moreover, there are no reports that indicate that either *Neisseria* or any other bacteria are able to synthesize sucrose from other metabolites. Thus, accumulation of glycogen in *Neisseria* and in other bacteria grown on other carbon sources besides sucrose cannot be mediated by amylosucrase action.

A number of enteric and other organisms when grown on maltose or maltodextrins as a carbon source can synthesize a low-molecular-weight α-1,4-glucan via transfer of the glucosyl moiety of maltose to a growing α-1,4-glucan chain (eqn [5]). The enzyme catalyzing this reaction is amylomaltase:

$$\text{maltose} + \alpha\text{-glucan} \Leftrightarrow \alpha\text{-D-glucose} + \alpha\text{-1, 4-glucosyl-glucan} \quad [5]$$

The polymer is of very low molecular weight and is quickly degraded by another enzyme, maltodextrin phosphorylase, which is also induced by the presence of maltose in the medium. The synthesis of these two enzymes, however, is repressed by glucose, and thus neither amylomaltase nor maltodextrin phosphorylase activity would be responsible for synthesis of glycogen when the organisms are grown on carbon sources other than maltose.

Glycogen phosphorylase, as well as maltodextrin phosphorylase, is found in many bacteria and can catalyze synthesis or phosphorolysis of α-1,4-glucosyl linkages either in maltodextrins or in glycogen:

$$\alpha\text{-1, 4-(glucosyl)}n + \text{P}_\text{i} \Leftrightarrow \alpha\text{-glucose 1-P} + \alpha\text{-1, 4-(glucosyl)}n - 1 \quad [6]$$

However, maltodextrin phosphorylase is usually induced only in the presence of maltose or maltodextrins. *E. coli* mutants deficient in this enzyme accumulate maltodextrins, indicating that maltodextrin phosphorylase is involved in degradation of the α-glucans rather than in synthesis. The glycogen phosphorylase activity found in organisms is usually insufficient to account for the rate of synthesis observed.

In summary, it appears that glycogen synthesis in bacteria occurs mainly by the sugar nucleotide pathway. Both *E. coli* and *Salmonella typhimurium* mutants, either glycogen deficient or containing glycogen in excess of that observed in the parent wild-type strain, have been isolated. They are affected either in their glycogen synthase or in their ADP-Glc synthetase activity, implying that in these organisms, the ADP-Glc pathway is the major, if not exclusive, route for glycogen formation.

Properties of the Glycogen Biosynthetic Enzymes

ADP-glucose Pyrophosphorylase

ADP-Glc PPase has been purified from a number of microorganisms, and, with one exception, the enzyme has been found to be homotetrameric in structure with a subunit molecular size of about 50 kDa. The one exception enzyme, in *Bacillus stearothermophilus*, is also a tetramer, but is composed of two subunits: GlgC, of 387 amino acids with a molecular size of 43.3 kDa, and GlgD, of 343 amino acids with a molecular size of 39 kDa. The *Bacillus* enzyme is thus similar to the higher plant ADP-Glc PPases, which also have been shown to be heterotetrameric, $\alpha_2\beta_2$. In the plant enzyme, the small subunit is also known as the catalytic subunit, and the large subunit is known as the regulatory subunit. The potato tuber ADP-Glc PPase catalytic subunit expressed alone in *E. coli* is highly active while the large subunit by itself is inactive. Expression of both the subunits of potato tuber ADP-Glc PPase together results in a heterotetramer having higher affinity for the allosteric activator and lower affinity for the inhibitor. Therefore the large subunit is regarded as the regulatory subunit. The *B. stearothermophilus* GlgC subunit shows a 42–70% similarity in amino acid sequence with other ADP-Glc PPases and when expressed alone in *E. coli* shows catalytic activity. The *Bacillus* GlgD subunit shows no activity, and its function is unknown. However, it seems to increase the V_{max} of the GlgC activity, and slightly increases the apparent affinity of GlgC for the substrates.

An important facet of both bacterial and plant ADP-Glc PPases is that they are allosteric enzymes, and the allosteric function is important for the regulation of bacterial glycogen and plant starch synthesis rates. More than 50 ADP-Glc PPases – mainly bacterial, but also plant – have been studied with respect to their regulatory properties. In almost all cases, glycolytic intermediates activated ADP-Glc synthesis while AMP, ADP, or P_i were inhibitors. Glycolytic intermediates in the cell can be considered as indicators of carbon excess and, therefore, under conditions of limited growth with excess carbon in the media, accumulation of glycolytic intermediates would be signals for the activation of ADP-Glc synthesis. For most of the ADP-Glc PPases studied, the activator

glycolytic intermediate increases the enzyme's apparent affinity for the substrates, ATP and glucose-l-P, and increasing concentrations of activator reverse the inhibition caused by the inhibitors, AMP, ADP, or P_i.

The activator specificity of the bacterial and plant ADP-Glc PPases can be categorized into seven groups (Table 1) on the basis of their specificity of activation by the various glycolytic intermediates. The variation of activator specificity observed correlates with the nature of carbon assimilation dominant in the bacterium or plant tissue. *E. coli* or *S. typhimurium* obtain their energy mainly through glycolysis. The primary activator for their ADP-Glc PPases is fructose 1,6-bisP, 5″-adenylate is the major inhibitor, and their ADP-Glc synthetic activity is regulated by the [fructose 1,6-bisP]/[AMP] ratio. In plants and in the cyanobacteria, the activator is 3-P-glycerate (3PGA) and the inhibitor is Pi. 3PGA is the primary product in the carbon assimilation pathways of these photosynthetic organisms/tissues.

In plants there can be different patterns of interaction between the activator 3PGA and inhibitor P_i. For most plants the interaction is simply increasing 3PGA concentrations reversing Pi inhibition or increasing Pi concentrations reversing 3PGA activation. However 3PGA is not an activator of wheat endosperm ADP-Glc PPase, but can reverse P_i inhibition. The maize endosperm ADP-Glc PPase as well as some CAM ADP-Glc PPases is not inhibited by P_i but does reverse or inhibit 3PGA activation. Barley endosperm is neither inhibited by Pi, nor activated by 3PGA. However they do affect the barley enzyme activity by 3PGA increasing the affinity for the substrate, ATP, and P_i reversing the 3PGA effect.

Evidence has accumulated to indicate, that the *in vitro* observed kinetic allosteric activation and the inhibitor effects occur *in vivo* in bacterial cells. There is a class of mutants of *E. coli* and of *S. typhimurium* LT2 affected in their ability to accumulate glycogen. This mutant class has ADP-Glc PPases with altered regulatory properties. Generally, the mutants with ADP-Glc PPases having higher affinity for the activator, fructose 1,6-bisphosphate, and/or a lower affinity for the allosteric inhibitor, AMP, accumulate glycogen at a faster rate than their parent wild-type strains; mutants with enzymes having a lower affinity for the activator accumulate glycogen at a lower rate than does the parent strain.

Table 2 summarizes the allosteric properties of the mutant ADP-Glc PPases that have been studied and their ability to accumulate glycogen in the stationary phase. With respect to *E. coli*, there is a direct relationship between the affinity of the enzyme for the activator and the ability of the mutant to accumulate glycogen. If the apparent affinity for the activator, fructose-1,6-bisP, is higher, glycogen accumulation by the mutant is higher. If the apparent affinity for activator is lower, as seen for mutant SG14 enzyme, glycogen accumulation is lower in the mutant than in the parent strain. The two *S. typhimurium* mutant strains have ADP-Glc PPases that are more affected in their affinity for the inhibitor. Both JP23 and JP51 enzymes have lower affinity for the inhibitor,

Table 1 ADPglucose pyrophosphorylases classified with respect to activator specificity for the specific organisms that have been studied

Activator(s)	*Microorganisms*	*Predominant carbon assimilation pathway*
Pyruvate	*Rhodospirillum* sp. *Rhodocyclus purpureus*	Reductive pyruvate cycle
3-P-Glycerate	Cyanobacteria (higher plants)	Photosynthetic calvin cycle
Pyruvate, fructose-6-P	Some anaerobic photosynthetic bacteria, *Agrobacterium*, *Arthrobacter*	Reductive pyruvate cycle entner-doudoroff pathway
Pyruvate, fructose-6-P fructose-1,6-bis-P	Some *Rhodopseudomonas* species	Reductive pyruvate cycle entner-doudoroff pathway glycolysis
Fructose-6-P, fructose-1,6-bis-P	*Rhodopseudomonas viridis*, aeromonads, *Mycobacterium smegmatis*	Glycolysis
Fructose-1,6-bis-P	Enterics	Glycolysis
None	*Serratia* sp., *Enterobacter hafniae*, *Clostridium pasteurianum*	Glycolysis

Table 2 Allosteric kinetic constants, glycogen accumulation rates, and mutation of wild-type *Escherichia coli* and *Salmonella typhimurium* LT2, and allosteric mutant ADP-Glc PPases

Strain	*Maximal glycogen accumulation*[a] *(mg/Gram-cell)*	*Fructose 1,6-bisP*[b] $A_{0.5}$ ($\mu moll^{-1}$)	*AMP*[c] I_{0n5} ($\mu moll^{-1}$)	*Mutation*
E. coli B	20	68	75	-
Mutant SG14	8.4	820	500	A44T
Mutant SG5	35	22	170	P295S
Mutant 618	70	15	860	G336D
Mutant CL1136	74	5	680	R67C
S. typhimurium LT2	12	95	110	
Mutant JP23	15	No activation	250	
Mutant JP51	20	84	490	

[a]The bacterial strains were grown in minimal media with 0.75% glucose and the data are expressed as maximal milligram of anhydroglucose units per gram (wet wt.) of cells in stationary phase.

[b]$A_{0\ 5}$ is the fructose 1,6-bis-P giving 50% of maximal activation.

[c]$I_{0.5}$ is the AMP concentration required for 50% inhibition.

Table 3 Amino acid residues and sequences involved in binding of substrates and effectors of ADP-Glc PPase

Organism/tissue	*Activator sites*	*Sequence*
E. coli	Fru-1,6-bisP	38N**K**RAKPAV
Anabaena PC7120	3-P-glycerate	
	Site 1	412SGIVVVL**K**NAV
	Site 2	375QRRAIID**K**NAR
Potato tuber	3-P-glycerate	
Small subunit	Site 1	434SGIVTVI**K**DAL
	Site2	397IKRAIID**K**NAR
	Inhibitor Sites	
E. coli	5′-AMP	113W**Y**RGTADAV
Anabaena PC7120	Phosphate Substrate Sites	291TRA**R**YLPPTK
E. coli	Glucose-1-P	190IEFVE**K**PAN
Potato tuber	Glucose-1-P	193IEFAE**K**PQG
E. coli	ATP	113W**Y**RGTADAV

The amino acids identified by chemical modification and site directed mutagenesis to be involved in binding are in bold and underlined.

and these mutants accumulate higher amounts of glycogen than does the parent strain. Of interest is that JP23 ADP-Glc PPase is fully active in the absence of activator. Addition of activator fructose-1,6 bisP causes no further increase in activity.

The above studies plus the one showing a direct relationship between the fructose 1,6-bisP concentration in the *E. coli* cell and the rate of glycogen accumulation clearly point out that fructose 1,6-bisP is an allosteric activator of ADP glucose pyrophosphorylase and a physiological activator of glycogen synthesis in *E. coli* and in *S. typhimurium*.

Chemical modification and site-directed mutagenesis studies of the ADP-Glc PPases have provided evidence of the location of the activator binding site, the inhibitor binding site, and the substrate binding sites. These experiments have used pyridoxal-P as an analogue either for the activator, fructose 1,6-bisP, or, as subsequently shown, for the substrate, glucose-1-P. For an ATP analogue, the photoaffinity reagent 8-azido-ATP ($8N_3ATP$) proved to be a substrate for the *E. coli* enzyme, whereas 8-azido AMP ($8N_3AMP$) was an effective inhibitor analogue. Since the *E. coli* ADP-Glc PPase gene, *glgC*, had been cloned and its sequence determined, the identification of the amino acid sequence of the modified residue enabled one to determine the location of the modified residue in the primary structure of the enzyme. The amino acid residue involved in binding the activator was Lys39 and the amino acid involved in binding the adenine portion of the substrates (ADP-Glc and ATP) was Tyr114. Tyr114 was also the major binding site for the adenine ring of the inhibitor, AMP. Lys195 is protected from reductive phosphopyridoxylation by the substrate, ADP-Glc. It was proposed that Lys195 is also a part of the substrate binding site. Moreover, Asp142 has been shown to be the catalytic residue of the *E. coli* ADP-Glc PPase.

Similar experiments were also conducted to elucidate the activator, 3PGA, inhibitor, P_i, and substrate, Glc-1-P sites in *Anabaena* PC7120 and in the potato tuber and spinach leaf ADP-Glc PPases. Table 3 shows the amino acid sequences of the substrate and allosteric sites identified via chemical modification in the bacteria, *E. coli* and *Anabaena*, as well as in the higher plant enzymes. The sequence for the Glc-1-P substrate site is highly conserved for both bacterial and higher plant ADP-Glc PPases.

The ADP-Glc PPase of *Agrobacterium tumefaciens* has been cloned and expressed in *E. coli*. This enzyme has as activators, fructose-6-P and pyruvate, and an activator specificity different from that of the *E. coli* ADP-Glc PPase. Mutagenesis of the homologous Arg25 in the enzyme from *A. tumefaciens* yields an enzyme with an activity reduced by two orders of magnitude. Generally, it is expected that more dramatic effects would result from mutation of catalytic residues. Most probably in *A. tumefaciens* ADP-Glc PPase the catalytic residue equivalent to the *E. coli* enzyme Asp142 is Asp135.

Cloning of ADP-Glc PPases with Altered Allosteric Properties from *E. Coli* Mutants Affected in Glycogen Synthesis

As indicated above (Table 2), a class of mutants of *E. coli* and of *S. typhimurium* with altered rates of glycogen accumulation was found to have ADP-Glc PPases that were affected in their allosteric properties. To gain insight with respect to amino acid residues or domains involved in maintaining allosteric function the allosteric mutant ADP-Glc PPases were cloned.

Table 2 shows the various amino acid substitutions in the allosteric mutants that have been cloned and analyzed. Of interest is that the mutations causing large changes in the allosteric properties of the enzyme occur throughout the sequence of the ADP-Glc PPase. These changes of amino acids in the enzyme affect not only the affinity of the allosteric effectors (Table 2), but also the apparent affinities for the substrates ATP and Mg^{2+}. Thus, there are many domains affected by the mutations.

Site-directed mutagenesis of the ADP-Glc PPase gene and analysis of various allosteric mutant genes have provided much information in the structure–function relationships of the substrate and catalytic sites. What is needed for greater clarification is knowledge on the three-dimensional structure of the enzyme. Information on the three-dimensional structure of an ADP-Glc PPase is currently available for the plant enzyme, potato tuber ADP-Glc PPase, but not for the bacterial ADP-Glc PPase. However, several methods have been utilized to predict the ADP-Glc PPase enzyme structure. A 'hydrophobic cluster analysis' (HCA) was applied to several ADP-Glc PPases from different sources representing different classes, for example, *E. coli*, *Anabaena*, *Chlamydomonas*, potato

(*Solanum tuberosum* L.) tuber 'small' subunit, and different 'large' subunits from maize embryo, maize shrunken 2 and *Arabidopsis thaliana*. The cluster analysis strongly indicated that ADP-Glc PPases are extremely similar in the distribution and pattern of the clusters, in both bacterial and plant enzymes. Thus it is certainly possible that the ADP-Glc PPases share a common folding pattern, even though there is a different quaternary structure as seen for $\alpha_2\beta_2$ in plants and α_4 in bacteria.

A model has been proposed in which the conserved amino acids known to have specific roles in the binding of substrates (Tyr114, Lys195 in *E. coli*) and activators (Lys39 in *E. coli*, Lys382, Lys419 in *Anabaena*) are located in loops. The residues Pro295 and Gly336 seem to be located in a region important for the regulation of the *E. coli* enzyme and are also in loops. The amino acid residue Asp142 in the *E. coli* enzyme was identified as a catalytic residue and is also situated in a loop.

A structure that is observed in proteins that bind nucleotides is also predicted in this model. Region 1 of the ADP-Glc PPase has a Gly-rich loop after a β-sheet, which is similar to a 'P loop' in protein kinases or nucleotide binding sites, and region 2 has three β-sheets and helices that are compatible with the Rossman fold. Thus, regions 1 and 2 comprise a putative domain or subdomain that binds ATP. Moreover, Tyr114, which was shown to be reactive with the azido analogue of ATP, is present in this region.

The first pyrophosphorylase domain crystallized and solved was present in a bifunctional enzyme that is the expression product of the gene *glmU*. One domain of the GlmU protein is a UDP-*N*-acetylglucosamine pyrophosphorylase, and the other is an acetyltransferase. Later, other pyrophosphorylase domain structures were solved. All these studies verify the predicted secondary-structure model of the ADP-Glc PPase. The idea that the sugar-nucleotide pyrophophorylases share a similar catalytic domain is supported by the observation that the homologous Glc1-P site is present in the GDPMan PPase from *Pseudomonas aeruginosa*.

Glycogen Synthase

The bacterial glycogen synthases are specific for the sugar nucleotide ADP-Glc. In cases where it has been studied, the enzyme subunit size is about 50 kDa, and the enzyme in its native form is either a dimer or homotetramer.

An affinity analogue of ADP-Glc, adenosine diphosphopyridoxal (ADP-pyridoxal), was used to identify the ADP-Glc-binding site. Incubation of the enzyme with the analogue plus sodium borohydride led to an inactivated enzyme. The degree of inactivation correlated with the incorporation; about 1 mol of analogue per mole of enzyme subunit resulted in 100% inactivation. After tryptic hydrolysis, one labeled peptide was isolated, and the modified Lys residue was identified as Lys15. The sequence, Lys-X-Gly-Gly, where lysine is the amino acid modified by ADP-pyridoxal, has been found to be conserved in the mammalian glycogen synthase and the plant starch synthases.

The structural gene for glycogen synthase, *glg*A, has been cloned from *E. coli*, *S. typhimurium*, *A. tumefaciens*, and *B. stearothermophilus*, and the nucleotide sequences of these organisms have been determined. The *E. coli* glycogen synthase sequence consists of 1431 bp specifying a protein of 477 amino acids with a molecular weight of 52 412 Da. Its availabilty has enabled researchers to perform site-directed mutagenesis experiments to determine structure–function relationships for a number of amino acids in the *E. coli* glycogen synthase. Substitution of other amino acids for Lys at residue 15 suggested that the Lys residue is mainly involved in binding the phosphate residue adjacent to the glycosidic linkage of the ADP-Glc and not in catalysis. The major effect on the kinetics of the mutants at residue 15 was the elevation of the K_m of ADP-Glc, of about 30- to 50-fold, when either Gln or Glu was the substituted amino acid. Substitution of Ala for Gly at residue 17 decreased the catalytic rate constant, k_{cat}, about three orders of magnitude compared with the wild-type enzyme. Substitution of Ala for Gly 18 only decreased the rate constant 3.2-fold. The effect on the K_m of the substrates, glycogen and ADP-Glc, was minimal. It was postulated by the researchers that the two glycyl residues in the conserved LysX-Gly-Gly sequence participated in the catalysis by assisting in maintaining the correct conformational change of the active site or by stabilizing the transition state.

Since there still existed binding of the ADP-Glc and appreciable catalytic activity of the Lys15Gln mutant, the ADP-pyridoxal modification was repeated and in this instance about 30 times higher concentration was needed for inactivation of the enzyme. The enzyme was maximally inhibited about 80%, and tryptic analysis of the modified enzyme yielded one peptide containing the affinity analogue and with the sequence Ala-Glu-Asn-modified Lys-Arg. The modified Lys was identified as Lys277. Site-directed mutagenesis of Lys277 to form a Gln mutant was carried out, and the K_m for ADP-Glc remained essentially unchanged but k_{cat} decreased 140-fold. It was concluded that Lys277 was more involved in the catalytic reaction than in substrate binding.

A cysteine-specific reagent 5,5′-dithiobis(2-nitrobenzoic acid) was shown to inactivate the *E. coli* glycogen synthase. To determine the responsible residue, all cysteine residues present in the enzyme, Cys7, Cys379, and Cys408, were substituted with Ser. 5,5′-Dithiobis(2-nitrobenzoic acid) modified and inactivated the enzyme only if Cys379 was present. The inactivation was prevented by the substrate ADP-Glc. Mutations C379S and C379A increased the S_{05} for ADP-Glc 40- and 77-fold, whereas the enzyme-specific activity was decreased 5.8- and 4.3-fold, respectively. Studies of inhibition by glucose 1-phosphate and AMP indicated that Cys379 was involved in the interaction of the enzyme with the phosphoglucose moiety of ADP-Glc. Other mutations, C379T, C379D, and C379L, indicated that this site is intolerant for larger side-chains. Because Cys379 is in a conserved region, other residues were scanned by mutagenesis. Replacement of Glu377 by Ala and Gln decreased V_{max} more than 10 000-fold without affecting the apparent affinity for ADP-Glc and glycogen binding. Mutation of Glu377 by Asp decreased V_{max} only 57-fold, indicating that the negative charge of Glu377 is essential for catalysis. The activity of the mutation E377C, on an enzyme form without other Cys, was chemically restored by carboxymethylation. Other conserved residues in the region, Ser374 and Gln383, were analyzed by mutagenesis but were found not to be essential. Comparison with the crystal structure of other glycosyltransferases suggests that this conserved region is a loop that is part of the active site. Thus, the results ofthis study indicate that this region is critical for catalysis and substrate binding, with Glu377 being the catalytic residue.

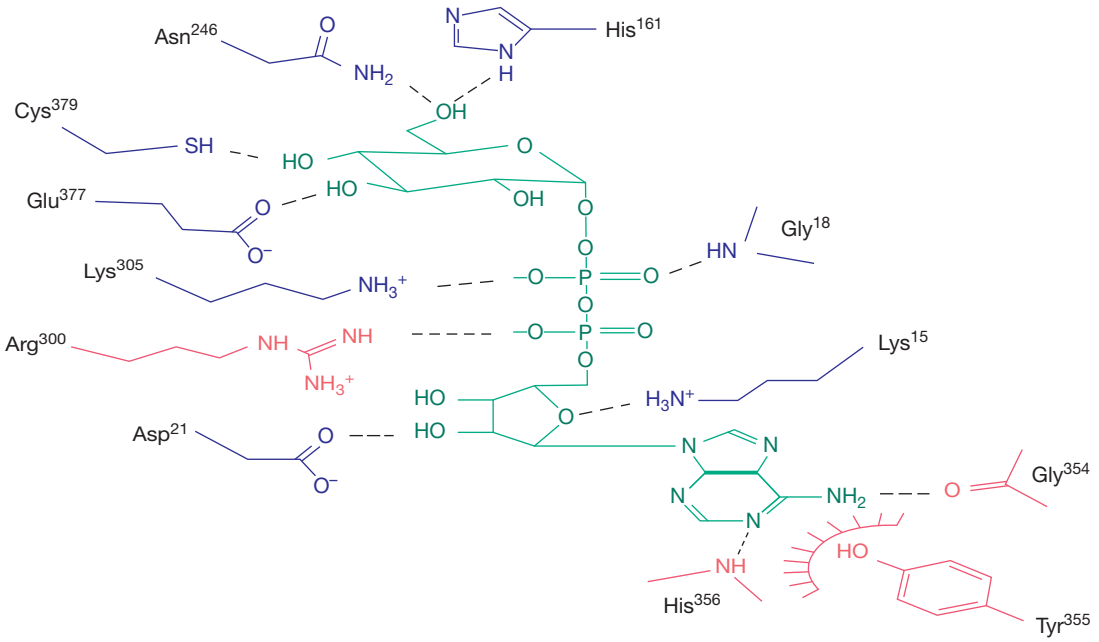

Figure 1 Schematic representation of the putative binding site of ADP-glucose in the glycogen synthase. The residues that are observed interacting with the ADP-Glc structure are in blue and red color. The ADP-Glc structure is in green.

Glycogen synthases are glucosyl transferases that retain the anomeric configuration of glucosyl linkage of ADP-Glc to the nonreducing end of glycogen. The *E. coli* glycogen synthase was therefore modeled based on three other glycosyl linkages retaining glycosyltransferases having a GT-B fold. Comparison between this model and the structure of the active sites of crystallized glycosyl linkages retaining GT-B glycosyltransferases identified conserved residues in glycogen synthase with the same topology. These residues were studied in the *E. coli* glycogen synthase by site-directed mutagenesis to confirm their importance predicted by the model. Mutations D137A, R300A, K305A, and H161A decreased the specific activity 8100-, 2600-, 1200-, and 710-fold, respectively. None of these mutations increased the K_m for glycogen, and only H161A and R300A had a higher K_m for ADP-Glc of 11- and 8-fold, respectively. These residues were essential, validating the model that shows a strong similarity between the active site of *E. coli* glycogen synthase and the others retaining GT-B glycosyltransferases, known to date.

The alignment of the amino acid residues essential for catalysis in the enzymes retaining glycosyltransferases – trehalose-P synthase, maltose phosphorylase, and a muramoyl *N*-acetylglucosaminyl transferase, AceA – in the glycogen synthase has been proposed. Recently, the glycogen synthase crystal structure has been elucidated and confirms the correctness of the proposed model. Figure 1 shows a schematic representation of the putative binding site for ADP-Glc in the glycogen synthase.

Branching Enzyme

The structural genes of various branching enzymes (BE) have been cloned from many bacteria. The nucleotide and deduced amino acid sequences of the *E. coli glg*B gene consisted of 2181 bp specifying a protein of 727 amino acids and with a molecular weight of 84 231 Da.

The amino acid sequences of BEs and amylolytic enzymes such as a-amylase, pullulanase, glucosyltransferase, and cyclodextrin glucanotransferase have been compared, and there is a marked conservation of the amino acid sequence of the four catalytic regions of amylolytic enzymes in the BEs irrespective of whether they are of bacterial, plant, or mammalian origin. As shown in Table 4 four

Table 4 Comparison of primary structures of various branching enzymes with the four best-conserved regions of the α-amylase family

Enzyme	*Region 1*	*Region 2*	*Region 3*	*Region 4*
B. subtilis α-amylase	100**D**AVIN**H**	171GF**R**F**D**AAKH	204FQYG**E**ILQ	262VTWVES**HD**
Maize endosperm BE I	277**D**VVHS**H**	347GF**R**F**D**GVTS	402TVVA**E**DVS	471IAYAES**HD**
Potato tuber BE	355**D**VVHS**H**	424GF**R**F**D**GITS	453VTMA**E**EST	547VTYAES**HD**
Rice seed BE 1	271**D**VVHS**H**	341GF**R**F**D**GVTS	396TIVA**E**DVS	462VTYAES**HD**
E. coli glycogen BE	335**D**WVPG**H**	400AL**R**V**D**AVAS	453VTMA**E**EST	518VFLPLN**HD**

The sequences have been derived from references cited in the text. Just α-amylase of the enzymes from the α-amylase family are compared with *E. coli* glycogen BE. However, Svensson and her colleagues show comparisons of over 40 enzymes ranging from amylases, glucosidases, various α-1,6-debranching enzymes as well as 4 cases of branching enzymes. The invariant amino acid residues are in bold letters

regions that putatively constitute the catalytic regions of the amylolytic enzymes are conserved in the plant branching isoenzymes and the glycogen BEs of *E. coli*. Analysis of this high conservation in the a-amylase family has been pointed out and greatly expanded by B. Svensson and H.M. Jesperson and colleagues with respect to sequence homology and also in the prediction of (β/α)8-barrel structural domains with a highly symmetrical fold of eight inner, parallel β strands, surrounded by eight helices, in the various groups of enzymes in the family. The (β/α)8-barrel structural domain was determined from the crystal structure of some a-amylases and cyclodextrin glucanotransferases.

The conservation of the putative catalytic sites of the α-amylase family in the glycogen and starch BEs would be expected as the BE catalyzes two consecutive reactions in synthesizing α-1,6-glucosidic linkages by cleavage of an α-1,4-glucosidic linkage in a 1,4-α-D-glucan to form a non-reducing-end oligosaccharide chain that is transferred to a C-6 hydroxyl group of the same or another 1,4-α-D-glucan. The eight highly conserved amino acid residues of the α-amylase family are also functional in BE catalysis. The regions of the C- and N-terminus are dissimilar in sequence and in size in the various branching isoenzymes. Recent studies suggest that these amino acid sequence regions are important with respect to BE specificity and with respect to substrate specificity (amylose or amylopectin) as well as in the size of chain transferred and the extent of branching. Furthermore, truncation of 113 amino acids of the N-terminal of the *E. coli* BE, causes it to branch longer branch-chains than do the wild-type enzyme, further indicating that the N-terminal region is involved in determining the size of chain transferred. Recently, the crystal structure of a truncated form of the *E. coli* BE has been elucidated.

Genetic Regulation of Glycogen Synthesis in *E. coli*

As indicated before, the glycogen biosynthetic enzymes are induced in the stationary phase. The rate of glycogen synthesis is inversely related to growth rate when growth is limited for certain nutrients, for example, nitrogen. Consistent with this are the findings that the levels of glycogen biosynthetic enzymes in *E. coli* increase as cultures enter the stationary phase.

When *E. coli* or *S. typhimurium* cells are grown in an enriched medium and 1% glucose, the specific activities of ADP-Glc PPase and glycogen synthase increase 11- to 12-fold, while glycogen BE increases 5-fold, as cultures enter stationary phase. However, when the organisms are grown in a defined medium, the ADP-Glc PPase and glycogen synthase activities are relatively high in the exponential phase. BE in defined media is fully induced in the exponential phase, with only about a twofold increase in specific activity of the ADP-Glc PPase and glycogen synthase occurring when the cells reach the stationary phase. These experiments suggest that the gene encoding the BE is regulated differently from the genes for ADP-Glc PPase and glycogen synthase. The addition of inhibitors of RNA or protein synthesis to prestationary phase cultures prevents the enhancement of glycogen synthesis in the stationary phase, and this is expected for a pathway that is under transcriptional control.

Location of the Structural Genes for Glycogen Biosynthesis

The structural genes for glycogen biosynthesis are clustered in two adjacent operons, which also contain genes for glycogen catabolism. The structural genes for glycogen synthesis were shown to be located at approximately 75 min on the *E. coli* K-12 chromosome, and the gene order at this location was shown to be *glg*A-*glg*C-*glg*B-*asd*. These genes encode the enzymes glycogen synthase, ADP-Glc PPase, glycogen BE, and are close to *asd*, the structural gene for the enzyme aspartate semialdehyde dehydrogenase (EC 1.2.1.11).

The *E. coli glg* structural genes were cloned into pBR322 via selection with the closely linked essential gene *asd*. Among several *asd* + plasmid clones isolated, pOP12 was found to contain a 10.5-kb *Pst*I fragment which encoded the structural genes *glg*C, *glg*A, and *glg*B. The arrangement of genes encoded by pOP12 has also been determined by deletion mapping experiments, and the nucleotide sequence of the entire *glg* gene cluster has been resolved. The genetic and physical map of the *E. coli* K-12 *glg* gene cluster is shown in Figure 2. The continuous nucleotide sequence of over 15 kb of this genome region includes the sequences of the flanking genes *asd* and *glp*D (glycerol phosphate dehydrogenase; EC 1.1.99.5; 1.1.1.8).

Nucleotide sequence analysis indicated that in addition to the *glg*C, *glg*A, and *glg*B genes, pOP12 contains *glg*X, located between *glg*B and *glg*C, and *glg*P, located downstream from *glg*A. The gene *glg*X has recently been shown to express isoamylase activity.

The *glg*P gene is identified by homology with rabbit muscle glycogen phosphorylase. This gene encodes glycogen phosphorylase as shown via the expression and characterization of its gene product. Neither *glg*X nor *glg*P is needed for glycogen synthesis, suggesting that both may be more involved in glycogen catabolism.

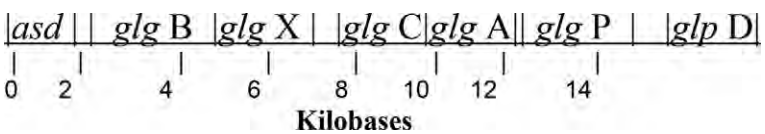

Figure 2 Structure of the glycogen cluster in *Escherichia coli*. The restriction map is constructed from known contiguous sequences. All of these genes are transcribed from left to right (counterclockwise on the genome), except *glp*D.

The organization of the gene cluster suggests that the *glg* genes may be transcribed as two tandomly arranged operons, *glg*BX and *glg*CAP (Figure 2) The coding regions of *glg*B and *glg*X overlap by one base pair, *glg*C and *glg*A are separated by two base pairs, and genes *glg*A and *glg*P are separated by 18 base pairs. The close proximity of these genes suggests translational coupling within the two proposed operons. Studies of the regulation of the *glg* structural genes, with *lac*Z translational fusions and other approaches are consistent with a two-operon arrangement for the *glg* gene cluster, in which the *glg*CAP and *glg*BX operons may be preceded by phase-regulated growth promoters. Transcriptions initiating upstream of *glg*C have been analyzed by S1 nuclease mapping.

Genetic Loci Affecting Glycogen Biosynthetic Enzyme Levels in *E. coli*

Studies of glycogen-excess *E. coli* B mutants SG3 and AC70R1, which exhibit enhanced levels of the enzymes in the glycogen synthesis pathway, suggested that glycogen synthesis is under negative genetic regulation. The mutations in these strains *glg*R and *glg*Q affect *glg*A and *glg*C transcription respectively, although these mutations have not been isolated and sequenced.

Four 5′ termini *in vivo* transcripts were identified within 0.5 kb of the upstream region of the *glg*C coding region by S1-nuclease protection analysis (Figures 2 and 3). Three of these transcripts were mapped to high resolution and their sequences are as shown in Figure 3.

The *glg*R mutation is closely linked to the glycogen structural genes by P1 transduction analysis, and the mutation results in 8- to 10-fold higher levels of ADP-Glc PPase and 3- to 4-fold higher levels of glycogen synthase in exponential phase, but does not alter the level of BE in minimal media. Analysis of RNA transcripts for *glg*C in strain SG3 having the *glg*R mutation reveals an increase in transcript B only (Figures 2 and 3). Therefore, it appears that the *glg*R mutation may alter a *cis*-acting site involved in the regulation of transcript B. This effect might be mediated via a negative regulatory site, but the current experimental evidence also is consistent with an overexpressed phenotype or a higher affinity CRP-binding site.

The *glg*Q mutation is not linked to the glycogen gene cluster in P1 transduction, and results in 11-, 5.5-, and 2-fold increases in ADP-Glc PPase, glycogen synthase, and glycogen BE, respectively. Therefore, *glg*Q appears to affect one or more *trans-acting* factors for the expression of the genes in the two glycogen operons. Levels of the four transcripts for the *glg*C gene are elevated in the *glg*Q mutant, AC70R1 (Figures 2 and 3). Transcript A was affected the most dramatically, with approximately 25-fold higher levels being present in AC70R1 versus the wild-type *E. coli* B or the SG3 strain. Since the levels of BE are also elevated in AC70R1, it was not considered likely that *glg*Q was a mutation in the cAMP-CRP or ppGpp regulatory systems (see below), which do not affect *glg*B expression. The expression of the chromosomal *lac*Z gene in AC70R1 (*glg*Q mutant) and in the wild-type *E. coli* B strain was also similar, providing further evidence for the idea that glgQ affects a different regulatory system for the *glg* genes. In summary, *glg*Q and *glg*R mainly affect Transcript A and Transcript B, respectively.

Sigma Factor E σ70 Transcribes *glg*CAP

The DNA sequences immediately preceding the 5′-ends of the three transcripts shown in Figure 4 are weakly related to consensus sequences for *E. coli* and *S. typhimurium* promoters. Although positively regulated promoters typically show weak similarity to the consensus sequence, it is also possible that one or more of the *glg* promoters is recognized via an alternative sigma factor. Therefore, the dependence of *glg*C expression on three sigma factors was tested in coupled transcription translation, using monoclonal antibodies that selectively inhibit transcription by specific and selective recognition of the sigma factors. A monoclonal antibody

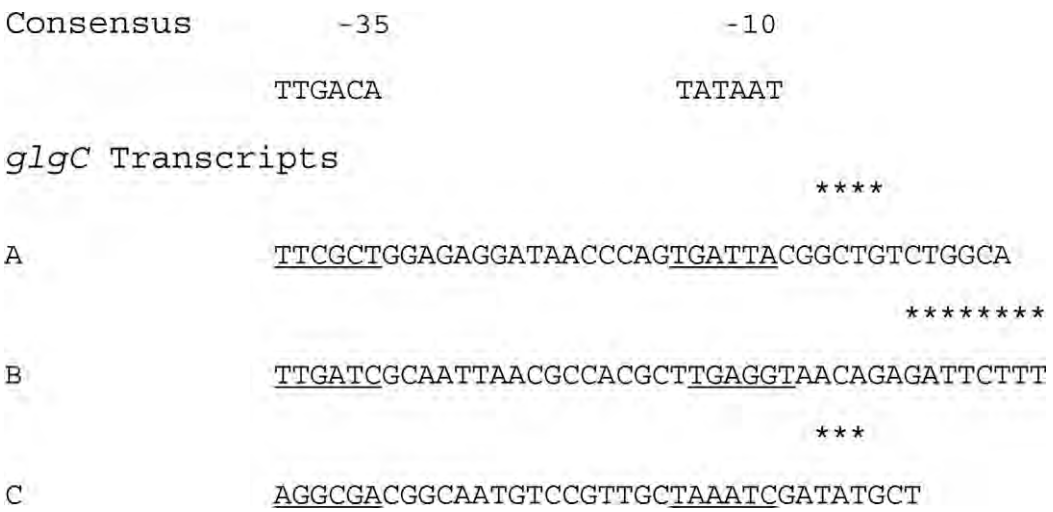

Figure 3 The cAMP-CRP-binding sites and the upstream sequences of the *glg*C gene of *Escherichia coli* and *Salmonella typhimurium*. The asterisks indicate the nucleotide identities between the proposed CRP-binding sites.

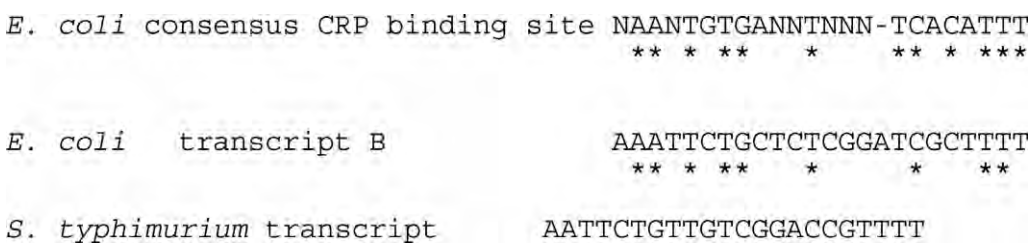

Figure 4 Comparison of the 5′ flanking regions of the *Escherichia coli glg*C transcripts with the consensus sequence of the *E. coli s· 70* promoter. The *glg*C transcripts, A, B, and C are located respectively, at −60, −130, and −245 from the *glg*C initiation codon. The asterisks indicate the 5′ termini of the transcripts. The best −10 and −35 regions are underlined.

directed against *E. coli* σ70 inhibited up to 85% of the *glg*C expression, while an antibody directed against σ54 or σ 32 did not inhibit expression of *glgC*, relative to *gln*AP 2 (σ54-dependent) and dnaK (σ32-dependent) controls. Therefore, even though nitrogen limitation enhances glycogen synthesis *in vivo*, the expression of *glg*C is not regulated by the nitrogen starvation, σ54-dependent transcriptional controls. This conclusion agrees with the experimental result that *Ntr*C and *Ntr*A (σ54) did not enhance expression of *glg* genes in S-30 experiments. The heat shock regulatory system (σ32-dependent) also appears to have no involvement in the control of *glg*C expression. Therefore, the major active form of RNA polymerase (E. σ70) is apparently utilized for *glg* expression in the S-30 transcription translation system. However, the S-30 extracts used in the experiments were prepared from exponential cells to obtain optimal translational activity, and would not have contained endogenous activity from a sigma factor or an accessory factor synthesized in stationary phase. Therefore, expression via one or more other possible *glg*C transcripts may not have been detected by the S-30 experiments.

The Three Global Regulatory Systems Regulating Glycogen Biosynthesis

Glycogen biosynthesis is under the direct control of at least three global regulatory systems: catabolite repression (stimulation of *glg*ABC expression by cAMP); stringent response (stimulation of *glg*ABC expression by ppGpp); and repression of the *glg*ABC expression by the *csr*A gene product, a 61-amino acid polypeptide.

Regulation by cAMP

The genes *cya*, encoding adenylate cyclase (EC 4.6.1.1), and *crp*, encoding cAMP receptor protein (CRP), are required for optimal synthesis of glycogen. Exogenous cAMP can restore glycogen synthesis in a *cya* strain but not in a *crp* mutant. cAMP and CRP are strong positive regulators of the expression of the *glg*C and *glg*A genes, but do not affect *glg*B expression. The addition of cAMP and CRP to S-30 extracts having *in vitro* coupled transcription-translation reactions and containing pOP12 as the genetic template, resulted in up to 25- and 10-fold increase in the expression of *glg*C and *glg*A, respectively. cAMP and CRP also enhanced the expression of *glg*C and *glg*A encoded by either plasmids or restriction fragments in *in vitro* reactions of protein synthesis. A restriction fragment that contained *glg*C and 0.5 kb of DNA from the upstream noncoding region of *glg*C was sufficient to permit cAMP–CRP regulated expression, suggesting that the *glg*C gene contains its own cAMP-regulated promoter(s).

Evidence of a CRP-binding site on a 243 bp restriction fragment from the upstream region of *glg*C was obtained using gel retardation analysis. There are also potential consensus CRP-binding sequences within the *glg*C upstream region preceding the *glg*C genes in both *E. coli* and *S. typhimurium*.

Regulation by ppGpp

Glycogen biosynthesis in *E. coli* is positively regulated by ppGpp as *rel*A strains are glycogen-deficient. Expression of the *glg*C and *glg*A genes is stimulated by ppGpp. Expression of *glg*C in transcription-translation reactions increased three- to fourfold in the presence of ppGpp; *glg*A expression exhibited approximately a twofold enhancement. The expression of *glg*B was not affected by ppGpp.

Cyclic AMP and ppGpp also have independent effects on *glg*CA expression in *in vitro* transcription-translation experiments. Actually, their combined effects on *glg*C expression in such experiments can be synergistic. The addition of cAMP-CRP or ppGpp results in an increase of 6.3- or 1.6-fold, respectively, in the expression of *glg*C over the basal or unactivated level of expression, while their addition together leads to an 18.8-fold stimulation.

Evidence for positive regulation of *glg*C expression *in vivo* by ppGpp was obtained using the *glg*C′-′*lacZ* translational fusion in pCZ3-3. This gene fusion was introduced into strains comprising an isogenic series that varied in basal levels of ppGpp owing to increasingly severe mutations in *spo*T. The *spo*T gene affects the level of ppGpp in the cell.

Regulation by *csr*A Gene Product via Regulating the *glg* Operons

A gene from *E. coli* K-12, *csr*A, encoding a negative factor for *glg* transcription has been identified. A mutant TR1-5 accumulated about 24-fold more glycogen than an isogenic wild-type strain. The gene affected by the TR1-5 mutation, *csr*A (for 'carbon storage regulator'), has been cloned, sequenced, and mapped on the *E. coli* genome, and some of its regulatory effects have been studied.

The TR1-5 mutation was also shown to affect glycogen levels by causing elevated expression of genes representative of both glycogen operons, *glg*C and *glg*B. Levels of ADP-Glc PPase expressed from the chromosome were approximately 10-fold higher in the TR1-5 mutant than in an isogenic *csr*A + strain in the stationary phase. The TR1-5 mutation affects glycogen levels and expression of the *glg*B and *glg*C genes in the exponential as well as the stationary phase.

The *csr*A gene also appears to regulate the expression of the gluconeogenic enzyme PEP carboxykinase (EC 4.1.1.38). Expression of a PEP carboxykinase operon fusion (*pck*A′-′*lac*Z) increased about twofold in exponential and stationary phases in the TR1-5 mutant, suggesting that gluconeogenesis may also be under negative control by *csr*A. When several isogenic strains were grown on synthetic media, it was found that *csr*A + and *csr*A::*kan*R strains (transductants with the TR1-5 mutation) were capable of growth on a wide variety of carbon sources. However, a strain that contained the functional *csr*A gene encoded on a multicopy (pUC19-based) plasmid pCSR10, could grow on glucose and fructose, but not on any of the gluconeogenic substrates – succinate, glycerol, pyruvate, and L-lactate. When strains were plated on a richer defined medium, support for growth of a pCSR10-containing strain using some gluconeogenic substrates, including acetate, as a major carbon source did occur. However, the pCSR10-containing strain formed only pinpoint colonies on succinate, whereas each of the other strains grew well. The *csr*A gene may affect succinate utilization or transport, independently of its effect on gluconeogenesis.

The *csr*A gene is located at 58 min on the physical map of the E. *coli* K-12 genome. It appears between the gene *ala*S, which encodes alanyl tRNA synthetase (EC 6.1.1.7), and the *ser*V operon of tRNA genes, and is transcribed counterclockwise on the chromosome. The *csr*A ORF (open reading frame) encodes a 61-amino-acid polypeptide, which was strongly expressed from the plasmid pCSR10 in S-30 transcription-translation experiments. Deletion mapping experiments of the plasmid-encoded *csr*A gene demonstrated that it is required to mediate the inhibitory effects on the glycogen synthesis phenotype *in vivo*.

The *csr*A gene product negatively modulates posttranscriptionally by facilitating decay of *glg*CA mRNAs. The *csr*A gene product is a specific mRNA-binding protein binding to *csr*B RNA. Binding of the *csr*A gene product to *csr*B RNA inhibits the repression of the *glg*CA operon and the *glg*B genes. Since *csr*B RNA increases in stationary phase, it is believed that repression of the glycogen biosynthetic genes is relieved by the binding of *csr*A gene product by the *csr*B RNA.

Analysis of *glg*C transcripts by S1-nuclease protection mapping showed that the steady-state levels of four *glg*C transcripts (Figure 5) are elevated in the TR1-5 mutant and are severely depressed in a pCsr10-containing strain, indicating that *csr*A affects the transcriptional regulation of *glg*C.

Proposed Integrated Model for the Genetic Regulation of the Glycogen Biosynthetics Pathway in *E. coli*

Regulation of glycogen metabolism involves a number of factors coordinating the glycogen synthetic rate with the physiology of the cell. Genetic regulation of the glycogen biosynthesis pathway by cAMP and ppGpp allows *E. coli* to adjust its metabolic capacity for converting available carbon substrate into glycogen in response to the availability of carbon, energy, or amino acids, respectively.

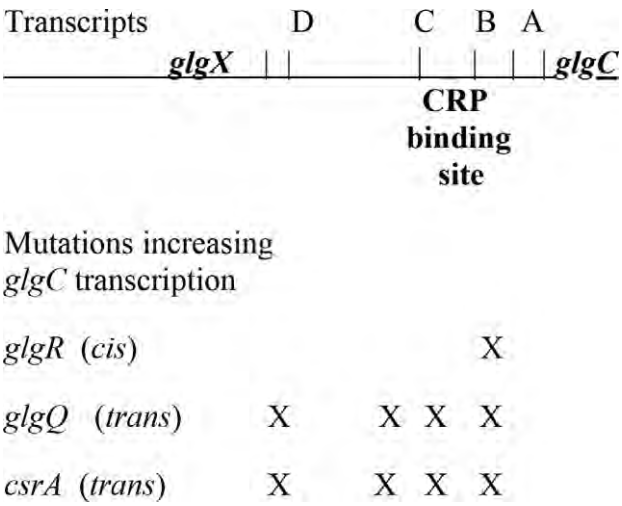

Figure 5 Regulatory sites for transcription of *glg*C. The location of the CRP-binding region and the *glg* upstream transcription sites. Reviewed in Preiss J (1996) Regulation of glycogen synthesis. In: Neidhardt FC (editor in chief) *Escherichia coli and Salmonella typhimurium*: *Cellular and Molecular Biology*, 2nd ed., Vol. 1, pp. 1015–1024. Washington, DC: American Society for Microbiology.

Table 5 Genes affecting glycogen metabolism in *Escherichia coli*

Gene	*Gene product*	*Map. site (min)*	*Comments*
Regulatory			
cya	Adenylate cyclase	8	Regulates catabolite repression
crp	Cyclic AMP receptor protein	74	Regulates catabolite repression
*rel*A	(p)ppGpp synthase I	60	Mediates stringent response and response to carbon/energy
*spo*T	(p)ppGpp3′-pyrophosphohydrolase	60	
*csr*A	6.8 kDa polypeptide (*csr*A)	58	Regulation of gluconeogenesis, *glg*CA, and *glg*B transcription
*glg*Q	Transacting factor (unidentified)	(?)	Transcriptional regulation of *glg*CA and *glg*B
*glg*R	*cis*-acting site	75	Transcriptional regulation of *glg*CA
Structural Biosynthetic			
*glg*C	ADP-Glc pyrophosphorylase	75	Synthesis of glucosyl donor
*glg*A	Glycogen synthase	75	α-1,4-Glucosyl transferase
*glg*B	Glycogen branching enzyme	75	α-1,6 branch chain synthesis
Degradative			
*glg*P (*glg*Y)	Glycogen phosphorylase	75	Glycogen phosphorolysis
*amy*A[a]	α-Amylase	43	Glycogen hydrolysis
*glg*X	Glucan hydrolase/transferase	75	Isoamylase

[a]Raha M, Kawagishi I, Muller V, Kihara M, and Macnab RM (1992) *Escherichia coli* produces a cytoplasmic α-amylase, AmyA. *Journal of Bacteriology* 174:6644–6652.

When cells are rapidly mutiplying, the levels of the enzymes are repressed, and although the energy and carbon are available for glycogen synthesis, the glycogen synthesis rate is low. Upon nutrient deficiency, synthesis of ADP-Glc PPase and glycogen synthase are induced, and the capacity for glycogen synthesis is greater. The level of glycogen that is ultimately accumulated will be dependent upon carbon availability and is subject to allosteric regulation of the ADP-Glc PPase activity.

Genetic regulation thus determines the capacity for glycogen synthesis, and this can be distinguished from the regulation of absolute glycogen levels. For example, the glycogen biosynthetic enzymes are induced in stationary phase when cells are grown on enriched medium lacking glucose. Yet, glycogen synthesis does not occur because of lack of available carbon. But in media with excess glucose and limiting nitrogen, the expression of the genes for the biosynthetic enzymes is somewhat weaker as cAMP is at a lower level. However, the glycogen synthesis rate is relatively greater because of the carbon availability, and the conditions are such that allosteric activation occurs. Carbon availability allows the activator concentration to be relatively high, the ATP levels high while maintaining the AMP levels (the allosteric inhibitor) low.

Mutants that are affected in either negative (*glg*R, *glg*Q, *csr*A) or positive control systems (*cya*, *crp*, *rel*A, *spo*T) for *glg*CA gene expression clearly demonstrate that the genetic regulation of the levels of glycogen biosynthetic enzymes is most important in determining the ultimate level of glycogen synthesized and accumulated under any given physiological condition.

The structural and regulatory genes involved in glycogen metabolism in *E. coli* are listed in Table 5; the effects of both positive and negative regulatory factors which control the expression of the *glg* genes of the glycogen biosynthetic pathway are listed. Many important questions remain to be solved, particularly the regulatory role of factor *csr*A. The physiological states to which the *csr*A regulatory system responds are yet to be determined. Moreover, the actual functions of *csr*A and many carbon starvation-induced genes in glycogen synthesis at the biochemical and molecular level also remain to be established.

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Green Algae: Chlorophyta and Streptophyta

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Glossary

Archaeplastida Eukaryotic supergroup comprising photosynthetic organisms with a primary plastid. The group contains the Viridiplantae (green plants), red algae and glaucophytes.
Autospore Non-motile spore produced within a parent cell, and which has the same shape as the parent cell before release.
Diplohaplontic life cycle Sexual life cycle with two vegetative phases, one diploid and one haploid.
Haplontic life cycle Sexual life cycle containing only a haploid vegetative phase, with the only diploid cell being the zygote.
Isokont Flagella of a cell that are similar in structure, but may differ in length or behavior.
Mixotrophic Having partly autotrophic and partly heterotrophic nutrition.
Monophyletic group (clade) A group of organisms that contains all the descendants of a common ancestor.
Oogamous reproduction Sexual reproduction involving the fusion between a motile male gamete and a large, non-motile female gamete.
Paraphyletic group A group of organisms that has evolved from a common ancestor but which does not contain all descendants of that ancestor.
Phagotroph Heterotrophic or mixotrophic organism that ingests nutrients by engulfing solid particles.
Phragmoplast Array of microtubules oriented perpendicularly to the plane of cell division, determining the formation of the cell plate and new cell wall.
Phycoplast Array of microtubules oriented parallel to the plane of cell division, determining the formation of a new cell wall.
Picoplankton The fraction of the plankton composed by cells between 0.2 and 3 μm.
Plasmodesmata Cytoplasmic threads running through the cell wall and connecting the cytoplasm of adjacent cells.
Pyrenoid A spherical structure in the chloroplast, containing the enzyme RuBisCO, and associated with the formation of reserve polysaccharides.
Siphonein, siphonoxanthin Xanthophyll accessory pigments found in some prasinophytes and Ulvophyceae.
Zoospore A flagellate spore.

Abbreviations

CCW	Counter clockwise oriented basal bodies
CW	Clockwise oriented basal bodies
Cytb$_6$f	Cytochrome b$_6$f complex
DO	Directed oppositely oriented basal bodies
mya	Million years ago
PSI	Photosystem I
PSII	Photosystem II
UTC	Ulvophyceae, Trebouxiophyceae and Chlorophyceae

Defining Statement

Green algae are a large and ecologically important group of oxygenic photosynthetic eukaryotes. They are diverse in terms of species number, morphology, biochemistry and ecology. Together with the embryophytic land plants, they form the Viridiplantae or green plants (also known as Chlorobionta or Chloroplastida). The Viridiplantae comprise two clades: the Chlorophyta and the Streptophyta. The Chlorophyta include a wide variety of marine, freshwater and terrestrial green algae. The Streptophyta include freshwater green algae (also known as charophytes) and the embryophytic land plants. As such, the green algae with the exclusion of the land plants form a paraphyletic group. Monophyly of the green plants, on the other hand, is well established based on ultrastructural, biochemical and molecular data.

The green algae possess the following unifying traits, most of which are also shared with the land plants:

- Chloroplasts are enclosed by a double membrane, and include stacked thylakoids. Many species have pyrenoids, which are embedded in the chloroplast, penetrated by thylakoids, and surrounded by starch.
- Photosynthetic pigments include chlorophyll a and b, along with accessory pigments such as carotenes and xanthophylls.

- The most important reserve polysaccharide is starch, which is clustered around the pyrenoids or scattered through the chloroplast stroma.
- Many green algae are flagellate unicellular organisms, or have flagellate cells in some stage of the life cycle. Flagellate cells typically possess two (or a multiple of two) flagella, which are similar in structure (isokont), although they may differ in length or behavior.
- The region between the flagellum and its basal body (flagellar transition zone) is typically characterized by a star-shaped (stellate) structure, which is a nine-pointed star linking nine pairs of microtubules.
- Cell walls, when present, are generally composed of a cellulose fiber matrix.

Morphological and Ecological Diversity

The green algae contain around 500 genera and an estimated 15,000 extant species. They display several degrees of organismal complexity, ranging from the smallest unicellular eukaryote (*Ostreococcus*) to large and complex organisms. Vegetative morphological types include unicellular motile cells (flagellates), unicellular non-motile cells (coccoids), motile or non-motile colonies, unbranched or branched filaments, blades, parenchymatous thalli, siphonocladalean thalli (composed of multiple, multinucleate cells) and siphonous thalli (composed of a single, multinucleate giant cell) (Fig. 1).

Green algae are globally distributed from tropical to arctic regions, occurring in a wide range of aquatic habitats. They are widespread in freshwater environments, including lakes, ponds, streams, estuaries and wetlands. Some groups are abundant in marine environments, as plankton in nearshore or oceanic environments or attached in benthic coastal habitats. Green algae can form nuisance blooms under conditions of nutrient pollution in freshwater and marine environments. Several green algae are terrestrial or aerophytic, living on moist soil, rocks, and tree trunks. Some species occur in hypersaline environments, such salt pans. Others have adapted to living in snow or ice. Several species engage in symbiosis with other eukaryotes, and some others are heterotrophic parasites.

The embryophytic land plants, which will not be discussed further in this article, include an estimated 400,000 species and are dominant in terrestrial habitats, although some species have adapted secondarily to freshwater or marine environments.

Cell Structure, Mitosis and Cell Division

The cell structure of green algae is essentially similar to that of other photosynthetic eukaryotes. Cells are enclosed by a plasma membrane, and contain a typical eukaryotic cytoskeleton, one to many nuclei, mitochondria, plastids, and other organelles. Many (but not all) green algae have a cell wall or other types of cell covering, such as organic body scales. Some green algal cells have a large vacuole. The fine structure of green algal cells, and processes of mitosis and cell division have been extensively studied, and some features, such as the ultrastructure of the flagellar apparatus, have been regarded as important characters for defining the main groups of green algae (Table 1).

Plastids

All green algae, with a few exceptions, are autotrophic with cells containing one to many chloroplasts. Chloroplasts have a double membrane and stacked thylakoids, which contain the photosynthetic pigments chlorophyll a and b. These pigments are generally not masked by differently colored accessory pigments, hence the characteristic green color of most species. However, some green algae (e.g., *Haematococcus* species, *Chlamydomonas nivalis*, and *Trentepohlia* species) accumulate photoprotective carotenoid pigments under high light conditions, masking the green color and appearing orange or red in color.

Chloroplast number and shape is highly variable. Most green algae have cells with a single chloroplast, which can be cup-shaped, ring-shaped, star-shaped, or plate-shaped. Giant celled green algae contain hundreds to millions of chloroplasts per cell, which can be isolated (e.g., in siphonous green algae), or organized in a netlike structure (e.g., in siphonocladalean green algae). Some green algae are heterotrophic (e.g., *Prototheca*, *Helicosporidium*, *Polytoma*) and contain non-photosynthetic plastids, with different metabolic functions. Several green algae are capable of supplementing photosynthesis by uptake of exogenous dissolved organic carbon, such as sugars, amino acids and other small molecules (osmotrophy), or by ingestion of living or dead particles (phagotrophy).

Most green algae have chloroplasts containing one to several pyrenoids. These structures are involved in the production of the photosynthetic reserve polysaccharide starch, which accumulates in plates around the pyrenoid (Fig. 2). The pyrenoid is often penetrated by several thylakoids. Starch formation also occurs elsewhere in the chloroplast, with starch granules scattered through the chloroplast stroma. Chloroplasts may also contain plastoglobules, which are lipoprotein particles coupled to thylakoid membranes and containing biosynthetic enzymes.

Flagellate cells often have an eyespots or stigma, which has a function in light perception, and determines the swimming direction of the cell. Depending on the intensity of the light, this can be either towards the light (positive phototaxis) or away from the light (negative phototaxis). The stigma is located inside the chloroplast, and comprises 1–8 rows of red-colored, carotenoid-containing globules, positioned between thylakoids (Fig. 2). The photoreceptor itself is rhodopsin (which is also the universal

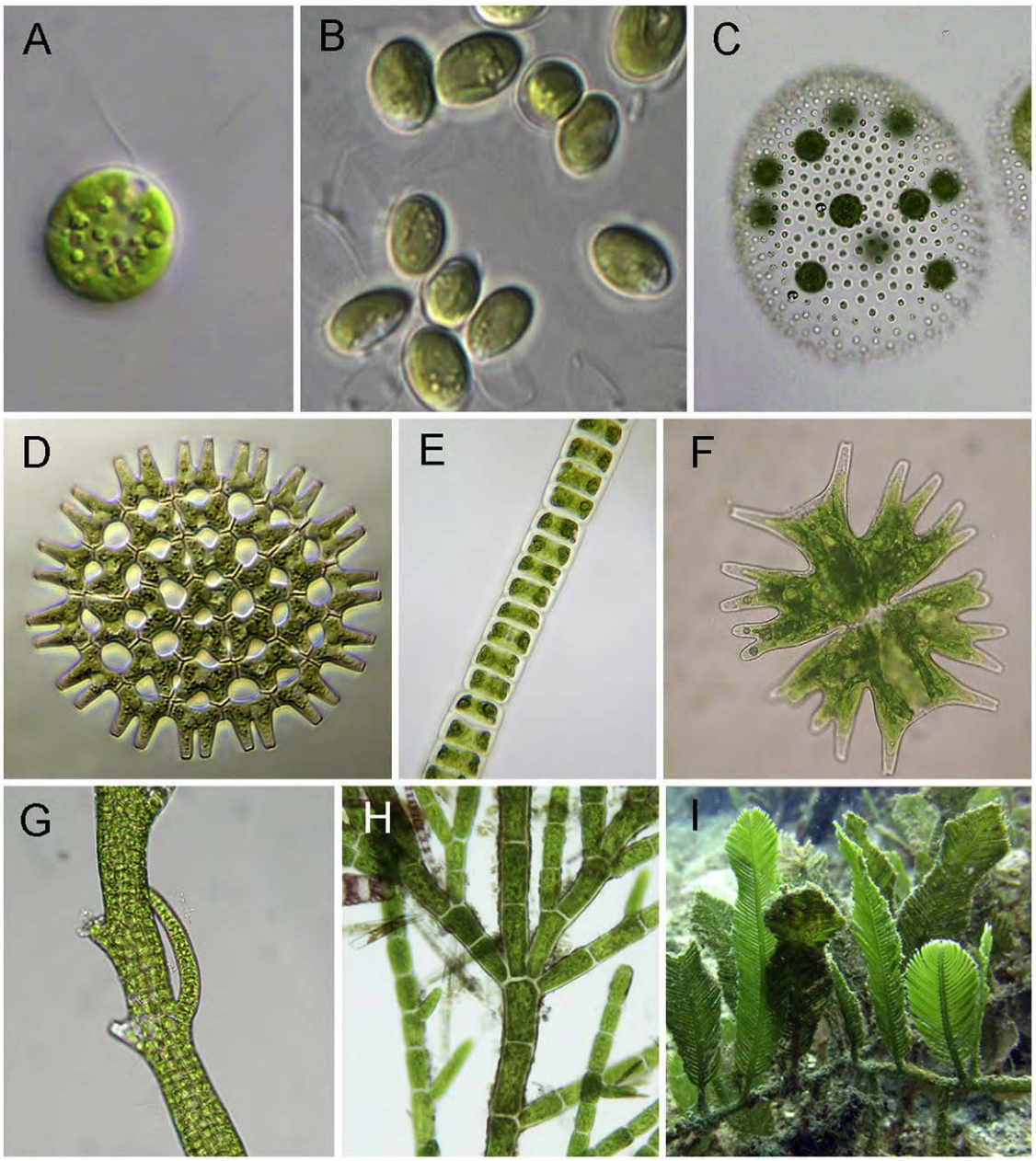

Fig. 1 Morphological diversity of the green algae. (A). *Chlamydomonas globose* (Chlorophyceae), unicellular flagellate with two flagella. (B). *Coccomyxa subellipsoidea* (Trebouxiophyceae), coccoid cells. (C). *Volvox reticuliferus* (Chlorophyceae), motile colony. (D). *Pediastrum* (Chlorophyceae), colony of non-motile cells. (E). *Ulothrix* (Ulvophyceae), unbranched filament. (F). *Micrasterias* (Zygnematophyceae, Desmidiales), non-motile unicellular alga constricted in two parts with ornamented cell wall. (G). *Ulva prolifera* (Ulvophyceae), multicellular tubular thallus composed of uninucleate cells. (H). *Cladophora albida* (Ulvophyceae), multicellular, branched filamentous thallus composed of multinucleate cells. (I). *Caulerpa sertularioides* (Ulvophyceae), macroscopic thallus composed of a single, multinucleate siphonous cell. (A) *Chlamydomonas globosa*, by Picturepest, licensed under CC BY 2.0, https://www.flickr.com/photos/picksfromoutthere/13263097835/in/album–72157629497519723/, (B) *Coccomyxa subellipsoidea*, by Tatyana Darienko et al. licensed under CC BY 4.0, https://doi.org/10.1371/journal.pone.0127838, (C) *Volvox reticuliferus*, by Nozaki et al. licensed under CC BY 4.0, https://doi.org/10.1371/journal.pone.0142632, (D) *Pediastrum*, by Giuseppe Vago, licensed under CC BY 2.0, https://www.flickr.com/photos/giuseppevago/4906329255/in/photolist–7fQw5p–7fUsD5–7MRMc6–8tydKH–48SNWp, (E) *Ulothrix*, by Giuseppe Vago, licensed under CC BY 2.0, https://www.flickr.com/photos/giuseppevago/4906329425/, (F) *Micrasterias*, by Ajburk, released into the public domain, https://commons.wikimedia.org/wiki/File:Micrasterias_.jpg, (G) *Ulva prolifera*. Photo: Frederik Leliaert, (H) *Cladophora albida*. Photo: Frederik Leliaert, (I) *Caulerpa sertularioides*. Photo: Frederik Leliaert.

visual pigment in animals), and is located in the plasma membrane. The function of the carotenoid globules is to shield the photoreceptor for strong light, to enhance the contrast, or to increase the light intensity.

The photosynthetic apparatus is highly similar to that of land plants. The primary reactions of photosynthesis are mediated by three protein complexes embedded in the thylakoid membranes of chloroplasts. These complexes are Photosystem II (PSII),

Table 1 General characteristics of the main groups of green algae

	Flagellar apparatus	*Type of mitosis*	*Cell division*	*Morphology*	*Main habitat*
Prasinophytes	Cruciate roots, some with MLS	variable	Furrowing	Unicellular	Marine planktonic, some freshwater
Trebouxiophyceae	Cruciate roots, CCW orientation	Semi-closed, non-persistent spindle	Furrowing, with phycoplast	Unicellular, colonial and simple multicellular	Freshwater to terrestrial
Chlorophyceae	Cruciate roots, CW or DO orientation	Closed, non-persistent spindle	Furrowing, with phycoplast	Unicellular, colonial and simple multicellular	Freshwater to terrestrial
Ulvophyceae	Cruciate roots (some with MLS), CCW orientation	Closed, persistent spindle	Furrowing, without phycoplast (one group with phragmoplast and plasmodesmata)	Unicellular to complex multicellular and giant-celled thalli	Mainly marine benthic, some freshwater or terrestrial
Charophytes	Asymmetric roots, MLS	Open, persistent spindle	Furrowing, some with phragmoplast and plasmodesmata	Unicellular to complex multicellular	Freshwater or terrestrial

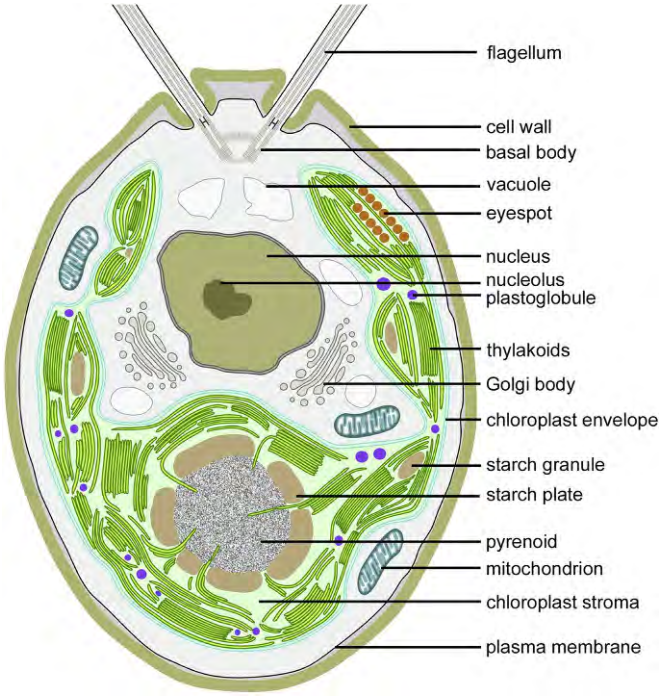

Fig. 2 Ultrastructure of the flagellate cell of *Chlamydomonas*, showing the main intracellular structures. Modified from Ultrastructure of the flagellate cell of *Chlamydomonas*, by Engel et al. licensed under CC BY 4.0, https://doi.org/10.7554/eLife.04889.001.

the cytochrome b_6f complex (Cytb_6f), and Photosystem I (PSI), which are connected in series through the photosynthetic electron transport chain.

Flagella

Most green algae are flagellates in their vegetative phase, or have flagellate cells in some stage of the life cycle (with few exceptions, such as the classes Zygnematophyceae and Palmophyllophyceae). Flagellate cells generally have two (or a multiple of two) flagella, which are generally inserted apically or subapically, or more rarely laterally (some prasinophytes and *Mesostigma*). The flagella of one cell are similar in structure (isokont), although they may differ in length or behavior. Few green algae have a single flagellum only (e.g., the prasinophytes *Micromonas* and *Monomastix*). A rare type of flagellate cells is characterized by a ring of flagella around the anterior end of the cell (stephanokont zoospores), and occur in asexual reproductive cells of Oedogoniales (Chlorophyceae) and *Derbesia* (Ulvophyceae).

The ultrastructure of green algal flagella has a 9 + 2 arrangement of microtubules (axoneme), similar to flagella and cilia of other eukaryotes. Towards the base of the flagellum, just above where it emerges from the cell, the axoneme passes into a transition zone, which typically contains a structure that appears star-shaped (stellate) in cross section, and H-shaped in longitudinal section, visible using an electron microscope (Figs. 2 and 3). The flagella terminate inside the cell by basal bodies, which are composed of nine interconnected triplets of microtubules, and function as template for assembly of the flagellar axoneme. In most species of Chlorophyta, the flagella are anchored in the cell by four microtubular roots, which are connected to the basal bodies and run beneath the plasma membrane towards the bottom of the cell. These microtubular roots are symmetrically arranged in a cross (cruciate root system) when the cell is viewed from the top. Two of these roots contain two microtubules, the two others contain three to eight microtubules. In the Streptophyta and some prasinophytes the flagellar roots are arranged asymmetrical, with basal bodies positioned parallel to one another, and a broad band of approximately 60 microtubules (termed multilayered structure) anchoring the flagella in the cell. The relative position of the basal bodies varies between green algal groups, and can be positioned directed oppositely (DO), shifted clockwise (CW), counter clockwise (CCW), or lay parallel to each other. Other flagellar structures include two robust fibers that connect the flagella (present in most green algae), and a contractile striated fiber (rhizoplasts, common in the Chlorophyta, but rare in the charophytes) that connects the flagellar apparatus to the nucleus (e.g., *Chlamydomonas*) or the chloroplast (e.g., *Pyramimonas*).

Cell Walls

The cells of many green algae are covered by a cell wall. Other green algae either lack a cell wall (naked cells), or have other types of cell covering, such as organic body scales. Most green algal cell walls have a polysaccharide fibrils-matrix organization. Some species have cell walls composed of proteins only. In Chlorophyta, the cell wall is often composed of cellulose or chitin-like polysaccharides, xylan or mannan. The cell walls in prasinophytes are highly diverse. Many prasinophytes are naked (e.g., *Ostreococcus*, *Micromonas*), while others (e.g., *Pyramimonas*, *Nephroselmis*) are covered by one to several layers of organic body scales of various forms, including plate-like, hair-like, and complex, three dimensional structures. These organic structures are mainly composed of monosaccharides and produced within the Golgi apparatus. Cells of in the Chlorodendrophyceae are covered by a coherent wall composed of fused body scales. The Chlorophyceae include a wide diversity of cell walls ranging from cellulose-pectin complexes to walls composed of hydroxyproline-rich glycoproteins. Cell wall composition may remain identical or change during the cell life-cycle. Cell walls in the Ulvophyceae are also highly variable and are composed of cellulose, mannans, glucan, xylans, and sulfated and/or pyruvylated polysaccharides.

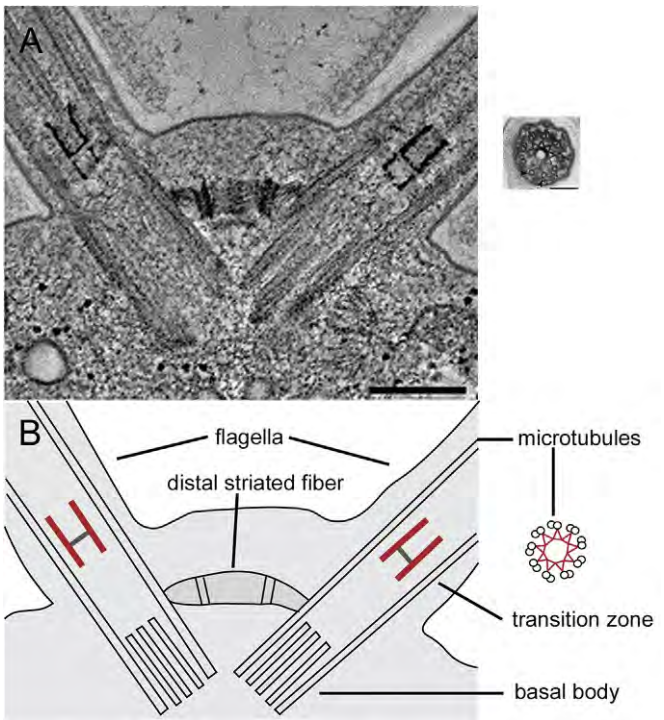

Fig. 3 Flagellar apparatus of *Chlamydomonas*. (A). Electron micrographs of a longitudinal section of the flagellar apparatus, and stellate structure of the transition zone in cross section. (B). Schematic representation of the flagellar apparatus. Flagellar apparatus of *Chlamydomonas*. Image top-left, by Dutcher and O'Toole, 2016, licensed under CC BY 4.0, https://doi.org/10.1186/s13630-016-0039-z. Image top-right, by Lechtreck et al., 2013, licensed under CC BY 4.0, https://doi.org/10.1186/2046-2530-2-15.

In the charophyte classes Charophyceae, Zygnematophyceae and Coleochaetophyceae, cell walls contain assemblages of polymers similar to land plants, including cellulose, pectins, hemicelluloses, arabinogalactan proteins, extensin, and lignin.

Some members of the ulvophycean orders Bryopsidales (e.g., *Halimeda*) and Dasycladales (e.g., *Neomeris*) and some species of Charophyceae (e.g., *Chara*) are calcified by depositing calcium carbonate on the outside of the cells, forming a structural defense against grazing.

Mitosis and Cell Division

The processes of mitosis and cytokinesis are variable among green algae, but generally characteristic for the main groups (Table 1). During early prophase the basal bodies duplicate and move towards the poles of the future mitotic spindle where they function as centrioles. An unusual type of mitosis is present in species of Trebouxiophyceae, where the centrioles are not positioned at the poles of the mitotic spindle but at the metaphase plate of the chromosomes (metacentric mitosis). Mitosis is either closed, in which the nuclear envelope remains nearly intact during metaphase (characteristic for most Chlorophyta), or open, in which the nuclear envelope is broken down during nuclear division (characteristic for most charophytes). Also the behavior of the mitotic spindle during telophase differs among green algal groups. The spindle may degenerate quickly during telophase (non-persistent telophase spindle), or persist, resulting in a typical dumbbell-shaped telophase configuration.

Mitosis is followed by cell division (cytokinesis) in most green algae, except in groups with multinucleate cells (e.g., Cladophorales and Bryopsidales), where mitosis is uncoupled from cytokinesis. In most green algae, cell division is accomplished by furrowing (Fig. 4), in which a cleavage furrow is usually already present at the early prophase, but only develops further during telophase. Several groups of the core Chlorophyta (including the Trebouxiophyceae and Chlorophyceae) produce a phycoplast, which is an array of microtubules oriented parallel to the plane of cell division, determining the formation of a new cell wall, which is either formed through cell wall ingrowth or outgrowth. The charophytic classes Charophyceae, Zygnematophyceae and Coleochaetophyceae have a phragmoplast mediated cell division, in which an array of microtubules is oriented perpendicularly to the plane of cell division, determining the formation of the cell plate and new cell wall. This type of cell division usually results in the formation of plasmodesmata, which are channels through the cross walls that enable intercellular communication, and the development of complex tissues.

Reproduction and Life Cycle

Green algae reproduce sexually or asexually. Two main types of sexual life cycle are distinguished: haplontic and diplohaplontic (Fig. 5). In haplontic life cycles the vegetative body is haploid and produces haploid gametes that fuse and form the zygote, which is the only diploid phase in the life cycle. The formation of the zygote is immediately followed by a meiotic division. Diplohaplontic life cycles contain two free-living vegetative phases, one diploid and one haploid. The diploid sporophytic phase arises through the growth and division of a diploid zygote. The haploid gametophytic phase develops from a haploid meiospore, produced following meiosis in a cell of the diploid phase. The type of life cycle is taxonomically dependent, but also largely coincides with the environment (marine versus freshwater). The marine Ulvophyceae mainly have diplohaplontic life cycles, while most freshwater green algae have a haploid vegetative phase and a single-celled, often dormant zygote as the diploid stage. In several green algal groups (most prasinophytes, the early-diverging streptophytic lineages Mesostigmatophyceae, Chlorokybophyceae and

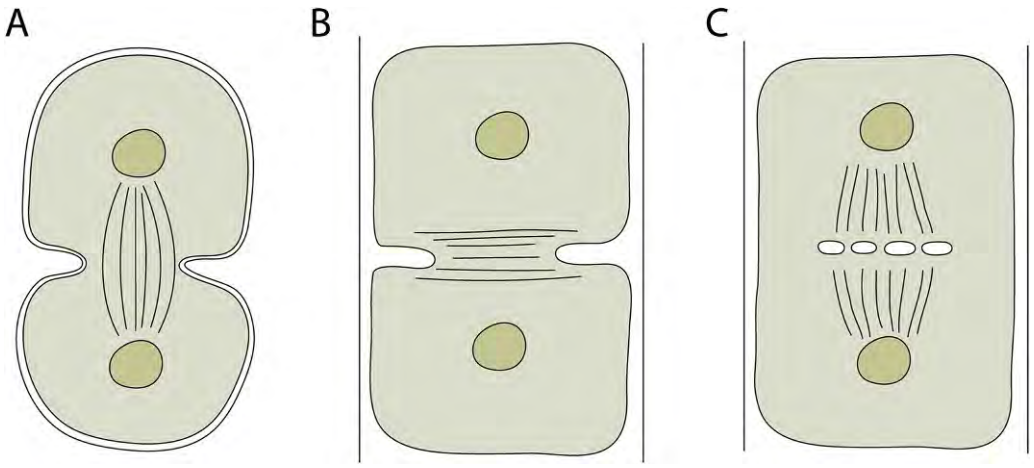

Fig. 4 Types of cell division. (A). Microtubule independent cleavage furrow. (B). Phycoplast microtubules oriented perpendicular to the spindle axis are involved in cell wall formation through cell wall ingrowth. (C). Phragmoplast microtubules are oriented parallel to the spindle axis associated with centrifugal vesicle fusion leading to the development of a cell plate with plasmodesmata.

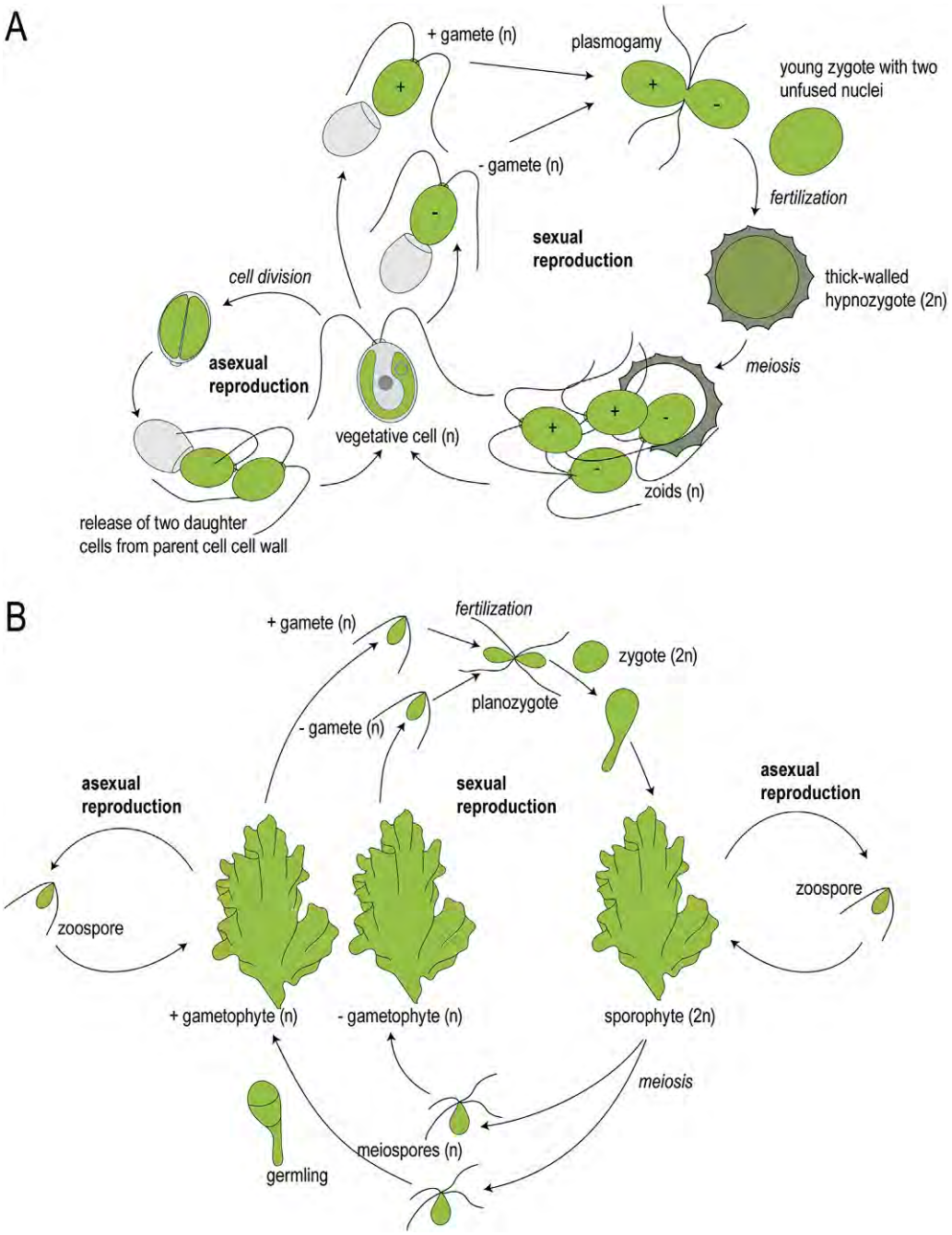

Fig. 5 Two main types of life cycles in the green algae. (A). Haplontic life cycle of *Chlamydomonas* (Chlorophyceae). (B). Diplohaplontic life cycle of *Ulva* (Ulvophyceae).

Klebsormidiophyceae, and several terrestrial members of the core Chlorophyta), sexual reproduction is apparently absent or has been rarely documented. Asexual reproduction occurs by binary fission, fragmentation or production of flagellate cells known as zoospores, or non-motile autospores.

Evolutionary History

Fossil Record

The green algae are ancient, and have played an important role in aquatic ecosystems for hundreds of millions of years. The oldest fossils attributed to green algae date from the Precambrian, but these fossils are scant, and their taxonomic affinities often

contentious. The resistant outer walls of prasinophytic cysts (phycomata) are well preserved in fossil deposits and especially abundant and diverse in the Paleozoic (ca. 250–540 mya). A filamentous fossil from middle Neoproterozoic deposits (*Proterocladus*, ca. 750 mya) has been attributed to siphonocladous green algae (Cladophorales), while the oldest reliable records of the siphonous seaweeds (Bryopsidales, Dasycladales) and stoneworts (Charophyceae) are from the Paleozoic.

Primary Endosymbiosis: Origin of the Archaeplastida

The green plants are one of the three clades of Archaeplastida, which originated following an endosymbiotic event, where a heterotrophic eukaryotic cell engulfed a photosynthetic cyanobacterium-like prokaryote (related to the present-day *Gloeomargarita lithophora*) that became stably integrated and ultimately evolved into a membrane-bound organelle, the plastid. This single event, which probably took place in the Palaeoproterozoic, around 1.9 billion years ago, marked the origin of oxygenic photosynthesis in eukaryotes and gave rise to three autotrophic clades with primary plastids: the Viridiplantae, the red algae (Rhodophyta) and the glaucophytes (Glaucophyta or Glaucocystophyta), which together form the Archaeplastida.

From this starting point, photosynthesis spread widely to other eukaryotes through secondary endosymbiotic events that involved the capture of either green or red algae by diverse non-photosynthetic eukaryotes, thereby transferring the primary plastids across distantly related eukaryotic lineages. Some of these secondary endosymbiotic partnerships have in their turn been captured by other eukaryotes, known as tertiary endosymbiosis. Three groups of photosynthetic eukaryotes now have plastids derived from a green algal endosymbiont: the chlorarachniophytes (Rhizaria), a small group of mixotrophic algae from tropical seas, the common freshwater euglenophytes (Excavata), which are common in freshwater, and some green dinoflagellates (Alveolata). A much wider diversity of photosynthetic eukaryotes, including the dinoflagellates, haptophytes, cryptophytes, chrysophytes, diatoms and brown seaweeds have obtained plastids from a red algal ancestor.

Diversification of the Two Green Plant Clades: Chlorophyta and Streptophyta

Molecular clock analyses, calibrated with the fossil record, have estimated the origin of the green algae around 1.2 billion year ago. An early split in the evolution of the green algae gave rise to its two main clades: Chlorophyta and Streptophyta (Fig. 6).

The Chlorophyta diversified as unicellular planktonic algae in the oceans of the Meso- and Neoproterozoic, and gave rise to the present-day prasinophytes, which form a paraphyletic group of early diverging clades of Chlorophyta. One of these prasinophytic lineages gave rise to the core Chlorophyta somewhere in the Neoproterozoic, which diversified in freshwater, terrestrial, and marine coastal environments, thereby evolving a wide diversity of morphologies, and different life cycle strategies.

The Streptophytic green algae or charophytes probably originated in the Neoproterozoic and diversified as unicellular algae in freshwater environments. The earliest charophytes were unicellular or simple multicellular organisms. Two important groups of multicellular charophytes diversified during the Paleozoic: the conjugating green algae (Zygnematophyceae) and stoneworts (Charophyceae). Ancestral charophytes invaded the land during the mid-Ordovician and early Silurian (480–430 million years ago), initiating the evolution of land plants.

Taxonomic Overview

Chlorophyta

The Chlorophyta include marine, freshwater and terrestrial green algae with diverse vegetative morphologies. Motile cells (when present) typically have a cruciate root system; a few groups have an asymmetrical flagellar apparatus. The orientation of the basal bodies – directly opposed (DO), counter-clockwise (CCW) or clockwise (CW) – has served as an important character for defining the main groups of Chlorophyta. Mitosis is generally closed, with a persistent or non-persistent spindle. Most species contain peroxisomes with the photorespiratory enzyme, glycolate dehydrogenase.

Prasinophytes

The early diverging clades of the Chlorophyta are collectively termed the prasinophytes (Fig. 6). Prasinophytes form a heterogeneous assemblage of mostly unicellular algae with diverse cell shapes that are naked, covered by walls and/or organic body scales; flagella are present or absent. Mitotic processes, biochemical features (including photosynthetic pigments and photorespiratory enzymes) are equally diverse, reflecting the paraphyletic nature of the group. Prasinophytes are predominantly found in marine environments, but several species also occur in freshwater. Most clades are relatively species-poor compared to the core Chlorophyta.

The **Mamiellophyceae** is the morphologically and ecologically most diverse clade of prasinophytes, including approximately 20 species. It includes the order Mamiellales and two smaller orders, Monomastigales (genus *Monomastix*) and Dolichomastigales (genera *Crustomastix* and *Dolichomastix*). The Mamiellales are comprised marine and freshwater flagellates and coccoid species, including *Ostreococcus* and *Micromonas*, which are among the smallest eukaryotes known (cell sizes of 0.5–2 μm), and form important components of marine picoeukaryotic communities. Indirect evidence for sexual reproduction in *Ostreococcus* and *Micromonas* is based on the occurrence of sex-related and meiosis-specific genes in their genomes.

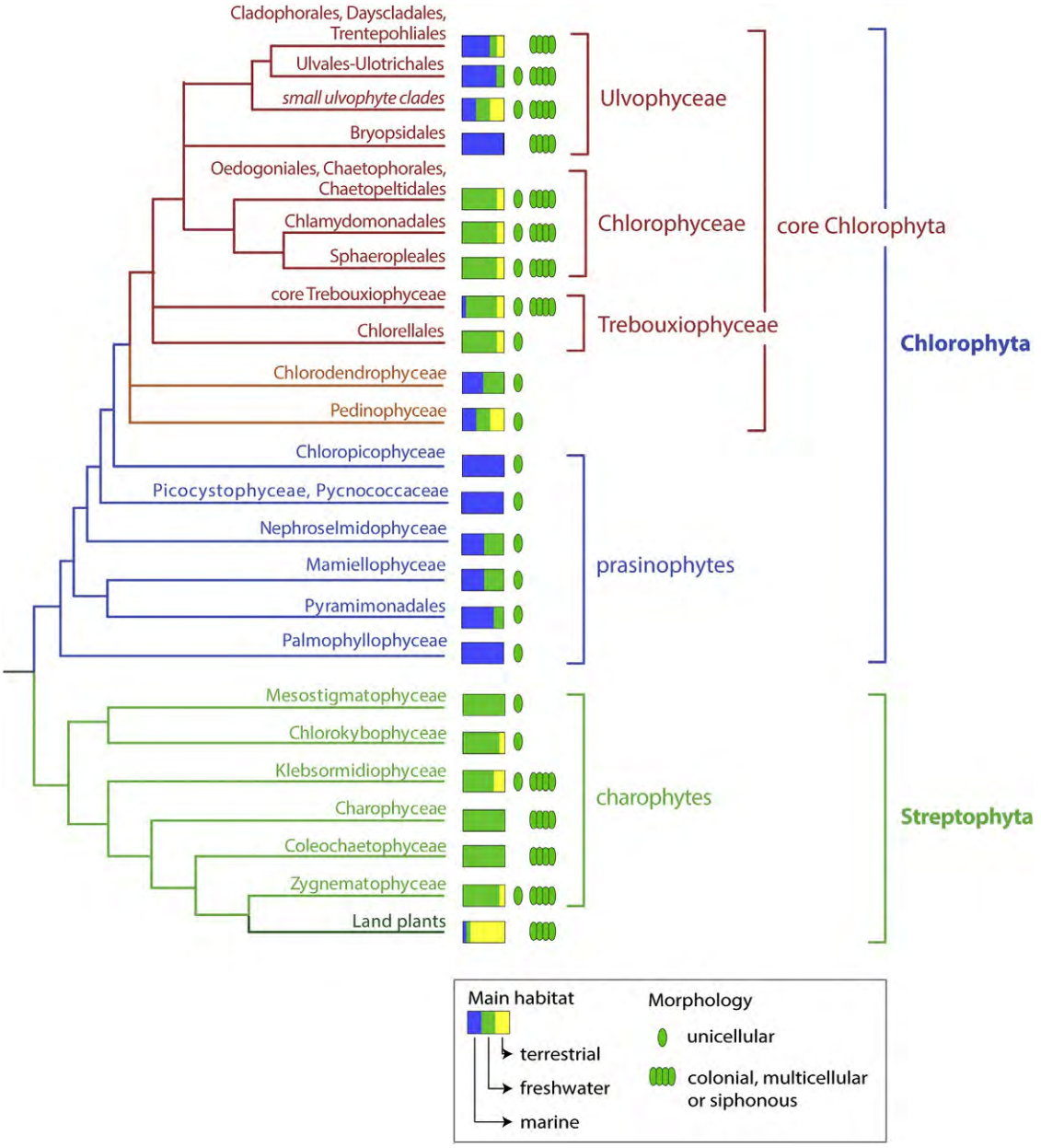

Fig. 6 Phylogenetic relationships among the main clades of green algae, and indication of the main types of habitats and morphological forms.

The **Pyramimonadales** includes relatively large flagellates with 4, 8 or 16 flagella, with cells covered by complex body scales in multiple layers. About 50 species are known from marine and freshwater habitats. Some species of *Pyramimonas* and *Cymbomonas* are mixotrophic and possess a food uptake apparatus. Some species produce resting cysts (phycomata), which possibly result from sexual reproduction. These phycomata are abundant in the fossil record from the Cambrian onward.

The **Nephroselmidophyceae** includes relatively large, asymmetrical flagellates with two laterally inserted, unequal and heterodynamic flagella. The flagellar apparatus is atypical in only having three flagellar roots. Cells and flagella are covered by diverse scales in multiple layers. About 30 species are known from marine and freshwater habitats. *Nephroselmis* is one of the few prasinophytes where sexual reproduction has been observed in culture.

The **Chloropicophyceae** comprises scale-less coccoid picoplanktonic algae surrounded by a thin cell wall. The class includes 6 known species, several of which are important components of marine phytoplankton, especially in moderately oligotrophic waters.

The **Palmophyllophyceae** is the deepest-branching clade of the Chlorophyta (Fig. 6), and include the marine coccoid Prasinococcales (e.g., *Prasinococcus*), and the Palmophyllales (e.g., *Palmophyllum*), which are macroscopic green algae growing in deep and low-light benthic marine habitats. These algae have a unique type of multicellularity, forming macroscopic bodies composed of isolated cells in a firm gelatinous matrix. Flagellate stages are absent.

Other prasinophytic clades include the **Picocystophyceae**, containing the coccoid species *Picocystis salinarum* from saline lakes, the **Pycnococcaceae**, including marine flagellates (*Pseudoscourfieldia*) and coccoids (*Pycnococcus*), and a couple of clades that are known only from environmental sequencing.

Core Chlorophyta

The core Chlorophyta include three large and diverse classes, Ulvophyceae, Trebouxiophyceae and Chlorophyceae (UTC), and two smaller classes, Pedinophyceae (marine and freshwater uniflagellates) and Chlorodendrophyceae (marine and freshwater quadriflagellates, including *Tetraselmis*) (Fig. 6). The UTC classes include a wide variety of morphological forms and are found in diverse environments. Trebouxiophyceae and Chlorophyceae mainly occur in freshwater and terrestrial habitats. Ulvophyceae are most abundant in benthic marine habitats. Several members of the Trebouxiophyceae and Chlorophyceae live as symbionts in protists (e.g., ciliates) and in animals (e.g., nudibranchs and anemones). These endosymbionts are commonly referred to as zoochlorellae, but belong to phylogenetically diverse lineages.

The classes Trebouxiophyceae, Chlorophyceae and Chlorodendrophyceae are characterized by cell division mediated by a phycoplast, which is absent in the Ulvophyceae.

Unlike the prasinophytes, where sexual reproduction has rarely been observed, the core Chlorophyta exhibit various sexual life cycle strategies. Marine members of the Ulvophyceae generally have diplohaplontic life cycles, while the freshwater Chlorophyceae and Trebouxiophyceae generally have a haplontic life cycle.

The **Trebouxiophyceae** include a wide diversity of about 900 species, including flagellates, coccoids (Fig. 1B), and colonies, as well as multicellular filaments and blades. Cells are generally uninucleate, are surrounded by a cell wall, but lack body scales. Motile cells have a cruciate flagellar root system and basal bodies in a CCW orientation. The sexual life cycle is haplontic, but many species reproduce asexually by zoospores or autospores. Species mainly live in freshwater or terrestrial environments, but some members (e.g., Prasiolales) occur in brackish or marine habitats. Several species are photosynthetic symbionts in various protists (e.g., ciliates), and invertebrates (e.g., scleractinian corals, sea anemones, sponges, green hydras). Species in several genera (e.g., *Trebouxia*, *Asterochloris*, *Asterococcus* and *Coccomyxa*) form symbionts with lichen fungi. Some species have lost photosynthetic capacity and have a heterotrophic free-living or a parasitic lifestyle. The heterotrophic alga *Prototheca*, which grows in sewage and soil, can cause infections in humans and animals known as protothecosis. *Helicosporidium* is an obligate parasitic alga found in the gut of insects. Genome data of *Chlorella variabilis*, an endosymbiont of the ciliate *Paramecium bursaria*, indicate that expansion of protein families containing protein–protein interaction domains and adhesion domains were involved in adaptation to symbiosis.

The **Chlorophyceae** include flagellates, coccoids and various colonial and multicellular forms. Approximately 3600 species are known, mainly from freshwater and to a lesser extent terrestrial habitats. Most species have uninucleate cells, with cell walls. Motile cells have cruciately arranged flagellar roots with basal bodies in DO or CW orientation. Sexual life cycle is generally haplontic, but many species reproduce asexually by cell division, or production of zoospores or autospores. Five main orders have been recognized. The species-rich and diverse orders Sphaeropleales (e.g., *Scenedesmus*, *Pediastrum*, *Hydrodictyon*) and Chlamydomonadales (e.g., *Chlamydomonas*, *Volvox*, *Dunaliella*) include some of the most common freshwater phytoplankton species. The Chlamydomonadales include two important model organisms. The unicellular flagellate *Chlamydomonas* (Fig. 1A) has been studied as a model for photosynthesis, chloroplast biogenesis, flagellar assembly and function, and cell cycle control. The colonial *Volvox* (Fig. 1C) has served as a model for the evolution of multicellularity, cell differentiation, and colony motility. The halotolerant species *Dunaliella salina* is among the most commercially important microalgae (see below). The smaller orders, Chaetophorales (e.g., *Uronema*), Oedogoniales (e.g., *Oedogonium*) and Chaetopeltidales (e.g., *Floydiella*) mainly include filamentous and colonial forms. Species in the Oedogoniales have a specialized form of oogamous sexual reproduction involving the production of stephanokont motile cells.

The **Ulvophyceae** include approximately 2000 species of marine macro-algae (green seaweeds) that grow in diverse coastal habitats, including rocky shores, coral reefs and tropical lagoons. A smaller diversity (about 200 species) occurs in freshwater or terrestrial habitats. Macroscopic forms include branched or unbranched filaments, blades or tubular forms, and giant-celled forms, including siphonocladous (Fig. 1H) and siphonous thalli (Fig. 1I). Cell division is by furrowing, without a phycoplast. Motile cells have cruciately arranged flagellar roots with basal bodies in a CCW orientation. Cell walls are diverse and are composed of cellulose, mannans, xylans, and sulfated polysaccharides. Ulvophyceae exhibit a wide diversity of life cycles. Species reproduce asexually or sexually. Sexual reproduction involves a heteromorphic or isomorphic diplohaplontic life cycle, or a haplontic life cycle. Monophyly of the Ulvophyceae is uncertain due to the lack of derived morphological and ultrastructural characters, and has not been convincingly established in molecular phylogenetic studies. Ten orders have been described of which the Ulotrichales, Ulvales, Cladophorales, Bryopsidales, Dasycladales, and Trentepohliales are the most speciose. The Ulotrichales and Ulvales mainly include multicellular thalli composed of uninucleate cells. A well-known representative of the Ulvales is *Ulva* or sea lettuce, which is globally distributed and can form extensive, free-floating blooms, known as green tides. *Ulva* is a model organism for studying morphogenesis in green seaweeds, and is also economically important in aquaculture. The Cladophorales include siphonocladous thalli, which are multicellular bodies composed of multinucleate cells with nuclei having a fixed position. The Bryopsidales and Dasycladales have siphonous thalli, which are macroscopic thalli composed of a single giant tubular cell with a single giant nucleus or thousands of nuclei, and cytoplasmic streaming enabling transportation of organelles, nutrients and transcripts across the giant siphonous cell. Well-known representatives of the Bryopsidales are *Caulerpa* and *Codium*, of which some species are invasive. The Trentepohliales is an atypical class with respect to ultrastructure and ecology,

occurring exclusively in terrestrial habitats. Several species of Trentepohliales (e.g., *Trentepohlia*) are symbiotic algae within lichens, and *Cephaleuros* includes pathogens of vascular plants.

Streptophyta

The Streptophyta include a paraphyletic assemblage of green algae (charophytes) and the land plants (Fig. 6). The morphology of charophytes ranges from unicellular to complex multicellular organisms. Species occur in freshwater or damp terrestrial habitats. Streptophyta share a number of unique traits, including motile cells (when present) with laterally inserted flagella, parallel basal bodies, asymmetrical flagellar roots, and one flagellar root with a characteristic multilayered structure. Mitosis is open with generally a persistent mitotic spindle. Motile cells generally lack body scales. Charophytes have peroxisomes with glycolate oxidase. Six classes of charophytes are recognized: Mesostigmatophyceae, Chlorokybophyceae, Klebsormidiophyceae, Zygnematophyceae, Charophyceae and Coleochaetophyceae. The Zygnematophyceae have been identified as the sister clade to the land plants based on molecular phylogenetic data (Fig. 6). The early-diverging classes, Mesostigmatophyceae, Chlorokybophyceae, and Klebsormidiophyceae, are characterized by cell division by furrowing, and apparent lack of sexual reproduction. The later-diverging classes, Zygnematophyceae, Charophyceae and Coleochaetophyceae, are characterized by a phragmoplast-mediated cell division, presence of plasmodesmata (absent in Zygnematophyceae), and sexual reproduction.

The **Mesostigmatophyceae** and the **Chlorokybophyceae** form the earliest-diverging lineages of the Streptophyta (Fig. 6). The Mesostigmatophyceae include the single species *Mesostigma viride* from freshwater habitats, characterized by motile cells covered with diverse organic body scales, and two laterally inserted flagella. The Chlorokybophyceae include the single species, *Chlorokybus atmophyticus*, which grows in freshwater or moist terrestrial environments, and forms packets of a few coccoid cells that produce asexual biflagellate zoospores with scales. The Klebsormidiophyceae are a small class of approximately 40 species from freshwater and terrestrial environments, characterized by unbranched filaments that produce biflagellate zoospores (e.g., *Entransia*, *Interfilum*, and *Klebsormidium*).

The **Zygnematophyceae** or conjugating green algae are a large class of about 4000 species that are widespread and abundant in freshwater environments, damp terrestrial habitats, snow and ice. The group is morphologically diverse, including non-motile unicellular algae, small colonies, and branched or unbranched filaments. Flagellate stages are absent. Sexual reproduction occurs by a unique process of conjugation, involving fusion of amoeboid gametes, and production of thick-walled resistant zygotes. Traditionally two groups are distinguished, the Zygnematales and the Desmidiales, but this separation is not supported by molecular data. The Zygnematales contain unicellular or filamentous algae. Species of Desmidiales are usually unicellular algae composed of two semicells joined by a narrow isthmus (Fig. 1F). The highly diverse Desmidiales (desmids) are particularly characteristic of oligotrophic freshwater environments of low pH, such as bog pools.

The **Charophyceae** or stoneworts, include about 700 species of freshwater algae with complex macroscopic bodies composed of a main axis formed by large, multinucleate cells, and whorled branches. Growth is by an apical meristematic cell. Some species are calcified. Sexual reproduction involves fusion between a motile male gamete with complex morphology, and a large, non-motile female gamete (oogamous reproduction). Male and female gametes are surrounded by sterile cells. Well-known genera are *Chara* and *Nitella*. Stoneworts are well represented in the fossil record, with a large diversity extending back to the Silurian.

The **Coleochaetophyceae** is a small class of approximately 20 species from freshwater habitats (e.g., *Coleochaete*, *Chaetosphaeridium*). They include branched filaments and discoid pseudoparenchymatous or parenchymatous forms, with specific cells having hair-like extensions. Sexual reproduction involves fusion between a motile male gamete, and a large, non-motile female gamete (oogamous reproduction). Some species of *Coleochaete* have corticated zygotes that are retained on the mother plant from which they receive nourishment via placental transfer cells with wall ingrowths, similar to land plants.

Economic Importance

Several green algae are commercially harvested as sources of food or food supplements for humans and animals, or are potentially useful for the production of sustainable and renewable biofuels. Green microalgae are cultured for food supplements on industrial scales in open air systems or closed bioreactors. *Dunaliella salina* (Chlorophyceae) is among the most commercially important microalgae because of its high β-carotene production carotene. *Chlorella* species (Trebouxiophyceae) are sources of β-1,3-glucan. *Haematococcus pluvialis* (Chlorophyceae) is cultivated for its production of astaxanthin, a carotenoid that is used as an antioxidant food supplement or as a food coloring, for example in salmon aquaculture. Some green microalgae naturally produce high amounts of lipids, and have potential value for biofuels production, including *Dunaliella salina* and *Ettlia* (*Neochloris*) *oleoabundans* (Chlorophyceae), and *Botryococcus braunii* (Trebouxiophyceae). Seaweeds have been used as a food source for humans and animals, and as fertilizer for agriculture for centuries. Some marine green macroalgae are cultivated at a commercial scale for utilization in various food preparations, mainly in East and South East Asia, including *Ulva reticulate*, *Monostroma oxyspermum* (green laver or aonori), and *Caulerpa lentillifera* (sea grapes or lato). Species of Cladophorales (Ulvophyceae) have highly crystalline cellulose cell walls with unique physical properties, which have potential for various high-tech industrial applications, including reinforcement fibers, filter membranes, and conductive cellulose composites. Some seaweeds, including species of *Caulerpa* and *Halimeda*, are sold as ornamental algae for marine aquaria, which brings a risk for introduction of alien species.

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Gut Microbiota in Human Health and Diseases

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Glossary

Commensalism Symbiotic relationship in which one species is benefited while the other is unaffected.
Diversity The degree of variation of organisms in an ecosystem based on the number of types of species present and their relative distribution in the assemblage.
Dysbiosis Imbalances in the composition of microbial communities colonizing the human body.
Evenness The distribution of organisms in a given community.
Microbiome The totality of microbes, their genomes, and interactions within a given ecosystem.
Microbiota The community of microbes residing in an ecosystem.
Mutualism Symbiotic relationship in which each of the organisms benefit.
Prebiotic Food compound refractory to the human digestive process that is selectively fermented by the gut microbiota resulting in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) on host health.
Probiotic Live microorganism that when administered in adequate amounts confers a beneficial health effect on the host.
Richness The number of types of species present.
Symbiosis Prolonged and close association between two or more different species.

The healthy human body is home to a vast number of phylogenetically and functionally diverse microbes assembled in communities of varying degrees of complexity. In fact, all external body sites and mucosal surfaces examined to date in healthy individuals are colonized by normally benign microorganisms in a typically symbiotic relationship. Compositionally distinct bacterial communities of commensal or mutualistic bacteria have been described for the skin (Grice and Segre, 2011), female reproductive tract (Ravel et al., 2010), oral cavity (Kolenbrander, 2000), and the respiratory (Lemon et al., 2010), as well as the gastrointestinal (GI) tract of healthy humans (Backhed et al., 2005). Since humans and their microbial colonists have coevolved, it is not surprising that a mutually beneficial relationship has emerged. Indeed, the ability of the human host to recognize and respond appropriately to the vast diversity and sheer numbers of organisms in sites such as the GI tract, which harbors up to 10^{14} bacterial cells, is a clear and impressive example of this coevolution. Advantages of this symbiotic relationship from a microbial perspective are easy to fathom: constant temperature, regular flow of nutrients, and protection from environmental insults. Benefits to the human host include a dramatically expanded metabolic capacity, vitamin production, pathogen exclusion, lymphatic development, and immune regulation, to name but a few (Kau et al., 2011). Most of the commensal bacteria that inhabit humans are well adapted to their respective niche; some are so highly specialized with respect to nutritional and environmental requirements that as of yet they have not been cultured under current standard laboratory conditions. Their presence in these sites is known only because of their detection using culture-independent methods that can identify microbial species without recourse to culture. With the advent and evolution of such technologies and several large-scale United States, European, and Asian funding initiatives to probe the diversity and function of microbial life associated with humans, we are beginning to better understand the interdependence of the human host and its microbial inhabitants, not least in the highly populated GI tract.

The Healthy Human Gut Microbiota

The notion that the bacteria residing in the intestine play an important role in human health and well-being is not new. Early attempts to identify and characterize these microbes were based on culturing bacteria from fecal samples. Using this approach, few species were identified and the microbial diversity within this niche appeared to be low. Microscopic analysis, however, indicated a much greater diversity suggesting that the majority of fecal bacteria could not be cultured under standard laboratory conditions (Suau et al., 1999). Following the advent of polymerase chain reaction (PCR) to amplify specific target genes of interest, microbial ecologists harnessed this technology to develop culture-independent techniques for microbial identification. This approach permitted identification of bacteria not based on their morphology and phenotypic characteristics, but on the sequence of particular biomarker genes. Moreover, using this approach, the identity and relative abundance of multiple organisms present in a mixed-species community of microbes could be determined, essentially generating a microbial fingerprint of the species present. A particularly useful genetic marker for the identification of bacteria is the 16S ribosomal ribonucleic acid (rRNA) gene (Woese and Fox, 1977). This gene encodes the small subunit of the bacterial rRNA, is thus present in all bacteria, and is not found in fungi or

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higher organisms. The gene itself is useful as a bacterial biomarker since it possesses regions of gene sequence that are highly conserved across all bacteria; these regions flank highly variable sequence domains. The conserved regions may be used to design PCR primers to amplify regions of the 16S rRNA gene encompassing the hypervariable domains from the majority of bacteria in a given sample. The sequence of the variable domains in a given sample is then used to classify and identify the microbiota members present. The proportion of sequence reads for each organism in a given sample permits assessment of their relative distribution within that sample; these distributions can be compared across samples to determine relative contraction or expansion of specific groups of organisms across, for example, treatment groups. Recent advances in high-throughput technologies have dramatically accelerated microbiome research (Figure 1). Next-generation sequencing (NGS) and phylogenetic microarray analyses permit high-resolution, 16S rRNA-based microbiota profiles to be generated (Paliy and Agans, 2012; Petrosino et al., 2009). More recently, application of high-capacity NGS to DNA and RNA extracted from microbial communities has produced profiles of the microbial pan-genome and transcriptome, respectively (Maccaferri et al., 2011). Moreover, a variety of mass spectrometry approaches have been used to generate metabolic and proteomic profiles associated with microbial communities (Jacobs et al., 2009). Such approaches, particularly when applied in parallel, permit an unprecedented ability to analyze the composition, functional capacity, and expression profile of the organisms within these complex communities (Turnbaugh and Gordon, 2008). Their more recent application to human samples has fundamentally increased our understanding of how, at a local and systemic level, microbial communities that inhabit the human host impact human health and disease.

Structure and Composition

The human digestive tract – from the oral cavity to the rectum – is home to a variety of distinct, niche-specific microbial communities, including highly complex communities in the lower GI tract that contain all three domains of life – bacteria, Archaea, and eukaryotes as well as viruses. Even at a gross anatomical level, the various compartments of the digestive tract differ physiologically and, as a result, represent distinct ecological niches that house specific microbial populations (Figure 2). For example, a low burden and diversity of bacteria exists in the stomach and the upper small intestine due to low pH, rapid peristalsis, and high bile concentrations. In comparison, the number of prokaryotes residing in the colon far exceeds that of all other microbial communities associated with the body's surfaces and is estimated to contain over 70% of all the microbes in the human body and to outnumber human cells by approximately 10-fold (Figure 3) (Savage, 1977).

Although large with respect to number of organisms, the GI microbiota exhibits a relatively low level of diversity at the phylum level compared to other ecosystems such as soils that harbor similar bacterial densities (Figure 4). This suggests that strong selective pressures and coevolution have resulted in a highly specialized microbial consortium in the lower GI tract of humans. Indeed, only nine of the 55 bacterial Ribosomal Database Project defined phyla, and a single Archaea phylum has been described to date in this

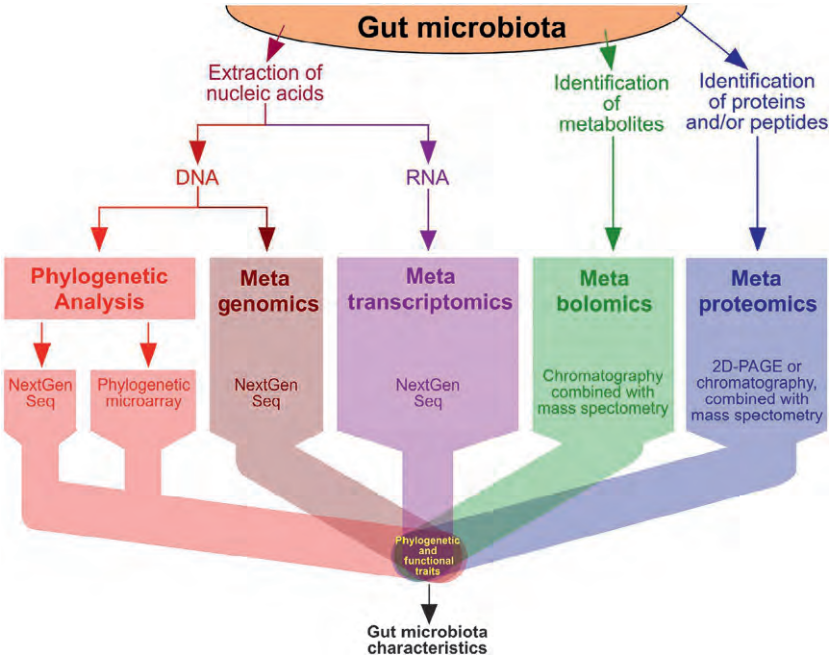

Figure 1 State-of-the-art technologies commonly employed to profile microbial communities. A variety of high-throughput molecular approaches permit stand-alone analysis of phylogenetic membership and relative abundance (phylogenetic analysis), the pan-genome of the dominant members of the community (metagenomic analysis), as well as transcriptional (metatranscriptomic), metabolic (Metabolomic), and proteomic (metaproteomic) expression profiles of the assemblage. Parallel use of these approaches permit phylogenetic and functional characterization of the gut microbiota in unprecedented detail. NextGen Seq, next-generation sequencing; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

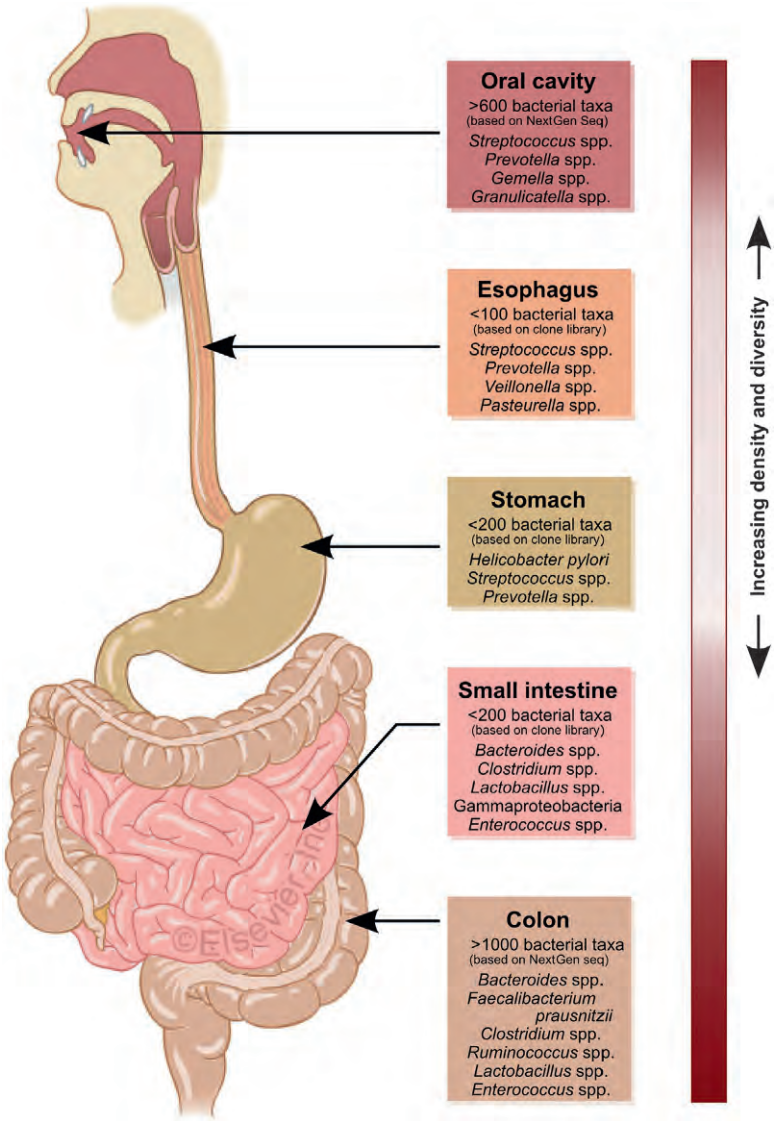

Figure 2 Niche-specific variation in microbial populations across physiologically distinct sites within the GI tract. The human GI tract consists of distinct partitions with characteristic physiological conditions that affect density, diversity, and stability of the microbiota present at each site. Commensals colonizing these compartments are well adapted to coping with exogenous factors such as peristaltic activity; food molecules and xenobiotics; gastric, pancreatic, biliary, and mucin secretions; as well as challenges by transient bacteria and antimicrobial peptides (Manson et al., 2008). Shown here are bacterial genera identified by culture-independent methods in samples obtained from the various sections of the GI tract. Data for this figure was obtained from Dewhirst, F.E., Chen, T., Izard, J., Paster, B.J., Tanner, A.C.R., Yu, W.-H., Lakshmanan, A., Wade, W.G., 2010. The human oral microbiome. J. Bacteriol. 192, 5002–5017 (oral cavity); Fillon, S.A., Harris, J.K., Wagner, B.D., Kelly, C.J., Stevens, M.J., Moore, W., Fang, R., Schroeder, S., Masterson, J.C., Robertson, C.E., Pace, N.R., Ackerman, S.J., Furuta, G.T., 2012. Novel device to sample the esophageal microbiome–the esophageal string test. PLoS One 7 (9), e42938 (esophagus); Bik, E.M., Eckburg, P.B., Gill, S.R., Nelson, K.E., Purdom, E.A., Francois, F., Perez-Perez, G., Blaser, M.J., Relman, D.A., 2006. Molecular analysis of the bacterial microbiota in the human stomach. Proc. Natl. Acad. Sci. U.S.A. 103, 732–737 (stomach); Wang, X., Heazlewood, S.P., Krause, D.O., Florin, T.H.J., 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. 95, 508–520 (small intestine); and Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., Knight, R., 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489, 220–230 (colon). Note that data based on the construction of clone libraries may underestimate the bacterial diversity present.

niche. The phyla Firmicutes and Bacteroidetes dominate these microbial ensembles, at least in the GI tracts of humans residing in Western nations, and account for more than 70% of all bacterial taxa found. Phyla representing smaller proportions of these consortia include Actinobacteria and Proteobacteria (Dethlefsen et al., 2007; Ley et al., 2006; Turnbaugh et al., 2009a). Although few phyla are described in the GI tract, a relatively large diversity of genus and species are found in these communities. Conservative calculations estimate the presence of at least 800 bacterial species but recent studies suggest that the true number could be as high as 40 000 individual bacterial species residing in the human gut (Backhed et al., 2005; Claesson et al., 2009; Frank and Pace, 2008).

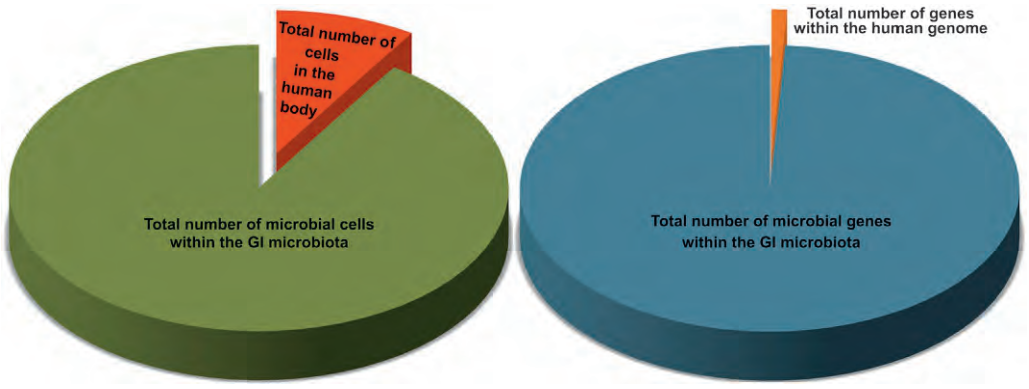

Figure 3 Relative frequency of microbial versus host cells and genes in humans. The GI microbiota is estimated to be composed of 10^{14} commensal bacteria, representing a 10-fold increase over the total number of human cells. Moreover, the sheer number of genes encoded by these species exceeds the number of genes identified within the human genome by about 100-fold. Given this diversity and the astounding metabolic capability contributed by the resident intestinal bacteria to human metabolism, it is easy to understand how significant they are to human health and how disturbances within the GI microbiota may induce or contribute to disease development.

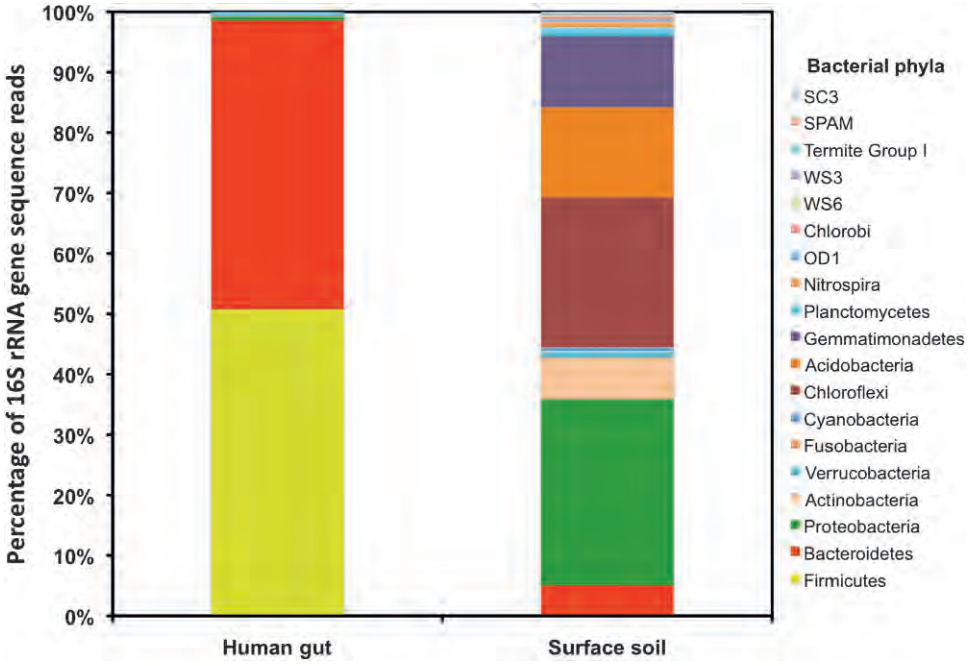

Figure 4 Phylogenetic diversity of bacteria identified in the healthy human gut compared to agricultural soil. Shown is the numerical distribution of bacterial phyla in each habitat, based on data published by Eckburg et al. (2005) for the human gut and by Tringe et al. (2005) for the agricultural surface soil, both using clone libraries to assess diversity within these ecosystems. Reproduced from Turroni, F., Ribbera, A., Foroni, E., Van Sinderen, D., Ventura, M., 2008. Human gut microbiota and bifidobacteria: from composition to functionality. Antonie Van Leeuwenhoek 94, 35–50 with permission.

The composition of the intestinal microbiota varies significantly between hosts. Even though the human gut microbiota is most similar among family members, when compared to unrelated individuals, each individual's GI microbiome is unique and differs substantially from each other in the specific bacterial lineages present (even across pairs of fraternal twins) (Turnbaugh et al., 2009a). This suggests that environmental influences trump genetic forces in shaping these consortia. Because of these substantial differences between individuals, defining a core gut microbiota, shared by all humans, even within a defined population, e.g., in habitants of Western nations, has so far been elusive. However, metagenomic analyses, which permits a profile of the pan-genomic composition of a given microbial community to be determined, have indicated that despite interindividual compositional variation, an extensive, identifiable 'core microbiome' defined across human populations exists at the functional gene level (Turnbaugh et al., 2009a). This suggests a vast level of functional redundancy across microbial species that populate the GI tract and has identified core functions encoded by the microbiome that characterize healthy individuals. More recently, investigations have described at least two robust and distinct types of microbiomes (referred to as 'enterotypes') that are primarily distinguished by relative abundance of *Bacteroides* and

Prevotella genera (both belonging to the phylum Bacteroidetes). These two enterotypes differ in species composition and functional capacity and appear to span several nations and continents, although clearly larger studies including discrete populations with distinct microbial exposures are necessary to corroborate such findings (Arumugam et al., 2011; Wu et al., 2011).

Development and Function

The Development of the Gut Microbiota over the Course of Life

The GI tract of humans is presumed to be sterile at birth. The first microbial colonizers of the intestine are acquired during and immediately after birth when infants are inoculated with species in their immediate environment. Vaginally delivered infants acquire bacterial communities resembling their maternal vaginal microbiota, dominated by *Prevotella*, *Sneathia*, or *Lactobacillus* species. Cesarean-delivered infants harbor bacterial communities similar to those found on the skin surface, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. and intestinal colonization by *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* species is delayed (Dominguez-Bello et al., 2010). Hence, the mode of delivery during the birth process has a significant impact on the earliest microbial colonizers and community composition of the nascent gut microbiome. There is some evidence that these early-life pioneer microbial colonizers may substantially influence the trajectory of the infant GI microbiota development and influence predisposition to subsequent disease development in child- and adulthood (Johnson and Versalovic, 2012; Vael and Desager, 2009). For example, evidence indicates that early-life (3 weeks) fecal bacterial colonization patterns, specifically colonization with *Escherichia coli* and *Clostridium difficile*, are associated with a significantly higher risk of allergic disease development, while colonization with *Lactobacillus* and *Bifidobacteria* species lowered this risk (Kalliomaki et al., 2001a; Penders et al., 2006). Presumably, these physiologically distinct pioneer colonizers shape the GI ecosystem, perhaps through antimicrobial production or competitive colonization of the mucosal surface, leading to distinct patterns of colonization, which in turn impact immune maturation and subsequent allergic disease outcomes. However, more studies examining gut microbiota of infants from birth through the first few years of life are necessary to confirm this hypothesis.

Over the first year of life, the GI microbiome undergoes a temporal and seemingly chaotic program of development. Beginning with a low bacterial burden and few pioneering colonizers at birth, the numbers and types of bacteria that occupy this niche rapidly expands during early infancy and exhibits extensive variation across individuals (Palmer et al., 2007). Infant microbiota exhibit dramatic perturbation on antimicrobial administration characterized by reductions in bacterial density and profoundly altered community composition. Although these assemblages reassemble within a relatively short period, the short-term recovery is incomplete (Fouhy et al., 2012). Typically, the infant microbiome resembles an adult microbiome somewhere between 1 and 3 years of age, following the introduction of solid food. The healthy adult gut microbiota is relatively stable over time within individuals and surprisingly resilient toward disturbances that occur during the adult life. Nonetheless, disturbances such as antimicrobial administration have been shown to deplete the microbiome of species that are never again detected in these individuals (Dethlefsen and Relman, 2011). Therefore, it is conceivable, given the obvious evidence for functional redundancy in these consortia, that early perturbations during the period of immune development or indeed cumulative loss over time of key functions encoded by such species in established communities lead to instability and dysfunction within these communities and ultimately disease development. Certainly, there is evidence to support this hypothesis; children who receive antibiotics in the first year of life have been shown to be at a higher risk of atopy (Alm et al., 2008), while risk factors for the development of inflammatory bowel disease (IBD) include three or more antimicrobial administrations in the 24-month period prior to disease development (Shaw et al., 2011).

In addition to compositional and functional changes elicited through acute perturbations, the gut microbiota exhibits a gradual evolution over the lifetime of individuals, and, in the elderly, in parallel with increasing immune senescence. Senior (>65 years) stool microbiome differs from that of younger individuals in that the variability between individuals is even greater (O'Toole, 2012). Despite this, distinct changes at the phylum, genus, and, in particular, the relative abundance of *Clostridium* are common among most elderly. In seniors, the Bacteroidetes:Firmicutes ratio is substantially different from that of younger adults with a significantly lower proportion of Firmicutes. The GI microbiota of the elderly also varies significantly in the abundance of Proteobacteria, Actinobacteria (which includes *Bifidobacterium* spp.) and butyrate-producing Firmicutes including the genus *Faecalibacterium* (which includes *Faecalibacterium prausnitzii* thought to confer antiinflammatory properties) (O'Toole, 2012). Collectively, although the number of studies performed to date is relatively low, they are cobbling together a scenario of GI (and likely other sites such as skin) microbiome genesis, development, and evolution over the lifetime of a human. It is likely not coincidence that the observed alterations in immune function both in the very young and in the very old parallel the observed changes in these assemblages and provide further evidence, in human populations, that immune function is intricately interlaced with host microbial colonization patterns.

Functional Characteristics of the Intestinal Microbiota

The gut microbiota bestows a multitude of key functions to the human host (Figure 5). Their enormous metabolic potential plays an active role in energy harvest, in that they increase their host's caloric uptake not only by degrading indigestible dietary polysaccharides into absorbable monosaccharides but also by fermenting complex carbohydrates to short-chain fatty acids (SCFAs), which are then assimilated by the host, accounting for about 10% of daily energy intake in humans (Turnbaugh et al., 2006). The relevance of microbially derived SCFA production extends beyond energetic contribution to the host; SCFAs, such as acetate, butyrate, and propionate enhance the proliferation and differentiation of epithelial cells and modulate immune responses through their antiinflammatory properties (Tappenden and Deutsch, 2007). In addition to digestion of food, the intestinal microbiome also regulates energy storage and provides essential vitamins for the host (Backhed et al., 2004; Hill, 1997). Finally, the gut microbiota

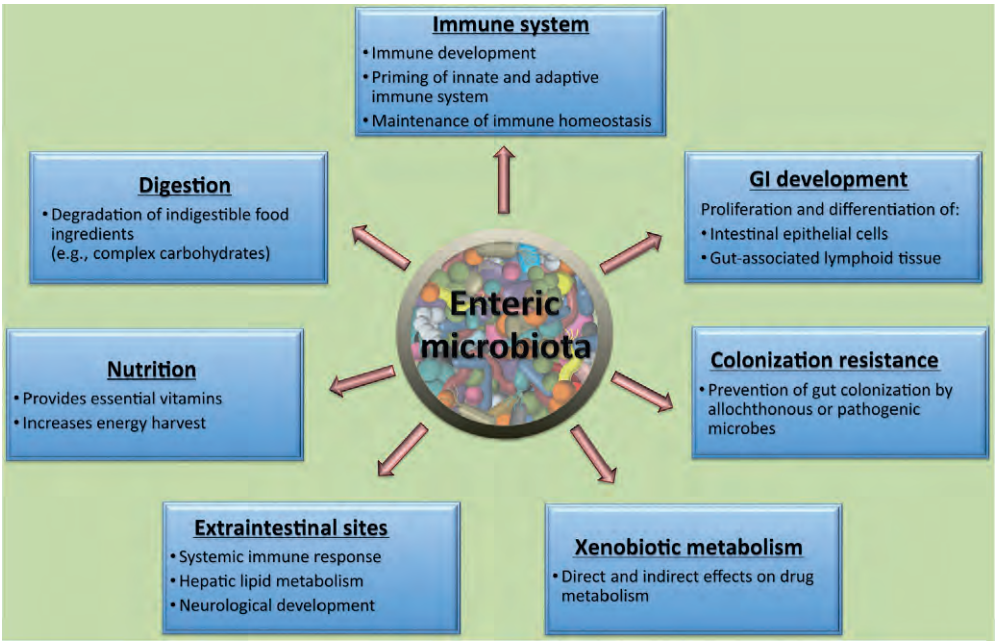

Figure 5 Key functions that the enteric microbiota bestows upon the human host. The community of commensals colonizing the GI tract has a substantial influence on host physiology and human health. Their effects are observed both locally and at sites remote from the GI tract.

also contributes to the metabolism of xenobiotics, i.e., compounds usually foreign to a living organism, and as such influence directly or indirectly the efficacy of drugs (Haiser and Turnbaugh, 2012).

Another key feature of the gut microbiota is prevention of pathogen overgrowth. Resident (autochthonous) intestinal bacteria inhibit invasion and colonization of foreign (allochthonous), potentially pathogenic bacteria by competing for the same nutrients and by occupying available niches in the gut, a phenomenon known as 'colonization resistance' (Stecher and Hardt, 2008). Disruption of the intestinal microbial community, for example, through (prolonged) antibiotic treatment, may result in a destabilized microbiota susceptible to invasion by obligate or opportunistic pathogens. For instance, epidemiologic data suggests that treatment with antibiotics is one of the predisposing factors for nosocomial infections with multiple-drug resistant *Enterococcus* species (Huycke et al., 1998). Antibiotic treatment has also been implicated in the etiology of GI infection with *C. difficile*, as is evident from patients suffering from *C. difficile*-associated diarrhea (DuPont et al., 2008). Indeed, mice exhibit enhanced susceptibility to *C. difficile* infection once their gut microbiota is perturbed with antimicrobials and these animals become highly contagious supershedders of this pathogen (Lawley et al., 2009). Similarly, mosquitoes (*Anopheles gambiae*), which naturally transmit *Plasmodium falciparum*, a protozoan parasite causing malaria in humans, become susceptible to this pathogen if their hindgut microbiota has been perturbed with antimicrobials (Dong et al., 2009). Collectively, these findings point to a role of the gut microbiome in protecting this niche from pathogen overgrowth and imply that loss of such species, particularly through antibiotic administration, results in increased susceptibility to infection.

The gut microbial community also has a significant impact on the development and morphology of the intestinal tract and contributes to angiogenesis and fortification of the intestinal epithelial barrier (Gustafsson et al., 1970; Hooper et al., 2001). Apart from the digestive tract, the enteric microbiota has also been shown to influence host physiology at extraintestinal sites. A relatively small but growing body of literature implicates a role of enteric bacteria on mammalian brain development (Heijtz et al., 2011) and hepatic lipid metabolism (Claus et al., 2011). Besides this, and with significant consequences for human health, is the intestinal microbiota's role in locally and systemically shaping and modulating the host's adaptive and innate immune system. Enteric microbes, through the secretion of metabolites and peptides, or via cell-associated ligands, constantly prime the innate immune system, thus facilitating a rapid response to pathogens (Clarke et al., 2010). Of particular importance is the GI microbiota's influence during the critical period of postnatal development and immune maturation. A growing body of evidence is focused on the complex interplay between initial events in GI microbiota assembly and the host's innate and adapted immune system and whether manipulation of these communities during this key developmental stage can impact the outcome of a number of inflammatory diseases such as asthma (Fujimura et al., 2010).

Influence of Diet on Microbiota Composition

From early life, the structural and functional composition of the intestinal microbiota is tremendously influenced by diet (Muegge et al., 2011; Turnbaugh et al., 2009b). Substantial differences exist in the gut microbiota composition of breastfed and formula-fed babies. The GI microbiota of breast-fed infants is dominated by lactobacilli and members of the genus *Bifidobacteria* (Adlerberth and

Wold, 2009). *Bifidobacteria* obtain their growth advantage from the utilization of complex human milk oligosaccharides, which are abundant in human milk, while *Lactobacillus* species utilize the abundant human milk sugar lactose as the primary carbon source. Both commensals shape the environmental conditions within the gut by increasing acidity through the production of lactic and/ or acetic acid. This modulation of the intestinal niche likely defines which species cocolonize and may prevent colonization and overgrowth by pathogenic species for which these conditions are not optimal for multiplication. In contrast, formula-fed babies tend to have higher counts of Clostridia, *Bacteroides*, Enterococci, and Enterobacteriaceae such as *Klebsiella* and *Enterobacter* (Adlerberth and Wold, 2009). However, how these microbiome members shape the GI ecosystem from both a metabolic and immune standpoint remains to be determined.

Dietary habits continue to shape the GI microbiota throughout life. The fecal microbiota of European children raised on a typical high-fat, low-fiber 'Western' diet was found to differ significantly in richness and diversity from their African counterparts raised on a diet high in plant polysaccharides and fiber (De Filippo et al., 2010). Polysaccharidedegrading bacteria were enriched in the African study group and absent in the European cohort. Moreover, the relative ratio of the two dominant phyla detected in the GI microbiota (Bacteroidetes and Firmicutes) was also reversed: compared with western-diet fed children who exhibit a 3:1 ratio of Firmicutes to Bacteroidetes, African children possessed gut microbiota that were significantly enriched in Bacteroidetes and reduced in members of the Firmicutes. Additionally, significantly more SCFAs were found in samples from the latter population. This may allow these individuals to maximize energy intake from plant-based fibers while also protecting them from inflammation and infectious colonic diseases. More recently, a study has demonstrated that long-term dietary habits are strongly associated with microbial enterotypes (Wu et al., 2011). A diet rich in protein and animal fat is associated with a *Bacteroides*-driven enterotype. In contrast, the consumption of primarily plant-based carbohydrates is linked to an enterotype characterized by the abundance of *Prevotella*. A controlled-feeding intervention study showed that enterotype identity remains stable even though the microbiome composition changed detectably within 24 h of initiating a high-fat/low-fiber or low-fat/high-fiber diet, suggesting that the composition of the GI microbiome is largely defined by long-term dietary habits.

Enteric Microbes are Essential for the Development of Appropriate Immune Function

Evidence pointing to the importance of early GI colonization events on immunological maturation includes studies of germ-free mice, which demonstrated deficiencies in immune development in the absence of GI microbial colonization (Hrncir et al., 2008; Macpherson et al., 2000). Several other animal models and comparisons of human gut communities have reinforced the key role that appropriate microbial colonization plays in the development of the gut-associated lymphoid tissue and subsequent immune system development (Cebra, 1999; Hill and Artis, 2010). Intestinal microbial colonization instigates the innate as well as the adaptive immune system and triggers a multitude of responses that are critical for maintaining host–microbial homeostasis. Examples include stimulation of the production of effector molecules such as secretory IgA (Macpherson and Uhr, 2004), activation of the epithelial antimicrobial protein RegIIIg expression (Lehotzky et al., 2010), induction of proliferation of specific T-cell subset, e.g., proinflammatory T_H17 cells or antiinflammatory regulatory T (T_{Reg}) cells (Ivanov et al., 2009; Round and Mazmanian, 2010). IgA delays the gut microbiota diversification in infants and acts as a decoy ligand preventing colonization by pathogenic species, suggesting that IgA, particularly maternal IgA transferred through breast milk, may significantly impact early colonization events in the GI tract, and at least partially explain the microbial colonization patterns observed in breast-fed babies. T_H17 cells recruit neutrophils to the site of inflammation by producing interleukin-17, while T_{Reg} lymphocytes, when activated, counteract inflammatory mediators by suppressing immune response and inflammation.

The implications of influencing early events in GI microbiota assembly are underscored by the fact that maintenance of homeostasis between helper T lymphocytes expressing proinflammatory T_H1 cytokines, activating cellular immunity required for efficient response to intracellular pathogens (e.g., viruses), and counteracting T_H2-associated cytokine expression, which stimulates a humoral antibody-based response, is essential to appropriate immune functioning. Humans are born with a bias toward a T_H2-mediated immune response. The foundation for the T_H1/T_H2 balance is then laid during postnatal immune maturation in early infancy, the period in which the GI microbiota develops progressively until an assemblage resembling that of an adult emerges sometime after the first year. There is substantial evidence now indicating that a protective T_H1 response is induced by early-life exposure to microbes (Yoo et al., 2007). Importantly, it has been demonstrated that the presence of a GI microbiota is fundamental for establishing and maintaining the delicate equilibrium between these two subsets of effector T cells (Bowman and Holt, 2001; Mazmanian et al., 2005). The emerging hypothesis is that early stimulation of the immature immune system by a diversity of appropriate commensal microbes during the crucial stage of immune maturation is required for the development of a balanced immune system.

Diseases and Disorders Associated with Aberrations in the GI Microbiota

The development and progression of many digestive tract as well as systemic disorders and diseases are being linked to the GI microbiota (Fujimura et al., 2010). This is not surprising in view of the intimate relationship between the human body and its microbial symbionts and the multidimensional effects commensal gut microbes have on physiology and immune system development and maturation of the host. Diseases associated with the GI microbiota include the more obvious conditions such as colon cancer (Marchesi et al., 2011), irritable bowel syndrome (Salonen et al., 2010), and IBD (Nagalingam and Lynch, 2012) and also less obvious disorders such as obesity (Ley et al., 2006), type 1 diabetes (Wen et al., 2008), rheumatoid arthritis (Vaahtovuo et al., 2008),

progression and severity of human immunodeficiency virus infections (Gori et al., 2008), development of nonalcoholic fatty liver disease (Spencer et al., 2011), cardiovascular disease (Wang et al., 2011), autistic spectrum disorders (Parracho et al., 2005), and allergy and asthma (Sjogren et al., 2009). Some of these diseases, such as obesity, IBD, or allergies, have been linked to aberrations in the composition of the gut microbial community, a condition called 'dysbiosis.' Dysbiosis is frequently accompanied by overgrowth of pathogenic microbial species, in conjunction with significant loss of microbial diversity and, presumably, as aforementioned, loss of key functional groups necessary to protect the mucosal surface and maintain metabolic and immune homeostasis. Factors that can impact the composition and stability of the GI microbiota include dietary changes, antibiotic use, psychological and physical stress, and altered GI tract peristalsis. For example, antibiotic therapy can give rise to antibiotic-associated diarrhea (e.g., due to *C. difficile* overgrowth). Current efforts by multiple research groups are examining how such perturbations may give rise to chronic disease.

Inflammatory Bowel Disease

The term 'inflammatory bowel disease' describes a range of GI disorders characterized by chronic inflammation of the mucosal layer of the digestive system. The two diseases under the umbrella of IBD, most commonly diagnosed in patients residing in Western nations are, ulcerative colitis (UC), typically restricted to the colon, and Crohn disease (CD), which can manifest anywhere along the digestive tract (from mouth to anus). The characteristic of UC is a contiguous inflammation of the superficial mucosal and submucosal intestinal layers. In contrast, CD patients exhibit healthy regions alternating with patches of inflamed tissue in a typically 'cobblestone-like' pattern. Transmural ulcerations causing fissures that may perforate the GI wall are also indicative for CD (Nagalingam and Lynch, 2012). Although the etiology of IBD remains unknown, perturbations in the GI microbial consortium have been associated with the onset and progression of the disease (Fava and Danese, 2011). More specifically, key members of the bacterial microbiota (i.e., *Clostridium* IXa and IV groups, *Bacteroides*, *Bifidobacteria*) are typically decreased in fecal samples from IBD patients while, in parallel, potentially detrimental bacteria (i.e., sulfate-reducing bacteria and *E. coli*) become more prevalent. The observed dysbiosis is concomitant with defective innate immunity and bacterial killing (i.e., reduced mucosal defensins and IgA, malfunctioning phagocytosis) and overaggressive adaptive immune response (due to ineffective T_{reg} cell populations and antigen-presenting cells), which are considered the basis of IBD pathogenesis (Fava and Danese, 2011). Moreover, at a functional level, bacteria producing butyrate such as *Faecalibacterium prausnitzii* are significantly depleted in the GI microbiota of IBD patients (Sokol et al., 2008). Butyrate and other SCFAs are known to have important antiinflammatory properties. Whether dysbiosis is the cause or result of IBD remains to be determined. Nonetheless, independent studies of microbiome collapse and the ability of specific GI species to induce subsets of inflammatory T cells associated with IBD implicate microbiome perturbation, failure of the community to reassemble, and pathogenic species proliferation as an obvious trigger for inflammation. This in turn likely further depletes microbiome diversity and protective functions within the community, permitting pathogen dominance and subsistence in the niche leading to chronic inflammation.

Enteric Microbes and Atopic Diseases

Atopic disorders such as allergies, allergic rhinoconjunctivitis, atopic eczema, and asthma are characterized by an imbalance in the T_H1/T_H2 equilibrium that is skewed toward an overexuberant T_H2-mediated immune response (Robinson, 2010). The deficiency in mounting an appropriate T_H1 balance has been linked to a lack of early-life exposure to microbial antigens or inappropriate intestinal colonization (Okada et al., 2010). This paradigm is an extension of the 'hygiene hypothesis' first postulated by Strachan in 1989 in an attempt to explain the rising prevalence of asthma and childhood eczema in Western nations (Strachan, 1989). Several broad ecological studies have since demonstrated significant differences in the composition of the infant GI microbiota between populations with different rates of allergic disease development (Penders et al., 2007; Watanabe et al., 2003). More specifically, development of atopy was correlated with low GI microbial diversity and an increase in abundance of specific bacterial species such as *Clostridia*. In contrast, higher counts of *Lactobacillus* species have been described in the feces of infants with a reduced risk for the development of allergies (Sepp et al., 1997). As discussed above, multiple factors affect the composition of the infant gut microbiome, e.g., host genotype, mode of delivery (vaginal delivery versus Cesarean section), diet, and antibiotic therapy, and influence disease outcome later in life. Differences in the microbial succession patterns in the gut such as delayed intestinal colonization by *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* species in infants born by Cesarean section may be the result of differences in initial community composition (e.g., lack of exposure to vaginal-associated *Lactobacillus* species in Cesarean-delivered babies), and these modifications will persist over time and ultimately may increase the likelihood of developing allergic traits. Supporting this hypothesis is the demonstration that 'like begets like,' in that communities with certain species present, e.g., *E. coli*, are more likely to permit 'invasion' by other similar species, e.g., *Shigella* species (Stecher et al., 2010). This suggests that assembly of the GI microbiota may be largely influenced by initial colonization events in this niche. Secondary to this argument is the supposition that if an aberrant microbiota establishes early in life during the critical period in which the immune system is primed by its microbial encounters, the resulting immune disequilibrium may also play a strong selective pressure in maintaining the abnormal microbiota and preventing colonization by beneficial species.

Microbiota Restoration

Although our understanding of the complex interplay between the human host and the intestinal commensal microbes as well as of the importance of maintaining the intricate balance between members of the gut microbiota is still in its infancy, various concepts

exist to treat or prevent dysbiosis and subsequent pathogen overgrowth or disease development by restoring microbiota homeostasis. Both fecal transplantation and probiotic supplementation have been used in an attempt to combat or prevent gut microbiota dysbiosis (Reid et al., 2011).

Fecal Transplantation

The transplantation of fecal material from a healthy individual into the GI tract of a patient recipient has been used to treat several intestinal as well as extraintestinal diseases with varying success. Donors are usually healthy relatives without a recent history of taking antimicrobial drugs. This is based on the assumption that their gut microbiome resembles most closely that of the patient prior to the development of dysbiosis. However, whether the patient's relationship to the donor affects outcome remains a contentious issue in the field. A fecal sample is collected from the donor and resuspended in a suitable diluent such as nonbacteriostatic saline prior to administration. The preferred route of transplantation is either through colonoscopy since this allows inoculation of the entire colon and ileum or through the application of a retention enema. Alternatively, the use of nasogastric or nasoduodenal tubes has also been described (Aroniadis and Brandt, 2013).

Fecal microbiota transplantation as an alternative to conventional antibiotic therapy has been highly successful for the treatment of chronic or recurring *C. difficile* infections (RCDI) (Gough et al., 2011). *Clostridium difficile* overgrowth and RCDI occur when the balance between the members of the gut microbiota is disrupted and pathogen exclusion is lost, e.g., due to the prolonged use of broad-spectrum antibiotics. Characteristic for patients suffering from RCDI is a low microbial diversity in the GI tract and a reduction in the numbers of Bacteroidetes and Firmicutes. Several reports describe that within 2 weeks of fecal transplantation, the recipient's intestinal microbiota closely resembles that of the donor. In fact, some recent studies have reported cure rates for RCDI using fecal microbiota transplantation at 92% (Gough et al., 2011).

Although relatively limited numbers of cases have been reported, the transplantation of fecal microbiota through retention enema has also successfully been used for the treatment of UC (Aroniadis and Brandt, 2013). Efficacy has been high with cessation of all symptoms within a few weeks after transplantation and without relapse. Remission was prolonged and up to 13 years postfecal transplantation in one case (Aroniadis and Brandt, 2013; Borody et al., 2003). The transplantation of fecal matter has also been described for the treatment of extraintestinal diseases such as autoimmune and neurological disorders (e.g., multiple sclerosis), obesity, chronic fatigue syndrome, and autism. However, the number of participants has been extremely small and the success rate highly variable. Yet, fecal microbiota transplantation for the restoration of appropriate diversity and, as a consequence, microbial function within the GI tract represents a viable and promising approach to the treatment of several refractory diseases.

Probiotics

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a beneficial health effect on the host" (Sanders et al., 2011). In contrast, a prebiotic is a food ingredient refractory to the human digestive process that is selectively fermented by the gut microbiota resulting in specific changes in the composition and/or activity of the GI microbiota, thus conferring benefit(s) on host health (Wallace et al., 2011). Although the concept of ingesting live microbes (primarily in the form of dairy products) to benefit human health and to abate diseases has been around for centuries, little is known about the immediate and prolonged effects probiotics have on the structure and function of the gut microbial community. By colonizing the gut and becoming part of the intestinal microbiota, probiotics are thought to alter the target microbiome, for instance, by introducing new metabolic capabilities such as the digestion of unusual carbohydrates. Colonization, however, may not be essential to induce shifts in the gut microbial community. Transient probiotic strains, when present in sufficiently high viable cell counts, may also significantly impact the structure of the intestinal microbiota by modulating key environmental factors such as pH or by producing antimicrobial substances (e.g., bacteriocins) or degrading proinflammatory cytokines.

Probiotic supplementation may improve IBD symptoms (e.g., induction or extension of disease remission periods) by restoring community composition and, thus, reconstituting functional capacity and the diverse beneficial effects the enteric microbes have on intestinal immune homeostasis. Several trials have been performed to test the efficacy of probiotic therapy on disease status and outcome of IBD. Some studies of probiotic supplementation indicate favorable outcomes in UC patients, while others report no significant improvement in the condition of CD patients (Haller et al., 2010). However, these preliminary findings are based on low-powered studies and the results have yet to be confirmed in controlled large-scale trials in which microbiome profiling is performed to determine the impact of bacterial supplementation on microbiome composition in patients with distinct disease and treatment courses.

Although several extrinsic and intrinsic factors likely contribute to the development of allergic disease, given the emerging importance of appropriate GI colonization in infancy, probiotic supplementation may be a useful strategy for the primary prevention of atopic disease. Several studies focus on newborns and infants and use various *Lactobacillus* strains as probiotic supplements. A randomized, placebo-controlled double-blind study of more than 100 newborns at risk for allergy development (based on family history) found that early feeding (daily for the first 6 months of life) of *Lactobacillus rhamnosus* GG (LGG) decreased the rate of atopic dermatitis at the age of 2 years by 50% (Kalliomaki et al., 2001b). In addition, follow-up studies after 4 and 7 years found that such effects are sustained past infancy, supporting the hypothesis that establishment of an appropriate GI microbial community during infancy via probiotic supplementation sets up a selfsustained community and presumably immune homeostasis that protects against allergic disease development (Kalliomaki et al., 2003, 2007). Others have, however, published

conflicting results. Kuitunen and colleagues studied the effect of postnatal supplementation for 6 months with a mixture of four probiotics (LGG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii* JS) along with prebiotic galacto-oligosaccharides in preventing allergic diseases (Kuitunen et al., 2009). At the 2-year followup, they found that probiotic intervention significantly prevented eczema and in particular atopic eczema, but had no substantial effect on the cumulative incidence of all allergic diseases. After 5 years, there was also no more significant difference detectable in the frequency of eczema between the probiotic and the placebo groups suggesting that supplementation with probiotics achieved no allergy-preventive effect that extended to the age of 5 years. Interestingly, however, they found that Cesarean-delivered infants supplemented with probiotics had significantly less IgE-associated allergic disease at the age of 5 years compared to Cesarean-delivered babies who had received placebo only. The fact that Cesarean-delivered infants benefited most from probiotic supplementation suggests that their GI microbiota may more amenable to probiotic manipulation. The bacteria found in the GI tract of Cesarean-delivered newborns are usually skin-associated bacteria that are likely to be ill-equipped to successfully colonize the intestine and, thus, represent unstable consortia readily invaded by species more adapted to these niches. In another negative study, Rose et al. (2010) reported that oral supplementation with LGG of infants at high risk for atopy had no clinical effect on atopic dermatitis or asthma-related events. However, this study enrolled children who were already 6 months old and had at least two previous wheezing episodes. It is therefore possible that these children had passed the critical period of intervention and their relatively developed intestinal microbiota was already skewed toward an unfavorable composition.

Future Directions

Through the effort of several large-scale research programs launched in recent years in the United States, Europe, and Asia and by employing state-of-the-art technology, scientists have not only taken stock of the microbes colonizing various sites of the human body in unparalleled detail but also begun to uncover how the human microbiome and in particular the microbes residing in the GI tract shape human health and disease. Despite these significant achievements and largely because of substantial interindividual differences in the gut microbiome composition, there is no consensus yet on what defines a 'normal,' i.e., healthy GI microbiota, although key factors that influence these consortia, e.g., diet and antibiotic use, have been identified. More research is necessary to precisely determine key organisms and functional characteristics distinguishing healthy and unhealthy microbiomes and, moreover, to uncover the causal relationship between various disease and these assemblages. Of equal interest are long-term studies to describe the development of the human microbiome over the life span of an individual and to assess how changes correlate with health and disease states. Finally, attempts to determine how lifestyle and environmental exposures, particularly those associated with Western or developing nations, affect the composition and function of the microbiome and contribute to disease burden are of obvious importance and require large studies across geographically and demographically distinct populations. Findings from such studies will likely provide transformative insights into diseases and disorders more prevalent in one population compared to others and open up novel approaches or improve upon existing therapeutic strategies to treat disease.

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Haemophilus Influenzae☆

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Glossary

Biofilm A surface-associated community of bacteria, usually surrounded by an exopolysaccharide matrix.
Competence The ability to take up extracellular DNA.
Lipooligosaccharide (LOS) Component of the outer membrane of the Gram-negative mucosal pathogens. Differs from the lipopolysaccharide (LPS) of most other Gram-negative in that LOS lack the repeating oligosaccharide component that comprises the O antigen. LOS are important in virulence.
Phase variation Reversible alteration of surface antigens and virulence factors that often aids in the evasion of the immune response.
Pili Filamentous projections from the cell surface that consist of a repeating protein subunit. Often involved in adherence, biofilm formation, and twitching motility.
Planktonic Single bacterial cells capable of floating in liquid that do not reside within a biofilm.
Sialic acid Carbohydrate that is acquired from the host and used in the decoration of the LOS and biofilm matrix of nontypeable *Haemophilus influenzae* (NTHi). Sialic acid confers a net negative charge to the bacterium and protects from complement-mediated killing.

Abbreviations

ABC	ATP-binding cassette
cAMP	Cyclic AMP
ChoP	Phosphorylcholine
COPD	Chronic obstructive pulmonary disease
CRP	cAMP receptor protein
ECM	Extracellular matrix
Hep	Heptose
Hib	*Haemophilus influenzae* serotype B
KDO	Ketodeoxyoctanoate
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
NAD	Nicotinamide adenine dinucleotide
Neu5Ac	Sialic acid
NTHi	Nontypeable Haemophilus influenzae
PAFR	Platelet activating factor receptor
PRP	Polyribosylribitol phosphate
PTS	Phosphoenolpyruvate:fructose phosphotrans- ferase system

☆*Change History:* December 2014. WA Szymczak and MH Levi changed a considerable amount of the text and the sections. Updated references and added 1 figure.

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Defining Statement

Haemophilus influenzae is a Gram-negative, strict human pathogen. Despite the development of a vaccine against the most invasive serotype of *H. influenzae*, serotype B (Hib), infections caused by strains with other capsular serotypes, nontypeable *H. influenzae* (NTHi), and HiB infection in unvaccinated populations remain to be of major medical importance. The features of *H. influenzae* that contribute to colonization, persistence, and invasion are discussed within.

Introduction

In 1892, Richard Pfeiffer isolated small, straight Gram-negative bacilli from the nasopharynx of patients with influenza during a pandemic. The frequency of occurrence of this organism in patients with influenza and its alleged virtual absence in normal individuals led to the erroneous conclusion that this bacillus was the cause of the influenza pandemic of 1890 and thus the organism was named the 'influenza' bacillus. In 1920, the bacterium was designated a species of Haemophilus by Winslow et. al.

H. influenzae gains access to the respiratory tract after host inhalation of respiratory droplets or contact with secretions containing the bacterium. NTHi strains of *H. influenzae* lack the genes for encapsulation and frequently colonize the upper respiratory tract without causing disease. However, NTHi can cause acute conjunctivitis, acute otitis media, acute sinusitis, exacerbation of chronic obstructive pulmonary disease (COPD) and pneumonia in some individuals. In contrast, encapsulated strains of *H. influenzae* are rarely recovered from the nasopharynx of asymptomatic individuals and are more likely to invade the bloodstream. There are six antigenically distinct serotypes (a through f) of encapsulated *H. influenzae*. By 1935, it was recognized that Hib strains were the most common isolate of Haemophilus isolated from blood and CSF.

In 1987, the first glycoconjugate vaccine for use in humans against Hib was licensed. Vaccination has significantly reduced the incidence of Hib meningitis and also provided a framework for the development of vaccines against other infections requiring the enhanced antibody response that can be elicited by conjugation of a protein carrier to bacterial carbohydrates. In 1995, the genome sequence of *H. influenzae* KW-20 Rd was published, becoming the first free-living organism to have its genome completely sequenced. Access to the full genome of the organism continues to drive research into the virulence factors of the species today.

Taxonomy, Culture Conditions, and Laboratory Testing

H. influenzae is a member of the family Pasteurellaceae in the Gamma subdivision of Proteobacteria. The organisms are non-spore forming, non-motile, pleomorphic Gram-negative coccobacilli. On Gram stained smears, the organism may be overlooked because of its small size and inconsistent uptake of counterstain.

H. influenzae is a facultative anaerobe and a strict human pathogen. The bacterium requires protoporphyrin IX (factor X) and nicotinamide adenine dinucleotide (NAD, factor V) as supplements for aerobic growth. *H. influenzae* will grow on chocolate agar, which contains adequate amounts of factor X and V. The organism grows optimally at 35–37 °C in the presence of 5–7% CO_2. Colonies 0.5–2 mm in diameter can be seen 24–48 h after inoculation. The colonies are smooth, low, convex, grayish and translucent. In contrast, blood agar plates do not support the growth of the organism because most NAD is sequestered intracellularly within red blood cells and blood contains enzymes that degrade extracellular NAD. The organism will only grow on blood agar plates as tiny, pinpoint 'satellite' colonies around organisms such as Staphylococcus aureus that release NAD and a toxin into the media that lyses red blood cells.

Factor X and V requirements along with sugar fermentation and utilization of other biochemical substrates can be used to distinguish *H. influenzae* from other species of Haemophilus that colonize the upper respiratory tract. Haemophilus parainfluenzae, which comprises approximately 75% of the Haemophilus species found in the nasopharynx, only requires V factor for growth and can ferment mannose. Although H. parainfluenzae rarely causes disease, the bacterium has become increasingly recognized as a cause of endocarditis. Haemophilus haemolyticus, another nasopharyngeal organism that rarely causes disease can be more difficult to distinguish from *H. influenzae* as it also requires both X and V factors and biochemical utilization is similar. Differentiation is often based on the ability of H. haemolyticus to hemolyze blood agar plates, but 10–40% of H. haemolyticus strains are not hemolytic, thus misidentification is common.

Other species of Haemophilus that can cause disease in humans include *Haemophilus influenzae* biogroup aegyptius and Haemophilus ducreyi. *H. influenzae* biogroup aegyptius can cause purulent conjunctivitis, or 'pink eye,' in children and is also the causative agent of Brazilian purpuric fever, a systemic illness that occurs subsequent to conjunctivitis. Like *H. influenzae*, *H. influenzae* biogroup aegyptius requires both X and V factors for growth but can be distinguished because it does not produce indole. H. ducreyi causes chancroid, a sexually transmitted infection characterized by a painful genital ulcer. The organism only requires factor X for growth, but isolation of H. ducreyi can be difficult because the organism dies quickly once removed from the lesion and recovery of the organism requires the preparation of media that limits growth of other bacterial species and inhibits the effects of toxic substances carried over from the genital ulcer. Two human pathogens formerly classified as Haemophilus species, Haemophilus aphrophilus and Haemophilus paraphrophilus, have been re-categorized as one species, Aggregatibacter aphrophilus. Although A. aphrophilus rarely causes disease, it can be a cause of sepsis, bone and joint infection, spondylodiscitis, and

endocarditis. The bacterium is a member of the HACEK group (Haemophilus parainfluenzae, Aggregatibacter spp, Cardiobacterium spp., Eikenella corrodens, and Kingella spp.). These organisms are found in the nasopharyngeal and oral cavities and all can cause endocarditis. A. aphrophilus does not require X or V factors for growth. Other Haemophilus species, H. suis, H. somnus, H. avium, H. equigenitalis, H. gallinarum, and H. paragallinarum, are pathogens of animals.

Haemophilus species isolated from blood or other sterile sites indicate disseminated infection and should always be considered significant. *Haemophilus influenzae* isolated from specimens originating from the lower respiratory tract in predominating amounts, from typanocentesis fluid, or from the eye may also be clinically significant. *Haemophilus influenzae* can be identified directly from CSF or urine specimens using a commercially available latex agglutination assay, but the assay lacks both sensitivity and specificity so it is not routinely performed in most clinical laboratories. Once recovered on media, the bacteria can be identified by X and V factor requirements as described above and by commercially available biochemical-based identification systems. MALDI-TOF mass spectrometry has emerged as a rapid means to identify bacterial species. MALDI-TOF identification can be used to identify Haemophilus species from plated media and can also be used to directly identify organisms isolated from positive blood culture bottles, but the ability to accurately identify H. haemolyticus is dependent on the instrument and reference database that is utilized. 16S ribosomal sequencing can also be employed for Haemophilus identification, although this is not performed routinely in most clinical laboratories. If further strain typing is needed to investigate strain epidemiology, pulsed-field gel electrophoresis is the gold standard.

An agglutination assay using polyclonal antisera is usually performed to determine if the bacterium is encapsulated and if so, the serotype. However, molecular methods that detect the Cap locus are more accurate at differentiating unencapsulated verse encapsulated strains. Clinically significant isolates of *H. influenzae* should also be tested for the ability to produce β-lactamases. Production can be assessed by performing a rapid chromogenic test for the ability to degrade a nitrocefin substrate. If positive, the isolate should be considered resistant to amoxicillin and ampicillin, but will usually remain sensitive to β-lactam/β-lactamase inhibitors, cephalosporins, and carbapenems. Further susceptibility testing may also be necessary depending on the source of the isolate and local epidemiology. For example, isolates of *H. influenzae* can be resistant to ampicillin as a result of altered penicillin binding proteins, not β-lactamase production. These strains are rare in the US but are increasing in Japan. Resistance to levofloxacin is also low in the United States but has increased in Taiwan between 2004–2010. Approximately 20% of *H. influenzae* strains in the US are resistant to trimethroprim sulfamethoxazole, and *H. influenzae* is intrinsically resistant to macrolide-lincosamide-streptogramin B antibiotics.

Infections Caused by *H. influenzae*

Meningitis and Sepsis

Prior to the development of the vaccine, *H. influenzae* was the commonest cause of meningitis in children between the ages of 2 months and 2 years, with 95% of these cases attributable to Hib. In the United States, introduction of the protein-conjugated Hib capsular vaccine has significantly reduced the incidence of Hib meningitis in both children and adults since 1990 by >95%. Today, the incidence in the United States is less than 1 case per 100 000 children and infection is usually present in children who were only partially or not vaccinated. In adults, *H. influenzae* is a relatively uncommon cause of meningitis. Hib meningitis in adults is usually associated with previous head trauma, neurosurgery, sinusitis, otitis, or CSF leak. The clinical presentation of meningitis due to *H. influenzae* is otherwise no different from that of meningitis due to other bacterial pathogens. Hearing impairment and neurological sequelae will develop in 15–30% of individuals with meningeal disease. In the United States, the Hib vaccine series is begun at 2 months of age. Vaccination is also recommended for adults with predisposing conditions such as splenectomy, HIV, primary immunodeficiency, or immunosuppression resulting from cancer treatment or stem cell transplant.

In countries with high Hib vaccination rates, NTHi causes the majority of *H. influenzae* infections resulting in bacteremia. Patients with invasive disease usually have other co-morbidities due to conditions such as alcoholism, cardiopulmonary disease, HIV, immunosuppression, complement deficiency, recurrent bacterial infections, viral infection or cancer. In adults, the respiratory tract is usually the source of NTHi. In addition to Hib, the other encapsulated strains are also capable of causing sepsis and meningitis. In North America and Europe, serotype F strains are responsible for most cases of invasive disease caused by encapsulated strains. The incidence of serotype A invasive infections is also believed to be increasing in some areas. In Alaska, serotype A invasive disease was not reported in the pre-Hib vaccine era, but beginning in 2002, serotype A has emerged as a major cause of invasive disease in native children.

Haemophilus influenzae, most frequently Hib, can cause cellulitis and septic arthritis. Hib septic arthritis occurs primarily in children less than 2 years of age and cellulitis caused by Hib is also more commonly seen in young children. *H. influenzae* biogroup aegyptius is the cause of Brazilian purpuric fever. This illness, first recognized in Brazil, is a serious systemic illness in children. The disease, which is preceded by purulent conjunctivitis, is characterized by fever, petechia, purpura, and shock.

Pneumonia and Tracheobronchitis

Immunization has reduced the carriage of Hib in the nasopharynx, and Hib is a rare cause of pneumoniae today. However, NTHi remains only second to Streptococcus pneumoniae as a cause of community-acquired bacterial pneumonia. Approximately 80% of Haemophilus species causing pneumoniae are NTHi. Disease can be caused by strains of NTHi that have asymptomatically

colonized the upper respiratory tract for months or from the acquisition of new NTHi strains. Unlike encapsulated strains, NTHi are antigenically and genetically diverse, and some strains are associated with a greater ability to cause disease because of the virulence determinants possessed. Host factors are also associated with the development of disease, as patients with infections caused by NTHi are generally elderly and have a history of chronic lung disease or a long smoking history. Patients with HIV infection have been shown to be susceptible to serious respiratory infection due to Hib and NTHi. The clinical features are indistinguishable from those of other bacterial pneumonias, with fever, cough, and purulent sputum, usually of several days' duration. The radiologic picture is not distinctive, showing either lobar or patchy involvement. HIV-infected patients may have an increased rate of bacteremia accompanying respiratory infections with Hib.

The role of NTHi as an etiologic agent in acute exacerbations of chronic bronchitis resulting from chronic obstructive pulmonary disease (COPD) was once controversial, but is generally accepted today. The organism can be found in serial cultures of sputum in the majority of patients with chronic bronchitis during quiescent periods. Thus, the mere presence of the organism does not imply a pathogenic role for the organisms in this setting. However, the isolation of NTHi from sputum during exacerbations, the development of serum antibodies to NTHi after exacerbations, and the response of patients to antibiotics strongly suggests that NTHi is the etiologic agent in many acute exacerbations of chronic bronchitis. In addition, a study of an unlicensed whole-cell NTHi vaccine reduced the number and severity of COPD bronchitis episodes. In March of 2014, a new 10-valent pneumococcal conjugate vaccine that includes NTHi protein D as a carrier protein was licensed. Whether the vaccine will protect against NTHi pneumonia and invasive infection in COPD patients remains to be determined.

Otitis Media, Sinusitis and Conjunctivitis

Otitis media is the most common cause for office visits resulting in antimicrobial treatment and surgery for children. NTHi is the second leading cause of acute otitis media in children behind S. pneumoniae and also causes disease in adolescents and adults. The peak incidence for disease is between 6 and 24 months of age. By 3 years of age, more than two-thirds of children have had one or more episodes of acute otitis media. Australian Aboriginal and American Inuit children are especially susceptible to NTHi otitis media. A high incidence of hearing loss is common in these populations. Preliminary studies suggest that the new 10-valent pneumococcal NTHi-D vaccine may reduce acute otitis media caused by NTHi. In an efficacy study of the 11-valent prototype NTHi-D vaccine, there was 57% efficacy against pneumococcal and 35% efficacy against NTHi-mediated otitis media.

Encapsulated strains of *H. influenzae* also cause acute otitis media. In one study in Thailand, where <5% of the population is vaccinated, 62% of Haemophilus isolates from tympanocentesis fluid were Hib. Another study in Venezuela also demonstrated that 31% of Haemophilus isolates causing acute otitis media were caused by encapsulated strains.

H. influenzae can be a cause of conjunctivitis and sinusitis. Bacterial conjunctivitis is more common in children than in adults and is caused by NTHi in approximately 50% of cases. NTHi conjunctivitis can occur simultaneously with NTHi-mediated otitis media. In children with acute purulent sinusitis, S. pneumoniae or *H. influenzae* often predominate over other nasopharyngeal flora.

Obstetrical and Neonatal Infections

Obstetrical infections due to *H. influenzae* are a serious complication in pregnancy as both the mother and the child become infected, and there appears to be maternal– fetal transmission. The isolates are almost invariably NTHi. Symptoms in both mother and child occur within 24 h of birth and there is a high frequency of prematurity associated with these infections. Infant mortality is high.

Adult Epiglottitis

Hib is a cause of epiglottitis in children (usually age 2–7) and in adults. In one large retrospective review, the incidence of acute epiglottitis in adults was 9.7 cases per million population. The average age of the patients was 44 years with an equal sex distribution. Patients presented with an elevated temperature, sore throat (90%), dysphagia (80%), respiratory difficulty (45%), and hoarseness or drooling. Indirect laryngoscopy visualization of an inflamed epiglottis has proven to be the more reliable in making the diagnosis (positive in 86% of cases) than soft tissue X- rays of the neck. Laryngoscopy can induce laryngospasm, particularly in children, and clinicians should be prepared to maintain the airway with tracheosotomy when this procedure is performed. Mortality in this study was 7%, with all deaths related to airway obstruction. Based on this complication, it has been suggested that an airway should be established at the time of presentation rather than waiting for a worsening clinical picture. Antibiotic therapy is primarily directed against *H. influenzae*, group A Streptococci, and S. pneumoniae, which have also been implicated as etiologic agents in adult epiglottitis.

Other Infections

A variety of other less common manifestations of *H. influenzae* infection in adults have been documented, primarily in the form of case reports. These include empyema, pericarditis, osteomyelitis, endocarditis, cholecystitis, intra-abdominal infections, urinary tract infections, mastoiditis, and aortic graft infection.

Virulence Factors

H. influenzae possesses many virulence factors that are modulated in a complex manner to aid in colonization, persistence, and invasion. Virulence factors that are beneficial during early infection but are not advantageous during later stages can be rapidly turned on or off as required. Many *H. influenzae* virulence determinants are involved in adhesion to respiratory epithelial cells, inhibition of the host complement pathway, and subversion of the humoral immune response. The bacterium also possesses factors involved in nutrient acquisition that are required for virulence, and *H. influenzae* can form a biofilm in the respiratory tract and in the middle ear. Some of the major virulence factors of the bacterium are described below.

Polysaccharide Capsule

Encapsulated strains, or typeable *H. influenzae*, are separated into six capsular serotypes (a, b, c, d, e, and f). Each of these six capsule types is antigenically distinct, and thus no cross-protection against other serotypes of *H. influenzae* occurs in individuals vaccinated against Hib. The Hib capsule is composed of polyribosylribitol phosphate (PRP). Strains of Hib that have been genetically engineered to lack the PRP capsule do not cause invasive disease in animal models of infection. The capsule functions as a virulence factor by protecting the pathogen from complement deposition and subsequent complement-mediated lysis and phagocytosis. Hib strains expressing increased copy numbers of the Cap b locus, which is responsible for capsule production, exhibit increased serum resistance.

Antigenic Variation

During the course of infection, a successful pathogen adapts to evade the immune response or adjust to a new location within the host. This often involves the variation of cell surface antigens. Frequently, a surface protein or component provides a benefit for the pathogen in certain situations but may be detrimental when the pathogen disseminates to another location or the host immune response changes. To escape from the host immune response, pathogens have evolved mechanisms that allow for the expression of antigens to be turned on or off. When the genes encoding these antigens are rapidly turned on or off in a bacterial population at a high frequency and in a reversible manner, this process is referred to as phase variation. Phase variable genes in *H. influenzae* exhibit repeated nucleotide sequences that are prone to slip strand mispairing during replication. The result is insertions or deletions of base pairs into the gene, which can shift the open reading frame. Translation may be prematurely terminated and the gene product not expressed or transcription can be altered if the promoter region of the gene is affected. During infection, organisms that are expressing the most advantageous phase for a certain environment will be selected. In *H. influenzae*, many of the phase variable genes encode enzymes involved in lipooligosaccharide (LOS) biosynthesis. The role of phase variation in virulence is discussed in more detail in the sections below.

Lipooligosaccharide

Lipopolysaccharides (LPS) and LOS are major components of the outer membrane of Gram-negative bacteria. LPS and LOS differ in the length of the polysaccharide chain that extends from the lipid A inner core. LPS are characterized by the presence of the O antigen, a long region of repeating oligosaccharide units. O antigen accounts for the resistance of enterics to detergents and bile salts like deoxycholate. LOS have just one oligosaccharide unit and are sensitive to deoxycholate. LOS is produced by a subset of Gram-negative bacteria inhabiting airway mucosal surfaces that includes Neisseria, Bordetella, Haemophilus, and Moraxella spp. The LOS of *H. influenzae* is a major factor in virulence. This is especially true for NTHi. Because NTHi lack a polysaccharide capsule, the LOS is exposed to the harsh conditions that are encountered in the host and thus must provide the first line of defense against the innate immune response.

The inner core region of *H. influenzae* LOS is composed of lipid A, ketodeoxyoctanoate (KDO), and three conserved heptoses (**Figure 1**). The lipid A core of LOS is hexaacylated. Hexaacylation is critical for virulence. HtrB is an acyltransferase that performs one of the late acylation reactions in the biosynthesis of the lipid A core. NTHi and *H. influenzae* A2 insertional htrB mutants have the expected loss of lipid A acylation producing a mixture of pentaacyl and tetraacyl lipid A. Hib htrB mutants are attenuated for colonization and virulence in an infant rat colonization model. In human cells, htrB mutants elicit significantly less production of inflammatory cytokines from macrophages and airway cells and htrB mutants are unable to colonize human airway xenografts or immortalized human bronchial epithelial cells. In addition, htrB was identified as being transcriptionally upregulated during host cell colonization using a differential-display PCR strategy for the identification of *H. influenzae* genes upregulated during host cell infection. Immunochemical comparison of lipid A structures using a lipid A-specific monoclonal antibody has suggested that considerable heterogeneity exists among lipid A expressed by different *H. influenzae* strains. Moreover, studies have indicated that htrB itself is temporally expressed during host colonization and may be a determinant of the expression of other LOS biosynthetic genes.

Extending from the inner core heptose molecules are the highly variable oligosaccharides to which non-carbohydrate molecules can also be attached (Figure 1). This outer core region can be composed of glucose, galactose, N-acetylglucosamine, phosphorylcholine (ChoP) and/or sialic acid (Neu5Ac) but incorporation is dependent on expression of genes that are subject to phase variation. The result is often an LOS structure that mimics human glycosphingolipids including human blood group antigens. Neisseria and Haemophilus spp. can express a digalactoside (Gal-a 1, 4-Gal) that is contained within the human pK blood group antigen (Gal-a 1,4-Gal-,8 1,4-Glc-ceramide), which humans do not make antibodies against. Addition of the digalactoside to the LOS is dependent on the phase-on expression of *lic2A* and *lgtC*, which are required for the addition of the proximal and distal galactose respectively. A recent study demonstrated that serum resistant *lic2A* and *lgtC* phase-on variants were selected for after serial passage of NTHi serum sensitive strains, and expression of both *lic2A* and *lgtC* was required for optimal serum resistance. Furthermore, expression of the digalactoside blocked IgG binding and subsequent complement-mediated killing. Thus, the digalactoside likely contributes to virulence directly through molecular mimicry of host proteins and indirectly by blocking antibody access to binding sites on the bacterium.

Other saccharide motifs in the outer core of LOS are also associated with virulence. The transferase LpsA is required for the addition of either a glucose or a galactose molecule onto the third heptose (HepIII) of the LOS. A threonine at position 151 of LpsA results in galactose attachment while a cysteine, alanine, or methionine results in the addition of glucose. If a glucose is attached, the resulting HepIII-β1,2-glucose is a target for naturally acquired IgM, unless the epitope is shielded by extension of the oligosaccharide chain by Lic2A. NTHi strains that express the HepIII-β1,2-glucose motif bind more IgM and are more susceptible to neutrophil-mediated killing. The predicted expression of the galactose or absence of saccharide on HepIII in invasive strains of NTHi was 3-fold more frequent than in carriage strains, suggesting that organisms without the HepIII-β1,2-glucose may have a greater propensity to cause invasive disease because the bacteria are able to avoid recognition by host antibody. Serum resistance has also been associated with phase-on expression of *lex2A*. The expression of *lex2A* results in the substitution of glucose in the position where Lic2a would otherwise attach a galactose molecule. The outcome of phase-on expression of *lex2A* was similar to *lic2A* in that IgG binding is reduced.

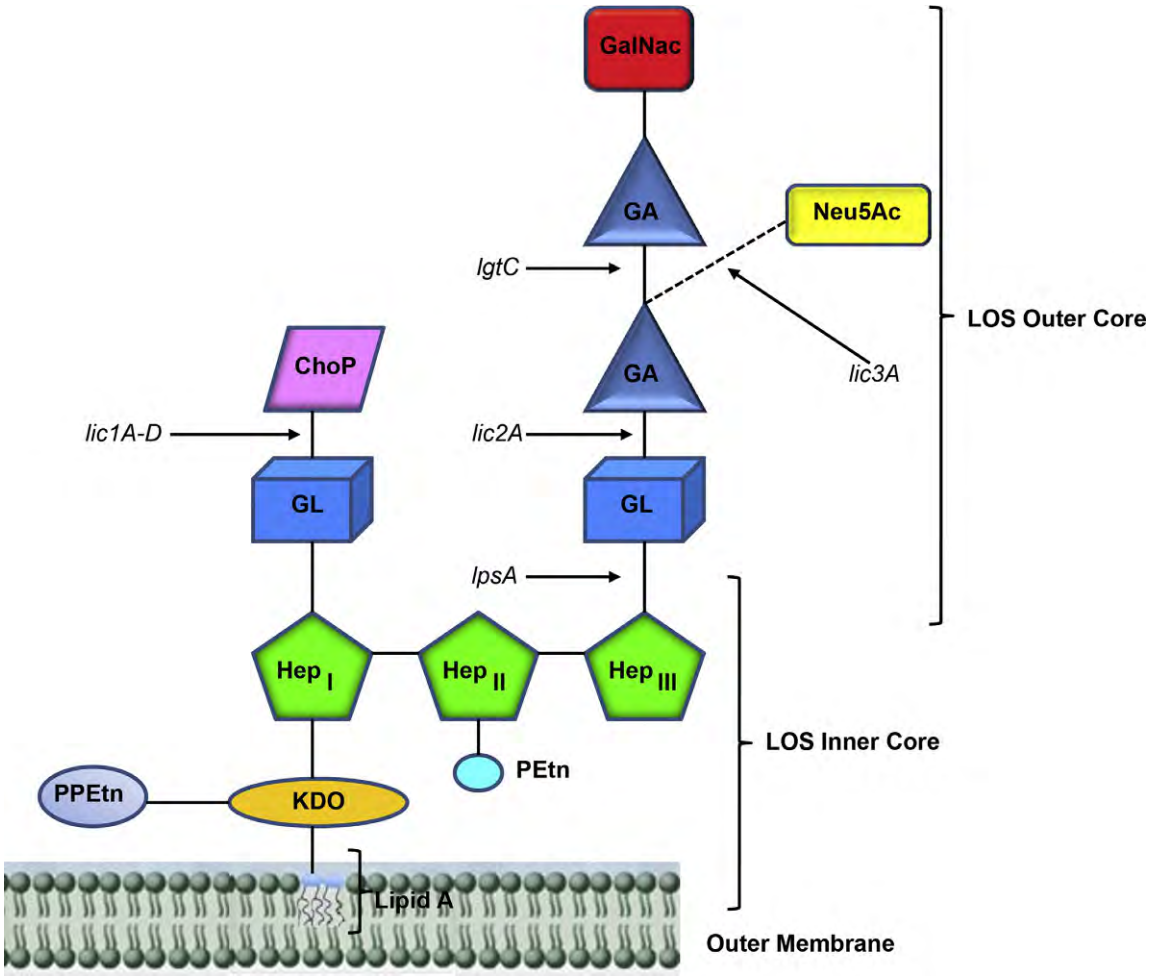

Figure 1 Schematic representation of the structure of *H. influenzae* strain Rd lipooligosaccharide (LOS). Extension of the LOS is dependent on the expression of phase variable genes. Abbreviations: ChoP, phosphorycholine; Neu5Ac, sialic acid; GalNac, acetylgalactosamine; GA, galactose; GL, glucose; Hep, Heptose; PPETN, pyrophosphoethanolamine; Petn, phosphoethanolamine. KDO, ketodeoxyoctanoate.

The addition of sialic acid as a terminal structure upon the LOS is widely conserved among mucosal pathogens. *H. influenzae* isolates can sialylate terminal galactose structures on their LOS. The organism cannot synthesize sialic acid and must acquire it from the human host via a highly regulated and complex transport system (**Figure 2**). It also appears that *H. influenzae* can remove sialic acid from human glycoproteins. Once transported into the cell, sialic acid is activated by SiaB, enabling it to be used for sialylation. The addition of sialic acid to the LOS contributes to protection from complement-mediated killing initiated via the alternative pathway. It is unknown exactly how sialic acid prevents complement-mediated killing of NTHi although its role in virulence is clear. SiaB mutants that are unable to activate sialic acid are attenuated in the chinchilla otitis media model. These mutants are virulent in animal models when the complement has been depleted using cobra venom factor, confirming the role of sialic acid in the protection from complement-mediated killing. In Neisseria gonorrhoeae, sialylation blocks the alternative pathway of complement activation by binding factor H, a key pathway regulator. Sialylation has not been shown to affect the binding of factor H by NTHi.

Genomic analyses have identified three potential sialyltransferases in *H. influenzae* strains. One of these sialyltransferases, *lic3A*, is an a-2,3-sialyltransferase with homology to a sialyltransferase in Campylobacter jejuni. Colonization of the nasopharynx with Hib in an infant rat infection model leads to the selection of *lic3A* phase-on variants. Interestingly, pathogen populations in the blood and cerebrospinal fluid of rats that have been infected via intraperitoneal inoculation did not select for phase-on variants. In this infection model, the relative distribution of the variants was similar to the inoculum used in the study. This appears to indicate that the expression of *lic3A* may be more crucial to the colonization of the upper respiratory tract rather than more invasive infections. However, in a study that investigated phase variable gene expression in paired NTHi isolates from the nasopharynx and middle ear of children, it was found that *lic3A* phase-off was the preferred state in vivo for 15 of the 21 isolates, regardless of infection site. More studies in humans are necessary to determine if *lic3A* expression is required for initial colonization and to understand under what circumstances expression of *lic3A* is not favorable.

The LOS can be further modified with the addition of phosphorylcholine (ChoP). ChoP decoration of the cell surface is also shared by many mucosal pathogens, including Gram-positive pathogens like S. pneumoniae. Analyses of *H. influenzae* strains from patient samples or animal infection models have repeatedly demonstrated a significant enrichment for ChoP variants. ChoP binds to the platelet activating factor receptor (PAFR), functioning as an adhesin. Binding to PAFR stimulates a host cell signaling cascade that may function to initiate invasion. ChoP also provides protection against certain antimicrobial peptides and bactericidal IgM antibodies. However, a drawback of expressing ChoP on the cell surface is the activation of complement via the C-reactive protein, thus increasing susceptibility of the pathogen to complement-mediated killing. These opposing roles of ChoP are controlled in part by phase variation. Expression of ChoP requires the products of the lic1 locus. This operon encodes for a choline kinase (*licA*), a choline transporter (*licB*), a pyro- phosphorylase (*licC*), and a choline transferase (*licD*). Phase variation of ChoP expression is controlled by tandem tetrameric repeats found in the 5′ end of *licA*. Infection models using Hib strains have demonstrated that *lic1* is predominately phase-on in colonization of the upper respiratory tract but this switches to a phase- off conformation at nonmucosal sites such as the blood and cerebrospinal fluid. NTHi strains expressing ChoP are better able to colonize the nasopharynx of chinchillas and promoted the development of otitis media. In a mouse infection model, ChoP expressing bacteria exhibited reduced antibody binding and greater outer membrane integrity, which was associated with increased bacterial survival. ChoP expressing variants were not selected for in SCID mice, demonstrating the requirement for selective pressures from the host adaptive immune response to induce phase variation. More recently, *licA* phase-on expression was found to significantly increase after experimental colonization of human volunteers. While only 3.8% of the inoculating NTHi population used in the study was phase-on, 18.8% of the population was phase-on three days after colonization and by day six there was an increase to 25.2%. These

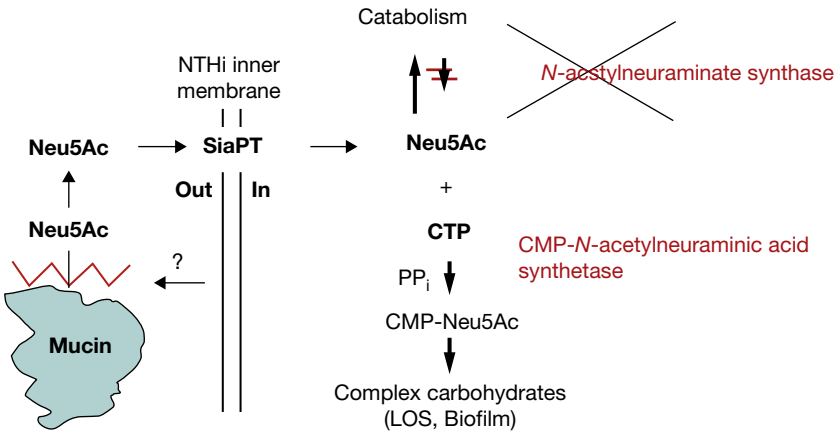

Figure 2 A diagram describing the uptake of sialic acid by nontypeable *Haemophilus influenzae* (NTHi). NTHi cannot synthesize sialic acid and must acquire it from the human host. Sialic acid is released by an unknown mechanism from mucin, moves through the outer membrane porin, and is captured in the cytoplasmic space by a specific binding protein, SiaP, which delivers the sugar to a transporter, SiaT. SiaT is an ATP-independent TRAP transporter and the sialic acid is thought to move through it driven by proton motor force. Once in the cytoplasm, sialic acid can be either directed to the catabolic pathway as a nutrient or incorporated into the lipooligosaccharide and biofilm after conversion to the nucleotide sugar.

studies suggest that ChoP provides a protective benefit in a mucosal environment but expression must be turned off when the pathogen disseminates to nonmucosal sites. ChoP expression is also selected during biofilm growth in otitis media infections.

Pili

There are two types of pili found in strains of *H. influenzae*: hemagglutinating pili and type IV pili. The hemagglutinating pili are primarily found in Hib strains, although they appear to be present in a few NTHi strains (approximately 15% of strains). Expression of the pilus requires a five-gene operon (hifABCDE) that encodes for the structural proteins and proteins that assist in transport and assembly. Hemagglutinating pili promote adherence to respiratory epithelial cells and respiratory mucus, assisting in the colonization of the upper respiratory tract. The pili likely recognize sialylated lactosylceramide derivatives found in some human gangliosides. After initial colonization, the expression of pili is turned off by phase variation. Early studies of the hemagglutinating pili of Hib strains observed that isolates from the nasopharynx were predominately piliated, while isolates from more invasive sites did not express pili. These nonpiliated strains could readily revert to piliated upon *in vitro* passage and enrichment. This phase variation has also been observed in NTHi strains that express hemagglutinating pili, with nonpiliated organisms isolated from the middle ear. Phase variation of pili is controlled by stretch AT dinucleotide repeats that are found in the overlapping promoter region of *hifA* and *hifB*, which encode for the major structural subunit of the pilus and a chaperone required for assembly, respectively. Variation in the number of dinucleotide repeats alters the spacing between the −10 and −35 regions for both promoters, affecting transcription. Three conformations result in full expression (10 repeats), no expression (9 repeats), or intermediate expression (11 repeats). Often, isolates associated with more invasive disease such as meningitis are nonpiliated. Phase variation likely functions to prevent the effectiveness of a host antibody response. This is believed to be necessary to allow for the persistence and dissemination of the pathogen. Hemagglutinating pili are also antigenically diverse, especially those found in NTHi strains, thus reducing the utility as a vaccine component.

Type IV pili are primarily associated with NTHi strains. Type IV pili have been identified and characterized in Pseudomonas aeruginosa, Vibrio cholerae, N. gonorrhoeae, and other Gram-negative pathogens. As in other pathogens, NTHi type IV pili have been shown to be involved in biofilm formation, are required for twitching motility, and DNA uptake. Type IV pili found in NTHi biofilms likely contribute to the structural stability of the biofilm matrix and are also involved in adherence to human respiratory epithelial cells and the colonization of the respiratory tract of chinchillas. In NTHi, PilA is the type IV pilus that is expressed at highest levels. Genes in the *pilABCD* and *comABCDEF* are operons that control expression of type IV pili, and all gene products are necessary for optimal adherence to epithelial cells, biofilm formation, and competence.

Adhesins

In addition to pili, other adhesins are present in *H. influenzae* strains (**Table 1**). The first of the *H. influenzae* autotransporter adhesins to be described were HMW1 and HMW2. These proteins are found in approximately 45–75% of NTHi strains but are rarely present in encapsulated strains. Named for their high molecular weight (125 kDa for HMW1 and 120 kDa for HMW2), they were initially identified because they were able to elicit a strong antibody response during NTHi infections. They both share significant homology to the filamentous hemagglutinin of Bordetella pertussis. HMW1 and HMW2 are involved in *in vitro* adherence to various cell lines. The two adhesins promote adherence to different cell lines and thus are likely to recognize different receptors. Adherence is sensitive to the pretreatment of the host cells with proteinases, removal of oligosaccharides and sialic acid, and the incubation with lectins, indicating that the receptor is likely a sialylated glycoprotein. HMW1 has also been shown to be glycosylated.

As with hemagglutinating pili, phase variation of the adhesins HMW1 and HMW2 likely serves to prevent the host from mounting an effective antibody response. When chinchillas that had been immunized with either adhesin were infected with a strain expressing the corresponding protein, depressed expression of HMW was observed. This was found to be due to phase variation. As with hemagglutinating pili, phase variation is controlled by repeats found in the promoter region of structural gene. A 7 bp sequence is found with copy numbers ranging from 1 to 4 with decreasing mRNA and protein levels as the number of repeats increases. In a study that examined serial isolates of *H. influenzae* from individuals with COPD, the authors found that HMW1 and HMW2 expression decreased over time in most isolates, which correlated with increased numbers of 7 bp repeats and high serum levels of anti-HMW1/2 antibodies. Taken together, these studies suggest that the pathogen balances the benefits of expressing the adhesin early during infection by modulating expression of antigens once targeted by the humoral immune response.

The *H. influenzae* adhesin, or Hia, is found in 80% of NTHi strains that lack HMW1/HMW2. Hia shares significant homology with Hsf, an adhesin typically found in Hib and other encapsulated strains. Hia and Hsf are conserved at the N-terminal and C-terminal ends. Hsf is considerably larger than Hia (240 and 115 kDa, respectively). This difference is due to the presence of three copies of an internal protein domain in Hsf while Hia contains only one copy. Hia and Hsf are found to be flanked by the same open reading frames in their respective strains and thus appear to be in the same location on the genome. Hsf forms fibrils on the cell surface that are visible when negatively stained cells are viewed by transmission electron microscopy. These fibrils are not present on Hia-expressing strains. Escherichia coli strains expressing either Hia or Hsf exhibit similar adherence profiles to a panel of human cell lines, suggesting that the proteins bind to the same receptor, although the receptor has yet to be identified. Hsf was recently shown to bind to host vitronectin. Vitronectin is found both in the extracellular matrix (ECM) and in plasma and is an inhibitor of the host complement membrane attack complex. In addition to inhibiting complement, Hsf binding to vitronectin

Table 1 *H. influenzae* virulence factors involved in adhesion to respiratory epithelial cells

Virulence factor	*Function*	*Phase variable expression*
ChoP	Binds to PAFR on epithelial cells, involved in invasion, limits antibody and antimicrobial peptide binding, biofilm formation, activates complement	Yes; tetrameric repeats in *licA*
Hemaglutinating pili	Binding to epithelial cells and mucus	Yes; dinucleotide repeats in the *hifa* and *hifb* promoter
Type IV pili	Binding to epithelial cells, biofilm formation, twitching motility, competence	No
HMW(1,2)	Binding to epithelial cells	Yes; 7 bp repeats in *hmw(1,2)* promoter
Hia	Binding to epithelial cells	No
Hsf	Binding to epithelial cells and the ECM protein/complement inhibitor vitronectin	No
Hap	Binding to epithelial cells, bacterial aggregation and microcolony formation, binds the ECM proteins laminin and collagen IV and the ECM protein/complement inhibitor vitronectin	No
Protein E	Binding to epithelial cells, binds the ECM proteins vitronectin, laminin, and plasminogen, also binds hemin	No
Protein F	Binding to epithelial cells, binds the ECM protein laminin and the ECM protein/complement inhibitor vitronectin	No
P5	Binds to ICAM-1 and CEACAM-1 on epithelial cells and the complement inhibitor Factor H, limits IgM binding	No
Protein D	Binding to and invasion of epithelial cells and monocytes, increases expression of Chop, inhibition of cilia function	No

promotes bacterial adherence and invasion of respiratory epithelial cells, thus Hsf may mediate attachment via direct adhesion and through indirect interaction with vitronectin.

Similar to Hsf, many proteins involved in H. influenza adhesion inhibit the host complement pathway and/or simultaneously bind to host ECM proteins, which often become exposed after damage to the lung epithelial cells from smoking or inflammation. Hap mediates adherence to host cells and also binds to the host ECM proteins fibronectin, laminin, and collagen IV. Hap has also been implicated in the promotion of bacterial aggregation and microcolony formation, an important early step in biofilm formation. The autoproteolysis of Hap is prevented by a leukocyte protease inhibitor that is produced by the host, preventing the release of the passenger domain from the cell surface. This mechanism allows the expression of Hap on the cell surface to be influenced by its local environment. Protein E is an outer membrane lipoprotein and Protein F is a membrane exposed ATP-binding cassette (ABC) transporter that are both involved in adherence of the organism to respiratory epithelial cells and both proteins bind to vitronectin and laminin. Protein E also binds plasminogen, which further enhances adhesion to the ECM and when the active plasmin protease is produced after host proteolytic processing of plasminogen, plasmin degrades the extracellular matrix proteins. Degradation possibly promotes dissemination of the bacterium. Plasmin bound to *H. influenzae* also degrades the complement component C3b. The adhesin P5 binds to ICAM-1 and CEACAM-1 on host epithelial cells and also binds the host inhibitor of the alternative complement pathway, Factor H. Additionally, P5 reduces IgM binding, thereby inhibiting activation of the classical complement pathway.

Protein D, the carrier protein in the new 10-valent pneumococcal vaccine is ubiquitously expressed in encapsulated Haemophilus and NTHi. The protein promotes adhesion to respiratory epithelial cells, but may do so indirectly by increasing ChoP decoration of the LOS. NTHi must obtain choline for ChoP biosynthesis from the host and Protein D has glycerophosphodiesterase activity that is thought to be involved in the cleavage of host choline-containing molecules. Protein D mutants exhibit both profound reductions in ChoP decoration of the LOS and adherence defects, thus it is likely that Protein D-mediated adherence is through ChoP binding to PAFR. However, adherence and internalization of NTHi by human monocytes is enhanced by Protein D and internalization is not associated with increased ChoP expression suggesting direct binding to this cell type. A role for Protein D in virulence has been demonstrated in both the rat and chinchilla models of otitis media.

IgA Protease

The immunoglobulin IgA1 is a significant component of the mucosal immune response. The *H. influenzae* protease genes *igaA* and *igaB* encode IgA1 proteases that neutralize these antibodies to aid in colonization and survival. Although IgA1 proteases appear to contribute to virulence, the proteases do not seem to be essential. IgA1 proteases are commonly found in N. gonorrhoeae, Neisseria meningitidis, S. pneumoniae, and other pathogens that colonize human mucosal surfaces but are usually absent in nonpathogenic Haemophilus species. Most strains of *H. influenzae*, both encapsulated and NTHi, possess at least one IgA1 protease; however, rare isolates that lack this proteolytic activity have been identified. In a human NTHi colonization study, there was a significant increase in the percentage of the NTHi population that was *igaB* phase-on after 6 days of nasopharyngeal colonization (12.5%) compared to the inoculating population (3.8%) suggesting that expression is beneficial during early colonization. A clonal group of strains expressing both *igaA* and *igaB* have been isolated from the respiratory tract of individuals with COPD, which raises the possibility that strains expressing both proteases are adapted for colonization of these hosts. *In vitro* studies have demonstrated that *igaA* promotes NTHi invasion of respiratory epithelial cells and *igaB* promotes intracellular persistence, but the mechanisms remain to be identified.

IgA1 protease is a secreted autotransporter protein. The N-terminal signal sequence of the 169 kDa precursor protein promotes transport to the periplasmic space. Next, the C-terminal outer membrane domain forms a 8-barrel in the outer membrane through which the internal passenger domain translocates to the cell surface. The serine protease activity of the protein then releases the protease domain from the surface, and an additional autoproteolytic event results in the mature protease. The IgA1 protease cleaves the hinge region of both the serum and the secreted forms of IgA1. Cleavage of the hinge region prevents the agglutinating activity of IgA1 by separating the antigen-binding Fab domains from the Fc portion of the antibody.

Acquisition of Iron, Zinc and Heme

Iron and heme acquisition are essential for survival during infection. In the host, iron is primarily stored intracellularly. Extracellular iron is sequestered in the serum by transferrin and in mucosal environments by lactoferrin, both of which are host iron-binding proteins. Pathogens have evolved mechanisms for releasing and acquiring iron from these sources. *H. influenzae* lack siderophores, the high-affinity iron chelators that are released by organisms into the environment, but an operon has been identified that may function in the capture and utilization of siderophores released by other organisms. *H. influenzae* utilize transferrin-binding proteins (Tbp1 and Tbp2) that are very similar to the transferrin-binding proteins of Neisseria species. In addition to Tbp1 and Tbp2, the cytoplasmic membrane protein TonB is required for the release of iron from transferrin. Tbp1, in complex with TonB, mediates the release of iron into the periplasm of the bacteria. Once in the periplasm, an ABC transporter moves the iron molecule across the cytoplasmic membrane. The ABC transporter consists of a periplasmic solute binding protein (FbpA), a permease protein (HitB), and an ATP- binding protein (HitC). FbpA binds the iron molecule and brings it to the HitB permease. The cleavage of ATP by HitC provides the energy necessary for the transport of the iron molecule into the cytoplasm.

Unable to synthesize heme, *H. influenzae* must also acquire it from the host. As with iron, heme is rarely found freely and is usually found bound to hemopexin and in hemoglobin/haptoglobin complexes. While some of the proteins involved in heme acquisition have been identified, a detailed model has not been elucidated. The HxuABC system is TonB-dependent and acquires heme from hemopexin. Other heme and hemoglobin binding proteins have been identified including HbpA, (heme-binding) and HgpA (hemoglobin binding). Transport via these receptors requires a lipoprotein that is encoded for by *hel*, which has a role in the transport of heme across the outer membrane. Transport of heme across the inner membrane requires Sap ABC transporters. The transporter permease SapBC and heme periplasmic binding protein SapA are required for transport into the cytoplasm. SapF is involved in heme utilization and was found to be important for biofilm formation, nasopharyngeal colonization, and acute otitis media in chinchillas. Recently, a study demonstrated that the adhesin Protein E binds hemin *in vitro*. The bound hemin could later be donated to *H. influenzae* that were starved of hemin, suggesting that Protein E may act as a storage reservoir for heme that can be released at times of limited availability.

Acquisition and regulation of zinc is critical for the survival and virulence of many organisms. Zinc is a cofactor for more than 300 enzymes in microorganisms and is reduced in host plasma during the acute phase response. Consequently, pathogens must be able to sufficiently obtain and utilize zinc during infection when host zinc is limited. Two zinc transporter systems have been identified in *H. influenzae*, ZnuABC and ZevAB. NTHi *zeva* and *zevb* mutants exhibit reduced ability to colonize lungs of mice.

Phasevarion

A regulatory system has been described for NTHi in which phase variation of the type III restriction– modification system controls multiple genes. The modA locus associated with this system encodes a DNA methyltransferase, that when expressed affects the methylation and subsequent expression of multiple phase variable genes. In Haemophilus, restriction activity has been lost or inactivated and it is certain that DNA restriction is not the function of the phase variable restriction-modification system. Rather, it is proposed that this system enables random switching of the organism between two different cell types with altered expression of virulence factors. Similar systems have been identified in N. gonorrhoeae, N. meningitidis, Moraxella catarrahalis and Helicobacter pylori.

Competence

H. influenzae is naturally competent and readily takes up extracellular DNA from the environment. *H. influenzae* can also acquire DNA that is carried within and coating the outside of vesicles that have been secreted by other *H. influenzae* bacteria and possibly other bacterial species. In NTHi, studies have shown that expression of the type IV pilus is required for DNA uptake. Once inside the cell, the DNA can be degraded and the nucleotides utilized for DNA replication or for metabolism. Alternatively, DNA may be incorporated into the genome through homologous recombination, which likely plays a considerable part in the genetic diversity of NTHi strains. However, competence may have initially evolved to function as a means for obtaining nucleotides for DNA synthesis rather than recombination. This viewpoint is supported by the fact that the regulation of competence is controlled by nutritional cues. The primary signaling molecule for competence induction is cyclic AMP (cAMP). cAMP is an intracellular signaling molecule produced by adenylate cyclase (CyaA). In *H. influenzae*, CyaA interacts with a phosphoenolpyruvate:fructose phosphotransferase

system (PTS), a transporter of fructose. When fructose is unavailable, CyaA produces cAMP, which is bound by the cAMP receptor protein (CRP). CRP is a global transcriptional regulator that regulates the expression of genes required for competence development as well as the acquisition and utilization of various carbon sources. Additional studies have determined that the availability of nucleic acid precursors also influences competence development. Competence can be induced by the addition of cAMP, upon entry into stationary phase growth, or by transferring cells to a defined starvation media. The latter approach is most commonly used in the genetic manipulation of *H. influenzae*. In combination with the publication of the complete genome sequence of *H. influenzae* KW-20 Rd, competence allows *H. influenzae* to be easily manipulated genetically, facilitating studies into virulence and cellular processes. The development of many genetic tools has greatly enhanced the capacity for advancements in *H. influenzae* research.

Colonization

H. influenzae has been found to have a high carrier rate in adults. When the human airway epithelium is compromised, *H. influenzae* can establish an infection. Nasopharyngeal colonization is the first step in pathogenesis. The bacterium must adhere to the respiratory epithelium and evade a number of host defenses including mucociliary clearance, secretory IgA, lysozyme, complement, lactoferrin, and antimicrobial peptides. Adherence of the bacterium to mucin may actually aid in clearance. Impairment of mucociliary clearance is a key step in the infective process. This can be achieved by a viral infection, cigarette smoke, cystic fibrosis, or a number of other causes. The bacterium can inhibit the function of cilia on nasopharyngeal cells by a Protein D dependent mechanism. Additionally, the LOS is toxic to ciliated epithelial cells and exacerbates impairment. With the mucociliary escalator impaired, the pathogen is now able to interact with nonciliated cells via one or more of its many adhesins. Next, the pathogen either invades the epithelial cell and passes to the subepithelial layer via transcytosis or aggregates and forms a biofilm.

It is unknown how *H. influenzae* establishes carriage, although invasion of respiratory epithelial cells, monocytes, and macrophages may be involved. While *H. influenzae* was not traditionally known as an intracellular pathogen, viable bacteria have been found within host cells suggesting that the organism invades host cells to escape the immune response. In one study, individuals with COPD exacerbations were found to have more intracellular NTHi than individuals with stable disease and no intracellular bacteria were found in healthy adults. The mechanisms leading to invasion are not completely understood. Invasion has been observed *in vitro* using cultured human epithelial cells and was found to be initiated by the binding of PAFR by ChoP on the LOS, but in a recent study, PAFR binding was found to be dispensable for respiratory epithelial invasion. It was found that NTHi invades by a mechanism that is dependent on host microtubule assembly, lipid rafts, and activation of phosphatidylinositol 3-kinase. Discrepancies in studies may originate from the differences in NTHi strains used and may indicate multiple mechanisms for adherence and invasion. NTHi IgaA and IgaB proteases participate in epithelial cell invasion and persistence respectively. The host β-glucan receptor has been implicated in adherence and invasion of *H. influenzae*, but further studies are needed to understand the pathogen-host interactions that mediate internalization. Macropinocytosis by bronchial epithelial cells also results in bacterial internalization. This mechanism involves extension of microvilli and the formation of lamellipodia, which engulfs the bacterium, and subsequent actin rearrangement.

In addition to intracellular invasion, *H. influenzae* are able to pass between cells to reach the subepithelial space. Translocation across the epithelium is enhanced after activation of a host transcriptional repressor of tight junction proteins by both *H. influenzae* and S. pneumoniae suggesting that both pathogens exploit the host immune response to gain access to the underlying tissue. Studies have found NTHi between the epithelial cells of bronchi and also in clusters in the submucosa. This phenomenon has also been observed in cultured cell lines. Often, cells can be observed in vacuoles of phagocytes in the epithelial layer. Currently, only one bacterial gene has been linked to promoting transcytosis, HI0638, which encodes for a putative lipoprotein of unknown function.

Modulation of the inflammatory response is an important aspect of colonization. Binding of the bacterium to epithelial cells can initiate inflammation through multiple pathways. The induction of cytokines promotes the recruitment of neutrophils, monocytes, eosinophils, and macrophages. The mucosal inflammatory response is primarily responsible for containment of the infection. Hosts in which inflammation is depressed are more likely to have invasive and recurrent infections. However, inflammation can be disadvantageous to the host, particularly in chronic lower respiratory tract infections and infections in cystic fibrosis patients. Increased inflammation damages epithelial cells, increases the production of mucus, prevents opsonophagocytosis, and recruits neutrophils, further impairing mucociliary clearance. Bacteria are then able to proliferate and disseminate.

Many bacteria, including *H. influenzae*, secrete outer membrane vesicles that have various effects on the immune response. *H. influenzae* secretes vesicles that contain LOS, DNA and proteins, many of which are associated with virulence. *H. influenzae* vesicles stimulate the production of antimicrobial peptides and IL-8 by respiratory epithelial cells, but whether secretions play a role in diverting the immune response away from bacterial cells has not been demonstrated.

Avoidance of complement and antibody are critical for *H. influenzae* persistence. Antibody-antigen complexes activate complement resulting in complement-mediates lysis of bacteria, and bound antibodies and complement promote phagocytosis of bacteria by host cells. Complement components are present in serum but have also been shown to be present at lower levels at the lung mucosal surfaces, especially during times of inflammation when the permeability of the mucosa increases. The polysaccharide of encapsulated strains provides a physical barrier that shields the bacterium from the effects of complement, siaylation and other LOS extensions inhibits complement activation, and many adhesins bind to host inhibitors of the complement system to exploit the inhibitors for their own protection. Multiple *H. influenzae* adhesins bind the host complement inhibitors vitronectin and Factor

H and the bacterium can also bind to the inhibitor C4BP although the binding partner has not been identified. The importance of complement in the host response is evidenced by individuals with C2 and C3 deficiencies whom are at increased risk of Haemophilus infection.

The bacterium utilizes multiple strategies to escape recognition by antibodies which include production of proteases that cleave IgA1, modification of the LOS to block antibodies from reaching their target, molecular mimicry of host antigens, antigenic variation of surface proteins, and intracellular invasion of host cells. Modification of the *H. influenzae* cell membrane has also been shown to reduce antibody binding. The NTHi transporters VacJ and Yrb inhibit IgM binding through removal of phospholipids from the outer membrane, which results in increased density of the oligosaccharides in the LOS and reduced access to IgM epitopes. Furthermore, transcription of both VacJ and Yrb was found to be increased in serum resistant clinical strains. Limiting IgM binding is likely critical for invasion of the bacterium from the upper respiratory to the lower respiratory tract. NTHi isolates recovered from the lower respiratory tract of patients with COPD bind less IgM and are more resistant to serum than isolates recovered from the upper respiratory tract. *H. influenzae* may also subvert the host humoral response through polyclonal activation of B cells. *H. influenzae* vesicles bind to the IgD receptor of B cells, which results in polyclonal activation and antibody secretion that is not specific to the bacterium. Similarly, NTHi has been shown to activate tonsilar B cells in a polyclonal manner. In a recent study, NTHi was isolated from 51% of tonsils from children undergoing tonsillectomy, where it was identified as the predominant bacterial species associated with tonsilar hypertrophy and was found to reside intracellularly. NTHi activated and was internalized by the tonsilar B cells in an IgD receptor-dependent manner, but the antibody produced by the B cells was not directed towards NTHi. Polyclonal B cell activation could be a mechanism utilized by NTHi to delay the development of an appropriate humoral response to *H. influenzae*. The host humoral response is critical in limiting disease. Individuals with primary antibody deficiencies have multiple exacerbations of NTHi disease over time with some strains persisting for up to 43 months after colonization.

Biofilm Formation

A biofilm is defined as a community of surface-associated bacteria that are usually surrounded by an exopolysaccharide matrix. Bacteria in a biofilm are more resistant to antimicrobial peptides and antibiotics than free-living planktonic bacteria. The matrix of the biofilm also plays a role in the protection from the host innate immune response. Pathogens can decorate components of the matrix to mask the biofilm and present it as 'self' to the host. Biofilms contribute to persistent and recurring infections. The protective properties of the biofilm make it difficult for the immune response to clear the pathogen and present a challenge in the treatment of biofilm-associated infections. There are several well-defined steps to biofilm formation (**Figure 3**). First, a bacterium adheres to a surface. Additional adhesins then interact with receptors and the bacterial cell forms a more intimate association. Additional cells are recruited and a microcolony is formed. The bacteria begin the production of the biofilm matrix and the biofilm continues to grow. Mature biofilms will have defined water channels that allow for the flow of nutrients and the release of waste throughout the biofilm. Conditions in the biofilm can vary significantly, depending on the ultrastructure. For example, oxygen levels will be higher closer to the surface of the biofilm while conditions are more anaerobic in the interior environment. It is possible that these types of changes impact the effectiveness of antibiotics. **Figure 4** shows a scanning electron microscopic image of an *H. influenzae* biofilm over airway epithelial cells.

The involvement of biofilm formation in NTHi infections has been well established, particularly in otitis media. Some patients presenting with chronic otitis media will produce sterile effusions, leading to the conclusion that the infection is not a bacterial infection but a sterile inflammatory process. However, bacterial DNA and mRNA can be detected in these effusions indicating that a bacterial pathogen is responsible. The failure to culture bacteria from the effusion is likely due to the bacteria to be associated to a biofilm, rather than free-living. It appears that antibiotic treatment contributes to the absence of bacteria in effusions, but other causes or mechanisms cannot be dismissed. The chinchilla model of otitis media has been used to demonstrate the role of NTHi biofilms in sterile infusions. More recently, bacterial biofilms were observed in middle ear mucosa biopsy specimens from children that were being treated for recurring otitis media infections.

In addition to middle ear infections, there is evidence that NTHi strains may form biofilms in the upper respiratory tract. Evidence of bacterial biofilms can be observed in bronchoalveolar lavage of cystic fibrosis patients. A study of patients with chronic rhinosinusitis confirmed the presence of bacterial biofilms on the sinus mucosa. *H. influenzae* was the predominant bacterial species in these biofilms. Interestingly, bacterial biofilms are also observed on healthy control samples suggesting that biofilm formation may be beneficial to the host in some circumstances.

Biofilm formation by *H. influenzae* appears to primarily be a function of unencapsulated NTHi strains. In one study, Hib strains were found to produce small amounts of biofilms *in vitro*, but production was reduced compared to NTHi strains and encapsulated strains have not been demonstrated to produce biofilms in vivo. Capsule may prevent the tight association required for the bacterial cell to adhere to a surface and interact with other bacteria.

A number of factors are involved in biofilm formation by NTHi. Many of the protein adhesins discussed above are also found in the extracellular matrix of the biofilm. Antibodies to Hap and HMW proteins have been used to detect these proteins in the matrix. However, since Hap and HMW are cell surface proteins, it is not surprising to find them associated with biofilms. Hap has been demonstrated to mediate microcolony formation by promoting aggregation, thus contributing to the early stages of biofilm formation. Strains lacking the type IV pili exhibit impaired biofilm formation *in vitro*. Type IV pili likely contribute to the

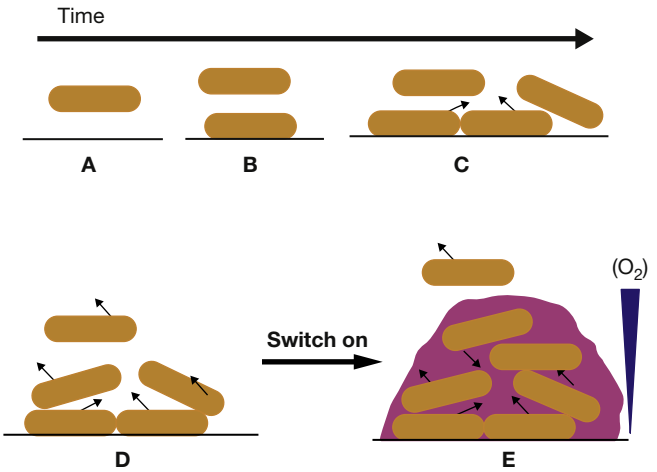

Figure 3 Steps in biofilm formation. The arrows designate production of quorum sensing molecules by the bacterium that triggers a coordinated response within the biofilm. Steps A, B, and C represent the early stages in microcolony formation on the biological surface followed by formation of the biofilm and the accompanying matrix. Bacteria are continually shed from the biofilm as planktonic cells, which can then establish new biofilms.

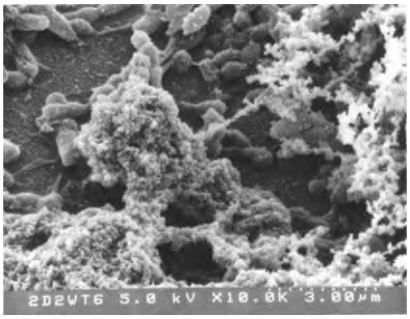

Figure 4 Nontypeable *Haemophilus influenzae* forming a biofilm over human airway epithelial cells in culture. Organisms covered with matrix can be readily seen.

maintenance of the biofilm structure. In addition to proteins, DNA may also provide structural stability to NTHi biofilms. In a detailed microscopy study of in vivo biofilms, it was observed that DNA forms a dense network of strands and dense rope-like structures.

Carbohydrate structures also play a key role in the biofilm structure. The precise nature of the NTHi exo- polymeric matrix is unknown, but certain features have been defined. Sialic acid is found to be a terminal non- reducing sugar of matrix glycoconjugants. An NTHi strain that expresses a truncated LOS lacking the acceptor for sialic acid, can still produce a biofilm that contains sialic acid. This indicates that sialic acid is used to decorate the biofilm matrix, which likely provides protection from the innate immune response of the host. Inactivation of various sialyltransferases or the CMP-Neu5Ac synthetase results in defective biofilm formation in a chinchilla infection model. WecA is an enzyme involved in the synthesis of the sialylated structure of the biofilm matrix. Inactivation of WecA results in the inability to form a biofilm in vivo.

ChoP also contributes to the persistence of NTHi biofilms. ChoP is found on the LOS and its expression is phase variable, as discussed above. Despite the increased susceptibility of ChoP expressing strains to complement- mediated killing, these strains are predominant in animal infection models, indicating that ChoP provides a beneficial function to the pathogen. In biofilm formation, the ChoP content of the LOS is elevated and this LOS is less inflammatory. A strain that is unable to express ChoP provokes an increased early inflammatory response and is unable to establish a robust biofilm in a chinchilla model for otitis media. Clearly, expression of ChoP on the LOS allows for the establishment of a biofilm, contributes to persistence, and illustrates how NTHi evade the host immune response.

Bacterial quorum sensing promotes formation and remodeling of bacterial biofilms. Quorum sensing involves the release of small molecule autoinducers by bacteria within biofilms in response to density. In NTHi, quorum sensing enhances biofilm formation, but it is not required. Production of the AI-2 autoinducer, a furanosyl borate diester, is dependent on *luxS*. NTHi *luxS* mutants can form biofilms but the thickness and density are decreased. Studies in the chinchilla otitis media model revealed that survival of *luxS* NTHi mutants is reduced, but decreased ChoP expression in the *luxS* mutant may contribute to the decrease in virulence. Mutation of *rbsB*, the gene encoding the NTHi periplasmic receptor for AI-2, results in decreased uptake of AI-2 by NTHi

and also results in reduced biofilm formation. Additionally, the QseB/QseC two-component system is involved in biofilm formation, but in an AI-2 independent manner.

Bacteria in biofilms are known to be more resistant to antibiotics, but in recent studies, antibiotics have been found to enhance biofilm production for a number of bacterial species as a result of altered gene expression. Similarly, sub-inhibitory concentrations of the β-lactam antibiotics amoxicillin, ampicillin, and cefuroxime were found to modulate gene expression and induce NTHi biofilm formation. Antibiotic treatment with suboptimal dosages of β-lactams resulted in biofilms with increased density and biomass *in vitro*. Prior antibiotic exposure also increased resistance to a lethal dose of cefuroxime.

NTHi and S. pneumoniae are found together within middle ear biofilms of children with chronic otitis media. However, only a few studies have investigated how polymicrobial biofilm formation contributes to pathogenesis. In the chinchilla otitis media model, mixed infection with NTHi and S. pneumoniae results in larger middle ear biofilms compared to infection with either species alone. Increased biofilm formation was associated with reduced systemic spread of S. pneumoniae, suggesting that mixed infection could be of some benefit to the host. Co-infection may also be advantageous to both bacterial species. In another study in chinchillas, co-infection was associated with the enhanced recovery of NTHi compared to mono-NTHi infection. In addition, co-infection with either a β-lactamase producing or a non-producing strain of NTHi resulted in reduced amoxicillin-mediated killing of S. pneumoniae within biofilms, although only the β-lactamase producing NTHi strain was capable of reducing killing of planktonic S. pneumoniae. This study suggests that NTHi promotes S. pneumoniae resistance to amoxicillin directly through β-lactamase production and passively through enhanced biofilm formation and that S. pneumoniae provides reciprocal protection to NTHi. More studies examining the genetic modulation of bacteria within mixed biofilms and the effects of the resident microbiota on biofilm formation are necessary to better understand the impact of multispecies biofilms in the host.

Conclusion

H. influenzae is a strict human pathogen that utilizes multiple virulence factors to persist in the host. While some host factors that predispose to the development of disease have been identified, disease can also develop in healthy individuals. Further studies are needed to understand the complex interactions that occur between *H. influenzae*, the microbial flora, and the host.

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Halophilic Archaea

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Glossary

Alkaliphiles Microorganisms that require high pH for growth.
Archaeol Core lipid consisting of two phytanyl (C20) chains bound to glycerol by ether bonds.
Bacteriorhodopsin A membrane-bound protein containing a retinal moiety, serving as a light-driven proton pump.
Compatible solutes Small organic molecules accumulated inside the cell to provide osmotic balance.
Glycolipids Lipids that contain bound sugar moieties.
Haloalkaliphiles Microorganisms that require both high salt concentrations and high pH for growth.
Halorhodopsin A membrane-bound protein containing a retinal moiety, serving as a light-driven chloride pump.
Methanogens Anaerobic archaea that produce methane.
Nanohaloarchaea A group of very small (~0.6 μm) Euryarchaeota living in hypersaline waters, only remotely related to the Halobacteria class.

Defining Statement

The halophilic archaea of the class *Halobacteria* (phylum *Euryarchaeota*) form a large and metabolically diverse group of prokaryotes that strictly depend on the presence of high salt concentrations much above that of seawater, and many can grow in salt-saturated brines. They are found in salt lakes and other hypersaline environments worldwide. Halophiles are also found in two other lineages of *Euryarchaeota*: the methanogenic *Methanosarcinaceae* family and the 'Nanohaloarchaea', a group of which no representatives have yet been cultivated.

Introduction

When in the late 1970s Carl Woese first used 16S rRNA sequence information to deduce the phylogenetic relationships between different groups of prokaryotes, it was quickly realized that the group of generally red-pigmented aerobic extremely halophilic prokaryotes classified in the family *Halobacteriaceae* belong to the newly defined group of the Archaebacteria, now named Archaea, together with the methanogens and certain hyperthermophilic and thermoacidophilic prokaryotes. This result was first described in a paper published in 1978 entitled 'Are extreme halophiles actually bacteria?', based on a study of the small subunit ribosomal RNA of *Halobacterium halobium* and *Halobacterium cutirubrum*, isolates later reclassified as strains of *Halobacterium salinarum*.

Indeed, hypersaline environments worldwide with salt concentrations up to saturation are inhabited by halophilic Archaea of the *Halobacteria* class, and these pigmented microorganisms are often present in sufficiently high densities to color the waters of salt lakes red. An example of such pink-red brines in a crystallizer pond of a solar saltern is shown in Fig. 1. The pigmented Archaea generally coexist with other groups of microorganisms adapted to life at high salt concentrations. These include a few types of Bacteria and unicellular eukaryotic algae of the genus *Dunaliella*. Most members of the *Halobacteria* live by aerobic respiration, but some a few representatives of the group prefer an anaerobic life style.

Among the methanogenic Archaea a few species are adapted to life at high salt concentrations. Most of these belong to the family *Methanosarcinaceae*. Some members of the *Halobacteria* also prefer an anaerobic life style. Cultivation-independent metagenomic studies of the biota of hypersaline environments have recently led to the discovery of a third group of halophilic Archaea: the 'Nanohaloarchaea'. These organisms are characterized by very small cells. All halophilic Archaea known thus far belong to the Euryarchaeota phylum. No representatives of the Crenarchaeota have yet been discovered in high-salt environments.

Most of this article deals with the biology of the *Halobacteria*. The halophilic methanogens and the 'Nanohaloarchaea' are discussed in the last sections of this article.

The Halobacteria

Taxonomy

The class *Halobacteria*, affiliated with the archaeal phylum *Euryarchaeota*, currently (May 2018) encompasses 3 orders and 6 families with a total of 59 genera and 246 species (Table 1). The class was circumscribed on the basis of small subunit rRNA gene sequence similarity, the high salt requirement of its members, and their physiological and chemotaxonomic features.

Fig. 1 A saltern crystallizer pond near Eilat, Israel, colored pink by halophilic Archaea (*Haloquadratum* and others). Unicellular algae (*Dunaliella salina*) and pigmented Bacteria (*Salinibacter*) also may contribute to the color of the brine. Photograph by the author.

Until 2015 the members of the *Halobacteria* were classified in a single order, the *Halobacteriales* and a single family, the *Halobacteriaceae*. The nomenclatural type is the genus *Halobacterium* with type species *Halobacterium salinarum*. Based on a comparative analysis of 129 sequenced genomes to identify shared molecular characteristics, including conserved signature insertions/deletions and conserved signature proteins, improved phylogenetic trees were constructed that provide a better understanding of the relationships between the organisms than can be deduced from 16S rRNA gene-based trees. This led to the splitting of the class into three orders, the *Halobacteriales* and two new orders: the *Natrialbales* and the *Haloferacales*, and a total of six families: the *Halobacteriaceae*, the *Haloarculaceae*, the *Halococcaceae* (order *Halobacteriales*), the *Haloferacaceae* and the *Halorubraceae* (order *Haloferacales*), and the *Natrialbaceae* (order *Natrialbales*). Haloalkaliphilic species that require high pH values (9–10 and higher) in addition to the requirement for high salt concentrations are found mainly in the orders *Natrialbales* and *Halobacteriales*; only few members of the *Haloferacales* are adapted to life at high salt and high pH. The order *Halobacteriales* contains a few representatives that are moderate acidophiles, preferring life at pH ~4.5, as well as a few species that prefer an anaerobic life style.

The chromosomes are between 2.0 and 4.5 Mb in length. Many species of *Halobacteriaceae* contain, in addition to the main chromosome, additional DNA in 'minichromosomes', 'megaplasmids' or plasmids. An extreme case is *Haloarcula marismortui* with nine circular replicons: two 'chromosomes' (3.1 and 0.28 Mb) and 7 plasmids. In some species 25%–30% of the genetic material is found outside the main chromosome. The number of rRNA operons encoded by these genomes varies between 1 and 3. Especially in the genera *Haloarcula* and *Halomicrobium* the multiple 16S rRNA genes can differ greatly in nucleotide sequence. Thus, the *rrnA* and *rrnB* genes of *Haloarcula marismortui* show substitutions in 74 positions, i.e., ~5% sequence difference. Both genes can be expressed so that each individual cell may contain ribosomes with either types of 16S rRNA.

The genome of *Haloquadratum walsbyi*, an unusually shaped flat square archaeon (Fig. 2), is of special interest: it has the lowest G+C content of all members of the class (47.9 mol%), and coding density is low: 76%, as compared to 86%–91% in other haloarchaea, a phenomenon caused by a very large average intergenic spacing and more than a thousand intergenic regions. It also encodes the largest protein identified in the group: the 9159 amino acids long halomucin, a protein associated with the cell envelope.

Chemotaxonomy and Physiology

The cells of most members of the *Halobacteria* lyse when suspended at salt concentrations below 100–150 g l^{-1}. Thus, high salinities are required not only for growth, but also for the maintenance of structural stability and integrity. With the exception of a few coccus-shaped genera (*Halococcus*, *Natronococcus*), the cell shape is maintained by an S-layer cell wall composed of a high molecular weight glycoprotein. The cell wall glycoprotein of *Halobacterium salinarum* (molecular mass ~120 kDa) consists of an 87-kDa core protein rich in acidic amino acids with attached acidic and neutral saccharide chains. The primary structure of the protein backbone and the mode of glycosylation vary among the species. Similar to most other proteins encoded by the genomes of the *Halobacteria*, the acidic cell wall protein denatures at low salt. As a result, suspension of the cells in fresh water leads to dissolution of the cell wall.

In contrast, members of the genus *Halococcus* (the single genus in the family *Halococcaceae*) possess a thick sulfated heteropolysaccharide cell wall that is not damaged in the absence of salt. The alkaliphilic coccoid genus *Natronococcus* (family *Natrialbaceae*) has a rigid cell wall of a different composition: it is built of repeating units of a poly(L-glutamine) glycoconjugate. Also these cells do not lyse when suspended in low salt solutions.

Table 1 The orders, families and genera of halophilic Archaea in the phylum *Euryarchaeota* with validly published names (as of May 2018)

Order	Family	Genus	Number of species	Type species	Comments
Halobacteriales					
	Halobacteriaceae				
		Haladaptatus	4	*Hap. paucihalophilus*	Cells remain viable for prolonged times at low salt concentrations
		Halalkalicoccus	3	*Hac. tibetensis*	Some species are haloalkaliphilic
		Halanaeroarchaeum	1	*Haa. sulfurireducens*	Anaerobic, oxidizing acetate with elemental sulfur as the electron acceptor
		Halarchaeum	6	*Hla. acidiphilum*	Some species are slightly acidophilic
		Haloarchaeobius	5	*Hab. iranensis*	
		Halobacterium	5	*Hbt. salinarum*	Type genus of the family
		Halocalculus	1	*Hcl. aciditolerans*	Slightly acidophilic
		Halodesulfurarchaeum	1	*Hda. formicium*	Anaerobic, oxidizing formate or hydrogen with elemental sulfur or thiosulfate as the electron acceptor
		Halomarina	3	*Hmr. oriensis*	
		Halorubellus	3	*Hrb. litoreus*	
		Halorussus	4	*Hrs. rarus*	
		Halostella	1	*Hsl. salina*	
		Halovenus	3	*Hvn. aranensis*	
		Natronoarchaeum	4	*Nac. mannanilyticum*	One species is moderately alkaliphilic
		Salarchaeum	1	*Sar. laminariae*	
		Salinirubrum	1	*Srr. litoreum*	
	Haloarculaceae				
		Halapricum	1	*Hpr. salinum*	
		Haloarcula	9	*Har. vallismortis*	Type genus of the family
		Halomicroarcula	3	*Hma. pellucida*	
		Halomicrobium	3	*Hmc. mukohataei*	
		Halorhabdus	2	*Hrd. utahensis*	One species preferentially grows anaerobically by fermentation and is not pigmented
		Halorientalis	3	*Hos. regularis*	
		Halosiccatus	1	*Hsc. urmianus*	
		Halosimplex	4	*Hsx. carlsbergense*	
		Natronomonas	3	*Nmn. pharaonis*	Haloalkaliphilic
		Salinirubellus	1	*Srb. salinus*	
	Halococcaceae				
		Halococcus	9	*Hcc. morrhuae*	Type genus of the family. Cocci that do not lyse in distilled water
Haloferacales					
	Haloferacaceae				
		Halobellus	8	*Hbs. clavatus*	
		Haloferax	13	*Hfx. volcanii*	Type genus of the family
		Halogeometricum	4	*Hgm. borinquense*	
		Halogranum	4	*Hgn. rubrum*	
		Halopelagius	3	*Hpl. inordinatus*	
		Haloplanus	7	*Hpn. natans*	
		Haloprofundus	1	*Hpd. marisrubri*	Isolated from a deep-sea brine
		Haloquadratum	1	*Hqr. walsbyi*	Flat square to rectangular cells
	Halorubraceae				
		Halobaculum	3	*Hbl. gomorrense*	
		Halobium	2	*Hbm. palmae*	
		Halohasta	2	*Hht. litorea*	
		Halolamina	6	*Hlm. pelagica*	
		Halonotius	1	*Hns. pteroides*	
		Haloparvum	2	*Hpv. sedimenti*	
		Halopenitus	3	*Hpt. persicus*	
		Halorubrum	37	*Hrr. saccharovorum*	Type genus of the family; also contains a few haloalkaliphilic species
		Salinigranum	2	*Sgn. rubrum*	
Natrialbales					
	Natrialbaceae				
		Halobiforma	3	*Hbf. haloterrestris*	Some species are haloalkaliphilic
		Halopiger	6	*Hpg. xanaduensis*	
		Halostagnicola	4	*Hst. larsenii*	Some species are haloalkaliphilic

(*Continued*)

Table 1 Continued

Order	Family	Genus	Number of species	Type species	Comments
		Haloterrigena	10	*Htg. turkmenica*	
		Halovarius	1	*Hvr. luteus*	
		Halovivax	4	*Hvx. asiaticus*	
		Natrialba	6	*Nab. asiatica*	Type genus of the family. Not all species are pigmented
		Natribaculum	2	*Nbl. breve*	
		Natrinema	8	*Nnm. pellirubrum*	
		Natronobacterium	2	*Nbt. gregoryi*	Moderately or extremely haloalkaliphilic
		Natronococcus	4	*Ncc. occultus*	Cocci that do not lyse in distilled water. Some species are haloalkaliphilic
		Natronolimnobius	3	*Nln. baerhuensis*	Haloalkaliphilic
		Natronorubrum	6	*Nrr. bangense*	Moderately or extremely haloalkaliphilic
		Salinarchaeum	2	*Saa. laminariae*	Moderately haloalkaliphilic
		Saliphagus	1	*Spg. infecundisoli*	
Methanomicrobiales					Not all members of the order are halophilic
	(unassigned)				
		Methanocalculus	6	*M. halotolerans*	Only one species is moderately halophilic
Methanosarcinales					
	Methanosarcinaceae				The family also contains non-halophiles
		Methanohalobium	1	*M. evestigatum*	
		Methanohalophilus	4	*M. mahii*	Not all species are halophiles
		Methanosalsum	2	*M. zhilinae*	Some species are haloalkaliphilic

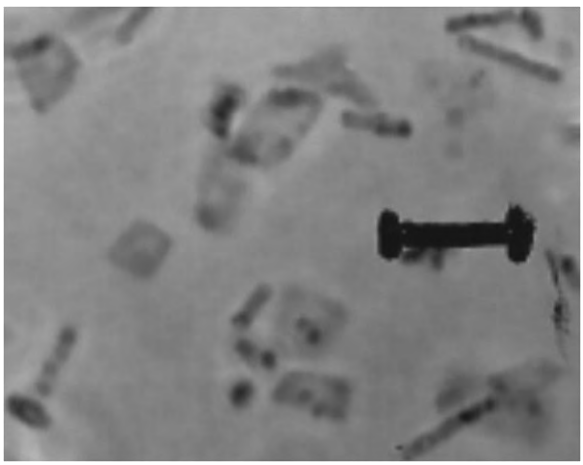

Fig. 2 Square and rectangular halophilic archaea (*Haloquadratum walsbyi*) in the brine of a saltern crystallizer pond near Eilat, Israel. Phase-contrast; bar=10 μm. Cells were concentrated by centrifugation, causing collapse of the gas vesicles. Photograph by the author.

The polar lipids of the *Halobacteria* are based on branched 20-carbon (phytanyl) and in some genera also 25-carbon (sesterterpanyl) chains, bound by ether bonds to glycerol. Fig. 3 shows the diether core lipids that forms the basis for most of the polar lipid structures: 2,3-di-*O*-phytanyl-*sn*-glycerol ($C_{20}C_{20}$) (archaeol) and 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol ($C_{25}C_{20}$). The hydrophobic chains are generally fully saturated, but unsaturated phytanyl ('phytenyl') chains were documented in *Halorubrum lacusprofundi*, a facultative psychrophilic species isolated from Deep Lake, Antarctica, that can to grow at temperatures down to 4°C.

Substitution of the free hydroxyl groups on the glycerol moiety enables the formation of a variety of polar lipids, including phospholipids, sulfolipids, and glycolipids. The types of polar lipids present differ among the genera of *Halobacteria*. All genera contain the diphytanyl diether derivatives of phosphatidylglycerol and the methyl derivative of phosphatidylglycerol phosphate. Phosphatidylglycerol sulfate is found in many genera. The alkaliphilic *Natronococcus occultus* has an unusual phospholipid with a cyclic phosphate group. Glycolipids are found in the neutrophilic species, but they are generally absent in the alkaliphilic members. Neutrophilic species may possess a variety of glycolipids with two, three or four sugar moieties, some of which may carry one or more sulfate groups. Not all glycolipids of the *Halobacteria* have yet been fully characterized.

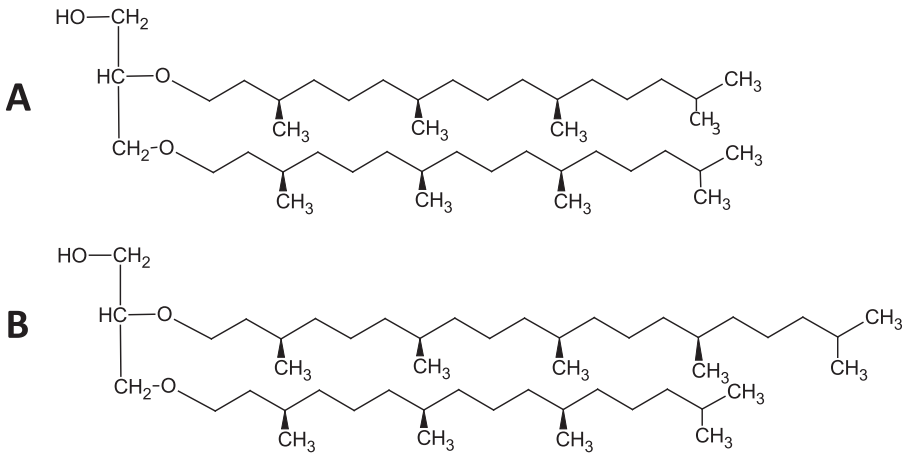

Fig. 3 The core lipids of halophilic archaea of the class *Halobacteria*: (A) 2,3-di-*O*-phytanyl-*sn*-glycerol ($C_{20}C_{20}$) and (B) 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol ($C_{20}C_{25}$) (not present in all genera). The free hydroxy group can bind different substituents to form phospholipids, glycolipids and sulfolipids.

The pink-red color of the cells of most species of *Halobacteria*, as shown in Fig. 1, is mainly caused by the presence of carotenoid pigments in their cell membrane. Only a few species are devoid of pigments. One example is *Natrialba asiatica* ('*alba*' in Latin means 'white'), isolated from beach sand in Japan. Species with an anaerobic life style such as *Halorhabdus tiamatea* isolated from a deep-sea brine on the bottom of the Red Sea and the obligate anaerobe *Halanaeroarchaeaum sulfurireducens* isolated from hypersaline lakes in Altai, Russia, are not pigmented. Some *Haloferax* species are pigmented when grown at relatively low salinity (~150 g l^{-1} NaCl) but can be almost colorless when grown at higher salt concentrations (250 g l^{-1}). The dominant carotenoids in the cell membrane of *Halobacteria* are generally the open-chain 50-carbon compounds α-bacterioruberin and different derivatives such as monoanhydrobacterioruberin and bis-anhydrobacterioruberin. C_{40} carotenoids such as lycopene and β-carotene are sometimes present in small amounts. The primary function of the carotenoid pigments appears to be to protect the cells against oxidative damage at high light intensities. Thus, it was shown that at high light intensities pigmented cells of *Halobacterium salinarum* out-competed a carotenoid-less mutant. The bacterioruberin pigments may also have additional functions such as the stabilization of the cell membrane structure.

An additional source of pigmentation of many members of the class *Halobacteria* can be the presence of retinal-containing proteins in their membranes. The best known retinal protein that can contribute a purple color to the cells is bacteriorhodopsin. It is a 27-kDa protein with seven trans-membrane helices and carrying retinal (a 20-carbon carotenoid derivative) as a prosthetic group. Bacteriorhodopsin serves as a light-driven proton pump. Upon excitation (maximum light absorbance at 570 nm) the molecule undergoes a complex photocycle during which also the stereochemistry of the retinal moiety changes as one of the double bonds changes from the *trans* to the *cis* conformation, and the Schiff base that binds the retinal to a lysine residue of the protein is deprotonated and protonated. As a result of these light-driven events a proton is transported from the inside of the cell to the outer medium. Thus, a proton gradient is established that can be used for the generation of ATP or for other energy-requiring processes. Light can supply the energy needed to drive anaerobic growth in *Halobacterium salinarum* cells that contain bacteriorhodopsin. However, photoautotrophic growth was never yet shown in this group: autotrophic CO_2 fixation is not possible, so that the cells depend on organic carbon sources. In *Hbt. salinarum* bacteriorhodopsin occurs in specialized patches of the cell membrane ('purple membrane'). Expression of the protein is induced by low oxygen tension and by light. Not all members of the *Halobacteria* possess the genes for the formation of bacteriorhodopsin and other retinal proteins.

Many members of the group have a second retinal protein, halorhodopsin. Its structure is similar to that of bacteriorhodopsin, but it acts as a light-driven chloride pump, transporting chloride from the outside medium to the cytoplasm. Inward chloride transport is important to maintain the proper ionic balance, and is essential for cell growth.

Motility by means of flagella is found in many species of *Halobacteria*, and such motile cells often show phototaxis so that cells can position themselves at optional light intensities and optimal light spectral quality for the operation of the light-driven retinal-containing pumps. Additional types of retinal proteins can serve as light sensors. Thus, *Hbt. salinarum* contains two sensory rhodopsins. Sensory rhodopsin I is a receptor for green light to which the cells are attracted, sensory rhodopsin II, also named phoborhodopsin, is a receptor for blue light that acts as a repellent. *Haloquadratum walsbyi*, which does not have flagella and therefore cannot be phototactic, has bacteriorhodopsin and halorhodopsin, but lacks sensory rhodopsins.

Some members of the *Halobacteria*, including *Halobacterium salinarum*, *Haloferax mediterranei*, *Haloquadratum walsbyi*, *Halogeometricum borinquense*, and *Halorubrum vacuolatum*, produce intracellular gas vesicles. Gas vesicles are made by some other groups of prokaryotes as well, including many cyanobacteria and some members of the *Proteobacteria*. Thus, this property is not a unique feature of the halophilic Archaea. The presence of gas vesicles decreases the density of the cells and increases buoyancy. This can be favorable when it enables the cells to float to the surface of the brine where the availability of light (as energy source, mediated by the bacteriorhodopsin proton pump) and oxygen for respiration may be increased. However, there is little evidence to show that in their natural environments cells indeed float to the brine surface thanks to their ability to produce gas vesicles.

Microorganisms that live at high salt concentrations need to keep their cytoplasm at least isosmotic with their environment. As biological membranes are permeable to water, maintenance of a dilute cytoplasm is not possible as the cell would rapidly dry out. Therefore all halophilic and halotolerant microorganisms need to adjust the osmotic pressure of their cytoplasm to that of the saline or hypersaline medium in which they live. This can be achieved in different ways. One strategy is the accumulation of inorganic ions for osmotic balance. This 'salt-in' strategy was adopted by a few specialized groups of halophiles only: the Archaea of the class *Halobacteria*, some aerobic Bacteria belonging to the *Bacteroidetes* (genus *Salinibacter* and relatives), and anaerobic halophiles affiliated with the *Firmicutes* (order *Halanaerobiales*). Such microorganisms contain intracellular chloride concentrations comparable to those of their medium, but sodium ions are excluded from the cells as much as possible. Cells that use the 'salt-in' strategy instead contain high concentrations of potassium ions.

An alternative way to achieve osmotic adaptation is by the synthesis (or accumulation from the medium, if available) of small organic, generally uncharged or zwitterionic compounds. Most halophilic and halotolerant microorganisms use such organic osmotic solutes or 'compatible solutes', as they are named because they are compatible with cell metabolism. No far-going changes in the properties of the intracellular enzymatic machinery are needed to enable the cells to function at high salt. Examples of such osmotic solutes are glycerol in the alga *Dunaliella*, glycine betaine, ectoine and hydroxyectoine in many Bacteria, some Archaea, and even a few protists (eukaryotes). In the past it was assumed that organic osmotic solutes do not play a role in the physiology of the halophilic Archaea of the class *Halobacteria*. However, already two decades ago, 2-sulfotrehalose was found to be accumulated within the cells of a number of haloalkaliphilic members of the group such as *Natronococcus occultus*, and the compound functions there, together with high concentrations of KCl, to provide osmotic balance. In recent years, analysis of genome sequences of many members of the group has shown that the ability to synthesize trehalose and sulfotrehalose is more widespread among the Archaea than previously assumed.

Potassium chloride at high concentrations is not as 'compatible' to cellular metabolism as are the uncharged or zwitterionic osmotic solutes mentioned above. Therefore an organism that uses the 'salt-in' strategy for osmotic adaptation needs extensive adaptation of the entire intracellular enzymatic machinery to function in the presence of molar concentrations of KCl. This the reason why most of the proteins encoded by the genomes of the *Halobacteria* have a strong acidic nature: they possess a large excess of negative charges due to their high content of the acidic amino acids glutamate and aspartate as compared to the positively charged amino acids lysine and arginine. Such acidic enzyme proteins are soluble and active in the presence of high salts, but many denature in low-salt solutions. As a result, the halophilic Archaea of the class *Halobacteria* cannot live in low-salt environments. As explained above, also the S-layer protein that makes up most of the cell envelope of most species needs high salt for stabilization, so that the cells lyse at low salt.

Species within the class *Halobacteria* generally lead an aerobic heterotrophic life style, and they are commonly grown in complex media containing high concentrations of yeast extract, casamino acids, and similar rich sources of nutrients. Amino acids and simple organic acids such as acetate, succinate, and pyruvate are among their favorite carbon and energy sources. Glycerol often stimulates growth. Many species can grow on defined media with a single organic carbon compound and inorganic sources of nitrogen, sulfur and phosphorus; others have complex nutritional demands.

Some members of the group are inhibited by high concentrations of organic substrates and can only be cultivated in nutrient-poor media. *Halosimplex carlsbadense*, isolated from a salt mine, only grows in defined medium with acetate and glycerol, acetate and pyruvate, or pyruvate alone. The flat square gas-vacuolated *Haloquadratum walsbyi* is another representative that only grows in low-nutrient media. Also here pyruvate is an important component of the media that enable the growth of this interesting organism that had defied all attempts toward its cultivation for nearly a quarter of a century since its discovery in 1980.

Carbohydrate metabolism was first investigated in *Halorubrum saccharovorum*. When grown on glucose as carbon and energy source, this organism degrades its substrate using a modified Entner-Doudoroff pathway in which the phosphorylation step is postponed: glucose is oxidized via gluconate to 2-keto-3-deoxygluconate, followed by phosphorylation to 2-keto-3-deoxy-6-phosphogluconate, which is then split into pyruvate and glyceraldehyde-3-phosphate. Members of the genera *Haloferax* and *Haloarcula* are also known for their ability to use simple sugars. Not all species grow on carbohydrates, and those that do often oxidize the substrate incompletely, leading to the formation of acids such as acetate, lactate, and sometimes also pyruvate.

The ability to produce exoenzymes such as proteases, amylases, lipases and nucleases is widespread among the halophilic Archaea, enabling them to use macromolecules such as proteins, starch, lipids and nucleic acids as sources of nutrients.

There are records of the isolation of halophilic Archaea, especially members of the genera *Haloferax* and *Haloarcula*, which can degrade aliphatic and aromatic hydrocarbons and other aromatic compounds. Compounds reported to be degraded include tetradecane, hexadecane, heptadecane, eicosane, heneicosane, the aromatic hydrocarbons acenaphthene, phenanthrene, anthracene, 9-methylanthracene, and other aromatic compounds such as benzoate, cinnamate, 3-phenylpropionate, and p-hydroxybenzoate. However, none of the type strains of the species that are available from culture collections is known to degrade such compounds.

As the solubility of gases in water decreases strongly with the increase in salt concentration, availability of oxygen may easily become a limiting factor for the development of halophilic Archaea. Some members of the group possess different modes of anaerobic growth. One of those mechanisms is based on the use of alternative electron acceptors. Nitrate is reduced to nitrite by many members of the *Halobacteria*, but this reaction is not coupled to anaerobic growth. Only in few species was anaerobic growth by denitrification documented. Thus, *Haloferax denitrificans*, *Haloarcula marismortui*, *Haloarcula vallismortis*, and *Halogeometricum borinquense* can grow anaerobically using nitrate as an electron acceptor. Gaseous nitrogen and/or nitrous oxide are the products of nitrate reduction. Nitrate is not abundantly found in salt lakes and salterns, also because of the lack of activity of autotrophic

nitrification at high salt concentrations. Therefore it is not clear to what extent this type of metabolism is used by these organisms in their natural environments. Some species can grow anaerobically by reduction of dimethylsulfoxide, trimethylamine-*N*-oxide or fumarate, with dimethylsulfide, trimethylamine and succinate as the reduction products, respectively. Also for these processes the ecological relevance is not clear.

An interesting, recently discovered type of anaerobic respiration that is probably relevant ecologically is the use of elemental sulfur and other sulfur compounds as the electron acceptors by the strictly anaerobic acetotrophic *Halanaeroarchaeum sulfurireducens*. This species is a rare example of an obligatory anaerobic member of the *Halobacteria*. It was isolated from sediments and brines collected from hypersaline lakes in the Kulunda Steppe (Altai, Russia). Acetate and pyruvate are the only electron donors used, and elemental sulfur is the only electron acceptor that supports growth. In contrast to nearly all other members of the *Halobacteria* it lacks pink-red carotenoid pigments.

Even anaerobic lithoheterotrophic growth is possible in the group. The recently isolated *Halodesulfurarchaeum formicicum* (family *Halobacteriaceae*) grows anaerobically using hydrogen gas or formate as the electron donors and elemental sulfur, thiosulfate or dimethylsulfoxide as the electron acceptors. Organic nutrients are required as carbon source. This type of organism is probably widely distributed in hypersaline inland lakes, solar salterns, lagoons and deep submarine anoxic brines, and it may well play an important role in the biogeochemical cycle of sulfur in such environments.

The ability of *Halobacterium salinarum* and possibly a number of additional members of the group to grow photoheterotrophically in the absence of oxygen based on the light absorbed by the outward proton pump bacteriorhodopsin was mentioned above. However, sustained anaerobic growth under these conditions is not possible as a minimal amount of oxygen is still needed for the biosynthesis of the retinal prosthetic group by splitting of its precursor β-carotene.

Anaerobic fermentative metabolism was documented in a few members of the *Halobacteria* class. *Halobacterium* species grow anaerobically in the dark when L-arginine is added to the medium. Fermentation of arginine to citrulline, ammonia and CO_2 via ornithine and carbamoylphosphate provides the ATP necessary for growth. However, this ability was seldom found in other genera. Other types of fermentative metabolism may be possible in some species, but the pathways responsible for ATP generation were not documented. For example, this is the case for *Halorhabdus tiamatea* (*Haloarculaceae*), a non-pigmented, extremely halophilic archaeon isolated from the brine–sediment interface of the Shaban Deep, a hypersaline anoxic basin in the northern Red Sea. It uses yeast extract and starch as carbon and energy source.

Ecology

Massive presence of halophilic Archaea of the class *Halobacteria* in hypersaline lakes is often conspicuous thanks to their red-pink pigmentation. This phenomenon can be seen in the northern basin of Great Salt Lake, Utah, in Lake Urmia in Iran, and in other natural lakes at or approaching salt saturation. The same is true for man-made hypersaline environments: saltern crystallizer brines such as shown in Fig. 1 typically contain between 10^7 and 10^8 such red cells per milliliter. Other types of pigmented microorganisms (bacteria, unicellular algae rich in carotenoids) may also contribute to the coloration of the waters. Such red microbial blooms are not restricted to NaCl-dominated brines of neutral pH: they can be seen also in hypersaline soda lakes in eastern Africa and elsewhere where the pH is 9–10 or even higher. Alkaliphilic members of the family such as *Halorubrum vacuolatum*, *Natronomonas pharaonis*, *Natrialba magadii*, *Natronobacterium gregoryi* and *Natronococcus occultus* are characteristic inhabitants of hypersaline soda lakes.

Occasional blooms of red Archaea were also reported from the Dead Sea on the border between Israel and Jordan, a magnesium chloride-dominated lake with a slightly acidic pH. Some isolates from the Dead Sea such as *Haloferax volcanii*, *Halorubrum sodomense* and *Halobaculum gomorrense* are markedly tolerant to high concentrations of divalent cations such as Mg^{2+} and Ca^{2+}, and they have a relatively low requirement for Na^{+}.

There are very few known acidophilic or acid-tolerant members of the *Halobacteria*. *Halarchaeum acidiphilum* is the only truly acidophilic representatives of the group. It only grows within the narrow range between pH 4.1 and 4.8 with an optimum at pH 4.4 in the presence of 200 g l^{-1} salt. Curiously, it was isolated from a sample of solar salt that had a slightly alkaline reaction. Temperatures up to 55°C are tolerated by many species. One cold-tolerant species is known: *Halorubrum lacusprofundi* isolated from Deep Lake, Antarctica. Its optimum growth is at 31–37°C, but it still can grow at temperatures as low as 4°C.

The first isolates described in the literature more than a hundred years ago, which are strains of the genera *Halobacterium* and *Halococcus*, were obtained from salted fish, salted meat and salted hides whose quality was negatively affected by the growth of red microorganisms. The source of those organisms may well have been the crude solar salt used for the preservation of the products. In other cases growth of such organisms is encouraged: during the preparation of certain salted fermented food products, e.g., nam pla, a fish sauce from Thailand, the proteases excreted by the Archaea contribute to the production process.

As high salt concentrations are necessary for growth of the halophilic Archaea, as well as for the stabilization of the S-layer cell walls of most species, we do not normally encounter such organisms in environments of seawater salinity and below. Still, viable cells of *Halococcus*, a genus that has a rigid polysaccharide cell wall, could be recovered from Mediterranean seawater. *Halococcus* species were also cultivated from the salt-excreting glands in the nostrils of certain seabirds. It is well possible that such birds can carry cells over long distances to colonize new hypersaline environments. Some other representatives of the *Halobacteria* have been found in environments with salinities similar to that of seawater or even below. Examples are *Halogeometricum pallidum* and *Haladaptatus paucihalophilus*, isolated from a low-salt, sulfide-rich spring in Oklahoma.

Members of the *Halobacteria* were recovered from rock salt deposited millions of years ago. Examples are *Halococcus salifodinae* and *Halosimplex carlsbadense*. Some halophilic Archaea may retain their viability for very long periods when trapped in fluid inclusions within halite crystals.

The Halophilic Methanogenic Archaea

Halophilic Archaea are not only found in the class *Halobacteria*. Also among the methanogenic Archaea we find true halophiles (Table 1). All methanogens are strict anaerobes. Most biogenic methane on Earth is produced by reduction of carbon dioxide by hydrogen and by splitting of acetate to yield methane and carbon dioxide. No aceticlastic methanogens are known to grow in hypersaline environments, probably because the amount of energy obtained in the reaction may be insufficient to supply the additional energy needed for osmotic adaptation. The most salt-tolerant methanogen known that derives its energy from CO_2 reduction with hydrogen is *Methanocalculus halotolerans* (order *Methanomicrobiales*). It was isolated from an oil well in Alsace, France. It can also use formate as energy source, and grows optimally at 50 g l^{-1} NaCl. No growth is possible above 120 g l^{-1}.

The preferred substrate for growth of the truly halophilic methanogens is trimethylamine. This compound can be expected to occur in anaerobic hypersaline environments as a degradation product of glycine betaine, a compatible solute used for osmotic stabilization by many types of microorganisms. Trimethylamine is degraded to methane, carbon dioxide and ammonia. Dimethylsulfide, a degradation product of another osmotic solute, dimethylsulfoniopropionate, is another substrate for some halophilic methanogens. Methanogens convert it to methane, carbon dioxide and hydrogen sulfide.

The halophilic trimethylamine degrading methanogens all belong to the family *Methanosarcinaceae*, order *Methanosarcinales*. *Methanohalobium evestigatum*, *Methanohalophilus portucalensis* and *Methanosalsum zhilinae* grow at salt concentrations up to 240–250 g l^{-1} salt. For osmotic stabilization they produce organic osmotic solutes: glycine betaine, β-glutamine, β-glutamate, and Nε-acetyl-β-lysine.

The Nanohaloarchaea

Metagenomic studies have recently revealed the existence of an additional group of extremely halophilic Archaea with very small cells, not yet represented by cultivated representatives. The novel group of *Euryarchaeota* known as 'Nanohaloarchaea', only distantly related to the *Halobacteria*, was found in different sites worldwide. One of those is Lake Tyrrell in NW Victoria, Australia (290 g L^{-1} salt). These organisms are about 0.6 μm in diameter. Based on the analysis of their genomes, these nanohaloarchaea probably lead an aerobic photoheterotrophic life style, as the genes of biosynthesis of the bacteriorhodopsin protein and the retinal prosthetic group are present. Genes for the degradation of polysaccharides are also found. Two lineages of nanohaloarchaea from Lake Tyrrell were named '*Candidatus* Nanosalinarum' and '*Candidatus* Nanosalina'. In a 190 g l^{-1} salinity saltern evaporation pond in Alicante, Spain, a related organism was characterized by single-cell genomics and was named '*Candidatus* Haloredivivus'.

Conclusion

We now have a good understanding of the biology of the aerobic halophilic Archaea of the class *Halobacteria*. Many different types have been cultured, and they are quite diverse with respect to their morphology, chemotaxonomic properties, growth conditions and possible modes of metabolism. Some representatives have become popular model organisms for the study of the group. Examples are *Halobacterium salinarum* strains used for genomic analysis, *Haloarcula marismortui* for protein structure, and *Haloferax volcanii* for genetic studies. We have cultured relatives closely related to nearly all those phylotypes belonging to the group that can be recognized in 16S rRNA gene libraries and in metagenomes prepared from most hypersaline environments. This is also true for the long-elusive flat square gas-vacuolated *Haloquadratum walsbyi*, recognized as a prokaryote in 1980 but first brought into culture only in 2004. Genomic comparisons have in recent years led to a highly improved taxonomic classification of the different members of the group, which is now divided into three orders and six families.

The *Halobacteria* are the best-known group of halophilic Archaea, but the archaeal domain contains additional members that are adapted to life at high salt concentrations. The family *Methanosarcinaceae* of methanogenic Archaea contains a number of halophilic representatives that can grow at salt concentrations close to saturation. Most members of this family are not halophilic, and the halophiles do not form a phylogenetically separate group as do the aerobic *Halobacteria*.

The recent discovery of the 'Nanohaloarchaea' during cultivation-independent studies based on DNA isolated from biomass collected from different hypersaline environments shows that the diversity of Archaea adapted to life at high salt concentrations is larger than assumed in the past. Based on the elucidation of their genome sequence we can deduce some information about their possible modes of life. The next challenge will be to obtain them in culture so that the organisms themselves, and not only their DNA, will become available for study.

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Heavy Metal Pollutants: Environmental and Biotechnological Aspects☆

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Glossary

Biomineralization The collective processes by which organisms form minerals.
Biosorption The removal of metal or metalloid species, compounds and particulates, radionuclides, and organometal(loid) compounds from solution by physico-chemical interactions with microbial or other biological materials.
Desorption Non-destructive elution and recovery of, for example, metal or metalloid species, radionuclides, and organometal(loid) compounds from loaded biological material by physico-chemical treatment(s).
Heavy metals An ill-defined group of biologically essential and inessential metallic elements, generally of density >5 g cm^{-3}, exhibiting diverse physical, chemical, and biological properties with the potential to exert toxic effects on microorganisms and other life forms.
Metal resistance The ability of a microorganism to survive the toxic effects of heavy metal exposure by means of a detoxification mechanism, usually produced in response to the metal species concerned.
Metal tolerance The ability of a microorganism to survive the toxic effects of heavy metal exposure because of intrinsic properties and/or environmental modification of toxicity.
Metallothioneins Low-molecular-weight cysteine-rich proteins capable of binding essential metals (e.g., Cu and Zn) as well as inessential metals (e.g., Cd).
Organometallic compound A compound containing at least one metal–carbon bond, often exhibiting enhanced microbial toxicity. When such compounds contain 'metalloid' elements (e.g., Ge, As, Se, and Te), the term 'organometalloid' may be used.
Phytochelatins Metal-binding γ-Glu-Cys peptides of general formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n generally 2–7); now designated as class III metallothioneins that are atypical, nontranslationally synthesized metal thiolate polypeptides. Cd-binding γ-Glu-Cys peptides from some yeasts are also called cadystins.
Siderophores Low-molecular-weight Fe(III) coordination compounds excreted by microorganisms, which enable the accumulation of iron from the external environment.

Abbreviations

γ-Glu-Cys	γ-glutamyl-cysteinyl
DMSe	Dimethyl selenide
EPS	Extracellular polymeric substances
SRB	Sulfate-reducing bacteria

Defining Statement

This article describes the roles of microorganisms in the transformation of heavy metals, as well as metalloids, organometals, and radionuclides, between soluble and insoluble phases, and the environmental and biotechnological importance of these transformation processes in biogeochemical cycles and in new biotechnologies for metal biorecovery and the treatment of metal, metalloid, and radionuclide pollution.

Introduction

Heavy metals comprise an ill-defined group of more than 60 metallic elements, of density higher than 5 g cm^{-3}, with diverse physical, chemical, and biological properties, but generally having the ability to exert toxic effects toward microorganisms. Many metals are essential for microbial growth and metabolism at low concentrations (e.g., Cu, Fe, Zn, Mg, Ni, Mo, Ca, Co, and Mn) but they are toxic in excess amounts, and both essential and inessential metal ions may be accumulated by the microbial cells by

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physico-chemical and biological mechanisms. Thus, 'toxic metals' and 'potentially-toxic metals' are useful general terms. In this article, the term 'heavy metal' is used in a broad sense, and the discussion will include actinides, metal radionuclides, and organometal(loid) compounds. All these substances have a common potential for microbial toxicity and bioaccumulation, are of environmental significance as pollutants or because of introduction as biocides and other substances, and many are of great economic and industrial importance.

Environmental Aspects of Heavy Metal Pollution

Heavy Metals in the Environment

Although elevated levels of toxic heavy metals can occur in natural locations (e.g., volcanic soils and hot springs), average environmental abundances are generally low with most of that immobilized in sediments and ores being biologically unavailable. However, anthropogenic activities have disrupted natural biogeochemical cycles, and there is increased atmospheric release as well as deposition into aquatic and terrestrial environments. The major sources of pollution include fossil fuel combustion, mineral mining and processing, nuclear and other industrial effluents and sludges, brewery and distillery wastes, biocides, and preservatives including organometallic compounds. In fact, almost every industrial activity can lead to altered mobilization and distribution of heavy metals in the environment. Because of the fundamental microbial involvement in biogeochemical processes, as well as in plant and animal productivity and symbioses, toxic metal pollution can have significant short- and long-term effects and ultimately affect higher organisms, including humans, for example, by accumulation and transfer through food webs.

Effects of Heavy Metals on Microbial Populations

Metals exhibit a range of toxicities toward microorganisms, depending on physico-chemical and biotic factors. While toxic effects can arise from natural activities, toxic effects on microbial communities are more commonly associated with anthropogenic contamination or redistribution of toxic metals. This can arise from aerial and aquatic sources, as well as agricultural practices, industrial activity, and domestic and industrial wastes. In some cases, microbial activity can result in remobilization of metals from other wastes and transfer into aquatic systems. It is commonly accepted that toxic metals (and their chemical derivatives and related substances) can have significant effects on microbial populations, and almost every index of microbial activity can be affected.

For toxicity to occur, heavy metals must directly interact with microbial cells and/or indirectly affect growth and metabolism by interfering with, for example, nutrient uptake, or by altering the physico-chemical environment of the cell. A variety of non-specific and specific mechanisms (e.g., biosorption and transport, respectively) determine the entry of mobile metal species into cells, and if toxic thresholds are exceeded, cell death will result unless mechanisms for detoxification are possessed. The plethora of intracellular metal-binding ligands ensures that many toxic interactions are possible. Thus, practically every index of microbial activity can be adversely affected by toxic metal concentrations, including primary productivity, methanogenesis, nitrogen fixation, respiration, motility, biogeochemical cycling of C, N, S, P, and other elements, organic matter decomposition, enzyme synthesis and activity in soils, sediments, and waters.

Despite potential toxicity, many microorganisms still survive, grow, and flourish in apparently metal-polluted locations, and a variety of mechanisms, both active and incidental, contribute to resistance and tolerance. However, general conclusions about heavy metal effects on natural populations are difficult to make because of the complexity of metal speciation, toxicity in the environment, and the morphological and physiological diversity encountered in microorganisms. Furthermore, environmental perturbations associated with industrial metal pollution (e.g., extremes of pH, salinity, and nutrient limitations) may also have adverse effects on microbial communities. Nevertheless, it is commonly assumed that microbes are able to respond to metal contamination and maintain metabolic activity through changes in microbial community structure and selection for resistance. Resistance and tolerance are arbitrarily defined, frequently interchangeable terms, and often based on whether particular strains and isolates can grow in the presence of selected heavy metal concentrations in laboratory media. It is probably more appropriate to use 'resistance' to describe a direct mechanism resulting from heavy metal exposure, for example, bacterial reduction of Hg^{2+} to Hg^{0}, or metallothionein synthesis by yeasts. 'Tolerance' may be a result of intrinsic biochemical and structural properties of the host, such as possession of impermeable cell walls, extracellular slime layers or polysaccharide, metabolite excretion, as well as environmental modification of toxicity. However, distinctions are difficult in many cases because several direct and indirect mechanisms, both physico-chemical and biological, can contribute to microbial survival. Thus, although heavy metal pollution can qualitatively and quantitatively affect microbial populations in the environment, it may be difficult to distinguish metal effects from those of environmental components, environmental influence on metal toxicity, and the nature of microbial resistance/tolerance mechanisms involved. Although some gross generalizations are possible regarding toxic metal influence on microbial communities, individual cases are likely to be site-specific and potentially complex.

Environmental Modification of Heavy Metal Toxicity

The physico-chemical characteristics of a given environment determine metal speciation and, therefore, chemical and biological properties of heavy metals. Concentration and speciation of metals in solution is governed by many processes, including inorganic and organic complexation, oxidation–reduction reactions, precipitation-dissolution, adsorption-desorption, some of these being mediated by microbial activities. Because major mechanisms of metal toxicity are a consequence of strong coordinating properties, a

reduction in bioavailability may reduce toxicity and enhance microbial survival. Such parameters as pH, temperature, aeration, soluble and particulate organic matter, clay minerals, and salinity can influence heavy metal speciation, mobility, and toxicity. Metals can exist in solution as free cations (e.g., Cu^{2+}, Cd^{2+}, and Zn^{2+}), as soluble complexes with inorganic or organic ligands (e.g., $ZnCl^{+}$, $CdCl^{3-}$, and metal citrates), or in association with colloidal material. Common inorganic ligands that can complex metals include SO_4^{2-}, Cl^{-}, OH^{-}, PO_4^{3-}, NO_3^{-}, and CO_3^{2-}. Organic complexing agents include humic and fulvic acids, aromatic and aliphatic compounds, and carboxylic acids. Acidic conditions may increase metal availability, although H^{+} may successfully compete with and reduce or prevent binding and transport. Environmental pH also affects metal complexation with organic components and inorganic anions (e.g., Cl^{-}). With increasing pH, there may be formation of hydroxides, oxides, and carbonates of varying solubility and toxicity. Some hydroxylated species may associate more efficiently with microbial cells than the corresponding metal cations. The oxidation state of several metals also determines solubility, for example, Cr(VI) being soluble and toxic and Cr(III) being immobile and less toxic. Such reductive transformations may be mediated by microbes, with accompanying consequences for survival. Colloidal materials of significance in affecting metal bioavailability and transport include iron and manganese oxides, clay minerals, and organic matter. Metals can precipitate as solid phases, for example, $CdCO_3$, $Pb(OH)_2$, ZnS, CuS, as well as mixed compounds. Toxic metals may also substitute for other metals in indigenous minerals, for example, Cd and Sr may substitute for Ca in $CaCO_3$. In addition, toxic metals may sorb onto preexisting minerals. A reduction in toxicity in the presence of elevated concentrations of anions such as Cl^{-}, CO_3^{2-}, S^{2-}, and PO_4^{3-} is frequently observed. Mono-, di-, and multivalent cations may affect heavy metal toxicity by competing with binding and transport sites. Other synthetic and naturally produced soluble and particulate organic substances, including microbial metabolites, may influence toxicity by binding and complexation. Anionic contaminants, such as arsenic, selenium, and chromium oxyanions like AsO_4^{3-}, AsO_2^{-}, SeO_4^{2-}, SeO_3^{2-}, and CrO_4^{2-}, can sorb to positive charges on insoluble organic matter and iron, manganese, aluminum oxides, and carbonates. The removal of heavy metal species by intact living and dead microbial biomass, by physico-chemical and/or biochemical interactions, may also be significant in some locations. In more general terms, microbial growth and activity is influenced by environmental parameters, including the availability of organic and inorganic nutrients, and this can clearly affect the responses to potentially toxic metals.

Mechanisms of Microbial Heavy Metal Detoxification

Extracellular metal complexation, precipitation, and crystallization can result in detoxification. Polysaccharides, organic acids, pigments, proteins, and other metabolites can remove metal ions from solution and/or convert them into less toxic species. Iron-chelating siderophores may chelate other metals and radionuclides and possibly reduce their toxic effects. Oxalate excretion by fungi can result in precipitation of insoluble metal oxalates, e.g., Ca, Cu, Ni, Mn, Mg, Zn, Pb, Cd and La. The production of H_2S by microorganisms, for example, by *Desulfovibrio* sp., results in the formation of insoluble metal sulfides and also disproportionation of organometallics to volatile products as well as insoluble sulfides, for example:

$$2CH_3Hg^{+} + H_2S \rightarrow (CH_3)_2Hg + HgS$$

$$2(CH_3)_3Pb^{+} + H_2S \rightarrow (CH_3)_4Pb + (CH_3)_2PbS$$

Many other examples of metal crystallization, biomineralization and precipitation are known, and mediated by processes dependent and independent of metabolism. Some of these are of great importance in biogeochemical cycles and involved, for example, in microfossil formation, iron and manganese deposition, silver and uranium biomineralization, and the formation of stable calcareous minerals.

Decreased accumulation, sometimes as a result of efflux, and impermeability may be important survival mechanisms. Impermeability may be a consequence of cell wall and/or membrane composition, lack of transport mechanism, or increased turgor pressure. Bacterial plasmids have resistance genes to many toxic metals and metalloids, for example, Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Sb^{3+}, TeO_3^{2-}, Tl^{+}, and Zn^{2+}. Related systems are frequently located on bacterial chromosomes, for example, Hg^{2+} resistance in *Bacillus* spp., Cd^{2+} efflux in *Bacillus* spp., and arsenic efflux in *Escherichia coli*. Copper tolerance genes are generally genome-located. General conclusions in bacterial metal resistance include the following: (1) plasmid-determined resistances are highly specific; (2) resistance systems have been found on plasmids in all bacterial groups tested; and (3) resistance mechanisms generally involve efflux from the cells or enzymatic detoxification. However, other less-specific interactions, for example, sorption, may contribute to the overall response. Many bacterial metal resistance mechanisms, for example, Cd, Cu, and As, depend on efflux. Efflux pumps, determined by plasmid and chromosomal systems, are either ATPases or chemiosmotic systems, with mechanisms often showing similarity in different types of bacteria. Cd^{2+} resistance may involve (1) an efflux ATPase in Gram-positive bacteria, (2) cation-H^{+} antiport in Gram-negative bacteria, and (3) intracellular metallothionein in cyanobacteria. Arsenic-resistant Gram-negative bacteria have an arsenite efflux ATPase and an arsenate reductase (which reduces arsenate [As(V)] to arsenite [As(III)]), which comprise the underlying biochemical mechanism. A Cd^{2+} efflux ATPase is widely found in Gram-positive bacteria, including species of *Bacillus*. Systems for Hg^{2+} resistance occur on plasmids from Gram-positive and Gram-negative bacteria with component genes being involved in the transport of Hg^{2+} to the detoxifying enzyme mercuric reductase, which reduces Hg^{2+} to elemental Hg^{0}. The enzyme organomercurial lyase can break the C-Hg bond in organomercurials. The large plasmids of *Alcaligenes eutrophus* (now *Cupriavidus metallidurans*) have several toxic metal resistance determinants, for example, three for Hg^{2+}, one for Cr^{6+}, and two for divalent cations, *czc* (Cd^{2+}, Zn^{2+}, and Co^{2+} resistance) and *cnr* (Co^{2+} and Ni^{2+} resistance). *Czc*

functions as a chemiosmotic divalent cation/H^+ antiporter. In *Enterococcus hirae* (previously *Streptococcus faecalis*), copper resistance is determined by two genes, *copA* and *copB*, which determine uptake and efflux P-type ATPases, respectively. Plasmid-determined Cu^{2+} resistance has been described in *Pseudomonas* sp., *Xanthomonas* sp., and *E. coli*. Chromosomal genes also affect Cu^{2+} transport and resistance by determining uptake, efflux, and intracellular Cu^{2+} binding. Bacterial arsenic resistance is plasmid-mediated in Gram-positive bacteria, and several mechanisms of plasmid-mediated tellurite resistance have been suggested, including reduction, reduced uptake, and enhanced efflux, although, as with Ag^+, resistance does not appear to depend on reduction to the elemental form (Te^0).

As with bacteria, intracellular metal concentrations in fungi may be regulated by transport, including efflux mechanisms. Such mechanisms are involved in normal metal homeostasis but also have a role in the detoxification of potentially toxic metals. Reduced heavy metal uptake has been observed in many tolerant microbes, including bacteria, algae, and fungi, although this is dependent on environmental factors, including pH and ion competition. However, some resistant strains may accumulate more metal than sensitive parental strains because of more efficient internal detoxification. Inside the cells, metal ions may be detoxified by chemical components, which include metal-binding proteins, or compartmentalized into specific organelles. Metal-sequestering organic and inorganic molecules, for example, polyphosphate, have been implicated in several microbial groups, whereas metal-binding peptides and proteins, including metallothioneins and γ-Glu-Cys peptides (phytochelatins, cadystins), have been detected in all microbial groups examined. Metallothioneins are small, cysteine-rich polypeptides that can bind essential metals (e.g., Cu and Zn), in addition to non-essential metals (e.g., Cd). Metal-binding γ-glutamyl-cysteinyl peptides are short peptides of general formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$. Peptides of $n = 2$–7 are most common, and these are important detoxification mechanisms in algae as well as several fungi and yeasts. In *Schizosaccharomyces pombe*, the value of *n* ranges from 2 to 5, whereas in *Saccharomyces cerevisiae*, only an *n*2 isopeptide has been observed. Although $(\gamma EC)_nG$ can be induced by a wide variety of metal ions, including Ag, Au, Hg, Ni, Pb, Sn, and Zn, metal binding has only been shown for a few, primarily Cd and Cu. For Cd, two types of complexes exist in *S. pombe* and *Candida glabrata*. A low-molecular-weight complex consists of $(\gamma EC)_nG$ and Cd, whereas a high-molecular-weight complex also contains acid-labile sulfide. The $(\gamma EC)_nG\text{-Cd-}S^{2-}$ complex has greater stability and higher Cd-binding capacity than a low-molecular-weight complex, and consists of a CdS crystallite core and an outer layer of $(\gamma EC)_nG$ peptides. The higher binding capacity of sulfide-containing complex confers tolerance to Cd. In *S. pombe*, evidence has also been presented for vacuolar localization of $(\gamma EC)_nG\text{-Cd-}S^{2-}$ complexes. The main function of *S. cerevisiae* metallothionein (yeast MT) is cellular copper homeostasis. However, induction and synthesis of MT as well as amplification of MT genes leads to enhanced copper resistance in *S. cerevisiae*. The fungal vacuole also has an important role in the regulation of cytosolic metal ion concentrations and the detoxification of potentially toxic metal ions. Metals preferentially sequestered by the vacuole include Mn^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, Ni^{2+}, and the monovalent cations K^+, Li^+, and Cs^+. The absence of a vacuole or a functional vacuolar H^+-ATPase in *S. cerevisiae* is associated with increased sensitivity and largely decreased capacity of the cells to accumulate Zn, Mn, Co, and Ni, metals known to be mainly localized in the vacuole.

Chemical transformations of metal and metalloid species by microorganisms may also constitute detoxification mechanisms, for example, bacterial Hg^{2+} reduction to Hg^0. However, plasmid-determined chromate resistance appears unconnected with chromate [Cr(Vl)] reduction to Cr(III), resistance depending on reduced CrO_4^{2-} uptake. Similarly, plasmid-mediated Ag^+ resistance appears not to involve Ag^+ reduction to Ag^0. In addition to these, other examples of reduction are carried out by bacteria, algae, and fungi (e.g., Au^{3+} to Au^0). Methylated metal and metalloid species may be volatile and lost from a given environment, for example, dimethyl selenide (DMSe). Methylation of Hg^{2+}, by direct and indirect microbial action, can result in the formation of CH_3Hg^+ and $(CH_3)_2Hg$. Arsenic methylation can be mediated by many organisms with compounds having the general structure $(CH_3)_nAsH_{3-n}$, and mono-, di-, and trimethylarsine ($n = 1$–3, respectively) being major volatile compounds. The reduction of arsenic oxyanions by reductase enzymes is also frequent and a determinant of As resistance. However, there appears no involvement of such reductases in biomethylation.

Organometallic compounds may be detoxified by sequential removal of alkyl or aryl groups. Organomercurials can be degraded by organomercurial lyase, whereas organotin detoxification involves sequential removal of organic groups from the tin atom:

$$R_4Sn \rightarrow R_3SnX \rightarrow R_2SnX_2 \rightarrow RSnX_3 \rightarrow SnX_4$$

It should be stressed that abiotic mechanisms of metal methylation and organometal(loid) degradation also contribute to their transformation and redistribution in aquatic, terrestrial, and aerial environments. The relative importance of biotic and abiotic mechanisms is usually difficult to establish.

Biotechnological Aspects of Heavy Metal Pollution

Microbial Processes for Metal Removal and Biorecovery

Certain microbial processes can solubilize metals, thereby increasing their bioavailability and potential toxicity, whereas others immobilize them and thus reduce their bioavailability (Fig. 1). The relative balance between mobilization and immobilization varies depending on the organisms and their environment. As well as being an integral component of biogeochemical cycles for metals, these processes may be exploited for the treatment of contaminated solid and liquid wastes. Metal mobilization can be achieved by autotrophic and heterotrophic leaching, chelation by microbial metabolites and siderophores, and methylation, which can result in volatilization. Similarly, immobilization can result from sorption to cell components or exopolymers, transport and

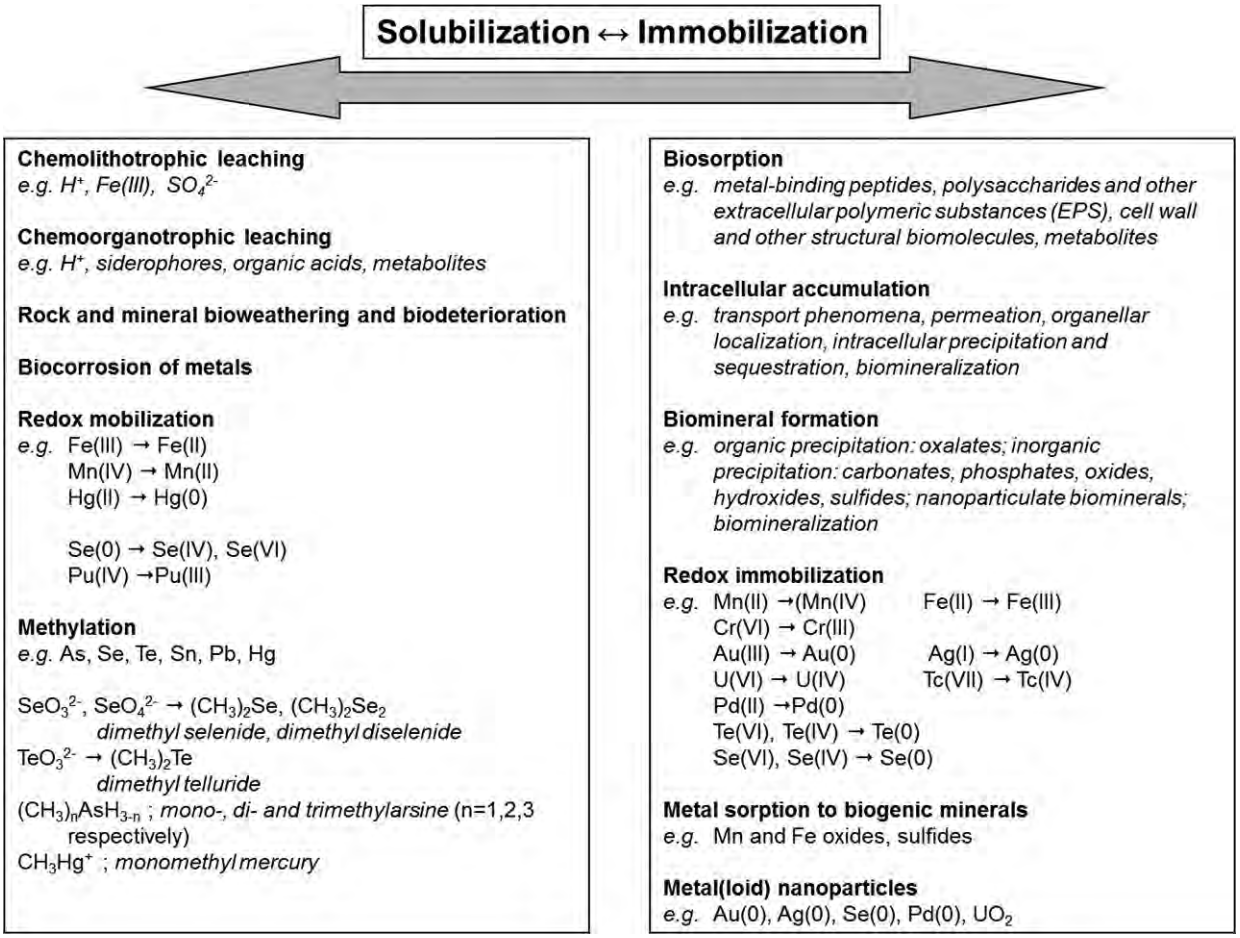

Fig. 1 Diagram depicting the major mechanisms of microbial metal transformation between soluble and insoluble metal species. The relative balance between such processes will depend on the environment and associated physico-chemical conditions, the microorganism(s) involved as well as relationships with plants, animals, and anthropogenic activities. Chemical equilibrium between soluble and insoluble phases is influenced by abiotic components, including dead biota and their decomposition products, as well as other physico-chemical components of the environmental matrix, for example, pH, water, inorganic and organic ions, molecules, compounds, colloids, and particulates. Solubilization can occur by chemolithotrophic (autotrophic) and chemoorganotrophic (heterotrophic) leaching; siderophores and other complexing agents; redox reactions; methylation and demethylation; biodegradation of organoradionuclide complexes. Immobilization can occur by biosorption to cell walls, exopolymers, other structural components, and derived/excreted products; precipitation can be a result of metabolite release, for example, sulfide, oxalate, or reduction; transport, accumulation, intracellular deposition, localization and sequestration; adsorption and entrapment of colloids and particulates. The overall scheme is also affected by reciprocal interactions between biotic and abiotic components of the ecosystem, such as abiotic influence on microbial diversity, numbers and metabolic activity; ingestion of particulates and colloids (including bacteria) by phagotrophs; biotic modification of physico-chemical parameters including redox potential, pH, O_2, CO_2, other gases and metabolites, temperature, and nutrient depletion.

intracellular sequestration, or precipitation as insoluble organic and inorganic compounds, for example, oxalates, carbonates, sulfides, or phosphates. In addition, microbiologically mediated reduction of higher valency species may effect either mobilization, for example, Mn(IV) to Mn(II), or immobilization, for example, Cr(VI) to Cr(III), and U(VI) to U(IV). In the context of bioremediation, solubilization of metal contaminants provides a means for removal of metals from solid matrices such as soils, sediments, and industrial wastes. Alternatively, immobilization processes enable metals to be transformed in situ and in bioreactors into insoluble, chemically inert forms, as well as providing a means of metal biorecovery. The latter is especially important for valuable metals that are of great industrial and economic significance but where their security of supply is inhibited because of, e.g., natural shortages, inadequate recycling, inefficient extraction methods and geopolitical factors. Biotechnological development of microbial systems may provide an alternative or adjunct to conventional physico-chemical treatment methods for contaminated effluents, leachates and wastewaters. Growing evidence suggests that some microbe-related processes are economically competitive with existing treatments in mining and metallurgy.

Metal Solubilization

Chemolithotrophic (autotrophic) leaching

Most chemolithotrophic metal leaching is carried out by chemolithotrophic, acidophilic bacteria, which obtain energy from oxidation of Fe(II) or reduced sulfur compounds and solubilize metals because of the resulting production of Fe(III) and H_2SO_4.

The microorganisms involved include sulfur-oxidizing bacteria (e.g., *Acidithiobacillus thiooxidans*), iron- and sulfur-oxidizing bacteria (e.g., *Acidithiobacillus ferrooxidans*), and iron-oxidizing bacteria (e.g., *Leptospirillum ferrooxidans*). As a result of sulfur and iron oxidation, metal sulfides are solubilized concomitant with the pH of their immediate environment being decreased, therefore resulting in solubilization of other metal compounds, including metals sorbed to soil and mineral constituents. Chemolithotrophic leaching of metal sulfides is well established for industrial-scale biomining processes, but it has also been used to solubilize metals from sewage sludge as well as remediate other metal-contaminated solid materials, including soil and red mud, the main waste product of Al extraction from bauxite. One two-stage soil treatment process used a mixture of sulfur-oxidizing bacteria to acidify metal-contaminated soil before treatment of the metal-loaded leachate with sulfate-reducing bacteria (SRB).

Chemoorganotrophic (heterotrophic) leaching

Many chemoorganotrophic (heterotrophic) fungi (and bacteria) can leach metals from industrial wastes, low-grade ores, and metal-bearing minerals. This occurs as a result of proton efflux, siderophores (for Fe(III)), and organic acids, for example, citric and oxalic. Organic acids provide a source of protons and a metal-complexing anion, for example, citrate, oxalate, with complexation being dependent on the metal/anion concentrations, pH, and metal complex stability constants. Organisms such as *Aspergillus niger* and *Penicillium simplicissimum* have been used to leach Zn, Cu, Ni, and Co from a variety of solid materials, including industrial filter dust, copper converter slag, lateritic ores, red mud, manganiferous minerals, and municipal waste fly ash. Citrate and oxalate can form stable complexes with a large number of metals. Many metal citrates are highly mobile and not readily degraded. Oxalic acid can act as a leaching agent for metals that form soluble oxalate complexes, including Al and Fe.

Siderophores are highly specific Fe(III) ligands (formation constant often $>10^{30}$) that are excreted by microorganisms to aid iron assimilation. Such assimilation may be improved by attachment to solid Fe oxides in soil. Although primarily produced as a means of obtaining iron, siderophores are also able to bind other metals such as magnesium, manganese, chromium (III), gallium (III), and radionuclides such as plutonium (IV).

Metal Immobilization

Biosorption

Biosorption (defined here as the microbial uptake of organic and inorganic metal, metalloid, and radionuclide species, both soluble and insoluble, by physico-chemical mechanisms) may be influenced by metabolic activity (in living cells), and may also provide nucleation sites for the formation of stable minerals, including phosphates, sulfides, and oxides. Crystallization of elemental gold and silver may occur as a result of reduction, whereas the formation of hydrolysis products can enhance precipitation of U and Th. All biological macromolecules have an affinity for metal species with cell walls and associated materials being of the greatest significance in biosorption (Table 1). Moreover, mobile cationic species can be accumulated by cells via transport systems of varying affinity and specificity, and internally bound, transformed, precipitated, localized within organelles, or translocated to specific structures depending on the metal concerned and the organism.

Biosorption by cell walls and associated components

In bacteria, peptidoglycan carboxyl groups are the main cationic binding sites in Gram-positive species, with phosphate groups contributing significantly in Gram-negative species. Chitin, phenolic polymers, and melanin are important structural components of fungal walls, and these are also effective biosorbents for metals and radionuclides. Fungi can be efficient sorbents of metal ions over a wide range of pH values, and although they may take up less metal per unit dry weight than clay minerals (the most important metal-sorbing component in soil), they are more efficient sorbents per unit surface area. Variations in the chemical behavior of metal species as well as the composition of microbial cell walls and extracellular materials can result in wide differences in biosorptive capacities (Table 1). Extracellular polymeric substances (EPS), a mixture of polysaccharides, mucopolysaccharides, and proteins, can bind significant amounts of potentially toxic metals and entrap precipitated metal sulfides and oxides. One process uses floating mats of cyanobacteria, the metal-binding process being due to large polysaccharides (>200 000 Da).

The ability of surface-associated macromolecules to effect the immobilization of aqueous metal(loid) species may be of great importance, particularly where organisms grow as surface-attached biofilms, enmeshed in a matrix of EPS. The biofilm mode of growth is now widely accepted to be the predominant form in which natural microbial populations occur, and it appears that natural mixed-species biofilms can act as sinks for precipitated minerals, including potentially toxic metals, in aqueous environments. The biofilm–EPS matrix can act as a direct adsorbent of dissolved metal ions, with the ionic state and charge density of EPS components determining the ionic binding and electrostatic immobilization properties. Bacterial EPS are dominated by polysaccharides, but secreted polymers also include proteins, nucleic acids, peptidoglycan, lipids, and phospholipids. This heterogeneous matrix generally has a net negative charge, with polyanionic moieties acting as an ion-exchange matrix for metal cations. Well-characterized examples include the propensity of uronic acid-containing polysaccharides to bind with carboxyl groups and thus bind metals, whereas neutral carbohydrates can bind metals by the formation of weak electrostatic bonds around the hydroxyl groups. Cross-linking of extracellular polysaccharides by metal ions themselves may alter the mechanical and chemical properties of EPS.

The biofilm growth mode appears to further enhance metal removal in various ways. Biosorption and bioprecipitation can be interrelated phenomena, such that ionic concentration by sorption at low-energy cellular, or EPS surface sites within biofilms can

Table 1 Examples of microbial metal and actinide accumulation to industrially significant levels. Data is derived from a number of sources and presented without reference to important experimental conditions, for example, metal and biomass concentration, pH, and whether freely suspended, living, dead or immobilized, or the mechanism of accumulation. In most cases, highest uptake levels are due to general biosorptive mechanisms

Microorganism		*Metal*	*Accumulation (% dry weight)*
Bacteria			
	Streptomyces sp.	Uranium	2–14
	Streptomyces viridochromogenes	Uranium	30
	Acidithiobacillus ferrooxidans	Silver	25
	Bacillus cereus	Cadmium	4–9
	Zoogloea sp.	Cobalt	25
		Copper	34
		Nickel	13
	Citrobacter sp.[a]	Lead	34–40
		Cadmium	170
		Uranium	900
	Pseudomonas aeruginosa	Uranium	15
	Mixed culture	Silver	32
Cyanobacteria			
	Anabaena cylindrica	Cadmium	0.25
	Anacystis nidulans	Nickel	1
	Spirulina platensis	Gold	0.52
	Plectonema boryanum	Zirconium	0.16
	Nostoc sp.	Cadmium	1
Algae			
	Chlorella vulgaris	Gold	10
		Lead	8.5
	Chlorella regularis	Uranium	15
		Zinc	2.8
		Manganese	0.8
	Scenedesmus sp.	Molybdenum	2.3
	Euglena sp.	Aluminum	1.5
	Sargassum natans[b]	Gold	25
		Lead	8
		Silver	7
		Uranium	4.5
		Copper	2.5
		Zinc	2
		Cobalt	6
		Cadmium	8.3
	Ascophyllum nodosum[b]	Gold	4
		Cobalt	15
		Cadmium	10
Fungi			
	Phoma sp.	Silver	2
	Penicillium sp.	Uranium	8–17
	Rhizopus arrhizus	Lead	0.6
		Cadmium	3
		Lead	10
		Uranium	20
		Thorium	19
		Silver	5
		Mercury	6
	Aspergillus niger	Thorium	19
		Uranium	22
		Gold	6–18
		Zinc	1–10
		Silver	10
	Saccharomyces cerevisiae	Thorium	12
		Uranium	10–15
		Cadmium	7
		Copper	1–3
	Ganoderma lucidum	Copper	1
	Mucor miehei	Zinc	3.4
		Uranium	18

[a]Phosphatase-mediated metal removal.
[b]Macroalgae (seaweeds).

initiate mineral formation and immobilization within biofilms. Mineral precipitates formed in the bulk solution may also be physically entrapped or chemically adsorbed by the biofilm EPS matrix.

Biosorption by free and immobilized biomass

Both freely suspended and immobilized biomass from bacterial, cyanobacterial, algal, and fungal species have received attention with immobilized systems appearing to possess several advantages over 'free' biomass, including higher mechanical strength and easier biomass/liquid separation. Living or dead biomass of all groups has been immobilized by encapsulation or cross-linking using supports, which include agar, cellulose, alginates, cross-linked ethyl acrylate-ethylene glycol dimethylacrylate, polyacrylamide, silica gel, and cross-linking reagents, such as toluene diisocyanate and glutaraldehyde. Immobilized living biomass has mainly taken the form of bacterial biofilms (see "Biosorption by cell walls and associated components") on inert supports, and has been used in a variety of configurations, including rotating biological contactors, fixed-bed reactors, trickle filters, fluidized beds, and airlift bioreactors.

Metal desorption

Biotechnological exploitation of biosorption may depend on the ease of biosorbent regeneration for metal recovery. Metabolism-independent processes are frequently reversible by nondestructive methods, and hence can be considered analogous to conventional ion exchange. Most work has concentrated on nondestructive desorption, which should be efficient, cheap, and result in minimal damage to the biosorbent. Dilute mineral acids (~0.1 M) can be effective for metal removal, although more concentrated acids or lengthy exposure times may result in biomass damage. It may be possible to apply selective desorption of metalloid species from a loaded biosorbent using an appropriate elution scheme. For example, metal cations (e.g., Cu^{2+}, Cr^{3+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, and Co^{2+}) were released from algal biomass using eluant at pH 2, whereas at higher pH values, anionic metal species (e.g., SeO_4^{2-}, CrO_42, and MoO_4^{2-}) were removed. Au^{3+}, Ag^{+}, and Hg^{2+}, however, remained strongly bound at pH 2, and these were removed by the addition of ligands that formed stable complexes with these metal ions. Carbonates and/or bicarbonates are efficient desorption agents with potential for cheap, non-destructive metal recovery. Operating pH values for bicarbonates cause little damage to the biomass, which may retain at least 90% of the original uptake capacity.

Metal-binding proteins, polysaccharides, and other biomolecules

A diverse range of specific and nonspecific metal-binding compounds are produced by microorganisms. Non-specific metal-binding compounds are metabolites or by-products of microbial metabolism and range from simple organic acids and alcohols to macromolecules, such as polysaccharides, humic and fulvic acids. Specific metal-binding compounds may be produced in response to external levels of metals. Siderophores are low-molecular-weight Fe(III) coordination compounds (500–1000 Da) excreted under iron-limiting conditions by iron-dependent microorganisms. Although specific to Fe(III), siderophores can also complex Pu(IV), Ga(III), Cr(III), scandium (Sc), indium (In), nickel, uranium, and thorium. Specific, low-molecular-weight (6000–10 000 Da) metal-binding metallothioneins are produced by animals, plants, and microorganisms in response to the presence of toxic metals. Metal-binding γ-Glu-Cys peptides (phytochelatins and cadystins) contain glutamic acid and cysteine at the N-terminal position, and have been identified in plants, algae, and several microorganisms. The metal-binding abilities of siderophores, metallothioneins, phytochelatins, and other similar molecules may have potential for bioremediation of waters containing low metal concentrations, although few examples have been rigorously tested.

Transport and accumulation

Microbial metal transport systems are of varying specificity, and essential and non-essential metal(loid) species may be accumulated. The rates of uptake can depend on the physiological state of cells as well as the nature of environment or growth medium. Integral to the transport of metal ions into cells are transmembrane electrochemical gradients, for example of H^{+}, resulting from the operation of enzymatic pumps (ATPases) that transform the chemical energy of ATP into this form of biological energy. ATPases are also involved in ion efflux in a variety of organisms and organellar ion compartmentation in eukaryotes via operation across vacuolar membranes. Metals may also enter (and leave) cells via pores or channels. With toxic heavy metals, permeabilization of cell membranes can result in exposure of intracellular metal-binding sites and increase passive accumulation. Intracellular uptake may result in death of sensitive organisms, unless a means of detoxification is possessed or induced (Fig. 2). Other mechanisms of microbial metal accumulation include iron-binding siderophores and co-transport of metals with organic substrates.

Metal precipitation and biomineralization

Precipitation by redox processes: Metal-reducing bacteria and iron oxidizers

A diverse range of microorganisms can use oxidized metallic species, for example, Fe(III), Cr(VI), or Mn(IV), as terminal electron acceptors. Many use more than one metal or anion, such as nitrate or sulfate. Fe(III) and Mn(IV) appear to be the most commonly utilized metals as terminal electron acceptors in the biosphere. However, since the solubility of both Fe and Mn is increased by reduction, other metals have been targeted in waste treatment, for example, molybdenum(VI) and Cr(VI). The reduction of, for example, Cr(VI) to Cr(III), by organisms including *Enterobacter cloacae* and *E. coli*, may facilitate removal by biosorption or (bio) precipitation. One potential application of dissimilatory biological metal reduction is uranium precipitation by reduction of soluble U(VI) compounds to U(IV) compounds, such as the hydroxide or carbonate, which have low solubility at neutral pH. Strains of *Shewanella* (*Alteromonas*) *putrefaciens* and *Desulfovibrio* sp. can produce a very pure precipitate of U(IV) carbonate. Such

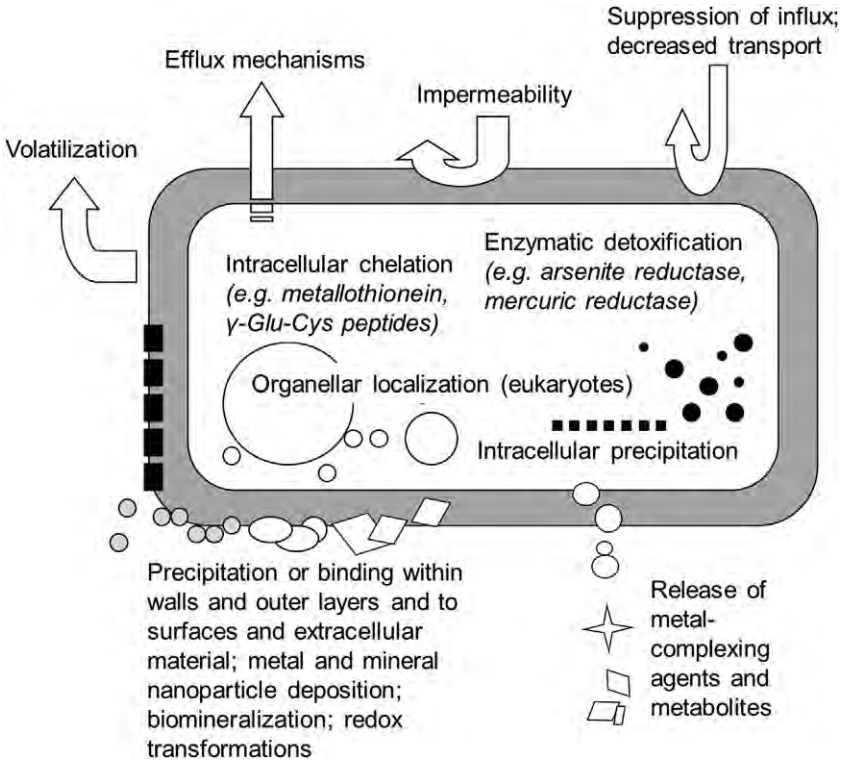

Fig. 2 Mechanisms involved in the detoxification of metals including mechanisms that restrict entry into the cell and intracellular detoxification or organellar compartmentation, the latter occurring in some eukaryotes, for example, fungi. Operation of several mechanisms is possible depending on the organism and the cellular environment, some dependent and/or independent of metabolism. A variety of mechanisms may be involved in transport phenomenon contributing to decreased uptake and/or efflux. A variety of specific or nonspecific mechanisms may also affect redox transformations, intracellular chelation, and intracellular precipitation.

bacterial uranium reduction can also be combined with chemical extraction methods. The solubility of some other radionuclides, for example, Ra and Pu, may be increased by reduction, which may favor removal from, for example, contaminated soil.

Bacterial Fe oxidation is ubiquitous in environments with sufficient Fe^{2+} and conditions to support bacterial growth such as drainage waters and tailing piles in mined areas, pyritic and hydric soils (bogs and sediments), drain pipes and irrigation ditches, and plant rhizospheres. Iron oxidizers commonly found in acidic soil environments are acidophilic chemolithotrophs, such as *A. ferrooxidans*, significant for their role in generating acid mine drainage. Fungi, too, oxidize metals in their environment. Desert varnish is an oxidized metal layer (patina), of few millimeter thickness, found on rocks and in soils of arid and semi-arid regions, and is believed to be of fungal and bacterial origin.

Sulfate-reducing bacteria

SRB are strictly anaerobic heterotrophic bacteria found in environments where carbon substrates and sulfate are available. These utilize an energy metabolism in which the oxidation of organic compounds or hydrogen is coupled to the reduction of sulfate as the terminal electron acceptor, producing sulfide that often reaches significant concentrations in sediments or bioreactors:

$$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O$$

Sulfur in S(VI) oxidation state is stoichiometrically reduced to S(-II) and, under circumneutral conditions in which SRB are generally encountered, the main product is bisulfide (HS^-), with a small proportion of volatile H_2S. Bisulfide is a highly reactive species, with the propensity to bind with metal cations in solution-forming metal sulfide solids. This is the main mechanism whereby SRB remove toxic metals from solution, for example,

$$M^{2+} + SO_4^{2-} + 2CH_3CH_2OH \rightarrow 2CH_3COOH + 2H_2O + MS\downarrow$$

$$M^{2+} + SO_4^{2-} + 2CH_3CHOHCOOH \rightarrow 2CH_3COOH + 2CO_2 + 2H_2O + MS\downarrow$$

The solubility products of most heavy metal sulfides are very low, in the range of 4.65×10^{-14} (Mn) to 6.44×10^{-53} (Hg), so that even a moderate output of sulfide can remove metals to safer levels permitted in the environment. SRB can create extremely reducing conditions, which can chemically reduce metals such as uranium(VI). In addition, sulfate reduction partially eliminates

acidity from the system, which can result in further precipitation of metals, for example, Cu, Al, as hydroxides as well as increasing the efficiency of sulfide precipitation.

Secondary metal(loid) removal by adsorption on SRB-produced metal sulfides deposited within, or immobilized by, the biofilm matrix can also contribute to overall removal. SRB-generated metal sulfides can adsorb a range of cations and anions. Fe(II) sulfides can bind a range of metals, allowing the level of metals in solution to be reduced from original concentrations in the order of mg l^{-1} to μg l^{-1}.

Processes utilizing metal sulfide precipitation

Sulfate reduction can provide both in situ and ex situ metal removal from acid mine drainage and, together with other mechanisms such as biosorption, it contributes to the removal of metals and acidity in artificial and natural wetlands. Large-scale bioreactors have also been developed using bacterial sulfate reduction for treating metal-contaminated waters. The best known commercial application involving metal sulfide precipitation is the THIOPAQ technology, developed and marketed by Paques Bio Systems B.V., Balk, the Netherlands (See Relevant Website section), and first applied in 1992 for the treatment of contaminated groundwater at the Budelco zinc refinery in the Netherlands. The basic THIOPAQ system consisted of a two-stage biological process in series: anaerobic sulfate reduction to sulfide followed by aerobic sulfide oxidation to elemental sulfur. Since the solubilities of most metal sulfides are much lower than those of their hydroxides, an advantage of the THIOPAQ system is that considerably lower effluent metal concentrations can be achieved than in the neutralization processes, which immobilize metals by hydroxide precipitation. In addition, the metal sulfide precipitate formed may be reprocessed in a smelter or a refinery.

Electron donors suitable for small-scale THIOPAQ installations were ethanol, various fatty acids, and organic waste streams. For large-scale applications, where more than 2.5 tons of hydrogen sulfide are produced per day, hydrogen gas is preferentially used as a reductant. Hydrogen gas can be produced on-site by cracking methanol or by steam-reforming natural gas or LPG. The main reaction that occurs in a reactor operated with H_2 is:

$$H_2SO_4 + 4H_2 \rightarrow H_2S + 4H_2O$$

Hydrogen sulfide in the reactor gas (3%–15%, v/v) can be employed for metal precipitation. Compared to the addition of a NaHS or Na_2S solution, an advantage with the use of H_2S is that sodium is not introduced into the system. It is also possible that careful control of the pH and the redox potential of the process liquid may allow selective recovery of metals. Thus, sulfide precipitation makes it possible to separate copper from zinc, arsenic from copper, iron from nickel, and so on, in multiple reaction stages at different pH values. Alternatively, metals may also be precipitated as sulfides inside the anaerobic bioreactor.

At the Budelco zinc refinery in Budel-Dorplein, the Netherlands, a THIOPAQ system capable of processing approximately 300 m^3 h^{-1} of polluted groundwater has been in operation since 1992. Its products, a metal sulfide sludge (mainly ZnS) and sulfur slurry, are fed back to the roasters in the refinery. The capacity of the installation was increased to 400 m^3 h^{-1} (in 1998), with the feed also including a mixture of groundwater and process water. Another THIOPAQ installation (called Budelco II, in operation since 1999) treated several bleed streams and process water: sulfate reduction occurs in a 500 m^3 bioreactor where hydrogen is used as the electron donor.

Another process, integrating bacterial sulfate reduction with bioleaching by sulfur-oxidizing bacteria, was developed to remove contaminating toxic metals from soils. In this process, sulfur- and iron-oxidizing bacteria were employed to release metals from soils by the breakdown of sulfide minerals and production of sulfuric acid, which liberates acid-labile forms such as hydroxides, carbonates, or sorbed metals. Metals are liberated in the form of an acid sulfate solution, which enables both the large proportion of acidity and almost the entirety of metals to be removed by bacterial sulfate reduction. Precipitation efficiency is further increased by the addition of flocculating agents.

In confined systems, SRB can also bring about significant increases in bulk pH, which can enhance sulfide precipitation and lead to precipitation of hydroxides and carbonates of transition metals. When an organic substrate acts as the electron donor, bicarbonate is also generated:

$$2CH_3CHOHCOO^- + {SO_4}^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + HS^- + H^+$$

This has useful implications for the use of SRB in the remediation of acidic metal-processing waters and mine wastes, particularly where they are active within suspended or surface-attached mesophilic biofilms. SRB are reported to contribute significantly to metal removal in constructed wetlands as well as in alkalization of acidic mine wastes. Both sulfide generation and pH-related precipitation appear to be important. However, some studies have questioned the contribution of SRB in these broad-scale systems, arguing that Fe(III)-reducing bacteria make a greater contribution where carbon is limiting, in terms of both metal removal and ameliorating low pH.

There is also evidence that extremely reducing conditions that develop during sulfate reduction can lead to chemical conversion of oxyanions into cationic species, which can be more easily precipitated or biosorbed. The indirect chemical reduction of Cr(VI), as soluble chromate (CrO_4^{2-}), to much less soluble Cr(III) cationic species by sulfide and/or Fe^{2+} in SRB culture appears to be at least partially responsible for the removal of chromate from solution by SRB.

Phosphatase-mediated metal phosphate precipitation

In this process, metal or radionuclide accumulation by bacterial (e.g., *Citrobacter* sp.) biomass is mediated by a phosphatase enzyme induced during metal-free growth, which liberates inorganic phosphate from a supplied organic phosphate donor molecule, for example, glycerol 2-phosphate. Metal/radionuclide cations are then precipitated as phosphates on the biomass to high levels. In addition, metal precipitation by secreted phosphate generated from polyphosphate hydrolysis has also been suggested as a mechanism to remove metals and actinides from aqueous waste streams. Fungi are also capable of mediating metal precipitation as phosphates, e.g., U, Pb, from organic or inorganic phosphate sources.

High-gradient magnetic separation

Metal ion removal from solution has been achieved using bacteria rendered susceptible to magnetic fields. 'Non-magnetic' bacteria can be made magnetic by the precipitation of metal phosphates (aerobic) or sulfides (anaerobic) on their surfaces, as described previously. For those organisms producing iron sulfide, it has been found that this compound is not only magnetic but also an effective adsorbent for metallic elements.

Biomineralization

Apart from sulfides, microbes can also mediate formation of several inorganic and organic biominerals, e.g., oxalates, phosphates, oxides and carbonates, which lead to metal immobilization. Biomineralization refers to the collective processes by which organisms form minerals. Mineral synthesis can be categorized into biologically-induced mineralization (BIM) and biologically-controlled mineralization (BCM). Biologically-induced mineralization is where an organism modifies its local microenvironment creating conditions such that there is extracellular chemical precipitation of mineral phases. The organism does not appear to control the biomineralization process in BIM while a great degree of control over biomineralization is exerted by the organism in BCM, e.g., magnetosome formation in magnetotactic bacteria, and complex cellular biomineral structures in certain eukaryotes. Biomineralization provides other potential means of metal biorecovery, detoxification or bioremediation.

Most biomineralization examples of relevance to environmental biotechnology relate to biologically-induced mineralization. This can result from microbial oxidation or reduction of a metal species (as mentioned above), metabolite excretion, e.g., sulfide, oxalate, and other metabolism-dependent transformations of metal species, with microbial surfaces providing chemically-reactive sites for sorption ($\equiv$ biosorption) which can also lead to the nucleation and formation of mineral precipitates. Microbial cell surfaces can bind metal ions, inorganic anions, and even preformed nanominerals leading to fine-grained mineral phases on cell surfaces.

Calcium oxalate is the most common form of oxalate encountered in the environment, mostly occurring as the dihydrate (weddellite) or the more stable monohydrate (whewellite). Fungi can also produce other metal oxalates on interacting with a variety of different metals and metal-bearing minerals, e.g., Ca, Cd, Co, Cu, Mg, Mn, Sr, Zn, Ni and Pb. Many bacterial species can oxidize manganese which is then deposited on cells, sheaths, or spores as oxides. Some promote oxidation non-enzymatically, others enzymatically with possible involvement of a multicopper oxidase system, and include spore-forming and non-spore-forming rods, sheathed and appendaged bacteria as well as the usual morphological forms of Gram-positive and -negative bacteria from a diverse range of freshwater, marine and terrestrial ecosystems. Several fungi can also promote Mn(II) oxidation to $Mn(IV)O_2$ including *Acremonium* spp. In many cases, fungal oxidation is probably non-enzymatic and due to interaction with a metabolic product (e.g., a hydroxy acid) or a cellular component although the involvement of laccase and/or multicopper oxidases have been shown in ascomycetes. Mn oxide phases have high sorption capacities for numerous metal cations (e.g., Ni, Zn, Cu, Co, Mn, Pb, and Cd), and also serve as strong oxidants for inorganic [e.g., As(III) to As(V); Cr(III) to Cr(IV)] and organic compounds such as humic substances. Microbes can play a role in the formation of phosphate minerals such as vivianite ($Fe_3(PO_4)_2{\cdot}8H_2O$), strengite ($FePO_4{\cdot}2H_2O$), and variscite ($AlPO_4{\cdot}2H_2O$). Secondary mycogenic uranium mineral precipitates on fungal mycelia growing in the presence of uranium oxides or depleted uranium were found to be uranyl phosphate minerals of the meta-autunite group, uramphite and/or chernikovite.

Metal, Metalloid, and Organometal Transformations

Microorganisms can transform certain metal, metalloid, and organometallic species by oxidation, reduction, methylation, or dealkylation. Biomethylated derivatives are often volatile and may be eliminated from a system by evaporation. The two major metalloid transformation processes described are reduction of metalloid oxyanions to elemental forms and methylation.

Microbial Reduction and Oxidation of Metalloid Oxyanions

The reduction of selenate (Se(VI)) and selenite (Se(IV)) to elemental selenium can be catalyzed by numerous microbes, which can result in a red precipitate deposited around the cells and colonies. Some bacteria use SeO_4^{2-} as a terminal e^- acceptor in dissimilatory reduction as well as reduce and incorporate Se into organic components, for example, selenoproteins (assimilatory reduction). Selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) can be reduced to Se^0, with SeO_3^{2-} reduction appearing more ubiquitous than

SeO_4^{2-} reduction. However, only SeO_4^{2-} can support bacterial growth under anaerobic conditions: SeO_4^{2-} reduction to Se^0 is a major sink for Se oxyanions in anoxic sediments. Anaerobic SRB like *Desulfovibrio desulfuricans* can reduce selenate/selenite to Se^0, but neither oxyanion could be used for respiratory growth. Reduction to Se^0 can be considered a detoxification mechanism. Reduction of TeO_3^{2-} to Te^0 is also a means of detoxification found in bacteria and fungi, with Te^0 being deposited in or around the cells, resulting in black colonies. The opposite process of Se^0 oxidation can occur in soils and sediments. It is possible that Se^0 oxidation is similar to S oxidation, and may be mediated by heterotrophs and lithotrophs. In aerobic soil slurries, Se^{4+} is the main product with lower amounts of Se^{6+} being produced; heterotrophic and autotrophic thiobacilli were believed to be the active organisms.

The extreme reducing conditions that SRB create can also result in indirect chemical reduction of metal(loid) and radionuclide species. This appears to be the case for U(VI), which is reduced chemically to U(IV) under highly reducing, sulfidic conditions. The ability of SRB to enzymatically reduce uranium is now established, and there has been some debate as to the relative contribution of chemical and enzymatic uranium reduction, with chemical reduction rates appearing relatively low. Nevertheless, while enzymatic reduction is effective in non-metabolizing cells, some studies appear to support a role for chemical reduction in the presence of growing cells.

Technetium (which, as ^{99}Tc, is a long half-life product of the nuclear fuel cycle) is present in many environments as Tc(VII) in the form of highly mobile pertechnetate ion (TcO_4^-). Chemical and enzymatic reductive precipitation of Tc has been demonstrated in SRB, in that chemical precipitation appears to be more efficient for uranium, with sulfide as a reductant. Under sulfidogenic conditions, chemical precipitation is operated in preference to enzymatic reduction, and *D. desulfuricans* was able to precipitate Tc extracellularly, probably as sulfide.

In contrast to reductive precipitation of metal(loid) described above, arsenic reduction frequently increases the solubility of this toxic element. Microbial dissimilatory reduction of As(V) to As(III) has been identified as an important route for increased As toxicity in the environment. This capacity appeared, for some time, to be phylogenetically and metabolically separate from dissimilatory sulfate reduction, but a *Desulfotomaculum* strain has the capability to simultaneously reduce arsenic and sulfate and to stimulate the precipitation of As(III) sulfide. The capacity of SRB to reduce and solubilize As and for soluble As(III) to precipitate with sulfide has further potential for bioremediation.

Methylation of Metalloids

Microbial methylation of metalloids to yield volatile derivatives, for example, dimethyl selenide, dimethyl telluride, or trimethylarsine, can be effected by a variety of bacteria, algae, and fungi. Bacteria and fungi are the most important Se-methylaters in soil, with the most frequently produced volatile being dimethyl selenide. Selenium methylation appears to involve the transfer of methyl groups as carbonium (CH_3^+) ions via the S-adenosyl methionine system. Arsenic compounds such as arsenate (As(V), AsO_4^{3-}), arsenite (As(III), AsO_2^-), and methylarsonic acid ($CH_3H_2AsO_3$), can be methylated to volatile dimethylarsine ($(CH_3)_2HAs$) or trimethylarsine ($(CH_3)_3As$). Environmental factors that affect microbial activity can markedly affect Se methylation, for example, pH, temperature, organic amendments, Se speciation; however, the addition of organic amendments can stimulate methylation. The opposite process of demethylation can also occur in soil and water systems. Anaerobic demethylation may be mediated by methylotrophic bacteria.

Microbial Metalloid Transformations and Bioremediation

In situ immobilization of SeO_4^{2-}, by reduction to Se^0, has been achieved in Se-contaminated sediments. Microbial methylation of selenium, resulting in volatilization, has also been used for in situ bioremediation of selenium-containing land and water at Kesterson Reservoir in the United States. Selenium volatilization from soil was enhanced by optimizing soil moisture, particle size, and mixing, while waters it was stimulated by the growth phase, salinity, pH, and selenium concentration. Se-contaminated agricultural drainage water was evaporated to dryness until the sediment selenium concentration approached 100 mg Se kg^{-1} dry weight. Conditions such as carbon source, moisture, temperature, and aeration were then optimized for selenium volatilization, and the process continued until selenium levels in sediments declined to acceptable levels. Some potential for ex situ treatment of selenium-contaminated waters has also been demonstrated.

Mercury and Organometals

Key microbial transformations of inorganic Hg^{2+} include reduction and methylation. The mechanism of bacterial Hg^{2+} resistance is enzymic reduction of Hg^{2+} to nontoxic volatile Hg^0 by mercuric reductase. Hg^{2+} may also arise from the action of organomercurial lyase on organomercurials. Since Hg^0 is volatile, this could provide one means of mercury removal. Methylation of inorganic Hg^{2+} leads to the formation of more toxic volatile derivatives; the bioremediation potential of this process (as for other metals and metalloids, besides selenium, capable of being methylated, e.g., As, Sn, and Pb) has not been explored in detail. In addition to organomercurials, other organometals may be degraded by microorganisms. Organoarsenicals can be demethylated by bacteria, while organotin degradation involves sequential removal of organic groups from the tin atom. In theory, such mechanisms and interaction with bioremediation possibilities described previously may provide a means of detoxification.

The Mycorrhizosphere

Nearly all land plants depend on symbiotic mycorrhizal fungi. Two main types of mycorrhizas include endomycorrhizas where the fungus colonizes the interior of host plant root cells (e.g., ericoid and arbuscular mycorrhizas) and ectomycorrhizas where the fungus is located outside plant root cells. Mycorrhizal fungi are involved in proton-promoted and ligand-promoted metal mobilization from mineral sources, metal immobilization within biomass, and extracellular precipitation of mycogenic metal oxalates. Mycorrhizal associations may also be used for metal clean-up in the general area of phytoremediation. Mycorrhizas may enhance phytoextraction directly or indirectly by increasing plant biomass, and some studies have shown increased plant accumulation of metals, especially when inoculated with mycorrhiza isolated from metalliferous environments. Mycorrhizal metal immobilization around plant roots, including biomineral formation, may assist soil remediation and revegetation. Naturally occurring soil organic compounds can stabilize potentially toxic metals like Cu, Cd, Pb, and Mn. The insoluble glycoprotein, glomalin, produced in copious amounts on hyphae of arbuscular mycorrhizal fungi can sequester metals, and could be considered a useful stabilization phenomenon in the remediation of polluted soils.

Concluding Remarks

Microorganisms play important roles in the environmental fate of toxic metals, metalloids, and radionuclides with physico-chemical and biological mechanisms effecting transformations between soluble and insoluble phases. Such mechanisms are important components of natural biogeochemical cycles for metals and associated elements, for example, sulfur and phosphorus, with some processes being of potential application to the treatment of contaminated materials. The removal of such pollutants from contaminated solutions by living or dead microbial biomass and derived or excreted products may provide a means for element recovery and environmental protection. Although the biotechnological potential of some of these processes has only been explored in the laboratory or pilot scale, some mechanisms, notably bioleaching, biosorption, and precipitation, have been employed at a commercial scale. Of these, chemolithotrophic leaching is an established major process in mineral extraction but has also been applied to the treatment of contaminated land. There have been several attempts to commercialize biosorption using microbial biomass but success has been short-lived, primarily due to competition with commercially produced ion exchange media. Bioprecipitation of metals as sulfides has achieved large-scale application, and this holds out promise for further commercial development. Exploitation of other microbiological processes will undoubtedly depend on a number of scientific, economic, and political factors. A recent driver for fuller understanding and development of microbial processes for metal biorecovery arises from the need to safeguard the security and supply of valuable elements critical to new technologies in energy, the digital economy, computing and electronics, and for production of novel nanoscale biomineral and biometal forms with useful electrochemical and catalytic properties.

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Helicobacter pylori☆

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Introduction

More than half the world's population carries *Helicobacter pylori*. This Gram-negative organism colonizes specifically the stomach. Coexistence with humans may provide the host with benefits, especially in childhood, but prolonged infection frequently leads to disease including chronic gastritis and peptic ulcer disease. *H. pylori* infection was identified as a major risk for the development of gastric non-cardiac adenocarcinomas, and MALT (mucosa-associated lymphoid tissue) lymphoma. The World Health Organization classified *H. pylori* as a type I carcinogen in 1994 due to the established connection to gastric cancer. Eradicative antibiotic therapy to cure *H. pylori* infections has become a standard treatment, yet the long-term consequences of eliminating *H. pylori* are not known. Indeed emerging evidence points to a protective role for *H. pylori* in gastroesophageal, pulmonary, and metabolic diseases. Thus *H. pylori* research now takes an unexpected turn where its potential benefits for the human host are investigated. The outcome will determine whether we need to reassess our relationship to *H. pylori* and the relative benefit of eradication.

Microbiology and Genome

Genus *Helicobacter* comprises >35 species; many of them non-culturable. *H. pylori* is the major medically relevant pathogen, but other species have been detected in human clinical samples. Non-Helicobacter pylori Helicobacter (NHPH) of human or animal origin have drawn recent attention due to their possible involvement in inflammatory bowel disease (IBD) and importance in animal health. Gastric and enterohepatic NHPH isolated from humans include *H. hepaticus*, as well as *H. cinaedi*, and *H. suis*, which pose risk for immunocompromised patients. *H. felis*, *H. bizzozeronii* and *H. salomonis* have been associated with chronic gastritis and peptic ulcers in humans. Strains of *H. hepaticus*, *H. bilis* and *H. mustalae* show carcinogenic potential in animals. Well-characterized strains of *H. felis*, *H. mustalae*, and *H. hepaticus* are finding use in gastric mouse colonization models.

H. pylori belongs to the epsilon subdivision of proteobacteria. The bacterium has been (re)named many times beginning with *Vibrio rugula*, then Campylobacter-Like Organism (CLO), *Campylobacter pylroidis*, and subsequently *C. pylori* until the new genus *Helicobacter* was established in 1989. It is a spiral shaped, highly motile bacterium with a unipolar bundle of 2–6 flagella. Production of catalase, oxidase and particularly urease enables the organism to persistently colonize the human gastric mucosa. Urease activity neutralizes gastric acidity by generating ammonium from urea. Optimal growth of *H. pylori* in vitro requires rich media supplemented with blood, serum, or cyclodextrin, 37°C and a microaerobic (5% oxygen) atmosphere. Colonies become visible after 2–10 days of incubation. Isolation and cultivation of *H. pylori* from gastric biopsies is still challenging due to their fastidious ex-vivo growth behavior. Selection medium for *H. pylori* ideally contains vancomycin, trimethoprim, cefsulodin, and amphotericin B. Incubation of biopsies with trypsin prior to plating can facilitate *H. pylori* isolation. The bacterium's morphology can change from spiral shaped (gastric biopsies) to rod-like (cultivation plates) and finally coccoidal cells, which are viable but not culturable. Alterations are detected after prolonged incubation on plates, or exposure to subinhibitory concentrations of certain antibiotics or other hostile conditions. The coccoid forms (found also in stomach biopsies) have not been linked to virulence but strong evidence indicates that these represent persister cells, able to subvert treatment, the immune system, and allow environmental passage.

The first whole genome sequence of *H. pylori* (strain 26695) was released in 1997. A 5,300-year-old *H. pylori* genome could be retrieved from human remains preserved in glacier ice and made available in 2016. In total NCBI currently lists 577 genome reports for *H. pylori*, 84 of them are finished sequences and plasmids are frequent. Genome properties are very constant with an average genome size of about 1.6 kbp, an average GC content of 39%, and approximately 1,500–1,600 predicted coding sequences per strain. Depending on the methods and strain pool analysed, the core genome is calculated with 1,063–1,300 genes and the pan-genome with 2,070, which, however is considered "open" and may extend to 5,000 genes. Data from transcriptome, methylome, and proteome analyses have provided important insights to gene regulation and virulence properties of *H. pylori*. The *H. pylori* genome is highly methylated and exhibits strain-specific variation. *H. pylori* possesses an unusually high number of restriction modification (R-M) systems of all four types. Generally, methylation of recognition site prevents DNA cleavage. Some of the R-M systems are subject to phase variation. Analysis of the primary transcriptome revealed an unexpected complexity in the gene regulation of *H. pylori*. Many transcriptional start sites were located within operons and opposite to annotated genes signifying uncoupling of polycistrons and antisense transcription. *H. pylori* encodes more than 60 small RNAs including the 6S RNA previously thought to be absent. Small RNAs are important mediators of post-transcriptional regulation under stress and during virulence. The data also underlines the complexity of the fairly small genome of this highly adapted mucosal colonizer.

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Population Genetics and Evolution

H. pylori is cohabitating with humans probably since the early development period of the genus *Homo* but definitely predating the human migration out of Africa (approx. 60,000 years ago). The phylogeographic structure of *H. pylori* is a result of coevolution with humans and immensely valuable to study migration of prehistoric and recent populations. Currently, 7 ancestral *H. pylori* populations and 14 subpopulations are described. Human genetic diversity decreases with distance from East Africa and *H. pyloris* parallels this, indicating a close association and co-evolution. In general, strains from family members are highly similar and strains isolated from unrelated persons show extensive differences in nucleotide sequence and gene content. It is also apparent that one person can be colonized by multiple *H. pylori* strains. Mixed infections of one stomach are major drivers of allelic diversification due to intra- and inter-strain recombination events and gene shuffling. Horizontal gene transfer contributes substantially to *H. pylori* evolution. The bacterium is naturally competent for DNA uptake via transformation and it frequently carries conjugative plasmids and transferable chromosomal elements. Slip strand mispairing at dinucleotide repeats and homopolymeric tracts affects protein expression generally and has been described for genes encoding surface structures like lipopolysaccharide (LPS). During infection *H. pylori* experiences multiple types of DNA damage and in contrast to earlier contentions, several DNA repair pathways are available to promote survival in the acidic gastric niche. The mutation rate in *H. pylori* is high and most genes are under negative selection. Notably, however, several genes encoding surface structures and the virulence factor cytotoxin-associated gene A (CagA) are under diversifying selection, which allows adaption to the host and persistence by immune evasion.

Epidemiology and Transmission

50% of the world's human population is infected. Current major determinants of prevalence are socioeconomic conditions and age. Improved hygiene and use of antibiotics has brought about rapidly declining colonization rates in industrialized countries that can be as low as 10% compared to 90% in developing countries. In high-risk areas (developing countries) and high-risk populations (i.e. indigenous) almost everyone acquires *H. pylori* by age 5, whereas in developed countries the overall prevalence is 40–60%. Frequency of *H. pylori* is equal in women and men. The infectious dose of *H. pylori* is unknown but there is strong evidence for fecal-oral, oral-oral, and vomitous-oral routes of acquisition. Contaminated water is a probable risk factor but possibly can be neglected as an important route since transmission within family members (mainly mothers to children) is well established and also occurs within communities where people live in close contact.

Pathogenesis

Research with this pathogen over nearly four decades has applied in vitro experiments as well as animal models (mice, Mongolian gerbils and rhesus macaques). Despite many gaps in knowledge and difficult challenges, detailed understanding of *H. pylori* pathogenesis mechanisms and its interaction with the immune system is emerging. Successful colonization, persistent infection, and onset of disease pathogenesis are accomplished in several steps. The bacterium's corkscrew shape and flagellar movement allow it to efficiently swim from the lumen to the epithelium and penetrate the thick, viscous layer of mucus to initiate colonization of the stomach. Production of urease helps to neutralize gastric acidity and lower viscosity of the mucus. The mucus-associated bacteria then become cell-associated to avoid displacement from the stomach by peristalsis and gastric emptying. Intracellular *H. pylori* have been observed in vitro (cell culture) and in vivo (biopsies) although at low frequencies. *H. pylori* stimulates gastric epithelial cells and thus induces an innate response characterized by the production of chemokines, which leads to the recruitment of myeloid cells, including macrophages, in the gastric mucosa. The pathogenesis of *H. pylori*-induced diseases is mediated by the infiltration, activation, and persistence of cells of the innate and chronic immune response. A sophisticated adaptation to the immune response is key to persistent colonization.

Immune Responses, Immune Evasion, and Host Factors

H. pylori interferes with many immunological pathways and disease outcome is strongly dependent on how and at which state host immune cells are manipulated. The interaction is very dynamic over time and many immunological pathways and cascades are constantly stimulated by the bacterium. Successful chronic colonization and bacterial persistence depends on multiple factors and the bacterium has developed a wide repertoire of strategies to evade host defenses.

When *H. pylori* enters the stomach it immediately interferes with the host's non-specific defense program against pathogens and the innate immune response. *H. pylori* swims through the mucus towards the epithelial cells where adherence, invasion and toxin release leads to disruption of tight junctions, altered cell polarity, cytoskeletal rearrangements and induction of apoptosis. Adhesins, urease, CagA and VacA are key manipulators of gastric epithelial cells, which react by activation of NFkB, cytokine release (Interleukin (IL)-10, IL-8, IL-6, IL-1β), followed by infiltration of immune cells including macrophages and dendritic cells (DC) towards the injured tissue. IL-10 is an immune suppressant important for chronic colonization and levels of cytokines vary depending on the state of infection (acute or chronic).

In epithelial cells and incoming macrophages, urease secreted by *H. pylori*, but not LPS, triggers the production of nitric oxide (NO), a free radical synthesized by the enzyme NO synthase (NOS) through the oxidation of L-arginine. In contrast to many enteropathogens *H. pylori* is less well equipped to resist direct effects of oxidative and nitrosative stress. Nonetheless, *H. pylori* successfully counters the deleterious effects of NO by expressing protective reductases, by L-arginine depletion of the extracellular milieu and most importantly, by blocking host NO production through transcriptional and translational CagA dependent down-regulation of NOS2 expression.

Macrophages and DCs typically express toll-like receptors (TLR) on the surface. An important evasion strategy of *H. pylori* is to avoid recognition by TLR. *H. pylori* flagellin is not recognized by TLR5 and, in contrast to other bacteria, CD14 and TLR4 poorly recognize *H. pylori* LPS. Phase variation in the Lewis antigens (see below) allows efficient evasion by minimizing the activation of cascades such as the complement pathways necessary for pathogen clearance. Moreover, *H. pylori* can prevent phagocytic killing in a CagA- and VacA-dependent manner and can induce apoptosis of macrophages. However, if macrophages are activated they produce IL-6, IL1β, IL-12 and TNFα, which help to initiate a Th1 type response.

T-cells and DCs play an important role in immune recognition and response to *H. pylori*. The urease subunit B (UreB) together with LPS activates the NLRP3 inflammasome in DCs via MyD88, NF-kB, TLR4 (LPS), and TLR2 (UreB) leading to caspase 1 activation and secretion of cytokines IL-1β and IL-18. IL-1β drives Th1 or Th17 differentiation, whereas IL-18 allows T regulatory (Treg) cell differentiation ($CD4^+$ $CD25^{hi}$). Thus, the T-cell response invoked varies between immune tolerance (Treg)—resulting in reduced disease incidence and probably to life-long bacterial persistence – and infection control (Th1/Th17). The latter leads to increased gastric inflammation, tissue injury and disease induction. Asymptomatic carriers generate *H. pylori* specific Tregs and patients with peptic ulcers display a pathogenic T-cell response. The interaction of *H. pylori* with DCs is a key factor in persistent colonization since *H. pylori* exposed "tolerogenic" DCs fail to induce a Th1/Th17 response but convert naïve T-cells into Tregs in a contact-dependent way directing the T-cell response away from the pathogenic response.

In chronic human colonization, macrophages, neutrophils, DC, mast, B-cells and a higher count of $CD4^+$ T-cells compared to $CD8^+$ T-cells are usually present. The existing $CD4^+$ T-cells are typically Treg cells, which suppress both innate and adaptive immune responses and suppress the induction and proliferation of effector T-cells. Moreover, numerous cytokines, including gamma interferon (IFN-γ), tumor necrosis factor (TNF), IL-1β, IL-6, IL-7, IL-8, IL-10, and IL-18, are elevated in the stomachs of *H. pylori*-colonized humans compared to uninfected humans.

In terms of the humoral immune response it is notable that *H. pylori* induces a strong B-cell response and colonized patients normally display a classical and long-lasting immunoglobulin- (Ig) A, IgM and IgG titer. Immunodominant factors are UreB, CagA, and VacA. However, this response is not sufficient to clear infection. In contrast, the induction of autoantibodies by *H. pylori* might be counterproductive and influences disease.

Generally, the immune response eclipsed by *H. pylori* is dependent on host factors like genetic background and age, and on the genetic versatility of the colonizing strain. Also environmental factors like smoking, food and high salt intake or dietary antioxidants play an important role. To add complexity, mouse studies showed that colonization not only interferes with immune cells in the stomach but also impacts immunity in distal tissue like the lung and the microbiota, which is linked to immune development.

The response to *H. pylori* is versatile and since so many host pathways and genes are affected, it is not surprising that host genetics are directly linked to disease outcome. Inter-individual variations in cytokine response due to polymorphisms are particularly important contributors do the diversity of clinical outcomes. Higher risks for gastric cancer in many populations were reported to be due to differences in genes coding for IL-1β, TNFα, IL-8, IL-10, and IL-18, as well as variations in their receptor antagonists. Moreover, polymorphisms in genes of the TLR signaling pathways influence the risk of gastric cancer.

Host Interaction (Virulence) Factors

Adhesion

Once *H. pylori* reaches the epithelial cell layer, adhesion is crucial to avoid shedding and to allow directed manipulation of host cells. *H. pylori* have >30 genes encoding *Helicobacter* outer membrane porins (Hop) and related adhesins. The blood-antigen binding protein A (BabA) and the sialic acid-binding adhesin (SabA) are most crucial for cell attachment. BabA binds to the ABO/$Lewis^b$ antigen exposed on the surface of the gastrointestinal mucosal epithelial cells and red blood cells. SabA binds to sialylated and sulfated glycans. The inflammatory response induced during infection results in up-regulation of these negatively charged glycans and reinforces bacterial attachment to host cells. Components of the *cag*-encoded pilus interact with integrins (described below). Recent work has identified a key interaction between the surface exposed adhesion HopQ and human apical carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) that is glycan independent. The purpose of this interaction is not yet clear, but lack of *hopQ* disrupts T4SS mediated delivery of CagA.

cag Pathogenicity Island and the Cytotoxin Associated Gene A (CagA) Protein

H. pylori appears to have acquired the *cag*-pathogenicity island (*cag*) via horizontal gene transfer and the island has been steadily maintained in virulent strains. Presence of *cag* is a major determinant of disease outcome due to expression of the CagA protein, which is able to interact with a wide range of host signaling proteins and undergo tyrosine phosphorylation modifications. CagA is delivered directly into the cytoplasm of the gastric epithelium and immune cells by a multicomponent bacterial type IV secretion

system (T4SS) that spans both bacterial and host cell membranes. The protein components required for secretion are encoded together with *cagA* on the pathogenicity island. These genes can be upregulated in vitro by acidic or high salt conditions and upon contact with epithelial cells. Their expression in vivo was confirmed in animal models. The T4SS forms an extracellular pilus, establishes contact with the host cell via integrin receptors and delivers the multifunctional CagA effector protein. Phosphorylation of CagA by c-SRC and c-ABL family kinases on the protein's Glu-Pro-Ile-Tyr-Ala (EPIYA) motif occurs after translocation. Both CagA and its phosphorylated forms interact with multiple host proteins resulting in broad dysregulation of cell signal transmission and cellular processes.

The *cag* T4SS and CagA activate transcription factor NF-kB via multiple pathways resulting in increased expression and release of proinflammatory IL-8 to the gastric lumen, as well as enhanced expression of other cytokines, enzymes, regulatory factors and angiogenic factors ultimately causing chronic gastric inflammation. CagA phosphorylation independent signaling alters epithelial cell to cell junctions by interaction with junctional adhesion molecules, zonula occludens, PAR1 and E-cadherin. CagA activity also causes loss of cell polarity, cytoskeletal rearrangements and formation of elongated cells characteristic of the *hummingbird* phenotype. Activation of the ERK pathway is a phosphorylation independent activity of CagA resulting in increased proliferation of epithelial cells.

Substantial research in recent years has been committed to understanding the mechanistic basis of pathogen-induced disease and the significance of *cag* using in vitro systems and experimental infection models in mice and Mongolian gerbils. CagA is not essential for stomach colonization and strains often lose their ability to translocate CagA during mouse colonization studies. Comparison of infection by *cag* + and *cag* deficient *H. pylori* strains in Mongolian gerbils clearly link CagA to expansion of gastric adenocarcinoma, and in transgenic animals (mouse, zebrafish, *Drosophila*) expressing CagA systemically, elevated JNK activation and Wnt target-gene upregulation resulted in intestinal cell proliferation, adenocarcinoma and small cell carcinoma. The sum of data clearly demonstrates CagA's cancer-promoting capability, allowing CagA to be identified as the first bacterial oncoprotein. The majority (60–70%) of Western *H. pylori* strains and almost 100% of East Asian strains express CagA.

Vacuolating Cytotoxin A (VacA)

Secretion of the pore-forming toxin VacA supports persistence and survival of *H. pylori* in the human stomach. The *vacA* gene is present as a single chromosomal copy in all *H. pylori* strains, yet mosaicism is common. Distinct *vacA* alleles encode toxin molecules with different activity and receptor affinity. The 140 kDa protoxin is processed to a 88 kDa monomer for secretion. Activation of the toxin requires acid exposure and oligoimerization. VacA targets primarily gastric epithelial cells yet translocation of the bacterium to the lamina propria exposes immune and parietal cells to VacA as well. In vitro, VacA disrupts epithelial barrier function and endosomal compartments allowing toxin-dependent ion influx and formation of acidic intracellular vacuoles. VacA unleashes multiple cellular effects. Access to immune cells supports its immunosuppressive phenotype. Interaction with dendritic cells promotes immune tolerance. Moreover, VacA interferes with T cell proliferation as well as IL-2 production and secretion.

Colonization Factors

Motility and Chemotaxis

Efficient motility is essential for persistent colonization of the gastric mucus layer. *H. pylori* is highly motile compared to other bacteria and strains exhibit different numbers of flagella of varying lengths. A higher degree of flagellation correlates with increased swimming speed. More than 40 proteins are involved in the biosynthesis and operation of the polar, sheathed flagella. Conserved *fla*, *fli*, and *mot* genes are sequentially expressed and subject to complex regulation on multiple levels including transcriptional, posttranscriptional, posttranslational mechanisms and phase variation. The flagellum is divided into the hook-basal body (HBB) complex and the extracellular filament. The HBB spans from the cytoplasm to the surface-localized hook. The inner part of the HBB is comprised of a functional type III secretion system that secretes many components of the flagellum and other proteins. MotA and MotB form the stator complex and the higher than average number of stator units present in *H. pylori* might generate higher torque and efficient motility. *H. pylori* senses and responds to chemical cues. Flagellar rotation can turn clock- or counterclockwise in response to chemosensory directed signals. In the stomach the bacterium prefers to swim towards injured tissue. The three membrane-bound chemoreceptors TlpABC and the cytoplasmic sensor TlpD sense different environmental conditions and interact with the Che-system. CheW, CheA, and CheY form the core-signaling complex and interact with the flagellar motor to control the direction of rotation. Mutants in the chemotaxis system are less virulent and in total up to 100 genes contribute to forming a chemoresponsive flagellum. As a final note, part of the capacity of *H. pylori* to escape detection is that flagellin is not recognized by TLR5, and thus fails to induce nuclear factor (NF)-kB. Moreover, flagellin fails to induce IL-8 production in gastric epithelial calls.

Urease Activity

H. pylori is a neutrophile that survives stomach acid using a urease-based acclimation mechanism. The enzyme hydrolyzes urea to produce ammonia, which is released to the cytoplasm and periplasm, neutralizing the local pH. The urease gene cluster comprises 7 genes where *ureA* and *ureB* encode catalytic subunits and *ureE-H* specify accessory proteins important to chaperoned assembly of the apoenzyme and nickel cofactor. Intrabacterial urease activity is regulated by a proton-gated channel UreI, which imports urea under

acidic conditions. Cytoplasmic urease prevents collapse of the transmembrane potential difference by maintenance of a constant internal and periplasmic pH. Surface bound urease increases the pH of the local extracellular milieu. Uptake and homeostasis of the metal cofactor nickel is well regulated. Transcription of *ureA* and *ureB* is controlled by NikR, a common regulator of nickel-dependent enzymes. Nickel uptake is governed by NixA and an ABC transporter. *H. pylori* has an elaborate system to store nickel via Ni2+ binding proteins Hpn and HpnI and further regulates nickel homeostasis via chaperonin HspA (GroES). A succession of transfer reactions passages the metal from Hpn and HpnI to higher affinity binding proteins like HypA and HypB, which then transfer the metal to the urease apoenzyme. Urease production is not only important for colonization, it also leads to tissue injury by provoking the NO response, manipulates immune cells and modulates phagosome pH to support *H. pylori* survival within macrophages.

LPS and Lewis Antigens

Lipopolysaccharide (LPS) is the main outer membrane component of Gram-negative bacteria and the LPS presented by pathogens usually has strong immunostimulatory and inflammatory capacities. It is composed of the endotoxic lipid A, the core oligosaccharide, and the O-antigen. To evade the innate immune response, *H. pylori* masks the negative charges on its lipid A and removes phosphate groups from the backbone to avoid binding by cationic antimicrobial peptides (CAMPs) and activation of hTLR4-MD2. Moreover, *H. pylori* LPS poorly binds to CD14 and TLR4 thus macrophage activation is avoided. *H. pylori* also promotes immune evasion and chronic infection by host mimicry of Lewis (Le) antigens expressed by gastric epithelial cells. The O-antigen of *H. pylori* is decorated by two types, $Le^{a/b}$ (type I) and $Le^{x/y}$ (type II), to avoid host recognition. Finally, phase variation of the Le antigens contributes to the heterogeneity of *H. pylori* LPS and immune tolerance.

Diseases Associated With *H. pylori*

Host Response to *H. pylori*

Initial infection by *H. pylori* usually remains unnoticed and is rarely diagnosed. In some cases colonization triggers nondiagnostic symptoms such as dyspepsia, abdominal cramps and vomiting. Gastritis is the inflammation of the inner lining of the stomach and follows mucosal injury. The acute ("active") inflammation is associated with neutrophilic infiltration, while the chronic inflammation is characterized by the presence of mononuclear cells, primarily lymphocytes, plasma cells and macrophages. Colonization with *H. pylori* classically results in a response of the gastric tissue that is termed chronic active gastritis. This inflammatory response is absent in *H. pylori*-free persons. A valid point increasingly debated, however, asks which situation should be considered as "normal"– the presence of an organism that has coevolved with humans from the beginning of mankind, or its absence? It is important to keep in mind that the inflammatory response to *H. pylori* might be beneficial in the stimulation and education of our immune system. The host response varies in its intensity and anatomic distribution and is dependent on the colonizing strain as well as on host genetic susceptibility and environmental factors. Only an excessive inflammatory response drives pathological processes in the gastric epithelium heightening risk for symptomatic gastritis, ulcer disease or cancer but importantly, 80–90% of infected individuals remain asymptomatic throughout their lifetime.

Ulcer Disease

Peptic ulcer disease (PUD) refers to painful sores that develop in the lining of the stomach, lower esophagus, or small intestine. Only ~10% of chronically infected patients, particularly those that manifest increased acid secretion, are predisposed to develop peptic ulcer disease. *H. pylori* has clearly been linked to duodenal ulcers since elimination of *H. pylori* substantially reduces ulcer relapses. Moreover, the incidence of PUD has declined as *H. pylori* disappears from human populations. Studies in Asian populations correlate diminished *H. pylori* prevalence and reduced occurrence of duodenal ulcers from 21.1% to 5.0% and that of gastric ulcers from 11.9% to 9.9% within 20 years. For appropriate management of patients with gastic ulcers, medication (non-steroid anti-inflammatory drugs (NSAIDs)) might be more important than *H. pylori*, as NSAID in combination with *H. pylori* colonization increases the risk of peptic ulcer bleeding. Generally, this underscores the recommendation is to eliminate *H. pylori* in all patients with ulcer disease.

Gastric Carcinoma

The most common cancer of the stomach is adenocarcinoma (neoplasia of epithelial tissue with glandular origin). Gastric adenocarcinoma is the third leading cause of cancer-related deaths in the world. Chronic inflammation induced by long term colonization with *H. pylori* is a major risk factor leading to the induction of stem cell hyperplasia, morphological changes in gastric mucosa and gastric cancer development. 1 to 3% of persons colonized with *H. pylori* develop gastric adenocarcinoma and the outcome is often poor with a less than 10% 5-year survival rate globally. Infection, inflammation, and antitumor immunity are key elements of disease progression. Factors like colonizing strain, host genetics (e,g. increased NOS2 gene expression and polymorphism in modulatory cytokines or TLR related genes), host microbiota, as well as environmental influences including diet, play a role in the pathologic outcome of *H. pylori* colonization.

Gastric Lymphoma

The stomach is the most frequent site of extranodal lymphoma. Gastric lymphoma originating from mucosa-associated lymphoid tissue (MALT) typically originates from B-cells in the marginal zone of the MALT. These non-Hodgkins´s lymphomas (NHL) (no Reed-Sternberg cells) are relatively rare, that is 2-3% of all malignancies in the world are NHL and MALT lymphomas comprise approximately 5% of all NHLs. These malignancies are associated with chronic inflammation and the development of lymphoid follicles in the submucosa due to *H. pylori* colonization. *H. pylori* infection protects splenic B-cells from apoptosis possibly having consequences for the lymphoma. *H. pylori* eradication may induce tumor remissions and important factors in response to *H. pylori* eradication therapy are tumor stage, depth of invasion, and localization, as well as geographic region. *H. pylori* is also linked to diffuse large B-cell lymphoma (DLBCL) with and without signs of MALT. Pure DLBCL (no signs of MALT) is normally treated with systemic chemotherapy however, full pathologic remission has been documented after *H. pylori* eradication therapy in *H. pylori*-positive pure DLBCL and DLBCL(MALT).

Extra Gastric Disease and Potential Benefits

H. pylori leads to infiltration of immune cells in the stomach, affects distal immune responses, and there is also evidence that the presence of *H. pylori* influences the gastric and intestinal microbiome, thus it is conceivable that *H. pylori* may variably cause, influence or protect from extra gastric disease. Currently, it is controversially reported and discussed whether *H. pylori* can induce or negatively influence cardiovascular and neurologic disorders, or influence dermatologic disorders, head and neck disease, or complications during pregnancy, as well as diabetes mellitus. A link has been established between *H. pylori* infection and a negative impact on idiopathic thrombocytopenic purpura, sideropenica anemia, and vitamin B12 deficiency. By contrast evidence indicates that *H. pylori* has a protective role in the development of early onset asthma (see below). Moreover, early childhood exposure to *H. pylori* might protect from gastroesophageal reflux disease (GERD) and Barrett´s esophagus, allergic rhinitis, eczema, skin sensitization, and inflammatory bowel diseases. The current status of research clearly demands further studies given the high impact of these diseases on public health.

Treatment, Antibiotic Resistance and Vaccine Development

Current guidelines suggest that the decision to test for *H. pylori* should be made with therapeutic intent, thus *H. pylori* should be eradicated if detected. Despite susceptibility to many antibiotics in vitro, no single agent achieves eradication rates above 55%. Efficacy is improved with acid suppression; therefore the standard triple therapy combines proton-pump inhibitors (PPI) and two antibiotics. Antimicrobial eradication levels are decreasing (as low as 60%) and are inversely correlated with antibiotic resistance rates. First line therapy in regions with low clarithromycin resistance classically combines PPI, clarithromycin, and either amoxicillin or metronidazole for 14 days. In regions with high clarithromycin resistance failed eradication using second- and third-line therapies may require cultivation of *H. pylori* and clarification of susceptibility. Regimes with the highest success rates include rifabutin, therefore this drug is best reserved as a third-line salvage therapy. Quadruple therapy with or without bismuth increases treatment success but new (non-antibiotic) strategies for eradication are clearly desired. So far, efforts to develop an effective vaccine were not successful. Several strategies triggering protective T cell-mediated immunity instead of humoral immunity have revealed useful antigens, adjuvants, and delivery routes, but effective protective immunity in humans was not achieved. Vaccine research is certain to continue. Nonetheless as our understanding of the beneficial functions of *H. pylori* also moves forward it may be prudent to reassess the value of eradicating human infection.

H. pylori and Asthma

Epidemiological studies in human populations have documented an inverse association of *H. pylori* colonization and the risk of developing allergic asthma, atopic rhinitis and other allergic diseases. These observations suggested that an exposure to *H. pylori* during childhood protects from diseases like early-onset asthma. In a comprehensive mouse asthma model, colonization of neonatal and adult mice confirmed the protective role of *H. pylori*, highlighting also the difference between early-in-life and late-in-life exposure to antigens. While adult mice develop chronic gastritis and preneoplasic lesions, mice infected early-in-life mimic the asymptomatic human carrier and are protected against gastric pathology. Neonatal mice develop a tolerance towards *H. pylori* that is characterized by the suppression of specific T-effector cells. VacA supports this response by blocking IL-2 production resulting in suppression of T-cell activation and proliferation (shown in humans). γ-glutamyl transpeptidase (GGT) prevents T-cells proliferation by arresting them in the G1 cell cycle phase. Moreover, both secreted factors indirectly prevent T-cell immunity by re-programming of DCs (only observed in mice). Exposure of DCs to *H. pylori* proteins forces these cells into to a semi-mature state characterized by high levels of MHCII but low or no costimulatory molecules. These DCs produce IL-10 and IL-18 thereby supporting T-cell differentiation towards Treg cells while supressing the development of Th1 and Th17 cells. As a side effect of this tolerogenic DC/Treg axis, protection against asthma is observed in the mouse model.

Conclusions

H. pylori is medically relevant and has become one of the most investigated bacteria world-wide. *H. pylori* can be genetically engineered, animal models are available and to some extent the bacterium can be studied in its natural niche—the human stomach. Co-evolution with the human host allowed *H. pylori* to acquire fascinating features. This organism is conceivably one of the best candidates we currently have to study and understand the dual (commensal/pathogen) role bacteria can establish with the human host. As such researchers in the field have recognized the opportunity to move beyond the singular view of negative consequences of infection to pursue the bigger picture. It follows that *H. pylori* research opens further opportunities to compare and relate these findings to other human colonizers, which might have a similar dual role in human health.

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Hemorrhagic Fever Viruses☆

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Glossary

Apoptosis The elimination of a cell through a process of programmed self-destruction, initiated by the binding of external mediators to the cell surface or disruption of normal metabolism.
Dendritic cells Cells of the immune system that specialize in taking up foreign material, processing it, and presenting it on their surfaces to lymphocytes, to initiate an antigen-specific immune response.
Disseminated intravascular coagulation A hemorrhagic syndrome that results from extensive activation of the coagulation system, with consumption of coagulation factors and platelets and production of fibrin degradation products.
Endothelium The layer of cells that lines the inner surface of blood and lymphatic vessels.
Inflammation The complex of changes that occur in a tissue and its blood vessels in response to an injury or microbial invasion, stimulated by products released by macrophages and other cells, which result in increased blood flow, transudation of proteins, and an influx of cells capable of attacking microorganisms.
Interferons A class of proteins produced by several cell types in response to viral infection that induce a battery of intracellular countermeasures against viral replication.
Macrophages Cells derived from precursors in the bone marrow, which are widely distributed throughout the tissues of the body, which ingest and destroy microbes and induce inflammatory changes through the release of mediators.
Zoonosis An infection shared by humans and animals; the term is usually applied to situations in which the animal host maintains the infectious agent while showing little sign of disease.

Abbreviations

DRC	Democratic Republic of the Congo
HF	Hemorrhagic fever
L	Large
M	Middle
NSs protein	Nonstructural protein
S	Small

Defining Statement

The hemorrhagic fever viruses are a group of RNA viruses that are maintained in animals and/or arthropod vectors, but cause severe illness in humans characterized by coagulation defects, increased vascular permeability, and shock. They share the ability to replicate in macrophages, suppress interferon responses, and induce a massive release of proinflammatory mediators.

Introduction

The hemorrhagic fever viruses are members of four different RNA viral families that cause a disease in humans characterized by coagulation defects that can result in bleeding and increased vascular permeability that can lead to a fall in blood pressure, shock, and death. The term 'viral hemorrhagic fever' was first used in the early 1950s to designate an illness that occurred sporadically among soldiers fighting in the Korean War, which is now known to result from exposure to the excretions of local rodents chronically infected by a hantavirus. Over subsequent decades, some 15 additional viral infections, ranging from the ancient plague of yellow fever to the highly lethal disease caused by the newly discovered microbes, Marburg and Ebola virus, have been found to meet the criteria for the hemorrhagic fever syndrome (Table 1).

☆*Change History:* September 2014: M Holbrook and M Bray updated the entire article with addition of Alkhurma and Lujo viruses to the text and a separate section for vaccines.

This article is an update of S. Suerbaum, M.J. Blaser, *Helicobacter pylori*, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 163–169.

Table 1 Taxonomic classification, geographic distribution, natural reservoirs, and vectors of the causative agents of viral hemorrhagic fever

Virus	*Disease*	*Geographic distribution*	*Reservoir host*	*Arthropod vector*
Arenaviridae				
Lassa virus	Lassa fever	West Africa	Rodents	None
Lujo virus	Lujo HF	Southern Africa	Unknown	Unknown
Junin virus	Argentine HF	Argentina	Rodents	None
Machupo virus	Bolivian HF	Bolivia	Rodents	None
Guanarito virus	Venezuelan HF	Venezuela	Rodents	None
Sabia virus	Sabia HF	Brazil	Rodents	None
Whitewater Arroyo virus	Whitewater Arroyo HF	California, USA	Rodents	None
Bunyaviridae				
Crimean-Congo HF virus	Crimean-Congo HF	Southern Eurasia, Balkans, Africa	Hares, birds, livestock	Ticks
Rift Valley fever virus	Rift Valley fever	Sub-Saharan Africa	Livestock, unknown	*Culex*, *Aedes* mosquitoes
Hantaviruses (Old World)	HF with renal syndrome	Northern Eurasia, China, Korea	Rodents	None
Hantaviruses (New World)	Hantavirus pulmonary syndrome	North and South America	Rodents	None
Filoviridae				
Ebolavirus	Ebola HF	West Africa	Unknown	Unknown
Marburgvirus	Marburg HF	Africa	Unknown	Unknown
Flaviviridae				
Yellow fever virus	Yellow fever (urban)	Africa, South American cities	Humans	*Aedes aegypti* mosquitoes
	Yellow fever (sylvan)	Africa, South American forests	Monkeys	*Haemagogus*, *Aedes* mosquitoes
Dengue virus	Dengue fever, dengue HF	Tropics worldwide	Humans	*Aedes aegypti* mosquitoes
Kyasanur Forest disease virus	Kyasanur Forest disease	Mysore State, India	Monkeys, rodents, birds	*Haemaphysalis* ticks
Alkhurma virus	Alkhurma HF	Saudi Arabia, Southern Egypt	Unknown	*Ornithodoros* ticks, other?
Omsk HF virus	Omsk HF	Russia	Rodents, muskrats	*Dermacentor* ticks

All of the hemorrhagic fever viruses have single-stranded RNA genomes, which differ in the polarity of the genome and in the number of segments into which it is divided (positive-sense, one segment for the flaviviruses; negative-sense, one segment for the filoviruses; negative-sense, two segments for the arenaviruses; and negative-sense, three segments for the bunyaviruses). They also differ in morphology: The arena-, bunya-, and flaviviruses are spherical, while the filoviruses are long and filamentous. All of the hemorrhagic fever viruses are 'enveloped' – that is, their surface glycoprotein molecules are anchored in a lipid bilayer derived from host cell membranes.

Almost all types of viral hemorrhagic fever are 'zoonoses': their causative agents infect various animal species, but can be transferred to humans through direct contact with an animal, exposure to its excretions, or the bite of a blood-feeding arthropod. The geographic distribution of each disease therefore corresponds to the range of the host species or arthropod vector. Because these viruses have no history of adaptation to humans, they have not acquired efficient mechanisms of person-to-person transmission. Spread of infection therefore either does not take place at all, or requires direct contact with virus-containing body fluids. In endemic areas, these diseases tend to occur either as single cases or as small clusters of infections that quickly 'burn out'. Dengue virus is not a zoonotic virus as it has adapted to a cycle of person-to-person transmission by blood-feeding mosquitoes that enables them to persist among human populations without the support of an animal reservoir. Yellow fever virus has also adapted to mosquito-driven person-to-person transmission in urban settings, but maintains a zoonotic transmission cycle in jungle settings where non-human primates serve as the reservoir host.

Because viral hemorrhagic fever occurs almost exclusively in regions of the world that lack a medical research infrastructure, few data on the pathogenesis of these diseases have been obtained by studying sick people. Instead, most of the current knowledge of the underlying mechanisms of illness has been obtained from experiments on laboratory animals. As discussed later under 'Pathogenesis of viral hemorrhagic fever', this article shows that despite differences in their basic replication strategy, geographic distribution, and the identity of their maintenance hosts, the hemorrhagic fever viruses appear to interact with the human immune system in a similar manner. All of them replicate in monocytes and macrophages, and they also infect dendritic cells, which have primary responsibility for initiating an adaptive immune response by presenting antigens to lymphocytes. Suppression of type I interferon responses permits rapid virus dissemination, and the release of large quantities of proinflammatory mediators plays a critical role in the development of the major signs and symptoms of illness.

Clinical Features and Laboratory Tests

All types of viral hemorrhagic fever can be associated with a significant case-fatality rate, but they differ in average severity. For example, roughly 5% of cases of dengue hemorrhagic fever end in death, but mortality in large outbreaks of Ebolavirus

and Marburgvirus infections in Africa has ranged from 50% to 90%. Because these diseases resemble each other in their clinical presentation and in the results of standard laboratory tests, a specific diagnosis requires the performance of specialized assays.

Signs and Symptoms

Viral hemorrhagic fever usually begins with the abrupt onset of fever and malaise, accompanied by headache, muscle and joint pain, nausea, vomiting, abdominal pain, and diarrhea. The vasodilatation and diffuse 'vascular leak' that are the major physiologic abnormalities leading to shock sometimes produce cutaneous flushing and soft tissue edema, but are manifested most clearly through a fall in blood pressure. Coagulation defects may cause the development of a maculopapular rash, petichiae, ecchymosis prolonged bleeding from needle puncture sites, conjunctival hemorrhages, and easy bruising. Major hemorrhage from the gastrointestinal and urinary tracts is generally seen only in severely ill patients, as a terminal phenomenon.

Despite the syndrome's name, bleeding is rarely the cause of death. Instead, the life-threatening lesion is a profound alteration of vascular function induced by high circulating levels of proinflammatory mediators, which combines the diffuse dilatation of small vessels with the increased permeability of their endothelial linings, permitting fluid and proteins to move out of the plasma into the interstitial space. The resulting reduction in effective blood volume produces circulatory insufficiency, stupor, coma, multiorgan failure, shock, and death (see 'Pathogenesis of viral hemorrhagic fever'). For most types of viral hemorrhagic fever, changes in neurologic function reflect a decrease in circulatory perfusion, rather than viral invasion of the central nervous system, so they resolve as the patient recovers.

Findings in Standard Laboratory Tests

The numerous physiologic changes induced by host responses to infection produce a wide range of abnormalities on standard laboratory tests. Increased vascular permeability results in diminished plasma volume, which is reflected in a rising hemoglobin concentration and hematocrit in the complete blood count and in a fall in albumin concentration, while the total protein may remain normal. Diminished circulatory volume also impairs renal function, as manifested by rising urea nitrogen and creatinine levels. As regards the complete blood count, high levels of circulating chemokines evoke the release of leukocytes from the bone marrow, producing an increased percentage of immature granulocytes in blood smears. At the same time, the lymphocyte count may fall as a result of the loss of these cells through apoptosis over the course of infection (see 'Pathogenesis of viral hemorrhagic fever').

For most viral hemorrhagic fevers, the shift to a procoagulant state that accompanies systemic inflammation also leads to a consumption of coagulation factors, which will eventually be manifested in a prolongation of the prothrombin and partial thromboplastin times and a fall in the platelet count. As the patient's condition worsens, fibrin split products and D-dimers will appear in the plasma, consistent with disseminated intravascular coagulation. The spread of infection to hepatic parenchymal cells is reflected in the appearance of elevated levels of liver-associated enzymes, such as aspartate and alanine aminotransferase, in the plasma, and may also contribute to deficits in coagulation factors.

Diagnosis

The physical findings and laboratory abnormalities described earlier are not unique to any one type of viral hemorrhagic fever, or even to the syndrome in general, but are also observed in many other infectious processes, including Gram-positive and Gram-negative bacterial sepsis, toxic shock, meningococcemia, plague, typhoid fever, leptospirosis, rickettsial infections, malaria, severe measles, and hemorrhagic smallpox. Making a firm diagnosis therefore requires the performance of specific microbiological and immunological procedures, such as antigen or antibody detection by enzyme-linked immunosorbent assay, identification of unique viral sequences by polymerase chain reaction, or the growth of the causative agent in cell culture. Because all types of viral hemorrhagic fever are characterized (from the earliest phase of illness) by the release of viral antigens and infectious virus from infected monocytes and macrophages into the plasma, blood is the most appropriate sample for testing.

Variation among Individual Types of Viral Hemorrhagic Fever

By definition, all types of viral hemorrhagic fever are characterized by fever, altered vascular function, and coagulation abnormalities, but individual agents also induce other physical changes that reflect specific virus–host interactions. For example, severe hepatic injury leading to jaundice is commonly seen in yellow fever (whence its name), but is rare or absent in the other syndromes. Some Rift Valley fever patients develop neurologic or ophthalmic complications that are not seen in the other diseases, while comparatively few develop hemorrhage. Lassa fever often shows an insidious onset and a lack of hemorrhagic complications. Renal insufficiency is characteristic of infection by the Old World hantaviruses, while the New World agents cause rapidly worsening cardiorespiratory failure, with the rapid onset of pulmonary edema. Despite the development of vascular leakage that leads to pulmonary edema, New World hantaviruses are not generally considered "true" hemorrhagic fever viruses.,

Pathogenesis of Viral Hemorrhagic Fever

As noted earlier, few data have been obtained from patients to elucidate underlying disease mechanisms. The only exception is dengue hemorrhagic fever, which has been studied in hospitalized patients in large tropical cities, but detailed research on the pathogenesis of the illness (and on that of the classic dengue) has still been hampered by the lack of good models of the disease in laboratory animals. At present, the most thoroughly studied form of viral hemorrhagic fever is the severe disease caused by the filoviruses, Ebola and Marburg, which has been studied to a limited extent in humans, but examined in great detail in laboratory animals, particularly non-human primates.

The fulminant illness caused by Zaire ebolavirus displays all of the classic features of the hemorrhagic fever syndrome. Exposure to the agent is usually followed within 3–7 days by the abrupt onset of high fever, malaise, and a variety of nonspecific signs and symptoms. These early changes are soon followed by a progressive fall in blood pressure, which in almost all cases leads to intractable shock and death. All patients show evidence of coagulation abnormalities, and bleeding from the gastrointestinal and genitourinary tracts is frequently seen during the terminal phase of the illness. Survival appears to require the rapid mobilization of a specific immune response: Those patients who develop anti-Ebola IgM or IgG antibodies during the second week of illness are likely to recover, while the persistence of high levels of circulating virus in the absence of a detectable antibody response is predictive of death.

Zaire ebolavirus is even more virulent for laboratory primates than for humans, causing uniformly fatal disease. The introduction of a few viral particles into a cynomolgus or rhesus macaque by injection, placement in the mouth or eye, or delivery as a small-particle aerosol initiates an inexorable process that appears to differ from the human illness only in that it proceeds more quickly and is uniformly fatal. Versions of the same virus that have been adapted to mice or guinea pigs through sequential animal-to-animal passage also cause a rapidly fatal illness that shares major signs and symptoms with the disease in humans and nonhuman primates. For the mouse-adapted variant, the lethal dose is a single virion.

Detailed time-course studies in these animal models have shown that four different mechanisms (discussed later in greater detail) act together to produce the clinical features of Ebola hemorrhagic fever (Figure 1). First, the virus replicates productively in

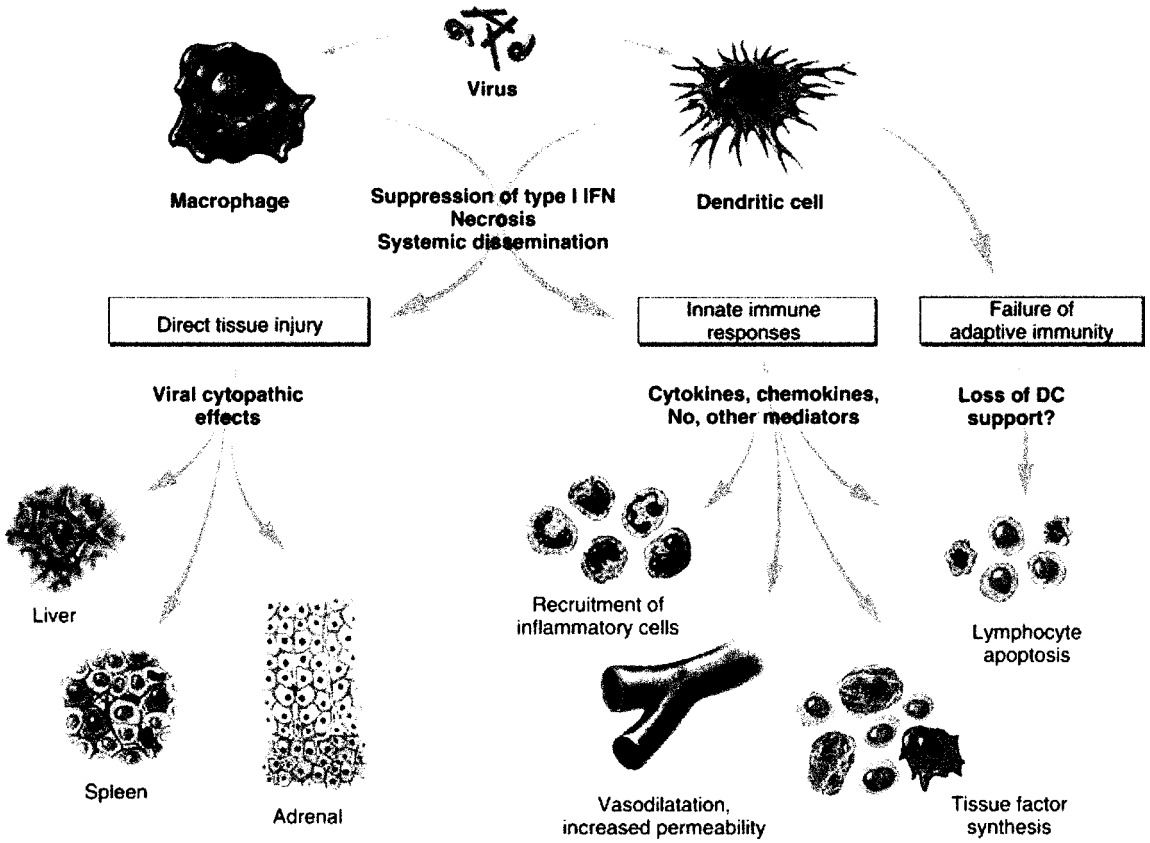

Figure. 1 Pathogenesis of filoviral hemorrhagic fever. Ebolavirus or Marburgvirus infection of macrophages leads to the generation of large numbers of new virions, release of proinflammatory mediators, and synthesis of cell-surface tissue factor. These agents also replicate in dendritic cells, impairing their ability to present antigens to the immune system. Suppression of type I interferon responses facilitates rapid viral dissemination. The direct effects of filoviral infection consist of the extensive tissue damage that results from the spread of virus to cells in the liver, adrenal glands, and other organs. Indirect effects include the induction of vasodilatation and increased vascular permeability by proinflammatory mediators and the massive apoptotic death of uninfected lymphocytes (see text).

monocytes and macrophages, inducing the release of large numbers of new virions and the production of proinflammatory mediators and tissue factor, which in turn cause vasodilatation, increased vascular permeability, and disseminated intravascular coagulation. Second, suppression of type I interferon responses by virus-encoded proteins supports the rapid dissemination of virus to monocytes, macrophages, and other cell types throughout the body. Third, the filoviruses are able to infect and kill a wide variety of parenchymal cells, resulting in extensive organ damage. Finally, the failure of adaptive immune responses, through the impaired function of virus-infected dendritic cells or macrophages and the massive destruction of lymphocytes through 'bystander' apoptosis, leads to a terminal state of 'immune paralysis' resembling that seen in septic shock.

Infection with filoviruses clearly results in the highest frequency of the most extreme form of the hemorrhagic fever syndrome, but it is reasonable to assume that the other types of viral hemorrhagic fever are produced by similar physiologic mechanisms. The following discussion of the four elements of pathogenesis is therefore based on the filovirus model, but also takes note of the extent to which the same phenomena have been identified in other diseases.

Proinflammatory Responses of Virus-Infected Macrophages

All of the hemorrhagic fever viruses make use of macrophages and their precursors, the monocytes, as their principal sites of replication. These ubiquitous 'sentinels' are the body's first-line defense against infection, charged with detecting invading microbes and initiating defensive responses through the production of a variety of vasoactive and proinflammatory mediators. A variety of pattern recognition systems, including the Toll-like receptors, are basic to this process. Macrophages also play a critical role in antigen presentation to activate adaptive immune responses.

In the case of RNA viral infections, the presence of single-stranded and double-stranded viral RNA in the cytoplasm is probably the principal stimulus leading to macrophage activation, resulting in an increased capacity for microbial killing and the synthesis and release of a variety of proteins and small molecules that alert the body to the presence of an invader and lay the groundwork for nonspecific and virus-specific countermeasures. The former include the release of proinflammatory mediators such as interleukin-1-β, interleukin-6, tumor necrosis factor-α, and nitric oxide, a potent stimulant of vasodilatation. Activated macrophages also synthesize and display tissue factor on their surfaces, triggering the extrinsic coagulation pathway. In addition to this set of nonspecific responses, macrophages help to initiate an antigen-specific immune response by processing the microbes they have ingested and presenting peptide fragments in association with major histocompatibility complex molecules for recognition by CD4+ lymphocytes.

When triggered by a localized process, such as the entry of bacteria through a break in the skin, mediators released by macrophages produce a variety of effects: dilatation of blood vessels, increased permeability of their endothelial linings, initiation of coagulation leading to fibrin deposition, and activation of endothelial cells in a manner that favors the adhesion of platelets and an influx of neutrophils, monocytes, and other inflammatory cells into the affected region. These physiologic changes are responsible for the swelling, heat, redness, pain, and pus formation that characterize an infected wound or other localized inflammatory process. Fever and other systemic signs and symptoms may also occur, depending on the extent to which inflammatory mediators produced at the site of infection enter the plasma.

When restricted to a small portion of the body, the changes in vascular function induced by activated macrophages and other inflammatory cells are beneficial, since they permit antibodies, complement, neutrophils, and other effector cells to leave the bloodstream and enter the affected area. In viral hemorrhagic fever, by contrast, the release of the same mediators by monocytes and macrophages in lymphoid organs and tissues throughout the body has a catastrophic effect, causing diffuse dilatation and increased permeability of blood vessels that result in hypotension and shock. The fundamental role of host responses in causing these basic signs and symptoms of illness has been demonstrated to varying degrees for many forms of viral hemorrhagic fever, including Crimean-Congo hemorrhagic fever, hantaviral infections, yellow fever, and dengue.

When the hemorrhagic fever syndrome was first described in the 1950s, increased vascular permeability and hemorrhage were assumed to result from direct viral infection of endothelial lining cells of blood vessels or their injury by virus-encoded products. Early designations for the disease therefore included such expressions as 'capillary toxicosis'. In the current scenario, by contrast, the hypotension and coagulopathy of viral hemorrhagic fever are seen as normal physiologic responses of endothelial cells to circulating inflammatory mediators – a system-wide version of the same changes that occur in a localized inflammatory response. The fact that vascular dysfunction is a response to host mediators does not mean, of course, that viral invasion of the vascular lining cannot also occur. Infection of endothelial cells has in fact been identified, but as a late consequence, rather than a causative mechanism of illness. Thus, in cynomolgus macaques infected with Ebola Zaire virus, careful examination of blood vessels in groups of animals killed on each day postinfection has found virus-infected cells only toward the end of the disease course. Infection of the endothelium has also been seen in tissues collected at autopsy of humans dead from other diseases, including Crimean-Congo hemorrhagic fever. These findings suggest that changes in endothelial cell function induced by inflammatory mediators over the course of illness render them susceptible to viral infection.

Suppression of Type I Interferon Responses

Once a virus has entered a cell, the type I interferon system is the primary barrier to its further dissemination. Triggered by Toll-like receptors and other pattern recognition molecules, a complex web of intracellular signaling leads to the expression of an array of proteins that collectively produce an 'antiviral state', inhibiting viral replication. At the same time, the infected cell secretes

interferons, inducing a similar resistance to viral infection in neighboring uninfected cells. The ability of the hemorrhagic fever viruses to spread rapidly from their initial site of entry therefore implies that these agents are able to evade or suppress the type I interferon system, and research has shown that this is in fact the case. Ebola virus, for example, encodes two different interferon antagonists: one protein that suppresses the initial production of interferon by the infected cell and another that prevents the cell from responding to exogenous type I or type II interferon. A number of agents from the other three viral families, including Rift Valley fever virus, dengue virus, and certain arenaviruses, have also been shown to block interferon responses, through a variety of mechanisms.

The importance of the interferon system to the control of viral dissemination helps to explain why all of the hemorrhagic fever agents are RNA viruses. Such agents generate double-stranded RNA molecules, a strong stimulus for interferon production, during the course of gene transcription and genome duplication. To be able to persist in their natural reservoir species, it is therefore essential that these viruses block interferon responses to at least the degree necessary to ensure their replication, dissemination, and transmission to a new host. When humans encounter a new RNA virus, the outcome of infection will depend in part on whether the viral interferon antagonist proteins are able to interact with and inhibit the interferon machinery of a human cell. Given the myriad of viruses that exist in nature and the relative rarity of severe viral zoonoses, it appears that the majority of cross-species transfers do not result in suppression of the human interferon system. In the case of the hemorrhagic fever viruses, however, it appears that virus-encoded interferon antagonist proteins suppress the human interferon system so effectively that the agents are able to disseminate rapidly, causing systemic infection of monocytes and macrophages and a massive inflammatory response.

Direct Tissue Damage

All of the hemorrhagic fever viruses infect human monocytes and macrophages, but they differ considerably in their ability to infect other types of cells. At one end of the spectrum, dengue virus principally infects only those two cell types, plus dendritic cells, without causing their death. At the other extreme, Ebola and Marburg viruses are apparently capable of infecting virtually every type of cell in the body except for lymphocytes and neurons. This lack of host cell specificity appears to reflect the ability of the filoviral surface glycoprotein to bind to widely distributed cell-surface lectins. Material released from dying cells is itself a stimulus for inflammation, contributing further to the fulminant systemic illness.

Most types of viral hemorrhagic fever produce a degree of tissue damage intermediate between dengue and filoviral hemorrhagic fever, with the liver as the principal target. Hepatic involvement probably begins with the spread of virus through the bloodstream to fixed macrophages (Kupffer cells) in sinusoids, from which infection then extends to parenchymal cells, giving rise to increased levels of 'liver-associated' enzymes in the plasma. Hepatic injury is a prominent feature of yellow fever, causing the jaundice that gave the disease its name, and is seen in some cases of Rift Valley fever. It also occurs in Crimean-Congo hemorrhagic fever and some other infections, but without producing the high levels of bilirubin that lead to jaundice.

Failure of Adaptive Immune Responses

Because the initial signs and symptoms of viral hemorrhagic fever are not caused by the replicating virus, but by host inflammatory mediators, they all begin in a similar manner. As infection progresses, however, specific virus–host interactions come into play that determine the precise nature of the illness. Of critical importance to the final outcome is the host's ability to generate the antigen-specific immune responses that are needed to contain and eliminate a viral infection. Two factors appear to determine whether such responses can take place quickly enough and strongly enough to ensure survival: (1) the ability of dendritic cells or macrophages to present viral antigens to lymphocytes and (2) the extent to which the lymphocytes themselves are destroyed through programmed cell death.

Dendritic cells are the major antigen-presenting cells of the body, and they are also a site of replication for hemorrhagic fever viruses. In a number of cases, such as Lassa fever and Ebola virus infection, it has been shown that infected immature dendritic cells are unable to mature, migrate to lymph nodes, present antigens, and stimulate naïve lymphocytes to proliferate. By contrast, such impairment does not appear to take place in milder forms of hemorrhagic fever, such as dengue, helping to explain its lower lethality.

The second phenomenon, the massive death of lymphocytes through apoptosis, is now recognized to be a component of septic shock resulting from Gram-negative bacterial infections, and studies suggest that it is a feature of all severe infections. Even though they remain uninfected by virus, natural killer cells and T cells appear to be especially vulnerable to programmed cell death during the course of illness, a process that is apparently driven by the action of such proapoptotic mediators as tumor necrosis factor-α and Fas ligand, and perhaps by changes in cellular metabolism that accompany severe disease. The fact that impairment of dendritic cell function and a massive loss of lymphocytes both occur during the course of Ebola hemorrhagic fever helps to explain why so few patients recover from the disease.

Antiviral Therapy

Of the many antiviral drugs now in clinical use, only the nucleoside analogue ribavirin has proven to be active against some (but not all) of the hemorrhagic fever viruses. Although it inhibits the replication of a wide range of viruses *in vitro*, ribavirin is licensed for the treatment of respiratory syncytial virus infection in infants, although it is only used in extremely critical cases, and for hepatitis C, in

combination with interferon. Limited clinical experience has indicated that the drug is also beneficial for the treatment of Lassa fever and for the Old World hantaviral infection, hemorrhagic fever with renal syndrome. As is generally true of antiviral therapy, treatment is most effective when begun early in infection. The drug produces a reversible hemolytic anemia, and evidence of teratogenicity prevents its use in pregnant women. Studies in nonhuman primates suggest that ribavirin would also be of value for the therapy of New World arenavirus infections. Surprisingly, it has no effect on filoviral replication. Although active against yellow fever in a rodent model, the drug had no impact on infection in macaques.

Ribavirin acts as a guanosine analogue. Its mechanisms of action include decreasing the size of intracellular guanosine di- and triphosphate pools, inhibiting 5′ cap formation on viral messenger RNA, and interfering with RNA-dependent RNA polymerase activity. Possibly because of these multiple mechanisms of action, no development of viral drug resistance has ever been observed. Ribavirin's ability to block the replication of some RNA viruses, but not others, remains unexplained.

Favipiravir (T-705) is a recently developed compound now under development for the treatment of influenza, that has also shown efficacy against hemorrhagic fever virus infections in a number of animal model studies. Favipiravir inhibits the viral RNA-dependent RNA polymerase by acting as a purine nucleotide analog. While favipiravir is not licensed for use in humans, it has been shown effective in animal models against Ebolavirus, Rift Valley fever virus, South American arenaviruses Junin and Machupo viruses, hantaviruses and yellow fever virus in addition to being tested in clinical trials for the treatment of influenza virus infection.

Human convalescent plasma has been widely used for the postexposure prophylaxis or treatment of viral hemorrhagic fever in countries where these diseases are endemic, but no controlled trials have been conducted to assess its actual efficacy. The best evidence of a beneficial effect has been obtained for Argentine hemorrhagic fever, for which immune plasma is currently the standard treatment. High-titer convalescent plasma was effective in treating laboratory primates for Lassa fever, but the results of limited trials in humans were not encouraging.

Causative Agents of Viral Hemorrhagic Fever

Arenaviruses

The arenaviruses (from the Latin word *arena* meaning 'sand') are spherical, lipid-enveloped viruses that frequently entrap host ribosomes during their formation, giving them a 'sandy' appearance on electron microscopy. The genome consists of two separate RNA molecules, which are ambisense, such that proteins are encoded in both directions on the same strand. The large (L) strand encodes the RNA-dependent RNA polymerase and a Z protein, which is thought to play a structural role, linking the nucleocapsid to the lipid membrane. The small (S) strand encodes a nucleoprotein, which binds to the genomic RNA, and the two surface glycoproteins, G1 and G2.

The arenaviruses that cause hemorrhagic fever are divided into two groups: (1) 'Old World' agents, including Lassa fever virus, which is found in west Africa and Lujo virus which recently emerged in southern Africa and (2) a number of 'New World' viruses that cause severe disease in areas of South America and in the southwestern United States. Each virus is named according to the location where it was first isolated. All of them are maintained in rodents, among which they spread through direct contact or excretions; none is transmitted by an arthropod vector. Human infection occurs through the handling, butchering, or ingestion of infected animals or through exposure to their aerosolized urine or feces. The epidemiology of each disease is therefore determined by the frequency and pattern of human–rodent contact.

Lassa fever virus

Although the actual numbers are not known, serosurveys suggest that as many as 100 000 people develop Lassa fever each year in west Africa, principally in the three coastal countries of Liberia, Guinea, and Sierra Leone. The causative agent is maintained through continuous transmission in the multimammate mouse (*Mastomys natalensis*). The high incidence of human infection reflects frequent contact with these animals, which infest living areas and are sometimes used as a source of food.

The majority of cases of Lassa fever are asymptomatic or mild, but among those patients sick enough to require hospitalization, the case-fatality rate is 15–20% or higher. Outbreaks have occurred in west African hospitals where the lack of proper infection control measures have allowed contact with body fluids of patients or the reuse of contaminated syringes. The occasional entry of infected travelers into Europe or the United States has not led to secondary transmission.

In contrast to other types of viral hemorrhagic fever, Lassa fever usually develops gradually, after a 1–3-week incubation period. The syndrome includes fever, sore throat, headache, and myalgias, which may progress to severe illness characterized by neurologic dysfunction and shock. Occasional cases show signs of meningitis, and virus has been isolated from the cerebrospinal fluid of a limited number of patients. Unlike the New World viruses described later, however, Lassa fever virus does not produce a delayed neurologic syndrome. Although hemorrhage may occur in severe cases, it is not a prominent part of the syndrome. Deafness is a major complication of the disease.

Virus may be isolated from the blood throughout the course of Lassa fever, and it is also present in urine, pharyngeal secretions, and other body fluids. The prognosis at the time of hospital admission is strongly determined by the level of virus in the blood; patients with high levels are at greatest risk of death. The severity of hepatic involvement, as indicated by the serum aspartate aminotransferase level, also correlates with a fatal outcome. A majority of patients already have IgM antibody to Lassa virus at the time of hospital admission, and about half have IgG. Antibody and virus may cocirculate throughout the course of the illness, with no evident relationship to the eventual outcome. The induction of cell-mediated immunity appears to be a more important factor than the humoral response in controlling and eliminating viral replication.

A large study in Sierra Leone that compared Lassa fever patients treated with ribavirin with previous patients with a similar severity of illness demonstrated a significant benefit of oral or intravenous therapy. For those with high viremia or significant liver involvement, treatment with oral or intravenous ribavirin reduced mortality from roughly 50% to <20%. Therapy was most effective if begun within 6 days after the onset of illness. Immune plasma was effective in treating Lassa virus–infected laboratory primates, either alone or in combination with ribavirin, but did not provide any benefit in limited clinical trials, perhaps because titers of neutralizing antibodies were too low to produce an effect. A number of experimental vaccines are effective in laboratory animals, but none has advanced to human studies. At present, control measures focus on reducing exposure to the virus by eliminating rodents from inhabited areas.

Lujo virus

Lujo virus was identified in an outbreak of hemorrhagic fever cases that originated in Lusaka, Zambia but carried over to Sandton, South Africa where the index case was transferred for treatment. There were a total of five cases with four fatalities. In contrast to Lassa fever, which is rarely transmitted person to person, three patients acquired their infection through exposure to the index patient or a secondary case, and one through contact with contaminated materials. The one survivor was treated with ribavirin following disease onset. Lujo virus was determined to be genetically similar to Old World arenaviruses, but within a distinct genetic branch that was more similar to New World viruses.

Argentine hemorrhagic fever (Junin) virus

Argentine hemorrhagic fever, caused by Junin virus, is the most common human arenaviral infection in the Western Hemisphere. Until vaccination was introduced for populations at greatest risk, up to 600 cases were diagnosed each year in grassland areas of Argentina. The maintenance host is a field vole (*Calomys* species) that lives along the margins of cultivated fields. Unlike in Lassa fever, exposure to Junin virus occurs during farming activity, most commonly during the harvest season. Infection does not spread from person to person.

The majority of Argentine hemorrhagic fever patients suffer a severe illness; about one-third develop life-threatening hemorrhagic or neurologic complications. The overall case-fatality rate is ~15%. Compared to other types of viral hemorrhagic fever, there is little hepatic involvement. Patients tend to develop a relatively low viremia, and the resolution of illness is accompanied by the development of neutralizing antibodies. In a small percentage of patients, neurologic signs and symptoms develop 4–6 weeks after resolution of the acute illness. This encephalitic phase tends to resolve uneventfully, but is occasionally fatal.

Treatment with pooled plasma collected from survivors reduced mortality from Argentine hemorrhagic fever in a large clinical trial, provided it was initiated before the eighth day of illness. Therapy has since been standardized, based on the titer of neutralizing antibodies in the plasma and the weight of the recipient. Although antiserum treatment reduces the severity of the acute illness, it does not prevent development of the delayed neurologic syndrome.

A live, attenuated variant of Junin virus (Candid 1) has been in use as a single-dose vaccine in Argentina for more than a decade. Its efficacy was demonstrated in a placebo-controlled trial involving 6500 agricultural workers, in which only one new case occurred among vaccine recipients, compared to 22 among controls. It has since been administered to several hundred thousand adults at risk of infection, and it is now being used in children. Evidence from laboratory animals indicates that the vaccine also cross-protects against Machupo virus.

Other new world arenaviruses

Three other arenaviruses cause severe illness in South America, but are responsible for far fewer cases per year. Bolivian hemorrhagic fever, which occurs in savannah regions of that country, is caused by Machupo virus. The agent is maintained in a field vole (*Calomys callosus*). As in the case of Lassa virus infection, human exposure occurs through household exposure to excretions of infected animals. Its incidence has been reduced by rodent trapping efforts. The overall case-fatality rate is about 15%. Severe hemorrhage is more common in Bolivian than in Argentine hemorrhagic fever, while the latter shows a greater incidence of neurologic damage.

Another severe arenaviral disease was recognized in 1990, when a patient in Sabia, Brazil, died from an illness initially believed to be yellow fever. A virologist investigating the case became infected in the laboratory, but survived after a prolonged febrile illness. A second accidental infection occurred in 1994, when an investigator in the United States became infected through a centrifuge accident. Treatment with intravenous ribavirin was started 2 days after the onset of fever, and he recovered uneventfully. The third agent, Guanarito virus, was identified in 1989, when an outbreak of severe hemorrhagic fever occurred in central Venezuela. The virus is maintained in cotton rats and cane mice, and male agricultural workers are at greatest risk of infection. Variation in annual disease incidence appears to reflect changes in the rodent population. More than 200 cases of Venezuelan hemorrhagic fever have so far been reported, with a case-fatality rate of about 33%. Arenaviruses have also been isolated in two areas of the United States. The Whitewater Arroyo virus, first recognized in the mid-1990s, has been responsible for scattered cases of illness in California, but the Tamiami virus, identified in rodents in Florida, has not been linked to human disease.

Bunyaviruses

The bunyaviral genome consists of three separate RNA molecules, which for most members of the viral family are all of negative polarity. The large (L) segment encodes the RNA-dependent RNA polymerase, the middle (M) the two virion surface glycoproteins, and the small (S) a nucleoprotein that binds to the viral genome to form the nucleocapsid. The S segment also encodes a small

nonstructural (NSs) protein that, at least in the case of Rift Valley fever virus, has been shown to act as an inhibitor of type I interferon production by blocking the general host transcription of new messenger RNA in the nucleus, in part by promoting degradation of the dsRNA-dependent protein kinase (PKR).

The bunyaviruses that cause hemorrhagic fever have the widest geographic distribution of the agents discussed in this article. Most are transmitted to humans by the bites of arthropods, with the important exception of the hantaviruses, which are spread through the aerosolized excretions of infected rodents. With the exception of Rift Valley fever, which can occur as large mosquito-borne epidemics, most cases of bunyaviral hemorrhagic fever are sporadic and solitary. Recent years, have seen a marked increase in cases of Crimean-Congo hemorrhagic fever in Turkey and Iran, where almost all infections appear to result from exposure to infected ticks. In addition to the fever, vascular leak and rapid development of shock that characterize all types of viral hemorrhagic fever, some bunyaviral infections have unique features: renal disease, in the case of Old World hantaviral infections; ophthalmic and neurologic involvement in Rift Valley fever; and fulminant respiratory failure in the case of New World hantaviral disease.

Crimean-Congo hemorrhagic fever virus

As its name implies, Crimean-Congo hemorrhagic fever occurs over a vast geographic area. The virus was first isolated during an outbreak in the Crimean peninsula of the Soviet Union during World War II. During the 1950s, another novel agent was recovered from a patient in Congo, and testing subsequently showed that the two pathogens were identical. Crimean-Congo hemorrhagic fever has since been found in a huge arc extending from western China across southern Asia into eastern Europe, and from Turkey southward to the tip of Africa. In all of these areas, the virus is maintained through continuous infection of ticks, which carry it to a variety of animals, including rodents, hares, cattle, goats, sheep, and birds, all of which may serve as amplifying hosts. With the possible exception of ostriches, none of these animals show signs of illness. The wide geographic distribution of Crimean-Congo hemorrhagic fever virus appears to be explained by the role of migratory birds in carrying infected ticks.

Humans are exposed to the virus in several ways. Most cases occur singly, when inhabitants of rural areas are bitten by infected ticks. Small outbreaks can also take place when slaughterhouse employees handle meat from infected animals or are exposed to aerosolized body fluids. Interestingly, there is no report of human infection through meat purchased in a market, suggesting that the virus is inactivated soon after the death of its animal host. Human-to-human transmission through contact with body fluids takes place most often in hospital settings, before the nature of a patient's illness has been recognized. Spread to health care workers can be prevented through standard barrier nursing measures.

The clinical course of Crimean-Congo hemorrhagic fever resembles that of other types of severe hemorrhagic fever. After a 3–6-day incubation period, it typically begins with the sudden onset of high fever, severe myalgias, abdominal pain, and vomiting. Gastrointestinal symptoms are sometimes so prominent that patients undergo unneeded surgery, exposing the hospital staff to infection. The presence of hyperemia of the face and chest, conjunctival congestion, and a petechial rash on the trunk may help to distinguish the disease from other febrile syndromes. Extensive bruises on the extremities and oropharyngeal and gastrointestinal bleeding often appear during the first week. As the illness progresses, a fall in blood pressure leads to shock, multiorgan failure, and coma. The case-fatality rate has variably been reported to range from 5% to 50%.

Laboratory tests during the course of Crimean-Congo hemorrhagic fever show shifts in white blood cell counts, as described earlier, with rising levels of liver enzymes in the plasma and the development of disseminated intravascular coagulation. Patients with the most severe liver involvement and coagulopathy are most likely to die from the disease. A specific diagnosis can be made through antigen-capture enzyme-linked immunosorbent assay or polymerase chain reaction testing of serum samples. Virus-specific IgM and IgG can generally be detected within 10 days after the onset of illness.

Ribavirin therapy appears to be beneficial, based on reports of its use in hospital-based outbreaks, but no controlled trials have been performed. Research on the disease has been hindered by the fact that the virus does not cause illness in nonhuman primates or other laboratory animals, except for suckling mice. Limited autopsy data have shown that the disease shares many features with Ebola hemorrhagic fever, including extensive infection of macrophages, infection and necrosis of hepatocytes, and a massive loss of lymphocytes.

Rift Valley fever virus

Rift Valley fever virus causes disease in livestock and humans across the entire extent of sub-Saharan Africa, extending north along the Nile Valley into Egypt. Both isolated cases and large mosquito-borne outbreaks occur. An epidemic in 1977 involved more than 200 000 human infections in Egypt, with a case-fatality rate of 0.3%. The recent spread of infection across the Red Sea to Yemen, carried by the sheep trade, has raised concern that the virus may become established in the Middle East. The virus is maintained through cyclic infection of *Culex* mosquitoes and rodents. Large epidemics are associated with increases in vector populations, when *Aedes* mosquitoes and biting flies carry the virus to farm animals, particularly sheep, and to humans, both of which develop high viremia and thus serve as amplifying hosts for further transmission. Current methods of satellite imaging and rainfall prediction are facilitating advanced planning for disease outbreaks. The virus induces abortion in livestock, and placental tissues are a source of infection for farmers. People who butcher animals may also acquire the disease through contact with contaminated blood or tissues.

Rift Valley fever is highly varied in its clinical manifestations. The majority of patients develop a nonspecific illness with fever, headache, myalgias, and gastrointestinal symptoms, which usually resolves within 1–2 weeks. About 5% develop hemorrhagic fever, in which a petechial rash and mucosal bleeding, accompanied by signs of liver involvement, develop within the first few days of illness. Severely ill patients show elevated circulating liver enzymes and jaundice, and may die from hepatic failure. Others develop central nervous system disease, in which neurologic abnormalities appear during the second week of illness beginning with

headache and progressing to severe or fatal encephalitis. A disabling complication unique to Rift Valley fever is ocular involvement, manifested as retinal inflammation and hemorrhage, often bilateral, which can result in partial or complete loss of vision in up to half of cases. These changes may develop days to weeks after the acute illness has resolved.

There is no specific therapy for Rift Valley fever. Ribavirin has been protective in animal models of the disease, but has given equivocal results in humans. A formalin-inactivated, cell culture–derived vaccine is in widespread use in Africa, and is available in the United States as an investigational new drug. Control measures during outbreaks focus on vector control and vaccination of livestock to restrict the spread of infection.

Old world hantaviruses

The expression 'hemorrhagic fever with renal syndrome' designates a spectrum of diseases caused by various species of hantaviruses that are found across Eurasia, from the Korean peninsula and China to Scandinavia and the Balkans. The agents are maintained through chronic infection of various rodent species. The most severe form of illness occurs in the eastern portion of the former Soviet Union, Korea, and China, where it has been recognized for centuries. When it came under intensive investigation as a result of the unexpected occurrence of severe illness among UN soldiers during the Korean War, the disease became known as Korean hemorrhagic fever. It is estimated that more than 100 000 cases occur annually in rural areas of China. Its causative agent, Hantaan virus, is carried by the field mouse *Apodemus agrarius*. Seoul virus, which chronically infects urban rats, causes a less severe form of disease. In Scandinavia, the Puumala species of hantavirus also causes a milder illness, designated nephropathia endemica. Dobrava virus, a hantavirus indigenous to the Balkans, produces a more severe disease that resembles Korean hemorrhagic fever.

The epidemiology of hemorrhagic fever with renal syndrome is determined by the frequency with which humans come into contact with virus-infected animals. Farmers, soldiers, and others whose daily activities bring them into close proximity with rodents or with materials contaminated by their excretions are at greatest risk of infection. Contact may also occur within homes, especially when the coming of winter causes rodents to move indoors in search of warmth. Laboratory colonies of mice and rats were at one time a source of infection, but screening programs have now eliminated infected animals.

Hemorrhagic fever with renal syndrome develops through five stages: febrile, hypotensive, oliguric, diuretic, and convalescent. Following an incubation period of 1–4 weeks, it begins with the abrupt onset of high fever, chills, malaise, headache, and muscle pain. Flushing of the skin and conjunctival hemorrhages may be noted on physical examination. Laboratory tests show an increase in the total white blood cell count, with mild to moderate thrombocytopenia. After about a week, a hypotensive stage begins, which may last from hours to days; it is during this phase that most patients seek medical attention. A specific diagnosis can usually be made, based on the presence of virus-specific IgM in the serum. Hemorrhagic phenomena, including mucosal bleeding, may occur, and some 10–15% of patients go into shock.

The stage of hypotension is also characterized by the first signs of renal dysfunction, including proteinuria and a loss of urine concentrating ability. With the onset of the subsequent oliguric phase, the blood pressure may become elevated, urea nitrogen and creatinine levels increase, and the urine output falls. The combination of hypertension and impaired coagulation may lead to intracerebral hemorrhage. The introduction of hemodialysis has reduced the case-fatality rate. Recovery is heralded by the onset of the diuretic phase, usually toward the end of the second week of illness. The final convalescent phase lasts days to weeks; anemia and loss of urine concentrating ability may persist beyond that time. Ribavirin therapy can improve the outcome of illness, provided it is initiated early in the disease course.

In contrast to the severe illness just described, nephropathia endemica (discussed earlier) has a shorter, biphasic course and a lower case-fatality rate. It generally begins with the sudden onset of fever, chills, headache, and myalgias. As the fever resolves, renal dysfunction appears, with proteinuria, loss of urine concentrating ability, and elevated blood urea nitrogen and creatinine. A brief period of oliguria is followed within days by polyuria and the resolution of illness. Hemorrhage is not observed.

New world hantaviruses

Up until 1993, hantaviruses were known only as the causative agents of hemorrhagic fever with renal syndrome in Europe and Asia. In that year, however, an outbreak of an acute febrile illness with rapidly progressive respiratory failure and a high case-fatality rate was reported in the Four Corners area of the American Southwest. To the great surprise of investigators, a previously unknown species of hantavirus, now designated Sin Nombre virus was isolated from patients. The agent is now known to chronically infect local deer mice, and retrospective studies found that the disease has occurred sporadically in the region for decades. The surge of cases that brought the disease to medical attention in 1993 was an indirect effect of increased rainfall the previous year, associated with an El Niño phenomenon, which produced an abundant food supply for local deer mice, a massive expansion in their population, and greater human exposure.

Because the principal disease manifestation of patients in the Four Corners area was rapidly progressive respiratory failure, the newly recognized condition was designated hantavirus pulmonary syndrome. Over the subsequent two decades, many additional cases have been diagnosed in numerous sites in North, Central, and South America. Within each geographic area, the local hantavirus is maintained in a unique rodent host. Many of these viruses are now recognized as discrete species, named according to the location where they were first isolated. Most produce a similar illness, with the exception of the Andes virus, found in Chile and Argentina, which is the only agent for which person-to-person transmission has been clearly demonstrated.

As in the case of the Old World hantaviral diseases, humans become infected with the agents of hantaviral pulmonary syndrome through exposure to the excretions of infected rodents, probably in the form of aerosols of dried urine or feces. Following an incubation period of 1–3 weeks, there is an abrupt onset of fever, chills, myalgias, headache, and nonspecific gastrointestinal

symptoms. After 3–4 days, the patient develops respiratory distress, which rapidly worsens. The chest X-ray is normal during the early, febrile phase of illness, but bilateral pulmonary edema, typical of acute respiratory distress syndrome, develops rapidly once respiratory symptoms appear. The majority of patients who are hospitalized at this point require intubation and mechanical ventilation. Blood tests show an increased white blood cell count, with immature granulocytes and immunoblasts, and a drop in the platelet count. About half of patients develop irreversible lactic acidosis and shock and die within 2–4 days, while the rest undergo a slow, but complete recovery. Although ribavirin is active against these viruses in cell culture, clinical studies have failed to demonstrate any benefit from treatment, probably because patients are already too sick at the time of hospital admission for an antiviral drug to modify the further course of illness. The use of Extracorporeal Membrane Oxygenation (ECMO) has been shown to be effective at reducing predicted mortality in patients demonstrating advanced shock or respiratory failure due to onset of pulmonary edema.

The underlying physiologic abnormality in hantaviral pulmonary syndrome appears to be a vascular leak that is largely limited to pulmonary blood vessels, caused by the effects of inflammatory mediators on endothelial lining cells. Autopsy studies have revealed viral antigens in endothelial cells of the lungs, where interstitial mononuclear cell infiltrates and hyaline membranes are also present. Research on the disease has benefited from the recent development of a model in hamsters infected with the Andes hantavirus.

Filoviruses

The genus name of the filoviruses (from the Latin *filum* meaning thread) reflects their unique filamentous structure. The two species, Marburg and Ebola, are identical in morphology. Virions average 80 nm in diameter, but may be up to 1 μm in length. The genome consists of a single 19-kb strand of negative-sense RNA. Transcription of viral genes and replication of the genome are performed by a complex of the RNA-dependent RNA polymerase and three other virus-encoded proteins. Marburg and Ebola viruses replicate in an identical manner, except that the primary glycoprotein gene product of all Ebola species is a C-terminally truncated protein ('sGP') that is secreted into the extracellular medium. Production of the full-length membrane GP requires the post-transcriptional addition of a nucleotide to its messenger RNA. No role has been identified for sGP in the pathogenesis of Ebola hemorrhagic fever in humans, but it might contribute to viral persistence in the maintenance host.

Ebola and Marburg viruses infect a far broader range of host cells than any other agent of viral hemorrhagic fever. The virus initially disseminates to monocytes and macrophages in lymph nodes, liver, spleen, and other tissues, from which it spreads further to infect parenchymal cells. Only lymphocytes and neurons are known to remain free of infection. Replication results in the formation of large nucleocapsid inclusion bodies and the budding of hundreds to thousands of new virions from the cell surface. Infected cells undergo death through necrosis.

The filoviruses are without doubt the most virulent of the causative agents of viral hemorrhagic fever, and they are certainly the most mysterious. The animal reservoir in which they are maintained in nature has not yet been demonstrated conclusively. Marburg virus has been isolated from bats in central Africa, and evidence suggests that these animals also harbor Ebola viruses, though it is not certain that they are the actual animal reservoir. The route by which humans and great apes become infected has also not been conclusively identified. Until 1989, all evidence indicated that the homeland of the filoviruses was central Africa, but in that year a novel variant of Ebola virus was unexpectedly discovered in nonhuman primates held in a quarantine facility in Reston, Virginia, after importation from the Philippines (see 'Ebola virus').

Marburg virus

Marburg was the first filovirus to be discovered, when the inadvertent importation of infected monkeys from Uganda into Europe in 1967 caused outbreaks of hemorrhagic fever among vaccine plant workers in Marburg, Germany, Belgrade, and Yugoslavia. Exposure to infected monkeys or their tissues resulted in severe disease in 31 people, of whom 7 died. Eight doctors and nurses caring for the patients also acquired the infection, but no further transmission occurred.

Since that first outbreak, all cases of Marburg hemorrhagic fever have occurred in Africa. For more than three decades, these consisted of a total of only six sporadic infections, for which no source could be determined. In 2000, however, a large epidemic came to light in Watsa, in northeastern Democratic Republic of the Congo (DRC). More than 150 cases were eventually confirmed, with a fatality rate of >80%. Genetic analysis of viral isolates showed that the epidemic had resulted from multiple independent introductions of virus into the local community of gold miners, with little secondary spread of infection. Because the miners were exposed to vast numbers of bats and their excreta during their time underground, this outbreak provided support for the proposal that bats were the reservoir host. However, no infected animals were identified.

In 2005, Marburg virus appeared unexpectedly in Angola, far from any previous site of human infection, causing some 250 cases of illness with a case fatality rate that approached 90%. Unlike in the Watsa epidemic, only a single strain of virus was isolated. The source of infection was again not known. The first known cases occurred in the pediatric ward of a provincial hospital, where transmission was amplified through the reuse of contaminated blood transfusion equipment.

Ebola virus

Ebola virus first came to the attention of the world scientific community in 1976, when two large epidemics of hemorrhagic fever occurred almost simultaneously in Zaire (the present DRC) and Sudan. The causative agents of the two outbreaks were found to be distinct, and are now recognized as separate viral species. The Zaire epidemic was centered at a mission hospital, where treatment

routinely included injections of drugs or vitamins, using syringes that were rinsed in water between patients. The inoculation of an unidentified index case of Ebola hemorrhagic fever apparently was followed by the inadvertent transmission of virus by contaminated syringes to nearly 100 other patients, all of whom developed fatal disease. Many of the doctors, nurses, and family members who cared for these patients also became infected; the final death toll was 280 of 318 cases. Another large outbreak caused by the Zaire ebolavirus occurred in Kikwit, DRC, in 1995, with a case-fatality rate exceeding 80% among more than 300 patients. Amplification in a hospital once again contributed to the large size of the epidemic: One of the initial patients developed severe abdominal pain and underwent surgery, spreading the infection to the entire operating team, apparently through exposure to aerosolized blood. Beginning in the spring of 2014, a significant outbreak of Ebola hemorrhagic fever with over 10,000 cases in Guinea, Liberia and Sierra Leone extended the endemic area of Zaire ebolavirus far to the northwest of its previously known range.

The origin of the 1976 outbreak caused by Sudan ebolavirus, which involved almost 300 patients, was also unidentified. The within-hospital transmission of virus from the initial patients to the family members and health care workers who cared for them without gloves or other forms of protection again played a major role in the large size of the outbreak. In contrast to epidemics caused by Zaire ebolavirus, the case-fatality rate for that epidemic and for two more in 1979 and in 2000 were all 50%. The 2000 outbreak, which occurred in Gulu, Uganda, was the largest yet recorded, with 425 patients.

Beginning in 1996, a new epidemiologic pattern was observed in central Africa, in which humans became infected with Zaire ebolavirus through contact with sick or dead chimps or gorillas. In the first known outbreak of this type, 19 Gabonese villagers who butchered and ate a chimp found dead in the forest developed fulminant hemorrhagic fever, and most of them died. The virus has spread among populations of great apes in Gabon and neighboring Republic of Congo, causing both a massive die-off of these animals and repeated epidemics among local residents. Because nonhuman primates die quickly from infection, they cannot serve as a natural reservoir for Ebola virus. It has been proposed that bats are the original source of infection, but conclusive proof that bats are the sole reservoir is still lacking. However, serological studies have suggested evidence of Ebola virus, or a closely related virus, in bats in China and Bangladesh suggesting that filoviruses are more widespread than has been thought. A third Ebola viral species, Tai Forest ebolavirus (formerly known as Ivory Coast), caused the death of a chimp in that country in the 1990s, and almost killed the scientist who performed its necropsy. The virus has not been detected since.

In fall 2007 and outbreak of hemorrhagic fever virus occurred in Western Uganda with 192 suspected and 42 laboratory confirmed cases of Ebola virus infection. This outbreak had a lower case fatality rate (~34%) than previous outbreaks of Ebola hemorrhagic fever. The causative agent for this outbreak was subsequently determined to be a novel virus species which was subsequently named Bundibugyo ebolavirus.

The fifth species of Ebola virus, the enigmatic Reston agent, made its world debut in 1989, not in the rain forests of Africa, but in a monkey quarantine facility in suburban Virginia, where it caused an outbreak of lethal illness in macaques recently imported from the Philippines. Several similar episodes occurred at primate holding facilities over the next few years, until the source company in the Philippines was shut down. Reston ebolavirus re-emerged in 2008 in pig populations in the Philippines. Subsequently, pigs have been shown to be a significant potential reservoir for Reston ebolavirus with bats playing a critical role in the dissemination of the virus within and between pig populations. Fortunately, there is no evidence that Reston ebolavirus infects humans as there have been no reports of human cases among people working with infected animals. These data suggest that Reston ebolavirus is avirulent for humans. However, it seems prudent to conclude that its pathogenic potential for humans is not known.

Flaviviruses

The flaviviruses have a positive-sense genome that is capped but is not polyadenylated, thus resembling a strand of messenger RNA. Following fusion of a virion with the cell membrane, the genome is released into the cytoplasm, where it is translated into a single polyprotein that is cleaved by host cell enzymes, and by a protease encoded toward the end of the viral genome, to form individual proteins. A complex of several viral nonstructural proteins then uses the viral genome as a template to synthesize negative-sense 'antigenomes', which in turn serve as templates for new genome formation.

Of the numerous flaviviruses that infect humans, five can produce a hemorrhagic fever syndrome. Two of them, Omsk and Kyasanur Forest viruses, resemble many of the viruses discussed earlier, in that they are maintained in rodents. The reservoir for Alkhurma virus, which is closely related to Kyasanur Forest disease virus, has not yet been determined. Yellow fever virus is maintained through infection of forest primates in Africa and South America, but is capable of maintaining itself through mosquito transmission from person to person. In contrast, dengue has evolved from an infection of wild primates in Southeast Asia into a disease that can persist solely in human populations. As will be discussed later, the degree of human adaptation appears to correlate inversely with the severity of the disease: Yellow fever virus can cause full-blown hemorrhagic fever, while primary dengue infections are relatively mild. The occurrence of dengue hemorrhagic fever/shock syndrome is based on a unique mechanism that reflects the virus's evolution into four distinct serotypes.

Yellow fever virus

Despite the existence of a highly effective vaccine since the late 1930s, tens of thousands of cases of yellow fever still occur each year, principally in rain forest regions of west Africa, where the virus is maintained through mosquito passage among wild primates. In the New World, where the virus was carried by the slave trade, it now occupies a similar ecological niche among monkeys in the rain forest of Brazil and adjacent countries. Sporadic cases of yellow fever occur in rural areas where sylvatic mosquitoes carry the virus to forest workers. In contrast, large outbreaks of urban yellow fever can take place when an infected individual travels to a city,

where the virus can be carried from person to person by *Aedes aegypti* mosquitoes. Fortunately, a significant urban outbreak of yellow fever has not occurred for several decades. However, historical records document major urban outbreaks in South America and North America including the southern gulf coast of the United States and as far north as Philadelphia and Boston. The capacity of yellow fever virus to leave its animal host and maintain itself in a human population suggests that its geographic distribution could eventually resemble that of dengue virus, which is found in tropical areas across the world.

As noted earlier, the partial adaptation of yellow fever virus to humans is reflected in a broad spectrum of disease severity, ranging from mild illness to full-blown hemorrhagic fever with hepatic failure and shock. Limited data from patients in west Africa and South America have shown that severe illness is marked by high levels of circulating proinflammatory cytokines, elevated liver enzymes in the serum, and disseminated intravascular coagulation. Patients develop a petechial rash, followed by mucosal and gastrointestinal hemorrhage, renal failure, and shock, leading to death 1–2 weeks after the onset of illness. Treatment is supportive. Although ribavirin inhibits yellow fever virus in cell culture and was protective in a hamster model of infection, it did not alter the course of illness in nonhuman primates.

Prevention of yellow fever is based on a combination of vaccination and mosquito control. The live, attenuated 17D chick embryo vaccine, first used in the field in 1937, is solidly protective against the disease. Travelers to endemic areas must provide evidence of vaccination within the past 10 years. The vaccine is unfortunately employed to only a limited extent in the poor and remote regions of Africa and South America where infection is most prevalent. The major mosquito vector, *A. aegypti*, breeds in collections of water associated with human habitation. It once carried yellow fever as far north as Boston, but aggressive eradication programs eliminated the mosquito and the disease from urban areas of the Western Hemisphere by the early 1930s. The subsequent abandonment of these efforts has permitted the return of *A. aegypti* to these regions, as evidenced by epidemics of dengue fever, but urban outbreaks of yellow fever have not yet reappeared.

Dengue virus

Dengue virus is transmitted from person to person by mosquitoes of the genus *Aedes* in tropical regions across the world, causing tens of millions of infections each year. Although the agent can cause silent infection in various nonhuman primate species, these animals do not play a role as a natural reservoir. It seems likely that dengue fever was at one time a geographically localized zoonosis, similar to yellow fever, but the growth of large tropical cities provided it with the 'culture flask' needed for it to evolve into an agent capable of sustained transmission among humans. The spread of dengue virus throughout the tropics has been accompanied by the development of four distinct serotypes that now circulate continuously in Southeast Asia and the Americas. Previous infection with one dengue serotype provides lifelong immunity against that type, but a subsequent infection by a different serotype may result in a severe illness, dengue hemorrhagic fever/shock syndrome.

Probably because of the virus's history of adaptation to humans, most cases of primary dengue fever amount to no more than an unpleasant flu-like illness. After an incubation period of up to a week, there is an abrupt onset of fever, headache, and muscle and bone pain, reflecting the release of proinflammatory cytokines from virus-infected monocytes and macrophages. A morbilliform rash may be present. Laboratory evaluation shows leukopenia and mild thrombocytopenia, but blood coagulation remains normal. By the time of symptom onset, virus is no longer circulating in the blood, and the illness resolves uneventfully.

A small percentage of dengue patients develop a hemorrhagic fever syndrome, in which the symptoms just described are followed by a fall in blood pressure and coagulation defects that may lead to bleeding and shock. In contrast to the classic dengue, virus is present in the blood after the onset of illness, accompanied by high levels of proinflammatory mediators such as interleukin-6 and tumor necrosis factor-α. Although some of these cases may reflect an encounter with an unusually virulent viral strain, most of them result from a second infection by a different serotype, and are thus are a consequence of the genetic heterogeneity of the virus.

Two immune mechanisms appear to be responsible for the occurrence of dengue hemorrhagic fever. First, in a person with a previous history of dengue virus infection, nonneutralizing antibodies in the plasma can enhance the severity of a heterologous infection by binding to circulating virions, then linking them to the surface of monocytes through attachment to surface Fc receptors, leading to fusion and virus uptake. This 'virion delivery system' increases the number of infected cells, the number of viral particles that enter each cell, and the magnitude of the resulting cytokine response. Second, cross-reactive memory $CD8^+$ T cells remaining from an earlier infection may recognize viral epitopes expressed on the surface of infected monocytes and attack them, triggering an explosive inflammatory response. Both mechanisms increase the release of vasoactive mediators far above that seen in ordinary dengue, causing a hemorrhagic fever syndrome. However, because tissue damage is minimal and adaptive immune responses remain intact, patients who are treated promptly with intravenous fluids can recover within a matter of days.

Efforts to develop effective dengue vaccines have been under way for many years, but field trials have been hindered by the concern that a partially protective vaccine might elicit only nonneutralizing antibodies, rendering subjects susceptible to hemorrhagic fever on subsequent exposure to a natural infection. In the absence of vaccines, prevention efforts include mosquito avoidance, spraying, and destruction of breeding sites. The recent introduction of *A. albopictus*, a vector that can breed in swamp and woodland areas in cooler climates, into the Western Hemisphere has extended the potential range of the disease into temperate regions and made control strategies much more problematic.

Other flaviviruses

The other three flaviviral agents of hemorrhagic fever are more typical zoonoses, in that the viruses are maintained in animals and transmitted to humans within confined geographic areas. Omsk hemorrhagic fever occurs in western Siberia, in the regions around

Omsk and Novosibirsk. The causative agent is maintained in water voles, in which it apparently does not cause illness, but it can cause severe or lethal infection in muskrats, which have recently been introduced to the area. Humans become infected through direct contact with muskrats during trapping or by tick bite, and apparently may also acquire the illness by drinking contaminated water. The virus can also spread to sheep and goats and be transmitted through their milk. The case-fatality rate of Omsk hemorrhagic fever is in the range of 1–3%. The agent shows antigenic cross-reactivity with tick-borne encephalitis virus, so that locally produced vaccines against the latter provide a degree of cross-protective immunity.

Kyasanur Forest disease is found primarily in Mysore State, India, where several hundred cases occur each year. The virus is maintained in rodents and transmitted by *Hemaphysalis* ticks to monkeys and other animals; humans usually become infected through a tick bite. Kyasanur Forest disease generally presents as a flu-like illness but can occur as a biphasic disease with the second phase more severe with occasional evidence of hemorrhage, although this is not a frequent manifestation of the disease. The case fatality rate for Kyasanur Forest disease is around 4% with those recovering from the disease suffering few long-term sequelae. A formalin-inactivated vaccine is licensed for use in India.

Alkhurma (aka Alkhumra) hemorrhagic fever was first identified in abattoir workers in the Saudi Arabian city of Jeddah in the mid-1990s. This virus was initially identified as being among the tick-borne encephalitis serocomplex of flaviviruses and was subsequently shown by genetic analysis to be closely related to Kyasanur Forest disease virus. Infection with Alkhurma virus has been documented in about 50 human cases, primarily in Saudi Arabia. However, evidence suggested that Alkhurma virus may also be found in southern Egypt as reports of two exported cases to Italy indicates that both patients visited camel markets in Egypt. Alkhurma virus has been isolated from *Ornithodoros savignyi* and *Hyalomma dromedarii* ticks in Saudi Arabia suggesting that ticks are the principal vector for this virus.

Vaccines

The 17D yellow fever vaccine is the only vaccine for protection against a hemorrhagic fever virus that is licensed worldwide. However, a number of vaccines are approved for use in individual countries or are currently in clinical trials.

Arenaviruses

Junin virus

The Candid #1 vaccine for protection against Junin virus infection is the only available vaccine for an arenavirus and is only available for use in Argentina. It is a live, attenuated vaccine that was generated through serial passage of wild-type strain XJ 44. The Candid #1 vaccine is well tolerated and Junin virus specific neutralizing antibodies can be detected within 15 days postvaccination with over 95% of vaccinees seroconverting within 60 days of vaccination. Vaccination campaigns in Argentina have significantly reduced the number of cases of Argentine hemorrhagic fever.

Bunyaviruses

Crimean-Congo hemorrhagic fever virus

A vaccine for protection against Crimean-Congo hemorrhagic fever has been in use in Bulgaria since 1974 to protect individuals living or working in endemic areas. This is the only actively used vaccine for Crimean-Congo hemorrhagic fever. This vaccine is an inactivated mouse brain preparation that was originally developed in the Soviet Union in 1970. There is very little clinical use data for this vaccine and its safety and efficacy are unclear. However, reports indicate that there has been a marked decrease in the number of Crimean-Congo hemorrhagic fever cases in Bulgaria since initiation of vaccine efforts.

Rift valley fever virus

The live, attenuated Smithburn strain of Rift Valley fever virus is used in sub-Saharan Africa to prevent or limit outbreaks in livestock. This virus was first reported in 1949 following serial passage of a wild-type virus through mouse brains. While the use of the Smithburn vaccine has met with some success, there are reports of adverse responses, including abortions, in vaccinated animals. The MP-12 vaccine was generated by serial passaging of wild-type virus ZH548 in MRC-5 cells in the presence of the mutagen 5-flurouracil. The MP-12 has subsequently been tested for safety and efficacy in a number of agricultural species including sheep and cattle. MP-12 has also been shown to protect non-human primates in direct virus challenge studies. A phase II clinical trial assessing vaccine safety and immune response stimulation in human volunteers has also been completed.

Filoviruses

While there are no licensed vaccines for protection against filovirus infection, there are a number of candidates that have shown some promise in animal studies. Perhaps the two leading candidates include an adenovirus-based vaccine that has been tested in several clinical trials in Africa and a vesicular stomatitis virus (VSV) vectored vaccine that has yet to be included in clinical trials. In addition to showing protective efficacy in primate challenge studies, the VSV-based vaccine has also shown potential as a post-exposure option if given shortly after exposure. A third live-virus based vaccine is a recently developed vaccine using the live-attenuated rabies vaccine strain as the vector for a potential "double hit" vaccination against rabies and ebolavirus which would be

beneficial in human and non-human primate populations in Africa. Given the size of the outbreak in West Africa, in late 2014 the adenovirus and VSV vaccines were pushed into phase I and phase II/III clinical trials.

Flaviviruses

Yellow fever virus

The live, attenuated yellow fever vaccine strain 17D was first developed in 1937 and is still in use world-wide. It was generated by extended serial passage origination the original yellow fever virus isolate Asibi. Over 500 million doses of the 17D vaccine have been given with very few instances of vaccine failure or vaccine-associated illness. A single dose of the vaccine provides protective immunity within 10 days postvaccination in nearly all vaccinees and evidence suggests that immunity is life-long. Current recommendations require a booster vaccination every 10 years.

Dengue virus

Development of a vaccine to protect against dengue virus infection has been one of the greatest challenges in virus vaccinology, because dengue virus exists as four distinct serotypes, and epidemiologic studies have shown that antibodies elicited by one serotype may induce enhanced disease upon secondary infection by a different serotype. A successful dengue vaccine must therefore produce protective neutralizing antibody titers against all four serotypes. A number of attenuated virus and recombinant virus or subunit vaccines have been tested over the years with limited success. There are currently several tetravalent dengue vaccines that show considerable promise, but additional clinical trials are necessary.

Kyasanur Forest disease virus and Alkhurma virus

As mentioned above, a formalin-inactivated vaccine is available in India for protection against Kyasanur Forest disease virus. There are also several vaccines available for tick-borne encephalitis virus that are licensed in Europe and Russia. Kyasanur Forest disease virus and Alkhurma virus are both members of the tick-borne encephalitis serocomplex of flaviviruses and share significant amino acid sequence homology with other members of the serocomplex. Although not conclusively proven or disproven, antigenic similarity between viruses within the tick-borne encephalitis serocomplex suggests that the licensed vaccines should provide some level of cross-protection.

Conclusion

The hemorrhagic fever viruses constitute a small subset of the vast number of different viruses that are encountered by humans over the course of life. Adapted through coevolution to persist in animals, these agents are fortuitously capable of replicating to high yield in human macrophages and suppressing type I interferon responses, permitting their rapid systemic dissemination. The resulting release of large quantities of proinflammatory mediators into the circulation causes a syndrome of coagulopathy and vascular collapse resembling septic shock.

Because the host response to hemorrhagic fever virus infection appears to be largely self-destructive, it is tempting to conclude that the human immune system is ill-equipped to deal with microbial threats. In fact, a defensive strategy based on the automatic release of proinflammatory mediators by macrophages whenever they encounter a microbe works well for the majority of infectious processes, which are localized in nature, and fails only when the unrestricted spread of a pathogen causes inflammation to occur 'everywhere at once'. Although the resulting severe illness is a disaster for the affected individual, it may actually benefit the larger population by quickly immobilizing (and often killing) the sick person, reducing the likelihood of further disease transmission. From an evolutionary point of view, inflammatory responses may therefore be seen as a 'double-edged sword', useful both to control minor infections and to hinder the cross-species transfer of novel pathogens.

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Hepatitis Viruses☆

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Glossary

Adjuvant Substance added to a vaccine to help stimulate the body's immune response to the target antigen, by increasing the antibody response and providing longer protection.
Alanine transaminase (ALT, formerly SGPT) An enzyme found predominantly in liver, released into the serum as a result of liver injury. In most liver diseases, ALT is elevated to a greater level than the aspartate transaminase (AST), and in acute viral hepatitis levels of >1000 IU mL^{-1} are not uncommon.
Alkaline phosphatase (ALP) An enzyme that is made in the liver, bone, and the placenta and can be measured in serum. It is increased in the setting of diseases in which bile secretion is impaired, and to a lesser degree in association with hepatocellular injury.
Angioedema A skin eruption similar to urticaria but affecting both the dermis and the subcutaneous tissue (deeper skin layer).
Ascites Abnormal accumulation of fluid in the abdominal cavity. Ascites is associated with advanced liver disease complicated by portal hypertension but can be seen in other conditions including abdominal malignancies.
Aspartate transaminase (AST, formerly SGOT) An enzyme found in many tissues including the liver, heart, muscle, kidney, and brain. It is released into serum when any one of these tissues is damaged, for example, after a myocardial infarction or muscle injury. Although not a highly specific indicator of liver injury, levels of >500 IU mL^{-1} suggest acute hepatitis.
B cells These lymphocytes mature in the bone marrow and are responsible for humoral immunity.
Bilirubin A chemical that results from the breakdown of red blood cells. Increased levels in serum result from increased red cell destruction (hemolysis), decreased uptake by the liver, or decreased excretion into the bile. High levels in the serum are associated with jaundice.
Cholestasis Caused by obstruction to bile flow within the liver (intrahepatic) or outside the liver (extrahepatic). The obstruction causes bile salts, the bile pigment bilirubin, and fats (lipids) to accumulate in the blood instead of being eliminated normally.
Cirrhosis The final common histological pathway for a wide range of chronic liver diseases, the hallmark of which is the formation of microscopic or macroscopic nodules of normal liver tissue separated by bands of fibrous tissue. Injury to the liver cells results in an inflammatory response that leads to the formation of scar or fibrous tissue. The liver cells that do not die replicate to replace the cells that have died, resulting in clusters of newly formed liver cells (regenerative nodules) within the fibrous tissue.
Cytopathic Pertaining to or characterized by pathological changes in cells.
Cytopenias A reduction in the number of cells circulating in the blood, which can take several forms; anemia is a decrease in red cell count, leucopenia is a decrease in white cells, and thrombocytopenia is a reduction in platelets. When all three classes of blood cells are decreased, the condition is known as pancytopenia.
Cytotoxic T lymphocytes Lymphocytes that mature in the thymus gland and generate cell-mediated immune responses, directly destroying cells that have specific antigens on their surface recognizable by these T cells.
Deoxyribonucleic acid (DNA) The genetic material of all cellular organisms and most viruses. A molecule of DNA consists of two strands composed of nucleotides, linked together to form a chain in the form of a double helix. DNA carries the information needed to direct protein synthesis and replication.
Enteral Entry of a substance or organism (such as a virus) into the body via the gastrointestinal tract.
Epitope Also known as the antigenic determinant, this is the specific part of an antigen that can be recognized by an antibody.
Glomerulonephritis An inflammation of the glomeruli, bunches of tiny blood vessels inside the kidneys. The damaged glomeruli cannot effectively filter waste products and excess water from the bloodstream to make urine.
HBV cccDNA Hepatitis B virus covalently closed circular DNA is a continuous double-chain ring, which serves as the template for all viral RNA transcription.
Hepatic portal system A portal system is a capillary bed draining into another capillary system through veins before returning to the heart. The hepatic portal system refers to the circulation of blood from parts of the gastrointestinal tract, via the hepatic portal vein into the hepatic sinusoids.
Hepatic steatosis One of a spectrum of liver diseases termed nonalcoholic fatty liver disease (NAFLD) that have in common the accumulation of excess fat in liver cells. These conditions range from simple steatosis (excess fat without inflammation), to

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nonalcoholic steatohepatitis (NASH), through to cirrhosis. The term nonalcoholic is used because NAFLD occurs in individuals who do not consume excessive amounts of alcohol, but the appearance of the liver microscopically is similar to that seen in alcoholic liver disease. NAFLD is associated with insulin resistance and the metabolic syndrome.

Hepatomegaly Enlargement of the liver.

Humoral immune response The production of antibodies that circulate through the blood and other body fluids, binding to antigens, and helping to destroy them.

Insulin resistance (IR) The uptake of glucose into tissues is stimulated by insulin, in the setting of IR, tissues have an impaired responsiveness to the actions of insulin. In an effort to maintain normal glucose levels, the pancreas secretes more insulin, leading to high plasma insulin levels. Over time the pancreas is unable to overcome IR through hypersecretion and overt diabetes develops.

International normalized ratio (INR)/prothrombin time These laboratory tests are measures of the extrinsic pathway of coagulation, and are a sensitive method of assessing liver function. The INR is the ratio of a patient's prothrombin time to a normal (control) sample.

Jaundice The yellowish coloration of the skin and sclerae (the whites of the eyes) observed when bilirubin levels are increased above a certain level. Also referred to as icterus.

Lipoprotein Classes of conjugated proteins in which proteins are combined with a lipid (fat) such as cholesterol. These complexes are the form in which lipids are transported in the circulation. Lipoproteins are classified by their density and chemical properties.

Lymphocyte Specialized white blood cells whose function is to identify and destroy invading antigens, are subdivided into B and T cells.

Major histocompatibility complex (MHC) A large cluster of genes located on the short arm of chromosome 6, which is traditionally divided into the class I, II, and II regions, each containing groups of genes with related functions. Many, but not all of the genes in this complex play important roles in the immune system.

Metabolic syndrome This syndrome can be defined as a number of related conditions, including obesity, hypertension, abnormalities of lipid metabolism, and type 2 diabetes, that are associated with IR and compensatory hyperinsulinemia.

Parenteral Entry of a substance or organism (such as a virus) directly into the bloodstream, via a device such as needle or catheter.

Polyarteritis nodosa A disease of unknown etiology, possibly due to hypersensitivity to an unknown antigen, causing inflammation and necrosis of medium-sized muscular arteries, with secondary ischemia of tissue supplied by affected vessels.

Portal hypertension Elevated blood pressure in the portal vein and its branches, resulting from intrahepatic or extrahepatic portal venous compression or occlusion. In the United States and Europe, the commonest cause is increased resistance to blood flow caused by extensive scarring of the liver in cirrhosis. Increased pressure in the portal circulation causes the formation of new veins called collaterals that develop at specific places, most importantly at the lower end of the esophagus and upper part (fundus) of the stomach.

Purpura Hemorrhages in the skin and mucous membranes having the appearance of purplish spots or patches.

Ribonucleic acid (RNA) A molecule of nucleic acid that differs from DNA by containing ribose rather than deoxyribose. RNA is formed on a DNA template. Several differing molecular classes of RNA are produced (messenger, transfer, and ribosomal) that play roles in the synthesis of protein and other cell functions. It reflects the exact nucleoside sequence of the genetically active DNA.

Splenomegaly Enlargement of the spleen.

Transient liver elastography A noninvasive technique for the evaluation of fibrosis in chronic liver disease, transient elastography uses a special probe that consists of an ultrasound transducer array mounted on the axis of a motor vibrator. Liver hardness (or stiffness) is evaluated by measuring the velocity of a vibration wave (also called a "shear wave") generated by the probe placed on the skin overlying the liver. Shear wave velocity is determined by measuring the time the vibration waves takes to travel to a particular depth inside the liver. As fibrous tissue is harder than normal liver, the degree of fibrosis can be inferred from liver hardness.

Urticaria A skin eruption consisting of localized wheals and erythema ("hives") affecting only the dermis (superficial skin layer).

Varices Abnormally enlarged and convoluted veins, prone to bleeding, seen in the lower esophagus and stomach in association with portal hypertension.

Vasculitis A group of diseases in which the primary pathology is inflammation of the blood vessels. Each of these diseases is differentiated by the characteristic distributions of blood vessel and organ involvement, and laboratory test abnormalities. Underlying immune system abnormality is a common feature.

Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine transaminase
Anti-HBs	Hepatitis B surface antibodies
Anti-HCV	Hepatitis C antibody
AST	Aspartate transaminase
Bili	Bilirubin
cccDNA	Covalently closed circular DNA
DAA	Direct acting antiviral
DNA	Deoxyribonucleic acid
EIA	Enzyme immunosorbant assays
EMC	Essential mixed cryoglobulinemia
HAV	Hepatitis A virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBIg	Hepatitis B immune globulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis delta virus
HEV	Hepatitis E virus
HVR	Hypervariable region
IFN	Interferon
Ig	Immunoglobulin
IgM anti-HAV	Hepatitis A IgM antibody
IgM anti-HBc	Hepatitis B core IgM antibody
IL-12	Interleukin-12
IM	Intramuscularly
INR	International normalized ratio
IR	Insulin resistance
IVDU	Intravenous drug user
LCMV	Lymphocytic choriomeningitis virus
LT	Liver transplantation
MHC	Major histocompatibility complex
NAFLD	Nonalcoholic fatty liver disease
NANB	Non-A, non-B
NASH	Nonalcoholic steatohepatitis
NHL	Non-hodgkin's lymphoma
NRTI	Nucleoside reverse transcriptase inhibitor
ORF	Open reading frame
RBV	Ribavirin
RIBA	Recombinant immunoblot assay
RNA	Ribonucleic acid
SENV	SEN virus
SVR	Sustained viral response
TNF	Tumor necrosis factor
TTV	Torque teno virus

Defining Statement

We discuss the pathogenesis of acute and chronic viral hepatitis, and the structure, replication, epidemiology, and clinical features of each hepatitis virus (A, B, C, D, and E). Current therapies for chronic HBV and HCV, vaccination against HAV, HBV, HEV, and newly identified hepatotropic viruses will also be covered.

Introduction

Hepatitis is a nonspecific term meaning inflammation of the liver (from the Greek *hepar* for liver + *itis* for inflammation) and does not necessarily imply a viral etiology. Many viruses can cause a systemic infection that may involve the liver with an acute hepatitis (e.g., cytomegalovirus, Epstein-Barr virus, and yellow fever virus). Hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis delta virus (HDV), and hepatitis E virus (HEV) are the five hepatotropic viruses responsible for most cases of acute viral hepatitis. HBV, HCV, and HDV can also cause chronic hepatitis, with the risk of progression to cirrhosis, and hepatocellular carcinoma (HCC). HEV has the potential to cause chronic hepatitis, but only in immunocompromised individuals (Table 1).

Before the identification of individual viruses, acute hepatitis was classified by British hepatologist F.O. MacCallum in 1947 as either infectious (transmitted via the fecal-oral route from person-to-person) or serum (acquired from the transfusion of blood and blood products). With the introduction of diagnostic tests in the 1960s and 1970s, HBV and HAV were identified as the major cause of serum and infectious hepatitis, respectively. In 1977, HDV was identified as a defective ribonucleic acid (RNA) virus that is dependent on HBV to replicate. However, not all individuals with acute infectious or serum hepatitis tested positive for HAV or HBV and it was strongly suspected there were additional causative viral agents. Non-A, non-B (NANB) hepatitis was characterized epidemiologically as either parenterally or enterally transmitted until two additional viruses were discovered in the 1980s: HCV and HEV. HCV was identified in 1989 as the major cause of parenterally transmitted NANB, and HEV in 1983 as the major cause of enterically transmitted NANB hepatitis. Some patients continue to have typical signs and symptoms of acute viral hepatitis, without serologic evidence of infection with any of the currently identified hepatitis viruses (non-ABCDE hepatitis).

HAV and HEV are spread via the fecal-oral route, HEV can also be transmitted from mother to baby, and via blood transfusion. HBV, HDV, and HCV are spread predominantly through parenteral exposure; sexual contact and vertical transfer from mother to infant are generally significant routes of transmission only for HBV. Until recently, sexual transmission of HCV was not considered a significant route of infection; however, there is now accumulating evidence that high-risk sexual activity is a potential HCV risk factor for men who have sex with men.

There is wide geographical variation in the prevalence of these agents, with HAV and HEV endemic in developing countries reflecting an absence of safe, clean water supplies. HBV is highly endemic in sub-Saharan Africa and Asia, and it is estimated that about 5% of the world's population are chronically infected.

Common symptoms of acute hepatitis include fever, malaise, and anorexia (decreased appetite) in the "prodromal" period, followed by the onset of dark-colored urine, pale stools, and jaundice. Although the majority will recover completely, a small proportion may develop severe fulminant hepatitis that carries a high mortality (50%) unless the individual undergoes liver transplantation (LT). Fulminant liver failure due to acute viral hepatitis is the most common emergency indication for LT. Chronic HBV and HCV infections are usually asymptomatic, unless there has been progression to cirrhosis when individuals can present with complications such as ascites and bleeding esophageal varices. Chronic HCV-related end-stage liver disease (ESLD) has been the leading indication for LT in the Western world over the past decade, however improvements in HCV therapy have already made an impact in reducing the numbers of candidates requiring LT. Nonalcoholic fatty liver disease, including the more aggressive variant of nonalcoholic steatohepatitis (NASH), is now the most prevalent chronic liver disease worldwide, paralleling the global epidemic of obesity and diabetes. NASH can progress to cirrhosis and ESLD, and is predicted to overtake chronic HCV as the commonest indication for LT within the next few years.

Histopathology of the Liver

Microscopically, the smallest functional anatomical unit of the liver is the "lobule" (Fig. 1). Each lobule consists of a branch of the hepatic vein (terminal hepatic venule) from which "plates" of hepatocytes radiate toward a number of peripheral "portal tracts," each tract composed of a biliary ductule, terminal hepatic arteriole, and terminal portal venule (the portal triad). In acute viral hepatitis, lobular rather than portal abnormalities dominate, whereas in chronic hepatitis changes are observed in the portal and periportal areas. Histological examination of the liver is not generally required in the setting of acute infection. In chronic viral hepatitis, liver histology is useful in assessing disease severity.

Table 1 The major hepatitis viruses

	Hepatitis A	*Hepatitis B*	*Hepatitis C*	*Hepatitis D*	*Hepatitis E*
Family	Picornovirus	Hepadnae	Flavi virus	Unclassified	Hepeviridae
Genus	*Hepatovirus*	*Orthohepadnavirus*	*Hepacivirus*	*Deltavirus*	*Hepevirus*
Genome	RNA	DNA	RNA	RNA	RNA
Main transmission routes	Fecal-oral	Parenteral/sexual	Parenteral	Parenteral	Parenteral/fecal-oral
Materno-fetal transmission	No	High risk	Low risk	No	High risk
Chronicity	No	Yes	Yes	Yes	Yes (in immunocompromised)
Commercially available vaccine	Yes	Yes	No	Prevented by hepatitis B vaccination	No

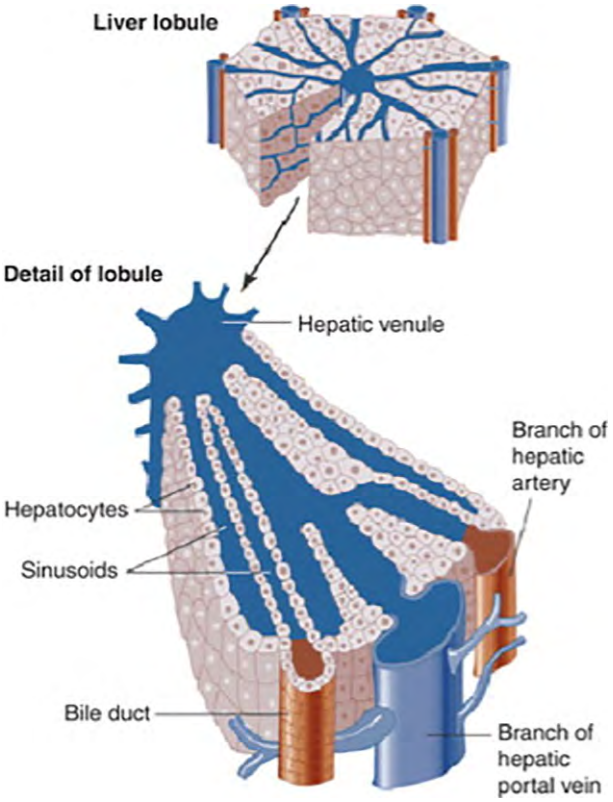

Fig. 1 The structure of the liver's functional units, or lobules. Blood enters the lobules through branches of the portal vein and hepatic artery, then flows through small channels called sinusoids that are lined with hepatocytes. Reproduced from Cunningham, C. C. and Van Horn, C. G. (2003). Energy availability and alcohol-related liver pathology. *Alcohol Research and Health* **27**(4), 281–299, with permission.

Acute Viral Hepatitis

The etiology of an acute hepatitis caused by any of the five hepatitis viruses cannot be distinguished solely by clinical or biochemical features, and requires serological testing. Common prodromal symptoms include malaise and anorexia (loss of appetite), followed by dark-colored urine, pale stools, and icterus (jaundice). Although most cases of acute viral hepatitis resolve uneventfully, some patients with acute HBV and HCV become chronically infected. A minority of patients with acute HAV, HBV, and HDV (and also with acute HCV but far less likely) will develop fulminant hepatitis with acute liver failure. Affected individuals can also be asymptomatic, identified only after the infection has resolved, by positive serology performed incidentally (e.g., before blood donation).

Histology of Acute Viral Hepatitis

The histological picture is similar in acute hepatitis caused by any of the major hepatitis viruses. Characteristic findings include lobular disarray, diffuse hepatocyte injury, ballooning, eosinophilic degeneration, and necrosis, together with a predominantly mononuclear inflammatory response in the parenchyma and portal tracts. The inflammatory infiltrates consist mainly of T-cell lymphocytes, reflecting the role of cellular immunity in the pathogenesis of hepatitis. Although "interface hepatitis," formerly referred to as "piecemeal necrosis" (the destruction of hepatocytes at an interface between liver parenchyma and connective tissue), is the defining feature of chronic hepatitis, it is also seen in acute hepatitis, particularly with HBV. Cholestasis may also be observed. As the reticular framework of the liver is usually preserved in acute hepatitis, hepatocyte regeneration and complete restoration of the liver normally occur after resolution of infection.

Typical features of acute HAV infection include hepatocellular injury and necrosis that predominates around the portal tracts, and a portal and periportal inflammatory infiltrate that contains abundant plasma cells. In adult-acquired acute HBV, histological findings are characterized by severe centrilobular necrosis and inflammation. Portal lymphoid aggregates and bile duct lesions of the Poulsen-Christoffersen type are characteristic of acute HCV, occurring rarely in other types of acute viral hepatitis (Fig. 2).

Acute Viral Hepatitis—Pathogenesis

Most hepatitis viruses are non cytopathic, liver damage in the acute as well as the chronic stages reflects the host immune response, largely controlled by CD4 T-helper lymphocytes. These immune responses are directed at viral- or self-antigens expressed on the

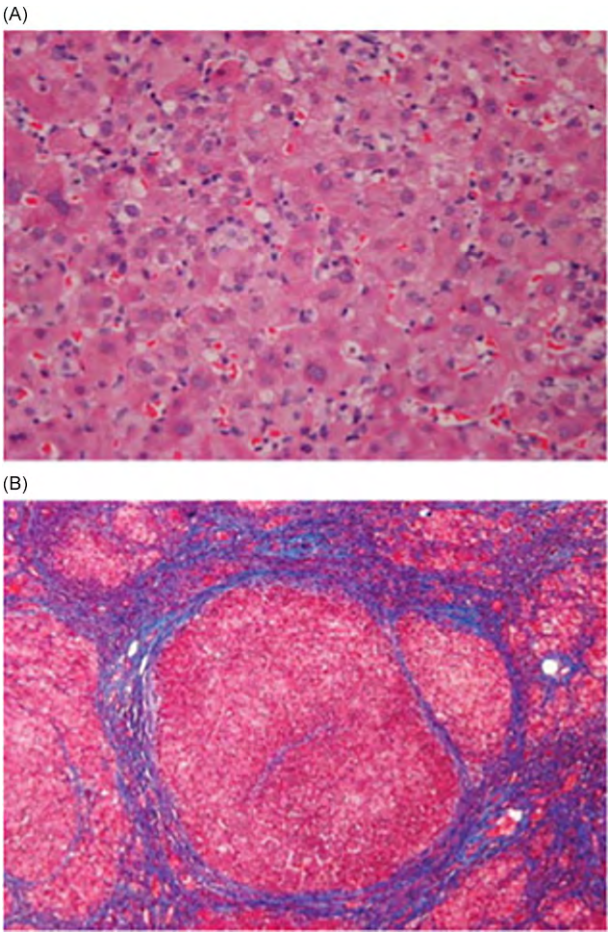

Fig. 2 Histological features of (A) acute recurrent hepatitis C in a liver allograft showing diffuse necro-inflammatory changes in the parenchyma with acidophilic bodies, inflammation, and ballooning degeneration of hepatocytes; and (B) chronic hepatitis C with nodular cirrhosis. Courtesy of Dr. S. Thung, Mount Sinai School of Medicine, New York.

surface of infected hepatocytes via the major histocompatibility complex (MHC). In acute HBV, the antiviral cytotoxic T-lymphocyte response is directed against multiple epitopes within the HBV core, polymerase, and envelope proteins. The mechanism of cytotoxic T-lymphocyte destruction of HBV-infected hepatocytes has been investigated in a mouse model, with the number of infected hepatocytes killed by direct interaction between cytotoxic T lymphocytes and their targets insufficient to explain the extent of damage observed in acute hepatitis. It has therefore been postulated that much of the injury is due to secondary antigen-non-specific inflammatory responses induced by the response of the cytotoxic lymphocytes (e.g., due to release of tumor necrosis factor (TNF), free radicals, and proteases), and also possibly due to the involvement of other immune cells such as natural killer T cells.

It is believed that the immune response to one or more viral proteins is responsible for both viral clearance and liver injury during infection.

Acute Viral Hepatitis—Clinical Features

Parenterally transmitted hepatitis viruses tend to have longer incubation periods than those transmitted via the enteric route. An incubation period of between 40 and 160 days is observed in HBV and HDV infection, 15–160 days with HCV, 14–60 days for HEV, and 10–50 days for HAV.

In symptomatic individuals, after a typical viral prodrome, symptoms of anorexia, right upper abdominal discomfort, with dark urine and pale stools, precede the onset of icterus by several days. On examination, the liver is usually enlarged/palpable and mildly tender, although marked hepatomegaly or splenomegaly is uncommon. Once the patient is icteric, fever resolves and constitutional symptoms generally improve. The duration of jaundice can vary from a few days to several weeks. In the majority of cases, acute viral hepatitis is a self-limited disease with patients recovering completely by 2–8 weeks after the onset of jaundice. Occasionally a patient may experience "biphasic hepatitis," where initial clinical improvement is followed by a relapse of signs and symptoms. This is most often seen in HAV infection; if this situation occurs in a patient with acute HBV, the possibility of acute HDV should be considered. Although wide fluctuations in alanine transaminase (ALT) occurring over weeks to months can be seen in acute HCV, a true "biphasic" course is uncommon. Approximately 10% of subjects with acute HBV initially develop a serum sickness-like syndrome

(characterized by skin rash, angioedema, and arthritis) due to circulating immune complexes of viral particles and antibody with complement activation (see "Extrahepatic Manifestations of Viral Hepatitis").

Fulminant Acute Viral Hepatitis

The majority of cases of fulminant hepatitis (>50%) are due to acute HBV (with and without HDV superinfection). Fulminant disease is only seen in a small percentage of patients with HAV (this is more likely if the disease is acquired by older adults), and very rarely associated with acute HCV. Between 1% and 2% of cases of HEV can also become fulminant, particularly when acquired by females in the later stages of pregnancy when the percentage increases to over 20%. Individuals with underlying chronic liver disease, such as HCV-related cirrhosis, who are infected acutely with HBV, HAV, or even HEV, are at increased risk for a fulminant course. In patients with chronic HBV, hepatitis flares and rarely fulminant hepatitis can accompany changes in immunological response to the virus, for example, reversion from nonreplicative to replicative infection (hepatitis B e antigen (HBeAg-to-anti-HBe seroconversion), and can also be seen in individuals with previously quiescent chronic HBV undergoing immunosuppressive or cancer chemotherapy. Although only a small percentage of patients with acute viral hepatitis develop fulminant hepatitis, altered mental status in an icteric patient should prompt referral to a transplant center because of the high mortality rate.

Acute Viral Hepatitis—Laboratory Features and Diagnosis

Acute viral hepatitis can usually be differentiated from other causes of acute jaundice by the marked elevations of two liver enzymes measured in serum, ALT, and aspartate transaminase (AST). ALT is typically higher than AST, but absolute levels correlate poorly with clinical severity. Serum transaminases begin to increase during the prodromal period, preceding the onset of jaundice, and can reach a peak level of $>1000^{\circ}\text{IU}^{\circ}\text{L}^{-1}$ (normal range 10–40 IU L^{-1}). The serum bilirubin (Bili) is mainly conjugated and reflects the severity of the hepatitis; however, large elevations in transaminases can be seen without significant elevations in Bili, particularly in acute HCV. Alkaline phosphatase (ALP) is usually only mildly to moderately elevated, marked elevation suggests extrahepatic cholestasis and should prompt imaging, for example, with ultrasound. Occasionally, there may be a cholestatic phase (most commonly seen with HAV infection) when Bili and ALP levels remain elevated in the face of decreasing transaminases, with the patient exhibiting symptoms of severe pruritis (itching) and jaundice. Assessment of the synthetic function of the liver with albumin and coagulation tests, that is, prothrombin time and International normalized ratio (INR), provides the most sensitive measure of liver injury. Increasing prolongation of the INR implies possible hepatocellular failure and evolution to fulminant hepatitis.

The most useful initial serological tests are hepatitis B surface antigen (HBsAg), hepatitis A IgM antibody (IgM anti-HAV), hepatitis B core IgM antibody (IgM anti-HBc), and hepatitis C antibody (anti-HCV) (Fig. 3). Acute HAV is confirmed by IgM anti-HAV. Acute HBV is indicated by the presence of HBsAg with IgM anti-HBc. Unless there is documented anti-HCV seroconversion after a discrete HCV exposure, such as a needlestick injury, acute HCV can be difficult to diagnose conclusively. Viral replication can be confirmed by the detection of HCV RNA in serum, but does not distinguish acute from chronic infection. If HEV infection is suspected, the diagnosis can be confirmed with serology. Both IgM and IgG anti-HEV can be seen in acute infection.

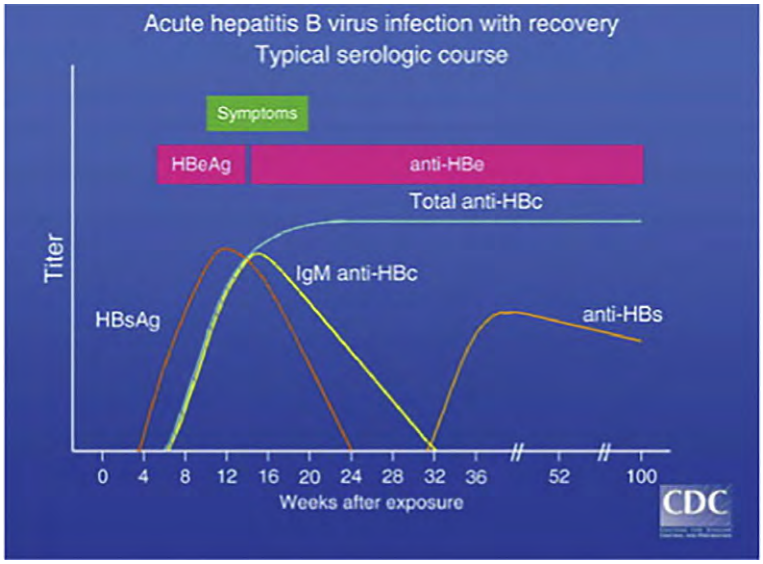

Fig. 3 Graphical representation of evolution of serological markers in acute hepatitis B infection. Reproduced from Center for Disease Control and Prevention, USA, with permission.

Acute Viral Hepatitis—Treatment

Most cases of acute viral hepatitis resolve spontaneously and require no specific treatment other than supportive care. First line antivirals (Tenofovir or Entecavir) should be considered in acute HBV infection complicated by fulminant hepatitis and liver failure. There is a high-risk of progression to chronicity in acute HCV infection, with up to 75% of those exposed failing to clear the virus. Individuals with detectable HCV RNA in the serum 6 months postexposure are defined as having chronic infection, although a small number may still clear the virus spontaneously after this time. With older treatment regimens using Interferon (IFN), efficacy was much greater when instituted early (within 6 months of exposure), rather than waiting until chronic infection was clearly established. However, newer non-IFN, oral combinations of "Direct Acting Anti-viral" drugs, or DAAs (see "Therapy of Chronic Hepatitis C" section) have high response rates when used in chronic infection, thus removing the impetus for early therapy. Monitoring for a minimum of 24 weeks before starting DAA therapy is currently recommended, although there is emerging data on the use of short DAA regimens in the acute setting.

Extrahepatic Manifestations of Viral Hepatitis

Extrahepatic manifestations of acute and chronic viral hepatitis are most frequently seen with acute or chronic HBV with immune complex-mediated tissue damage. A "serum sickness"-like syndrome sometimes seen in acute HBV is thought to be due to the deposition of circulating immune complexes of HBsAg-anti HBs in blood vessel walls, leading to the activation of the complement system and depressed serum complement levels.

Other types of immune-complex disease may be seen in chronic HBV, including membranous glomerulonephritis and, rarely, polyarteritis nodosa. Deposition of HBsAg, immunoglobulin (Ig), and C3 has been described in the glomerular basement membrane; membrano-proliferative glomerulonephritis has also been reported with chronic HCV. HBV is implicated in up to 20% of cases of childhood membranous nephropathy.

Essential mixed cryoglobulinemia (EMC), initially associated with hepatitis B, has subsequently been shown to be more frequently associated with chronic HCV (>90% cases). Cryoglobulinaemia is characterized by the presence of Igs in the blood (cryoglobulins), which are precipitated into the microvasculature at low temperatures, and then redissolve at temperatures of 37°C. In EMC, cryoprecipitable immune complexes of more than one Ig class are found in the serum; these immune complexes contain HCV RNA. Clinical features of EMC include arthritis, cutaneous vasculitis (palpable purpura), and occasionally glomerulonephritis. EMC can be associated with overt lymphoproliferative disorders of B-cell lineage. A link between HCV infection and B-cell non-Hodgkin's lymphomas has been observed in epidemiological studies, most strongly with marginal zone lymphomas, with reports of patients with HCV and indolent lymphomas responding to antiviral therapy alone.

A variety of other extrahepatic diseases such as Sjögren's syndrome, autoimmune thyroiditis, porphyria cutanea tarda, and lichen planus have been described in chronic HCV. The mechanism by which HCV causes these extrahepatic diseases is still unclear. These conditions may improve or resolve with therapy directed against HCV.

Chronic Viral Hepatitis

Acute infections with HAV do not become chronic, and therefore there is no risk of long-term liver damage. Although most commonly causing acute hepatitis, HEV can also cause chronic infection in the immunosuppressed, such as the recipients of organ transplants. Acute infection with HBV, HDV, and HCV, however, can become chronic, with persisting viral replication 6 months after initial infection. Chronic infection with HBV occurs most commonly in individuals who acquire the virus perinatally or during early childhood when the immune response is muted, whereas exposure in later life through sexual contact or IV drug use leads to chronic infection in less than 5% of cases. Both viral and host factors are thought to contribute to the high rate of chronicity with HCV infection (~80%), which unlike HBV is not related to age of exposure. Neither HBV nor HCV is predominantly cytopathic; hence, inflammation and injury in chronic infection are probably immune-mediated. Chronic HBV and HCV infections are often asymptomatic, patients may complain of vague right upper abdominal discomfort, and nonspecific symptoms such as chronic fatigue and malaise. The natural history of chronic HBV is complex, but recent data suggest that progression to cirrhosis and HCC is correlated with serum HBV DNA levels and HBV genotype. In HCV, up to one-third of chronically infected individuals, if untreated, will progress over a variable time period (usually 20–30 years) to cirrhosis; however, in contrast to HBV, viral factors such as HCV RNA level and genotype do not appear to influence disease progression. The rate of fibrosis progression in chronic HBV and HCV infections is also dependent on host variables, for example, age, immunogenetics, and alcohol consumption. Once cirrhosis develops, additional manifestations include portal hypertension with bleeding esophageal varices and ascites, and impairment of hepatocellular function. The risk of HCC is also increased in cirrhosis, whatever the underlying cause, although the risk is particularly high if the individual has chronic HBV (relative risk increased by >200). However, unique among most causes of liver disease, HCC can occur in chronic HBV infections in the absence of cirrhosis.

LT in Chronic Viral Hepatitis

There is currently no treatment other than LT to offer individuals with chronic HBV or HCV and advanced liver disease with complications such as portal hypertension and ascites. However, recurrence of the original infection after LT is an important cause of

morbidity and mortality. The early results of LT for HBV were disappointing due to the high rates of graft loss from severe cholestatic recurrent HBV infection. LT became a viable option once hepatitis B immune globulin (HBIg) had been shown to reduce recurrence rates to below 10%, and subsequently the combination of HBIg with an antiviral drug (lamivudine) further reduced recurrence to <5% at 5 years. More recently lamivudine has been superceded by the more potent antivirals Tenofovir and Entecavir, which have a high genetic barrier to resistance and minimal side effects, and are therefore better suited to long-term use. Selected patients, with low viral burden prior to transplant can now be managed with HBIg free prophylactic regimens using these potent antivirals. Individuals with HCV viraemia at the time of LT are at a high risk of recurrent infection, which although variable in severity can result in some individuals progressing to cirrhosis within 3–5 years of transplant. In a large US database (>11,000 patients), 5-year survival rates after LT were reduced in HCV-positive compared with HCV-uninfected individuals (69.9% vs. 76.6%). Before the advent of all oral DAA regimens, there was no effective therapy available to prevent recurrent infection with HCV posttransplant, older IFN based regimens being contraindicated in patients with decompensated liver disease due to toxicity and low rate of response. DAAs have been evaluated in clinical trials in the pre and posttransplant setting, and shown to be well tolerated, with high efficacy. Following viral clearance, long term posttransplant survival of HCV positive recipients can be expected to be comparable with HCV negative recipients.

Histology of Chronic Viral Hepatitis

HBsAg-positive "ground glass" hepatocytes are characteristic of chronic and not acute HBV infection. Identification of cytoplasmic HBsAg is possible with special stains (Shikata orcein or Victoria blue stains) or immunohistochemically. Accumulation of hepatitis B core antigen (HBcAg) in hepatocyte nuclei produces an appearance known as "sanded" nuclei. HBcAg is best identified using immunohistochemical staining. Coinfection with HBV and HDV produces the same histological picture as with HBV alone, but often with a more severe degree of necro-inflammation. Hepatitis D antigen can be visualized using immunohistochemical staining.

Characteristic histological features of chronic HCV infection include dense portal lymphocytic infiltrates, often with lymphoid aggregates and sometimes with follicle formation accompanied by varying degrees of interface hepatitis. Mild degrees of biliary epithelium damage (the "Poulsen lesion") can also be seen. There is currently no commercial immunohistochemical stain for HCV. Hepatic steatosis can be observed in up to 70% of liver biopsies from individuals with chronic HCV (74% in genotype 3 vs. 50% in non-3 genotype) as compared with up to 18% of patients with chronic HBV infection. The high prevalence is due to a combination of the direct steatogenic effect of HCV (particularly genotype 3) and the prevalence of metabolic risk factors in the HCV population. Steatohepatitis has been shown to enhance fibrosis progression in chronic HCV.

Chronic Viral Hepatitis—Pathogenesis

After acute infection with HBV or HCV, impairment of hepatitis virus-specific T-cell responses can lead to failure to clear the virus, with the establishment of chronic infection. Individuals who spontaneously clear HBV and HCV maintain durable virus-specific $CD4^+$ and $CD8^+$ T-cell responses that can be easily detected in the blood for decades, whereas those who progress to chronic viral hepatitis typically display narrowly focused and weak HBV- and HCV-specific T-cell responses. Virus-specific T cells in these subjects show reduced proliferation and production of cytokines, and there is reduced cytotoxicity of $CD8^+$ T cells. The gradual loss of T-cell function is termed "exhaustion," and is thought to be secondary to the frequent exposure to a high viral and antigenic load, for example, in chronic HBV, large quantities of HBeAg are secreted into the blood and may be the cause of neonatal T-cell tolerance and in altering the reactivity of HBe-specific $CD8^+$ T cells. In HCV, it has been demonstrated that other antigen-specific mechanisms are involved in the downregulation of cellular immune responses, even at low antigen concentrations; for example, recombinant HCV core protein has been shown to downregulate interleukin-12 (IL-12) production by macrophages in vitro.

Reduction of effector T-cell function occurs in the following order: IL-2 production being affected first, then cytotoxicity and the production of TNF-α, with IFN-γ production usually preserved until last. In addition, the functional potential of $CD8^+$ T cells can be negatively impacted if help from $CD4^+$ cells is reduced or unavailable. Finally, viral mutations (which are more frequent in HCV than in HBV infection) also contribute to $CD8^+$ T-cell impairment by affecting the intracellular processing of T-cell epitopes, their binding to major histocompatability molecules, and the stimulation of T-cell receptors. These mutations also affect recognition by specific antibodies, so that all arms of the adaptive immune response are downregulated.

A small number (<2% per annum) of chronically infected HBV patients spontaneously clear HBsAg and develop neutralizing antibodies. In these individuals, HBV-specific T-cell responses become detectable in the blood just before seroconversion. This implies that latent, immune-mediated clearance mechanisms can become spontaneously activated in chronically infected subjects. It is not known whether such immune responses can be induced therapeutically by vaccination and or antiviral therapy in patients with chronic HBV. Studies in animal models of related chronic viral infections (e.g., the mouse model of chronic infection with the 1 hepatotropic lymphocytic choriomeningitis virus, LCMV) suggested that proliferative $CD8^+$ T-cell responses can be partially restored if viral load was reduced with antivirals before vaccination. However, results of studies of HBV peptide and protein vaccination in adults and children with HBV have been disappointing, and although therapy with nucleoside analogs results in a transient recovery of HBV-specific $CD4^+$ and $CD8^+$ T-cell function in peripheral blood, this does not persist for greater than 6 months. Antiviral therapy in chronic HCV is not associated with even a transient increase of HCV-specific T-cell responses. Extrapolating on data obtained from the LCMV-infected mouse model, it would appear that virus-specific $CD8^+$ T cells develop an intrinsic defect during chronic infection that prevents a vigorous proliferative response to vaccination even if the virus is removed.

HAV

HAV is a nonenveloped positive-strand RNA virus transmitted via the fecal-oral route. Although globally distributed, risk of infection decreases as levels of hygiene and sanitation improve, and an effective preventative vaccine is available.

Virology

First visualized by immune electromicroscopy in 1973, HAV has been classified within a separate genus of the *Picornaviridae* family, the genus *Hepatovirus* (Fig. 4). The virus is nonenveloped, with a diameter of 27–32 nm, with a linear RNA genome The 3′-end terminates with a poly (A) tail of 40–80 nucleotides The genome contains a single, large open reading frame (ORF), which codes for a large polyprotein and which is subsequently cleaved by a viral protease, forming three capsid proteins and several nonstructural proteins. The capsid is composed of 60 copies of each of three major structural proteins, VP1, VP2, and VP3.

Once the virus enters the hepatocyte, there is loss of the protein coat releasing the positive-sense RNA strand into the cytoplasm. Genomic replication takes place by a mechanism involving an RNA-dependent RNA polymerase. HAV then enters the biliary tract and exits the body via the feces. Several viral genotypes have been identified, but with only one serotype, with one immunodominant neutralization site, a property that facilitated the development of an effective vaccine.

Epidemiology

HAV is transmitted via the fecal-oral route, by ingestion of contaminated food or water or direct person-to-person transmission; it can occur either sporadically or in large outbreaks. The highest prevalence of this infection occurs in countries with lack of access to clean water supplies. In endemic areas, infections occur early in life, such that most children by the age of nine are seropositive for HAV. In contrast, HAV is mainly a disease of adults in most industrialized nations, who are much more likely to have symptomatic infection. Despite high seroprevalence rates in highly endemic populations, HAV perpetuates in these regions due to its ability to survive in the environment for prolonged periods, and, once ingested, to withstand the acid environment of the stomach. Food and water can become contaminated with HAV by the use of human feces as fertilizer, and the pollution of water supplies by raw sewage. Consumption of raw or undercooked shellfish is associated with infection as certain mollusks can concentrate HAV from polluted waters. Parenteral transmission of HAV is possible, with cases reported in intravenous drug users, hemophiliacs, and rarely via blood transfusion. Sexual oro-anal contact can also result in transmission. Recent data indicates that the global epidemiology of the disease is changing, mainly due to improvements in living conditions in developing countries, with fewer children becoming infected. This results in a larger population of adults without protective antibodies who are at risk of symptomatic infection. In Europe and the United States, outbreaks of HAV have been described in men who have sex with men (MSM), and there have also been large food-borne outbreaks recently reported associated with consumption of contaminated frozen berries and soft fruit.

Clinical Features

The incubation period is from 10 days up to 7 weeks, during which time high levels of HAV are present in liver tissue, bile, and feces (virus can also be detected in blood in lesser amounts); the infected individual is generally asymptomatic and highly infectious. After the 4th or 5th week of infection there is an immune response accompanied by the first symptoms (the prodromal or preicteric period), which may include fatigue, fever, anorexia, nausea and vomiting, diarrhea, and right-sided abdominal pain. During this

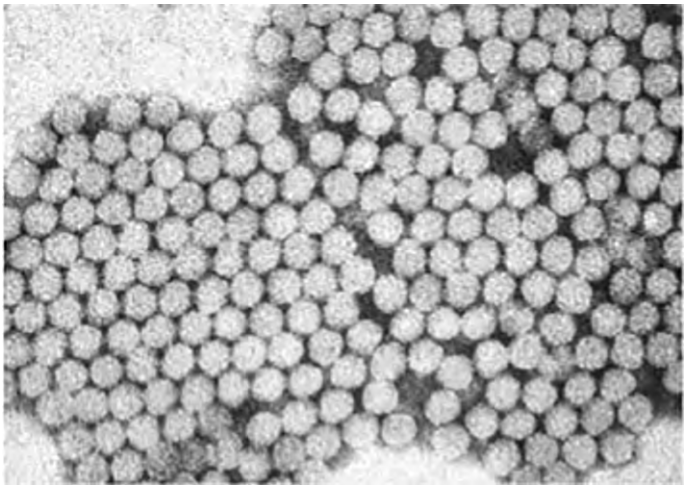

Fig. 4 Electron micrograph of hepatitis A virus particles. Reproduced from Center for Disease Control and Prevention, USA, with permission.

period, ALT and AST are increasingly elevated. Generally, within 10 days of the onset of symptoms the patient enters the icteric phase of infection, with resolution of fever and marked decrease in viremia, although virus is still shed in the feces for another 1–2 weeks. Extrahepatic symptoms are uncommon, although urticaria can occur. Jaundice usually resolves over 1–2 weeks, and a complete recovery can take up to 6 months. The neutralizing antibodies produced in an infected individual can be used therapeutically to prevent infection in an exposed individual (hyperimmune or γ-globulin).

Diagnosis

Routine diagnosis is usually made by the detection of IgM anti-HAV. Experimentally, infection with HAV can be confirmed by the detection of virus in the blood or feces. Antibodies to HAV of the IgM class can be detected approximately 3 weeks after exposure, and these increase in titer over the next 4–6 weeks before disappearing from the blood. Shortly after the appearance of IgM anti-HAV antibodies, IgG anti-HAV antibodies can be detected; these persist for years after resolution of acute infection, conferring lasting immunity. As the presence of IgG anti-HAV in the serum can indicate either prior or current infection, it is therefore important to request both IgM anti-HAV and IgG anti-HAV to establish the diagnosis of acute HAV.

Vaccines

There are four inactivated commercial vaccines currently available for the prevention of HAV. These are safe, highly effective, and provide long-term protection (for at least 20 years) against infection; however, a booster vaccination after 10 years is recommended. Although generally used preexposure, mass postexposure vaccination has been successfully utilized to contain the spread of HAV in established outbreaks. Dosing schedules vary, but generally two doses of vaccine are administered intramuscularly (IM) 6 months apart. This will produce protective levels of antibody within 1 month of the first dose in almost 100% of those vaccinated. These vaccines can be coadministered with others, including live attenuated vaccines, without affecting immunogenicity.

Vaccination strategies differ between areas of high and low endemicity. In most developing countries, HAV is acquired in early childhood and tends to be asymptomatic, and therefore HAV is not a major public health issue requiring universal immunization. As nations develop public sanitation, the age at which individuals will become infected is delayed until adulthood, and subsequently the risk of symptomatic illness is much higher. In such countries, groups at high-risk of infection due to lifestyle or occupation, and travelers to areas of high endemicity, are targeted for vaccination. One study from Ireland showed that if HAV immunity in a population is greater than 45%, screening followed by vaccination of nonimmune individuals is the most cost-effective strategy; if less than 45%, vaccination without prior screening should be used.

Ig

This is more often used in the setting of postexposure prophylaxis or in situations where immediate protection is required. Nonimmune individuals should be given 0.02 mL kg^{-1} of Ig within 2 weeks of exposure, in which case it will either prevent or attenuate the severity of the infection. Ig can safely be given to children, pregnant, and lactating women, and the immunocompromised, and a single dose of 100 IU given IM will provide protection for up to 6 months. Ig should not be coadministered with live attenuated vaccines as this may interfere with the immune response to these vaccines, but can be given with HAV vaccine (at a different site) for maximal protection after an exposure.

HBV

Uniquely among DNA viruses, HBV replicates within infected hepatocytes through a process called reverse transcription. Infection with HBV can result in different clinical scenarios, ranging from fulminant hepatitis to asymptomatic chronic infection, cirrhosis, or HCC. Integration of HBV viral DNA into the hepatocyte nuclear DNA is a key event in hepatocarcinogenesis. HCC resulting from chronic HBV is one of the most common malignancies worldwide.

Virology

HBV is a member of the family Hepadnaviridae, with a double-stranded circular DNA genome that replicates through an RNA intermediate. Although HBV infects only human and higher primates, related viruses are common in rodent (e.g., woodchuck) and bird species (e.g., duck). Hepadnaviridae preferentially infect liver cells, with trace amounts of HBV DNA found in kidney, pancreas, and mononuclear cells without causing disease at these sites. HBV DNA does not integrate into host cellular DNA as part of the viral replication cycle, but may persist in the host cell for many years even after clinical resolution of infection. During this time, HBV DNA may be integrated into cellular DNA and it is thought that integration plays a role in the subsequent development of HCC.

Each HBV particle consists of a 3.2-kb double-stranded circular DNA genome and DNA polymerase enclosed by a viral envelope, containing three related surface proteins, and an icosahedral nucleocapsid of approximately 30 nm diameter.

HBV replication proceeds through a unique and complex mechanism utilizing an RNA intermediate, with viral RNA transcripts being generated from a covalently closed circular DNA (cccDNA) transcriptional template found in the nucleus of the host cell in the

form of a viral mini chromosome. The genome encodes four ORFs, which when translated produce the precore and core, polymerase, small, medium, and large envelope proteins, and the transcriptional regulator protein X. The core gene can also produce a small-molecular-weight protein called HBeAg by an alternate start codon and post-translational modification. The HBV polymerase has a number of functions in the viral life cycle, including the initiation of minus-strand DNA synthesis (priming), the synthesis of DNA using either RNA or DNA templates (polymerase activity), the degradation of the RNA strand of RNA–DNA hybrids (nuclease activity), and the packaging of the RNA pregenome into nucleocapsids.

There are eight HBV genotypes (A–H) so designated on the basis of a genomic variation of at least 8%. Coinfection with different genotypes has been reported. Genotype A is the most widely distributed, being frequent in northwest Europe, sub-Saharan Africa, India, and North, Central, and South America; B and C are common in Southeast Asia and Oceania, and D is prevalent around the Mediterranean, Central Asia, and South America. Genotype E is found only in West Africa, F is limited to Central and South America but is also found in Native Americans, G has been described in the United States and France, and H in Central America.

There is accumulating evidence that HBV genotypes may influence HBeAg seroconversion rates, mutational patterns in precore and core promoter regions, and the clinical severity of liver disease. For example, infection with genotype A HBV may be more likely to become chronic, but causes milder liver disease than other genotypes; it also has a better response to treatment with IFN. In contrast, genotype C HBV appears to be associated with a more aggressive clinical course and faster progression to cirrhosis. The predominant mutation in the precore region of the HBV genome, which completely aborts the production of HBeAg, is rarely seen with HBV genotypes A and F, but is found mainly in genotypes B–D.

The lifecycle of HBV begins with viral attachment and entry into the hepatocyte followed by internalization. Recently, there have been advances in the understanding of this process following the identification of sodium taurocholate cotransporting polypeptide (NTCP) as a cellular entry receptor. Once in the cytoplasm, viral uncoating occurs and HBV DNA is transported to the nucleus where it is converted to cccDNA, a stable template for transcription of both subgenomic messenger RNA (for translation into viral proteins) and pregenomic RNA (for reverse transcription into genomic DNA). HBV cccDNA is responsible for viral persistence. After binding of the HBV polymerase and core Ag to the pregenomic RNA, encapsidation occurs. Synthesis of the negative DNA strand by reverse transcription and partial synthesis of the positive-strand is performed by HBV polymerase within the nucleocapsid, which is then either transferred to the endoplasmic reticulum where it assembles with HBsAg molecules to form the virion, or returns to the nucleus to amplify the cccDNA reservoir. The intact virion exits the cell by budding and vesicular transport. The surface proteins can also bud in the absence of capsids, forming both spherical and tubular (or "filamentous") particles approximately 22 nm in diameter consisting of HBsAg and host cell-derived lipids (Fig. 5). These particles are also secreted into the blood, as they do not contain viral nucleic acid and they are not infectious, but are highly immunogenic.

The turnover of virions is high, with estimates in the order of 10^{11} virions produced per day—much higher than that for other DNA viruses, HCV, or HIV. The HBV DNA polymerase does not have proofreading or editing capacity, and this together with the high rate of virion production makes replication errors inevitable. It has been calculated that over the period of 1 day, an estimated 10^{14} nucleotides are replicated, with the potential for 10^{7} base pairing errors.

The consequence of this high mutational rate is the formation of a large pool of "quasispecies" with the virus, with the best replicative "fitness" becoming the dominant species. Selection pressure, either from the host immune response or from antiviral therapy, can result in vaccine/Ig escape mutants and drug-resistant variants.

Mutations of the HBV envelope, precore, core, and polymerase regions occur. Several precore mutations have been identified in HBeAg-negative patients. The most common mutation observed is a G→A substitution at nucleotide 1896 (G1896A) in the precore region, creating a premature stop codon. Translation of the precore protein is prevented, which aborts the production of HBeAg.

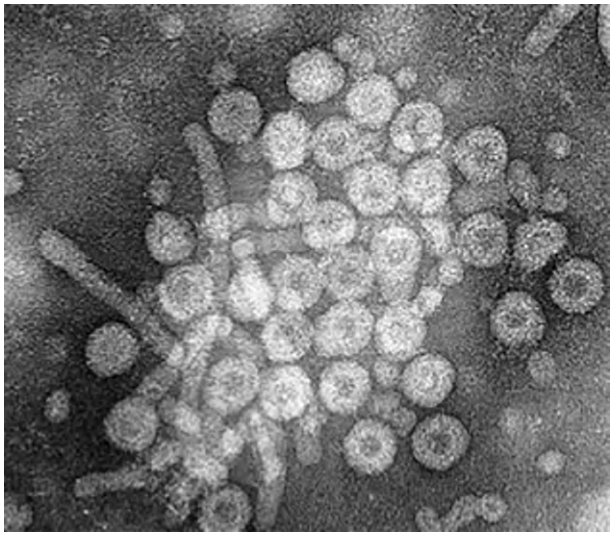

Fig. 5 Electron micrograph of hepatitis B virus showing spherical, tubular, and double-shelled forms. Courtesy of Dr. Arie Zuckerman.

Epidemiology

There are an estimated 350 million people in the world with chronic HBV, with prevalence varying widely between geographical areas. HBV is efficiently transmitted horizontally by intimate contact, by parenteral exposure as well as vertically from mother to infant. However, in about a one-third of adults with acute infection, no risk factors are identified, probably reflecting HBV's ability to survive outside the body for up to a week as well as its ubiquitous presence in body fluids. Vertical transmission occurs peripartum or in infancy rather than in utero. The risk of vertical transmission is increased if the mother is HBeAg-positive with high serum HBV DNA.

Countries with low prevalence in the general population (i.e., carriage rates of HBsAg of less than 2%) include the United States, Canada, Northern, Western, and Central Europe, and Australia; however, within these countries higher prevalence rates are seen in high-risk populations, for example, in intravenous drug users (IVDUs) and in individuals with multiple sexual partners. Intermediate carriage rates (2%–8%) are observed in Eastern Europe, the Mediterranean, Russia and the Russian Federation, Southwest Asia, and Central and South America. The highest prevalence of HBsAg positivity (8% and above) occurs in China, Southeast Asia, Alaska, and tropical Africa. The age at which most HBV transmission occurs varies according to the prevalence rate. The majority of individuals with chronic HBV in countries with high prevalence of HBV acquire infection vertically or horizontally during early childhood. In low prevalence areas, the highest incidence of infection occurs in teenagers and young adults, reflecting sexual and parenteral transmission. Iatrogenic transmissions from contaminated blood transfusions, use of unsterile needles for injections, or vaccinations remain risk factors in the less-developed world.

Clinical Features

Acute infection

The incubation period for HBV infection ranges from 6 weeks to 6 months. Clinical presentation reflects host age and immune status of the host. Acute perinatal or childhood infection is generally asymptomatic. Children older than 5 years and adults are symptomatic in 33%–50% of infections with typical signs and symptoms of acute hepatitis. Fulminant hepatic failure occurs in approximately 1% of patients with acute HBV. Most adults with acute HBV recover completely without sequelae, although in $\leq$5% infection persists into chronicity.

Chronic infection

The natural history of chronic HBV is dynamic, with several phases characterized by differing levels of serum ALT, HBeAg, and HBV DNA. Chronic HBV acquired in infancy or in early childhood often has a prodromal "immune-tolerant phase," which can last up to young adulthood, characterized by HBeAg positivity, high levels of serum HBV DNA, but with normal levels of ALT and minimal inflammation on liver biopsy. In the late teens or early adulthood, a transition occurs to a more active phase of "immune clearance," during which time serum HBV DNA levels fall with increasing ALT levels and worsening liver inflammation (classic HBeAg-positive chronic HBV). This phase of active replication is usually asymptomatic, but can be prolonged, with ultimate progression to cirrhosis, although it ends in HBeAg-to-anti-HBe seroconversion at a rate of approximately 10%–15% of individuals per annum. Increased immune responsiveness can be accompanied by an abrupt "flare" in ALT levels, which may even mimic fulminant hepatitis. The third phase, "low-replicative" or inactive carrier state, is characterized by detectable HBsAg and anti-HBe, the absence of HBeAg, and low ($<10^5$ copies per mL) levels of HBV DNA, with a reduction in necro-inflammation on biopsy. However, spontaneous reactivation, with an increase in markers of disease activity (HBV DNA and ALT) without the reappearance of HBeAg, occurs in up to a one-third of individuals (classic HBeAg-negative chronic hepatitis B) owing to mutations in the HBV genome, which abolish HBeAg production but allow a return of HBV replication. HBeAg-negative chronic HBV is characterized by fluctuating ALT levels and progressive liver injury and reflects a later stage of the disease.

Diagnosis of HBV Infection

There are a number of laboratory tests that can be used to diagnose hepatitis B infection, and also to determine whether the individual is in the acute or chronic phase (Table 2).

Therapy of Chronic HBV Infection

Achieving a "cure" in chronic HBV infection with currently licensed therapies is uncommon, due to the persistence of viral DNA in the form of cccDNA within the nucleus of infected hepatocytes. Even individuals achieving clearance of HBs Ag with seroconversion to anti HB s are at risk of viral reactivation if they receive prolonged therapy with immunosuppressive agents such as CD20 antibodies (e.g., Rituximab) or high dose steroids. Therefore, the goal of current HBV therapy is to suppress viral replication with the aim of preventing progressive liver disease and the development of complications such as cirrhosis and HCC. Large, prospective, cohort-based studies following a large Taiwanese population of HBsAg-positive patients for a median of 11 years have concluded that serum HBV DNA level is a key predictor of progression to cirrhosis and HCC, independent of ALT and HBeAg status. In 2004, a landmark study from this Taiwanese cohort was the first to demonstrate that an oral antiviral (lamivudine) could suppress HBV replication and modify the natural history of chronic HBV in individuals with advanced liver fibrosis or cirrhosis. In comparison to

Table 2 HBV serology and interpretation of diagnostic tests

Test	*Acute HBV*	*Past exposure (immunity)*	*Previous immunization*	*Chronic HBV infection ($HBeAg^+$)*	*Chronic precore mutant ($HBeAg^-$)*
HBeAg	+	−	−	+	−
Anti-HBe	−	−/+	−	−	+
HBsAg	+	−	−	+	+
Anti-HB^s	−	+	+	−	−
Anti-HBc	+	+	−	+	+
IgM anti-HBc	+	−	−	−[a]	−
HBV DNA	+	−	−	+	+
ALT	Elevated	Normal	Normal	Normal or elevated	Normal or elevated

[a]May become detectable in severe reactivations of chronic HBV.

Adapted from Hepatitis B Management Algorithim, Hepatitis Resource Network, with permission.

patients receiving placebo, a significant reduction in the rates of hepatic decompensation and HCC was observed in the cohort receiving lamivudine.

The decision as to which individuals with chronic HBV should be treated, which therapy to use, and when treatment should be initiated can be complex, and is outside the scope of this article, however, in general, the most important criteria used are levels of HBV DNA and ALT, and the degree of liver necro-inflammation and fibrosis. Treatment guidelines from the United States and Europe differ slightly in the threshold of HBV DNA and ALT used to initiate therapy, and recommendations also depend on whether the individual is eAg-positive or negative. Increasingly, elevated serum HBV DNA is being proposed as the major criteria to initiate antiviral therapy. Although depending on patient characteristics different thresholds for HBV DNA and ALT may be relevant, in general, noncirrhotic individuals with chronic HBV, elevated ALT, and HBV DNA levels of $\geq$20,000 IU mL^{-1} in eAg-positive disease and $\geq$2000 IU mL^{-1} in eAg-negative disease (>2000 IU mL^{-1} for both eAg-positive and negative individuals in European guidelines) should be offered treatment if there is evidence of moderate to severe necro-inflammation and/or at least moderate fibrosis on liver biopsy or validated noninvasive marker of fibrosis (such as transient liver elastography). Most authorities would recommend that individuals with cirrhosis or family history of HCC should be considered for treatment regardless of level of HBV DNA or ALT, as frank hepatic decompensation may occur with a spontaneous flare in cirrhotics. A number of end points are used to assess treatment response, normalization of elevated ALT (biochemical), achieving undetectable HBV DNA in the serum using a sensitive assay, and/or loss of HBV surface and eAg (virological), and improvement in liver necro-inflammation and fibrosis (histological).

Seven agents have been licensed for the management of chronic HBV: IFN-α 2b, pegylated IFN-α 2a, and the oral antivirals lamivudine, adefovir, telbivudine, entecavir and tenofovir, although only peg-IFN-α 2a, tenofovir and entecavir are currently considered "first line" drugs. IFN-α has been used to treat chronic HBV since the mid-1980s. A longer acting formulation, pegylated interferon (peg-IFN) has superceded standard IFN since 2005 when Peg-IFN-α 2a was approved for use in chronic HBV. Whereas IFN is contraindicated in individuals with decompensated cirrhosis, the oral agents have the advantage of being safe and well tolerated in this patient population. Efficacy of IFN therapy is strongly dependent on HBV genotype in eAg-positive disease, with best results seen in genotype A and B infections. The advantage of IFN is its finite duration of treatment (48 weeks), absence of selection of HBV resistance mutations, and potential for more durable response, however it has a large number of potential side effects. Lamivudine, a nucleoside analog reverse transcriptase inhibitor (NRTI), was originally developed for the treatment of HIV infection, but was found to also inhibit the replication of HBV and was subsequently licensed for use in chronic HBV in 1998. NRTI's interfere with viral replication by causing premature termination of the DNA chain; other recently licensed members of this drug class include entecavir and telbivudine. Adefovir, introduced in 2002, also works by DNA chain termination but is a nucleotide analog. Tenofovir, a structurally similar nucleotide to adefovir, but with higher potency, was licensed for use in patients with HBV in 2008, having been previously approved to treat HIV infection in 2001. The development of resistance to antivirals such as lamivudine, adefovir and telbivudine with prolonged use has previously been an area of concern, recalling the situation seen in the early days of antiretroviral therapy for HIV, and strategies to address this issue, such as the use of two antivirals from different drug classes (i.e., a nucleoside plus a nucleotide analog) have been evaluated. However as tenofovir and entecavir, which are more potent and have a much higher threshold for resistance, are now considered the preferred options for oral HBV therapy (and have largely replaced earlier agents such as lamivudine) this issue is no longer relevant. In large scale registration trials with long-term follow up less than 1% of patients on entecavir have developed mutations associated with viral breakthrough, and there have been no clinically significant resistance mutations detected in patients treated with tenofovir. There is a commercially available test that detects the key genotypic mutations in HBV polymerase associated with resistance to lamivudine, adefovir, and entecavir. Individuals with HBV who have developed resistance to lamivudine can be successfully treated with tenofovir.

Although these drugs effectively suppress HBV replication, they have much less effect on clearing the "reservoir" of cccDNA in hepatocytes and viral relapse will usually occur once therapy is discontinued. In selected individuals with eAg-positive chronic HBV who achieve seroconversion with the loss of eAg and production of anti-HBe maintained for at least 12 months, an attempt at discontinuation of antiviral therapy can be made, with low chance of relapse. In patients with eAg-negative disease, it is impossible to recognize a treatment-induced seroconversion, and therefore therapy may need to be continued indefinitely. HBs Ag levels are a

surrogate marker for cccDNA and there is evolving data on the use of quantitative HBs Ag levels as an aid to predict response to therapy with Peg-IFN. In a recent study by Marcellin et al., on treatment HBsAg decline during peg-IFN therapy was significantly associated with sustained immune control and HBsAg clearance in a cohort of eAg-negative patients. Rates of HBs Ag clearance 5 years post IFN therapy were highest in patients achieving at least a 10% decline in sAg levels at weeks 12 and 24 of treatment as compared to baseline. There are ongoing studies on whether HBsAg levels may also be useful to determine when individuals on long-term nucleos(t)ide therapy may be able to be discontinue therapy without risk of relapse.

In recent years there has been a resurgence of interest in the development of potentially curative agents for chronic HBV. Broadly speaking, new therapies can be divided into the following classes; immune modulators, inhibitors of gene expression and replication, cccDNA targeting drugs, and drugs blocking entry of HBV into the hepatocyte.

There are over 30 novel therapies currently in clinical development, the detailed discussion of which is outside the scope of this article. Immunomodulatory agents aim to restore an appropriate immune response to the virus. Specific T and B cell responses to HBV are crucial for elimination of the virus and the cccDNA "reservoir," and one approach is to target the adaptive immune response, through stimulation via therapeutic vaccines and toll like receptor 7 agonists, and blockade of immune inhibitory pathways. Viral targets other than HBV polymerase in the life cycle have been identified (see Fig. 6) and are the focus of new drug development, such as capsid inhibitors, which interrupt HBV replication via blocking pregenomic RNA encapsidation. This initial stage of the HBV lifecycle has gained attention as a potential target for inhibition following the identification of NTCP as a cellular entry receptor. There are currently a number of NTCP inhibitors in development, including Myrcludex-B, which has shown promise in preclinical studies, and has now progressed to phase 2 clinical trials. It is likely that future approaches to HBV eradication will involve the use of "combination therapy," using agents from different classes.

Vaccines

Several preventative HBV vaccines are currently available; these rely on the use of one of the viral envelope proteins (HBsAg) and are now made using recombinant technology. HBV vaccines are available in monovalent formulations and in combination, for example, with HAV vaccine. Two recombinant vaccines are available; both are safe, even during pregnancy. Three 1.0 mL IM injections are given into the deltoid or anterolateral thigh, at time 0, 1, and 6 months, although there is flexibility and an accelerated schedule can be used. Children are given lower doses (0.5 mL), and immunosuppressed patients and patients on hemodialysis are given higher doses.

Immunization strategy varies depending on the level of endemicity, with the ideal goal being universal childhood vaccination. Additional strategies include prevention of perinatal HBV infection through routine HBsAg screening of all pregnant women, with immunoprophylaxis given to all babies of HBsAg-positive mothers. As soon as possible after delivery, the infant is given 30 IU of HBIg and an HBV vaccination course is started. Even with prompt administration of HBIg and vaccine, there is still a risk of

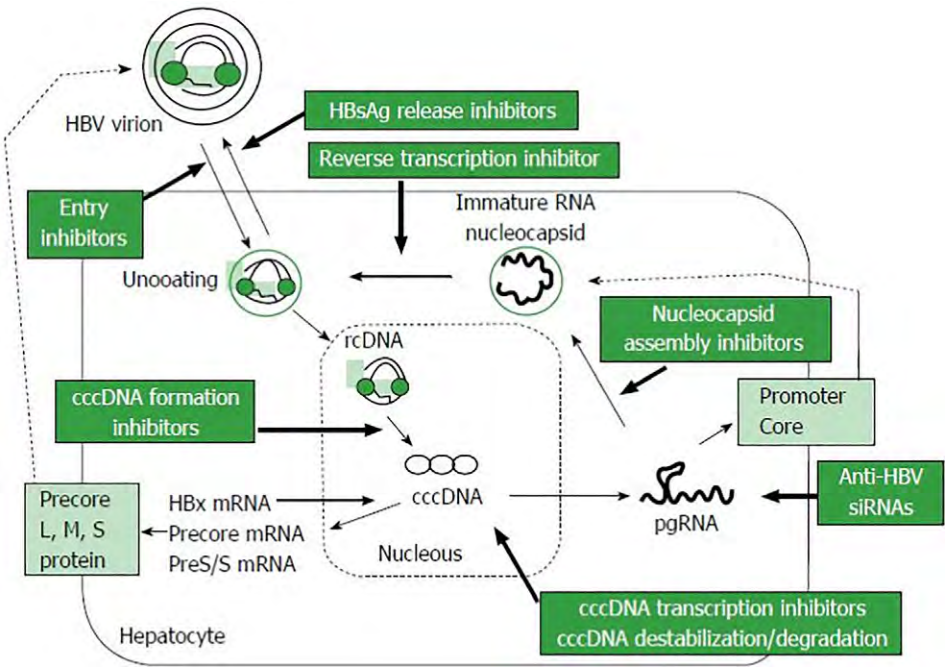

Fig. 6 Simplified schema of the hepatitis B virus life cycle and possible targets of therapy. *HBV*, hepatitis B virus; *cccDNA*, covalently-closed circular DNA; *HBsAg*, hepatitis B virus surface antigen; *rcDNA*, relaxed circular DNA; *siRNAs*, small interfering RNAs. From Tajiri et al. "New Horizon for radical cure of chronic hepatitis B virus infection." *World Journal of Hepatology*.

transmission to the neonate; this risk is increased if the mother is HBeAg-positive and has HBV DNA levels $>10^7$ IU mL^{-1}. In countries with low or intermediate endemicity, a higher proportion of chronic HBV is acquired by older children and young adults, and "catch-up" vaccination for those who were not vaccinated at birth should be considered, particularly for individuals at increased risk for exposure to HBV, such as healthcare workers and persons engaging in high-risk sexual activity.

HCV

HCV is a parenterally transmitted RNA virus, a characteristic feature of which is a propensity to cause chronic infection, with only 15%–20% of acutely infected individuals clearing infection spontaneously. This is explained in part by the extreme genetic diversity of HCV, a feature it shares with other RNA viruses. HCV was first identified in 1989, although clinically its existence had been suspected for many years as the agent responsible for parenterally transmitted "NANB" hepatitis. HCV is globally distributed, with over 170 million individuals estimated to be infected. Decompensated cirrhosis secondary to chronic HCV is still currently the leading indication for LT worldwide, although this is likely to change given that more effective antiviral therapy is now available.

Virology

HCV is a small enveloped positive-sense, single-stranded RNA virus, which is classified as a member of the family Flaviviridae, genus *Hepacivirus*. Within an infected individual, HCV exists as a population of closely related yet distinct "viral quasispecies," which may differ with respect to replicative capacity, cell tropism, immunologic escape, and antiviral resistance. HCV has six known genotypes (1a/b, 2b, 3a, 4, 5, and 6) and at least 30 subtypes with differing geographical distributions. Genotypes 1, 2, and 3 are distributed worldwide, genotype 4 is found in the Middle East and Africa, genotype 5 in South Africa, and genotype 6 is the predominant genotype in Southeast Asia. Coinfection with more than one genotype is not infrequently seen, and "superinfection" has been reported.

The genome of HCV contains a single, large ORF flanked by 5′- and 3′-untranslated regions, which codes for a polyprotein of approximately 3000 amino acids, which, when cleaved by viral and host peptidases, produces a number of functional proteins, namely three structural proteins, core, and two envelope glycoproteins (E1 and E2), six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a protein (p7). The viral nucleocapsid comprises multiple copies of the core proteins in complex with genomic RNA (Fig. 7). Apart from humans, HCV infects only chimpanzees, and characterization of the molecular mechanisms involved in viral replication and immune response to HCV was until recently impeded by the lack of a good in vitro model. Alternative tissue culture systems have now been developed, including recombinant HCV envelope glycoproteins, HCV-like particles, HCV retroviral pseudoparticles, and, most recently, cell culture-derived infectious HCV. Although these models have certain limitations, they have greatly advanced the understanding of the early steps of viral infection and host-virus interactions, and have facilitated the development of several new classes of antiviral drugs specific for HCV.

Circulating HCV is physically associated with lipoproteins or antibodies. The major target cell is the hepatocyte, but it is thought that HCV may be able to infect other cells, such as lymphocytes, monocytes, and dendritic cells. Both EI and E2 are essential for entry into the host cell. Within the E2 envelope, glycoprotein sequence hypervariable regions (HVR) have been identified, which differ more than 80% among HCV genotypes, and within subtypes of any one genotype. HVR-1 is a HVR that is important for host cell recognition and attachment. A number of potential entry receptors have been identified, including the tetraspanin CD81, scavenger receptor class B type 1 (SR-B1), heparin sulfate, low-density lipoprotein receptor, and, most recently, claudin-1, a protein involved in the maintenance of cell structures called "tight junctions" found in several epithelial tissues and most prevalent in the liver. Binding of viral envelope glycoproteins to the hepatocyte cell surface triggers endocytosis of the HCV virion, and the viral nucleocapsid enters the cytosol and is transported to the endosome where uncoating, IRES-mediated translation, and RNA replication occur. The viral RNA is translated into a polyprotein which is cleaved by both host and viral-encoded proteases into 10 mature viral proteins, including the nonstructural (NS) proteins (see Fig. 7). Synthesis of new viral RNA occurs in a highly structured replication complex that consists of NS3, NS4A, NS4B, NS5A, and NS5B. NS5B is an RNA-dependent RNA polymerase that is essential for viral replication, NS5A has a presumptive role in the organization of the replication complex. One of the viral proteases involved in posttranslational processing is a heterodimeric complex of the NS3 and NS4A proteins (NS3/NS4A). NS3 possesses the proteolytic activity and NS4 is a membrane protein that acts as a cofactor. The serine protease and RNA polymerase enzymes have been the focus for the successful development of a large number of oral antiviral agents, and inhibitors of the NS5A protein are also being studied. Once fully elucidated, entry receptors will also provide an important additional target.

Epidemiology

HCV infection is endemic in most parts of the world, with an estimated overall prevalence of 3%. The virus is predominantly transmitted via the parenteral route, although vertical and sexual transmission is recognized. In about 10% of cases, a source of infection cannot be identified. HCV has be shown to remain viable on surfaces outside the body for up to 16 h, and transmission may occur through sharing of toothbrushes or razors contaminated with infected blood.

Before the introduction of serological tests for HCV, infection with contaminated blood or blood products was a frequent source of infection. Use of contaminated pooled clotting factors led to prevalence rates of over 80% in the hemophiliac population in the

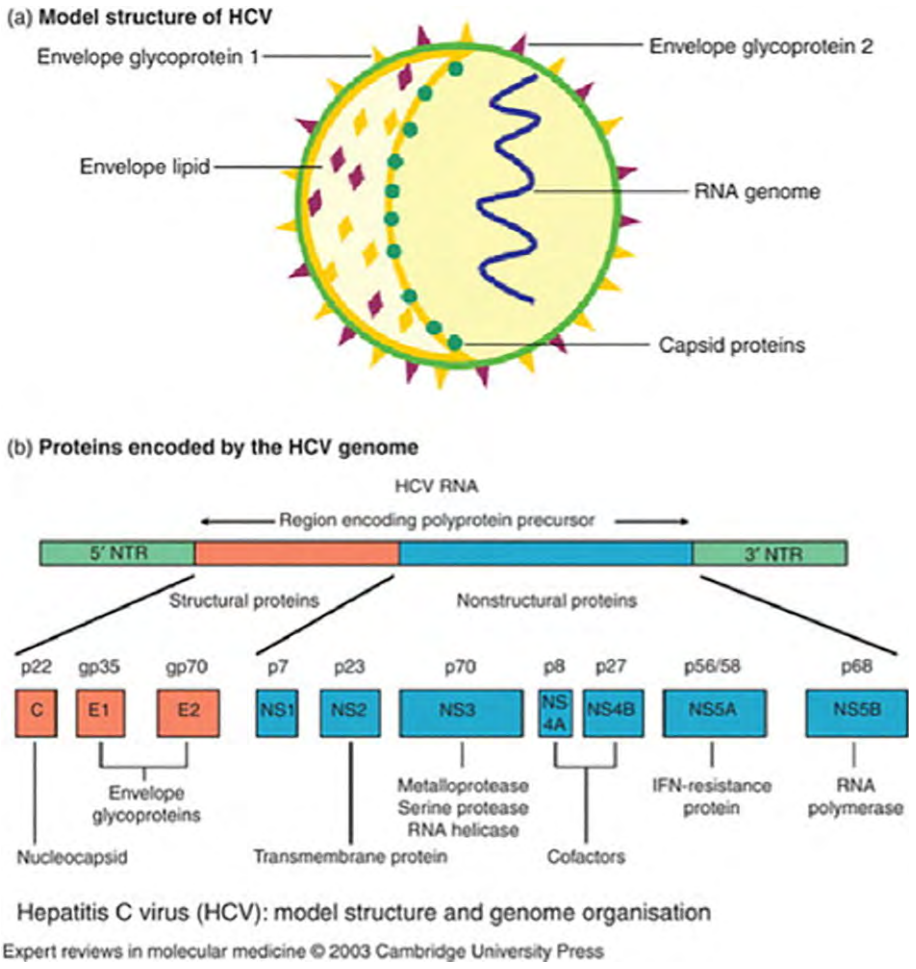

Fig. 7 Structural and genomic organization of the hepatitis C genome. Reproduced from Expert Reviews in Molecular Medicine, 2003, Cambridge University Press.

West, many of whom were also infected with HIV. Following the introduction of screening of blood donors for infection, the risk of transmitting HCV by blood products is presently at 1/200000 units distributed. Transmission from nonsterile injecting practices in healthcare settings is no longer a significant risk for acquisition of HCV, although sporadic cases have been reported in association with poor disinfection procedures for invasive medical equipment such as colonoscopes. HCV has been transmitted through tattooing and body piercing in establishments reusing needles between clients, and rarely infection has been reported to have contracted through manicures and pedicures. In developed countries, IVDUs are currently the only group at significant risk for acquiring HCV. Prevalence rates of HCV approach 80% in the IVDU population, with most becoming infected within 1–2 years of embarking upon intravenous drug use. Incidence of new infections can be reduced by harm-minimization strategies such as needle exchange programs. Health professionals are at risk following needlestick exposures from an infected source, as there is no equivalent of HBV Ig, and currently no vaccine is available.

Vertical transmission is uncommon (seen in <5% cases), the risk is proportional to the maternal HCV RNA level, with higher levels carrying greater risk. Higher HCV viral loads are usually seen in individuals coinfected with HIV, and increased rates of vertical transmission are seen in HIV-positive mothers.

The role of sexual transmission is controversial. Most data come from studies with HCV-positive hemophiliacs and their female partners, and indicate extremely low risk. Most authorities do not routinely recommend the use of "safe sex" practices in HCV sero-discordant heterosexual couples. However, there have been several recent reports from the United States and Northern Europe of outbreaks of acute HCV infection in HIV-positive MSM. Permucosal transmission of HCV-related to high-risk traumatic sexual and drug practices has been implicated as the infection route in these cases.

Although HCV infection is found worldwide, there is marked geographic variance in prevalence and incidence, and also between age groups within individual countries. In countries where IVDU is the major risk factor for HCV acquisition, such as North America, Northern Europe, and Australia, the highest prevalence rates are seen in adults aged 30–49, which would indicate an exposure in early adult life. In countries such as Italy and Japan, the majority of affected individuals are older, reflecting remote inadvertent transmission during medical interventions. In Egypt, prior mass parenteral therapy for schistosomiasis with contaminated needles is responsible for high rates of HCV prevalence in the general population.

Acute Hepatitis C—Clinical Features

Depending on the size of the inoculum, HCV RNA can be detected in serum from 6–7 days to 8 weeks after exposure, with the first biochemical evidence of infection. Approximately 80% of acutely infected individuals fail to clear the virus and develop chronic infection. Clinical factors favoring spontaneous resolution include age <40 years, female gender, and icteric presentation.

Following initial infection, HCV may become established despite the induction of a humoral immune response targeted against various epitopes of the HCV envelope glycoproteins. Strong and persistent proliferative responses of $CD4^+$ T cells and the production of IFN-γ are associated with spontaneous viral clearance, whereas weak or transient responses are associated with progression to chronic infection. The role of $CD8^+$ T cells in controlling acute HCV infection is less clear. It is known that IFN-treated individuals who have cleared HCV have decreased HCV-specific immune responses as compared with those achieving spontaneous clearance. There is some evidence that host genetic factors influence the outcome following HCV infection. Certain HLA class II alleles—HLA-*DRB1*1101-DQB1**0301, *DRB1*1104-DQB1**0301, and *DRB1*0401-DQB1**0301 haplotypes—have been associated with a significantly increased likelihood of viral clearance, predominantly in Caucasian populations.

Chronic Hepatitis C—Clinical Features

Insulin resistance (IR) and the other components of the metabolic syndrome (obesity, hyperglycemia, hypertriglyceridemia, increased blood pressure, and low high-density lipoprotein cholesterol levels) and type II diabetes mellitus are more prevalent in individuals with chronic HCV than in healthy controls or patients with chronic HBV. The natural history of chronic HCV infection is variable, ranging from indolent to rapid progression to cirrhosis. Cohort studies have shown that 20% of individuals will progress to cirrhosis over 20 years.

Increased fibrosis progression is associated with male sex, longer duration of infection, acquisition of infection at age >40 years, excess alcohol consumption, immunosupression, that is, from HIV coinfection or following organ transplantation, and coinfection with HBV, but it does not appear related to HCV genotype. Increased body mass index, visceral adiposity, and coexisting IR and hepatic steatosis may also increase the risk of fibrosis progression, and reduce response to IFN therapy, but these factors do not appear to have a negative impact on response rates with newer DAA regimens.

Diagnosis

Persistence of HCV RNA in the serum 6 months after initial infection indicates chronic HCV, although there are rare reports of spontaneous clearance up to 2 years after exposure.

Current commercially available tests for the detection of HCV antibody are based on either enzyme immunosorbant assays (EIA), which detect HCV-specific antibodies, or on recombinant immunoblot assays (RIBA). EIA's can diagnose more than 95% of chronic infections but can only detect 50%–70% of acute infections. RIBA assays identify antibodies that react with individual HCV antigens and are used as a supplemental test for confirmation of a positive EIA result.

Therapy of Chronic Hepatitis C

Unlike chronic HBV in which there is a "reservoir" of viral cccDNA in host cells with the potential for viral reactivation, there is no such reservoir in chronic HCV infection, and therefore HCV can potentially be eradicated with therapy. The previous "gold standard" of therapy for chronic HCV was the combination of pegylated IFN-α (Peg-IFN) injected subcutaneously once weekly, together with daily oral weight-based RBV, administered for 24 weeks to most patients with HCV genotype 2 or 3, and for 48 weeks to all other genotypes. The goal of therapy was the achievement of a sustained viral response or SVR, defined as undetectable serum HCV RNA levels (with sensitive PCR-based assays) 6 months after the completion of therapy. Mechanisms of action of IFN-α include the induction of an antiviral state that either protects cells from infection or attenuates the production of viral progeny in already infected cells, and, indirectly, the activation of the host adaptive immune response. RBV Both Peg-IFN and RBV have significant side effects, such as depression, fatigue, and cytopenias, and may be poorly tolerated. Efficacy of treatment is highly dependent on viral kinetics, HCV genotype, and ethnicity, with other factors such as gender, age, degree of liver fibrosis, and presence of HS and IR also playing a role. HCV genotype 1, African-American ethnicity, male gender, age >50 years and, advanced liver fibrosis with or without steatosis on liver biopsy are all negatively associated with response to Peg-IFN and RBV.

Owing to poor tolerability and low efficacy of combination Peg-IFN and RBV therapy, this regimen has been superceded by a novel approach to HCV therapy using "Direct Acting Anti-virals": small-molecule inhibitors of the HCV encoded proteins that are vital to the replication of the virus (see HCV-virology). DAAs can be classified by their mechanism of action and therapeutic target into NS3/4 protease inhibitors, NS5A inhibitors and NS5B RNA polymerase inhibitors These potent inhibitors of viral replication rapidly lower the serum HCV RNA levels to undetectable levels within a few weeks of starting therapy in the majority of patients. The probability of achieving SVR positively correlates with rapid reduction of plasma HCV RNA, as a prolonged period of aviremia while on therapy is beneficial because it allows a longer time for residual virus-infected cells to be eradicated. Data from phase 1 clinical trials showed that DAAs used as monotherapy quickly resulted in the virus developing mutations leading to drug resistance, (reminiscent of the experience seen in the development of antiretroviral drugs for HIV therapy). To prevent the emergence of resistance, DAAs therefore need to be given in combinations of two or more drugs with different viral targets, for example a NS5B

RNA polymerase inhibitor with a NS5A inhibitor. With DAA based regimens, SVR has been redefined as undetectable serum HCV RNA levels (with sensitive PCR-based assays) 3 months, rather than 6 months, after the completion of therapy, as clinical trials have shown that risk of viral relapse is negligible after this time point.

Over a decade ago the NS3 protease inhibitor BILN 2061 was the first DAA to be tested in humans, and although its development was halted at an early stage due to toxicity in animals, it provided "proof of concept" for this strategy. In 2011 the "first generation" NS3 protease inhibitors (PI) Telaprevir and Boceprevir were introduced, to be quickly superceded in 2013 by "second generation" drugs such as Sofosbuvir, Daclatasvir, and Simeprevir (by convention, the drug names of NS5B RNA polymerase inhibitors have the suffix *buvir*, NS5A inhibitors *asvir* and NS3/4 protease inhibitors *previr*). Sofosbuvir was the "first in class" NS5B RNA polymerase inhibitor, changing the landscape of HCV therapy owing to its unique properties, which included efficacy across all genotypes, once daily dosing, few side effects or drug–drug interactions, and a high genetic barrier against viral resistance.

Drug development has proceeded at pace, and in the United States and Europe many IFN free, all oral DAA drug combinations are now available, with minimal side effects and high rates of viral clearance with 8–12 weeks of therapy. Many are co formulated single tablet, once daily regimens, such as Sofosbuvir with Ledipasvir or Velpatasvir (second and third generation NS5A inhibitors respectively), Elbasvir with Grazoprevir (a third generation PI combined with a NS5A inhibitor), and the triple class combination of Paritaprevir/Ombitasvir and Dasabuvir. There are subtle differences between regimens with regards to drug–drug interactions and relative efficacy for different HCV genotypes, but overall these drugs are well tolerated and have SVR rates of over 95%. With the advent of more effective DAA regimens, the combination of Peg-IFN and RBV is no longer "standard of care" and has been removed from guidelines. RBV is still included in certain DAA regimens, depending on viral factors such as viral subtype (e.g., genotype 1a vs. 1b), "high" HCV RNA level, the presence of viral resistance associated mutations (RAS), and host factors such as cirrhosis. Current trials with the next generation of DAAs are ongoing, with the aim of reducing length of therapy down to 4 or 6 weeks, whilst maintaining SVR rates close to 100% irrespective of factors such as viral genotype, "high" HCV viral load, the presence of RAS, or cirrhosis.

Due to the overwhelming success of DAA therapy, it is postulated that HCV could be eradicated in the near future, using treatment as prevention and targeting populations in which HCV transmission is ongoing, such as IV drug users. Unfortunately due to the high cost of these regimens, the availability of these drugs in resource poor, but high prevalence countries is likely to be limited, although lower cost versions of certain DAAs have been made available through partnerships with generic manufacturers in these countries. The global eradication of HCV seems a tangible goal, but may still prove challenging.

Vaccines

Currently, no vaccine is available to protect against infection with HCV. During HCV replication, HCV sequences are continually evolving due to the error prone NS5B RNA dependant RNA polymerase, and the resulting viral diversity (quasi—species) creates obstacles for vaccine development with regards to the selection of target antigens, and the potential of escape from vaccine induced immune responses. Vaccine development has also been hampered by the lack of a suitable small animal model with which to assess the immunogenicity and protective efficacy of HCV candidate vaccines. Efficacy data has been obtained from chimpanzee models which have shown that it is possible to induce immune responses with vaccines that could inhibit viral replication, even if persistent infection was not prevented in all vaccinated animals. Prophylactic vaccines can be divided into those designed to induce T-cell responses, targeting HCV nonstructural proteins, and those designed to induce neutralizing antibodies targeting the envelope glycoproteins E1 and E2. New approaches, including peptide, recombinant protein DNA and vector vaccines have been evaluated in Phase 1/11 human clinical trials. A vaccine using recombinant E1 E2 proteins adjuvanted with MF59 has been tested in healthy volunteers and found to be safe, immunogenic, and to induce antibodies that neutralize HCV in vitro. Further development of this vaccine appears to be focusing on the induction of both neutralizing antibodies and T-cell responses by combining the recombinant E1 E2 protein with adenovirus vectors expressing the envelope glycoproteins of HCV. An immunocompetent, genetically humanized mouse model expressing the human orthologues of HCV has recently been developed that can support HCV entry but not full viral replication, and may aid future vaccine studies.

HDV

HDV, a defective RNA virus also referred to as Delta agent, infects nearly 20 million people worldwide, with a varying geographical distribution. It is only found in association with HBsAg—either as a coinfection in acute hepatitis B or as a "super infection" in an individual with chronic HBV infection.

Virology

First identified in 1977, HDV is deemed "defective" as it is unable to replicate by itself, requiring HBsAg as a protein "coat" for the HDV genome. HDV virions are 36–43 nm, roughly spherical, enveloped particles with no distinct nucleocapsid structure. The outer envelope contains lipid and all three forms of HBsAg (S, M, and L, but predominantly small). The genome of HDV (cloned and sequenced in 1986) consists of a compact (approximately 1.7 kb in length) circular single-stranded negative RNA molecule, which contains several sense and antisense ORFs, only one of which is functional and conserved. The RNA genome replicates via an RNA

intermediate, the antigenome, both of which can function as ribozymes to carry out self-cleavage and self-ligation reactions. A third RNA, complementary to the genome and found in the infected cell, is responsible for synthesis of the delta antigen. To date, there have been three genotypes and two subtypes of HDV have been characterized, but emerging data suggest that the genetic variability of the HDV genome is more complex than previously thought. Genotype 1 is mainly found in North America, Asia, Middle East, and Europe; genotype 2 in East Asia; and genotype 3 in South America. Genotype 2 is thought to cause less severe disease than the other two genotypes.

HDV genomic replication is not acutely cytopathic, and does not occur in cells other than hepatocytes; hence, the liver is the only organ affected. HBV is essential for the evolution of the hepatocellular necrosis and inflammation seen with HDV infection. Both humoral and cellular immune mechanisms are thought to be involved.

Epidemiology

Like HBV, HDV is transmitted by blood-borne and sexual routes, but in contrast perinatal transmission is rare. Infection with HDV has a global distribution, with two distinct epidemiologic patterns. First, high prevalence areas for HDV coincide with certain areas of high prevalence for chronic HBV infection, including the Mediterranean, Middle East, Central Africa, and the Amazon Basin in South America (Fig. 8). In these areas, viral transmission takes place predominantly by nonpercutaneous means, especially close personal contact. It is not known why HDV coinfection is not seen in other countries with a high prevalence of HBV, such as Southeast Asia and China. The second pattern is of parenteral transmission in subjects with multiple parenteral exposures, primarily injection drug users and hemophiliacs, as is seen in nonendemic areas such as Northern Europe and the United States. Worldwide, HDV infection is declining due to control of HBV. Even in Italy, an HDV-endemic area, public health measures introduced to control HBV infection there resulted in a significant reduction in the prevalence of HDV infection.

Clinical Features

The outcome of infection with HDV depends on whether HDV and HBV infect simultaneously (coinfection) or if HDV is acquired by an individual with chronic HBV (superinfection). Mortality from HDV infection is 10 times higher than for HBV alone.

In coinfection, both acute HBV and HDV hepatitis occur. Whether one or two bouts of clinical hepatitis are seen depends on the relative titers of HBV and HDV. The incubation period of acute HDV is between 3 and 7 weeks, with a preicteric phase lasting 3–7

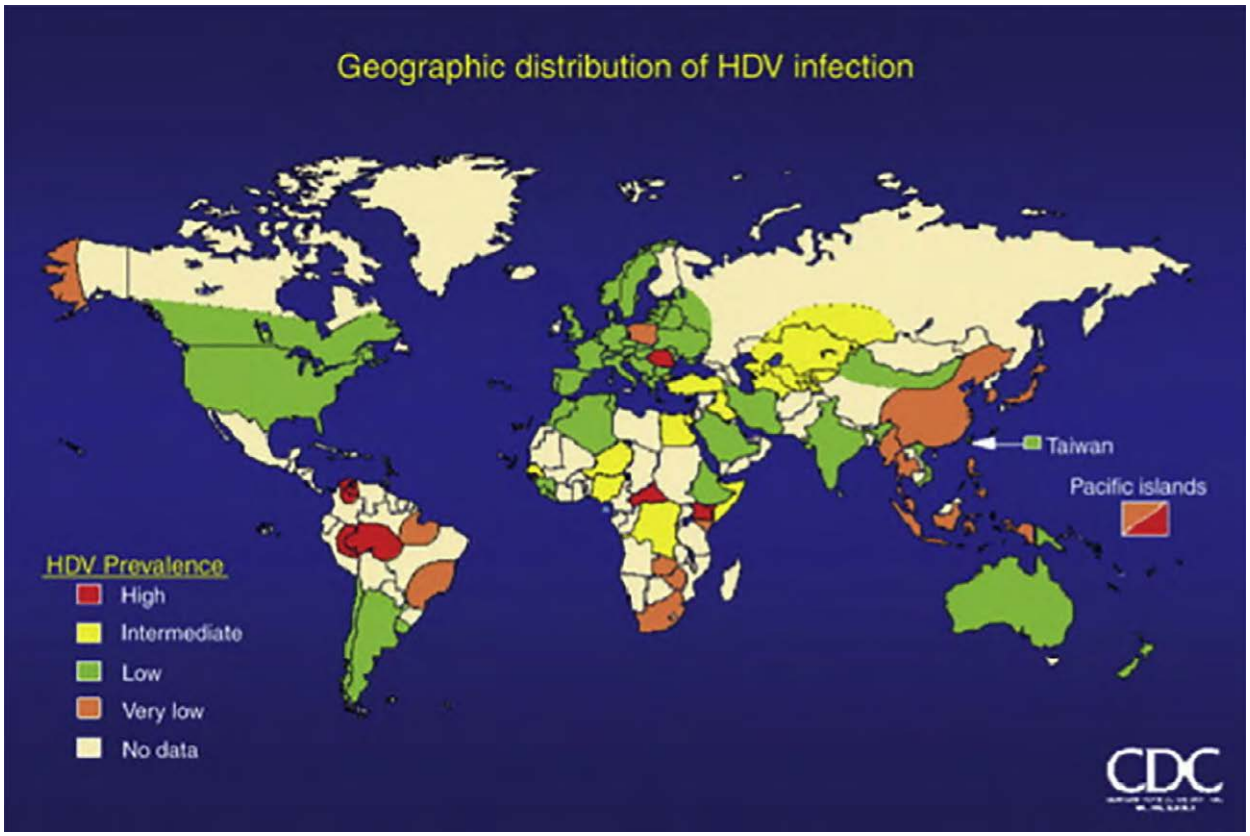

Fig. 8 Geographic distribution of hepatitis D. Reproduced from Center for Disease Control and Prevention, USA.

days, during which time the classic symptoms of acute hepatitis are manifested (fatigue, lethargy, anorexia, and nausea), and ALT and AST become elevated. This phase is followed by jaundice, which can last for several weeks. The severity of the resulting illness is typically greater than with HBV monoinfection, with a higher incidence of fulminant hepatitis, and low risk of chronic infection (<5%). During the acute phase of HDV infection, synthesis of both HBsAg and HBV DNA is suppressed until HDV is cleared.

Hepatitis D superinfection is frequently associated with severe acute hepatitis with progression to chronic HDV in up to 80% of cases. Fulminant hepatitis also occurs. Superinfection can transform inactive or mild chronic hepatitis B into severe, progressive chronic hepatitis, and result in faster progression to cirrhosis than seen with chronic HBV alone. Up to 70% of patients with chronic HDV will develop cirrhosis.

Diagnosis

Anti-HD IgM, HDV RNA, and HDAg are the most sensitive markers for the diagnosis of acute HDV, although anti-HDIgG is the only test commercially available. Detection of serum HDV RNA by PCR is the "gold standard" for confirming infection.

In acute HBV–HDV coinfection, HBsAg precedes the appearance of HDAg and HDV RNA. Anti-HDIgM develops late in the acute phase, and is accompanied by the disappearance of HDAg. In about 15% of patients the only evidence of HDV infection may be the detection of either IgM anti-HDV alone during the early acute period of illness or IgG anti-HDV alone during convalescence. Both IgM and IgG anti-HD antibodies decline after recovery to subdetectable levels within months to years, and it can sometimes be difficult to demonstrate past HDV infection as there is no serologic marker that persists to indicate that the patient was ever infected with HDV. These immune responses are thought to be protective, as second cases of HDV have not been reported.

With HBV–HDV superinfection, HBsAg titers decline with the appearance of HDAg in the serum. HDV RNA levels reach a peak between 2 and 5 weeks after exposure, declining over a period of 1–2 weeks. High titers of IgM and IgG anti-HD are detectable in the acute phase, and can persist for months or even indefinitely. Most superinfections result in chronic HDV, demonstrated by the persistence of HDV RNA, and high levels of IgM and IgG anti-HD. HDV viremia is associated with active liver disease.

Therapy

High-dose IFN-α (up to 9 million units three times weekly for 48 weeks) has been evaluated in individuals with chronic HDV, although standard IFN has now been superceded by Peg-IFN. Interferon therapy does not achieve viral eradication in the majority of those treated, although improvement in biochemical and histological parameters has been observed. Prolonging therapy for greater than 12 months may be of benefit in certain patients if eradication of HBV sAg is used as an endpoint. The development of specific antiviral therapy against chronic HDV has been impeded due to the lack of specific enzymatic targets in the virus. HDV virion assembly requires prenyl lipid modification (or prenylation) of its nucleocapsid—like protein large HDV antigen. Prenylation inhibitors are currently in clinical development and other viral processes which are potential targets for inhibition include acetylation, phosphorylation and methylation. Lonafarnib (a farnesyl transferase inhibitor) is being evaluated in combination with Peg-IFN in clinical trials. Treatment of the associated HBV infection with oral antiviral drugs does not alter the course of HDV infection.

Vaccines

No vaccine is available specifically for delta virus; however, as this virus is only acquired in association with HBV, vaccination against HBV will also prevent against the transmission of HDV. HBIg will not protect individuals with chronic HBV from infection with HDV.

HEV

HEV is an enterically transmitted RNA virus responsible for outbreaks of hepatitis in developing countries and sporadic cases of acute hepatitis in endemic areas such as Southeast and Central Asia (Fig. 9). Locally acquired hepatitis E in individuals who have not traveled to endemic areas is, however, becoming an emerging problem in European countries.

Virology

Although recognized as a clinical entity in the 1980s, the virus responsible for acute enterically transmitted NANB hepatitis was not identified until 1983. HEV has recently been classified in the genus *Hepevirus* of the family Hepeviridae.

Cloned in 1990, the HEV genome is a nonenveloped, single-stranded, positive-sense RNA molecule 7.2 kb in length, with three ORFs. ORF 1 codes for viral enzymes methyltransferase, proteases, helicase, and replicase; ORF 2 codes for the major HEV capsid protein, the function of the protein coded for by ORF 3 has not yet been well characterized, although it appears to be involved in virus–host interactions. Four major genotypes have been identified, geographically distinct, but all share at least one major serologically cross-reactive epitope despite substantial genomic variability, which is an important observation that may facilitate the development of a protective vaccine.

Fig. 9 Geographic distribution of hepatitis E. Reproduced from Expert Reviews in Molecular Medicine, 2003, Cambridge University Press.

Viral replication, transcription, virus–host interactions, and pathogenesis of HEV are poorly understood due to the lack of a cell culture system and a practical animal model for HEV. Subgenomic expression strategies have been used to fractionally characterize HEV-encoded proteins. However, in common with other hepatitis viruses, the mechanism of liver injury is probably immunological, as the onset of ALT elevation and histopathological findings of acute hepatitis correspond with the appearance of antibodies to HEV and decreasing levels of HEV antigen in hepatocytes.

Epidemiology

Outbreaks of acute HEV infection affecting several thousand people have been reported from Asia, the Middle East, Northern and Western parts of Africa, and Central America. HEV is transmitted through the fecal-oral route and most epidemics have been associated with contaminated drinking water. Although like HAV, HEV is detectable in the stools of infected individuals, person-to-person transmission is less commonly observed, and sexual transmission is debatable. Parenteral transmission of HEV is possible, with reports of individuals infected from receiving contaminated blood products. Risk of infection is linked to the amount of virus in the donor's blood, and the volume of plasma transfused. Materno-fetal transmission is possible, with recent data indicating maternal infection is associated with increased risk of preterm delivery, miscarriage, stillbirth or neonatal death. In contrast with perinatally transmitted HBV, there have not been reports of chronic HEV infection in children born to mothers with HEV.

HEV has four genotypes, with differences in epidemiology being observed between genotypes; genotype 1 is predominant in developing countries, causing community level outbreaks, whilst genotype 3 is generally responsible for isolated infections in developed nations. The spectrum and magnitude of disease caused by HEV genotype 3 is still being characterized.

HEV is a common cause of sporadic hepatitis in endemic areas, accounting for almost 50%–70% of acute hepatitis cases in India. In industrialized nations, acute HEV infections are infrequent, accounting for $<1\%$ of cases of acute viral hepatitis, and are usually associated with a history of travel to an endemic area. However, there have been an increasing number of reports of nontravel-associated HEV from Europe, United States, and Japan, and in these cases it has been postulated that HEV may have been acquired from a viral reservoir in swine or possibly rodents. In support of this theory, high seroprevalence of anti-HEV has been reported in pig veterinarians, and farm workers in contact with pigs. In Japan, cases of acute infection associated with the consumption of inadequately cooked pig liver have been reported. HEV may therefore be viewed as a new emerging pathogen with zoonotic potential.

There is evidence that subclinical infection may be common in endemic areas; one study reports HEV seroprevalence rates of 30%–50% in healthy subjects. Another study from Southeast Asia followed up a cohort of healthy individuals over an 18-month period and found that 66% of those who seroconverted to anti-HEV had no history of jaundice, suggesting that such "subclinical" cases may act as reservoirs for infection in endemic populations and also as a source for cases of sporadic acute HEV.

Clinical Features

As for hepatitis A, the attack rate is highest for young adults aged between 15 and 40. Work with susceptible primate models and infected individuals reveals that the incubation period is approximately 21 days, with the expression of HEV antigen in the serum and stool occurring 7 days before the onset of symptoms, and persisting for approximately 2 weeks. ALT and AST begin to rise a few days before the onset of symptoms, but generally return to normal within 1–2 months after the peak severity of the disease has passed. Anti-HEV (IgM) appears shortly after the onset of clinical illness and persists only for a few months. IgG anti-HEV appears a few days after IgM and persists for up to 3 years, and may afford protection against reinfection.

Clinical features are similar to other acute viral hepatitides. Acute HEV infection is often mild and self-limiting in immunocompetent individuals, but can cause fulminant liver disease in persons with underlying cirrhosis and pregnant women, particularly during the third trimester of pregnancy when mortality rates of up to 25% have been reported. Surveillance data from rural Bangladesh indicates that acute hepatitis, most likely due to HEV, is responsible for approximately 10% of pregnancy associated deaths. Disease severity may correlate with HEV genotype, genotype 3 appears to be associated with milder disease. In Japan where 3 and 4 are the most prevalent HEV genotypes, two studies found that patients with genotype 4 experienced higher peak ALT levels and longer hospital stays as compared with patients with genotype 3. Chronic infection with HEV genotypes 3 and 4 has been described in the setting of solid organ transplantation and potent immunosuppression, such as used in stem cell transplants. Chronic HEV can cause significant liver disease. As transmission of the virus via contaminated blood is possible, several countries now screen all donated blood for HEV, whilst others, including the United Kingdom, only screen blood products for recipients most at risk of developing chronic infection (solid organ and stem cell transplant patients, neonates and infants under the age of 12 months).

Acute and chronic HEV infection may be associated with a number of acute neurological syndromes, including Guillain Barre syndrome (acute inflammatory demyelinating polyradiculopathy), neuralgic amyotrophy, meningo encephalitis, and transverse myelitis. HEV RNA has been demonstrated in the serum and cerebrospinal fluid in some affected individuals, implying a direct effect of the virus, but causality is not fully elucidated. It is not clear if damage to the central nervous system is mediated by an abnormal immune response triggered by the virus, or secondary to local viral replication. In the majority of these cases jaundice is not present, and there is only mild derangement of liver transaminases, making diagnosis difficult.

Diagnosis

Infection with HEV can be confirmed by the detection of IgM and IgG anti-HEV antibodies or the presence of HEV RNA in the serum detected by RT-PCR, HEV RNA can also be detected in stool. IgM anti-HEV antibodies can be detected during the first few months after HEV infection whereas IgG anti-HEV antibodies represent either recent or previous infection. Presence of HEV RNA in serum indicates current infection, either acute or chronic. Although commercial antibody assays are available, they can vary markedly in their sensitivity and specificity. Improved validation of existing assays, and the development of new assays with better performance characteristics is required.

Treatment

Some patients with chronic HEV may respond to a reduction in immunosuppression. If this approach is unsuccessful, antiviral therapy can be considered. Peg-IFN and RBV have previously been evaluated, but the use of Peg-IFN may lead to graft rejection in solid organ recipients and is not recommended in this setting. Although data is limited, RBV monotherapy for at least 3 months is the preferred option. In one retrospective multicentre case series of 59 patients treated with RBV monotherapy for a median duration of 3 months (range 1–18 months), a viral clearance rate of 78% was achieved.

Vaccines

There is currently no globally available licensed vaccine against HEV. In 2007, Shrestha and colleagues evaluated a recombinant HEV vaccine in (mostly male) volunteers in the Nepalese Army; the protective efficacy against clinically overt HEV infection was 95.5% in subjects receiving all three vaccine doses and 85.7% after two doses. Although the vaccine was efficacious and well tolerated, it was not developed commercially. In 2005, researchers in China began working on a new recombinant HEV vaccine. Results from a randomized clinical trial with over 100,000 participants indicated the efficacy of the vaccine was greater than 99% in those who received the full three dose series. The vaccine was subsequently approved and licensed by Chinese authorities in 2012, but it is not approved outside China.

"New" Hepatitis Viruses

Several recently discovered viruses, namely torque teno virus (TTV), SENV, and hepatitis G, although potentially hepatotropic, have yet to be conclusively shown to be pathogenic to the liver.

In 1994, an agent (initially named hepatitis F virus) was isolated from a stool sample and found to be transmissible in primates. Subsequent researchers have not been able to confirm the existence of this agent, and there is currently no confirmed HFV.

TTV

TTV, family Circoviridae, genus *Anellovirus*, has a single-stranded circular DNA genome. Related viruses are also found in primates, chickens, pigs, cows, sheep, and dogs. This virus was first identified in 2001 in a patient presenting with non-A–E acute recurrent hepatitis with no history of drug or alcohol abuse and negative markers of viral or autoimmune disease. Transmitted parenterally and probably also enterically, the TTV family of viruses is present in over 90% of adults worldwide. Human pathogenicity, particularly regarding the liver, has yet to be clearly established.

SEN Virus

Named after the initials of the patient with HIV from which it was first isolated, SEN virus (SENV) is a novel circular single-stranded DNA virus distantly related to TTV and also classified within the family Circoviridae, which has been associated in some studies with post-transfusion hepatitis. Variants designated A–H have been identified, which have differing geographical prevalence, for example, SENV-D is highly prevalent in Japan. Although it has been found more frequently in individuals with liver disease than the normal population there is no firm evidence that infection causes hepatitis or worsens the course of liver disease.

HGV

In 1995 and 1996 several new flaviviruses, GB-A, GB-B, and GB-C, related to but distinct from HCV, were identified. GB-A and GB-B infect tamarin monkeys but GB-C can infect humans and has been implicated in some cases of acute and chronic hepatitis. Another group identified a virus which they termed HGV; based on genomic sequence comparisons, HGV and GB-C can be considered the same virus. Another virus in this family, GBV-D, infects bats. GB viruses have recently been reclassified, due to similarities in genome organization and pathogenic features, into the tentative genus Pegivirus within the family Flaviviridae.

HGV is a positive-stranded RNA virus, which has at least four major genotypes. It is thought to be lymphotropic, replicating predominantly in the spleen and bone marrow. Molecular epidemiological studies have demonstrated that about 1%–1.4% of the healthy population in developed countries are chronically infected with HGV. Parenteral is the most frequent mode of infection, and HGV has been implicated in a small number of cases of posttransfusion hepatitis. There is also a higher prevalence of HGV in drug users and patients on hemodialysis. Whether HGV is a significant cause of human hepatitis is still unconfirmed. Although relatively common, infection with HGV may cause a mild acute hepatitis but does not seem to cause clinically significant chronic liver disease. In one US study evaluating causes of acute viral hepatitis, HGV RNA was detected in 9% of patients with acute non-A–E hepatitis, 20% of those with HCV, 25% of those with HAV, and 32% of those with HBV. Another study examined serum samples from blood donors, transfusion recipients and controls. Prevalence of HGV in the nontransfused individuals ($n = 657$) was 1.4%. There were 35 HGV infections among the 357 transfusion recipients, with only three having HGV as the only agent detected. All these subjects had a mild hepatitis and did not develop clinically apparent chronic hepatitis (although liver biopsy was not performed).

A French study retrospectively examined 228 subjects with chronic HCV for HGV coinfection; 21% of the cohort and 32% of the subset who were IVDUs were coinfected. Another French study found that 57.5% of a cohort of 61 hemodialysis patients tested positive for HGV RNA; however, only four of these subjects had viremia associated with elevated serum ALT.

Several studies in HCV coinfected individuals, although with small numbers of subjects, have established that severity of liver disease, serum HCV RNA levels, and response to IFN therapy are not influenced by HGV coinfection. HGV RNA levels were observed to decrease with IFN therapy, but most subjects became detectable again once IFN was discontinued.

Future Prospects

Prospects for lessening both mortality and the socioeconomic burden resulting from viral hepatitis are optimistic, because safe and effective vaccines are currently commercially available for HAV and HBV, and a vaccine for HEV is available, albeit currently only in China. Improvements in sanitation and dissemination of simple public health information will lessen the transmission of HAV and HEV in endemic areas. Pretransfusion screening of blood and blood products for infectious agents is now routine in the majority of developed countries, thus lessening the iatrogenic transmission of HBV, HDV, and HCV. In Asia, where vertical transmission of HBV constitutes the major route of infection, increasing maternal HBV screening, and use of both active (administration of HBV vaccine) and passive (administration of hyperimmune globulin) immunoprophylaxis for infants at high-risk will prevent most neonatal infections, leading to a decreased incidence of chronic HBV-related complications (such as HCC) in these populations over time. Owing to particular viral characteristics, development of a vaccine for HCV remains a scientific challenge with no current indication of when such a vaccine might be available, however prospects are improving due to advances in vaccine technology and the understanding of HCV immunity and replication, and the availability of novel tools to test antibody responses. Fortunately, the incidence of HCV, at least in the Western world, is declining because of routine screening of blood and blood products before transfusion, and the implementation of risk minimization strategies, for example, needle exchange programs, in populations such as IVDUs. In chronic HBV, although long-term viral suppression can be maintained in the majority of patients using currently available oral therapies, achieving viral eradication is problematic. There are a large number of drugs in early clinical development, ranging from immunostimulatory agents to new antiviral targets and HBV entry inhibitors, which aim to bring about eradication of viral reservoirs with finite therapy. The treatment of chronic HCV has evolved dramatically, firstly with the introduction of novel protease inhibitor therapies in 2011, and the arrival in 2013–14 of highly efficacious and well tolerated "second generation" direct acting antivirals. Such is the potency of these agents that there is the potential to cure the majority of individuals with HCV, providing that access to these therapies globally is not impeded by the high costs of these regimens.

Many cases of acute hepatitis occur without any identification of a causative agent, viral or otherwise, which would imply that there are additional pathogenic hepatotropic agents. The search for these agents will provide a continuing diagnostic and therapeutic challenge.

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www.nice.org.uk/—National Institute for Health and Care Excellence Clinical Guideline 165 (Hepatitis B).
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Herpesviruses☆

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Glossary

Antiviral drugs A class of medication used specifically for treating viral infections. As with antibiotics for bacterial infections, specific antivirals are used for specific viruses.
Immunocompetent An individual with a normal immune system; the individual is capable of developing an immune response to an infection.
Immunocompromised An individual whose immune system is compromised in some way, the individual lacks the ability to mount a normal immune response to an infection and is often unable to resist or fight off infection.
Latency A quiescent period of infection when most or all of the viral lytic genes are silenced, and the virus is not making progeny virus.
Lytic A type of infection in which viral lytic genes are switched on and the virus is actively undergoing replication and making progeny virions. This is also referred to as a productive infection.
Seropositive A situation where an individual has antibodies against a certain pathogen in their blood, being seropositive indicates that the individual was previously exposed to that pathogen.
Vaccine A preparation that contains an antigen, consisting of an organism or a part of an organism, that is used to confer immunity against the disease that the organism causes. Vaccines can be natural, synthetic or derived from recombinant DNA technology.

Abbreviation

ACV	Acyclovir
BL	Burkitt's lymphoma
CAEBV	Chronic active EMV
CD21	Complement receptor 2
CD8$^+$	Cluster of differentiation 8
E genes	Early genes
EBNA	EBV-coded nuclear antigens
EBV	Epstein-Barr virus
ES	Exanthem subitum
HCF	Host cell factor
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HHV-4	Human herpesvirus 4
HHV-6	Human herpesvirus 6
HHV-8	Human herpesvirus 8
HPC	Hematopoietic progenitor cells
HSE	Herpes simplex encephalitis
HSK	Herpetic stromal keratitis
HSV	Herpes simplex virus
IE genes	Immediate early genes
IFN-γ	Interferon-γ
IM	Infectious mononucleosis
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L genes	Late genes
LATs	Latency-associated transcripts
LCV	*Lymphocryptovirus*
LMPs	Latent membrane proteins

☆*Change History:* September 2014. AL vant Lint and DM Knipe updated the text and further readings to this entire article.

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MCMV	Models with murine CMV
MHC	Major histocompatibility complex
NHANES	National Health and Nutrition Examination Survey
NK cells	Natural killer cells
NPC	Nuclear pore complexes
Oct-1	Octamer binding protein 1
PEL	Primary effusion lymphoma
PHN	Post-herpetic neuralgia
PTLD	Posttransplant lymphoproliferative disease
RDV	*Rhadinovirus*
TK	Thymidine kinase
VAHS	Virus-associated hemophagocytic syndrome
VHS	Virion host shutoff
VP16	Viral protein number 16
VZV	Varicella-zoster virus
XLP	X-linked lymphoproliferative

Defining Statement

The family *Herpesviridae* includes over 100 different species of DNA viruses, eight of which are currently known to infect humans. These viruses are discussed with relation to the diseases they cause, their ability to establish latent infections, their biology, replication and pathogenesis, and the treatment options available for each.

Family *Herpesviridae*

The family *Herpesviridae* is a family of large DNA viruses containing over 100 different virus species that infect hosts ranging from humans to birds to reptiles. Classification of a virus as a member of the family *Herpesviridae* is based on a shared virion structure: a linear, double-stranded DNA genome is contained within a central core, surrounded by an icosahedral capsid. This capsid is in turn surrounded first by an amorphous protein layer, known as the tegument, and then by an envelope containing viral glycoprotein spikes (Figure 1). Herpesviruses also share four significant biological properties:

1. They encode a large number of enzymes involved in nucleic acid metabolism, DNA synthesis, and processing of proteins.
2. The synthesis of viral DNAs and caspid assembly occurs in the nucleus of the infected cell. During infection, virus-specific compartments are assembled within the nucleus of the infected cell, commonly referred to as replication compartments (Figure 2). It is within these compartments that viral DNA replication, late viral gene expression, and encapsidation of progeny viral genomes occur. These compartments lead to the formation of basophilic nuclear inclusion bodies, which are diagnostic of herpes virus infection.
3. Production of infectious progeny virus is generally accompanied by the destruction of the infected cell.
4. The viruses are able to establish a latent infection in their natural hosts.

There are currently eight herpesviruses that are known to infect humans: herpes simplex virus (HSV)-1 and HSV-2, human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV) and human herpesvirus (HHV)-6 and HHV-7. These viruses, along with the majority of herpesviruses that infect other

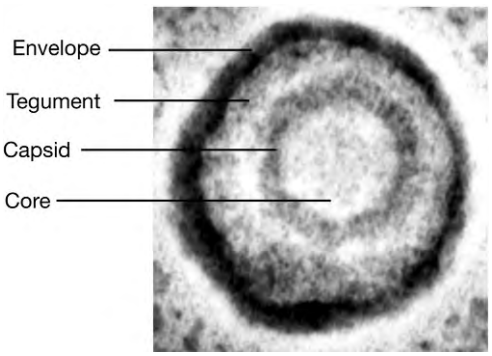

Figure 1 Structure of the herpes simplex virus (HSV) virion. An electron micrograph of a negative-stained HSV-1 virion. The envelope, tegument, capsid, and core are indicated. Micrograph provided by Dr. Travis Taylor.

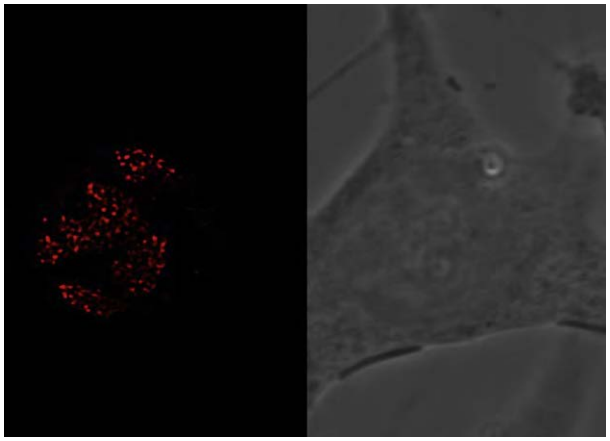

Figure 2 Replication compartments in the nucleus of a herpes simplex virus (HSV)-infected cell. Shown is an immunofluorescence image illustrating the localization of ICP8 to replication compartments within the nucleus of an infected cell. ICP8 staining is shown on the left, and the location of the nucleus within the cell can be seen in the phase-contrast micrograph on the right. Figure provided by Lindsey Silva.

mammals and birds, have been divided into three subfamilies, alpha, beta, and gamma, based on the biological properties of the viruses. HSV-1, HSV-2, and VZV are members of the *Alphaherpesvirinae* subfamily, EBV and KSHV are both members of the *Gammaherpesvirinae* subfamily, while the remaining viruses, HCMV and HHV-6A, HHV-6B, and HHV-7 are all members of the *Betaherpesvirinae* subfamily. Despite the many similarities in structure and biological properties shared by herpesviruses, it is not surprising that in a group of this size there are also many differences. Host range, length of replicative cycle, cell type in which latency is established, and clinical manifestations of disease all vary among the different members of the family.

The *Alphaherpesvirinae* Subfamily

Members of the *Alphaherpesvirinae* subfamily are characterized by a variable host range, short reproductive cycles, the ability to spread rapidly in culture and efficiently destroy infected cells, and the capacity to establish latent infections primarily, although not solely, in sensory ganglia. The members of the *Alphaherpesvirinae* subfamily that infect humans are HSV-1 and HSV-2, and VZV.

Herpes Simplex Virus

Disease

The two HSV species, HSV-1 and HSV-2, are capable of causing a variety of diseases within an infected host. The most common of which are orolabial lesions, commonly referred to as cold sores or fever blisters and most often caused by HSV-1, and genital herpes which is most often caused by HSV-2. The viruses are extremely widespread throughout the world's population. The US National Health and Nutrition Examination Survey (NHANES) conducted between 2005 and 2010 has put seroprevalence rates in the United States at 54% for HSV-1 and 16% for HSV-2. Seroprevalence rates in Europe tend to be slightly higher for HSV-1 and slightly lower for HSV-2 when compared to the United States, although there are large intercountry differences. In developing Asian countries, the seroprevalence of HSV-1 and HSV-2 appear to match those seen in Europe and the United States. However, in Africa and Central and Southern America, the picture is quite different. In these regions, HSV-1 is becoming an almost ubiquitous pathogen with greater than 90% of the population seropositive by their fourth decade of life. HSV-2 seroprevalence rates range from 30% to 80% in women and 10–50% in men in sub-Saharan Africa, and between 20% and 40% in women in Central and South America. Universally, HSV-2 seropositivity is higher in women than in men. Changes in sexual practices also mean that HSV-1 is becoming a more common cause of genital infection than it once was.

In an immunocompetent host, primary infections with HSV can be asymptomatic, with an individual only realizing that they have been infected when a recurrent infection occurs at a later point. However in some cases, primary infections can be symptomatic, and in these instances disease is usually more severe than that seen with recurrent infection. During symptomatic primary HSV infection, individuals can present with fever, malaise, and large quantities of painful vesicular lesions at and around the site of infection, lasting for a period of up to 3 weeks. Recurrent infections, at either the orofacial or genital site, generally involve a much smaller number of vesicular lesions that persist for 7–10 days.

Along with the common mucosal herpetic lesions associated with orofacial and genital infections, HSV is also associated with a number of more severe complications. In immunocompetent individuals, the most serious complications are herpetic stromal keratitis (HSK) and herpes simplex encephalitis (HSE). Ocular infection with HSV can lead to HSK, the leading cause of infectious corneal blindness. Initially, recurrent infections within the cornea can produce ulcers that result in pain, light sensitivity, and blurred vision. Repeated episodes of recurrent disease can lead to involvement of the underlying stroma, resulting in HSK, which can eventually lead to blindness due to corneal scarring and vascularization. HSE, while extremely rare, has a high risk of mortality if left untreated (>70%). It is usually caused by HSV-1 and results in inflammation and swelling of the brain tissue, with patients

presenting with weakness, visual disturbances, and seizures. Although antiviral drugs can be used to decrease mortality, almost 50% of patients fail to regain complete normal function.

As is common with herpes viruses, individuals with compromised or absent immune responses are at high risk of HSV complications. Patients with atopic dermatitis, where the immune response in the skin is skewed toward a Th2 response, can develop a disseminated HSV infection throughout the skin known as eczema herpeticum. Evidence from HIV-positive patients and people undergoing immunosuppressive therapy has demonstrated the increased severity of HSV infection in such populations, with these patients also more prone to chronic or atypical infections. Finally, HSV infection in neonates is associated with increased mortality and morbidity. Infection in this setting usually results from transmission from mother to child during delivery and is estimated to occur at a rate of one in every 3–5000 deliveries. Localized infections of the skin, eyes, and mouth are rarely fatal. However, children with disseminated infections or those involving the central nervous system are at high risk of mortality or ongoing neurological impairment. Prompt antiviral treatment has been able to reduce mortality rates; however, neurological sequelae remain high in children who recover from either disseminated HSV or HSV encephalitis.

Virus and Biology

The genome of HSV-1 is approximately 152 kbp in length, while that of HSV-2 is approximately 154 kbp. The two viruses have approximately 50% nucleotide sequence identity and encode protein products with high levels of amino acid sequence identity. Both viruses have the same genome structure of a unique long and unique short region flanked by inverted repeats and share the common herpesvirus virion structure described previously. The high level of shared protein sequence results in antigenic cross-reactivity between the two viruses but despite this they have different neutralization patterns and tend to produce different clinical symptoms.

One biological property, common to both HSV-1 and HSV-2, that influences the ability of these viruses to cause disease in humans is neurovirulence. The ability of the virus to invade and replicate within the host nervous system primarily enables the virus to establish a latent reservoir of virus within this site, from which reactivation and subsequent transmission can occur. However, it also provides a situation whereby the virus can produce severe disease within the host, such as is seen in cases of HSV encephalitis.

Replication

In permissive cells, the process of HSV viral replication takes 18–20 h (Figure 3). The initial step in this process is the attachment to and entry of the virus into the target cell, a process that involves five viral glycoproteins – gB, gC, gD, gH, and gL. The initial attachment is mediated by contact between glycoprotein C (gC) and/or gB with heparan sulfate on the surface of the cell. Viral

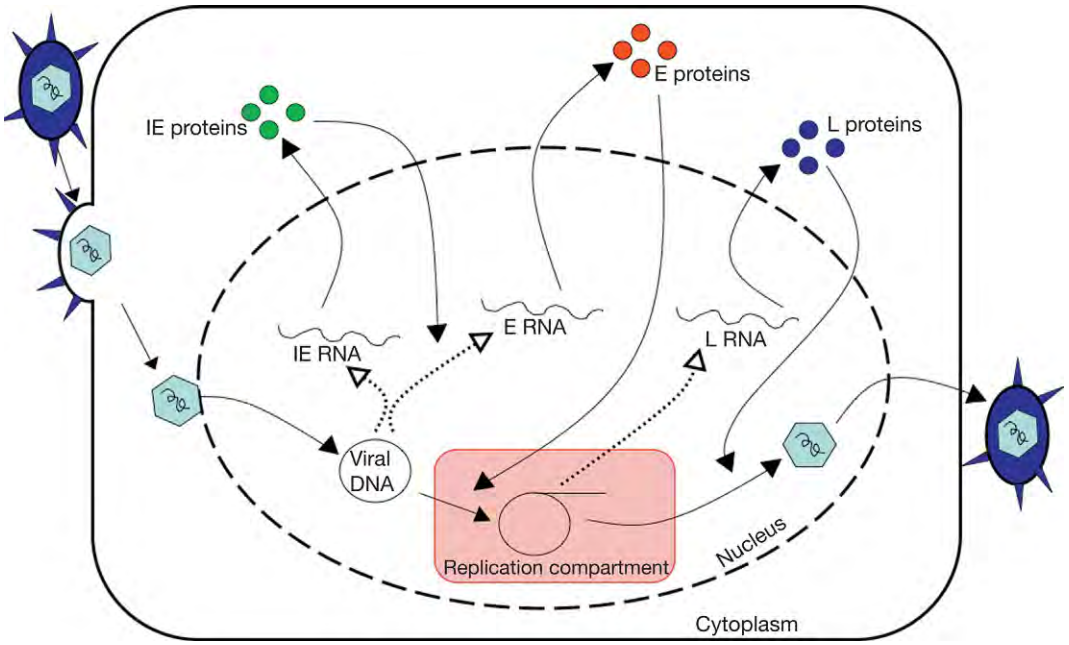

Figure 3 Replication cycle of herpes simplex virus (HSV). The virus binds to the cell surface and the capsid and tegument are released into the cytoplasm following fusion of the viral envelope with the plasma membrane. The capsid is transported to the nuclear pore, and the viral DNA is released into the nucleus and becomes circularized. The host RNA polymerase II transcribes the immediate early (IE) genes, and the mRNAs are transported into the cytoplasm where translation occurs. The IE proteins activate transcription of the early (E) genes that are involved in viral DNA replication. Viral DNA replication occurs within replication compartments and stimulates transcription of the late (L) genes. The L proteins are involved in assembling the capsids within the nucleus. Progeny viruses exit from the cell by one of three potential mechanisms described in the text.

attachment is closely followed by interaction between gD and one of the several cell surface receptors that facilitate entry into the cell; three different classes of receptors have been identified for gD. Viral entry can occur via two pathways, the well-characterized method of direct penetration of the cellular membrane via fusion with the viral envelope and the less well-characterized method of endocytosis. This second method of entry has to date only been demonstrated using *in vitro* systems with several different cell lines. Hence, the importance of endocytosis in a natural infection remains to be determined. The kinetics of both modes of viral entry appears to be similar, with the transition from attachment to penetration occurring within minutes.

Following entry into the cell, the de-enveloped viral capsid is transported through the cytosol to the nuclear pore complexes (NPC) where the viral DNA is released into the nucleus and takes on a circularized form. Movement of the capsid through the cytosol is rapid, reaching the nucleus within approximately 1 h. This transport is mediated by microtubules. Once the viral DNA has entered the nucleus, the host RNA polymerase II is then used to transcribe the viral genome in a sequential fashion, resulting in the expression of over 80 viral proteins. The viral proteins are preferentially translated within the infected cell, in part due to the action of the viral protein VHS (virion host shutoff). This tegument protein remains in the cytoplasm as the viral capsid is transported to the nucleus. It induces destabilization of host mRNA and causes a rapid cessation of host protein synthesis, resulting in the loss of the cellular mRNA pool and the preferential translation of viral mRNAs. The sequential pattern of viral gene expression results in the HSV genes being divided into three categories, loosely based on the timing of their expression in infected cells. The categories are the immediate early (IE) or α genes, the early or β genes, and the late (L) or γ genes.

IE Genes

The IE genes are expressed within 2–4 h of infection and include six different viral proteins. These genes can be expressed in the absence of *de novo* viral protein synthesis, using the viral tegument protein VP16 (viral protein number 16) as a transactivator. VP16 is transported to the nucleus with the cellular host cell factor (HCF) protein. Once within the nucleus, the VP16–HCF complex binds to a second cellular factor, Oct-1 (octamer binding protein 1) and forms a stable transcription regulatory complex called the VP16-induced complex. This complex recuits transcription factors, histone-modifying enzymes and chromatin-remodeling complexes to stimulate expression of the IE genes.

The IE genes ensure the orderly expression of subsequent viral genes and the evasion of the cellular responses to the infection. To do this, many of the IE genes perform multiple functions, and a variety of forms of posttranslational processing is employed to enable these proteins to fulfil the roles required. In brief, ICP4 is required for the expression of all early (E) and L genes; ICP0 acts as a nonspecific or 'promiscuous' transactivator, capable of stimulating the transcription of α, β and, γ genes; ICP27 is required for the transcription of the L viral genes and some E genes; ICP47 is involved in immune evasion; and ICP22 and $U_S1.5$ are thought to be involved in promoting the expression of some L genes.

E Genes

The E genes are maximally expressed between approximately 5–7 h after infection. The E proteins include those that make up the viral DNA replication machinery. Thus, the expression of the E genes signals the initiation of viral DNA replication. However, the expression of certain E genes is also involved in downregulating IE gene expression, in particular, ICP8 has been shown to downregulate expression of the IE gene ICP4.

L Genes

The L genes are the final group of viral genes to be expressed within the infected cell. Some L genes, such as gB and gD, are actually expressed early in the infected cell, and their expression simply increases with the onset of viral DNA replication. Alternatively, other L genes, including gC and U_S11, are only expressed following viral DNA replication. Many of the L genes encode viral structural proteins, and their expression enables the production of progeny virion particles.

Viral Egress from Infected Cells

Following the expression of the L genes, viral capsids are assembled within the nucleus. These capsids, predominantly made up of four viral proteins, are then filled with viral DNA, a process that utilizes viral proteins. It is generally accepted that the nucleocapsids then bud through the inner nuclear membrane and upon doing so acquire an envelope. However, the subsequent events that lead to the egress of the newly formed virus particle from the infected cell are not yet fully understood. Three competing theories exist, each with varying amounts of supporting evidence. (1) The enveloped virus fuses with the outer nuclear membrane, leaving the de-enveloped nucleocapsid to bud into the Golgi apparatus, regaining an envelope, and then to travel to the surface via vesicular movement. In this model, the tegument could be acquired in the nucleus or the cytoplasm, with work supporting this model demonstrating that the majority of virions gain their tegument and envelope in the cytoplasm. (2) The enveloped nucleocapsid buds through the inner nuclear membrane and is transported to the surface by vesicular movement through the Golgi apparatus;

thus, in this model, the tegument would be acquired in the nucleus. (3) Capsids exit the nucleus via nuclear pores and then bud through Golgi membranes.

Release of virus from an infected cell results in the shedding of newly formed virions, enabling the virus to spread to susceptible individuals. However, within the body, the virus can also spread directly from cell to cell. This process involves the viral glycoproteins gE and gI, which form a heterodimer, and is facilitated by cell contact. In general, cells infected with replicating HSV do not survive the infection, due to the cytopathic effects of viral infection.

Latent Infection

HSV persists for the life of the host by establishing a latent infection in sensory neurons. During a primary infection, virus enters the sensory nerve endings in the epithelium and travels to the neuronal cell body. Animal models have suggested that within the neuronal cell body viral replication can occur initially. However, within several days, no replicating virus can be detected. Concurrent with the short-lived lytic infection within the ganglia, the virus also establishes latency and, following clearance of the replicating virus, latent virus persists. Evidence from animal models and human studies has indicated that the latent HSV genome most likely exists as an extrachromosomal circular episome, with human studies suggesting a latent viral burden of between 1 and 10 viral copies per neuron, remembering that not all neurons within a ganglion will harbor latent virus. Viral replication within the nerves or even at the primary site of infection is not required for the establishment of latency. However, lack of viral replication appears to reduce the quantity of latent virus within a latently infected ganglion through a reduction in the number of latently infected cells.

The traditional view of latency is that lytic viral gene expression is shut down and only the latency-associated transcripts (LATs) are produced. The primary LAT is an 8.5 kb transcript that is cleaved to produce the 2.0 and 1.5 kb major LATs, referred to as such based on their abundance, and several microRNAs. LATs may play a role in silencing lytic genes, preventing cell death, or exerting other effects during latent infection.

As mentioned previously, latent virus can reactivate periodically. It is thought that only a small percentage of latently infected neurons will reactivate at any given time. Following reactivation within the ganglion, either virions or viral components such as the nucleocapsid and the glycoproteins travel down the neuroseparately via anterograde axonal flow and are assembled into virus particles prior to virus emergence into the periphery. Within the periphery, reactivated virus can result in either asymptomatic shedding or a clinical recurrent lesion. Although the mechanisms are not fully understood, a number of stimuli are known to induce reactivation including nerve damage, stress, ultraviolet (UV) light, menstruation, and hormonal imbalances. The fate of virus-infected neurons continues to be contentious, with some believing that neurons can survive a lytic HSV-1 infection and others arguing that they cannot.

Pathogenesis

Transmission of HSV requires close, personal contact between the susceptible individual and an individual secreting the virus, enabling the virus to come into contact with either mucosal surfaces or abraded skin. As previously mentioned, HSV-1 is generally the cause of orofacial infections while HSV-2 is usually transmitted via genital contact, and thus causes genital infections, although it should be noted that both viruses are capable of infecting either sites. At each site, the virus replicates in the epithelium and infects the innervating sensory nerve endings. Virus travels along the neuronal axon to the innervating sensory ganglion, the trigeminal ganglion in orofacial infections, and the sacral ganglia in genital infections. Within the sensory ganglion, HSV establishes a lifelong latent infection in which viral gene expression is silent except for transcription of the LAT.

A major factor in the pathogenesis of HSV is the ability of the virus to reactivate from latency. Although we do not yet fully understand the mechanisms through which this occurs, it has been demonstrated that the frequency of reactivation correlates with the severity of the primary infection. When reactivation occurs, progeny virions are produced within the ganglion and travel back down the neuronal axon to be released into the epithelium at or near the site of initial infection. Such reactivation events, which can be either symptomatic or asymptomatic, provide the virus with the opportunity to spread to other susceptible individuals. Interestingly, HSV-1 is more likely to reactivate within a trigeminal ganglion than a sacral ganglion, while the opposite is true for HSV-2. In this way, the tissue tropism of each virus appears to be coupled with a site-specific frequency of reactivation.

Drugs and Vaccines

A number of nucleoside analogues have been used effectively to treat herpes infections, including acyclovir (ACV), famciclovir, and valacyclovir. These drugs exploit the fact that viral enzymes recognize certain molecules that the endogenous cellular enzymes do not, enabling the drugs to target only virus-infected cells. One of the most successful antiviral drugs, ACV, a guanine base attached to an acyclic sugar-like molecule, is used to block HSV lytic replication (Figure 4). ACV is highly specific because it targets two viral enzymes, the virus-encoded thymidine kinase (TK) and the viral DNA polymerase. The viral TK phosphorylates ACV to the monophosphate form whilecellular enzymes phosphorylate it to the di- and triphosphate forms. The HSV DNA polymerase then incorporates the monophosphate form of ACV into the growing DNA chain, but the nascent DNA chain cannot be extended because

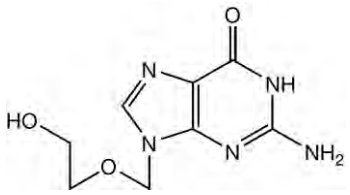

Figure 4 The chemical structure of acyclovir.

ACV lacks a 3′-hydroxyl group. Viral DNA synthesis is thereby inhibited. Minimal toxicity is observed because uninfected host cells lack the two enzymes needed for ACV incorporation into DNA.

ACV effectively blocks productive infection but does not affect latent infection. Resistance to ACV is uncommon except in individuals who are immunocompromised, such as AIDS patients, and those undergoing immunosuppression. In such cases, viral replication occurs at a high level, and mutant viruses that are resistant to ACV can arise.

Drugs such as ACV, when given promptly, have proved effective in reducing the mortality and morbidity associated with HSV encephalitis and ocular and neonatal infections. However, given the emerging link between HSV-2 and the acquisition and progression of HIV, a method of preventing HSV infection is needed. To this end, a number of different vaccine approaches have been investigated, including killed virus vaccines, subunit vaccines, and genetically engineered live virus vaccines. On the whole, killed virus vaccines have proven ineffective at preventing acquisition of HSV, and of two subunit vaccines that have been through human trials, only one showed some efficacy in a subgroup of patients. At this point, the use of replication-defective mutant viruses as vaccines appears to be the most promising approach. A current candidate, *dl*-529, has been rendered replication-defective through deletions in both U_L5 and U_L29 and has been shown to induce both high titers of neutralizing antibodies and strong cell-mediated responses.

Varicella-zoster Virus

Disease

VZV is the causative agent of varicella zoster or 'chickenpox', a common childhood disease with the highest prevalence occurring in the 4–10 years age group, and herpes zoster or shingles, a disease most often seen in older individuals. The virus is highly communicable, thought to enter susceptible individuals via the respiratory tract. From here, it spreads to the lymphoid system before producing the characteristic vesicular rash in the skin 10–21 days later. A fever and general feeling of malaise accompany the appearance of the rash. Most individuals become infected with VZV as children. However, approximately 10% of young adults remain susceptible. It should be noted that the incidence of varicella in the United States, particularly in children aged 1–4 years, has declined by approximately 85% since the introduction of a vaccine into the United States pediatric immunization schedule in 1995.

Following a primary infection, VZV establishes a latent infection in the sensory ganglia, with 10–20% of people experiencing a recurrent infection after several decades. Recurrent VZV infection manifests as a vesicular rash in the dermatome innervated by the latently infected ganglion and is referred to as herpes zoster or 'shingles'. It first presents with a prodrome, a painful burning sensation throughout the soon-to-be-affected dermatome. Several days later, the characteristic vesicular rash appears in the skin, which is generally accompanied by flu-like symptoms and can last up to 7–10 days. It is common for the burning, piercing pain to continue after the resolution of lesions in the skin, and in cases where pain persists for longer than 30 days after such resolution the patient is deemed to have post-herpetic neuralgia (PHN). PHN is generally self-limiting and most patients are pain-free by 6 months post-zoster, although pain can be significant until resolution.

The fact that herpes zoster is more common among the elderly and immunocompromised individuals supports the idea that the immune response plays an important role in determining whether VZV is able to reactivate from latency or not and whether symptomatic disease ensues. For those aged 20–50 years, the incidence of herpes zoster is around 0.25%, this rises to 0.8% in people aged over 60 and can reach as high as 50% in people who reach 85 years of age. Individuals with compromised immune systems are also at an increased risk of herpes zoster. People with HIV, leukemia, Hodgkin's and non-Hodgkin's lymphoma, and those who have undergone either bone marrow or renal transplants are at risk. It should also be noted that in immunocompromised individuals, the cutaneous rash is usually more extensive and widespread viremia is also common.

Virus and Biology

VZV shares the common herpesvirus virion structure of a core, containing a single copy of the 125 kbp linear, double-stranded DNA genome, surrounded by a nucleocapsid, a proteinaceous tegument layer, and an outer envelope. It also shares the ability to establish lifelong latent infections in the sensory ganglia with the other human alphaherpesviruses HSV-1 and HSV-2. However, unlike the HSVs that are able to replicate in cells from a wide range of hosts, VZV has a very narrow host range, restricted to selected cell types of

human and simian origin. Another difference between VZV and HSV relates to the spread of the virus through the host. HSV is generally confined to the epithelium infected during lytic infection and the neuronal cells within the sensory ganglia, where latency is established. VZV on the contrary has the ability to disseminate widely through the bloodstream of the infected host, infecting skin, mucous membranes, and visceral and nervous system tissues.

Replication

The infectious cycle of VZV is similar to that seen with HSV-1 and HSV-2. The virus uses surface glycoproteins, definitely gB and possibly gC, to attach to cellular surface glycosaminoglycans, such as heparan sulfate. Several other viral glycoproteins, gH, gE, and gI are also involved in the attachment and penetration process, following which the viral nucleocapsid, along with several tegument proteins, is transported to the nuclear surface and the viral genome is inserted into the nucleus. It is thought that, like VP16 during HSV infection, the VZV tegument proteins may be involved in initiating transcription of viral genes. The transcription of viral genes occurs in a sequential pattern with IE, E, and L genes, designated as such by the timing of their transcription. Each class of genes is transcribed in the nucleus, the mRNAs are then transported into the cytoplasm where they are translated, and the resulting proteins are then transported back into the nucleus. The IE genes are involved in the regulation of transcription of IE, E, and L genes and each VZV IE protein has homology to one of the HSV IE proteins, although the exact roles played by these proteins are not always homologous. The E proteins are involved in viral DNA replication, and the L proteins are generally structural, used to produce the capsids for progeny virions. The newly formed capsids travel out of the nucleus, becoming enveloped in the process, and are then transported to the cytoplasmic membrane where they are released from the infected cell. In cell culture systems, the entire process can occur in as little time as 8–16 h.

Pathogenesis

VZV is transmitted via inhalation of infectious respiratory secretions or skin-to-skin contact with infectious vesicular fluid and manifests as a vesicular rash throughout the skin 10–21 days later. Due to a lack of animal models for VZV, the pathogenesis of this virus was originally modeled on that of mousepox. Based on this model, it is thought that the inhalation of the virus enables infection of regional lymph nodes, resulting in a primary viremia that enables the virus to disseminate throughout the body, spreading to reticuloendothelial organs such as the liver. Within these organs, it is hypothesized that a phase of viral amplification occurs followed by a second round of viremia during which the virus is transported to the skin.

During primary infection, VZV is able to establish latency within sensory ganglia; VZV DNA is widely detected in the trigeminal ganglia and in many dorsal root ganglia of infected individuals. It is widely accepted that neuronal cells are the primary reservoir of latent virus, infected either hematogenously or by virus transported along neuronal axons from the skin via anterograde axonal transport. VZV latency differs from the situation during HSV infection in two main ways; first, unlike HSV infection, a number of VZV genes may be transcribed and translated while the virus is in a latent state, and second, VZV latency lacks the frequent episodes of asymptomatic reactivation seen with HSV infection. This later observation is thought to be due to the ability of the host immune response to quickly control reactivation events and maintain the virus in a latent state.

Many aspects of the host immune response come into play when dealing with a VZV infection. The innate response in epidermal cells plays an important role in slowing the spread of virus through the skin. NK cells and interferon (IFN-γ) also play a role in the innate defense against VZV infection, helping to contain the virus prior to the development of the adaptive response. In terms of adaptive immunity, VZV induces both cell-mediated and humoral immunity, with T cells thought to be particularly important in clearing infectious virus and helping to maintain VZV in a latent state.

Drugs and Vaccines

As with HSV, nucleoside analogues such as ACV and related drugs have been useful in treating VZV infections. In cases of varicella, oral ACV can be given to healthy individuals to reduce the severity of symptoms, while intravenous ACV is given to immunocompromised individuals to reduce the risk of disseminated infections. In cases of herpes zoster, ACV can be given to immunocompetent individuals to shorten the period of lesion outbreaks, the healing period, and the severity of acute neuropathic pain. Similarly for immunocompetent individuals, intravenous ACV can be used to shorten the period of disease and prevent disseminated infection.

VZV is the first HHV for which there is a licensed vaccine, a live attenuated virus vaccine derived from a clinical viral isolate known as the Oka strain. The Oka strain was attenuated by passage into guinea pig fibroblasts, producing a virus with reduced efficiency for replication in human skin. In clinical trials, a single dose of the vaccine was sufficient to induce seroconversion rates of 90% or greater in children 12 years and under and provided complete protection from disease in approximately 85% of exposures. The vaccine induces strong T cell responses with a single dose and also achieves high antibody titers when a second dose is administered and is now recommended for routine vaccination of infants and susceptible older children and adults in the United States. A recent study has also demonstrated that vaccination of healthy adults aged 60 years and older can significantly reduce the frequency and morbidity of herpes zoster, suggesting that a vaccination regimen in this population may also be useful.

The *Gammaherpesvirinae* Subfamily

Gammaherpesviruses are classified as such based on their ability to replicate in epithelial cells, establish latency in lymphocytes, and their oncogenic effects. Within the gammaherpesvirus subfamily, there are two genera: the *Lymphocryptovirus* (LCV) genus, which includes the human pathogen EBV, and the *Rhadinovirus* (RDV) genus, which includes KSHV. It is thought that the viruses within the LCV genus likely evolved from those in the RDV genus.

Epstein–Barr Virus

Disease

EBV, also known as HHV-4, is a widespread human pathogen with 90% of adults testing seropositive. In developing countries, most children are infected within the first 3 years of life, while in developed countries around 50% of individuals remain seronegative through childhood. It is estimated that 25% of people who then acquire EBV during adolescence or young adulthood will present with acute disease called infectious mononucleosis (IM), although it should be noted that childhood cases of IM are common in Asian populations and are possibly underdiagnosed in other parts of the world. Patients with IM can present with symptoms ranging from a mild and transient fever to a period of malaise and pharyngitis lasting several weeks. This period of EBV disease is closely linked to the emergence of the cytotoxic T cell response to infection, and it is widely accepted that IM is mostly an immunopathologic disease, with the proinflammatory cytokines secreted by active T cells thought to be responsible for many of the IM symptoms. In a small subset of patients, IM is poorly controlled and can lead to more severe, possibly fatal, outcomes. Males with X-linked lymphoproliferative (XLP) syndrome are highly sensitive to EBV infection. In these patients, primary EBV infection results in severe IM-like symptoms and can rapidly result in mortality, thought to be due to an uncontrolled T cell response to infection. Virus-associated hemophagocytic syndrome (VAHS) and chronic active EBV infection (CAEBV) are two other serious outcomes of EBV primary infection. Both involve EBV infection of T cells, resulting in virus-driven proliferation of these cells (much like the proliferation of B cells during a classical primary infection). The infected T cells release huge amounts of proinflammatory cytokines, which results in hemophagocytosis, where macrophages begin phagocytosing red blood cells, platelets, leukocytes, and other cells. The risk of mortality with either syndrome is high.

Following primary infection, EBV establishes a latent infection in B cells, which is generally maintained as such for the life of the host with no clinical manifestations. However, this is not always the case, and EBV is associated with a number of different human malignancies. In immunocompetent individuals, usually following several decades of EBV latency, EBV is associated with the development of certain types of Hodgkin's lymphoma, several B-lymphoproliferative lesions, T cell and NK cell nasal lymphomas, and gastric and nasopharyngeal carcinomas. In immunocompromised individuals, the virus is capable of rapid tumor development, with some cases of tumorigenesis evident within months of EBV infection. In transplant settings, a link has been demonstrated between EBV and posttransplant lymphoproliferative disease (PTLD), with EBV association as high as 100% in early onset cases and 80% with late onset cases. AIDS patients show a heightened risk of B cell lymphoma with approximately 50% linked to EBV, and most settings involving immunosuppression have demonstrated a link between EBV and smooth muscle cell tumors. EBV is also associated with endemic Burkitt's lymphoma (BL), the most common childhood cancer in equatorial Africa which is geographically linked to areas of holoendemic malarial infection, although the mechanisms through which EBV contributes to BL are not fully understood.

Virus and Biology

EBV shares the common herpesvirus virion structure and has a 184 kbp genome. There are two strains of the virus, types 1 and 2 or types A and B, which circulate in most populations. Individuals can be infected with both types and this is a common occurrence in immunocompromised individuals. Type 1 is generally more prevalent in developed countries while type 2 is dominant in equatorial Africa and New Guinea. The main differences between the two strains are seen in the nuclear protein genes that encode EBV-coded nuclear antigen (EBNA)-LP, EBNA-2, EBNA-3A, EBNA-3B, and EBNA-3C.

Replication

EBV is known to infect both B cells and epithelial cells during primary infection, with infection of epithelial cells thought to result in a lytic infection and infection of B cells thought to generally result in a latent infection. In B cells, the virus binds to CD21 and MHC class II on the surface of target cells using glycoproteins in the virus envelope. A cell surface receptor for epithelial cell infection has yet to be found, and it is hypothesized that virus may be transferred to epithelial cells directly from lytically infected B cells. The entry of virus into each cell type differs in that virus enters B cells via the endocytic pathway while virus entering epithelial cells does so at the cell surface, in both cases viral glycoproteins are involved in facilitating fusion and entry of the virus into the target cell. Following entry of the virus into the target cell, the nucleocapsid is transported to the nucleus into which the viral genome is inserted.

If a lytic infection ensues, as is thought to occur in epithelial cells *in vivo*, the sequential expression of IE, E, and L genes, common to herpesviruses, is seen. The IE proteins are primarily involved in activation of E gene expression, the E proteins are primarily involved in viral DNA replication, while the L proteins are involved in the production of progeny virions. At this time, it is thought that newly formed nucleocapsids initially acquire an envelope at the inner nuclear membrane, are de-enveloped as they are released into the cytoplasm, and then reacquire an envelope as they bud through the plasma membrane.

It is widely accepted that initial infection of B cells results in a latent or persistent infection. In fact, *in vitro* studies demonstrated that infection of B cells with EBV resulted in a latent infection that was capable of causing perpetual B cell proliferation, helping to confirm the oncogenic properties of EBV. During latency, the viral genome is maintained in the nucleus of the infected cell in an episomal form with variable levels of gene expression possible. There are four different forms of EBV latency: latency 0, I, II, and III. These stages are classified as such based on the level of expression of the EBNAs and latent membrane proteins (LMPs), with latency 0 having no viral antigens expressed and latency III having all latency proteins expressed.

These different forms of latency are used by the virus to ensure the maintenance of the viral genome within progeny B cells, and as such the virus uses cellular differentiation controls to determine which level of latency is required at any given time. For example, during a primary infection, EBV is present in infected B cells in latency III form, and the expression of viral genes at this stage is used to drive the proliferation and differentiation of these cells into a latently infected memory pool. Once the memory pool is established, and in order to prevent further detection by virus-specific cytotoxic T cells, EBV switches off all gene expression, thus entering latency 0. At times when memory B cells are induced to divide by homeostatic signals, the virus reactivates to latency I to ensure the viral genome is not lost during such cell division. In this way, the virus is able to establish a balance between avoiding immune detection and maintaining the viral genome. At times of cell proliferation, the viral EBNA-1 protein tethers the viral chromosome to the cellular chromosome to ensure maintenance of the viral genome within daughter cells.

Pathogenesis

EBV infection is transmitted via the oral route and is generally asymptomatic. As such, knowledge of the primary infection has come from the study of IM. These individuals shed virus in saliva and throat washings. However, the source of this virus remains contested. It is generally assumed that because B cell-deficient individuals show no sign of EBV infection in the throat, initial infection of a naïve host is B cell-dependent. However, it is becoming more widely accepted that epithelial cells may also be sites of viral replication, with studies suggesting that virus bound to the surface of a B cell is highly efficient at infecting epithelial cells. Interestingly, recent evidence further suggests that virus released from B cells is defective for B cell infectivity but shows enhanced infection of epithelial cells, while virus released from epithelial cells has the opposite phenotype.

EBV establishes a latent infection in B cells at the site of primary infection, the tonsillar tissue. Studies suggest that latently infected B cells express a memory phenotype, and it has been proposed that infection of naïve B cells with EBV mimics the process of B cell differentiation, resulting in activation and proliferation of the infected cell population, and thus the production of an expanded pool of latently infected memory cells. It is generally accepted that the cell-mediated immune response brings the proliferating B cells under control. The memory pool of latently infected B cells, which circulates through the body, is able to disperse the latently infected cells throughout the lymphoid system. Individuals latently infected with EBV will have peripheral blood B cells that harbor virus and, following clearance of the primary infection, these individuals will continue to shed low levels of infectious virus via the oral cavity. This virus comes from the latent B cell reservoir. It is thought that memory B cells containing latent virus may undergo reactivation when they receive an activation signal, and that such cells, which localize near mucosal surfaces, would be capable of transmitting lytic virus to epithelial cells where viral replication and subsequent shedding can occur.

In a healthy individual with an intact immune system, EBV persists in this form for the life of the host with no clinical manifestations. The latent pool is constantly maintained by the virus moving forward and backward through the various forms of latency as required, and infectious virus is sporadically shed from the oral cavity with the potential of infecting other susceptible hosts. However, when immune suppression occurs, either through disease or drug intervention, this balance is destroyed and the individual is put at risk of EBV-associated disease.

Drugs and Vaccines

To date, attempts to develop preventative vaccines against EBV have been largely unsuccessful. The finding that the major viral envelope protein gp350 was the dominant target of the neutralizing antibody response led to attempts to develop a gp350 subunit vaccine. However, some evidence suggests that neutralizing antibodies alone are not sufficient to protect against EBV, with clinical trials showing that the gp350 subunit vaccine failed to prevent primary infection, although it was able to reduce the incidence of IM symptoms. It is now widely believed that an integrative approach is required, where a vaccine to prime the antibody response (such as the gp350 subunit vaccine) would be given in combination with a vaccine aimed at priming the $CD8^+$ T cell response. However, it should be noted that even this integrative approach would probably be most successful at limiting rather than preventing infection.

In parallel with efforts to design a preventative vaccine, efforts are also being aimed at developing immunotherapeutics for EBV-associated malignancies. Current strategies being developed include adoptive transfer of activated T cells specific for viral antigens expressed on EBV-associated tumors and the development of vaccines that can boost the host T cell response to these same antigens. Both strategies are aimed at increasing T cell recognition and subsequent destruction of EBV-associated tumors.

Kaposi's Sarcoma-Associated Herpesvirus

Disease

KSHV, also known as HHV-8, is the most recently identified HHV. The virus was originally identified because of its association with Kaposi's sarcoma (KS), an endothelial neoplasm. It was later recognized as a member of the LCV genus within the gammaherpesvirus subfamily of herpesviruses.

Infection rates with KSHV in the United States and in Europe are relatively low with approximately 3% of the population infected. In Africa, a different picture emerges, with KSHV reaching endemic rates of infection of between 40% and 60%. The various strains of KSHV can be divided into four major groups or clades: A, B, C, and D, with each virus within a given clade sharing a single common ancestor. A and C tend to cluster together and are more prevalent in Europe and in the United States, B is the most commonly isolated in infected individuals in sub-Saharan Africa, and D is the dominant clade seen in South Asia and Australia. The pattern of distribution and the evolutionary relationship between viruses in the different clades suggest that KSHV entered the human population at about the time when modern man emerged in Africa, with the different clades being produced as different groups moved out of Africa to Europe and Asia, respectively. The fact that the distribution of these clades seems to have been maintained over millions of years also suggests that, particularly in areas of high seroprevalence, transmission of KSHV is primarily familial, moving vertically from parent to child and horizontally between different members in a family unit possibly via salivary exchange. It should be noted that in areas of low seroprevalence, such as Europe and the United States, the pattern of infection appears to follow that of a sexually transmitted disease, and virus has been successfully isolated from both saliva and genital secretions.

The most common malignancy associated with KSHV is KS. KS is a complex, angioproliferative, and inflammatory lesion. It was historically a disease of elderly Mediterranean men until it emerged as the most common neoplasm seen as a complication of HIV/AIDS. In both settings, the disease is a slow progressing malignancy, although it can result in death in AIDS patients if organ involvement is present. Unlike a classical tumor, KS lesions contain many different cell types with the driving cell being a KSHV-infected spindle cell (an elongated endothelial cell). The spindle cells produce proinflammatory and angiogenic products and may actually require factors released from proinflammatory cells for survival and growth. It is possible for KS lesions to be locally or systemically invasive, requiring chemotherapy or radiotherapy.

While strong evidence suggests that KSHV is necessary for KS development, it is certainly not sufficient. Within the general population, only 1 in 10 000 infected individuals will develop KS annually. Therefore, it is assumed that there are other cofactors involved in the development of KS. In AIDS-related KS, the assumption is that HIV infection is the cofactor. It has been proposed that an HIV protein may act as a growth factor for KSHV or that the immunodeficiency seen during HIV infection may enable KSHV to disseminate more widely through the host, increasing the chances of endothelial cell infection. The cofactor in non-AIDS-related KS remains unknown.

Along with KS, KSHV has been implicated in two B cell diseases, primary effusion lymphoma (PEL) and Castleman's disease. PEL is a rare disease seen in end-stage AIDS patients and is characterized by proliferation of B cells primarily in body cavities such as the pleura, pericardium, and peritoneum. Unlike KS, PEL is a classical malignancy with every cell in the tumor harboring KSHV DNA. Castleman's disease is a rare, lymphoproliferative lesion that is seen in both HIV-positive and HIV-negative individuals. In HIV-negative individuals, Castleman's disease generally presents as a benign tumor localized to a single lymph node. This form of Castleman's disease does not involve KSHV and is usually treated by excision of the involved tissue. Multicentric Castleman's disease is a more aggressive, systemic illness characterized by sustained fever, sweats, and weight loss. This form of Castleman's disease is seen with increased frequency in patients with AIDS and, in this setting, is almost always linked to KSHV infection.

Virus and Biology

KSHV shares the standard herpesvirus virion structure described above. The virion contains a double-stranded linear DNA genome of between 165 and 170 kbp in length that contains four blocks of highly conserved genes, many of which encode replication proteins common to alpha- and betaherpesviruses. The genome also encodes several small noncoding miRNAs, the function of which remains unknown, several of which are expressed during latency and one of which is expressed during lytic infection.

Replication

The replication cycle of KSHV follows a pattern similar to that seen with the other herpesviruses and thus will not be discussed in detail here. Virus attachment and entry are facilitated by several viral glycoproteins, following which the viral genome and several tegument proteins are delivered into the nucleus of the infected cell. If, at this point, the virus enters the lytic cycle, the sequential expression of over 90 viral genes is initiated. These genes are divided into IE, delayed early (DE), and L genes. The mechanism of KSHV egress has yet to be fully elucidated.

Despite the obvious ability of the virus to induce a lytic infection, as demonstrated by the intermittent shedding of virus from infected individuals, studies in cell culture systems suggest that the default pathway in KSHV infection is latency. From *in vitro* work, it appears that only a small number of cells (1–3%) will enter the true lytic pathway and that this will subside following several days of infection. In the majority of cells, a defective version of lytic infection arises, which is quickly terminated and latency established.

In these cells, several lytic cycle genes are expressed during the first 12 h of infection. However, the expression of these genes ceases by 24 h postinfection and the genes are not expressed in their correct sequential order, thus providing evidence of the defective nature of this 'lytic' infection. Latency is then quickly established in these cells. The role that the faulty lytic infection plays in the overall virus infection remains unknown, although it has been proposed that the transient expression of some of the viral immune evasion mediators may be beneficial during the early stages of infection.

Once latency is established, the viral genome is replicated as an episome. The viral LANA protein tethers the viral episome to the cellular chromosome so that the viral genome is distributed to progeny cells during cell division. At this time, only a few of the 90-plus viral genes are expressed. However, the exact roles that these proteins play during the latent infection still require much investigation. Work to date would suggest that they may be involved in maintenance of the viral genome in dividing cells, prevention of apoptosis and, surprisingly, upregulation of proinflammatory responses. The switch from the latent to the lytic phase of infection is thought to be facilitated by the so-called lytic switch protein, known as RTA. This protein is a viral transcriptional activator that is capable of inducing lytic gene expression on its own, but becomes even more efficient when bound to one of several different HCFs. In experimental systems, deletion of RTA prevents both spontaneous and chemically triggered induction of the lytic cycle.

Pathogenesis

Primary infection of a susceptible host is followed quickly by the establishment of latency, primarily in B cells. In most individuals, the latent infection is asymptomatic and is accompanied by intermittent, clinically silent viral reactivation that enables shedding of virus in the saliva. Given the apparent absence of an extended primary lytic infection, asymptomatic reactivation during latency would appear to play a major role in virus transmission.

The exact role of KSHV in the pathogenesis of KS is currently unknown. It appears that both latent and lytic stages of infection are important, with KSHV latency much less potent than EBV latency at inducing cell immortalization. Most KS spindle cells are latently infected but a small percentage demonstrates lytic infection. It would appear that this low level of lytic replication is important in the development of KS, possibly enabling the reinfection of spindle cells that have lost the KSHV genome, providing a reservoir of newly infected cells to replace cells within the tumor mass that have died, or even providing some of the inflammatory and angiogenic signals that play a role in KS pathogenesis.

Drugs and Vaccines

Ganciclovir may have a role in preventing KSHV spread and KS development but does not affect an established tumor. Because in most individuals, the immune response is adequate to control the virus and prevent virus-associated disease, it is common with immunocompromised patients to treat the underlying cause of immunosuppression or to attempt to treat the malignancy itself as well as the viral infection.

The *Betaherpesvirinae* Subfamily

The betaherpesviruses are characterized by a restricted host range, a long productive cycle, and the ability to establish latent infections in secretory glands, lymphoreticular cells, and kidneys. The betaherpesviruses have the highest level of evolutionary and genetic diversity of the three herpesvirus subfamilies, which can make the use of animal models to study human pathogens within this subfamily difficult. There are four genera within the betaherpesvirus subfamily: the cytomegaloviruses, the muromegaloviruses, the roseoloviruses, and the probosciviruses (which has only a single member).

Human Cytomegalovirus

Disease

HCMV is a ubiquitous human pathogen, infecting those in developing countries in their youth and those in developed countries across a slightly wider timeframe. In most individuals, HCMV causes an asymptomatic infection, with disease generally only seen in those unable to mount a cellular response to infection, such as neonates or individuals with some form of immunosuppression. As is characteristic of herpesviruses, HCMV establishes a latent infection within the host, although interestingly this is also accompanied by what can be called a chronic infection, with infected individuals shedding virus sporadically from their bodily fluids for life.

HCMV can be classified as an opportunistic pathogen, only causing disease in situations where the immune response is severely compromised (such as in AIDS) or absent (such as congenital infection). In most healthy individuals, infection with HCMV is clinically silent, although it should be noted that in a small number of cases a short bout of fever and malaise can occur, similar to the mononucleosis caused by EBV.

Congenital Infection

Transmission of HCMV from mother to fetus or newborn is a very common occurrence and can occur via three routes: transplacental, intrapartum, and via human milk. HCMV is the only herpesvirus known to exhibit natural transplacental transmission, and it is this congenital route of transmission that causes serious morbidity. That said, intrapartum and transmission via breast milk, while not associated with the morbidity of congenital infections, both play an important role in viral epidemiology. These newborn children infected with HCMV will continue to shed virus capable of infecting susceptible hosts for many years after the primary infection.

Congenital infection can occur when the mother has either a primary or reactivated infection during pregnancy, with evidence from those undergoing a primary infection suggesting that transmission to the fetus can occur in 20–40% of cases. Although less than 1% of live births involve a child with congenital HCMV, the long-term sequelae for these children make it a serious disease, with HCMV estimated to be the leading cause of infectious brain damage in the United States. Approximately 5–10% of those born with a congenital HCMV infection will be symptomatic, showing clinical manifestations such as hearing loss, seizures, jaundice and brain abnormalities, with long-term sequelae such as mental retardation, cerebral palsy, and impaired vision. In 10% of cases, symptomatic congenital HCMV infection will be fatal. Even the 90% of congenitally infected children born without symptoms remain at risk of long-term CNS sequelae such as hearing loss.

Infection in an Immunocompromised Host

Patients with compromised or suppressed immune systems are at greater risk of CMV-associated disease than healthy individuals, with the severity of disease often matching the level of immunosuppression. In patients with HIV or those undergoing solid organ or hematopoietic stem cell transplants, HCMV can disseminate into a number of different organs, causing clinical manifestations such as pneumonitis, retinitis, and hepatitis. In organ transplant patients, HCMV has also been shown to cause dysfunction of the transplanted organ and put the patient at greater risk of fungal and bacterial infections. The thorough screening of transplant patients and the use of antivirals in this setting and HAART in the HIV setting has reduced the morbidity and mortality associated with HCMV infection in immunocompromised individuals.

Virus and Biology

Compared to the other HHVs, HCMV is a very large virus. With a genome between 196 and 241 kbp, the virus encodes in excess of 166 gene products (less than half of which are conserved in all betaherpesviruses), and while HCMV shares the common herpesvirus virion structure, the actual size of the virions is larger than that of the other HHVs.

Replication

The replication cycle of HCMV follows a similar pattern to that described for the other herpesviruses. The virus uses heparan sulfate as an initial binding receptor on target cells and then enters the cell via either fusion of the viral envelope with the cellular membrane or the endocytic pathway. The viral genome is released into the nucleus and the lytic viral genes are expressed in a sequential manner: IE, DE, and L. Strong evidence argues that HCMV undergoes a two-stage envelopment/de-envelopment/re-envelopment process in order to exit an infected cell. Newly synthesized nucleocapsids are enveloped as they pass through the inner nuclear membrane and then deenveloped as they pass through the outer nuclear membrane. The envelope-free nucleocapsid is thus released into the cytoplasm where it reacquires an envelope at the ERGIC membranes before being transported out of the infected cell via the cellular exocytic pathway.

In terms of replication, the main difference between HCMV and other HHVs is the length of the replication cycle. DE gene expression begins at 6 h postinfection and continues through 18–24 h when viral DNA synthesis is initiated. From initial attachment to the initiation of progeny virion release, the complete infectious cycle takes between 42 and 78 h. During this time, the virus has a profound effect on the infected cell, blocking IRF-3 activation, IFN signaling, and apoptosis responses and interrupting the cell cycle in such a way that infected cells are able to survive for several days of productive infection.

Pathogenesis

In an immunocompetent host, HCMV infection is generally asymptomatic. Primary infection is usually initiated in the mucosal epithelium following direct contact with infectious secretions from another individual, aerosol transmission does not occur. A systemic phase of infection then follows with a leukocyte-associated viremia. Animal models with murine CMV (MCMV) suggest that the virus uses immature leukocytes from the bone marrow to facilitate dissemination to the salivary glands, kidneys, and other tissues. This systemic phase of infection is associated with high levels of persistent viral shedding in the saliva, urine, breast milk, and genital secretions and continues for a long time after the onset of the adaptive immune response. It can last for months in adults

and for years in young children, supposedly due to a less effective cellular immune response in younger patients. The ability of the virus to persist in the face of the cellular immune response is thought to be due, in part, to the fact that more than 25 viral genes have been found to play a role in modulating the host response to infection.

When virus is cleared following primary infection, HCMV is maintained in a latent state in hematopoietic progenitor cells (HPC). However, unlike the human alphaherpesviruses, this latent infection canbe accompanied by what can be called a chronic infection in epithelial cells of the salivary glands and kidneys, which results in sporadic shedding of virus in the bodily fluids for the life of the host. Reactivation of virus from latency, as opposed to the sporadic viral shedding achieved by the persistent infection in the salivary glands and kidneys, seems to be an issue only in situations of immunosuppression.

Immune responses to HCMV are well maintained for years beyond the primary infection, at levels not seen with other herpesviruses or persistent infections. Innate immune responses such as IFN and NK cells are important during the early stages on infection and may play a role in containing the infection until the adaptive immune response develops. T cell responses appear to be of greater importance than antibody responses, although in certain settings antibodies play a crucial protective role. Despite the persistence of the primary viremia in the face of an active cellular immune response, the fact that viral reactivation is only seen in cases of immunosuppression suggests that the immune response plays an important role in helping to maintain the virus in a latent state and preventing CMV-associated disease.

Drugs and Vaccines

There are currently four drugs approved for the treatment of HCMV infection in immunocompromised individuals: Ganciclovir, Valganciclovir, Foscarnet, and Cidofovir. All four have been shown to reduce or eliminate viremia, reduce viral shedding, and prevent or control CMV disease. However, due to risks of severe toxicity, the drugs are only used when a patient is at risk of serious disease. At this time, no drugs are approved for the treatment of congenital CMV, although a small Ganciclovir trial did produce some positive results.

Given the seriousness of congenital CMV infection and the difficulty in preventing maternal infections, a preventative vaccine would have a large public health benefit. Several different vaccine approaches have been tested to date. However, a CMV vaccine is yet to reach the market. Strategies that have reached clinical trials have included a live attenuated vaccine, a gB subunit vaccine, a canary pox vector expressing CMV gB and pp65, and a DNA vaccine with antibody and CTL epitopes. Several of these vaccines have shown promising results in inducing strong immune responses.

HHV-6 and HHV-7

Disease

HHV-6 was first isolated in 1986 and is classified into two variants, A and B. HHV-6B is the major causative agent of exanthem subitum (ES), while HHV-6A has not been clearly linked with any disease. HHV-7 was first isolated in 1990 and is also a causative agent of ES as well as being associated with febrile convulsions in young children. Both HHV-6 and HHV-7 are ubiquitous pathogens, with greater than 90% of adults seropositive for both.

ES or roseola is a classical childhood disease (sixth disease). It initially presents as a fever lasting for 3–4 days. As the fever clears, a rash appears, first on the trunk and hands and then on the lower limbs, lasting several days. HHV-6B is the major cause of ES, with the magnitude of viral replication correlating with the severity of disease. HHV-7, while also a cause of ES, has a lower frequency of disease as compared to HHV-6B. It is possible for children to have successive bouts of ES caused by one virus and then the other. Most cases of ES are benign and are associated with other symptoms such as diarrhea, cough, and febrile convulsions. However, it is possible for HHV-6 infection to result in encephalitis, meningitis, and hepatitis, which can be fatal.

Following primary infection, both HHV-6 and HHV-7 persist in a latent state. As with other herpesviruses, reactivation is generally only a problem in situations where the immune system is compromised. In bone marrow transplant recipients, asymptomatic HHV-6 reactivation is common. However, reactivation has also been linked to bone marrow suppression, encephalitis, colitis, pneumonitis, and graft-versus-host disease. In solid organ transplant recipients, HHV-6 reactivation has been associated with kidney rejection.

Virus and Biology

HHV-6A, HHV-6B, and HHV-7 are members of the roseolovirus genus of the betaherpesvirus family, sharing the common characteristics of growth in T cells, high prevalence, and association with febrile rash illness. HHV-6A and HHV-6B are closely related but actually meet the requirements for recognition as two separate viruses – they differ in cell tropisms, interactions with cells and the immune system, DNA sequences, and epidemiology. As with the other human betaherpesvirus, HCMV, both HHV-6 and HHV-7 have protracted replication cycles and share some beta-subfamily-specific genes.

The virion structure of HHV-6 and HHV-7 follows the common herpesvirus structure of a dsDNA genome within an icosahedral capsid that is surrounded by a tegument layer and finally a lipid bilayer envelope. HHV-6 has a genome of up to 170 kbp, while the HHV-7 genome is 145 kbp in length.

Replication

HHV-6 and HHV-7 have a similar replication cycle to those of the other HHVs. Following virus attachment and entry, the viral genome is delivered to the nucleus where viral gene transcription is initiated in a sequential manner: IE, E, and L. Egress of newly formed virions from the infected cell follows the same path as that used by HCMV: envelopment/de-envelopment/re-envelopment.

Pathogenesis

Transmission of HHV-6 and HHV-7 is not fully understood. It is thought that transmission during infancy occurs horizontally, possibly via saliva during close personal contact. However, it is also thought that the viruses can be transmitted across the placenta, during delivery, or even intrauterine. The exact site of primary infection is also yet to be determined. It is currently thought that infection is initiated through respiratory pathways, however, the exact cells that are infected are not known.

Both HHV-6 and HHV-7 establish latent infections within their hosts. It is thought that HHV-6 establishes latency in monocyte or macrophage cells and certain stem cells. HHV-6 DNA and antigens can also be detected in a range of other sites including the saliva, brain, and lung, suggesting a concurrent persistent infection. HHV-7 establishes a latent infection in $CD4^+$ T cells while maintaining a persistent infection in the salivary glands and a variety of other tissues.

Drugs and Vaccines

Several drugs approved for use against CMV have been shown to be effective against HHV-6 and HHV-7 *in vitro*, these include Ganciclovir, Foscarnet, and Cidofovir. IFN-α and IFN-β have also been shown to inhibit HHV-6 replication *in vitro*. However, no drugs are currently approved for use in the treatment of HHV-6 or HHV-7 infection.

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Historical Plague ☆

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Overview

The history of bubonic plague generates considerable popular and scientific interest, but it is more difficult to assess how plagues independently affected the course of human events. Within the enormous body of work devoted to this broad topic, we can identify patterns of scientific concern with past plagues, often occurring when newly available scientific technologies or theoretical perspectives invite reconsideration of long-unsolved questions. Plague scientists themselves made bold historical claims during the opening years of 20th century, after a new pestilence appeared in East Asian port cities in the mid-1890s. The disease was highly lethal and its victims had classic inguinal and axillary lymph node swellings, which bore strong similarities to historically famous epidemics in Europe centuries before. At the same time historical accounts did not readily explain why new outbreaks of a nearly-banished disease now suddenly appeared. Like the earlier, intermittent outbreaks of cholera in the 19th century, and the 'Russian influenza' of 1889–91, this plague became a pandemic, and it quickly spread to every continent except Antarctica.

An impressive array of early investigators from many different nations unraveled the basic biological and ecological parameters of the disease before 1900. While the clinical description of human plague infection seemed a secure point of contact between past and present, the biology of the pathogen did not reflect plague's reputation as a human contagion. Instead plague principally involved rodent populations and was spread by means of their fleas, evidence that formed a central paradox within plague history for the next century. Classically educated European scientists were especially anxious to link historical narratives of catastrophic past mortality events to the evidence of plague in modern times. Most also wrote histories that celebrated European scientific or cultural triumphs, explaining the defeat of a fierce ancient foe rather than confront the unpredictable reappearances of great pandemic mortality. Drawing upon public health tradition, plague histories touted generally helpful contagion-based epidemic containment strategies as responsible for plague's successful control in centuries past. To explain why earlier plagues were so deadly, the scientists' histories credited the large-scale urban environmental sanitation achievements that western industrial nations initiated during the 19th century. Non-western cultures and peoples were seen as plague-prone because these peoples did not embrace similarly high standards of public and private cleanliness.

The next generation of plague scientists also produced influential, comprehensive summaries of plague history. These works offered a parallel summary of plague biology and advanced an ever-more detailed historical framework that singled out two massive historical plague epidemics in the past, events caused by trade with regions outside Europe. The earliest recorded pandemic coincided with the reign of Byzantine Emperor Justinian in late antiquity; the other began with the 'Black Death' of 1347–53, understood as the first in a series of recurrent plagues in Europe. All of these works claimed that plague subsequently retreated to the margins of the Western world, from whence it came, lingering longer in medieval Europe than in the pandemic of late antiquity. These authors well understood that plague ordinarily appeared in remote rural foci, and so these histories acknowledged that the disease displayed different epidemiological characteristics in previous pandemics.

Historians in the post-war era relished the wide-ranging challenges that scientific histories of plague presented. Many turned to the retrieval and study of non-narrative historical sources; others provided more detailed background analysis of plague literature. The shortcomings of older science-driven plague history came to the foreground from the 1960s to 90s, as historians examined a wider range and depth of evidence about individual plague outbreaks, or focused on patterns of plague experience in particular locations or time periods. By the end of the century prominent historians of medicine argued that it was impossible to make any modern diagnosis from the observations of persons writing before the germ theory era. Early in the current century, several influential historians of the Black-Death epidemic and its recurrences even denied that *Yersinia pestis* could ever have existed in Europe, believing plague's requisite hosts and vectors were non-European species. Moreover, this argument went, whatever disease caused the extraordinary mortality crises of the later Middle Ages and early modern centuries, it was spread person-to-person, thus contagious, and was disseminated at a great a speed across Europe, distinctly unlike a vector-borne infection.

Two broad avenues of inquiry now lead plague history in new directions, because we now know with certainty that Y. pestis was present in Europe in both earlier pandemics. Once again, scientific work on plague's history intensifies. Recent genetic and archaeological methodologies permit the retrieval of ancient pathogen DNA from human remains, and multidisciplinary computerized data analysis can assemble and compare local historical and environmental evidence in ways not previously possible. Secondly, many historians have shifted away from small-scale microhistorical studies to regional and global history, wishing to prioritize geographically and culturally more inclusive analysis. *Yersinia pestis* confirmed, the older view that European responses to plague were either progressive or successful responses to plagues must be revised. Why plague disappeared from Europe becomes again an open question. New historical research overturns long-standing assumptions about the experience of non-Europeans, views no longer acceptable. Other work from social scientists and archaeologists helps to document the patterns and magnitude of

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discrete mortality events and move analysis beyond a reliance on views expressed by literate witnesses. Such research frameworks invite comparison of mortality crises in human communities. Some historical evidence can be aggregated and analyzed with very large data sets, opening the prospect of multidisciplinary, multi-scale investigation of past plagues.

Plague History from Plague Science: 1890s–1970s

Alexandre Yersin first isolated the plague pathogen *Yersinia pestis* in Hong Kong, 1894, naming it *Pasturella pestis* to honor Louis Pasteur. Hong Kong's plague became the opening drama to what came to be called the 'third pandemic'. Throughout the 19th century other plague outbreaks had occurred in southern Russia, southwestern China and some remote regions of Manchuria, attracting venturesome scholars to travel to outbreak sites, then publish their direct local observations. During the 1890s plague spread quickly and directly from southwestern Chinese uplands, to Hong Kong, to southern and southeastern Eurasian port cities, then on to many previously unaffected regions around the globe. Telegraphed news of plague fed the fears and anxieties of people in western industrializing nations, and journalists built on expose´s of other deadly epidemics that circuited the globe: influenza in 1889–91, cholera in 1892. With international attention trained on their actions British local administrators in Hong Kong feared political unrest as much as disruption to trade, and thus used draconian sanitation procedures to contain plague, measures focused on the desperately poor area where most of the native Chinese labor force lived. Similarly officials in nearly every port city where plague first appeared over the next two decades similarly tried to control plague by identifying politically powerless racial, cultural and ethnic groups as the primary threat to urban health and good order. The political and material costs of such panic responses were enormous, and became increasingly difficult to justify alongside the steady production of new bacteriological knowledge about plague.

By the late 1890s, urban rats were identified as the primary plague carriers and their fleas shown to transmit the bacillus to both rats and humans. By 1910, public health authorities in most nations had gradually adopted policies informed by bacteriological plague research, involving both mammologists and entomologists in their efforts to interrupt rat/flea plague transmission. By the time Western Europe swept the whole world into war, anti-plague procedures targeted commensal rodents and redesigned disinfection protocols, particularly at harbors and on large maritime vessels. In 1910–11, the combined research of Russian imperial commissions and a young Cambridge-educated Chinese investigator, Wu Liande [or, Wu Lien-Teh] verified plague transmission from Manchurian marmots to hunters and trappers in the sparsely settled Chinese-Russian frontier, shifting explanations about plague persistence and control away from an exclusive focus on urban *Rattus* species. Although Wu and others blamed the behaviors and habits of migrant laborers, specifically southern Chinese 'coolies', for spreading plague, from this point forward an important component of ongoing plague research investigated endemic plague transmission in remote regions, involving many different mammalian hosts and insect vectors.

Plague histories of the early 20th century struggled to follow the pace of plague science, as is evident from the famous 1911 edition of the *Encyclopaedia Britannica*. The 1880s entry was reprinted, then a second entry, written by new authors, was added to summarize evidence harvested since the mid-1890s. To explain why an ancient threat suddenly reappeared and spread globally, the authors of the updated addendum stressed the disappearance of plague from western Europe during the 17th and 18th centuries, setting up a view that has dominated much plague history ever since: European nations designed and implemented policies that banished plague.

During the first half of the 20th century a dominant multi-century history of plague emerged, well supported among participants in infectious disease research and control but profoundly Eurocentric. By the interwar decades the world's biomedical community had concluded that bubonic plague best accounted for the epidemic in Constantinople during the reign of Emperor Justinian (in the 540s ce), the peerless Black Death mortality wave, 1347–1353, the great plague of London in 1665–66, and the massive 1720–22 plague of Marseille. Vivid and distinctive historical accounts of victims with painful swellings around groin, axilla, or cervical lymph nodes linked the two earlier plague eras to the clinical features of victims in the third pandemic. Literary accounts of pestilences from medieval and early modern Europe were also used to support a view that progressive medical and public health ideas conquered recurrent plague, even though the first pandemic's retreat could not be similarly explained, and these specific measures failed in the third pandemic everywhere they were used without targeted rat and flea control.

Centering plague's pre-modern past around literary accounts led to histories that disproportionately reflected urban experience and even swayed the direction of some important investigations. Seeing epidemic plague as an urban phenomenon, investigators in British India produced a series of influential studies, 1897–1915, which identified a ubiquitous commensal rodent, *Rattus rattus*, and one particular cosmopolitan flea, *Xenopsylla cheopis*, as the principal culprits in human plague outbreaks. Some part of this work transmitted a stigmatizing characterization of the 'Indian rat', the 'Oriental rat flea', and the plague itself as a 'tropical' disease, views that deviled plague history for the rest of the 20th century. Meanwhile, two classic studies based on evidence gathered during the 1906 plague in Mumbai (then Bombay) became lasting tenets of plague epidemiology. In 1914, A.W. Bacot and C.J. Martin, both at the Lister Institute in London, claimed that *X. cheopis* was the flea responsible for human plague epidemics because it fed indifferently on humans and rodents and because their research showed that it became 'blocked', unable to feed, when infected with the plague bacillus. A starving blocked flea fed frantically, accelerating plague transmission to new hosts. The 1927 study of A.G. McKendrick and W.O. Kermack instead devised a mathematical model to explain the spread of human-to human infectious diseases, work known in introductory epidemiology texts as the SIR (susceptible–infected–recovered/removed) model. Unfortunately they used human clinical plague data collected in 1906 Mumbai to illustrate the model, thus nurturing a view that human plague transmissions were often contagious or airborne.

Description of great epidemics in Europe's past was not the only available perspective on prior plagues. For nearly two millennia written testimonies also suggested that 'endemic plague' existed, causing malignant lymphatic swellings but less dramatic mortality crises. The novel scientific work that best accounted for plague outside urban centers, first coalesced during the 1920s, in research conducted for the League of Nations' health division, the *Office International d'Hygiène Publique*. Portuguese delegate Ricardo Jorge during the 1920s headed a commission that synthesized evidence for *Y. pestis* in mammals other than commensal rodents, spread by fleas other than *X. cheopis*. Jorge himself had long experience managing plague. Vilified for his aggressive plague containment measures in Porto, 1899, he later became Portugal's inspector general of public health, having to confront why plague was difficult to eradicate in Madeira and the Azores, islands vital to Portugal's economy. Jorge offered the term *peste silvatique* [sylvatic plague] to characterize plague maintained in regional *foyers* (or enzootic foci), in a wide array of non-urban rodents and lagomorphs that transmitted plague year to year through a wide variety of fleas. But he and all the contributors to the plague commission of the League of Nations believed that only *R. rattus* infection propagated plague pandemics. Russian investigators became leaders in this field of plague studies. In the 1920s older Tsarist anti-plague institutes and field stations were still struggling after the Revolution, but the USSR over the following decades would steadily expand the mission and reach of plague research, producing many leaders and training programs in the study of endemic, or wild plague. E.N. Pavlovsky's model of plague 'focality', studying naturally endemic plague zones with attention to ecological and environmental drivers of plague outbreaks dominated ongoing plague control approaches after the end of the Second World War.

For English-speaking audiences some dedicated plague investigators of the interwar period, Robert Pollitzer, Wu Liande, Karl F. Meyer, and L. Fabian Hirst, ventured historical reflections on plague history that paired reflections on remote sporadic plague outbreaks alongside the dominant narrative of great historical plague pandemics. All argued that endemic rodent plague existed in Eurasia and Central Africa from 'time immemorial', thus history might be able to identify what kept plague within these 'uncivilized' areas, and what fostered its escape and expansion outside plague endemic 'foci'. Pollitzer speculated that there were physical, geographical barriers and 'sterile zones' separating endemic plague from populous regions. Pandemics occurred following human innovations in long distance trade and travel. Wu argued that peoples who had long lived in plague enzootic zones developed cultural and religious practices that helped them to avoid or contain human plague infections; unwary intruders stirred and spread infection abroad. Wu attributed much of the enormous urban mortality of great historical plagues to pneumonic plague. Meyer, the leading figure in the ecology of sylvatic plague in the western United States, from 1934 until the 1950s emphasized that most wild rodents were susceptible to plague, and that the particular characteristics of surrounding landscapes determined the extent and severity of colony infections. His eclectic historical work instead focused on European quarantine and disinfection of mail and commodities in stopping the spread of plague, an implicit argument for plague's steady disappearance from Europe. Hirst devoted far more attention to the independent agency of fleas and rodents in spreading plague, singling out historical evidence that spoke to environmental corruption as stoking plague outbreaks. Attributing importance to ubiquitous human fleas and squalid environments for the great mortality in urban plague, Hirst devoted considerable attention to progressive European ideas and the men who debated the control of plague and other epidemic diseases during the 16th through 19th centuries, emphasizing wherever possible a contrast with non-western societies.

As the international character of plague science generated new historical ideas and evidence about plague as scientific questions, practices and methods converged. Work at France's far-flung Pasteur Institutes during the 1940s through 1970s documented the passive movement of infected animals and/or their fleas, particularly along transports that brought grain to cities to explain recurring smaller-scale urban epidemics, but the temptation to reinforce the grander plague story was considerable. In 1951, Rene´ Devignat, a French bacteriologist working near Lake Albert in the Belgian Congo (now Democratic Republic of the Congo), aligned the three great multicentury waves of plague in history to three biochemically variant strains of *Y. pestis*. Believing that the first recorded pandemic of the 540s ce, originated somewhere in equatorial Africa, Devignat labeled his modern central African isolate *antiqua*. He then ventured that medieval and early modern European plagues, from the Black Death (1347–53) until the horrific 1720–22 plague of Marseille, were caused by biovar *mediaevalis* originating in the central Eurasian steppes. Devignat used biochemically distinctive isolates that Soviet investigators had collected and analyzed from Ukraine and the southern Caucasus region, but he did not borrow their views of the Eurasian origins of plague, nor their concept of 'focality'. Devignat's historical model made plagues erupting in southeastern Eurasia during the 1890s part of a 'modern' third pandemic wave attributed to a biovar *orientalis*. The third pandemic biovar supposedly originated in the Yunnan highlands of southwestern China.

This Soviet system of anti-plague research and surveillance stations ringing the USSR was by this time elaborate, their institutions comparable in geographical reach and sophistication to the French and British colonial stations. Russian investigators agreed with conclusions that Wu Liande and Robert Pollitzer offered: plague originated in central Eurasia, reaching eastern Africa at some later point in time, and this work supported an idea of the 'telluric' (in the soil) persistence of plague. The USSR's southern provinces spanned a broad region plague where outbreaks were observed continuously from the 1870s to his own time, so the idea that the Second Pandemic had ever ended was an inherently problematic historical claim. For Hirst biochemical differences in the bacillus reflected races, and lack of immunological immunity among hosts explained why plagues were uniquely devastating in Europe. Russian investigators believed instead in a broader evolutionary view, that variation and divergence among plague strains was caused by the pathogen's adaptation to new host and vector species, thus reflected how plague had survived in different geographical/ecological regions.

As a simple, elegant generalization about plague's past, Devignat's claim that the pathogen itself supplied useful historical evidence proved irresistible to historians and scientists alike. Reference to one or more of these classic three pandemics typically opens even recent scientific work on plague, even when the specific results better support a multi-local ecological model of plague

hotspots. Influential historians writing in the 1970s and 1980s borrowed from plague science to argue that infectious disease experience determined larger historical patterns and outcomes. Most historians investigated the social, political, demographic, or intellectual impacts of one or more discrete epidemics in a given locality, but two scholars offered highly influential books integrating biologists' views of plague into world history: William H. McNeill in *Plagues and Peoples*; and physician Jean-Noël Biraben, in *Les Hommes et la peste*. Biraben's masterwork has never been translated into English, but remains a staple for historical demography and epidemiology. McNeill, prominent in the field of world history, used Pollitzer's ideas of barrier zones and regionally shared infectious disease experience to make an argument why western Europeans came to dominate world events during the last 500 years. The plague-related aspects of his history drew upon triumphant public health histories such as C.E.A. Winslow's *Conquest of Epidemic Disease* (1943), Hirst's *The Conquest of Plague* (1953), and George Rosen's *A History of Public Health* (1958). Meanwhile newer trends in plague science and newer plague history increasingly diverged.

Historical Study of Plague in the Past: 1970s–2000

During the later 20th century historians with many different areas of expertise—temporal, geographical, methodological, and theoretical—challenged aspects of the science-driven history of plague. In general new historical research moved away from multicentury and multi-millennial timescales, seeking to analyze the causes and consequences of epidemic disease outbreaks within specific historical settings, and over much narrower temporal intervals. A keen awareness of our patchy understanding of earlier mortality crises initially led historical demographers to quantify and compare patterns of births and deaths, culling evidence from non-narrative archival sources. While the overall magnitude and extent of the initial Justinianic plague mortality is not quite calculable, sustained attention to the demographic impacts of the Black Death epidemic and a few of its aftershocks provides sobering confirmation that many millions died in fewer than two decades. Population decline continued for over a century. There was no common European demographic response to recurrent plagues to account for long-term demographic and economic shifts. Close study of different outbreaks showed that plague's unique and specific effects were regionally heterogeneous and everywhere difficult to characterize in their lasting historical impacts. Aggregated and diverse data streams led some to tackle a longstanding question usually assumed in earlier science-driven histories: did plague play an important role in shaping the rise of Western global dominance? Or, with plague's retreat, did the decline of recurring outbreaks in the 18th century somehow fuel the beginning phases of the modern rise of human population numbers? In the quantitative historical subfields investigators took a long multicentury perspective. Those interested in the short to medium term causes and effects of plague also disproportionately studied the second pandemic. Here, too, European archival documents permitted both in depth analysis of the political and ideological views of those in control, and some evidence the experiences and views of persons and places previously invisible within earlier plague histories.

Examining epidemics at the regional or local scale, later 20th century historians upended many of the broad historical generalizations about plague that had gone unchallenged for nearly a century. Neither the murderous plagues of late antiquity or the extraordinary Black Death epidemic of the mid-14th century independently altered the course of human history. At a middle temporal range, from decades to a century, or at a multi-regional spatial level, the supposed history-shaping influence of plague becomes equivocal. Plagues more usually resembled wars in causing temporary social and political crises, disruptions in trade and production. Despite its reputation as an indiscriminate killer, plague typically spared most of community leaders, leading to continuity of political and social power across plague events.

Urban authorities invented an array of discrete responses to the threat of plague—quarantine, the isolation of plague victims in specialized hospitals, trade and travel bans, and the disinfection of infected human locations or belongings through fire or chemicals—all of which once anchored a progressive story of plague as the 'great teacher' in the history of epidemics. Otherwise, plague did not alter European or world history to the same extent that other events may have done. In textbooks the second pandemic overlapped changes credited with a far greater role in human history: the Italian Renaissance, Protestant Reformation, print culture, overseas exploration and colonization, the rise of nation states with standing armies, early guns, early industrialization—a list that could go on. Even in many histories of medicine and science before the 1850s, theoretical discussions of plague causes or transmission had no comparable significance to the Scientific Revolution. Under the historical microscope evidence about plague in the Second Pandemic mostly revealed enduring aspects of power and poverty, modes of governance, the slow secularization of European society, and the importance of religion and art in mitigating fear.

Relatively few written documents survive from the first pandemic. Thus decades earlier than did historians of later eras ancient historians stressed the importance of identifying plague impacts across and beyond the Mediterranean littoral, incorporating wherever possible archaeological evidence about settlement decline and trade patterns. A longer-term, spatially broader understanding of intervals of crisis mortality became obligatory to understanding the decline of all Eurasian and North African societies. The first synthetic work to place the Justinianic plague and its recurrences appeared in 2007, a collection edited by Lester Little, *Plague and the End of Antiquity*. As with general European histories that explained how and when Europeans came to dominate global economies, general histories of classical antiquity and the 'fall' of Rome prior to this volume accorded plague minimal importance in shaping world history from the 500s to c. 1000 ce. This particular book's contributors engaged the complexities of making a multi-century, multi-local historical assessment of plague's impact, and greatly enlarged the geographic compass and interdisciplinary foundations of older first pandemic studies. No collection could at that point, a decade ago, include any coverage of contemporary events in South or Eastern Eurasia, or offer new evidence about plague in sub Saharan Africa or the Arabian peninsula

because written sources from these regions are exceedingly rare before the Islamic era. But the collection marked the beginning of the recent historical trend that promotes longer-term multidisciplinary, collaborative global history.

With far more written evidence available to those who study 19th and early 20th century plague than is available to secondpandemic historians, the social and ideological contexts in which plague decisions and observations were made can be examined in detail, and be compared with equivalent evidence from place to place. The inquiries reveal that modern-seeming statistics are nonetheless problematic as data. In other words, clinical and epidemiological evidence from the third pandemic urban outbreaks needs to be understood as historical, not contemporary, testimonials of plague. Early 20th-century conclusions about the vulnerabilities or dangers of plague are inseparable from their prejudices concerning race and class aspects of victimization, providing ample reason to doubt some of their findings. For example, very high case fatality rates occurred among hospitalized or otherwise isolated plague victims in Asian cities controlled by European colonial administrators. The recorded deaths almost exclusively occurred among those of non-European origins, but few of these patient diagnoses were confirmed by laboratory isolation of the plague pathogen. Two important and distinctive historical features of this pandemic for its historians are instead the scientists' adoption of maps as a fundamental analytical tool for understanding plague spread over time and space, and the use of photography to convey observations not easily mapped or tabularized. Interestingly there are very few known photographs of the pathognomonic indicator of acute bubonic plague, the bubo, but we have many cityscape images of urban slums. Cameras documented the supposed danger zones within cities and the often-uniformed authorities who dealt with the less biologically specific fear that plague provoked.

The belief that early 20th century plague publications can inform us about first and second pandemic outbreaks may also require review as the global history of plague is rewritten. At the opening of the present century two respected medieval historians offered strikingly different, conflicting histories of the Black Death, in books that are widely cited and consulted among plague scientists. Both scholars are fully confident in the factual reliability of scientific observations on plague made in the opening decades of the third pandemic. Ole Jørgen Benedictow assembled historical studies and evidence about the initial pandemic wave as few others could do; his linguistic command exceeds that of most solitary investigators. Benedictow described the Black Death's spread across Europe, guiding his historical research with detailed knowledge of the work of British investigators in Mumbai a century earlier. He assumed that *Rattus rattus* and *Xenopsylla cheopis* were widely prevalent in Western Europe during the medieval centuries, and that plague spread in 'metastatic' fashion through the passive transport of these hosts along lines of human communication, leaping over rural areas to reach the next populous settlements. Samuel K. Cohn, Jr., instead pored over the clinical and epidemiological reports of British investigators in India, finding few salient resemblances between their descriptions of plague and eyewitness or medical accounts of the to 14th-century disease. Cohn's work effectively translated many historians' doubts about our ability to trust disease diagnoses made with pre-modern medical or cultural perspective. Arguing that the word 'plague' in the past never signified an infection caused by a specific microorganism, Cohn was addressing a dominant perspective in the cultural history of medicine since the 1990s, that the early germ theory era in scientific thinking created an epistemological break with our collective understanding of written historical sources. But Cohn went much further, arguing that late medieval records convincingly revealed that modern bubonic plague never existed in Western Europe. After 2010, it was no longer possible to support his skepticism about either the first or second plague pandemic.

New Plague History From New Plague Science: Archaeogenomics

In 2010 a team of genetic and molecular archaeological investigators reported finding fragments of ancient *Y. pestis* DNA in a few individuals who died in London during the Black Death. The rapid technological innovations that led to the important 2010 publications. Bos and colleagues were assisted by historical documentation affirming that the East Smithfield cemetery was used only during this first epidemic of the Second Pandemic. Collaboration with professional historians and archivists matters to current scientific research on past plagues. Another international team (Morelli and colleagues) offered the first complete phylogeny of *Y. pestis*, plague's global family tree on which the London evidence could be located. These two publications ended a tense and highly competitive decade during which the drama of plague took place in laboratories around the globe and involved investigators with markedly different disciplinary skills and institutional settings. In a now-classic 2011 article, historian Lester Little summarized the frantic, not always civil competition to retrieve *Y. pestis* aDNA from first and second pandemic victims. The technological changes to our ability to know about the evolution of major human pathogens is a part of the recent history that did not principally concern Little, who considered instead how plague science demanded historical attention to examine large questions at global or continental scale, and over much longer temporal intervals. Despite massive attention to the cultural, social, intellectual and economic issues of plagues past, the new scientific studies exposed the relative lack of historical attention to climate, environment, human interactions with possible hosts and vectors, living conditions, and activities along pathways of human trade, travel and communication (rather than a selective focus on the nodes and endpoints of such exchanges).

The 2010 breakthroughs built upon specific archaeogenomic advances, 1996–2006. Since the late 1990s, genetic analysis of the evolution of *Y. pestis* placed its geographical origins somewhere in the vast terrain of central Eurasia, confirming the earlier conclusions of both Chinese and Soviet plague scientists. In 1998 Marseille-based investigators, Michel Drancourt and Didier Raoult, isolated ancient *Y. pestis* DNA from individuals buried during the 1720–22 plague. They reasoned that aDNA bacterial fragments could survive in dental pulp, because the end stage of most mammalian plague infection involves blood-borne dissemination of the pathogen. Dental pulp did not decay along with other soft tissue, being lodged in an area of the skeletal

remains that was not exposed to soil contamination. Raoult and Drancourt used PCR (polymerase chain reaction) techniques to amplify fragments of bacterial aDNA, and isolated fragments of one distinctive *Y. pestis* plasmid, thus showing the presence of pestis rather than the ubiquitous and mostly benign precursor, *Y. pseudotuberculosis*. Accused of reporting results that reflected laboratory contamination, the Marseille team and the archaeologists working with them refined and revised their methods, with which an entirely different group of Munich researchers vindicated the primary claim by isolating *Y. pestis* aDNA from humans buried during the Justinianic plague era. Two different technical innovations of the early 2000s, "next generation sequencing" of aDNA and the development of a rapid field test for DNA associated with one of the virulence-conferring plasmids of *Y. pestis* (developed by Pasteur Institute plague control teams in Madagascar around 2004), increased confidence in the isolation of *Y. pestis* from human skeletal remains.

In other pioneering work in the late 1990s, Mark Achtman and colleagues showed that Y. pestis was a recent emergent pathogen at most a few thousand years old. This line of historical-scientific investigation led to the breakthrough work of Yujun Cui and colleagues in 2013, identifying the approximate time and broad geographical location where the medieval plague pandemic likely emerge: a polytomy, described by popular terms such as a "big bang" event. Their complex research is accessible to a non-specialist audience by medievalist Monica Green. We know that all known pathogenic strains of *Y. pestis* emerged in somewhere in central Eurasia, from an ancestral strain of soil dwelling microorganism, *Y. pseudotuberculosis*. The new species has three plasmids, two of which confer the ability to replicate in fleas and survive in mammals, genetic changes that made *Y. pestis* an extraordinary human pathogen. Knowing something of plague archaeogenomics will lead historians and archaeologists to new research on multi-millennial human-driven changes to landscapes and use of natural resources.

Nothing in the new genomic history of plague has as yet either confirmed or refuted the older historical construction of three pandemics. Historian Robert Hymes used Chinese sources and the new genetic findings to identify catastrophic epidemics in the Qinghai/Tibet plateau, perhaps a century before the Black Death reached Europe. Otherwise the question of plague's origins brings newly unsettled historical issues to the foreground, and it exposes the inadequacy of any global history of plague written solely from Western historical evidence or perspective. 20th-century research on plague ecology was not incorporated into earlier historical work that addressed one or all of the three pandemics. A prominent medieval economic historian, Bruce Campbell very recently identified the environmental and climatological changes across the Northern Hemisphere that could explain the Central Asia *Y. pestis* polytomy of the 1200s ce. Campbell aggregated human-generated documentation from European archival sources, looking to commodity prices, the volume of trade across Eurasia and North Africa, and work on demographic and settlement patterns across the 13th through 16th centuries. He then temporally aligned various historical climatological measures of regional precipitation and aridity to the historical evidence. More work of this sort is underway with investigations on the first pandemic.

Secondly, historians' study of the third pandemic outbreaks once reflected professional interest in processes related to modern globalization. Ongoing research on this pandemic exposes the near absence of in-depth historical analysis of plague in regions outside Europe and the Mediterranean littoral, for either of the earlier pandemics. In other words, plague history is finally becoming an important historical topic within global history. Demand for a more inclusive, expansive understanding of the past arose independently among professional historians, while the archaeogenomic revolution was underway. Varlık's very recent study of plague in the early Ottoman empire, from the Black Death to the late 16th century, uses largely untapped Ottoman archives to undermine assumptions about the role of Anatolia and the Levant as regions that repeatedly seeded plague outbreaks in Western Europe. Plague in Anatolia instead expanded as Ottoman contacts with Venice and other western port cities increased; plague became endemic within Ottoman lands after, not before, the Black Death, through the physical infrastructure that a powerful, centralizing state built to connect governors to the governed.

Specialization and collaboration across disciplinary divides is a promising approach to next-generation plague history. A subset of exceptionally well educated, western-trained plague investigators a century ago could once devote free time to doing plague history. Even then important scientific research from Soviet scientists mid-century and Chinese investigators in later decades, uncovered important evidence that was not easily integrated with settled history. We now need a global framework for investigating multicentury, multiregional phenomena. The half-century's work spent dismantling some of old assumptions about plague in human history has created a more confident and informed generation of historians, discontent with static re-presentations of early science-driven history. As in the scientific community, new studies in history must be cast and argued simultaneously at local and global scale. Re-understanding the environmental drivers of catastrophic plague outbreaks should engage historians and the scientific community alike, for historical sources document the destructive potential and extraordinary virulence of pre-modern plague crises, causing losses nearly unimaginable today. A broader and multidisciplinary approach to historical plagues becomes necessary to understand whether human activities were related to the pathogen's emergence, spread, and variable factors governing persistence.

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Historical Smallpox☆

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Glossary

case-fatality rate The number resulting from dividing the total number of cases caused by a disease by the total number of deaths of the disease.

inoculation (sometimes called 'variolation') The introduction of pustular or scab material containing variola virus, the causative agent of smallpox, into the skin. This usually induced a localized infection that protected against natural smallpox but sometimes proved fatal.

poxviruses Members of a large family of viruses, each of which tends to be specific to a particular species (e.g., canarypox, mousepox, and raccoonpox). Only four are known to infect humans: variola, vaccinia, cowpox, and monkeypox.

vaccination The introduction of pustular or scab material containing vaccinia or cowpox virus into the skin.

variola The word is used almost interchangeably with 'smallpox'. It is derived from the Latin word 'pock' or 'pustule'. The term 'great pox' was used to describe syphilis, the lesions of which are much larger.

Abbreviations

WHO World Health Organization

Defining Statement

The history and nature of smallpox is summarized beginning in earliest historic times. A devastating disease, it remained uncontrolled until the discovery of a vaccine in the eighteenth century. Disease control was superseded by a global program of eradication which resulted in the last cases occurring in 1977. The smallpox virus now remains in only two research laboratories (U. S.,Russia) under high biosecurity conditions overseen by WHO scientists.

Introduction

Throughout history, until its eradication in 1980, smallpox was the most serious and feared of all the pestilential diseases with a history dating back to the time of the Pharaohs. It was a disease caused by the variola virus, which infected only humans. All countries with no regard to climate once experienced the disease. The severe form of smallpox, variola major, killed some 30% of its victims, but among the unusually susceptible Amerindian populations, case-fatality rates as great as 60–80% were recorded. There was no treatment for this disease. Smallpox was transmitted directly from person to person. Those who recovered from the disease were immune from acquiring a second infection. The disease was marked by high fever and the development of a characteristic pustular rash. As the disease progressed, scabs formed over the pustules and eventually separated leaving permanent, deeply pitted scars, which were most prevalent over the face. Some victims were permanently blinded. Of all the infectious diseases, none had the capacity both to spread so widely and to inflict such a high mortality as did smallpox.

In the eighteenth century England, it was widely recognized that milkmaids, in particular, seldom acquired smallpox and some attributed this to their having experienced a pustular infection of their hands resulting from contact with pustules on cows. As we now know, these infections were caused by the cowpox virus, which is closely related to variola. In 1796, Edward Jenner, a physician, demonstrated that this pustular material could be deliberately transferred from person to person by inoculation into the skin. He showed that individuals so treated were protected from acquiring smallpox when they were later challenged. This was the world's first vaccine and its discovery is acknowledged as one of the most important in the history of medicine.

As vaccine use increased and more potent and heat-stable vaccines became available, smallpox steadily receded until, in 1967, with only 31 countries still infected, the World Health Organization (WHO) launched a global campaign to eradicate the disease. This proved to be a success. The last naturally occurring case was a Somali resident who became ill on 26 October 1977. In 1980, smallpox was declared to have been eradicated and vaccination ceased everywhere. Except for some workers in orthopox virus laboratories.

☆*Change History:* August 2014. DA Henderson has updated the text and further reading.

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History

Smallpox was an ancient disease and affected specifically humans. Those who became ill could transmit infection only during the 2 to 3–week period of acute illness; second attacks of smallpox were rare. For the virus to survive, it was necessary for it to infect one person after another in a continuing chain of transmission. Thus, to sustain the chain of infection, a moderately large population in reasonably close contact with each other was required. For these reasons, it is assumed that the disease must have arisen less than 10 000 years ago, a time when agricultural settlements were initially developing. Three Egyptian mummies from the 18th to 20th dynasties (1570–1100 BC), apparent victims of the disease, all show the characteristic pustules of acute smallpox.

The subsequent spread of smallpox is difficult to trace because of the paucity of historical records that describe disease symptoms and epidemics in sufficient detail to identify them. Accounts of the disease in India suggest its existence there for almost as long a period as in Egypt. From India, it appears to have spread across China (about 250 BC) and to other parts of Asia. Periodic outbreaks occurred in Europe but not until the eighth century did smallpox became firmly established throughout the continent. By the seventeenth century, smallpox killed more than 400 000 people in Europe every year.

The saga of smallpox in the Americas represents one of the most catastrophic, yet little known chapters in the history of mankind's struggle against disease. In 1507, soon after contact with the New World, the Spaniards brought smallpox to Hispaniola. By 1541, the island's population, estimated to have originally been between 300 000 and 1 000 000 persons, numbered only 500. Several diseases contributed to this disaster but smallpox was the main offender. In 1520, Cortes, with an army of 500, was sent to Mexico to capture additional slaves. Smallpox traveled with him and soon spread throughout Mexico and Middle America and eventually into the vast Incan Empire of the Andean Region. By the end of the sixteenth century, populations throughout Central and South America were estimated to have decreased by 80–90%. North American Indians fared no better.

In all parts of the world, smallpox was known and feared. Special deities were found among populations in many parts of Asia and Africa. However, in India, sometime early in the Christian era, an unusual practice was discovered which served to protect against fatal smallpox. It was called inoculation. Pustular or scab material was taken from a smallpox lesion and inoculated into the skin of a susceptible person. Smallpox infection induced in this manner rather than by the usual route of inhalation, usually resulted in a much less serious disease. Customarily, the recipient would experience fever and malaise and after 5–7 days would develop a pustular lesion at the inoculation site. Most persons recovered uneventfully and were thereafter immune to acquiring smallpox. However, the inoculated individual could transmit infection to others and, in some, a more severe illness occurred which was sometimes fatal.

The practice of inoculation spread to China and Southwest Asia although it is uncertain as to what proportion were so treated. In 1721, the practice was introduced into England and later into other parts of Europe as well as North America. Although it was accepted in some areas, the practice was not enthusiastically embraced everywhere, in part because of the sometimes fatal outcomes and in part because of the occurrence of new smallpox outbreaks developing as a result of spread of the virus from inoculees.

The concept of inoculation as a protective measure effectively established this technique as a common practice. Thus, Edward Jenner utilized a form of inoculation in his now famous experiment in which he took pustular material from a cowpox lesion on the hand of a dairymaid and inserted it into the arm of a young boy, James Phipps. The use of cowpox was suggested by local lore, which held that dairymaids did not acquire smallpox because of having had a cowpox infection. Jenner attempted to inoculate Phipps after some weeks with pustular material from a smallpox lesion and demonstrated that he could not be infected. He published this and other observations in 1798 and soon thereafter the cowpox vaccine (an orthopoxvirus, like smallpox) was shipped to countries around the world. Jenner's discovery was acclaimed at the time and is recognized even now as one of the most important of all medical discoveries.

However successful vaccination, its widespread application proved difficult. Until late in the nineteenth century, vaccination was conducted by the arm-to-arm transfer of the vaccine material. When a vaccination was unsuccessful and no other material was available, fresh material had to be obtained. Cowpox, however, was found only in Europe and occurred only sporadically. The reason for this, as we now know, is that the natural reservoir of cowpox is actually small rodents. Thus, although the virus was continuing to circulate, cows themselves only occasionally acquired infection from the rodents but this was not known at the time. When efforts were made to ship new material, it often would deteriorate en route. Eventually, it was discovered that syphilis and hepatitis B could also be transferred by inoculation. Incidents occurred, in fact, in which cutaneous lesions of syphilis were thought to be vaccinial pustules and large-scale syphilis outbreaks resulted when the pustular material was transferred.

Late in the nineteenth century, cowpox began to be grown on the scarified flank of a calf or sheep, thus assuring a more reliable and ample supply of vaccine. In the 1920s, French and Dutch scientists showed that a dried preparation could be produced which was stable at ambient tropical temperatures. However, methods for drying the vaccine frequently resulted in a final product that was heavily contaminated with bacteria. Finally, a commercial process for freeze-drying the vaccine, perfected in England in the early 1950s, opened the way for widespread vaccination throughout the world and eventually for the global eradication program. Beginning in 2002, the vaccine began to be grown in tissue cell culture.

Smallpox, The Disease

Etiology

The causative agent of smallpox, variola virus, belongs to the genus *Orthopoxvirus*, subfamily *Chordopoxvirinae*, family *Poxviridae*. Three other members of this genus can also infect humans: monkeypox, cowpox, and vaccinia. However, none of the three are readily transmitted from person to person. The poxviruses are the largest and the most complex of all viruses. The virion is a

brick-shaped structure with a diameter of about 200 nm. Its lipoprotein outer membrane (envelope) encloses a single linear, double-stranded DNA. Replication of the poxviruses occurs in the cytoplasm. Unlike other DNA viruses, the poxviruses encode the dozens of enzymes required for transcription and replication of the viral genome. The viral core is released into the cytoplasm after fusion of the virion with the plasma membrane. Restriction endonuclease maps of the genome definitively identify the species of the *Orthopoxvirus* genus.

Pathogenesis

Two distinct forms of smallpox have been observed over the years: variola major with a case-fatality rate of 20–30% and variola minor with a case-fatality rate of 1% or less. Variola major appears to have predominated throughout the world at least through the nineteenth century. A much less virulent form of the disease, usually called variola minor or alastrim, was first definitively identified in South Africa toward the end of the nineteenth century. The rash of variola major is indistinguishable from that of variola minor. This form of smallpox may have been present in some areas of the world before this time but few records are available which set forth numbers both of cases and deaths along with a reasonable description of the disease. Variola minor appeared in the United States at the beginning of the twentieth century and spread rapidly across the country eventually displacing variola major. From the United States, variola minor spread to Latin America and to Britain.

Throughout Asia, variola major was the only type of smallpox seen, whereas in Africa, other variants of variola major occurred resulting in case-fatality rates of between 10 and 15%. Although epidemics of variola minor with the very low case-fatality rates were found in Ethiopia beginning in the mid-1970s, historical reports suggest that before this time, case-fatality rates more closely resembled those found elsewhere in Africa.

Natural infection occurred by the implantation of variola virus on respiratory mucosa. After migration to and multiplication in regional lymph nodes, viremia developed followed by multiplication of virus in the spleen, bone marrow, and lymph nodes. A secondary viremia with fever and toxemia began about the 8th day. The virus, contained in leukocytes, then localized in small blood vessels of the dermis and mucosa. This process resulted in formation of the characteristic vesicular and pustular lesions. Hemagglutinin-inhibiting (HI) and neutralizing antibodies could be detected beginning about the 6th day of illness. Neutralizing antibodies were long lasting, whereas HI antibodies declined to low levels within 5 years. Little is known about the development of cell-mediated immunity. Vaccinia-induced antibody responses developed more rapidly, accounting for the fact that complete or partial protection of individuals was possible even among those vaccinated several days after exposure. Secondary bacterial infection was not common, and death, when it occurred, probably resulted from the toxemia associated with circulating immune complexes and soluble viral antigens.

As the patient recovered, the scabs separated and the characteristic pitted scarring gradually developed. The scars were most evident over the face and resulted from the destruction of sebaceous glands followed by shrinking of granulation tissue and fibrosis.

Symptoms

After an incubation period of 7–12 days, the patient experienced a 2–5-day period of high fever, malaise, and prostration with headache and backache. Those with variola major were usually sufficiently ill to want to stay in bed. A maculopapular rash then began to develop at which time the patient first became contagious to others. The rash appeared first on the face and forearms and on the mucosa of the mouth and pharynx. It then spread to the trunk and legs. Within 1–2 days, the rash became vesicular and then pustular. The pustules were characteristically rounded, tense, and deeply embedded in the dermis. Crusts began to form about the 8th or 9th day. When they separated, they left pigment-free skin and eventually scars. The eruption was characteristically more extensive on the face and distal parts of the arms and legs, and lesions were occasionally found on the palms and soles. Although the case-fatality rate among unvaccinated victims of variola major was usually about 30%, a more severe form accompanied by extensive hemorrhage occurred in about 1 case in 20 and was invariably fatal.

Persons with variola minor usually experienced a less extensive rash and less prostration than with infection caused by variola minor but the rash was indistinguishable from that of variola major. A milder form of disease was also seen among those who had previously been vaccinated, the rash in such persons tended to be more scant and atypical and the evolution of lesions more rapid.

Supportive therapy, maintenance of fluids and nutrition, and good nursing care were the best that could be offered to most patients. On the infrequent occasions when bacterial infections supervened, antibiotics were appropriate. Antiviral drugs, most notably methisazone and the arabinosides, were reported in some early studies to be effective in early treatment or prophylaxis, but confirmatory studies showed otherwise.

Laboratory Diagnosis

Diagnosis of a poxvirus infection is confirmed rapidly by electron microscopic identification of virus particles in vesicular or pustular fluid or scabs. All orthopoxvirus virions have the same appearance. The four orthopoxviruses, which infect humans, grow and produce a cytoplasmic effect in cultured cells derived from many species but they cannot usually be differentiated from each other in most preparations. For diagnostic purposes, PCR techniques are now widely available which readily identify the different orthopoxvirus species, each of which has a distinctive DNA map.

The Global Eradication Campaign

A global campaign for the eradication of smallpox was proposed by the Soviet Union in 1958 and agreed by the World Health Assembly. However, over the succeeding 8 years, little progress was made. At the time, WHO and many of the smallpox-endemic countries were fully preoccupied with a global malaria eradication program which had begun in 1955 and which consumed a substantial proportion of the WHO's budget, personnel, and time. As the years passed, many began to express skepticism that malaria or, for that matter, any disease could actually be eradicated.

Soviet Union delegates, joined by others, expressed increasing displeasure that the WHO did not assign a higher priority to the smallpox effort, and demanded finally that steps be taken to work out a definitive plan and cost estimates for an intensified program. Meanwhile, in recognition of International Cooperation Year, the United States, in November 1965, announced its intention to provide support to 18 countries of Western and Central Africa in an effort to eradicate smallpox and control measles. At the 1966 World Health Assembly, the United States joined the Soviet Union in arguing for the development of a greatly intensified global eradication campaign. Some countries, as well as the Director General of WHO, opposed the concept of smallpox eradication as being technically impossible; others objected to increasing the WHO budget by $2.5 million per year to provide a portion of the needed financial support for the effort. Eventually, by the margin of only two votes, the decision was taken to intensify the eradication effort. Assembly delegates proposed a 10-year target.

In 1967, the year the program began, 46 countries reported smallpox of which 31 were considered to have endemic disease while 15 recorded imported cases. Estimates later showed that some 10–15 million cases and 2 million deaths occurred the same year.

The basic strategy for the program was twofold: population-wide vaccination and a surveillance-containment program. These were to be initiated simultaneously at the outset of each program. The vaccination program was intended to reach 80% of the population in each of the endemic countries and in neighboring countries at risk of imported cases. Those directing the program reasoned that a substantial level of vaccination immunity would sufficiently diminish the rapidity of virus spread so as to permit the surveillance-containment program to be fully effective. The latter program required that a reporting system be developed such that each hospital and health center would report each week as to the number of smallpox cases diagnosed. When cases were reported, a surveillance team would go to the field, seek to find other cases, and vaccinate household and village contacts in hopes of stopping the chain of transmission of infection.

The strategy of surveillance and containment was soon discovered to be far more effective than was expected based on the conventional wisdom of the time which held that smallpox spread rapidly and widely. Through field epidemiology, it soon became clear that a given patient seldom infected more than two to five others, usually household members or close friends and they normally did not develop illness until 7–12 days after exposure. Thus, smallpox usually spread comparatively slowly and cases tended to cluster within cities and within geographic areas. This greatly facilitated the containment activities.

Many observations of importance gradually shaped the program. It was found that with good planning and involvement of community resources, vaccinators could regularly average 500 or more vaccinations per day and vaccination coverage of more than 90% was to be expected. In contrast, vaccination coverage that relied solely on clinics and hospitals for dispensing vaccine seldom exceeded 60%.

Quality control was essential. To assure performance in vaccination, independent assessment teams checked a sample of villages vaccinated by the mass vaccination teams to assure that both coverage and vaccine efficacy standards were met. Where they were not, the entire area was revaccinated. The supply of vaccine was a special problem. As the program began, it was discovered that not more than 10% of the vaccine then in routine use met accepted international standards. Smallpox vaccine was then being produced in more than 40 different laboratories scattered throughout the world. Few undertook to perform routine quality controls of their product. Given the fact that the WHO allocation for smallpox eradication was too meager to purchase required vaccine from established laboratories, the decision was made to foster improvement in vaccine quality in the existing production facilities. Two laboratories, one in the Netherlands and the other in Canada, undertook to perform independent testing for all vaccine to be used in the program. WHO convened a panel of expert consultants from the major production laboratories to develop a standard manual of procedures. Many consultants then traveled to the various laboratories for purposes of training and evaluation. Remarkably, within 4 years, all vaccine met international requirements and more than 80% of the vaccine was able to be produced in the endemic countries themselves.

Research made important contributions. A new method for vaccination resulted in more successful vaccinations utilizing much less vaccine. In 1967, a new vaccination needle (the so-called bifurcated needle) was in the late experimental stages at Wyeth Laboratories in the United States. With Wyeth approval, WHO undertook additional studies with the needle, altered the technique for its use so that it was given by multiple punctures, and introduced its use worldwide. Using the needle, only one-fourth as much vaccine was required as was needed using the standard techniques. Training of vaccinators could be done within a matter of minutes and the result was a higher proportion of successful takes. The needles were fabricated from stainless steel and could be boiled and reused many times.

A second important finding derived from field epidemiological studies, which revealed that the protection afforded by primary vaccination was not the 3–5 years that most textbooks asserted but, rather, showed a 90% efficacy rate at 20 years. With this discovery, it was possible to focus efforts on assuring that every person had a vaccination scar rather than assuring that everyone had been vaccinated within the preceding few years.

Rapid progress was made in the campaign so that by 1973, smallpox was largely confined to four countries of South Asia and, in Africa, to Ethiopia. However, in Asia, efforts such as had been successful in the Americas and in Africa proved far less efficacious. The problem was that with a more extensive infrastructure for road and rail travel in India and Pakistan, people traveled frequently and

over considerable distances, often returning as a family to a home village when one of the families became ill. The result was that smallpox spread more rapidly than in other countries and over longer distances. In the summer of 1973, it was decided with Indian Government authorities to assign large numbers of health personnel to undertake a 10-day search throughout all the villages, towns, and cities of the country. It was hoped that this would permit cases to be found much earlier so that surveillance teams could move rapidly to vaccinate household and village contacts and so prevent further spread of the disease. The first search found tens of thousands of previously unreported cases, many more than had been expected, but early containment was then possible. Every few months the searches were repeated with increasing precision, eventually reaching every household in every village in India. Less than 2 years elapsed before India and all of Asia became smallpox-free.

As the numbers of cases decreased, it became the policy to offer a small reward to anyone reporting a case that proved to be smallpox. As the cases became fewer, the reward was increased so that by the end of the program, a reward of $1000 was being offered for the report of a case that could be confirmed as smallpox. This technique proved to be exceedingly valuable in the closing phases of the program in which each country had to demonstrate to an independent commission that it had a surveillance network that had been functioning effectively for at least 2 years after an endemic area was free of smallpox and that surveillance was sufficiently sensitive to detect cases if present.

The last naturally occurring case of smallpox occurred on 26 October 1977 in Somalia, albeit two further cases occurred, both in Birmingham, England, in 1978, as the result of a laboratory accident. As smallpox incidence declined, an independent international commission was created to review the work of the individual national commissions and to define additional studies and data that it required to assure itself that eradication had been achieved. The Committee last met in December 1979 and, at that time, pronounced that it was satisfied that eradication had been achieved. The Chairman of the Committee reported its findings to the Director General and the World Health Assembly in May 1980. The Assembly, in a specially convened session, passed a resolution indicating that eradication had been achieved and recommended that all countries cease vaccination.

Posteradication

A Possible Natural Reservoir of Variola Virus

From the inception of the program, eradication program staff had been concerned about the possibility, however remote it may have seemed, that there might be an unsuspected natural reservoir of smallpox. Not to be forgotten was the fact that a yellow fever eradication program had to be abandoned in 1932 when it was discovered that there was an unsuspected jungle reservoir. Thus, special efforts were made throughout the course of the eradication program to identify the source of all smallpox cases that were reported from countries that were believed to be free of the disease. It was believed that, if there were a natural reservoir of some sort or if it were possible for people to be infected from scabs or other material still remaining in the environment, smallpox cases would be found for whom the source of infection was not apparent. No cases of this type were found.

Monkeypox, a Disease in Man Similar to Smallpox

Monkeypox is caused by a virus that is a member of the *Orthopoxvirus* genus and so is closely related to smallpox. In man, it resembles smallpox clinically and is associated with a case-fatality rate of 10–15%, similar to that of smallpox in central African countries. Initially, there was concern that it might adapt to spread in humans.

Human monkeypox cases were first detected in 1970 and studied intensively by WHO teams throughout the 1980s. They documented the fact that the natural monkeypox virus reservoir was actually ground squirrels and that man only infrequently became infected. However, the teams discovered that the virus could be passed from person to person although it was much less contagious than was smallpox. Moreover, the studies indicated that it was highly unlikely that the virus could continue to spread even in populations that had never been vaccinated.

During 1997–98, reports of large outbreaks of human monkeypox cases in the Democratic Republic of the Congo raised questions as to whether monkeypox might be spreading so readily from person to person as to replace smallpox as a dangerous contagious disease. The reports in 1997 of large numbers of human monkeypox cases were widely publicized and caused alarm. However, as investigations progressed, it became clear that a large proportion of the cases were chickenpox and that there was little evidence to suggest that the smallpox virus had changed its character.

Destruction of Remaining Smallpox Virus Stocks

Following the 1980 declaration of smallpox eradication, a WHO expert committee was constituted to consider additional steps which might be taken to assure confidence in the fact of eradication and to advise regarding the continuation of a vaccine stockpile among other matters. One important question was to decide whether the known remaining stocks of variola virus should be destroyed.

As a result of WHO recommendations, 74 of 76 laboratories known to have stocks of variola in 1980, had destroyed their stocks or sent them to one of two WHO reference laboratories, located, respectively, in Moscow, Russia, and in Atlanta, Georgia.

At meetings in 1986 and 1990, the expert committee affirmed the desirability of destroying the variola stocks but advised that this be deferred until virus strains could be appropriately mapped, cloned, and sequenced. By 1994, this work had been completed

and endorsements supporting the destruction of the virus strains had been obtained from five prominent scientific bodies including the International Union of Microbiological Societies, the Russian Academy of Medical Sciences, and the board of scientific counselors of the US National Center for Infectious Diseases.

At its 1996 meeting, the World Health Assembly voted to accept the recommendations of the expert committee and proposed that the virus strains at the two laboratories be destroyed in June 1999. However, at subsequent meetings of the World Health Assembly, the last in 2014, several countries argued that it was important to retain the virus so as to permit studies to be undertaken to develop a more attenuated vaccine than that which had been routinely used and to develop antiviral drugs that would be suitable for treating smallpox cases should cases recur. On each occasion that a postponement in destruction has been agreed upon by the Assembly, it has added the provision that research studies using smallpox virus must be approved in advance by a designated WHO Committee and reported to the Assembly and that each of the two laboratories be visited by WHO-appointed experts to provide independent reassurance to all member countries that adequate measures were being taken to preclude smallpox virus from being released.

The rational for retaining the virus has continued to be viewed with skepticism by the majority of countries and many scientists who believe that the argument for virus destruction is a compelling one as it would make it less likely that the virus would be released either by accident or for malicious purposes.

Smallpox as a Terrorist Weapon

There is increasing concern that biological weapons might be deployed by terrorists either acting as a dissident group or under state sponsorship. Until the early 1990s, biological weapons had not been considered to be a threat. The primary reason was that all countries had agreed in 1975 to abide by a Biological Weapons Convention which required all countries to cease work on organisms for use as biological weapons and to destroy such stocks as they possessed. Smallpox, in any case, was considered an unlikely agent because of the high level of population immunity, the availability of a vaccine, and the knowledge that vaccination of contacts can rapidly and effectively control epidemics. Thus, vaccination programs ceased in 1980, all smallpox vaccine production stopped, and the facilities for producing vaccine were converted into other uses.

During the 1990s, it became known that the Soviet Union, ever since the Biological Weapons Convention had come into force, had been engaged in a massive research effort to develop biological weapons, to develop new mechanisms for their dispersal, and to produce them in large quantity. Such information was provided by a high-ranking defector from the program. President Yeltsin later confirmed publicly that the program had indeed existed. He took measures to close the civilian component of the program but the status of the military component is unknown. In all, more than 50 000 persons had been involved in more than 50 of the most advanced biological laboratories. Smallpox virus was considered to be among the most effective of the possible biological weapons and, of the smallpox strains, one that had been recovered in India in 1966 was considered by Russian scientists to be the most pathogenic. Special studies were undertaken to determine the possibility of developing recombinants of smallpox with Venezuelan equine encephalitis virus and with Ebola virus, and a major smallpox virus production unit was built that was capable of producing tons of smallpox virus.

Subsequent to the break-up of the Soviet Union, many of the former biological weapons laboratories have had to sharply reduce the sizes of their staffs. Some of those previously engaged have emigrated to Western countries and some are believed to have consulted and worked in laboratories in countries that are involved in fostering terrorism. It is unknown whether some or any have carried their knowledge or strains of smallpox or other agents elsewhere.

Because of these factors, smallpox vaccine production continued in at least two laboratories; a number of countries have stockpiles of vaccine; a WHO reserve stockpile is in process of being augmented; new diagnostic tests for smallpox have been developed and distributed; and special teams have been trained for immediate deployment should an outbreak be suspected. Studies are continuing to develop a new less reactogenic vaccine and anti-viral drugs. As of 2014, none proved to be fully satisfactory.

Further Reading

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History of Microbiology

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Glossary

adaptation Change in the ability of a microbe to utilize a specific nutrient after exposure to that nutrient.
animalcules Term given to microscopic organisms first described by Leeuwenhoek.
antisepsis Practice of using chemical agents to kill microbes during surgical or other procedures.
asepsis Practice of surgical or other procedures carried out in the absence of microbes.
attenuation Reduction in the virulence of a microbe by certain growth conditions or chemical or physical treatments.
chemotherapy Treatment for disease with specific drugs of known composition, usually a natural or synthetic organic compound, sometimes including the toxic, antimicrobial natural products of other organisms, termed antibiotics.
contagion The belief that an illness can pass from one individual to another by contact or by indirect transfer of germs.
cyclogeny A theory of life cycles of bacteria, usually in terms of an entire population of microbes.
dissociation The phenomenon by which bacteria with different properties, usually related to virulence, arise from a homogeneous population of bacteria. Now attributed to gene mutations.
episome An extrachromosomal genetic element in cells, which can sometimes become associated with the chromosome.
fermentation Classically, the process of conversion of sugar into alcohol.
infusion A suspension resulting from soaking or heating organic material in water, for example, tea.
inoculation Deliberate transfer of material from one source, such as a sick animal or a laboratory culture, into another.
miasma Concept of contagion by some atmospheric influences, 'bad airs', or emanations, not perceivable by the senses.
microbe Generic term for living organisms too small to be seen by the unaided eye. ***monomorphism*** Nineteenth century doctrine that bacteria are of a fixed form or morphology and that the different forms indicate distinct bacterial species.
mutation An abrupt change in a bacterial character, which is stably heritable. Now known to be the result of a change in the genetic code of an organism.
operon A concept applied to a group of genes for functions that are regulated coordinately by means of control of the transcription, or messenger RNA synthesis, of those genes.
plasmid An extrachromosomal, self-replicating genetic element, which may or may not become associated with the chromosome. More general term than episome.
pleomorphism Nineteenth-century doctrine that bacteria are of variable form or morphology and that the different forms are not indicative of distinct bacterial species, in contrast to the concept of monomorphism.
poisson distribution A statistical function that predicts the probability of occurrence of all-or-none rare events, such as mutations, under the assumption that their occurrence is random.
putrefaction The process of breaking down, decomposition, or rotting of organic matter, similar to, but often contrasted with, fermentation.
septicemia A bacterial infection carried in the blood of the affected organism.
spontaneous generation The process whereby living organisms arise from nonliving matter. The matter may be inorganic (abiogenesis) or organic matter from previously living organisms (heterogenesis).
transformation A change from one form of bacteria to another, usually in a 'single property', mediated by exposure to some material, now known to be DNA, from bacteria exhibiting the second property.
vaccine A preparation (made in various ways) from an infectious organism that induces immunity, but not a full infection, when inoculated into a susceptible animal.
virulence The capacity of a specific organism to cause disease when inoculated into a susceptible host.
vitalism The concept that all living organisms possess a 'life force' or 'vital principle' which accounts for the distinct properties of life, beyond the chemical and physical organization of the organism.

Abbreviations

HIV	human immunodeficiency virus
MALT	mucosa-associated lymphoid tissue
PPLO	pleuropneumonia-like organisms

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RTF resistance transfer factors
TMV tobacco mosaic virus

Defining Statement

This article surveys the key landmarks in both the conceptual and the practical history of microbiology.

Introduction

The field of microbiology is defined, more or less, by the physical scale of the living objects of its study. In this sense, it began with the invention of instrumentation to visualize objects below the limits of the human eye, that is, the microscope. In another sense, however, microbiology also includes the study of properties of these objects which can be observed macroscopically or indirectly, for example, the metabolic consequences, the diseases, and the products of microbial activity. In this latter sense, the history of microbiology includes fermentations and other processing of foods, preservation of materials from microbial decay, and concepts of disease and contagion, to mention just a few examples.

The term 'microbe' is a broad and somewhat general one, meant to embrace the biological organisms that are characterized principally by their small size. It was first suggested by Maximilien-Paul-Émile Littré (1801–81), the great French lexicographer and linguist who was also trained in medicine, and proposed to the *Académie impériale de Médecine* by the French surgeon Charles Se´ dillot (1804–83) and quickly adopted by the French school. 'Microbe' allows an agnostic stance until one is sure of the precise biological nature of the organism under discussion, a particular virtue in the early days of microbiology. Bacteria, molds, viruses, protozoans, and sometimes even small multicellular parasites and mammalian cells in culture are subsumed under this designation. For historic reasons, no doubt, the singlecell algae seem to have remained the province of the botanists and are rarely considered as microbes. Also, although resistance and responses to microbial infections are of great importance in microbiology, many of these phenomena have become the domain of immunology and will not be included here. This article will focus on the history of microbiology since the invention of the microscope for two reasons: first, the new mechanistic philosophy of the seventeenth century provided a context in which to understand microbes, and second, the ability to observe microbes, albeit indirectly with an optical instrument, was essential to the further development of the subject.

Since any brief survey must be selective, this article will emphasize those events and discoveries that are considered common to all microbiological investigations. Some areas have been omitted or treated with less attention that they deserve (e.g., parasitology and mycology), and the history of some newer topics (such as microbial evolution) have been left to the text articles on those subjects. The bibliography includes standard monographs of historical interest, key textbooks that give historical accounts of various topics, and a few references to the literature of the history of biological science and medicine. Articles on the history of microbiology are included in such searchable databases as Medline and History of Science and Technology (Research Libraries Group), both accessible on the Internet.

Microscopy in the Seventeenth Century

The invention of the telescope at the beginning of the seventeenth century quickly led Galileo to his revolutionary astronomical discoveries, and shortly thereafter experimenters in optics produced the first microscope, reportedly demonstrated for the first time in Paris in 1620. These first microscopes were 'simple' in that they had a single lens of high curvature, often a glass bead, and were first exhibited as curiosities. By the mid-seventeenth century, several serious investigations were underway, which employed the microscope to examine the invisible structures, if any, of matter. This interest in the structure of matter was stimulated by the broad seventeenth-century philosophical debates on the mechanistic concept of the universe, which included speculation that matter, animate and inanimate, is made of small particles, some of which function as tiny machines to impart function to the whole. Notable among the seventeenth-century microscopists were Robert Hooke (1635–1703), Nehemiah Grew (1641–1712), Marcello Malpighi (1628–94), Jan Swammerdam (1637–80), and Antony van Leeuwenhoek (1632–1723).

It was Leeuwenhoek who is most remembered today for his voluminous investigations and for the superior quality of his self-made instruments, and who was most concerned with observations on suspensions that contained objects we now consider as the subjects of microbiology. Leeuwenhoek was a local cloth merchant in Delft, Holland, who had little formal education but who developed a passion for lens-making and microscopic observations and descriptions. Through the Delft anatomist Reinier de Graaf, Leeuwenhoek came to the attention of Henry Oldenburg, the secretary of the newly formed Royal Society of London for the Improvement of Natural Knowledge ('The Royal Society'), who began publishing Leeuwenhoek's descriptive studies in 1673. Leeuwenhoek's 'Letters' to the Royal Society span over 50 years of microscopic observations on infusions, body fluids, and a wide range of other materials. His skill as a lens-maker became widely recognized, and his reports were appreciated for his careful descriptions as well as for his technical virtuosity. His studies on human semen and description of spermatozoa (his own) contributed to the seventeenth-century debates on animal generation and the demise of various preformation theories of

reproduction. In his own time, Leeuwenhoek was best known for these contributions, and his observations on microorganisms, now celebrated as the beginning of microbiology, were of relatively little interest and significance in his own time.

Robert Hooke was the other microscopist most credited as a founder of the field of microbiology. Hooke's famous book, *Micrographia*, published in 1665, astounded people with the details of the invisible world revealed by the new microscopes, some of which were devised by Hooke himself. This book contains for the first time illustrations drawn by Hooke of his microscopic observations. The most famous of these, showing the microscopic structure of cork, has been taken as the beginning of the cell theory of life, later developed by Schleiden and Schwann in the nineteenth century. Hooke was able to confirm and extend Leeuwenhoek's results in the presence of members of the Royal Society, thus establishing the credibility of the rather reclusive Dutch cloth merchant.

While Leeuwenhoek's reports on what he called 'animalcules' (little animals) in rainwater and in various infusions indicate that he had indeed seen various common protozoa, yeasts, and some bacteria, he did not extend these observations very much beyond his careful descriptions. Throughout the eighteenth century, dedicated microscopists pursued the study of these objects and refined their descriptions, but all were limited by the optics of the simple microscope: chromatic and spherical aberration, and relatively low magnification. Specimen preparation methods, such as sectioning and staining, were primitive. For example, prior to the introduction of the mechanical microtome in 1770, thin sections had to be cut freehand.

Contemporary ideas about life, matter, and disease did not allow seventeenth- and eighteenth-century microscopic observations to be easily interpreted. Even in the late eighteenth century, Linnaeus was puzzled as to how to treat these little animals in his grand classification schemes. He assigned many of them to the genus and species: *Chaos infusoria*, perhaps indicative of the ambiguous status of microbes at that time.

Spontaneous Generation and Microbes

In parallel with microscopic studies, complementary debates on the origin of living things were intense during the seventeenth and eighteenth centuries. Although these two topics started from quite different points, they would become intimately connected in the nineteenth century, so much that we now think of them as part and parcel of one tradition. Common observations of the apparently spontaneous appearance of insects, worms, and other small creatures (even mice) suggested to many observers that these living beings could arise from nonliving sources such as mud or putrefying vegetable or animal matter. Even the famous chemist and philosopher Jan Baptist van Helmont (1577–1644) believed that mice could arise from grain stored in granaries.

In 1668, Francesco Redi (1626–97) published his investigations to test the belief that maggots and flies arose spontaneously from meat. Redi was a courtier and natural philosopher under the patronage of the Medici Grand Duke in Tuscany and, as such, was expected to present 'observations' and 'demonstrations' for the enlightenment and amusement of the court. At one point, he conducted a series of observations on rotting animal flesh, some portions of which had been protected from the air or from other sources of infection. The interpretation of these 'experiments' was that the maggots, flies, and ova seen in the exposed samples but rarely in the protected samples, did not have their origin from the meat itself, but rather the rotting flesh only served as suitable nest for the growth and nourishment of the eggs of the animals that were deposited there. While Redi is often cited in textbooks as having used or even invented the 'controlled experiment', when his work is viewed in the context of his own time, it is clear that his view of 'experiment' was quite different from the modern interpretation. For Redi, experimentation meant visual demonstration of already 'known' truths.

Despite work such as that of Redi and others, the idea that living beings were continuously being created from more basic material persisted. Georges-Louis Leclerc, Compte de Buffon (1707–88), a famous French naturalist, in his monumental *Histoire naturelle* (1749 et seq.), described his beliefs that all living things contain an indestructible vital principle. Buffon incorporated newer ideas about the chemistry of 'molecules' (small globules or particles) into his theories and regarded spontaneous generation as the result of the intrinsic properties of these organic 'molecules'. The noted British naturalist, John Turberville Needham (1713–81) employed microscopic studies of various infusions, both heated and unheated, both open and closed to the air, and consistently observed the development of many of Leewenhoek's little animals. He interpreted such results in terms of Buffon's hypotheses and was a strong supporter of the notion of spontaneous generation. When Needham's work was repeated and extended by the Italian naturalist Lazzaro Spallanzani (1729–99), Spallanzani found that heating was quite effective in preventing the appearance of the little animals, and furthermore, that Buffon's ideas about the vitality of the material present in the infusions, thought to be the precursor of the little animals, were untenable. Spallanzani did many careful experiments with heated sealed and unsealed flasks from which he concluded that exposure to air was the source of the organisms that appeared in the infusions after heating. Neither Needham nor Spallanzani had the microscopical techniques that might have allowed resolution of their disputes. Furthermore, the biological status of the 'little animals' remained unclear, with the result that well into the nineteenth century, the questions of spontaneous generation and 'vital principles' remained unresolved.

As discussed below, motivated by practical concerns with fermentations and disease, Pasteur and others pursued the questions already framed by their eighteenth-century predecessors with newly developed techniques, concepts, and instruments and by the end of the nineteenth century, serious discussion of spontaneous generation waned. The belief in Buffon's vitalism, however, has been more durable, and even the most current textbook writers feel a need to exorcise the ghosts of this eighteenth- and nineteenthcentury doctrine by proforma reference to Wohler's famous synthesis of the 'organic' compound urea, from an 'inorganic' cyanate (a reductionistic view that claimed that only living organisms, because of the vital principle, could produce 'organic' compounds). Even in twenty-first century popular culture one can find strains of these eighteenth century ideas.

Classification of Microbes

The biological nature of the animalcules began to attract the attention of naturalists in mid-eighteenth century with the classification of many of these forms by the Danish naturalist Otto Friderich Müller (1730–84), who devised some of the terminology still in use today. He grouped the animalcules into two major groups: those without visible external organs and those with such structures. The first group was divided further into two groups, one of which included five genera, two of which (*Monas* and *Vibrio*) contained bacterial forms. One of Müller's species, *Monas termo*, would become the subject of most nineteenth-century attention, much as the colon bacillus, *Escherichia coli*, has been the paradigmatic species in the late twentieth century.

The science of optics was sufficiently developed by the 1820s to allow construction of compound microscopes with achromatically corrected lenses and some correction for spherical aberrations. Better microscopy resulted in more detailed observations of the microbial world. In some sense, modern classification in microbiology started in 1838 with the work of Carl Gustav Ehrenberg (1795–1876), who published *Die Infusionsthierchen als vollkommene Organismen*, a massive folio of 574 pages with an atlas of 64 hand-colored plates. Many of his drawings reflect his belief that 'Infusoria' (a category that included bacteria, protozoa, rotifers, and diatoms) were small animals and were organized on the same principles as larger animals; thus, he believed all these little organisms had tiny stomachs and were perfect and complete (*vollkommene*) animals. Three of Ehrenberg's 22 families of Infusioria include organisms that are recognizable as bacteria: Monadina, Cryptomonadina, and Vibronia. Vibrionia was composed of five genera: *Bacterium*, *Vibrio*, *Spriochaeta*, *Sprillium*, and *Spirodiscus*. These groups were defined by gross morphology: for example, *Bacterium* included filaments or threads of rod-like forms, which showed transverse divisions. Although Ehrenberg gave detailed descriptions and drawings of many of his observations, it is difficult, if not impossible, to determine with certainty how these organisms would be classified today.

Subsequently, many classifications of microorganisms were proposed, based predominantly on morphologic characteristics with some attempts to incorporate growth characteristics such as growth by transverse division or budding. After Ehrenberg, the next important classification scheme was that of Ferdinand Cohn (1828–98). In 1854, Cohn published an important work in which he suggested that Ehrenberg's Vibroinia should be regarded as belonging to the vegetable kingdom rather than to the animal kingdom, and suggested that bacteria were very similar to the well-known microscopic algae. He suggested that bacteria be classified as Mycophyceae or 'Wasserpilze' (water fungus). Cohn's major work in bacteriology, *Untersuchungen über Bacterien* (1872, 1875, 1876), was highly influential and established the outlines of modern bacteriological thought. While Cohn was arguing for the establishment of a taxonomy for bacteria that positioned them within the known biological realm, that is the kingdom of plants, another German biologist, Carl von Na¨geli (1817–91), maintained that microscopic fungi may arise spontaneously. Na¨geli was an important figure in nineteenth-century biology, and his views gave strong support to an alternative view of bacterial classification, namely the idea that there would be little or no constancy of form in bacteria, which were produced spontaneously from animal or plant precursors. This belief in the multiplicity of forms became known as pleomorphism, and was supported by observations that many fungi seemed to change form depending on conditions and stage in its growth cycle. The late nineteenth century saw intense investigation and debate about these changes or alteration of forms among the fungi and bacteria. In contrast to the pleomorphic hypothesis was the view of Cohn and his followers that each kind of organism had a definite and fixed form. This view became known as 'monomorphism'. Needless to say, the outcome of the monomorphism– pleomorphism controversy was crucial to the future of bacterial classification and taxonomy. An example of this confusion is observed in the work of Wilhelm Zopf (1846–1909), who sided with Na¨geli in supporting the pleomorphic doctrine, but implicitly followed the monomorphists in his widely influential classification scheme published in 1885 (*Die Spaltpilze*).

Toward the end of the nineteenth century the classification of microbes was influenced by three major developments: improved morphologic methods (both better instruments and staining methods), pure culture techniques, and better knowledge of the functional activities of bacteria (e.g., pathological actions and fermentations). By the turn of the century, classifications such as that proposed by Migula and Orla-Jensen included properties of bacteria beyond morphology, such as pathogenicity, growth requirements, and staining properties.

The filamentous fungi as well as the protozoa were also included in these early classification schemes, and their distinctive growth patterns, natural histories, and pathologies were recognized. The spore formation by fungi, the colonial growth forms, and the life cycles were characteristics that allowed classification of these organisms to proceed more surely than that of the smaller bacteria. Many of the animalcules of Leeuwenhoek were first called infusoria. The first genus for these infusoria (*Paramecium*) was introduced in 1752 and in 1817 the generic term protozoa was employed by Goldfuss.

While classification methods, stemming from the work of Ehrenberg and culminating in the early work of David H. Bergey (1860–1937) and the Society of American Bacteriologists (*Bergey's Manual of Determinative Bacteriology*), have been of great pragmatic use in categorizing and recognizing bacterial isolates, another goal of classification and taxonomy is to delineate evolutionary relationships. At the light microscopic level, the observable structures of bacteria are relatively uninformative, however, and there has been little confidence that simple size and shape determinations carry much evolutionary weight.

Only with the more detailed investigations and classification of bacteria using biochemical and genetic analyses (genomics) has it been possible to devise classifications that go significantly beyond those of the beginning of the twentieth century. Direct comparisons of the DNA of different organisms became possible with the discovery of nucleic acid 'hybridization' as a method for testing the relatedness of DNA sequences. Prior to the invention of DNA sequencing methods, the extent and rate of DNA helix formation from complementary DNA strands from different microbial species was used, first by Schildkraut, Marmur, and Doty

(1961) to compare the genomes of bacterial isolates. Substantial improvements in gene-based classifications were achieved when Woese (1977) showed that the comparison of the sequences of RNA in the smaller (16S) ribosomal subunit gave meaningful classificatory information. Since the aim of most systems of classification is to reflect evolutionary relationships, such gene-based schemes were highly satisfying. The comparisons of 16S RNA sequences (RNA sequencing was technically more advanced than DNA sequencing methodologies in the 1970s) allowed Woese to propose a novel group of evolutionarily ancient microbes, which he termed Archaea. He proposed that all life be classified into six kingdoms (Eubacteria, Archaebacteria, Protista, Fungi, Plantae, and Animalia). With the development of rapid methods for the determination of entire genomic DNA sequences since the late 1980s, the comparison and classification of microorganisms has become an industry, based almost entirely on gene relatedness. By 1990, Woese had revised his classification to three 'Domains' rather than 'Kingdoms' (Bacteria, Archaea, and Eukarya). Viruses, however, still resist attempts at rational classification into a single, consistent system.

Contagion

The periodic and widespread occurrence of disease in populations has been noted by historians for several millennia. Both popular and medical accounts abound with descriptions, explanations, and prophylactic advice about epidemic diseases. Many of these accounts suggest that disease is transmissible from an afflicted person to a healthy person, that is, the disease is contagious. Although 'contagious', in modern thinking, almost always implies 'infectious', this is not at all the way the notion of contagion should be read in these older accounts. Without the knowledge of microbes and their relation to disease, the concept of 'infection' is especially problematic. Instead, contagion sometimes implied active maliciousness such as the 'evil-eye', hex-casting, or other such magical practice. Alternatively, the contagion could reside in local conditions such as 'foul' air, or on the clothing or other belongings of the afflicted person, or emanate from decaying material or earth. Thus, the notion of contagion was broad and flexible.

While much has been written on the history of epidemics from the Plague of Athens in 430–425 BCE (Thucydides) to AIDS and SARS, and many writers have tried their hands at retrospective diagnosis and search for precursors to modern ideas of infectious disease, the small book usually called *De Contagione* by Girolamo Fracastoro (1478–1553) is usually taken as the first, more or less clear, recognition that a contagious disease might be transmissible by some sort of 'infectious' agent. Fracastoro treats three types of contagion: by contact alone, by fomites (things 'which are not corrupted themselves, but are able to preserve the original germs and give rise to their transference to others'), and by contagion at a distance. In spite of Fracastoro's rather clear exposition, however, his ideas seem to have lain fallow for several centuries. Contagionist doctrines, while held by many scientists, did not develop in the direction of infectionist beliefs until a plausible mechanism, that is microbes, was known.

Germ Theories

While many of the early microscopists speculated that their little animals might be related to disease, fermentations, and putrefaction, such ideas did not take hold partly because the existing concepts of these phenomena had no way to incorporate microbes into their explanatory schemes. While the diligent historian can find many examples of writings that appear to be precursors to modern germ theories, it is quite clear that they represent a rather silent or ignored tradition in medicine and biology in the seventeenth and eighteenth century.

As the biological understanding of bacteria developed in the nineteenth century, and as scientists turned more and more to physiology and 'animal chemistry' for understanding of biology and medicine, the newer findings about bacteria found their applications. No longer content with explanations of diseases based on miasmas, supernatural retribution for sinful behaviors, and simple environmental conditions, late eighteenth- and early nineteenth-century thinkers looked for causes of disease based within the body itself, in its function, normal, and deranged. These explanations often incorporated specific aspects to account for the contagiousness of certain diseases. The process of fermentation was so important and widely studied that it formed the model by which other biological processes were conceived. Thus, digestion was seen as analogous to fermentation. Likewise, the processes of putrefaction, and by extension, tissue degradations in abscesses, for example, were thought to be related to fermentation. The great German chemist, Justus von Liebig (1803–73), conceived of disease as a putrefaction of tissue produced by nonliving (yet 'vital') substances (which we now recognize as enzymes, *ferment*s in French) with the production of toxins. These toxins, when transferred to another individual, could induce further putrefaction 'by contact'. Such a mechanism could explain both contagion and physiological effects without the need for living, infectious agents. In Liebig's theory, fermentations, too, could be brought about by introduction of the nonliving *ferments* to suitable circumstances, for example, in wine-making.

Debate on the theories of contagion thus evolved into investigation of the biological processes of both fermentation and digestion in the mid-nineteenth century. As might be inferred, too, these debates were intimately related to the controversies over spontaneous generation and vitalism mentioned above. Did fermentation (and by extension, disease) require the presence of living organisms? Was fermentation a strictly chemical process, or did it require some essentially 'vital' component? If fermentations always required living organisms (yeast), where did the yeast come from in the apparently spontaneous fermentations seen in wine- and beer-making?

From his studies on the fermentation of beer and wine, Louis Pasteur (1822–95) concluded that the agent that is responsible for alcoholic fermentation is a living organism, yeast, which must be introduced either accidentally or intentionally. Occasional accidents of fermentation ('diseases' of wine and beer) occur when the wrong organism is present. This pathway of experimentation led Pasteur to strongly attack the belief in spontaneous generation. His vigorous experimentation and even more vigorous rhetorical activities led to his fame as the slayer of the doctrine of spontaneous generation. The English physicist, John Tyndall (1820–93), provided strong support for Pasteur's ideas by his investigation of dust and small particles in the air as a source of the contaminations in experiments, which claimed to show the existence of spontaneous generation.

Along with studies of 'diseases' of fermentations, Pasteur undertook the study of a disease of silkworms that was causing severe economic difficulties for French silk producers, and in 1866 found the 'cause' of this disease to be a specific microbe. By the mid-1870s, Pasteur had come to believe that all contagious diseases were caused by specific microbes, a grand generalization that had many versions, which now go under the rubric of 'germ theories of disease'.

Pasteur devised methods for preparation of pure liquid cultures of bacteria by serial transfers at high dilution, and exploited such pure culture techniques to investigate the role of bacteria in disease and later to devise protective vaccines.

A related problem also engaged the attention of nineteenth-century physicians: the nature and cause of diseases variously called putrid intoxication, septicemia, surgical fever, and pyemia. Experiments based on early belief in toxins showed that these diseases were often transmissible by inoculation of blood from one animal to another. Pus from a human wound could induce disease in animals, for example. The precise relationships between pus globules, white blood cells, and other components of blood were unclear. Casimir-Joseph Davaine (1812–82) examined the blood of animals with anthrax and septicemia and reported that bacteria were only present in the blood of diseased animals. Much work of this sort from 1850 onward supported the association between the presence of bacteria and the occurrence of disease. Davaine extended this work to deliberate inoculation experiments with graded injections of material from sick animals and showed that the transmissible agent (called the 'virus' in nineteenth-century terminology) could be titrated and that different animal species varied in their susceptibility to the virulent agent. Davaine's work attracted great interest in France and elsewhere, yet some investigators interpreted this work to show that the bacteria were considered a consequence of the disease rather than a cause.

Theodor Albrecht Klebs (1834–1913), working in military hospitals in Karlsruhe, studied septic deaths from gunshot wounds and found bacteria of different forms present in almost every case he examined. He added much to the knowledge of wound infections and devised several approaches to the study of wound infection, which were soon adopted by Koch.

Robert Koch (1843–1910) was a medical practitioner in the small town of Wollstein in Pozen, where he undertook the study of wound infections to determine if they were of parasitic (bacterial) origin. He conducted animal experiments and self-consciously considered just what kind of experiments he must do to prove, as conclusively as possible, that the wound infections that he produced in animals by injection of pus were, indeed, caused by the bacteria found in the pus. Koch was aided in his work by newly available aniline dyes for staining samples, by the newly available Abbe´ microscope illuminator, and by the fine oil-immersion lenses made in Jena by Carl Zeiss. In his publication, *Untersuchungen über die Aetiologie der Wundinfectionskrankheiten*, he clearly raised the standards for the study and description of the relationship between bacteria and disease. In spite of the minor stir that this publication caused, many physicians remained unconvinced. Similar investigations were carried out by many other workers, including Alexander Ogston (1844–1929) in Scotland and Daniel E. Salmon (1850–1914) in America.

Koch undertook the study of the life history of the anthrax bacillus in which he noted the formation of spores, which were especially heat-resistant. He immediately realized the relevance of the spore form both from an experimental point of view as well as from the epidemiological standpoint. Koch communicated this work to Ferdinand Cohn, a leader in the German academic world, who recognized its importance and arranged for its publication. From this work on anthrax, Koch became widely recognized as a masterful experimentalist and creative thinker.

Early in his research, Koch recognized the need for better cultivation and identification methods in bacteriology and he worked hard to develop the tools he saw as essential. Both his new staining methods and his improvements of the methods of solid surface cultures led to major new advances in his research work. He devised fixation methods, which preserved the morphology of the bacteria and thus was able to study the organisms in a nonmotile form. He tested a wide variety of fixatives and stains (much of this work based on the early studies of Paul Erhlich) and was able to obtain preparations far superior to any previously available. Koch also pioneered the use of photomicrography, which had an important role in helping spread his theories as well as convince his critics.

As early as 1865, there had been prior attempts to obtain pure cultures of bacteria by growth of individual 'colonies' on solid medium but these were generally unsatisfactory. In 1881, Koch reported that he could grow individual colonies of bacteria on the sterilized cut surface of a potato slice and soon he followed up on prior work by Oscar Brefeld, who laid the theoretical and practical foundation for work with pure cultures. Koch realized that what was needed was a medium that was sterile, transparent, and solid. Initially, he found gelatin to be very useful and later employed the more stable carbohydrate, agar–agar, as the gelling agent in the medium. At first the medium was simply poured on a glass plate and allowed to gel, but soon Koch's assistant Richard Julius Petri (1852–1921) made slight modifications and introduced the flat dish with an overhanging lid (Petri dish), still in use today.

The solid surface culture of bacteria was a revolutionary advance in technique that had major consequences. With this method for pure culture isolation, many of the ambiguities in the monomorphism–pleomorphism debate were resolved. Bacterial identification and classification became easier and more certain. Most importantly, Koch was finally able to affirm the identity of a specific bacterial type with the virulent agent in the blood, pus, or tissue extracts in his animal inoculation experiments.

Koch and several other thoughtful advocates of germ theory doctrines of disease were concerned about the knowledge claims they were making: what experimental evidence was needed to prove that a specific bacterium was the cause of a specific disease? Over a period of several years (1876–82), Koch evolved a set of criteria for such evidence. In his 1878 paper on wound infections, he gave a set of three rather weak criteria, but in 1882 in his landmark paper on the etiology of tuberculosis, he stated: "To prove that tuberculosis is a parasitic disease, that it is caused by the invasion of bacilli and that it is conditioned primarily by the growth and multiplication of the bacilli, it was necessary to isolate them [free] from any disease-product of the animal organism which might adhere to them; and, by administering the isolated bacilli to animals, to reproduce the same morbid condition which, as known, is obtained by inoculation with spontaneously developed tuberculous material." Later versions by Koch and textbook authors have combined this statement with some of Koch's other writing to synthesize three criteria which have come to be known as 'Koch's postulates'. (Apparently, however, he never referred to these criteria as 'postulates'.)

In America, Daniel Salmon was investigating the cause of hog cholera and had developed his own set of criteria for disease causation by bacteria. In addition to the criteria that Koch proposed, Salmon believed that the causal connection required the added demonstration that the killing of the bacteria was curative of the disease.

In the final decades of the nineteenth century, with the new methods available and the conceptual framework of Koch, Pasteur, Davaine and others, advocates of germ theories of disease were hard at work 'hunting microbes'. With each success of associating another disease with a specific pathogenic organism, the rush to find bacterial causes for all disease increased. In addition to bacterial causes of cancer, there even were reports of bacterial causes for mental disorders.

Applications of the Germ Theories

With a clearer understanding of the causes of contagious diseases, there was a strong impetus to use this knowledge in ways to treat or prevent the disease. These efforts took several forms: sanitation and public health, asepsis and antisepsis, and preventive vaccinations. Specific chemotherapy came later.

Germ theories of disease fit well with the program of the nineteenth-century public health movement with its emphasis on 'filth' as the cause of disease. From the mid-century work of the lawyer-sanitarian Edwin Chadwick (1800–90) in England, it was believed that mortality rates, and perhaps health in general, could be affected by sanitary reform. Bacteriologists noted that the very places and conditions considered 'filthy' were generally the places and conditions where bacteria were likely to flourish. It is no surprise, then, that many of the leading nineteenth-century bacteriologists were interested in water supply sanitation, food quality control, and sewage and waste treatment and disposal. In America, William T. Sedgwick (1855–1921) was the most accomplished sanitary scientist and water bacteriologist of his day, and as chair of the Harvard – MIT School for Public Health Officers, he educated a generation of public healthoriented bacteriologists.

In England, Joseph Lister (1827–1912) was the most active advocate for the application of Pasteur's germ theories to the practice of surgery. In 1868, he reported on his use of antisepsis (not asepsis) during surgery to prevent the occurrence of surgical wound infections. He employed phenol (carbolic acid) in an oil suspension. His results and his approaches to surgical cleanliness initiated a new era in surgical practice and led to a dramatic decline in postsurgical septic mortality. Lister's work led others to study antiseptics in detail, and Koch soon was able to make the important distinction between agents that simply arrest bacterial growth without killing (bacteriostatic agents) and those that are able to kill bacteria (bacteriocidal agents).

Germ theories did not find application only in medicine. Crop diseases, especially those involving fungi, were recognized as infectious in origin. As early as 1767, Giovanni Targioni-Tozetti (1712–83) proposed that rusts of cereals might be caused by infection with microscopic fungi and by 1807, Isaac Be´ne´dic Pre´vost (1755–1819) had demonstrated experimental smut infections and showed that copper sulfate solutions could be used to disinfect plant seeds. The biological study of fungi led to better understanding of their life cycles and of the alternation of hosts required by some organisms. For example, by 1865 Anton de Bary (1831–88) explained the role of barberry plants as an intermediate host in wheat rust, and recommended that rust epidemics could be controlled by elimination of the practice of using barberry hedges near wheat fields. Ergot of rye was another important pathogen, which led to widespread human illness in certain years when the infection was prevalent.

Several fungi (mildews) were recognized as pathogenic to grape vines by the mid-nineteenth century and direct treatment of vineyards with agents known to kill the organisms were developed. Copper sulfate and lime suspended in water were widely employed and became known as Bordeaux mixture.

Human diseases caused by fungi and protozoa were also recognized in the nineteenth century. Skin diseases (dermatomycoses) such as favus (Scho¨nlein, 1839) and thrush (Langenback, 1839) were shown to be related to specific fungi (*Achorion schonleinii* and *Candida albicans*, respectively), and the monograph *Histoire Naturelle de Ve´ge´taux Parasites* (1853) by Charles-Phillippe Robin (1821–85) was a landmark summary of early medical mycology. In 1910 when Sabouraud devised a medium on which pathogenic fungi could be easily cultivated, the development of medical mycology greatly accelerated.

The role of protozoa in disease was suggested early by observations such as Alphonse Laveran's (1845–1922) discovery of a specific organism in the blood of malaria patients in 1880. Later Giovanni Battista Grassi (1854–1925) and Ronald Ross (1857–1932) were able to discover the role of the mosquito in the transmission of malaria (1890s). Ross and his collaboration with Patrick Manson (1844–1922) exemplify the way the field of tropical medicine developed from the need to better understand newly encountered diseases during nineteenth-century European colonial expansion.

Other forms of microorganisms were found to be the cause of disease as well. For example, in the early twentieth century, small, bacteria-like, obligate intracellular forms now known as *Rickettsia* were found to be the infectious agent in diseases such as louse-born epidemic typhus (Charles Nicolle, Howard Ricketts, and Stanislaus Prowazek) and Rocky Mountain spotted fever (Ricketts). Chlamydiae, another group of obligate intracellular pathogenic forms, thought to be distantly related to Gram-negative bacteria, were recognized in 1952 as distinct from large viruses.

Mycoplasmas, the smallest known free-living microbes, are a pleomorphic and widespread group of organisms. Since they often pass through conventional filters, they were originally classified as filterable viruses. The first known mycoplasma disease was pleuropneumonia of cattle, which was studied by such luminaries as Pasteur (1883), Nocard (1898), and Bordet (1910). From this example, mycoplasmas came to be called 'pleuropneumonialike organisms' or PPLO until recently. One isolate from primary atypical pneumonia in humans was propagated in chicken embryo tissue and was known eponymously as the Eaton agent (1944).

Perhaps the most dramatic advances that followed the new understanding of germ theories of disease were those involving preventive vaccination. The general phenomenon of resistance and immunity had been recognized for a long time, but beyond general knowledge that a prior smallpox attack protected the individual in a subsequent epidemic, and that some diseases seemed to be species-specific, there seemed to be no way to understand or manipulate these phenomena except in the unusual case of smallpox.

Smallpox had long been deliberately transmitted by contact from a sick person to a healthy person with the intent of inducing a (hoped for) mild case of the disease, which would then result in lifelong immunity. This practice was called 'variolation', and was introduced to England and America in the early eighteenth century apparently from the Middle East, although this practice was widespread in India and East Asia before that. In 1878, Edward Jenner (1749–1823) started his studies on the role of cowpox infection in humans as a protective experience against smallpox. He was following an apparently well-known local folk belief, but through his careful work and clear exposition in his report of 1798 he gave wide attention to the effectiveness of this procedure, called 'vaccination' (Latin: vacca, cow), in protection against smallpox.

Pasteur seemed to have been influenced by notions of such cross-immunity resulting from similar but slightly different diseases, and when he was called upon to study chicken cholera in France, he saw an opportunity to apply this notion to another disease. While studying the bacteria associated with chicken cholera, Pasteur transferred the cultures serially and noted that the virulence of the cultures for killing inoculated chickens decreased with the number of laboratory culture transfers. He termed this decrease 'attenuation'. Apparently, he sought to 'challenge' some birds that had recovered from an inoculation of an attenuated culture with a highly virulent form of the bacterium and discovered that the attenuated inoculum had produced strong immunity to the lethal form of the disease. From these observations, Pasteur went on to formulate rather elaborate and complex theories of immunity and attenuation. These theories are no longer accepted, but they stimulated much important research on vaccines, immunity, and the nature of bacterial virulence. Out of this work came Pasteur's famous rabies vaccine as well as his famous work on anthrax vaccine, which resulted in highly publicized trials of his vaccine on a herd of sheep, some cows, and a goat at Pouilly-le-Fort in 1881.

Vaccine development has proceeded from this time partly along the empirical approach used by Pasteur and his colleagues, and partly along the more theory-based approaches developed by Paul Ehrlich (1854–1915), Emil von Behring (1854–1917), and later immunologists. In trying to explain the mechanisms by which vaccines induce immunity, early research focused on the interaction of the bacterium and the blood. Richard Pfeiffer (1858–1945) noted that the serum of immune animals was able to cause lysis of the specific bacterium against which the immunity was directed, and later Jules Bordet (1870–1961) discovered that multiple serum components (complement) are involved in the immune cytolysis (cell lysis) phenomenon. The antibody molecules induced in response to vaccine treatment were studied intensely, and Ehrlich explained the specificity of the antibody-antigen interaction in terms of the structural features of each of these components. The science of immunology took new directions with the landmark studies of Karl Landsteiner (1868–1943) on the specificity of serological reactions.

While germ theories of disease gradually gained adherents in the last two decades of the nineteenth century, and hunts were underway for microbes in every conceivable situation, doubts remained. For example, the discovery of the healthy carrier state in cholera by Koch and his colleagues provided a serious challenge to germ theories. When Max Von Pettenkofer (1818–1901), a major critic of the germ theories, drank a pure culture of cholera vibrios and remained healthy, Koch registered his worry that the germ theory had suffered a serious setback. Vaccination campaigns were not always readily accepted by the public and the validity of vaccination for a variety of diseases was doubted not only by many layperson, but also by many physicians as well. The new science of microbiology did not simply march triumphantly into the clinics and up to the bedsides to take over medicine by force of reason, superior science, and undeniable successes.

Antimicrobial Therapies

Following on the work of Lister on antiseptics, many microbiologists undertook searches for agents, which could be used to kill bacteria *in vivo*. Various attempts to use chloroform, iodine, thymol, and many other disinfectants were reported. Paul Ehrlich reasoned that just as there were dyes as well as antibodies that are specific for certain bacteria, there must be other kinds of chemicals that can bind to, and inactivate, specific microbes. His search for such compounds led to the discovery of several drugs useful in the treatment of protozoal and spirochetal diseases between 1905 and 1915. The arsenical compound, arsphenamine (salvarsan, compound 606), was used to treat syphilis until the advent of penicillin. In a continuation of Ehrlich's approach, another product of

the dye industry, prontosil, was introduced as an antibacterial agent by Gerhard Domagk (1895–1964) in 1935. This compound was metabolized to sulfanilamide, a fact which when recognized, led to the development of many new sulfa drugs.

While investigating lysozyme, a bacteriolytic enzyme present in some body fluids and abundant in egg white, in 1929 Alexander Fleming (1881–1955) noted that cultures of the mold *Penicillium* produced a substance (penicillin) that inhibited the growth of *Staphylococcus* on culture plates. Although Fleming was unable to purify and more fully characterize penicillin, an accomplishment of Howard Flory and Ernst Chain in 1940, his finding suggested to others that saprophytic fungi might be a useful source of antimicrobial agents. Rene´ J. Dubos (1901–82) discovered one such product, tyrothricin, which has the peptide gramacidin as its active component. This success marked the beginning of a search for microbial products that can be used as antimicrobials. The discovery of streptomycin by Selman A. Waksman (1888–1973) and Albert I. Schatz (1920–2005) in 1944 was the beginning of a cornucopia of useful antibiotics. Potent microbial toxins, such as the highly carcinogenic aflatoxin from *Aspergillus flavus*, cholera toxin and botulinus toxin, among many others, have been discovered.

Public Health

The origins of public health microbiology are rooted in the earlier concerns of public health with sanitation and with control of epidemic diseases. The linkage of epidemiology and microbiology was a natural outgrowth of germ theories of disease and the general understanding of the distribution of microbes in the environment. W. T. Sedgewick wrote a key text in 1902, *Principles of Sanitary Science and Public Health*, which described the examination of drinking water for microscopic forms (bacteria, diatoms, algae, and infusoria), the use of coliform counts to assess the effectiveness of water filtration and the processing of sewage involving microbial actions.

Major problems in public health included food microbiology, such as testing milk cows for tuberculosis (1889), epidemics, exemplified in *Sources and Modes of Infection* published in 1910 by Charles V. Chapin (1856–1941), and sanitation testing through the establishment of government microbiological laboratories.

Partly in response to the cholera epidemics in the United States in the nineteenth century, several major cities established permanent, active boards of health by the end of the century. The US Public Health Service established the Laboratory of Hygiene in a Marine Hospital on Staten Island in 1887, to serve as a cholera study unit. This laboratory evolved into the National Institute of Health in 1930. Governmental laboratories, both municipal and state, served as diagnostic centers for physicians and public health officers to carry out microbiological isolations, diagnostic identification, and community surveillance. In New York, for example, the Public Health Laboratory under Herman Biggs, Haven Emerson, and William H. Park provided diagnostic services, bacteriological screening of food handlers and of school children, and later supervised immunization programs. These public health laboratories combined diagnostic microbiology, epidemiology, field work, quarantine, and research to define the current scope of public health microbiology.

Clinical Microbiology

Microbiology in medicine often was in the professional domain of pathologists, because in the period before effective antimicrobial therapies, many infections were studied in the autopsy room after the demise of the patient. Within the hospital, then, microbiology grew up along with pathology, and until the middle of the twentieth century these two disciplines were frequently practiced in the same department, usually called 'pathology and bacteriology' recognizing the seniority and dominance of pathology. Starting in the early twentieth century, however, bacteriology began to claim its independence from pathology. Educational programs in bacteriology were separated from pathology, laboratory practices differentiated bacteriology from anatomic pathology, and professional boundaries began to develop.

The birth of medical microbiology in association with pathology probably made it relatively easy for microbiology to take on service roles in examination of clinical specimens as had pathology. Many of the early microbiologists had been trained as physicians and they could relate well to their clinical colleagues and argue for the importance of increased bacteriological study of patients prior to death. Especially with the discovery of antimicrobial therapies, starting with serums and vaccines, and later with bacteriophage and then sulfas and antibiotics, the role of the bacteriological laboratory in clinical medicine became central to the practice of medicine. With the advent of specific antimicrobial therapy, it became crucial to know the identity and antibiotic sensitivities of the infectious agent isolated from the patient. This demand for accurate and rapid microbiological analysis of clinical samples continues to the present, with great emphasis put on technological innovation and the rapid processing of massive numbers of samples.

Agricultural Microbiology

Microbiology developed from an early stage around both the practices and the products of agriculture. The study of the microbes in the soil, the microbiology of animal health, and the processes of food production all were important from the very beginning of the field.

While soil microbiology has developed into its own disciple, it originated in questions relating to the chemistry and fertility of soil and broad questions about the role of soils in chemical processes involving nitrogen. The sources of ammonia and nitrates in the soil puzzled chemists such as Pierre-Eugéne-Marcelin Berthelot (1827–1907) who soon surmised that it was the microbes in the soil which were responsible for the 'fixation' of nitrogen into ammonia and nitrates. Other nineteenth century microbiologists found that the root nodules in legumes were symbiotic colonies of bacteria with the special capacity to reduce atmospheric nitrogen to ammonia and other forms that were then available to the plants for assimilation. Sergei Winogradsky (1856–1953), the Great Russian microbiologist, focused on soil microbiology and through his work and that of his research school, developed our current understanding of microbial nitrogen fixation, a process essential for life on earth. The Dutch school of general and comparative microbiology, founded by Martinus W. Beijerinck (1851–1931) and his protege, Albert Jan Kluyver (1888–1956), grew from this same interest in soil and environmental microbiology.

Both the diseases and special biology of animals and plants provided important opportunities for crucial advances in microbiology. Pasteur refined his concepts of the role of microbes both in fermentation and in disease by his studies on the 'diseases' of beer and wine, and in his study of diseases of silkworms, beehives, and chicken flocks, not to mention the famous case of anthrax. Such diseases afforded chances to carry out investigations on animals that would not have been possible in analogous human diseases.

Animals also provided novel biological opportunities because of their specialized physiology.

The symbiotic relationship between bacteria and digestion in ruminants was an important advance in the understanding of animal husbandry. The production of silage by microbial action furthered the knowledge of both animal nutrition as well as the science of fermentation.

The role of microbes in the processing and preservation of foods provided another path of investigation in microbiology. Both the use and the avoidance of microbes in food technology has been crucial to improving food yields, reducing spoilage, and avoiding illnesses associated with food contamination. Food preservation by microbial action is ancient, familiar examples being wine, beer, and cheese making. The use of vinegar, a product of bacterial action, and salt to inhibit contamination in pickling is also an ancient practice. Smoking and drying fish and meat are traditional practices to limit bacterial growth.

Virology

One of the basic methods for 'microbe hunting' from the very early period of microbiology was filtration. Very fine filters were designed to remove small particles such as microbes to sterilize fluids and to characterize the particulate nature of infectious materials. Two widely used filters were the Chamberland filter and the Berkfeld filter. The former, named for Charles Chamberland (1851–1908) of the Pasteur Institute, is a tube of unglazed porcelain (a 'candle' filter) through which liquids can be passed under pressure. The latter is a column of diatomaceous earth (Kieselguhr), named for the owner of the mine near Hannover, which produced the material. If an infectious agent was not retained by such a filter, it was termed 'filterable' (sometimes spelled filtrable) or 'filter-passing'. Of course, both particulate and soluble substances could be 'filterable'.

The term 'virus' was initially used to designate the component of an inoculum, which was the causative agent of the disease (Latin: *virus*: poison, venom). The term was generically applied to bacterial agents as well as other organisms. One of the main goals of the early germ theorists was to isolate and identify the virus that was present in blood, tissue extract, sputum, or stool sample, which could transmit disease when inoculated into susceptible hosts. Soon it was found that some of these viruses of disease could pass through the filters, which were known to retain bacteria. These agents of disease came to be known as 'filterable viruses' to distinguish them from all the other viruses. Since the nature of the filterable viruses was obscure in the early part of the twentieth century, the term 'filterable virus' persisted in the literature until the 1930s; for example, the classic 1928 text, edited by Thomas M. Rivers (1888–1962), was entitled *Filterable Viruses*. Shortly thereafter, common usage came to drop the qualifier 'filterable' in favor of simply 'virus' to designate the filter-passing forms of infectious agents.

The first disease that was recognized as being caused by a filter-passing agent was tobacco mosaic disease. This disease was economically devastating to Dutch tobacco growers and its cause was actively studied in Holland starting with the work of Aldof Mayer (1843–1942) in 1879 who was able to transmit the infection but failed to find the causative virus, believed to be a bacterium. Martinus W. Beijerinck worked on tobacco mosaic disease off and on for over a decade and by 1898 he found that the virus was 'filterable', that it would diffuse through agar, and that it was serially transmissible. For the virus of tobacco mosaic disease, Beijerinck proposed a new category of agent, living and nonparticulate, a *contagium vivum fluidum*. A few years earlier, Dimitri Ivanovski (1864–1920) had shown the filterabilty of the agent of tobacco mosaic, but because he was able to transfer the disease via bacterial colonies (probably contaminated with residual filterable virus), Ivanovski believed that tobacco mosaic was a bacterial disease.

The nature of filterable viruses as represented by the tobacco mosaic virus (TMV) was controversial. Some thought of the agent as chemical, perhaps a cellular component, while others thought of it as an 'ultramicrobe', an organized living being, too small to be seen in the microscope.

Subsequent attempts to study TMV were hampered by the inability to grow the agent outside of the infected plant, and by difficulties in its detection and quantification. Some of these difficulties were overcome by the preparation of large amounts of infected plant material by Carl G. Vinson (1927) and an improved quantitative assay by Francis O. Holmes (1928). In 1935 Wendell Stanley (1904–71) reported that he had crystallized TMV, a startling observation that forced reconsideration of the concept

of 'living', 'infectious', and 'microbe'. Stanley and his coworkers, based on their chemical analyses at the time, believed that TMV was composed only of protein. Since crystallization was the standard criterion for purity of organic compounds and by extension, of proteins, this work was interpreted to show that filterable viruses such as TMV were self-replicating, infectious proteins. Indirectly, of course, this conclusion strengthened the belief that genes, too, were protein molecules. The RNA component of TMV (about 7%, by weight) was soon found by Frederick Bawden (1908–72) and N. W. Pirie (1907–97) in England. Still, the controversy over viruses remained. How did they replicate? Where did they originate? Were they autonomous or part of the cell? Should they be conceived as macromolecules or as microbes?

From the beginning of the twentieth century, filterable viruses were found as the causes of many contagious diseases that had resisted bacteriological etiologies: Herpes labialis (fever blisters), influenza, and poliomyelitis, to name a few human diseases; hog cholera, rabies, and cowpox among the mammalian diseases; and leukemia and sarcomas in birds. It was eventually realized that one of the defining, although 'negative', characteristics of all these viruses is that they are obligate intracellular agents, and they cannot be grown independently of their host or at least host cells. This realization finally led to searches for better ways to grow and assay viruses apart from animal or plant inoculations. The tissues of the embryo in the chicken egg became a convenient and standardized growth and assay medium for many viruses and are still in use today for some purposes. Egg cultures of many viruses were studied by Ernest Goodpasture (1886–1960), who perfected this method in the 1930s. By the 1950s the ability to grow explanted mammalian cells in various culture media ('tissue culture' or 'cell culture') provided a new and improved way to grow, assay and study many viruses. In particular, the growth of poliovirus in monkey kidney cells in culture in 1949 by John Enders and colleagues led to better understanding of this virus and its ultimate control through preventative immunizations first with a killed preparation of poliovirus (the Salk vaccine) and later a live, attenuated orally effective vaccine (Sabin).

A special class of filterable agents was discovered in the first decade of the twentieth century, which deserve special mention: the cancer-causing viruses. This group of viruses, not related by structure, pathogenesis, or genealogy, has provided important insights into the interplay between genes, viruses, and cells. In 1911, Peyton Rous (1879– 1970) observed that a cell-free filtrate prepared from a chicken sarcoma could induce similar sarcomas when inoculated into other chickens. Likewise, in 1908 Ellerman and Bang suggested that leukemia in fowl could be caused by inoculation with a filterable agent. The notion that cancer might be an infectious disease was so potentially frightening to the public that Rous tried to avoid any unnecessary publicity of his work, and eventually abandoned it. In searches for the causes of cancer, there were many reports of bacteria associated with various malignancies, but only the filterable viruses seemed to emerge as possible causal agents for animal cancers. (One possible exception is the mucosa-associated lymphoid tissue (MALT) lymphoma, associated with *Helicobacter pylori* infection of the stomach. In plants, the causative role of *Agrobacterium tumefaciens* is causing tumor-like proliferation in well established.) By the mid-1950s several classes of viruses had been identified, which could induce cancers in experimental animals. However, it was not until much later that candidate human tumor viruses were isolated. Most of these tumor viruses have RNA genomes, which can be copied into DNA and integrated into the cell genome to reside there in symbiosis while causing a neoplastic transformation in many cases. Interestingly, the organism associated with AIDS, human immunodeficiency virus (HIV), first identified in the 1980s, turned out to be in this same retrovirus group of viruses, although HIV does not cause tumors.

Bacteriophage are now recognized as viruses that infect bacteria; however, originally they were not believed to be similar to the filterable viruses. In 1915 F. W. Twort (1877–1950) in England reported 'glassy transformation' of micrococci, which contaminated his attempts to grow vaccinia virus on cell-free culture media. This phenomenon of glassy transformation was serially transmissible and killed the bacteria. Twort's interpretation of this phenomenon was unclear and ambiguous. In 1917, Félix d'Herelle (1873–1949), a French Canadian working in Paris, independently observed lysis of dysentery cultures, and noted that the lytic principle was filterable, and that something in the lysed culture could produce clear spots (plaques) on confluent bacterial cultures. This lytic principle was serially transmissible and by d'Herelle's interpretation, particulate. He conceived of this agent as a microbe, which parasitizes the bacteria, that is a virus of bacteria. Not only did he devise the quantitative plaque counting method, but he also worked out the basic life cycle of this agent, which d'Herelle termed bacteriophage (although long known by the noncommittal term 'Twort-d'Herelle Phenomenon'). The biological nature of bacteriophage was hotly debated for about 20 years, with the majority view in opposition to d'Herelle's ultravirus hypothesis and in favor of some sort of endogenous autocatalytic process. Only when uniform virus particles were observed by electron microscopy in 1940 did the particulate view of bacteriophage become widely accepted.

D'Herelle and Twort engaged in a 10-year polemic over the issue of priority of discovery of bacteriophage which tarnished the reputation of both scientists. Twort did not pursue phage research, and d'Herelle focused mostly on the application of phage as therapy and prophylaxis for infectious diseases in the era before antibiotics. While this application seemed to offer promise and is now being reexamined, it was eclipsed by the marvels of the new antibiotics in the early 1940s.

The study of phage from the biological point of view has been central to the development of molecular biology. Viruses in general, and phage in particular, were recognized as very useful 'probes' for cellular processes which they exploit during their life cycles. The problem of gene duplication, especially, seemed amenable to study with phage. Both in the United States and in France, phage research since the 1940s was directed at understanding what happens during the half hour or so that it takes for one infecting phage to produce a hundred progeny in an infected bacterium. This was the basic research program that Max Delbrück (1906–81) set for the American Phage Group. The work of this loosely defined school of research has been instrumental in deciphering much about the nature of the gene, mutagenesis, recombination, and gene expression and regulation.

In virology the electron microscope has had a major impact. Prior to about 1940, viruses were defined by their small size, by filtration and diffusion properties, and their invisibility in the light microscope. The electron microscope was invented in Germany

in 1931 by Max Knoll and Ernst Ruska, and by the late 1930s had sufficient resolving power to demonstrate the particulate nature of several viruses, including bacteriophage. The RCA company designed and constructed the first electron microscopes in the United States, and in 1942 Thomas Anderson (1911–91) and Salvador Luria (1912–91) used such an instrument to examine phage in detail. The ability to visualize viruses at last (albeit indirectly) brought some long-awaited unity to virology, and bacteriophage were finally accepted as viruses of bacteria.

Microbial Physiology

With the recognition of the role of microbes in ancient processes such as fermentations and other types of food processing (cheese, bread, soy sauce, and silage), a better understanding of these uses of microbes and their by-products was possible. Antibiotics represent just one aspect of this exploitation of microbial metabolism. Several groups of researchers, including Sergei Winogradsky and Martinus Beijerinck, emphasized the diversity of microbial forms and metabolism and pioneered the fundamental study of the physiology of bacteria. These studies led directly to the use of microbes to produce useful 'secondary metabolites', compounds such as glycerol, lactic acid, pigments, and other intermediates that are products of the metabolism in specific organisms. Soil and dairy microbiology developed in colleges of agriculture and in the agriculture experiment stations established in the United States, in the late nineteenth century. This work promoted the understanding of microbial processes used in cheesemaking, such as the use of specific molds to achieve the best products, in spoilage of foods, and in the use of manure, legumes, and composts to fertilize the soil. By the 1930s this work on bacterial metabolism was central to the new science of biochemistry, representing, for example, a major focus in Gowland Hopkins' department in Cambridge under the leadership of Marjory Stephenson (1885–1948).

Interest in animal and plant nutrition, especially work on growth factors and vitamins, was extended to microbes in the 1920s and 1930s. In Paris, André Lwoff (1902–94), for example, investigated the growth requirements of protozoa, while in England, Stephenson, B. C. J. G. Knight (1904–81) and Paul Fildes (1882–1971) studied bacterial growth requirements, and in America, Edward Tatum (1909–75) turned to fungal biochemistry. These kinds of investigations led to the concepts of essential nutrients and specific metabolic reactions in microbes.

Microbial Genetics

When George Beadle (1903–89) and Tatum were investigating the genes for eye color in the fruit fly (*Drosophila melanogaster*), they saw an opportunity to examine the biochemistry of gene action in a more tractable system, the biosynthesis of vitamins in fungi. In 1941 they produced pyridoxine-requiring mutants of *Neurospora crassa* by X-ray mutagenesis, and showed that these mutants behaved in a Mendelian fashion in mating experiments. This result suggested to Beadle and Tatum that genes were more or less directly involved in the control of metabolic steps, that is of enzymes. While the exact chemical nature of the gene was unclear at the time (many scientists thought that the genes were the enzymes themselves), they formulated a theory of the gene that came to be known as the 'one gene:one enzyme hypothesis', a basic principle of modern molecular genetics (notwithstanding the additional complexities introduced later by discovery of messenger RNA, splicing, operons, and oligomeric enzymes). This approach, employing both genetic and biochemical studies, was remarkably fruitful and in a few years the notion that genes controlled all the biochemical pathways in cells was widely (although not universally) accepted.

While some of the literature on cyclogeny (a theory based on bacterial growth cycles) suggested that mating, with the formation of zygotic pairs, might occur in bacteria, and some data were presented to support this concept, none of these studies led to new understanding and further progress in the genetics of bacteria. However, after the successful discovery of nutritional mutants in *Neurospora*, C. H. Gray and Tatum in 1944 were able to produce nutritional mutants in bacteria, and in 1947, Joshua Lederberg (1925–) and Tatum carried out experiments with two nutritionally defective mutants of the K-12 strain of *E. coli* (a clinical isolate that had been used in the student laboratory at Stanford University where Tatum had recently taught), and they found that they could obtain recombinant forms of *E. coli* K-12 in a clear demonstration of sexual mating in bacteria. By means of bacterial recombination, a genetic map of *E. coli* K-12 was constructed and by 1952 William Hayes (1913–94) clarified this process of conjugation by his explanation of unidirectional transfer of genetic material. Lederberg and Cavalli formulated bacterial mating as a rudimentary form of sexuality with the donor cell called F+ (fertility plus, 'male'), the recipient called F – (fertility minus, 'female'), and the recipient cell with the donated genes called the zygote. The transfer of the genetic material from donor cell to recipient cell could be interrupted by mechanical agitation of the culture, and François Jacob (1920–) and Élie Wollman (1917–) were able to investigate the nature and sequence of the gene transfer by this 'interrupted mating' ('coitus interruptus') experiment. Since some of the genes they studied did not seem to be associated with the bulk of the cell genes, they hypothesized that some genes exist on extrachromosomal elements, not always present in all cells, which they termed 'episomes' (1958).

While the understanding of microbial genetic processes was increasing during the 1940s and 1950s, the understanding of the nature of the gene itself and of the processes involved in gene stability and change, that is mutation, were also maturing, but from a somewhat different tradition. By the mid-1920s, two strands of investigation converged to direct attention to the problem of heredity in microbes. First, many studies on the virulence of bacterial isolates suggested, even from the time of Pasteur's work on attenuation of virulence, that supposedly pure strains of bacteria could give rise to variants with altered virulence. Second, many bacteria with recognizable colony morphologies, for example, smooth or rough colonies, pigmented or nonpigmented, were noted

to throw off variants of the other type now and then. This phenomenon in which a 'pure' culture 'dissociated' into a mixture of two types was called 'bacterial dissociation' and was widely studied.

Interestingly, explanations of dissociation first centered on the ideas that cultures had 'life cycles' and that the different forms of bacteria represented stages in the life cycle of the culture. Contemporary work on sporulation, protozoal development, 'phase variation' in some bacteria such as *Salmonella*, and fungal growth variations all supported this concept of bacterial 'cyclogeny' as it was termed. The theory of cyclogeny led to a resurgence of the pleomorphism concept of the nineteenth century and was a rather widely held belief in the period between World War I and World War II. The more recent success in the alternative explanation, genetic variation, has all but obliterated memory of the heyday of cyclogeny.

While some bacteriologists, such as Paul Henry DeKruif (1890–1971), better known as a science writer and the author of *Microbe Hunters*, advocated genetic mutation of individual cells as the explanation for bacterial dissociation, the continued focus on the entire culture rather than the individual cell obscured the genetic basis for this phenomenon. Indeed, the genetic status of bacteria and some other microorganisms was unclear. The science of genetics arose from breeding experiments, both in plants and in animals, and its focus was on reassortment and sexual transmission of characters from parent to offspring. Without easily visible chromosomes and without a sexual phase of reproduction, bacteria did not fit into the existing genetic paradigms. As late as 1942, the eminent British biologist Julian Huxley (1887–1975) wrote about bacterial heredity: "One guess may be hazarded: that the specificity of their composition is maintained by a purely chemical equilibrium, without any of the mechanical control supposed by the mitotic (and meiotic) arrangements of higher forms." This view was no doubt strongly influenced by Huxley's contact with Cyril Hinshelwood (1897–1967) and his school which saw bacterial physiology and genetics in terms of chemical kinetics, and which minimized the centrality of the gene in the life of the bacterium.

Perhaps because of the well-known phenomena of adaptation and 'training' of bacteria to different growth conditions, much of the discussion of mutation in bacteria during the 1930s and early 1940s has a strong neo-Lamarckian quality: the exposure of the organism to the agent somehow provoked the observed changes. Exposure of bacteria to bacteriophage led to the emergence of cultures resistant to the phage and often displaying new properties such as altered virulence, changed colony morphology, and different antigenic types. The 'adaptation' of cultures upon exposure to antiseptics, drugs, extremes of pH, and so on, were often seen as purposeful and directed responses.

A minority view in the 1930s was, however, that the dissociation phenomenon was happening independently of the exposure to the agent used to detect the change, that is the phage, drug, or chemical. The major problem in this work was one of experimental design: how to observe a rare event that happened in a huge population prior to the selection for the outcome of that event? In a particularly clear and convincing work, Isaac M. Lewis (1873–1943) examined the mutational change from the inability to ferment lactose to the ability to use this sugar source in the *E. coli* strain *mutabile* (in 1907 Rudolf Massini (1880–1955) reported on a variant of *Bacterium coli* (*E. coli*), which had lost its ability to utilize lactose but which frequently regained this ability and called it *B. coli mutabile*). Lewis spread the parental (lactose-negative) bacteria on glucose-containing plates and also on lactose-containing plates. About one in a million of the bacteria grew on the lactose-containing plates and when retested, all the cells in these lactose-utilizing colonies bred true and could use lactose. By laboriously picking colonies from the glucose plates and testing them for their ability to utilize lactose, he estimated the frequency of lactoseusing bacteria in the culture in the absence of exposure to lactose. This frequency was similar to that determined by plating of the mass culture on lactose medium, so he concluded that the mutations to lactose utilization occurred prior to the selection, not as a consequence of exposure to the selective conditions. This elegant and clear approach, however, did not change many minds, and it was not until the 1940s that two related experimental approaches gave results that comprise the canonical account of bacterial mutation.

In their work on the reproduction of bacteriophage, Max Delbrück and Salvador Luria were aware that bacteria often developed resistance to phage. If a bacterial culture was infected with phage and lysed, sometime later a 'secondary' growth of bacteria would often appear, and these bacteria were resistant to infection with the original phage. While the occurrence of resistant bacteria was a drawback to the use of phage as an antibacterial therapeutic agent, it afforded Luria and Delbrück (1943) an opportunity to study the change in another hereditary property of bacteria. They were also aware of the problem in the interpretation of bacterial mutation experiments. No doubt because of their routine use of statistical models in their work on the inactivation of bacteria and phage by radiation, as well as their backgrounds in atomic physics, they devised a statistical approach to show that phage-resistant mutants exist in the bacterial population prior to exposure to the lethal effects of phage. Their method ('fluctuation test') was based on the properties of the Poisson distribution and the arrangement of the experiment to analyze the frequency of occurrence of mutants in numerous replicate cultures of bacteria. If the occurrence of the mutants took place prior to the analysis (i.e., prior to the application of selective conditions), the Poisson distribution predicted that the frequencies of mutants would fluctuate widely among the replicate cultures. Conversely, if all the mutations occurred during the analysis (i.e., induced by the selective conditions), the frequencies of mutants would be very similar among the replicate cultures. The frequencies fluctuate as predicted by the Poisson distribution; however, Luria and Delbrück showed that this fluctuation could be used to estimate the mutation rate, that is, probability of a given type of mutation per cell division.

The method of Luria and Delbrück and a related procedure devised in 1949 by Howard B. Newcome were indirect, mathematical, and subject to suspicion. However, in 1952 Joshua Lederberg and Esther Lederberg (1922–2006) devised a simple and direct method to demonstrate that mutations were occurring in a random way, independent of the selection procedures. They reasoned that the approach of Lewis was logically correct, but that the methods for its execution had to be improved. They devised a method for transferring very large numbers of colonies from one plate to another by the use of velvet cloth as a transfer tool. The tiny fibers that stand out from the velvet acted as small inoculating needles and picked up some bacteria when pressed against the surface of a

culture plate studded with colonies. When the velvet was pressed on a fresh, sterile plate, this plate was inoculated with bacteria in exactly the same pattern as the original plate. Thus, this 'replica plate' could be used to test colonies in great numbers for mutant properties. Lederberg and Lederberg (1952) applied this technique to the study of phage resistance as well as to the streptomycin resistance, and in both cases it was clear that the mutants had appeared before the application of the selective agent.

Although by mid-century it was generally recognized that bacteria have some sort of genetic apparatus, since they do not have cytologically visible nuclei and chromosomes, the exact organization of the genes was unclear. Perhaps influenced by the widespread interest in cytoplasmic inheritance in higher organisms, there was much discussion about the possibility that bacterial heredity was carried on plasmagenes or that bacteriophage were 'raw genes' from bacteria. Joshua Lederberg (1952) introduced the term 'plasmid' to explain and encompass the wide variety of genetic determinants, which had been observed or hypothesized to exist in microbes. Thus, examples of cytoplasmic inheritance in protozoa, lysogeny in phage, and sometime later, bacterial fertility factors, came under this rubric. This concept, in a variant form described by Jacob and Wollman (1959) under the name of 'episome' nicely explained the genetic studies with fertility factors, specialized transfer of one or a few markers (e.g., the lactose-utilization genes) in some mating experiments, and later on, the 'infectious' nature of antibiotic resistance (resistance transfer factors, RTF) and the variation in virulence and toxin production in many pathogens. Today, the term plasmid has come to signify a circular, extrachromosomal DNA molecule, which autonomously replicates in the cell, and is not essential for that species (although culture conditions, such as the presence of certain nutrients or antibiotics, can make the plasmid essential under specific conditions).

The plasmid concept has facilitated the understanding, too, of the 'gene flow' between the chromosome proper and exogenous agents such as viruses. The ability of some plasmids to undergo genetic recombination with the chromosome explained the mechanisms by which genes can move between species in nature and also established some of the key steps in the process of lysogeny of bacteria by certain bacteriophage. As such, the plasmid concept has been important in understanding the genetics of cancer, as well as certain schemes for evolution of microbes. Of course, the role of plasmids as vectors for gene transfer in current biotechnology is too well known to need elaboration here.

Bacterial Transformation and DNA

With the beginning of the understanding of the process of bacterial mutation as well as the discovery of ways of manipulation of bacterial genes by conjugation, the field of microbial genetics entered the modern period. Two disparate strands of research led to the current understanding of the nature of genes in bacteria. One strand comes from work on bacterial virulence and pathogenesis in pneumonia, and led to the identification of DNA and the chemical stuff of which genes are composed. The other strand comes from the study of bacteriophage replication.

In 1928, Frederick Griffith (1877–1941) was investigating the virulence of the organism that is responsible for pneumonia (*Streptococcus pneumoniae* or 'pneumococcus'), and he found that heat-killed bacteria of one form of pneumococcus could somehow convert live bacteria of another form to exhibit some of the antigenic and virulence properties of the heat-killed form. While these results were of no apparent interest or relevance to the few bacteriologists interested in microbial heredity, they were of real importance to the pathologists interested in pneumonia. Oswald T. Avery (1877–1955), working at the Rockefeller Institute, was one of several scientists who confirmed and followed up Griffith's work with a research program on what he called 'transformation' of antigenic types. Work in his laboratory, first by Martin Dawson followed by James L. Alloway, and then by Colin MacLeod (1909–72) and Maclyn McCarty (1911–2005), eventually led to characterization of the transforming material from the heat-killed bacteria as DNA itself. The final characterization, published in 1944, relied on the use of the newly purified and characterized enzymes, DNase and RNase, as well as several well-known proteolytic digestive enzymes. Still, appreciation of DNA was not universal: at the mid-century meeting of the Genetics Society of America, DNA was hardly mentioned.

Because proteins exhibited the diversity expected of genes and the chemistry of DNA as it was then understood suggested its 'information content' was too low to have genetic potential, DNA was not taken seriously as the genetic material. Two major works soon challenged that belief: using new analytical methods resulting from wartime research, Rollin D. Hotchkiss (1911–2004) showed that the base compositions of DNAs from different organism were not identical and Erwin Chargaff (1905–2002) noted certain regularities in the analyses of all DNAs: the number of purines always equal the number of pyrimidines, and the ratios of adenine to thymine and guanine to cytosine were always very near to one.

While the chemistry of the 'transforming principle' in pneumococcus was being established, a second line of work was going on to understand the process of gene duplication. Max Delbrück saw bacterial viruses (bacteriophage) as a simple model for the process of gene replication and he recruited a group of like-minded associates to attack this problem. In the early stages of their work, the American Phage Group (as Gunther Stent has named Delbrück's school) treated the host bacterium more or less as a 'black box' and studied phage replication as a simple input–output process. In a widely cited experiment, in 1952 Alfred Hershey (1908–97) and Martha Chase (1927–2003) labeled bacteriophage with two isotopes, ^{32}P in the DNA, and ^{35}S in the protein, and in an attempt to follow the fate of the two major components of the phage through one life cycle, they noted that most of the ^{32}P label entered the cell and a significant fraction ended up in progeny phage, while very little of the ^{35}S label entered the cell and even less ended up in the progeny phage. While this result is often described in texts and reviews as if the results were 'all or none', the experimental results given in the original paper, while certainly supportive of the 'only DNA' hypothesis, are far from conclusive.

Another member of the American Phage Group was James D. Watson (1928–), a student of Luria in Indiana University. Watson's thesis was on the radiobiology of bacteriophage and he firmly believed that DNA was the chemical substance of the gene. Watson

and Francis Crick (1916–2004), working at the Cavendish Laboratory in Cambridge England, devised a plausible model for the three-dimensional structure of DNA, which finally provided the much-needed explanatory framework for the genetic role of DNA (1952). Accounts of this landmark research abound, and do not need repetition here.

Physiological Genetics

While the understanding of the role of the gene in the hereditary transmission of properties from parent to progeny organisms and in the explanation of the mutational process derived from research in diverse areas of microbiology as pneumonia research, microbial growth factors, and bacteriophage reproduction, the role of the gene in the growth, development, and physiology of microorganisms was studied in different contexts.

Although as early as Pasteur microbiologists investigated microbial physiology, this field developed rapidly in the 1930s. In parallel with the general advances in biochemical understanding, microbial physiology was important in the discovery of growth factors, in understanding the complexities of nutrition in animals with symbiotic flora (e.g., ruminants), and in appreciating the role of bacteria in conversion of atmospheric nitrogen to organic forms of nitrogen in the process of 'nitrogen fixation' that occurs in the bacteria-packed root nodules of leguminous plants.

A particularly interesting problem in bacterial physiology, one that represented a general phenomenon in microbes, was that of 'enzymatic adaptation'. It was widely observed that cultures growing on one substrate (e.g., glucose) and then shifted to another substrate (e.g., lactose) exhibited a short lag in growth and then 'adapted' to the new substrate by the appearance of an enzyme (or several enzymes), which function to metabolize the new substrate (e.g., lactose-splitting enzymes). The mechanism by which the adaptation occurred was subject of intense discussion and debate up to the mid-1950s. By that time, the work of Jacques Monod and colleagues at the Pasteur Institute had focused on the specific case of the response of *E. coli* to the shifts between glucose and lactose as the sole carbon source for growth. Their early approach was classically physiological, that is, they studied the rates of growth, the kinetics of the adaptations, the levels of the relevant enzymes, and the concentration of substrates involved. A key question arose: when the enzyme appeared in the adaptation (termed 'enzyme induction'), was it made *de novo*, perhaps upon the 'instruction' of the inducer (lactose), or did the enzyme exist in a preformed state, which was somehow stabilized or activated by the inducer? These basically physiological questions were brilliantly reformulated by the group at the Pasteur Institute into genetic terms by the reconceptualization of the process as involving an intermediate system for synthesis of the enzyme, which was distinct from the enzyme itself, and by exploiting mutations affecting the induction phenomenon to identify the components in this hypothetical enzyme-forming system. This work resulted in the operon concept of gene function as first described in 1960 by Jacob, Perrin, Sanchez, and Monod and fully elaborated by Jacob and Monod in 1961. This synthesis of physiology and genetics provided a broad explanation of the biology of a bacterium, *E. coli*, yet it unified as well much of the rest of biological thought. It ended the long estrangement between transmission genetics (Morgan and the 'nuclear monopoly') and the Continental embryologists, who viewed genes primarily as the agents directing growth and development, and rejuvenated interest in what is now known as cell biology.

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History of Virology

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The history of virology can be traced as the personalities involved have described their concepts and published their experimental results. Although infections we now know as, e.g., rabies, yellow fever, smallpox, etc. were clinically evident in early human history, the initial isolation of individual viruses and their assignment to specific diseases did not occur until about 1898, 120 years ago, a proverbial drop in the bucket of time. Just one lifetime ago, Peter Medawar, awarded the Nobel Prize in Medicine and Physiology in 1960, defined viruses as a piece of nucleic acid surrounded by bad news.

The early pioneers in virology had a very limited armamentarium on hand to classify or identify viruses as microbial agents. At the time of their initial discoveries, microscopes of sufficient power to see viral agents were not yet invented; cell cultures to grow viruses were unknown, and probes like antibodies or nucleic acids to mark an infectious agent were not available. Further, biophysical apparatuses like centrifuges or acrylamide gels to concentrate or separate viral proteins or nucleic acids did not exist. Eventually, the will for discovery caused such techniques to emerge. Their use not only defined modern virology and its classifications, but as virology developed it also provided insights that created contemporary cell and molecular biology, protein and structure/function study, immunology, epidemiology for infectious disease analysis, and emergence of treatment. A triumph of modern pharmacology is the formulation of vaccines and, recently, antiviral drugs. Vaccines changed the landscape of virology. Devastating infectious diseases like smallpox, which once killed or maimed millions of humans, have been eliminated, whereas others like poliomyelitis, measles, rubella (German measles), yellow fever, mumps, etc. can be well controlled and others, like influenza, managed. Antiviral therapeutics against HIV now markedly lessen the almost universal mortality rate formerly associated with AIDS. Further, viruses cause at least 20% of all cancers, and vaccines now routinely control liver cancers caused by hepatitis B virus as well as uterine, cervical, and penile cancers caused by several papilloma virus strains. Vaccines have provided the greatest punch-per-buck of any therapeutic regimen in medicine.

Now it is possible, in hindsight, to judge how correct or flawed were the initial concepts formed by early and even recent virologists about the diseases and causes they studied. For this article on the history of virology, selected principles and concepts are reviewed, together with the influences on and reasons for their evolution. Detailed descriptions of viruses that did or do cause plagues and their histories can be gleaned from the publications of McNeill (1976), Oldstone (2010), and Waterson and Wilkinson (1978).

The identification of viruses as infectious agents followed on the heels of pioneer work in the mid-1800s by Louis Pasteur and associates. During that period, the laboratory culturing process was developed for bacteria, which could be grown on enriched agar preparations or broths and were identifiable when the bacteria were fixed on glass slides, stained, and examined under the microscope. Bacteria were isolated by their retention on filters with specific pore sizes (Figure 1(a)). After their collection on filters, growth on agar and identification, specific bacteria could be linked with individual diseases by using Koch's (Henle–Koch) postulates (**Figure 1(a)**). It was with this framework that the first viruses were uncovered (Figure 1(b)). In 1898, Dmitri Losifovich Ivanovski (Ivanovski, 1899), in Russia, and Martinus Beijerinck (Beijerinck, 1899), in the Netherlands, demonstrated that the material responsible for a disease of tobacco plants, instead of being retained, passed through the then smallest pore-sized filter used in the Pasteur–Chamberland apparatus without losing infectivity. Thus, the material at the bottom of the filter-containing flask was smaller than bacteria and proved to remain infective when transferred to an uninfected recipient (plant) of the type from which it was first retrieved (Figure 1(b)). Ivanovsky and Beijerinck identified the first virus, a plant virus they named the tobacco mosaic virus. Almost concurrently, Friedrich Loeffler and Paul Frosch (Loeffler and Frosch, 1898), in Germany, utilizing a similar approach, concluded that the agent causing foot-and-mouth disease of cattle also passed through porcelain filters and induced symptoms of disease when inoculated back into healthy cattle (Figure 1(b)). These observations, highly controversial at the time, provided the basis for defining viruses as subcellular entities that caused distinct forms of tissue destruction, which became hallmarks of specific diseases. The uniqueness of this detective work is even more dramatic when one considers that the infectious virus particles were too small to see and could not grow on the culture media available (Table 1). Visualization of viruses awaited the use of electron microscopy in the mid-1930s, and the culturing of living cells necessary for viral replication was not possible until the late 1940s to early 1950s. Figure 1(a) illustrates the beginning of microbiology, and Figure 1(b) illustrates the first viral isolations and lists the conceptual problems such virologists had while proving Koch's postulates. Conceptually, a viral replicating agent was distinguishable from a toxin when dilution of the supernatant material that passed through the Pasteur– Chamberland filter lost potency on injection into the appropriate host (then it was a toxin). In contrast, if the diluted material retained potency after injection, it was a replicating agent. Yet, not all material obtained from an infected source and passed through a Pasteur–Chamberland-like filter followed by transmission of infectious supernatant material proved to be a virus (Figure 1(c)). In this illustration, filter passage-infected material from scrapie-infected sheep caused a similar disease when inoculated back into healthy sheep, the natural host. However, not until the 1980–1990 did research show that this infectious material did not contain nucleic acids but rather that disease was transmitted by the misfolded abnormal prion protein (termed PrPres or PrPsc) from infected sheep (Table 1).

Many viral infections cause acute illnesses. That is, the causative virus enters the body, multiplies in one or more tissues, and spreads locally through the blood, along nerves, or along the respiratory or gut tracts. The incubation period of 2 days to 2 or 3

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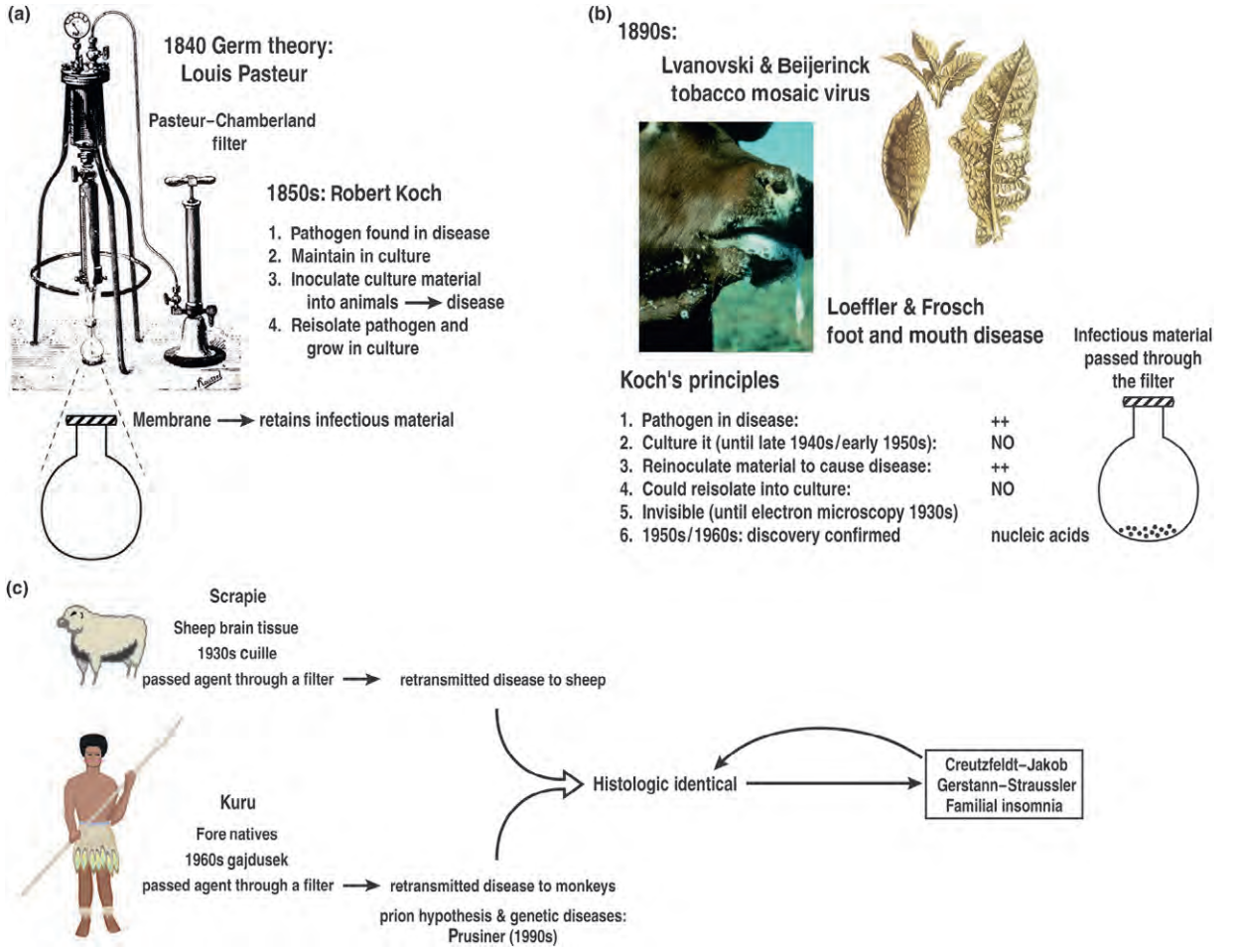

Figure 1

weeks is followed by signs and symptoms of disease and local or widespread tissue damage. Viruses can be isolated from the patient's blood (serum or blood cells), secretions or washes for a short time just before and after the appearance of symptoms or from their infected tissues. Afterward, the infected host either recovers from the infection, and is often blessed with lifelong immunity to that virus, or dies during the acute phase of illness. Although the majority of life-threatening and debilitating acute virus infections are now controlled through vaccination, it is worthwhile considering the consequences of acute infections like smallpox, measles, and yellow fever in the prevaccine era. Smallpox, before its eradication in 1980, killed over 300 000 000 persons just in the twentieth century and caused a mortality rate of approximately 33% (reviewed in McNeill, 1976, and Oldstone, 2010). In the sixteenth century, when Native Americans were inadvertently exposed to infectious viruses, often carried by Europeans colonizing the New World, the result was a reduction of the native population in Mexico and Latin America from an estimated 20 million to 2 million. This devastating depopulation came primarily from smallpox and measles infections, since these viruses had never before existed in the New World (Oldstone, 2010). Smallpox has now been eliminated from our planet and measles virus controlled, at least in most developed countries. However, measles virus infection was and remains a serious disease today with approximately one per thousand infected persons developing severe destruction of the central nervous system requiring institutionalization. Yellow fever spread up the Mississippi River, carried by infected passengers incubating the disease as they traveled on riverboat(s), embarking from the Caribbean to New Orleans on 24 July 1878, moving on to Vicksburg a few days later and arriving in Memphis, where the first case occurred by 14 August (reviewed in Oldstone, 2010). At that time, no one knew that the infection was transmitted by the bite of Anopheles *Aedes aegypti* mosquitoes. The mosquito sucks blood from an infected person, then transmits the virus by biting an uninfected susceptible person. At the time of the yellow fever outbreak in Memphis, the city population was approximately 98 000 persons. By the end of the two-month epidemic, 20 000 people had fled Memphis. Of those who stayed, 77% were infected and 70% died. The economic result was the destruction of Memphis as a commercial capital in the American South, to be replaced by Atlanta. As reported in the *New York Herald* newspaper by writer Robert Blakeslee, on assignment from New York City to Memphis to cover the yellow fever epidemic, "The city was almost deserted . . . We had not gone far, however, before the evidence of the terrible condition of things became apparent. The first thing in the shape of a vehicle that I saw was a truck (wagon), loaded with coffins, going around to collect the dead. As this was within four blocks of the depot you may imagine how

Table 1 Selected milestones for and in virology

Date(s)	*Virologists/investigators*	*Discovery*
400 BCE	Hippocrates	Greek physician, father of medicine, epidemiologic observations of many viral diseases
1796	E. Jenner	Application of cowpox virus for vaccination against smallpox, inflammation origins of virology and immunology
1846	P. Panum	Immunologic memory (protection) against reinfection (measles). Conceptual basis for vaccine strategy
1857	L. Pasteur, R. Koch	Founding of microbiology
1858	C. Darwin, A. Wallace	Concepts of evolutionary progression, natural selection
1865	G. Mendel	Founding of genetics
1883	E. Metchnikoff, P. Ehrlich	Founding of immunology
1885	L. Pasteur, E. Roux	Development of rabies vaccine
1892–98	D. Ivanovsky, M. Beijerinck	First demonstrations of a filterable plant virus: tobacco mosaic virus
1898	F. Loeffler, P. Frosch	First demonstration of a filterable animal virus: foot-and-mouth disease virus
1900	J. Carroll, J. Lazear, A. Agramonte, W. Reed, C. Finlay, W. Gorgas	First human virus: yellow fever virus, first use of consent form for human clinical investigation, identify mosquito as a transmitting agent, control of virus by elimination of mosquito breeding sites
1904–08	V. Ellermann, O. Bang, H. Vallee, H. Carre	First demonstration of a leukemia-causing virus, retrovirus
1908	C. von Pirquet	First report of virus causing immunosuppression: measles virus
1909	K. Landsteiner, E. Popper	Isolation of poliomyelitis virus
1911	J. Goldberger	Discovery of measles virus
1911	P. Rous	First demonstration of a solid tumor virus: Rous sarcoma virus
1915	F. Twort	Discovery of bacteriophages
1917	F. d'Herelle	Bacteriophages, plaque assay
1919	A. Löwenstein	Discovery of herpes simplex virus
1923–28	A. Carrel, H. Maitland, M. Maitland	Tissue culture of embryo explants and first tissue culture cultivation of virus: Rous virus, vaccinia virus
1928	R. Lancefield, E. Lennette, others	Beginning of viral disease diagnosis
1931	J. Furth	Use of mice as a host for viruses
1931	A. Woodruff, E. Goodpasture	Use of embryonated hen's eggs as a host for viruses
1933	W. Smith, C. Andrewes, P. Laidlaw	Isolation of human influenza virus
1933	R. Shope	Rabbit papillomavirus: first DNA tumor virus
1933	A. Tiselius	Development of electrophoresis
1936	J. Cuillé, P.-L. Chelle	Transmission of scrapie to normal sheep by cell-free material from diseased sheep
1936	P. Rous, J. Beard	Rabbit papillomavirus induces carcinomas in a different species
1936	C. Armstrong, T. Rivers, E. Traub	Discovery of lymphocytic choriomeningitis virus: the first arenavirus
1937–51	M. Theiler and others	Development of 17D strain human yellow fever vaccine: made in animal and embryonic cultures
1939	G. Kausche, P. Ankuch, H. Ruska	First electron micrograph of a virus: tobacco mosaic virus
1939	M. Debbruck, E. Ellis	Identification of the one-step virus growth curve
1941	G. Hirst	Discovery of influenza virus hemagglutination, hemagglutination inhibition test
1941	M. Gregg	Discovery of rubella virus congenital abnormalities
1944	O. Avery, C. MacLeod, M. McCarty	Identification of DNA as the material of inheritance
1945	T. Francis, J. Salk, G. Hirst, F. Davenport, E. Kilbourne, others	Development of inactivated influenza vaccines
1946	W. Stanley, J. Summer, J. Northrop	Preparation of a viral protein in a pure form: tobacco mosaic virus
1948–55	J. Enders, T. Weller, F. Robbins, H. Eagle	Routine use of tissue culture to grow and study viruses, development of optimal media for growing cells
1953	J. Watson, F. Crick, M. Wilkins, R. Franklin	Discovery of the structure of DNA
1953	A. Coons	Development of immunofluorescence
1954	J. Salk, J. Youngner, T. Francis, others	Development of inactive poliomyelitis vaccine
1954	B. Sigurdsson	Development of the concept of slow viruses (maedi-visna virus)
1954	W. Rowe	Role of thymus in immune responses to virus
1954–61	J. Salk, A. Sabin, H. Koprowski, J. Enders, S. Katz, S. Krugman	Development of poliomyelitis virus vaccine, development of measles virus vaccine: made in tissue culture
1956	H. Fraenkel-Conrat, B. Singer	Discovery of the infectivity of viral RNA (tobacco mosaic virus)
1956	M. Smith, W. Rowe, T. Weller	Discovery of cytomegalovirus
1957	A. Isaacs, J. Lindenmann	Discovery of interferon
1957	J. Enders, M. Hilleman, A. Gershon, S. Katz, S. Plotkin, M. Takahashi, others	Development of vaccines against measles, mumps, rubella, hepatitis A, hepatitis B

Table 1 Continued

Date(s)	*Virologists/investigators*	*Discovery*
1959	A. Sabin, H. Cox, H. Koprowski	Development of attenuated live-virus poliomyelitis vaccine
1959	S. Brenner, R. Horne	Invention of negative stain electron microscopy
1959	R. Porter, G. Edelman, A. Nisonoff	Discovery of the structure and molecular function of antibodies
1959	R. Yalow, S. Berson, F. Dixon	Development and use of radioimmune assays
1950s–70s	S. Luria, M. Delbruck, J. Monod, A. Lwoff, F. Jacob, S. Brenner, M. Nirenberg, S. Ochoa, H. Khorana, E. Wollman, A. Hershey, S. Benzer, S. Cohen, D. Nathans, R. Dulbecco, D. Baltimore, H. Smith, W. Arber, J. Kates, H. Temin, P. Sharp, others	Quantitative plaque assay, origins of molecular biology, and molecular virology
1950s–80s	B. Sigurdsson, B. Blumberg, C. Gajdusek, S. Prusiner, others	Persistent, latent, and slow virus infections, prions
1960s–70s	G. Palade, A. Claude, K. Porter, C. DeDuve	Description and techniques for fine structure and biochemistry of cellular organelles
1962	A. Klug, D. Caspar	Discovery of the principles of icosahedral virus structure
1967	J. Maizel, U. Laemmi	Discovery of SDS polyacrylamide gel electrophoresis
1969–76	R. Huebner, P. Vogt, M. Bishop, H. Varmus, R. Weinberg, others	Oncogenes
1970s–2000s	R. Zinkernagel, P. Doherty, M. Oldstone, B. Fields, B. Moss, A. Notkins, R. Ahmed, F. Chisari, N. Nathanson, others	Major histocompatibility restriction and cytotoxic T lymphocytes, immune-mediated viral diseases, molecular pathogenesis
1973	D. Nathans	Completion of the restriction map of a viral genome (SV40)
1974	G. Köhler, C. Milstein	Development of monoclonal antibodies
1975	B. Blumberg, B. Larouze, W. London, others	Discovery of the relationship of hepatitis B virus with hepatocellular carcinoma
1976	T. Diener	Discovery of viroids (infectious naked RNA molecules)
1976	K. Johnson, P. Webb, J. Lange, F. Murphy, J. McCormick, others	Discovery of Ebola virus
1977	World Health Organization, D.A. Henderson, F. Fenner, and many health workers and virologists	Eradication of smallpox as a disease that killed over 300 million people in the twentieth century
1978–83	S. Harrison, A. Olson, J. Hogle, M. Rossman, R. Rueckert	First atomic structure of a plant (tomato bushy stunt) and animal (poliovirus, rhinovirus) virus
1979	D. Lane, L. Crawford, D. Linzer, A. Levine	SV40T-antigen-p53, virus host cell interaction-tumor suppressors
1980s–90s	J. Strauss, E. Strauss, and others	Molecular analysis, structure/function of yellow fever
1980s–2000s	B. Roizman, J. Subak-Sharpe, E. Kieff, P. Spears, E. Wagner, J. Nelson, E. Goodpasture, J. Stevens, A. Notkins	Genetic map and function of genes of herpes viruses, herpes latency
1980s–2000s	H. zur Hausen, D. Galloway, D. Lowy, I. Frazer, others	Recognition of subtypes of papillomaviruses associated with cervical and penile cancer and development of papillomavirus vaccine
1981	J. Skehel, D. Wiley, I. Wilson, others	Structure/function of influenza virus hemagglutinin, first atomic structure of a glycoprotein
1981–84	R. Gallo, F. Barre-Sinoussi, L. Montagnier, Y. Hinuma, J. Chermann, others	First human retrovirus
1984–2000	M. Hillemann, others	First molecular recombinant virus vaccine: hepatitis B virus, first vaccine to successfully treat cancer: hepatitis B virus-induced liver cancer
1985	K. Mullis, others	Invention of polymerase chain reaction (PCR)
1987	S. Broder, H. Mitsuya, M. Fischl, D. Richman, others	Development of first anti-HIV drug approved by the FDA
1987	M. Capecchi, M. Evans, O. Smithies	Development of knockout and other genetically manipulated mice
1988–2000s	M. Eigen, C. Weissmann, J. Holland, E. Domingo, J.C. de la Torre, R. Adino, E. Koonin, A. Gibbs, others	Development of modern concepts in viral evolution and quasispecies
1991	C. Venter, H. Smith	Invention of shotgun cloning methods
1993	S. Falkow	Molecular criteria for proof of viral disease causation: revisiting the Henle–Koch postulates
1994–2000s	K.-K. Conzelmann, M. Schnell, T. Mebatsion, P. Palese, J.C. de la Torre, Y. Kawaoka	Development of reverse genetics for negative-strand RNA virus
1990–present	R. Ahmed, M. Bevin, M. Slifka, and others	Kinetics, cell, and molecular basis of T cell and B cell generation and immunologic memory
1998–2006	R. Ahmed, A. Zajac, E.J. Wherry, M. Oldstone, D. Brooks, others	Persistent virus infections associated with T cell exhaustion and caused by negative immune response regulators PD-1 and IL-10
1990–present	W.I. Lipkin, J. diRossi, D. Ganem, H. Virgin, and others	Modern molecular detection of new viruses

(*Continued*)

Table 1 Continued

Date(s)	*Virologists/investigators*	*Discovery*
2003	C. Urbani, J. Peiris, S. Lai, A. Osterhaus, others	Discovery of SARS coronavirus, the first viral pandemic of the twentyfirst century
2005	J. Taubenberger, P. Palese, T. Tumpey, A. Garcia-Sastre, Y. Kawaoka, others	Molecular reconstruction, sequencing of the 1918–19 influenza virus; determining viral genes associated with pathogenesis
2005	T. Wakita, C. Rice, F. Chisari	Manipulation of virus and cells for first *in vitro* hepatitis C virus culture system
2000s	B. Beutler, J. Hoffmann, S. Akari, C. Janeway, R. Medzhitov	Discovery of cell-surface molecules activated by innate immunity and pathways involved
2000s	R. Steinman, Z. Cohn, others	Discovery of the dendritic cell and its role in adaptive immunity
2000–2015	World Health Organization – many health workers and virologists	Planned eradication/elimination of poliomyelitis virus and measles virus (by 2015) as a disease

soon I came to a realizing sense of the desolation. Two blocks further on, coffins were piled on tiers on the sidewalk in front of the undertaker's shop, and we were compelled to walk between them . . . Everyone was thoroughly frightened, a young doctor said to me. 'It takes a man of great moral courage to stay in this place. You talk with a man tonight and tomorrow hear that he is in the grave'" (reviewed in Oldstone, 2010). To those who consider this part as ancient and perhaps nonrelevant contemporary history, it is well to recall that during the 1918–19 influenza epidemic in the USA, over 600 000 people died in less than 2 years. In the city of Philadelphia and some other locales, the supply of coffins ran out causing the dead to be buried in mass graves. As recently as the 1980s–90s, a similar scenario played out in parts of Africa when the AIDS epidemic caused by HIV resulted in widely publicized mass deaths and still do in parts of Africa and Asia.

Now these and many other acute virus infections are controlled by vaccination or antivirals and public health policies when and where they are instituted.

Distinct from those acute infections are persistent infections, a major health problem today. In persistent infection, the immune response fails to completely remove viruses from the body, and those viruses remaining then persist in their host for months or years. For infections like those by hepatitis B and C viruses and HIV, viruses can be recovered from the patients' blood for years, during the long course of infection. Although all components (antibodies and T cells) of the immune response are generated during those infections, the T cell response is compromised (by exhaustion or hyporesponsiveness) and is not capable of eliminating the infectious agent.

Table 1 lists events that have impacted the science of virology and its influence on not only diseases but also on the larger milieu of basic research. A more extensive list of important events, observations, and highlights in the discipline of virology has been compiled by Fred Murphy, a long-time contributor to the field, and is accessible at http://www.utmb.edu/virusimages/

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Relevant Website

http://www.utmb.edu/virusimages/.

HIV and Retroviruses

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ATL	Adult T cell leukemia/lymphoma
CA	Capsid
cART	Combination antiretroviral therapy
cDNA	Complementary DNA
DC	Dendritic cell
DIS	Dimerization initiation site
DLS	Dimer linkage structure
dsDNA	Double-stranded DNA
DU	dUTPase
ER	Endoplasmic reticulum
ERV	Endogenous retrovirus
ESCRT	Endosomal sorting complexes required for transport
gRNA	Genomic RNA
HAM/TSP	HTLV-associated myelopathy/tropical spastic paraparesis
IN	Integrase
INSTI	Integrase strand transfer inhibitor
IRES	Internal ribosomal entry site
LTR	Long terminal repeat
MA	Matrix
NC	Nucleocapsid
NNRTI	Nonnucleoside reverse transcriptase inhibitor
NPC	Nuclear pore complex
NRTI	Nucleoside reverse transcriptase inhibitor
ORF	Open reading frame
PBS	Primer binding site
PIC	Preintegration complex
Pol II	RNA polymerase II
PPT	Polypurine tract
PR	Protease
RRE	Rev-response element
RT	Reverse transcriptase
RTC	Reverse transcription complex
ssDNA	Single-stranded DNA
SU	Surface
TM	Transmembrane

Introduction

The retroviruses (Retroviridae) comprise a unique and fascinating family of RNA viruses found throughout vertebrate life, characterized by the two defining features of their life cycle: reverse transcription and integration. The process of reverse transcription, where the viral genomic RNA is reverse-transcribed to genomic DNA by the enzyme reverse transcriptase, was believed to be impossible prior to the discovery of retroviruses. While reverse transcription has since been described in other viruses, such as hepadnaviruses and caulimoviruses, the discovery of reverse transcriptase in retroviruses fundamentally transformed the field of microbiology. Integration of the viral genome into the host cell DNA is unique to retroviruses and endows them with the capacity to establish persistent infections and, if a germline cell becomes infected, permanently alter the host genome and achieve vertical transmission as an endogenous retrovirus. Integration into the host genome implies the possibility of altering host cell gene expression, as is the case with oncogenic activation and tumor formation observed in some retroviruses, but also makes retroviruses a promising vector for gene therapy, with the capacity to permanently introduce desirable transgenes. In this chapter, we will discuss

the classification of retroviruses, the structure of these viruses and their life cycle, as well as their capacity to cause disease and their promise as gene therapy vectors.

Taxonomy and Classification

All retroviruses share three genes that are essential to the viral life cycle: *gag*, *pol*, and *env*. *Gag* encodes the structural proteins required for virion formation, *pol* encodes the viral enzymes, and *env* encodes the transmembrane envelope protein, which mediates receptor binding and entry into the target cell. Retroviruses share a common genomic organization, in which Gag and a Gag-Pol polyprotein are generated via various mechanisms from the full-length genomic RNA (gRNA), and Env is produced from a singly-spliced subgenomic RNA. The viral protease (Pro), an enzyme required for viral particle maturation and infectivity, is often referred to as part of the *pol* gene, although it can be produced from a separate open reading frame (ORF) from *pol*, or as part of *gag*. Retroviruses encoding only *gag*, *pol*, and *env* are considered simple retroviruses, while those that encode additional genes are known as complex retroviruses. The retrovirus family is divided into two subfamilies: orthoretrovirinae, which is further divided into six genera, and spumaretrovirinae, which includes only the spumavirus genus (Table 1).

Orthoretrovirinae

Alpharetroviruses

Alpharetroviruses are simple retroviruses in which Pro is encoded in the *gag* ORF, and Pol is produced by a ribosomal frameshift. The virus particle assembles at the plasma membrane and has a central, symmetrically-placed, spherical core (C-type morphology). The genome is approximately 7.2 kb in size, and the Avian leukosis virus (ALV) and Rous sarcoma virus (RSV) are members of this genus. Alpharetroviruses are known to cause malignancies, and many oncogene-containing acute transforming retroviruses belong to this genus.

Betaretroviruses

Betaretroviruses were originally classified as simple retroviruses, but some, such as mouse mammary tumor virus (MMTV), encode the Rem protein, which facilitates gRNA nuclear export. *Gag*, *pro*, and *pol* are each encoded in a different ORF, with Pro and Pol produced following two independent ribosomal frameshift events. The virions of betaretroviruses assemble in the cytoplasm before budding from the plasma membrane, and some members have a round, eccentric core while others have a cylindrical core. The genome is approximately 8–10 kb in length, and other members of the genus include Jaagsiekte sheep retrovirus and Mason-Pfizer monkey virus (MPMV).

Gammaretroviruses

Gammaretroviruses are simple retroviruses which encode *gag*, *pro* and *pol* in the same ORF. Production of the Gag-Pro-Pol polyprotein requires translational readthrough of a stop codon at the end of *gag*. Gammaretroviruses, which include the murine

Table 1 Common retroviruses from each genus

Genus	*Species*	*Abbreviation*
Alpharetrovirus	avian leukosis virus	ALV
	Rous sarcoma virus	RSV
	Avian myelocytomatosis virus 29	MC29
Betaretrovirus	Mouse mammary tumor virus	MMTV
	Jaagsiekte sheep retrovirus	JSRV
	Mason-Pfizer monkey virus	MPMV
Gammaretrovirus	Murine leukemia virus	MLV
	Feline leukemia virus	FeLV
Deltaretrovirus	Human T lymphotropic virus	HTLV
	Bovine leukemia virus	BLV
Epsilonretrovirus	Walleye dermal sarcoma virus	WDSV
	Snakehead retrovirus	SnRV
Lentivirus	Human immunodeficiency virus type 1	HIV-1
	Human immunodeficiency virus type 2	HIV-2
	Simian immunodeficiency virus	SIV
	Feline immunodeficiency virus	FIV
	Caprine arthritis encephalitis virus	CAEV
	Maedi-visna virus	MVV
Spumavirus	Simian foamy virus	SFV
	Bovine foamy virus	BFV

and feline leukemia viruses (MLV and FLV), have a C-type morphology and a genome of approximately 8.3 kb, and they cannot infect dividing cells. Viruses from this genus can cause malignancies, immunosuppression, neurological disorders, and many oncogene-containing acute transforming retroviruses belong to this genus.

Deltaretroviruses

Deltaretroviruses are complex retroviruses with *gag*, *pro*, and *pol* encoded in three distinct ORFs, requiring two successive ribosomal frameshifts to produce the Gag-Pro-Pol polyprotein. These viruses also encode two additional proteins, Rex and Tax, from an alternatively-spliced subgenomic RNA. The genome is approximately 8.3 kb in length, and the virion assembles with a C-type morphology. This genus includes human T lymphotropic virus 1, 2 and 3 (HTLV-1, HTLV-2, and HTLV-3) and bovine leukemia virus (BLV), and does not include any known endogenous retrovirus members.

Epsilonretroviruses

Epsilonretroviruses are complex retroviruses encoding *gag*, *pro*, and *pol* in a single ORF, with production of the Gag-Pro-Pol polyprotein requiring translational readthrough of a stop codon at the end of *gag*. These viruses also include three additional ORFs, termed ORF A, B, and C. ORF A encodes a homolog of the host Cyclin D protein, and the functions of ORFs B and C are unknown. The viral genome is 11.7–12.8 kb in length, and the virion assembles with a C-type morphology. Epsilonretroviruses are exogenous viruses of fish and reptiles, including walleye dermal sarcoma virus and snakehead retrovirus, and some epsilon-like endogenous retrovirus fragments have been described. This genus remains poorly understood, and further investigation may warrant its division into two or more separate genera.

Lentiviruses

Lentiviruses are complex retroviruses encoding *gag* in one ORF and *pro-pol* in another. Production of the Gag-Pro-Pol polyprotein requires a ribosomal frameshift at the end of *gag*. Lentivirus particles assemble at the cell membrane and have distinctive conical cores, and the viral genome is approximately 9.3 kb in length. Lentiviruses include HIV-1 and HIV-2, SIV, caprine arthritis encephalitis virus, and visna virus. They encode several additional proteins, which vary among members of the genus. Lentiviruses are capable of infecting nondividing cells, and are named for the characteristically slow onset of the associated disease. HIV-1 and HIV-2 are the etiologic agents of acquired immunodeficiency syndrome (AIDS) and are the retroviruses responsible for the greatest disease burden in humans.

Spumaretrovirinae

Spumaviruses

Spumaviruses are the only genus of the subfamily spumaretrovirinae and are distinct from orthoretroviruses in many ways, including two defining characteristics: Pro-Pol is expressed from a singly-spliced subgenomic RNA instead of as a polyprotein with Gag from the full-length genomic RNA, and there is a significant amount of reverse-transcribed DNA present in the mature virion. Spumaviruses are complex retroviruses, as they also encode two additional proteins: Tas/Bel-1, a transcriptional trans-activator, and Bet. Both Tas/Bel-1 and Bet are produced from RNAs transcribed from an internal promoter within the *env* gene, which further distinguishes spumaviruses from orthoretroviruses. They assemble in the cytoplasm, budding into the ER or at the plasma membrane, and have a unique morphology with an uncondensed core and conspicuous spikes. Gag is processed by Pro to a lesser extent than in the orthoretroviruses, with only a single cleavage occurring near the C-terminus. The genome is approximately 11.6 kb in length, and the genus includes the simian, bovine, equine, and feline foamy viruses, named for the drastic cytopathological changes they cause in vitro. Spumaviruses are not associated with any disease, and while rare infection of humans with simian foamy virus has been observed, no human-human transmission is known to have occurred.

Structure and Composition

Genomic RNA and Protein Nomenclature

The retroviral genome is packaged into viral particles as a dimer of single-stranded, positive-sense, linear RNA molecules. The genomic RNA (gRNA) is produced by the host RNA polymerase II (Pol II) machinery and processed as a host mRNA, with addition of a 5′ methyl cap and a 3′ poly-A tail. The gRNA includes many important regulatory sequences, shown in Fig. 1. The coding region encodes the Gag, Pol, and Env polyproteins, which are further processed to produce the functional products listed in Table 2.

Structure of Virions

Retroviral virions are initially assembled when unprocessed Gag and Gag-Pol precursors multimerize within the cytoplasm or at the plasma membrane, which is likely mediated by initial interactions between Gag and dimerized gRNA. The immature virion is spherical and appears translucent by electron microscopy. The virion matures when the viral protease (Pro) cleaves Gag and Gag-Pol to the functional products listed above. The mature virion is roughly spherical, but the cleaved CA forms a more ordered viral core around the highly condensed gRNA, which is coated with NC. The viral particle typically contains 1500–2000 Gag precursors, with

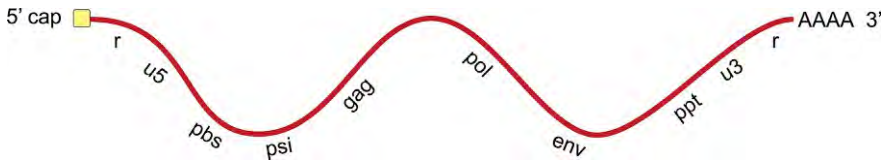

Fig. 1 *Diagram of the retroviral Genomic RNA (gRNA) highlighting important features.* The components of the gRNA are shown from 5′ to 3′. A 5′ methyl cap is present, as with all host mRNA molecules. R is the repeated sequence found at both ends of the gRNA. U5 is the unique 5′ sequence containing an "att" site for integration. PBS is the primer binding site, an 18 nucleotide tRNA hybridization sequence which is complementary to the tRNA primer that initiates reverse transcription. The Psi element (Ψ) is the encapsidation signal for packaging of the gRNA. *Gag*, *pol*, and *env* are the coding regions of the retroviral proteins, and complex retroviruses have additional coding regions in this section of the genome. Ppt is the polypurine tract, the initiation site for plus-strand DNA synthesis. U3 is the unique 3′ sequence containing an "att" site for integration. A 3′ poly-A tail is present, as with all host mRNA molecules.

Table 2 Protein products common to all retroviruses

Precursor	*Protein*	*Abbreviation*	*HIV-1*
Gag	Matrix	MA	p17
	Capsid	CA	p24
	Nucleocapsid	NC	p9
Pol	Protease	PR	p10
	Reverse Transcriptase	RT	p65/p51
	Integrase	IN	p31
	dUTPase	DU	–
Env	Surface	SU	gp120
	Transmembrane	TM	gp41

All retroviruses generate Gag, Pol, and Env precursor proteins, which are then cleaved to generate the functional protein products. These products are named in common for all retroviruses with the shown two-letter abbreviations, and the specific names for HIV-1 are also shown.

5%–10% as much Gag-Pol, although this ratio varies depending on the mechanism by which the Gag-Pol polyprotein is generated. The capsid core is made up of primarily hexamers of CA, while some pentamers stabilize the structure. The capsid core is surrounded by a sphere of MA proteins, which is surrounded by a lipid bilayer envelope acquired during budding from the host cell membrane. The lipid envelope includes host transmembrane proteins as well as the heavily glycosylated viral SU and TM proteins, which exist as trimers. SU is entirely extracellular, but remains associated with the envelope through interactions with the single-pass transmembrane TM protein. The number of Env trimers varies among retroviruses, with Gammaretroviruses having as many as 400, while lentiviruses have as few as 2–10. (Fig. 2).

Life Cycle

See Fig. 3.

Receptor Binding and Membrane Fusion

The retroviral life cycle begins when SU trimers on the outside of the virion envelope bind with high specificity to a protein or proteins on the surface of the target cell, which act as the viral receptor. Many different host proteins can act as the viral receptor, although viruses of the same genus often use the same or similar proteins. Receptor usage is determined by the structure of the SU protein, and thus SU is responsible for defining the range of host cells that are susceptible to infection. The SU protein of HIV (gp120) binds to the viral receptor CD4, as well as one of two coreceptors, either CXCR4 or CCR5. As CD4 is predominantly expressed on a subset of T cells, as well as to a lesser extent on macrophages and hematopoietic progenitor cells, these cells represent the primary targets of HIV infection. Upon receptor binding, the SU-TM trimers undergo a structural change that exposes the N-terminal fusion peptide of TM. TM mediates fusion of the viral envelope with the target cell plasma membrane, and the viral core is released into the target cell cytoplasm.

Internalization and Capsid Core Disassembly

The initial stages following membrane fusion and internalization of the capsid core are poorly understood. Most retroviruses do not depend on endosomal acidification to complete this process, although there is evidence that MLV and some alpharetroviruses may require acidic endolysosomal pH for entry. After internalization, it remains unclear precisely what must take place with the capsid core, made up of CA hexamers and pentamers, for productive infection. Maturation of Gag precursors is essential for reverse

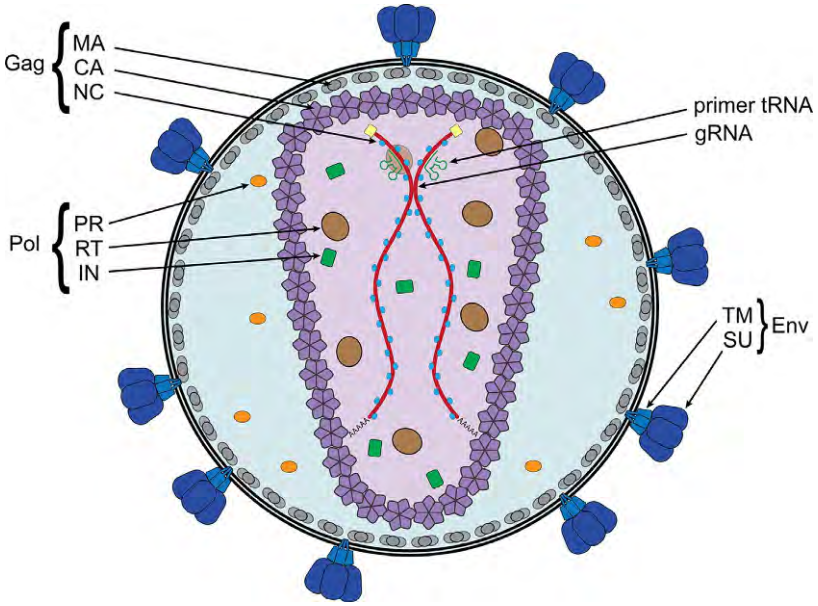

Fig. 2 *Diagram of the mature retroviral virion.* The essential components of mature retroviral virions are depicted, with the protein products labeled in groups according to their unprocessed precursor proteins, Gag, Pol, and Env.

transcription to proceed, and mutations that destabilize or hyperstabilize the capsid core severely hinder reverse transcription. The capsid core may also play a role in shielding the cytoplasmic viral DNA from recognition by innate immune sensors. Recently, it has been described that CA can be detected in the nucleus, suggesting that some amount of CA remains associated with the viral genome throughout the reverse transcription process. Furthermore, CA is the major determinant that allows HIV-1 and other lentiviruses to infect nondividing cells, and many interactions between nuclear import machinery and domains of CA that are only present when it is assembled in the capsid core are essential for this to occur. Thus, it is likely that some remodeling of the capsid core is required for reverse transcription to proceed, without complete disassociation of CA from the reverse transcription complex (RTC).

Reverse Transcription

Upon entry into the target cell, the virus must complete the reverse transcription of its RNA genome into a double-stranded DNA genome, allowing the virus to integrate into the host cell DNA and generate new viral progeny. Reverse transcription is a complex but remarkable process, proceeding through a series of essential steps facilitated by the reverse transcriptase (RT) enzyme that ultimately produces the reverse transcribed complementary DNA (cDNA). Reverse transcription begins shortly after the viral core enters the cytoplasm, although the precise signals regulating the initiation of reverse transcription are unknown, and proceeds within the reverse transcription complex (RTC). The RTC is likely enclosed in a partially-assembled capsid core containing the gRNA dimer and 50–100 RT enzymes. Unlike the orthoretroviruses, spumaviruses undergo reverse transcription during the late stages of infection in the producer cell (Fig. 4).

Initiation of minus-strand DNA synthesis

Reverse transcription begins when the 3′-terminal 18 nucleotides of the primer tRNA hybridize with complementarity to the 18-nucletoide pbs near the 5′ end of the gRNA. Each virus uses a preferred tRNA molecule as the primer, which vary among the different retroviruses. The 3′-OH of the tRNA serves as the primer for the initial DNA synthesis, which proceeds toward the 5′ end of the gRNA. This generates the U5 and R sequences of the minus-strand DNA, known as the minus-strand strong-stop DNA.

First translocation

The RNase H activity of RT degrades the region of the gRNA complementary to the newly-synthesized DNA, which exposes the DNA as a single strand. The R sequence of the minus-strand DNA is translocated and anneals to the 3′ r sequence of the gRNA, leaving a 5′ overhang of U5 and the tRNA primer. This translocation is facilitated by NC, and can result in annealing to either of the two gRNAs packaged in the virion.

Long minus-strand DNA synthesis

Synthesis of the minus-strand DNA proceeds along the length of the gRNA, beginning with the strong-stop 3′-OH as the primer. Thus, the entire minus-strand DNA copy of the gRNA is generated. As the DNA polymerase activity of RT synthesizes the

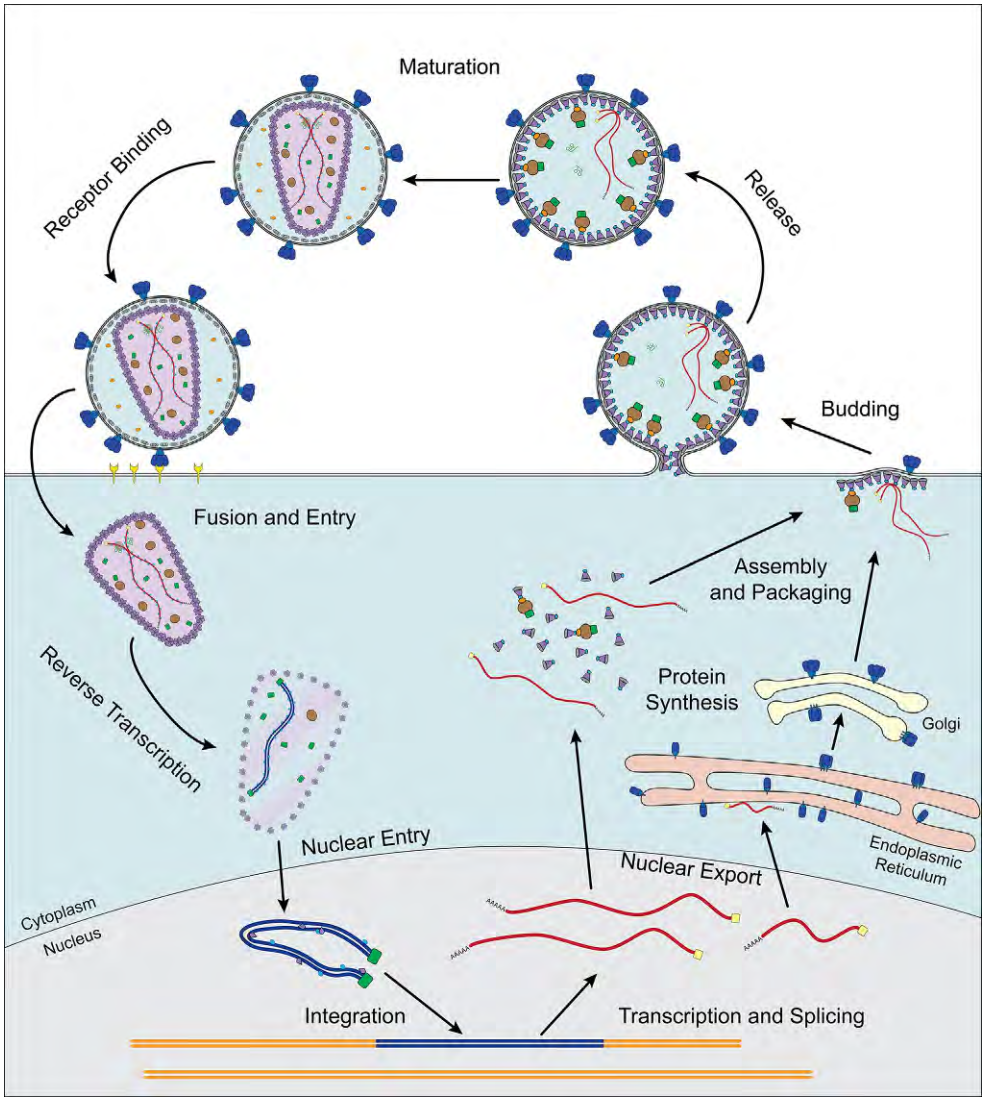

Fig. 3 *Diagram of the retroviral life cycle.* The critical stages of the retroviral life cycle within a single host cell are depicted, from the infection of a target cell with a mature virus particle to the production of mature virus particles from the infected cell.

minus-strand DNA, the RNase H activity of RT degrades the gRNA from the RNA–DNA hybrid that is generated. This can be performed by the same RT enzyme that is synthesizing the DNA, with degradation occurring 17–18 base pairs behind the elongating 3′ end, or by a separate RT enzyme.

Initiation of plus-strand DNA synthesis

In order for synthesis of the DNA plus-strand to begin, a 3′-OH primer is required. The ppt, a purine-rich region of the genome located near the 3′ end of the gRNA, is resistant to RNase H degradation and remains intact and bound to the fully-synthesized minus-strand DNA as an RNA–DNA hybrid. The ppt RNA oligonucleotide serves as the primer for initial plus-strand DNA synthesis, which proceeds through U3, R, and U5, continuing up to base 19 of the primer tRNA. The first 18 base pairs of the tRNA are copied, generating the PBS and the plus-strand strong-stop DNA product. The RNase H activity of RT degrades the ppt, which is susceptible to degradation after deoxynucleotides are added. In addition to the ppt near the 3′ end of the gRNA, spumaviruses and lentiviruses encode a central polypurine tract that acts as a second primer for plus-strand DNA synthesis, allowing for more efficient production of the plus-strand DNA.

Removal of tRNA and second translocation

The RNase H activity of RT removes the tRNA, exposing the 3′ end of the plus-strand DNA, which contains the PBS. The exposed PBS at the 3′ end of each DNA strand anneal, generating a circular intermediate with both 3′ ends ready for elongation. Each strand serves as the template for the other strand.

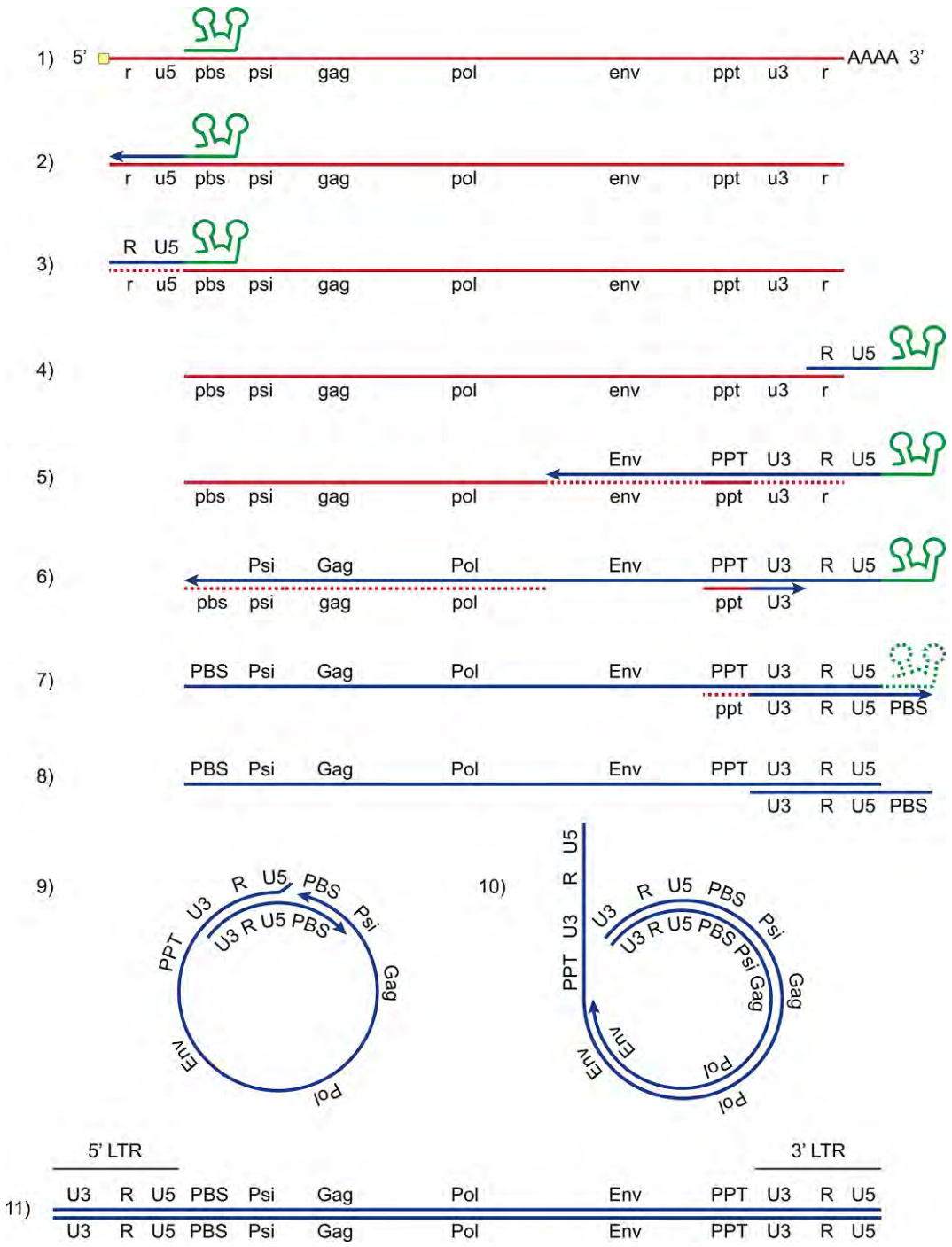

Fig. 4 *Schematic of the process of reverse transcription.* The viral genomic RNA is depicted in *red*, the primer tRNA in *green*, and the cDNA in *blue*. (1) gRNA with primer tRNA bound. (2) Initiation of minus-strand synthesis. (3) Formation of the 3′ strong-stop DNA and degradation by RNase H. (4) First translocation. (5) Minus-strand elongation and generation of the ppt primer. (6) Completion of minus-strand synthesis and initation of plus-strand synthesis. (7) Completion of 5′ strong-stop DNA and degradation of ppt and tRNA primer. (8) Linear product prior to second translocation. (9) Second translocation and formation of circular intermediate with simultaneous minus- and plus-strand synthesis. (10) Completion of minus- and plus-strand synthesis. (11) Finished cDNA product differing from gRNA with the presence of LTRs at both ends.

Completion of both strands

Elongation of the minus strand continues from the PBS through U5-R-U3, displacing the plus strand as it proceeds, creating a ssDNA template for synthesis of the plus-strand 3′ LTR. Simultaneously, elongation of the plus strand proceeds along the minus strand from the PBS, through the viral genome, and through the PPT-U3-R-U5 region at the 5′ end of the minus strand. Lentiviruses, which

have also initiated plus-strand elongation from a central ppt, generate breaks in the plus-strand DNA with flaps of overlapping sequence. These are eventually corrected by the host cell DNA repair machinery prior to integration.

Upon completion of reverse transcription, it is important to note that the cDNA genome is modified relative to the template gRNA. The overhangs from the two translocations result in the duplication of U5 and U3. The viral DNA now has 2 LTRs, one at each end of the genome, composed of U3-R-U5.

Biochemistry and structure of RT

The amino-terminal domain of RT has DNA polymerase activity, which is unique among DNA ploymerases in its ability to use either RNA or DNA as the primer or the template. The DNA polymerase activity of RT is slow relative to host DNA polymerases, ranging from 1 to 100 nucleotide additions per second, with estimates for minus-strand synthesis around 70 nucleotides per minute. It also possesses poor processivity, releasing frequently from the template, and is relatively low-fidelity, generating 10^{-4}–10^{-5} mutations per base. This low fidelity is exacerbated by the fact that RT lacks any proofreading activity. RT also has a helicase activity that is capable of displacing a DNA strand that is annealed to the template being used, which is essential during synthesis of the minus-strand 5′ LTR. The carboxy-terminal domain of RT possesses RNase H activity, which generates oligonucleotides with a 3′-OH, creating a suitable primer from the ppt to initiate plus-strand synthesis. RNase H only degrades RNA in duplex form, either with DNA or RNA, and can act in concert with the DNA polymerase activity of RT.

RT exists as a 75–80 kDa monomer in the gammaretroviruses, which contains both DNA polymerase and RNase H activities. RT forms a heterodimer in the alpharetroviruses, with the alpha subunit containing DNA polymerase and RNase H, and the larger beta subunit also containing integrase (IN). Lentiviruses also produce RT as a heterodimer, which for HIV-1 is composed of a p66 subunit with both DNA polymerase and RNase H, and a p51 subunit, which lacks the RNase H domain but is essential for proper positioning of the p66 subunit for RNase activity.

Recombination between the two copackaged virion gRNAs happens frequently during reverse transcription and contributes to the generation of genetic diversity within the viral population. In order for recombination between the two gRNAs to cause significant changes in the viral genome, the producer cell must be coinfected with multiple viral genomes. Usually the two gRNAs that are packaged are identical, limiting the effects of recombination events. Recombination is most common during template switching, and is thus a frequent event during minus-strand synthesis, when the template routinely switches back and forth between the two RNA strands. This switching can occur when RNase H degrades the template and allows annealing to the other RNA strand, which may occur more frequently at sites with RNA secondary structures that slow RT DNA polymerase activity, or when nicks in the template strand force a switch to the other strand. Template switching requires that the two gRNAs be similar enough for the 3′ end of the growing DNA strand to anneal, and similar enough for the two gRNAs to successfully dimerize and be copackaged into a single virion. Recombination between two gRNAs only occurs between the pairs of a packaged dimer, suggesting that each virion performs its own reverse transcription reaction, and the gRNAs packaged in other virions are not accessible for the duration of reverse transcription.

Nuclear Entry

After reverse transcription has been completed, the RTC transitions to become the preintegration complex (PIC), as the next important step of the viral life cycle is the integration of the double stranded genomic DNA into the host cell genome. The PIC is too large for passive transport across the nuclear envelope, and thus most retroviruses are only able to infect dividing cells after the nuclear envelope has broken down. The lentiviruses, however, can infect nondividing cells by entering the nucleus through the nuclear pore complex (NPC), and this capacity is likely mediated by the lentiviral CA protein, despite previous studies which implicated HIV-1 Vpr, IN, or MA for this function. The spumaviruses are also capable of infecting nondividing cells, although the mechanism is unknown.

Many host proteins are involved in the nuclear import of lentiviruses. NUP358 (RANBP2) is located on the cytoplasmic side of the NPC and interacts with HIV-1 CA to facilitate docking of the PIC at the NPC. NUP153 is located on the nuclear side of the NPC and interacts with residues on CA that are only present when CA is assembled in multimers as in the capsid core, and this interaction is important for HIV-1 integration. TNPO3 also binds CA and facilitates nuclear import of the PIC, and may be involved in removing CA from the PIC once it enters the nucleus. CPSF6 binds CA and is essential for proper trafficking of the PIC to the nucleus and nuclear import, as well as maintaining the characteristic integration site preferences of HIV.

Integration

Integration is the truly unique and defining feature of retroviruses, as it is not an essential function of any other family of viruses. Despite being present in the nucleus, unintegrated retroviral cDNA is a very poor substrate for transcription, and thus an integrated provirus is essential for persistent and efficient expression of retroviral genes. Integration allows retroviruses to occasionally enter the germline and become transmitted vertically, and is also responsible for retroviral-mediated mutational and insertional oncogenesis. Integration is mediated by the IN enzyme, which forms a dimer of dimers structure called the intasome via interactions in the N-terminal oligomerization domain. The catalytic core domain contains three acidic residues that interact with two Mg^{2+} ions, and is highly conserved. The integration reaction proceeds via two steps.

3′-end processing

To initiate the integration reaction, IN recognizes an "A-T-T" sequence at each end of the viral cDNA and removes 2–3 nucleotides (2 for HIV-1) on the 3′ strand at each blunt end of the cDNA in a concerted manner, with both ends being trimmed simultaneously. The cleavage occurs at a highly conserved "C-A" sequence in the cDNA, releasing the dinucleotide and leaving C-A-OH 3′-hydroxyl ends. This generates short 5′ overhangs at each end of the cDNA.

Strand transfer

After 3′ end processing, the target capture complex is formed. The free 3′-OH from the trimmed ends attack the target DNA in a concerted manner, with the two cDNA strands attacking the two strands of the host DNA simultaneously and only a few base pairs apart. This generates two nicks in the host DNA on opposite strands. The two nicks leave an equal number of unpaired complementary bases on either side of the integrated viral DNA, which were annealed to each other prior to the integration reaction. Host DNA polymerase fills in the empty bases on either side of the viral genome, generating a short duplication of the host integration site sequence. The length of the duplication is characteristic for each retrovirus: for instance, 4 bp by MLV and FLV, 5 bp by HIV, and 6 bp by alpharetroviruses. 5′ flap endonuclease removes the short 5′ overhang generated on the viral DNA during 3′ end processing, and DNA ligase seals the gaps to complete the integration of the retroviral provirus. Thus the integrated provirus contains a 2–3 bp deletion from the end of each LTR, as well as a 4–6 bp duplication of the host integration site sequence found immediately adjacent to each LTR.

Integration sites

The integration site has the potential to dramatically affect host cell function by altering gene expression or disrupting functional genomic elements. Integration near oncogenes can cause increases in oncogene expression and drive tumor development. Retroviral integration sites are not sequence-specific, and for the alpharetroviruses and betaretroviruses, the integration site is essentially random through the entire host genome. However, despite the absence of a specific integration site sequence, other retroviruses demonstrate preferential integration into certain locations in the host cell genome. Gammaretroviruses tend to integrate in promoters and DNase 1 hypersensitive regions, which are characteristic of open chromatin and likely enhance the likelihood that the viral genes will be expressed. On the contrary, spumaviruses puzzlingly appear to disfavor integration in genes and sites of active transcription. Lentiviruses, including HIV, preferentially integrate within expressed genes, intron-rich regions, and in chromatin located near the nuclear envelope, while avoiding chromatin in lamina associated domains.

Transcription

The integration of the virus into the host cell DNA, establishing a permanent provirus within the genome of the infected cell, marks the end of the early phase of retrovirus infection, shifting the focus to the production of new virus particles. The first step in virion production is the initiation of transcription from the promoter found within the 5′ LTR, which is responsible for producing both the mRNAs needed to encode the viral structural proteins and the gRNA. Initiation of transcription at the 5′ LTR is stochastic, although the U3 region contains a promoter, with both a TATA box and CCAAT box, as well as a range of enhancers. U3 enhancer regions recruit several transcription factors, including NFκB, AP-1, NFAT, SP-1, and CBF1, among others. Transcription of the provirus is performed by the host Pol II enzyme and initiates at the U3-R border.

Many complex retroviruses encode additional trans-activating proteins to enhance expression of the viral genes. HTLV encodes Tax, which recruits CREB to three cAMP response elements in the LTR to enhance LTR-driven transcription. HIV encodes Tat for a similar purpose, although Tat functions by binding to the TAR element, a secondary structure near the 5′ end of the nascent viral RNA. Tat then recruits P-TEFb, increasing the processivity of Pol II, which tends to stall early in the transcription of the HIV genome, by phosphorylating the Pol II C-terminal domain.

Transcription proceeds through the 5′ LTR and the coding region of the viral genes and concludes in the 3′ LTR, where transcription stops at the R-U5 border. A 5′ methyl cap is added, and the 3′ end is poly-adenylated. This generates the full-length viral RNA, which serves as both the packaged gRNA and as the mRNA template for translation of Gag and Gag-Pol polyproteins. In addition to the promoter in the 5′ LTR, which is responsible for driving all orthoretrovirus gene expression, the spumaviruses have an additional internal promoter, which drives expression of the proteins Tas and Bet.

Splicing and Nuclear Export

Upon generation of the full-length viral RNA, simple retroviruses require the successful nuclear export of two RNA forms: a singly-spliced variant that is used to produce Env proteins, and the full-length RNA for production of Gag-Pol and packaging into nascent virions. Complex retroviruses include other alternatively-spliced or multiply-spliced subgenomic RNAs. The splicing of the singly-spliced variants uses host cell machinery and behaves similarly to any cellular mRNA. Export of unspliced or incompletely spliced RNA, however, is inefficient, as host nuclear export factors are often recruited during splicing, and splicing factors that bind introns provide signals to prevent nuclear export. Thus, most host RNAs are properly and completely spliced prior to nuclear export.

For simple retroviruses, which encode no proteins in addition to Gag, Pol, and Env, export of the unspliced viral RNA can take advantage of several mechanisms. Many retroviruses, including MPMV, contain sequences called constitutive transport elements

(CTEs), which promote export by directly binding to the host export factor TAP/NXF1. Retroviruses also possess low-efficiency 3′ splice sites, which can prevent binding of splicing factors that act as negative regulators of nuclear export of incompletely spliced RNAs. Mutations to improve the efficiency of these splice sites inhibit the replicative fitness of retroviruses. The inefficient splice sites act in concert with negative regulators of splicing (NRS) sequences, which further prevent splice factor binding, to preserve the unspliced, full-length RNA for nuclear export. For RSV, the Gag protein can facilitate nuclear export of the full-length RNA. Gag has nuclear localization signals (NLS) in both the NC and MA domains, which allow Gag to enter the nucleus. The interaction between NC and the Psi element of the viral RNA exposes a nuclear export signal (NES) in the p10 domain of Gag, allowing the Gag-gRNA complex to exit the nucleus.

With the benefit of encoding additional genes, the complex retroviruses have evolved additional methods for promoting the export of partially spliced or unspliced RNA. Splicing of HIV is very complex, with at least four 5′ splice donor sites and eight 3′ splice acceptor sites. The major splice donor site is used in generating each of the spliced RNA products. Thus, there are many incompletely spliced subgenomic RNAs, as well as the unspliced gRNA, that must be exported. HIV encodes Rev to facilitate this process. Rev binds to a specific element on the viral RNA, the Rev-response element (RRE), and then binds to CRM1, which is responsible for the export of proteins containing a NES. HTLV Rex and MMTV Rem function similarly and for the same purpose.

Translation and Protein Processing

The Gag, Pol, and Env proteins are essential to the production of infectious virions for all retroviruses. Each of these proteins is translated as a precursor that is eventually cleaved and processed into the functional subunits that form a mature virus particle. Producing precursors likely benefits retroviruses in many ways, including producing several functional proteins from a single ORF, guaranteeing that proteins are produced and packaged in the appropriate ratio, and targeting many proteins to the site of virion assembly simultaneously.

Gag

The Gag precursor is produced from the full-length RNA. Some retroviruses employ an internal ribosomal entry site (IRES) near the *gag* start codon to prevent the need for translational initiation at the 5′ cap, which requires an elongated scanning through the R-U5 region of the genome. The major form of Gag is myristylated by myristyl CoA transferase, increasing the hydrophobicity of Gag and facilitating membrane association and packaging into the virion.

Pro-Pol

The viral protease is also produced from the full-length RNA and exists between the *gag* and *pol* genes. It can be fused to the 3′ end of gag, the 5′ end of pol, or neither, and is often produced along with Pol, which includes RT, IN, and dUTPase (DU) for some retroviruses, by translational readthrough or frameshifting.

Gammaretroviruses and epsilonretroviruses use translational readthrough, as Gag-Pro-Pol is encoded in a single ORF. A UAG stop codon between *gag* and *pro-pol* is translated as glutamine 5%–10% of the time due to the existence of a purine-rich region and pseudoknot structure downstream of the stop codon, which promote ribosomal slippage. This results in the Gag-Pro-Pol polyprotein being 10–20-fold less abundant than Gag, which is needed at higher quantities than the viral enzymes.

Alpharetroviruses and lentiviruses require a single −1 frameshift to occur during the junction between *gag* and *pol*, as they exist in separate ORFs. This is facilitated by a slippery region near the *gag* stop codon, which includes homopolymeric bases such as poly-A or poly-U and a large hairpin, stem-loop, or pseudoknot structure. The −1 frameshift results in an out of frame *gag* stop codon, and translation continues through the *pol* ORF. This occurs in 5%–10% of translation events, resulting in Gag-Pol being 10–20-fold less abundant than Gag. For alpharetroviruses, *pro* is in the *gag* ORF and Pro is produced equimolar to Gag, whereas in lentiviruses *pro* is in the *pol* ORF and Pro is produced equimolar to Pol.

The betaretroviruses and deltaretroviruses require two −1 frameshifts, the first at the end of *gag* to avoid the *gag* stop codon and enter the *pro* ORF, and the second at the end of *pro* to avoid the *pro* stop codon and enter the *pol* ORF. Each of these frameshifts can occur up to 30% of the time, resulting in a decreasing abundance of Gag, Gag-Pro, and Gag-Pro-Pol precursors.

Spumaviruses are unique in their production of the Pro-Pol precursor from a spliced, subgenomic mRNA. Thus, Pro-Pol for spumaviruses is not synthesized as a polyprotein with Gag and must be packaged into the mature virion independent of the Gag packaging signal.

Env

All retroviruses produce the Env precursor from a singly-spliced subgenomic RNA, with a 5′ leader sequence joined directly to the *env* coding region after *gag-pol* is removed as an intron. Env is synthesized as a single-pass transmembrane protein in the ER, where it is folded, glycosylated, and oligomerizes to form Env trimers. Unlike the other retroviral precursor proteins, the Env precursor is not processed by the viral protease, but rather by host furin proteases in the Golgi, which separate the SU and TM subunits. SU is wholly extracellular and remains associated with the virion via interactions with TM. It is heavily glycosylated, protecting the virion from neutralizing antibodies by shielding antibody epitopes, and contains hypervariable regions that determine receptor binding. TM has extracellular, transmembrane, and cytoplasmic domains, with the extracellular N-terminus containing the fusion peptide, which is essential for membrane fusion during entry into the target cell. TM also contains the major contacts for oligomerization. Some mannose residues are removed and *N*-acetyl glucosamine is added in the Golgi, and trimers of SU-TM heterodimers are transported to the cell surface for assembly.

Assembly and Packaging

Assembly of retroviral virions is predominantly mediated by Gag precursors, as these are sufficient for production of virus-like particles in the absence of any other viral factors. The C-type viruses assemble at the plasma membrane, with Gag targeted there by hydrophobic residues, basic residues, and myrsitic acid. For HIV, the MA domain of Gag, which has a myristic acid added to the N-terminal glycine, embeds in the plasma membrane and forms stable anchors only upon binding to $PI(4,5)P_2$, which is specifically localized to the plasma membrane. This prevents improper virion assembly at internal membranes. The MA domain of Gag also localizes HIV to budding lipid rafts and incorporates the glycosphingolipid GM3 into the envelope, which is later recognized by CD169/Siglec-1 on dendritic cells (DCs) to enhance trans-infection of $CD4^+$ T cells. The Gag proteins aggregate, causing curvature in the plasma membrane and initiation of budding. Some retroviruses, including betaretroviruses, assemble in the cytoplasm. The assembled virions are then transported to the plasma membrane for envelopment and release.

The SU and TM trimers of heterodimers are incorporated via contacts between the cytoplasmic tail of TM and the N-terminus of Gag, although there are likely additional mechanisms involved. HIV incorporates Vpr into virions at a high level, roughly equimolar with Gag. The recruitment of Vpr into virions requires the Gag p6 domain, although the function of Vpr remains unclear. Host proteins are also incorporated into virions, including Cylophilin A, which has many different effects on Gag stabilization and disassembly and can promote or reduce retroviral infectivity depending on the experimental context.

The proper packaging of the gRNA represents a challenge for retroviral assembly, as the gRNA comprises only a small fraction of the total cytoplasmic RNA in the host cell, and many viral subgenomic RNAs are also present and must be excluded from the assembling virion. The gRNA is packaged as a dimer, which is usually a homodimer of two identical gRNAs, except in rare cases where coinfection or expression of an endogenous retrovirus causes packaging of an RNA heterodimer. Packaging is mediated by the Psi (Ψ) sequence in the 5′ UTR, which interacts specifically with the NC subunit of the Gag precursor. In the absence of a Psi signal, virions package a random sampling of host mRNAs, and non-gRNAs can be packaged if they are engineered to contain a Psi sequence. HIV-1 splices the Psi sequence out of the subgenomic RNAs to prevent packaging interference with the gRNA. HIV-2 and ALV subgenomic RNAs retain Psi and must regulate packaging by other means. It has been proposed that a Gag precursor protein binds to the gRNA from which it was translated immediately posttranslation, thus ensuring that Gag assembly only occurs around unspliced, Gag-encoding gRNAs. The gRNA binds the Gag precursor protein in different subcellular locations for different retroviruses. MLV and RSV bind Gag in the nucleus, MMTV and MPMV in the pericentriolar region, FIV binds Gag at the nuclear membrane, and HIV-1 binds Gag in the cytoplasm. Gag is then responsible for the trafficking of gRNA to the assembly site for packaging.

Psi functions via cis-acting structural elements of the RNA, including stem loop structures containing purine-rich loops. For HIV-1, stem-loops 1 and 3 (SL1 and SL3) are especially important for packaging. For MLV, the 4 stem loops of Psi are referred to as A-D, where A and B facilitate dimerization of the gRNA, while C and D interact with the NC subunit of Gag precursors to encourage encapsidation.

Dimerization of the genome

Dimerization of the two packaged gRNAs is mediated by interactions in a complementary sequence near the 5′ end of the genome called the dimerization initiation site (DIS). For HIV, the DIS is in SL1. The DIS and *gag* start codon are both near the 5′ end of the viral RNA and determine whether the RNA dimerizes for packaging as a gRNA or is translated to produce Gag or Gag-Pol proteins. Changes in the secondary structure of this region occur in many retroviruses, allowing either the DIS or the *gag* start codon to be accessible, but not both, distinguishing the gRNA from the mRNA. When the DIS of two gRNAs bind, they form the dimer linkage structure (DLS). The NC subunit of the Gag precursor protein binds the DLS with high specificity, initiating Gag multimerization and assembly. These interactions are more specific than the generic affinity of NC for RNA, ensuring that dimerized gRNAs are packaged. Other domains of the Gag precursor may be involved in this interaction as well. The dimerized gRNAs are noncovalently associated and do not form significant base-pairing interactions, and dimerization is stabilized during virion maturation after release from the producer cell.

The timing of gRNA dimerization varies among the retroviruses. MLV dimerization initiates in the nucleus shortly after transcription, which increases the likelihood that identical gRNAs are copackaged as a homodimer. HIV-1 dimerization, on the other hand, initiates in the cytoplasm, increasing the likelihood that the dimerizing gRNAs will not be identical, and increasing the rate of recombination for HIV-1 up to 10-fold relative to MLV.

Dimerization has several purported benefits for retroviral replication. Nicks in the gRNA are common, and proper completion of reverse transcription would be impossible from a single nicked genome. Copackaging a second copy can greatly increase the fidelity of reverse transcription by increasing the likelihood that an intact template exists for each region of the genome. Dimerization also distinguishes the gRNA from spliced mRNA, as only dimerized gRNA has the DLS as a signal for Gag recruitment and assembly. Furthermore, recombination can drive evolution, as there are approximately 3–10 strand transfers per replication cycle. Retroviruses have higher rates of recombination than other RNA viruses, which may contribute to the adaptability of the virus. As spumaviruses perform reverse transcription prior to packaging, it is unclear whether they require dimerization.

Budding and Maturation

Gag precursors assemble at the plasma membrane, inducing membrane curvature and initiating the budding process. The p6 domain of the HIV-1 Gag precursor recruits the endosomal sorting complexes required for transport (ESCRT) machinery by binding

to TSG101 and ALIX, and the NC domain of Gag may be involved in ESCRT recruitment as well. ESCRT machinery is responsible for release of the immature virion.

Upon virion release, the viral PR enzyme cleaves itself out of its precursor form, which varies depending on the retrovirus. PR acts as a homodimer and subsequently cleaves the remaining Gag and Gag-Pol precursors in hydrophobic regions, triggering a number of events that generate the mature, infectious virion. Prior to PR processing of the precursors, retroviral virions are immature and noninfectious. Thus, mutations to or inhibition of PR can prevent retroviral infection.

Processing of Gag precursor

The MA protein remains bound to the inner face of the membrane via the N-terminal myristyl group. It may interact with TM for proper incorporation of Env trimers into the virion, and is referred to as p17 in HIV-1. The CA protein contains the major homology region and is conserved among all retroviruses. Upon cleavage, CA undergoes a dramatic rearrangement that is visible by electron microscopy, forming the condensed inner capsid core. The shape of the capsid core depends on the individual virus, but it is made up of CA-CA interactions that form primarily hexamers and some pentamers. For HIV-1, the CA protein is called p24. The NC protein is small and highly basic. After release from the Gag precursor, the basic NC coats the entire gRNA, with one NC protein for every 5–8 nucleotides. This is important for successful reverse transcription, as NC likely helps RT to proceed through regions with significant secondary structures, and also facilitates the various strand transfers that take place. NC processing results in pbs remodeling, exposing the 18 nucleotides with perfect complementarity to the 3′ end of the primer tRNA and allowing binding of the primer tRNAs to the pbs of each gRNA. This sets the stage for reverse transcription upon infection of a new target cell. For HIV-1, NC is referred to as p7.

Processing of Gag-Pro-Pol precursor

The Gag-Pol precursor is processed by PR into RT, IN, and the Gag subunits described above in all retroviruses, and additionally DU in some retroviruses. The functions and structures of RT and IN have been described above. DU is an enzyme that prevents the incorporation of dUTP during viral DNA synthesis by hydrolyzing dUTP to dUMP, reducing the amount of available dUTP relative to other dNTPs. DU is part of the *pro* ORF for betaretroviruses and the *pol* ORF of the nonprimate lentiviruses. Bovine lentivirus encodes an inactive DU, while the primate lentiviruses do not encode DU.

Host Response to Retroviral Infection

As retroviruses have been circulating in vertebrate genomes throughout evolutionary history, the host species have evolved a number of mechanisms to defend against retroviral infections, involving both innate and adaptive immune responses. Likewise, retroviruses have evolved to counteract these defense mechanisms, which is the role of many of the accessory proteins encoded by the complex retroviruses. This evolutionary race determines the tropism of the retroviruses, as each species is able to prevent infection by all but a few specific viruses that have evolved to overcome these restrictions in a species-specific manner.

Innate Immunity

The APOBEC3 family of host proteins are packaged into retroviral virions in a NC-dependent manner and restrict multiple retroviruses, including HIV and HTLV, by inducing hypermutation. Although APOBEC3G is the most widely-studied, other APOBEC3 family members, including 3B, 3D, 3F, and 3H are also active against HIV. APOBEC3G is a cytidine deaminase that deaminates cytidine nucleotides, generating uracil in the minus-strand ssDNA after RNA degradation but before plus-strand synthesis, resulting in a G-A mutation in the plus-strand DNA. It is expressed at high levels in human $CD4^+$ T cells and macrophages, the primary targets of HIV infection. This may explain the presence of the central PPT of HIV-1, which hastens positive-strand synthesis and reduces the exposure of the minus-strand DNA to APOBEC3. Vif, an accessory protein encoded by almost all lentiviruses, counteracts APOBEC3 proteins by inducing their polyubiquitination and proteasomal degradation, preventing their packaging in budding virions.

During reverse transcription, retroviruses generate RNA-DNA, ssDNA, and dsDNA in the cytoplasm of the host cell, none of which are present in the cytoplasm during normal host cell processes. As with other viruses, many sensors of retroviral nucleic acids have been described, including cGAS and IFI16. Cytosolic DNA is detected by cGAS, which interacts with STING to signal the production of interferon-β (IFN-β) via IRF3. HIV evades cGAS, however, by recruiting the host TREX1 protein to degrade viral DNA products that are susceptible to sensing by cGAS. IFI16 also senses cytoplasmic DNA, acting via STING to induce IFN-β. For HIV-1, this pathway may contribute to loss of $CD4^+$ T cells and progression to AIDS, as pyroptotic $CD4^+$ cell death following abortive HIV-1 infection requires IFI16.

SAMHD1 is a host protein that restricts HTLV and HIV-1 in some cell types, such as DCs, macrophages, and resting $CD4^+$ T cells, by depleting the cellular pool of dNTPs needed for cDNA synthesis. With reverse transcription unable to proceed to completion, the intermediates can be recognized by innate immune receptors like cGAS to activate an antiviral response. SAMHD1 is also likely to play a role in preventing retrotransposon activity. HIV-2 and SIV overcome this restriction by encoding the accessory protein Vpx, which recruits the E3 ubiquitin ligase DCAF1-DDB1 to induce degradation of SAMHD1. Vpx is absent in HIV-1, allowing SAMHD1 to reduce HIV-1 infection of SAMHD1-expressing cells, including DCs, resting $CD4^+$ T cells and, to a lesser extent, macrophages.

SERINC3 and SERINC5 were recently discovered as membrane-bound restriction factors of HIV-1, which are incorporated into the virion to reduce particle infectivity. HIV-1 counteracts this restriction with the accessory protein Nef, which redirects SERINC3 and SERINC5 away from the cell surface to prevent their incorporation into virions. Nef also facilitates HIV-1 infection in a number of other ways, including redirecting MHC-I to the lysosome for degradation to prevent antigen presentation to $CD8^+$ T cells and internalizing CD4 from the cell surface to prevent superinfection and Env interactions with CD4 during virion budding.

The TRIM5α protein, which was previously called Ref1 and Lv1, binds to the retroviral capsid core and accelerates uncoating in a proteasome-dependent manner, inhibiting reverse transcription and formation of a functional PIC. Trim5α proteins vary from species to species, and the species-specificity of primate lentiviruses is largely determined by their ability to evade restriction by TRIM5α in their native host species, but not in other primates. Fv1 is an additional restriction factor found in mice, which targets residues of Gag in gammaretroviruses. The restriction occurs after reverse transcription but before integration.

Tetherin, also known as BST2, tethers budding retroviral virions to the cell surface, preventing virion release and leading to internalization and degradation. HIV-1 overcomes the tetherin restriction with the accessory protein Vpu, which prevents tetherin from reaching the cell surface and leads to its degradation. HIV-2 and SIV do not encode Vpu, but SIV Nef facilitates the AP-2-dependent internalization of tetherin at the cell surface, while the HIV-2 Env protein is sufficient for HIV-2 to overcome tetherin restriction.

Other restriction factors that have recently been described for HIV-1 include MxB/Mx2, which is IFN-inducible and inhibits nuclear import or uncoating, and IFITM1, which is a plasma membrane protein preventing fusion and entry. The specific mechanisms of action for these recently-discovered restriction factors are still being elucidated.

Adaptive Immunity

$CD8^+$ T cells represent the primary antiviral effectors of cellular adaptive immunity, and massive HIV-specific $CD8^+$ T cell responses are observed shortly after the initial detection of HIV RNA in the plasma. While these responses shape the viral landscape and motivate viral evolution, they are unable to successfully control the infection in most individuals, and fail to clear the virus entirely. These $CD8^+$ T cells eventually become exhausted, losing their ability to control the evolution and spread of the virus. HIV-1 and HIV-2 also encode the accessory protein Nef, which removes MHC-I from the surface of infected cells to prevent presentation of viral peptides and recognition by HIV-specific $CD8^+$ T cells. A small subset of individuals known as elite controllers naturally suppress HIV-1 viral load and prevent progression to AIDS, and this control is due to their unusually effective $CD8^+$ T cell responses.

Humoral immunity to HIV-1 and SIV is also observed, with both neutralizing and nonneutralizing antibodies generated against the virus within the first 2 weeks of infection. The earliest detectable antibodies recognize Gag antigens, followed by those from Nef, Rev, and Env. Neutralizing antibodies predominantly target the HIV-1 Env receptor binding domains: the V1, V2, and V3 loops and the CD4-binding domain of the HIV-1 gp120 (SU). They interfere with the ability of the virus to interact with the receptor and prevent entry into the target cell. As V1, V2, and V3 are hypervariable regions of gp120, the virus rapidly evolves to avoid these neutralizing antibody responses, which fail to control spread and suppress viral loads. Neutralizing antibodies isolated from an infected individual often successfully neutralize previous isolates of virus from that individual, but fail to neutralize the virus that is currently circulating. Nonneutralizing antibodies can mediate antibody-dependent cell cytotoxicity (ADCC) and facilitate killing of infected cells by NK cells and macrophages, although the significance of this mechanism in controlling viral replication remains poorly understood.

Some neutralizing antibodies that have undergone extensive somatic hypermutation following years of exposure to high viral loads have developed the capacity to neutralize a broad range of HIV isolates and are known as broadly neutralizing antibodies. While the existence of broadly neutralizing antibodies offers some promise for HIV-1 vaccine development, various vaccine strategies have failed to elicit these responses.

Retroviral Diseases

Cancer

Retroviruses were first discovered when Rous sarcoma virus (RSV) was identified as the etiologic agent of a sarcoma in chickens, which was also the first established link between a viral infection and cancer. Since then, many cancer-causing retroviruses have been described, which can be divided into two categories: acute transforming retroviruses and nonacute transforming retroviruses.

Acute transforming retroviruses

The acute transforming retroviruses are distinguished by the rapid onset of cancer following viral infection, caused by mutated versions of cellular proto-oncogenes carried in their genomes. These genes usually alter cell cycle regulation or susceptibility to apoptosis, and include growth factors, growth factor receptors, tyrosine kinases, G proteins, transcription factors, and other types of genes. RSV encodes *v-src*, the viral oncogene derived from the cellular proto-oncogene *c-src*, whereas MC29 encodes *v-myc*, derived from *c-myc*. The viral oncogenes can be acquired by different means, but the process usually involves insertion of the retroviral genome upstream or in the middle of the transduced gene. Readthrough transcription can generate a chimeric RNA that is spliced to contain only the exons of the cellular proto-oncogene and is subsequently packaged into the virion. Nonhomologous

recombination during reverse transcription results in the acquisition of the cellular proto-oncogene as a viral oncogene. To accommodate the viral oncogene within the size limitations of retroviral genomes, viral components essential for replication are often lost, and the oncogene is often fused to *gag, pol,* or *env*. Despite the fact that many acute transforming retroviruses are replication incompetent on their own, they always retain the necessary cis elements for replication, including LTRs, the pbs, ppt, and Psi sequence. Thus, in the presence of a helper virus, which coinfects the same cell and expresses the essential viral proteins that are lacking, acute transforming retroviruses can be packaged and produce particles capable of single-round infection. In rare circumstances, as with RSV, the viral oncogene can be acquired with no loss of replication competency and no requirement for coinfection with a helper virus.

Nonacute transforming retroviruses

The nonacute transforming retroviruses differ from the acute transforming retroviruses in that they do not encode oncogenes, and thus do not rapidly induce transformation upon infection. Rather, during the course of a persistent infection or multiple infection events, as the virus continues to replicate and integrate proviral genomes into various host cells, random proviral insertional mutagenesis can occur. When the proviral genome integrates near or within a host proto-oncogene or tumor suppressor gene, it can alter the expression or functionality of the gene and ultimately lead to cancer development. There are multiple mechanisms by which this can occur, most often involving the promoters and enhancers in the viral LTR stimulating nearby host cell gene expression in addition to viral gene expression. If the virus integrates immediately upstream of a proto-oncogene, the 3′ LTR promoter can help drive expression of the host gene, generating an altered transcript containing the viral R-U5 sequence at the 5′ end. Proximal insertion to a proto-oncogene can also result in enhancer insertion, where the LTR enhancers facilitate transcription from an endogenous host promoter, generating the native transcript in higher frequencies. Insertional mutagenesis can also transform cells by altering posttranscriptional modifications of host products, or by readthrough transcription from the 5′ LTR, which is most common in mutated proviruses lacking the 3′ LTR stop signal. ALV causes lymphoid tumors in chickens after integrating upstream of *c-myc*, while MLV and FeLV cause leukemias following insertion upstream of a number of described genes. MMTV causes mammary carcinomas and integrates in the vicinity of growth factor genes, including *wnt* and *fgf-3*.

AIDS

By far the most significant retroviral disease burden in humans is the acquired immunodeficiency syndrome (AIDS), which is caused by untreated infection with either HIV-1 or HIV-2. AIDS was first recognized in the United States in 1981, a series of investigators from 1983 to 1984 identified that retroviruses may be the cause of AIDS, and by 1986 these retroviruses were identified as HIV, which was subsequently named HIV-1 upon the discovery of HIV-2. HIV-2 has only 45% sequence homology to HIV-1. HIV-2 differs from HIV-1 in that it encodes Vpx, which counteracts SAMHD1. In addition, HIV-2 lacks Vpu, which downregulates tetherin and CD4, and is less pathogenic than HIV-1. HIV-1 is separated into four groups: M, N, O, and P, of which M is responsible for the majority of infections worldwide. Group M is separated into nine subtypes, of which subtype B is most common in Europe and North and South America, while subtype C is by far the most prevalent subtype worldwide. HIV-2 is divided into eight groups, named A-H. Most clinical HIV research is done on HIV-1, group M, subtype B viruses.

HIV is transmitted primarily through sexual contact or exposure to blood, such as during blood transfusions or intravenous drug use. It can also be transmitted perinatally between mother and child during pregnancy, labor, or breastfeeding. During acute infection, some individuals can experience flu-like symptoms. After an initial peak in plasma viremia is established within a few weeks of exposure, immune responses primarily dependent on HIV-specific $CD8^+$ T cells begin to control the virus, suppressing the plasma viral load to the viral set point, but failing to completely prevent viral replication or clear the infection.

The virus requires its receptor, CD4 plus a coreceptor, either CCR5 or CXCR4, to enter cells. Thus, the virus replicates primarily in $CD4^+$ T cells, which express high levels of CD4. HIV-1 can also infect cells with lower levels of CD4, including macrophages and hematopoietic stem and progenitor cells. Infection of $CD4^+$ T cells leads to cell death, and persistent viremia leads to a steadily decreasing $CD4^+$ cell count during the course of infection. This depletion occurs gradually during a prolonged asymptomatic phase of infection, which can last for several years. AIDS is associated with a suite of rare opportunistic infections and tumors that are typically suppressed by normal immune responses, and these opportunistic infections and tumors are responsible for most AIDS-related deaths.

In addition to infecting CD4-expressing cells, HIV infection can affect other cell types as well. $CD8^+$ T cells and B cells can both be killed by bystander activation. While dendritic cells (DCs) aren't productively infected by HIV-1, as they express high levels of SAMHD1 to restrict the infection, they are susceptible to infection by HIV-2 and SIV, which encode Vpx to counteract SAMHD1. In the context of HIV-1, DCs can still contribute to infection through trans-infection of T cells, in which DCs capture viral particles, maintain their infectivity, and transfer them to T cells at the virological synapse. Initial studies implicated DC-SIGN and similar receptors as essential for trans-infection via DCs, although recent studies have shown that GM3 incorporated in the HIV-1 envelope is recognized by CD169/Siglec-1 to facilitate trans-infection. DCs can also produce interferon during acute infection, and are especially sensitive to HIV-2, where DC infection proceeds sufficiently for cGAS recognition of viral DNA.

Throughout the world, and even within a single individual, HIV demonstrates tremendous genetic diversity. This genetic diversity allows the virus to rapidly evolve to evade host adaptive immune responses, as well as antiretroviral monotherapies. The RT enzyme has a particularly low fidelity among DNA polymerases, which is likely the major contributor to HIV mutagenesis. HIV is especially variable in the exposed regions of the envelope protein. This variability likely benefits the virus in evading the host humoral response, which is directed predominantly against exposed residues of gp120 (SU) and gp41 (TM).

Antiretroviral therapy

The HIV/AIDS pandemic has been radically transformed by the development of effective combination antiretroviral therapy (cART). While HIV rapidly evolves to evade antiretroviral monotherapy, combination therapy is effective in suppressing viral load and dramatically prolonging survival, allowing infected individuals to live relatively healthy lives with an infection that until recently meant almost certain death. Current antiretroviral therapies target several aspects of the viral replication cycle. Nucleoside analogs (NRTIs) function as reverse transcriptase inhibitors by their capacity to be incorporated by RT during DNA synthesis. Since these nucleoside analogs lack the 3′-OH, DNA synthesis cannot proceed upon NRTI incorporation. Resistance to NRTIs occurs when mutations associated with increased efficiency of incorporation of the natural substrates relative to the nucleoside analog are accumulated. Other mutations can allow RT to gain the capacity to excise the unnatural nucleoside after incorporation. While the NRTIs inhibit reverse transcription by mimicking the natural substrates of RT, nonnucleoside reverse transcriptase inhibitors (NNRTIs) function as small, hydrophobic molecules that inhibit RT by an allosteric mechanism. NNRTIs are only effective against RT from subtypes B and C of HIV-1, as most retroviral RT enzymes, including those of HIV-1 group O, HIV-2, and SIV, lack the NNRTI binding pocket and are naturally resistant. Even in HIV-1 subtypes B and C, resistance mutations to NNRTIs develop easily. Integrase strand transfer inhibitors (INSTIs) target HIV integrase, preventing successful integration of the viral DNA genome and promoting the formation of nonproductive intermediates, such as 2-LTR circles. Protease inhibitors mimic the transition state for normal HIV protease substrates and block the enzyme active site. These inhibitors, however, carry particularly burdensome side effects, and thus are not utilized in first-line cART in most cases.

Latency

Despite the success of cART, it does not represent a cure, and infected individuals must remain on cART for the remainder of their lives. Although viral loads are suppressed below clinically detectable levels, viral rebound occurs within a few weeks of cessation of cART. Thus, despite the capacity of cART to virtually eliminate the pool of replicating virus in an individual, latent reservoirs of the virus exist in which an integrated provirus remains transcriptionally silent, evading detection by host immune responses or ART. These latent reservoirs are established early during the course of infection and represent a major barrier to curing HIV-infected individuals.

The current approaches to developing a cure for HIV involve reactivating these latent reservoirs in a so-called shock-and-kill approach. Once the previously latent proviruses are "shocked" to be reactivated, they can be "killed" by viral cytopathic effect or by the host immune system while cART prevents infection of new cells and reseeding of the latent reservoir. There are many such approaches to reactivate latent proviruses, including inhibitors of histone deacetylases, T cell activators, and activators of NF-κB and P-TEFb, although to date none of these approaches have proved successful in significantly reducing the size of the latent reservoir and substantially delaying rebound upon cessation of cART.

HTLV Diseases

HTLV-1 can cause adult T cell leukemia/lymphoma (ATL) or HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in a minority of infected individuals. Despite similarities with HTLV-2, −3, and −4, only HTLV-1 is known to cause disease. HTLV is a complex deltaretrovirus, encoding Tax to enhance gene expression by recruiting CREB to the viral promoter, as well as Rex to enhance export of unspliced and incompletely spliced RNAs. Tax can also be deleterious to the virus when over-expressed, as it is the dominant antigen recognized by effective $CD8^+$ T cell responses. These responses successfully control the infection in most carriers, although deficiencies in $CD8^+$ T cell lytic capacity are observed in HAM/TSP patients. Thus, the virus has evolved to suppress *tax* mRNA transcription with the HBZ accessory protein, while also suppressing nuclear export of *tax* transcripts with p30.

HTLV-1 infects T cells, B cells, and monocytes, and disease is mediated by Tax, which activates NFκB to induce immortality and growth, inhibits p53, p16, and MAD1 to prevent apoptosis, and inhibits tumor suppressive TGF-β signaling, all of which promote oncogenesis. HBZ may also contribute to disease maintenance. This can lead to the onset of adult T cell leukemia/lymphoma, as well as inflammatory disease such as HAM/TSP. Leukemia enhances the spread of the virus, which replicates poorly within the host due to effective $CD8^+$ T cell responses, by causing clonal expansion of infected cells. Rather than free virus particles, which are low in abundance in infected individuals, infected cells mediate transmission of the virus through sexual contact, blood transfusion, needle sharing, or breast milk. Other deltaretroviruses, such as bovine leukemia virus and simian T cell leukemia virus, can also cause lymphoid malignancies, similar to HTLV-1.

Endogenous Retroviruses (ERVs)

Due to their fascinating and unique capacity to integrate the viral DNA genome into the infected cell genome, retroviruses have a distinct potential for endogenization, the process of incorporating viral genetic material into the endogenous host genome through infection of germline cells. Once a retrovirus has been endogenized, it can be transmitted vertically through the population. Upon germline insertion, endogenous retroviruses (ERVs) can drive evolution through insertional mutagenesis, as well as immediately providing new genetic information in the form of the viral genome. Natural selection dictates that only ERVs with nondeleterious insertions persist in the population, and over time ERVs accumulate mutations to lose their pathogenic potential. Eventually some endogenous retroviral sequences can be hijacked for the benefit of the host, as in the example of the syncytin genes, which are derived from retroviral *env* genes and are essential for the formation of the mammalian placenta. The retroviral provirus shares many

similarities with the retroelements found in the genomes of virtually all living things. ERVs represent a common form of retroelement in vertebrates, where hundreds of thousands of copies of ERVs make up 1%–10% of vertebrate genomes. By comparing ERV sequences and integration sites across species, valuable insight into the evolutionary relationships among vertebrate species can be inferred. As retroviral integration site selection is virtually random, identical integration sites of ERVs between species or individuals imply that the exogenous retrovirus was endogenized in a common ancestor.

ERVs that still contain the *pol* gene can be classified based on the sequence of reverse transcriptase and divided into three classes: Class I ERVs are related to gammaretroviruses and epsilonretroviruses, Class II ERVs are related to alpharetroviruses, betaretroviruses, and lentiviruses, and Class III ERVs are related to spumaviruses. Most ERVs are grossly defective, containing numerous stop codons or frameshift mutations, and they are frequently transcriptionally silenced by various host mechanisms, including heavy methylation of the surrounding DNA. The vast majority of ERVs, known as solo-LTRs, have experienced a drastic recombination event between the two LTRs, removing all of the viral sequence and preserving only a single LTR.

Defective ERVs can still be transcribed to produce RNAs under conditions of cellular stress or differentiation, and these RNAs can be packaged in the context of an exogenous retroviral infection, so long as they maintain the necessary cis-acting packaging signals. ERVs that maintain some functionality can replicate if the essential missing functions are provided in trans by other ERVs, LTR retrotransposons, or exogenous retroviruses. The frequency with which this occurs is dependent on the ERV. Human ERVs are predominantly silent, with no actively-replicating members described. Koala retrovirus, however, is currently in the process of endogenization, with both exogenous and endogenous virus transmitted through the population, and with endogenous viruses likely spreading by both vertical and horizontal transmission.

Gene Therapy Vectors

The capacity of the viral genome to stably integrate into the host cell genome has made retroviruses a particularly attractive candidate for gene therapy delivery vectors. Successful integration can lead to sustained, persistent expression of the transgene throughout the life of the cell and all its progeny. Retroviruses are also particularly amenable to pseudotyping with different envelope proteins, which allows for selection from a wide range of tropisms to deliver the vector to particular target cells within the patient. Early clinical trials using MLV vectors to treat X-linked severe combined immunodeficiency (X-SCID) were successful, but one fourth of the patients developed clonal T cell leukemias resulting from integration-induced activation of a cellular proto-oncogene. The recent determination that MLV preferentially integrates near transcription start sites (TSS), increasing the likelihood of such outcomes, has led to the design of retroviral vectors that minimize undesirable outcomes.

Lentiviruses tend to integrate in the middle of actively transcribed genes, limiting the risk that they will activate transcription of a silent gene or integrate near a TSS. In addition, lentiviruses can infect nondividing cells, which makes them a preferred vector for targeting nonproliferative cell types. Thus, lentiviral vectors are the predominant retroviral gene therapy vectors in development today. These efforts focus on the use of replication-incompetent viruses, lacking some combination of *gag*, *pol*, and *env*, which are provided in *trans* in the producer cells to generate gene delivery vectors capable of only a single-round infection. However, the risk remains that recombination could occur in the producer cell, especially with cross-packaging of endogenous retroviral genomes, which could result in the production of a replication-competent virus. While such events are exceedingly rare, the risk of infecting an individual with a replication-competent lentivirus is an important consideration in developing safe retrovirus-based gene therapy approaches.

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Horizontal Gene Transfer: Uptake of Extracellular DNA by Bacteria☆

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Glossary

Extracellular DNA Extracellular DNA (also called naked, free, ambient, or environmental DNA) are DNA fragments released into the environment from decomposing cells, disrupted cells, or viral particles, or via excretion from living cells.
Heterogamic/interspecific transformation Heterogamic/interspecific transformation leads to recombination between chromosomal DNA from different species.
Homogamic/intraspecific transformation Homogamic/intraspecific transformation leads to recombination between chromosomal DNA within species.
Recipient Recipient is a bacterial cell that has the potential to acquire DNA by natural transformation.
Synteny Synteny describes the observation of shared presence and order of genes on the chromosomes of related species. Disruption of synteny due to genetic rearrangements may reduce recombination rates within and between the species.
Transformant Transformant is a bacterial cell that has successfully acquired DNA by natural transformation.
Transformation frequency Transformation frequency is usually given as the number of transformant bacteria divided to the total number of recipient bacteria of a given species over a given time period.

Abbreviations

CDS	Coding sequence
GMM	Genetically modified microorganism
GMO	Genetically modified organism
HFIR	Homology-facilitated illegitimate recombination
HGT	Horizontal gene transfer
MEPS	Minimum efficient processing segment
MR	Mismatch repair
RM	Restriction modification
SSBP	Single-strand binding proteins
USS	Uptake signal sequences

Defining Statement

Here we present experimental models that examine the uptake of chromosomal DNA in bacteria and discuss some of the advantages and limitations of the models used. The relationship between DNA uptake frequencies versus selection is examined, and the implications for the open release of genetically engineered microbes are discussed.

Process of Natural Uptake of Extracellular DNA in Bacteria

Extracellular DNA released from a donor organism can be "horizontally" acquired by bacteria (recipients) through the process of natural transformation. Natural transformation occurring with chromosomal DNA fragments can be divided into several steps (Thomas and Nielsen, 2005):

☆*Change History:* February 2016. S Domingues and KM Nielsen added an abstract and keywords and revised and updated text throughout chapter. References to more than 170 scientific studies were added. A new Table (1) is included and the previous Table 1 is now Table 2. The sub-sections "HGT in bacteria belonging to the microbiome" and section "Clinical relevance of HGT and antibiotic resistance" were added and the section on gene transfer in food was expanded with input from M. Woegerbauer. The many revisions lead to changes in authorship and we thank P. J. Johnsen and J. L. Ray for contributions to the earlier version of this chapter.

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1. Development of competence (physiological state for DNA uptake) in the recipient bacterium simultaneously with exposure to transforming DNA;
2. DNA uptake/active translocation into the bacterial cytoplasm;
3. Heteroduplex formation of transforming DNA with similar DNA sequences in the recipient chromosome;
4. Homologous recombination-mediated integration of the transforming DNA strand into the recipient bacterium's chromosome;
5. Expression of the acquired trait(s) leading to an altered recipient phenotype; and
6. Stable inheritance and maintenance of the acquired trait(s) at the individual recipient cell level and at the bacterial population scale level.

Although the last two steps are not strictly part of the transformation process, they are usually an integral part of most natural transformation studies in bacteria. Extracellular DNA is only accessible to naturally transformable bacteria when they are in a competent state. Competence is a genetically encoded physiological state in which bacteria express protein complexes that facilitate uptake of DNA into their cytoplasm (Dubnau, 1999; Chen and Dubnau, 2004; Claverys and Martin, 2003). In bacteria such as *Streptococcus pneumoniae* and *Bacillus subtilis*, competence is under tight regulation by a cell-to-cell signalling pathway; for other bacteria, such as *Neisseria gonorrhoeae* and *Helicobacter pylori*, DNA can be taken up during all phases of growth (Seitz and Blokesch, 2013). Competence development is usually linked to particular growth conditions or perturbation of those conditions. For instance, *Acinetobacter baylyi*, a soil and water bacterium, achieves maximum competence for DNA uptake during growth at early and late exponential phase (Palmen et al., 1993; Ray and Nielsen, 2005). In *B. subtilis*, random peak levels of the ComK global regulator protein trigger transition to a competent stage (Maamar et al., 2007). Bacteria that can express natural genetic competence are found in diverse habitats, and it is suspected that discovery of more naturally competent species and strains will continue with further improvement of culture- and nonculture-based methods of detection of DNA uptake (Claverys and Martin, 2003; Johnsborg et al., 2007). The reasons for the existence of DNA uptake/integration mechanisms in bacteria are still debated; incoming DNA fragments may be used for nutrition after further degradation, as a template for recombinational repair of DNA, for movement or for the recombination of existing variation in the population and generation of new genetic variation of which some may accelerate adaptation (Redfield, 1993; Chen and Dubnau, 2004; Bakkali, 2013). Natural transformation may serve different functions in different bacterial species, as evidenced by expression of competence at dissimilar stages in their life cycles and to varying extents (Baltrus and Guillemin, 2006; Dorer et al., 2010; Johnston et al., 2014).

The model organisms *A. baylyi*, *B. subtilis*, *Haemophilus influenzae*, *H. pylori*, *N. gonorrhoeae*, *S. pneumoniae*, and *Vibrio cholerae* have provided most of the information about the molecular aspects of the natural transformation process (Johnston et al., 2014). The DNA translocation apparatus is a putative pore-forming multimeric protein complex that spans the inner membrane/periplasm/outer membrane in Gram-negative bacteria, and the inner membrane/cell wall in Gram-positive bacteria (Averhoff and Graf, 2008; Chen and Dubnau, 2004; Kruger and Stingl, 2011). The DNA uptake machineries of Gram positive and Gram negative bacteria are remarkably similar. Both DNA uptake and processing relies on a comparable arsenal of competence (*com*) genes. The expression of *com* regulons is nevertheless controlled by a variety of competence regulators suggesting different triggers of competence development (Johnston et al., 2014). Extracellular DNA first binds to the translocation apparatus on the cell surface by a poorly understood mechanism. While *N. gonorrhoea* (Graves et al., 1982) and *H. influenzae* (Sisco and Smith, 1979) require specific uptake signal sequences (USS) for successful DNA binding and translocation, the majority of bacteria will bind extracellular DNA non-discriminatory with respect to sequence. Natural transformation can occur by both linear and circular donor DNA (Thomas and Nielsen, 2005). Structural proteins associated with extracellular DNA do not hinder the DNA-binding or uptake process, as bacterial cell lysates also efficiently transform competent bacteria (Nielsen et al., 2000a). Although the DNA is initially double-stranded, it enters the bacterial cytoplasm in single-stranded (ssDNA) form, providing evidence for an endonuclease within, or associated with, the membrane-bound translocation apparatus that degrades one strand during DNA uptake (Chen and Dubnau, 2004; Johnston et al., 2014). The uptake of DNA by competent cells is directional (Barany et al., 1983), and occurs at rates of up to 100 bp s^{-1} (Mejean and Claverys, 1993). Studies on the size of chromosomal DNA substrates in bacteria have demonstrated that the initial DNA fragment size is not correlated to uptake efficiency but to integration efficiency. Longer DNA fragments are more likely to yield detectable transformants in *in vitro* assays than shorter fragments (Palmen and Hellingwerf, 1997; Pifer and Smith, 1985). In some cases, part of the translocated DNA can be degraded in the cytoplasm prior to chromosomal integration; for example, approximately 500 and 1500 bp at each end of the linearized strand are degraded in *A. baylyi* (Palmen and Hellingwerf, 1997) and *H. influenzae* (Pifer and Smith, 1985), respectively.

Few details are available on the molecular interactions of ssDNA once it enters the cytoplasm of studied bacterial species. There is evidence that single-strand binding proteins (SSBP) or other proteins (eg, RecA) bind to the DNA fragment and provide protection from rapid nucleolytic degradation (Claverys et al., 2009). Although the majority of DNA molecules that enter the bacterial cytoplasm will be degraded, some of the DprA and RecA-associated ssDNA may subsequently pair with the bacterial chromosome by homology-guided base-pairing at the cognate locus in the recipient (Berge et al., 2003; Johnston et al., 2014). For heterogamic transformation (ie, recombination between chromosomal DNA from different species), the formation and stability of the heteroduplex molecule formed by donor strand base-pairing is determined by the degree of sequence similarity between the donor and recipient DNA. If the incoming chromosomal DNA is less than approx. 30% divergent from the recipient genome, integration by homologous recombination may occur into the host genome (de Vries and Wackernagel, 1998; de Vries et al., 2001; Majewski and Cohan, 1998; Majewski et al., 2000; Matic et al., 1997; Vulic et al., 1997). Lack of DNA similarity and, hence, stable heteroduplex formation is, for example, the most likely hindrance to interspecific recombination in *Bacillus* spp. (Roberts and Cohan, 1993), *Streptococcus* spp. (Majewski et al., 2000), and *Acinetobacter* spp. (Ray et al., 2009).

The most common type of recombination in bacteria is thought to occur when sequence similarity between the donor and recipient DNA is uniformly present over the entire heteroduplex region. In cases where the donor and recipient sequences are identical along their entire lengths, recombination with the donor DNA strand will not produce a detectable genetic or phenotypic change. In cases where some minor sequence dissimilarity between donor and recipient exists, successful transformation events yield the acquisition of donor polymorphisms in transformants (ie, allelic replacement). This process is called substitutive recombination, as the resulting DNA sequence in a transformant originates by substitution of the recipient sequence with (parts of) the donor sequence. Such substitutive recombination leads to mosaic gene formation when the recombining regions are of limited size. Such recombination events do not introduce major insertions or deletions and typically do not disrupt the overall coding sequence (CDS) and reading frame if a protein-encoding gene is present in the recombined region.

A second type of recombination process occurring in natural transformation is called additive integration. Such a recombination process occurs when a defined DNA sequence present only in the donor is flanked on both sides by sequences common to both donor and recipient bacteria. Heteroduplex formation occurring at flanking regions with high DNA similarity results in recombination of those sequences, as well as integration of the intervening foreign sequence, into the recipient chromosome. Similarly, recombination at homologous flanking segments can lead to loss of DNA (eg, IS elements or prophages) in the recipient.

Additive or substitutive integration may also occur in the presence of only one-sided homology/DNA similarity between donor and recipient. Such homology-facilitated illegitimate recombination (HFIR) allows additive integration of an incongruous DNA sequence via a recombinational anchor of homologous/similar sequence at one end of the invading DNA strand and a random microhomology (3–10 nucleotides) at the other end. HFIR events often result in nonspecific deletion of a recipient DNA sequence located between the anchor and the downstream microhomology. HFIR has been demonstrated as a mechanism of foreign DNA integration by natural transformation in *A. baylyi* (de Vries and Wackernagel, 2002), *S. pneumoniae* (Prudhomme et al., 2002), and *Pseudomonas stutzeri* (Meier and Wackernagel, 2003). The activity of the DNA maintenance machinery, combined with mutations and selection of the host chromosome, may over time produce a nucleotide composition including codon usage of the integrated fragment resembling that of the recipient. Also, a gradual elimination of DNA stretches may occur as a result of the combined outcome of recombination and population scale processes (mutations-deletions, genetic drift and negative selection).

A fourth type of recombination, illegitimate recombination, can occur also in the absence of DNA sequence similarity (eg, <70% DNA similarity) between the donor and the recipient bacteria (Hulter and Wackernagel, 2008). However, in contrast to many eukaryotic systems, illegitimate recombination of nonmobile chromosomal DNA in competent bacteria is rarely reported. Random insertion of foreign DNA in bacterial genomes most often results in a reduction in the relative fitness of the transformant (Elena et al., 1998). It can, therefore, be hypothesized that such events are extremely rare in bacterial populations and the forces modulating recombination frequencies in bacteria are complex (Fig. 1).

Natural transformation by DNA fragments encoding their own mobility and stabilization functions (eg, mobile DNA such as plasmids) may occur independent of RecA and recombination with the bacterial chromosome as these can recircularize and replicate in the bacterial cytoplasm. The stable uptake of plasmid DNA in competent bacteria usually occurs at lower frequencies than the integration of chromosomal DNA fragments due to reassembly constraints on the plasmid fragments in the cytoplasm (Skippington and Ragan, 2011; Thomas and Nielsen, 2005). Recently, it was also shown that very short DNA fragments (<100 bp) could recombine with the bacterial genome in a recA independent manner (Overballe-Petersen et al., 2013). Additionally, transposition of mobile elements like transposons and composite-transposons can also occur in the bacterial cytoplasm after uptake of DNA fragments containing these mobile genetic elements (Domingues et al., 2012c). Thus, recent studies suggest several types of DNA fragments can become heritably stable after uptake in the bacterial cytoplasm even in the absence of DNA sequence similarity, RecA or an origin of replication.

The resulting recombined locus in the transformant bacterium may contain genetic alterations in existing noncoding (eg, regulatory) or protein-coding sequence, or larger alterations such as changed gene composition, gene order (synteny), and so on. It is noted that single DNA exposure event/transformation period may lead to multiple recombination events in a single bacterial genome (Mell et al., 2011). This can occur when the DNA source is for instance genomic DNA from the same bacterial species as the competent recipient bacterium. The various combinations of these factors may result in favourable, unfavourable, or

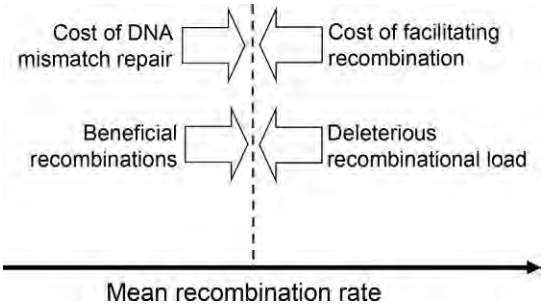

Fig. 1 Recombination frequencies may vary according to the metabolic (fitness) costs and benefits for the transformant. Recombination events can increase transformant fitness via rare acquisition of novel beneficial traits. Recombination rates are kept low by the high deleterious recombinational load associated with accumulation of deleterious sequences and the costs associated with production and maintenance of DNA translocation/recombination machinery. Recombination rates may be raised by the metabolic costs associated with frequent removal of unsuccessful recombination intermediates by DNA mismatch repair.

near-neutral fitness changes to the transformed bacterium that will ultimately determine the impact and the survival of the transformant in a dynamic bacterial population over time (Andersson, 2003).

Uptake of Extracellular DNA by Bacteria Present in Various Environments

Extracellular DNA molecules are released into the environment from decomposing cells, disrupted cells, or viral particles, or via excretion from living cells (Thomas and Nielsen, 2005). Release of intact DNA from decomposing cells depends on the activity and location of intracellular nucleases and reactive chemicals. DNA released from dead cells will be usually associated with the constituents of the cell cytoplasm, membrane, and DNA-binding proteins. Additionally, there are some bacterial species that trigger active release of DNA by killing noncompetent cells, a phenomenon called fratricide (Claverys et al., 2007; Johnsborg et al., 2008).

The first indication of the capacity of bacteria to take up extracellular DNA was published in 1928 by Griffith, when avirulent nonencapsulated *Streptococcus pneumoniae* in mice were "transformed" into the virulent, capsulated form after exposure to a capsulation "factor" from heat-killed virulent cells (Griffith, 1928). The experiments of Avery, MacLeod, and McCarty in 1944 identified as the transforming factor in Griffith's experiments (Avery et al., 1944). Natural transformation studies of *B. subtilis* (Anagnostopoulos and Spizizen, 1961; Spizizen, 1958) and *H. influenzae* (Alexander and Leidy, 1951; Goodgal and Herriott, 1961) followed. Researchers then expanded studies to investigate interspecific genetic exchange in *Streptococcus* spp. (Majewski et al., 2000; Perry and Kuramitsu, 1981), between *Haemophilus* spp. (Albritton et al., 1984), and between different species of *Bacillus* (Harford and Mergeay, 1973; Majewski and Cohan, 1999). The ability to take up extracellular DNA by natural transformation has since been detected in a range of divergent subdivisions of bacteria, including representatives of the Gram-positive, cyanobacteria, *Thermus*, *Deinococcus*, green-sulfur bacteria, and numerous Gram-negatives (Johnsborg et al., 2007; Lorenz and Wackernagel, 1994; Paget and Simonet, 1994). Examples of naturally competent bacterial species are presented in Table 1. There is also evidence that some archaea could undergo transformation (Johnsborg et al., 2007), as well as the single-celled eukaryote *Saccharomyces cerevisiae* (Nevoigt et al., 2000). The phylogenetically widespread ability to acquire genetic information by natural transformation suggests that it may be functionally important in the environment. The limited number of transformable species identified may be explained by an overall lack of competence in the test populations, inability to obtain sufficient testable population sizes and time scales in the laboratory, or inability to recreate competence-inducing conditions as they occur under natural conditions (Johnsborg et al., 2007).

Uptake of DNA by Soil Bacteria

A number of divergent bacterial species present in soil, including *A. baylyi*, *B. subtilis*, *P. stutzeri*, and *Thermoactinomyces vulgaris* are known to be naturally transformable (Hopwood and Wright, 1972; Paget and Simonet, 1994). So far, all the published studies with soil-derived bacteria have been conducted in laboratory microcosms with sterile or nonsterile soil samples. Often, the soils have been amended with clay minerals or nutrients prior to or during the transformation experiments. Natural transformation of bacteria in the field remains to be shown, possibly due to the experimental challenges in both identifying and quantifying low-frequency events occurring in bacterial communities reaching 10^9 bacterial cells per gram of soil (Mercier et al., 2006; Paget and Simonet, 1994). Some of the first transformation studies on soil reported that the commonly occurring Gram-positive bacterium *B. subtilis* takes up chromosomal DNA when grown in autoclaved potting soil (Graham and Istock, 1978, 1979). Many subsequent studies have investigated natural transformation of *B. subtilis* cells in soil with chromosomal and plasmid DNA (Gallori et al., 1994, 1998). Often, the addition of clay minerals (1–10%, w/w) such as montmorillonite has been shown to increase DNA stability and transformation frequencies (Lee and Stotzky, 1990, 1999). Natural transformation of the Gram-negative *P. stutzeri* cells has been shown in sterile soil slurry with chromosomal DNA (Paget and Simonet, 1997), and in a nonsterile soil microcosm with plasmids, chromosomal DNA, and intact bacterial cells (Sikorski et al., 1998). While most studies in soil microcosms have relied on the addition of purified DNA, natural transformation of the Gram-negative *A. baylyi* has also been shown after the addition of isogenic cell lysates or lysates from *Pseudomonas fluorescens* or *Burkholderia cepacia* (Nielsen et al., 2000a). The detection of DNA uptake was based on recombinational repair of a partially deleted neomycin phosphotransferase gene (*npt*II) in the recipient with a functional *npt*II gene transferred from the donor bacteria. While most studies of natural transformation in soil have relied on the addition of competent cells to the microcosms, some studies have also shown that competence for natural transformation can develop *in situ* (Nielsen and van Elsas, 2001; Nielsen et al., 1997; Nielsen et al., 2000a, b).

Several investigations have explored the possibility that plant transgenes, such as antibiotic resistance markers, can be released from decaying genetically modified plants and be exposed to competent soil- and plant-associated bacteria. Short fragments of plant DNA have been shown to remain stable for up to several years in agricultural soil (Nielsen et al., 2007). Soil microcosms have been used to investigate the possible transfer of the kanamycin and hygromycin resistance genes (*npt*II, *hpt*) from tissue homogenates of transgenic tobacco (*Nicotiana tabacum*) plants into indigenous soil bacteria (Becker et al., 1994). However, no bacterial transformants could be recovered. This investigation hypothesized that any integration of the plant marker genes into the genome of exposed soil bacteria would take place after illegitimate recombination. The only studies that show uptake of DNA fragments isolated from transgenic plants into a soil-residing bacterium utilize a recipient strain with inserted sequence similarity to the plant marker gene *npt*II (de Vries and Wackernagel, 1998; Nielsen et al., 2000b). For example, using this system, Nielsen and colleagues (Nielsen et al., 2000b) have shown that the uptake of the plant marker gene in the bacterium *A. baylyi* in a sterile soil microcosm occurred at frequencies of 1×10^{-7} transformants per plant-harboured copy of the *npt*II gene. The amount of DNA added was

Table 1 Some examples of bacterial species that have been reported to be naturally transformable

Bacterial species	*Reference*
Achromobacter spp.	Juni and Heym (1980)
Acinetobacter baumannii	Ramirez et al. (2010)
Acinetobacter baylyi (previously *Acineobacter calcoaceticus*)	Vaneechoutte et al. (2006)
Acinetobacter nosocomialis	Carruthers et al. (2013)
Actinobacillus actinomycetemcomitans	Tonjum et al. (1990)
Actinobacillus pleuropneumoniae	Bosse et al. (2004)
Aggregatibacter aphrophilus (previously *Haemophilus aphropilus*)	Tonjum et al. (1990)
Azotobacter vinelandii	Page and Sadoff (1976)
Bacillus amyloliquefaciens	Coukoulis and Campbell (1971)
Bacillus licheniformis	Gwinn and Thorne (1964)
Bacillus mojavensis	Roberts et al. (1994)
Bacillus subtilis	Graham and Istock (1978)
Bradyrhizobium japonicum (previously *Rhizobium japonicum*)	Mareckova (1969)
Campylobacter coli	Wang and Taylor (1990)
Campylobacter jejuni	Wang and Taylor (1990)
Cardiobacterium hominis	Tonjum et al. (1985)
Chlorobium limicola	Ormerod (1988)
Chlorobium tepidum	Frigaard and Bryant (2001)
Deinococcus radiodurans (previously *Micrococcus radiodurans*)	Moseley and Setlow (1968)
Dichelobacter nodosus	Kennan et al. (2001)
Eikenella corrodens	Tonjum et al. (1985)
Escherichia coli	Woegerbauer et al. (2002)
Gallibacterium anatis (previously *Pasteurella anatis*)	Kristensen et al. (2012)
Haemophilus influenzae	Goodgal and Herriott (1961)
Haemophilus parainfluenzae	Sisco and Smith (1979)
Haemophilus parasuis	Bigas et al. (2005)
Helicobacter pylori (previously *Campilobacter pylori*)	Tsuda et al. (1993)
Kingella denitrificans	Weir and Marrs (1992)
Kingella kingae (previously *Moraxella kingae* or *M. kingii*)	Bovre and Froholm (1972)
Legionella pneumophila	Stone and Kwaik (1999)
Leuconostoc carnosum	Helmark et al. (2004)
Methylobacterium organophilum	O'Connor et al. (1977)
Moraxella catarrhalis (previously *Branhamella catarrhalis*)	Luke et al. (2004)
Moraxella ovis (previously *Neisseria ovis*)	Bøvre (1967)
Moraxella atlantae	Bøvre et al. (1976)
Moraxella bovis	Juni et al. (1988)
Moraxella osloensis	Juni (1974)
Mycobacterium smegmatis	Norgard and Imaeda (1978)
Neisseria elongata	Bovre and Holten (1970)
Neisseria flava	Catlin and Cunningham (1961)
Neisseria flavescens	Catlin and Cunningham (1961)
Neisseria gonorrhoeae	Dougherty et al. (1979)
Neisseria lactamica	Hoke and Vedros (1982)
Neisseria meningitidis	Catlin (1960)
Neisseria perflava	Catlin and Cunningham (1961)
Neisseria sicca	Catlin and Cunningham (1961)
Neisseria subflava	Catlin and Cunningham (1961)
Nostoc muscorum	Trehan and Sinha (1981)
Oligella urethralis (previously *Moraxella urethralis*)	Juni (1977)
Porphyromonas gingivalis	Tribble et al. (2012)
Pseudomonas sp. WJT-1C (previously *Vibrio* WJT-1C)	Frischer et al. (1996)
Pseudomonas alcaligenes	Carlson et al. (1983)
Pseudomonas fluorescens	Demaneche et al. (2001)
Pseudomonas mendocina	Carlson et al. (1983)
Pseudomonas pseudoalcaligenes	Carlson et al. (1983)
Pseudomonas stutzeri	Carlson et al. (1983)
Psychrobacter immobilis	Juni and Heym (1986)
Ralstonia solanacearum (previously *Pseudomonas solanacearum*)	Bertolla et al. (1997)
Rhizobium meliloti	Courtois et al. (1988)
Rhizobium radiobacter (previously *Agrobacterium tumefaciens*)	Demaneche et al. (2001)

(*Continued*)

Table 1 Continued

Bacterial species	*Reference*
Staphylococcus aureus	Morikawa et al. (2012)
Streptococcus anginosus	Havarstein et al. (1997)
Streptococcus bovis	Mercer et al. (1999)
Streptococcus constellatus	Havarstein et al. (1997)
Streptococcus crista	Correia et al. (1996)
Streptococcus gordonii (Challis)	Pakula and Walczak (1963)
Streptococcus infantis	Ween et al. (2002)
Streptococcus intermedius	Havarstein et al. (1997)
Streptococcus milleri	Havarstein et al. (1997)
Streptococcus mitis	Bensing et al. (2001)
Streptococcus mutans	Perry and Kuramitsu (1981)
Streptococcus oralis	Ronda et al. (1988)
Streptococcus pneumoniae	Avery et al. (1944)
Streptococcus sanguinis (previously *Streptococcus sanguis*)	Gaustad et al. (1979)
Streptococcus thermophilus	Blomqvist et al. (2006)
Streptomyces kasugaensis	Roelants et al. (1976)
Streptomyces virginiae	Roelants et al. (1976)
Synechococcus elongates (previously *Anacystis nidulans*, *Synechococcus* sp. PCC 7942 and PCC 6301)	Shestakov and Khyen (1970)
Synechococcus sp. PCC 7002 (previously *Agmenellum quadruplicatum*)	Stevens and Porter (1980)
Synechocystis sp.	Grigorieva and Shestakov (1982)
Thermoactomyces vulgaris	Hopwood and Wright (1972)
Thermosynechococcus elongatus	Onai et al. (2004)
Thermus aquaticus	Koyama et al. (1986)
Thermus caldophilus	Koyama et al. (1986)
Thermus flavus	Koyama et al. (1986)
Thermus thermophilus	Koyama et al. (1986)
Thiobacillus thioparus	Yankofsky et al. (1983)
Vibrio cholerae	Meibom et al. (2005)
Vibrio parahaemolyticus (previously *Beneckea parahaemolytica*)	Frischer et al. (1990)
Xylella fastidiosa	Kung and Almeida (2011)

several orders of magnitude higher than the concentrations expected to be released from plants under their natural growth cycle. There is no evidence to date for stable incorporation and inheritance of plant DNA in bacteria in the absence of supplied DNA sequence similarity.

Soil is a spatially and structurally heterogeneous environment in which numerous microhabitats exist. The current empirical knowledge of natural transformation in soil has been exclusively collected in soil microcosms. Most often, the studies have relied on external addition of high concentrations of DNA, bacterial cells, nutrients, or clay minerals to either sterile soil (all studies prior to 1997) or nonsterile soil samples. Moreover, the transformation frequencies recorded in soil represent so far a broad distribution across a heterogeneous milieu. The detection of rare natural transformation events under more realistic conditions depends on advances in methodology to allow more sensitive identification and quantification in the locations in soil that are conducive to natural transformation.

Uptake of DNA by Plant-Associated Bacteria

Both transduction and conjugation have been shown to occur on plant surfaces (Kidambi et al., 1994; Lilley and Bailey, 1997). However, few studies have examined horizontal gene transfer (HGT) by natural transformation on plant surfaces or plant tissues. A growing interest in gene transfer mechanisms active in these environments has been stimulated by the presence of engineered genes (transgenes) in genetically modified plants. Candidates for exposure to plant transgenes are bacteria that interact closely with plant cells, for example, epiphytes, endophytes, rhizosphere bacteria, and plant pathogens. Several studies have examined the potential of DNA uptake in plant-associated bacteria (Nielsen et al., 2001). Uptake of plant marker genes has been examined in the plant pathogen *Agrobacterium tumefaciens* in, for example, tobacco crown-galls (Broer et al., 1996), in transgenic potato tubers infected with a pathogenic *Erwinia chrysanthemi* strain (Schluter et al., 1995), and *Ralstonia solanacearum*-infected tomato plants (Bertolla et al., 1999, 2000). Kay et al. (2002) reported successful transformation of *A. baylyi* cells with plastid DNA transplastomic tobacco plants. Transformation occurred *in situ* under greenhouse conditions in plants cocolonized with the bacterial pathogen *Ralstonia solanacearum*. Host plant DNA uptake was later also directly visualized with genes encoding green fluorescent protein

(Rizzi et al., 2008; Pontiroli et al., 2009). The general 1000-fold larger size of a plant genome, as compared with a bacterial genome, effectively dilutes the concentration of particular genes in plant DNA such as the selectable gene examined for transfer (Arumuganathan and Earle, 1991; Bertolla et al., 2000). Transgenes inserted into plant organelles may have higher copy numbers and higher sequence similarity to bacterial chromosomes than transgenes inserted into the plant chromosomes. Recent studies have shown that bacteria can access plant DNA during colonization of plants, or after mechanical disruption of plant tissues, if regions of high DNA similarity exist between the plant and bacterial DNA (Rizzi et al., 2008; Pontiroli et al., 2009). Thus, the main mechanistic barrier to the uptake and integration of plant transgenes in bacteria seems to be the limited DNA exposure and opportunity for homologous recombination to occur due to the limited extent of sequence similarity between plant transgenes and bacterial genomes. If such gene transfer occurred under natural conditions, constraints to efficient sampling makes it unlikely it would be detected in the short term (Nielsen and Townsend, 2004; Pettersen et al., 2005; Townsend et al., 2012).

Uptake of DNA by Bacteria in Water and Sediment

Marine environments can contain significant concentrations of dissolved DNA (Deflaun et al., 1987). Several studies have examined the ability of both introduced and native bacteria to take up DNA by natural transformation in water and sediment microcosms (Day, 2000). The studies can be divided into those that introduce defined bacterial inoculants (recipients) or purified selectable DNA (eg, containing an antibiotic resistance marker gene) into marine microcosm environments to detect DNA uptake events, and those that expose selected members of the indigenous population to DNA containing selectable markers *in vitro*. In studies utilizing introduced bacterial recipients, natural transformation has been shown in the *Vibrio* spp. strain WJT-1C (Paul et al., 1991) (later reclassified as a pseudomonad (Frischer et al., 1996)) with plasmid DNA in small-scale marine water and sediment microcosms sampled from the Eastern Gulf of Mexico; in marine sediments inoculated with the *P. stutzeri* and added chromosomal DNA (Stewart and Sinigalliano, 1990; Stewart et al., 1991); in *A. baylyi* with chromosomal DNA in groundwater samples from a drinking water well, sterile aquifer material, and in sterile groundwater microcosms (Chamier et al., 1993; Lorenz et al., 1992); and in *B. subtilis* with chromosomal and plasmid DNA bound to mineral aquifer material (Romanowski et al., 1993).

In the first experiments to describe natural transformation in open systems, an introduced *A. baylyi* strain was capable of being transformed to prototrophy by bacterial cell lysates or live donor cells in different river systems (Williams et al., 1996). The recipient *A. baylyi* bacteria and transforming DNA were immobilized on filters secured to stones on the riverbed. Transformation frequencies of 10^{-6} to 10^{-3} were reported.

In native bacterial populations, natural transformation of marine bacteria has been recorded after exposure to plasmid DNA or DNA in cell lysates of rifampicin-resistant *Vibrio* strains (Frischer et al., 1994). Three out of 30 marine bacterial isolates were found to be competent for uptake of the plasmid DNA in *in vitro* assays. These included isolates of the genera *Vibrio* and *Pseudomonas*. Moreover, 15 out of 105 sensitive isolates obtained from Tampa Bay (FL, USA) were found to acquire rifampicin resistance in *in vitro* assays. In another set of experiments, plasmid DNA exposure of 14 different whole bacterial communities sampled from a variety of marine environments, such as sediment, surface, and deep water, and from various organisms revealed that bacteria present in 5 out of the 14 communities examined could take up DNA (Frischer et al., 1994). This study estimated that between 0.000 05 to 1 transformant occurred per litre of water per day, suggesting that natural transformation may be an important mechanism for plasmid transfer among marine bacterial communities. In general, experimental studies suggest that DNA present in freshwater, in marine water, and on sediment surfaces is available for natural transformation of competent bacteria of the genera *Acinetobacter*, *Bacillus*, *Pseudomonas*, and *Vibrio*, albeit at variable frequencies that depend on environmental conditions and the type of DNA present. The introduction of defined recipient bacteria into an environmental sample results in competition between the introduced recipient population and indigenous communities for nutrients, habitats, and transforming DNA. This presents a challenge in the design of experiments that accurately reflect the environmental conditions, species compositions, and concentrations encountered by recipient bacterial strains when present in natural habitats.

Uptake of DNA by Bacteria Present in the Digestive System

The potential for competence has been identified in few bacteria isolated from the digestive system. Most studies have focused on members of the Gram-positive genus *Streptococcus* that are ubiquitous in the human oral cavity, as well as in rumen of many higher animals including cattle and sheep (Frandsen et al., 1991; Jarvis et al., 2001; Jayne-Williams, 1979; Savage, 1977). Several microcosms and *in vitro* systems have been applied to detect DNA uptake in *Streptococcus* spp. Sampling the teeth of 70 human individuals, it was found that at least 20 out of 129 isolates identified as *Streptococcus mutans*, which forms biofilms in dental plaques, could develop competence for acquisition of chromosomally borne streptomycin resistance *in vitro* (Westergren and Emilson, 1983). In a more recent study (Li et al., 2001), high natural transformation frequencies were reported, reaching up to three transformants per 100 exposed recipients, using *S. mutans* cells grown in biofilms on polystyrene microtiter plates and saturating concentrations of DNA. The transformation frequencies of the surface-bound cells were 10- to 600-fold higher than those observed for planktonic *S. mutans* cells. Natural transformation was also observed when *S. mutans* cells were exposed to biofilms of a heat-killed isogenic strain harbouring an erythromycin resistance gene (Li et al., 2001). Natural transformation of *Streptococcus gordonii*, also found in the human oral cavity, has been reported by plasmid or chromosomal DNA in saliva samples at frequencies up to 7×10^{-4} transformants per recipient cell (Mercer et al., 1999b). Naturally transformable bacteria can therefore, potentially take up DNA from food sources within the mouth. The natural transformation of a ruminal bacterium was first reported in 1999 with

Streptococcus bovis cells at a frequency of 1×10^{-5} transformants per microgram plasmid in a liquid culture medium (Mercer et al., 1999a).

Currently, only a few bacterial species localized to the digestive system of higher animals have been found to express competence *in vitro* and none have been found *in situ* (Nordgard et al., 2012). Few studies have examined or reported on the potential development of competence by bacteria colonizing invertebrates (Deni et al., 2005; Ray et al., 2007). The scarcity of available data may be due to insufficient incentive and methods to investigate limited bacterial transformability in such habitats. Although several members of the genus *Streptococcus* have been found to develop competence *in situ*, and other bacteria inhabiting the animal digestive tracts like *H. pylori* and *Campylobacter* spp. can develop competence *in vitro*, the biological significance of horizontal acquisition of extracellular DNA in the digestive system remains unclear.

Uptake of DNA by Bacteria Present in Food

Food provides an excellent growth substrate for many types of microorganisms. Some evidence exists for the uptake of extracellular DNA by bacteria residing in food. Natural transformation of *B. subtilis*, a common contaminant in milk, ranges from 10^{-4} to 10^{-3} transformants per recipient, with the highest frequency of 3×10^{-3} obtained in chocolate milk after an incubation time of 12 h at 37°C (Brautigam et al., 1997). Additional studies have also demonstrated the successful transformation of *B. subtilis* in milk products (Wittke et al., 2002; Zenz et al., 1998). Plasmid transfer by natural transformation of *Escherichia coli* in various foods, including milk, soy drink, tomato juice, carrot juice, vegetable juice, supernatants of canned cabbage, soy beans, shrimps, and various mixtures of canned vegetables, has also been shown to occur (Bauer et al., 1999). Natural transformation occurred in all growth substrates, although at variable frequencies. Typically, fewer than 10^{-8} transformants per recipient cell were observed, at frequencies three or four orders of magnitude lower than those of transformation experiments conducted under optimized laboratory conditions. No correlation was found between the content of divalent ions such as Ca^{2+}, which are considered prerequisites for artificial transformation of *E. coli*, in the foods and transformation frequencies. Natural transformation of *E. coli* on solid surfaces simulating biofilm conditions could be established on vegetables (eg, cucumber, potato, onion, tomato, carrot, leek), fruits (orange, pear, pineapple, apple, melon, grape, persimmon), raw meat and fish (beef, chicken, pork, tuna, salmon) and processed foods (tofu, orange gelatin, strawberry jam, pudding, curry, butter) obtaining transformation frequencies between 10^{-6} and 10^{-8} (Maeda et al., 2004). The above studies exemplify that food sources may provide the conditions required for natural transformation to occur and that the bacterial contaminants of food can experience competence-inducing growth conditions.

Development of competence and the uptake of plasmid-borne DNA in spring and mineral water were demonstrated for laboratory strains of *E. coli* (Baur et al., 1996). Calcium concentrations as low as 1–2 mM were sufficient to obtain transformants. These amounts of calcium ions were readily available in natural aqueous habitats of calcareous regions (spring water: 3.1–3.3 mM; mineral water: 1.9–11.3 mM; tap water: 1.5 mM; river water: 0.3–2.4 mM) and in mammalian body fluids (saliva: 2.3 mM; ileum: 4.2 mM; urine: 5.9 mM) (Baur et al., 1996). Variation of the incubation temperature (ie, temperature shifts as usually required for artificial transformations of *E. coli*) was no prerequisite for induction of natural competence. Upon prolonged incubation of laboratory strains of *E. coli* in spring and bottled mineral water (ie, under naturally occurring conditions) transformation frequencies as high as 10^{-2} to 10^{-5} were observed.

Laboratory strains of *E. coli* laboratory used for cloning purposes are mutants usually with inactivated DNA endonucleases and not representative for the DNA maintenance machinery active in naturally occurring bacterial populations of this species. Transformation frequencies using wild type strains of *E. coli* from clinical specimens under similar conditions as described above were strikingly strain-dependent (Woegerbauer et al., 2002). Some strains were highly transformable showing transformation frequencies up to 10% of the laboratory strain comparator; some were not transformable at all or expressed fluctuating transformation phenotypes. This strain-dependent variation in transformability was also observed in other bacterial taxa (Bosse et al., 2009; Maughan and Redfield, 2009). Water with Ca^{2+} concentrations between 1 and 2.9 mM served as matrix for the generation of wild type transformants if a temperature shift (25-37°C) was applied. No transformants could be detected at constant incubation temperatures. Transformability of wild type strains was, thus, more stringently controlled by the kind of temperature shifts compared to laboratory strains (Woegerbauer et al., 2002). Induction of competence was rapid process: a 1-min incubation in water supplemented with $CaCl_2$ was sufficient for the formation of transformants. As transformation limiting factors, DNase-dependent DNA degradation, a low poly-β-hydroxybutyrate (PHB)-calcium-polyphosphate complex production and a low C/N ratio (ie, nutrient starvation) could be identified (Lorenz and Wackernagel, 1994; Baur et al., 1996).

These observations provided indications for an internal — physiological — regulation of competence in *E. coli* rather than a dependence on a physiochemical induction via non-physiological concentrations of divalent cat-ions and heat shock as applied during cloning experiments (Baur et al., 1996). The experimentally obtained data were subsequently complemented by the detection of competence genes (Cameron and Redfield, 2006) and competence regulator homologs in the *E. coli* genome (Sinha et al., 2009). *E. coli* thus contains a functional DNA uptake machinery (Sinha and Redfield, 2012). *E. coli* was shown to develop competence even on Ca^{2+}-deficient solid media and agar was identified as trigger (Maeda et al., 2004; Sun et al., 2006, 2009, 2013). The reluctance to accept inducible competence-specific natural transformability of *E. coli* may have been caused by the difficulty to identify the exact cue for the induction of the competence regulator protein (Sinha and Redfield, 2012).

HGT in Bacteria Belonging to the Microbiome

Both the oral cavity (Roberts and Kreth, 2014) and the gut (Modi et al., 2014) are rich in naturally competent bacteria, and these environments offer the opportunity for natural transformation within the local microbiome or between the microbiome and transient bacteria (Huddleston, 2014). In fact, the human intestinal tract is the largest natural reservoir for antibiotic resistance genes (Huddleston, 2014). It has been shown that bacteria present in fecal samples are mainly resistant to antibiotics that have been in use for longer periods and that are used in livestock (Forslund et al., 2013). Antibiotic treatment selects for resistant bacteria and offers opportunity for HGT (Allen et al., 2014; Modi et al., 2014), which ultimately leads to treatment failure.

Bioinformatics based studies suggest HGT events are 25 times more likely to occur in human-associated bacteria than bacteria from other environments (Smillie et al., 2011). For instance, the animal digestive system is hypothesized to be an environmental hot spot for bacterial gene transfer due to the high concentrations of nutrients, actively growing bacteria, and surface (Huddleston, 2014; Modi et al., 2014; Roberts and Kreth, 2014; Salyers, 1993). The microbiota of people with cystic fibrosis also comprise several species, including the naturally competent *H. influenzae*, offering the opportunity for horizontal transfer (Sherrard et al., 2014); *in vitro* experiments have shown the conjugal transfer of antibiotic resistance determinants present in *H. influenzae* isolates collected from cystic fibrosis patients (Roberts et al., 2011). Conjugation involving the local microbiota has also been shown in the gastrointestinal tract of humans (Huddleston, 2014). The human microbiota comprises numerous naturally transformable bacterial species (Modi et al., 2014). However, probably due to methodological constraints and other undetermined factors, little information is available on DNA uptake processes by natural transformation active in the digestive system of animals (Huddleston, 2014; Mercer et al., 1999b; Rizzi et al., 2012), although DNA fragments can persist in the gastrointestinal tract of mammals (Rizzi et al., 2012). Transformation of the microbiota in the lower gastrointestinal tract, including the stomach, small intestine, cecum and colon, of rats has not been detected in experimental studies, even in the presence of high similar DNA (Nordgard et al., 2012).

Some Limitations of the DNA Uptake Model Systems Used

The data generated from transformation assays used to determine the availability and uptake of DNA in bacterial recipients are linked to the specific laboratory conditions employed. The informative value of such data on the DNA uptake processes occurring under natural conditions must be assessed case by case. Some technical constraints often embedded in the studies include the practice of adding bacterial recipients of a single strain, and often also DNA and nutrients, to a laboratory-maintained microcosm under semi-artificial conditions (Ashelford et al., 1997; Pearce et al., 1997). With few exceptions, studies of DNA uptake by natural transformation have been conducted *in vitro*, or in microcosms intended to represent soil, plants, or the gastrointestinal tract. The ability to establish representative bacterial habitat locations and population sizes after introducing externally cultured bacterial recipients into structurally organized microcosm models containing heterogeneously distributed indigenous bacteria is questionable (Bundt et al., 2001; Hallmann et al., 1997; Ranjard and Richaume, 2001). Few, if any, studies have examined DNA uptake processes in indigenous microbial communities with the DNA naturally present and spontaneously released on site. Moreover, the concerted action of DNA uptake and selection in determining the genetic compositions of bacterial populations undergoing adaptation to environmental changes is rarely studied in combination.

Several explanations for the dearth of such studies can be found. Most importantly, the range of gene transfer frequencies that can be practically measured in the laboratory is around 1 transformants per 10^8–10^9 exposed cells. The lower relative cell densities of a given species that are usually present in natural bacterial communities effectively limit the gene transfer frequencies that can be measured (Nielsen and Townsend, 2004). Another limitation is that only a fraction of the indigenous bacteria from any given environment can be cultivated for subsequent selection, making DNA uptake difficult to verify in uncultivable bacteria. Moreover, the use of selectable marker genes as a tool for DNA uptake identification is sometimes problematic due to frequently encountered background resistance to most antibiotics and a limited ability of rare transformants to adequately express the marker gene (Nielsen et al., 1998). Only a minor portion of bacteria from any given complex environment is, therefore, susceptible to positive selection by antibiotics and will take part in any screen for competence development. Because most detection methods of DNA transfer in bacteria are based on the uptake of resistance genes, the enumeration procedures usually performed on selective media disturb the sites and conditions that induced the gene transfer. Methods are now being developed for the *in situ* detection of discrete bacterial cells that allow potential DNA uptake events to be identified and quantified *in situ*. For instance, fluorescent protein (eg, green fluorescent protein) markers have been used to monitor horizontal acquisition of genetic material *in situ* (Dahlberg et al., 1998; Rizzi et al., 2008).

Most studies on natural transformation have been performed with monocultures with high population densities of the bacterium grown under laboratory conditions. Transforming bacterial species may, however, have specific requirements for competence development and resource utilization in complex environments. Hence, conditions conducive for gene transfer by natural transformation vary and the conditions established in the laboratory may not be those promoting competence development under natural conditions. For instance, transformation of *P. fluorescens* was not detected under several *in vitro* conditions, while such phenomenon could be observed *in situ* (Demaneche et al., 2001). It, therefore, follows that species currently thought to be nontransformable may show competence under yet unmonitored conditions.

Factors Affecting the Stable Uptake of DNA in Single Bacterial Cells

Molecular barriers define the sexual isolation or the degree to which two bacteria are prevented from exchanging genetic material by recombination. Sexual isolation may be expressed in terms of observed recombination frequencies between two bacteria, or more specifically as the ratio of homogamic recombination within species to heterogamic recombination between species (Majewski, 2001). Moreover, recombination frequency depends on the chromosomal location of the phenotypic marker, rendering sexual isolation between two strains locus-specific (Ray et al., 2009). Molecular barriers act upon chromosomal DNA uptake from its first interaction with the outside of the cell through its integration in a heritable state (Majewski, 2001).

Studies on natural transformation with various degrees of divergent DNA (Table 2) show that low DNA sequence similarity is a strong barrier to the integration of chromosomal DNA in bacteria. Indeed, studies have consistently demonstrated an absolute requirement for sequence similarity for detectable acquisition of chromosomal DNA by homologous recombination (Majewski, 2001). For some bacteria, the minimum length of 100% DNA similarity necessary to facilitate efficient DNA integration has been experimentally determined and is referred to as the minimum efficient processing segment (MEPS) (Matic et al., 1996; Shen and Huang, 1986). The minimum amount of base-pairing required for a single region to initiate heteroduplex formation with linear DNA substrates is 153 bp in *S. pneumoniae* (Prudhomme et al., 2002) and 183 bp in *A. baylyi* (de Vries and Wackernagel, 2002). Fewer details are available for the exact minimum requirements for double-sided DNA similarity for heteroduplex formation to occur. Such quantification is also not straightforward as it is likely to depend on the species and strain, and the specific nucleotide composition (GC%, etc.) of the recombining regions. For circular DNA intermediates (present in double-stranded form in the cytoplasm), DNA similarities of as little as 25 bp can mediate a single-crossover event and integration into the bacterial chromosome (Shen and Huang, 1986).

Base pairing of the single-stranded donor DNA with the complementary recipient strand within the heteroduplex molecule results in the formation of a double-stranded region that is recognized and acted upon both by host restriction modification (RM). Transforming DNA becomes single-stranded during the translocation process, which should theoretically render it insensitive to immediate digestion by restriction enzymes (Majewski, 2001; Matic et al., 1996). Nevertheless, studies indicate that restriction systems can reduce heterogamic transformation in *B. subtilis* (Cohan et al., 1991) and in *P. stutzeri* (Berndt et al., 2003).

The DNA mismatch repair (MR) systems of bacteria contribute to sexual isolation by binding to mismatches in heteroduplex regions formed during DNA-strand invasion, resulting in abortion of heterospecific recombination events. While the contribution of MR to sexual isolation in some bacteria (eg, *E. coli*) is large, it appears to be less important in naturally transformable bacteria such as *B. subtilis* and *S. pneumoniae* (Majewski, 2001). In bacteria, defective MR can lead to increased rates of heterogamic recombination in addition to increased rates of mutation, as cells are unable to identify/repair mismatches in heteroduplex DNA formed during heterologous strand invasion. For instance, inactivation of the *mutS* gene, encoding the protein that recognizes and binds to mismatches, results in about 100-fold increase in heterogamic transformation and recombination with DNA substrates of 24% sequence divergence in *A. baylyi* (Ray et al., 2009), threefold in *B. subtilis* (at 14.5% sequence divergence) (Majewski and Cohan, 1999), and sixfold in *P. stutzeri* (at 14.6% sequence divergence) (Meier and Wackernagel, 2005). These results are in contrast to plasmid–phage recombination systems in *E. coli* or *Salmonella enterica* subspecies Typhimurium, where *mutS* deletions increase heterogamic recombination rates by 1000- to 10 000-fold (Majewski, 2001). Reasons for this dichotomy in MR inhibition of heterogamic recombination are unknown, but it may be due to the molecular machinery of the individual bacterium or due to the nature of DNA interacting with the recipient chromosome (single-stranded in *B. subtilis*, *P. stutzeri*, and *A. baylyi* vs. double-stranded during phage or plasmid integration in *E. coli* and *S. enterica*).

Table 2 Some examples of interspecies/heterogamic recombination in bacteria

Transformation-mediated recombination in chromosomally localized loci	*Sequence divergence of recombining DNA molecules (%)*	*Reference*
Bacillus licheniformis and *B. mojavensis*	17	Zawadzki et al. (1995)
Streptococcus intermedius and *S. pneumoniae*	18	Majewski et al. (2000)
Acinetobacter sp. strain 01B0 and *Acinetobacter* sp. strain ADP1	20	Young and Ornston (2001)
Rhodococcus erythropolis and *Acinetobacter* sp.	24	Stratz et al. (1996)
Other recombination systems in nonchromosomally localized loci		
In vitro recombination between M13 and fd phage	3	DasGupta and Radding (1982)
Conjugal gene transfer between *Escherichia coli* and *Salmonella typhimurium*	17	Matic et al. (1995)
S. pneumoniae and various cloned Streptococcal fragments located on plasmids	18	Humbert et al. (1995)
E. coli and *S. typhimurium* genes located on compatible multicopy plasmids	25	Cossart et al. (1986)
λ phage – plasmid cointegration in *E. coli*	35[a]	Shen and Huang (1986)

[a]263/405 bp

Considerations of the Long-Term Persistence of Horizontally Acquired DNA in Bacterial Populations

The observed frequencies of DNA uptake, and prevalence and diversity of transformants in a bacterial population are determined by (1) the molecular barriers to natural transformation (see above); (2) the limited access to relevant DNA as determined by the nucleotide diversity present and accessible (physical and geographic isolation) (Majewski, 2001); and (3) the reduced fitness of bacterial transformants, after uptake of most random DNA fragments, that could lead to their removal from the bacterial population by purifying selection (Bjorkman and Andersson, 2000; Kuo and Ochman, 2009).

Experimental transformation studies have most often focused on quantifying the frequencies at which particular gene segments may be taken up by bacteria at one or a few chromosomal loci. The DNA uptake rates quantified are often described in the scientific literature as "low" to "high." It is important to note that such grading only reflects the range of frequencies that can be practically measured in limited sample sizes in the laboratory (Rizzi et al., 2008). The grading is not, as often erroneously implied, linked to the subsequent biological impact of the acquired DNA in the bacterial population. It may be that frequencies below those that can be observed in a 24–48-h DNA uptake experiment in the laboratory are significant in the long run (Nielsen et al., 2014). Most successful HGT events identified through comparative DNA analysis of bacterial genomes are estimated to have occurred over a timescale of millions of years (Croucher et al., 2014; Lawrence and Ochman, 1998). Therefore, experimentally measured DNA uptake frequencies collected over minute timescales in the laboratory may not necessarily provide relevant information to understand the occurrence and biological impact of infrequent DNA uptake events taking place in larger bacterial populations over several years.

In addition to considerations of the relevant timescale of DNA uptake events, the timescale of transformant population expansion may also be considerably longer than what can be practically measured in the laboratory. The long-term fate of any DNA uptake event/transformant is determined by selection and genetic drift and not the DNA uptake frequencies themselves. This is because it is the population trajectories of the descendants of the primary transformants that will determine the long-term survival and impact of the transformant (Thomas and Nielsen, 2005). For instance, single DNA uptake events may take place within the time span of a few bacterial generations. Nevertheless, it may subsequently take many thousands of generations and, hence, many years before the trait will become widespread in the overall bacterial population after clonal division and directional selection of the transformant. The latter process can only be approximated through modelling (eg, probabilistic or deterministic) of bacterial populations, if the strength of the selection of the transformant is known (Moradigaravand and Engelstadter, 2014; Starikova et al., 2012). Additionally, the spatial structure of the populations is important in the prediction of the dynamics of natural transformation (Moradigaravand and Engelstadter, 2014). The timescale needed to understand DNA uptake processes as they occur naturally depends on the objective of the study, the anticipated directional selection of the transformant bacteria, and their population structure, size, and generation time.

Predictors of DNA Uptake in Bacteria and Implications for the Release of Genetically Modified Microorganisms

Genomic information and laboratory evidence support both the occurrence of genetic recombination between bacteria of variable genetic relatedness and the existence of mechanistic and selective barriers that maintain divergence between bacterial lineages. A challenge in microbiology is to reconcile these two observations to assess the recombinogenic potential and impact thereof between different bacterial entities. The ability to accurately predict the potential for two separately evolving bacterial lineages to undergo genetic exchange remains, however, in its "adolescence." This is illustrated by our inability to predict the impact of future HGT events on bacterial genome evolution, although data on DNA uptake frequencies can be extracted from many experimental model systems. Knowledge of the factors governing horizontal transfer and selection of transformed bacteria is, nevertheless, crucial to the evaluation of the potential for unintended spread of recombinant DNA from genetically modified microorganisms (GMMs) (Keese, 2008). GMMs can offer benefits in, for example, improving food production and utilization and in disease prevention (Aguilera et al., 2013; Gilsdorf and Zilinskas, 2005). A potential hazard arising from the indiscriminate use of GMMs is adverse alteration of indigenous bacterial populations after horizontal transfer of recombinant DNA (recDNA) (de Carcer et al., 2007; De Leij et al., 1995).

Most countries and many international organizations have developed legislation and recommendations on the types of assessments necessary prior to the commercial use of GMMs (Aguilera et al., 2013). For instance, the Codex Alimentarius Commission of the United Nations have developed principles for risk analysis and guidelines for safety assessments of foods derived from modern biotechnology, including recDNA microorganisms (Codex Alimentarius Commission, 2003). In most assessments of genetically modified organisms (GMOs), the starting point is the familiarity and the history of safe use/behaviour/consumption of the parent organism. The concept of "substantial equivalence" is used to structure the assessment relative to the conventional counterpart and to focus the assessment on the determination of similarities and differences. Thus, risk assessment is based on the introduced biological changes in the modified organism and does not usually address the biological safety of the parent microorganism itself (Aguilera et al., 2013; Waigmann et al., 2012). In the context of unintended horizontal transfer of recDNA, some general safety recommendations for the construction of GMMs have been made (Codex Alimentarius Commission, 2003):

1. The genetic modification performed should be limited to the intended trait, and the final GMM or product thereof should not contain unnecessary DNA sequences, for example, antibiotic marker genes, DNA sequences that confer or stimulate genetic mobility of the recDNA, or DNA sequences that can confer or affect pathogenic properties.
2. A chromosomal location of the recDNA is desired because extrachromosomal elements such as plasmids are often self-transferable or mobilizable between cells and species. Plasmids also harbour replication functions that ensure their stability in an extracellular state over bacterial generation.
3. The use of recDNA (genes) that mediate a selective advantage to unintended bacterial recipients should be avoided to prevent dissemination of recDNA in bacterial communities.

The general recommendations made above are precaution-based and seek to minimize both the likelihood of uptake of recDNA in bacterial populations, and the potential positive selection of unintended bacterial transformants carrying recDNA, if rare DNA uptake events occurred. To reduce the likelihood of occurrence of unintended HGT, the recDNA may be inserted into the chromosome in the absence of sequences conferring mobility. Empirical and theoretical data provide a basis for the identification of parameters of importance for limiting the transfer potential of chromosomal DNA between bacteria. Nucleotide differences in DNA sequence between bacterial strains and species are probably the most important barrier to recombination of chromosomal DNA in bacteria (as discussed above). The lack of DNA sequence similarity between the donor and the recipient DNA preclude the ability of the transforming (rec)DNA fragment to form a heteroduplex with the recipient chromosome. In general, experimental studies in transformable bacteria have shown that DNA substrates with more than approximately 30% sequence divergence will not be successfully integrated in the recipient genome. Knowledge of the sequence divergence between species can be a useful predictor of their overall recombination potential. Different approaches are available to estimate the overall DNA sequence similarity between bacteria, including DNA–DNA hybridization, whole genome comparisons, or comparisons of housekeeping genes, including 16S rRNA (Goris et al., 2007; Wayne et al., 1987; Woese, 1987). While predictors of overall genomic sequence relatedness can be useful to understand the potential for chromosomal recombination between species, knowledge of local sequence divergence and conservation in the area spanning the recDNA will more accurately reflect its recombination potential into unintended recipients.

The technical approaches to limit the potential of unintended recDNA transfer from GMM are required because a precise understanding of bacterial fitness of unintended recipients of recDNA is lacking. As discussed above, it is not the transfer frequencies of chromosomal DNA that will cause a biological impact, but directional positive selection of transformants carrying recDNA. Integrated DNA fragments (including recDNA constructs) are likely to cause phenotypic changes that negatively affect the transformant fitness relative to the larger bacterial population and communities. Transformants carrying recDNA that are not competitive in growth and cell division will not remain in the larger bacterial population for long. Identifying conditions that may promote positive selection of transformants is, therefore, essential to understand the fate of recDNA in any bacterial population. The use of recDNA (genes) that is likely to mediate a selective advantage to unintended bacterial recipients should be avoided to prevent unchecked dissemination of recDNA in bacterial communities. Unfortunately, available methodology does not readily facilitate the collection of empirical data on the selection coefficients of GMMs and unintended bacterial recipients of recDNA in agricultural settings or the gastrointestinal tract. Expert evaluation and inference from a history of safe use and expected behaviour (of both the recDNA donor[s] and the recDNA recipient bacterium), therefore, remain an essential component in the case-by-case assessment of GMMs.

Clinical Relevance of HGT and Antibiotic Resistance

The range of pathogenic species that are able to express competence determines the clinical relevance of natural transformation. It was recently demonstrated that genetic diversity is linked with competence (Jorth and Whiteley, 2012), and natural transformation is expected to greatly contribute, together with other HGT mechanisms, to the dissemination of antibiotic resistance determinants and traits (Domingues et al., 2012a, b; Ochman et al., 2000; Roberts and Kreth, 2014). Most often, the intercellular gene transfer mechanism responsible for that pathogenic bacteria acquire new virulence or antibiotic resistance traits remains unknown. This is because all known gene transfer mechanisms are able to transfer chromosomal DNA fragments, plasmids and mobile genetic elements. Moreover, the gene transfer frequencies are alone rarely indicative of the effects (Feil et al., 2001; Pettersen et al., 2005). The acquisition of new genetic traits can transform bacteria from harmless to pathogenic. We now have bacteria that are virtually resistant to all know classes of antibiotics (Magiorakos et al., 2012), such as the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens (Boucher et al., 2009). The decline in the antibiotics development by the pharmaceutical industry in the last years seems to lead us to a post-antibiotic era, where antibiotics are not effective and the rate of development of new ones is extremely low (Fowler et al., 2014). Alternatives to antibiotics must be considered (Allen et al., 2014).

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How Microbial Pathogens Subvert Host Innate Immune Defenses☆

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The Skin and Mucosae Protect Us From Most Microbes

All organisms must interact with the outside world and regulate two-way communication. Each tissue in contact with the environment has a protective barrier, either skin or mucosa, through which this communication occurs. In addition, the brain, eyes, and testes are encased in specialized membranes to protect them from the rest of the body, including circulating immune cells that can cause damage. The first step to becoming a pathogen is to breach one or more of these barriers.

How Pathogens Cross the Skin

Skin is mainly composed of the epidermis and dermis layers. The outer layer, the epidermis, is made up mostly of cells called keratinocytes that produce the intracellular protein keratin that aids in providing durability. The outermost layer of skin consists of dead cells that form a dry, acidic, and rigid wall covered with chemicals that limit microbial colonization. These chemicals derive from glands producing sweat and sebum or are secreted by keratinocytes. Antimicrobial lipids and peptides from these same sources limit the kind and numbers of microbes that survive on skin. Some of the microbes that can survive on the skin, particularly the bacterium *Staphylococcus aureus*, are commensals that effectively prevent new microbes from finding binding sites through which they could access water and nutrients. Thus, our skin, while warm, is not a welcoming environment for most microbes.

Microbes that cross the skin barrier may do so passively on the surface of a damaging agent, such as gravel, at the time and site of damage. The vast majority of these microbes are not pathogens and are quickly killed by a healthy immune system. Some pathogens passively cross the skin with assistance from an insect or animal. The causative agent of Lyme disease, the bacterium *Borrelia burgdorferi*, is delivered into tissue with saliva via an insect bite. Similarly, animal bites transfer the Rabies virus from saliva of an infected animal across the skin barrier of a victim. In addition to saliva, animal and insect feces can harbor pathogens that are passively transferred across our skin. The eukaryotic parasite that causes Chagas disease, *Trypanosoma cruzi*, is deposited on our skin by the Kissing Bug in feces. When we scratch the site, *T. cruzi* is delivered across the skin barrier through the feces left by the Kissing Bug. These are just a few examples of the many different pathogens of all kinds that cross the skin barrier with assistance from either other organisms or our own actions.

A skin commensal can become a systemic pathogen

A human commensal that normally resides on and protects the skin, *Staphylococcus aureus*, may become a pathogen if it passively crosses the skin barrier. Normally, tissue damage triggers a signaling cascade that recruits neutrophils, immune cells that are expert at killing microbes. However, some *S. aureus* strains appear to benefit from the signaling cascade, including inflammatory cytokines and chemokines that normally protect our damaged skin. These bacteria preferentially colonize inflamed skin, such as a surgical wound, and cause a localized infection that may become systemic. *S. aureus* has even been observed to replicate in the presence of neutrophils and multiple antimicrobial chemicals we produce that normally inhibit bacterial survival. The molecular mechanisms by which *S. aureus* exploits our inflammatory signaling to colonize wounds require further understanding to facilitate the development of new treatments.

The Mucosal Barrier is a Key Host Defense

Mucosal tissues are more involved in communication with the environment than the skin. These tissues exchange water, light, gasses, nutrients, waste, pheromones, and gametes with the environment, and can be found in the airway, urogenital tract, and eyes. Conditions that allow for exchange also expose mucosal surfaces to large numbers of diverse microbes. We protect our mucosa with an aqueous and/or fatty acid layer: tears, saliva, mucus. Mucus is constitutively produced by specialized cells in the mucosal epithelium, such as the goblet cells of the gut. In response to stress or damage, the volume of mucus that we generate increases rapidly. Most microbes are limited to the surface of a mucous layer due to the high viscosity and an abundance of antimicrobial chemicals and peptides contained in the mucus. The vast majority of these microbes are not pathogenic and help defend the host from pathogens, for instance, by simply taking up physical space and by efficiently utilizing available nutrients.

☆*Change History*: December 2018. JL Dombach and CS Detweiler have made minor changes in the Text and the References.

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Crossing the mucosal barrier

Pathogens use a number of strategies to cross the mucous layer. The bacterial pathogen *Helicobacter pylori* swims through gastric mucus toward the underlying epithelium using flagella. Further down the gastrointestinal tract, the eurytopic protozoan pathogen, *Entamoeba histolytica*, forges a path through colon mucus by secreting proteases that cleave mucins, a major saccharide component of mucus. Mucus also contains a potent host protease, lysozyme, which degrades bacterial cell walls. The bacterium *Listeria monocytogenes* fights back by chemically modifying peptidoglycan, a key component of the cell wall, to be less sensitive to lysozyme. This reduction in lysozyme sensitivity enables L. *monocytogenes* to survive transit through the mucous layer and to subsequently live within macrophages.

Changing the local environment to outcompete other microbes

Pathogens can outcompete commensals using molecular tricks to make the environment more beneficial for them. One major pathogen strategy is to change the host environment in a manner that benefits the pathogen and restricts commensals. For instance, when *Salmonella enterica* serovar Typhimurium colonizes the intestine, it stimulates inflammation. Incoming inflammatory cells and fluids radically change the intestinal environment in a manner that *Salmonella* has evolved to exploit. In particular, *Salmonella* takes nutritional advantage of the availability of a different set of electron acceptors and carbon sources, enabling the pathogen to out-grow commensals.

Binding to Host Cells and Surviving Host Defenses at the Epithelium

When a pathogen finally reaches the epithelial layer, it needs to adopt new strategies to survive. One strategy is to adhere to epithelial cells. Bacteria use pili, long thin protein filaments that extend out from the bacteria to reach epithelial cells. At the ends of pili are proteins called adhesins that bind to specific host cell surface proteins. Another trick microbes use to attach to the surface of an epithelial cell involves inserting their own adhesive protein into the host cell membrane. For instance, some pathogenic *Escherichia coli* use a secretion system to inject a bacterial protein directly into the membrane of a host cell. *E. coli* then uses a protein on its own cell surface to bind the target protein it has just placed on the host membrane.

Mammals defend themselves against bacterial adherence to epithelial cells by increasing the turnover rate of these cells, a process that leads to more rapid shedding of the microbe back into the mucosal lumen. In turn, some microbes have evolved mechanisms to slow down the division rate of the stem cells within our intestine that divide to replace shed epithelial cells. This benefits the microbe by increasing the time it has to reside at the epithelial layer, before either breaking through this layer or being shed back into the lumen.

Breaking Through the Epithelial Layer

Pathogens use diverse molecular strategies to cross the host epithelial layer. Some microbes translocate across epithelial cells. This process does not typically damage the epithelial cell, as the pathogen can engineer its own uptake from the lumen or apical side of the epithelial cell within a host vesicle. This vesicle, called a phagosome, is transported via the cytoskeleton and motor proteins across the epithelial cell to fuse with the membrane on the basal side, releasing the pathogen. Another way to cross the barrier involves secreting proteins into the host cell that disrupt the tight junctions between epithelial cells. For example, the bacterium *Vibrio parahaemolyticus* delivers a protein to the host cytoplasm that modifies a host protein (i.e., catalyzes the covalent addition of AMP to Rho GTPases). Modifying the host protein leads to the disruption of the actin cytoskeleton and the rounding of the epithelial cells such that the bacterium can cross over. Alternatively, microbes such as *Vibrio cholerae* secrete a protease which cleaves the extracellular domain of a host tight junction protein to allow for the bacterium to pass between cells (Fig. 1).

A more dramatic way to cross the epithelium is for a microbe to either kill or exploit the death of an epithelial cell, and then pass through the gap before other cells migrate into the area to seal the break. However, the death of an epithelial cell typically alerts the immune system, radically changing the local biology in a manner that may benefit or harm the pathogen. Microbes can alternatively cross the epithelial layer in a manner that exploits the immune system but does not cause outright tissue damage: within a professional phagocyte. Dendritic cells are a type of professional phagocyte that is noted for reaching cytoplasmic projections called podia between the tight junctions of epithelial cells into the mucosal lumen, capturing microbes, and then retracting podia and pathogens back through the tight junctions to the basal side of the epithelium. The microbe then has to either be able to survive within, or kill and escape from, the professional phagocyte.

At least one microbial pathogen has been reported to use as many as three of the methods outlined above to cross the epithelial barrier. After adhering to host cells, the yeast fungal pathogen *Cryptococcus neoformans* transcytoses across epithelial cells, either causing or exploiting the death of the epithelial cell and thereby gaining access to the basal side, or is captured by a professional phagocyte that takes the microbe across the epithelial barrier without damaging host cells.

Underneath the Epithelial Barrier—The Lamina Propia

Mucosal tissue is underlain by the lamina propia, an area of loose connective tissue in which cells of the innate and adaptive immune systems coordinate responses to damage caused by pathogens. Underneath the lamina propia is a layer of smooth muscle that contracts regularly to keep fluids moving through our mucosal tissues. Smooth muscle also causes the involuntary contractions

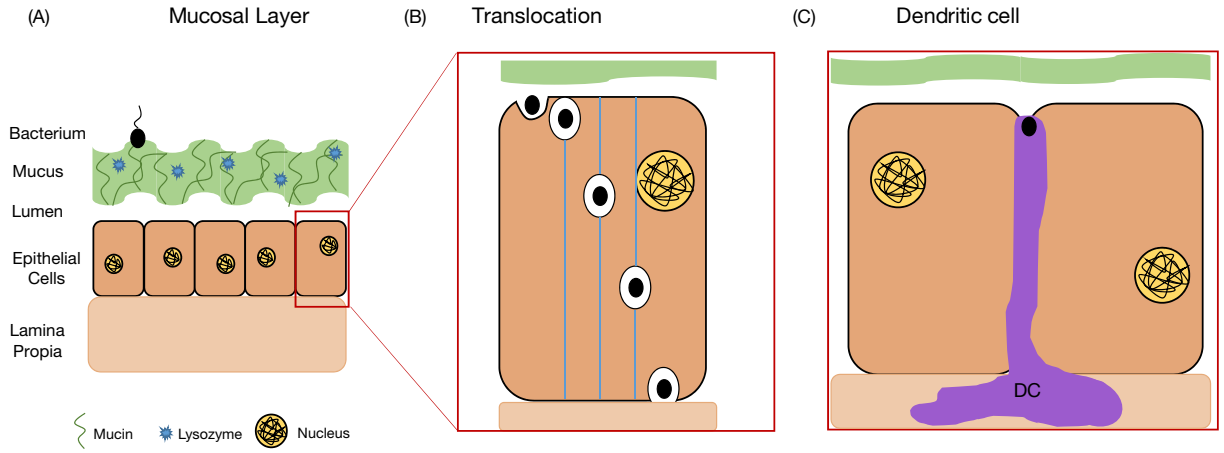

Fig. 1 Two ways pathogens cross mucosal barriers: translocation and dendritic cell. (A) The layers of the mucosae. First pathogens must cross a layer of mucus that is highly viscous because it is made up of mucins and is toxic to bacteria because it contains the host protease lysozyme, which degrades bacterial cell walls. Some bacteria cross the mucus layer by using their flagella. Once past the mucus, the pathogen must then cross the epithelial layer to reach the lamina propia. (B) Some pathogens are taken up by epithelial cells and then translocated across the cytosol to the basal layer in a phagosome. The phagosome is then brought to the basal side of the epithelial side by the cytoskeletal actin filaments (*blue*). Once the phagosome reaches the basal side, it fuses with the cell membrane and releases the pathogen into the lamina propia. (C) Pathogens can cross the epithelial layer by being taken up by the podia of dendritic cells (DC) that has inserted itself between epithelial cells. The podia then retracts, bringing the pathogen with it.

that suddenly force mucus out of the body, through a sneeze, for instance. Ideally, any pathogens residing in the tissue are expelled along with the mucus.

Avoiding, Evading or Destroying Host Soluble Defenses

Molecular defenses that are soluble are present all over the body, including on the skin and in mucus, in all tissues, blood, and interstitial fluid. Defenses such as complement, antimicrobial peptides (AMPs), and molecules that sequester nutrients from microbes are crucial for innate immunity and form a bridge to adaptive immunity. In addition, multiple aspects of adaptive immunity that are soluble are outlined below.

Complement

Complement is a host defense system that tags bacteria for recognition and engulfment by professional phagocytes, or, for recognition by a series of proteins that create holes in bacterial membranes, a process called the Membrane Attack Complex (MAC). Many microbes such as *E. coli*, defend themselves against complement by secreting saccharides which assemble into an exterior coat that makes it difficult for complement proteins to bind the pathogen surface. Before complement proteins can bind a bacterial surface, they have to trigger a proteolytic cascade that serially activates the proteins that will tag the microbe. Pathogens have evolved to disrupt this cascade, for instance, by secreting proteases that inactivate critical steps. Inactivation of critical steps effectively prevents the pathogen from being tagged and then recognized by a phagocyte. Some pathogens alternatively capture serum host proteases that cleave the tag attached to the bacterial surface. These host proteases evolved to protect host cells from complement, which deposits indiscriminately on friend and foe. When a pathogen hijacks a host protein to destroy the complement tag on the bacterial surface, the pathogen is effectively protected from recognition by a phagocyte and from the subsequent molecular steps that would allow for the MAC to assemble. For instance, *Neisseria gonorrhoeae* evades complement by using their pili to capture host anticomplement factors, such as the CD46 protein, and transport them to the bacterial membrane for protection. These are just a few of the many ways by which microbes evade recognition by the host complement system (Fig. 2).

Antimicrobial Peptides (AMPs)

AMPs are a diverse group of molecules present in all body fluids, including the fluids that bathe mucosal tissues. These peptides are typically positively charged and thereby able to interact electrostatically with the negatively charged surface of bacteria, fungi, and viruses to cause membrane damage and often destroy the microbe. Microbes detect membrane stress caused by AMPs and respond in multiple ways. For example, since many AMPs are cationic, microbes typically alter their surface charge to repel AMPs. For Gram-negative bacteria, this involves modifying the lipopolysaccharide (LPS) in the outer leaflet of the outer membrane. Gram-positive

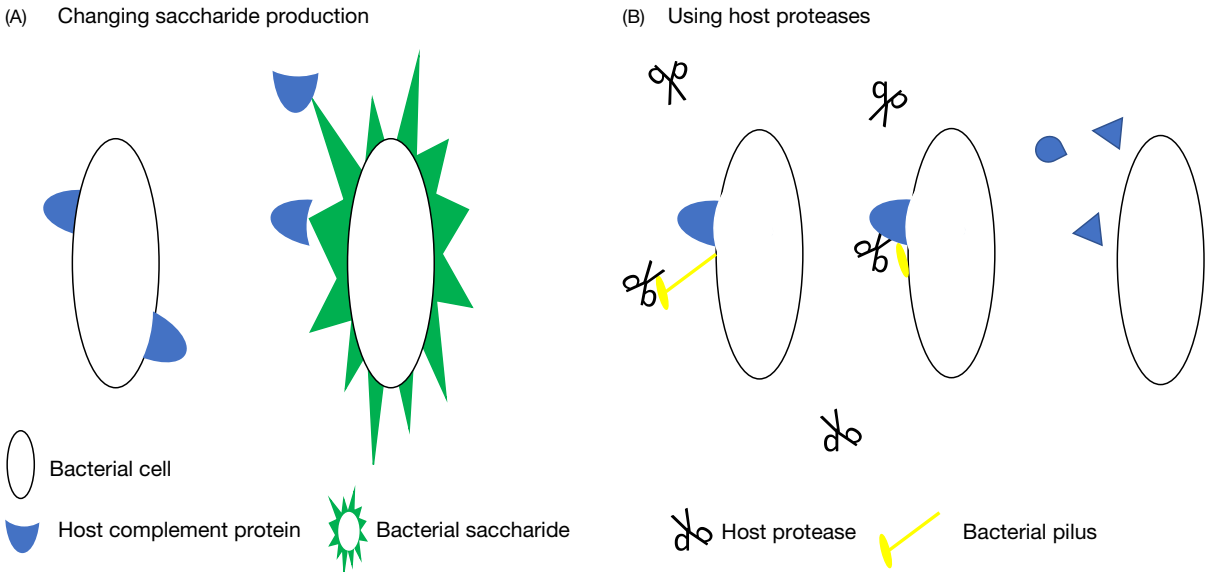

Fig. 2 Pathogens escape host complement using various methods. (A) Some bacteria excrete saccharides that inhibit the binding of host complement proteins to the bacterial membrane. (B) Pathogens can capture host proteases that are present in serum to prevent host cells from being tagged for complement. Through the use of the host protease the complement protein is cleaved and the pathogen has escaped recognition by complement.

bacteria modify their cell wall lipo teichoic acids (LTA) to evade AMPs. Both kinds of bacteria induce expression of enzymes that modify their surfaces, by adding or removing chemical groups to LPS or LTA. A second strategy for dealing with AMPs is to cleave them with secreted proteases and thereby detoxify them. Microbes also sequester AMPs to prevent them from reaching the bacteria. For example, Gram negative bacteria release outer membrane vesicles that trap AMPs in the extracellular environment. Microbes also simply export AMPs out of the cytoplasm or periplasm using efflux pumps, an energy intensive process. More recently, the role of biofilms in microbial protection from many kinds of toxic molecules including AMPs has been described. Since biofilm formation requires a critical mass of microbes and often includes microbes from multiple kingdoms or species, it is a good example of the role of social behavior in evading host defenses.

Biofilms

Biofilms are complex structures that consist of microbial cells, from one or multiple species of bacteria or fungi, that adhere to a surface. The microbes within the biofilm are embedded in an extracellular matrix mainly composed of polysaccharides and proteins produced by the bacterial cells, as well as extracellular bacterial DNA. This matrix provides support to cells in the biofilm, as well as increased protection from the environment. Many microbial species form biofilms during infection to evade harsh environments, antibiotics, and/or host defenses. One species that forms a biofilm to evade antibiotics, as well as host defenses, is the bacterium *Staphylococcus epidermidis*. *S. epidermidis* usually resides on the skin as a commensal, but has recently become a common nosocomial, or hospital acquired, infection. *S. epidermidis* enters through wounds but lacks virulence factors like *S. aureus*, and because of this *S. epidermidis* only colonizes a host when it has formed a biofilm on implanted medical devices. In addition to holding the biofilm together, the major polymer in *S. epidermidis* biofilms increases bacterial resistance to AMPs. Recent evidence also shows that some strains of *S. epidermidis* produce an enzyme that activates biofilm formation, as well as proteases that target neutrophils, and may use the cleaved neutrophil products as part of the biofilm matrix (Fig. 3).

Nutritional Immunity

Body fluids and mucus contain host defense molecules aimed at depriving microbes of specific nutrients. The best studied mechanisms deprive microbes of metals, such as iron and zinc, that are essential cofactors for enzymes. To ensure that cells have the cofactors they require, bacteria produce proteins called siderophores that capture local iron or other essential metals and deliver them to the bacteria. However, hosts can produce proteins that inhibit siderophores from functioning. For instance, once Salmonella infects a human gut, a cascade of signals triggers inflammation of the gut as well as the production of host proteins which bind and inactivate siderophores, thereby inhibiting bacterial acquisition of iron and zinc which are required for various essential metabolic activities. These host proteins are not specific to siderophores of pathogens, thus commensal species are also targeted. However, Salmonella has evolved a mechanism so that its siderophore is not bound by these host proteins. Salmonella adds a sugar group to its siderophore which prevents recognition by the host proteins, allowing the siderophore to bind iron and be taken up by Salmonella for its metabolism. Because commensal organisms often cannot compete with the host to acquire essential cofactors, the ability of Salmonella to modify its siderophore in this environment gives it a competitive advantage over commensals. In addition to metal sequestration, Salmonella is able to take advantage of the inflammation that is triggered by its

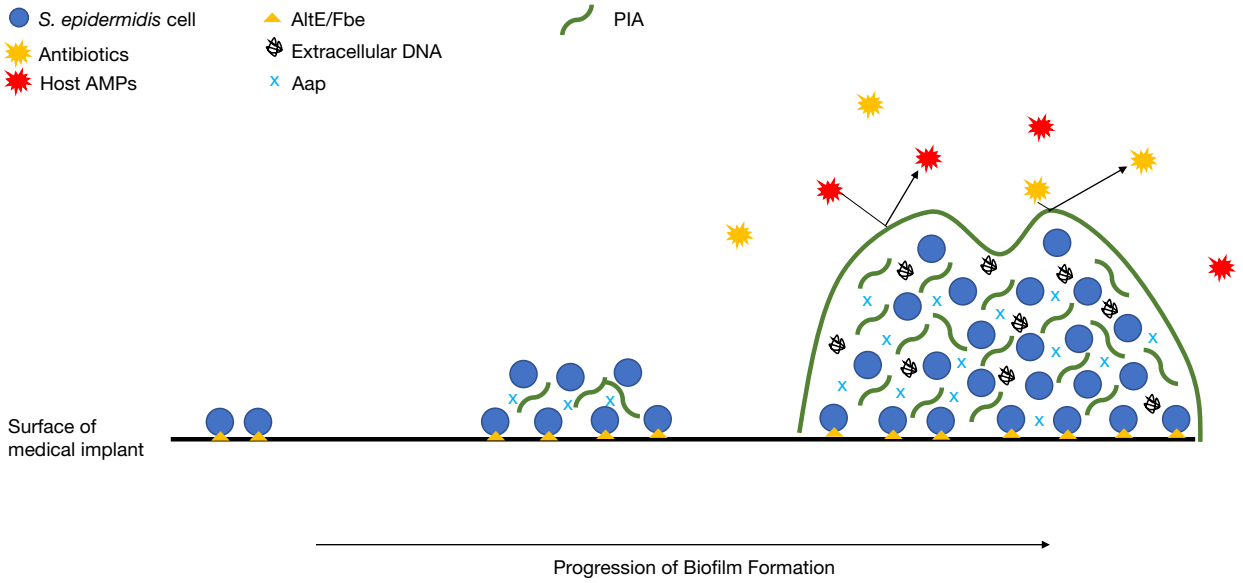

Fig. 3 Some species, like *Staphylococcus epidermidis* form biofilms to evade host defenses. To form a biofilm *S. epidermidis* produces numerous proteins and polymers. To initiate biofilm formation *S. epidermidis* must express the proteins AltE and Fbe which aid in attachment of the cell to the substrate. The surface protein Aap must be made and activated by cleavage. Once cleaved, Aap promotes interactions between *S. epidermidis* cells. Many strains also produce the polymer PIA which aids in holding cells together in the biofilm. As the biofilm matures extracellular DNA becomes stuck the biofilm matrix, which provides further structure. The coating of cells in the biofilm matrix protects them from many host defenses such as AMPs, as well as antibiotics.

presence in the gut. Inflammation changes the nutrients and potential electron acceptors that are available in the environment. Unlike most commensals, Salmonella is able to use this set of nutrients and electron acceptors giving it an additional advantage and allowing it to grow and outcompete commensals (Fig. 4).

Residing in Tissues Protected From the Immune System

Some microbes are able to evade host defenses by escaping to organs, such as the brain or the eyes, where immune cells are not normally present because an endothelial membrane barrier keeps them out. *Toxoplasma gondii* is an obligate intracellular, eukaryotic

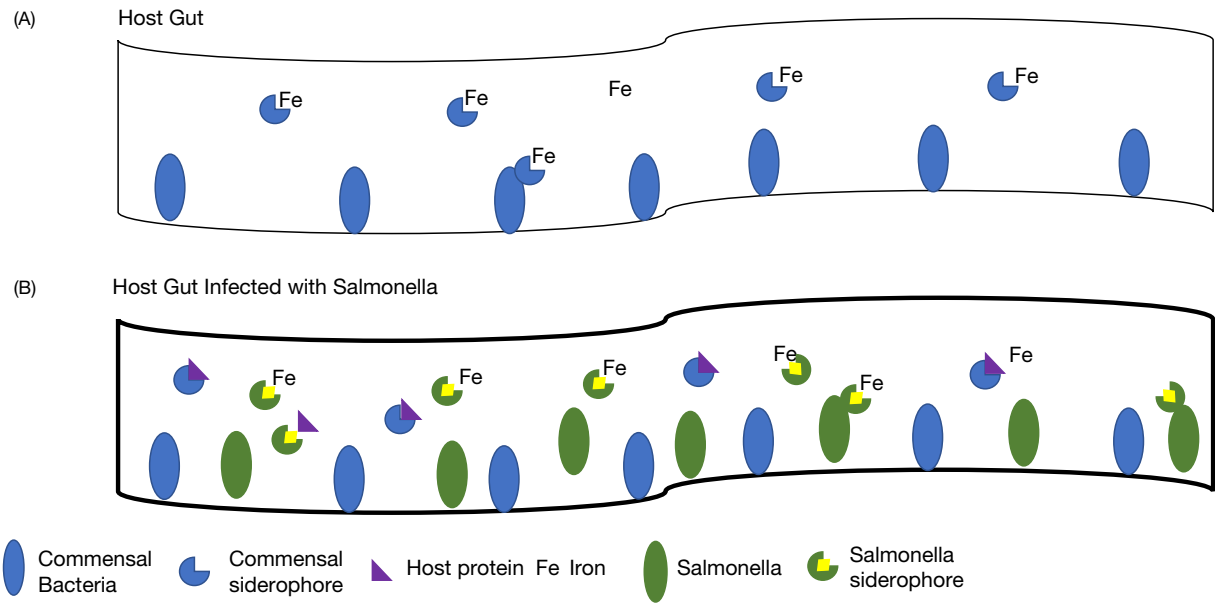

Fig. 4 *Salmonella enterica* acquires essential nutrients in the host gut. (A) Commensal bacteria in the gut of mammals produce siderophores to capture iron and bring it back to the bacterial cell to be used as essential cofactors by enzymes. (B) Upon colonization of the host by Salmonella the gut becomes inflamed and proteins are produced that bind bacterial siderophores inactivating them. Salmonella is unaffected by these proteins due to a sugar modification on their siderophore that inhibits recognition by host proteins. Because commensal siderophores are affected, but siderophores of Salmonella are not, Salmonella is able to acquire iron and thrive.

parasite that can cross the barrier that protects the brain, the blood–brain barrier (BBB). *Toxoplasma* is spread to mammals through contaminated cat feces. Once ingested, the parasite quickly replicates and is targeted by the immune system. However, as infection progresses *T. gondii* transitions into a slowly replicating form that becomes enclosed in a cyst. In humans and rodents this transition to the encysted form occurs in the brain. To enter the brain *T. gondii* must cross the BBB which is responsible for keeping out pathogens and immune cells. *T. gondii* can use three different methods to cross the BBB. Unlike many organisms that use flagella for swimming motility, *T. gondii* uses cytoskeletal motors to propel itself forward in a gliding manner. Using its gliding motility, *T. gondii* is able to move across the endothelial cell barrier of the BBB by gliding between cells. Another method is to bind to endothelial cells and be taken up by the cell. Inside theendothelial cell the parasite replicates and is then lysed out on the basal side, adjacent to the brain. The final method to cross the BBB is by infecting an immune cell which then crosses the layer of endothelial cells in the BBB. While immune cells cannot normally cross the BBB, research has shown that both macrophages and dendritic cells can cross the BBB after infection by *T. gondii*, and that *T. gondii* likely uses these cells to cross the BBB. Recent work has shown dendritic cells exhibit a hypermotility phenotype when infected with *T. gondii* and that this hypermobility may aid in crossing the BBB.

Soluble Adaptive Immunity—Antibodies

Each of the defenses listed above are considered elements of the innate immune system, which is active at all times but increases in strength the moment a host detects a pathogen, or damage caused by the pathogen. A major function of the innate immune system is to induce adaptive immunity, which generates specific responses to the invading pathogen. While the adaptive immune system is beyond the purview of this article, a key aspect of adaptive immunity, once generated, functions very much like innate immune system components, particularly complement, and therefore is briefly described. Adaptive immune cells (B cells) produce *antibodies*, large soluble proteins that circulate until they bind to pathogens. Antibodies effectively tag pathogens and mark them for capture and destruction by host phagocytic cells and are in this way conceptually analogous to complement. Complement and antibodies work together during an infection, and even on the same bacterium, to ensure that pathogens are rapidly cleared.

Host Cellular Defenses

The classic cellular players of innate immunity are professional phagocytes, largely consisting of neutrophils and macrophages. In addition, NK cells are now recognized to be critical to defending the host prior to the full activation of adaptive immunity. For these reasons, pathogens have evolved strategies to avoid or thwart each of these cell types.

Avoiding Phagocytosis

While the bacterium *Staphylococcus aureus* is generally a human commensal (see above), when it becomes pathogenic it employs numerous methods previously described to evade host defenses such as resistance to AMPs and lysozyme. However, *S. aureus* also has a mechanism to avoid phagocytosis. The cell wall of *S. aureus* has protein A anchored into it. Protein A is known to bind host antibodies (IgG) that would target the cell for phagocytosis. However, when IgG binds to protein A, it is bound in an orientation that neutrophils cannot recognize, allowing the cell to escape phagocytosis.

Inactivating and/or Adapting to the Phagosome

Some bacteria are able to evade the immune system by living and replicating within the phagosome. For example, the bacterium *Mycobacterium tuberculosis* is taken up by macrophages in phagosomes and is able to replicate inside. Under normal conditions a phagosome would recruit vacuolar H^+ ATPase to acidify the phagosome and then fuse with the lysosome. However, *M. tuberculosis* contains many proteins that inhibit acidification of the phagosome and fusion with the lysosome. For instance, the *M. tuberculosis* cell wall protein CpsA has been shown to inhibit degradation by the lysosome and the protein PtpA has been shown to permeate the phagosomal membrane and bind a subunit of the host vacuolar H^+ATPase. After PtpA binds, the H^+ATPase cannot bind the phagosome and thus inhibits the acidification of the phagosome and fusion with the lysosome.

Escaping the Phagosome to Live in the Cytosol

Some organisms are able to escape the phagosome and replicate in the cytoplasm of macrophages. The bacterium *Listeria monocytogenes* escapes the phagosome by lysing it through the production of the toxin lysteriolysin O (LLO) and lipases. Once outside the phagosome the bacterium replicates in the cytoplasm. In addition to being phagocytosed by macrophages, *L. monocytogenes* can be taken up by epithelial cells where they can also reach the cytoplasm to replicate. To further evade host defenses, *L. monocytogenes* produces a protein that hijacks the host cell cytoskeleton to form actin filaments that propel the bacterium into membrane/vacuolar protrusions and then into the neighboring cell. Once in the neighboring cell, *L. monocytogenes* lyses the vacuoles releasing bacterial cells into the cytoplasm without ever coming into contact with the extracellular environment. In the cytosol, *L. monocytogenes* evades host defenses such as complement and antibodies and further promotes infection by altering how organelles function to promote infection (Fig. 5).

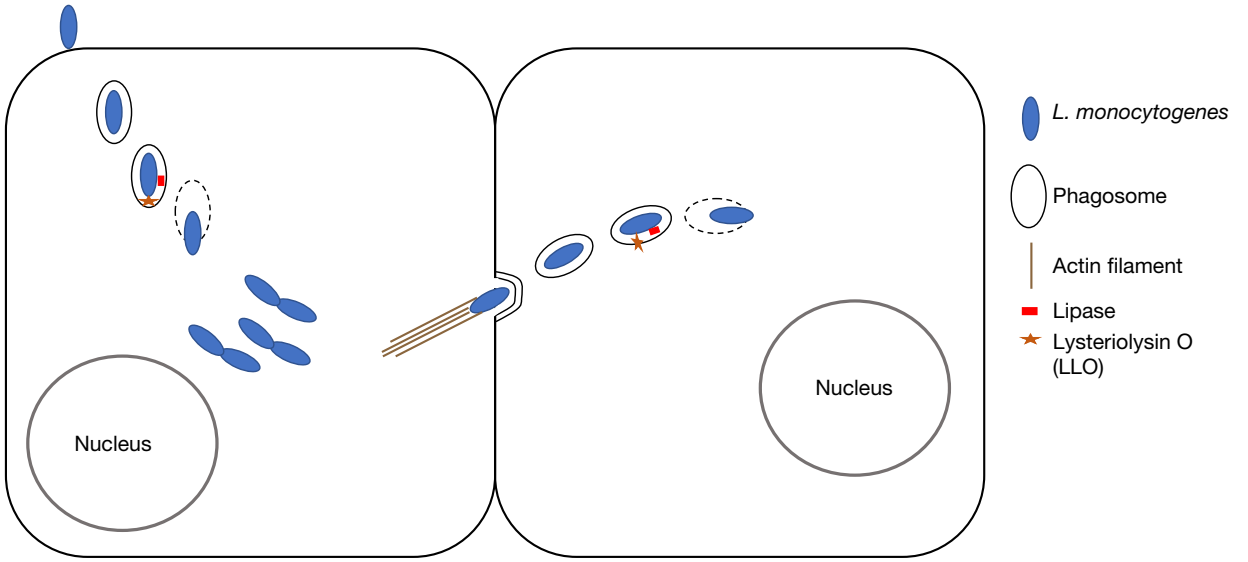

Fig. 5 *Listeria monocytogenes* evades host defenses by surviving in the cytosol of host cells. *L. monocytogenes* enters host cells through a phagosome. Once inside the host cell, *L. monocytogenes* produces lipases and the toxin lysteriolysin O (LLO) to degrade the phagosome membrane and escape into the cytosol. In the cytosol, the bacteria replicates and expresses the protein ActA. ActA interacts with the host complex Arp2/3 to polymerize actin filaments to push the L. *monocytogenes* cell into the neighboring host cell without coming into contact with the extracellular environment. Once in the new host cell, the cycle can repeat.

Nonmammalian Hosts: Evading Plant Defenses

Microbes have evolved methods to evade not only mammalian hosts, but also plant hosts. Plants do not have immune cells that circulate throughout the organism like mammals and must instead rely upon the innate immunity of each cell, and proper systemic signaling to occur from wound sites through which pathogens enter. Like mammals, plants use transmembrane receptors to recognize potential pathogens. When a plant receptor recognizes a microbe by its microbe-associated molecular pattern (MAMP) it can trigger an immune response. However, if a pathogen successfully evades recognition, it will produce effector proteins that can have a wide range of functions, but overall contribute to virulence. In addition to receptors that recognize MAMPs, plants also have a set of receptors that recognize a wide variety of pathogen effector proteins, leading to the activation of immunity. To avoid being recognized by plant receptors, microbes are continually evolving their effector proteins by acquiring mutations or additional effectors. Similar to mammals, once an immune receptor recognizes a potential pathogen, a signaling cascade occurs that will result in a range of responses to get rid of the pathogen, such as production of reactive oxygen and nitrogen species, calcium fluxes, or antimicrobial peptides. However, in addition to this immune signaling pathway, plants use small compounds such as the plant hormones jasmonic acid and ethylene to modify their immune response. As of now, there is no known similar mechanism in the immune response of mammals.

The most successful plant pathogens use numerous methods to evade the plant immune response. The bacterial pathogen *Pseudomonas syringae* has evolved many methods to get around the plant immune system. Of the 60 strains that have been identified, all have been found to use a type 3 secretion system (T3SS) to inject effector proteins that block plant receptors from identifying *P. syringae*, thus escaping degradation. Other effector proteins of *P. syringae* have been shown to inhibit immune signaling by interacting with the host cytoskeleton and prevent signaling molecules from getting to their proper destination. Some strains of *P. syringae* produce toxins and enzymes to degrade host cell walls, or are even able to produce compounds that mimic plant hormones to interfere with immune signaling. This variety in mechanisms to evade the plant immune system is likely why *P. syrinage* can infect most crop species and is one of the most common plant pathogens.

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Human Fungal Infections

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Fungal Pathogens and Fungal Infections in Humans

Fungi are eukaryotic microorganisms that are classified traditionally and morphologically into yeast and filamentous forms. Fungi, however, are often pleomorphic and the human fungal pathogens have been categorized into three groups: the yeast, filamentous, and dimorphic fungal pathogens (Figure 1). Dimorphic fungal pathogens exhibit a filamentous (hyphal) form at ambient temperatures below 37 °C and a yeast form at temperatures above 37 °C, during human infection. Dimorphic fungi tend to be restricted to specific geographic regions and are responsible for so-called endemic mycoses. This probably reflects adaptation of the filamentous fungi to certain climates or soils rather than mammalian hosts.

Fungi are responsible for a wide spectrum of diseases in humans, ranging from acute, self-limiting pulmonary manifestations and superficial infections in immunocompetent hosts to life-threatening invasive infections, especially among immunocompromised or hospitalized individuals with serious underlying diseases. Invasive fungal infections are usually severe, involving infections of the blood, deep tissues, and organs. In the past three decades, these infections have emerged as a major cause of human diseases. Most fungal pathogens of humans are ubiquitous in the environment and humans are exposed by inhaling spores. Yet, others are components of the normal human flora and take advantage of immune disequilibria to cause infection. This article will survey the fungal pathogens responsible for invasive human infections, distinguishing those causing opportunistic infections from those responsible for endemic infections.

The opportunistic invasive fungal infections that occur in immunocompromised or weakened hosts almost always represent health care–associated infections and may be either of nosocomial origin, occurring upon hospitalization, or community acquired. The prevailing agents of opportunistic mycoses are *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans, Pneumocystis jirovecii*, and several species of Mucorales (Figure 1). On the other hand, endemic mycoses are due to fungi restricted to specific geographic regions constituting their natural habitat. In contrast to opportunistic pathogens, these fungi also may infect and cause invasive diseases in immunocompetent individuals. Yet, these infections are exacerbated in individuals whose host immune functions are compromised or suppressed. The main pathogens recognized as agents of endemic mycoses are *Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis, Sporothrix schenckii, and Penicillium marneffei.*

Opportunistic Fungal Infections

The frequency of invasive infections due to opportunistic fungal pathogens has increased drastically during the recent years. This is in particular due to a growing population of immunosuppressed patients, including hospitalized patients, especially those from the intensive care unit; stem cell and solid organ transplant recipients; patients treated with immunosuppressive treatments; those showing advanced human immunodeficiency virus (HIV) infections or other acquired immunodeficiency conditions; and patients with organ failure syndromes (Alangaden, 2011; Pfaller and Diekema, 2007). Opportunistic fungi are a continuously evolving pathogen group that plagues these vulnerable patients (Figure 1). In hospitalized patients with serious underlying diseases, a number of fungi, including yeasts and filamentous fungi, may cause nosocomial invasive infections, *Candida* spp., *Aspergillus* spp., and species of Mucorales being the most prevalent pathogens (Perlroth et al., 2007; Pfaller and Diekema, 2010; Alangaden, 2011; Lass-Florl, 2009). In contrast, infections due to *P. jirovecii* and *C. neoformans* are communityacquired infections resulting from the inhalation of aerosolized fungi from the environment (Catherinot et al., 2010; Park et al., 2009).

Infections Due to *Candida* Species

Candida spp. are ubiquitous yeasts commonly isolated from the environment. Current taxonomies list almost 200 species of ascomycetous, asexual yeasts in the genus *Candida*. Among these, a few are encountered in the commensal flora of human beings (Calderone, 2002). *Candida albicans* and other *Candida* spp., such as *Candida glabrata* or *Candida tropicalis*, may be present in the normal gastrointestinal and genital flora of healthy humans, while *Candida parapsilosis*, *Candida famata*, and *Candida guillermondii* are commensals of the skin.

Candida spp. are opportunistic pathogens that have the ability to cause various superficial and systemic infections when the host's resistance to infection is impaired locally or systemically. The most frequent clinical manifestations are superficial candidiasis, including cutaneous, oropharyngeal candidiasis, and vulvovaginitis. These infections are frequent and usually benign in immunocompetent hosts. *Candida* sp., however, also can cause life-threatening infections, mainly in hospitalized hosts. Neutropenia, neutrophil dysfunction, parenteral nutrition, and disruption of mucosal barriers are the main risk factors for disseminated

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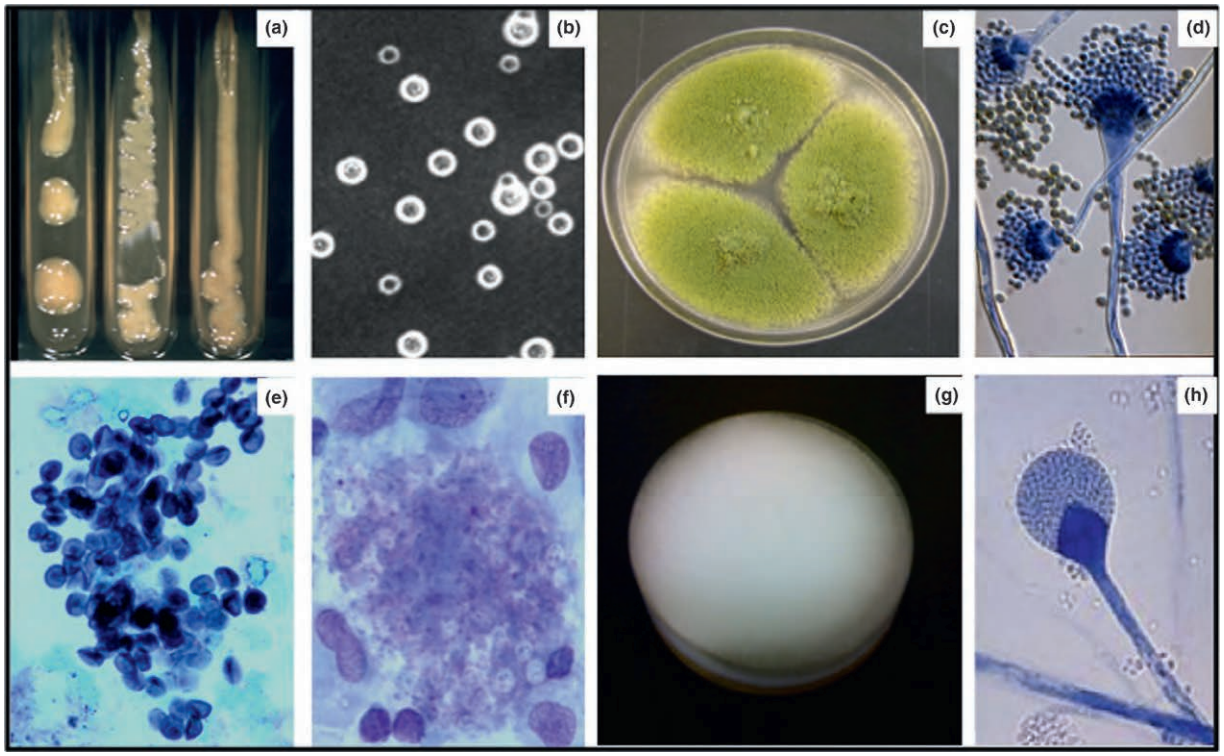

Figure 1 Examples of major morphological aspects seen in opportunistic fungal pathogens of humans. (a) Morphology of Cryptococcus neoformans in culture and (b) using India ink staining that allows easy visualization of the capsule. (c) Morphology of *Aspergillus* spp. in culture and (d) microscopic feature of *Aspergillus* spp. Lactophenol cotton blue staining shows spores that grow in chains from phialides that emerge from a central vesicle. (e) Morphology of *Pneumocystis jirovecii* upon direct microscopic examination using Grocott's methenamine silver stain (Grocott) and (f) Giemsa stain. (g) Morphology of *Lichtheimia ramosa* in culture and (h) in direct microscopic examination. Lactophenol cotton blue staining shows a globose intact sporangium with sporangiospores.

infections; translocation from the gastrointestinal tract and intravascular catheters are the two main portals of entry for disseminated *Candida* infections. These infections have emerged as a major cause of infectious complications in hospitalized patients with severe underlying diseases, as well as those with complicated surgical abdominal procedures or critical illnesses that need aggressive diagnosis or treatment procedures. Recent and comprehensive epidemiological surveys have reported that infections due to *Candida* spp. account for approximately 75–88% of all nosocomial invasive fungal infections (Azie et al., 2012; Jarvis, 1995; Perlroth et al., 2007). Notably, *Candida* spp. rank fifth among microorganisms causing nosocomial bloodstream infections in US and European hospitals and *C. albicans* is the most frequently isolated species (Gudlaugsson et al., 2003; Lass-Florl, 2009; Pfaller and Diekema, 2007; Bougnoux et al., 2008). Invasive *Candida* infections remain associated with a dramatically high mortality, in the range of 40%, despite progresses in their diagnostics and therapeutic advances (Andes et al., 2012; Pfaller and Diekema, 2007). Recent studies suggest a possible improvement of the patient outcomes depending on therapeutic management strategies. In particular, early antifungal treatment has been associated with an improved overall response, suggesting that therapy was more efficient when the fungal burden was low (Garey et al., 2006; Pfaller and Diekema, 2010; Morrell et al., 2005).

Infections Due to Aspergillus Species

Aspergillus spp. are filamentous fungi belonging to the Ascomycota phylum. More than 250 species within the genus *Aspergillus* have been described to date (Geiser et al., 2007). Currently, the genus *Aspergillus* is divided into seven subgenera that are subdivided into several sections composed of related species. Most *Aspergillus* spp. have asexual reproduction, but a sexual form has been identified for some of them (Figure 1(c) and 1(d)). These fungi, which are soil saprophytes, play a significant role in the decomposition of organic materials, recycling environmental carbon and nitrogen. During asexual reproduction, abundant conidia are produced and released into the atmosphere allowing the fungi to spread within the environment.

Exposure to *Aspergillus* conidia in the environment may cause a large spectrum of diseases related to host factors, including allergic manifestations, chronic necrotizing aspergillosis, and invasive infections (Figure 2(d) and 2(e)). Although *Aspergillus* conidia spread in the air are from several species, relatively few species are known to cause disease in humans. *Aspergillus fumigatus* is the leading etiological agent of aspergillosis followed by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans*.

In immunocompetent individuals, *Aspergillus* species can induce allergic responses, such as sinusitis and asthma. It might be the consequence of an immune reaction to the colonization of airways by *Aspergillus* spp. in patients who are likely atopic. Allergic

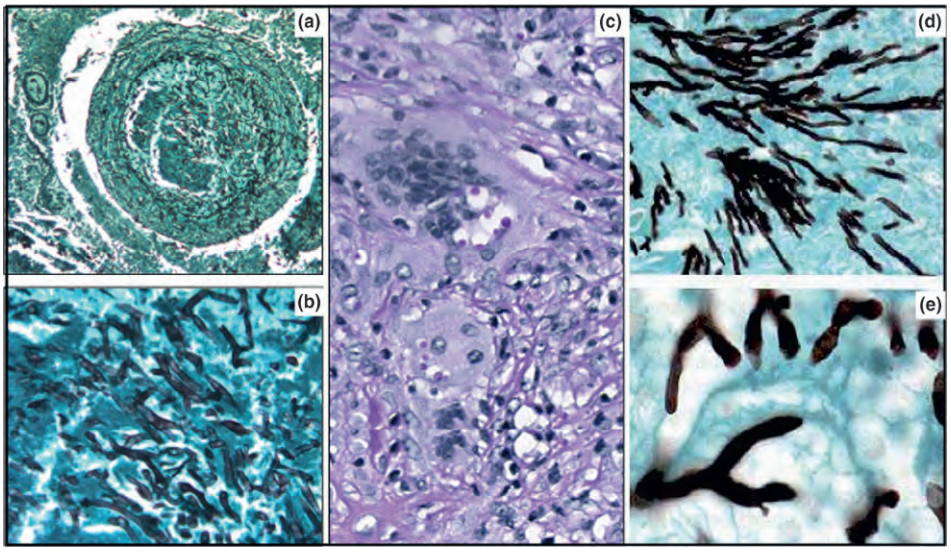

Figure 2 Appearance of Mucorales, *Cryptococcus neoformans*, and *Aspergillus* spp. in infected tissues using the two major stains used to detect fungi histopathologically, Periodic acid-Schiff's stain (PAS) and Grocott's methenamine silver stain (Grocott). (a) Lung infection due to Mucorales. Grocott staining of a lung biopsy shows the fungi stained in black (×100). (b) Further magnification (×400) shows the typical structure of Mucorales hyphae in tissue: distorted, nonseptate, and broad hyphae with right-angle branching. (c) Infection due to *Cryptococcus neoformans*. Nodular biopsy shows granulomatous reaction to fungus with 'empty' lacunar spaces containing rounded fungal organisms stained in bright red with PAS (×400). The cryptococcal capsule is not colored. (d) Lung infection due to *Aspergillus* spp. Grocott staining of a lung biopsy shows the fungi stained in black (×100). (e) Further magnification (×400) shows the typical structure of *Aspergillus* hyphae with dichotomous 45-angle branching.

bronchopulmonary aspergillosis is a severe allergic disease that occurs in 1–2% of the patients with asthma and in 1–15% of the patients with cystic fibrosis (Stevens et al., 2003). *Aspergillus* spp. also can proliferate within a preformed cavity in the lungs of patients who are immunocompetent, generating a fungal mass, called aspergilloma. Prevalence of aspergillomas may be high in post-tuberculous cavities and in patients with bronchiectasis or lung abscess.

Acute invasive aspergillosis is a rapidly progressing, often fatal disease that occurs in highly immunocompromised patients. Invasive pulmonary aspergillosis (IPA) is a common infectious complication and a major cause of severe morbidity and mortality in patients with hematologic diseases (Bougnoux et al., 2011). IPA is the leading form of invasive fungal infections in hematopoietic stem cell transplant patients, accounting for 43% of invasive fungal infections (Kontoyiannis et al., 2010). Invasive aspergillosis is confined mostly to the lungs, but sinus and the central nervous system also may be affected. The main defects in host defenses strongly associated with an increased risk for IPA are (1) prolonged neutropenia, (2) qualitative defects in phagocyte function, and (3) deficit in cell-mediated immunity (Patterson et al., 2000; Segal, 2009). Absolute neutrophil count $<100\,mm^{-3}$ and a duration >10 days of neutropenia are major risk factors for IPA. Despite the availability of new antifungal agents, mortality rates range from 30 to 50% (Herbrecht et al., 2002; Neofytos et al., 2010, 2009). Several factors, including the severity of the immunodeficiency secondary to the underlying disease and its treatment, contribute to this extremely poor outcome.

Infections Due to Mucorales

The fungi of the order Mucorales (class Zygomycetes) are filamentous fungi, ubiquitous in decaying soil and vegetation. The pathogenic Mucorales in humans include approximately 10 genera, the most common being *Rhizopus*, *Lichtheimia* (formerly known as *Absidia*; Figure 1(g) and 1(h)), *Mucor*, and *Cunninghamella*. Most human infections result from the inhalation of fungal spores released in the air or from direct inoculation of organisms into disrupted skin. These infections, referred to as Mucormycoses, occur mainly in immunocompromised or diabetic patients (Petrikkos et al., 2012). The importance of these opportunistic fungal infections has risen in the past few years, as the number of patients with predisposing factors for mucormycosis (e.g., diabetic ketoacidosis, hematologic malignancy, renal failure, and corticosteroid or deferoxamine therapy) has increased dramatically.

Mucorales species are vasotropic, with the hyphae invading blood vessels during infection and causing tissue infarctions and necrosis. The mucormycosis spectra encompass cutaneous, rhinocerebral, and pulmonary infections as well as disseminated infections (Figure 2(a) and 2(b)). Despite antifungal therapy associated with surgical debridement, the overall mortality rate for mucormycosis is extremely high, especially in immunocompromised hosts, ranging from 50 to 100% (Spellberg et al., 2005).

Many other opportunistic filamentous fungi have been reported to be responsible for invasive infections in immunocompromised patients, especially those with hematologic diseases and solid organ transplant recipients. Filamentous fungi, such as *Fusarium* spp., *Scedosporium* spp., or *Geosmithia argillacae*, give rise to refractory infections in part due to their poor susceptibility to the available antifungal agents (Caira et al., 2008; Valentin et al., 2012; Kontoyiannis et al., 2004).

Infections Due to *C. neoformans*

Cryptococcus spp. are encapsulated yeasts from the Basidiomycota phylum (Figure 1(a) and 1(b)). Two species can cause infections in humans: *C. neoformans* and *Cryptococcus gattii*. *Cryptococcus neoformans* is an environmental species from tree and plants isolated worldwide. Pigeons are known to contribute to its propagation and spread (Mitchell and Perfect, 1995). *Cryptococcus gattii* differs significantly in its geographic distribution and ecologic niches. It is found mostly in tropical and subtropical regions and is associated with Eucalyptus trees. Since an outbreak of *C. gattii* occurred in North America about 12 years ago, however, it has become evident that this species also can be isolated from other environmental samples and in temperate regions (Datta et al., 2009). Cryptococcosis is a lifethreatening infection usually acquired by inhaling aerosolized cells of *C. neoformans* or *C. gattii*. Cryptococcal pneumonia can be the initial form of the disease, but meningoencephalitis is the most common and serious clinical presentation (Figure 2(c)). Cryptococcosis due to *C. neoformans* occurs only in immunocompromised patients, whereas those due to *C. gattii* occur more often in immunocompetent hosts. *Cryptococcus neoformans*, the most prevalent species, causes an estimated 1 million cases of meningoencephalitis globally per year, in patients with acquired immunodeficiency syndrome (AIDS), leading to approximately 625 000 deaths (Park et al., 2009). Administration of highly active antiretroviral therapy (HAART) has resulted in a decrease in the number of cases of AIDS-related cryptococcosis in industrial countries. In developing countries where HAART is not readily available, however, *Cryptococcus* remains a major concern. Other patients at particular risk for Cryptococcal infections include those with neoplastic disorders, mainly Hodgkin lymphoma, those receiving corticosteroids, organ transplant, or immunosuppressive therapy. Cryptococcosis is currently the third most common invasive fungal infection in organ transplant recipients (Neofytos et al., 2010; Pappas, 2010; Pappas et al., 2010).

Infections Due to P. *jirovecii*

Pneumocystis spp. are classified within the phylum Ascomycota, in a unique class, order, and family (Pneumocystidomycetes, Pneumocysttidales, and Pneumocystidacae, respectively) (Redhead et al., 2006). These are atypical fungal microorganisms unable to grow *in vitro* in culture media, thus greatly complicating their study. *Pneumocystis* is ubiquitous throughout nature, infecting a variety of mammalian species with strict host specificity. In this respect, *P. jirovecii* (formerly *Pneumocystis carinii* f. sp. *hominis*) is restricted to humans (Stringer et al., 2002), causing severe pneumonia in patients having an impaired immune system (Figure 1(e) and 1(f)). CD4+ T-lymphocytes are essential for the control of *Pneumocystis* pneumonia (PCP) and a CD4+ T cell defect is a primary risk factor for development of PCP. A B-cell defect and lowered antibody production, however, also may predispose a patient to PCP. The patients at risk of PCP are mainly HIV-infected persons whose CD4+ cell count is <200 cells per μl and whose viral replication is not controlled; other non-HIV immunocompromised hosts, such as those with hematological malignancies and solid tumors, solid organ, and bone marrow transplant recipients; and patients with collagenvascular disorders (Catherinot et al., 2010). The incidence of PCP has increased in non-HIV immunocompromised hosts while it has declined in HIV-infected individuals since HAART became available. No exosaprophytic form of *P. jirovecii* has been identified so far and the latent human sources may be represented by all infected or colonized individuals (Totet et al., 2003). Host-to-host transmission of *P. jirovecii* via the airborne route has been suspected in humans through the occurrence of clusters of PCP cases in hospitals (Choukri et al., 2010; Le Gal et al., 2012). *Pneumocystis* spp. is highly susceptible to trimethoprim-sulfatmethoxazole (TMP-SMX), which is the first-line agent for the treatment of PCP in HIV and non HIV-infected patients. TMX-SMX is also the first-choice prophylaxis in HIV and non-HIV-infected hosts.

Endemic Fungal Infections

Infections Due to *Blastomyces dermatitidis*

Blastomyces dermatitidis is a dimorphic fungus endemic to the central and southeastern United States and also to some regions of Canada. Blastomycosis is acquired by inhalation of aerosolized conidia. Most infected individuals are asymptomatic or develop a self-limited respiratory illness. The dominant clinical manifestation of blastomycosis is a pneumonia that can be acute, subacute, or even chronic. The infection may disseminate to skin and bones or, less commonly, to the central nervous system and the meninges. The disease is more severe in patients with AIDS.

Infections Due to *Coccidioides Species*

Coccidioidomycosis is caused by the soil-dwelling fungi *Coccidioides immitis* and *Coccidioides posadasii*, which are localized to relatively arid regions of the Western hemisphere. Areas of highest endemism in North America are the San Joaquin Valley of California, the south-central region of Arizona, and northwestern Mexico. The number of reported cases of coccidioidomycosis has risen over the past decade: In Arizona and California, for instance, the incidence rates have increased by more than 90% from 2001 to 2006 (Hector et al., 2011). The vast majority of cases of coccidioidomycosis are acquired by inhalation. Approximately 60% of these infections are asymptomatic (Galgiani et al., 2005). Acute pulmonary coccidioidomycosis is not specific and the pulmonary syndrome mimics other community-acquired pneumonia syndromes or upper-respiratory-tract infections (Valdivia et al., 2006). It sometimes may be distinguished from other community-acquired pneumonia by the presence of hilar adenopathy, peripheral

blood eosinophilia, severe fatigue, night sweats, and erythema multiforme or erythema nodosum. In endemic regions, coccidioidomycosis might be responsible for nearly one-third of the patients presenting with lowerrespiratory-tract symptoms.

Infections Due to *Histoplasma capsulatum*

Histoplasma capsulatum is a dimorphic fungus reported in at least 60 countries worldwide, but it is endemic in the United States (especially in the Mississippi and Ohio river valleys) and in Central and South America. The natural habitat of this fungus is a soil with a high nitrogen content, such as those contaminated with bird or bat guano. Inhalation of aerosolized conidia and hyphal fragments from polluted soils is considered to be the main source of human contamination. The severity of illness after inhalation depends on both the intensity of exposure and the host immune status. Although the attack rate reaches 100% in certain areas, most cases remain asymptomatic and are detected only by skin tests (Kauffman, 2007). After exposure to a heavy inoculum, most individuals exhibit various symptoms from self-limited acute pulmonary histoplasmosis to disseminated histoplasmosis. Disseminated disease is much more common in young children and in immunocompromised adults, such as AIDS patients, organ transplant recipients, and patients receiving immunosuppressive chemotherapy. The mortality associated to disseminated histoplasmosis is high, up to 50%. The optimal treatment of histoplasmosis varies according to the patient's clinical syndrome (see Table 1) (Wheat et al., 2007).

Innate Immunity, Adaptive Immunity, and Fungal Infections

Fungal diseases are linked intimately to dysfunction of the immune system. Indeed, local or systemic, genetic, pathologic, or drug-induced alterations of the immune system will modify the equilibrium established between environmental or commensal fungi and the human body and favor fungal development and disease. Inversely, fungi can trigger an uncontrolled inflammatory response that has pathological consequences. In this respect, both innate and adaptive immunity play important roles in maintaining homeostasis with fungi (Romani, 2011). The knowledge that is being gained on these particular aspects of fungal infections certainly will contribute to their better management through the use of therapies aimed at modulating the immune system, the development of vaccines, or the genetic testing of patients to identify those at risk of developing some forms of fungal infections.

Innate immune mechanisms are used to sense and respond to fungal pathogens in a relatively unspecific manner. These mechanisms are effective at various sites of interaction with fungi. They include barriers, such as the skin and the mucosal surfaces of the respiratory, gastrointestinal, and genitourinary tracts (Romani, 2004). They also involve constitutive defense mechanisms, such as defensins, collectins, and the complement system. Fungi also harbor so-called pathogen-associated molecular patterns (PAMPs), whose recognition by host cell pattern recognition receptors (PRRs) triggers the activation of signaling cascades, the activation of the immune system, and fungal clearance. A majority of the known fungal PAMPs are constituents of the fungal cell wall that are shared by almost all fungi: chitin, a polymer of *N*-acetylglucosamine, and β-glucan, a polymer of D-glucose linked by β-(1,3) bonds and branched through β-(1,6) bonds, which together form the inner layer of the cell wall; mannose polymers (mannans), which decorate cell wall glycoproteins at *O*- and *N*-glycosylation sites and form the outer layer of the cell wall; α-glucan, a polymer of D-glucose linked by α-(1,4) bonds; and β-(1,2)-oligomannosides, which also are found on cell wall glycoproteins as well as cell wall lipopeptides (Munro and Richard, 2012). Although *N*-linked mannan, glucans, and chitin are recognized by *C*-type lectin receptors, *O*-linked mannan (as well as fungal RNA and DNA) are recognized by Toll-like receptors and β-(1,2)-oligomannosides are recognized by galectin-3 (Netea et al., 2008). Recognition of the PRRs results in activation of the nuclear factor κB (NF-κB) and the inflammasome. A consequence of this activation is the production of defensins, chemokines, and cytokines as well as phagocytosis and killing of fungal propagules (Romani, 2011).

Contribution of innate immunity components to antifungal defenses is highlighted by the increased susceptibility to fungal infections of patients receiving treatments that dampen innate immunity at different levels. Cancer chemotherapy, for instance, that affects the levels of neutrophils and alters the integrity of the intestinal epithelium is well known to trigger translocation of *C. albicans* from the gastrointestinal lumen to the bloodstream (Koh et al., 2008). Similarly, neutropenia is the main predisposing factor to invasive aspergillosis (Patterson et al., 2000; Segal, 2009). More specifically, mutations in genes that encode innate immunity components have been shown to predispose some individuals to fungal infection (Romani, 2011). Dectin-1 is a *C*-type lectin receptor that is responsible for the recognition of β-glucans (a major fungal cell wall component; see the next section) and subsequent activation of NF-κB (Brown, 2006). Mutations in the Dectin-1 gene that result in the production of a shorter form of the protein and impair its ability to bind β-glucans and trigger the production of cytokines confer increased susceptibility to chronic mucocutaneous candidiasis, colonization of the gastrointestinal tract by *C. albicans*, and invasive aspergillosis (Ferwerda et al., 2009; Plantinga et al., 2009; Cunha et al., 2010). Similarly, mutations in Toll-like receptors that recognize fungal RNA and DNA and *O*-linked mannan predispose to different forms of aspergillosis and to *C. albicans* infections (Romani, 2011).

Recognition of fungal pathogens by monocytes, macrophages, and neutrophils as well as epithelial and endothelial cells mainly results in their phagocytosis and killing. In contrast, phagocytosis of fungal pathogens by dendritic cells (DCs) initiates T cell–mediated immune responses that can contribute to the clearance of fungal infection or promote fungal pathogenesis and cause tissue damage. Indeed, maturation of DCs triggers the differentiation of naïve T cells into effector T helper (T_H) cell subtypes that have different properties. Although T_H1 and T_H17 cells contribute to protective immunity against fungi, T_H2 cells favor fungal infection, allergic responses, and disease relapse. Notably, a predominant T_H1 response is associated with effective fungal

Table 1 Summary of recommendations for the treatment of invasive fungal infections

Disease condition	*Therapy*		*Comments*
	Primary	*Alternative*[a]	
Candidiasis (Pappas et al., 2010)			
Candidemia nonneutropenic adults	Fluconazole (800 mg loading dose day 1, then 400 or 6 mg kg^{-1} day^{-1}) or caspofungin (70 mg loading dose day 1, then 50 mg day^{-1}) or micafungin (100 mg day^{-1})		
Candidemia, clinically unstable and unknown species neutropenic patients	Lipid-based amphotericin B (3–5 mg kg^{-} 1 day^{-1}) or caspofungin (70 mg loading dose day 1, then 50 mg day^{-1}) or micafungin (100 mg day^{-1})	Fluconazole (800 mg loading dose day 1, then 400 or 6 mg kg^{-1} day^{-1}) or voriconazole (6 mg kg^{-1} per 12 h × 2, then 3 mg kg^{-1} per 12 h) or high-dose fluconazole (800 mg day^{-1} or ∼12 mg kg^{-1} day^{-1})	
Aspergillosis (Walsh et al., 2008)			
Invasive aspergillosis	Intravenous voriconazole (6 mg kg^{-1} every 12 h for 1 day, followed by 4 mg kg^{-1} every 12 h) until improvement, followed by oral voriconazole (200 mg every 12 h)	Intravenous liposomal amphotericin B (3–5 mg kg^{-1} day^{-1}) or caspofungin (70 mg day 1 and 50 mg day^{-1}) or micafungin (100–150 mg day^{-1})or posaconazole (200 mg four times daily, then 400 mg twice daily) or itraconazole (400–600 mg day^{-1}) until resolution of disease	
Chronic necrotizing (semi-invasive) pulmonary aspergillosis	For mild to moderate disease, voriconazole (200 mg every 12 h) or itraconazole (400–600 mg day^{-1})until resolution or stabilization of all clinical and radiographic manifestations		
Mucormycosis (Limper et al., 2011)			
	Lipid formulations of amphotericin B (5 mg kg^{-1} day) or amphotericin B deoxycholate (0.7–1.0 mg kg^{-1} day^{-1})	Posaconozole (400 mg orally twice daily or 200 mg orally four times per day) (only some strains fully susceptible)	Exact dose and duration of treatment are not precise
Infections due to Trichosporon spp. (Limper et al., 2011)			
	Voriconazole	Posaconazole	
Infections due to Fusarium spp. (Limper et al., 2011)			
	Voriconazole or posaconazole or lipid formulation of amphotericin B		
Infections due to Scedosporium apivospermum (Limper et al., 2011)			
	Voriconazole (200 mg intravenously or orally twice daily) or posaconozole (200 mg four times daily)		
Cryptococcosis (Perfect et al., 2010)			
Cryptococcal meningoencephalitis HIV-infected individuals	Amphotericin B IV (0.7–1.0 mg kg^{-1} day^{-1}) plus flucytosine (100 mg kg^{-1} day^{-1}) for 2 weeks, followed by fluconazole (400 mg day^{-1} (6 mg kg^{-1} day^{-1}) orally for 10 weeks) or liposomal AmB (3–4 mg kg^{-1} per day IV) plus flucytosine (100 mg kg^{-1} day^{-1}) for 2 weeks, followed by fluconazole (400 mg day^{-1} (6 mg kg^{-1} day^{-1}) orally for 10 weeks	Amphotericin B IV (0.7–1.0 mg kg^{-1} day^{-1}) plus fluconazole (800 per day orally) for 2 weeks, followed by fluconazole (800 per day orally) 8 weeks	

(*Continued*)

Table 1 Continued

Disease condition	*Therapy*		*Comments*
	Primary	*Alternative*	
Pneumocystis jirovecii pneumonia (Limper et al., 2011)			
	Trimethoprim 15–20 mg kg^{-1} plus sulfamethoxazole 75–100 mg kg^{-1} daily for 3 weeks	Primaquine plus clindamycin or atovaquone	
Blastomycosis (Limper et al., 2011; Chapman et al., 2008)			
Mild to moderately ill patients with pulmonary and nonmeningeal disseminated blastomycosis	Itraconazole (200 mg twice daily for 24 weeks)		
Life-threatening severe blastomycosis	Liposomal amphotericin B (3–5 mg kg^{-1} day^{-1}) or amphotericin B (0.7–1.0 mg kg^{-1} day^{-1}) for 1–2 weeks, followed by itraconazole (200 mg twice daily for 6–12 months)		
Coccidioidomycosis (Limper et al., 2011)			
Diffuse pulmonary	Liposomal amphotericin B (3–5 mg kg^{-1} day^{-1}) or amphotericin B (0.7–1.0 mg kg^{-1} day^{-1}) until clinical improvement, followed by fluconazole (400 mg day^{-1}) or itraconazole (400 mg day^{-1}) for at least another year		
Disseminated, nonmeningeal (including bone disease)	Fluconazole (400 mg day^{-1}) or itraconazole (400 mg day^{-1})		
Meningitis	Fluconazole (400–1,000 mg day^{-1}) or itraconazole		
Histoplasmosis (Wheat et al., 2007)			
Mild pulmonary histoplasmosis	Itraconazole (200 mg twice daily for 12 weeks)		Treatment is usually unnecessary
Moderately to severely ill pulmonary histoplasmosis	Amphotericin B (0.7 mg kg^{-1} day^{-1}) +/− corticosteroids for 1–2 weeks, then itraconazole (200 mg twice daily for 12 weeks)		
Progressive disseminated histoplasmosis	Lipid formulation amphotericin B (3–5 mg kg^{-1} day^{-1}) for 1–2 weeks, followed by oral itraconazole (200 mg twice daily for 12 months)		

[a]Alternative (salvage) therapy for patients refractory to or intolerant of primary antifungal therapy.

vaccination. The balance between these responses is a consequence of the levels of different cytokines that are produced by DCs as well as macrophages and epithelial cells upon their interaction with fungal pathogens and their combined PAMPs.

The contribution of T_H1 and T_H17 cells to protective immunity against fungi is highlighted by the increased susceptibility to fungal infections of patients with mutations in genes that encode components of these two arms of adaptive immunity. For instance, deficiency in the recognition of interferon-γ, a landmark of the T_H1 response, is associated with disseminated coccidioidomycosis (Vinh et al., 2009). On the other hand, the study of kindreds with cases of autosomal recessive or dominant chronic mucocutaneous candidiasis disease showed that diseased individuals had mutations in genes with roles in T_H17 cell responses such as those encoding the interleukin IL-17, its receptor or STAT1, a transcription factor that is involved in inhibiting T_H17 cell responses (Liu et al., 2011; Puel et al., 2011; van de Veerdonk et al., 2011). Inversely, mutations in genes related to T_H2 cell responses often are associated with fungus-associated allergic responses. An example of such mutations includes those resulting in increased IL4 production, a landmark of T_H2 cell responses, that favor recurrent vulvovaginal candidiasis and those in the IL4 receptor that favor allergic bronchopulmonary aspergillosis (Babula et al., 2005; Knutsen et al., 2006).

Fungal Pathogenesis from the Prism of Molecular Biology

Major progresses have been made over the recent years in our molecular understanding of the mechanisms involved in fungal pathogenesis. These progresses have been driven partly by the availability of genome sequences for a majority of the fungal pathogens of humans (Table 2). Indeed, transcriptomic and proteomic approaches to these organisms are now possible, allowing to identify genes and proteins whose expression levels are influenced under conditions relevant to the interaction with the host (*ex vivo* and animal models of infection) (Wilson et al., 2009; Kniemeyer et al., 2011). These progresses are also a consequence of the availability of methods for the manipulation of the genomes of fungal pathogens. These methods allow the construction of knockout mutants, overexpression mutants, and strains with gene fusions that can be used to assess transcriptional changes at the single-cell level in a host–pathogen interaction context (Noble and Johnson, 2007; Idnurm and Williamson, 2011). In some species in which the engineering of mutant strains through homologous recombination is tedious, alternative strategies to knock down gene expression have been developed taking advantage of RNA interference (Rappleye and Goldman, 2006). Currently, the combination of genome sequences and methods for genome engineering is driving the establishment of systematic collections of molecularly tagged knockout mutants and overexpression strains for the major fungal pathogens of humans in a way that allows the simultaneous monitoring of many strains in a single experiment (Roemer et al., 2003; Liu et al., 2008; Noble et al., 2010). These approaches already have provided insights in fungal pathogenesis and identified virulence factors or antifungal targets (Xu et al., 2007a; Liu et al., 2008; Noble et al., 2010).

As mentioned earlier, most fungal pathogens of humans are opportunistic rather than obligate pathogens and their ability to cause disease is a direct consequence of local or systemic alteration of the immune system. It ensues that these fungi have evolved to thrive in their natural environments rather than cause infection in humans, an aspect that should be taken into consideration when one discusses their virulence factors (Casadevall and Pirofski, 2007). For instance, soil fungi, such as *C. neoformans*, because of their frequent encounters with amoeboid predators, are likely to have evolved escape mechanisms that also allow them to counteract human phagocytes and contribute to their ability to persist in the host and cause disease (Steenbergen et al., 2001).

A number of human pathogenic fungi are able to transit between different morphological forms, a property that is linked intimately to their virulence potential. For instance, *C. albicans* can grow in yeast, pseudohyphal, and hyphal forms

Table 2 Genomes of the major fungal pathogens of humans

Species	*Reference*
Candida albicans	Jones et al. (2004); Butler et al. (2009)
Candida tropicalis	Butler et al. (2009)
Candida lusitaniae	Butler et al. (2009)
Candida parapsilosis	Butler et al. (2009)
Candida guillermondii	Butler et al. (2009)
Candida dubliniensis	Jackson et al. (2009)
Candida glabrata	Dujon et al. (2004)
Aspergillus fumigatus	Nierman et al. (2005)
Histoplasma capsulatum	http://genome.wustl.edu/genomes/view/histoplasma_capsulatum/
Coccidioides immitis	Sharpton et al. (2009)
Blastomyces dermatiditis	http://www.broadinstitute.org/annotation/genome/blastomyces_dermatitidis/MultiHome.html
Cryptococcus neoformans	Loftus et al. (2005)
Cryptococcus gattii	D'Souza et al. (2011)
Malssezia globosa	Xu et al. (2007b)
Rhizopus oryzae	Ma et al. (2009)
Pneumocystis carinii	http://pgp.cchmc.org/
Encephalitozoon cuniculi	Katinka et al. (2001)

(Sudbery et al., 2004). The switch between these forms is triggered by a variety of environmental cues, including temperature, serum, CO_2, pH, and surface contact. Several signaling pathways, notably those involving the secondary messenger cyclic adenosine monophosphate (cAMP) and the cAMP-dependent protein kinase, have been described that transduce these cues and allow *C. albicans* yeast cells to initiate a transcriptional program that leads to the expression of so-called hypha-specific genes, the reorganization of cell polarity and the inhibition of cytokinesis (Sudbery, 2011). Locking *C. albicans* in the yeast or hyphal forms generally is associated with virulence defects, and it has been shown that while yeast cells contribute to dissemination and surface colonization, hyphal cells contribute to invasion of host cells and tissues and to biofilm formation (Saville et al., 2003; Sudbery, 2011). *Candida albicans* hyphal cells are characterized by an arsenal of cell wall–anchored surface proteins (Munro and Richard, 2012). These proteins include cell wall remodeling enzymes, detoxifying enzymes, and adhesins that mediate the association to a variety of host and microbial proteins (Munro and Richard, 2012). It is notable that one of these proteins, Als3, can trigger clathrin-mediated endocytosis of the hyphal cells by host epithelial and endothelial cells (Phan et al., 2005; Moreno-Ruiz et al., 2009). Yet hyphal morphogenesis is not a prerequisite to *Candida* spp. pathogenesis as other species in this genus are unable to form hyphae. As mentioned, elevated temperatures favor the transition from the yeast to hyphal form in *C. albicans*. This contrasts with other fungal pathogens, such as *B. dermatitidis*, *C. immitis*, *H. capsulatum*, *P. brasiliensis*, *S. schenkii*, and *P. marneffei*, that grow in the hyphal form at low temperatures and in the yeast form at body temperatures (Gow et al., 2002). The ability of these fungi to switch to the yeast form appears crucial to pathogenesis and the expression of virulence genes (Nemecek et al., 2006; Nguyen and Sil, 2008). Apart from the yeast-hypha dimorphism, several pathogenic fungi can undergo other forms of morphogenetic switches that favor virulence. For instance, *C. immitis* is forming endosporulating spherules that contribute to its persistence in the host (Hung et al., 2007). *Candida neoformans* forms giant cells that result from endoreplication and consequently are polyploid. These giant cells may be part of the defense strategies that allow *C. neoformans* to evade the initial host immune response, establish lung infection, and thus prolong survival within the host (Zaragoza et al., 2010; Okagaki et al., 2010).

Key to the virulence of human pathogenic fungi are mechanisms that protect fungal cells from the host defense arsenal. For instance, fungal pathogens produce enzymes, such as catalases and superoxide dismutases, that contribute to the detoxification of reactive oxygen and nitrogen species produced by immune cells (Chauhan et al., 2006; Brown et al., 2007). Moreover, some species produce extracellular appendages that prevent them from being recognized by the host or from the toxicity of reactive oxygen species. Infecting *C. neoformans* cells harbor a capsule composed of glucuronoxylomannan and galactoxylomannan polysaccharides that is essential for virulence. Both polysaccharides have immunomodulatory activity and are likely, upon their release from the cells during infection, to circumvent the host immune response (Janbon and Doering, 2011). Similarly, *A. fumigatus* spores (conidia) harbor an external layer that is composed of hydrophobic proteins called hydrophobins. Conidia that lack the hydrophobin layer are recognized by the immune system, indicating a role of this layer in protecting *A. fumigatus* and possibly other airborne fungi against immune attack (Aimanianda et al., 2009). Environmental fungal pathogens also harbor melanin in their cell wall that protects them from phagocytosis and killing by reactive oxygen species (Jahn et al., 1997; Trofa et al., 2011). In this respect, in ascomycetes, melanin is the product of one of several gene clusters that are responsible for the production of secondary metabolites. Another example of such secondary metabolites is gliotoxin, which is produced by *A. fumigatus*. Gliotoxin has immune modulatory and apoptosis-promoting activities and yet does not appear to play a major role in *A. fumigatus* virulence (Scharf et al., 2012). *Aspergillus fumigatus* mutants that are defective for the expression of multiple secondary metabolism gene clusters have reduced virulence, suggesting that one or several additional secondary metabolites contribute to fungal pathogenesis (Bok et al., 2005; Perrin et al., 2007).

Fungal pathogens of humans are generally well equipped with nutrient acquisition systems: extracellular proteases and lipases for degradation of host proteins and lipids, oligopeptide transporters and aminoacid permeases, and metal ion scavenging systems (Naglik et al., 2004; Schaller et al., 2005; Nierman et al., 2005; Butler et al., 2009; Almeida et al., 2009; Kronstad et al., 2011). Notably, comparative analyses of the genomes of pathogenic and nonpathogenic hemiascomycetous yeasts (the branch that includes *Candida* spp.) have revealed that gene families for these nutrient acquisition systems are expanded in the pathogenic species, consistent with a role in virulence (Butler et al., 2009). This is supported by the characterization of a variety of mutants although the contribution to virulence of these systems remains difficult to establish directly because of the large size of some gene families whose members are functionally redundant (Naglik et al., 2004). In particular, iron scavenging has been shown to be essential to virulence of several fungal pathogens of humans, such as *C. albicans*, *A. fumigatus*, *H. capsulatum*, and *C. neoformans* (Ramanan and Wang, 2000; Jung et al., 2006; Hwang et al., 2008; Schrettl et al., 2010). In this latter species, expression of many virulence genes is actually under the dependency of iron (Jung and Kronstad, 2011). This is one example of the ability of fungal pathogens to adapt to changing environments. Other examples include the regulation of the *C. albicans* yeast-tohypha switch by a variety of cues, reflecting the ability of this species to adapt to different environments through the production of hyphae (see earlier) and the ability of several fungal species to adapt to hypoxic environments, an adaptation that is often essential for virulence (Willger et al., 2008; Askew et al., 2009; Ernst and Tielker, 2009).

Antifungal Strategies and Mechanisms of Resistance

Modes of Action, Activity Spectra, and Therapeutic Applications of Antifungal Agents

This section focuses on the antifungal strategies used for treatment of invasive mycoses. The overall number of antifungal agents currently available to treat the large spectrum of invasive fungal diseases occurring in humans remains limited to less than 15. These

antifungal agents belong to four different chemical classes: polyenes, pyrimidin analogues, azoles, and echinocandins. The recommendations for the treatment of invasive mycoses using these agents are summarized in Table 1, taking into account the disease manifestations for each infection and alternative options when they are available.

Polyenes

Polyene drugs (amphotericin B (AmB) deoxycholate and its lipid-associated formulations) act by binding to ergosterol, the major sterol component of fungal membranes, forming tiny holes or channels into the membrane. The formation of channels allows small molecules to diffuse across the membrane and leads to impaired cellular functions, resulting in cell death (Lemke et al., 2005). AmB is active against most yeast and filamentous and dimorphic fungi. AmB desoxycholate is administered intravenously, and is associated with a broad range of side effects and toxicity, above all nephrotoxicity. The lipid-associated formulations of AmB are less toxic than the parent compound and reduce renal toxicity (Dupont, 2002). Polyenes administered intravenously remain a major treatment option for severe fungal infections, particularly life-threatening illnesses, including aspergillosis, cryptococcosis, candidiasis, and severe cases of histoplasmosis, blastomycosis, coccidioidomycosis, and infections due to Mucorales.

Azoles

Like polyenes, azoles exert their action on the fungal membrane. Azole drugs target the ergosterol biosynthetic pathway by inhibiting a key enzyme, the lanosterol 14-alpha demethylase. This inhibition takes place through the binding of the free nitrogen atom of the azole ring to the iron atom of the heme group of the enzyme. The resulting accumulation and metabolism of 14-alpha methylated sterol species leads to the synthesis of toxic compounds, which are unable to successfully replace ergosterol (Vandeputte et al., 2012). Azoles are cyclic organic molecules that are divided into two groups depending on the number of nitrogen atoms in the azole ring: The imidazoles contain two nitrogen atoms and the triazoles contain three nitrogen atoms. Only the triazole agents are used for the treatment of invasive fungal infections. They include itraconazole, fluconazole, voriconazole, and posaconazole. The structural differences between triazole molecules dictate antifungal potency and spectrum, bioavailability, and drug interactions (Odds et al., 2003). Azoles are effective against dimorphic fungi. Itraconazole, voriconazole, and posaconazole have good activity against most yeast and filamentous fungi, in contrast to fluconazole whose activity is limited mostly to yeasts. Furthermore, itraconazole and fluconazole are ineffective against some emerging pathogens, such as *Scedosporium* spp. and *Fusarium* spp. Finally, azoles have no activity against Mucorales, with the exception of posaconazole, which is effective against some strains of Mucorales.

Echinocandins

The echinocandins belong to the most recent class of antifungal drugs. Echinocandins are synthetic derivatives of lipopeptides, which disrupt fungal cell wall through inhibition of the β-1,3-glucan synthase complex. This complex is responsible for β-1,3-glucan synthesis, a major component of the cell wall. Echinocandins are fungicidal against most *Candida* spp. and fungistatic against *Aspergillus* spp. These molecules have no activity against *C. neoformans* and Mucorales.

Antifungal Drug Resistance Mechanisms in Fungal Pathogens

As mentioned, several fungal pathogen species are intrinsically tolerant to a subset of antifungals. The mechanisms that underlie this tolerance are not understood, but they possibly involve the structure of the cell wall, the structure of the plasma membrane, or the constitutive activation of drug efflux mechanisms. Notably, antifungal tolerance contributes to a shift in the epidemiology of species, causing invasive fungal infections with antifungal-tolerant species progressively surpassing in frequency antifungal-sensitive species. For instance, *C. glabrata* is to some extent tolerant to azoles and the prevalence of invasive candidiasis due to this species currently is increasing whereas infections due to azolesensitive *C. albicans* are decreasing (Hachem et al., 2008). Acquisition of resistance to antifungals by fungal pathogens is another cause for the inefficacy of these drugs. Resistance can be achieved through mutations in genes encoding antifungal targets, thereby increasing the antifungal target levels in the cells or decreasing its affinity for antifungal molecules. This is the case in *C. albicans* in which mutations in the *ERG11* and *FKS1* genes have been shown to affect the affinity of the Erg11 lanosterol 14-alpha demethylase for azoles and the Fks1 β-1,3-glucan synthase catalytic subunit for echinocandins, respectively (Sanglard and Odds, 2002; Perlin, 2007). This is also the case in *A. fumigatus*, where a change in the number of tandem repeats in the promoter of the *cyp51A* gene encoding lanosterol 14-alpha demethylase confers azole resistance (Snelders et al., 2008). Alternatively, antifungal resistance can result from increased drug efflux. This is especially the case for azole drugs that are effluxed from fungal cells through two types of pumps, the ATP-binding cassette transporters – such as *C. albicans* Cdr1 and Cdr2 – and the major facilitators – such as *C. albicans* Mdr1 (Sanglard et al., 1997, 1995). Increased expression of the genes encoding these pumps results in decreased sensitivity to azoles. Interestingly, mutations in transcription factors that control the expression of genes involved in azole biosynthesis or drug efflux have been shown to contribute to the acquisition of azole resistance (Coste et al., 2006; Morschhauser et al., 2007; Dunkel et al., 2008) and it is notable that genome rearrangements, such as loss of heterozygosity, aneuploidies, and the formation of isochromosomes can enhance the effect of these mutations and, consequently, the level of azole resistance (Coste et al., 2007; Selmecki et al., 2006). In contrast, drug efflux does not appear to play a role in the sensitivity of fungal

cells to echinocandins, possibly because they appear to act on the extracellular side of the plasma membrane, where the β-1,3-glucan synthase resides, and resistance to polyenes rarely is seen, possibly because these drugs act by directly targeting the fungal membrane. Finally, because horizontal gene transfer is rare in fungi, antifungal drug resistance is probably of lesser concern than antibiotic resistance as seen in bacterial species. Yet, the limited number of antifungals available dictates that all efforts should be made to limit the emergence of resistance to these drugs.

Another source of antifungal tolerance results from the ability of numerous fungal pathogens to form so-called biofilms. Biofilms are three-dimensional microbial structures that develop on biotic or abiotic surfaces. Biofilm cells are embedded within an extracellular matrix and are highly tolerant to antimicrobial and immune insults (Donlan and Costerton, 2002). Fungal pathogens of the genus *Candida* form biofilms on catheters and prosthetic devices. *Candida* biofilms constitute an important pitfall in the management of disseminated *Candida* infections because of their intrinsic tolerance to almost all antifungals in clinical use (Kojic and Darouiche, 2004). *Candida* biofilms are especially resistant to azoles and amphotericin B but remain sensitive to the echinocandins (d'Enfert, 2006). Recently, major progress has been made in understanding the molecular mechanisms implicated in the building of *C. albicans* biofilms (Finkel and Mitchell, 2011). Additionally, major progress has been made in understanding the basis of antifungal tolerance of *C. albicans* biofilms. Notably, drug efflux does not appear to play a major role in antifungal tolerance of biofilms except at early phases of biofilm formation (Mukherjee et al., 2003). In contrast, entrapment of antifungals in the biofilm matrix made up of β-1,3-glucans seems central to the antifungal tolerance of biofilms because altering the matrix enzymatically or genetically dramatically enhances the antifungal susceptibility of *C. albicans* biofilms (Nett et al., 2007, 2010; Taff et al., 2012). Additionally, C. albicans biofilms contain persister cells that are highly tolerant to antifungals and may contribute to the overall antifungal tolerance of the biofilm (Lafleur et al., 2006).

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Industrial Biotechnology (Overview)[☆]

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Glossary

Bioprospectin The exploration of novel applications, processes or products in living systems.
Bioremediation Processes of exploiting living systems, usually microorganisms or their products, to clean the environment spoiled by contaminants, in a natural way.
Directed evolution A method by which DNA or proteins are made to evolve artificially in a short time using rounds of random mutagenesis followed by selection of variants with desired qualities.
DNA shuffling Also known as sexual polymerase chain reaction (PCR), a technique where similar but nonidentical DNA sequences are reassembled to obtain novel combinations of related sequences, to rapidly propagate beneficial mutations leading to directed evolution.
Genetic engineering Also referred to as genetic modification or gene splicing, this is a technique of altering the genes of an organism, to change the amount or type of proteins an organism produces.
Metabolic engineering A technology aimed at increasing the cellular production of a desired substance by modifying pathway flux using genetic means.
MPSS (massively parallel signature sequencing) A method to determine the expression of genes by measuring mRNA levels.
Pharming A process by which transgenic animals are made, using genetic engineering, and exploited to mass-produce pharmaceuticals and nutraceuticals.
SAGE (serial analysis of gene expression) A method to determine gene expression using conventional cloning and sequencing procedures.
Strain improvement A strategic approach of using mutagenesis and/or modern genetic manipulations to develop superior strains in terms of yields of desired products.
Therapeutic proteins Proteins produced by recombinant DNA for pharmaceutical use. transcriptional profiling A technique used to study the pattern of expression or regulation of genes, which indicates the function of genes.
Transgenic plants Also known as genetically modified (GM) crops, plants that have foreign genes inserted in them artificially to obtain plants with desired traits.
Whole-genome shuffling A technique combining multiparental crossing through DNA shuffling and recombination of entire genomes, which results in improved strains in a very short time.

Abbreviations

AIDS	Acquired immunodeficiency virus
BAC	Bacterial artificial chromosome
CHO	Chinese hamster ovary
DHAP	Dihydroxyacetone phosphate
EPO	Erythropoietin
FDA	Food and Drug Administration
GM	Genetically modified

[☆]*Change History*: October 2016. Preeti Vaishnav and Arnold L. Demain updated the title of the article, author affiliations, and following sections "Introduction," "Medical Biotechnology," "Antibiotics," "Therapeutic Proteins," "Microbial Cell Systems," "Higher Cell Systems," "Biopharmaceutical Markets," "Primary Metabolites," "Enzymes," "Bioplastics and Biopolymers," and "Biofuels."

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GMP	5-Guanylic acid
GRAS	Generally recognized as safe
IMP	5-Inosinic acid
mAbs	Monoclonal antibodies
MPSS	Massively parallel signature sequencing
PCR	Polymerase chain reaction
PDO	1,3-propanediol
PHA	Polyhydroxy alkanoate
PHB	Polyhydroxybutyrate
PH3B	Poly(3-hydroxy-butyrate)
PHC	Polyhydroxycaproate
PHV	Polyhydroxyvalerate
PMP	Plant-made pharmaceutical
SAGE	Serial analysis of gene expression
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
USDA	US Department of Agriculture
VEGF	Vascular endothelial growth factor
WGS	Whole-genome shuffling

Defining Statement

This overview of industrial biotechnology discusses the use of genetics in the production of medical pharmaceuticals and other products. It includes medical biotechnology (antibiotics, therapeutic proteins, protein production systems, and biopharmaceutical markets), primary metabolites, enzymes, biopolymers and bioplastics, biofuels, bioremediation, and agricultural biotechnology.

Introduction

Mankind has sought refuge in biotechnology for survival and betterment of human life for millennia. Selective breeding of plants and animals, discovering microbes and eventually harnessing them to produce cheese, bread, wine, vaccines and antibiotics, have all been initial strides taken toward the biotechnological journey. Today, we manipulate the genetic makeup of microbes to suit our requirements and use them as factories to produce hormones, antibiotics, enzymes, monoclonal antibodies, therapeutic proteins, plastics, silk, and biofuels, to name a few.

Many discoveries in genetics in the late 20th century laid a strong foundation in the field of biotechnology, for example, genetic engineering, and have empowered humankind to exert greater control over its life and well-being. An epic finding was that of Avery, MacLeod, and McCarty in 1944 that DNA, rather than proteins, was the carrier of genetic information. This was followed in 1953 by the elucidation by Franklin, Watson, and Crick of the structure of double-stranded DNA. In 1956, Rich and Davies discovered the RNA double helix and RNA hybridization. Discovery of restriction enzymes in 1962 by Arber broadened the prospects of genetic manipulation. Nathan and Smith showed that restriction enzymes cut DNA in a sequence specific manner. In 1967, DNA ligase was isolated, while in 1970, the first restriction enzyme, reverse transcriptase, which cuts DNA molecules at precise locations, was isolated by Temin, Baltimore, and Delbecco. These findings opened the way for a major breakthrough in biotechnology, that is, the discovery of recombinant DNA (rDNA) by Berg, Boyer, and Cohen in 1972–73. Recombinant DNA technology allowed the discovery of a series of synthetic proteins manufactured in microbial factories. The first one was the human protein somatostatin in 1977, followed by human insulin produced in *Escherichia coli* in 1978. In 1982, the first rDNA animal vaccine was approved for sale in Europe while in the same year, the first rDNA pharmaceutical (human insulin) was approved for sale in the United States and United Kingdom. Another outstanding discovery was that of phage display technology, first described by Smith in 1985. This technique is used in cloning a gene when an antibody against that gene product is available. Mullis discovered the polymerase chain reaction (PCR) in 1983, which is of incredible value in diagnostics and forensic medicine. In 1984, the entire human acquired immunodeficiency virus (AIDS) gene was cloned and sequenced, and Jeffreys introduced the technique of DNA fingerprinting for genetic identification. In 1986, the first genetically engineered human vaccine, Recombivax HB, was approved for the prevention of hepatitis B. In 1995, the bacterium *Haemophilus influenzae* had its entire genome sequenced. The first complete animal genome, that is of the worm *Caenorhabditis elegans* was sequenced in 1998. In that year, Thomson and Gearhart individually developed a technique for culturing embryonic human stem cells. In 2000, Golden Rice containing vitamin A was produced. Researchers sequenced the DNA of rice in 2002, the first crop to have its genome decoded. Marshall and Warren discovered the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer, while Fire and Craig discovered RNA interference and gene silencing by small pieces of double-stranded RNA. Later, in 2003, the sequencing of the human genome was completed by Venter, Collins, and Lander; this threw much light on the field of medicine based on genetics.

Industrial biotechnology can be defined as the application of scientific and engineering principles to the processing of materials by microorganisms (bacteria, fungi, algae, protozoa, and viruses), plant cells and animal (insects and mammals) cells to create useful products or processes. The microorganisms used may be natural isolates, laboratory-selected mutants, or microbes that have been genetically altered using rDNA methods. Most antibiotics come from microbial fermentations involving a group of organisms called actinomycetes, which are filamentous bacteria. Yeasts are used in baking, in the production of alcohol for beverages and fuel (bioethanol), and heterologous proteins (especially mammalian proteins). This is due to the fact that knowledge of yeast genetics is more advanced than that of any other eukaryote. Microbial products include primary metabolites (amino acids, vitamins, purine nucleotides, organic acids, etc.) and secondary metabolites (antibiotics, antitumor agents, immunosuppressants, cholesterol-lowering agents, antiparasitic drugs, etc.), which microorganisms produce as part of their natural metabolism. The level of these metabolites produced by microbes is however very low, enough to satisfy their own requirements for life in the environment. Biotechnologists, utilizing mutational and genetic engineering techniques, modify the biosynthetic pathways leading to the desired product and develop strains that give rise to greatly magnified yields as compared to those of their parents. Molecular manipulation techniques such as recombinant DNA technology, hybridoma technology, and protein engineering provide us with tools to manipulate the genetic makeup of microbes, higher plants, and animals. Recombinant protein production can be improved using bacterial artificial chromosomes (BACs). Biotechnology has a key role to play in the healthcare, diagnostics, and agricultural industries along with others such as mining, petroleum, textile, food, and chemicals. Many medical products such as antibiotics, other low-molecular-weight pharmaceuticals, DNA vaccines, and human biologicals (e.g., insulin, growth hormone, and monoclonal antibodies) have been commercialized. Meanwhile, research continues to improve the production and decrease the costs using genetic engineering techniques. Diagnostic assays use monoclonal antibodies, DNA chips and marker enzymes, and so on, for the early detection of a myriad of diseases such as cancer, AIDS, hepatitis, and other viral diseases. Diagnostic kits including DNA chips, also known as DNA microarrays, have been commercialized using novel DNA sequencing methods such as MPSS (massively parallel signature sequencing) and SAGE (serial analysis of gene expression) to minimize production costs. The Human Genome Project revealed susceptibility genes for many diseases in individuals and allows humans to avoid the incidence of disease through preventive measures. The agricultural industry is largely benefitted by products of genetic engineering such as transgenic plants. These allow magnified yields, pest and weed resistance, and can be used by malnourished populations in developing countries. Genetically modified (GM) plants and animals can bring bewildering concepts to fruition. Already today, or on the near horizon, we have environmental-friendly plastics being made in *Arabidopsis thaliana* plants, pharmaceuticals being produced in corn, Golden Rice containing vitamin A, anti-allergic cats, and insect-resistant cotton. Developments in molecular biology have stretched beyond one's imagination, but commercialization of transgenic organisms does involve regulatory issues, and much caution has to be exercised to avoid mixing of genetically engineered organisms with wild-type organisms.

A novel approach of using synthetic biology is that of assembling biomolecular parts into systems that would carry out useful functions for human health, energy and the environment. Of importance has been the engineering of designer zinc-finger proteins that can bind to almost any DNA sequence.

Enzymes hold promising applications in industries such as foods, textiles, detergents, paper, and in sewage treatment. Enzymes and cells are of immense significance in biotransformations, also known as bioconversions. rDNA techniques help in designing enzymes that have improved stability, specificity, and activity. The commercial production and processing of a wide array of foodstuffs such as cheese, butter, chocolate, yoghurt, pickles, alcohols, food thickeners, flavoring agents, preservatives, artificial sweeteners, cured foods, and food supplements (vitamins and amino acids) require microbial activity. Some bacteria produce natural polymers and can be engineered to produce a range of thermostable polymers that can be used in all areas ranging from surgicals to packaging to plastics.

Isoprenoids, the largest group of plant chemicals, are produced naturally by plants and some microbes in very small amounts. Some like carotenoids, dolichols, ubiquinones, sterols, artemisinin and taxol can be produced by genetically-engineered microbes. They find application in pharmaceuticals, fragrances, solvents and recently, advanced biofuels.

Metallic nanoparticles have been used for catalysis, environmental remediation, gene therapy, drug delivery, imaging, as biomarkers, sensors, and energy. They can be synthesized by microbes. Bacteria can produce metal nanoparticles of different sizes and shapes. Mainly emphasized are silver, gold, palladium, copper, platinum, tellurium and titanium nanoparticles. The most important microbes for these purposes are species of *Pseudomonas*, *Enterobacter*, *Bacillus* and sulfate-reducing strains. Most of the synthesis is extracellular. Nanoparticles are important in fields such as physics, electronics, energy, chemistry, biology, pharmaceuticals, cosmetics, medicine, food industry, manufacturing, analytical chemistry, engineering, automotive, agriculture, and biological and environmental research. They can be used as catalysts, adsorbants, membranes, water disinfectants, and additives to increase catalytic activity and capability due to their high specific surface area and nanosize. Microbial synthesis of nanoparticles is new and of great interest, especially since chemical synthesis requires extreme conditions of pH and temperature, and the chemicals used may have environmental and human health impacts.

Medical Biotechnology

Genetic engineering using rDNA techniques is currently widely employed in the pharmaceutical industry and in human medicine. Drugs produced by biotechnological processes are used to treat invasive fungal infections, pulmonary embolisms, kidney transplant

rejection, infertility, growth hormone deficiency, diabetes, cancer, AIDS, and other serious disorders. In the future, drug therapy will become more personalized through the use of pharmacogenomics.

Recombinant immunotherapeutics are recombinant DNA products which have been useful in fighting cancer. The products generally kill the tumor and stimulate the immune cells to respond to the cancer cells. They include immunostimulants, vaccines, antibodies, immunotoxins, and fusion proteins. They were needed because conventional treatments (surgery, conventional chemotherapy, radiotherapy) were ineffective for half of the cancer patients. The new approach, called immunotherapy, is based on recombinant products and/or engineered cells that stimulate the immune system and reverse the tolerance provoked by cancer cells. Viruses are being studied as a means of killing tumors and training the immune system to combat cancer. There is one licensed oncolytic virotherapy which is used in China. Several late stage candidates, for example, Amgen's T-Vec and SillaJen's Vexa-Rec, are in late stage clinical trials.

Antibiotics

Molecular biology has a long history of contributions in the production of microbial pharmaceuticals, especially antibiotics. Antibiotics in 2004 had a market of $26 billion of which the major groups were cephalosporins ($7.3 billion), fluoroquinolines ($6.2 billion), penicillins and macrolides ($4.7 billion each), and others ($3.1 billion). Most antibiotics are secondary metabolites, produced by bacteria (e.g., *Bacillus*, *Nocardia*, and *Streptomyces*) or fungi (*Aspergillus*, *Penicillium*, and *Cephalosporium*). Antibiotic production involves fermentation. Levels of antibiotics produced in nature are very low; hence, continuous efforts had to be made since the discovery of penicillin to commercialize these products. Classical strain improvement involves mutational techniques, wherein the producing culture is mutagenized using chemicals or irradiation or sometimes a combination of the two. Then, the mutagenized population is screened for antibiotic production and the best strain is chosen for further study, including nutritional optimization and further mutagenesis. This procedure of mutagenesis and screening ("brute force" strain improvement) is laborious and time consuming, but it works well.

The aims of mutagenesis programs include: (1) increasing titers, (2) allowing efficient assimilation of inexpensive, complex raw materials, (3) altering product ratios, (4) eliminating side products, (5) improving excretion of products, and (6) shortening of fermentation duration.

For 40 years, genetic recombination was ignored due to very low frequencies of recombination. However, protoplast fusion changed the scenario markedly. Protoplast fusion increased the frequency of recombination in *Streptomyces* from 10^{-6} to 10^{-1}. Selected mutant lines were recombined genetically by protoplast fusion to produce improved strains. Soon, transformation, conjugation, and recombinant DNA technology became routine in strain improvement along with mutation techniques. These technologies helped to (1) remove bottlenecks of rate-limiting reactions. For example, the expandase promotor of the penicillin biosynthetic pathway was replaced by the much more active ethanol dehydrogenase promoter. The new technology was also employed to (2) eliminate feedback regulation. This was seen in *Corynebacterium glutamicum* where the threonine dehydratase gene *ilvA* was replaced by a feedback resistant *ilvA* gene from *E. coli* to increase isoleucine production. The technology was also used to (3) manipulate control genes. In actinomycetes, extra copies of positive-control genes were inserted to improve production of actinorhodin, undecylprodigiosin, and spiromycin, whereas negative regulatory genes were deleted or inactivated in producers of methylenomycin, tetracenomycin, jadomycin, and duanorubicin. Another application was that of (4) perturbing central metabolism. For example, the first or second enzyme of the pentose phosphate pathway was deleted in *Streptomyces lividans* to increase actinorhodin production. (5) Blockage of competing pathways is another application, for example, by blocking tyrosine formation, an increase in phenylalanine titer was achieved. The technology was also used to (6) enhance product excretion; for example, *lysE* encoding the protein involved in lysine excretion was overexpressed to increase lysine production. (7) Another application is that of decreasing the wasteful conversion of the desired product to a less active or inactive compound. In this category, genes *dnrX*, *dnrH*, and/or *dnrU*, which are involved in the conversion of the desirable doxorubicin into other compounds, were disrupted, thus increasing doxorubicin titer by threefold. Finally, (8) increasing the dosage of biosynthetic genes often results in an increase in production. For example, increasing the dosage of early genes of the spinosyn pathway increased production by three- to four-fold.

Many newer genetic engineering techniques are used to increase production and thus decrease costs. These include transcriptional profiling, DNA shuffling, whole-genome shuffling (WGS), directed evolution, massive parallel signature sequencing, association analysis, genome-based strain reconstruction, and metabolic engineering. Transcriptional profiling was used for strain improvement of the industrial lovastatin producer, *Aspergillus terreus*. Dosage of seven biosynthetic and regulatory genes, *lovF*, *creA*, *fadA*, *ganA*, *gna1*, *gna3*, and *gpa1* was increased. Of the seven genes, *lovF* was found to be crucial for increasing lovastatin titer. The *lovF* promoter was fused with the bleomycin resistance gene *ble* and bleomycin-resistant mutants were isolated. Of these, one doubled lovastatin production.

DNA shuffling, a method of *in vitro* recombination was used to increase the titers of doramectin. The parent strain produced avermectins B1 and B2 in the ratio of 0.6:1, respectively; B1 being the favorable member of the pair. Random mutation of gene *aveC* was carried out by PCR, and a mutant with the more favorable ratio of 2.5:1 was produced. Finally, DNA shuffling was applied and the ratio was markedly increased to 15:1.

WGS combines the advantage of multiparental crossing allowed by DNA shuffling with recombination of the entire genome. This method was successfully used to improve tylosin production by *Streptomyces fradiae*. Classical strain improvement methods in industry took 20 years to enhance tylosin production sixfold. WGS gave similar results in just 1 year.

The above-described novel molecular manipulations of genetic engineering combined with the classical approach thus make strain improvement much faster. This strategy not only reduces the time but lowers the amount of labor needed for random mutational screening. When the entire biosynthetic pathway leading to the desired product is elucidated and all the regulatory aspects are known, only genetic engineering is required to increase production. However, if the pathway is not clear, a combination of mutagenesis, protoplast fusion, and genetic engineering can still be applied for strain improvement. Classical mutagenesis and screening used without modern molecular technology possess a major disadvantage of accumulating secondary mutations that are detrimental to performance.

In the postgenomic era, the technique of genome-based strain reconstruction is an attractive option. This technique has been used for the reconstruction of superior strains of L-lysine-producing *C. glutamicum*. By comparative genomic analysis, mutations in three genes were determined to be of significance in lysine production. They were a Val-59Mala mutation in the homoserine dehydrogenase gene (*hom*), a Thr-311Mile mutation in the aspartokinase gene (*lysC*), and a Pro-458Mser mutation in the pyruvate carboxylase gene (*pyc*). The *hom* and *lysC* mutations were introduced individually into the wild-type strain by allelic replacement. This resulted in accumulation of 8 and 55 g/L of L-lysine, respectively. Reconstitution of both mutations into the wild-type genome resulted in a sharp rise in production to 75 g/L of L-lysine due to a synergistic effect. Further introduction of the *pyc* mutation resulted in a further rise to 80 g/L of L-lysine within only 27 h. Thus, the titers as well as speed were increased, raising the overall efficiency of the fermentation process.

Directed evolution is a method to improve the properties of enzymes by evolving proteins with desirable properties not found in nature. For example, the enzyme deacetoxycephalosporin C synthase of *Streptomyces clavuligerus* was modified by directed evolution for enhancement of penicillin G expansion into phenylacetyl-7-aminodeacetoxycephalosporanic acid, an important intermediate in the industrial manufacture of cephalosporin antibiotics.

The biosynthetic pathways leading to formation of desired products can be modified and improved by carrying out metabolic engineering. The overall flux through a metabolic pathway depends on several steps, not on just a single rate-limiting reaction. When all the enzymes of a pathway are identified, a number of branch points are revealed which lead to other parts of metabolism. The flux can be increased by increasing the enzyme concentrations without perturbing the branch points, thus leaving other functions such as growth and cellular metabolism unchanged. For example, the overexpression of pyruvate carboxylase or aspartate kinase increased lysine production by *C. glutamicum* only marginally. But overexpression of both the genes increased lysine production by 250%.

Metabolic engineering has been applied to antibiotic production. The increases in metabolic flux were carried out by enhancing enzyme activity, manipulating regulatory genes, enhancing antibiotic resistance, and heterologous expression of novel genes. Techniques involving overall analysis of gene expression, that is, genomics, proteomics, and metabolomics have gone a long way in developing the strain to yield best results. Mutational techniques generate different desirable phenotypes. The mutants are then compared with the parent strain by functional genomic techniques such as (1) genomic DNA sequencing, (2) DNA sequencing of selected genes, (3) transcriptional profiling, (4) proteome analysis, (5) metabolic profiling, and (6) comparative flux analysis. In the application of inverse metabolic engineering, mapping of differences between strains with different degrees of a certain desired phenotype is done. Subsequent identification of factors conferring that phenotype is an essential part. Therefore, the tools of functional genomics have the potential to promote and expand inverse metabolic engineering. Gene expression analysis offers valuable insights toward increasing metabolite production. The commonly used methods are microarray technology, direct cDNA technology, SAGE, and MPSS. SAGE employs conventional cloning and sequencing procedures. MPSS is a method for counting innumerable mRNA molecules from a given sample. It uses a set of new technologies for cloning and sequencing a 16–20 base signature sequence from more than a million molecules simultaneously.

Considerable progress has been made in genomic sequencing of microbes. As of December 2006, there were 470 published genome sequences, and 991 bacterial, 631 eukaryotic, and 56 ongoing archaeal sequence projects. The presence of complete genome sequences has clearly allowed better targeting of genetic modifications. Fungal genomes that have been sequenced include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus niger*, and *Neurospora crassa*. *S. cerevisiae* has 6294 genes, whereas actinomycetes have many more, for example, *Streptomyces coelicolor* with 7825 genes and *Streptomyces avermitilis* with 7574 genes. These numbers are about twice that possessed by *E. coli* (4289 genes) or *Bacillus subtilis* (4099 genes). *S. avermitilis* is the producer of avermectin, a commercial antihelminthic agent. Its genome sequencing revealed 30 gene clusters encoding pathways of secondary metabolism, including four clusters for melanin pigments, one for a carotenoid, one for a siderophore, three for type-1 polyketides, two for type-2 polyketides and non-ribosomal peptides, and four for ter-penes. The total length of these clusters includes 271 genes, which is equal to 6.6% of the genome.

By 2002, over 22,000 bioactive compounds were described from microbes. These included 20,000 antibiotics produced mainly by the actinomycetes (45%). Fungi produced 35% and unicellular bacteria 17%, chiefly by species of *Pseudomonas* and *Bacillus*. Of the actinomycete antibiotics, about 80% are made by members of the genus *Streptomyces*. Genome mining of *Streptomyces ambofaciens* revealed production of new secondary metabolites over and above its known metabolites spiramycin and congocidine. They include kinamycins, stambomycins (macrolides) and antimycins, all showing antitumor activity. Other promising antibiotics are platensimycin and platensin produced by *Streptomyces platensis*.

In 1996, the anti-infective market reached $23 billion and of these compounds, 160 were antibiotics. The market increased to $44 billion by 2005. The antibiotic market reached $30 billion in 2000 and $31 billion in 2005. Despite these impressive figures, many large pharmaceutical companies have deserted the antibiotic field and transferred their interests to lifestyle drugs and drugs

that the public would use daily for the rest of their lives. Thus, the antibiotic discovery field is in the midst of a serious crisis at present. There is a growing need to develop or discover new antibiotics. Microorganisms are evolving rapidly to attain resistance to the prevailing antibiotics.

Antibiotic resistance poses serious threats to human and animal health. It is known that pathogens can be attacked by new or modified antibiotics, produced by microbial flora belonging to diverse sources such as soil and oceans. The bacteria follow Darwin's principle of survival of the fittest by being able to thrive despite adversity and to emerge stronger every time they survive an antibiotic dose. Frequent use of antibiotics and incomplete courses of treatment boost resistance in bacteria. Antibiotic resistance occurs by gene mutation and by transfer of resistance genes. Even unrelated bacteria can gain resistance from their neighbors through a phenomenon called horizontal gene transfer. This can occur by conjugation, transformation, or transfection. We know that resistant bacteria emerged just 4 years after drug companies began mass-producing penicillin in 1943. The first such bacterium was *Staphylococcus aureus*. Antibiotic resistance occurs mainly in "opportunistic pathogens" with moderate to low disease-causing potential. These organisms usually are naturally present in healthy humans. They only cause a disease when they infect parts of the body where they do not normally live, either through physical injury or surgery, or in patients with immature or defective immune systems. In some cases, the situation has become alarming, with the emergence of strains resistant to virtually every antibiotic. Some multiply-resistant strains of *S. aureus*, for example, cause serious hospital-acquired infections. One of the only compounds that can be used effectively against most of them is an older antibiotic, vancomycin. The development of multidrug-resistant strains poses a serious threat to human health. Until recently, new drugs have been developed in time to treat resistant bacteria. The scenario is now changing. Since 1998, as few as ten new antibiotics were approved by the Food and Drug Administration (FDA) and only two were truly novel. In 2002, around 89 new medicines emerged in the market, but none of these was an antibiotic. Particularly disturbing is the fact that there is no antibiotic class for which a bacterial resistance mechanism does not exist. In fact, it is alarming to know that DNA from soil samples when checked for antibiotic resistance showed positive results. However, recent discovery suggests a way toward tackling antibiotic resistance. The soil fungus *Aspergillus versicolor* produces aspergillomarasmine (AMA) which turns off a gene in bacteria that normally leads to antibiotic resistance. Together with a carbapenem antibiotic, AMA inactivates the gene New Delhi Metalloprotease 1 in *E. coli*, *Acinetobacter* and *Pseudomonas*. NDM-1 requires zinc and AMA removes zinc from the enzyme. The combination of AMA and the carbapenem has shown its effect in mice and in human cell culture.

In addition to antibiotic resistance development, other threats include the evolution of new pathogens (Ebola virus, HIV, etc.), toxicity of some known antibiotics and antitumor agents, and the existence of certain pathogens that have never been eliminated by antibiotics, for example, the *Pseudomonas aeruginosa* strain that infects patients suffering from cystic fibrosis.

New antibiotics can be discovered from sources such as microbial flora from oceans, other waters, and soil, collected from various areas around the world. There is an incredible diversity of microorganisms in our environment, but the problem lies in the difficulty of cultivating them. Microbial life in oceans is far more diverse than ever imagined, with more than 20,000 different kinds of bacteria living in 1 L of seawater. Researchers predict that the total biodiversity of bacteria in the oceans is probably greater than 5 million different kinds of microorganisms. One gram of soil contains about 10 billion microorganisms of more than 17,000 species. However, it is estimated that only 1% of bacteria and 5% of fungi have been cultivated in the laboratory. This problem is being actively studied by a number of groups. Some success is being achieved in growing uncultured microorganisms by a number of strategies: (1) using very low nutrient concentrations, (2) adding signaling molecules, (3) adding inhibitors of known microbes, (4) employment of long incubation periods, (5) use of growth conditions resembling natural environments, (6) protection from natural peroxides, (7) addition of humic acid, (8) employment of hypoxic or anoxic environments, (9) encapsulation of cells in gel microdroplets coupled with detection of microcolonies by flow cytometry, and (10) use of high CO_2 concentrations.

Another technique used to tap into biodiversity is that of metagenomics, also referred to as environmental or community genomics. Metagenomics proves to be of great use in genomic studies of uncultivable microbes. DNA is isolated from nature and cloned into *E. coli* by using bacterial artificial chromosomes (BACs). The size of DNA isolated from soil is usually up to 70 kb and that from water, up to 40 kb. BACs are capable of carrying inserts of up to 350 kb in size. Then, the *E. coli* clones are screened for biological activity. Of the secondary metabolites detected from metagenomic DNA, about half were known molecules but the other half were new compounds. Novel genes and gene products discovered through metagenomics include the first bacteriorhodopsin of bacterial origin; novel small molecules with antimicrobial activity; and new members of families of known proteins, such as an $Na^þ(Li^þ)/H^þ$ antiporter, RecA, DNA polymerase, and antibiotic resistance determinants.

The Epstein group at Northeastern University first used a diffusion chamber incubated in situ in which diffusion provided the microbes with their naturally occurring growth factors. Many more cultures were isolated from Nature by this method. They then devised an isolation chip (called the IChip) composed of several hundred miniature diffusion chambers. The IChip recovered many more isolates from Nature than even the diffusion chamber. Many of the species isolated have never been isolated before by standard techniques. Isolation from seawater was only 4% of inoculated cells which formed colonies in Petri dishes, as compared to 20% with the diffusion chamber and 34% with the IChip. The figures from soil were 7, 31 and 33%, respectively. By use of the IChip, 10,000 isolates were isolated from Nature. 25 potentially new antibiotics were found from these isolates. One of the microbes, isolated by IChip method, *Eleftheria terrae*, produced a new antibiotic. This organism is a new species of β-proteobacteria. By genome sequencing, it was found to be a member of a new genus related to Aquabacteria which are Gram-negative organisms, not known to produce antibiotics. The antibiotic was named teixobactin. It is an unusual depsipeptide containing enduracididine, methyl phenyalanine, and four D-amino acids. Its biosynthetic gene cluster consists of two large non-ribosomal peptide synthetase-coding genes. No mutants of *S. aureus* or *Mycobacterium tuberculosis* were resistant to teixobactin. It has excellent activity against Gram-positive pathogens including strains that are drug-resistant. Potency versus most Gram-positive species was below 1 μg/mL. It is exceptionally active versus *Clostridium difficile* and *Bacillus anthracis*. Its killing activity of *S. aureus* was greater than that of

vancomycin. It was ineffective versus most Gram-negative bacteria except an *E. coli* strain with a defective outer membrane permeability barrier. No toxicity to mammalian cells was observed. It is an inhibitor of peptidoglycan synthesis with no effect on label incorporation into DNA. Its activity against *S. aureus* includes MRSA. It inhibits many other Gram-positive bacteria. Resistance to teixobactin could not be developed. It is active in the presence of serum. It is stable, has good microsomal stability and low toxicity. The antibiotic inhibits cell wall synthesis via binding to a highly conserved motif of lipid II (precursor of peptidoglycans) and lipid III (precursor of cell wall teichoic acid). Its target, lipid II, is a polyprenyl-coupled cell envelope precursor which is readily accessible on the outside of Gram-positive bacteria. The target is the pyrophosphate-sugar moiety of lipid II. The producer of teixobactin is a Gram-negative bacterium and its outer membrane prevents teixobactin from reentry into the producing organism. Teixobactin is distinct from glycopeptides, lantibiotics and defensins. It shows no cross-resistance with vancomycin which also is a lipid II binder. It showed no bad effects in toxicological in vitro investigation, i.e., hemolysis, cytotoxicity, hemolysis, etc.

New antibiotics can be created by combinatorial bio-synthesis, that is, by introducing genes encoding the antibiotic biosynthetic pathway into a foreign host, by changing the order of genes of an individual pathway in its native host, or by mixing genes from different path-ways. Hybrid antibiotics were first produced by gene transfer between strains producing the isochromanequinone antibiotics actinorhodin, granaticin, and medermycin. Since then, many new hybrid antibiotics have been produced.

A protein central to both the development and transmission of antibiotic resistance is RecA, which is found in almost all bacteria. Researchers are trying to create a drug that would cause bacteria to become RecA-deficient, opening up a number of therapeutic possibilities. This research could create a new class of antibiotics or make bacteria more susceptible to existing antibiotics. An alternative approach would be to develop drugs that would impair the bacterium's ability to develop resistance.

Among the marine microbes, the genus *Salinispora*, belonging to the actinomycete family, has been discovered in five different oceanic systems around the world. These strains possess antibiotic and anticancer behaviors. Approximately 35% of discovered *Salinispora* strains demonstrate an ability to kill pathogenic bacteria and fungi. A compound produced by members of this genus is salinosporamide A, which affects tumor cells by preventing them from functioning. It prevents their proteosomes from destroying old proteins. Thus, these proteins pile up in the cell and hinder the cancer cell's performance. Marine organisms such as corals, sponges, fishes, and bryozoans are also promising candidates for discovering new drug molecules. In many of these cases, the secondary metabolite is produced by a microbe living synergistically with the higher life form.

Mycophenolic acid, produced by *Penicillium*, was never commercialized as an antibiotic but its 2-morpholino-ethylester was approved as a new immunosuppressant for kidney transplantation in 1995 and for heart transplants in 1998. Other immunosuppressants are cyclosporine, tacroli-mus (FK506), and sirolimus (rapamycin). Some natural enzyme inhibitors such as the statins (compactin, lovastatin, and pravastatin) act as cholesterol-lowering agents. The statins, including the lovastatin-derivative Zocor and the synthetic Lipitor, have become the most economically important group of pharmaceuticals, exceeding sales of $20 billion per year. Other bioactive natural molecules are also very important, such as antiparasitic agents (avermectin), bioherbicides (bialaphos), plant growth regulators (gibberellins), biopesticides (polyoxins), bioinsecticides (spinosyns), and antitumor agents (doxorubicin, mitomycin, and bleomycin).

Therapeutic Proteins

The biopharmaceutical industry began with the establishment of the Cetus Corp. in Berkeley, California, in 1971. Within the next 2 years, Berg, Cohen, and Boyer discovered in vitro genetic recombination. In 1975, the first monoclonal antibodies were produced by Kolter and Milstein. In 1976, Genentech was founded. Yeast DNA was replicated and expressed for the first time in 1976, and in 1977, the first human gene was expressed in bacteria. Rutter and Goodman cloned the rat insulin gene, while Boyer's laboratory produced somatostatin in 1977. In 1978, Biogen was founded, Itakura produced recombinant human insulin, Baxter produced the first human growth hormone, and Fink inserted bacterial DNA into yeast chromosomes. In 1979, Baxter reported cloning the gene for human growth hormone. In 1981, scientists produced the first transgenic animals by transferring genes from other animals into mice. In 1986, the first genetically engineered human vaccine, Chiron's Recombivax HB, was approved for the prevention of hepatitis B. Genentech's tissue plasminogen activator (tPA), sold as Activase, was approved as a treatment for heart attacks in 1987. In 1997, the FDA approved Rituxan, the first antibody-based therapy for cancer. In 2003, the human genome was sequenced, stimulating researchers all over the world to develop new gene-based treatments for disease.

Proteins that are engineered in the laboratory for pharmaceutical use are known as therapeutic proteins. Most of biopharmaceuticals marketed to date are recombinant therapeutic protein drugs. These products have changed the life of humans as diabetics are now safe using human insulin instead of insulin of animal origin. Children with chronic granulomatous disease can lead a normal life due to interferon therapy. Patients undergoing cancer chemotherapy can recover quickly and suffer fewer infections by use of granulocyte colony-stimulating factors. These are only a few of the many benefits brought about by rDNA technologies.

The biotherapeutic proteins are being produced in microbial and mammalian cell systems. However, insect cells, transgenic plants, and transgenic animals are being developed as competitive processes.

Microbial Cell Systems

Recombinant proteins from the bacterium *E. coli* and the yeast *S. cerevisiae* make up about 40% of the therapeutic protein production market. Owing to scalability and well-characterized genetics, microbial host systems remain the best option for low-complexity and moderate-volume protein production at relatively low cost.

S. cerevisiae is a convenient host for producing pharmaceutical proteins especially of mammalian origin, due to many reasons: (1) it has attained GRAS (generally recognized as safe) status by the FDA; (2) it grows rapidly; (3) it secretes heterologous proteins extracellularly; and (4) knowledge of its genetics is greater than for any other eukaryote. Mammalian genes, such as those encoding human interferon, human epidermal growth factor, and human hemoglobin, have been cloned and expressed in *S. cerevisiae*. Also the production in this yeast of genes encoding surface antigens of the hepatitis virus resulted in the first safe hepatitis B vaccine. A major problem for *S. cerevisiae* is over-glycosylation. Other expression systems are the methanol-utilizing yeasts *Pichia pastoris* and *Hansenula polymorpha*. *P. pastoris* has been engineered to produce human-type glycosylation of recombinant proteins and will be very important in the future, especially for production of monoclonal antibodies.

With *E. coli*, the produced protein is not glycosylated, and if glycosylation is important, the bacterium cannot be used. Also, recovery can be challenging as the target protein is produced intracellularly. Robust recovery of recombinant proteins requires gentle lysis of cells after expression of the protein in the microbe's periplasm.

In addition to the above-mentioned microbes, other microbes have been used to make recombinant proteins. They include lactic acid bacteria, bacilli, corynebacteria, brevibacteria, streptomycetes, Nocardia, mycobacteria, halophilic bacteria, pseudomonads, caulobacteria and fungi.

Major problems with heterologous protein production in filamentous fungi are due to factors that influence production, that is, transcription, translation, secretion, and extracellular degradation. Different strategies have been developed to overcome these shortcomings, including the construction of protease-deficient strains, employment of strong fungal promoters, and increasing gene dosage. The introduction of a large number of gene copies, the use of efficient secretion signals, expression cassettes with strong fungal promoters, and fusions with a gene that encodes part of or an entire well-secreted protein have markedly improved production of recombinant proteins in fungi. Another problem lies in the postproduction recovery of proteins. Use of a secretory signal peptide fused to the N-terminus of the target protein can ensure efficient secretion of the protein. This eliminates the need for cell disruption and reduces interactions with host proteins. However, dilution of protein can be a shortcoming and proteins with complicated folding requirements and posttranslational modification steps cannot be improved with such a system.

Higher Cell Systems

Mammalian cells are used extensively for the production of complex human glycoproteins. It offers advantages such as proper protein folding assembly and posttranslational modifications. About 60% of therapeutic proteins, especially antibodies, are produced by mammalian cells. Cell lines generally used are Chinese hamster ovary (CHO), baby hamster kidney, human embryo kidney, and human retinal cells. Cell lines of human origin are now preferred as they allow production of target protein with human glycosylation patterns. The mammalian systems do suffer from high manufacturing costs however.

Production of recombinant proteins by the baculovirus-insect cell expression system has been used to produce tissue plasminogen activator, enzymes, viral proteins and parasitic proteins. Seven protein-based products have been commercialized. Four are veterinary vaccines, i.e., Porcelis Pesti from Merck, Bayovac from Bayer, Circo FLEX from Boehringer Ingelheim, and Porcilis PCV. The first two are for protection against swine fever virus and the last two are to protect against the porcine circovirus type 2. Three are for human parental use. They include Cervarix from GSK used to protect against cervical cancer caused by the human papilloma virus. Another is Provenge from Dendreon for treatment of prostate cancer. A third, FluBloc from Protein Sciences Corp., is used for immunization against disease caused by influenza virus subtypes A and B. Other vaccines are in development against many diseases.

Use of transgenic plants to make pharmaceuticals employs self-pollinating crops such as rice and barley for protein production. The major drawbacks include low levels of expression and environmental concern about contamination of nongenetically modified plants. Plant-made pharmaceuticals (PMPs) are produced in crops such as corn, tobacco, rice, and soy. The plant genes are manipulated to produce proteins with improved purity and activity. Transgenic plants offer benefits of being cost-effective, reduced chance of microbial contamination and increased production capacities. The first plant-made vaccine was approved by the US Department of Agriculture (USDA) in January 2006, validating the safety and efficacy of plant-made vaccines. Ventria Bioscience has developed an electrolyte solution using rice that is genetically altered to produce two breast milk proteins, lactoferrin and lysozyme. This plant-based electrolyte solution helped significantly in reducing the incidence of diarrhea and infections in children. Considerable efforts are being made to produce plant-based insulin using genetically altered safflower. This can meet the enormous supply needs and also reduce the capital costs and product costs substantially.

The first plant-based biopharmaceutical for humans to be approved by FDA was Elelyso (taliglucerase alfa) made by the Protalix company in Israel. It is used for the heritable Gaucher's disease. There are only two existing drugs for this disease but they are extremely expensive (up to $300,000 per year). One of these is Cerezyme made by Genzyme and produced in modified hamster cells. Problems with Cerezyme manufacture have limited its supply. Eleyso is made in carrot cells. Taliglucerase alfa replaces the malfunctioning enzyme which causes Gaucher's disease. The malfunction leads to fat accumulation resulting in bone deterioration and anemia.

Attempts are being made to develop transgenic farm animals that produce pharmaceuticals and nutraceuticals. This is also referred to as pharming. Transgenic animals can be used to produce therapeutic proteins, especially for those that are complex, difficult to express, and require mass production. Among GM animals, goats are preferred because of their well-developed transgenic technology and high yield per liter of milk. More than 85 proteins including human blood proteins, for example,

antithrombin, 1A trypsin, 1 antitrypsin, and fusion antibodies have been produced in goats. Transgenic animals have a major benefit of low-cost production once they are developed, but disadvantages exist such as difficulty and the length of time required in establishing such engineered animals.

Biopharmaceutical Markets

The market for therapeutic proteins reached $47.4 billion in 2006. Today, therapeutic proteins are used to relieve patients suffering from many conditions, namely cancer (monoclonal antibodies, interferon), heart attacks, strokes, cystic fibrosis, Gaucher's disease (enzymes, blood factors), diabetes (insulin), anemia (erythropoietin (EPO)), and hemophilia (blood clotting factors). The leading classes of therapeutic proteins in 2003 were EPO, monoclonal antibodies, and interferons.

Biopharmaceuticals made by mammalian cell cultures, especially monoclonal antibodies, continue to grow as an important class of pharmaceutical products. In 2012, biopharmaceuticals reached sales of $130 billion which was 13% of the total market for pharmaceuticals. 34 were "blockbusters" (i.e., sales over $1 billion) and more than 60% were produced by mammalian cell cultures. Of the ten best-selling pharmaceuticals, five are biopharmaceuticals and four of the five are monoclonal antibodies (Humira, Enbrel, Remicade and Rituxan).

The market for EPO grew from $200 million to $2 billion in a span of 5 years from 1990 to 1995. It grew further to $3.5 billion by 2000 and to $11.8 billion by 2004. Sales of other polypeptides such as human insulin (Novolin and Humulin) reached $12 billion, while that of interferon-(Intron A) amounted to $3.8 billion, the white blood cell stimulant Neupogen (filgrastim) $3 billion, interferon-la (Avonex for multiple sclerosis) $2.2 billion, human growth hormone $1.8 billion, recombinant hepatitis B vaccine and somatropin (Humatrope and Neutropin) $0.9 billion, and cerezyme $0.7 billion.

The fastest growing therapeutic protein class is that of monoclonal antibodies (mAbs), with a 2012 market of $18 billion. Monoclonal antibodies have diagnostic applications (determination of ovulation, pregnancy, and identification of infectious agents) and therapeutic uses (specific drug delivery in cancer therapy, destruction of platelet-catalyzed blood clots in heart disease therapy). They act as magic bullets by binding target proteins or by blocking the binding of target proteins. They bring a drug or a radioisotope to a designated target. ReoPro was the first successful therapeutic mAb approved in 1994 and reached a market of $384 million by 2002. By inhibiting platelet aggregation, it successfully prevented complications of angioplasty such as heart attack and death. Rituxan, used for non-Hodgkin's lymphoma, achieved sales of $5.9 billion. Enbrel, binding tumor necrosis factor (TNF), a protein involved in inflammation, and Remicade and Humira used for rheumatoid arthritis reached $1.5 billion in sales in 2002. Avastin is the first antiangiogenesis drug acting against vascular endothelial growth factor (VEGF), an angiogenic factor. Today, more than 20 mAbs are on the market and more than 100 are under clinical trials for allergy, asthma, autoimmune diseases, cancer, cardiovascular diseases, transplantation, and viral infection.

In 2012, the biopharmaceutical market was constituted by over 300 proteins and monoclonal antibodies with sales of over $100 billion. Hormones sold for over $11 billion and growth factors over $10 billion. Biopharmaceuticals have the fastest growth rate on the market, i.e., 19%. About 40% are produced by mammalian cell cultures, mainly CHO cells which have glycosylation patterns similar to that of human proteins. *E. coli* produces another 30% of products and *S. cerevisiae* produces 20%.

In 2012, twelve biopharmaceuticals were approved in the United States and/or Europe. This compared to ten in 2008, 20 in 2009, 13 in 2010, and six in 2011. Of the twelve were three enzymes, three antibodies, two fusion proteins, a gene therapy product, a regulatory peptide, a blood product, and a colony stimulating factor. The systems for production were mammalian cells, mainly CHO, for five of the products, and three in *E. coli*, including recombinant human glucocerebrosidase, used as replacement therapy for Gaucher disease. A new process was developed to make eicosapentaenoic acid (EPA), which is a long chain polyunsaturated fatty acid (PUFA), one in *S. cerevisiae*, one in *P. pastoris*, and one in an engineered plant. The plant-based product was Elelyso (taliglucerase alfa). The process uses an engineered strain of *Yarrowia lipolytica*. The oil produced has much higher levels of EPA than natural oils. EPA is important for the anti-inflammatory activity of fish oils, thus contributing to cardiovascular and joint health. The product is being commercialized by DSM. The yeast was engineered by transformation with 21 heterologous genes encoding five different activities.

Production of biopharmaceuticals by *S. cerevisiae* includes insulin and insulin analogs. Other products are human serum albumin, hepatitis vaccines, and virus-like particles used for vaccination against human papilloma virus. Other areas of current active research in the field of therapeutic biotechnology include research on vaults, that is, cell organelles made of ribonucleoproteins. These naturally occurring nanocapsules could provide a whole new class of delivery vehicles for therapeutic drugs and DNA. Indeed, vaults could be used for a wide range of applications in nanobiotechnology such as DNA delivery to correct mutations, enzyme delivery to replace or insert missing enzymes, and targeted drug delivery. Recently, genes that have a role in longevity and youthfulness have gained a lot of attention. Sirtuins are a family of genes that may be the master regulators of survival mechanisms at the time of stress, and understanding how they produce their health- and longevity-enhancing effects could lead to disease treatments and eventually longer disease-free human life spans. The biotechnology industry has now taken major strides by encompassing ribozymes, antisense molecules, monoclonal antibodies, gene and cell therapy, genomics, proteomics, metabolomics, pharmacogenomics, drug delivery, nanobiotechnology, bioinformatics, combinatorial chemistry, and developmental biology. These developments in genetic engineering have made tremendous impacts on almost all aspects of life.

Viruses are being studied as a means of killing tumors and training the immune system to combat cancer. There is one licensed oncolytic virotherapy which is used in China. Several late stage candidates, for example, Amgen's T-Vec and SillaJen's Vexa-Rec, are in late stage clinical trials.

Primary Metabolites

New processes for production of amino acids, flavor nucleotides, vitamins, and organic acids have been developed by recombinant DNA technology. *E. coli* strains were constructed with plasmids bearing amino acid biosynthetic operons. Plasmid transformation was accomplished in *Corynebacterium*, *Brevibacterium*, and *Serratia* and, as a result, recombinant DNA technology has been routinely used to improve such commercial amino acid-producing strains. New technologies which have proven to be very useful for increasing production of primary metabolites include genome-based strain reconstruction, metabolic engineering, and whole genome shuffling. Processes improved by genetic engineering include those for L-lysine, L-threonine, L-valine, L-tryptophan, L-proline, L-leucine, L-isoleucine, 5-guanylic acid (GMP), 5-inosinic acid (IMP), riboflavin, biotin, ascorbic acid, citric acid, gluconic acid, acetic acid, D- and L-lactic acid, and succinic acid, among many others.

The worldwide production of amino acids amounts to 4.5 million tons per year. L-tryptophan is produced at 14,000 tons per year, and L-phenylalanine at 30,000 tons per year.

High levels of primary metabolite production are as follows (in g/L): glutamic acid: 146; pyruvic acid: 135; citric acid: 250; isopropanol: 143; isobutanol: 111; 1,3-propanediol: 135; L-tyrosine: 50; isoprene: 60; farnesine: 100; poly(3-hydroxybutyrate):141; polyhydroxyalkanoate: 100; 3-hydroxypropionic acid: 42; muconic acid: 36; 3-methyl-1-butanol: 9.5; amorphadiene: 40; artemisinic acid: 25; lipids: 25; isobutyl acetate: 17; parahydroxy styrene: 17; hydroxybutyrate 9.

Amino acids are a leading category of primary metabolites produced commercially. Metabolic engineering has increased the microbial production of L-valine. Engineered *E. coli* can produce 60 g/L with a productivity of 2 g/L h and a yield of 0.34 moles of valine per mole of glucose. Engineered *C. glutamicum* can make 172 g/L of L-valine with a yield of 0.63 moles per mole of glucose. L-valine is used in human and animal nutrition, manufacture of pharmaceuticals, as a moisturizing compound in cosmetics, and for chemical synthesis of antibiotics, antivirals, and herbicides.

Activities of L-arginine include stimulation of secretion of growth hormone, prolactin, insulin and glucagon, promotion of muscle mass, and enhancement of wound healing. High production of L-arginine has been achieved with metabolically-engineered *C. glutamicum* at 92.5 g/L. The arginine derivative L-ornithine can be produced at 51.5 g/L by another genetically-engineered strain of *C. glutamicum*.

Putrescine production has reached 24.5 g/L with metabolically-engineered *E. coli*. Fermentative production of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) has been accomplished. GlcN is used for relief of osteoarthritis. Current global sales amount to $2 billion. Recombinant *E. coli* can produce 110 g/L of GlcNac. GABA (gamma amino butyric acid), used in pharmaceuticals and functional foods, is produced by genetically engineered *C. glutamicum* at a level of 29.5g/L.

Carboxylic acids are made mainly by catalysis from petroleum-based precursors but interest in microbial production is increasing. Annual production of these organic acids is as follows (kt/year): acetic acid:10,000; glycolic acid: 40; 3-hydroxypropionic acid: 3600; acrylic acid: 4200; lactic acid: 450; fumaric acid: 200; malic acid: 60; succinic acid 37; itaconic acid 80; citric acid 1600; adipic acid 3000; gluconic acid 87; glucaric acid: 42. Acetic and lactic acids are used as food preservatives.

Polyunsaturated fatty acids (PUFAs) are used as nutraceuticals and include (a) gamma linoleic acid (GLA;18:3 omega-6) from *Mucor circinelloides*, (b) docosahexaenoic acid (DHA; 22:6 omega-3) from *Crypthecodinium cohnii* and *Schizochytrium/Thraustochytrium spp*, (c) arachidonic acid (ARA; 20:4 omega-6) from *Mortierella alpine*, and (d) eicopentaenoic acid (EPA) from genetically-modified *Y. lipolytica*. They represent a multi-billion dollar industry, mainly ARA and DHA for infant formulas. Microbial fermentation can be used for their production. Microbial oils can be made by 30–40 species of yeast; also by molds and algae. They are called oleaginous microbes. Fungi can accumulate 70% of their biomass as oils. DHA is produced at 2000 tonnes per year and has a market of $317 million. It is produced by algae including a group of *Schizochytrium*, *Aurantiochytrium* and *UIbongichytrium* species. EPA-producing microbes are microalgae. *Nitzchia laevis* makes 1 g/L of EPA.

Carotenoids are terpenoids, composed of hydrocarbons (beta-carotene and lycopene) or oxygenated derivatives known as xanthophylls, for example, lutein, zeaxanthin, astaxanthin. The carotenoid market in 2010 was $1.2 billion. Astaxanthin is very efficient as a scavenger of reactive oxygen species. Beta-carotene is more potent against reactive nitrogen species. *E. coli* can produce 220 mg/L of lycopene. A recombinant strain of *Pseudomonas putida* can produce 239 mg/L of zeaxanthin. The market for beta-carotene was $223 million in 2010 and that for astaxanthin reached $225 million. Zeaxanthin is produced by microalgae and marine bacteria. A high level of 512 mg/L is produced by the marine bacterium *Flavobaterium multivorum* It can also be made by metabolically-engineered *E. coli* at 1.6 mg/g DCW .

Microbes produce vitamin B_{12} at high levels, for example, the aerobe *Pseudomonas denitrificans* at 214 mg/L and the *anaerobe Propionibacterium shermanii* at 300 mg/L. Chemical synthesis requires more than 60 steps.

Enzymes

In all living systems, most of the chemical reactions are mediated by enzymes – the biocatalysts. Enzymes are extensively used in the food industry (production of cheese and clarification of apple juice), textile industry (development of more efficient laundry detergents), paper industry, and in treatment of sewage. Living cells make enzymes and the strains are improved for better yield, and improved characteristics such as specificity, stability, and performance in industrial processes. Although the concentration of

individual enzymes in cells is typically less than 1%, this can be increased by use of gene amplification techniques. The enzyme market reached $1.6 billion in 1998 for the following application areas: food 45%, detergents 34%, textiles 11%, leather 3%, and pulp and paper 1.2%. This does not include diagnostic and therapeutic enzymes. The market for these non-pharmaceutical proteins reached $5 billion in 2015.

Recombinant fungi are one of the main sources of enzymes for industrial applications. More than 60% of the enzymes used in the detergent, food, and starch processing industry are recombinant proteins. Amylases, isolated mainly from *Bacillus licheniformis*, are used in sizing starch by the textile industry. Pectolytic enzymes are utilized for preparation of textiles such as hemp and flax. Catalases are used for removal of hydrogen peroxide prior to dying during textile processing. A number of enzymes are used for stain removal and allow mild washing conditions, thereby protecting the fabrics. The detergent industry employs cellulases for biopolishing of fabrics, and proteases isolated from *B. licheniformis* and *Bacillus amyloliquefaciens* for wools. Microbial lipases have a huge potential in areas such as food technology, and biomedical sciences, since they are stable in organic solvents, possess broad substrate specificity, do not require cofactors, and exhibit high enantioselectivity. In the food industry, lipases are commonly used in the production of fruit juices, baked foods, to form desirable flavors in cheeses, and interesterification of fats and oils to produce modified acylglycerols. There are three fungal recombinant lipases currently used in the food industry. They are from *Rhizomucor miehei*, *Thermomyces lanuginosus*, and *Fusarium oxysporum*, and are produced in *Aspergillus oryzae*. Lipases are employed in leather processing where they are combined with proteases, peroxidases, and oxidases. To be effective in detergents, lipases need to remain functional under harsh conditions such as the presence of surfactants and oxidants, temperatures above 45°C, pH values of about 10, and should be alkalophilic. In 1994, Novo Nordisk introduced lipolase, the first commercial recombinant lipase for use in a detergent by cloning the *Humicola lanuginosa* lipase gene into the *A. oryzae* genome.

Enzymes have great use in biotransformations. Either intact cells, an extract from such cells, or an isolated enzyme may be used as the catalytic system for a specific reaction. Biotransformation is highly cost-effective as compared with chemical transformation. Enzymes are employed in biotransformations for production of organic acids, steroid hormones, and antibiotics, in precious metal recovery (*Chlorella vulgaris*) and bioleaching of metals (*Thiobacillus ferrooxidans*), providing recovery of up to 70% of copper from low-grade ores.

Recombinant DNA has widened the scope of enzyme production and its applications. Plant and animal enzymes can be produced by microbial fermentations. It also enables production of enzymes from microorganisms difficult to grow or handle genetically by fermentation. Enzyme productivity can be enhanced by introducing multiple gene copies, introducing strong promoters, or using efficient signal sequences. Enzymes from pathogens or toxin producers can be produced in safe hosts. Designer enzymes with excellent selectivity and turnover rates can be created using directed evolution and rational design. Molecular modeling facilitates a detailed understanding of the catalytic mechanism and determinants of an enzyme–substrate reaction, which further helps in rational designing of enzymes.

Genetic engineering is used to produce microorganisms with altered enzymes or enzyme activities to achieve better yields of desired precursors, intermediates or products, such as polyketides, nonribosomal peptides, steroids, vitamins, and unnatural amino acids in an efficient manner. The use of directed evolution has rapidly emerged as the method of choice for the development and selection of mutated enzymes with improved properties. A variety of such methods have been used to alter the activity, stability, and availability of an array of enzymes. Currently, research is focused on bioprospecting for enzymes that fit specific applications. A majority of enzymes isolated from natural sources cannot meet the stringent demands of industrial and biotechnological applications, posing an exciting challenge for scientists. Modern biotechnological techniques enable modification of enzyme structure and function to exacting specifications. Directed evolution has allowed the generation of enzymes with greatly improved characteristics, and in some examples, with completely novel substrate specificities. It can help to design enzymes according to their potential applications. About 640 enzymes were structurally characterized by crystallization by 2000 and 3000 enzymes were identified by 2003.

Many natural enzymes have low stability, lack specificity and have low catalytic efficiency. This is being corrected by enzyme engineering techniques, i.e., chemical modification, phage display, rational design and directed evolution. The most commonly used are rational design and enzyme evolution.

Directed evolution has been successful in tailoring enzymes to improve their applications by changing substrate selectivity and evolving function de novo. It involves iterative mutagenesis followed by screening or selection. It can improve enzyme properties and functions even without much knowledge of enzyme structure. Important applications of directed evolution include modulating properties of enzymes acting on starch and alpha-glucans, and in organic synthesis, such as ketoreductases, monooxygenases, lipases and aldolases for improvement in activity, stability and enantioselectivity. Enzymes modified include those involved in biosynthesis, modification and degradation of carbohydrates, for example, ADP-glucose pyro-phosphorylase, amylosucrase, cytochhrome P450, aldolase, sugar kinase, cellulase, amylase, xylanase and glucose dehydrogenase. Other improved enzymes are those involved in pharmaceutical production such as (R)-transaminase, keto-reductases, glucose dehydrogenase and haloalkane dehydrogenases used to make pharmaceutical ingredients. Success stories include (a) alcohol dehydrogenase with a ten-fold improvement over the wild-type enzyme at low temperature, and (b) keto-reductase/glucose dehydrogenase activity in which 160 g/L of substrate was converted with over 99.9% ee and 95% isolated yield in 8 h. Organisms used as enzyme producers include *E. coli*, *B. subtilis*, *Bacillus megaterium*, *Bacillus brevis*, *Lactobacillus lactis*, *P. pastoris*, *S. cerevisiae*, *H. polymorpha*, *Kluyveromyces lactis*, *S. pombe*, *Y. lipolytica*, *Aryula adeninivorans*, *Candida boidini*, *A. oryzae*, *A. niger*, *Trichoderma reesei*, *Trichoderma atroviride*, *Penicillium sordida*, *Penicillium griseoroseum*, *Penicillium purpurogenum and Rhizopus oryzae*. Large proteins (>100 kd) are usually expressed in eukaryotes while small proteins (<30 kD) are expressed in prokaryotes.

Bioplastics and Biopolymers

Plastics are traditionally manufactured using fossil fuels. These petrochemical plastics have high durability but can-not be degraded easily, and eventually increase the bulk in landfills. This, along with the escalating costs of fossil fuels, prompt the plastic industry to adapt nature's resources to replace petroleum-based synthetics with bioplastics. Materials produced by living species, such as bacterial polyesters, lactic acid, and triglycerides are being polymerized to produce bioplastics. The kind of microbial plastic produced depends on the polymer produced by bacteria, which, in turn, is related to the carbon source used. Acetate and butyrate yield polyhydroxybutyrate (PHB), caproate yields polyhydroxycaproate (PHC), and valerate yields polyhydroxyvalerate (PHV).

Polyhydroxy alkanoates (PHAs) are naturally produced by many bacteria as energy reserves. These compounds have properties similar to synthetic thermoplastics and elastomers but are completely and rapidly degraded by bacteria in soil or water. PHAs were produced at 350,000 metric tons in 2006. Most biopolymers are produced by chemical synthesis from non-renewable resources or by extraction from plants, animals, or algal biomass. Microbial production of the prebiotic poly-hydroxybutyrate (PHB) costs 5–10 times more than for synthesis of petrochemical polymers. The gamma-polyglutamic acid polymer made by *Bacillus* spp has a titer of 101 g/L and that of xanthan gum is 62 g/L. Total market for biopolymers was $600 million in 2011.

The most abundant PHA is poly(3-hydroxy-butyrate) (PH3B), which bacteria synthesize as a part of their natural metabolism, from acetyl-CoA. Growing on glucose, the bacterium *Ralstonia eutropha* can accumulate up to 85% of its dry weight as PHB, which makes this micro-organism a miniature bioplastic factory. However, a major limitation in the commercialization of such bacterial plastics is their cost, being 5–10 times more than that for petroleum-based plastics. Much effort has therefore gone into reducing production costs with the aid of modern genetics, by developing superior bacterial strains in terms of polymer production. Recently, a novel alternative of generating transgenic plants that synthesize PHAs has emerged, which seems to be an economical and environmentally friendly approach to mass-produce bioplastics. The transgenic *A. thaliana* plant constructed had *R. eutropha* genes encoding two enzymes involved in the conversion of acetyl-CoA to PHB. Biocompatibility and biodegradability make PHB fibers ideally suited for surgical use; sutures made from PHB are only slowly degraded by the body's enzymes.

A new environmental-friendly and economical bioplastic is polytrimethylene terephthalate (3GT polyester; 3Gþ; Sorono), made by reacting terephthalic acid with 1,3-propanediol (PDO; trimethylene glycol; 3G). Scientists at Genencor International and DuPont jointly worked to develop a genetically engineered *E. coli* culture, by introducing eight new genes to convert dihydroxyacetone phosphate (DHAP) into PDO. These included yeast genes converting dihydroxyacetone into glycerol and *Klebsiella pneumoniae* genes converting glycerol into PDO. This recombinant *E. coli* grows on glucose and produces PDO at 135 g/L, with a yield of 51% and a rate of 3.5 g/L/h. They improved production by modifying 18 *E. coli* genes, including regulatory genes. PDO is also used as a polyglycol-like lubricant and as a solvent. Likewise, nylon has been traditionally made from a polymer of PDO. Another environmentally-friendly plastic is polylactide, made chemically from fermentation-derived L-lactic acid.

Improving efficiency is a major concern for the production of bioplastics. Only a few processes have emerged that actually use less energy in the production process. Therefore, researchers are still working on refining the processes used in order to make bioplastics viable alter-natives to petrochemical plastics. Apart from energy use, other areas of concern are the sharing of agricultural space, since plants are also needed to fulfill the basic requirement of food, or as a raw material for other applications. Researchers are looking into creating a plant that can be used for food, but also as feedstock for plastic production. Transgenic corn engineered to produce bioplastics is currently under study. Eventually, it is hoped to engineer the plant in a way which would restrict plastic production to the stem and leaves of the plant. Hence, such plants would serve a dual purpose by providing food through the edible part while plastic could be extracted from the nonedible parts of the plant.

Xanthan gum was the first biopolymer to be made industrially and is the most significant microbial polymer. This polysaccharide was made at 90,000–100,000 metric tons in 2011 with a price of $3–5 per kg. Ten to twenty thousand tons of xanthan are produced annually for use in the oil, pharmaceutical, cosmetic, paper, paint, and textile industries. Genetic manipulation has increased titers of xanthan by two-fold and increased pyruvate content, a desired characteristic, by 45%. Cloning genes, which complement xanthan-negative mutants, into wild-type *Xanthomonas campestris* increased xanthan production by 15%.

Cellulose is produced as an extracellular polysaccharide by a number of different bacteria in the form of microfibrils that can be used to produce molded materials of relatively high strength, such as high-fidelity loudspeaker cones and diaphragms.

Biopolymers having promising applications in wound healing are polysaccharides such as chitin, alginate, dextran, and hyaluronic acid. Although fungal cell walls form an important source of chitin and chitosan, these polymers are, at present, manufactured from sea food (shellfish) wastes. Researchers are trying to make use of intact fungal filaments as they prove to be an inexpensive source for wound dressings and other novel materials. Additional biopolymers, not previously available on a large scale, are now coming onto the market, thanks to biotechnology. One such example is hyaluronic acid, a polydisaccharide of D-glucuronic acid and *N*-acetyl glucosamine, found in the connective tissue matrices of vertebrates and also present in the capsules of some bacteria. Earlier, hyaluronic acid was produced by a laborious method using large quantities of rooster combs, while now, fermentation makes the process fast and efficient and obliterates the need of rooster combs.

1,3-Propane diol (1,3-PDO) produced by microbes is a non-toxic polyhydric alcohol used to produce polytrimethylene terephthalate, which is employed in the carpet industry, and to make highly durable and stain resistant textile fibers, thermoplastics, films, automobiles, apparel and coatings. Since the chemical processes are costly, 1,3-PDO is produced using biotechnology. Microbial production uses glycerol or glucose as the main substrate. Cell immobilization is an excellent way to produce 1,3 PDO from glycerol. Immobilized *K. pneumoniae* cells produced 83 g/L of 1,3 PDO, and *Clostridium butyricum* produced 93.7 g/L from pure

glycerol and 76.2 g/L from crude glycerol. The highest production was by recombinant *E. coli* at 104 g/L. In 2011, commercial production amounted to153,600 tonnes. Chemical production by Shell Oil Co. amounted to 72,600 tonnes per year and by Evonik Degussa was 50,000 tonnes per year, the total representing 60% of the market. Biologically-produced 1,3-PDO by DuPont Tate and Lyle Bioproducts was at 66,000 tonnes or 33% of the total. Biological production generates 40% less greenhouse gas emissions than that from petrochemistry.

A group of biopolymers that particularly interest bio-technologists are proteins, because of their ability to utilize the new genetic manipulation techniques. Thus, genes for animal and plant proteins (e.g., collagen and various silks) can now be transferred into suitable microbial hosts and the proteins produced by fermentation. Defense services are interested in developing spider silk as a high-performance fiber for use in products such as bullet-proof vests.

Isoprene, a highly volatile liquid that can be polymerized to rubber, is presently made by chemical synthesis or extractively distilled from crude oil. However, these procedures require high pressure, consume too much energy and create pollution. It would be useful to be able to produce isoprene microbiologically. Engineering the methylerythritol 1-phosphate (MEP) pathway in *E. coli* resulted in the production of 19.9 mg/L isoprene.

Biofuels

Industrial biotechnology is heavily involved in the production of energy from renewable resources and biomass. Bioethanol is an alcohol made mainly from sugar and starch crops such as corn by fermenting the sugar components of biomass. Recent advances in technology make the cellulosic biomass of trees and grasses worth using as feedstocks for ethanol production. Waste matter such as agricultural residues from harvested crops, forestry wastes such as chips and sawdust from lumber mills, dead trees, and tree branches, municipal solid waste such as household garbage, and industrial wastes should be used as feedstocks for ethanol production besides energy crops (fast-growing trees and grasses such as switchgrass) developed for this purpose. Ethanol can be used as a fuel for cars in its pure form. In fact, Henry Ford's first car ran on ethanol. However, it is usually used as a gasoline additive (called E10, E15 and E85) to increase octane and improve vehicle emissions. These US motor fuels have 10–85% of ethanol derived from corn sugar.

The main components of biomass are cellulose, hemicellulose, and lignin. Two reactions important in the conversion of biomass into bioethanol are hydrolysis and fermentation. Starch, from corn, potatoes, sugarcane, and wheat, is used to produce ethanol as a substitute for gasoline. However, turning starch into ethanol is neither the most environmentally nor economically efficient method, as growing plants for ethanol production involves the use of herbicides, pesticides, fertilizers, irrigation, and machinery. Agricultural waste and cellulosic materials from trees and grasses seem to be a superior option as a feedstock for bioethanol production. A great deal of effort is concentrated on developing more effective bacterial cellulases that can break down agricultural waste into simple sugars to create a more plentiful and cheaper raw substrate for the production of ethanol.

Cellulose is a possible source of ethanol, but instead of containing only glucose, cellulose also contains C5 sugars such as xylose and arabinose. The best C5 utilizer is *Pichia stipitis* which can produce ethanol and clean up concentrated toxins liberated from lignocellulose degradation. It can produce ethanol from pretreated sources of biomass such as red oaks, wheat straw, sugarcane bagasse, rice straw, corn cobs, corn stover, aspen wood, pinewood, and poplar wood. From aspen wood chips, ethanol titer was 41 g/L with a yield of 0.47 g/g. In chemically-defined medium, 61 g/L can be made.

In 2005, the total world production of bioethanol was 46 billion liters. The producers of bioethanol are using a variety of microorganisms; the most favored being the yeast *S. cerevisiae* for large-scale fermentation processes. Researchers have also metabolically engineered the bacterium *Zymomonas mobilis* to augment its ability to ferment xylose and arabinose along with its innate ability of fermenting glucose. The purchase and pretreatment processing costs of the raw material feedstock govern the economy of bioethanol production processes. The most commonly used feedstocks, by far, have been corn and sugarcane, but alternative feedstocks rich in lignocellulose are required for sustainability and self-sufficiency of bioethanol production. Metabolic engineering is being used to develop production strains that can process the most abundant pentose sugar, that is, xylose, in hemicelluloses, hardwoods, and crop residues. This effort involves the introduction of key enzymes (xylose reductase, xylitol dehydrogenase, xylulokinase, or xylose isomerase) into ethanol-producing strains. Such metabolic engineering efforts have improved xylose consumption efficiency, with small decreases in growth rate and ethanol yield as compared with glucose utilization. Additional areas of bioethanol process development include identification of thermostable cellulases development of ethanologenic bacteria, and further metabolic engineering of *S. cerevisiae* to decrease formation of by-products, such as glycerol and organic acids. Bacteria such as the anaerobic and thermophilic clostridia could play a major role in the future production of bioethanol. *Clostridium thermocellum*, an anaerobic thermophile, converts waste cellulose (i.e., biomass) or crystalline cellulose directly into ethanol, without the need for fungal cellulase. A large amount of genetic work has been done on the clostridia and they are ready for a major application of rDNA technology to increase titers and ethanol tolerance, and to eliminate production of by-products, such as acetate and lactate.

The bacteria that naturally metabolize cellulose have gained much interest in recent years since they can utilize lignocellulosic waste and generate ethanol, a rare property among living organisms. Indeed, their cellulase systems mediate hydrolysis of lignin-containing materials, such as hardwood pretreated with dilute acid, as well as model substrates not containing lignin. In principle, the concept of anaerobic ethanol fermentation is a very simple one, that is, a single fermentation with five biologically mediated events involved in ethanol production: (1) cellulase and hemicellulase formation, (2) cellulose and hemicellulose hydrolysis, (3)

uptake of sugars and oligosaccharides, (4) hexose fermentation, and (5) pentose fermentation, all consolidated in a single process step. An advantage of such an anaerobic process is the reduced need for power-consuming agitation/aeration of reaction vessels for making biomass-degrading enzymes. Proponents of anaerobic thermophiles as ethanologens offer the following reasons: (1) thermophiles appear to be robust and contain stable enzymes; (2) anaerobes generally have a low cellular growth yield, hence more of the substrate can be converted to ethanol; (3) thermophilic fermentations run at 60°C are less prone to detrimental effects of contamination; (4) growth at higher temperatures may facilitate the removal and recovery of volatile products such as ethanol; (5) in situ cellulase production is much more economical than addition of cellulase; (6) rates of metabolism of cellulose and hemicellulose are high; and (7) a reduced need for power involved in cooling of fermentors.

Clostridia and other anaerobic bacteria break down cellulose with the formation of cellobiose and cellodextrins as main products. The cellodextrins and the cellobiose can be further utilized by the organism; indeed, the cellodextrins are taken up more easily by *C. thermocellum* than the disaccharide. The final end products are ethanol, acetic acid, lactic acid, hydrogen, and carbon dioxide. The major economic obstacle is the production of the side-products acetate and lactate, which limits conversion yield. rDNA technology research is overcoming this bottleneck by inactivation of the genes encoding acetate- and lactate-producing enzymes.

Biofuel companies have already begun production of cellulosic ethanol. They include (a) DSM/Poet's plant in Emmetsburg, Iowa, expecting to produce 20 million gallons per year, (b) Beta Renewables in Crescentino, Italy, also a 20 million gallon/year operation. (c) Gran/Bio began production of cellulosic ethanol at 21 million gallons per year in San Miguel de Campos, Brazil. The cellulosic sources are agricultural wastes, i.e., straw and bagasse. It also uses technology from Beta Renewables, cellulase from Novozymes in Denmark, and yeast from DSM in Holland. (d) Abengoa opened its cellulosic ethanol plant in Hugoton, Kansas in October 2014. It is the largest one established so far, capable of producing 25 million gallons per year.

Ethanol can be produced from seaweed. The substrates are sugar from glucose, sulfated polysaccharides, mannitol, alginate, agar and carrageenan. Seaweeds contain low to zero levels of lignin, making carbon sources easier to use without delignification. They have a higher biomass productivity per year than lignocellulolytic biomass such as switchgrass, corn stover, Eucalyptus, poplar and willow. Fermentation of *Laminaria japonica* (brown sea weed) yielded 40 g/L of ethanol whereas that of *Gellidium elegans* (red seaweed) reached 55 g/L of ethanol.

Butanol is another alcohol which could help solve the problem of overdependence on petroleum as a motor fuel. Cloning of its *ace* operon genes *adc* (encoding acetoacetate decarboxylase), *ctfA*, and *ctfB* (two genes encoding coen-zyme A transferase) on a plasmid containing the *adc* promoter into *Clostridium acetobutylicum* resulted in a 95% increase in production of acetone, a 37% increase in butanol and 90% increase in ethanol, a 50% increase in solvent yield from glucose, and a 22-fold lower production of acids. Elimination of acetone and ethanol production by genetic means could increase biobutanol production further and simplify purification.

Biodiesel is made up of fatty acid alkyl esters made from vegetable oils, animal fats or recycled greases. Biodiesel can be used as a fuel for vehicles in its pure form, but it is generally used as a petroleum diesel additive to reduce levels of particulates, carbon monoxide, hydrocarbons and air toxics from diesel-powered vehicles. Biodiesel, also known as biologically-derived diesel substitute, can be made from rapeseed (canola) or soybean oil, palm oil, jatropha, or recycled cooking oils by heating with a chemical catalyst. Animal fats, other vegetable oils, and other recycled oils can also be used to produce biodiesel, depending on their costs and availability. In the future, blends of all kinds of fats and oils may be used to produce biodiesel. The main reaction for converting oil into biodiesel is called transesterification. Genetically-modified bacteria can produce biodiesel from plant materials.

As is the case with bioethanol, biodiesel proves to be environmentally friendly as it can help make up for greenhouse gas emissions because it is produced from organisms that remove CO_2 from the atmosphere as a part of their natural metabolism. Biodiesel is a good alternative energy source and a substitute for petroleum-based diesel fuel. However, the current method of production is still not economical. Alexander Steinbuechel created a new form of biodiesel with colleagues at the University of Muenster in Germany. He and his group created a fuel-refining strain of *E. coli* by modifying it with genes taken from two other bacterial species. The modified *E. coli* strain was cultured in a mixture of glucose and the main constituent of olive oil, which it processed into a fatty acid diesel-substitute called microdiesel. The German team used two genes from the bacterium *Z. mobilis* to give *E. coli* the ability to produce alcohol from the sugar. A third gene, taken from the bacterium *Acinetobacter baylyi*, enabled the *E. coli* to then combine the alcohol with plant oils to produce microdiesel. Unlike regular biofuels, microdiesel is produced without toxic chemicals.

Genetic engineering has been utilized to produce biodiesel. The principal organisms for biodiesel fermentation are called "grease microorganisms," i.e., oleaginous microbes which supply fatty acids and alcohols and convert them to biodiesel. They include microalgae (*C. vulgaris, Botryococcus braunii, Navicula pelliculosa, Scenedesmus acutus, C. cohnii, Dunaliella primolecta, Monallanthus salina, Neochloris oleoabundans, Phaedactylum tricornutum and Tetraselmis sueica*). Genetic engineering has been used to convert the following into biodiesel producers: *E. coli* and *S. cerevisiae*. *E. coli* was converted into a biodiesel producer by overexpression of ethanol production genes from *Z. mobilis* and the wax ester synthase/acyl-CoA-diacylglycerol acyl transferase (WS/DGAT) gene from *A. baylyi* strain ADPI. Of potential importance are *Rhodococcus opacus* and *Y. lipolytica* for microbial biodiesel production, as well as cellulolytic microbes for biomass digestion, after plasmid insertion for FAEE production.

In 2010, 23 billion gallons of ethanol and 6 billion gallons of biodiesel were produced globally.

Renewable fuels and chemicals can also be produced using 2-keto acid-based biosynthetic pathways. 2,3-Butanediol (2,3-BD) (also known as 2,3-butylene glycol and dimethylene glycol) is a 4-carbon diol. They can be converted to the liquid fuel additive methyl ethyl ketone and also to 2,3-butadiene which is polymerized in the manufacture of synthetic rubber. Methyl ethyl ketone is also a good organic solvent for resins and lacquers. 2,3-BD can also be made at 83 g/L from crude glycerol (from the biodiesel

process) by *B. amyloliquefaciens* in the presence of beet molasses as co-substrate. The yield was 0.42 g/g and productivity was 0.87 g/L/h. Genomatica Inc (San Diego) has used their integrated biotechnology platform to develop an *E. coli* strain producing 120 g/L of 1,4-butanediol (BDO) from sugars. The rate of production is over 3 g/L/h.

Research is also exploring the engineering of an artificial microorganism which would be an ideal synthetic energy producer. This microorganism would be designed to produce excess hydrogen by depriving it of the genes required for sugar formation since they normally use hydrogen ions. Hence, the microorganism would devote all its energy toward excess hydrogen production.

Bioremediation

Bioremediation can be defined as any process that uses bacteria, fungi, green plants or their enzymes to return the environment altered by contaminants to its original condition. Bacteria that can degrade some soil contaminants, such as chlorinated hydrocarbons, are used in bioremediation. They are even employed in cleaning up oil spills, which is done by addition of nitrate and/or sulfate fertilizers, which helps in the decomposition of crude oil by bacteria. Genetic engineering facilitates creation of organisms specifically designed for bioremediation. The bacterium *Deinococcus radiodurans* (the most radioresistant organism known) has been engineered to digest toluene and ionic mercury from highly radioactive nuclear waste. Certain bacteria (cyanobacteria, pseudomonads, corynebacteria, and mycobacteria), green algae, and fungi (several molds and yeasts), can remove nearly 80% of hydrocarbons from oil spills within a short span of one year by oxidizing hydrocarbons at aerobic water/oil interfaces. Isolating or developing microbial strains capable of degrading and detoxifying hydrocarbon and halogenated hydrocarbon waste of industrial, agricultural, or military origin is essential in waste management. Understanding the role of microorganisms in the degradation and transformation of chemicals in the environment is a key factor in assessing the environmental safety of new and existing chemicals. Engineering microorganisms may solve contamination and recycling process problems. Another interesting role bacteria play is in recovering oils, minerals, and metals from their sources. Oil recovery may be facilitated by the development of unique bacteria which produce a surfactant that forces trapped oil out of rocks. Extraction of minerals from low-grade ores is enhanced by some bacteria (microbial leaching). In addition, selective binding of metals by biohydrometallurgical processes is important in recycling of metals such as silver and uranium.

Agricultural Biotechnology

Agricultural biotechnology, also referred to as green biotechnology, is biotechnology applied to agricultural processes. Since the advent of agriculture 10,000 years ago, farmers have used biological technology inadvertently in the refinement of methods of farming and crops through cross breeding, and various hybridization techniques. Later, the genetics of plants were modified by sophisticated plant breeding programs which resulted in the "Green Revolution." Thus, farmers were enabled to grow high-yield crops in order to satisfy the increasing food requirements of a growing population, and prevented starvation in many parts of the world. Today, using genetic engineering, scientists are able to produce plants with enhanced nutritional content, desired texture, color, flavor, growing season, yield, impart disease resistance, and improve other properties of production crops. Current agricultural research is focused on producing genetically engineered plants (transgenic plants). These are plants which contain one or more genes that have been artificially inserted instead of acquiring them through the natural process of pollination. Modern genetics assists in improving the growth, health, vigor, and other qualities of agriculturally important mammals, poultry and fish. With the advances in molecular biology, major areas of interest in plant biotechnology are plant tissue culture, plant genetic engineering, and plant molecular marker-assisted breeding. Conventional and rDNA technology help in improving microbial inoculants to be used to control plant pests, as fertilizer supplements, and to aid in atmospheric nitrogen fixation. Transgenic Bt corn is an outstanding example of successful application of genetic engineering in agriculture. It produces its own insecticide and contains a gene from the bacterium *Bacillus thuringiensis*. The use of Bt varieties has dramatically reduced the amount of chemical pesticides applied to cotton. Plants that have one or more foreign genes inserted, instead of their acquiring them naturally through pollination, are called genetically modified or GM crops. These crops are superior in terms of yields, herbicide tolerance, and pest-resistance, and have dominated agricultural biotechnology in the last decade. The most important transgenic crop planted is soybean, followed by corn, cotton, and canola. One of the biggest adversities farmers encounter in crop production is weed control, since there is a sharp reduction in crop yield and quality with poorly controlled weeds. Many GM crops are inserted with herbicide tolerance gene so that a broad-spectrum herbicide sprayed to the fields would kill all crops except the GM crops. Nutritionally-enhanced transgenic crops are produced using genetic engineering techniques. A striking example of this is Golden Rice, which is produced by inserting two genes from daffodil and one gene from a bacterium into rice plants so that the rice becomes capable of synthesizing beta-carotene, the precursor of vitamin A. This rice is produced with the aim of benefiting millions of people suffering from vitamin A deficiency, especially those living in developing countries.

Transgenic plants which are currently being developed are tomatoes, rice, canola grapes, tobacco, tea, coffee, and some trees. Transgenic tomato varieties with enhanced lycopene content and delayed ripening, to develop improved nutrition and flavor, are being developed. Transgenic canola with enhanced vitamin E levels is a subject being studied. Food crops engineered to produce edible vaccines against infectious diseases would make vaccination more child-friendly around the world. Bananas have received considerable research attention of late as a vehicle for vaccine delivery, because of their palatability and adaptation to tropical and

sub-tropical environments. Transgenic bananas are produced and are being evaluated for use as vehicles for cholera, hepatitis B and diarrhea vaccines. Some other GM crops being studied are decaffeinated tea and coffee and nicotine-free tobacco. To develop modified trees with reduced lignin content for the paper industry and the bioethanol industry is also an intriguing area of research in agricultural biotechnology.

Traditional biotechnological processes have applications in almost all spheres of life, ranging from pharmaceuticals to food production, industrial processes, waste and wastewater treatment, bioremediation, bio-leaching, biofuels, and biopolymers. These applications will undoubtedly continue, but recombinant DNA technology is expected to expand horizons of biotechnological research and industry and, at the same time, give insights for a more specific and controlled use of microbes and microbial products. Used efficiently, after appropriate risk assessment and with effective and enforced regulation, biotechnology has enormous potential to improve the quality of life and to enhance our capacity to conserve and protect the environment.

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Industrial Production of Glycosaminoglycans

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Glossary

cGMP cGMP refers to "current good manufacturing practice", a set of regulatory principles designed for managing pharmaceutical manufacturing processes and facilities.

Fed-batch fermentation Fed-batch fermentation is used for high cell-density cultivation of recombinant cells. The fermentation begins with seeding cells in a fermentation medium in a bioreactor. The fermentation medium could be complex, enriched media (e.g., terrific broth) or defined media (e.g., phosphate-citrate broth, supplemented with chemicals such as carbon source and trace metals). Throughout the culture, the cells are fed with various nutrients, including a carbon source and nitrogen sources (such as amino acids). Usually, the feeds are concentrated so that the fermentation volume does not increase excessively.

Introduction

Glycosaminoglycans (GAGs) are naturally occurring polysaccharides, composed of alternating sugar units; these include an amino sugar (e.g., *N*-acetyl glucosamine, GlcNAc or *N*-acetyl galactosamine, GalNAc) and either a galactose (Gal) or an uronic acid (GlcA or IdoA) (Fig. 1). The carbon backbone of the GAG chain may undergo no further modifications (e.g., hyaluronan) or may be further modified through sulfation, de-acetylation, and/or epimerization (e.g., heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate). Simple non-sulfated GAGs (e.g., hyaluronan) and the precursors of highly sulfated GAGs (e.g., heparosan and chondroitin) are produced in certain bacteria as well as in animals (Cress et al., 2014) These simple GAGs are thought to contribute to pathogenicity of the host bacteria. Simple, non-sulfated GAGs as well as the highly modified sulfated GAGs (e.g., heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate) are produced in all animals, including humans. In humans, hyaluronan is part of the connective tissue extracellular matrix (ECM) and is involved in biological functions such as lubrication of joints. Highly sulfated GAGs have tissue-specific sulfated domains that are binding sites for various proteins (Capila and Linhardt, 2002) and, therefore, play critical roles in biological functions such as homeostasis, cell growth, cell migration, development, morphogenesis, tissue repair, and angiogenesis (Linhardt and Toida, 2004; Linhardt, 2003).

The current industrial production of GAGs can be broadly classified into three categories: (1) industrial production from animal source, (2) industrial production using microbial cells, and (3) industrial production using eukaryotes. Various GAGs have been routinely extracted and purified from animal sources, for example, hyaluronan from rooster combs, and heparin from pig intestines or bovine lung. An increased knowledge of GAG biosynthesis, as well as the advances in metabolic engineering strategies, bioprocess optimization, downstream processing approaches and analytical tools have led to the production of GAGs from microbial cells and exploration of eukaryotic cells, for example, CHO cells, toward heparin production.

Glycosaminoglycans

The glycosaminoglycan family includes: (1) hyaluronan (hyaluronate, hyaluronic acid); (2) heparin/heparan sulfate; (3) chondroitin/dermatan sulfate; and (4) keratan sulfate. Each GAG family has a common carbon backbone that can be variously modified (Table 1, Fig. 2) and may also have a variety of different molecular weight properties. The structural characterization of GAGs has been possible due to advances in biochemical and analytical tools, such as gel permeation chromatography, high-performance liquid chromatography (HPLC), ion-exchange chromatography, capillary electrophoresis, mass spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy (Sun et al., 2016; Schlessinger et al., 2000; Xiao et al., 2010; Cai et al., 2013, 2012; Laremore et al., 2007; Fu et al., 2016; Zhang et al., 2015; Volpi et al., 2009; Moon et al., 2012; Edens et al., 1992; Desai et al., 1993; Yang et al., 2011; Kailemia et al., 2013; Li et al., 2014; Thanawiroon and Linhardt, 2003; Liu et al., 2014; Chen et al., 2017; Volpi et al., 2012; Thanawiroon et al., 2004; Zhao et al., 2013; Lohse and Linhardt, 1992; Leach et al., 2012; Guerrini et al., 2009; Wang et al., 1991; Mikhailov et al., 1997; Li et al., 2012). These analytical tools have aided GAG structural analysis and improved insight into the GAG structure-function relationships. In parallel, metabolic engineering tools, knock-in/out animal models and mammalian cell culture models, have also contributed to deciphering the GAG biosynthetic pathway and GAG functions (Lin et al., 2000; Sarrazin et al., 2011; Pönighaus et al., 2007; Xu and Esko, 2014; Esko and Selleck, 2002; Thacker et al., 2014; Bame and Esko, 1989; Bai and Esko,

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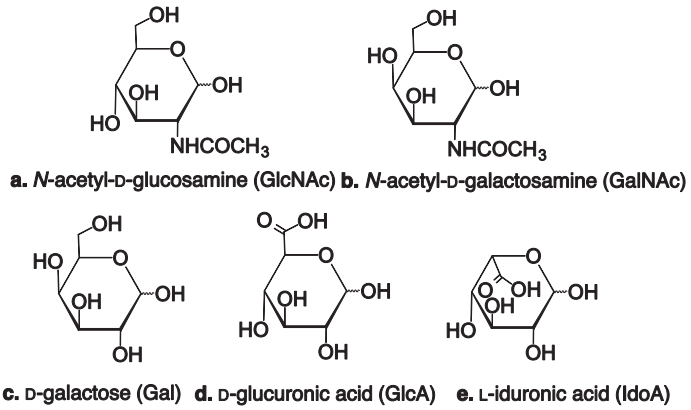

Fig. 1 Building blocks of glycosaminoglycans (a) *N*-acetyl glucosamine, (b) *N*-acetyl D-galactosamine, (c) D-galactose, (d) D-glucuronic acid, and (e) L-iduronic acid. These sugar moieties may be further modified through the action of specific enzymes, such as *N*-deacetylases, sulfotransferases, and epimerases.

Table 1 Glycosaminoglycans

Type	*Composition*	*Chemical Formula*
Hyaluronan	Linear polymer of alternating D-glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc).	→4)β-D-GlcA (1→3) β-D-GlcNAc(1→, with no sulfation
Family of heparin, heparan sulfate and heparosan	Linear polymer of alternating D-glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc). Heparosan is modified through the actions of tissue specific sulfotransferases, epimerases to form heparan sulfate and heparin.	→4) β-D-GlcA or α-L IdoA(1→4) β-D-GlcNAc/S(1→, with sulfation
Family of chondroitin sulfate and dermatan sulfate	Linear polymer of alternating D-glucuronic acid (GlcA) and N-acetylglucosamine (GalNAc). Chondroitin modified through the actions of tissue specific sulfotransferases to form chondroitin sulfate; dermatan sulfate is sulfated as well as the GlcUAGlcA moiety of dermatan sulfate is epimerized into iduronic acid.	→4) β-D-GlcA or α-L-IdoA(1→3) β-D-GalNAc(1→, with sulfation
Family of keratan sulfate	Linear polymer of alternating galactose (Gal) and N-acetylglucosamine (GalNAc), with sulfation, for example, sulfation at the carbon 6 of galactose and N-acetylglucosamine.	→3) β-D-Gal (1→4) β-D-GalNAc(1→, with sulfation

1996; Aikawa et al., 2001; Fuster et al., 2007; Grobe et al., 2002; Bame et al., 1991). For example, metabolic engineering of suspension CHO cells through the up-regulation of exogenous Golgi-targeted 3OST1 revealed formation of distinct antithrombin binding sites; antithrombin binding sites directly correlate to anticoagulant activity of CHO derived engineered HS (Datta et al., 2013). Metabolic engineering tools together with analytical tools synergistically contribute to GAG research.

Hyaluronic Acid

Hyaluronan (hyaluronic acid; hyaluronate) is a naturally occurring non-sulfated glycosaminoglycan (GAG), composed entirely of alternating GlcA and GlcNAc moieties (Table 1, Fig. 2) (Vigetti et al., 2014). Hyaluronan synthase (HAS) polymerizes linear HA polysaccharide from UDP-sugars by adding alternating GlcNAc and GlcA moieties to the growing HA carbon chain (Vigetti et al., 2014; Bodevin-Authelet et al., 2005; Fraser et al., 1997; O'Regan et al., 1994; Itano and Kimata, 2002; Itano et al., 1999). HAS enzymes occur in certain bacteria as well as mammals. *Streptococcus sp.*, such as *S. zooepidemicus* express bacterial HAS and biosynthesize hyaluronan (O'Regan et al., 1994; Hascall et al., 2016; DeAngelis, 2015) which aids in bacterial pathogenesis.

Cellular biosynthesis of hyaluronan in mammalian cells occurs through the action of a family of hyaluronan synthases (HAS1-3), which are found in the plasma membrane (Itano and Kimata, 2002). In humans, hyaluronan is synthesized primarily in mesenchymal cells (e.g., fibroblasts, endothelial cells); however, it is found on the cell surfaces in many tissues. In humans, hyaluronan has several important roles (Hascall et al., 2016). It forms a vital constituent of the extracellular matrix and connective tissue systems, including, skin, synovial fluid in the joints, vitreous humor of the eye, heart valve, lungs, and umbilical cord (Vigetti et al., 2014; Bodevin-Authelet et al., 2005; Fraser et al., 1997; Itano and Kimata, 2002; Hascall et al., 2016; Robert et al., 2010; Inatani and Tanihara, 2002; Carrino et al., 2000; Oksala et al., 1995; Temple-Wong et al., 2016). Due to its physicochemical properties, hyaluronan can provide a framework for cell migration and is capable of retaining moisture (Papakonstantinou et al., 2012). Aging skin shows reduced expression of GAGs including hyaluronan and reduced moisture retention (Papakonstantinou et al., 2012). Reduced hyaluronan expression and activity in part correlates with decreased HAS1, increased hyaluronan lyases (HYAL1-3), and decreased hyaluronan receptors (CD44 and RHAMM).

A number of medical and personal care applications for exogenous hyaluronan have been developed in recent years with research ongoing, many to address physiological and pathological changes that occur with aging. Hyaluronan has been used in skin-cosmetics (Carrino et al., 2000; Naylor et al., 2011; Tzellos et al., 2009; Anderegg et al., 2014; Oh et al., 2011). Due to its

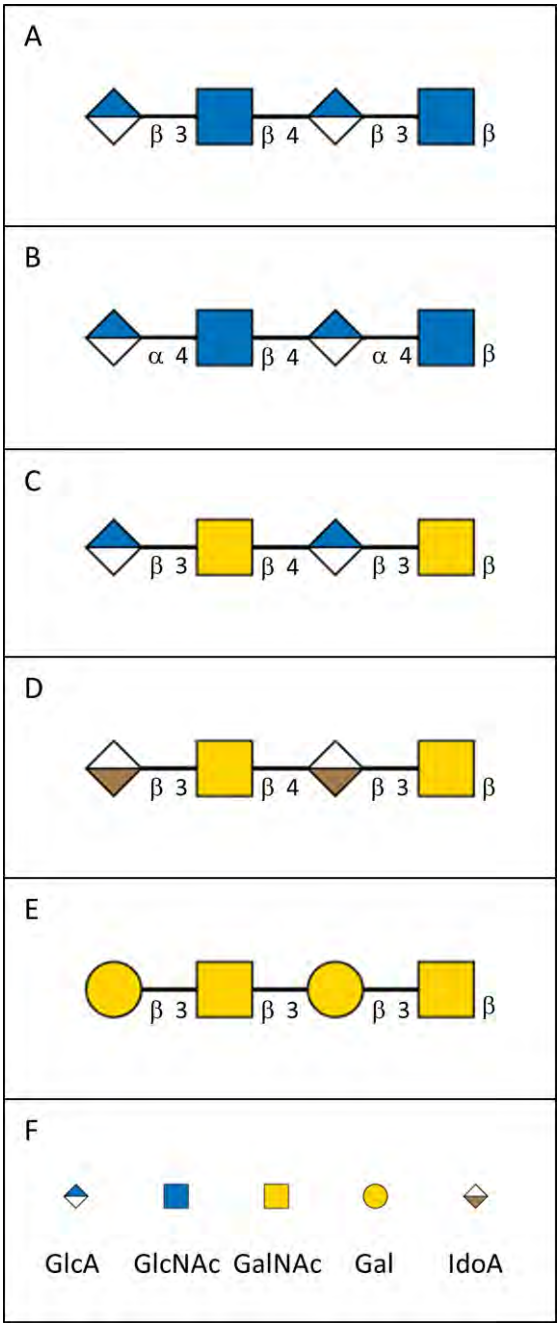

Fig. 2 Sugar moiety backbone of glycosaminoglycans (A) hyaluronan, (B) family of heparin, heparan sulfate and heparosan, (C) family of chondroitin and chondroitin sulfate, (D) dermatan sulfate, (E) family of keratan sulfate, and (F) sugar moieties, namely, glucuronic acid (GlcA), N-acetylglucosamine (GlcNAc), N-acetylglucosamine (GalNAc), galactose (Gal), and Iduronic acid (IdoA). Hyaluronan (A), heparosan (B), and chondroitin (C) are unmodified linear sugar moiety backbone chains. Keratan sulfates (E) have O-sulfated galactose and N-acetylglucosamine moieties. The sugar moieties of heparosan (GlcA and GlcNAc) may be further modified through actions of tissue-specific N-sulfotransferases, C5 epimerases and O-sulfotransferases, to form sulfated heparin and heparan sulfate. The sugar moieties of chondroitin (GlcA and GalNAc) may be further modified through actions of tissue-specific O-sulfotransferases, to form sulfated chondroitin sulfate. The sugar moieties of chondroitin (GlcA and GalNAc) may be further modified through actions of tissue-specific epimerases (that transform GlcA to IdoA), and O-sulfotransferases, to form sulfated dermatan sulfate. Figures were drawn from Cheng, K., Zhou, Y., Neelamegham, S., 2017. DrawGlycan-SNFG: A robust tool to render glycans and glycopeptides with fragmentation information. Glycobiology 27 (3), 200–205.

mucoadhesive behavior and contribution to the elastoviscous behavior of soft connective tissues, for example, in synovial fluid in the joints and vitreous humor of the eye (Robert et al., 2010; Nishida et al., 1991; Yokoi et al., 1997), hyaluronan has been used in eye drops to lengthen the pre-corneal retention time (Salzillo et al., 2016). The synovial fluid of the joints is majorly composed of hyaluronan and functions in lubrication and knee movement. During osteoporosis, cartilage degradation in joint and reduced synovial fluid results in pain and reduced movement. High molecular weight (1.0–2.9 million Dalton) ultra-pure hyaluronan has

been utilized to treat pain in osteoarthritis of the knee. Other applications of hyaluronan include wound healing (Oksala et al., 1995), a potential marker for tumors or cancers (Stern, 2009), a drug delivery vehicle (Brown and Jones, 2005), and a biomaterial scaffold for tissue engineering (Dicker et al., 2014; Shu et al., 2004). Currently, hyaluronan is used in a variety of pharmaceutical products including Euflexxa, Healon, Synvisc, Orthovisc, Seprafilm, and Restylane (Hascall et al., 2016; DeAngelis, 2015; Schiraldi et al., 2010; Boeriu et al., 2013; Cimini et al., 2017).

Heparin and Heparan Sulfate

Heparin and heparan sulfate are naturally occurring, sulfated GAGs composed of alternating GlcA and GlcNAc moieties (Table 1, Fig. 2). During their biosynthesis, these GAGs are modified through the action of specific enzymes, *N*-deacetylases, sulfotransferases, and epimerases (Carlsson and Kjellén, 2012; Garg et al., 2005). Heparan sulfate is present on cell surfaces and binds to various protein ligands, resulting in the regulation of a variety of biological processes such as cell growth and angiogenesis. Heparin is predominantly present in mast cells and is commercially used as a biopharmaceutical anticoagulant drug (Carlsson and Kjellén, 2012; Garg et al., 2005).

In humans, heparan sulfate/heparin are biosynthesized as proteoglycans, attached to specific core proteins, such as syndecan, glypican, and serglycin. Heparan sulfate/heparin share a similar biosynthetic pathway. Biosynthesis begins with the formation of a tetrasaccharide linker -xylose-galactose-galactose-GlcA on serine residues of the core protein. Next, α-*N*-acetyl glucosaminyl transferase adds α-GlcNAc to the non-reducing terminal GlcA of this tetrasaccharide linker. The family of EXT enzymes (Exostoses, EXT1-2) sequentially add GlcNAc and GlcA, forming a linear non-sulfated heparosan polysaccharide (Lin et al., 2000; Busse et al., 2007; Wuyts et al., 1998; Okada et al., 2010). The heparosan chain is modified through the action of various sulfotransferases and epimerases to produce sulfated heparan sulfate and highly sulfated heparin. Briefly, the GlcNAc residues are N-deacetylated and N-sulfated by a family of *N*-deacetylase/*N*-sulfotransferase (NDST) enzymes to form GlcNS (Saribaş et al., 2004). The GlcA residues may be epimerized into IdoA by C5-epimerase (GLCE) enzymes (Feyerabend et al., 2006) and the uronic acid residue O-sulfated by 2-*O*- sulfotransferase; the GlcNS moieties may be O-sulfated by families of 6-*O*-, and 3-*O*-sulfotransferases (Moon et al., 2012; Carlsson and Kjellén, 2012; Garg et al., 2005; Anower-E-Khuda et al., 2013; Sasisekharan and Venkataraman, 2000). There are tissue-specific isoforms of the NDSTs and *O*-sulfotransferases, and these generate tissue-specific sulfated domains. The 3-*O*-sulfotransferse isoform-1 (3OST-1), for example, is critical for the production of anticoagulant heparin and is present in mast cells, which chiefly biosynthesize anticoagulant heparin (Forsberg et al., 1999). Heparan sulfate is characterized by partially sulfated domains, and these domains provide the specific ligand binding characteristic to heparan sulfate, for example, cell growth, cell development, angiogenesis, and metastasis (Sarrazin et al., 2011). Heparin is characterized by completely, or nearly completely sulfated domains, and has specific antithrombin III (ATIII)-binding sites. The ATIII-binding site of the heparin interacts with antithrombin (AT; a serine protease inhibitor). This interaction leads to a conformational change in the AT and amplifies inhibition of thrombin (factor IIa) and factor Xa via the action of AT. This results in anticoagulation and use of heparin as an anticoagulant drug (Onishi et al., 2015).

Chondroitin Sulfate and Dermatan Sulfate

Chondroitin/dermatan sulfate belong to a family of naturally occurring linear, sulfated GAGs made of repeating sugar moieties, namely GlcA and GalNAc residues (Tables 1 and 2, Fig. 2). Chondroitin sulfate/dermatan sulfate in higher animals are highly sulfated, and the sulfated domains act as ligands for proteins and mediate biological functions.

In humans, chondroitin sulfate-type A (e.g., CSA) is predominant in connective tissues and ECM, for example, cartilage, cornea, bone, skin, arterial walls. Chondroitin sulfate provides a framework, and promotes elasticity in cartilage. These properties have made chondroitin sulfate-type A a popular dietary supplement. Dermatan sulfate is predominant in skin, heart valve, tendons, and

Table 2 Disaccharide units of chondroitin sulfate and dermatan sulfate

CS/DS	*Major disaccharide units and their sulfation pattern*	*Chemical formula*
Chondroitin (CS-DS)	Unsulfated glucuronic acid and unsulfated N-acetylgalactosamine	→4) β-D-GlcA(1→3)- β-D-GalNAc(1→
Chondroitin sulfate A (CS-A); chondroitin−4-sulfate	Unsulfated glucuronic acid and N-acetylgalactosamine sulfated at carbon 4	→4) β-D-GlcA(1→3)- β-D-GalNAc4S(1→
Dermatan sulfate (CS-B)	L-iduronic acid and N-acetylgalactosamine sulfated at carbon 4	→4) α-L-IdoA(1→3)- β-D-GalNAc4S(1→
Chondroitin sulfate C (CS-C); chondroitin−6-sulfate	Unsulfated glucuronic acid and N-acetylgalactosamine sulfated at carbon 6	→4) β-D-GlcA(1→3)- β-D-GalNAc6S(1→
Chondroitin sulfate D (CS-D); chondroitin−2,6-sulfate	Glucuronic acid sulfated at carbon 2 and N-acetylgalactosamine sulfated at carbon 6	→4) β-D-GlcA2S(1→3)- β-D-GalNAc6S(1→
Chondroitin sulfate E (CS-E); chondroitin−4,6-sulfate	Unsulfated glucuronic acid and N-acetyl galactosamine sulfated at carbon 4 and 6	→4) β-D-GlcA(1→3)- β-D-GalNAc4S6S(1→

blood vessels. Chondroitin sulfate/dermatan sulfate also play regulatory roles in cell proliferation, cell development, cell adhesion, homeostasis, cardiovascular disease, tumorigenesis, infection, wound repair and fibrosis (Hardingham, 1998; Maeda et al., 2011; Linhardt and Hileman, 1995; Sugahara and Mikami, 2007; Uebelhart, 2008; Vallières and du Souich, 2010). In addition, dermatan sulfate may form complexes with heparin cofactor II, and may act as an anticoagulant. Chondroitin sulfate proteoglycans are potential targets for malaria vaccines (Dinglasan et al., 2007) as *Plasmodium falciparum* (the dominant African malaria parasite)-infected erythrocytes express proteins, such as variant surface antigen 2-CSA that bind to chondroitin sulfate A and mediate malarial pathogenesis. Commercial applications for both chondroitin sulfate/dermatan sulfate have been growing, such as the use of chondroitin sulfate in bone repair and dermatan sulfate in wound repair. Other applications of chondroitin sulfate, with other biopolymers such as hyaluronan and collagen, include designing slow and controlled biodegradable scaffolds for wound healing.

In humans, chondroitin sulfate also exists as proteoglycans, synthesized and attached to specific core proteins (Mikami and Kitagawa, 2013). Like heparan sulfate/heparin, the biosynthesis of chondroitin sulfate begins with the formation of the tetrasaccharide linker xylose-galactose-galactose-GlcA on serine residues of the core protein. Next, a β1–4 linked GalNAc is added to the non-reducing GlcA. Chondroitin synthase sequentially adds GalNAc and GlcA, forming a linear non-sulfated chondroitin. The polysaccharide-backbone is further modified through the action of various sulfotransferases to produce sulfated CS. Epimerization of GlcA into IdoA and subsequent sulfation of sugar moieties results in dermatan sulfate (Table 2).

Keratan Sulfate

Keratan sulfates are naturally occurring linear sulfated GAGs made of repeating Gal and GlcNAc residues (Table 1, Fig. 2) (Pomin, 2015). The polysaccharide chain can be further modified through the action of 6-*O*-sulfotransferases (GlcNAc6ST) that act on both the GlcNAc and Gal moieties (Uchimura, 2015). Keratan sulfate is an important ECM component and has been found in cornea, cartilage, and brain (Funderburgh, 2000).

Industrial Production of Glycosaminoglycans: Current State and Future Directions

Industrial scale production of commercially relevant GAGs can be broadly grouped as (1) production from animal sources, (2) production from cultured microbial cells, and (3) production from cultured eukaryotic cells (Schiraldi et al., 2010; Boeriu et al., 2013; Cimini et al., 2017).

Industrial Production of GAGs From Animal Sources

Industrial production of GAGs from animal sources is performed for industrial production of hyaluronan, heparin, and chondroitin sulfate. GAGs are abundant in animal tissue. Specific animal tissues have been used for GAG extraction, for example, (1) hyaluronan from rooster combs and bovine/porcine eyes, (2) chondroitin from animal cartilage, (3) dermatan sulfate from mucosal tissue and animal hide/skin, and (4) heparin from porcine/bovine mast cells that are found in intestine and lung tissues. The extraction of GAGs is performed in non-cGMP slaughter houses (Boeriu et al., 2013). The extracted GAGs are then purified in cGMP facilities. However, concerns such as the presence of undesirable animal products and contamination risks (viruses, prions) have motivated development of methods for industrial production of GAGs through fermentation or cell culture (Fu et al., 2016). In addition, the ability to create tailored GAGs with specific or novel functionality creates another impetus for controlled production (Oduah et al., 2016).

Industrial Production of GAGs From Microbial Sources

Certain bacteria have the necessary enzyme machinery for producing simple, non-sulfated GAGs, such as hyaluronan, heparosan, and chondroitin (Fig. 3). Industrial production of GAGs from microbial sources is performed for production of hyaluronan from *Streptococcus sp.* and chondroitin sulfate from *E. coli* K4.

Hyaluronan is a simple non-sulfated GAG. Bacteria such as the gram-positive *Streptococcus sp*. and gram-negative bacteria *Pasteurella multocida*, can naturally produce hyaluronan (Schiraldi et al., 2010; Cimini et al., 2017). The hyaluronan exists in the mucoid capsule of these bacteria and aids in the pathogenicity. In *S. zooepidemicus*, genes required for the hyaluronan biosynthesis are encoded in the HAS operon (*hasA*, *hasB*, *hasC*, *hasD*, and *hasE*) (Table 3). In the presence of magnesium ions (e.g., $MgCl_2$), bacterial HAS enzymes polymerize the hyaluronan chain using UDP-GlcNAc and UDP-GlcA as substrates. Under certain conditions, the *S. zooepidemicus* may produce 6–7 g/L of hyaluronan. The hyaluronan from the fermentation broth is recovered using ultrafiltration, and precipitation with chemicals such as cetylpyridinium chloride. The final product is sterilized and tested for activity and endotoxin levels. Recent advances in exploring alternative bioengineered microbial hosts toward hyaluronan production include *E. coli*, *Bacillus* sp., *Agrobacterium* sp., and *Lactobacillus lactis*. *Escherichia coli* JM109 co-expressing two genes, pmHas gene from *P. multocida* and kfiD gene from *E. coli* K5 strain, was able to produce 0.5 g/L hyaluronan in shake-flask culture. The fed-batch fermentation and optimization of fermentation parameters, such as the addition of glucosamine supplements, led to the production of 2.0–3.8 g/L hyaluronan in fed-batch fermentation using the bioengineered *E. coli* JM109 strain. L. *lactis*, co-expressing genes *hasA*, *hasB*, and *hasC* from *S. zooepidemicus*, was able to produce 1.8 g/L hyaluronan in a batch bioreactor. *Agrobacterium* sp. ATCC

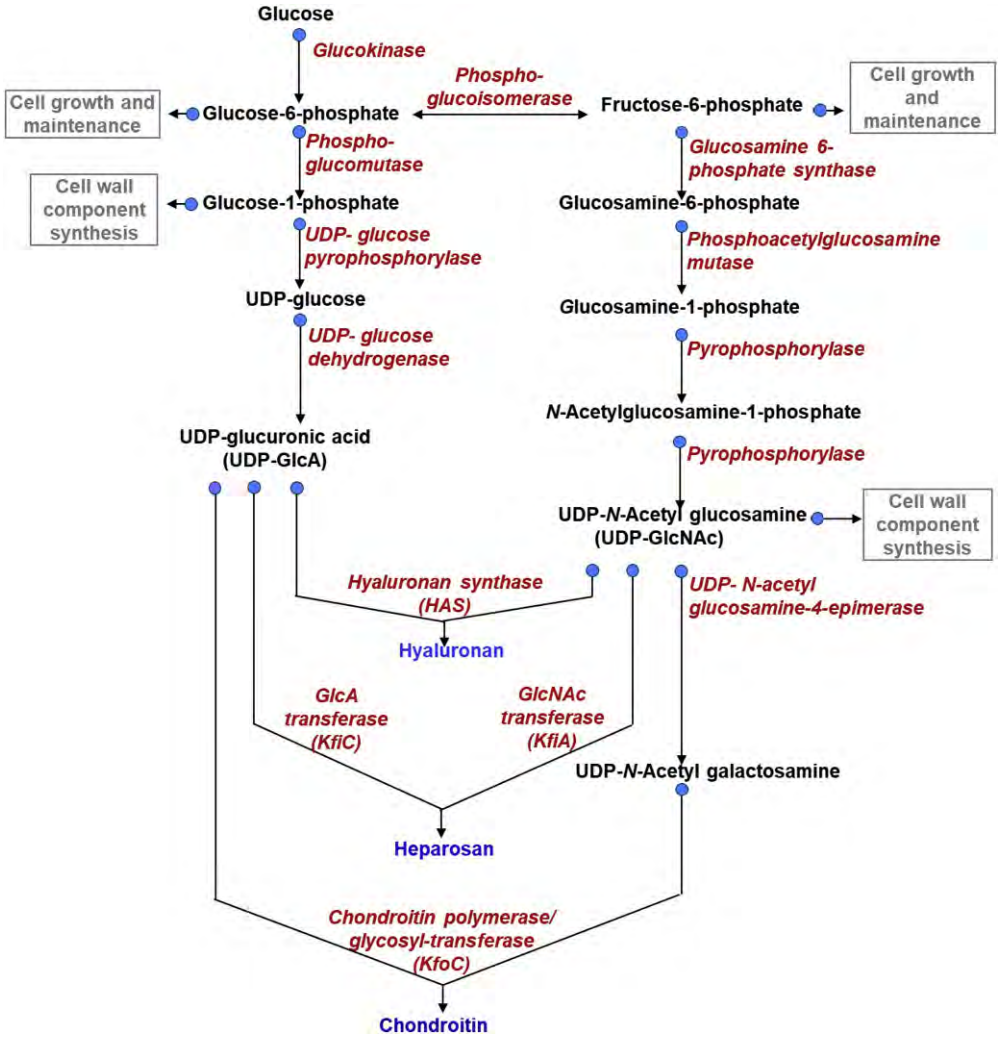

Fig. 3 Biosynthetic pathways for hyaluronan, heparosan and chondroitin in microbial species Reproduced from Schiraldi, C., Gatta, A. La, Rosa, M. De, 2010. Biotechnological production and application of hyaluronan. Biopolymers. 387–412; Cimini, D., Iacono, I. Dello, Carlino, E., *et al*., 2017. Engineering S. equi subsp. zooepidemicus towards concurrent production of hyaluronic acid and chondroitin biopolymersof biomedical interest. AMB Express 7 (1), 61; Cress, B.F., Greene, Z.R., Linhardt, R.J., Koffas, M.A.G., 2013a. Draft genome sequence of Escherichia coli strain ATCC 23502, serovar O5:K4:H4. GenomeAnnouncements 1 (2); Cress, B.F., Greene, Z.R., Linhardt, R.J., Koffas, M.A.G., 2013b. Draft genome sequence of Escherichia coli strain ATCC 23506 (Serovar O10:K5:H4). GenomeAnnouncements 1 (2), 1–2; He, W., Fu, L., Li, G., *et al*., 2015. Production of chondroitin in metabolically engineered *E. coli.* Metabolic Engineering 27, 92–100; Wang, Z., Ly, M., Zhang, F., *et al*., 2010. *E. coli* K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. Biotechnology and Bioengineering 107 (6), 964–973; Wang, Z., Dordick, J.S., Linhardt, R.J., 2011. *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. Bioengineered Bugs 2 (1), 63–67; Chavaroche, A.A.E., Van Den Broek, L.A.M., Boeriu, C., Eggink, G., 2012. Synthesis of heparosan oligosaccharides by *Pasteurella multocida* PmHS2 single-action transferases. Applied Microbiology and Biotechnology 95 (5), 1199–1210; Zhang, F., Lee, K.B., Linhardt, R.J., 2015. SPR biosensor probing the interactions between TIMP-3 and heparin/GAGs. Biosensors 5 (3), 500–512; Xu, P., Vansiri, A., Bhan, N., Koffas, M.A.G., 2012. EPathBrick: A synthetic biology platform for engineering metabolic pathways in *E. coli.* ACS Synthetic Biology 1 (7), 256–266; Zhao, S., Jones, J.A., Lachance, D.M., *et al*., 2015. Improvement of catechin production in Escherichia coli through combinatorial metabolic engineering. Metabolic Engineering. 28, 43–53; Xu, P., Koffas, M.A.G., 2013. Assembly of multi-gene pathways and combinatorial pathway libraries through ePathBrick vectors. Methods in Molecular Biology 1073,107–129.

Table 3 Hyaluronan biosynthesis genes in bacteria *Streptococcus zooepidemicus*

Hyaluronan genes	*Gene (product) and function*
hasC	*hasC* (gtaB, UDP-glucose pyrophosphorylase) is required for conversion of glucose−1-phosphate to UDP-glucose
hasB	*hasB* (tuaD, UDP-glucose dehydrogenase) is required for conversion of UDP-glucose to UDP-glucuronic acid
hasE	*hasE* (pgl, phosphoglucoisomerase) is required for reversible conversion of glucose−6-phosphate to fructose−6-phosphate
hasD	*hasD* (gcaD, Acetyl transferase/UDP-GlcNAc pyrophosphorylase) is required for conversion of GlcN−1-Pto UDP-GlcNAc
hasA	*hasA* (hyaluronan synthase) is required for polymerization of UDP-GlcA and UDP-GlcNAc

31749, co-expressing *pmHAS* gene from *P. multocida* and the kfiD gene (encodes UDP-glucose dehydrogenase) from *E. coli* K5 strain, was able to produce 0.3 g/L hyaluronan in shake-flask cultures.

Industrial production of sulfated GAGs, such as chondroitin sulfate and heparin would require production of chondroitin or heparosan, respectively, in bacteria, followed by purification and subsequent chemoenzymatic synthesis of chondroitin sulfate and heparin. Certain bacteria, for example, *E. coli* K4 and *E. coli* K5, are able to synthesize chondroitin and heparosan, respectively (Cress et al., 2013a,b). Bacterial chondroitin and heparosan are part of their capsular polysaccharides.

Recently, industrial production of food grade chondroitin sulfate from microbial fermentation of *E. coli* K4 has been developed (see Relevant Websites section). Briefly, *E. coli* K4 fermentation and subsequent purification produced crude chondroitin sodium. Regioselective sulfation of the chondroitin sodium, followed by purification produced chondroitin sulfate sodium (Mythochondro™). In a parallel experiment, metabolic engineering of *E. coli* BL21 StarTM (DE3) strain with ePathBrick vector, encoding K4 *CS-O* genes, namely, *KfoF, KfoA* and *KfoC*, resulted in 2.4 g/L in a fed batch fermentation (He et al., 2015).

Production of pharmaceutical heparin is estimated at almost 100 metric tons annually, with an annual sale of over $3 billion (Fu et al., 2016; Onishi et al., 2015). Currently, pharmaceutical heparin is extracted and purified from pig or cow mucosal tissues. Animal-sourced heparin production begins in non-cGMP animal slaughterhouses, followed by extraction and purification in cGMP facilities. However, concerns such as extraction of heparin in non-cGMP slaughterhouses, contamination risks (virus, prions), and the possibility of adulteration in sites not under USFDA or EMEA supervision, have necessitated alternate routes for heparin production (Fu et al., 2016). Understanding the heparin biosynthetic pathway and the key enzymes required for biosynthesis of heparin has provided strategies for industrial production of heparin from non-animal sources, such as chemoenzymatic synthesis of heparin from microbial heparosan and metabolic engineering of mammalian cells (Cai et al., 2013; Fu et al., 2016; Bhaskar et al., 2015, 2012; Masuko and Linhardt, 2012; Higashi et al., 2011; Wang et al., 2010, 2011; Jin et al., 2016). Knock-in and knock-out animal- and cell-culture studies demonstrated that HS/HP enzymes, NDST2, 2OST, C5epi (GLCE), 6OSTs and 3OST1 are important for the production of antithrombin binding sites on heparin (Fig. 4) (Lin et al., 2000; Pönighaus et al., 2007; Esko and Selleck, 2002; Bame and Esko, 1989; Bai and Esko, 1996; Aikawa et al., 2001; Bame et al., 1991; Gasimli et al., 2014). The chemoenzymatic synthesis of heparin uses microbial systems for the production of heparin precursors (i.e., heparosan) followed by chemoenzymatic modification using chemicals and/or the critical enzymes NDST2, 2OST, GLCE, 6OSTs, and 3OST1. For example, bacteria such as *Pasteurella multocida* type D strain and *E. coli* K5 naturally produce capsular heparosan (Wang et al., 2010, 2011; Chavaroche et al., 2012). Production of heparosan in *P. multocida* type D strain involves sequential addition of GlcNAc and GlcA, through the actions of glucosyltransferases (PmHS1) (Chavaroche et al., 2012). Production of heparosan in *E. coli* K5 involves sequential addition of GlcNAc and GlcA, through the actions of glucosyltransferases (KfiA and KfiC) (Wang et al., 2010, 2011). Fermentation process optimization in *E. coli* K5 in a fed-batch fermentation in a glucose-based defined medium at 37°C, with 30% dissolved oxygen, resulted in 15 g/L crude heparosan (Wang et al., 2010, 2011). Recently, researchers have metabolically engineered an *E. coli* BL21 strain with *E. coli* K5 glucosyltransferases and demonstrated heparosan production in the engineered BL21 strain

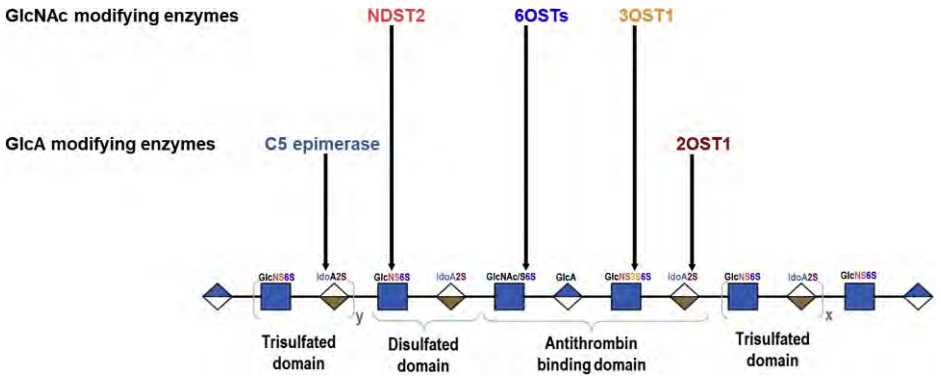

Fig. 4 HS/HP enzymes, NDST2, C5epi, 2OST1, 6OSTs, 3OST1 are important for biosynthesis of antithrombin site on anticoagulant heparin. Lin, X., Wei, G., Shi, Z., *et al.*, 2000. Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice. Developmental Biology 224 (2), 299–311; Pönighaus, C., Ambrosius, M., Casanova, J.C., *et al.*, 2007. Human xylosyltransferase II is involved in the biosynthesis of the uniform tetrasaccharide linkage region inchondroitin sulfate and heparan sulfate proteoglycans. Journal of Biological Chemistry 282 (8), 5201–5206; Esko, J.D., Selleck, S.B., 2002. Order out of chaos: Assembly of ligand binding sites in heparan sulfate. Annual Review of Biochemistry 71, 435–471. Available from: papers http://626fef3a-d60d-49b2-89f3-1999b93e20cf/Paper/p2885; Bame, K.J., Esko, J.D., 1989. Undersulfated heparan sulfate in a Chinese hamster ovary cell mutant defective in heparan sulfate N-sulfotransferase. The Journal ofbiological chemistry 264 (14), 8059–8065. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2524478; Bai, X., Esko, J.D., 1996. An animal cell mutant defective in heparan sulfate hexuronic acid 2-O- sulfation. Journal of Biological Chemistry 271 (30), 17711–17717; Aikawa, J.I., Grobe, K., Tsujimoto, M., Esko, J.D., 2001. Multiple isozymes of heparan sulfate/heparin GlcNAc N-deacetylase/GlcN N-sulfotransferase. Structure and activity ofthe fourth member, NDST4. Journal of Biological Chemistry 276 (8), 5876–5882; Bame, K.J., Lidholt, K., Lindahl, U., Esko, J.D., 1991. Biosynthesis of heparan sulfate: Coordination of polymer-modification reactions in a Chinese hamster ovary cell mutantdefective in N-sulfotransferase. Journal of Biological Chemistry 266 (16), 10287–10293; Datta, P., Li, G., Yang, B., *et al.*, 2013. Bioengineered Chinese hamster ovary cells with golgi-targeted 3-O-sulfotransferase-1 biosynthesize heparan sulfate with anantithrombin-binding site. Journal of Biological Chemistry 288 (52), 37308–37318; Gasimli, L., Glass, C.A., Datta, P., *et al.*, 2014. Bioengineering murine mastocytoma cells to produce anticoagulant heparin. Glycobiology 24 (3), 272–280.

(Zhang et al., 2012). The heparosan thus obtained from microbial sources, could be modified chemoenzymatically to produce anticoagulant heparin (Bhaskar et al., 2015). In another approach, mammalian cells, such as CHO cells which naturally produce heparan sulfate, could be metabolically engineered toward the production of heparin (Baik et al., 2012).

Optimization of GAG production in bacteria requires availability of genome sequence combined with advances in analytical and metabolic engineering tools. Genome sequencing has revealed gram-positive and gram-negative bacteria capable of producing simple non-sulfated GAGs, such as hyaluronan, chondroitin and heparosan. The biosynthesis of hyaluronan, heparosan and chondroitin share a carbon source that is also required for maintaining cell growth and biosynthesis of cell wall components. For example, glucose, the precursor for of GlcNAc and GlcA, is utilized for the biosynthesis of glucose-6-phosphate (Fig. 3). The glucose-6-phosphate is utilized for cell growth via glycolysis and the pentose phosphate pathway, as well as biosynthesis of cell wall components (Wang et al., 2011). Commercial production of GAGs from bacterial sources will require studies on metabolic control analysis and metabolic flux analysis. These methods may be utilized to evaluate correlations between cell metabolism and GAG production, in response to internal cues (e.g., genetics) and external cues (e.g., media composition). For example, it has been postulated that UDP-glucose 6-dehydrogenase and production of UDP-GlcA could be limiting factors for GAG biosynthesis. High throughput gene screening may reveal genes that steer metabolites toward production of GAGs without affecting cell growth. ePathbrick (Xu et al., 2012) vectors have been used to metabolically engineer a chondroitin pathway in BL21, resulting in CS yield comparable to CS produced from *E. coli* K4 (2.9 g/L) (He et al., 2015; Xu et al., 2012; Zhao et al., 2015; Xu and Koffas, 2013). In parallel, CRISPathBrick (Cress et al., 2015) can be utilized to tune expression of exogenous genes and repress endogenous genes in *E. coli* (Cress et al., 2015).

Conclusion

The medical and non-medical uses of GAGs (e.g., heparin, hyaluronan, chondroitin sulfate, dermatan sulfate) have led to a need for industrial-scale production of GAGs from Generally recognized as safe (GRAS) organisms. Collaborative research toward the improvement of current strains that produce simple GAGs, exploring new organisms (bacterial, simple eukaryotes, and mammalian systems, such as CHO cells), as well as improvement and development of analytical and metabolic engineering tools will play significant roles in the industrial production of simple and complex GAGs.

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Infectious Waste Management☆

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Glossary

Animal by-products (ABP) Any part of an animal carcass, or any material of animal origin, not intended for human consumption.
Biowaste (BW) Biological waste from human, animal, or plant origin as from toilets, households, restaurants, animal production, slaughter houses, food, and beverage industry, and so forth.
BSE Bovine spongiform encephalopathy, the mad-cow disease.
Emerging diseases New, reemerging, or drug-resistant infections with increasing incidence.
Pathogen pollution Spread of pathogens in the environment owing to the use of BW or due to other reasons.
Vector animals Insects, birds, and some wild mammalian species that transfer pathogens, directly or indirectly, to animals and humans.
Zoonoses Diseases and infections that are naturally transmitted between vertebrate animals and man.

Abbreviations

ABP Animal by-products
BSE Bovine spongiform encephalopathy;
BW Biowaste
EFSA European Food Safety Authority
FMD Foot and mouth disease
VTEC Verotoxin-producing *Escherichia coli*

Defining Statement

Incineration or landfilling of infective waste is almost a safe solution in the context of biosecurity. However, the desire to move toward a sustainable society is increasing the interest in recycling today. Treatment options for biological waste vary in their sanitization effect. After application to land, in fertilization purpose, some pathogens may persist in the environment for extended periods of time.

Sources of Infectious Waste

Biological Waste

Biological waste (BW) may be of agricultural, municipal, or industrial origin and must always be assumed to contain infectious agents of human, animal, or plant origin. It is debatable whether manure, sewage sludge, and several other BW should be classified as a waste or as a resource. However, concerning the biosecurity aspects and handling and treatment regimes, the considerations are very much the same as for BW in general, and therefore these products are classified as BW here.

Agriculture is a large producer of BW, both of animal origin, such as manure and animal by-products (ABP), and of plant origin, such as residues from harvest, animal feed residues, and plant material from greenhouses. In some regions, fish farming may also produce large amounts of BW. The definition and handling of ABP have been of special interest since the problem with bovine spongiform encephalopathy (BSE), or the mad-cow disease, became widespread in Europe during the 1990s. According to the present EU legislation (EC 2011/142), in Europe, ABP is divided into three risk categories depending on the risk of presence of prions and other pathogens, with different treatments prescribed for different categories. Category 1 ABP, the highest risk category, includes mainly material from ruminants, which may be suspected to be infected with prions. Category 2 includes, for example, carcasses of animals other than ruminants and manure for sale. Category 3 is mainly low-risk slaughterhouse waste.

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Municipal sewage treatment plants produce huge amounts of sewage sludge, but other handling systems for toilet waste also produce voluminous quantities of BW. Other origins of BW may be categorized as ABP from handling, preparation, and consumption of food, for example, from grocery stores, households, restaurants, and schools, and from international transport and trade. BW of plant origin comes from gardens, park areas, and so on as well as from food handling and production.

Origin of industrial BW is mainly the food and beverage industry. Another large source of BW is the technical industry, which make use of products of biological origin, such as the tanning industry and the production of industrial fat. When such BW is of animal origin, it should be treated and handled as ABP.

Mixing of BW and Nonorganic Compounds

Concerning recycling and the society's efforts to achieve a balanced interplay between urban and rural areas, it is beneficial if BW and other waste products are sorted so that they can be appropriately treated. Proper BW is much easier to process and recycle than BW contaminated with nonorganic compounds. However, source separation of BW is not always performed or even when performed may be unsuccessful. BW from sources lacking professional source separation, for example, households and public places, is often mixed with nonorganic compounds. Another example is the effluents from toilets, as sewage is a mixture containing many contaminants. Spreading of information and education may be beneficial for successful source separation of solid waste and for treatment of effluents of toilet systems, and the quality of the end product may increase. Separation of mixed wastes at a processing facility may also be possible followed by treatments for potential recycling, although this will increase the overall cost of treatment. Another way to handle the problem of mixed waste is to process the BW first by, for example, composting, followed by separation of nonorganic compounds. However, the end product may be of low quality, concerning its use on arable land. Mixed types of solid wastes that cannot be recycled and that are not suitable for incineration must be land-filled in a safe way. Sewage is often a mixture of BW, industrial effluents, and stormwater from streets, and so on and therefore rather complicated to handle and recycle. Agricultural use may lead to pollution of soil with heavy metals, nonorganic substances, and other unwanted substances.

Health Risks from Pathogens in BW

Infections in BW Can Be More or Less Severe

It must be assumed that almost all kinds of pathogens may be present in BW and that BW is therefore potentially hazardous to animal and human health. Most pathogens found in BW originate from human or animal feces. Sewage sludge and manure reflect the health status of a population and have in some studies been used as an easy way to get fecal samples from large populations. A broad spectrum of pathogens can be found in toilet waste, but the severity of gastrointestinal infections varies. Diarrhea is one of the most common causes of death worldwide, especially among children. In developing countries, infectious diseases of both humans and animals are more common than in developed countries, resulting in a heavy load of pathogens in BW. Pathogens that are rare in the developed world may be commonly seen in developing countries, parasitic diseases being of special interest. Infectious diseases are now spreading geographically much faster than at any previous time. Owing to intensive travel and trade, an epidemic or epizootic disease outbreak in any part of the world is not far from becoming a threat somewhere else. Exotic infections can quickly spread across borders and even across continents. The vulnerability is universal, and globalization increases the possibility of new pathogens being spread by BW. From highdensity livestock areas or areas of intensive agriculture, excess manure, plant residues, and so on may have to be transported to other areas to be used as fertilizers. This involves a considerable increase in the biosecurity risk, as diseases not indigenous to a region may be introduced.

We have to also consider and handle both known and unknown diseases. New diseases may arise from nowhere, and well-known diseases may change in character concerning severity, epidemiology, incidence, and other characteristics. Furthermore, just about half of the bacteria in the human intestine and most of the environmental bacteria have not yet been cultured or characterized.

Exposure to Pathogens Originating from Infective Waste

Direct exposure to infective agents in BW may occur at collection, transport, treatment, storage, use, and in other ways of BW. When these are in the hands of well-educated professionals who use suitable personal protective equipment, practice basic personal hygiene, and avoid direct contact with the waste, the risk for disease transmission is generally low. However, in some developing countries, it is common for poor people to make a living from collection of waste or manual sorting of untreated waste at dumps. Such direct exposure is a high-risk behavior as regards contracting infections from the waste. The risk is further increased if such individuals are in poor physical condition or are already suffering from other diseases. Another exposure problem here is the inhalation of endotoxins.

Indirect exposure to infective agents owing to pathogen pollution of the environment may originate from BW (Figure 1) in the form of leakage or aerosols while handling, storage, or dumping of BW. Pathogen pollution may also follow the use of fertilizers originating from BW. Agricultural use of BW of municipal or industrial origin creates new routes of disease transmission between animals, humans, and the environment and can lead to the contamination of the environmental elements, such as drinking water sources or arable soil. Infections may result directly from the soil or vegetation when domestic or wild animals are grazing on, or

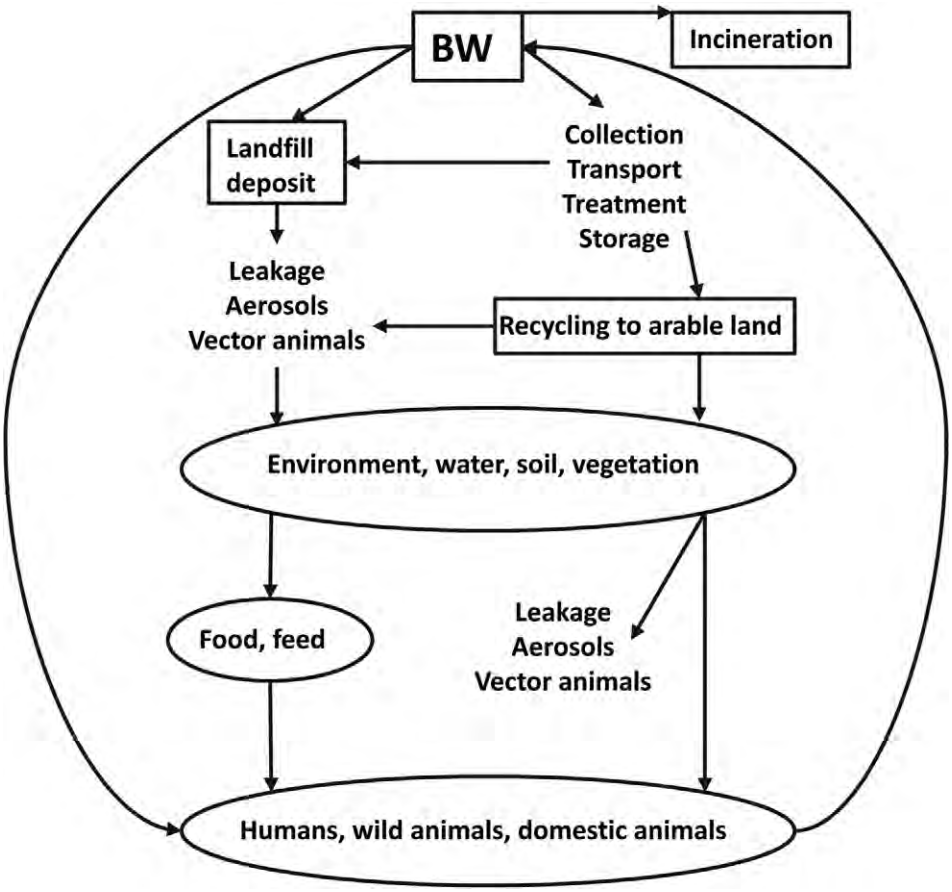

Figure 1 Possible circulation of pathogens between biological waste (BW), animals, humans, and the environment. Squares indicate destiny of BW; ellipses indicate destiny of pathogens.

passing over, the land. Humans may become infected, for example, during recreation activities and exposure to a contaminated environment or water. Pathogens can also be transmitted to humans and animals on dust particles transported by wind, in surface water or groundwater, or in food or feed harvested from the treated land. Water used for irrigation of food crops may be a particularly significant route of disease transmission. Vector animals, for example, insects, birds, and some wild mammalian species, may also spread pathogens to animals and humans, directly from BW produced or through treated BW that has been spread on arable land. Vectors may transmit pathogens themselves directly to a new host by biting or sucking blood, or indirectly by contaminating their food, water reservoirs, or environment. In addition, predators or humans may consume vectors.

The number of microorganisms needed to cause infection in man and animals varies considerably between pathogen species and also depends on the infection route (e.g., oral, inhalation, and through wounds). The susceptibility of an individual also varies depending on age, nutritional status, medical treatment, and other diseases. For some organisms, very low numbers may cause an infection even in healthy individuals, for example, just one embryonated helminth egg may be enough. The proportion of the human population regarded as being immunosuppressed has increased over recent decades. Malnutrition and HIV may be the main reasons for this in the developing world, whereas an ageing population and postoperative medication of transplant patients may be the main causes in the developed world. It is important that the processes involved in handling and treatment of infective waste provide sufficient protection even for these vulnerable groups. In addition, a dose–response relationship exists after exposure to a pathogen. In general, individuals become infected more from a single large dose than from the same quantity in small doses spread over time.

Zoonoses

Many of the actual pathogens in BW are zoonotic agents, which may be transmitted between animals and man. It should be realized that as much as 70% of infectious diseases currently affecting humans are caused by zoonoses and many of the most troublesome pathogens are zoonotic agents. Emerging pathogens may be defined as new, reemerging, or drug-resistant infections with increasing incidence. Of the emerging infectious diseases, 75% are due to zoonoses. Examples can be found among the most serious diseases during recent years, such as BSE, avian influenza, and anthrax. Ingestion of contaminated food or water is the main route of infection in humans for many zoonotic agents, but people can also become infected through inhalation, by direct contact with animals, or from contaminated soil or bathing water.

Salmonella mainly causes gastrointestinal disturbances and is a pathogen well-known all over the world. The genus consists of many serovars; most serovars can colonize the intestine of an extraordinarily broad spectrum of animal species as well as humans. *Salmonella* prevalence in animal production and in the society in general, as well as the strategies to control the infection, varies markedly between countries and regions. In most countries, *Salmonella* is frequently present in many kinds of BW, for example, sewage sludge, manure, and ABP. Verotoxin-producing *Escherichia coli* (VTEC) also causes serious gastrointestinal disturbances and even renal failure in humans. In many developed countries, the infection is endemic among cattle, even though they do not show clinical symptoms. When manure or ABP are not properly sanitized, such products can be an important cause of spread of this infection.

BSE has been extraordinarily important concerning the regulations and procedures for handling ABP, and is also an interesting example of a disease caused by inadequate recycling. Insufficiently heat-treated carcasses of scrapie-infected sheep as well as of the first wave of infected cattle were used as a component of cattle feedstuff. Since the incubation period is several years, the disease had spread widely before the source of the infection became known. BSE among cattle in the United Kingdom has now declined and so has the number of people infected after consumption of infected meat.

Traditional virology has long believed that viruses are quite host-specific, but there are many recent examples of viruses isolated from diseased humans that may have animal origins, for example, hepatitis E virus, and some strains of rotavirus and norovirus. These viruses are all excreted through feces and may therefore be found in certain kinds of BW. It should be borne in mind that viruses are diverse and complex and have the ability to infect different hosts by genetic changes and by the expression of different phenotypic properties.

Zoonotic helminths and protozoa of potential importance in BW include several subspecies of *Ascaris*, *Cryptosporidium*, *Taenia*/*Cysticerca*, *Giardia*, *Tricinella*, and *Toxoplasma*. Most of these agents need only a few organisms to cause infection. *Cryptosporidium* and *Giardia* are a threat in drinking water sources frequently causing epidemics, even in developed countries. Such disease outbreaks often follow flooding or heavy rainfall causing surface runoff or lack of capacity in sewage treatment plants, so sewage is let out untreated to recipient waters.

Human Diseases

Besides all zoonotic *Salmonella*, also some species-specific *Salmonella* serovars such as Typhi and Paratyphi may be of interest in BW sanitation. These cause gastrointestinal symptoms together with other more serious symptoms. Several bacterial species that may be persistent in BW (e.g., *Enterococcus*, *Proteus*, *Streptococcus*, *Pseudomonas*, *Micrococcus*, *Actinomyces*, *Meisseria*, *Bacillus*, and *Klebsiella*) may cause wound infections, sepsis, and even death. Numerous species of intestinal parasites such as *Entamoeba*, *Strongyloides*, *Strongyle*, and *Schistosoma* may also be present in BW. In addition, several virus species can be shed with feces – and infect by the fecal–oral route, for example, norovirus, hepatitis A virus, and rotavirus – and therefore be present in BW.

Animal Diseases

Epizootic diseases can seriously affect animal welfare and cause severe production losses, and therefore epizootic outbreaks are handled very strictly. Eradication commonly involves slaughter of all infected animals and destruction of the carcasses in all infected and infection-suspected herds. This, together with export restrictions on animal products, can have enormous economic consequences for both the farmer and the society.

Classical swine fever is one of the most serious epizootics affecting swine (Figure 2). This togavirus is highly resistant and may remain viable for a long time in the environment or in the gut of people who have consumed infected meat, as well as in BW composed of food scraps. Foot and mouth disease (FMD) is characterized by fever and vesicular eruptions and is caused by an extremely contagious enterovirus affecting cloven-footed animals. Feeding food scraps to swine has caused many outbreaks of the disease, as has handling of ABP. Another animal infection of significance is blackleg caused by *Clostridium chauvoei*, spores of which may persist in soil for many years and infect grazing cattle. Helminths and protozoa of significance to animals are mainly a problem concerning the handling and spreading of manure or if manure is mixed with other kinds of BW.

Figure 2 The virus causing classical swine fever, one of the worst epizootics affecting swine, is highly resistant in the environment as well as in biowaste.

Plant Diseases

Plant pathogens affecting field crops have great economic importance and warrant widespread and frequent use of pesticides. Besides more common ways of infection of the crop plants, pathogens and weed seeds may also be introduced by agricultural use of BW. The inactivation kinetics of plant pathogens in different kinds of hygiene treatment of BW is, in general, the same as that for human and animal pathogens. However, fungal pathogens may develop resting spores, await more advantageous conditions, and survive for many years in, for example, the soil. They are also rather resistant to a number of treatment methods. Some of them may also use many different plant species as hosts. Examples of long-living fungus include white mould (*Scelotinia sclerotiorum*) and club root of crucifers (*Plasmodiophora brassicae*). Furthermore, some plant viruses are hard to be inactivated using heat, for example, tobacco mosaic virus. Plant pathogens are not discussed further in this article.

Antibiotic Resistance

Antibiotic residues/metabolites and antibiotic-resistant bacteria present in sewage sludge, manure, and some other kinds of BW are an emerging public-health concern. The levels and incidence vary according to human and animal medical-care practices and drug-handling routines in the country or region, although antibiotic resistance is a global problem. A favorable environment for resistance development and transmission to other microbes may be present in BW and soil. Antibiotic-resistant bacteria may transmit their resistance genes to better-adapted indigenous bacteria, increasing the resistance reservoir. Handling strategies for BW and more conservative use of antibiotics may help to retain powerful antibiotics in human and veterinary medicine for future generations. Drug resistance is evident in, for example, the treatment of diarrhea-related diseases.

Waste Management Strategies

Produce Less

The volume of waste produced is increasing, but in a society aiming to be sustainable there must be an intention to reduce the burden of waste in both the production and consumption stages. Environmental awareness and education can enhance this trend. An ethical aspect in a long-term perspective concerning the destiny of BW is that it is only reasonable that each generation takes care of its own waste and transforms it into an environmentally harmless or stabilized form. Sorting and reuse or recycling reduce the waste burden, so this is one direction to go for the future (Figure 3). For paper, cardboard, metals, and plastics, recycling is often the producer's responsibility. The at-source management of BW may lower the cost of waste treatment, collection, and transport and possibly generates profits from the by-products or energy produced.

The rendering industry is responsible for the handling of ABP and is an example of a branch where efforts are being made to minimize the production of BW. Humans consume less than one half of a slaughtered animal, so the remaining parts have to be disposed of. Waste material from food processing (animal meat) is frequently heavily loden with microorganisms, the content of pathogens varying widely between different countries or regions. The rendering industry converts huge volumes of ABP from a waste to a resource. ABP is used for many purposes: as feed ingredients for domestic and companion animals; in aquaculture, tanning industry, and technical products; and in heat and energy production. Continuous adaptation to new regulations warranted by biosecurity or environmental reasons, as well as to the market, is essential.

Figure 3 Source separated biowaste aimed for anaerobic digestion. This reduces the waste burden but may be a biosecurity risk if not sufficient hygiene treatment is performed before recycling on arable land.

Get Rid of It

The management strategy for waste in many cases may simply be to get rid of it at the lowest possible cost. Hopefully environmental aspects such as pollution, eutrophication, and greenhouse gas emissions are also considered. Producing renewable energy from waste in the form of heat or methane is one desirable option, but investment needs are generally, but not always, high. Storage of waste, including BW, before such energy recovery may be a problem, although possible to deal with by, for example, baling technology for waste and drying.

Incineration of BW is an attractive solution concerning the biosecurity aspect and also results in a marked reduction in volume. Energy recovery and heat production are possible, but then more advanced treatment systems are generally needed. One of the drawbacks is that gas emissions from treatment facilities may cause air pollution and negative climatic effects. Such gas emissions can be reduced using advanced particle filters. Through the use of ash, minor recycling of plant nutrients may be possible. As for all handling methods of waste, strategies differ between countries or regions or for different kinds of waste. For example, in Japan 100% incineration of combustible waste has been a national waste-management policy. In Sweden, in contrast, recycling of waste before incineration, for example, biodegradation of BW in closed digesters, is proposed. According to the EU legislation for ABP, incineration is compulsory for the highest risk category owing to biosecurity reasons. As long as the temperature during combustion is above 850 °C, even extremely resistant prions are destroyed. Incineration of whole animal carcasses is costly since there is a high energy demand, as well as a demand for special incineration equipment. Production of meat and bone meal is widely practiced, and before the BSE crisis, it was largely used as an ingredient in animal feed. An advantage compared with untreated ABP is that meat and bone meal can be conveniently stored while waiting for incineration. The rendering industry is continuously developing new technologies. In Sweden, a significant proportion of ABP is processed as Biomal, and the energy-demanding processing of the raw material into fat respectively meat- and bone meal is removed. The ABP is crushed and ground and then pumped to a fluidized-bed boiler where it is combusted together with a base fuel such as peat, wood chips, or municipal waste. In the EU, Biomal is regarded as a renewable biofuel not contributing to global warming and can replace fossil fuels for heat and energy production. The heat production from Biomal is about the same as that from incineration of wood chips.

Open dumping of solid waste remains a widely used waste-disposal method around the world. The method is believed to be cheap, but is a generator of ill health. In developed countries, the number and also area of landfills have generally been reduced. The change from open dumping to integrated waste treatment is due to the new and stricter rules for landfilling warranted by environmental and biosecurity reasons, concurrent land use, recycling, and so on. Leachate from landfills, if not collected and treated, can cause environmental problems in surrounding surface-water bodies and groundwater. The European Union has laid down strict requirements for landfills to prevent and reduce such negative effects (1999/31/EC). Examples of individual countries as are making efforts to reduce the use of landfill is Singapore where the long-term goal is towards zero landfill, and in Sweden landfill of organic waste has been prohibited since 2005. This development creates a need for increased investment; as many small local landfills are being shut down. Also longer transport distances of waste to larger facilities may follow.

BW may also be landfilled in biocells, closed compartments from which biogas is recovered at an early stage. Beneficial here is the mitigation of methane (a greenhouse gas) emissions. If degradation variables such as moisture and gas movement are optimized, it is possible to degrade BW within a few years. Such cells may have separate leachate collection, and the fluid may be treated in constructed wetlands. Such treatment may provide the opportunity to digest wastes that are not sufficiently sanitized to be used on agricultural land.

Disposal method for carcasses in many parts of the world, and previously also in Europe, is burial, composting, or feeding to carnivores or birds of prey (domestic or wild). In the United Kingdom, there has been concern about the possible threat to groundwater supplies from buried carcasses of BSE-infected cattle due to the long-lasting nature of the prions, the infective agent. Close to 200 000 cases have been confirmed in cattle, and during the first years of the epizootic, deposit in landfill sites or burial of carcasses was frequent. In the developed parts of the world, well-adapted routines may be present for treating ABP produced at slaughterhouses and also for dealing with sporadic cases of on-farm deaths. However, burial is still used worldwide to get rid of single or numerous carcasses in cases of on-farm deaths, nationwide die-offs, or mass culling of infected animals during outbreaks of epizootic diseases. This method is associated with problems such as contamination of water sources and several biosecurity issues. In Europe, it is used just as one exception to the EU legislation concerning ABP, but sometimes it may be the only way to handle an acute situation. Furthermore, for some extremely contagious diseases such as FMD, transport of infectious carcasses is avoided for biosecurity reasons. At burial, special care should be taken to deposit at sufficient depth (>2 m), in nonporous soil and well separated from groundwater and surface water sources.

Recycle in the Food Production Chain

Treated BW may be a valuable resource as a fertilizer on arable land, in forests, or for other purposes. However, besides the desirable plant nutrients, undesirable pollutants such as infective agents and antibiotic-resistant bacteria are found in BW. Heavy metals, pharmaceutical residues and nonorganic pollutants may also be present but these problems will not be further discussed here.

It is strongly desirable that the handling of BW be viewed in a holistic way with respect to aspects concerning the environment, the economy, and animal and human health. Recycling of BW in the food chain as fertilizer to arable land or as a feedstuff may be beneficial for society and agriculture, as well as the environment. BW transformed from waste to a resource helps to reduce the need for fossil fuel and chemical fertilizers. For organic farming, BW can be very useful as a fertilizer complementary to manure.

In developing countries too, where chemical fertilizers are not regularly available or affordable, use of BW may be highly desirable to increase food and feed yield. It has been observed that in areas where human urine has become a valuable fertilizer, tanks need locks as otherwise the urine is stolen. ABP may be recycled as plant nutrients in the food chain according to EU legislation if pressure-cooked (133 °C for 20 min at 3 bar for risk category 2)Manure also has to be sterilized, although several exceptions exist. Sterilization at high temperature is costly and also diminishes the energy efficiency of treatment, so alternative sanitation methods are highly desirable. Pasteurized (70 °C for 60 min) is enough for risk category 3 but also alternative treatments are permitted if individual member states can validate that such treatment have a hygiene effect equivalent to that of pasteurization. So far, hygiene validation of such new, alternative treatment methods in a scientifically based, generally accepted way is not unquestionably available. According to regulations concerning treatment of ABP and previous opinions from the European Food Safety Authority (EFSA), the pasteurization treatment should reduce the number of relevant pathogenic bacteria by at least 5 $\log_{10}$, the infectious titre of the relevant thermo-resistant viruses by at least 3 $\log_{10}$ whenever they are a relevant hazard, and the number of viable parasitic stages by at least 3 $\log_{10}$ in the given exposure time. So, BW needs to be sufficiently treated before agricultural use. Previous practices, where health drawbacks are now obvious, have included the use of, for example, untreated cow and sheep blood, untreated gut contents from slaughterhouses, or insufficiently treated sewage sludge or other kinds of toilet waste as soil improvers on arable land.

Traditionally, BW including food waste has been fed to domestic animals such as pigs and poultry, while ruminants have been fed with meat and bone meal produced from rendered carcasses and litter from chicken houses. However, this has caused concern since such reuse can spread serious diseases to the animals and further on to humans. The BSE crisis should be still prominent in our minds. Another concern that has prompted the present EU legislation is the ethical (but also biosecurity) aspect that a species should not be forced to eat remains from its own species members. However, use of BW as feed for both companion and certain species of food-producing animals may still be possible, if careful sorting and sanitation treatment are performed first.

Treatment Options for BW to be Recycled to Arable Land

No Best Options Exist

Although it is technically possible to decontaminate BW completely, that is, to sterilize it, before agricultural use, this is an unrealistic proposal, for both practical and economic reasons. Extremely strict regulations will tend to discourage the reuse of BW on arable land, so a balance needs to be maintained between health risks and treatment costs. It must also be borne in mind that the treatment of BW is just one measure, however important, quantifiable, and controllable, against the introduction of infective agents in the environment. Other measures such as restricted spread or use of BW on farmland are discussed further below. Many different treatment options exist for BW to be recycled for agricultural use, and several more are still at the experimental stage. These options include small- or large-scale and low- or high-tech solutions. Universal solutions are unlikely, since a wide variation of situations exists across the world. There is a wide range of opinions and any treatment method may be tested as to its applicability to the local situation. Cost of treatment, possible energy production or consumption, environmental effects, equipment and skill, need for and availability of transport, reduction of smell, possible value and use for the end-product, and so on are factors that should be considered. Traditions (social and religious) and regulations for organic production also may need to be considered. To be successful, suitable treatment methods must combine biosecurity aspects with all these other factors. An efficient hygiene treatment of BW is essential, as agricultural use of BW can inadvertently spread infectious diseases to animals and humans, although opinions differ concerning the risk levels. Advantages of coprocessing BW of municipal, agricultural, or industrial origin include opportunities for the efficient use of large-scale procedures and addition of plant nutrients to agriculture. Some type of risk assessment tool enables treatment methods to be evaluated according to the risk of exposure, dose and type of pathogen, and other measures used.

Possible treatment systems for BW include many different processes based around a biological, physical, or chemical step, some examples of which are given below. Combinations of these methods may also be used. Here the focus is on the sanitation efficiency of the treatment. Acceptable hygiene quality of the end product can be achieved in different ways, among which a temperature/time-dependent method is the most common. The effectiveness of a sanitation treatment may also depend on the pH, the moisture content and the volatile fatty acid content of the treated material, oxygen availability, microbial antagonism, and possible production of for example, antibiotic, exoenzyme, and toxic breakdown products.

Hygiene Assessment of Treatment

The effect of treatment has to be continuously monitored by checking the process, to follow processing parameters such as temperature, pH, treatment time, and composition of the material. Additional information on hygiene level may be obtained by sampling of the end product. However, sampling of nonhomogenous material is complicated, and efficient sample distribution and sampling techniques are essential in obtaining representative samples to perform an accurate hygiene assessment. However, such sampling procedures for large volumes have not yet been standardized. Therefore, it is often easier, and safer, to specify a process rather than the end product. Clear distinctions need to be made between process standards and product standards.

Additional hygiene tests could check directly for the presence of certain pathogens, or comprise a more indirect analysis based on indicator organisms. It is often difficult to quantify pathogens in BW as their incidence is irregular. It should also be borne in mind that in direct analyses, pathogen numbers might be underestimated or undetected in cases where the pathogen is hidden by organic material and/or fat or oil. Another way around is to add well-defined pathogens, for example, teabags of permeable

polyamide containing *Ascaris* eggs, to the process and repeatedly monitor those. However, any kind of covering of microorganisms may cause an overestimation of their potential to persist during the treatment, making them too unreliable as indicators of sanitation effect.

An indicator organism should be easy to detect and common in the material of interest. It should be able to stay alive for an extended period, but should not be able to vigorously multiply. *Enterococcus*, coliforms, thermo-tolerant coliforms, *E. coli*, clostridia spores, *parvovirus*, and *Ascaris* eggs are some commonly used examples. The use of indicators can become complicated since they seldom meet each one of these criteria in the same organism. Indicators can also be added to the material, for example, bacteriophage have been used as virus indicators. The types of checks performed, and the frequency and number of analyses of the end product, vary for different kinds of BW and treatment processes.

The survival of bacteria during different treatment processes is multicausal, and therefore results from different studies are often different and difficult to be compared with one another. In most assessments of hygienic quality, kill-off in the case of bacteria is defined as the level at which the bacteria are no longer detected, most often using conventional culturing techniques on agar plates. However, many bacterial species such as *Salmonella* and *Campylobacter* can enter a viable but nonculturable state and thus prolong survival during periods of nutritional or thermal stress. Animals have been shown to become infected after oral administration of such bacteria. Viable but not culturable bacteria are also able to revert to culturable form if environmental conditions improve. The detection of such bacteria in a substrate or in BW is problematic.

Another consideration is that despite adequate treatment of BW, a risk for multiplication by posttreatment regrowth exists for some bacteria. Growth may be possible in the treated material if stabilization, whereby easily degradable organics are degraded, is not complete. However, neither parasites nor viruses have the capacity for regrowth. Bacterial regrowth may follow when complete elimination of some bacteria is not achieved during treatment, or when reintroduction by, for example, contaminated equipment or vector animals occurs while nutrients are still available. It is therefore essential that treated BW is handled, stored, and transported with great care. Stabilization, biological or chemical, also decreases the risk of methane emission from the end product.

Biological Treatments

Aerobic Digestion

Aerobic digestion, or composting, is a commonly used treatment method for BW of different origins or a mixture of such wastes. Composting may be used for small-scale household kitchen BW to large-scale; centralized windrow composting. It can also be differentiated into open or closed systems, often combined in different steps. Composting may be performed for solid or liquid material. For example, manure, when handled in solid form (>30% dry matter content, feces and bedding material from animal production, with or without excreta in liquid form), makes composting convenient. However, slurry (2–10% dry matter content, feces and bedding material from animal production, mixed with urine and water) may be successfully composted by forced aeration. Another way is to first use liquid–solid separation by mechanical methods and/or polymer flocculation.

Composting relies on thermal inactivation of microorganisms; most of the material must achieve sufficiently high temperature (Figure 4). The compost also needs to be repeatedly turned, 3–5 times for solid material, and/or thoroughly mixed to provide sufficient oxygen and thereby maintain optimal conditions for biodegradation. Addition of structural or bulking material, for example, straw, wood chips, or sawdust may help to absorb moisture and provide carbon and porosity, which will facilitate airflow for proper aeration during composting. An insulation layer above and below the compost or more technically advanced practices, such as preheating of incoming air, can also decrease cold subvolumes within compost heaps. Reactor composting, if the reactor walls are insulated, generally decreases the volume of cold zones compared with open composting. The smaller the volume of cold zones, the more enhanced the degree of sanitization, and therefore fewer turnings/less mixing of the material is needed to reach the sanitization goal. The length of time the compost has to sustain a specific temperature, mostly in the range of 55–65 °C, to become sanitized varies from a few hours to several weeks. According to recommendations given by WHO, sanitization of fecal matter is achieved if >50 °C is maintained for a minimum period of 1 week. At lower temperatures *Ascaris* spp. generally have a high survival rate, whereas at temperatures above 50 °C there is a higher survival rate of dsRNA viruses, for example, Rotavirus.

Figure 4 Composting relies on thermal inactivation of microorganisms; the material needs to be repeatedly turned or thoroughly mixed to provide sufficient oxygen and thereby maintain optimal conditions for biodegradation.

The main environmental concern is that most of the ammonia released during degradation of BW will be lost as an acidifying and eutrophying emission during composting. The high target treatment temperature increases this effect. Controlled reactor-composting offers possibilities to minimize gaseous emissions via condensing or biofilter treatment of the outgoing gas.

Anaerobic Digestion

Anaerobic digestion is today commonly used for biogas production and stabilization of a variety of different BW, including municipal sewage sludge, manure, food waste, and ABP. Large-scale centralized biogas plants may use a mixture of these substrates, although many municipal sewage sludge treatment plants just digest sewage sludge. When practiced at farm level, manure is the main substrate, but cotreatment with, for example, municipal BW may occur. Farm-level anaerobic digesters have been used in, for example, China for centuries, and a growing interest is seen today in some European countries.

Anaerobic digesters are commonly operated at either mesophilic (30–40 °C) or thermophilic (50–60 °C) temperatures. Operations at mesophilic temperatures do not ensure a sanitized material without a preceding sanitization step. During mesophilic anaerobic digestion many unwanted bacteria, as well as some viruses, need more than 2 days for a 1 $\log_{10}$ reduction, according to EFSA. In a survey of anaerobic-digestion plants for sewage sludge in Sweden, *Salmonella* were frequently detected (55%) in treated sludge. Thermophilic digestion (50–58 °C) of BW in a large-scale continuous process sufficiently reduces indicator bacteria and *Salmonella*. Separate pasteurization of substrate in a batchwise step prior to digestion is a reliable sanitization treatment. It also increases the biodegradability of the BW so that more biogas is produced. If a pasteurization step is not used, pathogen reduction has to rely on sanitization in the digestion chamber. Since most large-scale biogas plants use a continuous process and the minimal retention time in the digester may be very short, this is not reliable regarding sanitization. However, unwanted organic pollutants are shown to be more efficiently degraded by mesophilic than by thermophilic digestion.

In Sweden, recontamination of digested residues during postdigestion storage and transport has been observed as a problem. Despite proper sanitization by pasteurization, increased time since pasteurization increases the occurrence and density of unwanted bacteria such as indicators (coliforms and *Enterococcus*) and pathogens (*Salmonella* or VTEC) in digested residues.

Biogas can be used as fuel for kitchen stoves, and therefore hygiene evaluation of biogas from large-scale commercial biogas plants and digesters for sewage sludge has been warranted. The levels of microorganisms in biogas are comparable to the levels in natural gas (10–100 cfu m^{-3}). An evaluation of the domestic use of biogas indicates a low risk regarding biosecurity, especially when compared with other risks involved in handling the gas.

Anaerobic digestion is a complex system with environmental benefits, as valuable energy is produced, and the digestate is rich in plant nutrients and is an excellent fertilizer for agriculture. However, depending on the system design, large amounts of methane can escape to the atmosphere during digestion and subsequent storage and handling.

Storage

Simple storage may be classified as a nontreatment concerning pathogen reduction, but some decline in the total number of microorganisms will take place over time. This decline is difficult to predict, and some bacteria may both increase and decrease in numbers in a cyclic manner. As in BW aimed for composting, when the conditions for microbial activity are poor, the temperature will not rise. Some pathogens, for example, VTEC, can persist in liquid manure for several months; the lower the temperature, the longer the survival. Some bacteria (e.g., *Salmonella* and VTEC) can proliferate significantly if conditions are favorable, for example, as regards nutrient availability, so the regular transfer of fresh manure from livestock buildings to slurry tanks may help sustain populations of these pathogens during storage. Levels of indicator organisms such as coliforms and *Enterococcus* vary over time during storage, and pathogen levels are likely to follow a similar pattern. A long storage period without input of fresh material is generally impossible for manure, since storage capacity at farm level is largely determined by the need to store manure until disposed of/used for crop production. Anaerobic lagooning of liquid manure from intensive farming systems is commonly used in certain areas, since it gives rise to low cost and low maintenance-management efforts. Large-scale lagooning may be a potential environmental hazard in cases of faulty design, floods, or earthquakes. Sewage sludge may also be stored for longer or shorter periods of time. Furthermore, long-term storage has a negative impact on the environment if not special caution is taken since emission of the greenhouse gas methane and of ammonia continues throughout the storage period.

Wastewater Treatment

Wastewater treatment is a science on its own, with examples ranging from simple sedimentation in lagoons to high-tech treatment of sewage sludge in biogas reactors. Composting of sludge with or without the addition of other BW and long-term storage are other examples of biological treatment of sludge. As for other BW treated with these methods, the hygiene standard of the treated sludge is in most cases temperature/time-dependent.

Constructed wetlands may be used not only for a third-step cleaning of sewage, but also for treatment of effluents from animal production, landfills, and so on and plants are used for denitrification of such water. If wetland/pond vegetation is harvested and used as a fertilizer in agriculture or for energy production in biogas plants, some degree of recycling of plant nutrients can be achieved. The wetland area may also be intermittently dried and the stored load of plant nutrients used in agriculture, but the

biosecurity risk must then be considered. In addition, constructed wetlands mostly attract vector animals, so the risk of spread of infective agents must also be recognized.

Physical Treatments

Heat Treatment

Heat treatment owing to a temperature rise from microbial activity or by way of external supply of energy is a common and safe hygienic treatment, as long as both the temperature and the treatment time are sufficient. The temperature and the time required depend on the type and strain of organism, the structure of the material, and the moisture content. The higher the temperature, the shorter the time needed for treatment; heat resistance for microorganisms also generally decreases with increasing water activity. Pasteurization at 70 °C for 60 min gives a satisfactory reduction in pathogens for most kinds of BW. However, the reduction of heat-resistant viruses is limited, and spore-forming bacteria are not reduced at all. So if problems are being experienced with a disease caused by these agents, some kind of extended heat treatment or sterilization is necessary. For prions, incineration is a better choice.

Other Physical Techniques

Freezing-thawing is a variant of storage, which has been practiced for sewage sludge in cold climates. The sludge is exposed to repeated freezing–thawing cycles during storage in thin layers. The extent of cell damage caused by the process varies owing to a complex set of factors, and the reducing effect on pathogens from this treatment is variable. The process parameters are difficult to predict, as is the sanitization effect.

Desiccation or air drying to moisture levels $<1\%$ has been successful in inactivation of pathogens in municipal BW and in soil. Mortality of dried bacteria is generally enhanced by oxidation processes, since sensitivity to oxygen increases. Drying of animal manure is a widely practiced treatment in certain regions, but the pathogen inactivation effect is not well documented. From laboratory studies, it is known that bacteria dried with their culture medium survive fairly well, whereas suspensions of bacteria from agar surfaces or of washed bacteria generally have only a small percentage of survival. Freeze-drying under controlled conditions is an extensively used method for long-time preservation of bacteria at laboratories.

Chemical Treatments

Chemical sanitization may be achieved by use of, for example, lime or ammonia. Such sanitation requires a closed treatment system, for example, roofed tanks; otherwise, a marked loss of nitrogen by gaseous emission occurs. Lime or other alkaline treatment has been used for, for example, manure and sewage sludge. A pH of >12 for more than 2 h of contact is required. However, alkaline treatment may be effective in reducing most pathogens, although *Ascaris* eggs are rather resistant, if pH 12 is not reached and, according to standard recommendations, maintained for as long as 3 months. In addition to pH and time, factors such as composition of material, storage, and handling conditions are also important. Achieving a homogenous mixture of lime and the material to be treated is not easy, and the handling may cause health problems for workers. The high pH aimed for is also a work hazard. The cost of the treatment may be repaid by an increased value of the fertilizer owing to the added lime.

Ammonia treatment both stabilizes and sanitizes BW. The sanitization effect is achieved at considerably lower pH (9–10) than for regular treatment with bases. The ammonia treatment requires uncharged ammonia for inactivation of microorganisms and a reduction in the viability of *Salmonella* has been noted from 15 m mol l^{-1} NH_3 at 4 °C. The total ammonia concentration, pH, and temperature regulate the concentration of uncharged ammonia, and therefore it is possible to optimize the treatment. Ammonia is added to, for example, manure or fecal matter either as aqueous ammonia solution or as granulated urea. The environmental and economic cost of ammonia treatment is low, as the ammonia used can be recycled as a fertilizer. An interesting use for ammonia sanitization may be in a mesophilic anaerobic digestion process. If the incoming material is of high protein content, enough ammonia may be released during the anaerobic degradation of proteins for a direct sanitization process to occur. The process needs to be adapted for high ammonia content (8 g l^{-1}) at a pH close to 8, and a high ammonia level can be maintained by restricted feeding of the reactor with a high-protein substrate, for example, pig manure.

Spread and Persistence in the Environment of Infective Agents Originating from BW

Environmental Spread after Pathogen Pollution

Although pathogens occur naturally in the environment and reduction over time will occure, new transmission routes or movement of pathogens to new regions must be avoided. Once pathogen pollution has taken place in the environment, it is impossible to control the spread of the infective agents either in time or in space. Therefore it is of utmost importance to avoid introduction of pathogens to the environment. When BW is to be recycled as a fertilizer, the risk of spread of infections is dependent on a whole set of factors. The initial concentration of the pathogen in BW is of relevance, as is the sanitization efficiency during treatment and the other barriers employed to avoid exposure to animals and humans. Treatment of BW may be absent or inadequate, and other barriers may not be used, leading to problems connected with pathogen pollution.

Other measures complementary to treatment may help decrease the risk of further environmental spread of pathogens and infection of the crops or individuals directly. An example of this may be careful selection of what crop to fertilize, as hygiene-sensitive crops such as vegetables to be consumed raw require a high level of hygiene, whereas for energy crops, a more basic level may be acceptable. The time of the year when BW should be spread on land and the method of application used are other important aspects. Plowing-in or injection reduces pathogen spread and animal exposure, but persistence may be prolonged within soil compared with surface application. An extra safety precaution may be to introduce a restriction time, or quarantine period, before harvest of a crop or grazing of animals. During and after application of BW to land, it is important to avoid its transport to water, which may occur via surface run-off, leakage to groundwater, or flooding (Figure 5). Besides the risk for further spread of pathogens with surface run-off, this may also have negative environmental impacts such as eutrophication and altering of aquatic microenvironments. Aerosols may also result from application of liquid BW, and thus equipment with a low trajectory should be used for spreading to prevent aerosols drifting into adjacent pastures or watercourses. Fine droplets may also be swept by strong winds from the surface of stored liquid BW, with the duration of their ability to hover depending on particle size, relative humidity, and air velocity. Bacteria attached to dust particles are generally better protected against the detrimental influences of solar radiation than those enclosed in aerosol droplets. Depending on the type of soil, strong winds may cause the spread of some pathogens through dust particles. Dust particles may be transported for very long distances, for instance, particles from sandstorms in Africa have been found several hundred kilometers out in the Atlantic sea.

Determining the source of infection for disease outbreak among humans or animals is generally difficult, and the source may not be found. It is usually especially difficult to track the pathogens from an outbreak of a disease – if the pathogen is picked up from the environment – back to the source of the infection.

Vector Animals

Wild animals may acquire a pathogen, and the affected species may act as a disease reservoir for a prolonged time without displaying clinical symptoms. However, wildlife also can be negatively affected, as pathogen pollution may cause infection of immunologically naive wildlife and markedly reduce whole populations. Wildlife and insects may also act as vectors (Figure 6). Vectors are a potential hazard for the spread of pathogens from BW handled at landfill sites, home composting, different treatment facilities, and so on to the surrounding population of humans and domestic- or wild-animal species. BW attracts vectors, and after contact with BW, vectors may carry pathogens on their bodies or in their guts. If BW is sufficiently sanitized and stabilized, the risk of vectors becoming infected is reduced, and the attractiveness of the material for potential vectors is also reduced. The presence of vector animals in places where BW is handled should be reduced to a minimum. However, fencing out wild animals efficiently is associated with obvious problems. Fresh BW needs to be covered instantly to prevent birds and insects feeding on it. The range of vector habitats, migration patterns, and so on may change owing to extreme weather events such as floods, changing ecosystems due to ongoing climate change, deforestation, or other reasons or without obvious reason. Such events may lead to an increase in vector-borne infectious diseases.

Insects may breed in BW, or feed on it. Practically, every enteric pathogen has at some time been isolated in a viable state from insects. Insects may migrate actively, that is, by their own efforts, or be passively transported by wind or by some other kind of vehicle. For example, insects of the species *Psycoidae* have been trapped in a village 13 km away from a dump where they were labelled. This distance is well within the range of active migration.

Figure 5 During and after application of biowaste to land, it is important to avoid its transport to water, which may occur via surfaces run-off or flooding.

Figure 6 Biowaste attracts vectors and insects may carry pathogens on or within their bodies. Vectors are a potential hazard for the spread of pathogens to humans and animals.

Birds such as gulls (*Laridae*) and crows (*Corvidae*) and birds of prey such as owls, hawks, and buzzards may feed on BW and/or visit landfill sites. Eagle owls (*Bubo bubo*), in particular, are often seen on landfill sites, as they feed on small rodents. Not surprisingly, *Salmonella* is more often isolated from gulls in areas with lower hygiene standards than those in areas with better standards. When gulls use landfill sites to feed, they tend to visit the site during the daytime and thereafter stay in the surrounding area. Since birds have a daily need for water, bird droppings may contaminate water supplies in the surrounding environment. However, birds should be perceived not only as a local problem for the handling and treatment sites of BW and their immediate environment; long-distance transmission of pathogens with birds can also occur, especially during the winter season when many birds migrate to other regions or countries.

Some mammalian species such as red fox (*Vulpes vulpes*), hare (*Lepus*), roedeer (*Capreolus capreolus*) and, not the least, different species of rodents may be more or less permanent visitors on, for example, landfill sites. Wild animals acting as a reservoir for contagious diseases is a well-recognized problem in many parts of the world. For example, wild boars (*Sus scrofa*) have been found to be infected with classical swine fever in Eastern Europe. Wild boars infected from contact with BW may then transmit the infection to domestic swine, causing an *epizootie*.

Persistence

The ability of an organism to persist in the environment is critical to its transmissibility, as the opportunity to come into contact with a susceptible host increases. Agents that can infect many different species are more likely to find a suitable host compared with species-specific agents. Both the survival and growth potential of pathogens in the environment vary considerably between different species and subtypes of microorganisms. Parasites, spore-forming bacteria, prions, and some types of viruses (e.g., Parvo) generally persist for the longest periods of time. Eggs of some helminths, especially *Ascaris*, are very persistent and have been shown to be still viable even after 15 years. Helminths typically require time in the environment to develop into an infectious stage and may pass through one or more intermediate hosts, for example, *Schistosoma*. Spore-forming bacteria such as *Bacillus* and *Clostridia* species are extremely persistent in the environment and may stay viable for decades buried in dry soil, where microbial activity is minimal. Several examples of unexpected infection of cattle have been reported after digging in places where unknown anthrax-infected carcasses had been buried decades previously. In the surface layer of soil, in competition with other organisms, anthrax spores may disappear within a few years. In Germany, it was noted in connection with World War I that losses of cattle to anthrax on pastures flooded by water from rivers into which tanneries had drained their spore-contaminated sewage drastically diminished. Cattle losses increased subsequently once contaminated hides were again imported after the war. Other interesting observations concerning the potential of *Bacillus anthracis* to multiply in soil have been made during periods of major climatic and ecological changes in which the soil microenvironment is altered in such a way that spores start to germinate and multiply. Thawing of permafrost in East Siberia is today causing awareness of increasing numbers of anthrax cases. In the beginning of 1900 massive outbreaks of the disease caused 1,5 million dead deer, most of them buried in the permafrost. Permafrost warming may lead to the release of viable anthrax spores.

Other bacteria may also survive in the environment for periods over 1 year, if temperature, humidity, type of soil, and so on are favorable. Different soil types affect microbial survival in different ways owing to their texture, pore space, surface activity, moisture-holding characteristics, presence of essential nutrients, types of autochthonous microorganisms, and so on. Some pathogenic bacteria such as *Salmonella* and VTEC can multiply in favorable circumstances, such as after rainfall. To increase persistence, different *Salmonella* serovars adjust themselves to the surrounding circumstances in different ways. For many pathogens such as new viral pathogens, there is little information available about their persistence in the environment or their removal during treatment of BW. Prions may most likely persist for a very long time, although there is no evidence that BSE infection in animals occurs directly from the environment. For other transmissible spongiform encephalopathie agents such as scrapie in sheep and chronic wasting diseases in cervids, their persistence in the soil is thought to play an important role in animal-to-animal transmission. Extremely hard efforts are being practiced in the western world to avoid environmental pollution with prions.

In general, the survival rate of microorganisms is lower in terrestrial environments compared with aquatic environments, mainly due to higher microbial activity and antagonism in soil. Natural inactivation factors on plant surfaces, in soil, and in feed and foodstuff also vary considerably due to climate, season, vegetation, soil type, and so on. Physicochemical factors such as sunlight, temperature, desiccation, dilution and pH, and biological factors such as antimicrobials produced by *Microbiota*, predation, bacteriophage, and nutrient competition reduce pathogenic microorganisms over time. However, this type of reduction in pathogens is unreliable and should not be overestimated. In general, survival is prolonged in cold climates, a fact that must be taken into consideration if a quarantine period is established as a safety precaution/barrier between spread of BW and crop harvest/grazing. Ongoing climate change may change the possibilities for persistence of some pathogens in the environment. For example, parasite survival may be favored if winters become warmer and humidity increases, but negative effects may follow if the protective layer of snow is sparse. Vegetation may also provide a protective environment and enhance pathogen survival.

Conclusions

Widespread efforts are being made to reduce the burden of waste on the society, but further improvements are needed. Management strategies include producing less waste, reusing and recycling, and simply disposing of waste by dumping or incineration. Recycling of BW is desirable for several reasons, but it must be done in such a way that it does not constitute a risk to human and/or animal

health or to the environment. BW must always be assumed to contain infectious agents of animal, human, or plant origin. More or less severe infections are found, including epidemic, epizootic, and zoonotic infections of significance. Recycling of BW in agriculture enables reuse of plant nutrients and reduces the need for artificial fertilizers and nonrenewable energy. Sufficient hygiene treatment before the spread of BW as a fertilizer is essential to avoid ecosystem contamination with pathogens. Additional barriers such as a restriction period between spread of BW and harvest/grazing may also help. Pathogens can persist for long time in the environment and be transported for long distances. Vector animals and water are central to the spread of many infective agents. In addition, today's intensive travel and trade make the vulnerability to infectious diseases a universal problem.

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Insecticidal Toxins from *Photorhabdus* and *Xenorhabdus*[☆]

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Introduction

The Biology of *Photorhabdus* and *Xenorhabdus*

Bacteria, nematodes, and insects

Photorhabdus and *Xenorhabdus* bacteria live in association with nematodes from the families Heterorhabditidae and Steinernematidae, respectively (Forst et al., 1997). Both genera bacteria are members of the Enterobacteriaceae, and are found as symbionts within the guts of the infective juvenile nematodes (ffrench-Constant et al., 2003). These infective juvenile nematodes seek out and invade insects, whereupon the bacteria are released from the mouth of the nematode directly into the insect blood system or hemocoel (Forst et al., 1997). The bacteria then multiply rapidly within the insect, apparently unaffected by the insect immune system (Silva et al., 2002). In the case of *Photorhabdus*, the bacteria first colonize the gut and grow rapidly in the hemocoel only, later colonizing all the other insect tissues (Silva et al., 2002). The nematodes also multiply within the insect cadaver, feeding off both the bacteria and the degraded insect tissues (Forst et al., 1997). Finally, a new generation of infective juvenile nematodes reacquires the bacteria and leaves the insect in search of new hosts.

During the course of this infection process the insect dies and the bacteria are inferred to secrete a range of toxins capable of killing the insect or attacking specific tissues (ffrench-Constant et al., 2003). For the purposes of this article, it is important to note that during this process the bacteria lie within the hemocoel of the insect or within the insect tissues. Therefore, any toxin action on the gut in vivo is likely to be from the hemocoel side of the gut. The existence of toxins acting from the lumen side of the gut is therefore, unexpected from the biology of the organism (Bowen et al., 1998). In this respect, this article is divided into sections on the orally active Toxin complexes (Tc's) and on the injectably active Makes caterpillars floppy (Mcf) toxins.

Bacterial nomenclature

As this article involves the description of toxins from a wide range of *Photorhabdus* and *Xenorhabdus* strains, some description of current bacterial taxonomy is necessary to clarify subsequent discussion. The genus *Xenorhabdus* contains a number of species including *Xenorhabdus nematophilus*, *Xenorhabdus beddingii*, *Xenorhabdus bovienii*, and *Xenorhabdus poinarii* (Forst et al., 1997). The taxonomy of the genus *Photorhabdus*, however, is more complicated and has recently been revised. In this revision (Fischer-Le Saux et al., 1999), the genus was recently split into three species, *Photorhabdus luminescens*, *Photorhabdus temperata*, and *Photorhabdus asymbiotica*. Two of these genera have also been subdivided into new subspecies. Thus, the *P. luminescens* group has three subspecies, *luminescens*, *akhurstii*, and *laumondii*, and the *P. temperata* group has one subspecies, *temperata*. The third species group, *P. asymbiotica*, which consists of isolates from human wounds recovered in the apparent absence of a nematode vector (Gerrard et al., 2003), has not yet been subdivided. Most of the genetic analyses have been performed on a limited number of strains: *P. luminescens* subsp. *akhurstii* strain W14, *P. luminescens* subsp. *laumondii* strain TT01, and *P. temperata* strains K122 and NC1. For brevity these strains will be referred to as *Photorhabdus* strains W14, TT01, K122, or NC1, or simply W14, TT01, K122, and NC1, except where reference to their different specific designation is relevant to the discussion.

The Need for Alternatives to *Bacillus thuringiensis*

To date, the majority of transgenes deployed in insect resistant crops are *Cry* genes from *Bacillus thuringiensis* or "Bt." Despite the diversity of *Cry* genes cloned from *B. thuringiensis*, only a few Cry proteins are toxic toward specific pests. This observation has led to concerns about the development of resistance to specific Cry toxins and over the subsequent management of cross-resistance to other toxins occupying the same or similar binding sites (Ives, 1996; McGaughey et al., 1998). These concerns are increasing as specific cases of laboratory-developed Bt resistance are documented in pest insects (McGaughey, 1985; Gahan et al., 2001). *B. thuringiensis* has also proved to be a useful source of other non-*Cry* genes such as the vegetative insecticidal proteins or "Vips" (Estruch et al., 1996; Yu et al., 1997); however, this organism clearly has a limited capacity to produce further toxins. The work described here on the isolation and characterization of insecticidal toxins from *Photorhabdus* and *Xenorhabdus* has been performed partly in the search for alternative novel insecticidal proteins for insect control.

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The Toxin Complexes

Discovery of the Toxin Complexes

Purification and cloning of Photorhabdus *toxins*

Despite the fact that *Photorhabdus* is released directly into the insect hemocoel by its nematode host, the culture supernatant of *Photorhabdus* strain W14 shows unexpected oral toxicity to the lepidopteran model *Manduca sexta* (Bowen and Ensign, 1998). A toxic high molecular weightprotein fraction was purified from the W14 supernatant by sequential ultrafiltration, dimethyl aminoethyl (DEAE) anion-exchange chromatography, and gel filtration (Bowen and Ensign, 1998). As a final purification step, high-performance liquid chromatography (HPLC) anion-exchange chromatography was used to separate four peaks or "Toxin complexes" A, B, C, and D (Bowen et al., 1998). Purified Toxin complex A (Tca) has a median lethal dose of 875 ng cm^{-2} of diet against *M. sexta*, and is therefore as active as some Bt Cry proteins (Bowen et al., 1998). The histopathology of orally ingested Tca shows the primary site of action to be the midgut, where cells of the midgut epithelium produce blebs as the epithelium itself disintegrates (Blackburn et al., 1998). Interestingly, injection of purified Tca also results in destruction of the midgut with a similar histopathology, suggesting that Tca can act on the gut from either the lumen or the hemocoel (Blackburn et al., 1998).

Each of the HPLC purified complexes migrates as a single or double species on a native gel but resolves into numerous different polypeptides on a denaturing sodium dodecylsulfate (SDS) gel (Bowen et al., 1998). The *toxin complex* (*tc*) encoding genes were cloned by raising both monoclonal and polyclonal antisera against the purified complexes and using these to screen an expression library. Each of the four complexes Tca, Tcb, Tcc, and Tcd is encoded by four independent loci *tca*, *tcb*, *tcc*, and *tcd* (Bowen et al., 1998). Each of these loci consists of an operon with successive open reading frames, for example, *tcaA*, *tcaB*, *tcaC*, and *tcaZ* (Fig. 1). These open reading frames encode the different polypeptides that can be resolved from the native complexes as confirmed by N-terminal sequencing of the individual polypeptides resolved on an SDS gel (Bowen et al., 1998). Genetic knockout of each of the *tca*, *tcb*, *tcc*, and *tcd* loci in turn showed that both *tca* and *tcd* contribute to oral toxicity to *M. sexta* and that removal of both these loci in the same strain renders the double mutant nontoxic (Bowen et al., 1998).

Similar independent purification approaches to the supernatant of strain W14 identified two high molecular weight complexes, confusingly termed "toxin A" and "toxin B," with oral activity to the coleopteran *Diabrotica undecimpunctata howardi* (Guo et al., 1999). These two toxins correspond to the species TcdA and TcbA, respectively, therefore confirming that both Tcd and Tcb also have activity against Coleoptera. The native molecular weights of TcdA and TcbA were estimated at 860 kDa, leading to the suggestion that they are tetramers of the 208 kDa species observed on an SDS gel (Guo et al., 1999). These species can also be proteolytically cleaved by proteases found in the culture supernatant and an increase in insecticidal activity associated with the cleaved form of the toxin was reported (Guo et al., 1999). However, the nature of the protease responsible and the relevance of the cleavage in the biological activity of the toxins remain obscure.

Cloning of Xenorhabdus *toxins*

Supernatants of some *Xenorhabdus* strains also show oral toxicity to insects. Oral insecticidal activity against another lepidopteran, *Pieris brassicae*, was used to identify *tc* gene homologs from *X. nematophilus* PMF1296 (Morgan et al., 2001). In this study, a cosmid

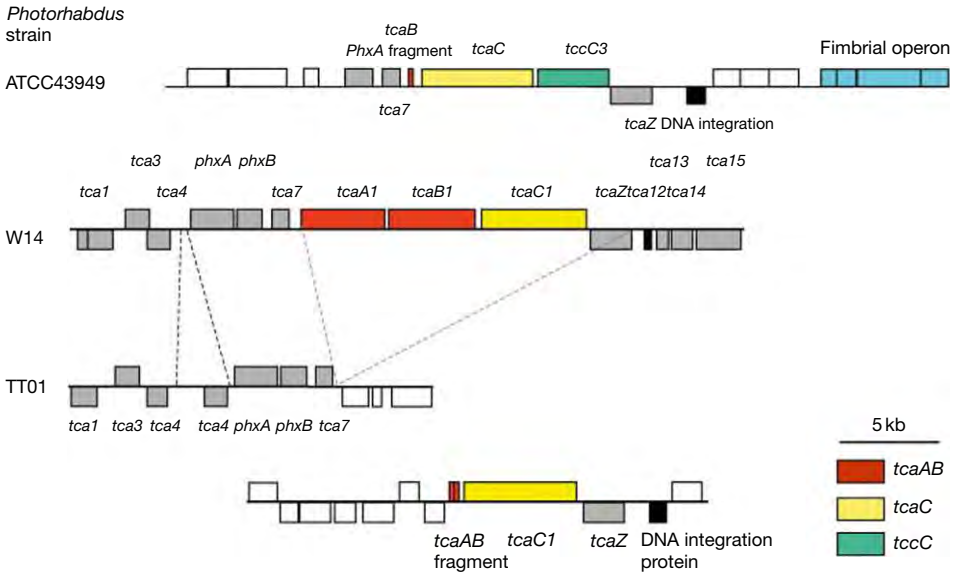

Fig. 1 The *toxin complex a* (*tca*) locus from three different *Photorhabdus* strains highlighting the three different color-coded elements *tcaAB*, *tcaC*, and *tccC* (see key). Note that this locus is only complete in strain W14 (in which it confers oral toxicity) and that in TT01 the locus is deleted from the equivalent location in the genome and found as a partially complete copy elsewhere (see text for discussion).

library was made in *Escherichia coli* and individual cosmids were screened for oral activity. Two overlapping cosmids with oral activity were recovered and one of these was completely sequenced. Transposon mutagenesis of the cosmids was then carried out to discover which open reading frames were involved in oral toxicity (Morgan et al., 2001). The genes encoding insecticidal activity were termed *Xenorhabdus protein toxins* (*xpt*) and insertions within the large gene *xptA1* were associated with a loss of oral activity (Morgan et al., 2001). The predicted amino acid sequences of the *xpt* genes show high similarity to the *tc* genes of *Photorhabdus* and their closest homologs have been indicated in a revised map of the open reading frames found in the cosmid (Fig. 2). The orally toxic *Xenorhabdus* cosmid therefore contains two *tcdA* homologs transcribed in opposite directions (*xptA1* and *xpta2*) with *tcaC* (*xptC1*) and *tccC* (*xptB1*) homologs between them. Expression of one *tcdA*-like gene (xptA1) alone in *E. coli* was sufficient to reproduce oral activity in *E. coli* (Morgan et al., 2001). This data suggests that the two different genera of nematode symbionts use similar toxin complex-like genes.

Genomic organization of Photorhabdus tc *genes*

Extended sequencing of the *tcd* locus in strain W14 revealed that clusters of *tcdA*-like genes were present in the *Photorhabdus* W14 genome (Fig. 3). Further, these multiple *tcdA* and *tcdB*-like genes are inserted next to an AspV tRNA in a potential pathogenicity island (Waterfield et al., 2002). This island is a region of DNA inserted in putative "core" sequence, i.e., sequence similar to core sequences in *E. coli* and *Yersinia pestis*. The island contains multiple copies of *tccC*-like sequences, which may promote recombination, and also enteric repetitive intergenic consensus (ERIC) sequences (Waterfield et al., 2002). The region also contains numerous directly duplicated open reading frames and a fragment of a *tcdA*-like gene, suggesting that it may be prone to rearrangement. Finally, a comparison of pathogenicity islands or phage inserted at the Asp tRNA in other related bacteria shows similar genomic islands carrying other toxin genes and rhs elements in place of *tccC*-like elements (Fig. 4). All these factors combine to support the concept that the *tcd*-island is a pathogenicity island. Moreover it is interesting to note that this island is adjacent to a gene *ngrA* implicated in nematode symbiosis (Ciche et al., 2001). Thus regions containing insect pathogenicity genes and nematode symbiosis factors may be linked in the W14 genome.

Homologs in Other Bacteria

Homologs of *tc* genes have now been found in other bacteria, both those pathogenic to insects and also bacteria with no obvious insect association (Waterfield et al., 2001b). Gene homologs are found in *Serratia entomophila*, a free-living bacterium causing "amber disease" in the New Zealand grass grub, *Costelytra zealandica* (Hurst et al., 2000). Larval disease symptoms after oral ingestion of *S. entomophila* include cessation of feeding, clearance of the gut, amber coloration, and eventual death. In *S. entomophila*, the disease determinants are encoded on a 115 kb plasmid designated pADAP, for amber disease-associated plasmid (Glare et al., 1993). Introduction of pADAP into *E. coli* is sufficient to confer oral toxicity on the whole bacteria (Hurst et al., 2000). The genes encoding the amber disease toxins were mapped to a 23 kb fragment of pADAP and then identified by insertional mutagenesis. The three disease-associated genes were termed *S. entomophila* pathogenicity (*sep*) genes (Hurst et al., 2000). These predicted products of

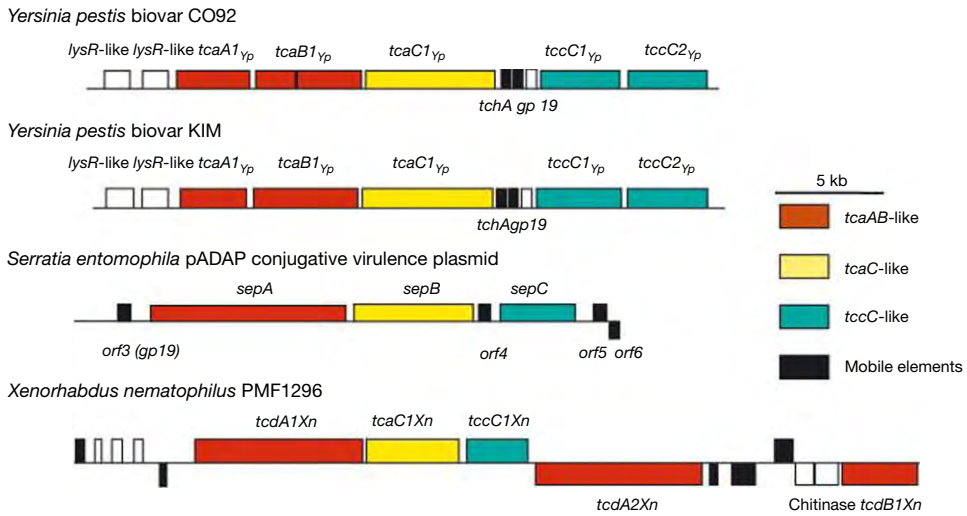

Fig. 2 Comparison of *tc*-like loci in entomopathogenic bacteria. *Yersinia pestis* is vectored by the flea and carries a *tca*-like locus, complete in biovar KIM but with *tcaB1* disrupted in biovar CO92. *Serratia entomophila* is a free-living bacteria that infects grass grubs. In this species the *tc*-homologous sepABC is carried on a conjugative plasmid required for insect pathogenicity. In *Xenorhabdus nematophilus*, vectored by a nematode, *tc*-homologous genes lie on a cosmid clone recovered on the basis of oral toxicity to caterpillars. Note again that the color coding indicates homology with the three central *tcaAB*, *tcaC*, and *tccC*-like elements (see key).

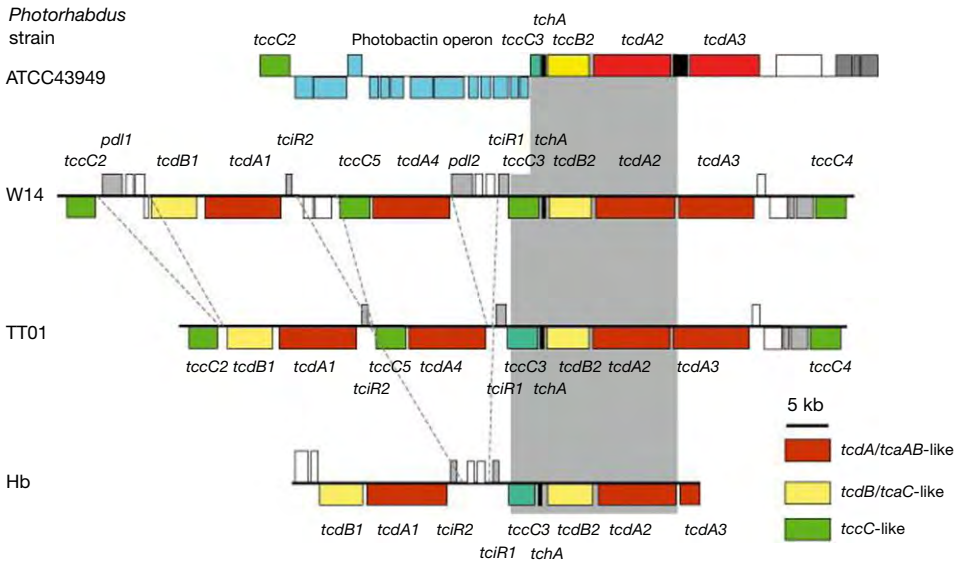

Fig. 3 The *toxin complex d* (*tcd*) locus from four different *Photorhabdus* strains highlighting the three different color-coded *tcaAB/tcdA*, *tcaC/tcdB*, and *tccC*-like elements (see key). Comparison of the four loci shows a conserved core (boxed in gray) and then localized deletions within and between the different *tcd* homologs (see text). Note that the *tcd* locus from W14 can confer oral toxicity on recombinant *Escherichia coli*.

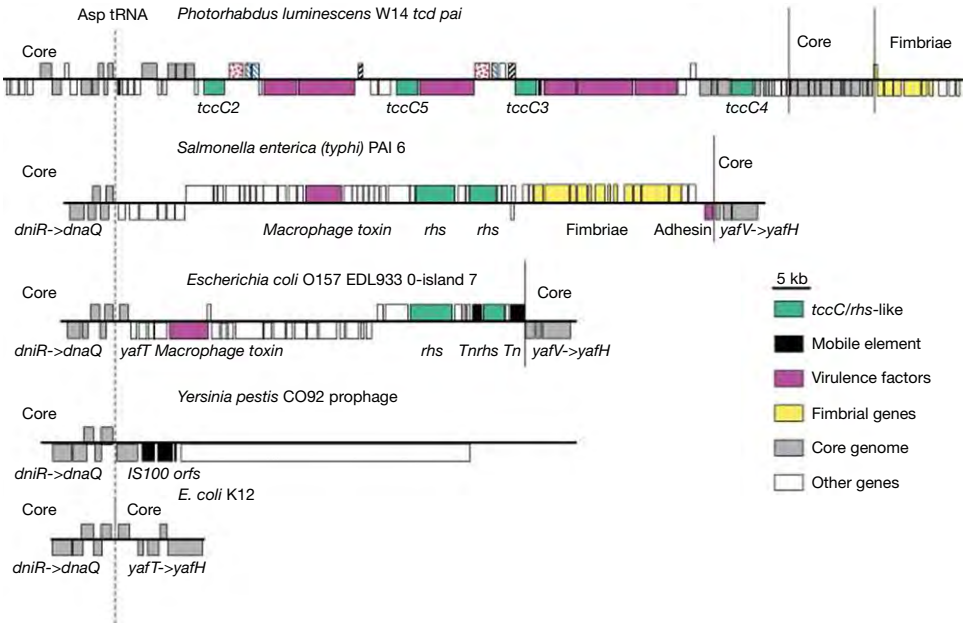

Fig. 4 Comparison of *Photorhabdus* W14 tcd pathogenicity island with pathogenicity islands also inserted at the Asp tRNA in other related bacteria. The "core" sequence flanking the inserted island is shown. Note the common elements of the pathogenicity islands sharing insertion near a tRNA, toxin genes and mobile elements (see key).

these genes show high similarity to those of the *tc* genes and are again clearly homologs. Thus *sepA* is *tcd*-like, *sepB* is *tcaC*-like, and *sepC* is a *tccC*-like gene (Fig. 2). The finding of *tc*-like genes in a bacterium with no known association with a nematode shows that this class of genes is not specific to nematode symbionts. Further, the presence of the sep genes on a plasmid supports the concept, derived from the *tcd* island of *Photorhabdus* W14 (Waterfield et al., 2002 ;ffrench-Constant et al., 2003), that the *tc* genes can be found on unstable or mobile stretches of DNA.

More recently, *tca*-like genes have also been discovered in both the sequenced genomes of *Y. pestis* (Parkhill et al. 2001; Deng et al., 2002), the causative agent of bubonic plague (Perry and Fetherston, 1997). Although highly pathogenic to man, *Y. pestis* is vectored by fleas and is therefore insect associated for part of its life cycle. Following uptake of an infected blood meal the

midgut of the flea becomes "blocked," a process facilitating transmission of the infected blood to the next mammalian host (Hinnebusch et al., 1996). The hypothesis that the *tca* homolog in *Y. pestis* may be involved in midgut blockage was investigated by making a knockout mutant of the gene and examining its effect on blood feeding in the flea. However, no direct effect of the tca mutant on midgut blockage was seen (ffrench-Constant, Perry, and Hinnebusch, unpublished data). The functional role of this gene in *Y. pestis* therefore remains unknown. The observation that one of the *tca* open reading frames is apparently disrupted in strain CO92 led Parkhill et al. (2001) to suggest the alternative hypothesis, that loss of *tca* function may actually be required for persistence of *Y. pestis* in the flea gut. Further, the presence of an intact *tca* homolog in the recent putative ancestor of *Y. pestis*, *Y. pseudotuberculosis* (S. Hinchcliffe, personal communication), supports the concept that *tca* function may have recently been lost.

Gene homologs can also be detected in the genomes of bacteria with no currently known associations with insects. In *Pseudomona syringae* pv tomato, there is a locus with *tcaC*, *tccB*, and *tccC* homologs (Fig. 5). Although this bacterium has no known association with insects, as a plant pathogen it may have an undescribed insect vector. Finally, and most recently, *tc* homologs have been found in the genome of *Fibrobacter succinogenes*, a commensal of ruminants with no obvious insect association. This growing range of noninsect associated bacteria with *tc* gene homologs may suggest that this class of genes is widely found in Gram-negative bacteria and that insect toxicity has been acquired in only a limited subset of *tc* gene homologs. The inferred ancestral function of *tc* homologs, however, remains obscure.

Molecular Biology of the *Toxin Complex* Genes

Toxin complex *gene organization*

The previous survey of *tc* gene homologs found in a range of bacteria leaves us with a confusing array of gene names and homologies. However, several aspects of the genomic organization of these genes in different species begin to indicate which may be the minimal functional units of insect toxicity. First, a color-coded comparison of *tc* loci in different bacteria shows that the different loci are mainly variations on a simple theme of three basic gene types: (1) *tcdA*-like genes, (2) *tcaC*-like genes, and (3) *tccC*-like genes (Figs. 1–3). Second, where isolated pieces of DNA can confer oral toxicity, such as the pADAP plasmid of *S. entomophila* (Hurst et al., 2000) and the cosmids isolated from *X. nematophilus* (Morgan et al., 2001), only these same three gene types are required for full toxicity. This effectively gives us a working hypothesis for attempts to recombinantly express *Photorhabdus* toxins, i.e., that combinations of these three types of genes are required for the expression of full oral toxicity.

Expression and structure of Photorhabdus *toxins*

Following the demonstration that knockout of either *tca* or *tcd* in *Photorhabdus* W14 decreases oral toxicity and in the absence of orally toxic cosmids carrying these loci, it was required to define the minimal components necessary for full oral toxicity in *E. coli*. In these experiments plasmids were inserted into *E. coli* carrying either (1) *tcdA* alone, (2) *tcdA* and *tcdB*, or (3) *tcdA*, *tcdB*, and *tccC* together. Only the plasmid carrying all three genes confers oral toxicity when expressed in *E. coli* and toxicity is increased by induction of the culture with mitomycin, suggesting that Tc production may be related to stress induced by this phage-inducing agent. Electron microscopy of high molecular weight particle preparations of the recombinant protein showed the Tc complexes to be "ball and stick" shaped particles of 25 nm in length (Waterfield et al., 2001a). Further, despite the fact that the presence of *tccC* is necessary for full oral toxicity, the particles themselves can be made by *tcdA* and *tcdB* alone. This suggests that TccC plays a critical role in the activation or export of the complex but does not change the physical structure of the *tcdAB*-derived structure.

Finally, despite the unexpected oral activity of the Tc toxins, how does *Photorhabdus* itself employ these toxins in a normal infection? Immunocytochemistry using an anti-TcaC antibody in insects infected with *Photorhabdus* W14 shows that Tca is produced during infection of the insect midgut (Silva et al., 2002). During infection, the bacteria occupy specific grooves in the midgut,

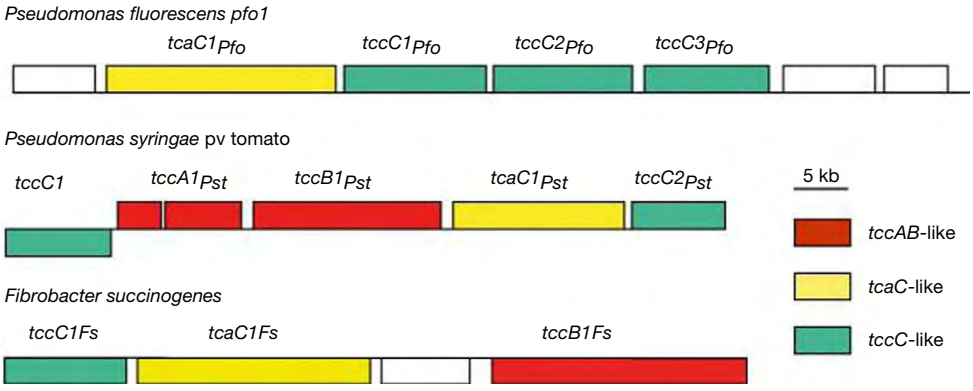

Fig. 5 Comparison of *tc*-like loci in bacteria with no known insect association showing that *tc*-like genes can also be found in plant pathogens with no known insect vector like *Pseudomonas syringae* and even ruminal commensals like *Fibrobacter succinogenes* (see text for discussion).

penetrating under the sheathing extracellular matrix to lie directly underneath the midgut epithelium. Within this sheltered niche the bacteria produce Tca, much of which appears to be associated with the bacterial outer membrane (Silva et al., 2002). In this fashion the gut active toxin can be delivered in extreme proximity to the midgut epithelium itself.

The Makes Caterpillars Floppy Toxins

Discovery of Makes Caterpillars Floppy

Cloning of mcf1

Although the Tc's account for the oral toxicity of some *Photorhabdus* supernatants, the nature of the toxins used by the bacteria growing within the hemocoel to actually kill the insect host remain unclear. To investigate potentially injectably active toxins individual cosmids were employed but this time injecting *E. coli* cultures carrying each cosmid individually into *M. sexta* caterpillars (Daborn et al., 2002). By injecting 2×107 cells per culture, 300 clones were injected and a single cosmid that resulted in death of the insect was recovered. As well as killing the insect, the cosmid also induced a rapid loss of body turgor, termed the "floppy" phenotype (Daborn et al., 2002). The cosmid was mutagenized with 126 single transposons to look for insertions showing the loss of either phenotype. The same insertions were also used as entry points to sequence the complete cosmid and identify open reading frames. Out of 126 insertions, 39 negated both phenotypes and all of these lay within a single 8.8 kb gene. This gene also caused both death and loss of body turgor when *E. coli* carrying this gene alone were injected into carterpillars. Therefore, this open reading frame was termed the *makes caterpillars floppy* gene or *mcf*. This open reading frame predicts a high molecular weight protein of 324 kDa. However, the predicted protein shows little homology to other proteins in the databases. Three regions of limited homology are present (Fig. 6). First, the C-terminus shows similarity to a repeated sequence found in RTX-like toxins (Schaller et al., 1999) potentially involved in toxin secretion. Second, part of the central domain is similar to the translocation domain from toxin B of *Clostridium difficile* (Hofmann et al., 1997). Finally, a region in the N-terminus matches the consensus sequence for a Bcl-2 homology domain 3 (BH3) domain. Vertebrate proteins involved in apoptosis that contain only this domain are proapoptotic (Budd, 2001), leading to the working hypothesis that Mcf itself may also promote apoptosis. Interestingly, if proved, this would be the first BH3 domain found outside of eukaryotes.

The observation that *E. coli* expressing Mcf alone can survive in the presence of the insect immune system is unexpected. Normally, *E. coli* carrying a pUC18 plasmid are cleared from the insect, presumably via the action of both antibacterial peptides and the insect phagocytes or hemocytes. In contrast, *E. coli* carrying pUC18-*mcf* and a second green fluorescent protein (GFP) expressing plasmid persist longer in the hemolymph. Dissection of infected animals shows that the Mcf and GFP expressing bacteria can colonize tissues in the insect and that the hemocytes fail to encapsulate the bacteria in melanin. Therefore, it was speculated that this failure to encapsulate Mcf expressing bacteria was due to hemocytes, dying via Mcf induced apoptosis. To investigate whether Mcf can promote apoptosis in hemocytes, the hemocyte monolayers were exposed to recombinant Mcf. The results show that exposed hemocytes, disintegrate rapidly, within 6 h, by producing numerous characteristic blebs. The residual actin cytoskeleton also shows a characteristic punctate pattern of staining. This proapoptotic effect on hemocytes helps to explain how recombinant *E. coli* can persist in the insect but fails to explain how the insects lose body turgor and die.

To examine the cause of the floppy phenotype, the histopathology of insects infected with *E. coli* expressing Mcf was investigated. Given that both the Malpighian tubules and the midgut are responsible for osmoregulation, destruction of either of these organs

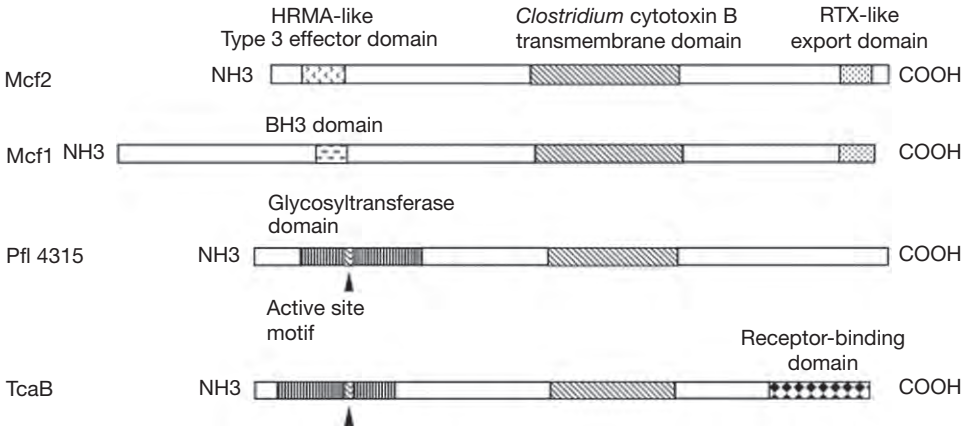

Fig. 6 The Makes caterpillars floppy (Mcf) toxins 1 and 2 compared to toxins from *Pseudomonas fluorescens* (Pfl 4315) and *Clostridium* (confusingly termed TcaB but not a Tc toxin). Both Mcf1 and Mcf2 have domains in their N-termini putatively associated with apoptosis, namely a BH3 and HRMA-like domain, respectively. Two regions of similarity within this class of toxins are highlighted. First a transmembrane domain found in the *Clostridium* toxin and second an RTX-like export domain found in the C-terminus of both Mcf proteins.

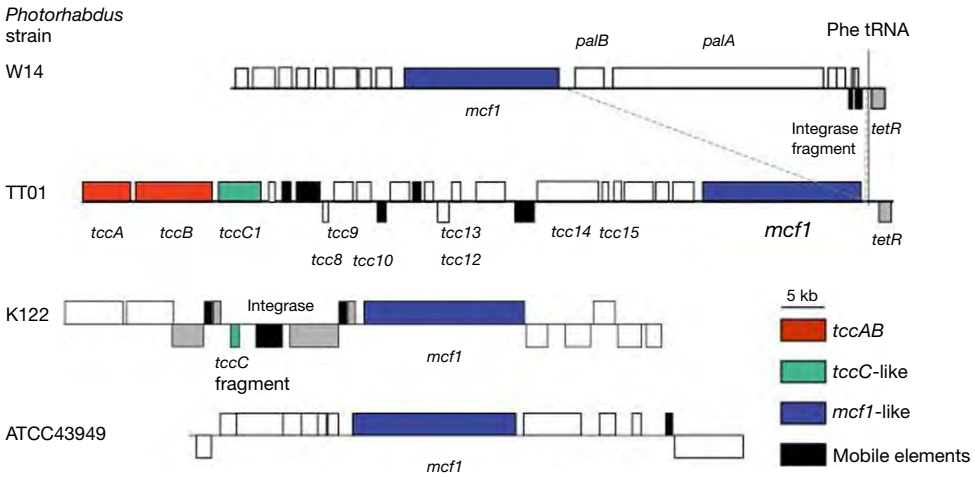

Fig. 7 Comparison of *mcf1* containing loci from four different *Photorhabdus* strains. Note that *mcf1* lies alongside a Phe tRNA in both W14 and TT01; however, W14 also carries *palBA*. In the other two strains sequenced, K122 and ATCC43949, *mcf1* appears to occupy different genomic locations suggesting that the toxin is mobile within the *Photorhabdus* genome.

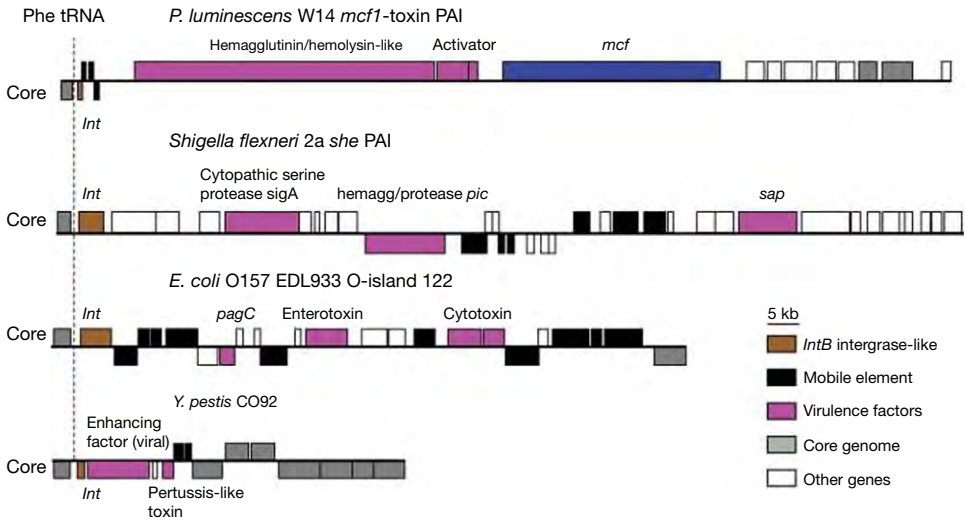

Fig. 8 Comparison of *Photorhabdus* W14 *mcf1* pathogenicity island with pathogenicity islands also inserted at the Phe tRNA in related bacteria. The "core" sequence flanking the inserted island is shown. Note the common elements of the pathogenicity islands sharing insertion near a tRNA, toxin genes and mobile elements (see key). Note also the similarities in genomic organization with the *tcd* island in Fig. 4.

could result in a rapid loss of body turgor. Sections of infected caterpillars showed that the Malpighian tubules were intact at the time of death but cells of the midgut epithelium were severely affected as early as 12 h post injection. Both goblet and columnar cells shed circular blebs, often including the nucleus, into the midgut lumen. Nuclei within the dying cells appear pycnotic and stain terminal deoxynucleotidyl transferase-mediated duTP end labeling (TUNEL) positive, suggesting that they are apoptotic. These observations are consistent with Mcf having a proapoptotic function and with its primary site of action being the insect midgut. However, some caution is necessary in interpreting these results as insects ingesting *B. thuringiensis* Cry of Vip toxins also show a similar histopathology of the midgut. More specific experiments to show that Mcf is proapoptotic are therefore needed.

Comparison of the genomic location of *mcf1*-like genes between *Photorhabdus* strains confirms two important points (Fig. 7). First, *mcf1*-like genes appear to be present in all strains of *Photorhabdus*, as would be expected if Mcf1 is a dominant toxin. Second, like the *tc* toxins, the location of *mcf1* differs between genomes and is often associated with either a Phe tRNA or integrase genes. In fact in strain TT01, *mcf1* is adjacent to a Phe tRNA on one side and the downstream of the *tccC* locus, suggesting that in this case both a *tc* and *mcf* locus may be found together on the same pathogenicity island. Finally, comparison of the relative genomic location of the island containing *mcf1* in *Photorhabdus* strain W14 with other islands inserted at the Phe tRNA in related bacteria again show a similar pattern with other toxins being inserted alongside integrase genes or integrase gene fragments (Fig. 8).

This confirms that *Photorhabdus*, like related enteric bacteria, carries a range of toxin encoding pathogenicity islands inserted at tRNA genes.

Cloning of mcf2 *homologs*

Sample sequencing of the *P. luminescens* W14 genome revealed the presence of a second *mcf*-like open reading frame termed *mcf2*. The predicted Mcf2 protein is similar to Mcf1 (Fig. 6) but is significantly shorter at the N-terminus. Further, rather than carrying BH3-like domain, part of the N-terminus shows similarity to HrmA from *Pseudomonas syringae pv. syringae* (Fig. 6). The *hrmA* gene encodes a protein that has an avirulence (avr) phenotype in plants, travels a type III (Hrp) secretion system, and also elicits cell death when transiently expressed directly in tobacco cells. This suggests that HrmA is a type III delivered effector that can cause apoptosis in plant cells. The similarity of HrmA with part of Mcf2 suggests that Mcf2 may also be involved in inducing apoptosis but via a different mechanism than Mcf1. In this case, it is interesting to note how a type III effector protein such as HrmA, which is normally delivered directly into the cell, has become fused within the N-terminus of Mcf2 to be delivered within the high molecular weight protein via an undescribed mechanism. Comparisons of available sequence data from different *Photorhabdus* strains suggests that copies of *mcf1* are always present, suggesting it may be the dominant insect killing toxin (Fig. 7). However, we remain uncertain about the distribution of *mcf2* and although this toxin is present in W14 and TT01, this gene is not present in currently available sequence from the *P. asymbiotica* isolate being sequenced at the Sanger Center. It is therefore not clear if *mcf2* is a required toxin present in all *Photorhabdus* strains like *mcf1* or if it is only present in a subset of strains.

Toxin Genomics

Microarray Analysis

The widespread distribution of the *tc* genes between different bacteria raises interesting questions as to their origins and transmission. To examine these questions in *Photorhabdus* strains the simple question should be answered: is oral toxicity monophyletic? In other words, do all *Photorhabdus* strains with orally toxic supernatants belong to the same taxonomic group? To address this question, two parallel approaches were used. First, phylogenetic analysis of orally toxic and nonorally toxic strains using 16S DNA sequences was carried out. Second, hybridized genomic DNA from each strain to a microarray carrying 96 putative virulence factor genes from the orally toxic *Photorhabdus* strain W14 was done to investigate the minimal subset of *tc* genes required for oral toxicity.

The phylogenetic analysis showed that the six orally toxic strains examined fell into two distinct taxonomic groups. The first (IS5, EG2, IND, and W14 itself) lies within the previously recognized *P. luminescens* subsp. *akhurstii* subspecies. The second (Hb1 and Hm1) represents a different subgroup, *P. luminescens* subsp. *luminescens* (Fig. 9). The microarray analysis also revealed groups of genes either conserved or variable between the strains examined (Fig. 10). Conserved genes included genes such as lux, which produces light, a phenotype common to all *Photorhabdus* (Forst and Nealson, 1996). A series of putative virulence factors also appear conserved in most, or all, strains, including the toxin encoding gene *mcf1* (Daborn et al., 2002), *rtxA1* and *rtxA2*-like genes, the operon encoding the *prtA* protease, and an *attachment and invasion locus* (*ail*) homolog (ffrench-Constant et al., 2003). The type III secretion system (Waterfield et al., 2002), often associated with virulence in other Gram-negative bacteria (Galan and Collmer, 1999), is also present in all strains. Other conserved genes include those encoding catalase, chitinase, ferrochetalese, and flagellae. Variable genes include bacteriocins, toxins/hemolysins, insertion elements, pili, and genes involved in iron acquisition. Some toxin and hemolysin/hemagglutinin genes are also variable between strains. These include *pnf*, a *Photorhabdus necrotizing factor* that is found within a recently acquired region of the W14 genome (Waterfield et al., 2002) which is absent from TT01, and the *palA* and *palB* genes, encoding a hemolysin/hemagglutinin and its export activator (Waterfield et al., 2002). In W14, *palBA* is immediately downstream of the *mcf1* toxin gene and adjacent to a Phe tRNA (Waterfield et al., 2002) whereas in TT01 (Fig. 7), *palBA* is deleted from the equivalent location, as predicted. In contrast, a second two-component hemolysin locus phlAB (Brillard et al., 2002), is predicted to be similar in all strains, and indeed potentially duplicated in strain Hm.

The *tc* genes correspond to variable genes, whose distribution is strikingly split between orally toxic and nontoxic strains (Fig. 11). Orally toxic strains carry all three genes in the *tca* operon (*tcaA*, *tcaB*, and *tcaC*) whereas those lacking toxicity lack *tcaA* and *tcaB*. The genes of the *tca* operon are the only genes to show a perfect correlation with oral toxicity, and genes from *tcb*, *tcc*, and *tcd* are all variable across orally toxic strains. For the *tca* locus (Fig. 1), a comparison of W14 and TT01 shows two important findings. First, the W14 *tcaABC* operon is absent from the equivalent location in TT01. Second, a *tca*-like locus is present elsewhere in TT01 but lacks most of *tcaA* and *tcaB*, which have been deleted, and retains only a *tcaC1*-like gene. These observations confirm that the presence of *tcaAB* is necessary for oral toxicity of the bacterial supernatant. Further, the fact that *tca*-like loci can be found at different locations in different genomes supports the concept that the *tca* locus is mobile, as suggested by the presence of either a transposase, in W14, or an integration protein, in TT01, adjacent to both *tca*-like loci (Fig. 1). For the *tcb* locus, the suggestion that *tcbA* is lacking from TT01 is again confirmed by analysis of the genomic sequence, as *tcbA* is deleted from the equivalent location in TT01 and a transposase left in its place. Again comparing W14 and TT01, the array suggests that the *tcc* locus should be completely conserved, as supported by an examination of the genomic sequence. Finally for *tcd*, the genomic sequence (Fig. 3) confirms that all the *tc* genes of the island are present in both W14 and TT01, including both *lysR*-like regulators. Further, the predicted loss of *tcdA4* in group 2 orally toxic strains is confirmed by an

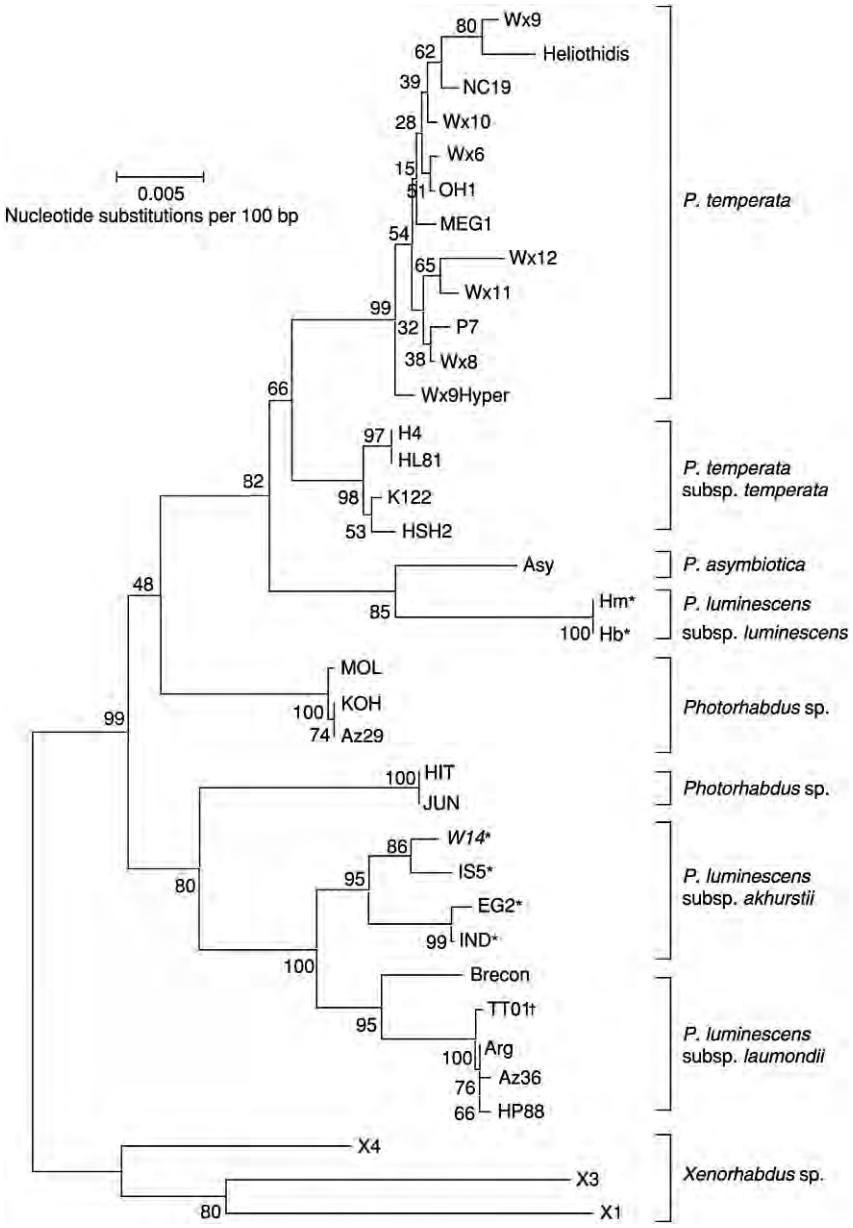

Fig. 9 Phylogenetic analysis of orally and nonorally toxic strains used in the microarray analysis based on 16s DNA sequencing. Note that orally toxic strains (*) fall into two well-supported phylogenetic groups. Strain TT01 (†) has recently been completely sequenced (see text).

examination of this locus in the Hb strain. The array even predicts the loss of *pdl1* and *pdl2* from within the *tcd* island of TT01, again confirmed by the sequence (Fig. 3). The localized deletion of *tcdA4*, and the apparent rearrangements mediated by *tccC*-like genes, again support the hypothesis that the *tcd* genes are encompassed in an unstable pathogenicity island. However, the consistent maintenance of four genetic elements within this island (*tcdA2*, *tcdB2*, a *gp13*-like holin, and the conserved region of *tccC3*), and the conserved organization of similar *tcd*-like genes in other bacteria (such as *sepA*, *sepB*, *orf4*, and *sepC* in Serratia), suggest that these genes form an invariant, but not orally toxic, core.

Previous analysis of strain W14 showed that either *tca* or *tcd* can independently contribute to oral toxicity. Thus plasmid clones of either *tca* or *tcd* from W14 confer recombinant oral toxicity when expressed in other nonorally toxic *Photorhabdus* strains, such as K122. This data is consistent with the current observation that the presence or absence of *tcaAB* is perfectly correlated with toxicity of the supernatant. However, all the tc genes within the tcd island are also present in some strains, such as TT01 (Fig. 3), which lack orally toxic supernatants. To investigate the apparent lack of oral toxicity associated with *tcd* in TT01, the toxicity of the bacterial cells was examined independently of their supernatant and it was found that toxicity is associated with the cells rather than the supernatant (Fig. 12). Confirmation that this novel cell-associated toxicity is *tcd* related now requires knockout or heterologous expression of this locus. In conclusion, even a very limited microarray can provide a powerful and predictive tool for correlating

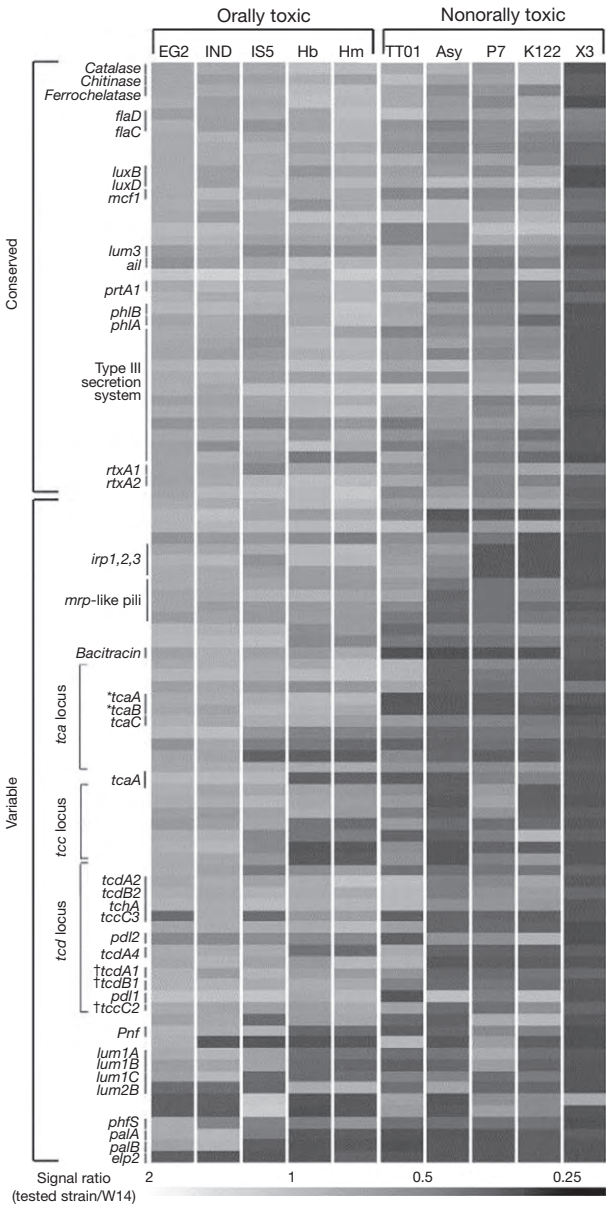

Fig. 10 Diagrammatic representation of microarray results where DNA from orally and nonorally toxic strains was hybridized to 96 W14 virulence factor genes. Genes have been divided into conserved (showing a consistent hybridization signal in all strains) and variable (those showing a variable signal between strains). Note, for example, that the type III secretion system appears present in all *Photorhabdus* strains, whereas members of the *tc* genes are variable (see text). The two loci implicated in oral toxicity are indicated: *tcaAB* (*) and *tcdAB* (†).

observed phenotypes with bacterial genotypes. Similar microarrays may therefore be useful in investigating other *Photorhabdus* phenotypes involved either in insect pathogenicity or symbiosis with their nematode hosts.

Conclusions

With the publication of the full genome sequence of strain TT01 and the ongoing sequencing of a *P. asymbiotica* strain at the Sanger Center, we are entering an era of comparative *Photorhabdus* genomics. As this article has indicated this now allows us to begin to determine where given toxin genes might have come from, and how they are passed between different strains. In the case of the tc genes, the appearance of orally toxic *tca* genes in divergent strains and the finding that these genes can occur at different locations in the genome support the hypothesis that they are mobile between strains. Microarray analysis of more toxin genes in a wider array of strains may also cast light on the origins of other toxin genes such as *mcf1* and *mcf2*. Despite this recent explosion in sequence data, perhaps the most important biological questions remain largely unanswered. Thus, what is the biological role of the array of toxins

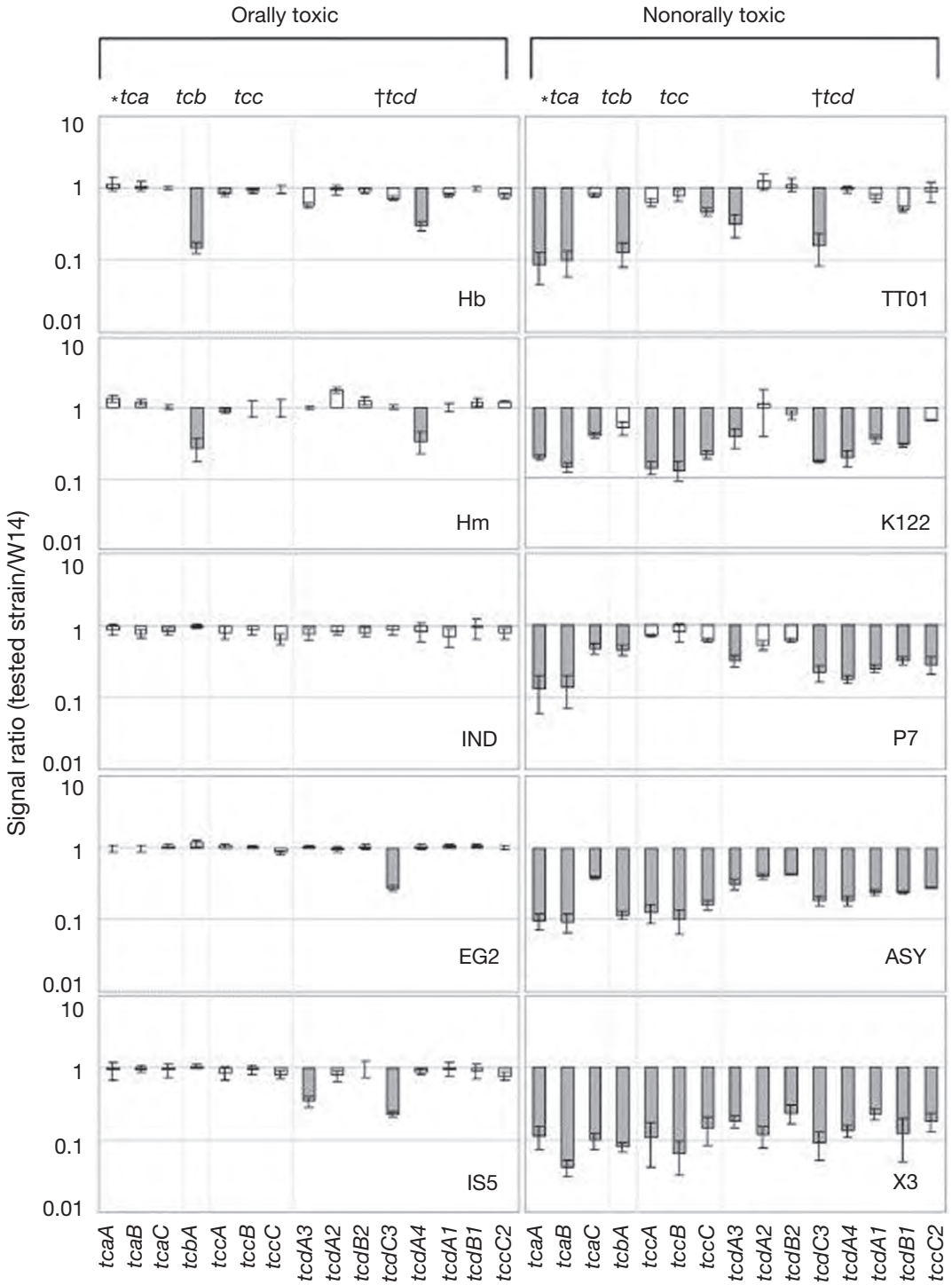

Fig. 11 Detailed analysis of the array results for *tc* genes only. In this diagram ratios close to 1 predict the presence of a gene and ratios greater than 0.5 (shaded histograms) predict it to be absent. Note that only the presence of tcaAB (*) is perfectly correlated with the presence of oral toxicity in the different strains (see text) and that tcd (†) is variable between strains.

that *Photorhabdus* encodes? Although it is easy to infer that these toxins may be necessary to kill the insect host, assessing the role of individual toxins via genetic knockout may be hampered by their functional redundancy. Thus, *tc* gene knockouts may show no oral activity but they can still kill the insect host. Further, mutants lacking mcf1 take a longer time to kill insects but are still lethal. The current challenge is therefore to dissect out the individual roles of each toxin gene, even when they are members of large and rapidly growing gene families.

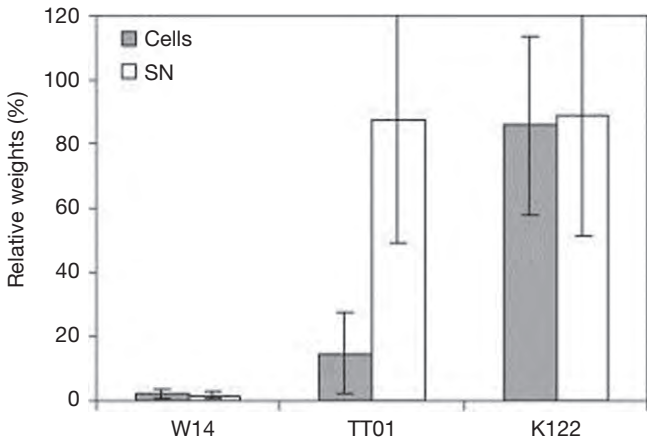

Fig. 12 Comparison of supernatant (SN) versus cell-associated oral toxicity for three different *Photorhabdus* strains. Note that strain TT01 containing a complete copy of the tcd locus has no oral toxicity associated with the bacterial supernatant but does show oral toxicity associated with the bacterial cells (see text for discussion).

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Intracellular Structures of Prokaryotes: Inclusions, Compartments and Assemblages☆

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Glossary

Archaea A phylogenetic domain of prokaryotes consisting of methanogens, halophiles, hyperthermophiles, and extreme acidophiles.
Autotroph An organism capable of using carbon dioxide (CO_2) as a sole source of carbon.
Carbonic anhydrase An enzyme involved in the transport and release of CO_2 from bicarbonate in physiological processes.
Carboxysomes Polyhedral microcompartments (PHMCs) (simple organelles) found in many autotrophic prokaryotes and capable of fixing CO_2 using the enzyme ribulose bisphosphate carboxylase.
Chlorosomes The cigar-shaped organelles of green bacteria enclosed in a nonunit membrane containing the light-harvesting bacteriochlorophyls.
Gas vesicle A proteinaceous, gas-filled, cylindrical or conical inclusion (compartment) found in many prokaryotes. When present in sufficient numbers confers buoyancy to the cell.
Ladderanes Cyclobutane-containing lipids of planctomycetes.
Operon A prokaryotic transcriptional unit consisting of several different genes cotranscribed from one promoter.
Organelle (prokaryotic) A simple, but metabolically functional structure in prokaryotes that is surrounded by a barrier of some sort (protein shell or coat, a lipid monolayer, lipid–protein monolayer, or a unit membrane.
Planctomycetes Unusual members of Bacteria that lack peptidoglycan and contain membrane enclosed compartments inside the cell.
Proteasomes Large, nanocompartmentalized proteolytic machines that couple ATP hydrolysis with the unfolding and hydrolysis of protein substrates in the maintenance of protein quality and regulation of cell function.

Abbreviations

4HB	4-Hydroxybutyrate
ADRP	Adipose differentiation-related protein
atf	Acyltransferase
BChl	Bacteriochlorophyll
CDF	Cation diffusion facilitators
CGP	Cyanophycin grana protein
CP	Core particle
Csm	Chlorosome membrane
DAPI	4′-6′-Diamino-2-phenylindole
eGFP	Enhanced green fluorescent protein

☆*Change History:* August 2014. E Gantt has been deleted and DA Bryant and D Schüler address has been changed. Shively et al. introduced small edits in the text of the article including Figures 2 and 5–7 and Further Reading.

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FMO	Fenna–Matthews–Olson
HCR	High CO_2-requiring
L_{CM}	Core-membrane linker
MAI	Magnetosome island
Mam	Magnetosome membrane
MM	Magnetosome membrane
Mms	Magnetic particle membrane-specific
MTB	Magnetotactic bacteria
Mtx	Magnetotaxis
Ntn	N-terminal nucleophile
PBP	phycobiliprotein(s)
PBS	phycobilisome(s)
Pdu	Propanediol utilization
PHA	Polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHMCs	Polyhedral microcompartments
PPKs	Poly P kinases
PPXs	Exopolyphosphatases
PS	Photosystem(s)
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SLD	Small lipid droplets
TAG	Triacylglycerol
WE	Wax esters

Defining Statement

Intracellular prokaryote inclusions, compartments and assemblages represent certain aspects of an organism's metabolic capability. Each of the structures, based on their primary function, is placed into one of three categories: (1) structures as metabolic machinery, (2) structures as contributors to cell mobility, (3) structures as metabolic reserves.

Introduction

The first observation of a prokaryote intracellular structure, a 'spherical body,' was in a 'colorless egg-shaped alga' in 1786, but the inclusion was not shown to be sulfur until nearly 100 years later. Other intracellular structures, gas vacuoles, and granules of cyanophycin, glycogen, lipid, and polyphosphate were observed toward the end of the nineteenth century, but were not extensively characterized until well into the twentieth century. Their clear-cut demonstration, isolation, and characterization awaited the availability of electron microscopy and the introduction of numerous techniques from a variety of disciplines including biochemistry, cell biology, genetics, microbiology, and molecular biology. The development of more sophisticated methodologies and technologies also gave rise to the discovery and characterization of many new structures. Discoveries and characterizations continue to this century.

The term 'inclusions' has been used for many years to categorize a wide variety of intracellular structures in prokaryotes. Although the term still appears to be applicable to some of the intracellular structures, the detailed examination and characterization elucidating the complexity of many of the structures, including some referred to as inclusions, has necessitated the introduction of new, more descriptive terms, for example, protein complexes, nano- and microcompartments, and simple organelles. In this article, a brief, up-to-date overview of each of the 14 intracellular structural types is given. Each of the structures, based on their primary function, is placed into one of three categories: (1) structures as metabolic machinery, (2) structures as contributors to cell mobility, and (3) structures as metabolic reserves. For more details on the structures as well as information on less common or less studied structures the reader is directed to 'Further reading.'

Structures as Metabolic Machinery

Carboxysomes and Other Polyhedral Microcompartments

Polyhedral microcompartments (PHMCs) are regularly shaped proteinaceous complexes 90 to over a few hundred nm in diameter that consist of a thin (3–4 nm) protein shell and a core of metabolically active enzymes. They are believed to be functionally analogous to eukaryotic organelles and have been classified into at least seven different types, depending on the enzymes they

sequester, but their exact role in various aspects of microbial metabolism in which they are thought to participate have not yet been fully elucidated. On the basis of comparative genomic analysis, PHMCs are predicted to exist in a wide range of bacterial lineages. Apart from their distinct polyhedral appearance and despite their diverse proposed metabolic roles, all PHMCs share two families of genes that are required for the formation of their shells. One of these (Pfam 00936) encodes the major shell proteins, which assemble into hexamers that form the facets of the polyhedral PHMC shell. The proteins encoded by the other family (Pfam 03319) are minor shell components that are thought to occupy the vertices of the polyhedra. The similar amino acid sequences of the shell proteins belonging to these families in different PHMCs suggest a common role in metabolic organization that serves to optimize and perhaps regulate metabolic flow through the particles by an as yet poorly understood molecular mechanism.

The PHMCs that were identified first were the carboxysomes. These organelles were originally detected by electron microscopy as 'polyhedral bodies' in thin sections of cyanobacteria and have since been shown to be present in all cyanobacteria and in many chemoautotrophs (Figure 1(a)–1(c)). Biochemical analysis of purified carboxysomes from the chemoautotrophic sulfur oxidizer *Halothiobacillus neapolitanus* demonstrated that their lumen is filled with the CO_2-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the key enzyme of autotrophic carbon metabolism (Figure 1(d)). This discovery led to the term carboxysomes for the microcompartments of autotrophic bacteria. Pure *H. neapolitanus* carboxysomes were subsequently dissected molecularly into multiple polypeptides, which are present in the microcompartment in defined stoichiometric relationships. Partial sequence determination of individual carboxysome proteins led to the identification of their genes. The manner in which most of the carboxysome genes are arranged in the genome of *H. neapolitanus* and in many other autotrophs suggests that they form an operon consisting of *cbbL* and *cbbS*, the genes for the large and small subunit, respectively, of RuBisCO; *csoS2*, a gene that specifies a shell protein of unknown function; *csoS3*, which encodes the carboxysomal carbonic anhydrase; and *csoS1A*, *-B*, and *-C*, three paralogs that code for the Pfam 00936 shell proteins. The *csoS4A* and *-B* genes encode the Pfam 03319 vertex proteins.

Mutants of cyanobacteria and chemoautotrophs that lack carboxysomes display a high CO_2-requiring (HCR) phenotype and either grow poorly or not at all at ambient CO_2 concentrations. On the basis of these observations, a model has been proposed in which carboxysomes function as the final component of a carbon dioxide (CO_2)-concentrating mechanism that improves the CO_2 fixation ability of the sequestered RuBisCO. Most autotrophic bacteria concentrate inorganic carbon as cytosolic bicarbonate to levels as much as 1000 times higher than those in their extracellular environment. Since RuBisCO is only able to utilize CO_2 as a

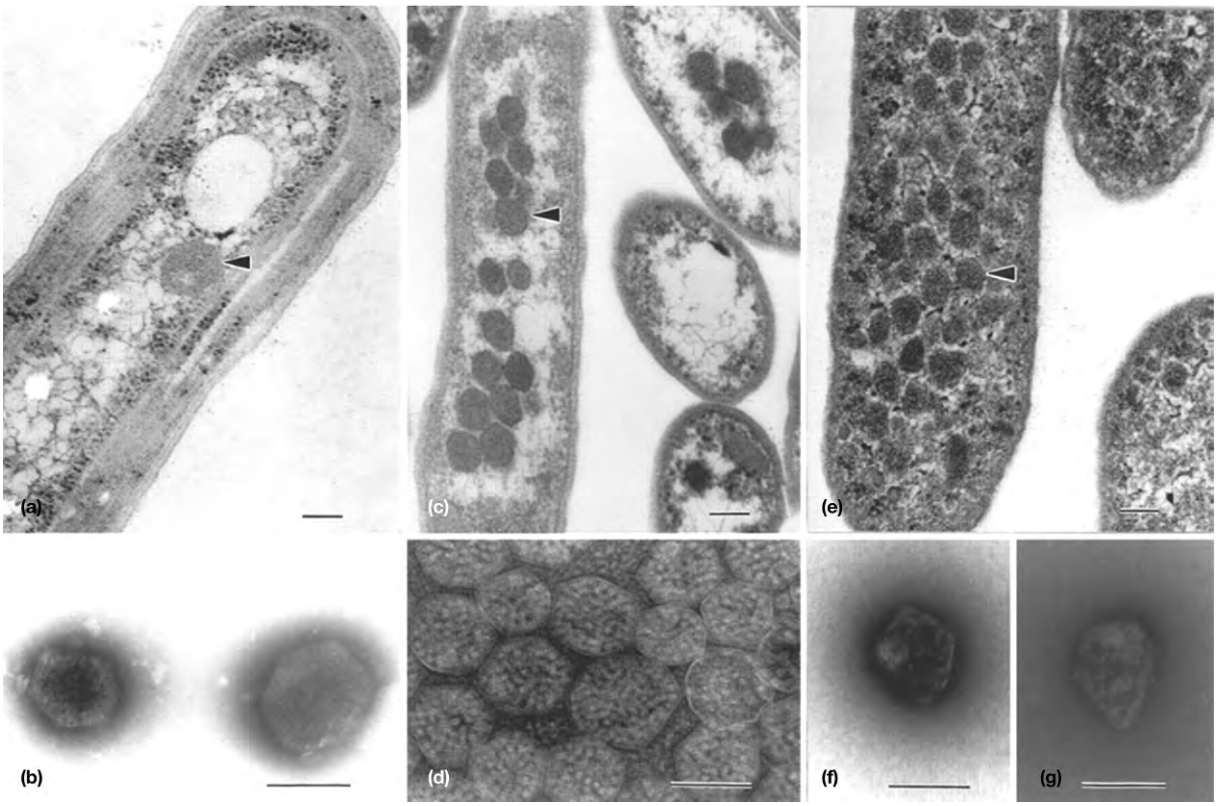

Figure 1 Electron micrographs of polyhedral microcompartments (PHMCs). (a) Thin section of *Synechococcus* PCC7942. The arrow indicates a typical cyanobacterial carboxysome. (b) Negatively stained carboxysome from a lysed cell preparation of *Synechococcus*. (c) Thin section of *Halothiobacillus neapolitanus* with arrow indicating carboxysomes. (d) Negative stain of purified carboxysomes of *H. neapolitanus*. Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) molecules are visible inside the particle. (e) Thin section of *Salmonella enterica* grown on propanediol with arrow indicating PHMCs. (f and g) Negative-stained isolated PHMCs from *S. enterica*. Bar = 100 nm. Reprinted with permission from Cannon GC, Bradburne CE, Aldrich HC, et al. (2001) Microcompartments in prokaryotes: Carboxysomes and related polyhedra. *Applied Environmental Microbiology* 67: 5351–5361.

substrate, the carboxysomal carbonic anhydrase is required to rapidly convert HCO_3^- to CO_2, thereby effectively raising its local concentration in the vicinity of the sequestered RuBisCO. The structure of the major shell proteins suggests that the shell may actively regulate access of small molecules to the carboxysome interior and may thereby limit the competitive inhibition of RuBisCO activity by oxygen.

PHMCs in heterotrophic bacteria have been referred to as enterosomes, polyhedral organelles, or metabolosomes. The particles from propanediol-grown *Salmonella enterica* (Figures 1(e)–1(g)) contain multiple enzymes that participate in propanediol degradation; their shells are formed by orthologs of the two common PHMC shell proteins. The proteins are encoded by the 23 genes of the propanediol utilization (*pdu*) operon. The organelle may prevent release of the toxic intermediate propionaldehyde into the cytoplasm. Similar particles that are formed by *S. enterica* grown on ethanolamine contain ethanolamine ammonia lyase and are believed to prevent the escape of acetaldehyde, a volatile intermediate of the ethanolamine degradation pathway.

Judging from the data emerging from the ever-growing list of sequenced bacterial genomes, homologues of genes that encode PHMC shells are widespread among the bacteria, and it appears that organization of key enzymes by carboxysome-like shell proteins is a general mechanism used by bacteria to enhance or control crucial metabolic steps. In recent years, the potential of PHMcs for synthetic biology purposes has led to multiple interdisciplinary research efforts that have tackled the molecular mechanism of enzyme sequestration and PHMC assembly, the physical characteristics of the shell, and the molecular mechanism behind PHMC function.

Anammoxosomes

Anammoxosomes are unique metabolically significant compartments performing the **anammox** process found so far only within anaerobic planctomycetes. These bacteria carry out **An**aerobic **Amm**onium **Ox**idation, a chemolithotrophic and usually autotrophic metabolism of significant environmental importance. They comprise *Candidatus* genera 'Brocadia,' 'Kuenenia,' 'Scalindua,' all autotrophs, and 'Anammoxoglobus,' which exhibits some heterotrophic oxidation of propionate as well as autotrophic ammonium oxidation. These have been mostly characterized from wastewater treatment bioreactors and none are yet in pure culture. Representatives also occur widely in marine sediment and other marine anaerobic habitats. During anammox metabolism, ammonium is oxidized under anaerobic conditions using nitrite as the electron acceptor with molecular dinitrogen as the final product. The anammox process is of major significance for the global nitrogen cycle, especially from their activity in anoxic marine water and sediment. Carbon dioxide is fixed when anammox organisms grow anaerobically on ammonium and some nitrite is oxidized anaerobically to nitrate and this may supply electrons for carbon dioxide reduction via reverse electron transport. Anammox planctomycetes thus need only ammonium, nitrite and carbon dioxide as major growth substrates. In recent models the mechanism is thought to involve nitric oxide generated from nitrite reduction which oxidizes ammonium to yield hydrazine which is in turn oxidized to dinitrogen. Like the cells of other planctomycetes, all named anammox species possess a planctomycete-characteristic cell plan involving a single-membrane-bounded pirellulosome compartment containing the nucleoid as well as a paryphoplasm region surrounding the outer rim of the cell. Within the pirellulosome however they possess another single-membrane-bounded compartment, the anammoxosome, unique to anammox planctomycetes (Figure 2(a)). The anammoxosome envelope divides the cell cytoplasm into two compartments, the riboplasm outside the anammoxosome and the contents of the anammoxosome. The anammoxosome harbours at least one specialized enzyme, a hydrazine oxidoreductase functional in the mechanism of anaerobic ammonium oxidation and important to formulation of models for anammox metabolism.

The anammoxosome is wrapped in a single-membrane envelope possessing unique cyclobutane-containing lipids termed ladderanes (Figure 3), which may confer relatively high density and impermeability to the anammoxosome membrane. Intriguingly from an evolutionary perspective, the ladderanes occur in both ether-linked and ester-linked forms in these mesophiles. This envelope has been hypothesized to have specialized functional roles e.g. providing protection from toxic intermediates of anammox metabolism (such as hydrazine) and responsibility for vectorial proton gradient formation related to bioenergetic mechanisms of anammox metabolism.

The anammoxosome and its envelope have formed an important part of models of anaerobic ammonium oxidation in these bacteria. According to these models (Figure 4), during electron transport occurring along with ammonium oxidation, protons are consumed in the riboplasm and protons generated inside the anammoxosome, so that a proton gradient is generated relative to an alkaline riboplasm. An ATPase/synthase embedded in the anammoxosome envelope would then be responsible for ATP synthesis chemiosmotically from the proton gradient generated by electron transport during ammonium oxidation. Such an ATPase/synthase is detectable via immunogold electron microscopy associated with anammoxosome membrane, and a pH gradient detected via NMR, consistent with this model. The anammoxosome membrane also may be the site of cytochrome c maturation. The anammoxosome has other characteristics which may relate more to cell biology, since within the anammoxosome tubules have been demonstrated which in some cases form distinct arrangements suggesting a cytoskeletal function (Figure 2(b)), and nucleoid is often in contact with anammoxosome envelope. The anammoxosome has the potential to be the first planctomycete compartment for which a specialized functional role will be demonstrated. This may occur with help of data from the '*Candidatus* Kuenenia stuttgartiensis' genome project, which is among the first such projects to sequence the complete genome of a bacterium not yet available in pure culture. A near-complete 4.2 Mb genome sequence for *Candidatus* Kuenenia stuttgratiensis is available and has yielded insights about models for metabolism catalyzed by the anammoxosome and how ladderane lipids may be synthesized.

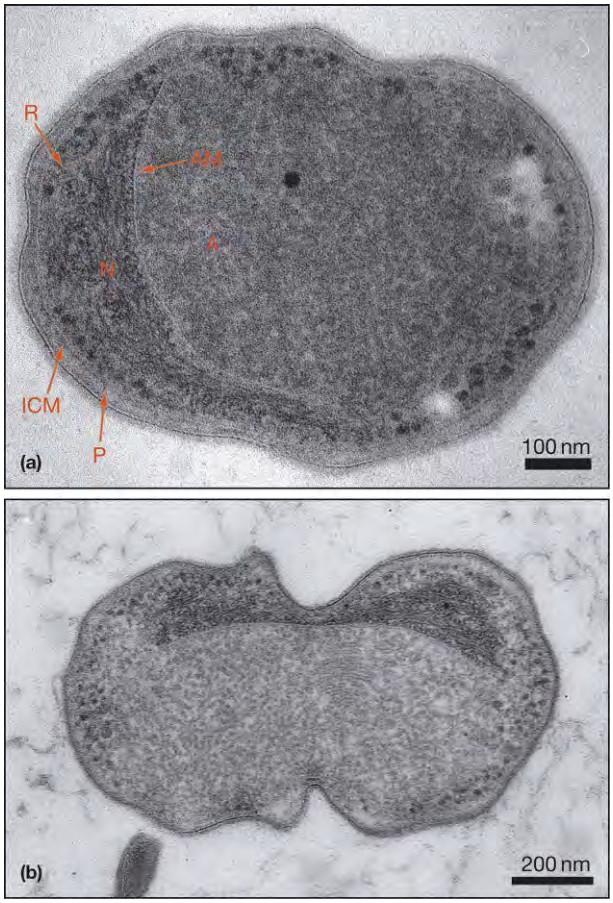

Figure 2 (a) Transmission electron micrograph of thin section of cryosubstituted cell of *Candidatus* Brocadia anammoxidans, from a bioreactor performing anaerobic ammonium oxidation. A central anammoxosome (A) is surrounded by a single membrane (AM). The cytoplasm external to the AM contains the nucleoid (N) and ribosome-like particles and is surrounded by the intracytoplasmic membrane (ICM) defining the pirellulosome. A paryphoplasm (P) lies at the rim of the cell between the ICM and the cytoplasmic membrane closely apposed to the cell wall. Tubule structures are visible inside the anammoxosome. Bar = 0.1 μm. (b) Transmission electron micrograph of thin section of cryosubstituted cell of *Candidatus* Brocadia anammoxidans, from a bioreactor performing anaerobic ammonium oxidation. An apparently dividing cell in which the central anammoxosome contains tubules, some of which are seen in longitudinal section oriented perpendicular to the plane of division; a nucleoid is seen in each half of the dividing cell accompanying the corresponding anammoxosome half section. Bar = 200 nm. Courtesy of J. A. Fuerst, R. Webb and M. Strous. Both (a) and (b) reproduced from Fuerst JA, (2005) Intracellular compartmentation in planctomycetes. *Annual Review of Microbiology* 59: 299–328, with permission from Annual Review of Microbiology (Annual Reviews).

Chlorosomes

Chlorosomes are light-harvesting organelles that that are tightly appressed to the inner surface of the cytoplasmic membranes of green chlorophototrophic bacteria (Figure 5(a)). Green bacteria are a loose assemblage of organisms belonging to three bacterial phyla: *Chlorobi*, *Chloroflexi*, and *Acidobacteria*. Green bacteria share two defining properties: they synthesize either bacteriochlorophyll (BChl) *c*, *d*, or *e*, and they assemble these BChls into chlorosomes. Chlorosomes can accurately be described as 'sacs containing supramolecular aggregates of BChl *c*, *d*, or *e*′ (Figure 5(b)). The aggregates formed from BChl *c* and *d* in chlorosomes cause a red-shift of 70–80 nm in the Q_y absorption, which allows cells to absorb near-infrared light at ~730–750 nm that is not absorbed by other Chls, especially Chl *a*. Aggregates formed from BChl *e* gain an absorption band at ~525 nm that allows organisms producing this pigment to harvest blue light very efficiently, and thus these organisms can grow at great depths in aquatic environments. The light energy absorbed by BChls in chlorosomes is rapidly and efficiently transferred to homodimeric type-1 photochemical reaction centers in the cytoplasmic membranes via BChl *a* molecules associated with chlorosome baseplate protein, CsmA (Figure 5(c)), and the BChl *a*-binding Fenna–Matthews–Olson (FMO) protein in chlorosome-producing members of the *Chlorobi* and *Acidobacteria* (Figure 5(c)). In chlorosome-producing members of the *Chloroflexi*, a membrane-associated BChl *a*-binding light-harvesting complex (B808–866) replaces FMO. Similar to light-harvesting complex I (LH-I) in purple bacteria, this complex forms a ring that surrounds the type-2 photochemical reaction centers found in chlorophototrophic members of the *Chloroflexi*.

Chlorosomes of the green sulfur bacterium *Chlorobaculum tepidum* (phylum *Chlorobi*) have been most extensively characterized. A single *Cba. tepidum* chlorosome can contain up to 250 000 BChl *c* molecules, and because a cell can have up to ~200 chlorosomes,

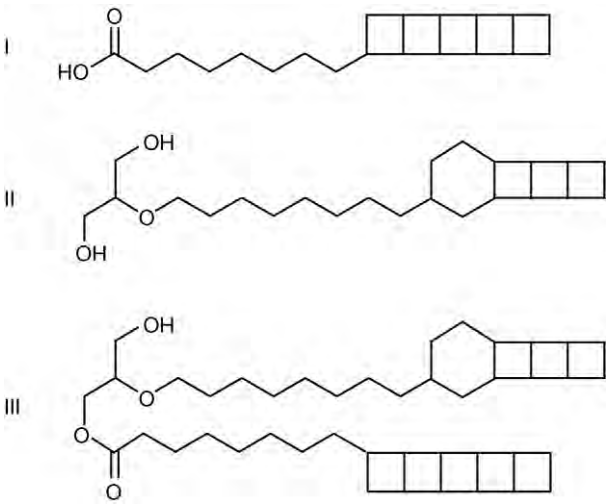

Figure 3 Structures of characteristic ladderane lipids of anammox planctomycetes, I ladderane fatty-acid-containing ring system Y with ester link II ladderane monoalkyl glycerol ether-containing ring system X and III a ladderane glycerol ether/ester containing both ring systems X and Y. Reproduced from van Niftrik LA, Fuerst JA, Sinninghe Damsté JS, et al. (2004) The anammoxosome: An intracytoplasmic compartment in anammox bacteria. *FEMS Microbiology Letters* 233: 7–13, with permission from FEMS Microbiology Letters (Elsevier).

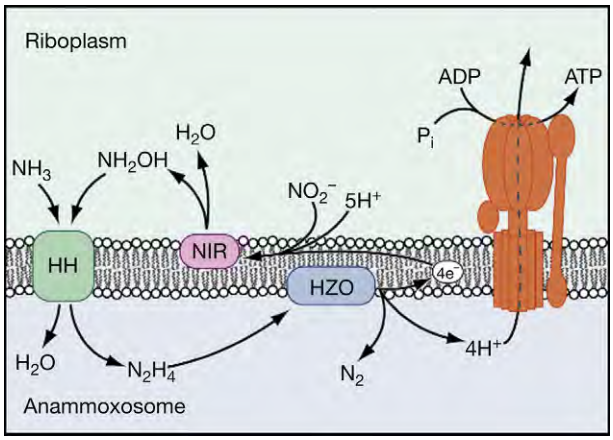

Figure 4 Model for anaerobic ammonium oxidation coupled to the anammoxosome membrane in anammox bacteria, resulting in a proton motive force and subsequent ATP synthesis via membrane-bound ATPases. HH: hydrazine hydrolase, the hydrazine-forming enzyme; HZO: hydrazine-oxidizing enzyme; NIR: nitrite-reducing enzyme. Note that in one alternative, nitric oxide can substitute for the place of hydroxylamine in the model shown here. Reproduced from van Niftrik LA, Fuerst JA, Sinninghe Damsté JS, et al. (2004) The anammoxosome: An intracytoplasmic compartment in anammox bacteria. *FEMS Microbiology Letters* 233: 7–13, with permission from FEMS Microbiology Letters (Elsevier).

a *Cba*. *tepidum* cell may contain ~50 million BChl *c* molecules (i.e., BChl *c* comprises ~30% of the cell carbon). In addition to BChl *c*, *Cba*. *tepidum* chlorosomes contain about 2500 BChl *a* molecules, 5000 protein molecules (all are found in the envelope, about half of which are the small BChl *a*-binding baseplate protein, CsmA), 20 000 carotenoids, 18 000 chlorobiumquinone and menaquinone molecules, and 20 000 lipid molecules (glycolipids, phospholipids, and wax esters). Chlorosomes are surrounded by a protein-stabilized, glycolipid 'monolayer' envelope that is ~3 nm thick. This envelope is an asymmetric bilayer membrane, in which proteins and glycolipids form the outer leaflet, and the tails of the BChl molecules inside form the inner leaflet (Figure 5(c)). The envelope of *Cba*. *tepidum* chlorosomes contains ten proteins belonging to four structure motif families: 1, CsmA/CsmE; 2, CsmB/CsmF/CsmH; 3, CsmC/CsmD/CsmH; and 4, CsmI/CsmJ/CsmX. Mutants lacking individual proteins except CsmA or up to four different proteins have been constructed. CsmA (and probably CsmE) forms dimers which in turn are organized into a paracrystalline baseplate structure that attaches the chlorosome to underlying FMO complexes (Figure 5(c)). Each CsmA polypeptide binds a single BChl *a* molecule and 1 or 2 carotenoid molecules as well. The proteins of motif families 2 and 3 help to determine the shape of the chlorosome and possibly influence the organization of the BChl aggregates inside. CsmI/CsmJ/CsmX bind [2Fe-2S] clusters; these proteins transfer electrons to reduce a quenching species, thought to be oxidized quinones, to reactivate energy transfer after cells or chlorosomes have been exposed to oxygen (see Figure 5(c)).

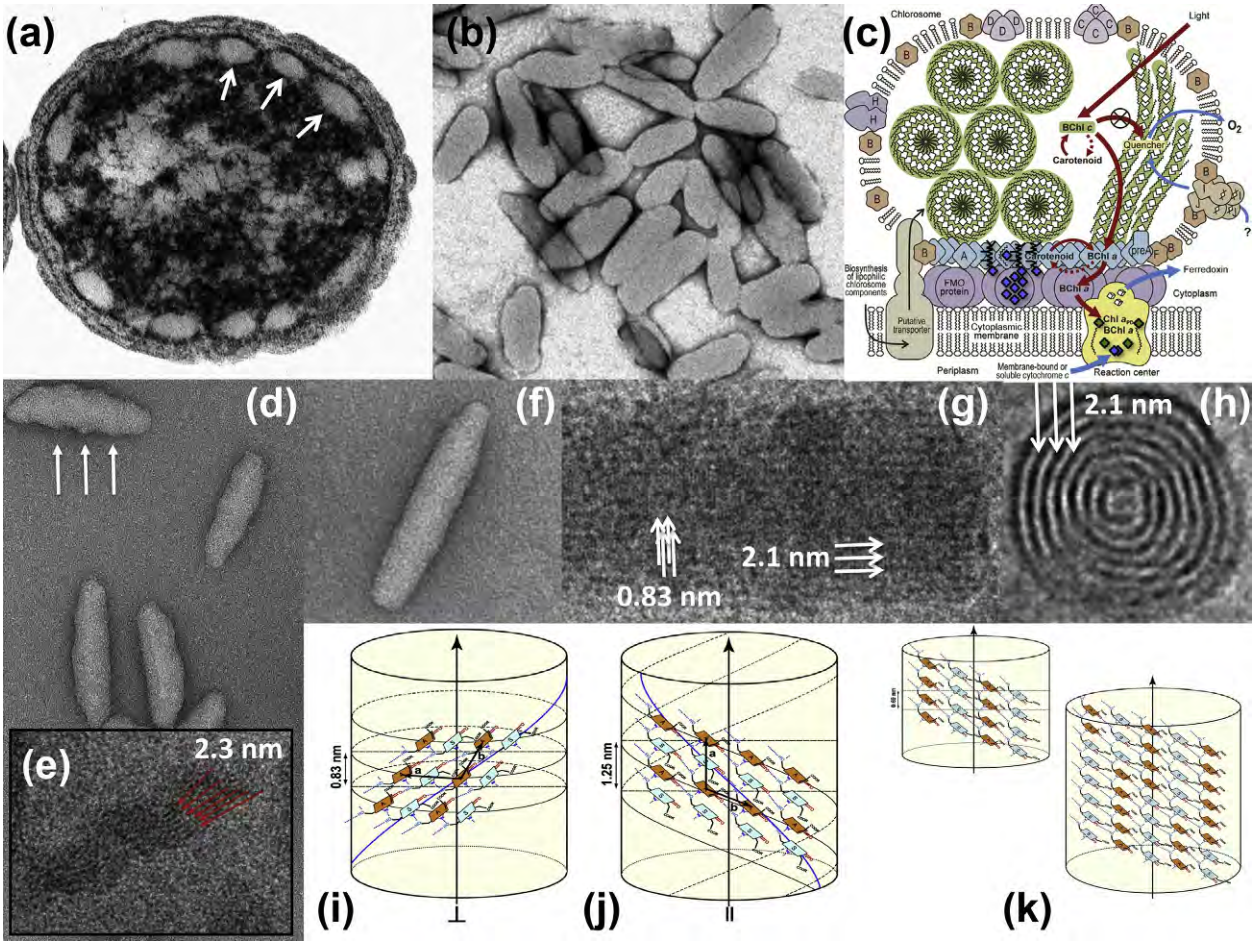

Figure 5 Structures of chlorosomes from the green sulfur bacterium *Cba. tepidum* and *Cab. thermophilum*. (a) Thin-section electron micrograph showing chlorosomes (white arrows), the electron transparent ovoids on the inner surface of the cytoplasmic membrane. (b) Electron micrograph of negatively stained chlorosomes from wild-type *Cba. tepidum*. (c) Structural model of chlorosomes from *Cba. tepidum*. BChls form lamellar nanotubes and arched lamellae. Possible energy transfer pathways are indicated by red arrows, and electron transfer pathways are indicated by blue arrows. (d) Electron micrograph of negatively stained chlorosomes from *Cab. thermophilum*. The three vertical white arrows show ridges on the cytoplasm-facing surface of the chlorosome. (e) Cryo-electron micrograph showing the 2.3-nm lamellar spacings (red arrows) between BChl *c* surfaces in chlorosomes of *Cab. thermophilum*. (f) Electron micrograph of a single, negatively stained chlorosome from the *bchQ bchR bchU* mutant of *Cba. tepidum*. (g) Cryo-electron micrograph showing the structure of [8-Et, 12-Me]-BChl *d* molecules in chlorosomes of a *bchQ bchR bchU* mutant of *Cba. tepidum*. The 2.1-nm lamellar spacings between BChl *d* surfaces are indicated by three horizontal white arrows, and the 0.83-nm spaces between the *syn-anti* BChls of the dimer stacks are indicated by four vertical white arrows. (h) Cryo-electron micrograph showing an end view of a single chlorosome from the *bchQ bchR bchU* mutant of *Cba. tepdium*. The three vertical white arrows indicate the lamellar surfaces that are formed from the *syn-anti* monomer stacks of [8-Et, 12-Me]-BChl *d* and that are separated 2.1 nm. (i–j) Schematic drawings showing the organization of the *syn-anti* dimer stacks of [8-Et, 12-Me]-BChl *d* in the chlorosomes of the *bchQ bchR bchU* mutant of *Cba. tepdium* (i) and of BChl *c* molecules in wild-type chlorosomes (j). BChls in *syn* configuration are show in orange and BChls in *anti* configuration are shown in aqua. (k) Schematic drawing showing the overall organization of [8-Et, 12-Me]-BChl *c* molecules in a *bchQ bchR* mutant of *Cba. tepidum*. The BChls form parallel monomer stacks of all-*syn* and all-*anti* configuration, which can adopt different suprastructures as shown.

The length and diameter of chlorosomes are highly variable and may vary considerably as a function of organism, light intensity, and growth conditions (Figure 5(b), 5(d) and 5(f)). This variability arises from the mechanism of chlorosome assembly, but is probably regulated in response to light and other parameters as well. Cryo-electron microscopy of chlorosomes from wild-type *Cba. tepidum* showed that the internal organization of the BChls in each chlorosome is unique: i.e., no two chlorosomes are structurally alike. Because of the heterogeneity in size, composition and internal structure, structural studies on chlorosomes were quite challenging, and they were the last class of light-harvesting complexes to be characterized in this way. End-on views of chlorosomes revealed that the BChl *c* molecules form coaxial, concentric nanotubular suprastructures with arched, interconnecting lamellae, The surface layers formed by the tetrapyrrole head groups of the BChls are separated by about 2.1 nm (Figure 5(g) and 5(h)), which are established by the hydrophobic interactions of the farnesol tails of the BChls in two adjacent layers, in a manner similar to the way that hydrophobic interactions of fatty acids occur in a lipid bilayer membrane. Solid-state NMR studies established that the BChls in chlorosomes of a mutant producing only [8-Et, 12-Me]-BChl *d* (Figure 5(i)) as well as in wild-type *C. tepidum* (Figure 5(j)), form *syn-anti* monomer stacks arranged in shallow helices. The farnesol tail of one BChl *c* molecule extends above, and of the other below, the

plane formed by the head groups. In a mutant that produces mostly [8-Et, 12-Me]-BChl *c*, the BChls form adjacent all-*syn* and all-*anti* parallel monomer stacks (Figure 5(k)). In all cases, hydrophobic interactions produced by the tails and the BChls and ligation of adjacent BChls by the C-3^1 hydroxyl group are key features that drive the self-aggregation of the BChls.

The chlorosomes of *Cfx. aurantiacus* ($120 \times 44 \times 30$ nm; 84 000 nm^3) and *Cab. thermophilum* ($99 \times 40 \times 31$ nm; 65 000 nm^3) are significantly smaller than those of *Cba. tepidum* ($133 \times 57 \times 36$ nm; 141 000 nm^3), and correspondingly must contain fewer BChl *c* molecules. The baseplate of *Cfx. aurantiacus* chlorosomes also forms a distinctive paracrystalline lattice with a 3.3-nm spacing, which is probably also formed from CsmA dimers. With the exception of CsmA, the proteins of the chlorosome envelope in *Cfx. aurantiacus* exhibit very limited sequence similarity to those of *Cba. tepidum*, but proteins of the same four motif families appear to be present. Chlorosomes of *Cab. thermophilum* differ structurally from those of *Cba. tepidum* and *Cfx. aurantiacus* (Figure 5(d)). Cryo-electron microscopy has demonstrated that the BChl *c* suprastructure of these chlorosomes has a lamellar spacing of ~2.3 nm (Figure 5(e)), a little larger than other chlorosomes; however, the BChl *c* molecules clearly do not form concentric nanotubes. Instead, the BChls seem to form twisted, sheet-like structures, which in turn form irregular subdomains within the chlorosomes. Two envelope proteins in *Cab. thermophilum*, CsmA and a protein with a [2Fe-2S] binding domain, share sequence similarity to chlorosome envelope proteins of *Cba. tepidum* and *Cfx. aurantiacus*. However, the other five proteins exhibit no sequence similarity with chlorosome envelope proteins in members of the *Chlorobi* or *Chloroflexi*. The structural similarity of chlorosomes in three of distantly related taxa that synthesize BChls, two of which have homodimeric type-1 reaction centers, suggests that chlorosomes are an ancient invention of nature, which may have evolved shortly after the evolution of type-1 reaction centers.

Phycobilisomes

Phycobilisomes (PBS) are very large (5–30 MDa) antenna protein complexes that occur in most cyanobacteria and the chloroplasts of red algae. PBS are composed of water-soluble phycobiliproteins (PBP) and 'linker' proteins, which commonly lack chromophores and direct the assembly of PBP into PBS. PBP complexes absorb visible light wavelengths for use in photosynthesis that are not efficiently absorbed by chlorophyll *a*. PBS are mostly associated with Photosystem (PS) II, which has only 35 chlorophyll *a* molecules per monomer (PS I has 96 chlorophyll *a* molecules per monomer), but PBP also transfer energy to PS I under some light conditions. Because these PBP complexes can absorb most of the visible spectrum, they are highly beneficial to organisms by increasing both their total absorbance capacity as well as enabling the use of additional, specific wavelengths of light that can be used for photosynthesis.

PBS are tightly associated with the cytoplasmic surface of the thylakoid membrane and project into the stromal region. When viewed by electron microscopy of thin sections of cells, they often form densely packed, interdigitating rows on the surfaces of adjacent thylakoids (Figure 6(a)–6(c)). Five structural classes of PBP antenna structures have been described, which vary in size, shape, and compositional complexity. These include: (1) hemi-ellipsoidal PBS; (2) hemi-discoidal PBS; (3) bundle-shaped PBS; (4) block-shaped PBS; and (5) rod-shaped PBS. Hemi-ellipsoidal and block-shaped PBS, which are mostly found in red algal chloroplasts, are the largest known PBS and can have sizes up to ~30 MDa. Most cyanobacterial PBS are smaller, hemidiscoidal structures with masses of 4.5–15 MDa (45–70 (w) × 30–50 (h) × 17 nm (d)). Hemi-discoidal PBS can add 300–800 chromophores to the absorption cross-section of PS II, which helps to balance the electron throughput between PS II and PS I. *Gloeobacter violaceus*, the only cyanobacterium that lacks thylakoid membranes, has large, bundle-shaped PBS that form a cortical layer by standing perpendicular to the inner surface of the cytoplasmic membrane. These PBS are formed from a hexagonally packed bundle of six PBP rods that are 50–70 nm in length with a diameter of 10–12 nm (i.e., the diameter of a trimeric ($\alpha_3\beta_3$) PBP disc). Finally, simple, rod-shaped PBP aggregates have been shown to occur in some cyanobacteria. These seem to be composed of one PBP (phycocyanin) or sometimes two PBP, and they include a specialized, rod-membrane linker protein (CpcG2 of *Synechocystis* sp. PCC 6803 or CpcG3 of *Nostoc* sp. PCC 7120) that has a hydrophobic, α-helical domain that anchors the rod complex to PS I. A long-standing mystery surrounding PBS is that they dissociate into their constituent PBPs and linker proteins when cells are disrupted in aqueous buffer at low ionic strength. The isolation of intact PBS requires the use of cross-linking agents and/or high-molarity phosphate buffer (0.5–0.75 M phosphate at near-neutral pH). Release of PBS from the thylakoid membrane usually requires the complete dissolution of the thylakoid membrane using detergents.

PBP are a superfamily of brilliantly colored, water-soluble proteins that are derived from an ancestral protein by gene duplication and divergence. Crystallography has shown that the major PBP phycoerythrin (red-colored, green-absorbing), phycoerythrocyanin (fuchsia-colored, yellow-absorbing), phycocyanin (blue-colored, orange-absorbing) and allophycocyanin (aqua-colored, red-absorbing) proteins all form torroid-shaped ($\alpha_3\beta_3$) trimers comprised of (αβ) protomers or face-to-face $(\alpha\beta)_6$ hexamers. The α and β subunits of allophycocyanin each carry one phycocyanobilin chromophore, which is covalently attached to residue cysteine 82 by a single thioether linkage. The α and β subunits of phycocyanin carry one and two phycocyanobilin chromophores, respectively. The alpha subunit chromophore is attached to cysteine 84, and the β subunit chromophores are attached to cysteines 82 and 153. Phycoerythrin subunits carry two (α) and three (β) three phycoerythrobilin chromophores. Three bilin lyases are sufficient to attach phycocyanobilin to allophycocyanin and phycocyanin subunits except ApcE. The CpcE/CpcF bilin lyase attaches phycocyanobilin to cysteine 84 of the apo-α subunit of phycocyanin; CpcT attaches phycocyanobilin to the cysteine 153 of the β subunit; and a homodimer ($CpcS_2$) or a heterodimer (CpcS/CpcU) attaches phycocyanobilin to cysteine 82 of all other PBP. ApcE, which is the core-membrane linker of the PBS core, is the only PBP that auto-ligates phycocyanobilin to cysteine 186 in its biliprotein domain. Two additional chromophores, phycoviolobilin (α subunit of phycoerythrocyanin) and phycourobilin (some phycoerythrins), can be introduced into some cyanobacterial PBP by isomerization reactions that occur during chromophore attachment by so-called isomerizing lyases.

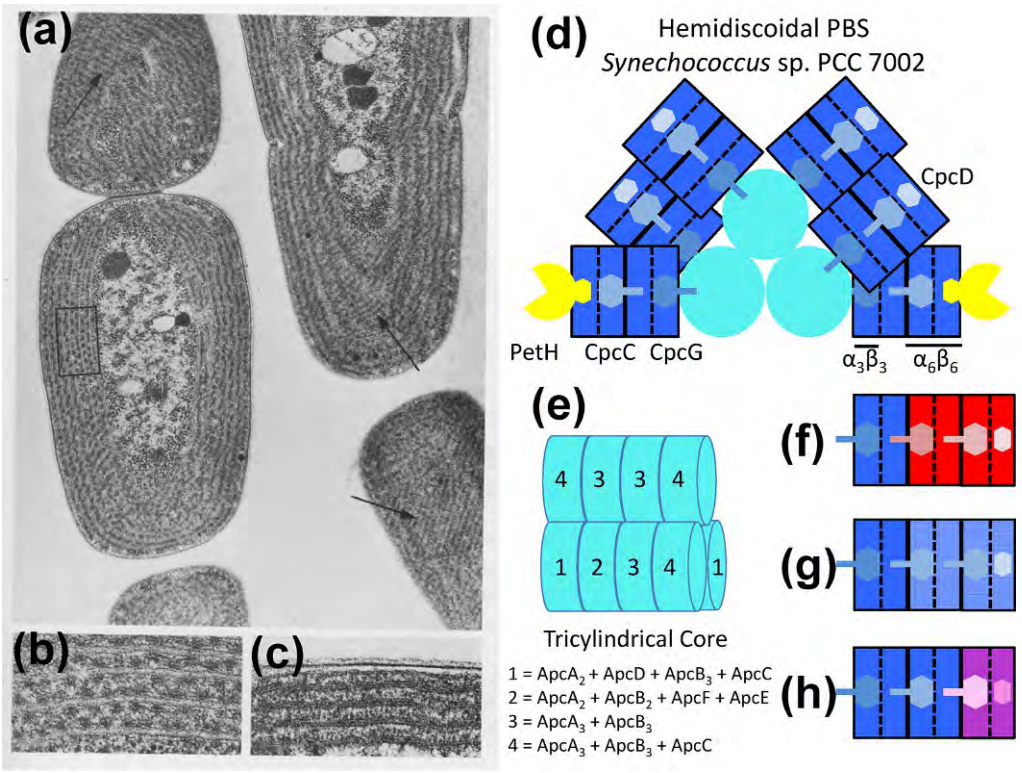

Figure 6 Phycobilisomes. (a) Electron micrograph of a thin section of the cyanobacterium *Pseudanabaena* sp. PCC 7409. The three arrows point to rows of PBS. (b) Enlarged view of the boxed region of panel (a), which shows a cross-section of the interdigitating rows of PBS on the stromal surfaces of the paired thylakoid membranes. (c) Longitudinal section of rows of PBS which are standing perpendicular to the thylakoid membranes. (d) Diagram showing the structural organization of the hemidiscoidal PBS of the cyanobacterium *Synechococcus* sp. PCC 7002. The tricylindrical core is mostly comprised of allophycocyanin (aqua; ApcA and ApcB); the six peripheral rods are each composed of two phycocyanin $(\alpha\beta)_6$ hexamers (blue; CpcA and CpcB), which are assembled by linker proteins CpcC, CpcD, and CpcG. Ferredoxin:$NADP^+$ oxidoreductase (PetH; yellow) has a CpcD domain and occurs at ~2 copies per PBS. (e) Compositional details of the subcomplexes of a tricylindrical PBS core. The complexes of the two lower cylinders have an anti-parallel configuration. (f–h) Examples of other peripheral rod complexes. The complexes shown in (f) and (g) occur in PBS of *Pseudanabaena* sp. PCC 7409 when cells are grown in green or red light, respectively. (f) Peripheral rod composed of one phycocyanin hexamer (blue) and two phycoerythrin hexamers (red). (g) Peripheral rod composed of one hexamer of phycocyanin-1 and two hexamers of phycocyanin-2. (h) Peripheral rod composed of two hexamers of phycocyanin (blue) and a hexamer of phycoerythrocyanin (violet).

The PBS of the cyanobacterium *Synechococcus* sp. PCC 7002, which forms simple hemidicoidal structures from twelve gene products, are principally comprised of peripheral rods composed of phycocyanin and a tricylindrical core substructure composed of allophycocyanin (Figure 6(d)). Each of six peripheral rods is formed from two phycocyanin $(\alpha\beta)_6$ hexamers (CpcA = α; CpcB = β), and three linker proteins: CpcD, the rod-terminating linker; CpcC, the peripheral rod linker; and CpcG, the rod-core linker. In PBS, CpcD is sometimes replaced by PetH, ferredoxin:$NADP^+$ oxidoreductase, which also has an N-terminal CpcD domain. This presumably places PetH in close proximity to PS I and limits the diffusion distance required for ferredoxin to deliver electrons to $NADP^+$. The core substructure is composed of six proteins: ApcA, ApcB, ApcC, ApcD, ApcE and ApcF. ApcE is the core-membrane linker (L_{CM}), which acts as a scaffold for assembly of the core substructure. In PBS with bicylindrical cores (e. g., *Synechococcus* sp. 7942), this ~75-kDa protein has an N-terminal PBP domain that binds one phycocyanobilin and two REP (linker) domains, with intervening spacer sequences. This protein can direct the assembly of a core with two allophycocyanin cylinders. PBS with tricylindrical cores (e.g., *Synechococcus* sp. PCC 7002 (see Figure 6(e))) have larger, 99-kDa ApcE proteins with three REP domains, and PBS with pentacylindrical cores (e.g., Nostoc sp. PCC 7120) have 120-kDa ApcE proteins with 4 REP domains. Two ApcE polypeptides are required to produce one core, the remainder of which is mostly made up of $(\alpha\beta)_3$ trimers of allophycocyanin (ApcA, ApcB). ApcC is a small, cylinder-terminating core linker protein; ApcF is a beta-type subunit that is the assembly partner of the PBP domain of ApcE; and ApcD encodes an α-type subunit that is found in the outer most trimer of the bottom cylinders of the core (one copy for each cylinder on average). ApcE and ApcD are often referred to as 'terminal emitters,' because they are associated with the longest wavelength absorption (665 and 670 nm, respectively) and because they are responsible for the fluorescence emission of isolated PBS. ApcE is the primary pathway for energy transfer to PS II via PsbC *in vivo*, and ApcD is principally responsible for energy transfer to PS I via PsaA. The degree to which energy is directed to the two photosystems is controlled by a process known as 'state transitions,' which alters the coupling of PBS to PS II and/or PS I.

PBPs are arranged within the PBS in a downhill energy transfer cascade such that shorter-wavelength-absorbing proteins of higher energy occur at the periphery of the PBS and transfer energy to longer-wavelength absorbing species of lower energy in the

PBS core. This arrangement ensures rapid, efficient and largely unidirectional energy transfer to PS I and PS II complexes in the thylakoid membrane. Thus, when they occur in PBS, phycoerythrin or phycoerythrocyanin are located at the core-distal ends of the peripheral rods (see Figure 6(f)–6(h)). Such peripheral rods always contain some phycocyanin, which is located proximal to the core and which facilitates efficient energy transfer to allophycocyanin in the core. In turn, the core components and especially the terminal emitters promote efficient energy transfer to the chlorophylls associated with photosystems in the thylakoids. Some cyanobacteria can alter the composition of their PBS to match the incident light, a process known as complementary chromatic acclimation. A variation of this phenomenon occurs in some marine cyanobacteria, which respond to blue light by reversibly isomerizing the phycoerythrobilin chromophores to phycourobilin chromophores.

Proteasomes

Proteasomes are large, multicatalytic proteases that are widespread in archaea, eukaryotes and bacteria of the phylum Actinobacteria. Proteasomes are important in general protein turnover, the removal of damaged and foreign proteins as well as regulatory proteolysis. Protein modification pathways (i.e., ubiquitylation in eukaryotes, pupylation in mycobacteria and sampylation in archaea) that mediate the covalent attachment of ubiquitin or ubiquitin-like proteins to protein targets are regulated and often mark the tagged protein for destruction by proteasomes. Proteins specifically directed to proteasomes for proteolysis encompass a variety of functions, including those associated with cell division, metabolism, DNA repair, translation, transcription and pathogenesis, making proteasomes and their associated protein tagging mechanisms attractive targets for chemotherapy, pathogen control, and biotechnology applications.

Proteasomes have a nanocompartmentalized architecture that is characterized by the sequestration of proteolytic active sites on the interior of a chamber-like structure. This arrangement minimizes the uncontrolled hydrolysis of cellular components and promotes the proper selection, unfolding and translocation of substrate proteins for degradation by proteasomes through energy-dependent processes.

Proteasomes that mediate the energy-dependent degradation of proteins are composed of 20S core particles (CP) that associate with ATPases of the AAA+(triple-A or ATPases associated with various cellular activities) superfamily (Figure 7). The CPs are formed by the stacking of four heptameric rings of related α- and β-type subunits into a cylindrical complex of 28 subunits in an α7β7β7α7 configuration that is approximately 15 nm in length and 11 nm in width. The outermost rings are of α-type subunits and the innermost rings are of β-subunits. In eukaryotes, seven different α- and β-type proteins form the CP, while in prokaryotes, only one to two different α- and β-type proteins are needed. Narrow, gated openings on each end of the cylinder provide portals for substrate entry into the central channel of the CP that harbors two antechambers and one central proteolytic chamber. The two antechambers are formed at the α- and β-ring interfaces and provide a site of initial substrate entry to the central chamber formed by the two β-rings that harbor the proteolytic active sites. The ends of the CP cylinder are in contact with hexameric rings of the AAA+ATPases. The AAA+ATPases select, unwind and translocate protein substrates into the chambered interior of the proteolytic nanomachine. Proteasomal CPs and AAA+ATPase subtypes of distinct biological function and different subunit composition based on cell conditions are identified.

Once protein substrates access the central chamber of the proteasome, hydrolysis of their peptide bonds occurs through nucleophilic attack mediated by N-terminal threonine residues of the CP β-type subunits. These active site threonine residues are exposed by autohydrolysis and removal of the N-terminal propeptides from the β-type proteins during CP assembly. The high concentration of active sites within the CP (up to 14 active sites) facilitates the processive cleavage of substrate proteins into peptide fragments of 3–24 amino acids in length.

A number of AAA+(triple-A) ATPases are identified that associate with CPs and serve as unfoldases or segregases to facilitate the degradation of proteins by proteasomes. For example, the 26S proteasomes of eukaryotes are formed by association of CPs with 19S regulatory particles or caps that are composed of 18–20 subunits including six regulatory particle triple-A (Rpt1–6) subunits. Rpt1–6 form the hexameric ATPase rings of the 19S cap that are in close contact with the ends of the CP cylinder and important for energy-dependent proteolysis of ubiquitylated proteins. Rpt subunit homologs are also identified in archaea named proteasome-activating nucleotidases or PANs that associate with and stimulate the energy-dependent degradation of structured proteins by CPs. Substrate binding to archaeal PANs activates ATP hydrolysis, which successively promotes substrate unfolding and opening of the

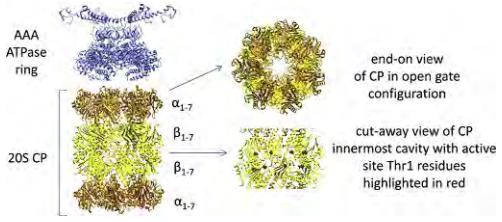

Figure 7 Proteasomes are composed of 20S core particles (CP) and regulatory ATPases of the AAA+(triple-A or ATPases associated with various cellular activities) superfamily. The CPs are nanocompartmentalized with their proteolytic active sites sequestered on the interior of a cylindrical barrel-shaped complex. The AAA+ATPase rings facilitate the energy-dependent selection, unwinding and translocation of protein substrates into the chambered interior of the CP for degradation.

CP gates. PAN also facilitates substrate translocation into the central proteolytic chamber of the CP. Cdc48 (p97) is another example of an AAA+ATPase that facilitates energy-dependent movement of protein substrates to proteasomes for hydrolysis in eukaryotes and archaea. Cdc48 complexes can also segregate ubiquitylated substrates from unmodified partners, an activity crucial for certain proteasome-mediated degradation pathways. Mpa and ARC are closely related AAA+ATPases of mycobacteria and actinobacteria that also associate with CPs and facilitate protein degradation.

We now understand that mechanisms, such as ubiquitylation, that are used to generate isopeptide linkages for targeting proteins for destruction by proteasomes are not restricted to eukaryotes. Prokaryotes form ubiquitin-like isopeptide bonds on target lysine residues of proteins by at least two distinct mechanisms that are reversible and regulated. In actinobacteria, the small, disordered Pup (prokaryotic ubiquitin-like protein) is known to be deamidated at its C-terminal Gln and isopeptide linked to proteins by enzymes (Dop and PafA) related to carboxylase-amine ligases. In archaea, the ubiquitin-fold SAMPs (small archaeal modifier proteins) are isopeptide-linked to target lysines by an E1-type mechanism that appears similar in chemistry, but streamlined, compared to E1-E2-E3 enzymes of ubiquitylation. SAMPs are members of a small group of Ub-fold proteins that function not only in protein modification but also in sulfur-transfer pathways associated with tRNA thiolation and molybdopterin biosynthesis. These multifunctional Ub-fold proteins are thought to be some of the most ancient of Ub-like protein modifiers.

Structures as Contributors to Cell Mobility

Magnetosomes

Magnetosomes are specific prokaryotic organelles, which serve as navigational devices in magnetotactic bacteria (MTB) and consist of membrane-enclosed intracellular crystals of a magnetic iron mineral. The chain-like arrangement of magnetosomes confers a magnetic dipole moment to the cell, which enables it to orient and migrate along geomagnetic field lines, a behaviour known as 'magnetotaxis.' Magnetotaxis, in combination with chemotaxis, aerotaxis, and even phototaxis, is thought to assist the cell in finding and maintaining an optimum position within vertical chemical gradients in stratified aquatic environments, such as freshwater and marine sediments.

MTB represent a heterogeneous group of motile aquatic prokaryotes with a variety of morphological types (Figure 8(a)–8(f)), and magnetosome formation occurs in several distinct phylogenetic lineages of Gram-negative bacteria, including representatives from the Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, and the *Nitrospirae* phylum. Some MTB have unusual morphologies, as for example the giant rods with more then 1000 magnetosome particles as well as multicellular magnetotactic forms. Most MTB are recalcitrant to axenic cultivation and only very few species have been isolated in pure culture. All magnetosome particles consist of inorganic ferrimagnetic crystals of either monocrystalline magnetite (Fe_3O_4) or the iron sulfide greigite (Fe_3S_4), which typically fall within the stable single magnetic domain range between 30 and 120 nm. Magnetosome crystals display a variety of uniform species-specific morphologies, which are mostly unknown from inorganic systems (Figure 8(g)–8(m)).

Crystals of the magnetic mineral magnetite are synthesized by a highly controlled biomineralization process within the magnetosome membrane (MM) vesicles, which are formed by invaginations from the cytoplasmic membrane. In all MTB analyzed so far the MM is a phospholipid bilayer associated with proteins. In *Magnetospirillum gryphiswaldense* and related MTB approximately 30 specific polypeptides were identfied in the MM, which were designated as Mam (*magnetosome membrane*), Mms (*magnetic particle membrane-specific*), and Mtx (*magnetotaxis*) proteins. Most of these proteins are encoded by several operons within a genomic magnetosome island (MAI) of 100–130 kb, which is conserved between several MTB, and which may have been acquired by horizontal gene transfer. Cloning and transfer of all of the about 30 relevant genes was shown to confer the ability of magnetosome biosynthesis to foreign, hitherto non-magnetic bacteria.

Although the functions of individual magnetosome-associated proteins have not been fully revealed yet, some of them could be assigned specific roles in iron uptake, control over nucleation and growth of crystals. Other proteins assembly, positioning and partitioning of magnetosome chains. For example, Two other proteins, MamJ and MamK, are involved in the assembly, intracellular positioning and partioning of the highly ordered magnetosome chain, which has an immanent tendency to collapse without some form of support. For example, the deletion of MamJ resulted in cells, which do no longer assembly chains of magnetosomes, but instead magnetosome particles are arranged within clusters. It is thought that MamJ attaches the magnetosome particles in a 'beads-on-a-string'-like configuration to a unique cytoskeletal structure, the magnetosome filament, which is formed by the actin-like MamK protein (Figure 9).

Magnetosomes produced by MTB represent magnetic nanoparticles with unique crystalline, magnetic, and chemical characteristic, which might be useful in a number of various biotechological applications. Size and morphology of magnetosome crystals are under genetic control, which can be modified by genetic engineering yielding magnetic nano-crystals with 'tailored' characteristics. The encapsulation of the magnetic crystal within the MM provides a natural coating, which can been addressed for modification and functionalization of the particles, for instance for the magnetosome-specific display of biomolecules such as fluorochromes, enzymes and antibodies coupled to the MM.

Gas Vesicles

Gas vesicles are gas-filled protein structures found in many bacteria (mainly phototrophs but also heterotrophs) and Archaea (halophiles and one methanogen). They affect the buoyancy and allow the cells to maintain a position in the water column where

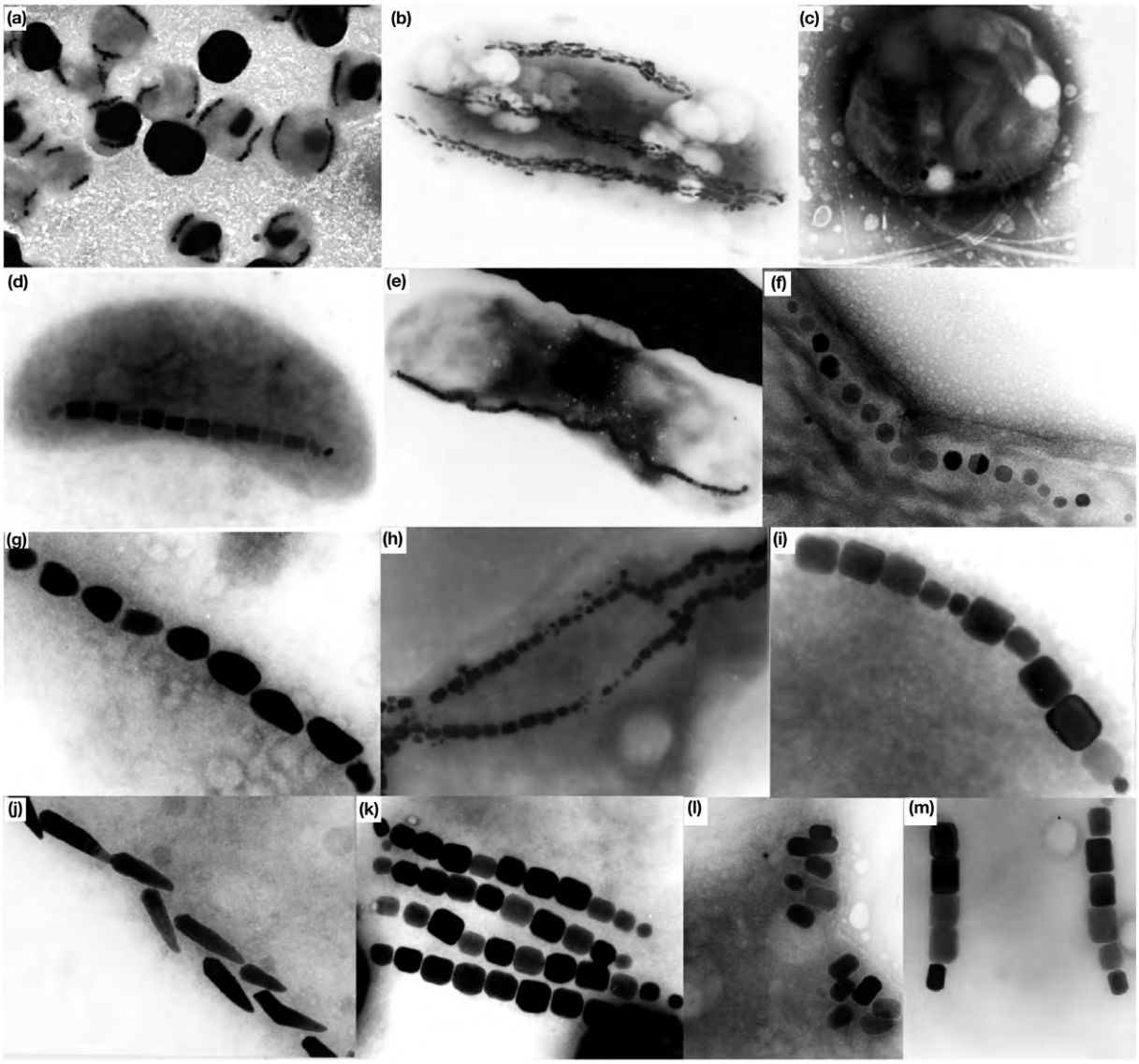

Figure 8 Diversity of magnetotactic bacteria (MTB) and magnetosomes. (a–e) Transmission electron micrographs of a variety of morphological forms found in MTB, including coccoid cell forms (a, c), large rod-shaped bacteria with one or several magnetosome chains (b, e), and magnetic *Vibrio* (d). (f–m) Electron micrographs of crystal morphologies and organization of magnetosomes found in various MTB. Micrographs by Gruska M and Plitzko JM, Max Planck Institute, Martinsreid, Germany. Reproduced from Scheffel A and Schüler D (2006) Magnetosomes of magnetotactic bacteria. In: Shively JM (ed.) *Microbiology Monographs* 2 *Complex Intracellular Structures in Prokaryotes*, pp. 167–192. Berlin, Heidelberg: Springer-Verlag, with permission from Springer Science and Business media.

the conditions are optimal for growth. Gas vesicles are recognized as white, light-reflecting particles within the cells by phase-contrast microscopy, and single particles are visualized by electron microscopy (Figure 10(a)). These structures irreversibly collapse when higher pressure is imposed on the cells. Isolated gas vesicles are spindle- or cylinder-shaped structures consisting of 4.6 nm ribs running perpendicular to the long axis of the vesicle (Figure 10(b)). Gas vesicles of haloarchaea are larger in width (diameters of 200–250 nm) compared to gas vesicles of bacteria (33–75 nm), but the maximal lengths are very similar (up to 1 μm). The reason for the larger diameter in haloarchaea is that these organisms possess a negligible turgor pressure because they accumulate K^+ in a molar range to maintain an iso-osmotic equilibrium with the environment.

Detailed investigations on gas vesicle structure, formation, and also on the genetic regulation have been performed with cyanobacteria and haloarchaea. Gas vesicles develop from hollow bicones that grow in length through the assembly of a cylindrical midsection. Their wall is solely composed of protein; neither lipids nor carbohydrates are found. The major constituent is the 7–8 kDa GvpA, one of the most hydrophobic proteins known. Hydrophobic interactions are responsible for the extremely tight contacts of GvpA proteins that aggregate to form a helical structure of low pitch seen as 4.6 nm ribs. Atomic force microscopy indicates regularly spaced subunits along the ribs that slope in an angle of 54° to the rib axis. The resulting gas vesicle wall is extremely resistant to solubilization by detergents. Gases like O_2, N_2, H_2, or CO_2 freely diffuse inside and are in equilibrium with

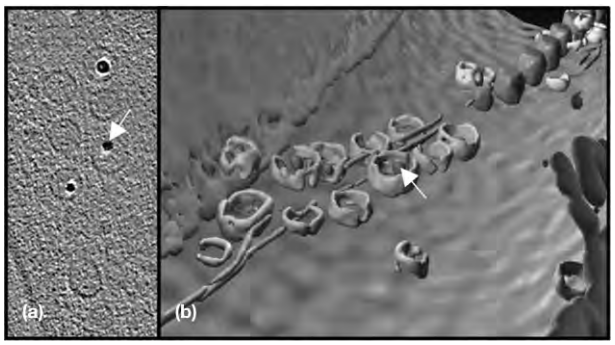

Figure 9 Tomographic reconstruction of a *Magnetospirillum gryphiswaldense* cell. (a) A section in *z*-direction from the reconstructed volume showing empty magnetosome membrane (MM) vesicles, and those which are partially filled with growing, immature magnetite crystals (white arrow), are closely attached to the magnetosome filament, which represents a cytoskeletal structure that stabilizes magnetosome chains. (b) Three-dimensional visualization of a part of a *M. gryphiswaldense* cell with the cytoplasmic membrane (blue), empty vesicles (yellow), growing and mature magnetite crystals (red), and the magnetosome filament (green). Visualization by Gruska M and Plitzko JM, Max Planck Institute, Martinsreid, Germany. Reproduced fromScheffel A, and Schüler D (2006) Magnetosomes of magnetotactic bacteria. In: Shively JM (ed.) *Microbiology Monographs* 2 *Complex Intracellular Structures in Prokaryotes*, pp. 167–192. Berlin, Heidelberg: Springer-Verlag, with permission from Springer Science and Business media).

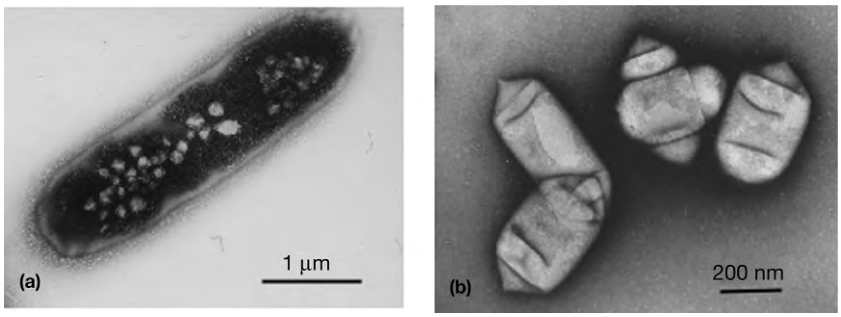

Figure 10 (a) Gas vesiculated cells of *Halobacterium salinarum* as viewed by electron microscopy. (b) Isolated intact gas vesicles.

those in the cytoplasm; no storage of gas occurs. Water molecules are presumably able to enter the gas vesicle, but the surface tension at the hydrophobic inner surface excludes the formation of liquid water. A second protein, GvpC, attaches at the outside surface and strengthens the gas vesicle structure. The sequence of the 30–40 kDa GvpC exhibits several 33-amino acid repeats (4–5 in cyanobacteria and 6–7 in haloarchaea). It has been proposed that each 33-amino acid repeat spans a rib formed by GvpA, enabling GvpC to clamp adjacent ribs and strengthen the gas vesicle wall.

Genetic analyses indicate that additional Gvp proteins are involved in gas vesicle formation. In haloarchaea 12–14 *gvp* genes are present and up to 10 in cyanobacteria. The *gvp* genes of *Halobacterium salinarum* and *Haloferax mediterranei* are arranged as two oppositely oriented clusters, *gvpACNO* and *gvpDEFGHIJKLM*. Mutation analyses using the gas-vesicle-negative species *Haloferax volcanii* as host reveal that a deletion of *gvpC*, *−D*, *−E*, *−H*, *−I*, or *-N* still yields gas-vesiculated transformants. Gas vesicles formed in the absence of GvpC, GvpI, or GvpH have altered morphologies, whereas the deletion of *gvpN* results in very small bicone structures. GvpE and GvpD are involved in the regulation of *gvp* gene expression, with GvpE acting as a transcriptional activator and GvpD having a repressing function. The remaining eight Gvp proteins are essential and could be minor gas vesicle structural components or take part in their assembly as chaperones.

Gas vesicles are formed only under certain environmental conditions. In cyanobacteria, darkness stimulates whereas UV light appears to repress gas vesicle formation. In haloarchaea, gas vesicle formation is strongly reduced when cells are grown in media with salt concentrations below 17%, or under anaerobiosis. How these environmental factors are signaled and influence *gvp* gene expression still needs to be explored. Transcription of the *gvp* gene cluster is 7- to 10-fold reduced under these conditions, leading to the lack of the accessory proteins GvpF through GvpO.

Structures as Metabolic Reserves

Polyphosphate Granules (Acidocalcisomes)

Polyphosphate (polyP) granules or acidocalcisomes are membrane-bounded organelles that are conserved from bacteria to higher organisms and whose main function is the accumulation of polyP and cations.

The granules were first described in bacteria in 1895 and initially referred to as metachromatic or volutin granules. The names derived from their property to stain red when bacteria were treated with toluidine blue, and for their presence in the bacterium *Spirillum volutans*, respectively. Subsequent investigations found volutin granules in algae, yeasts, and eukaryotic parasites. Eventually they were named polyP granules when this polymer, a linear chain of inorganic phosphates linked by high-energy phosphoanhydride bonds, was found to be present in high concentrations. More recently, based on newly discovered properties, the granules of eukaryotic parasites were named acidocalcisomes. Acidocalcisomes were found to possess a limiting membrane and have also been found in green algae, slime molds, eggs from different species, human platelets (dense granules), and mast cells (mast cell granules). The discovery that acidocalcisomes were membrane-bounded organelles led to the investigation of the presence of a limiting membrane in polyP granules of bacteria. Two bacteria, *Agrobacterium tumefaciens* and *Rhodospirillum rubrum*, have been found to possess membrane-bounded acidocalcisomes. Evidence has also been presented for the presence of these organelles in Archaea.

Acidocalcisomes are spherical, from 15 to about 200 nm in diameter, and sometimes located at one pole of the bacteria. They appear empty when observed by conventional electron microscopy or as electron-dense granules when observed by direct transmission electron microscopy. In addition to polyP, they accumulate a variety of cations, for example, calcium, magnesium, sodium, and zinc. They can also accumulate heavy metal cations from the environment, thus having a detoxifying role. Acidocalcisomes stain well with 4′-6′-diamino-2-phenylindole (DAPI), which stains polyP, as well as with acidotropic fluorescent dyes. A proton pump, a vacuolar H^+-pyrophosphatase (V-H^+-PPase), has been detected in the membrane lining the organelle from *A. tumefaciens* and *R. rubrum* (Figure 11).

In bacteria the mobilization of polyP is mainly due to the action of membrane-bound enzymes that catalyze the synthesis and degradation of this polymer, the polyP kinases (PPKs) and the exopolyphosphatases (PPXs), respectively. Two PPKs, PPK1 and PPK2, are present in different bacteria and catalyze the transfer of phosphate residues from ATP to polyP, while the PPXs split phosphate from the end of long-chain polyP in a processive manner.

The functions of acidocalcisomes are those of their main constituents, cations and phosphorus compounds, in particular polyP. PolyP has several functions in bacteria. In addition to its role as phosphorus store, energy source to replace ATP, and cation sequestration and storage, polyP is also important for cell membrane formation and function, transcriptional control, regulation of enzyme activities, response to stress and stationary phase, and to maintain the structural integrity of channels and pumps. By manipulating the expression of the genes involved in polyP metabolism and the cellular levels of their products, different investigators have created phenotypes that provided clues to the role of polyP in the physiology and development of bacteria. Mutant *Escherichia coli* lacking polyP kinase were deficient in polyP; failed to survive the stationary phase; were less resistant to heat, oxidants, and osmotic challenge; and were deficient in adaptation to nutritional deprivation. Disruption of the polyP kinase gene in other bacteria reduced their motility, their ability to develop biofilms, and decreased their virulence in animal models. PolyP levels have also been reduced by overexpression of PPX in *E. coli*, leading to decreased resistance to H_2O_2. PolyP has an important function as a primordial chaperone in bacteria. It has been established that polyP stabilizes proteins *in vivo*, diminishes the need of bacteria for other chaperone systems to survive proteotoxic stress conditions, and protects a wide variety of proteins against stress-induced unfolding and aggregation. These results help explaining the long known but largely unexplained role of polyP in protecting bacteria against stress conditions.

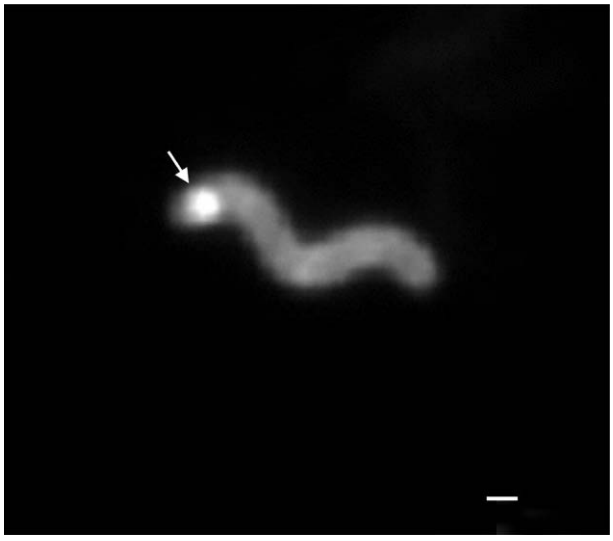

Figure 11 Confocal immunofluorescence microscopy of *Rhodospirillum rubrum* vacuolar H^+-pyrophophatase (arrow) as detected using polyclonal antibodies against *Arabidopsis thaliana* V-H^+-PPase. Methods as described in Seufferheld M, Lea C, Vieira M, et al. (2004) The H^+-pyrophosphatase of *Rhodospirillum rubrum* in predominately located in polyphosphate-rich acidocalcisomes. *Journal Biological Chemistry* 279: 51193–51202. Bar = 0.5 μm.

Sulfur Globules

Sulfur can accumulate in the form of water-insoluble globules as a transient or the final product during the oxidation of reduced sulfur compounds (sulfide, polysulfides, thiosulfate, polythionates, and elemental sulfur) by either chemotrophic or phototrophic sulfur-oxidizing bacteria. While sulfur is deposited extracellularly by part of these organisms, others form sulfur globules within the confines of the cell wall (Table 1), i.e. sulfur is present as a bacterial inclusion senso strictu. Internal sulfur globules are easily recognized even via light microscopy as they are highly light refractive, up to 2 μm in diameter and can comprise 20–34% of the of the cell dry mass. Cultures of cells containing sulfur globules exhibit a characteristic milky appearance.

Those organisms forming intracellular sulfur globules for which the systematic affiliation has been unequivocally determined belong to the α-, β-, γ- and ε- Proteobacteria (Table 1). They include the anoxygenic phototrophic purple sulfur bacteria of the family Chromatiaceae as well as *Thiomargarita namibiensis* and the filamentous sulfur-oxidizing bacteria of the genera *Beggiatoa* and *Thioploca*, some of the largest and most conspicuous bacteria in nature. In thin-section electron micrographs, sulfur deposits appear as conspicuous, empty and electron-lucent space. The exact intracellular localization of sulfur globules has not been resolved in all cases. Often, sulfur inclusions appear to be separated from the cytoplasm by a unit membrane which may be continuous with the cytoplasmic membrane depending on the organism. Topologically, these sulfur globules are periplasmic.

In many cases, sulfur inclusions have been observed to be bounded by a protein envelope which usually consists of a 2–5 nm single layer. Pentalaminar envelopes of 12–14 nm thickness have also been described. The envelopes are fragile in fixatives used for transmission electron microscopy therefore, their lack in electron micrographs is not a valid proof for their absence *in vivo*. Analysis of the sulfur globule proteome in the purple sulfur bacterium *Allochromatium vinosum* revealed that the envelope consists of four different, extremely hydrophobic proteins (SgpA, SgpB, SgpC, SgpD) of 8.5–20.8 kDa. Relative transcript abundances for all four of these genes strongly increase upon exposure of the cells to sulfide, thiosulfate and elemental sulfur compared to photoorganoheterotrophic growth on malate in the absence of reduced sulfur compounds. All four proteins are synthesized with cleavable N-terminal signal peptides mediating Sec-dependent transport into the periplasm. *A. vinosum* mutants lacking SgpB and SgpC are no longer able to oxidize sulfide and to form sulfur inclusions from it. SgpC is important for expansion of the globules and is less abundant than the other three proteins. All sulfur globule proteins share highly repetitive amino acid sequences rich in regularaly spaced proline residues and the prediction to be structural proteins. SgpD appears to be a coiled-coil protein. In general, these proteins are characterized by superhelical twisting of two or more alpha helices around one another. They can form rod-like tertiary structures and include the intermediate filaments of the metazoan cytoskeleton as well as bacteria specific cytoskeletal proteins.

X-ray absorption near edge structure spectroscopy of intact cells revealed at least three main different forms of sulfur in bacterial sulfur globules reflecting the different ecological and physiological types of sulfur bacteria: polythionates in the extracellular sulfur formed by *Acidithiobacillus*, *cyclo*-octasulfur (S_8) in *Thiomargarita* and *Beggiatoa*, and long sulfur chains probably terminated by organic residues (monoorganyl/bisorganyl polysulfanes) in phototrophic sulfur bacteria.

Sulfur deposition is comparatively well characterized in the purple sulfur bacterium *A. vinosum*. In this organism, the oxidation of sulfide is catalyzed in the periplasm by flavocytochrome *c* and two different sulfide:quinone oxidoreductases (SqrD and SqrF). Polysulfides are the first readily detectable product of this step. It is unknown how polysulfides are converted into sulfur globules. When thiosulfate is oxidized, only its sulfane group is stored as sulfur; the sulfone sulfur is immediately excreted as sulfate. Thiosulfate oxidation is catalyzed by the periplasmic Sox proteins (SoxXAK, SoxYZ, SoxB, SoxL). Proteins related to most of these are

Table 1 Occurrence and properties of bacterial sulfur inclusions

Organism/group	*Main substrates for sulfur globules*	*Properties of sulfur globules*
α-Proteobacteria		
Magnetotactic bacteria	H_2S	Intracellular
β-Proteobacteria		
Thermothrix	$S_2O_3^{2-}$	Intra- and extracellular
Macromonas	H_2S	Intracellular
γ-Proteobacteria		
Achromatium	H_2S	Intracellular
Beggiatoa	H_2S, $S_2O_3^{2-}$	Periplasmic, *cyclo*-octasulfur
Thiomargarita	H_2S	Periplasmic, *cyclo*-octasulfur
Thioploca	H_2S	Intracellular
Thiothrix	H_2S, $S_2O_3^{2-}$	Periplasmic
Thioalkalivibrio	$S_2O_3^{2-}$	Extracellular, some strains intracellular in periplasm
Bacterial endosymbionts of invertebrates	H_2S	Intracellular
Phototrophic members of the Chromatiaceae	H_2S, S^0, polysulfide, some $S_2O_3^{2-}$	Intracellular, periplasmic, organic polysulfanes
ε-Proteobacteria		
Thiovulum	H_2S	Intracellular
Unclear phylogenetic affiliation		
Thiobacterium	H_2S	Intracellular
Thiospira	H_2S	Intracellular

part of the widely distributed Sox multienzyme system that oxidizes thiosulfate directly to sulfate. The intermediate formation of sulfur globules in organisms like *A. vinosum* is probably related to the lack of SoxCD, a protein proposed to oxidize SoxY-bound sulfane sulfur. In its absence, the latter may instead be transferred to growing sulfur globules. A complicated mechanism that requires the presence of many different enzymes encoded in the *dsr* (dissimilatory sulfite reductase) gene cluster, is necessary for oxidation of stored sulfur. Low molecular weight organic persulfides like glutathione amide persulfide probably serve as carrier molecules transferring sulfur form the periplasmic sulfur globules into the cytoplasm. Here, a sulfur relay system sets in that transfers sulfur atoms via a sulfur mobilizing rhodanese, to the proteins TusA and DsrEFH and finally to DsrC, which then serves as the direct substrate for reverse acting sulfite reductase, DsrAB. The product of this step is sulfite which is finally oxidized to sulfate either directly via the membrane-bound iron-sulfur molybdoenzyme SoeABC or by an indirect pathway involving formation of adenosine-5′-phosphosulfate (APS) catalyzed by APS reductase snd ATP sulfurylase. Under anoxic conditions sulfur globules can also serve as an electron acceptor reserve that allows a rudimentary anaerobic respiration leading to production of sulfide. The underlying enzymatic mechanisms are unresolved.

Glycogen Reserves

The occurrence of glycogen-like reserves has been reported in over 50 different bacterial species. These inclusions may be naked or membrane-enclosed. Naked inclusions are 20–100 nm in diameter. In *Clostridium pasteurianum*, membrane-enclosed polyglucose inclusions are found to be 160–300 nm in diameter.

Three reactions are involved in the synthesis of bacterial α-glucan inclusion bodies. First is the synthesis of the glycosyl donor adenosine diphosphate–glucose (ADP–Glc) that is catalyzed by ADP–glucose pyrophosphorylase (ADP–Glc PPase; EC 2.7.7.27). ADP–Glc is then utilized for synthesis of a new α-1,4-glucosidic bond in the glycogen molecule and this reaction is catalyzed by glycogen synthase (EC 2.4.1.21). After extensive elongation of the α-1,4-glucosidic chain, branching enzyme (EC 2.4.1.18) then catalyzes formation of the α-1,6-linked branch chains.

The synthesis of ADP–Glc is the first committed step in bacterial glycogen synthesis and the enzymatic step is allosterically activated by glycolytic intermediates. The nature of the activator is dependent on the major carbon assimilatory pathway dominant in the organism. For example, enteric bacteria that utilize glycolysis for glucose assimilation have fructose-1, 6-bisphosphate as an activator for its ADP–Glc PPase while the cyanobacteria being oxygenic photosynthesizers have 3-phosphoglycerate (3-PGA) as the enzyme activator. The allosteric inhibitors for the ADP–Glc PPases are AMP, or P_i or ADP, indicating that ADP–Glc and glycogen synthesis are also controlled by the energy status of the cell. The regulatory properties of ADP–Glc PPase, together with the fact that ATP is one of the substrates of the enzyme, indicates that storage polysaccharides in bacteria are maximal when cellular carbon and energy are in excess.

Strong experimental evidence supports the view that ADP–Glc PPase is a regulatory enzyme in the bacterial glycogen synthetic pathway. In *E. coli* and *Salmonella typhimurium*, mutants have been isolated that were affected in their ability to accumulate glycogen, and their ADP–Glc PPases display altered regulatory properties. Also, in the unicellular green alga *Chlamydomonas reinhardtii* ADP–Glc PPase is activated by 3-PGA and its inhibitor is P_i. Starch-deficient mutants were isolated and were shown to have ADP–Glc PPases that could not be activated by 3-PGA. Regulation of ADP–Glc synthesis in bacteria therefore agrees with the generalization that a biosynthetic pathway is effectively regulated at its first unique step. The enzymatic and structural properties of the glycogen biosynthetic enzymes have been extensively reviewed and the regulatory properties of the various bacterial ADP–Glc pyrophosphorylases discussed and compared.

An alternative pathway to glycogen synthesis has recently been demonstrated in mycobacteria and is known as the GlgE pathway and involves the α-1,1 diglucoside trehalose (α-D-glucopyranosyl- (1 → 1)- α - D- glucanopyanoside). Trehalose is converted by the enzyme TreS to the α-1,4 disaccharide, maltose which is then converted by Pep2 (maltokinase) with ATP to maltose 1-phosphate. The maltose-1-P can then be transferred the matosyl residue to the end of exterior chains of the glycogen molecule by GlgE. The GlgE enzyme is thus a maltosyl transferase. In summary,

1. Trehalose → Maltose (Tres; trehalose synthase)
2. Maltose + ATP → Maltose-1-P + ADP (Pep2; maltose kinase)
3. Maltose −1-P → Maltosyl- α -1,4 Glycogen + Pi (GMPMT; glycogen, maltose-1-P maltosyl transferase)

Genetic regulation of glycogen synthesis in *E. coli* K12 and in *S. typhimurium* has been studied. The structural genes for glycogen biosynthesis are clustered in two adjacent operons and contain genes for glycogen catabolism. They are located approximately 75 min on the *E. coli* K12 chromosome, and the gene order at this location is *glgP–glgA–glgC–glgX–glgB–asd*. *glgA* encodes glycogen synthase, *glgC*, the ADP–Glc PPase, and *glgB*, the glycogen-branching enzyme. *glgX* encodes an isoamylase and *glgP*, glycogen phosphorylase.

Glycogen biosynthesis is under the direct control of at least three global regulatory systems: catabolite repression (stimulation of *glgABC* expression by cAMP), stringent response (stimulation of *glgABC* expression by pGpp), and repression of the *glgABC* expression by the *csrA* gene product, a 61-amino acid polypeptide. Transcription of *glgCA* appears to involve initiation events at four or more separate sites upstream from the genes and the *glgC* and *glgA* gene products; ADP–Glc pyrophosphorylase and glycogen synthase are in higher relative concentration in stationary phase versus exponential phase.

Mutations leading to higher levels of the biosynthetic enzymes also result in enhanced accumulation of specific transcripts.

The *csrA* gene maps to 58 min on the *E. coli* chromosome and its gene product negatively modulates posttranscriptionally by facilitating decay of *glgCA* mRNAs. The *csrA* gene product is a specific mRNA-binding protein binding to *csrB* RNA. Binding of the

csrA gene product to *csrB* RNA inhibits the repression of the *glgCA* operon and the *glgB* genes. Since *csrB* RNA increases in stationary phase, it is believed that the binding of *csrA* gene product relieves repression of the glycogen biosynthetic genes by the *csrB* RNA.

Polyhydroxyalkanoate Granules

Cytoplasmic granules consisting of PHAs have been described in a wide, taxonomically diverse range of prokaryotes and are probably the most frequent storage compounds for carbon and energy in bacteria making up to 90% of the cell dry weight (Figure 12). Poly(3-hydroxybutyrate) (PHB) is the most common PHA, and their granules and biosynthesis have been studied in most detail.

Approximately 150 other hydroxyalkanoates and also mercaptoalkanoates have been identified as constituents of these polyesters beside 3-hydroxybutyrate. The constituents are distinguished with regard to length and structure of the alkyl side chain or the position of the hydroxyl group. Bacteria accumulate PHAs when a carbon source is provided in excess or under nutrient or oxygen limitation when supply of carbon is still adequate. PHAs are of interest for several technical applications because they represent thermoplasts and elastomers that are in contrast to most biodegradable, compostable, and resorbable synthetic polymers. Therefore, several attempts were made by the chemical industry to produce various PHAs on a large scale.

The first step in PHB biosynthesis is the condensation of two acetyl-CoA molecules to acetoacetyl-CoA by β-ketothiolase (PhaA). Acetoacetyl-CoA is reduced to *R*-(−)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB), which is then converted into PHB by PHA synthase (PhaC). In *Ralstonia eutropha* and many other bacteria the genes for these enzymes constitute the *phaCAB* operon. Several other pathways have been described for biosynthesis of various PHAs. Unspecific PHA synthases are the key enzymes of PHA biosynthesis. They polymerize a large variety of different hydroxyalkanoates if the respective hydroxyacyl-CoA thioester is available. *R. eutropha* produces, for example, also the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), which was commercialized for use as a biodegradable packaging material. This bacterium is also capable to synthesize copolyesters containing 4-hydroxybutyrate (4HB) or poly(4HB) homopolyesters, which are of interest for applications in medicine and pharmacy. Pseudomonads synthesize PHAs consisting of 3-hydroxyalkanoic acids of medium carbon-chain length like 3-hydroxyoctanoate or 3-hydroxydecanoate. Biosynthesis of these PHAs depends either on fatty acid *de novo* biosynthesis plus an acyltransferase (*atf*)-transferring the hydroxyacylmoiety from the acyl carrier protein to coenzyme A if they are synthesized from glucose or other carbohydrates, or on fatty acid β-oxidation if they are synthesized from fatty acids, oils, or even hydrocarbons.

A thin layer consisting of regularly arranged small, amphiphilic proteins called phasins (PhaP) and eventually also phospholipids surrounds PHA granules. Granule formation is initiated in the cytoplasm when soluble PHA synthase starts synthesis and remains covalently linked to the growing polyester chain (Figure 13). Micelles of amphiphilic enzyme–PHB complexes with the enzyme at the surface and the hydrophobic polymer in the core are formed. When the micelles enlarge, only a minor fraction of the hydrophobic surface remains covered by PhaC, which remains attached to the surface. Phasin PhaP1, whose formation can be induced in *R. eutropha*, mostly covers the surface. Mutants lacking PhaP1 contain only one, large PHB granule because the granules

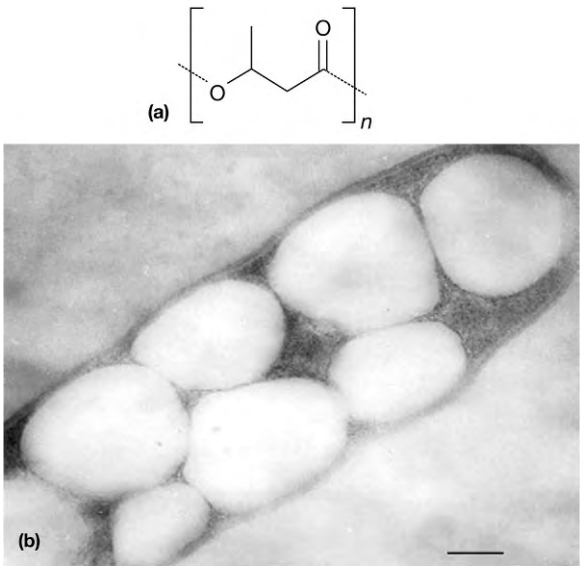

Figure 12 (a) Structural formula of poly(3-hydroxybutyrate) (PHB). (b) Transmission electron micrograph of a thin section of the Gram-negative bacterium *Ralstonia eutropha* showing PHB granules. Cells were grown in a mineral salts medium containing 1.5% sodium gluconate and had accumulated PHB to about 70% of the cell dry matter. Bar = 0.2 μm. Reproduced from Markus Pötter and Rudolf Reichelt, Westfälische Wilhelms-Universität Münster, with permission from Shively, 2006a and Shively, 2006b Bacterial inclusions. In: *Encyclopedia of Life Sciences*. Chichester: John Wiley & Sons, Ltd. http://www.els.net/ [doi: 10.1038/npg.els.0004268].

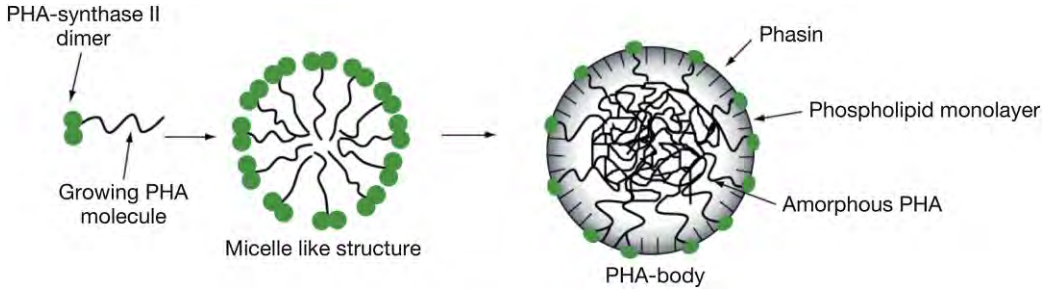

Figure 13 Model proposed for poly(3-hydroxybutyrate) (PHB) granule formation in bacteria. Phasins are amphiphilic proteins. This figure is based on studies done in *Ralstonia eutropha*. Reproduced from Wältermann M, Hinz A, Robenek H, et al. (2005) Mechanism of lipid-body formation in prokaryotes: How bacteria fatten up. *Molecular Microbiology* 55: 750–763, with permission from Blackwell. For further information see this reference.

coalesce during enlargement. In *R. eutropha* there are three PhaP1 homologues expressed, which might play a role together with PHA depolymerases for intracellular degradation of PHB. The transcriptional repressor PhaR strictly controls the expression of PhaP1.

Fusions of the phasin protein to other proteins were also detected at the surface of PHA granules in the cells. Phasins on the other side bind also to lipid granules. When PhaP1 and C-terminal fusions of PhaP1 with enhanced green fluorescent protein (eGFP) or with *E. coli* β-galactosidase (LacZ) were separately expressed in the triacylglycerol (TAG)-accumulating actinomycetes *Rhodococcus opacus* PD630, and *Mycobacterium smegmatis* mc^2155 (see below), PhaP1 became associated with the TAG inclusions and was mainly located on the surface. This provides various possibilities to generate functionalized granules or nanoparticles for various applications.

Triacylglycerol and Wax Ester Granules

TAG, that is, trioxoesters of glycerol and long-chain fatty acids, and wax esters (WE), that is, oxoesters of an alcohol and a fatty acid both of long carbon-chain length, that occur as insoluble cytoplasmic inclusions serve less frequently than PHAs as carbon and

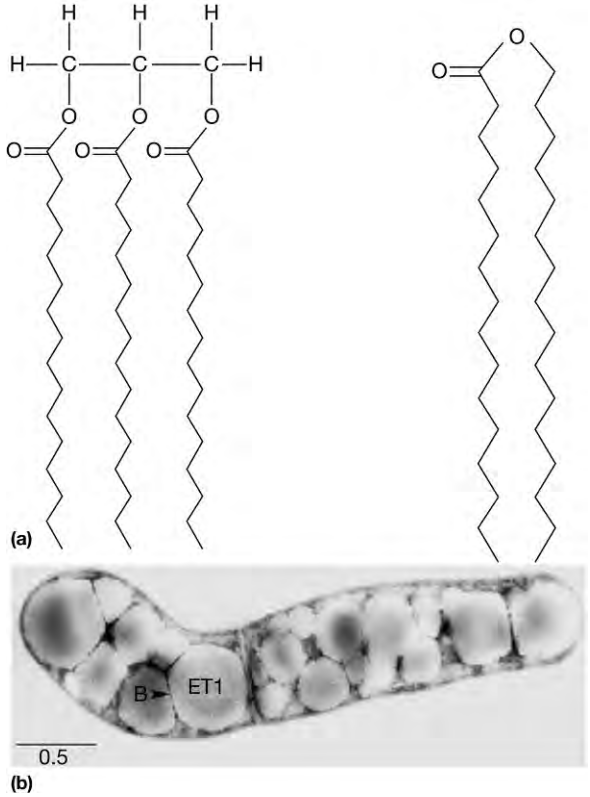

Figure 14 (a) Structural formulas of triacylglycerol (TAG) and wax ester (WE). (b) Transmission electron micrograph of a cell of the Gram-positive bacterium *Rhodococcus opacus* strain PD630 showing TAG granules. Cells were grown in a mineral salts medium containing 1.5% (wt. vol^{-1}) sodium gluconate and had accumulated various TAGs to about 80% of the cell dry matter. Reproduced from Alvarez HM, Mayer F, Fabritius D, and Steinbüchel A (1996) Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. *Archives of Microbiology* 165: 377–386, with permission from Springer Science and Business Media.

energy storage compounds in bacteria (Figure 14(a)). Whereas the chemical structure of TAGs is similar to those accumulated in most plant and animal cells, the chemical structure of WEs of most bacteria is similar to those produced by jojoba and the sperm whale (*Physeter macrocephalus*). The latter consists mainly of in total 32–36 carbon atoms, with saturated and monoenoic C16 and C18 fatty acid and fatty alcohol residues. Similar to the accumulation of PHAs, TAG and WE accumulation occur during growth limitation due to a lack of a suitable nitrogen source and imbalanced growth.

TAGs as the main storage lipid in bacteria have mainly been described in Actinomycetales like *Actinomyces* sp., *Arthrobacter* sp., *Mycobacterium* sp. including *Mycobacterium tuberculosis*, *Nocardia* sp., *Rhodococcus* sp., *Gordonia* sp., and *Dietzia* sp., and in streptomycetes such as *S. coelicolor*, *S. lividans*, and *Micromonospora echinospora*. Species of the Gram-negative genera *Acinetobacter* and *Alcanivorax* also frequently accumulate TAGs. These species accumulate only minor amounts of TAGs besides major amounts of WEs, which serve as the predominant storage lipid. In addition to these bacteria, WEs were also described to occur in members of the genera *Corynebacterium*, *Micrococcus*, *Moraxella*, *Mycobacterium*, and *Nocardia*. WEs do not occur exclusively as intracellular storage compounds, since some strains of *Acinetobacter* sp., for example, *A. borkumensis* and *A. jadensis*, and several other marine bacteria were also reported to synthesize extracellular WEs from hydrocarbons or to secrete lipids from the cells into the environment. However, the origin and possible function of these extracellular WEs remain to be elucidated. So far, TAGs and WEs have never been reported to occur in Archaea.

In *R. opacus* strain PD630, one of the best-studied model organisms with respect to its neutral lipid metabolism, TAGs can amount up to 80% of the cell dry matter (Figure 14(b)). In *Acinetobacter baylyi* strain ADP1, a novel type of a promiscuous enzyme, the wax ester/acyl-CoA:diacylglycerol acyltransferase (WE/DGAT), shortly referred also to as *atf*, catalyses the final step in TAG and also in WE biosyntheses. It can therefore be considered as the key enzyme for the biosynthesis of these lipids. Mutants with a defective *atf* gene are mostly or even completely impaired in WE and TAG biosyntheses. This enzyme is unique to bacteria, and it does not occur in eukaryotes. It synthesizes a wide range of different lipids. Interestingly, all bacteria capable of synthesizing these storage compounds among those whose genomes have been sequenced contain at least one or even several genes coding for homologues of this *atf*. The genome of *M. tuberculosis* contains 15 genes coding for *atf*homologues. Therefore, in bacteria, it is likely that TAGs and WEs are generally synthesized by this type of enzyme.

Although WE and in particular TAG inclusions are in most cases indistinguishable from PHA granules by microscopic techniques, significant differences from the latter with regard to surface structure and biogenesis were found. Lipid biosynthesis starts at the cytoplasmic membrane to which the *atf*is attached (Figure 15). At the very beginning small lipid droplets (SLD) are formed, which remain bound to the membrane-associated *atf*. They form a thin lipid layer at the inner leaflet of the cytoplasmic membrane. The SLDs increase in size and conglomerate subsequently to lipid prebodies that still remain associated with the membrane. They are released only in a later stage into the cytoplasm where they mature due to further coalescence of the SLDs inside the prebodies. Conclusive evidence exists that these lipid inclusions are surrounded by a phospholipid monolayer and that proteins homologous to phasins are absent.

It was recently shown that the mammalian lipid body proteins, perilipin A, adipose differentiation-related protein (ADRP), and tail-interacting protein of 47 kDa (TIP47), which comprise the so-called PAT family of proteins, are targeted to prokaryotic TAG bodies *in vivo* as revealed by analysis of fusions of these proteins to eGFP and by immunocytochemical labeling of ultrathin cryosections and freeze-fracture replicas. In contrast, the maize (*Zea mays* L.) oleosin could not be directed to the surface of lipid inclusions when heterologously expressed in the recombinant actinomycetes *R. opacus* PD630 and *M. smegmatis* mc^2155.

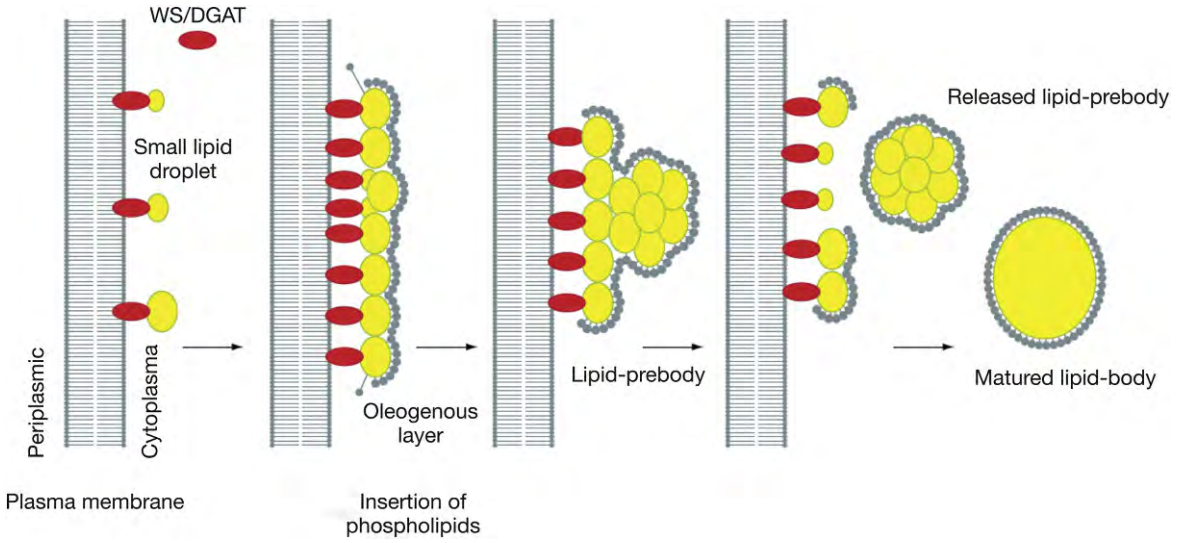

Figure 15 Model proposed for triacylglycerol (TAG) and wax ester (WE) granule formation in prokaryotes. WS/DGAT is wax ester/acyl-CoA:diacylglycerol acyltransferase. This figure is based on studies done in *Acinetobacter calcoaceticus* and *Rhodococcus opacus*. Reproduced from Wältermann M, Hinz A, Robenek H, et al. (2005) Mechanism of lipid-body formation in prokaryotes: How bacteria fatten up. *Molecular Microbiology* 55: 750–763, with permission from Blackwell.

Cyanophycin Granules

Cyanophycin is the third known polymer, the others being polyglutamate and polylysine, consisting of a restricted number of amino acids, that is synthesized by a nonribosomal synthetase. Cyanophycin is a comb-like polymer with α-amino-α-carboxy-linked L-aspartate residues representing the poly(aspartate) backbone and with L-arginine residues linked to the β-carboxylic groups of aspartate (Figure 16(a)). It is also referred to as cyanophycin grana protein (CGP) or multi-L-arginyl-poly-L-aspartate in the literature. Lysine and some other amino acids, for example, ornithine or citrulline, may in some bacteria partially replace arginine in the cyanophycin synthesized in the cytoplasm or during *in vitro* biosynthesis of cyanophycin using the purified enzyme. It is found in the cytoplasm as insoluble membrane-less inclusions (Figure 16(b)). Bacteria accumulate cyanophycin as storage compound for nitrogen, carbon, and energy when growth is limited by another nutrient.

Cyanophycin is synthesized by many cyanobacteria and also several nonphotosynthetic bacteria like *Acinetobacter calcoaceticus* independently of ribosomal protein biosynthesis. The key enzyme of cyanophycin synthesis is cyanophycin synthetase encoded by *cphA*. The enzyme catalyses the consecutive addition of aspartate and arginine residues to a primer or to the growing polymer chain and requires one ATP for addition of each constituent. In addition, the enzyme is dependent on the presence of K^+, Mg^{2+}, a (cyanophycin) primer, and a thiol reagent such as β-mercaptoethanol in the reaction mixture. Cyanophycin is intracellularly degraded by cyanophycinases encoded by *cphB* if growth resumes yielding β–Asp–Arg dipeptides that are hydrolysed by a peptidase to the monomers. Cyanophycin is extracellularly also degraded by cyanophycinases secreted into the medium by some bacteria. Proteases seem generally not to be capable of hydrolyzing cyanophycin.

So far technical applications are not known for cyanophycin. However, due to the availability of cyanophycin synthetase genes from several cyanobacteria and the occurrence of cyanophycin also in heterotrophic bacteria that grow much faster and to many higher cell densities than cyanobacteria, cyanophycin has become much more readily available. It is foreseen that the material properties and applications will now be intensively investigated. However, cultivation of heterotrophic bacteria for production of large amounts of cyanophycin in the cells seems to be a burden for the organism due to the competition of the ribosomal protein biosynthesis machinery and the cyanophycin synthetases for the amino acids aspartate and arginine. This can be overcome by using amino acid rich complex media or by directly feeding both amino acids. However, this increases the production process costs. A further alternative is to use an addiction system. A recombinant strain of *R. eutropha* has been recently constructed for the

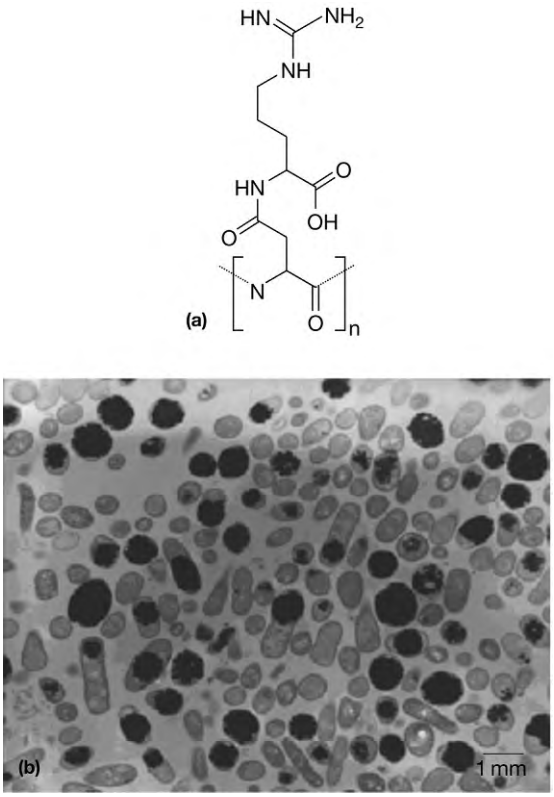

Figure 16 (a) Structural formula of cyanophycin. (b) Transmission electron micrograph of cells of the Gram-negative bacterium *Acinetobacter baylyi* showing cyanophycin granules. Cells were grown in a mineral salts medium containing 75 mmol l^{-1} arginine and 10 mmol l^{-1} ammonium sulfate and had accumulated cyanophycin up to about 40% of the cell dry matter. Bar = 1 μm. Micrograph courtesy of Yasser Elbahloul and Rudolf Reichelt, Westfälische Wilhelms-Universität Münstery, with permission from Shively, 2006a and Shively, 2006b Bacterial inclusions. In: *Encyclopedia of Life Sciences*. Chichester: John Wiley & Sons, Ltd. http://www.els.net/ [https://doi.org/10.1038/npg.els.0004268].

production of cyanophycin with a defective chromosomal KDPG aldolase gene (*eda*), which is essential for growth of the cells if, for example, fructose is used as carbon source. Instead, the strain contained a plasmid containing not only a cyanophycin synthetase gene (*cphA*) but also an intact *eda* gene. Thereby, *cphA* was coupled to *eda*, and therefore the cells could not lose the plasmid if they grew on fructose.

Inclusions of Bacillus Thuringiensis

The insecticidal bacterium, *Bacillus thuringiensis* (Bt) Berliner, consists of approximately 60 subspecies, many that occur commonly in habitats such as soil, leaf litter, plants, insect feces, and the midguts (stomachs) of many insect species. All subspecies of Bt are characterized by the production of one or more crystalline parasporal inclusions during sporulation, each of which typically contains one or more insecticidal proteins (Figure 17). At the end of sporulation, the host cell lyses, releasing the spore and parasporal inclusions. Many of the parasporal proteins are highly and selectively insecticidal for certain insect species. These proteins are protoxins activated by proteolytic cleavage in the insect midgut after ingestion. Activation typically requires a highly alkaline pH of 8–11. Once activated, the proteins bind to and destroy midgut cells, paralyzing and killing sensitive insects within 2 days of ingestion. In natural hosts of Bt like the larvae of certain moths that attack grains, mosquito larvae, and larvae of certain beetles, the spores germinate in the midgut as the larvae become intoxicated. Subsequently, vegetative Bt cells invade the moribund and dead larvae, colonize these, and sporulate. A typical increase in Bt cells in natural hosts ranges from 300- to 3000-fold. Without the insecticidal proteins, spores are poor insect pathogens and rarely kill insects. Thus, the role of these proteins is to intoxicate and contribute to the death of natural hosts, thereby providing a rich source of nutrients for reproduction of Bt.

The insecticidal proteins produced by Bt are so effective that several subspecies have been developed as commercial insecticides, some of which have been used for decades. In addition, through genetic engineering, important crop plants in the United States and several other countries such as Australia, China, and India have been developed that produce one or more Bt proteins to control important lepidopteran and beetle pests. Among these pests are corn borers, corn rootworms, and important cotton pests such as the pink bollworm and cotton budworm.

The most widely used Bt is *B. thuringiensis* subsp. *kurstaki*, a subspecies that typically produces four inclusion proteins, Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa, packaged into two crystalline parasporal inclusions. The Cry1 proteins cocrystallize forming a bipyramidal crystal, whereas Cry2Aa forms a separate cuboidal crystal (Figure 17). This subspecies is the active ingredient of numerous commercial insecticides used to control lepidopteran pests of field and vegetable crops, and forests.

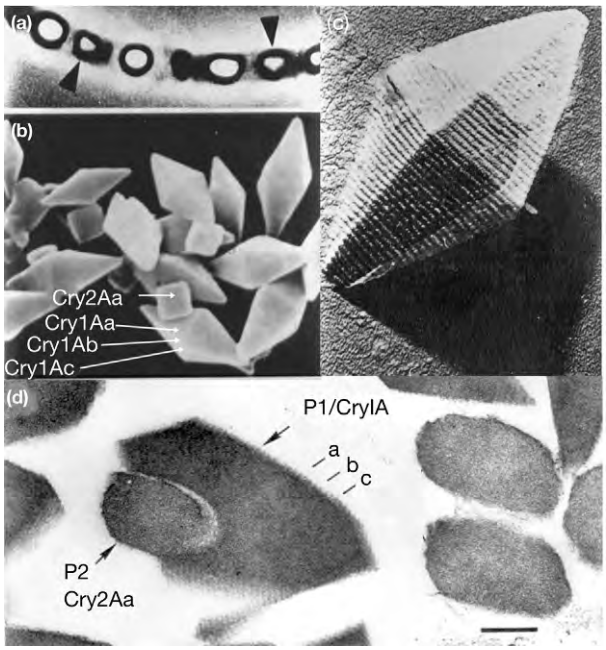

Figure 17 Sporulated cells of *Bacillus thuringiensis* and parasporal protein crystals. (a) Phase contrast micrograph of cells from a sporulated culture of *B. thuringiensis* just before lysis. Parasporal protein crystals (arrowheads) lie adjacent to oval spores. (b) Scanning electron micrograph of typical Cry1 and Cry2 crystals purified from a sporulated culture of *B. thuringiensis* subsp. *kurstaki*, isolate HD1. The parasporal body of this isolate consists of a bipyramidal crystal that contains Cry1Aa, Cry1Ab, and Cry1Ac, which cocrystallize, and a separate cuboidal crystal composed of Cry2Aa molecules. (c) Carbon replica of a typical bipyramidal Cry1 type protein crystal exhibiting the lattice of Cry1A molecules that compose the crystal. The HD73 isolate of *B. thuringiensis* subsp. *kurstaki* only expresses a single *cry1Ac* gene, and its parasporal body contains only a single crystal, such as this one, which measures approximately 1 mm from point-to-point along the longitudinal axis. (d) Transmission electron micrograph through a parasporal body of the HD1 isolate of *B. thuringiensis* subsp. *kurstaki* illustrating the embedment of the cuboidal Cry2A crystal (P2) in the bipyramidal crystal (P1). Bar in (d) = 200 nm. Micrograph in (c) by Hannay CL.

Another successful Bt is *B. thuringiensis* subsp. *israelensis*, used to control larvae of many mosquito and blackfly species. This subspecies produces a spherical parasporal inclusion that contains four major proteins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa. The Cry proteins are related to those of *B. thuringiensis* subsp. *kurstaki*, but are only active against mosquitoes, blackflies, and related dipterans. Cyt proteins are unrelated to Cry proteins, but synergize some of these. Several commercial products based on this subspecies are used to control both nuisance and vector mosquitoes and blackflies. This subspecies proved particularly important in the World Health Organization's Onchocerciasis Control Program in West Africa, where it was used during the 1980s and 1990s in rotation with chemical insecticides to control larval populations of the blackfly vector, *Simulium damnosum*, of the filarial worm that causes this disease.

A third Bt subspecies that has been developed commercially is *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis), which is used to control beetle pests such as the Colorado potato beetle. The key proteins in this and related strains are known as Cry3 proteins. These are essentially nontoxic to lepidopteran and dipteran pests, but highly effective against many beetle species.

One of the most remarkable features of Bt inclusion proteins is their remarkable safety record, whether used in bacterial insecticides or transgenic crops. Hundreds of millions of people have eaten these proteins over the past 40 years, as they occur commonly on many vegetable crops, and now in many transgenic crops. But not a single human is known to have become ill due to eating these proteins. The reason for this high degree of safety is that these proteins have evolved to only bind to the stomachs of certain types of insects. As recombinant DNA techniques are improved further, it is likely that Bt proteins will be used to protect many other crops from insect damage, including such important crops as rice and soybeans. Since the initial release of so-called Bt cotton in 1996, which produces the Cry1Ac protein to control certain caterpillar pests, several other Cry proteins (Cry1Ab, Cry2, and Cry3B) have been genetically engineered into important commodity crops such as corn and soybeans, to help protect these crops from other caterpillar and beetle pests. These crops, i.e., Bt corn and Bt soybeans, along with the earlier and newer versions of Bt cotton, now make up most of acreage of cotton and corn crops grown in the United States, as well as in several other countries including Argentina, Australia, Brazil, China, and India. In the future, it is likely that genes encoding Cry proteins will be engineered into other crops such as sorghum and even coffee, and possibly even vegetable crops, and will be used virtually worldwide owing to their high degree of efficacy, non-target safety and lack of detrimental environmental effects compared to synthetic chemical insecticides.

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Intracellular Symbionts and Parasites☆

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Introduction

Definitions

An organism is defined as intracellular where it is entirely enclosed within the cell membrane of the cell of another organism. In other words, the habitat of an Intracellular microorganism is a cell of another organism (Fig. 1(a)). These associations, by definition, comprise organisms of unequal size, and the partners are generally known as the (smaller) symbiont within the (larger) host. The description of the intracellular microorganism as a symbiont derives from the original definition of symbiosis as any persistent association between different species, irrespective of the impact of the association on the fitness of the participating organisms. The specific cells of a multicellular host (e.g., plant or animal) that are colonized by intracellular symbionts are known as host cells.

Impact of Intracellular Symbioses on the Host

In terms of function, intracellular symbionts are diverse and often variable (Fig. 1(b)). Some intracellular symbionts are deleterious to the host. These taxa are commonly described as pathogens or parasites, and include taxa that are obligately intracellular (e.g., *Rickettsia* agents of typhus fever, and the sexually-transmitted *Chlamydia trichomatis*), as well as taxa with a required intracellular stage of their life cycle (e.g., the merozoite stage of the malaria agent *Plasmodium* within erythrocytes of the vertebrate host) or that are facultatively intracellular (e.g., *Salmonella*, *Shigella*). Other intracellular symbionts are beneficial and, in many cases, required by the host. For example, cockroaches are absolutely dependent on their intracellular symbiont *Blattabacterium cuenoti*, and the fungus *Rhizopus microsporus* requires its intracellular symbiont *Paraburkholderia rhizoxinica*, while many leguminous plants have facultative associations with rhizobia (including the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium*). The impact of some intracellular symbionts on their host is, however, variable, depending on host genotype or species, physiological condition (especially immunocompetence) and environmental circumstance. For example, *Mycobacterium tuberculosis*, the bacterial agent of tuberculosis in humans, can persist for extended periods in macrophages of the lung without causing any disease symptoms; and the green alga *Chlorella* promotes the survival of its ciliate host *Paramecium bursaria* under a light-dark cycle, but is deleterious to the ciliate host in the dark.

All intracellular symbionts are predicted in impose some cost on their host. This is because all the resources utilized by a symbiont for maintenance and growth are derived, of necessity, from the host cell (Fig. 1(a)). Furthermore, all symbiont waste products, including end-products of metabolism that do not contribute to growth, cell wall fragments etc., are deposited into the host cell, potentially perturbing host cell function. In the many associations that promote host fitness, these costs are outweighed by other beneficial symbiont traits, which may include the consumption of host waste products and the release of metabolites that are utilized by the host (Fig. 1(a)).

Taxonomic Diversity of Intracellular Symbioses

As the preceding section illustrates, intracellular associations are taxonomically diverse. Nevertheless, distinct taxonomic patterns are evident (Table 1). Most conspicuously, nearly all known hosts of intracellular symbionts are eukaryotes, and all phyla of Eukaryotes studied in detail include representatives with intracellular symbionts. The propensity of eukaryotes to engage with intracellular symbionts is reinforced by the overwhelming evidence that the common ancestor of all extant eukaryotes had mitochondria, which are derived from intracellular α-proteobacteria. Interestingly, vertebrate animals are colonized by a diversity of intracellular pathogens, but just one beneficial intracellular symbiont is known, the alga *Oophila* associated with embryos of the salamander *Ambystoma maculatum*. The intracellular partners include many different Bacteria, for example representatives of the phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Cyanobacteria (the latter include the ancestor of plastids in algae and land plants); and multiple eukaryotes, including various protists (e.g., *Toxoplasma*), fungi (e.g., *Cryptococcus*) and algae, notably the dinoflagellate *Symbiodinium* in corals are other marine animals. By comparison, intracellular representatives of the Archaea are dominated by just one functional group, methanogens of the class Methanomicrobia.

☆*Change History*: March 2019. Angela E. Douglas updated the text and references. This is an update of Douglas, A.E., 2009. Endosymbionts and intracellular parasites. In: Schaechter, M. (Ed.), Encyclopedia of Microbiology, third ed. Academic Press, pp. 128–141.

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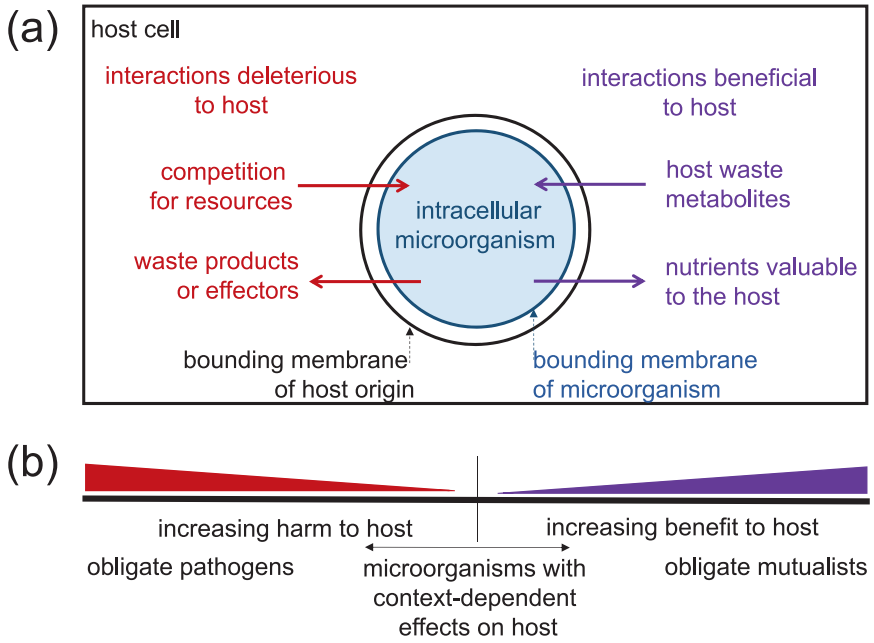

Fig. 1 Intracellular microorganisms. (a) The intracellular microorganism is enclosed within a host cell. Many intracellular microorganisms are in the cytosol, often localized to a compartment that is bounded by a membrane of host origin (shown as black circle) and variously known as a vacuole, vesicle or symbiosome. Some intracellular microorganisms are localized to sites other than the cytoplasm, including the nucleus (e.g., *Candidatus* Berkiella aquae in freshwater amoebae) and between the two membranes of mitochondria (e.g., the α-proteobacterium *Candidatus* Midichloria mitochondrii in *Ixodes* ticks). Intracellular microorganisms obtain all their requirements from the surrounding host cell, and thereby compete with the host cell, except where the inputs to the microorganism are host waste metabolites. Similarly, waste metabolites and other outputs of an intracellular microorganism may perturb host cell function. An effector is a molecule (usually a protein) of microbial origin that has evolved in the context of the association with the host and functions specifically to perturb host cell function, to the benefit of the microorganism. Many intracellular microorganisms that are beneficial to the host overproduce and release nutrients that contribute to host metabolism and growth. (b) Variation in the impact of intracellular microorganisms on host fitness. Some intracellular microorganisms are invariable either deleterious or beneficial to the host (obligate pathogens and mutualists, respectively), and the impact of other microorganisms on host fitness various with genotype and environmental factors.

Gaining Access to the Host Cell Habitat

The principal route of access to hosts is by phagocytosis, i.e., the microbial cell is engulfed by a portion of the cell membrane of the host cell. The phagocytozed microorganism is, thus, in the cytoplasm of the host cell but separated from the cytoplasmic contents by a host membrane (Fig. 1(a)).

Some host cells are highly phagocytic, and internalize a wide range of particles; for these systems, microbial access to the host cell appears to involve no specific molecular recognition events between the partners. This applies to phagocytically-active protists and the gut epithelium of various lower invertebrates (e.g., Cnidaria, Turbellaria) that internalize food particles by phagocytosis for intracellular digestion. (In most animals, including mammals, digestion is predominantly extracellular in the gut lumen.) Photosynthetically-active algal symbionts are acquired by this route, for example *Symbiodinium* in scleractinian corals and *Chlorella* in freshwater ciliates and hydra. Internalization by phagocytosis is also observed for macrophages of the animal cellular immune system. For example, several bacterial pathogens, including *Legionella pneumophila*, *Coxiella burnetii* and *Mycobacterium tuberculosis* gain entry to mammalian macrophages by passive phagocytosis. The usual fate of microbial cells internalized by these cells is delivery to the lysosome and digestion for host nutrition or anti-microbial defense, and the mechanisms by which persistent intracellular microorganisms evade this fate are considered in the following section (Persistence of Intracellular Microorganisms in the Host Cell).

Other intracellular microorganisms gain entry to eukaryotic cells by inducing engulfment by nonphagocytic cells. This involves microbe-induced perturbation to the membrane and underlying cytoskeleton of the host cell. Research on interactions between bacterial pathogens and mammalian cells has identified two broad mechanisms: trigger and zipper. Many bacterial taxa display either one or the other mode of internalization, for example the trigger reaction by *Shigella* and zippering by *Listeria* and *Yersinia*. Exceptionally, *Salmonella enterica* has the capacity for multiple routes of entry, including both zipper and trigger mechanisms.

The trigger mechanism involves the transfer of bacterial effector proteins into the target host cell, resulting in major reorganization of the cytoskeleton of the host cell. For example, the effector proteins SipA and SipC of *Salmonella enterica* are delivered via a Type-III secretion system (T3SS, encoded by SPI-1) into epithelial cells of the intestine, causing polymerization and reorganization of host cell actin filaments. Together with other cytoskeletal-modifying *Salmonella* proteins (e.g., SopA, SopB, SopD, SopE), these proteins induce membrane ruffles that engulf the bacterial cell by a process most accurately described as macro-pinocytosis.

Table 1 Survey of intracellular microorganisms. This survey is not comprehensive, and includes some widespread and notable examples

	Examples of Intracellular Microorganisms			
Host	*Bacteria*	*Archaea*	*Protists*	*Fungi*
Eukaryotes				
Protists				
[a]Trypanosomes	β-proteobacteria			
Foraminifera			Various dinoflagellates	
Amoebae	Various including *Chlamydia*, *Burkholderia*, *Legionella*		[b]*Parkinsela* (kinetoplastid) in *Paramoeba*	
Ciliates		Methanogens in various anaerobic ciliates	*Chlorella* in *Paramecium bursaria*	
Fungi	Burkholderiaceae in arbuscular mycorrhizal fungi and in *Rhizopus microsporus*			
Plants				
Legumes	Rhizobia			
Gunnera	Cyanobacteria (Nostocaceae)			
Animals	Various, including *Chlamydia*, *Rickettsia* Various chemosynthetic bacteria in molluscs, annelids *Wolbachia* and bacteriocyte symbionts in insects		Various, e.g., Apicomplexa, trypanosomes, *Entamoeba* Dinoflagellate algae (*Symbiodinium*) and *Chlorella* in sponges, cnidarians, turbellarians and mollusks *Oophila* in *Ambystoma maculatum* (salamander)	Microsporidia, *Cryptococcus* [d]"yeast-like" mycetocyte symbionts in some insects
Bacteria				
[c]*Tremblaya princeps*	Various γ-proteobacteria (e.g., *Moranella*)			

[a]In many species, e.g., of genus *Crithidia*, but not reported in trypanosome parasites of humans.

[b]Only known intracellular protist within a protest.

[c]Intracellular symbionts restricted to specialized cells known as bacteriocytes (housing bacteria) or mycetocytes (housing fungi).

[d]*Tremblaya* is an intracellular symbiont in mealybug insects.

In the zipper mechanism, the interaction is between surface proteins of the bacterium and target host cell. Specific outer membrane proteins bind tightly to receptors on the host cell membrane, inducing localized changes in the organization of the membrane and underlying cytoskeleton and internalization. The protein-protein interactions that induce zippering have been characterized in various pathogen-host cell interactions. These include Rck-invasin of *Salmonella* which binds to the host cell epidermal growth factor receptor (EGFR), and two Internalin (Inl) proteins of *Listeria monocytogenes*: InlA which interacts with the host cell adhesion molecule E-cadherin and InlB which binds to the hepatocyte growth factor receptor (Met).

A further example of active invasion of host cells is displayed by apicomplexan parasites, including *Toxoplasma* and *Plasmodium*. Being eukaryotes, the apicomplexans possess an actomyosin cytoskeleton, which has been shown to be crucial for penetration of the host cell. The key structures are the cytoskeleton associated with the inner membrane complex that lies internal to the cell membrane of the apicomplexan cell, together with proteins and other products discharged from specialized secretory organelles known as rhoptries and micronemes at the cell apex. These proteins induce tight adhesion to the host cell membrane and then rapid internalization. For example, *Plasmodium* merozoites can make contact with mammalian erythrocytes, re-orientate for apical contact with the host cell membrane, and invade the host cell within just 2 min.

Large-scale reorganization of host cell membrane and cytoskeleton is also exhibited by the root cells of leguminous plants that come into contact with rhizobia bacteria. The rhizobia are trapped at the tip of a root hair. This interaction induces local invagination of the root hair cell membrane, and then the progressive ingrowth of the membrane, forming an infection thread that penetrates ultimately to the inner cortex of the root. The bacterial and plant determinants of this process have been identified by genetic analysis, and include the sustained release from the rhizobial cells of both an acylated chitin oligomer known as the Nod factor and exopolysaccharide, both with side groups that determine compatibility between specific bacterial strains and host plant species. When the infection thread reaches in the inner cortex, each bacterial cell is internalized into a cell, enclosed within the membrane derived from the infection thread.

The mode of entry of some intracellular microorganisms is unknown. Particularly intriguing is the remarkable intracellular symbiosis within the bacterium *Tremblaya princeps* (Table 1). *Tremblaya* is an intracellular symbiont within cells of mealybugs (insects of the family Coccidae) and different lineages of this bacterium are known to contain one of several γ-proteobacteria. Phylogenetic data suggest that the different γ-proteobacteria do not represent multiple, independent acquisitions but replacements of one taxon by another in certain lineages of *Tremblaya*. Further sampling is required to assess whether *Tremblaya* is particularly susceptible to invasion by γ-proteobacteria (or certain taxa within the γ-proteobacteria), and the molecular basis of this interaction.

Persistence of Intracellular Microorganisms in the Host Cell

At the point of internalization into a host cell, intracellular microorganisms are bounded by a membrane of host cell origin (Fig. 1(a)). The default fate of these membrane-bound structures is fusion with host cell lysosomes, exposing the internalized microbial cells to the very low pH and hydrolases of the lysosomal contents. Some intracellular microorganisms, including the bacteria *Legionella pneumophila* and *Coxiella burnetti*, are remarkably tolerant of the conditions in the phagolysosome. On internalization into the host cell, these bacteria release protein effectors via a type-IV secretion system (T4SS) that delay phagosome-lysosomal fusion until the bacterial cell has made the necessary physiological changes for transition from an extracellular to intra-lysosomal habitat. However, most persistent intracellular microorganisms are not trafficked to the lysosome, and they avoid this fate by various mechanisms, notably by suppressing maturation of the phagosome, such that it fails to fuse with a lysosome, and by escape from the phagosome.

Various approaches have been adopted to investigate the molecular mechanisms by which many intracellular microorganisms evade trafficking to the lysosomes. In particular, the host membrane has been scored for its Rab protein content because these GTP-binding proteins define the developmental stage of vesicles involved in membrane trafficking in eukaryotic cells. For example *Chlamydia trachomatis* is bounded by a Rab6-bearing membrane, while Rab5 is associated with the membrane bounding *Mycobacterium tuberculosis*, Rab7 with *Salmonella enterica* and the homolog of human Rab3C with the alga *Symbiodinium* in the sea anemone *Aiptasia pallida*. Although this approach has diagnostic value for each individual association, there is an increasing appreciation that microbial perturbation of membrane function yields atypical Rab protein patterns, so limiting comparative functional interpretation. Other analyzes of the protein and lipid composition of the host membrane bounding various intracellular microorganisms are also indicative of mixed affinities of the membrane. In particular, the vacuole bounding *Legionella pneumophila* fuses with secretory vesicles from the endoplasmic reticulum, a process is driven by bacterial effectors secreted by the T4SS Dot/Icm of *Legionella*, rendering the membrane incapable of fusing with the lysosome; and the symbiosomal membrane bounding rhizobia in cells of the plant root nodule includes proteins that are diagnostic of both the cell membrane and endoplasmic reticulum in uninfected plant cells, as well as various unique proteins.

A minority of recently-internalized microorganisms transfer from the phagosome into the cytosol. Where studied, this process is mediated by microbial proteins. For example, the pathogen *Listeria* secretes a protein toxin, lysteriolysin O (LLO) which creates pores in the membrane, permitting the escape of the bacterial cells. Although LLO has broad cytotoxicity, it is rapidly denatured and degraded, so avoiding generalized damage to *Listeria*-infected host cells. Once in the cytosol, some bacteria nucleate the actin, generating actin "comet tails" that power bacterial movement through the cell. These actin structures are produced by the Arp2/3 complex, which is recruited to the bacterial via specific bacterial proteins, e.g., IcsA/VirG of *Shigella*, ActA of *Listeria*.

The long-term persistence of intracellular microorganisms requires the sustained growth and proliferation of the microbial cells within the host cell and spread, either by partitioning with host cell division or (especially for terminally-differentiated host cells in multicellular hosts) by transfer from infected host cells to uninfected cells. Where host cells bear one or small numbers of intracellular microorganisms, e.g., 1–7 *Symbiodinium* cells in gastrodermal cells of corals, host and symbiont division tend to be synchronized and the microbial partner is actively partitioned between host daughter cells, although the mechanisms remain to be identified. Coordinated host-symbiont cell division is unnecessary in host cells with hundreds or thousands of microbial partners (as, for example, for many intracellular symbioses in insects) because the probability that a host daughter cell would be symbiont-free by chance is vanishingly small.

Microbial manipulation of host cell growth and division has been demonstrated, most notably in the apicomplexan *Theileria* which, on taking up a position close to the host cell nucleus, induces high rates of host cell division and coupled proliferation of the parasite. A different strategy for population expansion is adopted by the apicomplexan *Toxoplasma gondii*, which, on depleting the resources in one host cell, spreads via an intermediate extracellular phase to previously-uninfected cells. First, the *Toxoplasma* parasite secretes a perforin-like protein TgPLP1, which permeabilizes the vacuole membrane; and then the cell membrane is ruptured by processes that are not fully understood but appear to involve parasite-mediated activation of host calpain-proteases and consequent perturbation of the host cell cytoskeleton.

Transmission of Intracellular Microorganisms Between Hosts

All living hosts are finite, in both space and time. The long-term persistence of intracellular symbionts depends on their transmission from one host to another. Two broad routes of transmission are available: horizontal and vertical transmission. Vertical transmission comprises transfer from parent host to offspring; in sexually-reproducing hosts with anisogamy (gametes of unequal size), the microorganisms are usually transferred to the larger gamete, e.g., to the oocyte of animals, leading to maternal inheritance. For horizontally-transmitted symbionts, the relatedness of the donor and recipient hosts is not defined, and some horizontally-transmitted microorganisms can have an extended free-living phase in the external environment (e.g., rhizobia in the soil). Vertical transmission has far-reaching evolutionary consequences for the host-microbial relationship. Specifically, the fitness of a vertically-transmitted symbiont is promoted by the vigor and fecundity of its host, and this overlap in selective interest between host and symbiont is particularly strong where horizontal transmission is limited or blocked. Thus, vertical transmission tends to reduce the virulence of microbial partners, and this trait is widespread in hosts that require their microbial partners.

Some horizontally-transmitted intracellular microorganisms have no apparent adaptations that facilitate long-range contact with the host. For example, various pathogens that colonize the gut epithelial cells of humans and other mammals gain entry to the gut lumen in food and water. Similarly, taxa that colonize macrophages and other cells of the mammalian lung are transmitted between hosts via aerosols, especially when the infected host coughs or sneezes with the 'explosive' release of pathogen-bearing droplets. Motile cells of other horizontally-transmitted taxa, however, display chemotaxis towards their host. For example, free-living cells of the dinoflagellate alga *Symbiodinium* swim towards the plume of ammonia emitted from alga-free cnidarian host, *Cassiopea*, and various rhizobia display chemotaxis towards root exudates of host legumes. For *Sinorhizobium meliloti*, which associates with alfalfa roots, nonmotile cells display both elevated minimum inoculum density for nodule formation and time to nodule formation. *mcp* (methyl accepting chemotaxis protein) genes of the bacterial partner play an important role in mediating rapid and effective colonization of the host this trait. For example, *mcpU* in *S. meliloti* promotes chemotaxis towards the amino acid proline, a major component of host plant root exudates.

Vertical transmission in asexually-reproducing protists is mediated by the retention of the intracellular partners through mitosis and cytokinesis of the host cell, and at sexual reproduction through meiosis to the gametes. In animal hosts, intracellular microorganisms that are transferred to the germ line early in animal development are, similarly, partitioned to the gametes and transmitted vertically. For example, *Wolbachia* gains access to the oocytes of its insect hosts by this route. Research on *Drosophila* fruit flies indicates that *Wolbachia* interacts with the products of key genes in insect germ-line stem cell proliferation and differentiation. In particular, *Wolbachia* infection has been implicated in the rapid sequence evolution of the gene *bam* (bag of marbles), and can partially restore fertility in female *D. melanogaster* with *bam* null mutations. Other intracellular symbionts in animals are restricted exclusively to somatic cells, e.g., gastrodermis cells of Cnidaria (corals, hydra etc), and bacteriocytes (whose sole function appears to be the housing and maintenance of the intracellular symbionts) in various insects. Generally, these intracellular microorganisms are eliminated from the somatic cell, and transferred via an extracellular phase to the host oocytes, where they are engulfed by the developing oocyte. For example, *Chlorella* in hydra pass from the gastodermal layer through the mesoglea to the developing oocytes in the ectoderm of the hydra body column, and the bacterial symbionts are expelled from the stomach disc of reproductive female anopluran lice and transit through the insect body cavity to the ovaries, although their mode of locomotion of the bacterial cells has not been established.

Patterns of transmission observed over short timescales, especially in laboratory conditions, can give an incomplete picture of the diversity of transmission modes available to some intracellular microorganisms. For example, the horizontal transmission of some *Symbiodinium* is likely facilitated by passage through the gut of mobile coral-feeding fish, such that viable *Symbiodinium* cells are distributed across the reef via fecal pellets. In addition, some intracellular symbionts display vertical transmission with higher

fidelity in benign laboratory conditions than in the field (*Hamiltonella* in aphids is a likely example). The occasional horizontal transmission of intracellular bacteria between taxonomically-divergent hosts can be evident as incongruence between the phylogeny of the host and symbiont. This is evident, for example, for both *Wolbachia* and *Hamiltonella* in insects.

Nutrient Exchange Between Intracellular Microorganisms and Their Hosts

As Fig. 1(a) illustrates, intracellular microorganisms derive their entire nutritional requirement from the surrounding host cell. Energy acquisition is a key requirement for intracellular symbionts. The dominant energy source for many taxa is host-derived sugars, especially glucose, but other sources include pyruvate utilized by *Shigella*, glycerol by *Listeria* and dicarboxylic acids utilized by rhizobia. The obligate intracellular parasite *Rickettsia prowazekii* obtains host ATP in exchange for bacterial ADP via an ADP/ATP translocase, Tlc1; this bacterium has additional *tlc* genes, whose protein products mediate the uptake of other nucleotides. Host-derived fatty acids and lipids are also utilized by various intracellular microorganisms, as a source of energy; long-chain and unsaturated fatty acids are also essential nutrients for some intracellular microorganisms.

An indication that intracellular microorganisms have access to a diversity of host metabolites is that they are generally auxotrophic for many nutrients, including the fatty acids mentioned in the previous paragraph as well as amino acids, nucleotides and often cofactors. The evidence comes from genome annotations and, for cultivable microorganisms, analyzes of the growth requirements in culture. Bacterial amino acid auxotrophy can compete particularly acutely with host requirements in animals and various protists that are also auxotrophic for a subset of amino acids (particularly the 9–10 essential amino acids that contribute to protein but cannot be synthesized de novo by animals). For example, various intracellular parasites require multiple amino acids that are either essential or of high value to the host, e.g., the 7 amino acids required by *Legionella* include 5 animal-essentials (leucine, isoleucine, valine, methionine and threonine) as well as arginine, synthesized by mammals at low rates, and the sulfur-amino acid cysteine. In contrast, beneficial intracellular microorganisms tend to be auxotrophic for amino acids that are synthesized by the host. This is illustrated by *Buchnera aphidicola*, whose genetic capacity for amino acid synthesis almost perfectly complements that of its aphid host: *Buchnera* produces the 10 essentials and just two of the non-essentials produced by the host, glycine and the sulfur-amino acid, cysteine.

For various systems, the microbial transporters that mediate uptake of host-derived nutrients have been characterized. However, inferences from genome data for some intracellular bacteria, e.g., *Buchnera* lead to many predicted metabolic inputs without annotated transporters for their acquisition. Multiple processes may contribute to resolution of this inconsistency: errors in gene annotation (especially in relation to transporters), host-to-microbe transfer via membrane vesicles, and non-specific permeability of the microbial cell membrane. Cysteine-rich peptides of host origin enhance the permeability of rhizobia (as well as promoting terminal differentiation of these bacterial cells), and similar peptides are expressed in the cells bearing intracellular bacteria of some insects.

The host membrane that separates many intracellular microorganisms from the surrounding host cell contents can represent a major barrier to nutrient acquisition by the microbial cells. For legume-rhizobia associations, the symbiosomal membrane substantially restricts rhizobial access to sugar pools in the host cell cytoplasm and regulates the supply of dicarboxylates to the rhizobial cells. In some associations, the microbial partner can modify the transport capabilities of the bounding membrane. The acquisition of arginine by *Mycobacterium* and *Salmonella* is mediated by the recruitment of the host cationic amino acid transporter SLC7A1 to the vacuole membrane; and the vacuole membrane bounding *Toxoplasma* has enhanced permeability, permitting the nonspecific leakage of many cytosolic metabolites to the parasite. However, *Toxoplasma* do not have free access to all nutrients. In particular, host cell mitochondria accumulate around the parasite and oxidize fatty acids at high rates, thereby restricting parasite access to these host nutrients.

Intracellular microorganisms release various metabolites into the host cell. These include the waste products of metabolism and, in some microorganisms, the products of metabolic pathways that are both absent from the host and advantageous to the host. For example, rhizobia release ammonium, the product of nitrogen fixation, *Chlorella* algae release the sugar maltose, and *Buchnera* releases essential amino acids. In other systems, the identity of the released products is uncertain or variable (e.g., glucose, fatty acids and glycerol have all been implicated as release products of *Symbiodinium*, and the compounds released from chemoautotrophic bacteria in various invertebrates are not characterized with confidence). Various mechanisms have been implicated in the selective release of metabolites from intracellular microorganisms. Maltose release from symbiotic *Chlorella* is induced by low pH (conditions that pertain within the symbiosome membrane) and the intracellular pool of maltose is very small or undetectable under all conditions, suggesting that the proton gradient may trigger the synthesis and release of this compound. Ammonium release from rhizobia is facilitated by an ammonium transporter that is localized to the symbiosome membrane and closely associated with a cytosolic host glutamine synthetase, resulting in the efficient assimilation of symbiont-derived ammonium into glutamine and maintenance of a steep ammonium concentration gradient from symbiont to surrounding host cell cytoplasm (Fig. 2(a)). The synthesis and release of the essential amino acid methionine by *Buchnera* is driven by supply of the host-derived substrate cystathionine (Fig. 2(b)), raising the possibility that essential amino acid production in this bacterium may generally be regulated by substrate supply; however, the molecular and metabolic basis of this putative process remains to be established.

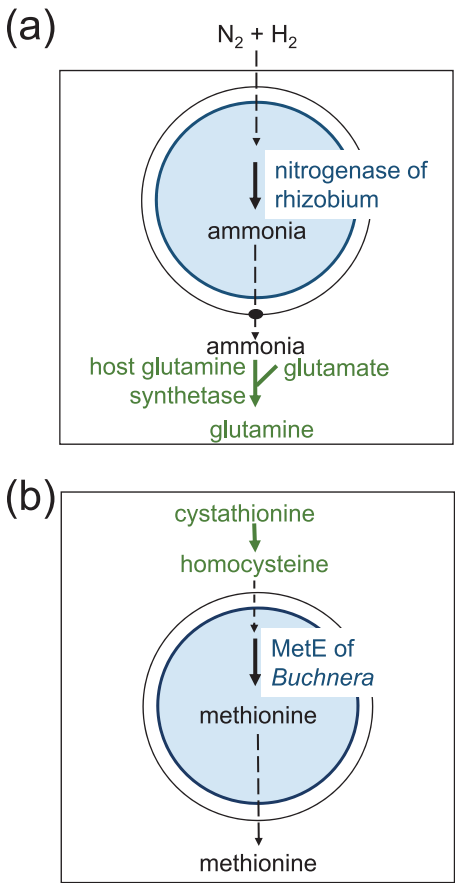

Fig. 2 Metabolic processes that promote nutrient overproduction by intracellular microorganisms. The intracellular microorganism (blue circle) bounded by a membrane of host origin (black circle) within the host cell (black rectangle) with metabolic reactions (solid lines) and transport reactions (dashed lines) shown. (a) Intracellular rhizobium (including the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*) in infected cell (host cell) of the root nodule of leguminous plants. Release of ammonia (comprising NH_3 and NH_4^+), the product of nitrogen fixation, is maintained by the high concentration gradient of ammonia across the rhizobium-host cell interface, maintained by: first, association of host glutamine synthetase with the ammonium transporter on the symbiosome membrane (shown), resulting in the efficient assimilation of ammonia into glutamine in the host cell cytoplasm; and, second, repression of rhizobium glutamine synthetase, the major route of ammonia assimilation in the rhizobia (not shown). (b) Intracellular *Buchnera* in bacteriocyte (host cell) of an aphid. Overproduction of the essential amino acid methionine is driven by substrate supply, specifically the concentration of cystathionine in the bacteriocyte. *Note*: a single intracellular microorganism is shown (the infected cell of legumes and bacteriocyte of aphids contain large numbers of rhizobia and *Buchnera* cell, respectively).

Genome-Scale Consequences of Intracellular Symbiosis

The genome of many intracellular microorganisms is much-reduced, in comparison to related taxa, with some genomes less than 1 Mb (Fig. 3). This condition is not unique to intracellular microorganisms (for example, the genome of the extracellular bacterium *Stammera* in midgut ceca of the tortoise beetle *Cassida* is just 0.27 Mb). There is wide agreement that genome reduction in these various microorganisms is driven by relaxed selection on genes not required in the host habitat and accumulation of deleterious mutations by drift in microorganisms with very small effective population size. Once genes involved in DNA repair and recombination start to decay, genetic degradation and gene loss can accelerate, leading to large genome deletions and very rapid genome reduction in some lineages.

Genome reduction in some intracellular microorganisms has been accompanied, and possibly promoted, by compensatory changes in the host genome and the acquisition of helper microorganisms. For example, the loss of the gene *ilvE* coding the terminal transaminase reaction in branched chain amino acid synthesis in *Buchnera* is compensated by the selective expression of an aphid gene *BCAT*, which can mediate the same reaction, in the host cell containing *Buchnera* (Fig. 4(a)). In this way, the synthesis of branched chain amino acids in the aphid-*Buchnera* symbiosis is shared between the two partners. Some shared essential amino acid synthesis pathways involve genes acquired horizontally by the insect genome from bacteria (e.g., *argGH* from bacteria of the order Enterobacteriales in the whitefly *Bemisia tabaci*) (Fig. 4(b)). In other instances, multiple bacterial taxa contribute to essential amino acid synthesis, either mediating the production of different essential amino acids (e.g., *Sulcia* and

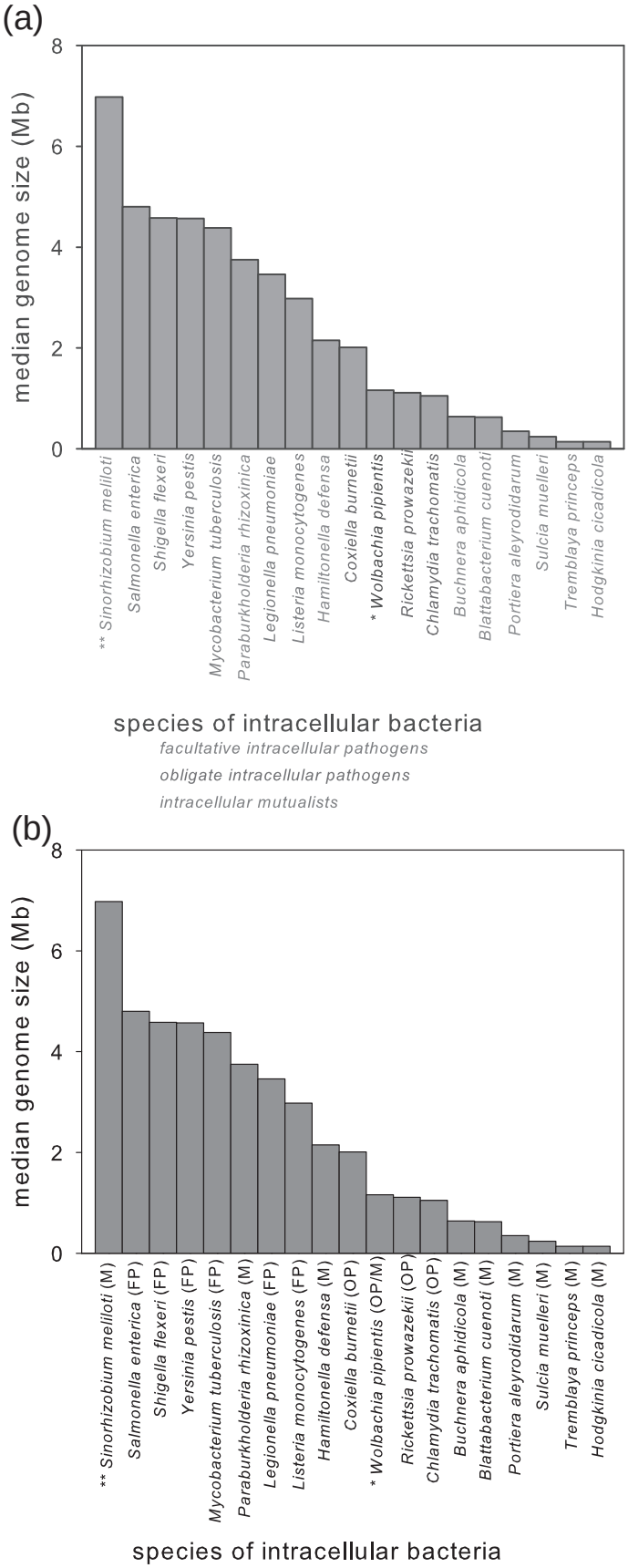

Fig. 3 The genomes of intracellular bacteria. (M): intracellular mutualist; (FP): facultative parasite; (OP) obligate parasite * *Wolbachia pipientis* is a reproductive parasite that induces cytoplasmic incompatibility, parthenogenesis and male killing in various insects and other arthropod hosts, but it can also enhance host fitness, especially by conferring protection against virus infections. ** The large genome size of *Sinorhizobium meliloti* and other rhizobia can be linked to its lifestyle as both a mutualist of legumes and saprophyte in soils; other intracellular mutualists included in this figure are unknown or rare in the external environment. Data collated from NCBI Genomes database on 9 May 2018.

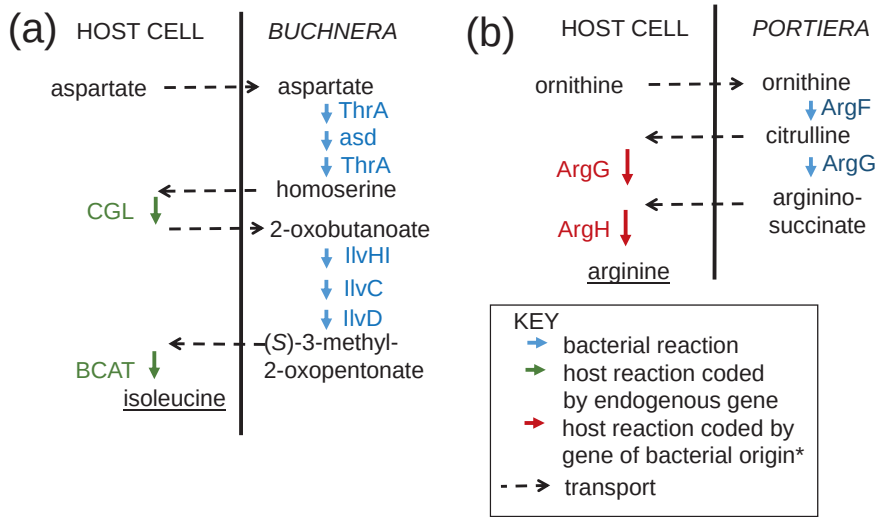

Fig. 4 Coevolved metabolic pathways between intracellular bacteria and their host. Host reactions compensate for gene loss from intracellular bacteria subject to genomic decay. (a) Synthesis of isoleucine in the aphid-*Buchnera* association involves two aphid enzymes: cystathionine-γ-lyase (CGL) and branched chain amino acid aminotransferases (BCAT). (b) Synthesis of arginine in the whitefly-*Portiera* association involves two enzymes of bacterial origin, argininosuccinate synthetase (ArgG) and argininosuccinate lyase (ArgH), transferred horizontally from Enterobacteriaceae to the whitefly genome (these genes are not derived from the intracellular symbiont *Portiera*, which is a member of the Halomonadaceae).

Hodgkinia in cicadas) or different reactions in the synthesis of an individual amino acid (e.g., *Tremblaya* and *Moranella* in mealybugs). Generally, the intermediate metabolites are transferred between the different host and microbial compartments mediating the various metabolic reactions, but the mechanisms underlying metabolite transfer and multi-partner coordination of metabolic function are not known.

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Iron Metabolism[☆]

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Glossary

Bacterioferritin (BFR) A subclass of prokaryotic ferritin that contains heme.
Ferritin (FTN) A large intracellular iron storage protein, present in both prokaryotic and eukaryotic cells.
Haptoglobin (Hp) A serum protein that scavenges hemoglobin.
Heme (Hm) A metal ion, customarily iron in the Fe(II) state, chelated in a tetrapyrrole ring.
Hemopexin A serum protein that strongly binds heme.
Hemophore (Hbp) A secreted bacterial protein that shuttles environmental heme to the bacterial surface.
Lactoferrin (LF) A glycoprotein present in the mucosal secretions and phagocytic cells of vertebrates that binds and transports iron.
Siderophore A small molecule secreted by bacteria and fungi that chelates environmental Fe(III) and, as the ferrisiderophore, then binds to the microbial surface.
Transferrin (TF) Iron-binding and transport protein found in the serum and lymph of vertebrates.

Abbreviation

BFR Bacterioferritin
Fes Ferrienterobactin esterase
FTN Ferritin
Hbp Hemophore
Hm Heme
Hp Haptoglobin
LF Lactoferrin
OH Hydroxyl radical
TF Transferrin

Defining Statement

Bacteria are able to obtain iron from diverse sources by employing a variety of transporters. These assimilation systems are described, as is the necessity for their precise regulation and the mechanisms by which this is achieved. The roles of iron in pathogenicity and primary fueling reactions are also summarized.

Introduction

A knowledge of iron metabolism is essential for a complete understanding of bacterial growth and survival and pathogenesis of bacterial pathogens. Although bacteria need relatively low concentrations of iron (5 μmol l^{-1}) for maximum growth and survival, iron functions as cofactors of enzymes in forms of ferrous ion, ferric ions, Fe-S clusters, and Hm. Thus, iron is necessary for processes such as electron transport, nitrogen fixation, removal of toxic forms of oxygen, synthesis of DNA precursors, tRNA modifications, ribosome modification, and syntheses of certain amino acids and tricarboxylic acid cycle intermediates; some bacteria can even oxidize iron to obtain energy (and, conversely, other bacteria use iron as a terminal electron acceptor). Iron is such a valuable and versatile nutrilite that only a few prokaryotes, such as the lactic acid bacteria, can manage without it.

The redox couple, Fe(II)/Fe(III), can have a range of potentials of −300–+700 mV, depending on the nature of the ligands and the environment surrounding the coordinated iron ions.

[☆]*Change History:* December 2014. B Lei updated the sections of Iron Uptake and Iron-Dependent Regulation, expanded the further reading list, and added Figure 2.

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A major consideration in iron metabolism is that, although iron is abundant on the surface of the earth, it is relatively unavailable. At neutral pH and in an oxidizing environment, which includes many common microbial habitats, iron exists in the +3 valence state and, as such, is extremely insoluble. For a microbe inhabiting an animal host, iron availability is restricted by the presence of iron storage and transport proteins, such as ferritin (FTN), lactoferrin (LF), and transferrin (TF). Thus, although relatively low concentrations of iron (5 μmol l^{-1}) are generally sufficient for maximum growth yields, bacteria frequently find themselves in iron-deficient environments and must devote a significant amount of their resources to obtaining this metal. Also important is the fact that it is possible for bacteria to suffer from iron overload and iron assimilation must, therefore, be precisely controlled. This article covers prokaryotic iron metabolism only. Descriptions of fungal iron assimilation are included in the reviews by Guerinot, 1994 and Leong and Winkelmann, 1998.

Iron Uptake

Bacterial iron assimilation occurs by a great variety of pathways, presumably because (1) transport systems were unnecessary for iron uptake until relatively late in evolution, when the atmosphere became oxidizing and iron became insoluble, and (2) a number of sources can serve as iron suppliers. In addition, many bacteria have multiple iron uptake systems. This enables them to obtain iron in diverse environments and from several sources. It is not rare to find bacteria with six or more high-affinity iron transport systems, and genome sequencing has shown additional systems may be present but defective. The large number and types of transport systems and variations among closely related bacteria make generalizations regarding iron uptake difficult, even within a species.

Bacteria can obtain iron from a variety of sources but, regardless of its origin, iron must be transported through the several microbial surface layers to reach the cytoplasm. For Gram-negative bacteria, these layers minimally include an outer membrane, a layer of peptidoglycan, and an innermost cytoplasmic membrane. The peptidoglycan cell wall is located in the periplasm, the space between the outer and inner membranes. Gram-positive cells, in contrast, may contain only a thick, highly crosslinked peptidoglycan cell wall external to the cytoplasmic membrane. Because of these distinct extracellular structures, Gram-positive and Gram-negative bacteria employ different mechanisms to relay iron and iron complexes through the surface layer(s). However, regardless of iron sources or forms and transport systems, uptake processes usually involve specific ATP-binding cassette (ABC) transporters that bring iron ions, ferrisiderophore, or Hm across the cytoplasmic membrane in both Gram-negative and Gram-positive bacteria. ABC transporters consist of a peripheral cytoplasmic membrane ATPase, present in two copies and possessing a defining ATP-binding site motif, and two hydrophobic cytoplasmic membrane proteins. The latter proteins can be similar, distinct polypeptides, one large fusion protein, or a homodimer. In addition, iron transporters have a component exterior to the cytoplasmic membrane. For Gram-negative cells, the component is a periplasmic binding protein, which recognizes the iron substrate and presents it to the cytoplasmic membrane complex. In Gram-positive cells, the periplasmic protein is replaced by a lipoprotein tethered in the cytoplasmic membrane but protruding into the peptidoglycan region.

Iron and Heme Uptake in Gram-Negative Bacteria

General pathway in gram-negative bacteria

A great variety of iron transport systems can be distinguished on the basis of the iron source and the form in which iron is mobilized but, in general, they follow a generalized pattern (Figure 1). Thus, for iron complexed to a carrier, first, passage through the outer

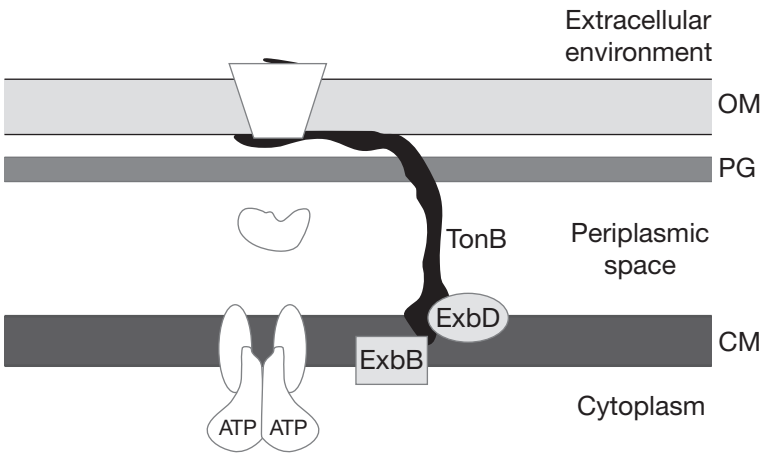

Figure 1 Generalized high-affinity iron transport system of Gram-negative bacteria. The three basic components are shown; they include: (a) an outer-membrane receptor protein; (b) a TonB system for energizing the receptor protein; and (c) a periplasmic binding protein-dependent ABC transporter, located in the cytoplasmic membrane. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane. See text for details.

Table 1 Consensus sequences related to iron

TonB box[a]	*Asp/Glu*		*Thr*			*Hyd*			*Val*		*Val*		*Thr*			*Ala*		*Glu*	
Fur binding site (Fur box, iron box)	G	A	T	A	A	T	G	A	T	A	A	T	C	A	T	T	A	T	C
DtxR binding site	T	A T	A	G	G	T	A	A	G	C G	T	A	A	C	A T	T	A		

[a]Positions two and five are the most conserved. Position three is the most variable; Hyd indicates hydrophobic amino acid (Ile, Leu, Val, Met, Phe).

membrane requires an outer membrane receptor protein whose synthesis is iron-regulated. A receptor protein has specificity for and binds a given iron-carrier complex and is sometimes synthesized in abundance only when that particular iron complex is available. In the best-studied cases, receptor proteins have been shown to be gated pores. Second, to permit entry of complexed or free iron into the periplasm, cytoplasmic membrane proteins TonB, ExbB, and ExbD are required. These proteins function as a group to utilize the electrochemical potential of the cytoplasmic membrane to open the gated receptor pores, permitting iron and iron complexes to pass into the periplasm. Generally, each bacterium has just one set of TonB/Exb proteins, capable of interacting with multiple receptors, but *Vibrio cholerae* and *Pseudomonas aeruginosa* have two distinct TonB systems. TonB is anchored in the cytoplasmic membrane but spans the periplasmic space, so as to be able to physically contact all receptors that require it. ExbB has three transmembrane domains and is present primarily in the cytoplasm, while ExbD has only one transmembrane segment and extends into the periplasm. Determination of the details by which TonB and its accessory proteins act is an area of active research. A straightforward mechanism would have ExbB sense the proton-motive force and, with ExbD, use it to induce a conformational change in TonB, which, in turn, could bring about by physical contact allosteric changes in outer-membrane receptors. TonB-dependent receptors have a heptapeptide (TonB box) near their N-termini that is thought to serve as a recognition sequence (Table 1). Third, transport across the cytoplasmic membrane employs an ABC transporter.

In summary, entry of iron into the cytoplasm of Gram-negative bacteria requires an outer-membrane receptor protein, a TonB system, and an ABC transporter. Passage through the outer and cytoplasmic membranes depends on the proton-motive force and ATP, respectively. Outer-membrane receptor proteins bind just one specific iron complex, TonB systems have broad specificity, and ABC transporters often specifically recognize different iron complexes.

Siderophore-mediated systems

A major mechanism by which bacteria obtain iron is through the production and secretion of small (600–1000 Da) iron-chelating molecules termed siderophores. More than 500 different siderophores have been isolated and characterized, but they can generally be classified as being either of the catecholate or of the hydroxamate type. A recent surprise was the observation that in some bacteria the common siderophore enterobactin is further modified by the addition of one or two sugar (glucose) molecules. Glucosylated derivatives of enterobactin and its breakdown products are termed salmochelins. Siderophores are synthesized in iron-deficient environments; after release from cells, siderophores bind iron and the siderophore–iron complex is subsequently internalized using the general type of transport system that has been described.

Siderophore biosynthesis is an interesting and growing area of research. Siderophores, generally, are built up of common amino acids, nonprotein amino acids, and hydroxy acids and, although they contain amide bonds, their synthesis does not involve ribosomes. Instead, a thiotemplate process with strong similarities to that employed for the synthesis of certain peptide antibiotics is used. Nonribosomal peptide synthetases link activated precursors to the enzyme-bound cofactor 4′-phosphopantetheine (P-pant). The thioesterified precursors are then covalently linked by amide bonds in the sequence determined by their position on the synthetase. The synthetase enzymes can be huge; in some cases, molecular weights of greater than 350 000 have been reported. Also, studies on siderophore biosynthesis resulted in the discovery of a new class of enzymes, the phosphopantetheinyl transferases, which donate the P-pant to the peptide synthetases. This essential posttranslational modification of synthetases permits them to bind activated precursors and then join the precursors together to yield the final product.

Other molecules, which do not meet the rigorous definition of siderophores, can also provide iron to cells by means of specific outer-membrane receptor proteins acting in concert with binding protein-dependent ABC transporters. Citrate, when present in environmental concentrations of greater than 0.1 mmol l^{-1}, is one such molecule, as are dihydroxybenzoic acid, a precursor of the common siderophore enterobactin, and dihydroxybenzoylserine, a breakdown product of enterobactin.

How newly synthesized siderophores are secreted is the least-known aspect of the siderophore cycle. In the cases where the process is somewhat understood, cytoplasmic membrane efflux pumps are required. There are a variety of such pumps, most of which are powered by the proton-motive force, such as members of the MFS and RND families, but the ABC transporters use ATP. Representatives of all three of these families have been implicated in siderophore release. Specifically, species of the genera *Erwinia*, *Vibrio*, *Bordetella*, and *Legionella* use MFS exporters; *Pseudomonas* may use an RND exporter and siderophore secretion in *Mycobacteria smegmatis* requires the ExiT protein (an ABC transporter). Efflux pumps generally have broad substrate specificity, and this makes it possible that in some bacteria several pumps may be able to extrude a given siderophore. Such redundancy may pertain in *E. coli*, where two proteins, EntS and TolC, affect enterobactin secretion. EntS is a member of the MFS family of transporters. Secretion of enterobactin but not its breakdown products is reduced in cells bearing an *entS* mutation. TolC is an

outer-membrane protein that is necessary for the functioning of many efflux pumps. *E. coli tolC* strains secrete no enterobactin. A current model postulates that there are two paths for enterobactin release and that both require TolC. The less commonly used path secretes enterobactin directly from the cytoplasm to the environment. Any of the several efflux pumps can probably be used to move enterobactin through a continuous channel that terminates with TolC. The more frequently used route may employ two steps; EntS acts first to place enterobactin in the periplasm and a second transporter, probably of the RND family, completes the secretion process via TolC.

Uptake of ferrous and ferric ions

Ferrous iron transport is useful to microbes capable of inhabiting oxygen-restricted environments, such as swamps, intestines, and marshes, or acidic locales, where reduced iron is stable and soluble. Fe(II) can enter the periplasm by means of pores in the outer membrane, and from there, bacteria have a variety of systems capable of transporting it through the inner membrane. Some of these cytoplasmic membrane transporters have broad specificity for transition metals and only low affinity for ferrous iron. However, systems that function only with Fe(II) as substrate are known. One such system that is essential in some bacteria is encoded by the *feo* operon (*feoABC*). FeoB is a large cytoplasmic membrane protein that hydrolyzes GTP and functions optimally in conjunction with its two smaller coregulated proteins, FeoA and FeoC. Many bacteria have *feo* genes, as does *Methanococcus jannaschii*. The latter finding is of interest in that *M. jannaschii* is classified in the domain Archaea, not in the domain Bacteria. Another widely distributed group of Fe(II) transporter proteins are members of the OFeT (oxidase-dependent iron transporter) family first observed in lower eukaryotes. Details regarding Fe(II) transport are lacking for both the Feo system and the bacterial members of the OFeT family. Finally, certain aerotolerant bacteria, such as the Gram-positive organism *Streptococcus mutans*, obtain iron by using a reductase exposed on the cell exterior to convert surface-bound Fe(III) to Fe(II). A ferrous ion transporter then delivers the iron to the cytoplasm.

A ferric iron acquisition system of the ABC type is present in a number of Gram-negative genera including *Serratia*, where it was discovered and termed Sfu type transport, *Haemophilus*, *Yersinia*, *Actinobacillus*, and *Neisseria*. A periplasmic binding protein accepts Fe(III) and presents it to the cytoplasmic components of the transporter, which internalize the iron. Ferric iron transporters can also function in concert with uptake systems that have outer-membrane components. In these cases, they become the terminal portion of the assimilation path. Thus, iron is removed from TF and LF at the outer membrane (see following), a process which requires a receptor and active TonB system, and then translocated into the cytoplasm by the ferric transporter. Also, iron can be transferred from citrate to a periplasmic binding protein for entry into the cytoplasm.

Uptake of iron from heme

The vast majority of iron in animals is present intracellularly as heme (Hm). Hm, in turn, serves as a prosthetic group of proteins, primarily hemoglobin (Hb), but also myoglobin and Hm-containing proteins such as cytochromes. Cell lysis is necessary for the release of these proteins. Freed Hb, such as that present following hemolysis of erythrocytes, is bound by the serum glycoprotein haptoglobin (Hp). Hb not bound by Hp can oxidize, in the process releasing Hm from globin. This extracellular Hm is bound by the plasma protein hemopexin and, less specifically, by serum albumin. Therefore, extracellular Hm, available as a possible source of iron (and Hm), occurs infrequently by itself as it is usually bound to Hb, Hb–Hp complexes, or hemopexin. In no case has a siderophore been able to scavenge iron from Hm. However, as will be described, each Hm source is capable of being utilized by one or another group of bacteria. These uptake systems vary greatly.

Iron assimilation pathways that recognize free Hm resemble those for iron–siderophore complexes; they require (1) an outer-membrane receptor protein that is TonB-dependent and (2) for cytoplasmic membrane passage, an ABC transporter. For several of these systems, including those of *Yersinia enterocolitica*, *V. cholerae*, and *Shigella dysenteriae*, it has been deduced that the entire Hm molecule enters the cytoplasm. In these cases, Hm supported porphyrin growth requirements, as well as provided iron. Pathogenic *E. coli* strains can often obtain iron from Hm but *E. coli* laboratory strains cannot. However, laboratory strains have an ABC transporter for dipeptides that can also recognize Hm. Therefore, recombinant laboratory strains that have a foreign outer-membrane receptor for Hm can use Hm as an iron source. Presumably, this dipeptide transporter can serve an auxiliary role in transporting Hm in bacteria that have a designated Hm transporter.

Several genera can remove Hm from Hb. *Neisseria* and *Haemophilus* spp. have TonB-dependent Hb-binding proteins in their outer membranes. Remarkably, both of these genera have additional TonB-dependent receptors that function in iron acquisition from Hb–Hp complexes. Each of these Hb–Hp receptors may consist of two different proteins. A different mechanism for the initial steps in obtaining iron from Hm or Hb is found in *Serratia marcescens*. This organism uses an ABC transporter to secrete a small protein (HasA) that functions as a hemophore (Hbp). That is, HasA is an extracellular Hm-binding protein that is necessary for the uptake of Hm, either free or bound to Hb. It shuttles its bound Hm to an outer-membrane receptor (HasR). *E. coli* strains harboring the virulence plasmid pColV-K30 also secrete an Hbp. Unlike HasA, this Hbp is autotransported out of the cell and is bifunctional. It has protease activity, degrading Hb as well as binding and transporting the released Hm. A chromosomally encoded outer-membrane receptor protein (ChuA), required for the well-known *E. coli* pathogen O157:H7 to use Hb or Hm, may be the Hbp–Hm receptor.

Only *Haemophilus* strains are known to utilize Hm associated with hemopexin. The system is not well characterized but three genes are required and one appears to encode a large secreted Hbp (HxuA). HxuA binds Hm–hemopexin, removes the Hm, and carries Hm to an outer-membrane receptor. The other two genes encode proteins concerned with the secretion of HxuA. Like all

Haemophilus Hm transport systems (free Hm, Hb:Hp, Hm:albumin), the Hm–hemopexin system requires a functional TonB protein.

Acquisition of iron from transferrin and lactoferrin

TF and LF are extracellular iron transport molecules present in the fluids of many vertebrates; each of these related glycoproteins can bind two ferric ions. Many siderophores are capable of removing iron from these glycoproteins. Members of the Neisseriaceae, Pasteurellaceae, and Moracellaceae produce no siderophores, however, and instead they have specific TonB-dependent outer-membrane receptors that bind these transport proteins. An ABC transporter necessary for all nonheme iron uptake pathways (TF, LF, or iron chelates) is present in *Neisseria*; iron from these sources passes through the outer membrane and is bound by periplasmic protein FbpA, prior to entry into the cytoplasm. Outer-membrane TF and LF receptors are unusual in that they appear to be bipartite; one protein has the characteristics of a typical TonB-dependent receptor, while the second is a surface-exposed lipoprotein. These receptors in *Neisseria* and *Haemophilus* show high specificity for glycoproteins of their normal hosts, such as humans.

Low-affinity iron transport

The high-affinity iron transport systems described above are generally not expressed in environments containing more than 5–10 $\mu mol\ l^{-1}$ iron. Remarkably, the means by which bacteria assimilate iron in such iron-replete environments, oxic or anoxic, is not known. This so-called low-affinity iron uptake may first require that iron be in the Fe(II) form. Ascorbic acid, which reduces Fe(III), stimulates low-affinity iron uptake. Fe(II) could be assimilated either (1) by the repressed levels of the *feo* system proteins or (2) by a cytoplasmic membrane transporter with broad specificity for divalent cations, such as the CorA protein of *E. coli* and *Salmonella typhimurium*. Also, several types of small molecules (monocatecholates, α-keto acids, and α-hydroxy acids) can, in certain genera, function as siderophores; they could mobilize extracellular ferric iron and, using a variety of transporters, provide iron.

Iron and Heme Uptake in Gram-Positive Bacteria

Gram-positive bacteria do not have the outer membrane but has a thick cell wall envelope. Because of this distinct extracellular structure, Gram-positive bacteria have evolved iron and heme acquisition processes that have distinct features from those in Gram-negative bacteria. Most notably, acquisition of ferrisiderophores only requires ABC transporters, and cell wall-linked proteins are produced to relay heme through the thick cell wall envelope to heme-specific ABC transporters in Gram-positive bacteria.

Siderophore-mediated iron acquisition

Specific ABC transporters appear to be sufficient to acquire ferrisiderophores in Gram-positive bacteria. Apparently, ferric siderophore complexes can diffuse through the cell wall to reach their ABC transporters. Siderophore-mediated iron acquisition is common in Gram-positive bacteria. *Staphylococcus aureus* produces two hydroxycarboxylate-type siderophores, staphyloferrin A and staphyloferrin B, which are transported by the ABC transporters HtsABC and SirABC, respectively. *B. subtilis* produces a catechol-type siderophore bacillibactin, and its ferric complex is taken up by the ABC transporter FeuABC. *Bacillus anthracis* produces two catecholate siderophores, petrobactin and bacilibactin, and petrobactin acquisition involves lipoprotein FpuA and redundant permeases FpuB and FatCD and ATPases FpuC, FpuD, and FatE. *Mycobacterium tuberculosis* produces mycobactins and carboxymycobactins.

Siderophore production is not detected in some streptococci, including Group A *Streptococcus* and *Streptococcus pneumoniae*. *Streptococcus equi* subsp. *equi* produces a siderophore. However, the capacity of *S. equi* to produce siderophore is acquired from an integrative conjugative element. These findings support the notion that bacteria in the Streptococcaceae family do not have a siderophore-producting system. However, members of the Streptococcaceae family produce specific ABC transporters to take up certain ferrisiderophores. Group A *Streptococcus*, Group B *Streptococcus*, and *Streptococcus pneumoniae* produce FtsABCD, FhuABCD, and PiaABCD, respectively, to acquire ferric complexes with hydroxamate-type ferrisiderophores, such as ferriferichrome. Even though streptococci usually do not have the capacity to produce siderophores, they have the capacity to acquire iron using siderophores produced by other bacteria in the same habitats.

Utilization of transferrin iron in gram-positive bacteria

Whether Gram-positive pathogens are able to use ferric transferrin as an iron source depends on whether they produce siderophores. *S. aureus* can use transferrin iron for growth, which can be extracted by either staphyloferrin A or staphyloferrin B and transported through their cognate ABC transporters. In contrast, ferric transferrin cannot support Group A *Streptococcus* and S. *pneumoniae* growth *in vitro*. However, pathogens without siderophore production may be able to use transferrin iron by using exogenous siderophore in infection with multiple bacterial species

Heme uptake pathways

Hm is sequestered tightly in proteins. Free Hm and hemoproteins to be used as iron sources appear to be unable to diffuse through the cell wall envelope to reach specific ABC transporters in Gram-positive bacteria. Thus, Gram-positive bacteria produce surface proteins to assimilate heme from hemoproteins and relay it to heme-specific ABC transporters.

Significant progress has been made in the last decade in the understanding of the machinery, pathway, and biochemical mechanism, and structural basis for heme acquisition process in Gram-positive bacteria, including *Group A Streptococcus*, *S. aureus*

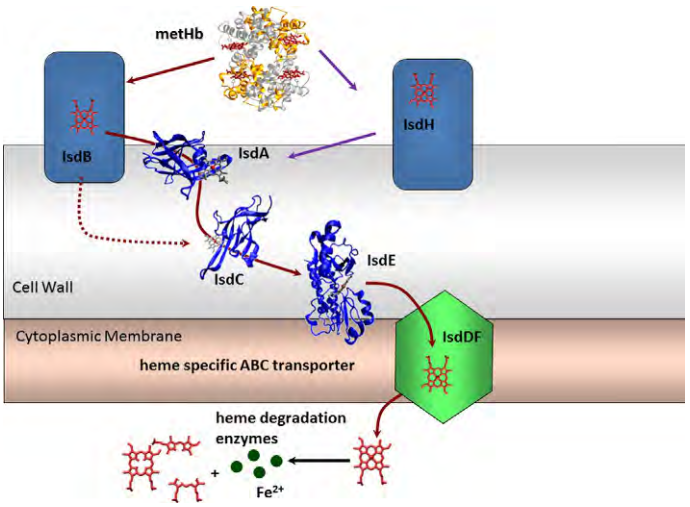

Figure 2 The proposed pathway of Hm acquisition from metHb by the Staphylococcus aureus Isd system. The arrows indicate the direction of direct heme transfer. Heme transfer from IsdB to IsdC represented by the dotted arrow may be prevented *in vivo* by their physical locations in the cell wall. The structure models of the proteins were from the coordinates 2Q8Q, 2ITF, 2O6P, and 1HHO. This figure was originally published in Journal of Biological Chemistry 283 (2008) 18450–18460.

and B. anthracis. The heme acquisition machinery of Group A *Streptococcus* consists of the surface proteins Shr and Shp and heme-specific ABC transporter HtsABC. The heme acquisition system in *S. aureus* is composed of the iron-regulated surface determinant (Isd) proteins, including the surface proteins IsdA, IsdB, IsdH, and IsdC and ABC transporter IsdDEF. Group A *Streptococcus* HtsA and *S. aureus* IsdE share a high level of sequence homology. However, Shr and Shp have no overall homology with the Isd surface proteins, and Shp can rapidly and directly donate its heme to the HtsA homologue of *Streptococcus equi* but not to *S. aureus* IsdE. The heme acquisition systems of Group A *Streptococcus* and *S. aureus* must have diverged enough to be considered as two distinct systems even they might have evolved from a common ancestor. *B. anthracis* has the homologues of *S. aureus* IsdC and ABC transporter IsdDEF and two other surface proteins IsdX2 and BslK but the *B. anthracis* system uses secreted hemophore IsdX1, thereby representing a distinct heme acquisition system in Gram-positive bacteria.

Relay of Hm from hemoglobin to ABC transporter by surface proteins has been demonstrated *in vitro* for the Group A *Streptococcus* and *S. aureus* heme acquisition systems. Hm is directly and rapidly transferred from human Hb to IsdB, from IsdB to IsdA and IsdC, from IsdA to IsdC, and from IsdC to IsdE, and IsdB and IsdC at catalytic levels dramatically enhance heme transfer from metHb to IsdE. These findings support the heme acquisition pathway of Hb → IsdB → IsdA → IsdC → IsdDEF in *S. aureus* (Figure 2). IsdH is also a hemoglobin receptor and is able to rapidly extract heme from metHb and donate it to IsdA. Thus, IsdB and IsdH apear to be redundant. The heme acquisition pathway of Hb → Shr → Shp → HtsABC in Group A *Streptococcus* is established based on the findings that rapid and direct heme transfer is observed only in the Hb/apoShr, holoShr/apoShp, and holoShp/apoHtsA reactions. In the *B. anthracis* system, IsdX1 can scavenge heme from metHb and transfer heme to IsdX2 and IsdC, and IsdX2 can also scavenge heme from Hb. Whether IsdC transfers heme to IsdE in *B. anthracis* is not known yet. Thus, the Hm acquisition pathway in *B. anthracis* has not been fully established yet.

Surface proteins and the lipoprotein of Hm-specific ABC transporters involved in Hm acquisition bind Hm at extremely high affinity. Hm-binding surface proteins usually contain one or more the NEAr-Iron Transporter (NEAT) domain. NEAT proteins were first identified in Gram-positive pathogens *Group A Streptococcus*, *S. aureus*, *B. anthracis*, *Clostridium perfringens* and *Listeria monocytogenes* and in non-pathogens *Bacillus jalodurans* and *Listeria innocua*. Characterized NEAT proteins have one or more NEAT domains, which bind Hm or interact with other proteins. A recent bioinformatic analysis identified 343 putative NEAT domains within 185 proteins. These NEAT proteins are mostly encoded by Gram-positive bacteria of the phlym Firmicutes, and analysis of metagenomic data from the Human Microbiome Project suggests that NEAT domain proteins are generally restricted to pathogenic species in human. However, NEAT proteins are also present in environmental and soil organisms. NEAT protein-mediated heme acquisition may occur in environmental and soil Gram-positive bacteria.

Heme transfer mechanisms

Kinetic, structural, and functional analyses indicate that Hm is transferred rapidly and directly from one protein to another along Hm acquisition pathways. Hm transfer follows a concerted two-step kinetic mechanism in the Shp/HtsA and IsdA/IsdC reactions. In this mechanism, the heme donors Shp and IsdA first form an activated complex with HtsA and IsdC, respectively, to allow direct Hm transfers that are >4000 times faster than simple Hm dissociation from these Hm donors. In the Shp/HtsA reaction, the axial ligand residues Met79 and His229 for Hm iron in HtsA specifically displace the axial ligand residues Met66 and Met153 of Shp, respectively, and the HtsA axial residues are involved in the cleavage of the axial bonds of Hm iron in Shp.

The Hb receptor IsdH of *S. aureus* has three NEAT domains. A truncated fragment of IsdH containing NEAT 2 and NEAT 3 and the linker between them is able to acquire Hm from Hb *in vitro*. Based on the crystal structure of a complex of Hb with the NEAT 2-linker-NEAT 3 IsdH fragment, NEAT 2 binds α/β globin through a site distant from the globin Hm pocket and positions NEAT 3 perfectly for the Hm pocket in NEAT 3 to acquire Hm from Hb. A similar mechanism is most likely followed in the reaction between Hb and IsdB. IsdB has two NEAT domains. The first NEAT domain (NEAT 1) in the N-terminal region interacts with Hm, and the second NEAT domain (NEAT 2) is the Hm-binding domain. A disordered loop in NEAT 1 in solution is critical for extraction of Hb Hm by IsdB both *in vitro* and *in vivo*. The other non-NEAT domains in the N-terminal regions critically contribute to the rapid kinetics and/or favorable equilibrium of the Hb/IsdB reaction. It is proposed that the non-heme-binding domains of IsdB interact with Hb and NEAT 2 to bring the Hm pockets of Hb and IsdB together to facilitate direct extraction of Hm from Hb by the Hm pocket in NEAT 2.

Cytosolic Iron Release from Siderophore and Hm

Prior to cellular usage iron is released from high affinity complexes with siderophores and protoporphyrin after ferric siderophore and Hm are transported across the cytoplasmic membrane. Release of iron from siderophores can be achieved through two kinds of enzyme-catalyzed reactions: hydrolysis of siderophore and reduction of ferric ion into ferrous ion. Pathogenic *salmonella* and *E. coli* produce ferrisiderophore esteases Fes and IroD to hydrolyze ferric enterobactin and its glucosylated derivatives called salmochelins, respectively, for the release of ferric ion from enterobactin and salmochelins. Gram-positive *Bacillus subtilis* produces ferric bacillibactin esterase BesA that can hydrolyze both ferric bacillibactin and ferric enterobactin whereas Fes cannot hydrolyze ferric bacillibactin.

Ferrisiderophore reductases convert high affinity ferric siderophore complexes into low-affinity ferrous complexes from which iron is moved to other iron binding sites of equal or higher affinities in the cytosol. Reduced flavin generated by flavin reductases or reduced flavin cofactor in nitroreductases can reduce various ferrisiderophores *in vitro*. These flavin reductases and nitroreductases belong to the NAD(P)H:flavin oxidoreductase superfamily. These enzymes have broad specificity and their abundance is not affected by iron availability. Bacteria can also produce specific ferrisiderophore reductases. *E. coli* ferric hydroxamate reductase FhuF uses a [2Fe-2S] cluster as a cofactor to reduce ferric hydroxamates but not ferric enterobactin. FchR produced by Gram-positive *Bacillus halodurans* catalyzes the reduction of ferric hydroxamates. Specific ferric triscatecholate reductases are also described recently. ViuB, which belongs to the NAD(P)H:flavin oxidoreductase superfamily, is required for ferric vibriobactin utilization in *V. cholerae* and can complement the function of Fes in *E. coli* though Fes does not complement a ViuB mutant of *V. cholerae*. ViuB homologs are present in various Gram-positive and Gram-negative bacteria and may play a critical role in iron assimilation for bacteria that utilize high affinity catecholate siderophores.

Like conventional Hm oxygenases in eukaryotes, Hm oxygenases in *C. diphtheriae*, *N. meningitides*, and *Pseudomonas aeruginosa* catalyze the degradation of Hm, forming biliverdin, ferrous ion, and CO. The Hm oxygenases, such as IsdG and IsdI in *S. aureus* and *B. anthracis* and MhuD in *Mycobacterium tuberculosis* also oxidize Hm to release ferrous iron. Unlike the conventional Hm oxygenases, the IsdG/I proteins convert heme to a chromophore called staphylobilin with the release of iron and formaldehyde, and MhuD degrades protoporphyrin to a product called mycobilin that keeps the meso-carbon as an aldehyde.

Intracellular 'Free' and Stored Iron

Two types of iron storage proteins, FTN and bacterioferritin (BFR), are found in bacteria. BFRs are a subfamily of FTNs, distinguished by the fact that they contain Hm units. Like eukaryotic FTNs, the bacterial proteins are large (~500 000 Da) and composed of 24 subunits.

The actual role of these proteins is uncertain. Unlike eukaryotic FTNs, which can accommodate over 4000 iron atoms, maximum iron contents for FTN and BFR are much less. BFR is synthesized primarily during periods of slow or no growth and may serve as an iron donor upon resumption of growth. FTN, on the other hand, is present at a constant low level throughout the growth cycle and, for at least *E. coli* and *Campylobacter jejuni*, has been shown to protect against iron-catalyzed oxidative damage.

A major uncertainty regarding bacterial iron metabolism is the distribution and form of much of the intracellular iron. Even the quantity of iron in a bacterium is unclear; for the well-studied organism *E. coli*, estimates of the number of iron atoms per cell range from 100 000 to 750 000. In a variety of bacteria, Hm-containing proteins account for less than 10% of the total iron, in contrast to the situation in animals. Iron–sulfur proteins also contain 10% or less of the bacterial iron, as do FTN and BFF when cells grown under iron-deficient conditions are examined. The remainder of the bacterial iron, the majority, exists in a mobile pool about which little is known. One report has suggested that the ions of the mobile Fe(II) pool are bound by a complex of phosphorylated sugars. It is this mobile iron that is presumably responsible for iron regulation and for the toxic effects of iron overload. Whether or not iron passes through this pool before being placed by ferrochelatase into protoporphyrin *IX* to form Hm, before incorporation into Fe–S clusters of proteins by unknown means, or before being converted into Fe(III) by the ferroxidase activity of BFR and FTN and deposited in the core of these molecules is unknown.

A most unusual role for intracellular iron occurs in the magnetotactic bacteria. These organisms contain internal magnets termed magnetosomes. Magnetosomes are 30–120 nm crystals of magnetite (Fe_3O_4) or greigite (Fe_3S_4) enclosed by a membrane; they exist

as chains and enable magnetotactic bacteria to sense and orient along geomagnetic fields. Magnetotactic bacteria are a diverse group but all are flagellated Gram-negative cells that favor microaerophilic environments in stratified natural waters. In conjunction with other tactic responses, magnetosomes are thought to assist these bacteria in finding their optimal environmental niche by guiding the cells downward following magnetic lines of force. The biogenesis of magnetosomes is not well documented but what is known indicates that it has several unusual aspects. The magnetosome membrane originates as an invagination of the cytoplasmic membrane, and is thus a phospholipid bilayer, and attains a distinctive protein complement that includes 20 magnetosome-specific proteins, most of which are encoded on a 130 kb magnetosome genomic island. Although magnetotactic bacteria have normal iron assimilation components, the uptake and biomineralization of iron for magnetosomes may occur by a distinct pathway that functions only in microoxic or anoxic conditions. Nonmagnetic mutants of a marine vibrio were found to be damaged in a gene for a periplasmic copper-containing protein, which was hypothesized to be part of a complex that transported iron for magnetite synthesis. Also, the assembly of magnetosomes into chains apparently requires cytoskeletal filaments composed of a new species of actin-like protein. Iron can constitute 4% of the dry weight of magnetotactic bacteria.

Iron-Dependent Regulation

Regulation of Iron Uptake

Genes encoding proteins necessary for high-affinity iron uptake are regulated by iron availability. The key regulatory protein in most bacteria, Gram-negative and Gram-positive bacteria whose DNA has a low GC content, is Fur. Fur, a small, histidine-rich polypeptide, is an aporepressor; in the presence of its corepressor Fe(II), it binds DNA. The holorepressor binds operators termed iron boxes, which are AT-rich 19 bp sequences with dyad symmetry (Table 1). Iron boxes are located in promoters of iron-regulated genes, such that steric hindrance prevents the repressor and RNA polymerase from binding simultaneously. Adequate intracellular iron supplies thus prevent transcription of genes for transport proteins and siderophore biosynthetic enzymes. A typical bacterium will have 50–100 such Fur-regulated genes.

Gram-positive organisms whose DNA has a high GC content, like *Corynebacterium* and *Streptomyces*, have no Fur but instead accomplish iron-regulated transcriptional control with DtxR proteins. The protein sequence of DtxR is unlike that of Fur but DtxR nonetheless functions in a Fur-like manner. Fe(II)–DtxR binding sites are 19 bp palindromes (Table 1) and are positioned in the promoters of genes they regulate.

Fur-like and DtxR-like proteins have repeatedly been selected to ensure that bacteria have appropriate levels of specific metals. Genome sequencing has demonstrated that many microbes have several proteins related to Fur and DtxR. Bacteria with Fur-regulated iron metabolism often have DtxR-controlled manganese uptake.

Some iron transport systems are produced only in iron-deficient environments that contain their cognate ferrisiderophores. That is, the systems are synthesized only if the intracellular iron concentration, as sensed by Fur, is low and if their specific ferrisiderophore is bound to the cell. As established in the ferridicitrate system of *E. coli*, the specific outer-membrane receptor must be present, as must a functional TonB system. Binding of ferrisiderophore to the receptor transmits a signal to a cytoplasmic membrane protein that has both periplasmic and cytoplasmic domains. The signal is then passed to a cytoplasmic sigma factor-like protein that, upon activation, associates with RNA polymerase and stimulates transcription of the necessary transport genes. Unique regulatory elements for these systems include an outer-membrane receptor capable of interacting with the cytoplasmic membrane protein, the cytoplasmic membrane protein, and its partner sigma-like factor. Among Pseudomonads, this dual regulation of specific iron transport systems by Fur and surface binding of the relevant ferrisiderophore is common.

Posttranscriptional Regulation of Intracellular Iron Usage

When iron is limited, numerous iron-containing proteins are down-regulated, including succinate dehydrogenase, aconitase, cytochrome C, and biosynthetic enzymes for heme, cysteine, and branched chain amino acids. The repression of iron-using enzymes in *E. coli* is mediated by a Fur-controlled small RNA, RyhB. RyhB synthesis is negatively regulated by Fur in iron-replete conditions. When iron becomes scarce, RyhB is expressed and stabilized by the RNA chaperone Hfq. The RyhB sRNA pairs with an mRNA target to block translation, and the sRNA-mRNA complex is recognized and degraded by the multi-protein complex RNA degradosome. RyhB-like small RNAs have been identified in many bacterial species, such as *P. aeruginosa*, *V. cholera*, *Shigella flexneri*, *N. meningitides*, *S. enterica*, and *B. subtilis*.

Regulation of Responses to Various Environment

In addition to directly controlling genes for iron assimilation and storage and indirectly controlling genes for iron-using enzymes in metabolism, Fur contributes to responses to and survival in a particular environment. Fur is involved in the defenses against oxidative stress. Iron mediates the cytotoxic effect of reactive oxygen species. H_2O_2 reacts with iron to form the reactive and damaging hydroxyl radical via the Fenton reaction, and superoxide is converted to H_2O_2 and increases iron concentration of iron by attacking iron-sulfur proteins. Fur deletion mutants are more sensitive to hydrogen peroxide and show increased oxidative DNA damage and mutations under aerobic conditions. H_2O_2 and superoxide activate the regulators OxyR and SoxS, respectively. Besides

inducing transcription of a set of antioxidant genes, OxyR and SoxS also enhance the expression of Fur. Superoxide dismutase (Sod) is commonly regulated by Fur. SodA, SodB, and SodC utilize Mn(II), Fe(II), and Cu(II) or Zn(II), respectively, as their metal cofactor. *N. gonorrhoeae sod* is activated directly by Fur. In *E. coli*, *sodA* is directly repressed by Fur-iron whereas *sodB* is indirectly activated by Fur through the action of RyhB. In *H. pylori*, *sodB* is directly repressed by Fur in the absence of iron. Fur transcripts are also affected by regulators that sense carbon source (cAMP–CAP) and oxygen availability (ArcC). Fur is also involved in the acid tolerance response.

Fur and Fur-like proteins play a role in colonization and virulence in diverse pathogens. Diphtheria toxin belongs to a family of ADP-ribosyltransferase toxins that transfer ADP-ribose to eukaryotic proteins to inactive target proteins and promote bacterial pathogenesis. The diphtheria toxin gene is directly regulated by DtxR. Shiga-like toxin and hemolysin in *E. coli* are repressed by Fur. Fur negatively regulates hymolysis production and an outer membrane virulence determinant IrgA in *V. cholerae*. In *N. gonorrhoeae*, Fur directly interacts with the promoters of 11 outer membrane proteins that aid in adherence to and invasion of host cells. MtsR, an iron/manganese-dependent metalloregulator in Group A *Streptococcus*, directly regulates streptokinase that activates host plasminogen to dissolve fibrin for pathogen dissemination. In *S. aureus*, many virulence factors are positively regulated by Fur. These virulence factors include coagulase, superantigen exotoxins, and two virulence factors known as Eap and Emp that are involved in biofilm formation. Fur is required for the expression of the two-component regulatory system SaeRS, which regulates the expression of many virulence factors of *S. aureus*.

Iron in Primary Fueling Reactions

Some bacteria can use the oxidation of iron compounds as their primary energy source. Bacteria capable of using inorganic, rather than organic, molecules for their fueling reactions are termed chemolithotrophs, and iron-oxidizing bacteria are a major group in this nutritional category. Iron-oxidizing bacteria typically live in acidic, aerobic environments rich in both reduced-iron and -sulfur compounds; they grow poorly at pH values greater than 4. Among other things, low pH is critical in keeping Fe(II) from being spontaneously oxidized to Fe(III).

Thiobacillus ferrooxidans, the best-studied microbe in this group, oxidizes iron using proteins in the cell envelope. An outer-membrane complex oxidizes iron to the ferric form, and a periplasmic protein transfers the electrons to cytochromes in the cytoplasmic membrane; these, in turn, pass the electrons to oxygen, the ultimate electron acceptor, in the cytoplasm.

T. ferrooxidans is a major cause of water pollution, specifically, acid mine drainage. Ferrous sulfide is common in many coal and ore sites, and its chemical and bacterial oxidation leads to both acidification and addition of dissolved metals to the water. Downstream, as the pH of the mine water becomes less acidic, insoluble ferric precipitates form.

In contrast to the relatively few bacteria that can oxidize iron as the initial step in generating energy, in the absence of oxygen many bacteria, notably Shewanella species and members of the Geobacteriaceae family, can use Fe(III) as the terminal electron acceptor in fueling reactions. This dissimilatory reduction of iron is a form of anaerobic respiration and is of interest for several reasons. It has the geochemical significance of solubilizing iron, and it is likely to represent a very early form of respiration, as all of the last common ancestors of modern organisms can reduce Fe(III) (using dihydrogen as the electron donor). Also, dissimilatory iron reduction is widespread among hyperthermophilic archaea, which is additional evidence for the ancient origin of this form of respiration. This extracellular respiration in which electrons are transferred from the cell to external substrates occurs in a manner similar to the electron flow in iron-oxidizing bacteria but in reverse. Electrons are thought to pass from menaquinone in the cytoplasmic membrane to small, soluble periplasmic electron-carrier proteins and then to terminal reductases, generally *c*-type cytochromes, in the outer membrane. From there electrons can be transferred directly or indirectly to minerals. Two types of direct transfer are known; the terminal reductases either bind or interact directly with extracellular iron and, alternatively, electron transfer takes place by means of surface appendages. The latter process has been reported in *Geobacter sulfurreducens* and *Shewanella oneidensis*, which are believed to produce specialized pili (nanowires) through which electrons pass to reduce insoluble Fe(III) compounds. Indirect electron transfer to extracellular iron can employ (1) metal chelators, such as citrate and nitrilotriacetic acid, to solubilize ferric iron and transport it to the cell for reduction or (2) electron shuttles, such as flavins, which transfer electrons from the cell to the static Fe(III) oxide substrates. Mechanistic details for all of the external reductive reactions are being determined. Interest in extracellular electron transfer to Fe(III) (and Mn(IV)) oxides is great because of the environmental significance and possible practical applications in bioremediation and electricity generation. The latter involves transferring electrons from organic compounds to electrodes via bacteria and their nanowires. Also, iron reduction by *Shewanella oneidensis* is remarkable in that surface appendages called pili are required. The electrons that reduce extracellular insoluble Fe(III) compounds pass through the pili, in the process generating a detectable current. The term 'electricigens' is used to describe bacteria that produce electricity.

Iron and Pathogenicity

A major stimulus to current studies on microbial iron metabolism is their medical significance. A brief review of the genera studied in the section titled 'Iron Uptake' will emphasize this fact.

Bacterial pathogens find themselves in an iron-deficient environment when they invade a eukaryotic host; conditions facilitating their acquisition of iron would be expected to increase virulence. Several general observations support this idea. Humans with higher than normal iron levels show enhanced susceptibility to bacterial infections. Similarly, at least 20 bacterial pathogens are more virulent when their animal host is injected with iron compounds prior to infection. Because excess iron can have a number of effects, including some that are detrimental to host immune defenses, these data are somewhat ambiguous. They are bolstered, however, by *in vitro* experiments showing that the antibacterial effects of body fluids can be uniquely reversed by iron supplementation.

Bacteria must synthesize an appropriate iron uptake system to overcome the bacteriostatic conditions in their hosts. Pathogens that multiply extracellularly, such as in blood or on mucosal surfaces, and that do not lyse host cells must be able to obtain iron from TF or LF. This can be accomplished by siderophores or by TF- or LF-specific receptor proteins. Septicemic bacteria with defective siderophore systems are, in fact, less virulent. On the other hand, the major source of iron for intracellular pathogens is Hm, and siderophore-deficient mutants of these organisms are still virulent. The required iron is taken from Hm compounds in this case.

For many pathogens, it has been difficult to identify specific iron uptake systems that are essential for their virulence. The multiplicity of means for assimilating iron in every species studied complicates such attempts. However, when all high-affinity systems are inactivated by the use of mutations in *tonB*, *S. typhimurium*, *V. cholerae*, and *Haemophilus influenzae* are avirulent. That a specific iron assimilation pathway can be a virulence factor has now been demonstrated for a number of pathogens. The type of transport system critical for virulence varies with the pathogen and can be species-specific. Thus, *N. meningitidis* lacking its outer-membrane hemoglobin receptor (HmbR) is attenuated for meningococcal infection in infant rats. *N. gonorrhoeae* with no functional TF receptor is unable to initiate urethritis in humans. High-affinity ferrous iron transport (FeoB-mediated) is important for *H. pylori* colonization of the gastric mucosa of mice and a functional siderophore system is required for *Yersinia pestis* virulence in mice. *Y. pestis* strains unable to either synthesize or transport the siderophore yersiniabactin are avirulent. A siderophore system is also a virulence factor in the fish pathogen *Vibrio anguillarum*. This marine microbe infects salmonid fish; approximately ten bacteria per fish are sufficient to cause vibriosis, a fatal disease characterized by hemorrhagic septicemia. A plasmid-encoded iron uptake system that uses the siderophore anguibactin is necessary for virulence.

Finally, many pathogens utilize the low iron concentrations in the host as an environmental signal to synthesize virulence factors. Fur and DtxR-like proteins control not only iron acquisition systems but also the synthesis of toxins and hemolysins in these organisms.

Conclusion

Interest in microbial iron metabolism continues to expand. Initial work in the field provided evidence regarding the diverse means by which bacteria obtain iron, began the elucidation of the critical role of iron in bacterial metabolism, and uncovered the means and necessity for the precise regulation of iron assimilation. The importance of iron in bacterial pathogenesis became well established and is now considered in all studies of prokaryotic pathogens. Basic studies utilizing *E. coli* and its relatives, while still incomplete and still yielding unexpected results, such as the existence of glucosylated derivatives of enterobactin, have given way to studies of less understood, and more difficult to cultivate, organisms. Studies of magnetotactic bacteria and particularly magnetosome biogenesis promise to uncover processes for controlling biomineralization and to provide insight into organelle formation. Possible biotechnology ramifications of investigations of dissimilatory iron reduction are likewise exciting. The recognition of the broad and central role played by the environmental interactions between bacteria and iron has led to the entry of geologists, ecologists, environmental and chemical engineers, and physicists as well as other disparate professions into the field. There is every possibility of multiple fascinating new discoveries.

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Legionella and *Bartonella*☆

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Glossary

Alarmone Small guanine nucleotide molecules (i.e., ppGpp and pppGpp) that are produced by bacteria under stress and regulate numerous cellular activities in response. These alarmones are signals for the stringent response that occurs in the presence of amino acid starvation.
Atypical pneumonia Infection of the pulmonary airspace that fails to produce the sudden onset and rapid clinical course associated with pneumococcal ("typical") pneumonia.
BCYE-a Buffered charcoal yeast extract agar supplemented with a-ketoglutarate. This medium is enriched for the isolation of *Legionella* species, and it also contains supplemental iron salts and cysteine.
Carrio´n's disease A geographically isolated disease transmitted by sandflies infected with *Bartonella* bacilliformis, including a severe febrile illness with hemolysis (Oroya fever) and chronic disseminated skin lesions (verruga peruana).
Endocarditis An infection of the internal surface of the heart, usually manifested as a collection of fibrin, platelets, and microorganisms deposited on one of the valve leaflets.
Endocytic pathway The process by which substances that are ingested by the cell are directed to their final intracellular destination. In the case of ingested bacteria, this refers to the steps in the delivery of the phagosome to the lysosomal compartment.
Hematophagous Literally, 'blood-eating'; referring to the behaviors of some biting arthropods.
Hemolytic anemia A loss of erythrocytes in the bloodstream by virtue of a process that causes their rupture or inappropriate removal from the circulation.

Abbreviations

ACES	N-(2-Acetamido)-2-aminoethanesulfonic acid
BA	Bacillary angiomatosis
CDC	Centers for Disease Control & Prevention
CSD	Cat scratch disease
ER	Endoplasmic reticulum
IFA	Indirect fluorescent antibody
T4SS	Type IV Secretion System
TLR	Toll-like receptor

Introduction

This article focuses on two bacterial genera, *Bartonella*, and *Legionella*. These bacteria are both fastidious Gram-negative pathogens, each of which requires specialized conditions for *in vitro* cultivation. However, as pathogens, they demonstrate distinct pathophysiologic and epidemiologic features.

Specifically, *Legionellae* live in aquatic environments and soil, and grow within resident protozoa. They also exist within biofilm in fresh water plumbing systems, providing one route of aerosol exposure to susceptibility humans. When inhaled in the form of

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aerosols from environmental water sources, they use their profoundly adaptive machinery evolved to parasitize numerous types of protozoa to grow intracellularly within alveolar macrophages in human lungs and thereby adventitiously cause a life threatening pneumonia. Quite often *Legionella* pneumonia occurs in an outbreak setting linked to a common source exposure. In contrast, *Bartonella* organisms are arthropod-borne pathogens that cause chronic systemic infection in mammals and in part persist through colonization of host red blood cells. Human infection occurs through exposure to infected animals or associated blood sucking arthropod vectors. Disease manifestations depend on the immune status of the host, and may range from febrile illness to induction of angioproliferative tumors.

Hence these two pathogens represent two unique disease causing entities, an environmental bacteria (*Legionella*) that fortuitously sets up a dead-end infection in humans and a zoonotic pathogen (*Bartonella*). The remainder of this article focuses on the specific factors that mediate the distinct life cycle and pathophysiologic features of these two bacterial species.

Legionella

The Organism

The Legionellaceae are aerobic, Gram-negative bacilli that use amino acids rather than carbohydrates as their preferred energy source. There are nearly 50 species within the genus *Legionella*, the majority of which have been isolated only from environmental, rather than clinical sources. In the environment, these organisms may inhabit complex communities composed of multiple bacterial species that grow within biofilms. They have been isolated from waters with temperatures ranging from 5 to 50 °C; however, they can grow to abundance at the warmer end of this spectrum, particularly in water distribution systems, water heaters, whirl pool baths, and most notoriously evaporative cooling towers. *Legionella* bacteria are natural parasites of free-living protozoa that dwell in lakes, ponds, or streams and graze on bacteria in biofilms. When *Legionella* are taken up by protozoa, such as the amoebal species Hartmannella or Acanthamoeba, they are able to evade intracellular degradation and grow within specialized vacuoles in these single-cell eukaryotes. The coexistence of *Legionella* bacteria and a competent protozoan hosts in potable water systems appears to be a prerequisite for the creation of *Legionella* containing aerosols, which may result in human infections.

Legionella pneumophila is the most frequently encountered and the best-studied species, as it is associated with most human infection, usually in the form of an atypical pneumonia designated Legionnaires' disease (see 'History'). There are 16 distinct serogroups of L. pneumophila, with serogroup 1 accounting for the majority of recognized Legionnaires' disease cases in the United States, Europe and Japan. Interestingly, *Legionella longbeachae* causes approximately half of the cases in Australia and New Zealand, and is also prevalent in Southeast Asia. In contrast to L. pneumophila, infection by this species is highly associated with exposure to potting soil and composts. At least twenty-four other species have caused human infections (e.g., *Legionella micdadei*, *Legionella dumoffi*, and *Legionella bozemanii*), but they are much less prevalent and most often isolated from hospitalized or severely immunocompromised patients. All of the potential pathogens must be able to survive and grow within macrophages in order to cause productive infection in the lungs of mammals, presumably by exploiting similar pathogenic mechanisms used to parasitize their natural protozoan hosts. Interestingly, dependence on the intracellular niche for growth is a feature also shared by the most closely related non-*Legionella* species, *Coxiella burnetii*, a pathogen that also causes atypical pneumonia.

The Legionellaceae are fastidious in their growth requirements *in vitro*. They are susceptible to noxious substances generated during the autoclaving of agar. Growth is also inhibited by concentration of sodium ions found in almost all standard bacterial growth media. Thus, semisolid media are typically prepared with added charcoal to absorb toxic substances and an organic buffer to avoid addition of sodium salts. Specifically, in addition to charcoal, the standard *Legionella* growth media contains yeast extract, to supply amino acids; ACES, an organic buffer; and lastly supplemental L-cysteine and soluble iron, required for growth. α-ketoglutarate is also often added as a growth enhancer. Media is buffered to a pH of 6.9 with addition of potassium hydroxide either prior to or after autoclaving. Typically, L. pneumophila colonies can be isolated on this buffered charcoal yeast extract α-ketoglutarate agar media (BCYE-α) in 2–5 days. As most normal flora grow abundantly on this rich media, antibiotics may be added to help select for *Legionella* spp. when culturing non-sterile respiratory specimens. Liquid culture of *Legionella* may be performed in ACES Yeast Extract (AYE) medium prepared with the same constituents as BCYE-α with the exception that insoluble activated charcoal is not added, and the medium is filter sterilized rather than autoclaved to avoid generation of toxic growth inhibitors.

History

Awareness of the Legionellaceae began after a large outbreak in a downtown Philadelphia hotel that hosted an American Legion Convention in 1976. Over 200 people were affected, and the case-fatality rate was 15%. The severe respiratory disease affecting the conventioneers thus became known as "Legionnaires' disease." However, the etiologic agent, L. pneumophila serogroup 1, was not isolated and characterized until the following year at the Centers for Disease Control and Prevention (CDC). The organism was initially passed from postmortem clinical specimens into guinea pigs, subsequently on embryonated chicken eggs, and finally on specialized, enriched media. The association of this organism with disease was suggested by the development of antibodies to this organism in affected patients, detected by using an indirect fluorescent antibody (IFA) test with the fixed bacteria as the capture antigen. The use of the IFA test also permitted CDC investigators to screen stored human sera associated with prior unexplained outbreaks of respiratory infection, several of which were retrospectively diagnosed as Legionnaires' disease, one of which had occurred at a different convention at the same Philadelphia hotel.

In addition to severe pulmonary disease, outbreaks of self-limited, flu-like illnesses were associated with L. pneumophila serogroup 1 seroconversion. This relatively benign syndromic response to *Legionella* organisms has been named 'Pontiac fever' (see below for details). Since 1977, the list of L. pneumophila serogroups and non-L. pneumophila species has gradually lengthened, as new organisms were isolated from patients and from environmental sources.

Epidemiology

With refinements in the media and serology, it was possible to show that Legionnaires' disease occurs both in discrete outbreaks and in sporadic cases. Water distribution systems were shown to be the predominant environmental sources of human infection (e.g., potable water sources, showers, sprayers, decorative fountains, and evaporative cooling towers). When outbreaks occur from these sources, exposure of humans is usually very common, but disease among exposed individuals is relatively rare. Accordingly, attack rates in these outbreaks are typically ~5%, suggesting that most humans can clear an inhaled inoculum of L. pneumophila without developing disease. In contrast, most individuals who develop Legionnaires' disease in outbreak situations have one or more underlying predispositions (e.g., advanced age, smoking history, lung disease, or immunosuppression).

Pathogenesis – Intracellular Infection

Intracellular infection of free-living amoebae in the natural aquatic environment appears to be a strategy that permits *Legionella* organisms to survive and grow among complex bacterial communities that are frequently grazed upon by protozoa. Mutants that cannot grow in protozoa have been isolated. Whether these mutants could survive in the aquatic environment is unknown. However, it is clear from laboratory animal experiments that infection in the lungs requires the capacity to infect and to grow in alveolar macrophages. Mutants that cannot grow in eukarytotic cells cannot cause disease. Because *Legionella* bacteria are not transmitted between mammals, it is presumed that the mechanisms that permit growth and development in macrophages evolved in protozoa.

Transmissive and Replicative Phases

L. pneumophila alternate between two distinct phases to complete their intracellular life cycle. The regulation of these phases occurs through a complex cascade of gene expression or suppression, reviewed by Molofsky and Swanson. A key determinant of the phase is the abundance of free amino acids, as amino acids are the primary nutrient source for L. pneumophila, and alterations of fatty acid metabolism thought to occur during the terminal stages of intracellular growth. When nutrients are abundant, the posttranscriptional regulator CsrA represses traits associated with transmission and cellular invasion (e.g., flagella synthesis and motility, lysosomal evasion, stress resistance, and cytotoxicity) and promotes bacterial division. In contrast, when nutrients are scarce, *Legionella* are triggered to increase production of the stringent response alarmone, (p)ppGpp, through an altered balance of synthase and hydrolase activities for this signaling nucleotide. This alarmone in turn increases the level of the alternative sigma factor, RpoS, and stimulates a virulence-associated two-component system, LetA/LetS. The latter system induces expression of small noncoding regulator RNAs that bind to and effectively inactivate, CsrA, a transcriptional repressor of transmissive phase genes. Together with the abundance of RpoS, these regulators induce the expression of the transmissive traits mentioned above and inhibit bacterial replication.

Similar to log-phase growth in nutrient broth, the intracellular niche provides abundant amino acids to the bacteria. Consequently, in both log-phase and intracellular growth, the organisms replicate rapidly, but they do not express flagellar motility or any of the traits required to establish infection in host cells. If harvested from these growth states, bacteria are unable to initiate cell infection de novo. As a broth culture enters stationary phase, or as the intracellular bacteria exhaust available amino acid and fatty acid stores of the cell, (p)ppGpp is produced, the bacteria stop growing, and they express a phenotype that will permit productive infection of a new host cell – that is motility, invasion, and host cell altering virulence factors that promote productive infection. Hence, bacteria grown to stationary phase in broth culture or harvested from host cells during terminal stages of infection are efficient in establishing infection in new host cells.

Intracellular Life Cycle

Flagellate L. pneumophila (in the transmissive phase) are promptly taken up by macrophages on contact (**Figure 1**, step 1). Several types of phagocytosis have been described including uptake into spacious phagosomes, uptake by a zippering mechanism, and more rarely a novel coiling mechanism in which a pseudopod wraps itself around the bacterium, drawing the organism into the host cell (**Figure 2(a)**). During and after ingestion, the bacterium alters the normal endocytic maturation pathway and in doing so avoids trafficking to the digestive pit of the host cell, the lysosome. Instead, the L. pneumophila-containing vacuole matures by acquisition of membranous intracellular organelle components from the exocytic rather than the endocytic pathway. (**Figure 1**, steps 2 and 3). Specifically, the cellular proteins, Arf1 and Rab1, which normally attract vesicles from the endoplasmic reticulum (ER) to the cis-Golgi, are instead recruited to the membrane of the L. pneumophila-containing vacuole. Within a few hours, the vacuolar membrane enlarges by fusion with redirected ER vesicles. In addition, the membrane becomes studded circumferentially with ribosomes (**Figure 2(b)**). In essence, the *Legionella pneumophila* phagomsome becomes part of the ER. Importantly, the ER vesicles contain nascent proteins, and

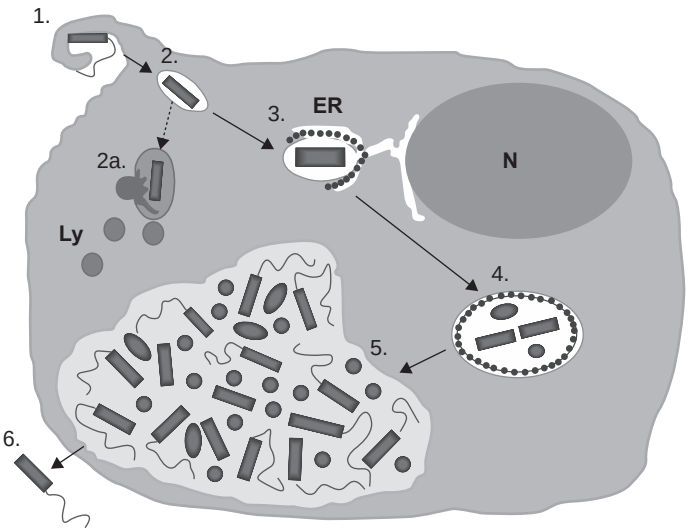

Figure 1 Schematic diagram of events in the *Legionella* pneumophila life cycle in macrophages. Flagellate bacteria in the transmissive phase are taken up by the cell, sometimes by a process of 'coiling' phagocytosis (1). Virulent bacteria within the early phagosome, (2) evade phagolysosomal fusion (2a), and associate with other membranous organelles (3). Endoplasmic reticulum (ER) bearing ribosomes associate with the *L. pneumophila*-containing vacuole, the bacteria are converted into the replicative phase, and intracellular replication begins (3 and 4). At 24 h, large vacuoles displace the cytoplasm of the cell, and the bacteria are converted again into the transmissive phase (5) before they are released from the cell to swim free to the next host cell (6). N.C. Engleberg *Legionella*, *Bartonella*, *Haemophilus*, Encyclopedia of Microbiology, 2009, 170–181, http://dx.doi.org/10.1016/B978-012373944-5.00194-2.

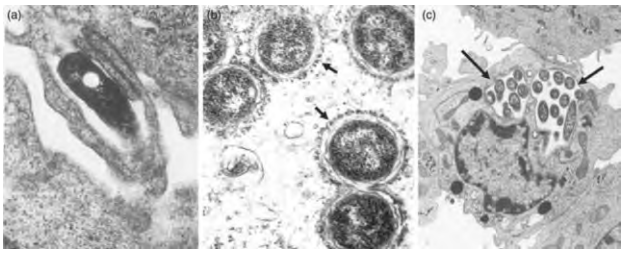

Figure 2 Electron micrographs showing key events in L. pneumophila replication in macrophages. (a) An electron-dense bacterium is seen surrounded by a pseudopod coil as it is taken into the cell. (b) Bacteria are seen in individual phagosomes studded with ribosomes (arrows) after endoplasmic reticulum (ER) vesicle recruitment. (c) As intracellular replication proceeds, increasing numbers of bacteria are seen in enlarging vacuoles (arrows). N.C. Engleberg *Legionella*, *Bartonella*, *Haemophilus*, Encyclopedia of Microbiology, 2009, 170–181, http://dx.doi.org/10.1016/B978-012373944-5.00194-2.

it is speculated that the ribosomes may provide polypeptides that the bacteria cleave proteolytically (secreted protease being abundantly liberated) and utilize as nutrients. Presumably, amino acids are likely to be abundant at this stage, since the bacteria convert into the nonflagellated, replicative phase and begin to grow and to divide. As replication proceeds and bacterial numbers increase, some fusion with lysosomes may occur; however, late in the course of intracellular replication, there is little deleterious effect of lysosomal contents on bacterial viability. After 24 h, the cell contains large membrane-bound compartments filled with bacteria, many of which have converted again into the transmissive phase (**Figure 1**, step 5; **Figure 2(c)**). Presumably, the resources of the host cell available to the intracellular bacteria are exhausted at this point, leading to an increase in the alarmone, (p)ppGpp, which triggers the phase transition. Bacteria in the transmissive phase produce a cytotoxin that induces the release of bacteria from the cell. Finally, the motile progeny are then free to seek out a new host cell (**Figure 1**, step 6).

Mechanisms of Intracellular Survival

Survival of L. pneumophila in both macrophages and amoebae depends on a type IV secretion system (T4SS), designated as Dot/Icm, and on the effectors that this system translocates into the host cell cytoplasm. Mutations that abrogate the functions of this secretion system result in the inability of an individual bacterium to escape the endosomal–lysosomal pathway, and instead the ingested bacteria are directed to the lysosome within 5 min (**Figures 1** and **2(a)**). Approximately 300 substrate proteins are now known to be transported by this system, and the functions of some are either known or strongly suspected (**Table 1**). Substrate proteins have been identified using various screens for T4SS-dependent protein translocation. Alternatively, other investigators devised genetic screens to detect specific functions that are thought to mediate the intracellular life cycle events. For example, a genetic screen was used to identify mutants that are unable to recruit Rab1 to the L. pneumophila-containing vacuole. Another

Table 1 Selected substrates of the Dot/Icm type IV secretion system

Function	*Protein*	*Mechanism*
Vacuolar modification	RalF	Exchange factor for Arf GTPase
	DrrA	Exchange factor for Rab1 GTPase
	LidA	Binds to Rab1 GTPase
	SidJ	Facilitates recruitment of ER vesicles
Inhibition of lysosome fusion	VipA	Inhibits lysosomal trafficking (in yeast)
	VipF	Inhibits lysosomal trafficking (in yeast)
	VipD	Inhibits lysosomal trafficking (in yeast)
Prevention of host cell apoptosis	SdhA	Forestalls host cell apoptosis
	SidF	Forestalls host cell apoptosis
Release from the host cell	LepA	Facilitates lytic release of bacteria from protozoa
	LepB	Facilitates lytic release of bacteria from protozoa; inactivates Rab1

method identified genes encoding proteins that bind to Rab1. A third approach identified mutations that interfere with membrane trafficking in yeast cells. Lastly, translocated effectors could sometimes be identified from genomic sequence based on homology with eukaryotic functional domains. In fact, all told, about 10% of the *Legionella* genome appears to be "eukaryotic" in nature and likely devoted to cooptation of host cells.

Examples of identified effectors include two Dot/Icm-dependent proteins that redirect secretory vesicles to the L. pneumophila vacuole (RalF and DrrA; **Table 1**). Other Dot/Icm substrates have been shown to interfere with phagosome–lysosome fusion; stimulate host cell antiapoptotic factors to postpone cell death until bacterial replication is exhausted; and facilitate the release of progeny bacteria from the cell (**Table 1**). Many of the substrate proteins translocated by Dot/Icm have redundant functions, presumably to ensure functionality in diverse protozoan hosts. The functions of many translocated effectors remain yet to be determined. As *Legionella* have been evolving creative ways to coopt the host cell for eons, discovery of new translocated effector functions will undoubtedly yield novel insights into host cell biology.

Consequences of Infection (Legionnaires' Disease)

Infection of humans with L. pneumophila is usually acquired by the inhalation of aerosolized bacteria directly into the lower airways. Aspiration of contaminated drinking water is also considered a means of inoculating organisms deep within the lung. The minimal infectious inoculum and form of the infectious particle (e.g., individual bacteria; biofilm fragments; protozoa; protozoan cysts; or isolated protozoan vesicles containing bacteria) are unknown. Of interest, L. pneumophila within amoebae are more infectious than plate-grown bacteria alone, when equivalent inocula were administered by intratracheal injection in laboratory animals. However, relative infectivity of free organisms living in their natural, relatively nutrient poor fresh water environment was not studied. Therefore, the jury is still out on the importance of different forms of *Legionella* for human infection.

After the initial infection of pulmonary alveolar macrophages with L. pneumophila, inflammatory cells (monocytes and neutrophils) are recruited into the lung. Interestingly, *Legionella* appears in contrast to other bacterial agents of acute pneumonia to induce a relatively macrophage rich inflammatory response - i.e., recruiting the exact type of inflammatory cell required for propagation in the host. Eventually inflammation in alveoli coalesce into microabscesses which may spill through the pores of Khan and distal bronchiole connections to extend the pneumonia within lobules and/or lobes of the lung.

Most of the lung damage is likely to be a product of the vigorous inflammatory response. However, bacteria may contribute directly to damage through specific toxic activity. For example, the organisms produce a lipopolysaccharide that is mildly endotoxic. They also elaborate a major extracellular protease that is potentially cytotoxic; however, experimentally, protease knockouts mutants cause similar disease making suspected contributions of this protease less likely. Another characteristic lung pathology in Legionnaires's disease is leukocytoclasis: visually, fields of chewed up pieces of inflammatory cells, likely both apoptotic and necrotic cell in origin. This detritus may represent the outcome of the prominent contact-dependent macrophage cytotoxicity noted *in vitro* in the presence of modestly high infection ratios. Interestingly, this cytotoxicity, although initially thought to be directly related to the action of the dot/icm type IV secretion on host cell membranes, appears in large part to be a secondary event. Specifically bacterial flagellin adventitiously translocated into the host cell via the same T4SS leads to host cell inflammosome activation and consequent host cell self-immolation (technically known as pyroapoptosis) through host cell-actuated perforation of its own cell membrane.

Legionella pneumonia may be severe, and locally destructive, and lead to permanent structural damage to the lung (fibrosis or scarring) after resolution. Ultimately, the outcome of infection is dependent on the balance of pathogen virulence, host response to infection, and appropriate treatment.

Host Response to Infection

The host can mount an innate response to L. pneumophila. At a cellular level in mouse macrophage hosts cells, this response is, in part, dependent on the presence of assembled flagella that trigger cytosolic pattern recognition receptors (e.g.., naip-5) and inflammasome

activation as mentioned above. Inflammasome activation, if not leading to direct pyroaptosis of host cells, may limit infection through several less drastic mechanisms: (1) increased targeting of pathogens to lysosomal compartments; (2) enhanced degradation of organisms; and (3) and a cascade of cytokine release that ultimately leads to interferon gamma (IFN-γ) production and resulting sequestration of intracellular iron so vital to the fecundity of this organism. The relative importance of inflammasome activation in controlling human infection is a matter of current investigation. Interestingly, *L. pneumophila* also produces anti-apoptotic factors that directly oppose the self-sacrifice of individually infected cells that might otherwise limit availability of an intracellular growth compartment. Cell surface pathogen recognition receptors, specifically Toll-Like Receptors (TLR), must also play an important role in the host response. Supporting this notion, several TLR polymorphisms have been strongly implicated in disease susceptibility. Lastly, we should not understate the importance of innate mechanisms of a more macroscopic nature. Specifically, normal hosts with good mucociliary clearance may merely sweep inhaled organisms out of the lungs.

Once infection has been established, the growth of L. pneumophila within macrophages must be controlled by a Th1-dependent adaptive immune response with IFN-γ and tumor necrosis factor alpha (TNF-α) as major effectors. The restriction of intracellular growth caused by IFN-γ is a result of the sequestration of intracellular iron induced by this cytokine, as mentioned above, and can be overcome experimentally by providing abundant transferrin-iron. The role of humoral immunity in Legionnaires' disease appears secondary (as *Legionella* is primarily an intracellular pathogen) and at all events would require prior exposure to have an impact on acute disease. However, when present, specific antibodies may help target any extracellular bacteria to neutrophils, where intracellular replication does not occur, rather than mononuclear phagocytes, where infection may flourish.

Taken together, these observations may explain why Legionnaires' disease tends to occur in a subset of smokers or individuals with chronic lung disease (altered physical clearance); and those with suboptimal innate or adaptive immune responses including the elderly and immunosuppressed patients.

Pontiac Fever

After the discovery of L. pneumophila in 1977, an outbreak of illness at the Pontiac, Michigan county health department in 1968 was retrospectively found to be associated with seroconversion to L. pneumophila serogroup 1. The epidemic illness in Pontiac, that affected 95% of the building occupants, was remarkably different from Legionnaires' disease. It was noted that the patients reported self-limited, flu-like symptoms that lasted for only 2–5 days. Both healthy and high-risk individuals were exposed and became ill, but none died. In more recent outbreaks of Pontiac fever, *Legionella* bacteria have not been isolated from affected patients, suggesting that illness may result from inhaling nonviable organisms. The exact pathogenesis of Pontiac fever remains a mystery.

Diagnosis, Treatment, and Prevention

Clinically, Legionnaires' disease presents as a pneumonia that may demonstrate focal, lobular or lobar consolidation on chest X-ray; bibasilar lower lobe pneumonia is typical. Respiratory symptoms are often preceded by a flu-like prodome. While symptomatically not readily distinguishable from other causes of pneumonia, patients do have an unusual predilection to develop low blood sodium levels (hyponatremia) and diarrhea. The reasons for these seemingly quixotic physiological associations are unknown.

Legionnaires' disease can be diagnosed definitively by the culture isolation of *Legionella* bacteria from the respiratory tract, although sensitivity of culture diagnosis is suboptimal. Moreover, from a practical point of view, culture diagnosis is often not helpful clinically, because colonies generally do not appear until 3–4 days of incubation on selective media. A brief acid treatment of sputa samples has been reported to increase yield from respiratory samples by reducing competition from the much faster growing normal respiratory flora, but has not been widely adopted in clinical settings. Colonies isolated on BCYE-α media or BCYE-α selective media with appropriate speckled opalescent appearance can be presumptively confirmed as *Legionella* based on several *in vitro* phenotypes. Specifically, *Legionella* stain faintly as Gram negative rods with a typical safrinin counterstsain or more strongly with a basic fuchsin counterstain. Importantly, *Legionella* is one of the rare bacteria that fail to grow on other standard clinical laboratory medium (chocolate and blood agar), based on the aforementioned inhibitors in autoclaved agar and the strong inhibition provided by levels of sodium chloride present. Therefore, a Gram negative organism with appropriate colonial characteristics and Gram stain morphology can be presumptively reported out as *Legionella* if it fails to grow on passage on chocolate and/or blood agar, or on BCYE medium lacking L-cysteine. Serotyping and/or speciation can then be accomplished by immunofluorescence or agglutination, and molecular analysis, respectively. Of note, cysteine-dependent *Francisella tularensis* can grow particularly well on BCYE-α and may not passage well on blood agar. Identification of this dangerous alternative pathogen may be suspected based on characteristic Gram stain showing minute coccobacilli.

Currently, infection with L. pneumophila serogroup 1 can be diagnosed rapidly and with great sensitivity by a urine antigen test that detects serogroup-specific lipopolysaccharide epitopes. This specific assay is useful in clinical practice because it is rapid and accurate and also because serogroup 1 accounts for approximately 70-80% of Legionnaires' disease in most locations. Newly introduced nucleic acid amplification tests offer the ability to rapidly detect *Legionella pneumophila*, potentially in the context of multiplex respiratory panels, that simultaneous detect viral and bacterial causes of atypical pneumonia.

Although *Legionella* organisms are susceptible to most antibiotics *in vitro*, many agents (e.g., all β-lactams and aminoglycosides) are of little or no use in vivo because they do not efficiently penetrate into cells where *Legionella* bacterial replication takes place. Antibiotics that are concentrated intracellularlly to high levels – fluoroquinolones rifampin, and advanced-generation macrolides (e.g., azithromycin) – are clinically useful. There is currently no vaccine against Legionnaires' disease.

Environmental testing for *Legionella* may be warranted to locate the source of human exposure in an outbreak setting. Of special concern are water sources in hospitals where especially susceptible patient populations are often present. Special testing methods that make use filtration of one liter volumes of suspect water sources and swabs of suspect piping, and subsequent culture on selective media are generally used to isolate environmental *Legionella*. Typing of environmental and patients strains, for example, through pulsed field gel electrophoresis or multi-locus sequencing typing is then used to establish that the environmental *Legionella* and patient isolates are clonal, thereby providing impetus for environmental remediation. Environmental remediation may be accomplished through hyperchlorination and/or thermal pasteurization, that is raising water temperatures either intermittently or permanently, to kill off organisms and their protozoan hosts.

Bartonella

The Organism

The *Bartonellae* are Gram-negative bacilli of the class α-proteobacteria, within the order, Rhizobiales, and therefore most closely related to Brucella, Rhizobium, and Agrobacterium spp. *Bartonella* species have evolved to infect and establish carriage in specific mammalian host species, although they may also cause disease when transferred to a nonnatural host. In the native host, the specific anatomic sites contributing to long-term persistence is not known; however, the bacteria may persist in the bloodstream without producing overt sepsis by replicating and surviving for long periods within erythrocytes. Their presence in the bloodstream (either extracellular or within erythrocytes, so called hemotropic infection) enables transmission between natural hosts via blood-sucking arthropod vector intermediates. Numerous *Bartonella* species have been identified, but only a handful have been observed to infect humans. The most well-known and prevalent causes of human disease are *Bartonella* henselae, *Bartonella* bacilliformis, and *Bartonella* quintana. However, *Bartonellae* are notoriously difficult to culture. With recent advent of more sensitive culture and molecular techniques there has been increasing awareness of zoonotic infection with diverse species, some of which may be specific to certain locales and zoonotic exposures, for example B. tamiae infection in Thailand likely related to exposure to rodents or their ectoparasites, and B. *vinsonii susp. berkhoffii* infections in veterinarians exposed with frequency to canines.

History

In parts of Andean South America, a warty skin disorder known as verruga peruana (Peruvian wart) was recognized since pre-Columbian times. An epidemic of febrile illness associated with hemolytic anemia (known as Oroya fever) occurred among railroad workers in a similar geographic distribution during the late nineteenth century. The common etiology of these two disorders was established in a famous auto-experiment performed by a Peruvian medical student. Daniel Carrión injected himself with lesional material from a patient with verruga peruana; he then developed Oroya fever and died, thereby establishing verruga peruana and Oraya fever as manifestations of the same disease. Subsequently, the biphasic disease caused by this organism has been designated "Carrión's disease" in recognition of Daniel Carrio´n's heroic experiment. In 1909, the erythrocyte-associated bacteria that cause Oroya fever were observed by microbiologist Alberto Barton and later designated as *B. bacilliformis*. *B. bacilliformis* was the only recognized member of the genus *Bartonella* for the next 80 years.

Until 1990, bacillary angiomatosis (BA) was an unexplained opportunistic infection of patients with advanced AIDS. It is a disease that causes persistent fever, malaise, and skin lesions reminiscent of verruga peruana. The focal lesions of BA feature abundant proliferation of small blood vessels, infiltration of inflammatory cells, and abundant clumps of silver-staining bacteria within expansive extracellular matrix. The identity of the bacteria in these lesions was revealed by using a set of universal bacterial primers to amplify 16S rDNA from human tissues affected by BA. The sequence of the amplified DNA was then compared with a databank of known 16S RNA sequences. The closest match was with Rochalimaea quintana, the cause of Trench fever, a louse-borne infection encountered primarily during World War I. Consequently, the related agent associated with BA was initially placed in the same genus, as Rochalimaea henselae. However, when the close identity of both R. quintana and R. henselae with B. bacilliformis and other zoonotic *Bartonella* species was appreciated, all of these agents were reassigned to the genus *Bartonella*, and the genus Rochalimaea was eliminated.

Perhaps the most intriguing consequence of the identification and eventual cultivation of B. henselae was the discovery that it also caused a more common and benign disease in immunocompetent individuals, that is, CSD. For decades, the etiology of CSD was obscure and widely debated. However, after B. henselae was isolated from the tissues of BA patients, it was possible to demonstrate a serologic response to this agent in virtually all patients with CSD. B. henselae was also detected in lymph nodes of patients with CSD and in the blood of healthy cats both by cultivation *in vitro* and by PCR. Ironically, the unification of these two disparate pathological entities is like a twentieth century echo of the unification of verruga peruana and Oroya fever, demonstrated by Daniel Carrio´n's tragic sacrifice in the name of science 100 years earlier.

Epidemiology

The occurrence and range of human *Bartonella* infection depend on the geographical location of the natural hosts and the vectors of transmission (**Table 2**). Humans are thought to be the exclusive natural hosts for B. bacilliformis and B. quintana. B. bacilliformis

Table 2 Representative *Bartonella* species, their hosts, and arthropod vectors

Species	*Natural host*	*Arthropod vector*	*Human disease*
Bartonella bacilliformis	Humans	Sandflies	Oroya fever, verruga peruana
Bartonella quintana	Humans	Lice	Urban trench fever, endocarditis, bacillary angiomatosis (BA)
Bartonella henselae	Cats	Fleas	Cat scratch disease (CSD), bacillary angiomatosis (BA), retinitis, endocarditis
Bartonella vinsonii subsp. berkhoffii	Dogs,	Ticks?	Bacteremia, endocarditis

infection is limited geographically to the Western Andean slopes, probably by the distribution of competent sandfly vectors. B. quintana is transmitted by the human body louse; the infection is therefore restricted to regions and populations where human infestation with this ectoparasite is common, such as among inner-city homeless populations, refugee encampments, and historically during trench warfare (so called trench or quintan fever during World War I). Because B. henselae primarily infects domestic cats and is transmitted by cat fleas, the disease is cosmopolitan in distribution, wherever cats are kept as pets. There are numerous additional *Bartonella* species that primarily infect other small mammals and some large ones too, especially ruminants. Prevalence of infection in some hosts may exceed 50%. However, human contact with these animals and their ectoparasites is either uncommon, or transmission to humans does not occur efficiently. Therefore, human infection is relatively rare.

Pathogenesis

Infection in the natural host

In their natural hosts, most of the *Bartonella* species cause minimal disease manifestations or no disease at all. A notable exception is B. bacilliformis infection in humans in which a fatal hemolytic anemia (Oroya fever) can occur. Yet, even with this potentially dangerous infection, there are selected human hosts who become bacteremic, but remain minimally symptomatic. It is not known what factors determine whether a human host will suffer a severe and fatal illness or remain asymptomatic and become part of the reservoir.

The natural host typically becomes infected by the bite of an infected blood-feeding arthropod (Figure 3). Adherence of the organisms to both endothelial and epithelial cells is mediated by trimeric autotransporter adhesins, exemplified by the BadA adhesin in B. henselae and the VompA-VompD proteins in B. quintana. These proteins are members of the so-called type V secretion systems. The same proteins also mediate adherence to various extracellular matrix components including collagens, fibronectin and laminin. These proteins are remarkably large, indeed for a time BadA was the largest bacterial protein yet described. Interestingly too, they show extensive variation and may become truncated or deleted during infection, potentially as a way to undermine immunological recognition of these prominent, environmentally exposed proteins.

For most species, the primary site of infection may be vascular endothelium at the site of inoculation. This assumption is based on the observation that *Bartonella* bacteria readily proliferate in contact with endothelial cells (the cell type that forms the inner

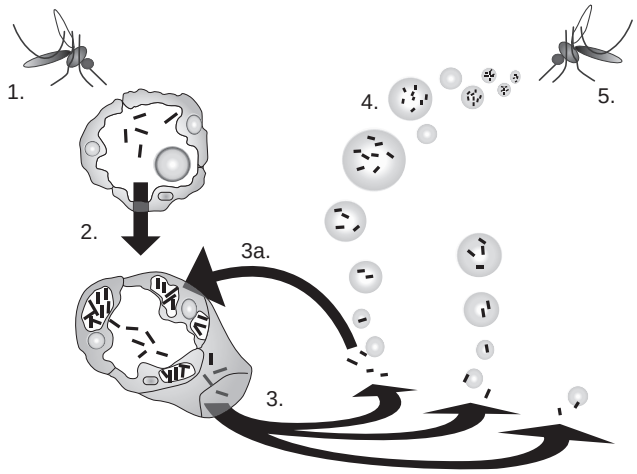

Figure 3 Infection of *Bartonella* in the natural host. The organism is inoculated into the susceptible host by a biting hematophagous arthropod, for example, sandfly, flea, louse, or ked, depending on the *Bartonella* species. (1) Bacteria adhere to and invade endothelial cells at or near the site of inoculation, (2) creating a primary nidus of intra- or extra-cellular growth and source of synchronized release of bacteria into the circulation every few days. Circulating *Bartonella* organisms infect red blood cells. (3), and grow to large numbers in the bloodstream. (4). Alternatively, some bacteria may reenter endothelial cells or extravascular space to maintain the primary nidus of infection (3a). Infected red blood cells serve as a source for transfer to an uninfected vector arthropod (5) to complete the cycle. N.C. Engleberg *Legionella*, *Bartonella*, *Haemophilus*, Encyclopedia of Microbiology, 2009, 170–181, http://dx.doi.org/10.1016/B978-012373944-5.00194-2.

lining of blood vessels) *in vitro*, and they are taken up into a membrane-bound vesicle within these cells. Furthermore, organisms are observed preferentially in cat scratch nodes or angioproliferative lesions extracellularly in clusters around small blood vessels, although this colocalization may merely represent organisms caught in transit into and out of the bloodstream.

As with the L. pneumophila–host cell interactions described above, *Bartonella*–endothelial cell interactions in most species are mediated by the activity of a type IV secretion system (T4SS). However, the T4SS used by *Bartonella* and *Legionella* are only very distantly related, and are generally considered to fall within two distinct subsets: the type IVa (used by *Bartonella*, Brucella, Rickettsia, Anaplasma, Agrobacterium) and IVb (used by *Legionella*, *Coxiella*, Ricketsiella) systems, respectively. *Bartonella* henselae's T4SS system and a set of translocated effector proteins (BepA-G) are involved in the induction of cytoskeletal rearrangements, modulation of intracellular infection, and suppression of apoptosis in endothelial cells. Interestingly, a separate T4SS is essential for the establishment of erythrocyte infection in many *Bartonella* species including B. henselae and B. quintana. A species-specific T4SS-encoded pilus appears to act as a tether to host-specific red blood cell receptors, thereby permitting efficient red cell invasion, only if pathogen tether and host species receptor match. Therefore, for example, B. henselae efficiently invades into red cells of cats but not humans. After invasion, the bacteria persist in erythrocytes until the red bloods cells become senescent and are removed from circulation. This is quite a long time since the average life span of a red cell is on the order of four months. In this way, *Bartonellae* can maintain a high enough concentration in blood to facilitate efficient uptake by hematophagous arthropod vectors during a blood meal, and ensure continuation of the transmission cycle.

Notably, *Bartonella* are highly unusual in that they can cause prolonged and relatively high level bloodstream infection without obviously damaging or septic consequences for the host. This is largely without precedence amongst other bacterial pathogens. Several phenomena may contribute to this capability. First, the bacteria may remain sequestered within erythrocytes and in doing so have diminished contact with Toll-like receptors (TLR) on circulating leukocytes. Second, the *Bartonella* lipopolysaccharide is modified to be less capable of triggering innate cytokine induction. Third, at least some *Bartonella* induce the expression of the anti-inflammatory cytokine interleukin-10, which dampens cell-mediated immune responses and facilitates bacterial persistence. Interestingly, in some hosts, a detectable antibody response also fails to develop, despite chronic bacteremia. Taken together, these capabilities and almost certainly others not yet discovered must underlie the highly unusual ability of *Bartonella* to cause silent bacteremia in their natural hosts. Again, this silent infection contrasts markedly with symptomatic chronic bloodstream infections caused by Brucella (the nearest pathogenic relative) and other causes of acute and chronic bacterial bloodstream infection.

Interestingly, B. bacilliformis, a species that lacks T4SS, causes overt disease in its natural human host. In the absence of help from T4SS, red cell invasion by B. bacilliformis relies instead on a flagellar motor-dependent mechanism. Possibly related to this distinct mode of red cell colonization, and in contrast to other *Bartonella* species, B. bacilliformis causes contact-dependent lysis of red blood cells *in vitro* and as mentioned previously a severe hemolytic anemia in humans (Orroya fever). Therefore, T4SS-dependent processes may have evolved to allow a more harmonious and perhaps evolutionarily more successful relationship between pathogen and host. Notable in this regard is that the acquisition of T4SS capabilties has been associated with an explosive adaptive radiation of *Bartonella* within mammals.

Infection in an accidental host

Erythrocyte infection is not typically a feature of infection in the accidental host. Consequently, the accidental host may have a localized symptomatic infection at the site of inoculation or a primary infection with septicemia or endocarditis or both. In the case of human B. henselae infection, it appears that the immune competency of the host is the most important determinant of disease. Most immunocompetent individuals who are inoculated by a cat scratch (or exposure to cat fleas) develop a minor, transient lesion at the site of inoculation and a highly inflammatory reaction to infection in one or more nearby regional lymph nodes. The host response in lymph nodes may take the form of stellate micro-abscesses surrounded by histoycytes (tissue macrophages); necrotizing granulomas (granulomas with dead cellular material in their center); or suppurative inflammation (pus). The immune response is exuberant and limiting, and consequently bacteria are often difficult to find by microscopy. In CSD, the infection may persist for weeks to months, but it usually is contained at the level of the regional lymph node and ultimately self-limited.

In contrast to CSD, B. henselae infection in an immunocompromised host presents a very different picture. Patients with AIDS cannot contain the infection at the level of the lymph node. Bacteria reach the general circulation, inducing persistent fevers and causing metastatic infection in the skin and internal organs. The same manifestations also occur with infection by B. quintana in AIDS patients. A notable feature of disseminated *Bartonella* henselae and quintana infection in the immunocopromised host is vascular proliferation at sites of local bacterial infection, a process named bacillary angiomatosis. Lesions teem with bacteria appearing in aggregates or microcolonies, and these bacteria have the capacity to induce small blood vessels to proliferate. The result is the development angioproliferative tumors in skin, other tissues, or organs. In addition, endothelial-lined blood-filled cysts in the liver may form in association with microcolonies of bacteria, a disease process known as bacillary peliosis. Vascular proliferation accounts for the warty appearance of verruga peruana (caused by B. bacilliformis) and the similar appearance of B. henselae or B. quintana bacilliary angiomatosis lesions in patients with AIDS.

Occasionally, *Bartonella*, either in immuncompetent or immunocompromised hosts, cause localized infection of the eye (Parinaud's oculoglandular syndrome, a granulomatous conjunctivitis with accompanying lymphadenopathy; retinitis), heart values (endocarditis) or brain (encephalitis; meningitis). *Bartonella* infection should always be considered in the differential of "culture negative" endocarditis, especially with the appropriate zoonotic exposures.

Diagnosis, Treatment, and Prevention

The *Bartonella* species are not obligate intracellular pathogens and can therefore very rarely be isolated on routine clinical laboratory culture media. However, although culture on standard blood agar plates is sometimes feasible, it is very insensitive and untimely. The development of colonies may take weeks of incubation. New insect cell culture-based medium holds promise in increasing recovery of organisms. However, because of the insensitivity of bacterial culture and unavailability of optimal culture medium in most diagnostics settings, almost all cases are diagnosed by serologic and/or molecular testing. Notably, serological tests are commonly available only for B. henseale and quintana. Therefore, zoonotic disease caused by other *Bartonella* species is certainly under-diagnosed. Studies in veterinarians for example suggest zoonotic exposure and infection by diverse *Bartonella* species.

Bartonella bacteria are susceptible to tetracyclines and macrolide antibiotics. Treatment of patients with BA or immunocompromised individuals is essential. However, in most cases of CSD, it is difficult to ascertain whether antimicrobial therapy has any beneficial effect on the duration or outcome of infection. There is no *Bartonella* vaccine for cat owners or their pets, but acquisition of the disease can be prevented by the treatment of pet cats and kittens for fleas and by avoidance of contacts that may break the skin or inoculate mucous membranes when handling these pets.

Conclusion

The two fastidious Gram-negative pathogens described in this article share elements of their basic structure and the potential to cause disease in humans. They are otherwise distinct in their preferred hosts and strategies for survival. Like many bacterial pathogens, these species cause particularly serious disease in impaired or immunocompromised hosts in whom the pathology may differ dramatically from that in healthy exposed individuals. Avoidance of infection by these species depend on public health measures, proper design and maintenance of water systems, and/or limiting zoonotic exposure especially for those at greatest risk for serious disease.

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Leishmania☆

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Glossary

Amastigote The aflagellated stage of *Leishmania*. Amastigotes are obligate intracellular parasites of mononuclear phagocytes of the vertebrate host.
Complement system A large number of small plasma proteins that act in concert, as part of the innate immune system of vertebrates. Complement actions include enhancing phagocytosis, inducing inflammation, and potentially effecting the lysis of microbes and some viruses.
Delayed-type hypersensitivity (DTH) reaction A cell-mediated immune reaction in the skin in response to foreign antigens, that is indicative of prior sensitization to those antigens.
Macrophage Large, actively phagocytic mononuclear cell that is found in tissues and blood.
Membrane attack complex (MAC) A large macromolecular complex of complement proteins that forms on foreign cell surfaces, creating a pore in the plasma membrane and leading to cell lysis.
Metacyclogenesis Developmental and biochemical transformation, occurring within the sand fly midgut, of the noninfectious procyclic promastigote into the highly infectious metacyclic promastigote.
Phagolysosome Organelle resulting from the fusion of a phagocytic vacuole with a lysosome.
Promastigote Flagellated insect stage of *Leishmania*. Promastigotes exist in the sand fly vector as either the replicative procyclic form (noninfectious) or the highly infectious (and nonreplicative) metacyclic form.
Reticuloendothelial system Part of the mammalian innate defense system, consisting of mononuclear phagocytic cells found in the lymph nodes, liver, spleen, and bone marrow.
Subclinical Infection without any overt clinical signs of disease.
Zoonotic disease An infectious disease in which the transmission cycle in nature is primarily within an animal population, and humans are only incidentally infected.

Abbreviations

CR	Complement receptor
DCL	Diffuse cutaneous leishmaniasis
DTH	Delayed-type hypersensitivity
ER	Endoplasmic reticulum
GPI	Phosphatidylinositol
IFN-γ	Interferon-gamma
IL	Interleukin
LCL	Localized cutaneous leishmaniasis
LPG	Lipophosphoglycan
MAC	Membrane attack complex
ML	Mucosal leishmaniasis
PFR	Paraflagellar rod
PPG	Proteophosphoglycan
TNF-α	Tumor necrosis factor-alpha
VL	Visceral leishmaniasis

Defining Statement

Leishmania are intracellular protozoan parasites of vertebrates that are transmitted by the bite of a sand fly vector. Their fascinating life cycle, cell biology, novel mechanisms of RNA processing and gene regulation are discussed. *Leishmania* are also an important model of intracellular parasitism and host–parasite immune interactions. The leishmaniases are a diverse set of zoonotic diseases of

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global importance, including diseases of the skin, mucosa, and a potentially fatal systemic disease; these are discussed along with current approaches to treatment and control.

Classification and Morphology

Leishmania are eukaryotic protozoan parasites of vertebrates in the family Trypanomasomatidae (order Kinetoplastida). Characteristic of members of the order Kinetoplastida is the presence of a conspicuous Feulgen stain-positive (i.e., DNA-containing) kinetoplast (see 'Other membrane-bound organelles'). All members of the family Trypanosomatidae are parasitic for vertebrates or invertebrates and undergo morphological changes during transition between stages of their life cycle. Of the many genera in this family, only species of *Leishmania* and *Trypanosoma* are human pathogens. Two subgenera of *Leishmania*, *L.* (*Leishmania*) and *L.* (*Viannia*) are recognized, and 20 *Leishmania* species are pathogenic for humans.

Leishmania exist as morphologically distinct forms. In mammals, amastigotes are an obligate intracellular parasite of mononuclear phagocytes. The elongated motile promastigote form is found in female sand flies, which are the only known vectors for *Leishmania*. Promastigotes possess a single nucleus and are variable in length (15–25 μm) and shape (ellipsoid to slender). The most prominent features of stained promastigotes are the nucleus, the kinetoplast, and the flagellum (Figure 1); the kinetoplast and the origin of the flagellum define the anterior region of the parasite (see 'Flagellum'). Amastigotes are round to oval in shape with a 2–10 μm diameter. This stage is aflagellar (i.e., the flagellum does not extend past the cell boundary), but the kinetoplast and nucleus remain visible in stained amastigotes.

Life Cycle and Ecology

Leishmania spp. have a complex life cycle (Figure 2) with two morphologically distinct forms, the amastigote (mammalian stage) and the promastigote (invertebrate stage) (Figure 1). The taxonomy of sand flies is complex and varies according to different authors. In the Old World the genera *Phlebotomus* and *Sergentomyia* are of medical importance and they include several species. In the New World *Lutzomyia* is the most important genus (Table 1). The female phlebotomine sand fly vector becomes infected during a blood meal when the fly bites (often repeatedly) an infected vertebrate host, creating a pocket of pooled blood. Upon drinking the bloodmeal, the fly ingests amastigotes, and within the blood meal itself, the amastigotes transform into the promastigote forms. These early stage promastigotes, termed procyclics, actively replicate within a chitin-containing gut lining (the peritrophic membrane) secreted by fly midgut epithelial cells (the members of the *Viannia* subgenus replicate in the hindgut, known as the pyloric triangle). About 3 days after being ingested by the fly, procyclic forms escape the peritrophic membrane and travel to the anterior portion of the midgut, where they attach to epithelial cells. This marks the early stage of metacyclogenesis, where procyclics transform into the nonreplicating, infectious, metacyclic promastigotes. Metacyclics are thinner and faster moving than procyclic forms, but more importantly, about 5 days after the bloodmeal, biochemical changes occur in the surface coat (see LPG in 'Plasma membrane and surface molecules'), which cause the release of metacyclics from midgut epithelial cells. The newly released, and now highly infectious, metacyclic promastigotes are free to migrate and attach to the stomodeal valve in the foregut. When sandflies attempt to feed on a new host to procure blood necessary for egg development these infectious promastigotes are egested into the wound. The accumulation of infective promastigotes in the stomodeal valve may create mechanical problems during feeding, forcing sand flies to probe in different skin sites, thereby introducing *Leishmania* promastigotes in multiple sites. Upon contaminating the wound of the vertebrate host, promastigotes are phagocytosed by tissue

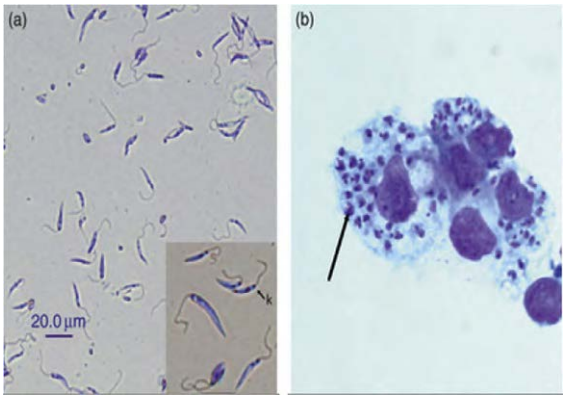

Figure 1 Morphological characteristics of *Leishmania*. In (a) (including insert), the elongated forms of cultured *L. donovani* promastigotes are shown (stained with Giemsa, bar in insert = 10 μm). An arrow points to a kinetoplast (k) in the insert. (b) Shows the intracellular *L. donovani* amastigotes infecting hamster spleen cells after staining with Giemsa. Each of the darkly stained bodies within the cell's cytoplasm (arrow) is an amastigote. Photo of promastigotes kindly provided by Gary B. Ogden, amastigote photo by EYO.

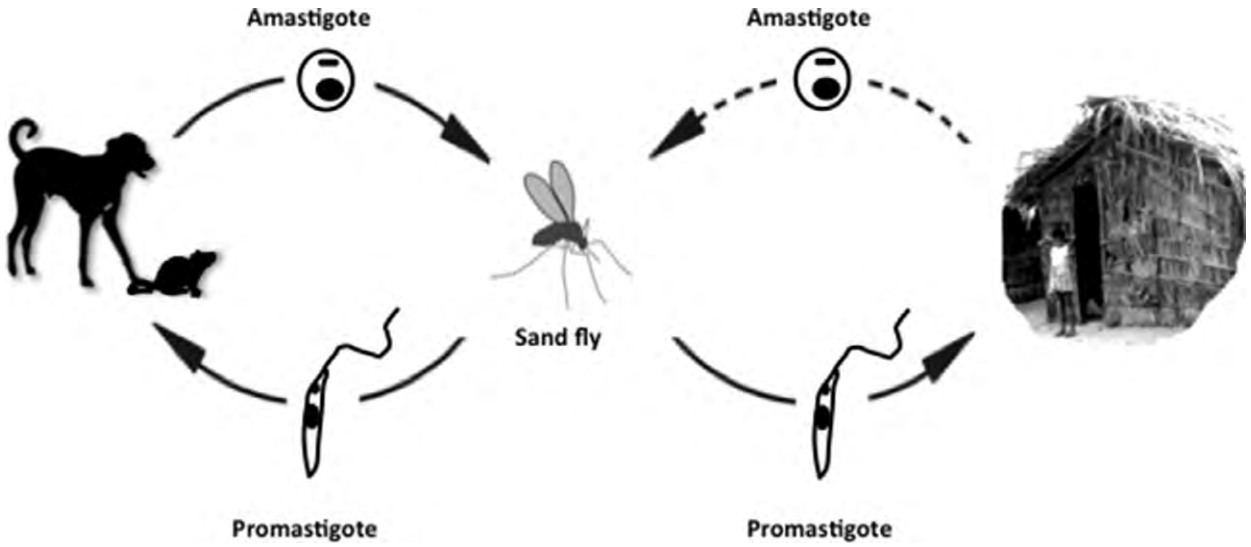

Figure 2 Life cycle of *Leishmania*. *Leishmania* have two morphologically distinct forms, the amastigote and the promastigote. In this schematic diagram, transmission of the infectious promastigotes is shown to occur from the infected female phlebotomine sand fly vector to the two main reservoir animals (i.e., dogs or rodents). The infectious metacyclic promastigotes then infect mononuclear phagocytes in the animal reservoir and within the phagolysosome transform into non-motile amastigotes. The amastigotes replicate within the phagosome and eventually lyse the host cell. The cycle is completed when a sand fly ingests amastigotes from the reservoir during a blood meal. As is typical in zoonotic transmission, humans (frequently children) are incidentally infected; however, as indicated by the broken arrow, humans can (uncommonly) serve as reservoirs for the parasite (see full description of life cycle in 'Pathogenesis and host response').

phagocytic cells (neutrophils and macrophages) responding to the damaged tissue. Within the macrophage, promastigotes inhibit maturation of phagosome and rapidly transform into non-motile amastigotes. The amastigotes replicate within the acidic and hostile environment of the phagolysosome, eventually lysing the macrophage and releasing amastigotes to infect other macrophages. The cycle continues when a sand fly ingests free amastigotes or amastigote-infected macrophages during a blood meal.

The transmission of *Leishmania* in most endemic areas is maintained through a zoonotic cycle during which humans are only incidentally infected. In general, the species that cause cutaneous disease are maintained in small mammal reservoirs, whereas the domestic dog is the usual reservoir for the species that cause visceral disease. In most instances, multiple mammalian species are found to be infected with a given parasite, but usually there is a principal reservoir and the other animals are incidental hosts that play a secondary role in the transmission cycle. The transmission between reservoir and sand fly is highly adapted to the specific ecology of the endemic region. For instance, the reservoirs for *L. major* in the Middle East are desert rodents and the sand fly vectors cohabit the rodent burrows where they are protected from the extreme heat and low humidity of the desert environment. In the New World, the zoonotic cycle of parasites of the *Viannia* or *Leishmania* subgenus typically involves small mammals and sand flies that inhabit dense tropical and subtropical forests. Human infections occur when their activities bring them in contact with the zoonotic cycle. Anthroponotic transmission, where humans are the presumed reservoir, occurs with *L. tropica* in some urban areas of the Middle East and with *L. donovani* in India, Bangladesh and Nepal.

Cellular Biology

Plasma Membrane and Surface Molecules

Plasma membrane

Leishmania possess a bileaflet plasma membrane similar to the membranes of metazoan cells (i.e., multicelled eukaryotes). A membrane indistinguishable in appearance from the plasma membrane itself also surrounds the flagellum. During development of promastigotes these membranes undergo biochemical changes, which modulate parasite attachment to host tissues (see the following section). The plasma membrane that lines the flagellar pocket actively acquires nutrients by pinocytosis, and pinocytotic vesicles have been observed to travel to the cytoplasm, where they fuse with lysosomes. The flagellar pocket is also active in secretion by exocytosis.

Lipophosphoglycan

The entire surface of promastigotes, including the flagellum, is covered by a cell coat or glycocalyx composed predominantly of lipophosphoglycan (LPG), as well as lesser amounts of gp63 (see the following section), proteophosphoglycans (PPGs), and other

Table 1 Major species of *Leishmania* and their geographic distribution

Parasite	*Disease form*	*Reservoirs*	*Vectors*	*Distribution*
Old World Leishmaniasis				
L. (*L.*) *major*	LCL	Desert rodents (*Psammomys, Rhombomys, Meriones, Gerbillus*)	*Phlebotomus papatasi, P. Caucasicus, Phlebotomus duboscqi*	North Africa, the Middle East, Central Asia,and the Indian subcontinent
L. (*L.*) *tropica*	LCL	Humans, Rock hyrax, Golden jackal, red fox	*P. sergenti*	North Africa, the Middle East, Central Asia,and the Indian subcontinent
L. (*L.*) *aethiopica*	LCL, DCL	Bush and rock Hyraxes	*P. pedifer P. longipes*	Ethiopian highlands, Kenya
L. (*L.*) *infantum*	VL, LCL	Domestic dog, wild canines, hares	*P. perniciosus P. ariasi P. tobbi P. langeroni*	Mediterranean basin, Middle East, and Central Asia
L. (*L.*) *donovani*	VL	Humans, possibly animals	*P. argentipes P. orientalis P. martini P. chinensis*	Kenya, Sudan, India, Pakistan, and China
New World Leishmaniasis				
L. (*L.*) *mexicana*	LCL, DCL	Forest rodents (*Ototylomys, Peromyscus, Heteromys*)	*Lutzomyia olmeca olmeca Lu. cruciata, Lu. shannoni*	Southern Texas through Mexico and northern Central America
L. (*L.*) *amazonensis*	LCL, DCL	Forest spiny rats (*Proechymis*)	*Lu. faviscutellata*	South America in the Amazon basin and northward
L. (*L.*) *pifanoi*	LCL, DCL	Probably rodents	Unknown	Venezuela
L. (*L.*) *garnhami*	LCL	Unknown	*Lu. youngi*	Venezuela
L. (*L.*)		*venezuelensis*	LCL	Unknown
Lu. olmeca bicolor		Venezuela	*L.* (*V.*) *braziliensis*	LCL, ML
Forest rodents, Opossums, sloths, domestic dogs, donkeys, horses		*Psychodopygus wellcomei* and several *Lutzomyia* species	South America from the northern highlands of Argentina and northward to Central America	
L. (*V.*) *panamensis*	LCL, ML	Sloths	Several *Lutzomyia* species	Panama, Costa Rica, Colombia
L. (*V.*) *guyanensis*	LCL	Sloths, lesser anteater	*Lu. umbratilis*	Guyana, Surinam, northern Amazon Basin
L. (*V.*) *peruviana*	LCL	Unknown	*Lu. peruensis, Lu. caballeroi, Lu. ayacuchensis*	Peru
L. (*L.*) *infantum* (=*chagasi*)	VL, LCL	Domestic dogs, wild canids, opossum, forest rodents	*Lu. longipalpis, Lu.evansi, Lu. cruzi*	Mexico (rare) through Central and South America

molecules. LPG is anchored to the parasite via a phosphatidylinositol (GPI) linkage, as are most other surface molecules (including gp63 and PPGs). This type of lipid anchor is unusual in animal cells. In addition to the GPI lipid anchor, LPG has three other domains: a glycan core, repeats of a saccharide–phosphate region, and an oligosaccharide cap. The glycan core and lipid anchor are conserved among all species of *Leishmania*, whereas the carbohydrates in the repeat region and the cap are highly variable in composition.

Early in the development of *L. major* promastigotes in the sand fly, the terminal sugars of the LPG cap are predominantly galactose residues, but during metacyclogenesis these sugars are replaced by arabinopyranose. Because of these changes, procyclic promastigotes can attach to receptors on the epithelial layer of the sand fly midgut, allowing developing promastigotes to remain in the gut during digestion and excretion of the bloodmeal. Once the developmental transformation to the infectious metacyclic stage has taken place, changes in LPG composition enable the parasites to be released for passage to the sand fly's mouth parts to infect the next host. Similar events occur in other species of *Leishmania*, although the specific changes in LPG composition may differ. Certain species of sand fly are competent hosts for particular species of *Leishmania*. Variations in LPG structure, which accompany varying ability to attach to sand fly epithelial cells, seem to determine, at least in part, what species of sand fly are suitable hosts for a particular species of *Leishmania*. LPG also mediates protection against hydrolytic enzymes found in the digestive tract of the invertebrate host.

The creation of mutants unable to synthesize LPG, by targeted gene disruption, has helped to establish LPG's role in *L. major*'s virulence. LPG is thought to contribute to *L. major*'s virulence in key ways – providing resistance to oxidative damage in the

phagolysosome and protecting the parasite from the effects of human serum complement (LPG may not be needed for complement resistance in mice). Infective *L. donovani* or *L. major* metacyclic promastigotes possess a thickened cell coat because of an increase in the number of phosphorylated saccharides in the LPG. The LPG molecules in metacyclic promastigotes of *L. major* are almost double the length of those found in procyclics, long enough to prevent the membrane attack complex (MAC) of the complement system from reaching the plasma membrane and lysing the parasite. Noninfective procyclic promastigotes have a thinner LPG coat and are readily lysed by host complement; LPG is almost absent from amastigotes. Interestingly, *L. mexicana* LPG mutants behave differently, as they show little or no reduction in virulence in macrophages or in mice, demonstrating that *Leishmania* virulence factors may be species specific.

Attachment and entry of promastigotes into macrophages may require the interaction of several parasite surface molecules with macrophage receptors. Identification of the specific molecules required for uptake remains to be determined. LPG plays a role in the uptake of complement-opsonized parasites into macrophages; however, because LPG mutants are efficiently endocytosed by macrophages, LPG is not required for uptake (see the following section). The macrophage CR1 and CR3 surface complement receptors, which bind C3b and inactivated C3b (iC3b), respectively, mediate adhesion of the promastigotes to macrophages. Attachment via complement receptors does not trigger the oxidative burst during phagocytosis (the oxidative burst in the phagolysosome is required for efficient intracellular killing of *Leishmania*). Consistent with these observations, *in vitro* experiments with *L. major* have demonstrated the increased intracellular survival of complement-fixed metacyclic promastigotes in macrophages. LPG also transiently impairs phagosomal maturation and provides protection against degradation by lysosomal enzymes.

Glycoprotein 63

The major protein found on the surface of promastigotes in all pathogenic species of *Leishmania* is a 63-kDa phophotidylinositol-linked glycoprotein designated gp63 (gp63 synthesis is downregulated in amastigotes). Biochemical and molecular analysis has identified gp63 as a type of zinc-dependent metalloprotease. Like LPG, the surface expression of gp63 is increased during metacyclogenesis. Surface exposure of gp63, however, is largely blocked by the elongated metacyclic LPG. Gp63 may play a secondary role in the binding of *Leishmania* to the macrophage surface and subsequent entry by receptor-mediated endocytosis. As C3b is converted to iC3b by gp63, it has been suggested that an accumulation of iC3b on the parasite's surface would promote phagocytosis by CR3; as mentioned earlier, this would prevent a triggering of a more destructive oxidative burst during phagocytosis. GP63 also promotes intracellular infection by degrading or altering the function of several macrophage signaling molecules, including kinases, transcription factors, and phosphatases.

Flagellum

The flagellum originates from the anterior of the cell, arising from an invaginated region termed the flagellar pocket, and pulls, rather than pushes, the promastigote. In addition to serving as a means of locomotion, electron microscopy reveals that the *Leishmania* flagellum sometimes functions as a tether, attaching the developing promastigotes to the tissues lining the midgut of the sand fly vector (see LPG in 'Plasma membrane and surface molecules'). In the mammalian host, the functional role of the flagellum is curtailed, as the flagellum in amastigotes does not extend beyond the flagellar pocket. The flagellum has the typical $9+2$ axoneme structure of metazoan cells, possessing a cytoskeletal core or axoneme of nine microtubule doublets surrounding two unattached inner microtubules. In addition, there is a complex cytoskeletal structure running parallel to the axoneme, termed the paraflagellar rod (PFR). The PFR is unique to kinetoplastids, euglenoids, and some dinoflagellates. Little is known about the role of the PFR, but *Leishmania* with mutations in PFR proteins have reduced swimming velocity. The basal bodies at the base of the flagellum are structurally similar to centrioles found in animal cells.

Nucleus

The *Leishmania* nucleus is spherical or slightly ovoid in shape, with a diameter of approximately 1.5–2.5 μm. It is unusual in that the nuclear membrane remains intact during nuclear division. During division, the nucleus elongates and then constricts, splitting the nuclear membrane into two. Spindle microtubules are believed to elongate and push the dividing nucleus apart, but how the genetic material is partitioned is unknown. The nuclear membrane itself is typical in appearance, possessing a double unit membrane studded with 65–100 nm diameter nuclear pores. The nucleus contains chromatin, but the chromosomes do not condense or become visible during nuclear division.

Other Membrane-Bound Organelles

Mitochondrion and kinetoplast

A single mitochondrion is present and is functional in both the insect and mammalian forms of *Leishmania* (i.e., cyanide-sensitive respiration is found in both forms of the parasite). Within the mitochondrion is the kinetoplast, one of the defining morphological features of members of the order Kinetoplastida. This structure lies at the base of the flagellum, close to the basal bodies from which the flagellum arises, and is visible by light microscopy. The kinetoplast is a large rod-shaped assemblage of kinetoplast DNA (kDNA) located within the mitochondrion. The kDNA itself is unusual, as spreads of purified kDNA visualized under the electron microscope appear predominantly as thousands of 'minicircles' and a few 'maxicircles' catenated (i.e., interlocked) into a large DNA network. This contrasts sharply with the mitochondrial DNA found in metazoan cells, which typically exists as a single circle of DNA. Moreover, maxicircles encode the usual mitochondrial genes but pre-mRNA transcripts for these genes must be extensively

edited (by uridine insertion and deletion) to generate a translatable form of mRNA. The 'RNA editing' process is dependent upon guide RNAs (gRNAs) acting as templates for editing, and these gRNAs are encoded by the minicircles (see RNA editing, 'mRNA processing').

Endoplasmic reticulum and Golgi apparatus

The endoplasmic reticulum (ER) is similar in appearance to that found in metazoan cells. It is contiguous with the outer nuclear envelope and may be rough (containing attached ribosomes) or smooth (lacking attached ribosomes) in appearance. Closely associated with the ER is the Golgi apparatus, which appears as flattened membranous structures frequently stacked at the base of the flagellar pocket (in addition to being a site of endocytosis, the flagellar pocket is active in exocytosis).

Molecular Biology and Control of Gene Expression

Genomic Organization

Recently, the genomes of several of the *Leishmania* (including *L. major*, *L. infantum*, *L. donovani*, *L. L. amazonensis*, and *L. V. braziliensis*) have been sequenced and analyzed. *Leishmania* spp. have 34–36 linear chromosomes, with each genome measuring about $3.0–3.8 \times 10^7$ nucleotides. This is approximately eight times the size of the genome of the bacterium *Escherichia coli*. The estimates for the number of putative protein-encoding genes found are very similar for the three species of *Leishmania* ranging from about 8000 to 8300 genes. Many of these genes are repeated multiple times in tandem, forming large gene clusters. About 50% of the genes identified have no known function; however, 3–4% of these genes encode leucine-rich repeat sequences that are thought to bind to complement receptor CR3. The *Leishmania* genomes have a high degree of synteny. Despite wide geographical distribution, unique ecological niches, and diverse clinical manifestations, more than 90% of the genes are shared among the different pathogenic species. There are remarkably few genes that are unique to a given species, although differences in gene copy number may impact the parasite-host interaction and disease expression. Notably, the genomes of the Old World species contain transposable elements and RNA interference machinery that is lacking in *L. braziliensis*. *Leishmania* spp. appear to be primarily asexual, however sexual crosses were recently discovered to occur in promastigotes within the invertebrate host prior to metacyclogenesis. Analysis of multiple field isolates suggests a high level of endogamous sexual recombination that likely contributes to strain diversity within an endemic area.

mRNA Processing

RNA editing

Two of the most uncommon types of messenger RNA (mRNA) processing, namely, RNA editing and trans-splicing, were first discovered in studies on trypanosomatid protozoa. One of these, RNA editing, has changed our understanding of how gene expression may be controlled in eukaryotes. The kDNA maxicircles mentioned earlier encode genes for ribosomal RNA, and certain proteins necessary for oxidative phosphorylation and electron transport in the mitochondrion. The study of many of these genes revealed that the mature mRNA sequences are different from the genomic DNA sequence from which they are transcribed. Moreover, many of the genomic sequences do not code for a functional protein. These observations were reconciled when it was discovered that a posttranscriptional process, now termed RNA editing, resulted in the site-specific deletion or addition of uridine (U) residues in the mRNA, which created functional initiation sites for protein translation and/or created an open reading frame for protein translation. RNA editing, in effect, repairs 'defects' in certain protein-coding regions in the DNA at the RNA level, enabling the expression of a functional protein from the processed mRNA. gRNAs are known to be critically important to this process. The gRNAs carry the template for proper mRNA editing and may also contribute U residues to the editing process. It is not clear why the trypanosomatids rely on this unusual form of gene regulation.

Trans-splicing

It is believed that within any species of trypanosomatid protozoa all mature mRNAs possess an identical 5′ exon sequence. This 5′ sequence, termed the 'spliced leader' or 'mini-exon,' is encoded by a gene separate from the genes corresponding to the mRNA transcripts. The mechanism for linking the mini-exon to the mRNA, termed trans-splicing, was first discovered in *Trypanosoma brucei* and is now known to also occur in about 10–15% of nematode mRNAs and in some flatworm mRNAs. The biochemistry of trans-splicing is similar to conventional cis-splicing where one exon is fused to a second downstream exon by two sequential transesterification reactions, resulting in the covalent joining of the two exons and the excision of the intervening or 'intron' sequence (in the form of a partially circular molecule termed a 'lariat structure') from the precursor mRNA. Although both mechanisms use small nuclear RNA–protein complexes (i.e., snRNPs) in catalyzing this reaction, the crucial difference between cis- and trans-splicing is that the two exons linked in trans-splicing are not encoded on the same pre-mRNA molecule, but rather are transcribed from separate genes. As a consequence of splicing two separate transcripts, the trans-splicing reaction releases a branched or 'Y-shaped' intron instead of a lariat. In all *Leishmania* spp. studied, the spliced leader RNA is transcribed from clusters of tandemly repeated genes (ranging from about 90 to 200 copies) on the genome. In *L. enrietti*, an 85 nt spliced leader RNA is transcribed from a 440 bp long gene. At the 5′ end of this transcript is the 35 nt mini-exon sequence found at the 5′ end of all mature *L. enrietti* mRNAs.

Control of Gene Expression

For most eukaryotes and bacteria, gene expression is controlled primarily by regulating the initiation of transcription. In this paradigm, transcriptional promoters on the genome largely dictate where, and how often, initiation of transcription takes place. No RNA polymerase II promoter sequence has been clearly identified in any *Leishmania* spp. Transcription in *Leishmania* appears to be nonstringent, as promastigotes transfected with plasmids containing the gene for neomycin resistance (but containing no *Leishmania* sequences) were shown to actively transcribe the neomycin gene. All that was required was the incorporation of a signal for trans-splicing in the neomycin gene sequence. So far, other evidence for a true *Leishmania* promoter has been merely suggestive or inconclusive. Thus, gene regulation in *Leishmania* and other kinetoplastids depends largely on posttranscriptional mechanisms. Protein-coding genes are arranged in long head to tail tandem repeats and are transcribed as polycistronic mRNAs. These are converted to monocistrons by trans-splicing at the 5′ end and polyadenylation at the 3′end. The polyadenylation of an upstream gene is functionally coupled to trans-splicing of a downstream gene. The formation of mature mRNA may then be controlled by regulating splicing and/or polyadenylation. Tandemly repeated genes may enable the cell to increase the expression of a protein (by increasing gene copy number) without modulating the initiation of transcription. Changes in the transcriptome, most evident during life cycle differentiation, are most heavily influenced by RNA stability. Trans-acting factors (RNA binding proteins) are thought to bind to the 5′ or 3′ ends to selectively stabilize or destabilize the mRNAs.

DNA Transfection and Gene Targeting

A major contribution to the molecular analysis of *Leishmania* was the development of functional plasmid expression vectors in 1990. Two groups used an *E. coli* plasmid in combination with the intergenic region of the α-tubulin gene or the flanking region of the *dhfr-ts* gene to construct a *Leishmania* plasmid. More recently, other plasmids have been made: several selectable markers are available for stable transfection (e.g., neomycin, hygromycin, bleomycin, and puromycin) and a number of phenotypic markers are available for transient assays (e.g., β-galactosidase, luciferase, and β-glucuronidase). Negative selection is also possible using the thymidine kinase gene. Important questions about the pathogenesis of *Leishmania* have been answered using these plasmids. As mentioned earlier, transfection of gp63-variant strains with gp63-expressing plasmids has helped to elucidate the role of gp63 in infection. Similarly, targeted mutations in LPG have contributed to our understanding of this molecule's role in virulence. Moreover, homologous recombination between a plasmid and the *Leishmania* genome has proved to be a powerful tool in studying the function of particular genes in these parasites. Researchers may now specifically target and eliminate the activity of a gene or add a gene to a species' genome. Although gene knockout experiments are potentially powerful in studying *Leishmania*, difficulties may arise, as many *Leishmania* genes are present in tandem repeats on the chromosome.

Pathogenesis and Host Response

The *Leishmania* parasite has multiple ways in which it adapts to, and survives within, both the invertebrate and vertebrate hosts. Within the sand fly, promastigotes undergo metacyclogenesis to the infective stage (see 'Life cycle and ecology'). When the sand fly takes a bloodmeal, the infective promastigotes are deposited in the host dermis to initiate the infection. Host factors (genetic background, concomitant disease, nutritional status), parasite factors (virulence, size of the inoculum) and possibly, vector-specific factors (sand fly genotype, immunomodulatory salivary constituents) influence the subsequent development and evolution of disease. As noted earlier (see 'Cellular biology'), the parasite's surface plays an important role in the entry and survival of *Leishmania* in the mammalian host cell by conferring complement resistance and by facilitating entry into dermal resident macrophages and dendritic cells without triggering a respiratory burst. As consequence of the infective insect bite, inflammatory mediators induced by the tissue trauma, complement C3, and/or components or microbiota in the sand fly saliva attract neutrophils that phagocytize the promastigotes. Neutrophils can kill the intraphagosomal parasite by fusion of myeloperoxidase-containing and tertiary granules, decreasing the intraphagosomal pH, and production of reactive oxygen radicals. When infected neutrophils become apoptotic they are phagocytosed by macrophages, which take on a tissue repair phenotype that is permissive to *Leishmania* infection. Inflammatory monocytes are recruited to the infection site by cytokines and chemokines released by neutrophils, platelets and fibroblasts. Recruited monocytes differentiate locally into monocyte-derived dendritic cells or into macrophages and facilitate the trafficking of parasites to the draining lymph node. The parasitized phagocytes may induce and adaptive immune response by presenting *Leishmania* antigen to lymph node T cells, or provide a permissive site for parasite replication, long-term survival and systemic spread.

There is extensive evidence from experimental animal models that adaptive cellular immune mechanisms mediate resistance to *Leishmania* infection. The knowledge of immunological mechanisms related to resistance and susceptibility are largely derived from experimental models and are summarized in Figure 3. Resistance to leishmanial infection is associated with the capacity of $CD4^+$ T cells (Th1 subset) to generate interferon-gamma (IFN-γ) in response to the parasite. Tumor necrosis factor-alpha (TNF-α) may contribute to protective responses by augmenting IFN-γ-mediated macrophage activation. Interleukin (IL)-12, which stimulates T cells and NK cells to produce IFN-γ, promotes Th1 subset expansion, suppresses Th2 cells (IL-4), and plays a critical role in the

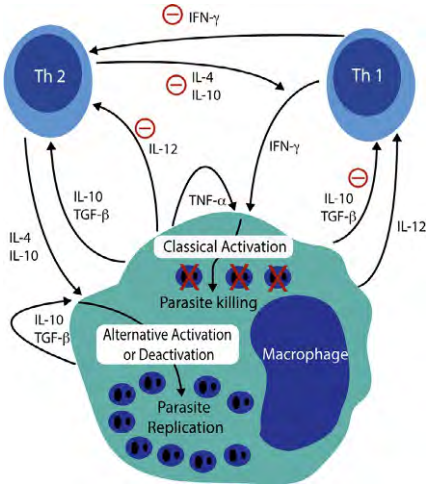

Figure 3 Immunopathogenesis of *Leishmania* infection. The balance between the Th1 and Th2 cell response and the effect on macrophage activation and parasite killing is shown schematically. Inhibitory effects are noted by a (−). When Th1 cells are activated by *Leishmania* antigens, IFN-γ is produced, which downregulates the Th2 response and promotes classical macrophage (M1) activation with nitric oxide production. TNF-α, produced by macrophages, synergizes with IFN-γ to induce macrophage activation and parasite killing. If *Leishmania* antigens induce a Th2 response with IL-4 and/or IL-10 production, the Th1 response is inhibited and the macrophage is activated through an alternative (M2) pathway, making it permissive for parasite replication. The infected macrophage itself may also produce IL-10 and TGF-β, which promote parasite survival by deactivating the macrophage.

generation of a protective immune response. The killing of intracellular *Leishmania* is mediated by the generation of reactive oxygen and nitrogen intermediates. The latter mechanism is dominant in rodent models of leishmaniasis, but it has a less prominent role in killing of *Leishmania* by human macrophages. IFN- γ also drives the expression of a large number of antimicrobial effector genes, though the roles of these genes in the antileishmanial defense are largely unknown. CD8+T cells may contribute to protective response through cytotoxic activity and IFN-γ production, but IL-10-producing CD8+T cells may be involved in immunosuppression and immunopathology.

Susceptibility to leishmanial infection and progression of disease is associated with the production of IL-4 and IL-10 by T cells, and IL-10 and TGF-β by infected macrophages (see Figure 3). IL-4 is a cross-regulatory cytokine that inhibits the expansion of the Th1 subset of T cells. IL-10 inhibits the production of cytokines by Th1 cells by suppression of accessory cell function and IL-12 production.

Infected macrophages have a diminished capacity to initiate and respond to an inflammatory response, thus providing a safe haven for the intracellular parasite. IL-10 and TGF-βare potent inhibitors of the antimicrobial activity of macrophages, and the expression of IL-10 mediates the persistence of parasites in the infected tissue. In addition to the production of deactivating cytokines, infection of the macrophage, especially in the presence of the type 2 cytokines (IL-4 and IL-13), can lead to an alternative activation phenotype characterized by blunted IFN-γ-mediated activation, low NO production, high arginase activity, increased production of prostaglandin E_2, and a gene expression profile that includes increased expression of phagocytic receptors, IL-1 receptor antagonist, and certain chemokines. Infected macrophages may also inhibit antigen presentation to effector T cells and there may be expansion of regulatory T cell populations. Both $CD4^+CD25^+Foxp3^+$ regulatory T cells and IL-10-producing $CD4^+CD25^-Foxp3^-$ T cells have been shown to promote chronic disease. Direct parasite-induced disruption or activation of a number of signaling pathways within the macrophage also contributes to macrophage permissiveness to infection.

CD4+ and CD8+ regulatory T cells and Th17 cells may play roles in both protection and pathology. $CD4^+CD25^+Foxp3^+$ regulatory T cells and IL-10-producing $CD4^+CD25^-Foxp3^-$ T cells have been shown to promote chronic disease. Leishmania-specific subsets of T regulatory cells may control inflammation and participate in resolution of CL. Th17 cells, a type of pro-inflammatory CD4+ T cell, are associated with both protection and pathological tissue destruction mediated by recruitment of neutrophils. Macrophages secrete IL-27 to control development of the Th17 subset, and collaborate with IL-21 to induce regulatory T cells to produce IL-10.

The cytokine response in human leishmaniasis has a mixed phenotype, driven by both innate (dendritic cells, natural killer cells, macrophages) and adaptive (T cells, B cells) immune cells. Abundant pro-inflammatory cytokines and chemokines (IL-6, IL-1, IL-8, TNF-α, IFN-ɣ, IL-12, CXCL10) are secreted together with anti-inflammatory cytokines (IL-10, IL-4, IL-13, IL-27 and TGF-ß) that attempt to control the infection and excessive inflammation. The anti-inflammatory mediators may block classical activation by IFN-γ and TNF-α and down regulate IL-12, MHCII and co-stimulatory molecules in macrophages and dendritic cells. This results in deficient antigen presentation and impaired acquired immune response. The balance of pro-inflammatory and anti-inflammatory signals largely determines the outcome of infection, tissue pathology and disease resolution.

Patients who develop localized cutaneous leishmaniasis (LCL, see following text) generally exhibit strong protective antileishmanial T cell (Th1) responses and spontaneously heal within 3–6 months. Failure to clear the parasite or resolve inflammation may lead to chronic non-healing lesions. Patients with mucosal leishmaniasis (ML, see 'epidemiology and disease') exhibit a hyperresponsive cellular immune reaction, with prominent Th17-mediated neutrophilic inflammation, which leads to the extensive tissue destruction. These patients appear to have ineffective counterregulatory mechanisms, such as impaired IL-10 responsiveness, that contributes to the excessive inflammation. Patients with active disseminated disease (diffuse cutaneous leishmaniasis (DCL) or visceral leishmaniasis (VL) see see 'epidemiology and disease') demonstrate minimal or absent *Leishmania*-specific cellular immune responses and low expression of IFN-γ by T CD4 + cells at the site of pathology. In non-healing LCL or VL local T cell dysfunction is may be due to depletion of L-arginine by expression of arginase in parasites and host myeloid cells. In contrast to the suppressed T cell responses, patients with VL display a polyclonal activation of B lymphocytes and high circulating pro-inflammatory cytokines characteristic of systemic inflammatory response syndrome and sepsis.

Epidemiology and Disease

The leishmaniases are highly prevalent, estimated to affect more than 10 million people in endemic tropical and subtropical regions on all continents except Australia and Antarctica. Over the past decade, an increased number of cases have been reported throughout the world, either as new sporadic cases in endemic regions or new epidemic foci. It is estimated that the global annual incidence of visceral leishmaniasis ranges between 200 000 and 400 000 cases while cutaneous leishmaniasis affects approximately 690 000 to 1 200 000 persons each year. The disease has emerged in new foci because of the following factors: (1) movement of susceptible people into existing endemic areas, usually because of agro-industrial development, (2) migration of infected individuals to new eco-epidemiologically high-risk regions due to social conflicts, (3) an increase in the vector and/or reservoir populations because of agriculture development projects, (4) heightened anthroponotic transmission because of rapid urbanization in some foci, and (5) increased sand fly density because of scaled-back malaria vector control programs.

The different leishmaniases are distinct in their etiology, epidemiology, transmission, and geographical distribution. These features are summarized in Table 1. With only rare exceptions, the *Leishmania* species that primarily cause cutaneous disease do not visceralize. Cutaneous leishmaniasis may be localized to one or a few skin ulcers, which occur at the site of the sand fly bite (LCL), or in rare instances the parasite may disseminate to involve large areas of skin (DCL). ML, an uncommon complication of LCL, is a destructive, disfiguring infection of the nasal and oropharyngeal mucosa. VL is the most serious form because there is progressive parasitism of the visceral reticuloendothelial organs (liver, spleen, and bone marrow).

In most instances, when *Leishmania* are transmitted to humans, the infection is controlled by the host response and overt disease does not develop. Field studies in endemic areas reveal people who have no history of disease often have evidence of infection by virtue of a positive leishmanin (delayed-type hypersensitivity, DTH) skin test. In most endemic areas, subclinical infection (no overt disease) occurs 5-10-fold more frequently than active disease. Epidemiological studies indicate that individuals with previous active cutaneous leishmaniasis or subclinical infection are usually immune to a subsequent clinical infection. Within endemic areas the prevalence of DTH skin test positivity (related to previous subclinical or clinical infection) increases with age and the incidence of clinical disease decreases with age, indicating that immunity is acquired in the population over time.

LCL (also called oriental sore) can affect individuals of any age but children are the primary victims in many endemic regions. It typically presents as one or a few raised or ulcerated lesions located on skin that is exposed to the sand fly bite, for example, the face and extremities. Cases of more than a 100 lesions have rarely occurred. The lesions develop at the site of the sand fly bite and increase in size and often ulcerate over the course of several weeks to months. The lesions remain localized to the skin and the patient generally has no systemic symptoms. In general, the lesions are painless unless they develop secondary bacterial infections. Lesions caused by *L. major* and *L. mexicana* usually heal spontaneously after 3–6 months leaving a depressed scar. Lesions on the ear pinna caused by *L. mexicana*, called Chiclero's ulcer because they were common in chicle harvesters in Mexico and Central America, often follow a chronic, destructive course. In general, lesions caused by *L. (Viannia)* spp. tend to be larger and more chronic.

DCL is a rare form of leishmaniasis caused by organisms of the *L. mexicana* complex in the New World and *L. aethiopica* in the Old World. DCL manifests as nonulcerating skin lesions, which often involve large areas of skin and may resemble the lesions of leprosy. The face and extremities are most commonly involved. Dissemination of the parasite from the initial lesion usually takes place over several years. It is thought that an immunological defect underlies this severe form of cutaneous leishmaniasis.

ML (also called espundia in Latin America) is an uncommon but serious manifestation of leishmanial infection resulting from the spread of the parasite from the skin to the nasal or oropharyngeal mucosa. ML is usually caused by parasites in the *L. (Viannia)* complex. In individuals who have, or have had, untreated skin lesions caused by *L. (V.) braziliensis* the lifetime risk of developing ML is estimated at 2–10%. In patients infected with *L. (V.) panamensis* the lifetime risk is about 5%. Patients with ML most commonly have nasal mucosal involvement, which results in symptoms of nasal congestion, discharge, and recurrent nosebleeds. In severe cases over the course of several years, there is marked soft tissue, cartilage, and even bone destruction, which lead to visible deformity of the nose or mouth, perforation of the nasal septum, and even obstruction of the airways.

VL (also called kala azar) typically affects children less than 5 years of age in the New World and Mediterranean region (*L. infantum* = *L. chagasi*), and older children and young adults in Africa and Asia (*L. donovani*). After inoculation of the organism

into the skin by the sand fly, the child may have a completely asymptomatic infection (no clinical signs of disease), or 2–9 months later show signs of active VL. There is typically no lesion at the site of the sand fly bite. The classic clinical features of VL include high persistent fever, enlargement of the liver and spleen, and severe cachexia (weight loss and muscle wasting). There is loss of bone marrow function, so the leukocyte and erythrocyte counts fall. Malnutrition is a risk factor for the development and more rapid evolution of active VL. Without the institution of drug therapy death occurs in more than 90% of patients. VL has been increasingly recognized as an opportunistic infection associated with HIV infection. Most cases have occurred in southern Europe (Mediterranean basin) and Brazil. Although anti-retroviral therapy has decreased the incidence of co-infected VL cases there is potential for many more cases as the endemic regions for HIV and VL converge. Disease may result from reactivation of a long-standing subclinical infection.

Diagnosis, Treatment, and Control

A definitive diagnosis of leishmaniasis rests on the microscopic detection of amastigotes in tissue specimens, or isolation of the organism by culture (performed only in specialized laboratories). A positive culture enables speciation of the parasite (usually by isoenzyme analysis by a reference laboratory). Serological tests are useful for the diagnosis of VL because of the very high level of antileishmanial antibodies, but not for ML or LCL where the antibody response is minimal or highly variable. PCR-based diagnosis is being increasingly used in secondary and tertiary health facilities but is still unaffordable in many endemic areas of developing countries.

Specific antileishmanial therapy is not routinely needed for uncomplicated LCL caused by strains that have a high rate of self-healing (*L. major*, *L. mexicana*). Lesions that are extensive or located where a scar would result in disability or disfigurement should be treated. Cutaneous lesions suspected or known to be caused by members of the *Viannia* subgenus (New World) should be treated because of the low rate of spontaneous healing and the potential risk of developing mucosal disease. Likewise, patients with lesions caused by *L. tropica* (Old World), which are typically chronic and non-healing, should be treated. All patients with VL or ML should receive therapy.

The pentavalent antimony compounds (sodium stibogluconate and meglumine antimoniate) have been the mainstay of antileishmanial chemotherapy for more than 40 years. These drugs are difficult to administer and have moderate toxicity. Using the recommended regimen, cure rates of 80–100% for LCL, 50–70% for ML, and 80–100% for VL can be expected. Failure of antimony therapy is more common in some parts of the world, and is particularly problematic in young children. Relapses are common in patients who do not have an effective antileishmanial cellular immune response, such as patients who have DCL or are co-infected with HIV. These patients often require multiple courses of therapy. Several alternative therapies have been used in the treatment of the leishmaniases, including the antifungal agent Amphotericin B and its related lipid-associated compounds, parenteral paromomycin (aminosidine) and pentamidine. Recently, single doses of Amphotericin B in a liposomal formulation was demonstrated to be effective in treating visceral leishmaniasis in India. Recombinant human IFN-γ has been successfully used as an adjunct to antimony therapy in treatment of refractory cases of ML and VL. Oral miltefosine has been used with success in treating VL in India and is currently used as second line treatment for cutaneous leishmaniasis in the Americas. The development of significant resistance may be a problem with miltefosine. The oral antifungal drugs ketoconazole and fluconazole have been effective in treating some patients with LCL. Topical cream formulated with paromomycin–gentamicin achieved cures rates >80% in patients infected with *L. major*. This formulation also has been proposed as an alternative for treating New World CL.

Leishmaniasis is best prevented by avoiding exposure to sandflies and use of insect repellents. Where transmission is occurring near homes, community-based insecticide (deltamethrin) spraying indoors or utilization of insecticide-impregnated bednets has had some success in reducing human infections. Control or elimination of infected reservoir hosts (e.g., seropositive domestic dogs) has had limited success. Where anthroponotic transmission is thought to occur, the early recognition and treatment of cases is essential. Because humans acquire long-standing immunity following infection with *Leishmania*, prevention of the disease through vaccination is a possible approach. No vaccines for humans are currently available but immunization studies involving experimental animal models are promising. Vaccination of humans, or domestic dogs to prevent transmission of VL, may have a role in the prevention of leishmaniasis in the future.

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Lipid Biosynthesis☆

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Glossary

Acyl carrier protein (ACP) Low molecular weight cytosolic protein functioning as the acyl group carrier for all the intermediates in fatty acid biosynthesis.
Bacillus subtilis The most intensively characterized Gram-positive bacterium.
Escherichia coli Paradigmatic Gram-negative bacterium that has been used for the vast majority of work on bacterial lipid metabolism.
Fatty acids Carboxylic acids with a long aliphatic carbon chain. Carbon atoms of fatty acids are numbered starting at the C-terminus. The position of the double bond in unsaturated fatty acids is represented by the symbol Δ followed by a superscript number. For example, cis-Δ9 means that there is a cis double bond between carbon atoms 9 and 10.
Phospholipid Major constituents of biological membranes, composed of two fatty acids, esterified to the first two positions of a glycerol backbone, and a polar headgroup attached to the third position via a phosphodiester bond.

Abbreviations

ACC	acetyl-CoA carboxylase
ACP	acyl carrier protein
acyl-CoA	acyl-coenzyme A
CDAGS	CDP-diacylglycerol synthetase
CFA	cyclopropane fatty acid
CL	cardiolipin
CLS	CL synthase
Cti	*cis-trans* isomerase
DHAP	dihydroxyacetone phosphate
FASII	type II fatty acid synthase
G3P	glycerol-3-phosphate
G3PD	glycerol-3-phosphate dehydrogenase
LPA	lysophosphatidic acid
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidilcholine
Pcs	phosphatidylcholine synthase
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGP	phosphatidylglycerol phosphate
PGPP	PGP phosphatase
PGPS	PGP synthase
PS	phosphatidylserine
PSD	phosphatidylserine decarboxylase
PSS	PS synthase
SAM	S-adenosylmethionine
SFA	saturated fatty acid
UFA	unsaturated fatty acid
UGT	UDP-glucosyltransferase

☆*Change History:* August 2014. D de Mendoza and GE Schujman introduced small edits in the text of the article, updated further readings, added the section "Engineering of Fatty Acid Biosynthesis for biofuel production", and added new Figure 5.

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Defining Statement

Lipids are important components of the plasma membrane of all bacteria and play a key role in cell viability. The major membrane lipids are phospholipids consisting of fatty acids esterified to glycerolphosphate derivatives and are called phosphoglycerides. Biochemical, genetic, and structural studies permitted to understand in great detail the phosphoglyceride metabolism in model bacteria.

The Type II Fatty Acid Biosynthetic Pathway

Fatty acid synthesis is essential for the formation of membranes and hence for the viability of all cells except Archaea, in which the membranes are composed of glycerol–ether lipids instead of glycerol–ester lipids and are based on isoprenoid side chains.

Fatty acid synthesis in bacteria is achieved by a highly conserved set of genes that each encodes an individual step in the type II fatty acid biosynthetic pathway (FASII) (Figure 1). Each protein of the pathway is located in the cytosol, and reaction intermediates are covalently attached to acyl carrier protein (ACP). The ACPs are a group of highly related small acidic proteins with molecular weights of about 9.0 kDa. The acyl intermediates of fatty acid biosynthesis are bound to ACP through a thioester linkage attached to the terminal sulfhydril of the 4′-phosphopantetheine prosthetic group.

Malonyl-CoA is required for all the elongation steps of the fatty acid biosynthetic pathway and is formed by carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). ACC is constructed from four separate proteins and requires biotin as a cofactor. Malonyl-CoA is made available to the enzymes of fatty acid biosynthesis by its conversion to malonyl-ACP by malonyl transacylase

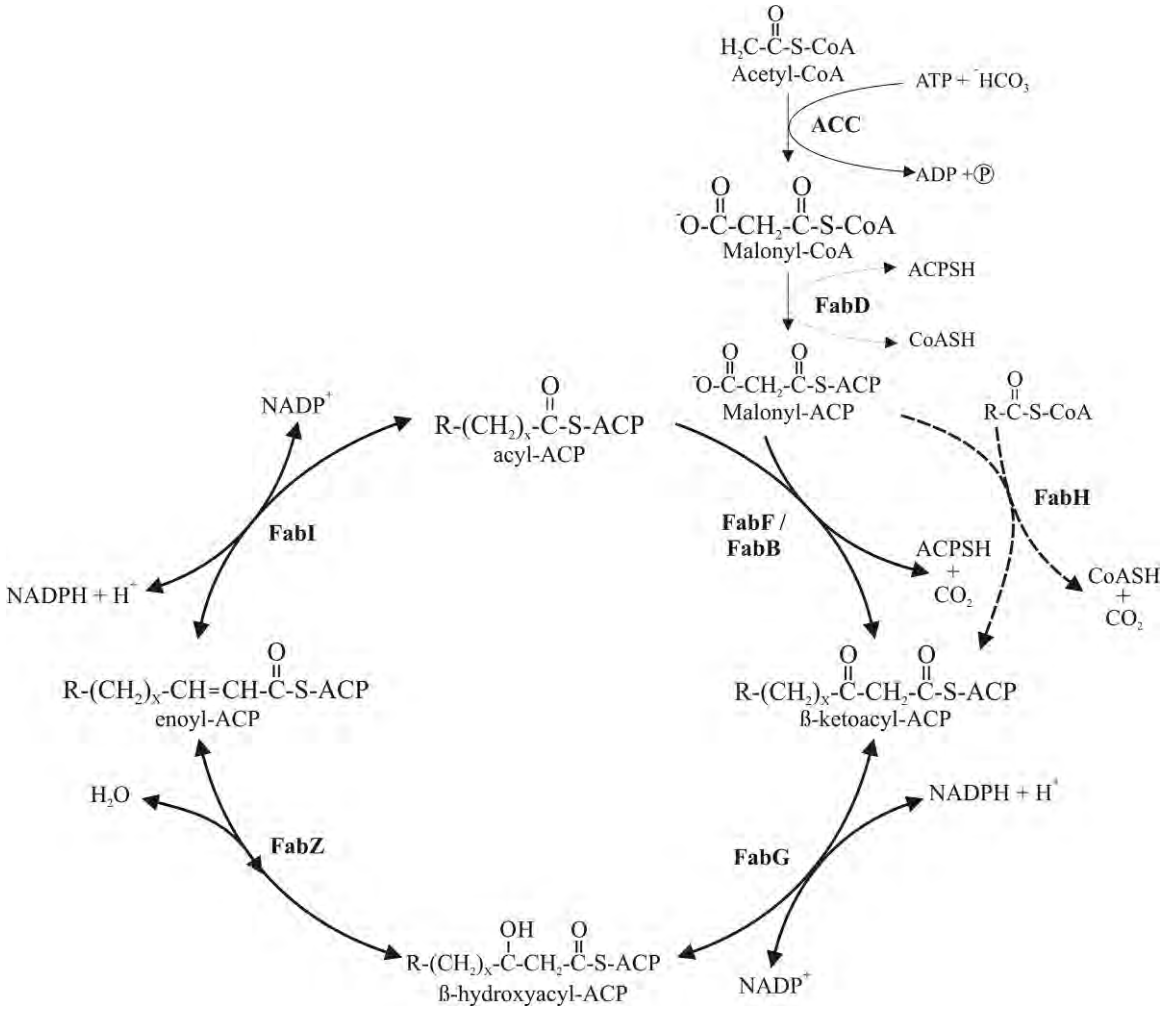

Figure 1 Initiation and elongation cycle of fatty acid biosynthesis. Malonyl-CoA, the product of the acetyl-CoA carboxylase (ACC) reaction, is converted to malonyl-ACP by malonyl-CoA transacylase (FabD). The following step is catalyzed by β-ketoacyl ACP synthase III (FabH), which is able to utilize short acyl-CoA primers as substrates. The elongation of the growing acyl chains is accomplished in four successive steps catalyzed by the following enzymes: β-ketoacyl ACP synthase I or II (FabB or FabF, respectively), β-ketoacyl ACP reductase (FabG), β-hydroxyacyl-ACP dehydrase (FabZ), and enoyl reductase (FabI).

(FabD). The chain elongation step in fatty acid biosynthesis consists of the condensation of acyl groups, which are derived from acyl-ACP or acyl-coenzyme A (acyl-CoA), with malonyl-ACP by the β-ketoacyl-ACP synthases (often referred as condensing enzymes). These enzymes are divided into two groups. The FabH class of condensing enzymes is responsible for the initiation of fatty acid elongation and utilizes acyl-CoA primers. *Escherichia coli* produces straight chain and unsaturated fatty acids (UFA), and *E. coli* FabH selectively uses acetyl-CoA to initiate the pathway. In contrast, *Bacillus subtilis* produces mainly branched-chain fatty acids and contains two FabH isozymes (named FabHA and FabHB) that differ from the *E. coli* enzyme in that they are selective for branched-chain acyl-CoAs. The FabF–FabB class of condensing enzymes is responsible for the subsequent rounds of fatty acid elongation in the pathway. These enzymes condense malonyl-ACP with acyl-ACP to extend the acyl chain by two carbons. The FabF isoform is universally expressed in bacteria, and in *B. subtilis* is the sole condensing enzyme able to carry out the subsequent elongation reactions in fatty acid synthesis. *E. coli*, and some other bacteria, also have the FabB isoform, which plays a special role in the synthesis of UFA (see 'Biosynthesis of UFAs'). FabG is the β-ketoacyl-ACP reductase and only a single isoform of this enzyme has been identified so far in bacteria. The next step in the elongation cycle is the dehydration of β-hydroxyacyl-ACP to *trans*-2-enoyl-ACP. Two isoforms, FabZ and FabA, catalyze the dehydration of β-hydroxyacyl-ACPs, albeit with different substrate specificities. FabZ is universally expressed in bacteria, whereas FabA is restricted to organisms that use the FabA/B system to introduce double bonds into the growing acyl chain (see 'Biosynthesis of UFAs'). Although most FAS II enzymes are relatively conserved in bacteria, an exception is the last step of the elongation cycle, where a saturated acyl-ACP is formed by a NAD(P)H-dependent reduction of the enoyl-ACP double bond. In *E. coli*, this reaction is catalyzed by FabI, while *B. subtilis* contains two isozymes, a FabI homologue and FabL that, like FabI, is a member of the short chain reductase superfamily. *Vibrio cholerae* contains FabV, a NADH-dependent member of the short chain reductase superfamily, while *Streptococcus pneumoniae* contains a single enoyl reductase, FabK, unrelated to this last superfamily of enzymes.

E. coli and mostly of Gram-negative bacteria synthesize straight-chain fatty acids, but there can be a major variation of this paradigm. Branched-chain fatty acids are produced in several Gram-positive bacteria, such as *Bacillus*, *Streptomyces*, and *Listeria*, and exist in two forms that are characterized by the presence of either iso and anteiso branches at their termini. The primer carbons for the synthesis of branched-chain fatty acids are 2-ketoacids derived from valine, leucine, and isoleucine. While isoleucine is the precursor of anteiso-branched-chain fatty acids, leucine and valine give raise to the primers for iso-branched chain fatty acids. The enzyme responsible for the formation of the branched-chain acyl-CoAs is a specialized branched-chain α-ketoacid dehydrogenase complex. These branched-chain acyl-CoAs are then introduced into the FASII pathway by FabH enzymes of the appropriate substrate specificity.

Biosynthesis of UFAs

Bacterial growth requires an appreciable fraction of acyl chains of the membrane lipids to be in a disordered state (fluid). A disordered state is imparted by presence of either *cis*-unsaturated or terminally anteiso-branched-chain fatty acids both of which act to offset the closely packed ordered arrangement of the lipid bilayer acyl chains that is imparted by straight chain saturated acyl chain. In this section, we discuss the basic features of biosynthesis of UFA in organisms in which the proposed biosynthetic mechanisms have been well characterized. In *E. coli*, synthesis of the normal UFA content requires three enzymes, FabA, FabB, and FabF (Figure 2(a)). FabA, β-hydroxydecanoyldehydrase, is the key enzyme of the classic anaerobic pathway of UFA synthesis and introduces a *cis* double bond into a 10-carbon intermediate. FabA is a bifunctional enzyme that catalyzes both the removal of water to generate *trans*-2-decenoyl-ACP and the isomerization of this intermediate to *cis*-3-decenoyl-ACP. This *cis*-10 carbon intermediate is then elongated by FabB and later by FabF to form the two major UFA found in *E. coli*: C16:1Δ^9 and C18:1Δ^9. FabB and FabF have distinct and nonoverlaping roles in *E. coli* UFA synthesis. FabB is thought to elongate the product of the FabA enzyme to the C12 unsaturated intermediate, whereas FabF is required to convert C16 unsaturated species to the C18 species (Figure 2(a)).

Expression of the key UFA synthetic gene *fabA* from *E. coli* is positively regulated by FadR which was hitherto thought to function only as a repressor of the ß-oxidation pathway of fatty acids (see 'Fatty Acid β-Oxidation'). Positive regulation of *fabA* expression is neutralized both *in vivo* and *in vitro* by long-chain acyl-CoAs that are small ligands which regulate the binding of FadR to the *fabA* promoter. FadR is also a positive regulator of *fabB*, the second UFA biosynthetic gene, although the changes in *fabB* expression in *fadR* mutants are not as great as with *fabA*. Thus, FadR acts as a repressor of ß-oxidation genes and as an activator of the two genes required for UFA synthesis. FabR is a second *E. coli* regulator of UFAs synthesis. It behaves as a repressor acting downstream of FadR operator in the regulation of *fabA* and *fabB*. The ligand regulating FabR activity is still unknown.

There is a variation of the FabA/FabB bacterial strategy for UFA synthesis, discovered in *Enterococcus faecalis*. This organism possesses a special FabZ enzyme (called FabN) that has a FabA-like activity and a unique FabF (called FabO) that replaces FabB. However, other organisms, such as *S. pneumoniae*, compensate FabA absence with an enzyme called FabM that is capable of isomerizing the *trans*-unsaturated bond at the key 10-carbon intermediate to its *cis*-isomer.

In the examples mentioned above, the proteins FabA, FabM, or FabN anaerobically introduce the double bond into a C10 intermediate and either FabB or FabF, in the organisms lacking FabB, channel this intermediate to the mainstream of the fatty acid synthetic pathway. In contrast, other bacteria like *Bacillus* and Cyanobacteria have completely separate systems for the synthesis of UFA and saturated fatty acids. These organisms use fatty acid desaturase enzymes, which require molecular oxygen and reducing equivalents obtained from an electron transport chain, to introduce the double bond into previously synthesized saturated fatty acids (Figure 2(b)).

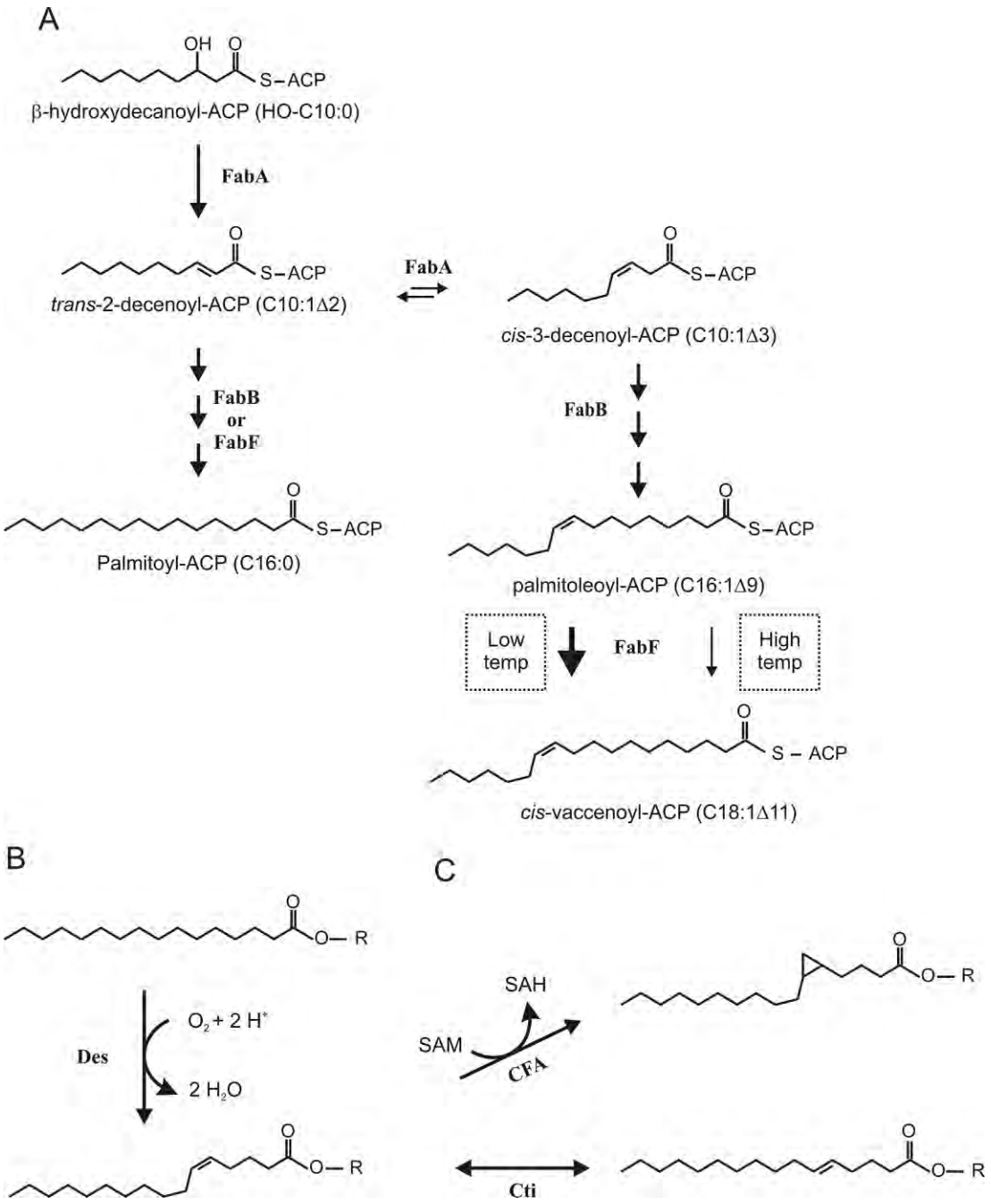

Figure 2 Unsaturated fatty acid (UFA) biosynthesis and modification. (a) In *Escherichia coli* and related bacteria, FabA catalyzes the key step in UFA production, introducing the double bond into the acyl chain at the 10-carbon intermediate. FabA is a bifunctional enzyme that catalyzes both the removal of water to generate *trans*-2-decenoyl-ACP and the isomerization of this intermediate to *cis*-3-decenoyl-ACP. The FabB enzyme is required for the elongation of these unsaturated acyl-ACP intermediates, and FabF participates in SFA synthesis and in the elongation of C16:1Δ9 palmitoleoyl-ACP to C18:1Δ11 *cis*-vaccenoyl-ACP. The reactivity of this enzyme toward C16:1Δ9 palmitoleoyl-ACP is increased after a temperature downshift. (b) Reactants and products of a representative fatty acid desaturase reaction. This is a $2e^-$- and O_2-dependent dehydrogenation at an unactivated position of the fatty acyl chain, resulting in *cis*-double-bond formation. (c) Modification of the acyl-chain double bonds of bacterial lipids. The *cis* acyl-chain double bond can be isomerized to a *trans* acyl-chain double bond or methylenated to form a cyclopropane ring. The configuration of the double bond is conserved in the cyclopropanation reaction. SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

The organisms mentioned above each have only a single pathway for the synthesis of the UFAs required to make functional membrane lipids. In marked contrast, UFA biosynthesis in *Pseudomonas aeruginosa* proceeds by three distinct pathways. In this organism, the FabA–FabB pathway does the bulk of UFA synthesis under all growth conditions. However, *P. aeruginosa* has two fatty acyl desaturases (DesA and DesB) that supplement the FabA–FabB pathway under aerobic conditions and thereby allows faster

growth. DesA introduces the double bond into the acyl chains of intact phospholipids, whereas the substrates of DesB are acyl-CoAs that are derived from exogenous fatty acids. DesB is inducible and is regulated by DesT, a transcriptional regulator that has the property of being able to sense the fatty acid composition of the long chain acyl-CoA pools to adjust the expression of the desaturase.

Phospholipid Synthetic Pathways and Headgroup Diversity

The type and proportion of each complex lipid vary greatly according to the bacterial genus. In the model organisms, *B. subtilis* and *E. coli*, the phospholipids consist largely of the common bacterial lipids phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), while in *B. subtilis* almost 30% of membrane lipids are composed of the glycolipids, mono-, di-, and triglucosyldiacylglycerols, absent in *E. coli*. The Gram-negative bacterium contains important amounts of cardiolipin (CL), which is only minor in *B. subtilis*.

In all organisms, phospholipid biosynthesis begins with two acylation steps of sn-glycerol-3-P (G3P) to form phosphatidic acid (PA) (Figure 3). The synthesis of G3P from dihydroxyacetone phosphate (DHAP) is catalyzed by GpsA (G3P dehydrogenase [NAD+]), and similar proteins are encoded in a wide diversity of bacterial species. Therefore, the GpsA reaction likely provides the phospholipid 'backbone' of most bacteria. Two pathways have been described for the first acylation of G3P. The most ubiquitous one, present in all but one family of proteobacteria, involves two biochemical steps: first, PlsX catalyzes the synthesis of fatty-acyl-phosphate from acyl-ACP and inorganic phosphate and then the acyltransferase PlsY transfers the fatty acid from the activated acyl intermediate to the 1-position of G3P. In the second pathway, present in the γ-proteobacteria, the acyltransferase PlsB catalyzes the acylation of G3P to give lysophosphatidic acid (LPA). This enzyme utilizes either long-chain acyl-ACP or acyl-CoA as the acyl-donor.

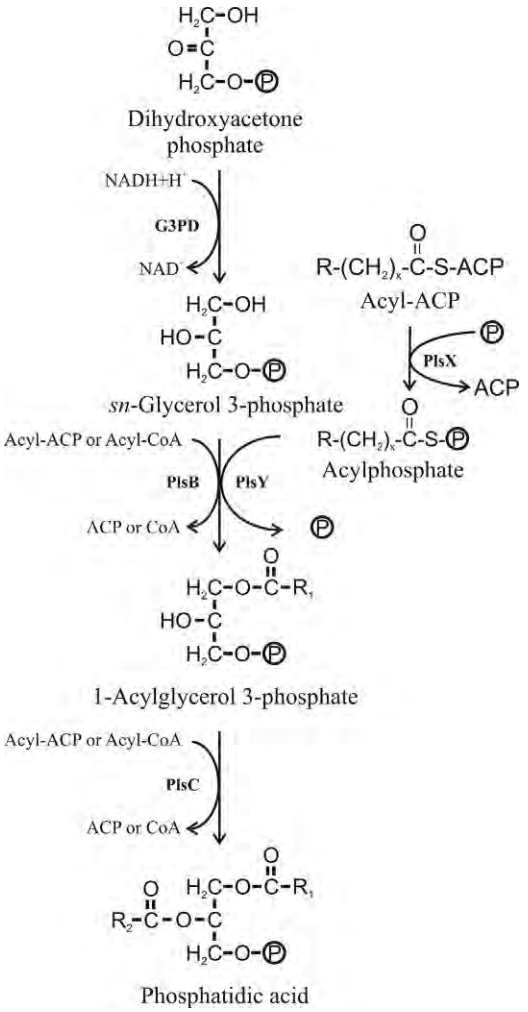

Figure 3 Initiation of phospholipid biosynthesis in bacteria. Glycerol-3-phosphate (G3P) is synthesized by the product of the *gpsA* gene, glycerol-3-phosphate dehydrogenase (G3PD). In *Escherichia coli* and related bacteria, PlsB catalyzes the formation of 1-acylglycerol 3-phosphate (lysophosphatidic acid (LPA)) from glycerol 3-phosphate (G3P) and acyl-ACP (or acyl-CoA). In most bacteria, two enzymes replace PlsB activity: first, PlsX catalyzes the synthesis of fatty acyl-phosphate from acyl-ACP; second, PlsY transfers the fatty acid from the activated acyl intermediate to the 1-position of G3P. LPA is then acylated to phosphatidic acid by PlsC.

Acylation of 2-position of LPA to give phosphatidic acid is carried out by PlsC (Figure 3). PlsB, PlsC, and PlsY are intrinsic membrane proteins, while PlsX is a soluble enzyme that *in vivo* is anchored to the membrane by a still unknown mechanism.

Most sequenced bacterial genomes encode a protein having strong homology to CDP-diglyceride synthase, the *E. coli* enzyme encoded by the *csdA* gene. This enzyme catalyzes the conversion of PA to the activated intermediate, CDP-diglyceride, which can then be utilized in the synthesis of either PE or PG and CL. These two branches of the pathway present different pictures. The enzymes of the pathway to PE, the major phospholipid of *E. coli* and many other bacteria, are semiconserved. The first enzyme of the *E. coli* pathway, phosphatidylserine synthase (PssA), is poorly conserved outside of the alpha and gamma proteobacteria, whereas protein homologous to the second enzyme, phosphatidylserine decarboxylase (Psd) is widely distributed among bacteria. In contrast to *Bacillus* spp. where PssA is a membrane-associated protein, the PssA from *E. coli* is a soluble protein that is able to associate to the membrane (Figure 4).

The other branch of the pathway, which leads to PG and CL, begins with the *pgsA*-encoded-CDP-diacylglycerol-G3P 3-phosphatidyltransferase, which, like GpsA and Cds, is widely conserved. The next step in the pathway, removal of the phosphate moiety from PG phosphate, is indeterminate because at least three different enzymes (PgpA, PgpB, and an unknown protein) are thought to catalyze this hydrolytic step. The condensation of two molecules of PG to give CL and glycerol is catalyzed in *E. coli* by the *cls* gene product, CL synthase (Figure 4). Like *pgsA*, this gene is widely conserved. It seems that PG, but not CL, is essential for viability of *B. subtilis*. This situation is similar to that in *E. coli* where PG has important physiological roles in various cellular processes, such as initiation of DNA replication and protein secretion.

A variation of the biosynthetic pathways detailed above is the formation of glycolipids by the reaction of a UDP-sugar with diacylglycerol. Glucosyl- and diglycosyl-diacylglycerol are abundant lipids in many Gram-positive bacteria, and these lipids function as membrane anchors for the lipoteichoic acid constituents of the cell wall. For instance, the *ypfP* gene of *B. subtilis* codes for a UDP-glucosyltransferase (UGT), which catalyzes the addition of glucose residues to 1,2-diacylglycerol (Figure 4). The enzyme is able to sequentially add up to four glucose residues to 1,2-diacylglycerol, but *in vivo* the diglucosylacylglycerol is the main glycolipid found. This enzyme is specific for the sugar donor and acceptor and appears to be bound to membrane. In addition to glycolipids, there are membrane components that are not phospholipids. For example, sulfolipids and ornithine-derived lipids are produced by some bacteria when phosphorous availability is scarce.

Phosphatidilcholine (PC) is the major phospholipid in eukaryotes where it fulfills important structural and signaling functions. In contrast, only about 10% of all bacterial species are predicted to produce PC. It is notable that many of these bacteria are either symbionts or pathogens of animals and plant hosts. However, the advantage of having PC in their envelope, presumably in the inner and outer membrane, is unclear. PC in bacteria can be synthesized via two alternative routes. A pathway unique to bacteria comprises the direct condensation of choline and CDP-diacylglycerol catalyzed by a phosphatidylcholine synthase (Pcs). In the methylation pathway, PC is produced by the threefold methylation of PE by one or more phospholipid *N*-methyltransferases using *S*-adenosylmethionine (SAM) as methyl donor.

Phospholipid Modifications in Bacteria

The acyl chains of bacterial membrane phospholipids are often altered after synthesis and incorporation into membrane lipid bilayers. All the three major acyl chain modifications involve double bonds (Figures 2(b) and 2(c)). In cyclopropane fatty acid (CFA) synthesis, a methylene carbon is added across the double bond to form a three-membered ring. In *cis–trans* isomerization, the double bond is reconfigured and, as discussed above, in desaturation a double bond is introduced into a saturated acyl chain.

Double bonds exist in two diasteroisomers: *cis* in which the two hydrogen atoms are located on the same side of the bond and *trans* in which the hydrogen atoms are on opposite sides of the bond. Both isomers kink the acyl chains in which they reside, although the kink of the *cis* isomer is much larger. Therefore, the *trans*-UFA mimics the physical properties of phospholipids that contain saturated fatty acid (SFA). Virtually all the double bonds in naturally occurring fatty acids are *cis* isomers, although the occurrence of *trans* fatty acids is well documented in a restricted number of bacteria, such as pseudomonads, vibrios, and methyltrophs. The *cis–trans* isomerization reaction leads to membranes that have higher phase transition, increased rigidity, and decreased permeability to solutes. Although this consideration argues that *cis–trans* isomerization is a mechanism to adapt the membranes to higher temperatures, most work has been made in *Pseudomonas putida*, an organism of interest for detoxification of soils and waters contaminated with organic solvents. The synthesis of *trans* fatty acid by *P. putida* has been interpreted as a mechanism to decrease penetration of solvents through the inner membrane. In this bacterium, it has been demonstrated that the *cis–trans* isomerization reaction is carried out by a periplasmic, haem-containing *cis–trans* isomerase (Cti).

In contrast to *cis–trans* isomerization, cyclopropanation is very widely distributed among bacteria. Unlike Cti, CFA synthase is a cytosolic enzyme, as expected from its dependence of the methyl donor SAM. The cyclopropane bond retains the *cis* double bond configuration of the acyl chain, and therefore phospholipids that contain CFAs have similar biophysical properties to those that are composed of *cis* UFA. However, the cyclopropane bond is more stable than a double bond and is physiologically useful, for example, cyclopropanation increases *E. coli* resistance to acid stress. In addition, the inactivation of a homologue of the *E. coli cfa* gene in *Mycobacterium tuberculosis*, in which a cyclopropane ring is normally introduced into mycolates, results in *M. tuberculosis* being unable to establish a persistent infection, which indicates that CFAs have an important role in the survival of bacterial pathogens.

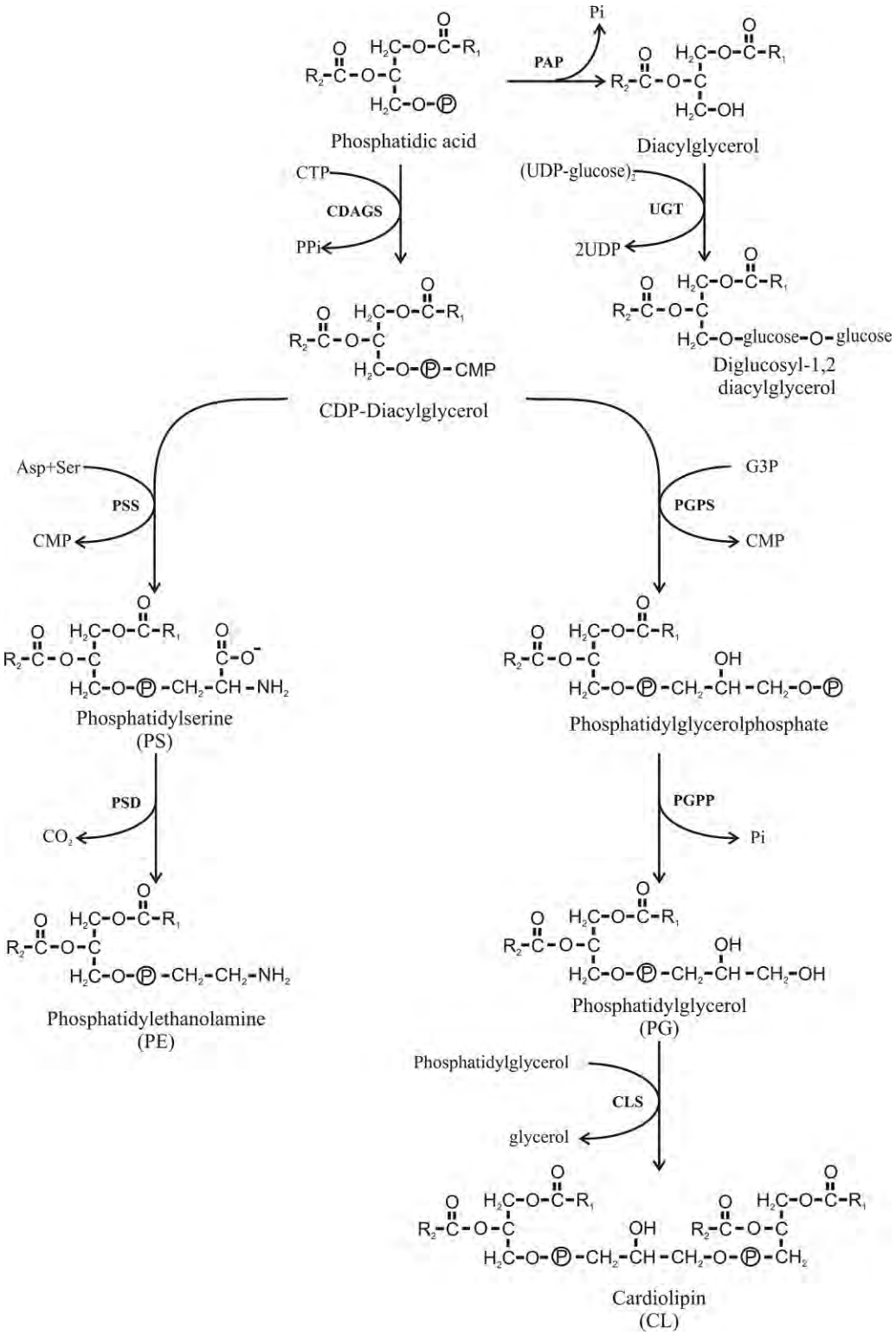

Figure 4 Complex lipid biosynthesis. The first step is the activation of phosphatidic acid to CDP-diacylglycerol by CDP-diacylglycerol synthetase (CDAGS). Biosynthesis of phosphatidylethanolamine (PE) is achieved by the sequential catalysis of phosphatidylserine synthase (PssA) followed by phosphatidylserine decarboxylase (PSD). PG is formed by the condensation of CDP-diacylglycerol with G3P, catalyzed by PGP synthase (PGPS), followed by removal of the phosphate by PGP phosphatase (PGPP). CL is formed by the condensation of two molecules of PG catalyzed by the CL synthase (CLS). Diacylglyceride is synthesized by dephosphorylation of phosphatidic acid by phosphatidic acid phosphatase (PAP). The diacylglyceride is then glucosylated by one or two transfers of glucose from UDP-glucose in a sequential reaction catalyzed by UDP-glucosyltransferase (UGT).

Control of Fatty Acid Biosynthesis

Fatty acid synthesis is coordinately regulated with phospholipid, macromolecular synthesis, and growth as part of the response to changes in the environment. This control is crucial for membrane homeostasis, because the biophysical properties of membranes are determined in large part by the composition that results from *de novo* lipid biosynthesis. Many of these processes are rapid responses of the integrated biochemical network and do not involve changes in gene expression. An important recent development is the identification and characterization of transcription factors that globally coordinate the expression level of genes that function in lipid biosynthesis.

Biochemical Control of FASII

In *E. coli*, the long chain acyl-ACP end products have emerged as important negative effectors of the activity of FASII. The importance of long-chain acyl-ACP regulation was determined through the demonstration that acyl-ACP hydrolysis by thioesterases leads to dysregulated fatty acid biosynthesis and to the release of fatty acids into the medium. The enzymes that are inhibited *in vitro* by acyl-ACPs are ACC, the enzyme that catalyzes the conversion of acetyl-CoA in malonyl-CoA, FabH that catalyzes the first step of the pathway, and FabI that catalyzes the completion of acyl-ACPs elongation (Figure 1).

Recent evidence showed that PlsX, the peripheral enzyme that catalyzes the first committed step in the biosynthesis of phospholipids in *B. subtilis*, plays an important role in the coordination of the production of fatty acids and phospholipids. In this organism, the inactivation of PlsX leads to the cessation of both fatty acid and phospholipid synthesis. The inhibition of total lipid synthesis in PlsX-depleted cells was attributed to the accumulation of acyl-ACPs due to the block in its conversion to acyl-phosphate. This observation suggests that acyl-ACP may play a regulatory role in *B. subtilis* similar to acyl-ACP regulation of FabH and ACC in *E. coli*.

Transcriptional Control of FASII

A major advance in our understanding of the transcriptional control of bacterial lipid synthesis occurred following the identification of FapR, a global transcriptional repressor that controls the expression of the *fap* regulon involved in the biosynthesis of fatty acids and phospholipids in *B. subtilis*. The binding of FapR to its DNA targets is specifically inhibited by malonyl-CoA, an essential intermediate of fatty acid synthesis in all living cells, the cellular pools of which provide a mechanism for sensing the status of fatty acid biosynthesis and for adjusting the *fap* regulon accordingly. FapR is highly conserved in many Gram-positive organisms, many of which are human pathogens, including *Bacillus anthracis*, *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The consensus binding sequence of FapR is also highly conserved in the putative promoter regions of the *fapR* gene in these species. These observations indicate that the regulation mechanism observed in *B. subtilis* is probably conserved in many other organisms.

The recent crystal structure of the effector binding domain of FapR, alone and complexed with malonyl-CoA, has provided the first insight into the ligand-induced transcriptional regulation of fatty acid and phospholipid synthesis. FapR is a homodimeric protein that displays the typical 'hot-dog' fold characteristic of the thioesterase enzyme family. Binding of malonyl-CoA promotes an order–disorder transition, which transforms an open ligand-binding groove into a long tunnel occupied by the effector molecule in the complex. This ligand-induced modification propagates to the helix-turn-helix motifs, impairing their productive association for DNA binding. It was recently reported the structural characterization of full-length FapR from *Staphylococcus aureus* (*Sa*FapR), a major Gram-positive pathogen causing several human infections. The crystal structures of *Sa*FapR have been obtained from the protein alone and for the complexes with cognate DNA and malonyl-CoA. Structure based mutations that disrupt the *Sa*FapR interaction is lethal for *S. aureus* suggesting that this homeostatic signaling pathway is a promising target for novel chemotherapeutic agents against Gram-positive pathogens (see 'Antibacterial Agents that Target Fatty Acid Biosynthesis').

The second global regulator, FabT, was identified in the human pathogen *S. pneumoniae*. In this bacterium, the 12 genes required for fatty acid biosynthesis are located together in a single cluster. The second gene in the cluster encodes the FabT transcriptional repressor, which regulates all these genes except *fabM* (involved in UFA biosynthesis, see 'Biosynthesis of UFAs'). A similar genetic organization is found in other Gram-positive bacteria, such as *Enterococcus*, *Clostridium*, and *Lactococcus*. The ligand that enhances FabT binding to DNA is long-chain acyl-ACP. Upon binding to acyl-ACP, the affinity of FabT for the DNA binding site is significantly increased and the FabT-acyl- ACP complex docks on the DNA and represses transcription of the FAS cluster. *B. subtilis* lacking FapR and *S. pneumoniae* deficient in FabT do not produce increased quantities of lipid, despite overexpression of the pathway enzymes. This observation strongly argues for the presence of biochemical regulatory mechanisms in both organisms that override transcriptional control to prevent excess production of phospholipids and maintain the appropriate lipid:protein ratio in the membrane.

Control of Membrane Fluidity

Bacteria can encounter a wide range of environments and must adapt to new conditions in order to survive. As the selective barrier between living cells and their environment, the plasma membrane plays a key role in cell viability. The barrier function of the cytoplasmic membrane is known to depend critically on the physical state of lipid bilayers, making it susceptible to changes in

environmental temperature. In fact, it has been established that normal cell function requires membrane lipid bilayers that are largely fluid; indeed, the bilayers of most organisms are entirely or mostly fluid at physiological temperatures. However, at lower temperatures, membrane lipid bilayers undergo a reversible change of state from a fluid (disordered) to a nonfluid (ordered) array of the fatty acyl chains. The temperature at the midpoint of this transition is called the transition temperature, and the change of state accompanying an increase in temperature is called the lipid phase transition, the gel–liquid crystalline transition, or most properly, the order–disorder transition. The transition temperature is a function of the membrane lipid composition and, in organisms deficient in cholesterol, mainly depends on the fatty acid composition of the membrane lipids. The (overly simplified) rule of thumb is that phospholipids that contain UFAs have much lower transition temperatures than those lipids made of saturated fatty acids. The effect is due to different packing of the two types of phospholipid acyl chains. Saturated fatty acid acyl chains can pack tightly, but the steric hindrance imparted by the rigid kink of the *cis* double bond results in much poorer chain packing of UFAs, even below the phase transition temperature. From these considerations, it seems clear that bacteria must regulate their phase transition in response to temperature. Without regulation, an organism shifted from a high to a low temperature would have membrane lipids with suboptimal fluidity, resulting in subnormal membrane function. The mechanism of regulation in all of the cases examined seems to occur via the incorporation of proportionally more UFAs (or fatty acids of analogous properties) as the temperature decreases. This regulatory mechanism, called thermal control of fatty acid synthesis, seems to be a universally conserved adaptation response allowing cells to maintain the appropriate fluidity of membrane lipids regardless of the ambient temperature. In this section, we discuss the basic features of thermal regulation of membrane lipid fluidity in *E. coli* and *B. subtilis*, in which the proposed mechanisms are firmly based on both genetic and biochemical evidences. There are two fundamental differences between the *E. coli* and *B. subtilis* models that account for the formation of UFAs and the regulation by growth temperature of the synthesis of these fatty acids. First, *B. subtilis* lacks the *fabB* and *fabA* genes that are essential for UFA synthesis in *E. coli*, and this property accounts for the absence of UFA production by the Gram-positive synthase. Instead, *B. subtilis* makes UFAs via an oxygen-dependent desaturase that uses existing phospholipids as substrates to introduce a double bond at the 5 position of the fatty acyl chain. Second, *B. subtilis* uses a two-component system composed of a membrane-associated kinase, DesK, and a transcriptional regulator, DesR, which stringently control the transcription of the gene coding for the desaturase. Induction of this pathway is brought about via the ability of the molecular thermosensor, DesK, that sense changes in the lipid bilayer structure triggered by a sudden decrease in temperature. In contrast, in *E. coli*, there is a biochemical mechanism that allows the elongation cycle of fatty acid synthesis to respond to the environmental temperature. In this organism, the UFA synthesized in greater quantity at low temperatures is C18:1Δ^{11}. This regulatory response is due to the properties of FabF that converts C16:1Δ^{9} to C18:1Δ^{11}. This enzyme is present at all temperatures but is more active at low temperature (Figure 2(a)). Thus, a cytosolic thermosensor governs the temperature-dependent adjustment on membrane fluidity in *E. coli*, and probably in related bacteria, while in *B. subtilis* this control is exerted by a membrane-associated thermosensor.

Fatty Acid β-Oxidation

Many bacteria are capable of degrading exogenous fatty acids as a carbon source for growth. *E. coli* can use fatty acids with various chain lengths as sole carbon and energy sources. After uptake, fatty acids can either be degraded via the β-oxidation pathway or used as precursors for membrane phospholipids biosynthesis. The degradation pathway is catalyzed by the enzymes encoded by the *fad* regulon, which are responsible for the transport and activation of long-chain fatty acids, and their β-oxidative cleavage into acetyl-CoAs. The biochemistry of the inducible enzymes of β-oxidation has been extensively studied and the pathway for degradation is defined. Transcriptional control of the fatty acid degradation genes occurs via the FadR regulatory protein, which is also an extensively studied fatty acid-responsive transcription factor (see 'Biosynthesis of UFAs'). When long-chain fatty acids of more than 14 carbons are present in the growth medium, they are internalized and converted to acyl-CoAs, which bind to FadR, causing release of the regulatory protein from the operator and thus derepression of transcription of the *fad* genes. Furthermore, FadR negatively regulates the expression of two gene products that are involved in anaerobic degradation of fatty acids. Thus, FadR in *E. coli* functions as a repressor of many genes mainly involved in growth on fatty acids.

B. subtilis cannot grow well on acetate as the sole carbon source, probably due to its lack of the glyoxilate shunt enzymes. Growing *B. subtilis* cannot degrade long-chain fatty acids added to the culture medium. These results suggest that fatty acid degradation through β-oxidation is insignificant. However, *B. subtilis* possesses a considerable number of genes involved in oxidation of fatty acids. This conservation suggests that the β-oxidation of fatty acids has some functions under certain physiological functions, such as differentiation, since a *fad* operon is induced at the onset of sporulation. Five *fad* operons are repressed by FadRBs. In a similar manner to *E. coli* FadR, the binding of FadRBs to its DNA targets is inhibited by long-chain acyl-CoAs.

Antibacterial Agents that Target Fatty Acid Biosynthesis

The fatty acid biosynthetic pathway is an attractive but still largely unexploited target for the development of new antibacterial agents. Several compounds empirically selected as inhibitors of bacterial growth owe their antibacterial to inhibition of fatty acid synthetic enzymes, being the most important example the antitubercular drug, isoniazid, which specifically inhibits the InhA enoyl-ACP reductase (FabI) from Mycobacterium tuberculosis (see 'The Type II Fatty Acid Biosynthetic Pathway'). The antiseptic agent

triclosan also targets FabI. However, a drawback to the development of FabI inhibitors as broad spectrum antimicrobial is the existence of a nonhomologous isozyme, FabK, in clinically important pathogens, such as *S. pneumoniae* (see 'The Type II Fatty Acid Biosynthetic Pathway'). It should be noted that there are many examples of natural products, such as thiolactomycin, cerulenin, and platensimycin that have evolved to target the FabF and FabB condensing enzymes. Although fatty acid synthesis inhibitors may not be effective against some Gram-positive bacteria that are supplemented with extracellular fatty acids; there is compelling evidence that such inhibitors are useful against many Gram-positive and all Gram-negative organisms even in the presence of extracellular fatty acids.

Engineering of Fatty Acid Biosynthesis for Biofuel Production

In the last years, bio-based sustainable production of fuels has been attracting increasing interest. Fatty acid derived compounds like alkanes, alkenes, ethylesters and fatty alcohols are of high energy content, and similar to the petro-based fuels currently in use. Production of these molecules has been achieved in genetically modified microorganisms, principally in *E. coli*, employing plant derived sugars and byproducts of agro-industrial processes. To this end, thioesterases, acyl-CoA ligases, several reductases and wax synthases have been expressed in different combinations in *E. coli*, as depicted in Figure 5. Improvements, like overexpression of

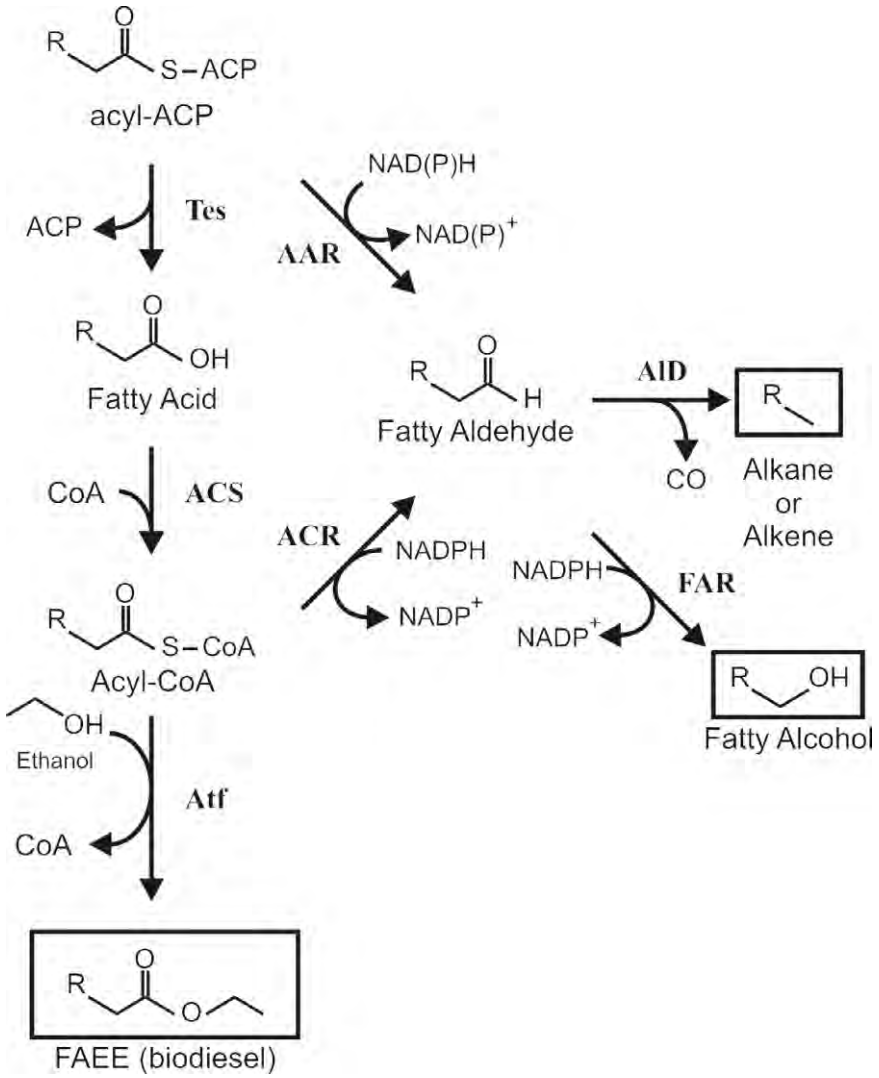

Figure 5 Biosynthesis of fatty acid-derived biofuels. Acyl-ACPs are reduced to fatty aldehydes by acyl-ACP reductases (AAR). Alternatively, the acyl-moiety can be released by the action of thioesterases (Tes). Free fatty acids are in turn activated to acyl-CoAs by acyl-CoA synthase (ACS). In biodiesel production the acyl-CoAs are esterified with ethanol by an acyltransferase (Atf), generating ethylesters of fatty acids. Acyl-CoAs can be also reduced to fatty aldehydes by Acyl-CoA reductases (ACR). Fatty aldehydes can be reduced again to fatty alcohols in a reaction catalyzed by ACR or fatty aldehyde reductase (FAR). Decarbonylation of fatty aldehydes by aldehyde decarbonylase (AID) produces alkanes (when the acyl group R is saturated) or alkenes (if R is unsaturated).

ACC and knock down of β-oxidation pathway, have increased production yields. The adequate election of the thioesterase employed permits to control the length of the fatty acyl chain. Although associated costs are still high and current efficiency does not allow massive production of these biofuels, several fermenting pilot plants have been constructed and started to work recently, showing promising results.

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Lipopolysaccharides (Endotoxins)☆

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Lipopolysaccharide Structure

Lipopolysaccharide (LPS) is an essential component within the outer membranes (OM) of almost all Gram-negative bacteria. The LPS glycolipid is less abundant than glycerophospholipids but approximately 2×10^6 molecules cover ~75% of the cell surface of bacteria like *E. coli*. LPS is often called "endotoxin," a term first introduced in the 19th century to describe the component of Gram-negative bacteria responsible for the pathophysiological phenomena associated with Gram-negative bloodstream infections. Early structural analyses of LPS were driven, in part, by the desire to resolve the identity of the molecule responsible for the endotoxic effect. The development of techniques for the extraction and isolation of LPS by O. Westphal, O. Lüderitz, C. Galanos and colleagues in the 1950s and 1960s represented a key breakthrough and most current methods are still based on the original hot phenol-water and aqueous phenol–chloroform–petroleum ether extraction procedures. This pioneering early work led to the understanding that LPS is responsible for the endotoxic activity, and, equally importantly, that different Gram-negative bacteria possess LPS molecules with similar overall compositions. Biophysical methods (nuclear magnetic resonance spectroscopy and mass spectrometry) now offer high resolution insight into the range of structural diversity.

The prototypical LPS of *E. coli*, *Salmonella enterica* and other members of the family *Enterobacteriaceae* is organized into three distinct structural domains: lipid A, core oligosaccharide (core OS) and O-antigen polysaccharide (OPS) (Fig. 1). This tripartite LPS structure is known as "smooth LPS" (S-LPS), taking its name after the "smooth" or shiny colony morphology displayed by enteric bacteria that have S-LPS on their cell surface. Mutants with defects in OPS or core OS assembly produce truncated LPS molecules but their growth in vitro is unaffected. For example, the widely used *E. coli* K-12 strains carry a defect in OPS biosynthesis. The resulting colonies lack the smooth character, and, therefore, the truncated LPS are widely known as "rough LPS" (R-LPS) (Fig. 1A and B). Preparations of LPS from bacteria that produce S-LPS contain a heterogeneous mixture of molecules with differing OPS chain length and always have a variable amount of truncated R-LPS. This molecular heterogeneity is clearly evident when LPS preparations are examined by SDS-PAGE (Fig. 1B). Some bacteria, particularly mucosal pathogens, naturally lack OPS chains in their LPS. Their LPS contains oligosaccharide extensions attached to various residues within a typical inner core OS, in a form known as lipooligosaccharide (LOS) (Fig. 1A). The role of lipid A in establishing the essential barrier function of the OM places constraints on the extent of its structural variation. In contrast, the outer part of the LPS molecule interacts with environmental factors, such as components of the host immune response and LPS-specific bacteriophages. These selective pressures may have played a significant role in the diversification of outer LPS structures.

While LPS has been considered as a biomarker for Gram-negative bacteria, some bacteria lack this molecule. In *Sphingomonas* sp. glycosphingolipids likely serve as functional replacements for lipid A. The situation in endosymbionts (e.g., *Wolbachia*, *Borrelia* species) is less clear. In LPS-producers, it is generally thought that lipid A is essential for viability. However, the universality of this assumption was challenged by the identification of a viable *Neisseria meningitidis* mutant containing a defect in an essential step in the lipid A biosynthesis pathway. The recent identification of "LPS-less" clinical isolates of *Acinetobacter baumannii* offers fascinating insight; these mutants have elevated contents of surface lipoproteins, suggesting an alternative strategy to form a viable OM.

Lipid A

Despite the challenges imposed by its heterogeneity and amphipathic properties, lipid A structures from many bacterial species have now been resolved, revealing a family of structurally related glycolipids based on common architectural principles. Free lipid A generally does not exist on the surfaces of bacteria, due to constraints within LPS assembly and export processes (see below). *Francisella tularensis* provides an exception, with only small quantities of the total cellular lipid A found in a complete LPS. The prototypical lipid A from *E. coli* possesses a carbohydrate backbone composed of two (β-1,6-linked) glucosamine (GlcN) residues,

☆*Change History*: November 2018. Chris Whitfield and Bradley R Clarke prepared the update. *Modified sections* "Lipopolysaccharide Structure, LipidA, Core Oligosaccharides, O Polysaccharides, Lipooligosaccharides, Biosynthesis and Assembly of Lipopolysaccharides, Biosynthesis of Lipid A-Core OS, Synthesis of O-Polysaccharides." *Added*: "LPS Translocation-MsbA and the Lpt Machine, Functions and Biological Activities of Lipopolysaccharides *changed to* Protective Functions of LPSRole of LPS in Outer Membrane Integrity, O-Polysaccharides as a Protective Barrier" *replaced with* "Resistance to Polycations *and* Protective Roles of O-Polysaccharides" *and* "Molecular Mimicry, Lipopolysaccharide and Gram-negative sepsis *replaced*" *with* "Biological (Endotoxic) Activities of Lipopolysaccharides, Lipid A Modification Systems *and* Phase Variation and Molecular Mimicry in LPS, and LOS *and* Phase Variation and Molecular Mimicry in LPS and LOS" were incorporated into other sections. *Removed* "Biotechnological Applications Involving LPS". Figures: figs 1 and 2 were combined and modified, fig 3 now fig 2 and legend modified, fig 4 now fig 3 and modified, fig 5 now fig 4 and modified, fig 6 now fig 5 and modified, fig 7 now fig 6 and modified, fig 8 now fig 7 and modified, fig 9 removed.

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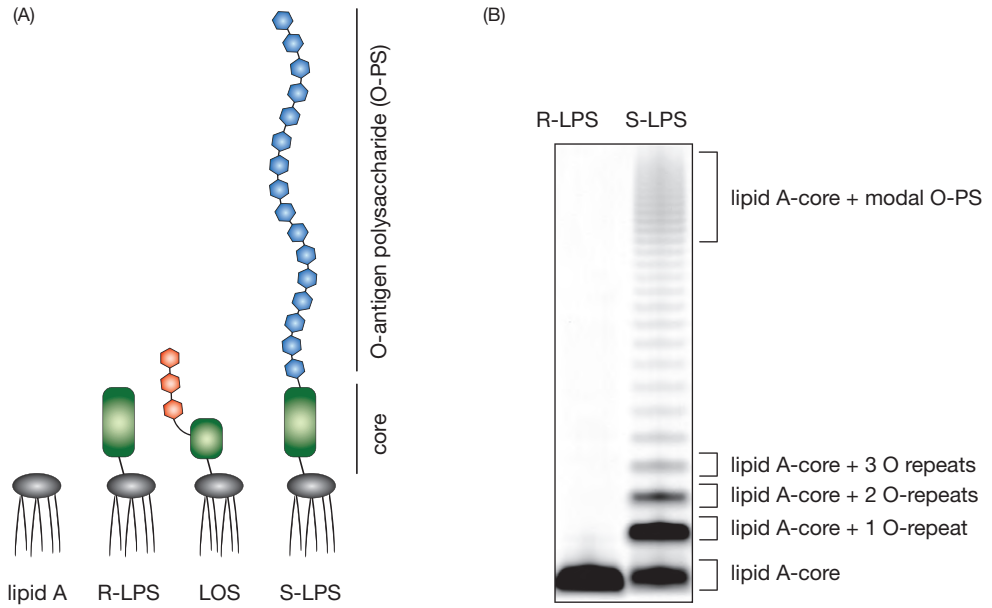

Fig. 1 (A) Schematic diagram showing different forms of LPS molecules. The tripartite S-LPS structure is typical of LPSs produced by members of the *Enterobacteriaceae*, *Pseudomonadaceae* and *Vibrionaceae*. These bacteria also produce a variable amount of R-LPS that lacks OPS and, in some cases, part of the core OS. Mucosal pathogens such as *Neisseria* and *Haemophilus* spp. lack OPS but instead may have phase-variable oligosaccharide extensions attached to the core OS, to form LOS. Only a few bacteria produce free lipid A. (B) Characteristic SDS-polyacrylamide gel profiles of R-LPS and S-LPS preparations.

which are phosphorylated at positions 1- and 4′ (Fig. 2). However, in some cases (e.g., *Brucella abortus*, *Campylobacter jejuni*, *Leptospira interrogans*, *Rhizobium* and *Bradyrhizobium* species), a diamino-derivative (2,3-diamino-2,3-dideoxy-glucose; GlcN3N)) can replace one or both GlcN residues. The backbone is acylated with ester and amide-linked 3-hydroxyl saturated fatty acids (3-OH-C14:0). In *E. coli* and *Salmonella*, the fatty acyl chains on the nonreducing GlcN residue are substituted by nonhydroxylated fatty acids, creating an asymmetric arrangement in the classic hexaacyl structure. However, the precise acylation pattern is species specific (Fig. 2). In some nitrogen-fixing plant symbionts (e.g., *Rhizobium* and *Bradyrhizobium*), the fatty acids of the nonreducing GlcN3N residues are substituted with very long chain fatty acids (VLCFAs; 26–34 carbons). One or both VLCFAs are additionally substituted with hopanoid residues, complex pentacyclic triterpenoid lipids that also occur in the free form in the membranes of these organisms. VLCFAs can span the lipid bilayer and, together with the hopanoids, stabilize the outer membrane and protect against detrimental compounds within the root nodule. Mutants, defective in hopaniod synthesis are unable to persist as symbionts.

Within a given species structural variants arise due to modifications such as the addition of sugars including 4-aminoarabinose (Ara4N), galacturonic acid (GalA), GlcN, hexoses (mannose) and phosphoryl ethanolamine (PEtN) to the phosphate residues. Additional modifications include addition or removal of acyl chains. These modifications are constitutive in some bacteria but, in others, they are the result of complex environmental regulation. The additional glycoforms are not essential for viability in the laboratory but play important roles in host-bacterium interactions.

Core Oligosaccharides

For the purpose of discussion of structure-function relationships, the core OS is often conceptually divided into inner and outer core regions (Figs. 1 and 3). In all known LPS structures, α-linked 3-deoxy-D-*manno*-octulosonic acid (Kdo) or variants including, D-*glycero*-D-*talo*-octulosonic acid or 8-amino-3,8-dideoxy-Kdo, provide the linking sugar between the 6′-position of lipid A and the rest of the carbohydrate backbone. While Kdo is generally considered to be a diagnostic marker of LPS, this sugar is also now known to be present in some OPSs and (in its β-linked form) in a linker attaching a terminal lipid to some capsular polysaccharides in Gram-negative bacteria. Another common component of the inner core is L-*glycero*-D-*manno*-heptose (Hep). In the *Enterobacteriaceae*, the base inner core region is highly conserved and the phosphorylated glycan backbone is nonstoichiometrically modified by PEtN and additional Kdo and rhamnose residues (Fig. 3). In contrast, the outer core shows more diversity; for example, there are 5 outer core OS structures in *E. coli* reflecting variations in glycose components and linkages. In most cases, the core OS carries a net negative charge that is important for its function in establishing a stable OM permeability barrier (see section "Protective Functions of LPS"). The charge is contributed by the carboxyl groups of Kdo and phosphate in *E. coli* and *Salmonella* but galacturonic acid residues (GalA) achieve this role in *K. pneumoniae*.

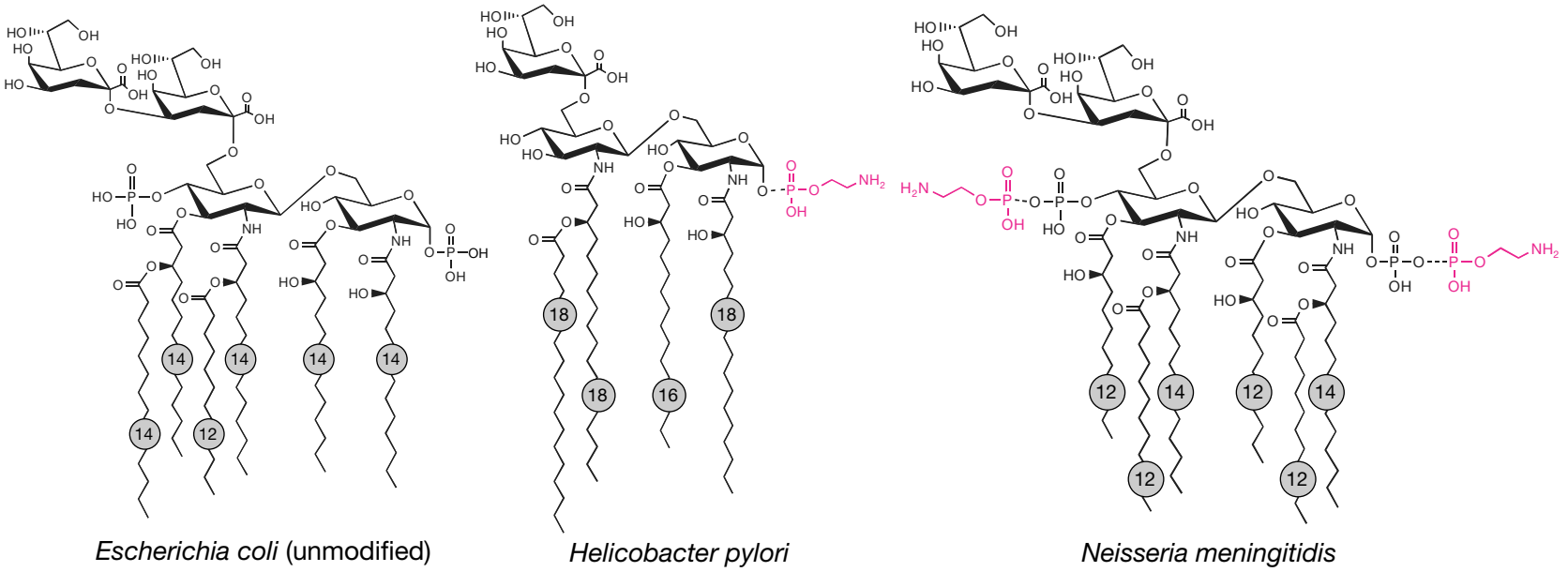

Fig. 2 Structural diversity of representative lipid A molecules found in pathogenic bacteria. The major lipid A species from *Helicobacter pylori* and *Neisseria meningitidis* are compared to the unmodified "basic" lipid A of *Escherichia coli* K-12. Regulated covalent modifications (*red*) are indicated with dashed bonds. The lengths of the fatty acyl chains are indicated with the numerals enclosed by *circles*.

O Polysaccharides

OPS is the most variable portion of the LPS molecule. A remarkable array of novel structures arises from alterations in constituent sugars, linkages, and both complete and partial substitutions with nonsugar residues. The simplest OPSs contain a repeat unit with a single monosaccharide constituent and a single glycosidic linkage type. Complex homopolysaccharides can result from larger repeat units defined by a specific sequence of glycosidic linkages. At their most complex, OPSs can be heteropolysaccharides in which the repeat units contain several component sugars, together with nonsugar substituents, such as *O*-acetyl groups and amino acids. The remarkable diversity in OPS structures has been exploited in serological classification of isolates from a given bacterial species. In *E. coli*, there are approaching 200 distinct O serogroups. While the LPSs from most bacterial strains tend to have a single OPS type in the same clone, there are a growing number of bacteria being identified for which the lipid A-core serves as an acceptor for two or more different polymeric structures that are coexpressed. The length of the OPS attached to lipid A-core is heterogeneous but the distribution of chain lengths is both strain- and growth-condition dependent. This is best reflected in the patterns of LPS molecules revealed by SDS-PAGE analysis (Fig. 1B).

Lipooligosaccharides

The LOS of many mucosal pathogens is smaller than S-LPS (Figs. 1 and 3). An example of the LOS structure from *Neisseria meningitidis* is given in Fig. 3. The oligosaccharide chains linked to the inner core OS provide distinct antigenic epitopes giving rise to seven (L1-L7) immunotypes. A common feature in these bacteria is the phenomenon of genetic phase variation, where the LOS epitopes are differentially expressed. The resulting structures are subject to intense selective pressures in the host and can significantly influence host-pathogen interactions. Phase variation of LOS is well studied in mucosal pathogens such as *Neisseria* spp., *Campylobacter jejuni*, and *Haemophilus influenzae*. For example, the LOS of *N. gonorrhoeae* isolates typically contain low amounts

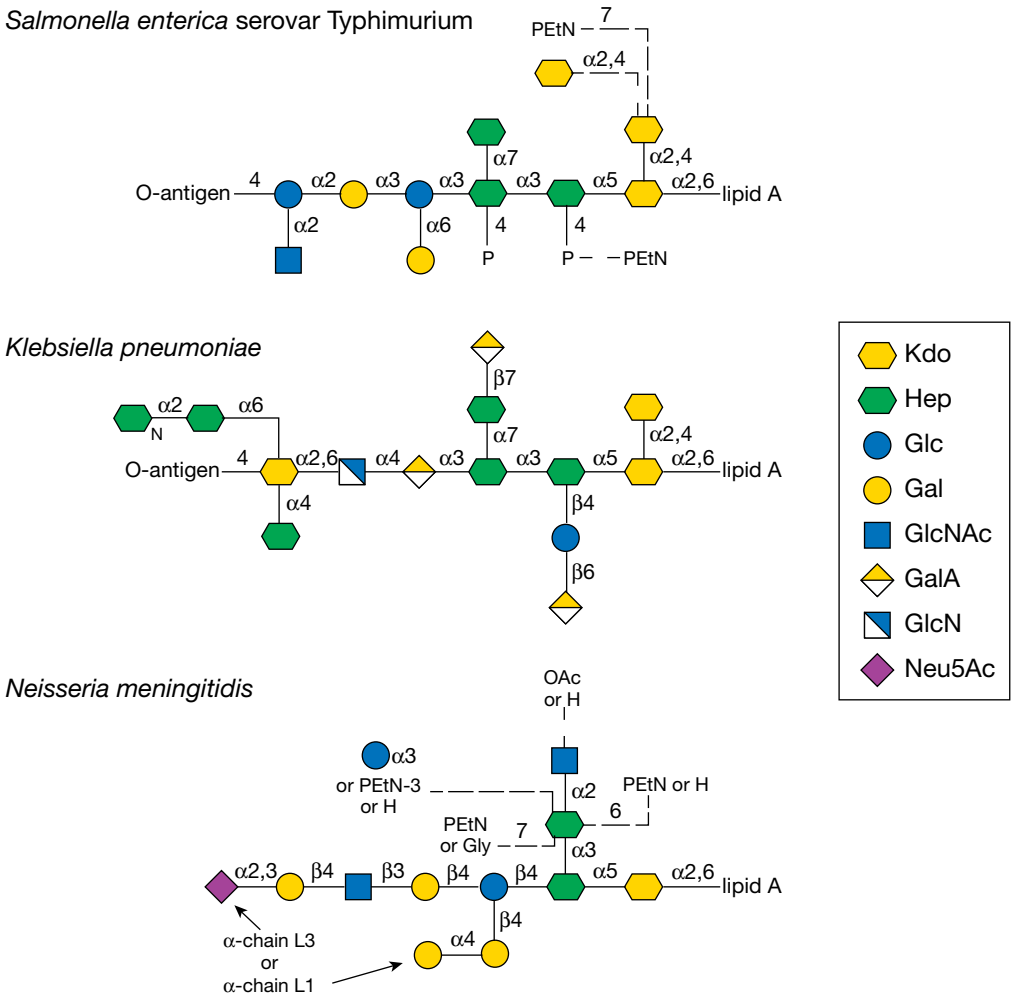

Fig. 3 Structural relationships in the core oligosaccharides from *Salmonella enterica* serovar Typhimurium and *Klebsiella pneumoniae* and the lipooligosaccharide of *Neisseria meningitidis*. The structures are represented using the graphical symbol nomenclature. *Dashed lines* indicate variable modifications.

of sialic acid, and this is important for entry of the bacterium into human mucosal epithelial cells. In contrast, variants with highly sialylated LPS resist killing by complement and antibodies but fail to enter the epithelial cells. The complex glycoforms preclude precise determination of LOS structures unless phase-locked variants are available. Some bacteria (e.g., *Bordetella* spp.) can form both S-LPS and LOS.

Biosynthesis and Assembly of Lipopolysaccharides

As might be anticipated from the structure of LPS, the processes involved in LPS biosynthesis are complex (Fig. 4A). A further complication arises from the fact that most LPS precursors are found in the cytoplasm and the completed molecule must be assembled in a process that traverses the inner membrane, the periplasm, the peptidoglycan layer and the outer membrane. In overview, lipid A-core is synthesized in consecutive steps and is then exported across the inner membrane by MsbA, an ATP-binding cassette (ABC) transporter. OPS is assembled on the lipid acceptor, undecaprenol-phosphate (und-P; the same carrier used in peptidoglycan and teichoic acid biosynthesis), and becomes available at the periplasmic face of the inner membrane in the und-PP-linked form. The LPS molecule is completed by transfer of the OPS to the lipid A-core before its translocation to the cell surface by the multiprotein Lpt machine. These processes establish a basal LPS structure but a variety of postsynthetic reactions can remodel these products in order to fit a particular lifestyle or respond to changing environmental cues. These remodeling reactions are described later in the context of their impact on LPS properties and functions (see sections "Protective Functions of LPS" and "Biological (Endotoxic) Activities of LPS").

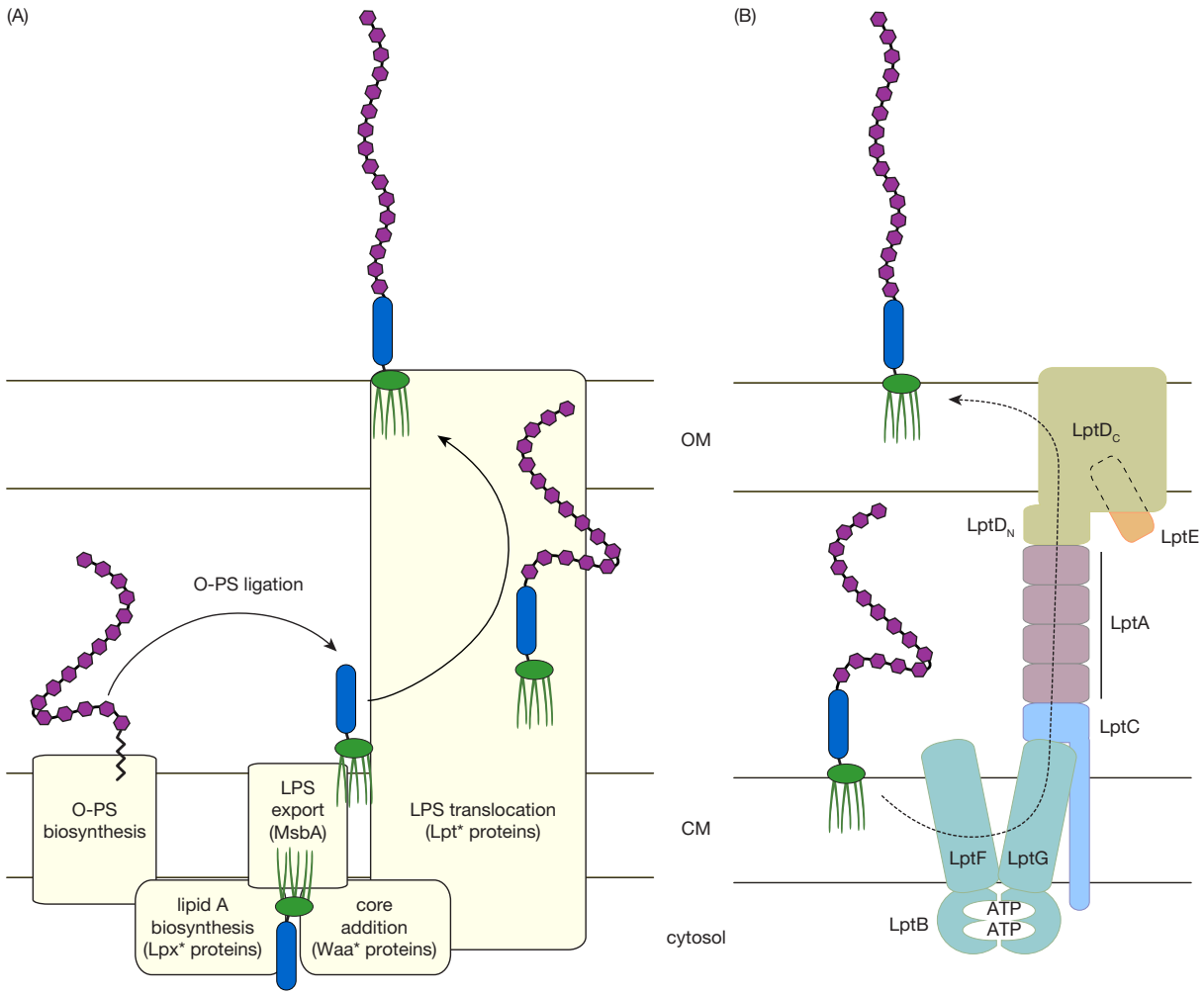

Fig. 4 (A) Overview of LPS biosynthesis. The lipid A-core domain is synthesized at the cytoplasmic face of the inner membrane and is exported to the periplasm by MsbA, an ABC transporter protein. The O-polysaccharide (O-antigen) is assembled separately on a lipid carrier (undecaprenol-phosphate) and is ligated to lipid A-core by WaaL in the periplasm. Both completed S-LPS molecules and R-LPS molecules are transported to the cell surface by the envelope-spanning Lpt protein complex. (B) The transenvelope Lpt complex. Lpt proteins extract nascent LPS molecules from the cytoplasmic membrane and translocate them via a periplasmic scaffold to the outer membrane. The translocation process is driven by ATP hydrolysis.

Biosynthesis of Lipid A-Core OS

The pathway for the biosynthesis of lipid A was established through systematic efforts in the laboratory of Chris Raetz; the pathway is widely referred to as the "Raetz pathway" (Fig. 5). The nine Lpx enzymes of the constitutive Raetz pathway are highly conserved and a single copy of each *lpx* gene can be found in the genome of most Gram-negative bacteria and, interestingly, in some plants. The biochemistry of each step is resolved and structures are available for the key enzymes. The pathway begins in the cytoplasm of the cell with the reversible acylation of the sugar nucleotide UDP-*N*-acetylglucosamine (UDP-GlcNAc), catalyzed by LpxA. UDP-GlcNAc is also a precursor for peptidoglycan biosynthesis. The second (and first committed) step of the pathway, catalyzed by LpxC, is well-regulated. LpxC is a metalloamidase with a unique structure, making it an attractive target for drug discovery. Hydroxamate compounds (e.g., CHIR-090; *N*-aroyl-L-threonine hydroxamic acid) are potent inhibitors. LpxC-catalyzed deacetylation allows the addition of a second fatty acyl chain catalyzed by LpxD, forming UDP-2,3-diacylglucosamine. LpxH then cleaves the pyrophosphate bond of the UDP-2,3-diacylglucosamine intermediate to yield UMP and lipid X. Elucidation of the reaction sequence was made possible by the initial discovery of this intermediate, more than 20 years ago. The disaccharide synthase, LpxB, forms the characteristic β-(1,6) glycosidic linkage in the disaccharide backbone and is followed by phosphorylation at the 4′-position by a specific kinase, LpxK, to generate lipid IV_A, a key lipid A precursor. A distinctive feature of the Gram-negative bacterial LPS is the presence of Kdo at the 6′-position of the disaccharide backbone. In *E. coli* and *Salmonella*, transfer of the Kdo sugars is catalyzed by the bifunctional Kdo transferase, WaaA. The Kdo transferase from other organisms can be monofunctional (e.g., *Haemophilus* and *Vibrio* species) or trifunctional (e.g., *Chlamydia* species). In *E. coli* and *Salmonella*, the synthesis of Kdo_2-lipid A (Fig. 5) is completed by the addition of two fatty acyl chains by the so-called "late" or secondary acyltransferases, LpxL (lauroyl, C12) and LpxM (myristoyl, C14).

The completed Kdo_2-lipid A provides an acceptor for glycosyltransferases that act sequentially to assemble the core OS, as well as those that add the oligosaccharide chains of LOSs. Assignments of glycosyltransferases that synthesize core OS and enzymes such as kinases that modify the backbone have primarily been made by approaches where specific genes are individually mutated and the resulting LPS structure is resolved by chemical and physical analysis. In some cases, these mutations have complex effects since they alter the structure of the acceptors for additional enzymes, that is, a single mutation can give a phenotype suggesting multiple defects. For some enzymes detailed biochemical properties and solved structures are now available.

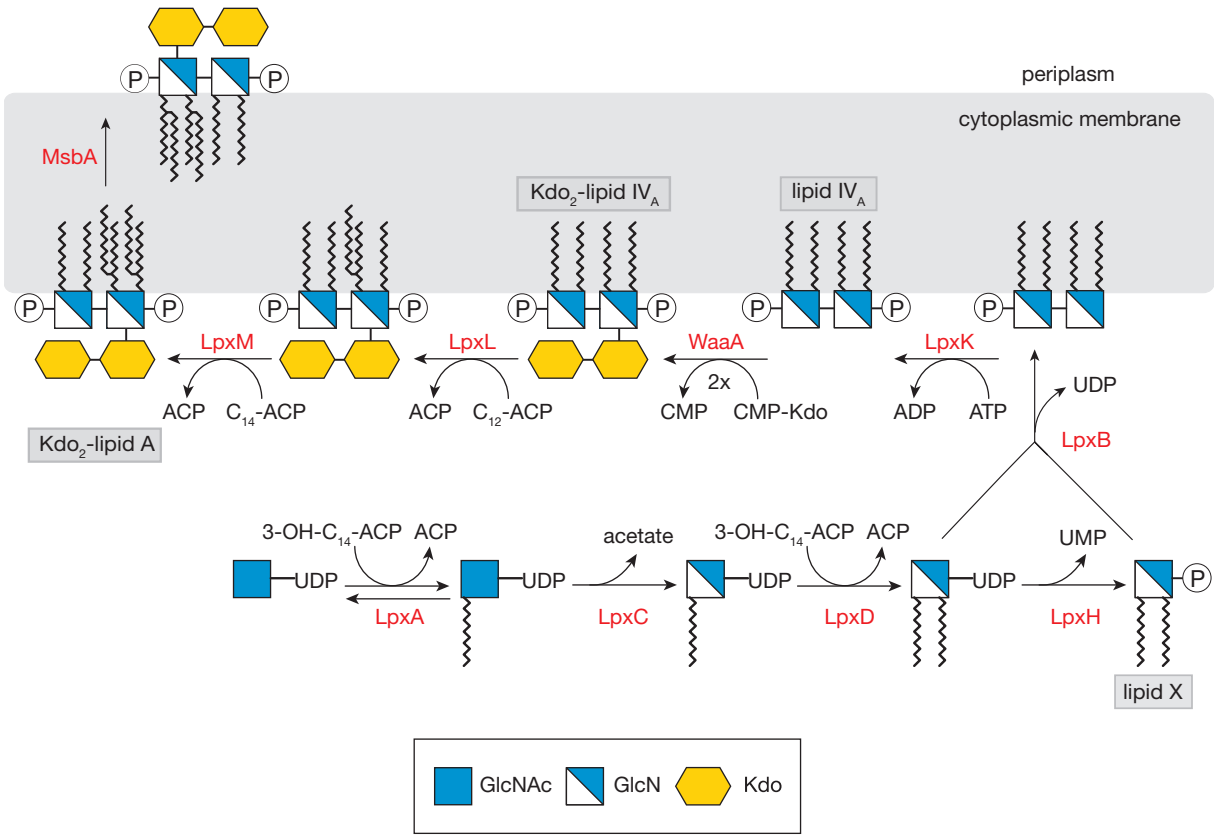

Fig. 5 The pathway for biosynthesis of the Kdo_2-lipid A domain of LPS in *Escherichia coli* K-12. The enzymes (*red*) responsible for each step in the pathway are indicated. All have been identified by genetic and biochemical approaches. The completed Kdo_2-lipid A can serve as an acceptor for sequential assembly of the core oligosaccharide at the inner face of the cytoplasmic membrane. Acyl-acyl carrier proteins (ACP) are the obligate donors for acylation in the pathway. The ABC transporter MsbA is responsible for exporting the complete Kdo_2-lipid A (or core-lipid A) molecule across the cytoplasmic membrane.

Synthesis of O-Polysaccharides

Despite the diversity in OPS structures, only three mechanisms are known for their assembly. OPS synthesis begins at the cytoplasmic face of the inner membrane with activated precursors (sugar nucleotides; NDP-sugars) and the process terminates with a nascent OPS at the periplasmic face (Fig. 4A). Undecaprenol phosphate (und-P) is an obligatory carrier in all three OPS assembly pathways. As a result, all are initiated by polyprenol phosphate phosphoglycosyltransferases (PGTs) that transfer a sugar-1-phosphate from UDP-hexose or UDP-acetamidohexose donors to Und-P. The ligase (WaaL) from *E. coli* K-12 will ligate structurally distinct OPSs formed by any pathway, indicating that the form in which nascent OPS is presented for ligation is conserved. The three pathways for assembly of O antigens vary in the components required for polymerization, in the cellular location of the polymerization reaction, and in the manner in which material is exported across the inner membrane (Fig. 6). Similar mechanisms are identified in the biosynthesis of capsular polysaccharides in Gram-negative and Gram-positive bacteria.

The earliest known pathway for OPS synthesis is distinguished by the involvement of the putative "OPS polymerase" enzyme, Wzy. The "Wzx-Wzy-dependent" system (Fig. 6) is the classical pathway first described in *S. enterica* in the 1960s by H. Nikaido, M.J. Osborn, P. Robbins, A. Wright and others. The OPSs synthesized by this pathway are all heteropolymers and often have branched repeating unit structures. Und-PP-linked O-repeat units are assembled by glycosyltransferase enzymes at the cytoplasmic face of the inner membrane by the sequential action of an integral membrane PGT and additional peripheral glycosyltransferases. The Wzy polymerization reaction occurs at the periplasmic face of the membrane and utilizes und-PP-linked OPS-repeat units as the substrate. These are supplied by an integral membrane protein transporter, Wzx, a member of the Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) family. Polymerization of the OPS repeat units minimally involves the putative polymerase (Wzy) and the OPS chain length regulator (Wzz) and genetic data suggest critical interactions between Wzx, Wzy and Wzz. Polymerization occurs in a block-wise process where the growing glycan is transferred from its lipid carrier to the newly exported und-PP-linked repeat unit. A *wzy* mutant is unable to polymerize OPS and its LPS comprises a single O-repeat unit attached to lipid A-core (sometimes called "semirough" SR-LPS). In contrast, a *wzz* mutant makes S-LPS but loses the characteristic modal distribution (Fig. 1B) of OPS-chain lengths evident in SDS-PAGE analysis. The presence of additional Wzz proteins in some bacteria can generate LPS species with multiple discrete clusters of O-PS sizes.

In the "ABC-transporter-dependent" pathway (Fig. 6) the OPS is synthesized exclusively inside the cytoplasm and once complete, it is exported to the periplasm via an ATP-binding-cassette (ABC) transporter. OPSs from this pathway typically lack glycose side branches, although these can be added later (see section "Protective Roles of O Polysaccharides"). As in the Wzy-dependent pathway, synthesis is initiated at the cytoplasmic face of the inner membrane by a PGT enzyme and peripheral glycosyltransferases then act sequentially to elongate the und-PP-linked intermediate at the nonreducing terminus to form a fully polymerized und-PP-OPS. In some examples, polymerization is terminated by the addition of a residue that caps the chain. In such cases, the assembly system employs an extended coiled-coil structure that separates the capping enzyme from the polymerizing enzymes. This serves as a molecular ruler to establish OPS chain length. The cognate ABC transporter possesses a carbohydrate-binding module that selects only capped chains for export. In systems lacking the capping strategy, chain-length determination is less precise and is dictated by protein: protein interactions involving the biosynthesis machinery and the ABC transporter. The mechanism by which an ABC transporter can translocate a long chain OPS linked to und-PP across the cytoplasmic membrane is intriguing. The recent structure of a putative O-antigen ABC transporter suggests a processive model in which multiple rounds of

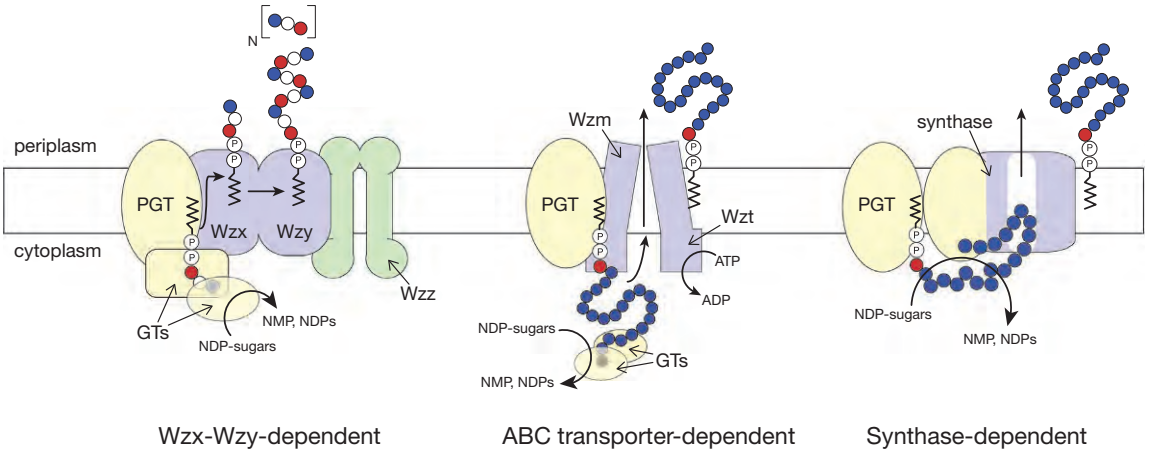

Fig. 6 The three known pathways for the biosynthesis of O-polysaccharide (OPS). Each of the assembly systems initiate at the cytoplasmic face of the inner membrane with an undecaprenyl pyrophosphoryl (und-PP)-linked intermediate. The initiating reaction proceeds by addition of a sugar-1-phosphate from a nucleoside diphosphosugar (NDP-sugar) and is catalyzed by a phosphoglycosyltransferase (PGT). Subsequent additions by glycosyltransferases (GT) involve only the sugar residues from NDP-sugars. The enzyme complexes include both integral and peripheral membrane proteins and key components are indicated by name. The pathways differ in the mechanism and location of O-PS polymerization and in the manner by which OPS (or O-repeat units) are transferred across the cytoplasmic membrane. Termination of OPS synthesis occurs with ligation of the nascent OPS to preformed lipid A-core OS at the periplasmic face of the membrane.

ATP binding and hydrolysis are required to complete the task. A model based on PglK, a bacterial ABC transporter of Und-PP-oligosaccharides for protein N-glycosylation proposed only the glycan enters the central cavity of the transporter complex and the same is likely true for OPS.

The "synthase-dependent" pathway for OPS biosynthesis is, so far, confined to the one homopolymeric O antigen (factor 54) of *S. enterica* serovar Borreze. The model for this pathway (Fig. 6) proposes that the initiating PGT forms an und-PP-sugar acceptor that is elongated and exported by a single multifunctional synthase enzyme, in a manner analogous to cellulose synthases and hyaluronan synthases.

LPS Translocation—MsbA and the Lpt Machine

Export of lipid A–core across the membrane to the periplasm is achieved by the conserved ABC transporter, MsbA. MsbA is essential for viability in *E. coli* and depletion of its activity in temperature-sensitive mutants results in the accumulation of Kdo_2-lipid A and glycerophospholipids in the inner membrane. Crystal structures of MsbA transporters from several species are available but a key question remains—how does MsbA handle an amphipathic export substrate; that is, what is the transport solution for a molecule with a highly hydrophobic acylated domain and a polar carbohydrate backbone? MsbA is highly selective for the hexaacylated LPS forms, but full acylation only occurs after the addition of 2 Kdo residues to a tetraacyl lipid A in *E. coli*. Thus, defects in Kdo addition are manifested as blocks in LPS export and the growth defects can be overcome by compensatory mutations in MsbA. MsbA specificity therefore defines the minimal LPS structure required for growth of *E. coli* and *Salmonella*; Kdo_2-lipid A (sometimes referred to as chemotype *Re*-LPS). In other bacteria where the order of Kdo addition and secondary acylation is not conserved, the minimal structure may differ.

Translocation of LPS to the cell surface is completed by the LPS transport machine, composed in *E. coli* of seven conserved proteins that form subcomplexes in the inner and outer membranes with a connecting bridge (Fig. 4B). The functional complex and stable bridge have been reconstituted in proteoliposomes, resolving any lingering debate about whether transport across the periplasm involved a contiguous structure or soluble chaperones. LptBFG form an ABC-transporter complex that appears to extract LPS molecules from the cytoplasmic membrane. The LPS is then passed to the outer membrane via a bridge composed of the periplasmic domain of LptC, several copies of LptA, and an N-terminal domain of LptD. These proteins/domains share a common β-jellyroll fold, where the acyl chains of the LPS molecule are sequestered from the aqueous periplasm between the two β-sheets of each component. At the OM, the LPS enters the LPS translocon (LptDE) which possesses a lateral gate to allow the release of the LPS into the outer leaflet of the OM. The unidirectional translocation system is driven by ATP turnover by the ABC protein complex with successive LPS molecules pushing their predecessors through the conduit in a model analogous to a PEZ candy dispenser.

Protective Functions of LPS

LPS is a crucial component of the OM and is well known for its major role in establishing a permeability barrier. However, each part of the LPS molecule performs protective functions against different environmental factors.

Role of LPS in the Outer Membrane Integrity

OM asymmetry (i.e., LPS in the outer leaflet and glycerophospholipids in the inner leaflet) is essential to the barrier properties. When this is not achieved, and glycerophospholipids migrate to the outer leaflet, the resulting areas of the outer membrane become permeable to large hydrophobic antibiotics, detergents such as SDS and bile salts (sodium cholate and deoxycholate) that normally only affect Gram-positive bacteria. Secondary acyl chains are implicated in maintaining a low degree of fluidity, a condition that is critical to outer membrane function. Although secondary acyl chains are not essential for viability, under-acylation of lipid A often results in growth defects. The normal tight packing of saturated acyl chains induces a network of hydrophobic interactions that help maintain the integrity of the outer leaflet of the outer membrane through van der Waal's forces. Stability also involves lateral interactions between adjacent LPS molecules and between LPS and OM proteins. These interactions are dependent on negatively charged elements, including the terminal phosphates of lipid A, core OS phosphates and carboxyl groups from residues such as Kdo or galacturonic acid. These may participate in divalent cation bridges that minimize electrostatic repulsion while promoting strong lateral interactions. An illustration of the importance of these lateral interactions is provided by "deep-rough" mutants of *E. coli* and *Salmonella*, which show hypersensitivity to hydrophobic compounds and other physiological changes. The phenotype is conferred by loss of core OS heptose and loss of phosphorylation catalyzed by the WaaP kinase. In *Pseudomonas aeruginosa*, addition of core OS phosphates is essential for viability and nonphosphorylated molecules are not efficient substrates for the Lpt system. Core OS phosphorylation may provide further avenues for therapeutic intervention.

Resistance to Polycations

Polycations including host-produced cationic antimicrobial polypeptides (CAMPs) and antibiotics such as polymyxin B and colistin exploit negative charges in the LPS structure to breach the outer membrane. As a result, bacteria exploit a variety of postsynthetic strategies to reduce the charge content and several examples are now well documented. Many changes simultaneously

result in alterations in endotoxic activity (see section "Biological (Endotoxic) Activities of Lipopolysaccharides"). Some modifications are constitutive. At a simplest level, some bacteria reduce the complexity of their LPS species. While the Raetz pathway and the specificity of MsbA imposes a minimal structural requirement for export (Kdo_2-lipid A), *Helicobacter pylori* removes two acyl chains and the phosphate groups to protect against CAMPs. In *F. tularensis*, Kdo is removed to yield free lipid A. A different constitutive modification occurs in *Vibrio cholerae* O1, where the classical and El Tor biotypes are distinguished by the resistance of El Tor to polymyxin B. This is mediated by glycine (or diglycine) moieties added to the acyl chain at the 3′ position of lipid A by a process resembling nonribosomal peptide synthetases.

In other bacteria, exposure to polycations results in a highly-regulated LPS remodeling response. *S. enterica* provides a classic example and has contributed to much of our understanding of lipid A remodeling processes in general. The PhoP/Q and PmrA/B two-component phosphorelay regulatory systems respond to environmental cues (e.g., low Mg^{2+} and CAMPs, or mildly acidic pH, Fe^{3+}, respectively) and transcriptionally regulate a variety of remodeling genes. Many changes involve the acylation pattern. For example, PhoP/Q signaling upregulates PagP, an outer membrane protein that transfers a palmitoyl group from glycerophospholipid to the acyl chain at position 2 on lipid A. PagP is important for CAMP resistance and PagP homologues are essential for virulence of a variety of bacteria. In addition, PhoP/Q-regulated deacylases (PagL and LpxR) remove ester-linked fatty acids. In *Klebsiella pneumoniae*, a common pathogen of immunocompromised individuals, LPS is modified in a tissue-specific manner. In the lungs only, LpxO adds a 2-hydroxyacyl moiety on the lipid A to confer CAMP resistance and this is accompanied by resistance to colistin, a drug of last resort for treating multidrug resistant *K. pneumoniae*. Addition of colistin to growth media triggers this lipid A modification outside the host. One of the best characterized modifications in *S. enterica* is the addition of Ara4N to the lipid A phosphates. As this modification occurs in the periplasm, the direct glycosylation donor is not a nucleotide diphosphosugar. Instead, a three-enzyme system synthesizes Und-P-Ara4N, exports it to the periplasm, and then transfers the Ara4N to lipid A. Interestingly, the same modification is constitutive in *Burkholderia cepacia*, where the specificity of the LptBFG complex ensures that only appropriately modified LPS species are translocated to the surface. In *Francisella* and *Bordetella* species, a comparable modification process adds galactosamine and GlcN, respectively. These examples illustrate the varied applications of related processes in different pathogens. Another common LPS modification is the addition of PEtN to lipid A and Kdo residues, or (in *Campylobacter jejuni*) to Hep. The enzymes in *S. enterica* are differentially regulated. This addition is particularly important for CAMP resistance in *N. meningitidis*, which cannot add Ara4N. Plasmid-encoded resistance to the last resort drug, colistin, is major healthcare threat. The MCR-1 enzyme responsible for resistance is a PEtN transferase that modifies lipid A.

Protective Roles of O-Polysaccharides

Molecular modeling of LPS structure and its organization in the outer membrane suggests that the OPS lies partially flat on the cell surface, where the crossover of multiple chains forms a "felt-like" network. Since OPS chains are flexible, they can also extend a significant distance from the surface of the outer membrane and cryo-electron microscopy reveals a significant OPS layer on the cell surface. It is therefore not surprising that many properties attributed to the OPS are protective. These are particularly important for pathogens to withstand the effects of host innate immune components including complement and bactericidal/permeability-inducing protein (BPI), an antibacterial product found in polymorphonuclear leukocyte-rich inflammatory exudates. BPI has multiple roles including lysis of bacterial cells, neutralizing LPS for clearance, and opsonization of bacteria for phagocytosis by neutrophils. OPS chain length plays a key role in resistance to both complement and BPI. Serum proteins in the complement pathway interact to form a membrane attack complex (MAC) that can integrate into lipid bilayers, leading to cell death. MACs can be formed via a "classical" pathway, initiated by surface antigen-antibody complexes, or by the "alternative" pathway, where the complement component C3b interacts directly with the cell surface in the absence of antibody. S-LPS does not prevent C3b deposition but the longest OPS chains preferentially bind C3b, so the resulting MAC is unable to insert into the OM. In addition to OPS chain length, complement-resistance is influenced by the extent of coverage of the available lipid A core with OPS. As is often the case, there are exceptions to such generalizations. For example, there are some *E. coli* strains with S-LPS that are serum-sensitive unless an additional capsular polysaccharide layer is present. Although R-LPS variants of *E. coli* and *Salmonella* are almost invariably serum-sensitive, other bacteria (including many with LOS) use alternate strategies to achieve resistance.

OPS can also mask surface molecules used as receptors by bacteriophages. However, OPS themselves provide receptors for some phages and this may be one of the biggest drivers of OPS diversity. In some bacteria, OPS structure is modified by activities encoded by lysogenic phages, presumably to prevent superinfection. A range of modifications have been recognized including acetylation, addition of PEtN and glycosylation. The glycosylation processes are well documented in members of the genera *Escherichia*, *Klebsiella*, *Salmonella* and *Shigella*. In these bacteria, three-enzyme modification systems use Und-P-Glc or Und-P-Gal to modify OPS in the periplasm in processes analogous to Ara4N modification of lipid A. The acceptors for modification are Und-PP-OPS molecules produced in Wzy- or ABC-transporter dependent pathways.

Molecular Mimicry

Molecular mimicry, a process where LPS substructures are identical or very similar to host glycans, is frequent in LPS and LOS. For example, the O-chain of *H. pylori* LPS contains fucosylated OS and Lewis antigen epitopes, which mimic human blood group antigens. Variable expression of these epitopes facilitates evasion of the host immune response and influences autoimmune responses, cell adhesion, and long-term colonization of the bacteria. The LOS of *C. jejuni* and *Brucella melitensis* contain

phase-variable ganglioside mimics and these may again provide a mechanism for avoiding the host immune response. However, they are also implicated in the development of an autoimmune response that underlies Guillain-Barré syndrome.

Biological (Endotoxic) Activities of Lipopolysaccharides

Mammals have evolved innate mechanisms for detecting and clearing microorganisms. These processes detect conserved microorganism-associated molecular patterns (MAMP) and elicit an immune response and bacterial killing through the production of inflammatory mediators such as tumor necrosis factor (TNF-α), interferons, and interleukins. However, continued stimulation of this response by invading pathogens can lead to life-threatening sepsis which is often manifested by fever and hypotension and can lead to disseminated intravascular coagulation and organ failure. In the United States, Gram-negative sepsis accounts for nearly 200,000 deaths each year. LPS (endotoxin) is the predominant MAMP exhibited by Gram-negative bacteria. Since free LPS is required to initiate the inflammatory process, treatment with antibiotics and the ensuing bacterial lysis can exacerbate the problem.

The key elements of the complex system by which mammalian cells respond to LPS have been identified (Fig. 7). Circulating LPS interacts with a variety of LPS-binding proteins including BPI and LPS-binding protein (LBP). LBP is an acute phase protein which delivers LPS to cell surface receptors to initiate signaling for production of inflammatory mediators. The signaling is initiated by LPS binding to CD14; CD14-knockout mice are 10,000-fold less sensitive to LPS in vivo. CD14 occurs as a glycosylphosphatidylinositol (GPI)-anchored glycoprotein attached to the membrane (mCD14) but nonmyeloid (endothelial and epithelial) cells respond to LPS via a soluble form of CD14 (sCD14). CD14 has a hydrophobic pocket that accommodates the acyl chains of lipid A. The slow and rate-limiting binding kinetics is overcome by LBP delivering LPS to CD14. LPS is then transferred to a cell-surface signaling complex of Toll-like receptor 4 (TLR-4) and myeloid differentiation factor 2 (MD-2). These proteins are both essential for LPS responsiveness. A crystal structure of TLR-4/MD-2 complexed with *E. coli* LPS has been solved. TLR-4 and MD-2 form a heterodimer and LPS binding induces an additional interaction between two TLR-4/MD-2 complexes.

TLR-4/MD-2 can promote two different signaling responses to LPS depending on lipid A structure and the source of the cells; mouse and human cells may respond differently to a given LPS. An immediate and strong inflammation response occurs through the myeloid differentiation primary response protein 88 (MyD88) pathway, leading to production of pro-inflammatory cytokines such as TNF, IL-6, and IL-12. The second response occurs after endocytosis of the TLR-4/MD-2 complex and involves the TRAM (TRIF-related adaptor molecule) and TRIF (TIR domain-containing adaptor inducing IFN) proteins in a pathway resulting in production of type I interferon which induces genes involved in a milder inflammatory response. Phosphorylated hexa-acylated lipid A signals both TLR-4-dependent responses. However, the LPSs of numerous pathogens such as, *Helicobacter pylori*, *Yersinia pestis*, *Francisella tularensis*, *Legionella pneumophila*, *Porphyromonas gingivalis*, and *Chlamydia trachomatis* all have lower biological activities. The ability of these pathogens to cause severe disease in humans may be due, in part, to their ability to preferentially promote TRAM/TRIF-dependent inflammation or to circumvent TLR-4 signaling altogether. These alternative biological effects may be a result of variations in binding properties of lipid A to the TLR-4/MD-2 complex and the ability to promote dimerization of the signaling complex.

The structure of lipid A generally dictates the biological (endotoxic) properties of LPS. Importantly, partial structures (including lipid IV_A) and some naturally occurring LPS are biologically inactive and can act as antagonists, abrogating the biological effects of "toxic" LPS. Understanding the structure-activity relationships, and the underlying biosynthetic foundation, affords the possibility of generating LPS species with predicted effects for vaccine adjuvants and therapeutics. The key structural principles underlying biological activity have been resolved. In the toxic hexaacyl lipid A of *E. coli*, the phosphates and the position and lengths of acyl chains are critical. MD-2 accommodates five of the acyl chains of lipid A with the sixth chain interacting with TLR-4. This is important for receptor dimerization. In contrast, lipid A molecules that show low biological activity typically possess only four or five acyl chains, some which are 16–18 carbons in length, and they contain only one phosphate group attached to lipid A. However, the presence of fewer acyl chains is not an absolute signal of reduced biological activity. In *B. caenocepacia* the Ara4N modification on lipid A allows exposure of the fifth acyl chain to promote receptor dimerization and a similar principle may underpin the enhanced activity of GlcN-modified lipid A from *B. pertussis*. While the lipid A moiety alone is usually responsible for TLR-4/MD-2 binding and activation, other regions of the LPS molecule are important for inducing inflammation in some bacteria. *Capnocytophaga canimorsus* is an oral commensal in dogs but can cause severe sepsis in humans. Its lipid A-core molecule is 20,000-fold more toxic than free (pentaacyl) lipid A and the carboxylate group of the core OS Kdo residue is critical for MD-2 interaction. Lipid A of *Brucella melitensis* is a poor agonist of the TLR-4/MD-2 pathway. The core OS contains a GlcN-rich side branch that is proposed to shield the lipid A portion and prevent efficient binding to the host cells. LPS lacking the side branch showed enhanced pro-inflammatory responses and reduced the ability of the bacteria to survive in bone marrow dendritic cells. Certain OPS structures (e.g., polymannose OPS of some isolates of *Hafnia alvei* and *E. coli*) also influence cytokine production. In this case, the OPS binds to Dectin-2 on the surface of myeloid cells and enhances the TLR-4/MD-2 response to lipid A. This synergy was only observed using complex S-LPS and was attributed to juxtaposition of Dectin-2 and TLR-4 following LPS binding.

Most studies have focused on the effect of LPS from a single organism. However, recent studies have reinforced the need to consider collateral effects in the microbiome. LPS from gut bacteria is generally considered to produce a strong TLR4-dependent response but recent work has shown that total LPS from gut populations is immunosuppressive. The underacylated lipid A structure in the *Bacteroidales* is believed to be responsible for immune silencing in the gut microbiome, to facilitate tolerance

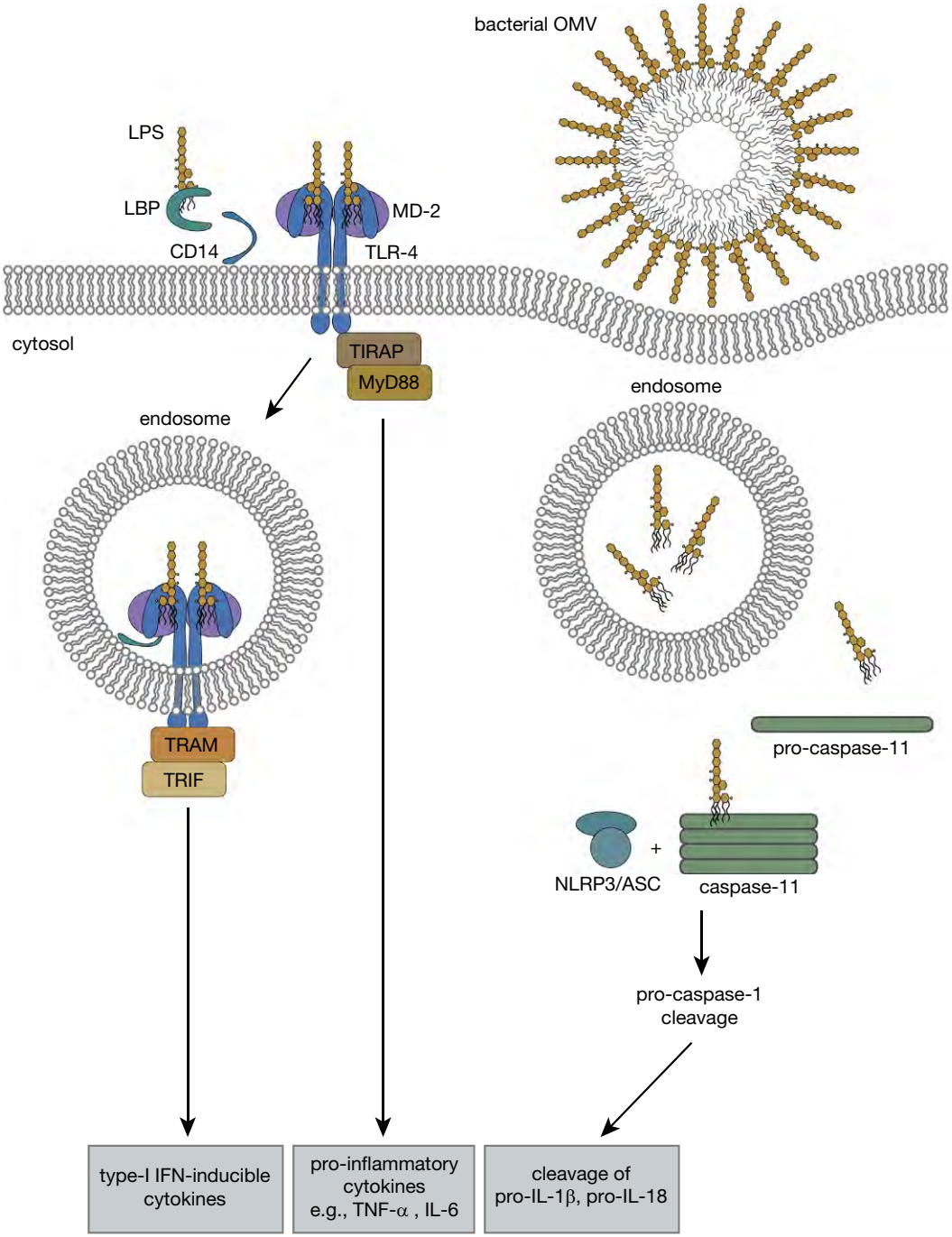

Fig. 7 The pathways involved in stimulation of myeloid and epithelial cells by LPS. LPS-binding protein sequesters LPS from bacterial membranes or micelles and delivers it to CD14. Subsequently, LPS is transferred to the TLR-4/MD-2 complex and induces TLR-4/MD-2 dimerization. The cytosolic domains of TLR-4 can promote a strong and immediate inflammatory response through recruitment of TIRAP/MyD88 and/or a mild response after endocytosis of the TLR-4 complex and recruitment of the TRAM/TRIF proteins. Alternatively, bacterial OMVs or invasive Gram-negative bacteria can induce inflammation by activation of the caspase-11-dependent inflammasome pathway following escape of LPS from the early endosome.

of the gut microflora. Alterations of this critical hoemostasis to favor an inflammatory response can have wide-reaching effects in human health.

While the receptor-mediated signaling of external LPS has been intensively investigated, an additional noncanonical caspase-dependent inflammasome pathway detects LPS in the cytosol of mammalian cells. Intracellular pathogens such as *Salmonella*, *Burkholderia* and *Shigella* species can introduce LPS either directly into the cytosol, or by release from the endosome. However, most pathogens do not invade host cells and these organisms release LPS-containing outer membrane vesicles during growth. These are

taken up by host cells through clathrin-mediated endocytosis and released to the cytosol from the early endosome. This signaling pathway activates the caspase-1 protease, which cleaves and activates inflammatory cytokines IL-1β and IL-18. Inflammasome activation can also lead to pyroptosis, a programmed cell death presumably aimed at eliminating invading bacteria. Oligomerization of caspase is central to the process and nontoxic LPS forms can bind to caspase without promoting oligomerization, consistent with their antagonistic effects in LPS-induced toxic shock.

The coverage here has focused on the LPS molecule as a signaling molecule for the innate immune system. However, a fascinating recent development has revealed the importance of heptose-1,7-bisphosphate, an intermediate in biosynthesis of a critical LPS precursor (ADP-heptose), also acts as a PAMP. Active release of heptose-1,7-bipsphosphate has been documented in *Neisseria* and enters host cells by endocytosis, where it is recognized by a cytosolic pathway via the host tumor necrosis factor receptor-associated factor (TRAF)-interacting protein with forkhead-associated domain (TIFA) protein. In *Helicobacter pylori*, the same metabolite is translocated by the Cag type 4 secretion system and the resulting inflammatory response may have implications for stomach cancer.

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Listeria monocytogenes☆

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Defining Statement

L. monocytogenes is a facultative intracellular Gram-positive bacterium able to invade, survive and replicate in phagocytic cells and non-phagocytic cells. *L. monocytogenes* has become a model to study the intracellular lifestyle of bacteria, and a powerful tool to study basic cellular biology processes.

Listeriology: An Historical Perspective

Historically, the first officially deposited strain of the human pathogen *L. monocytogenes* was isolated in 1924, by E.G.D. Murray, from rabbits and guinea pigs in animals exhibiting severe mononucleosis. Listeriosis was long considered as a zoonosis contracted through bacterial oral contamination. During the 1970s and 1980s, a number of outbreaks occurred in industrialized countries and led to the conclusion, in 1983, that *Listeria monocytogenes* was indeed the causal agent of food-borne listeriosis.

Several seminal discoveries or new approaches have changed our view on *Listeria*, and have contributed to make this organism one of the most studied bacterial pathogens. Following studies performed in the 1960s, *L. monocytogenes* became one of the primary pathogens used to study the T-cell immune response, due to its propensity to induce a rapid and protective cellular immune response after infection. Furthermore, the humoral immune response does not play a critical role in recovery from primary infection or protection against a secondary infection. The ability of *L. monocytogenes* to invade cells was first highlighted in 1970s. The molecular details underlying this ability have since been studied in great detail. In the mid 1980s, the use of molecular biology tools combined with cell biology approaches permitted the study of virulence factors, and the effect of *Listeria* on infected cells allowed researchers to unravel key concepts in cell biology. In 2001 the sequencing of the genomes of *L. monocytogenes* and *L. innocua* launched the still flourishing era of post genomics including comparative studies between *Listeria* species and between *L. monocytogenes* strains. More recently, the advent of and ever increasing accessibility of next generation sequencing and RNA-seq technology has contributed to more precise genome annotation and analysis of the bacterial transcriptional landscape in a variety of conditions both *in vitro* and *in vivo*.

Taxonomy

Listeria monocytogenes was first named *Bacterium monocytogenes* by E.G.D. Murray in 1926. It then received the name *Listerella hepatolytica* (Pirie, 1927). In 1940 Pirie suggested the name *Listeria monocytogene*s which became the name in the Approved Lists of Bacterial Names.

The genus *Listeria* belongs to the Firmicutes phylum and group with a low G+C content (38%). This sub-group includes *Clostridium*, *Bacillus*, *Enterococcus*, *Streptococcus* and *Staphylococcus* species. Furthermore, comparative analysis of *L. monocytogenes* EGDe and *L. innocua* CLIP 11 262 genomes revealed a conserved, co-linear organization of these two genomes and an unexpected synteny with the genomes of *B. subtilis* and *S. aureus*.

On the basis of DNA-DNA hybridization, multi-locus enzyme electrophoresis, rRNA gene restriction patterns and 16s rRNA sequencing, the genus *Listeria* includes ten currently recognized species and two subspecies: *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii* subsp. *ivanovii*, *Listeria ivanovii* subsp. *londoniensis*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria grayi*, *L. marthii*, *L. rocourtiae*, *L. weihenstephanensis* and *L. fleischmannii*. Only *L. monocytogenes* and *L. ivanovii* are pathogenic, but *L. ivanovii* is strictly described as an animal pathogen, that is particularly associated with abortion in ruminants.

Bacteriological Characterization

General Characters of the *Listeria* Genus

Listeria spp. are small Gram-positive, non-spore forming, non-capsulated bacilli, facultative intracellular, aero-anaerobic bacteria. They possess 1 to 5 flagella that confer motility at low temperature (20°C). Many *Listeria* strains are nonmotile at 37°C due to a lack

☆Change History: July 2014. L Radoshevich and P Cossart updated Abstract and the entire text. Figures were unchanged and added new references in 'Further reading'.

This article is an update of L. Dortet, E. Veiga-Chacon, P. Cossart, *Listeria monocytogenes*, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 182–198.

of flagellin expression at this temperature. The genome G + C percentage is between 36% and 42%. The *Listeria* peptidoglycan is of the type A1γ (presence of meso-diaminopimelic acid in position 3 of the tetrapeptidic unit), which is the same as in most Gram-negative bacteria. It also contains techoic and lipotechoic acids. (Figure 1)

Growth Conditions: Temperature, pH, Salts

Listeria spp. are free-living, ubiquitous, environmental bacteria commonly found in soil, water, plants and decaying vegetation. However, the natural habitat or the preferred niche (e.g. amoeba) of *Listeria* has not been identified yet. *Listeria* spp. have also been isolated as commensal bacteria from the intestinal flora of bovids, birds and humans.

Listeria spp. are non-fastidious bacteria, able to grow in classical broth media such as Brain Heart Infusion (BHI), blood added gelosis or Luria broth (LB). In these media, *Listeria* growth is stimulated by addition of glucose (0.2 to 1). On solid media containing 5% of sheep, horse, rabbit or human blood, beta-hemolysis is observed around colonies of *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*.

One of the hallmarks of *L. monocytogenes* is that it is able to grow at a wide range of temperatures (1 to 45°C), with optimal growth obtained between 30°C and 37°C. *Listeria* are killed at 60°C, making pasteurization an effective technique to eradicate bacteria from dairy products.

L. monocytogenes survives extreme growth conditions relatively well; from pH 4.5 to 9 and into 10% NaCl, but optimal growth occurs at neutral pH and 0.5% NaCl.

Isolation and Identification

L. monocytogenes is ubiquitous and its presence in food, environmental and clinical samples can be detected using classical or more recently developed rapid tests. Identification traditionally involved culture methods based on selective enrichment (for polycontaminated samples, such as food products) and plating on solid selective media followed by characterization of *Listeria* spp. based on colony morphology, sugar fermentation, and hemolytic properties. The characteristics of *Listeria* species identification are shown in Table 1.

For clinical samples, specific identification of *L. monocytogenes* strains can be obtained by plating on chromogenic solid media (Rapid'L.mono, BCM chromogenic agar test, CHROMagar *Listeria* test and ALOA).

These methods are the gold standard but are time-consuming and may not be suitable for testing short-lived products. This is why more rapid tests were developed based on antibodies (ELISA) or molecular techniques (PCR or DNA hybridization). These tests possess equal sensitivity and can be completed within 48h. More recently, real time PCR (RT-PCR) or nucleic acid based sequence amplification (NASBA) were developed to provide not only a measure of cell viability but also a quantitative analysis.

Serologic tests cannot be used directly for diagnosis because of an antigenic cross-reactivity between *L. monocytogenes* and other Gram-positive bacteria. However detection of anti-listeriolysin antibodies, after adsorption on streptolysin O of *Streptococcus pyogenes*, can be a good indicator of infection.

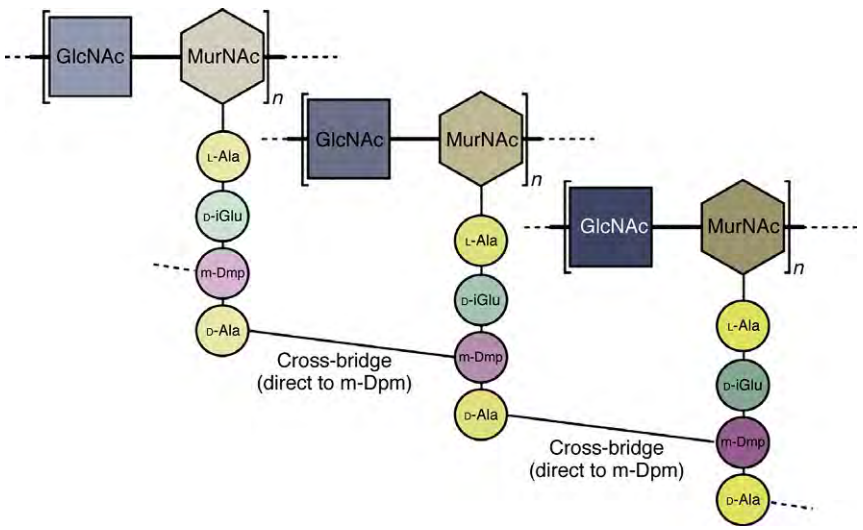

Figure 1 Schematic representation of the structure of L. monocytogenes peptidoglycan. GlcNAc, N-acetylglucosamine acid; MurNAc, N-acetylmuramic acid; Ala, alanine; iGlu, iso-glutamate; m-Dmp, meso-diaminopimelic acid.

Table 1 Characters allowing *Listeria* species identification.

		Listeria monocytogenes	*Listeria in nocua*	*Listeria ivanovii*	*Listeria seeligeri*	*Listeria welshimeri*	*Listeria grayi*
B-Hemolysis		+	−	+	+	−	−
Catalase		+	+	+	+	+	+
Oxidase		−	−	−	−	−	−
Fermentation of	L-Rhamnose	+	±	−	−	±	±
	D-Mannitol	−	−	−	−	−	+
	D-Xylose	−	−	+	+	+	−
	α-methyl-D-mannoside	+	+	−	−	+	+
	Ribose	−	−	±	−	−	±

Identification Methods Used in Epidemiology

Only *L. monocytogenes* is regarded as a human pathogen, and only three of the 13 serotypes of *L. monocytogenes* are implicated in major food-borne listeriosis outbreaks. Therefore, epidemiological investigations must use techniques that are able to discern closely related *L. monocytogenes* strains in order to confirm the source of outbreak, establish patterns of transmission, or determine and monitor reservoirs of epidemic strains. Typing of *L. monocytogenes* strains is either based on phenotypic markers, such as somatic O- and flagella H-factors for serological typing, phage receptors (phage typing), and enzymes (multilocus enzyme electrophoresis). More recently, typing based on molecular variations within the bacterial genome was used. Molecular typing techniques are based on DNA hybridization, PCR, restriction digest analysis or direct sequencing of DNA. Direct sequencing of DNA is the most accurate way of comparing genetic differences or similarities. However, it is also the most expensive and time-consuming method, and currently cannot be applied to high throughput testing. Hence, methods that give a relatively accurate reflection of genetic variation as well as high sample throughput in a rapid timeframe were developed. These methods are aimed at establishing the degree of allelic variation of particular genes, which then forms the basis of measuring genetic relatedness of *Listeria* strains. Some important molecular techniques are well established and are routinely used, such as ribotyping, macrorestriction digests, pulse-field electrophoresis (PGFE), PCR-fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD). Other techniques such as single strand conformational polymorphism (SSCP) or multilocus sequence typing (MLST) are currently becoming established as typing sequences for *L. monocytogenes* and show great promise. In particular MLST seems to be a good candidate to replace PGFE, however there are still certain strains of *Listeria* between which it is difficult to differentiate using MLST. Laboratories are currently gathering large data sets for these methods and they will probably be considered for routine testing in the near future. Next generation sequencing will eventually overtake these techniques as well but it remains too costly for routine identification at the moment.

Inhibitory Agents and Antibiotics

Virulent *Listeria* strains are resistant to bile (up to 40%) on agar plates. This is partly due to expression of a bile salt hydrolase, encoded by the *bsh* gene (see below) and other bile tolerance genes encoded by the locus *btlA*. Classical disinfectants are active on *Listeriae*, allowing efficient treatment of surfaces in contact with food, but serum and milk reduce their bactericidal activities, emphasizing the importance placed on hygiene in food industries.

Most *Listeria* spp. are sensitive to many antibiotics such as, penicillin G, ampicillin, amoxicillin, imipenem, aminoglycosides, tetracyclines, macrolides, glycopeptides, sulfamides and chloramphenicol. A natural resistance is observed against large spectrum third generation cephalosporins (cefotaxime or cefepime), aztreonam, nalidixic acid, ofloxacin and fluoroquinolones. *Listeria*'s natural resistance to nalidixic acid, as well as growth at low temperature, is exploited to select for the pathogen, and most enrichment media contain this antibiotic. There is a discrepancy between *in vitro* resistance and the *in vivo* therapeutic efficacy of fosfomycin. This phenomenon is due to differential *in vitro-in vivo* expression of Hpt virulence factor that mediates uptake of fosfomycin in *L. monocytogenes* during infection (see below). Listeriosis treatment generally consists of penicillin G or amoxicillin and gentamicin. Trimethoprim-sulfamethoxazol association is a good alternative treatment for patients who are allergic to β-lactam antibiotics. Because of the unique intracellular life style of *L. monocytogenes* during infection of its mammalian hosts, it is impossible to employ a phage therapy approach to treat listeriosis.

In contrast, phages have been recently used in the food industry to target *Listeria*. In 2005, the virulent bacteriophage P100, which can infect and kill a majority of *L. monocytogenes* strains, was characterized and evaluated. This phage P100 was shown to be an effective and safe measure for the control of *L. monocytogenes* contamination in soft cheese and production equipment. Recently, a comprehensive study of the effect of the phages A511 and P100 on *L. monocytogenes* in various foodstuffs treated under different conditions has been completed. Both phages were highly effective in reducing or eradicating *Listeria* contamination from almost all food types tested. More recently, in July 2007, the Listex P100®, a phage-based decontamination treatment in food processing, was

approved as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and the US Department of Agriculture (USDA).

Listeriosis

Pathophysiology

L. monocytogenes infects humans through the ingestion of contaminated food. It is able to cross the intestinal barrier and is thought to disseminate from the mesenteric lymph nodes to the spleen and the liver. The extent of intraluminal multiplication, as well as the precise location or locations at which it crosses the intestinal barrier are still debated. If not controlled properly by the immune system notably at the liver and spleen levels, *L. monocytogenes* infection may cause prolonged and asymptomatic bacteremia. It may then reach the brain or the placenta, resulting in meningitis or encephalitis mostly in immunocompromised patients, abortions in pregnant women, and generalized infections in infected neonates (granulomatosis infantiseptica).

Digestive Manifestations

Highly contaminated food products ingested by healthy individuals may result in self-limited gastroenteric symptoms. The enteric phase of the infection was previously considered to be almost always clinically silent. However, it was demonstrated that it might lead to the development of digestive symptoms such as nausea, aqueous or bloody diarrhea, abdominal pain, and fever - accounting for genuine gastroenteritis, with a rate of up to 70% of symptomatic cases, in cases of high ingested inocula. These digestive manifestations which strike healthy as well as in immunocompromised individuals are usually self-limited and resolve spontaneously.

Bacteriemia

In the population at risk for invasive listeriosis, that is immunocompromised individuals, generalized infection can frequently occur after an incubation period that can be very long (10–70 days), rendering identification of the source of contamination difficult for sporadic cases. *L. monocytogenes* bacteremia might be responsible for an influenza-like febrile illness, with myalgia, arthralgia, headache and backache, but might also be clinically silent.

Pregnancy-Associated Listeriosis

The specific complications of listeriosis during pregnancy lie in the ability of *L. monocytogenes* to cross the maternofetal barrier, leading to villitis and placental abscesses, chorioamnionitis, and ultimately systemic infection of the fetus. Maternofetal listeriosis is responsible for premature labor, stillbirth, abortion, and neonatal infection with high mortality. Disseminated infection of the fetus is also called *granulomatosis infantiseptica* and is characterized by the widespread presence of microabscesses and granulomas in the liver, spleen and skin.

Infection of the Central Nervous System

In addition to its ability to cross the intestinal barrier and the maternofetal barrier in pregnant women, *L. monocytogenes* has the capacity to breach the blood–brain barrier and infect the central nervous system, resulting in both meningitis and parenchymal brain infection (encephalitis or intraparenchymal brain abscesses). Among all bacterial meningitides, *L. monocytogenes* meningitis has the highest mortality rate (22%).

Other Clinical Manifestations

In immunocompromised patients, *L. monocytogenes* has been isolated in several other clinical contexts, such as endocarditis, rare localized infections (conjunctivitis, skin infection or lymphadenitis), digestive diseases (peritonitis or cholecystitis), or hematogenous seeding (macroscopic abscesses of liver and spleen, pleuropulmonary infections, joint infection and osteomyelitis, pericarditis, myocarditis, arteritis or endophthalmitis).

Epidemiology

L. monocytogenes more commonly infects individuals with attenuated cell-mediated immunity. Populations at risk include pregnant women and neonates, adults with underlying diseases (including cancer patients, organ transplantation recipients, people with AIDS, chronic hepatic disorder, diabetes) and the elderly.

A limited geographical distribution of listeriosis is observed, as it is mainly reported from industrialized countries (Table 2) with very few reports from Africa, Asia and South America. Whether this reflects different consumption patterns, dietary habits, host susceptibility, or lack of testing facilities is not known.

L. monocytogenes has been isolated from many types of food all of which have been associated with major outbreaks, but milk and more specifically dairy products have been repeatedly involved (Table 2).

Within the species differences in virulence among strains of *L. monocytogenes* seem to exist. Epidemiological data indicate, that not all strains of *L. monocytogenes* are equally capable of causing diseases in human. Isolates of only three (1/2a, 1/2b, 4b) of the 13 serovars identified within this species are responsible for 98% of the human listeriosis cases reported. Furthermore, the major

Table 2 International foodborne disease outbreaks of invasive listeriosis, 1980–2005.

Year	*Location*	*No. of cases*	*Perinatal cases*	*No. of deaths*	*Suspect/implicated vehicle*	*Serotype*
1981	Nova Scotia, Canada	41	34	18	Coleslaw	4b
1983	Massachusetts, USA	49	7	14	Pasteurized milk	4b
1985	California, USA	142	94	48	Mexican-style cheese	4b
1983–87	Switzerland	122	65	34	Vacherin Mont d'Or cheese	4b
1987-89	United Kingdom	366	?	?	Patè	4b
1989-90	Denmark	26	3	7	Blue mold cheese	4b
1992	France	279	0	85	Pork tongue in jelly	4b
1993	France	38	31	10	Rillettes	4b
1998-99	Multiple states, USA	108	?	14	Hot dogs	4b
1999	Finland	25	0	6	Butter	3b
1999–2000	France	10	3	3	Rillettes	4b
1999–2000	France	32	9	10	Pork tongue in aspic	4b
2000	Multiple states, USA	30	8	7	Delicatessen turkey ready-to-eat meats	1/2a
2000	North Carolina, USA	13	11	5	Homemade Mexican-style cheese	4b
2002	Multiple states, USA	54	12	8	Delicatessen turkey ready-to-eat meats	4b
2002	Quebec, Canada	17	3	0	Cheese made from raw milk	
2003	Texas, USA	12	?	?	Mexican-style cheese	4b

?, undetermined.

food-borne outbreaks of listeriosis, as well the majority of sporadic cases, have been caused by serovar 4b strains suggesting that strains of this serovar may possess unique virulence properties (Table 2).

The characterization of a large number of *L. monocytogenes* strains by several molecular epidemiological tools, such as pulse-field electrophoresis (PGFE) or multilocus enzyme electrophoresis (MLEE) was able to identify three lineages within this species. These lineages correlate with serovars: lineage I includes strains of serovar 1/2a, 1/2c, 3a; lineage II comprises strains of serotypes 1/2b, 4b, 4e; and lineage III includes strains of serotypes 4a and 4c. This classification is highly robust, because it is also reproduced when using other molecular typing methods such as ribotyping, Multi Locus Sequence Typing (MLST) and DNA-array hybridization. These findings led to the idea that the three lineages are of ancient origin and the congruence of various methods highlights the genomic stability of *Listeria* species.

L. monocytogenes is a relatively rare human disease (232 cases of invasive infections in France in 2006, including 48 cases of meningitis). *L. monocytogenes* is first and foremost an animal pathogen, in which it may be responsible for sporadic cases as well as outbreaks in wild as well as domesticated animals.

Animal Models

It would be ideal to use animal species that are naturally infected by *L. monocytogenes* as model organisms to study the physiopathology of human listeriosis. This approach has not been feasible because the animals that develop the disease most similar to human listeriosis are not classical laboratory animals but rather farm animals such as sheep, cattle and goats. The common clinical features of zoonotic listeriosis are abortion, central nervous system infection, digestive disease and bacteriemia. In contrast, in rat and mouse models a relatively mild infection is observed, on the other hand epidemic septicemiae are frequently observed in captive and wild colonies of guinea pigs, chinchilla or gerbils.

Moreover, it was shown that *L. monocytogenes* possesses stringent species specificity in terms of cellular invasion. This species specificity affects two major virulence factors of *L. monocytogenes*, InlA and InlB, which are able to recognize their respective receptors in humans, but not in all other species. InlA and InlB gain entry into cells by binding E-cadherin and Met, respectively (see V.B. Major virulence factor implicated in cellular life of *L. monocytogenes*). InlA does not engage mouse E-cadherin, due to a single amino acid substitution in comparison with human E-cadherin. A novel animal model was thus developed to study the role of InlA *in vivo*: it is a transgenic mouse expressing human E-cadherin in the intestinal enterocytes. In the same vein, InlB does not recognize or activate guinea pig or rabbit Met (Figure 2).

Human explants and cells remain the best system to study *Listeria* infection. For example, human explants have been successfully used to determine the mechanism by which *L. monocytogenes* interacts with the maternofetal barrier.

Caenorhabditis elegans and *Drosophila melanogaster* can be also infected by *L. monocytogenes*. Indeed, *L. monocytogenes* can establish lethal infections in adult fruit flies and larvae with extensive bacterial replication occurring before host death. Bacteria were found in the cytosol of insect phagocytic cells, and were capable of directing host cell actin polymerization. Bacterial gene products necessary for intracellular replication and cell-to-cell spread within mammalian cells were similarly found to be required within insect cells, and although *L. monocytogenes* virulence gene expression normally requires temperatures above 30°C, bacteria within insect cells were found to express virulence determinants at 25°C. Mutant strains of *Drosophila* that were compromised for innate immune responses demonstrated increased susceptibility to *L. monocytogenes* infection. Thus *Drosophila* has the potential to serve as a genetically tractable model that will facilitate the analysis of host cellular responses to *L. monocytogenes* infection. In addition,

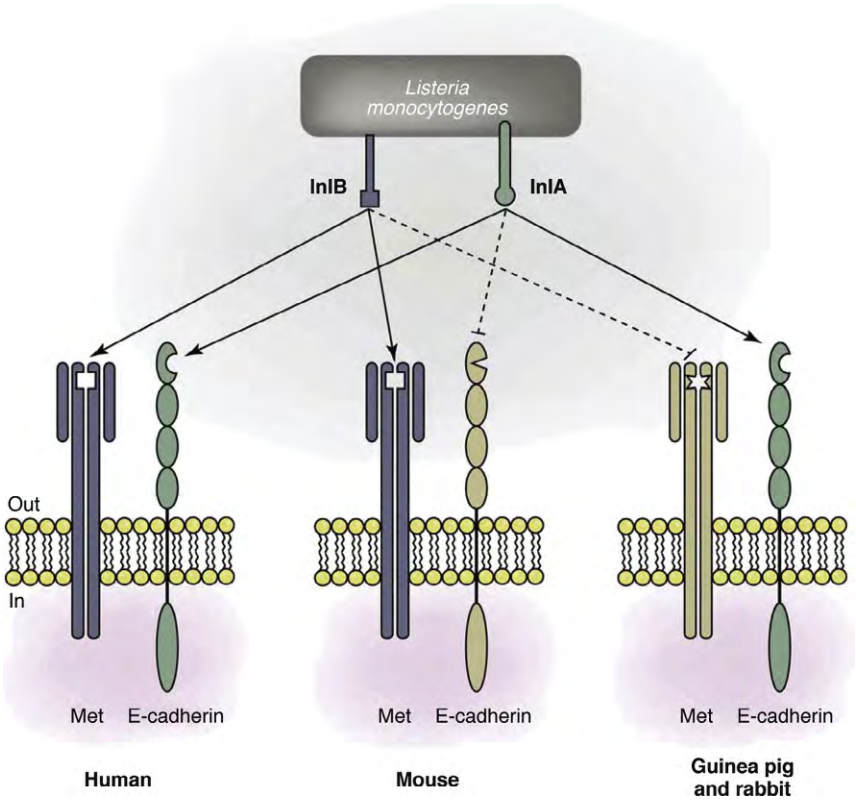

Figure 2 Species specificity of *Listeria monocytogenes* InlA and InlB. InlA and InlB bind and induce entry of *L. monocytogenes* into human cells that express the respective cell-surface receptors, E-cadherin or Met. However, a single amino-acid change in E-cadherin (at position 16; see text) prevents InlA from binding mouse E-cadherin. InlB cannot recognize guinea pig or rabbit Met.

zebrafish larvae have been successfully infected with *L. monocytogenes*. Intravenous infection leads to the capture of bacteria by macrophages, escape from the vacuole and even instances of cell-to-cell spread and actin comet tail formation *in vivo*. Most importantly, zebrafish larvae are an excellent system for *in vivo* live imaging of infection in real time.

Infection Cycle of *L. monocytogenes* and Molecular Mechanisms of Cell Subversion

Overview of *L. monocytogenes* Infectious Cell Cycle

The ability of *L. monocytogenes* to invade and replicate within mammalian host cells involves an amazing diversity of interactions between bacterial and cellular proteins. Intriguingly, many bacterial effectors provoke normal processes in the host cell suggesting some sort of evolutionary conversion between bacterial proteins performing roles in bacteria and also having secondary functions in virulence in mammalian cells.

L. monocytogenes has the ability to infect phagocytic and non-phagocytic cells such as fibroblasts, enterocytes, hepatocytes, epithelial and endothelial cells. While entry into phagocytic cells, such as macrophages or dendritic cells is bacteria-independent, entry into non-phagocytic, e.g. epithelial cells, is mediated by the bacterium (Figure 3). Invasion involves two critical *Listeria* internalins: internalin A (InlA) and InlB that interact with E-cadherin and Met (or HGF-R, the hepatocyte growth factor receptor), respectively. Depending on the cell type, *L. monocytogenes* may use E-cadherin, Met or both receptors to internalize. Both interactions (InlB/Met, or InlA/E-cadherin) trigger intracellular signalling cascades that induce the actin polymerization required to provide the necessary force to engulf the bacteria. Internalized bacteria reside for approximetly 30 min in a vacuole that is lysed by the concerted effort of the pore-forming toxin listerolysin O (LLO) and two secreted phospholipases PlcA and PlcB. Then, bacteria are released in the cytoplasm, start to multiply and polymerize actin. The listerial surface protein, ActA, triggers polymerization of actin at one of the bacterial poles giving rise to the structure known as actin tail or actin comet. This event propels the bacterium through the cell cytosol. Actin-propeled bacteria can push the cytoplasmic membrane, generating protrusions that invade neighbouring cells. *L. monocytogenes* is therefore able to pass from one cell to another without being exposed to the external milieu, avoiding contact with circulating antibodies and other extracellular bactericidal compounds. *L. monocytogenes* then lyses the double membrane vacuole (one membrane originating from the first infected cell and the other from the second infected cell) by the action of PlcB and LLO, and a second infection cycle can begin.

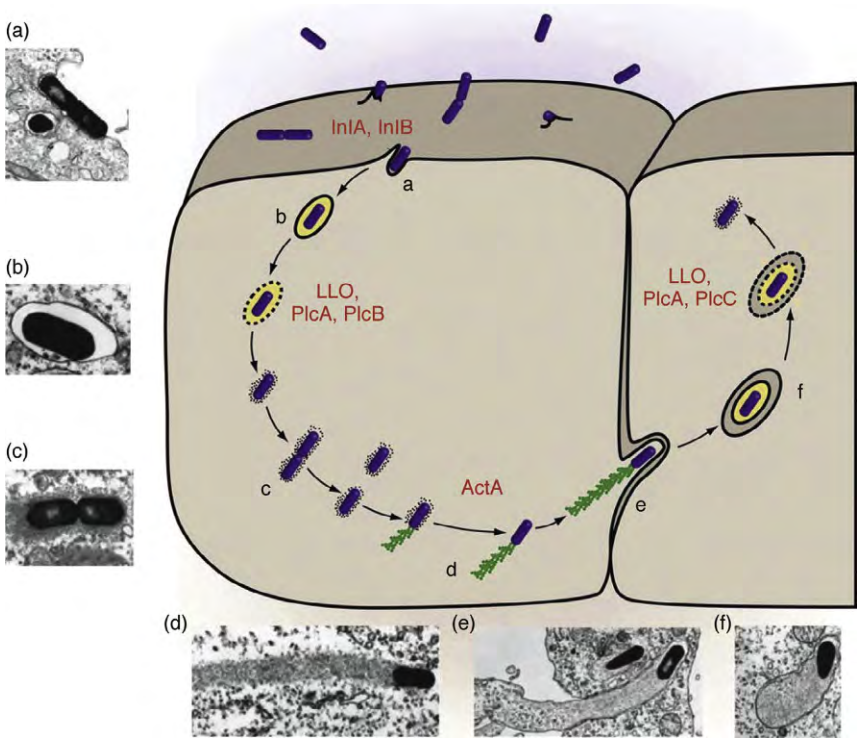

Figure 3 *Listeria monocytogenes* life cycle. (a) *L. monocytogenes* induces its entry into a non-professional phagocytes via InlA or InlB. (b) Bacteria are internalized in a vacuole. (c and d) The membrane of the vacuole is disrupted by PlcA, PlcB and LLO. Bacteria reach the cytoplasm, where they multiply and polymerize actin, as observed by the presence of the characteristic actin tails induced by ActA. (e) Actin polymerization allows bacteria to pass into a neighbouring cell by forming protrusions in the plasma membrane. (f) Upon entry into the neighbouring cell, bacteria are present in a double-membrane vacuole, from which they can escape using PlcB and LLO to perpetuate the cycle.

Major Virulence Factors Implicated in the Cellular Life of *L. monocytogenes*

InlA, InlB and other internalins: InlA and InlB were the first two *L. monocytogenes* effectors described to promote host cell invasion. Both proteins are necessary and sufficient to induce bacterial entry in host cells that express their respective receptors, E-cadherin for InlA and Met for InlB. InlA and InlB are two members of the internalin family which is comprised of proteins with an N-terminal leucine rich repeat (LRR) domain composed of tandem repeats of 20–22 amino acids structured in a solenoid-like shape. Each repeat is formed by a five amino acid β-strand followed by a seven-residue loop, a five-residue 3_{10} –helix, and a second five-residue loop, adds a new turn to the LRR solenoid structure. The β-strands forms the concave face normally implicated in protein-protein interactions. The LRR domain is flanked by a cap domain corresponding to the N-terminus of the mature protein and an Ig-like interrepeat (IR) region, structurally related to immunoglobulin (Ig) domains (Figure 4). The 2001 genome sequence of *L. monocytogenes* EGDe revealed the presence of 25 internalins. There are more internalins in *L. monocytogenes* than in any other Gram-positive bacterium.

InlA Promotes L. monocytogenes *Internalization by Mimicking Adherens Junctions Formation*

InlA mediates invasion by interacting with cellular E-cadherin, a transmembrane protein required for the formation of a type of cell-to-cell adhesion structure called adherens junctions. The formation of the adherens junction involves the N-terminal domain (EC-1) of two E-cadherin molecules that mediate cell to cell adhesion by homophilic interactions in a Ca^{2+} dependent manner. One key residue in this interaction is Trp2, which forms an EC-1/EC-1 inter-molecular bridge. Trp2 can also insert intra-molecularly in a "closed" conformation which abrogates intermolecular interactions and hence does not allow formation of adherens junctions. The intracellular part of E-cadherin mediates the interactions of the adherens junction complex with the actin cytoskeleton. InlA also binds to the E-cadherin EC-1 domain, but through a different zone than that which is involved in E-cadherin/E-cadherin interactions. The LRR domain of InlA specifically wraps around EC-1 (Figure 5). The Pro16 residue in EC-1 is critical to the InlA/E-cadherin interaction as revealed by mutagenesis and confirmed by the structure of the InlA/E-cadherin co-crystal. Replacement of Pro16 in human E-cadherin by glutamate in mouse E-cadherin (which shows 85% identity and superposable structure with the human E-cadherin) prevents InlA from binding mouse E-cadherin and renders mice resistant to InlA-triggered bacterial infections. Despite the lack of homology between InlA and E-cadherin, InlA binding to human E-cadherin, triggers the recruitment of the proteins that form the adherens junctions complex, including β and α catenins. Similar to E-cadherin during junction formation, InlA promotes cortactin- and Arp2/3-dependent polymerization of actin. However, the actin rearrangements induced by

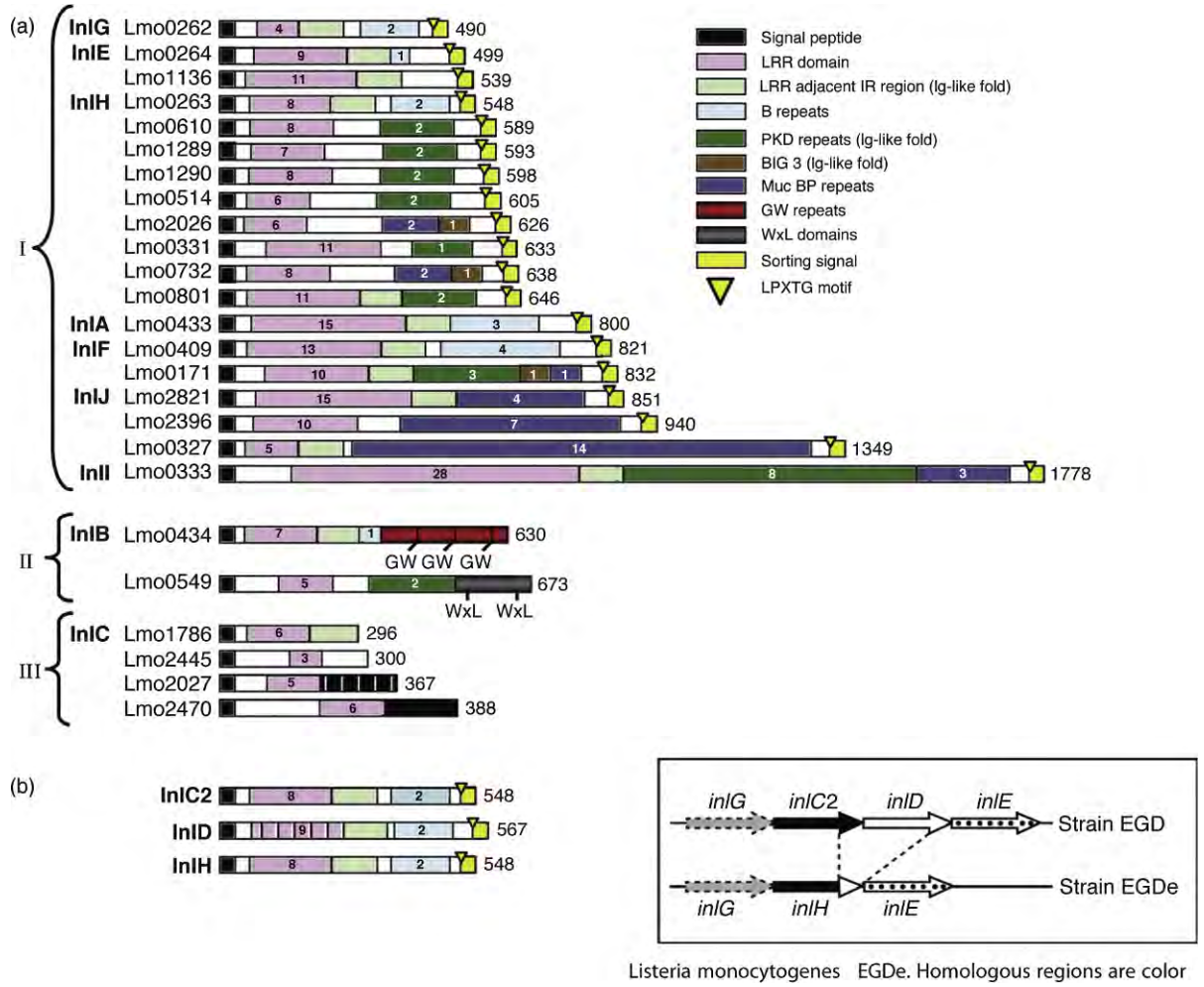

Figure 4 Schematic domain organisation of the 25 internalins from *L. monocytogenes* EGDe. Homologous regions are colour coded with legend provided in boxes on the right. Numbers inside boxes indicate the number of repeats. (a) Internalins can be separated in three groups by reference to their association with the bacterial surface : I, LPXTG-internalins covalently attached to the cell wall by sortase A; II, GW- and WxL-internalins non-covalently attached to the cell wall lipoteichoic acids; III, Secreted internalins. (b) InlH results from a recombination event between InlC2 and InlD. Domain organisation of InlC2, InlD and InlH is shown on the right, while organisation of their respective gene clusters in EGD versus EGDe is squared on the left. The C-terminal part of *inlG* is present in EGD, suggesting that whole gene is present.

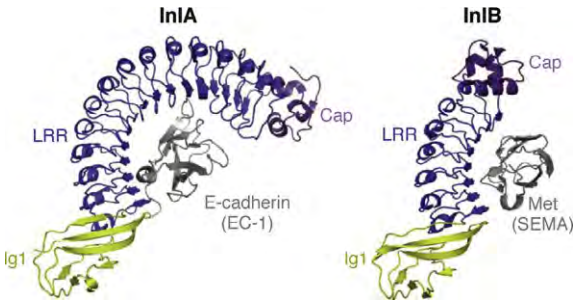

Figure 5 Co-crystals InlA/E-cadherin and InlB/Met. InlA, is an 800 amino acid internalin with 15 leucine rich repeats (LRR), and hence more curved than that of InlB which is formed by 7 repeats. The LRR binding zones of the E-cadherin (EC-1) and Met (a fragment of the SEMA domain) are shown. Note that InlB interacts with Met through its LRR domain but also with its Ig-like (Ig) 1 domain (not shown). 3D Images were performed using pyMol (DeLano Scientific LLC) from the PDB structures 1O6S and 2UZX.

L. monocytogenes during its internalization and during junction formation as well as the role of α-catenin in these processes are not fully understood. *L. monocytogenes* entering by the InlA pathway appears as a useful tool to study the cellular signalling promoting the formation of adherens junctions. The validity of using *L. monocytogenes* as a tool for adherens junction study was confirmed by a report that identified the Rho-GTPase activating protein 10 (ARHGAP10) as essential for bacterial entry and as a novel component in the adherens junction formation.

InlB Promotes L. monocytogenes *Internalization by Mimicking HGF, the Hepatocyte Growth Factor*

InlB mediates invasion by specific interaction with Met, a receptor tyrosine kinase normally involved in cellular signalling after HGF stimulation. The extracellular domain of Met includes an N-terminal semaphorin (Sema) domain, a small PSI domain (named for its presence in plexins, semaphorins and integrins) and four immunoglobulin (Ig)-like domains. HGF binding to the Sema domain of Met triggers a cellular response involving Met autophosphorylation, as well as activation of the phosphatidylinositol 3-kinase (PI3K) and the Ras–mitogen-activated protein kinase (MAPK) pathways. HGF-induced signalling leads to diverse cellular functions such as scattering, invasion, proliferation, morphogenesis and angiogenesis. It is also implicated in a large number of human cancers, correlating closely with metastasis and poor prognosis. These tumorigenic activities occur when Met is overexpressed, or when Met signalling after ligand (HGF) contact is not downregulated. Down regulation is achieved by endocytosis and degradation of activated Met. The LRR domain and the intergenic IR region of InlB interact with the first Ig domain (Ig1) and the Sema domain of Met (Figure 5), respectively, but not at the same site as HGF. The lack of competition between InlB and HGF for Met is in perfect agreement with the finding that they bind to different zones in the receptor. After binding to InlB, Met adopts a signalling-competent conformation, which is distinct from from the conformation after binding to HGF. Despite the lack of structural similarity between InlB and HGF, the different domains in Met recognized by InlB or HGF and the different conformations adopted by InlB- or HGF-activated Met, the signalling after InlB or HGF stimulation is very similar. InlB-activated Met also autophosphorylates, and induces activation of PI3K and MAPK pathways. However, differences exist in signalling after InlB, or HGF activation of Met. InlB induces stronger activation of the Ras-MAPK pathway than HGF at equimolar concentration. These differences could be explained, at least in part, by the fact that InlB can recruit other molecules that could act as co-receptors, for example gC1qR, the receptor for the globular part of the complement component C1q, or GAGs the glycosaminoglycans.

Both InlB and HGF induce actin cytoskeleton rearrangements that in the case of InlB are necessary for bacterial internalization. InlB-induced actin remodelling is mediated by the actin-nucleation complex, Arp2/3, which promotes actin nucleation and polymerization. Arp2/3 activation involves the small GTPases Rac and CDC42, and two neural WASP (N-WASP) and WAVE proteins of the Wiskott–Aldrich syndrome protein (WASP) family. Proteins of the Ena/VASP family (enabled homologue/vasodilator-stimulated-phosphoprotein family), which promote actin-filament elongation, are also involved in bacterial internalization. In addition, cofilin, which is essential for depolymerization of actin filaments, functions successively as a stimulator and a downregulator of actin rearrangements that occur during the internalization process. Remodelling of the actin cytoskeleton after HGF activation of Met is involved in cell spreading, motility and migration, and the pathway leading to actin remodelling is very similar to that induced by InlB. Both, InlB and HGF induce the clathrin-dependent internalization of Met and it has been recently described that *L. monocytogenes* hijacks the clathrin-dependent endocytic machinery to invade host cells showing that clathrin can permit the internalization of objects much larger that previously known (a bacterium is far larger than the maximal size of vesicles known to use that clathrin-dependent endocytosis). In 2011 and 2012, the precise molecular steps required for internalization downstream of either InlA or InlB-mediated bacterial uptake were characterized in detail. The process begins with recruitment of the clathrin adaptor Dab2, followed by the clathrin heavy and light chain complex, phosphorylation of clathrin heavy chain then precedes Hip1R recruitment and finally the force generated to complete internalization requires Myosin VI. In addition, a similar cellular machinery is required to form junctions between cells, which is yet another example of what can be learned about cell biological processes from *L. monocytogenes*. These data show that *L. monocytogenes* is not only a useful tool to study growth factor induced signalling but also an exceptional tool to study basic features of cellular biology. The signalling induced by InlB is presented in Figure 6.

Other Internalins Playing a Role in L. monocytogenes *Infection*

In addition to InlA and InlB, three other internalins (InlC, InlH and InlJ) interact with host cells and are involved in the infectious process. None of them however acts as an invasin.

InlC. The secreted internalin InlC is extremely abundant within infected cells to the degree that it has recently been used as a surrogate to quantify infection. The *ΔinlC* mutant had an increased LD_{50} compared to the wild type illustrating that is important in infection *in vivo*. In 2010, InlC was found to play a critical role during infection by dampening the innate immune response to *L. monocytogenes*. Cells with intracellular InlC prevent NF-κB translocation to the nucleus and dampen proinflammatory cytokines Furthermore, another group characterized that it plays a role in cell-to-cell spread through its interaction with TUBA.

InlH. InlH has an atypical LPXTG sortase recognition motif (LPTAG). However, it is anchored to the *Listeria* cell wall through a sortase A–dependent mechanism (see below). *ΔinlH* mutants are attenuated in bacterial colonization of spleen and liver following oral and intravenous infection of mice. The precise role of InlH during infection remains unclear. The InlH gene belongs to the operon encoding InlG, InlH and InlE in the sequenced strain EGDe. This operon has limited synteny with the operon of other *L. monocytogenes* strains, for example, *L. monocytogenes* EGD serotype 1/2a, with i*nlC2* and *inlD* instead of *inlH* (Figure 4(b)). *inlH* seems to be a fusion gene containing the internalin domain of *inlC2* and the B-repeats and the sorting signal from *inlD*. But in contrast to *ΔinlH*, the *ΔinlC2* shows no defect in virulence.

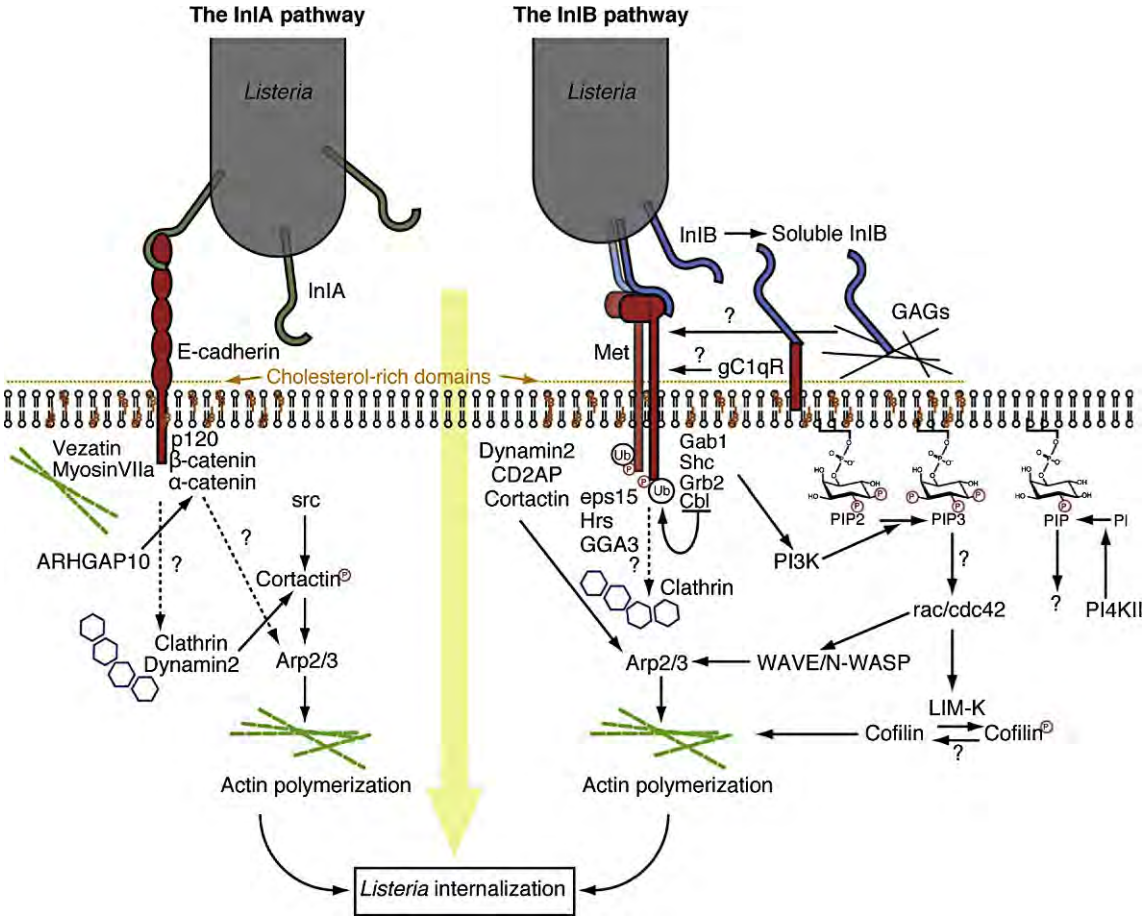

Figure 6 InlA (a) and InlB (b) induced signalling cascades after binding to E-cadherin and Met respectively.

InlJ. InlJ is a member of the LPXTG internalin family with a cysteine-containing LRR motif. The *ΔinlJ* mutant is significantly attenuated in virulence in mouse infections. However its role during infection remains elusive.

LLO and Phospholipases C. Listeriolysin O (LLO), the major virulence factor of *L. monocytogenes*, is a member of a large family of pore-forming toxins, the cholesterol dependent cytolysins (CDC). These toxins are secreted by many bacteria of different genera (e.g., *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus*, and *Arcanobacterium*). Similar to other CDCs, LLO binds to the host membrane as monomer and multimerizes into large complexes composed of up to 50 subunits that insert into membrane forming pores of ~200–300 Å diameter. Unlike other members of the CDC family, LLO requires a low pH for optimal lytic activity. This property facilitates the disruption of the membrane surrounding internalized bacteria and minimizes damage to the host plasma membrane. In addition, LLO is secreted before entry of the bacteria into host cells and functions extracellularly as a potent signalling molecule that activates multiple signalling pathways. LLO induces MAPK and calcium signalling in a pore-forming-dependent manner and NF- κB signalling in a pore-forming-independent manner by mechanisms that remain unclear. Recently it has been shown that extracellular LLO modulates host gene expression by inducing post-translational histone modifications. During early steps of infection, secreted LLO from extracellular *L. monocytogenes* induces a dramatic dephosphorylation of Ser_{10} on H3 and deacetylation of H4. These modifications correlate with modification of the transcription levels of a subset of host genes, notably with the decreased expression of key immunity factors e.g. CXCL2, DUSP4. CXCL2 is a chemoattractant chemokine with proinflammatory function and its down-regulation could reduce the recruitment of polymorphonuclear cells, which are key players in the clearance of *L. monocytogenes* during infection. DUSP4 is usually up-regulated upon activation of the MAPK pathway as a feedback mechanism. Its LLO-dependent down-regulation might suggest down-regulation of the MAPK pathway, a mechanism commonly used by many bacteria during infection to reduce the inflammatory response. Strikingly, dephosphorylation of Ser10 is a feature shared by at least two other CDC toxins that is *Clostridium perfringens* perfringolysin (PFO) and *Streptococcus pneumoniae* pneumolysin (PLY), revealing a general mechanism of epigenetic regulation used by unrelated extracellular bacteria. LLO was also found to be responsible for a global deSUMOylation event following infection. This deSUMOylation occurred due to a specific degradation of the sole E2 from the SUMOylation conjugation system, Ubc9. This event leads to more efficient infection as overexpression of SUMO prior to infection had the opposite effect.

Even though LLO is absolutely required for lysis of membrane vacuoles formed during bacterial internalization in macrophages, *L. monocytogenes* can escape from these vacuoles in the absence of LLO in several human epithelial cell lines. In addition to LLO, *L. monocytogenes* secretes two phospholipases (a phosphatidylinositol-specific phospholipase C (PI-PLC or **PlcA**) and a broad-range PLC (PC-PLC or **PlcB**)) that help the bacterium escape from the primary phagosomes and from the two-membrane vacuole resulting from cell-to-cell spread of *L. monocytogenes*. Although mutations in *plcA* or *plcB* resulted in small increases in mouse LD_{50}, deletions in both genes resulted in a 500-fold increase in LD_{50}. PlcB catalyzes the cleavage of the membrane lipid phosphatidylinositol (PI) into inositol phosphate and diacylglycerol (DAG) which activates the host protein kinases C (PKC). In the absence of LLO, PlcB activity is not only required for lysis of primary vacuoles in human epithelial cells but is also necessary for efficient cell-to-cell spread.

ActA: Actin-based bacterial propulsion. L. monocytogenes lyses the membrane of the vacuole and enters the host cytoplasm where it polymerizes actin at one bacterial pole thereby propelling the bacteria through the cell and into neighbouring cells. The bacterial surface protein ActA, which mediates polymerization of host actin was the first protein identified to promote actin nucleation. ActA has been an outstanding tool to understand the molecular mechanism behind actin polymerization, which is involved in numerous cellular processes e.g. cellular motility, phagocytosis, endocytosis or intracellular trafficking. ActA was shown to mimic host proteins of the WASP family (which include N-WASP and WAVE). The N-terminal region of ActA, which is essential for actin-based motility, has homology to the C-terminal region of WASP, which binds the actin-nucleation complex Arp2/3. ActA and WASP proteins function as nucleation-promoting factors (NPFs) forming multi protein-protein complexes composed by NPF, actin and the Arp2/3 complex. The Arp2/3 complex comprises seven subunits, including Arp2 and Arp3, which share structural similarity to actin and are thought to function as template for actin polymerization. The discovery of the Arp2/3 complex, which is the major component of the actin polymerization machinery, came from studies looking for ActA partners. ActA has also been useful for highlighting the function of VASP, the other main ligand of ActA. VASP binds the central proline-rich domain of ActA and promotes efficient actin-based motility by recruiting the actin-binding protein profilin, which promotes polymerization at actin-filament barbed ends. Although their mechanistic role is not fully elucidated, proteins of the Ena/VASP family are important in the formation of actin fibers, filopodia and the lamellipodial leading edge. In addition to actin polymerization, ActA was reported to play a role in bacterial internalization, a property which might depend on its capacity to bind heparan sulfate proteoglycans. However the role and the mechanism of ActA as an invasin remain to be elucidated. Another group recently characterized a role for ActA in bacterial aggregation and biofilm formation. ActA makes intramolecular bridges between bacteria. Though this property is unrelated to its ability to polymerize actin, it seems important for bacterial dissemination, in particular mutants lacking ActA are shed in the feces of the animal much more rapidly than wild-type bacteria. This likely leads to less dissemination to the environment and as such fewer new infections. ActA also seems to play a role in the stress response to ROS during *L. monocytogenes* growth in the environment. Taken together, these studies highlight another example of selection pressures in the environment and in host cells that lead to distinct but essential roles of the same protein.

Vip. Vip was identified '*in silico*' as a *L. monocytogenes* specific protein by the comparison of the genome sequences of *L. monocytogenes* and that of the related non-pathogenic species *L. innocua*. Vip is a surface exposed protein anchored to the *L. monocytogenes* cell wall via its LPXTG motif and involved in bacterial internalization in several cell lines. Its cellular receptor, Gp96, a chaperone normally localized in the endoplasmic reticulum has been described to modulate the immune response by affecting the intracellular trafficking of several molecules, including Toll-like receptors (critical mediators of the innate immune response). Whether *L. monocytogenes* also affects the immune response by hijacking Gp96 remains unknown.

Auto, Ami, P60, NamA. Several autolysins from *L. monocytogenes* have been involved in virulence, including Auto, Ami, P60 and NamA. Bacterial autolysins hydrolyze distinct components of the bacterial cell wall to enable *de novo* biosynthesis of the peptidoglycan layer and are involved in various biological processes including bacterial growth, protein secretion, bacterial division, competence, or sporulation. Ami is an autolytic amidase with an N-terminal domain similar to the alanine amidase domain of the Atl autolysin of *Staphylococcus aureus* and a C-terminal GW cell wall anchoring domain. Ami is exposed at the *Listeria* surface and like other GW autolysins, the molecule is likely to be secreted and targeted to the bacterial surface via its GW domain. Ami contributes to the attachment of *L. monocytogenes* to eukaryotic cells. *Δami* mutants lacking InlA or InlB or both are five to ten times less adherent than the parental strains in various cell types. The gene encoding Auto was identified by comparison of the *L. monocytogenes* and the *L. innocua* genomes. Auto possesses an N-terminal autolysin domain and a C-terminal cell wall-anchoring GW domain. Auto is required for entry of *L. monocytogenes* into cultured non-phagocytic eukaryotic cells. The Δ*auto* mutant also shows reduced virulence, compared with its wild type parental strain, following inoculation of mice and guinea pigs. P60, a 60-kDa secreted autolysin, is known to be involved in cell division and is associated with invasion into several mammalian cell lines. *Δiap* (the deleted gene for encoding for P60) mutants are attenuated in mouse infections. P60 was characterized as a murein hydrolase based on its homology to a repeat domain of an autolysin in *Enterococcus faecium* and the observation that P60 overexpression induces autolysis in *L. monocytogenes*. **NamA** (MurA) is another *L. monocytogenes* autolysin involved in virulence. A *ΔnamA* mutant shows reduced bacterial virulence *in vivo*. Coordinated peptidoglycan hydrolysis by p60 and NamA activities is predicted to generate glucosaminylmuramyl dipeptide, which is known to regulate host inflammatory responses. Both p60 and NamA are secreted out of the bacterial cell in a SecA2-dependent manner (see below). SecA is responsible for general protein secretion and drives the transport of a variety of extracellular proteins in *L. monocytogenes*. SecA2 is involved in the secretion of proteins lacking a signal sequence. SecA2 homologues have also been described in other Gram-positive bacteria such as *Mycobacterium* spp. and *Streptococcus* spp. The *L. monocytogenes ΔsecA2* mutant is defective in the secretion of P60, NamA and at least 15 additional cell wall-associated or secreted proteins.

PgdA and OatA. The peptidoglycan is an essential feature of the bacterial cell wall and is an important target recognized by the innate immune system. The peptidoglycan from *L. monocytogenes* is particular as its N-acetylglucosamine residues are partially deacetylated by PgdA, a peptidoglycan N-deacetylase first identified "*in silico*". A *ΔpgdA* mutant shows increased bacterial sensitivity to host lysozyme and decreased bacterial virulence in mouse models of infection. Within macrophage vacuoles, the mutant is rapidly destroyed and induces a massive IFN-beta response in a TLR2 and Nod1-dependent manner. Interestingly, *L. monocytogenes* makes use of a second peptidoglycan modification to mask the bacteria from innate immune recognition. O-acetyl modification of peptidoglycan is mediated by its O-acetyltransferase, OatA and is present on 23% of *L. monocytogenes* peptidoglycan. Upon deletion of this gene bacteria are much less virulent both *in vitro* and *in vivo* and furthermore are more susceptible to lysozyme and other antimicrobial compounds. Interestingly, a different subset of cytokines is induced by the two mutants suggesting that deacetylation by PgdA and O-acetylation by OatA play complementary roles in camouflaging L. monocytogenes from the innate immune response. Furthermore, the presence of deacetylase genes in other pathogenic bacteria indicates that peptidoglycan N-deacetylation could be a general mechanism used by bacteria to evade the host innate immune system.

Bsh. Bile salts are end products of cholesterol metabolism and are primarily synthesized in hepatocytes. In response to food ingestion, bile salts are released into the duodenum helping to absorb fat. In addition to this role, bile salts have antimicrobial properties acting as detergent on bacterial membranes. Some bacteria, in particular commensal bacteria, have developed the capacity to inactivate the detergent action of bile salts with a reaction catalysed by the enzyme bile salt hydrolase (Bsh). *Listeria monocytogenes* Bsh is involved in bacterial survival in the intestinal lumen and liver. Deletion of *bsh* results in decreased resistance to bile *in vitro*, and reduced virulence in mice and guinea pigs models. The *bsh* gene is positively regulated by PrfA, the transcriptional activator of known *L. monocytogenes* virulence genes, confirming the role of Bsh as virulence factor involved in intestinal and hepatic phases of listeriosis.

Hpt. Host cell hexose phosphates can be used by *L. monocytogenes* as a carbon source allowing rapid bacterial intracellular growth. Hexose phosphate uptake is mediated by Hpt, a bacterial homologue of the mammalian translocase for glucose-6-phosphate from the cytosol into the endoplasmic reticulum in the final step of gluconeogenesis and glycogenolysis. The master regulator of virulence in L. monocytogenes, PrfA, regulates the expression of the Hpt permease. A *Δhpt* mutant is impaired in intracytosolic proliferation and attenuated in virulence in mice. Hpt is another example of *Listeria* mimicry of eukaryotic host cells physiological mechanisms. Hpt is upregulated inside cells and allows for the uptake of the antibiotic fosfomycin that can attack intracellular bacteria expressing Hpt (see above section III E).

FbpA. FbpA (fibronectin binding protein) is required for intestinal and liver colonization in mouse models of infection. FbpA binds to human fibronectin *in vitro* and increases adherence of wild-type *L. monocytogenes* to cultured epithelial cells in the presence of exogenous fibronectin. FbpA lacks a conventional secretion/anchoring signal and is exported to the bacterial surface via SecA2.

MnSOD. Superoxide dismutases (SODs) are enzymes that protect organisms from reactive oxygen species (ROS) which are major mediators of microbicidal activity in phagocytic cells. *L. monocytogenes* express one SOD, (MnSOD) which can be phosphorylated on serine and threonine residues in the bacterial cytoplasm. These phosphorylations result in decrease of SOD activity. The non-phosphorylated form of MnSOD is more active than the phosphorylated form and can be secreted, via the SecA2 pathway, in culture supernatants or in infected cells, where it becomes phosphorylated. A *Δsod* deletion mutant is impaired in survival within macrophages and is dramatically attenuated in mice, showing that the capacity to counteract ROS is an essential component of *L. monocytogenes* virulence. MnSOD was the first example of a bacterial SOD post-translationally controlled by phosphorylation, potentially highlighting a possible new host innate mechanism to counteract a virulence factor.

LntA. A new class of *L. monocytogenes* virulence factors was discovered thanks to comparative genomics. The so-called "nucleomodulins" are proteins which are secreted from the invading bacteria and subsequently translocate to the nucleus to mediate their effects on host-gene transcription. *Listeria* nuclear-targeted protein A or LntA was also discovered based on its absence in *L. innocua*. When expressed in mammalian cells, it translocates to heterochromatin rich regions and it interacts with a repressor of chromatin, BAHDI. Bacteria that lack the protein are less virulent *in vivo* and paradoxically bacteria that overexpress the protein are also impaired in virulence. This is due to a temporally controlled tuning of interferon signaling which if deregulated by the absence or untimely overabundance of the protein leads to less efficient infection. It is likely that other secreted factors of *L. monocytogenes* could also bring about changes in gene expression that could have a long-term effect on host cell memory.

Regulation of Virulence Factors

L. monocytogenes is a facultative intracellular pathogen that can live both inside and outside its host. This bacterium has therefore evolved sophisticated regulatory mechanisms to ensure that virulence factors are optimally expressed when they are required.

PrfA : A Thermosensing Regulation

The majority of virulence proteins that have been identified in *L. monocytogenes* are under the control of a the transcriptional regulator, PrfA, which itself is tightly regulated by environmental conditions (Figure 7).

PrfA is synthetized from two transcripts: a monocistronic transcript driven by two promoters ($P1_{prfA}$ and $P2_{prfA}$) situated in the *plcA-prfA* intergenic region, and a plcA-prfA bicistronic transcript driven by P_{plcA} promoter (Figure 7). During exponential growth, *prfA* is primarily transcribed as a biscistronic mRNA. By contrast, during stationary phase, a monocistronic mRNA is produced.

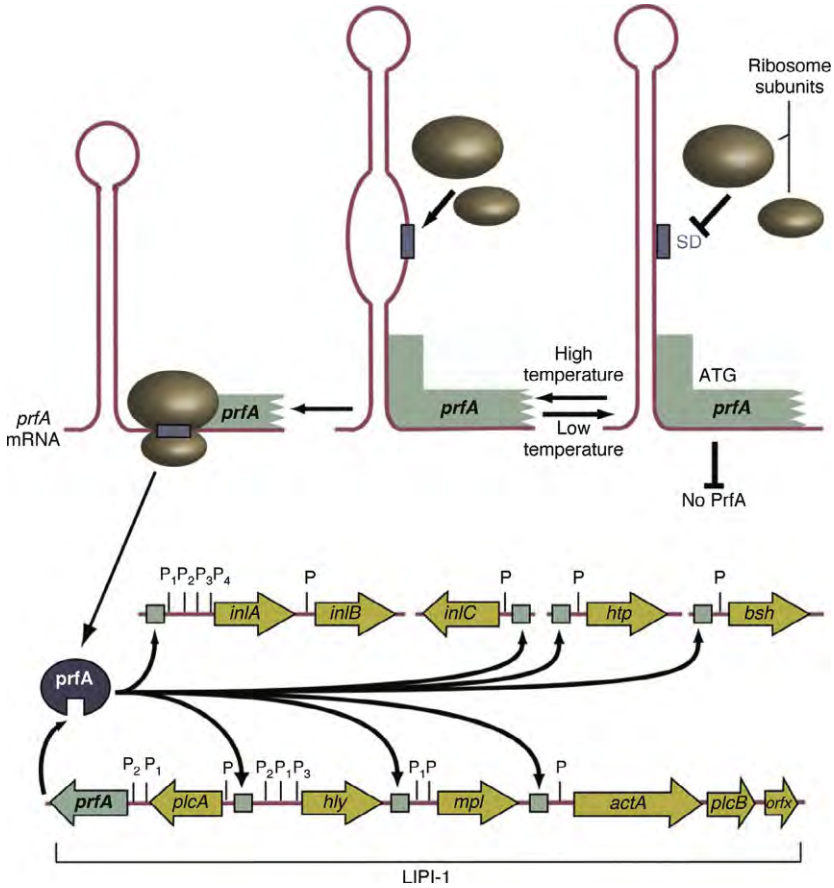

Figure 7 The PrfA regulator. Mechanism that controls the thermoregulated expression of PrfA. At low temperature ($\geq$30°C), a secondary structure forms in the untranslated region of *prfA*, and this prevents ribosome binding and therefore expression of PrfA. At high temperature ($\geq$37°C), melting of the prfA untranslated region allows ribosome to bind Shine-Dalgarno sequence (SD), and PrfA expression occur. PrfA interacts with PrfA-boxes (green squares) situated upstream of regulated genes (yellow arrows).

Similar to many other pathogens, *L. monocytogenes* can sense various conditions in the mammalian host and respond by expressing virulence genes. $P1_{prfA}$ is a σ^A vegetative promoter leading to a low level of *prfA* mRNAs synthesis during normal bacterial growth. The 5′ untranslated region (UTR) of these constitutive transcripts forms a secondary structure that acts as a thermosensor. At low temperatures (<30°C), the secondary structure masks the ribosome-binding site and prevents the translation of the downstream *prfA* message. However, at mammalian host temperature (37°C), this structure partially melts to expose the ribosome-binding site, thereby allowing translation to occur (Figure 7). Indeed, the PrfA protein is not produced at low temperature even though monocistronic *prfA* transcripts are present. Thermoregulation of *prfA* translation provides an elegant explanation for restricting costly virulence factor production in the environment, and for mediating rapid synthesis of PrfA from a pool of untranslated transcripts as soon as the pathogen invades a warm-blooded host.

$P2_{prfA}$ is situated downstream from $P1_{prfA}$ and is regulated by both α^A and the stress-responsive alternative sigma factor σ^B. σ^B-dependent $P2_{prfA}$ occurs during the stationary phase.

The role of $P1_{prfA}$ and $P2_{prfA}$ is to provide basal levels of *prfA* transcripts, ensuring that a minimal amount of PrfA protein is continuously present in the bacterial cytosol. This basal amount appears to be sufficient to stimulate the PrfA-hypersensitive *hly* (coding for LLO) and *plcA* genes and to mediate escape from the phagocytic vacuole, but not to stimulate other genes requiring a more intense PrfA stimulus, such as those involved in cell-to-cell spread.

The P_{plcA} promoter is PrfA-dependent, so that through the plcA-prfA bicistronic transcript, PrfA can stimulate its own synthesis by positive feedback.

Three groups of genes have been identified as PrfA-dependant by a transcriptomic approach. Group I comprises twelve genes including ten major virulence genes (*inlA*, *inlB*, *inlC*, *htp*, *plcA*, *hly*, *mpl*, *actA*, *plcB* and *orfX*), *lmo2219* and *lmo0788*. These genes are strongly induced in active-PrfA background and during intracellular infection. A putative PrfA box precedes these genes. They are organized into several transcriptional units, of which four are clustered in the pathogenicity island (LIPI-1) essential for *L. monocytogenes* virulence (Figure 7). Group II comprises eight genes encoding a putative sugar transport system that is negatively regulated by PrfA, indicating that PrfA can also act as a repressor. Only one of them (*lmo0278*) is preceded by a putative PrfA box and

the remaining seven genes (*lmo0178-lmo0184*) are organized in an operon. Group III comprises fifty-three PrfA-upregulated genes of which only two (*lmo0596* and *bsh*) are preceded by a putative PrfA box.

VirR : Global Regulation of Surface Component Modification

VirR is the response regulator of the two-component system VirR/VirS. Deletion of *virR* decreases virulence in mice as well as invasion in cell-culture experiments. By comparing the transcriptomes of *virR* deletion versus wild type bacteria, twelve genes seem to be regulated by VirR. These genes include the *dlt*-operon, involved in modification of cell wall teichoic and lipoteichoic acid, that are both also important for *L. monocytogenes* virulence. Another VirR-regulated gene is homologous to *Staphylococcus aureus mprF*, which encodes a protein that modifies membrane phosphatidyl glycerol with L-lysine and is involved in resistance to human defensins.

VirR thus appears to control virulence by a global regulation of surface component modification. These modifications may affect interactions with host cells, in particular components of the innate immune system, such as Toll-like receptors. Interestingly, although controlling the same set of genes as VirR, the cognate histidine kinase of VirR, VirS was not shown to be essential for virulence. Furthermore, VirR can be activated independently of VirS. Finally, as is the case of PrfA, VirR-regulated promoters were shown to possess a DNA-binding consensus site (CTNACAwwwTGTNAG), and specific interaction between this consensus sequence and purified VirR protein has been demonstrated.

Sigma B

L. monocytogenes is predicted to contain only five sigma factors in comparison to 18 in *B. subtilis* and 13 in *Mycobacterium tuberculosis*. The *L. monocytogenes* sigma factors are similar to *B. subtilis* SigA, SigB, SigH, SigL (RpoN, sigma 54) and ECF-type sigma factors.

The alternative sigma factor sigma B modulates the stress response of several Gram-positive bacteria, including *Bacillus subtilis* and the food-borne human pathogens *Bacillus cereus*, *L. monocytogenes* and *Staphylococcus aureus*. In all these bacteria, sigma B is responsible for the transcription of genes that can confer protection against adverse conditions. Upon exposure to stress a regulatory cascade is triggered leading to the activation of sigma B and subsequently to transcription of sigma B-regulated genes. In *L. monocytogenes*, sigma B plays a role in growth and survival at low temperatures, acidic conditions and to the stress of the intracellular environment.

The deletion of *sigB*, however, has limited effects on virulence, as shown in animal models, even though several listerial virulence factors are under the control of sigma B. Interestingly, the *bsh* gene encoding the bile salt hydrolase and two genes from the internalin family, which contribute to bacterial entry are partially sigma B-dependent and also PrfA-regulated. The central role of sigma B in the regulatory network of the bacterium is further illustrated by the fact that sigma B can control other important regulatory proteins like the RNA-binding protein Hfq in *L. monocytogenes*. Hfq is thought to play a crucial role in the post-transcriptional regulation of gene expression by small non-coding RNAs and the dependence of this regulator on sigma B adds another level of complexity to the function of sigma B in *L. monocytogenes*.

Genomics and Post Genomics

Genome Sequencing of *Listeria monocytogenes* EGDe and *Listeria innocua* CLIP11262

In 2001, the determination of the complete genome of the pathogen *L. monocytogenes* (strain EGDe) and that of the closely related non-pathogenic species *L. innocua* (strain CLIP11262) were achieved by a consortium of 10 European laboratories. In 2004, The Technical Institute of Genomic Research (TIGR) determined the sequence of another *L. monocytogenes* strain (*L. monocytogenes* serovar 4b, strain F2365). Currently, ten *Listeria* species have been identified and sequenced (*L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. marthii*, *L. rocourtiae*, *L. weihenstephanensis* and *L. fleischmannii*) two of which are pathogenic: *L. monocytogenes* and *L. ivanovii* (for ruminants). The availability of these different genome sequences of *Listeria* spp. paves the way for comparative genomics studies and the identification of unknown virulence factors. There are currently thirty-nine complete genome sequences of *Listeria monocytogenes*, with more being sequenced daily, including the recent sequencing of the frequently used strain EGD.

The *L. monocytogenes* (strain EGDe) genome is 2.9 Mb long with an average G+C content of 39% and 2853 predicted protein-coding genes. The *L. innocua* genome is 3.0 Mb with an average G+C content of 37% and 2981 predicted protein–coding genes. Comparative genome analysis identified that 2587 of 2853 genes of *L. monocytogenes* EDGe possess an orthologous gene in the *L. innocua* genome.

The most striking feature of these genomes is that they encode an exceptionally large number of surface proteins (4.7% of all predicted genes of *L. monocytogenes* EGDe), especially proteins that belong to the internalin family (Figure 4). The ability of *Listeria* spp. to colonize and grow in a broad range of ecosystems correlates with an abundance of transport proteins (11.6% of all predicted genes of *L. monocytogenes* EGDe), in particular proteins dedicated to carbohydrate transport mediated by phosphoenolpyruvate-dependant phosphotransferases. Furthermore the extensive regulatory repertoire (7.3% of all predicted genes of *L. monocytogenes*

EGDe) identified correlates with the varied environmental conditions faced by *L. monocytogenes*. This high proportion of regulatory genes is second only to that of *Pseudomonas aeruginosa*, another ubiquitous pathogen.

In contrast to many other bacterial genomes, the *L. monocytogenes* genome contains only three copies of one insertion sequence and that of *L. innocua* does not contain any. Both genomes contain bacteriophages, but these do not seem to play a major role in acquisition of virulence genes as has been shown for other bacteria such as pathogenic *E. coli*. A perfect conservation in the order and in the relative orientation of orthologous genes was observed between *L. monocytogenes EGDe* and *L. innocua* genomes. This co-linear organization may be related to the low occurrence of insertion sequences. Surprisingly, comparison of the two *Listeria* genomes with related bacterial genomes that have low G+C, such as *Bacillus subtilis* or *Staphylococcus aureus* reveals a similar organization, indicating that genomes of this group of bacteria are particularly stable.

Sequence analysis of the two *Listeria* genomes revealed a close relationship to *B. subtilis*, suggesting a common origin of the three species. *L. monocytogenes* subsequently acquired multiple non-contiguous DNA fragments, including the virulence locus LIPI-1 (Figure 7), which is absent in the genome of *L. innocua* and was probably lost.

Post Genomic Studies

Following the sequencing of *L. monocytogenes* strain EGDe, several post genomic studies, based on genome comparison, have been performed to identify new virulence mechanisms and new genetic features. In 2012, a new study generated genome wide Transcription Start Site (TSS) maps of *L. monocytogenes* and *L. innocua* coupled with a browser to compare transcriptomic and RNA-seq data in a variety of conditions to assess differential regulation of genes and allow direct comparison between the non-coding genome of the two strains.

This approach yielded highly interesting comparisons between the genomes, and more specifically the identification of noncoding RNAs (ncRNAs) in *L. monocytogenes*. Previously, computer-based method analysis of the intergenic regions of the EGDe strain led to the identification of twelve ncRNAs. Among these 12 new ncRNAs, nine ncRNAs named *Rli* genes (*RliA* to *RliI*) appear to be specific to the genus *Listeria* with no homologous counterparts detected in other bacterial genomes. *RliA*, *C*, *F* and *G* are present in *L. monocytogenes* and absent in *L. ivanovii* and in non-pathogenic *L. innocua*. *rliB* is duplicated in *L. ivanovii*, an ovine pathogen, but is absent in *L. innocua*. As with protein coding genes, ncRNAs absent in *L. innocua* species might be involved in infection. The four other *Rli* family genes (*RliD*, *E*, *H* and *I*) were identified in all *Listeria* species, suggesting these ncRNAs genes would control non-infection related functions. Furthermore, a novel computational approach was developed to search mRNA targets of ncRNAs in bacteria. mRNA targets were predicted for five (RliB, D, E, H and I) of the nine Rli ncRNAs. Finally, *in vitro* experiments demonstrate pairing between ncRNAs and each predicted mRNA target suggesting a role for these 'new regulatory factors'.

Post genomic studies were also performed to investigate the genetic diversity of *L. monocytogenes* strains with varying virulence potential. An epidemic serovar 4b strain was partially sequenced and compared with the complete genome of the non-epidemic *L. monocytogenes* EGDe serovar 1/2a. This approach identified unexpected genetic divergences between the two strains, as about 8% of the sequences were serovar 4b specific. These sequences included seven genes coding for surface proteins with the LPXTG motif, six transport proteins, three transcriptional regulators, one phosphotransferase system component and twenty-six unknown proteins. All of these newly identified proteins might be important to different steps of the infectious process. Furthermore, the comparison of the gene content of 113 *Listeria* strains was performed by using an array containing the flexible part of the sequenced *Listeria* genomes. Hybridization results showed that all identified virulence factors were present in the ninety-three *L. monocytogenes* strains tested. However, distinct patterns of the presence or absence of other genes were identified among different *L. monocytogenes* serovar and *Listeria* species.

The genome analysis of *L. monocytogenes* EGDe revealed the presence of forty-one genes encoding surface proteins bearing an LPXTG motif, two surface proteins containing an NXZTN motif and two sortases (SrtA and SrtB). Sortases are enzymes that anchor surface proteins to the cell wall of Gram-positive bacteria by cleaving a sorting motif located in the C-terminus of the protein substrate. The best-characterized motif is LPXTG, which is cleaved between the T and G residues. Non-gel proteomic approaches identified a total of thirteen LPXTG-containing proteins anchored in the peptidoglycan by SrtA, six of which (InlA, InlG, InlH, Lmo1413, Lmo1666 and Lmo2085) have no ortholog in the closely related non-pathogenic *L. innocua*. Two surface proteins, Lmo2185 and Lmo2186 were identified only when SrtB was active. The analysis of the peptides identified in these proteins suggests that SrtB of *L. monocytogenes* may recognize two different sorting motifs, NXZTN and NPKXZ. A mutant *ΔsrtA* is strongly attenuated in virulence, while no impact in virulence could be detected with the *ΔsrtB* mutant. In contrast the SrtB ortholog in *Staphylococcus aureus* is involved in heme capture and virulence.

Another example of the efficiency of comparative genome studies was the discovery of an auxiliary protein secretion system (SecA2) present in *L. monocytogenes* and eight other Gram-positive pathogens that cause severe or lethal infection in humans. In *L. monocytogenes*, a proteomic approach identified seventeen SecA2-dependent secreted and surface proteins. The two most abundant of these proteins, the p60 and N-acetylmuramidase (NamA), hydrolyze bacterial peptidoglycan and contribute to host colonization. Coordinated peptidoglycan hydrolysis by p60 and NamA activity generates bacterial cell wall fragments. Thus, SecA2-dependent secretion may promote release of muramyl peptides that may modify host inflammatory responses and subvert host pattern recognition.

Taken together post-genomic comparative studies are paving the way to discovery of novel virulence factors and may help to understand pathogen evolution.

Conclusion and Perspectives

The *Listeria* genus is composed of six species among which *L. monocytogenes* is the only human pathogen. This food-born pathogen is responsible for listeriosis after ingestion of contaminated food. Listeriosis is characterized by a high mortality rate (>30%) especially in susceptible populations (immunocompromised patients, pregnant women, and the elderly). These reasons explain the absolute requirement for *L. monocytogenes* detection in food products and its eradication as soon as possible in food storage and processing.

L. monocytogenes is one of the most well-studied bacterial pathogens. This facultative intracellular bacterium has in the last twenty years become a paradigm to study host-pathogen interactions and bacterial adaptation to the mammalian host. Analysis of *L. monocytogenes* has provided considerable insight into how bacteria invade cells, move intracellularly, and disseminate in tissues, as well as tools to address fundamental processes in cellular biology. Thanks to its capacity to provoke cell-mediated immunity, *L. monocytogenes* was also used as an efficient tool to study the induction of the T-cell immune response, and this bacterium is being developed as a vaccine vector for antigen delivery, with potential a potential impact in infection and cancer. Continued research into the molecular biology of *Listeria* infection coupled with the availability of the complete genome sequences of *L. monocytogenes* and of the related non-pathogenic *L. innocua*, will continue to be fruitful to both basic microbiology and cellular biology.

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Taxonomy, Epidemiology and Identification

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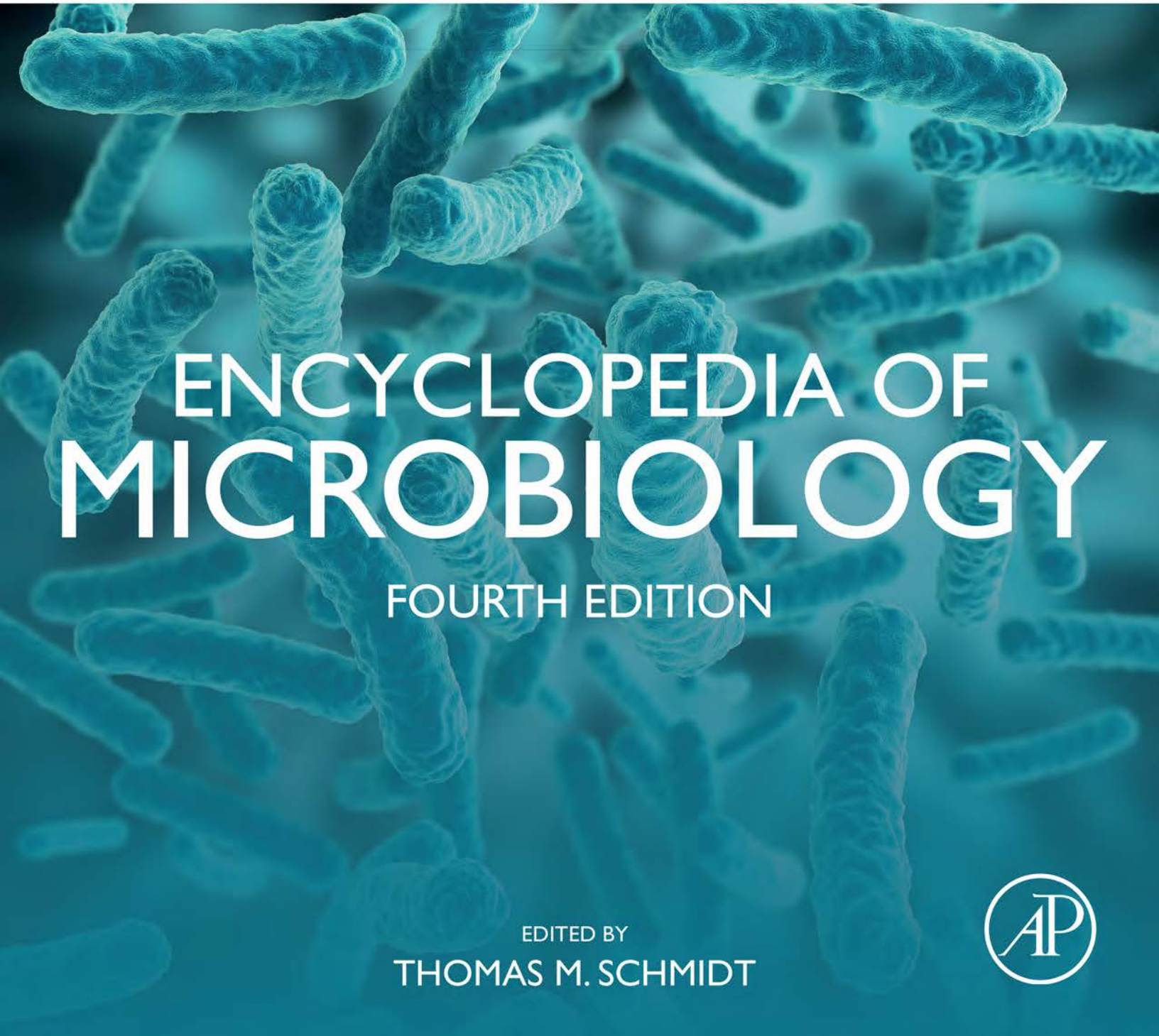

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Fourth Edition

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Thomas (Tom) Schmidt is a microbial physiologist and ecologist who studies diverse microbes and microbial communities. Tom received a PhD from The Ohio State University and conducted postdoctoral research at Scripps Institute of Oceanography and Indiana University. He spent much of his career studying the ecology of microbes in soil that are responsible for the exchange of greenhouse gases with the atmosphere. More recently, he joined the University of Michigan and focused his research on the human microbiome. His joint appointment in the Departments of Internal Medicine and Ecology & Evolutionary Biology reflects his expertise in applying ecological and evolutionary principles to understand the functioning of complex microbial communities.

Tom is a fellow of the American Academy for Microbiology and was director of the Marine Biological Laboratory's Microbial Diversity Course in Woods Hole. Through that course and in his laboratory, he has helped numerous scientists incorporate molecular approaches into traditional strategies for studying the microbial world. He currently teaches a university course that merges his research and teaching goals by engaging students in a coordinated study of the effects of diet on the gut microbiome, and he directs a graduate program that combines laboratory sciences and modeling. Tom was a section editor for previous editions of the *Encyclopedia of Microbiology* and was delighted to assume the role as editor-in-chief to help bring the microbial world to an expanding audience.

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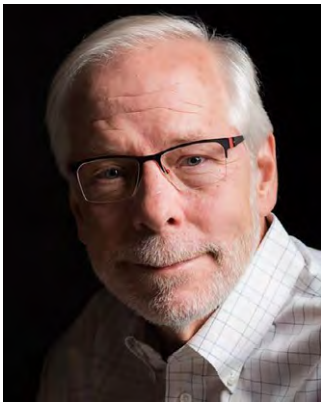
Bianca Brahamsha is a research microbiologist at Scripps Institution of Oceanography at the University of California, San Diego. She was a biology and history major at Goucher College, earned her PhD in microbiology at Cornell University, and carried out postdoctoral work in molecular genetics as an American Cancer Society fellow at the University of Chicago. Her research interests have centered on bacterial motility, the genetics and physiology of marine cyanobacteria, and on the interactions between cyanobacteria and their eukaryotic predators.

James (Jim) Brown was born in Atlanta, Georgia, and lived in Dade City (Florida), Bloomington (Indiana), Lima (Peru), and Muncie (Indiana) while growing up. From the beginning, he had an intense interest in nature, including anything found in the woods, rivers, beach or ocean that were always nearby.

Jim attended Ball State University beginning in 1976. A single lecture on microbial diversity in a general microbiology class, followed by the announcement of the discovery of the Archaea (George Fox and Carl Woese, 1977), sparked his lasting interest in microbiology. This led to undergraduate research examining *Beggiatoa* in a sulfur spring in French Lick, Indiana. After receiving his BS in biology in 1980, he joined the graduate program in microbiology at Miami University, where he worked on plant tissue culture mRNAs with Prof. Ronald Treick. After obtaining an MS degree in 1982, he moved to the MCD Biology Program at The Ohio State University to work on the molecular biology of methanogenic Archaea in the Department of Microbiology with Prof. John Reeve. While there, Jim worked on polyadenylation of mRNAs, RNA polymerase, and promoters in Archaea, and received his PhD in 1988. Jim then went to Indiana University for 5 years of postdoctoral work in Prof. Norman Pace's lab on the comparative analysis of the structure of a bacterial ribozyme, RNase P.

In January of 1994, Jim started as an assistant professor in the Department of Microbiology and moved to North Carolina State University. Research in Jim's lab focused on the comparative sequence and biochemical analysis of RNA, and in particular RNase P in Archaea. Students in the Brown lab developed a high-resolution model for the structure of this RNA, how it changed over the diversification of the Archaea, the protein subunits associated with the RNA, and how they contribute to the function of the holoenzyme. Jim developed, teaches, and wrote the textbook for a senior-level undergraduate lab course in microbial diversity. Jim is now Professor Emeritus in the Department of Biological Sciences at NC State University.

Jim has a son and two daughters, and is married to Melanie Lee-Brown, professor of biology and director of Research and Creative Endeavors at Guilford College. For fun, Jim enjoys skin and scuba diving, driving (i.e., working on) his 1968 Lotus Super Seven, and restoring vintage racing bicycles. Jim and Melanie have been renovating an abandoned fishing lodge on North Caicos Island (Turks and Caicos Islands, BWI), which will be reopened soon as a bed and breakfast.



Larry Forney is a university distinguished professor and a member of the American Academy of Microbiology with academic appointments in the Department of Biological Sciences and Bioinformatics and Computational Biology at the University of Idaho. Dr. Forney is an evolutionary ecologist who conducts research on the complex array of factors that influence the function, composition, structure, and temporal dynamics of bacterial communities in a wide array of habitats. In recent years he has largely focused on the community ecology of the human vagina across a woman's lifetime with an eye on understanding how changes in community structure and function affect a woman's risk to bacterial vaginosis, sexually transmitted infections, and pre-term birth, and how host factors shape these communities. His research extends to understanding the mutation-selection processes that govern the occurrence and persistence of genetic diversity of bacterial populations in spatially structured environments such as microbial biofilms and porous media. These studies have shown that spatial structure creates conditions in which mutation frequencies are high and selective sweeps are protracted, which leads to extraordinary within species diversity that increases the resilience of populations to environmental changes.

Robert Haselkorn was the F. L. Pritzker distinguished service professor of molecular genetics and cell biology at the University of Chicago, retiring several years ago. He was an undergraduate at Princeton, a graduate student at Harvard, and a postdoctorate in Cambridge, England. He started his teaching career at Chicago in 1961 in biophysics, extending later to microbiology, biochemistry, and chemistry. His research interests have centered on heterocyst differentiation in nitrogen-fixing cyanobacteria, in bacterial genomics, and in the enzyme acetyl-CoA carboxylase in plants, parasites, and people. He is a member of the National Academy of Sciences, a fellow of the American Academy of Arts and Sciences, and a member of the American Philosophical Society. Among other external activities he was a founder and adviser for 20 years to the International Center for Genetic Engineering and Biotechnology, located in Italy and India. For the past 15 years Haselkorn and his wife Margot have been working on selection and supporting a speaker for the Haselkorn Lecture at the University of Chicago, an award they have endowed. Many of the Haselkorn Lecturers have been Nobel Prize winners or will be soon.

Jennie C. Hunter-Cevera received her PhD from Rutgers University in 1978 (microbial physiology and biochemistry), an MS in microbial ecology in 1972, and a BS in biology from West Virginia University in 1970. She is the founder of Hunter and Associates, a consulting firm focusing on finding integrative solutions to complex problems involving sustainability in the life sciences arena. From July of 2009 to August 2012, she was the executive vice president of Discovery and Analytical Sciences (DAS), Corporate Development and Government Relations. She has 22 years of experience in the pharmaceutical and biotechnology industries (E. R. Squibb and Sons, Cetus Corporation, GeoBiotics, and Universal Foods). She was the co-founder of The Biotic Network and BlueSky Laboratory that contracted with biotechnology and pharmaceutical companies on basic and applied natural product research. Dr. Hunter-Cevera was an

employee for 5 years with the Department of Energy as the head of the Lawrence Berkeley National Laboratory's Center for Environmental Biotechnology. She worked in academia for 10 years as the president of the University of Maryland Biotechnology Institute and 2 years as the Interim Provost of Mount St. Mary's University. She also served as project manager for UC Davis's CIFAR (Center for Industrial Food and Agricultural Research). She has published many papers and chapters, and served as senior editor of the *Journal of Industrial Microbiology and Biotechnology* for 10 years. She is a co-section head of microbiology for Faculty of 1000. She holds 15 patents and specializes in areas of screen design for the discovery of natural compounds in the area of human therapeutics, biodefense, sustainable agriculture, bioremediation, and biocatalysis for industrial processes in the food and clothing industries.

While in Maryland, she served as a member of the Executive Committee for Governor Erhlich's transition team, his Committee on science, technology, engineering, and maths, and was the Technology Representative for Governor Glendenning on the Southern Governor's Association, and she served on the Technology Economic Development Corporation, TEDCO (Board of Directors for 4 years and was Chair for 2 years). Dr. Hunter-Cevera also served on the Advisory Board for the Maryland Industrial Partnerships, the MDBio's Board of Directors, and the MDBio Foundation for 10 years and on the BioIT Coalition for 5 years. She served as an Entremed Board Member for 10 years and served on the Executive Committee for 2 years. She was a board member for Patients with Power, a software company that designs programs for cancer patients to make the best decisions for their treatments. Jennie also served as acting secretary for Maryland's Higher Education Commission in 2015.

She is a member of several professional societies and has served as president of the Society for Industrial Microbiology, the International Marine Biotechnology Association, and the United States Federation of Culture Collections. Dr. Hunter has served on many national committees and commissions and was Chair of the National Research Council's Committee on Large Scale Production of Biofuels from Algae. She also chaired two other NRC Committees: Standing Committee on DOD's Translational Medicine and the DOE's Genome to Life for Biofuels.

She is the recipient of several awards and honors including Maryland's Top 50 Influential People (2007, 2009) and Maryland's Top 100 Women (2003, 2006, 2009), American Society for Microbiology's Porter Award for distinguished research in microbial systematics and taxonomy, elected as a SIM fellow, a member of the ASM Academy of Microbiology, and an AAAS fellow. She is also a WVU Distinguished Alumni Awardee and Nath lecturer and served as an entrepreneurial coach for the UNC Executive MBA program. She was awarded an Honorary Doctorate from West Virginia University, May 2013, and the Rutgers Cook College Dennis M. Fenton Distinguished Graduate Alumni Award in 2014.

She currently serves on the International Advisory Council for Brazil's Fundação Dom Cabral which is a world-class Brazilian business school that develops strategic thinking skills of executives, entrepreneurs, and public-sector managers. In addition, Jennie served 6 years on the Edison Awards Steering Committee and currently serves on the Edison Awards Advisory Board.

Stanley Maloy is a professor of microbiology and associate vice president for research and innovation at San Diego State University. Stanley's research has focused on bacterial and phage genetics and physiology, evolution of infectious diseases, and development of antimicrobials and vaccines. In addition, he has consulted with large and small companies in multiple sectors, played a role in starting several Biotech companies, and served leadership roles in multiple start-up companies.

Dr. McCormick earned her PhD in microbiology in the topic area of intestinal ecology, and completed postdoctoral training at Harvard Medical School. She remained on the faculty of Harvard Medical School where she was an associate professor of pediatric gastroenterology, and director of research for the Mucosal Immunology and Biology Research Center at Massachusetts General Hospital. In 2008, she joined the University of Massachusetts Medical School where she is professor and vice chair of the Department of Microbiology and Physiological Systems. Dr. McCormick is also the founding executive director of the University of Massachusetts Center for Microbiome Research, which she established in 2014. Dr. McCormick is one of the original pioneers in the field now known as cellular microbiology. Her work provided the first evidence that epithelial cells in response to pathogen contact orchestrate a pro-inflammatory program, which recruits inflammatory cells. Dr. McCormick has since identified new, previously unidentified and unexpected virulence mechanisms that are key to the inflammatory response, leading to both novel biological principles of host–microbe interactions and therapeutic intervention strategies for the treatment inflammatory bowel disease, and cancer. Her work continues to identify novel ways in which microbes interact with the intestinal epithelium, publishing over 100 original research papers and opinion pieces in this area. Dr. McCormick is an elected fellow of the American Academy of Microbiology, is on the Board of Editors for *Gastroenterology*, and *Gut Microbes*, and serves as a member of four Editorial Review Boards.

Dr. Mobley received his BS degree in biology from Emory University in 1975 and his PhD degree in microbiology and immunology from University of Louisville in 1981. He conducted postdoctoral training in biological chemistry and then bacterial genetics in the Center for Vaccine Development at the University of Maryland School of Medicine. He served on the faculty at the University of Maryland School of Medicine from 1984 until 2004 in the Division of Infectious Diseases (1984–97) and then the Department of Microbiology and Immunology (1997–2004) where he led the graduate program. During that time, he held a joint appointment in the Department of Biochemistry and Molecular Biology and trained graduate students in that program. In 2004, Mobley moved to the University of Michigan to chair the Department of Microbiology and Immunology and was installed as the inaugural Frederick G. Novy Collegiate Professor of Microbiology and Immunology.

Dr. Mobley's research interests focus on the molecular mechanisms of bacterial pathogenesis and on the fundamental basic research that will lay the groundwork for future therapeutics and vaccines. His lab studies virulence mechanisms of uropathogenic *Escherichia coli* and *Proteus mirabilis* that cause uncomplicated and complicated urinary tract infections, respectively, and, in the recent past, *Helicobacter pylori* that causes gastritis and peptic ulcer disease. For *E. coli* and *Proteus mirabilis*, his lab is focused on identifying surface-exposed proteins that are both synthesized by the bacteria during a urinary tract infection and conserved among uropathogenic *E. coli and Proteus* strains. Using these conserved antigens, his lab is determining the efficacy of candidate proteins as components of a multivalent subunit vaccine to protect against urinary tract infection.

Dr. Mobley is a fellow of the American Academy of Microbiology and a fellow of the American Association for the Advancement of Science and a member and past president of the Association of Medical School Microbiology & Immunology Chairs. He was the recipient of the inaugural University of Michigan Postdoctoral Association Excellence in Mentorship Award in 2012. He is a member of the editorial review boards of *Infection and Immunity and Microbiology Spectrum* and has served as a study section member for the National Institutes of Health. Dr. Mobley was awarded and named a distinguished university professor in 2015. This is the University's most prestigious professorship established to recognize senior faculty with exceptional scholarly achievements, national and international reputations for academic excellence, and superior records of teaching, mentoring, and service.

Dr. Mobley has published 255 peer-reviewed articles which have been cited in the literature over 18,000 times as of June 2019, as well as 49 book chapters and 5 books. He has trained 29 PhD students and 38 postdoctoral fellows, and has delivered 232 invited lectures in 21 countries.

Carlos Pedrós-Alió graduated in biology at the Autonomous University of Barcelona and got his PhD in bacteriology at the University of Wisconsin–Madison. After a postdoctoral stay at the Autonomous University he became an assistant professor of microbiology. He moved to the Marine Sciences Institute (CSIC, Barcelona) in 1989 where he was a research professor since 2000. In 2016 he moved to the National Center for Biotechnology (CSIC, Madrid). Dr. Pedrós-Alió's interest is to understand the ecology of aquatic microorganisms. Around 2005 he started to use genomics as a tool to generate hypotheses that could later be tested experimentally. He also likes to study extreme environments such as hypersaline systems, thermal springs, or polar waters. Another interest is in finding the mechanisms maintaining a large number of rare bacteria in aquatic ecosystems. He is also interested in outreach, relationships between art and science, biology of spirituality, fiction writing, and birding.

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PREFACE

As with previous editions of the *Encyclopedia of Microbiology*, the 4th edition takes on the challenge of providing a contemporary overview of microbes—the most abundant and diverse forms of life on Earth. These single-celled organisms were the first life forms to inhabit Earth and they transformed the planet and its atmosphere. They continue to maintain Earth's atmosphere, drive essential processes in terrestrial and aquatic ecosystems, and form intimate relationships with all plants and animals. Microbes can reproduce by doubling every 20 minutes or can remain dormant for many thousands of years. They colonize every known habitat on Earth, accounting for approximately half of the living biomass on our planet. Microbes also cause the most devastating diseases known to humans and are winning the antibiotic war we wage against them. Yet, we cannot live without microbes as symbionts of humans and drivers of Earth's biosphere.

Despite the central roles of microbes, we are only just becoming aware of our limited knowledge of interactions among microbes, other organisms, and the environment. Molecular techniques continue to lead a revolution in our understanding of microbial diversity and function, with the new "big data" sciences of genomics, transcriptomics, proteomics, and metabolomics now being applied to communities of microbes. These techniques provide the ability to identify and study complex communities of microbes in diverse environments including the human gut, forest soil, water-treatment biofilters, and the open ocean.

Given the remarkable physiological and phylogenetic diversity of microbes, their capacity for rapid evolution, and the pivotal role of host-associated and environmental microbiomes, it is difficult, if not impossible, to identify contemporary questions in biology that are not influenced by microbes. It is also increasingly difficult to assemble an encyclopedia that provides comprehensive coverage of the vast universe of microbes. Despite these challenges, we have engaged the expertise of scientists around the globe to provide an exceptional overview of and perspective on the microbial world. This edition of the *Encyclopedia of Microbiology* will help readers develop a framework for understanding microbes and will provide references directing readers to the primary literature that is needed for a more thorough appreciation of specific topics.

We have included a number of articles which provide a historical perspective of microbiology that is focused on disease—one of humanity's earliest acknowledgment of microbes. These help frame more contemporary issues in microbiology that have moved far beyond the relatively small collection of microbes that cause disease. Rather than simply alphabetize topics as in traditional encyclopedias, articles in the 4th edition are also listed under the following themes in a Subject Classification: Historical, Microbial Diversity, Physiology and Genomics, Ecology and Evolution, Pathogenesis and Immunology, and Technological Advances and Applied Microbiology. We hope this helps readers navigate the work more effectively.

On behalf of an insightful and collegial editorial team, I hope that you emerge from your forays into the *Encyclopedia of Microbiology* with some of the awe that we share for the elegance and power of the microbial world.

Thomas M. Schmidt
Editor-in-Chief

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INTRODUCTION: MICROBIAL DIVERSITY

It is what we think we know already that often prevents us from learning.

—Claude Bernard

One of the difficult lessons for any professional scientist is that, as much as we know, as good as our tools and questions are, our knowledge is crude and far smaller than we imagined when we were students. Particularly troublesome is that most fundamental aspects of various scientific fields are simply not very well understood. The physicist wonders what, exactly, "time" is, how space is structured, and why the Planck Constant is $6.62607004 \times 10^{-14}$ m^2 kg/s instead of any other number you might care to choose. Chemists argue about the nature of a covalent bond, or whether or not transition states exist in reality. Biologists are at a loss to describe how life originated, or even what exactly life (in a general sense) actually is. In the most important book of modern times, "On the Origin of Species," Charles Darwin clearly (but not concisely) deconstructs the idea of the biological "species," and despite what many of us learned in school, our view of the "species" even for multicellular sexually reproducing creatures remains murky. The modern reader of "On the Origin of Species" might be forgiven for concluding that the reason for this is that it is the actual *biology* of species, and by extension "life," our origin, etc., rather than our concepts of them, that are fundamentally disordered.

The subject of this section of the Encyclopedia is Microbial Diversity. What does this mean? What *is* "microbial diversity?"

Let us start with "microbial." Literally speaking, the term "microbial" means small living things, usually understood to mean life too small to see with the unaided eye. The scientific field of microbiology, however, mostly emerged from our need to solve crucial health dangers created by a tiny fraction of microbes. As a result, "microbiology," and so "microbes," refer to Bacteria (and in modern times the Archaea), fungi, a scattering of problematic single-celled or small multicellular eukaryotes, and the physiological systems of the human body that manages our interactions with these creatures. This sells the microbial world very short. For 85% of the history of our planet, life was *entirely* microbial, and it remains *predominantly* microbial. In the words of Stephen J. Gould, "*We live now in the 'Age of Bacteria.' Our planet has always been in the 'Age of Bacteria,' ever since the first fossils—bacteria, of course—were entombed in rocks more than 3 billion years ago. On any possible, reasonable or fair criterion, bacteria are—and always have been—the dominant forms of life on Earth.*" Another view of this comes from the phylogenetic perspective. If you scrutinize any objective/quantitative "Tree of Life," based on molecular sequence analysis or any other criterion of choice, you will quickly discover that nonmicrobial life is limited to the tips of a small number of otherwise un-noteworthy branches. Only from our self-centered anthropocentric viewpoint is microbiology distinguishable from biology as a whole.

And what of "diversity?" Living things and their morphology, structure, physiology, ecology, internal mechanisms, behavior, interactions, etc., are far more diverse than most of us have been led to believe. For example, if you think you know how mitosis works, look it up in *Giardia* and be amazed. The notion of what constitutes a "gene" in the kinetoplast of *Trypanosoma* requires you to throw almost everything you think you know about molecular genetics away. Then have a look through the genome of the archaeon *Nanoarchaeum.* These are just the tips of icebergs; these and many more can be found within the chapters that follow. And yet . . . all of this diversity rests over an almost entirely uniform biochemistry that speaks to the shared ancestry of all living things on Earth. How, then, do we assess the "diversity" of living things? Historically, this meant comparing the morphology of different living things; this was the origin of Linnaean taxonomy and, to be fair, is very useful for organisms on the human size scale. More recently molecular phylogenetics has allowed us to more clearly understand how creatures are related, and draw an objective and quantitative graph of these

relationships; the molecular "Tree of Life" is a more useful roadmap of biological diversity. But it is clear that an even more sophisticated view of biological diversity is required, for example to describe how horizontal gene transfer impacts "diversity," and many of these questions emerge from microbiology. Perhaps the most important fundamental question in microbiology today is "What is a microbial species?" In other words, how do we conceive of a "species" in organisms that do not reproduce sexually? This is not a trivial or parochial question; remember that the concept of biological evolution, and so the foundation of the science of biology, emerged directly from exploration of what a "species" is and how it originates, in plants and animals. But this was over 150 years ago. Broadening our view of microbial diversity, how it is structured and its history and origins, is the key to new and deeper biological insight.

And so, as is usual in science, attempt to answer a simple question that raises many more questions and provides few answers. What *is* microbial diversity? I dunno, but let us have a look. . ..

James Brown

INTRODUCTION: PHYSIOLOGY AND GENOMICS

The first two editions of EOM were edited entirely by Dr. Joshua Lederberg. The third edition was also planned largely by Dr. Lederberg, but he died during that phase, in 2008, and was replaced by Dr. Moselio Schaechter, promoted from the ranks of associate editors. Dr. Thomas Schmidt was also an associate editor for that edition and moved up to editor-in-chief for the fourth edition.

The time that has passed from the first to the fourth edition has seen remarkable advances in understanding the variety of microbes. Complete genome sequences were determined for 18 microbes by October 1998 and published in the second edition in 2000. By 2019, a single company had determined the complete annotated genome sequences of several thousands of microbes. Comparative genomics, including the ancillary "omics," as well as the application of systems biology to reconstruct metabolic networks, have provided new insights into many aspects of microbial physiology and genetics. The advent of cryo EM and of single particle cryo EM has revolutionized how we understand the structure of cells and macromolecules. For this fourth edition, we were guided by the splendid organization of the third and additional areas in which considerable advances have been made. The fourth edition now includes articles describing additional features of microbiology: cryo-electron microscopy, CRISPR/*cas*, and metabolic systems biology, as examples of completely new methods to study microbes, circadian rhythms as an important aspect of the physiology of at least one group of microbes, and new developments in our understanding of the many ways in which bacteria and archaea move. We have also made use of updates of chapters that were included in the third edition.

Bianca Brahamsha
Robert Haselkorn

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INTRODUCTION: ECOLOGY AND EVOLUTION

Evolution

Seven articles deal with different aspects of evolution. Two articles analyze the remains of the past evolution of microbes in the fossil record. Three other look at theories, mechanisms, and experimental procedures. Two more examine specific aspects of the evolution of viruses and of a ciliate. Finally, one article bridges ecology and evolution looking at the emerging field of evolutionary ecology of microbes.

Ecology

The ecology section contains a few general articles about what microbial ecology is about, and the particularities of the ecology of viruses and rare microorganisms.

The remaining articles can be placed in two complementary approaches: habitat oriented or process oriented. In the first approach there is a lot of attention devoted to extreme environments. These environments are usually exclusively microbial and show the capacity of microbes to deal with really difficult conditions. Besides, the relative simplicity of such ecosystems allows somewhat easier manipulations than in "normal" ecosystems. Several of the latter are also examined in articles ranging from the deep ocean to plastics. Special attention is given to animal and plant habitats and how microbes interact with their hosts.

The process-oriented approach shows, on the one hand, adaptive traits of microorganisms such as the ability to live in diluted environments or to use chemotaxis to find the optimal conditions. One promising and novel avenue to study adaptations is the analysis of the genomes of uncultured microorganisms. On the other hand, several articles review processes relevant at the ecosystem level, from feeding strategies such as myxotrophy to element cycles such as methane formation and oxidation and, finally, to the use of models in microbial ecology.

We hope this collection of articles provides an overview of the exciting times that microbial ecology is going through, with many new discoveries appearing every few years, and a fascinating and invisible world to be explored.

Carlos Pedrós-Alió
Larry Forney

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INTRODUCTION: PATHOGENESIS AND IMMUNOLOGY

This section of the encyclopedia focuses on the relatively narrow slice of all microbes that infect living hosts and how the hosts combat these microbes using innate and adaptive immunity. In addition, we examine how antimicrobial agents including unique metabolites and even bacteriophages have been enlisted to combat these infections. This section takes a broad look at infectious agents ranging from prions, viroids, and satellites to the more traditional etiologic agents like bacteria, viruses, fungi, and selected parasites. Also addressed are epidemiologic and diagnostic techniques used to identify and track the spread of infectious microbes including the most advanced diagnostic methods used by the clinical microbiology laboratory. A closer look at pathogenic mechanisms includes groups of virulence factors such as adhesins, iron acquisition, exotoxins of bacteria and fungi, and bacterial endotoxin (lipopolysaccharide). Strategies adopted by bacteria and fungi to colonize implanted foreign bodies such as catheters are also discussed. Plus, infections of the gastrointestinal tract, lung, oral cavity, and the skin are examined. In addition to pathogenic microbes, some articles examine the protective effect of the microbiome and commensal organisms. With respect to immunity this section also focuses on the interplay of the host immune system with pathogenic microbes covering evasion immune strategies in humoral and cellular immunity as well as innate immunology and the complement immune system.

Beth McCormick
Harry Mobley

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INTRODUCTION: TECHNOLOGICAL ADVANCES AND APPLIED MICROBIOLOGY

This section focuses on the development and refinement of the tools commonly used for both basic and applied microbiology. The articles in this section are written by authors who personally have worked on developing and fine-tuning technology for research in microbiology. Many of the products and processes described have multiple uses in a variety of disciplines such as health, the environment, food and agriculture, and industrial biocatalysis. The advances made in sequencing, protein analysis, and bioinformatics have given microbiologists new insights into old problems. These advances have also enabled the rapid identification of microorganisms in studies of biowarfare, infectious disease, and pollution of our water sources. At the same time the integration of mathematics and physics with biology and chemistry has provided instrumentation that enables a greater depth of analysis than ever before. Never in the history of microbiology have so many technological advances been developed and commercialized for use by scientists. Microbiologists today are equipped with improved "tools" within the toolbox to push the boundaries of not only elucidating the relationship of microbes to humans, plants and animals but also moving basic research much faster from the bench to development of new products and processes that benefit society and the planet. The renaissance of applied microbiology powered by technological advances has been both exciting and promising in all fields of study.

Jennie C Hunter-Cevera
Stanley Maloy

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Magnetotaxis[☆]

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In memory of Ulysses Lins (1969–2017): Ulysses Lins, a professor at the Microbiology Institute of the Federal University of Rio de Janeiro (UFRJ) Brazil, unexpectedly passed away at age 48. Lins' passion in microbiology was the magnetotactic bacteria, in particular, a unique, uncultured group of these organisms referred to as the "multicellular magnetotactic prokaryotes." The impact of Lins' outstanding work in biomineralization and magnetotactic bacteria cannot be overstated based on numerous important discoveries in his field. For his dedication to microbiology and his sincerity and generosity, his students and colleagues will always remember Professor Ulysses Lins with admiration and respect.

Glossary

Biologically controlled mineralization Highly regulated process in organisms that produces relatively complex materials with specific biological functions and well-organized structures.
Biologically induced mineralization The precipitation and deposition of minerals resulting from secondary chemical interactions between metabolic processes of organisms and the surrounding medium.
Biomineralization The formation of inorganic minerals by biological organisms.
Electron holography Electron interference technique that enables the extraction of amplitude and phase information for an electron wave scattered by a thin electron transparent specimen, in a transmission electron microscope.
Magnetosome Hallmark organelle of magnetotactic bacteria. A magnetosome consists of a nano-sized iron oxide or sulfide magnetic crystal enveloped by a lipid-bilayer membrane containing numerous proteins.
Magnetotactic bacteria Motile bacteria propelled by flagella that are capable of aligning in a magnetic field and navigating along magnetic field lines.
Magnetotaxis The term used to describe the magnetic response observed in magnetotactic bacteria and protists. It consists of the passive orientation and the active motility of the cell along magnetic field lines.

Abbreviations

BCM	Biologically controlled mineralization
BIM	Biologically induced mineralization
MMA	Magnetotactic multicellular aggregates
MMO	Magnetotactic multicellular organisms
MMP	Many-celled magnetotactic prokaryotes
PCR	Polymerase chain reaction

Introduction

Many bacteria are motile and swim using flagella and as they do, their swimming pattern changes from being random to being directed toward a more favorable environment. This more favorable environment, which varies from species to species, is tracked on

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the basis of sensing changes in a range of stimuli that includes both chemicals like nutrients and toxins and physical parameters like pH, light, and temperature. This behavioral response to directional stimuli is called taxis; more specifically, if the organism swims toward the stimulus, it is called positive taxis; and if away from the stimulus, it is called negative taxis. Among the most-studied stimuli and tactic responses in bacteria are: chemicals and chemotaxis; and light and phototaxis. This article deals with an unusual bacterial behavior in magnetic fields called magnetotaxis. It should be pointed out, however, that magnetotaxis is very different than other types of taxis in that magnetotactic bacteria do not swim toward or away from a magnetic field and only passively align along magnetic field lines like a compass needle.

The geomagnetic field is considered to be one of several important environmental cues for navigation by many different biological organisms. The Earth's geomagnetic field is important for life on this planet for several reasons including the fact that it creates a planetary shield against the solar wind and that it appears to be responsible for the orientation and migration by a relative large number of biological species. Different organisms that have been shown to respond in some way to the Earth's geomagnetic field include homing pigeons and other migratory birds, trout, sea turtles, social insects such as honeybees and ants, and the worm *Caenorhabditis elegans*. Among the microbes, the motility of several types of bacteria and protists is affected by magnetic fields. This behavior is referred to as magnetotaxis and the organisms as being magnetotactic. Magnetotactic microbes represent the best-understood example of magnetically influenced, biological navigation on Earth.

The first report of magnetotactic bacteria was made by Salvatore Bellini in 1963 in a publication of the Microbiology Institute (Instituto di Microbiologia) of the University of Pavia, Italy. His microscopic observations of freshwater sediments revealed large numbers of bacteria that swam in a single, consistent direction. Bellini realized that the bacteria swam toward the North Pole and hence called them magnetosensitive bacteria (batteri magnetosensibili). He presumed that some sort of biomagnetic compass was present in the bacteria. His idea proved to be true. Richard P. Blakemore, then a Microbiology graduate student at the University of Massachusetts at Amherst, reported in the journal Science in 1975, similar magnetosensitive behavior of bacteria in sediments collected from a freshwater swamp in Woods Hole, Massachusetts. He discovered that these magnetosensitive bacteria contained electron-dense, iron-rich crystalline inclusions. Blakemore also realized that these microorganisms were swimming toward the geomagnetic north in the Earth's magnetic field and named them magnetotactic bacteria and their behavior magnetotaxis. Since then, many multidisciplinary studies have been done on these microorganisms, many of which include recent advances toward understanding the molecular mechanisms underlying the biomineralization and ultrastructural organization of magnetosomes in bacteria. The controlled synthesis and organization of lipid-bilayer membrane-bounded organelles, the magnetosomes, using specific cytoskeleton elements has caused rethinking on some of the traditional boundaries used to distinguish eukaryotic cells from their prokaryotic counterparts, as prokaryotes were thought to not contain lipid-bilayer membrane-bounded organelles or an organized cytoskeleton. Magnetotactic bacteria are increasingly becoming the cell biology model for understanding the molecular mechanisms underlying the basic rules that govern the intracellular ultrastructural organization of prokaryotes.

Despite the efforts of a number of different research groups since 1980, relatively few representatives of magnetotactic bacteria have been isolated and cultivated in axenic culture. Due to a number of important improvements in developing growth media for these organisms, approximately 25 pure cultures of magnetotactic bacterial species have been isolated although only a subset of these have been validly named or available in microbial repositories for study. Difficulties in culturing members of this group are related to their fastidiousness as to specific growth requirements and their overall diversity among species. As a consequence, relatively little is known regarding their metabolic/physiological characteristics and versatility; many studies on their diverse morphology and phylogeny as well as mineralogy of their magnetosomes have relied on culture-independent methods.

General Features of Magnetotactic Bacteria

The term "magnetotactic bacteria" has no taxonomic significance and represents a heterogeneous group of bacteria with various cell morphologies/phylogenies/physiologies. The operational definition of magnetotactic bacteria is that they are a heterogeneous collection of prokaryotes that share the traits of magnetotaxis and the biomineralization of magnetosomes. The magnetosome is defined as an intracellular organelle consisting of a single-magnetic-domain crystal of a magnetic iron mineral enveloped by a lipid-bilayer membrane that contains proteins that are unique to it. Despite their heterogeneity, there are other features shared by these microorganisms.

Magnetotactic bacteria are morphologically diverse, comprising cocci, rods, spirilla, vibrios, and multicellular forms. All possess a Gram-negative cell wall and are phylogenetically associated with Gram-negative groups of prokaryotes (the *Proteobacteria* and the *Nitrospirae* phyla; the candidate phylum "Omnitrophica" of the *Planctomycetes–Verrucomicrobia–Chlamydiae* (PVC) superphylum and, possibly the candidate phylum "Latescibacteria") in the domain *Bacteria*. Although magnetotactic bacteria appear to be restricted to the domain *Bacteria*, there are no known specific reasons why there are no magnetotactic representatives phylogenetically associated with the *Archaea* and *Eukarya* domains. Eukaryotic microbes (protists) that respond to an applied magnetic field similarly to magnetotactic bacteria have been reported. However, this behavior appears to be associated with the ingestion of magnetotactic bacteria by these protists, leading to a temporary response to magnetic fields. Until now, there is no evidence to fully support autonomous magnetosome biomineralization in eukaryotes.

All currently known magnetotactic bacteria are motile by means of flagella. Because most studies on uncultivated magnetotactic bacteria relied on the use of a magnetic field for retrieving cells from water and sediment samples, it is possible that nonmotile bacteria that synthesize magnetosomes exist that would not swim toward the magnet and thus would not be collected and observed.

By definition, the latter organisms would be magnetic rather than magnetotactic (the term tactic here implies motility). Flagellation patterns in magnetotactic bacteria vary greatly and include polarly monotrichous, bipolar, or lophotrichous (possessing two tufts or bundles of flagella) representatives. The number of flagella per cell ranges from one in the marine vibrio, *Magnetovibrio blakemorei* strain MV-1, to over a thousand in the many-celled magnetotactic prokaryotes (MMPs). The swimming velocity also varies greatly between species and ranges from about 40 μm per second in members of the genus *Magnetospirillum* to over 1000 μm per second in some of the magnetotactic cocci. Although motility is a key factor in magnetotaxis, little is known about the structural and molecular components of flagella in magnetotactic bacteria. In some cases, flagella in many magnetotactic bacteria (e.g., the magnetococci) are shorter than those of many other bacteria (e.g., *Escherichia coli*) (Fig. 1). Tufts or bundles of flagella are typical of the magnetotactic cocci and originate from a disk-shaped depression or pit on the cell. Targeted disruption of the flagellin gene *flaA* was found to eliminate flagella formation and motility in *Magnetospirillum gryphiswaldense*. Large numbers of flagella are distributed only on one side of each of the cells of the MMP; the part of the cell exposed to the environment and thus the entire organism can be considered to be peritrichously flagellated.

Recently several studies with the magnetotactic coccus *Candidatus Magnetococcus massalia*, strain MO-1, isolated from the Mediterranean Sea and capable of swimming 300 μm per second, revealed some interesting features of the flagella properties of the magnetotactic cocci. In this strain, the flagella are powered by proton-motive force and a sodium ion gradient. The flagellar bundle is enclosed by a sheath described as a six-part, right-handed helical tubular structure with a diameter of about 100 nm and a helical pitch of about 260 nm. This sheath consists of a glycoprotein called a "sheath-associated protein" (Sap) and shows some conserved amino acid sequence with some known bacterial adhesins and eukaryotic cadherin. Every bundle contains seven flagella, with six individual flagella organized as a hexagon and the seventh in the middle. Strain MO-1 contains 14 homologs of flagellin genes encoded in the genome. In general, the genomes of magnetotactic bacteria contain more flagella-associated genes than the genomes of nonmagnetotactic bacteria. This characteristic might be associated with special swimming patterns or even the very high swimming velocities observed in the magnetotactic cocci.

Most magnetotactic bacteria swim in helical tracks. The flagella force the cell body of the bacterium to rotate around its axis while the cell is propelled by the flagella. Under the influence of the geomagnetic field, the consequence of the action of the short flagella is a misalignment of the trajectories that enables the cells to change the trajectory direction when they, for example, hit an obstacle in the sediment. The small misalignments during movement might be essential for certain magnetotactic bacteria to escape from physical "traps" present in sediments where they thrive. Recently, high-speed imaging showed that the trajectory of a magnetococcus was linear rather than helical as previously reported.

Magnetotactic bacteria are distributed worldwide in aquatic habitats that are neutral or close to neutral in pH. In general, they inhabit many aquatic environments where there is strong chemical stratification, that is, where vertical chemical (e.g., O_2) concentration gradients exist. In general, magnetotactic bacteria are obligately microaerophilic and/or anaerobic organisms and display a negative tactic and/or growth response to high concentrations of O_2 but also show a positive aerotactic response to low concentrations of O_2 (these two responses are generally referred to as aerotaxis). In natural environments they appear to prefer to be located at the oxic–anoxic interface where they are found in their highest numbers. This interface can occur in the water column or in the uppermost millimeters to centimeters of unconsolidated sediments depending on the environment.

In general, most magnetotactic bacteria biomineralize a single type of mineral in their magnetosomes: either the iron oxide magnetite (Fe_3O_4) or one or more iron sulfide, for example, greigite (Fe_3S_4) and its chemical precursors. The shape of the

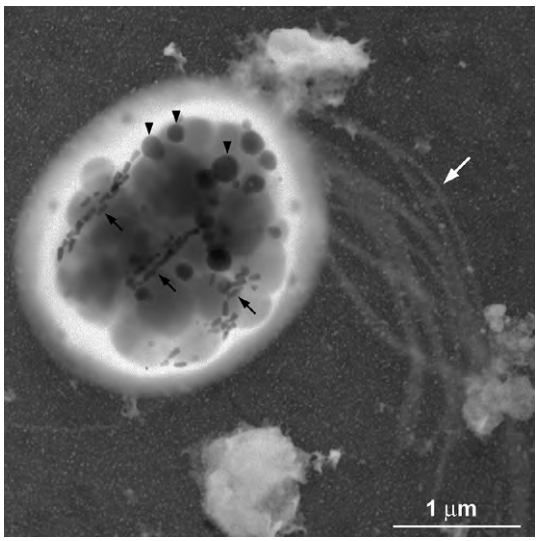

Fig. 1 Transmission electron micrograph of a freshwater magnetotactic bacterium. Three rows or chains (*arrows*) of bullet-shaped magnetosomes can be seen aligned parallel to the main axis of the cell. A flagella bundle is present at one end of the cell (*white arrow*). Several intracellular (internal) granules, possibly sulfur-rich inclusions, are also present (*arrowheads*).

magnetosome crystals is also considered to be species-specific. Currently described magnetosome crystal shapes include cuboctahedral (roughly cubic), elongated prismatic (appear rectangular in projection), bullet- or tooth-shaped, and pleomorphic (irregularly shaped more typical for greigite magnetosomes). Magnetite producers are usually found at the oxic–anoxic interface proper or slightly above it while some magnetotactic bacteria, particularly those that produce the iron sulfide greigite, are generally found below the oxic–anoxic interface in the anaerobic zone where sulfide is also present. It was thought that all freshwater magnetotactic bacteria and some marine, estuarine, and salt marsh morphotypes produce magnetite. However, greigite-producing magnetotactic bacteria, only previously found in brackish to marine environments, have been recently reported in freshwater environments. Many magnetotactic rod-shaped magnetotactic bacteria biomineralize both magnetite and greigite magnetosomes (sometimes in the same chain) and have also been reported from freshwater environments. The recently formally named *Desulfamplus magnetovallimortis* strain BW-1, isolated from a brackish spring at Badwater Basin, Death Valley National Park, California (United States) is an excellent cultured example of a magnetotactic bacterium that biomineralizes both minerals.

All magnetotactic strains in pure culture produce magnetite magnetosomes except *Desulfamplus magnetovallimortis* strain BW-1 which biomineralizes both mineral types in culture depending on conditions. Metabolically, all cultivated strains of magnetotactic bacteria have a respiratory metabolism and are unable to ferment except for *Desulfovibrio magneticus* strain RS-1 which has a solely chemoorganoheterotrophic metabolism and ferments pyruvate to acetate, CO_2, and H_2 in the absence of a terminal electron acceptor. Most known magnetotactic bacteria are mesophilic with respect to growth temperatures. Only recently have extremophilic MTB been described, including alkaliphilic, psychrophilic, halophilic and moderate thermophilic species. *Candidatus* Thermomagnetovibrio paiutensis strain HSMV-1 is an uncultured, moderately thermophilic magnetotactic bacterium found in Great Boiling Springs located in Nevada, United States, at temperatures close to 63°C but not higher. Three different magnetotactic strains were isolated from highly alkaline environments in California, United States. These alkaliphilic strains, designated as ML-1, ZZ-1 and AV-1, show optimal growth at pH values between 9.0 and 9.5. Psychrophilic magnetotactic bacteria, predominantly cocci, have been described in sediments from the Antarctic maritime region. The sequenced genomes of most cultivated magnetotactic bacteria contain the *nif* genes responsible for the fixation of atmospheric N_2 and almost all strains that have been tested exhibit nitrogenase activity. Because N_2 fixation is an O_2-sensitive process, microaerophilic magnetotactic bacteria might be further restricted to the oxic–anoxic interface in nitrogen-limited environments.

Characterization of Magnetotactic Bacteria in Pure Culture

All cultured magnetotactic bacteria phylogenetically belong to the *Proteobacteria* phylum (Table 1), most affiliated with the *Alphaproteobacteria* class. *Magnetospirillum* species make up most of the currently cultured magnetotactic bacteria and are freshwater, facultatively anaerobic microaerophiles that show varying tolerances to O_2 and biomineralize cuboctahedral crystals of magnetite in their magnetosomes. They are chemoorganoheterotrophs that use organic acids (e.g., succinic) as a source of electrons and carbon but several have the potential for chemolithoautotrophy as at least one strain has been shown to grow autotrophically and as many strains possess the gene for ribulose-1,5-bisphosphate carboxylase/oxygenase, a key enzyme of the Calvin-Benson-Bassham cycle for autotrophy, in their genomes. In addition to oxygen, most strains can use nitrate as an alternate terminal electron acceptor for

Table 1 Examples of cultivated magnetotactic bacteria

Phylum *Class*	*Species*	*Cell morphology*	*Magnetosome composition*	*Magnetosome shape*
Proteobacteria	*Magnetospirillum gryphiswaldense* strain MRS-1	Spirillum	Magnetite	Cuboctahedral
Alphaproteobacteria	*Magnetospirillum magneticum* strain AMB-1	Spirillum	Magnetite	Cuboctahedral
	Magnetospirillum magnetotacticum strain MS-1	Spirillum	Magnetite	Cuboctahedral
	Magnetospirillum caucaseum strain SO-1	Spirillum	Magnetite	Cuboctahedral
	Magnetospirillum marisnigri strain SP-1	Spirillum	Magnetite	Cuboctahedral
	Magnetospirillum moscoviense strain BB-1	Spirillum	Magnetite	Cuboctahedral
	Magnetococcus marinus strain MC-1	Coccus	Magnetite	Elongated, octahedral
	Magnetofaba australis strain IT-1	Coccus	Magnetite	Elongated, octahedral
	Magnetococcus massalia strain MO-1	Coccus	Magnetite	Elongated, octahedral
	Magnetovibrio blakemorei strain MV-1	Vibrio	Magnetite	Elongated, octahedral
	Magnetospira thiophila strain MMS-1	Spirillum	Magnetite	Elongated, octahedral
	Magnetospira sp. strain QH-2	Spirillum	Magnetite	Elongated, octahedral
Proteobacteria	*Desulfovibrio magneticus*	Vibrio	Magnetite	Bullet-shaped
Deltaproteobacteria	strain RS-1			
	Desulfamplus magnetovallimortis strain BW-1	Rod	Magnetite or greigite	Bullet-shaped or irregular
Proteobacteria	Strain SS-5	Rod	Magnetite	Elongated, octahedral
Gammaproteobacteria	Strain BW-2	Rod	Magnetite	Cuboctahedral

anaerobic growth. Thus far, there are seven validly described species of *Magnetospirillum*: *M. magnetotacticum* strain MS-1, *M. gryphiswaldense* strain MSR-1, *M. magneticum* strain AMB-1, *M. caucaseum* strain SO-1, *M. marisnigri* strain SP-1, *M. moscoviense* strain BB-1, and *M. bellicus* strain VDY. Of these seven strains, cells of *M. bellicus* are unusual in that they are apparently unable to biomineralize magnetosomes. A number of other strains of this genus have also been cultivated and partially characterized but not validly named. Interestingly, several related nonmagnetotactic spirilla with ~95% 16S rDNA sequence similarity to known species of *Magnetospirillum* have also been isolated. Tractable genetic systems have been developed for *M. gryphiswaldense* and *M. magneticum*.

Cultured marine species belonging to the *Alphaproteobacteria* include: the marine vibrio *Magnetovibrio blakemorei* strain MV-1; two species of cocci, *Magnetococcus marinus* strain MC-1 and *Magnetofaba australis* strain IT-1; and the spirillum *Magnetospira thiophila* strain MMS-1 and *Magnetospira* sp. strain QH-2. All of these strains are facultative chemolithoautotrophs also being able to grow chemoorganoheterotrophically. *Magnetovibrio blakemorei* strain MV-1 and *Magnetospira thiophila* strain MMS-1 utilize the Calvin-Benson-Bassham cycle for autotrophy, while *Magnetococcus marinus* strain MC-1 and *Magnetofaba australis* strain IT-1 utilize the reverse or reductive tricarboxylic acid cycle for autotrophy. All four strains oxidize reduced sulfur compounds (e.g., sulfide and/or thiosulfate) as a source of electrons and produce intracellular sulfur-rich globules. In marine, chemically stratified environments where an oxygen: sulfide double inverse concentration gradient exists, oxygen and sulfide generally overlap at the oxic–anoxic interface. Because these marine species require both compounds for energy, these organisms are restricted to the oxic–anoxic interface. Interestingly, many uncultivated cells of magnetite-producing magnetotactic bacteria collected from these environments also contain intracellular sulfur-rich globules suggesting that they also oxidize reduced sulfur compounds as a source of electrons. Strain MV-1 is a facultatively anaerobic microaerophile, capable of anaerobic respiration on some nitrogen oxides (e.g., nitrate, nitrous oxide) while strains MC-1, IT-1 and MMS-1 appear to be obligate microaerophiles. All four species biomineralize elongated prismatic crystals of magnetite in their magnetosomes.

A few magnetotactic bacterial strains from proteobacterial classes other than the *Alphaproteobacteria* have also been isolated in axenic culture. These include strains from the *Gamma-* and *Deltaproteobacteria*. Gammaproteobacterial strains include BW-2 and SS-5. Both are rod-shaped and are chemolithoautotrophs that oxidize reduced sulfur compounds for electrons and use the Calvin-Benson-Bassham cycle for autotrophy. Cells of strain BW-2 and SS-5 biomineralize cuboctahedral and elongated prismatic crystals of magnetite, respectively. Cultivated magnetotactic strains from the *Deltaproteobacteria* are all chemoorganoheterotrophic and dissimilatory sulfate reducing bacteria and include: *Desulfovibrio magneticus* strain RS-1 a curved rod-shaped sulfate-reducing bacterium that can also reduce fumarate as a terminal electron acceptor and has recently been shown to grow microaerobically; *Desulfamplus magnetovallimortis* strain BW-1, an obligately anaerobic bacterium; and several strains of obligately anaerobic, alkaliphilic bacteria isolated from strongly alkaline habitats all very closely related to the bacterium *Desulfonatronum thiodismutans*. All deltaproteobacterial magnetotactic bacteria biomineralize bullet-shaped magnetite crystals with the exception of *D. magnetovallimortis* strain BW-1 which biomineralizes greigite as well as bullet-shaped magnetites.

Characterization of Uncultured Magnetotactic Bacteria

The presence of magnetosomes and the trait of magnetotaxis allow for the magnetic manipulation and easy retrieval of uncultured magnetotactic bacteria from environmental samples with the aid of a magnetic field. Our current knowledge regarding the diversity of magnetotactic bacteria is based more on microorganisms harvested from the environment than on the isolation and characterization of pure cultures. Thanks to modern molecular techniques including the polymerase chain reaction (PCR), it is relatively easy to obtain and analyze the sequence of their 16S rRNA genes and therefore be able to determine phylogenetic relationships of these organisms.

The most commonly found morphotype of magnetotactic bacteria found in marine and freshwater environments are the magnetococci and thus numerous culture-independent studies have been focused on these organisms (Table 2). In addition, the magnetotactic cocci, unlike many other magnetotactic morphotypes, persist for long periods in microcosm experiments. All known magnetotactic cocci are phylogenetically affiliated with the *Alphaproteobacteria* class of the *Proteobacteria* phylum but are not closely related to any other alphaproteobacterium and appear to form a unique lineage within the group. All magnetotactic cocci biomineralize elongated prismatic crystals of magnetite although the arrangement of the crystals is variable including those with multiple chains and those with a clump (rather than a chain) of magnetosomes at one side of the cell (Fig. 2).

The techniques of gold labeling in conjunction with in situ hybridization and transmission electron microscopy revealed the presence of at least three different types of uncultured magnetococci in the Itaipu Lagoon in Brazil (Fig. 3). In this case, polyribonucleotide probes were labeled with digoxigenin or fluorescein and detected by immunolabeling with antifluorescein and antidigoxigenin antibodies bound to 10 and 15 nm gold particles. The use of this technique allowed for the correlation of phylogenetic association with a specific magnetosome morphology and organization; that is, to determine which 16S rRNA gene sequence belonged to which of the three cocci. The cocci were distributed as two clusters, referred to as Itaipu-1 and Itaipu-2, and were closely related based on 16S rRNA gene sequences which also showed they belonged to the *Alphaproteobacteria*.

A number of as yet uncultured magnetotactic bacteria are phylogenetically affiliated with the *Nitrospirae* phylum. Morphotypes of MTB phylogenetically associated with this phylum include: large rod- and ovoid-shaped cells and vibrios (Table 2).

Large numbers of a barbell-shaped magnetotactic bacterium were discovered in a chemically stratified coastal salt pond that displayed unusual magnetotactic behavior (discussed in the Magneto-aerotaxis section). This microorganism appears to consist of a

Table 2 Uncultivated magnetotactic bacteria

Phylum *Class*	*Bacteria* *Sampling site*	*Cell morphology*	*Magnetosome composition*	*Magnetosome shape*
Proteobacteria Alphaproteobacteria	Uncultured coccus Itaipu-I Brazil	Coccus	Magnetite	Elongated, prismatic
	Uncultured coccus Itaipu-III Brazil	Coccus	Magnetite	Elongated, prismatic
Proteobacteria Deltaproteobacteria	*Candidatus* Magnetoglobus multicellularis Brazil	Spherical MMP	Greigite	Irregular
	Ca. Magnetomorum litorale Germany	Spherical MMP	Magnetite	Bullet-shaped
	Ca. Magnetomorum rongchengroseum China	Spherical MMP	Magnetite or greigite	Bullet-shaped or irregular
	Ca. Magnetananas tsingtaoensis China	Elliptical MMP	Magnetite	Bullet-shaped
	Ca. Magnetananas rongchenensis China	Elliptical MMP	Magnetite	Bullet-shaped
	Uncultured barbell-shaped bacterium United States	Coccus	Not determined	Not determined
Proteobacteria Gammaproteobacteria	North-seeking bacterium strain NS-1 Brazil	Vibrio	Magnetite	Elongated, prismatic
Nitrospirae	*Ca.* Magnetobacterium bavaricum Germany	Rod	Magnetite	Bullet-shaped
	Ca. Magnetoovum mohavensis United States	Ovoid	Magnetite	Bullet-shaped
	Ca. Thermomagnetovibrio paiutensis strain HSMV-1 United States	Vibrio	Magnetite	Bullet-shaped
	Ca. Magnetobacterium bremense Germany	Rod	Magnetite	Bullet-shaped

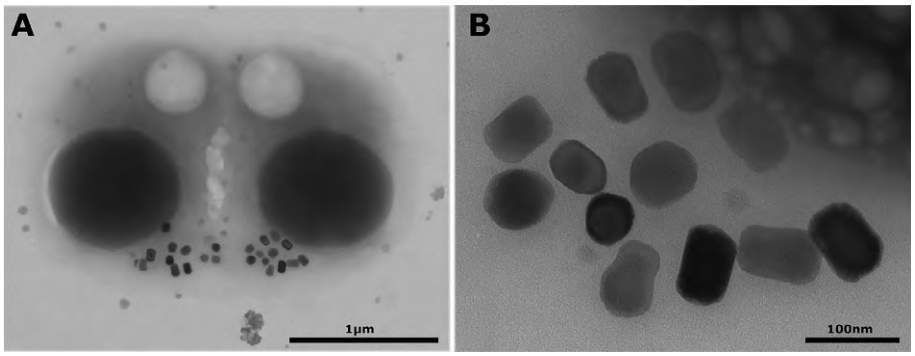

Fig. 2 Transmission electron microscopy image of a magnetotactic coccus containing a "clump" of apparently disorganized magnetosomes at one side of the cell rather than an organized chain. (A) Image of the magnetotactic coccus showing the presence of magnetosomes and granules. (B) Higher magnification image of magnetosomes.

chain of two to five cocci and belongs phylogenetically to the *Deltaproteobacteria*. Unfortunately, the composition of the magnetosome mineral was not determined.

An uncultured, relatively large (~5 μm long) slow-moving rod was retrieved from the chemically stratified Salt Pond, Woods Hole, United States and was found to be a member of the *Gammaproteobacteria* class of the *Proteobacteria* although there is some doubt as to the phylogenetic affiliation of this organism (Table 2).

Although one magnetotactic bacterium capable of producing both magnetite- and greigite-containing magnetosomes has been isolated in pure culture (discussed in the previous section), a number of uncultured magnetotactic bacteria have been described that are capable of biomineralizing both minerals and partially characterized using culture-independent techniques, including genomics. Evidence for the common ancestry of magnetite and greigite biomineralization in magnetotactic bacteria was revealed through the partial sequencing of the genome of an uncultured greigite-producing multicellular magnetotactic bacterium from sulfide-rich environment after magnetic concentration and separation. This approach allowed for the identification of specific genes considered unique for magnetite magnetosome synthesis.

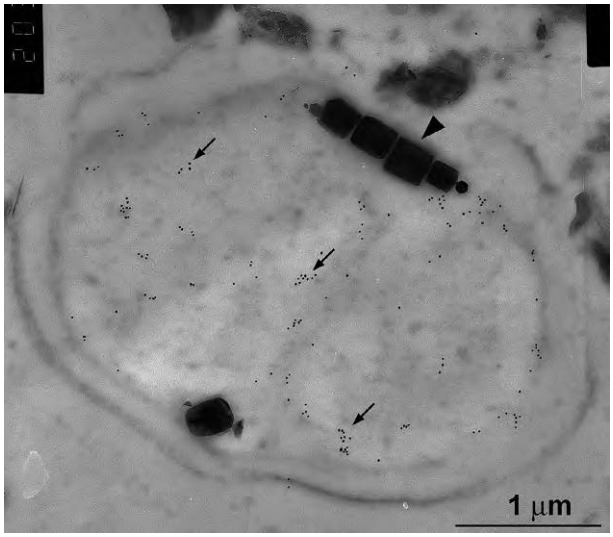

Fig. 3 Transmission electron microscope image of an ultra-thin section of a cell of a magnetic coccus collected from the Itaipu Lagoon in Brazil classified as Itaipu-1. Section was hybridized with a 16S rRNA gene-digoxigenin probe and detected by antidigoxigenin antibodies conjugated with 10 nm gold particles (*arrows*). Magnetosomes can be seen (*arrowhead*).

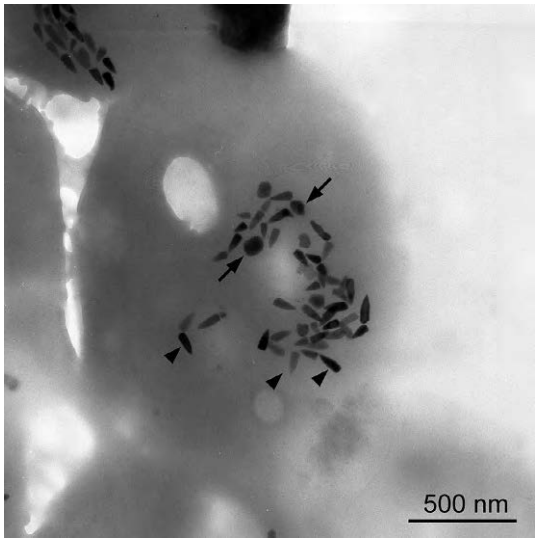

Fig. 4 Transmission electron microscopy image of a cell of a multicellular magnetotactic bacterium containing chains of bullet-shaped magnetite (*arrowheads*) and rounded greigite magnetosomes (*arrows*).

Morphologically conspicuous, multicellular magnetotactic bacteria have been reported from a number of different brackish, marine, or hypersaline environments in both the Northern and the Southern Hemispheres, usually in sulfide-rich environments very likely to be anaerobic (Table 2). These very unusual organisms are assemblage of Gram-negative cells that generally contain chains of greigite-containing magnetosomes. These organisms are not a consortium of cells of different species, but an aggregate of genetically identical cells. All those examined are phylogenetically affiliated with the *Deltaproteobacteria* class of the *Proteobacteria* phylum (Table 2). For many years, because of their unique morphology, these multicellular bacteria were thought to represent a single species. However, phylogenetic analyses of a collection of these organisms from a natural population present in a salt marsh (Falmouth, MA, United States) showed that this group of multicellular prokaryotes had a number of different 16S rRNA gene sequences separated by at least 5% divergence, suggesting that they are not a single species but represent a number of distinct species within a single genus. An unaffiliated multicellular magnetotactic bacterium capable of producing both greigite and magnetite magnetosomes was also reported (Fig. 4). Although several ellipsoidal-shaped types containing both magnetite and greigite have been characterized (Fig. 5), most multicellular magnetotactic bacteria are spherical in shape (Fig. 6).

These organisms have not yet been validly named (largely because they have not been isolated and grown in pure culture) and confusing and imprecise terminology has been used for them including magnetotactic multicellular aggregates (MMAs), many-celled magnetotactic prokaryotes (MMPs), and magnetotactic multicellular organisms (MMOs).

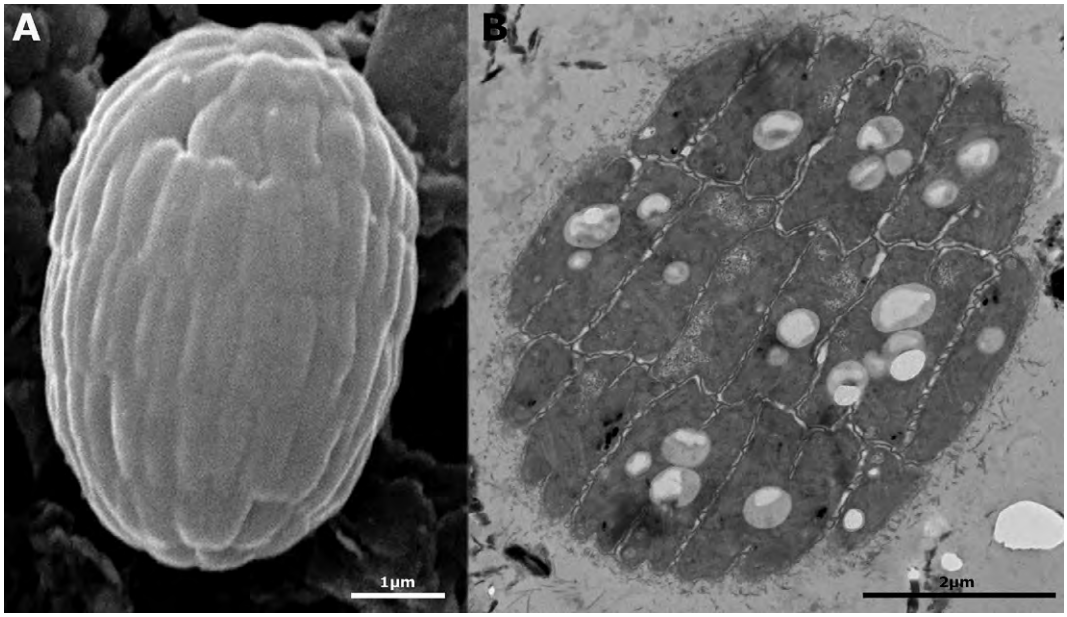

Fig. 5 Electron microscopy of ellipsoidal MMPs. (A) Scanning electron microscopy image of the microorganism showing cells organized as an ovoid-shape structure. (B) Transmission electron microscopy image of an ellipsoidal MMP showing cell arrangement inside the microorganism. Image in (A) was kindly given by Prof. Tian Xiao and Prof. Long-Fei Wu.

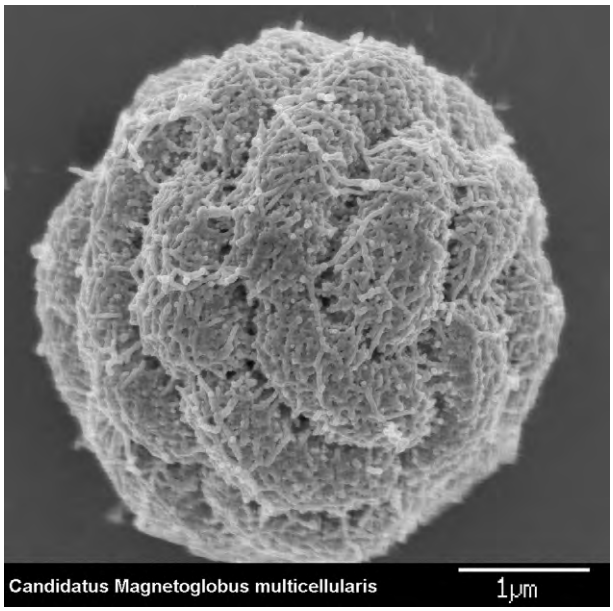

Fig. 6 Scanning electron microscopy image of *Candidatus* Magnetoglobus multicellularis showing its multicellular nature and its overall spherical morphology.

Like other spherical multicellular magnetotactic prokaryotes, *Candidatus* Magnetoglobus multicellularis is a member of the *Deltaproteobacteria* (Table 2). The most conspicuous structural feature of these microorganisms is the fact that its cells appear to be precisely organized and must live as a "colony" or unit and are unable to live individually (Fig. 7). Each cell faces both the external surrounding environment and the internal portion of the aggregate. If a cell leaves the assemblage, for example, when the organism dies, it loses its ability to swim and to orientate along magnetic field lines.

Candidatus Magnetoglobus multicellularis exhibits a unique method of reproduction. This has been studied in some detail and the following growth stages have been proposed: (1) individual cells grow in size thereby increasing their volume and their number of magnetosomes; (2) the cells then divide doubling the number of cells in the microorganism; (3) individual cell within the microorganism then reorganize; and (4) the microorganism divides into two new and identical units.

On the outer surface of the microorganism, cells of multicellular magnetotactic prokaryotes are covered with numerous flagella that are responsible for its coordinated complex movement. Four different types of motility have been observed in *Candidatus*

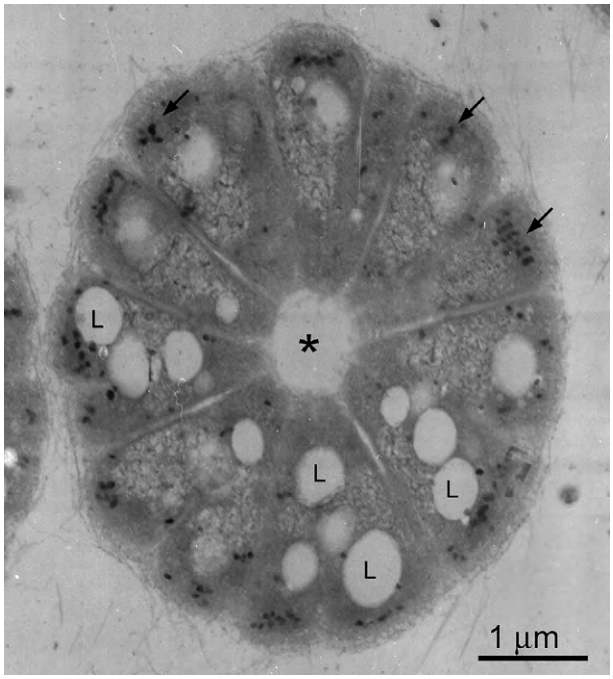

Fig. 7 Transmission electron microscopy image of an ultra-thin section of *Candidatus* Magnetoglobus multicellularis showing the general organization of cells and the internal compartment (at *asterisk*). All cells have a cell wall surface structure of Gram-negative bacteria and face both the external environment and the internal compartment. The numerous magnetosomes occur mostly at the periphery of the cells (arrows). Lipid or polyhydroxyalkanoate inclusions are marked (L).

Table 3 Motility of multicellular magnetotactic prokaryotes

Motility	*Description*
Free motion	The microorganism swims at speed up to 100 μm s^{-1} in either straight or helical trajectories along magnetic field lines in a uniform magnetic field
Rotation	At the edge of a drop under atmospheric conditions, the microorganism spins around an axis that passes through its center
Walking	At the air–water interface on the top of the drop, the microorganism swims freely in a complex trajectory, maintaining the same sense of body rotation
Escape	This movement consists of a backward movement (north-seeking in the Southern Hemisphere or south-seeking in the Northern Hemisphere) for tens or even hundreds of micrometers, followed by a forward regular movement. In the backward movement, the microorganisms decelerate continuously with time, whereas in the forward movement that follows, they display uniform acceleration. Also known as "ping-pong" response

Magnetoglobus multicellularis: free motion, rotation, walking, and escape motilities (Table 3). Coordination among cells in the multicellular prokaryote are considered necessary for all types of motility. A negative photo response has been observed in a multicellular magnetotactic prokaryote when illuminated with high intensity UV light (365 nm), violet-blue light (395–440 nm filter) of about 80 W m^{-2} of intensity and blue light (450–490 nm filter) of about 200 W m^{-2} of intensity. For longer wavelengths no photo response was observed. Photo kinesis has been observed in this multicellular magnetotactic prokaryote, decreasing their velocity when illuminated with green light (517 nm, 0.46 W m^{-2}) and increasing their velocity when illuminated with red light (628 nm, 0.16 W m^{-2}), both respectively to the velocity observed when illuminated with blue light (469 nm, 0.8 W m^{-2}). That effect is related to the presence of a constant magnetic field and it can be canceled in the presence of radio-frequency electromagnetic field at the Zeeman resonance frequency, showing the involvement of a radical pair mechanism, a very well-known magnetoreception mechanism used by migratory birds.

Unusual ellipsoidal-shaped multicellular magnetotactic prokaryotes were discovered in sediments and water from the intertidal zone of the Yellow Sea in Qingdao (Tsingtao) City, China and were present along with spherical types (Table 2). Magnetosomes were organized in chains close to parallel to the long axis of the organism. Motility in the ellipsoidal multicellular magnetotactic prokaryotes was similar to that of spherical types, showing the characteristic "escape" motility and also negative phototaxis for UV, violet and blue light.

Spherical multicellular magnetotactic prokaryotes change their behavior with magnetic field intensity in a way that cannot be explained by the simple compass model used for magnetotaxis in unicellular bacteria. This unexpected behavior led to the hypothesis that multicellular magnetotactic prokaryotes have a sort of magnetoreception mechanism in magnetic fields higher in intensity than that of the Earth's geomagnetic field. This magnetoreception mechanism and the unusual "escape" motility might imply transference of information from the magnetic interaction of the magnetosome chains to the flagellar motors. There is, however, no scientific evidence for any type of magnetic "sensor" such as this in magnetotactic bacteria.

Magnetosomes

Many prokaryotic microorganisms facilitate the formation of a large number of various minerals, including magnetosome crystals, through one of two biomineralization processes. These include biologically induced mineralization (BIM) and biologically controlled mineralization (BCM). There are several important differences between BIM and BCM. The most important differences relate to the control of the synthesis of the biominerals and the functionality of the biomineralized products. Generally, in BIM the microorganism exerts little control over the biomineral, which has no known recognized function for the bacterium. In BCM, the microorganism exerts strict crystallochemical control over the nucleation and growth of the biomineral, which in some cases has a somewhat defined function. Because of these features, BCM processes are considered to be under specific biochemical and genetic control. Examples of products of BCM in higher organisms are bones, teeth, or shells. In the microbial world, most known biominerals are produced by bacteria through BIM. The mineral particles produced by bacteria in BCM are characterized as well-ordered crystals with narrow size distributions, high chemical purity, and specific particle morphologies (Fig. 8). Generally, biomineralized particles produce through BIM lack these qualities. The most characterized example of BCM in bacteria is the magnetosome.

Magnetosomes are iron oxide or sulfide magnetic crystals, each enveloped by a lipid bilayer membrane (i.e., the magnetosome membrane). As previously stated, there are two compositional types of magnetic minerals in magnetosomes: magnetite (Fe_3O_4) and greigite (Fe_3S_4). Additional nonmagnetic iron sulfide minerals, including mackinawite (tetragonal FeS) and a cubic form of FeS, have been found in some greigite-producing magnetotactic bacteria and are assumed to represent precursors of greigite. In most

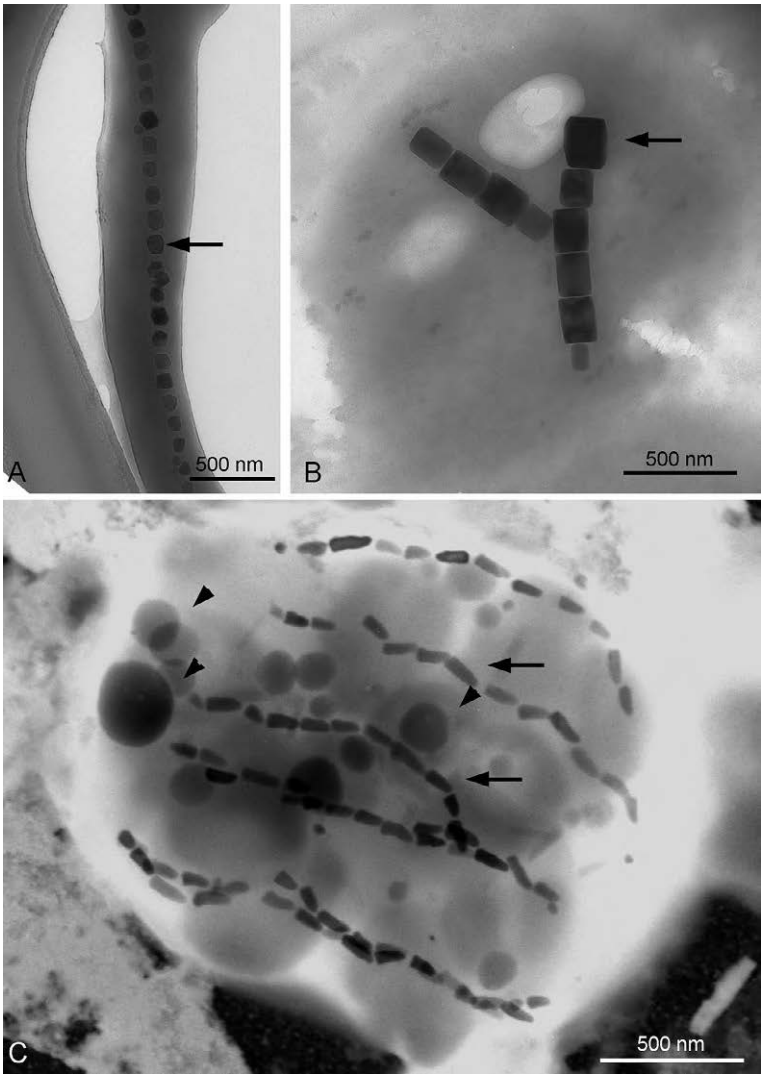

Fig. 8 Transmission electron microscopy images of magnetosome magnetite crystal morphologies. (A) Cell of *Magnetospirillum magnetotacticum* containing a chain of cuboctahedral magnetosomes (*arrow*), (B) an uncultivated marine coccus with two chains of elongated prismatic magnetosomes (*arrows*), (c) uncultured freshwater magnetotactic bacterium with rows of bullet-shaped magnetosomes (*arrows*) and sulfur-rich inclusion (*arrowheads*).

Fig. 9 High-resolution transmission electron microscopy image of a bullet-shaped magnetite magnetosome biomineralized by magnetotactic bacteria.

magnetotactic bacteria, magnetosomes are organized in a chain or chains within the cell. This has important physical significance and is discussed later.

The nature of the magnetosome membrane has been studied in several magnetite-producing *Magnetospirillum* species. The magnetosome membrane appears to originate as an invagination of the cell membrane and contains a number of proteins unique to it that are thought to be responsible for regulating the size, the chemical purity, and the morphology of the magnetosome crystal.

The crystalline phase of the magnetosome has been extensively studied using a number of forms of electron microscopy. Magnetotactic bacterial magnetite typically has cubic {100}, octahedral {111}, and dodecahedral {110} faces in crystals that have quasi-rectangular projected shapes in the horizontal plane or are bullet-, tooth-, or arrowhead-shaped (Fig. 9). Morphologies of greigite crystals are similar and include the same forms as magnetite but, in contrast, the crystals often appear "wrinkled" and have an irregular border. The number of magnetosomes varies from a few to thousands per cell.

Regardless of mineral type, magnetosome crystals are generally in the size range of 35–120 nm, which corresponds to the single-magnetic-domain size range for magnetite or greigite. However, magnetosomes up to 250 nm have been reported. The significance of the size of the mineral crystals is discussed in the next section.

Biophysics of Magnetotaxis

As previously stated, in general, the sizes of magnetosome crystals in magnetotactic bacteria are within the permanent single-magnetic-domain size range for magnetite (~35–120 nm), meaning that they are uniformly magnetized with the maximum magnetic dipole moment per unit volume. Magnetosomes larger than single magnetic domains are not uniformly magnetized because of the formation of domain walls, resulting in multiple magnetic domains. The overall effect of being larger than the single-magnetic-domain size range is the significant reduction of the magnetic dipole moment. On the other hand, very small particles (<35 nm) are superparamagnetic, which means that they are still uniformly magnetized but their magnetic dipole moments are not constant because of thermally induced spontaneous reversals. This results in a net magnetic moment of zero over time. Thus, the single-domain size range is the optimum particle size for the maximum magnetic dipole moment in a magnetosome.

Magnetosomes are anchored to the cell membrane in most, if not all, magnetotactic bacteria and are thus fixed in place within the cell. In most magnetotactic bacteria, the magnetosomes are arranged as a chain or multiple chains. Experimental evidence

involving *Magnetospirillum* species shows that magnetic interactions between the crystals are not sufficient to align them in a chain. The initial step in the biomineralization process is the invagination of the cytoplasmic membrane to form the magnetosome membrane vesicle. Magnetite or greigite are biomineralized within these vesicles and may possibly nucleate even while the magnetosome membrane is still an invagination with a connection to the periplasm. In most magnetotactic bacteria, at some point, it is thought that the membrane invagination detaches from the cytoplasmic membrane, forming a separate magnetosome vesicle. However, in cells of *Magnetospirillum magneticum* strain AMB-1, the magnetosome membrane may remain as cell membrane invaginations. In both cases, cytoskeletal filaments composed by the protein MamK, an actin-like MreB homologue, are necessary to organize the magnetosomes in chain in the cytoplasm and attach this structure to the cellular framework. This results in an efficient transfer of torque to the cell body when the cell is in a magnetic field.

A number of the genes thought to be involved in the formation of the magnetosome membrane and biomineralization of magnetite/greigite have been studied. Several different genes have been identified that encode magnetosome-associated proteins using cultured *Magnetospirillum* species as a model (mainly *Magnetospirillum gryphiswaldense* strain MSR-1). Most genes associated thought to be involved with magnetosome biogenesis are unique to magnetotactic bacteria. In *Magnetospirillum gryphiswaldense* these genes are clustered in a region of the genome that is flanked and interrupted by transposase and tRNA genes. These are features typical of what is referred to as a genomic island and thus, this genomic region in *Magnetospirillum gryphiswaldense* is referred to as a magnetosome island (MAI). In the magnetotactic *Alphaproteobacteria*, set of approximately 30 genes have been described and named the *mam* (*ma*gnetosome *m*embrane) and *mms* (*m*agnetic particle *m*embrane *s*pecific) genes. The following steps have been described for the BCM biomineralization process of magnetosomes based on studies using *Magnetospirillum* species: (1) invagination of the cytoplasmic membrane to form the magnetosome vesicle; (2) iron transport into the vesicle by the action of the cation transporters MamM and MamB; (3) nucleation and controlled growth of the magnetic crystals;(4) formation of magnetosome chains through the interaction of MamJ on the magnetosome vesicle with the cytoskeletal filament protein MamK and positioning the magnetosome chain in the middle of the cell.

The magnetosome chain(s) within the cell impart a magnetic dipole moment to the cell that overcomes the thermal forces that tend to randomize the position of the cell in the aqueous environment in which the bacteria live. The magnetosome chain causes the cell to behave as a miniature magnetic compass needle, continuously causing the cell to passively align along local magnetic field lines. This alignment is not perfect however and disturbed mostly by the thermal fluctuations of the aqueous medium. Thus the alignment of magnetotactic bacteria along geomagnetic field lines depends on the ratio the magnetic to thermal energies, or MB/k_BT, where M is the magnetic dipole moment of the cell, B is the geomagnetic field strength, k_B is the Boltzmann constant, and T is the ambient temperature. When magnetosomes are arranged in a linear chain within the bacterial cell, the total magnetic dipole moment of the cell is the sum of the magnetic dipole moments of the individual crystals. The organization of magnetosomes in chains causes the individual magnetic dipole moments of the single-magnetic-domain magnetosome crystals to orient parallel to each other along the chain. In this case, the permanent magnetic dipole moment for the entire chain is optimum, that is, it approaches the maximum value possible. Electron holography in the electron microscope, magnetic force microscopy and pulsed-magnetic-field remanence measurements on individual cells have demonstrated the permanent magnetic microstructure of the magnetosome chain(s).

At ambient temperature, 10–20 magnetosomes (~50 nm in diameter) in a chain would be sufficient for passive orientation of a magnetotactic bacterium along the Earth's magnetic field lines. The efficient orientation in the geomagnetic field results in the migration of the cell along the magnetic field lines as it swims. If the magnetic field is increased (e.g., by exposing the cells to a bar magnet), the time-averaged orientation of the cell along the field is increased and the migration rate of the cell in the magnetic field direction is increased, even though the swimming speed of the cell remains unchanged.

Intriguingly, many magnetotactic bacteria seem to produce more magnetosomes than is necessary for orientation suggesting that magnetosomes might be responsible for other roles, perhaps physiological, than simply the magnetic orientation of the cell. Unusually large magnetite-containing magnetosomes (up to ~250 nm in length) were reported in an uncultured magnetotactic coccus, named Itaipu-1, collected from the Itaipu Lagoon, near Rio de Janeiro, Brazil. Because of the large size of the magnetic crystals, they would be expected to be outside the single-magnetic-domain range. However, electron holography measurements demonstrated that the crystals in fact behave as single-magnetic-domains while they are organized in chains. Breaking or removal of these magnetosome crystals from the chains results in a complicated magnetic microstructure with domain walls.

The functional significance of the unusually large volume of the magnetosomes is unknown. It has been speculated that the large crystals in Itaipu-1 occur in order to compensate for the anomalous low magnetic field strength (0.23 gauss) of the South Atlantic Magnetic Anomaly region, which includes Itaipu Lagoon. However, a calculation of the total magnetic dipole moment for a typical Itaipu-1 cell results in $MB/k_BT = \sim 250$, which is over 10 times the minimum value required for efficient magnetotaxis, suggesting there may be other functions of the magnetosomes in Itaipu-1.

Magnetotaxis

It is important to understand that magnetotaxis is somewhat of a misnomer and does not represent a true taxis response as in phototaxis or chemotaxis. Magnetotactic cells do not swim toward or away from a magnetic field source. Instead, magnetotaxis is the combination of the passive orientation and active motility by flagella, of magnetotactic bacterial cells along magnetic field lines. All known magnetotactic bacteria generally swim bidirectionally although some are known to "loop" while swimming. None exhibit run-and-tumble motility as do some nonmagnetotactic bacteria (e.g., *Escherichia coli*). Most magnetotactic bacteria swim

following an helical-like trajectory whose characteristics depends on the relative inclination of the magnetosome chain and the flagella, according to theoretical studies done on mono- and bi-flagellated bacteria.

Magnetotaxis is considered advantageous to bacteria in vertical chemical concentration gradients (e.g., O_2) in locating and maintaining an optimum position in the gradient because magnetotactic bacteria use the Earth's magnetic field lines as a fixed reference to swim, unlike other bacteria. Presumably, if a magnetotactic bacterium is displaced from its optimal position, it would use geomagnetic field lines to more efficiently return to its optimal position. Magnetotactic bacteria, after aligning along geomagnetic field lines, would have a one-dimensional search problem in locating their optimal position in a vertical chemical concentration gradient whereas nonmagnetotactic bacteria would have a three-dimensional search problem, presumably expending more time and more energy.

The original model of magnetotaxis stated that all magnetotactic bacteria had one of two possible swimming polarities: north-seeking and south-seeking. The swimming polarity depends ultimately on the direction of motion of the cell under oxic conditions. This was based on the observation that in the Northern Hemisphere, magnetotactic bacteria predominantly swim parallel to the magnetic field toward the north, hence the term north-seeking. In the Southern Hemisphere, the opposite was observed and south-seeking bacteria swim antiparallel to the field. Because, the geomagnetic field is inclined downward from the horizontal in the Northern Hemisphere and upward in the Southern Hemisphere, with the magnitude of the inclination increasing from the equator to the poles, the outcome of this situation is that in both hemispheres the bacteria swim downward toward the region of the sediment with little oxygen or no oxygen. In the original model, once a bacterium reached a layer of the sediment with suitable oxygen concentration, it would stop moving and possibly adhere to a sediment particle.

Two important observations were inconsistent with this magnetotaxis model: (1) the observation that north-seeking magnetotactic bacteria in pure culture (e.g., *Magnetococcus marinus* strain MC-1) form horizontal bands at certain positions below the meniscus in culture tubes containing an O_2 concentration gradient, instead of swimming persistently to the bottom of the culture tube; and (2) some populations of uncultured magnetotactic bacteria were observed in the water column in chemically stratified aquatic environments, not in the sediments. Should the original model be true, all magnetotactic bacteria would swim to the bottom of culture tubes and to the sediment in natural aquatic environments. An expansion of the magnetotaxis model was then proposed based on the observation that magnetotaxis appears to function in conjunction with aerotaxis. This is known as the magneto-aerotaxis model.

Magneto-Aerotaxis

Originally, two different magneto-aerotactic mechanisms were proposed: axial and polar magneto-aerotaxis. In axial magneto-aerotaxis, cells do not show a polar preference and the magnetic field defines the axis but not the direction of swimming. This mechanism is typical of cells of *Magnetospirillum* species grown in liquid media. Cells spontaneously reverse their swimming direction in an applied magnetic field. Thus, the distinction between north-seeking and south-seeking does not apply to axial magneto-aerotactic spirilla. Recent studies on cells of 12 strains of cultured magnetotactic bacteria of various morphotypes, including spirilla, in microcapillary tubes containing an O_2 concentration gradient and placed in a homogeneous magnetic field showed six different behaviors for north-seeking magnetotactic bacteria when the O_2 gradient and the magnetic field were parallel, and not simply polar and axial magneto-aerotaxis as described above. It was proposed from the results, that there are three general magneto-aerotactic mechanisms: axial, dipolar (formerly called polar) and unipolar magneto-aerotaxis.

In dipolar magneto-aerotaxis, the most common mechanism observed in natural populations of magnetotactic bacteria, cells show a polar preference in their swimming direction. The magnetic field provides both direction and axis for the magnetotactic behavior. This is based on experimental findings of the cultured coccus *Magnetococcus marinus* strain MC-1. In the magneto-aerotaxis model, magnetotactic bacteria can be either in an oxidized or in a reduced state. In the oxidized state, when in the oxic zone of a vertical concentration gradient of O_2, cells swim downward. They do this by rotating their flagella counterclockwise (Fig. 10). However in the reduced state, their flagella would rotate clockwise and the bacteria would reverse direction without turning around (they would still be aligned along magnetic field lines) and thus move upward, again toward the optimal O_2 concentration. In this model, magnetotactic bacteria still benefit from having a fixed axis for swimming.

The dipolar magneto-aerotaxis model can be extended to redoxtaxis in habitats in which spatially separated reservoirs of reductants and oxidants coexist. In these cases, some magnetotactic bacteria would swim into anoxic zones of their habitat to accumulate reduced compounds possibly as a source of electrons for growth (e.g., reduced sulfur compounds) and then swim back to zones in which oxidized compounds (e.g., O_2) are present and perhaps required for growth.

Unipolar magneto-aerotaxis is similar to the dipolar magneto-aerotaxis, although cells swim persistently in one direction (toward the anoxic region or toward the oxic region) using the geomagnetic field as a guide. Once the persistent anoxic unipolar magnetotactic bacteria reach an oxic region, they reverse their swimming direction, reaching the anoxic zone again and vice-versa.

There is evidence that magneto-aerotaxis does not explain the behavior of all magnetotactic bacteria particularly those in natural environments. For example, a population of mainly south-seeking magnetotactic bacteria (the barbell-shaped bacterium discussed in an earlier section) has recently been discovered in a chemically stratified environment in the Northern Hemisphere. In the Southern Hemisphere, a population of north-seeking magnetotactic bacteria was also characterized in a lagoon in the South Hemisphere. These microorganisms seem to represent a deviation from the magneto-aerotaxis model for magnetotactic bacteria as they were found in the anoxic zone of their environment and putatively swam in the theoretically "wrong" direction, that is, they

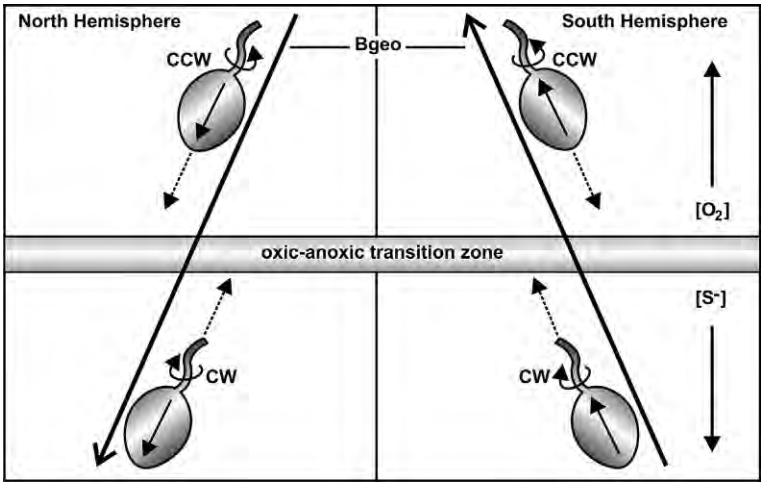

Fig. 10 Schematic drawing showing how magneto-aerotaxis may help magnetotactic bacteria to locate and maintain an optimal, preferred position in natural habitats with vertical chemical concentration gradients (e.g., O_2). See text for details.

had the "wrong" magnetic polarity. In these situations, if cells follow the polar magneto-aerotaxis principle, they would swim persistently to a less favorable/optimal environment.

For cells of *Magnetospirillum gryphiswaldense*, magneto-aerotaxis experiments showed that an increase of the geomagnetic field intensity 10-fold or a tilt of 45 degree of the magnetic field relative to the O_2 concentration gradient did not significantly change the magneto-aerotaxis efficiency, although it was drastically reduced when the magnetic field was null or when the tilt was 90 degree. These results show that magneto-aerotaxis efficiency is not proportional to the cosine of the tilt angle, as assumed by classical models of magneto-aerotaxis.

In both polar and dipolar magnetotactic bacteria, the swimming polarity can be switched from north-seeking to south-seeking and vice-versa by exposing cells to demagnetizing magnetic fields of 50 Hz and above 0.1 T, or even at pulses of 1 to 5 ms at about 0.07 T. These observations were used to show that magnetosome magnetite crystals are single-magnetic-domains. There is now evidence that redox gradients combined with manipulations of the magnetic field direction can also change the swimming polarity of magnetotactic bacteria. In cultures of *Magnetospirillum gryphiswaldense*, it has been shown that the swimming behavior of cells can be switched from axial to dipolar in the presence of O_2 concentration gradients and magnetic fields oriented parallel or antiparallel to the gradient. In addition, sudden increases in the local O_2 concentration results in the conversion of cells to the opposite polarity. Collecting and/or concentrating magnetotactic bacteria from mud and water samples using strong magnets can also reverse their swimming polarity and thus caution must be used in determining the polarity of magnetotactic bacteria in environmental samples. In addition, a high number of chemotactic receptor genes are present in many magnetotactic bacterial genomes (e.g., 56 genes appear to encode for chemotactic receptors in *Magnetospirillum gryphiswaldense* strain MSR-1) in comparison to other bacterial genomes (e.g., five genes encoding chemotactic receptors in *Escherichia. coli*), possibly indicating that chemotaxis is more complex in magnetotactic bacteria possibly suggesting that the interaction between chemotaxis and magnetotaxis is also complex. Thus, additional models of magnetotaxis, possibly associated with chemical concentration gradients other than O_2 (e.g., sulfide), are likely needed to explain the behavior of some magnetotactic bacteria.

Magnetotactic Eukaryotes

A magnetotactic response was reported in some eukaryotic microbes (protists) collected from coastal areas. Transmission electron microscopy revealed intracellular iron-rich crystalline inclusions identical to bacterial magnetosome magnetite crystals in some of these organisms. Intact magnetotactic bacteria were not observed in the cells. Two possibilities exist for the origin of these magnetosomes. First, the protist itself might biomineralize magnetosomes. This seems to be the case for the magnetotactic euglenoid alga *Anisonema platysomum* found to be present in a marine habitat in Brazil. This protist produces rows of chains of bullet-shaped magnetosomes in an elaborate pattern along the long axis of the cell. The morphology of the crystals is uniform and the magnetotactic response is consistent with the magnetic polarity of magnetotactic bacteria from the same environment as they show a directional swimming preference in the magnetic field.

Second, bacteria-grazing protozoa may ingest magnetotactic bacteria and their magnetosomes, which may be stable or partially or fully solubilized. They might be incorporated into the cell or present for a short time in food vacuoles, the likely site for solubilization. This seems to be the case for certain biflagellates, dinoflagellates, and ciliates detected in coastal areas. The significance and implications of this, as well as details of protozoal grazing of magnetotactic bacteria, is discussed later. Regardless of the origin of magnetite or greigite crystals in protists, some of these eukaryotic microbes contain a significant amount of intracellular iron.

Geochemical, Geophysical, and Astrobiological Aspects of Magnetotactic Bacteria

Cultured magnetotactic bacteria are known to facilitate a number of important geochemical processes and thus it is likely they play important roles in these processes in natural environments. These include sulfide oxidation, sulfate reduction, denitrification, nitrogen fixation, and iron redox reactions (iron cycling) through magnetosome biomineralization and possibly through iron oxidation not related to magnetosome synthesis. In addition, all cultured marine strains are facultatively autotrophic through either the Calvin-Benson-Bassham or the reverse (or reductive) tricarboxylic acid cycles. The freshwater magnetospirilla show potential for autotrophy at least one species has been shown to grow autotrophically and as most appear to contain in their genomes an intact ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) gene, which encodes for one of the key enzymes in the Calvin-Benson-Bassham cycle. Thus magnetotactic bacteria have great potential in regulating important geochemical reactions and cycles involving several important elements including sulfur, nitrogen, iron, and carbon.

Protozoa are known to be important components of the marine ecosystems as the primary consumers of bacteria and recyclers of certain nutrients, including iron. Magnetotactic protists might play an important role in iron cycling either through direct biomineralization or through grazing on magnetotactic bacteria and solubilizing magnetosomes. Iron is a limiting factor in primary production in some oceanic and coastal areas. This element, which is often present in seawater in particulate and colloidal forms, can be digested in the food vacuoles of protozoans during grazing of particulate and colloidal matter. This process might generate more bioavailable iron for other species in the food chain. The chemical stability and thus the persistence of magnetosomes and magnetosome crystals depend on its surrounding environment. Magnetite magnetosomes appear to be quite stable in the bacterial cell possibly due to the magnetosome membrane and there is no evidence that magnetotactic bacteria can reuse iron in magnetosomes for another purpose even when cells are starved for iron. Is it possible for protozoans grazing on magnetotactic bacteria to make ingested magnetosome iron more bioavailable through solubilization? One possible mechanism of conversion of biologically unavailable forms of iron into more soluble forms is through the action of acidic food vacuoles on solid or colloidal forms of iron in protozoa. This has been observed to occur with colloidal iron and with greigite magnetosomes from magnetotactic bacteria.

The dissolution of greigite magnetosomes by the ciliate *Euplotes vannus* has been demonstrated in in vitro experiments. The ciliates ingested greigite-producing magnetotactic bacteria, the multicellular magnetotactic prokaryote discussed earlier, which became enveloped in food vacuoles known to have an internal acidic pH. Transmission electron microscopy (Fig. 11) and X-ray microanalysis of thin-sectioned ciliates showed that within the acidic vacuoles, the greigite crystals dissolved at different rates after the magnetosome membrane was digested. The experiments with *Euplotes* suggest that protozoans that graze on magnetotactic bacteria in natural environments are likely important in iron cycling as they may help to solubilize iron trapped in the magnetosomes, making it bioavailable to other organisms. The impact of magnetite magnetosome digestion by protozoans remains unknown, but, based on the experiments with greigite magnetosomes, there is potential for magnetosome magnetite to also be solubilized by protozoans.

A simple calculation may help estimate the potential of the recycling of magnetosomes produced by magnetotactic bacteria. Each multicellular magnetotactic prokaryote contains about 30 cells with approximately 100 magnetosomes per cell, which leads to each aggregate containing about 3000 magnetosomes. Using an average magnetosome size of 100 nm, which results in a cubic volume of 106 nm^3, and a greigite density of 4.1 g/cm^3, each multicellular unit contains an estimated total amount of 1.2×10^{-11} g

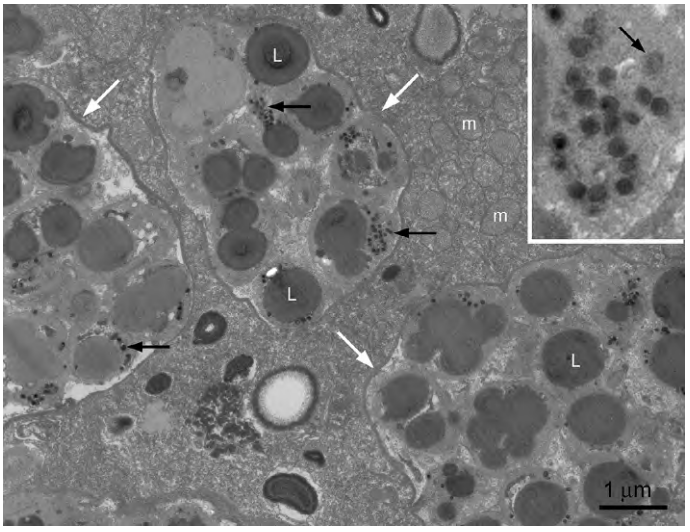

Fig. 11 Transmission electron microscopy image of an ultra-thin section of a ciliated protozoan that has ingested a number of magnetotactic multicellular bacteria. The bacterial cells (*white arrows*) are in vacuoles where they appear to be in the process of being digested. Magnetosome clusters can be seen (*dark arrows*). The inset shows a high-magnification image of a cluster of magnetosomes in different degrees of dissolution. No magnetosome membrane is seen in heavily digested crystals (*arrow*); *m*, mitochondria; *L*, Lipid inclusions.

of greigite that could potentially be solubilized within the ciliates and eventually released to the environment. Marine magnetotactic bacteria are known to reach population densities of 104 cells ml^{-1} in some natural environments. Taking into consideration the large amount of iron concentrated by magnetotactic bacteria in intracellular magnetosomes (10^{-13} to 10^{-15} g iron per cell) and their estimated population density, in some habitats, nanomolar concentrations of iron may be sequestered by magnetotactic bacteria and then solubilized by grazing protozoans that ingest them, thereby recycling significant amounts of iron.

Magnetotactic bacteria are considered to be a main source of the magnetization of sediments in a number of freshwater and marine environments. In many of these environments, magnetosome crystals, particularly magnetite, is released from dead, lysing cells and accumulate in sediments and persist relatively unaltered for long periods of time. In some cases, even magnetosome chains persist. Because magnetosomes are single-magnetic-domain particles, they form ideal carriers of paleomagnetic information as they cannot be demagnetized. Nanometer-sized magnetite grains have been recovered from a number of soils and lacustrine and modern and ancient deep sea sediments. In many cases, these particles were identified as biogenic by their shape and size, which were similar to magnetite crystals in magnetotactic bacteria and were therefore referred to as "magnetofossils." Putative fossil magnetotactic bacterial magnetite crystals have been found in stromatolitic and black cherts from the Precambrian, some dating as far back as 2 billion years. If these crystals are actually from magnetotactic bacteria, they represent some of the oldest bacterial fossils on Earth. Many of the crystals, however, found in these rocks were partially degraded, showed indistinct edges, and underwent partial oxidation to maghemite. The crystals have been used as supporting evidence that free O_2 had begun to accumulate in the atmosphere before 2000 Ma and that the present intensity of the Earth's magnetic field strength had appeared by 2000 Ma. If magnetosomes are biomineralized by protists or retained by protists after ingestion of magnetotactic bacteria, then these eukaryotes, like the magnetotactic bacteria, might also directly contribute to the magnetization of sediments.

The cuboctahedral crystal habit is the equidimensional form of magnetite and it is commonly found in inorganically produced magnetites. The elongated, nonequidimensional, pseudo-hexagonal prismatic and bullet-shaped forms of magnetite are very unusual. Both have been found in magnetic separates of sediments and it would seem only the latter elongated forms of magnetite might represent reliable magnetofossils of magnetotactic bacteria and could therefore be used as biomarkers of the past presence of magnetotactic bacteria.

The presence of ultrafine-grained magnetite, pyrrhotite, and greigite in the rims of carbonate inclusions in the Martian meteorite ALH84001 was considered one of the lines of evidence for life on ancient Mars. This meteorite, estimated now to be ~4.1 billion years old, contains magnetite crystals about 10–100 nm in size with cuboid, teardrop-shaped, and irregular morphologies. About 25% of the magnetite crystals present have an unusual morphology referred to as truncated hexa-octahedral, which is essentially the same morphology of magnetite crystals found in the marine magnetotactic vibrio, *Magnetovibrio blakemorei* strain MV-1.

The elongated magnetite crystals from *Magnetovibrio blakemorei* strain MV-1 have been studied in great detail and show a number of different distinctive properties putatively not found in inorganically produced magnetite crystals. These properties have been considered by some to be suitable criteria for the determination of whether magnetite crystals are biogenic or not. These properties include (1) narrow size range (a non-log-normal size distribution with the mean centered in the single-magnetic-domain size range); (2) restricted width-to-length ratios; (3) high chemical purity; (4) few crystallographic defects (crystals are generally defect-free with the exception of occasional crystal twinning); (5) crystal morphology with unusual truncated hexa-octahedral geometry; and (6) elongation along a specific crystallographic axis of the crystal. The 25% of the magnetite crystals in ALH84001 that resemble those of *Magnetovibrio blakemorei* seem to fit the above criteria. However, there are several other hypotheses regarding the origin of the magnetite crystals in meteorite ALH84001 that do not involve biology but only chemistry. Thus, some disagree that these criteria are robust enough to distinguish between biogenically and abiogenically produced magnetite crystals and so the debate continues.

Biotechnological Applications of Magnetotactic Bacteria, Magnetosomes, and Magnetosome Crystals

The controlled biomineralization of magnetosomes and their single-magnetic-domain particles of relatively high structural perfection and purity as well as consistent species-specific crystal morphologies suggest that there is great potential for biotechnological applications. In general, the amount of magnetite and magnetosomes from magnetotactic bacteria is relatively low especially considering the amount needed for specific applications. Thus, in order to produce enough cells, magnetosomes, and magnetite crystals for these applications, cells must be grown in mass culture where the conditions for growth and magnetite synthesis must be optimized. This was a major obstacle to research in this area for years. In the last decade, techniques including those involving continuous culture methods have been devised for the successful mass culture of various *Magnetospirillum* species and *Magnetovibrio blakemorei* strain MV-1. For example, for *Magnetospirillum gryphiswaldense* strain MSR-1, a chemostat culture technique based on pH-stat feeding allowed the production of a high cell density in an auto-fermentor. Using this technique, it was possible a magnetosome yield of about 83 mg L^{-1} and a productivity of about 55 mg L^{-1} day^{-1}.

Cells of magnetotactic bacteria have proven useful in many interesting applications. For example, living north-seeking cells of polar magnetotactic bacteria have been used to determine south magnetic poles in meteorites and rocks containing fine-grained (<1 μm) magnetic minerals. Cells of magnetotactic bacteria have also been used in a number of medical applications including the magnetic separation of cells such as granulocytes and monocytes that phagocytized magnetotactic bacteria. Recently, the possibility of using magnetotactic bacteria in the removal of heavy metals and radionuclides from wastewater was investigated. The advantage here again is that if the cells can take up significant amounts of heavy metals they can be separated magnetically and the contaminant can be eliminated from an aquatic system. Cells of the sulfate-reducing magnetotactic bacterium, *Desulfovibrio magneticus* strain RS-1, have been used in cadmium recovery using magnetic separation. A recently described application is the trapping of magnetotactic bacteria using a commercial magnetic recording head. This method may be useful in counting

magnetotactic bacteria cells in water samples or to detect magnetically labeled bacteria or magnetosomes. Magnetotactic bacteria have been manipulated as nanorobots through a directional magnetic field that force them to push 3 μm beads along preplanned paths and creating structures as pyramids.

Magnetosomes contain single-magnetic-domain crystals that have been shown to have useful magnetic and physical properties. In addition, the organic phospholipid membrane that envelopes the crystals allows for the immobilization of biological molecules such as nucleic acids or nonmagnetosome proteins on their surfaces. Magnetosomes have thus been used in the immobilization of specific enzymes, some of which show higher enzymatic activity when attached to magnetosomes than when attached to commercially synthesized magnetite particles. Antibodies linked to magnetosomes or magnetosome crystals have been developed and have proven useful in various immunoassays involving the detection of allergens, certain cancer cells, and the quantification of immunoglobulins.

Magnetotactic bacterial magnetite crystals have been used to detect single nucleotide polymorphism based on a fluorescence resonance energy transfer technique in which double-stranded labeled DNA is synthesized by PCR and immobilized to the magnetite crystals hybridize to target DNA and a fluorescence signal is detected. Magnetosome membrane proteins on the surface of bacterial magnetite particles have been used as anchor proteins for the assembly of foreign proteins on the surface of magnetite magnetosomes. These so-called protein displays have been designed using the magnetosome membrane proteins MagA, MpsA, Mms16, and Mms13 (MamC). Magnetotactic bacterial magnetite particles have been modified using compounds such as hyperbranched polyamidoamine dendrimers or amino silanes for the extraction of DNA. Biotin attached to a monolayer-modified substrate was detected by streptavidin immobilized to bacterial magnetite particles using a magnetic force microscope showing the potential of magnetosomes in the detection of biomolecular interactions in medical and diagnostic analyses. In the case of antitumor treatments, magnetosomes have been used as potential drug carries and as targets for hyperthermia. Finally magnetosomes and/or magnetosome crystals are being currently used to attempt to enhance magnetic resonance imaging (MRI).

Perspectives

Magnetotactic bacteria and magnetosomes are a subject of an active and interdisciplinary field of research that includes topics such as microbiology, cell biology, geomicrobiology, palaeomagnetism, microscopy, biomineralization, biophysics, and biochemistry to name a few. New and exciting findings are constantly being reported on these microorganisms. New directions will come that will inspire research groups in this highly multidisciplinary field of microbiological research.

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Marine Deep Biosphere[☆]

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Glossary

AOM Anaerobic oxidation of methane, carried out by a consortium of anaerobic methane-oxidizing (ANME) Archaea and sulfate-reducing bacteria (see 'gas hydrates').
Basalt Gray to black extrusive volcanic rock, forming the basement underneath the marine sediments (see 'marine basement').
Cretaceous shales Organic-rich marine sediments deposited under oxygen-free conditions in the Cretaceous period 84–140 million years ago (see 'sapropel').
Crustal fluid Circulating water in the basement rock.
Gas hydrate Crystalline water-based solids physically resembling ice, in which gases (typically methane) are concentrated inside 'cages' of water molecules.
Igneous rocks Rocks formed by solidification of cooled magma.
Lithotrophy/lithotrophic organisms Use of an inorganic substrate (e.g., H_2, NH_4^+, Fe^{2+}, H_2S) to obtain reducing equivalents for aerobic or anaerobic respiration.
Marine basement Oceanic crust; part of the Earth's crust formed at the mid-ocean ridges that becomes buried by sediment with age (see 'basalt' and 'crustal fluid').
Pyrolysis The chemical decomposition of organic materials by heating in the absence of oxygen or any other reagents.
Sapropel Periodically occurring organic matter-rich sediment layers of the Eastern Mediterranean and Black Sea that were deposited under conditions of bottom water anoxia (see 'Cretaceous shales').
Subsurface The upper boundary of subsurface sediments is not clearly defined. A feasible definition is the anoxic layers beneath the zone of bioturbation by borrowing macrofauna.
Weathering Decomposition of rocks, soils, and their minerals. Can be driven chemically or by microorganisms.

Abbreviations

AOM anaerobic oxidation of methane
IODP International Ocean Discovery Program
mbsf meters below sea floor

Defining Statement

Marine sediments cover about 70% of the Earth's surface and contain the largest reservoir of organic matter. These sediments have been found to be a major microbial habitat to at least 2.5 km depth. Specific prokaryotic groups able to cope with extreme limitation dominate deep sediments, using ancient organic matter and geosphere energy sources.

Introduction and Background

Oceans cover around 70% of the surface of the Earth and are underlain by extensive sediments with an average depth of about 500 m, but up to 10 km deep in places. Within the oceans between 5 and 10×10^9 tons of particulate organic matter are constantly sinking, but only about 1 % of gross photosynthetic production finally reaches the sediment surface. Most of this sedimentary organic matter is degraded within the uppermost sediment layers and only a very small fraction survives and is buried. However, this buried organic carbon represents one of the largest carbon reservoirs on Earth ($15{,}000\times10^{18}$ g). On geological timescales the burial of organic matter had a significant effect on the global biogeochemical cycles, allowing atmospheric oxygen levels to increase and acting as a sink for carbon dioxide and hence exert significant influence on climate and the habitability of the planet.

[☆]*Change History*: September 2018. Henrik Sass and Gordon Webster have completely rewritten and updated the text. All paragraphs have been rewritten. The further reading list was updated and modified. Outdated information has been removed, including Tables 1–3. Fig. 1 has been updated using the latest data available and replaces the old Fig. 1. Figs. 2, 3 and 7 have been removed. Previous Fig. 4 becomes new Fig. 5; previous Fig. 5 becomes new Fig. 4; Fig. 6 stays as it is. Previous Fig. 8 becomes new Fig. 2 and previous Fig. 9 becomes new Fig. 3.

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The oceans have an average depth of 3800 m and thus an average bottom pressure of 38 MPa (this is about 380 times higher than atmospheric pressure), while at the maximum ocean depth (11,000 m, Marianas Trench) pressure reaches 110 MPa. Pressure increases further with depth into the sediments. This high pressure in deep-sea sediments in combination with low temperatures (around 2°C at the sediment surface), little organic matter to provide an energy source, and with porosity and nutrients decreasing with depth, resulted in conditions that were once considered to be too extreme for life. Subsurface sediments were usually not even considered as a possible habitat. Researchers investigating the presence of microorganisms in deep-sea sediments by cultivation-based methods in the 1950s seemingly confirmed this absence of life. As no microorganisms were cultured from the deepest (7.47 mbsf, meters below sea floor) and oldest layers, they then concluded that they had reached the lower limit of the biosphere. Hence, almost nothing seemed to happen in deep subsurface sediments until temperature (average increase 30 K per km) reached 100–150°C at a few kilometers depth, and when buried recalcitrant kerogen (preserved macromolecular organic matter) pyrolyzed to produce oil and gas. However, more recently a number of findings led to question whether subseafloor sediments are really devoid of life: It turned out that only a small fraction of environmental bacteria can be grown in the laboratory on standard microbiological media, questioning the validity of previous results. The discovery of hyperthermophilic microorganisms thriving at temperatures above 100°C significantly extended the temperature range for life and hence the potential depth range. In addition, geochemical studies found indications for biogenic methanogenesis some tens of meters beneath the sediment surface, far below the previous 'lower limit' of the biosphere.

Comprehensive microbiological investigations since 1986 have consistently shown the presence of significant prokaryotic populations in marine subsurface sediments. The presence of these prokaryotes was confirmed by a combination of different methods directly corroborating each other, including microscopic detection, presence of culturable prokaryotes (including anaerobes), direct activity measurements (using radiolabeled substrates), presence of high molecular weight prokaryotic DNA, RNA and lipid biomarkers, and correlation with geochemical changes characteristic of prokaryotic activity. Population estimates vary among different researchers, depending on which data are used for their calculations, but are generally so large that global estimates suggest the marine deep biosphere accounts for between 1% and 30% of all biomass and for up to 70% of all prokaryotes on Earth. However, the marine deep subsurface is part of a global deep biosphere that includes other deep subsurface environments such as aquifers, consolidated Cretaceous shales, igneous rocks, glaciers and ice sheets, lignite and coal formations, mud volcanoes, and marine and terrestrial oil reservoirs.

Prokaryotic Cell Concentrations and Distributions in Subseafloor Sediments

A major concern for early subsurface microbiology research was potential contamination of deep sediments with prokaryotic cells from shallow sediment layers or from seawater during the coring process. Chemical tracers and fluorescent microspheres (0.5 μm diameter to mimic cells) have been added to drilling fluids to directly detect potential contamination during the drilling process. This approach has confirmed that contamination is usually restricted to the outer layers of the core and thus contamination can be avoided by the normal practice of sampling from the core center.

Microbial cells in sediments are usually quantified after staining with a DNA dye and by counting recognizable cells using an epifluorescence microscope. Counting cells directly within fixed sediment slurries has a detection limit of about 10^4 cells cm^{-3}, and hence, even if no cells are detected a population between zero and 10^4 cells cm^{-3} may actually be present and nonetheless represent a considerable prokaryotic community. Recently, a number of modifications to the original approach have increased sensitivity, for example detachment of cells from sediment particles, allowing larger volumes to be analyzed. However, these methods still rely on subsequent quantification by epifluorescence microscopy or flow cytometry.

Sediment cell counts generally decrease with depth (Fig. 1), a correlation that can be explained by the rapid degradation of easily degradable organic matter, leaving increasingly recalcitrant material behind for microorganisms in deeper layers. It may therefore not be surprising that in most sediments cell numbers decrease exponentially with depth, and although at a few hundred meters depth cell counts have decreased about 1000-fold, in some sediments more than 10^6 cells cm^{-3} are still present which represents still a significant amount of biomass. Cell numbers at the sediment surface can vary substantially between sites and the amount of microbial biomass seems to directly correlate with the amount of organic matter input into the sediment. The latter is controlled by the trophic state of the water column, and primary production, but also by water depth and hence sedimentation time, during which the organic matter is exposed to degradation by pelagic microorganisms. Sediments with a high organic matter input (e.g., Peru margin, 150 m water depth) can have cell numbers at the surface around 10^9 cells cm^{-3} or significantly higher (Baltic Sea), whereas numbers in surface sediments of the ultra-oligotrophic South Pacific Gyre (more than 4000 m water depth) are almost 1000-times lower. This suggests that organic matter controls overall numbers of subsurface prokaryotic cells and represents their main energy supply.

The lower boundary of the deep biosphere has not been found so far. Viable cells were detected in coal layers over 2000 mbsf, and a number of IODP cruises detected cells though the entire sediment column and into the underlying oceanic crust. This suggests that living cells will be present as long as the physicochemical conditions permit (the current upper temperature limit for life is 122°C). It may be surprising to find cells in basaltic rocks, but their densities appear to be low (up to 10^5 cells cm^{-3}) and cells mostly confined to crustal fluids, fractures, and altered glassy basalts. However, because of the large volume of the oceanic crust (10^{18} m^3), the crustal biosphere is estimated to represent at least 1% of the global biomass.

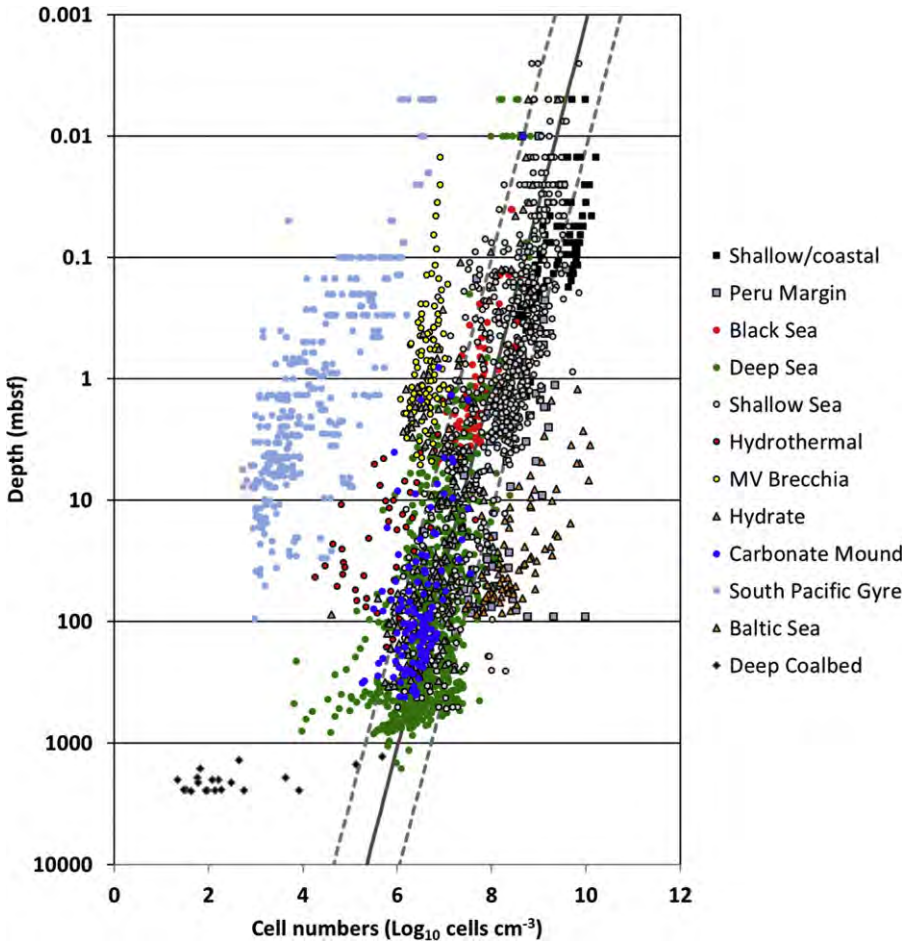

Fig. 1 Distribution of microbial cells in sub-seafloor sediments at 108 locations worldwide, including 21 ODP/IODP Expeditions. The solid line is the regression line and the dotted lines either side are 95% upper and lower prediction limits.

Deep Biosphere Hot Spots

Deep sediments with elevated cell numbers can be seen as deep biosphere 'hot spots'. There are, however, different reasons for these increased cell numbers. As previously described, prokaryotic populations are consistently elevated in layers high in organic matter despite the latter being often millions of years old. One example is sapropels that are discrete organic matter rich (up to 30% OM) layers that occur in some marine sediments, including the Mediterranean where they alternate with hemipelagic carbonate-rich sediments. When compared to these 'normal' hemipelagic sediments, sapropels showed significantly elevated cell numbers and strongly enhanced potential activities (e.g., exoenzyme activity and glucose degradation). The presence of such discrete zones of high cell concentrations and the preservation of individual sapropel layers also demonstrates that drilling disturbance during sample collection is minimal.

More numerous than the discrete sapropel layers are the high organic matter layers of Cretaceous shales, that were deposited some 84–140 Mya during major oceanic anoxic events, that resulted in enhanced global accumulation of sedimentary organic matter. Almost 100 Mya after their deposition these sediments still provide substrates for continuing prokaryotic activity not only in deep marine sediments (e.g., Equatorial Atlantic, Demerara Rise) but also when uplifted onto land. However, these shales are often highly compacted by the uplifting process and porosity and permeability are low. This restricts organic matter degradation within the shales to fermentative processes with the produced organic acids and hydrogen slowly diffusing into adjacent more permeable sediments (Fig. 2) where oxidation can be completed using electron acceptors supplied to the more permeable layers. This has been shown for lignite- or clay shale–sandstone interfaces where prokaryotic activity and cell numbers are clearly elevated (e.g., Waikato coalfield, New Zealand and Cerro Negro, New Mexico).

Cretaceous shales also have high petroleum generation potential when deeply buried. When temperatures reach 100–150°C thermogenic breakdown of kerogen produces oil and gas. Kinetic modeling predicts increasing kerogen reactivity with depth and abiological thermogenic processes are confirmed *in situ* by increases in methane and ethane. However, before temperatures for thermal breakdown are reached, kerogen reactivity already incrementally increases, potentially enabling its utilization by

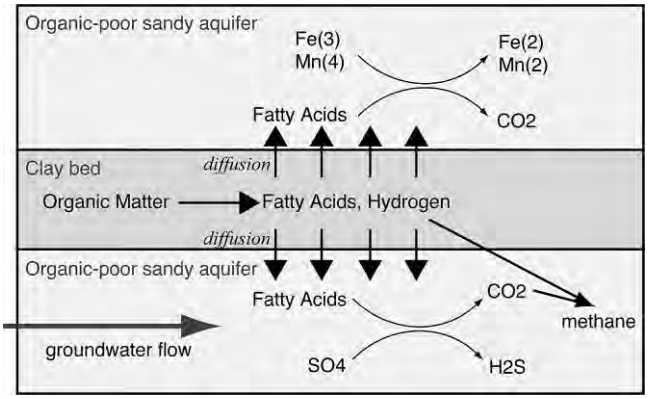

Fig. 2 Schematic of prokaryotic metabolisms and interactions in subsurface, interbedded clay/Cretaceous shale and sand layers.

prokaryotes which means that prokaryotes are present when the thermal breakdown starts, as indicated by the presence of cells and intact phospholipids (Fig. 3). This demonstrates an overlap of biogenic and thermogenic processes in deep, hot sediments and the potential for high-temperature processes to feed the base of the deep biosphere. Thermogenic breakdown of kerogen produces a range of potential substrates for prokaryotes including low molecular weight hydrocarbons, organic acids, and hydrogen. Likewise, many subsurface oil reservoirs have considerable prokaryotic activity. In some high-temperature (70–110°C) North Sea oil reservoirs prokaryotic activity is so high that between 3 and 16 kg of prokaryotic cells is expelled per day with production fluids.

Gas hydrates contain globally twice the amount of carbon as other fossil fuels combined. They are only stable at low temperatures and high pressures, conditions typical for most marine subsurface sediments where a zone of gas hydrate stability exists. However, for the formation of sufficiently large quantities of methane to form hydrates, sulfate and other electron acceptors need to be depleted. Therefore, methane hydrates are usually found in relatively deep sediments that are organic matter rich, for example those underlying upwelling regions. Above the stability zone, gas hydrates are unstable due to low pressures and beneath

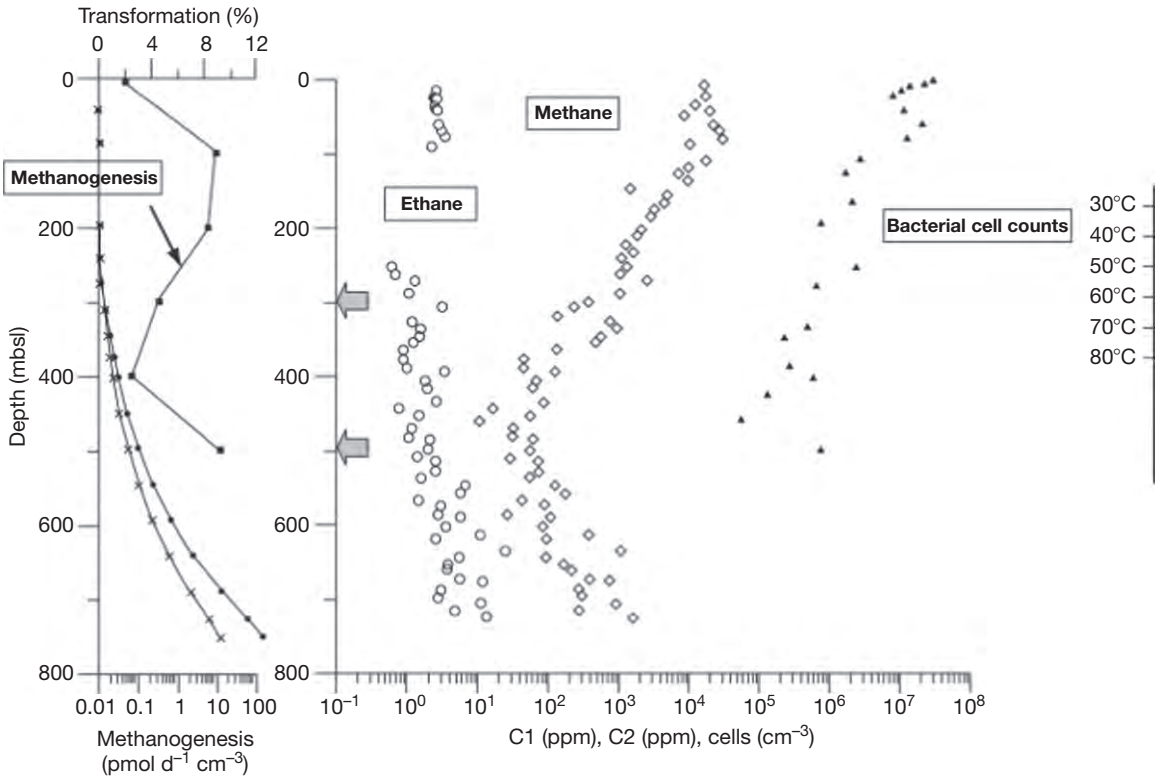

Fig. 3 Deep biosphere interaction with high-temperature thermogenic processes in Nankai Trough Sediments, Pacific Ocean. Left panel: Curves with crosses and dots (representing different sediment samples) show modelled estimates of increasing organic matter (kerogen) reactivity (as % transformation into volatile hydrocarbons) and measured rates of prokaryotic methanogenesis, relative to sediment depth (mbsf). Right panel: Depth distributions of ethane (circles), methane (diamonds), and total prokaryotic cells (triangles). The gray arrowheads indicate depths where intact phospholipids were detected. Temperature range of sediments extreme right. Reproduced from Horsfield, B., Schenk, H.J., Zink, K., et al., 2006. Living microbial ecosystems within the active zone of catagenesis: Implications for feeding the deep biosphere. Earth and Planetary Science Letters 246, 55–69.

due to high temperatures that cause hydrates to melt. Hence, above and below the gas hydrate zone, free porewater methane concentrations can be elevated and potentially stimulate anaerobic oxidation of methane (AOM) (Fig. 4). For this, however, an electron acceptor needs to be available which usually is sulfate. Sulfate-methane transition zones (SMTZ) are characterized by a general stimulation in prokaryotic activity and by elevated cell numbers (Fig. 4). While the upper SMTZ is usually the depth where sulfate is depleted, a lower SMTZ beneath this can be found in areas where sulfate is supplied to the sediments by deep fluid flow, like in sediments of the Peru Margin. Cell numbers at this deep SMTZ (at about 90 mbsf) are as high as at the sediment surface. Microbial community composition showed a significant change at this depth indicating that the deep sediment prokaryotic communities here are active and dynamic, as opposed to just being dormant. In some deep sediments nitrate may also be found from the stimulation of nitrification within the crust. In addition to electron acceptors, deep fluid flow can also provide substrates/electron donors like hydrogen or organic compounds synthesized by abiotic reactions between seawater and basement basalts at temperatures between about 100 and 300°C.

Diversity of Subsurface Prokaryotes

Early molecular surveys of deep subsurface sediments revealed a microbial community that strongly differed from that of surface sediments. The shift from a community dominated primarily by *Proteobacteria*, *Actinobateria* and *Bacteroidetes* to a community consisting of novel phylotypes typical for subsurface sediments was usually found between one and a few meters into the sediment. The dominant bacterial groups in the deep biosphere were found to be certain subphyla of the *Chloroflexi*, and the *Gammaproteobacteria*, and the candidate phylum *Atribacteria*. These can be detected in the majority of subsurface sediments and represent the majority of bacterial phylotypes. *Deltaproteobacteria* are also widespread but numerically not as numerous. While the detected *Gammaproteobacteria* in most cases are fairly closely related to cultured organisms, the majority of the deltaproteobacterial sequences are only distantly related to known organisms. Other bacterial groups, e.g., *Alphaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, the Aminicenanthes (candidate division OP8), and the NT-B2 cluster were less frequently detected.

Like for the Bacteria, most archaeal sequences found in deep subsurface sediments were generally not closely related to cultured organisms. The molecular surveys targeting Archaea revealed an unexpected high diversity of *Bathyarchaeota*, *Euryarchaeota*, *Thaumarchaeota* and *Lokiarchaeota*. In almost all subsurface sediments studied so far, the *Bathyarchaeota* (including the Miscellaneous Crenarchaeotal Group, MCG) is by far the dominant group. Several other groups are also widespread but numerically less abundant, for example, the thaumarchaeotal Marine Group I (MG-I), the lokiarchaeotal Deep-Sea Archaeal Group (DSAG), and the euryarchaeotal *Hadesarchaea* (including the South African Gold Mine Euryarchaeotal Group, SAGMEG), Marine Benthic Group

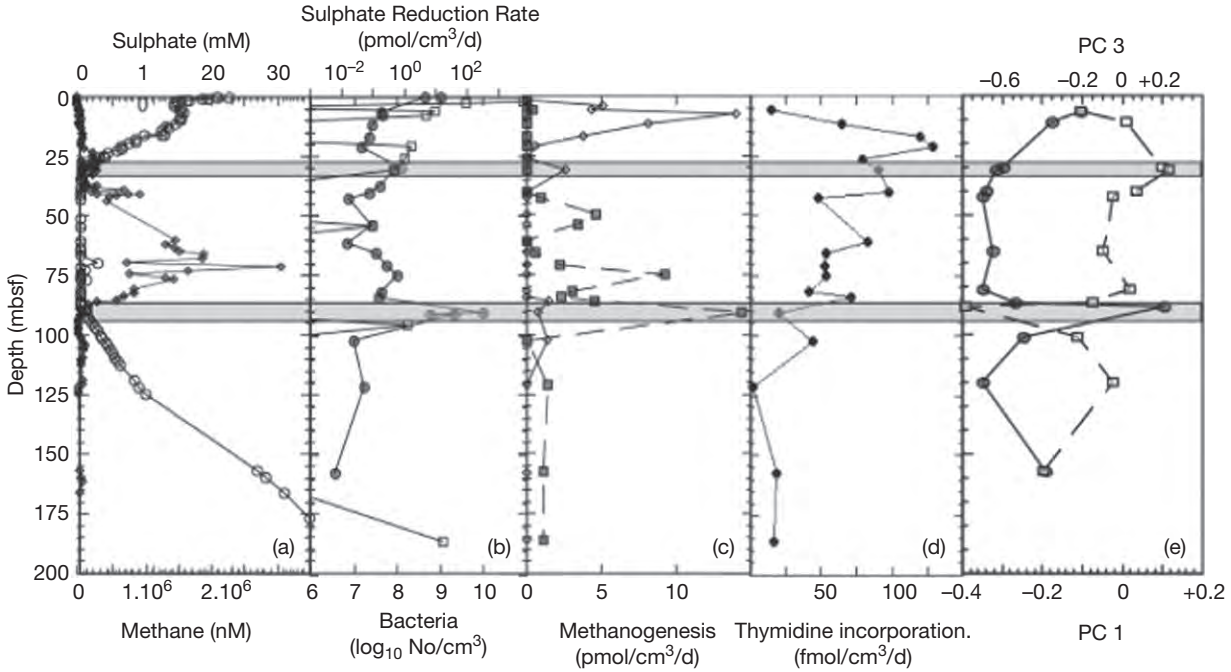

Fig. 4 Biogeochemical processes and prokaryotic biodiversity profiles in Peru Margin sediments (ODP 1229). (a) Geochemistry: O pore water sulfate (mmol l^{-1}), ♦ CH4 (nmol l^{-1}), (b) □ sulfate reduction rates, ● total cell numbers, (c) ◄ methanogenic rates, H_2/CO_2, ▪ acetate, (d) ● heterotrophic prokaryotic growth rates (thymidine incorporation), (e) Principal components profile of diversity of Bacteria from DGGE analysis of 16S rRNA gene sequences: ● Component 1 (56% of variation), □ Component 3 (9% of variation); Component 2 (24% of variation) has a similar profile to Component 1. Shaded boxes highlight elevated prokaryotic processes and sulfate: Methane interfaces. Reproduced from Parkes, R.J., Webster, G., Cragg, B.A., et al., 2005. Deep sub-seafloor prokaryotes stimulated at interfaces over geological time. Nature 436, 390–394.

D (MBG-D), and Terrestrial Miscellaneous Euryarchaeotal Group (TMEG). Other archaeal groups (e.g., *Thermococcales*, *Archaeoglobales*, deep-sea hydrothermal vent euryarchaeota) are less frequently found and represent a small percentage of all sequences.

Sulfate-reducing and methanogenic prokaryotes are the most important terminal oxidizers in anoxic environments and were expected to be amongst the dominating physiological groups present in subseafloor sediments. However, known sulfate-reducing genera were very rarely detected by molecular biological approaches, even if primers specific for sulfate-reducing prokaryotes were used. Methanogens were generally more often detected with the most abundant sequences affiliating with the well-known genera *Methanosarcina*, *Methanobrevibacter*, and *Methanococcoides*.

These early molecular studies were restricted by problems in DNA extraction and purification and by the resolution of the methods used. Clone libraries and denaturing gradient gel electrophoresis (DGGE) were the most commonly used methods and allowed only a limited number of sequences to be identified. However, new methods that became available during the last decade, particularly high-throughput sequencing and metagenomic analysis, significantly increased phylogenetic resolution, allowing the identification of up to several thousand operational taxonomical units (OTUs) within a single sample. This improved detection limit and resolution. It turned out that those OTUs that were novel and considered specific for subsurface sediments (e.g., *Chloroflexi*, *Atribacteria*, *Bathyarchaeota*, or *Hadesarchaea*) are by no means restricted to the deep, but also present in near-surface environments, although representing here only a minor fraction of the total community. However, these OTUs represent a core set of prokaryotes that are present throughout the entire sediment column and becomes dominant at depth, whereas 'typical' surface phylotypes disappear. This was seen as an indication that these subsurface OTUs are particularly well adapted to energy limitation.

The model of a residual ('left over') community is supported by the low growth rates observed in subsurface sediments. In a coastal subsurface sediment average cell generation time increased from 0.2 to 40 years along the upper seven meters. If generation times in these organic matter rich sediments are already so long, they can be expected to be even higher in deeper or organic matter poor sediments. This seems to be confirmed by reports that indicate generation times of a few hundred years in laboratory incubations of ultradeep (over 2000 mbsf) sediments.

Prokaryotic Metabolic Processes and Energy Sources

The metabolism of subseafloor prokaryotes is considerably diverse and reflects the range of prokaryotic activities found in near-surface sediments (Fig. 5). The major substrate for subseafloor prokaryotes is recalcitrant macromolecular organic matter, that is

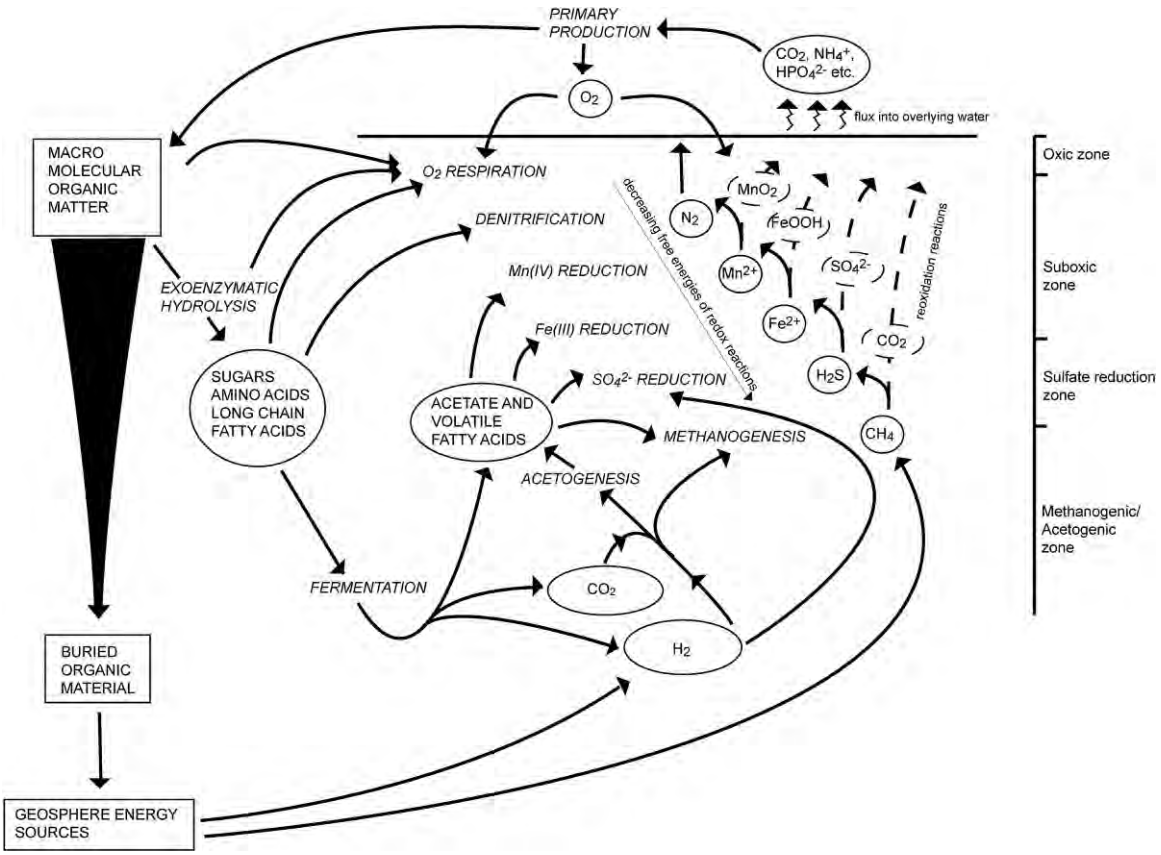

Fig. 5 Schematic of prokaryotic processes of organic matter degradation in subseafloor sediments.

initially hydrolyzed and, once oxygen is removed, fermented into low molecular weight compounds, particularly acetate and other organic acids, and hydrogen. These fermentation products are then oxidized by the terminal oxidizers using a range of electron acceptors (Fig. 5) in order of decreasing energy yield: Nitrate followed by manganese oxides, iron oxides, sulfate; and carbon dioxide (methanogenesis). In shallow sediment with its high cell densities anaerobic respiration seems to follow this predicted sequence with depth. In deep sediments, however, cell densities, organic matter contents and reaction rates are much lower, and minerals containing metal oxides with different reactivities may be available. This allows a number of anaerobic reactions to occur simultaneously (Fig. 5), with sulfate reduction and methanogenesis considered to be the major terminal oxidation processes in subseafloor sediments.

A special subset of macromolecular organic matter is necromass. As shown above cell numbers significantly decrease with depth and these disappearing cells serve as a potential substrate for the surviving *in-situ* community. The role of necromass is disputed with some studies suggesting that it is only important in the upper sediment layers, whereas other studies estimate turnover times and contribution to sediment metabolism for thousands of years and suggest a significant role for necromass in maintaining subseafloor life.

Hydrogen appears to be a particularly important substrate as it can diffuse effectively within sediments, eliminating the need for prokaryotes to actively move toward substrates. Hydrogen is a major fermentation product, but in deep sediments and the oceanic crust hydrogen can also be produced from inorganic reactions. This includes radiolysis of water by the decay of radioactive minerals and, particularly as temperatures increase with sediment depth, weathering of iron silicates. More recent studies suggest that elemental hydrogen can be produced even from silicate minerals, which depends on mechanochemical processes, and is stimulated by microbial activity. Abiological hydrogen formation and its utilization under anoxic conditions is extremely important, as it provides the potential for autotrophic prokaryotic processes and indicates the potential for some subsurface prokaryotic habitats being independent of photosynthetically produced organic matter (Slime – subsurface lithotrophic microbial ecosystem).

While hydrogenotrophic methanogenesis is well documented, evidence for homoacetogenesis, a process considered typical in deep freshwater lake sediments and in animal intestines, has only recently been found in deep sediments. Homoacetogenesis uses hydrogen and carbon dioxide to form acetate which provides a similar amount of energy to hydrogenotrophic methanogenesis. It has recently been implied that acetate can be used in the formation of both ethane and propane. Interestingly, these compounds have previously been considered to be of thermogenic origin and their potential prokaryotic origin suggests a wider microbial role in geochemical processes in deep marine sediments.

In deep coal-bearing sediments (over 2000 mbsf) methylotrophic methanogenesis was detected but surprisingly also heterotrophic methylotrophy was found (i.e., the oxidation to carbon dioxide). Methylotrophy uses one-carbon compounds, e.g., methylamine or methanol, but also methyl groups connected to larger molecules. Some of the methylotrophic substrates are released during kerogen maturation at elevated temperatures as volatiles. What acts as electron acceptor during the heterotrophic methylotrophs is unclear.

Analysis of the subsurface transcriptome seemingly corroborated organotrophy as the major energy metabolism in the marine deep biosphere. In sediments of the Peru Margin most RNA transcripts were of genes involved in the anaerobic metabolism of proteins, carbohydrates and lipids. Genes involved in cell growth and division were usually only found in layers with peaks in cell numbers. This is hardly surprising since subseafloor microbes are living under severe energy constraints. The subseafloor biosphere is a habitat that is under severe energy limitation and microorganisms present need to make use of any potential substrate available. It is likely that most of the *in-situ* microbial community has insufficient energy to even provide conventional amounts of energy for cell maintenance considering the large cell numbers present. However, subseafloor prokaryotes may be well adapted to their habitat where energy supply is on geological timescales. In addition, there may be a greater range of energy sources available than we are currently aware of.

The phylogenetic diversity of the yet-to-be-cultured bacterial and archaeal groups, for example the *Chloroflexi*, *Atribacteria*, Aminicenanthes, *Bathy-* and *Thaumarchaeota*, suggests that each of these phyla harbors a wide range of metabolic types. This also is to be expected as they form an anaerobic foodweb in which the different roles need to be occupied. *Bathyarchaeota*, for example, possess the genes for the extracellular hydrolysis of proteins and amino acid fermentation. The genomes of *Atribacteria* showed potential for fermentative and syntrophic metabolism, but no indication for a respiratory chain as it would be required for aerobic and anaerobic respiration. The *Chloroflexi* is the only of these phyla that contains a number of families with cultured representatives. These cultures use anoxygenic photosynthesis, fermentation or reductive dehalogenation as energy metabolism. Single cell genomes of *Chloroflexi* indicated in addition the ability for lithotrophic growth and the reductive acetyl-CoA pathway, which is involved in homoacetogenesis. The potential for acetogenic growth and methanogenesis has also been found in cells belonging to the *Bathyarchaeota*.

Possible Contribution of Endospores to the Deep Biosphere

Many members of the bacterial phylum Firmicutes are able to form spores, resting stages that are inactive, strongly dehydrated, and highly resistant to environmental stresses. The stability and resistance of spores may help to explain the absence of spore-forming bacteria in molecular surveys, as it has been shown that most nucleic acid extraction procedures do not include spores, and that these require a much harsher treatment. Spores do contain enough energy for germination and are specifically adapted to quickly respond to substrate availability and formation of a vegetative cell able to replicate. When fresh *Desulfotomaculum* cultures,

containing either only vegetative cells or spores, were analyzed by dilution culturing, the spores showed almost 100% culturability, which was three to four order of magnitude higher than that of the vegetative cells. Therefore, spores may be able to easily outgrow other organisms after transfer to microbiological media, explaining their repeated isolation from subsurface sediments. As they do not exhibit active metabolism and do not require energy, it can be expected that they accumulate during sediment burial. Estimation of endospore numbers in deep marine sediments using the endospore-specific biomarker dipicolinic acid (DPA), suggested that spores are present in similar numbers to vegetative cells. However, what happens to these spores during extended burial is unknown; whether they degrade and contribute to necromass or survive and eventually germinate when conditions are favourable.

Cultivation of Deep Biosphere Prokaryotes

Cultivation efficiencies in subsurface sediments are generally very low, in most cases less than 0.1% of the cells can be stimulated to grow in microbiological media. This low culturability might be explained by the strongly energy-limited nature of deep sediment prokaryotes. Starved cells are often severely harmed (substrate shock, substrate-accelerated death) by a sudden exposure to high substrate concentrations as present in most standard microbiological media. Higher viable counts were obtained in more active layers, like sapropels or with deep layers that receive fluids from the underlying ocean crust that supply fresh growth substrates. In these layers up to 3% of all cells were stimulated to grow. However, this 'culturability' is still one order of magnitude lower than in coastal surface sediments when the same media are used.

Isolates obtained from deep subsurface sediments so far belong mostly to the alpha, beta, gamma, delta, and epsilon subgroups of the *Proteobacteria*, to the *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Fig. 6). Although these are the same phyla as obtained from marine surface sediments, cultured diversity in subsurface sediments is generally lower. This reflects the decreased diversity with depth and the presence of a core microbial community found by molecular biological methods. Microorganisms isolated from these deep sediments must be able to survive under energy limitation, but to switch to fast growth if conditions change, like in microbiological medium. This resilience and longevity have been demonstrated for members of *Actinobacteria* (*Micrococcus*) and *Firmicutes* (*Bacillus*) that have been isolated from several million years old amber.

The presence of some of the cultured genera in subsurface sediments, for example, relatives of the alphaproteobacterium *Rhizobium radiobacter*, or the gammaproteobacterial genera *Pseudomonas*, *Halomonas*, and *Acinetobacter*, has also been confirmed by molecular biological techniques. For example, by quantitative real-time PCR it was shown that *R. radiobacter*-like bacteria can

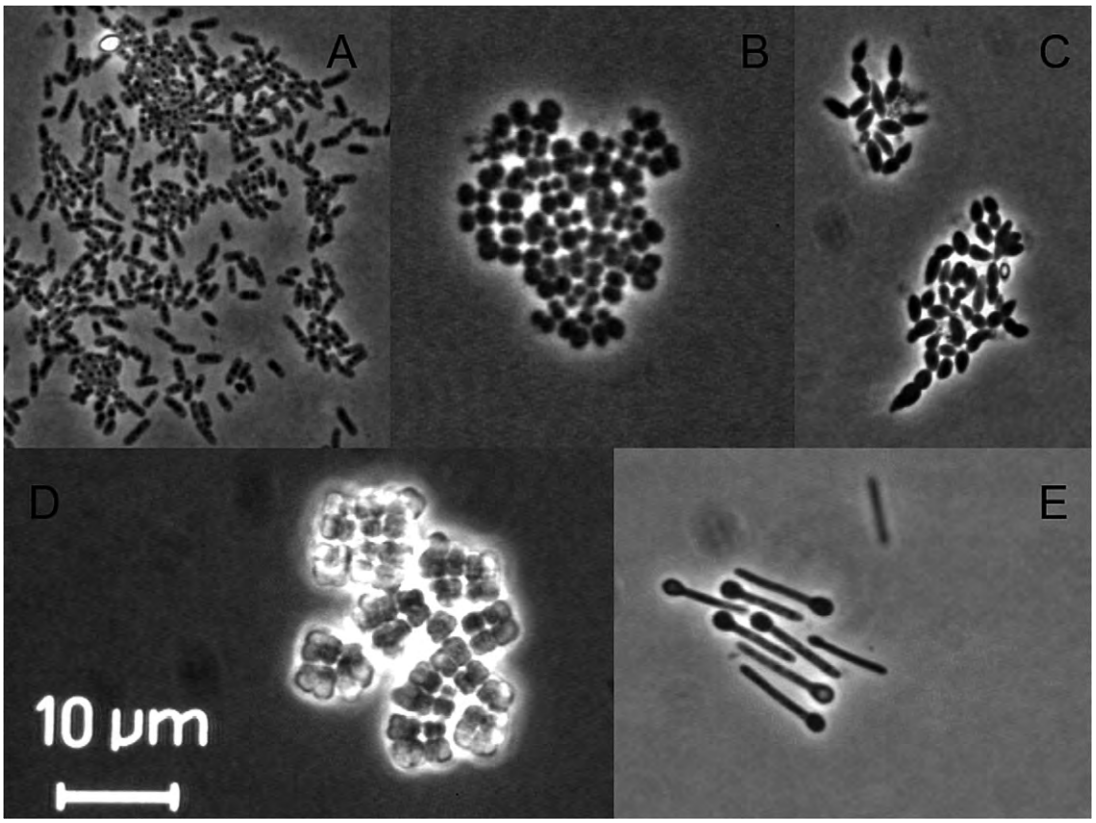

Fig. 6 Phase contrast micrographs of bacterial isolates from Eastern Mediterranean sapropels (a–c) and carbon-poor hemipelagic sediment layers (d–e). (a) *Halomonas* sp. S6BA, (b) *Alteromonas* sp. S8FS1, (c) Bacillus sp. S6BB, (d) *Micrococcus* sp. SM3, (e) *Clostridium* sp. SO1.

represent up to 5% of the *in situ* microbial community within Eastern Mediterranean sapropels. The presence of some of these cultured bacterial types in deep sediments, however, seems surprising, particularly for bacteria related to the putative soil bacterium *Rhizobium*, but these isolates grew well in marine medium under anoxic conditions, indicating that they may actually thrive in the deep sediments. These 'deep' bacteria, however, show only little differences to their surface counterparts in standard physiological tests (e.g., substrate tests, temperature range) and currently it is not clear what allows them to thrive or survive in deep sediments.

Cultivation-based studies have been widely criticized for not reflecting the actual *in-situ* diversity by missing the dominant phylotypes. One reason may be that the supposedly slow-growing subsurface prokaryotes are overgrown by opportunistic fast-growing bacterial types like some *Proteobacteria* or that they fail to form visible colonies. However, although there are no pure cultures of the subsurface *Chloroflexi* and *Atribacteria*, members of both phyla have been successfully enriched and maintained for extended periods of time in the laboratory. Since some *Chloroflexi* were growing in 'typical' anoxic microbiological media, a fermentative metabolism appears likely, which has been confirmed by single-cell genome analysis.

Physiological Adaptations of Deep Biosphere Isolates

Generally, there is relatively little information available about the physiological adaptations of deep subsurface organisms to their habitat due to the low number of fully characterized isolates. However, a few isolates have been fully investigated and may have characteristic adaptations for deep sediments (Table 1). Some of these isolates have an extraordinary broad temperature range for activity and growth, such as *Desulfovibrio profundus* covering a span of 15–65°C. This can be seen as a prerequisite to colonize large parts of the deep biosphere, since temperature increases with depth. As pressure increases with water and sediment depth, it is obvious that bacteria in subseafloor sediments have to be pressure-tolerant. As an example, the optimum pressures of sulfate-reducing bacteria (e.g., *D. profundus*) from subsurface sediments of the Japan Sea reflect well the *in-situ* pressures as the isolates show no decrease in activity between atmospheric pressure and 20 MPa and are still active at pressures between 30 and 40 MPa. However, to date, all samples used for the enrichment and isolation of sulfate-reducing bacteria from subsurface environments have been decompressed during sampling and isolates obtained under atmospheric pressure, thereby potentially selecting for less barotolerant types. Although sampling devices can be recovered slowly to reduce the speed of pressure change, bacteria may still not be able to adapt quickly enough, and barophilic prokaryotes might still be damaged. Recently, coring and sediment handling systems that constantly maintain high pressures (e.g., DeepIsoBug) have been developed. These have been used to enrich microorganisms without decompression from gas hydrate containing sediments from a range of sites and the resulting cultures compared to those that were obtained with depressurization during core recovery. Both assays (undecompressed and decompressed) resulted in the enrichment of similar phylotypes. However, none of the sites sampled was deeper than 2000 m (estimated water pressure 20 MPa) and it seems likely not deep enough to see an effect of decompression during sample recovery.

Many of the isolated Bacteria obtained from subsurface environments are facultative anaerobes. At first sight, this may seem surprising, as subsurface sediments are generally anoxic. However, due to the low metabolic activities and consequently low production of reduced compounds like hydrogen sulfide, the redox potential in many deep-sea and subsurface sediments is not highly reduced. For example, in the Mediterranean Sea nitrate, oxidized manganese, or iron species are present at least as deep as the sapropel S1, which is approximately 12,000 years old. In some abyssal sediments, for example within the South Pacific Gyre, organic matter degradation rates are so low, that oxygen is only depleted several meters below the sediment surface, if at all, and no truly anoxic conditions establish anywhere in the sediment column. In addition, deep circulation of fluids may provide oxidants to subsurface sediments.

Significance of the Deep Biosphere

Estimates of the magnitude of the subseafloor biosphere are up to 30% of all biomass on Earth. This deep biosphere must have a profound effect on the global biogeochemical cycles, by controlling the fate of sedimentary organic matter, but particularly by affecting deep and long-term processes previously considered to be purely geological. Prokaryotic activity probably continues with

Table 1 Pressure, temperature and salinity range for growth of some bacterial and archaeal isolates from marine subsurface sediments

Strain	*Origin depth* *Water*	*[mbsf]* *Sediment*	*Temperature* in situ *[°C]*	*Pressure range* *[MPa]*	*Temperature range* *[°C]*	*Salinity range* *[%]*
Shewanella profunda DSM 15900^T	4791	4.2	1–2	0.1–50	4–37	0–6
Rhodoferax ferrireducens DSM 15236^T	n.a.[a]	5.5	0–8	n.a.	4–30	n.a.
Photobacterium. sp. S14	2150	4.5	13–16	n.a.	4–35	1–7.5
Marinilactibacillus piezotolerans DSM 16108^T	4791	4.2	1–2	0.1–30	4–50	0–12
Desulfovibrio profundus DSM 11384^T	900	500	16	0.1–40	15–65	0.2–10
Methanoculleus submarinus DSM 15122^T	950	247	15–16	n.a.	10–50	0.6–9

[a]Coastal subsurface sediments, n.a.: data not available.

depth until maximum temperatures are reached (currently at 122°C). This means that biosphere processes overlap with geosphere processes, and may occur even within the 'oil window' (100–150°C). Prokaryotes gain additional energy from these thermogenic degradation products and may make a significant contribution to methane gas reserves in the process. However, deep microbial activity can both produce and consume methane, contributing to the formation or dissolution of gas hydrate deposits. Sediments around gas hydrates are one of several subseafloor 'hot spots' characterized by elevated microbial activity and biomass. Deep sediments require physiological and biochemical adaptations that enable survival under extreme conditions (e.g., high pressure, temperature extremes, limited energy supply, existence with extremely slow growth, recalcitrant, and ancient organic matter supply) and are a potentially untapped resource for biotechnological applications. In addition, the potential response of the deep biosphere to anthropogenic activity, such as sequestration of carbon dioxide, fracking and the deep storage of spent nuclear fuels needs to be seriously considered. A range of abiological hydrogen generation ('dark energy') processes (radiolysis, mechanochemistry, serpentinization) have been considered to be important in supporting an anaerobic deep biosphere that would be completely independent of surface photosynthesis. Interestingly, some of these processes appear to be stimulated by microbial activity. This has implications for the origin of life, and potential life on other planets, even those with an inhospitable surface.

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Medically Relevant *Mycoplasmas* and *Ureaplasmas*[☆]

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Defining Statement

Mycoplasma and *Ureaplasma* species are bacteria that lack a cell wall. These organisms evolved in close association with their eukaryotic hosts, resulting in an extreme genome reduction. In this article, the biology of the mollicutes is discussed with special emphasis on their pathogenic features, disease associations, cell and molecular biology.

Introduction

Mycoplasma and *Ureaplasma* are two important genera of the bacterial group called mollicutes. The name mollicutes – *soft skin* – reflects the major collective characteristic of these bacteria which is the lack of a cell wall, leaving the cells enclosed only by a trilaminar plasma membrane. This property distinguishes them from all other bacteria. In nature, mollicutes are never found as free-living organisms. Hosts are either animals including humans (*Mycoplasma*, *Ureaplasma*) or plants and insects (*Spiroplasma*, *Phytoplasma*) (Table 1). *Mycoplasma* species that infect humans usually cause respiratory diseases (*Mycoplasma pneumoniae*) or urogenital infections (*Mycoplasma genitalium*, *Mycoplasma hominis*, and *Ureaplasma* species), though many species exist in the respiratory or urogenital tracts as harmless commensals.

The close association of mollicutes with eukaryotic hosts and their adaptation to habitats with a good nutrient supply and relatively constant growth conditions led to a remarkable process of reductive genome evolution. The organism with the smallest known genome capable of independent life when provided with rich artificial medium is *M. genitalium*. This organism has a genome size of only 580 kbp and encodes about 480 proteins, as compared to about 4 million kbp and 4000 genes for bacteria such as *Escherichia coli*. These small genomes have made the mollicutes important tools for the new discipline of synthetic biology.

The Systematics of the Mollicutes

Evolution of Mollicutes

The analysis and comparison of 16S rRNA sequences revealed that the mollicutes have a distinct lineage from the Gram-positive bacteria with genomes of low GC content. It is believed that the first mollicutes emerged some 600 million years ago and that significant loss of ancestral genomic sequences was a major force in their evolution.

The mollicutes can be subdivided in several ways. Traditional classifications rely on their physiological and phenotypic properties, whereas more recent classification schemes are based on the similarity of their 16S rRNA (Figure 1) or conserved protein families. One conventional way of classifying the mollicutes is based on their requirement for sterols. Most genera need sterols for growth, whereas this is not the case for the members of the genera *Acholeplasma*, *Anaeroplasma*, and *Mesoplasma* (Table 1). However, this requirement can only be determined for those mollicutes that can be cultivated, and many representatives have not yet been cultured, including all species of the genus *Phytoplasma*. The mollicutes may also be classified according to whether they ferment glucose, utilize arginine, or hydrolyze urea. Another peculiarity of most mollicutes is that many species use the UGA codon to specify tryptophan rather than as a stop codon as in the universal genetic code. Only the genera *Acholeplasma* and *Phytoplasma* use UGA as a stop codon. Because this is the ancestral property, it can be assumed that *Acholeplasma* and *Phytoplasma* represent the more ancestral mollicutes. For practical reasons, the mollicutes are grouped in four orders that do not represent the phylogenetic relationships. An overview of these taxa is provided in Table 1. The two major genera of significance as human pathogens are *Mycoplasma* and *Ureaplasma*.

Genus *Mycoplasma*

The genus *Mycoplasma* is a paraphyletic collection of mollicutes that are widespread in nature as parasites of humans, mammals, birds, reptiles, and fish. It is the largest genus in the class *Mollicutes* and contains at least 118 species, 17 of which have humans as their primary host, though not all are considered pathogenic. Organisms in the genus *Mycoplasma* can be distinguished from the other mollicute genera because they are non-helical, require sterols, do not hydrolyze urea, are not obligate anaerobes, have optimum growth temperature of 37°C or higher, and have genomes of 580–1350 kbp.

The first representative of the genus *Mycoplasma* was identified in 1898 as the causative agent of contagious bovine pleuropneumonia (*Mycoplasma mycoides*). The human pathogens *M. hominis* and *M. pneumoniae* were discovered in 1937 and 1944, respectively.

[☆]*Change History:* February 2015. KB Waites has modified and updated all the sections. Deleted Table 2 and Section Plant Pathogenic Mollicutes.

This article is an update of J. Stülke, H. Eilers, S.R. Schmidl, Mycoplasma and Spiroplasma , *Encyclopedia of Microbiology (3rd Edn)*, edited by Moselio Schaechter, Academic Press, 2009, pp. 208-219

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Table 1 The systematic groups of the mollicutes

Order	*Genus*	*Genome size*	*Sterols required*	*G + C content of DNA (mol%)*	*Characteristics*	*Habitat*
Mycoplasmatales	*Mycoplasma*[a] (118 species)	580–1350 kb	Yes	23–40	Growth optimum: 37°	Humans, animals
	Ureaplasma[b] (7 species)	760–1140 kb	Yes	25–32	Urea hydrolysis	Humans, animals
Entomoplasmatales	*Entomoplasma* (6 species)	870-900 kb	Yes	27–34	Growth optimum: 30°C	Insects, plants
	Mesoplasma (11 species)	825–930 kb	No	26–32	Growth optimum: 30°	Insects, plants
	Spiroplasma (38 species)	780–2220 kb	Yes	24–31	Growth optimum: 30–37° Helical motile filaments	Insects, plants
Anaeroplasmatales	*Anaeroplasma* (4 species)	1500–1600 kb	Yes	29–33	Obligate anaerobes	Bovine/ovine rumen
	Asteroleplasma (1 species)	1500 kb	No	40	Obligate anaerobes	Bovine/ovine rumen
Acholeplasmatales	*Acholeplasma* (18 species)	1500–1650 kb	No	26–36	Growth optimum: 30–37°	Animals, plants, insects
	Phytoplasma[c] (35 species)	640–1185 kb	Not known	23–29	Uncultured *in vitro*	Insects, plants

[a]The total number includes subspecies.
[b]*U. urealyticum* and *U. parvum*, formerly considered biovars of *U. urealyticum*, are now classified as two separate species.
[c]Phytoplasmas are *Candidatus* species of mollicutes of plants and insects genetically related to the *Acholeplasmatales*.

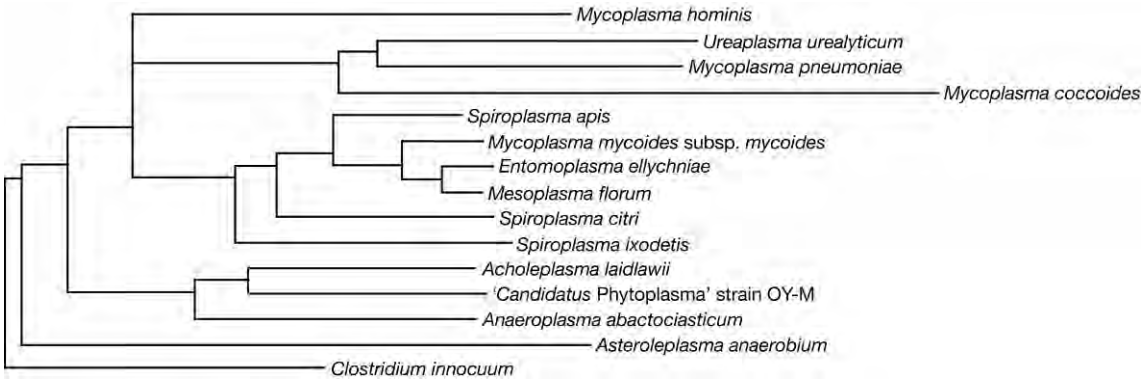

Figure 1 Phylogenetic grouping of the class *Mollicutes* based on 16S rRNA analyses of the type genera plus representatives of other major clusters. Adapted from Bergey's Manual of Systemic Bacteriology, Phylum XVI. Tenericutes Murray 1984a, 356VP (Effective publication: Murray 1984b, 33.), Figure 105, by D.R. Brown, 2011 and reproduced with kind permission from Springer science and Business Media.

In 1981, *M. genitalium* was isolated from a patient suffering from nongonococcal urethritis. *Mycoplasma amphoriforme*, the most recently reported human mycoplasmal species, was first described in 2005. It has been recovered from the respiratory tracts of several persons with antibody deficiency and various types of chronic lung diseases, but its role as a primary pathogen of humans has not been well established.

The genus *Mycoplasma* now includes several *Candidatus* species of cell wall-less uncultivated parasitic bacteria based on 16S rRNA gene sequence analysis. Some of these organisms were previously classified as *Rickettsia* in the genera *Haemobartonella* and *Eperythrozoon*. Most mycoplasmas exhibit a rather strict host and tissue specificity, probably reflecting their highly specific metabolic demands and their parasitic lifestyle. For example, *M. pneumoniae* and *M. genitalium* are preferentially detected in the respiratory and urogenital tracts of humans, respectively.

The complete genome sequences of several species of the genus *Mycoplasma*, including all of the human pathogenic species, have been determined. This large interest in the variability of the *Mycoplasma* genetic complement is stimulated by the interest in creating artificial organisms based on the *Mycoplasma* species. The genome sequences revealed the reason for the complex nutritional requirements of the mycoplasmas: they lack the genes for many biosynthetic pathways and are thus dependent on their host or on the artificial medium to provide these required nutrients. Another interesting feature revealed by genome sequences is that very few

known regulatory proteins are present. Again, this is reflective of their close adaptation to one single natural habitat and a result of the reductive evolution. A metabolically versatile bacterium such as *Pseudomonas aeruginosa* that is capable of thriving in a wide variety of environments reserves as much as 10% of its genome for regulatory genes, but only a small number of these genes can be found in the mycoplasmas.

Genus *Ureaplasma*

Shepard provided the first description of T-strain mycoplasmas, eventually known as ureaplasmas in 1954 when he isolated them from men with urethritis. Originally, there was only a single species, *U. urealyticum*, named in 1974 and eventually shown to consist of at least 14 serovars. Eventually the species was divided into 2 biovars that have now been given separate species status. *U. parvum* includes serovars 1,3,6, and 14 while *U. urealyticum* includes the remaining serovars (2,4,5,7,8,9,10,11,12, and13). There are at least 5 other *Ureaplasma* species that infect cats, dogs, cattle, or birds. This genus is unique among mollicutes in that the organisms have a unique and obligate requirement for urea from which they generate metabolic energy through urease enzymes. Their genomes range from 750–1140 kbp.

Cytology of the Mollicutes

The lack of a cell wall is caused by the absence of genes encoding enzymes for peptidoglycan biosynthesis and is closely linked to another characteristic feature of the mollicutes which is their cellular pleomorphism. Individual cells vary in shape from coccoid to flask-shaped cells or helical filaments, reflecting their flexible cytoskeleton. The mollicutes differ from other bacteria not only because they lack a cell wall but also by their small cell sizes. For example, a typical cell of *M. pneumoniae* is 1–2 μm long and 0.1–0.2 μm wide (Figure 2). In contrast, a typical rod-shaped bacterial cell (such as *E. coli*) is 1–4 μm in length and 0.5–1 μm in diameter. *M. pneumoniae* produces spherical colonies on SP4 agar that may be approximately 100 μm in diameter, whereas *M. hominis* produces characteristic fried egg colonies (Figure 3) and ureaplasmas form very small fried egg type colonies on agar of about 15–60 μm.

The absence of a cell wall has serious consequences for the osmotic stability of the mollicute cells. They are much more sensitive to changes of the osmotic conditions than bacteria possessing a cell wall. The parasitic lifestyle of the mollicutes may be directly related to their osmotic sensitivity. The hosts provide them with osmotically constant conditions that would not be found in the external environment. For example, *M. genitalium* is a parasite of the human urogenital tract, and its transmission by sexual contact ensures minimal exposure of the bacteria to an external, osmotically variable, environment.

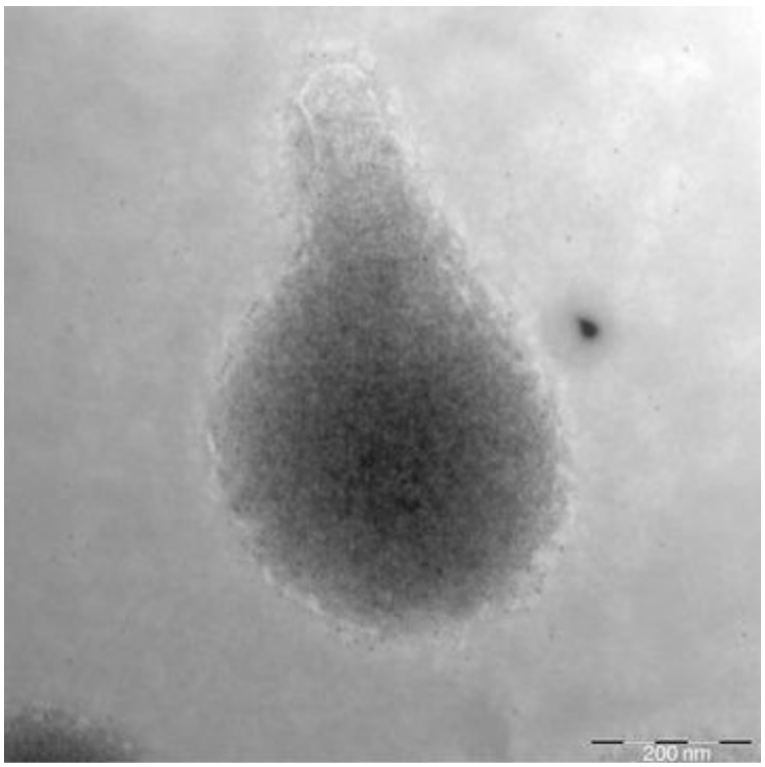

Figure 2 Electron micrograph of a cell of *Mycoplasma pneumoniae*. The terminal organelle (also called the tip structure) is visible in the upper part of the cell. Scale bar = 200 nm.

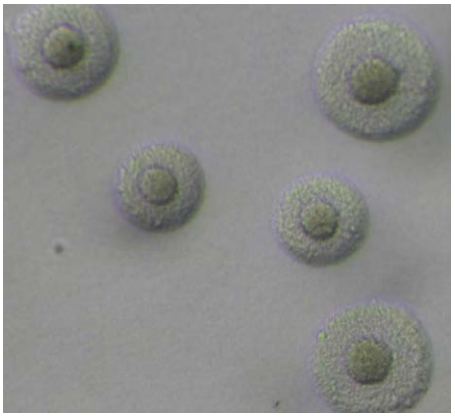

Figure 3 Colonies of *Mycoplasma hominis* growing on A8 agar (126X). Colonies are typically 200–300 μm in diameter and are best visualized using a stereomicroscope. *M. hominis* demonstrates the typical fried egg appearance characteristic of many species in the genus. Adapted from Mycoplasmas and Ureaplasmas as Neonatal Pathogens, Figure 4, by K.B. Waites, B. Katz, and R.L. Schelonka, *Clinical Microbiology Reviews* 18:757–789, 2005. Reproduced with permission from ASM Press.

Some species such as *M. pneumoniae* and *M. genitalium* and *M. penetrans* have a flask- or clublike tip structure (Figure 2). This tip structure is a complex and specialized attachment organelle that has evolved to facilitate the parasitic existence of the mycoplasmas. The tip structure is made up of a network of adhesins, interactive proteins, and accessory proteins, which cooperate structurally and functionally to mobilize and concentrate adhesins at the tip of the cell. The major adhesin of *M. pneumoniae* is the 170 kDa P1 protein that is responsible for the interaction of the bacteria with the host cells. In addition, the tip structure is important for the internalization of intracellular mollicutes such as *M. genitalium*. This may protect the bacterial cells against the host immune system.

Mycoplasma species are able to glide on solid surfaces with the help of their terminal attachment organelle. Terminal organelles that are detached from the body of the *M. pneumoniae* cell are released by some mutants. These detached organelles are still capable of gliding, demonstrating that this organelle acts as a novel engine that allows cellular movement.

As other bacteria, the mollicutes divide by binary fission. Again, the terminal organelle seems to be very important for this process. Cell division in *M. pneumoniae* is preceded by the formation of a second tip structure adjacent to the existing one. The two terminal organelles then separate leading eventually to cytokinesis.

Biochemistry and Metabolism of the Mollicutes

The reductive evolution of the mollicutes is reflected in their limited metabolic properties and energy-yielding systems. Many common biosynthetic systems are partially or completely lacking and there are considerable differences among the various species. No quinones or cytochromes have been found in any representative. The electron transport system is flavin-terminated. Thus, ATP is produced by substrate-level phosphorylation, a less efficient mechanism as compared to oxidative phosphorylation.

Both *M. pneumoniae* and *M. genitalium* utilize glucose as their primary metabolic substrate; whereas *M. hominis* utilizes arginine and *Ureaplasma* spp. hydrolyze urea for generation of metabolic energy. Glycolytic kinases of some mollicute species have functions in addition to that in glycolysis. These enzymes can use not only ADP/ATP but also other nucleoside diphosphate/triphosphate couples. Thus, these enzymes (phosphofructokinase, phosphoglycerate kinase, pyruvate kinase, and acetate kinase) compensate for the lack of the normally essential *ndk* gene encoding nucleoside diphosphate kinase that is required for nucleotide biosynthesis. The complete set of genes encoding components of the Embden-Meyerhoff-Parnas glycolysis pathway is complete in *M. genitalium* and *M. pneumoniae*, which are able to generate ATP through glucose hydrolysis, but *M. hominis* lacks a single gene in the pathway.

Glycolysis is not the only source of ATP formation by substrate level phosphorylation in the mollicutes. Pyruvate can be oxidized to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA can be further catabolized by phosphotransacetylase and acetate kinase in an additional substrate level phosphorylation resulting in the formation of acetate. An alternative pathway of pyruvate consumption is its reduction to lactate, leading to the regeneration of NAD^+.

In addition to utilization of glucose, *M. pneumoniae* can utilize glycerol and fructose. Interestingly, arginine is not used even though the genetic equipment to utilize this substrate is present in the genome. In the case of *M. pneumoniae*, the enzyme is believed to be inactive due to a frameshift mutation leading to a premature stop in the *arcA* gene.

The arginine dihydrolase pathway can also be found in *M. hominis*. Arginine hydrolysis by this pathway results in the production of ornithine, ATP, CO_2, and ammonia. The pathway uses three enzymes: arginine deiminase, ornithine carbamoyl transferase, and carbamate kinase. The degradation of arginine is coupled to equimolar generation of ATP by substrate-level phosphorylation.

Mollicutes possess very limited metabolic and biosynthetic activities for amino acids, carbohydrates, and lipids as compared to 'conventional' bacteria. *M. pneumoniae* scavenges nucleic acid precursors and does not synthesize purines or pyrimidines de novo. These may be provided by RNA and DNA that have been degraded by potent mycoplasmal nucleases. Furthermore, both

M. genitalium and *M. pneumoniae* lack all the genes involved in amino acid synthesis, making them totally dependent on the exogenous supply of amino acids from the host or from the artificial culture medium. The mycoplasmas have also lost most of the genes involved in cofactor biosynthesis; therefore, to cultivate them *in vitro*, the medium has to be supplemented with essentially all the vitamins.

Being dependent on the exogenous supply of many nutrients would predict that mycoplasmas need many transport systems. Surprisingly, *M. genitalium* and *M. pneumoniae* possess a only small number of transport proteins compared to the 281 transport and binding proteins annotated in *E. coli*. The apparent low substrate specificity of some of the mollicute transport systems, such as those for amino acids, may also contribute to the significant gene reduction observed.

Although mollicutes produce hydrogen peroxide, *M. pneumoniae* and *M. genitalium* lack the genes dealing with oxidative stress, such as those encoding catalase, peroxidase, and superoxide dismutase. A thioredoxin reductase system, identified in the mycoplasmas, may protect them from reactive oxygen compounds.

A major problem for the research with mollicutes is the difficulty of cultivating them *in vitro*. Only a minority of the mollicutes existing in nature have been cultivated so far. For example, none of the phytoplasmas infecting insects or plants has been cultivated *in vitro*. To overcome the metabolic deficiencies of the mycoplasmas, complex media are used for their cultivation. The media are usually based on beef heart infusion, peptone, yeast extract, and serum with various supplements. Serum has been shown to provide, among other nutrients, fatty acids and sterols that are required for membrane synthesis. For most mollicutes, ureaplasmas being a notable exception, the pH is adjusted to a slightly alkaline value, conditions that imitate those in the eukaryotic host.

Pathogenesis and Disease Associations

Pathogenicity has been most intensively studied with *M. pneumoniae*. This organism is a common cause of tracheobronchitis and community-acquired pneumonia in persons of all ages and is particularly prone to spreading in closed populations such as military camps, boarding schools, and prisons. Following inhalation, organisms are opsonized by complement or antibody, macrophages are activated and migrate to the site of infection and neutrophils, plasma cells, B and T lymphocytes infiltrate the lung. The organism attaches to ciliated respiratory epithelium using its specialized attachment organelle through a complex process involving numerous transmembrane as well as cytoskeletal proteins, including a 170-kDa P1 protein which is present over the entire membrane surface, but is concentrated in the attachment organelle. Numerous accessory proteins are also involved in cytadherence, including HMW1, 2, and 3, and P90, P 40 and P30. Once the polar structure is established, an independently assembled complex of proteins B, C, and P1 complete formation of the organelle. The importance of P1 in cytadherence is demonstrated by the fact that mutants lacking P1 are unable to adhere to cells and antibodies directed against P1 can block adherence. An ADP-ribosyl transferase, similar to pertussis toxin, known as the community-acquired respiratory distress syndrome (CARDS) toxin binds to surfactant protein A and enters the host cell by means of clathrin-mediated endocytosis. The toxin leads to ciliostasis and nuclear fragmentation and stimulates the production of proinflammatory cytokines and acute cellular inflammation, ultimately leading to airway damage. The amount of toxin produced correlates with disease severity. It has been suggested that cytokine production by the host response to *M. pneumoniae* infection may lead to chronic pulmonary diseases such as bronchial asthma.

Another major factor contributing to cytotoxicity and thus to pathogenicity of this mycoplasma is the formation of hydrogen peroxide. The hydrogen peroxide formed by *M. pneumoniae* acts in concert with endogenous toxic oxygen molecules generated by the host cells and induces oxidative stress in the respiratory epithelium. The effects of the peroxide on the host cells include loss of reduced glutathione, denaturation of hemoglobin, peroxidation of erythrocyte lipids, and eventually the lysis of the cells. Hydrogen peroxide also confers hemolytic activity. A nuclease produced by *M. pneumoniae* has the ability to enter host cells and induce apoptosis. Intracellular localization has been described in tissue culture models and if it occurs *in vivo*, may contribute to establishment of chronic infection by protecting the organisms from direct contact with cells of the host immune system, antibodies, and complement. Immunomodulation of the host immune response may occur through stimulation of B and T lymphocytes. Many of the clinical manifestations of mycoplasmal respiratory disease as well as extrapulmonary complications can be caused by autoimmune immunologic and inflammatory effects produced by the host in response to antigenic mimicry of eukaryotic structures by functional sites of microbial components, including the cytoadhesins. A strong humoral immune response typically results from a primary infection with *M. pneumoniae* in an immunocompetent host. Antibodies are directed against proteins and lipids, including the cytadhesins. IgM can be detected after about one week, followed by IgG about 2 weeks later. IgM can sometimes persist for several weeks to months and may not be detectable in subsequent infections in middle aged or older adults who have had several previous infections. Surface antigen rearrangements may be responsible for host susceptibility to repeated infections over time.

Acquired antimicrobial resistance to macrolides through point mutations in 23S rRNA has become a major problem in *M. pneumoniae* over the past several years in Asia. Greater than 90% of clinical isolates from some regions of Japan and China are now highly-resistant to drugs such as erythromycin, clarithromycin, and azithromycin. Macrolide resistance is now being reported from Europe and North America, but rates are not nearly as high as those in Asia. Most of the reported resistance has involved children thus far, but adults may also be affected. Resistant organisms tend to cause more severe and prolonged illness that often necessitates substituting alternative antimicrobials such as fluoroquinolones or tetracyclines.

M. hominis is a common commensal organism in the lower urogenital tract of healthy persons and is often isolated in conjunction with *Ureaplasma* spp. Despite the frequency with which *M. hominis* is detected in healthy persons, it may cause

conditions such as pyelonephritis, pelvic inflammatory disease, post-partum endometritis, bacterial vaginosis, as well as systemic infections in newborn infants including congenital pneumonia and meningitis. Bacteremia, wound infections, and arthritis sometimes occur as systemic manifestations of *M. hominis* infections in adults, especially in persons with dysfunction in the humoral immune system. *M. hominis* does not have a prominent attachment tip, but it is able to adhere to various types of host cells. A complex of four cytoadhesive surface membrane proteins has been described in *M. hominis*. This complex includes lipoprotein P60 and P80, which has an uncleaved amino-terminal signal protein mediating membrane anchoring. The lipoprotein P50, known as the variable adherence-associated antigen (Vaa) displays high-frequency phase and size variation and may undergo antigenic variation and participate in evasion of the host immune response. An additional surface protein P100, known as OppA, the substrate-binding domain of the oligopeptide permease, is also involved in *M. hominis* cytadherence and apoptosis. Release of NH_3 as a product of arginine hydrolysis has been suggested as a virulence factor of *M. hominis*, resulting in cytotoxic effects. Reports of hyperammonemia in immune suppressed persons with systemic infection with *M. hominis* provide support for this observation. Tetracycline resistance mediated by the *tetM* transposon occurs to a variable degree in *M. hominis*. Moreover, fluoroquinolone resistance caused by mutations in DNA gyrase and/or topoisomerase IV has also been reported.

M. genitalium is now known to be a significant cause of male urethritis as well as female cervicitis, and pelvic inflammatory disease. Whether it is an important pathogen affecting pregnancy outcome and neonates has not been established. In contrast to *M. hominis* and *Ureaplasma* spp., *M. genitalium* is less commonly detected in the lower urogenital tracts of healthy persons. The terminal structure of *M. genitalium* is composed of at least seven proteins, two of which, MgPa and P110 are necessary for cytadherence, in association with other accessory proteins. The MgPa adhesin shows significant homology with P1 in *M. pneumoniae*, and like P1 is a strong immunogen. Another protein P32 involved in cytadherence also has homology to an *M. pneumoniae* adhesin, P30. Following cytadherence, *M. genitalium* can invade epithelial cells and localize in the nucleus.

Repetitive elements in the *M. genitalium* adhesin gene have strong sequence similarity to repetitive DNA elements in *M. pneumoniae*. Typical of other human mycoplasmal infections, antigenic variation of membrane proteins in *M. genitalium* encoded by *mgpB* and *mgpC* in the MgPa operon due to recombination and slipped-strand mispairing with nine partial non-coding MgPars loci is believed to facilitate chronic infection. A calcium-dependent membrane-associated nuclease MG186 enables the organism to degrade host nucleic acid, thus providing a source of nucleotide precursors for its own replication. Of major concern is the acquisition of genes that mediate macrolide and sometimes fluoroquinolone resistance by *M. genitalium*, making infections with this organism difficult to treat. Most data concerning acquired antimicrobial resistance are based on small numbers of cases, so the extent to which it occurs is not known, but it is apparently due to selective antibiotic pressure in treatment of sexually transmitted infections. For many years, cross reactivity in the complement fixation serological tests between *M. pneumoniae* and *M. genitalium* caused diagnostic confusion because of their antigenic similarities. This became less important when other more specific serological methods such as enzyme immunoassays became more widely used.

M. fermentans received considerable attention several years ago when it was thought possibly to play a role as a mediator or cofactor in development of AIDS, as a cause of Gulf War Syndrome, and Chronic Fatigue Syndrome. Subsequent studies however, have failed to prove these disease associations and now it is appreciated that this mycoplasma behaves as an opportunistic pathogen causing systemic infections in persons with HIV, as well as those who are HIV-negative. *M. fermentans* has been recovered from the throats of children with pneumonia and from adults with influenza-like illnesses. There is no evidence that *M. fermentans* causes urethritis or cervicitis, but it has been detected by PCR in placentas with chorioamnionitis, suggesting venereal transmission is possible. This mycoplasma has also been isolated from the joints in persons with inflammatory arthritis, and there has been speculation that it may be involved in rheumatoid arthritis, though this has not been conclusively proven. *M. fermentans lacks* a well-defined attachment organelle, but it is still able to invade host cells. Glycoglycerol lipid (GGPL-III) and lipoprotein P29 are believed to be responsible for *M. fermentans* cytadherence to host cells as well as for triggering an inflammatory response. Understanding the role of *M. fermentans* as a possible opportunistic pathogen in humans is complicated because it is known to colonize mucosal surfaces in some healthy persons, as is the case with *M. hominis* and *Ureaplasma* spp.

Ureaplasmas may be isolated from the lower urogenital tract in as many as 80% of healthy adult women and may also be detected in the urethras of a significant proportion of healthy men. Ureaplasmas are readily transmitted vertically in utero or at time of delivery. Most studies have reported that *U. parvum* is more common as a colonizer than *U. urealyticum*. As is the case with other mollicutes, ureaplasmas reside primarily on mucosal surfaces with preference for the urogenital tracts of adults and respiratory tracts in neonates who acquire the organisms from their mothers *in utero* or at delivery. A number of clinically significant illnesses have been attributed to ureaplasmas, despite their common occurrence in healthy persons. These include urethritis, urinary calculi, postpartum endometritis, and chorioamnionitis, and preterm labor, in adults and bacteremia, meningitis, abscesses, congenital pneumonia, and chronic lung disease in infants, mainly affecting those born preterm. Systemic spread to the bloodstream, central nervous system, joints and other body sites rarely occurs outside of the neonatal period except in persons with defects in the humoral immune system.

Infection with ureaplasmas produces a robust inflammatory response in conditions such as chorioamnionitis. Inflammation is enhanced by upregulation of pro-inflammatory cytokines such as tumor necrosis alpha and interleukin-8. Although no toxins have been detected in ureaplasmas to date, they can adhere to erythrocytes, spermatozoa, neutrophils, and urethral epithelial cells. Their adhesins are surface proteins for which the receptors may be sialyl residues and/or sulfated compounds, similar to what have been described for *M. pneumoniae* and other mycoplasmas. Some experimental data suggest the presence of several ureaplasma adhesins, some of which are species- or serovar-specific and antigenic. The 96 kDa surface-expressed antigen may be one such adhesin. Surface antigen variation may be a means for persistence and repeated infections with these organisms. Another means by which

ureaplasmas may evade the host immune response to produce chronic infections may involve suppression of endogenous antimicrobial peptides that are an integral part of the innate host immune response by downregulation of expression of their respective genes. Ureaplasmas can produce IgA protease and hemolysins that are believed to be encoded by *hlyC* and *HlyA*. Ureaplasmas release NH_3 through urea hydrolysis mediated by ureases and this is the mechanism by which these organisms produce struvite urinary stones and hyperammonemia that has been reported in lung transplant recipients. At least 15 potential nucleases have been identified in *Ureaplasma* genomes. Acquired antimicrobial resistance has been described in both *Ureaplasma* spp. for macrolides, fluoroquinolones, and tetracyclines. Depending on the population tested and the history of antibiotic exposure, resistance to tetracycline may be fairly common, affecting as many as half of all isolates, mediated by the *tetM* transposon. Mutations affecting activities of DNA gyrase and/or topoisomerase IV mediating resistance to fluoroquinolones and mutations in 23S rRNA affecting susceptibility to macrolides appear to be uncommon, affecting less than 5% of clinical isolates of *Ureaplasma* species in the southeastern United States.

Since the very first descriptions of *Ureaplasma* serovars and designation of the two biovars as individual species, there has been much speculation regarding whether there was differential pathogenicity at the serovar or species level. Evidence available to date supports the concept that *U. urealyticum* is more pathogenic than *U. parvum* in male urethritis, but data are much less convincing for other conditions. There is no credible evidence that differential pathogenicity exists at the serovar level, primarily because of extensive horizontal gene transfer among the 14 known serovars that are divided between the two species.

Various commensal mycoplasmal species of the respiratory and urogenital tracts of humans can occasionally be detected as causes of systemic infections, usually in immunosuppressed hosts with antibody deficiencies, as can mycoplasmal species that normally infect animals. These uncommon occurrences underscore the importance of a functional humoral immune system in protection against serious mollicute infections.

Genetics of the Mollicutes

Gene Expression

The basic mechanisms of gene expression have not been studied extensively in the mollicutes. These organisms possess a conventional bacterial RNA polymerase, but unlike most other bacteria, they encode only one sigma factor of the RNA polymerase. Thus, diversity of promoters and RNA polymerase holoenzymes are not used for regulatory purposes in the mollicutes. The transcription start sites have been identified for several *M. pneumoniae* genes, and it turned out that the −10 region of these promoters is similar to that recognized by the housekeeping sigma factors of other bacteria such as *E. coli*. In contrast, there is no conserved −35 region. These observations were confirmed by an analysis of the sequence determinants that are required for promoter activity in front of the *M. pneumoniae ldh* gene encoding lactate dehydrogenase. The −10 region is essential for transcription initiation, whereas the −35 region could be mutated without any consequences. Thus, the single *M. pneumoniae* RNA polymerase holoenzyme recognizes only the −10 region for promoter recognition.

Another peculiarity of the *M. pneumoniae* transcription machinery is the lack of the termination factor Rho, and correspondingly, the absence of Rho-dependent transcription terminators. Surprisingly, a bioinformatic analysis of bacterial genomes and the free energy values of RNAs around the end of open reading frames suggest that the mollicutes also do not contain functional Rho-independent transcription terminators. This raises the important question of how transcription is terminated in the mollicutes or whether it is terminated at all. The answer came from Northern blot experiments aimed at the identification of *in vivo* transcripts, and this answer is ambiguous. Indeed, defined transcripts were observed in a few cases, such as the *M. genitalium* and *M. pneumoniae ftsZ* gene clusters or the *M. pneumoniae ptsH* gene. The existence of these defined transcripts implies that there are also defined transcription terminators present. However, these terminators may be very rare. This might explain the observation that unrelated genes are expressed as parts of one transcription unit in the mollicutes. Moreover, most attempts to determine transcript sizes by Northern blot analysis in the mollicutes have failed. This is probably the result of mRNA length polymorphisms, which prevent the detection of clearly defined RNA species.

Intrinsically fluorescent proteins such as the green fluorescent protein (GFP), the yellow fluorescent protein (YFP) and their derivatives have been used to study protein localization, interactions, cell division and gene expression. These proteins have been used in *M. pneumoniae* to demonstrate the subcellular localization of the proteins HMW2, P65, P41, and P24 and to confirm them as components of the attachment organelle. Moreover, P41 and P24 appeared to be cytoskeletal proteins.

Most genes in the mollicutes have the same orientation on the chromosome and the intergenic regions are usually quite short, when present. The transcription of most of these large gene clusters is colinear with replication. This genome organization also favors polycistronic transcription of large gene clusters.

The lack of defined mRNA species results not only from the absence of transcription terminators, but also from the weak conservation of sequences that mediate transcription initiation. Indeed, the −10 regions predicted from the analysis of many start points occur about 2900 times in the 816 kbp genome of *M. pneumoniae*. This large number of possible transcription initiation sites is also reflected by the observation of substantial antisense transcription in both *M. genitalium* and *M. pneumoniae*.

M. pneumoniae contains 8 known transcription regulators. The low number of transcription factors in the mollicutes and the weak stringency of transcription signals might therefore reflect their close adaptation to specific habitats that provide a good supply of nutrients and protect the bacteria from harmful environmental conditions. Moreover, the good supply of nutrients from the host may abolish the need for transcription regulation, thereby switching off the expression of genes if their products are not required.

Translation is one of the most prominent activities of the mollicute cell. As much as 15% of the genome of the mollicutes is devoted to translation-related functions. The principal mechanisms of translation in the mollicutes are identical to those found in other bacteria. Because of the low genomic GC content, the codon usage is strongly biased toward AT-rich codons. The molecular mechanisms of translation initiation in *M. pneumoniae* and its close relatives still await elucidation.

Posttranslational Protein Modification

Post-translational protein modification plays an important role in protein secretion. Several types of protein modifications have been described which include (1) cleavage of signal peptides; (2) acetylation; (3) endoproteolytic cleavage of secreted proteins; and (4) phosphorylation. Much of the existing information about protein modification in mollicutes pertains to *M. pneumoniae*. Among 693 predicted open reading frames in the *M. pneumoniae* genome, 620 have been identified by proteome studies and functions for about 70% of the proteome have been assigned thus far. Mpn 142 is one of 3 genes in the operon that includes the P1 adhesin (Mpn 141) and Mpn 140. Mpn 142 undergoes endoproteolytic cleavage to generate the amino terminal 40-kDa P40 and the carboxy terminal 90-kDa P90 proteins that reside on the cell surface and complex with P1 and other proteins in the terminal organelle. There is also evidence that *M. pneumoniae* has signal peptidase activity and that lipoprotein families are also subject to endoproteolytic cleavage. Many cleavage fragments remain surface bound in the mycoplasmal cell membrane. Additionally, proteins associated with glycolysis, components of translational machinery, and adhesins can be phosphorylated and/or lysine acetylated. Such modifications alter the cellular proteome and influence protein interaction pathways.

Considerable work has been devoted to the biochemical characterization of the protein kinase HPrK from *M. pneumoniae*. As in related proteins, it contains an essential Walker A motif for ATP binding. Mutations in this region severely affect both the kinase and the phosphatase activities of the protein. Fluorescence studies revealed that the *M. pneumoniae* HPrK has a significantly higher affinity for ATP than any other HPrK studied so far. This may explain why it is active even at low ATP concentrations. The *M. pneumoniae* HPrK was crystallized and its structure determined. As observed for homologous proteins, it forms a hexamer with the C-terminal domains in the active center.

An analysis of the *M. genitalium* proteome revealed that each identified protein is present at an average of 1.22 spots on a 2-D gel, suggesting posttranslational modification of about 25% of all proteins. Given the importance of protein phosphorylation in all other living organisms, it seems safe to assume that a large portion of these modified proteins is actually phosphorylated. A phosphoproteome analysis of *M. genitalium* and *M. pneumoniae* identified 5 and 3% of the total protein complement of these bacteria, respectively, as phosphoproteins. Among these proteins are not only enzymes of central carbon metabolism such as enolase and pyruvate dehydrogenase subunits, but also several cytoskeleton and cytadherence proteins.

Genomic Comparisons in the Mollicutes

One scientific question that has been of interest to humans is the problem of what constitutes life. Only today, in the era of genomics research, are we able to attempt an answer to this question. A major milestone in defining life was the identification of key features that characterize all living things. Among these features are metabolism, autonomous replication, communication, and evolution. With the availability of genome sequences, it is has become possible to determine the genetic equipment required for independent life. The mollicutes are of special interest in this respect because they have the smallest genomes that allow independent life under laboratory conditions.

Genome research with the mollicutes was begun in the 1990s and has been driven by two major challenges: (1) the identification of the minimal set of genes that is required for independent life and (2) the creation of artificial organisms that are based on this minimal gene set. The simplicity of the mollicutes and the broad body of knowledge on their biology makes them ideal starting points for these research areas.

Several different strategies have been applied to identify the minimal gene set required for life. The simplest approach is based on the comparison of sequenced genomes of different organisms. It seems safe to assume that those genes that are conserved in different organisms are more important than those that appear only in certain species. The smallest genome of any independent living organism known so far is that of *M. genitalium*. This bacterium has a genome of 580 kbp with 482 protein-coding genes. *M. pneumoniae* has a genome of 816 kbp with 779 genes coding for proteins. A comparison of the two genomes based on the initial descriptions of *M. genitalium* G37 (ATCC 33530) and *M. pneumoniae* M129 (ATCC 29342) reveals an overlap of 477 genes common to both species. This suggests that *M. pneumoniae* is an 'extended version' of *M. genitalium*. It is tempting to speculate that *M. genitalium* is further advanced on the pathway of reductive genome evolution. Indeed, some genes present in *M. pneumoniae* but not in *M. genitalium* such as the mannitol utilization genes are known to be nonfunctional in the former organism. Thus, *M. genitalium* seems to be very close to a true minimal organism.

Evaluations of 15 *M. pneumoniae* genomes at the University of Alabama at Birmingham by Next-Generation Sequencing using the Illumina MiSeq platform, that included previously sequenced strains M129 (ATCC 29342) and FH (ATCC 15531) demonstrated that greater than 99% sequence identity, despite the facts that the strains were collected over a span covering 1944 to 2009 from the United States, Europe, and Asia, and including both of the major subtypes, P1 and P2. The CARDS toxin gene had very little variation among the 15 strains, with only a single nucleotide polymorphism distinguishing P1 and P2 subtypes. Evidence for

horizontal gene transfer was minimal. The major region of variability was within the P1 and open reading frame 6 genes associated with the adhesin complex.

Only *M. hominis* type strain PG 21 (ATCC 23114) has been sequenced and reported, demonstrating it to be the second smallest human mollicute with a total of 665 kbp and 537 coding sequences. Comparative analyses indicated the occurrence of horizontal gene transfer between *M. hominis* and *U. parvum*, serovar 3 which had been sequenced earlier. This is not unexpected since these species occupy the same urogenital niche in humans. Despite many common genes, the pathogenic *Mycoplasma* species, *M. pneumoniae*, *M. genitalium*, and *M. hominis*, have distinct energy-generating pathways as well as different cytadherence and virulence genes. Interestingly, when genomes of the urogenital species M. *hominis*, *M. genitalium*, and *U. parvum were compared*, there were 220 genes specific for *M. hominis*, 172 specific for *M. genitalium*, and 280 specific for *U. parvum*. Thus, only a subset of their genes is part of a common gene pool.

Additional recent studies have undertaken to compare the genomes of 19 *U. urealyticum* and *U. parvum* strains, including all 14 known serovars and several clinical isolates. *U. parvum* genomes had about 750–780 kbp with an average of 608 genes, of which 201 encode hypothetical proteins, while *U. urealyticum* genomes had 840–950 kbp with an average of 664 genes, of which 230 encode hypothetical proteins. The ureaplasma pangenome has1010 protein coding genes of which 758 have orthologs in at least one other strain and 515 genes are universally conserved among all 19 strains, thereby comprising the ureaplasma core genome. The number of genes found on only a single serovar was 262. Despite the fact that IgA protease activity has been demonstrated in several *Ureaplasma* serovars, the presence of the gene encoding this enzyme could not be identified in any of the genomes. Thus, it is possible that the gene may be there but was not recognized as such in the computational genome analysis.

Some very interesting findings evolved from comparative analyses of *Ureaplasma* spp. genomes involving the multiple banded antigens (MBA) which are immunogenic and initially provided the basis for which the 14 serovars were established. Genomic analysis showed the *mba* genes to be part of a large complex superfamily which is phase variable with some serovars having identical sets of *mba* genes. These studies further support the concept that horizontal gene transfer occurs, especially with the more pathogenic *U. urealyticum,* and the organisms exist as quasi-species rather than being comprised of stable serovars.

A major achievement in genomics research in recent years was the chemical synthesis and assembly of the *M. genitalium* chromosome. Thus, an artificial chromosome can be synthesized and this DNA can be introduced into a living cell to provide the environment for the expression of this genome. This achievement has led to the replacement of one genome by another, a process called genome transplantation, which was demonstrated in mollicutes when a *Mycoplasma mycoides* genome cloned in *Saccharomyces cervisiae* was transplanted into *Mycoplasma capricolum* recipient cells. These species were chosen as opposed to *M. genitalium*, which has the smallest genome, because of its extremely slow growth and difficult cultivation *in vitro*.

Molecular Biology and Genetic Tools

The detailed genetic analysis of the mollicutes has been hampered by the lack of genetic tools that allow the efficient expression of mollicute genes in heterologous hosts for purification and subsequent biochemical analysis, the stable introduction of foreign genetic material into a mollicute cell, and either the targeted construction or the targeted isolation of desired mutant strains. During the past few years considerable progress has been made in the field of mollicute genetics, making these organisms more accessible for genetic studies.

The occurrence of UGA codons in the genes of mollicutes has often prevented their expression in heterologous hosts for detailed biochemical analysis because they serve as stop codons in *E. coli* and other expression hosts. To circumvent this problem, a variety of different strategies had been employed, including the expression of UGA-containing genes in opal suppressor strains of *E. coli* that also read UGA as a tryptophan codon. As long as only few UGA codons are present in a gene, their sequential replacement by standard site-directed mutagenesis strategies might also be taken into consideration. However, the latter approach is time-consuming and cost-intensive with an increasing number of UGA codons. Recently, a strategy referred to as multiple mutation reaction (MMR) allowing the simultaneous replacement of multiple UGA codons in a single-step reaction was developed. This strategy is based on the use of 5′-phosphorylated oligonucleotides containing the desired mutations in a polymerase chain reaction (PCR). During the elongation steps, the external amplification primers are extended. As the mutation primers are designed to hybridize more strongly to their targets, the elongated amplification primers can then be ligated to the 5′ ends of the mutation primer by a thermostable DNA ligase, yielding a DNA strand that contains the desired mutation. With this strategy, the simultaneous introduction of up to nine mutations in one single step is possible.

In combination with smart screening systems, transposons have been used for the disruption of genes but also as carriers for the introduction of genetic material into the chromosome. The transposons Tn*916* and Tn*4001* and their improved derivatives can be used in mollicutes. These transposons were originally isolated from *Enterococcus faecalis* and *Staphylococcus aureus*, respectively, and have a broad host range. Tn*916* is a conjugative 18 kb transposable element that contains the *xis-Tn*/*int-Tn* genes for excision/integration, followed by the *tetM* tetracycline resistance determinant and a set of genes (*tra*) required for intercellular transfer. Tn*916* does not generate target duplications at its integration site, because it transposes by an excision/integration mechanism that is based on staggered nicks in the donor DNA. Tn*4001* is a 4.5 kb composite transposon consisting of two identical IS*256* elements flanking the gentamicin/kanamycin/tobramycin resistance conferring *aac-aphD* gene. Tn*4001* has been used for transforming several

Mycoplasma species. To increase the stability of transposon insertion mutants, mini-transposons on the basis of Tn*4001* were constructed that have the transposase gene outside the transposable elements to prevent reexcision of the transposon after the first transposition event.

Targeted construction of gene knockout mutants via homologous recombination has been reported in a few mollicutes such as *M. genitalium*, *Mycoplasma gallisepticum*, *Mycoplasma pulmonis*, and *Acholeplasma laidlawii* to enable some investigation of the role of individual genes in pathogenesis. In the absence of homologous recombination, the only remaining way to obtain gene knockouts is transposon mutagenesis. Because of the randomness of integration, the screening of large transposon mutant libraries for the loss or gain of a specific phenotype is required to isolate a gene knockout of interest. If no screenable phenotype can be expected to be associated with a gene of interest, the only known feature of the desired gene knockout is the specific DNA junction between the gene of interest and the transposon. Based on this idea, a strategy referred to as 'haystack mutagenesis' has been designed that allows the targeted isolation of any viable transposon insertion strain out of an ordered library of transposon mutants. The concept of haystack mutagenesis is based on a saturating transposon mutagenesis to ensure that each dispensable gene is disrupted at a desired confidence level. Once the required number of transposon mutants has been isolated, they are arranged in pools of a reasonable size. These pools can then be screened by PCR using a gene-specific oligonucleotide and another one specific to the transposon for identifying the pool that contains the desired insertion. Subsequently, a similar screen at the level of the individual clones of the positive pool will identify the mutant of interest. This strategy has already been used for the isolation of several *M. pneumoniae* mutants. Alternatively, transposon mutant libraries can be screened for mutants that exhibit an interesting phenotype, such as loss of gliding motility.

There is ample evidence for transposon delivery into *Ureaplasma* genomes in nature through the acquisition of tetracycline resistance mediated by the *tetM* transposon. Despite several failed attempts, transposon mutagenesis has now been used successfully to transform 3 serovars of *U. parvum* and a clinical strain of *U. urealyticum* with a *Tn4001*-based mini-transposon plasmid containing a gentamicin-resistance selection marker, leading to random disruption of genes and phenotypic changes in the organism. This finding suggests that it may be possible to determine non-essential genes, deliver and express exogenous genes, and identify key pathogenic markers in human ureaplasmas.

Laboratory Detection

Culture is useful for *M. hominis* and *Ureaplasma* spp. since they grow rapidly from clinical specimens inoculated into appropriate liquid and solid media such as 10B broth and A8 agar, usually forming colonies that can be visualized under a stereomicroscope within 1to 3 days. Ureaplasmas can be identified to genus level by tiny granular brown fried-egg colonies 15–60 μm in diameter on A8 agar. Rapidly growing mycoplasma species from urogenital specimens that produce larger fried egg type colonies on agar within 2 to 3 days can be identified to species level using PCR or alternative methods. However, *M. pneumoniae* and *M. genitalium* are quite fastidious and grow much more slowly. Culture of *M. genitalium* requires up several weeks, is rarely successful and need not be attempted. Serology is sometimes used for diagnosis of acute *M. pneumoniae* infections, but suffers from poor sensitivity and requires testing acute and convalescent sera for IgM and IgG for optimum results. Serology is not standardized, nor is it useful for detection of infections caused by any of the genital mycoplasmal species or ureaplasmas. For these reasons, molecular-based assays such as the PCR reaction are the most common means for rapid detection of *M. pneumoniae* and *M. genitalium*. PCR is also a suitable alternative for detection of *M. hominis* and *Ureaplasma* spp., but is generally more expensive than culture and less widely available due to the lack of commercialized assays in the United States. Other types of molecular-based assays used for detection of the various mycoplasma species have included loop-mediated isothermal amplification (LAMP), nucleic acid sequenced-based amplification (NASBA), and transcription-mediated amplification (TMA). Molecular-based assays are available through many reference laboratories for detection of *M. pneumoniae*, *M. hominis*, *M. genitalium*, *U. parvum*, and *U. urealyticum*, although no assays are commercially sold in the United States except for *M. pneumoniae*. Real-time PCR has improved specificity compared to conventional PCR, partly because of the use of probes labeled with a fluorphore and a quencher designed to correspond to a sequence located between the forward and reverse primers. Labeled probes allow a signal to be detected only if the probe has annealed to its complementary target sequence, thus minimizing cross –reaction and detection of undesired amplicons. Another aspect of real-time PCR that contributes to its specificity is that amplicon melting temperature is determined at the end of the assay and can be used to verify whether the desired PCR product is being detected. A variety of gene targets have been employed for detection of the various pathogenic mycoplasmas and ureaplasmas of humans using real-time PCR. In our laboratory, the non-coding sequence *repMp1* has been a useful target for real-time PCR assays for *M. pneumoniae* since it is present in multiple copies for improved sensitivity. For *M. genitalium*, *gap*, encoding glyceraldehyde-3-phosphate dehydrogenase overcomes problems encountered with 16S rRNA and *MgPa* targets. Several different genes have been described as targets for real-time PCR for *M. hominis*, including 16S rRNA, *gap*, *fstY*, *yidC, and tuf*, but the presence of mutations and sequence variations have caused problems with analytical sensitivity. Our laboratory has developed and validated a multiplex real-time PCR assay for detection and speciation of ureaplasmas based on two genes. *UU063* encodes a conserved hypothetical protein identical in all 4 *U. parvum* serovars that is absent in *U. urealyticum*. UU10-0680 is an open reading frame that is conserved in all 10 *U. urealyticum* serovars, but absent in *U. parvum*. These assays perform very well and have an improved analytical and clinical sensitivity over quantitative culture.

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Further Reading

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Metal Extraction and Biomining[☆]

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Glossary

Biofilm A layer of adhesive exopolymeric substances attached to a surface and secreted by microorganisms forming colonies on it.
Bioleaching Refers to the microbial conversion of an insoluble metal (usually a metal sulfide or oxide) into a soluble form (metal sulfate).
Biomining The use of microorganisms to recover metals in industrial operations.
Bioremediation Use of microorganisms to remove toxic chemicals from the environment.
Biosorption Refers to the binding of metal ions by whole biomass (living or dead).
Chemolithoautotroph A microorganism that fixes CO_2 and obtains its energy by the oxidation of inorganic compounds.
Consortium A group of microorganisms living together and in which each individual benefits from the others.
Genome The complete set of genes present in an organism.
Genomics Refers to mapping, sequencing, and analyzing genomes.
Proteome The total complement of proteins present in a cell at any one time.
Proteomics Genome-wide study of the structure, function, and regulation of proteins in the cell.
Systems microbiology Considers microorganisms or microbial communities as a whole to create an integrated picture of how a microbial cell or community operates.

Abbreviations

AMD	Acid mine drainage
DGGE	Denaturing gradient gel electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
ESI-MS	Electron spray ionization MS
FISH	Fluorescence in situ hybridization
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometer
HPLC	High performance liquid chromatography
MS	Mass spectrometry
QS	Quorum sensing
SDO	Sulfur dioxygenase
SOR	Sulfite oxidoreductase
TEM	Transmission electron microscopy

Defining Statement

Microorganisms interact with heavy metals, transforming them by uptake, bioaccumulation, bioprecipitation, bioreduction, biooxidation and other mechanisms. Some of these activities result in the solubilization or extraction of metals which are successfully used in industrial biomining processess to recover valuable metals or in biorremediation to remove toxic metals from contaminated soils.

Microbial Transformations of Metals

Microorganisms interact with metals by several mechanisms, most of which are shown in Fig. 1. All bacteria require several metals that are essential for their functioning and the uptake of most of them is metabolism- or energy-dependent. For this bioaccumulation, they possess specific or general energy-dependent metal transporters to directly incorporate them or through chelation by means of organic compounds such as siderophores in the case of iron. Some of these transporters are used to bioaccumulate metals

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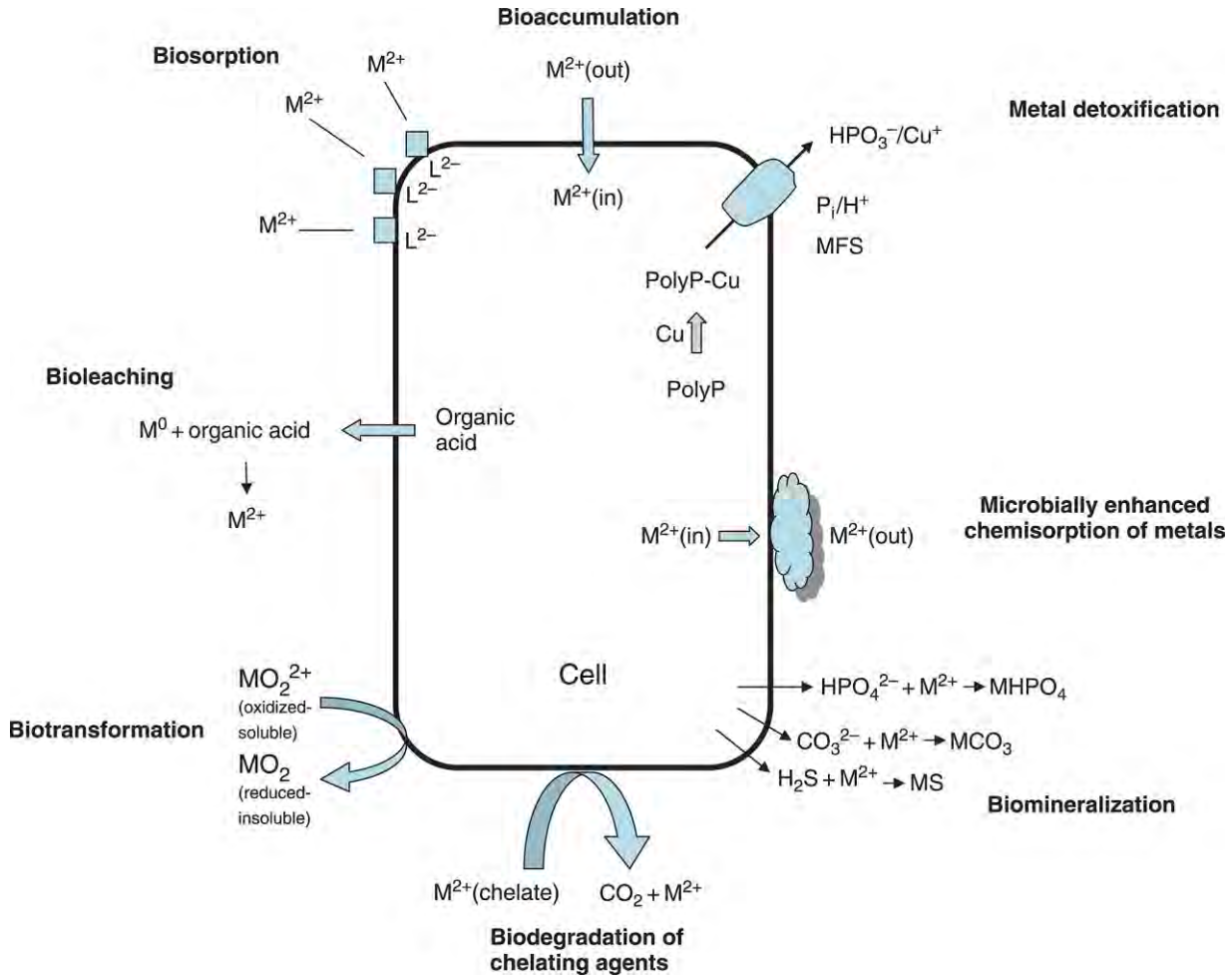

Fig. 1 A general scheme showing the most common bacteria–metals interactions. Modified from Lloyd et al. (2005).

inside the cell. This can be done by sequestering the metal by proteins rich in cysteine or histidines or they can be chelated by inorganic polyphosphates (polyP), which are long chains of phosphate molecules joined through phosphodiester bonds and are highly negatively charged at neutral pH. Microorganisms such as Acinetobacter spp. bioprecipitate metals in the form of metal phosphates via hydrolysis of stored polyP depending upon alternating aerobic (polyP synthesis) and anaerobic (polyP hydrolysis and phosphate release) periods.

The metabolism-independent sorption of heavy metals or biosorption refers to the binding of metal ions by whole biomass (living or dead). It can take place by adsorption in which metals accumulate at the surface of the biomass and by absorption or a rather uniform penetration of the metal ions from a solution to another phase. Biomolecules present in the biomass contain several chemical groups that act as ligands (L2− in Fig. 1) for the biosorption of the metal ions. The most common are the amino, carboxyl, phosphate, and sulfhydryl groups present in proteins, nucleic acids, and polysaccharides.

Microorganisms can also catalyze several biotransformations. They transform most toxic metals into less soluble or less volatile forms. One example is the reduction of metals such as Cr(vi) to Cr(iii), U(vi) to U(iv), Te (vi) to Te(0) and many others that results in the precipitation of the metal under physiological conditions. Another very well-studied biotransformation of a toxic metal is the bioreduction of Hg(ii) to the relatively nontoxic volatile elemental Hg(0).

Microorganisms also precipitate metals in the form of carbonates, hydroxides, or insoluble sulfides and phosphates. This constitutes a biomineralization, and due to the very low solubility products of these compounds formed, most of the soluble metals would be removed by precipitation in their liquid medium (Fig. 1).

Microorganisms That Solubilize Metals

A basic prerequisite for life is the existence of a chemical redox gradient to convert energy. This allows energy flow and its use in the biochemical reactions of the cells. Most cells use organic compounds like sugars as fuels (electron donors). Their oxidation in the

presence of oxygen or respiration provides carbon to the cell generating as end products CO_2 and H_2O and energy in the form of electrons. This energy is stored mainly in the form of ATP to be used in the cell metabolism. A group of special microorganisms (Bacteria and Archaea) known as chemolithoautotrophs are capable of using minerals as fuels. Their oxidation generates electrons to obtain ATP and the carbon is obtained by fixing CO_2 from the air. During this aerobic mineral oxidation metals are solubilized or bioleached.

Anaerobic respiration can also solubilize metals. In this case, the mineral acts as an electron acceptor and the metal is solubilized under reducing conditions. Several microorganisms are capable of reducing heavy metals and couple this reduction with the oxidation of energy sources such as hydrogen or organic compounds (formate, lactate, amino acids, and others). Characteristic examples are the reduction of Fe and Mn, which account for good part of organic carbon turnover in several environments (Lovley, 2000). Several of the dissimilatory metal-reducing bacteria not only reduce Fe and Mn but also other metals such as the toxic Cr(vi) and U(vi) and, therefore, microorganisms like *Shewanella oneidensis* could also be used for bioremediation.

The reactions just mentioned are part of normal biogeochemical processes in nature. They are performed under neutral conditions or as the majority of microorganisms do, at very high acidic values (pH 1–3 usually). This article will concentrate mainly on reviewing metals mobilization by acidophilic microorganisms.

Some of the general oxidation reactions that acidophiles are able to catalyze are given below.

$$\text{Ferrous iron}: \ 2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

$$\text{General metal sulfide}: MS + 2O_2 \rightarrow M^{2+} + {SO_4}^{2-}$$

$$\text{Pyrite}: 4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$$

$$\text{Chalcopyrite}: \ CuFeS_2 + 4Fe^{3+} \rightarrow 5Fe^{2+} + Cu^{2+} + 2S^o$$

$$\text{Sulfur}: S^o + 1\frac{1}{2}O_2 + H_2O \rightarrow H_2SO_4$$

These reactions not only solubilize the metals present in the minerals but also generate sulfuric acid. The acidophilic microorganisms, therefore, are able to stand not only low pH values but also very high metal concentrations. They possess heavy metal resistance or detoxification mechanisms. Neutrophilic bacteria have metal resistance mechanisms involving either an active efflux or a detoxification of metal ions by different transformations (Fig. 1). In the case of copper, for example, these include intracellular complexation, decreased accumulation, extracellular complexation, or sequestration in the periplasm. Neutrophilic bacteria are able to grow in a range of copper concentration between 1 and 8 mmol L^{-1} depending on the species. However, acidophiles such as *Acidithiobacillus ferrooxidans* or archaeons such as *Sulfolobus metallicus* can resist concentrations of copper up to 800 and 200 mmol L^{-1}, respectively (Orell et al., 2010). Most likely, they possess additional mechanisms to those present in neutrophiles, allowing them to have such dramatic metal resistance. It has been proposed that in microorganisms possessing large amounts of polyP, such as some chemolithoautotrophic acidophilic bacteria and archaea, these polymers may be actively involved in the elimination of toxic heavy metals such as Cu. This detoxification would take place through the enzymatic hydrolysis of polyP that would generate free phosphate that would bind the excess of cytoplasmic metal to form a metal–phosphate complex that is transported outside the cell through phosphate transporters (Fig. 1). These properties make these microorganisms very appropriate for their use in biomining and also for the bioremedation or removal of heavy metals from polluted places (see below).

Microbial Extraction of Metals From Ores and Biomining

The microbial solubilization of metals is widely and successfully used in industrial processes called bioleaching of ores or biomining, to extract metals such as copper, gold, uranium, and others (Brierley and Brierley, 2013). This process is done by using chemolithoautotrophic microorganisms. There is a great variety of microorganisms capable of growth in situations that simulate biomining commercial operations and many different species of microorganisms are living at acid mine drainage (AMD) sites (Hedrich and Schippers, 2016). The most studied leaching bacteria are from the genus *Acidithiobacillus*. *A. ferrooxidans* (Fig. 2(a)) and *Acidithiobacillus thiooxidans* are acidophilic mesophiles and together with the moderate thermophile *Acidithiobacillus caldus*, they belong to the Gram-negative γ-proteobacteria. Fig. 2(c) shows *A. ferrooxidans* cells growing in the form of a biofilm on the surface of an elemental sulfur particle, most likely as a monolayer. When this biofilm is removed from the solid particle by using a detergent (Fig. 2(d)), only those cells attached more strongly to the sulfur particle are remaining. They are seen as attacking the sulfur surface through a 'pitting' in which some cells are still tightly bound to the cavities and others have been released, leaving empty cavities. A similar attack has been observed on the surface of other minerals such as pyrite.

Members of the genus *Leptospirillum* are other important biomining bacteria that belong to a new bacterial division. Some Gram-positive bioleaching bacteria belonging to the genera *Acidimicrobium*, *Ferromicrobium*, and *Sulfobacillus* have also been described. Biomining using extremely thermophilic archaeons capable of oxidizing sulfur and iron (ii) has been known for many years, and the archaeons are mainly from the genera *Sulfolobus* (Fig. 2(b)), *Acidianus*, *Metallosphaera*, and *Sulfurisphaera*. Also some mesophilic iron (ii)-oxidizing archaeons belonging to the *Thermoplasmatales* have been isolated and described – *Ferroplasma acidiphilium* and *Ferroplasma acidarmanus*. In fact, a consortium of different microorganisms participates in the oxidative reactions, resulting in the

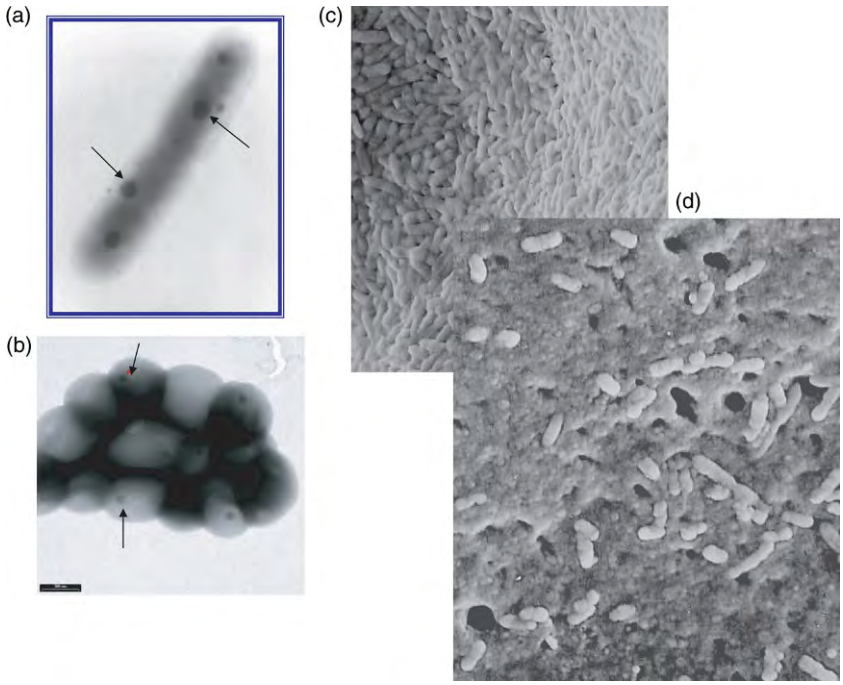

Fig. 2 Some examples of acidophilic microorganisms that participate in metal extraction through biomining. (a) *Acidithiobacillus ferrooxidans* cells. (b) A group of *Sulfolobus metallicus* cells. (c) A biofilm, possibly a monolayer of *A. ferrooxidans* cells growing on the surface of an elemental sulfur prill. (d) Most of the biofilm of *A. ferrooxidans* seen in (c) was removed from the solid particle by using a detergent and vigorous shaking of the sample. The remaining cells seen are those more strongly adhered to the particle. Cells in (a) and (b) were observed by transmission electron microscopy (TEM) of unstained preparations and those in (c) and (d) by scanning electron microscopy. The arrows in each case point to electron dense polyphosphate granules that may help these microorganisms in their extremely high metal tolerance.

extraction of dissolved metal values from ores (Hedrich and Schippers, 2016). Since chalcopyrite it is the most difficult mineral to solubilize by microorganisms, there is actually great interest in developing processes by using thermophilic biomining microorganisms for these recalcitrant minerals. The extraction of copper from chalcopyrite by using bioreactors can be faster and efficient, and temperatures higher than 60°C can be used with thermophilic archaea such as *Sulfolobus metallicus*, *Metallosphaera sedula* and *Acydianus brierleyi* (Norris et al., 2013; Wheaton et al., 2015).

On the other hand, although no psychrophilic acidophiles (optimal growth temperature lower than 15 °C) have been reported some psychrotolerant microorganisms such as *Acidithiobacillus ferrivorans* have been isolated that can catalyze metal dissolution from sulfide minerals at 5°C. These bacteria are of importance in biomining operations since many of them are present in high altitudes and polar regions and therefore can be used for biotechnological operations in cold environments (Dopson, 2016a).

Industrial biomining operations are of several kinds depending on the ore type and its geographical location, the metal content, and the specific minerals present (metal oxides, metal sulfides of different kinds) (Watling, 2006). One of the most used setups for the recovery of gold or copper is the irrigation type of processes. These involve the percolation of leaching solutions through the crushed ore that can be contained in a column, a heap or a dump. In Fig. 3, we can see a scheme in which the crushed ore to bioleach is transported to an agglomeration tank or drum where it is acidified. This process is the key one since the bigger ore particles are surrounded by the very fine particles that stick to them thus preventing all the particles especially the fine material sediment to the bottom of the heap. In this way irrigation and aeration of the heap takes place from the top to the bottom, allowing a much more homogeneous growth of the microorganisms and therefore a better metal solubilization. The heap can be 6–10 m tall and 100 or more meters long and wide and is constructed over irrigation pads lined with high-density polyethylene to avoid losses of the pregnant copper-containing solution (Fig. 3). This solution containing copper sulfate generated by the microbial solubilization of the insoluble copper sulfides present in the ore is subjected to solvent extraction to have a highly concentrated copper sulfate solution from which the metal is recovered in an electrowinning plant to generate electrolytic copper of high purity (Fig. 3). Since most mining operations are located in areas where water is scarce, the spent leach liquors or raffinates are recirculated to the heap for further irrigation. This has to be controlled because the liquor is being enriched in salts that can rather select for those microorganisms able to stand the high salt and are not necessarily the most fit for the biooxidation reactions.

Bioleaching bacteria can also be used for gold recovery. Gold is usually found in nature associated with minerals containing arsenic and pyrites (arsenopyrites). During gold bioleaching, the iron- and sulfur-oxidizing microorganisms attack and solubilize the arsenopyrite releasing the trapped gold particles. Following this release, the gold is complexed with cyanide according to standard gold-mining procedures. Instead of using big leaching heaps or dumps as in the case of bioleaching of copper ores, gold bioleaching is usually done by using highly aerated stirred tank bioreactors connected in series. Since these reactors are expensive to

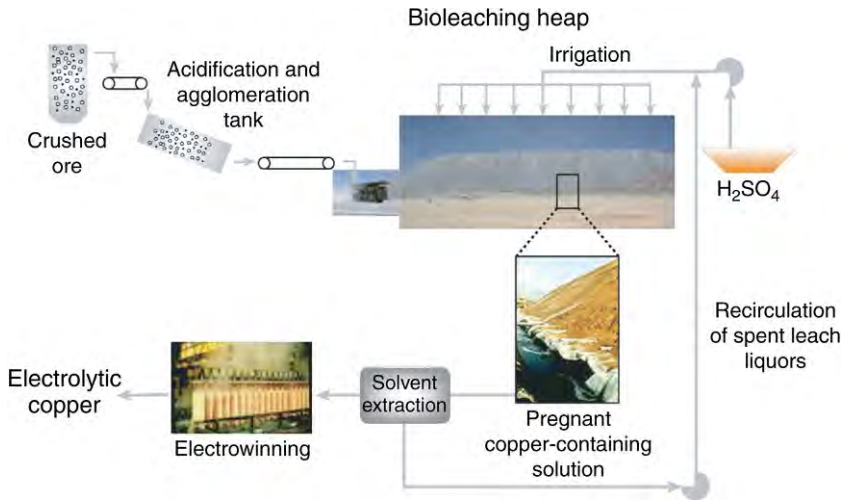

Fig. 3 A scheme showing the construction of a heap bioleaching process to obtain copper in a large scale.

build, they are used with high-grade ores or with mineral concentrates. The advantage of tank reactors over heaps and dumps, which are 'open bioreactors', is that in the tanks conditions can be controlled, thus having a much faster and efficient metal extraction process.

Currently, there are operations using both mesophilic and thermophilic microorganisms. Biomining has distinctive advantages over the traditional mining procedures (Harrison, 2016). For example, it does not require the high amounts of energy used during roasting and smelting and does not generate harmful gases such as sulfur dioxide. Nevertheless, AMD can be generated, which if not properly controlled, pollutes the environment with acid and metals. Biomining is also of great advantage since not only discarded low-grade ores from standard mining procedures can be leached in an economically feasible way but also some high-grade ores. In countries like Chile, which is actually the first world copper producer, many mining operations process from 10,000 to 40,000 tons of ore per day and produce between 10,000 and 200,000 tons of copper per year by using heap or dump bioleaching of minerals such as oxides, chalcocite, covellite, chalcopyrite, and others. Similar situations take place in United States, Australia, and other countries. The most successful ones have been those processing copper oxides and secondary copper sulfides. However, chalcopyrite is the most abundant copper sulfide in the world. Since it is the most difficult to be solubilized by microorganisms, there is actually great interest in developing processes mainly using thermophilic biomining microorganisms.

Acidiophilic Microorganism–Mineral Interaction

The microorganisms used in biomining belong to those known as extremophiles since they live in extremely acidic conditions (pH 1–3.0) and in the presence of very high toxic heavy metals concentrations. Considerable effort has been spent in the past few years to understand the biochemistry of iron and sulfur compounds oxidation, bacteria–mineral interactions, and the several adaptive responses that allow the microorganisms to survive in a bioleaching environment. All of these are considered key phenomena for understanding the process of biomining.

How do the bacterial cells recognize the site of attachment in the ores from which they will extract the metals? Do they have a specific way to detect or sense where the oxidizable substrate is present in the rocks? Ample evidence has shown that attachment of the bacterial cells to metal sulfides does not take place randomly. For example, *A. ferrooxidans* cells preferentially adhere to sites with visible surface scratches and it is seen to form pitting at the surface of minerals as already seen in Fig. 2.

Bacteria such as *Leptospirillum ferrooxidans* and *Acidithiobacillus thiooxidans* have been shown to possess a chemosensory system (Jerez, 2001) that allows them to have chemotaxis, that is, the capacity to detect gradients of oxidizable substrates being extracted from the ores such as Fe (ii)/Fe (iii) ions, thiosulfate, and others (Fig. 4). The response is the positive chemotactic attraction to the sites that will constitute specific favorable mineral attachment sites.

It is known that most leaching bacteria grow attached to the surface of the solid substrates such as elemental sulfur and metal sulfides. This attachment is predominantly mediated by extracellular polymeric substances (EPS) surrounding the cells and whose composition is adjusted according to the growth substrate (Fig. 4). Thus, planktonic or free-swimming cells grown with soluble substrates such as iron (ii) sulfate produce almost no EPS.

Biofilms are organized layers of bacteria associated to a solid surface by means of the matrix of EPS. The environment within this community favors intercellular interactions between bacteria (Orell and Guiliani, 2016). Thus, in the presence of oxygen or aerobiosis, *A. ferrooxidans* can respire this element and oxidize Fe (ii). Some of these microorganisms are also able to perform Fe (iii) respiration under anaerobiosis, regenerating Fe (ii) that can be used by those microbial cells closer to the oxygen within the biofilm structure. Other types of bacteria forming part of the microbial biofilm consortium will generate compounds useful to other

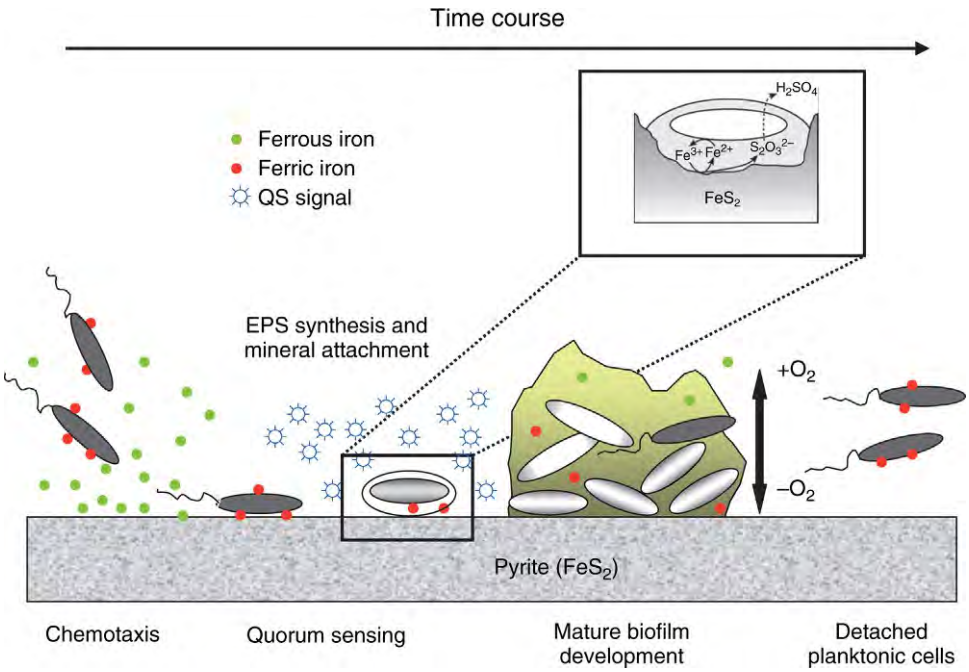

Fig. 4 A diagrammatic representation showing the main steps of the bacteria–mineral interaction. Details of the attack of the non-chemotactic *Acidithiobacillus ferrooxidans* on the mineral pyrite are shown in the amplified inset. The cell is embedded in an extracellular polymeric substances (EPS) biofilm in which the indirect mechanism generates ferrous iron and thiosulfate, which is finally oxidized to sulfuric acid. Inset Reproduced from Rohwerder et al. (2003).

members of the community and they can themselves benefit from the metabolic byproducts of other microbes present in the biofilm (Vera et al., 2013).

Several microorganisms are known to monitor their own population density through processes collectively described as quorum sensing (QS), and in several cases there is strong evidence indicating that biofilm formation is affected by QS. In most cases, specific genes within the bacterium are switched on at a defined population density (defined bacterial quorum) and the result obtained is the activation of functions under the control of a quorum sensor. In almost all cases, the capacity to detect a bacterium quorum depends on the release of a signal molecule from the microorganism that accumulates in proportion to the cell number (Fig. 4). Thus, QS represents a multicellular action in bacteria, where each cell communicates with each other to coordinate their behavior. It has been demonstrated that *A. ferrooxidans* not only contains quorum sensor signal molecules but also may induce the expression of genes related to QS and EPS production when grown attached to a solid substrate. Thus, modulation of the attachment of the microorganisms to ores through interferences of their QS responses can be envisaged as a new way to control metal extraction by these microorganisms (Farah et al., 2005).

A. ferrooxidans is also able to develop biofilm structures when growing in solid substrates such as elemental sulfur or metal sulfides and presents morphological modifications during the cellular adhesion process. For example, new proteins related to sulfur metabolism appear in the surface of *A. ferrooxidans* when grown in sulfur (Ramírez et al., 2002). This is clearly seen in Fig. 5, in

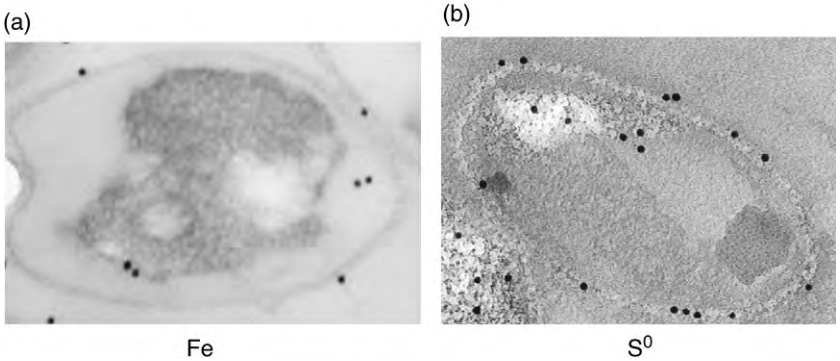

Fig. 5 Example of changes on the *Acidithiobacillus ferrooxidans* surface depending on growth conditions. It is known that *A. ferrooxidans* produces a protein in high amounts when the bacterium grows on elemental sulfur and not in ferrous iron (Fe). To see the location of that protein, an immunological system was used in which a gold particle (black dots) indicates the presence of the protein in the cells grown in ferrous iron (Fe) (a) or elemental sulfur (b).

which a primary antibody specific against a protein related to sulfur metabolism is bound to the surface of *A. ferrooxidans* cells grown in sulfur but is almost absent in ferrous iron-grown cells. The primary antibody bound to the protein was recognized by using a secondary antibody labeled with gold particles and specific to recognize the primary antibody bound to the sulfur-induced protein. The presence of the protein changing its expression is seen as the black dots of gold by transmission electron microscopy (TEM).

Mechanisms Involved in Metal Solubilization by Acidophiles

Traditionally, it has been proposed that microorganisms oxidize metal sulfides by either a direct or an indirect mechanism. In the first one, the bacteria have to attach to the mineral and the electrons would be directly extracted by an enzymatic reaction of the microorganism. However, there is no evidence how the bacteria break the metal–sulfide bond of a given mineral. In the indirect mechanism, the role of bacteria would be to oxidize ferrous ions present in the solution to ferric ions. Ferric ions are strong oxidants therefore will oxidize chemically the sulfide metals, being an indirect mode of attack. Actually, many researchers consider the existence of a contact mechanism, since the bacteria attaches to the surface of the mineral carrying Fe (iii) bound to its exopolysaccharides, and when the microorganism forms a biofilm being embedded in its EPS, this metal would chemically attack the metal sulfide generating, in the case of pyrite, ferrous iron that is reoxidized to Fe (iii) and thiosulfate that can be further oxidized to sulfuric acid (Fig. 4). The mechanism still is indirect (known also as indirect contact mechanism). This close contact of the bacterium with the mineral makes more efficient and specific the sulfide oxidation.

The insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron, the main role of the microorganisms being the reoxidation of the generated ferrous iron to obtain additional ferric iron (Fig. 4).

As already mentioned, *A. ferrooxidans* is a chemolithoauthotrophic bacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds and it has been considered as a model biomining microorganism. The reactions involved in ferrous iron oxidation by *A. ferrooxidans* have been studied in detail, however, the electron pathway from ferrous iron to oxygen has not been entirely established. The terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane. The transfer of electrons would occur through several periplasmic carriers, including at least the blue copper protein rusticyanin and cytochrome c552. Recently, a high molecular weight c-type cytochrome, Cyc2, has been suggested to be the prime candidate for the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen (Fig. 6). This pathway would be Cyc2 → rusticyanin → Cyc1(c552) → aa3 cytochrome oxidase. In addition, there is an apparent redundancy of electron transfer pathways via bc(1) complexes and terminal oxidases in *A. ferrooxidans* (Rawlings, 2005; Nitschke and Bonnefoy, 2016).

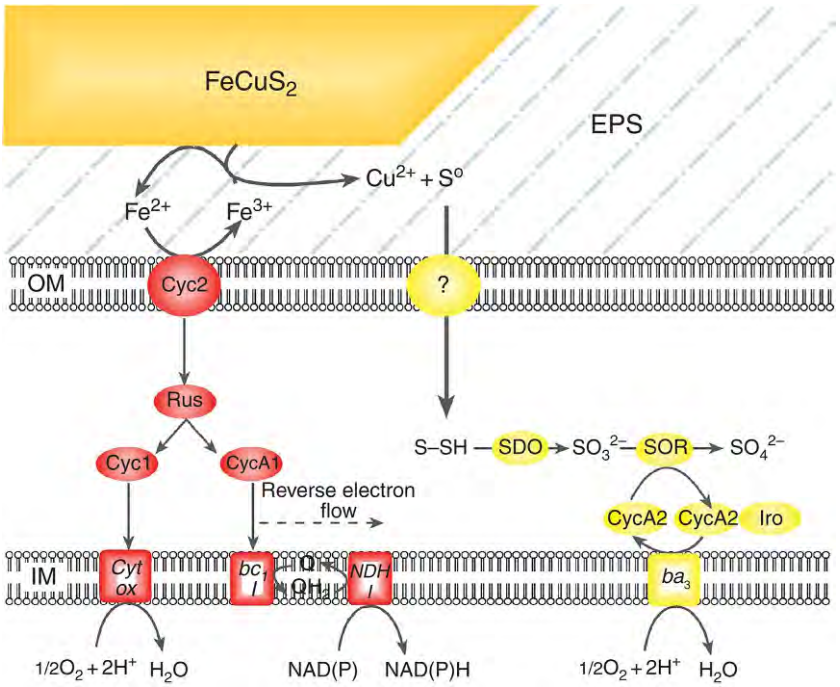

Fig. 6 Simplified model showing the main protein components (in red) present in the periphery of the cell and involved in the transfer of electrons from ferrous iron to oxygen and those oxidizing elemental sulfur (in yellow) and transferring the electrons to oxygen. This model represents the indirect mechanism of mineral attack by the ferric iron bound to the EPS of the *Acidithiobacillus ferrooxidans* cells. As an example, chalcopyrite is shown from which ferrous iron, copper (ii), and elemental sulfur are generated upon its oxidation. SDO, sulfur dioxygenase; SOR, sulfite oxidoreductase. Adapted from Rawlings, D.E., 2005. Characteristics and adaptability of iron-and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. Microbial Cell Factories 4, 13.

As already mentioned, thiosulfate has been postulated as a key compound in the oxidation of the sulfur moiety of pyrite (Fig. 4). Iron (iii) ions are exclusively the oxidizing agents for the dissolution. Thiosulfate would be consequently degraded in a cyclic process to sulfate, with elemental sulfur being a side product. This explains why only Fe(ii) ion-oxidizing bacteria are capable of oxidizing these metal sulfides. All reactions comprising this oxidation have been shown to occur chemically. However, sulfur compound-oxidizing enzymes such as the tetrathionate hydrolase of *A. ferrooxidans*, *A. thiooxidans*, or *T. acidophilus* may also be involved in the process.

The oxidation of some metal sulfides such as chalcopyrite generates elemental sulfur as a side product instead of thiosulfate (Fig. 6). The aerobic oxidation of elemental sulfur by *A. ferrooxidans* and other microorganisms is carried out by a sulfur dioxygenase (SDO) and a sulfite oxidoreductase (SOR) (Fig. 6).

It is important to remark here that the ultimate oxidizing agent for iron (ii) and reduced inorganic sulfur compounds is oxygen, since often the transport of dissolved oxygen is the rate-limiting step in commercial bioleaching operations.

Biomining in the Postgenomic Era

Systems microbiology, which is part of systems biology, is a new way to approach research in biological systems. By this approach, it may be possible to explore the new properties of microorganisms that arise from the interplay of genes, proteins, other macromolecules, small molecules, and the environment. This is particularly possible today due to the large numbers of genomic sequences that are becoming increasingly available. However, additional genomic sequences of the different biomining microorganisms will be required to define the molecular adaptations to their environment and the interactions between the members of the community.

The use of genomics, metagenomics, proteomics and metaproteomics (Fig. 7), together with metabolomics to study the global regulatory responses that the biomining community uses to adapt to their changing environment is just beginning to emerge. These powerful OMICS approaches will have a key role in understanding the molecular mechanisms by which the microorganisms attack and solubilize ores. Furthermore, they offer the possibility of discovering exciting new findings that will allow analyzing the community as a microbial system, determining the extent to which each of the individual participants contributes to the process,

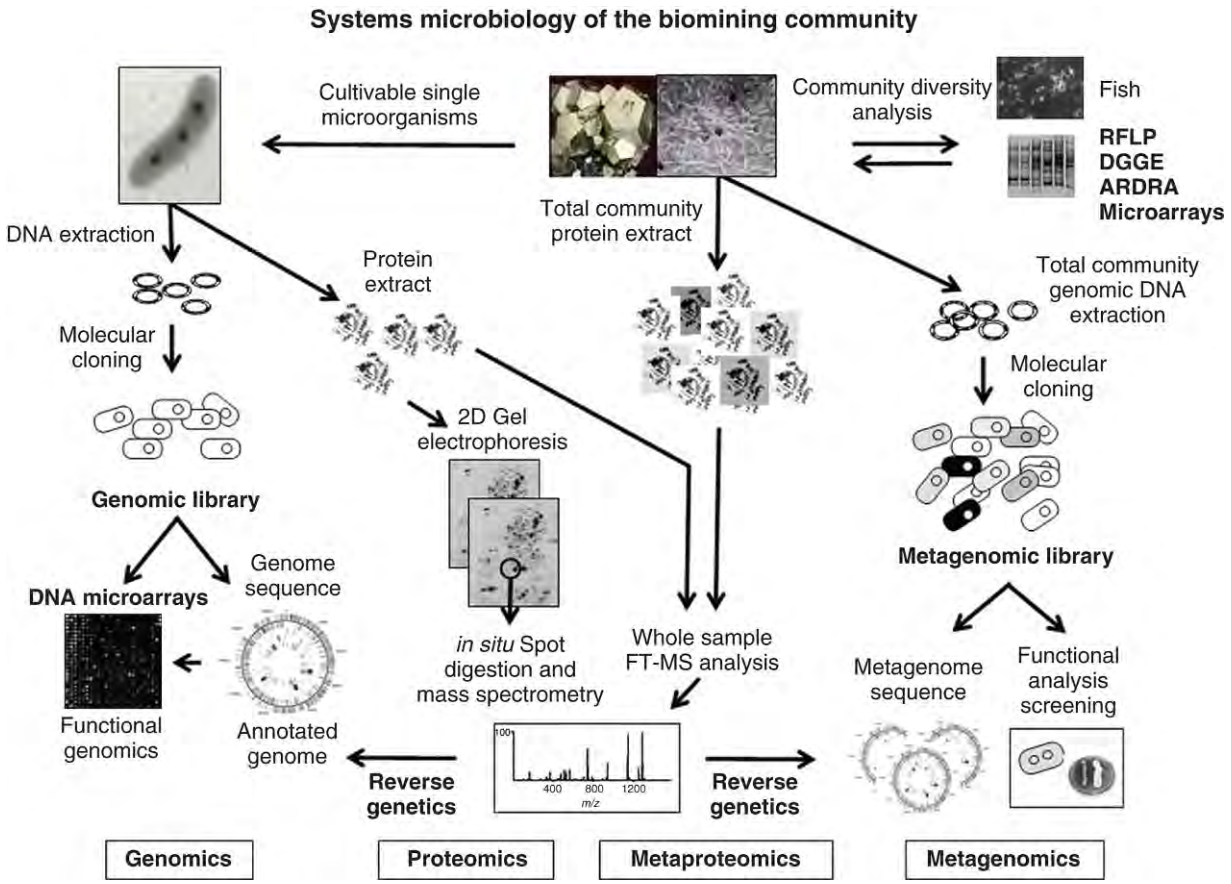

Fig. 7 Systems microbiology and the use of OMICS approaches to study microbial communities as applied to biomining microbes. Modified from Valenzuela, L., Chi, A., Beard, S., *et al.*, 2006. Genomics, metagenomics and proteomics in biomining microorganism. Biotechnol. Adv. 24, 197–211.

and how they evolve in time to keep the conglomerate healthy. This, taken together with the physicochemical, geological, and mineralogical aspects of the process, will allow improving the efficiency of this important biotechnology.

Biomining Community Diversity Analysis

Of extreme importance is not only to know the microorganisms present in a bioleaching operation, but to be able to monitor their behavior during the process, to determine the predominant species and the way they evolve in time with the changing environment as the metals are solubilized (Dopson, 2016b; Golyshina et al., 2016). In recent years, several molecular methods have been developed for other microorganisms and these have been successfully applied to many biomining operations. The most common techniques employed to explore the bacterial diversity use 16S rRNA and rDNA profiles and are culture-independent methods. Among these methods are included fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), real time PCR, DNA microarrays, and others (Fig. 7).

Genomics

A. ferrooxidans was the first biomining microorganism whose genome was sequenced by TIGR. This information has been very useful to do genome-wide searches for candidate genes for important metabolic pathways and several important physiological functions, which can now be addressed. Furthermore, predictions for the functions of many new genes can be done. The main focus of research has been the energy metabolism that is directly responsible for bioleaching. Genes involved in phosphate, sulfur, and iron metabolism, QS, those potentially involved in several other functions such as metal resistance and amino acid biosynthesis pathways, and those involved in the formation of EPS precursors have been studied. Having the genomic sequence of a given biomining microorganism is very important, since it is then possible to formulate hypothesis about the regulation of the expression of most of these genes under different environmental conditions. Metabolic reconstruction and modeling provides an important preliminary step in understanding the unusual physiology of this extremophile especially given the severe difficulties involved in its genetic manipulation and biochemical analysis. However, all these bioinformatic predictions will have to be demonstrated experimentally by using functional genomics, proteomics, and other approaches. The existence of paralog genes that show sequence similarities but may have different functions in the same microorganism becomes obvious only when the genome sequence is available.

Currently, there are over 55 bacterial and 36 archaeal complete genome sequences from microorganisms that are present in biomining or AMD places (Cárdenas et al., 2016; Siezen and Wilson, 2009; Martínez et al., 2015). Some of the acidophilic microorganisms usually found in biomining or AMD places are for the bacteria *A. ferrooxidans*, *Leptospirillum* group II, and *Acidiphilium cryptum*. The genome sequences of *A. thiooxidans* and *A. caldus* have also been determined. Amongst the archaea, *Metallosphaera sedula* and *F. acidarmanus* genome sequences amongst several others are also available. *Sulfolobus acidocaldarius* and *Sulfolobus tokodaii* are also known, although they have not been reported as having a role in bioleaching. The generation of new DNA microarrays to monitor not only all the microorganisms present in samples from industrial operations (Fig. 7) but also specific genes such as those indicating the nutritional or stress state of the microorganisms or those involved in iron or sulfur compound oxidation whose products should predominate during different stages of active bioleaching (Parro and Moreno-Paz, 2003).

Functional Genomics

One of the most used techniques to study differential genome expression at the level of mRNA synthesis, transcriptomics, or the functional analysis of new and characterized genes is the use of DNA microarrays. The generation of new DNA microarrays and high throughput RNA sequencing can be used to monitor not only all the microorganisms present in samples from industrial operations but also specific genes such as those indicating the nutritional or stressing state of the microorganisms or those involved in iron or sulfur compound oxidation whose products will be predominant during different stages of active bioleaching.

The use of microarrays based on the entire genome of *A. ferrooxidans* and other microorganisms will enable a nearly complete view of gene expression of the members of the microbial community under several biomining conditions, helping to monitor their physiological state and adjustment made during the bioleaching process. A genome-wide microarray transcript profiling analysis (approximately 3000 genes of the *A. ferrooxidans* ATCC 23270 strain) has also been performed (Quatrini et al., 2006). The genes preferentially transcribed in ferrous iron growth conditions or in sulfur conditions were studied. The results obtained supported and extended models of iron and sulfur oxidation (Fig. 6) and supported the possible presence of alternate electron pathways and that the oxidation of these two kinds of oxidizable substrates may be coordinately regulated. By using the same approach, the expression of the genes involved in carbon metabolism of *A. ferrooxidans* has been studied in response to different oxidizable substrates.

As already mentioned, some mining companies are currently interested in doing transcriptomic analysis of their newly isolated microorganisms with improved capacities to leach copper since they already have obtained their genomic sequences (Latorre et al., 2016).

A very interesting alternative approach can be used to analyze gene function in environmental isolates without knowing the sequence of the microorganism of interest. A random genomic library from the isolated microorganism can be printed on a microarray. Gene expression by using total RNA extracted from the microorganism grown under different conditions can be determined. With this approach, it is possible to select and sequence only those clones bearing the genes that showed an altered expression pattern. Shotgun DNA microarrays are very powerful tools to study gene expression with environmental microorganisms whose genome sequence is still unknown.

Metagenomics

Metagenomics is the culture-independent genomic analysis of microbial communities. In conventional shotgun sequencing of microbial isolates, all shotgun fragments are derived from clones of the same genome. To analyze the genomes of an environmental microbial community (Fig. 7), the ideal situation is to have a low diversity environment. Such systems were found when analyzing the microbial communities inhabiting a site of extreme AMD production, in which few types of organisms were present. Still, variation within each species might complicate assembly of the DNA fragments. Nevertheless, random shotgun sequencing of DNA from this natural acidophilic biofilm was used. It was possible to reconstruct the near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II and partially recover three other genomes. The extremely acidic conditions of the biofilm (pH about 0.5) and relatively restricted energy source combine to select for the small number of species found (Ram et al., 2005; Tyson et al., 2004; Baker and Banfield 2016).

The analysis of the gene complement for each organism revealed the metabolic pathways for carbon and nitrogen fixation and energy generation. For example, genes for biosynthesis of isoprenoid-based lipids and for a variety of proton efflux systems have been identified, providing insights into survival strategies in the extreme acidic environment. Clearly, the metagenomic approach for the study of microbial communities is a real advancement to fully understand how complex microbial communities function and how their component members interact within their niches. A full understanding of the biomining community also will require the use of all these current molecular approaches.

Proteomics

Proteomics provide direct information of the dynamic protein expression in tissue or whole cells, giving us a global analysis. Together with the significant accomplishments of genomics and bioinformatics, systematic analysis of all expressed cellular components has become a reality in the post genomic era, and attempts to grasp a comprehensive picture of biology have become possible.

One important aspect of proteomics is to characterize proteins differentially expressed by dissimilar cell types or cells imposed to different environmental conditions. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with mass spectrometry (MS) is currently the most widely used technology for comparative bacterial proteomics analysis (Fig. 7). The high reproducibility of 2D-PAGE is particularly valuable for multiple sample comparisons. In addition, it directly correlates the changes observed at the peptide level to individual protein isoforms (Remonsellez et al., 2016).

2D-PAGE separates a complex mixture of proteins such as those present in a bacterial cell extract based on the isoelectric point of the proteins in the first dimension (isoelectrofocusing gel). These proteins or groups of proteins are further resolved in a second dimension (SDS-PAGE), in which the proteins are all negatively charged and are separated based on their molecular masses (Fig. 7). Actual 2D-PAGE procedures can resolve around 1000 protein spots in a single run. One of the limitations of 2D-PAGE is that only the most abundant proteins in the cell can be detected. Therefore, to increase the resolution of the method, it is also possible to analyze a subproteomic cell fraction instead of the total cell proteins as shown for the periplasmic proteins from *A. ferrooxidans* (Chi et al., 2007).

The high reproducibility of 2D-PAGE is particularly valuable for multiple sample comparisons. In addition, it directly correlates the changes observed at the peptide level to individual protein isoforms. This is a typical 'reverse genetics' approach in which (after isolating from a 2D-PAGE gel) an individual protein is differentially expressed in a condition of interest, and the amino acid sequence of a peptide from the protein is obtained to identify its possible homologue in databases. With this information, its coding gene and genomic context can be searched using the genome DNA sequence (Fig. 7). Depending on these results, a suggested function could be hypothesized. It will be of great importance to demonstrate the expression of putative genes related to EPS synthesis in *A. ferrooxidans* cells grown on different metal sulfides and to find out if these genes are involved in cell attachment and biofilm formation. In this regard, *A. ferrooxidans* is known to form biofilms on solid substrates (Fig. 2). In an AMD biofilm analyzed by the proteomic approach, it was not known which microorganisms are responsible for the production of the polymer embedding the community. However, the presence of numerous glycosyltransferases and polysaccharide export proteins in the predominant bacterial species also suggests a role in biofilm formation.

Several studies have used 2D-PAGE to study changes in protein expression of *A. ferrooxidans* under different growth conditions. Proteins induced under heat shock, pH stress, phosphate limitation, or the presence of copper have been reported. A set of proteins that changed their levels of synthesis during growth of *A. ferrooxidans* in metal sulfides, thiosulfate, elemental sulfur, and ferrous iron has been characterized by using 2D-PAGE.

During growth of *A. ferrooxidans* in metal sulfides containing iron, such as pyrite and chalcopyrite, proteins upregulated both in ferrous iron and in sulfur compounds were synthesized, indicating that the two energy-generating pathways are simultaneously induced depending on the kind and concentration of the available oxidizable substrates.

The increasing number of sequenced genomes has provided good options for high-throughput functional analysis of proteomes. Proteomic studies are well advanced for diverse bacteria, such as the model bacterium *Escherichia coli* and others. Nevertheless, there is still a lack of data for identification of proteins from organisms with unannotated or unsequenced genomes, which makes large-scale microbacterial proteomics analysis a challenge. With the development of highly sensitive and accurate computational gene-finding methods, new microbial genomes could be explored and scientific knowledge of them could be maximized.

Traditional 2D gel electrophoresis coupled with MS is time consuming as a result of the nature of spot-by-spot analysis and it is biased against low abundance proteins, integral membrane proteins, and proteins with extreme pI or molecular weight (MW). Alternatively, solution-based approaches offer unbiased measurement of relative protein expression regardless of their abundance, subcellular localization, or physicochemical parameters (Fig. 7). This methodology, however, results in extremely complex samples. For instance, of the 4191 predicted genes in the complete genome of *E. coli*, 2800 of them are believed to be expressed at any one time. Additional complexity is introduced upon enzymatic digestion, which generates multiple peptide species for each protein. To obtain comprehensive protein expression information from the samples, a chromatographic separation step prior to MS protein analysis is often necessary. High performance liquid chromatography (HPLC) coupled with online electron spray ionization MS (ESI-MS/MS) has been proved to be a valid approach for analyzing protein expression in complex samples. Alternatively, Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) is well suited for the differential analysis of protein expression due to the high mass accuracy and high resolution as well as its inherent wide dynamic range. Peptide charge state can be readily derived with the accurate isotopic peak distribution information provided by FT MS experiment, and co-eluting species with the same nominal m/z ratio can be resolved. The differentially expressed m/z values identified can be assigned to the peptide sequences and, subsequently, differentially expressed proteins can be identified (Fig. 7).

In the periplasm of *A. ferrooxidans*, 216 proteins were identified, several of them changing their levels of synthesis when the bacterium was grown in thiosulfate, elemental sulfur, or ferrous iron media. Thirty four percent of them corresponded to unknown proteins. Forty one proteins were exclusively present in sulfur-grown and 14 in thiosulfate-grown cells. The putative genes coding for all the proteins were localized in the available genomic sequence of *A. ferrooxidans* ATCC 23270. The genomic context around several of these genes suggests their involvement in sulfur metabolism and possibly in sulfur oxidation and formation of Fe–S clusters (Chi et al., 2007).

Currently, quantitative proteomics has been quite useful with respect to better understanding global adaptations that acidophilic biomining microorganisms undergo in the presence of high metals concentrations and other stresses in their extreme environment (Remonsellez et al., 2016; Almárcegui et al., 2014; Martínez-Bussenius et al., 2017; Jerez, 2013).

Metaproteomics

The term 'metaproteomics' was proposed for the large-scale characterization of the entire protein complement of environmental microbiota at a given point in time. High-throughput MS has been used in a metaproteomic approach to study the community proteomics in a natural AMD microbial biofilm. Two thousand and thirty-three proteins from the five most abundant species in the biofilm were detected, including 48% of the predicted proteins from the dominant biofilm organism *Leptospirillum* group II. It was also possible to determine that one abundant novel protein was a cytochrome central to iron oxidation and AMD formation in the natural biofilm (Ram et al., 2005). This approach together with functional metagenomics can offer an integrated study of a microbial community to establish the role each of the participant plays and how they change under different conditions.

The goal of functional proteomics is to correlate the identification and analysis of distinct proteins with the function of genes or other proteins. With the discovery of a variety of modular protein domains that have specific binding partners, it has become clear that most proteins occur in protein complexes and that the understanding of a function of a protein within the cell requires the identification of its interacting partners. In the case of a biomining bacterium such as *A. ferrooxidans*, the identification of protein complexes involved in oxidative reactions is of high priority. What complexes are formed by cytochrome-like proteins such as rusticyanin (Rus in Fig. 6) with other proteins in the periplasm? Do some periplasmic proteins form a complex involved in sulfur compound oxidation in the periplasm? What other complexes of oxidative reactions are present in this microorganism? Proteomics may answer these and many other questions that will help to understand better the biomining process.

Some of the OMICS procedures briefly analyzed and summarized in Fig. 7 should be used in close conjunction with the known physiological functions of the microorganisms being studied. Metabolomics is also an important approach since it allows a global view of changes in the metabolism of cells under different conditions. This approach together with the other omics being used to study biomining microorganisms (Jerez, 2011) has been recently applied to industrially important bioleaching bacteria and unique metabolites found may be useful biomarkers to monitor and understand better the responses and adaptations of microorganisms to the stressing conditions present in industrial bioleaching operations.

It should be possible to better control the activity of the bacteria and archaea by giving them the appropriate nutritional and physicochemical conditions, and by interfering with some of these microbiological functions in order to enhance their action (for metal extraction) in the case of biomining or to inhibit their capacities to control AMD. As already mentioned (Fig. 4), some of these key physiological behaviors are chemotaxis, QS, and biofilm formation. Bacteria such as *A. thiooxidans* and *L. ferrooxidans* clearly

possess chemotactic systems and are attracted by a concentration gradient of thiosulfate or ferrous iron such as the one generated on the surface of pyrite (Fig. 4). This sensing ability is very important for the specific bacterial adherence at the places where the substrates to be solubilized through oxidation are present.

The development of efficient transformation or conjugation systems to introduce DNA to generate mutations affecting a gene of interest by using gene knockout systems is still almost entirely lacking for biomining microorganisms. These tools will be essential not only to perform functional genomics and have experimental demonstrations for the suggested gene functions based on bioinformatics analysis of the postgenomic data, but also to eventually improve some physiological bacterial capabilities. At the same time, the new OMICS methods are greatly helping to monitor more precisely the biomining consortia, and it is expected that providing the right physiological conditions to the community together with proper chemical or physical manipulations of the bacterial environment will further improve bioleaching rates in biomining operations.

Environmental Effects of Metals Solubilization and Bioremediation

The acidophilic microorganisms mentioned, mobilize metals and generate AMD, causing serious environmental problems. AMD should be remediated or abated. Often there is a sealing of the contaminated sites or the location of barriers to contain the acidic fluids (Jerez, 2009). Many approaches use prevention techniques to avoid further spillage of acidic effluents in the contaminated area. It can be controlled by chemical treatments such as the use of calcium oxide that neutralizes the acid pH. It is also possible to inhibit the acidophilic microorganisms responsible of the acid generation. This can be done by using certain organic acids, sodium benzoate, sodium lauryl sulfate, or quaternary ammonium compounds that will affect the growth of bacteria such as *A. ferrooxidans*.

Bioremediation or removal of the toxic metals from these contaminated soils can be achieved by a very interesting combination of two opposite biological activities: that of sulfur-oxidizing bacteria with the one of sulfate-reducing microorganisms. In a first step, the sulfur-oxidizing bacteria generate sulfuric acid which bioleaches or solubilizes the metals in the solid phase of the soil. The leachate metals are then precipitated in a second step by using a bioreactor in which the hydrogen sulfide generated by the sulfate-reducing bacteria under neutral and anaerobic conditions forms insoluble metal sulfides, which are eliminated (Fig. 8) (Lloyd et al., 2005). Metal contaminants such as Cu, Cd, Ni, and others can be efficiently leached from contaminated soils. The effluents obtained from such a process are clean enough of the metals that they can be reused in the environment.

Bioleaching microorganisms such as *A. ferrooxidans* can also have other uses to help avoiding metal contaminations in modern societies. For example, this bacterium has been successfully used to recover metals such as cadmium from spent batteries. By using bioreactors, *A. ferrooxidans* is grown attached on elemental sulfur. The bacteria generate sulfuric acid through the oxidation of sulfur that is then used for the indirect dissolution of spent nickel–cadmium batteries recovering after 93 days 90–100% of cadmium, nickel, and iron. Bioleaching of spent lithium ion secondary batteries, containing lithium and cobalt, has also been explored. These approaches are not only economically valuable but may be an effective method which could be considered the first step to recycle spent and discarded batteries preventing one of the many problems of environmental pollution (Jerez, 2009, 2011).

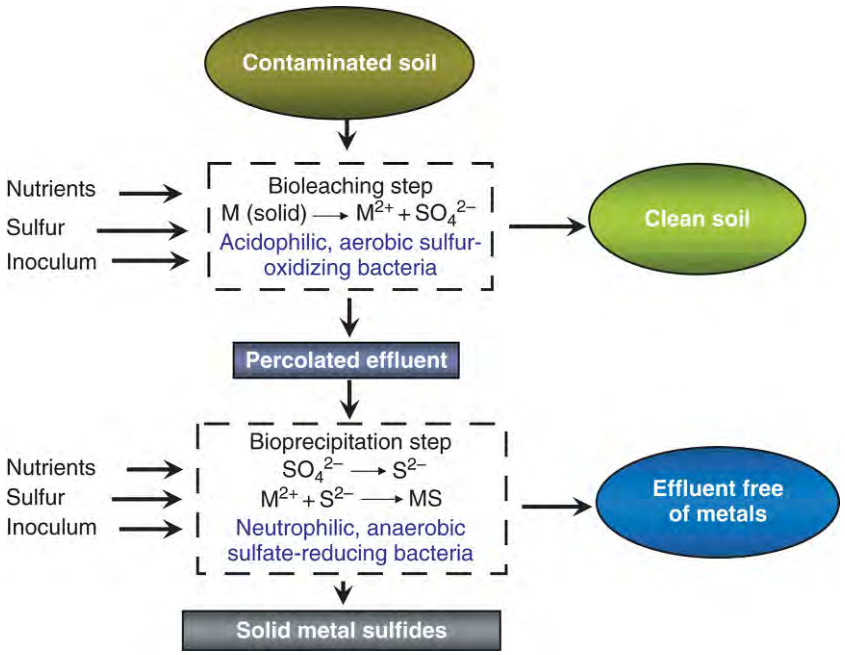

Fig. 8 A schematic diagram illustrating a process for the bioremediation of soils polluted with metals.

Conclusions

Microorganisms (Bacteria and Archaea) require several metals that are essential for their life. However, when the metal concentrations reach toxic levels, they also possess metal resistance mechanisms that involve active efflux or a detoxification of metal ions by different transformations. They can transform most toxic metals to less soluble or less volatile forms by intracellular complexation, decreased accumulation, extracellular complexation, or sequestration in the periplasm. Some of these activities result in the solubilization or extraction of metals, which are successfully used in environmental biotechnological applications.

The aerobic mineral oxidation or the anaerobic respiration of different microorganisms can result in the solubilization or extraction of metals. All these bacteria–metal interactions are part of the normal biogeochemical processes in nature.

The microbial solubilization of metals in acid environments is successfully used in industrial processes called bioleaching of ores or biomining, to extract metals such as copper, gold, uranium and others. On the contrary, the acidophilic microorganisms mobilize metals and generate AMD, causing serious environmental problems. However, bioremediation or removal of the toxic metals from contaminated soils can be achieved by using the specific properties of microorganisms interacting with metals.

Current approaches to study microorganisms consider the microorganism or the community as a whole, integrating fundamental biological knowledge with genomics, proteomics, metabolomics, and other data to obtain a global picture of how a microbial cell or a community functions. This new knowledge will help not only in understanding microbiological phenomena but it will be also useful to improve applied microbial biotechnologies.

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Relevant Website

http://cmr.jcvi.org—Comprehensive Microbial Resource.

Methanogenesis☆

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Glossary

Acetotrophic methanogen Methanogen that catabolizes acetate to CH_4 and CO_2.
Coenzyme M Coenzyme that serves as the carbon carrier during the final reduction of bound CH_3 to CH_4.
DNA/RNA polymerases Enzymes that catalyze the synthesis of DNA or RNA, respectively, from a DNA template.
H_2-consuming acetogen Microorganism that synthesizes acetate from H_2 and CO_2.
H_2-producing acetogen Microorganism that generates H_2 by oxidizing short-chain fatty acids or alcohols.
Hydrogenotrophic methanogen Methanogen that generates methane by the reduction of CO_2 with H_2.
Interspecies H_2 transfer Mutually beneficial transfer of H_2 from H_2-producing microorganisms to H_2-consuming microorganisms in an anaerobic ecosystem.
Methanofuran Coenzyme that serves as a carbon carrier during the initial reduction of CO_2 in the methanogenic pathway.
Methylotrophic methanogen Methanogen that generates methane by reduction of methyl groups.
Obligate syntroph Microorganism that must interact mutualistically with one or more other microorganisms to metabolize a specific substrate; specifically refers to interspecies H_2 exchange in this article.
Restriction endonuclease Enzyme that protects organisms by degrading ('restricting') foreign DNA that enters the cell.
Tetrahydromethanopterin Coenzyme that serves as the carbon carrier during sequential reduction of bound CO_2 to CH_3.

Abbreviations

BRE	B recognition element
CoA	Coenzyme A
CRISPRs	Clustered regularly interspaced short palindromic repeats
H_4MPT	Tetramethanopterin
H_4THF	Tetrahydrofolate
H_4SPT	Tetrahydrosarcinapterin
MCM	Minichromosome maintenance
MFR	Methanofuran
MT1	Methanol:5-hydroxybenzinidazolyl
MT2	Methylcobamide:CoM methyltransferase
NAD	Nicotinamide adenine dinucleotide
RNAP II	RNA polymerase II
TCA	Tricarboxylic acid
TFB	Transcription factor B
TFE	Transcription factor E
tRNA	Transfer RNA
5′-UTR	5′ untranslated region
%G + C	Percentage guanosine + cytosine

Historical Overview

The generation of combustible gas, presumably CH_4, has been reported by Pliny as early as during the Roman Empire. Legendary manifestations of methanogenesis include the will-o'-the-wisp, hypothesized to have resulted from the spontaneous combustion of marsh gas, and fire-breathing dragons, conjectured to have resulted from the accidental ignition of gas from CH_4-belching ruminants. The close association between decaying plant material and the generation of 'combustible air' was first described by the Italian physicist Alessandro Volta in 1776, when he reported that gas released after disturbing marsh and lake sediments

☆*Change History*: August 2014. K Sowers changed address to Institute of Marine & Environmental Technology, University of Maryland, updated and reduced length of abstract, updated text, references, tables, and figures.

This article is an update of K.R. Sowers, Methanogenesis, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 265–286.

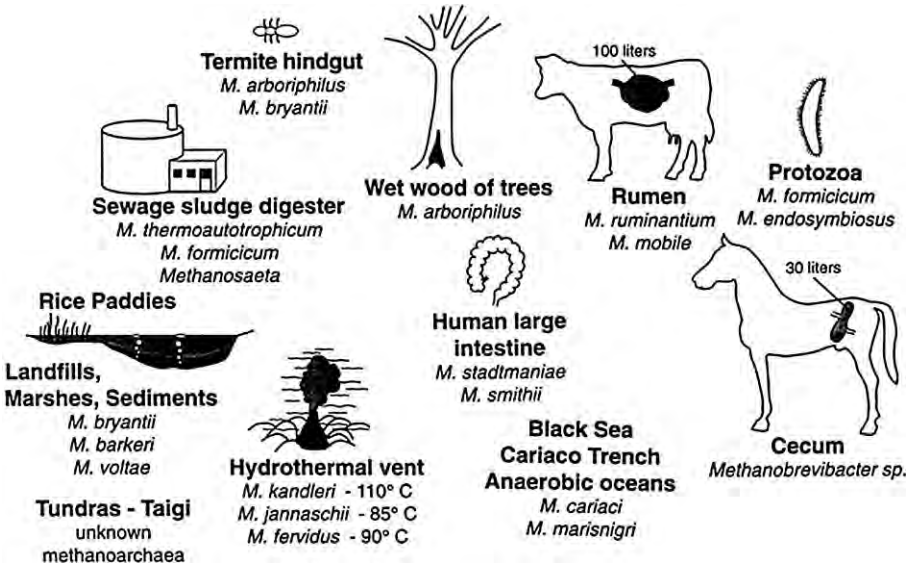

Figure 1 Habitats of the methanogenic Archaea. Reproduced from Wolfe RS (1996) *ASM News* 62: 529–534. ASM Press, with permission.

produced a blue flame when ignited by a candle. Bechamp, a student of Pasteur, was the first to establish that methanogenesis was a microbial process, which was corroborated by others throughout the remainder of the nineteenth and early twentieth centuries. Because of the methanogens' requirement for strict anaerobic conditions, the first isolates were not reported until the 1940s. The approach used for isolation, the shake culture, involved adding microorganisms to molten agar growth medium containing a chemical reductant, such as pyrogallol carbonate, to prevent O_2 from diffusing into the agar. However, this approach was not suitable for isolating and maintaining methanogens in pure culture for long periods of time, as the medium was not sufficiently anaerobic. It was not until 1950 that a simple, effective technique was developed that provided the rigorous conditions required for routine isolation and culturing of methanogenic *Archaea* (Figure 1). The technique, referred to as the 'Hungate technique', employs gassing cannula, O_2-free gases, and cysteine-sulfide reducing buffers to prepare a highly reduced, O_2-free medium. Boiling initially deoxygenates medium, and a cannula connected to an anaerobic gas line, such as N_2 or CO_2, is inserted into the vessel to displace air as the medium cools. The medium is dispensed into culture tubes or serum vials while purging with anaerobic gas and then the medium is sealed with a rubber stopper or septum. The vessel containing reduced medium and anaerobic gas effectively becomes an anaerobic chamber for culture growth. The development of the anaerobic glove box has further simplified culturing of methanogens by providing a means for colony isolation in Petri plates containing anaerobic medium solidified with agar (Figure 2). Inoculated plates are then transferred to an anaerobe jar that is purged with anaerobic gas and hydrogen sulfide to create conditions necessary for growth.

Diversity and Phylogeny

The methanogens are members of the *Archaea*, one of three domains of life proposed by C. Woese on the basis of 16S rRNA sequence, which also include the *Bacteria* and *Eukarya* (Figure 3). *Archaea* have morphological features that resemble the *Bacteria*; they are unicellular microorganisms that lack a nuclear membrane and intracellular compartmentalization. In contrast, several

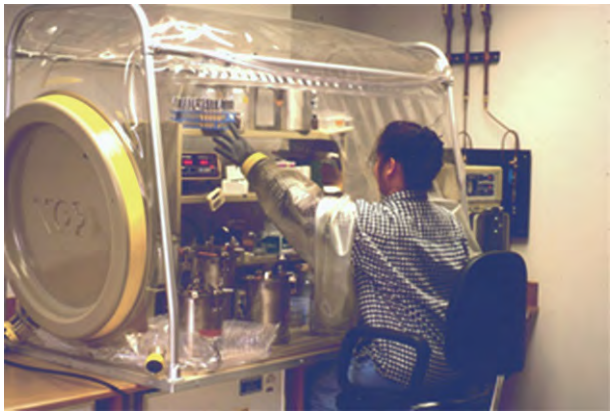

Figure 2 Anaerobic glove box used for growing methanogens. Materials are introduced into the glove box through an air lock located on the side.

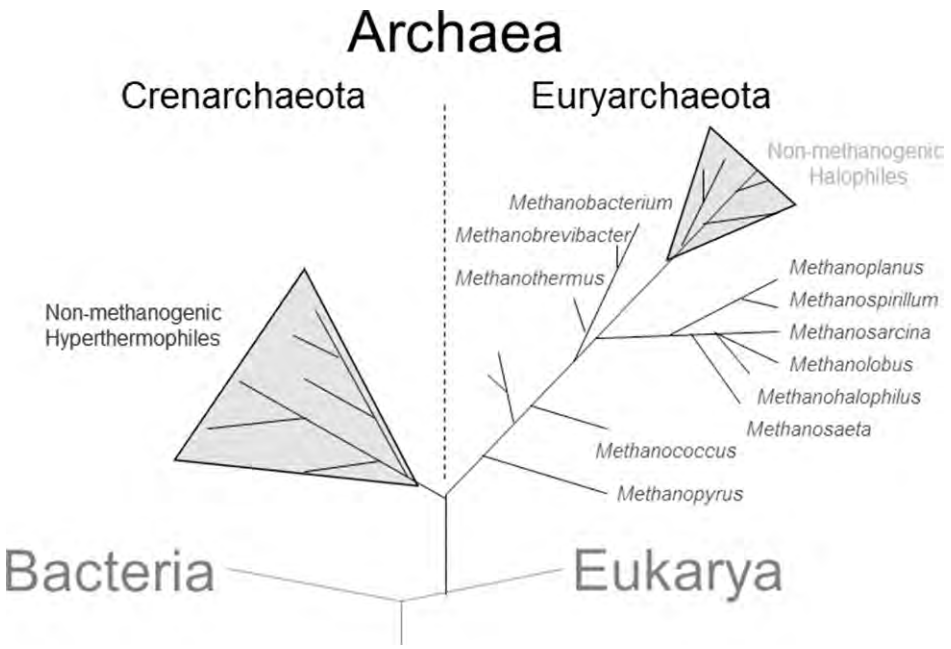

Figure 3 Phylogenetic tree based on 16S rRNA sequence showing selected genera of methanogens within one of the two major phyla of *Archaea*, the *Euryarchaeota*.

molecular features of the *Archaea* have similarity to the *Eukarya*; these features include histone-like DNA proteins, a large multicomponent RNA polymerase, and eukarya-like transcription initiation. Despite the similarities to the other domains, *Archaea* also have unique characteristics that distinguish them from the *Bacteria* and *Eukarya*. These distinguishing features include membranes composed of isoprenoids ether linked to glycerol or carbohydrates, cell walls that lack peptidoglycan, synthesis of unique enzymes, and enzyme cofactor molecules. An additional unifying characteristic among the *Archaea* is their requirement for extreme growth conditions, such as high temperatures, extreme salinity, and, in the case of the methanogens, highly reduced, anoxic environments.

Although the methanogens are a phylogenetically coherent group and have a limited substrate range, they are morphologically and physiologically diverse. They include psychrophilic species from Antarctica that grow at 1.7 °C to extremely thermophilic species from deep submarine vents that grow at 110 °C; acidophiles from marine vents that grow at pH 5.0 to alkaliphiles from alkaline lake sediments that grow at pH 10.3; species from freshwater lake sediments that grow at saline concentrations below 0.1 mol l^{-1} to extreme halophiles from solar salterns that grow at nearly saturated NaCl concentrations; autotrophs that use only CO_2 for cell carbon and methylotrophs that utilize methylated compounds. Despite the range and diversity of growth habitats where methanogens are found, methanogens have one common attribute: they all generate CH_4 during growth.

There are currently over 140 described species of methanogens in six orders within the archaeal kingdom *Euryarchaeota* (Figure 3). Characteristics of methanogenic *Archaea* are described in Table 1.

The order *Methanococcales* includes marine autotrophs that grow exclusively by CO_2 reduction with H_2 or in some cases use formate for growth and methanogenesis. Morphologically, these species form irregularly shaped cocci. Instead of a rigid cell wall, typical of most *Bacteria*, these species form an S-layer, composed of an array of protein subunits, and are subject to osmotic lysis at NaCl concentrations below seawater. This order includes several mesophilic *Methanococcus* spp., moderately thermophilic *Methanothermococcus* spp., and the extremely thermophilic genera *Methanocaldococcus* and *Methanotorris*.

The order *Methanobacteriales* is predominantly composed of rod-shaped cells that grow by CO_2 reduction with H_2. One exception is the genus *Methanosphaera*, which grows as cocci and uses H_2 to reduce the methyl group of methanol instead of CO_2. Other species can also reduce methyl groups with hydrogen, oxidize alcohols to reduce CO_2 or use formate for growth and methanogenesis. Cells have a rigid cell wall approximately 15–20 nm thick and, when stained for thin-section electron microscopy, resemble the electron-dense monolayer cell wall of Gram-positive bacteria. These archaeal cell walls are composed of pseudomurein, which is chemically distinguishable from bacterial murein by the substitution of *N*-talosaminuronic acid for *N*-acetylmuramic acid and substitution of β(1,3) for β(1,4) linkage in the glycan strands, and substitution of D-amino acids for L-amino acids in the peptide cross-linkage. *Methanothermus* spp. also have an additional cell wall layer, composed of glycoprotein S-layer that surrounds the pseudomurein. *Methanobrevibacter* spp. are all mesophilic; *Methanobacterium* includes mesophiles and moderate thermophiles with optimal growth temperatures as high as 65 °C; *Methanothermobacter* spp. are exclusively moderate thermophiles, and *Methanothermus* species are extreme thermophiles, with maximum growth temperatures as high as 97 °C.

Table 1 Description of methanogenic Archaea

			Optimum growth conditions		
Taxonomic epithet	*Morphology*	*Substrates*[a]	*pH*	*Temp (° C)*	*Isolation source*
Order *Methanobacteriales*					
Family *Methanobacteriaceae*					
Genus *Methanobacterium*					
aarhusense	Rod	H	7.5–8.0	45	Marine sediment
alcaliphilum	Rod	H	8.4	37	Alkaline lake sediment
arcticum	Roc	H, F	6.8–7.2	37	Arctic permafrost
beijingense	Rod	H, F	7.2–7.7	37	Beer waste digestor
bryantii	Rod	H, 2P, 2B	6.9–7.2	37–39	Sewage digestor
congolense	Rod	H	7.2	37–42	Cassava peel digestor
espanolae	Rod	H	5.6–6.2	35	Kraft mill sludge
ferruginis	Rod	H	6.5	35	Natural gas field
flexile	Rod	H, F	7.0–7.5	35–38	Lake sediment
formicicum	Rod	H, F, 2P, 2B	6.6–7.8	37–45	Sewage digestor
ivanovii	Rod	H	7.0–7.4	45	Sewage digestor
kanagiense	Rod/curved rod	H	7.5–8.5	40	Rice field
lacus	Rod	H/Me	6.5	30	Meromictic lake sediment
movens	Rod	H	7.2–7.5	35–38	Lake sediment
movilense		H, F, 2P, 2B	7.4	33	Subsurface lake
oryzae	Rod	H, F	7.0	40	Rice field
palustre	Rod	H, F, 2P, CP	7.0	37	Peat bog
petrolearium	Rod	H	6.5	35	Natural gas field
subterraneum	Rod	H, F	7.8–8.8	20–40	Deep granitic groundwater
thermoaggregans	Rod	H	7.0–7.5	65	Cattle pasture
uliginosum	Rod	H	6.0–8.5[b]	40	Marsh sediment
veterum	Rod	H, H/ME, H/MA	7.0–7.2	28	Arctic permafrost
Genus *Methanobrevibacter*					
acididurans	Coccobacillus	H	6.0	35	Alcohol distillery waste
arboriphilicus	Coccobacillus	H, F	7.8–8.0	30–37	Cotton wood tree
boviskoreani	Rod	H, F	6.5–7.0	37–40	Bovine rumen
curvatus	Curved rod	H	7.1–7.2	30	Termite hindgut
cuticularis	Rod	H, F	7.7	37	Termite hindgut
filiformis	Filamentus rod	H	7.0–7.2	30	Termite hindgut
gottschalkii	Coccobacillus	H	7	37	Horse feces
millerae	Coccobacillus	H, F			Bovine rumen
olleyae	Coccobacillus	H, F			Ovine rumen
oralis	Coccobacillus	H	6.9–7.4	36–38	Human subgingival plaque
ruminantium	Coccobacillus	H, F	6.3–6.8	37–39	Bovine rumen
smithii	Coccobacillus	H, F	6.9–7.4	37–39	Sewage digestor
thaueri	Coccobacillus	H	7	37	Bovine feces
woesii	Coccobacillus	H, F	7	37	Goose feces
wolinii	Coccobacillus	H	7	37	Sheep feces
Genus *Methanosphaera*					
cuniculi	Coccus	H/ME	6.8	35–40	Rabbit rectum
stadtmanae	Coccus	H/ME	6.5–6.9	36–40	Human feces
Genus *Methanothermobacter*					
crinale	Rod/curved rod/coccoid	H	6.9	65	Oil sands
defluvii	Rod	H, F	6.5–7.0	60–65	Methacrylic waste digestor
marburgensis	Rod	H	6.8–7.4	65	Sewage digestor
tenebrarum	Rod	H	6.9–7.7	70	Natural gas field
thermoautotrophicus	Rod	H, CO	7.2–7.6	65–70	Sewage digestor
thermoflexus	Curved rod	H, F	7.9–8.2	55	Methacrylic waste digestor
thermophilus	Rod	H	8.0–8.2	62	Digestor methane tank
wolfeii	Rod	H, F	7.0–7.7	55–65	Sewage/river sediment
Family *Methanothermaceae*					
Genus *Methanothermus*					
fervidus	Rod	H	6.5	83	Solfataric hot spring
sociabilis	Rod	H	6.5	88	Solfataric mud
Order *Methanococcales*					
Family *Methanocaldococcaceae*					

Table 1 (Continued)

Taxonomic epithet	Morphology	Substrates	Optimum growth conditions		Isolation source
			pH	Temp (°C)	
Genus *Methanocaldococcus*					
fervens	Irreg. coccus	H	6.5	85	Marine hydrothermal vent
indicus	Irreg. coccus	H	6.6	85	Marine hydrothermal vent
infernus	Irreg. coccus	H	6.5	85	Marine hydrothermal vent
jannaschii	Irreg. coccus	H	6.0	85	Marine hydrothermal vent
villosus	Irreg. coccus	H	6.5	80	Marine hydrothermal vent
vulcanius	Irreg. coccus	H	6.5	80	Marine hydrothermal vent
Genus *Methanotorris*					
formicicus	Irreg. coccus	H, F	6.7	75	Marine hydrothermal vent
igneus	Irreg. coccus	H	5.7	88	Marine hydrothermal vent
Family *Methanococcaceae*					
Genus *Methanococcus*					
aeolicus	Irreg. coccus	H, F	6.6	46	Marine sediment
maripaludis	Irreg. coccus	H, F	6.8–7.2	35–39	Marine marsh sediment
vannielii	Irreg. coccus	H, F	8.0	36–40	Marine sediment
voltae	Irreg. coccus	H, F	6.7–7.4	32–40	Estuarine sediment
Genus *Methanothermococcus*					
okinawensis	Irreg. coccus	H, F	6.0–7.0	60–65	Marine hydrothermal vent
thermolithotrophicus	Irreg. coccus	H, F	6.5–7.5	65	Thermal coastal sediment
Order *Methanomicrobiales*					
Family *Methanocalculaceae*					
Genus *Methanocalculus*					
chungsingensis	Irreg. cocci	H, F	7.2	37	Mariculture fish pond
halotolerans	Irreg. coccus	H, F	6.5–7.5	35	Marine waste leachate site
natronophilus	Irreg. coccus	H, F	9.0–9.5		Soda lake
pumilus	Irreg. coccus	H, F	7.6	38	Oil field
taiwanensis	Irreg. coccus	H, F	6.7	37	Estuarine sediment
Family *Methanocorpusculaceae*					
Genus *Methanocorpusculum*					
aggregans	Irreg. coccus	H, F	6.4–7.2	35–37	Sewage digestor
bavaricum	Irreg. coccus	H, F, 2P, 2B, CP	7.0	37	Sugar plant wastewater
labreanum	Irreg. coccus	H, F	7.0	37	Tar pit lake
parvum	Irreg. coccus	H, F, 2P, 2B	6.8–7.5	37	Whey digestor
sinense	Irreg. coccus	H, F	7.0	30	Distillery wastewater
Family *Methanomicrobiaceae*					
Genus *Methanoculleus*					
bourgensis	Irreg. coccus	H, F	7.4	37	Tannery waste digestor
chikugoensis	Irreg. coccus	H, F, 2P, 2B, CP	6.7–7.2	25–30	Rice field
horonobensis	Irreg. coccus	H, F	6.7–6.8	37–42	Groundwater
hydrogenitrophicus	Irreg. coccus	H	6.6	37	Wetland soil
marisnigri	Irreg. coccus	H, F, 2P, 2B	6.2–6.6	20–25	Marine sediment
palmolei	Irreg. coccus	H, F, 2P, 2B, CP	6.9–7.5	40	Palm oil wastewater reactor
receptaculi	Irreg. coccus	H, F	7.5–7.8	50–55	Oil field
submarinus	Irreg. coccus	H, F	4.8–7.7	45	Deep marine sediment
thermophilicus	Irreg. coccus	H, F	7.0	55	Thermal marine sediment
Genus *Methanofollis*					
aquaemaris	Irreg. coccus	H, F	6.5	37	Mariculture fish pond
ethanolicus	Irreg. coccus	H, F, 1P, 1B	7.0	37	Lotus field
formosanus	Irreg. coccus	H, F	6.6–7.0	37	Mariculture fish pond
liminatans	Irreg. coccus	H, F, 2P, 2B	7.0	40	Industrial wastewater
tationis	Irreg. coccus	H, F	7.0	37–40	Solfataric hot pool
Genus *Methanogenium*					
boonei	Irreg. coccus	H, F	6.4–7.8	19.4	Marine sediment
cariaci	Irreg. coccus	H, F	6.8–7.3	20–25	Marine sediment
frigidum	Irreg. coccus	H, F	6.5–7.9	15	Antarctic lake
frittonii	Irreg. coccus	H, F	7.0–7.5	57	Lake sediment
organophilum	Irreg. coccus	H, F, E, 1P, 2P, 2B	6.4–7.3	30–35	Marine sediment
Genus *Methanolacinia*					
paynteri	Irreg. rod	H, F, 2P, 2B, CP	6.6–7.2	40	Marine sediment

(*Continued*)

Table 1 (Continued)

			Optimum growth conditions		
Taxonomic epithet	*Morphology*	*Substrates*	*pH*	*Temp (°C)*	*Isolation source*
Genus *Methanomicrobium*					
mobile	Curved rod	H, F	6.1–6.9	40	Bovine rumen
Genus *Methanoplanus*					
endosymbiosus	Irreg. disk	H, F	6.6–7.1	32	Marine ciliate
limicola	Plate	H, F	7.0	40	Drilling swamp
petrolearius	Plate	H, F, 1P	7.0	37	Offshore oil field
Family Methanoregulaceae					
Genus *Methanolinea*					
mesophila	Rod	H, F	7.0	37	Rice field
tarda	Rod	H, F	7.0	37	Sludge digestor
Genus *Methanoregula*					
boonei	Rod	H, F	7.4	30–33	Beer waste digestor
formicica					
Genus *Methanosphaerula*					
palustris	Coccus	H, F	5.5	28–30	Fen peatland
Family *Methanospirillaceae*					
Genus *Methanospirillum*					
hungatei	Sheathed spiral	H, F	6.6–7.4	30–37	Sewage sludge
lacunae	Sheathed spiral	H, F	7.5	30	Soil
psychrodurum	Sheathed spiral	H	7.0	25	Wetland soil
stamsii	Sheathed spiral	H, F	7.0–7.5	20–30	Bioreactor
Order Methanocellales					
Family Methanocellaceae					
Genus Methanocella					
arvoryzae	Rod	H, F	7.0	45	Rice field
conradii	Rod	H	6.8	55	Rice field
paludicola	Rod	H, F	7.0	35–37	Rice field
Order *Methanosarcinales*					
Family *Methanosarcinaceae*					
Genus *Methanosarcina*					
acetivorans	Irreg. coccus, pseudosarcina	AC, ME, MA, DMS, MMP, CO	6.5–7.5	35–40	Marine sediment
baltica	Irreg. coccus, pseudosarcina	AC, ME, MA	6.5–7.5	25	Brackish sediment
barkeri	Irreg. coccus, pseudosarcina	H, AC, ME, MA, CO	6.5–7.5	30–40	Sewage digestor
horonobensis	Irreg. coccus	H, ME, MA, DMS	7.0–7.3	37	Groundwater
lacustris	Irreg. coccus	H, ME, MA	7.0	25	Lake sediment
mazei	Irreg. coccus, pseudosarcina	AC, ME, MA AC, ME, MA	6.5–7.2	30–40	Sewage digestor
semesiae	Irreg. coccus	ME, MA, DMS, MT	6.5–7.5	30–35	Mangrove sediment
siciliae	Irreg. coccus	AC, ME, MA, DMS, MMP	6.5–6.8	40	Marine sediment
soligelidi	Irreg. coccus	H, AC, ME	7.8	28	Permafrost
thermophila	Irreg. coccus, pseudosarcina	AC, ME, MA	6.0	45–55	Sewage digestor
vacuolata	Irreg. coccus, pseudosarcina	H, AC, ME, MA	7.5	40	Methane tank sludge
Genus *Methanococcoides*					
alaskense	Irreg. coccus	MA	6.3–7.5	23.5	Cold marine sediment
burtonii	Irreg. coccus	ME, MA	7.7	23.4	Antarctic saline lake
methylutens	Irreg. coccus	ME, MA	7.0	30–35	Marine sediment
Genus *Methanohalobium*					
evestigatum	Irreg. coccus	ME, MA	7.4	50	Salt lagoon sediment
Genus *Methanohalophilus*					
euhalobius					
halophilus	Irreg. coccus	ME, MA	7.4	26–36	Marine cyanobacterial mat
mahii	Irreg. coccus	ME, MA	7.4	35–37	Saline lake sediment
portucalensis	Irreg. coccus	ME, MA	6.5–7.5	40	Solar salt pond
Genus *Methanolobus*					

Table 1 (Continued)

			Optimum growth conditions		
Taxonomic epithet	*Morphology*	*Substrates*	*pH*	*Temp (°C)*	*Isolation source*
bombayensis	Irreg. coccus	ME, MA, DMS	7.2	37	Marine sediment
taylorii	Irreg. coccus	ME, MA, DMS	8.0	37	Estuarine sediment
oregonensis	Irreg. coccus	ME, MA, DMS	8.6	35	Saline alkaline aquifer
psychrophilus	Irreg. coccus	ME, MA, DMS	7.0–7.2	18	Cold wetland soil
profundi	Irreg. coccus	ME, MA	6.5	30	Natural gas field
tindarius	Irreg. coccus	ME, MA	6.5	37	Lake sediment
vulcani	Irreg. coccus	ME, MA	7.2	37	Submarine fumarole
zinderi	Irreg. coccus	ME, MA	7.0–8.0	40–45	Coal seam
Genus *Methanosalsum*					
zhilinae	Irreg. coccus	ME, MA, DMS	9.2	45	Alkaline lake sediment
Genus *Methanimicrococcus*					
blatticola	Irreg. coccus	H/ME, H/MA	7.2–7.7	39	Cockroach hindgut
Genus *Methanomethylovorans*					
hollandica	Irreg. cuccus	ME, MA, DMS, MT	6.5–7.0	34–37	Freshwater sediment
thermophila	Irreg. coccus	ME, MA	6.5	50	Paper mill wastewater digestor
Family *Methanosaetaceae*					
Genus *Methanosaeta*					
concilii	Sheathed rod	AC	7.1–7.5	35–40	Pear waste digestor
harundinacea	Sheathed rod	AC	7.2–7.6	34–37	Beer waste digestor
thermacetophila	Sheathed rod	AC	7.4–7.8	35–40	Thermophilic sludge digestor
thermophila	Sheathed rod	AC	6.5–6.7	55–60	Thermophilic sludge digestor
Family *Methermicoccaceae*					
Genus *Methermicoccus*					
shengliensis	Coccus	ME, MA	6.0–6.5	65	Oil field
Order *Methanopyrales*					
Family *Methanopyraceae*					
Genus *Methanopyrus*					
kandleri	Sheathed rod	H	6.5	98	Geothermal marine sediment

[a]H = hydrogen/carbon dioxide; F = formate; AC = acetate; ME = methanol; MA = methylamines; MT = methanethiol; CO = carbon monoxide; H/ME = methanol reduction with hydrogen; H/MA = methylamine reduction with hydrogen; E = ethanol; 1P = 1-propanol; 2P = 2-propanol; 1B = 1-butanol; 2B = 2-butanol; CP = cyclopentanol; DMS = dimethylsulfide; MMP = methylmercaptopropionate.

[b]Only one range reported.

The order *Methanomicrobiales* contains genera that are diverse in morphology and physiology. Most species grow as cocci and rods. In addition, *Methanoplanus* forms flat plate-like cells with characteristically angular ends. Another species, *Methanospirillum hungatei*, forms a helical spiral. The cell walls in this order are composed of a protein S-layer and are sensitive to osmotic shock or detergents. In addition to the S-layer, *M. hungatei* also has an external sheath that is composed of concentric rings stacked together. Species range from nonhalophilic to slightly halophilic. Temperature tolerances include the only currently described psychrophile, *Methanogenium frigidum*, mesophiles, and moderate thermophiles. Most species grow by CO_2 reduction with H_2, but some species also use formate or secondary alcohols as electron donors for CO_2 reduction.

The order *Methanosarcinales* includes the most catabolically diverse species of methanogens. Whereas most methanogenic species grow by obligate CO_2 reduction with H_2, species within this order can also grow by methyl reduction with H_2, aceticlastic fermentation of acetate, or methylotrophic catabolism of methanol, methylated amines, and dimethylsulfide. *Methanosarcina acetivorans* was recently reported to grow also with CO by conversion to acetate and formate, rather than methane, as the major metabolic end products. Whereas some species of *Methanosarcina* can grow by all five catabolic pathways, *Methanosaeta* species are obligate acetotrophs and all other genera are obligate methylotrophs. All species have a protein S-layer cell wall and most species grow as irregularly shaped cocci. However, several species of *Methanosarcina* also synthesize a heteropolysaccharide matrix external to the S-layer. This external layer can be up to 200 nm thick and is primarily composed of a nonsulfonated polymer of *N*-acetylgalactosamine and D-glucuronic or D-galacturonic acids. The matrix is called methanochondroitin because of its chemical similarity to a mammalian connective tissue component, known as chondroitin. At freshwater NaCl concentrations, *Methanosarcina* spp. that synthesize methanochondroitin grow in multicellular aggregates rather than as single cells, but when grown at marine salt concentrations or with high concentrations of divalent cations, such as Mg^{2+}, they no longer synthesize methanochondroitin and grow as single cells. *Methanosaeta* species have an external sheath that appears similar in structure to that described for *M. hungatei*.

Two orders presently include only one described genus. The order *Methanocellales* was proposed for species within a clonal lineage identified as 'rice cluster I'. This order currently includes three isolated species within the genus, *Methanocella*.

These hydrogenotrophic rod-shaped species have all been isolated thus far from rice fields. The order *Methanopyrales* is the most deeply branching methanogenic archaeon and presently includes only one species, *Methanopyrus kandleri*. This hyperthermophilic species is an obligate hydrogenotroph and grows as a rod with a pseudomurein cell wall surrounded by a protein S-layer, similar to that described for *Methanothermus*.

Habitats

Interspecies H_2 Transfer

As described above, methanogens utilize a limited number of simple substrates. In most habitats, they depend on other anaerobes to convert complex organic matter into substrates, which they can catabolize. Therefore, unlike aerobic habitats, where a single microorganism can catalyze the mineralization of a polymer by oxidation to H_2O and CO_2, degradation in anaerobic habitats requires consortia of interacting microorganisms to convert polymers to CH_4 and CO_2. These interactions are dynamic with the methanogens affecting the pathway of electron flow, and, consequently, carbon flow, by a process called interspecies H_2 transfer. In this association, the H_2-utilizing methanogens maintain a low H_2 partial pressure that allows certain reactions that do not occur under standard conditions to be thermodynamically favorable. One physiological group of microorganisms affected by this process is the H_2-producing acetogens. The reactions carried out by these microorganisms for growth are not thermodynamically favorable (i.e., $+\Delta G^{\circ}{}_{\bullet}$; under physiological growth conditions), as the H_2 they generate accumulates and growth is subsequently inhibited. However, in association with H_2-consuming microorganisms, such as the methanogens, the H_2 partial pressure is maintained at levels low enough to make the reaction thermodynamically favorable (i.e., $-\Delta G^{\circ}{}_{\bullet}$; under physiological growth conditions). Because of their dependence on the H_2-consuming microorganisms, the H_2-producing acetogens are often referred to as obligate syntrophs.

Another physiological group of microorganisms affected by interspecies H_2 transfer is the fermentative anaerobes that synthesize a hydrogenase. In many of these microorganisms, substrate oxidation is linked to the electron carrier nicotinamide adenine dinucleotide (NAD), which has higher redox potential (-320 mv) than H_2 (-414 mv) under standard conditions. However, if the H_2 partial pressure is maintained at a low level (<10 Pa) by H_2-utilizing microorganisms, then H_2 production from NAD becomes thermodynamically favorable. This enables the fermentative microorganisms to reoxidize NADH by reducing protons to form H_2 rather than reducing pyruvate to form dicarboxylic acids and alcohols. This synergistic process enables the fermentor to conserve ATP by synthesizing more acetate and less further reduced products. In addition, the net products, acetate and H_2, serve as substrates for growth by the methanogen. The result is that carbon and electron flow is directed toward more efficient degradation by the consortium to the gases CH_4 and CO_2. CO_2 reenters the carbon cycle directly and CH_4 is either oxidized by methylotrophs to CO_2 as it diffuses into the aerobic zone or it enters the atmosphere.

Synergistic interspecies substrate transfer has also been reported to occur with formate and acetate. Removal of formate by formate-utilizing methanogens and the removal of acetate by acetotrophic methanogens have been shown to create a thermodynamic shift that favors butyrate and propionate degradation in syntrophic cocultures of acetogenic *Bacteria* and methanogenic *Archaea*.

Freshwater Sediments

As organic matter accumulates on lake and river basins, the sediment becomes anaerobic, because oxygen is depleted by the activities of aerobic microorganisms. In eutrophic environments with high organic loading, the anaerobic region occurs immediately below the sediment surface and even into the water column if the activities of the aerobic microorganisms exceed the rate of oxygen diffusion. Once oxygen and alternative electron acceptors such as NO^{3-}, Fe^{3+}, Mn^{4+}, and SO_4^{2-} are depleted, methanogenesis becomes the ultimate degradative process. The flow of carbon in anaerobic freshwater sediments is shown in Figure 4. Fermentative bacteria that synthesize hydrolytic enzymes, such as cellulases, proteases, amylases, and lipases, catalyze degradation of complex polymers to soluble monomers. The fermentative bacteria then ferment the soluble products to H_2, CO_2, simple alcohols, and fatty acids, including significant generation of acetate, due to interspecies H_2 transfer. H_2-producing acetogenic bacteria then catalyze the oxidation of alcohols and fatty acids to H_2, CO_2, and acetate. The third consortium group of microorganisms, the methanogenic *Archaea*, utilizes the simple substrates H_2, CO_2, and acetate generated by the fermentative and acetogenic bacteria, to generate CH_4. The net result of this consortium is that carbon and electrons are directed toward the synthesis of CH_4 and CO_2, which then reenter the global carbon cycle. CH_4 is oxidized by aerobic methanotrophic bacteria as it diffuses through the aerobic regions of the water column and reenters the atmosphere as CO_2. However, some CH_4 escapes from shallow water bodies, such as rice fields, through the vascular systems of aquatic plants, and enters the atmosphere as a greenhouse gas.

Anaerobic Bioreactors

Methanogenic bioreactors are used for the conversion of organic wastes to CH_4 and CO_2. These anaerobic digestors are found in nearly all sewage treatment plants where wastes in the form of sewage sludge, polymeric particulate material generated by settling of raw sewage, are converted to CH_4 and CO_2 by a consortium of microorganisms in freshwater sediments similar to that previously described. In contrast to most sediment environments, microbial metabolism is higher in a bioreactor because of greater rates of

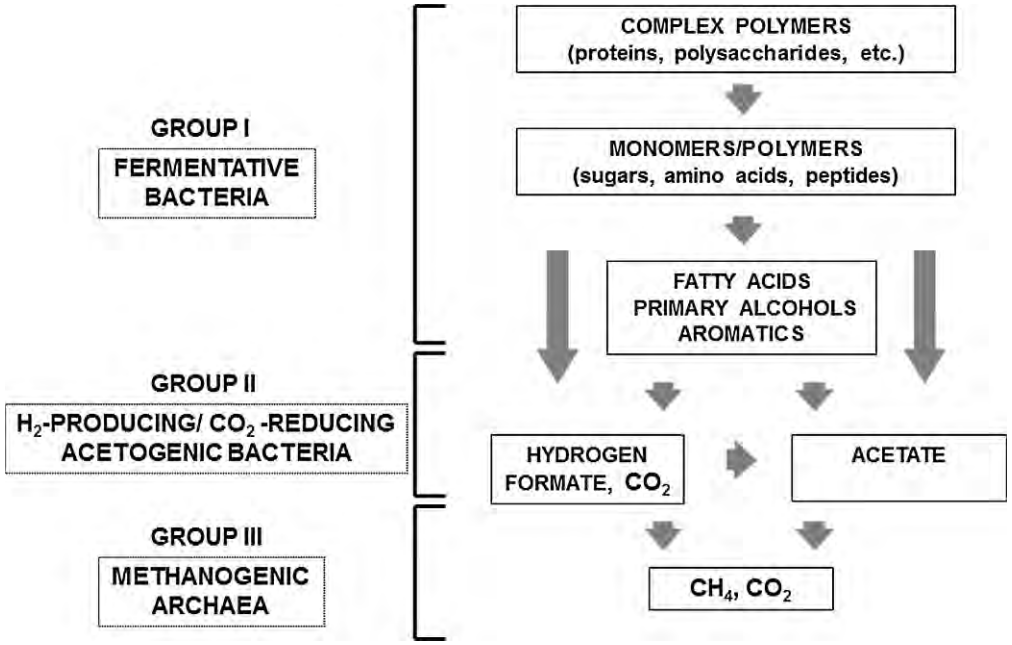

Figure 4 Carbon flow in an anaerobic microbial consortium from freshwater sediments.

organic loading and higher temperatures (35–40 °C) generated by heating the reactor, often by combusting the CH_4 generated in the degradative process. As a result, the rate-limiting factor in a bioreactor is the slow growth rates of the acid-consuming H_2-producing acetogens and the acetate-utilizing methanogens, which require retention times of 14 days or more, to compensate for their slow metabolic activities. Another critical factor is the requirement for H_2-utilizing methanogens to maintain H_2-partial pressures below 10 Pa. Perturbations in the process that result in inhibition and subsequent 'washout' of either of these metabolic groups will result in a drop in pH, resulting in acid accumulation and subsequent inhibition of the entire reactor process. Perturbations can include sudden overloading with a readily fermented organic substrate that results in rapid accumulation of H_2 and fatty acids or introduction of a toxic compound that disrupts the microbial balance.

Anaerobic bioreactors have also been tested as a low-cost method for treatment of other types of particulate organic wastes, including animal manure and crop wastes. Nonparticulate industrial wastes, including many food processing by-products, and organic solvents, such as chlorinated aliphatic and aromatic compounds, are often too dilute to be economically treated by standard bioreactor configurations, which would require long retention times. In order to decrease the hydraulic retention time of the waste without washing out the biomass, high-rate anaerobic bioreactor configurations have been developed. Examples of these reactors include fixed-film reactors, in which biofilms of microbial consortia are retained in the vessel on solid supports, such as plastic or ceramic matrixes, glass beads, or sand grains. Other designs exist, such as the anaerobic upflow sludge blanket process, in which biomass is immobilized by the aggregation of microbial consortia into distinct granules (Figure 5). The granules usually consist of three discrete layers of microorganisms that include acetate-utilizing methanogens in the interior, H_2-producing acetogens and H_2-utilizing methanogens in the middle layer, and fermentative microorganisms in the outermost layer. Settler screens separate the granules from the treated water and gas is collected at the top of the reactor. In contrast to particulate waste reactors, in which microbial biomass is generated at the same rate of hydraulic washout, requiring retention times as long as several days, growth of biomass in these high-rate reactors is uncoupled from retention time of the waste and can have retention times as short as 1 h. Hydraulic retention times have also been reduced further in anaerobic fermentors that operate at temperatures of up to 60 °C, since the metabolic rates of thermophilic microorganisms, including acetate-utilizing methanogens, are greater than those of mesophiles.

Marine Habitats

In marine habitats where substrates are often limited, sulfate-reducing bacteria outcompete methanogens as the terminal members of the anaerobic consortium (Figure 6). Their predominance is a result of the availability of the electron acceptor sulfate in seawater ($\sim$30 mmol l^{-1}). Their lower K_s for substrate utilization enables the sulfate-reducing bacteria to use low concentrations of H_2 and acetate at rates that are greater than those of methanogens. However, methanogenesis is predominant in marine environments where SO_4^{2-} has been depleted. These environments include the lower depths of sediments and elevated coastal marshes, where the rate of SO_4^{2-} reduction is greater than the rate of diffusion from seawater, and also sediments that receive large amounts of organic matter, such as eutrophic coastal regions and submarine trenches. In these regions, the three-member methanogenic consortium is similar to that in freshwater environments, but it is composed of halophilic and halotolerant species. Although the acetotrophic

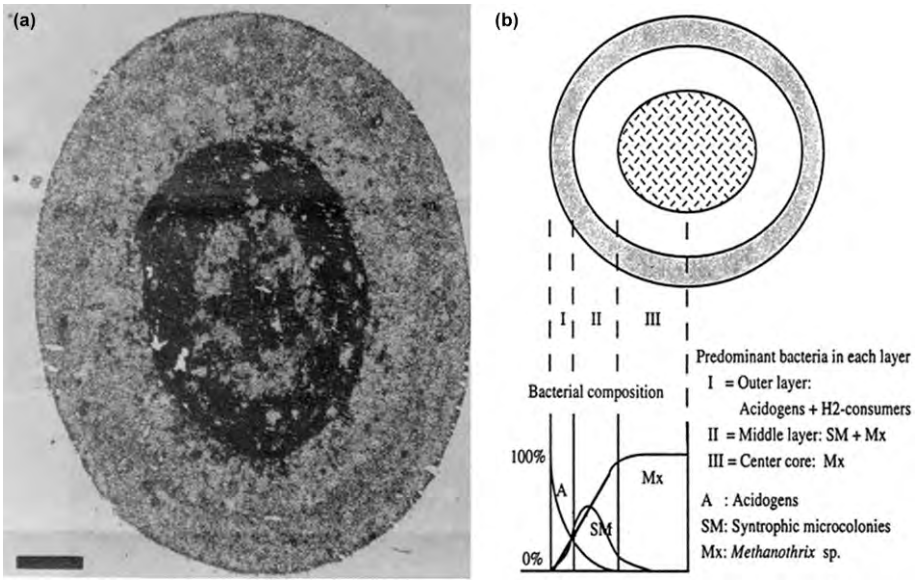

Figure 5 An anaerobic granule from a brewery wastewater digestor. (a) A thin-section photomicrograph of a granule under a bright-field microscope. Scale bar = 0.25 mm. (b) The bacterial composition of a granule. Reproduced with permission from Fang HHP, Chui HK, and Li YY (1994) *Water Science and Technology* 30: 87–96. Pergamon Press; and Fang HHP, Chui HK, and Li YY (1995) *Water Science and Technology* 31: 129–135. Pergamon Press.

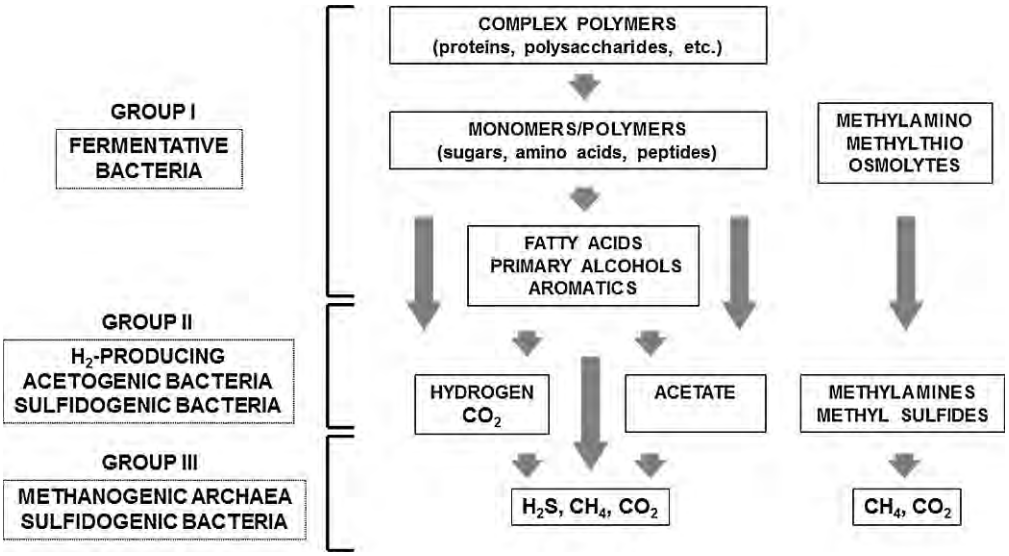

Figure 6 Carbon flow in an anaerobic microbial consortium from marine sediments.

methanogens *Methanosarcina* and *Methanosaeta* have been isolated from marine methanogenic enrichments, isotope studies performed in sediment suggest that most of the acetate is oxidized by an H_2-producing syntroph rather than by fermentation to CH_4. Methanogens also generate CH_4 from methylated amines and thiols, which are readily available in the marine environment as metabolic osmolytes. Since methylated amines are not used by SO_4^{2-}-reducing bacteria, this class of compound is 'noncompetitive' and can be used by methanogens in habitats that contain high SO_4^{2-} concentrations.

CH_4 generated in sediments is often consumed as it diffuses through the SO_4^{2-}-reducing region of the sediments, before reaching the aerobic regions. Although the microbes that catalyze this anaerobic oxidation have not been isolated, analysis by fluorescence *in situ* hybridization with species-specific probes indicates that the methane is oxidized by a consortium of two microorganisms: a methanogen that oxidizes the methane by reverse methanogenesis coupled by an unknown intermediate to sulfate reduction by a sulfate-reducing bacterium. The metagenome and mRNA expression analysis of the microbial consortia mediating the anaerobic oxidation of methane with sulfate (ANME) are consistent with acetate and formate as putative electron shuttles. Expression of putative secreted multiheme c-type cytochromes suggests direct electron transfer as an additional possible mode to shuttle electrons from ANME-1 to the bacterial sulfate-reducing partner, but a methanogenic isolate and confirmation of

the actual mechanism for this anaerobic process remain elusive. Although much of the CH_4 generated in sediments is consumed in the SO_4^{2-}-reducing regions, the water column in the open ocean is supersaturated with CH_4, compared with the atmospheric concentration. This may result from a combination of unoxidized CH_4 that escapes from sediments, the activity of microbial communities in planktonic aggregates known as marine snow, and methanogenic activity in the gastrointestinal tracts and fecal material of marine animals. Biologically generated CH_4 in some organic-rich buried sediments can accumulate as gas deposits. Since natural gas deposits generated abiotically are used as indicators of petroleum, these biologically generated CH_4 deposits can act as false indicators of petroleum deposits during oil exploration. Under high hydrostatic pressures generated in deep-ocean sediments, biogenic CH_4 can accumulate also as solidified CH_4 hydrates.

Ruminant Animals

Ruminant animals include both domestic (e.g., cows, sheep, and camels) and wild (e.g., deer, bison, and giraffes) animals. These animals have a large chamber, called the rumen, before the stomach, in which polymers, such as cellulose, are fermented by bacteria to short-chain fatty acids, H_2, CO_2, and CH_4. The rumen is similar to an anaerobic bioreactor, except that the short retention time created by swallowing saliva is less than the generation time of H_2-producing fatty acid oxidizers and acetate-utilizing methanogens (Figure 7). As a result, acetate, propionate, and butyrate are not degraded by the consortium, but are absorbed into the bloodstream of the host animal as carbon and energy sources. The volatile gases (e.g., CH_4, CO_2) are removed from the animal by belching. Acidification of the system by the acids is prevented by bicarbonate in the saliva of the animal. Carbon diverted to CH_4 and belched into the atmosphere represents a loss of energy to the animal and a significant global source of greenhouse gas emissions. Ruminant nutritionists have been attempting to increase feed efficiency by adding methanogenic inhibitors, such as monensin, to feed, thereby, diverting carbon flow to metabolites that can be utilized by the animal. One approach currently being tested is to target specific methanogen proteins with antibodies and harness the immune system of ruminants to increase feed efficiency and mitigate greenhouse gas emissions from agriculture.

Xylophagous Termites

All known termites harbor a dense microbial community of anaerobic bacteria, and, in the case of lower termites, they also contain cellulolytic protozoa that catalyze the digestion of lignocellulose from wood. As in the rumen, these microorganisms have a synergistic relationship with the termites by converting polymers to short-chain fatty acids that are used as carbon and energy sources for their host. The carbon flow in the hindgut of soil-feeding and fungus-cultivating termites is similar to that in rumen, but in wood- and grass-eating termites, most H_2-CO_2 is converted to acetate instead of CH_4. Generally, methanogenesis outcompetes H_2-CO_2 acetogenesis and the factors that cause the predominance of acetogenesis in some termites in not known. Three species of H_2-utilizing *Methanobrevibacter* have been isolated from the hindgut of the subterranean termite that exhibit catalase activity and are particularly tolerant to oxygen exposure. Oxygen tolerance may be important for recolonization by bacterial consortia after expulsion of the hindgut contents during molting. Reinoculation is achieved by transfer of hindgut contents from other colony members, which are exposed to air during the process.

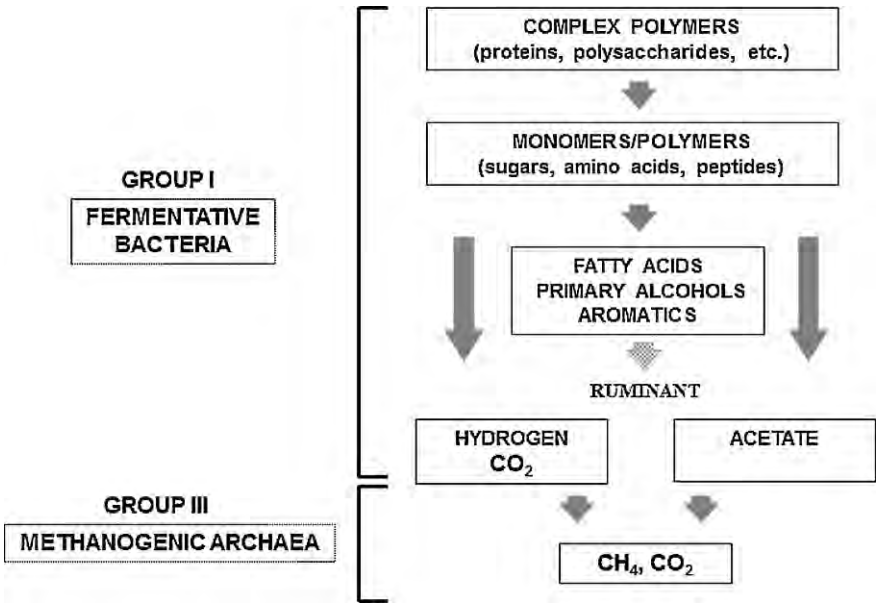

Figure 7 Carbon flow in an anaerobic microbial consortium from the rumen.

Protozoan Endosymbionts

Methanogens are present as endosymbionts in many free-living marine and freshwater anaerobic protozoa, where they are often closely associated with hydrogenosomes, organelles that produce H_2, CO_2, and acetate from the fermentation of polymeric substrates. The products of the hydrogenosomes are substrates for methanogenesis. It is conceivable that the methanogens have a synergistic role by lowering the H_2 partial pressure to create a favorable thermodynamic shift in the protozoan's fermentation reaction. Also, evidence suggests that excretion of undefined organic compounds by the methanogen provides an advantage to the protist host. Endosymbionts are also found in flagellates and ciliates that occur in the hindgut of insects, such as termites, cockroaches, and tropical millipedes. Although rumen ciliates do not harbor endosymbionic methanogens, many have ectosymbionic methanogens that may have an analogous function.

Colonization in Humans

The human colon serves as a form of hindgut where undigested polymers and sloughed off intestinal epithelium and mucin are dewatered, fermented by bacterial consortia, treated with bile acids, and held until defecation. Fatty acids generated by fermentation are absorbed into the bloodstream and can provide approximately 10% of human nutritional needs. Methanogenesis occurs in 30–40% of the human population, with the remaining population producing H_2 and CO_2 instead. Acetogenesis and SO_4^{2-} reduction from H_2-CO_2 also occur in the human colon, but studies with colonic bacterial communities suggest that these activities are only prevalent in the absence of methanogenic activity. The level of CH_4 produced by an individual corresponds to the population levels of *Methanobrevibacter*, but factors controlling the occurrence of methanogens in the human population are not known. Diet does not appear to have a significant role in determining whether an individual harbors an active methanogenic population, but hereditary and individual physiological factors might have a role. For example, methanogens might be absent from individuals who excrete higher levels of bile acids, which are inhibitory to methanogens. Methanogenic *Archaea* have also been isolated from the human vagina and oral cavity.

Epidemiological studies have revealed a relationship between the severity of periodontal disease and the relative abundance of *Methanobrevibacter*-like phylotypes. It has been suggested that the methanogens have a syntrophic relationship with periodontitus-causing fermentative bacteria that promotes their colonization. There have also been studies that show an increase in methanogenesis associated with colon cancer patients, but other reports contradict these conclusions. Although annotation of methanogen genomes reveals properties characteristic of many pathogenic bacteria including putative RelBE toxin/antitoxin genes and evidence of lateral gene transfer with bacteria, methanogens have not been shown unequivocally to cause or promote human disease.

Other Habitats

Habitats that have a source of organic carbon and high water content can become anaerobic as a result of respiratory depletion of oxygen, and, subsequently, support methanogenic communities. Examples include soils waterlogged by heavy rainfall, marshes, rice paddies, rotting heartwood of trees, and landfills. Most of the CH_4 generated in these habitats is released into the atmosphere. However, many landfills are now vented to prevent a buildup of potentially combustible CH_4 underground or in nearby dwellings, and some communities harvest the vented biogas for heat and energy.

Methanogenic habitats are also found in geo-hydrothermal outsources, such as terrestrial hot springs and deep-sea hydrothermal vents. Methanogens from these environments use geothermally generated H_2-CO_2 for methanogenesis and most are hyperthermophilic, requiring growth temperatures as high as 110 °C. In terrestrial sites, methanogens are usually associated with microbial mats composed of photosynthetic and heterotrophic consortia. However, in deep-sea hydrothermal vents, where there is no light available for photosynthetic production of organic carbon, the methanogens and other autotrophic bacteria serve as primary producers of cell carbon for a complex community of heterotrophic microbes and animals that accumulates in the vicinity of a hydrothermal vent.

Methanogenesis occurs in high-saline environments, such as Great Salt Lake, Utah, Mono Lake, California, and solar salt ponds. Methanogens from these environments generate CH_4 from methylated amines and dimethylsulfide, which are synthesized by animals and plants as osmolytes. Methanogenesis has been detected in subsurface aquifers over 400 m deep, where it has been proposed that H_2 generated by an abiotic reaction between iron-rich minerals in basalt and ground water is used as a substrate for methanogenesis. Methanogenesis has also been detected in deep subsurface sandstone, where it is hypothesized that methanogenic consortia use organic compounds that diffuse from adjacent organic-rich shale layers.

Physiology and Biochemistry

Catabolic Pathways

Methanogens generate CH_4 during growth by four catabolic pathways: autotrophic CO_2 reduction with H_2, formate, or secondary alcohols; methyl reduction with H_2; fermentation of acetate or pyruvate; and methylotrophic dismutation of methanol, methylated amines, or methylthiols. In addition, some species grow on CO and *M. acetivorans* was recently reported to grow nonmethanogenically on CO with acetate and formate as the end products. The reactions for methanogenesis are shown in Table 2. Six coenzymes serve as the principle carbon carriers in the methanogenic pathway (Figure 8). Methanofuran (MFR) is an analogue

Table 2 Reactions and free energy yields from methanogen substrates

Substrate	*Reaction*	$\Delta G^{0\prime}$ *(kJ mol^{-1} CH_4)*
Hydrogen/carbon dioxide	$4H_2 + HCO_3^- \rightarrow CH_4 + 3H_2O$	−135
Formate	$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O$	−145
Ethanol[a]	$2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$	−116
Hydrogen/methanol	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−113
Methanol	$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$	−105
Trimethylamine[b]	$4CH_3NH^+ + 9H_2O \rightarrow 9CH_3 + 3HCO_3^- + 4NH_4 + 3H^+$	−76
Dimethylsulfide[c]	$2(CH_3)_2S + 3H_2O \rightarrow 3CH_4 + HCO_3^- + 2H_2S + H^+$	−49
Acetate	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31
Pyruvate	$4CH_3COCOOH + 2H_2O \rightarrow 5CH_4 + 7CO_2$	−31
Carbon monoxide	$4CO + 5H_2O \rightarrow CH_4 + 3HCO_3^- + 3H^+$	−196
Carbon monoxide[d]	$4CO + 4H_2O \rightarrow CH_3COO^- + 2HCO_3^- + 3H^+$	−165
Carbon monoxide[d]	$CO + H_2O \rightarrow HCOO^- + H^+$	−16

[a]Other short-chain alcohols are utilized.
[b]Other methylated amines are utilized.
[c]Other methylated sulfides are utilized.
[d]Nonmethanogenic reaction.

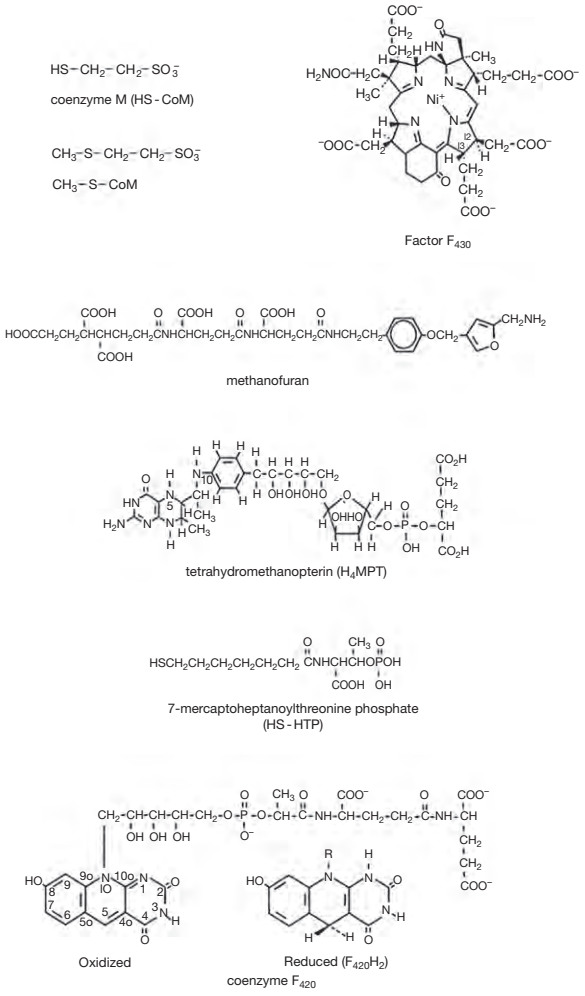

Figure 8 Structures of coenzymes that participate in the methanogenic pathway. Reproduced with permission from Rouviere PE and Wolfe RS (1988) *The Journal of Biological Chemistry* 263: 7913–7916. American Society for Biochemistry and Molecular Biology, Inc.

of molybdopterins, which occur in enzymes that catalyze similar reactions in the *Bacteria* and *Eukarya*. Tetramethanopterin (H_4MPT) is an analogue of tetrahydrofolate (H_4THF), which is also a one-carbon carrier in bacterial and eukaryal systems. Although H_4MPT was initially found to be unique to methanogens, it has since been detected in other *Archaea* and, more recently, enzymes catalyzing the methyl transfer for MFR and H_4MPT have been found to coexist with the H_4THF pathway in the CH_4-utilizing methanotrophic *Bacteria*. The methyl carrier coenzyme M (CoM-SH), 7-mercaptoheptanoylthreonine phosphate (HS-HTP), and cofactor F_{430} are currently unique to the methanogens.

Methanogenesis from H_2, formate, or secondary alcohols involves the sequential reduction of CO_2 with electrons generated by substrate oxidation (Figure 9(a)). The methanogenic sequence is initiated by a two-electron reduction of CO_2 and MFR by formyl-MFR dehydrogenase (a) to form formyl-MFR (Figure 10). The formyl group is then transferred to H_4MPT by formyl-MFR:H_4MPT formyltransferase (b) yielding formyl-H_4MPT. A homologue of H_4MPT, tetrahydrosarcinapterin (H_4SPT), found in *Methanosarcina* spp., differs by an additional glutamyl moiety in the substituted R group. Formyl-H_4MPT cyclization to methenyl-H_4MPT is catalyzed by N5,N10-methenyl-H_4MPT cyclohydrolase (c). N5,N10-methylene-H_4MPT dehydrogenase (d) and N5,N10-methylene-H_4MPT reductase (e) catalyze the sequential reduction of methenyl-H_4MPT by the electron carrier coenzyme F_{420} to methylene-H_4MPT and methyl-H_4MPT. The methyl group is then transferred to CoM-SH by N5-methyl-H_4MPT:CoM-SHmethyltransferase (f) forming methyl-S-CoM. Methyl-CoM reductase (g) catalyzes the terminal reduction of methyl-S-CoM by two electrons from HS-HTP to CH_4. CoM-SS-HTP is the product of the terminal reaction, which is subsequently reduced by heterodisulfide reductase to regenerate the reduced forms CoM-SH and HTP-SH.

The acetotrophic pathway for acetate catabolism proceeds by initial 'activation' of acetate by formation of acetyl coenzyme A (CoA). *Methanosarcina* spp. synthesize acetyl-CoA by sequential activities of phosphotransacetylase and acetyl kinase (Figure 9 (b)). In contrast, activation of acetate to acetyl-CoA by *Methanosaeta* spp. is catalyzed in a single step by acetyl-CoA synthase. In both

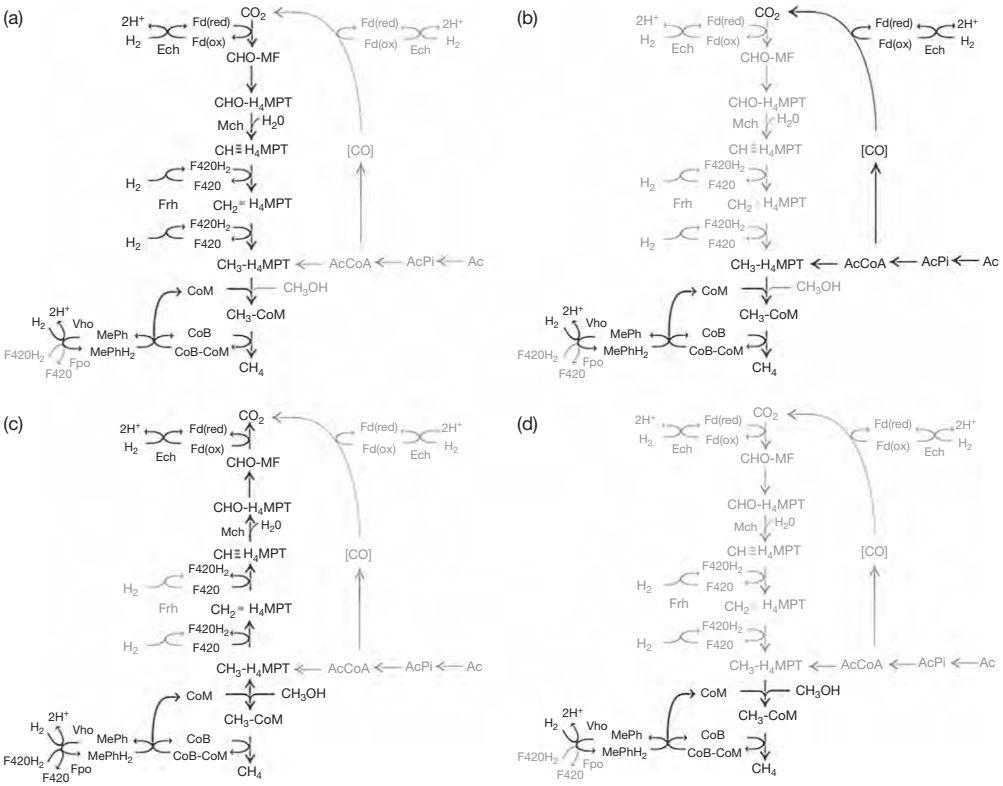

Figure 9 Four overlapping methanogenic pathways found in *Methanosarcina barkeri*. All four pathways share a common step in the reduction of methyl-CoM to methane; however, they differ in the pathway used to form methyl-CoM, and in the source of the electrons used for its reduction to methane. (a) Many methanogens reduce CO_2 to methane using electrons derived from the oxidation of H_2 via the hydrogenotrophic pathway. (b) In the aceticlastic pathway, acetate is split into a methyl group and an enzyme-bound carbonyl moiety. The latter is oxidized to CO_2 to provide the electrons required for reduction of the methyl group to methane. (c) In the methylotrophic pathway, C-1 compounds such as methanol or methylamines are disproportionated to CO_2 and methane; one molecule of the C-1 compound is oxidized to provide electrons for reduction of three additional molecules to methane. (d) In the methyl reduction pathway, C-1 compounds can be reduced using electrons derived from hydrogen oxidation. Ech, ferredoxin-dependent hydrogenase; Frh, F_{420}-dependent hydrogenase; Vho, methanophenazine-dependent hydrogenase; Fpo, F_{420} dehydrogenase; CHO-MF, formyl-methanofuran; CHO-H_4 MPT, formyl-tetrahydromethanopterin; $CH{\equiv}H_4$MPT, methenyl-tetrahydromethanopterin; $CH_2{=}H_4$MPT, methylene-tetrahydromethanopterin; CH_3-H_4MPT, methyl-tetrahydromethanopterin; CH_3-CoM, methyl-coenzyme M; CoM, coenzyme M; CoB, coenzyme B; CoM-CoB, mixed disulfide of CoM and CoB; Meph/$MephH_2$, oxidized and reduced methanophenazine; $F_{420}/F_{420}H_2$, oxidized and reduced factor 420; Fd(ox)/Fd(red), oxidized and reduced ferredoxin; Ac, acetate; Ac-Pi, acetyl-phosphate; Ac-CoA, acetyl-coenzyme A. Modified from Guss AM, Mukhopadhyay B, Zhang JK, and Metealf WW (2005) *Molecular Microbiology* 55: 1671–1680. Blackwell Publishing Ltd.

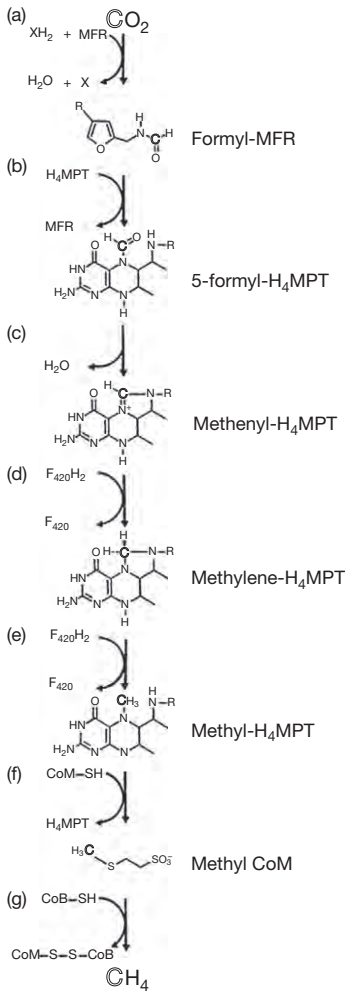

Figure 10 Methanogenic pathway in which CO_2 is sequentially reduced as coenzyme-bound intermediates to form CH_4. Details are described in the text. Reproduced with permission from Weiss DS and Thauer RK (1993) *Cell* 72: 819–822. Cell Press.

genera, cleavage of the C–C and C–S bonds of acetyl-CoA is then catalyzed by the acetyl-CoA decarbonylase/synthase complex, yielding enzyme-bound methyl and carbonyl groups. The complex contains CO:acceptor oxidoreductase, Co-β-methylcobamide: tetrahydropterine methyltransferase, and acetyl-CoA synthase activities. The five subunit complex consists of a two-polypeptide CO-oxidizing nickel/iron–sulfur component, a two-polypeptide corrinoid/iron–sulfur component, and a single polypeptide of unknown function. The nickel/iron–sulfur component catalyzes the cleavage of acetyl-CoA, the oxidation of the bound carbonyl to CO_2, and methyl transfer to the corrinoid/iron–sulfur component. The methyl group is sequentially transferred to H_4SPT by a currently unknown process and to HS-CoM by H_4MST:CoM-SH methyltransferases. Methyl-S-CoM is reductively demethylated to CH_4 by methylreductase as previously described. The enzyme-bound carbonyl group is oxidized to CO_2. *Methanosarcina* spp. and autotrophic methanogens growing on H_2/CO_2 synthesize acetate, presumably by reversing the direction of the acetyl-CoA decarbonylase/synthase complex in a reaction analogous to acetyl-CoA synthase in acetate-utilizing *Clostridia*.

Methylotrophic catabolism of methanol and methylated amines requires three polypeptides for the initial reaction. Methanol is catabolized by transfer of its methyl group to a corrinoid-binding protein, which is methylated by a substrate-specific methyltransferase, methanol:5-hydroxybenzinidazolyl (MT1). The methyl group is then transferred from the corrinoid protein to coenzyme HS-CoM by methylcobamide:CoM methyltransferase (MT2) (Figure 9(c)). Trimethylamine, dimethylamine, and monomethylamine each require a distinct corrinoid-binding protein, which is methylated by a substrate-specific methyltransferase. The methyl group is then transferred from the corrinoid protein to coenzyme HS-CoM by a common MT2 homologue. In contrast, catabolism of the methylthiols, dimethylsulfide, and methylmercaptopropionate is catalyzed by only two polypeptides: a corrinoid-binding protein tightly bound to a methylcobamide:CoM methyltransferase homologue of MT2. Methyl-S-CoM generated from methanol, methylated amines, and methylthiols is reduced to CH_4 in the methanogenic pathway, as has been described. A portion of the methyl groups generated from methylotrophic catabolism is oxidized in reverse sequence in a pathway identical to the CO_2 reduction pathway, after what appears to be a direct transfer of the methyl groups to H_4MPT. However,

the mechanism of this transfer is not yet known. This oxidative sequence generates electrons for the reduction of CoM-S-S-HTP in the methyl-S-CoM reductase system. Methylotrophic substrates can also be converted to methane by reduction of their methyl groups via hydrogen oxidation instead of partial oxidation of their methyl groups (Figure 9(d)).

Bioenergetics

The methanogenic *Archaea* derive their metabolic energy from autotrophic CO_2 reduction with H_2, formate, or secondary alcohols, cleavage of acetate, or methylotrophic dismutation of methanol, methylated amines, or methylthiols (Table 2). Currently, there is little evidence to convincingly support substrate-level phosphorylation. Evidence that redox reactions in the catabolic pathways are catalyzed, in part, by membrane-bound enzyme systems and are dependent upon electrochemical sodium ion or proton gradients indicates that electron transport phosphorylation is responsible for ATP synthesis. Both gradients generate ATP for metabolic energy via membrane ATP synthases. Several reactions in the methanogenic pathway are sufficiently exergonic to be coupled with energy conversion, including reduction of methyl-CoM ($-29\ kJ\ mol^{-1}$) and methyl transfer from H_4MPT/H_4SPT to HS-CoM ($-85\ kJ\ mol^{-1}$).

In addition, the oxidation of formyl-MF ($-16\ kJ\ mol^{-1}$) during methyl oxidation in the methylotrophic pathway and CO oxidation ($-20\ kJ\ mol^{-1}$) in the acetotrophic pathway are also exergonic.

During growth on H_2-CO_2 or H_2-methanol, the H_2:heterodisulfide oxidoreductase system catalyzes the H_2-dependent reduction of CoM-S-S-HTP to HS-CoM (Figure 9). Electron translocation across the membrane generates a proton gradient for ATP synthesis via an A_1A_0 ATPase. During methylotrophic growth of methanol or methylamines, heterodisulfide dehydrogenase is linked to F_{420} dehydrogenase instead of hydrogenase for CoM-S-S-HTP reduction and generation of a proton gradient. Reduced F_{420} is generated by methylene-H_4MPT dehydrogenase and methylene-H_4MPT reductase. A third type of heterodisulfide oxidoreductase system in the acetotrophic pathway is linked to acetyl-CoA decarbonylase via ferridoxin.

In addition to proton gradients, methanogens also use sodium ion gradients to drive endogonic reactions and generate ATP. During growth on H_2-CO_2 or acetate, vectoral Na^+ translocation is coupled to methyl-H_4MPT:CoM-SH methyltransferase. The Na^+ gradient generates ATP via an F_1F_0 ATPase. During growth on methanol and methylamines, a sodium ion gradient is formed by a Na^+/H^+ antiporter, and the methyl-H_4MPT:CoM-SH methyltransferase sodium pump is used in reverse to drive the endergonic methyl transfer from methyl-CoM to methyl-H_4MPT, for subsequent oxidation of the methyl groups.

Formyl-MFR dehydrogenase is involved in the bioenergetics of autotrophic growth on H_2-CO_2 or methylotrophic growth on methanol. During hydrogenotrophic growth, the exergonic CO_2 reduction to formyl-MF is driven by a hydrogenase-generated H^+ or Na^+ gradient. The reaction is reversed during CO_2 generation by methylotrophs, resulting in a net H^+ or Na^+ gradient.

Biosynthetic Pathways

Most methanogens assimilate carbon by CO_2 fixation or acetate uptake, and current evidence indicates that assimilation occurs via acetyl-CoA synthesis in a pathway analogous to the Ljungdahl–Wood pathway in acetotrophic clostridia. This conclusion is supported by (1) labeling studies; (2) the presence of acetyl-CoA synthase in autotrophic methanogens; and (3) the absence or partial absence of enzymes required for other routes of CO_2 fixation, including the Calvin cycle, reverse tricarboxylic acid (TCA) cycle, the serine pathway, and ribulose monophosphate cycle of the methylotrophs and the hydroxypropionate pathway of *Chloroflexus*. Acetotrophic methanogens likely utilize acetyl-CoA directly synthesized by acetate kinase and phosphotransacetylase in the catabolic acetate pathway. In the autotrophic methanogens, current evidence suggests that methyl-H_4MPT, formed by CO_2 fixation in the H_4MPT reductive pathway, likely donates a methyl group to a corrinoid protein containing an iron–sulfur center where it is subsequently transferred to HS-CoA by acetyl-CoA synthase. This pathway is a reversal of the acetotrophic pathway for acetate catabolism. During growth on methanol or methylated amines, methyl-H_4MPT, formed by direct methyl transfer to H_4MPT, is also the likely precursor for acetyl-CoA synthesis in methylotrophs.

Acetyl-CoA is reductively carboxylated by pyruvate oxidoreductase and enters the predominant biosynthetic pathways as pyruvate via an incomplete TCA. Both reductive and oxidative partial TCA cycles are detected in methanogens and their distribution among species is based on the bioenergetics of α-ketoglutarate synthesis. The oxidative incomplete TCA pathway requires a second acetyl-CoA and is restricted to acetotrophic methanogens, which readily synthesize acetyl-CoA from acetate via acetate kinase and phosphotransacetylase. In contrast, the reductive incomplete TCA pathway requires two additional reductive reactions and a reductive carboxylation to α-ketoglutarate synthesis. This pathway is commonly found in autotrophic methanogens, which have a steady reducing potential from H_2. Studies based on labeling and identification of specific enzymatic activities in selected species, thus far, indicate that biosynthesis of the aromatic aspartate, glutamate, histidine, pyruvate, and serine families of amino acids occurs by pathways similar to those described in the *Bacteria* and *Eukarya*. Genetic studies indicate that aromatic amino acids in some species are synthesized by the *de novo* via chorismate and by activation of aryl acids via the CoA thioesters indoleacetyl-CoA, phenylacetyl-CoA, and *p*-hydroxyphenylacetyl-CoA, which are then reductively carboxylated by indolepyruvate oxidoreductase to indolepyruvate, phenylpyruvate, or *p*-hydroxyphenylpyruvate, respectively. Biosynthesis of hexoses, required as precursors for cells walls and reserve polysaccharides, such as glycogen and trehalose, proceeds via gluconeogenesis from phosphoenolpyruvate. Pentoses are formed from fructose 1,6-phosphate and glyceride 3-phosphate via transketolase and transaldolase in *Methanobacterium* and *Methanococcus*. In contrast, *Methanospirillum* forms pentoses by oxidation of glucose-6-phosphate. Labeling studies in *Methanospirillum* and *Methanobacterium* indicate that pyrimidine and purines are synthesized by the expected pathways and they also

have a purine salvage pathway. The polar core lipids, which include diphytanyl glycerol ethers and diphytanyl diglycerol tetraethers, are synthesized via the mevalonate pathway for isoprenoids.

Molecular Genetics

Genome Structure

The methanogens have a single circular chromosome that ranges from 1.6×10^6 to 5.7×10^6 bp in size. The percentage guanosine + cytosine (%GC) ranges from 23% to 62% and high %GC content is not always associated with high growth temperatures. Tranposable elements are detected in all methanogen genomes. A three-way comparison between *M. acetivorans*, *Methanosarcina barkeri*, and *Methanosarcina mazei* reveals that genomes are well conserved with respect to the region proximal to the origin of replication, but the second half of the genomes most distant from the origin are disordered and marked by increased transposase frequency and a decrease in gene density and conserved colocalization (synteny). The apparent genome plasticity might contribute to these species' ability to adapt to a broad range of environments as a result of genome elongation and enrichment for favorable phenotypes. CRISPRs (clustered regularly interspaced short palindromic repeats), which can cause large-scale rearrangements, and all four known CRISPR-associated, or *cas*, genes have been detected in methanogen genomes, but they do not appear to be associated with large-scale DNA rearrangements.

Extrachromosomal DNA has been detected in methanogens in the form of large extrachromosomal elements, plasmids, viruses, and virus-like particles. At this time, two large extrachromosomal elements have been detected in *Methanocaldococcus jannaschii* (58.4 kb) and *M. barkeri* (36 kb) and 12 smaller plasmids, ranging in size from 4.4 to 20 kb, have been detected in four genera of the methanogenic *Archaea*. The large extrachromosomal element in *M. jannaschii* encodes a putative minichromosome maintenance (MCM) helicase and the element in *M. barkeri* encodes a homologue of the DNA replication initiation protein, Cdc6, in a region of highly repetitive sequence, which suggests replication of the large extrachromosomal elements is synchronous with cell division. In contrast homologues of site-specific recombinase and plasmid DNA replication initiator protein RepA detected in *M. acetivorans* suggests that this plasmid replicates by a rolling circle mechanism associated with many bacterial plasmids and phage. Other open reading frames annotated in methanogen plasmids include a type II DNA restriction–modification system, glycosylase transferase, and recombinases. However, genes involved in central metabolism have not yet been detected and the functions of the most methanogen plasmids are cryptic at this time. Lytic viruses have been detected in three strains of *Methanothermobacter* and one strain of *Methanobrevibacter smithii*. These viruses show a varying range of host specificities. A temperate virus-like particle has been isolated that can integrate into the chromosome of *Methanococcus voltae*.

In contrast to the similarities of DNA structure in methanogenic *Archaea* and *Bacteria*, DNA-modifying proteins in the methanogens share features common to both the *Bacteria* and *Eukarya* (Table 3). All cells must package their genomic DNA within the limited space of the cells. Although the *Bacteria* do not appear to have a conserved mechanism for DNA packaging, all *Eukarya* and some *Archaea* (one crenarchaeon and all Euryarchaeota) chromosomal DNAs are compacted by histones into defined structures called nucleosomes, which are further assembled to form chromatin. Methanogen histones form a structural motif called a histone fold, but lack N-and C-terminal sequences associated with higher order nucleosome polymerization and undergo modification in eukaryotes. Unlike the four histones in eukaryotes, methanogens typically encode 1–6 histones. Interestingly, *Methanosarcina* spp., which have the largest genomes, only encode one histone and it is not clear how this affects dimer polymerization. Archaeal histone-like proteins increase the melting temperature of linear DNA *in vitro* by as much as 25 °C and likely protect DNA from heat denaturation *in vivo*. Reverse gyrase, also detected in *Methanothermus fervidus*, may contribute to heat resistance of DNA by creating stable positive supercoils in inter-histone regions. Histone-like proteins isolated from mesophilic *Methanosarcina* species cause

Table 3 Molecular features of three phylogeneic domains

Feature	*Eukarya*	*Bacteria*	*Archaea*
Genome	Multiple linear	Single circular[a]	Single circular[a]
Chromatin	Histone-mediated	No conserved mechanism	Histone-mediated
Extrachromosomal DNA	Plasmids, viruses, IS units	Plasmids, viruses, IS units	Plasmids, viruses, IS units
DNA polymerase	Families A[b], B, and X	Families A, B, and C	Families B and X
RNA polymerase	Three classes, complex	One class $\beta\beta'\alpha_2\sigma$	One class, complex[c]
Gene structure	Single gene	Multiple gene operon	Multiple gene operon
Transcription promoter	TATA box	−35/−10 sequence	TATA box
Transcription terminator	AAUAAA sequence	Intrinsic, σ-dependent	Intrinsic, oligo T
Ribosomal RNA	28S-5.8S/18S	23S-5S/16S	23S-5S/16S
Translation initiation	5′ cap	Ribosomal-binding site	Ribosomal-binding site
Initiator transfer RNA	Methionine	Formylmethionine	Methionine

[a]Some species have multiple copies of the same genome.
[b]Mitochondrial.
[c]AB′B″C based on homology to eukaryal RNA polymerase II.

concentration-dependent inhibition or stimulation of gene transcriptions *in vitro*, which suggests that they might also have a role in gene regulation. Direct evidence that *M. jannaschii* histones does not have posttranslational modifications indicates that gene regulation by histone modification is unlikely, but this does not rule out the possibility that different sequence affinities might regulate genes by selective proximity of nucleosomes to regulatory regions of DNA.

DNA Replication, Repair, Modification, and Metabolism

Unlike replication in the *Bacteria*, which starts at a single origin, *in silico* analysis indicates there are up to three origins in some species of methanogens. Multiple origins have been experimentally determined in the *Crenarchaea*, which suggests that some species of methanogenic *Archaea* may also have multiple origins. At least one of the origins lies immediately upstream of genes encoding homologues of the eukaryal DNA replication initiation proteins Orc and Cdc6. The proximity of the replication factors to the origin suggests that the methanogenic *Archaea* use a bacterial-like mode of replication, but with eukarya-like machinery. DNA replication proteins in methanogens are similar to those of other members of the euryarchaeal kingdom and most similar to eukaryal enzymes. Studies on the replication machinery of a number of methanogenic *Archaea* show that most have homologues of the eukaryotic initiation proteins Orc/Cdc6, which are thought to function in origin recognition and helicase loading. All methanogenic *Archaea* also contain at least one homologue of the replicative helicase, MCM. The replicative machinery also includes the proteins needed for the elongation phase including primase, DNA polymerases (B-and D-type), the PCNA processivity factor and its loader RFC, the GINS complex, thought to function in coordination of leading and lagging strand replication, and ligase, Fen1 and RNase H needed for Okazaki fragment maturation.

DNA repair mechanisms have also been identified in methanogens. Annotation of genome sequences reveals putative genes for alkyltransferase, base-excision repair, photoreactivation, nucleotide excision repair, *trans*-lesion synthesis, mismatch repair, and homologous recombination among species of the methanogenic *Archaea*. A photoreactivation system in *Methanothermobacter thermoautotrophicus* is mediated by class II photolyase with homology to metazoan photolyases. Homologues of putative DNA include eukaryal repair proteins RAD2, RAD25, and RAD51; and bacterial DNA repair proteins uvrABC, RadA, mutL, and mutS. Although our understanding of DNA repair in the *Bacteria* and *Eukarya* is well advanced, there has been comparatively little functional analyses of these processes in the methanogenic *Archaea* at the present time.

Gene Structure and Transcription

The organization of methanogenic genes in tightly linked clusters is similar to the operon configuration found in the *Bacteria*. As in the *Bacteria*, the archaeal operons are transcribed from an upstream promoter into polycistronic RNAs such that individual mRNA molecules encode multiple genes and transcription and translation are coupled. However, archaeal genes are transcribed by a multicomponent RNA polymerase that is structurally homologous to eukaryal RNA polymerase and recognizes a promoter with high sequence homology to the consensus TATA motif found in eukaryal promoters and B recognition element (BRE) immediately upstream. Unlike the variable upstream distance of eukaryal promoters, the archaeal promoter element is located at a consistent distance, approximately 20–30 bp upstream from the transcription initiation site, a range similar to the conserved −35 bp region observed in the *Bacteria*. Site-directed deletion studies conducted with methanogenic *Archaea* by *in vitro* techniques indicate that efficient transcription and start-site selection is dependent upon a TATA promoter. This arrangement closely resembles the core structure of promoters recognized by RNA polymerase II (RNAP II) in the *Eukarya*. Purified archaeal RNAPs from *M. thermoautotrophicus* and *M. voltae* fail to initiate site-specific transcription without the addition of a protein that binds to the TATA promoter (TBP) and transcription factor B (TFB). Yeast and human TATA-binding proteins can substitute for TBP in a *Methanococcus*-derived archaeal cell-free transcription system, indicating that they are functionally homologous. An additional gene that has sequence similarity to a eukarya-like transcription factor E (TFE) has also been identified in the methanogenic *Archaea*. Site-specific transcription initiation in the *Archaea* is analogous to that in the *Eukarya*. The mechanism involves binding of the TBP to the archaeal TATA box, stabilization of the complex by association of TFB with TBP and interaction with the BRE, and recruitment of the RNAP TFE facilitates binding of TFB to RNAP and further stabilizes the initiation complex by closing the RNAP clamp on the DNA strand.

Mechanisms of transcriptional regulation in some highly regulated genes have been reported. Regulatory protein Ptr2 in *M. jannaschii*, an archaeal homologue of the bacterial leucine-responsive regulatory protein Lrp, is a transcriptional activator for expression of ferridoxin A and rubredoxin 2. However, unlike bacterial activators that interact with RNAP, Ptr2 activates transcription by recruiting transcription factor. Negative regulators have also been described in the methanogens. TrpY is an allosteric effector that both autoregulates itself and regulates transcription of the tryptophan biosynthesis operon. TrpY forms a dimer that inhibits TrpY expression by blocking RNAP recruitment. When tryptophan binds to TrpY a conformational change increases its affinity for an adjacent, divergent operator for the tryptophane encoding operon (*trp*), thereby competing with TBP binding to the promoter. NrpR regulates nitrogen fixation by repressing the operon encoding nitrogenase (*nif*) during growth with alternative nitrogen sources ammonia or alanine. In the absence of ammonia or alanine, an indicator of intracellular nitrogen level, 2-oxyglutarate, binds to NrpR and reduces its affinity for the *nif* operator, thereby derepressing transcription of the *nif* operon. Regulators involving the 5′ untranlated region (5′-UTR) of the mRNA have also been reported in the methanogens. A systematic approach using transcriptomic modeling of *M. maripaludis* under different growth condition in a chemostat identified two novel transcription factors in the regulation of phosphate-dependent repression of formate dehydrogenase.

A 113 nucleotide 5′-UTR upstream of an RNA helicase in the Antarctic species *Methanococcoides burtonii* contains bacteria-like regulatory elements that appear to be involved in cold adaptation. Long 5′-UTRs associated with methanol methyltransferase and carbon monoxide dehalogenase in *Methanosarcina* spp. have also been shown to be involved in regulation. Although the mechanisms of regulation have not been determined, five genes designated msrABCDE – identified by deletion analysis – are involved in repression or induction of methanol methyltransferase isozymes. In the case of carbon monoxide dehalogenase, expression is regulated in response to substrate by at least two mechanisms: differential transcription initiation and early elongation termination near the 3′ end of a 371-bp leader sequence. Genes encoding three distinct TBPs have been detected in the *M. acetivorans* genome, which raises the possibility that gene regulation is mediated by differential TBP–promoter pairing. Only TBP-1 is expressed during growth of *M. acetivorans*, but this does negate the possibility that the different TBPs might be differentially expressed under different growth conditions, such as a response to stress. This possibility is supported by evidence of differential TBP–promoter pairing in the closely related halophilic *Euryarchaeota*. Generally, transcription is terminated following an inverted repeat sequence located downstream of methanogen genes. Transcription termination sites are similar to the ρ-independent terminators in the *Bacteria*, and likely form a stem-loop secondary structure to mediate termination. A second type of transcription terminator, which consists of a single or several tandemly arranged oligo-T sequences, is found in hyperthermophilic methanogens. The occurrence of this terminator in hyperthermophiles suggests that the stem-loop structures characteristic of σ-independent promoters may be unstable at higher growth temperatures.

RNA Structure and Translation

The stable RNAs transcribed by methanogen genes have been investigated in some detail. Methanogen ribosomes resemble bacterial ribosomes. They are composed of two protein subunits of 30S and 50S and three rRNA components of 23S, 16S, and 5S, which, assembled, yield a ribosome of 70S. Archaeal and bacterial ribosomal proteins are functionally homologous and can be interchanged to create an active ribosome *in vitro*. Genes encoding rRNA are arranged in the order 16S-23S-5S and the number of operon copies varies in number from 1 to 4. The organization and order of genes encoding methanogen ribosomal proteins also resembles that found in the *Bacteria*. Methanogen transfer RNAs (tRNAs) contain the sequence 1-methylψ CG substituted for the sequence TψCG, typically found in the arm of bacterial and eukaryal tRNAs. Introns have been detected in genes encoding tRNA. Several methanogenic *Archaea* lack cysteinyl-tRNA synthetase. In these cases, a class II-type *O*-phosphoseryl-tRNA synthetase specifically forms Sep-tRNACys, which is then converted to Cys-tRNACys by Sep-tRNA:CystRNA synthase. Several methanogenic *Archaea* also synthesize two nonstandard amino acids: selenocysteine, found also in *Bacteria* and animals, and pyrrolysine, which is thus far unique to methanogenic *Archaea* with genes encoding dimethylamine or trimethylamine methyltransferases. Both selenocysteine and pyrrolysine are encoded by the RNA stop codons UGA and UAG, respectively. However, unlike selenocysteine that is synthesized on the charged tRNA, pyrrolysine is charged onto a pyrrolysine-specific tRNA.

Some methanogen mRNAs have poly-A^+ tails, but, as in the *Bacteria*, they average only 12 bases in length. Protein-encoding genes employ the same genetic code as *Bacteria* and *Eukarya*, and codon preferences reflect the overall base composition of the genome and the level of gene expression. The codon ATG is frequently used as an initiation codon as well as GTG and TGG. A ribosomal-binding site located upstream of structural genes, when transcribed, is complementary to the 3′ terminal sequence of methanogen 16S rRNA. Compared with *Bacteria* and *Eukarya*, there is little information on the mechanisms of translation based on biochemical experimentation. Computational analyses of methanogen genomes have identified six putative initiation factors, two elongation factors, and one release factor that are most similar to eukaryal homologues. Inteins have been identified by genomic sequencing of *M. jannaschii*, which suggests that protein splicing occurs in methanogens. RNA processing by endonucleolytic cleavage 12–16 nucleotides upstream of the translation start site of 30 protein-coding genes was identified in *Methanocaldococcus jannaschii*. In addition, evidence has been found for phosphorylation of proteins at a tyrosine residue, which is a mechanism of posttranslational control in the *Bacteria* and *Eukarya*.

Genomics and Gene

Function analysis

The genomes of strains from over 20 methanogenic genera have been completely sequenced and annotated. In general, similar genes are found for the CO_2-reducing catabolic pathways in all four genomes. Genes encoding multiple methyltransferases and acetyl-CoA decarbonylase/synthases are found only in the genomes of *Methanosarcina* spp., which is consistent with the ability of this species to also grow by methylotrophic and aceticlastic pathways. The large genome size of *Methanosarcina* spp. likely reflects these species' ability to use a greater range of substrates, adapt to a broader range of environments, and form complex multicellular structures compared with the more limited capabilities of the hydrogen-utilizing species. In contrast, the hyperthermophiles are more 'minimalist' exhibiting a paucity of genes encoding proteins for signaling and gene regulation, *grp*E–*dna*J–*dna*K heat-shock operon, proteasome–chaperonin, several DNA repair proteins, DNA helicases, nitrogenase subunits, ribonucleotide reductase, and proteases. Despite their sensitivity to oxygen, superoxide dismutase and catalase have been identified in methanogen genomes. Superoxide dismutase and catalase activities have been confirmed and characterized in methanogens, and genes encoding both enzymes are transcriptionally upregulated in *M. barkeri* upon oxidative stress. Interestingly, a putative cytochrome *d* has been identified in *Methanosarcina* genomes, but its role has not been determined. Overall, the majority of archaeal open reading frames with similarity to bacterial sequences include genes for small molecule biosynthesis, intermediary metabolism, transport, nitrogen

fixation, and regulatory functions. Archaeal open reading frames with similarity to eukaryal sequences include genes for DNA metabolism, transcription, and translation. The presence of Cdc6 homologues and histones suggests that DNA replication initiation and chromosome packaging is eukaryal, but detection of cell division gene *ftsZ* suggests that bacteria-type cell division occurs. Additional unique features include an archaeal B-type DNA polymerase with two subunits, putative RNAP A′ subunits that suggest possibility of additional mechanisms for gene selection, and two introns in the same $tRNA^{Pro}$ (CCC) gene that establishes a new precedent. A genomic-scale metabolic model was created for *M. acetivorans* that identified key knowledge gaps and accurately predicted wild-type phenotypes and knockout lethality as a tool for hypothesis testing by genetic analyses.

The availability of genome sequences of methanogens and current activities to sequence others make the development of archaeal gene-transfer systems essential for confirmation of gene function. Two gene-transfer systems have been developed for species of *Methanococcus* and *Methanosarcina*. Both systems utilize hybrid shuttle vectors derived from native archaeal plasmids. Since plasmids occur in low copy number in the methanogens, the DNA to be transferred is ligated into the vector, which is then amplified in *E. coli* with ampicillin as a selectable marker. The modified plasmid is then transferred into the methanogen using transformation methods that employ either cells made competent for DNA uptake by polyethylene glycol treatment (*Methanococcus* sp.) or uptake of DNA embedded in liposomes (*Methanosarcina* spp.). A second selectable marker in the plasmid, such as *pac* (puromycin acetyl transferase) controlled by a highly expressed methanogen promoter *mcr* (methyl CoM reductase), provides selection in the methanogen. Both autonomously replicating plasmids for introducing specific phenotypes and integration plasmids for disrupting genes by homologous recombination have been designed. These systems are highly efficient, yielding 10^7–10^9 transformants per μg DNA. Methods for gene disruption based on homologous recombination and transposon-mediated mutagenesis, gene reporters for expression analysis, and protein overexpression have also been developed. One current limitation is the limited number of selectable and phenotypic markers that are currently available. This has been addressed by the development of a 'markerless' system in which the genetic marker is removed by homologous recombination after selection. A transducing phage has been isolated from *M. thermoautotrophicus*, but it is not currently useful for gene transfer because of its limited burst size (~6/cell). Conjugation has not been observed in methanogens. Although gene-transfer systems are somewhat limited at this time, development of new and more sophisticated systems is ongoing.

Conclusion

The methanogenic *Archaea* have a pivotal role in the global carbon cycle by complementing aerobic processes that ultimately lead to the oxidation of organic carbon to CO_2. However, a steady increase in the levels of atmospheric CH_4 that has coincided with the increase in the human population is a cause for concern, since CH_4 is a greenhouse gas. Methane's contribution to global warming results from its high infrared absorbance and its role in complex chemical reactions in the stratosphere that affect the levels of ozone. Increased waste disposal activities, such as landfills, are a significant source of atmospheric CH_4. Another significant source results from agricultural activities, such as the increased use of domesticated ruminants for production of meat and dairy products and the increased development of rice paddies. Understanding the properties of methanogens and the roles they have in the global carbon cycle will have important implications in addressing the issue of global warming as the human population increases. The ability of geological hydrogen-utilizing CO_2-reducing methanogens to serve as primary producers of reduced carbon compounds in deep subsurface bedrock and submarine vents has significant implications also in astrobiology and the potential for extraterrestrial life.

The application of methanogens in biotechnology has been largely limited to waste management, which is often coupled to limited biogas production. Although the petroleum crisis of the 1970s led to an interest in methanogenic biogas production by fermentation of sources ranging from agricultural products to marine kelp, cost-effective technologies were never fully developed as interest waned with the drop in petroleum prices. Recent rises in petroleum prices and international concern about global warming have renewed interest in alternative energy sources. Methanogenic biogas is an attractive energy source for applications such as heating and electric generation plants as it requires minimal treatment and can be distributed in current infrastructures used for natural gas. Unlike petroleum, biogas is generated from organic waste and CO_2 released from combustion of biogas does not cause a net increase of greenhouse gases into the atmosphere. Similarities in the DNA expression machinery of mesophilic methanogens and *Eukarya* combined with recent advances in methanogen genetic systems provides a potential platform for functional analyses of mammalian gene and proteins. Other potential applications for methanogens include the production of novel pharmaceuticals, corrinoids, and thermo-stable enzymes. To date, methanogens have yielded only a few restriction endonucleases as commercial products. However, significant advances in our understanding of the physiology and biochemistry of methanogens over the past two decades, combined with the recent developments in gene transfer systems and advances in genome sequencing, make the application of methanogens for biotechnology more likely in the near future.

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Methods, Philosophy of

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Glossary

cause A condition that is necessary in the circumstances for bringing about an event.
model An idealized, usually causal, mechanism or collection of mechanisms that account for a biological outcome.
scientific method A collection of general principles of scientific inquiry, test, and evaluation.
strong inference A systematic attempt to devise mutually exhaustive alternative hypotheses and subject them to crucial experimental tests.
theory Generally, a collection of related principles that explain a domain.

Abbreviations

BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
IPTG	isopropylthiogalactoside
ONPF	*o*-nitrophenyl-fucoside
PrP	prion protein
PrP^{c}	cellular PrP
PrP^{sc}	scrapie PrP
TSE	transmissible spongiform encephalopathy

Defining Statement

Philosophy of method is a branch of the philosophy of science that investigates scientific methodology. Though 'scientific method' is largely implicit in microbiology, microbiologists do implement a number of methods of scientific inquiry that have been analyzed by philosophers, such as the methods of difference and comparative experimentation. In addition, these methods are put into practice against a backdrop of more general philosophical assumptions concerning scientific progress, scientific realism, scientific inference, and the nature of empirical evidence. The philosophy of method is best described using particular microbiological examples illustrating the application of specific methods of experimental inquiry.

Scientific Method in General

The search for a method that would articulate and codify the principles of scientific inquiry has a long history. Philosophers, in particular, have proposed a number of different approaches to scientific method, beginning with Aristotle. In more modern times, Descartes and Francis Bacon wrote on the subject, but the study of scientific method received its most systematic treatments in the work of the nineteenth-century philosophers and scientists William Whewell, Stanley Jevons, and John Stuart Mill. It was the last who forcefully re-presented the methods of agreement, difference, concomitant variation, and other methods that continue to have an influence among contemporary philosophers. Later in this article, some of the more current positions of such philosophers as Karl Popper and Thomas Kuhn will also be cited as contributing in important ways to this subject.

Though some philosophers studying scientific method, such as Francis Bacon, hoped to furnish a method of 'discovery', the majority of philosophical thinking in this area has tended to follow Popper's view that states

The initial stage, the act of conceiving or inventing a theory, seems to me neither to call for logical analysis or be susceptible of it. The question how it happens that a new idea occurs to a man – whether it is a musical theme, a dramatic conflict, or a scientific theory – may be of great interest to empirical psychology; but it is irrelevant to the logical analysis of scientific knowledge. The latter is concerned not with *questions of fact* but only with questions of *justification* or *validity*.

This view of Popper's has come under attack by a number of recent philosophers and artificial intelligence theorists. These proponents of a logic of scientific discovery have been able to develop discovery programs in a number of domains, including organic chemistry and mathematics, and active research in this area is continuing. Advances in this area are quite technical and

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typically domain specific, and, thus, it will not be possible in the context of the present article to discuss in any detail these various attempts to develop a logic of discovery; this selection will primarily be concerned with the logic of testing and of proof.

It should be noted that 'scientific method' has been largely 'implicit' in the writings of microbiologists. There are some occasional reflections on methodological issues, for example, Koch's development of his 'postulates' for assessing the causation of disease by a bacteriological agent and Peter Duesberg's recent appeals to methodological issues in connection with the cause(s) of HIV disease. Another interesting methodological debate is the recent controversy about the causative agent – putatively a prion – of the spongiform encephalopathies, such as 'mad cow disease'. Though these explicit debates are the exception, microbiologists do put into practice various philosophies of method, and it is the function of the present article to make explicit what figures in a largely silent way in microbiology.

Scientific Method in Biology; Objective Truth and Scientific Progress

Theory and Experiment in Physics Compared and Contrasted with Biology

Biology in general, and microbiology in particular, shares a number of common methodological assumptions with all the natural sciences, including the physical sciences. The life sciences, however, also posses some special methodological features that will be useful to distinguish between them. Because each of the natural sciences seeks reliable general knowledge, the methods mentioned briefly in 'Scientific method in general', such as the methods of agreement and difference, are widely employed in the life and nonlife sciences. These methods can be thought of as attempting to discern the causal structure of the world, and, in their application, scientists endeavor to identify possible confounding factors that can lead to spurious inferences about causes and effects. Thus, all natural scientists attempt to control for interfering and extraneous factors, frequently by setting up a control comparison or a control group. Such controls are a direct implementation of what Mill termed the method of difference and Claude Bernard the method of comparative experimentation, which will be reviewed in some detail in 'Specific but philosophically general methods of experimental inquiry'.

In the biological sciences, including microbiology, some added complexity is frequently encountered due to biological diversity and the number of systems that strongly interact in living organisms. It is the backdrop of evolutionary theory that allows one to understand why there can be both extensive and subtle variation in organisms and mechanisms, as well as why there may be narrowly defined precise mechanisms that are (nearly) biologically universal, such as the genetic code. Variations due to meiosis, mutation, and genetic drift, for example, predict that extensive variation should occur most frequently in evolving populations where strong selection pressures toward precise and universal mechanisms are not present. Alternatively, where variations would almost certainly be lethal, there exist strong pressures toward the fixation of (nearly) universal mechanisms. Thus, evolutionary theory, at a very general level, explains some of the specific and general features of other theories and models in microbiology.

Because broad and subtle variations may be encountered in microbiology, special attention frequently needs to be given to ensuring the (near) identity of the organisms under investigation, except for those differences that are the focus of the scientist's inquiry. Accordingly, the development of special strains of organisms and the identification and classification of populations (and subpopulations) assume an urgency that can be frequently ignored in the physical sciences, where, for example, all electrons are identical. It is in connection with the satisfaction of these urgent needs that the development of the techniques of pure culture becomes so vitally important.

Organism and subsystem variation do not only influence the experimental arena, but also figure in what constitutes the biological analogue of 'theory'. Genetic and environmentally produced variation frequently results in the need of biologists (and microbiologists) to focus on 'model' organisms and on prototypical systems, which are highlighted against a backdrop of similar but different organisms and mechanisms. Biologists, thus, often find themselves practicing what a 1985 National Academy of Sciences report called 'many–many modeling' in a complex 'biomatrix'. The report, which re-presented the results of a series of workshops directed by H. Morowitz, introduced a notion of the 'matrix' of biomedical knowledge: "The workshops demonstrated that the results of biomedical research can be viewed as contribution to a complex body, or matrix, of interrelated biological knowledge built from studies of many kinds of organisms, biological preparations, and biological processes at various levels." From within this multidimensional matrix, many–many modeling occurs, in which analogous features at various levels of aggregation are related to each other across various taxa. The report notes: "An investigator considers some problem of interest – a disease process, some normal physiological function, or any other aspect of biology or medicine. The problem is analyzed into its component parts, and for each part and at each level, the matrix of biological knowledge is searched for analogous phenomena.... Although it is possible to view the processes involved in interpreting data in the language of one-to-one modeling, the investigator is actually modeling back and forth onto the matrix of biological knowledge."

Method and Scientific Progress

P. Collard, in his historical monograph on the development of microbiology, distinguished four historical eras. The period of 'speculation' comprised the epoch from about 5000 BC until the work of the microscopist Leeuwenhoek around 1675 ushered in the era of 'observation'. Beginning in the mid-nineteenth century, the era of 'cultivation' began with Pasteur's studies of fermentation and Koch's development of pure culture methods employing solid media. Collard suggests that the modern physiological era, which is dominated by the elucidation of bacteriological and biochemical mechanisms, commenced about 1900. Implicit in Collard's chronology is the inference that the more recent methodologies are more scientifically sound and objective, in contrast to the earlier, more speculative inquiries in microbiology.

It may initially appear odd to scientifically trained microbiologists to even raise the question of whether there is any 'objective truth' in science. Over the past 25 years or so, however, serious questions have been raised about the nature of scientific 'truth' and 'progress' by Kuhn in his influential work, some philosophers of science (e.g., Feyerabend), and several sociologists of science (e.g., Latour and Woolgar), and a brief discussion of these issues may be helpful.

Truth and Progress in Science

Throughout this century, philosophers of science have engaged in vigorous disputes about the nature of scientific truth. An examination of the history of science in general, and microbiology in particular, would lead one to the conclusion that there have existed many 'good' scientific theories that have not survived to the present day. Kuhn's characterization of scientific revolutions provides a superb (if ultimately misleading) introduction to examples of these discarded theories. Such theories have gone through the stages of discovery, development, acceptance, rejection, and extinction. Further examination of extinct theories, however, would show that they possessed a number of beneficial consequences for science.

Incorrect and literally falsified theories have several explanatory functions, and have systematized data, stimulated further inquiry, and led to a number of important practical consequences. For example, the false Ptolemaic theory of astronomy was extraordinarily useful in predicting celestial phenomena and served as the basis for oceanic navigation for hundreds of years. Newtonian mechanics and gravitational theory, which is incorrect from an Einsteinian and quantum mechanical perspective, similarly served both to intelligibilize the world and to guide its industrialization. In the biological sciences, the false evolutionary theory of Lamarck systematized and explained significant amounts of species data, and in microbiology, Pasteur's false nutrient depletion theory of the immune response, nonetheless, served as the background for the development of the anthrax vaccine. Such examples lead one toward what has been termed an 'instrumentalistic' analysis of scientific theories (or hypotheses).

The basic idea behind such a position is to view theories and hypotheses as 'tools' and not as purportedly true descriptions of the world. For a thoroughgoing instrumentalist, the primary function of scientific generalizations is to systematize known data, to predict new observational phenomena, and to stimulate further experimental inquiry. Such an approach bears strong analogies to the 'constructivist' program of several sociologists of science, such as Latour and Woolgar, who conceive of many biomedical entities (e.g., neuroendocrine releasing factors) as being 'constructed' rather than as 'discovered'. To constructivists, such putatively 'real' microbiological substances are actually 'constructed socially' (as conceptualized entities), as part of a complex give-and-take among laboratory machine readings and discourse among laboratory scientists.

Though such positions as Kuhnian relativism, instrumentalism, and social constructivism are *prima facie* attractive, they are inconsistent with other features of scientific inquiry. The relation of scientific theory to 'observations' in the laboratory is exceedingly complex. In spite of the weblike connections between theoretical postulation and laboratory experiments, however, multiple empirical constraints on theoretical speculations generate a stability and 'stubbornness' of both data and theory, to use Galison's terms. For example, scientists view the distinction between what they term 'direct' and 'indirect' evidence as important. Even though, as shall be seen in the following, the distinction is relative, it is, nonetheless, significant to note that scientists 'behave' as if the distinction is important, and that 'direct evidence' would seem to support a more 'realistic' analysis of scientific theories (or hypotheses). A realistic type of alternative to the instrumentalist position would characterize scientific theories as 'candidates' for 'true' descriptions of the world. Though not denying the importance of theories' more instrumentalistic functions, such as prediction and fertility, the realist views these features as partial indications of a theory's 'truth'. The history of recent philosophy of science and the more general discipline of 'science studies' has seen an oscillation between these realist and instrumentalist positions, as well as the development of some interesting variants of these positions. The relativist reviews have been strongly criticized, especially by realismdefending scientists, the give-and-take of which has been described as the 1990s 'science wars'. This, however, is a subject that cannot be pursued within the limitations of this article.

Suffice it to say that, in spite of the variation in positions that scientists and philosophers of science have taken about the nature of ultimate scientific truth, these varying positions do not disagree about the need to report accurately and faithfully what the scientist has observed or reasoned to in his or her investigations.

Specific but Philosophically General Methods of Experimental Inquiry

This section provides a more systematic and in-depth review of the classical methods of experimental inquiry that were very briefly introduced in 'Scientific method in general'. It begins with John Stuart Mill's characterization of these experimental methods, since Mill's analysis is 'provisionally' adequate for our purposes and is also well known.

'Mill's' Methods

Mill's first experimental method is known as the 'method of agreement'. This was as follows:

> If two or more instances of the phenomenon under investigation have only one circumstance in common, the circumstance in which alone all the instances agree, is the cause (or effect) of the given phenomenon.

It should immediately be pointed out that this method is most difficult to satisfy in microbiological inquiry because of the complexity of organisms. To vary every relevant character save one is largely unrealizable, though it may occasionally be done in very narrowly circumscribed investigations in molecular biology, for example, in genetic codon analysis.

The applicability of the method of agreement is also suspect in complex biological organisms for two other general reasons, originally pointed out by Mill in his *System of Logic*. These general reasons were referred to by Mill as the 'plurality of causes' and the 'intermixture of effects'. In the former, Mill noted that in those complex cases where the same consequence could be the result of different jointly sufficient antecedents, the method of agreement yielded 'uncertain' conclusions. In complex and adaptable biological organisms, such a situation is often likely. The problem of the 'intermixture of effects' produced even greater difficulties for the method of agreement. This occurred in cases where effects were 'intermixed', that is, either they were not separable into clearly defined components, or the result was emergent and incalculable on the basis of the causal components. As examples of these two species of the intermixture of effects, Mill cited the vector addition of forces in mechanics and the production of water from oxygen and hydrogen. Mill also believed that biological properties were emergent with respect to the physicochemical, with attendant difficulties. Suffice it for now to note that the method of agreement possesses serious imperfections in its application to complex circumstances, as are found in biological organisms.

Mill's methods also include, besides the better known method of agreement and the method of difference, the method of residues and the method of concomitant variations. The latter, according to Mill, may be stated in canonical form as follows:

> Whatever phenomenon varies in any manner whenever another phenomenon varies in some particular manner, is either a cause or an effect of that phenomenon, or is connected with it through some fact of causation.

This method has important uses in biology and medicine, and an illustration of it will be considered in the following section on specific implementation of the methods. Again, as in the case of the method of agreement, the complexity of biological organisms makes a simple and straightforward application of the method of concomitant variations uncertain. When attempting to determine a major causal role which, say, an entity plays in a system, a simple recording of concomitant variations is almost always insufficient.

The method of residues is likewise suspect in its simple application to biological and microbiological causation. This method, in which one "subduct[s] from any phenomenon such part as is known by previous inductions to be the effect of certain antecedents", and considers "the residue of the phenomenon... [to be] the effect of the remaining antecedents", is similarly difficult to apply in complex systems in which the interfering residues are likely to be very large, both in number and in their interactions.

The method of difference supplemented in a number of cases with a statistical interpretation, which bears certain analogies to Mill's methods of concomitant variation, is often the most appropriate method of experimental inquiry to establish empirically and directly scientific claims. The method of difference was stated by Mill in the following manner:

> If an instance in which the phenomenon under investigation occurs, and an instance in which it does not occur, have every circumstance in common save one, that one occurring only in the former, the circumstance in which alone the two instances differ is the effect or the cause, or an indispensable part of the cause of the phenomenon.

The application of the method of difference, like the other methods, is not automatic in any experimental situation and, as has been observed by a number of thinkers, 'presumes' an analysis of the situation into all relevant factors that can be examined one at a time.

This rather demanding requirement can be ameliorated in a way that is reasonably faithful to Mill's approach by following some suggestions of the great nineteenth-century physiologist and philosopher of method, Claude Bernard. In his influential monograph *An Introduction to the Study of Experimental Medicine*, Bernard noted that these presumptions of an antecedent analysis of the experimental situation and the ability to examine factors one at a time were not necessarily valid in experimental medicine, and he urged that the method of difference be further distinguished into (1) the method of 'counterproof' and (2) the method of 'comparative experimentation'. According to Bernard, in the method of counterproof, one assumes that a complete analysis of an experimental situation has been made, that is, all complicating and interfering factors have been identified and controlled. Subsequently, one eliminates the suspected cause and determines if the effect in which one is interested persists. Like Karl Popper in the twentieth century, Bernard believed that experimental medical investigators avoided counterproof as a method, since they feared attempts to disprove their own favored hypotheses. Bernard defended a strong contrary position and maintained that counterproof was essential to avoid elevating coincidences into confirmed hypotheses. He argued, however, that those entities that fell into the province of biology and medicine were so complex that any attempt to specify all of the causal antecedents of an effect was completely unrealistic. As a remedy for this problem, he urged the consideration of 'comparative experimentation:'

> Physiological phenomena are so complex that we could never experiment at all rigorously on living animals if we necessarily had to define all the other changes we might cause in the organism on which we were operating. But fortunately it is enough for us completely to isolate the one phenomenon on which our studies are brought to bear, separating it by means of comparative experimentation from all surrounding complications. Comparative experimentation reaches this goal by adding to a similar organism, used for comparison, all our experimental changes save one, the very one which we intend to disengage.

Bernard referred to comparative experimentation as 'the true foundation of experimental medicine'.

Strong Inference

The use of counterproof, crucial experiments, and comparative experimentation finds unified application in what J. R. Platt termed 'strong inference'. Platt contended that rapid progress could be made in the biological sciences if well-formulated, alternative hypotheses were subjected to crucial experiments, designed to eliminate most of the alternatives. He characterized this approach as involving the following steps:

1. devising alternative hypotheses;
2. devising a crucial experiment (or several of them) with alternative possible outcomes, each of which will, as nearly as possible, exclude one or more of the hypotheses;
3. carrying out the experiment so as to get a clean result;
1.′ repeating the procedure, making subhypotheses or sequential hypotheses to refine the possibilities that remain; and so on.

In the next section, some examples of how this 'strong inference' approach is coupled with the methods of experimental inquiry in several recent examples in microbiology are discussed.

Microbiology: Its Scope and Subject Areas; Representative Illustrations of the Philosophy of Method

The scope of microbiology is astonishingly broad. The most recent edition of Zinsser's well-known textbook comments on this subject, stated as follows:

> With each passing year the term microbiology becomes a less satisfactory umbrella for the many disciplines that it attempts to cover. Bacteriology, immunology, virology, mycology, and parasitology have each long since become separate and independent disciplines.

Zinsser provides a continuing rationale for the subject area, however, noting that these numerous specialities "are treated together... simply because they deal with the agents that cause infectious diseases and with the mechanisms by which hosts defend against them". Be that as it may, any analysis of the philosophy of method of a domain that includes infection, bacteria, spontaneous generation and origin of life, the germ theory of disease, viruses, immunity, and chemotherapy must, of necessity, be selective. In the following paragraphs, several examples are discussed that are representative of methodology in microbiology. These illustrations will be used to provide a specific and accessible approach to the somewhat abstract topic of the philosophy of method in microbiology.

The Example of Koch's Postulates and AIDS Virology

As noted in the discussion earlier, self-conscious, explicit methodology is rare in the writings of microbiologists. One prominent and influential exception to this general rule are the Koch's postulates – more accurately termed the Henle–Koch postulates – developed to assess bacteriological causation of disease. About 10 years ago, these postulates returned to prominence at the center of a potentially momentous controversy in AIDS virology. The postulates can also be used as a framework for investigating another controversial example of disease causation – prions – as well as to point toward the limits of 'direct evidence' in microbiology. A brief account of the postulates' history and the philosophical presuppositions will facilitate an understanding of their role in the contemporary debate to be discussed later in this article.

The postulates have their proto-origin in Henle's work in 1840 and were then further developed by his pupil, the great microbiologist Robert Koch, in lectures in 1884 and 1890. There are several slightly different formulations of the postulates (see Evans, who follows Rivers as well as Zinsser), but the essence can be summarized as follows:

1. The infectious agent occurs in every case of the disease in question and under circumstances that can account for the pathological changes and the clinical course of the disease.
2. It must be possible to isolate the agent from all cases of the disease and to grow the agent in pure culture.
3. After being fully isolated from the diseased animals (or humans) and repeatedly grown in pure culture, it can induce the disease anew by inoculation into a suitable host.
4. In addition, some formulations add a fourth postulate:
5. The infectious agent occurs in no other disease as a fortuitous and nonpathogenic agent.

These are stringent conditions, and even at the time that Koch enunciated them, a number of etiological agents were known that did not fully meet the criteria, including bacteria isolated from typhoid fever and cholera. In the ensuing 100 years since the articulation of the postulates, several types of hitherto unrecognized forms of disease, including chronic diseases, cancer, and a number of viral diseases, have required the modification and extension of these postulates. In 1976, Evans attempted to bring together a number of these different themes into a 'unified' epidemiological conception of disease causation.

It is, however, the more traditional and stringent form of the postulates that was cited over the course of the past decade in an attempt by molecular virologist Peter Duesberg to question the causal relationship between HIV and AIDS. The theses of

Duesberg's work were that (1) HIV (the AIDS virus in either its HIV-1 or HIV-2 form) is neither necessary nor sufficient for AIDS, though HIV is a good 'marker' for American AIDS, and (2) AIDS is actually an autoimmune disease caused by as yet unknown pathogens, probably acting in concert with a variety of environmental insults, including hard psychoactive as well as medical drugs (AZT) and blood transfusion (particularly in the case of hemophiliacs). In the eyes of most scientists, Duesberg's principal arguments have been effectively answered, though Duesberg has continued to criticize the standard view in several recent books.

One of Duesberg's major arguments is that HIV fails to satisfy Koch's postulates. He wrote concerning the first of Koch's postulates that "there is no free virus in most – and very little in some – persons with AIDS, or in asymptomatic carriers". Though he admits that various viral elements (and, by definition of the disease, antibody to HIV) can be found in most or all of AIDS patients, he contended that HIV is not present in ways that can account for the loss of T cells or for the clinical course of the disease, which lags 8 or more years behind infection. Further, with respect to Koch's second postulate, Duesberg contends that there is an "often over 20% failure rate in isolation of HIV from AIDS patients". Finally, citing Koch's third postulate, Duesberg notes that HIV does not produce AIDS in any animal model (chimpanzees had been inoculated unsuccessfully), and the data from accidental inoculations of humans (through donor semen, laboratory accidents involving HIV researchers, and blood transfusions) are not consistent with the role of HIV as the sole cause of AIDS.

The response to Duesberg's arguments has been threefold. First, a number of commentators have pointed out that Koch's postulates listed earlier fail to be satisfied in a number of other diseases, primarily of the viral and immunological type encountered in AIDS, and that the postulates need to be taken as 'guidelines' and supplemented by broader epidemiological evidence. Second, when a broader, more epidemiological conception of causation is implemented using epidemiological data, that the evidence for HIV as the cause of AIDS is overwhelming. Finally, molecular investigations of HIV pathophysiology have deepened in recent years. New coreceptors for the virus have been identified that explain some resistance to HIV and may also help explain its oddly delayed pathogenesis. These receptors have also permitted better animal models of HIV/AIDS to be created. Duesberg has rejoined to this criticism, arguing that appeals to epidemiological considerations do not make the case that the proponents of HIV think they do and continues to aggressively defend his own theory.

It is not the purpose of this article to delve further into the details of the ongoing controversy regarding HIV and AIDS, but several points need to be highlighted that illustrate the role of the different philosophies of method involved. First, problems with the failure of HIV to satisfy Koch's first postulate have led to further speculations about the mechanism(s) of T-cell depletion. These speculations include syncytia formation, 'co-factor' pathogens, HIV as the trigger for an autoimmune disease, and, most recently, the proposal of a 'super-antigen' theory. Investigators generally believe that the evidence for these hypotheses are 'indirect', at best, and that more 'definitive or direct' proof is needed. Thus, the Henle–Koch postulates provide a framework and the failure to satisfy them in any simple way should provide the motivation for additional investigation, which may move the subject more in the direction of 'direct' evidence for explanatory models of AIDS pathogenesis. In the next illustration of the philosophy of method, another example of how ambiguous results that do not fully meet the requirements of Koch's postulates is examined, which raises the question of whether fully conclusive evidence is ever forthcoming in microbiology. The last example of just how 'direct' evidence can be obtained will partially answer this question affirmatively, with the continuing caveat, however, that in science, even direct proof continues to remain somewhat conditional.

Prions and the Question of the Causative Agents of the Transmissible Spongiform Encephalopathies

Modifications needed to extend Koch's postulates to take into account later microbiological developments are also a useful framework for a consideration of the cause of the transmissible spongiform encephalopathies (TSEs). These diseases include Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE), or 'mad cow' disease, in cattle. A number of investigators have attempted to determine the etiological agents and the details of the pathogenesis of the TSEs. At present, TSEs are generally thought to be caused by a novel infectious agent, a 'prion' – a term coined by S. Prusiner, to represent a 'proteinaceous infectious particle'. Prusiner received a Nobel Prize in 1997 for his accomplishments in this area and was cited for his 'discovery of 'Prions – a new biological principle of infection'y.' That prions are the fundamental cause of TSEs is still somewhat controversial, and an alternative viral causation hypothesis continues to have its defenders.

If one followed Koch's postulates, as outlined previously, in the TSE area, one would attempt to (1) show the (various) infectious agent(s) occurs in every case of the diseases and accounts for the pathogenesis, (2) isolate the agent and purify it, and (3) use the purified agent(s) to induce the disease(s) in new hosts, In addition, one might want to (4) show the agent does not occur in any other disease as a nonpathological agent. Though Prusiner does not explicitly use Koch's postulates in his various writings on prions and TSEs, the postulates can function as a framework to approach his various arguments. Prusiner's early work was directed at the sheep TSE, scrapie. Following work by Alper that suggested the infectious agent was a protein that did not include nucleic acid, Prusiner was able to show that scrapie prions contained a protein he called PrP, short for 'prion protein'. These proteins are also unusually resistant to degradation by protease enzymes. Working with Leroy Hood, the protein was then partially sequenced, and the sequence used to construct a probe that could identify the genetic source of the protein. Remarkably, it turned out that the *PrP* gene can be found in hamsters, mice, and humans, but, most of the time, cells that make PrP are not pathological. An unorthodox, unprecedented, but possible, explanations was that PrP could exist in two different tertiary forms with the same amino acid sequence: one normal, called 'cellular PrP' (PrP^c), and the other associated with scrapie, termed 'scrapie

PrP′ (PrP^{sc}). This heretical idea was subsequently confirmed and considerable work has been done that examines various conformational changes in PrP and their pathological effects.

Prusiner and his group initially attempted (implicitly) to conform to Koch's second postulate by using the *PrP* gene to generate pure copies of PrP. They thought they could then implement Koch's third postulate and, as they wrote, "inject the protein molecules into animals, secure in the knowledge that no elusive virus was clinging to them. If the infections caused scrapie, we would have shown that protein molecules could, as we had proposed, transmit the disease". But as Prusiner adds, "by 1986, we knew the plan would not work". It was difficult to induce the gene to make the high levels of PrP needed, and, in addition, the PrP that was made was the benign 'cellular' type.

An alternative strategy was then devised that looked to inherited, rather than transmissible, forms of the SEs. Gestmann – Straussler–Scheineker disease is a very rare familial human SE that Prusiner and other investigators were able to link with a point mutation in the *PrP* gene. This served as the backdrop for the creation of genetically altered mice carrying a mutated *PrP* gene. One could first examine whether the mutated gene in the transgenic mice produced scrapie and then whether brain tissue from these mice could cause scrapie in 'healthy' mice, albeit only healthy transgenic mice expressing a low level of mutant PrP. These events do, in fact, occur and are claimed by Prusiner in 1995 to be "solid evidence that the protein encoded by the mutant gene had been solely responsible for the transfer of the disease". Step back and examine this claim more carefully and also consider a critic's view of this 'solid' or 'persuasive' evidence.

Prima facie, the experiment seems to satisfy Koch's first postulate, since all of the transgenic mice making high levels of PrP did develop mouse scrapie. Transplanted infected brain tissue, however, caused only many (not all) of the recipients to develop the disease. A proponent of the competing viral hypothesis, B. Chesebro, finds the transgenic mouse experiment 'inconclusive'. He notes that the transgenic mice differ from all known TSE models, because no abnormal protease-resistant PrP – a hallmark of scrapie PrP – is detectable in the diseased brain tissue. Also, disease occurs only when the mutant gene is overexpressed, not when it is found in its normal site as a single copy. Finally, transmission by infected brain tissue is only successful in transgenic mice – the low-level PrP producers – and not in nontransgenic animals. Chesebro suspects that 'other molecules' from the diseased brains could be causative of the disease, and, further, doubts the utility of the transgenic model, because none of the transmitted materials has been demonstrated to have typical properties of TSE agents, such as resistance to inactivation by heat. Other investigators also believe that additional TSE experimental findings point toward something more than prions as the cause of these diseases, perhaps a virus.

The prion hypothesis and the competing viral hypothesis are, thus, not unambiguously and definitively tested by this transgenic model. But this is only one of a number of lines of evidence supporting the prion hypothesis, and this article cannot delve into the multiple arguments and counterarguments in support of and against the prion view, which continue to evolve (see, e.g., the very recent report by J. Safar and colleagues in *Nature Medicine*, that provides data that different protein conformations are responsible for variations in prions' 'strains'). This fragment of the story, however, does suggest that Koch's postulates can be a useful framework within which to analyze possible etiological agents of disease, albeit one that needs extensions. Such extensions include both new understandings of molecular biological processes never dreamed of by Koch, as well as powerful new experimental techniques, such as the use of transgenic and knockout animals. But the story also indicates how difficult it is to obtain conclusive or 'direct' evidence of a causal hypothesis in microbiology, particularly in the still comparatively early stages of an investigation of a novel model of infection, even with this new powerful molecular knowledge and associated techniques.

The Example of the Isolation of the Repressor

The third example illustrating the application of methods of experimental inquiry is drawn from molecular genetics and involves progress in clarifying the nature of the mechanism of genetic control in bacteria, more specifically, the development and testing of the operon model of genetic regulation. The model, which is principally due to the work of Jacob and Monod, developed over a number of years. The initial form of the mechanism dates from 1960 to 1961 and has had a most important impact on both experiment and theory construction in molecular biology (and in embryology). It has been extraordinarily well corroborated by a variety of experiments. It was further developed in the 1960s, 1970s, and 1980s.

The model proposed the existence of a new class of genes, termed regulator genes, which were responsible for the synthesis of a substance, later determined to be a protein of about 160 000 molecular weight, which was termed a repressor. The repressor, in inducible systems, such as the *lac* region of *Escherichia coli*, binds specifically with a DNA region termed the operator locus. This operator has adjacent to it several structural genes, which are under the operator's control. When the repressor is bound to the operator, the associated structural genes are not transcribed into messenger RNA, and, accordingly, no proteins or enzymes associated with those structural genes are synthesized. An inducer can specifically interact with the repressor, altering its three-dimensional structure, and thus render it incapable of binding to the operator. The enzyme RNA polymerase, which attaches to a DNA sequence called a promoter in the presence of a protein called CAP and which can transcribe the structural genes, is then no longer prevented from initiating transcription of the structural genes adjacent to the operator, and mRNA synthesis and then protein synthesis commence. Repressible systems employ a similar regulatory logic, only, in that case, the initially ineffective repressor is aided in its operator-binding capacity by an exogenously added corepressor. It has been often noted by biologists that such a regulatory system is most useful to the organism since it allows unnecessary genes to be turned off when the proteinaceous enzyme products of those genes are not required for a metabolic process. This results in energy saving and is conceived of as evolutionarily useful to the organism.

In spite of the breadth of evidence for the operon model when it was first proposed in 1961, it can be said that the evidence, at that time, was primarily of an indirect genetic type. Monod in his Nobel lecture suggested, for example, that in the early 1960s the repressor – the crucial regulatory entity – seemed "as inaccessible as the matter of the stars". The difficulty of being able to provide more direct biochemical evidence for the existence of the repressor and its postulated properties stimulated at least one alternative theory of genetic regulation, involving a mechanism different from that of the operon model.

In 1966, however, W. Gilbert and B. Müller-Hill reported that they had been able to isolate the elusive repressor, and, in 1967, the same authors were able to demonstrate that the repressor bound directly to the operator DNA. These experiments were most important for the field of regulatory genetics, and they were confirmed by Mark Ptashne's essentially simultaneous isolation of the repressor in the phage λ system and the discovery that it also bound to DNA. These experiments, and the analyses of the experimental results, illustrate both the application of Mill's methods and Platt's 'strong inference'.

Gilbert and Müller-Hill noted in their article reporting their results that though they felt the Jacob and Monod model to be the simplest, other models would also "fit the data" available. "Repressors", they continued, "could have almost any target that would serve as a block to any of the initiation processes required to make a protein. A molecular understanding of the control process has waited on the isolation of one or more repressors". Isolating the repressor was most difficult because of the hypothesized very low concentration of repressor in the *E. coli* cells. Gilbert and Müller-Hill solved this problem by looking for and finding a mutant form of the bacteria (i^t or tightbinding), which produced a repressor with about a ten fold increased affinity for the inducer. As an inducer, the chemical IPTG (isopropylthiogalactoside) was used, which induces the *lac* operon but which, in contrast to lactose, is not metabolized by the induced enzyme. The repressor was detected by placing an extract of the i^t mutant in a dialysis sac in a solution of radioactive IPTG (labeled to facilitate detection of very low amounts of it), and looking for an increased concentration of IPTG inside the sac after about 30 min (at 4°C).

Using the increased concentration as a sign of the presence of repressor, Gilbert and Müller-Hill purified the extract and analyzed the binding component. They discovered that the enzymes which would attack DNA and RNA would not destroy the inducer binding ability of the presumed repressor, but that an enzymes which attacked proteins would. Heating (above 50°C) also destroyed the ability to bind inducer. This was rather direct evidence (though still conditional on the hypotheses that (1) the enzymes possessed anti-DNA/anti-RNA capacity and (2) heat denatures proteins) that the repressor was a protein. The repressor (labeled with the radioactive inducer) was centrifuged and sedimented on a glycerol gradient. The 'profile' of the sedimented repressor indicated that it had a molecular weight of about 150 000–200 000.

Gilbert and Müller-Hill, in their article, cited what they termed 'negative and positive controls' on their supposition that they had isolated the repressor product of the *i* gene. The most important 'negative controls' involved examining the extracts from i^s (the superrepressed) and i^- (constitutive) mutants. The former type (i^s) presumably synthesizes a repressor that has lost the ability to bind inducer, and the latter (i^-) had been hypothesized to produce an (significantly) incomplete repressor. For both of these mutants, no binding of IPTG was observed. (Gilbert and Müller-Hill noted that "the various mutant forms of the *i* gene were put into identical genetic backgrounds, so that unknown variations from strain to strain could not confuse the issue".)

Gilbert and Müller-Hill plotted graphs representing the binding constants of the wild type and i^t mutant to demonstrate the essential identity of the repressors, which were used as positive controls. An examination of the binding ability of the repressor for various other substances, including galactose and glucose (and yielding a decreasing sequence of affinities), "gave further support for the [thesis that the isolated] material... [was] the repressor".

In 1967, Gilbert and Müller-Hill used this partially purified radioactive repressor to test for *in vitro* binding to the operator of *lac* DNA. In this experiment, they used strains of bacteriophage that carried different forms of the *lac* operon in their DNA.

By sedimenting mixtures of the various strains of the phage DNA with the radioactive repressor, Gilbert and Müller-Hill found the following:

1. In the absence of inducer, the radioactive repressor binds to the DNA.
2. In the presence of IPTG, which releases the repressor from the DNA, no binding is observed.
3. The effect of the IPTG is specific, for substitution by a chemical ONPF (*o*-nitrophenyl-fucoside; a substance that binds to the DNA but does not induce) has no effect.
4. Use of two mutant phage o^c strains, in which the affinity of the operator for the repressor is expected to be weaker by a factor of 10 and 200, yielded sedimentation profiles in rough quantitative conformity with these binding expectations.

These experiments, which appear to utilize the methods of comparative experimentation or difference (the 'negative control') and concomitant variation (the 'positive controls'), have been characterized as offering 'direct' evidence and 'proving' the hypotheses of the Jacob– Monod model. Clearly, they do so, however, with the aid of auxiliary hypotheses involving, among others (1) the effects of growth in radioactive media so as to specifically label the 'observed' entities and (2) the analysis of centrifugation and sedimentation techniques in density gradients. The experiments require a chain of reasoning. There is, in addition, (3), a very general negative auxiliary assumption that what is observed is not an artifact. It must be further emphasized that the direct proof of the Jacob–Monod operon model is contingent on the assumption that other, and perhaps more important and overriding, factors are not involved, that is, some competing, but as yet unarticulated, hypothesis that could account for all these results. The proposal of a novel mechanism of control that would account for the genetic data as well as Gilbert and Müller-Hill's findings (perhaps by showing some of them to be artifacts), but which would disagree with basic tenets of the operon model cannot be logically excluded. The existence of Gilbert and Müller-Hill's experiments are thus a 'conditional' direct proof, the conditions depending on (1) the truth of auxiliary hypotheses and (2) the nonexistence of an inconsistent competing theory or model that would account for the same data as the operon model.

Generalizing from this example, it is obvious that the 'directness' and force of the evidence is obtained by choosing one or more 'central' (roughly equivalent to 'essential') property(ies) of the hypotheses in the theory or model to be tested. In the operon model's case, these properties were initially suggested in Monod's work, namely, the repressor's 'recognition' of the inducer and the operator.

In addition, plausible competitor theories and models are scanned to determine if the test condition(s), if realized, would support the model under test while falsifying the competitors. Recall that 'genetic' evidence was insufficient to do this, whereas the Gilbert–Müller-Hill experiments (as well as Ptashne's) were. This exhibits Platt's 'strong inference' in specific ways.

The evidence was considerably strengthened by providing both positive and negative controls. These controls are essentially equivalent to the well-known Mill's methods of difference and concomitant variation, as discussed previously.

These three particular examples, the use of the Henle–Koch postulates to assess the causal role of HIV and the TSEs, and the employment of a strategy of strong inference implementing methods of difference and concomitant variation to identify and characterize the repressor, illustrate the application of several methodological themes found in microbiology. Each of these examples also indicates that the application of such methods requires creative insight coupled with detailed knowledge of the subject domain, as well as exemplary technique, in the case of laboratory investigations. In addition, the examples illustrate that the path from speculation and indirect evidence for scientific hypotheses to direct proof is difficult and frequently depends on background assumptions that themselves could, at some time, be called into question. Such a process of continued questioning of received assumptions, coupled with the proposal of new, precisely formulated hypotheses subject to experimental test and evaluation by rigorously applied scientific method, is the essence of excellent science, and though this process is typically implicit in microbiology, it is no less an important constituent of scientific progress in the subject.

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Methylation and other Modifications of Nucleic Acids and Proteins☆

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Glossary

Bacteriophage Virus specific for particular strain of bacteria.
Epigenetic A phenotypic but not genotypic change.
Methyltransferase Enzyme methylating particular residues in a macromolecule.
Modification Chemical change of DNA/RNA bases or amino acids.
Promoter Sequence at which RNA polymerase initiates transcription.
Restriction Inactivation of DNA by nucleolytic cleavage.
S-adenosyl-L-methinone Universal methyl donor used by methyltransferases.

Abbreviations

cAMP	cyclic AMP
Cap	Cyclic AMP binding protein
CcrM	Cell cycle-regulated methyltransferase
Dam	Adenine methyltransferase
Lrp	Leucine-responsive regulatory protein
OxyR	Oxidative damage response
Pap	Pyelonephritis-asociated pili
PTM	Post-translational modification
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosyl-L-methionine

Defining Statement

Methylation and many other covalent modifications occur in DNA, RNA, and protein. In DNA, methylated bases regulate chromosome replication, the cell cycle, DNA repair, and transcription of specific genes. RNA modification may be required for optimal ribosome function and translation. Protein modifications include methylation, acetylation and phosphorylation that regulate signal transduction during chemotaxis and response to other environmental cues, and play important roles in bacterial metabolism, pathogenesis and other cellular processes.

Introduction

Modification of polymers such as DNA, RNA, and protein generally occurs at the post-synthetic level. For example, methylation of bases in nucleic acids occurs after formation of the polymer rather than by incorporation of methylated precursors during synthesis. Methylation results from the action of enzymes, methyltransferases, which are very specific not only for the particular polymer but also for specific residues in defined sequences in the polymer. In some cases, the biological role for methylated residues of polymers has been elucidated. Although various modifications to particular polymers are possible, such as phosphorylation, thiolation, and acetylation, this review concentrates on methylation of macromolecules since this modification appears to be the most frequent and, currently, best understood. *Escherichia coli* will be used as a model because more information on the biochemistry and genetics of modification is available in this organism than any other.

☆*Change History:* January 2015. J-R Zhang, M Marinus, and H Deng added an Abstract and Figure 7, updated the sections of DNA methylation and other modifications (of proteins), and revised the reference section.

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DNA, RNA, and Protein Methyltransferases

Methyltransferases catalyze the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to specific residues in the polymer. For example, DNA adenine methyltransferase (Dam) from *E. coli* catalyzes the formation of N^6-methyladenine at adenines in the sequence 5′-GATC-3′. Only the N^6 position is modified and adenine residues in other sequences are not methylated nor are adenines in RNA. In double-stranded nucleic acids, methylation substrates are symmetric (5′-GATC-3′/3′-CTAG-5′) and two independent methyltransferase binding events are required.

All methyltransferases acting on polymers use SAM as the methyl donor, which results in the formation of *S*-adenosylhomocysteine (SAH) after catalysis. SAH is a powerful inhibitor of methyltransferase action and has a physiological role in limiting the action of this class of enzymes. Amino acid sequence comparisons of the DNA methyltransferases suggests that they evolved from a common ancestral protein by gene duplication followed by extensive divergence. DNA cytosine methyltransferases, however, retain conserved amino acid sequence motifs suggesting a similar basic mechanism of catalysis. This mechanism involves the transient and covalent binding of the enzyme through nucleophillic attack of cysteine-177 to the number 4 carbon atom of the pyrimidine ring. This leads to the activation of the number 5 carbon to receive the methyl group from SAM. After completion of methyl group transfer, SAH is released and the enzyme dissociates from the substrate sequence. A similar mechanism of catalysis has been proposed for RNA methyltransferases.

The atomic structures of several DNA methyltransferases have been solved recently and in some instances, the structure of the enzyme bound to DNA. These structures have shown that the enzyme rotates the target nucleotide about 180° into a concave catalytic pocket. This rotation is accomplished without any breakage of chemical bonds and has been termed 'base-flipping' (Figure 1). Although the number of demonstrated examples of base flipping is currently limited, this mechanism is surprisingly elegant and simple. It is tempting to speculate that this may be the basic mechanism of action for DNA methyltransferases in general.

DNA Methylation

Methylation has been found in three nucleotides of bacterial DNA: N6-methyl-adenine (m6A), C5-methylcytosine (m5C), and N4-methyl-cytosine (m4C) (Figure 2). m6A and m5C widely exist in the genomes of fungi, bacteria and some Archaea, whereas m4C is exclusively found in bacteria. While the bases of some bacterial genomes are highly methylated, certain bacterial species have not detectable methylation. In *E. coli* strains, m6A and m5C represent 1–2% of the total bases. Bacterial viruses (phages) also can contain modified bases in their DNA and in the T-even group, which infects *E. coli*, 5-hydroxymethylcytosine is present instead of cytosine. Furthermore, one or more glucose residues are attached to this modified base. It is possible that the viral DNA is modified in this way so that it is protected from viral enzymes that degrade host DNA during infection.

In *E. coli* K-12, strains lacking all endogenous DNA methyltransferases have been constructed by genetic means and these have no detectable methylated bases in DNA. Such strains are viable as long as their ability to recombine DNA is not impaired. Recombination is required to repair the double-strand breaks that occur in the absence of *dam* methylation as a consequence of DNA mismatch repair. In other bacteria such as *Vibrio cholerae*, however, Dam methylation is an essential function probably for the initiation of chromosome replication. Since endogenous DNA methylation in *E. coli* blocks the action of some restriction

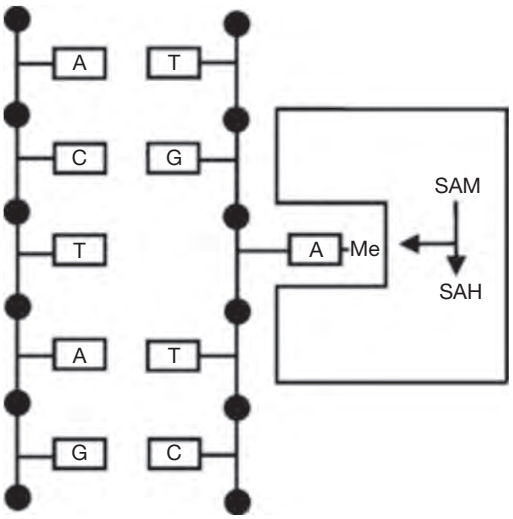
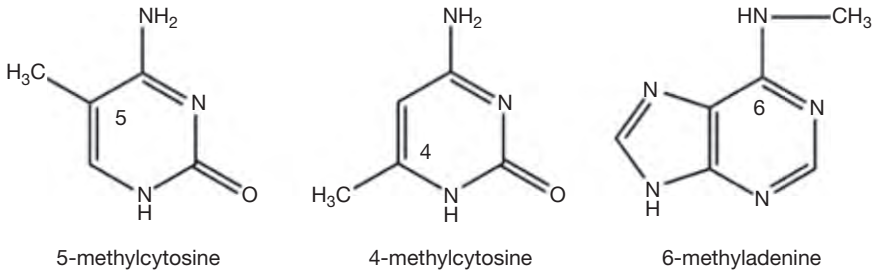


Figure 1 Methylation by base flipping. An adenine residue is flipped out of the DNA helix into the active site of Dam methyltransferase where a methyl group from SAM is added to the N^6 position of the base.



5-methylcytosine 4-methylcytosine 6-methyladenine

Figure 2 Common methylated bases in DNA. The structures of 5-methylcytosine, 4-methylcytosine, and 6-methyladenine are shown.

endonucleases used for recombinant DNA technology, plasmid DNA is often prepared from these methylation-deficient strains to avoid this problem.

DNA methylation is traditionally detected by biochemical methods such as thin restriction digestion, layer chromatography, high-performance liquid chromatography, mass spectrometry, and nanopore amperometry. Recent advancement in DNA sequencing technology has made it possible to characterize the methylation status of the entire genome, so called methylome. Treating genomic DNA with sodium bisulfite followed by parallel DNA sequencing (termed bisulfite sequencing) has allowed genome-wide detection of cytosine methylation sites. Single molecule, real-time (SMRT) sequencing can identify methylated adenines and other bases in a high throughput manner. While bisulfite sequencing is predominantly used in eukaryotic organisms, the SMRT sequencing method has been applied to detect the DNA methylations of the entire genome or methylomes of pathogenic *E. coli*, *Helicobacter pylori* and several other bacteria. These technologies have revolutionized the research on DNA methylation.

Restriction and Modification of DNA

DNA transfer of plasmid, bacterial virus or chromosomal DNA is thought to occur at a low level between bacterial strains and species. Most bacteria have surveillance systems that allow them to differentiate 'foreign' DNA from their own DNA. The basis of one of these systems is the possession of an endonuclease (e.g., *Eco*RI) that recognizes a specific sequence in DNA (5′-GAATTC-3′) and cleaves it only when unmethylated. A corresponding DNA methyltransferase (M.*Eco*RI) recognizes the identical sequence and modifies it to 5′-GAmeATTC-3′, where the second A is methylated. Therefore, bacteria possessing the *Eco*RI methyltransferase will have all susceptible sequences methylated and thus be immune to *Eco*RI endonuclease action. Introduction into the bacterium of homologous or heterologous DNA lacking the specific *Eco*RI modification leads to recognition and cleavage of such DNA and subsequent degradation by the endonuclease.

A very large number of restriction enzymes have been isolated from bacteria, each having a unique DNA recognition sequence. The availability of this battery of enzymes has been a major contributing factor to the success of recombinant DNA technology in allowing the mapping of genes and their isolation from chromosomes.

One other interesting aspect of restriction/modification is that it can be the basis of epigenetic behavior. For example, bacterial viruses containing DNA not modified at *Eco*RI sites will not kill bacteria, since their DNA is degraded upon entering the bacteria. However, at a low frequency, a few virus DNA molecules survive because they have been methylated and, when these are tested again, they now infect and kill the same bacteria at high efficiency. The virus has not mutated in any way; the only difference is that its DNA has become methylated and thus is now resistant to *Eco*RI endonuclease action. Since methylation is dependent on the host in which the virus was last propagated, it is not a heritable change but an epigenetic one.

A second system that bacteria possess to protect themselves from foreign DNA is the ability to detect 'foreign' modifications on such DNA and degrade it using specific endonucleases. For example, in the early days of recombinant DNA technology, it was difficult to isolate genes from animals and plants in the standard bacterial host, *E. coli* K-12. We now know that this failure was due to the destruction of the DNA because it contained methylated cytosine and was recognized by an endonuclease called Mcr (for methylcytosine restriction). This enzyme recognizes and cleaves any methylcytosine-containing DNA that does not have the *E. coli* K-12 signature cytosine methylation. Currently, *E. coli* strains lacking all known restriction systems are used to successfully isolate human and plant genes.

Dam Methylation

In some bacteria (and all eukaryotes) most methylated bases are not part of restriction/modification systems. The Dam and Dcm methylation systems of *E. coli* K-12 are the best characterized of these. The *dam* and *dcm* genes encode DNA adenine (Dam) and DNA cytosine methyltransferases (Dcm), respectively. Dam modifies the adenine residue in 5′-GATC-3′ sequences to N^6-methyladenine and Dcm modifies the second cytosine in 5′-CCAGG-3′ and 5′-CCTGG-3′ sequences to 5-methylcytosine. The combined action of these two methyltransferases is responsible for about 99% of the total methylated bases in DNA. The methyltransferase (M.*Eco*K) that is part of the modification/restriction system contributes the remaining small percentage of the total owing to the infrequent occurrence of the DNA sequence it recognizes.

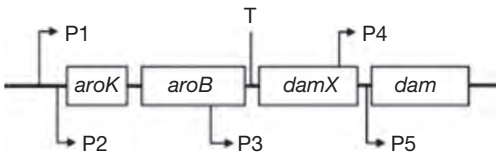


Figure 3 Promoters and terminators contributing to *dam* gene transcription. Five promoters (P) and one transcriptional terminator (T) contribute to transcription of the *dam* gene. The major contributing promoter is P2 which is growth-rate regulated. Reproduced from Marinus MG (1996) Methylation of DNA. In: Neidhardt FC, Curtiss R, Ingraham JL, et al. (eds.) *Escherichia coli and Salmonella*: *Cellular and Molecular Biology*, 2nd ed., pp. 861–886. Washington, DC, with permission of the American Society for Microbiology Press.

The *dam* gene is regulated at the level of transcription by at least five promoters and one terminator sequence (Figure 3). The major promoter, P2, is located about 3000 bases upstream of the gene and it produces a multigene transcript for the *aroK*, *aroB*, *damX*, *dam*, and *trpS* genes. This promoter is growth-rate regulated; the faster the growth rate the greater the frequency of transcript initiation. This is very logical because at slow growth rates, the Dam level should be lower than in fast growing cells to prevent premature methylation of hemimethylated DNA that might lead to increased spontaneous mutation and alteration of nucleoid structure (see below). The rationale for growth rate regulation for the *aro* (aromatic amino acids), *damX* (function unknown), and *trpS* (tryptophanyl-tRNA synthetase) genes is not known.

The mode of transcriptional regulation of the *dcm* gene is not known. The gene order is of interest because the last 20 bases of *dcm* overlap the beginning of the *vsr* gene, the product of which is an endonuclease necessary for very short patch (VSP repair; see 'Dcm methylation'). Dcm and Vsr proteins are translated from the same mRNA transcript in different reading frames but the mechanism by which this occurs has not been elucidated since ribosomal frameshifting has been excluded. Coupling translation of Vsr to Dcm may ensure that VSP repair is available when Dcm methylation is active.

Dam-Directed Mismatch Repair

The concentration of Dam in the cell is regulated to be less than that needed for rapid methylation of all available sites in the DNA. This results in under-methylation of the newly synthesized DNA chain relative to the parental strand, which is fully methylated. This difference in methylation state is exploited by a DNA repair system (Dam- or methyl-directed DNA mismatch repair) that removes errors generated by the replication machinery from the newly synthesized strand (Figure 4). If the replication machinery makes a mistake, it will be present in the newly synthesized (unmethylated) strand as a base mismatch and be recognized by the MutS protein. After specific recognition, MutL is recruited followed by MutH that activates its latent endonuclease activity to nick the unmethylated strand (Figure 4). The UvrD helicase enters at the nick and unwinds the DNA making the nicked strand available for exonuclease digestion. The gap so formed is filled by DNA polymerase III, the replicative enzyme, followed by ligation by DNA ligase and methylation of the GATC sites by Dam methyltransferase. Note that MutH can cut only if the substrate DNA is hemimethylated; fully methylated is not a substrate for MutH endonuclease action, thereby targeting repair to the region just behind the replication fork. Inactivation of this mismatch repair pathway increases the spontaneous mutation frequency 100- to 1000-fold relative to the wild-type strain indicating its importance in proofreading newly replicated DNA. In *dam* bacteria, discrimination between the new and old strands is lost and, therefore, half the time it is expected that mismatch repair introduces mutations into the DNA resulting from replication errors. This is reflected in the mutator phenotype associated with the *dam* mutant.

Initiation of Chromosome Replication

Chromosome replication in *E. coli* begins at a sequence called *oriC* (origin of replication), which has about tenfold more GATCs than expected on a random basis. Once chromosome duplication begins, it is important to suppress further initiation until the cell requires it. It appears that *dam* methylation is involved in the suppression mechanism. Upon replication, the *oriC* region becomes hemimethylated and is bound by SeqA protein and 'sequestered' as is the nearby *dnaA* gene. At a later stage in the cell cycle, SeqA dissociates and the free *oriC* region is methylated by Dam thereby preparing it for the next initiation event. Experimental evidence supporting this model includes the inability of hemimethylated DNA molecules to replicate in *E. coli dam* mutants. The SeqA protein binds with high affinity to hemimethylated DNA containing two or more GATC sites on the same face of the DNA and so only a subset of all GATCs are bound.

Dam methylation also affects the frequency of initiation at *oriC* by multiple mechanisms. First, initiation of unmethylated *oriC* DNA appears to be less efficient than with fully methylated DNA. Second, DnaA is the key protein acting at *oriC* to initiate the process and transcription of the *dnaA* gene is regulated, in part, by Dam methylation of its promoter region, and thus there may be a reduction in the effective concentration of this protein in a *dam* mutant. Third, in wild-type *E. coli* containing multiple copies of *oriC*, initiation occurs simultaneously at all origins. In *dam* mutants, however, initiation at multiple *oriC*s occurs at random throughout the cell cycle indicating that Dam methylation controls synchronization. All of these mechanisms may contribute to the reduced efficiency of chromosome initiation at *oriC* in *dam* mutants.

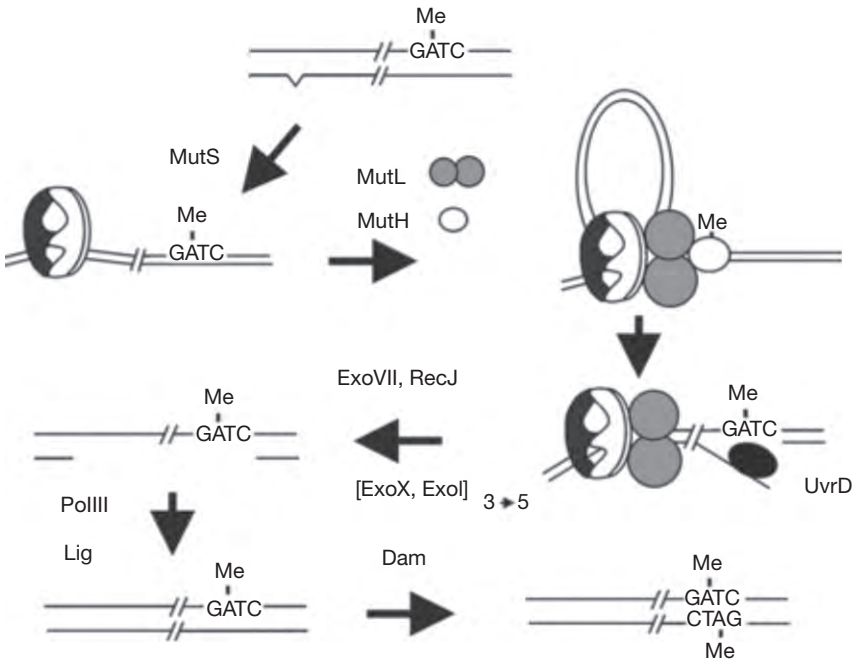


Figure 4 Dam-directed mismatch repair in *E. coli*. The top of the figure shows DNA immediately behind the replication fork in which the 'old' top strand is methylated and the 'new' strand is not; it also contains a base mismatch (carat) created as a replication error. The mismatch is recognized and bound by MutS followed by recruitment of MutL and MutH to form a ternary complex. The formation of this complex is thought to involve DNA looping to bring the mismatch and a GATC sequence in close proximity but the details are unclear. In the ternary complex, the latent nuclease activity of MutH is activated, which cleaves the new unmethylated strand 5′ to the GATC sequence. The nick created by MutH serves as an entry site for the UvrD helicase, which unwinds the DNA exposing single-stranded DNA which is digested by one or more of the following exonucleases: *Rec*J, *Exo*VII, *Exo*X, or *Exo*I. The exonuclease(s) used depends on the relative orientation of the mismatch to the GATC sequence; in the figure, the direction of UvrD unwinding is 5′ to 3′ and so either *Exo*VII or *Rec*J or both are needed. If the mismatch was to the 'right' of the GATC sequence, UvrD would unwind in the 3′ to 5′ direction and *Exo*X and/or *Exo*I would digest the single-stranded DNA. The gap created by nuclease digestion is filled in by DNA polymerase III. The resulting nick is closed by DNA ligase and eventual Dam methylation in the newly synthesized strand precludes any further repair. Reproduced from Marinus MG (2005) Dr. Jekyll and Mr. Hyde: How the MutSLH repair system kills the cell. In: Higgins NP (ed.) *The Bacterial Chromosome*, pp. 413–440. Washington, DC, with permission of the American Society for Microbiology Press.

Nucleoid Structure

Hemimethylated DNA occurs immediately behind the replication fork and three proteins are known to bind to such DNA: Dam, MutH, and SeqA. Overproduction of Dam leads to premature methylation of hemimethylated DNA and a high level blocks MutH action thereby preventing mismatch repair and increasing the frequency of spontaneous mutation. There is a substantial effect on SeqA binding even in low-level Dam-overproducing cells where Dam out competes SeqA for GATC binding. This is equivalent to SeqA not acting at all and leads to an alteration in supercoiling of the chromosome leading to a perturbed global transcription pattern. To maintain proper nucleoid structure, therefore, it is crucial that cells regulate the Dam/SeqA protein ratio.

Transcriptional Regulation of Gene Expression

Dam methylation can influence gene expression by two different mechanisms: direct and indirect. The direct mechanism involves RNA polymerase binding to promoter regions that have 5′-GATC-3′ in one of two critical regions; the '-10' and the '-35' hexamers. Methylation at these hexamers can increase, decrease, or have no effect on transcription initiation, depending on the specific promoter. Methylation of the *trpR* (tryptophan repressor) and *sulA* (suppressor of filamentation) gene promoter hexamers reduces transcription initiation while methylation of one of the two *dnaA* gene promoters increases it. The promoter regulating transposition of the tetracycline-resistance element Tn10 is active only in a hemimethylated state, which occurs during the brief period when it is replicated. Transposition is thereby coordinated with the cell cycle.

The second mechanism by which Dam methylation can control gene expression relies on competition for overlapping DNA binding sites between Dam and regulatory proteins such as transcriptional activators and repressors. Methylation of the sequence bound by the regulatory protein then locks gene expression into a particular mode. For example, Cap (cyclic AMP – cAMP binding protein), with cAMP, binds to specific sequences upstream of many promoters and acts to increase the basal level of transcription. The consensus 22 bp recognition site for Cap-cAMP is 5′-AATGTGATATCACATTT-3′ and a high-affinity site contains the TGTGATC sequence, which overlaps with the Dam recognition sequence. Methylation of this sequence can cause interference with Cap-cAMP binding, thus preventing high-level expression of the gene. In addition to Cap-cAMP, the transcriptional activators Lrp (leucine-responsive regulatory protein) and OxyR (oxidative damage response) have recognition sites that can overlap with-GATC-sequences

and show differential DNA binding depending on the methylation state of this tetra-nucleotide. For example, the *agn43* (antigen 43) gene of *E. coli* encodes an auto-transporter protein that causes cells to aggregate and form precipitates in culture media. The gene has three – GATC- sites in its promoter region and their methylation leads to full transcription initiation (Figure 5). During replication of the gene, Dam or SeqA or OxyR can bind the hemimethylated DNA. Dam binding to the GATCs allows continued transcription while SeqA binding is transient and favors its eventual replacement by Dam. OxyR binding, however, is tighter than SeqA and can exclude Dam so that after a second round of replication a fully unmethylated promoter region with bound OxyR is created and gene transcription cannot occur. Other examples of such regulatory circuits involving GATC sequences and differential DNA binding include the *pap* (pyelonephritis-asociated pili) operon of uropathogenic *E. coli*, the K88 pili associated with diarrheal *E. coli* infections and the Pef fimbriae of *Salmonella enterica*. These regulatory circuits can also include other DNA-binding proteins such as H-NS and the sigma factor RpoS. Note that, expression of genes controlled in this way constitutes epigenetic behavior.

High-level gene expression is achieved only if transcriptional activators, such as Lrp and Cap-cAMP, are continuously bound to the site preventing access to Dam and subsequent modification. In practice, such unmethylated sites are identified in chromosomes by their lack of Dam methylation and about 50 such sequences have been detected. The methylation state of such sites can change depending on the need for the gene products involved.

Posttranscriptional Regulation of Gene Expression

There are three examples of posttranscriptional gene regulation influenced by Dam. First, the VSP repair system corrects T/G mismatches arising from deamination of 5-methylcytosine (Figure 6). VSP repair is reduced in *dam* cells by an unknown indirect mechanism since there are no GATC sites in the presumed regulatory regions of the VSP repair genes. Second, a similar situation occurs in the *std* operon of *S. enterica*, which regulates production of fimbriae that are important for long-term carriage in mice. Third, the Tir effector of enterohemorrhagic *E. coli* is essential for virulence and is secreted from bacterium into human cells to form tight binding between them. In a *dam* mutant of this bacterium, the abundance of the Tir protein is dramatically increased but the mRNA level is not. In none of these three cases has the mechanism of posttranscriptional regulation been elucidated.

Control of Bacteriophage DNA Packaging into Virions

Bacteriophage P1 encodes its own *dam* gene and Dam methylation is essential for successful production of new bacteriophage after infection of the host. Introduction of bacteriophage DNA into the head (capsid) of the maturing virus particle is the critical step at which Dam methylation acts. The DNA is synthesized in long pieces (concatamers) that need to be cut into smaller pieces at *pac* sites (containing seven GATC sequences) in order to fit properly into the head. This process requires that the DNA be fully methylated at

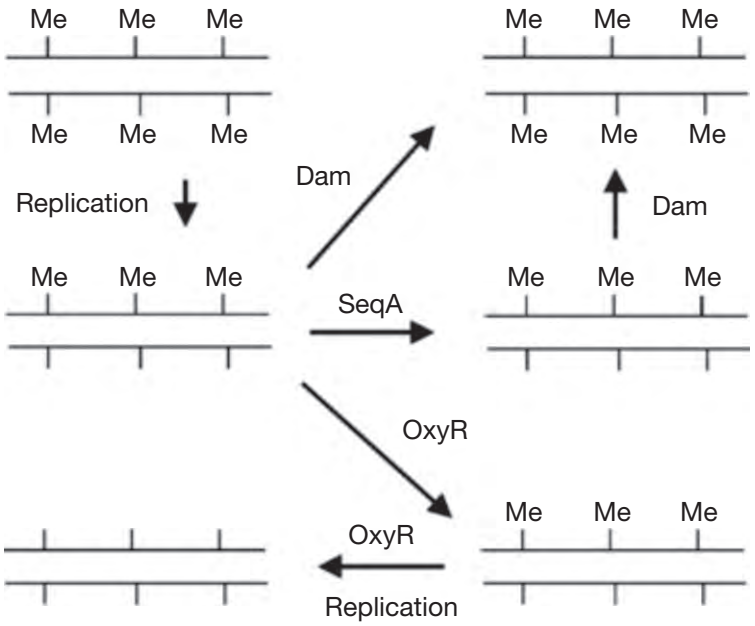


Figure 5 Regulation of *agn43* gene transcription in *E. coli*. The promoter region of the *agn43* gene contains three GATC sequences that must be methylated (Me) for the gene to be expressed. Replication of the gene will cause transient hemimethylation allowing one of three proteins can bind this DNA. Dam action will methylate the GATCs on the new strand thereby preserving expression of the gene. SeqA can also bind but is easily displaced by Dam resulting in methylation and continued gene expression. OxyR binding prevents Dam action and after a second round of replication the GATCs are unmethylated and transcription is prevented. Reproduced from the *Journal of Bacteriology* 184, 3338 (2002), with permission of Dr. Marjan van der Woude and the American Society for Microbiology Press.

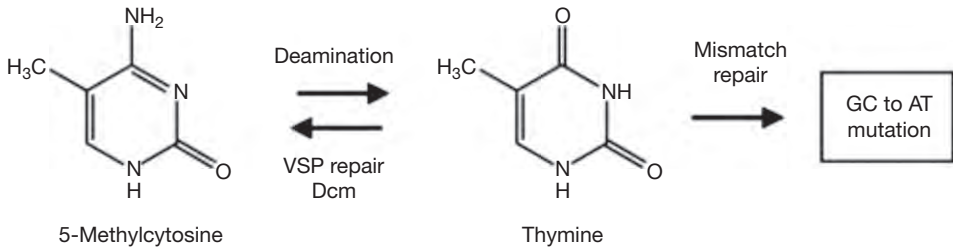


Figure 6 VSP repair of deaminated 5-methylcytosine. 5-Methylcytosine deaminates spontaneously to thymine thereby creating a T-G mismatch. This is acted upon by the VSP repair system, which removes the base by the action of the Vsr glycosylase and cleavage of the DNA backbone. DNA polymerase I is used to fill-in the missing base and DNA ligase seals the remaining nick. Dcm methyltransferase transfers a methyl group to the cytosine to restore the original methylated base. The T-G mismatch is infrequently also a substrate for Dam-directed mismatch repair which can convert the GC to an AT base pair creating a mutation.

pac sites since it is only in this state that the DNA can be cleaved by the viral p9 enzyme. On any one concatamer, however, all but one *pac* sites are hemimethylated due to binding, but not cleavage, of P9. Obviously, lack of methylation prevents successful encapsidation of DNA and the production of complete virus particles.

There are many bacteriophage that encode a *dam* or *dam*-like gene. At this time, other than bacteriophage P1, there is no known role for the function for these *dam* gene products.

Dam as a Virulence Factor in Pathogenic Bacteria

Loss of Dam methylation in certain strains of pathogenic bacteria, such as *Salmonella*, *Haemophilus*, and *Yersinia*, reduces virulence considerably in animal models, probably due to changes in gene expression in pathogenicity islands in the absence of methylation. In other pathogens such as *Shigella flexneri* and enterohemorrhagic *E. coli*, loss of Dam has no effect on virulence. In *Pasteurella multocida* and *Yersinia pseudotuberculosis*, virulence is altered when Dam is overproduced probably by preventing binding of SeqA to hemimethylated DNA and consequent alteration of chromosome structure leading to a change in global gene expression.

Plasmids bearing virulence determinants can be transferred from cell to cell by conjugation. The pSLT virulence plasmid of *Salmonella* can transfer at high frequency from a *dam* mutant because the transfer (*tra*) operon is de-repressed. Transcription of the *traJ* gene, which encodes a transcriptional activator of the *tra* operon, is normally reduced in wild-type due to GATC sites in the promoter region. In addition, transcription of *finP*, a small regulatory RNA that antagonizes *traJ* expression, is also decreased in *dam* cells.

Dcm Methylation

The DNA cytosine methyltransferase (Dcm) in *E. coli* is an orphan cytosine-5 DNA methyltransferase, which methylates the second cytosine in the sequence 5′CCWGG3′. Dcm-mediated methylation is highly conserved in *E. coli* isolates. Unlike Dam methylation, a physiological role of 5-methylcytosine is not completely clear. Given its widespread occurrence in bacteria, however, there must be some evolutionary advantage in retaining it. 5-Methylcytosine in DNA is spontaneously converted into thymine at a low level. This conversion produces a thymine-guanine base mispair and *E. coli* and other bacteria have evolved a repair system (VSP repair) that converts the thymine back into cytosine and then Dcm methylation to 5-methylcytosine (Figure 6). The Vsr glycosylase, which removes the thymine, is encoded by the *vsr* gene. *vsr* overlaps the *dcm* gene by seven codons and both are translationaly coupled. The thymine-guanine mispair is also a substrate for Dam-dependent mismatch repair, which can convert it into a thymine-adenine base pair thereby producing a mutation. Mutation at 5-methylcytosine residues is the most common C to T base pair alteration in *E. coli* and probably occurs most frequently by replication of the T-G mismatch before VSP repair. Dcm can influence drug resistance and the expression of ribosomal protein genes during stationary phase influence stationary phase gene *in E. coli*.

YhdJ Methylation

Annotation of the *yhdJ* gene of *E. coli* K-12 suggested that the gene product might encode a methyltransferase based on similarity of certain amino acid motifs that occur in this class of enzymes. Recently, the *yhdJ* gene was cloned and its product is indeed a DNA methyltransferase that methylates the 3′ adenine in the sequence 5′-ATGCAT-3′. The *yhdJ* gene, however, is not essential for growth of *E. coli* and is not expressed under normal laboratory growth conditions and consequently its biological role is unknown.

CcrM Methylation

Although the CcrM (cell cycle-regulated methyltransferase) and its homologues have been found in *Agrobacterium*, *Sinorhizobium*, and *Brucella*, the enzyme was discovered in *Caulobacter crescentus*; and it is in this organism where its properties have been most fully characterized. In all these organisms, CcrM is essential, and in *Caulobacter* it contributes to control the cell cycle. The *Caulobacter* life

cycle can be divided into nonmotile stalked and motile swarmer forms where chromosome replication occurs only in the former to produce a stalked and a swarmer daughter cell. Both nascent daughter cells, therefore, contain hemimethylated DNA and in the late stage of chromosome duplication, but before cell division, the *ccrM* gene is expressed and the methyltransferase methylates the adenine in 5′-GANTC-3′ sequences of the entire chromosome. Once the origin of replication (C*ori*) is fully methylated it is bound by the CtrA protein to prevent initiation of chromosome replication. In the swarmer cell, CtrA remains bound while in the stalked cell, CtrA is destroyed by proteolysis allowing initiation to proceed on a fully methylated *Cori*. After cell division, the CcrM protein is degraded preventing further methylation thereby ensuring the origin is hemimethylated and inert for further initiation.

Transcription of the *ccrM* gene occurs only in the hemimethylated state and is activated by CtrA, which binds to two 5′-GANTC-3′ sequences in the upstream regulatory region of *ccrM*. This arrangement ensures that the concentration of CcrM increases toward the end of the replication cycle. The synthesis of CtrA is also regulated by CcrM methylation. Two 5′-GANTC-3′ sequences also occur in the upstream regulatory region and one is close to the -35 hexamer of the promoter. This promoter also is active only when hemimethylated, and the expression of the gene is, therefore, co-coordinated with the cell cycle. The *ctrA* gene is located proximal to the origin relative to the *ccrM* gene thereby ensuring the proper sequence of gene expression and regulation. This arrangement is very reminiscent of the DnaA–*ori*C interaction described above for *E. coli*.

RNA Modification

More than 100 modifications have been found on four canonical nucleotides of RNA. Recent progress in high throughput detection of RNA modifications in eukaryotic organisms makes it possible to map some of RNA modifications at the genome scale, such as N^6-methyladenosine (m^6A) and 5-methylcytidine (m^5C). While there are extensive modifications in mRNAs and non-coding RNAs of eukaryotic organisms, our understanding on RNA modification in bacteria is currently confined to ribosomal and transfer RNA. The following sections will focus on RNA modifications in bacteria.

RNA Methylation

In contrast to DNA, ribosomal and transfer RNA are extensively modified and it has been estimated that there must be at least 80 RNA modification enzymes in *E. coli* and of these at least 20 are RNA methyltransferases. Only a few of these enzymes have been characterized to date. There are no reports of methylated bases in messenger RNA but this may be difficult to measure given the inherent instability of this class of molecules.

Two tRNA methyltransferases, TrmD and TrmU, are essential for cell viability and TrmD methylates guanine at position 37 and TrmU is responsible for 5-methylaminomethyl-2-thiouridine formation. The reason for the absolute requirement of these modifications for cell viability is not known but most likely reflects their contribution to the fidelity of translation through tRNA-mRNA codon interaction. This idea is supported by the observation that nonlethal point mutations in these genes lead to excessive ribosomal frameshifting during translation.

Although deletion of the genes for other known RNA methyltransferases does not lead to lethality, many of these strains show subtle effects on translation. For example, ribosomal subunits lacking dimethyladenine from the *ksgA* (kasugamycin-resistant) mutant strain have decreased affinity for each other and an increased requirement for a translation initiation factor.

Other RNA Modifications

Deletion of the genes for most known RNA modification enzymes does not result in loss of viability although multiple deletion of these genes encoding enzymes with overlapping specificities has not been tested. The single gene deletion mutants may show subtle effects on the rate of translation or the ability of tRNAs to bind to cognate codons. Mutant strains lacking ribosomal modifications are often resistant to antibiotics (kasugamycin, viomycin), again suggesting subtle alterations in the translation apparatus.

Protein Modification

Methylation

Methylation can occur at side chain nitrogens in arginine, lysine, *iso*-aspartic acid, histidine, glutamine, and glutamic acid residues in proteins. Only a few protein methyltransferases, however, have been purified and characterized.

Bacteria exhibit chemotactic behavior toward certain chemicals to which they are either attracted or repelled. Receptors in the cell membrane are used to monitor the concentration of such chemicals and to signal the flagella motor to turn on or off, which, in a cumulative manner over time, results in movement of the cell either toward or away from the chemical. Methylation/demethylation at four glutamic acid residues on the CheR membrane receptor protein is part of the signaling cascade. A methyltransferase and a methylesterase (which removes the methyl groups) have been implicated in this process. Inactivation of either one of these enzymes leads to loss of chemotactic behavior. Presumably, changes in the cell's environment alter the pattern of methylation/demethylation at the four glutamic acid residues and the subsequent response of the flagellar motor. CheR methylation/demethylation is the only known instance of a reversible methylation involved in regulating a protein's activity.

The *prmA* (protein methyltransferase) and *prmB* genes encode protein methyltransferases that form methyllysine on protein L11 and N^5-methylglutamine on protein L3, respectively. Although these methyltransferases are found in many bacterial species, *E. coli* strains deleted for each of these genes are viable indicating that these methylated derivatives are not essential for protein synthesis. The *prmC* gene of *E. coli* encodes a protein N-5 glutamine methyltransferase that methylates residues in release factors 1 and 2. Methylation of these release factors greatly increases the efficiency of the termination reaction of translation when proteins are released from the ribosome. Further evidence for the importance of this methyltransferase is that *E. coli* strains deleted for the gene are not viable.

Flagella are responsible for the bacterium's movement, and are composed of a structural protein called flagellin. The FliB protein methyltransferase produces *N*-methyllysine in flagellin. The physiological role for this methyltransferase, however, is unclear since *E. coli* bacteria missing this enzyme are not impaired in flagellar locomotion. Similarly, mutant strains of *E. coli* lacking methylated lysines and glutamic acids in ribosomal proteins show no impairment of growth. Spontaneous chemical degradation of aspartic acid to *iso*-aspartic can occur and subsequent methylation of the *iso*-aspartic residue is part of a protein repair pathway. Mutations eliminating the methyltransferase, however, appear to have no effect on *E. coli* viability.

Pili (fimbriae) are rod-like appendages the bacterial cell uses to attach to various cells. For example, pathogenic bacteria often use pili to attach to host cells in order to infect them. Pili are composed of pilin protein, and type 4 pilin has been reported to contain *N*-methylphenylalanine at its N-terminal end. Methylation may be necessary as a transport signal for pilin to reach its destination. However, there are no reports in the literature on how this methylated amino acid is formed, although the action of a specific methyltransferase would seem probable.

The translation elongation factor, EF-Tu, is monomethylated at lysine-56 in growing bacteria but is gradually converted into dimethyllysine as *E. coli* cells enter the stationary phase of growth. The physiological significance of this observation currently remains unknown.

Recent studies have shown that methylation plays a role in virulence of *Rickettsia*. Two protein lysine methyltransferases were identified that preferentially methylate outer membrane protein B (OmpB) to generate mono-, di-, and tri-methylated lysine residues. Using an *in vitro* methylation assay, the consensus motifs for methylation were characterized to be KX(G/A/V/I)N and KT(I/L/F). A correlation between the protein methylation states and virulence was found, indicating that lysine trimethylation is crucial for *Rickettsia* virulence.

Other Modifications

Over the last decade, proteomics technology has been rapidly developed for characterization of bacterial proteins. Many new protein post-translational modifications (PTMs) were identified including tyrosine phosphorylation. The most frequently observed PTMs include acetylation, carboxylation, citrullination, deamidation, glutathionylation, hydroxylation, methylation, methylthiolation, S-nitrosylation and nitration, phosphorylation, and proteolysis. Recent availability of proteomic tools allows a large-scale analysis to identify thousands of PTM sites in one experiment by searching mass spectrometric data against the sequences of the genome-wide encoded proteins. The large-scale identification of PTM sites is carried out by a combination of the enrichment of specific modified proteins/peptides and mass spectrometric analysis (Figure 7). For identification of acetylation in proteins, pan-antibodies (antibodies recognized modified amino acids or peptide) against acetylated peptides have been developed to enrich acetylated peptides. However, investigations into functions and regulations of a specific PTM are still needed.

Phosphorylation of proteins, predominantly at histidine residues, is a common mechanism of signal transfer in 'two-component' regulatory systems, such as that described for chemotaxis above. Stimulation of the surface receptor, Mcp, induces a phospho-relay system through several chemotaxis (Che) proteins, leading eventually to signaling the flagellar motor to rotate or not. Although histidine phosphorylation was discovered 50 years ago and was functionally important in central metabolism and bacterial cell signaling, the large-scale analysis of protein histidine phosphorylation is a difficult task due to the acid labile nature of the phosphohistidine residue. Recently, pan anti-phosphohistidine antibodies have been developed and successfully applied into immuno-detection and identification of histidine-phosphorylated proteins.

Phosphorylations at Ser/Thr/Tyr residues have been extensively characterized in different bacterial species. In *E. coli*, 81 phosphorylation sites from 79 proteins were identified with distribution of Ser/Thr/Tyr phosphorylation sites 68%/23%/9% respectively. In *Mycobacterium tuberculosis*, 516 phosphorylation sites were identified from 301 proteins. PknG, one of the 11 Ser/Thr protein kinases encoded by the *Mycobacterium tuberculosis* genome, has been extensively studied for its function and regulation. The autophosphorylation of PknG mediates the survival of pathogenic mycobacteria in host macrophages.

Tyrosine phosphorylation in bacteria is regulated by tyrosine kinases containing Walker A and B motifs and phosphatases including the eukaryotic-like phosphatases (PTPs), dual-specific phosphatases and the low molecular weight protein-tyrosine phosphatases (LMW-PTPs). In *E. coli*, both tyrosine kinase Wzc and phosphotyrosine-protein phosphatase Wzb are involved in exopolysaccharide synthesis, and in turn regulate pathogenicity of bacteria. Wzc also possesses an ATPase activity regulated by autophosphorylation. Quantitative phosphoproteomics has also been applied to characterization of the dynamics of the *E. coli* phosphoproteome showing that a global increase of protein phosphorylation levels occurs in late stationary phase.

Protein acetylation can occur at amino groups of the protein N-terminus or lysine residues, so called protein N-acetylation. Unlike the prevailing existence of protein N-terminal acetylation in eukaryotes, protein N-terminal acetylation was rarely observed in prokaryotes. Three N-terminal acetyltransferases in *E. coli*, Rime, RimJ and RimL, specifically modify ribosomal proteins S18, S5 and L12, respectively. The level of N-terminal acetylation is correlated with the growth phase in *E. coli*. Until

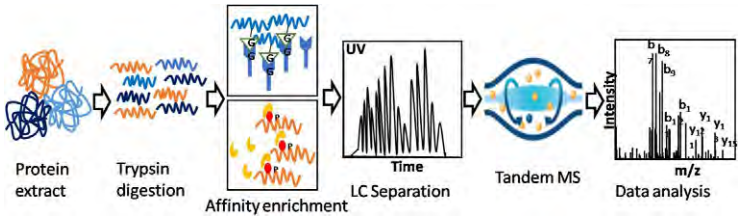


Figure 7 Proteomic workflow for identification of protein post-translational modifications. Proteins from bacterial samples are enzymatically digested; peptides with a specific modification enriched by affinity chromatography, followed by analysis with nano-flow chromatography coupled with tandem mass spectrometry.

recently, only a few acetylation events were observed in bacteria. Proteomic analysis has greatly increased the number of acetylation sites identified. Using a combination of immunoprecipitation and mass spectrometry analysis, hundreds of acetylation sites have been identified in *E. coli* and in *Salmonella*. Proteins targeted by lysine-acetylation mediate a variety of cellular processes. For example, acetylation of the chemotaxis response regulator CheY decreases its binding to the promoters of the targeted genes.

Protein acetylation plays a crucial role in regulation of cellular metabolisms. Many metabolic enzymes are acetylated mostly by Gcn5-related N-acetyltransferases (GNATs). Pat/YifQ, one of the extensively characterized GNAT in *Salmonella enterica* negatively regulates acetyl-CoA synthetase activity once it is acetylated at Lys609. Deacetylation of acetyllysine residues in proteins is carried out by sirtuin-like NAD-dependent deacetylases. CobB, the best-characterized sirtuin in bacteria, plays important roles in regulation of cellular metabolism. One of the best-characterized substrates of CobB is acetyl-CoA synthetase. Acetylation of acetyl-CoA synthetase inhibits its activities but the deacetylation by CobB reactivates the activities of acetyl-CoA synthetase. Acetylation and deacetylation of ribosomal proteins have been reported, suggesting acetylation may have a role in protein synthesis in bacteria.

Concluding Remarks

It will not have escaped the reader's attention that although there are modifications in DNA, RNA, and protein in *E. coli*, we know little about their functions. Some progress has been made for N^6-methyladenine in DNA in Enterobacteria but not for the more widespread cytosine methylated bases. In heavily modified RNA, we have only a vague notion as to their function. Protein phosphorylation relay systems are well established but other modifications have only been studied in sporadic instances. Research into the modification of polymers would seem to be a fertile field given all these unknowns. It is probable, however, that future insights into the role of these modifications will come from two areas other than traditional fundamental research in *E. coli*. The first is from other bacteria through computational comparative genomics of sequenced genomes where open reading frames encoding conserved predicted proteins with amino acid motifs and/or folds associated with binding of co-factors (such as SAM) identify potential modification enzymes. The second is through research on bacteria of medical, commercial, or environmental importance since it is in this area that most current research is focused. The rapid development of genomics and proteomics technologies has made it possible to identify chemical modifications on nucleic acids and proteins in a high throughput manner. The next challenge is to uncover the biologic meanings of these modifications.

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Microbial Adhesion☆

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Defining Statement

Microbial adhesion is crucial to the survival and lifestyle of many microorganisms. Both beneficial and pathogenic relationships forged between microbe and host depend on adhesive events and colonization. This article highlights the highly evolved microbial adhesion mechanisms and discusses the prevalence and implications of adhesion in diverse ecosystems.

Introduction

From the center of the earth and deep-sea vents to plant roots and the human intestine, microorganisms occupy remarkably diverse niches on our planet. These microbes include bacteria, archaea, fungi, and protista, and are found attached to rocks and soil particles, corals, and ocean sponges. Bacteria, for example, symbiotically colonize plants and humans as well as fish and squid, resulting in mutual benefit to both microbe and host. Pathogenic and unwelcome bacteria can egress from their native niche and adhere to and infect other sites and host tissues, leading to cellular injury and disease. Microbes also adhere to the hulls of ships and to machinery in food processing factories, resulting in contamination and adverse circumstances. Specific adhesion strategies have evolved in order to facilitate microbial attachment to diverse substrata in both symbiotic and pathogenic associations. Understanding the molecular mechanisms and functional implications of microbial adhesion is crucial for generating complete descriptions of our ecosystems, understanding and predicting ecosystem stability due to globalization and climate change, and attempting to control and prevent the unfortunate and often devastating consequences of infectious diseases. Thus, microbial adhesion is a fundamental component of the field of microbial ecology. This article will focus on the adhesive strategies employed specifically by bacteria, though many parallels can be found in the arsenal of adhesive strategies harbored by the other classes of microbes. We will highlight several exciting and up-to-date scientific discoveries as a platform to illustrate the biological significance and implications of microbial adhesion.

Biological Significance of Microbial Adhesion

The propensity for bacteria to associate with surfaces (living or abiotic) in nearly all ecosystems far exceeds the tendency to persist in suspension, living freely in a planktonic state. Attachment to surfaces allows bacteria to persist in advantageous locations where there may be high nutrient concentrations or to provide protection from hostile environments. In numerous instances, bacteria form biofilms – structurally complex and dynamic bacterial communities. The metabolic labor of acquiring nutrients is divided, sometimes according to spatial coordinates in the community, and distribution is promoted through an organized architecture of community members. Protection from harsh environmental conditions is a major benefit of life in a biofilm and the first line of defense is provided by members residing at the edges of the community. Under certain conditions, bacteria disperse from the biofilm, to seek a new environment and potentially re-adhere and colonize new niches. Adhesion events are crucial to biofilm formation, growth, and development. We set the stage for discussing the mechanisms of microbial adhesion by first illustrating a few examples across a broad landscape in which bacterial adhesion (often followed by biofilm formation) takes place.

Adhesion in the Water

The coral reefs are home to an enormous diversity of marine life, including charismatic and commercially important species of fish, mollusks and urchins, and the significantly smaller microorganisms with which they cohabit. Bacteria are, in fact, an integral constituent of the microbiota of healthy corals. They colonize distinct sites in coral tissue including the surface mucous layer and porous components in the coral skeleton where they fix nitrogen, decompose chitin, and provide organic compounds. The molecular mechanisms of adhesion and the sustained interactions between bacteria and their coral hosts are currently not well understood but are key questions being addressed in the emerging field of coral microbiology. Understanding the microbial interactions that promote health versus those that cause disease is important in efforts to preserve and prevent further destruction of coral reefs worldwide. Adhesion of bacteria in many water environments inevitably has detrimental consequences. Bacterial adhesion and biofilm formation on the hulls of ships creates resistance to water flow, increases drag, and thus decreases the efficiency of movement through the water. Microbial fouling is an economic and environmental burden in this way and in several industrial settings including drinking water pipes and oil pipelines, where the proliferation of sulfide-producing bacteria leads to the

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deterioration and corrosion of the steel surfaces. Understanding the mechanisms of adhesion is key to developing strategies to control and prevent these adverse and costly consequences.

Adhesion to Plants

Rhizobia are Gram-negative soil bacteria that adhere to and colonize the root cells of leguminous plants, including soybeans and alfalfa. Upon entry into a root hair, rhizobia traverse a distance to the center of the root hair cell and together with proliferating plant cells form a nodule. Here, rhizobia fix nitrogen, converting molecular nitrogen (N2) from the air into ammonia, nitrates, and other nitrogenous compounds to support plant metabolism. Rhizobia are particularly important to plants in nitrogen-deficient soils. In return, rhizobia receive carbon rich organic compounds, important for their own energy production, from the plant. Other beneficial symbionts include *Bacillus thuringiensis*. This bacterium is an important Gram-positive pathogen whose insecticidal properties have gained attention in the development of crops genetically modified to express the bacterium's potent toxin, now referred to as Bt transgenic crops. In the wild, *B. thuringiensis* colonizes the surface of some plants and exists naturally in some caterpillars. The bacterium produces a unique kind of endotoxin, a proteinaceous crystal that is lethal to several pests, including flies, mosquitoes, and beetles, upon ingestion. This symbiosis with plants is dependent on initial host–microbe adhesion events. The attractive chemical signals and ultimate adhesive interactions *of Agrobacterium tumefaciens* with wounded plants leads to the unfortunate development of tumors on the lower stems and main roots, the hallmark of Crown Gall diseases. Attachment is the first step in the pathogenic cascade and takes place in the soil around the roots –the rhizosphere. In a two-step adhesive process, initial weak binding interactions are followed by the bacterial expression of multiple gene products to synthesize cellulose and anchor the microbe to the host tissue, while enhancing adhesive interactions between bacteria in the microcolony. Adhesive plant proteins called vitronectins are also implicated in the adhesion process. Subsequent DNA transfer and integration of a specific fragment of DNA (the transfer DNA) from the bacterium to a plant cell results in the expression of several oncogenic genes and the formation of tumors.

Adhesion in the Human Host

The normal and healthy human body is composed of approximately ten times more bacterial cells than human cells. These bacteria comprise our microbiota and are colonized in distinct sites throughout the body, including the skin and mouth, the small intestine, and colon. In the mouth and small intestine, bacterial adhesion is critical to maintenance of microbial populations, where either salivary flow or movement of contents eliminates the nonadherent bacteria. Our microbiota is, in general, beneficial; for example, bacteria in the gut degrade some otherwise indigestible polysaccharides into carbon and energy sources. Recent results indicate that the balance of bacterial populations in the gut influence caloric intake through complex interbacterial metabolic networks and further study may help to understand and potentially control (decrease or increase) caloric uptake. Indeed, the microbiota is dynamic and shifts in balance that alter the relative numbers of different bacterial populations can also lead to proliferation of disease-causing opportunistic pathogens. In addition, the inoculation of the human host with bacteria from the environment is a common source of infectious disease, particularly in the hospital setting. The consequences of bacterial adhesion in human infectious diseases are numerous and will be addressed in more detail after the description of specific adherence mechanisms.

Mechanisms of Microbial Adhesion

General Physicochemical Factors Affecting Adhesion

Mechanisms of microbial attachment are incredibly diverse and can be generally classified as either general nonspecific interactions or specific molecular-recognition binding events that involve the presentation of specific adhesive proteins on the bacterial cell surface. Of course, multiple mechanisms can act cooperatively to promote adhesion. A successful adhesive event depends on properties of both the bacterium and the substratum. Nonspecific interactions are the primary form of attachment to abiotic surfaces in aquatic and soil environments. Van der Waals interactions are attractive, usually weak, noncovalent forces that can operate at large separation distances (>50 nm) between the bacterium and the surface. At smaller distances (10–20 nm), electrostatic interactions participate and compete as attractive and repulsive forces. The net surface charge of most bacteria is negative due to cell wall and cell membrane components including negatively charged phosphate groups, carboxyls, and other acidic groups, in addition to surface-exposed proteins. Thus, bacteria like to adhere to positively charged surfaces. Typical binding surfaces, however, have a net negative surface charge, creating electrostatic repulsion that must be overcome by other physicochemical factors. The entire binding process is akin to a tug-of-war. The ionic strength of the surrounding medium affects the electrostatic interactions, and the aforementioned repulsion is eliminated, for example, in most aquatic environments due to high ionic strength resulting from high salt concentration. In the range of near contact (0.5–2 nm), hydrophobic interactions are important for bacterial adhesion. Energetically, the association of nonpolar groups on a bacterial surface with hydrophobic surfaces compensates for the unfavorable displacement of water molecules at that surface. When separated by less than 1 nm, stronger interactions including hydrogen bonding and the formation of salt bridges contribute to surface adhesion.

Specific Adhesin–Receptor Mechanisms

On many biotic surfaces, the adhesive forces and interactions described above promote the formation of an initial interface, but require concomitant or subsequent specific adhesive interactions to enable firm adhesion. Adhesin is the term ascribed to the surface-exposed bacterial molecule that mediates specific binding to a receptor or ligand on a target cell. It is not unusual for bacteria to harbor several types of specific adhesive machinery to provide adhesive capacity to multiple receptor molecules or to permit adhesion under changing environmental conditions such as temperature, pH, or nutrient status, where one adhesive strategy may be more effective than another. Bacteria can produce a diverse array of adhesins with varying specificities for a wide range of host receptor molecules. Adhesion mechanisms can be classified according to the type of adhesin–receptor pair. Many bacterial adhesins function as lectins and the interactions between bacterial lectins and host cell carbohydrates are among the best-characterized attachment processes. Hallmark examples of carbohydrate recognition include *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* adhesion in the respiratory tract, *Escherichia coli* adhesion in the urinary tract and intestine, and *Helicobacter pylori* adhesion in the stomach. Other adhesins recognize specific amino acid-recognition motifs in proteins expressed on host cell surfaces. Extracellular matrix (ECM) proteins that are not directly integrated into the host cell also serve as attractive binding platforms for many bacteria, such as *Enterococcus faecalis* and *Staphyloccocus aureus*, and numerous adhesins bind to these components in order to indirectly hijack the host signaling pathways, often to enable host cell internalization. Another general category of adhesins includes nonproteinaceous molecules such as lipopolysaccharides and teichoic acids, synthesized by Gram-negative and Gram-positive bacteria, respectively.

Most adhesins are incorporated into heteropolymeric extracellular fibers called pili or fimbriae. Bacteria invest enormous cellular resources to assemble fimbriae in order to present adhesins at the right time and the right place to initiate attachment when conditions are favorable and to permit detachment when necessary. Indeed, hundreds of such fibers have been described in Gram-negative organisms, and although they have diverse functions, many appear critical to binding, invasion, and survival of pathogenic microorganisms in the human host. Distinct assembly mechanisms have emerged as the most well studied and include the chaperone–usher pathway (CUP pili), the general secretion pathway (type 4 pili), the extracellular nucleation–precipitation pathway (curli), and the alternate chaperone pathway (class 5 pili). Gram-positive pathogens also produce adhesive pili. Unlike their Gram-negative counterparts, Gram-positive pili are formed by covalent polymerization of pilin subunits, for example the endocarditis and biofilm-associated pili (Ebp) from *E. faecalis* and Pilus Island 1 (PI-1) from *S. pneumonia*. A representative set of fimbrial adhesins is provided in Table 1 for Gram-negative and Table 2 for Gram-positive.

Table 1 Representative Gram-negative fimbrial adhesins, adhesins, and disease association

Organism (s)	*Adhesin*	*Assembly proteins*	*Associated fiber*	*Associated disease(s)*
Escherichia coli	FimH	FimC/FimD	Type 1 pili	Cystitis
	PapG	PapD/PapC	P pili	Cystitis/pyelonephritis
	PrsG	PrsD/PrsC	Prs pili	Cystitis
	SfaS	SfaE/SfaF	S pili	UTI, newborn meningitis
	CooD	CooB/CooC	CS1 pili	Diarrhea
	DraE	CsgA (major subunit) CsgB (nucleator), CsgE/CsgF (assembly), CsgG (secretion) DraD and DraC	Curli	Sepsis
			Dr fimbriae	Cystitis, chronic pyelonephritis, Gestational pyelonephritis
Proteus mirabilis	AipA		MR/K pili	Pyelonephritis
	Taap		MR/P pili	UTI and pyelonephritis
			PMF pili	UTI
				UTI and pyelonephritis
				UTI and pyelonephritis
Salmonella typhimurium		PefD/PefC	Pef pili	Gastroenteritis
		LpfB/LpfC	Long polar fimbriae	Gastroenteritis
Salmonella enteritidis	AgfA	AgfC (nucleator)	Sef17 (thin aggregative fimbriae)	
Klebsiella pneumoniae	$FimH_{52}$	FimC/FimD	Type 1 pili	UTI, CAUTI
	MrkD	MrkB/MrkC	MR/K (type 3) pili	UTI, CAUTI, Pneumonia
Bordetella pertussis	FimD	FimB/FimC	Type 2 and 3 pili	Whooping cough
Yersinia enterocolitica		MyfB/MyfC	Myf fimbriae	Enterocolitis
Neisseria gonorrhoea	PilC	General secretion apparatus	Type 4 pili	Gonorrhea
Haemophilus influenzae	HifE	HifB/HifC	Hif pilus	Otitis media, meningitis

Table 2 Representative Gram-positive fimbrial adhesins, adhesin, and disease association

Organism (s)	*Adhesin*	*Assembly proteins*	*Associated fiber*	*Associated disease(s)*
Enterococcus faecalis	EbpA	SrtC, SrtA	Ebp pili	CAUTI, UTI,
	Ace	SrtA		Endocarditis
	Esp	SrtA		UTI, endocarditis
	ElrA	SrtA		UTI, endocarditis
	AS proteins	SrtA		Peritonitis
				Endocarditis
Staphylococcus aureus	ClfA	SrtA		Endocarditis, sepsis
	ClfB	SrtA		Sepsis
	FnbpA	SrtA		Sepsis
	FnbpB	SrtA		Sepsis
Streptococcus pneumonia	RrgA	SrtC-1, SrtC-2, SrtC3, SrtA	PI-1 pili	Pneumonia, bacteremia

Some bacteria present afimbrial adhesins on their surface, including Staphylococcal species, which solely use afimbrial adhesive factors and do not encode pili. Afimbrial adhesins are expressed as monomeric proteins or protein complexes that assemble at the cell surface and recognize host cell surface elements. Staphylococci have a variety of cell wall bound adhesins that binds a number of host factors, including fibrinogen and fibronectin. These adhesive factors are essential for infective endocarditis, systemic infection, and kidney abscess formation. *E. faecalis* also has a number of adhesins such as Ace, ElrA, and Esp; for example, Ace binds to collagen and laminin and important during UTI and endocarditis. Adhesive autotransporters represent a class of afimbrial adhesins expressed by a variety of unrelated microorganisms, including species of *Rickettsia*, *Bordetella*, *Neisseria*, *Helicobacter*, and many members of the family Enterobacteriaceae. *H. influenzae*, a causative agent of sinusitis, bronchitis, otitis media, and pneumonia, expresses an adhesive autotransporter termed Hap. Hap mediates binding to laminin, fibronectin, and collagen, all components of the ECM.

The most comprehensive descriptions of bacterial adhesion have emerged from studies of pathogenic bacteria involved in infectious diseases. Examples of these host–microbe interactions as well as some involved in the attachment of bacteria to plants, either as symbionts or as pathogens, are described in more detail below to highlight the remarkable diversity, specificity, and complexity among microbial adhesive strategies.

Selected Survey of Specific Adhesion Strategies

Pilus-Mediated Adhesion to Carbohydrates in the Urinary Tract

Uropathogenic *E. coli* (UPEC) colonize the gut as well as the genitourinary tract and produce numerous important adhesins and adhesive CUP pili organelles to mediate adhesion in these niches. A recent analysis of 35 *Escherichia* spp. genomes and 132 plasmids identified a total of 458 CUP operons, representing 38 distinct CUP pilus types based on usher phylogeny. Single *Escherichia* genomes can have as many as 16 distinct, intact CUP operons encoding adhesins with sterochemical specificity for their ligands, which have important influences on microbial tropisms. For example, FimH and PapG adhesins are presented at the tips of type 1 and P pili, respectively. FimH-presenting type 1 pili are required for *E. coli* to cause cystitis, or infection of the bladder, and PapG presenting P pili are associated with pyelonephritis, infection of the kidney. Type 1 and P pili are composite heteropolymeric structures, with a distal tip fibrillum joined to a thicker rigid helical rod and both are assembled by the chaperone–usher system. These systems are prototypes for the 100's of chaperone–usher systems that have been identified through comparative genome analyses of pathogens including *E. coli*, *Salmonella*, *Haemophilus*, *Klebsiella*, and *Yersinia*.

In each chaperone–usher system, pilus assembly requires a unique assembly protein pair (a chaperone and a usher) to facilitate the folding, transport, and ordered assembly of pilus subunits at the cell surface. This process begins in the periplasm after subunit expression and translocation by the general secretory pathway into the periplasm. Periplasmic pilus chaperones consist of two immunoglobulin (Ig)-like domains and bind to and assist folding of subunits to keep their interactive surface capped and prevent nonproductive subunit aggregation. Pilin subunits also have an Ig-like fold, but they lack the seventh β strand, thus exposing the hydrophobic core. In a process termed donor strand complementation, the chaperone's G1 β-strand serves as the pilin's seventh strand, catalyzing the folding of the subunit. Chaperone–subunit complexes are targeted to their cognate outer membrane usher to facilitate chaperone uncapping, translocation of subunits across the outer membrane, and pilus assembly. Each cognate usher consists of five functional domains: a 24 stranded transmembrane β-barrel translocation domain (TD), a β-sandwich plug (PLUG) that resides in the pore of the TD in the apo-usher, an N-terminal periplasmic domain (NTD) and two C-terminal periplasmic domains (CTD1 and 2). Binding of the chaperone-adhesin complex to the usher's NTD results in the PLUG domain being translocated into the periplasmic space and a conformational change in the TD from the apo, kidney shaped conformation (52 x 28 Å) to a circular form (44 x 36 Å). This conformational change likely facilitates the extrusion of folded pilins (~20–25 Å in diameter) across the outer membrane. After translocation into the periplasmic space the PLUG domain mediates a high affinity interaction with the NTD of the usher. Thus, the PLUG

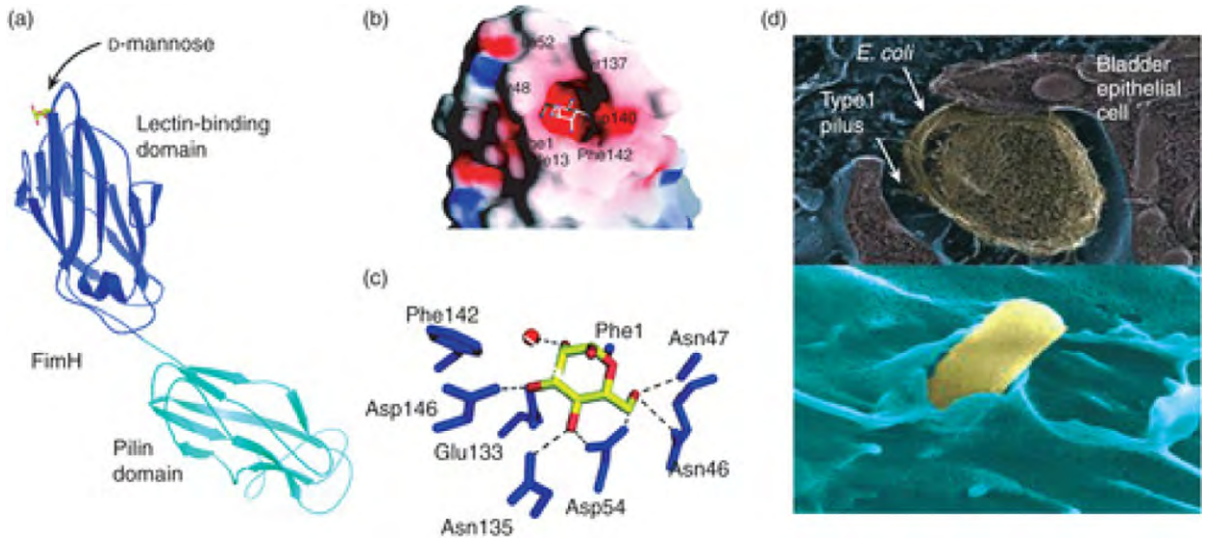

Figure 1 The FimH adhesin and type 1 pili-mediated adhesion of *E.coli*. (a) The ribbon representation of FimH (from the crystal structure of the FimCH complex). D-mannose is located at the top of the molecule. (b) Molecular surface representation in which the electrostatic potential surface with positively charged residues is shown in blue, negatively charged residues in red, and neutral and hydrophobic residues in white. Residues defining the hydrophobic ridge around the mannose-binding pocket are labeled. (c) The mannose-binding site with FimH residues. Mannose residues are shown with carbon atoms in yellow, oxygen atoms in red, and nitrogen atoms in blue. (d) Type 1 pili-mediated attachment of uropathogenic *E. coli* (UPEC) to the luminal surface of the bladder epithelium. (Top) High-resolution, freeze-fracture, deep-etch electron micrograph is from Mulvey MA, Lopez-Boado YS, Wilson CL, *et al.* (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic *E. coli*. *Science* 282: 1494–1497. Reprinted with permission from AAAS. (Bottom) Scanning electron micrograph of a bacterium entering the membrane of bladder epithelial cells is reprinted from Soto GE and Hultgren SJ (1999) Bacterial adhesins: Common themes and variations in architecture and assembly. *Journal of Bacteriology* 181: 1059–1071.

gates the TD, such that in the absence of pili the PLUG prevents large molecules from flowing freely across the outer membrane. The PLUG, the NTD, CTD1 and CTD2 work together in the assembly function of this molecular machine. This occurs via a process termed donor strand exchange, in which the G1 β -strand of the chaperone is replaced by an N-terminal extension of the next pilus subunit. Thus in the mature pilus, each subunit incorporates its neighbor's N-terminal extension as part of its own Ig fold. Subunits have distinct specificity for other interactive subunits and distinct roles in pilus adhesion, initiation, elongation, termination and regulation.

The FimH adhesin, incorporated at the tip of the type 1 pilus, consists of a pilin subunit and the receptor binding domain (Figure 1). The primary carbohydrate specificity of FimH is mannose. Interestingly, different *E. coli* isolates present FimH variants (specific allelic variations in protein sequence and structure) that exhibit varying specificities for monomannose and trimannose binding. FimH expressed by most commensal isolates of the intestine exhibit a higher specificity for trimannose presenting glycoprotein receptors, whereas urinary tract isolates encode for a FimH variant with higher affinity for monomannose. In the latter, FimH mediates adhesion to the monomannose-containing glycoprotein uroplakin Ia that is expressed on the surface of superficial facet cells –the epithelial cells that line the lumen bladder (Figure 1). Further, the FimH sequences of uropathogenic *E. coli* strains (UPEC) have invariant mannose binding pocket residues; however, there are some residues outside of the binding pocket that are under positive selection in clinical UPEC isolates that influence mannose binding by influencing a conformational switch. FimH can adopt two conformations, compact and elongated, and switch between them, and the allelic variation of these residues can favor one state or another. The elongated conformation has high mannose binding ability, which is important for binding to the mannosylated-urothelial cells; while the compact form has low mannose binding ability. However, recent studies have shown that FimH variants, that maintain the elongated conformation and appear unable to effectively switch between conformations, were attenuated during chronic bladder colonization arguing that switching between conformations is important for *E. coli* pathogenesis in the urinary tract. Presented at the tips of P pili, the PapG adhesin mediates binding to a different carbohydrate receptor, the α-D-galactopyranosyl-(1–4)-D-galactopyranoside moiety of glycolipids presented by cells predominantly in the kidney. PapG variants (G-I, G-II, and G-III) exhibit altered specificities for three Galα(1–4)Gal-containing isoreceptors: globotriaosylceramide, globotetraosylceramide (GbO4), and globopentaosylceramide (the Forssman antigen). The demonstrated allelic variation in PapG and FimH binding specificities supports the notion that, through bacterial evolution, pathoadaptive mutations are selected for increasing the fitness of pathogenic organisms in distinct niches in the host.

Adhesion to ECM Components

The ECM contains a diverse array of oligosaccharides, proteoglycans, and proteins and functions to provide structural support and adhesive interactions among cells. Prevalent components include collagen, fibrinogen, fibronectin, laminin, and vitronectin, as well as molecules such as heparin sulfate and chondroitin sulfate. Fibronectin is present in most tissues and fluids of the body and helps to create a cross-linked network between cells by presenting binding sites for other ECM components, a process that pathogenic organisms exploit to gain a foothold in host tissue. The ability to adhere to ECM components is a primary adhesion mechanism that contributes to the virulence of many pathogenic microorganisms. *S. aureus* is a significant cause of nosocomial and often persistent infections. Among other ECM-binding proteins, *S. aureus* expresses the fibronectin-binding proteins FnBP-A and FnBP-B that permit adherence to fibronectin that are bridged to cellular integrins. This crucial binding event leads to host cell cytoskeletal rearrangements and invasion. *Streptococcus pyogenes* is armed with more than 12 fibronectin- and collagen binding proteins. Like the FnBP-A adhesin in *S. aureus*, the major *S. pyogenes* adhesin, SfbI, and the *Yersinia* adhesin, YadA, bind to fibronectin and bridge the bacteria to integrins, leading to integrin clustering and eventual internalization. Fibrinogen is present in tissues and fluids throughout the human body and helps control proper blood homeostasis through clotting. Clotting, or blood coagulation, is a tightly controlled process initiated during injury or infection through the release of specific signals that activate the host protein thrombin. Thrombin then cleaves soluble fibrinogen into insoluble fibrin cables creating clots. *S. aureus* encodes numerous adhesin proteins that bind fibrinogen and fibrin cables, including clumping factor A and B (ClfA and ClfB), which are essential during initiation and establishment of infection. Additionally, *S. aureus* is able to circumvent the tightly controlled clotting cascade through the secretion of proteins that induce cleavage of fibrinogen to fibrin cables, providing a substrate for ClfA and ClfB to bind and establish infection at almost any body site. Similar to the most of staphylococcal surface adhesins, ClfA and ClfB are covalently linked to the cell wall by the housekeeping membrane-bound transpeptidase sortase A (SrtA). These adhesins contain a C-terminal LPXTG motif that is recognized and cleaved by SrtA between the threonine and glycine and the carboxyl group of threonine is then covalent linked to the amino group of peptidoglycan cross-bridges.

Similar *to S. aureus*, *E. faecalis* also binds to fibrinogen via Ebp pilus and this interaction is important for establishing a catheter-associated urinary tract infection and for binding to platelets. The Ebp pilus is composed of three structural proteins, EbpA (tip), EbpC (shaft) and EbpB (base), and its assembly and anchorage to the cell wall is dependent two sortases, SrtC (pilus-associated) and SrtA, respectively. All three subunits contain a LPXTG motif, which plays an important role for their incorporation and additionally, EbpC and EbpB contain a pilin-like motif, which important for polymerization of the pilus fiber. The pilin motif is located in the N-terminal domain of the protein and contains a lysine residue that is involved in the formation of an intermolecular bond between of an incoming subunit and the threonine residue of the LPTXG of the recently incorporated subunit. EbpA is the first subunit to be incorporated by SrtC, which cleaves the LPXTG motif and linked to the pilin motif Lys residue of EbpC and this step is followed by EbpC polymerization, which is mediated by SrtC. After polymerization of EbpC, EbpB, the base subunit, is incorporated into the growing fiber via its pilin motif, and then SrtA anchors the fully polymerized pilus fiber into the cell-wall.

Contrary to the previous examples, *Yersinia* has an adhesin, invasin that bypasses the ECM and binds directly to integrin transmembrane receptors. Other less-ubiquitous ECM components also serve as binding receptors for bacterial adhesins and their sites of expression often relate to the tissue tropism of a particular bacterial pathogen.

Curli-Mediated Multipurpose Adhesion

Curli are a unique class of adhesive extracellular amyloid fibers produced by Gram-negative bacteria, including *E.coli*. The highly homologous fibers produced by *Salmonella* species are called Tafi (thin aggregative fimbriae). The fibers mediate biofilm formation and attachment to host proteins including fibronectin, laminin, and plasminogen, and have been implicated in human sepsis. When expressed together with cellulose, curli and Tafi contribute to a remarkable aggregative phenotype characterized by a patterned assembly of cells radiating from the center when grown on a surface such as agar. Curli are assembled by the nucleation–precipitation pathway, and assembly requires specific molecular machinery encoded by the *csg*BA and *csg*DEFG operons (Figure 2). The major subunit protein (CsgA) and the nucleator protein (CsgB) are secreted to the cell surface in a CsgG dependent fashion. CsgE and CsgF are assembly factors required for the stabilization and transport of CsgA and CsgB. Transcriptional regulation of the curli operons is complex and responds to many environmental cues including temperature, pH, and osmolarity. The adhesive functionality is attributed to the main fiber subunit, CsgA. Curli are also implicated in the binding of *E. coli* strains to plant surfaces and are expressed by many strains associated with food-borne illness, including the prototype strain *E. coli* O157:H7, which has caused several foodborne outbreaks in the United States and around the world. Although the exact nature of binding is still under investigation, curli production is sufficient to permit laboratory strains of *E. coli* to bind plant tissues, such as alfalfa. However, among pathogenic strains such as *E. coli* O157:H7, there appear to be redundant adhesion systems, and under the conditions tested, curli are not required for adhesion. Indeed, external conditions in the environment and in the host may differ as a function of time, and bacteria may depend more on one adhesive system than another in certain circumstances.

The curli bacterial adhesive fiber machinery has gained considerable attention since the discovery of curli as amyloid fibers in 2002. The sticky nature of curli amyloid fibers is like that of amyloid aggregates and plaques associated with eukaryotic amyloid

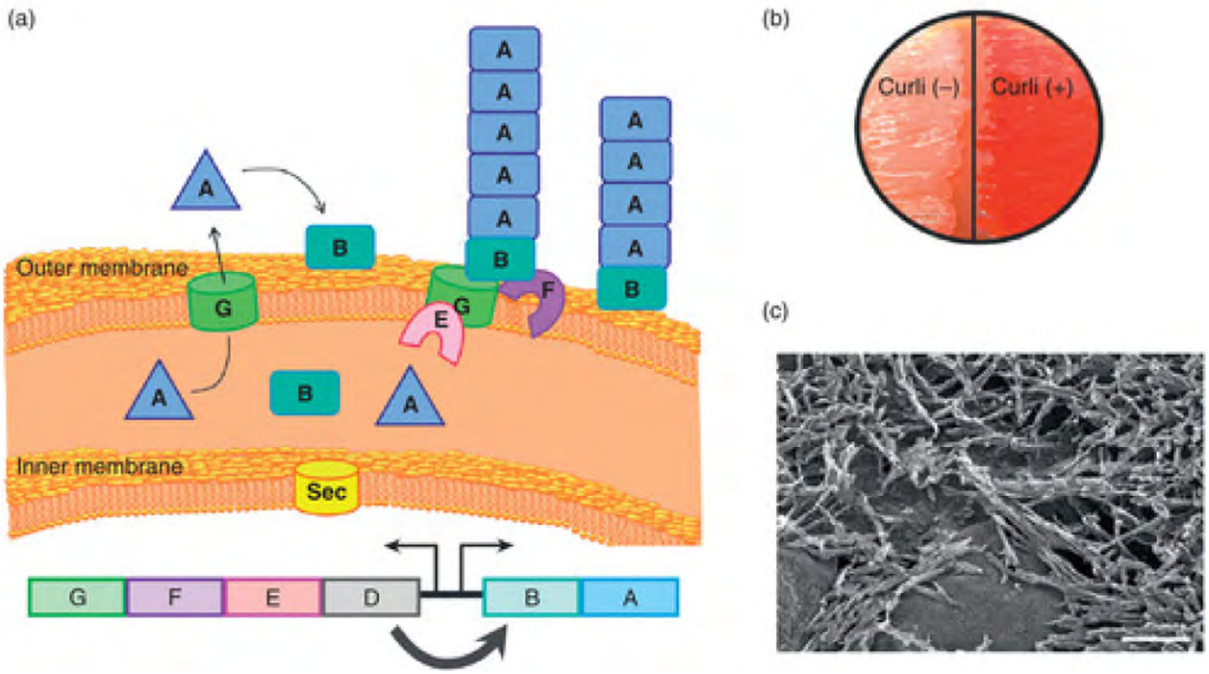

Figure 2 Curli biogenesis and biology. (a) Current model of curli biogenesis. Curli assemble through the nucleation–precipitation pathway. Polymerization of the major curli subunit protein, CsgA into β-sheet-rich amyloid fibers depends on the nucleating activity of the minor subunit, CsgB. Proteins CsgE, CsgF, and CsgG are assembly factors required for the stabilization and transport of CsgA and CsgB to the cell surface. (b) Congo red-binding phenotype. Curli are amyloid fibers and bind the hallmark amyloid dyes Congo red and thioflavin T. Curliated *E. coli* grown on Congo red-containing agar medium take up the dye and stain red. Noncurliated cells do not. (c) High-resolution deep-etch electron micrographs of curliated *E. coli*. From Chapman MR, Robinson LS, Pinkner JS, *et al.* (2002) Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science* 295: 851–855. Reprinted with permission from AAAS.

disorders such as Alzheimer's and Parkinson's diseases. Thus, ongoing curli research that aims to elucidate structural features of curli assembly and the functional implications of curli-mediated adhesion may also provide valuable information to the exciting field of amyloid fiber biogenesis and aggregation.

Consequences of Microbial Adhesion in Human Disease

The critical first step in most infectious diseases requires physical contact between a bacterium and host cell. Bacterial adhesins mediate this binding event through the sophisticated adhesion mechanisms described above and allow the pathogen to gain a foothold in the host, initiating complex signaling cascades in both the pathogen and the host. Binding events can lead to extracellular colonization and invasion into underlying host cells. Adhesion is the first step that promotes the cascading sequelae of infectious diseases, particularly important in the pathogenesis of chronic infections including urinary tract infection (UTI), chronic otitis media (middle ear infection), and chronic lung infections.

E. coli and UTI

UPEC engage in an incredibly coordinated and regulated genetic and molecular cascade to assemble type 1 pili, as described above. UTIs are among the most common bacterial infections and nearly 50% of women will be afflicted by at least one UTI in their lifetime, with many experiencing recurrent UTIs. Virtually all clinical UPEC isolates express type 1 pili, enabling them to bind the mannose-containing host receptors, which results in invasion of host bladder epithelial cells (Figure 3). Once inside the epithelial cells, UPEC is protected from innate immune defenses of the host, and a single bacterium can replicate to 10^4 or more bacteria within hours, forming intracellular bacterial communities (IBCs) that have biofilm-like properties. IBCs are transient in nature and upon maturation, bacteria from the IBC subsequently become filamentous as they disperse from the biomass and spread to neighboring host cells where they repeat the cycle. IBC formation is a general trait of UPEC and IBCs and the filamentous UPEC that emerge from them are significantly observed in urines from women with rUTI but not healthy controls, or in patients with a UTI caused by Gram-positive pathogens. Population dynamic studies revealed a strict bottleneck in acute infection. From an initial 10^7 inoculum, only $\sim 10^4$ UPEC invade the bladder tissue after infection and only one percent of those invaded bacteria go on to form

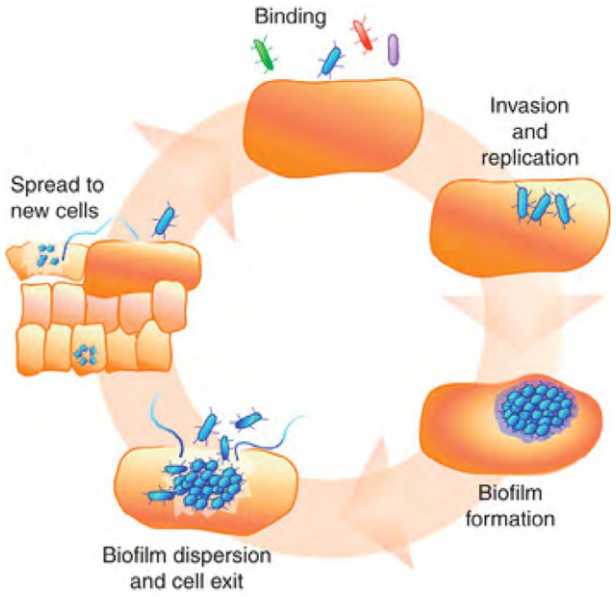

Figure 3 Pathogenic cascade of uropathogenic E. coli (UPEC). UPEC coordinate highly organized temporal and spatial events to colonize the urinary tract. UPEC bind to and invade the superficial umbrella cells that line the bladder lumen, where they rapidly replicate to form a biofilm-like intracellular bacterial community (IBC). In the IBC, bacteria find a safe haven, are resistant to antibiotics, and subvert clearance by innate host responses. UPEC can persist for months in a quiescent bladder reservoir following acute infection, and challenge current antimicrobial therapies. Quiescent bacteria can reemerge as pathogens from their protected intracellular niche and can be a source of recurrent urinary tract infections (UTIs).

IBCs, resulting in an average of 100 IBCs per infected mouse bladder. If this is extrapolated to human infection, this bottleneck likely prevents the majority of bacterial inoculation events into the bladder from leading to disease. However, the finding that a single infecting bacterium can replicate clonally within hours to 10^4 bacteria within a single host epithelial cell and then these bacteria can spread to neighboring epithelial cells to reinitiate the IBC process, suggests that introduction of even a single UPEC bacterium into the bladder can result in a UTI if it transcends the strict bottleneck. In humans, the ultimate outcome of UPEC infection of the UT ranges from asymptomatic bacteriuria to acute self-limiting infection to chronic/recurrent UTI. Placebo studies have shown that 50% of UTIs in humans do not resolve in the absence of effective antibiotic treatment, indicating the importance of clinical intervention for resolution of infection and highlighting the crisis that may ensue as multi-drug resistant pathogens continue to emerge, for example ST131. The outcome of experimental UPEC infection differs among inbred mouse strains, which is reflective of the different disease manifestations observed in humans. For example, in C57BL/6 mice, acute infection and bacteriuria resolves within 7–10 days of the initial infection. In contrast, mice belonging to the C3H and CBA backgrounds are susceptible in an infectious dose-dependent manner to long-lasting, chronic cystitis characterized by (i) high titers ($>10^4$ CFU) of bacterial burdens in the bladder tissue and persistent high titer bacteriuria ($>10^4$ CFU/ml) throughout infection; (ii) ongoing acute and chronic inflammation in the bladder, which prevents regeneration of the superficial facet cell layer; and (iii) a predisposition to severe rUTI upon subsequent bacterial challenge even months after antibiotic treatment to clear the initial chronic infection. In C3H mice, the induction of high levels of serum cytokines (IL-6, IL-5, G-CSF) and neutrophil chemokines (CXCL1 and CXCL2) at 24 hpi, is predictive of severe chronic cystitis.

P. aeruginosa and CF

P. aeruginosa has emerged as an opportunistic pathogen in several clinical settings, causing nosocomial infections such as pneumonia, UTIs, and bacteremia. *P. aeruginosa* adheres to the respiratory epithelium, leading to chronic lung infections in cystic fibrosis (CF) patients, responsible for the eventual pulmonary failure of most CF patients, typically by 37 years of age. Pilus-mediated adherence is important in the adhesion and early stages of epithelial colonization, and additional virulence factors contribute to the subsequent persistence in the lung. Alginate, for example, is a mucoid exopolysaccharide produced by *P. aeruginosa* that forms a matrix of 'slime' to surround a forming biofilm and anchors the cells to each other and to their host. Surrounded by alginate, the bacteria are protected from the host defenses and are often resistant to treatment with antibiotics.

S. aureus and Invasive Infections

S. aureus is an important human pathogen responsible for causing a wide range of invasive infections, including necrotizing pneumonia, bacteremia, infective endocarditis, shock following bacteremic UTI, among many other infections. *S. aureus* is able to establish such a wide range of infections because this pathogen encodes numerous adhesins, termed Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) that allow *S. aureus* to adhere to many different underlying host tissues and ECM proteins, including platelets, fibrinogen, fibronectin, and collagen. Interactions between *S. aureus* and ECM proteins allow the bacteria to form different types of community structures, including biofilm and agglutinates, which are important for establishment and persistence of disease. Biofilms form when *S. aureus* adheres to surfaces and develops attached communities that enable to the bacteria to persist during infection. *S. aureus* biofilms are particularly problematic during implant-associated infections such as those that can form on pace-makers, central lines, and prostheses, which are recalcitrant to treatment. These implants are typically coated with host ECM proteins upon implantation, providing a substrate for bacterial attachment and biofilm formation. Agglutination refers to the process by which individual bacteria interact through adhesin-ECM protein bridges creating tightly packed communities that can remain unattached to surfaces. These communities form in the absence of a traditional endothelial or epithelial substratum, such as in the blood stream or other body fluids, where high local concentrations and accessibility of ECM proteins provide a binding substrate for *S. aureus* adhesins. Agglutination in *S. aureus* occurs via interactions between the ClfA adhesin and the host ECM protein fibrinogen. ClfA specifically binds fibrinogen via the δ domains exposed on either end of the matrix protein, effectively linking fibrinogen to the cell surface. Two ClfA proteins binding δ domains on the same fibrinogen protein creates an ECM bridge and induces agglutination. The general ability of *S. aureus* to form tightly packed groups of cells aids survival in various host situations and these communities rely on adhesive protein interactions with host. ClfA is also an important immune modulator making this adhesin protein particular important for initiation and persistence of disease.

E. faecalis UTI and CAUTI

E. faecalis is able to cause ascending UTI and is a frequent causative agent of catheter-associated UTIs (CAUTI), and if untreated, can lead to serious complications including bacteremia and death. Urinary catheterization by itself causes histological and immunological changes in the bladder due to mechanical stress, resulting in a robust inflammatory response and edema of the lamina propria and submucusa, and mucosal lesion of the urothelium and kidney. Fibrinogen is released as part of the inflammatory response and accumulates in the bladder lumen, coating the urinary catheter. *E. faecalis* takes advantage of the availability of fibrinogen and uses it as bridge to form biofilm on the catheter. The interaction between *E. faecalis* and fibrinogen is mediated by the Ebp pilus adhesin, EbpA, which is important for establishment and persistence during CAUTI. Moreover, disruption of this interaction results in protection of the host.

Targeting Adhesion to Inhibit Bacterial Virulence

The ability to impair bacterial adhesion represents an ideal strategy to combat bacterial pathogenesis because of its importance early in the infectious process. In addition, adhesion is essential to the long-term persistence of bacteria in the pathogenic cascade of several infectious diseases. Moreover, the adhesion process can be targeted without placing life or death pressure on the bacterium, per se. Targeting bacterial virulence in this way is an alternative approach to the development of new therapeutics to disarm pathogens in the host that may offer reduced selection pressure for drug-resistant mutations. In addition, virulence-specific therapeutics could avoid the undesirable dramatic alterations of the host microbiota. Indeed, standard antibiotic treatment regimens may lead to the loss of symbiotic benefits and the proliferation of disease-causing opportunistic pathogens. As emphasized earlier, pathogens are capable of presenting multiple adhesins that can be expressed differentially to permit binding in specific sites and at specific times over the course of a complex infectious cycle. Thus, it may be difficult to develop a universal

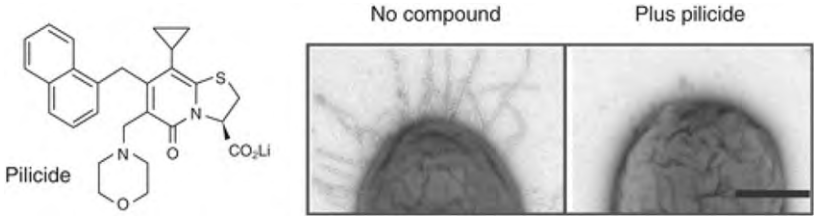

Figure 4 Targeting microbial adhesion. Rationally designed 'pilicides' inhibit pilus biogenesis by disrupting chaperone–usher protein interactions and reduce piliation levels dramatically. Electron micrographs reproduced from Pinkner JS, Remaut H, Buelens F, et al. (2006) Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. Proceedings of the National Academy of Sciences of the United States of America 103: 17897–17902. Copyright (2006) National Academy of Sciences, U.S.A.

class of antiadherence drugs. Nevertheless, several specific pathogenic adhesive strategies have emerged as hallmark requirements for virulence in certain infectious diseases, and represent amenable targets for drug discovery and development. Adhesion is sometimes just the first step of many in pathogenic cycles, yet targeting adhesion holds value even after an infection has been established. In biofilm-associated infections, for example, drug development strategies include attempts to induce the dispersal of bacteria from the biofilm and to inhibit the chemical signaling necessary to encourage new biofilm formation. In UTI, the fluxing bacteria are capable of re-adhering to naïve host cells, gaining a foothold and potentially invading a new cell to remain undetected until drug pressure subsides and conditions encourage replication and new intracellular biofilm formation. Thus, strategies to prevent microbial adhesion are being considered in combination therapies to both prevent and treat infectious diseases. Carbohydrate derivatives of host ligands have demonstrated efficacy in blocking the adhesive properties of *E. coli* expressing type 1 and also P pili in biophysical and hemagglutination assays. This approach of using soluble carbohydrates or mimics recognized by the bacterial lectin can be readily extended to other adherent organisms by tailoring the antiadhesive compounds to their receptor specificities.

'Pilicides' are a class of pilus inhibitors that target chaperone function. A new class of pilicides, based on a bicyclic 2-pyridone scaffold, inhibit the assembly of both type 1 and P pili in *E. coli* (Figure 4). The potent molecules inhibit an essential protein–protein interaction between chaperone and usher, required for pilus biogenesis. Chaperone–usher systems are highly conserved among various bacteria including *Salmonella*, *Haemophilus*, *Klebsiella*, and *Yersinia* and it is possible, although not yet demonstrated, that pilicides may exert broad-spectrum activity and be effective against several Gram-negative pathogens. Compounds have been identified that target the two-component signaling system, AlgR2/AlgR1, that controls the synthesis of alginate by *P. aeruginosa*. Alginate is a key component of the protective exopolysaccharides coat, critical to *P. aeruginosa* adherence, biofilm formation, and CF pathogenesis. The inhibitors of alginate synthesis could be therapeutically employed to render the pathogen more susceptible to host defenses or to standard antibiotics currently in use, and thus could be effective also in combination therapy. The ability to inhibit microbial adhesion and thus prevent subsequent pathogenic processes holds enormous therapeutic potential and promises to improve the treatment of numerous infectious diseases.

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Microbial Agents to Treat Cancer

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Introduction

An association between infection and cancer regression has been recognized for well over a century. In 1853, Sir James Paget, one of the founders of medical pathology, commented on the "... occasional withering of a cancer under the influence of some fever ..." (Onuigbo, 2012). His observations were followed by the recognition, by Louis Pasteur and Robert Koch, of bacteria as the causative agents of infection.

The use of microbes to treat cancer was pioneered by William B. Coley, a surgeon at the New York Hospital, in the late 19th century. Coley noted cases in which tumors regressed after patients developed bacterial skin infections, and he hypothesized that these infections activated the immune system to fight the tumor. To test this hypothesis, Coley injected live cultures of *Streptococcus* into tumors with regression of the tumors in some cases. However, this form of treatment led to development of severe infections in some patients. To address this, Coley began injecting tumors with lysates derived from cultures of *Streptococcus pyogenes* and *Serratia marcescens*. This form of treatment became known as Coley's toxins, and is considered the first example of cancer immunotherapy. Coley's toxins were used in the United States to treat tumors from 1893 to 1963. However, they had mixed success and their use was considered controversial among the oncological community (Coley, 1893; Coley, 1910).

There are three main ways by which Coley's toxins and other forms of microbial-based cancer therapy could work (Fig. 1). First, microbes or their products could be *directly* toxic to tumor cells. Second, microbes could *compete* with tumor cells and deprive them of essential nutrients. Finally, microbes could *indirectly* destroy cancer cells by activating an antitumor immune response. It is currently thought that the main mechanism by which Coley's toxins resulted in elimination of tumors was through indirect killing of tumor cells through induction of an antitumor immune response. As such, Coley's toxins are possibly the first instance of successful *cancer immunotherapy*, a field that has rapidly expanded in the past decade.

Microbes offer several potential advantages as cancer therapies when compared to traditional cancer treatments. First, microbes may be more specific for tumors rather than for normal tissue. This could be due to tropism of microbes for the tumor microenvironment. For example, anaerobic bacteria thrive in hypoxic conditions. The centers of many tumors are hypoxic, creating an immunosuppressive environment in which radiation and chemotherapeutic agents are less effective. However, this environment is ideal for Clostridia species (see below), which may grow within the tumors but remain unable to spread to the surrounding normal tissue. A tropism of microbes for tumors could also be related to the genetic changes associated with tumorigenesis. For example, BCG preferentially infected bladder cancer cells harboring mutations of the RAS or PI3K-PTEN pathways (Redelman-Sidi et al., 2013). Second, our immune system has evolved to recognize and deal with microbes. Microbes are, therefore, good substrates to activate an immune response. This immune response may potentially bypass the mechanisms used by tumors to escape attack by the immune system. Finally, microbes provide a plastic platform that can be manipulated to achieve desired phenotypes. Thus, microbes can be engineered to kill cancer cells more effectively, deliver cargo to the tumor environment, activate the immune system in a beneficial fashion, or have higher tumor-specificity and a reduced toxicity profile.

In the past decades, the use of microbes for the treatment of cancer has substantially expanded beyond the original Coley's toxins. Here, I will review the current state of the use of microbes as agents of cancer therapy.

Current Use of Microbial Agents as Cancer Treatment

Microbial agents used for the treatment of cancer can be divided into bacterial, viral or protozoal. A summary of the major microbial agents in current use or under investigation for the treatment of cancer is shown in Table 1.

Bacteria

Bacillus Calmette-Guerin (BCG)

BCG is an attenuated strain of *Mycobacterium bovis* that was originally developed as a vaccine against tuberculosis. The first clue suggesting that mycobacteria, such as BCG, might be valuable as treatments for cancer was provided by the biologist Raymond Pearl, who observed in an autopsy study that cancer was less common in patients who had lesions of active tuberculosis (Pearl, 1928). Subsequently, Lloyd Old, an immunologist at the Sloan Kettering Institute in New York, showed that mice infected intravenously with BCG were more resistant to the transplantation of tumors (Old et al., 1959). Following these groundbreaking studies, other investigators attempted to use BCG as therapy for various cancers, including leukemia and melanoma (Mathe et al., 1969; Morton et al., 1974). These studies were followed, in 1976, by the first report on successful use of BCG to treat patients with bladder cancer (Morales et al., 1976).

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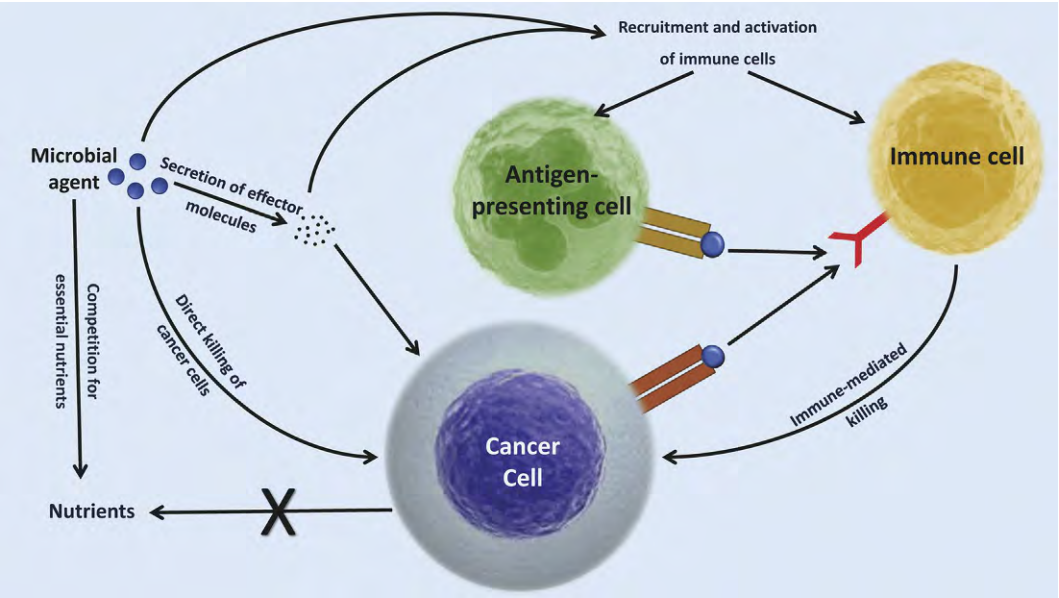

Fig. 1 Potential mechanisms for the efficacy of microbial agents.

Table 1 Microbial agents for the treatment of cancer

Agent	*Investigative stage*
Bacterial	
Mycobacterium bovis BCG	Approved for clinical use in patients with non-muscle-invasive bladder cancer
Salmonella typhimurium	Phase I clinical trials
Clostridium novyi	Phase I clinical trials
Listeria monocytogenes	Phase II clinical trials
Viral	
Talimogene laherparepvec (T-VEC)	Approved for clinical use in patients with nonresectable melanoma
Vaccinia virus	Phase III clinical trials
Adenovirus	Phase II clinical trials
Protozoal	
Toxoplasma gondii	Preclinical studies

While no longer in use for other cancers, BCG remains the standard of care for many patients with bladder cancer. These include patients with carcinoma in situ (stage Tis), high-grade papillary tumors (stage Ta), and lamina-propria-invasive tumors (stage T1) (Hall et al., 2007; Babjuk et al., 2011). These patients first undergo transurethral resection of their cancer with removal of any visible tumors. Once tumors are appropriately staged, patients go on to receive 6 weekly instillations of live BCG intravesically (into the bladder). This is followed, in some patients, by additional intravesical instillations of BCG (maintenance treatment) every several months.

When used as described above, BCG therapy decreases the risk of recurrence compared to transurethral resection of the tumor (Han and Pan, 2006; Shelley et al., 2001). It also reduces the risk of progression of the cancer to invasive disease (Sylvester et al., 2002). The clinical outcome of patients treated with BCG is generally better than that of patients treated with intravesical chemotherapy (Shang et al., 2011; Shelley et al., 2004). It is arguably the most successful microbial treatment of cancer in current clinical use.

The mechanism of action of BCG remains to be fully elucidated. Current evidence suggest that BCG success depends on attachment of live BCG to the inner lining of the bladder leading to activation of an immune response that involves neutrophils, T-lymphocytes and natural-killer cells. This immune response eventually leads to killing of the tumor cells. As such, BCG treatment of bladder cancer is considered one of the first immunotherapies of cancer (Redelman-Sidi et al., 2014).

BCG therapy can be associated with several toxicities. Most patients who receive BCG develop symptoms of bladder irritation, such as urinary frequency or burning upon urination, occasionally accompanied by fever. These symptoms usually begin within hours after instillation of BCG and resolve spontaneously within 48 h. Around 5% of patients develop more prolonged symptoms. These can include symptoms attributable to local spread of BCG causing cystitis, prostatitis, epididymitis, or due spread of BCG to other organs, causing renal abscess, pneumonitis, hepatitis or a sepsis-like syndrome. These complications usually occur within days

to weeks after BCG therapy, but can sometimes occur months or years after BCG is given. The treatment of these complications usually involves antimycobacterial antibiotics, sometimes in combination with corticosteroids (Decaestecker and Oosterlinck, 2015).

Clostridium novyi

The genus *Clostridium* comprises of anaerobic spore-forming bacteria that include nonpathogenic commensals species of the human gut as well as pathogenic, toxin-producing species, such as *Clostridium tetani*, *C. botulinum*, *C. perfringens*, and *C. difficile*. These bacteria form spores under conditions of normal oxygen tension and germinate in anaerobic conditions. As such, clostridia have a potential advantage for cancer therapy as they could germinate in the hypoxic milieu at the center of tumors but be unable disseminate to the well-oxygenated normal tissue outside of the tumor.

Several clostridial species have been tested as treatments for cancer, including *C. tetani*, *C. butyricum*, and *C. histolyticum*. These strains perform well in destroying cells within the poorly-oxygenated center of tumors, but do not effectively lead to complete regression of tumors, including their well-oxygenated areas. To address this issue, further studies have focused on combining Clostridia with chemotherapeutic agents or genetically modifying Clostridial species to improve their cytotoxic potential (Staedtke et al., 2016).

C. novyi is a soil-dwelling, toxin-producing clostridial species that is extremely sensitive to oxygen and cannot survive in oxygenated condition in its vegetative form. *C. novyi* has been associated with cases of human infection, including cases of necrotizing fasciitis or gas gangrene. In order to render *C. novyi* nonpathogenic, the phage carrying α-toxin, a major requirement for its pathogenicity, was removed. The resulting strain was named *C. novyi*-NT (Staedtke et al., 2016).

In animal tumor models, intratumoral injection of *C. novyi*-NT spores into a variety of solid tumors results in hemorrhagic necrosis with regression of tumors. Intravenous injection of *C. novyi*-NT spores results in dissemination of *C. novyi*-NT to the tumors and tumor regression in a substantial portion of treated animals. *C. novyi*-NT was relatively safe in most animals tested, through some developed abscesses and systemic signs of infection such as weight loss and lethargy (Roberts et al., 2014; Agrawal et al., 2004).

The mechanism of action of *C. novyi*-NT appears to be mostly direct cytotoxicity against cancer cells. Interestingly, in some of these animal models, *C. novyi*-NT results not only in destruction of the poorly-oxygenated center of tumors but also of the well-oxygenated regions. This mechanism appears to be immune-dependent, with animals successfully treated with *C. novyi*-NT exhibiting resistance to re-introduction of the same tumor (Agrawal et al., 2004).

Phase I studies are currently in progress to evaluate the safety of intratumoral *C. novyi*-NT injections, alone or in combination with systemic immunotherapy.

Salmonella typhimurium

Salmonella typhimurium is a motile, gram-negative facultative anaerobe that is a causative agent of gastroenteritis. Several features of *Salmonella typhimurium* make it an attractive cancer therapy agent when compared to Clostridia species. First, *Salmonella* is pliable to genetic engineering. Many anaerobic bacteria, including *Clostridium novyi*, are challenging to genetically manipulate. However, facultative anaerobes, such as *Salmonella*, can be easily transformed to express a gene of interest at the tumor site or to delete genes associated with virulence. Second, as a facultative anaerobe, *Salmonella* preferentially colonizes the hypoxic centers of solid tumors. However, it is also able to colonize oxygen-rich regions of the tumor. *Salmonella* appears to have a high specificity for tumor tissue, achieving, in animal models, far higher titers in tumors than in the liver or spleen (Ebelt and Manuel, 2017).

Due to the concern for toxicity with wild-type strains of *Salmonella typhimurium*, attenuated strains of *Salmonella* have been used in animal studies. The attenuation typically involves engineering of auxotrophic strains which are unable to grow in the absence of certain nutrients. In animal models, treatment with these attenuated *Salmonella* strains results in slowing of tumor growth and improved survival (Wang et al., 2016).

One attenuated strain of Salmonella, VNP20009, was tested in human patients with metastatic solid malignancies (mostly melanoma). While it was relatively well tolerated and was able to persist in some tumors, treatment with this strain did not lead to tumor regression in most cases. Current efforts are focused on engineering novel strains of Salmonella with improved tumor efficacy (Wang et al., 2016).

Listeria monocytogenes

Listeria monocytogenes is a saprophytic motile gram-positive rod. In humans, *Listeria* can cause gastrointestinal infections as well as meningitis and other severe infections. Like *Salmonella*, *Listeria* can be genetically manipulated relatively easily. *Listeria* strains used for cancer therapy have been attenuated to reduce their pathogenicity. For example, one of the *Listeria* strains used in cancer therapy is the Live Attenuated Double-Deleted (LADD) strain which has deletions of the virulence genes *inlB* and *actA*. As compared to wild-type *Listeria*, this strain is highly attenuated while maintaining its antitumor efficacy (Brockstedt et al., 2004).

In the host, *Listeria* is mostly found intracellularly. It can invade a variety of cells, including macrophages and epithelial cells. Once internalized, *Listeria* can escape the phagosome and then actively spread to adjacent cells (Wood and Paterson, 2014). Due to its ability to escape the phagosome, *Listeria* can deliver factors directly into the cell cytoplasm. Antigens secreted by *Listeria* in the cytoplasm are processed and presented by major histocompatibility antigen (MHC) class I and II molecules, resulting in

recognition of the antigens by CD8-positive and CD4-positive T-cells respectively, with development of a strong cellular immune response. In the setting of cancer therapy, this property of *Listeria* can be exploited by engineering strains that secret tumor associated antigens into the cytoplasm of cells, resulting in activation of tumor immunity. *Listeria* in this context acts as a tumor vaccine vector.

Several strains of *Listeria* expressing tumor antigens have been tested in animal tumor models. These have included models of cervical cancer, prostate cancer, breast cancer, pancreatic cancer, mesothelioma, and melanoma (Wood and Paterson, 2014). Some of these strains have advanced to clinical trials, though none is currently approved for clinical use (Flickinger Jr. et al., 2018).

Other Bacteria

Other bacterial species are under investigation as potential cancer therapy agents. Examples include *Escherichia coli*, *Clostridium beijerinckii*, and *Bifidobacterium infantis*. These species are currently still in preclinical stages of investigation and a detailed description of their use is beyond the scope of this review.

Viruses

Viruses have several advantages as agents of cancer treatment. They are highly effective at infecting cells, which they access by binding to surface receptors or by fusion with the plasma membrane. The specificity of each viral species to certain receptors can be exploited to increase the selectivity of the virus to malignant cells while sparing normal ones. Once cells are infected, viral proteins can be presented on MHC molecules on the surface of the cells, leading to killing of the cell by the immune system. Additionally, viruses may directly lyse the cells. For this reason, viruses used in cancer therapy are commonly referred to as oncolytic viruses.

A major limitation of the use of oncolytic viruses is that the antiviral immune response may dampen treatment efficacy. Particularly, the antiviral immune response may preclude systemic treatment with the virus, as it may neutralize the virus before it reaches the tumors. This issue is aggravated by repeated administration of the virus, as immunity may build over time, or if there is a preexisting immunity to the viral species used. Oncolytic viruses are, therefore, typically administered via intratumoral injection. In the setting of intratumoral injection, injected tumors respond to the virus through a combination of direct lysis by the virus and immune-mediated destruction, while distant tumors which have not been injected respond through development of an antitumor immune response (abscopal effect).

Talimogene laherparepvec

Talimogene laherparepvec (T-VEC) is the only viral agent currently approved for clinical use as a cancer therapy. It is an attenuated strain of Herpes Simplex Virus (HSV) which was genetically engineered to express granulocyte macrophage-colony stimulating factor (GM-CSF) (Hu et al., 2006). GM-CSF is a white blood cell growth factor that, under physiologic conditions, stimulates stem cells to produce granulocytes and monocytes and also activates mature neutrophils and macrophages. In the context of T-VEC, GM-CSF appears to enhance oncolysis by induction of an immune response though promotion of antigen presentation by dendritic cells.

An initial phase I trial of T-VEC administered by intratumoral injection in patients with a variety of tumors, including melanoma, showed a good safety profile with evidence of antitumor efficacy in some of the patients treated (Hu et al., 2006). A subsequent phase II study in patients with nonresectable melanoma showed an overall response rate of 26%, with a 58% 1-year survival (Senzer et al., 2009). These studies led to a phase III randomized-controlled clinical trial comparing intratumoral injections of T-VEC to subcutaneous GM-CSF in patients with unresectable stage 3B, 3C or 4 melanoma. Durable disease response (disease response lasting more than 6 months) occurred in 16.3% of patients in the T-VEC arm compared to 2.1% of those in the GM-CSF arm ($P < .001$). There was a trend toward improved overall survival in the T-VEC arm compared to the GM-CSF arm 23.3 months versus 18.9 months, respectively ($P = .051$). Importantly, the antitumor effect of T-VEC was also observed in distant tumor sites that were not injected (Andtbacka et al., 2015). These trials led to approval of T-VEC for the treatment of nonresectable melanoma.

With the advent of checkpoint blockade, current efforts are focused on combining T-VEC with checkpoint inhibitors, including cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and PD1/PDL1 antibodies. In a phase Ib trial, a combination of T-VEC with ipilimumab (CTLA-4 blocking antibody) in patients with advanced melanoma was associated with a 44% rate of durable response (lasting at least 6 months), and 50% progression-free survival and 67% overall survival at 18 months (Puzanov et al., 2016). These rates of response are higher than those seen in prior studies of either agent alone. In a phase II trial of patients with nonresectable melanoma, 39% of those receiving a combination of T-VEC with ipilimumab had an objective response as compared to 18% of those receiving ipilimumab alone ($P = .002$) (Chesney et al., 2018).

Trials are ongoing to determine the utility of combining T-VEC with checkpoint blockade. Additionally, T-VEC is being tested in other solid tumors, including pancreatic cancer, breast cancer, sarcoma, and nonmelanoma skin cancer. Beyond T-VEC, other genetically-engineered HSV strains are currently being tested in clinical trials (Fountzilas et al., 2017).

Vaccinia Virus

Vaccinia virus is a large, double-stranded DNA virus that was originally used as a vaccine against smallpox. Unlike HSV, its entire replication cycle takes place within the cytoplasm of the infected cell, and it does not enter the nucleus. Its large genome, which is approximately 192,000 base-pairs in length, allows insertion of large amounts of foreign DNA, making it an appealing option for gene delivery into tumors. It has a good safety profile and there is extensive experience using it, given that it has been used extensively in humans as a vaccine to eradicate smallpox (Haddad, 2017).

For a variety of reasons, Vaccinia virus has a tropism for tumors as compared to normal tissue (McCart et al., 2001). This is particularly true for Vaccinia virus strains that have been engineered for cancer therapy. These strains are typically deleted for the thymidine kinase (TK) gene, limiting their ability to synthesize nucleotides, and allowing them to replicate only in nucleotide rich cells, including cancer cells (Haddad, 2017).

Several Vaccinia virus strains have been tested in human cancers. JX-594, a vaccinia virus strain deleted for the TK gene and expressing GM-CSF, was tested by intratumoral injection in phase I studies in patients with melanoma and hepatocellular carcinoma (HCC). The strain had a good toxicity profile and response to treatment was seen in some of the patients treated (Park et al., 2008; Mastrangelo et al., 1999). Administration of JX-594 by the intravenous route was tested in a phase I study in patients with colorectal cancer. In this study, JX-594 was well tolerated, and 67% of the patients had radiographically stable disease (Park et al., 2015).

In a phase II clinical trial, intratumoral injections of JX-594 to patients with HCC were associated with responses of both the injected and the noninjected tumors, showing evidence of an abscopal effect of JX-594 injection. There was a dose-dependent response, with improved survival in those who received a high dose of JX-594 compared to those who received a low dose (Heo et al., 2013). There is currently an open phase III clinical trial in patients with HCC to compare JX-549 with sorafenib (angiogenesis inhibitor) to sorafenib alone.

Another Vaccinia virus strain in clinical testing is GL-ONC1, which was engineered by inserting the genes Ruc-GFP, β-glucuronidase, and β-galactosidase into Vaccinia virus. This virus was tested in two phase I clinical trials in patients with peritoneal carcinomatosis, where it was administered by intraperitoneal injection, and in patients with head and neck cancer, where it was administered intravenously. In both trials GL-ONC1 was found to be safe with some evidence of antitumor efficacy (Mell et al., 2017; Lauer et al., 2018). Phase II clinical trials of GL-ONC1 are currently underway.

Adenovirus

Adenoviruses are nonenveloped double-stranded DNA viruses. There are over 60 serotypes of adenovirus that can cause infections in humans. These strains can cause a variety of clinical manifestations, depending on the serotype involved, including respiratory, gastrointestinal, and urinary tracts and conjunctival infections. As compared to Vaccinia virus, adenoviruses have a much smaller genome, typically less than 40,000 base-pairs in length.

There are two main issues associated with adenovirus-based treatment of cancer. First, wild-type strains of adenovirus are not selective to tumors and may also infect normal tissue, particularly the liver for which they have a tropism. Second, they are highly immunogenic, potentially limiting their efficacy in vivo. To address these issues, oncolytic adenovirus strains have been modified to improve their tropism for tumors and to decrease their immunogenicity (Yamamoto et al., 2017).

Several strains of adenovirus have been tested in humans. ONYX-015 is a strain of adenovirus that can cause lysis in cells that have a mutation in the p53 oncogenes, making it selective for tumor cells carrying this mutation. This strain was tested in a phase II study via intratumoral injection in patients with head and neck cancer who received concurrent chemotherapy. There was complete response of the injected tumors in some of the patients who received intratumoral ONYX-015, but progression in all patients who received chemotherapy alone (Khuri et al., 2000). However, further studies utilizing this strain failed to replicate these results, and there are no current clinical trials involving ONYX-015.

CG0070 is an adenovirus strain expressing GM-CSF which was designed to be specific for cancer cells with alterations in the retinoblastoma pathway. In a recent phase II study, 45 patients with nonmuscle invasive bladder cancer who had failed to respond to BCG treatment or experienced recurrence after BCG were treated with CG0070. CG0070 was administered into the bladder of these patients at weekly intervals for a total of six administrations. The treatment was relatively-well tolerated and 47% of the patients had a complete response after 6 months Packiam et al., 2018).

DNX-2401 (delta-24-RGD) is another adenovirus strain that was designed to specifically infect cells with alterations in the retinoblastoma pathway. It was tested in a phase I trial in patients with recurrent brain gliomas. Patients were treated with a single intra-tumoral injection of DNX-2401. The treatment was generally well-tolerated, and 3 of 25 patients had a complete response (Lang et al., 2018).

A fourth adenovirus strain, called Enadenotucirev, was evaluated in a phase I study. In this study, Enadenotucirev was administered intravenously to patients with resectable tumors of various types. Enadenotucirev was detected in the resected tumors of most patients but was found to a much lesser degree in normal tissue. There were no adverse events related to tumor.

Other Oncolytic Viruses

Multiple other oncolytic viruses are in preclinical stages of investigation as agents of cancer therapy. These include oncolytic viruses base on Newcastle disease virus, reovirus, parvovirus, measles virus, and vesicular stomatitis virus, among others (Fountzilas et al., 2017).

Protozoa

Toxoplasma

Toxoplasma gondii is a protozoan parasite that can be transmitted via contact with cats. *Toxoplasma* infection is typically asymptomatic in immunocompetent humans; however, it can cause severe infections in immunocompromised individuals, including those with AIDS. *Toxoplasma* is an obligate intracellular organism, meaning that it can only replicate within cells.

For use in cancer therapy, Toxoplasma has been engineered to completely prevent its replication. This has been done by creating a strain of toxoplasma that lacks the pathway for synthesis of uridine 5′-monophosphate, resulting in a mutant that can only grow if supplied with exogenous uracil, a metabolite that is not available within the host. This mutant can invade host cells but is unable to replicate. It is highly immunogenic but nonpathogenic, making it an attractive vector of cancer immunotherapy (Fox and Bzik, 2002; Fox and Bzik, 2010; Dupont et al., 2014; Fox et al., 2016).

In preclinical animal models, vaccination with this attenuated *Toxoplasma* strain results in rejection of implanted tumors. The cured mice are also resistant to re-challenge with the same tumor cell line. Tumor rejection appears to be related to activation of tumor-associated T-cells with increased production of the T-cell chemokines CXCL9 and CXCL10 within the tumors as well as increased production of interleukin-12 (Fox et al., 2016; Baird et al., 2013a). The mechanism for this may be infection of suppressive myeloid derived cells, including dendritic cells, within the tumors with reversal of their suppressive functions (Baird et al., 2013b).

There are no current clinical trials evaluating *Toxoplasma* as a cancer treatment.

Conclusions

Microbial agents have been used to treat cancer for over a century, however it is only in the past two decades that scientific understanding of microbial genetics and tumor immunology have combined to yield novel microbial strains for use in the treatment of cancer. Some microbes, such as BCG and T-VEC are already approved for clinical use. Many others are in various stages of clinical and preclinical investigation. With the recent explosion of the field of cancer immunotherapy, future studies will likely test the combination of microbial agents with checkpoint blockade and other forms of immunotherapy. It is conceivable that these combinations will be synergistic and will improve treatment outcomes. As our understanding of cancer biology and immunology keeps expanding, future investigations are expected to yield novel genetically engineered microbial strains with higher specificity for cancer cells and better efficacy in the treatment of cancer.

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Microbial Biofilms☆

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Abbreviations

AHLs	Acyl-homoserine lactones
c-di-AMP	Cyclic diadenylate
c-di-GMP	Cyclic diguanylate
eDNA,	Extracellular DNA
EPS	Extracellular polysaccharide
NMR	Nuclear magnetic resonance
QS	Quorum sensing

Biofilms in Health and Disease

Biofilms are rigorously studied because they promote bacterial pathogenesis and drive infectious diseases. Recent microbiome studies reveal the crucial role of the biofilm microbiota in modulating health and disease. Although the human microflora is established not long after birth, studies suggest that the gut microflora, in particular, can be altered not only by antibiotics, but variations in diet (Tamburini et al., 2016). Interestingly, alterations in diet can prompt the gut microbiota to undergo metabolic switches and modulate host metabolites, and as a result, byproducts of microbial and altered host metabolism can impact overall health and disease. Disruptions in the gut microbiota have been implicated in the onset of obesity, irritable bowel syndrome, colon cancer, and cardiovascular disease, among other disorders (Cho and Blaser, 2012; Gilbert et al., 2016; Lewis et al., 2015). Using 'omics techniques (genomics, metabolomics, and proteomics), microbial metabolic pathways associated with health and disease can be identified. Similarly, next-generation sequencing of 16 s rRNA bacterial signatures enables the identification of specific genera of bacteria associated with health and disease in an effort to identify microbes specifically associated with health (Cho and Blaser, 2012; Gilbert et al., 2016). A metatranscriptomics study of the oral microbiome found clear differences in metabolism between health and disease-associated microbial communities and these differences were conserved between patients (Jorth et al., 2014). Altogether, current microbiome studies highlight the role biofilm communities play not only in active infectious diseases, but also in metabolic syndromes and other systemic diseases, which further highlights the impact biofilms have on human health.

Biofilm Composition, Structure and Function

In the biofilm community, bacteria are encapsulated within a highly structured, complex matrix. Typically, the matrix is comprised of water, microbial cells, extracellular polysaccharides, proteins, extracellular DNA (eDNA), lipids and small ions (Flemming and Wingender, 2010; Payne and Boles, 2016). Together, these biofilm components support a matrix that serves as protection for the bacteria while permitting the exchange of nutrients and oxygen. Biofilm formation occurs in 4 stages: 1) attachment 2) microcolony formation 3) maturation and 4) dispersal (Fig. 1). Attachment involves an initial stage when planktonic bacteria bind to a surface in a reversible manner, followed by a stage in which planktonic that incorporated into the biofilm are no longer able to detach. Cell surface associated proteins called fimbriae and pili, and exopolysaccharides have been demonstrated to be instrumental both in vitro and in vivo for attachment of bacteria to abiotic or biotic surfaces, in addition to facilitating the incorporation of other bacteria into the biofilm (Garnett et al., 2012; Ghafoor et al., 2011; Koo et al., 2010; Liang et al., 2011; Reardon-Robinson et al., 2014). The attachment of microbes to a surface induces the expression of genes that promote the biofilm mode of growth which results in microcolony formation or microbial aggregates that are spatially arranged in an exopolysaccharide matrix (Flemming and Wingender, 2010). As the biofilm matures, various environmental cues can signal the dispersal of the biofilm, including the depletion of nutrients, toxin build-up, oxygen availability, or stress (Flemming and Wingender, 2010).

Extracellular polysaccharides are a main constituent of many biofilm matrices. For example, the oral pathogen, *Streptococcus mutans* produces glucosyltransferases, which are enzymes that use dietary sugars to catalyze the production of sticky glucans that

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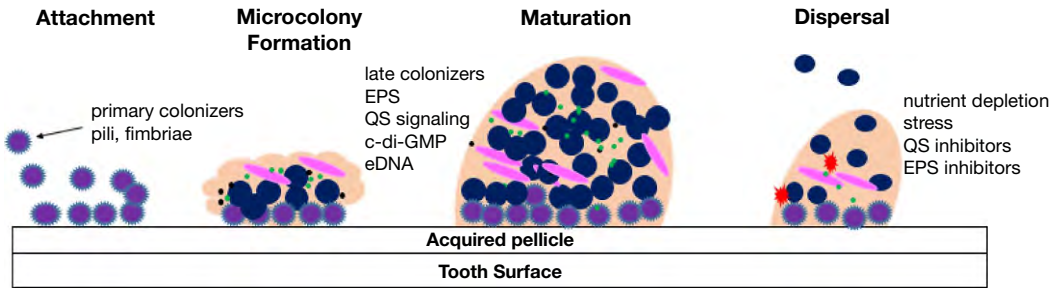

Fig. 1 Stages of biofilm development in the oral cavity. Attachment of planktonic cells, activation of signaling cascades (QS and c-di-GMP) that regulate EPS production, microcolony formation, maturation, and biofilm dispersal triggered by the depletion of nutrients, environmental stress, or inhibitors that target QS or EPS.

allow the bacterium to adhere to the tooth surface (Koo et al., 2010). The opportunistic pathogen *Pseudomonas aeruginosa* produces exopolysaccharides alginate, Pel, and Psl that are differentially regulated at different phases of biofilm development and in acute versus chronic isolates (Ma et al., 2012; Schurr, 2013; Scoffield and Silo-Suh, 2016). Pel has also been shown to cross-link with eDNA, which forms an unappreciated biofilm structural scaffold that may be conserved among various biofilms (Jennings et al., 2015). The production of biofilm matrix components is often controlled by coordinated community behavior known as quorum sensing or through secondary messenger activity. Quorum sensing (QS) is cell density-dependent communication between bacteria in which the production of autoinducers regulates certain community behaviors, such as exopolysaccharide production (Sakuragi and Kolter, 2007; Ueda and Wood, 2009). The requirement of QS signaling to control biofilm formation is well documented for *Vibrio cholera*, the causative agent of cholera, and *P. aeruginosa* (Kamruzzaman et al., 2010; Ueda and Wood, 2009). In addition to QS signaling, second messenger cyclic diguanylate (c-di-GMP) or cyclic diadenylate (c-di-AMP) plays crucial roles in regulating biofilm formation by many bacteria, including, *Bacillus cereus*, *P. aeruginosa*, and *S. mutans*, and *Klebsiella pneumoniae* (Fagerlund et al., 2016; Peng et al., 2016; Valentini and Filloux, 2016; Wilksch et al., 2011). Increased intracellular concentrations of c-di-GMP or c-di-AMP mediate biofilm formation via distinct signaling cascades, which can be explored to develop anti-biofilm drugs. Furthermore, QS signaling and c-di-AMP or c-di-GMP signaling may cross talk, which enrich and enhance the regulatory capacity of bacteria within biofilms.

Mechanisms of Adaptation and Persistence in Biofilms

Cells growing within biofilms often exhibit increased tolerance to antimicrobials compared to free living planktonic bacteria, and as a result, eradicating the biofilm bacteria has long been recognized as a persistent challenge in treating recalcitrant infections. In the biofilm lifestyle, bacteria have adopted several strategies that permit them to avoid clearance by antibiotics and evade the host immune response. Some of these strategies include the utilization of drug efflux pumps that prevent antibiotics from reaching high concentrations in the cytoplasm, the development of persister cells, which are dormant, drug-tolerant cells that persist subsequent to drug treatment, and the activation of stress response genes (Lewis, 2005; Liao et al., 2013; Mulcahy et al., 2010). A transcriptomic study in *P. aeruginosa* revealed that biofilms challenged with tobramycin or ciprofloxacin overexpressed genes controlled by the stress response sigma factor, RpoS, as well as genes that are devoted to hypoxia and osmotic stress (Stewart et al., 2015). Moreover, the SOS response system is required for biofilm, nutrient-deprived cells to acquire increased resistance to ofloxacin (Bernier et al., 2013). In addition, proteomic studies demonstrate that *Candida albicans*' persister cells exposed to antifungal drugs upregulate proteins involved in stress response (Li et al., 2015).

Historically, exopolysaccharides have been shown to play a role in bacteria resisting antimicrobials and the immune response. For instance, *P. aeruginosa* exhibits the mucoid phenotype caused by an overproduction of exopolysaccharide alginate, which prevents the penetration of antibiotics, and inhibits phagocytosis and the activation of complement. As a result, *P. aeruginosa* is able to resist clearing by therapeutic measures and the host immune system. New evidence suggests that another major *P. aeruginosa* polysaccharide, Psl, provides the "first line of defense" against antimicrobials by promoting biofilm tolerance to cationic and anionic antibiotics (Billings et al., 2013). In addition, a different *P. aeruginosa* polysaccharide, Pel, increases resistance to aminoglycoside antibiotics (Colvin et al., 2011). Moreover, the activity of two glycosyltransferases (*epa* I and *epa OX*), which are predicted to be involved in polysaccharide biosynthesis, are critical for the biofilm formation by *Enterococcus faecalis* in the presence of antibiotics (Dale et al., 2015). Similarly, fungal β-1, 3 glucans play a role in *Candida* species' resistance to antibiotics in single (Nett et al., 2010) and dual species biofilms with *Escherichia coli* (De Brucker et al., 2015). Taken together, current data suggest that polysaccharides not only function in maintaining biofilm structure, but serve as protection against antimicrobials.

Biofilm Microbe-Microbe Interactions and Pathogenesis

Many studies that examine factors involved in biofilm formation focus on studying single species models. However, in many cases, such as the oral cavity, chronic infections, or natural habitats, biofilms are always polymicrobial. Complex and dynamic mixed species biofilm communities can significantly alter health and disease outcomes. Emerging studies are now focusing on evaluating

the impact that polymicrobial biofilms have on the host, in addition to how diverse species of bacteria influence community behavior. In vitro and in vivo biofilm models have been developed to study biofilms containing two or more species. *P. aeruginosa* and *Burkholderia cenocepacia* are two bacteria that commonly co-infect the cystic fibrosis airway. Co-infection results in the promotion of *P. aeruginosa* biofilm and a heightened host immune response in a murine model of chronic infection (Bragonzi et al., 2012). Infection by a mixed species biofilm of *Staphylococcus aureus*, *P. aeruginosa*, *Enterococcus faecalis* and *Finegoldia magna* severely impaired wound repair. In addition, the bacteria within the mixed species infection exhibited higher tolerance to antimicrobials (Dalton et al., 2011). These data demonstrate that bacteria in a polymicrobial biofilm may display synergism in the host, which could exacerbate microbial infection.

Oral biofilms are another example of a polymicrobial environment. Oral biofilms are comprised of mostly commensal streptococci. Commensal streptococci such as *Streptococci sanguinis*, *Streptococci gordonii*, and *Streptococcus parasanguinis*, are primary colonizers of the tooth surface. These streptococci play a role in containing the attachment of the cariogenic pathogen *S. mutans* into the oral biofilm. *S. mutans* produces a sticky glucan matrix that assists the bacterium in adhering to the tooth surface and ultimately cause tooth decay. The balance between commensal streptococci and *S. mutans* is a key indicator in predicting the health and disease outcomes, and this balance is largely driven by antagonistic interactions within the dental biofilm. Commensals in dental plaque can maintain homeostasis, however, a disruption in the oral flora could lead to dysbiosis. Several studies have elucidated the interactions of commensals and pathogens in dental plaque. Colonization sequence, hydrogen peroxide production by commensal streptococci and bacteriocin production by *S. mutans* drive bacterial competition in the dental biofilm (Fujishima et al., 2013; Kreth et al., 2008). As a result, *S. mutans* must employ several mechanisms to resist oxidative stress in the oral cavity, which includes harboring the peroxide resistance protein (Dpr), superoxide dismutase (*sod*), and *perR* (Fujishima et al., 2013; Yoshida et al., 2015), which facilitate the ability of *S. mutans* to coexist in the presence of hydrogen peroxide producing streptococci. In contrast, arginine metabolized by commensal streptococci can rebalance oral biofilms by increasing pH and shifting the microbiota towards healthy flora (Kolderman et al., 2015; Nascimento et al., 2014). Commensals themselves do not cause disease, however, they can promote the virulence of pathogens. The oral commensal, *Streptococcus gordonii*, promotes fitness and virulence of the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (*Aa*) by producing the pathogen's preferred carbon source, controlling its spatial location at an infection site, and enhancing the bioavailability of oxygen to *Aa* (Ramsey et al., 2011; Stacy et al., 2014, 2016a,b). In contrast, the fitness of *Aa* is reduced when co-colonized with the oral commensal *Streptococcus parasanguinis*, and interestingly, *Aa* enhances biofilm formation by *S. parasanguinis* by modulating its hydrogen peroxide production (Duan et al., 2016). These studies suggest that specific commensals may exhibit either synergism or antagonism in the presence of pathogens. In addition to commensal-pathogenic bacterial interactions, there are examples of cross-kingdom interactions between bacteria and fungi where fungi can modulate bacterial colonization and virulence. For instance, *S. mutans* and *Candida albicans* are frequently detected together in dental plaque. Co-infection with *S. mutans* and *C. albicans* promotes biofilm formation and disease progression compared to a single infection with either species. In addition, co-infection with *C. ablicans* triggers the activation of several *S. mutans*' virulence genes (Falsetta et al., 2014). *C. albicans*, also co-infects the cystic fibrosis airway with *P. aeruginosa*, which can either stimulate or attenuate *P. aeruginosa* virulence and biofilm formation using various mechanisms (Chen et al., 2014; Lopez-Medina et al., 2015). *C. albicans* can also influence other fungal species by enhancing the expression of *Candida glabrata* cell wall adhesins and the colonization of *C. glabrata* on tongue tissue (Tati et al., 2016). Owing to the fact that multiple species of microbes are found at infection sites and engage in interactions that affect microbial fitness and virulence, it is critical to elucidate mechanisms of microbial synergism and antagonism in polymicrobial biofilms.

Biofilm Experimental Techniques and Emerging Biofilm Control Strategies and Therapeutics

Technological advances have facilitated the biofilm research. Use of the microtiter dish and crystal violet assay has long been a powerful tool to assess biofilm biomass (O'Toole, 2011). In addition, immunofluorescence and confocal microscopy has afforded high resolution imaging of single and mixed species biofilms. These imaging techniques permit the labeling of viable and dead bacteria in the biofilm, the visualization of matrix components such as glucan, 3D imaging of biofilms, and specific labeling of bacteria in mixed species biofilms using cell surface specific antigens. Moreover, solid-state nuclear magnetic resonance (NMR) has been used as an effective way to study cell surface associated proteins involved in *S. mutans*' adhesion and the extracellular matrix of *Aspergillus fumigatus* (Reichhardt et al., 2015 and Tang et al., 2016). New tools to study biofilms will assist the research field to identify and develop new anti-biofilm targets. More recently, super-resolution light microscopy has provided the unprecedented resolution to visualize the spatial organization of the extracellular matrix proteins (Berk et al., 2012). Understanding the spatial arrangement of microbes in a biofilm is important largely because the biogeography of microbes in polymicrobial infections is linked to virulence potential (Stacy et al., 2016a,b). In vivo fluorescent probes coupled with metagenomic sequencing allows the monitoring of the biogeographical distribution of bacteria on a micron scale (Mark Welch et al., 2016). Other tools combine segmentation and image analysis algorithms with a spinning-disk confocal microscope to image the evolving 3D architecture of *V. cholera* biofilms, which allows researchers to examine biofilms at single-cell resolution (Drescher et al., 2016). In addition, micro-3D printing and scanning electrochemical microscopy (SECM) can image bacterial aggregates in situ to examine QS signaling, behavior, and spatial organization within the biofilm (Connell et al., 2014). Collectively, these new technologies will drive the development of therapeutics that disrupt the biogeographical arrangement of polymicrobial biofilms.

In the post-antibiotic era much emphasis has been focused on developing novel non-antibiotic therapeutics to target biofilms. Due to the high level of drug resistance exhibited by recalcitrant pathogens, such as methicillin-resistant *Staphylococcus aureus*,

P. aeruginosa, *Acinetobacter baumanii*, in addition to robust biofilms produced by *S. mutans*, the efforts in developing anti-biofilm strategies have shifted towards more targeted approaches. Specific genes or pathways that play a direct role in biofilm formation are now being directly targeted in an effort to block the synthesis or activity of biofilm related constituents. Due to the fact that QS genes directly regulate biofilm formation in various bacteria, inhibitors of QS are now being considered as potential biofilm targets. Drug targets of the *las* and *rhl* QS systems of *P. aeruginosa* have been developed that target or degrade the acyl-homoserine lactones (AHLs) structure. These inhibitors have been shown to not only attenuate biofilm formation, but downregulate cytotoxic virulence factors in *P. aeruginosa* (Furiga et al., 2016; O'Loughlin et al., 2013) and *S. aureus* (Srivastava et al., 2015). Other techniques involved the use of small molecules that target DnaK in *E. coli*, cell wall biosynthesis in *Mycobacterium tuberculosis*, and dihydrofolate reductase in *S. mutans*, are also promising as they also inhibit biofilm formation in the respective bacteria (Arita-Morioka et al., 2015; Wang et al., 2013; Zhang et al., 2015). Targeting microbial geography by disrupting the exopolysaccharide matrix is another mean to disrupting biofilms. For example, PslG, a protein involved in the biosynthesis of the major *P. aeruginosa* biofilm polysaccharide, Psl, disrupts the Psl matrix and disassembles pre-existing biofilms when added exogenously (Yu et al., 2015). Moreover, nanoparticles that exploit the acidic pH of the oral biofilm can deliver farnesol to interfere with exopolysaccharides in the biofilm matrix (Horev et al., 2015). Similarly, nanocatalysts have been shown to degrade *S. mutans*' matrix and increase bacterial killing (Lizeng et al., 2016). Overall, the approach of using specific targets that inhibit QS signaling or biofilm matrix components appears to be a viable strategy for inhibiting biofilms.

Conclusion

Biofilm formation contributes to the microbial persistence and virulence. We are frequently uncovering new mechanisms utilized by microbes to form biofilms. More importantly, scientists are beginning to appreciate the significance of polymicrobial biofilms, since infections often involve multiple species of microbes and these microbes engage in complex and dynamic interactions that impact the outcomes of health and disease. Fortunately, the rapid development of new, powerful technologies and tools will provide the needed resolution to study intricate details of microbial biofilms, which will ultimately result in the development of novel and advanced anti-biofilm therapeutics.

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Microbial Cycling of Methane

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Introduction

Methane, produced both abiogenically, through processes in the Earth's crust (Sherwood Lollar et al., 2002, 2006; Etiope and Sherwood Lollar, 2013) and biogenically, through microbial methanogenesis (Singh et al., 2010), is an important component of the global carbon cycle. Microbes that consume methane, the methanotrophs, play a major role in methane emission mitigation (Conrad, 2009). Methane-metabolizing species have been recognized within the bacterial and archaeal domains of life, and it has been recognized that methane metabolism can proceed either aerobically or anaerobically (Chistoserdova and Lidstrom, 2013; Chistoserdova, 2015). This article aims to summarize the diversity and environmental distribution of methanotrophs and recent developments in the understanding of the metabolic details specific to each subgroup. We also touch on recent developments in the potential role of lanthanides as environmental regulators of methane metabolism, as well as on the increasingly recognized concept of syntrophies as parts of microbial methane metabolism.

The Methane Cycle

While methane is produced in essentially every ecosystem, from the deep ocean to the human gut, the scale of the global methane cycle, in the context of the global carbon cycle, is sometimes difficult to fully appreciate. This is partly due to the complexity and the variety of its sources and sinks, which we briefly summarize below. An annual input of methane carbon into the Earth's atmosphere, in the form of emitted methane, is calculated at 325–500 Tg (Tg=10^{12} g C) (Shindell et al., 2012; Tian et al., 2016). Approximately 70%–80% of the newly added methane is oxidized photo-chemically in the troposphere and the stratosphere, ~5%–10% contribute to the atmospheric methane pool, and ~20% are consumed via biological activities in terrestrial ecosystems (woodland soils, deserts, etc.) (Striegl et al., 1992; Bender and Conrad, 1995; Topp and Pattey, 1997; Shrestha et al., 2012). However, it is important to remember that the emitted methane is only a small portion of the annual biological methane cycle (Fig. 1). The global methane pool is built upon numerous atmospheric, biomass-based, fossil, and geothermal reservoirs. The abundance of methane in the Earth's atmosphere is slowly approaching 5 Gt of carbon (1 Gt=1000 Tg), making it the most abundant organic compound. As one of the main end products of biomass decomposition occurring in anaerobic (via methanogenesis) and aerobic (via methylphosphate degradation) conditions, methane is produced in every ecosystem where biomass decomposition occurs. The deposits of thermogenic, primary and secondary methane gas, and geological (abiotic) methane are estimated at 2500 Gt of carbon (Milkov, 2011; Etiope and Sherwood Lollar, 2013). Every year, a portion of fossil methane leaks to overlaying areas, stimulating chemosynthesis and providing additional sources to ecosystems (Chapelle et al., 2002; Thauer et al., 2008; Orcutt et al., 2013; Kietäväinen and Purkamo, 2015; Chronopoulou et al., 2017). Methanotrophs play a major role in methane consumption, thriving across environmental niches in which its production occurs: soils (Hanson and Hanson, 1996), marine and freshwater sediments (Tavormina et al., 2010; Trimmer et al., 2015), tissues of mollusks (Fiala-Médioni et al., 2002) and even human gut (Clemente et al., 2015).

Diversity of Methanotrophs

Beyond the use of methane as a carbon and energy source, methanotrophs have both numerous cellular features in common and significant metabolic differences between the functional types. The two main groups of methane-utilizing prokaryotes are the methanotrophic archaea (ANME) and the methanotrophic bacteria, the former typically associated with anaerobic metabolism of methane, while the latter can metabolize methane both aerobically and anaerobically (Figs. 2 and 3).

Methanotrophic Archaea

Geochemical evidence suggesting microbial anaerobic oxidation of methane linked to sulfate reduction has existed since the late 1970s (Reeburgh, 1976, 1980). However, it took another two decades to obtain molecular evidence that the microbes involved are Euryarchaeota archaea (ANME), a phyla closely related to *bona fide* methanogens and their syntrophic partners, sulfate-reducing bacteria (SRB) (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001; Knittel and Boetius, 2009). The biochemical pathways potentially involved in such cooperative metabolisms were teased out via metagenomics studies, as none of the partner organisms were available in culture. Sequence analysis strongly suggested that a reverse methanogenesis pathway must be involved in methane oxidation (Hallam et al., 2004; Fig. 3(B)). This hypothesis was further supported by high expression of methanogenesis genes in

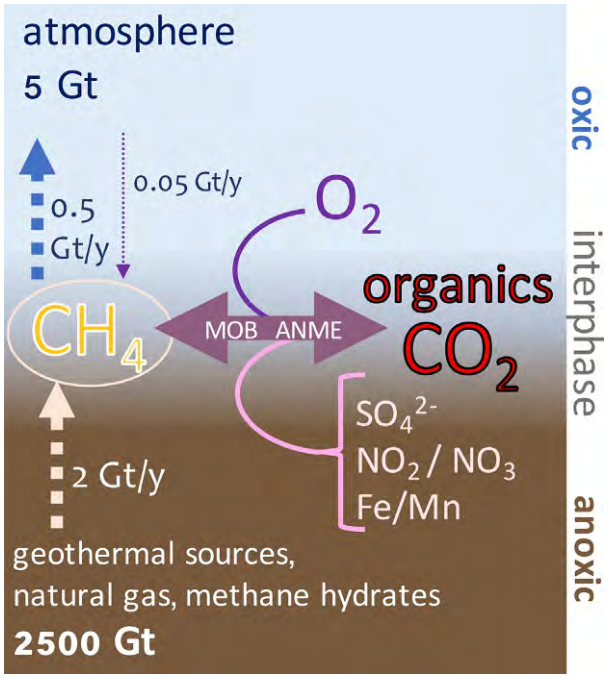

Fig. 1 (A). Overview of the global methane cycle. It could be estimated that methane-utilizing microbes are responsible for consumption of 2–2.5 Gt of methane carbon each year. Overall, methanotrophy plays an important role in local carbon turn-over in a variety of terrestrial ecosystems.

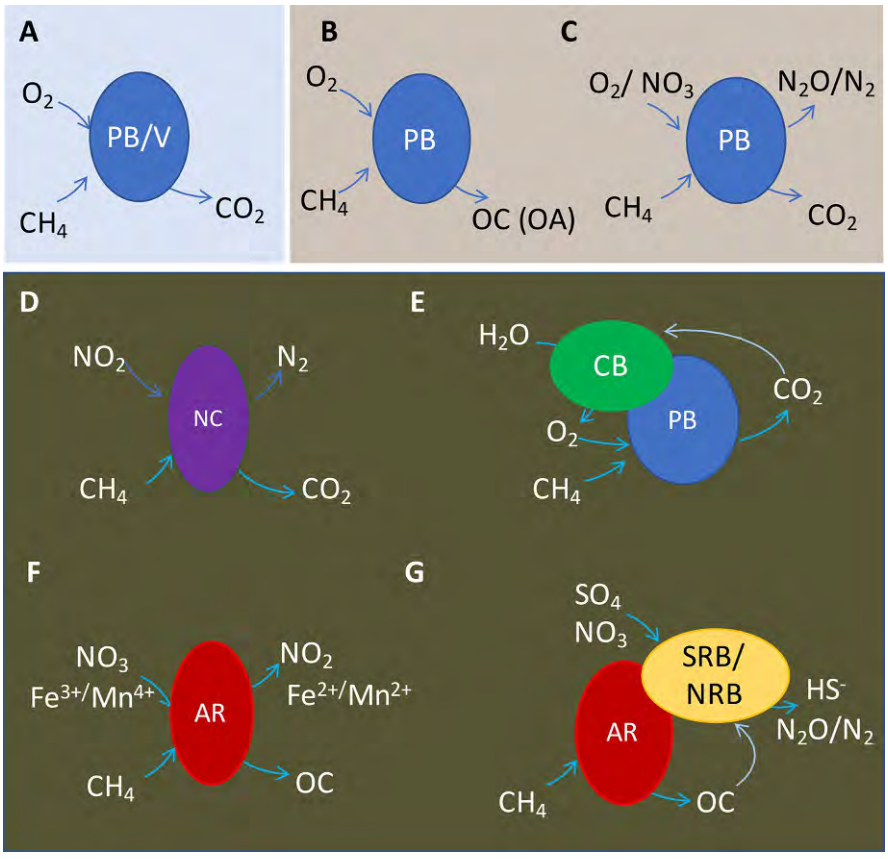

Fig. 2 Schematic illustration of metabolic coupling in methane oxidation. (A–E) Oxygen-dependent methane oxidation, which is now known to occur in aerobic, microaerobic, and anaerobic environments. Methanotrophic bacteria employ various strategies to cope with low oxygen tensions, including switch to fermentative lifestyle (B), denitrification (C–D), or close cooperation of oxygen-producing cyanobacteria (E); (F–G) Oxygen-independent methane oxidation is driven by anaerobic archaea that are capable of denitrification or metal reduction (F) and sulfate reduction (G). PB, proteobacteria; V, Verrucomicrobia; NC, NC10 bacteria; AR, archaea; SRB, sulfate-reducing bacteria; NBR, denitrifying bacteria; CB cyanobacteria; OC, organic carbon; OA, organic acids.

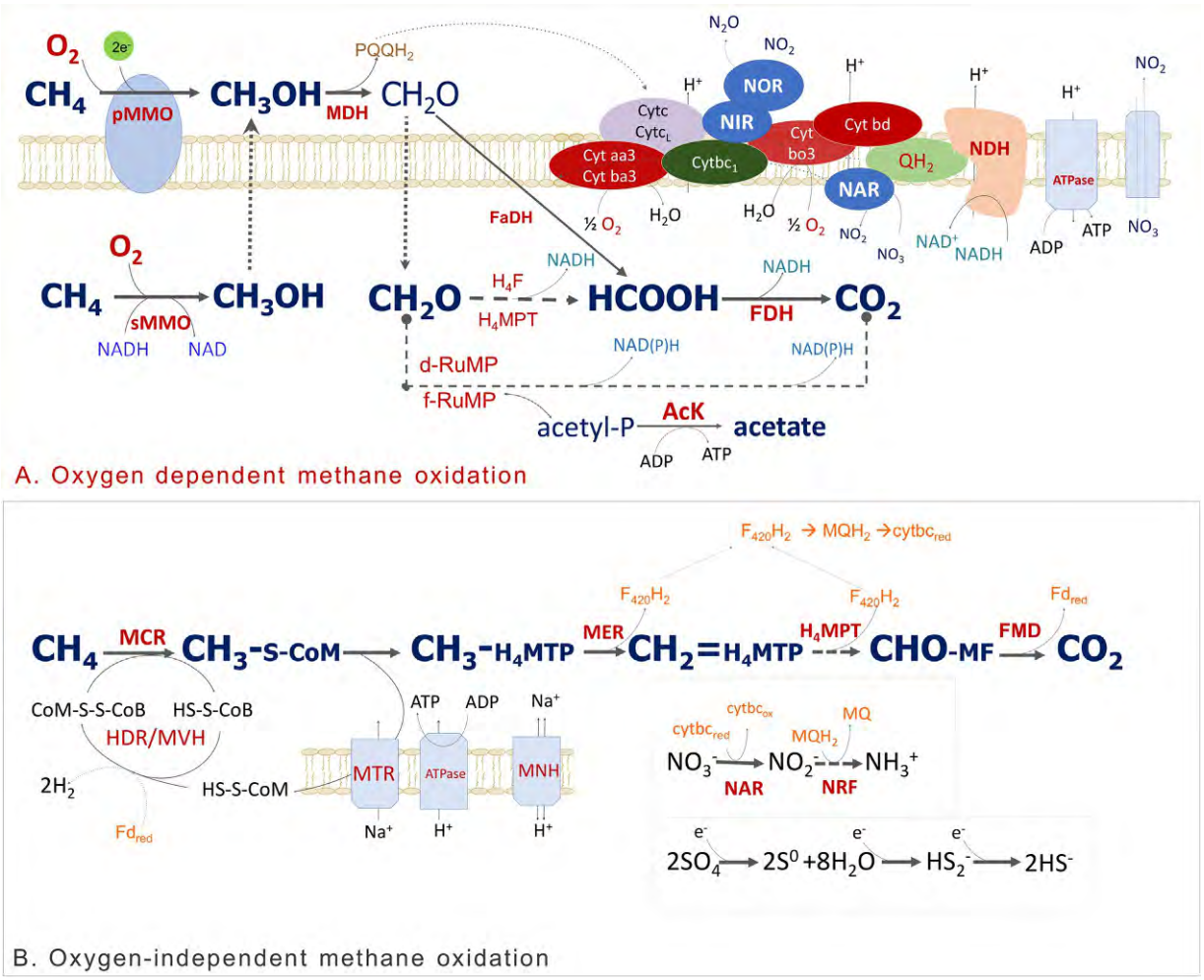

Fig. 3 Schematic illustration of methane oxidation. (A) aerobic and (B) anaerobic methane oxidation. pMMO, membrane-bound methane monooxygenase; sMMO, soluble methane monooxygenase; MDH (Mxa or Xox), PQQ-linked methanol dehydrogenases; H_4MPT, tetrahydromethanopterin-linked C1 transfer; H_4F, tetrahydrofolate-linked C1 transfer; FaDH, formaldehyde dehydrogenase; FDH, formate dehydrogenases; NDH, NADH:quinone oxidoreductase; d-RuMP, dissimilatory ribulose monophosphate cycle; f-RuMP, RuMP-phosphoketolase pathway for fermentation of methane; MCR, methyl-coenzyme M reductase; MTR, Methyl-H_4MPT:coenzyme M (CoM) methyl-transferase; HDR/MVH, coenzyme M-coenzyme B (CoM-S-S-CoB) heterodisulfide reductase and D subunit of F_{420}-nonreducing hydrogenase; FMD, Formyl-MFR dehydrogenase; MER, Methylene-H_4MPT dehydrogenase; Cyt, cytochrome; AcK, acetate kinase; NAR, nitrate reductase; NRF or NIR, nitrite reductases; NOR, nitrous oxide reductase.

ANME-dominated cultures (Meyerdierks et al., 2010; Stokke et al., 2012; Wang et al., 2014). The metagenomic data were also in agreement with data showing highly abundant proteins in ANME-dominated microbial mats, identified as subunits of methyl-CoM reductase (MCR) (Krüger et al., 2003). These metagenomic studies, lending strong support for reverse methanogenesis, have facilitated further progress in understanding the process of ANME, including mechanisms for methane activation and interspecies electron transfer. Validation of the involvement of MCR in methane activation has been demonstrated experimentally by showing reverse reaction activity for an MCR from a bona fide methanogen (Scheller et al., 2010). Additional validation was obtained through purification and crystallization of an MCR homolog from an ANME-enriched microbial mat (Shima et al., 2011). This enzyme revealed striking structural similarities with MCR enzymes involved in methanogenesis (Shima et al., 2011), further suggesting that methane production and methane oxidation may rely on the same enzyme acting in reverse. Indeed, it has been demonstrated that ANME organisms can both produce and oxidize methane, a conclusion based on quantification of gene transcripts in zones of methane oxidation and methane production separated across the depths of a sediment (Lloyd et al., 2011). Direct coupling of methane oxidation to sulfate reduction by ANME organisms without the requirement of SRB partners (Milucka et al., 2012) was proposed to resolve potential mechanisms for interspecies electron transfer. However, this proposal contradicted the well-documented co-occurrence and tight physical association of ANME and SRB (Boetius et al., 2000; Orphan et al., 2001). An alternative hypothesis of direct electron transfer (DIET) between ANME and SRB has been supported by data from several recent studies (McGlynn et al., 2015; Wegener et al., 2015; Krukenberg et al., 2016). Further support for DIET was obtained through decoupling methane oxidation from sulfate reduction using artificial electron acceptors (Scheller et al., 2016). Respiration

with solid electron acceptors via extracellular metal reduction could be one mechanism responsible for the described processes coupled to insoluble iron (III) and manganese (IV) (Beal et al., 2009).

Methane oxidation by ANME can also be linked to denitrification (Haroon et al., 2013; Arshad et al., 2015). This metabolism was proposed to involve a different syntrophic partner, the anaerobic ammonia-oxidizing bacteria of the genus *Kuenenia* (Planctomycetes) (Haroon et al., 2013). Recently, novel lineages of archaea were identified, including Thorarchaeota (Seitz et al., 2016), Bathyarchaeota and MSBL1 (Evans et al., 2015; Mwirichia et al., 2016; Lazar et al., 2016), for which a role in methane metabolism was predicted. However, whether these novel organisms are active in methane oxidation or in methanogenesis remains to be demonstrated.

For carbon assimilation, ANME, like the methanogens, utilize the Wood–Ljungdahl pathway (Hallam et al., 2004), the -omics based evidence being supported by *in-silico* metabolic simulations (Nazem-Bokaee et al., 2016).

Methanotrophic Bacteria

Methanotrophic bacteria are found among three phyla: Proteobacteria (Alpha- and Gamma-), Verrucomicrobia, and NC10 phylum ("Candidatus *Methylomirabilis oxyfera*") (Chistoserdova and Lidstrom, 2013). Methanotrophs are diverse in terms of cell shape: in addition to the common rod or coccoid shapes (Trotsenko and Murrell, 2008; Islam et al., 2008), spiral (Danilova et al., 2016), filamentous (Stoecker et al., 2006) and polygonal shapes (Wu et al., 2012; Gambelli et al., 2018) have been described. Frequently, the cells are decorated with surface layer (S-layer) proteins displaying hexagonal symmetry (Khmelenina et al., 2010; Shchukin et al., 2011). The function of these S-layers remains unknown. One unifying feature among proteobacterial methanotrophs is the presence of networks of intracytoplasmic membranes (ICMs) that fill the cells (Weaver and Dugan, 1975; Hyder et al., 1979). Similar structures have also been detected in some verrucomicrobial methanotrophs (van Teeseling et al., 2014). However, so far, they have not been observed in "*Methylomirabilis oxyfera*". Where present, the ICMs are assumed to house particulate methane monooxygenase (pMMO), the key enzyme involved in methane oxidation (Sirajuddin and Rosenzweig, 2015). Some methanotrophs encode a soluble type of methane monooxygenase (sMMO) (Sirajuddin and Rosenzweig, 2015). The mechanism for ICM formation remains unknown, but their presence has been correlated with the expression of pMMO that, in turn, depends on the presence of copper (Brantner et al., 1997; Murrell et al., 2000). Substrate availability also seems to be a factor in ICM formation, as starvation has been demonstrated to cause a higher content of ICMs per cell (Tavormina et al., 2017).

Methanotrophs are frequently observed to contain cytoplasmic inclusion bodies, which vary in composition between species, but which are typically formed of polymeric storage compounds. Polyhydroxyalkanoates (PHA) and glycogen are among such storage compounds (Dunfield et al., 2003; Khadem et al., 2012a), while other electron-dense inclusions have been identified as phosphate storage granules (van Teeseling et al., 2014).

As pMMO-encoding methanotrophs have high demand for copper, an essential cofactor of pMMO methane catalysis (Sirajuddin and Rosenzweig, 2015; DiSpirito et al., 2016; Semrau et al., 2018), some methanotroph species have been shown to secrete a chalkophore, methanobactin, which binds copper ions, which are then transported into cells in a form of the methanobactin-copper complex (Dassama et al., 2016; DiSpirito et al., 2016; Semrau et al., 2018).

The product of methane oxidation is methanol, which is further oxidized to formaldehyde (Anthony, 1982). One enzyme that carries out this function, a periplasmic pyrroloquinoline quinone (PQQ)-dependent, calcium-containing methanol dehydrogenase (MDH), has been identified over a half century ago and studied in detail for years (Anthony and Zatman, 1964, 1965, 1967a,b; Anthony, 2004). The enzyme consists of two subunits, large and small, MxaF and MxaI, and it is functionally linked to a dedicated cytochrome, MxaG (Williams et al., 2006). Rather surprisingly, a novel MDH has been recognized just recently, whose single type of subunit, XoxF, is homologous to MxaF. This enzyme requires lanthanides – rare Earth elements (REEs) – in its active site, instead of calcium (Pol et al., 2014; Keltjens et al., 2014). This discovery was remarkable not only in terms of demonstrating the existence of an enzyme overlooked for over a century of methylotrophy research, but also in terms of overturning the long-standing dogma of the biological inertia of REEs (Lim and Franklin, 2004). Genetic investigations in model organisms indicated that not only are REEs important for the activity of XoxF enzymes, but that they are also involved in the regulation of the alternative MDH enzymes, by decreasing expression of the *mxa* gene cluster and by increasing expression of *xox* genes (Vu et al., 2016; Chu and Lidstrom, 2016; Chu et al., 2016; Gu et al., 2016). High expression of *xoxF* genes and proteins in diverse environments, determined through metatranscriptomics and metaproteomics (Delmotte et al., 2009; Sowell et al., 2011; Mattes et al., 2013; Moitinho-Silva et al., 2014; Grob et al., 2015; Ramachandran and Walsh, 2015), also suggests that *xoxF*-encoding microbes must possess molecular mechanisms for solubilizing, chelating, and transporting REEs into cells; however, these mechanisms have yet to be discovered (Chiustoserdova, 2016).

Methanotrophic bacteria can use various pathways for formaldehyde oxidation, including direct oxidation via a formaldehyde dehydrogenase (Hou et al., 2008; Khadem et al., 2012b), cofactor-mediated C1-transfer pathways, and, in organisms operating the ribulose monophosphate (RuMP) cycle, the oxidative pentose phosphate pathway (Trotsenko and Murrell, 2008). In cases when formate is the final product of formaldehyde oxidation, formate dehydrogenases (FDH) complete the primary pathways. Several types of the FDHs have been described; however, two of them, including tungsten- and molybdenum-dependent enzymes are the most prevalent (Chistoserdova, 2011).

Three different metabolic pathways can contribute to C1-carbon assimilation, including the RuMP pathway, the serine cycle, and the Calvin–Benson–Bassham (CBB) cycle (Anthony, 1982, Fig. 4). The pathways have recently undergone a revision, through matching the modern *-omics* data to the established knowledge in combination with enzyme and metabolite characterizations.

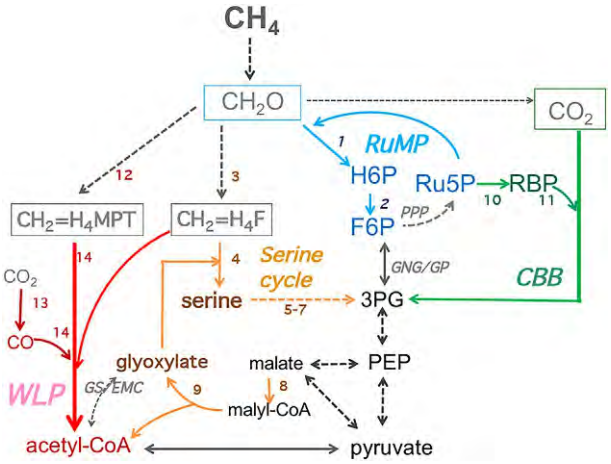

Fig. 4 Main pathways for carbon assimilation by methanotrophs. RuMP, assimilatory ribulose monophosphate pathway (unique enzymes 1–2); SC, serine cycle (unique enzymes/steps 3–9); CBB, Calvin cycle (unique enzymes 10–11); WLP, the reductive acetyl-CoA (Wood–Ljungdahl) pathway (unique enzymes/steps 12–14). 1. hexulosephosphate synthase; 2. hexulosephosphate isomerase; 3. H_4F, tetrahydrofolate-linked C1 transfer; 4. serine hydromethyltransferase; 5–7. serine glyoxylate aminotransferase, hydroxypyruvate reductase, and glycerate 2-kinase; 8. malate thiokinase; 9. malyl CoA lyase; 10. phosphoribulokinase; 11. ribulose-1,5-bisphosphate carboxylase; 12. H_4MPT, tetrahydromethanopterin-linked C1 transfer; 13. carbon monoxide dehydrogenase; 14. CO-methylating acetyl-CoA synthase. Additional pathways: PPP, pentose phosphate pathway; GNG, gluconeogenesis; GP, glycolytic pathway; the Embden–Meyerhof–Parnas (EMP) pathway and/or the Entner–Doudoroff (ED) pathway; EMC, ethylmalonyl-CoA pathway; GS, glyoxylate shun.

The pathway for glyoxylate regeneration by serine cycle methylotrophs not possessing the glyoxylate shunt (GS, among them the alphaproteobacterial methanotrophs) that had remained a mystery for half a century (Anthony, 2011) has been only recently determined to be the ethylmalonyl-CoA cycle (EMC, Erb et al., 2007). The discovery of this new pathway also prompted a reevaluation of the amount of carbon assimilated in the form of CO_2 through the multiple carboxylation reactions that are key to this metabolism (Erb et al., 2007; Yang et al., 2013). Interestingly, genomic evidence indicated that the gammaproteobacterial methanotrophs also encode enzymes for all key steps of the serine cycle but not the EMC or GS (Kalyuzhnaya et al., 2015a,b). Yet the enzymes are expressed and are functional (But et al., 2017). The role of this variant of the serine cycle in these methanotrophs and its significance for methylotrophy metabolism remains to be determined. Once assumed unlikely due to low efficiency (Chistoserdova et al., 2009), assimilation of carbon from methane through the CBB cycle has been demonstrated in certain methanotrophs, such as Verrucomicrobia and NC10 Phylum (Khadem et al., 2011; Rasigraf et al., 2014).

The dogma of the 'obligate' dependence of methanotrophs on single carbon compounds (methane or methanol) has also been recently overturned by the demonstration of growth on and metabolism of other organic compounds such as acetate and propane, at least in alphaproteobacterial methanotrophs (Crombie and Murrell, 2014; Dunfield and Dedysh, 2014). Analysis of the genomes of multiple methanotrophs indicated the presence of all of the genes for a complete tricarboxylic acid (TCA) cycle (Kalyuzhnaya et al., 2015a; Flynn et al., 2016), contrary to prior beliefs based on enzyme activity measurements (Anthony, 1982; Trotsenko and Murrell, 2008). ^{13}C-tracer metabolomics applied to gammaproteobacterial methanotrophs in combination with mutant analysis demonstrated that a significant flux of carbon occurs through the TCA cycle, identifying this pathway as a source of not only precursors for biomass synthesis but also of reducing power (Fu et al., 2017). The TCA cycle in gammaproteobacterial methanotrophs appears to be highly branched and mostly dedicated to the production of two key amino acids, aspartate and glutamate. However, glutamate could be returned into the TCA cycle by the way of succinate-semialdehyde dehydrogenase and glutamate carboxylase, while aspartate could be converted into fumarate via aminotransferase and aspartate lyase. Thus, the function of the TCA cycle may to provide a balance between anabolic reactions and to supply the metabolites necessary for fulfilling redox demands (Akberdin et al., 2018).

Finally, the RuMP pathway now appears as a very modular route of formaldehyde assimilation. The reactions downstream from the two key steps of formaldehyde incorporation into phosphosugars might include Embden–Meyerhof–Parnas (EMP pathway) reactions, Entner–Doudoroff pathway reactions, and the *Bifidobacterium* shunt (Akberdin et al., 2018). All three pathways are interconnected with the pentose phosphate pathway reactions for the regeneration of ribulose 5-phosphate. This metabolic flexibility supports a number of additional functional abilities of methanotrophic bacteria, including conversion of C_2-units from acetyl-CoA back into C_5 and C_6-sugars via a reverse phosphoketolase (Akberdin et al., 2018) and the proposed acetate fermentation pathway upon growth at low oxygen tensions (Fig. 2; Kalyuzhnaya et al., 2015b).

Oxygen-Dependent Metabolism of Methane in Anoxic and Hypoxic Environments

A novel mode of metabolism has been proposed for bacteria belonging to the Candidate Phylum NC10 (tentatively named *Methylomirabilis oxifera*), which oxidize methane in anoxic or hypoxic environments, but employ the classic methane oxidation

pathway (pMMO, MDH and H_4MPT-linked formaldehyde oxidation) and the CBB assimilatory cycle (Rasigraf et al., 2014). However, no external source of oxygen was considered for their metabolism, thus oxygen was proposed to be generated intracellularly, via a modified denitrification pathway (Raghoebarsing et al., 2006; Ettwig et al., 2010). So far, this metabolic mode remains unconfirmed in terms of enzyme identities or activities. Meanwhile, the more traditional methanotrophs have also been shown to express the denitrification pathway under hypoxia, suggesting that electron acceptors alternative to oxygen may indeed be involved in methanotrophy (Kits et al., 2015; Kalyuzhnaya et al., 2015a; Fig. 2). NC10-type bacteria, both terrestrial and marine, are typically detected in environmental niches characterized by high concentrations of methane and nitrate (Zhu et al., 2012; Deutzmann et al., 2014; Padilla et al., 2016). However, multiple environmental studies provide strong evidence for the activity of true 'aerobic' methanotrophs in hypoxic and anoxic conditions. *Methylobacter* species have been identified as the most active types in arctic soils and lakes (Yergeau et al., 2010; Martineau et al., 2010; Graef et al., 2011; Tveit et al., 2013, 2014; Crevecoeur et al., 2015) while the identity of gammaproteobacterial methanotrophs in non-arctic lakes remains uncertain (Oswald et al., 2016a). In some lakes, alphaproteobacterial methanotrophs have also been detected in both oxic and anoxic layers (Oswald et al., 2016b).

Compelling evidence has been presented for anaerobic methane oxidation by Proteobacteria in the absence of nitrate (Blees et al., 2014), and none of the other alternative electron acceptors were unequivocally proven to be involved, while stimulation of methane oxidation by Fe(III) and Mn(IV) has been observed in some samples (Oswald et al., 2016b). The exact mechanism that allows 'aerobic' methanotrophs to thrive in anoxic environments remains to be discovered. A recent study that characterized a novel microaerophilic methanotroph suggested that motility may play a key role in benefiting from the optimal methane/oxygen concentrations in natural environments (Danilova et al., 2016).

One mechanism for adaptation to anoxic conditions emerged from starvation experiments, in which methanotrophs were demonstrated to survive better in the absence oxygen. Fermentation metabolism was proposed to be responsible (Roslev and King, 1994, 1995). Indeed, such a pathway has been recently demonstrated in a gammaproteobacterial methanotroph (Kalyuzhnaya et al., 2013). The ability of 'aerobic' methanotrophs to ferment has implications beyond the occurrence and distribution of aerobic methane utilization, as it also suggests a potentially much larger contribution to local and global carbon cycling than previously thought. It is likely that methane oxidation by 'aerobic' methanotrophs is the main mode of methane metabolism in ecosystems with limited but constant input of oxygen (such as shallow anaerobic freshwater sediments, anaerobic soils surrounding plant roots, or deep sea). While in some of those niches the oxygen may be present under detectable levels, a low but constant flux of oxygen from photosynthesis (Miluka et al., 2015; Oswald et al., 2015; Hill et al., 2017) or radiolysis (Lin et al., 2005) may be supporting active methanotrophic communities. However, the scale and the distribution of fermentation-based methanotrophy need further investigation.

Syntrophy and Predation in Methanotrophy

As mentioned above, anaerobic methane oxidation by either ANME or NC10 bacteria appears to depend on syntrophic associations, as, so far, none of these organisms have been isolated in pure culture. In the case of ANME, the requirement for syntrophy is assumed to be due to energetic constrains of methanotrophy via reverse methanogenesis when coupled to sulfate reduction (Thauer and Shima, 2008) or the necessity for nitrite removal when coupled to denitrification (Haroon et al., 2013). The reasons for potential syntrophic dependencies of NC10 bacteria are unknown, and it is possible that they could be obtained in pure culture based on the notion that their metabolism follows the same scheme as the metabolism of proteobacterial methanotrophs. As to the latter, while proteobacterial methanotrophs have been isolated and maintained in pure cultures for over a hundred years (Kaserer, 1906; Söhngen, 1906), it appears that in nature they also tend to form consortia, and, while carbon from methane is a hard-earned currency, they share it generously with other bacteria (Kalyuzhnaya et al., 2008; Beck et al., 2013; Oshkin et al., 2015; Hernandez et al., 2015; Yu et al., 2017; Yu and Chistoserdova, 20162017). Some of the most commonly co-occurring species are non-methane-utilizing methylotrophs (Oshkin et al., 2015; Hernandez et al., 2015), which are proposed to feed on methanol leaked by the methanotrophs (Krause et al., 2017). Other co-occurring species include non-methylotrophic bacteria, most frequently members of Burkholderiales and Flavobacteriales (Oshkin et al., 2015; Hernandez et al., 2015; Yu et al., 2017), and these are likely feeding, respectively, on organic acids released by the methanotrophs (Kalyuzhnaya et al., 2013) and on the polymeric substances that are excreted by the methanotrophs and other community members (Yu et al., 2017; Yu and Chistoserdova, 2017). Little is known about what methanotrophs get in return. Among the most interesting observations is the oxygen – CO_2 exchange between methanotrophs and cyanobacteria (Miluka et al., 2015; Oswald et al., 2015; Hill et al., 2017).

Like many other bacteria, methanotrophs can be infected by viruses that may be species- to strain-specific (Tyutikov et al., 1983; Gambelli et al., 2016). Thus, virus predation is yet one additional mechanism controlling trajectories on methanotroph consortia in nature. Metabolic reprogramming of methanotroph genomes may also be mediated by viruses, based on the presence of prophages in their genomes (Kalyuzhnaya et al., 2015a; Flynn et al., 2016).

Conclusions and Future Perspectives

While the methanotrophy field is over a hundred years old, recent developments indicate that we are quite far from understanding the process precisely, and many challenging questions remain. Several long-held dogmas in methanotrophy have been overturned recently. The 'strictly' aerobic nature of the proteobacterial methanotrophs has been questioned, as well as the relevance of

laboratory performance to performance in natural environmental niches. Novel biochemistries have been recently (re)discovered, such as the XoxF-type MDH distributed across bacterial methanotrophs, the complete tricarboxylic acid cycle, and functional serine cycle in gammaproteobacteria and the EMC pathway in alphaproteobacterial methanotrophs. Gammaproteobacterial methanotrophs have been shown to be capable of fermentation. Can alphaproteobacterial methanotrophs ferment as well? Is methanotrophy different in 'anaerobic' NC10 and 'aerobic' proteobacterial methanotrophs? Is denitrification different among these species? Why do methanotrophs share their carbon with other bacteria and do they get anything in return? Are the communities formed randomly or as guild-specific or even species-specific syntrophies? We are in a good position to address these questions as we now have access to extensive genomic databases, -omics, and modeling tools. However, these challenging problems can only be solved by combining the information tools with experimental validation, for which we need appropriate model organisms. Some of the newly-emerged questions, such the role of community function in methane oxidation may require complex model communities rather than pure cultures. We look forward to the next hundred years of methanotrophy research.

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Conflict of Interest

The authors declare no conflicts of interest.

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Relevant Website

http://www.methanotroph.org–Methanotroph Commons.

Microbial Ecology of the Rumen

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Abbreviations

AD	Anaerobic digestion
IHT	Interspecies hydrogen transfer
USDA	United States Department of Agriculture
VFA	Volatile fatty acids

Introduction

The rumen is the first of four pre-intestinal digestive chambers of mammals within the order Artiodactyla. The rumen is located just upstream of a second chamber, the reticulum, with which it can exchange material and which has many of the similar functions (and thus is sometimes considered together as the 'reticulorumen'). The basic functions of the rumen are to facilitate the microbial fermentation of feedstuffs to produce usable energy sources for the host (primarily volatile fatty acids, 'VFA') and microbial protein for subsequent digestion or absorption. Central to the functioning of the rumen is the process of rumination, by which partially digested feeds are regurgitated to the buccal cavity for re-chewing (the so-called 'chewing of the cud') and then re-swallowing. The mechanical movements of the buccal cavity and the structure of the animal's dentition combine to delaminate the layers of forage leaves and stems in a manner that exposes additional degradable surface while at the same time retaining the relatively long feed particle sizes that stimulates further rumination and chewing. The net effect of rumination is to decrease the size and increase the surface area of feed materials to improve their degradability, and to stimulate production of saliva that serves as the major component of ruminal fluid.

The Rumen as a Microbial Habitat

Just as ruminant species span a size range from ~3 kg to ~1200 kg, the rumen also varies in size, but typically comprises one-fifth to one-tenth of the weight of the adult ruminant host. The contents of the rumen are stratified, with a mat of freshly ingested and partially digested feeds floating atop a sea of ruminal fluid that contains feed and forage particles of various sizes and densities. The concentration of solids averages ~12%–15%, and increases gradually with depth, although periodic muscular contractions associated with rumination and animal movement keep the contents moderately well-mixed. The mat layer, primarily fibrous digesta, serves as a partial refugia for motile ruminal protists, allowing them to be selectively retained in the rumen to a degree greater than if they were freely suspended in (and washed out with) the ruminal liquid. The ruminal liquid phase has an approximate retention time of 8–12 h, while the larger solids may have retention times exceeding 48 h. It is this long retention of solids that permits the high degree of fiber fermentation that is achieved in the ruminant animal.

The ruminal fluid has a high salts content (220–420 mOsmoles L^{-1}), and is highly reducing (reduction potential −170 to −220 mV), thus supporting the growth of strictly anaerobic microbes. pH is typically maintained in the range of 6–7, but is often lower in animals consuming diets high in rapidly fermented starches or sugars. High concentrations of bicarbonate contained within saliva (produced at up to 100 L d^{-1}) and substantial concentrations of VFA (60–180 mM) work together to provide effective pH buffering.

Ruminal Fermentation Pathways

Like all fermentations, that of the rumen involves the conversion of organic materials of intermediate oxidation state to a mixture of products of higher, lower, or similar oxidation state. Most of the fermentable substrates in feeds are carbohydrates, and their fermentation products include CO_2, formate, and succinate (more oxidized); acetate and lactate (redox-neutral); and C_3-C_6 VFA and methane (more reduced). The relative proportions of these products vary depending on the specific substrate and the species composition of the microbial community. In all cases, the organisms must maintain a redox balance, which is accomplished by directing the flux of fermentation intermediates through different catabolic pathways.

The ruminal fermentation has some similarities to another economically important mixed culture fermentation, the anaerobic digestion (AD) of organic wastes. In AD, long retention times (2–4 weeks) are employed to allow a nearly complete mineralization of organic material to methane and CO_2. VFA, the conventional intermediates of AD, are fermented to acetate and H_2 (a process known as proton-reducing acetogenesis), and methane is produced both by reduction of CO_2 with H_2 (hydrogenotrophic

methanogenesis), and by cleavage of acetate to methane and CO_2 (aceticlastic methanogenesis). In the rumen, retention times are much shorter than in AD – too rapid to allow establishment of the slow-growing VFA fermenters and aceticlastic methanogens. As a result, methane yields in the ruminal fermentation are much lower, and the unfermented VFA (primarily acetate, propionate and butyrate) remain available for absorption through the rumen wall, to serve as primary sources of energy and biosynthetic precursors for the host animal. Acetate serves primarily as a source for synthesis of lipids (including milk fat in the mammary gland). Propionate serves primarily as a substrate for gluconeogenesis in the liver. Butyrate serves primarily as an energy source for the absorptive cells of the rumen epithelium.

The rumen is additionally a site of protein fermentation by some members of the microbial community. Feed proteins are, to a variable extent, hydrolyzed to peptides and amino acids, which can either be fermented by this subpopulation as a source of energy, or used as anabolic precursors by the larger microbial community. The products of protein fermentation are primarily the above-mentioned VFA and CO_2, but also ammonia, and with very little H_2 or methane. Fermentation of amino acids is carried out both by metabolic generalists that also ferment carbohydrates (e.g., *Prevotella* and *Butyrivibrio*) and by obligate amino acid fermenting bacteria (e.g., *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *C. aminophilum*). The latter group can ferment amino acids at ten times the rate of the former, but their overall contribution to ammonia production is unclear, as they typically have very low abundance in the rumen. Although the ruminal community can synthesize large amounts of microbial cell protein from ammonia and VFA and thus recapture some of the protein lost to fermentation, the process of protein utilization in the rumen results in a net loss of feed protein, and thus contributes to the substantial inefficiency of N utilization by ruminants.

On the whole, then, the rumen can be regarded as an essential organ in which feeds that cannot be used directly by the host are converted to VFA, the usable currency for energy metabolism for the host animal. VFA production allows the ruminal fermentation to maintain its redox balance in a habitat where electron acceptors are generally lacking (CO_2 being a major exception). The overabundance of readily oxidizable organic matter in the ruminant diet also facilitates ready utilization of other electron acceptors that may become available, including unsaturated fatty acids (reduced to their corresponding saturated fatty acids); nitrates and nitrites (reduced to ammonia); and sulfate (reduced to sulfide, which at elevated levels can cause the neurodegenerative disease, polioencephalomalacia).

Culturable and Uncultured Ruminal Microorganisms

The development by Hungate and others in the 1940s of effective techniques for cultivating strictly anaerobic bacteria led to the isolation and characterization of many ruminal bacterial species that, in total, appeared to represent a substantial portion of the fermentative capabilities of the ruminal community. These isolates allowed microbiologists to elucidate the biochemical pathways of these fermentations and to quantify fundamental parameters such as growth yields and maintenance requirements. They also permitted, through studies of mixed cultures of defined species composition, the elucidation of metabolic interactions among these species. As it turned out, however, there was much more to the story.

By the late 1970s, fundamental studies on microbial phylogeny, gained largely through sequence analysis of chronometric macromolecules such as small-subunit ribosomal RNAs, revealed that species diversity in all microbial habitats was far greater than had been anticipated from studies that employed only culture-based methods. In some habitats, particularly low-nutrient ones such as pristine groundwater or oligotrophic lakes, fewer than 0.01% of the microbial population could be cultivated in the laboratory. For the rumen, it appears that the percentage is much higher – on the order of 10%. Even so, it is clear that the vast majority of the ruminal microbial community has resisted laboratory cultivation. So, while much of the metabolic activity of the rumen can be understood from earlier studies with cultured representatives of the community, elucidating the characteristics and capabilities of the larger, uncultivated community members is likely to provide a trove of new information that will inform our understanding of ruminal metabolism.

Bacteria are the most numerically dominant members of the ruminal microbiome, accounting for $\sim 10^9$–10^{10} cells mL^{-1} of ruminal fluid. Analysis of community composition using the techniques of molecular ecology is confounded somewhat by methodological issues, such as community DNA extraction method, PCR primer sequences, sequencing platform, and downstream bioinformatics package. Consequently, it is difficult to compare community compositions across studies. Nevertheless, some general trends on community composition have emerged. It is clear that the two most abundant bacterial phyla in the rumen are the Firmicutes and Bacteroidetes. The Firmicutes are dominated by the order Clostridia, particularly the genera *Butyrivibrio*, *Ruminococcus*, *Lachnospira* and *Succiniclasticum*. The Bacteroides are heavily dominated by the genus *Prevotella*, which may represent half or more of the individual bacteria in the rumen.

Archaea are much less abundant than bacteria ($\sim 10^7$–10^8 cells mL^{-1}), and consist almost entirely of methanogens. The hydrogenotrophic genus *Methanobrevibacter* is by far the most dominant genus, although there are significant abundances of the little-studied methylotrophic family Methanomassilliicoccaceae.

Protists (often called protozoa) are much less abundant than bacteria (10^5–10^6 cells/mL), but because of their large size (10–100 μm) can comprise 40% or more of total ruminal microbial biomass. The two most abundant groups in the rumen are the entodiniomorphs and the isotrichids. Both are motile via cilia, though the distribution of the cilia differs between the groups. Entodiniomorphs have tufts of cilia, and those near the mouth aid in engulfment of particulate matter – bacteria, starch granules, fiber and insoluble protein – that support protozoal growth. The engulfment and fermentation of bacteria and feed proteins result in ammonia production and contribute to nitrogen inefficiency in the host animal. On the other hand, the entodiniomorphs assist

in fiber degradation, and their engulfment of starch and its subsequent slow fermentation decreases the much more rapid degradation of starch by bacteria that can lead to lactic acidosis. The other major protozoal group, the isotrichids, have cilia evenly distributed on the cell surface, and subsist primarily on soluble sugars. When sugar is abundant, the isotrichids produce and store massive amounts of glycogen, often rendering the cells opaque. Isotrichids are also thought to assist in maintenance of anaerobic conditions in the rumen, as they can respire oxygen, albeit without coupling the respiration to ATP synthesis. Some protozoa harbor endosymbiotic methanogens (discussed in more detail below) and thus are indirect contributors to ruminant enteric methane production.

Ruminal fungi were originally thought to be protozoa, due to the presence of a motile zoospore stage, but their fungal nature was revealed by their chitin-containing cell walls; their filamentous growth habit during their non-motile stage; and, ultimately, their 18S rRNA gene sequences. While not particularly abundant (10^3–10^4 cells mL^{-1}), these fungi appear to be important in degrading plant cell wall material via a combination of physical splitting of fibers by growing appresoria (exposing fibers for bacterial colonization), and by production of small amounts of high-specific activity polysaccharide hydrolases. The ruminal fungi are presently restricted to family Neocallimasticaceae within the primitive, anaerobic phylum Neocallimastigomycota. Assessment of phylogenetic diversity within the fungi is complicated by the limited sequence diversity within the small-subunit rRNA genes, but examination of internally transcribed sequences between rRNA genes has revealed extensive diversity within the fungal group.

While the above characterizations of the ruminal community are averaged across the whole of the rumen, there are substantial differences in the community compositions of the solid phase (which contains feed particles in the process of degradation) and the liquid phase of ruminal contents. Samples collected directly from the rumen through a ruminal fistula are considered the gold standard for community composition analysis, as they allow for representative sampling of the rumen and separate analysis of the liquid- and solids-associated communities. Samples collected by stomach tubing or by buccal swabbing are more enriched in the liquid-associated community, but methodological improvements (such as directly hand-capturing the cud, or buccal swabbing during periods of active rumination) are likely to improve representative sampling, and thus allow analysis of a larger number of animals than would be possible by relying purely on fistulated animals.

In addition to the solids- and liquid-associated microbial communities, there is an important but relatively unstudied epimural community that is attached to the interior of the rumen wall. This community is the interface between the ruminal liquid phase and the ruminal epithelial cells that are involved in uptake of VFA, NH_3, CO_2 and other metabolites into the host circulatory system. Community analysis reveals that, relative to the liquid and solid phases of ruminal contents, the epimural community is underrepresented in genus *Prevotella* but overrepresented in *Butyrivibrio*, consistent with the importance of butyrate as a primary energy source for the ruminal epithelial cells.

Microbial community analyses have revealed that microbial taxa that are abundant in feeds are much less abundant in the rumen itself. For example, lactic acid bacteria such as *Lactobacillus* or *Enterococcus*, that are the dominant bacteria in silages, are essentially undetectable even in the rumens of cows fed silage as the main component of their diet. This is a powerful demonstration that ruminal conditions select against nonruminal microbes, even those from plant-based anaerobic habitats. However, some transient species can still be problematic for human and animal health. For example, the rumen is a known reservoir for low populations of enteropathogenic *E. coli*, and it has been shown that feeding of too much grain (which decreases ruminal pH) activates acid resistance in these pathogens; upon shedding from the ruminant GI tract, these strains are more able to survive passage through the acid stomach of human hosts, and thus more capable of colonizing the lower GI tract, where they can cause severe – and even fatal – toxicoses.

Interactions Among Ruminal Microbes

Negative Interactions: Competition, Amensalism and Predation

The ruminant diet, despite its complexity, contains a relatively modest number of fermentable monomeric components. Because the utilization of these substrates is distributed across a large number of different microbial species, it is inevitable that there is intense competition among these species for these substrates. There appears to be a relationship between the metabolic capabilities of the fermentative organisms and the abundance of the substrates. Widely available substrates such as glucose can be used by a large number of catabolically versatile species, while less abundant substrates (e.g., plant toxins such as mimosine or oxalic acid) are metabolized by a relatively small number of metabolic specialists.

Competition, an interaction in which there is a simultaneous inhibition of two organisms based on their shared utilization of a common substrate, is governed by the concentration of substrate and by the relative abundance and metabolic activity of the individual microbial species, including their maximal rates of substrate utilization and their saturation constants. Analysis has revealed that utilization rates for most substrates follow Michaelis–Menten kinetics, and are typically first-order with respect to substrate concentration. In the case of insoluble biopolymers (e.g., cellulose), degradation rates appear to depend on the accessible surface area. The most abundant and capable of the cellulolytic species require adherence to cellulose for effective hydrolysis (Fig. 1), so the outcome of competition is driven primarily by an organism's ability to rapidly colonize cellulose fibers as they are exposed by rumination.

Amensalism, an interaction based on the inhibition of one organism by another, occurs in the rumen via production of bacteriocins. Most bacteriocins are small, membrane-permeabilizing proteins that often show a high degree of specificity – some times inhibiting only closely related species or even other strains within the same species. While most studies of bacteriocins have been carried out using

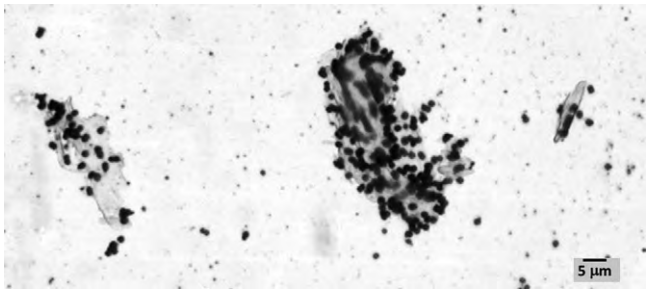

Fig. 1 Light microscopic image of three particles of microcrystalline cellulose colonized by cells of the ruminal cellulolytic bacterium *Ruminococcus albus* 8 (visualized here by staining with crystal violet dye). Effective cellulose hydrolysis requires adherence of the cells to the particles, and the adherent bacteria far outnumber the non-adherent (planktonic) cells, which will likely colonize an exposed cellulose surface upon contact. Physical adherence to cellulose allows the cellulolytic cells first access to the soluble products of cellulose hydrolysis, and helps them to compete effectively with the many saccharolytic but non-cellulolytic species within the diverse community of the rumen.

common intestinal bacteria such as *E. coli*, most of the known bacteriocin producers in the rumen are Gram-positive members of the Firmicutes, particularly within the genera *Butyrivibrio*, *Streptococcus* and *Ruminococcus*.

Predation of microbes in the rumen primarily involves engulfment of bacteria by flagellated and ciliated protists (protozoa). Feeding appears to be non-specific. The net result is that hard-won microbial protein is degraded by the ingesting protozoa, thus reducing the N efficiency of the ruminal fermentation. Artificial removal of protozoa from the rumen (a process termed 'defaunation') can be accomplished with certain chemical agents (e.g., alkanes or lauric acid), but the effects on animal performance have been somewhat contradictory. In general, defaunation decreases the wasteful process of methanogenesis and improves N efficiency, but can also result in decreased fiber digestion. Moreover, these effects gradually disappear over time (~10–12 wks) as the protozoa slowly recolonize the rumen. While protozoa are the primary agents of predation in the rumen, shotgun metagenomics sequencing of ruminal contents have revealed the presence of an extensive viral community, and also the presence of predatory bacteria such as *Vampirobacter*. The quantitative importance of these groups remains to be elucidated.

Mutualistic Interactions

Mutualistic interactions, in which both partners benefit from the association, are common in the rumen (Fig. 2). The best-known example is 'interspecies hydrogen transfer' (IHT), which was first characterized in the rumen but has subsequently been shown to be widely distributed in anaerobic habitats. In IHT, the H_2 produced by a fermentative microbe is used as an electron donor and energy source for a H_2 consuming microbe (methanogenic archaea, sulfate-reducing bacteria, etc.). The H_2 consumer obviously benefits from the availability of the electron donor. The H_2 producer benefits because continuous removal of H_2 permits enhanced flux of

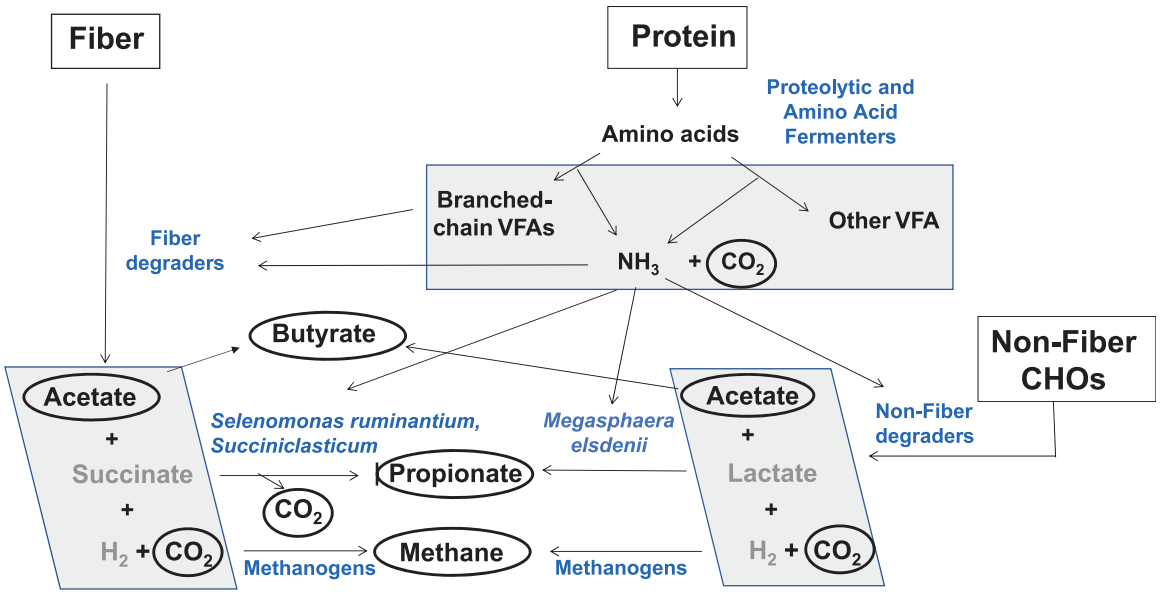

Fig. 2 Examples of interactions among trophic groups in the rumen. Major dietary inputs are in square boxes; major terminal end products are in oval boxes. Key intermediates that do not typically accumulate are in grey type. Exemplary microbial species or groups are in blue. The straight-chain C_4-C_6 VFA butyrate (shown) along with valerate, and caproate (not shown) are synthesized primarily via reverse β-oxidation by *M. elsdenii*, among others.

reducing equivalents toward the reduction of protons to H_2 ($2\ H^+ + 2\ e^- \rightarrow H_2$). This allows the fermentative bacterium to channel more carbon-containing catabolic intermediates toward production of acetate (a pathway that generates more ATP for growth) rather than toward production of reduced intermediates such as lactate or ethanol (pathways that have lower net ATP yields). A particularly interesting type of IHT occurs between carbohydrate-fermenting holotrich protists and endosymbiotic methanogens. Pyruvate produced by the protozoa is translocated to a specialized intracellular organelle, the hydrogenosome, where the pyruvate is converted to acetate, CO_2, and H_2. The H_2 diffuses through the hydrogenosome membrane, where the endosymbiotic methanogens hungrily await. The phenomenon can be visualized under a fluorescence microscope, due the autofluorescence of F_{420} electron carrier within the methanogenic cells.

The microbial use of another microbe's fermentation products in the rumen extends well beyond IHT. Succinate is an important intermediate in carbohydrate fermentation by some ruminal bacteria, such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Prevotella bryantii*, and *P. brevis*. However, succinate is rarely detected in ruminal fluid, due to the activity of succinate-fermenting bacteria such as *Selenomonas ruminantium* and *Succiniclasticum ruminis*, which decarboxylate succinate to produce propionate. It has been estimated that approximately half of the propionate produced in the rumen is derived from succinate decarboxylation (the other half is derived from conversion of lactate, via acrylate as an intermediate).

Cross-feeding of nutrients is not restricted to energy sources. One well-documented example involves peptide- and amino acid-fermenting bacteria and cellulolytic bacteria (Fig. 1). The former produce the branched-chain volatile fatty acids (BCVFA) isobutyrate, 2-methylbutyrate and isovalerate, which are required for synthesis of lipid precursors (long, branched-chain fatty acids) by the primary cellulolytic bacteria *Rumincoccus albus*, *R. flavefaciens* and *Fibrobacter succinogenes*.

The ruminal microbial community is also important as a producer of vitamins needed by the host animal. These include the common water-soluble vitamins as well as the fat-soluble vitamin K. Production levels are typically sufficient to meet the needs of the host, so in conventional ruminant rations only vitamins D and E are required. Many individual species of ruminal microbes require the water-soluble vitamins, but their needs are met via overproduction by other members of the microbial community.

Redundancy and Resilience of the Ruminal Microbial Community

As noted previously, there are likely hundreds of individual species of ruminal bacteria, based on community analyses of rRNA gene sequences in bulk ruminal contents. By contrast, there are likely a smaller number of potential fermentable substrates or hydrolyzable polysaccharide linkages. Simple division allows us to conclude that, on average, there are multiple species capable of exploiting any particular "degradation point", and many substrates such as glucose, are likely degradable by many dozens of species. Moreover, many ruminal species are catabolically versatile, being capable of fermenting dozens of substrates. On the whole, then, the ruminal community displays considerable overlap of metabolism, or a high degree of 'functional redundancy'. As a result, the ruminal community is less dependent on individual members, because if one such species should disappear, other community members, either individually or collectively, are likely able to assume the functional role of the displaced member. In fact, despite the large variation in the abundance of individual microbial species across individual animal units fed similar diets, there is a striking similarity in the proportions of the various fermentation products, i.e., there appears to be a distinct convergence of function among different communities.

Ruminal communities from individual cows display temporal variation in composition, particularly during the feeding cycle. Soon after feeding, rapidly fermented substrates are metabolized to yield organic acids at rates that outpace the ability of the host to absorb them, or the ability of salivary buffers to neutralize them. Nevertheless, despite these temporal variations, each rumen has a relatively unique range of microbial species compositions. This community displays remarkable resilience, as shown by the fact that near-total (~95%) exchange of ruminal contents across animals results in a gradual return, over days to weeks, to approximate the pre-disturbed composition. Some of this resilience is likely due to inherent differences in ruminal chemistry between animals (e.g., in pH dynamics or VFA concentrations), but the resilience has also been observed following exchanges in ruminal contents between animals having similar ruminal chemistries.

The redundancy of the ruminal community explains the behavior and usefulness of in vitro ruminal fermentations. Such fermentations are widely employed to assess the fermentability of feeds and feed components; to compare the digestibility of different forage cultivars; or to test the effects of ruminal modification agents. In vitro fermentations do not benefit from continuous VFA removal or from continuous salivary buffering, as occurs in the live animal. Consequently, in vitro fermentations can be viewed as a type of enrichment culture, and – as in all enrichment cultures – the species composition of the community begins to change immediately following inoculation. Nevertheless, the outcome of the fermentation, in terms of product distribution and microbial cell yield, is remarkably similar to that observed in the rumen itself. This is likely due to the above-mentioned convergence of function among the many species in the rumen, such that taxa which are enriched under in vitro conditions produce fermentation end products in similar proportions to the taxa they outcompete in vitro.

Community Development and Host Individuality

At birth the ruminant animal lacks a functional rumen, and from a nutritional standpoint resembles the non-ruminant animal in that nutrients are derived primarily from colostrum, and soon thereafter from milk. The rumen develops slowly over about a

two-month period, stimulated largely by a gradual increase in intake of solid feeds, including fibrous feeds. These anatomical and digestive changes are accompanied by dramatic changes in the composition of the microbial community. Although the 3 most abundant bacterial phyla are Firmicutes, Bacteroidetes and Proteobacteria, regardless of age group, the proportion of the different genera comprising these phyla change in an age-dependent manner. During the first few days after birth, the community is dominated by taxa associated with aerobic or facultatively anaerobic metabolism, including the genus *Bacteroides*. Over a period of about two weeks, the community shifts toward one dominated by strictly anaerobic bacteria; the genus *Prevotella* becomes numerically dominant, and this dominance is retained into adulthood. Compared to the mature rumen of a 2-year old animal, calves at 1 d and 3 d of age show less diversity and a greater degree of inter-animal variation in bacterial community composition than do the animals at 2, 12, or 24 months. It is notable, however, that some taxa that are abundant in the adult rumen make their initial ruminal appearance within a few days of birth. One particular example is the genus *Ruminococcus*, most of whose members are considered to be cellulolytic, but whose ruminal appearance long precedes the ingestion of fiber.

Protozoa are not found in newborn calves, and often do not make their appearance until the animals reach several months of age. Protozoa can be established earlier by direct inoculation of calves with ruminal contents (or cud samples) from adult ruminants.

Although the bacterial communities of different animals on the same diet converges somewhat in species composition over time, adult ruminants do maintain microbial communities that are generally individualized to the host. This individuality is most clearly reflected in ruminal contents exchange experiments, as described in the previous section.

Community Responses to Ruminal Environmental Conditions

Response to Dietary Inputs

Surveys of ruminal microbial communities across host species and geographic regions have indicated that diet is the main driver of ruminal bacterial composition, overriding differences between individual animals, breeds within ruminant species, and even ruminant species themselves. Different diets select for variations in the proportions of individual bacterial species, and their differential use of particular fermentation pathways results in end product ratios that profoundly affect animal performance. Diets rich in soluble sugars and starch, particularly in rapidly fermentable forms such as finely ground high-moisture corn or barley, yield lactate as a major primary fermentation product, and subsequent conversion of this lactate to propionate results in the characteristically low ratio of acetate:propionate (A:P) observed under these feeding conditions. Protist community composition does not appear to show as much dependence on diet as do the bacteria, perhaps because of their predatory lifestyle and/or nonspecific feeding behavior. Fungal populations in the rumen are generally low (~1% of total microbial biomass) in ruminants fed diets rich in concentrates or in forages from temperate regions, but are five-fold or more higher in ruminants grazing tropical forages. This is likely the result of the important role fungi have in physically splitting and enzymatically depolymerizing the more heavily lignified and recalcitrant cell walls in tropical forages.

While the microbial communities in forage-fed ruminants differ substantially from those fed primarily concentrates, it has proven difficult to identify more than a few genera that differ consistently with diet. It does appear that the most abundant genus, *Prevotella*, is even more abundant in concentrate-fed animals, while forage-fed animals show greater abundance of *Fibrobacter* and unclassified members of the Ruminococcaceae and Bacteroidales families. Within individual studies, it is apparent that even minor changes in diet can have observable effects on community composition. For example, a seemingly modest shift in grazing dairy heifers from orchardgrass pasture to orchardgrass hay results in an increase in the molar percentage of acetate and a decrease in the molar proportion of butyrate, along with a decrease in the abundance of *Butyrivibrio*, the major butyrate producer in the rumen.

Response to Ruminal pH

After feed composition, pH is arguably the most important effector of the ruminal fermentation. Ruminants evolved as grazing animals, during which they largely subsisted on forages whose bulk limited intake, and whose composition and structural anatomy resisted rapid fermentation. In most grazing ruminants, ruminal pH remains in the range of 6–7 most of the time, and this pH range seems ideal for most ruminal microbes. The introduction of grains and other rapidly fermented concentrates to the diets of domesticated ruminants has resulted in greater dry matter intakes and more rapid fermentation of carbohydrate and protein, and as a result mean ruminal pH can be relatively low (sometimes less than 5), and diurnal pH range can be substantial. Individual ruminants fed the same diet can vary substantially in mean pH and diurnal pH range, due in part do differences in meal patterning; volume of buffering saliva secreted; and efficiency of VFA absorption by the ruminal epithelia.

In general, carbohydrates such as sugar monomers and dimers, as well as highly processed starches (such as milled barley and finely ground high-moisture corn) are fermented more rapidly than resistant starches or plant cell walls rich in cellulose and hemicelluloses. The agents of such rapid fermentation include *Prevotella* species, *Streptococcus bovis* and *Selenomonas ruminantium*. *S. bovis* has been widely considered as a major producer of lactic acid, which is a much stronger acid than are the VFA (pK_a 3.8 versus 4.8). Because lactate is consumed relatively slowly in the rumen, its production in quantity can substantially decrease ruminal pH, sometimes to values below 4.5. The resulting acute lactic acidosis can disrupt ion balances in the rumen, and if imported into the bloodstream can cause a dangerous drop in blood pH. Interestingly, *S. bovis* is barely detectable in the rumen of cows under normal conditions, and its abundance even under acidotic conditions is not particularly high, suggesting perhaps that *S. bovis* can be highly

active but produces only a low cell yield, or that other bacteria may contribute to lactic acidosis. Ruminal acidosis is not only caused by lactic acid accumulation. Some cows that display chronically low ruminal pH do not contain measurable amounts of lactate, but do contain high concentrations of VFA (up to 0.25 mol L^{-1}), and often do not produce adequate amounts of saliva to effectively buffer ruminal pH; such cows often have ruminal dry matter contents of 18% or higher.

Certain members of the ruminal microbial community appear to be particularly sensitive to low pH. The predominant species of cellulolytic bacteria do not grow at pH values below 6.0. However, pH values below this minimum do not kill these bacteria, but rather arrest their growth, without inhibiting their cellulases. Thus, even in animals with atypically low pH values, fiber digestion is not inhibited as long as pH remains above 6.0 for long enough during the feeding cycle to allow the bacteria to grow at rates exceeding the average dilution rate of the solid portion of ruminant contents (to which the cellulolytic bacteria are attached). During the time period when pH is below 6 and cellulose hydrolysis is still occurring (albeit at a decreased rate), certain acid-tolerant noncellulolytic bacteria become the primary agents of fermentation of cellodextrin oligomers. As it happens, many of these species produce lactate, whose conversion to propionate results in an observed decrease in acetate:propionate ratio, a key effector of nutrient delivery to the host.

A second relatively acid-sensitive microbial group in the rumen is the methanogenic archaea. These organisms also grow poorly below pH 6, and this property is thought to be a major reason that ruminants fed diets high in concentrates produce lower amounts of enteric methane than do forage-fed ruminants.

Response to Toxic Compounds

Many plants produce anti-nutritive compounds to discourage consumption by animal and arthropod herbivores. Ruminants can often develop tolerance to these plant metabolites, primarily through the adaptation of the ruminal microbiota to metabolize these compounds. A classic example involves production of mimosine by the tropical legume, *Leucaena leucophila*. Mimosine is a non-protein amino acid that is metabolized to 3,4-dihydroxypyridine (3,4-DHP), a potent goitrogen. As a result, consumption of *Leucaena* by ruminants was problematic, until it was discovered that a certain herd of feral goats in Hawaii could consume this legume with impunity. The resistance was traced to the presence of a novel specialist ruminal bacterium (since named *Synergistes jonesii*), that could metabolize both mimosine and 3,4-DHP. Inoculation of ruminal contents from these goats, or with a pure culture of *S. jonesii*, conferred mimosine tolerance to other ruminants, including sheep and cattle.

A second example of bacterial detoxification is presented by oxalic acid. This small dicarboxylic acid (HOOC-COOH) can accumulate in some xerophytic plants (up to 40% of dry matter in the noxious weed, *Halogeton glomeratus*). Oxalate is a potent chelator of calcium, and upon ingestion can induce severe hypocalcemia in sheep or other livestock. Gradual adaptation to halogeton results in proliferation of *Oxalobacter formigenes*, a specialist bacterium whose sole known catabolic pathway is the dismutation of oxalate to formic acid (HCOOH) and CO_2.

Metabolism of plant toxins is not restricted to native microbial species. Inoculation of sheep with a recombinant strain of *Butyrivibrio fibrisolvens*, engineered to contain a *Moraxella* dehalogenase enzyme, has been shown to confer resistance to plants containing the potent TCA cycle inhibitor, fluoroacetate. Subsequent searches have revealed the presence of natural fluoroacetate-metabolizing bacteria in some Brazilian ruminants.

A common characteristic of these plant toxin-degrading bacteria is that their substrates (the plant toxins) are low molecular weight compounds that are produced abundantly in their plant hosts, and that are degradable by very short metabolic pathways. These characteristics have likely hastened the evolutionary development and maintenance of these pathways. By contrast, robust degradative pathways do not appear to have yet developed for compounds such as perloline or lysergic acid alkaloids (produced by mycorrhizal fungi associated with tall fescue, an important pasture grass in the southeastern USA); these potent poisons are more structurally complex and are produced in only small amounts, thus providing less selective pressure for development of microbial degradative pathways. However, given the high degree of adaptability of microbes and the large number of available habitats (i.e., the large number of individual ruminant animal units), the eventual development of such degradative pathways cannot be ruled out.

Prospects for Manipulating the Ruminal Microbiota

The desire to direct the ruminal community toward specific host physiological outcomes has long enchanted both microbiologists and animal scientists. In particular, there are substantial motivations from economic, social and environmental standpoints in three specific manipulations: Mitigation of methanogenesis; improvement in efficiency of feed utilization (especially nitrogen utilization); and improved utilization of low digestibility feeds (especially forage fiber). In addition, there has been substantial interest in introducing non-native probiotic microbial strains into the rumen to address the proposed manipulations, as well as others. The practicality of all such manipulations must be viewed through careful consideration of the principles of microbial ecology.

Methane Mitigation

Efforts to decrease enteric methane production are driven by the fact that animal agriculture accounts for ~15% of anthropogenic production of methane, a greenhouse gas much more potent than CO_2. As noted above, ruminal production of methane almost

exclusively proceeds via oxidation of H_2 with CO_2 as electron acceptor. Attempts to divert H_2 oxidation to other pathways have primarily focused on reductive acetogenesis ($4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH$), as the electron acceptor (CO_2) is already abundant, and the product acetate contributes to host nutrition as a lipid precursor and energy source. Although acetogens capable of the above reaction are found in low abundance in the rumen, they do not appear to produce significant amounts of acetate from the above reaction. The reasons for this appear to be both thermodynamic (lower energy yield than methanogenesis) and kinetic (lower affinity for H_2 by the acetogens' hydrogenase, when compared to the methanogens). Instead, these versatile acetogenic bacteria are likely metabolizing other energy sources (methoxylated plant components or carbohydrates) rather than H_2. Diverting H_2 oxidation toward other microbes also appears to be of limited practicability, due the requirement for feeding large (stoichiometric) amounts of alternative electron acceptors (e.g., nitrate, fumarate, or Fe^{+++}), and have been only partially successful under both in vitro and in vivo conditions. A more promising strategy may be targeted inhibition of enzymes involved in the methanogenic pathway; the compound 3-nitrooxypropanol appears to be particularly effective. Disruption of these pathways should allow a redistribution of reducing equivalents into other fermentation products (particularly propionate and butyrate, via existing metabolic pathways) whose production would provide energy to the host animal.

Improving Nitrogen Utilization Efficiency

The main motivation for domesticating ruminant animals was to harness their ability to convert low-quality forage and browse plants to proteins useful to their keepers, in the form of meat, milk and wool. The protein in the plant materials – often low in quality and quantity – were augmented with microbial cell protein synthesized by the ruminal microbial cells. This microbial cell protein was produced using amino acids from the original plant proteins, as well as from microbial anabolic reactions in which organic intermediates were combined with ammonia released from urea cycling and from amino acid fermentations in the rumen. As agriculture intensified over the last century, feeding of concentrated protein sources has helped improve production of ruminant animal protein. However, nitrogen utilization efficiency in ruminants has remained poor (e.g., less than 25% of the protein fed dairy cows is recovered in the milk, with most of the rest lost in manure). Many feed proteins are extensively fermented in the rumen before they can pass to the intestines. Moreover, a substantial fraction of proteins synthesized de novo by ruminal bacteria is lost due to protozoal predation on bacteria, and the resulting fermentation of amino acids by the protozoa yields ammonia, whose re-assimilation by bacteria is energetically costly.

Attempts to improve N utilization efficiency have focused on any of several strategies: (1) treatment of feeds to make them less degradable in the rumen (e.g., soybean roasting); (2) enhancing ruminal availability of readily utilizable carbohydrate to facilitate microbial protein synthesis; (3) decreasing density or activity of protozoa that ingest bacterial cells and ferment their protein; and (4) inhibiting microbial proteases or amino acid fermentations in the rumen. Of these, the last approach involves microbial activities most directly, but has proven the hardest to exploit, likely because of the large variety of proteolytic and amino acid-fermenting microbes (i.e., the functional redundancy of the microbial community).

Improving Fiber Digestibility

Many forages, particularly from tropical regions, are poorly digestible due to extensive lignification, high ash content, or the presence of anti-nutritive compounds. Regardless, even the most digestible forages are digested more slowly than are most grains and other feed concentrates. The rate-limiting step in the digestibility of most forages is the complex structure of polysaccharides in the plant cell wall. As noted in the Introduction, the ruminant animal uses the process of rumination to expose the degradable surface of cell wall polysaccharides to enzymatic and microbial attack. Digestibility of cellulosic feeds in vitro can be enhanced by grinding the feeds to a smaller particle size, but such an approach is not practical in vivo because it accelerates passage of the particles from the rumen, before they can be fully degraded. Chemical pretreatments can improve digestibility both in vitro and in vivo, but are inconvenient, costly, and often introduce waste disposal problems. As a result, there has been considerable interest in stimulating the microbiota to degrade forage fiber more effectively and completely. In practice, this has proven to be difficult, because the rate-limiting factor is substrate accessibility, not microbial numbers or microbial capabilities. This can readily be demonstrated by measuring rates of fiber degradation in vitro. Dilution of ruminal fluid does not decrease the rate of cellulose digestion in vitro until dilutions exceed 5- or 6-fold. Limitation by substrate is also shown by the fact that its digestion kinetics remain first-order with respect to substrate concentration. Because the process of fiber digestion is limited by substrate, improving the numbers, density or activity of the fibrolytic microbes are unlikely to improve fiber digestion, except if the microbes could disrupt specific linkages in the plant cell wall that would improve substrate accessibility.

Probiotics

Probiotics (sometimes called direct-fed microbials, DFM) are living microorganisms that enhance the health of the host animal after they are consumed. Probiotics are widely employed, with variable success, in humans and in non-ruminant domestic animals (e.g., swine and chickens). Successful probiotics demonstrate at least limited persistence in the host. The use of probiotics in ruminants is more sporadic, due in part to the challenge of effectively persisting in the rumen or in competing with the indigenous microbes of the rumen and/or hindgut. Indeed, few controlled studies with probiotics have included measurement of their persistence within the host animal. The mechanisms by which probiotics exert beneficial effects are not always apparent, and it likely that elucidation of these mechanisms will guide development of more effective probiotics.

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Microbial Forensics☆

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Introduction

Biothreats gained renewed attention as a result of the 2001 anthrax letter attacks. Less than a month after 9/11, a deliberate act of bioterrorism was committed by using the United States Postal Service as a dissemination vehicle to intentionally disperse *Bacillus anthracis* spores along the eastern seaboard, ranging from New York to Florida Bush et al., 2001; Hsu et al., 2002; Jernigan et al., 2001; Jernigan et al., 2002; Keim et al., 2011; National Research Council, 2011; Popović & Glass, 2003; Traeger et al., 2002. Letters were mailed to two senators, news anchor Tom Brokaw of NBC News, and the *New York Post*, each containing *B. anthracis* spores Hsu et al., 2002; Keim et al., 2011; National Research Council, 2011. The attack resulted in 22 infections, of which five deaths occurred, and caused substantial disruption across the nation Jernigan et al., 2002; Keim et al., 2011; National Research Council, 2011. Although biological weapons and the threat of their use as biowarfare, bioterrorism, and biocrimes have been employed throughout history, this act of bioterrorism spread new fears among the nation. Some of the earliest known cases using biological threats can be traced back thousands of years. The Romans used to contaminate their enemies water supplies with decaying animal carcasses Budowle et al., 2005a. In the fourteenth century, in the battle of Kaffa, Tatar soldiers threw the bodies of their infected dead over the walls of the city to infect the enemy with plague Christopher et al., 1997. Biological warfare has been used in wars such as the French and Indian war, WWI, and WWII, and a number of countries, including the United States, have had offensive biological warfare programs Christopher et al., 1997. Biological agents also have been used as weapons in cases, known as biocrimes, in recent history. For example, in 1984 followers of the Baghwan Sri Rajneesh cult intentionally contaminated local salad bars with *Salmonella typhimurium* in Dalles, Oregon hoping to influence the results of a local election; this biocrime resulted in 751 infected individuals Török et al., 1997. In 1996, a Dallas, TX hospital laboratory technician intentionally contaminated muffins with a laboratory stock of *Shigella* and placed them in a breakroom; 12 people were infected, four of which were hospitalized Kolavic et al., 1997. Anthrax, the disease caused by the bacterium *B. anthracis*, is probably the most infamous biothreat agent. *B. anthracis* spores were disseminated in Tokyo by the Aum Shinrikyo Japanese cult in 1993; luckily, no infections resulted as the spores were of the Sterne strain, a vaccine strain Keim et al., 2001. In contrast, the Ames strain, a strain not commonly found endemically in the United States, was used in the 2001 anthrax letter attacks Budowle, 2004. Biological agents that may cause harm can be relatively cheap, easy to obtain, and require little sophistication to disseminate compared with other forms of weapons of mass destruction. The historical and recent use of biological weapons demonstrates the continual threat of their use in cases of bioterrorism and biocrimes.

The 2001 anthrax attacks demonstrated the degree of fear, disruption and damage that can occur from a relatively small attack by contamination with only a handful of spore-laden letters. In addition, the investigative time and costs associated with the attack were substantial. The anthrax letter investigation, termed Amerithrax by the FBI (Federal Bureau of Investigation) National Research Council, 2011, spanned nearly a decade with an estimated economic impact of $6 billion Ellis, 2014, of which $320 million was associated with decontamination costs Schmitt & Zacchia, 2012. In 2001, the US government, public health service, and law enforcement agencies were largely unprepared for such an event, and the realization became evident of how vulnerable the country was to such an attack Breeze et al., 2005; Budowle et al., 2011. Although, prior to 2001 a need for an established microbial forensics field was predicted, a formal system had not been implemented Murch, 2003. As a result of the 2001 attack, the microbial forensics field was officially launched by necessity Breeze et al., 2005; Budowle et al., 2003; Budowle et al., 2005b; Budowle et al., 2005c; Budowle et al., 2006; Budowle et al., 2011; Morse & Budowle, 2006; Murch, 2003. Microbial forensics is the discipline of applying scientific methods for analyzing evidence from a bioterrorism attack, biocrime, hoax, or inadvertent release of a biological agent or toxin with attribution as the ultimate goal Budowle et al., 2003. Attribution of microbial evidence is to determine an associated source and perpetrator or group of individuals to the highest degree possible. The microbial forensics field is an interwoven network of scientists from multiple specialties (i.e., microbiology, genetics, bioinformatics, forensic science, immunology, population genetics, biochemistry, molecular biology, epidemiology, etc.) and the law enforcement, public health, policy, and intelligence communities.

Microbial forensic investigations center on the detection and characterization of both biological agents, in addition to non-biological evidence. Biological agents consist of bacteria, viruses, protists, fungi, and toxins. Non-biological evidence, such as additives, growth media, delivery devices, intelligence, etc., can be useful in microbial forensics, potentially providing investigative leads and helping to infer methods of manufacture and dissemination Velsko, 2011. Non-biological evidence analysis is an integral part of microbial forensics; however, the focus of this chapter is on the biological analytical methods. Microorganisms and their

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toxins are desirable weapons as they are relatively cheap to culture, can be easy to procure if endemic or occur naturally, and for many only small amounts of biological material can cause infection or even death. There is a wide variety of microbial species or strains that could serve as possible biothreats (human, plant, and animal pathogens). Indeed, over 1400 microbes are known to infect humans Taylor et al., 2001, although some are more harmful than others. However, microorganisms of the highest concern regarding public health and national security are listed as NIH NIAID (National Institutes of Health, National Institute of Allergy and Infectious Diseases) Priority Pathogens National Institutes of Health, 2013 (reviewed by the Department of Homeland Security (DHS) and the Centers for Disease Control and Prevention (CDC)) and select agents listed on the National Select Agent Registry National Select Agent Registry, 2014 (Table 1). Animal and plant pathogens also are important for biosafety and biosecurity. The agriculture and livestock industries provide substantial infrastructure to our food supply and economy. An attack on these industries can impact health, national/global economics, and political policy, as well as cause substantial disruption. For example, the 2001 foot-and-mouth disease outbreak, albeit a natural outbreak, in England had an estimated impact of more than $12 billion Cottam et al., 2006; Ferguson et al., 2001; Thompson et al., 2002. Natural animal, plant, and food-borne disease outbreaks occur regularly causing local to wide spread infections and death and potentially cripple sectors of the food and agriculture industries. The consequences of intentional attacks can be as or more serious.

Toxins are included on the NIAID Priority Pathogens and select agents lists National Institutes of Health, 2013; National Select Agent Registry, 2014. Toxins are natural products produced by bacteria, fungi, plants, and eukaryotes. Enterotoxins, produced by different strains of, for example *Staphylococcus aureus*, are some of the most common causes of food poisoning Marks, 2011. Botulinum toxin, produced by the bacterium *Clostridium botulinum*, is a powerful toxin that is lethal even in extremely small doses Marks, 2011. One of the most accessible toxins is ricin. Ricin is derived from the castor bean *Ricinus communis* and has been used in infamous cases of assassination Audi et al., 2005; Carus, 2002; Marks, 2011. The US postal system was used again in 2003, and more

Table 1 NIH National Institute of Allergy and Infectious Diseases Priority Pathogens National Institutes of Health, 2013[a]

Category A	*Bacillus anthracis* (anthrax)[b]
	Clostridium botulinum toxin (botulism)[b]
	Yersinia pestis (plague)[b]
	Variola major[b] (smallpox) and other related pox viruses
	Francisella tularensis (tularemia)[b]
	Arenaviruses (LCM, Junin, Machupo, Guanarito, Lassa Fever)
	Bunyaviruses (Hantaviruses, Rift Valley Fever)
	Flaviruses (Dengue)
	Filoviruses (Ebola[b], Marburg[b])
Category B	*Burkholderia pseudomallei*[b]
	Coxiella burnetii (Q fever)
	Brucella species (brucellosis)
	Burkholderia mallei (glanders)
	Chlamydia psittaci (Psittacosis)
	Ricin toxin (from *Ricinus communis*)
	Epsilon toxin of *Clostridium perfringens*
	Staphylococcus enterotoxin B
	Typhus fever (*Rickettsia prowazekii*)
	Food- and Waterborne Pathogens (Diarrheagenic *E. coli*, Pathogenic Vibrios, *Shigella* species, *Salmonella*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, Caliciviruses, Hepatitis A, *Cryptosporidium parvum*, *Cyclospora cayatanensis*, *Giardia lamblia*, *Entamoeba histolytica*, Toxoplasma, Microsporidia)
	Additional viral encephalitides (West Nile Virus, LaCrosse, California encephalitis, Venezuelan equine encephalitis, Eastern equine encephalitis, Western equine encephalitis, Japanese Encephalitis Virus, Kyasanur Forest Virus)
Category C	Nipah virus and additional hantaviruses
	Tickborne hemorrhagic fever viruses (Crimean-Congo Hemorrhagic fever virus)
	Tickborne encephalitis viruses
	Yellow fever
	Mycobacterium tuberculosis (Tuberculosis, including drug-resistant TB)
	Influenza
	Other Rickettsias
	Rabies
	Prions
	Chikungunya virus
	SARS-CoV
	Antimicrobial resistance microorganisms[c]
	Coccidioides immitis
	Coccidioides posadasii

[a]See reference National Select Agent Registry, 2014 for a list of all select agents, including animal and plant pathogens listed by the US Department of Agriculture.

[b]Top Tier 1 Agent as listed on the National Select Agent Registry.

[c]Excludes sexually transmitted organisms.

recently in April 2013, as a dissemination vehicle to send Ricin-contaminated letters addressed to the White House and other public figures Audi et al., 2005; Centers for Disease Control and Prevention, 2003; Federal Bureau of Investigation, 2013. The contaminated letters were intercepted in each case during routine mail screening and did not cause any harm Centers for Disease Control and Prevention, 2003; Federal Bureau of Investigation, 2013. The ease of access of castor beans and recipes to purify the toxin make it an easy biothreat to produce.

The ease and relatively inexpensive costs associated with the production and use of biothreats will remain an ongoing concern. Following the 9/11 terrorist attack and the anthrax letter attack, the US implemented new homeland security policies, including the formation of the Department of Homeland Security, and these new directives led to the initial policy regarding microbial forensics Pesenti, 2011. Since 2001, federal funding for civilian biodefense research (and related non-biodefense) and efforts have increased from $414 million in fiscal year 2001 Schuler, 2004 to $6.69 billion dollars in fiscal year 2014 Sell & Watson, 2013. Federal funding has helped initiate biodefense programs and research efforts to provide a forensic capability as well as further development of analytical tests to aid in public health and microbial forensic investigations and disease outbreak preparedness and response.

Biothreats used in acts of bioterrorism, biocrimes, and hoaxes are the main focus of the microbial forensics field. However, microbial forensics is used increasingly in other criminal and civil investigations, for example, in cases of disease transmission involving intentional exposure Bhattacharya, 2014; González-Candelas et al., 2013; Metzker et al., 2002; Sajantila, 2014; Scaduto et al., 2010 or sexual assault Hammerschlag & Guillén, 2010. Population genetics and phylogenetics form the bases for establishing viral or bacterial transmission in sexual assault or deliberate acts of infection with an infectious agent, such as HIV (Human Immunodeficiency Virus) Metzker et al., 2002; Scaduto et al., 2010. Phylogenetic evidence has been used in courts of law to provide interpretations regarding these types of crimes involving infectious microorganisms González-Candelas et al., 2013; Metzker et al., 2002; Scaduto et al., 2010. By constructing species and strain phylogenies and using additional information, such as times of infection, disease transmission can be inferred from one individual to another and also can be used to rule out individuals not infected by a potential source González-Candelas et al., 2013; Sajantila, 2014. Essentially microbial evidence can be used to evaluate transmission events as opposed to merely detecting the presence of a particular microorganism.

Microbial forensics is an emerging field and encompasses many specialties with collaborative efforts among scientists, public health, law enforcement, the intelligence community, and policy makers. Due to the diversity of the number of biothreats which potentially could be used in a bioterrorism attack or biocrime the development and validation of methods are continually ongoing as new methods are needed to address the variety of investigations that may be encountered. Therefore, it is imperative to have established standards, quality assurance guidelines, databases and biorepositories, and policy to provide the required infrastructure for a national, and even international, microbial forensic capability.

Forensic and Epidemiological Investigations

Disease outbreaks naturally occur every year throughout the world, and investigations into these outbreaks often include both epidemiology and microbial forensics investigations (Figure 1). Epidemiology studies the occurrence, features, and determinants of disease in populations. The same general principles of epidemiology for disease investigations apply to a bioterrorist attack or crime. Therefore, microbial forensics investigations are based on the same well-established principles of epidemiological investigations Butler et al., 2002; Morse & Budowle, 2006; Morse & Khan, 2005; Treadwell et al., 2003. Microbial forensics and public health share common interests regarding the identification and genetic characterization of the biological agent and how it was disseminated in the population. However, public health officials tend to focus on (1) determining that an outbreak has occurred, (2) defining the population at risk, (3) determining the method of spread and reservoir, and (4) characterizing the agent Morse & Budowle, 2006. A common thread of public health and microbial forensics is determining whether the outbreak is natural, accidental, or intentional. While microbial forensics and epidemiology are integrated disciplines, microbial forensic scientists and law enforcement concentrate on attempting to individualize the agent or toxin and how it was produced and disseminated (if applicable) for attributing the event to a person or group of persons while maintaining chain of custody for legal purposes or for decision makers and their response(s).

US public health has a well-developed system for disease outbreak surveillance. The laboratory response network (LRN) is a system of public health laboratories throughout the country designated as testing laboratories for outbreak investigations Morse et al., 2003. This system is overseen by the CDC, and it provides quality assurance guidelines and standardized protocols for pathogen detection Morse et al., 2003. In contrast, the microbial forensics field does not have a large battery of standardized methods, centralized databases or repositories. However, the National Bioforensics Analysis Center (NBFAC), part of the DHS, was created to serve as the nation's central laboratory for analysis of any bioterror or biocrime evidence Budowle et al., 2003. A single laboratory, though, cannot address all possible biothreats. Instead a hub and spoke model approach is employed where the NBFAC serves as the primary facility and national labs, other government agencies, academia, etc. are loosely tethered to the NBFAC to provide support as needed with testing and expertise for a quick and reliable response Budowle et al., 2003. The Amerithrax investigation was a collaborative effort of federal, private, and academic labs to develop new procedures and test the thousands of items of evidence. In 2002, the FBI formed the Scientific Working Group on Microbial Genetics and Forensics (SWGMGF) to create quality assurance guidelines for microbial forensics and identify gaps and direction to develop a robust microbial forensics capability Budowle, 2003; Budowle et al., 2005a. SGWMGF has since disbanded; however, the recommendations laid out by the working group are still valid and very much applicable today.

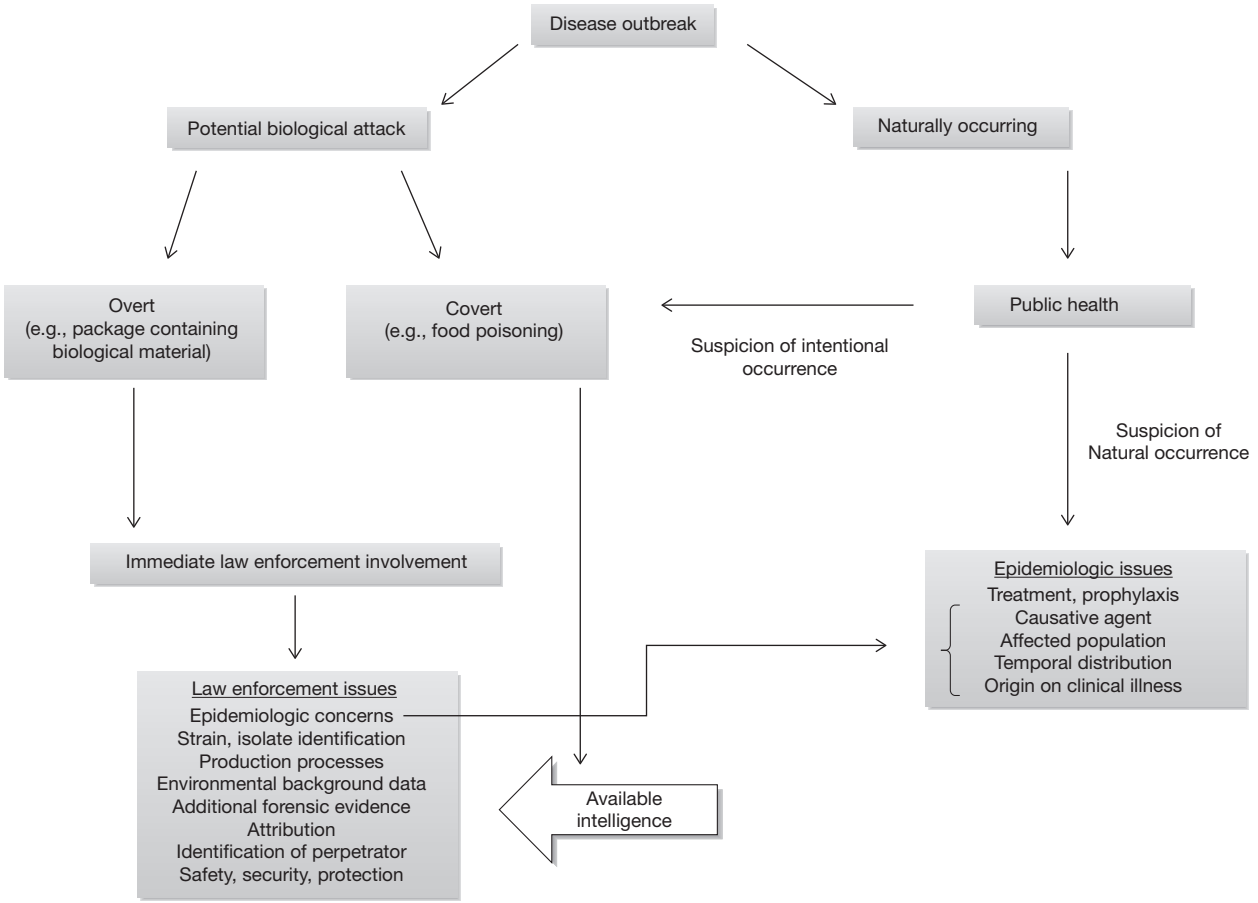

Figure 1 General schematic approach to epidemiological and microbial forensic investigations.

Detection, Characterization, and Emerging Technologies

Traditional Detection Methods and Genetic Typing

Attribution is the primary goal in microbial forensics by comparison of data obtained from evidentiary samples to reference samples. Both biological and non-biological signatures can be sought for attribution, investigative leads, or exclusionary purposes. Microbial forensic evidence may reside in a wide range of samples matrices, including food, water, air filters, swab and swipes, soil, animal tissue, and clinical samples (e.g., tissue, sputum, blood, stool, urine). Therefore, an analyst must have a variety of sample processing methods available to address the demands of myriad possible samples and scenarios, and methods need to be as robust as possible. In addition, traditional forensic evidence, such as fingerprints, human or animal DNA, and fibers and hair can be analyzed. So the analyst must consider collecting and analyzing evidence in a manner that preserves other forms of evidence beyond those of the purview of microbial forensics.

Detection methods in the microbial forensic workflow can range from culture, microscopy, immunoassays, mass spectrometry, real-time PCR, microarray, genetic typing, whole-genome sequencing, and beyond. While the focus of this chapter is on genetic signatures for biological threat agent identification, non-biological signatures, such as those that infer the culture method used, manufacturing processes, time of production, and methods of dissemination can be quite informative for developing investigative leads Velsko, 2011. For example, non-biological signatures pertaining to silica, growth media, and purity indicated that the anthrax spores in the 2001 attack were not weaponized in a sophisticated manner and likely were cultured from at least two batches, providing investigative value National Research Council, 2011. In addition, biological evidence other than genetic signatures of threat agents, such as host immune response, can provide invaluable information for investigative leads regarding if a suspected perpetrator took prophylactic antibiotics or other antidotal substances, inferring the handling, manufacture, or possession of a biothreat agent Schutzer, 2011. Culture is still considered the gold standard for pathogen detection Peters et al., 2004. However, culturing cannot provide resolution, many times beyond the genus or species level, and because there can be a substantial lag time due to growth requirements of the microorganism, it may not be efficient for response especially when the safety of individuals is an immediate concern. Moreover, about 99% of microorganisms cannot be cultured by current methods; therefore culturing is not a reliable method for fastidious and possibly novel/uncharacterized microorganisms. In addition, the microbes may have been

exposed to environmental insults and may no longer be viable. So even if the microorganism was one of the few that could be cultured, no information would be obtained if it were nonviable.

Ideally, attribution seeks characterization of biological threat agents with resolution at the strain/isolate level. While culture and immunoassays are sufficient methods for initial testing and sample screening, nucleic acid typing often is more resolving. MLVA (multi-locus variable number tandem repeat (VNTR) analysis) analyzes polymorphisms found in minisatellite regions within bacterial genomes and has been shown to be effective at discriminating among strains of highly monomorphic species, such as *B. anthracis* Keim et al., 2000; Keim et al., 2004 and *Yersinia pestis* Klevytska et al., 2001; Pourcel et al., 2004. MLVA was the method used to identify the Sterne strain used in the Aum Shinrikyo Anthrax release Keim et al., 2001 and the Ames strain used in the Amerithrax attack Keim et al., 2011. This level of characterization, although not sufficient for individualization of isolates obtained from evidence, did provide a good investigative lead, as the Ames strain is not typically found in nature and is far more prevalent as a laboratory strain Keim et al., 2004; Keim et al., 2011. Since these genetic markers cannot resolve at the isolate level, SNPs (single nucleotide polymorphisms) and other genetic signatures are sought for better attribution. One approach for SNP marker detection is use of microarrays, which consist of potentially large numbers of short oligonucleotide probes on a solid support. Microarrays, which can be highly efficient screening and characterization tools, have been developed specifically for bacterial and viral detection, and can achieve species to strain level identification Gardner et al., 2010; Leski et al., 2009. However, at the isolate level the variant sites, if they exist, on the genome are unknown and may not be detected with an *a priori* array design. An unbiased more comprehensive genome scanning method is needed to extract the most resolving information possible.

Whole-genome shotgun sequencing (WGSS) is one approach that may be able to identify those species/strain/isolate markers that would enable better attribution. WGSS is a sequencing approach which does not require any prior knowledge of the sequence being determined. Because WGSS is unbiased in its identification of markers, it can provide the capability to detect any number of genetic markers, such as SNPs, insertions, deletions, duplications, genome rearrangement, virulence genes, pathogenicity islands, plasmids, horizontally transferred elements, and evidence of genetic engineering. Initially, WGSS was performed using Sanger sequencing Sanger et al., 1977. This approach requires the use of cloning vectors, has relatively low-throughput, is time consuming and rather expensive. WGSS was performed to attempt to characterize different isolates of the Ames strain including an isolate from the first known victim of the 2001 anthrax attack Read et al., 2002; however no genetic differences were observed between the evidence and reference samples. It was not until the astute discovery of late-forming spore morphology variants by a microbiologist that a potential distinguishing characteristic could be exploited for attribution purposes Keim et al., 2011. Pure cultures of some of the morphology variants were prepared and sequenced enabling detection of genetic variants specific to each morphology variant Keim et al., 2011. Sequencing many samples was cost-prohibitive as it cost on average approximately $140,000 to perform WGSS on a single sample in 2002 Cummings & Relman, 2002. Therefore, based on genetic data from a limited number of sequenced samples, real-time PCR assays were developed to detect these different genetic signatures of the variants Keim et al., 2011. These PCR-based assays, being easier to perform and far less costly, were used to screen over a thousand (N = 1077) repository samples collected from laboratories inside and outside the US housing the Ames strain Keim et al., 2011. The results eliminated the vast majority of Ames samples collected and strongly indicated an association to a flask containing *B. anthracis*, known as RMR1029, at the USAMRIID (United States Army Medical Research Institute for Infectious Diseases) Keim et al., 2011; National Research Council, 2011. This flask contained a mixture of the same colony morphology variants as was seen in the Amerithrax evidence Keim et al., 2011; National Research Council, 2011. While real-time PCR enabled analysis of a large number of evidentiary samples, the approach was limited to only the few variants that the assay was designed to detect. Therefore, any other variants that may have existed within the approximately 5 million bases of the *B. anthracis* genome would go undetected with such a focused assay. This inability to scan the entire genome in a single assay was a limitation of the technology just a decade ago. Today, identifying genetic signatures and typing a large number of samples are more feasible with next-generation sequencing technologies.

Massively Parallel Sequencing

One of the most significant genetic typing tools to come to fruition in the last few years is high-throughput sequencing. Next-generation sequencing, or better described as massively parallel sequencing (MPS), has become a mainstream technology in many molecular biology and genetics laboratories. MPS allows for the generation of gigabases of sequence data in days at a substantially reduced cost than even a few years ago Wetterstrand, 2014. Larger genomes can be fully sequenced in a couple weeks and small genomes, such as a bacterial genome, can be fully sequenced within a few days. MPS provides greater coverage across a genome with higher depth of coverage at each site for increased confidence in base calls, and barcoding allows for multiplexing of samples (from a few to hundreds) in a single sequencing run. With the introduction of smaller, faster, and cheaper benchtop sequencers, the technology now makes it feasible for the capabilities of large genome centers to be transferred to the application-oriented laboratory. Thus, microbial forensic analyses are being driven in a new direction with the new capabilities provided by MPS. Full characterization of bacterial or viral genomes can be achieved in a number of days as compared with more limited traditional methods, such as culturing, MLST (multi-locus sequencing typing) and PFGE (pulsed-field gel electrophoresis), which can take several days to weeks depending on the microorganism. Most importantly, far more genetic information can be realized and thus attribution to a deep level may become a reality for a number of scenarios. In essence, MPS provides a high-throughput, culture-independent method for whole-genome sequencing and comparative genomic analyses.

MPS was first introduced in 2005 by 454 Life Sciences based on pyrosequencing technology Margulies et al., 2005. In the past decade, the MPS possibilities have exploded and a number of novel platforms have been introduced, such as: GS FLX+ and GS

Junior (454 Life Sciences, Roche, Branford, CT); HiSeq, NextSeq500 and MiSeq (Illumina, San Diego, CA); SOLiD® 5500 (Life Technologies, Foster City, CA); Ion Torrent's Proton and PGM (Personal Genome Machine) (Life Technologies). Each system employs a different sequencing chemistry, but all provide higher throughput at a reduced cost per base pair compared to traditional Sanger sequencing. The next generation of sequencing systems, from Pacific Biosciences (PacBio) Eid et al., 2009 and Oxford Nanopore Technologies (2012), focus on single-molecule sequencing strategies. These sequencing technologies hold great potential for microbial forensics with the ability to generate long sequence reads. For example, PacBio sequencing can produce greater than 30,000 bp reads Pacific Biosciences, 2014. Also, PacBio sequencing allows for the detection of specific base-pair modifications, such as methylation patterns, that can be used for further characterization of samples Flusberg et al., 2010. Single molecule analyses may offer the important features of increased sensitivity of detection and higher quality genome assembly provided by the increased read lengths.

MPS is being fully exploited by clinical microbiology labs Didelot et al., 2012; Köser et al., 2012a and offers several different applications for microbial forensics Budowle et al., 2013. Cummings et al. (2010) evaluated MPS as a microbial forensic and epidemiological tool to detect SNP and other genetic variants within the monomorphic select agents, *B. anthracis* and *Y. pestis*. They were able to detect genomic variants and differentiate among four different strains of *B. anthracis* and four strains of *Y. pestis* simultaneously within a single sequencing run Cummings et al., 2010. This study demonstrated the ability to detect low-level variants within a sample, in particular if known genetic variant regions were amplified by PCR and sequenced in parallel Cummings et al., 2010. For example, four 200 bp genetic variants amplified by PCR would result in 800 bp total length. Cummings et al. (2010) calculated that sequencing these samples on the SOLiD® system, with a 10GB throughput (the throughput available in 2010), would result in a yield of 12.5 million read coverage; if 256 samples were multiplexed read coverage would yield approximately 50,000× and would allow for minor variant detection of less than 1 in 10,000 within a mixed sample. Today, the highest throughput available with MPS, provided by the Illumina HiSeq, is around 1TB of sequence data on a dual flow cell run Illumina, 2014. Using the HiSeq, multiplexing the same 256 samples, would result in, on average, approximately 5 million read coverage. Obviously this level of coverage is not needed for variant detection, but it demonstrates the immense amount of data that can be obtained for potential low-variant detection and the degree of sample multiplexing that is possible. The length and number of genetic targets can be increased; for example, 256 samples at a desired 50,000× coverage on the HiSeq could be used to sequence about 78,000 base pairs per sample. Therefore, this technology has the capability to dramatically reduce the number of false negatives (particularly with low level or trace analyses).

Disease outbreak investigations are integral to clinical, epidemiological, and microbial forensic investigations. MPS has been used to investigate disease and food-borne illness outbreaks, such as the 2006–08 outbreak of *Mycobacterium tuberculosis* in British Columbia, CA Gardy et al., 2011 and a food-borne *Salmonella enterica* outbreak in 2009–10 spanning 44 states in the United States Lienau et al., 2011. While these investigations using MPS were retrospective, MPS has been used to detect and monitor disease outbreaks in near real-time, such as the 2010 Haitian Cholera outbreak Chin et al., 2011 and most notably the 2011 *Escherichia coli* O104:H4 outbreak in Europe Grad et al., 2012; Mellmann et al., 2011. From May–July 2011 an outbreak of *E. coli* O104:H4 occurred due to contaminated alfalfa sprouts in Germany and France, which ultimately led to over 4000 infections and 50 deaths Grad et al., 2012. During the outbreak MPS was employed, using the Ion Torrent PGM and supplemented using Optical Mapping technology, to produce a draft whole genome sequence assembly of the outbreak strain within 62 h, Mellmann et al., 2011 demonstrating the utility of MPS as a real-time epidemiological tool. MPS has been used on a more local level as a diagnostic tool for infections Hasman et al., 2014, including mixed infections Eyre et al., 2013, and to monitor nosocomial outbreaks within hospital units Eyre et al., 2012; Köser et al., 2012b. In addition, MPS has been used to monitor patient treatment therapies, such as in the case of stool substitute implantation as a treatment regimen for recurrent *Clostridium difficile* infections Budowle, 2013. Harris et al. (2012) demonstrated that the use of MPS provides far more genetic information regarding recombination in *Chlamydia trachomatis*, than current clinical typing methods. Thus, MPS provides substantial utility for clinical diagnostics and outbreak surveillance with increased coverage of more informative genetic regions for more accurate analysis than using certain current clinical typing methods. While these aforementioned studies focus on MPS applications for mainly clinical and epidemiological uses, these same methods and practices can be applied to a microbial forensics investigation (i.e., if these same outbreaks were intentional these sample methods could be employed) and can serve as retrospective studies to facilitate interpretation of results if an attack were to occur.

MPS technologies continue to improve at an exceedingly fast rate. DNA input requirements and run times are decreasing, while multiplexing capabilities and read lengths are increasing. MPS likely will become more sensitive and have higher throughput, which will make the technology applicable to more microbial forensic applications. In addition, new methods and technologies will provide novel microbial forensic investigation applications, such as metagenomics analyses and sample preparation enrichment strategies.

Metagenomics

Metagenomics, the application of sequencing DNA collected directly from environmental and other complex community samples, provides a culture-independent method for studying microorganisms from environments such as soil Mocali & Benedetti, 2010, water Biers et al., 2009, and human-associated samples Human Microbiome Project Consortium, 2012a; Human Microbiome Project Consortium, 2012b. There is an estimated 10^{30} bacteria on earth and the majority of these species cannot be cultured Sleator et al., 2008. Thus, metagenomics applications using MPS provide tools to sequence, in theory, all nucleic acids present in a given

environmental sample, most of which could not be detected using culture-dependent approaches. New capabilities are provided for microbial community profiling, novel microbial species and metabolic pathway discovery, and microbial-host interactions for applications in areas such as environmental and clinical microbiology. Several studies have demonstrated the applicability of metagenomic sample analyses for forensic investigations such as for human identification Fierer et al., 2010; Lazarevic et al., 2010; Mason et al., 2013, cause of death Kakizaki et al., 2012; Thèves et al., 2011, time since death Hyde et al., 2013; Pechal et al., 2014, biological fluid identification and characterization Benschop et al., 2012; Brenig et al., 2010; Giampaoli et al., 2012, disease outbreak investigations Loman et al., 2013, herbal supplement authenticity Coghlan et al., 2012 (non-microbial), biogeography of humans Yatsunenko et al., 2012 and environmental samples Heath & Saunders, 2006, and public bio-surveillance Robertson et al., 2013. In addition to targeted pathogen detection from samples for epidemiological and biodefense purposes, metagenomic analyses of whole microbial community profiling hold promise for microbial forensic utility.

The types and conditions of samples that may be encountered in a microbial forensic investigation are variable and many will be mixed with other microbes and/or background eukaryotic nucleic acid and at low abundance or trace levels in a sample, making detection of, for example, select agents very challenging. Microbial forensics typically focuses on the detection and comparative analyses of priority pathogens and select agents, and more readily from relatively pure or homogeneous samples. However, forensic metagenomics detects target microorganisms in complex samples. There are currently two main approaches for metagenomic sequencing, targeting the 16S rRNA gene or WGSS. The former provides better depth of coverage but tends to lack species-level resolution, which is imperative for microbial forensics purposes. The latter can provide species or even sub-species level identification but lacks the depth of coverage provided by targeted amplicon sequencing, which limits the sensitivity of detection of target microbes. However, WGSS is more desirable for microbial forensic metagenomics analysis as species level resolution is imperative. It would not be helpful to identify at the genus level that a sample contains, for example, Bacilli. There are many Bacilli species that are not harmful and some are even beneficial. Without more information on whether a harmful species is present, no action could be taken regarding health and safety, whether an investigation should proceed, or whether a response is warranted.

Bioinformatics is the application of computational methods to analyze biological data, such as MPS sequence data and is essential for interpreting MPS sequence data, phylogenetic reconstruction, statistical analyses, and visual representation of data. With the explosion of MPS and the onslaught of sequence data numerous bioinformatics software tools and data management systems have been developed such as software tools for metagenomic assembly Namiki et al., 2012, taxonomic classification Davenport et al., 2012; Huson et al., 2007; Segata et al., 2012, phylogenetic analysis Darling et al., 2014, entire metagenomic analysis pipelines Treangen et al., 2013, and database analysis and management systems Markowitz et al., 2012; Meyer et al., 2008; Sun et al., 2011. Software that use sequences specifically informative for species or sub-species level identification from shotgun sequencing data, such as Pathoscope Francis et al., 2013 and SIANN Minot et al., 2014 will allow better attribution. These programs were developed for species- and strain-level detection of pathogens or other target microorganisms from shotgun sequencing data with direct application for clinical diagnostics and/or microbial forensics. Customized bioinformatics tools and comprehensive databases for microbial forensic purposes are essential.

New Tools from Paleopathology Investigations

Ancient microbial analyses are another branch of forensic related efforts that expand the limits of analyzing challenged samples. Throughout history disease outbreaks have led to the deaths of millions of people throughout the world. For example, the Spanish Influenza pandemic killed more than 50 million people Johnson & Mueller, 2002, and multiple plague epidemics have afflicted different regions of the world throughout history leading to the deaths of significant portions of the human population in these areas, sometimes as high as 50% of the population Holmes, 2011. For some of these epidemics the underlying cause or causative microorganism is known; for some historical events, the causative agent remained a mystery or was controversial. New and improved extraction methods, more sensitive detection assays, and new sequencing technologies have been developed enhancing the capability to genetically characterize these ancient pathogens from skeletal remains and other sample types. These studies contribute new representative genomes to fill in missing diversity from the phylogenetic tree, thus aiding in the epidemiology of modern outbreaks of these same species.

Y. pestis, the causative agent of plague, remains an important health focus. Plague has been responsible for several of the most devastating pandemics, specifically the Justinian Plague, the Black Death, and the twentieth century pandemic Morelli et al., 2010. Therefore, knowledge of the causative strains from these outbreaks and reconstructing phylogenetic relationships with new and old strains can be used for modern outbreak investigations Yan et al., 2014. Recently researchers have utilized novel enrichment strategies coupled with MPS to extract and reconstruct the draft genomes of *Y. pestis* from victims of the Black Death Bos et al., 2011; Schuenemann et al., 2011 and the Justinian Plague Wagner et al., 2014. Due to the highly fragmented and damaged properties of ancient DNA, novel library prep methods and enrichment strategies using baits comprised of complementary nucleic acid sequences were used Bos et al., 2011; Schuenemann et al., 2011; Wagner et al., 2014. Enrichment strategies employed the use of probes, constructed from modern reference sequences, which are suspected to be highly similar to the ancient sequence, with biotin tags attached to streptavidin coated magnetic beads or probes attached to glass slides to retrieve target endogenous DNA. These same enrichment approaches could be used in more modern microbial forensic samples to capture sequences of interest (i.e., sequences from a biothreat) from highly complex samples, providing a new microbial forensic tool for metagenomic analyses. The historical epidemiology of other priority pathogens are of interest as well; a historic strain of *M. tuberculosis*, the bacterium which causes tuberculosis, was isolated and genotyped using enrichment strategies coupled with SOLiD® sequencing from nineteenth century

skeletal remains Bouwman et al., 2012. These studies demonstrate the capability to detect priority pathogens and select agents from highly degraded samples, which may be important in certain microbial forensic investigations.

Synthetic Biology

As molecular technologies advance, new tools will arise that benefit society and at the same time be exploited for criminal purposes. Synthetic biology, the ability to synthesize any genomic sequence both naturally occurring and artificial, is one such dual purpose technology. The ability to synthetize DNA of any sequence, transform bacteria and viruses with selected genes, recreate known but difficult to attain pathogens (including extinct ancient pathogens), and even create novel microbial genomes is a growing reality. Although most biosynthetic efforts concentrate on, for example, studying genes, altering cell lines to study diseases, generating therapeutic solutions, and bioenergy Khalil & Collins, 2010, this same biosynthetic capability could be used to generate a microorganism to use as a biological weapon or could create unintentional consequences of an accidental release. Pathogenic microorganisms and toxins naturally occurring in hosts and the environment will continue to be a main biothreat. However, it is reasonable to consider the threat of creating difficult to obtain microbes that reside in unknown reservoirs, such as Ebola, secured microbes, such as smallpox, or microbes no longer in nature, such as the Spanish influenza H1N1 virus Bügl et al., 2007; Tumpey et al., 2005. Bügl et al. (2007) have highlighted the significance of this capability and the need for a structured framework for DNA synthesis and biological security.

Regulations are in place regarding the creation of genomic sequences of select agents National Select Agent Registry, 2014, and recommendations have been made, for example, by the National Science Advisory Board for Biosecurity, regarding the need to block publication of reporting findings of infectious agents that could be used to cause harm by malicious persons or groups Berns et al., 2012. Recommendations need to be in place regarding the safety and biocontainment of synthetically created microorganisms, as an accidental release could cause just as much harm as an intentional release. Microbial forensic methods using MPS can be used to monitor and detect synthetically created microorganisms. Since MPS is not biased regarding marker or signature analyses, it can be used to determine if a gene(s) or plasmid(s) is inconsistent with the previously known microorganism genetic background, suggesting a deliberate attempt or success of synthetically creating a biothreat.

Interpretation of Microbial Forensic Results

Proper interpretation of microbial forensic evidence is imperative for establishing confidence, to withstand the scrutiny of the legal system and for making critical policy or response decisions. Microbial forensics, much like human forensic DNA interpretation, can support the conviction or exoneration of an individual. However, more dire consequences can occur or be prevented based in part on a microbial forensic result. In the case of a bioterror attack, attribution to a particular government or sovereign entity could result in diminished diplomatic relations or military retaliation. Therefore, proper guidelines, validations and quality assurance, and statistical support need to be in place for microbial forensic evidence interpretation. Microbial forensic analyses typically rely on the comparison of evidentiary samples to a known reference sample(s) or other evidentiary item. The three general types of interpretation, include: inclusion; exclusion; or inconclusive. An inclusion, or association, is stated when the evidentiary profile matches, or is highly similar, to the comparative profile. In microbial forensics an inclusion also can mean that the microorganism from the evidentiary sample and comparative microorganism share a recent common ancestor. An exclusion is stated when the profiles are sufficiently dissimilar such that they cannot have originated from the same common ancestor or from the same source. An inconclusive is stated when there is insufficient information to render an interpretation. When an interpretation of an association is made, a statistical assessment or weight is assigned to this interpretation. These results are combined with other metadata to determine whether there is support, for example, of an intentional attack and that a particular person or persons are the perpetrator.

When associations are made between an evidence and reference sample the significance of the weight of the results must be properly stated. Currently there are no standard interpretation guidelines for microbial forensic evidence. However, some recommendations have been made. Although standard interpretation guidelines are lacking in the microbial forensics field, phylogenetic analyses have supported associations and have successfully been admitted as evidence in legal criminal proceedings in the United States and abroad Bhattacharya, 2014. Reconstructing phylogenies has been used as a microbial forensic tool to convict individuals in cases of intentional infection with RNA viruses González-Candelas et al., 2013; Metzker et al., 2002; Sajantila, 2014; Scaduto et al., 2010. A well-known case in which phylogenetic analyses supported an investigation in a criminal matter was in the second-degree attempted murder case in which Dr. Richard J. Schmidt was accused of intentionally injecting his girlfriend, Janice Trahan, with HIV Metzker et al., 2002. On August 4, 1994, Dr. Schmidt allegedly injected his girlfriend with a mixture of HIV and hepatitis C virus (HCV) tainted blood from two of his patients Metzker et al., 2002. Subsequently, Janice Trahan was diagnosed with HIV Metzker et al., 2002. An investigation occurred and sequence data of two genes (*gp*120 and *RT*) were generated from samples from the HIV-positive patient, Janice Trahan, and a local population of HIV-positive patients (about 30 control samples and 2 database samples) Metzker et al., 2002. Phylogenetic analyses revealed that the HIV variants between the patient and the victim were more similar than those from the local population Metzker et al., 2002. This evidence was used in the conviction of Dr. Schmidt Metzker et al., 2002. Phylogenetic analysis also was used in the investigation and submitted as evidence in the case of a Spanish anesthetist, Juan Maeso, who allegedly infected 275 of his patients with HCV by injecting himself with some of the patients' morphine prior to

administering the drug to patients using the same needle Bhattacharya, 2014; González-Candelas et al., 2013. In February 1998, an HCV outbreak was investigated and led to the association of hundreds of cases linked to two hospitals where Maeso worked González-Candelas et al., 2013. Sequence data were generated from two genes (NS5B and E1-E2) from 322 patients and 44 local controls González-Candelas et al., 2013. These data led to the exclusion of 47 patients from the outbreak strain and association of 275 patients infected by Maeso González-Candelas et al., 2013. Maeso was convicted of professional malpractice González-Candelas et al., 2013. This case differed from other cases using phylogenetic analysis since this case spanned a longer time frame, nearly 25 years, and involved many victims; as such, a molecular clock analysis was used to infer the time of infection of some of the patients González-Candelas et al., 2013; Sajantila, 2014. These accounts of phylogenetic analysis and use of models, such as the molecular clock analysis, will provide insight into best practices of interpretation that should be considered for assessing statistical significance.

Endemicity can play a large role in microbial forensic investigations for weighing the probability of the presence of a microorganism being due to a natural or intentional outbreak. The lack of endemic data played a key role in the difficult response of a partial positive result for *Francisella tularensis* (a NIAID Priority Pathogen and select agent) from routine sampling in the BioWatch program in Washington, DC in 2005 Eshoo et al., 2011. On September 24–25, 2005 partial positive results for a low-level signal of *F. tularensis* were reported from routine air sampling in the Capital Mall area of Washington, DC Eshoo et al., 2011. This detection occurred during the time of an antiwar protest, attended by a large number of people Eshoo et al., 2011. *F. tularensis*, a highly infectious bacterium, is a naturally occurring microorganism endemic to many areas and has been found in samples such as air, water, and soil Barns et al., 2005; Kuske et al., 2006. Shortly after the partial positive result, the CDC issued a health advisory to inform public health officials and individuals of potential exposure; however, no infections were reported Eshoo et al., 2011. It is more likely that the large movements of the protestors stirred up natural dust and excrement from the ground that contained endemic *F. tularensis* as opposed to the result of an intentional bioattack.

Endemic data assist in source tracing of microorganisms and to trace the origin of a particular microorganism, especially in the case of a disease outbreak. In 2010 following the Haitian earthquake, there was an outbreak of cholera, a disease caused by the bacterium *Vibrio cholerae*, resulting in more than 470,000 cases and more than 6,600 deaths by 2011 Centers for Disease Control and Prevention, 2011. Source tracing of the *V. cholerae* strain, using MPS to sequence clinical isolates, determined the origin to be a strain from South East Asia Chin et al., 2011; Hasan et al., 2012, specifically Nepal Hendriksen et al., 2011, likely introduced by human activity such as humanitarian aid in response to the earthquake. Source tracing also can be used to investigate microbial contaminants to provide associations or indication of intentional or accidental contamination aiding in microbial forensic investigations. For example, in 2009 multiple cases of injectional anthrax were diagnosed among heroin users in Scotland Price et al., 2012. *B. anthracis* is not endemic to Scotland Price et al., 2012. The source of the infection was believed to be a batch of heroin Price et al., 2012. Canonical SNP genotyping and WGSS were used to determine the strain and origin of the *B. anthracis* spores, which were likely introduced in Turkey or surrounding areas Price et al., 2012. This source tracing method indicated likely contamination from an endemic source, possibly from using an animal-derived cutting agent and determined the likely drug trafficking route that was used to smuggle the drugs into Europe Price et al., 2012.

Microbial contamination was a potential signature in the 2001 Amerithrax case. Some of the letters contained a contaminant of the bacterium *Bacillus subtilis*, a non-pathogenic near-neighbor of *B. anthracis* National Research Council, 2011. Although, this contamination of *B. subtilis* did not provide any additional investigative lead value in the Amerithrax investigation, it was noted by the NAS (National Academy of Sciences) Review of the FBI's investigation that such co-cultured contaminants could be considered endemics of an area or of a laboratory and could be invaluable evidence in future cases National Research Council, 2011, to trace the origin of the biothreat producer or to trace routes of dissemination. Knowing the types of strains and the geographic areas they reside helped in determining that the Ames strain is not a common endemic in the United States and indicated a likely source would be laboratories. The Ames strain was used for research and a number of laboratories had access and housed stock cultures of this strain Keim et al., 2011.

Databases are an essential tool in microbial forensics. Microbial forensic interpretations require the comparison of evidentiary genetic data to fully characterized references comprised in databases. Databases must be as inclusive as possible and contain as many strains of a particular species as possible, in addition to near-neighbors and other microorganisms representative of a wide range of phylogenetic diversity. Population genetic data within databases can be analyzed to provide information for unique markers for genetic typing for assay development and other data such as mutation rates and diversity. In addition to genetic data, databases must contain associated metadata. Metadata are the information associated with a given sample such as collection site location, date of collection, tissue source, virulence, extraction and sequencing methods, assembly and annotation methods, etc., which can be used to determine endemicity and other information associated with quality control of data. Metadata are essential to epidemiological investigations providing information to aid in source tracing and therapeutics and essential to microbial forensic investigations providing invaluable supplementary information of investigative value.

Since the majority of microbial diversity is unknown, current databases may not represent the true range of diversity that exists and programs have been initiated to sequence more reference genomes Wu et al., 2009. However, in microbial forensics the focus is on microbes that are particularly infectious and/or pathogenic and many representatives have been sequenced and with continued improvements in MPS, the number of microbial genomes that will be available will also increase. For example, the HMP (Human Microbiome Project) Consortium initiated the task to sequence new reference genomes for enhancing interpretation of health vs disease-state microbiomes Human Microbiome Jumpstart Reference Strains Consortium, 2010, and the 100K Foodborne Pathogen Genome Project seeks to sequence 100,000 different food-borne pathogens to help with epidemiological investigations UC Davis

School of Veterinary Medicine, 2014. More inclusive databases with reference sequences of forensic relevance can help improve source tracing efforts and phylogenetic reconstructions to determine disease transmission in biocrime and other biothreat cases. Databases constructed for biosecurity purposes must be systematically curated, have high quality genome sequences and the number and quality of draft genomes included must be properly vetted. Recommendations and guidelines also must be in place for metadata, including security-related regulations Sjödin et al., 2013. International databases would improve the capability of determining strain level attribution and source tracking Yang & Keim, 2012; however, international databases likely will not come to fruition as it requires countries to share information that they may regard part of their own national security or may not want to release as outbreaks can have serious economic consequences.

Conclusion

Microbial forensics is an interdisciplinary field that involves scientists, public health, law enforcement, the intelligence community, and policy and decision makers. Together they provide the interconnected system that helps protect us from naturally occurring disease outbreaks and acts of biological terrorism and biocrime. New advancements in molecular techniques, especially sequencing technologies, provide tools for the microbial forensic scientist to extract more information at dramatically reduced costs and faster turnaround times than previously possible. It is imperative for researchers in the field to continue to pursue novel research in areas such as method and software development and comparative genomics, as well as further expand our virtual and physical databases and biorespositories. Continuous evaluation and updates to quality assurance and quality control practices should be maintained to uphold microbial forensics practices to the highest of standards. Interpretation of results in a microbial forensics investigation must meet rigorous criteria and proper validation. It is important to understand the limitations of a method so as not to overstate results especially in the cases of exigent circumstances during an imposing threat. New genomic technologies and data, inclusive databases with expanded reference genomes, extensive endemic data, and validated methods all contribute to the proper interpretation of results in a microbial forensic investigation. High quality and confidence of results are essential since microbial forensic interpretations can have a large impact on society, regarding safety, political policy, and economics. Challenges will continue to exist in microbial forensics, however, implementation of new technology and continued communication across the scientific, public health, law enforcement, intelligence and policy communities will contribute towards the advancement of the microbial forensics field.

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Microbial Mats: Impact on Geology

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Geobiology: An Evolving Discipline

Emphasis on interactions across traditional discipline boundaries rather than on the core disciplines lead that *Geobiology* is mostly concerned with exploring the interface and complex relationships between the biosphere and geosphere. This subject was discussed in a colloquium convened by the American Academy of Microbiology, held in 2000 in Arizona, where the interconnecting fields of biological and geological sciences was considered. Despite the living extent may seem to be unconnected from nonliving, these two realms form a single prolific domain. Geological and biological activities influence each other in intense ways. This interaction has formed the Earth and all organisms on it. In that sense, Nealson et al. (2001) described this interaction as a Geobiological Tango: *"Living creatures and the inanimate worlds they inhabit dance an intimate tango. As each partner steps to Nature's tune, the other responds in harmony... This interplay of Life with Earth has shaped our present environment."* Life evolved from chemicals and their activities modified the environment. In case, the gases we breathe have been created by geobiological interactions. So, the interaction at all scales in space and time is under the umbrella of this modern and fast evolving discipline.

Geobiology has recently been defined by Knoll et al. (2012) as the scientific discipline in which the principles and tools of biology are applied to studies of the Earth. How life began, how it evolved, and how environmental conditions have influenced these processes are studied by geobiologists. Therefore this discipline produces new discoveries that contribute to a better comprehension of Earth in modern and past times. Moreover, the search of life beyond the Earth (i.e., astrobiology) is extending and, in that sense, geobiological approaches provide the basis for studies that focus on the exploration for extraterrestrial life on other planets (Baumgartner et al., 2009; Cady and Noffke, 2009, and references there-in).

Since the past few years, several studies are concerned in knowing the ways in which the organisms affect the Earth and the interactions between biological and physical processes in order to infer their participation in the planet's history. This interaction is useful for geologists in the interpretation of ancient sedimentary rocks, which requires the comprehension of processes resulting in a particular sedimentary stratum. A major bridge that joins the past with the present is the world of microbiota, such as cyanobacteria, the only oxygenic phototrophs known to have existed from before 2 Ga up to the present (Knoll et al., 2012). When plants and animals evolved and, due to a rapid growth of grazing metazoan communities extensive microbial mats became scarce (Grotzinger, 1990). However, they are still present in many ecosystems and have a significant impact on global biogeochemical cycles. Today, extensive microbial mats only exist in special places where they have little competition from vascular plants or grazing animals (Pflüeger and Gresse, 1996). From a geological point of view, the interaction of organisms with their depositional environment is very important, because this interaction defines distinctive and preservable sedimentary signatures, giving a clear evidence for biological activities. A very useful proxy is the study of processes on modern settings as analogues of ancient environments. Although the processes are similar (not identical), they often provide useful hypotheses for the interpretation of the processes that occurred in the past.

From Biofilms to Microbial Mats

In most shallow-marine environments, sediments are densely populated by benthic microorganisms as pennate diatoms that form biofilms around individual sediment grains as an initial step. Then, continuing to its expansion to adjacent grains ultimately can form a laterally continuous organic layer (Fig. 1). Biofilms, thin cohesive microbial communities, and well adapted benthic consortia, in favorable nutrient-enriched conditions develop complex, multilayered microbial ecosystems called microbial mats (Castenholz, 2009). The benthic microbial communities are commonly dominated by photosynthetic prokaryotes, mainly cyanobacteria and other photosynthetic bacteria, and also by eukaryotic microalgae, such as epipelic diatoms. This microphytobenthos colonizes sediments in shallow marine or estuarine settings, forming biofilms or microbial mats. They are common in salt marshes and estuaries (Fig. 2(A)) that may extend their distribution even into the lower intertidal zone (Stal, 2010). However, they may also be found in peritidal environments, such as coastal lagoons (Javor and Castenholz, 1981), as well as in extreme environments with high temperatures and hypersaline conditions.

The formation of a microbial mat comprises the secretion of an exopolymer to allow the microorganism to adhere to the substratum (Decho, 2000). Sediments are commonly embedded in this thick mucilaginous matrix of extracellular polymeric substances (EPSs), which may consist of 90% or more of polysaccharides, forming sticky coatings on individual sediment particles (Fig. 2(B) and (C)). EPS aid in the organisms vertical migration in response to light and tidal conditions, and in turn this substance also bind sediment particles together and they can form a smooth layer on the sediment surface (Fig. 2(D)). Thus the sediment bed is highly influenced by EPS affecting their stability (Paterson, 1989, 1997). So, the movement of filamentous microorganisms between the sediment grains forms a dense, mesh-like meshwork (see Fig. 1(C)). Moreover, due to the presence of EPS, microbial

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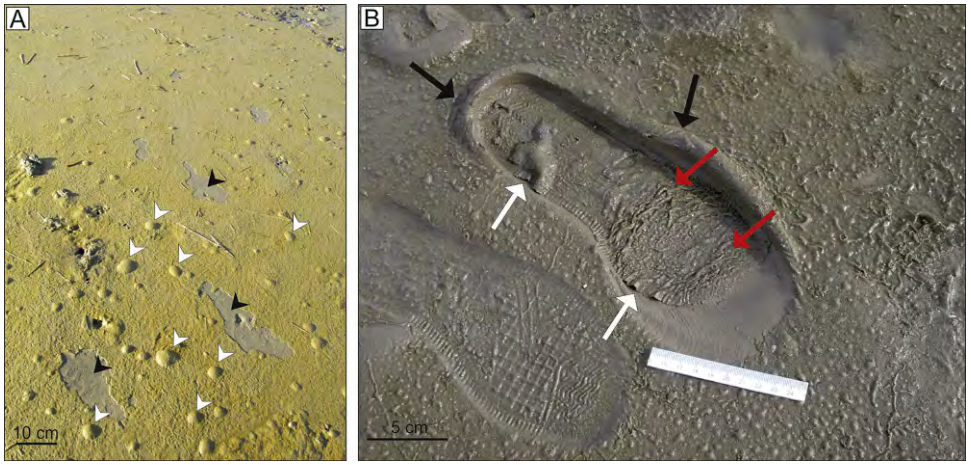

Fig. 1 Colonized sediments in Bahia Blanca Estuary tidal flat. (A) Extensive microbial biofilm as a protection of the substrate. Erosion of thin biofilm shows different colored sediment underneath (pointed by black arrows). Gas domes are formed (pointed by white arrows) by uprising gases of buried decaying organic matter and photosynthetic activity of microorganisms. (B) A footstep over a thin biofilm made by gliding over slippery fine sediment below resembling a waving cloth (white arrows) forming wrinkles (red arrows) on the biofilm. This fact shows the flexibility and cohesion aspect the biofilm carpet has. There is a protuberance (black arrows) at the border of the footstep inferring soft underlying sediment.

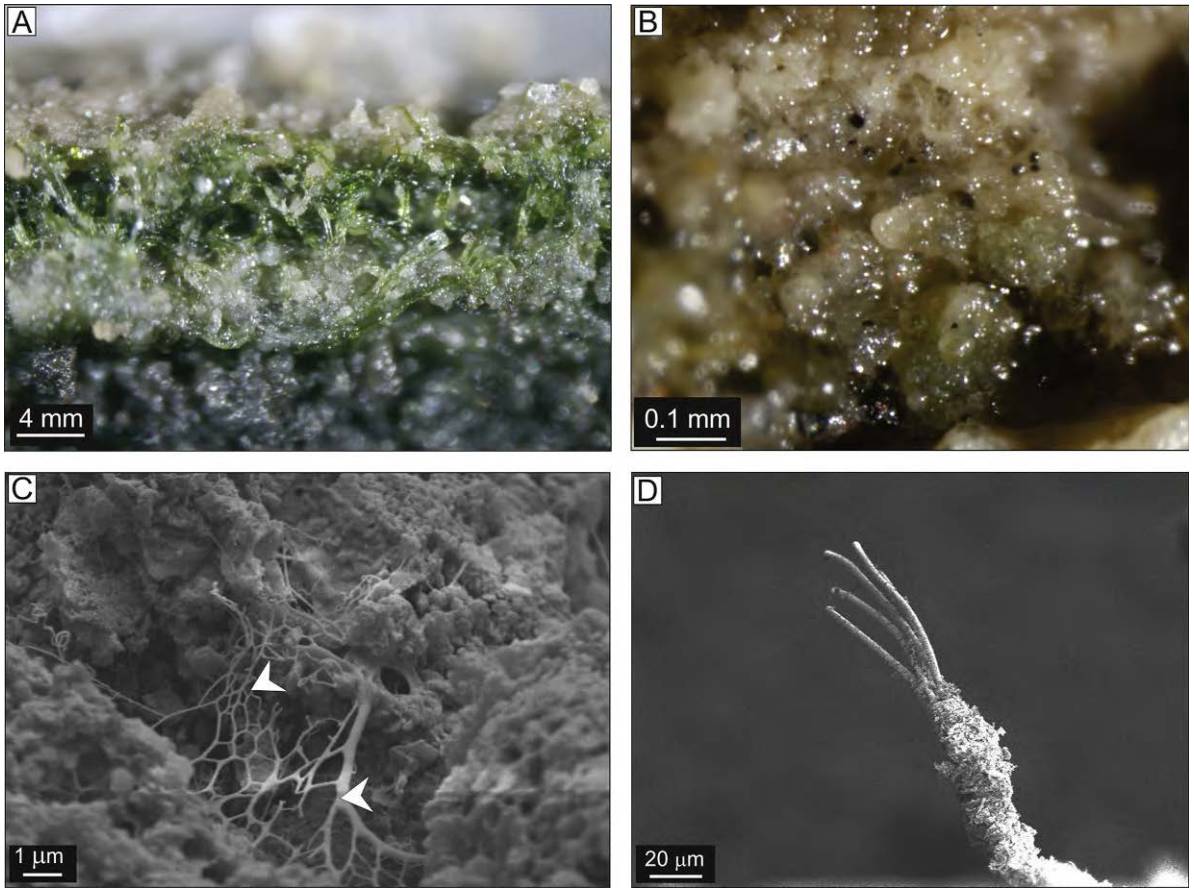

Fig. 2 Microbial mats. (A) Filamentous cyanobacteria (green filaments) interwoven sediment particles in the uppermost surface of a plain. Below there is an anoxic black layer due to bacterial activity. (B) Sediment grains coated by extracellular polymeric substances (EPSs) as a sticking glue giving cohesion to the sediment particles. (C) Scanning electron microscope (SEM) micrographs showing the EPS between the particles as polysaccharides net. (D) Bundle of trichomes of *Microcoleus chthonoplastes* in a common mucilaginous sheath coated by sediment particles, mainly platy-silty grains.

cells typically present a high potential for resiliency during periods of physical stress (e.g., changes in salinity and temperature, UV irradiation, desiccation, inundation) (Decho, 2000). This resiliency can also be extended to direct physical processes, such as water flow and wave activity, and where the colonized upper sediment establishing flat surface is erosioned by a flow current the non-colonized sediment beneath behaves in a different manner, i.e., forming ripples (see below erosional pockets).

Complex morphological features were produced by certain microbial behavior. By virtue of gliding motility of some filamentous cyanobacteria, the formation of reticulate structures occurs, being common in natural microbial mats as well as in the fossil record. They were found in different environments, such as extreme settings (Petroff et al., 2010; Bosak et al., 2012), closed coastal lagoon (Horodyski, 1977), coastal sabkha (Gerdes et al., 2000; Taj et al., 2014), closed coastal plain, and also they were studied under laboratory experiments (Shepard and Sumner, 2010; Bosak et al., 2012). The reticulate morphology comprises polygonal and elongated bulges over a lower topography where the intersections involve vertically growing microbial filaments forming a tuft

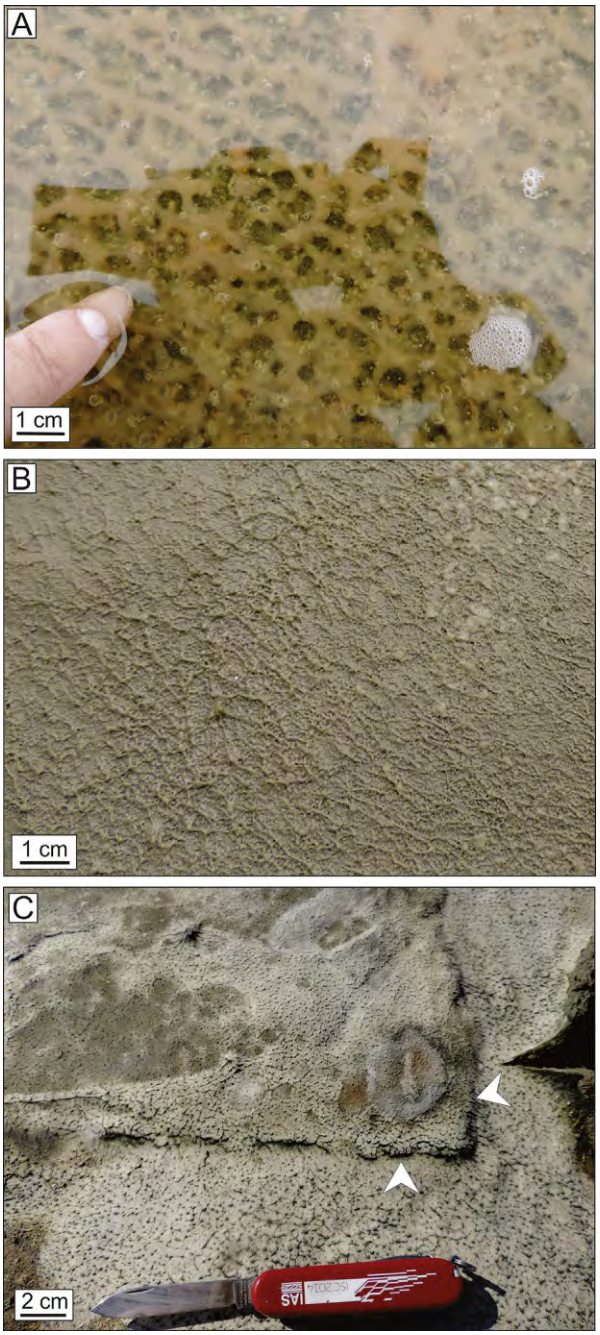

Fig. 3 (A) Initial formation of reticulate structures by the gliding motility of cyanobacteria in a shallow pond (2 cm of water depth). (B) Reticulate structures without water in an evolutionary stage. (C) Desiccated reticulate structures with small pinnacles and tufts after several days under solar radiation. Analogous structures can be found in fossil rocks.The arrows show the border of a recolonized fold-over mat.

texture (Fig. 3(A) and (B)). Cell motility is crucial for the development of reticulates and filaments organize into reticulates faster than cell division and growth. Shepard and Sumner (2010) used the motile strain *Pseudanabaena* for their experimental studies, while Fujisawa et al. (2010) studied the vigorous gliding motility of filaments of *Arthrospira*. *Phormidium*, *Lyngbya* sp., *Microcoleus chthonoplastes* change in grow direction into a vertical position and rise above the mat forming pinnacles (Gerdes et al., 2007). Laboratory experiments stated that in the early stages of formation of microbial reticulates, at least a lamina of water is required, pointing to the importance of calm conditions for their establishment (Fig. 3(A)). This is in agreement with the general insight of calm hydrodynamic conditions needed for the formation and preservation of microbial mats. However, after the aerial exposure that gives their desiccation (Fig. 3(C)), the reticulate structures can resist hydrodynamic regimes of high energy (Cuadrado and Pan, 2017). The consolidated pinnacles and tuft we found in the field was also reported for sedimentary textures for Precambrian rocks (Tice et al., 2011).

Geochemical Processes

All trophic groups (i.e., primary producers, consumers, and decomposers) are present in microbial mats, which is of great importance from an ecological perspective. Their populations are arranged into specific communities in different layers interacting with each other and their environment (Stolz, 2000). The top sedimentary layer is formed by cyanobacteria and diatoms. They are primary producers prompting organic material using sun light by photosynthesis. Organic matter of deceased primary producers is gradually decomposed by heterotrophic microbes that are established directly beneath the cyanobacteria–diatoms layer. Deceased cyanobacteria and EPS are transformed by chemoorganotrophic bacteria forming simpler chemical compounds. These large diversity of species within microbial mats support a wide spectrum of metabolic processes (Visscher and Stolz, 2005) resulting in coupled reactions (e.g., reduction and oxidation of essential elements, such as C, S, N; or, alternatively, stepwise oxidation or reduction of a compound) that support robust biogeochemical cycles (Fig. 4(A) and (B)). The result might be the formation of gases (Fig. 4(C)) and mineral precipitates.

Carbonate and Siliciclastic Environment

In a carbonate environment, the biogeochemical processes enhance precipitation of Ca_2CO_3 from seawater causing stromatolites, a huge biogenic system that is of significant interest to geologists, microbiologists, and ocean chemists. They reach some meters in height and are mainly formed by cyanobacteria and associated heterotrophic bacteria, which developed massive microbial reefs throughout shallow waters of Precambrian oceans and provide significant information on the microbiogeochemical processes interaction during their formation and the precipitation of $CaCO_3$. They are the assemblage of processes that promote the precipitation of $CaCO_3$ minerals, such as aragonite and calcite, and the trapping of sedimentary particles.

However, there is another sedimentary environment, the barely studied siliciclastic environment characterized by quartz sediments instead of carbonate sediments. In such siliciclastic environment, predominantly in cold waters, the chemical precipitation of minerals is less important than physical processes. Benthic microbiota colonizes the sedimentary plain and responds actively to the hydraulic movement of sediments being the microbial–physical interaction more important than biogeochemical processes (Noffke, 2010). This interaction displays a great variety of microbially induced sedimentary structures (MISSs) (after Noffke et al., 2001) that have the same significance for interpretation of Earth history as stromatolites.

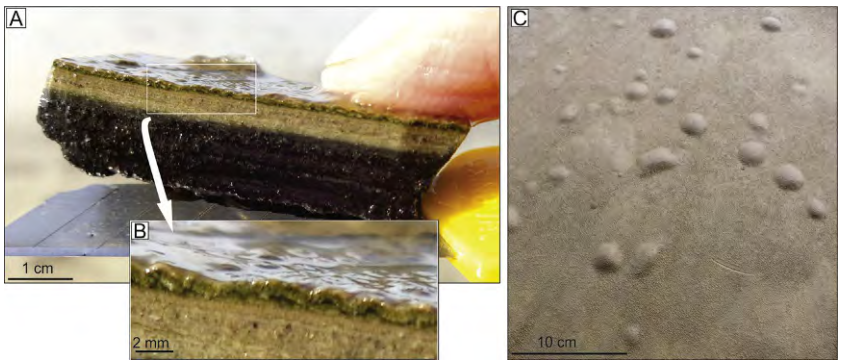

Fig. 4 (A) Vertical section of microbial mat showing, mainly two layers, an oxic one above and anoxic below. The oxic layer has a very thin green layer of cyanobacteria covered by a very thin layer of diatoms to protect the underlying filamentous cyanobacteria (inset (B)). These two uppermost layers present a wavy characteristic. (C) Example of sealing of the sedimentary surface forming gas domes that inhibit the exchange of gases between sediment and atmosphere.

Mineral Precipitation

Much of the studies on mineral precipitation by the influence of microbes have been carried out in calcium carbonate minerals. This is the case of the growing of build-ups stromatolites formed essentially by two processes: (1) the microbial communities responsible for successive "trapping and binding" of sediment; and (2) in situ precipitation of calcium carbonate. The precipitation of minerals led the lithification that preserves the biosedimentary structure. Two key and intimately joined components are involved in carbonate precipitation within microbial mats: (1) the alkalinity engine and (2) the organic matrix (Dupraz et al., 2011). The alkalinity engine changes the saturation index in two manners, intrinsically and extrinsically. Intrinsic alkaline engine refers to the microbial metabolism, the way the microbial communities acquire their energy and their source of carbon. While the mechanisms those microbial mats promote the precipitation of carbonate minerals involves: oxygenic photosynthesis, aerobic and anaerobic respiration, chemolithoautotrophy; the extrinsic alkaline engine comprises mainly two physicochemical processes: water evaporation and CO_2 degassing. In both cases, the organic matrix of EPSs, which embed the microbial communities, is the physical location where carbonate minerals nucleate and grow. Cyanobacteria are considered to be the major producers of EPS. However, many other bacteria can produce EPS matrix, in case anaerobic bacteria, especially sulfate-reducing bacteria, are known to produce large amounts of EPS (Bosak and Newman, 2005; Braissant et al., 2007).

The numerous studies on carbonate environments colonized by microbial mats has demonstrated that a rapid mineral precipitation and early cementation promote a quick lithification by the precipitation of calcium carbonate, enhancing thus the preservation potential of sedimentary rocks in the form of stromatolites. Unlike, as was mentioned above, studies in the siliciclastic tidal flats revealed that the lithification would be supported by the precipitation of hydrated alumino-silicates among others. This mineral precipitation is responsible for early cementation and subsequent preservation of sedimentary structures in an area where carbonates are scarce. Cuadrado et al. (2012) documented the precipitation of microscopic authigenic zeolites, identifying trigonal trapezohedron crystals of analcime, tabular clinoptilolite, elongate rods of mordenite, radiating clusters of phillipsite and chabazite (Fig. 5). This mineralization was associated with small (micrometer scales) crystals of framboidal pyrite and magnetite, which are frequently covered by EPS remains. The EPS substances synthesized by microorganisms within the microbial mats, may have acted as mineral nucleation sites favoring the precipitation of small zeolites, like the carbonate precipitation in analogous carbonate environments. It is worthy to mention that the dominant cyanobacteria in the microbial mats were the cyanobacteria *M. chthonoplastes* forming a tough coherent mat; while the pennates diatoms 40–100 μm in size were *Nitzschia* spp., *Diploneis*

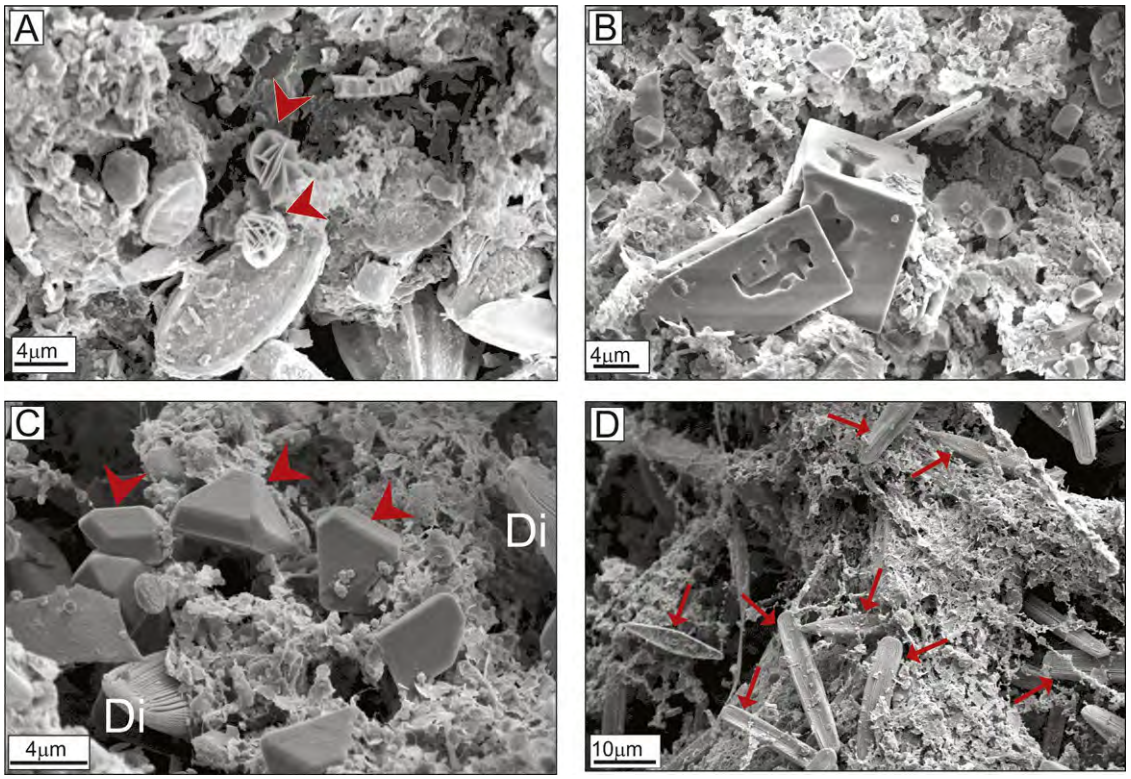

Fig. 5 Scanning electron microscope (SEM) micrographs showing the mineral precipitation in microbial mat in the Bahia Blanca Estuary. (A) In a mixed sediment and diatom assemblage, chabazite occurs as rosette-shaped crystal (arrows). (B) Halite cube crystals probably diluted by the sample preparation. (C) Clinoptilolite (pointed by arrows) with one or two sets of triangular or truncated faces on the edges. The crystals are immersed in a matrix formed by extracellular polymeric substances (EPSs) and mainly naviculoid diatoms (Di). (D) Matrix and filaments of EPS where the diatoms (red arrows) and sediment particles are immersed.

spp., *Amphora* spp., as the most abundant diatoms, while >100 μm pennates diatoms were *Pleurosigma* spp., *Gyrosigma* spp., less frequent (Pan et al., 2013).

Although in siliciclastic environments the mechanism of mineral precipitation is poorly documented, the activity of sulfate-reducing bacteria in the mat increases the alkalinity and reduces the pH, leading to desilicification of the diatom frustules in the mats (Kremer et al., 2008). Thereby, it can be achieved two essential conditions for zeolites precipitation: alkalinity and silica, so the potential role of microorganisms and EPS cannot be ignored.

Sediment Stabilization

One of the actions associated to the sediment colonization by microbial communities is the modification and stabilization of the sediment surface. Development of microbial mats requires an environment with low sedimentation rates that enable the vertical movement of photoautotrophic microorganisms toward the surface of the sediment to obtain optimum sunlight without been buried. Microbial mats are highly organized laminated communities, where cyanobacteria are usually recognized as the most important EPS producer. One of the most extensively distributed cyanobacteria, *M. chthonoplastes*, comprises filaments constituted by several trichomes, surrounded by a mucous hyaline sheath. These sheaths consist mostly of EPS, providing a mat resistance to degradation and erosion and where the fine particles of sediments are adhere (Fig. 6(A)). Newman et al. (2017) pointed that fine grains coat the sticky sheaths of filamentous cyanobacteria as *Nodosilinea* sp. and this fact contribute the fossilization potential of cyanobacteria without the preservation of interior cells. Moreover, benthic diatoms are also important EPS producers. The EPS occurs as a matrix or linkage of strands in the interparticle voids of the sediment (de Winder et al., 1999; Fig. 6(B)). The condensed meshwork made by diatoms and interweaving microbial filaments together with mucilaginous matrix of EPS contributes significantly to increase the cohesion of the detrital grains giving resistance to erosion and resulting in biostabilization (*sensu* Paterson et al., 1994). Sometimes, cyanobacteria layer (bluish green in color) is covered by a very thin diatoms layer (light brown in color) acting as a protection from UV radiation (Fig. 7).

During low-energy phases in high tide, cyanobacteria (mainly the filamentous *M. chthonoplastes*) move up the sediment in order to reach optimal light conditions (phototaxis), orienting their filaments vertically. This action, termed "baffling" by Noffke (2010), makes slower the sediment transport and so, suspended silty sediments deposit, and mats become enriched in silt-sized particles.

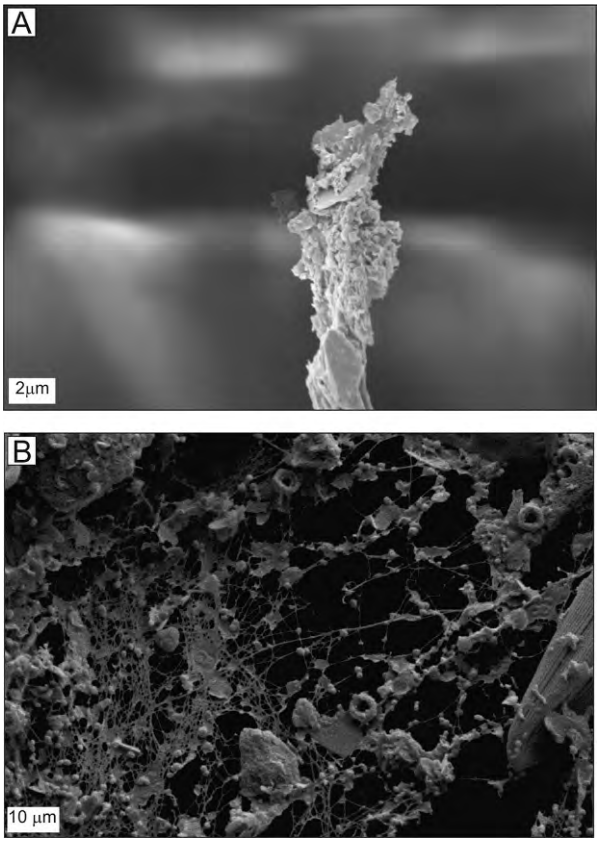

Fig. 6 (A) Filament of cyanobacteria coated by platy fine sediment due to the extracellular polymeric substances (EPSs) mucilage. (B) Dense and sparse filaments of EPS coated by platy sediments that contribute to the sediment biostabilization.

Fig. 7 Microbial mat showing the cyanobacteria layer below a thin layer of diatoms in Paso Seco (Argentina). *Microcoleus chthonoplastes*, the dominant cyanobacteria in the microbial mat, lacks of scytonemin, a pigment that provides a broad absorption in the UV and violet wavelengths. Contrarily, the microorganisms that inhabit the surface contain this pigment to withstand the solar radiation.

Moreover, biomass enrichment by means of cell replication, under favorable sunlight, temperature, humidity, and available nutrients, leads the growth of the microbial mats. By the other hand, "trapping" is the action of the sticky EPS glue small-sized particles to the mat surface, while *binding* is the formation of a mat fabric during calm conditions by the active movement of cyanobacteria generating a mesh-like network.

The lower intertidal zone in temperate siliciclastic tidal flats, with a short aerial expose period, is colonized only by a biofilm. The upper intertidal zone, which gets inundated twice every day with a longer aerial exposition, a microbial mat is formed in just a few hours, being the main builder the cyanobacteria *Oscillatoria limosa*. Single trichomes interweave with sand particles in the upper mm of the sedimentary surface. In contrast, the lower supratidal zone, inundated periodically only by spring tides and sporadically during storms events, is characterized by planar microbial mats, arising from the construction of a coherent network by *M. chthonoplastes* filaments in presence of abundant EPS on top of the sedimentary surface. Both types of cyanobacteria colonization (consequently the microbial mats they form) affect the biostabilization in a different manner. Microbial mats in the intertidal zone raise the erosional current velocity due to the mesh between the sand grains and the filaments, resulting in a rough sedimentary surface. However, microbial mats in the supratidal zone raise the erosive shear strength in 9–12 times compared with uncolonized sediments due to the slippery surface by the EPS richness (Noffke, 2010). These values were overpassed by wave shear stress calculated over a similar type of microbial mats during storms (Cuadrado et al., 2013).

By another point of view, the biostabilization also enhance the preservation of footprints as was demonstrate on a study carried out over the tidal flat of the Bahia Blanca Estuary (Carmona et al., 2011), where the morphologic modifications of several bird-footprints were recorded by the course of 10 months. This study revealed that footprints showed resistance to the regular tide and wind erosion and also to the heavy rains and storms that affected the tidal flat. This resistance is clearly associated with the presence of microbial mats, which biostabilize the sediment, and where the presence of authigenic minerals may also favor the consolidation of the footprints. The morphology of the footprints is also affected by the mat thickness, the tracks were deeply imprinted and the fine details were erased in areas with thick microbial mats. Contrarily, in zones colonized with thin microbial mats overlying cohesive mud, the traces preserved details, such as skin impressions and slipping marks (Fig. 8). Probably, this might be the way the delicate fossil impressions are preserved.

Microbially Sedimentary Structures

Due to the interaction of benthic microbiota with sediments, microbially sedimentary structures are formed and they are influence by the environment dynamics. The studies in modern settings allow inferring analogous process in the fossil record because sedimentary structures are preserved along great part of the Earth history. Eriksson et al. (2007) modified a previous scheme (Schieber et al., 2004) inferring genetic processes subject to microbial influences during deposition or diagenesis as the responsible for the sedimentary structures. The structures classification scheme they presented in geology is related to biostabilization, especially to mat grow, mat metabolism, mat destruction, decay and diagenesis for both, sandstone and mudstone. Therefore the detailed knowledge of the processes involved in the modern microbial mat colonization of sedimentary substrate is an essential tool for the correct geological interpretation of the paleoenvironment that led to such sedimentary deposits.

The fossil microbial structures found in the sedimentary rocks are consequence of taphonomic windows (Noffke, 2010). Two taphonomic stages are involved: the preservation processes that occur immediately at the time of the sediment deposition (primary process or syndepositional), and the preservation processes that occur after burial of sediment (secondary or postdepositional). Where the ecological and taphonomic windows overlap, the microbially sedimentary structures occur.

Fig. 8 Shorebird footprints over thin microbial mats showing delicate skin impressions. Due to biofilm formation the footprint details may be preserved in a sedimentary rock as fossil impression.

Different MISS were found in the modern environment and respond to particular process. In Bahia Blanca Estuary is common to find shrinkage cracks, mat domes, flipped-over mats, microbial chips, erosional pockets, and remnants (Cuadrado et al., 2012), as a consequence of estuarine process (Fig. 9). Shrinkage cracks constitute mat-destruction structures formed due to subaerial exposure and desiccation of the mats, typical of supratidal areas, where the upper surface of the mat undergoes greater contraction than the underlying layers (Fig. 9(A)). Typically, open cracks are favorable sites for the new growth of microbes, so when the surface gets wet again mat cracks are overgrown by the newly formed microbial mats and "healed" (Fig. 9(B)). Gas domes occur when gases trapped beneath the relatively impermeable microbial film accumulate, increasing the pressure and pushing the mat upward, producing a bulge and a hollow cavern underneath (Fig. 9(C)–(E)). Its formation requires gas production underneath or within the mat which involves organic matter decay, and also an impermeable sealing-mat. Each gas dome can last several days. Flipped-over mats are polygonal fragments of mat completely turned over at the edges, probably in the direction of tidal current, exposing the underlying substratum (Fig. 9(F)). The polygon is generally formed along weakness of crack network. Microbial chips are sedimentary features related to the physical mat-destruction that comprise eroded mat fragments, which reflect reworking and transport (Fig. 9(G)). Chips are irregularly shaped, flat-planar, often with rounded edges, and sometimes with flexible-cohesive behavior. These mat-chips are commonly seen spread all over the supratidal flat after storm events. Erosional pockets are derived from the mechanical destruction of the biostabilized sediment surface (Fig. 9(H)). These structures evolve from local destruction of the microbial mat that covered the surface sediment during high energy conditions generating irregular-shaped depressions with ripples features showing the difference in sediment colonization. Remnants are the flat-top surface portions biostabilizated by microbial mats.

Other significant microbial structures were found in a modern environment, in an ancient tidal channel flooded only by a storm-induced high tide, so an intermittent high energy occurred. The sedimentary plain is colonized by thick leathery microbial mats (Cuadrado et al., 2015). Mainly, nonheterocystous filamentous cyanobacteria grow conspicuously in the upper mm of the plain surface, in order to find the optimal light intensity for photosynthesis. The microbial biomass in this mat was dominated by *M. chthonoplastes*, with their strongly entangled meshwork with polysaccharide sheaths particularly capable of binding particles. The interwoven filaments, trichomes, of these cyanobacteria produced a highly coherent structure, providing a dense and coherent fabric for the binding of sedimentary particles of 1–2-cm-thick (Fig. 10), giving to the microbial mat a remarkable external leathery appearance (Fig. 11). Water currents are the triggering mechanism to create deformed microbial sedimentary structures. Folds, rolls ups and flip over microbial structures are formed as a resulting of the flexible behavior and mat cohesiveness, under high shear stress, forming the deformed microbial sedimentary structures.

Concluding Remarks

Modern microbial mat communities comprise species that can inhabit in extreme habitats. These associations are well-adapted to a range of harsh conditions that include extremes of temperature, atmospheric pressure, salinity, pH, and nutrient deprivation. One of the best adapted groups to various extreme conditions is cyanobacteria which constitute the uppermost layer of organisms in microbial mats. In this chapter, the microbial mats described comprise most of the cases that are under some form of stress most of the time by intermittent flooding. That fact involves daytime stress of too much light and UV radiation, and desiccation. Modern studies had stated that they had evolved various strategies to survive with the harmful effect of high solar radiation at midday. The bluish-green cyanobacteria migrate some millimeters below the mat surface being protected by diatoms that contains a yellowish-brown pigment called syctonemin that absorbs up to 97% of UVA radiation. Moreover, most microbial eukaryotes and mainly cyanobacteria are known for withstand moderate to severe dehydration for prolonged periods of time, and they are able to recover from this stress (Castenholz, 2009).

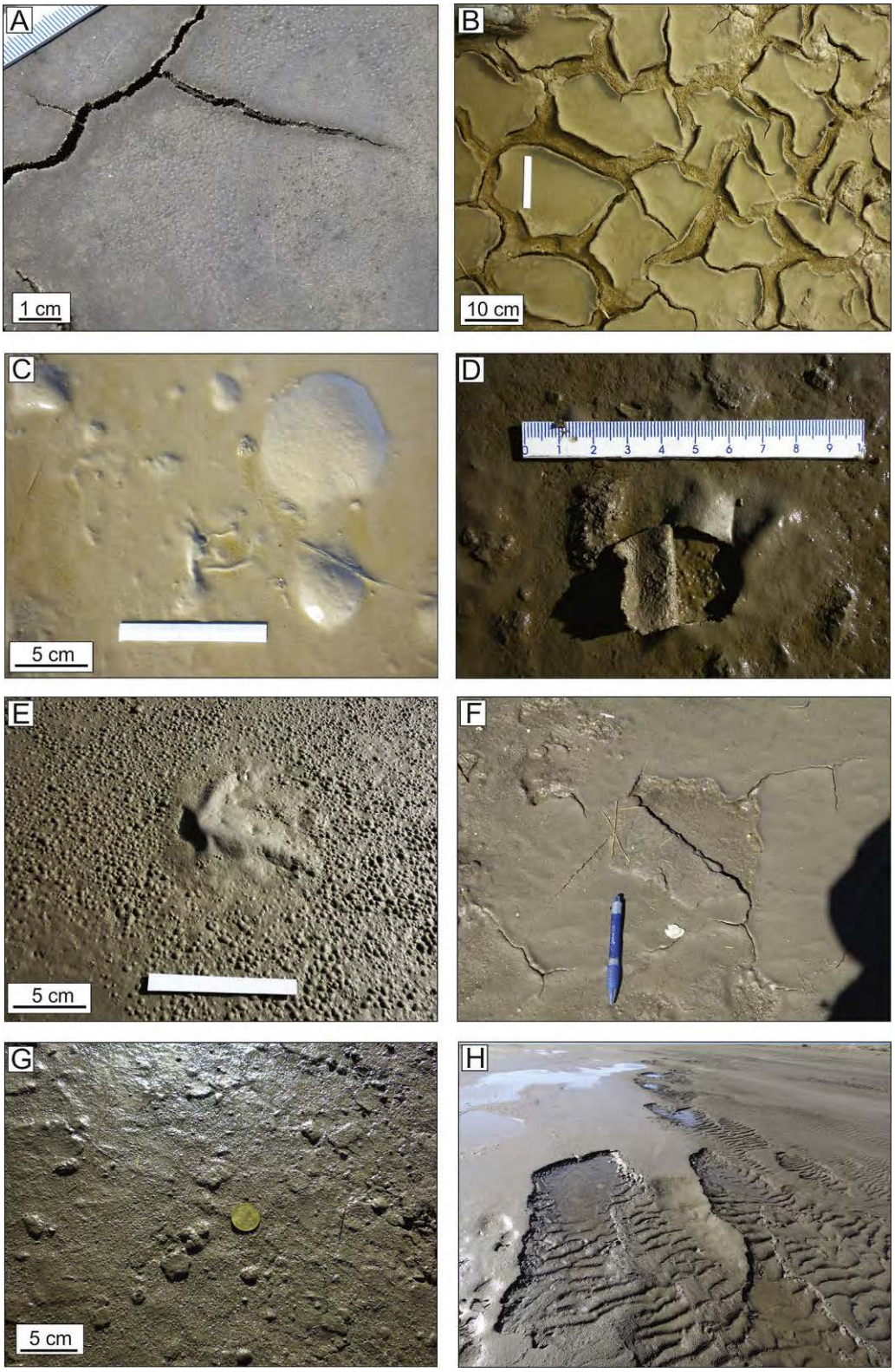

Fig. 9 Sedimentary microbial structures formed in a tidal plain by the colonization of sediments. (A) Desiccation or shrinkage cracks are formed in the supratidal zone due to the solar radiation during the exposure of the microbial mats over several days. The rupture of the mat sometimes is alike a torn cloth displaying the filaments at the border of the crack. (B) Recolonization of wide desiccation cracks after a subsequent water inundation. (C) and (D) Gas dome and a broken one showing the hollow below. In (D) new little bubbles are form over the sediment inside the dome due to gases from photosynthesis. (E) Desiccation of gas dome probably formed by organic matter decay, surrounding by photosynthetic bubbles. (F), (G), and (H) Features due to erosion. (F) Flipped-over mats are formed after an increase in energy mainly where a former crack was present. Pencil as scale is 14 cm long. (G) Microbial chips formed by the erosion of microbial mat, transported, and laid down over the plain to be recolonized. (H) Erosional pockets and remnants as a consequence of a high level of energy that broken some parts of the colonized microbial mats.

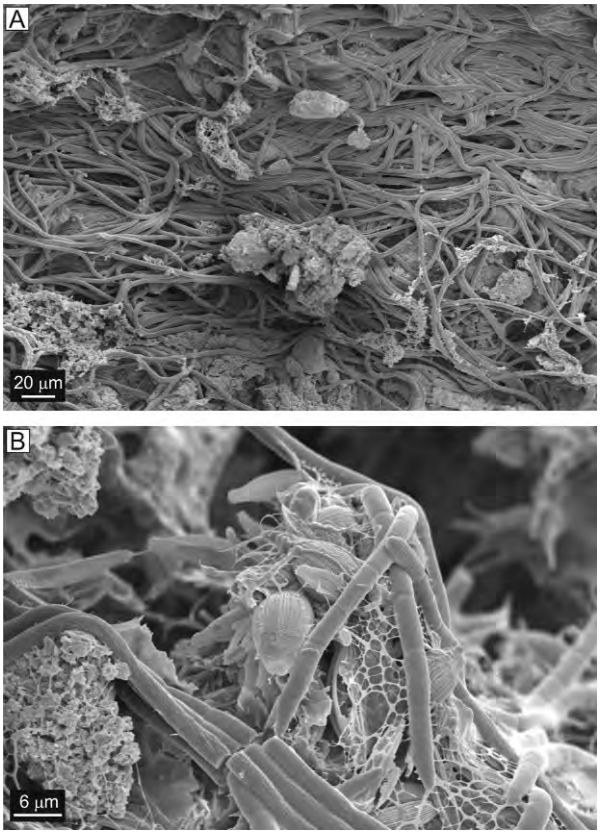

Fig. 10 Scanning electron microscope (SEM) micrographs of microorganisms forming thick microbial mats in Paso Seco (Argentina). (A) A dense mesh-like arrangement of cyanobacteria. (B) Cyanobacteria trichomes and thick extracellular polymeric substances (EPSs) threads interwoven with pennate diatom frustules.

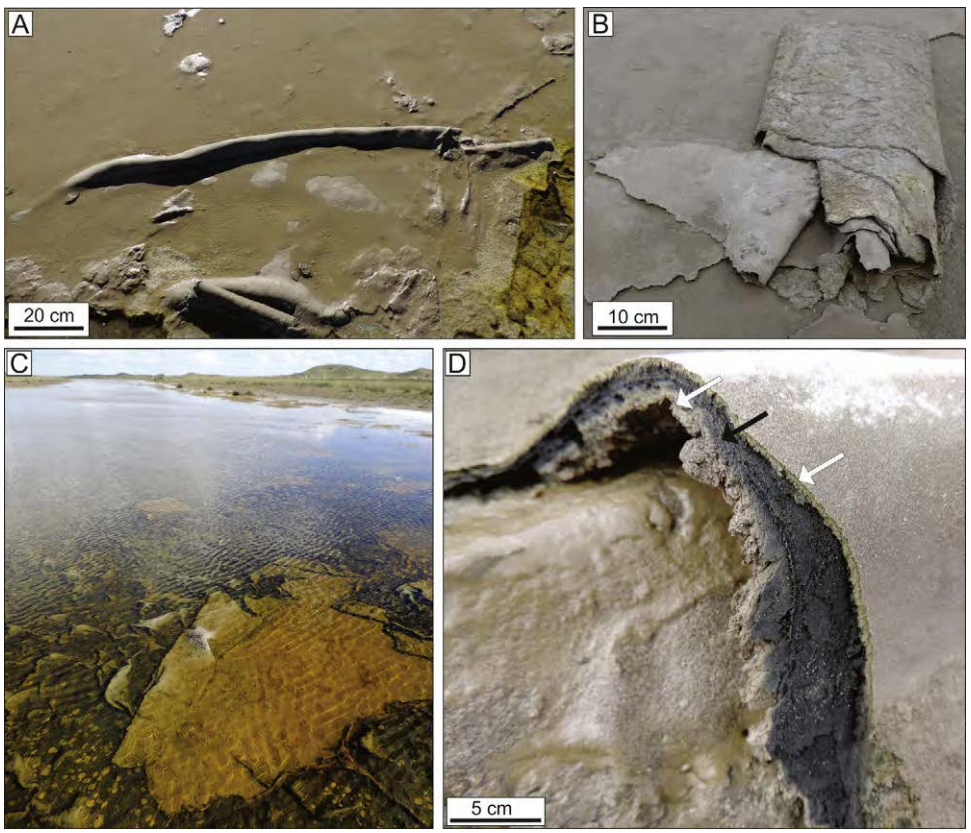

Fig. 11 Deformed sedimentary microbial structures present in Paso Seco (Argentina). (A) Microbial folds structures formed by the slippery thick and flexible microbial mat over the underlying sediment as a consequence of a high water current. (B) Microbial roll-up structure formed by the previously torn microbial mat and latter turn round by water current. Several involutions may happen. (C) View of large pond colonized by microbial mats where submerged flipped-over mats can be identified by the different color. The light brown mat is the recolonization of underlying sediment. The cracks present on the microbial mat can infer a previous subaerial exposure. (D) Close up of a fold microbial structure and the sediment underneath. The thick torn mat (1 cm thick) shows an oxic layer above (white arrow), an intermediate anoxic layer (dark layer, black arrow), and again an oxic layer below (white arrow) due to the air between the mat and the sediment underlying. Different laminae are forming the mat and the sediment below has the same form as the fold.

Biofilms and microbial mats work in sediments as cooperatives, not as solitary organisms, to respond to internal and external factors affecting the community (Noffke and Awramik, 2013). In fact, is in a cooperative way the manner they increase their survival possibilities, and they find strategies to subsist in a damaging environment. Although not with exactly the same microbial groups present today, biofilms and microbial had evolved from the ancient times of Earth. Such structures as stromatolites and MISSs have existed since Archean times, demonstrating that microorganisms have been sustained throughout the geological record of life (Costerton and Stoodley, 2003). It is necessary to study the geobiological processes that take place in modern environments in order to be able to correctly understand what has happened during the geological past.

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Microbial Solute Transporters☆

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Introduction

The cellular membrane is a selectively permeable barrier between the cell and the extracellular environment. Cytoplasmic membrane transporters are a group of membrane proteins embedded in the phospholipid bilayer. These proteins typically contain multiple α-helical segments of usually 18–25 hydrophobic amino acids. These proteins mediate the movement of molecules and ions across the cytoplasmic membrane, and play essential roles in fundamental cellular processes like the acquisition of organic nutrients, extrusion of toxic and waste compounds, maintenance of ion homeostasis, environmental sensing and cell communication in archaea, bacteria and eukaryotes. Other transport proteins, such as those in the outer-membrane of Gram negative bacteria span the membrane as β-barrels. Based on current analyses, approximately 1–20% of open reading frames (ORFs) in prokaryotic genomes are predicted to encode membrane transport proteins, emphasizing the importance of membrane transport to microbes living in diverse environments. This estimated percentage range is broader than that listed in a previous edition of this book (published 2009) at both the upper and lower limits, which reflects the larger volume of genome sequence data available and the diversity amongst the strains that have now been sequenced. Knowledge of the suite of membrane transporters present in an organism is important to understand fully its metabolism and physiology, as well as its adaptation to a particular ecological niche (Fig. 1).

Various transport systems differ in their mechanisms of transport, membrane topology, energy coupling mechanism and substrate specificities (Fig. 2). Membrane **channels** form pores through the membrane that facilitate diffusion of water, specific types of ions or hydrophilic small molecules down their concentration or electrical gradient. This process is not coupled to metabolic energy and cannot transport substrates against their concentration gradient across the membrane. The rate of transport mediated by channels can be rapid, but will be related to the substrate concentration gradient and/or the transmembrane electrical potential in the case of charged substrates. Channels can have varying degrees of selectivity. For example, the narrow pores in channels may restrict passage to ions of the appropriate size and charge. Many channels, such as ion channels are not permanently open, a property referred to as gating; they open transiently in response to specific stimuli, e.g., the binding of signaling molecules (ligand-gated channels), changes in electric potential across the plasma membrane (voltage-gated channels), or changes in membrane tension (mechanosensitive channels).

In contrast to channels, active transport proteins require energy to mediate the movement of solutes across biological membranes. **Primary active transporters** energize transport via utilization of a primary chemical energy source, typically adenosine triphosphate (ATP) (Fig. 2). Transport involves the binding of specific molecules from one side of the membrane, then the transporter undergoes a conformational transformation that allows the substrate to pass through the membrane and be released on the other side of the membrane. It exhibits a much lower rate of transport and higher substrate stereospecificity compared to the channels.

The **secondary active transporters** also mediate energy-dependent active transport process, but utilize a pre-existing source of energy (e.g. an ion/solute electrochemical gradient). Secondary transporters can be differentiated on the basis of their transport mechanism, i.e., the mode of movement of substrate(s) and coupling molecule(s); uniport, symport or antiport (Fig. 2). Uniporters transport a single molecule without the involvement of a coupling molecule. Symporters transport substrate and coupling molecules in the same direction. Antiporters mediate the exchange of one or more substrates for coupling molecules.

A final group of solute transporters are the **group translocation systems** which modify their substrates during transport, exemplified by the bacterial phosphotransferase system (PTS) transporters (Fig. 2). PTS transporters typically enable the uptake of sugars from the external environment and concomitant phosphorylation and release into the cytoplasm as sugar-phosphates, using phosphoenolpyruvate (PEP) as both phosphoryl donor and energy source.

A great variety of substrates can be transported across the membrane by transporters. These include inorganic molecules; carbon compounds; amino acids and derivatives; bases and derivatives; vitamins, cofactors, signaling molecules and their precursors; drugs, dyes, sterols, and toxic substances; and macromolecules. Fig. 1 shows the predicted metabolic and transport capabilities of the soil and plant-associated bacteria *Pseudomonas putida* and provides an example of the complexity of transporters found in

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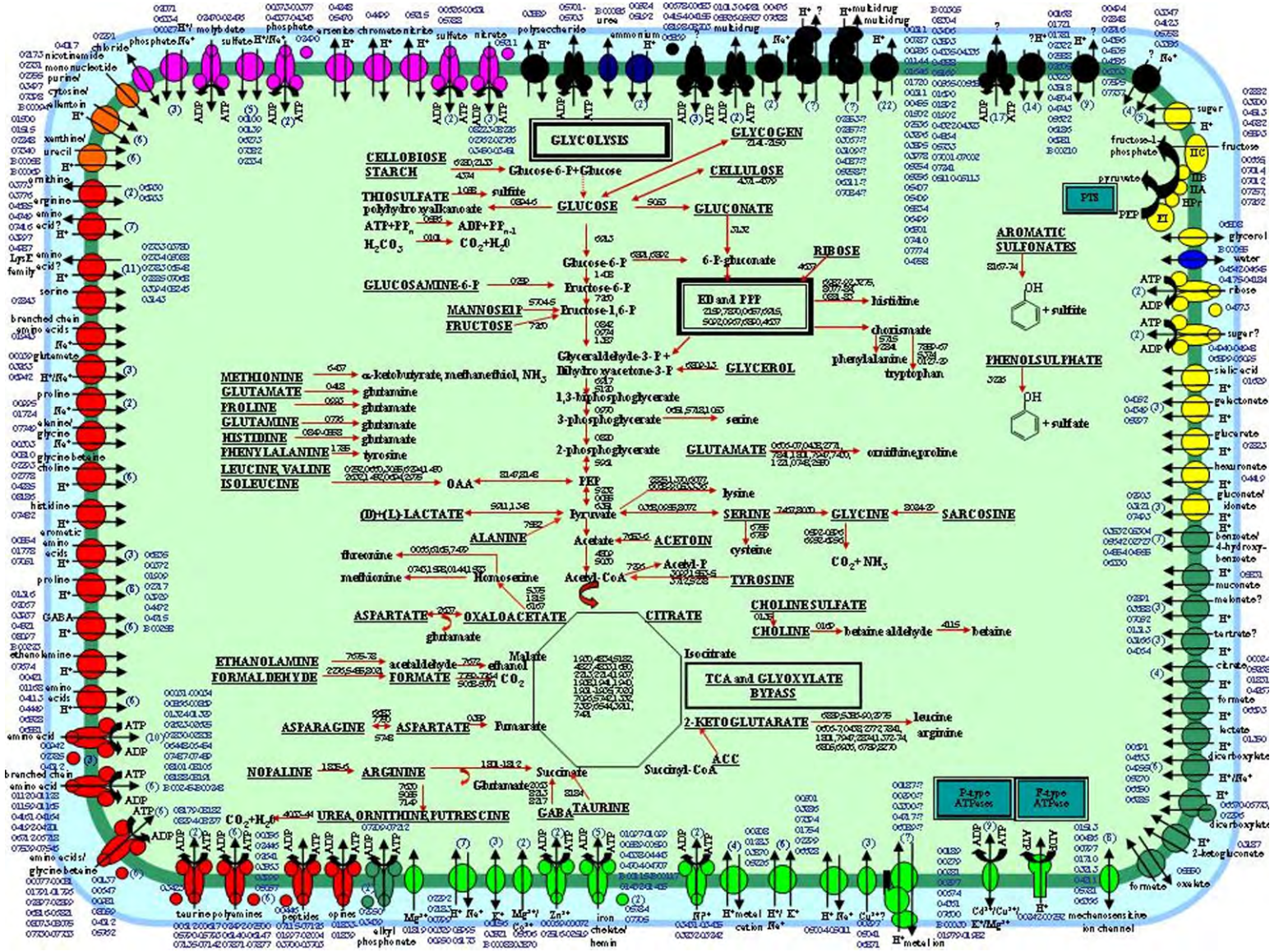

Fig. 1 Metabolic reconstruction of pathways present in the *Pseudomonas putida* genome. Transporters are grouped by substrate specificity as follows: inorganic cations (*green*), inorganic anions (*pink*), carbohydrate/carboxylates (*yellow*), amino acids/peptides/amines/purines/pyrimidines (*red*), and drug efflux and others (*black*). *Question marks* indicate uncertainty about the substrate transported. Export or import of solutes is designated by the direction of the *arrow* through the transporter. The energy-coupling mechanisms of the transporters are also shown: solutes transported by channel proteins are shown with a *double-headed arrow*; secondary transporters are shown with *two arrowed lines*, indicating both the solute and the coupling ion; ATP-driven transporters are indicated by the ATP hydrolysis reaction; and transporters with an unknown energy coupling mechanism are shown with only a *single arrow*. Components of transporter systems that function as multi-subunit complexes that were not identified are outlined with *dotted lines*. Where multiple homologous transporters with similar substrate predictions exist, the number of that type of transporter is indicated in *parentheses*. Systematic gene numbers (XXXXX) are indicated next to each pathway or transporter; those separated by a dash represent a range of consecutive genes. The outer and inner membrane are sketched in *dark blue* and *dark green* respectively, the periplasmic space is indicated in *light turquoise* and the cytosol in *light green*. Abbreviations are as follows: ADP, adenosine diphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; FucNAc, *N*-acetylfucosamine; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GluNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NeurNAc, *N*-acetylneuraminate; P, phosphate; PP, diphosphate; Pyr, pyruvate. Reproduced with permission from Environmental Microbiology. Nelson, K. E., Weinel, C., Paulsen, I. T., et al. (2003). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environmenttal Microbiology* **4**(12), 799–808.

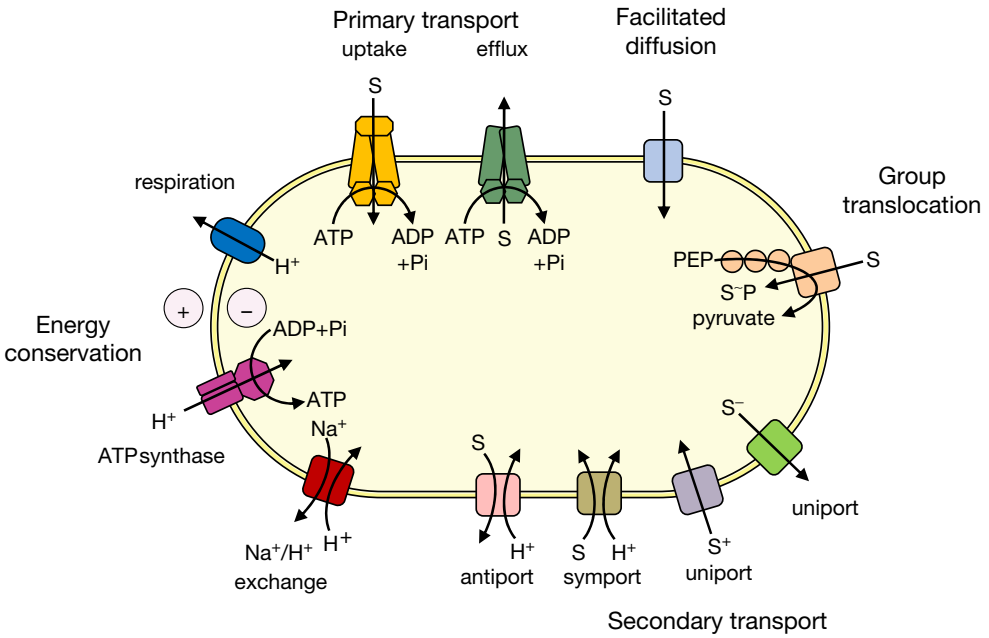

Fig. 2 Schematic representation of a bacterial cell showing variants of the four main types of transporters and their modes of energization. Energy is generated and stored across biological membranes through processes of "energy conservation". In many bacterial species, a respiratory chain (respiration) is used to generate a electrochemical proton gradient (outside positive and acidic). The nature of the respiratory chain is determined by the bacterial species and the environmental conditions. The proton gradient generated during respiration can be used for the generation of ATP via oxidative phosphorylation mediated by ATP synthase. The proton gradient can also be used to generate gradients of other cations, such as sodium or potassium, by exchange through proteins such as the Na^+/H^+ exchanger. There are four broad categories of transporter function, facilitated diffusion, primary transport, secondary transport and group translocators (text in *bold* corresponds to the labels in the figure). The simplest of these, **facilitated diffusion**, is where substrates (S) are moved down their concentration gradient across the membrane. **Primary transport**, where ATP hydrolysis is used to power the movement of a substrate against its concentration gradient. In bacteria, these proteins can be either uptake or efflux systems. **Secondary transport**, utilizes a concentration gradient of one solute, frequently a proton, to energize the uphill movement of a substrate. These proteins can be "antiporters", where the primary substrate is exchanged for the counter solute, "symporters" where the primary substrate and counter solute are moved in the same direction, or "uniporters", where only a single substrate is moved, but at the expense of an electrical gradient, i.e., a cation is moved inside the cell or an anion is moved out. The fourth common group of bacterial transport proteins are referred to as **group translocators**. These proteins facilitate the uptake of a substrate, typically a sugar, using chemical energy held in a high energy phosphate bond on phosphoenolpyruvate (PEP), and the substrate is phosphorylated during translocation.

microorganisms that are related to the environmental niche(s) that they may occupy. Informatic identification of the true biological substrate for many transporters remains difficult, with up to 37% of *Saccharomyces cerevisiae* transporters unable to be annotated bioinformatically. Even in best case scenarios such as *Halobacterium* sp. 5% of transporters remain unassigned to a substrate, and given that these annotations rely heavily on sequence matching (as opposed to any kind of structural analysis) it is likely that the real substrate is not reliably identified in many cases. Biochemical identification of substrate specificity also presents considerable technical difficulty, however recent high throughput methods utilizing thermostability assays should improve this situation.

The structural study of membrane transporters has lagged behind other types of proteins. Due to their amphipathic characteristics, membrane transport proteins have proven difficult to overexpress, purify and crystallize, as required for three-dimensional structural analysis by X-ray diffraction, NMR or cryo-EM. The percent of membrane transporter structures deposited into PDB is much less than the percent of transport proteins encoded in the average genome and proportionally lower than the structures available for soluble proteins. Since the last update of this chapter in 2014 there have been significant advances in the detectors used in cryo-EM that allow reconstruction of structures to high resolution through single particle analysis. Cryo-EM may be good for membrane proteins because gets around some of the bottlenecks encountered in crystallography, such as the need for significant amounts of purified protein, making the typical low yield of membrane proteins less of an issue. There may also be more flexibility in detergent use in cyro-EM compared to crystallography, since the detergent doesn't need to fit into the crystal matrix. However, cryo-EM does have some limitations, the proteins/protein complexes must be above approximately 100–200 kDa to have a good chance of obtaining sufficient resolution. In crystallography there have been advances in using lipid cubidic phases and in microcrystals that have also advanced our ability to obtain high-resolution structural information for membrane proteins.

High-throughput genomic and metagenomic analyses are important to facilitate the study of membrane transporters. Instead of focusing on one or several transporter genes at a time, this type of study emphasizes the big picture, i.e. the complete transporter profile of an organism or a community of organisms. Examples include systematic annotation and classification of transporters, comparative study of the fundamental differences of transporter features amongst organisms with different evolutionary background, the association of genome transporter features with evolutionary history, physiology and lifestyle, and the integration of

transporter reactions into metabolic networks. In metagenomic studies the transporter profile of a community can be viewed within the context of the environment in which the community exists, e.g. in light of carbon or nitrogen source availability, or underlying selective pressures, such as contaminants or frequent disinfectant exposure.

In this chapter, we will review the features and functions of major transporter types and families, with the focus on recent progress in both structural and functional studies. We will also overview the bioinformatic classification of transporters as well as other types of comparative genomic studies of membrane transporters.

Transporter Classification and Annotation

Transporter Classification System

Transporters with similar functions characteristically cluster together in phylogenetic analyses. Thus substrate specificity appears to be a relatively conserved evolutionary trait in transporters. This has led to the premise that phylogeny can provide a rational basis for functional assignment. The Transporter Classification (TC) system and associated database, which is maintained by Milton Saier's research group at UC San Diego, represents a systematic approach to classify membrane transporter families according to their mode of transport, energy coupling mechanism, molecular phylogeny and substrate specificity (www.tcdb.org). The TC system is analogous to the Enzyme Commission (EC) system for classification of enzymes, except that it incorporates both functional and phylogenetic information. Transport mode and energy coupling mechanism serve as the primary bases for the classification due to their relatively stable characteristics. There are four major characterized classes of solute transporters in the TC system: channels, secondary transporters, primary active transporters and group translocators (described above; Fig. 2). Additionally, there are three other categories to catalog: proteins involved in the movement of electrons, accessory proteins, and transporters of unknown mechanism or function. These are shown in Table 1.

Each transporter class is further classified into individual families or superfamilies according to their function, phylogeny and/or substrate specificity. A specific transport protein can be represented by a TC number that normally has five components as follows: V.W.X.Y.Z. V (a number) corresponds to the transporter type (i.e., channel, secondary transporters, primary active transporters, group translocators, etc.); W (a letter) corresponds to the transporter subtype, which in the case of primary active transporters refers to the energy source used to drive transport; X (a number) corresponds to the transporter family; Y (a number) corresponds to the subfamily in which a transporter is found, and Z corresponds to the substrate(s) transported or to a specific transport protein. For example, the well-characterized *Escherichia coli* LacY lactose permease is represented in the TC system as 2.A.1.5.1, where "2" indicates it is a secondary transporter, "A" indicates it is a uniporter/symporter/antiporter, "1" indicated it belongs to the major facilitator superfamily (MFS), "5" indicates it belongs to an oligosaccharide symporter subfamily within the MFS family, and "1" indicates it is a lactose/proton symporter.

Table 1 The TCDB schema of transporter classes

Transporter class	*Type*	*Description*
1	Channels/pores	Energy-independent transporters that transport water, specific types of ions or hydrophilic small molecules down the concentration or electric gradient with higher rates of transport and lower stereospecificity compared to other transporter classes. Most ion channels, referred to as gated channels, open only in response to specific chemical, electric or mechanical signals.
2	Electrochemical potential-driven transporters (secondary transporters)	Secondary transporters (also called carriers) utilize an ion or solute electrochemical gradient, e.g. proton/sodium motive force, to drive the transport process. Uniporters move a single type of molecule down its concentration gradient. Antiporters and symporters couple the transport of ion or molecule against its concentration gradient with the movement of one or more different ions or molecules down its gradient.
3	Primary active transporters	Use the energy derived from a primary source of chemical energy (e.g. ATP hydrolysis) and move substrates across a membrane against a chemical concentration gradient or electric potential or both.
4	Group translocators	Modify their substrates during the transport process. For example, the bacterial phosphotransferase system (PTS) phosphorylates its sugar substrates using phosphoenolpyruvate as the phosphoryl donor and energy source and releases them into the cytoplasm as sugar-phosphates.
5	Transmembrane electron carriers	Mediate the flow of electrons from an electron donor to an electron acceptor in a biological membrane, and use the released energy to drive ion transport across the membrane and thus influence the membrane potential.
8	Accessory factors involved in transport	Indirectly influence the transport of solutes. These proteins may interact with transporters, assist in energy coupling or regulation.
9	Incompletely characterized transporter systems	Transporter protein families for which insufficient information is available to allow classification in one of the defined classes above.

TCDB: A Transporter Classification Database

Transport system families included in the current TC system are available in database format on the web. The TCDB site (http://www.tcdb.org/) provides detailed information and references for each transporter class, subclass, family, subfamily and individual proteins. When possible, these families are grouped into superfamilies that define the evolutionary relationships between individual families. TCDB also provides web-based search tools and various bioinformatic software packages that allow users to search by key word, gene name, family, or protein sequence. There is also a major section devoted to poorly characterized families of transport or putative transport proteins where more studies are needed.

TransportDB: A Comprehensive Database Resource for Transporters

TransportDB (http://www.membranetransport.org/) is a relational database, developed by Ian Paulsen's research group at Macquarie University, that catalogs all transport proteins encoded by organisms with fully sequenced genomes. Transport proteins are identified using the Transporter Automated Annotation Pipeline (TransAAP), which surveys fully sequenced genomes for genes encoding transport proteins using a variety of searches. It was created as a comprehensive database resource of information on cytoplasmic membrane transporters and Gram negative outer membrane channels in organisms whose complete genome sequences are available. The complete set of membrane transport systems and outer membrane channels in each organism is annotated based on a series of experimental and bioinformatic evidence and classified into different types and families according to the TC system. User-friendly web interfaces have been designed for easy access, query and download of the data (www.membranetransport.org). Features of the TransportDB website include text-based and BLAST search tools against known transporter and outer membrane channel proteins; comparison of transporter and outer membrane channel contents from different organisms; known 3D structures of transporters, and phylogenetic trees of transporter families. On individual protein pages, users can find detailed functional annotation, supporting bioinformatic evidence, protein/nucleotide sequences, publications and cross-referenced external online resource links. Another feature of TransportDB is the TransAAP web server where users can submit their genome or transporter annotation utilizing the high-throughput transporter analysis pipeline, view automatic annotation and supporting evidence, as well as curate the transporter annotation, all of which are through a user-friendly web interface.

TransportDB has now been in existence for nearly 20 years and continues to be regularly updated with new evidence and data from newly sequenced genomes, as well as having new features added periodically. As of November 2018, the TransportDB databases contain data from 2760 species, including 2557 bacteria and 164 archaea. Over 900,000 transporter proteins from these organisms were identified and classified according to the family designations described by TCDB. Some of these transport protein families are very large superfamilies with tens or hundreds of thousands of members, such as the ATP-binding cassette (ABC) superfamily, and the major facilitator superfamily (MFS), both of which are widely distributed in eubacteria, archaea and eukaryotes (Table 2). Others are very small families with only a few members present in a narrow distribution of organisms. The following sections look in detail at some representative membrane transport families.

Major Solute Transporter Families

Channels

KcsA, a potassium ion selective bacterial ion channel, was amongst the first membrane transporters to have their high resolution structure solved. Its structure was first reported in 1998 by MacKinnon's group utilizing X-ray crystallography, and later, the refined structure was reported at 2.0 Å resolution. The KcsA channel is a homotetramer with a fourfold symmetry axis forming the potassium ion permeation pathway. The N and C termini of each subunit are located inside the bacterial membrane, and contain two integral transmembrane helices, which are connected by a P-loop. The P-loop consists of a short helix that spans about 10 Å into the membrane and a loop region. It forms the selectivity filter, which is responsible for the selectivity for potassium ion over other ions. The structural studies of the KcsA channel, as well as other potassium ion channels (MthK, KvAP and KirBac), suggest the involvement of protein backbone structures in the selectivity process rather than individual amino acid residues, and illustrate the mechanism of high flux rate and selectivity for small polar and charged molecules across cell membranes.

Primary Active Transporters

The ATP binding cassette superfamily

The ABC transporter superfamily is characteristically one of the largest protein families found in the genomes of both prokaryotes and eukaryotes, with hundreds of thousands of identified members (Table 2). Most ABC transporters consist of four structural domains: two hydrophobic membrane-spanning domains that contain a variable number of membrane transmembrane α-helices (TMH), and two hydrophilic cytoplasmic nucleotide-binding domains that are responsible for ATP binding and hydrolysis to drive the transport process. ABC efflux systems are found in all domains of life, whereas ABC import systems appear to be prokaryote-specific and typically require an additional substrate binding protein. In Gram negative bacteria, this substrate-specific binding protein is periplasmic, whereas it is an extracellular lipoprotein bound to the membrane in Gram positive bacteria. ABC uptake

Table 2 The top 12 largest transporter families within bacteria and archaea in the TransportDB database

Family ID	*Family Name*	*TC#*	*Occurrence*[a]	*Count*
ABC	The ATP-binding cassette (ABC) superfamily	3.A.1	B,A	392,744
MFS	The major facilitator superfamily (MFS)	2.A.1	B,A	77,601
SSPTS	Sugar specific PTS	4.A	B,A	28,302
DMT	The drug/metabolite transporter (DMT) superfamily	2.A.7	B,A	24,229
F-ATPase	The H^+- or Na^+-translocating F-type, V-type and A-type ATPase (F-ATPase) superfamily	3.A.2	B,A	23,351
APC	The amino acid-polyamine-organocation (APC) family	2.A.3	B,A	16,351
RND	The resistance-nodulation-cell division (RND) superfamily	2.A.6	B,A	16,222
SSS	The solute:sodium symporter (SSS) family	2.A.21	B,A	14,482
MOP	The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase superfamily	2.A.66	B,A	14,024
P-ATPase	The P-type ATPase (P-ATPase) superfamily	3.A.3	B,A	13,191
TRAP-T	The tripartite ATP-independent periplasmic transporter (TRAP-T) family	2.A.56	B,A	9704
IIISP	The type III (virulence-related) secretory pathway (IIISP) family	3.A.6	B	9399

Most of them are widely distributed in both bacteria and archaea, with the exception of IIISP (bacteria).
[a]B, bacteria; A, archaea.

transporters are involved in the import of a diverse spectrum of solutes, including inorganic anions and cations, carbohydrates, amino acids and peptides. In these uptake systems the membrane-spanning and nucleotide-binding domains are typically encoded by distinct genes, which are often organized together in gene operons or clusters. ABC exporters in both prokaryotic and eukaryotic species are involved in the extrusion of various drugs, metabolites, toxins, lipids and signal molecules. In these systems, the membrane and nucleotide-binding domains are often fused into a single polypeptide.

ABC transporters are particularly abundant in prokaryotes, constituting up to 14.5% of all open reading frames (ORFs) (Table 3). Thermophilic *Thermotoga* bacteria devote some of the highest proportions of their genome to encoding ABC transporters (14.5%). These organisms lack a complete TCA cycle and an electron transfer chain, and therefore obtain their metabolic energy

Table 3 Top organisms with highest percent of ABC superfamily transport proteins

Organism	*Number of ABC transporters*	*Total ORFs*	*ORF %*
Thermotoga lettingae TMO	295	2040	14.5
Verminephrobacter eiseniae EF012	697	4947	14.1
Agrobacterium radiobacter K84	941	6684	14.1
Ketogulonicigenium vulgare Y25	426	3213	13.3
Sphaerochaeta pleomorpha Grapes	418	3159	13.2
Ketogulonicigenium vulgare WSH001	401	3054	13.1
Sphaerochaeta coccoides DSM17374	233	1822	12.8
*Mesotoga prim*a MesG1Ag42	329	2574	12.8
Paenibacillus sp. Y412MC10	778	6238	12.5
Agrobacterium tumefaciens strC58	664	5355	12.4
Spirochaeta smaragdinae DSM11293	522	4219	12.4
Agrobacterium sp. H133	642	5345	12.0
Gardnerella vaginalis HMP9231	157	1317	11.9
Rhizobium leguminosarum bv viciae 3841	833	7143	11.7
Agrobacterium vitis S4	624	5389	11.6
Ochrobactrum anthropi ATCC49188	555	4799	11.6
Paenibacillus sp. JDR2	717	6213	11.5
Rhizobium leguminosarum bvtrifoliiWSM2304	730	6415	11.4
Beutenbergia cavernae DSM12333	464	4197	11.1
Gardnerella vaginalis ATCC14019	150	1365	11.0
Sinorhizobium meliloti Rm41	752	6844	11.0
Rhizobium leguminosarum bv trifoliiWSM1325	769	7001	11.0
Mesorhizobium ciceri biovar biserrulaeWSM1271	682	6264	10.9
Sinorhizobium medicae WSM419	676	6213	10.9
Petrotoga mobilis SJ95	206	1898	10.9
Caldilinea aerophila DSM14535NBRC104270	445	4119	10.8
Sinorhizobium meliloti 1021	671	6218	10.8
Sphaerochaeta globus str Buddy	325	3017	10.8
Pelagibacterium halotolerans B2	416	3881	10.7
Sinorhizobium meliloti 2011	670	6263	10.7
Mesorhizobium opportunistum WSM2075	685	6508	10.5

Table 3 (Continued)

Organism	*Number of ABC transporters*	*Total ORFs*	*ORF %*
Dictyoglomus thermophilum H612	201	1912	10.5
Sinorhizobium meliloti BL225C	667	6354	10.5
Azorhizobium caulinodans ORS571	489	4717	10.4
Mesorhizobium australicum WSM2073	600	5792	10.4
Dictyoglomus turgidum DSM6724	180	1744	10.3
Bifidobacterium asteroides PRL2011	171	1658	10.3
Thermotoga neapolitana DSM4359	198	1937	10.2

from the fermentation of carbohydrates. They probably generate ATP as their primary source of energy through substrate level phosphorylation, and therefore ABC transporters are most frequently used to drive nutrient uptake and maintain ion homeostasis. Alphaproteobacteria also encode high numbers of ABC transporter proteins as a proportion of their total coding potential, e.g., *Agrobacterium*, *Ketogulonicigenium* and *Sinorhizobium* spp. The expansion of the ABC transporter family in these α-Proteobacteria may reflect an organismal requirement for high affinity transport since ABC uptake transporters can show higher substrate affinities than most secondary transporters by virtue of their substrate binding proteins.

The high-resolution structures of many intact microbial ABC transporters have now been determined, including uptake systems, for small (sugars, ions, etc.), or larger molecules (vitamins, metal chelates), and export systems (Table 4). The maltose transport complex in *E. coli* was one of the first and best-characterized members of the ABC superfamily and can serve as a general model for ABC sugar and other small molecule importers. In *E. coli*, the maltose transport complex is composed of a periplasmic maltose-binding protein (MalE),

Table 4 Bacterial and archaeal ATP-binding cassette transporters of known structure[a]

Transport protein	*Organism*	*TCDB number*[b]	*PDB number*[c]
Solute uptake systems			
BtuCD(-F) vitamin B12 uptake transporter	*Escherichia coli*	3.A.1.13.1	1L7V; 2QI9; 4FI3
HI1470/1 metal-chelate ABC transporter	*Haemophilus influenzae*	3.A.1.14.11	2NQ2
MalFGK2(−MalE/MBP) maltose uptake transporter	*Escherichia coli*	3.A.1.1.1	2R6G; 3FH6; 3PV0; 3RLF; 3PUX; 3PUV; 3PUW; 4JBW; 4KHZ; 4KI0
MetNI methionine uptake transporter	*Escherichia coli*	3.A.1.24.1	3DHW; 3DHX
HmuUV heme transporter	*Yersinia pestis*	3.A.1.14.5	4G1U
ModB2C2-A molybdate transporter	*Archaeoglobus fulgidus*	3.A.1.6.8	2ONK; 2ONR; 2ONS
ModBC molybdate transporter	*Methanosarcina acetivorans*	3.A.1.8.2	3D31
FbpC ferric uptake—NBD	*Neisseria gonorrhoeae*	3.A.1.10.3	3FVQ
Art(QN) amino acid importer	*Cladanaerobacter tengcongensis*	3.A.1.3.27	4YMS; 4YMT; 4YMU; 4YMV; 4YMW; 4YMX
AlgM1M2SS	*Sphingomonas sp.*	3.A.1.1.10	4TQU; 4TQV
BhuU/BhuV—haem importer	*Burkholderia cenocepacia*	No TCDB ID	5B57; 5B58
Export systems			
Sav1866 Multidrug Exporter	*Staphylococcus aureus*	3.A.1.106.2	2HYD
MsbA Lipid A Flippase	*Salmonella typhimurium*	3.A.1.106.1	3B60; 3B5Y; 3B5Z
	Escherichia coli		3B5W; 5TV4; 6BPL: 5TTP; 6BPP
	Vibrio cholerae		3B5X
Atm1-type ABC exporter	*Novosphingobium aromaticivorans*	3.A.1.210.11	4MRN; 4MRP; 4MRR; 4MRS; 4MRV
Heterodimeric ABC exporter TM287-TM288	*Thermotoga maritima*	3.A.1.135.5	3QF4; 4Q4H; 4Q7K; 4Q7L; 4Q7M
TmrAB antigen transporter homolog	*Thermus thermophilus*	No TCDB ID	5MKK
McjD antimicrobial peptide transporter	*Escherichia coli*	3.A.1.118.1	4PL0; 5OFR; 5OFP
Pcat1	*Ruminiclostridium thermocellum*	3.A.1.112.9	4RY2; 4S0F
PglK lipid linked flippase	*Campylobacter jejuni*	3.A.1.106.15	5C78; 5C76; 5C73
Wzm-WztN—O-antigen transporter	*Aquifex aeolicus*	3.A.1.103.9	6AN7; 6AN5; 6AMX
PrtD Type 1 secretion system	*Aquifex aeolicus*	3.A.1.110.13	5L22
$LptB_2FG$ Lipopolysaccharide transporter complex	*Pseudomonas aeruginosa*	3.A.1.152.1	5X5Y
MacB	*Acinetobacter baumannii*	*3.A.1.122.18	5GKO
MacAB-TolC	*Escherichia coli*	3.A.1.122.1	5NIK
MacB	*Aggregatibacter actinomycetemcomitans*	No TCDB ID	5LIL; 5LJ7; 5LJ8; 5LJ9; 5LJA; 5NAA
MacAB-like efflux pump	*Streptococcus pneumoniae R6*	3.A.1.122.20	5XU1; 5UX0

[a]Adapted from the "Membrane proteins of known 3D structure" database maintained by the laboratory of Stephen White, UC Irvine (http://blanco.biomol.uci.edu/mpstruc/).
[b]Transporter Classification Database number (www.tcdb.org).
[c]Protein Data Bank reference number (http://www.pdb.org). PDB entries link to relevant references.

a transmembrane complex made up of the MalF and MalG proteins, and two copies of the ATPase subunit, MalK. Transport of maltose is initiated by interaction of substrate-bound MalE with the periplasmic loops of MalFG, which induces a conformational change that results in ATP hydrolysis at the MalK subunits and eventually in the translocation of substrates. It has been shown that the ABC family maltose transport systems are widely distributed amongst Gram positive bacteria, Gram negative bacteria and archaea. This may be because maltose derived from starches in decomposing plant materials in the environment or the human gastrointestinal tract is one of the major sources of carbon and energy for heterotrophic bacteria and some archaea. In Gram negative bacteria, maltose uptake requires an additional protein on the outer membrane, LamB (maltoporin), to mediate the diffusion of maltose and maltodextrin into the periplasm. The genes encoding the maltose transporter components are usually clustered into closely linked operons. However, the ATP-binding component is often missing, suggesting a single ATPase could function with several transporters with similar functions. For example, the ATPase, MsiK can function in the uptake of maltose and cellobiose mediated by different ABC transporters.

The periplasmic substrate-binding protein MalE in *E. coli* can bind a variety of α-1,4-linked oligoglucosides, including linear, cyclic, reduced, and oxidized maltodextrins. However, only a fraction of the bound ligands are subsequently transported into the cell. The binding of *E. coli* MalE to substrates that are actively transported results in an endothermic reaction that is entropy driven, while the binding to non-transported substrates is an exothermic reaction. MalE has been crystallized in the presence of maltose or maltodextrin. Similar to other substrate-binding proteins, MalE consists of two symmetrical lobes, with a substrate-binding site positioned in a cleft in between. The MalE protein undergoes conformational changes involving the bending of a hinge that joins the two lobes. There is an open conformation in which the binding site is accessible to the substrate. The binding of substrate initiates a closed conformation in which the two lobes move toward each other and trap the substrate inside the cleft. Binding proteins have two roles in the transport: they are responsible for the high substrate specificity of transport, and they stimulate the ATPase activity. MalG and MalF are membrane-integral subunits with 6 to 8 hydrophobic TMH. They usually are less conserved than the ATP-binding domains. The presence of long inter-helical periplasmic loops in MalG and MalF represents a general feature of ABC transporters.

The nucleotide-binding domain (NBD), MalK, is located on the cytoplasmic side of the membrane. Two MalK proteins form a dimer in the complex and dissociate through ATP hydrolysis. The energetic motion of this process is believed to be communicated to the transmembrane domains to drive the transport reaction in both importers and exporters. The NBDs of ABC transporters are highly conserved. Structural studies have confirmed that a wide variety of NBDs share similar structures and that there is no clear distinction between importers and exporters in NBD structure. NBDs possess Walker A and B motifs that are characteristic of all P-loop ATPases, as well as a C-loop ("LSGGQ") signature motif, a D-loop ("SALD"), a Q-loop ("Q") and a H-loop ("H"). The crystal structure of MalK has been determined as a dimer in both nucleotide-free and ATP-bound forms, making it possible to visualize directly the MalK dimer in different conformation states. The two NBDs of MalK dimerize upon the binding of ATP in a "sandwich" pattern, with ATP molecules bound along the dimer interface, flanked by the Walker A motif of one subunit and the LSGGQ motif of the other subunit. Residues in these motifs are involved in extensive interactions with ATP and are required to form the ATPase active sites. MalK, as well as several other bacterial sugar transporters share a distinct feature: they contain an additional C-terminal domain of about 135 residues called the regulatory domain, which mediates the subunit-subunit interactions and contributes substantially to the dimer interface. The regulatory domain of MalK also interacts with regulatory proteins like enzyme IIA^{Glu}, part of the glucose phosphotransferase system and the transcriptional regulator MalT. A structure of the *E. coli* enzyme IIA^{Glu} in complex with $MalFGK_2$ has been solved (see section on "The Phosphotransfer-driven family" below for further discussion of EIIA proteins). The C-terminal domains of MalK maintain their intersubunit contacts all the time, while the N-terminal NBDs of MalK are in close contact only in the ATP-bound state. The motion of MalK implied by these structures is tweezer-like. The regulatory domains represent the handle that holds the two halves together, and the Q loops are located at the tips of the tweezers where they can move apart or together.

The 3.1 Å crystal structure of a putative archaeal molybdate transporter ($ModB_2C_2$) in complex with its binding protein (ModA) has also been reported (Fig. 3). This structure involved a single ModA subunit with bound substrate attached to the external side of $ModB_2C_2$, which allows unidirectional transport into the cytoplasm. Each ModB subunit contains 6 hydrophobic transmembrane helices, which form a total of 12 transmembrane segments in the transporter. The ModB subunits present an inward-facing conformation; a large cavity is formed by the two subunits facing the cytoplasmic side, which narrows towards the external membrane boundary and is closed by a gate beneath the interface with the binding protein (Fig. 3). This cavity, only accessible from the cytoplasm, is suggested to represent the translocation pathway.

Similar to other NBDs, the ATPase ModC subunits contain the highly conserved P-loops and LSGGQ motifs involved in ATP binding and hydrolysis. They are oriented in a head-to-tail arrangement, with the P-loops of one subunit juxtaposed to the LSGGQ motif of the other. In the nucleotide-free open conformation state, these motifs are separated by a gap. Also, during the open conformation, the attached binding protein ModA aligns the substrate-binding cleft with the entrance to the translocation pathway. Binding of ATP triggers a closed conformation. The observed spacing of the P-loops and the LSGGQ motifs in ModC is consistent with biophysical studies of the MalK transporter in which the solvent accessibility to the fluorescently labeled ATP binding sites is increased in the absence of ATP. The ModC-ModB interface transmits critical conformational changes, which links the ATP binding and hydrolysis to the transport process. Residues around the Q-loop of ModC primarily contribute to this interface.

In addition to these small molecule uptake systems, structures have also been solved for ABC transporters mediating the uptake of larger molecules, such as vitamins and metal chelates (haem), e.g., BtuCD(-F), HmuUV and HI1470/1, as well as for efflux systems (Table 4). In comparison to small molecule uptake systems, transporters involved in the uptake of large molecules display differences within the transmembrane domains, which are typically composed of a great number of TMHs (typically 10 per transmembrane domain).

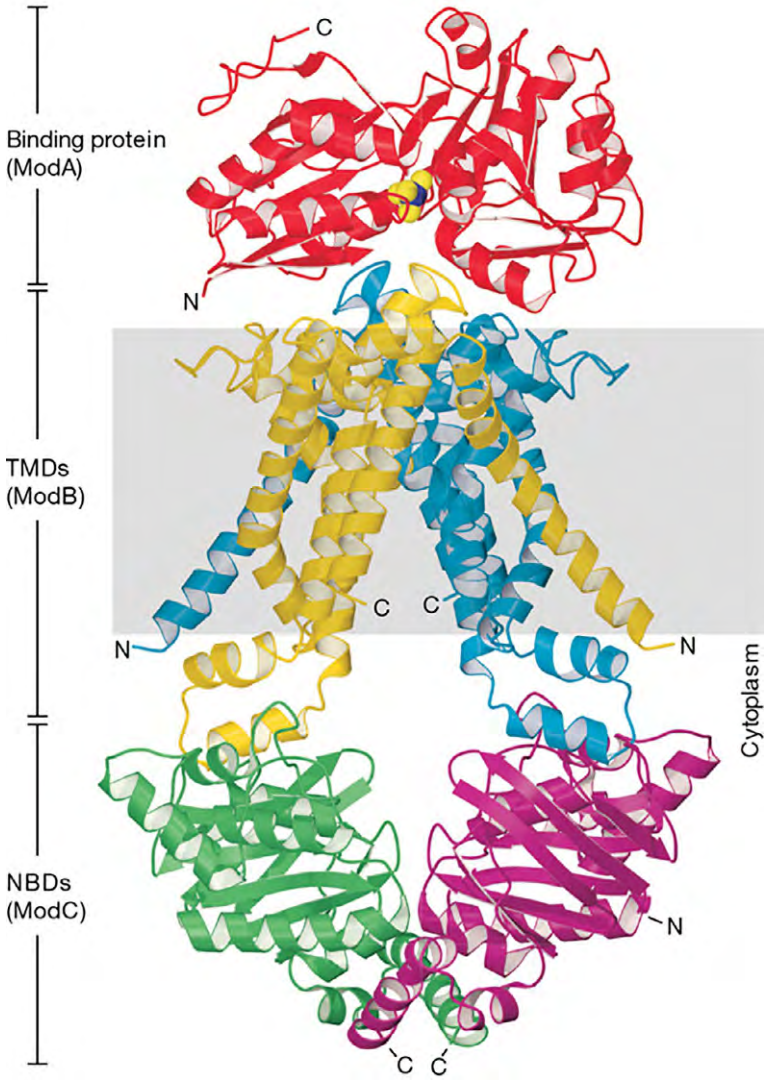

Fig. 3 Front view of the $ModB_2C_2A$ complex in ribbon representation, with the ModB subunits colored *yellow* and *blue*, the ModC subunits colored *green* and *magenta*, the binding protein ModA colored *red*, and with bound tungstate in van der Waals representation (*yellow* and *blue spheres*). The *gray box* depicts the probable location of the lipid bilayer on the basis of the hydrophobicity of the protein surface. N, amino terminus; C, carboxy terminus. Note that there is a vertical two-fold molecular and non-crystallographic symmetry axis for $ModB_2C_2$. Adapted by permission from Macmillan Publishers Ltd., Hollenstein, K., Frei, D. C., and Locher, K. P. (2007). Structure of an ABC transporter in complex with its binding protein. *Nature* **446**(7132), 213–216, copyright ©2007.

ABC efflux systems also display differences in their transmembrane regions compared with small molecule uptake systems. In these transporters the substrate specificity must come from the transmembrane domains themselves, unlike uptake systems, where this is dictated by substrate binding proteins. These exporters often function as multidrug transporters, capable of recognizing a broad range of structurally dissimilar lipophilic substrates. In line with this substrate recognition profile, the transmembrane domains can have been seen to create large central hydrophobic cavities when open inside to promote substrate binding, and hydrophilic cavities when open outwards to promote substrate release in the extrusion state.

Structural comparisons of ABC transporters of all these types share a common mechanism for ATP-driven transport, with binding of ATP promoting an outward-facing conformation (e.g., as in Sav1866) and dissociation of the hydrolysis products promoting an inward-facing conformation (e.g., as in $ModB_2C_2A$). Binding of two ATP molecules at the interface of the NBDs closes the gap between the conserved ATP-binding motifs. As this gap closes, so does the distance between the attached coupling helices, causing the TMDs to flip from the inward-facing to the outward-facing conformation. In uptake systems, this rearrangement may induce the conformation change of the attached binding protein, which force the two lobes to release the bound substrate. The substrate may then diffuse from the binding site through the open gate and into the translocation pathway formed by the transmembrane domain subunits.

Secondary Active Transporters

The major facilitator superfamily

The MFS is an ancient, large, and diverse superfamily of secondary transporters, which use an electrochemical gradient to drive substrate translocation across the membrane. They catalyze uniport in which one type of solute is driven across the membrane by its own substrate gradient, symport in which two or more types of solutes are pumped in the same direction simultaneously utilizing the electrochemical gradient of one of the solutes as the driving force, and antiport in which solutes are transported in opposite directions across the membrane. Most transporters in this family have 400–600 amino acid residues and usually possess either 12 or 14 putative TMH segments. MFS proteins exhibit specificity for a diverse range of substrates, such as sugars, sugar phosphates, polyols, drugs, neurotransmitters, metabolites, amino acids, peptides, osmolytes, iron siderophores, nucleosides, organic and inorganic anions.

The MFS transporters are very widespread across all three domains of living organisms. Like the ABC superfamily, MFS transporters are abundant in prokaryotes with more than 77,000 members identified to date in sequenced prokaryotic genomes, constituting up to 3.5% of ORFs (Table 5). The acidophile *Picrophilus torridus* encodes the highest percent of ORFs as MFS transporters (53, 3.5% of the total ORFs). Several other acidophilic archaea (*Thermoplasma acidophilum*, *Ferroplasma acidarmanus*), or acid-tolerant bacteria (*Lactobacillus fermentum*) were amongst the top five microbes in terms of MFS coding percentage (Table 5). Proton-driven transport mediated by the MFS may be energetically favorable for organisms in acidic environments. Only certain obligate intracellular organisms with highly compact genomes do not encode MFS transporters, for example *Phytoplasma asteris*, *Mesoplasma florum*, *Borrelia afzelii*, *Borrelia garinii*, *Treponema pallidum*, *T. whipplei*, *M. leprae*, and *Nanoarchaeum equitans*.

The tertiary structures of 13 bacterial MFS transport proteins (11 uptake and 2 efflux) have been solved, all of which are of the 12 TMH type (Table 6). The glycerol-3-P:P (GlpT) and the lactose-H^+ (LacY) transport proteins from *E. coli* were the first of these MFS transporters to have their high-resolution three-dimensional structures determined. Despite their functional differences as an antiporter (GlpT) and symporter (LacY) and the differences in their amino acid sequences these structures revealed a similar twofold symmetry in the arrangement of the N-terminal six-helix bundle and the C-terminal six-helix bundle of each protein. Subsequent structures of MFS proteins, such as the fucose-H^+ symporter or the EmrD antiporter from *E. coli* showed a similar overall protein fold, even revealing a further duplication of 3-TMH protein folds within each 6-TMH bundle.

Table 5 Top organisms with highest percent of MFS transport proteins

Organism	*Number of MFS transporters*	*Total ORFs*	*ORF %*
Picrophilus torridus DSM9790	54	1537	3.5
Thermoplasma acidophilum DSM1728	48	1484	3.2
Francisella cf. novicida Fx1	56	1818	3.1
Ferroplasma acidarmanus fer1	60	1951	3.1
Lactobacillus fermentum CECT5716	32	1051	3.0
Francisella tularensis novicida U112	52	1719	3.0
Francisella novicida U112	51	1719	3.0
Francisella tularensis subsp. holarctica LVS	52	1754	3.0
Francisella cf. novicida 3523	54	1854	2.9
Burkholderia sp. YI23	227	7804	2.9
Thermoplasma volcanium GSS1	43	1501	2.9
Francisella sp. TX077308	55	1976	2.8
Raoultella ornithinolytica B6	134	4909	2.7
Francisella philomiragia subsp. philomiragia ATCC25017	52	1915	2.7
Burkholderia cenocepacia HI2424	182	6919	2.6
Burkholderia cenocepacia AU1054	169	6477	2.6
Francisella tularensis subsp. holarctica F92	48	1842	2.6
*Burkholderia sp.*383	200	7716	2.6
*Burkholderia sp.*RPE64	168	6498	2.6
Burkholderia cepacia 383 (R18194)	198	7717	2.6
Rhodococcus sp. RHA1	183	7211	2.5
Francisella tularensis subsp. holarctica FSC200	36	1438	2.5
Francisella tularensis subsp. mediasiatica FSC147	35	1406	2.5
Burkholderia cenocepacia MC03	174	7008	2.5
*Burkholderia sp.*CGE1003	148	5988	2.5
Francisella tularensis subsp. holarctica FTNF00200	39	1581	2.5
Burkholderia multivorans ATCC17616	150	6111	2.5
Burkholderia cepacia AMMD	161	6572	2.4
Klebsiella variicola At22	123	5057	2.4
Burkholderia cenocepacia J2315	173	7116	2.4

Table 6 Bacterial major facilitator superfamily (MFS) transporters of known structure[a]

Transport protein	*Organism*	*TCDB number*[b]	*PDB number*[c]
Solute uptake systems			
XylE xylose:H^+ symporter	*Escherichia coli*	2.A.1.1.3	4GBY; 4GBZ; 4GC0; 4JA3; 4JA4; 4QIQ
LacY lactose:H^+ symporter	*Escherichia coli*	2.A.1.5.1	1PV7; 1PV6; 2CFQ; 2CFP; 2V8N; 2Y5Y; 4OAA; 4ZYR; 5GXB; 6C9W
FucP fucose:H^+ symporter	*Escherichia coli*	2.A.1.7.1	3O7Q; 3O7P
MelB melibiose:Na^+ symporter	*Salmonella typhimurium*	2.A.2.1.1	4M64
GlcP glucose/H^+ symporter	*Staphylococcus epidermidis*	2.A.1.1	4LDS
GlpT glycerol-3-phosphate transporter	*Escherichia coli*	2.A.1.4.3	1PW4
$PepT_{So}$ di/tri-peptide:H^+ symporter	*Shewanella oneidensis*	2.A.17.4.7	2XUT; 4TPH; 4TPG; 4TPJ; 4UVM
$PepT_{St}$ di/tri-peptide:H^+ symporter	*Streptococcus thermophilus*	2.A.17.1.6	4APS; 4XNJ; 4XNI; 5OXL; 5OXK; 5OXM; 5OXN; 6EIA; 5OXQ: 5OXP: 5OXO
POT proton-dependent oligopeptide transporter	*Geobacillus kaustophilus*	2.A.17.1.7	4IKV; 4IKW; 4IKX; 4IKY; 4IKZ
YbgH peptide transporter	*Escherichia coli*	2.A.17.14	4Q65
PepT dipeptide transporter	*Yersinia enterocolitica*	2.A.17	4W6V
PepTXc mammalian-like peptide transporter	*Xanthomonas campestris*	2.A.17	6EI3
$PepT_{Sh}$	*Staphylococcus hominis*		6EXS
NarU nitrate transporter	*Escherichia coli*	2.A.1.8.10	4IU9; 4IU8
NarK nitrate/nitrite exchanger	*Escherichia coli*	2.A.1.8.1	4JR9; 4U4V; 4U4W; 4U4T
Ferroportin Fe^{2+} transporter	*Bdellovibrio bacteriovorus*	2.A.100	5AYN; 5AYO; 5AYM
Export systems			
MdfA multidrug resistance transporter	*Escherichia coli*	2.A.1.2.19	4ZP0; 4ZOW; 4ZP2
EmrD multidrug transporter	*Escherichia coli*	2.A.1.2.9	2GFP
YajR drug efflux transporter	*Escherichia coli*	2.A.1.2.60	3WDO

[a]Adapted from the "Membrane proteins of known 3D structure" database maintained by the laboratory of Stephen White, UC Irvine (http://blanco.biomol.uci.edu/mpstruc/).
[b]Transporter Classification Database number (www.tcdb.org).
[c]Protein Data Bank reference number (http://www.pdb.org). PDB entries link to relevant references.

One group of MFS transporters are particularly important for sugar metabolism throughout all organisms from microbe to man. They are known as the "Sugar Porter" (SP) family, 2.A.1.1 in the TCDB and recognize substrates like glucose, galactose, xylose, arabinose, inositols. The structures of several of these proteins have been resolved, including several from bacteria, such as a xylose-H^+ symporter (XylE) from *E. coli*, and a glucose-H^+ symporter (GlcP) from *Staphylococcus epidermidis*. As expected from the similarities in their amino sequences, their structures are very similar to each other, with an overall fold of the 12 TMH like that of GlpT and LacY and other MFS proteins, but with an additional 4-helix bundle in the cytoplasm formed from highly conserved motifs in their sequences. Most importantly, for XylE more than one form of the protein opening towards the periplasm or towards the cytoplasm has been described. Also, the position of the sugar binding site was established towards the center of the protein. A sequence of events can be deduced (Fig. 4) in which the protein, initially oriented towards the outside, closes around the ligand to yield a form occluded to both sides that then opens to the inside.

Despite the relatively low resolution of the XylE structures the changes in conformation accomplishing the alternating access transport of the substrate from outside to inside (or its reverse) can be visualized as mainly due to a rigid body movement of each of the N-terminal and C-terminal sub domains with respect to each other rather than differences within each subdomain (Fig. 4). Specifically, the subdomains undergo a tilting motion around an axis of symmetry roughly perpendicular to the plane of the membrane, resulting in tighter interactions at the periplasmic side and a wide opening appearing at the cytoplasmic side. In addition, in the outward-facing XylE structure, a helix bundle, containing motifs that are highly conserved in the SP family on the inside face of the proteins, interacts with the TMHs mainly via salt bridges, closing the exit of the substrate to the inside of the cell; in the inward-facing forms of XylE, these interactions are seen to be broken highlighting the important contribution of the motifs to mechanism.

Based on the structures of XylE taken with both the structures of the closely related glucose transporters and those of the less related transporters like GlpT, LacY, FucP and EmrD and a large body of biochemical and biophysical evidence, a mechanism is proposed for MFS proteins in which the transporter operates via a single binding site, alternating access model sometimes referred to as a "rocker-switch" mechanism. The substrate-binding site is accessible from only one side of the membrane at a time. Initially, the substrate-binding site is accessible from the extracellular space in the outward-facing conformation. The binding of substrate to the binding site induces a conformational change that exposes the substrate-binding site to the cytoplasm in the inward-facing conformation for release. It is reasonable to think that other MFS transporters maintain a similar basic architecture and substitute key residues in the substrate-binding site to change the specificity of the transporter.

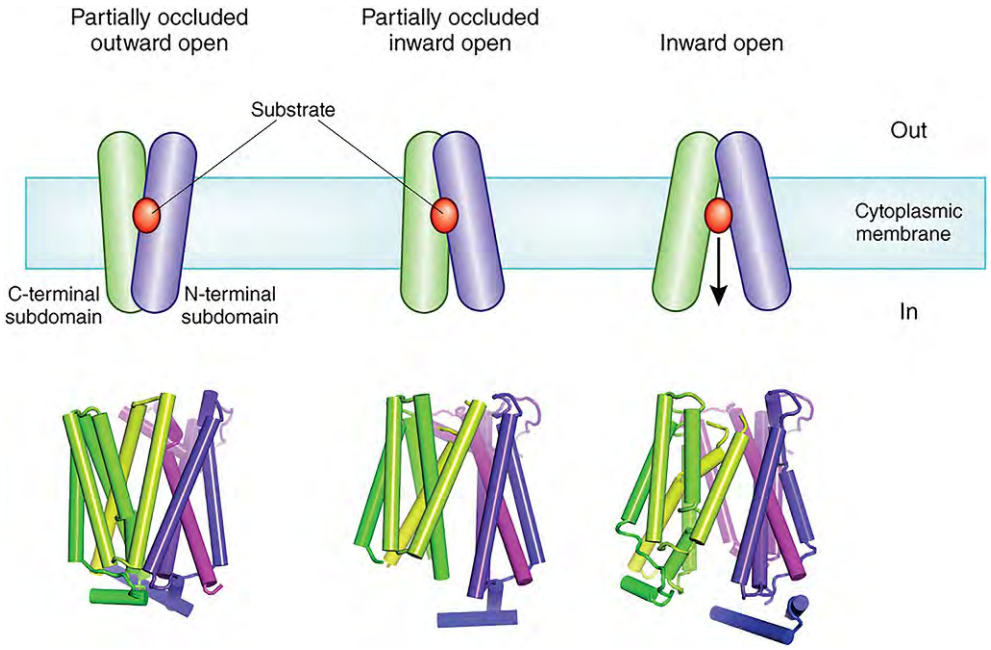

Fig. 4 Structures of the XylE D-xylose–H^+ transport protein of *Escherichia coli*. The occluded forms would contain bound substrates, whereas the inward-facing open conformation would release substrates. The structures in the bottom correspond to PDB 4GBY (partially occluded outward open), 4JA3 (partially occluded inward open) and 4JA4 (inward open); models generated by M. Breidenbach. Figure and caption taken from Henderson, P. J. F. and Baldwin, S. A. (2013). This is about the in and the out. *Nature Structural & Molecular Biology* **20**, 654–655.

The 5-Helix Inverted Repeat Superfamily of Membrane Transport Proteins

An important development since the last couple of editions of this article is the realization that the separate families of transporters listed in Table 7 actually comprise a highly diversified superfamily of secondary transporters that, like the MFS, use an electrochemical gradient to drive translocation of a very wide range of substrates across the membrane by both symport and antiport, often with cations, commonly Na^+ or H^+, and sometimes anions such as Cl^- and SO_4^-. They are variously known as the LeuT superfamily, the amino acid-polyamine-organocation (APC) superfamily, or the 5-Helix Inverted Repeat Transporter (5HIRT) superfamily. We prefer the last because it is based on what is likely to be a generic structural feature in all members (see below).

The 5HIRT transporters are found across all three domains of living organisms. They are abundant in prokaryotes, with more than 50,000 members listed in TransportDB, including more than 16,000 and 14,000 proteins contributed by the Amino Acid-Polyamine-Organocation (APC) family and the solute:sodium symporter (SSS) family, respectively. A Na^+-leucine transporter, LeuT, from *Aquifex aeolicus* was selected for detailed characterization and structural studies, because it is an easily overexpressed and crystallized homologue of human neurotransmitter transporters. Similarly, a bacterial homologue of human Na^+-glucose transporters, vSGLT, from *Vibrio parahaemolyticus*, and bacterial homologues of human amino acid transporters from *Escherichia coli* and *Methanocaldococcus jannaschii*, were characterized for structural studies. Additional structures have been determined for the sodium-hydantoin transporter, Mhp1, of the nucleobase cation symport-1 (NCS1) family from *Microbacterium liquefaciens* and for the sodium-betaine symporter, BetP, from *E. coli* of the betaine/choline/carnitine transporter (BCCT) family (Table 7). Despite profound differences in their primary sequence, proteins from different families within the 5HIRT superfamily display similar 3D structures. Furthermore, according to the TCDB each of the families has some members with sufficiently similar aligned sequences of amino acids between different families for them all to have a common evolutionary ancestor. Taken together with similarities in their hydropathic profiles all the proteins of all the families are conceived to be members of the 5HIRT Superfamily.

Like members of the MFS most of the 5HIRT proteins are predicted to have 12 TMH. However, unlike the arrangement of two opposing similarly-folded six-helix bundles in the MFS with an axis of pseudo symmetry approximately perpendicular to the plane of the membrane 10 TMHs in the LeuT-like proteins are arranged in an *inverted* repeat fold in which the first 5 TMHs are topologically equivalent to the second 5 and are related to one another by a pseudo twofold axis that runs roughly parallel to the plane of the membrane. The two halves of the protein are completely intertwined with both contributing to the ligand binding site, which is situated buried in the membrane approximately at the centers of the respective proteins. Both MFS and 5HIRT types of protein achieve an alternating access mechanism of transport, but by very different conformational changes in structure. In contrast to the MFS rocker-switch mechanism, transport in 5HIRT proteins occurs via a "rocking-bundle" motion.

Insight into the latter mechanism was gained from the first protein for which the structure was determined in three conformational states, open-outwards, occluded with bound ligand, and open-inwards, the sodium-benzylhydantoin transporter, Mhp1,

Table 7 Transport protein families now thought to comprise the 5-helix inverted repeat superfamily of membrane transport (5HIRT) proteins

Family	*3D Structure (PDB numbers)*[a]	*TCDB number*[b]
Amino acid-polyamine-organocation (APC)	– *Escherichia coli* AdiC arginine:agmatine antiporter, (3LRB, 3L1L, 3OB6, 5J4N) – *Salmonella enterica* AdiC arginine:agmatine antiporter (3NCY) – *Methanocaldococcus jannaschii* ApcT Na^+-independent amino acid transporter (3GIA, 3GI9, 3GI8) – *Escherichia coli* GadC glutamate-GABA antiporter (4DJK, 4DJI)	2.A.3[c]
Betaine/carnitine/choline transporter (BCCT)	– *Corynebacterium glutamicum* BetP glycine betaine transporter (2WIT, 4DOJ, 4AIN, 4C7R) – *Escherichia coli* CaiT carnitine transporter (3HFX, 2WSX) – *Proteus mirabilis* CaiT carnitine transporter (2WSW, 4M8J)	2.A.15[c]
Amino acid/auxin permease (AAAP) family	No structures yet determined?	2.A.18
The solute:sodium symporter (SSS)	– *Vibrio parahaemolyticus* vSGLT Sodium Galactose Transporter (3DH4, 2XQ2) – *Proteus mirabilis* SiaT sialic acid transporter (5NV9, 5NVA)	2.A.21[c]
Neurotransmitter:sodium symporter (NSS)	– *Aquifex aeolicus* LeuT leucine transporter (2A65, 2QEI, 2Q6H, 2Q72, 2QB4, 3F3A, 3F48, 3F3E, 3F3D, 3F4I, 3F3C, 2QJU, 3GJD, 3GJC, 3MPN, 3MPQ, 3QS4, 3QS5, 3USM3QS6, 3TT1, 3TT3, 3TU0, 3USG, 3USI, 3USJ, 3USK, 3USL, 3USO, 3USP, 5JAE, 5JAF, 5JAG)	2.A.22[c]
Alanine or glycine:cation symporter (AGCS)		2.A.25
Branched chain amino acid:cation symporter (LIVCS)		2.A.26
Cation-chloride cotransporter (CCC)		2.A.30
Anion exchanger (AE)		2.A.31
Nucleobase:cation symporter-1 (NCS1)	– *Microbacterium liquefaciens* Mhp1 benzylhydantoin transporter (2JLN, 2JLO, 2X79)	2.A.39[c]
Nucleobase:cation symporter-2 (NCS2)	– *Escherichia coli* UraA uracil/H^+ symporter (3QE7)	2.A.40
Hydroxy/aromatic amino acid permease (HAAAP)		2.A.42
Benzoate:H^+ symporter (BenE)		2.A.46
Sulfate permease (SulP)		2.A.53
Metal ion (Mn^{2+}-iron) transporter (Nramp)	– *Staphylococcus capitis* transition metal ion transporter (4WGV, 4WGW) – *Deinococcus radiodurans* (5KTE)	2.A.55
K^+ uptake permease (KUP)		2.A.72
Putative peptide transporter carbon starvation CstA (CstA)		2.A.114
Putative amino acid permease (PAAP)		2.A.120

[a]Structures listed at "Membrane proteins of known 3D structure" database maintained by the laboratory of Stephen White, UC Irvine. Protein Data Bank reference numbers are given in parentheses (http://www.pdb.org). PDB entries link to relevant references.
[b]Transporter Classification Database number (www.tcdb.org).
[c]Structural homologues of LeuT (from Cameron, Beckstein and Henderson).

from *Microbacterium liquefaciens* (Fig. 5). A mechanism was derived for switching from the outward-facing open conformation through an occluded structure to the inward-facing open state. In this a bundle of the four helices 3, 4, 8, 9 known as the hash motif moves as a relatively rigid body with respect to a bundle of helices 1, 2, 6, 7 to provide the major element, the thick gate, of the switch from open-outwards to open-inwards (Fig. 5). Additionally a reciprocating movement of helices 10 and 5 closes an external thin gate and opens an internal thin gate (Fig. 5). A similar mechanism of the switching between outward- and inward-facing conformations caused by a rigid-body movement of the four-helix bundle relative to the rest of the protein was, in fact, first proposed for LeuT based on the asymmetry of the crystal structure and investigated more thoroughly using a mutational analysis of the serotonin transporter.

Can the fundamentals of the mechanism proposed for Mhp1 be extended to all the members of the 5HIRT (5 helix inverted repeat transporter) superfamily? This question is to some extent addressed by the recent structures of the LeuT transporter, which show outward, occluded and inward facing conformations of this protein. Like the mechanism above for Mhp1, the major change between outward and inward facing conformations is a rotation of the bundle relative to the hash motif. However, the details of the conformational changes are rather different between the two proteins. In LeuT there is no independent movement of TM5 or TM10 and instead the bundle helices are more mobile with TMs 1 and 6 flexing upon ligand binding and TM1 bending considerably as the protein changes from outward to inward facing. Similarly, by comparing the outward-facing AdiC with the inward-facing ApcT from the APC family or the occluded BetP with the inward-facing CaiT from the BCCT family, the trend of the hash motif moving relative to the bundle can be observed but the mechanism of occlusion is different. For example, in AdiC, which has been solved in the outward-facing and occluded states TM10, TM2 and TM6 move upon binding the ligand. Thus the mechanism for Mhp1 is to some extent

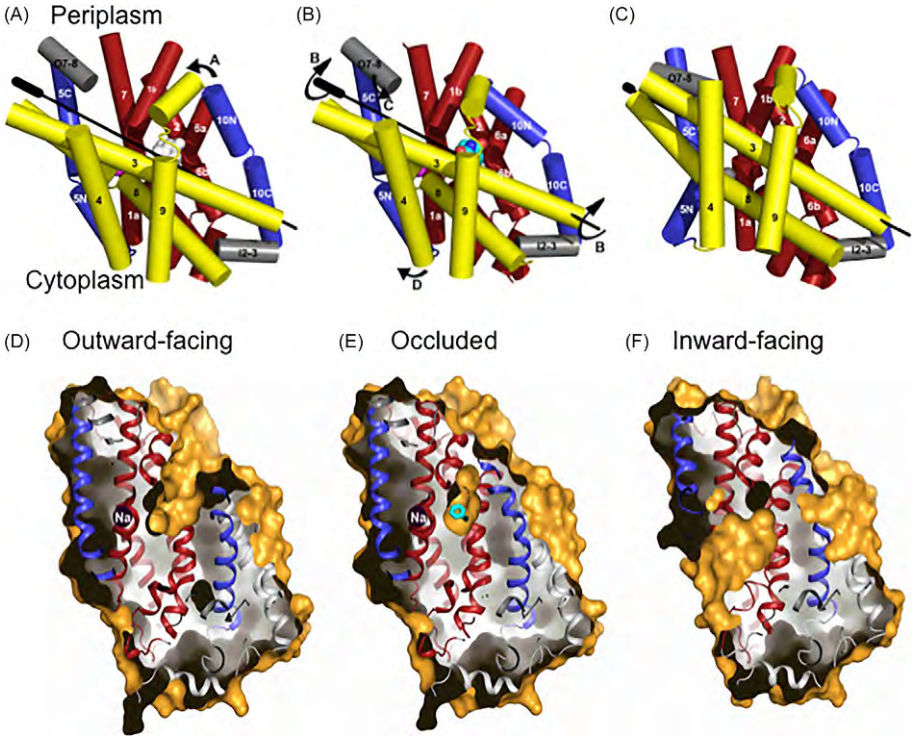

Fig. 5 The mechanism of Mhp1 taken from Shimamura et al. (2010). A, B, C The movements are delineated by *arrows*. (A) Helix 10 bends over the substrate. (B) The hash motif rotates by 30° around the rotation axis shown as a *black line*. (C) The small extracellular helix moves to seal completely the extracellular side of the protein. (D) Helix 5 bends to open the cavity on the intracellular side in a reciprocal manner to helix 10. (D–F) Surface representations of the three forms of Mhp1, showing the cavities in each. Figure taken from the supplementary material of Shimamura, T., Weyand, S., Beckstein, O., Rutherford, N. G., Hadden, J. M., Sharples, D., Sansom, M. S. P., Iwata, S., Henderson, P. J., and Cameron, A. D. (2010). Molecular basis of alternating access membrane transport by the sodium-hydantoin transporter Mhp1. *Science* **328**, 470–473.

observed in other members of the superfamily but the details vary, presumably reflecting the different substrates as well as the architecture of the non-core helices. Parallel studies of these proteins involving higher-resolution structures and mutational analysis combined with kinetic measurements are required if we are really to understand the general principles of the mechanism in this superfamily.

The Elevator Mechanism of Transport

The rocker-switch mechanism of transport, seen in MFS proteins, and the rocking bundle mechanism of transport, seen in 5HIRT proteins, such as Mhp1, are variations of the alternating access transport mechanism. In both mechanisms a substrate binding site that lies approximately mid-way through the membrane, is made alternately accessible to both sides of the membrane due to conformational changes that occur between protein domains to change the location of molecular gates around the substrate binding site. In the rocker-switch mechanism the domains surrounding the binding site are typically symmetrical, whereas the domains are usually asymmetrical in proteins operating via the rocking-bundle mechanism. Furthermore, the gates preventing access to the substrate binding site from one side of the membrane or the other are generally more complex in proteins operating via the rocking-bundle mechanism.

A third and distinctly different alternating access mechanism has been suggested to occur in a large number of transport proteins to have been structurally characterized over the past few years. This mechanism is referred to as the "elevator" mechanism of transport. In these proteins, the substrate binding site lies within one protein domain, which moves the substrate to the opposite side of the membrane past a centrally located molecular gate, formed against a second domain that is relatively immobile during the translocation cycle. The sodium-aspartate symporter from *Pyrococcus horikoshii*, Glt_{Ph}, was the first protein proposed to function via the elevator mechanism, and is supported by structural, biophysical and biochemical evidence. Remarkably, the substrate binding site in Glt_{Ph} appears to move approximately 18 Å across the membrane, relative to the immobile domain, during the translocation cycle.

Group Translocators

The Phosphotransfer-Driven Family

The phosphoenolpyruvate: sugar phosphotransferase systems (PTS) are complex enzyme systems functioning in the detection, transport and phosphorylation of various sugar substrates, including monosaccharides, disaccharides, amino sugars, polyols, and

other sugar derivatives. The phosphorylation of sugars in parallel with their uptake serves both to prevent their efflux and to prepare them for metabolism. The PTS family transporters use phosphoenolpyruvate (PEP) as the energy source and phosphoryl donor to carry out their catalytic function in sugar transport and phosphorylation. The basic components of the PTS (Fig. 6) are similar in all species studied. It is comprised of two general cytoplasmic components, enzyme I (EI) and phosphoryl carrier protein (HPr), which are common to all PTS carbohydrate transporters. The multi-domain enzyme II (EII) complex is sugar specific. Eubacteria usually encode many different EIIs. Each EII complex consists of one or two hydrophobic integral membrane domains (domains C and D) and two hydrophilic domains (domains A and B). The three or four domains together are responsible for the transport of the carbohydrate across the bacterial membrane as well as its phosphorylation. EII complexes can be formed either by distinct proteins or by a single multidomain protein. Likewise, fusion proteins that contain EI and/or HPr domains exist. A prominent example of the latter is FPr, which consists of HPr and an EIIA domain and mediates phosphoryl transfer during the uptake of fructose by *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Rhodobacter capsulatus*. The PTS transporters are found exclusively in Gram positive and Gram negative eubacteria, and are absent in archaea and eukaryotes. *Coriobacterium glomerans*, *Sebaldella termitidis* and *Lactobacillus casei* exhibit the highest number of PTS systems amongst all the sequenced prokaryotic genomes; each have about 50–60 EII complexes. About 15 different EII complexes are encoded in *E. coli* and *B. subtilis*. The properties of these enzymes have been established by various genetic, biochemical, and physiological studies. Paralogs of EI and HPr have also been identified in some species. For example, five paralogs of each general PTS protein were discovered in *E. coli*.

In a typical phosphoryl transfer reaction (Fig. 6), EI is phosphorylated using a phosphoryl group from PEP first, which initiates a chain of reactions. Phosphohistidyl-EI then phosphorylates a histidyl residue in HPr. This phosphorylation then transfers its phosphoryl group to a histidyl residue in EIIA of the sugar-specific EII complex. The phosphoryl group is then sequentially transferred to EIIB, and is finally transferred to the transported sugar bound to the membrane components, EIIC and/or EIID. All the phosphoryl derivatives in this multi-step phosphoryl transfer system exhibit similar energy. The phosphorylation occurs at either histidyl or cysteyl residues.

E. coli EI is a 63 kDa protein which contains about 570 residues, and is encoded by the *ptsI* gene. Sequence comparisons reveal significant similarities amongst EIs from various Gram positive and Gram negative eubacteria. EI is autophosphorylated in the presence of Mg^{2+} at the N-3 position of the imidazole ring of conserved histidine residue on the N-terminus of the protein, which also contains the binding site for HPr. The C-terminus of EI contains the PEP binding site and is required for dimerization. The structure of a full-length EI of *Staphylococcus carnosus* reveals that the N-terminal phosphohistidine and HPr-binding domain are

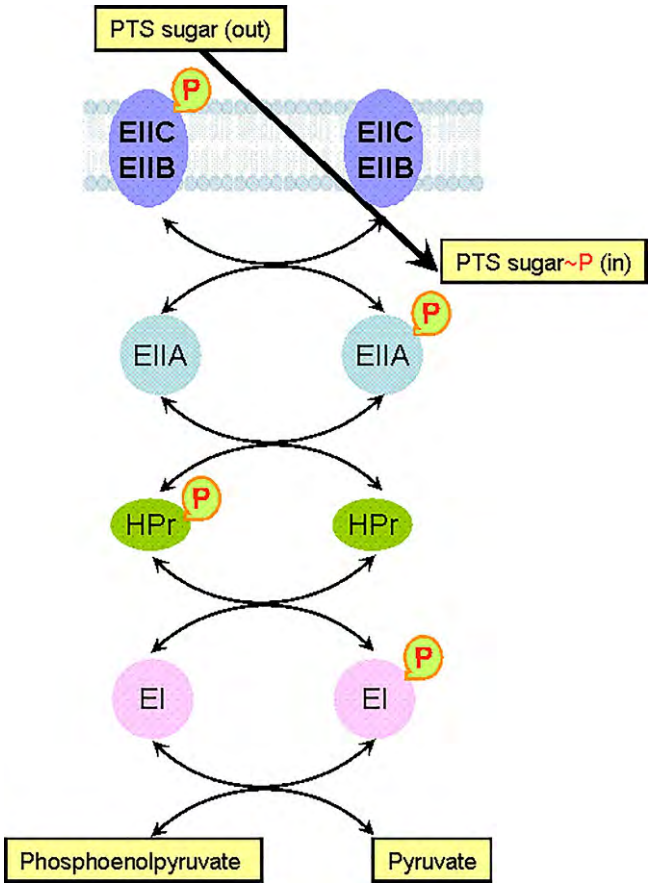

Fig. 6 General phosphoryl and sugar transport reaction catalyzed by the PTS. Sugars are transported and concomitantly phosphorylated by the PTS. See text for details.

clearly separated from the C-terminal dimerization and PEP binding domain. EI forms a homodimer that accepts the phosphoryl group from PEP. The inactive monomeric EI exhibits a relatively high structural variability. The dimerization and the binding of Mg^{2+} and PEP induce conformational changes that bring the C-terminal domain with the two bound ligands close to the active site, which is necessary to facilitate the phosphotransfer.

HPr is a small, heat-stable protein (about 90 residues, 9–10 kDa). It is encoded by the *ptsH* gene. HPr is phosphorylated at the N-1 position of the imidazole ring of a histidyl residue (His-15) by PEP and EI. In most low-GC Gram positive eubacteria and a few Gram negative organisms, HPr can also be phosphorylated by a regulatory ATP-dependent Hpr(Ser) kinase on a seryl residue (Ser-46). This is not part of the phosphoryl transfer to carbohydrates, but phosphorylation of the seryl residue slows the phosphoryl transfer from EI to HPr at least 100-fold. *E. coli* HPr contains a Ser-46 residue but lacks an HPr(Ser) kinase. However, replacement of Ser-46 by an aspartyl residue significantly lowers the affinity of EI for HPr. The three-dimensional structure of HPrs from *E. coli*, *B. subtilis* and several other species are known. It forms an open-faced β sandwich consisting of four antiparallel β sheets that is covered at one side by one short and two long α-helices. The active histidyl residue (His-15) is located in the N-terminal part of the first long α-helix and is exposed to the solvent. The β sheet curls back on itself so that the regulatory seryl residue (Ser-46) is close to the active histidyl residue. Thus, the presence of the negatively charged phosphoryl group at Ser-46 may inhibit the phosphorylation at the active site by electrostatic repulsion or by inhibition of EI binding.

The carbohydrate specificity of the PTS is dependent on the EII complexes, which consist of integral membrane domains/proteins (EIIC/EIID) and cytoplasmic domains/proteins (EIIA/EIIB) (Fig. 6). The PTSs were classified into four superfamilies with distinct evolutionary origins on the basis of the phylogenies of the EII complexes: (i) the glucose-fructose-lactose superfamily, comprised of the glucose/glucoside family (TC 4.A.1), the fructose/mannitol family (TC 4.A.2), the lactose/diacetylchitobiose-β-glucoside family (TC 4.A.3), and the glucitol family (TC 4.A.4); (ii) the mannose/fructose/sorbose family (TC 4.A.6); (iii) the ascorbate-galactitol superfamily, comprised of the L-ascorbate family (TC 4.A.7) and the galactitol family (TC 4.A.5); and (iv) the dihydroxyacetone family. The sequence-based classification of the various PTSs is supported by X-ray crystallography and nuclear magnetic resonance (NMR) studies, which clearly show that the structures of the EIIA and EIIB domains/proteins belonging to the various classes are quite different. A representative structure for the EIIC membrane bound component has been solved from the *Bacillus cereus* diacetylchitobiose PTS permease (TCDB: 4.A.3.2.8; PDB 3QNQ). The structure shows a homodimer with one molecule of diacetylchitobiose bound in each protomer. Each protomer contains 10 TM helices and the protomer interface is comprised of the N-terminal regions and the sugar binding sites are formed by the C-terminal domains.

PTS also carries out numerous regulatory functions. The four proteins/domains (EI, HPr, EIIA, and EIIB) form a PTS phosphorylation cascade (Fig. 6) that can phosphorylate or interact with numerous non-PTS proteins and thereby regulate their activity. Indeed a structure of the ABC superfamily maltose uptake transporter $MalFGK_2$ has been solved in complex with a glucose specific EIIA protein (Table 4; PDB 4JBW). In this structure two EIIA proteins are bound to the MalK nucleotide binding domains and stabilize the inward-facing conformation of the transporter by preventing structural rearrangements required for ATP hydrolysis. The PTS regulation network not only controls carbohydrate uptake and metabolism but also interferes with the utilization of nitrogen and phosphorus and the virulence of certain pathogens. In *E. coli*, nitrogen enzyme I (EI^{Ntr}), nitrogen HPr (NPr), and nitrogen IIA protein (IIA^{Ntr}), paralogues of EI, HPr, and $EIIA^{Fru}$ respectively, constitute a phosphoryl transfer chain that has been shown to exhibit little enzymatic cross-reactivity with the classical sugar-transporting phosphoryl transfer chain consisting of EI, HPr, and various sugar-specific enzyme II complexes. This nitrogen-related phosphoryl transfer chain presumably functions only in regulation. EI^{Ntr} homologues have been shown to cluster phylogenetically together, and distant from all other enzyme I homologues. EI^{Ntr} may serve a sensory function linking carbon and nitrogen metabolism. A mutation of the EI^{Ntr} gene resulted in impaired metabolism of poly-β-hydroxybutyrate, and diminished respiratory protection of nitrogenase under carbon-limiting conditions. In addition to EI^{Ntr} and NPr, *E. coli* encodes within its genome three additional EI paralogues and four additional HPr paralogues. The functions of most of these proteins are still unknown. A non-transporting enzyme II complex has been characterized in *E. coli*. It phosphorylates dihydroxyacetone (DHA) at the expense of PEP, using three soluble DHA-specific proteins (DhaK, DhaL, and DhaM), in addition to EI and HPr. The DhaM protein consists of three domains: an N-terminal IIA^{Dha} domain that is distantly related to IIA^{Man}, a central HPr domain, and a C-terminally truncated EI domain. All three domains can be phosphorylated by PEP with the EI and HPr as phosphoryl donors.

Genomic Analyses of Transporter Distribution and Function

Ongoing advancements in genome sequencing make it possible to conduct comparative analyses of essential cellular processes like transport in organisms across the three domains of life. Tens of thousands of prokaryotic genomes have been sequenced and deposited in the public databases. These genomes cover a broad range of microbial organisms from different phylogenetic groupings, allowing comparative genomic analyses across a diverse range of organisms and lifestyles. Genomic analyses of transport potential in a broad range of organisms has shown that both phylogenetic and environmental factors appear to influence transporter distribution patterns and databases such as TransportDB make this data accessible to all researchers. For example, queries made to TransportDB can identify groups of organisms that contain similar transporter profiles. These types of analyses have shown that organisms with a similar physiology or living environment, such as obligate intracellular organisms, plant/soil-associated organisms or autotrophs display similar transporter profiles. Each of these groups exhibit distinct patterns of transporter family distribution. The obligate intracellular organisms possessed the fewest types and number of transporters while plant/soil-associated organisms generally encode the largest variety and number of transporters of microorganisms examined. The cluster of

autotrophic organisms generally lack transporters for carbohydrate and organic nutrients while possessing a range of transporters for inorganic ions and molecules.

Beyond genome sequencing, functional genomic analyses, such as transcriptomics and proteomics, can be used to determine the relevance of different transport systems to physiological adaptations. This is based on the premise that transporters are generally held under tight regulatory control and are only expressed when required by the cell. These approaches can be particularly useful when the physiological carrier of a particular substrate is unknown, or when substrate(s) for given transporters, such us multidrug efflux systems, which are seemingly redundant in most microbes, are unknown. Additionally, in recent work transcriptomic analyses, coupled with biochemical and biophysical studies were used to identify a new family of drug efflux systems found mainly in proteobacteria (TCDB: 2.A.117.1: the (mainly) proteobacterial chlorhexidine efflux (PCE) family). An alternative approach for high-throughput functional characterization of transporters is via phenotypic profiling of libraries of mutant strains. This approach has been used to survey the substrates of putative uptake systems in mutant libraries of *Pseudomonas aeruginosa* and resulted in the identification of a novel histamine uptake system in this organism. Furthermore, high-throughput transposon mutant library screening approaches based on illumina sequencing, such as Transposon-directed insertion site sequencing (TraDIS), have allowed the identification simple genes mediating phenotypes of interest. Recent coupling of these approaches with flow sorting has led to systems that can identify transport proteins specifically involved in the transport of fluorescent substrates of interest.

Conclusions

The era of structural biology and genomics has opened new horizons in our understanding of complex biological questions. Three-dimensional transporter structures have provided us invaluable information on the mechanisms of transporter function. These structures have highlighted the alternating access model as being key to the mechanism of many diverse active transport protein families and the structures of these proteins in different conformational states have provided graphic detail of the intricacies of this process for several families. Comparative genomic approaches for the analysis of membrane transport systems have stimulated insights into how microbes adapt to their environment. The observations that organisms with similar lifestyles and/or ecological niches display similar transporter profiles despite their phylogenetic differences strongly suggest the influence of living environment on the complement of membrane transport genes in an organism. High-throughput functional analyses and functional genomic studies are being applied to address important gaps in our knowledge of transporter function and to identify novel substrates for the many putative transport systems identified in microbial genomes.

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Microbiology of Fermented Dairy Products

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Introduction

The dairy industry is one of the most relevant in the food sector. It is characterized by a high diversity of products, obtained by applying different technologies. Especially cheeses can be considered the result of the meeting between tradition and innovation, since artisanal procedures have been "industrialized" to meet the global consumer demand.

Undoubtedly, microorganisms have an essential role in the dairy sector, especially for products, for which it is necessary to promote an acidification step in the milk, such as yoghurts and cheeses.

Microbial ecology in dairy products is very diverse and depends from the raw materials (e.g., raw or pasteurized milk), the processing technology (e.g., curd cooking, stay of the curd in the whey and pressing of the loaf) and the type of product (e.g., fresh, semi-ripened or ripened cheeses). However, several groups of microorganisms can be generally found in most dairy products, belonging to lactic acid bacteria (LAB), yeasts and filamentous fungi. LAB are responsible for the conversion of lactose in lactic acid, resulting in a reduction of milk pH, and they also produce hydrolytic enzymes, active on the protein fraction of the milk, able to release precursors of aroma compounds. Yeasts and moulds are important for their ability to produce proteolytic and lipolytic enzymes, which degrade milk proteins and lipids producing important compounds contributing to the final sensory characteristics.

There is a large body of literature that has described the microbial populations relevant in the dairy sector and there is a general consensus, in which LAB are divided in starter LAB (SLAB) and non-starter LAB (NSLAB). While the first group is responsible for the initial acidification of the milk and for the production of proteolytic enzymes, NSLAB possess specific metabolisms able to produce secondary metabolic compounds, which enrich the organoleptic profile of dairy products, especially yoghurt, butter and cheese. Yeasts and moulds are not involved in the acidification and they usually participate in the aroma formation through their enzymatic production. In the case of the molded cheeses, filamentous fungi are also responsible for the typical aspect and pigmentation of the cheeses.

In this article we will describe these categories of microorganisms, taking into account their role and ecology in the dairy products. Moreover, we will also discuss the advancements in the understanding of the microbial diversity by using molecular biology methods, which often have been applied without prior cultivation, thereby avoiding the biases of the traditional microbiological methods.

Starter Lactic Acid Bacteria

Over the centuries, cheese makers have domesticated fermentation processes empirically, without a deep understanding of the multiple biochemical reactions governing the sensory qualities of their cheeses. Nowadays, the role of microbiota in terms of metabolic activities has been mostly understood and many dairy productions are piloted and optimized by using starter cultures to standardize the fermentation process and reach the targeted properties for the final products.

Natural starter cultures (NSC) are produced daily, at cheese plant, by some form of backslopping and/or by application of environment selective pressure. Because no actions are used to prevent contamination from cheese making environment and raw milk, NSC are continuously changing, undefined mixtures of several species and strains of LAB (Parente and Cogan, 2004). Biodiversity is considered a strength point in NSC and producers of traditional cheeses, as Grana Padano and Parmigiano Reggiano, accept slight variability in their products as compromise with more peculiar and individual characteristics coming from their ability to manage wild populations in raw milk and whey starters (Gatti et al., 2014).

The concept of selected starter culture (SSC) is well established in the dairy sector, referring to homofermenting lactic acid strains, which produce lactic acid giving rise, with added rennet to curd formation. The *meso*philic lactococci, mainly *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, generally make up around 90% of a mixed dairy starter population while thermophilic, mainly *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus*, are employed in cooked curds because of their heat tolerance. Mesophilic cultures are used in the production of, for example, Cheddar, Gouda, Blue, Camembert, Edam, while thermophilic cultures are used for high temperature cooked hard cheese such as Emmental, Gruyere, Grana Padano and Parmigiano Reggiano (Beresford et al., 2001).

As reported above, the homofermentative *L. lactis* is the species mostly employed as *meso*philic starter. The primary function of this microorganism, when inoculated into vat milk, is to produce acid, reducing the pH of milk to <5.3 in 6 h at 30–37°C. Moreover, *L. lactis* starter strains can be chosen also for their ability to metabolize citrate, during fermentation process, resulting in flavor production.

L. lactis is also involved in cheese ripening where its enzymes are involved in proteolysis and conversion of amino acids into flavor compounds (Beresford et al., 2001). The production of biopeptides by cell envelop-proteinases (CEP) has been studied in

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L. lactis and showed high strain-dependent variability resulting in the production of peptides associated to different aroma (bitter, sweet, acid etc.) (Thierry et al., 2015). *L. lactis* contributes to cheese ripening either as intact cells, in early ripening, by means of its proteolytic enzymatic apparatus, and as autolyzed cells, during late ripening. On the basis of recent insights it has been hypothesized a role of *L. lactis* in VNC state, in cheese ripening, (Ruggirello et al., 2016).

In the last years, additional characteristics have been taken into account for the selection of new *L. lactis* strains to be added to starter cultures. Bioprotective properties have been studied by different authors. Renes et al. (2014) showed that the use of selected autochthonous *L. lactis* cultures reduced the content of biogenic amines in ewe's milk cheese, while Dal Bello et al. (2012) studied the ability of bacteriocin producing *L. lactis* strains to limit *Listeria monocytogenes* growth in Cottage cheese manufacturing.

Thermophilic starter strains, mainly belonging to *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* species are chosen according to their ability to acidify milk in the range of 50–55°C. Likewise, other properties related to texture, flavor, health have been taken into account, for their selection, in the last years.

Fuglsang et al. (2003) studied the production of bioactive peptides in various LAB, and found that *Lb. helveticus* strains, *in vivo* conditions, were able to produce antihypertensive peptides in sufficient amount to efficiently decrease blood pressure.

Stretchabilty has been also widely studied for its importance in the production of cheeses like Mozzarella and Emmental. This property was correlated, in *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus*, to a low level of proteolysis accompanied by a low release of intracellular peptidases into the cheese aqueous phase (Sadat-Mekmene et al., 2013). Richoux et al. (2009) showed that qualitative more than quantitative aspects of proteolysis were determinants for the development of stretchability.

Finally, some authors investigated the ability of *S. thermophilus* strains to metabolize galactose. Galactose accumulation in cheese can favour the growth and CO_2 production by undesirable bacteria capable of galactose utilization. Despite most of *S. thermophilus* strains are unable to metabolize galactose, Umamaheswari et al. (2014) selected 6 strains Gal+ to be used to reduce the quantity of residual galactose in yogurt. Mukhurjee and Hutkins (1994) also ascribed the low residual galactose in mozzarella cheese using *S. thermophilus* in combination with *Lb. helveticus* in starter cultures.

Non Starter Lactic Acid Bacteria

Non starter lactic acid bacteria (NSLAB) are important constituents of the cheese microbiota during ripening. Conversely to SLAB, NSLAB are not involved in the milk acidification process or the curd formation, but are responsible for important biochemical modifications that take place during ripening of the cheese curd. They play an important role in aroma formation in hard cheeses while their presence may result detrimental for fresh or soft cheeses that do not undergo ripening. NSLAB most likely originate from raw milk and are thermo-tolerant enough to survive pasteurization and/or high temperatures used in curd cooking. Furthermore, they can colonize the dairy environment and equipment, also through biofilm formation, and therefore involuntarily inoculate milk and curd during cheese production.

During cheese ripening, complex and dynamic NSLAB consortia evolve. The kinetics of the different NSLAB populations depend on parameters such as initial load, environmental conditions (mainly temperature and humidity of ripening, salt concentration) and other technological aspects such as cooking, stretching of the curd, use of natural starters, ripening time (Gobbetti et al., 2015). NSLAB mainly consist of species belonging to the *Lactobacillus* genus. Oftentimes the term *meso*phlic lactobacilli is used to designate NSLAB and to distinguish the species mainly found during ripening from the thermophilic, SLAB responsible for the acidification. *Lactobacillus paracasei* and *Lb. plantarum* are considered predominant NSLAB species, but *Lb. rhamnosus*, *Lb. casei*, *Lb. fermentum*, *Lb. parabuchneri*, *Lb. curvatus*, *Lb. buchneri*, *Lb. brevis* and *Lb. pentosus* are important constituents of NSLAB consortia and frequently isolated from cheeses during ripening. The above mentioned *Lactobacillus* species are either facultative or obligate heterofermentative and have the metabolic capability to utilize both hexoses and pentoses or solely pentoses respectively. Although to lesser extent, other genera of LAB may be part of NSLAB consortia. *Enterococcus* spp. naturally occur in artisanal cheeses, mainly produced in Southern Europe (Giraffa, 2003). *Enterococcus* spp. are considered controversial microorganisms mainly due to demonstrated antibiotic resistance by members of this group, capacity to produce biogenic amines and presence of virulence factors within the genome of food associated *Enterococcus*. Furthermore, conversely to what has been proven for different *Lactobacillus*, no direct link between the presence of *Enterococcus* and specific modifications (chemical or other) in the product has been shown (Giraffa, 2003). Despite that, certain species, namely *E. faecalis*, *E. faecium* and to a lesser extent *E. durans*, have been found to dominate the NSLAB microbiota of cheeses and therefore are believed to contribute to the sensorial characteristics of the product. *Pediococcus* spp., mainly *P. acidilactici* and *P. pentosaceus*, have also been isolated from a variety of European ripened cheeses. *Leuconostoc* spp. are used as starter cultures for the production of Swiss type (for eye formation) and pressed ripened Dutch cheeses and to facilitate cheese openness in blue veined cheeses (Hemme and Foucaud-Scheunemann, 2004). Consequently, leuconostocs can be isolated from cheeses, produced with *Leuconostoc* – Containing starters, during ripening. Furthermore, they are isolated from a variety of raw-milk, as well as cooked curd and white-brined cheeses (Beresford and Williams, 2004). Isolates from raw milk cheeses mainly belong to *Leuc. mesenteroides* ssp, *mesenteroides*, *Leuc. mesenteroides* ssp. *dextranicum* and *Leuc. citreum* (Hemme and Foucaud-Scheunemann, 2004).

The cheese components that are potential substrates of NSLAB metabolism during ripening are: residual lactose, lactate and citrate, triglycerides that undergo lipolysis with liberation of free fatty acids (FFA) subsequently catabolized, and proteins (mainly the casein matrix) that undergo proteolysis to peptides and free amino acids (FAA) that, in turn, are further degraded (McSweeney and Sousa, 2000). The metabolic pathways and enzymatic reactions described above are not exclusively associated to NSLAB. SLAB, rennet added for the coagulation, the milk itself, as well as exogenous sources, contribute to the biochemical modifications

described during ripening. Residual lactose can be utilized by facultative heterofermentative lactobacilli as an energy source while citrate can be co-metabolized with sugars, by leuconostocs and *meso*philic lactobacilli. The main products of citrate metabolism are diacetyl (produced in limited amounts), acetoin and 2,3 – butanediol; important flavor compounds for some cheese varieties. For Dutch cheeses citrate metabolism results in CO_2 production that leads to eye formation. Finally, acetate can be produced from lactose, lactate or citrate metabolism and is considered important flavor compound for many cheeses (McSweeney and Sousa, 2000). FFA that originate from lipolysis are important flavor compounds, indispensable for the sensorial characteristics of many types of cheeses. Short chain fatty acids have a direct impact on cheese flavor and are also precursors for the production of acids, alcohols, aldehydes, esters and thioesters, that further enhance flavor development (McSweeney and Sousa, 2000). Generally, non-starter lactobacilli, enterococci and pediococci have shown low lipolytic activity although important variability for this phenotype has been observed (Giraffa, 2003). For hard and semi-hard –type cheeses, casein proteolysis attributable to rennet enzymes and the microbial proteolytic system is an important biochemical pathway that leads to flavor formation (Smit et al., 2005). Through proteolysis, LAB are able to satisfy their amino acid requirements. Casein is mainly degraded by chymosin, endogenous milk enzymes and cell-envelope proteinases of SLAB that liberate peptides and amino acids. NSLAB are mainly responsible for peptidolysis and liberation of free amino acids. Peptides are hydrolyzed by intracellular peptidases and a wide variety of peptidolytic specificities has been described in non starter lactobacilli, pediococci and *Leuconostoc* spp (Beresford and Williams, 2004). Both peptides and amino acids possess specific flavor characteristics, however it has been underlined that the rate limiting step in flavor formation is the conversion of amino acids rather than their release through casein degradation (Beresford and Williams, 2004; Smit et al., 2005). Amino acid conversion ability varies within LAB and these activities are linked to the ability to synthesize amino acids (Smit et al., 2005). An α-ketoglutarate-dependent transamination reaction is the first step in amino acid break down. The resultant keto-acids are substrate for enzymatic or chemical reactions that lead to hydro-acids, aldehydes, carboxylic acids and alcohols; many of them flavor intensive molecules. Branched chain and aromatic amino acid transaminase activities, that are essential for flavor formation, have been detected in non-starter lactobacilli (Beresford and Williams, 2004). The amino acid conversion potential of certain non starter lactobacilli has been further confirmed by *in silico* analysis and comparative genomics focusing on pathways of flavor formation from amino acids (Liu et al., 2008). Decarboxylation is an alternative catabolic pathway for the pool of free amino acids that are available to LAB in cheese. Amino acid decarboxylation is believed to play a crucial role in pH homeostasis and allows microorganisms to respond to low pH stress conditions. The products of amino acid decarboxylation are CO_2 and primary amines. Some of these amines are volatile compounds that contribute to flavor and some cheese types contain numerous volatile amines (Zuljan et al., 2016). Amino acid decarboxylation reactions in cheese may also have a food safety implication. Biogenic amines produced by amino acid decarboxylation, when ingested in high quantities, can cause health problems. The most common biogenic amines found in cheese are tyramine, histamine and putrescine and the main producers are NS-LAB strains of the *Enterococcus* and *Lactobacillus* genera (Zuljan et al., 2016). Production of biogenic amines is strain dependent; therefore, careful management of the microbiota (both SLAB and NSLAB) throughout production should reduce the presence of these compounds in cheese.

An interesting trait that is common among LAB is the ability to produce bacteriocins. Bacteriocins have been defined as bacterially produced, small, heat-stable peptides that are active against other bacteria and to which the producer has a specific immunity mechanism (Cotter et al., 2005). Bacteriocinogenic LAB have been the focus of intense research since they can potentially by applied as biopreservation agents, active against foodborne pathogens or spoilage microbes in cheeses. Bacteriocinogenic LAB, belonging to *Enterococcus* and *Lactobacillus* spp, have been isolated from cheeses and were shown to have inhibitory activity towards important foodborne pathogens (for example *List. monocytogenes*, *Staphylococcus aureus*) and spoilage agents (for example *Clostridium spp.* responsible for the late blowing of hard cheeses) (Favaro et al., 2015). The antifungal activity of several species of *Lactobacillus* and *Enterococcus* is also of particular importance since fungal spoilage of dairy products is common and is currently dealt with using chemical preservatives.

Given the multifaceted attributes of NSLAB that influence the overall quality of the final product, the dairy industry is particularly interested in controlling the ripening process to achieve desired standards. For this purpose, NSLAB are often added as secondary/adjunct cultures. Strain selection for the development of such cultures is today sophisticated and takes into consideration not only technological properties but also safety characteristics, protective and probiotic potential.

Secondary Microbiota

In the definition of cheeses, different microorganisms are involved: longer than LAB population, other groups play fundamental role in the final character of the different cheeses. They are defined as the secondary microbiota, but of primary importance in the definition of peculiar characteristics.

The microbial diversity of the cheese depends on both the milk microbiota and on practices of production. This biodiversity decreases in the core of the cheese where LAB are the prevalent species, but it persists on the surface where numerous species are detected. The difference between cheeses is due particularly to changes in the dynamics of the microbial population used in the production. Cheese microbial population still remains difficult to control due to their complex dynamics and to their interactions (Artini et al., 2015).

The secondary microbiota significantly contributes to cheese characteristics, together with milk composition and starter and non-starter LAB activity (Bockelmann and Hoppe-Seyler, 2001) they are responsible for color, flavor and texture development during cheese maturing (Rademaker et al., 2005).

Propionic acid bacteria (PAB) are widely used in Swiss-type cheeses to generate holes and specific flavor notes, and surface bacteria that contribute to the characteristic flavor and color of smear-rind cheeses. Many of these properties depend on the strain within a species, and the choice of a selected strain is thus a means to modulate the final cheese properties (Thierry et al., 2015). The formation of flavor results from the conversion of milk lactose, citrate, caseins and lipids into taste and aroma compounds during the fermentation of dairy products. Propionibacteria is one of the important class of organisms used for the production of the Swiss cheese varieties. Swiss type cheese is a generic term for hard cheeses that were initially produced in Emmen valley in Switzerland and they are easily identifiable by characteristic round regular eyes. In this group of Swiss cheeses are included Gruyere, Maasdammer, Leerdammer, Emmental etc. Propionibacteria are often added to milk as adjunct cultures, but sometimes they are indigenous to the raw milk (Thierry et al., 2005).

Propionibacteria are Gram positive, non spore forming, anaerobic or aerotolerant and they possess several metabolic pathway: one of the most important is the central carbon metabolic pathway through which they use lactate during growth and produce propionate, acetate and carbon dioxide beside different functional metabolites like B group vitamins, conjugated linolenic acid (CLA) etc. (Hugenholtz and Smid, 2002). They are used widely as ripening cultures and different studies have also defined them as potential probiotic bacteria. They are involved also in the food preservation being microorganism able to produce antimicrobial compounds as bacteriocin and organic acids involved also in the antifungal activity.

Propionibacterium spp. was first discovered in 1906, when the relationship between the presence of *Propionibacterium freudenreichii* and the formation of holes in Swiss Emmental cheese was described. Indeed, *Pr. freudenreichii* together with *Pr. acidopropionici* are the principally used as an adjunct culture for the production and considered the most industrially important species (Poonam et al., 2012).

Focusing the attention on their technological attributes, they are mainly used as an adjunct culture for manufacture of Swiss type cheese where they are responsible for the characteristic flavor and eye formation. Beside this activity, they dominate the cheese microbiota during the ripening period (Rehn et al., 2011). However, their growth and fermentation rates in cheese depend on the conditions, in particular, NaCl content of the cheese (Richoux et al., 1998).

Eye formation is the result of CO_2 production during their growth, creating holes. On the other hand, flavor production is due to the metabolism of lactate (produced by LAB), aspartate and amino acid catabolism and fat hydrolysis. Volatile flavor compounds as 3-methylbutanal, 2-methylbutanal, and 3-methylbutanoic acid are produced by the catabolism of branched chain amino acids, particularly from leucine, isoleucine and valine (Poonam et al., 2012; Thierry and Maillard, 2002). They also produce FFA through lipolysis of milk fat. *Propionibacterium* species can also be used in the manufacture of some cheeses without eyes to enhance flavor formation: they can also modulate the flavor of Cheddar (Fernandez-Espla and Fox, 1998), Raclette and Morbier cheeses (Thierry and Maillard, 2002; Thierry et al., 2005). The inclusion of PAB as starter affect substantially the volatile profile; their presence increase the levels of several compounds, such as branched-chain aldehydes, primary alcohols, branched-chain alcohols, diacetyl, acetoin, ethyl esters, branched-chain acids, and short-chain acids. The aroma formulation is connected to their inter- and intraspecies diversity in the production of aroma compounds as demonstrated by Yee et al. (2014).

As introduced above, *Pr. freudenreichii* in particular has a key role in the formation of the typical round holes (eyes).The correct formation of holes depends on physicochemical factors, such as the presence of nucleation sites for hole development, an appropriate cheese structure, a massive production of carbon dioxide that induces a local saturation of gas leading to the formation of holes. During the ripening in the warm room, *Pr. freudenreichii* produces CO_2 from lactate fermentation, thus producing the main part of the CO_2 formed in cheese, the remaining part being formed by facultative heterofermentative lactobacilli from citrate fermentation and amino acid catabolism (Thierry and Maillard, 2002).

In recent years, *Pr. freudenreichii* has been evaluated for its probiotic properties and it has been shown that it beneficially modulates the gut function and physiology (Thierry et al., 2011). Furthermore, Cousin et al. (2016), showed that *Pr. freudenreichii* can kill human colon cancer cells acting as a potential protective probiotic against gut cancer both *in vivo* and *in vitro*. The successful application of PAB as probiotics was recently demonstrated with milk fermented by *Pr. freudenreichii*, which induced apoptosis of HGT-1 human gastric cancer cells (Cousin et al., 2012). Recently (Angelopoulou et al., 2017) showed that *P. freudenreichii* could be used also in the production of Feta cheese.

Many cheeses are characterized by complex surface microbiota. Cheese rind ecosystem is in contact with the external environment and consequently differs from the cheese core in terms of microbial composition and biochemical characteristics (Irlinger et al., 2015). The surface microbiota has an important organoleptic impact on cheese thanks to its various enzymatic activities and its role as a barrier against pathogens and spoilage microorganisms. The structure of cheese rind communities differs between facilities and describes significant shifts in the microbiota between short- and long-ripened cheeses (Dolci et al., 2009; Schornsteiner et al., 2014).

During cheese ripening, complex microbial communities, generally referred to as smear, develop on the surface of some types of cheese. In fact, such cheeses, when exposed to air, naturally tend to develop a smear layer on the surface, typically consisting of yeasts and bacteria (Chapman and Sharpe, 1990). The composition of this bacteria, yeasts and moulds, depends on the cheese technologies, environmental conditions, such as temperature, humidity and salt, as well as on the microbiota of the brine and of the rooms in which cheese is ripened (Fontana et al., 2010). Due to extensive washing of the cheese surfaces with brine during ripening, bacteria-ripened cheeses are also known as washed rind cheeses and develop a red-brown glistening appearance called “red smear”. The most widespread cheeses with this characteristic are Tilsiter or Limburger (Dutch production), Baufort and Munster (French) and Italian cheeses as Taleggio and Fontina (Corsetti et al., 2001; Dolci et al., 2014). In these cheeses, yeasts and moulds initially dominate the surface post-manufacture because they are acid-tolerant and salt-tolerant. The development of yeasts during the first

few days of ripening, which metabolize the lactate completely into CO_2 and H_2O, and forming alkaline metabolites, such as ammonia, induces an increase in the surface pH from 5 to 6 (Bonaiti et al., 2004). In addition, the production of growth factors by yeasts appears to promote the development of a Gram positive, catalase positive, salt-tolerant microbial communities composed mainly of coagulase-negative cocci (CNC) belonging to genera such as *Staphylococcus*, *Micrococcus* (Bockelmann, 2002) and coryneform bacteria (genera: *Brevibacterium*, *Arthrobacter*, *Microbacterium* and *Corynebacterium*) (Bockelmann et al., 2017; Corsetti et al., 2001). Traditionally, growth of the surface microbiota is initiated with the help of mature cheese, which release part of their surface into the smear tank (Bockelmann, 2002). The disadvantage is that pathogens or contaminants are also transferred to the smear tank to all the cheeses: the control of smear development on cheese surface is considered essential during ripening to reduce the risk of surface contamination by spoilage and pathogenic microorganisms (Bockelmann and Hoppe-Seyler, 2001). The knowledge of the microbial composition is also a prerequisite for the development of surface starter cultures. The surface starter culture could help in the preservation of spoilage microorganisms.

Generally this smear layer is undesirable in many hard and semi-hard cheeses, but is fundamental for soft and semi soft cheeses (Bockelmann, 2002). These smear microorganisms play a role in the ripening process, both through the action of proteolytic and lipolytic enzymes and the formation of many alkaline products that penetrate the body of cheeses.

Brevibacterium linens is one of the important surface microorganism that is present in the smear of surface-ripened cheeses and is commonly regarded as the organism primarily responsible for the characteristic taste, aroma, and color of surface cheese. *B. lines* was the first commercial smear culture and is today sold by all major starter culture suppliers (Bockelmann et al., 2017). The metabolism and physiology of this microorganism determine its growth on smear surface-ripened cheeses and the effect of such growth on the characteristics of the cheese (Meile et al., 2008; Motta and Brandelli, 2008).

More in general *Brevibacterium* spp. is a non-motile, non-spore formed, gram-positive coryneform bacteria that tolerates high salt concentrations (8%–20%), and is capable of growing in a broad pH range with an optimum at pH 7.0. They also survive carbohydrate starvation and drying for extended periods. Some strains of *Brevibacterium* produce distinctive red-orange carotenoid-type pigments when exposed to light during growth. The color of the colonies varies from orange (*B. linens*), through gray-white (*B. epidermidis* and *B. casei*) to purple (*B. iodinum*) (Jones and Keddie, 1986; Motta and Brandelli, 2008). (carotenoids) of the type-strain is often light dependent.

The interest in *B. linens* is connected to their ability to produce the self-processing extracellular proteases (Rattray et al., 1995), high levels of volatile sulfur compounds (Dias and Weimer, 1998a,b), bacteriocin (Motta and Brandelli, 2008), cell membrane-associated carotenoid pigment, and for their aromatic amino acid metabolism (Arrach et al., 2001).

Other important bacteria involved in the cheese definition and in the surface, are CNC composed mainly by *Micrococcus* and *Staphylococcus* genera. They are considered as positive biota involved in the development of organoleptic characteristic of the end products thanks to their proteolytic and lipolytic activities.

Among staphylococci, *Staphylococcus equorum* is one of the more predominant in cheeses. High cell count of staphylococci were found in brines of several cheese factories, explaining that brines are a natural source for cheese surface (Jaeger et al., 2002).

Except *Staph. equorum*, other several species as *Staph. cohnii*, *Staphy. epidermidis*, *Staph. saprophyticus*, *Staph. xylosus* and *Staph. warneri* have been isolated from surface ripened cheeses (Bockelmann, 2002; Bockelmann and Hoppe-Seyler, 2001; Rea et al., 2007).

Staph. equorum was also isolated from milk and some types of cheese (Ercolini et al., 2003) and it was proposed as starter culture for Swiss semi-hard cheeses (Place et al., 2003) and smear-ripened cheeses (Bockelmann, 2002). *Staph. equorum* strains were isolated for their ability to produce bacteriocins and Bockelmann et al. (2017) showed that its cultures would be an ideal adjunct for cheese factories being able to inhibit *List. monocytogenes*.

The genus *Micrococcus* plays also important roles in cheese ripening due to its high proteolytic and lipolytic activities (Morales et al., 2006).

Yeasts and Moulds

As described above, dairy products are characterized by a high biodiversity in terms of bacterial populations, which are mainly responsible for the acidification and the production of aromas from proteolytic and lipolytic activities. However the complexity of these microbial ecosystems is enriched by the presence of eukaryotes, represented by yeasts and moulds (unicellular and multi-cellular[filamentous] fungi). Compared to the bacteria, the yeast and mould relevant to dairy products are relatively limited and comprise genera and species able to ferment and assimilate lactose, utilize lactic and citric acids, grow at low temperature and in presence of high salt concentration, and able to hydrolyze milk proteins and lipids. *Kluyveromyces marxianus* (anamorph *Candida kefyr*), *Kluyveromyces lactis*, *Debaryomyces hansenii* (anamorph *C. famata*), *Yarrowia lipolytica* (anamorph *C. lipolytica*), and *Saccharomyces cerevisiae* are the most isolated yeasts from dairy products (Frohlich-Wyder, 2003). Regarding filamentous fungi, *Penicillium camemberti* and *P. roqueforti* are used as starter cultures for the production of mould fermented cheeses (Haasum and Nielsen, 1998). Moreover, the yeast-like fungus *Geotricum candidum* is also relevant for Camembert cheese production and some semi-hard cheeses, in which specific surface smear is developed.

Yeast and moulds are, however, also responsible for spoilage in dairy products. The species may different or the same as the ones described above, moreover depending on the products from where they are isolated, they can be considered positive or negative.

Yeasts represent spoilage organisms in the dairy sector due to their ability to grow at low pH and low temperature. They are mainly involved in blowing of yogurt packages, through their fermentative metabolism, and they are responsible for anomalous

coloration of cheeses (mainly fresh), especially those producing pigments. Like yeasts, adventitious moulds should be considered spoiling agents of dairy products. They usually grow on the surface of the products due to their need of oxygen, thereby altering their aspect. However of specific concern is the capability of some species (e.g., *Aspergillus flavus*) to produce mycotoxins, which should be considered a risk for human health. The abundance and distribution of spoilage yeasts and moulds differ between products and within the same type of product manufactures have important role in the contamination. Even different batches of the same dairy product of the same manufactured can have different levels of spoilage yeasts and moulds (Banjara et al., 2015; Garnier et al., 2017).

Yeasts in dairy products are relevant to modulate the ecology of the cheese rinds, where they mainly metabolize lactic acid, thereby allowing the development of a complex microbial ecosystem composed by several bacterial groups, such as *Staphylococcus*, *Brevibacterium* and *Corynebacterium* (see above) and filamentous fungi. They are present in the raw materials, in the environment and the brine (smear water) represents an excellent carrier for their spread on the cheese surface. *Debaryomyces hansenii*, *C. versatilis*, *K. marxianus*, *Sacch. cerevisiae*, *Torulaspora delbrueckii*, *Trichospomn cutaneum* and *Y. lipolytica* are some of the most relevant species found in those ecosystems (Larpin-Laborde et al., 2011).

In fresh and short ripening cheeses, yeast ecology is represented by lactose positive species such as *K. lactis* and *K. marxianus*. In mould-ripened cheeses *D. hansenii* and *G. candidurn*, as well as *K. marxianus* and *Y. lipolytica* dominate the mycobiota (Frohlich-Wyder, 2003).

In ripened cheeses, they contribute to sensory characteristics through the production of aroma from proteolysis and lipolysis, and they also play an important technological role in blue-veined cheeses where they produce CO_2 from the fermentative metabolism, which helps to maintain an open structure, allowing oxygen to penetrate in the loaf allowing filamentous fungi development. *Y. lipolytica* is recognized to have a great lipolytic activity (Groenewald et al., 2014), releasing fatty acids from triacylglycerols, that are important precursors of volatile compounds. Proteolysis is also an important role of dairy yeasts. *K. lactis*, *K. marxianus*, *D. hansenii*, *Y. lipolytica*, *G. candidum* and *C. catenulata* have been all characterized by their strong extracellular proteolytic and or peptidolytic activity (Baur et al., 2015). Relevant to underline is their role in the brake-down of the bitter peptides, especially *G. candidum* (Auberoer et al., 1997).

Regarding the filamentous fungi, as already described above, they are mainly considered as spoilage agents, however some species are exploited in the dairy sector since they are taking part to the ripening of mould-ripened cheese. Specifically *P. camemberti* and *P. roqueforti* are used as starter cultures for the production of Camembert, Brie, Roquefort and Gorgonzola cheeses.

Penicillium camemberti in Camembert and Brie cheeses is essential for their characteristic appearance and sensory profile, since low-molecular-weight compounds produced by the fungus contribute significantly to taste. Volatile fatty acids, in particular, are produced from lipolytic activities important in soft cheeses, such as Camembert, where free fatty acids can reach up to 10% of the total fatty acids present. *P. camemberti* produces also proteases, involved as well in the development of the aroma profile. (Abbas and Dobson, 2011a).

Penicillium roqueforti is used as a fungal starter culture for the production of blue-veined cheeses such as Danablu, Gorgonzola, Roquefort, and Stilton. The fungus is involved in the production of both proteolytic and lipolytic enzymes. The proteolytic enzymes soften the curd and produce the desired body in the cheese. The water-soluble lipases hydrolyze the milk fat to free fatty acids, which contribute to the flavor of Blue cheeses (Abbas and Dobson, 2011b).

Culture-Dependent and Culture-Independent Approaches: What is Changing

Microbial communities in cheeses, at various stages of manufacturing and ripening, have been extensively studied by microbiological techniques based on the cultivation of microorganisms on selective media. This approach if allows, on the one hand, to obtain isolates to be studied for their phenotypic and genotypic characteristics, on the other hand, only a fraction of the community can be retrieved on cultural media. In fact, traditional agar-based and culture-dependent methods typically reveal the most commonly occurring microorganisms. These methods often underestimate the less abundant components of microbiota that could be equally important for cheese ripening and flavor development (Steel et al., 2006). To this regard, Neviani et al. (2009) optimized an innovative cheese agar medium able to recover minor populations, coming from milk, whey starter and fresh curd, in long ripened cheeses. Thus, alternative cultural approaches have to be investigated to obtain more reliable pictures of microbial community in dairy products.

The limits associated to culture-dependent techniques have led, in the last years, to the increasing use of culture-independent approaches, which depict a more reliable profiling (and also quantification) of microbial communities. Culture-independent methods overcome most of the cons associated to use of cultural media and they become essential in the study of microbial populations in physiological stressed state, included microorganisms in a viable but not culturable (VNC) state. Moreover, culture-independent techniques have shown high potentiality in the study of metabolic and functional genes strictly involved in the quality and safety of the final products.

Dairy Strains in Viable but not Culturable State

Bacteria in VNC state fail to grow on synthetic substrates on which they would normally grow and develop into colonies, but are alive and capable of metabolic activity. ATP levels have been found to remain high in VNC cells as also gene expression (Oliver, 2005). Thus, the role of microorganisms in VNC state has not to be underestimated and their presence has to be carefully taken into account.

LAB have been demonstrated to enter in VNC state under the stress conditions they can find during fermentation and ripening processes. In particular, sugar starvation has been recognized as a key stress for LAB (Ganesan et al., 2007). In those conditions, lactococci switch to the catabolism of amino acids and, potentially, contribute to flavor production during cheese ripening. Ganesan et al. (2007) have shown that *L. lactis* IL1403, in carbohydrate starvation, loses the ability to grow on solid media and expresses genes related to arginine catabolism. Thus, VNC cells, in ripened cheeses, remain intact and continue to metabolize peptides and amino acids to end products with a potential impact on cheese flavor (Weimer, 2011).

Studies on Swiss-Dutch-type cheeses showed that the physiological state of lactococcal starter cultures affect ripening and flavor development (Miks-Krajnik et al., 2013). Specifically, *L. lactis* populations, in VNC state, exhibited different metabolisms than in logarithmic growth phase and, around 30 days of ripening, their entering in VNC state was correlated to the simultaneously appearance of the branched-chain fatty acid C_{5iso}.

The presence of *L. lactis* cells in VNC state has also been hypothesized in some Italian cheeses, by Ruggirello et al. (2014). The same authors assessed, by culture-independent approaches, the persistence and viability of this microorganism throughout manufacturing and ripening of model cheeses where *L. lactis* populations were detected up to the sixth month of ripening (Ruggirello et al., 2016). On the contrary, from the analysis of the same samples, *L. lactis* was not found on selective media and this evidence, together with preliminary results obtained by resuscitation assays, corroborated the hypothesis it entered in a stressed physiological VNC state.

Community Profiling Methods: From PCR-DGGE to HTS

Since the 90s, methods in molecular microbiology have become a valid support to traditional techniques, in food analysis, and the trend towards culture-independent approaches have been considered a possible solution to problems associated with selective cultivation. Denaturing gradient gel electrophoresis (DGGE)-PCR has been, in the last twenty years, the most commonly used among culture-independent techniques, able to profile the diversity and dynamics of dominant microbial populations in many matrices, from environmental to food samples, included dairy products (Ercolini, 2004).

Genetic diversity of microbial communities, in dairy products, have been profiled by means of DNA-based protocols by different authors (Alegrìa et al., 2009; Bonetta et al., 2008; Casalta et al., 2009; Coppola et al., 2001; Ercolini et al., 2003; Flòrez and Mayo, 2006; Gala et al., 2008; Randazzo et al., 2010). PCR-DGGE technique has been carried out to fingerprint bacterial populations developing in raw milk cheeses manufactured without the addition of starter cultures, as in the case of Castelmagno PDO (Dolci et al., 2008, 2010) and Capo Verde cheeses (Alessandria et al., 2010). Moreover, it was also used in the study of the complex ecosystem of natural fermented Sicilian Ragusano-type cheese, giving useful information for starter culture design and preservation of artisanal cheese technology (Randazzo et al., 2002). The performance of selected and natural starters was also followed by PCR-DGGE in studies about whey cultures in Mozzarella cheese (Ercolini, 2004; Guidone et al., 2016) and in Caciocavallo silano cheese (Ercolini et al., 2008). This technique was also successfully used to determine the geographic origin of cheeses, such as Fontina, manufactured in alpine farms located at different altitudes (Giannino et al., 2009), Grana Trentino produced in different factories (Rossi et al., 2012) and Pecorino Crotonese from two areas (Randazzo et al., 2010). Mostly, PCR-DGGE and PCR-TGGE were performed to follow fate and dynamics of technological microbial populations; in addition, in a few studies, target microorganisms were related to cheese defects as late blowing (Cocolin et al., 2004; Le Bourhis et al., 2007).

Microbial RNA analysis has been also performed by RT-PCR-DGGE, but to a lesser extent, to obtain a picture of the species metabolically active at a particular sampling instant (Alessandria et al., 2010; Dolci et al., 2010, 2014; Masoud et al., 2011; Randazzo et al., 2002; Rantsiou et al., 2008). In particular, it was used to describe microbial communities characterized by a high complexity, as surface microbiota in Fontina PDO cheese (Dolci et al., 2013). In this study, RT-PCR-DGGE showed a major power than PCR-DGGE in describing biodiversity of Fontina PDO rind. Thus, the authors proposed that RNA molecules could have been considered a more informative target than DNA.

In the last years, several next-generation sequencing (NGS) technologies have been developed and applied to the study of dairy products reaching a high in-depth qualitative and quantitative profiling level, especially if compared to PCR-DGGE technique mainly focused on dominant populations (Ercolini, 2013).

Alessandria et al. (2016) explored the active microbiota during the manufacturing and ripening of a Grana-like cheese by beta-diversity analysis of the 16S rRNA sequencing data; Dalmasso et al. (2016) investigated the microbiota of Plaisentif, an artisanal antiqueue cheese manufactured in the Italian Alps during the violet's blooming season. In another study, the bacterial diversity and structure of Mexican Poro cheese was analysed by 454 pyrosequencing in order to gain detailed insight about changes in bacterial communities associated with the cheesemaking process (Aldrete Tapia et al., 2014). Dolci et al. (2014) investigated bacterial dynamics, by both RT-PCR-DGGE and high throughput sequencing (HTS), during Fontina PDO cheese manufacturing and ripening, in relation to the different cow lactation stages, in order to evaluate a possible correlation between microbiota and phase of lactation. Quigley et al. (2012) carried out HTS to reveal the highly diversity of bacterial populations present in Irish artisanal cheeses, and detected several genera not previously associated with cheeses, specifically, goats' milk cheeses. HTS approach was also performed to investigate the microbial ecosystem of two artisanal smear-ripened cheesemaking plants (Bokulich and Mills, 2013); in this study, the authors demonstrated how environmental microorganisms dominated the surface microbiota of washed-rind products even in inoculated cheeses. Dugat-Bony et al. (2015) also collected metagenomic and metatranscriptomic data for a detailed overview of surface-ripened cheese microbial community, involved in cheese maturation. Finally, O'Sullivan et al. (2013)

reviewed nucleic acid-based approaches to investigate microbial-related cheese quality defects and underlined the potentiality of NGS in providing greater insight into structural community interactions and metabolic activities.

Lastly, the composition of cheese surface microbiota has been studied by the application of conventional culture-based analysis, by using culture-independent methods based on direct extraction of DNA/RNA from the matrices and by the application of HTS technologies. As reported by Irlinger et al. (2015), considering *Firmicutes*, *Staphylcoccus* spp. was the most frequently genus followed by *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus* and among halophilic bacteria *Marinilactobacillus* and *Facklamia*. In the case of *Actinobacteria*, *Brevibacterium*, *Corynebacterium* and *Arthrobacter* were the most frequent genera followed by *Brachybacterium*, *Microbacterium*, *Agrococcus* and *Micrococcus*.

Technical issues in culture-independent methods concern nucleic acid extraction: nucleic acid yield, PCR inhibitors as various substances coming from cheese matrix itself, beside differential PCR amplification, are topics which have been widely reviewed (Ercolini, 2004; Wintzingerode et al., 1997). For these reasons, protocols have to be accurately optimized and adapted to extract nucleic acids from all different types of microorganisms and matrices. In particular, many efforts have been made to reach efficient RNA extraction from dairy matrices (Monnet et al., 2008). Authors agree on the necessity of high quality RNA from cheese as a prerequisite for reliable gene expression analysis in dairy microorganisms (Ulve et al., 2008; Ruggirello et al., 2014). In ripened cheeses, the presence of natural constituents such as lipids, proteins, carbohydrates and salt may render extraction very hard because they might act as PCR inhibitors.

DNA and RNA extraction optimization is fundamental when we move from the qualitative/semi-quantitative approach by PCR-DGGE, to the quantitative HTS (Quigley et al., 2012). The occurrence of each OUT can be negatively affected by an inefficient nucleic acid extraction in terms of quantitative estimation and proportions.

Furthermore, the choice of target molecule is critical for the success of any molecular profiling techniques. The target DNA sequence must include conserved sequences serving as anchors for PCR primers and, at the same time, it must be variable to differentiate microorganisms belonging to different species and genera (Bokulich and Mills, 2012; Jany and Barbier, 2008).

Bacterial community studies in cheese are based, mainly, on the analysis of 16S rRNA gene and 16 S–23 S intergenic region (Jany and Barbier, 2008). In particular, different sets of primers targeting the V3 hypervariable region have been extensively used (Delbès et al., 2007) and allowed identification at species or genus level. Differently, in fungi, the analysis of rRNA genes provides identification at genus or family level (Anderson and Cairney, 2004) and, thus, fungal internal transcribed spacers (ITS) are generally chosen as target for fungal community surveys inasmuch provide a greater taxonomic resolution than rRNA genes (Anderson and Cairney, 2004).

In HTS studies, when species assignment should be the target of the analysis, long sequence reads are required with as much as possible variable traits of 16S rRNA gene. Ercolini et al. (2012) studied the microbiota involved in the production of water buffalo mozzarella cheese by amplification of a 500 bp V1-V3 region and they reached identification at species level; differently, Alegria et al. (2012) used, as target, a 300 bp V5-V6 region to study Osypek cheese and the taxonomic resolution was at genus level. However, even with long reads, the 16S rRNA gene is not always heterogeneous enough for species discrimination in dairy bacteria, as observed in a research on Danish cheese where a 450 bp V3-V4 region was the target considered (Masoud et al., 2011).

In addition to the length of the target sequence, another technical issue is the copy number (Bokulich and Mills, 2012). For example, ribosomal RNA genes vary widely among taxa, skewing quantitative estimates. To circumvent this limit, alternative single-copy target sites, such as the *rpoB* gene in bacteria (Renouf et al., 2006), should be taken into account, despite the lack of representative sequence coverage poses a challenge for widespread implementation.

Quantitative PCR Approach: Detection and Quantification of Dairy Microorganisms and Study of Functional Gene Expression

In comparison with environmental microbiology, the use of molecular tools applied to the study of population dynamics and microbial gene expression in food is just beginning (Juste et al., 2008; Postollec et al., 2011). Quantitative PCR (qPCR) has become extremely popular in food studies, included researches on dairy products. Besides being faster than conventional culture-based methods, it is also highly sensitive and specific in the detection of microorganisms in dairy matrices; it has been also successfully applied in the study of sub-dominant populations even in absence of selective enrichment media.

An interesting multiplex qPCR protocol was optimized to simultaneously detect *Escherichia coli* O157:H7, *Salmonella* spp. and *List. monocytogenes* in milk samples (Omiccioli et al., 2009). Other interesting applications were in the detection and quantification of technological microorganisms, which positively affect the organoleptic properties of the final products. For example, Zago et al. (2009) followed sub-dominant populations of *E. gilvus* in cheese targeting *pheS* (phenylalanyl-tRNA synthase) gene; other authors (Furet et al., 2004; Grattepanche et al., 2005) quantified different LAB species in fermented milk, with detection limits between 10^2 and 10^3 colony forming units (CFU)/ml, in the presence of other dominant microbial populations. Quantitative PCR was also applied to study mycelial growth dynamics of *P. roqueforti* and *P. camemberti* (Le Dréan et al., 2010) whose presence is fundamental in mould surface-ripened cheeses. Despite the viable biomass was probably overestimated, in late ripening, the results allowed to monitor changes in fungal populations. The possibility to follow the growth, in real time, of specific microbial populations, in mixed dairy communities, opens the way towards industrial applications for the control of fermentation processes.

Beyond cell quantification, the detection of metabolic and functional genes, and also the study of their expression presents a novel use of qPCR; thus, for example, RT-qPCR is now being increasingly employed to follow microbial activity in dairy products.

The study of the expression of genes related to primary metabolism, or constitutively expressed in most of the cultural conditions, has been carried out by different authors. For example, Ulve et al. (2008) investigated seven genes of *L. lactis* inoculated in ultrafiltrated Cheddar cheeses, and demonstrated metabolic activity of lactococci even after several weeks of ripening. Similar results were obtained by Ruggirello et al., (2014, 2016) in the study of vitality and persistence, throughout cheese ripening, of commercial starters of *L. lactis* in both commercial and model cheeses by studying the expression of the housekeeping *tuf* gene. Moreover, the expression of *spxB* gene, encoding for pyruvate oxidase, was analysed by Sardaro et al. (2016) for monitoring the metabolically active community of *Lb.s casei* group involved in different ripening stages. RT-qPCR was also used in the elucidation of the dynamics, in Emmental cheese ripening, of *Lb. paracasei* and *Pr. freudenreichii* (Falentin et al., 2010). The results showed that *Lb. paracasei* began to grow in pressed curd and its metabolic activity reached a maximum during the first part of ripening, in cold room; *Pr. freudenreichii* began to grow from the beginning of ripening and its activity was maximum at the end of cold ripening while was stable during the first two weeks in warm room.

Quantitative PCR and RT-qPCR techniques has been applied to the research of metabolically active populations, either spoilage and pathogenic. For example, Martín et al. (2010) optimized an RT-qPCR protocol, based on *lacZ* gene, to detect coliforms in cheese, in a single reaction and within one day. In addition, other authors designed a qPCR protocol aiming to detect and quantify LAB carrying the histidine decarboxylase (*hdcA*) gene in milk and cheese (Fernandez et al., 2006). Despite LAB are non-pathogenic, however, in some environments, certain strains may produce undesirable compounds such as biogenic amines, which are responsible for food poisoning. Histamine is one of these compounds, resulting from histidine decarboxylation. Finally, a protocol to detect enterotoxin gene expression in *Staph. aureus*, artificially inoculated in cheese, was set up by Duquenne et al. (2010).

Thus, RT-qPCR revealed high potentiality in the elucidation of technological microorganisms roles in different stages, from cheese-making to ripening and, finally, conservation and storage of the final products, considering also the recent evidences related to the vitality of starter strains in late ripening (Dolci et al., 2010; Flòrez and Mayo, 2006; Rantsiou et al., 2008; Ruggirello et al., 2014; Ulve et al.2008). Moreover, the impact of the different technological steps and parameters (temperature, pH, etc.) on the expression of target genes, linked to both primary and secondary metabolisms, could give additional interesting information. Finally, the detection and quantification of transcripts predicting for the presence of undesirable molecules and/or pathogenic and spoilage microorganisms could represent an efficient tool for microbiological control and risk assessment.

Conclusions

As described above, dairy microbiology is characterized by a high level of biodiversity involving different microorganisms, which interact in the same ecosystem. It is extremely important to understand how those events are taking place in order to have the possibility to control microbial activities that have a direct impact on the final characteristics of the end products. The use of molecular methods has given the possibility to better determine the taxonomy of a specific dairy ecosystem, however the challenges that we are facing at the moment is how to interpret and exploit this new knowledge to improve the safety and the quality of dairy products. An exiting future is expected for dairy microbiologists, who should be able to take advantage of these approaches to fully understand microbial diversity, activity and interactions.

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Microbiology of the Cystic Fibrosis Airway

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Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive disorder and is the most common inherited life-shortening disease affecting Caucasians. It is a multi-organ disease caused by mutations in the CF Transmembrane Conductance Regulator (CFTR) gene on chromosome 7 which render the CFTR protein either ineffective or absent in those with CF (Welsh, 1986; Tsui et al., 1985; Riordan et al., 1989). The class II △F508 CFTR mutation is by far the most common CFTR mutation and is caused by a deletion of a single phenylalanine at position 508 of the CFTR protein (Lukacs et al., 1994). CF patients homozygous for △F508 (△F508/△F508) have a more severe disease phenotype including more frequent respiratory infections, an early onset of pancreatic insufficiency and decline in lung function (McKone et al., 2003).

The wild-type form of the CFTR protein is 1480 amino acids in length and is located on the apical surface of the epithelial cells of exocrine glands. The CFTR protein plays a vital role in osmotic balance in the lungs by mediating the translocation of negatively charged chloride ions out of epithelial cells while positively charged sodium ions translocate across the epithelial sodium channel (ENaC) (Ahmed et al., 2003). In CF chloride ion secretion is significantly reduced or absent, resulting in sodium hyperabsorption due to ENaC dysregulation (Stutts et al., 1995, 1997). The airway surface fluid becomes viscous, mucociliary clearance is impaired and the subsequent build-up of mucus creates an ideal environment for opportunistic pathogens to colonise and persist.

CF Airway Disease and Infection

Despite being a multi-organ disease, up to 95% of morbidity and mortality in CF is caused by airway infections and the associated inflammation (Zhao et al., 2012). CF is characterised by a cyclical pattern of infection, inflammation and airway obstruction. This leads to a build-up of irreversible lung damage and ultimately respiratory failure. The most common early symptoms include chronic cough, recurrent chest infections, breathlessness, and wheeze. Beyond the age of four months the pathological features in CF airways include inflammation, mucous plugging and bronchiectasis. Lung function is abnormal in CF infants shortly after birth (Kieninger et al., 2017). Early reductions in lung function are directly associated with microbial infection (Nixon et al., 2002) and microbial bioburden has been linked to increased inflammation. Dakin et al. (2002) Whether the lungs of people with CF are intrinsically inflamed from birth or become inflamed upon first exposure to microbes had been highly debated (Khan et al., 1995; Birrer et al., 1994; Balough et al., 1995). This stems largely from the detection of inflammation in the absence of culturable microorganisms from early CF sputum samples (Muhlebach et al., 1999) although more recent molecular detection techniques have shown that few CF airway samples are truly microorganism free.

Upon entry of bacterial cells to the healthy airways pathogen-associated molecular patterns (PAMPs) on bacterial cell walls are recognised by pathogen recognition receptors (PRRs) of circulating alveolar macrophages and lung epithelial cells which produce interleukin-6 (IL-6) and IL-8 (Bonfield et al., 1995), leading to infiltration of neutrophils (Conese et al., 2003). A cascade of chemokines, cytokines and activation of lymphocytes is triggered which elicits further inflammation. Alveolar macrophages and neutrophils can phagocytose bacterial cells and kill them internally within the phagolysosome. Neutrophils work to clear pathogens by secretion of proteases, such as neutrophil elastase (NE) and neutrophil extracellular traps (NETs), killing bacteria though causing epithelial cell damage in the process (Sorensen and Borregaard, 2016). Mass infiltration of neutrophils into the airways is a hallmark of CF disease and causes significant parenchymal damage (Ulrich et al., 2010). People with CF are not generally considered immunocompromised however the CFTR protein has been shown to act as a PRR for *Pseudomonas aeruginosa* (Pier et al., 1996) meaning this bacterium may be able to evade certain host responses. High levels of salt in the CF airway have been shown to impair activity of antimicrobial peptides (Smith et al., 1996; Goldman et al., 1997) and more recently CF neutrophils have been shown to have impaired function, including defective phagolysosomal fusion (Painter et al., 2006; Houston et al., 2013; Pohl et al., 2014). CF mast cells have been shown to release increased levels of IL-6 which can overstimulate neutrophil production (Ratner and Mueller, 2012). Inflammation and immunology of the CF airway is evidently very complex and is only briefly covered here. The following text gives a more thorough overview of defective immune responses in CF (Ratner and Mueller, 2012).

Microbiology of the CF Airway

Airway infections cause the burden of morbidity and mortality in CF (Gibson et al., 2003) and diagnosis of these infections forms the cornerstone of CF care. Diagnosis is largely based on the ability to culture these microorganisms from CF airway samples. The antibiotic resistance profiles of the cultured bacteria then informs therapeutic approach. Some of the most common bacteria isolated from the airways of people with CF are *Staphylococus aureus, P. aeruginosa, Haemophilus influenzae, Stenotrophomonas maltophilia* and *Achromobacter xyloxidans*. Cultivation data has revealed a general pattern of succession with *S. aureus* and

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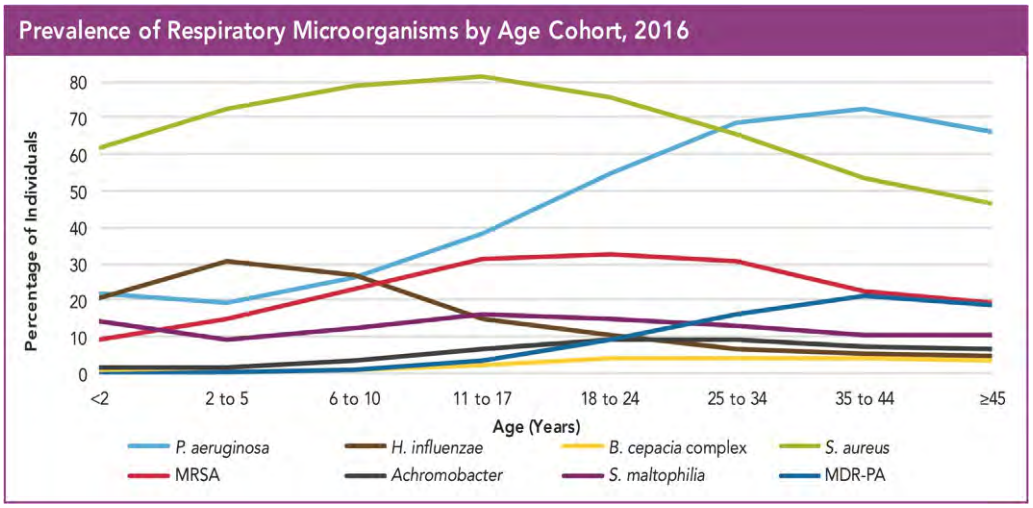

Fig. 1 The 2016 Annual data report from the US Cystic Fibrosis Foundation Patient Registry. Reproduced from CFF, 2016. Cystic fibrosis foundation patient registry. Prevalence (%) of most common CF bacteria by age.

H. influenzae colonisation common in childhood, while *P. aeruginosa*, *S. maltophilia* and/or *Burkholderia spp* being more common in adults (Harrison, 2007; Pettigrew et al., 2008) (Fig. 1).

P. aeruginosa in CF

Prevalence

P. aeruginosa is a Gram negative rod shaped bacteria that commonly colonises the CF airways (Doring et al., 2000; Pitt et al., 2003). There is a chronology to *P. aeruginosa* colonisation in CF (Fig. 1). The prevalence in children has been reported to be anywhere between 20% and 97.5% (CFF, 2016; Lebecque et al., 2006; Burns et al., 2001; CFRI, 2016; Trust, 2016) while in adults it is between 52.5% and >80% (CFF, 2016; CFRI, 2016; Trust, 2016; FitzSimmons, 1993). One study reported that 29% of children with CF have acquired *P. aeruginosa* in the first 6 months of their life (Li et al., 2005). There have been conflicting reports on whether the prevalence rate of *P. aeruginosa* is decreasing in the CF population. A study in the US of the 1995–2005 CFF patient registry reported a 4.3% decrease in prevalence of *P. aeruginosa* in CF patients over the 10 years (Razvi et al., 2009) while a similar study carried out in the UK between 1985 and 2005 found no increase or decrease in prevalence (Millar et al., 2009). The 2016 CFF patient registry reported a decrease in prevalence between 1990 and 2016 however MDR *P. aeruginosa* prevalence remains constant (CFF, 2016) (Fig. 2). Reported prevalence differs depending on the detection method used (culture-based, molecular, serological), the age and disease status of the patient cohort and the country of the study.

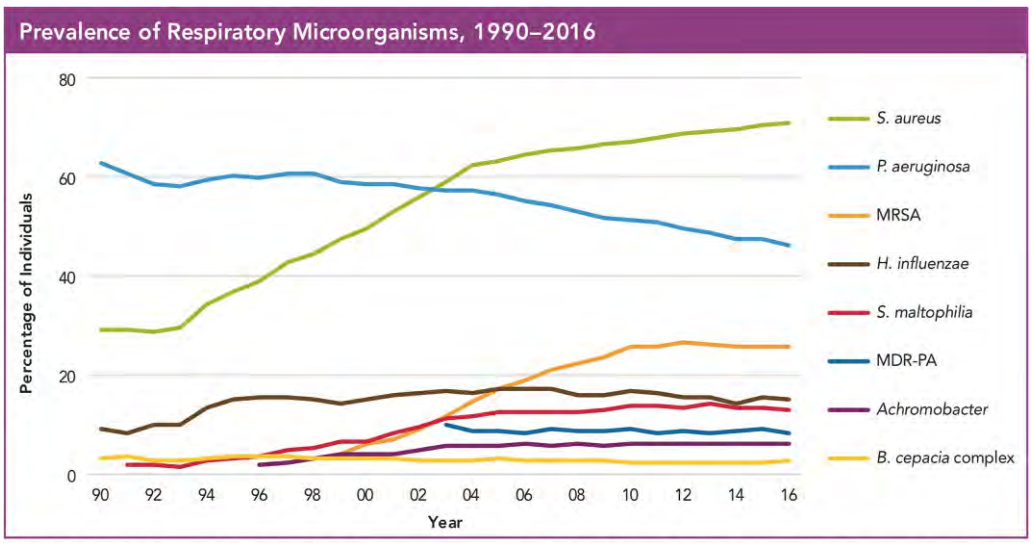

Fig. 2 The 2016 Annual data report from the US Cystic Fibrosis Foundation Patient Registry. Reproduced from CFF, 2016. Cystic fibrosis foundation patient registry. Prevalence (%) of most common bacteria in CF between 1990 and 2016.

Infection, biofilms and persistence

P. aeruginosa infection is known to transition from initial sporadic colonisation to an intermittent infection, before becoming established as a chronic infection (Smith et al., 2006). In 2016, 46.9% of patients in the CFF registry were positive for *P. aeruginosa* of which 29.1% were chronic and 17.8% were intermittent (CFF, 2016). *P. aeruginosa* can adapt to the CF airway environment during infection changing from a non-mucoid motile phenotype to a mucoid, antibiotic resistant, biofilm former at which point it becomes almost impossible to eradicate (Doring et al., 2000; Hogardt and Heesemann, 2010). In early intermittent colonisation, *P. aeruginosa* in its environmental, non-mucoid and thus more motile state, can penetrate into thickened hypoxic mucus zones in the CF airway. Conversion of *P. aeruginosa* to mucoidy growth and loss of motility are often seen as responses to stress (Deretic et al., 1994) and the physiological environment in the CF airway applies stresses such as osmotic imbalance, oxidative stress, immune cell responses, inter-species competition and antibiotic exposure. This results in diversification of *P. aeruginosa* genotypes in early and intermittent infection with the most favourably adapted descendants, usually mucoid biofilm formers, being selected for persistent colonisation (Jelsbak et al., 2007). Biofilms are assemblages of bacteria that adhere to and form microcolonies on surfaces, in this case CF epithelial cells or in mucus plugs, and are embedded in extracellular polymeric substances (EPS) which protect it from the surrounding environment. Biofilms are highly tolerant to the stressful environment of the CF airway (Smith et al., 2006; Jelsbak et al., 2007; Leid, 2009). Thus the ability of *P. aeruginosa* to adapt to the CF airway environment and grow in biofilms means it can evade host responses, avoid antibiotics and persist. Development of chronic *P. aeruginosa* colonisation is a significant disease milestone in CF and has been observed to mark the beginning of significant lung function decline (Pamukcu et al., 1995). Chronic *P. aeruginosa* colonisation is considered a major limiting factor in patient survival, with estimates of 10 years reduced life expectancy in chronically colonised patients (Li et al., 2005; Koch, 2002; Kosorok et al., 2001).

Virulence

P. aeruginosa has many virulence factors that help it invade and evade the host immune system. Secretion of phenazines and siderophores in addition to formation of biofilms have been known to result in poorer clinical outcomes (Jimenez et al., 2012). Pyocyanin is a phenazines produced by *P. aeruginosa* and is known to exert host inflammatory responses (Lau et al., 2004a,b; Fothergill et al., 2007) and act as an iron reducing agent (Pierson and Pierson, 2010). A study in CF mice reported an increase in pyocyanin levels during *P. aeruginosa* lung infection which was associated with more virulent infections mediated by tissue damage and necrosis (Lau et al., 2004b). A recent study investigating pyoverdine (a siderophore secreted by *P. aeruginosa*), pyocyanin and LasA (an elastolytic protease), concluded that high-virulence sub-populations of *P. aeruginosa* can persist in CF patients with chronic infections (O'Brien et al., 2017).

P. aeruginosa utilises its quorom sensing (QS) system to secrete virulence factors and to initiate biofilm formation by signalling between cells. In the early stages of infection *P. aeruginosa* utilises QS to determine high cell density and subsequently induces an upregulation of virulence factor-related gene expression (Bjarnsholt et al., 2010). Virulence factors are targets for immune cells, thus many strains of *P. aeruginosa* reduce their expression of virulence factors and extracellular toxins in chronic infections (Smith et al., 2006). Genes associated with QS, iron acquisition, phenazine biosynthesis, multidrug efflux and twitching motility have been found to be mutated to maximize survival and progression to chronic infection (Marvig et al., 2015). Under periods of severe stress subpopulations of *P. aeruginosa* biofilms can randomly convert to persisters, cells with reduced metabolic activity and increased stress tolerance. This is a vital survival mechanisms for *P. aeruginosa*. One study reported a 100-fold increase in drug-tolerant persister cells in late CF isolates when compared to the earlier CF isolates of the same clonal strain (Mulcahy et al., 2010). *P. aeruginosa* is an extremely adaptable microorganism and possesses a surprising number of virulence mechanisms for an opportunistic pathogen.

Epidemiology and transmission

Previously it was thought that CF patients carried their own individual strains of *P. aeruginosa* acquired from the environment however in 1996 a group in a CF centre in Liverpool reported that 55 of 65 paediatric CF patients harboured the exact same beta-lactam resistant epidemic strain of *P. aeruginosa*, the Liverpool Epidemic Strain (LES) (Cheng et al., 1996). Since then other epidemic strains have been reported in CF centres in the UK (Jones et al., 2001), Australia (Armstrong et al., 2003; O'Carroll et al., 2004) and most recently the priarie epidemic strain (PES) in Canada (Parkins et al., 2014). Clonal strains have been shown to be transmissible (Armstrong et al., 2003; McCallum et al., 2001), thus supporting the separation of *P. aeruginosa* positive and negative patient groups in the clinical setting. Many clonal strains of *P. aeruginosa* are multidrug resistant (MDR) (Jones et al., 2001; Ashish et al., 2012) and are associated with significant morbidity and mortality. CF patients colonised with LES have been shown to have greater annual loss of lung function than those not colonised with LES, but with unique strains (Al-Aloul et al., 2004). Somayaji R. and colleagues (2017) performed a 34 year clinical outcome study monitoring 274 CF patients and found PES was associated with a greater risk of death or lung transplant and the rate of lung function decline was greatest in the PES group (Somayaji et al., 2017).

Therapy and resistance

P. aeruginosa is intrinsically resistant to many antimicrobials due to its cell wall structure and its ability to express a wide variety of efflux pumps. *P. aeruginosa* can become resistant through mutation under selective conditions (such as the CF airway) and can also acquire resistance through uptake of plasmids, bacteriophage activity and transposons. *P. aeruginosa* possesses many multidrug-efflux pumps such as the MexAB-oprM pumps which extrude beta-lactams, quinolones and disinfectants, the MexXY-oprM pumps extruding aminoglycosides and the MexEF-oprN pumps extruding carbapenems and quinolones. Overexpression of the MexXY

pump is common in CF isolates (Singh et al., 2017) and a common mutational target in chronic CF airway infections is the multidrug efflux gene *mexZ* (Sobel et al., 2003).

Antibiotic sensitive strains of *P. aeruginosa* infections are usually treated with beta-lactams, such as ceftazidime, meropenem or an anti-pseudomonal penicillin combined with tobramycin or colistin (Trust, 2009). Colistin can often be reserved for treatment of more resistant strains of *P. aeruginosa*, as is often the case in chronic infection (Defez et al., 2004). MDR *P. aeruginosa* has been shown to arise due to exposure to antibiotics (Mohr et al., 2004). There is considerable susceptibility diversity within the *P. aeruginosa* population isolated from an individual sputum sample (and within colony morphotypes) (Workentine et al., 2013; Foweraker et al., 2005; Qin et al., 2018). This poses considerable complications when performing susceptibility testing in the clinical setting.

The ability of *P. aeruginosa* to adapt to the CF airway by increasing several virulence mechanisms and its intrinsic and adapted antibiotic resistance make it a very complex infection to treat. Early diagnosis and eradication are considered to be vital in delaying onset of chronic infection upon which morbidity and life expectancy are severely curtailed.

S. aureus in CF

S. aureus is the most common Gram positive bacteria detected in people with CF (CFF, 2016; CFRI, 2016; Goss and Muhlebach, 2011). It is often the first bacteria isolated and is more common in childhood (Fig. 1). The prevalence of methicillin-susceptible *S. aureus* (MSSA) in children with CF (≤19 years old) in 2016 in Ireland was approximately 56.3% (CFRI, 2016), which is higher than the prevalence in the UK and US, 31.5% and 55%, respectively (Trust, 2016; Razvi et al., 2009). An average of 37.6% of children with CF in Ireland were recorded to be chronically colonized with *S. aureus*, whereas only 22.1% of adults were chronically colonised (CFRI, 2016). Prevalence of *S. aureus*, both MSSA and MRSA, is on the rise in the US (CFF, 2016) (Fig. 2).

Therapy and resistance

Since the emergence and dissemination of penicillin resistance in *S. aureus*, methicillin, cloxacillin and flucloxacillin have become first-line therapies. Narrow spectrum antibiotics such as flucloxicillin can be used as prophylactic drugs and dose can be increased if required. Flucloxacillin prophylaxis for children with CF is widely debated with countries and even clinics within certain countries differing in their approach. Prophylaxis is currently recommended from infancy in the United Kingdom (UK, 2009), advised against in the United States (Lahiri et al., 2016) while in Ireland there is no consensus, with individual clinics differing in their approach. There has been an increase in studies attempting to determine the benefit of anti-staphylococcal prophylaxis however there are contradicting outcomes (Stutman et al., 2002; Smyth and Walters, 2003). One trial reported no significant differences in clinical outcome between the placebo group and a trial group treated with cephalexin (5–7 year course), however it was noted that children treated with *S. aureus* prophylaxis were more likely to become positive for *P. aeruginosa* (Stutman et al., 2002). Conversely, other work has proven effective clearance of *S. aureus* and improved lung function upon anti-staphylococcal treatment (McCaffery et al., 1999).

MRSA

There has been a rising prevalence of methicillin-resistant *S. aureus* (MRSA) in CF in the US (~26%) (CFF, 2016) (Fig. 2) in the past 2 decades but not in Canada, Australia (2.6%) and Europe (2.6%) (Parkins and Floto, 2015). Methicillin resistance is caused by mutations in the *mecA* gene encoding PBP2A (a transpeptidase involved in cell wall maintenance) resulting in reduced affinity for penicillin and other beta-lactams. The mecA gene is present on a mobile staphylococcal chromosomal cassette (SCC*mec*) which means it is transferrable and 11 SCC*mec* types have been identified to date. MRSA has been shown to cause similar lung function decline and inflammation as *P. aeruginosa* in CF (Gangell et al., 2011; Sagel et al., 2009). MRSA infection can contribute to morbidity in CF patients, with an infected paediatric cohort showing impaired growth and an increased requirement for intravenous antibiotics compared to a non-MRSA infected cohort (Miall et al., 2001). In a large decade long analysis of over 17,500 individuals in the Cystic Fibrosis Foundation (CFF) Patient Registry, a more rapid decline in lung function was noted in those with persistent MRSA infection relative to controls (Dasenbrook et al., 2008). MRSA infection has been identified as an independent risk factor for failure to recover lung function following pulmonary exacerbation (PEx) (Sanders et al., 2010). Compared to patients with chronic MSSA infection or transient MRSA infection, those with chronic MRSA have an increased risk of death (Dasenbrook et al., 2010). Risk factors for chronic MRSA infection in CF include patient-specific factors such as genotype, level of pancreatic insufficiency, antibiotic use and comorbidities such as diabetes.

MRSA is resistant to β-lactams and often aminoglycosides and macrolides (Leski et al., 1999), thus it is recommended that tetracyclines and clindamycin are used, with glycopeptides and linezolid advised for more severe infections (UK, 2009). Linezolid, ceftaroline and tigecyclin resistant MRSA isolates have now been detected from CF airway samples (Champion et al., 2014; Long et al., 2014). New treatment strategies for MRSA in CF are being explored such as eradication strategies however it is uncertain whether eradication prevents development of chronic MRSA colonisation later in life (Dasenbrook et al., 2008; Dasenbrook et al., 2010).

Transmission

The anterior nares are a reservoir for *S. aureus*. There is some evidence of transmission of MSSA and MRSA from patient-to-patient (Garbacz et al., 2018; Givney et al., 1997; Schlichting et al., 1993; Muhlebach, 2017), between family members (Goerke et al., 2000) and siblings of CF patients (Elizur et al., 2007). It has been suggested that current MRSA infection management in CF should focus on controlling transmission (Muhlebach, 2017; Bell and Flume, 2017).

H. influenzae and *H. parainfluenzae* in CF

H. influenzae and *H. parainfluenzae* are among the most common Gram negative bacteria identified from the CF airway and are more prevalent in childhood. Both *Haemophilus* species are fastidious, non-motile, rods (or coccobacillus) and can form capsules which are important in virulence. Upon formation of biofilms, this bacteria exhibits reduced susceptibility to antibiotics, and thus can become chronic and cause lung damage (Starner et al., 2006).

Approximately 30.6% of children (aged ≤19 years) were colonized with this bacteria in Ireland in 2016 (CFRI, 2016). *H. influenzae* is more prevalent in childhood than adulthood, with the prevalence for this bacteria dropping to an average of 7.9% in adults over 19 years of age (CFRI, 2016). This reflects colonisation patterns reported in the US (CFF, 2016) (Fig. 1). A 15 year study on 1528 *H. influenzae* and *H. parainfluenzae* isolates from 349 CF patients found that the prevalence of both bacteria and antibiotic resistance increased annually (Ebbing and Robinson, 2015). Cardines et al. (2012) found the majority of *H. influenzae* strains in their CF cohort to be nonencapuslated or nontypeable (NTHi), with many different genotypes identified. Genes involved in biofilm production and adhesion were persistent in *H. influenzae* colonising clones. There have been some reports of *H. influenzae* colonisation associated with lung function decline (Ramsey et al., 2014; Vandenbranden et al., 2012) while other studies have linked *H. influenzae* colonisation to preserved lung function (Hector et al., 2016). In a recent study *H. influenzae* was observed to have no link to inflammation in CF (Ratjen et al., 2016). The role of *H. influenzae* in exacerbation and disease progression is uncertain.

Colonisation with *H. influenzae* is generally treated with antibiotics such as co-amoxiclav or doxycycline for two to four weeks, to which most strains of *H. influenzae* are susceptible (UK, 2009). Resistances include azithromycin, amoxicillin and macrolides, while resistance to imipenem may be on the increase, though some resistant strains still remain susceptible to ampicillin (UK, 2009; Giufre et al., 2011).

S. maltophilia in CF

S. maltophilia is a Gram negative, MDR bacteria with a prevalence of up to 15% in CF patients in Ireland (CFRI, 2016) and a prevalence of 13.1% in the US in 2016 (CFF, 2016). It has been reported that prevalence of this pathogen is on the increase (CFF, 2016) (Fig. 2) however issues with the changing taxonomy of this species make these findings less reliable. CF patients can be persistently colonised with *S. maltophilia* over a number of years and can be colonised with numerous strain types at one time (Esposito et al., 2017).

S. maltophilia colonisation has been associated with poor clinical outcomes. One study found that CF patients positive for *S. maltophilia* had worse lung function than *S. maltophilia* negative patients (Goss et al., 2004). In a complimentary study IL-8, Tumour Necrosis Factor-alpha (TNFa) and *S. maltophilia* antibodies were higher while lung function was lower in patients with chronic *S. maltophilia* colonisation, compared to intermittently or non-*S. maltophilia* colonised patients (Waters et al., 2011). *S. maltophilia* colonisation has also been associated with a 3-fold increased risk of death and lung transplantation (Waters et al., 2013) and increased hospitalisation rates (Barsky et al., 2017).

Treatment of *S. maltophilia* is often based on co-trimoxazole due to its resistance to anti-pseudomonal drugs (UK, 2009). Though one study identified high rates of co-trimoxazole resistance *in vivo* and suggested doxycycline and a high concentration of colistin to be the most active against *S. maltophilia*, despite multidrug resistance becoming a common occurrence (San Gabriel et al., 2004).

A. xylosoxidans in CF

The true prevalence of *A. xylosoxidans* in CF is hard to establish largely due to disparities in taxonomy of the species. It has been named *Alcaligenes xylosoxidans* in the past and isolates currently defined as *A. xylosoxidans* may actually comprise multiple species. The CFF patient registry reported a prevalence of 6.3% in 2016 (Fig. 1) with prevalence remaining largely the same from 1990 to 2016 (Fig. 2) (CFF, 2016). Prevalence rates up to 11% have been reported (CFRI, 2016; Edwards et al., 2017).

Evidence suggests there is limited impact of *A. xylosoxidans* on clinical outcomes. Two studies have reported no association between *A. xylosoxidans* colonisation (either persistent or transient) and lung function decline (Edwards et al., 2017; De Baets et al., 2007). Edwards et al. (2017) observed no transmission events in a long-term genotyping study (Edwards et al., 2017) however transmission of a clone of *A. xylosoxidans* has been previously reported (Cools et al., 2016). Infection with *A. xylosoxidans* is generally transient with some reports of chronic infection.

Other CF Pathogens

Many other microorganisms are commonly found in the lung of CF patients including *Streptococci spp*, *Klebsiellae pneumoniae*, *Acinetobacter baumannii* and more rarely *Burkholderia cepacian complex* and *Mycobacteria spp*. Other bacteria detected in low frequency from CF airway samples include *Ralstonia spp*, *Pandoraea spp*, *Herbaspirillum spp*, *Bordatella pp*, *Moraxella spp*, *Serratia marcescens* and *Xanthomonas spp* (Coburn et al., 2015; Davies and Rubin, 2007; Lipuma, 2010; Rocha et al., 2018; Floto et al., 2016).

Streptococcus. This genus has been shown to be one of the core bacteria present in children with CF using molecular tools (Coburn et al., 2015; Zemanick et al., 2017). Sibley and colleagues identified several species in the *S. milleri* group (SMG) from CF airway samples (Sibley et al., 2010). In a recent large-scale single centre retrospective study of 381 CF adults, group B *Streptococcus*

(GBS) was found in only 3.5% of the patients (Skolnik et al., 2017). There were no shared strains among patients and patients colonised with GBS were not at increased risk of developing exacerbation and did not experience poorer lung function. The clinical significance of *Streptococcus* species in CF is uncertain although there have been some reports of SMG causing bronchopulmonary exacerbations (Parkins et al., 2008; Cade et al., 1999).

A. baumannii. *Acinetobacter* is becoming recognised as an emerging pathogen in CF, with one recent study identifying colonisation in 9% of samples from CF patients (Fernandez-Olmos et al., 2012). *A. baumannii* is phenotypically indistinguishable from those closely related species in routine laboratory technologies, thus the term Acb complex now covers *A. calcoaceticus*, *A. baumannii*, *A. seifertii* and *A. dijkshoorniae*. Recently over 50 different strains of *Acinetobacter* species were identified from CF sputum samples, many of which were MDR (Rocha et al., 2018). MDR carbapenemase-resistant strains of *A. baumannii* accounted for approximately 28% of all *A. baumannii* isolates in this study. These MDR strains may represent lineages which are a source of resistance genes with potential to spread to other pathogens in the CF airway. The current recommendation for treatment of *A. baumannii* infections is carbapenems, though a rising resistance has been documented (Karageorgopoulos and Falagas, 2008).

Burkholderia cepacia complex (Bcc) (Once called *Pseudomonas cepacian*) is a collection of phenotypically similar Gram negative bacteria, which differ by genotype and are divided into approximately nine species. Prevalence of Bcc in the US was 2.7% in 2016 (CFF, 2016). All nine species of *Burkholderia* have been detected in CF however *B. multivorans* and *B. cenocepacia* are the most common. Bcc infection can become chronic or even cause 'cepacia syndrome' which leads to a serious decline in lung function and can exhibit invasive disease (Mahenthiralingam et al., 2005). Epidemic strains have been identified in Canada (Johnson et al., 1994), the UK and Ireland (Pitt et al., 1996), the US (Chen et al., 2001) and several European countries (Drevinek et al., 2005; Campana et al., 2005). Implementation of appropriate infection control measures was demonstrated to eliminate transmission events (Chen et al., 2001). Bcc isolates from CF patients have been shown to be highly resistant to carbapenemases (Van Dalem et al., 2018) and ceftazidime-avibactam and ceftolozane-tazobactam (Van Dalem et al., 2018).

Mycobacteria. Non-tuberculosis mycobacterium (NTM) and most frequently *Mycobacterium avium complex* (MAC) (*M. intracellulare* and four *M. avium* subspecies) and *M. abscessus* complex (MABSC) (subspecies *abscessus*, *massiliense* and *bolletii*) are isolated from CF respiratory samples. In a large study of the UK CF patient registry an NTM prevalence of 3.8% in 2015 was reported and this had risen from 1.3% in 2010 (Gardner et al., 2018). A similar analysis of the US CFF patient registry from 2010 to 2014 reported 20% NTM prevalence of which 61% were MAC and 39% were MABSC (Adjemian et al., 2018). While the 2016 CFF registry report found 12.7% of patients in the US to be positive for Mycobacterial species (CFF, 2016). A recent whole genome sequencing study revealed that two MDR *M. abscessus subspecies massiliense* strains were transmissible among a CF population in the UK causing two outbreaks (Bryant et al., 2013). NTM can cause inflammation and a condition called 'NTM pulmonary disease' (NTM-PD) in CF but can also reside intermittently or persistently without causing any symptoms. MAC colonisation has been shown to negatively impact lung function in CF (Qvist et al., 2016). Considering the above and the difficulties in diagnosing NTM in CF, the CFF and European Cystic Fibrosis Society (ECFS) published a consensus recommendations document to standardise the management of NTM infection in individuals with CF (Floto et al., 2016).

Fungi in the CF Airway

Although much attention is given to the bacteria that colonise the CF airway, fungi are also important in CF lung infection. The most commonly isolated fungi from the CF airways include *Aspergillus fumigatus*, *Candida albicans*, *Scedosporium spp.*, *Pneumocystis jirovecii* and *Exophiala dermatitidis*. The prevalence of fungi among CF patients varies significantly, ranging from 11% to 60% for *A. fumigatus* (Bakare et al., 2003; Valenza et al., 2008; Ziesing et al., 2016; Reece et al., 2017), 38%–78% for *C. albicans* (Bakare et al., 2003; Valenza et al., 2008; Ziesing et al., 2016; Noni et al., 2015a), for 3%–17% *Scedosporium spp.* (Sedlacek et al., 2015; Blyth et al., 2010), 12%–38% for *P. jirovecii* (Pederiva et al., 2012; Hernandez-Hernandez et al., 2012) and 1%–17% for *E. dermatitidis* (Horre et al., 2004; Kondori et al., 2014). Reported prevalence of fungi tends to vary greatly between studies depending on the method of detection used.

A. fumigatus. Of the *Aspergillus* species to colonise the CF airways, *A. fumigatus* is the most common, followed by *A. niger*, *A. terreus*, and *A. flavus*. Allergic bronchopulmonary aspergillosis (ABPA) is the most recognised fungal disease in CF and is caused by a Th2-mediated hypersensitivity response to *Aspergillus*. The prevalence of ABPA in CF is between 2% and 25% (Armstead et al., 2014; Maturu and Agarwal, 2015). A 2010 study reported chronic *A. fumigatus* infection was associated with significantly lower FEV_1% predicted (Amin et al., 2010). Itraconazole treatment of *A. fumigatus* colonised patients was shown to improve lung function suggesting a pathogenic role for *A. fumigatus* in CF (Chotirmall et al., 2008). Numerous studies have now concluded that CF patients chronically colonised with *A. fumigatus* had a more rapid decline in FEV_1 and in general experienced worse lung function than non-colonised patients (Reece et al., 2017; Noni et al., 2015b; Saunders et al., 2016). Gangell et al. (2011) carried out a study with 215 paediatric patients with CF and found those that were *Aspergillus* positive had increased inflammatory responses (neutrophils, neutrophil elastase and IL-8) compared to those who were *Aspergillus* negative. Although there are contradictory studies unable to link *A. fumigatus* colonisation to worse disease (de Vrankrijker et al., 2011).

A. fumigatus infection and ABPA in CF can be treated using posaconazole prophylaxis (Karthaus, 2011) or exacerbation therapy with systemic corticosteroids and azoles, most commonly itraconazole (Coughlan et al., 2012; Mahdavinia and Grammer, 2012). However there is no consensus on the treatment of *A. fumigatus* colonisation in CF with some clinics opting to treat upon positive culture and other only treating if symptoms of ABPA develop.

C. albicans. *C. albicans* has been associated with decreased FEV_1 and body mass index (BMI) and increased hospital-treated exacerbations (Chotirmall et al., 2010; Navarro et al., 2001). Baseline FEV_1 was also significantly lower and annual decline was significantly higher in patients chronically colonised by *C. albicans* compared to non-colonised patients. However other studies have not observed any negative association between *C. albicans* colonisation and lung function or BMI (Hector et al., 2016). It is likely that frequent use of antibiotics and steroids predispose CF patients to colonisation with this species (Noni et al., 2015a). Options for treatment of *C. albicans* infections include fluconazole and amphotericin (UK, 2009).

The CF Airway Microbiome

Traditionally, bacterial identification and detection were reliant upon selective and differential cultivation techniques. These practices could not account for fastidious and low abundance bacteria. In the past two decades culture independent approaches have revolutionised CF airway microbiology. The CF airway is now known to harbour a diverse community of bacteria (Sibley et al., 2006; Rogers et al., 2003), fungi (Willger et al., 2014; Delhaes et al., 2012) and viruses (Willner et al., 2009). The 'microbiome' has been defined as *"the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes, and the surrounding environmental conditions"* (Marchesi and Ravel, 2015). Molecular techniques such as T-RFLP, 16S rRNA gene cloning, Sanger sequencing and more recently next generation sequencing (NGS) have improved detection of previously under-identified genera such as *Streptococcus* and have identified anaerobic genera such as *Prevotella* and *Veillonella* as not only common in the CF airway but highly abundant (Zemanick et al., 2017; Rogers et al., 2003; Bittar et al., 2008).

The core microbiota of both adult and paediatric CF patients were recently defined by Coburn et al. (2015). The genera listed in Table 1 are commonly identified from the CF airway using molecular tools. Among these highly abundant genera, many are

Table 1 The most common conventional and emerging genera identified from the CF airway using molecular approaches

	Genus	*Respiration*
Conventional genera	*Achromobacter*	Ae
	Burkholderia	Ae
	Haemophilus	Ae, F
	Pseudomonas	Ae
	Staphylococcus	Ae
	Stenotrophomonas	Ae
Emerging genera	*Actinomyces*	F, An
	Bacteroides	An
	Fusobacterium	An
	Gemella	F
	Granulicatella	F
	Leptotrichia	An, F
	Moraxella	Ae
	Mycobacterium	Ae, F
	Neisseria	Ae
	Porphyromonas	An
	Prevotella	An
	Rothia	Ae, F
	Serratia	F
	Streptococcus	F
	Veillonella	An
Other genera commonly detected	*Abiotrophia*	F
	Acinetobacter	Ae
	Atopobium	An
	Blautia	An
	Campylobacter	F
	Capnocytophaga	F
	Catonella	An
	Corynebacterium	Ae, F
	Inquilinus	F
	Mycoplasma	Ae, F
	Oribacterium	An
	Pandoraea	F
	Ralstonia	F
	Selenomonas	An
	Sphingomonas	Ae

Note: Ae = aerobic, An = anaerobic, F = facultative anaerobic.

considered non-classical CF pathogens and with a considerable amount being anaerobes. The relative abundances of these genera vary considerably depending on patient age (Coburn et al., 2015; Cox et al., 2010), different regions of the respiratory tract (Klepac-Ceraj et al., 2010; Renwick et al., 2014) and individual differences between patients (de Dios Caballero et al., 2017).

Patient increasing age is significantly correlated with decreased alpha-diversity (Cox et al., 2010; Klepac-Ceraj et al., 2010) and the risk of acquiring pathogenic organisms increases with age (Cox et al., 2010). The reducing community complexity in adult patients may be due to frequent disturbances to the ecological environment as CF patients are exposed to long-term, high dose antibiotics and indeed antibiotic usage has been associated with reduced diversity and *P. aeruginosa* invasion as patients age (Klepac-Ceraj et al., 2010). Some studies have shown that antibiotics impact the microbial community structure (Zhao et al., 2012; Klepac-Ceraj et al., 2010) while others have shown little influence of antibiotics on the overall diversity and community composition (Fodor et al., 2012). Antibiotic therapy directed towards *P. aeruginosa* has been shown to have little effect on *P. aeruginosa* bioburden but did significantly reduce the relative abundance of anaerobic species in the CF airway microbiome (Carmody et al., 2018). Repeated exposure to antibiotics most likely has an impact on the microbial community structure however whether this is transient or has long-term effects is still not known.

It is becoming increasingly evident that anaerobes are important in CF disease. In a 10-year study monitoring community composition fluctuations in 111 CF patients, diversity and the relative abundance of anaerobes increased during exacerbations and prior to antibiotic exposure in a disease stage dependent manner (Carmody et al., 2018). Conversely, a higher relative abundance of anaerobes has recently been linked to milder disease in CF with better nutritional status, pancreatic sufficiency and better lung function (Muhlebach et al., 2018) suggesting that these anaerobes may be beneficial in the CF airway. A study by Tunney and colleagues suggests a role for *Prevotella* species in shielding *P. aeruingosa* from antibiotics (Sherrard et al., 2016) which suggests a role for anaerobes in passive resistance.

Linking microbial community composition to measures of disease severity such as exacerbation and lung function is vital to establishing the significance of the microbiome in CF lung disease. Community composition fluctuations were monitored in a 10-year study of 111 CF patients (Carmody et al., 2018). Changes to the microbiome during exacerbation were shown to be largely dependent on community composition and diversity at baseline, with particular genera such as *Pseudomonas* and *Gemella* acting as important drivers of change (Carmody et al., 2013). In a climax-attack model established by Carmody et al. (2013) dominant pathogens in the microbiome, such as *P. aeruginosa*, were found to decrease with the onset of symptoms while overall diversity increased (Carmody et al., 2013). The climax or baseline microbiota consisting of typical CF pathogens and the attack/ exacerbation community being dominated by anaerobes such as *Prevotella*, *Veillonella*, *Fusobacterium* and microaerophilic *Streptococcus*. Alternatively other studies have shown there to be little change in microbial community composition in patients during exacerbation and when these patients were clinically stable (Fodor et al., 2012; Cuthbertson et al., 2016). While the link between exacerbation and microbial community composition is not clear, poor lung function is clearly correlated to reduced microbiome diversity as has been published in a number of studies (Delhaes et al., 2012; Acosta et al., 2017; Stressmann et al., 2012).

Microbial Interactions in the CF Airway Microbiome

We now have a more comprehensive view of the microbiology of CF airways and co-infections involving different species of bacteria probably represent the norm in the CF lung. Dominance of the CF airway by specific pathogens, particularly *P. aeruginosa*, has been shown to impact microbial community diversity (Klepac-Ceraj et al., 2010; Renwick et al., 2014). How *P. aeruginosa* colonisation changes the surrounding microbiome and indeed how diverse members of the community interact in general is not well understood. Some studies have begun to shed light on the polymicrobial interactions taking place among these bacteria (Table 2). These studies provide evidence that inter-species and cross-Kingdom interactions can influence not only pathogen virulence but also host responses. The impact of these interactions in CF airway disease are yet mostly unexplored.

Summary

CF airway microbiology is complex and while some microorganisms such as *P. aeruginosa*, have a clear role in inflammation and disease progression, the role of other microorganisms is less clear. With the increased sensitivity of molecular techniques the diversity of microbes colonising the CF airway is becoming apparent. Several studies have found anaerobes to be highly prevalent and abundant in the CF airway. While other studies are identifying an ever increasing number of "emerging species" previously not known to colonised the airway. Care must be taken in interpreting the significance of low abundance microorganisms as the lower airway microbiome is low density and this brings technical issues with distinguishing true microbiome from background noise and contamination. Nevertheless, the discovery of these complex communities has revolutionised CF airway microbiology and highlighted the potential for inter-species and cross-kingdom interactions to influence host immunity and thus disease progression. Many groups are now focusing on using bioinformatic tools and *in vitro* models to further tease out symbiotic, mutualistic, competitive and antagonistic relationships within microbial communities and how they may impact disease (Magnusdottir et al., 2017; Shah et al., 2016). It is evident that the CF airway breeds diverse, adapted and resistant microbial phenotypes and with this comes infections that are extremely challenging to treat. Understanding more about the complex airway microbiome in chronic lung diseases may provide alternative avenues for developing novel therapeutic approaches in the future.

Table 2 Studies reporting microbial interactions with *P. aeruginosa*

Species interacting with Pa	*Microbial/host response*	*Potential implications on disease*	*Ref(s)*
Gram positives	↑ lytic activity by Pa ↓ Gram+ in *in vivo* models	Pa more toxic in co-infections with Gram+	(Korgaonkar et al., 2013)
	↑ pyocyanin production by Pa	Pa mechanisms of dominance	(Korgaonkar and Whiteley, 2011)
S. aureus	Co-infection strains less competitive than mono-infection strains	Adaptation to coexistence in the lung	(Reece et al., 2018)
	Pa induces bronchial epithelial cells to produce phospholipase, sPLA2-IIA	Manipulation of host immune response, enhanced survival of Pa and killing of Sa	(Pernet et al., 2014)
	Pa EPS can affect mixed species biofilm architecture	Proximity of Sa and Pa in mixed biofilms	(Chew et al., 2014)
	↑ Pa siderophore production	Iron competition	(Tognon et al., 2017)
	Pa LPS inactivation mutations	Reduced recognition by immune system, persistence	(Tognon et al., 2017)
	↑ resistance of Pa to B-lactams	Resistance	(Tognon et al., 2017)
S. maltophilia	Co-colonise the CF airway	Opportunity to interact in airway	(Wainwright et al., 2009)
	↓ Sm growth	Altered virulence and persistence	(Pompilio et al., 2015)
	↑ Pa biofilm		(Ryan et al., 2008)
	↓ Adhesion of Pa to CFBE	Evasion of immune system and persistence	(Pompilio et al., 2015)
	↑ tobramycin resistance of Sm in mixed biofilms	Resistance	(Pompilio et al., 2015)
	↑ tolerance of Pa to polymixin	Resistance	(Ryan et al., 2008; Huedo et al., 2015)
A. fumigatus	Co-colonise the CF airway	Opportunity to interact in airway	(Armstead et al., 2014)
	Co-colonised patients have reduced lung function	Directly associated with disease outcome	(Reece et al., 2017)
	Pa SNs stimulate Af growth	Increased Af abundance in co-infections[a]	(Briard et al., 2016)
	Metacaspases from Pa SNs inhibit and damage Af biofilms	Reduced Af abundance in co-infections[a]	(Shirazi et al., 2016)
	↑ elastase production by Pa in presence of Af	More damaging pathology	(Smith et al., 2015)
	SNs from co-cultures more toxic to epithelial cells lines	More damaging pathology	(Smith et al., 2015)
	Mutually antagonistic	Competition beneficial to host?	(Reece et al., 2018)
	Gliotoxin produced by Af reduces Pa biofilm	Competition beneficial to host?	(Reece et al., 2018)
	Co-infections cause altered inflammatory response	Evasion of the immune system and persistence	(Reece et al., 2018)
	Pa dirhamnolipids induce Af ECM production	Inhibits Af growth[a] and facilitates Pa binding	(Briard et al., 2017)

[a]Contradictory findings.

Note: Pa = *P. aeruginosa*, Sa = *S. aureus*, Sm = *S. maltophilia*, Af = *A. fumigatus*, SNs = supernatants, EPS = exopolysaccharide, LPS = lipopolysaccharide, CFBE = cystic fibrosis bronchial epithelial cells, ECM = extracellular matrix.

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Mixotrophy Among Freshwater and Marine Protists

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Glossary

Autotroph An organism capable of synthesizing its own food from inorganic substances.
Bacterivory The ingestion of bacteria as food or as an energy supply.
Chlorophytes A group of microalgae that are coccoid or bi-flagellated, solitary or in colonies. They are green and possess chlorophylls *a* and *b* and store starch in the chloroplast. Very common in freshwater, but can also be found in marine waters.
Chloroplast Membrane-bound structure (=plastid) containing photosynthetic pigment found within a cell.
Chrysophytes A group of protists with two flagella (one short and one long) that belong to the Heterokonta. They have chloroplasts with chlorophylls *a* and *c*. They are yellowish due to carotenoids. They are common in both marine and freshwaters.
Ciliates A group of protists belonging to the Aveolata. They are characterized by the presence of hair-like organelles called cilia, which are identical in structure to eukaryotic flagella, but are in general shorter and present in much larger numbers.
Constitutive mixotrophs (CMs) Protists with built-in chloroplasts that are capable of phagotrophy.
Cryptophytes A group of protists in which most of the species contain chloroplasts with chlorophylls *a* and *c*, together with phycobili-proteins and other pigments; they vary in color (brown, red to blueish-green). Cells contain an extra reduced nucleus called a nucleomorph, which is derived evolutionarily from a previous endosymbiosis occasion.
Dinoflagellates A group of protists belonging to the Alveolata, very common in marine and freshwaters. They are bi-flagellated and possess a dinokaryon, a special nucleus characterized by the presence of a permanent nuclear envelope and chromosomes in a permanently compacted state. Half of the species lack built-in chloroplasts.
Immature successional stages A term used here to describe the colonization of pelagic waters by a few fast growing species. Immature successional stages are typically found during the spring bloom in temperate and polar regions, and upwelling areas where nutrient rich water is brought up from deep waters into the light.
Kleptoplastidy Nutritional strategy in which phototrophy is aquired by sequestration and subsequent utilization of plastids and other cell organelles from prey organisms.
Mature successional stages A term used here to describe the successional stages dominated by many slower-growing species. Mature successional stages are typically found during summer periods in temperate and polar regions, and year round in tropical open marine waters. Concentrations of inorganic nutrients are low during these stages.
Mixotroph As used here, a planktonic organism that mixes photosynthesis and food uptake via phagotrophy; sometimes referred to as a photo-phago-mixotroph.
Non-constitutive mixotrophs (NCMs) Mixotrophic protists which lack built-in chloroplasts. Some NCMs utilize ecto- or endosymbionts, while others sequester chloroplasts and sometimes other cell organelles from their prey. Species in the latter group are often referred to as klepto(chloro)plastidic.
Pelagic Describing or referring to organisms that are living in the water column in fresh and marine waters, either swimming or passively suspended; opposite to benthic (living on the bottom).
Phagotrophy Ingestion of particulate matter.

Introduction

A wide diversity of marine and freshwater unicellular eukaryotes (protists) are capable of acquiring energy, carbon and nutrients through both photosynthesis and phagotrophy of prey. Among these so-called photo-phago-mixotrophs, two major groups can be distinguished: (1) Protists with permanent, built-in chloroplasts, termed 'Constitutive mixotrophs (CMs)' (Fig. 1). These are often referred to as phytoplankton that can eat microbial prey (e.g., bacteria or other protists). (2) Protists that lack permanent chloroplasts (and often regarded as microzooplankton), but use photosynthetic machinery derived from their algal prey. These are termed, 'Non-constitutive mixotrophs (NCMs)'. Some NCMs keep the engulfed algal cells intact as ecto- or endosymbionts

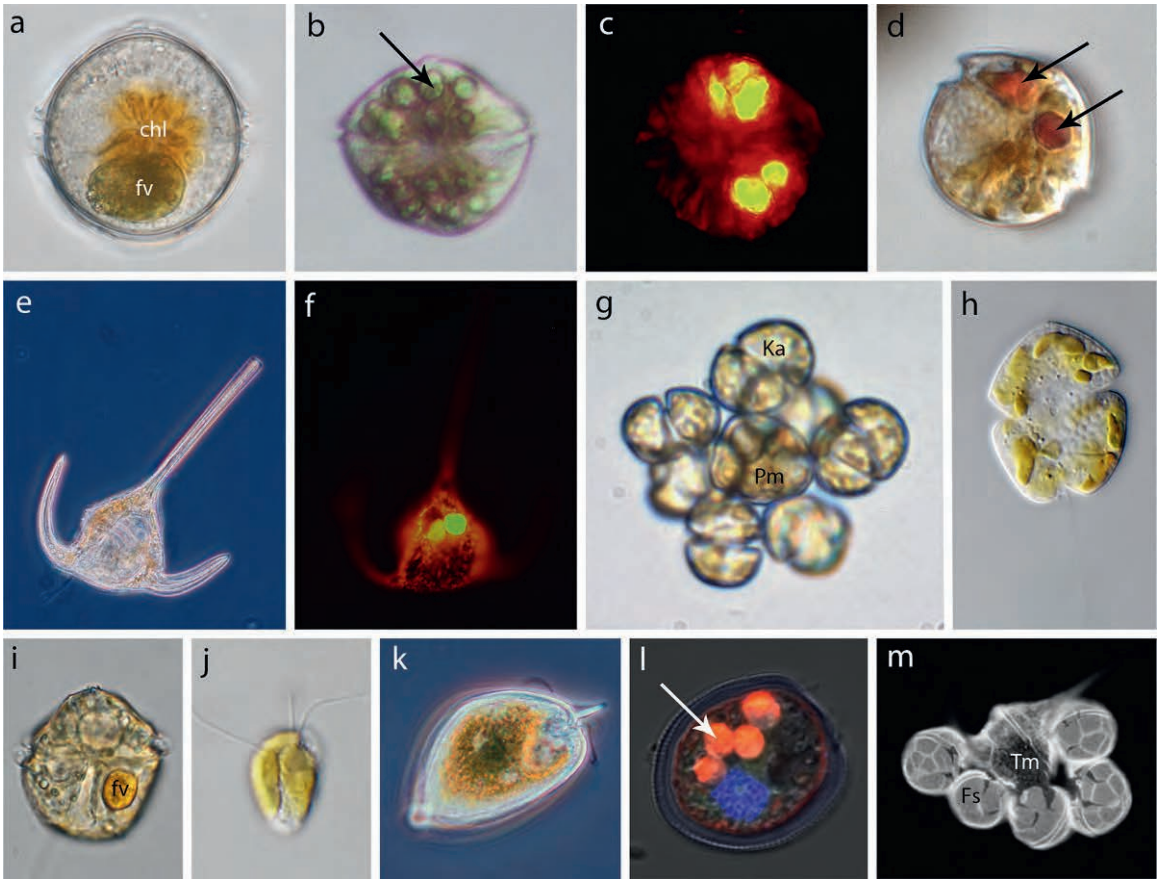

Fig. 1 A panel of light and epifluorescent microscopy images showing different marine CMs. (a) *Alexandrium* sp. (ca. 45 μm in width) found in Greenland with a large food vacuole (fv), and a reduced chloroplast (chl). Photo by Hannah Blossom. (b) *A. pseudogonyaulax* (ca. 40 μm in width) found in Denmark with many food vacuoles (arrow). Photo by Hannah Blossom. (c) Epifluorescent microscopy image of *A. pseudogonyaulax* (red), which had eaten *Teleaulax acuta* (visible as yellow food vacuoles). The food vacuoles (yellow) are easy to distinguish against the red autofluorescence of dinoflagellates. Photo by Hannah Blossom. (d) *A. andersonii* (ca. 20 in width) fed *Teleaulax* sp. which are visible as food vacuoles (arrows). Photo by Kyong Ha Lee, courtesy of Hae Jin Jeong. (e) *Tripos menueri* (= *Ceratium tripos*) (ca. 60 μm wide and 200–250 μm long). Photo by Per Juel Hansen. (f) *Tripos menueri* found in Denmark with two food vacuoles (yellow). Photo by Hannah Blossom. (g) *Karlodinium armiger* (Ka, ca. 18 μm in length) swarming around and feeding on *Prorocentrum minimum* (Pm). Modified from Berge, T., Hansen, P.J., Moestrup, Ø., 2008. Feeding mechanism, prey specificity and growth in light and dark of the plastidic dinoflagellate *Karlodinium armiger*. Aquatic Microbial Ecology 50, 279–288, with permission from Inter-Research. (h) *Karenia mikimotoi* (20 μm in width). Photo by Gert Hansen. (i) *Lingulodinium* sp. found in the field in Greenland with a food vacuole (fv). Photo by Hannah Blossom. (j) The haptophyte *Prymnesium parvum* (ca. 10 μm long). Photo by Gert Hansen. (k) *Prorocentrum minimum* (15 μm in length). Photo by Per Juel Hansen. (l) Confocal laser scanning micrograph image of *Prorocentrum minimum* (15–20 μm in length) fed with *Teleaulax amphioxei*. Four food vacuoles are visible, one of which is indicated with an arrow. Modified from Johnson, M.D., 2014. Inducible mixotrophy in the dinoflagellate *Prorocentrum minimum*. Journal of Eukaryotic Microbiology 62, 431–443, with permission from John Wiley and Sons. (m) Five *Fragilidium subglobosum* (Fs, 40 μm) engulfing *Tripos menueri* (Ct). Adapted from Skovgaard, A., 1996. Engulfment of *Tripos* spp. (Dinophyceae) by the thecate photosynthetic dinoflagellate *Fragilidium subglobosum*. Phycologia 35, 490–499. All these examples are dinoflagellates except *Prymnesium parvum* (j).

(Fig. 2), while other NCMs, often termed klepto(chloro)plastidic species, retain only the chloroplasts or the chloroplasts along with prey nuclei, mitochondria, and cytoplasm (Fig. 3). Some NCMs are prey specialists and can only exploit very specific types of prey (Specialist NCM = SNCMs), while others can exploit a wide range of prey (Generalist = GNCMs).

How Common are Photo-Phago-Mixotrophic Protists in Different Aquatic Environments Compared to Photoautotrophs and Heterotrophs?

Photo-phago-mixotrophic protists are common, and often very abundant, in both freshwater and marine environments ranging from eutrophic to highly oligotrophic, and from tropical to polar. However, the relative contributions of CMs and different NCM groups to standing protist biomass and production across different scales of time and space are mostly unknown. This knowledge gap is mainly explained by the difficulty in identifying which organisms are actively mixotrophic in the field. Below we summarize current knowledge.

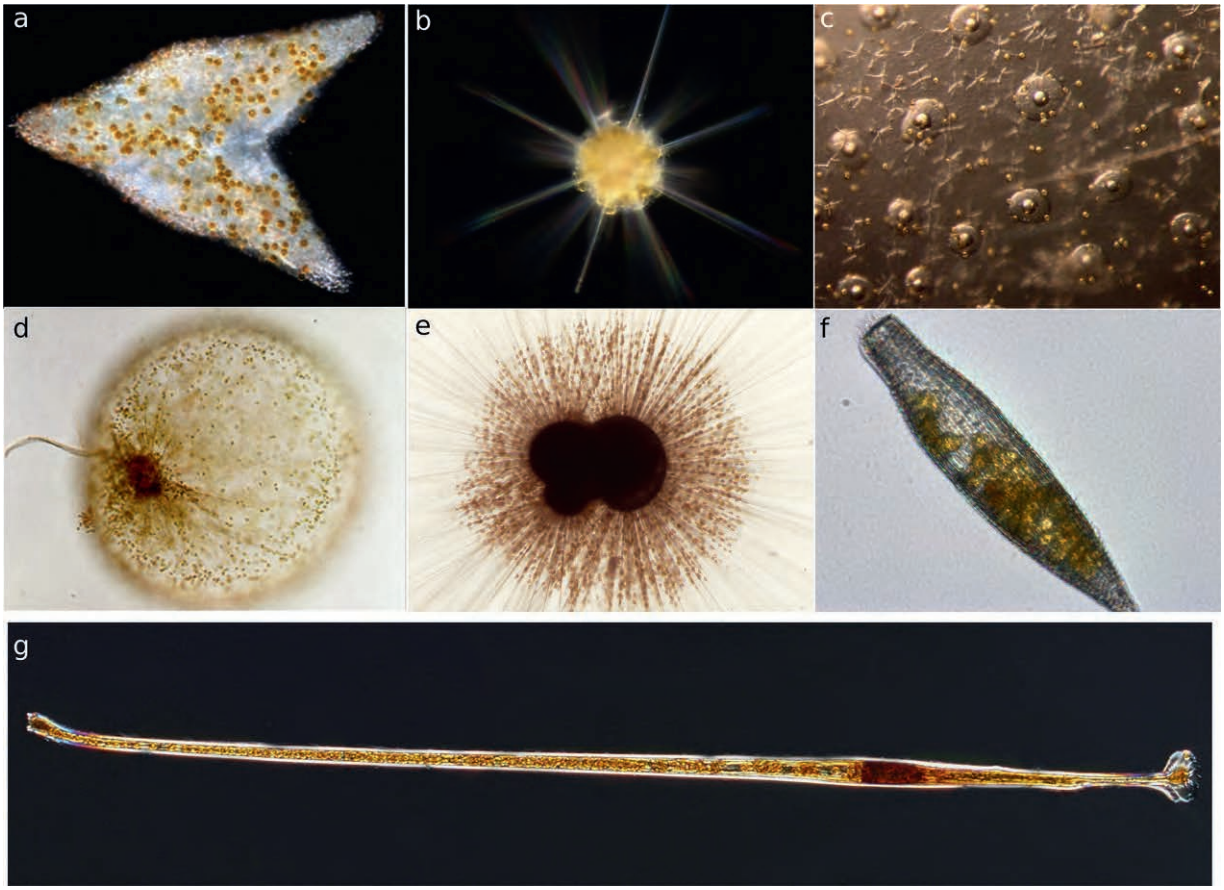

Fig. 2 A panel of light microscopy images showing different marine planktonic protists (NCMs) in symbiosis with microalgae, a common relationship in oceanic waters worldwide. (a,b,c) The rhizarian Radiolaria hosts symbiotic microalgal cells (yellow cells), which can be haptophytes or dinoflagellates. Radiolarians can build mineral skeletons of about 200–300 μm in size. (a) polycystines, (b) acantharians, (c) collodarians. Photos by Johan Decelle. (d) The green dinoflagellate *Noctiluca scintillans* (500–1000 μm in diameter), which can proliferate in Southeast Asia. Photo by Per Juel Hansen. (e) The rhizarian Foraminifera (*Globigerinoides sacculifer*) living with dinoflagellates (200 μm in diameter). Photo by Katsunori Kimoto. (f) The ciliate *Tiarina* sp. that builds a shell in calcium carbonate (80 μm in length) and hosts dinoflagellate symbionts (*Symbiodinium*, also found in corals) in oceanic waters. Photo by Johan Decelle. (g) The dinoflagellate *Amphisolenia* sp. that hosts pelagophyte endosymbionts along its 500 μm-long cell. Photo by John Dolan.

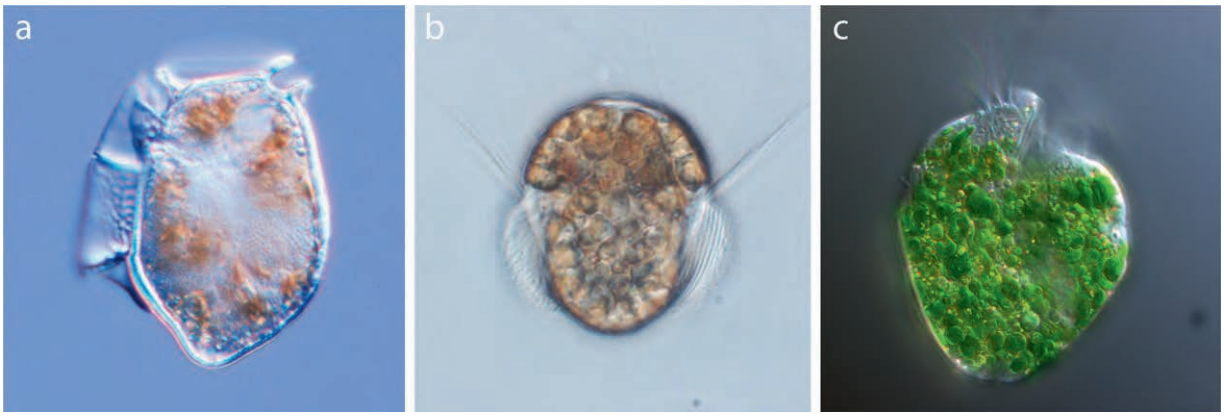

Fig. 3 A panel of light microscopy images showing different NCMs in marine ecosystems, which sequester the chloroplasts of their prey (kleptoplastidy). (a) The toxic dinoflagellate *Dinophysis acuta* (60 μm in length), which harbors chloroplasts of cryptophyte origin. Photo by Per Juel Hansen. (b) The ciliate *Mesodinium major* (50 μm in diameter), one of the red *Mesodinium* species. Photo by Niels Daugbjerg. (c) The oligotrich ciliate *Strombidium rassoulzadegani* (60 μm in length) with sequestered chloroplasts from different algal prey (green algae and cryptophytes). Modified from Schoener, D.M., McManus, G.B., 2012, Plastid retention, use, and replacement in a kleptoplastidic ciliate. Aquatic Microbial Ecology 67, 177–187, with permission from Inter-Research.

Constitutive Mixotrophs (CMs)

In principle, all eukaryotic microalgal lineages (chlorophytes, euglenophytes, cryptophytes, chrysophytes, haptophytes and dinoflagellates) have the capacity to take up prey, though it is not always present in all species of each lineage. Only diatoms are thought to lack phagotrophic capabilities as a group; but like all microalgae, they are able to utilize dissolved organic nutrients.

The major phytoplankton groups have a global distribution with smaller species present in greater proportion in oligotrophic waters, while larger species, such as dinoflagellates, will gain relative abundance in coastal waters. We can view the dominance of diatoms (autotrophs) vs CMs in an environment in the context of the supply and demand of nutrients and energy that can be fulfilled by phagotrophy. The balance between photoautotrophy and photo-phago-mixotrophy in plankton communities can be considered in significant part as determined by a combination of supply and demand of inorganic (dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP)) and organic nutrients (including prey availability). A schematic representation of the expected seasonal progression of non-mixotrophic plankton to one dominated by mixotrophs is shown in Fig. 4.

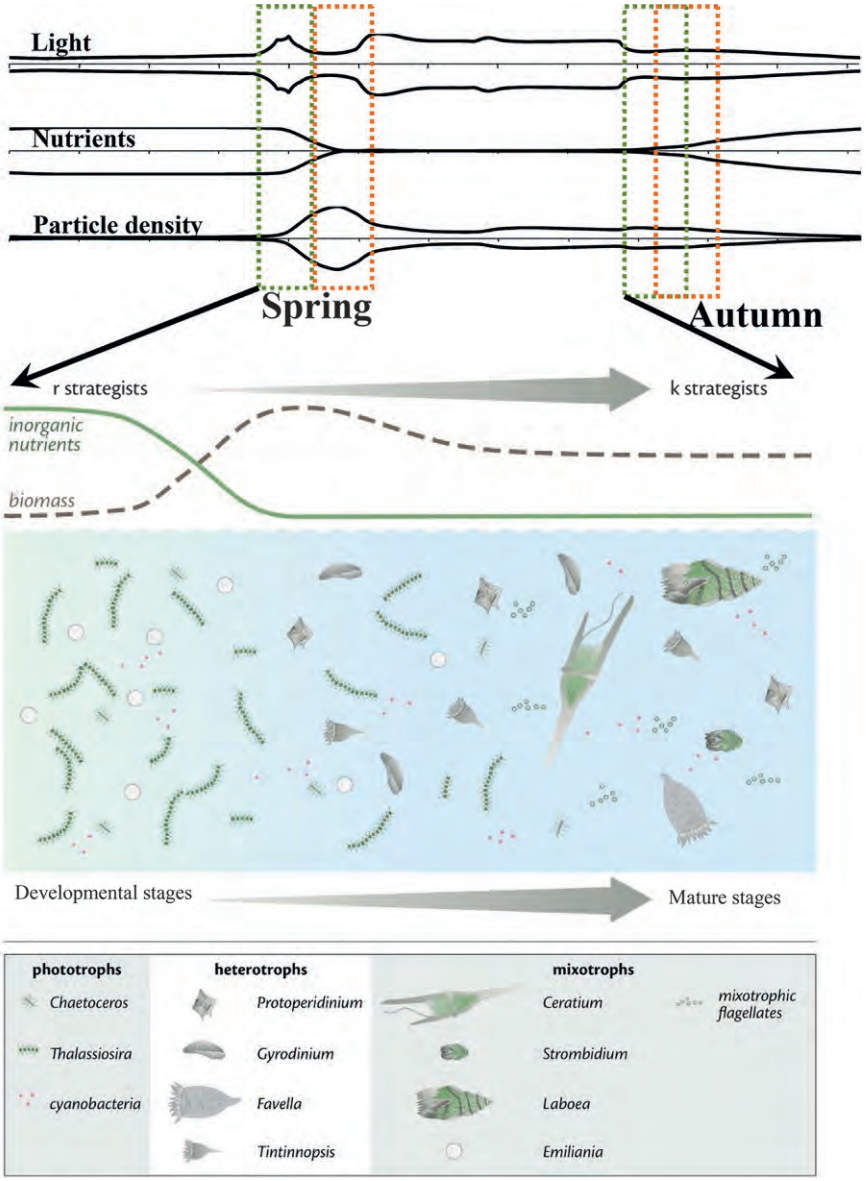

Fig. 4 Schematic diagram of the changes to the planktonic food web over a year, with transitions between immature and mature ecosystem states. The upper panels show changing patterns of light, inorganic nutrients and particle density (i.e., total plankton biomass) over the temperate year. Transitions from mature to immature (spring or autumn "blooms") to mature again, are as indicated; green dashed line indicting conditions optimal for phototrophy, orange dashed lines for phagotrophy. Other periods are sub-optimal for strict phototrophs and/or strict phagotrophs, and preferable for mixotrophs. The lower panel shows in detail the transition from immature to mature, with changes in selection priorities from so-called "r-select" phototrophs and phagotrophs in immature ecosystems, to a mature ecosystem more optimal for "K-select" mixotrophs. Reprinted from Mitra et al., 2014. The role of mixotrophic protists in the biological carbon pump. Biogeosciences 11, 995–1005, with permission from Copernicus Publications.

There are two significant challenges affecting our interpretation of the role of CMs in plankton ecology. The first is the quality of plankton survey data, which usually group diverse organisms into different size classes and only identify larger cells (>20 μm) with easily recognizable morphology. Small cells are not usually sampled or identified effectively. In consequence, we know very little even in quasi qualitative terms about the global distribution of organisms that we expect to be capable of photo-phago-mixotrophy. Secondly, we do not know the extent to which these phototrophic organisms are engaging in significant levels of mixotrophy. Experimental approaches on cultured representatives are required to actually measure rates of primary production and phagotrophy, but many planktonic organisms are not in culture. Coupled to this challenge is that a degree of feeding which may not appear significant in a community ecology context, could be essential for growth if it supplies a limiting nutrient.

Non-Constitutive Mixotrophs (NCMs)

These can be found mainly among ciliates, heterotrophic dinoflagellates, foraminiferans and radiolarians. The NCMs are often relatively large cells (20–1000 μm), and some can build carbonate or silicate mineral skeletons, such as the foraminiferans and radiolarians. Despite a high dependency on photosynthesis, some NCMs need to maintain heterotrophic activity. For others, the type of prey (specificity) and the contribution of predation to the overall metabolism is still poorly understood.

Different types of NCMs inhabit different biomes (Fig. 5), though collectively they are present across the global seas. Most inhabit mature ecosystems primarily due to the distribution of their prey, which can be very specific. Examples are large symbiotic NCMs like radiolarians and foraminiferans, which are the most abundant NCMs in the surface waters of the oligotrophic open ocean. However, some NCMs that maintain symbionts or sequester prey nuclei along with the chloroplasts can inhabit waters during less mature stages. Examples of the latter are the ciliate *Mesodinium rubrum*, which is heavily influenced by upwelling, and the green dinoflagellate *Noctiluca*, which responds to eutrophication.

Feeding Mechanism, Prey Selection and Toxin Assisted Feeding

Prey Capture and Feeding Mechanisms

Suspended and motile prey can be difficult for a single-celled organism to capture and consume, but many mixotrophic protists have come up with truly spectacular ways to achieve this. Many swim and catch prey upon collision, with the prey engulfed directly into a food vacuole. Others, typically slower swimming species, have evolved various sophisticated ways of capturing prey, using a haptonema (many haptophytes), a capture filament (many dinoflagellates) or a mucus trap (some dinoflagellates). Prey are usually taken up whole by direct engulfment, but some species use a feeding tube (a type of straw) to suck out the contents, thereby enabling these mixotrophs to exploit larger prey items (Fig. 6).

Toxin-Assisted Feeding

Some mixotrophic protists use toxins to immobilize swimming prey. Examples of these include the haptophyte *Prymnesium parvum*, which releases toxins to the water to immobilize swimming prey that it would otherwise not be able to catch. Excreted toxins from the dinoflagellate *Karlodinium armiger* have been shown to be involved in capturing prey organisms that would normally be their predators, thereby reversing the food web. Excreted toxins may also be used in combination with a mucus trap, as described in the dinoflagellate *Alexandrium pseudogonyaulax* (Fig. 6), to keep effective toxin concentrations high and in close proximity. Toxin-Assisted prey capture could be more common than we realize; it may not be coincidental that most toxin producing harmful algal bloom forming species are mixotrophic.

Prey Selection

Most mixotrophic protists are thought to feed on a wide range of prey and the diet usually includes bacteria and/or other protists, but it may in some cases also include copepods and mollusc larvae. In contrast, some protists are quite selective and rely on specific species of prey. For example, the ciliate *Mesodinium rubrum* utilizes a specific clade of cryptophytes, which are incorporated into the cell body of *M. rubrum* allowing the ciliate to rely on photosynthesis as its major carbon source. In turn, *M. rubrum* is specifically consumed by *Dinophysis* spp. (dinoflagellate) in order to acquire the cryptophyte chloroplasts from the ciliate.

Abiotic and Biotic Factors Influencing Photosynthesis, Growth and Feeding Rates in CM and NCMs

Light and Prey Concentration

CMs

Most studied CMs require light for sustained growth and will not grow in complete darkness even if fed. Irradiance is required to at least fix enough carbon to match respiration needs. At higher irradiances protist growth increases until saturation, as it does with pure phototrophs such as diatoms and cyanobacteria. If prey are abundant, some CMs can grow faster mixotrophically than

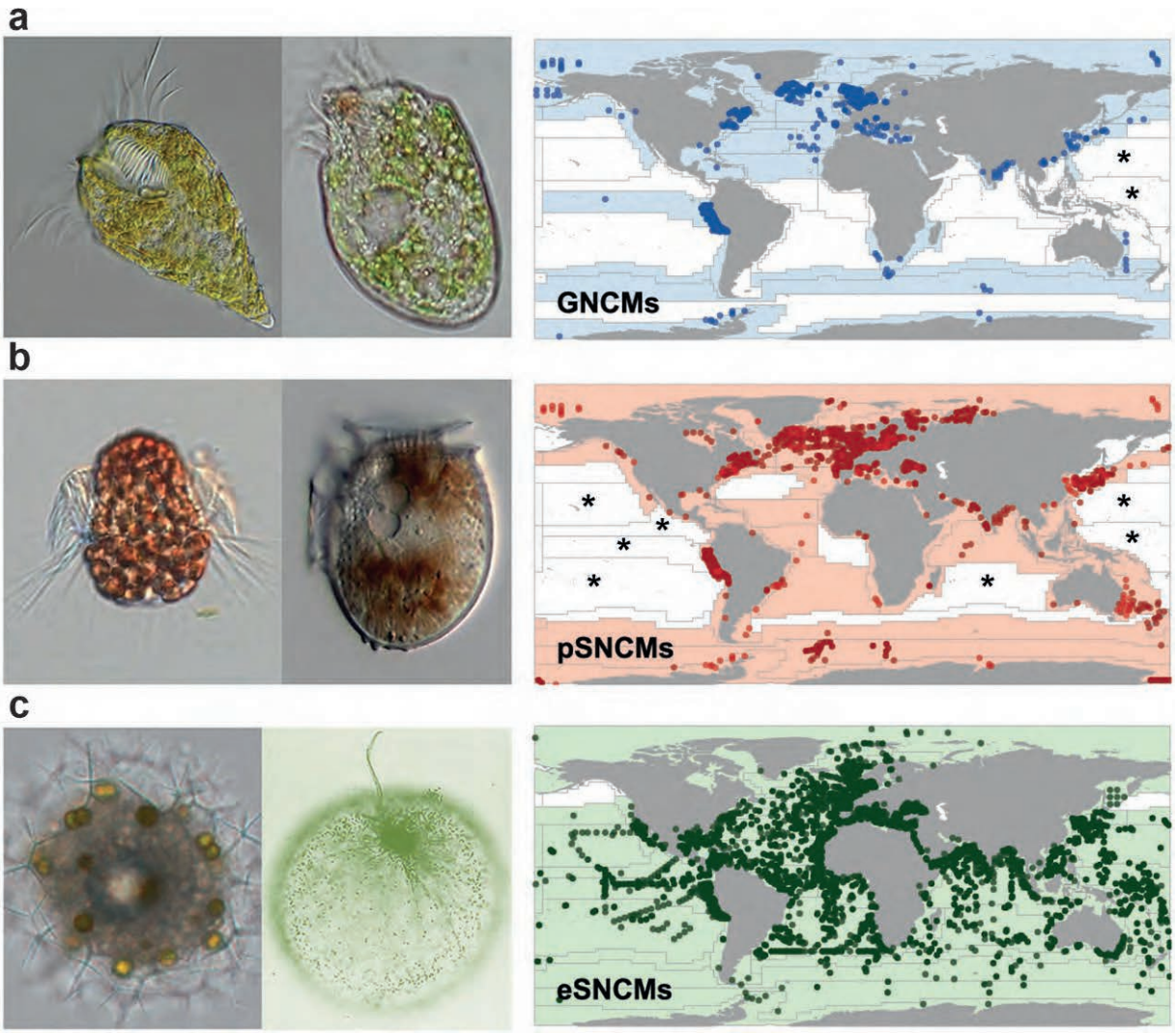

Fig. 5 Global distribution of protists with acquired phototrophy (non-constitutive mixotrophs, NCMs). Functional groups identify protists which acquire plastids from a variety of prey (generalist NCMs, GNCMs; blue (a)), from specific prey (plastidic specialist NCMs, pSNCMs; red (b)), or enslave entire specific autotrophic prey (size as length): (a) GNCMs *Laboea* (100 μm) and *Strombidinium* (50 μm); (b) pSNCMs, *Mesodinium* (60 μm) and *Dinophysis* (40 μm); (c) eSNCMs, *Sphaerozoum* (200 μm) and *Noctiluca* (500 μm). On maps, symbols correspond to the exact location where mixotrophic species/taxa were found (from more than 110,000 records); the grid indicates biogeographic provinces. Color-cast provinces indicate the presence of NCMs and white provinces correspond to the absence. Provinces marked with asterisk indicate that studies conducted in these areas did not record the presence of mixotrophic species; unmarked white provinces indicate a lack of field studies providing information on acquired phototrophy among microzooplankton. Reproduced with permission from Leles et al., 2017. Oceanic protists with different forms of acquired phototrophy display contrasting biogeographies and abundance. Proceedings of the Royal Society B 284, 20170664.

phototrophically at low irradiance (e.g., *Karlodinium veneficum*) and ingestion rates increase as a function of irradiance until it also satiates (Fig. 7). In consequence, mixotrophic growth rates of CMs are often much higher than when grown purely phototrophically. There are, however, exceptions. Some species can grow in complete darkness when fed a suitable prey (e.g., some chrysophyte species of the genera *Poterioochomonas*/*Ochromonas* and the dinoflagellate *Fragilidium* spp.) and light has a very limited effect on growth and prey uptake. In other cases the growth rate increases with irradiance despite the fact that ingestion is unaffected (e.g., the dinoflagellate *Gymnodinium resplendens*).

NCMs

Most NCMs are dependent on both light and prey for sustained growth, but some species can grow completely heterotrophically in the dark if prey is available (e.g., the dinoflagellate *Noctiluca scintillans*; the ciliate *Strombidium rassoulzadegani*). Similar to CMs, the growth rates of NCMs generally increase with irradiance until saturated (e.g., *Dinophysis acuminata*; *Mesodinium rubrum*), while ingestion rates may (*Dinophysis*) or may not (*Mesodinium*) increase as a function of irradiance.

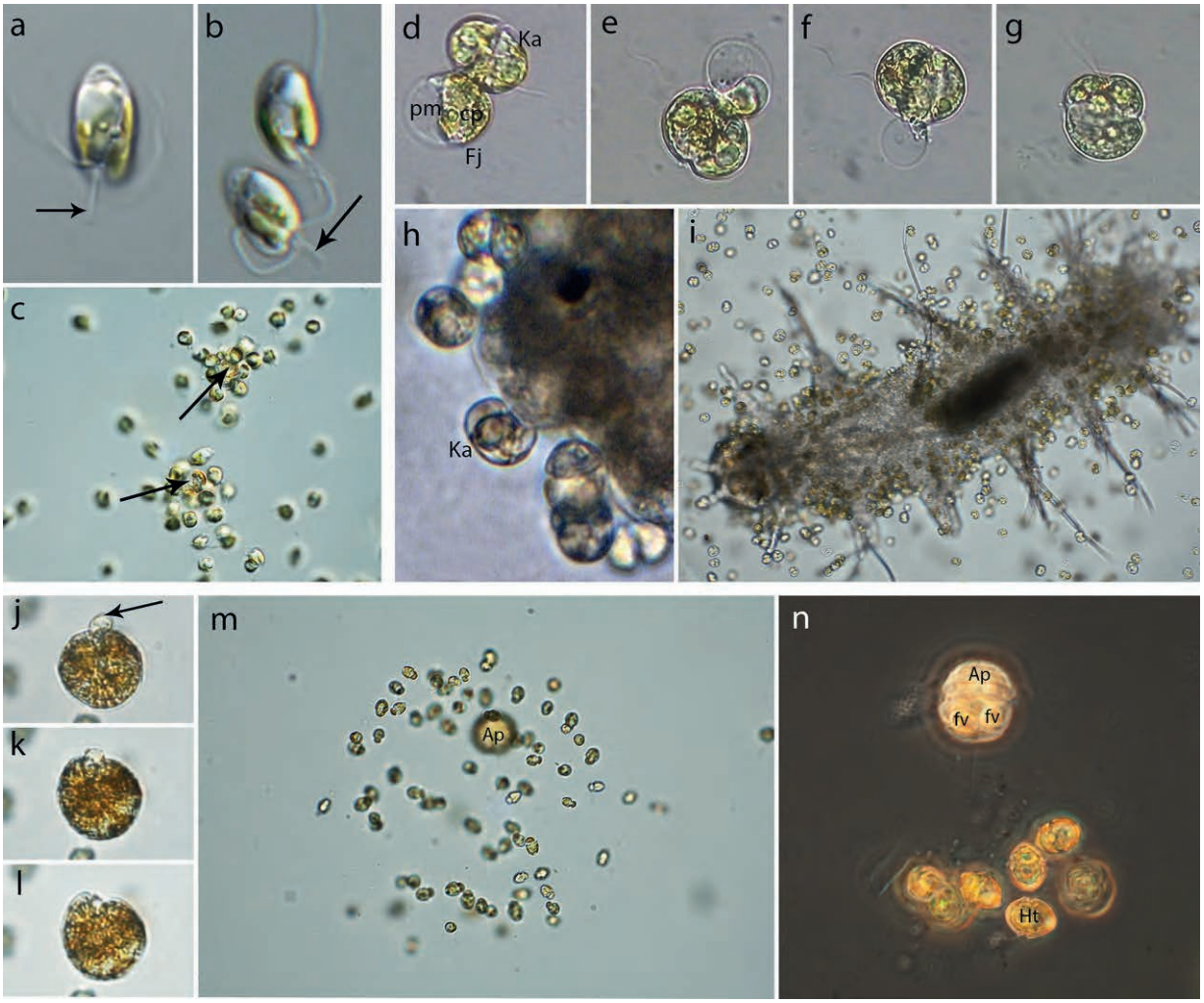

Fig. 6 A panel of light microscopy images showing examples of feeding mechanisms exhibited by mixotrophic protists. (a–c) *Prymnesium parvum* (10–12 μm long). Photos by Hannah Blossom. (a–b) *P. parvum* with arrows showing the characteristic short haptomena which prevents *P. parvum* from using a haptonema to catch mobile prey, unlike many other haptophytes. (c) Two *Teleaulax acuta* prey (arrow), which *P. parvum* swarm around and feed upon. *P. parvum* releases toxic substances which immobilize prey, prior to capture. (d–g) *Karlodinium armiger* (Ka; ca. 20 μm in length) using tube-feeding to suck the cytoplasm (cp) of the prey (in this case, *Fibrocapsa japonica;* Fj). The cytoplasm separates from the plasma membrane of the prey. (g) *Karlodinium armiger* after complete ingestion of prey. Modified from Berge, T., Hansen, P.J., Moestrup, Ø., 2008. Feeding mechanism, prey specificity and growth in light and dark of the plastidic dinoflagellate *Karlodinium armiger*. Aquatic Microbial Ecology 50, 279–288, with permission from Inter-Research. (h) *Karlodinium armiger* tube-feeding on a much larger organism, in this case an unidentified polychaete trochophore larva. Modified from Berge et al., 2012. Marine microalgae attack and feed on metazoans. The ISME Journal 6, 1926–1936, with permission from Springer-Nature. (i) *K. armiger* swarming and feeding on an immobilized polychaete larva (ca. 500 μm in length). *K. armiger* uses toxins during prey capture. Photo by Terje Berge. (j–l) *Alexandrium pseudogonyaulax* ingesting whole cell prey through the sulcus. *Heterocapsa rotundata* prey (arrow). Modified from Blossom, H., Daugbjerg, N., Hansen, P.J., 2012. Toxic mucus traps: A novel mechanism that mediates prey uptake in the mixotrophic dinoflagellate *Alexandrium pseudogonyaulax*. Harmful Algae 17, 40–53 with permission from Elsevier. (m) A mucus trap made by *A. pseudogonyaulax* to capture prey, in this case *Heterocapsa rotundata*. *A. pseudogonyaulax* (Ap) is attached, behind the trap. Photo by Hannah Blossom. (n) *A. pseudogonyaulax* (Ap) with two food vacuoles (fv) attached to its mucus trap with *Heterocapsa triquetra* (Ht) entrapped. Photo by Hannah Blossom. *A. pseudogonyaulax* is approximately 35–40 μm wide.

Major and Minor Nutrients and Complex Macromolecules

Major and minor nutrients

Nutrients are essential for the growth and biochemical reactions of protists; particularly, photosynthetic activity relies on pigments and proteins that require nitrogen and metals (iron). It is generally assumed that inorganic or dissolved organic sources are the preferred source of nutrients for most microalgal groups. Thus, their availability at concentrations limiting growth is thought to promote prey ingestion in mixotrophs (Fig. 8). Indeed, many CMs appear to consume prey exclusively to obtain nutrients, maintaining photosynthesis as their primary source of carbon. However, most studies have focused on general nutrient limitation, with organisms grown in diluted growth media until limitation hampers growth, but exactly which nutrient is limiting is unknown. The few studies available focusing on limitation for specific nutrients have demonstrated that inter- and intra-species variability may occur in the mixotrophic

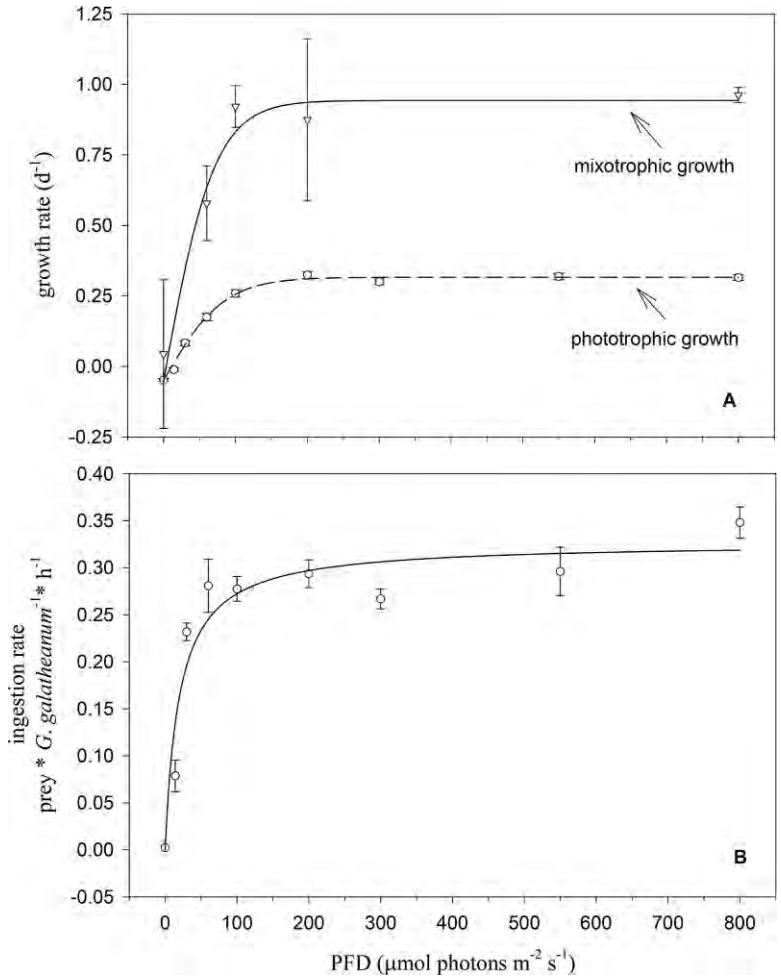

Fig. 7 The dinoflagellate *Karlodinium veneficum* (=*Gyrodinium galatheanum*). (a) Growth rate as a function of irradiance. *K. veneficum* was grown phototrophically in mono-specific cultures (circles) and mixotrophically in cultures with daily addition of cryptophyte prey *Storeatula major* (triangulars). (b) Ingestion rate of *K. veneficum* in food-saturated cultures as a function of irradiance. Error bars indicate ±1 SE, n=3. PFD: photon flux density. Adapted from Li et al., 1999. Feeding, pigmentation, photosynthesis and growth of the mixotrophic dinoflagellate *Gyrodinium galatheanum*. Aquatic Microbial Ecology 19, 163–176, with permission ©Inter-Research 1999.

response when limited for different nutrients. As both major (nitrogen and phosphorous) and minor (e.g., iron) nutrients are known to be important factors constraining planktonic primary production, the lack of clarity on interactions between inorganic nutrient stress and predation severely hampers our understanding of CM ecology. Nutrient uptake in NCMs is largely unexplored. However, uptake of inorganic and dissolved major nutrients has been shown recently in *Dinophysis* spp. and *Mesodinium rubrum*.

Complex macromolecules

Many CMs are known to be auxotrophic for complex vitamins, amino acids and fatty acids, due to the loss or absence of part or all of the genes required for synthesis. This phenomenon is so prevalent that certain vitamins, such as B_{12}, are routinely added to isolation and growth media to ensure microalgal growth. Paradoxically, very few studies have assessed the influence of complex macromolecules on feeding. For example, the haptophyte *Prymnesium parvum* has been shown to take up fatty acids from phagocytized ciliate and bacterial prey and is thought to use them as building blocks for membrane biosynthesis.

New Techniques in the Study of Mixotrophs to Assess Feeding and Nutrient Uptake

Omics

During the last decade, the application of omics techniques has revolutionized microbial oceanography. Detailed genome information is necessary to understand the evolutionary history of protists and important events in eukaryotic evolution such as lateral or horizontal gene transfer. Many groups of aquatic protists still have no, or only very few, genomes sequenced. The data available indicate that phagotrophy and photosynthesis occur in most major lineages, thus the capacity for mixotrophy among many aquatic protists would be an ancestral adaptation/capacity.

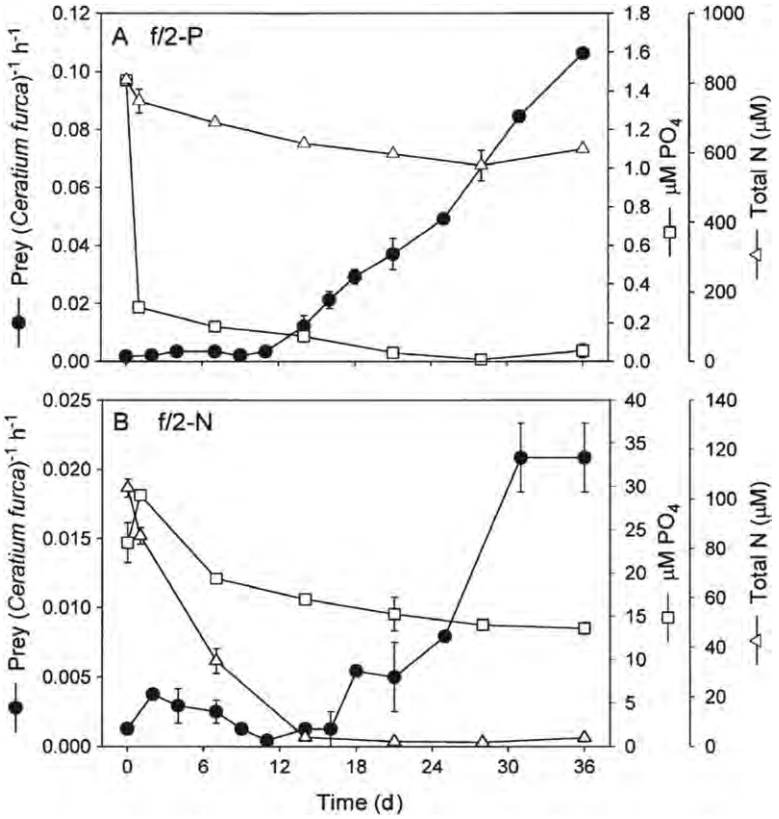

Fig. 8 The dinoflagellate *Tripos furca* (= *Ceratium furca*). Ingestion rates of cultures compared to inorganic phosphorus (PO_4) and nitrogen (NO_2+NO_3 and NH_4) concentrations over time. (a) f/2-P medium, (b) f/2-N medium. Data are presented as mean ± SE. Reproduced from Smalley et al., 2003. Feeding in the mixotrophic dinoflagellate *Ceratium furca* is influenced by intracellular nutrient concentrations. Marine Ecology Progress Series 262, 137–151, with permission ©Inter-Research 2003.

Transcriptomics is the analysis of gene expression. In this method, RNA is extracted from single cells or cultures and sequenced. As RNA is the messenger of genetic information from the nucleus to the cytoplasm of the cell, the sequenced RNA gives an overview over which genes are expressed at a certain point in time within single cells. This can improve our understanding of cell physiology, particularly how they respond to different environmental constraints. Using this method it was found that the availability of light has a minimal effect on population growth of the CM, *Ochromonas* sp. (chrysophyte). An upregulation of phototrophy- and phagotrophy-related genes was observed, while the availability of bacteria as prey led to major changes in carbon and nitrogen metabolic pathways. Further, transcriptomics of *Prymnesium parvum* showed that bacteria and ciliate prey were sources of organic carbon, macro- and micro-nutrients, organic nitrogen, and fatty acids. However, a large part of expressed genes in transcriptomes typically remains unassigned to a specific function, thus preventing a full understanding of physiological mechanisms.

High Resolution Chemical Imaging (NanoSIMS: Nanoscale Secondary Ion Mass Spectrometry)

Based on cell lysis and extraction of molecules, omics data only provide averaged information from cell population and their organelles. This is an especially important consideration in the study of interactions between heterotrophs and their algal symbionts or between heterotrophs and their sequestered chloroplasts, since it is generally difficult to identify the origin of a gene function/metabolite, and to distinguish the role of each partner and/or organelles. Thus, intra-cell spatial information is needed to further decipher the complex nutritional strategies of mixotrophs.

Recent advances in high-resolution single-cell chemical imaging coupled with stable isotopes now allow the observation and quantification of the metabolic activity within a single cell. In particular, nanoscale mass spectrometry imaging (nanoSIMS) is one of the most powerful chemical imaging platforms, available to biologists, with high mass and high spatial resolution capabilities (<100 nm) and excellent sensitivity (detection limit in the ppm or ppb range). NanoSIMS can document metabolic transfers between closely interacting cells and organelles, as well as the subcellular distribution of major nutrients (N, P and N, P and S) and metals. More specifically, following isotope incubation with ^{13}C or ^{15}N, nanoSIMS can elucidate the incorporation and flux of the isotope in cells at the subcellular level. This can be used to characterize and quantify the nutritional exchange between symbiotic partners, but also to quantify the contributions of photosynthesis and heterotrophy to the metabolism and growth of mixotrophs. As an example, nanoSIMS demonstrated that the chrysophyte *Ochromonas* sp. predominantly acquires carbon and nitrogen from bacterial prey when available, whereas inorganic C and N is assimilated primarily when bacterial

abundance is low. This technique should be applicable for determining the relative role of phagotrophy and photosynthesis in both CMs and NCMs over a range of conditions.

Measurements of "In Situ" Feeding Rates in Mixotrophs: Variation in Time and Space (Seasonality, Vertical and Diel Variations)

Experiments on cultured organisms are required to understand the ecology and physiology of aquatic protists. However, scaling results of these microcosm experiments to large-scale natural systems is challenging for a number of reasons: (i) In contrast to simplified and strictly controlled cultures, natural ecosystems are immensely complex, with protist behavior influenced by interacting and fluctuating factors. (ii) Some external drivers for protist behavior, such as small scale turbulence, are very difficult to mimic under laboratory conditions. (iii) Isolation and culturing selects for species that survive under standard culture conditions, but these do not necessarily represent the most important species in the field. (iv) Culture studies tend to be with single strains or species within a genus, potentially ignoring significant inter-strain and inter-species variability. To understand the dynamics of mixotrophic protists in aquatic systems, culture studies need to be complemented by *in situ* studies. Field studies provide generalized patterns of how natural communities respond to environmental shifts; while culture studies help us understand these patterns by concentrating on selected populations subjected to specific environmental cues.

Bacterivorous CMs and NCMs

Bacterivory rates in mixotrophs are most commonly measured by tracking the ingestion of fluorescently labeled bacteria (FLB). The assumption behind this technique is that mixotrophs feed indiscriminately, ingesting FLB at the same rate as co-occurring natural bacteria. This technique is relatively simple, with the possibility to target specific protist groups based on pigment signatures or gene probes. However, it has some caveats: (i) Not all mixotrophs feed indiscriminately, and selection for or against the FLB can occur, leading to biased results. (ii) Bacterivory rates can fall below detection limits when feeding rates are low. (iii) It is laborious and time-consuming. Alternative methods exist, but they present their own limitations, making the development of more accurate methods to determine mixotrophic bacterivory a major goal for future studies. As an example, food vacuole markers, such as acidotropic probes, can be used to determine the fraction of feeding cells in a population. This method is fast and highly sensitive, but cannot provide feeding rates since the number of ingested bacteria is unknown.

Despite these complications bacterivory by mixotrophs, primarily CMs, has been generally shown to account for 30%–60% of bacterial consumption in diverse freshwater and marine systems. Mixotrophic bacterivory can thus equal or surpass that of heterotrophic protists, the organisms previously held as the principal cause of bacterial mortality, along with viral lysis. The principal bacterivorous mixotrophs are small (2–10 μm diameter) phytoflagellates of diverse phylogeny. Haptophytes and chrysophytes are well established as major bacterivores, but there are increasing indications that cryptophytes and chlorophytes could also play a significant role. Most information available is extrapolated from punctual studies, with very few assessments existing of bacterivory along spatial and temporal scales. The relative importance and regulation of bacterivory by different algal groups and the spatio-temporal variability of bacterivory are major fields for future study, and vital information for correctly incorporating these organisms into the models that predict productivity of aquatic systems.

CMs and NCMs That Feed on Other Protists

Current methods fall short in measuring general in situ feeding rates of CMs and NCMs that feed on other protists, due to the much wider range of prey and predator sizes and the enormous differences in prey motility. Fluorescently labeled protistan prey can be used, but only to measure grazing rates of specific mixotrophs on very specific prey. This has been done to estimate grazing of *Tripos furca* (dinoflagellate CM) on small ciliates and of *M. rubrum* (ciliate NCM) on specific cryptophytes. However, this technique is not very useful for estimating the grazing rates of mixotrophs that ingest a variety of protistan prey, for example many of the CM dinoflagellates, plastid retaining oligotrich ciliates (NCMs), and mixotrophic foraminiferans and radiolarians (NCMs). Thus, a major future challenge is the development of methods to measure in situ feeding rates of these protists.

Incorporation of Mixotrophs in Food Web and Biogeochemical Models – Challenges for the Future

Models for support of fisheries, harmful algal bloom science, and biogeochemistry are based almost exclusively on the traditional paradigm of nutrient-phytoplankton-zooplankton (i.e., NPZ) with links to higher trophic levels. Having established that the bulk of the "phytoplankton" and half the "microzooplankton" (which as a collective form ca. 60% of "zooplankton") are photo-phago-mixotrophs, radically alters the conceptual framework for such simulation models. In theory, the "phytoplankton" are no longer simply restrained by nutrients and light, and the "microzooplankton" are less likely to starve. Mixotrophy has long provided a basis for explorations of trait-trade-offs in theoretical ecology. However, mixotrophs in such models have almost exclusively been configured as CMs, often including trade-offs that have not been substantiated by physiology studies. If such trade-offs are not

implemented, then modelled mixotrophs come to dominate; ironically, as we now better understand their physiology and ecology, that is indeed sometimes true.

Models including mixotrophs typically fail to describe the variety of mixotrophic formats and the relative roles of different nutritional modes operating under different conditions. This situation, in part, reflects the lack of data and understanding of mixotroph physiology, and also of their prey that concurrently inhabit the environment. This lack of understanding has led some to propose conceptual frameworks for plankton ecosystem modelling. One framework is based on scaling laws which indicates an allometric scaling for osmotrophy (bacteria), phototrophy (phytoplankton), mixotrophy and then particle feeding (zooplankton). The fallacy of the argument is revealed by the fact that some of the very smallest protist "phytoplankton", as exemplified by *Micromonas*, are mixotrophic. At the other extreme there are mixotrophs (foraminiferans, radiolarians, green *Noctiluca*) that are amongst the largest single celled organisms, and are larger than many metazoan zooplankton. We now know that mixotrophy is important in planktonic ecosystems and there is a drive for better marine ecosystem models for climate change prediction. However, we lack the physiological understanding and numeric data to properly construct and constrain simulation models. To get to where we need to be will take a significant and coordinated effort.

For field work we need survey protocols that adequately and routinely sample the plankton down to 2 μm. We also need experimental protocols that can provide data on the contributions of phototrophy and phagotrophy in different organism groups. How molecular biology can contribute to our understanding in the quantitative way required to support modelling is at present unclear. An allied issue is that molecular biology provides high resolution details down to sub-species or individual cell level, while models have to clump organisms together as functional groups. It is not yet clear how best to build those functional groups.

Laboratory experiments have significant potential to aid model construction, at least of the few species that are cultured. Notably such work requires simultaneous studies of both prey and mixotrophs growing alone, and together, with time series analysis of changes in nutrients, biomass, C:N:P and pigments. Fully integrated physiological-molecular-modelling studies likely offer the best route to enhancing conceptual, practical and numeric understanding; modelling efforts need to be fully embedded in the research effort and not left to the end. What we do have is sufficient conceptual understanding to build alternative structures, models that can be switched to better represent different physiological strategies, and that can be used to test against experimental data and to help us better interpret those data. Thus the "perfect beast" model, which is a simulation model (Fig. 9) has been used variously to explore CMs and NCMs in experimental, field and conceptual scenarios. Integrating molecular biology in such laboratory studies

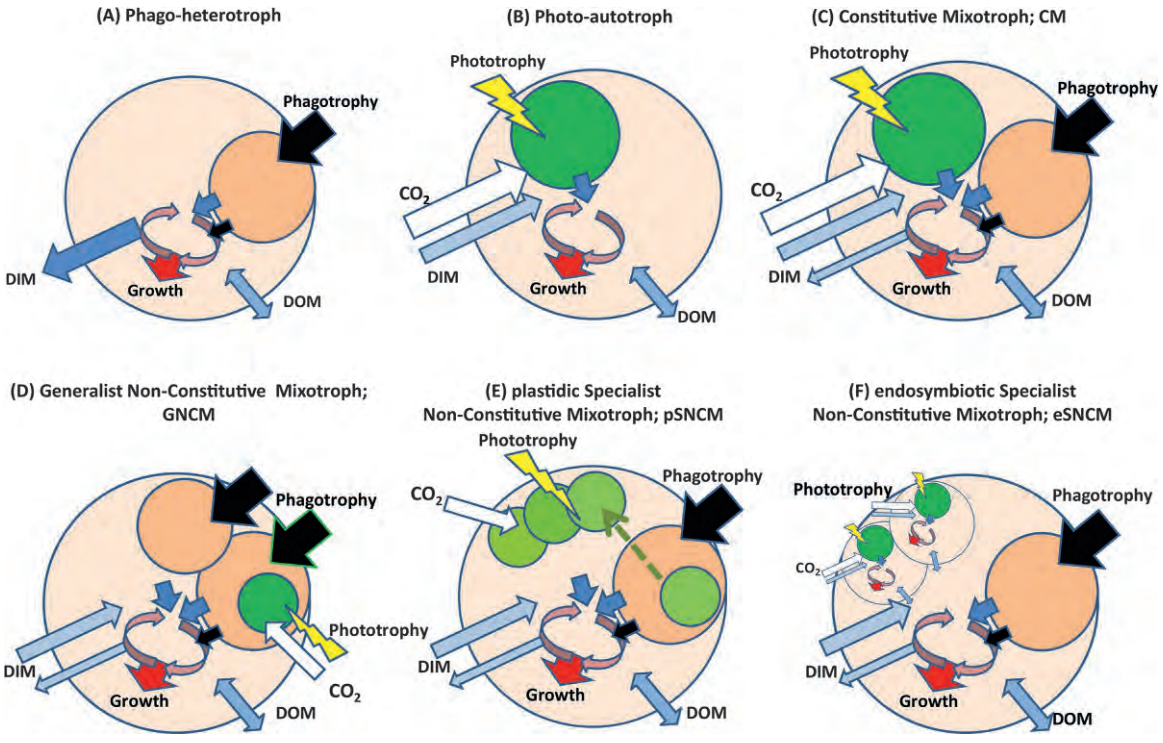

Fig. 9 Conceptual models for the four main photo-phago-mixotrophs. Constitutive mixotroph (CMs) contain their own innate ability to synthesize photosynthetic machinery, use inorganic nutrients, and eat prey. Non-constitutive mixotrophs (NCM) do not possess an innate ability to do photosynthesis but must acquire the ability; they follow three patterns. The generalists (GNCM) can exploit photosystems from many prey types but have little ability to maintain and control them. Plastidic specialists (pSNCM) acquire plastids and some allied nucleic material from very few prey species (though they can eat many prey types); they can achieve a much enhanced level of phototrophy maintenance and control compared to GNCMs. The endosymbiotic specialists (eSNCM) contain fully functional phototrophic algae; they operate in a way akin to a floating greenhouse. Of these, the "perfect beast" model of Flynn and Mitra (2009) can describe all but the eSNCM format. DIM – dissolved inorganic matter (nutrients); DOM – dissolved organic matter. Reproduced from Flynn, K.J., Mitra, A., 2012. Building the "perfect beast": Modelling mixotrophic plankton. Journal of Plankton Research 31, 965–992, with copyright permission from Oxford Academic.

would also help ground omics approaches, providing the missing links between RNA transcripts and function mentioned above. This would, in turn, improve the linkage between field studies and models which is essential for building robust simulation platforms.

What we can say for sure is that the complexity of the control of physiology within a given species (responses to light, nutrients, prey etc.), and differences in physiology between different mixotroph groups, argue strongly against attempts to deploy simple descriptions. Indeed, outputs from such models should likely be viewed with as much skepticism as we would now view simple NPZ models given what we now know about the role of mixotrophy in aquatic ecology.

Appendix A Supplementary Material

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/B978-0-12-809633-8.20685-7.

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Models in Microbial Ecology

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Introduction

The term *microorganism* or *microbe* refers to all unicellular organisms, which are microscopic in size (<1000 μm), including prokaryotes (bacteria and archaea) and unicellular eukaryotes (protists). Microbes are possibly the earliest evidence of life on Earth, approximately 3.700 million years ago (Dodd et al., 2017). Today, the vast majority of microbial prokaryotes (bacteria and archaea) on Earth are found in the open ocean (10^{29} cells), soils (10^{29} cells), deep oceanic and terrestrial subsurfaces (10^{30} cells). Bacteria are the largest contributor to global microbial biomass, followed by archaea, protists, and unicellular eukaryotes (Bar-On et al., 2018). The global biomass of bacteria on Earth is estimated to be 70 Gt Carbon, which constitutes 15% of global carbon biomass of all living organisms on Earth. This figure is dominated by terrestrial deep subsurface environments (60 Gt C) and marine deep subsurface environments (9 Gt C). The global biomass of archaea is estimated to be 7 Gt C, a figure dominated by terrestrial (4 Gt C) and marine deep subsurface (3 Gt C) environments. In open ocean environments, for instance, the abundance of bacteria is four times the abundance of archaeal cells (Bar-On et al., 2018), and the entire microbial food web, including protozoan microzooplankton, is generally from five to ten times the mass of all multicellular marine organisms (Pomeroy et al., 2007). The human gut is also a large reservoir of microbial prokaryotes, with the number of bacterial cells residing in the human body being of the same order as the number of human cells (*i.e.*, close to a 1:1 ratio) (Sender et al., 2016), although is not as species rich (on the order of 10^2 bacterial ecotypes) compared to soils or oceans (Faith et al., 2013). Freshwater microbial eukaryotic richness is estimated to be on the order of $2{\cdot}10^4$ species (Debroas et al., 2017).

Microbes can live as single-cells or form colonies. In addition to their ubiquity and important contribution to the global pool of carbon, microorganisms play fundamental roles in several biochemical processes and their ecological importance is, nowadays, unquestionable (Prosser et al., 2007b). Marine microbial communities are at the bottom of the trophic food webs that ultimately feed global fisheries, and about one half of Earth's primary production occurs in the ocean (Field et al., 1998). Microbes control many processes that sustain life on Earth: they are crucial for virtually all biogeochemical processes, perform photosynthesis, and decompose inert organic matter into simpler compounds that are the basis of many food chains (Falkowski et al., 2008; Thompson et al., 2017). Moreover, they frequently constitute the basis of food webs themselves, both in marine and terrestrial ecosystems (Field et al., 1998). Microbial communities often live in association with host organisms, both plants and animals, including humans, where they have a very profound impact in controlling host's health and several aspects of their life cycles, such as flowering time in plants or reproductive behavior in animals (Leitão-Gonçalves et al., 2017; Wagner et al., 2014). From a biotechnological perspective, microorganism consortia are used to purify wastewater, control catalytic reactions, including those involved with biofuels, and increase crop yields (Halan et al., 2012; Mueller and Sachs, 2015). Finally, even though most microbes are harmless, some species are responsible of diseases that affect multicellular organisms, including humans (Costerton et al., 1999).

The aim of this article is to provide a summary of the state-of-the-art regarding the use of mathematical models in microbial ecology and plankton dynamics. Unveiling the means by which microbial communities assemble, stabilize, and operate is essential to predicting and manipulating their functions, and thus likely to be among the most important questions modern ecology faces. Such relevance arises not only from the intellectual merit of the question *per se*, but also from its utility for coping with many of the challenges that will confront human society in the coming years, such as the management of natural resources and ecosystems or the palliation of the effects of climate change (Prosser et al., 2007a). Despite this well-founded importance, there are large gaps of knowledge about the rules governing the assembly of microbial communities. Such gaps partly stem from the difficulty of studying microbes in their natural ecological context, which has biased the development of microbiology towards studies in which populations are treated in lab-created setups that do not capture the complexities of the natural environments. In this scenario, the development of ecological models, as shown in this article, and recent experimental efforts aiming to quantify species interactions in increasingly complex communities, can improve our understanding of how microbial communities operate and affect ecosystem functioning.

Uptake Machinery

One of the main ways in which microbes interact with their environment is through their uptake machineries, which enable the harvesting of key resources that they require to grow. Autotrophic microbes, for example, harvest sunlight through their photosynthetic apparatus. Both autotrophs and heterotrophs capture key inorganic and/or organic nutrients using membrane transport

proteins, also called transporters (see Fig. 1) (Caperon, 1967). Models have considered uptake machineries in many different ways. The uptake of light by autotrophic microbes, for example, was initially approached simply as if it were one more nutrient (Jassby and Platt, 1976), although current models take into account the dynamics of cellular chlorophyll and the photosynthesis apparatus (Cullen, 1990). The classical representation for nutrient uptake, on the other hand, centers on the "Michaelis-Menten" (MM) functional form (Michaelis and Menten, 1913). This equation is based on theoretical considerations (see chapter "Enzyme Kinetics" of Lehninger's book (Nelson et al., 2008)) and has been supported by empirical observations leading to the "Monod model" of microbial growth (Monod, 1949).

If we call U (R) to the uptake rate (mmol cell^{-1} day^{-1}) for a particular nutrient or resource R (mmol m^{-3}), the *canonical* MM equation is usually written as:

$$U(R) = \frac{U_{max}\ R}{(U_{max}/A) + R} = \frac{U_{max}\ R}{K_R + R} \tag{1}$$

where U_{max} represents the maximum possible uptake rate (mmol cell^{-1} day^{-1}) of the cell; and A is the nutrient uptake affinity (m^3 cell^{-1} day^{-1}) of the cell. The ratio $K_R = U_{max}/A$ is called the half-saturation constant (mmol m^{-3}) because it represents the concentration of resources at which the uptake rate is half of its maximum ($U_{max}/2$). See Box 1 for a step-by-step derivation of the MM equation and its use in resource uptake kinetics. See also Aksnes and Egge (1991), Bonachela et al. (2011), Nelson et al. (2008) and Vallina et al. (2014b) for alternative derivations of the MM equation.

Sometimes it may be convenient to use instead biomass-normalized traits by defining $\alpha_\rho = A\ B^{-1}$ as the specific uptake affinity (m^3 mmol^{-1} day^{-1}) and $\rho_{max} = U_{max}\ B^{-1}$ as the specific maximum uptake rate (day^{-1}), where B is the cell's biomass (mmol cell^{-1}).

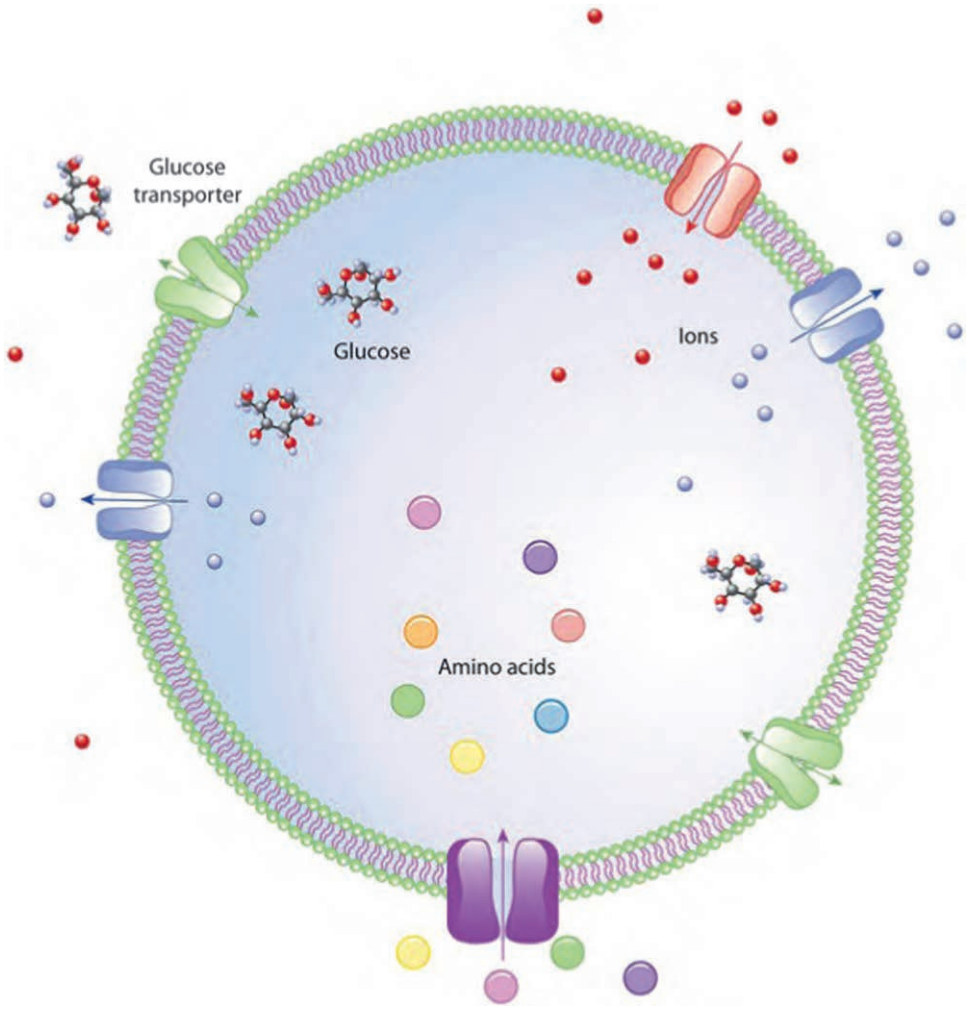

Fig. 1 Specialized proteins in the cell membrane regulate the concentration of specific molecules inside the cell. Picture credits: © 2010 Nature Education.

Box 1 Michaelis-Menten kinetics

- Enzyme-substrate reaction

The cannonical Michaelis-Menten equation was originally derived to describe enzyme-substrate kinetics (Briggs and Haldane, 1925; Michaelis and Menten, 1913). The MM equation describes the formation of a product (P) from a substrate (S), mediated by the binding interaction with an enzime (E) to form a complex enzime-substrate (ES) that finally releases the product. This two-step sequence of reactions (binding + catalysis) can be described by the following diagram:

$$[E] + [S] \underset{k_d}{\overset{k_a}{\rightleftarrows}} [ES] \xrightarrow{k_h} [E] + [P] \tag{4}$$

where [S] is the concentration (mmol m^{-3}) of substrate; [E] is the concentration (mmol m^{-3}) of free enzymes; [ES] is the concentration (mmol m^{-3}) of enzymes forming the enzyme-substrate complex (*i.e.*, non-free enzymes); [P] is the concentration (mmol m^{-3}) of the product; k_a is the forward reaction rate constant (m^3 $mmol^{-1}$ d^{-1}) to form complex ES; k_h is the forward dissociation rate constant (mmol $mmol^{-1}$ d^{-1}) of complex ES to form product P; and k_d is the reverse dissociation rate constant (mmol $mmol^{-1}$ d^{-1}) of complex ES back to free enzimes and substrate.

The law of mass action enables writing the reactions as a set of differential equations:

$$\frac{d[S]}{dt} = -F_a + F_d \tag{5}$$

$$\frac{d[E]}{dt} = -F_a + F_h + F_d \tag{6}$$

$$\frac{d[ES]}{dt} = F_a - F_h - F_d \tag{7}$$

$$\frac{d[P]}{dt} = F_h \tag{8}$$

$$F_a = k_a[E][S] \tag{9}$$

$$F_h = k_h[ES] \tag{10}$$

$$F_d = k_d[ES] \tag{11}$$

where F_i represents the fluxes (mmol m^{-3} d^{-1}) among state-variables in the system, each of them governed by a rate constant k_i (see arrows in Eq. (4)). The MM kinetics assumes a single binding site per enzyme, thus 1 mole of enzyme binds to 1 mole of substrate to form 1 mole of enzyme-substrate complex. The total concentration of enzymes $[E_T]$ is assumed to be constant (*i.e.*, conservation constrain) and is given by the sum of the free enzymes [E] plus the non-free enzymes forming the complex [ES]:

$$[E_T] = [E] + [ES] \tag{12}$$

Assuming steady-sate of the complex [ES], solve for [E] using Eq. (7):

$$F_a = F_h + F_d \tag{13}$$

$$[E][S]\,k_a = [ES](k_h + k_d) \tag{14}$$

$$\frac{[E][S]}{[ES]} = \frac{k_h + k_d}{k_a} \equiv k_m \tag{15}$$

$$[E] = k_m \frac{[ES]}{[S]} \tag{16}$$

where the parameter k_m is known as the Michaelis constant (mmol m^{-3}), a.k.a. half-saturation constant.

Substituting Eq. (16) into Eq. (12) and solving for [ES]:

$$[E_T] = k_m \frac{[ES]}{[S]} + [ES] = \frac{k_m + [S]}{[S]}[ES] \tag{17}$$

(*Continued*)

Box 1 (Continued)

$$[ES] = [E_T]\frac{[S]}{k_m + [S]} = \frac{[E_T][S]}{((k_h + k_d)/k_a) + [S]} \tag{18}$$

Substituting Eq. (18) into Eq. (8) and solving for the production rate of P:

$$V(S) \equiv \frac{d[P]}{dt} = \frac{k_h[E_T][S]}{((k_h + k_d)/k_a) + [S]} \tag{19}$$

The maximum production rate (mmol m^{-3} d^{-1}) occurs when all enzymes are tied to a substrate (*i.e.*, saturating conditions), which means that there are no free enzymes ([E] = 0) since they are all bound as enzyme-substrate complex ([ES] = $[E_T]$). Thus:

$$V_{max} = k_h[E_T] \tag{20}$$

Substituting Eq. (20) into Eq. (19) we obtain the final expression for the production rate V (mmol m^{-3} d^{-1}) of product P from substrate S:

$$\begin{aligned} V(S) &= \frac{k_h[E_T][S]}{((k_h + k_d)/k_a) + [S]} \\ &= \frac{V_{max}[S]}{k_m + [S]} \end{aligned} \tag{21}$$

- Resource uptake analogy

By direct analogy, we can translate the canonical MM equation to model the uptake rate of resources by microbes using the following change of variables:

$$[S] = [R] \tag{22}$$

$$[E_T] = n\,[N] \tag{23}$$

$$k_a = a \tag{24}$$

$$k_h = h \tag{25}$$

$$k_d << k_h \tag{26}$$

$$k_m = K_R \tag{27}$$

where [R] is the molar concentration of resource (mmol m^{-3}); [N] is the abundance of microbes (cells m^{-3}) in the population; n is the number of transporters per cell (sites $cell^{-1}$); a is the binding affinity per transporter (m^3 $site^{-1}$ d^{-1}) for the resource; and h is the handling rate per transporter (mmol $site^{-1}$ d^{-1}) for the resource.

Substituting Eqs. (24)–(29) into Eq. (21) gives the uptake rate of resource by the microbial population (mmol m^{-3} d^{-1})

$$V(R) = \frac{h\,n\,[N][R]}{(h/a) + [R]} \tag{28}$$

Dividing equation Eq. (28) by the biomass concentration (mmol m^{-3}) of the microbial population [C] = $B\times$[N], where B is the biomass per cell (mmol $cell^{-1}$), then leads to the MM equation for specific uptake rate (d^{-1}) of resource R by the microbial population, which was also previously defined in the main text (see Eq. (2)):

$$\begin{aligned} \rho(R) &\equiv \frac{V(R)}{[C]} = \frac{h\,n\,B^{-1}\,[R]}{(h/a) + [R]} \\ &= \frac{\rho_{max}\,[R]}{K_R + [R]} \end{aligned} \tag{29}$$

Thus, if we call $\rho(R)$ to the biomass-specific uptake rate (day^{-1}) for resource R (mmol m^{-3}), the *biomass-specific* MM equation becomes:

$$\rho(R) = \frac{\rho_{max}\, R}{(\rho_{max}/\alpha_\rho) + R} = \frac{\rho_{max}\, R}{K_R + R} \tag{2}$$

The Michaelis-Menten model for resource acquisition described in Eq. (2) effectively summarizes the main features of the nutrient uptake apparatus using just two primary traits: specific affinity α_ρ and specific maximum uptake rate ρ_{max}. Graphically, the specific affinity α_ρ represents the initial slope of the curve that relates the specific uptake rate $\rho(R)$ to the external concentration of resources (Button et al., 2004), while the specific maximum uptake rate ρ_{max} represents the horizontal asymptote of the uptake curve that is reached at infinite resource conentration (see Fig. 2). Ecologically, the specific affinity quantifies the competitive ability of microbes at low resource concentration, and the specific maximum uptake rate quantifies the competitive ability of microbes at high resource concentration. Formally speaking, the half-saturation constant is a composite trait of these two primary traits ($K_R = \rho_{max}/\alpha_\rho$). Extensions of Eq. (2) can be used to model the uptake of several substitutable forms of the same elemental nutrient (*e.g.*, nitrate, nitrite, and ammonium forms of nitrogen), and can be combined with other expressions (*e.g.*, Liebig's law of the minimum (de Baar, 1994)) to model the uptake of several non-substitutable nutrients (*e.g.*, phosphorous, nitrogen, iron, *etc*).

The specific Michaelis-Menten equation for resource uptake can also be expressed as a function of the number n of transporters specific to the focal nutrient (sites cell^{-1}); the handling h rate of the nutrient ions per transporter (mmol site^{-1} day^{-1}); and the affinity a constant per transporter (m^3 site^{-1} day^{-1}), which results from considering the area of the transporter (m^2 site^{-1}) times its mass transfer efficiency (m day^{-1}) for the nutrient. Lets now define $\rho_{max} = n\, h\, B^{-1}$, $\alpha_\rho = n\, a\, B^{-1}$, and $K_R = (\rho_{max}/\alpha_\rho) = (h/a)$, where B is the biomass of the cell (mmol cell^{-1}). That represents the MM uptake rate as a function of three major physiological traits of the cell, normalized by its biomass (B), providing further biological context to the MM uptake kinetics parameters. Then Eq. (2) becomes:

$$\rho(R) = \frac{(h\, n\, B^{-1})\, R}{(h/a) + R} = \frac{(h\, n\, B^{-1})\, R}{K_R + R} \tag{3}$$

Variations of Eq. (3) have been used to model the uptake of several nutrients by different transporters (see Section "Elemental Stoichiometry") or even the uptake of the same nutrient by several types of transporter (Button, 1998). Furthermore, Eq. (3) can be used to relate a "gleaner – opportunist" trade-off to the three physiological traits that the cell can manipulate (number of

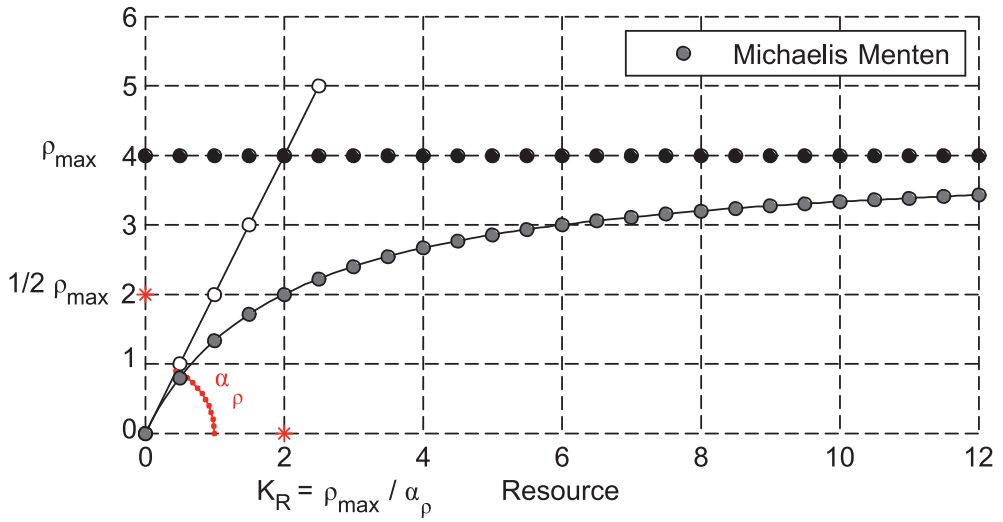

Fig. 2 Michaelis-Menten function (gray dots) that relates the specific uptake rate $\rho(R)$ (day^{-1}) to the external concentration of resources (mmol m^{-3}). The specific affinity α_ρ (m^3 mmol^{-1} day^{-1}) represents the initial slope of the curve at zero resource conentration (white dots). The specific maximum uptake rate ρ_{max} (day^{-1}) represents the horizontal asymptote (black dots) of the curve that is reached at infinite resource conentration. The lines for specific affinity and maximum uptake rate intersect at the half-saturation constant K_R. The specific affinity quantifies the competitive ability of microbes at low resource concentration, and specific maximum uptake rate quantifies the competitive ability of microbes at high resource concentration. Formally speaking, the specific affinity and specific maximum uptake rate are primary traits, while the half-saturation constant is a composite trait of these two primary traits ($K_R = \rho_{max}/\alpha_\rho$).

transporters, their affinity, and their handling rate) by imposing that $\alpha_\rho \times \rho_{max} = (n/B)^2\ (h\ a) =$ constant. See Section "Functional Trade-Offs" for more details.

The examples above, however, neglect the possibility for the cell to regulate the uptake apparatuses as a response to changes in the environment. This phenotypic plasticity has been documented in the past in a diversity of microbes and for a wide range of resources, including light as well as nutrients (Smith et al., 2009). Because they affect microbial growth, understanding these acclimation responses is essential to predicting reliably the role of microbes in ecosystem functioning (*e.g.*, global biogeochemistry). In the case of light acclimation, modelled responses range from changes in pigments that optimize light uptake to photo-protective mechanisms limiting damages caused by excess of light to the photosynthetic apparatus (Armstrong, 2006; Geider et al., 1998; Litchman and Klausmeier, 2008). In the case of nutrient uptake, cells can for example alter the number and handling rate of transporters used to take up the focal nutrient. Most models focus on the commonly observed negative correlation between nutrient availability and maximum uptake rate ρ_{max} by means of phenomenological expressions (Morel, 1987). More mechanistic models include interactions between different eco-physiological processes and cellular nutrient pools by assuming instantaneous acclimation time (Geider et al., 1998; Pahlow and Oschlies, 2009; Smith et al., 2009), or including explicitly a dynamic equation for transporter regulation (Bonachela et al., 2013; Bonachela et al., 2011). Such models have been employed to reproduce successfully laboratory and field patterns that were not explained by non-plastic models (Arteaga et al., 2016; Lomas et al., 2014; Smith et al., 2015). Including a dynamic number of transporters allows equation Eq. (3) to account for another important feature that this MM equations neglect: the so-called boundary layer for the cell (see Bonachela et al., 2011 and references therein for details). Mixing high- and low-affinity transport systems, where at least one system is inducible (*i.e.*, whose expression is genetically regulated), is an alternative way of implementing plasticity of nutrient uptake. Under nutrient-replete conditions, there is a low affinity transporter that is always expressed; but under nutrient starvation, the synthesis of a high affinity transporter is switched on. This leads to a varying mix of two different uptake curves, in different proportions depending on the numbers of each transporter type (Lin et al., 2016). However, phenomenologically this mix of the high and low affinity transporters can look like a single Michaelis-Menten uptake curve with phenotypic plasticity. Because in some well studied cases the genes of these regulatory systems are known (Martiny et al., 2006), this opens the venue for linking mechanistic modelling of uptake kinetics to ecological and evolutionary processes of microbes (see Sections "Ecological Selection and Community Assembly" and "Ecological Evolution and Community Adaptation").

Although more experimental information is becoming available, our understanding of the wide diversity of microbial acclimation processes remains limited. One aspect that remains unknown, for example, is the metabolic cost of plasticity in the uptake apparatus which, as models have shown, potentially plays an important role in whether responding to environmental changes is beneficial or detrimental to the microbial organism (Menge et al., 2011); also, the evolutionary cost and trade-offs that emerge from the use of, *e.g.*, the nutrient uptake apparatus as a gateway for viral infections by bacterio- and phyto-phages (Menge and Weitz, 2009). Finally, more and more models are now exploring the eco-evolutionary interactions that emerge from the overlap between acclimation in the uptake apparatus and microbial rapid evolution (Kremer and Klausmeier, 2013; Lomas et al., 2014), interactions that are key to predict reliably microbial dynamics and their role in their environment.

Elemental Stoichiometry

Understanding microbial elemental composition is essential to understanding the role that microbes play in local and global biogeochemistry (Bonachela et al., 2016; Geider and La Roche, 2002). Microbes require a wide range of micro- and macro- nutrients to grow and reproduce. Models for microbial growth typically rely on the Liebig's law of the minimum and focus only on the nutrient that limits most such growth (de Baar, 1994). However, the identity of this nutrient is highly species- and environment-specific. For example, whereas carbon is typically considered the limiting resource for bacteria such as *Escherichia coli* in terrestrial ecosystems, phosphorus typically limits microbes in fresh water, and nitrogen is limiting in marine environments (Moore et al., 2013; Proctor and Karl, 2007; Sterner and Elser, 2002). Iron is essential for all autotrophic microbes, whereas silica is only limiting for diatoms. Furthermore, in the absence of other mechanisms or competitive interactions, the number of elemental nutrients (*i.e.*, that cannot be substituted one for another) imposes an upper limit in the number of microbial populations that can coexist at equilibrium in a single homogeneous habitat. In other words, the maximum number of microbial ecotypes coexisting at steady-state cannot exceed the number of essential resources, in the absence of environmental heterogeneity (either spatial and/or temporal) and/or stabilizing interactions (Grover, 1997) (see Section "Food Web Dynamics").

Based on the observation that several parts of the ocean show a remarkably constant ratio of key nutrients such as carbon, nitrogen, and phosphorus (Redfield, 1934, 1958), models have classically approached microbial elemental composition by focusing on the most limiting nutrient and calculating cell content and uptake of the rest of nutrients using the so-called Redfield ratios. Such approach has been used in aquatic ecosystem models, both at the local and the global level (*e.g.*, Follows et al., 2007). However, more recent theoretical and field observations have shown that microbial elemental ratios are highly variable in the short and long term (Galbraith and Martiny, 2015), and that different microbial species manage such ratios in different ways in response to their environment (Klausmeier et al., 2004a; Martiny et al., 2013). Early models introduced the possibility of a dynamic ratio of, *e.g.*, nitrogen to phosphorus assuming total independence between the two (Klausmeier et al., 2004b; Legovic and Cruzado, 1997; Smith and Yamanaka, 2007). Cells in these models show limitation by either one or the other nutrient, which provides arguments for coexistence between species that, for the same environmental conditions, are limited by different nutrients. Under this situation,

a sufficient condition of stable coexistence is that each microbial ecotype must be a stronger competitor for the nutrient limiting its own growth than for the nutrients limiting its competitors' growth (Grover, 1997; Tilman, 1982). Except for extreme conditions such as high mortality rates, in these models the cellular elemental ratio matches that of the nutrients introduced in the environment, which is an homeostatic response that has been observed experimentally (*e.g.*, Rhee, 1978). These models also replicate early laboratory observations regarding the existence of one single input ratio for which growth is limited by the two nutrients, which represents an optimum as it is the point with the least limitation by either resource (Rhee and Gotham, 1980).

For microbes, however, nutrients such as phosphorus and nitrogen are not completely independent from each other; for example, phosphorus is essential for protein synthesis (*e.g.*, in the form of ATP), and proteins are mostly composed of nitrogen (Geider and La Roche, 2002). Models that have introduced interactions between the different cellular nutrient pools predict a much wider set of environmental conditions for which co-limitation is possible (Agren, 2004; Arteaga et al., 2016; Bonachela et al., 2013; Klausmeier et al., 2007; Pahlow and Oschlies, 2009). As a consequence, the patterns of coexistence predicted by these more realistic models change significantly with respect to those of early models, which in turn affects their predictions regarding microbial community composition. Modelling such nutrient interactions is, nonetheless, a highly non-trivial task due to the multiple layers at which they occur and how the dynamics of microbial stoichiometry are intertwined with other resources (*e.g.*, light, temperature (Moreno et al., 2018)) or cellular plastic responses to resource changes (*e.g.*, regulation of nutrient uptake proteins; see Section "Uptake Machinery"). For the same reasons, essential aspects of such dynamics and their overall emergent results remain unknown. For example, the extent to which the correlation between microbial maximum growth rate and phosphorus content (the so-called growth rate hypothesis (Elser et al., 1996)) holds across organisms and different environmental conditions is still a matter of debate (Moody et al., 2017). There is also very little empirical and theoretical information regarding the interactions between the dynamics of elemental stoichiometry and that of nitrogen fixation (Pahlow et al., 2013), the latter affecting not only the focal organism's stoichiometry but also that of the rest of its community. Although current laboratory work aims to fill the knowledge gap regarding species-specific responses to different environmental conditions (*e.g.*, Edwards et al., 2015; Mouginot et al., 2015), additional multidisciplinary efforts combining field, laboratory, and models are needed in order to unravel the dynamics of microbial stoichiometry.

Functional Trade-Offs

The efficiency of resource acquisition and utilization determines species competitive ability, and therefore contributes to its fitness. Several models have been proposed to mathematically encode nutrient uptake and its investment in population growth. One of the first attempts is the so-called Michaelis-Menten equation, in which cell growth is a saturating function of the concentration of external resources (Monod, 1949). The MM model assumes that resource uptake leads to growth *instantaneously*. Therefore, in this model there is no difference between both processes (uptake and growth) and they are perfectly coupled. However, even though this assumption provides a moderately accurate picture for constant or low-frequency fluctuating environments (Grover, 1990), the MM model may fail under high-frequency fluctuating conditions because it does not consider cell storage effects. A more detailed description is provided by the so-called Droop model of internal store (Grover, 1991), in which the level of nutrients stored in the cell (*i.e.*, the cell quota) is described explicitly.

Internal store models consider that resource uptake and cell growth are two sequential processes that are decoupled. Resource uptake rate (mmol cell^{-1} day^{-1}) is represented by a saturating function U (R) of the concentration of external resources R (mmol m^{-3}), such as the cannonical MM model (see Eq. (1)). Once absorbed, resources accumulate in the cell and determine the specific growth rate (d^{-1}) as a saturating function $\mu(Q)$ of the internal quota of resources Q (mmol cell^{-1}). In a seminal work (Droop, 1973), Droop proposed the following saturating dependence between growth rate and cell quota that has been widely used since:

$$\mu(Q) = \mu_\infty \left(1 - \frac{Q_{min}}{Q}\right) \tag{30}$$

where Q is the concentration of intracellular resources, μ_∞ the specific growth rate at infinite quota, and Q_{min} the minimum quota required for growth (Fig. 3(b)). The parameters in Eq. (1) and Eq. (32) define a spectrum of resource acquisition and utilization strategies, and thus impact the species fitness. Therefore, they can be understood as functional traits (Violle et al., 2007). Alternative forms of the Droop model can take into account the maximum storage capacity of the cell (Q_{max}) by replacing the constant μ_∞ by $\mu_\infty = \mu_{max}/(1 - Q_{min}/Q_{max})$, where $\mu_{\max}$ is the maximum specific growth rate at full storage of resources (Grover, 1997).

If parameters A, U_{max}, Q_{min}, Q_{max} and μ_{max}, in Eqs. (1) and (32) were independent from each other, it would be possible to evolve a super-species with the strongest competitive abilities regardless of the environmental conditions, often termed "Darwinian demon". However, due to physiological constrains, functional traits very often correlate with each other in a way that one trait cannot increase indefinitely without the decrease of another (Fig. 3(c)). These correlations lead to *trade-offs*, *i.e.*, situations in which improving in one task weakens the performance in another task, and thus to the evolution of ecological strategies that are only selected for in a narrow range of environmental conditions.

One of such trade-offs, experimentally measured in bacteria and phytoplankton (Button et al., 2004; Elbing et al., 2004; Healey and Hendzel, 1980), appears to exist between specific affinity α_ρ and specific maximum uptake rate ρ_{max} (Litchman et al., 2015b; Vallina et al., 2017) such that $\rho_{max} \times \alpha_\rho =$ constant, although the thermodynamic basis of this trade-off are yet unclear (Wirtz, 2002). This "affinity *versus* handling-rate" trade-off defines a spectrum of resource uptake strategies from gleaners to opportunists, each of

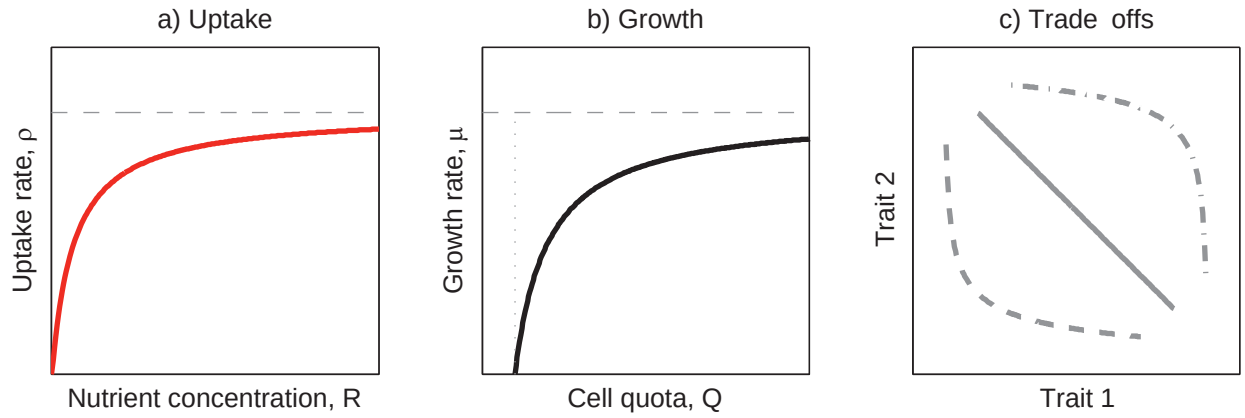

Fig. 3 (a) Uptake rate as a function of the external nutrient concentration as defined in Eq. (2) – the gray dashed line represents ρ_{max}; (b) Growth rate as a function of cell quota – the gray dashed line represents μ_{max} and the dotted line represents Q_{min}; (c) Three possible shapes for two-trait trade-offs depending on how changes in one of the traits impact the other.

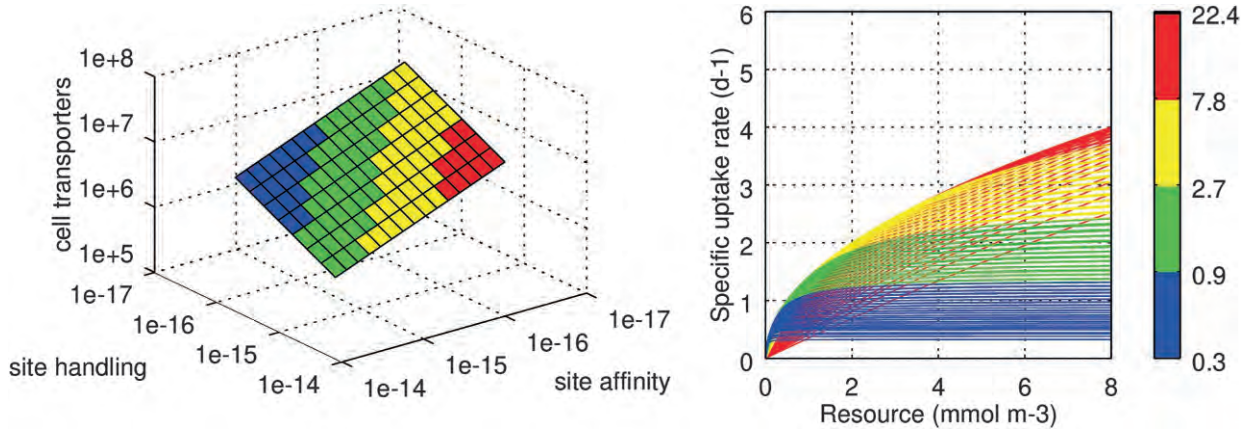

Fig. 4 A "gleaner *vs.* opportunist" trade-off of the type $\rho_{max} \times \alpha_\rho = constant$ leads to: (Left panel) a plane in a 3D space that relates three cell traits, specifically: affinity rate per transporter (m^3 $site^{-1}$ day^{-1}), handling rate per transporter (mmol $site^{-1}$ day^{-1}), and number of transporters per cell (sites $cell^{-1}$), none of which can be maximized without minimizing another. (Right panel) an array of uptake strategies that gradually go from gleaner ecotypes (blue color) to opportunist ecotypes (red color) as measured from their biomass-specific uptake rate $\rho(R)$ (day^{-1}). Under fluctuating conditions of resource concentration, each of these strategies will dominate the community at their optimal nutrient concentration that is given by $N_{opt} = \rho_{max}/\alpha_\rho = K_R$ (half-saturation constant – mmol m^3), where α_ρ is the specific affinity (m^3 $mmol^{-1}$ day^{-1}) and ρ_{max} is the specific maximum uptake rate (day^{-1}) of each microbial ecotype. Under steady-state conditions, the uptake strategy with lowest Rstar (see Eq. (34)) will tend to outcompete all other strategies asymptotically in time.

them adapted to different resource concentrations (see Fig. 4). High affinities (and hence low ρ_{max}) constitute gleaner strategies that are favored at low concentrations of resources, while low affinities (and hence high ρ_{max}) constitute opportunistic strategies that are better competitors at high concentrations of resources (see Fig. 4) (Fiksen et al., 2013; Grover, 1997). The root of this trade-off is the way uptake sites are packed in the finite cell surface (Aksnes and Egge, 1991) (see Eq. (3)). Assuming a fixed cell size: (1) for a constant handling rate per transporter (h), decreasing the number of transporters (n) while increasing their affinity (a) will lead to a gleaner strategy (and *vice-versa*); (2) for a constant affinity per transporter, decreasing the number of transporters while increasing their handling rate will lead to an opportunist strategy (and *vice-versa*); and (3) for a constant number of transporters per cell, increasing their handling rate while decreasing their affinity rate will lead to an opportunist strategy (and *vice-versa*); this last case provides the fastest transition from one strategy to the other (*e.g.*, from blue to red in Fig. 4). Finally, decreasing the number of transporters while increasing both their affinity and handling rates will lead to basically no change in the uptake strategy (see Fig. 4). The affinity of a transporter increases with its active area, and the handling rate of a transporter increases with the speed at which the carrier protein change its shape when it moves molecules across the membrane (see Fig. 1) although the exact mechanism is not well understood.

A variable cell size with a constant per-surface density of transporters (*i.e.*, the proportion of the cell's surface covered by uptake sites) can also affect biomass-specific uptake rates. For a constant per-transporter affinity (a) and handling rate (h), both the specific affinity (α_ρ) and maximum uptake rate (ρ_{max}) should in theory decrease with cell size due to a concomitant decrease in the surface-to-volume ratio (Aksnes and Egge, 1991). There is strong experimental support for these allometric relationships for marine

phytoplankton (Edwards et al., 2012) and the ecological implication is that the ability to compete for limiting nutrients should tend to decline with increasing cell volume in microbes (Chisholm, 1992). However other physiological limitations and trade-offs can appear with cell size, complicating this picture (Lindemann et al., 2016). For example, small cells (<10 μm) have several physiological constrains (*e.g.*, nonscalable components) that lead to low ρ_{max} (Marañón, 2015; Marañon et al., 2013). These limitations are less acute for larger cells because there is more internal space for catalytic and biosynthetic units, although other physiological constrains such as intracellular distances arise for much larger cells (Marañón, 2015). Large phytoplankton cells, such as diatoms, also have vacuoles that provide a high storage capacity for nutrients. These processes may help attenuate the negative effect of cell size on their biomass-specific effective growth rate and explain why relatively large cells (10–40 μm) usually have a dominant contribution (>50%) to total biomass under blooming conditions of opportunist phytoplankton (Marañón, 2015).

Since cell metabolism and functional traits are strongly linked to the microorganisms' size (Kempes et al., 2012; Marañón, 2015), they may also correlate with other traits, such as susceptibility to viral attacks or predation risk, that are influenced by cell size (Litchman and Klausmeier, 2008). Furthermore, due to the differences in species competitive ability at different external nutrient concentrations of the affinity – handling-rate (or gleaner – opportunist) trade-off defined earlier, if resources are provided in pulses rather than with a constant supply, the optimal uptake strategy changes as resources are depleted, leading to the formation of temporal niches that may favor the coexistence of competitors (Cermeno et al., 2011; Vallina et al., 2017). Functional traits and trade-offs are thus not only important at the level of the individual, determined by physiological constrains and controlling individual performance; they also have fundamental implications in species competitive ability and ultimately impact population dynamics and community composition (Litchman et al., 2015b; Litchman and Klausmeier, 2008; Tilman et al., 1982). Additional processes, such as spatial heterogeneities in nutrient availability or in population density also foster species coexistence and contribute to explaining global patterns of species distributions (Martínez-García and Tarnita, 2017; Tarnita et al., 2015; Vallina et al., 2014b).

In addition to the trade-off in resource acquisition discussed above, other trade-offs linking nutrient acquisition and utilization have been quantified in microbes. For instance, three major strategies have been defined for phosphorus acquisition in phytoplankton (Litchman and Klausmeier, 2008): (i) rate-adapted species, represented by high uptake rate (ρ_{max}) and high growth rate (μ_{max}); (ii) storage specialist, represented by high ρ_{max} but low μ_{max}; and (iii) affinity-adapted species, represented by low ρ_{max} and high affinity α_ρ (Crowley, 1975). Related trade-offs have been also found between resource use efficiency and growth rate or between the use of different resources (nitrogen *versus* phosphorous) in phytoplankton (Grover, 1997). Moreover, even though we have placed the focus here in competition for a shared resource as a fundamental driver of community composition, trade-offs have been also studied, both experimentally and theoretically, between different resource utilization abilities (Posfai et al., 2017). Finally, trade-offs also emerge in many other aspects of microbial lives, such as biofilm production (Nadell and Bassler, 2011), sporulation (Martínez-García and Tarnita, 2016; Wolf et al., 2015) or chemotaxis (Yi and Dean, 2016). They also control various ecological processes that underlie the composition, dynamics and stability of microbial communities, such as dispersal to new environments, dormancy or foraging (see also Section "Social Behavior in Microbes").

Food Web Dynamics

Models of microbial food webs focus on simulating the flux of mass among species at different trophic levels (Mostajir et al., 2015). The structure of such interactions is usually represented with a network in which each node represents a species and links represent the existence of interactions between them. Food webs are a subclass of ecological networks (Montoya et al., 2006; Xiao et al., 2017) in which species are connected by prey–predator interactions (Allesina et al., 2008; Allesina and Pascual, 2008; Thompson et al., 2012). Planktonic communities of unicellular organisms, dominated by protists (unicellular algae and protozoa), bacteria, and viruses (Smetacek, 2002) constitute one of the best studied examples of microbial food webs. They were traditionally seen as linear food chains (Steele, 1974) until the discovery of feedbacks between the species in higher and lower trophic levels, termed the *microbial loop*, emphasized the complexity of their structure (Azam, 1998; Pomeroy et al., 2007) (Fig. 5). Another example of microbial food webs are soil microbial communities, whose dynamics may shed light on the flows of energy that govern below-ground decomposition processes and nutrient cycling (Scheu, 2002). The existence of prey–predator interactions leads to negative correlations among predator and prey populations (*i.e.*, an increase in the abundance of predators leads to a decrease of their prey) (Wangersky, 1978). Population dynamics in food web models is remarkably rich and complex, ranging from equilibrium to oscillations and chaos (Fussmann and Heber, 2002; Huisman and Weissing, 1999; Murray, 2002b).

The general equation describing the dynamics of a food web, assuming linear Lotka-Volterra (LV) interactions, is:

$$\dot{x}_j = x_j\left(\sum_i \alpha_{ji}\, x_i - m_j\right) = x_j\, g_j(\mathbf{x}) \tag{31}$$

where $\dot{x}_j$ (mmol m^{-3} d^{-1}) represents the rate of change in the concentration of focal species j (mmol m^{-3}); α_{ji} (m^3 $mmol^{-1}$ d^{-1}) is the strength of the interaction of species i upon species j (which is positive when i is a prey of j and negative when i is a predator of j); and m_j (d^{-1}) gives the background mortality rate of species j (see Box 2). LV equations assume that the predator–prey (*i.e.*, resource–consumer) interactions do not saturate at high prey concentration because the biomass-specific handling rate of the predators is much higher than their biomass-specific affinity ($\rho_{ji}^{max} >> \alpha_{ji}$). Hence, the interaction is mainly governed by the affinity

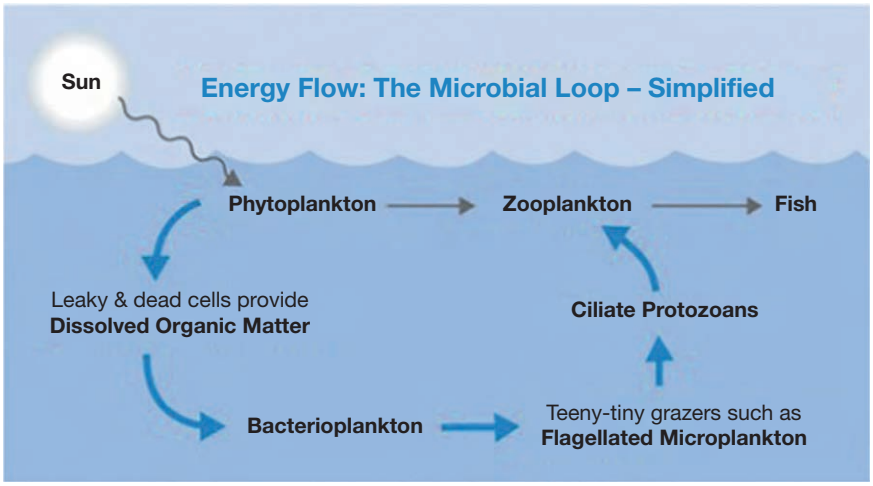

Fig. 5 Simplified marine food web incorporating both a food chain between different types of plankton (black arrows) and the microbial loop (blue arrows), mediated by the decomposed organic matter. Picture credits: © Moen, S., 2005. All plankton great and small. Journal of Great Lakes Research 492.

Box 2 Lotka-Volterra food webs – Steady states

Resource-ratio (R^* rule)

$$\frac{dx_j}{dt} = \text{NetGrowth} - \text{NaturalMort}$$

$$\left.\frac{dx_j}{dt}\right|_{x^*} = (\alpha_j R - m_j)x_j = 0$$

$$(\alpha_j R - m_j)x_j = 0$$

$$R^* = \frac{m_j}{\alpha_j}\left(\frac{\text{Mortality rate of consumer } x_j \text{ upon resource } R}{\text{Affinity rate of consumer } x_j \text{ upon resource } R}\right) \tag{32}$$

Food Chain ($h = j-1 < j < k = j+1$)

$$\frac{dx_j}{dt} = \text{NetGrowth} - \text{GrazingMort} - \text{NaturalMort}$$

$$\left.\frac{dx_j}{dt}\right|_{x^*} = (\alpha_j x_h - \alpha_k x_k - m_j)x_j = 0$$

$$(\alpha_j x_h - \alpha_k x_k - m_j)x_j = 0$$

$$x_h^* = \frac{m_j + (\alpha_k x_k^*)}{\alpha_j} = \left(\frac{\text{Mortality rate of predator } x_j \text{ upon prey } x_h}{\text{Affinity rate of predator } x_j \text{ upon prey } x_h}\right) \tag{33}$$

Food Web ($h = prey < j < k = predator$)

$$\frac{dx_j}{dt} = \text{NetGrowth} - \text{GrazingMort} - \text{SelfMort} - \text{NaturalMort}$$

$$\left.\frac{dx_j}{dt}\right|_{x^*} = \left(\sum_h \alpha_{jh} x_h - \sum_k \alpha_{jk} x_k - \alpha_{jj} x_j - m_j\right)x_j = 0$$

$$\left(\sum_i \alpha_{ji} x_i - m_j\right)x_j = 0$$

$$\sum_i \alpha_{ji} x_i^* = m_j \tag{34}$$

Matrix notation

$$\dot{x}_j = xj\left(\sum_i \alpha_{ji} x_i - m_j\right) = 0$$

$$\dot{\mathbf{x}} = \text{diag}(\mathbf{x})(\mathbf{A}\,\mathbf{x} - \mathbf{m}) = \mathbf{0}$$

$$\mathbf{x}^* = \text{inv}(\mathbf{A})\,\mathbf{m} \approx \left(\frac{\text{Mortality rate of all predators } j > i \text{ upon prey } x_i}{\text{Affinity rate of all predators } j > i \text{ upon prey } x_i}\right) \tag{35}$$

(see Eq. (2) after assuming that $\alpha_\rho/\rho_{max} \approx 0$). Furthermore, even if we relax this constrain, the assumption of a linear functional response between predator may still be valid when prey concentration is relatively low. For example, for prey *i* concentration values lower than the 25% of the predators *j* half-saturation constant $K_{ji} = \rho_{ji}^{max} / \alpha_{ji}$ (mmol m^{-3}) the linear approximation and the non- linear saturating curve are quite similar (see Fig. 2). This implies that the LV model assumes that the food web interactions are essentially limited by the encounter rate between predator and prey, either because of the fast handling rate of predators and/or because of the low concentration of prey.

The α_{ji} values are the elements of the Interaction matrix (**A**) of the LV food web. Element $\alpha_{ji} = \partial(\dot{x}_j/x_j)/\partial x_i$ (m^3 $mmol^{-1}$ d^{-1}) provides the linear effect that a small change in the concentration of species *i* has on the *biomass-specific* rate of change ($\dot{x}_j/x_j$) of species *j* (Novak et al., 2016). From a mathematical point of view, the Interaction matrix is related to the Jacobian matrix (**J**) of the LV model linearised around the steady-state equilibrium x^*, because $\mathbf{J} = \mathrm{diag}(\mathbf{x}^*)\,\mathbf{A}$ (see Box 3). The elements $\mu_{ji} = \partial\dot{x}_j/\partial x_i$ (d^{-1}) of

Box 3 Lotka-Volterra food webs – Stability analysis

The general form a multi-species food web ecosystem model is:

$$\frac{dx_j}{dt} \equiv \dot{x}_j = g_j(x_1, x_2, ..., x_N)x_j = g_j(\mathbf{x})x_j = f_j(\mathbf{x}) \tag{36}$$

where x_j (mol m^{-3}) refers to a given state-variable *j* in the ecosystem that can change with time ($x_j \equiv x_j(t)$), such as the concentration of one species when it refers to a biotic variable or the concentration of abiotic nutrients if they are explicitly resolved in the model. The integer value *N* gives the total number of variables in the ecosystem; and $g_j(\mathbf{x})$ gives the specific rate of change (d^{-1}) of x_j as a non-linear function g_j of the concentration of the relevant interacting variables specified as a vector $\mathbf{x} = x_1, x_2, ..., x_N$. When $g_j(\mathbf{x}) > 0$ the population x_j grows, and when $g_j(\mathbf{x}) < 0$ the population x_j decreases. Therefore $\dot{\mathbf{x}}_j = f_j(\mathbf{x})$ gives the absolute rate of change (mol m^{-3} d^{-1}) of variable x_j at any time as a function of its non-linear interactions with the other variables in the food web. Solving (integrating in time) this system of differential equations gives the time series of each variable x_j. The species concentration in the steady-state (x^*_j) are the *fixed points* of Eq. (36), which are obtained by setting all the rates of change $\dot{x}_j$ for all *j* equal to zero:

$$\dot{x}_j = f_j(\mathbf{x}^*) = 0 \tag{37}$$

A fixed point is defined as any vector $\mathbf{x}^*$ with coordinates (x^*_1, x^*_2, ..., x^*_N) in the phase space in which the concentration of the variables do not change with time (*i.e.*, the time series are flat). It is possible that a system has more than one feasible (*i.e.*, with positive concentrations) fixed point and therefore multiple steady-states. A steady state can be locally stable, neutrally stable, or unstable; depending on the dynamics of the system in the vicinity of the fixed point (see Fig. 6). Thus in order to study that dynamics, we add an infinitesimal perturbation to each steady-state concentration:

$$\mathbf{x}(t) = \mathbf{x}^* + \Delta\mathbf{x}(t) \tag{38}$$

where $\Delta\mathbf{x}(t=0) = \varepsilon\,\mathbf{x}^*$ with $\varepsilon << 1$, and calculate the change of the perturbation $\Delta\mathbf{x}(t) = \mathbf{x}(t) - \mathbf{x}^*$ over time. When $\Delta\mathbf{x}(t) > 0$ the perturbed community departs from its fixed point (which is thus unstable), and when $\Delta\mathbf{x}(t) < 0$ the perturbed community returns to its fixed point (which is thus stable). To study the change with time of this perturbed community $\Delta\mathbf{x}(t)$ by means of Eq. (36) we need to *linearise* the original non-linear system around the fixed point. This will give a linear approximation of the original non-linear system only valid for small departures from the steady-state. For larger perturbations, the behavior of the linear system can be significantly different to that of the original non-linear one, and the approximation may no longer be valid. To linearise Eq. (36), we obtain its Taylor expansion around the fixed point and neglect terms of second order (*i.e.*, $O(\varepsilon^2)$) and higher (May, 1974; Novak et al., 2016):

$$\Delta\dot{x}_j \approx \sum_{i=1}^{N} \frac{\partial f_j(\mathbf{x}^*)}{\partial x_i}\,\Delta x_i = \sum_{i=1}^{N} (\alpha_{ji}\,x_j^*)\,\Delta x_i = \sum_{i=1}^{N} (\mu_{ji}\Delta x_i) \tag{39}$$

$$\alpha_{ji} = \left.\frac{\partial(\dot{x}_j/x_j)}{\partial x_i}\right|_{\mathbf{x}^*} \quad \mu_{ji} = \left.\frac{\partial\dot{x}_j}{\partial x_i}\right|_{\mathbf{x}^*} \tag{40}$$

where α_{ji} (m^3 mol^{-1} d^{-1}) measures the effect of population *i* on the *biomass-specific* growth of population *j*, and $\mu_{ji} = \alpha_{ji}\,x_j^*$ (d^{-1}) measures the effect of population *i* on the *absolute* growth of population *j*; both at fixed point $\mathbf{x}^*$ in the linearised system. For example, the value $\mu_{jh} = \alpha_{jh}\,x^*_j$ defines the (linearised) interaction of prey at lower trophic level *h* on predator *j* (growth of *j* due to ingesting *h*); the value $\mu_{jk} = -\alpha_{jk}\,x^*_j$ defines the interaction of predator *k* on prey at lower trophic level *j* (mortality of *j* due to predation by *k*); and the value $\mu_{jj} = -\alpha_{jj}\,x_j^*$ defines the interaction of population *j* on itself (quadratic mortality of *j* due to intra-specific competition), if any. Note that $\alpha_{jh} = e_j\,\alpha_{hj}$, where $0 < e_j < 1$ is the assimilation efficiency of population *j*, which means that predator *j* has stronger negative effect on the growth rate of prey *i* than the positive effect that prey *i* has on the growth rate of predator *j*. This is because only a fraction of the prey ingested will be assimilated into predators' biomass, which leads to the skewed symmetry of the food web interactions. An equivalent but more compact matrix notation can be used for Eq. (39):

$$\Delta\dot{\mathbf{x}}(t) = \mathbf{J}\,\Delta\mathbf{x}(t) \tag{41}$$

where $\mathbf{x}$ is the ($N\times1$) column vector of x_j (mol m^{-3}), $\dot{\mathbf{x}}$ is their absolute rate of change (mol m^{-3} d^{-1}) and $\mathbf{J} = \partial\dot{\mathbf{x}}/\partial\mathbf{x}$ is the ($N\times N$) *Jacobian matrix* whose elements μ_{ji} (d^{-1}) are defined in Eq. (40). The off-diagonal elements μ_{ji} of **J** measure the inter-specific interactions, while the diagonal elements μ_{jj} measure the intra-specific interactions. Note that linearising a non-linear ecosystem leads to assuming linear Lotka-Volterra (LV) dynamics around its fixed point(s). The analytical solution of this linear ecosystem gives the change with time of the perturbed food web:

$$x_j(t) = x_j^* + \Delta x_j(t) = x_j^* + \sum_{i=1}^{N} (c_i v_{ji} e^{\lambda_i t}) \tag{42}$$

(*Continued*)

Box 3 (Continued)

where c_i are constants, and λ_i are the *eigenvalues* corresponding to the *eigenvectors* $\boldsymbol{v_i}$ of the Jacobian matrix such as $\mathbf{J}\, v_i = \lambda_i v_i$. This simply means that a linear transformation **J** applied to v_i (eigenvector *i*) may change is its length by a factor λ_i (eigenvalue *i*). Thus the eigenvectors of **J** can be stretched (unstable) or contracted (stable) by their eigenvalues, but they do not change direction because they are linearly independent among them. The eigenvalues may be complex numbers, but the system's resilience is given by their real part. For the system to be *stable* in the vicinity of a given steady-state, (the real part of) all eigenvalues of **J** must be negative. This can be easily understood by inspecting Eq. (42): if one (or more) eigenvalues is positive, the perturbation grows exponentially with time and thus that particular fixed point would be unstable. If we define the *dominant* eigenvalue (λ_d) as the largest eigenvalue, a necessary and sufficient condition for stability is that $\lambda_d < 0$. For non-positive eigenvalues if the real part of (at least) the dominant eigenvalue is zero, the steady-state is *neutrally stable* (the perturbation does not move along at least one eigenvector). The eigenvectors with negative eigenvalues form the stable eigenspace (*stable manifold*); the eigenvectors with positive eigenvalues form the unstable eigenspace (*unstable manifold*); the eigenvectors with zero eigenvalues form the neutrally stable eigenspace (*center manifold*). A summary of the several stability regimes of fixed points (*e.g.*, stable or unstable nodes, stable or unstable spirals, unstable saddle points, neutrally stable centers) and their relationship to the two major properties of the Jacobian matrix (its trace and determinant) can be found in Figs. 6 and 7. This asymptotic stability analysis must be performed around all the feasible steady-states of the system (there might be more than one) to obtain the whole picture of the stability properties of the food web ecosystem in its N-dimensional phase-space.

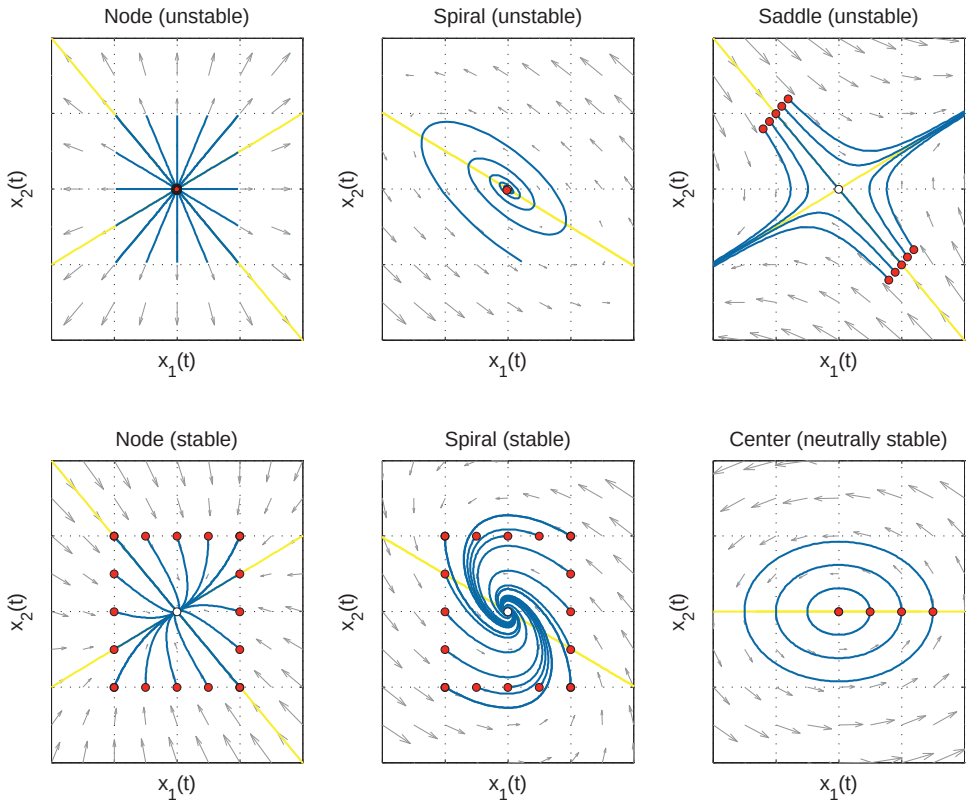

Fig. 6 Examples of the most common trajectory behaviors near fixed point singularities in the phase plane solutions of a two-dimensional continuous-time linear dynamical food web ecosystem (*e.g.* one predator – one prey) where the red dots are the initial conditions: [Upper left] Unstable node; [Upper middle] Unstable spiral; [Upper right] Saddle point (these are always unstable); [Lower left] Stable node; [Lower middle] Stable spiral; [Lower right] Center (these are neutrally stable).

the Jacobian matrix provide the linear effect of a small change in the concentration of species *i* on the *absolute* rate of change ($\dot{x}_j$) of species *j* at equilibrium (Berlow et al., 2004). That is, if a change in the concentration of a given species *i* does not affect the rate of change of another species *j*, then they are not linked (either directly or indirectly) in the food web (Novak et al., 2016). When predator *i* and prey *j* are connected, then $\alpha_{ji} > e_i\, \alpha_{ij}$, where $0 < e_i < 1$ is the assimilation efficiency of predator *i*. Therefore, the Interaction matrix is said to be "skew-symmetric" because the negative effect of predator *i* on prey *j* is larger (in absolute magnitude) than the positive effect of prey *j* on predator *i* by a constant factor. For the Jacobian matrix this is not necessarily the case because its elements are proportional to the concentration of the species at equilibrium, and inverted biomass pyramids (*i.e.*, top heavy) are possible (Barbier and Loreau, 2019). The Jacobian matrix is important when modelling microbial communities because its eigenvalues inform about the asymptotic stability (Allesina and Tang, 2012; Coyte et al., 2015) of the network to small perturbations of the steady state (*i.e.*, food web resilience – see Box 3). The diagonal elements of the Jacobian (μ_{jj}) represent the strength of

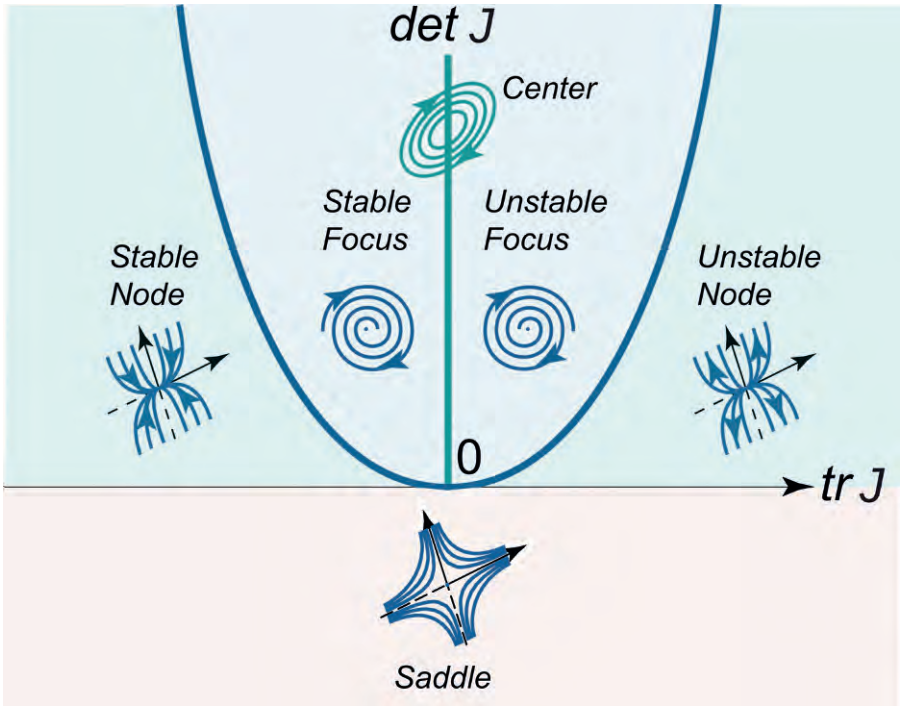

Fig. 7 Summary diagram showing how the trace **tr(J)** and determinant **det(J)** of the **Jacobian matrix (J)** define the stability types of fixed points in the phase plane of a two-dimensional continuous-time linear dynamical food chain (*e.g.*, one predator – one prey). Picture credits: © Math24.

the interaction of a species j with itself, and are usually considered to be negative (*e.g.*, intra-specific competition). Negative interactions in these diagonal elements of the Jacobian mimic the existence of a carrying capacity (*e.g.*, through quadratic mortality or self-regulation) for each species, leading to the *logistic equation* (Murray, 2002a). This promotes asymptotic stability of the network at equilibrium by preventing excessive growth of any microbial species (Haydon, 1994). While negative diagonal elements μ_{jj} are stabilizing, the off- diagonal elements μ_{ji} are destabilizing (Neutel et al., 2002). The Jacobian matrix ($\mathbf{J} = \mu_{ji}$) is said to be *negative diagonally dominant* and thus stable (Hofbauer and Sigmund, 1988; Logofet, 1993) if there is a set of positive numbers π_1, π_2,...,π_n, such that $\pi_j\,\mu_{jj} + \sum_{i\neq j}\pi_i\,|\mu_{ji}| < 0$. In other words, if for each species j in the network the intra-specific competition ($\mu_{jj} < 0$) is stronger than the sum of all the inter-specific interactions μ_{ji} affecting species j.

When essential nutrients are not explicitly included in the simulated food web, the nutrient competition among basal species must be implicitly assumed by imposing negative α_{ji} elements in the Interaction matrix. This is probably one of the major criticisms of classical food web models (see Eq. 36), because this simple approach lacks mechanistical foundations. Thus, it is usually advisable to add (at least) one essential abiotic nutrient N to the system by imposing a mass conservation constrain (that is, the total mass N_{tot} must be constant) such that $N = N_{tot} - \sum_j x_j$ (Grover, 1997). This basically means closing the system in terms of mass circulation and thus that the sum of all the differential equations is zero ($\dot{N} + \sum_j \dot{x}_j = 0$) When this constraint is explicitly included in the simulated food web, introduces a carrying capacity for the *whole* food web (*i.e.*, all nodes) because there is an upper limit to the whole community density given by the total mass N_{tot} in the system. Furthermore, the interaction of consumers that are not connected by predator–prey links but share the same resource(s), may be indirectly channeled through *resource competition* for the same pool of essential nutrients or common prey. That means that there is no need to impose non-zero elements in the Interaction matrix for their interaction. Another interesting feature of any food web (simulated or real) is the presence of indirect interactions among the species comprising the food web: even species that are not linked by direct predator–prey interactions (they don't prey upon each other) can be connected if they share a common predator, leading to *apparent competition* (Holt, 1977; Holt and Bonsall, 2017).

The temporal dynamics of food webs depends, therefore, on both bottom-up and top-down controls (de Ruiter et al., 1995). Resource supply acts as bottom-up control, while predator (or virus) induced mortality acts as top-down control. This mesh of interactions is usually composed by many weak and few strong linkages between species (Neutel et al., 2002). Top-down controls can be either stabilizing or destabilizing of food web dynamics depending on the functional form of the predatory response (Abrams and Allison, 1982; Haydon, 1994). While the LV equations assume linear functional relationships, more mechanistically driven models of microbial food webs use non-linear functional forms (Ward et al., 2012). In addition, food web models can include killing-the-winner (KTW) mortality of microbial communities, either by viral infection (Thingstad, 2000; Winter et al., 2010) or by preferential grazing (Vallina et al., 2014b). KTW is known to stabilize food web dynamics because it is an equalizing mechanism that "down-regulate the winners and up-regulate the losers" through density-dependent mortality

(*i.e.*, predator-mediated coexistence). KTW has a similar effect as density-dependent (*e.g.*, quadratic) self-mortality because it also makes the diagonal elements of the Jacobian to be more negative (Haydon, 1994). Since trophic relationships add potential interactions among species, both directly and indirectly, microbial food webs bring together population-, trophic-, and ecosystem-level processes. The existence of these connections across and within different levels opens the possibility for trophic cascades (Duffy et al., 2007) and positions species interactions as biological filters that can propagate environmentally-driven fluctuations in species densities throughout the community (Ives et al., 2003; Ives et al., 2000). Although food webs have thus been suggested to amplify environmental fluctuations (Barbier and Loreau, 2019; Ives et al., 2003), this theoretical prediction assumes linear interactions between species for mathematical simplicity and analytical tractability. More realistic interactions are known to be highly non-linear (*e.g.*, density-dependent interactions). Thus, due to non-linearities, food webs could be biological filters that actually dampen environmental perturbations throughout the community.

Loop length and loop weight analyses (LLA, LWA) are mathematical tools for exploring the structure and organization of trophic food webs by studying how the strength of feeding interactions affects the stability of the system. Loop length is defined as the number of different species visited through a closed pathway of interactions (*i.e.*, ending back to the starting species) without visiting other species more than once. Loop weight is defined as the geometric mean of the absolute values of the interaction strengths in a loop (Neutel et al., 2002). The skewed combination of many weak and few strong links of predator-prey interactions within long network loops has been suggested to increase the stability of food webs (Neutel et al., 2002). However, this prediction is based on rigid non-selective predator–prey interactions, which are known to lead to ecosystem instability if they are too strong (Neutel et al., 2007). The relative contribution of weak and strong links in microbial communities may be affected by more flexible KTW interactions. In addition to loop length and loop weight, other topological properties of networks related to stability are connectivity, interaction strength, centrality, modularity and nestedness (Berlow et al., 2004; Landi et al., 2018). Connectivity measures the density of links in the network; interaction strength measures how strong these links are; centrality measures how important a node is in the network (*e.g.*, keystone species), either because it is highly connected (to many other nodes), does so strongly (channels large flows of mass energy), or because it governs many indirect interactions and/or can trigger many secondary extinctions if the node is removed (Dunne et al., 2002); modularity implies that the network is composed of distinct and densely connected subsystems (Grilli et al., 2016); nestedness means that the diet of the most specialized species is a subset of the diet of the next more generalized species, and its diet a subset of the next more generalized, and so on (Montoya et al., 2006). These properties define the patterns of ecological networks and are used to evaluate their stability and robustness (Grilli et al., 2016; Montoya et al., 2006).

Despite the extensive research and the many models developed during the last four decades, the exact mechanisms that stabilize food webs are yet not fully understood. Early work focused on measures of stability at equilibrium (see Box 3) and predicted that more complex food webs should lead to more unstable ecosystems (Harrison, 1979; May, 1974; Pimm, 1984). However, these predictions are challenged by field observations in both terrestrial and aquatic systems, and thus newer theories have moved the focus to aggregated community-level propertiral equation describing the dynamics of a foes under non-equilibrium conditions (Loreau, 2010a). Yet, the analysis of microbial food web models in the steady-state is a powerful tool to predict the outcome of competition in microbial communities (*i.e.*, species persistence) subject to lab-controlled conditions such as chemostat bioreactors (Grover, 1997; Tilman, 1982). Assuming LV interactions (Lotka, 1920; Volterra, 1928), we can predict the steady-state abundance of the different populations that form the food web. For the simplest case of a trophic interaction composed of one abiotic resource shared by several consumers, the "*R*-star" rule applies. The concentration of the resource at equilibrium (R^*) in a mono-culture of the consumer is given by the ratio between the mortality rate (m_j) and the uptake affinity (α_j) of the consumer j (see Eq. (35) in Box 2). The consumer that is able to subsist at the lowest resource concentration after reaching equilibrium (*i.e.*, lowest R^*) will eventually outcompete all other consumers (Tilman, 1982). However, this theoretical prediction only holds in the absence of stabilizing mechanisms (*e.g.*, self-regulation), which can lead to some degree of species coexistence at steady-state (see above). The same rule applies to food webs, considering that the consumers j at lower trophic levels are the resources of consumers k at higher trophic levels. In the simplest scenario of linear food chains there is only one species per trophic level, and the concentration at equilibrium of the species at trophic level $(j-1)$ is given by the ratio between the mortality rate and the grazing affinity of its consumer at trophic level j (see Eq. (36) in Box 2). Note that the equilibrium of each species does not depend on their own physiological traits but on those of their predators. Food chains based on linear predator–prey interactions have been very influential in the development of ecological theory and understanding their dynamics serves as the cornerstone for studying more complex food webs and functional responses (see Barbier and Loreau, 2019 for a review). For the most complex case of a multi-species and multi-trophic level food web, considering that species in a given trophic level j may serve as resource of the consumers at several trophic levels above it ($k > j$), the steady-state of x_j will depend on the ratio between a combination of the mortality rates and grazing affinities of those higher-trophic level consumers feeding on him (see Eq. (38) in Box 2).

Assuming a more realistic Michaelis-Menten (MM) formulation for resource-consumer interactions (see Section "Uptake Machinery"), because it accounts for consumer saturation at high resource concentration, leads to a slightly different expression for the "R-star" expression:

$$R^* = \frac{m_i}{\alpha_i - m_i\left(\alpha_i/\rho_i^{max}\right)} \tag{43}$$

Most food web models are based on variants of the MM formulation for resource-consumer interactions (Gilljam et al., 2015), where the R^* rule still applies at equilibrium. However, the R^* rule cannot predict the outcome of competition under

non-equilibrium conditions when there is a trade-off between resource uptake affinity (α_i) and maximum uptake rate (ρ_i^{max}) (see Section "Functional Trade-Offs"). The best known example occurs when under pulsed nutrient supply, high-nutrient-adapted ecotypes (HNA – which are opportunists with high ρ_i^{max}) can outcompete low-nutrient-adapted ecotypes (LNA – which are gleaners with high α_i). This occurs even if LNA ecotypes have lower R^* than HNA ecotypes and would otherwise dominate the community at steady-state equilibrium (Cermeno et al., 2011). The MM formulation can simulate this trade-off, which happens when the uptake curves of two consumers intersect at some resource concentration (see Fig. 4). However, the LV formulation does not account for this trade-off because LV equations assume that $\rho_{ji}^{max} >> \alpha_{ji}$, and thus that the resource-consumer interactions are governed by just α_{ji} (*i.e.*, without saturation at high resource concentration). Therefore, in non-equilibrium environments it is advisable to use the MM equations to simulate resource–consumer interactions in microbial food webs, and restrict the use of the LV equations to the linear stability analysis of steady-states (see Box 3). Thus, the R^* rule derived for equilibrium conditions (either using LV or MM equations) is useful but does not always apply to most natural ecosystems, in which microbial communities are subject to non-equilibrium environmental conditions and physiological trade-offs are the norm (Barton et al., 2010; Litchman and Klausmeier, 2008). Furthermore, under non-equilibrium environmental conditions, classic equilibrium theory to compute ecosystem resilience (see Box 3) does not apply, and other metrics such as the standard deviation of the population time-series are more informative (Vallina and Le Quéré, 2011). Recognizing that most microbial ecosystems are subject to non-equilibrium environmental conditions has also opened the door to a reconciliation between earlier theoretical models, whose predictions assumed that populations are in the steady-state equilibrium, and more recent numerical models that enable predictions for non-equilibrium situations (Barton et al., 2010; Vallina et al., 2017).

Ecological Selection and Community Assembly

The concept of "assembly rules" was proposed almost 40 years ago and refers to the fundamental mechanisms that govern how species associate to form a community. This concept defines the theoretical conditions for species persistence depending on their trait values (Diamond, 1975; Grover, 1997) and it is thus related to what is known as "ecological selection" (see below). One of the most intriguing aspects regarding microbial community assembling is how to explain the maintenance of species and trait diversity – what are the mechanisms that allow so many different and competing species to coexist within the same ecological niche? The *competitive exclusion principle* states that two species that occupy the same ecological niche cannot coexist indefinitely (Hardin, 1960). The rate of competitive exclusion depends on the degree of niche overlap and the particular trait values of the competing species (Vallina et al., 2017). Large niche overlap and larger differences in trait values lead to faster exclusion rates than small niche overlap and smaller differences in trait values. When such niches are defined by shared resources (maximum overlap), the competitive exclusion principle translates into the *theory of limiting nutrients*, which states that there cannot be more coexisting species than the number of *essential resources* (Tilman, 1982) (see Section "Food Web Dynamics" – "R-star" rule).

Ecological selection is directly related to *invasion fitness* (see Box 4) and competitive exclusion (Gause, 1934), as it works upon a multi-species community until only the set of best adapted species (a fraction of the original pool) survive locally. Ecological selection leads to the "survival of the fittest" through competitive exclusion on ecological time-scales, *i.e.*, before longer-term evolutionary dynamics can take place (Falkowski and Oliver, 2007). The best adapted species to a particular environment or ecological niche will dominate the local community. Thus, the system may be dynamically unstable, even in the absence of environmental changes, because species extinctions may occur locally until a stable community is reached for the current environmental conditions. When the environmental conditions change, a new and different microbial community may self-assemble de-novo through ecological selection if there is exchange of species with other communities or if there is a local pool of rare (non-dominant) ecotypes available (Follows et al., 2007; Pedros-Alio, 2012). Ecological selection can thus be defined in the context of *niche space*, which can be categorized in two classes: fundamental niche and realized niche. The *fundamental niche* of a given species defines the environmental conditions that are suitable for growth in the absence of interactions with other species, while the *realized niche* defines the environmental conditions where a given species is actually observed when it is interacting with other species (Hutchinson, 1957b). Therefore, the realized niche may be narrower than the fundamental niche, due to the exploitative competition among the interacting species.

Community assembly in soil ecosystems can be affected by agricultural practices and thus there is a well developed theoretical framework to simulate the observed patterns at equilibrium (de Ruiter et al., 1993; de Ruiter et al., 1995; Kaunzinger and Morin, 1998; Neutel et al., 2002, 2007). Metabolic modelling under a reverse-ecology framework has also been used to successfully elucidate the assembly rules of the human microbiome at the community-level, suggesting that microbiome assembly is dominated by environmental filtering (Levy and Borenstein, 2013, 2014). Aquatic environments are among the best studied examples of how ecological selection may operate for the community assembly of microbial communities (see Fig. 8) (Follows and Dutkiewicz, 2011; Litchman and Klausmeier, 2008). Microbial cells such as bacterioplankton and phytoplankton, are suspended in a generally well-mixed medium and compete for the same essential resources from a common and spatially fairly homogeneous pool, which implies that all individuals are potentially interacting with each other (Sommer, 2002). However, field observations contradict theoretical predictions and show that there is a much larger number of coexisting species than of non-substitutable resources (*e.g.*, elemental nutrients) in the ocean, which has led to what is known as the "Paradox of the Plankton" (Hutchinson, 1961). Theoretical studies have suggested several hypotheses in order to account for this discrepancy between theoretical predictions and field observations. There are several conceptual frameworks that have been explicitly invoked to explain the assembly of microbial

Box 4 Ecological fitness.

$$\frac{\partial \mathbf{x_j}}{\partial t} = \text{NetGrowth} - \text{NaturalMort}$$

$$= \mu_j^{max}\left(\gamma_j^N\ \gamma_j^T\ \gamma_j^I\right)x_j - m_j\ x_j$$
$$= \mu_j\ x_j - m_j\ x_j$$
$$= (\mu_j - m_j)x_j$$
$$= \lambda_j\ x_j \tag{44}$$

where:

$$\gamma_j^N = \frac{N}{\left(\mu_j^{max}/\alpha_j\right) + N} \leq 1 \tag{45}$$

$$\gamma_j^T = \frac{\exp(-(T_j^{opt} - T)^2}{(2\sigma_T^2)} \leq 1 \tag{46}$$

$$\gamma_j^I = \frac{I}{I_j^{opt}}\exp\left(1 - \frac{I}{I_j^{opt}}\right) \leq 1 \tag{47}$$

The parameter $\lambda_j \leq \mu_j^{max}$ gives the specific rate of change of microbial ecotype j in units of inverse time (d^{-1}), and is a measure of their ecological (*i.e.*, invasion) fitness that depends on the environmental conditions affecting their maximum growth rate μ_j^{max}. The parameters γ_j^N, γ_j^T and γ_j^I determine the nutrient limitation, temperature limitation and irradiance limitation for microbial ecotype j, respectively. This model assumes that the effect of these three environmental factors on fitness is multiplicative, which implies that they are considered to be independent factors co-limiting growth (Fasham et al., 1990; Follows et al., 2007). The physiological traits of the different ecotypes will determine their optimal values of nutrient concentration (N_j^{opt}), optimal temperature (T_j^{opt}), and optimal irradiance (I_j^{opt}). These three coordinates (N_j^{opt}, T_j^{opt}, I_j^{opt}) define the environmental niches where each ecotype is the best competitor (see Fig. 9), and thus its location in the niche space (see Fig. 9, lower panel). Note that $N_j^{opt} = K_j^{sat} = (\mu_j^{max}/\alpha_j)$ when a gleaner-opportunist trade-off is assumed (see Section "Functional Trade-Offs"). Therefore, for a given set of environmental conditions, ecological selection will lead to a community dominated by the fittest ecotypes. Changes in the environmental conditions modify the fitness of each ecotype and also the composition of the community (*i.e.*, beta diversity; see Section "Biodiversity and Ecosystem Functioning").

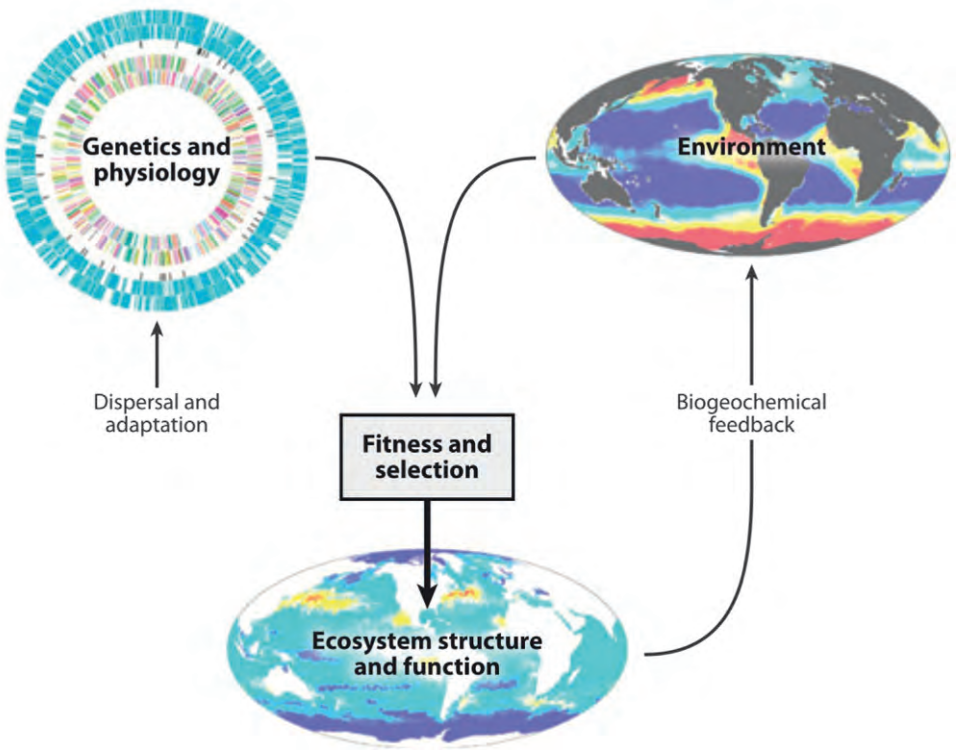

Fig. 8 The combination of different genotypes, different environmental conditions, and global ocean dispersal leads the "ecological selection" of the ecotypes with higher fitness locally, in either space or time. The resulting community assembly and species composition at each location will thus be different depending on the environmental conditions. Therefore, the ecosystem structure and functioning will vary. Furthermore, the environmental conditions will also be affected by the local community and its composition, mostly through nutrient depletion. Therefore there is a feedback between environmental conditions and the ecological selection of locally dominant ecotypes. Picture credits: © Follows, M.J., Dutkiewicz, S., 2011. Modeling diverse communities of marine microbes. Annual Reviews of Marine Science 3, 427–451.

communities using mechanistic ecosystem models, such as niche theory for marine algae (Barton et al., 2010; Follows et al., 2007) or the human microbiome (Levy and Borenstein, 2013); neutral theory for generic host-associated microbiomes (Zeng and Rodrigo, 2018); the resource-ratio hypothesis for freshwater algae (Tilman, 1982); the killing-the-winner hypothesis for bacteria (Maslov and Sneppen, 2017; Thingstad, 2000) and phytoplankton (Vallina et al., 2014b); the nutrient-load hypothesis for cyanobacteria and phytoplankton (Brauer et al., 2012); the high-order interactions hypothesis for bacteria (Grilli et al., 2017); and even chaos theory for phytoplankton (Huisman and Weissing, 1999, 2001); among others.

The *niche theory* suggests that species are ecologically unique, which implies that they occupy different environmental niches and fulfill different functional roles (MacArthur, 1968). Therefore, organisms can exploit and partition a set of resources along environmental gradients (*e.g.*, elemental nutrients, prey-size, ambient temperature, solar radiation) by decreasing their niche overlap through specialization (Loreau, 2010a). On the other hand, *neutral theory* suggests that species are ecologically equivalent, which implies that they have a similar competitive strength, slow exclusion rates, and thus can occupy the same environmental niches or fulfill the same functional roles for long periods of time. Under this view, community assembly is governed by random demographic processes such as the rate of immigration from the regional pool (Hubbell, 2001). The *killing-the-winner* hypothesis provides a simple mechanism for stable coexistence due to the presence of a trade-off between competition ability for growth and vulnerability to mortality among the competing species. Higher-abundance species will be proportionally more susceptible to phage or predatory attack, which can eventually result in greater fitness of the lower-abundance species (i.e., the fitness of a phenotype increases as it becomes rarer) (Maslov and Sneppen, 2017; Thingstad, 2000; Vallina et al., 2014b). The resource-ratio hypothesis describes the assembly rules by which species competing for several resources can coexist at intermediate levels of nutrient supply (Tilman, 1982). Finally, high-order interactions theory suggests that the presence of a given species influences the interaction between other species (Billick and Case, 1994; Grilli et al., 2017; Kelsic et al., 2015) and shapes the relationship between ecosystem diversity and stability (Bairey et al., 2016). Recent studies, both theoretical and experimental, have shown that this higher-order interactions are often established through cross-feeding, a class of interspecific interaction in which organisms belonging to one species use metabolites produced and secreted by the other species as energy or nutrient sources (Goldford et al., 2018; Goyal and Maslov, 2018; van Hoek and Merks, 2017) (see Section "Social Behavior in Microbes").

One fundamental feature of most microbial organisms is their great capacity of passive dispersal by external agents, which affects the population dynamics and community assembly (Cermeño and Falkowski, 2009). Dispersal influences the probability of species to reach distant environments and thus their potential to interact with many other species – hence the common say in microbial ecology "everything is everywhere, but the environment selects" (Baas-Becking, 1934; De Wit and Bouvier, 2006). For instance, aquatic microorganisms are subject to passive advection and turbulent diffusion by water currents; in terrestrial systems, wind plays a similar role. This is generally explicitly included in global ocean models (*e.g.*, Follows et al., 2007). Other means by which microbes are dispersed are within the microbiome or attached to larger organisms. Community assembly theory suggests that the two main processes affecting the spatial and temporal distribution of species trait values are *species competition* and *environmental filtering*. Environmental filtering occurs when a species arrives at a focal site but fails to survive even in the absence of neighbours; competitive exclusion occurs when a species arrives and can persist in the absence of neighbours but not in their presence (Kraft et al., 2015). These processes do not act in isolation, however, and it is the interplay of competition- and filtering-driven fitness that leads to the *ecological selection* of species. Competition leads to ecological differentiation, while filtering reduces the spread of trait values and leads to similar ecological tolerances (Cornwell et al., 2006). Therefore, functional traits determine the species distribution along environmental gradients as well as the species competition for resources within a given environmental niche (Ackerly and Cornwell, 2007). Coexistence between species is thus the result of a balance between stabilizing forces (*i.e.*, niche differences) and equalizing forces (*i.e.*, fitness similarity) (Chesson, 2000a). Stabilizing mechanisms are those that increase the strength of intra-specific competition relative to the strength of inter-specific competition, while equalizing mechanisms are those that reduce the magnitude of the fitness difference (Loreau, 2010a). Stabilizing niche differences facilitate coexistence whereas relative fitness differences drive competitive exclusion.

Thus the niche space can be seen as the hypervolume of environmental conditions under which each microbial ecotype persists (Hutchinson, 1957a). Microbes in natural ecosystems have traits to cope with several environmental gradients, such as nutrient concentration, ambient temperature, solar irrandiance, *etc* (see Fig. 9 upper panels). The patterns of functional trait similarity cannot easily be used to infer the relative contribution of environmental filtering *versus* competitive interaction in shaping the community assembly of microbial communities (Vallina et al., 2017). Competitive exclusion can be avoided by species that are functionally very dissimilar when the difference between the species traits are acting as stabilizing niche differences; *e.g.*, optimal values of temperature and/or solar radiation levels (see Fig. 9 upper panels). However, when differences in species traits directly translate into fitness differences, then competitive exclusion can eliminate the functionally weaker competitors within a given ecological niche (*i.e.*, lower resource exploitation ability) (Chase and Leibold, 2003). The role of species dispersal can further blur the line separating environmental filtering and species competition. Furthermore, microbial communities in aquatic systems live in a very dynamic fluid environment, which implies that their environmental niches are neither stationary nor spatially closed. Therefore, all ecotypes can potential migrate between different niches by passive dispersal and will dominate the community where the environmental conditions match their optimal values (*e.g.*, see Fig. S2 in Follows et al., 2007). A similar effect occurs by wind-mediated dispersal in terrestrial communities (Tarnita et al., 2015).

Dispersal can thus influence the microbial diversity at local and regional scales. The rate of dispersal compared to the rates of competition and/or environmental filtering will determine the actual community assembly and ecosystem structure over larger regions (Clayton et al., 2013). Spatial environmental heterogeneity, coupled to dispersal between different locations, may thus

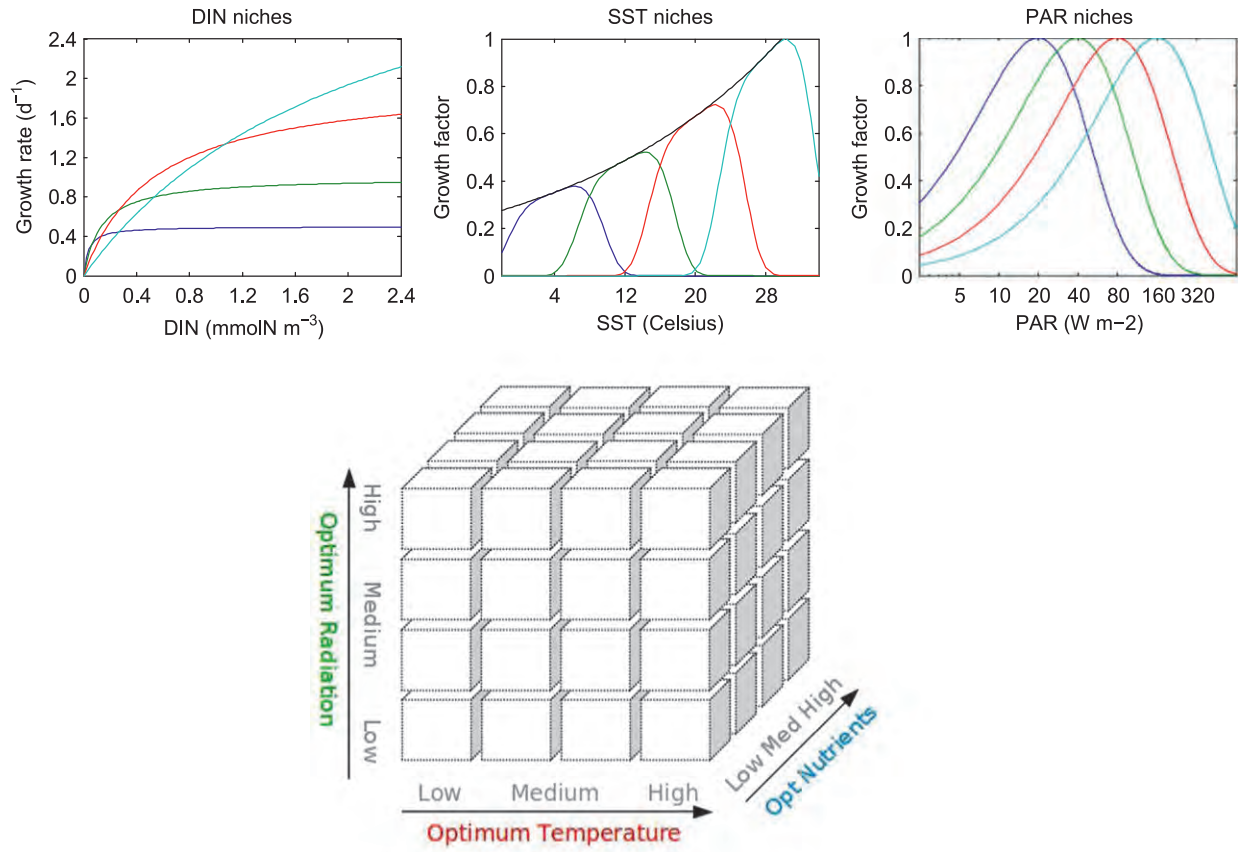

Fig. 9 Bottom: "Hypercube" setup of a model with 64 phytoplankton species each of them occupying a particular ecological niche given by some of their functional-traits (*i.e.*, optimal levels) for 3 environmnetal axes: dissolved inorganic nutrients (DIN), water temperature (SST), and photosynthetically active radiation (PAR); Upper: Growth curves and optimum values ± ecological tolerances of the 64 simulated marine phytoplankton species along those 3 environmnetal axes (DIN, SST, PAR).

foster species diversity. Temporal heterogeneity may also promote coexistence when alternating environmental conditions within a time period favor different species, which is sometimes called the *storage effect* (Chesson, 2000b; Vallina et al., 2017). Such heterogeneity is characteristic of many real ecosystems that are subject to the effect of seasonal trends in environmental parameters. However, in order to induce a temporal niche partition and thus lead to species coexistence, time-varying environmental conditions need to satisfy two additional requirements: (i) the rate at which populations decline together with the temporal scale of the environment avoids the extinction of non-favored species and (ii) the covariance between environment and competition intensity is opposite for high density and low density species, which allows species to have positive growth rates when they become less abundant and recover larger population sizes. The temporal storage effect was initially proposed to explain coexistence in annual plants, but recent models have extended the concept to sporulating microbes and suggested its potential importance in explaining diversity in certain terrestrial communities of microbes in which dispersal can be neglected (Martínez-García and Tarnita, 2017). The theoretical frameworks and ideas discussed here are the conceptual basis for the mechanisms included in most microbial ecosystem models that are used today.

Ecological Evolution and Community Adaptation

Microbial organisms have the capacity to evolve over hundreds to few thousands of generations in response to environmental variability, even when mutation rates are low, because of their fast replication rates and large population sizes (Elena and Lenski, 2003; Frank and Slatkin, 1990; Hellweger et al., 2014; Huertas et al., 2011; Van den Bergh et al., 2018). Microbial communities are subject to both (i) *ecological selection*, which acts through short-term competitive exclusion between species (see Section "Ecological Selection and Community Assembly"); and (ii) *adaptive evolution*, which provides inheritable change of species-traits and affects the organisms' fitness after several successive events of ecological selection. Ecological evolution thus refers to the arrangement of organisms along phylogenetic lines (Bews, 1927). The relative influence and interplay of both selection and evolution on the

dynamics of microbial communities is a challenging and fascinating research venue for modelers due to the overlap of temporal scales (Doebeli et al., 2017; Fussmann et al., 2007; Ghoul and Mitri, 2016; MacColl, 2011). Formally, however, there is no difference between ecological and evolutionary dynamics because both are based on the same fundamental birth-death processes (Doebeli et al., 2017). Given the random nature of discrete genetic mutations and their effects on the phenotype, these *eco-evolutionary dynamics* can be studied with stochastic differential equations in continuous time (*e.g.*, Focker-Planck equation) that approximate the mutation steps as infinitesimally small. Under this view, evolution can be visualized as an uphill walk on "Dynamic Fitness Landscapes" (DFL) that keep changing as a result of the evolution it engenders (Metz, 2011; Mustonen and Lassig, 2009; Richter, 2013; Richter and Engelbrecht, 2014). However DFL are *not* a driver of evolution, they are an emergent property of the underlying stochastic birth-death ecological process (Doebeli et al., 2017). Evolutionary changes in microbial communities thus respond to both local selection pressures as well as population dynamics, because ecological interactions create density-dependent selective pressures that affect gene frequencies (Cordero and Polz, 2014).

Eco-evolutionary dynamics are particularly important for microbial communities because their explicit inclusion of population dynamics into evolutionary models allows evaluation of the potential feedbacks between fitness and organisms (Dieckmann, 1999). Theoretical studies show that species will evolve to maximize their geometric mean fitness in environments that vary stochastically in time. Hence, evolutionary change is expected even if decadal-scale changes are smaller than inter-annual variability (Frank and Slatkin, 1990). An example in which eco-evo feedbacks are relevant is antibiotic-mediated interactions. Microbial evolution in a spatially structured landscape with environmental gradients of antibiotic concentration has even been shown in real time (Baym et al., 2016). The evolution of cancer cells may also impose limits to the predictability of precision cancer medicine (Lipinski et al., 2016). Several studies have addressed the effect of eco-evolutionary feedbacks under different microbial scenarios (Bonachela et al., 2017; Kotil and Vetsigian, 2018; Martin et al., 2016; Tarnita, 2018; Vetsigian, 2017). The introduction of such feedbacks in plankton models is, however, a more recent line of research than in other microbial systems (Chen et al., 2018; Merico et al., 2014; Norberg et al., 2012; Smith et al., 2016). However, in most current models of microbial ecosystems, species traits do not evolve nor adapt over time. For example, current model projections of plankton community structure under future climate change scenarios assume that species have fixed traits (environmental preferences) and will not adapt to the new conditions (Dutkiewicz et al., 2013). The need to include adaptive dynamics in the next generation of microbial models is widely recognized (Irwin et al., 2015) and several important contributions have been made in this direction (*e.g.*, Chen et al. (2018), Edwards and Steward (2018), Lomas et al. (2014), Merico et al. (2014), Norberg et al. (2001), Sauterey et al. (2017), Sauterey et al. (2015), Smith et al. (2016) and Williams and Lenton (2008)).

The ability to adapt to changes in resource concentration may be linked to mutations in genes that regulate cell size or shape, as well as the number and efficiency of membrane transporters (see Section "Uptake Machinery"). Likewise, the ability to adapt to environmental changes in temperature or irrandiance may be linked to mutations in the genes that regulate biochemical reactions involved in catalytic efficiency or light harvesting capabilities. Eco-evolution also applies to social behaviour in microbes, such as cellular adhesion to form aggregates with collective functioning (Garcia et al., 2015) (see Section "Social Behavior in Microbes"). This long-term evolutionary capacity of microbes to adapt to changing environments or social contexts on decadal scales may be more predictive than short-term physiological responses (or acclimation) in determining the winners and losers under future climate change scenarios (Dutkiewicz et al., 2013). The absence in most models of mechanisms allowing the *adaptive radiation* of species-traits resulting from random mutations combined with natural selection implies that once a dominant species is established, it will remain unchanged and with no possibility to be unchallenged by newly created species (Dieckmann, 1999; Geritz et al., 1998) (*i.e.*, resident phenotypes *versus* mutant phenotype PIP – see Fig. 10). Finding ways to include ecological evolution in models (*e.g.*, evolutionarily singular strategies and/or trait-diffusion frameworks) is an active field of research (Chen et al., 2018; Doebeli, 2002; Fritsch et al., 2017; Szappanos et al., 2016). Adding eco-evolutionary dynamics to microbial models may help understand fundamental hypotheses of species evolution such as the "Red Queen Dynamics" (Bonachela et al., 2017). Theoretical models can help improve our understanding of ecological evolution by testing hypothesis about the conditions for adaptive evolution, the waiting time to adaptation, the duration of adaptive processes and the different characteristics of the emerging species (Gavrilets, 2004). These features are expected to depend on different evolutionary factors and parameters, such as the rates of migration and mutation, the strength of selection for local adaptation, population size, or the spatial structure of the population.

Several "ecological evolution" (eco-evo) frameworks for modelling Adaptive Dnamics (AD) of traits based on evolutionarily singular strategies (ESS) and pairwise invasibility plots (PIP) (Dieckmann, 1997; Diekmann and Law, 1996; Geritz et al., 1998; Smith, 1982) or modelling Trait Diffusion (TD) based on solving the mean and variance of trait distributions (Norberg et al., 2001), were developed more than 20 years ago. These modelling frameworks for eco-evo are based on the notion of Dynamic Fitness Landscapes (DFL) that its rooted on the original concept of *adaptive landscapes* (Shpak, 2012; Wright, 1932) (see Figs. 11 and 12). Although the definition of the "fitness" metric is context dependent (Metz, 2018; Metz et al., 1992), for deterministic models the fitness of an ecotype under the local environmental conditions is usually measured as the biomass-specific growth rate (d^{-1}) of the population. DFL give the relationship between genotype and survival fitness for a given environmental condition (Loewe, 2016). The horizontal axes (independent variables) represent the *genotype space* (the set of all possible genotypes) and the vertical axis (dependent variable) represents the *genotype's viability* (probability of survival or growth rate) (see Fig. 12). Due to feedbacks between survival fitness and genotype frequency, the fitness landscape is not static but dynamic because of its density-dependency on gene frequencies, which vary through time (Doebeli et al., 2017; Mustonen and Lassig, 2009; Richter, 2013; Richter and Engelbrecht, 2014; Shpak, 2012). They also change in response to variations in the abiotic environmental conditions Thus the original view of evolution along static fitness landscapes is now recognized to be misleading since evolution happens along

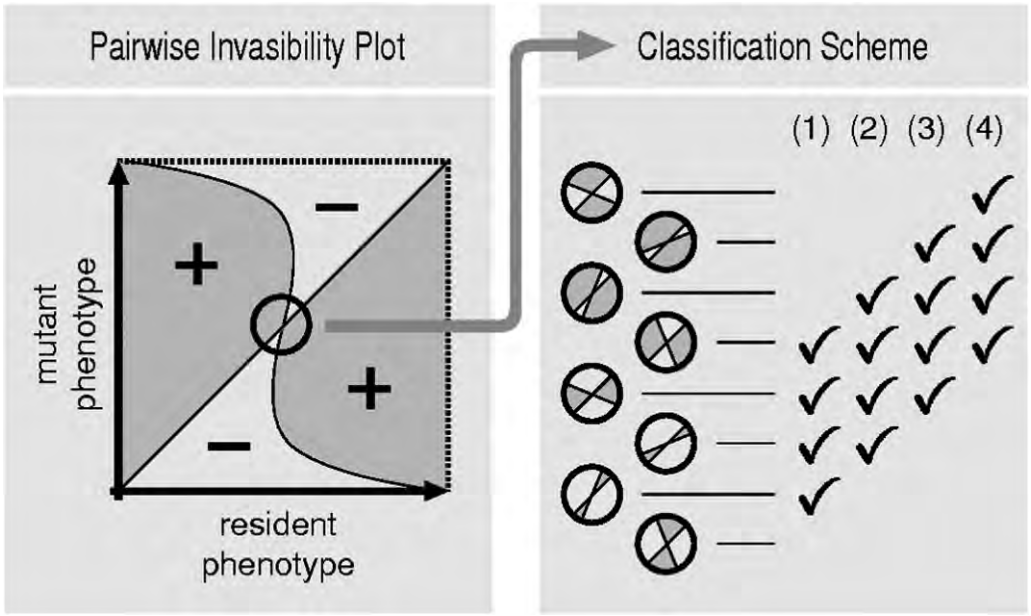

Fig. 10 Pairwise invasibility plots (PIP) and the classification of evolutionarily singular points (ESS). *[left-panel]* The horizontal axis defines the phenotype of the resident population given by the scalar value k_r of one trait (e.g., optimal temperature). The vertical axis defines the phenotype of a mutant population given by the scalar value k_m of the same trait. The invasion fitness λ_m (see Box 4) is defined as the specific growth rate (d^{-1}) of an initially rare mutant within local the environment (*e.g.*, ambient temperature) where a resident population is well established. The resident population is assumed to be a demographic attractor in steady-state and therefore has $\lambda_r = 0$. Shaded areas represent the trait-space combinations (k_r, k_m) for which a rare mutant with phenotype or trait value k_m can invade a resident population consisting of individuals with phenotype or trait value k_r; that is, the regions for which $\lambda_m > 0$. Clear areas represent the regions for which $\lambda_m < 0$, therefore where the rare mutant cannot invade the resident phenotype. The evolutionary process can be seen as a sequence of successfully established invasions, where the mutant phenotype k_m then becomes the new resident k_r. The strategy or trait value for which evolution can come to a halt are called *evolutionarily singular strategies* (ESS). Near such points the fitness landscape as experienced by a rare mutant is locally flat. ESS can be (i) a local fitness maximum representing a possible endpoint of evolutionary change; (ii) a local fitness minimum at which evolutionary branching can occur; or (iii) a degenerate case (these are without real-world significance). A given singular strategy can be evolutionarily stable (either convergent or non-convergent) or evolutionarily unstable (either convergent or non-convergent). *[right-panel]* The adaptive dynamics (AD) invasion function of a particular ecological system defines a PIP for resident and mutant phenotypes. When the invasion function is positive for a particular pair of phenotypes, the resident may be replaced by the invading mutant. Intersections of the invasion function's zero contour line with the 45° line indicate potential evolutionary end-points. Knowing the slope of the contour line at these singular points suffices to answer four separate questions: (1) Is a singular phenotype immune to invasions by neighboring phenotypes? (2) When starting from neighboring phenotypes, do successful invaders lie closer to the singular one? (3) Is the singular phenotype capable of invading into all its neighboring types? (4) When considering a pair of neighboring phenotypes to both sides of a singular one, can they invade into each other? Picture credits: © Dieckmann, U., 1997. Can adaptive dynamics invade? Trends in Ecology & Evolution 12 (4), 128–131.

dynamic fitness landscapes (Mustonen and Lassig, 2009). The basic idea of evolutionary ecology is that ecotypes in a community differ in fitness because their different genotypes make them suitable for different environments (Doebeli et al., 2017). This allows the community to track environmental changes and avoid catastrophic collapse (see Fig. 11). The relationship between genotype and fitness is mediated by the phenotype. Therefore, the conceptual framework of DFL can be applied to discrete genetic sequences (*i.e.*, number of loci and number of alleles per locus) that define the genotype, as well as to continuous quantitative traits (*e.g.*, organism size, optimum temperature, *etc.*) that define the phenotype. Either way, the population evolves to a state where its average fitness is maximized (Nagylaki, 1992). That is, the divergence of populations along DFL under the joint action of different selection pressures results in the evolution of traits and, ultimately, speciation (Geritz et al., 1997; Ostman et al., 2014). The presence of competitive trade-offs for the uptake of resources has been shown to lead to adaptive evolution and the gradual establishment of microbial ecotypes (Gudelj et al., 2007; Ostman and Adami, 2013; Ostman et al., 2014).

More broadly, the flows of genetic material in microbial populations and communities can be classified as *horizontal*, *i.e.*, moving from one organism to another that is not its offspring; and *vertical*, *i.e.*, moving from one generation to the next (see Fig. 13). Horizontal gene transfer is widespread among bacteria and archaea, because these prokaryotic microorganisms lack internal nuclear membranes to contain and protect their DNA. Thus, prokaryotes rapidly acquire genes from other prokaryotes and even from eukaryotes. Many researchers have therefore abandoned the term *species* and instead refer to different prokaryotic taxa as *ecotypes*. Cohan originally defined an ecotype as: "a set of strains using the same or very similar ecological niches, such that an adaptive mutant from within the ecotype outcompetes to extinction all other strains of the same ecotype; an adaptive mutant does not, however, drive to extinction strains from other eco-types" (Cohan, 2001). The stable ecotype model (SEM) of bacterial speciation (Cohan, 2006) thus gives a prominent role to natural selection regarding the formation and maintenance of separate genetic clusters (see Fig. 14) (Shapiro et al., 2012). In contrast, most gene transfer is vertical for eukaryotic microorganisms, such as green algae,

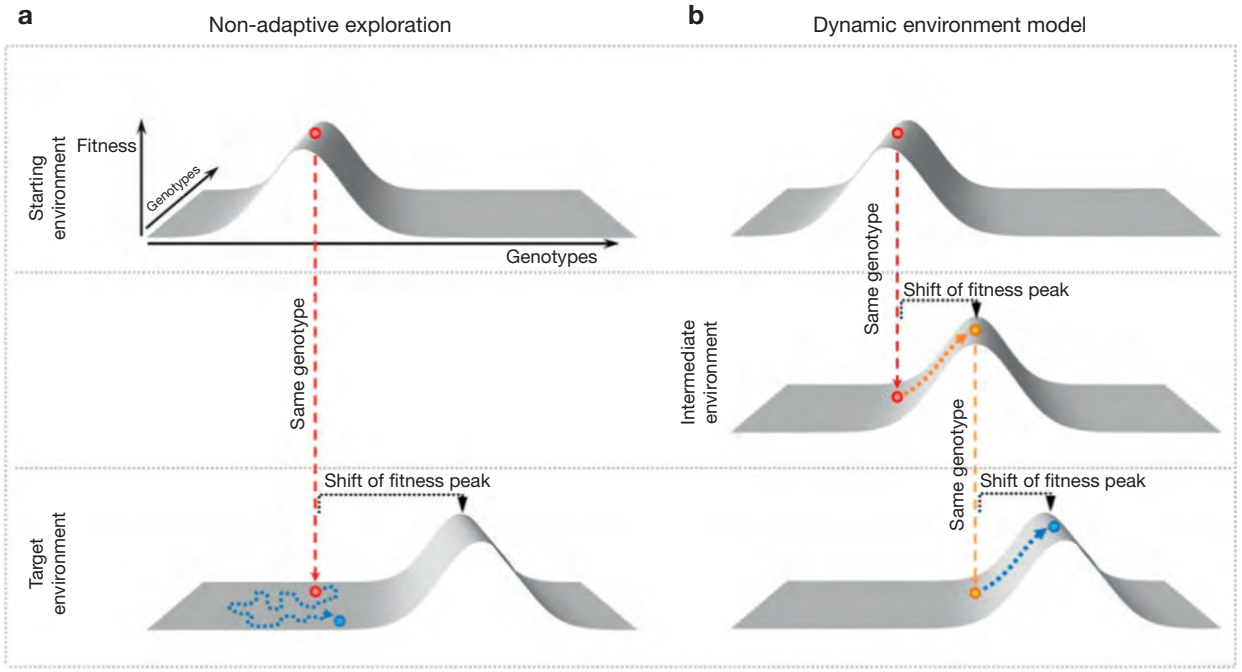

Fig. 11 Non-adaptive evolution (a) *vs.* adaptive evolution (b) of a simulated ecotype on a fitness landscape for a two-dimensional genotype space. The red genotype is well-adapted because it is located on the fitness peak of this starting Dynamic Fitness Landscape (DFL). A change in the local environment shifts the fitness peak, so that the red genotype is no longer of high fitness (bottom). (a) Abrupt (or fast) environmental changes requires the non-adaptive exploration (any direction) of the neutral part of the landscape. (b) Gradual (or slow) environmental changes allow for Adaptive Evolution (AD) through small mutation steps (hill climbing) as defined by the pairwise invasibility plots (see Fig. 10). Picture credits: © Szappanos, B., Fritzemeier, J., Csörgő, B., *et al.*, 2016. Adaptive evolution of complex innovations through stepwise metabolic niche expansion. Nature Communications 7, 11607. doi:10.1038/ncomms11607.

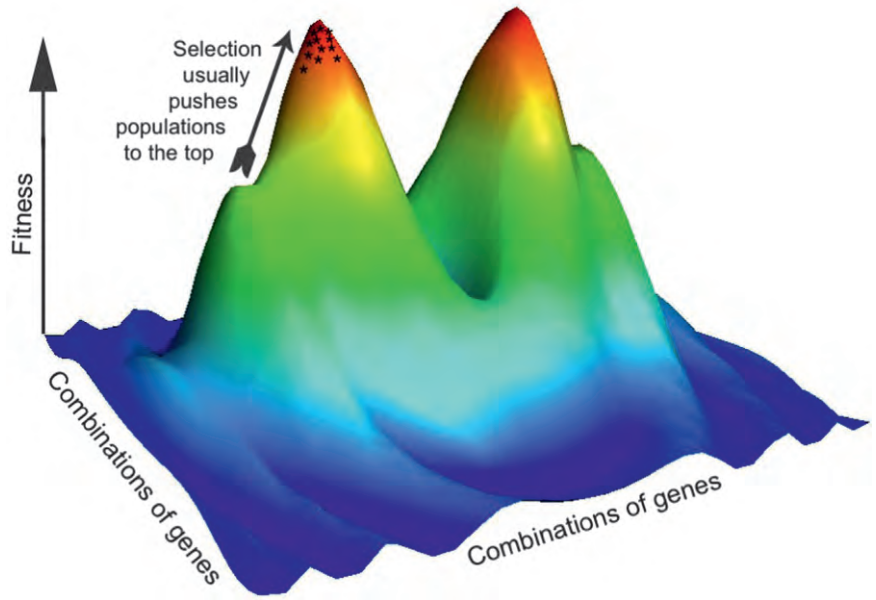

Fig. 12 Dynamic Fitness Landscape (DFL) in a 2D genotype space showing two peaks. Fitness peaks for a given genotype depend on the local environment, but environmental conditions (*e.g.*, nutrient concentration) may also be affected by population dynamics (*i.e.*, gene frequencies) over time so there is a feedback between fitness and organisms. Environmental changes can trigger perpetual changes in fitness, resulting in a constant need for adaptation. Picture credits: © Loewe, L., 2016. Systems in evolutionary systems biology. In: Kliman, R.M. (Ed.), The Encyclopedia of Evolutionary Biology, vol. 4. Oxford Academic Press, Elsevier, pp. 297–318. doi:10.1016/B978-0-12-800049-6.00184-0.

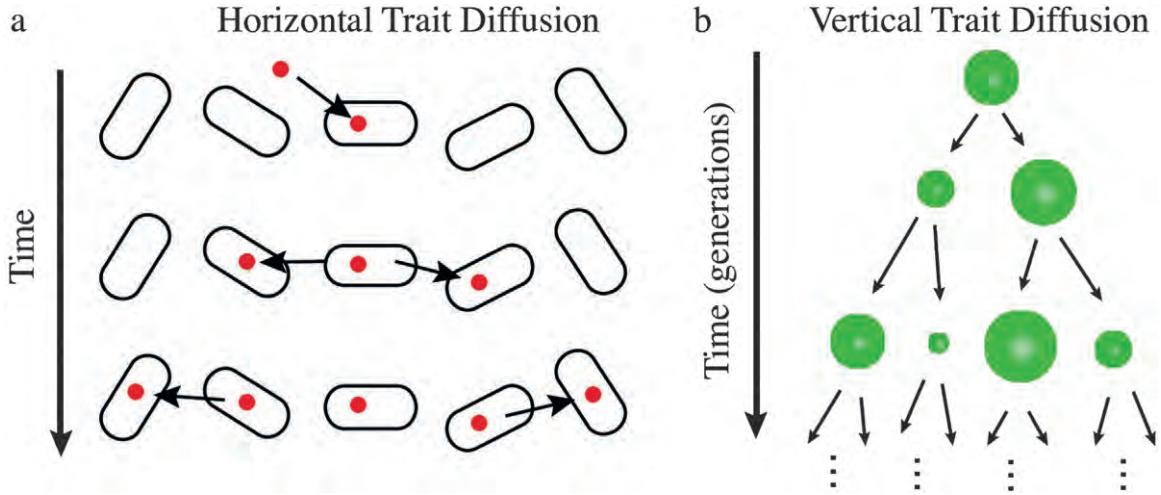

Fig. 13 Genes and associated traits can diffuse (a) horizontally, independent of any genetic relationship among the organisms (red dots denote a gene of interest moving between bacterial cells), and (b) vertically, *i.e.*, through inheritance from generation to generation, as denoted here for phytoplankton reproducing to produce offspring of differing size.

fungi and protozoa, because they have nuclear membranes isolating their genetic material. Many ecological models represent different ecotypes and strains. However, it has also proven useful to develop models and analyse data in terms of the *probability distribution of traits* using continuous functions, without explicitly representing ecotypes or strains (Follows and Dutkiewicz, 2011; Norberg, 2004). Such models have been applied to population genetics and evolution (McGill and Brown, 2007), and recently for understanding biogeographical distributions and Biodiversity Ecosystem Function (BEF) relationships of marine plankton (Chen et al., 2018; Smith et al., 2016) on ecological timescales (see Section "Biodiversity and Ecosystem Functioning").

Both horizontal and vertical gene flows generate genetic (and hence phenotypic) variations, and thus in effect they "diffuse" trait values through populations and communities. This maintains microbial biodiversity and sustains the adaptive capacity of microbial ecosystems. These trait variations are then *filtered* by competition and environmental factors to shape the compositions of populations and communities, and ultimately by ecological evolution to determine the course of evolution. The Trait Diffusion (TD) framework (Merico et al., 2014) simulates the diffusion of trait values through successive generations, as a result of various processes such as vertical gene transfer and/or de-novo mutations. TD can thus be viewed as a mean of representing in microbial models the maintenance of species-trait diversity *via* endogenous mechanisms. These mechanisms of adaptive evolution can include mutation or rapid evolution (Yoshida et al., 2003), which alter the genotype; as well as trans-generational phenotypic plasticity, which is independent of changes in the offspring genotype. The different ecotypes would evolve driven by the three major factors controlling evolutionary dynamics of microbes: random mutation, genetic drift, and natural selection. Therefore, the TD approach gives a simple yet powerful mathematical framework to parameterize these eco-evo factors without the need to simulate them explicitly (Smith et al., 2016). TD assumes that the rate of trait diffusion is directly proportional to growth rate and its derivatives. This framework could readily be applied to model the effects of trait diffusion on the eco-evolutionary dynamics of other eukaryotes (*e.g.*, fungi) and possibly also with some modification of prokaryotes. However, TD does not represent horizontal trait diffusion, which can dominate the flow of genes and traits among prokaryotes migrating within spatially heterogeneous environments (Niehus et al., 2015). Such migration is likely common given that micro-scale variability is commonly observed (Section "Micro-Scale Variability"). Rates of horizontal trait diffusion must depend on the presence of other organisms and phages (viruses) as well as growth rates.

Currently the TD framework is well developed for one trait (*e.g.*, cell size linked to nutrient uptake strategy); that is, for one-dimensional (1D) dynamic fitness landscapes of ecological evolution. However, the dimensionality of DFL is probably very large for most organisms, with different traits being important along different environmental gradients. For procaryotic microbes, two major environmental gradients dominating the ecology and evolution of the ecotypes are the concentration of resources (*i.e.*, uptake strategy) and the temperature of the environment (*i.e.*, thermal tolerance). For eukaryotic phytoplankton, a third environmental gradient is the solar irradiance (*i.e.*, light harvesting and photoprotection). Therefore, the simulated microbial ecotypes should be able to evolve by varying the value of their optimal traits (*e.g.*, N_{opt}, T_{opt}, I_{opt}) under an hypervolume of environmental conditions (see Fig. 9). Dynamic fitness landscapes are variable in space and time due to changes in the environmental conditions. Therefore, the rate of trait-diffusion (*i.e.*, a mathematical parameterization of the genotype mutation rate) of the microbes should be sufficiently fast to track the environmental changes, in order to capture the adaptive capacity of the population or community of interest. Furthermore, even in relatively stable environments, fitness landscapes can change with the genetic state (*i.e.*, ecotype frequencies) of the population as a result of density-dependent selection (Gavrilets, 2004). The evolutionary trajectories are expected to follow the changes in DFL resulting from (i) variability of the environment and (ii) inter-specific competition. Basically each population should try to "climb" the fitness gradient (including both biotic and abiotic factors) in order to maximize their

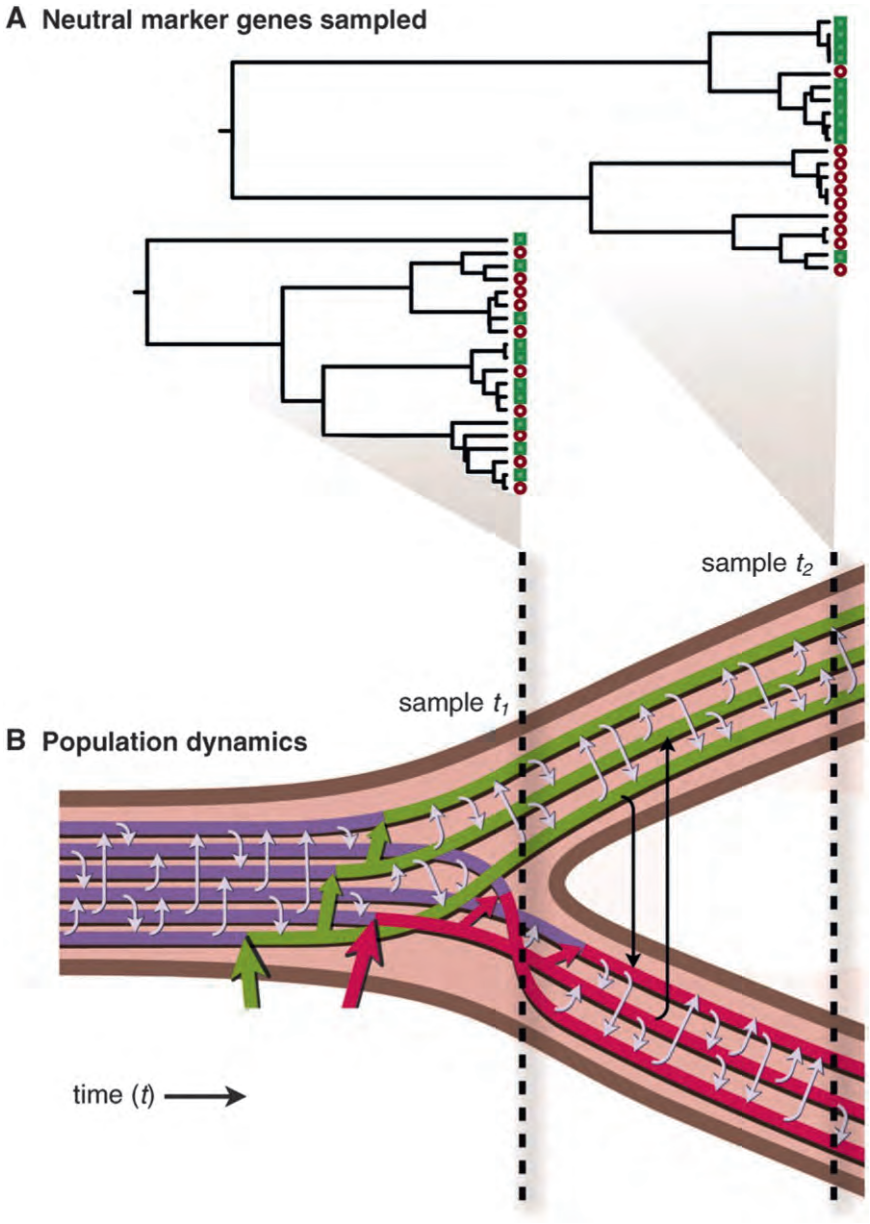

Fig. 14 Ecological differentiation in recombining microbial populations leading to evolutionary branching of two distinct ecotypes. (A) Example genealogy of neutral marker genes sampled from the community at different times. (B) Underlying model of ecological differentiation. Thin gray or black arrows represent recombination within or between ecologically associated populations. Thick colored arrows represent acquisition of adaptive alleles for red or green habitats. Picture credits: © Shapiro, B.J., Friedman, J., Cordero, O.X., *et al.*, 2012. Population genomics of early events in the ecological differentiation of bacteria. Science 336 (6077), 48–51. Available at: http://science.sciencemag.org/content/336/6077/48.

chances of survival (*i.e.*, attain the fastest possible biomass-specific growth rate of the population) under any given environmental condition. The resulting trajectories may be the result of a "tug of war" between two opposite directions: *convergent evolution* to maximize performance under the current environmental conditions and *divergent evolution* to avoid strong competition for a limited set of resources among phenotypically similar populations (Darwin, 1859).

Further work is needed to develop improved models and better understand the roles of trait diffusion in the eco-evolutionary dynamics of microorganisms. One particularly promising area is modelling horizontal trait diffusion and its effects among prokaryotes, which are important in epidemiology, the micro-biome of the human gut, and many commercial applications. Trait diffusion also needs to be considered in the context of the Complex Adaptive Systems (CAS), which define systems with the capacity of *self-organization* where (1) patterns emerge from the interaction among its components, and (2) these interactions are in turn affected by the patterns they produce (*i.e.*, there is feedback between the system's components and its emergent properties)

(Holland, 1992; Levin, 1998). Microbial ecosystems are good examples of CAS when organisms migrate within spatially heterogeneous environments (Norberg, 2004) (Section "Micro-Scale Variability"). Spatially explicit CAS models yield unexpected dynamics, and even without considering TD have already given new insights concerning the evolution of cooperation, restraint (Werfel and Bar-Yam, 2004), group selection and the meaning of fitness (Goodnight et al., 2008). Spatial heterogeneity is expected to be especially important in microbial communities in which mobility is limited, as for instance in soil-dwelling microbiomes where variability in the porosity of the soil constrains the diffusion of the different molecules that are present in the environment (see Section "Micro-Scale Variability").

Biodiversity and Ecosystem Functioning

Unveiling the relationship between biodiversity and ecosystem functioning is one of the major goals of ecological theory (Duffy et al., 2007; Hooper et al., 2005; Thompson et al., 2012), and it is becoming increasingly important in microbial systems (Glockner et al., 2012; Smith et al., 2016; Vallina et al., 2017). The simplest measure of biological diversity is the number of species that are present in a given place, but diversity can be measured using many indices and scales (Tuomisto, 2010; Whittaker et al., 2001). Species richness and the Shannon index (Shannon, 1948) are among the most commonly used due to their simplicity. However, these metrics do not always have a direct translation to *functional diversity* – from the point of view of ecosystem functioning, rather than the richness per-se it is the species identity and role that matters, both related to the species traits and trade-offs (Vallina et al., 2017). Scale is also an essential component of ecological theory because the response of dependent variables can change across spatial and temporal scales (Levin, 1992). There are three main terms for measuring biodiversity over spatial scales at a given time (snap shot): alpha, beta, and gamma diversity (Whittaker, 1972). *Alpha diversity* represents the mean diversity of species within a focal ecosystem or study region and is usually computed as the average value of the local species richness at each niche, habitat or site that the ecosystem contains. *Beta diversity* refers to the diversity of species that are unique to each habitat of the ecosystem and thus provides a measure of compositional heterogeneity. Finally, *gamma diversity* represents the total integrated diversity of the ecosystem from all habitats and thus provides a measure of its overall diversity.

Most microbial communities are important for ecosystem functioning, in particular soil microbial communities; plant-associated microbiomes (both microbes living within the roots or attached to them) (Loreau, 2010b); and planktonic communities, either bacteria (Fuhrman, 2009) or micro-algae (Litchman et al., 2015a). Since these habitats can have different sizes and the geographical limits among them are diffuse, there is no consensus on what spatial scales are the most appropriate to quantify alpha diversity. The suggestion is that it can be measured using habitats or sites of any scale that are delimited just for the purpose of analysis. Beta diversity can then be computed as the ratio between gamma over alpha diversity (Whittaker, 1972). These three definitions of biodiversity are also equally applicable over temporal scales at given location (focal point), or mixing space and time in the analysis. For example, the shape of the productivity-diversity relationship (PDR) for marine unicellular algae (*i.e.*, phytoplankton) at the scale of the global ocean using alpha diversity of species richness has been observed to be unimodal, with diversity peaking at intermediate levels of productivity (Irigoien et al., 2004). Numerical simulations showing the same global pattern when looking at weekly-averaged data suggested that predator-mediated coexistence by selective feeding (*i.e.*, killing-the-winner, see Section "Ecological Selection and Community Assembly") explains the positive slope at low nutrient supply, while transient competitive exclusion explains the negative slope at high nutrient supply of this unimodal PDR curve (Vallina et al., 2014a).

Diverse communities are often more efficient in resource exploitation than single species communities and there is no a-priori limit to the total number of species (increasing the packing and elaboration of axes of the niche hyperspace) (Loreau, 2010a). This also applies to temporally-variable environments through local selection and dominance of the most efficient ecotypes. For example, higher diversity of thermal preferences has a positive effect on ecosystem functioning on a seasonal basis because it leads to *niche complementarity* of the species, which allows covering the whole temporal gradient in temperature. Likewise, a higher diversity of uptake strategies has a positive influence on ecosystem functioning through a *sampling probability* effect: the more uptake strategies are potentially available, the higher the chance of sampling a faster-growing strategy (Hooper et al., 2005; Tilman et al., 1997). The stability of aggregate ecosystem properties like total production or biomass has thus been suggested to increase with species diversity either through functional redundancy or functional complementarity (de Mazancourt et al., 2013). Species are redundant when they occupy the same environmental niche and fulfill the same functional role, and are complementary when they occupy different environmental niches within the same ecosystem and fulfill different functional roles (Loreau, 2010a). Functional redundancy helps sustain ecosystem functioning in the face of species extinctions through the replacement of species with similar ones, while functional complementarity decreases the temporal variance of aggregated properties (*i.e.*, increases the stability at the community level) through the species asynchrony with environmental variability (Vallina et al., 2017). Different species that have distinct ecological niches would respond differently to environmental changes, leading to an asynchrony of individual population dynamics (Loreau and de Mazancourt, 2013). Asynchronous responses of the populations will thus have a "buffering effect" on aggregate properties, leading to the *insurance hypothesis of biodiversity* for ecosystem functioning (Yachi and Loreau, 1999). This is a major prediction of niche theory regarding biodiversity and ecosystem functioning (BEF) that seems to be well supported with observational data for terrestrial plants (Loreau, 2010a) and freshwater phytoplankton (Ptacnik et al., 2008).

Neutral theory (see Section "Ecological Selection and Community Assembly"), on the other hand, states that coexisting species are ecologically equivalent and therefore the rate of competitive exclusion becomes infinitely slow (Hubbell, 2001). Thus, in the view of neutral theory, diversity has no functional consequences (Loreau, 2010a). Under this modelling framework, the dynamics of

an isolated microbial community then becomes a slow random drift to extinction due to the stochastic probability of both birth and death processes, leading to transient non-equilibrium coexistence. However, for non-isolated communities this random drift can be counterbalanced by the immigration by dispersal into the local community of new species coming from an external pool of microbial communities or metacommunity, leading to permanent coexistence. The identity of species continuously change but the microbial community properties such as the species abundance distribution (SAD) remains invariant. The main drawback of neutral theory for microbial ecosystem modelling is that there is a large body of evidence that most species in natural ecosystem are not functionally equivalent – many studies have shown that microbial ecotypes have deterministic niche differences and that ecological displacements by competitive exclusion are common (Coyte et al., 2015; Follows and Dutkiewicz, 2011; Ghoul and Mitri, 2016; Litchman and Klausmeier, 2008). In addition, neutral theory in its current form is seen as incompatible with niche theory (Loreau, 2010a).

There is also the middle-ground possibility that both theories (niche and neutral) may be partially correct and be in fact complementary to explain microbial community assembly. Observational data show that rank-abundance distributions (RAD) of microbial communities are often highly skewed, with a few dominant ecotypes and a long tail of low abundant ecotypes that form the so-called *rare biosphere* (Fuhrman, 2009; Pedros-Alio, 2006, 2012; Ser-Giacomi et al., 2018). The tail of rare ecotypes is thought to be the result of bottom-up controls (being in sub-optimal environments for growth) and top-down killing-the-winner processes (being subject to relatively smaller mortality) (Pedros-Alio, 2006). Global ocean simulations resolving many ecotypes of phytoplankton based on niche theory, for example, also show the same pattern (Follows and Dutkiewicz, 2011). This suggests that: (i) the few dominant ecotypes of the RAD are in their optimal environmental niches and thus are outperforming most of the ecotypes by ecological selection; (ii) the many rare ecotypes of the RAD are far from their optimal niches and have been probably recruited through immigration by dispersal; and (iii) within either category but particularly for the long tail of rare ecotypes, coexistence is probably happening because ecotypes have very similar fitness and thus can be viewed as close to *competitively neutral*. Eventually, environmental conditions may change and some rare ecotypes could then become dominant (and *vice-versa*) (Pedros-Alio, 2006). Therefore, the rare biosphere of microbes can be seen as a "seed-bank" of biodiversity and way to store *potential* ecosystem functioning (Zorach and Ulanowicz, 2003).

Under temporally varying and/or spatially heterogeneous habitats, keeping a rare biosphere of microbes waiting to become dominant when conditions change may thus lead to what is known as *overyielding*. Overyielding occurs when the production of a multi-species community is higher than the production of each of its species as a mono-culture. Niche differentiation and functional complementarity are thought to lead to overyielding production on microbial ecosystems. There are two major mechanisms by which biodiversity can lead to overyielding: *sampling probability* effect and *niche complementarity* effect (for details see Fig. 2 in Hooper et al., 2005). These mechanisms explain the BEF response curve of simulated microbial communities such as phytoplankton when the species functional diversity is given by their range of uptake strategies and temperature tolerances (Vallina et al., 2017). An increase in the range of nutrient concentration niches (N_{opt}) leads to sampling probability, while an increase in the range of temperature niches (T_{opt}) leads to niche complementarity. Note that the BEF response curve for each of these mechanisms is different: sampling probability has no effect on the maximum values of the ecosystem functioning indicators (*e.g.*, productivity and stability), while niche complementarity increases both the maximum and minimum values of these indicators (see Fig. 15). These BEF responses for simulated microbes agree well with the general BEF curves predicted for terrestrial plants under theoretical scenarios of sampling probability and niche complementarity (Tilman et al., 1997).

Regarding *niche complementarity*, a microbial ecosystem with a spatial environmental gradient of temperature will be more productive if all its temperature niches are being occupied simultaneously by locally adapted ecotypes. This is because the *fundamental niches* for temperature (negative-skew gaussian bell curves) are *closed at both ends* and thus a single ecotype will generally not be able to cover (*i.e.*, survive at) the whole range of ambient temperatures under a mono-culture (see Fig. 9, upper-center). Regarding the *sampling effect*, a microbial ecosystem with a spatial environmental gradient of nutrient concentration will be more productive if *at least* one fast growing ecotype is present in the community. That is, there is no need to have a diversity of uptake strategies (*i.e.*, affinity-adapted *versus* rate-adapted) simultaneously as long as one rate-adapted (*i.e.*, opportunist) species is present. This is because the fundamental niches for nutrients (Michaelis-Menten curves, see Fig. 2) are *not closed on the upper side* since high nutrient concentrations are (usually) not harmful for growth, and thus a single ecotype will be able to cover (*i.e.*, survive at) the whole range of resource concentrations under a mono-culture (see Fig. 9, upper-left). In this case, the probability of sampling at least one rate-adapted ecotype increases with biodiversity. Therefore, having a high diversity of uptake strategies translates into having a higher chance of having a highly productive microbial community. The effect on ecosystem functioning of diverse uptake strategies is thus not the result of functional complementarity but the result of sampling probability (Tilman et al., 1997). The concept of *thermal niche* and *nutrient niche* are therefore quite different from the point of view of BEF in microbial communities because they lead to different mechanisms of overyielding (Hooper et al., 2005). The concept of *irradiance niche* for photo-autotrophic microbes falls somewhere in the middle of these two extreme categories if there is photo-inhibition at high solar radiation (see Fig. 9, upper-right). The same reasoning applies to temporal environmental gradients (see Fig. 15) (Vallina et al., 2017).

Micro-Scale Variability

Micro-scale variability plays a central role in determining the functioning of microbial communities and the various processes that they govern. It is especially important in contexts in which, due to limited mobility, cells explore a limited range of environments

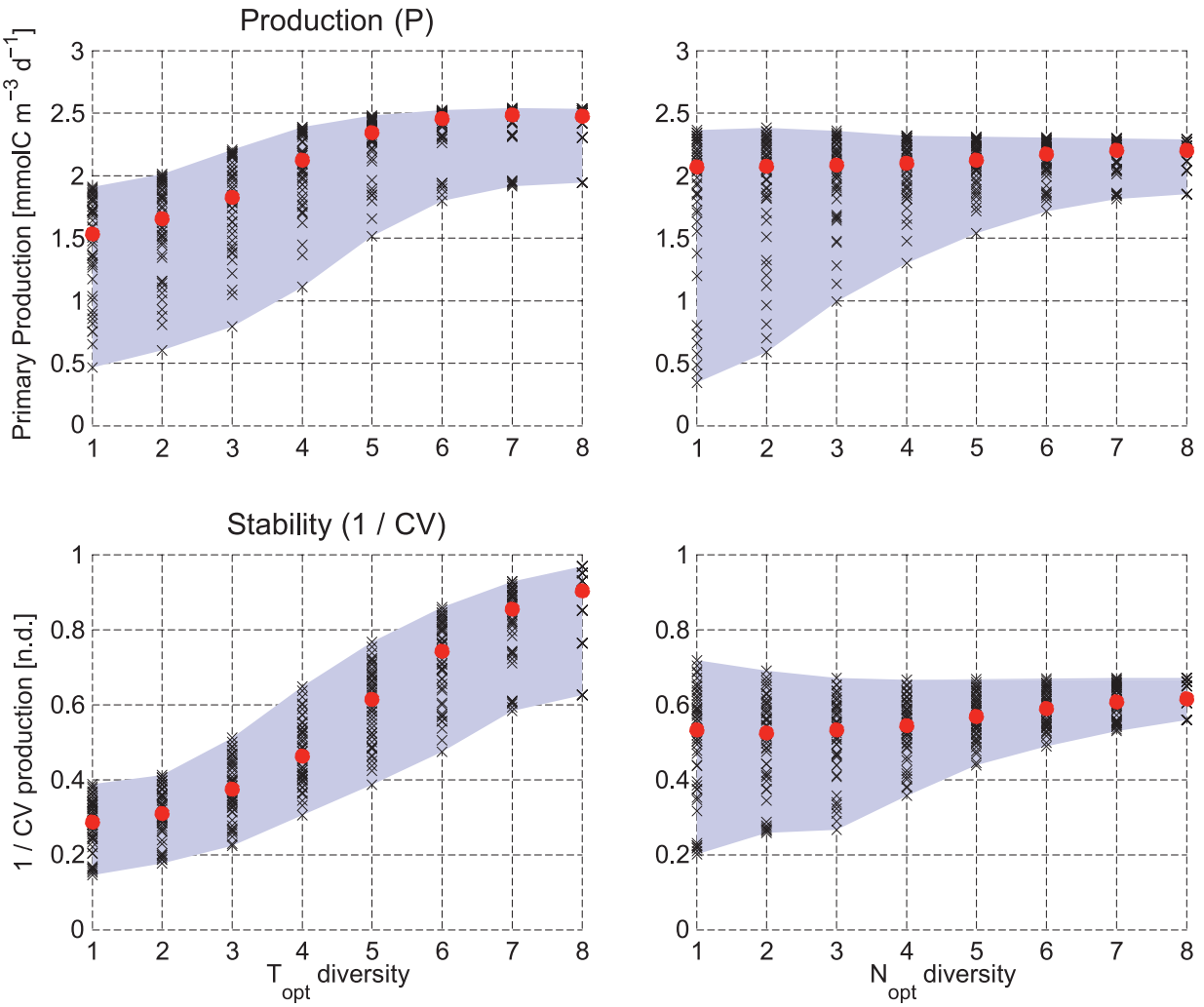

Fig. 15 Phytoplankton annual average community-level primary production (mmol C m^{-3} d^{-1}) and its temporal stability (inverse of the coefficient of variation normalized by its maximum value; n.d.) as a function of the diversity in thermal preferences (left panels) and the diversity in nutrient uptake strategies (right panels). The red dots correspond to the median value of the distribution. The gray shaded area spans from the minimum to the maximum value of the distribution. Increasing the diversity of thermal preferences of the phytoplankton community leads to a strong increase in the median, minimum and maximum value of the community-level production and its temporal stability over a seasonal cycle. Increasing the diversity of uptake strategies of the phytoplankton community leads to a strong increase of only the minimum value of the community-level production and its temporal stability over a seasonal cycle, to a weak increase of the median value, and has no effect on the maximum values of the distribution. Picture credits: © Vallina, S.M., Cermeno, P., Dutkiewicz, S., Loreau, M., Montoya, J.M., 2017. Phytoplankton functional diversity increases ecosystem productivity and stability. Ecological Modelling 361, 184–196. doi:10.1016/j.ecolmodel.2017.06.020.

and interact mostly with individuals in their closest neighborhoods. Nano- and micro-scale variability are well appreciated features of soil ecosystems (Fierer, 2017; Kuzyakov and Blagodatskaya, 2015). In terrestrial ecosystems, bacterial distributions in the soil show a high degree of clustering, due to the combined effect of extrinsic (*e.g.*, pore size, availability of organic matter) and intrinsic factors (*e.g.*, reproductive pair correlations) (Raynaud and Nunan, 2014). Regardless of the environmental conditions in which they thrive, bacterial biofilms are a paradigmatic example in which short-scale variability is well-known to be of paramount importance. Biofilms are dense and highly diverse communities in which cells remain encased in a secreted polymer matrix that holds them together and hence reduces their mobility dramatically. They are central for global scale processes, such as biogeochemical cycling, play a central role in the interaction between bacteria and multicellular organisms, and provide a myriad of potential biotechnological applications (Fux et al., 2005; Halan et al., 2012; Paerl and Pinckney, 1996). However, the contribution of biofilms to each of these processes is usually controlled by the secretion of different metabolites, whose efficiency is strongly determined by the spatial arrangement of the different species within the matrix (Nadell et al., 2016). Due to the importance that spatial structure has for biofilm functioning, several models have attempted to understand the means by which individual cells interact among them and with the environment during biofilm proliferation as well as the ecological and evolutionary implications of the nascent patterns of spatial variability (Nadell et al., 2010; van Gestel et al., 2015). Most of these models rely on individual-based approaches, in which each cell is treated as a discrete entity (Xavier and Bassler, 2005; Kreft et al., 2001; Martínez-García et al., 2018), but frameworks based on reaction-diffusion descriptions of the growth dynamics have been proposed as well (Horn and Lackner, 2014).

Less obvious has been the existence of micro-scale patterns of spatial variability in environments where microbes are subject to external mixing forces, such as the aquatic environment. Over 30 years ago, however, such microscale patchiness in the distributions of organic matter, bacteria, and other microorganisms was observed and appreciated as an important feature of marine ecosystems and the biogeochemical cycles that they mediate (Azam et al., 1994). More recently, patchiness in the distribution of marine phytoplankton has been widely observed and confirmed to impact the productivity of simulated plankton ecosystems (Brentnall et al., 2003; Mandal et al., 2014). Spatial heterogeneity of microbial distributions in aquatic systems arises from the clustering of organisms (see Fig. 16). Also, in the presence of potential mixing forces such as external flows and micro-scale eddy stirring, organisms can cluster in certain turbulent regimes (see Fig. 17). More generally, environmental heterogeneity at larger scales has long been known to impact ecological dynamics and enhance biodiversity by providing distinct environmental niches physically separated (*e.g.*, d'Ovidio et al., 2010; Levy et al., 2014). Micro-scale aggregates (<1 mm) of particulate organic matter, phytoplankton, and bacteria constitute microbial hotspots in the ocean (Azam et al., 1994), with distinct environmental niches that are important for aquatic ecology and biogeochemistry. Furthermore, because plankton undergoing limited migration (incomplete

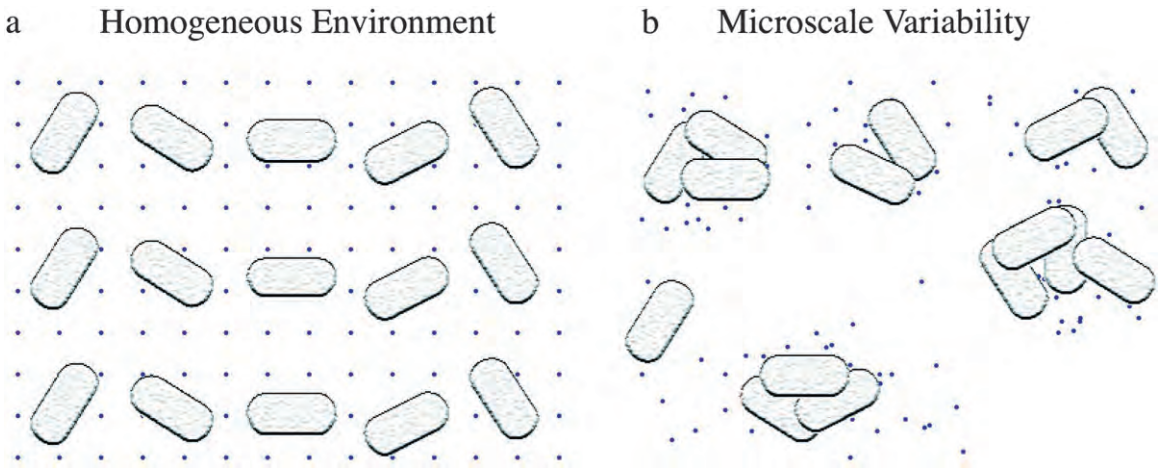

Fig. 16 Many theoretical models were first developed assuming (A) homogeneous environmental conditions as denoted here for the distribution of bacterial cells and their growth substrate (blue dots). (B) Now more and more models are accounting for spatial heterogeneity, as depicted here for a patchy distribution of bacteria and their substrate. Environmental heterogeneity is typical of many natural environments and in many cases yields substantially different predictions of both ecological and evolutionary dynamics.

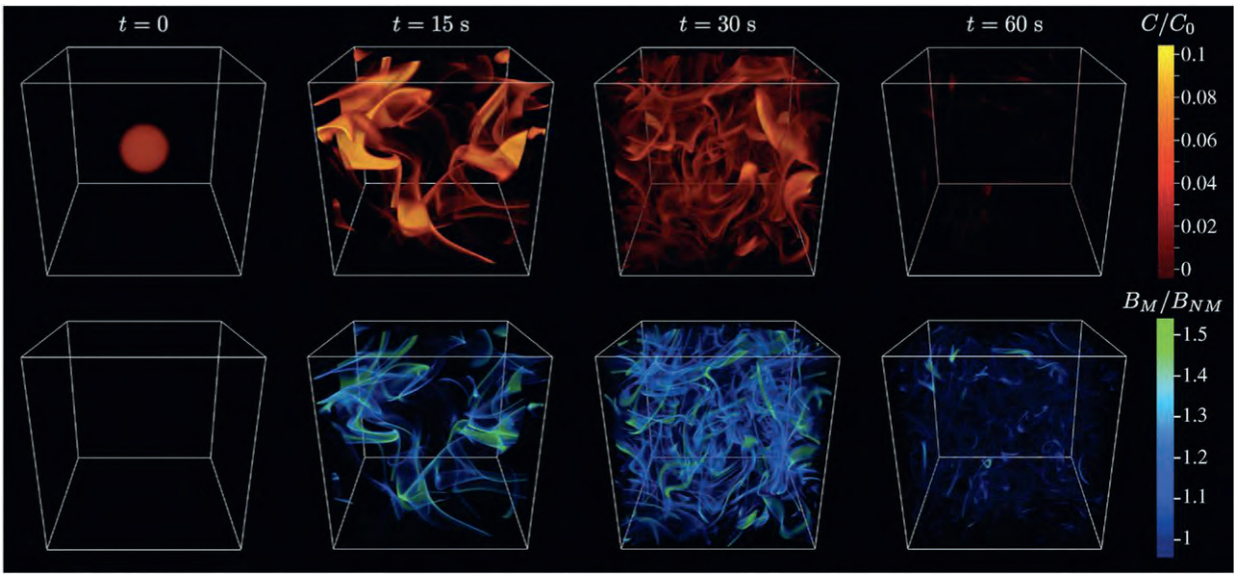

Fig. 17 Stirring of a nutrient patch and response of chemotactic bacteria within a domain of ≈0.5 m length using direct numerical simulations (DNS). As a nutrient patch is stirred by a turbulent flow (top row, showing nutrient concentration C), chemotactic bacteria respond by accumulating within nutrient filaments (bottom row, showing concentration of motile bacteria BM), thereby enhancing their uptake. Picture credits: © Taylor, J.R., Stocker, R., 2012. Trade-offs of chemotactic foraging in turbulent water. Science 338 (6107), 675–679. Available at: http://science.sciencemag.org/content/338/6107/675.

mixing) in aquatic environments constitute Complex Adaptive Systems (CAS), a CAS modelling approach for simulating the biodiversity and adaptive dynamics of such systems has recently been developed (Norberg, 2004). Although the emphasis was given on larger spatial scales, the same principle applies for migration between distinct micro-environments.

The ubiquity of micro-scale variability suggests that CAS dynamics are common in the eco-evolutionary dynamics of microorganisms. Also, recent modelling studies have revealed novel insights into the complex eco-evolutionary dynamics of microorganism in spatially heterogeneous environments. Evolution and maintenance of social cooperation, restraint, and altruism can be explained by considering eco-evolutionary dynamics with migration under environmental heterogeneity (Werfel and Bar-Yam, 2004) (see Section "Social Behavior in Microbes"). A similar model predicted complex dynamics for group selection (Goodnight et al., 2008). Migration between distinct habitats enhances substantially the degree to which horizontal gene transfer impacts the genetic diversity and evolution of prokaryotes (bacteria and archaea) (Niehus et al., 2015). Phytoplankton distributions in coastal waters have also been shown to display great vertical heterogeneity or intermittency, with layers of increased fluorescence and concentration, probably resulting from a fine balance between layer formation and destruction through mixing (Prairie et al., 2011). These peaks or anomalies in concentration over the background value can have disproportionate effects on the biological dynamics. They are usually modelled using the same approach employed in turbulence theory (Mandal et al., 2016).

Fractal patchiness at the micro-scale can occur when motile phytoplankton are exposed to turbulent flow, leading to increases in local concentration of an order of magnitude. They are the result of a balance between cell motility and fluid shear (Durham et al., 2013). Motile bacterioplankton can exploit ephemeral micro-patches of resources through chemotaxis in turbulent environments (see Fig. 17). This gives them an advantage over non-motile competitors, albeit subject to a trade-off against the energetic cost of locomotion. There exists therefore an optimal swimming speed of $\approx$60 μm s^{-1} for standard marine conditions (Stocker and Seymour, 2012; Taylor and Stocker, 2012). Compared to typical plankton ecosystem models based on mean-field (background) concentrations, the newly developed models that account for the micro-scale variability (anomalies) in the distributions of plankton and nutrient concentration have yielded substantially different simulated spatio-temporal patterns of biomass and productivity at macroscopic scales (Mandal et al., 2016). These models have also predicted that micro-scale variability may enhance trophic transfer and potentially also biodiversity (Priyadarshi et al., 2017).

The distribution of micro-scale aggregates can be represented either explicitly as in agent- or individual-based models (Goodnight et al., 2008; Werfel and Bar-Yam, 2004), by direct numerical simulations (DNS) of turbulence at high spatial resolution (Taylor and Stocker, 2012), or statistically in ecosystem models at low spatial resolution by decomposing the concentration fields into mean and fluctuating parts. The last approach is analogous to the Reynolds decomposition employed widely in fluid dynamics (Mandal et al., 2016; Mandal et al., 2014; Priyadarshi et al., 2017). Accounting for micro-scale variability compounds the already formidable challenge of modelling the combined effects of ecological dynamics and physical transport by advection and diffusion (Mandal et al., 2016), which are relevant to many microbial systems. Future studies that better account for micro-scale variability have much potential to yield new insights into the eco-evolutionary dynamics of microorganisms. Remaining challenges include developing models that are both sufficiently robust for application over wide environmental gradients (*e.g.*, in 3-D models of ocean circulation and marine ecosystems) and computationally efficient enough to be practically useful for simulation studies. Promising approaches have begun to incorporate the effects of heterogeneous environments as "interaction structure" within the compact Adaptive Dynamics equation that is already widely used to model eco-evolutionary trait dynamics (see Section "Ecological Evolution and Community Adaptation") (Allen et al., 2013b).

Social Behavior in Microbes

Microbes very often coordinate their behavior with each other in order to perform several tasks, such as foraging, dispersal, reproduction, or nutrient acquisition (Crespi, 2001). This vast variety of collective behaviors, together with their lab manageability, fast evolution, and the various possibilities of molecular and genetical manipulations, have turned microbes into a recurrent model to investigate social behaviors and inspired many theoretical models. Microbial collective behaviors are very often controlled by the secretion of substances to the extracellular medium, named exo-products. Exoproducts may serve to coordinate behavioral synchronization *via* quorum-sensing communication (Czárán and Hoekstra, 2009; Miller and Bassler, 2001), but they can also establish different types of ecological interactions among cells. For instance, mutualistic interactions may arise in mixed cultures in which each species benefits from the exoproducts of its partner in the mix (cross-feeding); antagonistic interactions have been also observed, as represented by the different forms of chemical warfare that facilitate the elimination of competing individuals in some bacterial species (McNally et al., 2017; West et al., 2007) (see Fig. 18(a)).

Especially interesting from an evolutionary perspective are cooperative interactions, actions that incur in a cost for the executor and whose benefit is shared with other individuals in the population. A paradigmatic example in which such dilemma arises is the synthesis of public goods, exoproducts that are costly to produce and benefit both the producer and its neighbours (see Fig. 18(a)). In a well-mixed population of public-good producers and non-producers, every individual interacts with each other, and therefore the payoffs for each species due to public-good production are:

$$f_{NP}(N_P) = rc(N_P/N) \tag{54}$$

$$f_P(N_P) = f_{NP} - c \tag{55}$$

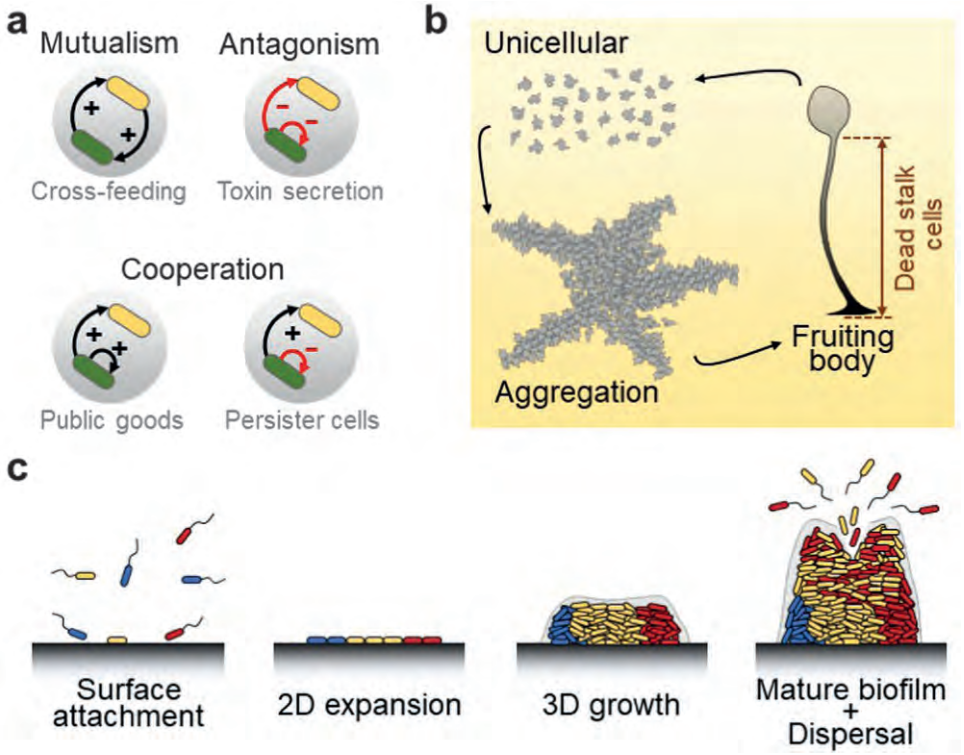

Fig. 18 (a) Cell secretions may establish different types of ecological interactions. Mutualistic interactions, as represented by cross-feeding, appear when each of the species in the mix benefits from the secretions of the other species in the mixes. Antagonistic interactions are represented, for instance, by chemical warfares in bacteria, in which exoproducts kill competing cells. The costs associated to the actor (green cell) in these behaviors may be just a reduced growth rate due to allocation of resources into chemical production, but sometimes the release of the substances requires cell death. Cooperative behaviors may be classified in two major categories depending on whether the actor also receives a benefit or only pays a cost without receiving a direct fitness benefit. Public good production is an example of the former, while persistent cells constitute an example of the later (see main text for a detailed explanation of both behaviors). The existence of these non-growing phenotypes alleviates the competition for resources, which favors the growth of conspecifics. (b) The life cycle of *Dictyostelium discoideum* is an example of division of labor in microbes. Starvation triggers the coordinated aggregation of free-living amoebae and the beginning of a developmental program that culminates with the formation of a fruiting body. Within the fruiting body, about 80% become dormant spores, whereas the other 20% facilitates spore dispersal by forming a stalk of dead cells. (c) Biofilms present a huge diversity of social behaviors, ranging from the secretion of exoproducts that establish mutualistic, competitive or cooperative interactions, programmed cell death, to quorum-sensing, to division of labor.

where the subscripts NP and P stand for non-producers and producers respectively. N_P is the number of producers within the population of total size N, r gives the benefits provided by the public good, and c is the cost of the cooperative behavior (*i.e.*, the investment in the public good). Therefore, the total revenue of the public good depends on the number N_P of producers, how much c they invest in the production, and the growth multiplication factor r of the public good. Additionally, each individual obtains and equal share from this total production, regardless of whether it contributed to the production or not, but producers have to cope with the additional production cost. In such simplified scenario, non-producers will always outcompete producers because they get their shared benefit of the cooperative behavior without bearing its cost, and any initial mix of species will converge to a extinction of cooperators. This result, hence, poses an interesting *evolutionary dilemma* that can be summarized in two questions. First, why would an individual decrease its own fitness to confer fitness benefits on other individuals? And second, what are the mechanisms that stop the invasion of non-cooperative mutants (represented here by non-producers) and avoid the extinction of cooperation?

Several models have proposed mechanisms that could turn cooperation evolutionarily stable (see Section "Ecological Evolution and Community Adaptation") and solve this dilemma: (1) the increase in population size due to cooperation makes the system more robust against extinctions induced by demographic fluctuations (Constable et al., 2016); (2) individuals that transiently neither execute nor benefit from the cooperative behavior are resistant to mutants and provide a pool to recover cooperation (Hauert et al., 2002); (3) the partial privatization of the benefits of cooperation by cooperators (Gore et al., 2009; Jin et al., 2018); (4) the effect of external forces, such as environmental flows (Drescher et al., 2014; Uppal and Vural, 2018); and (5) any form of assortment that makes cooperators more likely to interact with other cooperators in game-theoretic frameworks (Nowak, 2006). Some of the mechanisms to achieve assortment are: the existence of a spatial structure in the environment that, combined with individual limited mobility or cell properties such as adhesion and/or directed movement, keeps related cells together (Allen et al., 2013a; Nowak and May, 1992; Tarnita et al., 2009); recognition of cooperators and non-cooperators in order to choose the partner in the interactions (Antal et al., 2009; Pacheco et al., 2006); reciprocity *via* individual reputation or/and memory in the interactions

(Axelrod and Hamilton, 1981; Nowak and Sigmund, 2005); or multilevel selection, which considers that different selective pressures acting at the individual and the group level favor groups dominated by cooperators (Keller, 1999; Traulsen and Nowak, 2006). However, these models very often oversimplify the ecological context in which interactions occur, as well as the possible existence of eco-evolutionary feedbacks that emerge due to the rapid evolution of microbes and that can reshape their ecology (see Section "Ecological Evolution and Community Adaptation") (Estrela et al., 2018; Tarnita, 2017). The necessity of modelling the ecological context and eco-evolutionary feedbacks is supported by numerous experimental studies. For instance, the cooperative nature of Pyoverdin production (an iron-chelating molecule hypothesized to be a public good) in *Pseudomonas* strongly depends on the environmental conditions in which strains evolve (Zhang and Rainey, 2013). Hence, any sociobiological label assigned to these secretion behaviors is only meaningful when posed in the proper ecological context, which should be recognized in any modelling framework (Estrela et al., 2018; Tarnita, 2017).

Beyond the production and release of public goods and other exoproducts, cooperation relates to division of labor, whereby individuals in a population specialize in different tasks. This leads to fitness differences across cells, as illustrated by cell differentiation in *Dictyostelium discoideum* fruiting bodies: upon starvation, single-living cells aggregate and develop a multicellular fruiting body, in which only a proportion of cells become spores and eventually reproduce, while the rest die to form a stalk (see Fig. 18(b)). Other examples of cooperation are provided by persisters, cells that exist in a quiescent non-growing state, or programmed cell death. In both cases, the existence of non-growing phenotypes alleviates the competition for resources, which favors the growth of conspecifics (West et al., 2007). These fitness differences between phenotypes that are specialized in different tasks depend on the ecological context in which populations thrive, which emphasizes the necessity of developing more comprehensive models that include a description of the environmental dynamics. Persister cells, for instance, while growing slowly, show antibiotic resistance and could thus outcompete fast-growing susceptible phenotypes in antibiotic-rich environments. Considering that persistence is acquired through a reversible phenotypic switch (Balaban et al., 2004), a model that explicitly describes fluctuations in the concentration of antibiotics in the environment has shown that, rather than a cooperative behavior, persistence could constitute an adaptation that is tuned in stochastic environments by the statistical properties of changes in antibiotic concentration (Kussell et al., 2005).

Finally, all the behaviors discussed in this section frequently occur simultaneously and are hard to disentangle from each other. An example of such complex scenarios are biofilms, conglomerates of cells that often belong to hundreds of different species and grow attached to surfaces, subject to external flow stresses (Drescher et al., 2014; Kempes et al., 2014; Martínez-García et al., 2018; Persat et al., 2015) (Fig. 18(c)). Division of labor has been reported in *Pseudomonas* biofilms, with some phenotypes specializing in biofilm growth and others in dispersal. Quorum-sensing plays an important role in initiating and structuring the biofilm, as well as in regulating the secretion of several exoproducts. Cell cooperation is crucial in the secretion of the extracellular matrix that holds cells together, and in the production of numerous public goods that regulate various aspects of biofilm progression and functioning. The secretion of other substances, such as antibiotics or bacteriocins, establishes competitive interactions between cells. And, finally, programmed cell death aids in structuring the biofilms (see Nadell et al., 2016; West et al., 2007 for a review). Importantly, due to their reduced mobility, biofilm-dwelling bacteria mainly interact with cells in their local environment (see Section "Micro-Scale Variability"). This makes biofilms a very appropriate system to develop theoretical frameworks that couple ecological factors and the various forms of assortment that influence the outcome of social interactions (Martínez-García et al., 2018; Nadell et al., 2016).

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Mollicutes

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Introduction

Despite their notorious fastidiousness and difficulty growing in axenic culture, the first member of what became known as the class *Mollicutes* was isolated more than a century ago. In the early days of microbiology as a discipline, Edmond Nocard and Emile Roux set out to cultivate the agent of the devastating cattle disease, contagious bovine pleuropneumonia (CBPP), and eventually isolated the bacterial species now known as *Mycoplasma mycoides* subsp. *mycoides* (Manso-Silván et al., 2009; Nocard, 1990). It several decades before the first isolate from humans was collected, and even longer for its true nature to be recognized. Originally termed "Eaton's agent", the isolate collected from a patient with primary atypical pneumonia ("walking pneumonia") was thought to be viral because it could pass through small-pore filters and was unresponsive to treatment with penicillin (Brown et al., 2018). These features of what showed by what is now called *Mycoplasma pneumoniae*, are common to all *Mollicutes* and strongly related to their defining features. They are filterable because they are among the smallest free-living bacteria, and they exhibit intrinsic resistance to penicillins because they lack cell walls (May et al., 2014).

Members of the class *Mollicutes* have evolved degenerately. This streamlined nature makes them excellent systems in which to study major principles in biological sciences including those relating to genomics, synthetic biology, disease emergence, evolvability, gliding motility, and moonlighting proteins. The class also includes several agricultural and human pathogens whose associated diseases have major economic and adverse health impacts worldwide. This article will introduce the cellular biology, phylogeny (and taxonomic challenges), associated diseases and their pathogenesis, genetics/genomics, and societal impact of these unique bacteria.

Phylogeny and Cellular Biology

The class *Mollicutes* is the single member of its own phylum, *Tenericutes*, and is thought to have diverged from common ancestors about 605 Myr (million years) (Maniloff, 2002). Their phylogeny is surprising given their phenotypically distinct absence of a cell wall: they are descended from low G+C Gram positive organisms, with their closest living relatives being the *Firmicutes* (Brown et al., 2018). The class has four orders (class *i.e.*, *Mycoplasmatales*, *Entomoplasmatales*, *Acholeplasmatales*, and *Anaeroplasmatales*) based on aerobic vs. anaerobic growth, sterol requirement, and host range, but the *Mollicutes* on the whole can be subdivided into 5 main branches: spiroplasma, pneumoniae, hominis, anaeroplasma and asteroleplasma (Weisburg et al., 1989). The genus *Asteroleplasma*, which includes the single species *Asteroleplasma anaerobium*, is somewhat ambiguous and shares marginal relationships with phyla of other *Firmicutes* and may in the future be reassigned from the class *Mollicutes* (Johansson and Pettersson, 2002). The validly named genera in the class are noted in Table 1.

There are some examples of polyphyly within the *Mollicutes* due to the conflict between species named based on phenotypic characters that have been revisited using molecular techniques. The most striking example is the type species of the genus *Mycoplasma* and the first *Mollicutes* species ever described: *M. mycoides* subsp. *mycoides*. It exhibits the defining traits of the genus in that it is pleomorphic, lacks a cell wall, grows aerobically, requires sterols for growth, and parasitizes a vertebrate host (albeit with unusually high pathogenic potential). However, 16S rRNA gene based-taxonomy led to an unexpected finding: the type species of the genus *Mycoplasma* (along with a few of its close relatives) is not really a mycoplasma at all (Balish et al., 2019; Brown et al., 2018). Ribosomal RNA-based methods, multi-locus sequence typing, and genome-based methods all indicate that *M. mycoides* and its close relatives (collectively called the "*mycoides* cluster") make up a distinct group in the family *Entomoplasmataceae* (order *Entomoplasmatales*), and are most closely related to members of the genus *Spiroplasma* (Brown et al., 2018). It is no simple task to rename these organisms in a way that would resolve this taxonomic discrepancy. Being the first isolate and the type species, *M. mycoides* has taxonomic precedent over all other members of the genus *Mycoplasma*. According to taxonomic rules, the *mycoides* cluster organisms should retain the genus name *Mycoplasma*, and the other members of the genus should all be given new names. This presents an enormous practical challenge due to the nature of several species within and outside of the *mycoides* cluster. *M. mycoides* subsp. *mycoides* itself, along numerous other *Mycoplasma* species, are World Organization for Animal Health (OIE)-listed pathogens. Both international law and domestic regulations govern the shipping, transport, containment procedures, and reporting of detection for these organisms. Human mycoplasmas, while not reportable, require specific diagnostic and treatment strategies. Changing the names of these organisms would cause potentially catastrophic unintended consequences if careful regulations no longer legally apply. Attempts to resolve this taxonomic discrepancy have been made (Gupta et al., 2018a,b), but the committee that advises the International Committee for the Systematics of Prokaryotes has strongly rejected the proposed changes (Balish et al., 2019; May et al., 2013).

Thorough explorations into mollicutes morphology require electron microscopy due to their small size, although numerous protein localization and structural studies have been successfully carried out using immunofluorescent confocal microscopy and

Table 1 *Mollicutes* taxa

Order	*Defining trait*	*Family*[a]	*Defining trait*	*Genus*	*Defining trait*	*Type species*
Mycoplasmatales	Aerobic	*Mycoplasmataceae*	Culturable	*Mycoplasma*	No urea hydrolysis	*M. mycoides*
	Sterol requirement			*Ureaplasma*	Urea hydrolysis	*U. urealyticum*
	Vertebrate host	'Incertae sedis'	Non-culturable	*Haemobartonella*	Non-culturable	N/A
Entomoplasmatales	Aerobic	*Spiroplasmataceae*	Helical morphology	*Spiroplasma*	Helical morphology	*S. citri*
	Sterol requirement	*Entomoplasmataceae*	Non-helical morphology	*Entomoplasma*	No growth with PES[b]	*E. ellychniae*
	Invertebrate or plant host			*Mesoplasma*	Growth with PES	*M. florum*
Acholeplasmatales	Aerobic	*Acholeplasmataceae*	Culturable	*Acholeplasma*	Animal, plant hosts	*A. laidlawii*
	No sterol requirement animal or plant host	'Incertae sedis'	Non-culturable	"*Candidatus* Phytoplasma"	Plant hosts	N/A
Anaeroplasmatales	Anaerobic	*Anaeroplasmataceae*	Anaerobic	*Anaeroplasma*	Sterol requirement	*A. abactoclasticum*
				Asteroleplasma	No sterol requirement	*A. anaerobium*

[a]'Incertae sedis' indicates that the taxon is "of uncertain placement" due to lack of valid publication. In each case here, this is because the organisms in question cannot be grown in axenic culture.
[b]PES = Polyoxyethylene sorbitan.

cryo-electron tomography (May et al., 2014; Milne and Subramaniam, 2009). Because they do not have a cell wall, most mollicutes lack defined, consistent morphology and are referred to as pleomorphic. There are notable exceptions such all members of the genus *Spiroplasma*, who retain a distinctive elongated, helical form (Brown et al., 2018), and individual or small groups of species in the genus *Mycoplasma*, which can exhibit polar structures (Balish and Krause, 2006). The periodicity of coils varies among and between *Spiroplasma* species, and assuming the helical structure is tied to twitching and rotational motility (Brown et al., 2018). Acquisition of the genes encoding the linear motor driving spiroplasma motility and the tip structure potentially mediating adherence appears to have occurred at the point of divergence from the rest of the *Entomoplasmataciae*, and are present throughout the genus (Liu et al., 2017). Polar structures are also associated with motility in some mycoplasma, who exhibit gliding motility in the direction of their structure. Unlike the genes conferring spiroplasma morphology and motility, mycoplasma polar structures arose as a result of convergent evolutionary events following horizontal transfer of distinct gene families into a small number of species. The proteins composing the attachment organelles of the closely-related species *M. pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma gallisepticum*, *Mycoplasma testudinis*, *Mycoplasma amphoriforme*, *Mycoplasma pirum*, and *Mycoplasma imitans* are distinctly different in structure, sequence and origin from those composing the polar structure of *Mycoplasma mobile* (Balish and Krause, 2006). Additional gene families encode the ultrastructural proteins giving *Mycoplasma insons*, *Mycoplasma sualvi*, *Mycoplasma penetrans*, and *Mycoplasma pulmonis* their distinct morphologies (Balish and Krause, 2006; Kirchhoff et al., 1984).

Degenerate evolution has led to the loss of many bacterial structures and capacities, and these apparent deficits are often compensated for by utilizing host cell components. Mollicutes lack known mechanisms for DNA repair, nucleotide synthesis, amino acid synthesis, and numerous other functions retained by most free-living bacteria (May and Brown, 2015), undoubtedly lending to their obligate parasitism. Lacking a cell wall suggests that *Mollicutes* species must have unique cell membranes equipped to provide osmotic resistance. Species in the genera *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Entomoplasma* and *Candidatus* Phytoplasma incorporate host- (or growth medium-) derived sterols into their membranes (May et al., 2014). *Mollicutes* membranes are also unusually rich in sphingomyelin and/or phosphatidylcholine, and can include phosphatidylglycerol, cardiolipin, or glycolipids (Kornspan and Rottem, 2012; Maquelin et al., 2009; Rottem, 1980).

Mollicutes as Pathogens

To date, there has been infection (natural or experimental) by *Mycoplasma* or *Ureaplasma* demonstrated by isolation of pure cultures or molecular detection in over 100 different animal hosts, with humans, and mammalian, reptilian, avian, and piscine species represented (Brown et al., 2018). The volume of study likely represents the overrepresentation of mammals and birds in the known host range rather than a natural predilection for homeotherms, although this possibility cannot be fully excluded. Only 2 isolated, named species in the class *Mollicutes* (*M. mobile* and *Spiroplasma eriocheiris*) have fish or shellfish hosts. However, recent molecular

examinations of several (non-mammalian, non-avian) aquatic animals found sequences of unnamed *Mollicutes* species in high relative abundance to other members of the microbiota (King et al., 2012; May et al., 2014; Svanevik and Lunestad, 2011). Metagenomic studies have also challenged previous understanding of host range as a defining trait of taxa within the class, identifying mycoplasmas (defined by restriction to vertebrate hosts) in invertebrates (Hulcr et al., 2012; May et al., 2014). As the metagenomes of additional species and environments are characterized, our understanding of *Mollicutes* species host ranges will continue to evolve.

Transmission between hosts occurs primarily by direct contact (includes nose-to-nose contact, sexual contact, or milk feeding) (mycoplasmas, ureaplasmas, acholeplasmas, anaeroplasmas), fomite transmission (veterinary mycoplasmas, spiroplasmas [via plant surfaces]), or by insect vector (phytoplasmas, spiroplasmas, entomoplasmas, hemotropic mycoplasmas) (Brown et al., 2018; May et al., 2014). Aerosol transmission of respiratory mycoplasmosis between humans or animals housed in high density (*e.g.*, dormitory settings, military barracks, or livestock and poultry rearing) has also been observed (Atkinson et al., 2008; Feberwee et al., 2006; Tanskanen, 1987). Vertical and perinatal transmission of mycoplasmas, ureaplasmas, and spiroplasmas has been reported (May et al., 2014). The clinical signs of vertically transmitted mycoplasmosis are distinct from those displayed by the infected mother, as congenital infection with *M. hominis* and *Ureaplasma* species can pneumonia, bronchopulmonary dysplasia, meningitis, or encephalitis in (frequently premature) newborns born to mothers with either clinical or subclinical urogenital tract infections (Viscardi, 2010; Waites et al., 2005).

Infection of vertebrate hosts by *Mycoplasma* and *Ureaplasma* species often occurs at mucosal surfaces, and a small number of species can spread to distal tissues from there. Originally thought to exists only as surface pathogens, many species have now been demonstrated to have intracellular phases wherein they can evade adaptive immune responses, antimicrobials, or both. Histopathology generated during mycoplasmosis shows their disease process to be highly inflammatory, and lesions in naturally and experimentally infected animals exhibit hallmarks of chronic inflammatory responses. Mycoplasmosis is infrequently fatal with the notable exceptions of *M. mycoides* subsp. *mycoides*, *M. capricolum* subsp. *capripneumoniae*, and *Mycoplasma alligatoris* (May et al., 2014). The high level of mortality experienced by songbirds afflicted with conjunctivitis due to *M. gallisepticum* stems from an increased susceptibility to predation rather than an enhanced pathogenesis in this particular host (Adelman et al., 2017). The chronic inflammatory nature of most forms of mycoplasmosis makes them diseases of concern for livestock, who often exhibit decreased production and exacerbation of disease with other infectious agents (May et al., 2014) (see also, **Societal Impacts of *Mollicutes***) (Table 2).

Human mycoplasmosis can result from infection with *M. pneumoniae*, *M. genitalium*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Ureaplasma parvum*, and potentially *M. amphoriforme*. It is currently unclear if *Mycoplasma fermentans* and *M. penetrans* can serve as primary pathogens of humans. The majority of human disease involves the respiratory tract or the urogenital tract, but numerous atypical manifestations particularly of *M. pneumoniae* and *M. hominis* infection affecting the central nervous system, skin, eyes, ears, heart, circulatory system, liver, pancreas, kidneys, and joints (Adelman et al., 2017; Viscardi, 2010) (Table 2). Post-infectious complications following "walking pneumonia" due to *M. pneumoniae* and nongonococcal urethritis due to *M. genitalium* include development of asthmatic exacerbation, adult-onset asthma, pelvic inflammatory disease, spontaneous abortion/adverse pregnancy outcomes, and infertility. An association between *M. genitalium* infection and transmission of human immunodeficiency virus (HIV) is strengthening (May et al., 2014).

The genus *Spiroplasma* contains multiple plant, insect, and crustacean pathogens. Diseases of citrus, corn, honey bees, *Drosophila*, shrimp, and farmed Chinese mitten crabs have high potential for substantial economic losses (Brown et al., 2018) (Table 2). In contrast, reduced survival of *Spiroplasma taiwanense*-infected mosquitos have potential to serve as bioremediation tools. Spiroplasmas are occasionally implicated in cervid or human disease as potential causes of transmissible spongiform encephalopathies (TSEs), but fulfillment of Kock's postulates have yet to be carried out (Brown et al., 2018).

The genus "*Candidatus* Phytoplasma" is a unique taxon within the *Mollicutes* most closely related to the *Acholeplasmataceae*. No member of the genus has yet been reproducibly grown in axenic culture (Brown et al., 2018). Phytoplasmas preferentially infect the phloem sieve elements of plants, and are transmitted via insect vectors. General disease features of phytoplasmosis are variable and species-specific, but can include greening (virescence), phylloidy, stunting, leaf curling, floral sterility, yellowing, purpling, witches' broom formation, cupping, vein clearing, and failure to thrive. Numerous diseases of important crop species are caused by phytoplasmas (Table 3).

At this time, no members of the genera *Acholeplasma*, *Entomoplasma*, *Asteroleplasma*, *Anaeroplasma*, *Mesoplasma* are considered primary pathogens of their respective hosts. Major pathogens in the class *Mollicutes* are listed in Tables 2 and 3, and reviews addressing pathophysiology and virulence factors can be found in the suggested **Further Reading**.

Control of *Mollicutes* infection is challenging. Vaccination is a widely used control strategy for multiple veterinary mycoplasmoses with varying degrees of success. Several vaccines are commercially available for the protection of livestock and poultry against mycoplasmosis. The efficacy of these vaccines remains questionable in many cases, however, emphasizing the continuing issue of mycoplasmosis in veterinary medicine among production animals (May and Brown, 2019). Husbandry practices such as continuous monitoring of flocks and herds and culling of infected populations is an additional practice that is crucial to disease control, particularly for species with no available vaccine such as *S. eriocheiris*. Control of plant diseases due to *Mollicutes* species is reliant solely on husbandry practices such as control of insect vectors and early removal of infected plants (Maejima et al., 2014). Despite numerous experimental vaccines targeting *M. pneumoniae*, vaccination against human mycoplasmosis (respiratory or urogenital) is not currently available. Reduction of contact exposure during both respiratory and sexual transmission are the best measures of prevention.

Table 2 Diseases caused by *Mollicutes* species[a]

Species	*Host*	*Disease*	*Signs*
Agricultural (Animal) Pathogens			
M. alkalescens	Cattle	URTD	Nasal discharge, Lameness, Decreased milk production
M. arginini	Cattle	RTI	Infertility
M. bovigenitalium	Cattle	LRTD, URTD, RTI	Nasal discharge, Coughing, Infertility
Mycoplasma leachii	Cattle	Mastitis, arthritis	Lameness, Decreased milk production
M. bovis	Cattle	URTD, LRTD	Coughing, Head tilt, Purulent discharge, Lameness, Decreased milk production
M.bovoculi	Cattle	conjunctivitis	Ocular discharge
M.californicum	Cattle	Mastitis	Decreased milk production
M.canadense	Cattle	Mastitis	Decreased milk production
M. dispar	Cattle	LRTD	Coughing
M. leachii	Cattle	RTI	APO
M. mycoides mycoides	Cattle	CBPP	Coughing, Fever, Torticollis
M. verecundum	Cattle	conjunctivitis	Ocular discharge
M. wenyonii	Cattle	Hemolytic anemia	Malaise, Jaundice
M. gallsepticum	Galliform	CRD, IS, neurological disease, arthritis	Rales, Coughing, Fever, Sinus swelling, Ataxia, Ocular Discharge, Lameness
M. synoviae	Galliform	Arthritis, LRTD, RTI	Rales, Coughing, Sinus swelling, PEQ, Reduced hatchibility, Lameness
M. anatis	Duck	URTD	Nasal discharge
M. adleri	Goat	arthritis	Lameness
M. agalactiae	Goat	LRTD, RTI, contagious agalactia	Coughing, ocular discharge, decreased milk production, lameness, APO
M. capricolum	Goat	Contagious agalactia, LRTD, encephalitis	Lameness, decreased milk production, coughing, sudden death
M. capricolum capripneumoniae	Goat	CCPP	Coughing, Fever, Torticollis
M. mycoides capri	Cattle	Contagious agalactia, LRTD	Lameness, decreased milk production, ocular discharge, coughing
M. putrefaciens	Goat	Septicemia, contagious agalactia	Fever, Malaise, Cataracts, Blindness, Lameness, Decreased Milk Production
M. subdolum	Horse	RTI	APO, Infertility
M. haemolamae	Llama	Infectious anemia	Malaise
M. haemosuis	Pig	Hemolytic anemia	Malaise, Jaundice
M. hyopneumoniae	Pig	PEP	Coughing, Malaise, Arrhythmia
M. hyorhinis	Pig	URTD, LRTD	Nasal discharge, Coughing, Arrhythmia, Lameness
M. hyosynoviae	Pig	arthritis	Lameness
M. arginini	Sheep	LRTD, URTD	Nasal discharge, Coughing, Ocular discharge
M. conjunctivae	Sheep, chamois, ibex	conjunctivitis	Cataracts
M. ovipneumoniae	Sheep	URTD, LRTD	Nasal discharge, Coughing
M. ovis	Sheep	Hemolytic anemia	Malaise, Jaundice
M. meleagridis	Turkey	LRTD, RTI	PEQ, Reduced hatchability, Rickets, Skeletal deformity
M. iowae	Turkey	Arthritis, osteitis, RTI	PEQ, Reduced hatchability, Ocular discharge, Lameness, Skeletal deformity
M. pullorum	Turkey	RTI	Reduced hatchibility
S. eriocheiris	Ch. Mitten crab	Tremor disease	Tremor; death
S. apis	Honeybees	May Disease	Tremor; paralysis
S. melliferum	Honeybees	Colony loss	Sudden death of bees
Human Pathogens			
M. pneumoniae	Respiratory Tract	PAP; encephalitis; meningitis; conjunctivitis	Post-infections inflammatory complications can occur; additional body sites are rarely implicated
M. genitalium	Urogenital Tract	NGU; PID; cervicitis; orchitis	Infertility, pre-term labor, APOs
M. hominis	Urogenital Tract	BV; cerebral abscess; NGU	Infertility(?); APOs (?)
M. amphoriforme	Respiratory Tract	LRTI	Diagnostic differentiation from *M. pneumoniae* historically challenging
Ureaplasma spp. (adult)	Urogenital Tract	NGU; chorioamnionitis; endometritis; hyperammonemia	Strain-dependent pathogenicity; APOs; hyperammonemia occurs in immunocompromised patients only
Ureaplasma spp. (neonate)	Respiratory Tract; Central Nervous System	BPD; encephalitis; meningitis	Premature infants born to infected mothers
Wildlife Pathogens			
M. alligatoris	Alligator	LRTD, nephritis, ME, Fulminant inflammatory disease	Rapid death, Lethargy, Rapid death, Lameness

Table 2 (Continued)

Species	*Host*	*Disease*	*Signs*
M. felifaucium	Cheetah, Puma	gastroenteritis	Emesis, Wasting
M. agassizii	Desert tortoise	URTD	Nasal exudates
M. buteonis	Falcon	LRTD, Unclear	Uncoordinated movement, Lameness, Skeletal deformity
M. gallsepticum	Passerines (primarily finches)	conjunctivitis	Ocular Discharge
M. pneumoniae	Hominids (chimpanzee, rhesus monkeys)	PAP, URTD	Coughing, Malaise
M. iguanae	Iguana	Osteitis	Lameness
M. sphenisci	Jackass penguin	URTD	Halitosis, Choanal discharge
M. crocodyli	Nile crocodile	LRTD	Lameness
M. haemodidelphidis	Opposum	Infectious anemia	Malaise
M. orale	Orangutan	URTD	Rales, Nasal discharge
M. columborale	Pigeon	LRTD	Rales, Coughing, Lethargy
M. phocicerebrale	Seal, Human	Eye infection, Seal finger (hu)	Cataracts, Blindness
M. phocirhinis	Seal	URTD	rhinitis
M. zalophi	Sea lion	Necrotizing LRTD	Lameness, Coughing
M.conjunctivae	Sheep, Chamois, Ibex	Conjunctivitis	Ocular discharge, Cataracts
M. kahanei	Squirrel monkey	Hemolytic anemia	Malaise, Jaundice, Arrythmia
M. sturni	Starling	Conjunctivitis	Ocular discharge
M. mobile	Tench	Red gill disease	Ulceration, labored breathing
M. testudineum	Tortoise	URTD	Nasal discharge
M. corogypsi	Vulture	Abscess	Chronic exudate
M. vulturii	Vulture	LRTD	Rales, Coughing
Companion and Laboratory Animal Pathogens			
M. oxoniensis	Chinese hamster	conjunctivitis	Ocular discharge
M. cricetuli	Chinese hamster	conjunctivitis	Ocular discharge
M. canis	Dog	RTI, UTI, URTD	Frequency, coughing
M. cynos	Dog	pneumonia	Coughing, malaise
M. edwardii	Dog	Septicemia	Fever, Lameness
M. haemocanis	Dog	Infectious anemia	Malaise
M. maculosum	Dog	LRTD, UTI	Coughing, Frequency
M. spumans	Dog	arthritis	Lameness
M. haemominutum	Domestic cat	Infectious anemia	Malaise
M. felis	Cat, Horse	URTD (Fe), LRTD (h)	Nasal discharge, Labored breathing, Ocular discharge
M. gateae	Cat	URTD	Anorexia, lethargy, Ocular discharge, Lameness
M. heamofelis	Cat	Infectious anemia	Malaise
M. equigenitalium	Horse	RTI	APO, Infertility
M. equirhinis	Horse	Inflammatory airway disease	Nasal discharge, Coughing, Rales
M. coccoides	Mouse	Infectious anemia	Malaise
M. haemomuris	Mouse	Infectious anemia	Malaise
M. neurolyticum	Mouse	Rolling disease	Uncoordinated movement
M. pulmonis	Mouse, rat	Murine respiratory mycoplasmosis	Nasal discharge, coughing, anorexia, lameness
M. ravipulmonis	Mouse	Gray lung disease	Coughing
M. arthriditis	Rat	Septicemia, arthritis	Lameness
S. poulsonii	Drosophila spp.	Sex ratio abnormalities	Overabundance of males due to lethality in females

[a]Abbreviations: URTD = upper respiratory tract disease; LRTD = lower respiratory tract disease; RTI = reproductive tract infection; CBPP = contagious bovine pleuropneumonia; CCPP = contagious caprine pleuropneumonia; CRD = chronic respiratory disease; IS = infectious sinusitis; PEP = porcine enzoonotic pneumonia; APO = adverse pregnancy outcomes; PAP = primary atypical pneumonia; NGU = nongonococcal urethritis; PID = pelvic inflammatory disease; BPD = bronchopulmonary dysplasia; ME = meningoencephalitis; RTI = reproductive tract infection; UTI = urinary tract infection.

Diagnosis and treatment of infection by *Mollicutes* species remain distinct challenges. Commercial diagnostic tests have been available for livestock pathogens for several years, but sensitive and specific diagnostics for *M. pneumoniae* and *M. genitalium* have only become available recently. Diagnostic tests for mycoplasmosis of companion animals are not available. Commercial testing for phytoplasmosis and spiroplasmosis is not available. In the absence of commercial diagnostic tests, laboratory culture or molecular testing can be performed. However, specialized expertize is needed to reliably rule these organisms out, and there are a limited number of laboratories in the world with this capacity.

Treatment of infection with *Mollicutes* is inherently complex due to the absence of many antibiotic targets and their potential for facultative intracellularity. Treatment most frequently employs antibiotics inhibiting DNA replication or protein synthesis including

Table 3 *Mollicutes* diseases of food-producing plants

Species	*Plant host*	*Disease*
S. citri	Citrus (orange; grapefruit)	Citrus stubborn
S. kunkelii	Corn/maize	Corn stunt
Candidatus P. asteris	Aster family plants	Aster yellows disease; corn stunt; cabbage proliferation disease; aplle sessile leaf; strawberry green petal; blueberry stunt; tomato big bud; potato purple top
Candidatus P. americanum	Potato	Potato purple top
Candidatus P. autralasia	Papaya	Papaya yellow crinkle disease
Candidatus P. australiense	Grapevine; papaya; strawberry, peanut, apple, alfalfa, sweet potato	Australian grapevine yellows; papaya die-back; strawberry lethal yellows; peanut witches' broom; cocky apple witches' broom; sweet potato little leaf; alfalfa witches' broom
Candidatus P. mali	Apple	Apple proliferation disease
Candidatus P. prunorum	Stone-containing fruits	European stone fruit yellows
Candidatus P. lycopersici	Tomato	Hoja de perejil
Candidatus P. trifolii	Potato	Colombian potato purple top, potato witches' broom
Candidatus P. ziziphi	Cherry, nectarine	Nectarine yellows; cheery lethal yellows
Candidatus P. aurantifolia	Lime	Witches' broom disease of lime
Candidatus P. brasiliense	Alfalfa	Alfalfa witches' broom
Candidatus P. fragariae	Strawberry	Strawberry lethal yellows
Candidatus P. japonicum	Coconut, sugarcane, strawberry	Coconut lethal yellows; sugarcane yellows; strawberry green petal
Candidatus P. pyri	Pear, peach	Peach yellow leafroll; pear decline
Candidatus P. phoenicum	Almond	Almond witches' broom
Candidatus P. oryzae	Rice, sugarcane	Rice yellow dwarf; sugarcane white leaf
Candidatus P. spartii	Sweet potato	Sweet potato little leaf
Candidatus P. solani	Grapevine	Grapevine yellows
Candidatus P. omaniense	Blueberry, peach, sugarcane, grapevine, walnut, cherry	Blueberry proliferation; Canadian peach X; little peach; sugarcane yellows; Virginia grapevine yellows; walnut witches' broom; cherry buckskin disease ("Western X")

tetracyclines, macrolides, fluoroquinolones, aminoglycosides, and certain ketolides. Aminoglycosides, pleuromutilins, and phenicols are frequently used in veterinary medicine, but are not currently used to treat human mycoplasmosis under normal circumstances (Bebear and Kempf, 2005). Mycoplasmal infections can often require long-term antimicrobial therapy, as it is not unusual for disease to recur following the cessation of short-term treatment (Jensen, 2004). Because many *Mollicutes* infections of humans are treated empirically in the absence of readily available, point-of-care diagnostic tests, the need for long-term antibiotic therapy presents a unique challenge in the age of antimicrobial stewardship. Short courses, or even single-dose treatment, of walking pneumonia and nongonococcal urethritis are standard, and they are frequently effective against non-mycoplasma agents (Atkinson et al., 2008). The risk of recurrence of mycoplasmosis due to insufficient therapy, often resulting in intermediate- or resistant strains, must be weighed carefully against the desire to appropriately use antibiotics.

Genetics and Genomics

Mollicutes genomes are among the smallest of free-living organisms. Comparative genomics studies suggest that the small genome size stems from gene loss that occurred early in the divergence of *Mollicutes* from their common ancestor with *Firmicutes*. Ongoing gene loss is still occurring, however, and can be observed by comparing gene sets within each of the phylogenetic branches. Despite an overall trend toward gene loss, the genomes of several mollicutes show extensive evidence of gene gain via horizontal gene transfer (HGT) (Sirand-Pugnet et al., 2007). HGT events have occurred between several species that share common host habitats, and multiple single species show evidence of acquisition of traits from distantly related bacteria (May et al., 2014). HGT in the *Mollicutes* represents an opportunity for high impact discoveries, as *known* mobile genetic elements that mediate gene exchange are relatively uncommon across the class compared to other bacterial clades. Mobile elements seen in the class include insertion sequence elements, integrative conjugal elements (ICE), bacteriophage, and plasmids; however, bacteriophage and plasmids are largely restricted to spiroplasmas, entomoplasmas, and the *M. mycoides* cluster. Species across the class exhibit extensive evidence for HGT despite the relative paucity of known genetic elements that could mediate the exchanges.

Organisms in the class *Mollicutes* have been the basis for additional pioneering studies in the field of genomics. *M. genitalium* was among the first organisms to have its complete genome sequenced (Fraser et al., 1995) and its genome was the first to be made as a fully artificial chromosome (Gibson et al., 2010). The genome of *M. genitalium* was also the basis for studies on the concept of a

minimal cell (Glass et al., 2006; Koonin, 2000) and experimental attempts to generate a cell with the fewest possible genes while maintaining the capacity for axenic culture have been (Glass et al., 2006; Juhas et al., 2011; May et al., 2014). *Mycoplasma mycoides* subsp. *capri* was the first organism whose complete genome was created artificially and transplanted to result in a living cell, realizing the original vision behind the field of synthetic biology (Gibson et al., 2010; Lartigue et al., 2007).

Societal Impacts

Agricultural Losses

Mycoplasmosis of livestock and poultry and phytoplasmosis of several crop species lead to billions of dollars of economic loss each year. Several *Mollicutes* species cause diseases that are listed by OIE, the International Plant Protection Convention (IPPC), and the Food and Agriculture Organization of the United Nations (FAO) because of the potentially devastating impact on local economies, nutrition, and food availability. While not routinely fatal, the morbidity associated with mycoplasmosis of poultry, swine, cattle, and small ruminants results in decreased production in the forms of lowered feed conversion, downgraded carcasses, loss of egg production, loss of milk production, eggs or milk of inferior (*i.e.*, unable to be sold) quality, and infertility. The most common livestock and poultry diseases include porcine enzoonotic pneumonia due to *Mycoplasma hyopneumoniae*, chronic respiratory disease/avian mycoplasmosis due to *m. gallisepticum* and/or *Mycoplasma synoviae*, and bovine mastitis/contagious agalactia due to *M. agalactiae*, *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum*, and *M. putrefascens*. Over and above these diseases, potentially catastrophic economic losses can occur due to outbreaks of CBPP and contagious caprine pleuropneumonia (CCPP). Both diseases have been eradicated from Western Europe, the Americas, and Australia; however, their impact on the livelihood of sub-Saharan Africans in particular is widespread and multi-faceted. Cattle with CBPP are often culled or experience natural mortality. Survivors produce 90% less milk, provide substantially less meat, and are far less able to perform production work (Mariner and Catley, 2003; Tambi et al., 2006; Thiaucourt, 2008). Control of CBPP and/or CCPP during active outbreaks often manifests as quarantine, which is further devastating for pastoralists. Infected herds under quarantine have severely restricted movement, and animals may not be able to access grazing grounds or water sources. Quarantine also often prevents the animals from being brought to markets. Both the health of uninfected animals in the herd and the ability of famers to access the value of their cattle or goats (Barrett et al., 2004) is impacted by the practice of quarantine. The loss of a food source has a community-level negative impact in addition to the monetary burden on farmers (Thiaucourt, 2008). Both CBPP and CCPP are OIE-listed diseases, and cases of either one in a previously disease-free country can trigger freezing of exportation of animal products to non-endemic countries.

Several grain crops, fruit trees/bushes, nut trees, vegetables, and ornamental plants are affected by phytoplasma or spiroplasma diseases. The distribution of phytoplasmoses is worldwide, and affected crops include rice, corn, wheat, barley, onions, sweet potatoes, celery, carrots, lettuce, citrus, cherries, strawberries, coconut, vineyard grapes, peaches, walnuts, pecans, almonds, alfalfa, potatoes, papaya, apples, tomatoes, and many others. Phytoplasmosis is often slow to develop, making its detection in time to prevent spread challenging. Economic losses are substantial for producers of food crops and ornamental plants, and can border on catastrophic for viticulturalists should their grapevines exhibit bois noir disease ("grapevine yellows", "grapevine flavescence dorée"). Significant diseases of deciduous and coniferous trees are caused by phytoplasmas, and diseases of honeybees are associated with two spiroplasmas. These diseases, while not directly affecting food supplies, have the potential to exert profound ecological devastation with wide-ranging implications.

Complications and Consequences of Urogenital Mycoplasmosis in Humans

The association of urogenital mycoplasmosis with pelvic inflammatory disease, infertility, spontaneous abortion, and preterm labor is significant. *M. genitalium* infection is strongly associated with pelvic inflammatory disease, spontaneous abortion, and infertility in addition to its primary clinical presentation of nongonococcal urethritis (May et al., 2014). Clinical findings associated with *Ureaplasma* spp. and *M. hominis* are highly variable, likely in concert with strain- or patient-specific factors, but both organisms have been associated with spontaneous abortion or preterm labor (Viscardi, 2010). While spontaneous abortion and preterm labor occur during active infection, pelvic inflammatory disease and infertility persist even if infection is resolved. Tubal factor infertility due to *M. genitalium* is often permanent for women. Ureaplasmosis of pregnant woman leading to preterm labor/premature rupture of membranes is a significant risk factor neonatal respiratory tract or central nervous system infections. Chronic lung conditions often persist in surviving infants following the resolution of infection (May et al., 2014; Thiaucourt, 2008). Impaired fertility, chronic pain, and lifelong complications from premature delivery can have significant impacts on economic livelihood and quality of life.

Several reports indicate that there is a synergistic relationship between infection with certain *Mycoplasma* species and human immunodeficiency virus (HIV). *M. genitalium* infection leads to increased vaginal shedding of HIV in patients not on antiretroviral medications, indicating that this organism can have enormous public health consequences that extend beyond acute mycoplasmosis (Manhart et al., 2008; Perez et al., 1998). It has also been hypothesized that *M. penetrans* acts as a co-factor in the progression from subclinical HIV infection to the development of acquired immune deficiency syndrome (AIDS). *M. penetrans* strongly stimulates T cell proliferation and thus HIV replication (Blanchard, 1997; Sasaki et al., 1995) Initial *in vitro* results further supported this by demonstrating that the cytocidal effect of HIV is enhanced in the presence of contaminating mycoplasmas (Lo et al., 1991; Montagnier and Blanchard, 1993). Antibodies against *M. penetrans* are far more common in AIDS patients relative to HIV positive,

non-AIDS patients or HIV negative patients (Grau et al., 1998; Wang et al., 1992). In light of this somewhat tantalizing evidence, prospective studies are necessary to further evaluate the potential of *M. penetrans* as a driver of AIDS progression.

Conclusions

Members of the class *Mollicutes* are biologically unique organisms that serve as outstanding conceptual models for many biological principles. The class contains many important pathogens and organisms that have been developed as outstanding tools with which to explore fundamental biological questions. Biological, clinical, and applied research on *Mollicutes* is fostered by the International Organization for Mycoplasmology, and is presented at the organization's bienniel congress.

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Municipal Water Treatment☆

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Abbreviations

AOP	Advanced oxidation processes
BOD	Biochemical oxygen demand
CAS	Conventional activated sludge
COD	Chemical oxygen demand
CW	Constructed wetland
DBP	Disinfection by-product
FC	Fecal coliforms
GBH	Gravel bed hydroponic
HRT	Hydraulic retention time
LRV	Log reduction values
MBR	Membrane bioreactor
MLSS	Mixed liquor suspended solids
MWWTP	Municipal wastewater treatment plants
RO	Reverse osmosis
SC	Somatic coliphage
SF	Sand filter
SP	Stabilization ponds
SRT	Solid retention time
TC	Total coliforms
THM	Trihalomethanes
UASB	Upflow anaerobic sludge blanket
UF	Ultrafiltration

Introduction

Humans are in direct contact with many pathogens mainly via water used for drinking, washing, and cooking. One of the sources of pathogens in the environment is wastewater discharged with or without treatment or recycled for many purposes (Fig. 1). Therefore there is a need to control and monitor this entrance route of pathogens. The evaluation of pathogen removal by any treatment system and of the final pathogen content in the effluent is of importance in assessing the health risks of treated sewage. Sewage discharge is considered as a worldwide risk factor for the introduction of human pathogens in surface waters. Untreated or insufficiently treated wastewater has often been reported as the primary source of contamination in groundwater. Therefore, removal of pathogenic organisms is one of the important objectives of municipal wastewater treatment.

Occurrence of Pathogens in Municipal Wastewater

Several pathogens and parasites are commonly found in municipal wastewater and in treated wastewater. Some of the important pathogens found in municipal wastewater are listed in Table 1. Important bacterial pathogens found in wastewater are generally associated with diarrhoea, and include *Salmonella*, *Shigella*, *Vibrio cholerae*, *Campylobacter* and *Escherichia coli*. While diseases caused by *Salmonella and Vibrio cholerae* are prevalent in developing countries, *Campylobacter*-associated gastroenteritis is more common in developed countries including the United States.

Many viruses exist within wastewater, especially enteric viruses, which originate from the human gastrointestinal tract. More than 160 enteric viruses have been identified to be pathogenic to humans at low infectious doses. It is presumed that many outbreaks of waterborne diseases may be attributed to enteric viruses. Among the important enteric viruses, rotavirus is a common cause of

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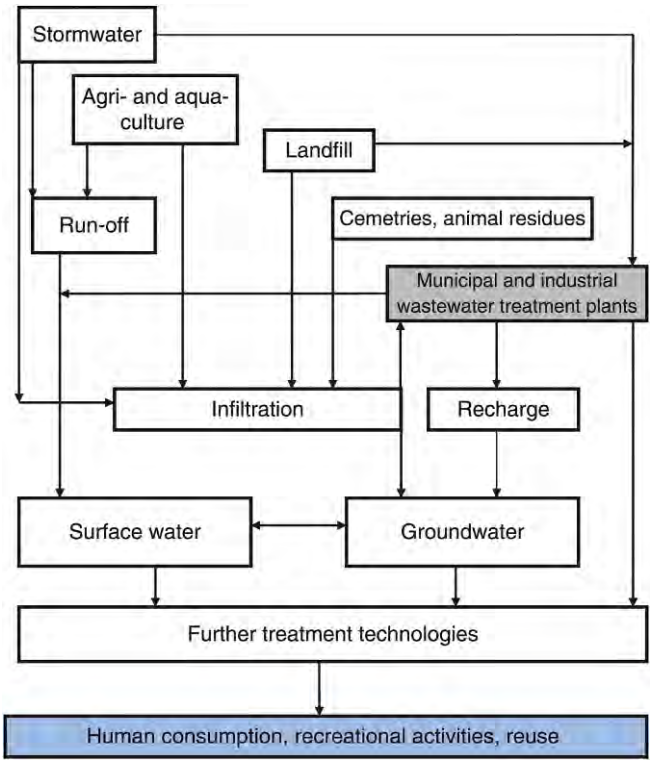

Fig. 1 Antrophogenic-based occurrence pathways of pathogens.

Table 1 Most frequently detected pathogens in municipal wastewater

Bacteria	*Protozoa*
Salmonella	*Cryptosporidium parvum*
Shigella	*Giardia lamblia*
Campylobacter jejuni	*Entamoeba histolytica*
Vibrio cholera	
Escherichia coli	
Yersina enterocolitica	
Legionella spp.	
Viruses	**Helminths**
Poliovirus	*Ascaris lumbricodes*
Hepatitis A virus	*Trichuris trichiura*
Rotoviruses	*Necator americanns*
Echoviruses	
Adenoviruses	

infection among children under the age of two in developing countries. Waterborne hepatitis by hepatitis A and E viruses is also common in these regions.

Among the protozoan pathogens, *Cryptosporidium parvum*, *Giardia lamblia*, and human virulent microsporidia are human anthropozoonotic enteropathogens that inflict considerable morbidity on healthy people, particularly children, and can cause mortality (in immunosuppressed individuals). *Cryptosporidium* and *Giardia* are very frequently transmitted via water. The transmissive stages, that is, oocysts, cysts, and spores, are environmentally robust and therefore ubiquitous in aquatic habitats. These enteropathogens are categorized as category B biodefense agents by the National Institute of Health, and microsporidian spores are on the Contaminant Candidate List of the US Environmental Protection Agency because spore identification, removal, and inactivation in drinking water is technologically challenging. Only low numbers of *Giardia* cysts and *Cryptosporidium oocysts* need to be ingested to cause infection. *Entamoeba histolytica* is a major cause of morbidity and mortality in developing countries, while waterborne transmission is rare in developed regions. Reported concentrations of pathogens found in feces are presented in Table 2. The concentrations of enteric pathogens in municipal wastewater are much higher in developing regions compared to developed countries due to higher rates of infection in the developing world.

Table 2 Concentration of pathogens in feces

Organism	*Concentration per g of feces*
Salmonella spp.	10^4–10^{10}
Shigella spp.	10^5–10^9
Vibrio cholerae	10^7
Campylobacter	10^7
Rotavirus	10^{10}
Adenovirus	10^{11}
Hepatitis A virus	10^6
Giardia lamblia	10^6
Cryptosporidium	10^6–10^7
Ascaris lumbricodes	10^4

Indicator Organisms

Since direct monitoring of pathogenic microorganisms in water and wastewater requires costly and time-consuming procedures and skilled manpower, indicator organisms have been used to assess the presence of fecal contamination and efficiency of wastewater treatment processes. Coliform group was first accepted as an indicator of fecal pollution drinking water in early twentieth century. Since then, various microorganisms have been used for indicating the presence of fecal contamination. Since it is now recognized that there is no direct correlation between bacterial indicators and pathogenic organisms, use of indicators is now defined by their intended purposes such as fecal contamination indicators and process efficiency indicators. Generally used and proposed indicators are the following: (i) total coliforms (ii) fecal coliforms (thermotolerant coliforms) (iii) *Escherichia coli* (iv) fecal streptococci (v) *Clostridium perfingens* (vi) bacteriophages. Bacteriophages are viruses that infect bacteria. Bacteriophage that infect *Escherichia coli* are commonly referred to as coliphages. Bacteriophages, which are commonly found in the feces of humans and warm-blooded animals, are considered by some researchers to be good indicators because they have fundamental properties and features, such as structure, composition, size, and replication mode, similar to enteric viruses. Coliphages have been detected wherever fecal contamination occurs. Many studies have proposed the use of coliphage as indicators of enteric viruses.

Table 3 presents the reported levels indicator organisms and pathogens in raw municipal wastewater. The occurrence of all these species can vary from one country to another, or even from one city to another, or during daily hours due to wastewater-generating characteristics. For example, the occurrence of parasite ova, cysts, and fecal coliforms (FC) in urban wastewater and their removal was determined after sewage treatment in gravel bed hydroponic (GBH) constructed wetlands (CWs) receiving conventionally treated wastewater at Abu Attwa, Ismailia, Egypt. Samples of raw wastewater and GBH influents and effluents were examined for eggs of intestinal helminths for over 2 months in 1995. All raw wastewater samples were found to be positive for *Ascaris lumbricoides* but eggs of *Hymenolepis* spp., *Trichuris* spp., *Ancylostoma duodenale*, and *Toxocara* spp. were also detected in raw and conventionally treated wastewaters. In Ismailia, the concentration of eggs of human intestinal helminths in raw wastewater ranged from 6 to 42/l. In the influent to the GBH beds, the concentration of helminth eggs was reduced and ranged from 0 to 11/l; eggs of Ascaris were found in 28% of the samples. No helminth eggs were recovered from GBH-treated effluents. Cysts of protozoa and FC bacteria were also removed by GBH beds to some degree. Raw wastewater contained cysts of the protozoa *Entamoeba coli*, *Entamoeba histolytica*, and *Giardia* spp. Although the density and diversity of species was reduced after treatment in the GBH beds, some amoebic cysts were found in the effluents. FC removal averaged 2–3 logs during sewage treatment in GBH beds but effluents did not satisfy WHO guidelines for unrestricted irrigation. This survey indicates that GBH beds have the capacity to remove pathogens from wastewaters. Improvement in wastewater quality after GBH treatment satisfied WHO microbiological quality guidelines for restricted irrigation. With a retention time of 6 h, GBH CWs have practical applications for wastewater treatment for safe reuse in Egypt.

Table 3 Reported levels of pathogens and indicators in raw sewage

Organism	*Concentration per 100 ml*
Total coliforms	10^7–10^9
Fecal coliforms	10^6–10^7
Fecal streptococci	10^5–10^6
Clostridium perfingens	10^4
Coliphages	10^2–10^3
Salmonella	10^2–10^4
Giardia cysts	10^1–10^3
Enteric viruses	10^1–10^5
Cryptosporidium oocysts	10^1–10^3

Removal of Microorganisms by Wastewater Treatment Processes

One of the important objectives of municipal wastewater treatment is the removal of pathogenic microorganisms. Therefore, treatment technologies for wastewater have been continuously improving due to stringent limits set including microbial control and limits set for treated wastewater reuse practices. Generally municipal wastewater treatment comprises three phases namely, primary, secondary and tertiary treatment. Fig. 2 shows the common approach for treatment of municipal wastewater with tertiary treatment processes, and the integration of advanced oxidation processes (AOPs). In general, the common treatment technologies are able to remove pathogens at different levels as shown in Tables 4, 5.

In primary treatment, large solid particles are physically separated from the wastewater in a settling tank. The settled solids are referred to as primary sludge. Primary sedimentation is effective for removal of larger pathogens such as helminthes. In addition, solid-associated pathogens are also removed to some extent. Total coliform removals in the range of 25–70% have been reported. Secondary treatment generally consists of biological processes such as activated sludge process, trickling filter, lagoon (pond) or anaerobic process such as upflow anaerobic sludge blanket reactor. Tertiary treatment is used to further reduce pathogens and other contaminants so as to protect water bodies or to reuse the treated wastewater. Physico-chemical processes such as coagulation, adsorption, filtration and disinfection are generally used for this.

Factors Affecting Microbial Removal in Municipal Wastewater Treatment Systems

A number of factors influence the microbial removal during wastewater treatment. Microbial aggregation and solids association increase settleability while it offers protection from disinfection. Higher temperature generally results in rapid inactivation of microbes as a result of denaturation by thermal effect, by increased enzymatic activity in biological processes or by faster reaction rates in chemical processes. However, elevated temperature may result in increased growth for some pathogens such as *Vibrio* spp. and *Legionella* spp. Biological activity can also reduce pathogens by predation mechanisms such as grazing, or by increased enzymatic activity by bacteria. Biofilm and floc formation also reduce pathogens by increased adsorption and accumulation. Sunlight influences the pathogen removal especially in pond systems.

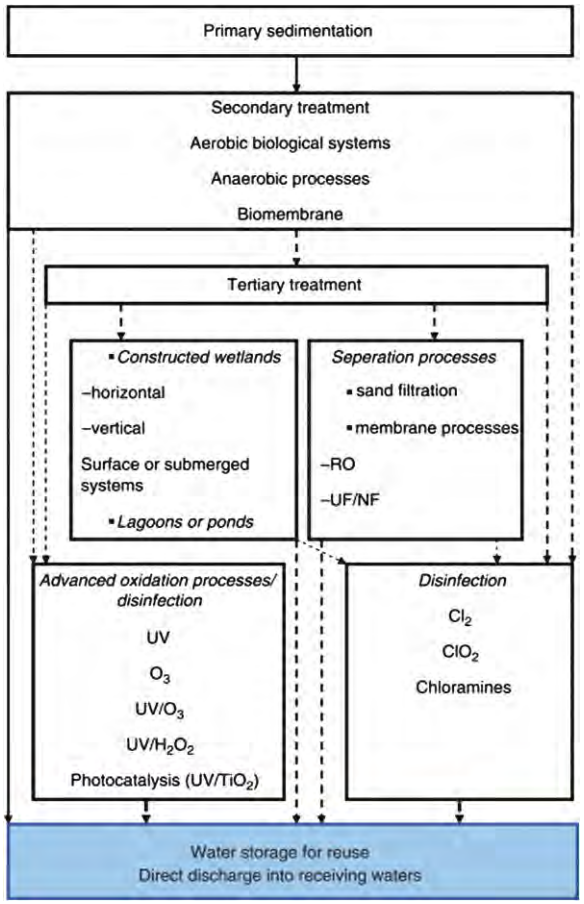

Fig. 2 Municipal wastewater treatment and disinfection technologies.

Table 4 Comparison of removal efficiency of some parameters for common treatment systems

	Removal efficiency (%)			
Treatment systems	*BOD*	*N*	*P*	*Coliforms*
Prechlorination	15–30			90–95
Primary settling	25–50			25–70
Coagulation and flocculation	50–85			40–80
Stabilization ponds	75–90	30–50	20–60	60–99
Activated sludge	85–95	30–40	30–45	60–90
Low rate trickling filters	85–90	30–40	30–45	60–90
Anaerobic treatment	60–80	10–25	10–20	60–90
Aerated lagoons	90–95			95–98
Postchlorination				98–99

Table 5 Removal of pathogens in stabilization ponds and constructed wetlands

Pathogens/pathogen indicators	*Average removal (%)*	*Final level*	*Note*	*References*
Fecal coliforms	99.6–99.95	10^2–10^3 CFU/100 ml	–	van Der Steen et al. (1999)
Escherichia coli	98	375150/100 ml	30 $gBOD_5/m^3/d$	Almasi and Pescod (1996)
E. coli	94	819471/100 ml	65 $gBOD_5/m^3/d$	Almasi and Pescod (1996)
E. coli	83	4861670/100 ml	100 $gBOD_5/m^3/d$	Almasi and Pescod (1996)
S. faecalis	95	46760/100 ml	30 $gBOD_5/m^3/d$	Almasi and Pescod (1996)
S. faecalis	96	165374/100 ml	65 $gBOD_5/m^3/d$	Almasi and Pescod (1996)
S. faecalis	86	439961/100 ml	100 $gBOD_5/m^3/d$	Almasi and Pescod (1996)
Vibrio cholerae	94	28 units/L		Oragui et al. (1993)
Giardia cyst	100		16 days (HRT)	Amahmid et al. (2002)
Total coliforms	>95		50 mm/d (*Typha latifolia*)	Ciria et al. (2005)
Fecal coliforms	>93			
Fecal streptococci	>81			

Note: For full details of the references cited in this table please refer to the Further Reading list at the end of the article.

Secondary Treatment Systems

Activated Sludge Process and Membrane Bioreactor

Pathogen removal by conventional activated sludge (CAS), which is widely used to treat municipal wastewater, is highly variable depending on the type of organisms and detention time. Up to 2 log reduction of organisms has been reported. Since activated sludge effluents have been shown to contain high levels of pathogens that may pose an unacceptable hazard to human health, particularly when the treated effluent is reused. In recent years, membrane bioreactors (MBR) technology has emerged as an alternative to conventional activated sludge process for municipal wastewater treatment. In MBR a permselective membrane is used in combination with a biological process. In MBR systems, secondary sedimentation is not needed for separation of solids since removal of solids is achieved by the membrane. The small pore size of the membrane also results in the physical removal of a wide variety of microorganisms.

In a study to compare the performance of MBR and CAS with tertiary treatment in removing microbial pathogens, TC and FC and somatic and F-specific coliphage were used as indicators of pathogenic bacteria and viruses. Each MBR pilot had a hydraulic retention time (HRT) of ~6 h with a solid retention time (SRT) of 18 days and mixed liquor suspended solids (MLSS) of ~10 g/L. Average concentration of TC and FC was 2.5×10^7 CFU/100 mL and 9.6×10^6 CFU/100 mL, respectively, while average concentration of somatic and F-specific coliphage was 2.9×10^5 PFU/100 mL and 1.79×10^5 PFU/100 mL, respectively. Up to 5.7 log removal of coliforms and 5.5 log removal of coliphage were observed in the conventional treatment process with advanced tertiary treatment (RBC, sand filtration, and chlorination). Complete removal of FC and up to 5.8 log removal of coliphage was observed in the MBR system. It was shown that the MBR system was capable of high removal of coliphage despite the variation in feed coliphage concentrations. Compared with the CAS process followed by tertiary treatment, the MBR pilot plants achieved better FC removal by more than 1 log unit. The MBR permeate was shown to be suitable for unrestricted reuse while the advanced tertiary system did not meet the pathogen removal limits. The results of this study indicate that the MBR process can achieve better microbial removal in fewer steps than a CAS process with advanced tertiary treatment.

The removal of human enterovirus (EV) and norovirus genogroup II (NoV GGII) was studied in a full-scale MBR wastewater treatment plant and compared with the removal of human adenovirus (HAdV) (Simmons et al., 2011). Samples were collected from four separate locations throughout the treatment process; influent, primary settling effluent, membrane influent and membrane effluent. EV was detected in all 32 samples with an average concentration of 1.1×10^7 and 7.8×10^1 viruses/L for the

membrane influent and membrane effluent, respectively. NoV GGII was detected in 20 of 32 samples (63%) with an average membrane influent and membrane effluent concentration of 2.8×10^5 and 1.2×10^1 viruses/L, respectively. HAdV was detected in all 32 samples with an average membrane influent concentration of 5.2×10^8 and 2.7×10^3 viruses/L in the membrane effluent. Results indicated that the full-scale MBR treatment was able to reduce the viral loads by approximately 5.1 and 3.9 and 5.5 log units for EV, NoV GGII and HAdV, respectively. This full-scale MBR system outperformed the removal observed in previous pilot and bench scale studies by 1–2 log units.

Purnell et al. (2015) assessed the removal efficacy of viruses in a full-scale membrane bioreactor (MBR) wastewater reuse system, using a range of indigenous and 'spiked' bacteriophages. Mean levels of fecal coliforms were reduced to 0.3 CFU/100 mL in the MBR product and were undetected in samples taken after the chlorination stage. The microbial removal values recorded were greater than those commonly reported for conventional activated sludge treatment. A large reduction (5.3 log) in somatic coliphages was also observed following MBR treatment. However, F-specific and human-specific (GB124) phages were less abundant at all stages, and demonstrated log reductions post-MBR of 3.5 and 3.8, respectively. Spiked phages (MS2 and B-14) displayed post-MBR log reductions of 2.25 and 2.30, respectively. The removal of these suspended phages, which are smaller than the membrane pore size (0.04 μm), also highlights the possible role of the membrane biofilm as an effective additional barrier to virus transmission. The study suggested that phage removal in MBR systems is highly variable and may be closely related on both the size and morphology of the viruses, and to whether or not they are attached to solids. It also showed that monitoring several phage groups would be required to assess the ability of MBR to remove enteric viruses.

Stabilization Ponds

Waste stabilization ponds (SP) have been extensively used for treatment of domestic wastewater all over the world, especially in small cities and towns of developing countries as a low-cost method of pathogen removal for wastewater reuse. The low cost and simplicity of their construction, operation, and maintenance has made them one of the most important wastewater treatment technologies, in particular when the effluent is land-applied. Sunlight is an important factor for the removal of microbes in these systems. Natural die-off, predation, sedimentation, and environmental parameters such as pH, dissolved oxygen and temperature also influence the pathogen removal.

In Spain, a waste stabilization pond (WSP) system consists of two anaerobic ponds, a facultative pond and a maturation pond with a total retention time of 6 days was evaluated for their efficiency in the removal of fecal indicator bacteria (total coliforms, *E.coli* and fecal streptococci), coliphages, helminth eggs and protozoan cysts (*Cryptosporidium* and *Giardia*) (Reinoso et al., 2011). The results showed that the pond system significantly reduced the concentration of pathogenic and indicator microorganisms from wastewater producing a final effluent suitable for agricultural irrigation. The overall removal of the system ranged from 1.4 log units for coliphages in the cold period to 5.0 log units for *E.coli* in the hot period. *Cryptosporidium* oocysts were reduced by an average of 96% and *Giardia* cysts by 98%. Complete removal of helminth eggs was also observed. The surface removal rates reflected that anaerobic ponds were the most effective in removing parasites followed by facultative pond and maturation pond. Sludge samples also revealed that anaerobic ponds were the most effective in parasite removal. However, sludge samples indicated that sedimentation was not the main removal mechanism. Other factors such as sunlight, pH, temperature and dissolved oxygen were comparatively more important in reduction of pathogens in this system (Reinoso et al., 2011).

The removal of TC, FC, and coliphage in waste SP functioning as a pilot system in the tropical climate of Maracaibo, Venezuela, was investigated. Sampling points included raw sewage and each pond effluent. Turbidity, pH, and temperature were recorded. The results for raw sewage showed average levels of 4.1×10^6 TC and 2.8×10^6 FC/100 mL. Temperature, pH, and turbidity ranges between 26 and 31°C, 6.2 and 9.5, and 15 and 98 NTU, respectively. Removal rate of microorganisms in the three systems ranged between 93% and 98%. Despite the high removal efficiency of microorganisms, the final effluents showed average counts of 5.4×10^4–1.4×10^5 TC and 5.2×10^4–1.3×10^5 FC/100 mL. This study shows that the microbiological quality of the final effluents did not achieve the WHO water quality requirement for FC (10^3/100 mL); therefore, they cannot be used for irrigation. Additional treatments, such as slow sand filtration, are needed in order to improve the quality of the water.

Verbyla and Mihelcic (2015) reviewed virus removal reported in the literature from 71 different wastewater treatment pond systems. Only a weak to moderate correlation of virus removal with theoretical hydraulic retention time was observed. On average, one log reduction of viruses was achieved for every 14.5–20.9 days of retention, but the 95th percentile value of the data analyzed was 54 days. Among the different mechanisms, sedimentation may not be a significant virus removal mechanism in some wastewater ponds. Direct and indirect sunlight-mediated mechanisms are not only dependent on pond water chemistry and optics, but also on the characteristics of the virus.

Anaerobic Systems

Upflow Anaerobic Sludge Blanket (UASB) reactors are the most widely used anaerobic technology for domestic wastewater treatment in tropical and semi-tropical conditions. Pathogen removals have been reported to be low during anaerobic treatment. Thus, UASB reactor effluent has to undergo post treatment in order to meet the discharge standards.

A combination of UASB reactor and downflow hanging sponge (DHS) anaerobic treatment reactors was compared with activated sludge process for removal of TC and FC. Raw sewage for both systems had coliforms in the order of 10^7–10^8 MPN/100 ml. There was only single order removal of coliforms in UASB reactor. However, they were removed by 3–4 orders after the

treatment by DHS. TC and FC removals were 4.0 and 3.7 log units, respectively, by the whole system. But a slight drop in coliform removal was observed during the winter season. Nevertheless, it was observed that UASB+DHS system outperformed ASP in pathogen removal as coliform count in the final ASP effluent was higher. In the final ASP effluent, the removal efficiencies were 3.9 and 3.4 log units for TC and FC, respectively. The higher amount of retained sludge in UASB+DHS system and longer SRT could be the reason for the better performance of the system compared to that of ASP.

The capacity of anaerobic processes for pathogens reduction was studied in a laboratory-scale UASB reactor treating domestic wastewater at low temperatures in Peru (Yaya-Beas et al., 2016). The average total helminth egg (HE) content in the influent wastewater varied between 166 and 256 egg/L and the most common specie was *Ascaris lumbricoides*. Results showed that the HE removal varied between 89% and 95% when the upflow velocity was varied in the range of 0.12–0.41 m/h, with no significant change within the range of upflow velocities studied. Fecal coliform and *Escherichia coli* removals varied in the range of 0.9–2.1 and 0.8–1.6 log respectively. The UASB effluent with a HE content varying between 5 and 35 egg/L did not meet the WHO standards for reuse and a post-treatment unit would be required to polish the effluent.

Constructed Wetlands

Constructed wetlands (CW) are well known as highly efficient systems for treating wastewater from different sources. This treatment system is cost-effective for reuse in desert areas. CWs, of either vertical or horizontal (i.e., free surface or subsurface) flow, are increasingly being used worldwide for secondary or tertiary treatment of municipal sewage. In many developing and developed countries, these reed bed systems discharge to drinking water or waters used for recreational purposes. Reed bed discharges can contribute to microbial buildup in reclaimed water, but these pathogens may not necessarily originate from the wastewater received by these wetlands. While CW systems facilitate nutrient removal, they may not provide substantial protozoan pathogen remediation, despite reported removal efficiencies. The bacterial removal in constructed wetlands can be influenced by a number of factors such as solar radiation, natural cell die-off, sedimentation, filtration and adsorption. Furthermore, predation by nematodes, protozoa, and bacteriophages, and competition for limiting resources may also inactivate bacteria. Virus attenuation is controlled mainly by adsorption and inactivation.

Table 5 gives a background survey of CWs and stabilization ponds for pathogens removal. The role of HRT and granular medium in FC and somatic coliphage (SC) removal in tertiary reed beds was investigated in a pilot plant with four parallel reed beds (horizontal subsurface flow CWs), each one containing a different type of granular medium (near Barcelona). Secondary effluent from the wastewater treatment plant was used as the influent of the pilot system. The microbial inactivation ranged between 0.1 and 2.7 log units for FC and from 0.5 to 1.7 log units for SC in beds with coarser granular material (5–25 mm), while it ranged between 0.7 and 3.4 log units for FC and from 0.9 to 2.6 log units for SC in the bed with finer material (2–13 mm). The microbial inactivation rises as the HRT increases until it reached a saturation value (in general at an HRT of 3 days). The value of the microbial inactivation at the saturation level depended on the granular medium contained in the bed. The specific surface area necessary to reach 2–3 log units of FC and SC was $\sim$3 m^2/people equivalent.

A continuous flow, free water surface (FWS) pilot wetland using the duckweed plant *Lemna gibba* L. was constructed at Israel, and operated on domestic primary effluents. Water quality and system efficiency were observed during the experiment for reuse purposes. Results indicated that hydraulic residence time averaged 4.267±0.61 days, average influent flow rate was 0.2347±0.027 m^3/day, and hydraulic load 0.227±0.03 m/day. Hydraulic efficiency in the system was high and allowed good settling conditions. Suspended solids and organic matter removals were the highest and effluent concentrations were 13.1±79.7 and 40.37±11.9 mg/L for total suspended solids and total BOD, respectively. Nitrogen removal was lower (10–20%) but increased slightly with higher nitrogen loads. Therefore, nitrogen content in the plants was high (4.3±0.5%/kg dry plant). Phosphorus removal was negligible. High removal for FC (95%) and effluent turbidity (>50%) were also observed.

Abreu-Acosta and Vera (2011) evaluated the removal efficiencies of fecal bacteria and enteric pathogens in two wastewater natural reclamation systems. These systems were constituted of a combination of anaerobic treatment, small sub-surface flow constructed wetland refilled of volcanic ashes and a final pond as water reservoir. Fecal coliforms, enterococci, *Escherichia coli*, somatic coliphages, *Salmonella* sp., *Campylobacter* sp., *Cryptosporidium* sp., *Giardia* sp. and helminth eggs were analyzed in constructed wetlands inlet and outlet and storage pond effluent. Low numbers of protozoan positive samples (4.54% and 19.05% for *Giardia* sp.) and an absence of helminth eggs were found. Both systems demonstrated efficient reduction of fecal contamination indicators in the wastewaters (removal rates values of 2 log). The natural systems for wastewater treatment used to be efficient in *Salmonella* abatement, this fact was confirmed in the reported systems, since enterobacteriaceae were found in only one of the effluents. *Campylobacter* species associated with the access of animals to storage ponds were detected in the reclaimed water. The presence of *Salmonella* and *Campylobacter* in the effluents of the evaluated systems implies a potential health risk, although the bacteria presence is minimal when EC are lower than 200 cfu/100 mL.

Tertiary Treatment Systems

Sand Filtration

In recent years, slow sand filtration (SSF) has gained increasing attention as a promising technology for treatment of secondary effluent with the purpose of reuse. Seeger et al. (2016) investigated the use of SSF for removal of pathogen indicators from

secondary effluent of constructed wetlands. With a view to improve SSF performance by an enhanced use of the biologically active *Schmutzdecke* layers, they compared the pathogen indicator removal efficiency of four different SSF designs: standard, recirculating, a static series of two SSFs, and a rotating cascade. All filter configurations gave substantial removal for *E. coli* (≤4.7 log), enterococci (≤2.4 log), *C. perfringens* spores (≤2.1 log), coliphages (≤2.8 log) and heterotrophic plate count (HPC) (≤1.5 log) with the tested operational conditions. Results showed that only rotating and static cascade systems complied with European irrigation water standards for both *E. coli* and enterococci at hydraulic loading rate=5 cm/h achieving mean log removal of 2.7–4.7 and 2.1–2.4, respectively. The standard filter showed considerable improvement of removal efficiency for *E. coli* and enterococci during an operation period of up to 1 ½ years. *C. perfringens* spore removal performance was good for all SSFs. Somatic coliphages were reduced to concentrations close to the detection limit, while F-specific RNA coliphage removal was ∼1.1 log. Removal of heterotrophic bacteria was generally low (0. 5–1.5 log) (Seeger et al., 2016).

The performance of rapid sand filtration (SF) was evaluated in three tertiary wastewater treatment plants in the State of Kuwait. These plants are located at Ardiya, Rikka, and Jahra, and receive municipal wastewater flows of 220,000, 95,000, and 42,000 m^3/day, respectively. The Ardiya plant uses a two-stage activated sludge process for the secondary treatment of wastewater, whereas both the Rikka and Jahra plants use the extended aeration process. The study was conducted over a period of 1 year, and the efficiency of the tertiary SFs was determined based on reductions in suspended solids (TSS), volatile suspended solids (VSS), BOD, and COD. Analysis of these records showed that the secondary treated effluent quality was highly variable. Seasonal variations were observed due to nitrification and denitrification that enhance the production of nitrogen gas and carry sludge solids in the effluent during summer, causing more frequent backwashing of the filters. The results obtained indicated significant improvements, at 95% and 99% significance levels, in solids (TSS, VSS) and organics (BOD, COD) removal by SF. They also showed that, in addition to improving effluent quality, tertiary filtration played an important role in the stability of effluent quality so as to dampen variations in the quality of secondary-treated effluent. The tertiary effluent consistently satisfied the water quality requirements for irrigation. TC were determined as 2 in Ardiya and 0 and 0 MPN/100 mL in Rikka and Jahra tertiary treated effluents, respectively, which met the guidelines for landscape irrigation (2.2 MPN/100 mL) in Kuwait. Based on current cost figures from wastewater treatment plants in Kuwait, the cost of producing treated effluent using filtration and chlorination for tertiary treatment was about US\$0.50/m^3 (\$0.20 wastewater collection plus \$0.30 treatment for reuse). This represented about one-third of the cost of producing 1 m^3 of desalinated water using the MSF process as an alternative source for irrigation water in Kuwait.

The occurrence and removal of Salmonellae and fecal indicators in four conventional municipal wastewater treatment plants (MWWTP) was investigated. In addition, the efficiency of a semitechnical-scale biological nutrient removal process and three pilot-scale tertiary filtration units in microbial removal were tested. All influent samples collected from MWWTPs contained *Salmonella* from 93 to 11,000 MPN/100 ml and indicator bacteria from about 10^7 to 10^8 CFU/100 ml. The reductions in *Salmonella* numbers achieved in full-scale biological–chemical wastewater treatment and semitechnical-scale biological nutrient removal processes were usually between 94% and virtually 100% (99.9%) and indicator bacteria reductions between 2 and 3 log units. Microbial numbers in MWWTP effluents could be modeled as a function of effluent residual organic matter, suspended solids, and total phosphorus concentrations. Pilot-scale tertiary treatment by rapid sand contact filter, chemical contact filter, and biological–chemical contact filter reduced *Salmonella* numbers below the detection limit and FC on average by 99%, 39%, and 71%, respectively. A total of 32 *Salmonella* serovars were identified among 197 *Salmonella* isolates from municipal wastewaters. These results, especially antibiotic-resistant *Salmonella* strains, indicated that conventional municipal wastewater treatment without efficient tertiary treatment, like filtration or disinfection, may constitute a risk for public health.

Membrane Processes

Two polyvinylidene fluoride microfiltration membranes with a nominal pore size of 0.22 mm were challenged with mixed microbial cultures present in Milli-QTM water and in secondary effluent, and with a Gram-negative model bacterium, SW8, to investigate bacterial passage. Total bacterial counts measured microscopically using the DNA Fluorochrome DAPI revealed that the small bacteria in Milli-QTM water passed MF membranes totally. The model bacterium, SW8, and bacteria from secondary effluent were mostly retained with a log removal value (LRV) of 4 and 3.5, respectively. Transmembrane pressure did not influence the levels of bacterial passage significantly. Pore size effects were investigated using track-etched membranes with nominal pore sizes of 0.2, 0.1, and 0.05 mm. The LRV of 0.2 mm membranes for SW8 and secondary effluent cells was 3 and 1.5, respectively (total counts). Both membranes with pore sizes smaller than 0.2 mm acted similarly; they still transmitted secondary effluent cells with LRV 2 log higher than 0.2 mm membranes, but for SW8 only 50% higher. In contrast to total count results, removal of bacteria was 100% with all membranes when assessed by cultureable counts, that is, the number of bacterial colonies recovered on R2A agar plates. Transmitted bacteria failed to grow on standard basal microbiology media most probably because they were injured during passage through the membranes to the extent that recovery in laboratory media did not occur. However, tests with CTC, an indicator of cell viability, indicated that approximately half of the cells of SW8 that passed the membranes had what appeared to be functional electron transfer chains in their membranes. All membranes had a pore size distribution that included pores larger than the nominal value. Field emission scanning electron microscopy provided evidence for entrapment of bacteria within the membrane matrix.

An MBR and reverse osmosis (MBR–RO) system was developed to assess potential reuse applications of municipal wastewater. The objective of the study was to examine the water quality throughout the system with a focus on waterborne pathogens,

disinfection by-products (DBPs), and nitrate. High-quality reuse water could be produced from municipal wastewater through the use of an MBR–RO system. The water meets California Title 22 reuse regulations for nonpotable applications and US EPA drinking water limits for trihalomethanes (THM) (80 mg L^{-1}), haloacetic acids (60 mg L^{-1}), chlorite (1.0 mg L^{-1}), TC (not detectable), viruses (not detectable), and nitrate/nitrite (10 mg N L^{-1}).

A field study on municipal wastewater reclamation for the irrigation of two experimental crops of tomato and fennel aimed at the evaluation of the performance of a membrane filtration pilot plant (productivity 0.7 m h) for tertiary treatment and the comparison between agronomic results (features of soil and crops) after irrigation with reclaimed wastewater versus conventional groundwater. Over long-term operation, the pilot plant performance results were very good in terms of suspended solids and bacterial removal. Referring to the agronomic results, no substantial differences were observed after 2 years, in terms of both microbiological quality of the crops and characteristics of the soil. The results indicate that membrane-filtered municipal effluent is a viable alternative water resource for irrigation. The performance of the MFPP in terms of microorganisms removal was evaluated by measuring the content of coliforms (total and fecal) and *E. coli* in wastewater samples collected before and after membrane filtration. Average removal efficiencies for TC, FC, and *E. coli* resulted in 3.7, 4.2, and 3.7 log, respectively. The average content of TC in membrane-filtered wastewater was below 1000 CFU/100 mL. In any case, the residual bacterial content of the membrane permeate did not reflect the claimed feature of this technology that, based on the nominal membrane pore size (0.03 mm), should remove 'all bacteria'. Interestingly, microbiological analyses on the conventional well water normally and legally used for irrigation showed much higher average concentrations of TC and FC than those measured in the membrane-filtered wastewater. The concentrations of *E. coli* were only measured during period 4 and were found to be comparable to those observed in the permeate (10 CFU/100 ml on average).

Advanced Oxidation Processes and Disinfection

Photocatalytic and membrane processes are strong candidates for improving conventional water treatment processes. These advanced treatment methods are capable of removing many pollutant chemicals as well as pathogenic microorganisms. The performance of small-scale UV/TiO_2 photocatalytic pilot plant process in treating secondary and tertiary treated effluent from a wastewater treatment plant in Bahrain was investigated. The performance was evaluated in terms of the efficiency of removing a chlorine-resistant parasite, namely, *Strongyloides stercolaris*, in addition to reducing the chemical oxygen demand (COD). The effect of pH and exposure time was considered. The performance of UV/TiO_2 was compared with small pilot-scale UF and RO membrane processes. Treatment of the samples with each of the UV/TiO_2, UF, and RO processes resulted in a complete removal of *S. stercolaris* parasites. Moreover, the COD was reduced by about 50%, 64%, and 86% with each of UV/TiO_2, UF, and RO. The specific power consumption of the UV/TiO_2 process was estimated to be about 4.0 kWh/m^3 for each of UV/TiO2 and seawater RO and 1.0 and 0.9 kWh/m^3 for each of brackish water RO and cross flow UF, respectively. The UV/TiO_2 process generates no waste stream, whereas the RO and UF processes generate waste streams, which are concentrated in the microorganisms, and suspended organic matter, which result in a disposal problem.

The experimental results of a pilot-scale (100 mVh) investigation, carried out at the West Bari (Southern Italy) municipal wastewater treatment plant, focused on parasite removal and DBP formation during the UV disinfection of clarified (CL) and clarified-filtered (F) secondary municipal effluents. The investigation demonstrated that parasites like *G. lamblia* cysts and *C. parvum* oocysts were both significantly affected by UV radiation and that potential UV-promoted formation of DBPs (nitro-phenols and N-nitroso-amines) did not occur according to GC/MS and LC/MS analytical evidences. Operational and maintenance costs ranged from ~17.5 up to euros 35/1000 m^3 for effluent F and CL, respectively.

Removal of bacteria by ozonation in combination with charcoal or slow sand filtration was compared with that by flocculation filtration in pilot scale at the sewage treatment plant Eriskirch, Baden-Wuerttembergm, Germany (Lüddeke et al., 2015). Influent and effluent of different treatment processes were analyzed in a culture-based approach for its content of *Escherichia coli*, enterococci and staphylococci and their resistance against selected antibiotics over a period of 17 months. Ozonation followed by a filter passage led to an additional reduction of total and antibiotic resistant *E. coli*, enterococci and staphylococci between 0.8 and 1.1 log-units as compared to the respective concentrations in treated sewage by only flocculation filtration. However, ozonisation led to an increased percentage of antibiotic resistant *E. coli* (16%) and staphylococci-isolates (5.5%) and a decrease of the resistance-level of enterococci (25.4%). Overall, advanced wastewater treatment by ozonation plus charcoal or sand filtration after common sewage treatment is an effective tool for further elimination of microorganisms from sewage before discharge in surface waters (Lüddeke et al., 2015).

Conclusions

It is clear that municipal wastewater harbors a variety of pathogenic organisms. A number of technologies are available today to treat wastewater for different intended uses. Treatment technologies for wastewater have been continuously improving due to the stringent limits set, including microbial control, and also due to reuse practices because of the increasing water scarcity in a number of regions of the world. It is necessary to continuously monitor the microbial quality of treated wastewater in order to ensure that the treatment objectives are achieved. Generally a combination of different processes only would ensure complete removal of pathogens from wastewater.

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Mycotoxins☆

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Glossary

Alkaloid Any of various natural bases derived from amino acids that contain one or more heterocyclic nitrogen atoms.
Mycosis (plural: mycoses) A mycosis is a disease produced by pathogenic invasion of a fungus on an animal host.
Mycotoxicosis (plural: mycotoxicoses) A mycotoxicosis is an animal disease produced by exposure to mycotoxins. Eating contaminated foods and feeds is the most common cause of mycotoxicosis; however, some mycotoxins cause disease upon dermal contact or inhalation.
Mycotoxin A low molecular weight natural product, produced by a filamentous fungus that is harmful to animals in low concentration. Curiously, toxic metabolites made by mushrooms and other macroscopic fungi usually are classified separately as 'mushroom poisons'.
Nonribosomal peptide A short peptide that is synthesized by a nonribosomal method, that is, peptide formation occurs in the absence of the usual translational machinery by nonribosomal peptide synthases. They often contain unusual amino acids.
Polyketide Any of a diverse group of natural products synthesized via linear poly-β-ketones, which are themselves formed by repetitive head-to-tail addition of acetyl or substituted acetyl units indirectly derived from acetate. The mechanism is similar to that for fatty acid biosynthesis but without the intermediate reductive steps.
Sclerotium (plural: sclerotia) A firm, compact mass of hardened mycelium with no spores in or on it. The sclerotia of *Claviceps purpurea* contain ergot alkaloids.
Secondary metabolite A small molecule usually synthesized after active growth has ceased by pathways not part of primary metabolism. Many secondary metabolites have strong biological activity.

Abbreviations

FAO Food and Agriculture Organization of the United Nations
IUPAC International Union for Pure and Applied Chemistry

Defining Statement

Mycotoxins are low molecular weight natural products produced by molds that are toxic to vertebrates in low concentrations. In most cases, mycotoxins are of limited taxonomic distribution, that is, they are made by only a few species within certain fungal genera. Animals are exposed to mycotoxins through ingestion, inhalation, and/or skin contact. The ensuing diseases produced by mycotoxin exposure are called mycotoxicoses; the vast majority of characterized mycotoxicoses are due to dietary exposure. Many mycotoxins are potent in low doses, that is, quite small amounts of these compounds can represent significant health effects. Since filamentous fungi are common and opportunistic organisms, mycotoxins are widespread. These toxins contaminate foods and feeds across the globe and are of increasing concern as possible contaminants of indoor environments. Some mycotoxins have been implicated as chemical warfare agents. As with all toxins, the route of exposure (i.e., how the substance enters the animal body), the dose (how much of the substance is present), and the duration (the length of time the mycotoxin is present) interact to affect the severity of the outcome. Mycotoxins are implicated in certain cancers as well as many different disorders affecting the gastrointestinal, urogenital, vascular, renal, respiratory and nervous systems. Some mycotoxins act as immunosuppressants, thereby reducing resistance to infectious disease. It is estimated that 25% of the world's crops, including many basic foods, are contaminated by mycotoxin-producing fungi. Although as yet unproven, there also is increasing concern that mycotoxins may be implicated in the adverse health effects associated with exposure to mold growth in damp indoor environments.

There is an ongoing need to protect the health of humans and susceptible animals by limiting their exposure to mycotoxins. Despite many years of research and the introduction of good practices in the chain of food production, storage, and distribution, mycotoxins continue to be a global problem. Many countries regulate mycotoxin levels in foods and feeds because of their public

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health significance and commercial impact. In recent years, certain internationally focused groups such as the Gates Foundation have increased support for research on mycotoxin control in Africa and other highly impacted parts of the world.

Poisons and Toxins

The vocabulary surrounding 'poison' and 'toxin' is not uniform. Some experts use the words synonymously, while others make a distinction. For example, some purists say poison describes any harmful substance, while toxins are a subset of poisons made by living systems. Many zoologists make a further distinction and limit the use of toxin to proteinaceous poisons such as snake venom. When used in adjective form, the distinctions blur. Both poisonous and toxic materials encompass all substances that can be injurious to living systems (e.g., the toxic mustard gases of chemical warfare and the poisonous darts of certain South American tribes). Both 'toxin' and 'poison' imply a concept of concentration – they must be present in sufficient quantity to cause damage, illness, or death.

The vocabulary used to describe the toxic metabolites of fungi is especially peculiar in that the toxic metabolites produced by macrofungi-like mushrooms and shelf fungi are usually called mushroom *poisons* while the toxic metabolites produced by microfungi (molds and mildews) are called myco*toxins*. The technical name for mushroom poisoning is 'mycetismus' while the technical name for mycotoxin poisoning is 'mycotoxicosis'.

In general, mycotoxins are not proteinaceous but, rather, one of a huge array of low molecular weight natural products. They are categorized together simply because they all are produced by molds and are all toxic to vertebrates in low concentrations. In most cases, individual mycotoxins are made by only a few species or genera of filamentous fungi. For example, aflatoxin production is limited to a few species of the genus *Aspergillus* while the trichothecenes are mostly produced by species within the genus *Fusarium*. Like all secondary metabolites, mycotoxins share the enigmatic property of cellular dispensability meaning that mycotoxigenic species can regularly grow and thrive, at least in the laboratory, without producing toxic secondary metabolites.

A Primer in Toxicology

One of the most famous statements in toxicology, attributed to Paracelsus, states "Everything is poison, there is poison in everything. Only the dose makes a thing not a poison." This concept is often stated as an aphorism: The dose makes the poison. In mycotoxicology, as in other branches of toxicology, the degree of harm caused by a mycotoxin will depend not only on an interaction between the nature and concentration of the chemical compound, but also on the metabolic status of the target organism, the route of exposure, and the potential for metabolic detoxification. Animals vary widely in their responses to a given mycotoxin dose based on age, sex, nutritional and gestational status, as well as their ability to detoxify. In general, the greater the amount of toxin exposure, the more severe an effect it will have. Since some mycotoxins are far more biologically active than others, there is a different threshold level (the lowest concentration that produces a harmful effect) for each compound. Further complicating the picture is that the threshold level may vary among animal species and even among individuals within a species population. Often the young are more sensitive to mycotoxin exposure than adult animals. Characteristics such as diet, pregnancy, general state of health, and drug interactions also impact mycotoxin sensitivity. Finally, illnesses that involve organs for detoxification, such as hepatitis, can increase the sensitivity of an organism to toxin exposure. Repeated exposure to low quantities of a mycotoxin over long periods of time also may cause serious harm. Thus, in assessing the health burdens associated with mycotoxins, it is important to distinguish between acute and chronic conditions. Acute reactions occur shortly after exposure to a high concentration of a given mycotoxin. Chronic conditions follow long exposure to low doses. It is almost always easier to discern the connection between exposure and disease for acute conditions than for chronic ones, especially when the outcome of chronic exposure is something like a subtle change in reproductive behavior or fertility.

Unlike the environmental toxins, where the most common mode of entry is inhalation or dermal contact, the most common mode of entry for mycotoxins is inadvertent ingestion from a contaminated food supply. Many mycotoxins are tasteless, so consumers are unaware that they are ingesting contaminated food. Even when mold growth is apparent, starving people will resort to eating infected commodities because the alternative choice is no food at all. Historically, the first recognized human mycotoxicoses are associated with the ergot alkaloid made by fungi in the genus *Claviceps* that infect grains such as rye. These ergot compounds are found in distinctive morphological structures called sclerotia and have both vasoconstrictive and neurological effects. The famous hallucinogen LSD is a semisynthetic ergot derivative. Outbreaks of human ergotism have been documented since medieval times. In addition, the historical veterinary literature is littered with reports of ill-defined outbreaks of animal toxicity in cattle, horses, poultry, and swine associated with feeding with moldy grains or bedding with moldy straw.

A Bewildering Diversity of Substrates, Mycotoxin-Producing Fungi, and Physiological Effects

The scientific literature surrounding mycotoxins is enormous and is published in a variety of disciplines from chemistry and mycology to molecular biology and environmental toxicology. The emphasis in the veterinary literature is different from that in the literature of human biology, which in turn differs from studies in analytical chemistry, classical mycology, or molecular genetics. For more detailed treatments, the reader is referred to the list of references given at the end of this article.

Because of the opportunistic nature of fungi, there is almost no foodstuff that has not, at one time or another, been found to be contaminated with a mycotoxin. They have been reported on cereals, dried seafood products, fruits, legumes, nuts, oil seeds, and spices. When farm animals eat mycotoxin-contaminated food, derivative mycotoxin residues can be found in meat and milk. Thus, aflatoxin M_1, a biotransformation product of the mold product aflatoxin B_1, has been found in sources as diverse as ice cream, yogurt, and human breast milk.

The Food and Agriculture Organization of the United Nations (FAO) estimates that global foodstuff losses due to mycotoxins are in the range of 10^9 tons per year. The incidence of contamination varies with crop, preharvest stress factors such as drought and insect damage, and weather at the time of harvest. Once harvested, almost all foods can become susceptible to mold contamination at some stage during transport, storage, and/or processing. Finally, mycotoxin contamination can occur in the home after cooking, especially when foods are not properly stored. This is a particular problem in underdeveloped regions of the world where refrigeration is lacking. Moisture is the single most important variable. Fungi need sufficient water and warmth in order to grow and synthesize secondary metabolites; therefore, harvested foodstuffs should be stored in cool and dry conditions.

Each mycotoxin group exerts its toxic effects by a different mechanism. For example, aflatoxins intercalate with DNA, fumonisin is an inhibitor of sphingolipid biosynthesis, and trichothecenes interfere with protein synthesis. For each mycotoxin, the symptoms induced will vary with the pharmacological action of the toxin, but will also depend on the target organism susceptibility and toxin concentration. The best-studied mycotoxins include aflatoxins, ergot alkaloids, fumonisins, ochratoxins, patulins, and the trichothecenes.

The disease states associated with mycotoxins are named rather haphazardly. Sometimes vague diagnoses such as 'aflatoxicosis', 'stachybotrycosis', or 'tremorgen intoxication' are given, and other times the pathologies are named after the general condition (e.g., liver cancer) or the specific symptom (e.g., convulsions). Some diseases, such as Balkan endemic nephropathy, have been attributed to mycotoxins based on correlative evidence, but this etiologywas disproven. Balkan nephropathy is due to a plant toxin called aristolochic acid that sometimes contaminates bread. Finally, certain obscure diseases have been postulated to be mycotoxin-based without any concrete evidence. For example, Onyalai is a rather mysterious disease entity encountered in central southern Africa that has been attributed to fungal-contaminated millet, sorghum, and/or maize. Nevertheless, even for well-studied mycotoxins, exposure and occurrence data are limited, so it is possible that world health officials are underestimating the general toxicological burden posed by these natural products while simultaneously being too quick to attribute rare disease entities to them.

The major mycotoxins are listed in Table 1, categorized by the fungal genera producing them. Important genera of mycotoxin-producing fungi include *Alternaria*, *Aspergillus*, *Chaetomium*, *Claviceps*, *Fusarium*, *Myrothecium*, *Penicillium*, and *Stachybotrys* as well as their teleomorphs (i.e., sexual stages). Because mold taxonomy is a challenging scientific enterprise, the literature is filled with erroneous reports or misidentifications of mycotoxin-producing fungi. An excellent compendium that lists correct species identifications, and documents the more egregious cases of misidentification, was published by Frisvad and colleagues in 2007.

In summary, mycotoxins – collectively – are a worldwide problem, although individual mycotoxins are most likely to be encountered in specific habitats, on specific foodstuffs, and under specific weather conditions. In general, mycotoxin contamination is worst among agricultural commodities kept under poor storage conditions with high temperature and high humidity. Aflatoxin is usually considered the most important mycotoxin, not only because aflatoxins are potent in extremely low concentrations but also because the producing molds colonize such a wide range of substrates across an equally wide range of habitats. Most experts believe that aflatoxin is a major factor contributing to the high rates of liver cancer in some parts of Africa and Southeast Asia.

Related Topics in Mycotoxicology

Zearalenone, a *Fusarium* polyketide metabolite, deserves special attention. This compound, often classified as a mycotoxin, is also called F-2. Zearalenone resembles estradiol and binds to estrogen receptors in mammals where doses as low as 1 $\mu g\ kg^{-1}$ may create a detectable uterine response in female swine. On the other hand, the lethal dose of zearalenone in guinea pigs is 5000 $mg\ kg^{-1}$. Accordingly, while biologically potent, zearalenone is not toxic in the usual sense of the term. This compound is better classified as an endocrine disrupter than as a mycotoxin, but because of the pathological estrogenic syndrome it causes in swine, zearalenone traditionally has been lumped together with other veterinary problems caused by toxic fungal metabolites. Chemical derivatives of zearalenone have found application as growth promoters in cattle, especially in the United States; their use has been banned in the European Union.

In recent years, mycotoxins have been implicated in the cause of an ill-defined syndrome called 'sick building syndrome' or 'damp building syndrome'. In the United States, popular fear of 'toxic molds in the environment' has been fueled by a sensationalist press and through unscrupulous insurance claims. Although macrocyclic trichothecenes have been demonstrated in the sera of individuals who had been exposed to *Stachybotrys chartarum* in indoor environments there is only circumstantial evidence linking molds and mycotoxins and their myriad symptoms to sick building syndrome. Documented adverse health effects of molds in indoor environments are largely associated with allergies to fungal spores, asthma and upper respiratory irritation. A great deal more research is needed about the possible health implications of mold growth on damp building materials and whether or not mycotoxins are contributing to the symptomology associated with the adverse health effects associated with time spent in damp indoor environments.

Table 1 Examples of major mycotoxins

Producing genus and name of compound	*Toxigenic effect*
Alternaria toxins	
Tenuazonic acid	Protein synthesis inhibitor
AA-toxins	Sphingolipid metabolism inhibitor
Aspergillus species	
Aflatoxins	Carcinogen, mutagen, teratogen
Cyclopiazonic acid[a]	Calcium transport disrupter
Gliotoxin	Immunosuppressant
Ochratoxin[a]	Nephrotoxin
Sterigmatocystin	Carcinogen, mutagen
Verruculogen	Tremorgen
Claviceps toxins	
Ergot alkaloids[a]	Vasoconstrictive agents, neurotoxins
Penitrem[a]	Neurotoxin
Fusarium toxins	
Butennolide	Fescue foot disease in cattle
Fumonisin	Sphingolipid metabolism inhibitor
Moniliformin	Chromosome damage, heart failure
Trichothecenes (over 200 have been identified)	Many suppress protein synthesis
Deoxynivalenol (n)	Emetic agent
Diacetoxyscirpenol	Hemorrhagic agent
Nivalenol	Hemotoxin
T-2	Immunosuppressant
Penicillium toxins	
Citreoviridin	Inhibitor of ATPase
Citrinin	Nephrotoxin, hepatotoxin, fetotoxin
Mycophenolic acid	Immunosuppressant
Patulin[a]	Immunosuppressant, neurotoxin
Penicillic acid	Nephrotoxin
Penitrem A	Tremorgenin
Rubratoxin	Hepatotoxin
Secalonic acid	Cleft palate induction in mice
Xanthomegnin	Hepatotoxin, nephrotoxin
Stachybotrys toxins	
Atranone	Inflammatory agent
Satratoxin	Protein synthesis inhibitor

[a]Also produced by other genera.

Finally, mycotoxins have also been implicated as chemical warfare agents. Their use in Yellow Rain during the Cambodian War has been largely discredited. However, there is strong evidence that Saddam Hussein stockpiled aflatoxin for use in missiles, even though aflatoxin is a poor agent for chemical warfare.

Biosynthesis, Molecular Biology, and Genomics

Fungi are known to be inventive chemists, so it is not surprising that mycotoxins encompass many chemically unrelated fungal metabolites that are synthesized by several different secondary metabolite biosynthetic pathways. Most known mycotoxins are polyketides, nonribosomal peptides, or derived from isoprene pathways. Often the chemical structures contain elaborate cyclic or branched cyclic architecture (Figures 1 and 2). Aflatoxin, alternariol, citreoviridin, citrinin, ochratoxin, patulin, penicillic acid, secalonic acid, sterigmatocystin, and xanthomegnin are all examples of polyketide mycotoxins. Gliotoxin is a nonribosomal peptide, while the penitrems and trichothecenes are isoprene-derived. Many mycotoxins; for example, cyclopiazonic acid, are formed from a combination of more than one pathway.

The biochemical, genetic and molecular characterization of mycotoxin biosynthesis has been an active field for basic research. In early research, the aflatoxin, sterigmatocystin, and trichothecene pathways were elucidated using isotopically labeled precursors, blocked mutants and gene cloning. The genes of the aflatoxin and sterigmatocystin pathways are chromosomally clustered ('linked'), allowing common regulatory elements to be shared. Gene clustering may influence gene expression and regulation through modulation of localized chromatin structure. In addition, recent advances in genomics have made it possible to detect the genetic signatures of polyketide synthases, nonribosomal peptides synthases, and other secondary metabolite-associated genes as

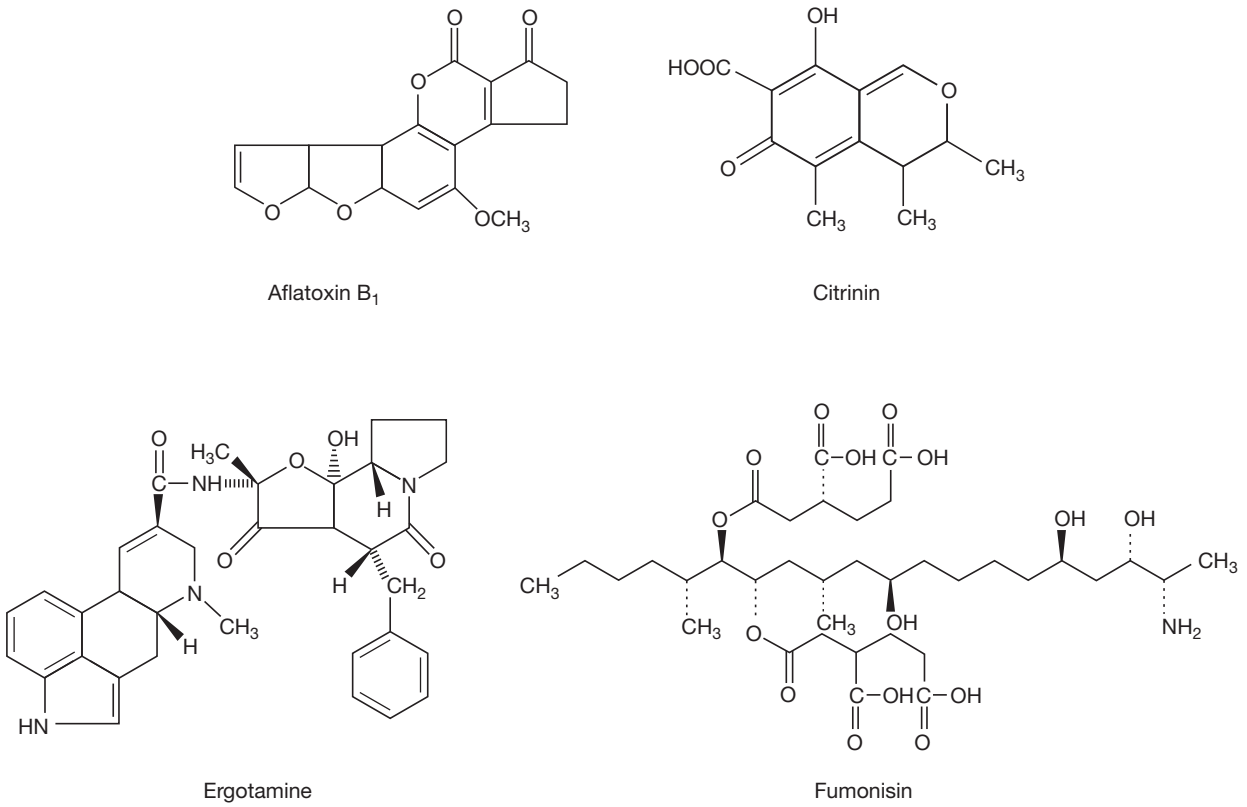

Figure 1 Chemical structures of aflatoxin B_1, citrinin, ergotamine, and fumonisin.

well as their associated gene clusters. Genomic, proteomic, and metabolomic advances have revolutionized the study of mycotoxins and other secondary metabolites. High throughput genomic technologies have increased our understanding of gene function, genetic regulation, signal transduction and the response of mycotoxigenic fungi to environmental stimuli. Nevertheless, these advances have not yet been translated into cognate advances in mycotoxin control.

Another important development in mycotoxin research involves the discovery of sexual stages in the life cycles of several fungi previously thought to reproduce only asexually (clonally). Additionally, genetic recombination within and among mycotoxigenic fungal species can result in the creation of novel chemotype profiles and inhibit their control. These discoveries open the way to conduct genetic research on gene regulation and to create recombinant strains that may be useful in mycotoxin control strategies.

Mycotoxin Detection, Prevention, and Regulation

The detection of mycotoxins is crucial for food safety and is of growing concern for air quality measurements in indoor environments. Regulatory limits, sometimes stated as a 'tolerable daily intake', are based on extrapolations from animal models and on human epidemiological data.

Several international agencies such as The European Union, the Institute of Public Health in Japan, and the U.S. Food and Drug Administration have set recommended guidelines, sample products for detection and measurement of mycotoxins, and currently maintain websites that are good sources for information on mycotoxins. The following are good mycotoxin resources: The Council for Agricultural Science and Technology, the American Oil Chemists Society Technical Committee on Mycotoxins, the International Union for Pure and Applied Chemistry (IUPAC) Section on Mycotoxins and Phycotoxins, and the U.S. Food and Drug Administration Committee on Additives and Contaminants.

A compendium summarizing international regulations on mycotoxins is available from the FAO. IUPAC has a Mycotoxin Working Group; publishes a newsletter; sponsors proficiency testing, collaborative trials, and workshops on quality assurance for mycotoxin testing laboratories; and posts regular listing of worldwide mycotoxin regulations. Most of these resources are easily accessed via the internet. In addition, print versions have been published such as The *World Mycotoxin Journal* which is available from Wageningen Academic Publishers in The Netherlands.

Mycotoxin analysis involves sampling, extraction, clean up and separation, detection, and quantitation. Sample preparation often remains a stumbling block because the methods used for the separation of toxins from the other compounds in the sample will depend on the nature of the sample as well as on the nature of the toxin. Thus, extracting and quantifying aflatoxin from corn is

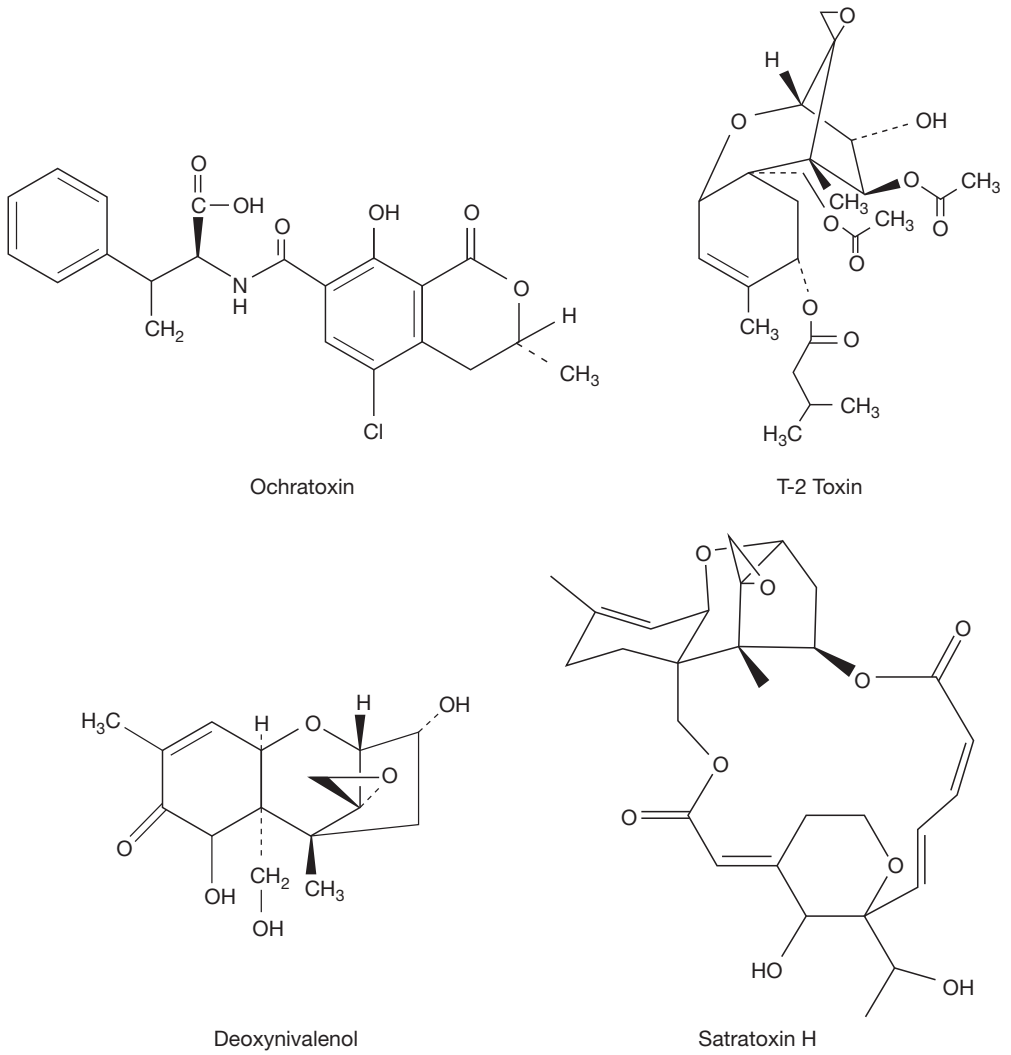

Figure 2 Chemical structures of ochratoxin, T-2 toxin, deoxynivalenol, and satratoxin H.

different from the same analysis involving human blood. The time between sampling and analysis is also critical because samples held in environmental conditions conducive to mold growth could easily produce mycotoxins within 1–2 days of sampling. The major methods used for separation are high performance liquid chromatography and thin layer chromatography. A variety of immunological mycotoxin assay kits are available commercially.

The main challenge in mycotoxin control is prevention. If the global climate is indeed warming, then there is the potential for increased incidences of mycotoxin contamination in agricultural commodities through the combined stress to plant hosts and favorable conditions for fungal infection. Although it is not reasonable to expect a mycotoxin-free food supply, many practices in tillage, handling, and storage can minimize contamination. More importantly, a number of international agencies support the education of farmers, especially in the developing world. In the case of aflatoxin control, non-aflatoxigenic *Aspergillus flavus* strains have shown promise as biocontrol against infection and contamination by aflatoxigenic fungi.

Conclusion

Significant economic losses are associated with mycotoxins due to their impact on human health and animal productivity. Apart from the bacterial, protozoan, and viral agents that cause 'food poisoning', world health authorities consider mycotoxins to be the most important human chronic dietary risk factor, higher than food additives, pesticide residues, or synthetic contaminants. Mycotoxin effects may be exacerbated by poor nutrition and health, so their toll is heaviest in regions of the world where malnutrition is prevalent and where the inhabitants are immunocompromised. Their burden in animal husbandry is even greater than that in human health because mold-damaged foodstuffs are often diverted into animal feed. Chronic mycotoxin exposure in

animal feeds leads to reduced growth rates, lower feed efficiency, infertility, and a general lowering of productivity. Although most of the emphasis in mycotoxin research has been in agriculture and veterinary medicine, mycotoxins are of great importance to many other areas of basic and applied research, such as cancer toxicology, food science, evolutionary biology, fungal molecular biology, and organic chemistry.

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Nanoarchaeota

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Diversity and Exploration of the Archaeal Domain

Karl Woese's 1977 discovery of the archaeal domain dramatically shifted and expanded our view of the universal tree of life. Prior to this discovery, the Archaea or "Archaebacteria" were thought to represent divergent members of the Bacteria. However, Woese's 16S ribosomal RNA (rRNA) gene trees revealed that the Archaea were very unique on the molecular level, often more similar to their eukaryotic relatives than to the Bacteria. Because of the small number of cultivated archaeal lineages, the Archaea were thought to be exclusively extremophilic or methanogenic, limited to a few specific niches across the planet. The archaeal domain was initially divided into two main kingdoms, which are now called phyla: the thermophilic Crenarchaeota and the Euryarchaeota, comprised of methanogens, halophiles, thermophiles and sulfate reducers.

Exploration into the third domain of life quickly began to reveal the true extent of archaeal diversity, both on the phylogenetic and physiological level. A third archaeal phylum, the Korarchaeota, was discovered in the early 1990s based on 16S rRNA gene sequences. Like the Crenarchaeota, the Korarchaeota are a thermophilic lineage, and were detected in a geothermal spring in Yellowstone National Park (YNP). However, korarchaeotal 16S rRNA genes sequences branched deep within the archaeal tree of life, prior to the split between Euryarchaeota and Crenarchaeota. The Korarchaeota represented the first phylum-level division in the Archaea to be proposed without a single cultivated representative, setting a precedent for many later-described lineages. Around the same time period, archaeal 16S rRNA gene sequences from marine environments were also amplified and sequenced, and a cold-loving archaeal lineage was found associated with a marine sponge, revealing that the Archaea were not solely limited to methanogenic and extremophilic lifestyles. Hints regarding the physiology of these moderate archaeal lineages came from a fosmid clone obtained from a soil sample, which contained both archaeal rRNA and genes typically involved in ammonia oxidation. Shortly thereafter, ammonia oxidation was demonstrated in a cultivated isolate. Although initially identified as cold Crenarchaeota, these temperate taxa were found to be widely distributed in marine and terrestrial environments and were later proposed to represent a novel mesophilic phylum, the Thaumarchaeota. The unique ability of some Thaumarchaeota to oxidize ammonia suggested that Archaea may play major roles in global biogeochemical cycling that were previously overlooked.

Yet another archaeal phylum, the Nanoarchaeota, was proposed in the early 2000s, once again expanding our understanding of archaeal diversity and physiology. These ultra-small Archaea were first identified in anaerobic enrichment cultures, where they were seen attached to the outer surface of hyperthermophilic Crenarchaeota. Sequencing revealed that *Nanoarchaeum equitans,* the first identified nanoarchaeote, harbored a highly unique 16S rRNA gene. This gene could not be amplified using traditional archaeal 16S rRNA gene primers, explaining why previous diversity studies failed to detect the lineage. Direct cultivation studies showed that *N. equitans* required direct cell-to-cell contact with its crenarchaeotal host for survival, although the host did not need any nanoarchaeotes. Whole genome sequencing also revealed that *N. equitans* contained a small, reduced genome with few biosynthetic genes, consistent with its host-dependent lifestyle. The Nanoarchaeota-host system became the first described interspecies symbiosis between two members of the Archaea, once again demonstrating the diversity and functional flexibility of the archaeal domain.

Since the identification of these first archaeal phyla, metagenomic and single-cell sequencing techniques have revolutionized the study of microbiology, leading to the proposal of many new archaeal phyla and higher-order divisions. Today, the Archaea are understood to be ubiquitous, thriving in both extreme and temperate environments across the globe. Key lineages of abundant, widely distributed Archaea have been discovered in subsurface and aquatic environments, contributing significantly to biogeochemical cycling, while other lineages of divergent Archaea have provided insights into the relationship between the archaeal and eukaryotic domains. As genetic material has continued to increase, the archaeal tree of life has also undergone significant restructuring, with several higher-order groupings proposed as a means to categorize major archaeal phyla.

In the time since the discovery of the Nanoarchaeota, several other archaeal lineages with reduced genomes have been identified, largely by metagenomics and single-cell genome sequencing. These taxa have been grouped with the Nanoarchaeota to form the DPANN superphylum, based on their tendency to cluster together in phylogenetic analyses. The DPANN superphylum was originally named for five key lineages (the Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanohaloarchaeota, and Nanoarchaeota) but now includes several additional phylum-level designations. Although they harbor relatively small genomes and often

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have small cell sizes, members of the DPANN are highly diverse, and DPANN sequences have been recovered from a wide range of environments, including hypersaline habitats, groundwater, alpine lakes and acid mine drainage. Although some Archaea in the DPANN superphylum are thought to be free-living, no organism from the group has been successfully isolated in axenic culture. Genomic evidence also suggests that particular DPANN lineages may rely on a host or partner for survival, like the Nanoarchaeota. *In situ* imaging and enrichment cultivation have revealed intimate associations between different members of the DPANN and Euryarchaeota, suggesting that interspecies dependence may be a hallmark of some taxa in this group.

Despite the recovery of many DPANN genome sequences, the placement of the entire superphylum in the archaeal tree of life, and the Nanoarchaeota in particular, remains unclear. It also continues to be debated whether or not the DPANN superphylum truly represents a monophyletic group. Nonetheless, the discovery of diverse DPANN taxa suggests that the host-dependent lifestyle of the Nanoarchaeota may be widely distributed across multiple archaeal lineages. As the first characterized symbiosis between two Archaea, the Nanoarchaeota and their hosts greatly expand our understanding of the nature and evolution of interspecies interaction in the third domain of life, and they may provide valuable insights into the adaptations that allow diverse, small archaeal lineages to thrive across the globe.

Global Distribution of Nanoarchaeotes

The first cultivated nanoarchaeote, *N. equitans*, was discovered in enrichment cultures from Kolbeinsey Ridge, a shallow marine hydrothermal vent site near the coast of Iceland. However, with the development of nanoarchaeotal-specific primers, sequences related to *N. equitans* were soon identified in geothermal sites around the world, including hot springs in YNP, New Zealand, Chile, Kamchatka and central Asia (Fig. 1). 16S rRNA gene diversity studies also identified nanoarchaeotal-like sequences in lower temperature environments, including hypersaline ponds and the photic zone of Yellowstone Lake. While it is possible that Nanoarchaeota survive in lower temperature environments, the sequences identified in the photic zone of Yellowstone Lake may also represent cells that have drifted away from geothermal vent sites on the lake floor. In either case, however, it appears that Nanoarchaeota predominantly inhabit high-temperature habitats. Nanoarchaeotal genomic signatures have also been identified at deep-sea hydrothermal vent sites along the Eastern Lau Spreading Center, Mid-Atlantic Ridge, Guaymas Basin and East Pacific Rise. A 16S rRNA gene sequence study at East Pacific Rise demonstrated that Nanoarchaeota and their hosts may be among the first organisms to colonize young deep-sea hydrothermal vent deposits, suggesting that Nanoarchaeota-host pairings may play a key role in the ecology of high-temperature hydrothermal systems. This study also recovered nanoarchaeotal sequences both in the presence and absence of *Ignicoccus*-like sequences, providing the first hint that nanoarchaeotes may associate with diverse hosts.

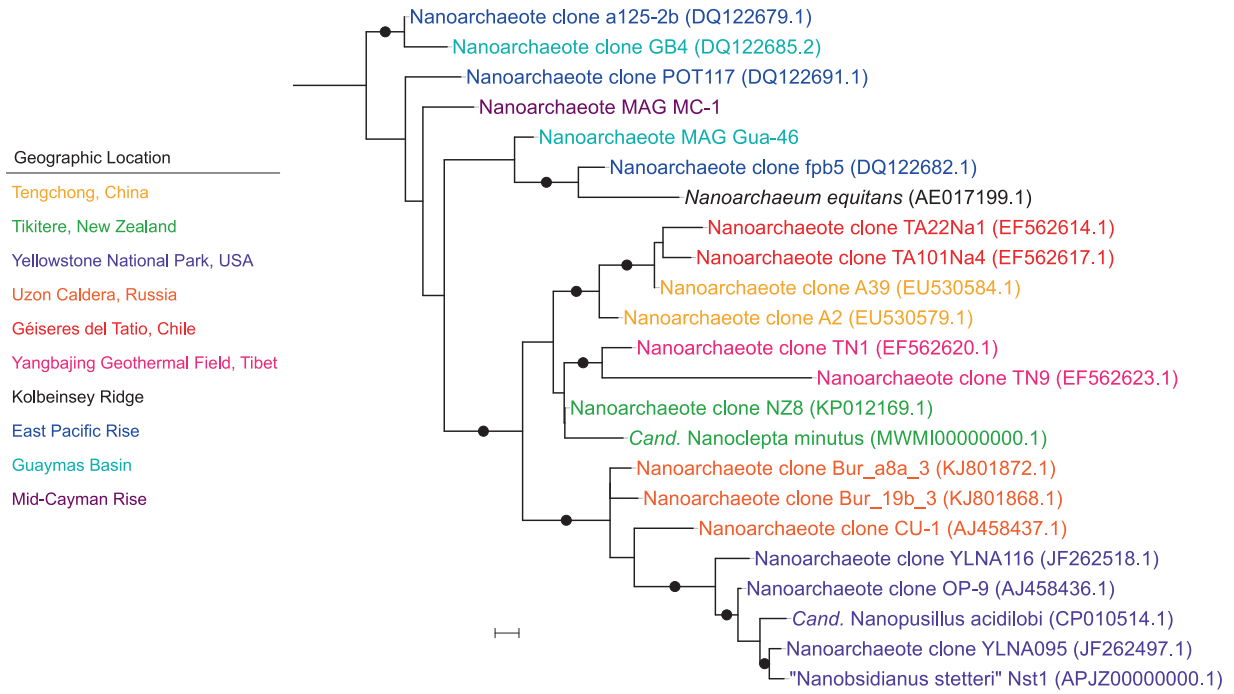

Fig. 1 Maximum-likelihood phylogenetic tree of nanoarchaeotal 16S rRNA gene sequences, inferred under the GTRGAMMA model. The tree was rooted with *Desulfurococcus mucosus* O7/1^T, *Staphylothermus marinus* F1^T and *Ignisphaera aggregans* AQ1.S1^T. Bootstrap support (based on 1000 replicate trees) is shown where 80% or greater. The scale bar indicates 0.01 nucleic acid substitutions per site. Nanoarchaeote sequences are colored based on geographical location.

Diversity Within Nanoarchaeote-Host Systems

Despite their broad distribution across geothermal environments, only three Nanoarchaeota-host systems have been successfully cultivated in the laboratory setting. Cultivated nanoarchaeotes vary in size, but they have consistently small cell diameters (~100–400 nm), and each nanoarchaeote relies on a distinct host from the Crenarchaeota (Table 1). The three cultivated nanoarchaeote-host pairings all appear to be specific, although the crenarchaeotal hosts are highly diverse.

N. equitans, the first described nanoarchaeote, associates with *Ignicoccus hospitalis*, a hyperthermophilic Crenarchaeota with a highly streamlined genome. *I. hospitalis* fixes carbon using the efficient dicarboxylate/4-hydroxybutyrate cycle and obtains energy via sulfur reduction. Members of the *Ignicoccus* are relatively unique among the Archaea due to the extensive double membrane surrounding their cells. *I. hospitalis* has a large periplasmic space between its inner and outer membrane, which often contains vesicles and/or protrusions. Energy generation in *I. hospitalis* takes place across the energized outer membrane, which houses an ATP synthase complex and enzymatic machinery needed to generate a proton gradient. This extensive endomembrane system likely has played a key role in the evolution of the *N. equitans*-*I. hospitalis* interaction.

A second and third cultivated nanoarchaeote-host system were isolated from geothermal springs in YNP and New Zealand. The YNP nanoarchaeote, *Candidatus* Nanopusillus acidilobi, was obtained from Cistern Spring, an acidic geothermal pool. *Cand*. Nps. acidilobi associates with the acidophilic lineage *Acidilobus* sp. 7A. Unlike autotrophic *I. hospitalis*, *Acidilobus* sp. 7A is a heterotroph that ferments protein- and carbohydrate-rich substrates. The New Zealand nanoarchaeote, *Candidatus* Nanoclepta minutus, was cultivated from a circum-neutral hot spring in New Zealand. *Cand*. Ncl. minutus lives on the surface of *Zestosphaera tikiterensis*, the first representative of a novel genus in the Crenarchaeota. *Z. tikiterensis* is also a heterotroph that degrades proteinaceous substrates and requires thiosulfate for growth. In contrast to *I. hospitalis*, neither of the cultivated terrestrial hosts have a double membrane system. Instead, both *Acidilobus* sp. 7A and *Z. tikiterensis* encode for an S-layer, a protein-based lattice that envelops the cell.

Several additional nanoarchaeote-host pairings have also been identified using single-cell genome sequences from hot springs in YNP. Because of the close relationship between the Nanoarchaeota and their hosts, single-cell genome sequencing techniques often recover both nanoarchaeotal and host DNA, providing a method of assessing host diversity. Putative nanoarchaeote hosts from YNP span several genera and include lineages related to the *Sulfolobales*, *Metallosphaera*, *Caldivirga*, *Thermocladium* and *Vulcanisaeta*. Despite this diverse array of hosts, however, single-cell amplified nanoarchaeotes show high 16S rRNA gene sequence similarity (>95%) to the cultivated lineage *Cand.* Nps. acidilobi, suggesting that YNP Nanoarchaeota may undergo frequent host-switching events. It is currently unclear what may be driving this rapid host diversification. However, both nanoarchaeote and host cell envelope structure may play a crucial role. An analysis of synonymous versus non-synonymous mutations in single-cell amplified YNP nanoarchaeotal genomes suggested that hypothetical cell-surface proteins in the Nanoarchaeota undergo rapid evolution, which may provide the novel mutations needed to facilitate new host associations. Additionally, several of the putative host lineages likely have S-layers, a similarity that may allow for more frequent host-switching events.

Although no Nanoarchaeota have been cultivated from deep submarine hydrothermal vent environments, three nanoarchaeotal metagenome-assembled genomes (MAGs) have been obtained from deep-sea hydrothermal vents sites across the world. However, without cultivation or single-cell sequencing approaches, it is nearly impossible to determine what host organism(s) associate with these Nanoarchaeota. No *Ignicoccus*-like bins were recovered from any of these deep-sea metagenomes, but one data set contained a bin closely related to the New Zealand host lineage *Zestosphaera*. This suggests that marine nanoarchaeotes may associate with multiple hosts, not just *I. hospitalis*, and some of these host organisms may resemble their terrestrial counterparts on the phylogenetic level.

Table 1 Various features of cultivated nanoarchaeota and their hosts

	N. equitans	*Cand. Nps. acidilobi*	*Cand. Ncl. minutus*
Location	Kolbeinsey Ridge	Cistern Spring, YNP	Cooking Pots, NZ
Geothermal feature	Shallow hydrothermal vent	Geothermal spring	Geothermal spring
Cultivation pH	5.5	3.6	6.0
Cultivation temperature (°C)	85–90	82	80–85
Host	*Ignicoccus hospitalis*	*Acidilobus* sp. 7A	*Zestosphaera tikiterensis*
Host family	Desulfurococcaceae	Acidilobaceae	Desulfurococcaceae
Host metabolism	Autotroph	Heterotroph	Heterotroph
Trans-spliced tRNA	+	−	−
Cis-spliced tRNA	+	+	+
RNase P	−	+	+
ATP synthase	P	−	−
RNase P	−	+	+
Split protein-coding genes	+	+	+
CRISPR-Cas cassette	+	−	+

Note: YNP, Yellowstone National Park; NZ, New Zealand; +, present; −, absent/not detected; P, partial.

Nanoarchaeote-Host Interaction Dynamics

Although all three cultivated nanoarchaeotes have an ectosymbiotic lifestyle, most of our understanding of Nanoarchaeota/host interactions comes from the *N. equitans-I. hospitalis* system, which has been studied for over 15 years and can be cultivated in large volumes using an anaerobic fermenter. Like all cultivated nanoarchaeotes, *N. equitans* largely relies on its host for the production of metabolic building blocks. Labelling studies have demonstrated that *I. hospitalis*-synthesized lipids and amino acids are transferred to *N. equitans*. Likely, this exchange takes place via a pore-like structure that connects *I. hospitalis* and *N. equitans*, where the cytoplasm of host and nanoarchaeote appear to meet. While studies of the *N. equitans-I. hospitalis* system are much more extensive, electron microscopy has also revealed a similar pore-type formation between terrestrial nanoarchaeote *Cand*. Ncl. minutus and its host and membrane stretching at the attachment site of *Cand*. Nps. acidilobi and *Acidilobus* sp. 7A. Due to the differences in host cell envelope, the mechanism of association in these terrestrial nanoarchaeote-host systems may be very different compared to that of *N. equitans* and *I. hospitalis*. Nevertheless, imaging studies suggest an equally intimate attachment between all cultivated Nanoarchaeota, which may involve cytoplasmic bridging.

In the laboratory setting, different nanoarchaeotes appear to have variable effects on their respective hosts. Although *N. equitans* does not significantly affect the growth rate or final population of *I. hospitalis* cells, the growth curves of the two organisms are not synchronized during cultivation. *N. equitans* cells continue dividing exponentially after *I. hospitalis* enters stationary phase, and nanoarchaeotes may eventually outnumber *I. hospitalis* cells by a factor of ten. Also, infection with nanoarchaeotes can hamper or even halt the ability of *I. hospitalis* to divide, indicating a high cost for individual host cells. In contrast, the growth of the YNP nanoarchaeote *Cand*. Nps. acidilobi is synchronized closely with that of its host, *Acidilobus* sp. 7A, with *Acidilobus* cells consistently outnumbering nanoarchaeotes. Growth profiles of pure culture and co-cultured *Acidilobus* sp. 7A also show no appreciable difference. Overall, this may suggest that *Cand*. Nps. acidilobi is less damaging to host growth and survival than *N. equitans*. In the *Cand*. Ncl. minutus-*Z. tikiterensis* system, direct counts of host cells and qPCR-derived counts of nanoarchaeotes also appear to be synchronous, pointing to a system more similar to the YNP nanoarchaeote/host symbiosis. Notably, *Cand*. Ncl. minutus is able to grow to much higher densities in complex enrichment cultures compared to co-culture with *Z. tikiterensis*. Although *Z. tikiterensis* functions as the obligate and primary host for *Cand*. Ncl. minutus, it is possible that other lineages provide nutrients or small molecules that encourage nanoarchaeote growth. To date, all cultivated nanoarchaeote-host pairings appear to be specific, and most cross-infection studies have been largely unsuccessful. Although rare host-switching events have been observed in the laboratory, none of these novel Nanoarchaeota pairings have been maintained. However, this does not rule out the possibility that nanoarchaeotes engage in a variety of interactions with diverse organisms in their natural geothermal habitats, all while maintaining their obligate host associations.

Proteomic data comparing pure *I. hospitalis* cultures and *N. equitans-I. hospitalis* co-cultures has also revealed some of the effects of Nanoarchaeota association on the host. *I. hospitalis* cells show differential changes in transcriptional regulator expression when cultivated with *N. equitans*, suggesting that nanoarchaeote association causes highly controlled, specific changes within the host. Over time, co-cultivation with *N. equitans* also causes increases in expression of host carbon fixation and energy generation genes, likely in response to the increased metabolic demands from the nanoarchaeotes. Stress proteins are shown to be highly expressed in the *I. hospitalis* proteome, although the organism does not appear to mount a significant defensive response to nanoarchaeotal infection. High levels of stress proteins were also detected in the proteome of YNP host *Acidilobus* sp. 7A when co-cultivated with its Nanoarchaeota. However, because no proteomic data from pure *Acidilobus* sp. 7A cultures is available, it is unclear whether or not these proteins are expressed in reaction to nanoarchaeotal infection, or if they represent a constitutive response to environmental stressors. In the future, comparative proteomic studies such as those performed on the *N. equitans-I. hospitalis* system will be pivotal in determining whether or not terrestrial hosts react to their respective nanoarchaeotes in a similar manner.

Shared Genomic Features of Cultivated Nanoarchaeotes

Like many obligate symbionts and parasites, cultivated Nanoarchaeota have reduced genomes, ranging in size from 0.491 (*N. equitans*) to 0.606 Mbp (*Cand*. Nps. acidilobi). Although described Nanoarchaeota have a fairly extensive set of genes used in cell replication, RNA transcription and protein translation, they have lost most biosynthetic capabilities. Without these core functions, Nanoarchaeota rely on host-produced cofactors, nucleotides, lipids and amino acids. Each of the three genomes also encode split protein-coding genes, which are in multiple open reading frames spread across the genome. At least some of these split genes likely maintain their functionality, as demonstrated for the split alanyl-tRNA synthase in *N. equitans*. Although the three cultivated Nanoarchaeota share a common set of split protein-coding genes, several unique split genes are also scattered across the three genomes. Some of these split genes have also been recovered in single-cell nanoarchaeotal genomes from YNP and Nanoarchaeota MAGs from deep-sea hydrothermal vents. These patterns of distribution suggest that the last common ancestor of described nanoarchaeotal lineages already had split protein-coding genes and that genomic fragmentation has continued to occur after these taxa diverged. Because of the fast-evolving nature of the reduced and fragmented Nanoarchaeota genomes, the positioning of the nanoarchaeotal lineage and the DPANN group in the archaeal tree remains unclear. However, split protein-coding genes in the Nanoarchaeota are typically seen as a derived feature, resulting from genome degradation due to host association.

In addition to their lack of biosynthetic genes, cultivated Nanoarchaeota do not have any apparent means of ATP production. Although *N. equitans* encodes a partial ATP synthase, the complex is quite divergent and cannot undergo conformational changes, which are necessary for enzyme functionality. Additionally, no ATP synthase complex genes could be identified in the genomes of

Cand. Nps. acidilobi or *Cand*. Ncl. minutus, suggesting this method of ATP generation is not functional across nanoarchaeotal lineages. The glycolysis pathway, which can produce minimal ATP through substrate-level phosphorylation, is also not complete in any of the three cultivated nanoarchaeotal genomes. One deep-sea Nanoarchaeota MAG contains genes for the pentose bisphosphate pathway, which may provide a way for the nanoarchaeote to produce minimal ATP after circumventing the early steps of glycolysis. However, this pathway is thus far restricted to one nanoarchaeotal MAG, and it is unknown whether or not these enzymes are functional *in vivo*. Without a widely-distributed means of synthesizing ATP, most Nanoarchaeota likely rely on their hosts for ATP production. Co-cultures of *N. equitans* and *I. hospitalis* have been shown to contain less ATP than pure *I. hospitalis* cultures, supporting this hypothesis and suggesting that nanoarchaeotes are utilizing host-produced ATP to fuel their own metabolic processes. Complete and partial dependence on host ATP has been identified previously in obligate intracellular symbionts and parasites, including pathogenic *Chlamydia* and the insect symbiont *Cardinium*. Both of these intracellular organisms contain ATP transporters to import host-produced ATP across the membrane, which have not been identified in the Nanoarchaeota. However, nanoarchaeotes may siphon ATP directly away from their hosts through the pore-like bridge connecting the two organisms.

Despite their small size and highly reduced gene content, the three cultivated Nanoarchaeota have efficient genomes with high coding density. Compared to obligate intracellular Bacteria, the nanoarchaeotes have maintained a higher level of genomic integrity and avoided extensive genome collapse. The three cultivated Nanoarchaeota have also retained their DNA repair mechanisms, which are often lost in endosymbiotic lineages, allowing them to avoid a buildup of deleterious mutations. Also, Nanoarchaeota have the added advantage of exposure to the outside environment, which provides them with avenues for horizontal gene transfer (HGT) from viruses and neighboring cells. Based on genomic analysis of the *N. equitans-I. hospitalis* system, HGT also appears to occur between the host and nanoarchaeotal symbiont, which may help Nanoarchaeota compensate for gene loss events. The differences between nanoarchaeotal genomes and those of obligate endosymbiotic Bacteria may point to the different selection pressures affecting genome size and structure in these diverse environments. In the case of endosymbionts, isolation within the host typically leads to massive gene loss and degradation, with few mechanisms for recovering lost functions. However, genome reduction in the Nanoarchaeota may be a product of both host dependence and the high-temperature geothermal habitat, which tends to select for smaller cell volumes, streamlined genomes and shorter gene variants, all while avoiding the extreme isolation of the intracellular environment.

Unique Features of Various Nanoarchaeotes

Although all cultivated Nanoarchaeota share a similar ectosymbiotic lifestyle, their genomes harbor some key differences. *N. equitans* has the smallest genome of any cultivated Nanoarchaeota and shows more extensive gene loss and reduction compared to its terrestrial relatives, which is especially evident in its RNA systems. *N. equitans* is lacking the RNase P complex, previously thought to be ubiquitous across all life. RNase P performs a crucial step in the generation of mature RNA molecules by trimming the 5′ leader sequence from precursor tRNAs. However, *N. equitans* can function without RNase P due to its shifted tRNA promoter regions, which allow precursor tRNAs to be transcribed without a leader sequence. Both cultivated terrestrial Nanoarchaeota appear to retain the enzyme, and RNase P protein subunits have been identified in multiple nanoarchaeotal MAGs from marine hydrothermal vents, suggesting that RNase P is typically maintained across nanoarchaeotal lineages. The loss of RNase P in *N. equitans* likely reflects the high level of genomic reduction found in this particular Nanoarchaeota.

Trans-encoded tRNA genes in the *N. equitans* genome also support a scenario of extensive genome rearrangement. These split tRNA genes are encoded in multiple pieces, which are fused into functional tRNAs after transcription using a nanoarchaeotal splicing endonuclease. Since their discovery in *N. equitans*, *trans*-spliced tRNA genes have also been identified in several lineages of free-living Crenarchaeota. Potentially, these split tRNA genes represent a defense mechanism used to avoid integration of viral DNA, which targets conserved tRNA gene sequences, or they may be the product of previous insertion events. Alternately, it has also been proposed that these *trans*-encoded tRNA genes represent an ancestral gene form, which carries implications for the evolutionary history of extant tRNA genes. To date, no split tRNA genes have been identified in any nanoarchaeotal lineage outside of *N. equitans*.

Genomic evidence suggests that diverse Nanoarchaeota interact with the abundant viral populations found in geothermal environments. CRISPR-Cas cassettes have been identified in *N. equitans* and *Cand*. Ncl. minutus, and *cas* genes have been discovered in deep-sea nanoarchaeotal MAGs. Integrated prophage DNA has also been found in single-cell amplified nanoarchaeotal genomes from YNP. However, no CRISPR-Cas regions were detected in YNP Nanoarchaeota genomes, suggesting that defense mechanisms against viral infection may not be distributed across all nanoarchaeotal lineages. While viruses may pose a threat to nanoarchaeotal cells, they may also provide a reservoir of genetic material which could serve to replace lost gene functions through HGT.

Compared to *N. equitans*, both terrestrial nanoarchaeotes have somewhat expanded genomes, and both contain genes that are lacking in their marine relative. Most notably, *Cand*. Nps. acidilobi and *Cand*. Ncl. minutus both encode genes for the archaeal flagellum, which are expressed in the proteome of *Cand*. Nps. acidilobi and are visible by electron microscopy in *Cand*. Ncl. minutus. Unlike most Archaea, however, the archaeal flagellar genes are not encoded in an operon, once again pointing to patterns of genome reshuffling in the Nanoarchaeota. Currently, the function of the archaeal flagella is unclear, especially given the apparent inability of cultivated nanoarchaeotes to produce ATP needed to fuel motility. However, it is possible that the Nanoarchaeota may use their archaeal flagella only when attached to their hosts, fueling motility with host-produced ATP. Alternately, the archaeal flagella may have a function in the association between Nanoarchaeota and their hosts. Both terrestrial nanoarchaeotes also encode genes for the gluconeogenic pathway, which are highly expressed in the *Cand*. Nps. acidilobi

proteome. Likely, these enzymes aid in the production of sugars for cell-surface glycosylation. This process may play a key role in the Nanoarchaeota-host association, potentially facilitating the wide diversity of nanoarchaeote-host pairings identified in YNP hot springs. Although archaeal flagella and gluconeogenic genes were not detected in the genome of *N. equitans*, their distribution does not appear to be governed by habitat type alone. Marker genes for gluconeogenesis and archaeal flagella were also identified in nanoarchaeotal MAGs from geographically distant deep-sea hydrothermal vents, suggesting these features may be widely distributed across the Nanoarchaeota. Once again, this pattern suggests that *N. equitans* represents a more highly reduced lineage compared to other described nanoarchaeotes. However, as more Nanoarchaeota-host pairings are identified, additional eco-physiological patterns may emerge that are not yet apparent.

Conclusions

The Nanoarchaeota represent a diverse, widely distributed lineage thriving in geothermal environments around the world. The highly specialized host-dependent lifestyle of the nanoarchaeotes provides unique insights into interspecies relationships across the Archaea. However, the three described Nanoarchaeota-host systems defy easy classification. Although the nanoarchaeotes initially resemble parasites, relying on their hosts for the majority of metabolic products, they cause fewer detrimental effects to the host than expected. Marine and terrestrial Nanoarchaeota also appear to interact with their hosts in different ways, as evidenced by unique growth patterns during cultivation. To date, no positive benefits of Nanoarchaeota infection have been demonstrated in the laboratory setting, although nanoarchaeotal association may provide yet unknown benefits to hosts in their diverse natural communities. Further cultivation and genome sequencing will be crucial to understanding the intricacies of the Nanoarchaeota-host interaction, and the role this symbiotic system plays in the ecology of hot springs and deep-sea hydrothermal vents.

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Nitrogen Assimilation in Bacteria

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Introduction

Nitrogen is a main component of living organisms, accounting for 10%–15% of the dry weight of bacteria, which represents ¼ of their carbon contents. In living matter, nitrogen is present in nucleic acids and proteins, the defining molecules of cells, and in bacteria it is also present in cell walls. Moreover, besides its importance for the organism that conducts the process, biological nitrogen assimilation is a crucial part of the global carbon and nitrogen cycles, and has thus a major impact on climate and planetary composition at global scales.

Nitrogen is found in different molecular forms in all natural environments in our planet, and these forms are interconverted with a massive participation of microorganisms (Fig. 1). In soils, nitrogen is found in organic forms, mainly derived from animal waste and organic matter decomposition, or inorganic compounds, mainly ammonium, although other compounds such as nitrates, nitrites and nitrogenous oxides are also present. The relative composition of nitrogen in land depends very much on the particular environment. In fresh waters the content of nitrogenous compounds is also variable and could be related to that of in-shore lands. In oceans, nitrate, ammonium and urea can reach micromolar concentrations. The main component of the Earth's atmosphere is N_2 (N≡N, 78%), which to a large extent is of biological origin.

The bulk of the biological transformations of nitrogenous compounds in land and water environments take place through the processes of: ammonification, carried out by bacteria and fungi and whereby organic nitrogen is transformed into ammonium; bacterial nitrification, which involves the conversion of ammonium into nitrites and nitrates; nitrate and nitrite assimilation, the intracellular conversion by plants and microorganisms of nitrate and nitrite into ammonium; and the anaerobic processes of bacterial denitrification and anammox, which involves the conversion of nitrate or ammonium, respectively, into N_2 gas, which is liberated to the atmosphere (Fig. 1). Although some transformation of atmospheric N_2 into nitric oxides and nitrate takes place through natural atmospheric phenomena such as lightning and volcanic activity, and into ammonium by industrial processes to produce fertilizers, the main pathway of transformation of N_2 nitrogen is through biological N_2 fixation. This process, which is exclusively conducted by prokaryotes, either free-living or in symbiosis with eukaryotes, represents the main way to introduce N into the biologically utilizable pool (Fig. 1). Thus, it is of primordial importance to maintain the global ecological stasis.

The oxidative processes of nitrification (by nitrifying bacteria) or anammox and the reduction of nitrate by denitrifying bacteria are used for the production of reductant and energy, while the dissimilatory reduction of nitrate and nitrite can be used to generate energy or serve detoxification purposes (e.g., to eliminate excess of reductant or nitrite). In contrast, the assimilatory reduction of nitrate, nitrite and N_2 is, together with the direct incorporation of ammonium or of some forms of organic N, directed to building organic matter for growth, often in an energy-dependent manner (Fig. 1). In the following sections, we describe these assimilatory processes (Fig. 2) and their regulatory control. Given their central nature, we first cover ammonium assimilation and its regulation. We then study the assimilation of alternative N sources and their specific regulatory mechanisms, and conclude emphasizing the global systems of N control.

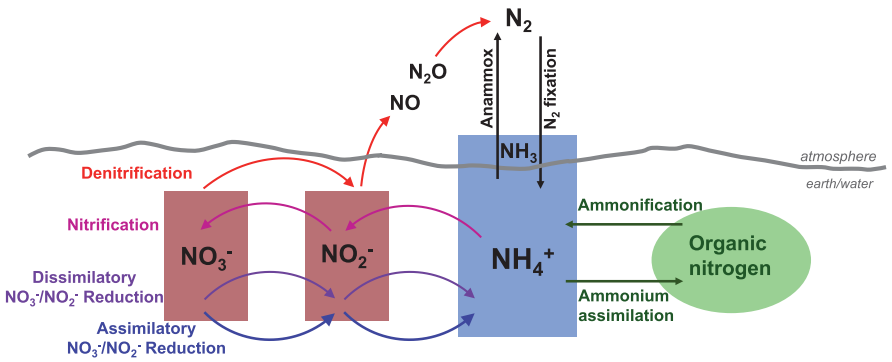

Fig. 1 Main paths of nitrogen cycling in Nature. The global processes accounting for the bulk of transformations of nitrogen forms taking place in natural habitats are represented. Except for nitrate/nitrite assimilation, in which plants and algae have an important contribution, and ammonification, in which fungi have a role, all others are carried out by bacteria and archaea.

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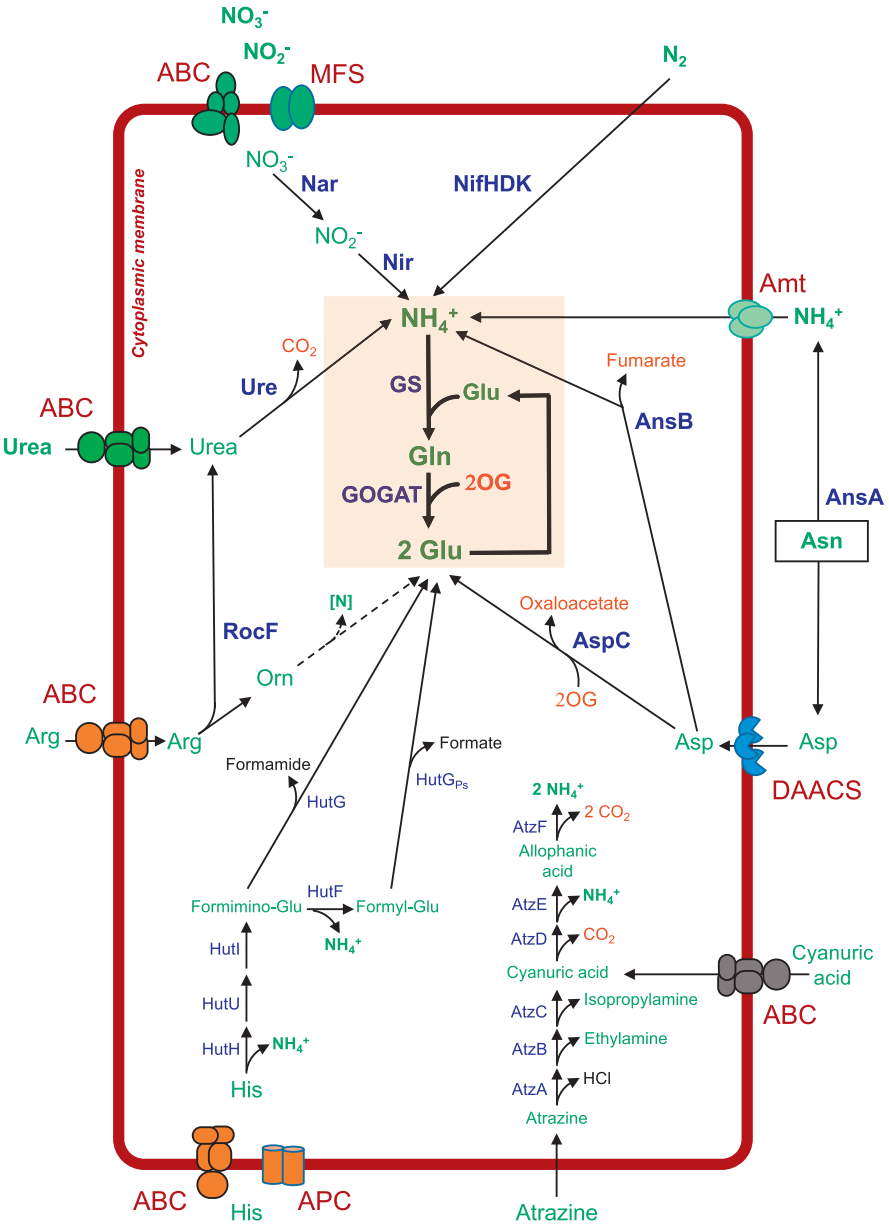

Fig. 2 Core and some peripheral nitrogen assimilation pathways in bacteria. The assimilation of ammonium through the GS-GOGAT cycle is at the core of nitrogen assimilation in bacteria. Ammonium can be taken up from the medium or derived from inorganic sources of nitrogen including nitrogen gas (dinitrogen), nitrate and nitrite, as shown in the upper part of the scheme. Nitrogen can be obtained also from organic nitrogen sources including the core amino acids glutamine and glutamate. Other organic nitrogen sources, shown here as examples, include urea, arginine, asparagine, aspartate, histidine and the xenobiotics atrazine and cyanuric acid. Amino acids that serve as excellent nitrogen sources can be catabolized producing glutamate and ammonium; atrazine and urea serve as nitrogen sources because they can be catabolized releasing abundant ammonium. The family to which each represented transporter belongs is indicated (for further information on transporter families, see the Transporter Classification Database at http://www.tcdb.org). (Transporters for glutamate and glutamine are not depicted in the figure.) The names of some enzymes or gene products that participate in the catabolism of the indicated nitrogen sources (amino acids in three letter code) are shown: AnsA, periplasmic asparaginase (*B. subtilis*); AnsB, aspartase (*B. subtilis*); AspC, aspartate aminotransferase; Atz, atrazine degrading proteins; GS, glutamine synthetase; GOGAT, glutamate synthase; Hut, histidine degrading proteins; Nar, nitrate reductase; NifHDK, nitrogenase complex; Nir, nitrite reductase; RocF, arginase (*B. subtilis*), also known as ArcA (*Agrobacterium tumefaciens*); Ure, urease. The dashed arrow in arginine catabolism denotes two different sub-pathways described in the text within pathway 1 and pathway 2 ([N], nitrogen conserved as glutamate in 1 or as ammonium in 2). Only substrates/products relevant for our discussion are indicated, and stoichiometry of the reactions is not specified.

Ammonium Assimilation

Ammonium Uptake

The ammonium ion (NH_4^+) is a key compound in the assimilation of nitrogen in numerous biological systems because it is the inorganic form of nitrogen that is incorporated into carbon skeletons. Ammonium present in the environment can be assimilated by many bacteria, yeast, fungi, algae, and plants. Ammonium solutions always contain ammonia (NH_3) (the pK_a of ammonium/ammonia is 9.25), which can diffuse through biological membranes. Diffusion of ammonia followed by trapping of intracellular ammonium by glutamine synthetase can represent a significant process for nitrogen acquisition, especially in organisms such as some bacteria that can grow in alkaline media. This process would be less important in fungi that grow in acidic media. However, in numerous organisms there is evidence for the presence of ammonium transport systems that can be conveniently probed with ^{14}C-labeled methylammonium (the pK_a of methylammonium/methylamine is 10.65). Genes encoding ammonium/methylammonium permeases were first identified in *Saccharomyces cerevisiae* (MEP) and *Arabidopsis thaliana* (AMT1). Of note, the Rhesus proteins in chordates are homologues of the ammonium transporters. Genes coding for proteins similar to MEP- and AMT1-encoded proteins are ubiquitous in bacterial genomes, where they are denoted *amtB* or a variation thereof. For instance, a gene that has been denoted *amt1* is widespread in cyanobacteria.

The Amt proteins

Bacterial Amt proteins contain 400–500-amino acid residues. They are integral membrane proteins that normally have 12 transmembrane segments. The N-terminal transmembrane segment is frequently part of a signal sequence that is removed after integration of the protein into the membrane. The crystal structures of the AmtB proteins from *Escherichia coli* and the euryarchaeon *Archaeoglobus fulgidus* are available, showing that AmtB forms trimers in which each monomer provides a hydrophobic pore to accommodate and conduct the substrate. Although the nature of the transferred substrate (NH_4^+ or NH_3) has been debated, recent work supports early conclusions based on physiological studies that the transferred substrate is the ammonium ion, although the mechanism of transport could involve a deprotonation step and parallel transfer of a proton. The net result of transport is concentration of ammonium inside the cells dependent on the membrane potential (negative inside the bacterial cells), and incorporation of ammonium into glutamine catalyzed by glutamine synthetase (GS). This has been clearly shown also in studies using [^{14}C]methylammonium as a substrate, in which intracellularly accumulated [^{14}C]methylammonium and [^{14}C]methylglutamine are detected.

Bacterial Amt proteins have a very high affinity for ammonium (K_s values below micromolar), and the expression of *amt* genes is commonly repressed by high concentrations of ammonium in a process that involves the global nitrogen regulatory system, e.g., Ntr in the enterobacteria or NtcA in cyanobacteria (see Section "Nitrogen Control"). Indeed, the Amt proteins are not needed for ammonium assimilation when ammonium is provided at high levels (e.g., in the millimolar range at a neutral pH), as demonstrated by the ability of *amt* knock-out mutants to grow using ammonium as a nitrogen source.

Regulation of amtB

The *amtB* gene is frequently clustered together with *glnK*, which encodes a P_{II}-like protein. As described in the section on N control, P_{II} proteins are sensors of the nitrogen status of the cell. In enterobacteria, P_{II} proteins are modified by uridylylation under low-nitrogen conditions. In the presence of a non-limiting ammonium concentration, the GlnK trimer is deuridylylated and binds the AmtB trimer, inhibiting ammonium transport (Fig. 3).

In conclusion, the Amt proteins function to scavenge ammonium when this substrate is found at low extracellular concentrations. Because of the paired presence of ammonium and diffusible ammonia in solution (respective concentrations depending on pH), when the concentration of the NH_4^+/NH_3 pair is too high, the cells respond repressing *amt* gene expression and frequently also blocking AmtB activity. These responses could impede the operation of a futile cycle in which the ammonium concentrated within the cells leaks out due to unavoidable ammonia diffusion.

Incorporation of Intracellular Ammonium Into Carbon Skeletons

We will show throughout this article that all N sources eventually yield intracellular ammonium. This is not surprising, since N in NH_4^+ is at the same oxidation state as the organic N present in biological molecules. Intracellular ammonium, either coming from NH_4^+ uptake or from the assimilation of alternative N sources, is incorporated into C skeletons by reaction with organic acids to yield amino acids. 2-oxoglutarate is the preferred ammonium acceptor, to yield glutamate and glutamine. Glutamate and glutamine provide the N for all the other amino acids, while glutamine provides N for nucleotide and amino sugar biosynthesis.

Enzymatic routes

Glutamate and glutamine are the main entry points of N to the organic pool in bacteria. Glutamine synthetases (GS) catalyze the amidation of glutamate to yield glutamine at the expense of one molecule of ATP. A second enzyme, glutamate synthase (GOGAT), produces two molecules of glutamate from one of 2-oxoglutarate and one of glutamine, consuming two reducing equivalents. Through the concerted action of the GS/GOGAT two-enzyme system (Fig. 2), ammonium is incorporated into 2-oxoglutarate with high affinity, but one ATP is consumed per glutamate formed. A third enzyme, glutamate dehydrogenase (GDH) can catalyze the reductive amination of 2-oxoglutarate to yield glutamate using a reduced pyridine nucleotide, but the reaction equilibrium is

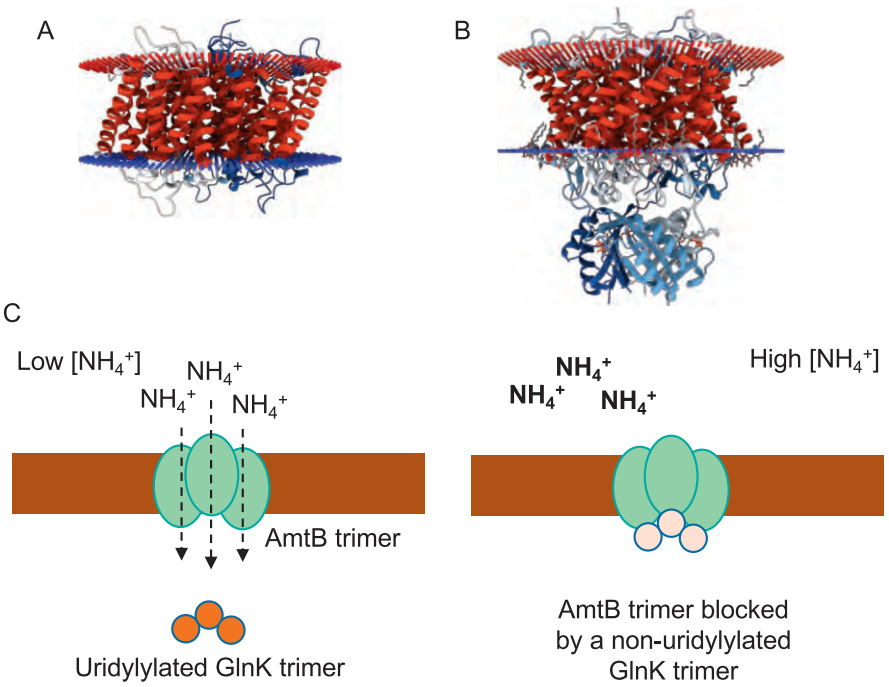

Fig. 3 Structure and scheme of AmtB and its inhibition by GlnK. Structure of the AmtB trimer (A) and the AmtB-GlnK complex (B) from *Escherichia coli* viewed in the membrane plane. Structure references are 1UTG for AmtB and 2NS1 for AmtB-GlnK. (C) Schematic of the blocking of AmtB (inserted in the cytoplasmic membrane) by GlnK that takes place when the ammonium concentration is high, which determines a low C-to-N balance that results in deuridylylation of GlnK as explained in the text.

heavily displaced in the opposite direction, and GDHs are usually catabolic. In enterobacteria and in many other bacteria, both systems coexist. Other bacteria can lack GDH or, more rarely, GOGAT. In enterobacteria, GS is a dodecamer of identical subunits, each with an active site. In other bacteria, GS can be much simpler. Still other bacteria contain more than one type of GS, which can increase the flexibility of their N assimilation pathways. In enterobacteria, GOGAT is a heterodimeric, iron-sulfur flavoprotein that uses NADPH. In other bacteria, NADH or even ferredoxin are preferred over NADPH, and it is not uncommon for bacteria to harbor more than one type of GOGAT.

Other, minor, ways of ammonium incorporation into organic skeletons exist, such as NAD^+ synthase or the amidation of aspartate catalyzed by asparagine synthetase, but one of them in particular, the synthesis of alanine from pyruvate and ammonium, catalyzed by alanine dehydrogenase, appears to be important in diazotrophic legume bacteroids of some rhizobia, both as a sink of fixed ammonium and as a N-delivery molecule to the plant.

Regulation

In many bacteria, the *glnA* gene is regulated by the availability of ammonium through the global N-control circuits. Additionally, in *E. coli*, the GS activity is regulated by a tight bicyclic regulatory cascade (Fig. 4), which shares components of N-regulation. In this circuit, each of the 12 GS subunits can be modified through adenylylation/deadenylylation by GlnE, a bifunctional adenylyl transferase/removing enzyme (ATase). Adenylylation reduces the catalytic activity of GS, and although high glutamine levels do not directly inhibit GS activity, they determine ATase-mediated adenylylation. Conversely, high levels of 2-oxoglutarate stimulate deadenylylation. In turn, ATase activity is under control of the P_{II}/uridylyl transferase (UTase, the *glnD* gene product) cascade: GlnB (and to a lower extent GlnK) stimulates adenylylation and GlnB-UMP (GlnK-UMP) stimulates deadenylylation. ATase appears to preferentially sense two forms of the P_{II} protein: trimers bound to one 2-oxoglutarate stimulate adenylylation, while fully uridylylated and 2-oxoglutarate saturated trimers stimulate deadenylylation.

Assimilation of Organic Sources of Nitrogen

A large diversity of nitrogenous organic compounds can be used as sources of nitrogen in the bacterial world. Relatively simple compounds that can be used by different bacteria include urea, some amino acids, amino sugars and simple amine-containing compounds such as methylamine, ethanolamine or some polyamines. Utilization of amino acids sometimes implies previous degradation of peptides that should be considered the actual nitrogen source. Other complex compounds such as heterocyclic

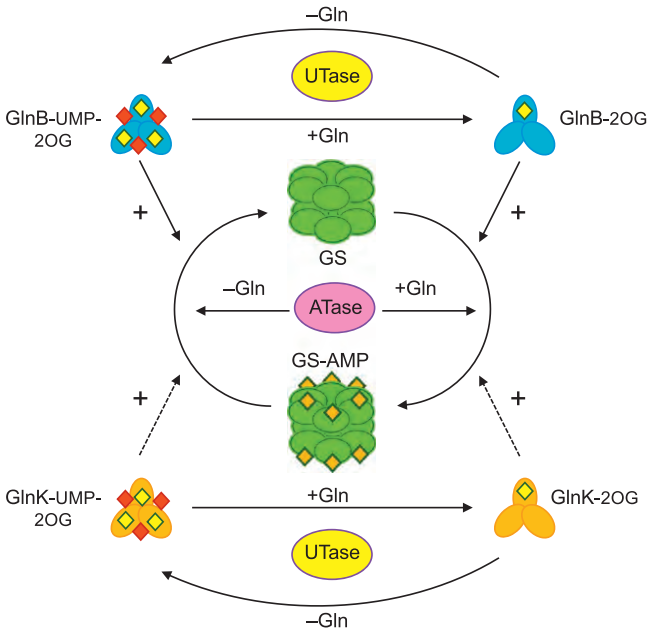

Fig. 4 Regulation of glutamine synthetase in *Escherichia coli*. Adenylylation of GS reduces its activity, and each of the twelve subunits can be independently adenylylated/deadenylylated by adenylyl transferase (ATase). High levels of glutamine favor adenylylation, while low levels result in deadenylylation. In addition, ATase activity is modulated by the P_{II} proteins, GlnB and GlnK. These proteins can bind 2-oxoglutarate (2OG) and be modified by uridylylation. Both uridylylation and deuridylylation are catalyzed by UTase. High levels of glutamine favor deuridylylation, while low levels result in uridylylation. Two forms of the P_{II} proteins are shown. When completely deuridylylated and bound to one 2OG, P_{II} proteins stimulate the adenylyl transferase activity of ATase. When completely uridylylated and bound to three 2OG, P_{II} proteins stimulate the adenylyl removing activity of ATase. GlnK has a much lower effect on ATase, and this is denoted by dashed lines. This cascade system responds, in a highly sensitive manner, to variations in the intracellular N status, since it senses the levels of both glutamine and its C precursor (2OG). Yellow diamonds, 2-oxoglutarate (2OG); red diamonds, UMP; orange diamonds, AMP.

nitrogenous compounds including, e.g., nitrogenous bases (pyrimidines and purines) or xenobiotics such as atrazine can also be used by some bacteria. The utilization of an organic nitrogenous compound as a nitrogen source requires the uptake of the substrate into the bacterial cell. Then, specific metabolic route(s) catabolize the substrate to produce ammonium that is incorporated into amino acids. In many cases, the bacteria completely mineralize the substrate releasing ammonium. Some amino acids, on the other hand, are catabolized producing glutamate, which is the major distributor of nitrogen in metabolism; however, degradation of the amino acid should also produce ammonium to be used by GS to synthetize glutamine (Fig. 2).

The myriad of bacterial pathways involved in the utilization of organic sources of nitrogen cannot be covered here. We will therefore outline examples of the pathways of utilization of a few substrates that can enlighten the reader about the variety of catabolic pathways displayed in the bacterial world.

Urea

Urea ($CO[NH_2]_2$) is a simple organic compound present in both ocean and fresh waters as well as in the soil (in part because of its use as fertilizer), frequently at micromolar concentrations or below, and it can be assimilated by bacteria belonging to different phylogenetic groups. Although urea at relatively high (millimolar) concentrations can permeate biological membranes permitting at least slow growth, it can also be incorporated into many bacteria by an active transporter. Once inside the cell, urea is degraded by urease to produce CO_2 and ammonium, which is incorporated into organic material (Fig. 2).

Genes and proteins

Active uptake of urea appears essential to assimilate this compound when found below micromolar concentration. Bacterial urea uptake is mediated by an ABC-type transporter that was first identified in cyanobacteria. ABC uptake transporters typically comprise one periplasmic solute-binding protein, two integral membrane (transmembrane or permease) proteins/domains and two nucleotide-binding proteins/domains that hydrolyze ATP in the cytoplasm. The urea transporter is encoded by five genes that form an operon with a typical structure for ABC transporter-encoding operons, including (5′ to 3′) the genes for the periplasmic substrate-binding protein (UrtA), the transmembrane proteins (UrtB, UrtC), and the nucleotide-binding proteins (UrtD, UrtE) (Fig. 5). This transporter shows a high affinity for urea (K_s about 0.1 μM in the cyanobacterium *Anabaena* sp. PCC 7120), keeping the concentration of urea in the cytoplasm to a level usable by urease.

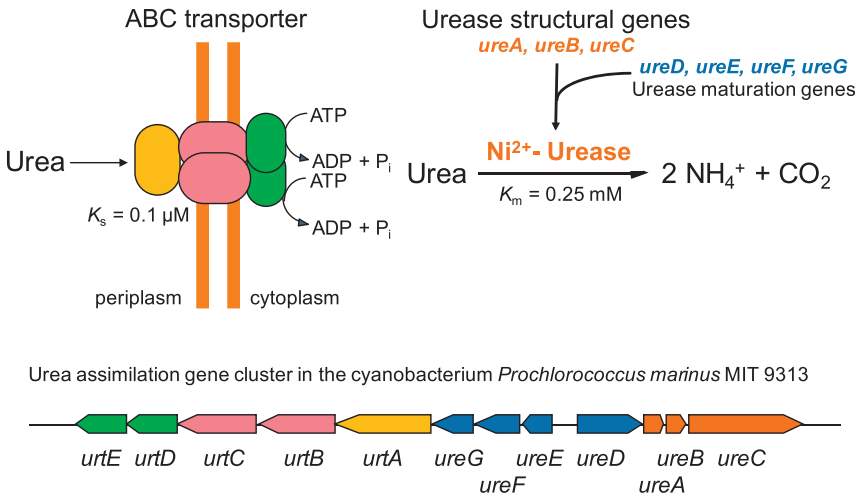

Fig. 5 The urea assimilation system. Urea is taken up into the cells with high affinity by an ABC transporter. These transporters are constituted by a periplasmic substrate-binding protein (yellow), two transmembrane proteins (pink) and two nucleotide-binding proteins (green) that hydrolyze ATP in the cytoplasm. Urea is concentrated inside the cell to a level that can be used by the urease complex, which has lower affinity for urea than the transporter. Urease is made up of three different subunits (genes depicted in brown) and bears a nickel-containing active center that is the product of a maturation process carried out by the products of urease-specific maturation genes (genes depicted in blue). All the genes required for urea assimilation are frequently clustered together in bacterial genomes, as exemplified by the urea assimilation gene cluster of the unicellular cyanobacterium *Prochlorococcus marinus* strain MIT 9113.

Urease (urea amidohydrolase) catalyzes the hydrolysis of urea to produce ammonia and carbamate, which spontaneously decomposes into another molecule of ammonia and carbonic acid. Urease is a trimer of a complex formed itself by three subunits encoded by the *ureA*, *ureB* and *ureC* genes. Each UreABC complex bears, located in UreC, a catalytic site that contains two nickel ions bridged by a carbamylated lysine and coordinated by histidine and aspartate residues. Synthesis of active urease is a complex GTP-dependent process that requires the products of accessory genes, *ureD*, *ureE*, *ureF* and *ureG*, which have a role in metallocenter assembly (Fig. 5).

The urease structural and maturation genes are generally clustered together in bacterial genomes and, interestingly, they are also frequently clustered with the urea ABC transporter-encoding genes, which may be found in the same or opposite orientation (Fig. 5). This arrangement may suggest that all these genes are subjected to concerted regulation, but also (or alternatively) that they have been received as a package by horizontal gene transfer in the bacterium where the cluster is found.

Regulation

The *urt* genes encoding the urease transporter are subjected to N control, so that they are expressed at high levels only under nitrogen deficiency. This is the case in, e.g., the cyanobacterium *Anabaena* sp. PCC 7120, where the *urt* operon is transcribed from an NtcA-dependent promoter, and *Mycobacterium smegmatis*, where the *urt* genes are under the control of the response regulator GlnR. In contrast, urease-encoding genes are expressed constitutively in some bacteria but regulated in others. Amongst the latter, the *Klebsiella pneumoniae ure* genes are transcribed from two promoters, one of which is strictly dependent on the nitrogen assimilation regulator Nac, which is a LysR-type factor that activates σ^{70}-dependent transcription in the absence of any effector (i.e., Nac is not regulated at the protein activity level). Instead, the expression of the *nac* gene is dependent on the Ntr system (see Section "Nitrogen Control", the *E. coli* cascade system).

In conclusion, the urea transporter appears to have an important role in urea scavenging, permitting the proliferation of urea-assimilating bacteria in natural habitats where urea may be found at low concentrations. A urease regulated in a similar way as the transporter would contribute to this function. On the other hand, in some bacteria urease participates in specific metabolic pathways (e.g., in arginine catabolism; see Fig. 2) that require constitutive expression or expression regulated independently of nitrogen control. Additionally, urease has an important role in virulence and/or adaptation to acidic environments in bacteria such as *Streptococcus* sp., *Proteus* sp. and *Helicobacter* sp., but this topic is outside the scope of this article.

Aspartate, Asparagine, Glutamate and Glutamine

These amino acids can be used as sources of nitrogen by many bacteria. The utilization of glutamate and glutamine is straightforward. Specific glutamate uptake systems of different transporter families are found in bacteria, including secondary H^+- or Na^+-dependent transporters such as the Na^+-dependent GltS of *E. coli*, or ABC transporters such as GluABCD from *Corynebacterium glutamicum*. There are also transporters for glutamate and related compounds such as the H^+-dependent symporters for aspartate or glutamate, TRAP (Tripartite ATP-independent periplasmic) transporters for dicarboxylates, or ABC transporters for aspartate/glutamate/asparagine/glutamine in numerous bacteria. Once inside the cells, glutamate can be used

directly in metabolism or deaminated by glutamate dehydrogenase, releasing ammonium. Regarding glutamine, it can be taken up by shared transporters and also by specific transporters such as the ABC transporter GlnHPQ of enterobacteria. Glutamine can be deaminated by specific glutaminases (glutamine amidohydrolases) producing glutamate and ammonium, but it can also be used to distribute nitrogen directly in metabolism through amidotransferases that bear glutaminase domains. The utilization of glutamine is however subjected to some nuances in organisms, such as the enterobacteria, in which glutamine is itself an indicator of the carbon-to-nitrogen balance of the cell. Hence, although abundant glutamine should be perceived as nitrogen excess (see Section "Nitrogen Control", the *E. coli* cascade system), growth of *E. coli* on glutamine as a nitrogen source induces the Ntr response.

Aspartate can be incorporated into bacterial cells by secondary transporters of the Dicarboxylate/Amino Acid:Cation (Na^+ or H^+) Symporter (DAACS) Family such as GltP (in *E. coli*) or GltT (in *B. subtilis*), or by ABC transporters such as GltIJKL (in *E. coli*). Once inside the cell, aspartate can be subjected to transamination to 2-oxoglutarate catalyzed by aspartate aminotransferase to produce oxaloacetate and glutamate, or to deamination catalyzed by aspartase (aspartate-ammmonia lyase) to produce fumarate and ammonium. Many bacteria, including phylogenetically distant organisms such as *E. coli* and *B. subtilis*, can metabolize aspartate with these two enzymes making nitrogen available for anabolism in two forms: glutamate and ammonium (Fig. 2).

Asparagine can provide nitrogen directly in the form of ammonium and, further, through aspartate catabolism. Asparaginases are hydrolytic enzymes that produce aspartate and ammonium. *B. subtilis* expresses two periplasmic asparaginases, one, termed AnsZ, that is subjected to nitrogen control and another, termed AnsA, that is induced by asparagine or aspartate. The *ansA* gene forms an operon with *ansB* that encodes an aspartase. Hence, in the presence of an inducer, *B. subtilis* can obtain two ammonium molecules from one asparagine molecule with the concourse of a periplasmic hydrolytic enzyme, a permease, and a cytoplasmic deaminating enzyme (Fig. 2). This pathway also involves the ammonium translocator AmtB (formerly known as NrgA in *B. subtilis*) to incorporate into the cytoplasm the ammonium produced in the periplasm.

The enterobacteria express two asparaginases: a cytoplasmic low-affinity enzyme (the *ansA* gene product) and a periplasmic high-affinity enzyme (the *ansB* gene product), which are not homologous. This permits metabolism of both asparagine incorporated into the cell and extracellular asparagine. Asparagine transporters are known in bacteria, including e.g., *E. coli* AnsP, a member of the Amino Acid-Polyamine-Organocation (APC) Superfamily of secondary transporters, and *Rhodobacter capsulatus* BztABCD, an ABC transporter for Glu/Gln/Asp/Asn. Aspartate produced by cytoplasmic asparaginase can be metabolized as described above for that taken up from the extracellular medium.

Regulation of expression of the genes encoding the elements for the different routes for asparagine and aspartate degradation can determine preferential utilization of these amino acids under certain conditions in particular bacteria. In *E. coli*, fumarate is a respiratory substrate, and under anaerobic conditions aspartate-fumarate antiporters (DcuA, DcuB) are induced that translocate fumarate to the periplasm, where it is used in respiration. Interestingly, under these conditions the periplasmic asparaginase is also induced, and asparagine is preferentially hydrolyzed in the periplasmic space, allowing the operation of the aspartate-fumarate antiporter. In contrast, in *Klebsiella* the periplasmic asparaginase is subjected to N control, which is consistent with a specific role in nitrogen nutrition. The utilization of asparagine and aspartate thus illustrates the plasticity of bacteria, which can use different types of transporters, including secondary transporters and high-affinity ABC transporters, and different enzyme localization strategies (periplasmic, cytoplasmic) to integrate assimilatory pathways and other important physiological processes of the bacterium such as respiration.

Arginine

Arginine is the amino acid richest in nitrogen, since it contains four N atoms within a six C atom skeleton. Hence, it is not surprising that arginine can be used as a source of nitrogen by many bacteria, which is reflected in the high number of different arginine catabolism pathways that can be found in the bacterial world. Whereas the nitrogen substrates discussed in this article generally traverse the outer membrane of Gram-negative bacteria through non-specific or low-specificity porins, a porin specific for basic amino acids, OprD, is involved in arginine utilization by *Pseudomonas aeruginosa*. Arginine can be taken up into bacterial cells by arginine-specific or basic amino acid ABC transporters, of which the HisJ/ArgT-HisMQP system of *Salmonella typhimurium* – which is involved in the transport of His, Lys, Arg and ornithine – is a well-known example, or by arginine/ornithine antiporters of the APC superfamily. At least seven arginine catabolism pathways are found in bacteria, but only three of them utilize efficiently the four N atoms contained in arginine:

(1) In the arginase pathway, well characterized in *Bacillus*, in which arginine is the second preferred nitrogen source (only after glutamine), urea is first removed from arginine by arginase (arginine ureohydrolase) with the concomitant production of ornithine (Fig. 2). Urea is then degraded by urease producing two ammonium molecules and CO_2, and ornithine is further catabolized to glutamate via glutamate semialdehyde/Δ^1–pyrrolyne-5-carboxylate, which involves a transamination to 2-oxoglutarate producing a second glutamate molecule. Hence, in this pathway, the four N atoms of arginine are conserved in two ammonium molecules and two glutamate molecules.

(2) In an alternative arginase pathway that has been well characterized in *Agrobacterium*, ornithine cyclodeaminase releases ammonium producing proline that is further catabolized to glutamate by the bifunctional enzyme proline oxidase, PutA. In this pathway, three ammonium molecules and one glutamate molecule are produced per arginine consumed.

(3) In a variation of arginine catabolism found in some bacteria including the enterobacteria and Pseudomonads, arginine is activated by addition of succinyl-coenzyme A to produce N^2-succinylarginine that follows a pathway similar to that of arginase to finally release succinate and glutamate. However, the second enzyme of this pathway, N^2-succinylarginine dihydrolase, releases two ammonium molecules and CO_2 instead of urea as arginase does. Hence, the arginine succinyltransferase pathway directly produces two ammonium molecules and two glutamate molecules per arginine consumed.

Other pathways make use of the arginine molecule with different purposes. Only the two most relevant of these pathways will be described here:

(4) In the arginine deiminase pathway, present in many phylogenetically different bacteria, arginine is first deaminated producing ammonium and citrulline, which is then used by catabolic ornithine carbamoyltransferase to produce ornithine and carbamoyl phosphate. Whereas carbamoyl phosphate is used by carbamate kinase to produce ATP, ornithine is excreted in exchange for arginine by an arginine/ornithine antiporter. Hence, this pathway only produces one ammonium molecule per arginine utilized, but additionally produces ATP, which makes it useful as an energy-producing pathway under anaerobic conditions.

(5) Arginine decarboxylase produces agmatine that is further catabolized by agmatine ureohydrolase (agmatinase, which is homologous to arginase) releasing putrescine and urea, which can be hydrolyzed by urease producing two ammonium molecules and CO_2. Putrescine is a polyamine and is itself a source for synthesis of other polyamines, including spermidine. Hence, although the arginine decarboxylase pathway releases N in the form of urea (and further on ammonium), its main role appears to be the production of polyamines that are essential in many organisms.

The arginine utilization pathways outlined here illustrate the efficient use of arginine as a nitrogen source, but also its use for other metabolic purposes such as energy-conservation and polyamine biosynthesis. Utilization of arginine as a nitrogen source is tightly regulated in some bacteria such as the enterobacteria, in which expression of the *ast* operon encoding enzymes of the arginine succinyltransferase pathway requires nitrogen limitation and is mediated by the Ntr regulatory system. Interestingly, however, the widely-distributed repressor of arginine biosynthesis, ArgR, a transcriptional regulator of the winged helix-turn-helix (wHTH) family of DNA binding proteins that binds DNA in the presence of arginine, is also involved in expression of the *ast* operon in the presence of arginine. ArgR-arginine binds to the *ast* promoter likely facilitating the action of NtrC. The other physiological functions of arginine catabolism involve specific regulations too. Thus, for instance, expression under anoxic conditions of the *arc* operon encoding the proteins of the arginine deiminase pathway in *Pseudomonas aeruginosa* is mediated by the global regulator of anaerobic metabolism, ANR (a transcriptional regulator of the CRP/FNR family).

Histidine

Histidine is rich in nitrogen (three N atoms in a six C atom skeleton) and can be assimilated by many, although not all bacteria. The histidine assimilation pathway is relatively complex but well conserved among bacteria. It involves histidine uptake and further degradation in four or five successive enzymatic steps, producing one glutamate and one or two ammonium molecules, respectively, per histidine molecule consumed. Because the imidazole side chain of histidine has a pK_a of 6.0, at a neutral pH histidine can be taken up through both neutral and basic amino acid ABC uptake transporters. Thus, the ABC basic amino acid transporter HisJ/ArgT-HisMQP of *Salmonella typhimurium* is involved in histidine uptake. Additionally, there are histidine-specific transporters such as the HutT permease of the Pseudomonads, a secondary transporter of the APC family.

Histidine is deaminated by histidase (the *hutH* gene product) producing ammonia and urocanate, which is subjected to two successive hydration steps mediated by urocanase (the *hutU* gene product) and imidazolone propionate hydrolase (the *hutI* gene product) forming imidazole propionate and formimino glutamate, respectively. In some bacteria (e.g., those of the genera *Bacillus* and *Klebsiella*), formimino glutamate is hydrolyzed by formimino glutamate hydrolase (the *hutG* gene product) releasing glutamate and formamide, which is not further catabolized, being instead excreted. In these bacteria, therefore, the pathway renders one glutamate molecule and one ammonium molecule per histidine consumed (Fig. 2). In other bacteria (e.g., those of the genera *Pseudomonas* and *Streptomyces*), formimino glutamate deiminase (the *hutF* gene product) produces ammonium and formyl glutamate, which is hydrolized by formylglutamate hydrolase (the product of a *hutG* gene that is not homologous to that of *Bacillus* or *Klebsiella*) releasing formate and glutamate. Hence, in these bacteria two molecules of ammonium and one molecule of glutamate are obtained per histidine consumed (Fig. 2), which represents an optimal efficiency in the utilization of histidine as a nitrogen source.

The *hut* genes encoding histidine catabolism enzymes and a transporter-encoding gene, *hutT*, are frequently clustered together constituting one or several transcriptional units. A gene, *hutC*, encoding a transcriptional repressor is also frequently found in the *hut* gene cluster. Because the biosynthesis of histidine is energetically expensive, the expression of the catabolic *hut* genes is subjected to tight regulation to avoid a futile cycle. Thus, the *hut* genes are only expressed if excess histidine is available and a shortage of nitrogen or carbon is perceived in the cells. Urocanate (the first catabolic product) is an inducer blocking the HutC repressor activity, thus ensuring that the *hut* genes are expressed only if histidine is abundant. An important variation in the mechanism of induction is found in *Bacillus*, where histidine-promoted anti-termination mediated by a protein termed HutP is in operation. Additionally, *hut* genes are subjected to catabolite repression and to N control. In enterobacteria, catabolite repression implies cAMP-CRP-mediated activation of expression of the *hut* genes, and their expression under nitrogen deficiency requires the Nac transcription factor. C- and N-regulation of *hut* genes in other bacteria such as those of the genera *Pseudomonas* or *Streptomyces* is carried out by their corresponding global regulatory systems.

Atrazine and Cyanuric Acid

Atrazine is very rich in nitrogen (five N atoms in an eight C atom skeleton), and an atrazine-mineralization pathway is encoded in plasmids found in some Pseudomonads, which make such organisms capable of using this xenobiotic as a nitrogen source. Cyanuric acid, an intermediate in the atrazine degradation pathway, can also be used as nitrogen source in these bacteria. Atrazine mineralization frequently occurs via a hydrolytic pathway that proceeds through the sequential elimination of the chlorine, ethylamino and isopropylamino substituents, to yield cyanuric acid that is cleaved and mineralized to CO_2 and ammonia (Fig. 2). Thus, mineralization of atrazine renders one ethylammonium and one isopropylammonium molecule, on one hand, and three ammonium molecules that can be directly incorporated into carbon skeletons, on the other. Not surprisingly, the capability to use atrazine is only expressed under nitrogen deficiency, but this is based in regulation of only part of the atrazine degradation pathway. Whereas the genes encoding the enzymes that catalyze the first three steps in the pathway (*atzA*, *atzB*, *atzC*) are scattered in the atrazine-catabolism plasmid and appear to be constitutive, the genes encoding the enzymes that catalyze the last three steps of the pathway (those involved in degradation of cyanuric acid) constitute an operon (*atzDEF*) whose expression is tightly regulated. The *atzDEF* promoter is a standard σ^{70}-dependent promoter that is activated by AtzR, a LysR-type transcriptional regulator that is encoded just upstream, divergently from the operon. The *atzR* gene is expressed from a σ^{54}-dependent promoter whose activation requires the general Ntr system of the bacterium, thus linking the capability to mineralize atrazine to nitrogen deficiency. The AtzR protein additionally requires cyanuric acid as a co-activator, and its activity is further enhanced by uridylylated GlnK. In addition to the only partial regulation of the atrazine degradation pathway, it is of interest that whereas the plasmid bearing the genes for atrazine/cyanuric acid degradation also has genes encoding an ABC transporter for cyanuric acid, the presence of a transporter for atrazine is unclear. Hence, atrazine is a nitrogen source whose utilization has likely involved the integration of genes specific for its degradation down to cyanuric acid with a pre-existing pathway for the utilization of this compound. Thus, degradation of atrazine and cyanuric acid exemplifies the adaptability of bacteria that have evolved to use xenobiotics as nitrogen nutrients.

The Assimilation of Nitrate

Nitrates are present at low concentrations (micromolar or below) in natural habitats including the oceans. However, nitrates are relatively abundant in local areas, especially those that hold extensive agricultural activity, because the use of excessive fertilization often leads to nitrate leakage into the environment. Nitrate is an excellent nitrogen source for many bacteria, fungi, algae and plants, and its assimilation involves entrance into the cell by an active transporter and its intracellular reduction to ammonium by two sequential reductive reactions, with ammonium being finally incorporated into organic material (Fig. 2).

Nitrate Uptake

Two types of active transport systems for nitrate, which can also accept nitrite, have been described in bacteria: ABC-type transporters and MFS permeases. Whereas the nitrate/nitrite transporters described in several bacteria (such as *K. oxytoca*) conform to the basic structure of ABC transporters, in cyanobacteria the Nrt transport system presents some peculiarities. First, the binding protein, NrtA, is anchored to the periplasmic side of the cytoplasmic membrane by post-transcriptional processing (including lipidation) by signal peptidase II and, second, the ATPase domain is made of two different subunits: NrtD, a standard ATPase subunit, and NrtC, a long protein with an N-terminal part similar to NrtD and a C-terminal part homologous to NrtA. The permease domain of Nrt is made of two NrtB subunits, each providing 5-transmembrane helices.

Major facilitator superfamily (MFS) permeases are made of dimers of a single gene product that normally contains 12 transmembrane segments and couple substrate transport or exchange through the cytoplasmic membrane to the dissipation of an electrochemical gradient. The *Bacillus subtilis* or *Paracoccus denitrificans* NasA, the *Synechococcus* sp. PCC 7002 NrtP and the *Trichodesmium* sp. WH9601 NapA belong to this family of transporters and couple nitrate/nitrite transport to a H^+ or Na^+ gradient.

Nitrate Reduction

Intracellular nitrate is first reduced to nitrite in a $2e^-$-transfer reaction catalyzed by nitrate reductase, and the resulting nitrite is then reduced to ammonium in a $6e^-$-transfer reaction catalyzed by nitrite reductase. In spite of this general scheme, the reductases largely diverge in structure and electron transfer paths depending on the organism. In cyanobacteria, which are characterized by a photoautotrophic mode of life relying on oxygenic photosynthesis, both nitrate reductase and nitrite reductase utilize photosynthetically reduced ferredoxin or flavodoxin as the electron donor. In most heterotrophic bacteria, and some anoxygenic phototrophs such as *Rhodobacter capsulatus*, both reductases accept electrons from reduced pyridine nucleotides (NADH), although some exceptions are found. For example, the nitrate reductase from *Azotobacter vinelandii* uses reduced flavodoxin, and the nitrate reductase from the chemolithoautotroph *Hydrogenobacter thermophilus* and the nitrate and nitrite reductases from the archaeon *Haloferax mediterranei* utilize reduced ferredoxin as electron donor.

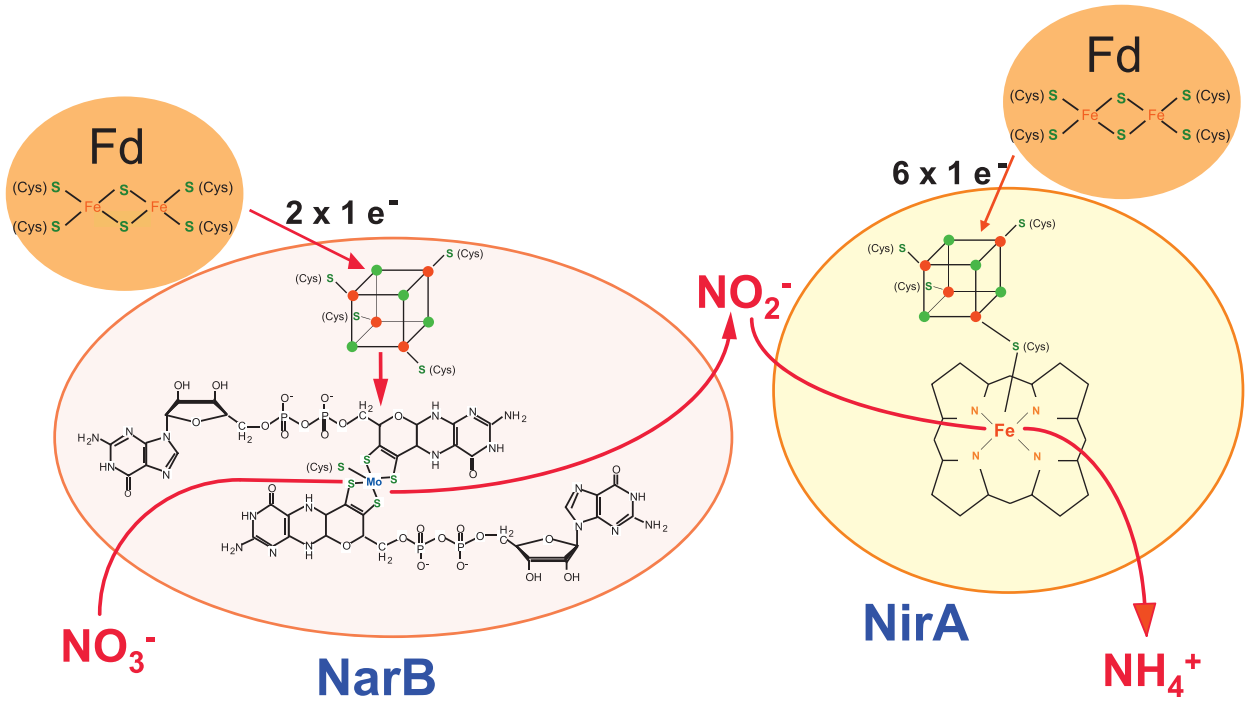

Fig. 6 Scheme of the electron transfer pathways in cyanobacterial nitrate reductase (NarB) and nitrite reductase (NirA). The cyanobacterial nitrate reduction system is especial in that both reductases use ferredoxin as an electron donor, but at the same time it is illustrative of the nitrate reduction process. Ferredoxin donates electrons (one at a time) to an iron-sulfur cluster in NarB, from which they follow a typical pathway to reduce nitrate to nitrite in a $2-e^-$ reduction reaction catalyzed in the Mo-*bis*-molybdopterin-guanine dinucleotide active center. NirA also uses reduced ferredoxin as an electron donor, and here the six electrons required to reduce nitrite to ammonium are transferred (one at a time) through an iron-sulfur center and a siroheme group, in which the reduction of the substrate takes place.

Nitrate reductase

The cyanobacterial nitrate reductase, as of that of *Synechococcus elongatus*, is a monomeric enzyme of ca. 80 kDa, the product of the *narB* gene, containing one [4Fe–4S] cluster, which receives electrons from the external electron donor, and a molybdenum-bis-molybdopterin guanine dinucleotide (Mo-bisMGD) cofactor, which represents the active site for nitrate reduction (Fig. 6). In contrast, NADH-dependent nitrate reductases frequently contain at least one additional reductase subunit, with FAD and sometimes Fe–S clusters, besides the Mo-bisMGD-containing catalytic subunit, which also includes one [4Fe–4S], and sometimes a [2Fe–2S], cluster. In the case of *Klebsiella oxytoca*, nitrate reductase is constituted by a 92-kDa catalytic subunit (NasA -please note, different from *B. subtillis* NasA above), which contains one Mo-bisMGD cofactor, one [4Fe–4S] and one [2Fe–2S] cluster, and a reductase subunit of 43 kDa (NasC), which contains FAD and transfers electrons from NADH to the catalytic subunit. Cyanobacterial Fd-dependent, *H. thermophylus* Fd-dependent and the catalytic subunit of NADH-dependent nitrate reductases, are all homologous proteins.

Nitrite reductase

Although appreciable differences can be found between ferredoxin- and NADH-dependent nitrite reductases, all of them exhibit considerable sequence and structural similarities as well. In particular, all nitrite reductases studied to date include [4Fe–4S] and siroheme groups similar to those of the sulfite reductase hemoprotein from enteric bacteria. The cyanobacterial enzymes are monomers, generally of ca. 55 kDa (NirA), where electrons from ferredoxin are transferred to the [4Fe–4S] cluster and then the siroheme (Fig. 6). Cyanobacterial nitrite reductase has been conserved in the chloroplasts and, hence, is an enzyme of universal relevance in photosynthetic organisms. Bacterial NADH-nitrite reductases may be monomeric, such as that of *K. oxytoca* (NasB), or, as in the case of *B. subtilis*, dimeric and constituted by a large catalytic subunit (NasD) and a small subunit (NasE) with similarity to the carboxy-terminal part of monomeric NasB. Both NasB and NasD include non-covalently bound FAD and two [2Fe–2S] clusters besides [4Fe–4S] and siroheme groups, the two latter as found in cyanobacterial nitrite reductase.

Genetics and Regulation

In cyanobacteria, the genes for the structural components of the nitrate and nitrite assimilation system (nitrate reductase, nitrite reductase and nitrate/nitrite transporter) are frequently clustered together with the order *nirA*-transporter gene(s)-*nirB*, although

some variants of this arrangement can also be found. In the well-studied cyanobacteria *Synechococcus elongatus* and *Anabaena* sp. PCC 7120, these genes constitute an operon that is preceded by additional genes involved in modulation of either the expression of the *nirA* operon or the activity of its encoded elements. Outside of the cyanobacteria, a cluster of nitrate/nitrite assimilation genes has been identified in all the nitrate-assimilating bacteria studied to date, which sometimes also includes regulatory genes and genes for electron donors and synthesis of the nitrite reductase cofactor siroheme or the nitrate reductase cofactor Mo-bisMGD.

Nitrate (and nitrite) assimilation is a costly process due to the energetic requirement for active transport, the high requirement for reducing equivalents and the synthesis of complex reductase cofactors. Thus, in most bacteria, nitrate assimilation is subjected to global N-control circuits (see Section "Nitrogen Control"). In addition, frequently, pathway-specific regulators – often encoded in the nitrate/nitrite assimilation gene cluster – influence the expression of nitrate assimilation genes, in some cases eliciting positive responses to the availability of nitrate or nitrite.

In cyanobacteria, nitrate and nitrite assimilation genes are under global N control exerted by the transcriptional regulator NtcA, and in the case of *Synechococcus*, the activity of the Nrt active transporter for nitrate and nitrite has been shown to be regulated by P_{II} (GlnB), which in cyanobacteria is phosphorylated in response to high 2-oxoglutarate levels. In addition, expression of the *nirA* operon is subjected to pathway-specific regulation by the LysR-type transcription factor NtcB. NtcB exerts a positive effect on the expression of nitrate assimilation genes in the absence of ammonium, which is of high magnitude in the case of *Anabaena* sp. PCC 7120. In this case, NtcB has been shown to bind DNA in the promoter region of the *nirA* operon, close to the binding site for NtcA, and the expression of the *ntcB* gene is itself activated by NtcA.

In many bacteria, responses to the presence of nitrate or nitrite appear to be mediated by transcription anti-termination mechanisms. In *K. oxytoca* and *A. vinelandii*, expression of the *nas* operon is regulated by the general N-control Ntr system. In addition, the factors NasR or NasT, respectively, bind the leader sequence of the *nas* operon mRNA avoiding premature termination, thus promoting transcription completion in the presence of nitrate or nitrite. NasT acts together with NasS, a protein homologous to cyanobacterial NrtA, which may represent a cytoplasmic nitrate/nitrite sensor.

Nitrogen Fixation

In order to enter the biosphere, atmospheric N_2 must be first reduced to a form that can be readily assimilated by organisms, usually ammonium. This reduction is known as nitrogen fixation. Nitrogen fixation is a highly energy-demanding process, given that the triple bond in dinitrogen is very stable and difficult to break down. As a direct result of the inertness of dinitrogen, nitrogen can become a limiting element in ecosystems and, particularly, in agriculture, where fixed nitrogen is the main component of fertilizers.

Biological nitrogen fixation is carried out exclusively by prokaryotes, both bacteria and archaea, collectively known as diazotrophs. Diazotrophy is not limited to any specific group, and it appears to be widely, but haphazardly, distributed throughout different prokaryotic groups. This distribution, together with the high conservation of nitrogen fixation systems (see later in this section) and their extreme oxygen sensitivity, suggest that diazotrophy is a very ancient capability. Ecologically, diazotrophy is also widely distributed. Substantial rates of nitrogen fixation have been observed in terrestrial and aquatic (both freshwater and oceanic) ecosystems, where diazotrophic prokaryotes are often in close, symbiotic associations with eukaryotic organisms, from unicellular algae to fungi, plants and animals.

Nitrogenases

Diazotrophic organisms carry out biological nitrogen fixation under normal conditions of pressure and temperature, compatible with life, through the catalytic action of a complex enzyme, nitrogenase. Although some nitrogenase variants exist, especially regarding the complement of transition metals at their active center, the available information on their structure and organization, as well as on their assembly and biochemistry, denotes a striking conservation of nitrogenases among diazotrophs.

Nitrogenases catalyze the reduction of dinitrogen to ammonia at the expense of ATP, protons and reducing power, according to the equation:

$$N_2 + 8H^+ + 8e^- + 16\text{MgATP} \rightarrow 2NH_3 + H_2 + 16\text{MgADP} + 16P_i$$

where ammonia is in the protonated ammonium form in solution.

Two striking characteristics of this equation are its high energetic cost, in terms of ATP and reducing power, and the stoichiometric synthesis of one molecule of hydrogen gas per molecule of dinitrogen reduced. The latter is a source of energy inefficiency in the reaction, since 2 e^- and 4 MgATP are used to make H_2 that can be lost as gas. This is an unavoidable outcome of the complex enzymatic mechanism required to reduce dinitrogen, whereby electrons must be pumped in rapid succession onto a dinitrogen molecule immobilized at the active center that undergoes stepwise reductions through short-lived, unstable intermediates. Nonetheless, many diazotrophs express hydrogenases that can oxidize the hydrogen produced in the nitrogenase reaction, recovering electrons and producing ATP.

The structural and biochemical characteristics have been studied especially in nitrogenases that contain the transition metal molybdenum (Mo-nitrogenases). Other, less common and less studied, variants replace vanadium (V-nitrogenases) for

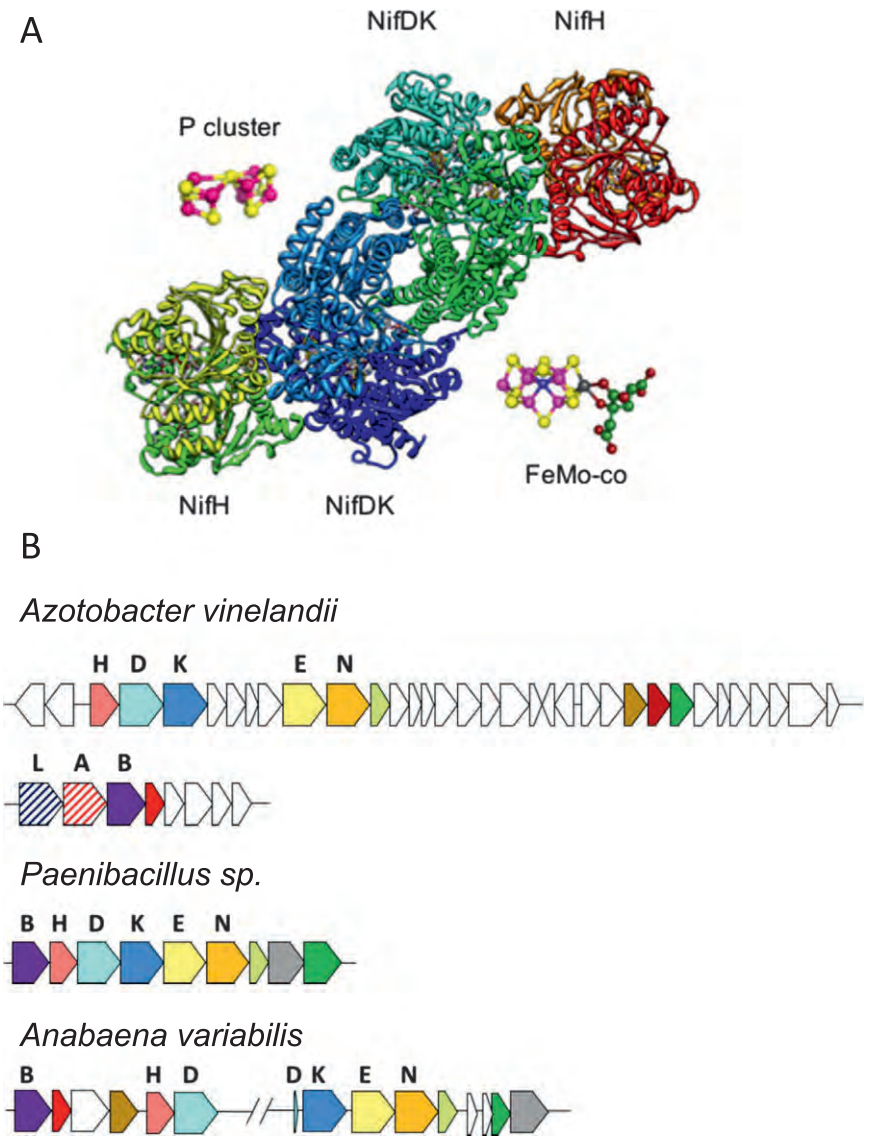

Fig. 7 Nitrogenase and nitrogenase genes. (A). Nitrogenase structure at 1.9 Å resolution. Two molecules of the dinitrogenase reductase NifH dimer are bound to a central dinitrogenase heterotetramer (2 NifDK). Substrate reduction takes place on FeMo-co (depicted), which is buried within each of the two α (NifD) subunits of dinitrogenase. Also shown is one of the P clusters that facilitate transfer of electrons from dinitrogenase reductase to the FeMo-co. (B). Genetic organization of *nif* (nitrogen fixation) genes in *Azotobacter vinelandii* and *Paenibacillus* sp., examples of *nif* clusters containing a large number or a minimal set of *nif* genes, respectively, and in *Anabaena variabilis*. In the latter, note the split *nifD* gene, interrupted by an intervening genetic element that is excised upon heterocysts differentiation. Common *nif* genes are identified: *nifHDK, nifEN and nifB*. Regulatory genes *nifLA* are also shown for *A. vinelandii*.

molybdenum. A third type of nitrogenase contains iron instead of Mo or V. Since all of the above variants also contain additional Fe, the latter are known as Fe-only nitrogenases.

Mo-nitrogenase is actually a complex of two metalloenzymes. The largest, dinitrogenase (also known as MoFe protein), is a ca. 250 kDa heterotetramer ($\alpha_2\beta_2$) organized as two αβ heterodimers (Fig. 7(A)). Each dimer contains a unique iron-molybdenum cofactor (FeMo-co) on the α subunit, the active site of substrate binding and reduction, and a unique Fe–S cluster, the P cluster, at the α-β subunit interface. The smaller enzyme, dinitrogenase reductase (also known as Fe protein), is a ca. 46 kDa α_2 homodimer (Fig. 7(A)). Each subunit contains an ATP/ADP binding site and both subunits are bridged by a 4Fe–4S cluster.

The role of dinitrogenase reductase is to deliver electrons to dinitrogenase, one at a time. In turn, dinitrogenase reductase is reduced by a cellular source of high-energy electrons, often ferredoxins or flavodoxins, that varies among diazotrophs and is often a reflection of its particular lifestyle. Once reduced, dinitrogenase reductase transiently associates with dinitrogenase and hydrolizes 2 molecules of ATP to ADP in order to transfer one electron to dinitrogenase. After transfer, the oxidized enzyme dissociates and replaces the ADPs by ATPs, getting ready to be reduced again. Electrons received by dinitrogenase are stored in the protein and eventually transferred to the substrate in FeMo-co through the P clusters. Therefore, eight interlocking catalytic cycles

of dinitrogenase reductase are required per cycle of dinitrogenase in what is one of the slowest enzymatic rates (ca. 1 cycle per second). Given these characteristics, and the fact that, despite the availability of high resolution structures, bound substrate and intermediates have not been unequivocally visualized, it is not surprising that details of the nitrogenase mechanism have yet to be ascertained.

The two complex metal cofactors of dinitrogenase, FeMo-co and P clusters, are unlike any other found in biological systems. Despite the fact that FeMo-co (and probably P clusters) can be extracted from the enzyme in active form, they are extremely labile, and their structure has only been solved within the enzyme. FeMo-co contains a [4Fe–3S] subcluster and a [3Fe–Mo–3S] subcluster separated by a C atom, probably a carbide, and a molecule of (R)-homocitrate coordinated to Mo through its 2-hydroxy and 2-carboxyl groups. FeMo-co synthesis and assembly require a large number of specific proteins, and its mechanism has been unraveled through the use of sophisticated in vitro systems with purified components and precursors. P clusters are [8Fe–7S] clusters that originate by specific, in situ reductive fusion of two standard [4Fe–4S] clusters. Both FeMo-co and P clusters confer oxygen sensitivity to dinitrogenase, which is rapidly inactivated and denatured upon exposure to air. Even higher oxygen sensitivity has been observed with dinitrogenase reductase, despite the fact that its [4Fe–4S] cluster is similar to clusters present in other, oxygen-resistant proteins.

Although fragmentary, the available evidence suggests that V-nitrogenases and Fe-only nitrogenases are evolutionarily related to the more widespread Mo-nitrogenases, that their general architecture, synthesis and mechanism are similar, and that they may reflect adaptations to diazotrophy under conditions where Mo may become limiting for nitrogen fixation. In this regard, several considerations could be taken into account: (a) the catalytic inefficiency of nitrogenase (see above) is often compensated by the synthesis of very high levels of the enzyme, that can amount to over 20 % of the total cell protein; (b) Mo-depleted ecosystems exist, such as some acidic soils and open oceanic waters; (c) the catalytic efficiency of V- and Fe-nitrogenases is lower than that of Mo-nitrogenases; and (d) one or both of these "alternative" systems can be found in diazotrophs that also possess a Mo-nitrogenase. In those cases, the more efficient Mo nitrogenase is expressed when Mo is available, and the several systems can share some of their accessory components, such as those related to metal cofactor synthesis.

Genetics and Regulation

The genetic derminants for nitrogenase and nitrogenase maturation proteins have been studied in a few model systems, notably *K. pneumoniae* and *A. vinelandii* (Fig. 7(B)). Comparative DNA sequence analyses, stimulated by the recent explosion in prokaryotic genome sequencing, have shown that many of the *nif* (nitrogen fixation) determinants, notably those encoding structural proteins, are strongly conserved in diazotrophs. Analysis of their functions has been facilitated by their organization in tight gene clusters containing co-regulated operons. The number of *nif* genes in diazotrophs is variable, from over 30 in *A. vinelandii*, 20 in *K. pneumoniae*, and down to nine in Firmicutes (Fig. 7(B)) and some archaea, with a minimum set of six *nif* genes conserved throughout. These include *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, and *nifB*. The first three encode the dinitrogenase reductase structural subunit and the α and β subunits of dinitrogenase, respectively, while the last three are involved in FeMo-co synthesis: NifE and NifN are structural homologues of NifD and NifK, respectively, and form a $\alpha_2\beta_2$ heterotetramer that acts as a scaffold on which FeMo-co is assembled from a precursor cluster (NifB-co) synthesized by NifB. Other *nif* genes are involved in Mo metabolism, synthesis of Fe–S cluster precursors and homocitrate, on one hand, and nitrogenase assembly and maturation, electron transfer to nitrogenase and regulation, on the other. Some of these functions, such as the first three, can be provided by the housekeeping cellular machinery, although it is not uncommon to find *nif*-specific determinants of those functions, such as in the case of Fe–S cluster synthesis, in view of the demands that high levels of nitrogenase synthesis can impose on housekeeping elements.

As is the case with electron transfer to nitrogenase -highly dependent upon specific metabolic lifestyles- different models of nitrogen fixation regulation exist, so as to adapt nitrogen fixation expression to specific cellular contexts. Besides the high energy requirements, the enzyme is strongly and irreversibly inactivated by oxygen. Therefore, it is not surprising that diazotrophs prefer to use fixed nitrogen sources over dinitrogen, and that nitrogenase is usually tightly regulated by the nitrogen and oxygen status of the cell, either directly or indirectly. Regulation of nitrogen fixation occurs mainly at the transcriptional level rather than at the activity level. Contrary to many biosynthetic enzyme systems, nitrogenase is not subject to end-product inhibition, and oxygen simply inactivates the enzyme irreversibly.

Transcriptional regulation of nitrogen fixation has been studied in detail in proteobacteria (*K. pneumoniae*, *A. vinelandii* or the Rhizobia). Promoters of *nif* genes are dependent on an alternative sigma factor (σ^{54}) that recognizes consensus sequences at positions −24 and −12. σ^{54}-RNA polymerase holoenzyme can initiate transcription only after σ^{54} interacts with a transcriptional activator bound to upstream enhancer sequences. In the case of *nif* promoters, this activator is NifA, a homodimeric protein that belongs to the ATPase AAA+ superfamily and that, using ATP hydrolysis, determines a conformational change in σ^{54} that results in the formation of a transcriptionally competent RNA polymerase-promoter open complex. This strategy is advantageous in that it ensures that no low-level transcription can occur in the absence of NifA, and in that it provides a target, NifA, for signal transduction. In some organisms, notably the Rhizobia, NifA is able to sense the oxygen status directly, becoming inactivated under aerobic conditions, and even the N status. In others, such as in *K. pneumoniae* and *A. vinelandii*, *nifA* is cotranscribed with *nifL*. The NifL protein has the ability to bind to NifA, inactivating it in response to environmental signals, such as the redox, N and C status of the cell. In that respect, the NifLA pair resemble two-component regulatory systems. However, although it is possible that they may have evolved from such a system (the identifiable remnants of a histidine-protein kinase domain are present in NifL), their ability to integrate these disparate environmental signals, not only within NifL, but in some cases also within NifA, makes this an unusual

regulatory pair. Transcription of *nifA* (or *nifLA*) can, in turn, be subjected to environmental signal control in a cascade-like manner, thus ensuring an amplification of the signal compatible with the need for a tight, highly responsive regulation of nitrogen fixation.

This specific picture of nitrogen fixation regulation, drawn from studies of proteobacteria, is not shared by many other diazotrophs. Thus, although detailed knowledge of specific regulatory mechanisms is lacking in many cases, proteins homologous to NifA proteins are missing from the genomes of diazotrophs as diverse as the archaea and the Firmicutes, among others. However, the basic tenets of expression in the absence of fixed nitrogen sources and oxygen still hold in most cases. The *nif* genes from methanogenic archaea and Firmicutes appear to be regulated by transcriptional repression, rather than induction. Cyanobacteria also lack NifA and appear to use a redox-sensitive transcriptional activator, CnfR. In strains without heterocysts (cells specialized in N_2 fixation), CnfR is expressed under N starvation, while in heterocyst-forming strains, it is expressed only in heterocysts (see Section "The heterocyst").

A further aspect of regulation concerns the alternative V- and Fe-nitrogenases, especially when they coexist with a Mo-nitrogenase system, such as in *A. vinelandii* and the cyanobacterium *Anabaena variabilis*. *A. vinelandii* is unique in containing all three, Mo-, V-, and Fe-only nitrogenase systems. The alternative systems are regulated by NifA orthologues, VnfA and AnfA, respectively, in response to N and O_2 signals, and by a further, hierarchical regulation with preference for Mo-nitrogenase > V-nitrogenase > Fe-only nitrogenase. Rather than sensing the transition metal directly, regulation appears to respond to the efficiency of the nitrogenase system in a not well-understood cross-talk that implicates NifA, VnfA and AnfA. *A. variabilis* contains two Mo-nitrogenases and one V-nitrogenase. The main Mo- and the V-nitrogenase systems are hierarchically expressed in heterocysts, while the second Mo-nitrogenase is only expressed under anaerobic conditions, and thus in all the cells in the filament.

Finally, it is worth noting that a post-transcriptional regulatory mechanism of nitrogenase activity operates in some α-proteobacteria, such as *Azospirillum brasilense*, and purple photosynthetic bacteria. In these bacteria, dinitrogenase reductase is reversibly inactivated by ADP-ribosylation in response to ammonium or lack of energy (darkness in photosynthetic bacteria or anaerobiosis in *A. brasilense*).

Mechanisms of Protection of Nitrogenase Against Oxygen

The extreme oxygen sensitivity of nitrogenase proteins and cofactors is probably a reflection of the fact that they evolved before the Great Oxygenation Event. Nonetheless, the physiological and ecological advantages afforded by the ability to fix nitrogen in fixed N-depleted environments, even in the presence of air, have resulted in the acquisition of a wide range of adaptations to protect nitrogenase from oxygen. The simplest case is that of the obligate anaerobes, such as archaea and the clostridia. These diazotrophs cannot live in the presence of oxygen even if they are not fixing nitrogen, therefore no special adaptations of nitrogenase to oxygen is required or has been selected. Facultative anaerobes, such as enterobacterial diazotrophs, can fix nitrogen only under fermentative conditions, but their respiratory systems have been shown to help scavenge oxygen by using it as electron acceptor.

A large number of chemoorganotrophic, diazotrophic bacteria are dependent on respiration with oxygen as terminal electron acceptor to obtain energy, and can only fix nitrogen under decreased oxygen tension, where the combined effect of physical barriers to oxygen diffusion and respiratory consumption of the diffused oxygen result in near-anoxic conditions compatible with nitrogenase expression and activity. This is the case of the Rhizobia, that associate with legumes to fix nitrogen within a special root organ, the nodule, where root and bacterial respiration contribute to decrease oxygen levels. However, the obligatory need for oxygen as terminal electron acceptor in the Rhizobia has resulted in specific molecular adaptations of the symbiosis, such as the accumulation of a legume hemoglobin, leghemoglobin, that provides the bacteria with a steady supply of oxygen at low levels, or the expression of a high-oxygen-affinity terminal respiratory oxidase that allows the bacteria to obtain energy at these low oxygen levels.

A. vinelandii exemplifies the best-studied case of strictly aerobic diazotrophy in unicellular bacteria. Complete protection of nitrogenase against oxygen is provided, again, by a combination of structural oxygen diffusion barriers (cell envelopes, exopolysaccharides) and respiratory protection (increased respiration mediated by several redundant terminal oxidases with high capacity and high affinity towards oxygen), which together result in an adequate decrease in dissolved oxygen levels and diffusion rates. It is worth noting that one of these high capacity oxidases -a quinol oxidase- can become uncoupled from ATP synthesis and thus work as a combustion machine, provided that reducing power is not limiting. A further adaptation to diazotrophy under air is the presence in *A. vinelandii* of the Fe/S II protein, which can bind to nitrogenase forming an oxygen-resistant complex that can preserve the integrity of nitrogenase under transitory episodes of increased oxygen.

Protection in oxygenic phototrophs (Cyanobacteria)

In cyanobacteria, the issue of protection of the nitrogenase system from oxygen is specially demanding because, as oxygenic phototrophs, they not only have to cope with ambient oxygen but also with that produced intracellularly by oxygenic photosynthesis. Nevertheless, the capacity to fix N_2 is widespread in the cyanobacterial phylum, and different strains have evolved different strategies to fix N_2 in oxic environments, although some strains can do so only under micro-oxic or anoxic conditions. As an example of the latter, cyanobacteria of the genus *Oscillatoria* can fix N_2 while performing H_2S- and photosystem I-dependent anoxygenic photosynthesis under anaerobic conditions. Besides that, well known strategies for protection against intracellular O_2 are based on temporal or spatial separation of the processes of N_2 fixation and oxygenic photosynthesis. Thus, in many filamentous and unicellular cyanobacteria, N_2 fixation is restricted to the dark periods when subjected to a diel cycle, and likely under natural conditions (as shown for the marine unicellular organism *Chroccosphaera* sp.). In the dark period, N_2 fixation

can coincide with active respiration, which has a dual function in provision of energy and oxygen scavenging. As an example, in the unicellular *Cyanothece* sp., photosynthesis, ATP synthase, CO_2-fixation and glycogen biosynthesis genes are expressed in the light periods, whereas glycogen degradation, respiration and *nif* genes are expressed in the dark periods. This pattern of expression involves circadian regulation. In contrast, in the filamentous cyanobacterium *Trichodesmium* sp., a globally-relevant N_2 fixer in the oceans, N_2 fixation peaks in the light periods during diel cycles. Although the mechanism of nitrogenase protection is not clear, it is debated whether N_2 fixation is confined to cells in a central stretch of the filament that would have reduced oxygen content, perhaps aided by temporal segregation of CO_2 and N_2 fixation during the day. Further specialization is found in some filamentous cyanobacteria, included in a defined phylogenetic clade, that can produce heterocysts, which are cells specialized for the fixation of N_2. In these strains, heterocysts are the only cells of the filament that, under oxic conditions, express nitrogenase (Fig. 8).

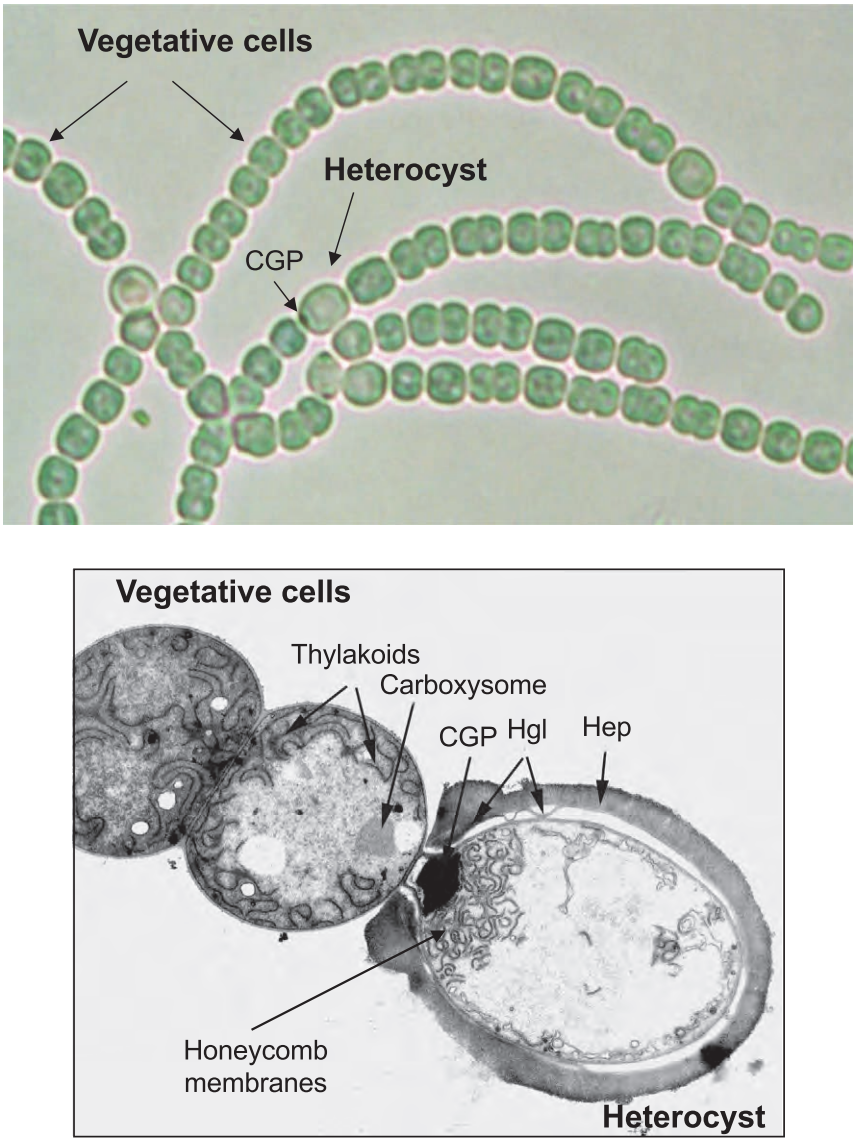

Fig. 8 Heterocyst-forming cyanobacteria. (Top) Light micrograph of filaments of *Anabaena* sp. strain PCC 7120 showing chains of vegetative cells, which perform CO_2 fixation through oxygenic photosynthesis and divide, and intercalary heterocysts, terminally differentiated cells that carry out N_2 fixation. (Bottom) Transmission electron micrograph of a terminal heterocyst and two adjacent vegetative cells. The intracellular membranes in vegetative cells (thylakoids) are the sites of oxygenic photosynthesis, and the carboxysomes are the sites of CO_2 fixation. In the heterocysts, carboxysomes are missing and the intracellular membranes are largely reorganized to form the "honeycomb" membranes, which are sites of active respiration and anoxygenic photosynthesis. The heterocyst has, deposited outside of the outer membrane, a special cell envelope that contains heterocyst-specific glycolipids (Hgl) and polysaccharides (Hep). At the heterocyst poles, cyanophycin (multi-L-arginyl-poly [L-aspartic acid]) accumulates as a large cell inclusion known as cyanophycin granule (CGP) that is also observed by light microscopy (see above).

The heterocyst

The differentiation of a vegetative cell into a heterocyst takes place in response to N deprivation and involves extensive structural and biochemical changes to provide suitable sites for efficient N_2 fixation. Morphological changes that take place during differentiation include the deposition of specific glycolipid and polysaccharide cell envelope layers to limit gas, including oxygen, penetration into the cell cytoplasm. Indeed, it has been proposed that the main path for N_2 entrance into the heterocyst is a differentiated, membrane protein structure at the heterocyst poles that could modulate gas diffusion rates. Also, conspicuous reorganization of the photosynthetic membranes, which is concomitant with loss of photosystem II function (and thus of oxygenic photosynthesis), takes place during differentiation.

Biochemically, the photoautotrophic metabolism of the vegetative cell is turned into a heterotrophic metabolism in the heterocyst. This transition is based on the loss of photosynthetic CO_2 fixation (which is catalyzed by ribulose-bisphosphate carboxylase/oxygenase [RubisCo]), expression of a specific invertase for sucrose catabolism, increased levels of the oxidative pentose phosphate cycle enzymes, and expression of specific terminal respiratory oxidases. Indeed, the heterotrophic metabolism of heterocysts relies on sugars donated by the neighboring vegetative cells, which also provide carbon skeletons for the incorporation of the ammonium resulting from N_2 reduction. In turn, heterocysts transfer fixed nitrogen in the form of amino acids (including a dipeptide, β-aspartyl-arginine) to the vegetative cells. Thus, under diazotrophic conditions the organismic unit of these cyanobacteria is a filament constituted by different cell types with specialized metabolic functions that exchange nutrients and regulatory molecules (see later in this section), representing true pluricellular bacteria (Fig. 8).

At the genetic level, heterocyst differentiation involves the sequential action of the products of multiple regulatory and structural genes, leading to the establishment of a gene expression pattern differing conspicuously from that taking place in the vegetative cells. Two transcription factors are required for triggering differentiation: the global N-regulator NtcA (see Section "Nitrogen Control"), and HetR. At the initiation of differentiation, basal levels of NtcA are activated by the increased levels of 2-oxoglutarate that result from an increased C-to-N cellular balance upon N deficiency, and the genes *ntcA* and *hetR* are activated in a mutually- and self-dependent manner, which leads to the establishment of high levels of both regulators. Remarkably, activation of the expression of the genes encoding these two main regulators, as well as of many of their regulated genes, takes place in the specific cells of the filament engaged in differentiation. Thus, many genes involved in heterocyst differentiation and function present spatiotemporal specificity for activation, including the *nif* genes, which are expressed only in mature heterocysts. Molecular events contributing to this pattern include the remarkable occurrence of complex promoter regions for many of those genes, which bear several successive promoters with different requirements for NtcA and HetR, and the participation of secondary regulators that are expressed at precise stages during differentiation, some of which increase the expression or activity of NtcA or HetR (Fig. 9).

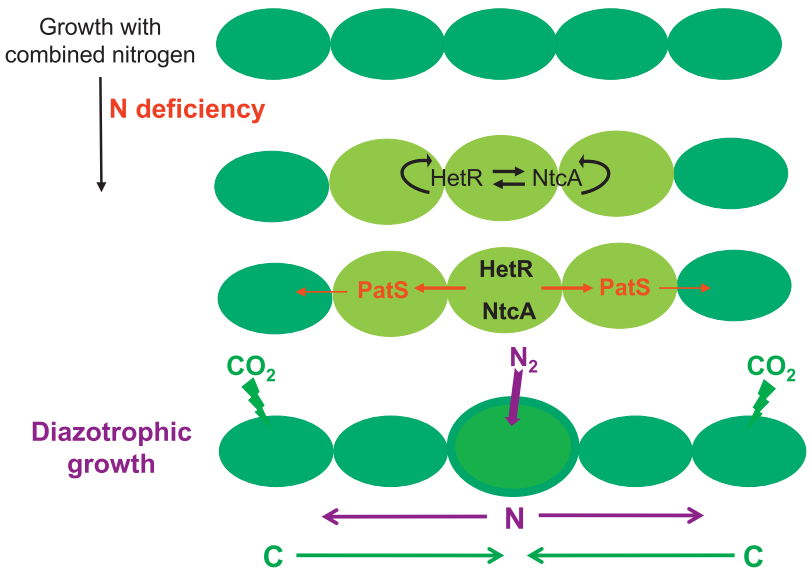

Fig. 9 Regulation of heterocyst differentiation. Nitrogen deprivation elicits an increase in the 2-oxoglutarate cellular levels, and 2-oxoglutarate binds and activates NtcA, which was present at low concentration during growth with combined nitrogen, initiating a loop of mutual and auto-induction of the genes *hetR* and *ntcA*, which initially takes place in clusters of cells. Concomitantly, the *patS* gene is expressed in specific cells that have initiated differentiation, and the primary gene product is processed to render the PatS morphogen, which is transferred to neighboring cells inhibiting their differentiation and leading to single cell selection. Structural proteins and secondary regulators are sequentially expressed, with a direct or indirect requirement for NtcA and HetR, that promote differentiation into heterocysts. In the diazotrophic filament of *Anabaena*, heterocysts, which fix N_2, are separated by stretches of ca. 10 vegetative cells, which fix CO_2. Heterocysts donate organic nitrogen to the vegetative cells, while the vegetative cells transfer organic C to the heterocysts.

Heterocysts are not randomly-distributed in the cyanobacterial filament. In the case of strains such as those of the genera *Anabaena* or *Nostoc*, heterocysts are flanked by stretches of ca. 10 vegetative cells, an adequate pattern to fulfill the nutritional interdependence between the two cell types. The PatS morphogen is a key element in establishing the heterocyst distribution pattern. The *patS* gene is induced early in differentiating cells and encodes a primary product of 17 amino acids that is processed to render the active molecule (a C-terminal peptide of likely seven residues), which is a HetR (and thus a differentiation) inhibitor. Indeed, PatS is transferred from source cells, where the *patS* gene is expressed, to neighboring cells, setting a concentration gradient with higher levels in the cells adjacent to the source cell, within which the concentration remains negligible, and diminishing with distance from there on (Fig. 9). The gradient of PatS peptide away from the heterocyst appears to create an inverse gradient of HetR to determine the pattern of cellular differentiation in each filament.

Nitrogen Storage

Although scarcely studied, nitrogen storage could take place in different bacteria. Only one specific nitrogen reservoir is known, the cyanophycin granules that will be discussed here. Nonetheless, the general protein pool of a bacterium, or specific proteins such as phycobiliproteins in the case of cyanobacteria, can constitute nitrogen reservoirs made available by protein turnover under nitrogen deficiency.

In most cyanobacteria, cyanophycin represents a dynamic reservoir of nitrogen that is accumulated in the form of cytoplasmic granules. Cyanophycin accumulates under conditions of unbalanced growth that do not involve N starvation, e.g., under phosphate or sulfur starvation or high CO_2 supply. In diazotrophic strains that temporarily separate oxygenic photosynthesis and N_2 fixation, cyanophycin accumulation may coincide with the periods of N_2 fixation. In filamentous heterocyst-forming strains, cyanophycin is conspicuously accumulated at the polar regions of the heterocysts (Fig. 8), representing a pool of fixed N *en route* to the vegetative cells. First thought to belong only to cyanobacteria, genes encoding cyanophycin-metabolism enzymes have later been identified in the genomic sequence of many other bacteria with different metabolic options including phototrophy, aerobic or anaerobic respiration, chemolithoautotrophy or fermentation. As in cyanobacteria, cyanophycin is accumulated under phosphate limitation in, e.g., *Acinetobacter* sp. Interestingly, cyanophycin is considered a source of products with potential interest in the chemical and pharmacological industries (biopolymers, natural food additives, therapeutic amino acid supplements), and projects aimed at increasing the accumulation of cyanophycin in several bacteria, as well as the expression of cyanophycin synthesizing enzymes in heterologous hosts, including yeast and plant plastids, have been developed.

Cyanophycin Metabolism

Cyanophycin is made of polypeptide chains of variable length that contain arginine residues linked to a polyaspartate backbone in a nearly 1:1 (Arg:Asp) ratio. The cyanophycin polypeptides are water insoluble and form granules. Cyanophycin is non-ribosomally synthesized by cyanophycin synthetase, which catalyzes both the elongation of the backbone by addition of L-Asp forming an α-peptide bond, and the addition of L-Arg to the β-carboxyl group of Asp making an isopeptide bond. Both reactions require ATP. The enzyme is a homodimer, the product of the *cphA* gene, with two predicted catalytic sites: one C-terminal site for the incorporation of Asp, and one N-terminal site for the addition of Arg. Cyanophycin degradation takes place in two sequential steps. First cyanophycinase, a C-terminal exopeptidase, a dimer of the product of the *cphB* gene, excises a β-Asp-Arg dipeptide. The dipeptide is further hydrolyzed to the constituent amino acids by isoaspartyl dipeptidase, which is a dimer of two different subunits resulting from autoproteolytic cleavage of the precursor gene product, and which is similar to plant asparaginases. While the *cphA* and *cphB* genes are frequently clustered, the gene encoding isoaspartyl dipeptidase is located in a different chromosome location.

Some bacteria are able to use cyanophycin as a carbon source for growth in a process that involves extracellular cyanophycinases. The enzyme from *Pseudomonas anguilliseptica* (CphE) has been purified and shows high specificity for cyanophycin to produce also a β-Asp-Arg dipeptide, although it exhibits only ca. 27% identity to the intracellular cyanobacterial cyanophycinase.

Regulation of Cyanophycin Accumulation

In both the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 and the filamentous heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120, the P_{II} protein factor (GlnB) influences cyanophycin accumulation. In the former case, the effect has been shown to involve activation of N-acetylglutamate kinase (NAGK) -a key enzyme of the arginine biosynthesis pathway- by unphosphorylated P_{II}, which leads to increased production of arginine under conditions of low C-to-N balance.

In several strains of *Anabaena* grown under diazotrophic conditions, cyanophycin synthetase and cyanophycinase activities are higher in heterocysts than in vegetative cells. In *Anabaena* sp. PCC 7120, the transcriptional regulator NtcA has been shown to orchestrate this spatial regulation from a complex promoter arrangement. In contrast, aspartyl dipeptidase levels are higher in vegetative cells than in heterocysts. Accordingly, cyanophycin synthesized at the expense of fixed N in the heterocyst is transiently accumulated in polar granules and hydrolyzed to β-Asp-Arg dipeptide, which is transferred to the vegetative cells to be further hydrolyzed to Asp and Arg to be used for metabolism. Thus, the dipeptide represents a nitrogen vehicle in the diazotrophic filament.

Nitrogen Control

In many bacteria, a hierarchy in the order of assimilation of nitrogen sources is established when more than one is available. In many cases ammonium represents the preferred nutrient, given that its assimilation implies a lower energetic cost than that of nutrients that have to be intracellularly transformed into ammonium. Indeed, in most bacteria ammonium exerts a negative effect on the expression of genes encoding elements of the pathways for assimilation of other nitrogen sources, a global regulatory circuit that is referred to as nitrogen control (N control). At the molecular level, both the array of protein factors (transporters, enzymes, pathway-specific regulators) that are subjected to N control, as well as the mechanism of ammonium-promoted down-regulation, vary among different bacteria. However, several basic schemes can be recognized, as described below. A key issue regarding N-control circuits deals with signaling of the N status of the cell to influence the activity of the N-control regulators. In general, high levels of 2-oxoglutarate which, as mentioned above, is a substrate for the incorporation of ammonium into glutamate and glutamine, are indicators of N deficiency, whereas additionally high levels of glutamine may be sensed as an indicator of N sufficiency. Whether both indicators or just 2-oxoglutarate are used depends on the metabolic characteristics of the different bacteria.

The *E. coli* Cascade System

The genetic and biochemical mechanisms of nitrogen control have been studied in detail in the *E. coli* model system, but some of its general characteristics are conserved in the Proteobacteria. In addition, the N-control system shares sensors and signal transduction mechanisms with those regulating the activity of the principal first enzyme for ammonium incorporation into C skeletons, glutamine synthetase (see Section "Incorporation of intracellular ammonium into carbon skeletons", Regulation).

In *E. coli*, the *glnA* gene encoding GS is organized in an operon together with the genes for a N-responsive, two-component regulatory system, the Ntr system (Fig. 10). The sensor/transmitter component, NtrB, is a protein kinase/phosphatase that can specifically phosphorylate or dephosphorylate the receiver/regulator component, NtrC. When phosphorylated, NtrC acts as a transcriptional activator of the main *glnA* promoter, while dephosphorylation inactivates it. NtrB is able to autophosphorylate at the expense of ATP, and to transfer this phosphoryl group to NtrC, therefore activating it in the absence of any other component. However, the NtrB activities are modulated by its interaction with the P_{II} factor GlnB.

P_{II} proteins are organized as trimers, and each subunit can bind one molecule of 2-oxoglutarate. Under N-excess most of the 2-oxoglutarate binding sites in P_{II} proteins will be empty, with the reverse being true under N-deficiency. In the case of GlnB, as a result of a cellular situation of N excess, many of the trimers will contain a single molecule of 2-oxoglutarate. This form of GlnB will bind to NtrB, with two consequences: (i) inhibition of NtrB autophosphorylation; and (ii) stimulation of the NtrB-mediated NtrC-P dephosporylation. As a result, the levels of NtrC-P will drop and transcriptional activation of the main *glnA* promoter will stop. A large number of N assimilation genes are also transcribed from NtrC-dependent promoters, which makes NtrBC a global regulatory system. As with NifA (see Section "Nitrogen fixation", Genetics and Regulation), NtrC-P binds to upstream activator sequences of σ^{54}-dependent promoters, thus ensuring that no transcriptional leakage occurs in the absence of the activator.

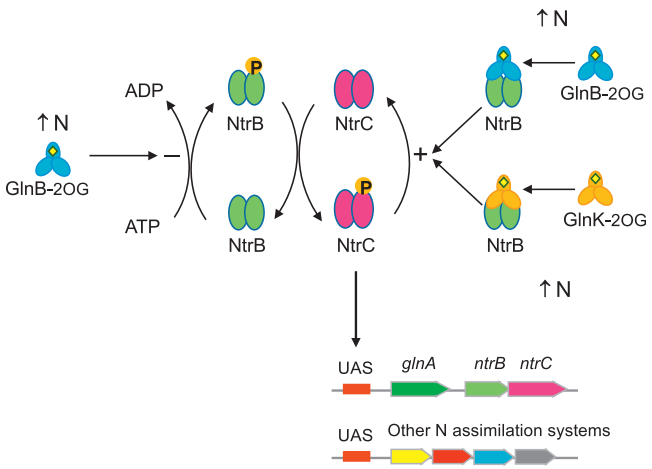

Fig. 10 The *Escherichia coli* cascade system of N control. Activity of the two-component Ntr regulatory proteins, NtrB and NtrC, is regulated by phosphorylation. NtrB can be autophosphorylated at the expense of ATP, and then transfer this phosphoryl group to NtrC (upper central part). NtrC-P can activate transcription through binding to upstream activating sequences (UAS) of N-regulated promoters, in particular the promoter of the *glnA-ntrBC*, but also those of operons whose gene products allow the cell to assimilate alternative nitrogen sources (lower part). The GlnB and GlnK P_{II} proteins control the levels of NtrC-P. The completely deuridylylated form of the proteins bound to one 2OG (GlnB-2OG and GlnK-2OG), found in the cell when levels of glutamine are high (N excess, Fig. 4), bind to NtrB and stimulate dephosphorylation, and hence deactivation, of NtrC (upper right part). In addition, GlnB-2OG inhibits the autophosphorylation activity of NtrB (upper left part), this ensuring that no further phoshorylation of NtrC-P can take place. Yellow diamonds, 2-oxoglutarate (2OG).

The GlnB > NtrB > NtrC > target promoter cascade is further controlled at the level of the GlnB sensor. In addition to binding 2-oxoglutarate, each monomer in the trimer can be covalently modified by uridylylation through the activity of the GlnD protein. GlnD is a bifunctional uridylyltransferase/uridylyl-removing enzyme (UTase) that can apparently sense the cellular levels of glutamine. High glutamine levels determine deuridylylation of GlnB, while complete uridylylation is observed at low glutamine levels. Since binding of 2-oxoglutarate -and probably ADP- to GlnB is required for uridylylation, GlnB appears to be a sensor where different signals (glutamine, 2-oxoglutarate, energy charge) can be integrated.

A further level of complexity arises from the existence of another P_{II} protein, GlnK, a GlnB paralog. While GlnB is always present in the cell, *glnK* transcription is under N control and GlnK is present only under N-deficiency. Both proteins can bind 2-oxoglutarate, can interact with NtrB, and be a substrate of UTase, while only GlnK regulates the AmtB channel activity (see Section "Regulation of *amtB*", above).

The NtcA Circuit of Cyanobacteria

The NtcA regulator was identified as the product of the gene inactivated in cyanobacterial mutants unable to use inorganic nitrogen sources (nitrate, nitrite or N_2) other than ammonium, and it turned out to have a universal distribution in the cyanobacterial phylum. NtcA belongs to the family of transcriptional regulators that also includes CAP (catabolite activator or cAMP receptor protein -CRP) from *E. coli*. As other members of this family, NtcA bears in its C-terminal part a helix-turn-helix motif for binding to DNA at sites with a palyndromic structure, which in the case of NtcA includes the sequence signature $GTAn_8TAC$. NtcA can act as a transcriptional activator or repressor depending on the location of the NtcA-binding site in the regulated promoter, and in the most common NtcA-activated promoters, the binding site is separated by ca. 22 nucleotides from a −10 promoter determinant (consensus: TAn_3T). This promoter structure is similar to that of Class II bacterial-activated promoters, in which the binding site of the regulator substitutes for the −35 determinant that is found in constitutive promoters. Besides this, in some other NtcA-activated promoters, the NtcA-binding site is located further upstream from the −10 determinant (Class I activated promoters) or present degenerate NtcA-binding sites requiring the participation of NtcA co-activators. Worth mentioning is the principal role of NtcA in determining the spatiotemporal specificity of activation of genes involved in heterocyst differentiation by acting on different types of NtcA-dependent promoters included in complex promoter regions, as mentioned above.

Because cyanobacteria lack 2-oxoglutarate dehydrogenase, 2-oxoglutarate is mainly used through the GS/GOGAT cycle (see Section "Incorporation of intracellular ammonium into carbon skeletons"). Thus, 2-oxoglutarate levels depend not only on CO_2 assimilation through the Calvin/Benson cycle but also on ammonium assimilation through the GS/GOGAT cycle. As such, 2-oxoglutarate levels, which are low in the presence of ammonium, are a good indicator of the C-to-N balance of the cell. Indeed, 2-oxoglutarate has been shown to bind to NtcA, to stimulate NtcA-binding to DNA in regulated promoters, and to be required at the step of promoter melting to establish the open promoter complex for transcription initiation in Class II promoters.

Regarding NtcA targets, a plethora of genes of different N-assimilation pathways and heterocyst differentiation have been individually characterized as regulated by NtcA. Besides that, a global study by chromatin immunoprecipitation has identified more than 2000 NtcA targets in the genome of *Anabaena* sp. PCC 7120, including promoter regions of genes involved in N assimilation but also in other cellular functions such as photosynthesis, central metabolism or DNA replication and repair. This exceptional abundance of DNA binding sites suggests that besides being a global trancriptional regulator, NtcA could serve a structural function as a genome-wide DNA shaper.

Gram-Positive Bacteria

The Gram-positive bacteria include organisms for which relevant information on nitrogen control is available. These organisms are *B. subtilis* and the Actinobacteria *Streptomyces coelicolor*, *Corynebacterium glutamicum* and *Mycobacterium* spp., including the non-pathogenic species *M. smegmatis*.

Bacillus subtilis

The soil, sporulating bacterium *B. subtilis* has been intensively investigated as a model of bacterial development and cell biology. In the presence of glucose as a carbon and energy source, the preferred nitrogen source of *B. subtilis* is glutamine followed by arginine and ammonium. *B. subtilis* expresses two MerR-family transcription factors, TnrA and GlnR, that execute N control in concert with glutamine synthetase (GlnA). MerR-family transcriptional regulators generally contain a helix-turn-helix motif for DNA-binding in their N-terminal region, followed by a coiled-coil domain and a C-terminal effector-binding region that is specific to the effector recognized. TnrA and GlnR bind to similar DNA sites of consensus sequence TGTnAnATTTTnTnACA, which results in an overlap of their respective regulons. However, while TnrA acts under conditions of nitrogen limitation, GlnR acts under conditions of nitrogen sufficiency. Under nitrogen deficiency, TnrA is positively auto-regulatory and regulates at least 35 transcriptional units. TnrA activates the expression of operons such as *nrgAB* encoding the ammonium-scavenging protein AmtB and its inhibitor GlnK, respectively, *ureABC* encoding urease or *nasBC* and *nasDEF* encoding nitrate assimilation proteins, and represses operons such as *gltAB* encoding GOGAT or *glnRA* encoding the GlnR repressor and glutamine synthetase. Under nitrogen sufficiency, glutamine-inhibited glutamine synthetase (GlnA-glutamine) binds to TnrA blocking its DNA-binding activity (and, hence, impeding its regulatory roles) and to GlnR promoting repression of operons such as *ureABC* or *glnRA* itself as well as the *tnrA* gene. GlnA-glutamine-activated GlnR can indeed bind to a wide number of genomic sites, implying that it is part of complex transcriptional

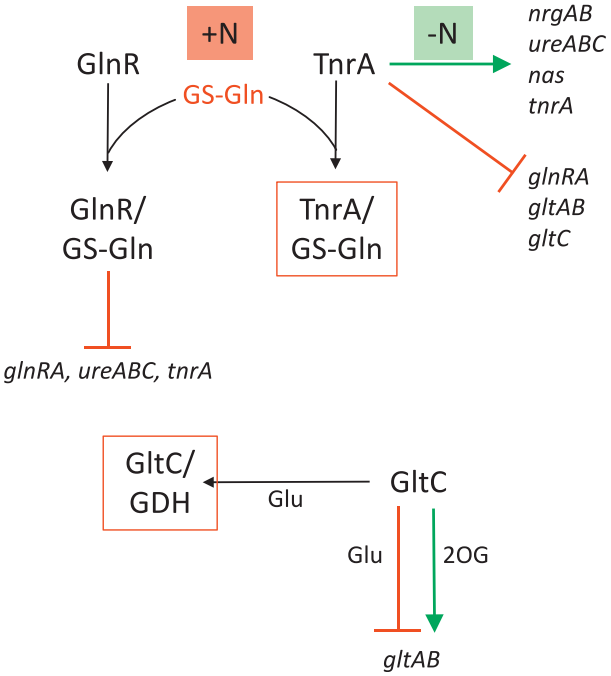

Fig. 11 Nitrogen control in *Bacillus subtilis*. (Top) Scheme of the basic actions of the MerR-family transcriptional regulators TnrA and GlnR. Under N deprivation, TnrA can act as an activator and a repressor of gene expression. Under nitrogen sufficiency (high glutamine cellular levels), a glutamine synthetase (GS)-glutamine complex binds to TnrA blocking its transcriptional activity and to GlnR allowing it to act as a repressor. (Bottom) The LysR-family transcriptional regulator GltC activates expression of the *gltAB* operon encoding glutamate synthase (GOGAT) under N deficiency (high 2-oxogluatarate [2OG] levels). In response to high glutamate levels, GltC acts instead as a repressor of *gltAB* and is blocked by binding of the catabolic enzyme glutamate dehydrogenase (GDH).

regulatory networks. GlnR binds DNA as a dimer, and the C-terminal domain of GlnR (which differs from that of TnrA) has a role inhibiting GlnR dimerization and DNA binding; interaction of this domain with GlnA-glutamine releases such inhibition permitting binding to DNA (Fig. 11). This regulation ensures that operons encoding proteins for the assimilation of nitrogen sources alternative to glutamine, such as *ureABC*, are expressed under nitrogen deficiency and fully repressed under nitrogen sufficiency.

Another layer of N control in *B. subtilis* is exerted by GltC, a LysR-family transcriptional regulator that alternatively binds 2-oxoglutarate or glutamate to activate or repress, respectively, the *gltAB* operon. Activation and repression involves binding to different DNA sites in the intergenic region between the divergent *gltAB* operon and *gltC* gene. Further, the *gltC* gene is negatively auto-regulated and also repressed by TnrA, and the GltC protein is inhibited by interaction with the enzyme glutamate dehydrogenase in the presence of glutamate (Fig. 11). This complex regulation ensures maximal expression of *gltAB* when 2-oxoglutarate is available and glutamate scarce.

This brief summary illustrates the complex system utilized by *B. subtilis* to respond to nitrogen availability and the C-to-N balance. Of special interest is the integration in this system of so-called trigger enzymes, proteins that perform both enzymatic and regulatory functions. Thus, the nitrogen metabolism enzymes glutamate dehydrogenase and glutamine synthetase interact with transcriptional regulators influencing their DNA-binding activities.

Actinobacteria

The Streptomycetes are the most important antibiotic-producing bacteria. A model strain for this bacterial group in laboratory work is *S. coelicolor*, which can use a wide range of nitrogen sources including ammonium, nitrate, urea, amino acids and some amino sugars. Nitrogen control is executed by GlnR, which is an orphan OmpR-family response regulator unrelated to *Bacillus* GlnR. The expression of *S. coelicolor glnR* is regulated by the availability of nitrogen, as it is induced under nitrogen limitation and repressed under nitrogen excess. The GlnR protein produced under nitrogen limitation acts as a dimer activating or repressing different genes. GlnR frequently binds to two tandem DNA boxes with consensus sequence gTnAc-n_6-GaAAc-n_6. Genes activated by GlnR include *amtB* and *glnA*, and genes repressed include *ureA* and *gdhA*, whose expression may be not necessary under nitrogen limitation in this bacterium. Interestingly, GlnR regulates the expression of many carbon assimilation as well as nitrogen assimilation genes.

C. glutamicum has importance in the biotechnological industry as a producer of amino acids, mostly glutamate and lysine. This bacterium shows the capability of assimilating a number of nitrogenous compounds including ammonium, urea or creatinine. Nitrogen control is executed in this organism by the TetR-family transcriptional regulator AmtR that, acting as a dimer, represses at least 33 genes. The AmtR-binding site has the consensus sequence tttCTATn$_6$AtAGat/aA. TetR-family proteins frequently respond to

small-molecule ligands that function as metabolic sensors. AmtR instead responds to the P_{II} protein GlnK. Under nitrogen deprivation, *C. glutamicum* GlnK is adenylylated in response to the increase of the cellular levels of 2-oxoglutarate. Whereas adenylylation/deadenylylation of GlnK is catalyzed by GlnD, the 2-oxoglutarate sensor appears to be GlnK itself. While an AmtR dimer binds to gene promoters repressing gene expression, adenylylated GlnK interacts with AmtR releasing it from DNA and, hence, preventing it from repressing gene expression.

Mycobacterium smegmatis contains both GlnR and AmtR orthologues, of which GlnR appears to be the general N-control protein. GlnR regulates the expression of over 100 genes by binding to about 50 DNA sites with consensus sequence $Gn_2ACn_6GnAACA$, within which ACn_9AC appears to be essential. In contrast, AmtR seems to have some specific roles including repression of the urea assimilation operon in competition with activation of the operon by GlnR. Although less studied than in *M. smegmatis*, the response to nitrogen stress in *M. tuberculosis* appears to be mediated also by GlnR.

In conclusion, the Actinobacteria present two interesting variations in the regulation of gene expression in nitrogen control. *Streptomyces* GlnR appears to be regulated just at the level of its own expression, and *Corynebacterium* AmtR is regulated by interaction with a universal N-to-C balance (2-oxoglutarate) sensor, GlnK.

Concluding Remarks

As with so many other metabolic processes, bacteria display a bewildering diversity in their nitrogen assimilation capabilities, and in the way they regulate them. Bacteria, as a group, are able to effectively assimilate most mineral and organic nitrogen sources, from the simplest, exemplified by dinitrogen, which is among the most inert N compounds and that only bacteria (and archaea) can attack, to complex, man-made xenobiotics, exemplified by atrazine. Many of these capabilities are often found within the same cell under strict regulatory controls governed by economy considerations: N assimilation can be quite demanding energetically. As a result, when given a choice, bacteria prefer the least expensive N source, often ammonium, and organize the regulation of the activity and expression of N assimilation pathways accordingly. Inorganic and most organic N sources eventually yield intracellular ammonium that is incorporated into C skeletons as glutamate and glutamine. The N status of the cell is usually sensed in terms of concentration of 2-oxoglutarate, or the ratio 2-oxoglutarate/glutamine. These signals may be transmitted to a cascade of effectors that finely control both the activity of the key ammonium assimilatory enzymes (notably GS and the ammonium transporter) and their expression and that of alternative N assimilation pathways, often in hierarchical terms.

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Nitrogen Cycle[☆]

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Glossary

Ammonification Generation of ammonium by decomposition of organic matter or by dissimilatory nitrate or nitrite reduction.
Ammonium assimilation Incorporation of ammonium into carbon skeletons to synthesize organic N-compounds for cell growth and metabolism.
Anammox Anaerobic oxidation of ammonium with simultaneous nitrite reduction to dinitrogen coupled to the generation of proton-motive force.
Comammox Complete ammonia oxidation to nitrate coupled to the generation of proton-motive force.
Denitrification Sequential respiratory reduction of nitrate to nitrite, nitric oxide, nitrous oxide and dinitrogen that generates proton-motive force.
Detoxification Transformation of a toxic compound into a less harmful or innocuous product.
Dissimilatory nitrate reduction to ammonium Reduction of nitrate, via nitrite, to ammonium for the generation of proton-motive force, redox balancing or nitrite detoxification.
Nitrate assimilation Reduction of nitrate, via nitrite, to ammonium for its incorporation into C-skeletons.
Nitrification Aerobic oxidation of ammonium to nitrite and nitrate coupled to the generation of proton-motive force.
Nitrifier denitrification Ammonia oxidation to nitrite coupled to nitrite denitrification performed by nitrifying bacteria or archaea.
Nitrogen fixation Reduction of dinitrogen to ammonium for its incorporation into C-skeletons.
Nitrogen mineralization Formation of inorganic nitrogen by degradation of organic N-compounds.
Respiration Process that couples electron transfer from a reduced compound to oxygen or an alternative electron acceptor with generation of proton-motive force.

The Biological Nitrogen Cycle

Nitrogen, an essential element for life, is present in proteins, nucleic acids and many biomolecules. In nature, nitrogen is distributed between three major pools, atmosphere, soil/groundwater and biomass, with oxidation states ranging from +5 in the most oxidized compound (nitrate) to −3 in the most reduced form (ammonia/ammonium). Ammonia (NH_3) predominates at alkaline pH whereas the protonated ionic form, ammonium (NH_4^+), prevails at neutral and acidic conditions. Interconversions of different N-compounds allow a complex nitrogen exchange in the ecosystems that constitutes the so-called nitrogen cycle (Fig. 1), with prokaryotes playing a predominant role. Biological N-cycle includes redox reactions catalyzed by enzymes with different metal cofactors (Table 1), but global N-cycle also includes abiotic processes like atmospheric N-oxide production by lightning and photochemical reactions.

Microbes may use the N-cycle reactions for assimilatory or respiratory/dissimilatory purposes. Assimilatory processes allow nitrogen incorporation into cell material (Fig. 2). Ammonium, the preferred N-source for microbial growth, is directly assimilated forming glutamine and glutamate, but nitrate assimilation in plants and microorganisms occurs through two sequential reductive reactions ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$) catalyzed by assimilatory nitrate and nitrite reductases. Dinitrogen constitutes about 78% of the atmosphere, but only a few free-living or symbiotic N_2-fixing prokaryotes may convert this stable molecule into ammonium through a high energy-consuming reaction catalyzed by nitrogenase.

Microbes also transform N-compounds by respiratory processes for energy conservation or by dissimilatory reactions used for redox balancing or N-detoxification (Fig. 3). Nitrification consists in two separate steps, ammonia oxidation to nitrite ($NH_3 \rightarrow NH_2OH \rightarrow NO_2^-$) performed by ammonia-oxidizing bacteria/archaea (AOB/AOA) and nitrite oxidation to nitrate ($NO_2^- \rightarrow NO_3^-$) carried out by nitrite-oxidizing bacteria (NOB), although some strains fulfill complete oxidation of ammonia to nitrate (comammox). Denitrification is the sequential reduction of nitrate to dinitrogen ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) coupled to proton-motive force (pmf) generation. This allows some prokaryotes to grow anaerobically using these compounds as electron acceptors, although also exist aerobic denitrifiers. Anaerobic ammonium oxidation (anammox) is performed by some planctomycetes that generate dinitrogen

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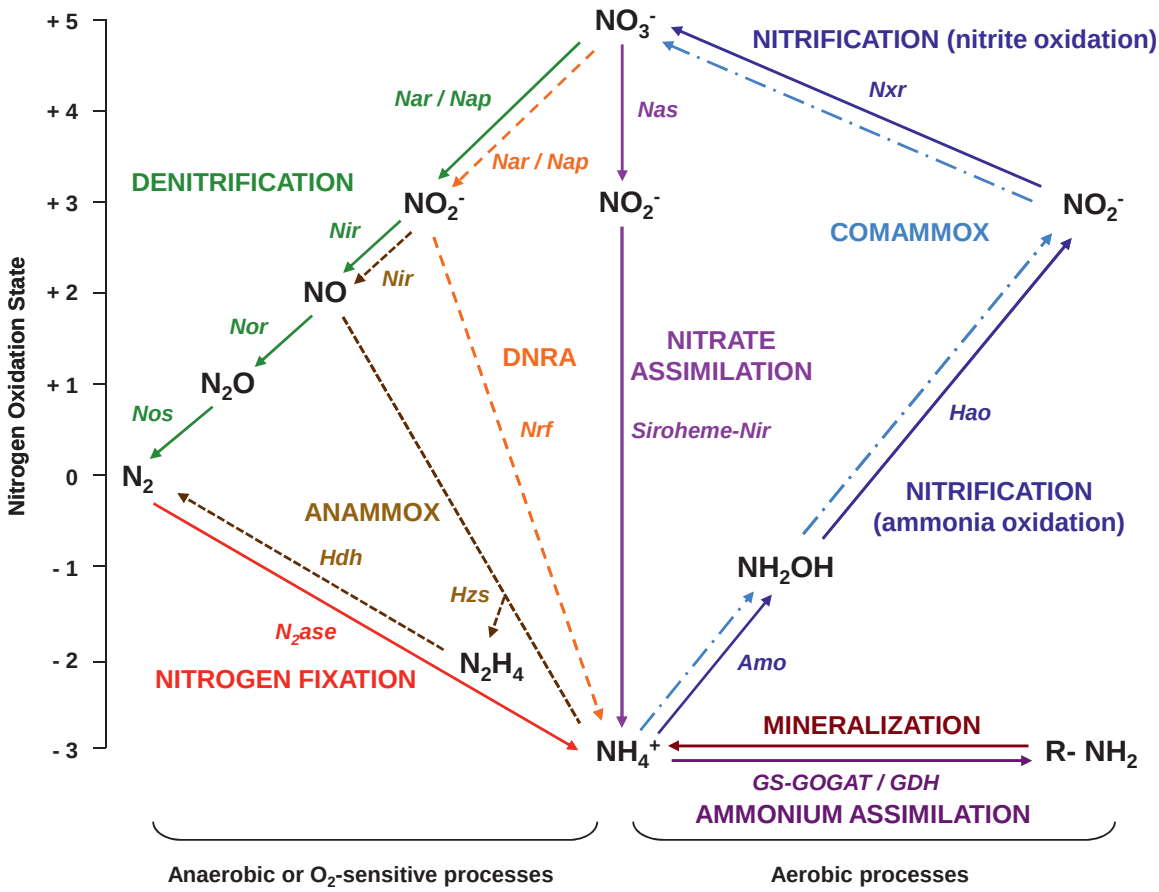

Fig. 1 Overview of the biological nitrogen cycle. The different N-compounds are arranged according to the N-oxidation state. Main oxidative or reductive N-cycle pathways (indicated by arrows), and their corresponding enzymes are shown with different colors. Anaerobic ammonia oxidation (anammox), dissimilatory nitrate reduction to ammonia (DNRA) and complete ammonia oxidation to nitrate (comammox) are indicated by dotted, dashed and dotted/dashed arrows, respectively. Anaerobic processes and oxygen-sensitive reactions are drawn in the left, and aerobic processes are shown in the right.

coupling ammonium oxidation to nitrite reduction ($NH_4^+ + NO_2^- \rightarrow N_2$). The combined action of nitrification, denitrification and anammox causes losses of fixed nitrogen from environments; by contrast assimilatory pathways make nitrogen available for organisms. Finally, different enzymes and pathways are involved in detoxification of harmful N-compounds like nitrite, nitric oxide, hydroxylamine and hydrazine.

In last decades, human activities have altered drastically the natural balance of the N-cycle. Annually, huge amounts of N-compounds enter in the ecosystems by anthropogenic processes such as production of synthetic fertilizers by the Haber-Bosch process and release of N-oxides by fossil fuel combustion.

Ammonium Assimilation

Ammonium assimilation requires the active transport of this ion through Amt permeases and its further incorporation into C-skeletons by the glutamine synthetase-glutamate synthase pathway or the glutamate dehydrogenase enzyme (Table 1). Once glutamine and glutamate are formed, other organic N-compounds are synthesized by a set of secondary N-transfer reactions (Fig. 2).

Glutamine synthetase (GS) catalyzes the ATP-dependent synthesis of glutamine from glutamate and ammonium. In most bacteria and archaea, GS is a dodecameric protein (*glnA* gene product) arranged in two hexagonal rings with a cylindrical aqueous channel, although octameric or hexameric isoenzymes are present in some bacteria. Glutamate synthase (glutamine:2-oxoglutarate amidotransferase, GOGAT) is an iron-sulfur flavoprotein that catalyzes the reductive transfer of the glutamine amide group to 2-oxoglutarate, yielding two molecules of glutamate. NADPH-GOGAT of heterotrophic bacteria is arranged in $\alpha_4\beta_4$ structure with one [3Fe-4S] center and FMN bound to α subunits (*gltB* gene product), FAD bound to β subunits (*gltA* gene product) and two additional [4Fe-4S] centers located in the subunit interface. Cyanobacteria may contain a dimeric NADH-GOGAT or a monomeric ferredoxin-GOGAT, which is similar to the large subunit of NADPH-GOGAT. Glutamate dehydrogenase (GDH) usually catalyzes the oxidative deamination of glutamate, but at high ammonium concentrations (mM) it may promote the reductive incorporation of ammonium to 2-oxoglutarate, acting as an alternative pathway for ammonium assimilation.

Table 1 Structure and cofactors of the main prokaryotic enzymes involved in the processes of the nitrogen cycle

Processes and enzymes	*Enzyme structure (frequent protein name)*	*Subunit molecular mass (kDa)*	*Cofactors*	*Subcellular location*	*Relevant organisms*
Ammonium assimilation					
Glutamine synthetase					
GSI	Dodecamer (GlnA)	50	Mg^{2+}	Cytoplasm	Heterotrophic bacteria, cyanobacteria, phototrophic bacteria, archaea
GSII	Octamer	45–50	Mg^{2+}	Cytoplasm	*Rhizobium*, *Frankia*, *Streptomyces*
GSIII	Hexamer	80	Mg^{2+}	Cytoplasm	*Bacteroides*, *Butyrivibrio*, cyanobacteria
Glutamate synthase					
NADPH-GOGAT	Octamer $\alpha_4\beta_4$ (GltBA)	150 (α, GltB) 50 (β, GltA)	FMN, [3Fe4S] FAD, 2x[4Fe4S]	Cytoplasm	Most bacteria. Archaea have truncated forms (α- or β-like subunits)
Ferredoxin-GOGAT	Monomer	170	FMN, [3Fe4S]	Cytoplasm	Cyanobacteria
Glutamate dehydrogenase					
GDH1, GDH2	Hexamer	45–55	NAD or NADP	Cytoplasm	Most bacteria and archaea
Nitrogen fixation					
Mo-nitrogenase	MoFe-protein (NifDK): Heterotetramer $\alpha_2\beta_2$	55–60 (α,β)	2xFeMoCo ($MoFe_7S_9C$), 2xP-clusters (Fe_8S_7)	Cytoplasm	*Klebsiella*, *Azotobacter*, *Clostridium*, *Rhizobium*, *Frankia*, *Rhodobacter*, cyanobacteria, methanogenic archaea
	Fe-protein (NifH): Dimer	64	[4Fe-4S]		
V-nitrogenase	VFe-protein (VnfDKG): $\alpha_2\beta_2\delta_2$	55–60 (α,β); 15 (δ)	2xFeVCo, 2xP-clusters	Cytoplasm	*Azotobacter chroococcum*, *Azotobacter vinelandii*
	Fe-protein (VnfH): dimer	64	[4Fe-4S]		
Fe-nitrogenase	FeFe-protein (AnfDKG): $\alpha_2\beta_2\delta_2$	55–60 (α,β); 15 (δ)	2xFeFeCo, 2xP-clusters	Cytoplasm	*Rhodobacter capsulatus*, *Azotobacter vinelandii*
	Fe-protein (AnfH): dimer	64	[4Fe-4S]		
Nitrate assimilation					
Nitrate reductase					
NADH-Nas	Heterodimer (Nas)	90–95 (catalytic) 45 (diaphorase)	[4Fe4S], MGD FAD	Cytoplasm	*Klebsiella*, *Bacillus subtilis*, *Rhodobacter capsulatus*, *Paracoccus denitrificans*
Ferredoxin-Nas	Monomer	80–105	[4Fe4S], MGD	Cytoplasm	Cyanobacteria, *A. vinelandii*, *Haloferax mediterraneii* (105- and 50-kDa dimer)
Nitrite reductase					
NADH-Nir	Heterodimer	87 (catalytic) 12 (NirD)	FAD, [4Fe4S], siroheme	Cytoplasm	*Klebsiella*, *Bacillus subtilis*, *Rhodobacter capsulatus*, *Paracoccus denitrificans*
Ferredoxin-Nir	Monomer	55–66	[4Fe4S], siroheme	Cytoplasm	Cyanobacteria, *Haloferax mediterraneii*
Nitrification					
Ammonia monooxygenase (Amo)	Heterotrimer (AmoABC)	27 (AmoA) 38 (AmoB) 32 (AmoC)	Cu, Fe	Cytoplasmic membrane	AOB (*Nitrosomonas*) and AOM. Some *Nitrospira* strains (comammox) with a different AmoA subunit
Hydroxylamine oxidoreductase (Hao)	Homotrimer	63	8xheme *c*	Periplasm	*Nitrosomonas* and AOB. In *Paracoccus* is a 20-kDa monomer with non-heme iron
Nitrite oxidoreductase (Nxr)	Heterodimer (NxrAB)	115 (NxrA)	MGD, FeS	Membrane (nNxr, active site facing cytoplasm; pNxr, active site outside)	*Nitrobacter* (nNxr), *Nitrospira* (pNxr), anammox bacteria (*Kuenenia*)
		65 (NxrB)	FeS		
	Heterotetramer (NxrABIC)		heme *b* (NxrI) and *c* (NxrC)		

(*Continued*)

Table 1 Continued

Processes and enzymes	*Enzyme structure (frequent protein name)*	*Subunit molecular mass (kDa)*	*Cofactors*	*Subcellular location*	*Relevant organisms*
Denitrification					
Nitrate reductase					
Nar	Heterotrimer (NarGHI)	140 (α, NarG) 60 (β, NarH) 25 (γ, NarI)	[4Fe4S], MGD [3Fe4S], 3x [4Fe4S] 2xheme *b*	Membrane-bound (active site facing cytoplasm)	*E. coli*, *Paracoccus denitrificans*, *Pseudomonas*. In archaea, the active site faces outside membrane (pNar)
Nap	Heterodimer (NapAB)	90 (NapA) 15 (NapB)	[4Fe4S], MGD 2xheme *c*	Periplasm	*E. coli*, *R. sphaeroides*, *Bradyrhizobium*, *Paracoccus*, *Desulfovibrio* (monomeric 70-kDa NapA)
Nitrite reductase					
cd_1-Nir (NirS)	Dimer	60	Heme *c*, heme d_1	Periplasm	*Pseudomonas*, *Paracoccus*, *Kuenenia*
Cu-Nir (NirK)	Trimer	40	Cu_I, Cu_{II}	Periplasm	*Rhodobacter sphaeroides*, *Alcaligenes*, *Achromobacter*, nitrifiers, *Jettenia*
Nitric oxide reductase					
cNor	Heterodimer (NorBC)	53 (NorB) 17 (NorC)	2xheme *b*, Fe_B heme *c*	Cytoplasmic membrane	*Pseudomonas stutzeri*, *Paracoccus denitrificans*, *Bradyrhizobium*
qNor	Monomer (NorB)	80	heme *b*, Fe_B	Cytoplasmic membrane	*Ralstonia eutropha*, *Pyrobaculum aerophilum*, *Neisseria*
Nitrous oxide reductase					
Nos	Homodimer (NosZ)	65	Cu_A, Cu_Z (heme *c* in clade II cNos)	Periplasm	*Pseudomonas stutzeri*, *Paracoccus denitrificans*, *Wolinella* (clade II)
Anammox					
Hydrazine synthase (Hzs)	Heterotrimer	61	8xheme *c*	Anammoxosome	*Kuenenia*, *Brocardia*, *Anammoxoglobus*, *Scalindua*
Hydrazine dehydrogenase (Hdh)	Heterodimer	62	8xheme *c*	Anammoxosome	*Kuenenia*, *Brocardia*, *Anammoxoglobus*, *Scalindua*
DNRA				**(ammonification)**	
Nitrite reductase NrfA	Heterotetramer NrfABCD Heterodimer NrfAH	60 (catalytic NrfA)	5xheme *c*	Cytoplasmic membrane	*E. coli*, *Desulfovibrio*, *Wolinella succinogenes*
Nitrite reductase NirBD	Heterodimer (NirBD)	90 (NirB) 12 (NirD)	FAD, [4Fe4S], siroheme [2Fe2S]	Cytoplasm	*E. coli*

GS, the primary ammonium-assimilating enzyme, is target of a complex regulation at both transcriptional (*glnA* gene expression) and post-translational (GS activity) levels. In enterobacteria, GS activity is regulated by reversible adenylylation/deadenylylation of a tyrosine residue of each subunit. Thus, under N-starvation (high 2-oxoglutarate; low glutamine) deadenylylated GS is fully active whereas under N-excess (low 2-oxoglutarate; high glutamine) GS is adenylylated to decrease its activity. The GS adenylylation state is controlled by the trimeric PII protein (GlnB), a 2-oxoglutarate sensor that is uridylylated/deuridylylated by an uridylyltransferase/uridylyl-removing bifunctional enzyme. Unmodified GlnB stimulates GS adenylylation (inactivation) whereas uridylylated GlnB promotes GS deadenylylation (activation). Additionally, *glnA* gene expression is controlled by the two-component general nitrogen regulatory system Ntr. At low nitrogen, the NtrB sensor phosphorylates an aspartic residue of NtrC, and phosphorylated NtrC activates transcription of *glnA* and other N-regulated genes, which are recognized by the RNA polymerase σ^{54} factor (RpoN). At high nitrogen, deuridylylated GlnB promotes NtrC dephosphorylation through NtrB, decreasing transcription of *glnA* and N-regulated genes.

This regulation model for ammonium assimilation is widely distributed among bacteria, although some bacteria lack Ntr system. In cyanobacteria the main nitrogen regulator is NtcA, a CRP-family transcription factor. NtcA activates *amt1* (ammonium permease) and *glnA* (GS), but represses the *gifAB* genes encoding small polypeptides of 7- and 17-kDa (IF7 and IF17) that inactivate GS under N-excess. Therefore, GS is not adenylylated but controlled by inactivating factors IF7/IF17. In addition, PII protein is phosphorylated rather than modified by uridylylation. Both PII and NtcA proteins bind 2-oxoglutarate, and their signaling and transcriptional activities seem to be mutually dependent.

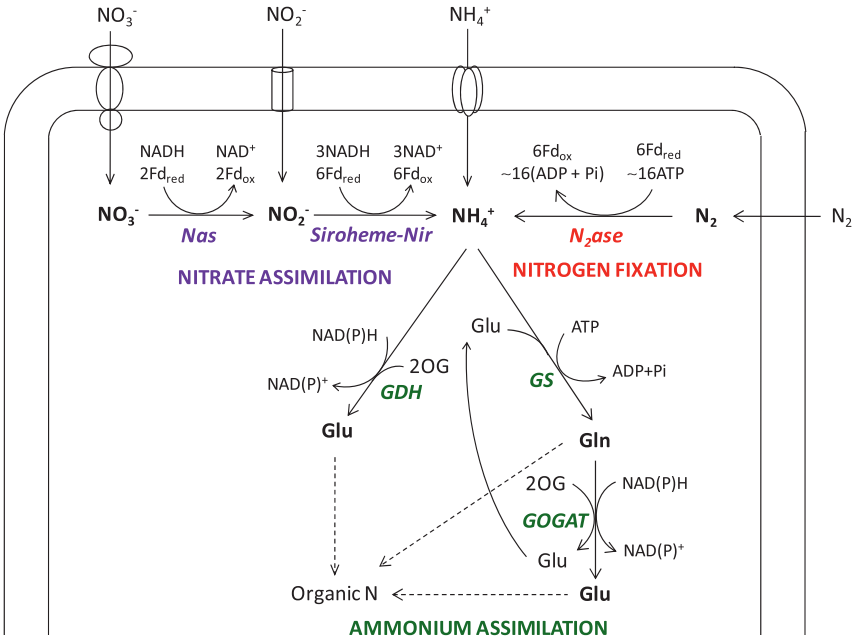

Fig. 2 Assimilatory N-cycle processes. Microbes may use different N-sources for growth, including both organic and inorganic N-compounds. In organic compounds, nitrogen is usually found in its fully reduced state, and therefore, ammonium assimilation only requires the transport of this ion inside the cells (Amt transporter) and its further incorporation into C-skeletons either by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway or the glutamate dehydrogenase (GDH) enzyme. Assimilation of oxidized N-compounds (nitrate, nitrite) requires the specific oxyanion uptake by ATP- or pmf-dependent transporters and their reduction by the Mo-containing nitrate reductase (Nas) and the siroheme-containing nitrite reductase (siroheme-Nir). These assimilatory enzymes use either NADH or reduced ferredoxin (Fd_{red}) as electron donor. In N_2-fixing bacteria and archaea, dinitrogen gas is reduced to ammonium by the enzyme nitrogenase (N_2ase). Ammonium formed by nitrate/nitrite reduction or nitrogen fixation is mainly incorporated into cell material by the GS-GOGAT pathway. 2OG, 2-oxoglutarate.

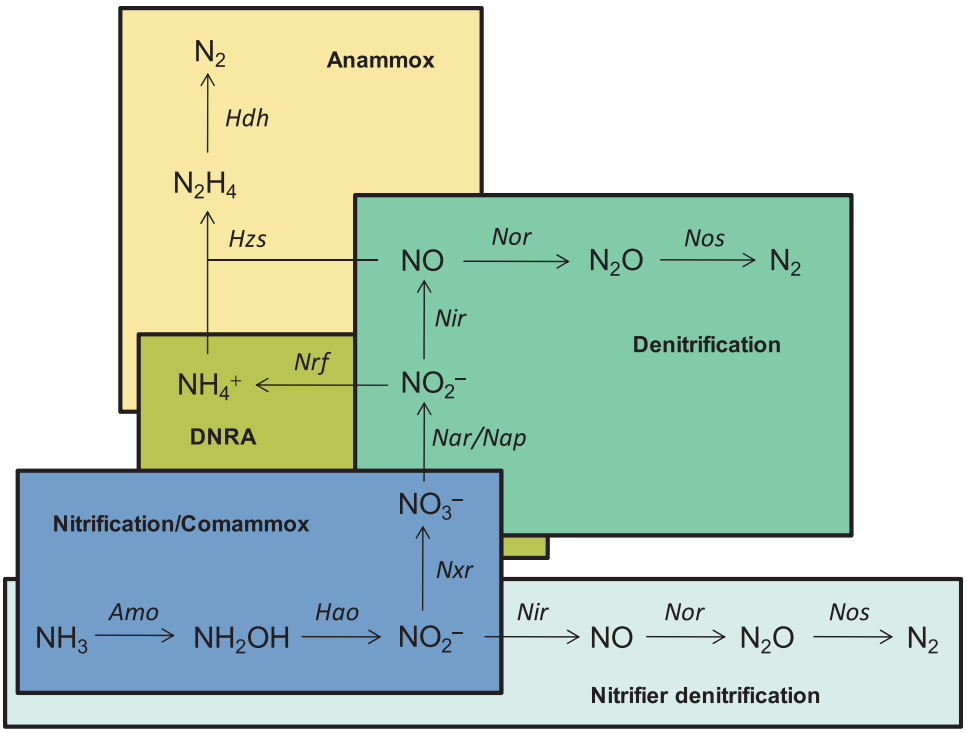

Fig. 3 Respiratory and dissimilatory N-cycle processes. Substrates, intermediates, products and enzymes of denitrification, nitrification, complete ammonia oxidation to nitrate (comammox), nitrifier denitrification, anaerobic ammonia oxidation (anammox) and dissimilatory nitrate reduction to ammonia (DNRA) are indicated. These processes are boxed in different colors.

Nitrogen Fixation

Biological nitrogen fixation is only performed by some bacteria and archaea, but provides a major source of available (fixed) nitrogen for organisms in both terrestrial and marine environments. Nitrogenase catalyzes the six-electron reduction of dinitrogen to ammonium, with a high consumption of ATP, and also forming H_2 during the process:

$$N_2 + 8e^- + 10H^+ + 16MgATP \rightarrow 2NH_4^+ + H_2 + 16MgADP + 16P_i$$

Prokaryotes may fix dinitrogen either free-living or in symbioses or associations with plants. Free-living diazotrophs may grow under anaerobic, microaerobic or aerobic conditions, but in this case, as nitrogenase is oxygen-labile, different mechanisms are used to protect the enzyme against oxygen. In the most representative *Rhizobium*-legume symbiosis, N_2-fixation occurs inside plant root nodules, specialized structures that allow bacterial growth under microaerobiosis. Actinobacteria (*Frankia*) establish symbioses with non-leguminous trees and cyanobacteria (*Nostoc*) with bryophytes, pteridophytes and angiosperms (*Gunnera*). Some crops also obtain part of their N-demand by association with N_2-fixing proteobacteria in the root surface (*Azospirillum*) or that colonize plant tissues as endophytes (*Azoarcus*, *Herbaspirillum*)

Three types of nitrogenases containing molybdenum, vanadium or iron are known (Table 1). Mo-nitrogenase, the main enzyme, consists of a catalytic MoFe-protein (dinitrogenase) and an electron transfer Fe-protein (dinitrogenase reductase), which binds ATP and also participates in MoFe-protein maturation. The $\alpha_2\beta_2$ tetrameric MoFe-protein (encoded by *nifDK* genes) contains 2 Mo, 30 Fe and 32 S atoms arranged in two pairs of special metallocenters, FeMo-cofactor and P-cluster. The FeMo-cofactors, composed of $MoFe_7S_9C$-homocitrate, are bound to the α-subunits and the P-clusters, composed of Fe_8S_7 ([4Fe-4S] and [4Fe-3S] centers sharing one S), are located at the α/β-subunit interfaces. The homodimeric Fe-protein (*nifH* gene product) has a single [4Fe-4S] center bridging both subunits. The catalytic mechanism involves a Fe-protein cycle consisting in complex formation, electron transfer to MoFe-protein, ATP hydrolysis and reduction of the oxidized Fe-protein with the physiological donor (ferredoxin or flavodoxin), and a MoFe-protein cycle that includes the reactions within the MoFe-protein for substrate reduction.

Nitrogen fixation is regulated at transcriptional and post-translational levels in response to ammonium and oxygen, although different regulatory mechanisms are involved depending on the organism. In *Klebsiella*, *nif* gene expression is controlled by the regulatory *nifLA* genes, which respond to the general Ntr system. NifA is a σ^{54}-dependent transcriptional activator and NifL is a flavoprotein that inactivates NifA in response to oxygen. In anoxygenic phototrophic bacteria, post-translational regulation of nitrogenase allows a rapid response to O_2 and NH_4^+ (switch-off/switch-on) through reversible ADP-ribosylation of Fe-protein by the DraT/DraG system, which in turn, is regulated by PII protein. In response to ammonium, DraT catalyzes the transference of ADP-ribose from NAD^+ to a Fe-protein subunit, preventing nitrogenase complex formation and leading to enzyme inactivation. At low ammonium, DraG removes the ADP-ribose group to activate nitrogenase.

Establishment of legume-rhizobia symbiosis requires the formation of a specialized organ, the nodule, where N_2-fixation occurs. Nodulation is a specific interaction in which a rhizobial strain infects a defined plant-host, with a close bacteria-plant coordination based on exchange of diffusible signal molecules. Plant-secreted flavonoids act as specific signals recognized by compatible bacterial strains, which respond by attaching to root-hairs and inducing nodulating (*nod*) genes that activate infection events. Cortical cell divisions generate the nodule primordium and bacteria enter the plant through an intracellular tubular structure, the infection thread, which grows and ramifies into the nodule primordium. Bacteria released into the cells are enclosed in the symbiosome, a compartment where they differentiate into N_2-fixing bacteroids. The nodules maintain low oxygen levels to avoid nitrogenase inhibition, and leghemoglobin plays an important role as O_2-scavenger. Bacteroids use dicarboxylic acids supplied by the plant to generate ATP and reducing power for N_2-fixation, and ammonia formed is assimilated in host cells by the GS-GOGAT pathway. Fixed nitrogen is exported through the xylem from nodules to the rest of the plant, either as amides (asparagine) or ureides (allantoin or allantoic acid).

Nitrate Assimilation

Higher plants, fungi, algae, archaea and bacteria may use nitrate as N-source for growth by a process that requires the transport of nitrate inside the cells and its further reduction to ammonium, which is incorporated into C-skeletons by GS-GOGAT. Assimilatory nitrate and nitrite reductases use either NADH or reduced ferredoxin as electron donor (Fig. 2, Table 1). Nitrate uptake may occur either by ATP-dependent ABC transporters that include a periplasmic substrate-binding protein, a homodimeric transmembrane protein and a homodimeric or heterodimeric cytoplasmic ATPase, or by pmf-dependent MSF permeases that act as H^+/NO_3^- symporters.

Prokaryotic nitrate reductases are metalloenzymes with a Mo-*bis*-molybdopterin guanine dinucleotide (Mo-*bis*-MGD) cofactor that catalyze the two-electron reduction of nitrate to nitrite, although cytoplasmic assimilatory nitrate reductases (Nas) are structurally and functionally different from the periplasmic (Nap) or membrane-bound (Nar) enzymes involved in dissimilatory/respiratory processes. Ferredoxin-dependent nitrate reductases are monomeric proteins that contain a [4Fe-4S] center and Mo-*bis*-MGD, but NADH-dependent Nas require an additional FAD-containing oxidoreductase that mediates electron transfer from NADH to the catalytic Mo-containing subunit. Assimilatory nitrite reductases catalyze the six-electron reduction of nitrite to ammonium and contain an iron-sulfur center and siroheme, a special porphyrin with eight carboxylic acid peripheral sidechains,

but NADH-dependent nitrite reductases also contain FAD. They are also completely different in structure and function to the cytochrome cd_1 (NirS) or copper (NirK) nitrite reductases involved in denitrification, or the dissimilatory pentaheme NrfA enzyme of some bacteria.

In *Paracoccus denitrificans*, the nitrate reductase catalytic subunit (NasC), the FAD-containing nitrite reductase (NasB) and a Rieske-type protein with a [2Fe-2S] cluster (NasG) form a functional NasBGC complex in which NasG mediates electron transfer from an unique NADH-binding site present in NasB to the nitrate and nitrite reduction sites located in NasC and NasB, respectively. Phylogenetical analyses suggest that a composite nitrate/nitrite reductase system similar to the NasBGC complex is widespread among bacteria.

Nitrate assimilation is usually regulated by nitrate induction (pathway-specific control) and ammonium repression (general N-control), but significant differences exist depending on the organism. In cyanobacteria, N-control is exerted by the NtcA transcription factor, which is influenced by the 2-oxoglutarate PII sensor, and the specific positive control by nitrate involves the LysR-family NtcB protein and other regulators. In *Klebsiella*, ammonium control is mediated by the NtrBC system, whereas nitrate induction requires the transcription antitermination protein NasR. Under N-limitation the Ntr system activates *nas* gene expression, but without nitrate transcription terminates prematurely. However, in presence of nitrate, NasR acts as transcription antiterminator binding to mRNA and allowing *nas* gene expression. In other bacteria nitrate control requires a two-component system composed of a nitrate sensor (NasS) and a response regulator (NasT), which also acts as transcriptional antiterminator. In *Paracoccus denitrificans*, ammonium and nitrate exert a complex control involving both transcriptional and translational mechanisms through the NtrBC and NasTS systems. The *nas* genes are transcribed and translated only when nitrate is present, and ammonium absent. In presence of ammonium the genes are not transcribed, and in absence of ammonium and nitrate the genes are transcribed but not translated.

Nitrification

Classical nitrification consists on the aerobic oxidation of ammonium to nitrate in two separate steps, the oxidation of ammonium to nitrite via hydroxylamine and the subsequent oxidation of nitrite to nitrate, carried out by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively (Fig. 3). These chemolithoautotrophic bacteria (represented by *Nitrosomonas* and *Nitrobacter* species) use energy released in ammonia or nitrite oxidation to support CO_2 fixation and growth. Electron transfer to oxygen generates an electrochemical gradient that drives ATP synthesis and NAD reduction by reverse electron transport. Different ammonia-oxidizing archaea (AOA) have been also described, in addition to *Rhodopseudomonas* and *Thiocapsa* strains that use nitrite oxidation for supporting anoxygenic photosynthesis, and heterotrophic and methanotrophic bacteria that oxidize ammonia without gaining energy for growth. Cross-feeding interactions and co-aggregation of AOB/AOA and NOB is frequently observed. Thus, reciprocal feeding allows the use of cyanate as nitrification substrate since NOB have a cyanase that converts cyanate into CO_2 and ammonia, which is oxidized by AOB to form nitrite used by NOB. However, until the very recent discovery of some *Nitrospira* strains that perform complete ammonia oxidation to nitrate by the so-called comammox process, all known nitrifiers were unable to fulfill both nitrification steps despite complete nitrification is energetically advantageous.

In *Nitrosomonas* and other AOB, ammonia is first oxidized to hydroxylamine by ammonia monooxygenase, a multimeric transmembrane complex (AmoABC) with Cu and non-heme iron. Hydroxylamine is further converted to nitrite by Hao, a periplasmic trimeric octaheme *c* hydroxylamine oxidoreductase (Table 1). Electrons are passed to the quinol pool through cytochrome c_{554} and a NapC-family membrane-anchored *c*-type cytochrome. Ubiquinol is oxidized by the cytochrome bc_1 complex, which transfers electrons to oxygen via an aa_3-type oxidase generating pmf, and by an NADH-ubiquinone oxidoreductase that generates NADH through reverse electron transfer. Ammonia-oxidizing archaea (AOA) contain *amo*-like genes but lack hydroxylamine oxidation/detoxification genes, suggesting a different mechanism for archaeal ammonia oxidation. Instead of hydroxylamine, archaeal Amo may produce nitroxyl (HNO), which is oxidized to nitrite by a putative nitroxyl oxidoreductase.

Nitrite oxidation in NOB requires a multimeric membrane-bound nitrite oxidoreductase (Nxr) with Mo-*bis*-MGD, iron-sulfur centers and heme groups. The Mo-containing catalytic subunit may have a periplasmic location (pNxr), contributing to pmf generation directly, or faces the cytoplasm and electrons from nitrite oxidation flow to an electrogenic aa_3-type cytochrome oxidase (nNxr). Both types of Nxr evolved independently (*Nitrospira* pNxr is related to DMSO reductases whereas *Nitrobacter* nNxr is similar to Nar) and were probably spread among NOB by lateral gene transfer.

Nitrite-oxidizers can be involved in complex associations with different microorganisms. NOB diversity was also revealed by the unexpected discovery of *Nitrospira* strains that perform complete ammonia oxidation to nitrate (comammox) using an ammonia monooxigenase with an AmoA subunit different from those of AOB/AOA. Therefore, identification of *Nitrospira*-like *amoA* genes provides evidence of comammox. Metagenomic analysis reveals that comammox is widespread in natural ecosystems and wastewater treatment plants sludge.

Denitrification

In denitrification, nitrate, nitrite and gaseous nitric and nitrous oxides are used as terminal electron acceptors for anaerobic respiration, generating dinitrogen as final product. This four-step pathway is sequentially catalyzed by the enzymes nitrate, nitrite,

nitric oxide and nitrous oxide reductases (Fig. 3, Table 1). Many bacteria and archaea denitrify under oxygen-limited conditions, but some organisms also perform aerobic denitrification. Other microbes are not considered true denitrifiers because they develop an incomplete process, releasing nitrite or emitting N_2O to the atmosphere.

Nitrate reduction, the first denitrification step, is catalyzed by membrane-bound respiratory (Nar) or periplasmic (Nap) nitrate reductases. Most bacterial Nar are heteromeric complexes including a catalytic protein with Mo-*bis*-MGD and one [4Fe-4S] cluster (NarG), an electron transfer protein with one [3Fe-4S] and three [4Fe-4S] centers (NarH), and a quinol-oxidizing dihem *b* membrane protein (NarI). In these systems (known as nNar) the NarG active site faces the cytoplasm and electrons pass from the membrane quinol pool to nitrate via NarI heme groups and NarH iron-sulfur clusters allowing pmf generation by a redox loop mechanism. Therefore, nitrate must be incorporated inside the cells whereas nitrite needs to be exported to the periplasm. The *Paracoccus denitrificans narK* gene encodes a transmembrane protein with a NarK1 domain that functions as NO_3^-/H^+ symporter to initiate respiration and a NarK2 domain acting as NO_3^-/NO_2^- antiporter. In archaea exists a similar nNar with the active site in the cytoplasm and a pNar with the active site located outside the membrane, coupled to a quinone-cycle for energy-conservation through diheme (NarC) and Rieske-type (NarB) proteins.

Periplasmic nitrate reductases were initially described in phototrophic and denitrifying bacteria, but they are widespread among proteobacteria. The Nap system is usually composed of a large catalytic subunit containing [4Fe-4S] and Mo-*bis*-MGD (NapA) that receives electrons from a diheme-*c* cytochrome (NapB). A quinol-oxidizing, membrane-anchored tetraheme-*c* protein (NapC) donates electrons to NapB, but some bacteria lack NapB or NapC and/or include a quinol-oxidizing iron-sulfur NapGH complex. In addition, NapF participates in the assembly of the [4Fe-4S] center into NapA, and NapD is involved in NapA maturation. Aerobic denitrifiers couple Nap enzyme to nitrite and N-oxide reductases, but besides of its role in denitrification, Nap also functions in redox balancing (dissipation excess reducing power).

Nitrite reductases involved in denitrification are periplasmic enzymes that catalyze the one-electron reduction of NO_2^- to NO. There are two types of denitrifying nitrite reductases, the homodimeric containing *c*-type (electron entry site) and d_1-type (catalytic site) heme groups, and the trimeric Cu-NirK which includes a type-I Cu site that mediates electron transfer to a catalytic type-II Cu. Both enzymes receive electrons from periplasmic *c*-type cytochromes or cupredoxins that are connected with the cytochrome bc_1 complex, making electron transfer from quinones to nitrite electrogenic. It was believed that denitrifying bacteria contain either NirS or NirK, but denitrifiers with both enzymes have been recently described.

Membrane-bound nitric oxide reductases catalyze the reduction of NO to N_2O. Most denitrifying bacteria have a heteromeric NO reductase (cNor) constituted by a small heme *c*-containing subunit (NorC) and a catalytic subunit (NorB) that binds heme *b* and a dinuclear heme b_3 and non-heme iron (Fe_B) center. Electrons from pseudoazurin or *c*-type cytochrome pass to heme *c* (NorC) and then to *b*-heme groups and Fe_B (NorB), where NO is reduced to N_2O. Some bacteria have a quinol-dependent monomeric enzyme (qNor) that contains only the dinuclear center (heme *b* and Fe_B). *Bacillus azotoformans* possesses an unusual Cu_A-containing dimeric variant (Cu_AqNor) that uses menaquinol or cytochrome *c* as reductant.

Reduction of N_2O to N_2 is catalyzed by a periplasmic homodimeric nitrous oxide reductase (NosZ) with two copper centers, a catalytic tetranuclear Cu_Z and a dinuclear Cu_A that contributes to enzyme stabilization and receives two-electrons from cytochrome bc_1 via cytochrome *c* or cupredoxin. Many non-denitrifying N_2O-reducing bacteria and archaea act as real N_2O sinks since they do not generate, but remove, this greenhouse gas. Genome sequencing reveals that NosZ from these organisms usually belongs to clade II, a phylogeny lineage distinct from NosZ of typical denitrifying bacteria (clade I). Clade II proteins are translocated to the periplasm by the Sec pathway whereas most clade I NosZ contain the twin-arginine motif for the Tat pathway. The *Wolinella succinogenes* NosZ (clade II) includes an additional monoheme-*c* domain that receives electrons from menaquinol through the membrane iron-sulfur proteins NosGH.

A proteomic-based interactomic approach in *Pseudomonas aeruginosa* revealed that all denitrification enzymes (NarGHI/NirS/NorBC/NosZ), nitrate transporter (NarK2) and regulatory or enzyme maturation proteins (NarXL, NirFMN, NosFL) form a functional membrane-attached supercomplex that interacts with NADH dehydrogenase, ATPase, TCA cycle enzymes, Sec-translocon components, and flagella-assembly proteins. This dynamic supercomplex probably optimizes electron transfer and energy conservation, reducing reactive oxygen species formation.

Denitrification is controlled by multiple regulatory systems that basically respond to oxygen, nitrate/nitrite and N-oxides, but showing differences among microorganisms. These regulators include FNR/CRP-superfamily oxygen (FNR, ANR), nitrate (NarR) and nitric oxide (NNR, DNR) sensors, oxygen-responsive (FixLJ, ArcAB) or nitrate-responsive (NarXL, NarQP) two-component systems, and NO-sensing Fe-S flavoproteins (NosR, NirI), non-heme iron σ^{54}-transcritional activators (NorR) or Rrf-family transcriptional repressors (NsrR).

Considering that nitrite is an intermediate of both nitrification and denitrification, ammonia oxidation to nitrite may be coupled to denitrification in the so-called nitrifier denitrification, which allows AOB to detoxify nitrite accumulated during nitrification (Fig. 3). Under oxygen-limitation AOB oxidize ammonia to nitrite via hydroxylamine, and nitrite is further reduced to N_2O or dinitrogen by denitrification enzymes ($NH_3 \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Ammonia-oxidizing archaea (AOA) also perform nitrifier denitrification, but they could generate N_2O by a different mechanism (via HNO instead of NH_2OH). When nitrifiers and denitrifiers are present in the same environment, nitrification and denitrification can be coupled to remove both ammonia and nitrate ($NH_3 \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). On the other hand, heterotrophic nitrification may occur in some denitrifiers like *Paracoccus denitrificans*, where a hydroxylamine oxidoreductase with non-heme iron catalyzes oxidation of hydroxylamine to nitroxyl radicals that dimerize to form N_2O (under anoxia) or react with oxygen forming nitrite (in aerobiosis). Methane oxidation coupled to denitrification has been also described. Metagenomic sequencing has revealed

that some denitrifying methanotrophs form N_2 without N_2O synthesis because a putative NO dismutase catalyzes the direct conversion of NO into N_2 generating oxygen ($2NO \rightarrow N_2 + O_2$) that supports methane oxidation.

Anaerobic Ammonium Oxidation

Anaerobic ammonium oxidation to dinitrogen with nitrite as electron acceptor (anammox) allows some chemolithoautotrophic bacteria (Planctomycetes) to grow using the exergonic reaction $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$ as primary source of energy (Fig. 3). Metagenomic studies have revealed that anammox bacteria significantly contribute to removal of fixed nitrogen in oceans, terrestrial environments and wastewater treatment plants. Genomics also provided information about the anammox pathway, which proceeds in three steps that generate two highly toxic intermediates, NO and hydrazine (N_2H_4). First, nitrite is reduced to NO by cd_1-NirS (*Kuenenia*, *Scalindua*), Cu-NirK (*Jettenia*) or a novel Hao-like nitrite reductase (*Brocadia*). Then, a heterotrimeric heme *c*-containing hydrazine synthase (Hzs) combines NO and ammonia to form hydrazine, which is finally oxidized to N_2 by a homotrimeric octaheme-*c* hydrazine dehydrogenase (Hdh) homologous to Hao of AOB (Table 1). Anammox bacteria have special ladderane lipids that confer impermeability and rigidity to the membranes of the anammoxosome, a compartment that internalizes the anammox enzymes reducing hydrazine losses. The four electrons released in the Hdh reaction are transferred to the proton-pumping cytochrome bc_1, yielding energy. Cytochrome *c* donates the three electrons required for the Hzs reaction and the single electron consumed in nitrite reduction. In addition, anaerobic oxidation of nitrite to nitrate by a Nxr-like nitrite oxidoreductase provides electrons required for C-fixation by the acetyl-CoA pathway. Anammox substrates are also formed by an Nrf-type nitrite reductase, which converts nitrite into ammonium, and a Hao-family multiheme hydroxylamine oxidase, which synthesizes NO from hydroxylamine.

Other Processes of the N-Cycle

Nitrogen mineralization is the process by which inorganic nitrogen is obtained by decomposition of organic N-compounds. Extracellular proteolytic enzymes of saprophytic bacteria and fungi are responsible for protein degradation to small peptides and amino acids, which are finally degraded releasing ammonium. Therefore, this process is also known as ammonification, although this term is used as well for other N-cycle ammonium-generating processes.

Dissimilatory nitrate reduction to ammonium (DNRA), or nitrate ammonification, is an anaerobic process in which nitrate reduction to nitrite by Nar or Nap enzymes is followed by the dissimilatory six-electron reduction of nitrite to ammonium catalyzed by a periplasmic pentaheme cytochrome *c* nitrite reductase (NrfA), which receives electrons from a membrane NapC-family cytochrome (NrfH) or a menaquinol-oxidizing complex (NrfBCB), or by a cytoplasmic NADH-dependent, siroheme-containing nitrite reductase (Table 1). The role of these enzymes is to detoxify nitrite formed in nitrate respiration rather than to generate ammonium for N-assimilation.

Many N-compounds are toxic for organisms. Therefore, a number of enzymes play a role in detoxification of these harmful molecules. Some of these enzymes detoxify NO by its oxidation to nitrate (flavohemoglobin Hmp) or its reduction either to N_2O (Nor, flavorubredoxin, and flavo-diiron proteins) or ammonium (nitrite reductase NrfA). Hydroxylamine detoxification is also achieved by reduction to ammonium (cd_1-Nir, NrfA) or oxidation to nitrite (Hao).

Environmental Aspects of N-Cycle, Human Impact and Perspectives

Human impact affects the N-cycle at global scale, with relevant environmental, agronomic and health implications. Actually, over half of the N-fixed that enters in ecosystems has anthropogenic origin, and human activities also modify composition and dynamics of microbial populations in terrestrial, freshwater and marine environments. The excessive use of synthetic fertilizers in agricultural practices leads to accumulation of nitrate and nitrite in groundwater causing eutrophication of aquatic ecosystems and human health risks, since consumption of drinking water with high nitrate may produce methaemoglobinaemia and gastric cancer. Fossil fuel combustion is also the major source of N-oxide emissions that contribute to global warming and other environmental hazards. Thus, N_2O is a stable gas with about 300-fold more greenhouse effect than CO_2 and also participates in photochemical reactions that destroy the stratospheric ozone layer. In addition, atmospheric NO may be oxidized to nitrogen dioxide (NO_2), which after hydration generates HNO_3 causing acid rain. Symbiotic nitrogen fixation provides a source of fixed N for plant growth without the need for fertilizers. Establishment of artificial symbiosis or associations between diazotrophic organisms and agronomic plants, or potential transfer of N_2-fixing capacity to mayor crops, could reduce the demand for chemical fertilizers. Nitrification, denitrification and anammox are the main pathways for N-losses in natural ecosystems and wastewater treatment plants, but these processes have also negative effects since significantly contribute to N_2O emissions. A better understanding of the major N-cycle processes and their relative contribution as N_2O sources or sinks will enable to improve ecosystem management and to develop tools for mitigation the negative effects of anthropogenic N-inputs.

Understanding of the role of microbial populations and consortia in N-cycle was limited by the fact that vast majority of bacteria present in an ecosystem are non-cultivable by traditional microbiology techniques. However, modern genomics and metagenomics

tools are changing our knowledge of these communities in natural habitats and their impacts on N-cycle. Comparative genomics reveals important networks of microbial populations, contributing to the identification of novel protein functions and metabolisms, and bringing to light that diversity of microorganisms involved in N-cycle and variety of their functions are much higher than previously thought. Thus, omic-based approaches have changed our view of N-cycle with the elucidation of the anammox pathway and the discovery of new processes like comammox, nitrifier denitrification and methane oxidation coupled to denitrification, among others.

Genome-resolved information allows the elaboration of new concepts on the biochemical origin of biological diversity, shifting our traditional knowledge based on particular processes and organisms (i.e., diazotrophs, nitrifiers, denitrifiers) into a global view that integrates the distinct gene/protein modules for microbial adaptation or survival in different environments. This emerging view emphasizes the importance of microbial interactions, metabolic by-products exchange and symbioses, and provides new inputs to translate this information into useful models for understanding global changes and predicting N-cycle fluxes to mitigate greenhouse gas emissions.

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No Bones About It: The Bacterial Cytoskeleton

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Glossary

Cytokinesis Splitting of the cytoplasm of a mother cell into multiple daughter cells.
Cytoskeleton A subcellular structure composed of assembled proteins that is important for organization of cellular components.
DAPI 4′,6-diamidino-2-phenylindole, a fluorescent dye that binds to DNA, excited by UV light and emitting in the blue wavelengths.
Immunofluorescence Fixed cells are incubated with a primary antibody against a protein, then a secondary antibody with a fluorescent tag that binds the primary antibody, which results in a fluorescent signal reflecting the location of the protein.
Immunogold Like immunofluorescence, except that the secondary reagents are nanometer-sized gold particles, which can only be observed with transmission electron microscopy.
Nucleoid The structured and packaged bacterial chromosome.
Segregation Movement of duplicated chromosomes or plasmids from the mother cell to each daughter cell; also called partitioning.

Introduction

As animal and protozoan cells have no wall, their cytoskeleton provides their required structure, organization and shape. Plant cells have walls, but their cytoskeleton also guides where the wall is synthesized and thus their growth and shape. These eukaryotic cells rely on three general classes of cytoskeletal proteins: tubulins, actins, and intermediate filaments. All three of these proteins form polymers. Because polymers can extend for large distances, they are capable of translating the structural organization of proteins at the molecular level (nanometers) into large scale organization at the cellular level (micrometers). For example, the regulation of tubulin polymerization into microtubules and their depolymerization back into tubulin monomers, along with the interaction among different microtubules, help to define the direction and forces during mitosis and cytokinesis, serve as tracks for intracellular transport, and provide structural strength needed for locomotion.

Because bacteria and archaea are small and have cell walls, and most have no obvious intracellular structures visible by electron microscopy, it was thought until recently that they had no equivalent of the cytoskeleton or cytoskeletal proteins. However, about 20 years ago this idea was swept aside by three concurrent revolutions. The first was the ability to sequence multiple bacterial genomes, which revealed striking similarity between certain bacterial proteins and eukaryotic actin and tubulin. The second was the large number of protein atomic structures that were solved, allowing proteins with limited sequence similarity to be compared for structural similarity. The third was the ability to visualize genetically-encoded fluorescent tags in intact bacterial cells. Fusion of a jellyfish green fluorescent protein (GFP) or its derivatives to any protein of interest results in a fluorescent pattern that reflects the localization of only that protein in a living cell. Consequently, it was discovered that proteins such as bacterial homologs of tubulin, actin and intermediate filaments localized in specific and dynamic patterns in bacterial cells, often as polymers. Genetic experiments on these proteins indicated that they play crucial roles in cell growth and shape, consistent with their hypothesized cytoskeletal functions, and biochemical studies confirmed that they could assemble into polymers in vitro. These findings and discoveries of uniquely bacterial cytoskeletal proteins have ushered in a new era of bacterial cell biology, providing unexpected insights into the evolution of the cytoskeleton.

The Bacterial Tubulin Cytoskeleton – FtsZ

Prior to 1991, little was known about subcellular organization of most bacteria, particularly those such as *Escherichia coli* that have no obvious internal structure. The breakthrough occurred when the FtsZ protein, essential for cell division in most bacteria, was successfully localized by immunogold labeling to a ring (called the Z ring) at the division site at the cell midpoint. This Z ring is comprised of polymers of FtsZ that are anchored to the inner surface of the cytoplasmic membrane at the cell center. FtsZ is a GTPase, and it assembles into protofilaments in a GTP-dependent manner. This GTP-dependent assembly, along with high structural similarity to tubulin confirmed by crystal structures and the sharing of a short sequence called the "tubulin signature", clearly indicates that FtsZ is a tubulin homologue. This was surprising at the time, given that animal cell cytokinesis uses a ring of actin, not tubulin. FtsZ is widely conserved throughout several kingdoms of life. Its homologs are present not only in most bacteria, but also in plant chloroplasts, where they aid in chloroplast fission, and in a large branch of the archaea (euryarchaea), where their roles are still unclear but may be involved in regulating cell shape.

Although it is a tubulin homologue, FtsZ is clearly a primitive version, as it does not form hollow microtubules, nor does it consist of two distinct alpha and beta subunits. Nevertheless, there is considerable evidence that FtsZ likely acts at a level higher than single protofilaments. For example, lateral interaction among FtsZ protofilaments is stimulated *in vitro* by several proteins, including Zaps (Z associated proteins) in *E. coli*, and there is evidence for FtsZ protofilament bundling *in vivo* by these proteins as well. Other bacterial species feature Zap-like proteins, or have FtsZ proteins with high intrinsic ability to laterally interact. Such interactions are likely regulated, as too much or too little lateral interaction between FtsZ protofilaments can interfere with normal cell division.

The Z ring is highly dynamic. When *E. coli* grows in rich medium, Z rings at mid-cell form shortly after the birth of newborn cells, but do not begin to constrict until considerably later in the cell division cycle, guiding the growing division septum behind it (Fig. 1). When the ring finally constricts, studies with FtsZ–GFP fusions indicate that FtsZ subunits are released from the ring into the surrounding cytosol. This suggests that constriction of the Z ring, and subsequent cytokinesis, is driven by the loss of subunits from protofilaments that are bound to the cytoplasmic membrane. In addition to this turnover during ring contraction, the rate of turnover of FtsZ subunits between the ring and cytosol is very rapid, with a half-time of <10 s. This suggests that the pool of cytoplasmic FtsZ is large and mobile, and it has been estimated that 60%–70% of the FtsZ in the cell is not in the ring.

There have been some recent breakthroughs in the mechanistic understanding of the Z ring and how its dynamics drive formation of the division septum. Super-resolution microscopy studies using 3D structured illumination microscopy (3D-SIM) and Photoactivated Localization Microscopy (PALM) suggest that the Z ring is not a continuous polymer or polymers, but instead comprised of several aggregates of FtsZ polymers distributed in a loose network (Fig. 2, Movie 1). Recently, super-resolution fluorescence microscopy was crucial for discovering that these FtsZ aggregates migrate processively around the circumference of the Z ring by a mechanism called treadmilling. Treadmilling, often observed with eukaryotic filamentous actin and microtubules, occurs when protein subunits are added preferentially to one end of a polymer while removed from the opposite end, and is a way

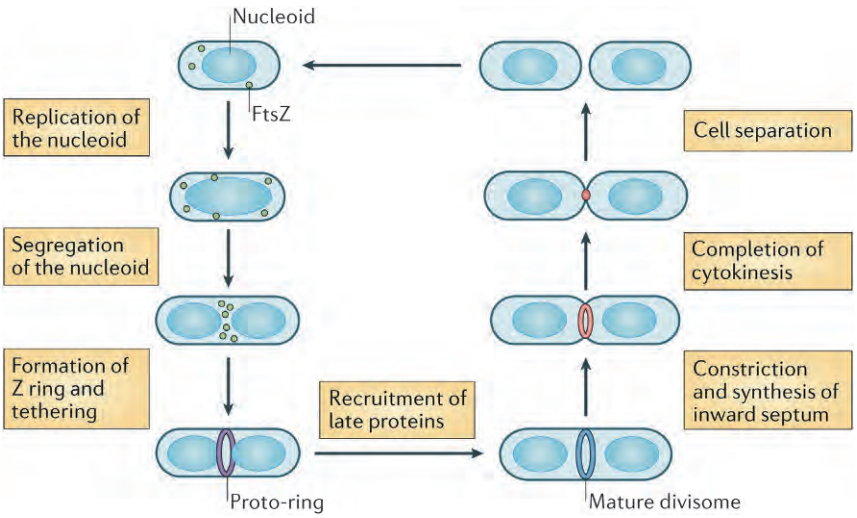

Fig. 1 Cell division cycle of *E. coli*. Reprinted from Haeusser, D.P., Margolin, W., 2016. Splitsville: Structural and functional insights into the dynamic bacterial Z ring. Nature Reviews Microbiology 14, 305–319. https://dx.doi.org/10.1038/nrmicro.2016.26.

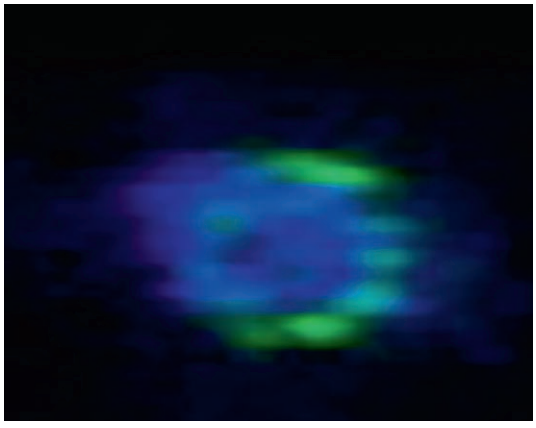

Fig. 2 FtsZ ring structure of *E. coli* in cross section. 3D-SIM image of a fixed *E. coli* cell expressing FtsZ-GFP (green) and stained with DAPI (blue) to visualize the nucleoid.

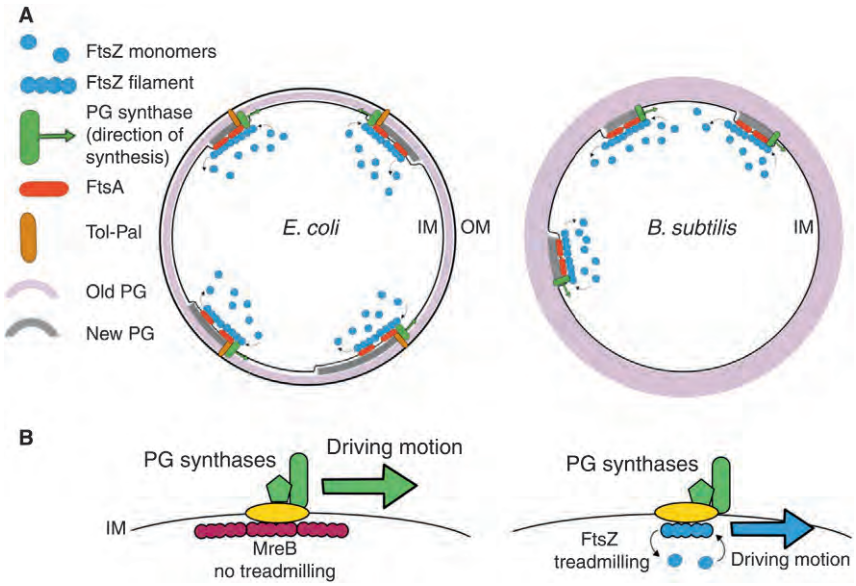

Fig. 3 FtsZ treadmilling helps to build the division septum in *E. coli* and *B. subtilis*. (A) cross-sectional view of the division ring, showing several treadmilling FtsZ/FtsA complexes that organize inward synthesis of the division septum and coordinate invagination of the outer membrane in *E. coli* using Tol/Pal proteins. (B) Detailed view of a sidewall synthesis complex organized by non-treadmilling MreB filaments (left) and a division septum synthesis complex organized by treadmilling FtsZ filaments (right). PG = peptidoglycan. Reprinted from Schoenemann, K., Margolin, W., 2017. Bacterial division: FtsZ treadmills to build a beautiful wall. Current Biology 27, 301–303. https://dx.doi.org/10.1016/j.cub.2017.03.019.

to achieve large-scale movement of a polymer by regulating monomer-monomer interactions at the nanometer scale. The treadmilling speed of FtsZ polymers is regulated by GTP hydrolysis and has an important role in regulating the dynamics of cell wall synthesis enzymes needed to form the division septum, which also comigrate with the FtsZ polymers around the cell circumference (Fig. 3). However, it is not yet known how the treadmilling FtsZ protofilaments coordinate with each other or interact with the many additional membrane-bound proteins attached to the Z ring. Other high resolution methods to explore the nanometer range such as whole-cell cryo-electron tomography (see below) will likely provide further structural insights into the structure of the FtsZ cytoskeleton.

Supplementary material related to this article can be found online at https://dx.doi.org/10.1016/B978-0-12-811736-1.90741-4.

Like microtubules, FtsZ assembly is the target of several inhibitors. Some are naturally occurring, such as viriditoxin; others are synthetic, including benzamide derivatives such as PC58538. Other FtsZ inhibitors include small proteins that are produced endogenously, such as SulA or MciZ of *E. coli* or *Bacillus subtilis*, respectively, in response to specific environmental conditions. In addition, peptides encoded by infecting bacteriophages, such as Kil from phage lambda, specifically inhibit FtsZ assembly and may confer a selective advantage during phage infection.

Alternative Tubulin-Like Cytoskeletal Elements

FtsZ is now just one prominent member of a family of bacterial tubulins (Fig. 4). Another family includes bacterial microtubules (bMTs) of the Verrucomicrobial family of bacteria, encoded by two genes, bTubA and bTubB. As the name implies, these tubulin homologs are much more like tubulin than FtsZ: they coassemble to form GTP-dependent heterodimers, and in the cell form long narrow microtubule-like structures consisting of 4–5 protofilaments instead of the 13 protofilaments that comprise eukaryotic microtubules. Interestingly, although eukaryotic tubulin requires chaperones for proper folding and function, these mini-microtubules do not. This suggests that bTubA and bTubB evolved from a more primitive microtubule system that was probably acquired by horizontal gene transfer. But like other microtubules as well as FtsZ, the mini-microtubules can treadmill, and even display rapid depolymerization (dynamic instability) typical of microtubules. The function of these mini-microtubules in the physiology of these organisms is not yet known, although their proximity to cellular projections in their prosthecate bacterial hosts suggests that they may be involved in growth and/or rigidity of these projections. Furthermore, the ability of the mini-microtubules to mimic eukaryotic microtubules without the need for chaperones will likely be very useful for synthetic biology applications, including constructing artificial microtubules.

Another bacterial tubulin family is encoded by large plasmids of the Bacillus group, including *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. The *B. thuringiensis* member of this family, called TubZ, is encoded by the pBtoxis plasmid that also encodes the well-known insect toxin. TubZ, which is a divergent form of FtsZ that also has significant similarity to tubulin, forms highly dynamic polymers that curve along the cytoplasmic membrane of both the native host and *E. coli* by treadmilling. Such behavior may reflect a role in plasmid partitioning. A protein similar to TubZ, called RepX, is encoded by pXO1, the toxin-encoding

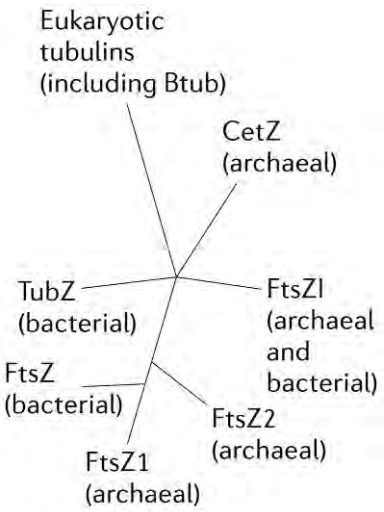

Fig. 4 Phylogeny of tubulins. Reprinted from Wagstaff, J., Löwe, J., 2018. Prokaryotic cytoskeletons: Protein filaments organizing small cells. Nature Reviews Microbiology 16, 187–201. https://dx.doi.org/10.1038/nrmicro.2017.153.

plasmid of *B. anthracis*. This protein is essential for plasmid maintenance, although its localization and dynamics have not yet been characterized. TubZ is double helical, which suggests that in contrast to FtsZ, TubZ has a mitotic function more similar to that of eukaryotic microtubules.

Another example of a mitotic-like function of a bacterial tubulin comes from a set of large bacteriophages that infect certain *Pseudomonas* species. These phages encode their own homologue of tubulin, called PhuZ, which assembles into a three-stranded polymer that forms a mitotic spindle-like structure extending the length of the cell during phage infection. The PhuZ filaments in this spindle display dynamic instability, which, as with microtubules, results in rapid depolymerization of the entire structure. This dynamic cell-spanning spindle nucleates at both cell poles, with each half growing unidirectionally and tethered to a developing nucleus-like compartment that harbors the phage replication factory at midcell. The pushing forces from this PhuZ spindle end up organizing and centering the replication compartment.

Finally, just as bacterial tubulins are not limited to FtsZ, FtsZ homologs are not limited to bacteria. A large branch of the archaea called euryarchaea carry two paralogs of FtsZ, both of which are likely involved in cytokinesis of these bacteria-like cells. Multiple paralogs of FtsZ are also widespread in plant chloroplasts, where they assemble as rings to divide and distribute these organelles. FtsZ paralogs are also present in the mitochondria of protists such as *Dictyostelium*. However, FtsZ is not present in archaea outside of the euryarchaea, nor is it present in mitochondria of higher eukaryotes.

The Bacterial Actin Cytoskeleton

Bacteria also have multiple homologs of actin that are involved in everything from cell division (FtsA), plasmid partitioning (ParM and AlfA), organelle organization (MamK), and regulation of cell growth and chromosome segregation (MreB) (Fig. 5). The most widely conserved of these are FtsA and MreB, and in species such as *E. coli*, these proteins are essential for viability.

FtsA is a divergent homologue of actin that is required for proper cell division in a wide variety of species, although it is absent in archaea and in some families of bacteria such as the Actinobacteria. It localizes to the Z ring, binds directly to FtsZ, and its localization is dependent on functional FtsZ. Unlike actin, FtsA has a C-terminal membrane targeting sequence, which is required for tethering dynamic FtsZ protofilaments to the inner surface of the cytoplasmic membrane. Purified FtsA from *Streptococcus pneumoniae* forms helical pairs of protofilaments, and purified *E. coli* FtsA binds tightly to lipid membranes and forms curved polymers on them, often as closed miniring forms (Fig. 6). Although FtsA binds ATP like actin, FtsA subunits structurally interact in a different orientation compared with subunits of F-actin, and the atomic structures of FtsA from *Thermotoga maritima* and *Staphylococcus aureus* already suggest that different FtsA proteins polymerize in distinct orientations from each other. The membrane targeting sequence also strongly influences FtsA's oligomerization properties, because when it is removed, FtsA forms straight bundled polymers that fail to bind to the membrane.

Recent high resolution studies with FtsZ and FtsA have shed light on how this primitive tubulin-actin interaction functions in the cell. When purified FtsZ is added to FtsA on lipid membranes in the presence of ATP, FtsZ polymers align and move by treadmilling, mimicking their behavior in the cell. Although it is not yet known if FtsA minirings exist in cells, when assembled on lipid monolayers they promote alignment of FtsZ protofilaments but inhibit them from directly interacting. Cryo-electron tomography of intact bacterial cells has shown that FtsA polymerizes closest to the membrane, with FtsZ polymers assembling parallel to the FtsA polymers. It should be emphasized that in addition to binding FtsZ and tethering it to the membrane, FtsA is truly multifunctional as it also acts to recruit other proteins to the cell division machinery.

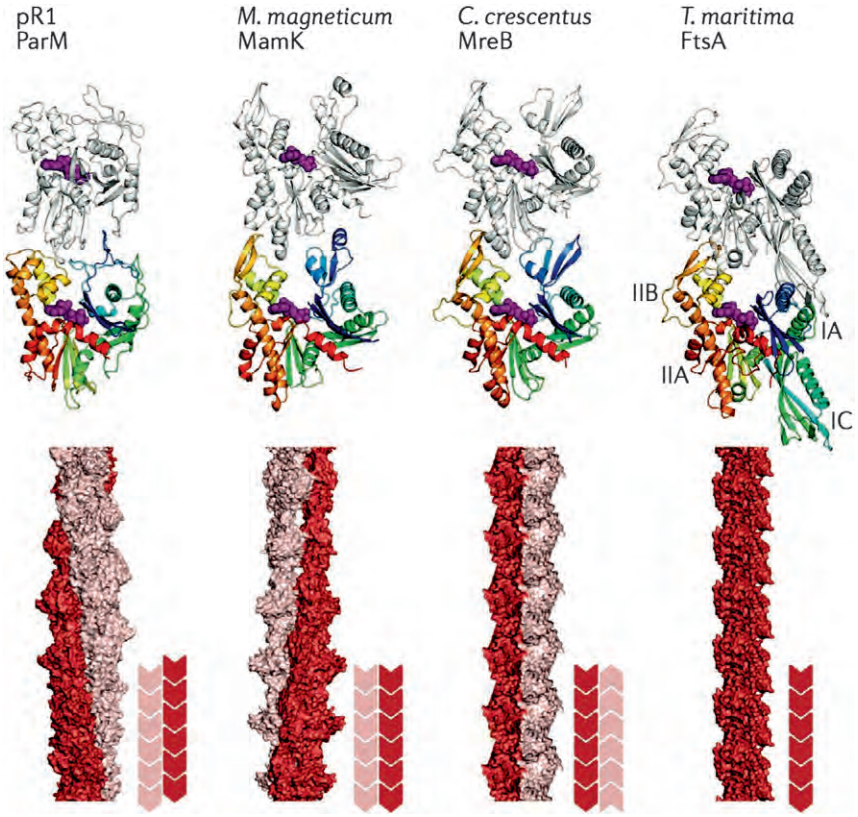

Fig. 5 Structure of diverse bacterial actins. Atomic structures of dimers are shown above schematics of protofilament structures and arrangements for each. Reprinted from Wagstaff, J., Löwe, J., 2018. Prokaryotic cytoskeletons: Protein filaments organizing small cells. Nature Reviews Microbiology 16, 187–201. https://dx.doi.org/10.1038/nrmicro.2017.153.

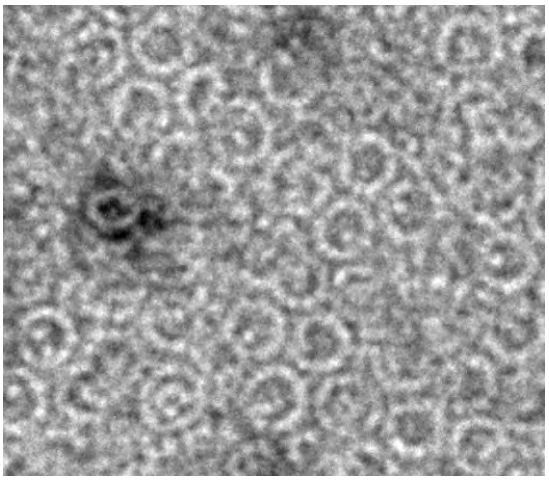

Fig. 6 Minirings formed by *E. coli* FtsA on a lipid monolayer. Transmission EM image of FtsA minirings on a lipid monolayer after negative staining by uranyl acetate.

MreB was originally identified by a mutation that caused normally rod-shaped *E. coli* cells to round up. The MreB protein is highly similar to actin, and like F-actin, MreB assembles into double stranded protofilaments in the presence of ATP. However, an important distinction is that MreB filaments are antiparallel, which should prevent them from net directional movement by treadmilling. In rod-shaped cells such as *E. coli* or *B. subtilis*, MreB was originally found to form a helix along the length of the cell. In *B. subtilis*, two other MreB-like paralogues also exist (Mbl and MreBH) that also appear to form helical filaments with somewhat different localization patterns. Recent high resolution microscopic imaging of MreB suggests that MreB forms mobile patches and

short filaments on the membrane instead of or in addition to continuous helices (Movie 2), and these patches move around the membrane circumferentially in response to cell wall biosynthetic activity. Although these short filaments depend on cell wall synthesis for their mobility, they function to target wall synthesis to regions of negative membrane curvature. It is thought that this self-reinforcing feedback mechanism results in maintenance of rod shape. Moreover, local insertion of peptidoglycan mediated by these MreB patches results in left-handed twisting of the entire cell during growth. During the cell cycle of *Caulobacter crescentus* or *E. coli*, a portion of MreB moves from the sidewall to the Z ring. The function of this relocalization may be to redirect cell wall biosynthesis to the area of septum growth. If MreB localization to the Z ring is driven by a direct interaction with FtsZ, which has been suggested for *E. coli*, it would be another example of interaction between a tubulin and actin in bacteria in addition to the FtsA–FtsZ interaction.

Supplementary material related to this article can be found online at https://doi.org/10.1016/B978-0-12-811736-1.90741-4.

The absence of MreB in cocci, as well as the loss of rod shape when MreB is inactivated, is entirely consistent with these functions of MreB in rod-shaped bacteria. Like FtsA, MreB interacts with the cytoplasmic membrane using an amphipathic helix, in this case at its N terminus. MreB also interacts with MreC and MreD, which are transmembrane proteins that are encoded within the same operon as *mreB*. Other proteins interact with MreB and colocalize with MreB. In *E. coli*, these include MurG, a key cell wall biosynthetic enzyme, and FtsZ itself (see below). In a striking evolutionary twist, the Chlamydia family of bacteria, which are obligately intracellular, lack FtsZ and instead use MreB to divide by binary fission.

Intriguingly, a number of rod-shaped bacteria such as the rhizobia, corynebacteria, and mycobacteria lack MreB homologs, prompting the question of how they achieve cylindrical growth without MreB as a guide. Labeling of nascent cell wall synthesis of *Corynebacterium glutamicum* by fluorescent vancomycin indicates that its new wall growth occurs solely at the cell poles, suggesting that this may be a mechanism for the other MreB-less rod-shaped species as well. Streptomycetes, which contain MreB homologs, also grow primarily at cell poles or by initiating branches, which are essentially new poles. Streptomycetes do not require MreB for vegetative growth but instead to coordinate growth during sporulation. In the case of streptomycetes and corynebacteria, a protein called DivIVA is crucial for proper polar growth. In both species, disruption of DivIVA causes severe shape abnormalities, and DivIVA localizes mostly at the cell poles, indicating that it orchestrates wall growth at the cell tips. Interestingly, DivIVA is cytoskeletal-like, as it has weak homology to myosins and forms oligomeric structures. Moreover, DivIVA can localize to cell poles in non-native species, including *E. coli* and even fission yeast, suggesting that it recognizes negative membrane curvature or a specific phospholipid domain that is enriched at that curvature. The common thread among rod-shaped bacteria that lack MreB seems to be polar growth, which would obviate the need for a cytoskeletal coordination of sidewall growth. Indeed, like their Gram positive corynebacterial counterparts, Gram negative rhizobia were shown to grow by unipolar extension by using recently developed fluorescent amino acid analogs such as HADA.

By analogy to actin, MreB is subject to inhibition by specific small molecules. One of these, called A22, rapidly abolishes the regular localization of MreB in cells, suggesting that it disassembles MreB polymers. Continued exposure to A22 eventually causes cells that depend on MreB for rod shape to round up. Interestingly, when A22 is added to *C. crescentus* cells, chromosome segregation is inhibited relatively rapidly, before any cell shape changes become visible. The inhibitory effect is mainly exerted on the chromosomal origins of replication, which fail to migrate properly when MreB is inactivated by A22. This suggests that MreB is involved in chromosome segregation, although any role of MreB in this process remains unclear as it has been difficult to rule out indirect effects of cell growth perturbation on replication origin segregation.

Bacterial Actin-Like Proteins are Ubiquitous

In addition to crenactin, which is a homologue of actin present in some crenarchaea (Thermoproteales) that is implicated in cytokinesis, diverse actin families in bacteria are still being discovered and characterized. The prototype for these is ParM, which is encoded by an *E. coli* plasmid, R1. ParM assembles into a left-handed double helical filament that extends bidirectionally between two replicated plasmids, helping them move apart into daughter cells. The mitotic spindle-like ParM filaments connect to the plasmid DNA via a kinetochore-like protein adaptor, ParR, which binds to a specific DNA sequence element (*parC*) on the plasmid. By themselves, ParM polymers are ATP-dependent and dynamically unstable, so like microtubules, they are prone to rapid depolymerization once their growing ends become destabilized. This rapid depolymerization occurs after ATP hydrolysis, and can be inhibited by the presence of a bound ParR-parC "cap" at the filament ends. The ParM/ParR/*parC* system has recently been reconstituted *in vitro*. The purified ParR/*parC* complex binds to the ends of ParM polymers, riding along the growing filament ends and inhibiting the dynamic instability of the polymers. It is likely that ParM filaments provide the mechanical force to push *parC*-containing plasmids apart.

Other ParM-like actin homologs are present in diverse species, and their overall mechanisms of action are similar. For example, AlfA promotes segregation of a low copy *B. subtilis* plasmid by forming a highly dynamic polymer bundle. Like ParM, AlfA uses an adaptor protein (AlfB) to bind to plasmid DNA in order to move plasmids apart in the cell. But unlike ParM, AlfA polymers can elongate in one direction and are thus able to move by treadmilling. The regulation of the AlfA spindle-like apparatus occurs through the activity of AlfB. When bound to its DNA target, AlfB permits AlfA to polymerize into a spindle. However, when unbound to DNA, AlfB instead destabilizes the AlfA polymer bundle, allowing AlfA to reassemble into a new spindle that will push the plasmids farther apart. AlpC is another well-studied actin homologue that forms a mitotic spindle-like structure, this time encoded by a prophage present in *C. glutamicum*. Like ParM, AlpC binds to specific sites on phage DNA (*alpS*) through an adaptor protein, AlpA, and undergoes dynamic instability. When AlpA is not bound to DNA, it promotes AlpC disassembly; however, when

bound to its DNA target, AlpC is free to polymerize into the spindle structure that spatially organizes phage DNA and promotes its replication.

In addition to mitotic spindle structures, actin-like proteins have been found to be important for forming and positioning organelles in certain bacteria. The best known of these is MamK, an actin homologue from the magnetotactic bacterium *Magnetospirillum magneticum*. These bacteria rely on the earth's magnetic field to guide their motility, and contain iron-containing organelles called magnetosomes that need to be anchored in place in order to align the cells properly with the magnetic field. MamK accomplishes this by assembling into dynamic, ATP-dependent filaments that stabilize the chain-like magnetosomes, preventing their diffusion, and ensuring their proper partitioning into daughter cells. MamK filaments form independently of MreB, and like those of many bacterial actins, these filaments extend for significant lengths within the cell and consist of double stranded parallel protofilaments (Fig. 5). Partitioning of the organelle occurs after the magnetosome chain is severed by cytokinesis and remains at the new poles of the daughter cells, at which point MamK helps to reposition the magnetosomes.

Bacterial Intermediate Filaments

The third major eukaryotic cytoskeletal element, intermediate filaments (IFs), is also represented in at least one bacterial species. *C. crescentus* is so named because of its vibrioid crescent shape. A protein called crescentin is required for this shape, because a null mutant in the gene it encodes results in straight, rod-shaped *C. crescentus* that otherwise retains its cell polarity. Crescentin localizes as a long polymeric structure to the concave side of the cell, suggesting that it is directly or indirectly associated with the inhibition of growth that causes the opposite side of the cell to curve outward. However, a function in membrane rigidity, analogous to the function of many intermediate filaments in eukaryotes, cannot be ruled out. Remarkably, when crescentin is produced in *E. coli*, it induces cells to become curved, although producing it in the more closely related *Agrobacterium tumefaciens* had no effect on its rod shape. This effect indicates that crescentin is sufficient to induce curvature, perhaps because both *E. coli* and *C. crescentus* contain MreB and grow by sidewall synthesis, in contrast to *A. tumefaciens*, which lacks MreB and grows by polar extension. Purified crescentin self-assembles into long filaments that are 10–20 nm wide and independently of nucleotides, characteristic of IFs such as vimentin. Crescentin filaments in cells are not dynamic, which makes their nucleation and distribution during cell division open questions. Another open question is how many bacterial IFs exist; IFs share low sequence similarity, so it is difficult to know how widely conserved such IF homologs might be in bacteria without directly testing candidate genes and proteins for activity.

Other bacterial proteins have IF-like properties. Like crescentin, some of them are involved in controlling cell growth and shape. For example, two IF-like proteins, Scy and FilP, localize near growth zones at the hyphal tips of *Streptomyces*, forming a protein machine along with DivIVA. As mentioned earlier, DivIVA is the scaffold for tip growth. Scy colocalizes with DivIVA, indicating that both proteins are present where nascent peptidoglycan is being synthesized. FilP, on the other hand, lags behind and localizes in its own sub-polar zone, distal from the pole and from DivIVA and Scy. It is thought that Scy is directly involved in wall synthesis at the tip, while FilP acts to provide a rigid scaffold for the growing tip. How FilP and Scy partition their activity and their localization, especially given their structural similarity, is not known.

Polymerizing Walker a Cytoskeletal Atpases

There are many other bacterial proteins that form filaments, including AglZ of myxobacteria and CTP synthase of *E. coli*, that are not homologs of eukaryotic cytoskeletal proteins. However, one family of ATPases that forms polymers and generally displays cytoskeletal behavior deserves mention: the Walker A cytoskeletal ATPases (WACA). These proteins share a deviant Walker A motif, KGGXXGKT, with two conserved lysines that are crucial for ATP binding and hydrolysis. These proteins generally assemble into polymers using ATP, and their ability to dynamically relocate in the cell in response to other factors has important roles in cellular organization. One founding member of this family is MinD, which in *E. coli* teams up with a small protein called MinE to oscillate from cell pole to cell pole. Like FtsA and MreB, MinD uses an amphipathic helix to bind to the inner surface of the cytoplasmic membrane, where it recruits an inhibitor of FtsZ assembly called MinC. Without MinE, MinD binds the membrane statically. But the binding of MinE to MinD stimulates the latter's ATPase activity, dislodging it from the membrane where it can diffuse, rebind ATP, and rebind the membrane at the opposite cell pole (Movie 3). The bipolar gradient of MinC and MinD resulting from the pole to pole oscillation promotes maximum FtsZ assembly at the midpoint between the cell poles, thus helping to define the cell division site. Another WACA is ParA, which powers the assembly of its DNA binding partner, ParB, on chromosomal DNA and helps to partition chromosomes in some bacterial species. Other ParA-like proteins include Soj and SopA, which oscillate over the nucleoid. Like MinD, their dynamic relocalization requires interaction with another protein factor. Both ParA and MinD form polymers in vitro, although the physiological relevance of this polymerization is still controversial. However, it is notable that MinC and MinD form alternating copolymers that are similar to those formed by septins, which are cytoskeletal GTPases that form ring structures in eukaryotic cells used for protein assemblies and cellular compartmentalization.

Supplementary material related to this article can be found online at https://dx.doi.org/10.1016/B978-0-12-811736-1.90741-4.

Other WACAs important for cellular organization in bacteria include MipZ of *C. crescentus* and PomZ of *Myxococcus xanthus*. Like MinD, both proteins play an important role in positioning the Z ring, and loss of function of either causes cell division defects. MipZ forms a bipolar gradient similar to that of MinD, except there is no pole to pole oscillation. Instead, like ParA, MipZ binds to ParB, which in turn binds to a region of chromosomal DNA near the origin of replication (oriC) that serves as a centromere-like sequence. MipZ is also an inhibitor of FtsZ assembly, like MinC. The result of these activities is that FtsZ is prevented from assembling near the

middle of *C. crescentus* cells until oriC is duplicated and partitioned to daughter cells. PomZ was discovered more recently and functions as a positive spatial regulator of Z ring assembly instead of a negative regulator. Along with its partner proteins PomX and PomY, PomZ localizes to the nucleoid near midcell prior to FtsZ and promotes Z ring assembly.

The Cytoskeleton of Wall-Less Mycoplasmas

This article has dealt mainly with cells that have walls, and use cytoskeletons to guide their growth and organization. Mycoplasmas, on the other hand, are very small gliding bacteria that lack cell walls. Their cytoskeleton assumes an even greater importance, as it is necessary for the distinctive behavior and morphologies of these organisms. Probably the most well-characterized part of their cytoskeleton is the terminal organelle. In *Mycobacterium pneumoniae*, this organelle is required for pulling the daughter cells apart during cytokinesis as well as for adherence to host cells. The organelle is an extension of the cell that by electron microscopy consists of paired parallel bars ending with a button-like structure. Several proteins are part of the organelle, including HMW2 and P28, which are part of the electron-dense core, and HMW3, a major component of the terminal button. Other proteins localizing to the terminal organelle include P1, P30, P41, and P65. Duplication of new terminal organelles takes place adjacent to an existing one, followed by migration of one organelle to the opposite side of the cell. Gliding temporarily stops during this process, then continues to help pull the cell poles apart during cytokinesis. Many mycoplasma species have an FtsZ homologue that is probably required for cytokinesis, but lack other homologs of cell division proteins. This suggests that FtsZ alone may provide the force to pinch mycoplasma membranes during cytokinesis. However, one species of mycoplasma, *Ureaplasma urealyticum*, lacks an FtsZ homologue, suggesting that it may use its own specialized cytoskeleton for cell division.

Conclusions and Outlook

From what was nothing 25 years ago, there is now an astonishing array of bacterial cytoskeletal proteins that have been characterized in detail, and many more still to understand. In addition to the major classes of proteins discussed here, there are others recently discovered but not as well characterized, such as bactofilins and bacterial homologs of dynamins and spectrins, which have important roles in cell growth, shape, and membrane dynamics. Other candidates for cytoskeletal functions include metabolic enzymes such as CTP synthases that form polymers. With the advent of cryo-electron tomography and other more powerful *in situ* techniques such as single molecule imaging and TIRF microscopy, along with the advances in proteomics, the goal of understanding how these cytoskeletal proteins cooperate to operate an entire cell seems more likely than ever, and it is likely that a bacterial cell will be the first.

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Nonflagellar Bacterial Motility

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Glossary

AglQ A component of the *Myxococcus xanthus* gliding motility motor that is related to flagellar motor protein MotB.
AglR The component of the *Myxococcus xanthus* gliding motility motor that is related to flagellar motor protein MotA.
AglS A component of the *Myxococcus xanthus* gliding motility motor that is related to flagellar motor protein MotB.
MreB Bacterial cytoskeletal protein that is involved in *Myxococcus xanthus* gliding.
Cryo-EM-tomography Electron microscopy technique used to view intact cells or cell fractions in a frozen, hydrated state at high resolution that can be used to generate 3-dimensional images.
Gas vacuole Clusters of gas vesicles.
Gas vesicle Protein shells filled with gas that provide buoyancy.
Gliding motility Active movement of bacteria over surfaces that does not involve flagella or pili.
Periplasm Region between the cytoplasmic membrane (inner membrane) and outer membrane of a Gram negative bacterial cell.
Pilin The protein that is the main component of the filament of a bacterial pilus.
PilB ATPase involved in type IV pilus assembly.
PilC Cytoplasmic membrane protein that is part of the type IV pilus motor.
PilQ Protein that makes the outer membrane pore for type IV pili.
PilT ATPase involved in type IV pilus retraction and disassembly.
Sliding motility Passive movement of bacteria over surfaces as a result of forces generated in a colony during cell growth.
SprB *Flavobacterium johnsoniae* gliding motility adhesin that is propelled rapidly along the cell surface.
Twitching motility Movement of bacteria over surfaces using type IV pilus extension and retraction.

Abbreviations

EM Electron microscopy
PilA Pilin protein
PMF Proton motive force
T9SS Type IX protein secretion system

Background and Experimental Approaches

The involvement of flagella in bacterial motility was suggested in the 1800s, and the functioning of these structures as rotary propellers was recognized in 1973 by Berg and Anderson. In contrast, all other forms of bacterial motility (nonflagellar motility) remained mysterious until more recently, and some are still poorly understood. In recent decades, a combination of experimental approaches including genetic analyses, electron microscopy (EM), fluorescence microscopy, biochemical approaches, and comparative genome studies have elucidated the basic mechanisms for several forms of bacterial nonflagellar movement. Genetic analyses involved the isolation of mutants that had lost the ability to move. This allowed identification of the genes and proteins required for movement. Many of the proteins that were required for motility were components of the novel motility machineries discussed below. The development of high resolution EM approaches, and most recently of cryo-EM-tomography, allowed the visualization of the previously invisible motility machines in intact cells or cell fractions. This approach was used to compare wild-type and nonmotile mutant cells to identify motility structures. Use of fluorescent molecules to label components of the motility machinery that had been identified by genetic or biochemical analyses allowed visualization of components of the motility machines in motion and the development of models for bacterial nonflagellar motility. Use of these approaches resulted in the recognition of the four different mechanisms for active bacterial movement over surfaces that are described below: type IV pilus-mediated motility, myxobacterial gliding motility, *Flavobacterium* gliding motility, and *Mycoplasma* gliding. Two forms of active nonflagellar swimming motility are also known. The movement of these bacteria has received less attention and many mysteries remain regarding their movements.

Type IV Pili and Twitching Motility

Type IV pili are long thin dynamic structures (about 6 nm in diameter and up to 10 μm long) that extend from the cell, usually from a cell pole. Type IV pili and twitching motility are most common in Gram negative bacteria. These include, for example, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, and *Myxococcus xanthus*. Type IV pili are also found in a few Gram-positive bacteria, such as *Clostridium perfringens*.

Type IV pili are comprised primarily of many copies of pilin proteins (PilA) with smaller numbers of adhesins present at the tip. An individual pilus spans the entire cell envelope, from the cytoplasmic membrane through the outer membrane (Fig. 1). The involvement of pili in twitching motility was first suggested in the 1980s by the realization that mutants that lacked pili were nonmotile and that antisera that bound to pili blocked motility. At that time, Bradley proposed that pilus retraction was responsible for cell movement, an idea that was later confirmed by genetic, molecular, biochemical, and microscopic studies.

Genetic and biochemical analyses identified dozens of proteins (at least 35 for *P. aeruginosa*) involved in twitching motility. These include at least 20 that are involved in pilus biogenesis. Among these, PilA is the primary structural subunit of the pilus, PilB is the ATPase that powers pilus assembly (extension), PilT is the ATPase that powers pilus disassembly (retraction) and PilQ is the outer membrane secretin that makes a pore through which the pilus protrudes. A key feature of the pilin protein PilA is its ability to exist in two forms, both of which rely on a hydrophobic α-helix. This hydrophobic region anchors the protein to the cytoplasmic membrane, with its hydrophilic portion apparently extending into the periplasm. During pilus assembly, pilin monomers are recruited from the membrane. During this process, the hydrophobic helices of the PilA proteins interact with each other and become buried at the center of the growing pilus cylinder. Biochemical and genetic analyses revealed that pilus assembly is catalyzed by the rotating pilus motor comprised of PilB and PilC. This ATP driven motor is thought to scoop PilA monomers from the membrane and add them to the base of the growing pilus. This process is repeated many times as the pilus grows. Importantly, this process is reversible. When PilT replaces PilB, ATP hydrolysis results in removal of PilA from the base of the pilus, returning the pilin protein into the cytoplasmic membrane, and resulting in retraction of the pilus.

How do type IV pili result in cell movement? At the tip of the pilus is an adhesin. When this adhesin attaches to a surface this is sensed by a poorly understood mechanism, and PilT initiates removal of PilA monomers from the base, melting them back into the cytoplasmic membrane. As the pilus shortens it remains attached at its tip to the external surface, and the cell is dragged toward this point (Fig. 2). Multiple pili can extend from the pole, and function together to pull the cell. PilA monomers in the membrane are apparently recycled to construct new pili, allowing the cell to continuously crawl in the same direction. Reversal of cell movement involves assembly and disassembly of pili at the opposite cell pole. For at least some bacteria secreted polysaccharides also play a role in twitching. These may coat the substratum and provide points of attachment for pili, or may alter the friction between the cells and the substratum allowing the cells to be pulled more effectively by pilus retraction.

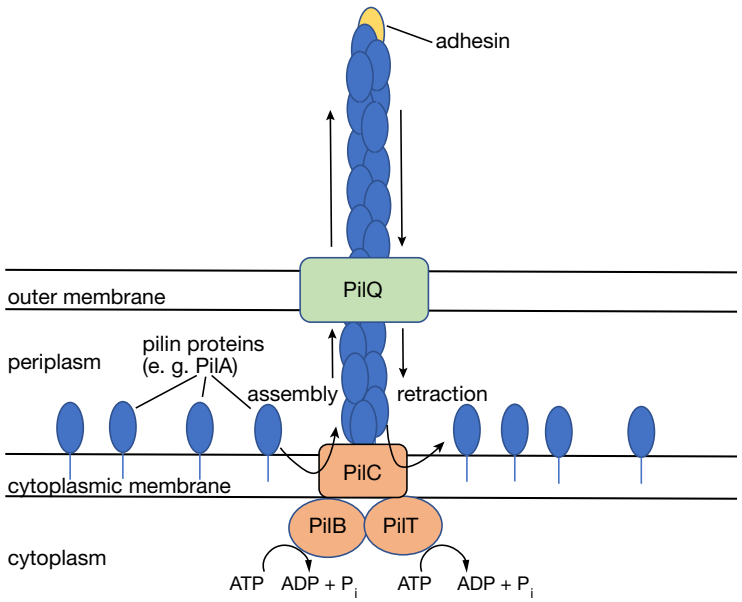

Fig. 1 The type IV pilus. The type IV pilus machinery spans the bacterial cell envelope. The pilus itself, which extends from this machine, is comprised primarily of many copies of the pilin protein PilA (*blue*) with smaller amounts of minor pilins. The pilus extends through an outer membrane channel comprised of PilQ proteins. Adhesins (*yellow*) are found at the distal tip of the pilus. An ATP-powered motor at the base of the machine pulls PilA monomers from the cytoplasmic membrane and inserts them into the base of the growing pilus. Disassembly of the pilus, also powered by ATP hydrolysis, results in retraction of the pilus, and returns the PilA proteins to the cytoplasmic membrane. Assembly and disassembly involves two ATPases (PilB and PilT respectively) that function with PilC. Other proteins involved in pilus biogenesis and function have been omitted from this model for simplicity.

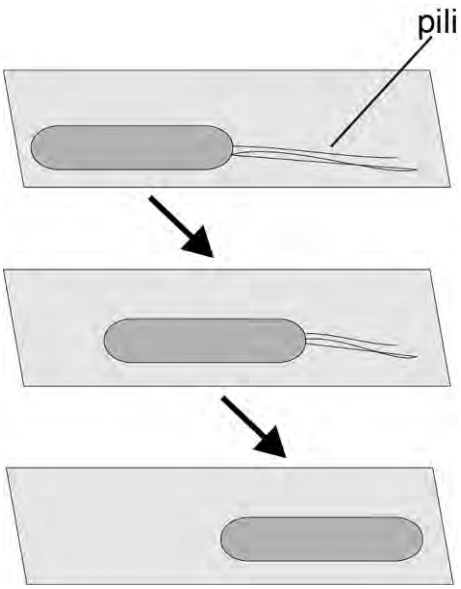

Fig. 2 Movement of a bacterial cell by type IV pilus-mediated twitching motility. Pili extend from the front pole of the cell and contact a surface. Retraction of the pili results in cell movement. Extension and retraction of pili from the opposite pole results in movement in the opposite direction. From McBride, M. J. (2001). Bacterial gliding motility: Multiple mechanisms for cell movement over surfaces. *Annual Reviews of Microbiology* **55**, 49–75.

Studies of *N. gonorrhoeae* and *P. aeruginosa* provided direct evidence for cell movement by pilus retraction. The most dramatic evidence was provided by Skerker and Berg in 2001 and involved examination of cells whose pili had been fluorescently labeled. Pili were observed to extend and retract from a stationary cell. Cells actively moved when pili that were attached to a surface retracted. Biophysical analysis of retraction of pili attached to latex spheres revealed that the pilus motor is one of the strongest biological motors known. In addition to the roles of type IV pili in cell movement, they are also involved in many other processes, including attachment of cells to surfaces, uptake of DNA by transformation and conjugation, and infection by viruses that use pili as receptors to attach to the cell. The dynamic nature of type IV pili appears to be important in each of these processes.

Recent cryo EM-tomography experiments provided stunning insights into the structure of type IV pili. Analyses of wild type and mutant cells allowed elucidation of the overall structure of the pilus and the localization of many of its components in intact cells. This includes not only the proteins mentioned above, but many others that form a series of rings in the cell envelope that support the growing pilus (Fig. 3).

A number of genes regulate the expression of pilus genes in response to environmental stimuli, so that pili are only produced and function when needed. Some of these control the location of the pili at the appropriate pole, or control extension and retraction of individual pili. For example, *P. aeruginosa* cells migrate in chemical gradients using a set of signal transduction proteins related to the well-studied chemotaxis proteins of swimming bacteria that control the direction of rotation of flagella. Exactly how these function to control pilus function is not yet known.

Myxobacterial Gliding Motility

Myxobacteria are rod-shaped, social gliding bacteria that crawl over surfaces in groups in search of food. Myxobacteria digest polymers such as polysaccharides and proteins. Many of them are predators that attack and digest bacteria and other organisms. The cells swarm over surfaces cooperatively digesting their prey. When they run out of food, thousands to millions of myxobacterial cells aggregate to form a fruiting body. Within the fruiting body the cells differentiate to become dormant myxospores. When the myxospores experience favorable conditions again, they germinate and generate a new feeding swarm. Myxobacteria are Gram-negative bacteria that belong to the δ proteobacterial phylum.

Myxobacterial motility has been most well studied for cells of *M. xanthus*, which use two independent machines to move over surfaces. As described in the previous section, they use type IV pilus extension and retraction to move by twitching motility. This allows *M. xanthus* cells to move in groups and is often referred to as "social motility" (S-motility). The second form of movement allows more efficient movement of individual cells and is called "adventurous gliding motility" (A-motility), or sometimes just "gliding motility."

The possibility of multiple *M. xanthus* motility machines was suggested in the 1970s by genetic analyses. Hodgkin and Kaiser isolated numerous mutants that were partially defective in cell movement, and grouped these into the "social" and "adventurous" categories mentioned above. Most completely nonmotile mutants had two mutations; one that disrupted the social motility machine, and another that disrupted the adventurous motility machine. These initial studies also implicated pili in social motility,

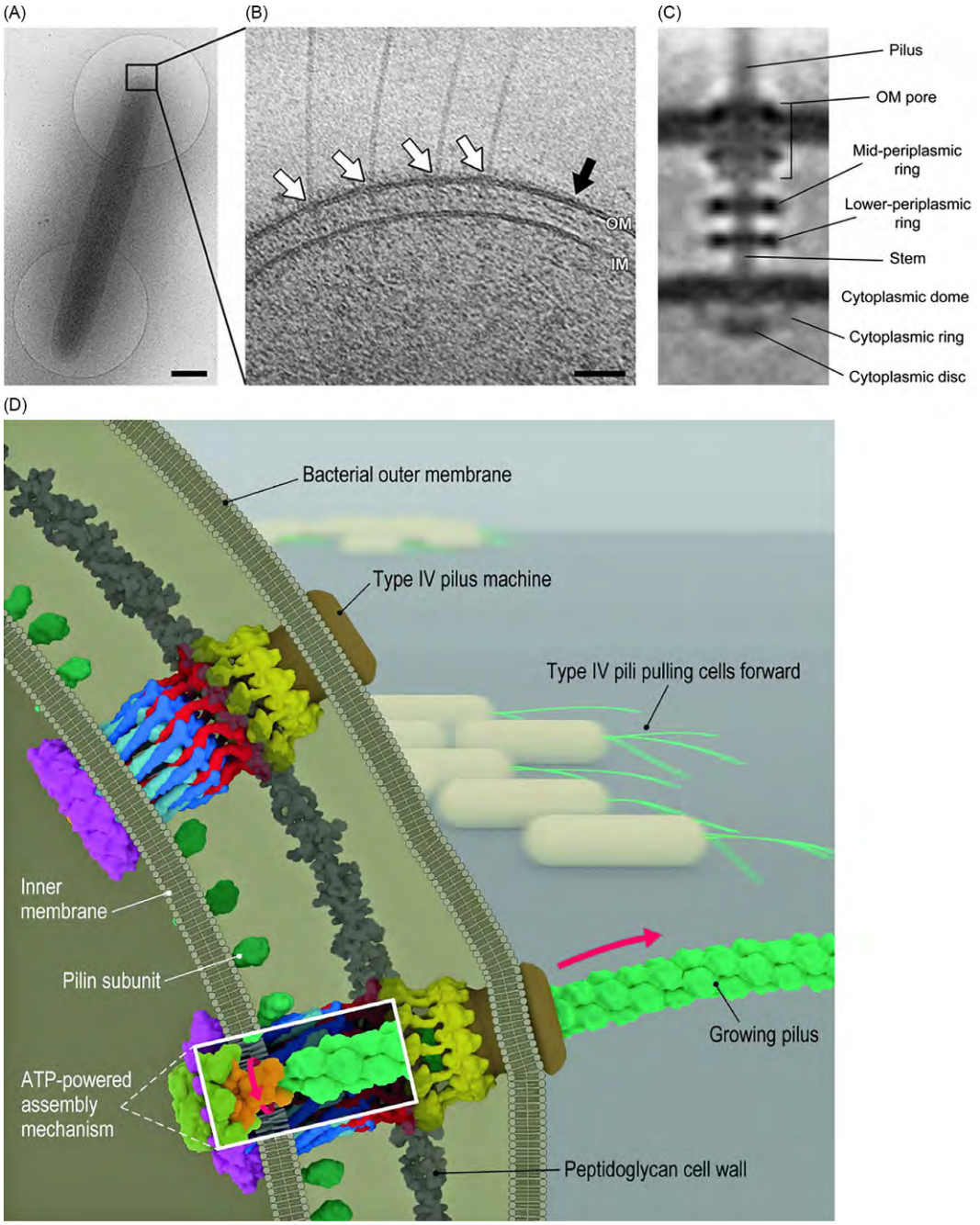

Fig. 3 Type IV pilus structure. Type IV pili were observed in intact *M. xanthus* cells by cryo-EM tomography. (A) An *M. xanthus* cell on an EM grid. *Bar* indicates 500 nm. (B) Higher magnification of the boxed region from (A). *White arrows* indicate type IV pilus machines with pili extending from the cell. *Black arrow* indicates an empty type IV pilus machine. Outer and inner membrane (OM and IM) are indicated and bar indicates 50 nm. (C) Pilus structure at higher resolution obtained by averaging images of multiple pilus machines. (D) Cartoon illustrating the structure of type IV pilus machines with and without pilus filaments. Type IV pili extend and retract to pull cells forward. ATP hydrolysis in the cytoplasm is thought to cause the PilC protein in the inner membrane (*orange*) to rotate and transfer pilin proteins from the inner membrane into the growing pilus. This process is reversed during pilus retraction. Modified from Chang, Y. W., Rettberg, L. A., Treuner-Lange, A., Iwasa, J., Sogaard-Andersen, L., and Jensen, G. J. (2016). Architecture of the type IVa pilus machine. *Science* **351**, aad2001.

since these were absent from cells of many social motility mutants. Molecular analyses identified the nature of the mutated genes and the proteins that they encode. Many of the social motility proteins were involved in type IV pilus assembly or function and were similar to those of other bacteria that use twitching motility. The adventurous motility proteins on the other hand were mostly novel. Analysis of A-motility mutants revealed that these proteins comprise a unique motility machine within the cell envelope.

The motor driving A-motility was recently identified. It is comprised of three cytoplasmic membrane proteins, AglQ, AglR, and AglS, that harvest the proton gradient across the membrane to power cell movement. This proton gradient is also referred to as the

proton motive force (PMF). AglQ, AglR, and AglS are related to components of other PMF-driven motors including the rotary flagellar motor (MotA and MotB) and the motor that energizes various outer membrane transport processes (TolQ and TolR). AglR is similar to MotA/TolQ and AglQ and AglS are both similar to MotB/TolR. Unlike the flagellar motor and the Tol complex, the Agl motor complex is apparently not anchored to the peptidoglycan cell wall, and instead it moves along the cytoplasmic membrane following a helical path. This was demonstrated by microscopic analysis of AglR and AglQ proteins fused to fluorescent proteins. The *M. xanthus* gliding motility apparatus also includes proteins in the cytoplasm, periplasm, and outer membrane. The Agl motors in the cytoplasmic membrane appear to transiently interact with cytoplasmic, periplasmic, and outer membrane components of the machinery to result in cell movement. The cytoskeletal protein MreB appears to be involved in motility and the motors may migrate along the helical cytoskeleton. Two models are currently being considered to explain cell movement (Fig. 4). The focal adhesion model for *M. xanthus* gliding suggests that this involves propulsion of cell surface adhesins that contact the substratum over which the cells crawl. In contrast, the helical rotor model suggests that the proteins that are propelled are not expressed on the cell surface but instead are confined to the periplasm of the cell. These proteins form a moving bulge in the outer membrane that transmits the force to the substratum resulting in cell movement. A novel feature of both models is the movement of the motors in the cytoplasmic membrane along the length of the cell following helical tracks. Secreted polysaccharides are also involved in movement. These are thought to facilitate the productive interaction of cell surface components of the motility machinery with the substratum.

As with twitching motility, described above, myxobacterial gliding is controlled by signal transduction proteins related to the chemotaxis proteins that control the direction of rotation of bacterial flagellar motors. Exactly what the myxobacterial cells sense is not known, but the sensory transduction system controls cell reversals, and thus facilitates migration toward preferred environments.

Flavobacterium (Bacteroidetes) Gliding Motility

Many members of the large and diverse phylum *Bacteroidetes* exhibit a unique form of gliding motility that appears to be restricted to this phylum of Gram negative bacteria. Flagellar motility is rare or nonexistent in the phylum, as are type IV pili, but members of many genera and species exhibit gliding motility. This type of gliding has been most well studied for the soil and freshwater bacterium *Flavobacterium johnsoniae* (Fig. 5). The long slender *F. johnsonaie* cells crawl over surfaces at speeds of about 2 μm per second. They occasionally reverse direction, with the "head" becoming the "tail." Cells may also undergo pivoting, flipping, and rotating movements when they are attached to the surface at a single point. The PMF across the cytoplasmic membrane powers these movements. As with most other gliding bacteria, the movement of individual cells results in the formation of thin spreading colonies (Fig. 5).

Genetic tools developed for *F. johnsoniae* in the 1990s were used to identify genes and proteins involved in gliding. This involved isolation of nonmotile mutants (Fig. 5) and identification of the mutated genes and the motility proteins that they encode. At least 19 proteins are involved in *Flavobacterium* gliding, and most of these are conserved in other gliding members of the phylum. One of these proteins, SprB, is a huge (669 kDa) cell surface adhesin. Microscopic analyses of cells labeled with fluorescent antibodies

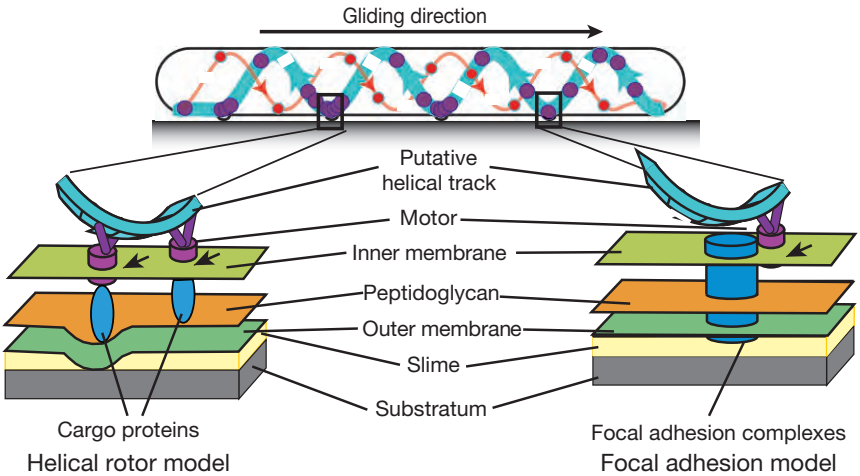

Fig. 4 *M. xanthus* gliding motility. Gliding is powered by motor proteins (AglQ, AglR, AglS) that harvest the PMF and move along helical tracks associated with the inner face of the inner membrane. The two models propose different mechanisms for force transmission to the substratum. In the helical rotor model (left) the motors (*purple*) carry periplasmic cargo (*blue*) as they migrate. The moving cargo deforms the cell surface which pushes against the substratum. The focal adhesion model (right) is similar except that the cargo carried by the motors extends to the outside of the cell, and the action of the motors on these surface-exposed proteins that have attached to the substratum results in cell movement. From Nan, B. and Zusman, D. R. (2016). Novel mechanisms power bacterial gliding motility. *Molecular Microbiology* **101**, 186–193.

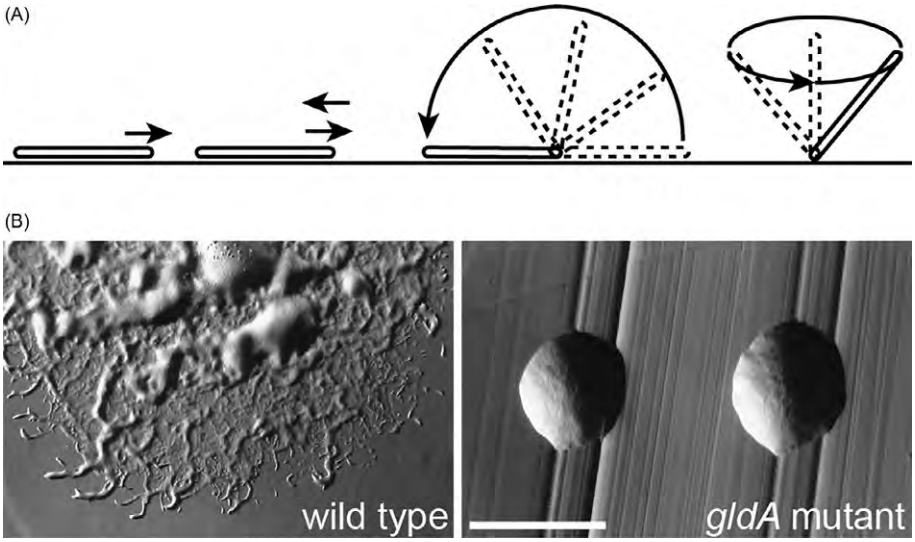

Fig. 5 Gliding of *F. johnsoniae* cells. (A) Characteristic movements of cells. (B) Spreading colony formed by millions of wild type cells, and nonspreading colonies formed by cells of a nonmotile *gldA* mutant. *Bar* in panel B corresponds to 1 mm and applies to both images. From McBride, M. J. and Nakane, D. (2015). *Flavobacterium* gliding motility and the type IX secretion system. *Current Opinion in Microbiology* **28**, 72–77.

against SprB revealed that it is propelled rapidly (approximately 2 μm per second) along the cell surface. SprB proteins appear to follow a helical path as they move from one pole of the cell to the other (approximately 5–10 μm), flip around the pole, and continue back toward their original location. SprB is not the only moving cell-surface adhesin involved in motility. RemA moves in a similar way and genome analyses suggest the presence of additional semiredundant motility adhesins that may allow the cells to crawl over many different surfaces. The exact nature of the machinery that propels motility adhesins such as SprB is not known, but it is probably comprised of the other motility proteins, most of which are referred to as Gld proteins. Some of these proteins also form the core of the type IX protein secretion system (T9SS), that delivers SprB and the other motility adhesins to the cell surface. This is reminiscent of the bacterial flagellum which has a type III secretion system at its core that is involved in its assembly. The *F. johnsoniae* T9SS has many roles besides motility and secretes dozens of soluble enzymes and cell-surface localized proteins. T9SSs are confined to but common in members of the phylum *Bacteroidetes*. They are even found in some members that are nonmotile, such as the human oral pathogen *Porphyromonas gingivalis*, which uses its T9SS to secrete virulence factors.

Many proteins required for *Flavobacterium* motility have been discovered, but fundamental mysteries remain to be explored. The PMF is needed for gliding, but the components of the motors that harvest this proton gradient to propel SprB along the cell surface remain uncertain. These motors appear to rotate, and they are probably anchored to the cell wall, unlike the *M. xanthus* gliding motors described above that move freely along the cytoplasmic membrane. The *Flavobacterium* gliding motors must span the cytoplasmic membrane to harvest the PMF. Two cytoplasmic membrane proteins, GldL and GldM, that are components of the T9SS and are required for gliding may comprise these motors, but this has not been directly demonstrated. The remaining motility proteins probably comprise the rest of the machinery that connects the motors with SprB and the other cell-surface adhesins. Some of these proteins are helically arranged in the cell envelope, and may constitute the tracks that SprB follows along the cell surface. In the model for *F. johnsoniae* gliding shown in Fig. 6, multiple PMF-powered rotary motors are attached to the cell wall and propel adhesins such as SprB and RemA along looped helical tracks on the cell surface. The action of the motors on adhesins that are attached to the substratum results in forward movement and rotation of the cell. A variation that requires fewer rotary motors per cell (not shown) postulates attachment of the adhesins directly to a helical track, and movement of the entire track or sections of track, in a conveyor-belt fashion. Still mysterious is the mechanism used to control the motors to allow migration in favorable directions. Unlike the myxobacteria described above, *F. johnsoniae* and other *Bacteroidetes* lack genes related to bacterial chemotaxis genes. They probably control their motility in response to environmental stimuli but the mechanism is completely unknown.

Movement of Bacteria That Lack Cell Walls: The Mycoplasmas and Spiroplasmas

The bacterial cell wall prevents osmotic lysis, and is thus critical for survival of most bacteria. Members of the genus *Mycoplasma* and related bacteria in the class Mollicutes lack peptidoglycan, a critical component of the bacterial cell wall of nearly all bacteria. Electron microscopic examination confirmed that mycoplasmas, most of which are parasitic, lack cell walls. Given the demonstrated importance of the cell wall to the survival of other bacteria, how do mycoplasmas manage in the absence of this structure? Apparently, they compensate for the absence of a cell wall by incorporating sterols into their cytoplasmic membranes and by the presence of glycoproteins on the cell surface. Both of these may help to stabilize the cells. They also have an unusual cytoskeleton that may contribute to stability.

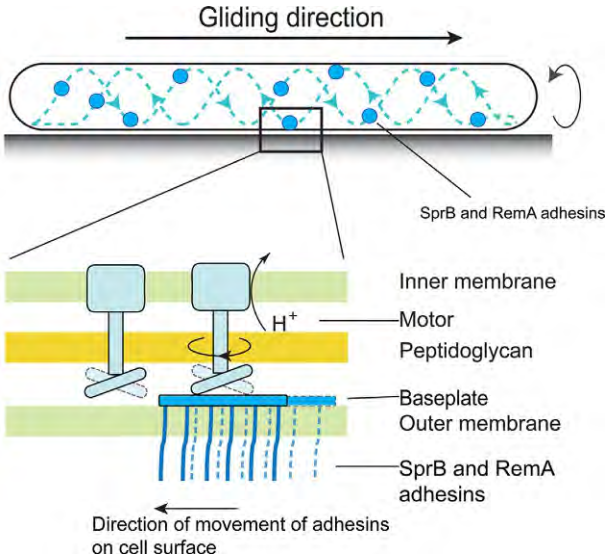

Fig. 6 Model of *F. johnsoniae* gliding. Gld proteins in the cell envelope form the PMF-powered rotary motors that are attached to the cell wall and propel adhesins such as SprB and RemA along looped helical tracks on the cell surface. The action of the motors on adhesins that are attached to the substratum results in forward movement and rotation of the cell. Two rotary motors are shown. In the model shown, rotation of one motor propels a baseplate carrying SprB and RemA adhesins and delivers it to the next motor. From McBride, M. J. and Nakane, D. (2015). *Flavobacterium* gliding motility and the type IX secretion system. *Current Opinion in Microbiology* **28**, 72–77.

The mycoplasmas and related bacteria have evolved several novel mechanisms for cell movement. Most common is mycoplasma gliding. This involves the movement of cells over surfaces. The cells are often flask shaped, comprised of the cell body, a thinner neck region, and a head region (Fig. 7). A complex cytoskeleton that is especially evident in the neck region is presumably responsible for this shape. Gliding cells always migrate toward the head. A clever experiment conducted by Uenoyama and Miyata in 2005 demonstrated that ATP is the energy source for this movement. They treated motile *Mycoplasma mobile* cells on a glass slide with detergent resulting in leakage of the cytoplasmic contents and formation of cell "ghosts." These ghosts failed to move. However, addition of ATP resulted in the immediate resumption of gliding motility. Apparently the remaining cytoskeletal and cell surface components of the motility machinery remained functional in the dead cell "ghosts," and only required addition of their energy source to resume active movement.

Genetic, biochemical and microscopic studies of *M. mobile* identified the cell surface proteins required for motility. Three large proteins (Gli123, Gli349, and Gli521) appear to form the external motility machines that may function as "legs" (Fig. 7). These legs were found primarily in the neck and head region of the cell. ATP hydrolysis in the cytoplasm is thought to cause conformational changes to cytoskeletal proteins that in turn push or pull on the many legs, resulting in the "centipede-like" walking of the mycoplasma cells. It remains unclear if the other gliding mycoplasmas, such as *Mycoplasma pneumoniae*, move in the same way. The surface proteins of *M. mobile* are not conserved in the other gliding mycoplasmas. *M. pneumoniae* may use a similar form of movement with other proteins replacing those of the *M. mobile* legs, or it may use its cytoskeleton and cell surface components to move by a different mechanism. One such proposed mechanism is repeated contraction and expansion of the cytoskeleton to

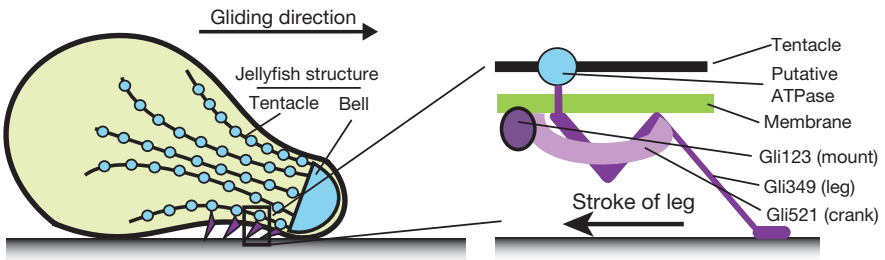

Fig. 7 "Centipede" model for *Mycoplasma mobile* gliding motility. The cartoons represent a *M. mobile* cell (left) and the cell-surface motility proteins (right). The "neck" region of the cell surface has many protein "legs" (*purple*). These are associated with a cytoskeletal structure on the inside of the cell (*blue*). This is sometimes called a "jellyfish structure" because it resembles a jellyfish with the body ("bell") at the head of the cell and the tentacles trailing behind. ATP hydrolysis in the cytoplasm is thought to result in conformational changes to the legs (stroke), resulting in cell movement. The legs repeat this process and alternate between sticking to the substratum and releasing to allow the cell to essentially "walk." From Nan, B. and Zusman, D. R. (2016). Novel mechanisms power bacterial gliding motility. *Molecular Microbiology* **101**, 186–193.

shorten and lengthen the cell respectively, resulting in "inchworm" like movement. Additional studies are needed to determine if there is a conserved motility mechanism shared by all gliding mycoplasmas.

The gliding mycoplasmas move over surfaces, but some other mollicutes evolved novel mechanisms to swim. Members of the genus *Spiroplasma* are long, slender, spiral-shaped cells that swim rapidly in viscous, aqueous environments. Spiroplasmas are common insect and plant pathogens. Unlike the similar looking spirochetes, which have flagella embedded in their cell envelopes, the spiroplasmas lack flagella or other obvious motility organelles. Like other mollicutes, they also lack cell walls. Electron microscopy studies revealed the presence of a prominent cytoskeletal system in *Spiroplasma* cells that confers the spiral shape and is also thought to be responsible for movement. The cytoskeleton filaments may function as a contractile linear motor. Conformational changes of components of the cytoskeletal filaments result in "kinks" that progress from one end of the cell to the other. These moving kinks are thought to push the surrounding fluid toward the back of the cell and thus propel the cell forward. Evolution of the unusual and diverse mollicute motility systems, not shared with other bacteria, may have required release from the constraints of the semirigid peptidoglycan layer that encases other bacteria.

Cyanobacterial Gliding Motility: Pili and Polysaccharides

Cyanobacteria (previously called "blue-green algae") are bacteria that carry out oxygenic photosynthesis. The cyanobacteria invented this process billions of years ago, and todays plants owe their ability to carry out photosynthesis to these bacterial metabolic pioneers. Active motility is common among cyanobacteria, but flagella are absent.

In 1803 Jean-Pierre Vaucher in his "Histoire des conferves d'eau douce" documented the rapid movement over surfaces of filamentous cyanobacteria. These movements are still easily observed today by light microscopic examination of samples from natural environments such as pond water. In the 1900s experiments were performed to understand the mechanism of this movement. Gliding cyanobacteria secrete polysaccharides, and some evidence pointed to this secretion as the propulsive force. However, it was never completely certain if the polysaccharides left behind the cell had caused the movement, or if they had just been deposited as the cells were propelled by another mechanism. Other proposed models involved the generation of waves in the cell surface, perhaps involving the action of contractile filaments in the cell.

Genetic experiments conducted in the 1990s demonstrated that the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 uses type IV pili to move over surfaces (Fig. 8). This is thought to involve extension and retraction of pili, as described above for bacterial twitching motility. In contrast, the mechanism of gliding of filamentous cyanobacteria remained mysterious until very recently. However, it is now clear that the gliding filamentous cyanobacterium *Nostoc punctiforme* moves by a modification of the mechanism used by single-celled cyanobacteria: type IV pilus extension and retraction. *N. punctiforme* grows as long filaments that consist of many cells linked together like beads in a chain. The long vegetative filaments are nonmotile. Under certain conditions shorter filaments (hormogonia) are produced that actively move over surfaces. Genetic analyses revealed that mutations in genes involved in synthesis or function of type IV pili resulted in loss of motility. Microscopic analysis of cells with fluorescently labeled pilin protein indicated that the pili extend from between the junctions of the individual cells of the filament, and suggested that pilus retraction pulls the entire filament across the surface. Polysaccharides also appear to be involved in this process since mutations in genes involved in polysaccharide synthesis also compromised motility. The polysaccharides may coat the substratum and enhance the function of pili by providing a point of attachment for the pili or by altering the friction between the cell and substratum to facilitate pilus-mediated movement. Genome analyses revealed that genes associated with type IV pilus assembly and function are also found in many other filamentous gliding cyanobacteria. Additional experiments are needed to determine if filamentous cyanobacteria have evolved any other gliding mechanisms, such as those postulated to involve propulsion by polysaccharide secretion or by generation of cell-surface waves.

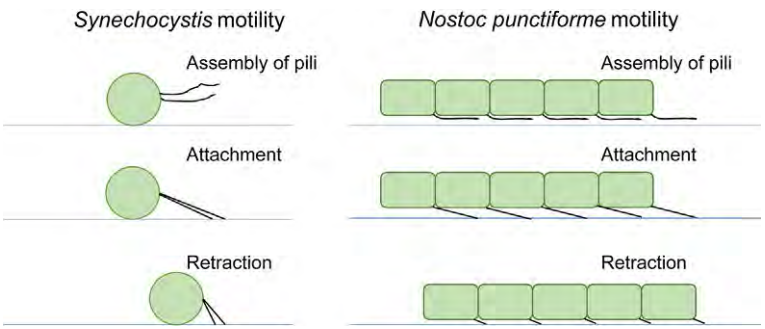

Fig. 8 Type IV pilus-mediated motility over surfaces of unicellular (*Synechocystis* sp. PCC6803, left) and filamentous (*N. punctiforme*, right) cyanobacteria. Movement of *Synechosystis* is similar to twitching motility of other bacterial cells, with assembly of pili, attachment to a surface, and retraction resulting in cell movement. Movement of *N. punctiforme* filaments is a slight variation of this process, with pili extending from locations near each cell junction of the filament. Polysaccharides secreted by *N. punctiforme* also play a role in motility, by either providing a point of attachment for the pili or by decreasing friction to allow the cell filaments to be pulled over the surface.

Gliding cyanobacteria control their movements using proteins that are related to those that control chemotaxis of flagellated bacteria. Both chemotaxis (migration in chemical gradients) and phototaxis (migration toward optimal light conditions) have been observed. Cyanobacterial phototaxis is remarkable in many ways. The individual spherical cells of phototactic *Synechocystis* cells function much like an eyeball to focus light on a single region of the cell, enabling these simple bacteria to identify the location of the light source and migrate directly toward or away from it as appropriate. Some filamentous cyanobacteria have the remarkable property that exposure of one end of a filament to light almost instantaneously alters the movement of the entire filament. How cells of the filament that are not directly exposed to the light source respond is not entirely understood.

Cyanobacterial Nonflagellar Swimming Motility

In the 1980s a novel form of bacterial motility was observed by Waterbury and colleagues in some strains of unicellular marine cyanobacteria belonging to the genus *Synechococcus*. These bacteria actively swim but they lack flagella. Microscopic observation and genome analyses revealed the absence of flagella and of the genes needed to construct flagella. Cells rotate as they swim and the sodium gradient across the cytoplasmic membrane is required for movement. The use of a sodium gradient rather than the more common proton gradient to power a motility machine is not unique to swimming cyanobacteria. Some flagellated marine bacteria also use this sodium motive force instead of a PMF to power their flagella. Large cell surface proteins (SwmA, SwmB) that are involved in motility have been identified by genetic and molecular analyses, but the mechanism of cyanobacterial swimming motility remains a mystery today. Electron microscopic studies revealed the presence of numerous thin structures (spicules) extending from the surface of cells of at least some strains of these bacteria, and these may have a role in movement. One model for cyanobacterial swimming involves generation of cell surface waves that progress from the front to back of the cell resulting in cell movement. These waves might be amplified by the large cell surface proteins and spicules mentioned above. Such waves could be generated by contraction and expansion of structures in the periplasm. Alternatively, motors in the cytoplasmic membrane may propel periplasmic cargo along a helical path to result in the surface waves, as has been suggested for *M. xanthus* gliding above. Additional studies will be needed to determine the exact mechanism of cyanobacterial swimming motility.

"Passive" Movement

The active motility processes described above all require a continuous supply of metabolic energy in the form of ATP or of ion gradients across the cytoplasmic membrane, such as the PMF. Several other forms of bacterial cell movement are less directly connected to such energy sources. These include sliding of cells in a growing colony, and the use of gas vacuoles to position cells optimally in the water column.

Sliding motility is a form of "passive" motility over surfaces. These bacteria produce surfactants that are important in this process. As cells grow and divide to form colonies, the pressures that build up in the crowded colony result in sheets of cells sliding away from the raised colony center. The bacteria form spreading colonies that superficially resemble those of gliding bacteria, but the individual cells lack the ability to move under their own power. Rather, they move when they are pushed by the other growing cells of the colony.

Gas vacuoles are found in some aquatic bacteria and archaea. They are particularly common in aquatic cyanobacteria. Gas vacuoles are comprised of multiple gas vesicles. Each gas vesicle is a protein shell filled with gas. The gas that fills the vesicles has similar composition as the surrounding atmosphere. Gas vesicles and gas vacuoles are not enclosed by lipid membranes. The presence of gas vacuoles alters the buoyant density of the cell, and allows cells to float higher in the water column. The balance between production of gas vacuoles and production of more dense cell components determines how rapidly the cells rise or fall in the water column. Cells with gas vacuoles can travel significant distances to reach a favorable zone in the water column. For photosynthetic bacteria, this may be the region where the light intensity is optimal.

"Parasitic" Movement

One form of nonflagellar bacterial motility occurs only within eukaryotic cells. These intracellular pathogens include Gram negative (e.g., *Shigella dysenteriae*) and Gram positive (e.g., *Listeria monocytogenes*) bacteria. What these bacteria have in common is the ability to enter the eukaryotic host cell cytoplasm and the production of a protein on their cell surface that facilitates the assembly of filaments of host cell actin. The actin-nucleating proteins are usually located at one pole of the bacterial cell. Assembly of actin filaments prevents backward movement and thus facilitates forward movement of the bacterium within its host cell. It can also result in migration of bacteria into neighboring cells, resulting in spread of the infection within the host. The movements rely on host cell actin and thus can only occur as part of this parasitic relationship. These bacteria can also grow outside of the host cell and some of them exhibit other forms of motility such as flagellar-based swimming under those conditions.

Summary

The most well understood form of bacterial movement involves rotation of flagella but bacteria have evolved numerous other ways to move. Many of these are specialized for movement over surfaces, and include the pulling of cells by pilus retraction (twitching motility), and various forms of gliding motility that involve either conveyor belt-like motion as a result of movement of proteins along cytoskeletal or cell-surface tracks, or the centipede-like motion of mycoplasmas. Some of these are powered by ATP hydrolysis (twitching motility, mycoplasma gliding) whereas others use the proton gradient across the cytoplasmic membrane (myxobacterial gliding and *Bacteroidetes* gliding). Other bacteria lack flagella but move through aqueous environments. Spiroplasmas and some marine cyanobacteria actively swim using novel and still poorly understood machines. Other bacteria migrate vertically in a water column using gas-filled protein shells to alter their buoyant density, or make use of host cell proteins to move within the cytoplasm of a eukaryotic cell. In spite of decades of research the mechanisms underlying many of these forms of bacterial motility are incompletely understood. Future research will likely reveal additional surprises regarding these diverse nonflagellar bacterial motility systems.

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Oral Microbiology☆

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Glossary

Acidogenic Refers to bacteria that produce acid from metabolism of sugars.
Aciduric The ability to withstand conditions of low pH.
Acquired salivary pellicle A layer of proteins and glycoproteins of salivary origin that covers the surface of teeth.
Calculus A mineralized deposit that accumulates on the surface of teeth, usually close to the gum margins.
Coaggregation Cell–cell recognition between genetically distinct cells mediated by complementary surface molecules on the two or more partner cell types.
Commensalism A symbiotic relationship between two organisms of different species in which one partner derives a benefit and the other suffers no harm.
Dental plaque The biofilm on tooth surfaces.
Gingival crevice The narrow space below the gumline between the tooth and the gum tissue (gingiva).
Gingival crevicular fluid (GCF) A fluid derived from serum that seeps across the epithelial barrier into the gingival crevice.
Oral biofilm The multispecies community of microorganisms and associated extracellular material that is present on the surface of teeth or soft tissues in the mouth.
Periodontium Tissues and supporting structures that hold a tooth in place.
Phylotype An evolutionarily related group of organisms. Phylotypes of oral bacteria are typically defined as sharing $\geq$99% sequence identity in the gene encoding the 16S subunit of ribosomal RNA.
Subgingival plaque Plaque that develops on tooth surfaces below the gumline and tends to be anaerobic.
Supragingival plaque Biofilm on teeth above the level of the gums.

Abbreviations

AI-2	Autoinducer-2
CSP	Competence-stimulating peptides
GCF	Gingival crevicular fluid
IE	Infective endocarditis
IL-1β	Interleukin-1β
LDL	Low-density lipoprotein
PRG	Proline-rich glycoprotein
PRP	Proline-rich protein
RANKL	Receptor activator for nuclear factor κB ligand
RPS	Receptor polysaccharides
S-IgA	Secretory immunoglobulin A
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
VAP	Ventilator-associated pneumonia
VEGh	Von Ebner glands protein

☆*Change History*: September 2018. N.S. Jakubovics updated all sections and added references throughout and Figures 1 and 7.

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Human Oral Microbiology

Like the vast majority of microbial populations in nature, microbial inhabitants of the oral cavity are predominantly found in surface-attached multispecies communities. However, the warm, moist character of the mouth combined with the relatively open access to the wider environment presents a unique niche for microorganisms. In fact, the oral cavity is a heterogeneous environment that contains numerous different surfaces for bacterial colonization, including gums, teeth, cheeks, tongue, and palate. Oxygen levels are not constant and range from fully aerobic in the outer layers of dental plaque to levels that are low enough to support the growth of strict anaerobes around the circumvallate papillae towards the back of the tongue and in deeper areas of dental plaque both above (supragingival) and below (subgingival) the gumline (Allaker et al., 2008; Hanioka et al., 2000). The microbial species diversity in the mouth is influenced by the presence of disease. For example, the bacterially produced acid responsible for dental caries selects for organisms that can tolerate low pH (Richards et al., 2017). Another major class of oral pathology is inflammatory gum disease, known as gingivitis (bleeding gums) or periodontitis. If left unchecked, periodontal disease can lead to the formation of pockets of up to 8–10 mm depth below the gumline and adjacent to the teeth. These periodontal pockets are protected from the flow of saliva and movement of the tongue, and are bathed in gingival crevicular fluid (GCF), an exudate derived from serum. The microflora in periodontitis is distinct from that in biofilms at other locations in the oral cavity (Meuric et al., 2017). Thus, oral surfaces whether they are hard (tooth) or soft (epithelial) are colonized by bacterial communities that differ and are site-specific (Eren et al., 2014).

Our understanding of oral microbiology has been greatly impacted by the "-omics" revolution that began in the 1990s (Nascimento et al., 2017). This term relates to the development of new methodologies that enabled the simultaneous analysis of large numbers of targets such as genes, proteins, metabolites or cells rather than focusing on one at a time. These methods have been particularly beneficial for complex systems such as oral biofilms, where there may be hundreds of different microbial species all metabolizing in different ways and each interacting with neighboring microbes or the host. The overall species composition in such an environment is known as the microbiome. Each member of this microbial community brings its own genome, the total genetic content of the cell. The prefix "meta-" is used to denote the content within a complex population rather than one individual, and thus the metagenome refers to the total genetic content of a mixed microbial population. The functions of oral microbial communities, and the ways in which they respond to individual stimuli or during the transition from health to disease, can be monitored by studying the metatranscriptome (messenger RNA transcripts present in the microbial cells), metaproteome (all the different proteins present), or metabolome (metabolites that have been produced). Of course, all of these microbial factors may be studied in relation to the host genome, transcriptome or proteome, to understand how microbes and the host interact with one another. Overall, these studies have begun to reveal how the microbial population changes from a health-associated state to a dysbiotic state in diseases such as dental caries, and how treatment can potentially recover the system to a stable "healthy" state (Gomez and Nelson, 2017; Nascimento et al., 2017).

In total, more than 700 phylotypes of bacteria have been detected in the human mouth, representing at least 300 different species. The most abundant genera in health include *Streptococcus*, *Haemophilus*, *Prevotella* and *Veillonella* (Human Microbiome Project Consortium, 2012). Viruses are also present, and the majority of these are bacteriophage that target a variety of different oral bacteria (Edlund et al., 2015). A relatively small number of fungi are found in oral biofilms (Diaz et al., 2017). Of these, *Candida* spp. are by far the most important in terms of their potential to cause disease. With just a few notable exceptions, oral diseases are caused by the combined actions of multiple species of microorganisms in polymicrobial communities. The great challenge for oral microbiologists is to understand how these microbes interact with one another and with the host in health and disease.

Characteristics of the Oral Environment

Host Surfaces Available for Microbial Colonization

Epithelial cells are constantly shed from soft tissues (desquamation) along with any bacteria attached to them. According to estimates, cells in the outer layer of epithelial surfaces in the oral cavity are shed as rapidly as every 3 h (Dawes, 2003). Therefore, bacteria that colonize oral epithelia must continually reinfect the surface to avoid being lost. Escaping detachment is also a problem for bacteria on the tooth surface because saliva shear and oral hygiene procedures such as tooth brushing remove attached bacteria. Gum margins and cracks and fissures in the teeth constitute natural areas of protection for bacteria. Artificial havens for bacteria are provided by orthodontic appliances such as braces, which often leave exposed areas of enamel that are difficult to access with a toothbrush. Areas that have not been fully cleaned act as reservoirs for a fresh supply of microorganisms to recolonize the tooth surface. Although new species are constantly introduced into oral biofilms, some strains may persist in a person's mouth for several months at least (Haraldsson et al., 2004; Kirchherr et al., 2005). Clearly, such strains have highly effective colonization and commensal mechanisms that prevent the strains from being cleared during oral hygiene procedures.

Mucosal surfaces

In fully dentate adults, teeth provide approximately 20% of the total surface area in the oral cavity and the remainder is soft tissue (Collins and Dawes, 1987). Keratinized epithelium is present in areas that are subject to high compression or friction such as the hard palate and the exposed surface of the gingiva. In contrast, the mucosa of areas that require flexibility such as the cheeks, floor

of the mouth, and the ventral surface of the tongue contain nonkeratinized epithelium. Certain species of streptococci preferentially attach to either keratinized or nonkeratinized tissues *in vitro* (Sklavounou and Germaine, 1980), and streptococci belonging to the *S. mitis/oralis/infantis* cluster are more abundant on keratinized oral epithelial tissues *in vivo* (Eren et al., 2014). However, in general, keratinized gingiva harbor a distinct population from other keratinized oral epithelial surfaces, indicating that other characteristics of oral epithelia are also important for directing microbial colonization. Interestingly, the microbial population on palatine tonsils is more similar to that on mineralized tissues, and subgingival dental plaque in particular, than on other epithelial surfaces (Eren et al., 2014). This may be a result of the reduced oxygen tension in the tonsillar crypts, which will select for anaerobic species. Anaerobic microorganisms on the tongue dorsum play an important role in generating volatile sulfur compounds and other malodorous chemicals that are associated with halitosis (bad breath) (Yang et al., 2013). It should be noted that the distribution of anaerobes on the tongue dorsum is uneven (Allaker et al., 2008), and the overall microbial population across the tongue dorsum is not significantly different from other oral epithelia (Eren et al., 2014). The microbial population on the tongue is dynamic and the relative abundances of the major species present changes significantly from week to week (Flores et al., 2014; Hall et al., 2017; Mark Welch et al., 2014). Despite these changes, the most abundant species, the "core microbiome," remain detectable on the tongue for weeks or months, whereas rare species in the community are frequently lost and replaced by others (Hall et al., 2017).

Mineralized tissues

The inner regions of teeth are composed of a central core of connective tissue (dental pulp) surrounded by a mineralized tissue, dentine (Fig. 1). At the roots, the dentine is enclosed within a thin layer of cementum. The crown of the tooth contains a hard enamel exterior and provides strong protection against oral bacterial challenge. Enamel is the hardest biological tissue and is almost entirely mineral in content. More than 95% of the mass of enamel is calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and the rest is organic material and water. The enamel layer is thickest at the occlusal part of the tooth crown, and becomes very thin toward the gingival margin. Dental caries is characterized by a localized loss of the outer mineral layer of the teeth caused by acids derived from bacterial sugar metabolism. Caries most commonly affects enamel, but root dentine can also become demineralized if it is exposed by recession of the gums (Takahashi and Nyvad, 2016). In the absence of an intact outer tooth layer, bacteria may invade the dentine and often cause painful endodontic infections.

Saliva is supersaturated with respect to calcium hydroxyapatite, and this prevents net demineralization of enamel in the absence of high concentrations of acids. However, large amounts of calcium phosphates in saliva can lead to the accumulation of unwanted mineral deposits (calculus) on tooth surfaces where plaque exists. Dying bacterial cells may contribute to calcification by accumulating minerals and ions, which then precipitate intracellularly (Moorer et al., 1993). Extensive calculus at the gingival margin irritates the gum tissue and correlates with gingival recession. Accumulation of subgingival calculus coincides with periodontal disease and, at least in some cases, calculus contributes to disease progression. Effective control of dental plaque is therefore fundamental for maintaining healthy enamel surfaces.

In health, many of the microorganisms present in dental plaque can also be detected in saliva or on oral epithelial surfaces (Human Microbiome Project Consortium, 2012). This is not surprising since saliva is responsible for transporting bacteria to tooth surfaces, and the majority of microbial cells in saliva are attached to desquamated epithelial cells (Dawes, 2003). Epithelial cells are commonly found in dental plaque, and it is likely that many bacteria reach the tooth surface by "hitchhiking" on host cells (Tinanoff and Gross, 1976). Nevertheless, the environment of the tooth surface selectively enriches certain bacteria and snapshots of the microbial population at different sites in the human mouth indicate that the microbiota of supra- and sub-gingival dental plaque is distinct from other sites in the mouth (Eren et al., 2014). For example, *Streptococcus gordonii*, *Streptococcus sanguinis*,

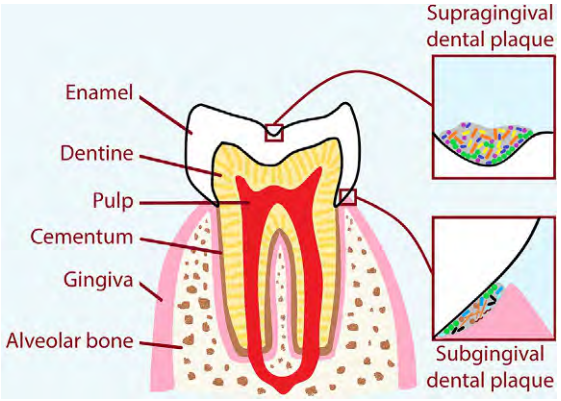

Fig. 1 Basic structure of a tooth and common areas of biofilm formation. Supragingival plaque forms on enamel above the gumline. Subgingival plaque develops between gum tissue (gingiva) and the hard tissue of the tooth (enamel or cementum). The underlying structures of the tooth are not normally exposed to bacteria in health. However, dental caries can lead to invasion of bacteria through the enamel into dentine and eventually to the pulp chamber.

Corynebacterium matruchotii and *Corynebacterium durum* are enriched in dental plaque, whereas *Streptococcus infantis* and *Streptococcus salivarius/vestibularis* are enriched on soft tissue surfaces (Eren et al., 2014; Mark Welch et al., 2016).

Artificial (synthetic) surfaces

Dental intervention procedures introduce a variety of materials into the mouth, which may be retained for months or, in many cases, years. These materials provide new surfaces for microbial colonization and frequently affect the surrounding tissues.

Restorations of damaged teeth form a major component of modern dentistry. The widespread use of silver-based amalgam alloys as filling materials can be traced back to the early decades of the nineteenth century. In many Western countries, amalgam restorations are gradually being replaced by composite resins and glass ionomer cements, which do not contain mercury and are aesthetically more pleasing. The occurrence of caries adjacent to the filling material, termed "secondary" or "recurrent" caries, is a significant risk factor for failure of the restoration. One approach to limiting secondary caries has been to include fluoride in the matrix of glass ionomer cements (Chau et al., 2015). The fluoride is released slowly over a period of weeks. Fluoride-releasing formulations inhibit the growth of a variety of bacteria *in vitro*. However, the presence of salivary flow appears to limit the antibacterial properties of these resins and there is little evidence that the current formulations provide long-term protection against secondary caries *in vivo* (Wiegand et al., 2007).

Signs of caries are a common problem during orthodontic treatment. A survey of children in England, Wales and Northern Ireland conducted in 2013 found that 9% of 12-year olds and 18% of 15-year olds wore orthodontic appliances (braces) (Rolland et al., 2016). Fixed braces are bonded to enamel surfaces and are typically worn for 18–24 months. These devices impede salivary flow and interfere with conventional oral hygiene procedures. White spot lesions, which occur at the early stages of caries, are commonly reported in the areas adjacent to the bonding material. In one study, around 40%–50% of buccal tooth surfaces that had been fitted with fixed braces (multibracket appliances) were found to have developed white spot lesions upon removal of the appliance (Beerens et al., 2015). Typically, the lesions do not progress once the orthodontic appliance is removed. However, remineralization of the lesions occurs slowly, and the consequences of microbial activity during orthodontic treatment therefore remain visible for many years.

The elimination of pathogenic microorganisms is a significant challenge during more invasive dental surgery. Where whole teeth are lost, restorations require the insertion of implants into the alveolar bone. Titanium is almost exclusively used for implants because of its ability to adhere and integrate into regrowing bone. In approximately 40% of implants, a gradual infection and inflammation will result in loss of supporting alveolar bone (peri-implantitis) (Derks and Tomasi, 2015; Derks et al., 2016), which may occasionally lead to failure of the implant (Chrcanovic et al., 2014). Lesions in the mucosa surrounding the implant (peri-implant mucositis) are also common, although it is difficult to estimate the prevalence due to the lack of a consensus for diagnostic criteria between different studies (Derks and Tomasi, 2015; Figuero et al., 2014). Peri-implantitis and peri-implant mucositis are essentially the same disease, distinguished only by a higher degree of alveolar bone loss in peri-implantitis patients. The etiology of peri-implantitis is thought to be similar to that of periodontal disease and previous experience of periodontal disease is a significant risk factor for implant failure (Monje et al., 2014). Therefore, a periodontal care program may be beneficial for patients receiving dental implants.

Where it is impractical to fix artificial dentition in the mouth, tooth function may be restored by wearing dentures. The use of dentures introduces a relatively large surface area into the mouth for prolonged periods, and dentures are thus susceptible to microbial colonization. The constant contact between denture biofilms and mucosal epithelia frequently results in infection, known as denture stomatitis. In epidemiological studies, the prevalence of denture stomatitis amongst denture wearers ranges from 15% to 70% (Gendreau and Loewy, 2011). *Candida* spp. are considered the primary etiological agents of denture stomatitis. However, as with other oral diseases of microbial origin, it is likely that other microorganisms contribute to pathogenesis, and indeed the microbiome associated with denture stomatitis is extremely complex (Shi et al., 2016). Reports of denture stomatitis in the absence of *Candida* spp. exist, and there is no evidence that antifungal treatments improve clinical outcomes (Emami et al., 2014). Therefore, further investigations on the etiology of denture stomatitis are required in order to identify improved approaches for treating the disease.

Body Fluids

Saliva

Saliva has a wide range of functions, many of which are related to protection against oral microorganisms. However, it is important to note that many oral microbes are well-adapted to exploit saliva as a key source of nutrients (Jakubovics, 2015). In fact, saliva is the most important nutrient source during the formation of supragingival dental plaque. It is only once plaque matures that nutrients from the host diet start to have a significant impact on the function of dental plaque (Bowden and Li, 1997). In some situations, components of saliva can coat surfaces, where they may promote adhesion and colonization of microorganisms. The roles of saliva in promoting and inhibiting microbial adhesion are discussed in detail in the section *Microbial Interactions in Oral Biofilms*.

The importance of saliva in controlling infections is apparent from patients suffering chronic hyposalivation, which is a side effect of many drugs, autoimmune disorders such as rheumatoid arthritis and Sjögren's syndrome, and of radiotherapy treatment for head and neck cancers (Millsop et al., 2017). These patients have an increased propensity for caries, periodontal disease, and opportunistic infections such as candidiasis (Billings et al., 2017; Chapple et al., 2017). Caries associated with hyposalivation has an

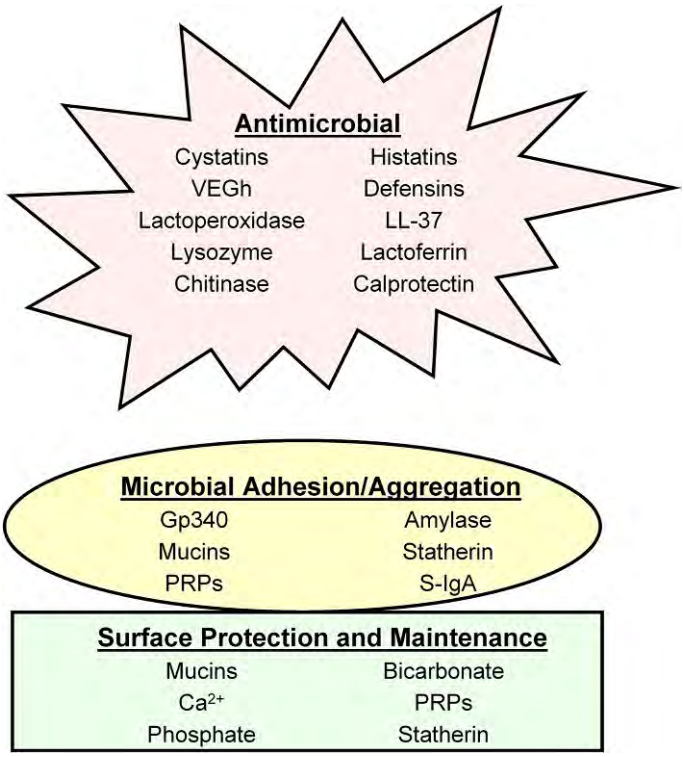

Fig. 2 Salivary components that influence microbial activity. Bactericidal (e.g., lysozyme, histatins, defensins, LL-37), fungicidal (chitinase, histatins, defensins), and antiviral (VEGh, mucins, cystatins, defensins) compounds reduce the overall microbial load in the mouth. Microorganisms are aggregated by Gp340 or mucins, and this is thought to aid their removal by swallowing. However, a number of bacteria can adhere to these same compounds when they are immobilized on surfaces leading to the initiation of a microbial biofilm. Bicarbonate plays a key role in buffering saliva and preventing acid demineralization of enamel. Other inorganic (Ca^{2+}, phosphate) and organic (mucins, PRPs, statherin) components of saliva help maintain a healthy mineral content in enamel.

unusual pattern and frequently occurs at the cervical margin, the area where the cementum of the tooth root meets the enamel of the crown (Boutsi et al., 2000; Silva et al., 2009). The infection control properties of saliva can be attributed to a number of factors that broadly group into (1) antimicrobial (bacteriostatic or bactericidal) molecules, (2) adhesion inhibitors, and (3) protection and maintenance of epithelial barriers (Fig. 2).

Whole saliva is a mixture of the products of all salivary glands together with proteins and carbohydrates of bacterial origin, remnants of foodstuffs, cellular debris, and serum exudate from gingival crevicular fluid (GCF). High concentrations of serum proteins such as albumin are indicative of gingival inflammation and leakage through the epithelial barrier (Henskens et al., 1993). The major components of saliva, more than 100 proteins and glycoproteins in total, are actively secreted by three major pairs of glands (parotid, sublingual, and submandibular) and several smaller glands. The composition of whole saliva depends to a great extent on the relative activities of the serous (parotid) and seromucous (submandibular/sublingual) glands (Jasim et al., 2016). The parotid glands are the largest salivary glands and, following mechanical or acid stimulation, contribute approximately 50% of the volume of secreted saliva in the mouth (Proctor, 2016). Approximately 70% of proteins in parotid saliva are proline-rich proteins (PRPs), a family of polypeptides that contains 35%–40% proline residues, and most of the remainder is α-amylase (Bennick, 1982). In the absence of stimulation, around two-thirds of whole saliva is derived from the submandibular gland. Secretions from this, and the sublingual gland, contain mucins and are consequently more viscous and viscoelastic than parotid saliva (Park et al., 2007; Zussman et al., 2007).

Mucins play a key role in protecting oral tissues from desiccation, mechanical insults, and microbial challenge. Human saliva contains two distinct classes of mucins, MG1 and MG2, which are produced by the MUC5B and MUC7 genes, respectively. Together these form the major component of the "mucosal pellicle" that covers oral epithelial surfaces (Hannig et al., 2017; Helmerhorst and Oppenheim, 2007). MG1 belongs to the classical mucins that cover epithelial surfaces throughout the human body. More than 80% of MG1 mucin is carbohydrate, and monomers of MG1 are disulfide-linked in a long thread-like structure with a total $M_r > 1000$ kDa (Thomsson et al., 2005). Even at low concentrations, MG1 forms a viscoelastic gel (Raynal et al., 2002; Wickström et al., 1998). In contrast, MG2 is a relatively small (150–200 kDa) molecule with low viscoelastic properties. The carbohydrate content of MG2, approximately 68% of the total mass, is lower than that of MG1. In addition, the carbohydrate side chains of MG2 are relatively short and uniform. Approximately 80% of the MG2 carbohydrates are Galβ1 → 3GalNAc with or without a terminal fucose or sialic acid residue (Nieuw Amerongen et al., 1995). Whereas MG1 has only been shown to bind a few species of bacteria such as *Haemophilus parainfluenzae* and *Helicobacter pylori*, MG2 appears to have a significant role in binding a broad range of

microorganisms (Frenkel and Ribbeck, 2015a, b and see below). Bacterial adhesion is mediated by protein and by carbohydrate structures of MG2. Although MG2 mucin is present in the mucosal pellicle, it is almost absent from the pellicle on tooth enamel (Hannig et al., 2017). Therefore, bacterial binding by MG2 may help to sequester bacterial cells away from the tooth surface. Mucin MG1 has also been shown to keep *Streptococcus mutans* in a planktonic state, in this case by repelling it from adhering to surfaces (Frenkel and Ribbeck, 2015a, b).

Preventing dissolution of tooth enamel by microbially produced acid requires strong buffering capacity in saliva and supersaturation with calcium and phosphate ions. Buffering is provided primarily by bicarbonate, which can reach concentrations in excess of 25 mM under stimulated conditions (Neyraud et al., 2009). Maintenance of calcium and phosphate is facilitated by acidic PRPs, histatin and statherin (Tamaki et al., 2002). These molecules bind calcium ions and inhibit precipitation of calcium phosphates.

Removal of specific acidogenic bacteria may be another strategy for protection against dental caries. Strains of *S. mutans*, a species strongly associated with dental caries, are aggregated by a salivary component termed salivary agglutinin, which has now been identified as glycoprotein gp340 (Jakubovics et al., 2005; Loimaranta et al., 2005). Aggregation of oral bacteria enhances clearance from the oral cavity by swallowing or phagocytosis (Itzek et al., 2017). In contrast, adhesion of *S. mutans* to gp340 adsorbed on enamel surfaces will promote colonization, as discussed in the following text. A number of other salivary molecules have been implicated in adherence and/or aggregation of oral bacteria, including PRPs, statherin, α-amylase, and secretory immunoglobulin A (S-IgA) (Rudney, 2000). The impact of adhesion versus aggregation on the colonization of tooth surfaces by bacteria is discussed in more detail below.

Saliva contains several products that kill or restrict growth of microorganisms. For example, cystatins are cysteine proteinase inhibitors that have been shown to inhibit growth of the periodontal disease-associated species *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Blankenvoorde et al., 1998; Ganeshnarayan et al., 2012) and to possess potent antiviral activities (Bjorck et al., 1990; Korant et al., 1985). Saliva also contains another class of cysteine proteinase inhibitor, the salivary lipocalin von Ebner glands protein (VEGh), which may play similar roles in inhibiting replication of bacteria and virus particles (van't Hof et al., 1997).

Bacteria and fungi require certain essential nutrients such as metal ions for growth, and saliva, like other body fluids, contains proteins that sequester metals. Lactoferrin is present in saliva, milk, and tears and binds iron with high affinity ($K_D \sim 10^{-20}$ M) (Rosa et al., 2017). Lactoferrin has a broad spectrum of antimicrobial properties against bacteria, fungi, and viruses, and not all of these are attributable to its ability to deplete free iron. It has been shown that lactoferrin binds cell walls of Gram-negative bacteria and *Candida* spp., and this appears to interfere with the integrity of the cell membrane (Nikawa et al., 1993). Manganese and zinc ions are important components of many bacterial enzymes. Chelation of Mn^{2+} and Zn^{2+} by calprotectin inhibits growth of *Staphylococcus aureus* in abscesses (Corbin et al., 2008). Calprotectin in saliva is derived primarily from GCF and epithelial cells and is upregulated in gingival inflammation, candidiasis and in Sjögren's syndrome (Farina et al., 2012; Sweet et al., 2001). Interestingly, polymorphisms in the calprotectin gene have been correlated with susceptibility to chronic periodontitis (Sun et al., 2011). Calprotectin expression by oral epithelial cells protects against *P. gingivalis* infection *in vitro* (Nisapakultorn et al., 2001). Therefore, metal binding proteins such as lactoferrin and calprotectin appear to play important roles in host defenses against oral diseases.

Microbicidal enzymes are important components of saliva. Under aerobic conditions, salivary lactoperoxidase enhances oxidative killing of microorganisms by converting thiocyanate and hydrogen peroxide (H_2O_2) to the strongly antibacterial molecule hypothiocyanite ($OSCN^-$). Certain oral streptococci secrete H_2O_2 during growth and there is some evidence that H_2O_2-producing streptococci such as *S. mitis* and *S. sanguinis* are better able to recover after exposure to $OSCN^-$ than *S. mutans* and *S. salivarius* strains that do not secrete H_2O_2 (Carlsson et al., 1983). $OSCN^-$ is believed to cause cellular damage by oxidizing sulfhydryl groups in intracellular proteins (Nagy et al., 2009). In contrast to H_2O_2, $OSCN^-$ does not cause DNA damage and is not thought to be harmful for the host cells that produce it. In saliva, uric acid may compete with thiocyanate to inhibit the production of $OSCN^-$ and it is not yet clear whether lactoperoxidase is an important antimicrobial defense mechanism naturally (Seidel et al., 2014). Nevertheless, an H_2O_2-generating system such as glucose oxidase, lactoperoxidase and thiocyanate has been used in oral care products and shows strong *in vitro* antimicrobial activity in the presence of saliva (Welk et al., 2011).

Lysozyme is present in all major body fluids and has well-characterized antibacterial activity. Lysozyme hydrolyzes glycosidic linkages in the peptidoglycan of some bacterial cell walls, resulting in cell lysis or weakening of the cell barrier. In addition, lysozyme can potentiate nonenzymatic cell degradation in some bacterial species, for example by acting as a cationic antimicrobial peptide (Herbert et al., 2007). Chitinase, which catalyzes the hydrolytic cleavage of chitin, has also been found in saliva (Prodan et al., 2015). Chitin is a component of *Candida* spp. cell walls and chitinase may therefore protect against yeast infections.

Antimicrobial peptides are a key component of innate immunity and it is clear that many peptides produced in saliva have antimicrobial activity. Most work on salivary antimicrobial peptides to date has focused on histatins, a family of neutral or cationic peptides that are rich in histidine residues. At least 25 different histatin peptides have been found in saliva, of which histatins 1 and 3 are encoded by distinct genes and others are generated by proteolysis (Castagnola et al., 2004). Histatins 1, 3, and 5 predominate in parotid saliva (Campese et al., 2009). Histatins are bactericidal against some strains of *S. mutans* and prevent hemagglutination by *P. gingivalis* (Murakami et al., 1992). However, in relation to other antimicrobial peptides throughout the body, the antibacterial properties of histatins are relatively weak. Histatins may be more important in defense against fungal infections because they are potent inhibitors of *Candida* spp. germination and growth (Xu et al., 1991). Human α-

and β-defensins and cathelicidin LL-37 have been identified in saliva: these cationic peptides have broad spectra of antimicrobial properties against fungi, Gram-negative and Gram-positive bacteria, and some enveloped viruses (De Smet and Contreras, 2005). Human β-defensins are expressed in duct cells of salivary glands and in oral epithelial cells (Sahasrabudhe et al., 2000). The α-defensins are expressed in salivary glands and it is likely that they are introduced into saliva by neutrophils that continually migrate across the epithelial barrier (Dale et al., 2006). Neutrophils are also likely to be a significant source of LL-37 in saliva. Individuals with deficiencies in α-defensin and LL-37 levels in neutrophils had no detectable LL-37 in saliva and had severe periodontal disease (Pütsep et al., 2002). In addition, it has been shown that low levels of α-defensins correlate with caries experienced in children (Tao et al., 2005).

Many antimicrobial peptides, including histatins and LL-37, are generated by proteolytic cleavage of larger molecules. The N-terminal regions of lactoferrin and mucin MG2 contain peptide elements that, when synthesized in isolation, display antimicrobial properties against a range of bacteria and fungi (Chapple et al., 1998; Wei et al., 2007). The N-terminal antimicrobial region of lactoferrin, designated lactoferricin-H, is released by pepsin digestion under acidic conditions, a reaction that occurs in the stomach. However, it is not clear whether the N-terminal domains of lactoferrin or MG2 possess antimicrobial activity in saliva. Irrespective of the natural function of these peptides, there is clear potential for exploiting their antimicrobial properties for therapies aimed at oral diseases.

Gingival crevicular fluid

In 1956, while tackling problems related to failures of dental implants in dogs, Neils Brill and Bo Krasse observed that significant amounts of fluid started to ooze from healthy regions of the gingival crevice immediately after drying the entire area (Krasse, 1996). Subsequent work showed that a dye injected into the hind leg of an animal appeared in the gingival crevice within 30 s. The serum-based exudate GCF is also produced in humans and bathes bacteria in gingival crevices and periodontal pockets and seeps gradually into saliva. GCF production is dramatically increased in periodontal disease, and periodontitis treatment protocols can reduce the amount of GCF present. Besides containing serum, GCF contains locally generated degradation/secreted products of tissues and bacteria, inflammatory mediators, and antibodies. Intensive efforts are under way to find diagnostic and prognostic markers of disease in GCF (Kinney et al., 2014).

Microbial Interactions in Oral Biofilms

Acquisition of the Oral Microbiota

Bacterial DNA can be detected in neonates right at the point of birth, and within hours *Staphylococcus* spp. and *Streptococcus* spp. can be cultured (Chu et al., 2017; Nelson-Filho et al., 2013). These genera were shown to be the most abundant over the first 8–21 days of life in a sample of six low birthweight infants (Costello et al., 2013). By 4–8 weeks, streptococci account for 55%–90% of all bacteria in breastfed or formula fed infants, and independent of delivery mode (vaginal delivery or caesarian section) (Al-Shehri et al., 2016; Barken et al., 2008). A study of five edentulous infants with a mean age ~4.6 months found that *Streptococcus* remained the dominant genus prior to tooth eruption and accounted for approximately 60% of oral bacteria (Cephas et al., 2011). Interestingly, many of the species that are commonly found in dental plaque such as *S. sanguinis* and *S. gordonii* (Eren et al., 2014) were present in the saliva of these infants, indicating that the presence of tooth surfaces is not essential for these species to thrive in the oral cavity. Between 3 months and 3 years of age, the species richness (total number of different species present) and the diversity of taxa (a measure that accounts for both the richness and the evenness of taxa) increases significantly (Lif Holgerson et al., 2015). Major events that lead to changes in the infant microbiome are thought to be tooth eruption and weaning. The oral microbiome continues to mature through childhood until at least the age of 18 years (Crielaard et al., 2011; Gomez and Nelson, 2017; Papaioannou et al., 2009).

There is evidence that early acquisition of *S. mutans*, or the presence of increased *S. mutans* load in infancy, is correlated with caries development in children (Lif Holgerson et al., 2015; Straetemans et al., 1998). In adults, *S. mutans* is commonly found in biofilms on enamel and is detectable at low levels in >95% of adults (Lindquist et al., 1989; Lindquist and Emilson, 1990). Culture-based approaches indicated that the acquisition of *S. mutans* occurs at a particular time during childhood (Caufield et al., 1993). This "window of infectivity" was found to occur between 19 and 31 months after birth with the median age for acquiring *S. mutans* being 26 months. Curiously, the median age for tooth emergence is 7.1 months; yet, the window of infectivity for this bacterium appeared to be at least a year later. More recent studies have now shown that the window of infectivity is not absolute. In one cohort of 104 subjects, 12% of infants were found to be colonized by *S. mutans* by just 4–6 weeks (Reed et al., 2014). Although tooth surfaces are clearly not essential for *S. mutans*, it has been demonstrated that there is a correlation between tooth eruption and the acquisition of mutans streptococci (Nelson et al., 2014).

The source of each person's individual strains of bacteria is unknown but the close contact with family members or other caregivers probably influences the species of bacteria that inoculate and colonize the oral cavity. This notion is supported by the studies using *S. mutans*, which is common in adult saliva. Unique patterns of bacteriocin production and sensitivity of isolates of *S. mutans* from mother–infant pairs were very similar, suggesting that intrafamilial transmission is a likely route for establishing the oral microbial populations of children (Berkowitz and Jordan, 1975). More recently, genetic fingerprinting techniques have confirmed that identical strains of *S. mutans* can be found in mothers and their children (Li and Caufield, 1995; Momeni et al., 2016).

Bacteria–Host Interactions

For successful colonization of surfaces in the oral cavity, bacteria must attach to soft or hard tissues, obtain energy from the available nutrients to grow and divide, resist or evade host immune responses, and compete or cooperate with neighboring bacterial cells to establish a stable biofilm community. Much attention has focused on the first step in colonization, bacterial adhesion to host tissues, because this holds great promise for proactive strategies that are aimed at preventing integration of harmful bacteria into oral biofilms and thus avoiding even the early stages of disease. Adhesion is a selective process that involves specific interactions between receptors immobilized on the surfaces of host tissues and adhesive proteins and glycoproteins (adhesins), expressed in the outer layers of oral bacterial cells.

Bacterial colonization of tooth enamel: Adhesion versus aggregation

All tooth surfaces in the oral cavity are coated with a layer of proteins and glycoproteins known as the acquired enamel pellicle (Siqueira et al., 2012). The pellicle starts to form within 1 min of introducing a clean enamel surface into the mouth and reaches equilibrium between dissociation and deposition of material at 90–120 min (Lee et al., 2013). After 2 h, the pellicle consists of a compact basal layer and a globular surface layer (Hannig, 1999). The activity of some enzymes is affected by immobilization on a surface. Nevertheless, it is clear that a wide range of salivary enzymes retain catalytic activity when adsorbed onto calcium hydroxyapatite (Hannig et al., 2005). Accumulation of salivary pellicle on enamel is a process of selective adsorption of salivary components, and the (glyco)protein composition of pellicle is thus distinct from that of unbound saliva. Because of the ubiquitous presence of salivary pellicle, bacteria that attach to teeth do not make contact with the enamel directly, but instead interact with pellicle components.

Investigations on the inhibitory role of S-IgA on adhesion of oral bacteria to enamel surfaces in the early 1970s led Ronald Gibbons and coworkers to propose that aggregation (agglutination) of bacteria might be an active mechanism for clearing bacteria from the oral cavity (Bammann and Gibbons, 1979; Bratthall and Gibbons, 1975). It was suggested that large aggregates coated with S-IgA would be less prone to attach to surfaces than single uncoated bacterial cells, and that aggregates would presumably be removed by swallowing. These seminal studies led to a new way of thinking about oral bacterial adherence as an interplay between factors that promote clearance from the oral cavity (including molecules that cause aggregation of bacterial cells) and components that aid bacterial adhesion to oral surfaces. It is now recognized that the abilities of different host receptors, including S-IgA, to modulate bacterial adhesion and/or aggregation are extremely complex. Like many other salivary proteins, S-IgA is present in both saliva and the acquired salivary pellicle and may therefore inhibit or promote bacterial adhesion, depending on the context (Stiefel, 1976). Bacteria that adhere to salivary pellicle, predominantly the oral streptococci, tend to have multiple specificities for host receptors and it is often difficult to define the roles of individual (glyco)proteins in adhesion (Jakubovics et al., 2005). Furthermore, aggregation may not necessarily lead to clearance from the oral cavity. *In vitro* studies indicated that large aggregates of *Streptococcus* spp. or *Actinomyces* spp. cells have reduced affinity for saliva-coated hydroxyapatite, but that small aggregates of two to three times a single adhering unit actually show enhanced capacity for binding (Liljemark et al., 1981). In many cases, recognition of salivary molecules by bacterial adhesins depends on whether the receptor is present in the fluid or the surface-immobilized phase. For example, strains of *A. naeslundii* and *S. mutans* bind acidic PRPs such as PRP-1 in acquired pellicle, but do not bind the same PRPs in saliva fluid (Ruhl et al., 2004). Physicochemical studies have shown that the conformation of PRP-1 is altered upon binding to hydroxyapatite (Elangovan et al., 2007). Presumably this conformational change upon adsorption unmasks a bacterial binding domain. Hidden binding domains such as these are termed "cryptitopes."

The major salivary proteins known to interact with oral bacteria are summarized in Table 1. Although there is undoubtedly some bias in the species that have been chosen for investigation, it is clear that streptococci possess the most diverse range of binding specificities for salivary proteins of all oral bacteria. This is perhaps not surprising because oral streptococci constitute approximately 60%–80% of the total microbial population in early supragingival dental plaque. *Actinomyces* spp., *Haemophilus* spp., and *Veillonella* spp. constitute most of the remainder of cells in plaque within the first 4–8 h after tooth cleaning (Diaz et al., 2006), and these bacteria coaggregate with streptococci or bind a more limited range of salivary components. *P. gingivalis* and *Fusobacterium nucleatum* require anaerobic conditions for growth and are not found at high levels in initial supragingival biofilms. The importance of oral colonization by *H. pylori* has yet to be properly evaluated, but there is concern that the survival of this species in oral biofilms could provide a reservoir for infection of the stomach. There have been several reports of *H. pylori* adhesion to salivary macromolecules and this is still an active area of research (Linden et al., 2008; Prakobphol et al., 2000; Veerman et al., 1997; Walz et al., 2009).

The most abundant enzyme in saliva is α-amylase, and this is the receptor for proteins produced by a number of species of oral streptococci. Surveys of isolated streptococci demonstrated that whereas strains of *S. mitis*, *S. cristatus*, *S. parasanguinis*, and *S. gordonii* bound α-amylase, no *S. sanguinis*, *S. oralis*, or *S. mutans* strains adhered (Douglas et al., 1990; Nikitkova et al., 2013; Scannapieco et al., 1994; Scannapieco et al., 1995). On this basis, α-amylase binding has been suggested as a useful test for discriminating between oral streptococci (Douglas et al., 1990). Adhesion to α-amylase has been most thoroughly studied in *S. gordonii*. This organism produces two amylase-binding proteins, AbpA (20 kDa) and AbpB (82 kDa) (Tanzer et al., 2003). α-Amylase remains active when bound to streptococci, and thus may provide oligosaccharides from dietary starch that could be used by the streptococci or by neighboring bacteria in biofilms (Nikitkova et al., 2013).

Compared with other oral streptococci, *S. mutans* expresses few different polypeptides on its cell surface. Mutans streptococci are not generally found in nascent supragingival plaque, and inhabit areas of the teeth such as cracks and fissures that are protected from fluid and masticatory shear forces. Once established, mutans streptococci are difficult to remove because they are

Table 1 Constituents of saliva that mediate adhesion or aggregation of oral bacteria

Salivary component	*Species of bacteria bound*	*Bacterial adhesin*
α-Amylase	*S. gordonii*	AbpA, AbpB
	S. mitis, *S. parasanguinis*, *S. cristatus*,*S. salivarius*	
Gp340	*Actinomyces* spp.	Type 1 and Type 2 fimbriae
	S. gordonii	AgI/II[a], GspB/Hsa
	S. mutans	AgI/II[b]
	H. pylori	SabA
MG1	*H. pylori*	SabA, BabA, AlpA, AlpB
	H. parainfluenzae	HifA and type b pili
MG2[c]	*S. gordonii*, *S. sanguinis*	GspB/Hsa/SrpA
	S. mutans	
	A. actinomycetemcomitans	
PRPs	*S. gordonii*, *S. mutans*	AgI/II[a,b]
	A. naeslundii	Type 1 fimbriae
	P. gingivalis	Long fimbriae
	F. nucleatum	
S-IgA	*S. sanguinis*, S. *mitis*	
Statherin	*A. naeslundii*	Type 1 fimbriae
	F. nucleatum	
	P. gingivalis	Long fimbriae

[a]Antigen I/II proteins of *S. gordonii* are SspA and SspB.
[b]*S. mutans* produces one antigen I/II adhesin, designated P1, PAc, antigen I/II or antigen B.
[c]Absent or at low concentration in acquired salivary pellicle.

often embedded in extracellular polysaccharide matrices, derived from the action of glucosyltransferase and fructosyltransferase enzymes on sucrose (Koo et al., 2013). The possibility of preventing adhesion by mutans streptococci is attractive and has been the subject of intensive research efforts. Both *S. mutans* and *S. sobrinus* adhere *in vitro* to pellicles on the surface of hydroxyapatite (Gibbons et al., 1986). However, these species appear to use different mechanisms for attachment because *S. sobrinus*, but not *S. mutans*, requires glucan polymers for adhesion. *S. mutans* is aggregated in the presence of parotid saliva by a high M_r (>300 kDa) protein termed salivary agglutinin (Carlen et al., 1998). An approximately 170 kDa adhesin on the surface of *S. mutans* was found to be critical for adhesion to salivary pellicle and saliva-mediated aggregation, because disruption of the coding gene abrogated adhesion by *S. mutans*. This protein has been variously described as antigen B, PAc, P1, and antigen I/II. The receptor for antigen I/II has now been identified as gp340, a molecule that interacts with surfactant protein D and has a role in innate immunity (Loimaranta et al., 2005, Prakobphol et al., 2000). A specific peptide corresponding to amino acids 1025–1044 of antigen I/II from *S. mutans* is highly effective at blocking binding of *S. mutans* to gp340 and has been suggested as a possible anti-caries treatment agent (Li et al., 2009). One or two copies of antigen I/II protein genes are present in many oral and extra-oral streptococci, including *S. cricetus*, *S. pyogenes*, and *S. gordonii* (Brady et al., 2010). In contrast to *S. mutans*, disruption of antigen I/II genes *sspA* and *sspB* in *S. gordonii* reduces adhesion to gp340 by only ~40% (Jakubovics et al., 2005). Residual binding is because of two families of cell surface adhesin polypeptides, the CshA/CshB proteins that form fibrillar structures and Hsa (GspB in *S. gordonii* M99) sialic acid-binding adhesin.

The sialic acid-binding adhesins of *S. gordonii* mediate binding to mucin MG2 (Takamatsu et al., 2006). However, similar to gp340, MG2 also has bacterial binding properties in the peptide backbone structure. For example, the N-terminal region of MG2 is recognized by *S. mutans* and *A. actinomycetemcomitans* (Ge et al., 2004; Liu et al., 2002). MG2 has not been detected in salivary pellicle, and may be more important for aggregation of bacteria than adhesion to surfaces. Mucin MG1 is found in the acquired pellicle, but is not bound by streptococci. Only *H. pylori* and *Haemophilus* spp. bind to MG1 (Linden et al., 2008; Veerman et al., 1995). *Haemophilus* spp. appear to recognize peptide motifs of MG1 and the large quantities of carbohydrates on MG1 mucin are not involved in binding. In contrast *H. pylori*, a persistent colonizer of the mucin-rich gut environment, expresses several lectin-like adhesins, including SabA, BabA, and AlpA/B, that combine to produce strong adhesion to many glycosylated polypeptides in the oral cavity.

PRPs, including proline-rich glycoprotein (PRG), have diverse bacterial binding properties when immobilized on surfaces. These molecules mediate attachment of antigen I/II-bearing streptococci including *S. mutans* and *S. gordonii* (Ruhl et al., 2004). Antigen I/II protein of *S. mutans* binds PRPs *in vitro* (Russell and Mansson-Rahemtulla, 1989). In addition, lectin-like interactions that are inhibited by carbohydrates have been demonstrated between *S. oralis* or *S. gordonii* and PRG (Murray et al., 1992). *F. nucleatum* and *A. naeslundii* also recognize PRG, and PRG promotes adhesion of these organisms to hydroxyapatite surfaces (Gillece-Castro et al., 1991; Ruhl et al., 2004). A small peptide (RGRPQ) that is released by proteolytic action of *S. gordonii* on PRP-1 inhibits adhesion and promotes desorption of *A. naeslundii* from immobilized PRP-1 (Drobni et al., 2006). Therefore, streptococcal protease activity may be important in competition for binding sites in salivary pellicle.

Like PRPs, statherin interacts with bacteria only when it is immobilized on surfaces. Statherin is unstructured in solution and folds upon adsorption onto hydroxyapatite to form a bacteria-binding motif in the C-terminus (Goobes et al., 2006). Type 1 fimbriae of *Actinomyces* spp. and long fimbriae of *P. gingivalis* recognize and bind to statherin (Amano et al., 1996; Li et al., 2001). Interestingly, these fimbriae also bind PRPs, although at least in the case of *P. gingivalis*, distinct regions of fimbrial proteins are required for interactions with PRPs and statherin (Amano et al., 1996). Surface-bound statherin also promotes adhesion of *F. nucleatum* and *C. albicans*, but retards adherence of *S. mutans* (Johansson et al., 2000; Nakagaki et al., 2010; Shimotoyodome et al., 2006).

Interactions with oral epithelia

As mentioned earlier, the healthy soft oral tissues are heavily populated by microorganisms (Aas et al., 2005). However, cell adherence and invasion are also common virulence factors of many pathogens. In the oral environment, cell adherence provides retention to the soft oral surfaces, without which the pathogen would be washed away by the saliva. Cell invasion can offer an escape mechanism from a hostile host immune system and from antimicrobial therapeutic agents. Therefore, it is not surprising that cell adherence and invasion capabilities are found in oral pathogens.

The periodontopathogen *A. actinomycetemcomitans* is recovered more frequently and in higher numbers from oral mucosal surfaces than from subgingival and supragingival plaque (Muller et al., 2001). This species has been isolated from mucosal surfaces in predentate children (Lamell et al., 2000). In addition, the transfer of *A. actinomycetemcomitans* from oral epithelia to tooth surfaces has been demonstrated in human volunteers, indicating that the oral mucosa is the initial site of colonization and the primary reservoir of *A. actinomycetemcomitans* in the oral cavity (Fine et al., 2010). Aae, a 90-kDa surface-exposed protein that is homologous to an epithelial cell adhesin (Hap) produced by *H. influenzae*, mediates weak binding of *A. actinomycetemcomitans* to buccal epithelial cells (Fine et al., 2005). A second *A. actinomycetemcomitans* adhesin involved in adhesion to buccal epithelial cells is ApiA, a 100-kDa protein also known as Omp100, a member of the *Yersinia* spp. YadA family of autotransporter adhesins (Yue et al., 2007). Combined gene knockout mutations of *apiA* and *aae* completely abrogated the ability of *A. actinomycetemcomitans* to bind human buccal epithelial cells. *A. actinomycetemcomitans* also invades gingival epithelial cells through actin-dependent or actin-independent mechanisms (Brissette and Fives-Taylor, 1999).

P. gingivalis is not only capable of cell adherence and invasion but also of transmission between different cell types (Yilmaz et al., 2006). This intercellular transmission is a likely mechanism for persistence of an inflammatory pathogen in host tissue. Cell invasion of atheromatous tissue has been suggested as a virulence mechanism linking *P. gingivalis* with cardiovascular disease (Olsen and Progulske-Fox, 2015).

At least two fusobacterial adhesins participate in attachment to mammalian cells. The FadA adhesin is a 12-kDa outer membrane protein, and the second adhesin is a galactose inhibitable protein called Fap2 that also mediates adhesion to *P. gingivalis* (Coppenhagen-Glazer et al., 2015; Fardini et al., 2011). Host cell adherence of *F. nucleatum*, mediated by Fap2, is also hypothesized to play a role in the involvement of this bacterium in preterm labor (Coppenhagen-Glazer et al., 2015). Further, Fap2 is involved in enrichment of *F. nucleatum* at sites of colon adenocarcinoma through adhesion to Gal-GalNAc receptors and protects tumor cells from immune attack by binding to and activating the human inhibitory receptor TIGIT (Abed et al., 2016; Gur et al., 2015). Thus, through the activities of Fap2, *F. nucleatum* may promote the development and/or progression of colon cancer. It is expected that identification of other adhesins involved in cell adherence by major oral pathogens could lead to important therapeutic strategies.

Bacteria–Bacteria Interactions

Coaggregation and coadhesion in spatiotemporal oral biofilm development

All oral bacteria coaggregate with at least one genetically distinct cell type (Kolenbrander and London, 1993). Coaggregation is the phenomenon in which a dense suspension of one cell type, for example *S. oralis*, when mixed with a dense suspension of another cell type, for example *A. naeslundii*, forms clumps or coaggregates and often within seconds these coaggregates settle to the bottom of the suspension (Kolenbrander et al., 2006). Coadhesion is a term often used to describe cell–cell recognition between a suspended cell type and one already attached to a surface. Coaggregation partners are usually from different genera, but some partnerships are intrageneric and intraspecific between strains. These latter two kinds are especially seen among viridans streptococci. This special property may be critical for the development of initial streptococcal communities on freshly cleaned teeth. In addition, most streptococci also bind to receptors in the acquired pellicle and may contribute to their being the most numerous bacteria found in the initial microflora (Nobbs et al., 2011). Their adherence to the surface provides numerous opportunities for other species and genera to bind to them and establish multispecies communities through growth and adherence of additional species, which facilitates the succession of species on tooth surfaces (Fig. 3).

Among the first reported examples of large-scale succession of bacteria was the evidence in the 1960s that progression from periodontally healthy sites to gingivitis was accompanied by a shift from predominately Gram-positive bacteria to predominately Gram-negative bacteria (Theilade et al., 1966). The "experimental gingivitis" model, in which volunteers abstain from oral hygiene for up to 21 days, continues to be used to monitor microbial changes upon transition from health to gingivitis, although the model has been enhanced by the application of more powerful analysis techniques. Such models continue to show enrichment of Gram-negative species in gingivitis such as *Fusobacterium nucleatum* subsp. *polymorphum*, *Lautropia* sp. HOTA94, and *Prevotella oulorum*, whereas certain Gram-positive species such as *Rothia dentocariosa* are associated with health

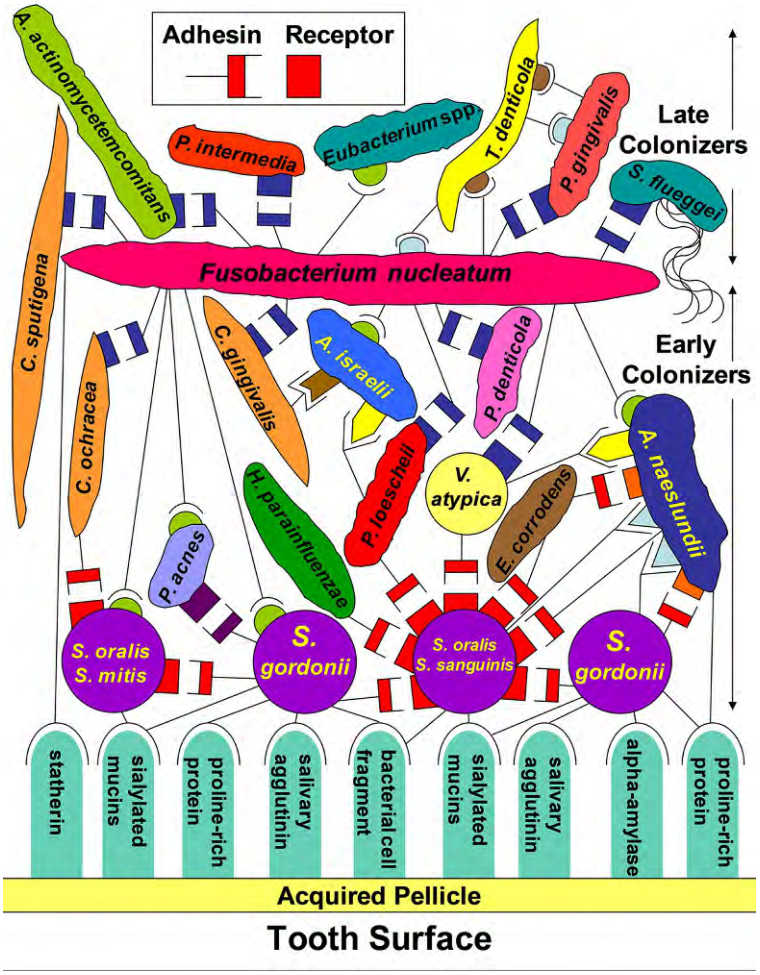

Fig. 3 Spatiotemporal model of oral bacterial colonization, showing recognition of salivary pellicle receptors by initial colonizing bacteria and coaggregations between initial colonizers, fusobacteria, and late colonizers of the tooth surface. Each coaggregation depicted is known to occur in a pairwise test. Collectively, these interactions are proposed to represent development of dental plaque. Starting at the bottom, initial colonizers bind via adhesins (round-tipped black line symbols) to complementary salivary receptors (blue-green vertical round-topped columns) in the acquired pellicle coating the tooth surface. Late colonizers bind to previously bound bacteria. Sequential binding results in the appearance of nascent surfaces that bridge with the next coaggregating partner cell. Several kinds of coaggregations are shown as complementary sets of symbols of different shapes. One set is depicted in the box at the top. Proposed adhesins (symbols with a stem) represent cell-surface components that are heat inactivated (cell suspension heated to 85°C for 30 min) and protease sensitive; their complementary receptors (symbols without a stem) are unaffected by heat or protease. Identical symbols represent components that are functionally similar but may not be structurally identical. Rectangular symbols represent lactose-inhibitable coaggregations. Other symbols represent components that have no known inhibitor. Communication among cells is favored by their close cell–cell contact. The bacterial species shown are *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), *Actinomyces israelii, Actinomyces naeslundii, Capnocytophaga gingivalis*, *Capnocytophaga ochracea*, *Capnocytophaga sputigena*, *Eikenella corrodens*, *Eubacterium* spp., *Fusobacterium nucleatum, Haemophilus parainfluenzae, Porphyromonas gingivalis, Prevotella denticola, Prevotella intermedia, Prevotella loescheii, Propionibacterium acnes, Selenomonas flueggei, Streptococcus gordonii, Streptococcus mitis, Streptococcus oralis, Streptococcus sanguinis, Treponema* spp., and *Veillonella atypica*.

(Kistler et al., 2013). The correlation is not absolute as the Gram-positive organism *Lachnospiraceae* [G-2] sp. HOT100 was also associated with the onset of gingivitis. Therefore, these data have not changed our understanding of the overall temporal succession and periodontal disease-associated progression of species from Gram-positive to Gram-negative bacteria. When cells from various Gram-positive and Gram-negative species were mixed pairwise for their ability to coaggregate, a nonrandom set of coaggregation partnerships emerged. Each cell type coaggregated with specific partner cell types. Importantly, those species that initially colonize the tooth such as many Gram-positive cell types are ones that coaggregate with each other (Fig. 3). Those species that appear much later such as many Gram-negative cell types generally do not coaggregate with the initial colonizers, but they often coaggregate with each other. Both initial and later colonizers coaggregate with fusobacteria, which acts as a coaggregation bridge that spans a temporal biofilm development on teeth (Fig. 3) (Kolenbrander et al., 1989; Nobbs et al., 2011). Integration of these data led to the hypothesis that coaggregation is a significant contributor to the succession of species in dental plaque.

Metabolic interactions and cell–cell signaling

The development of global profiling technologies such as microarrays and "-omics" techniques has heralded a new era for the study of interbacterial communication and interactions in complex biofilm systems such as those present in the oral cavity. Microarrays and RNASeq allow the measurement of an entire bacterial genome response to a relevant stimulus such as integration of cells into a biofilm or interaction with a different species of bacteria. These methods can be supplemented with the proteomics or metabolomics to investigate global changes in protein or metabolite concentrations, respectively. From these and other studies it has become clear that bacteria respond to contact with cells of other species by changing their patterns of gene expression and hence their phenotypes. Therefore, bacteria in multispecies biofilm communities are physiologically different from isogenic cells, which have been studied in laboratory monocultures. This has profound implications for oral microbiologists because studies exclusively focused on bacteria in isolation cannot be extrapolated to complex dental plaque communities without further empirical evidence. Some of the interactions that occur between bacteria in dental plaque are shown in Fig. 4.

Many species that are commonly found in dental plaque do not grow in axenic laboratory cultures when provided with unmodified saliva as the sole source of nutrients. It is likely that the growth of these species in the oral cavity is enabled by the metabolic activities of nearby bacteria. Beneficial interactions can arise through metabolic cooperation, where the combined action of enzymes from multiple species allows the efficient degradation of a complex substrate, or through nutrient exchange where the waste products of one species are used as a nutrient source for a microorganism with different metabolic requirements. Several studies have shown that microbial consortia are more efficient at degrading substrates found in saliva than single species of bacteria. For example, in chemostat cultures supplied with hog gastric mucin as the major source of carbon and energy, a five-member consortium grew but not efficiently. Introduction of new species with different glycolytic and proteolytic activities led to their stable integration into the community and increased the total yield of bacteria from the substrate (Bradshaw et al., 1994). A microbial consortium isolated from dental plaque consisting of *S. mitis* Biovar 2, *S. gordonii*, *S. cristatus* and *A. naeslundii* was able to degrade purified human mucin MG1, whereas these species in isolation were unable to catabolize this substrate (Wickström et al., 2009a, b). Degradation of mucins is a complex process and appears to be more efficient when mucins are in suspension than when they are bound to a surface (Wickström et al., 2009a, b).

Nutritional interactions influence the growth of periodontal pathogens. For example, mutualistic growth as a consequence of cross-feeding has been demonstrated between *P. gingivalis* and *T. denticola* (Grenier, 1992). Succinate produced by *T. denticola* serves as a nutrient source for *P. gingivalis*, whereas isobutyric acid, a product of *P. gingivalis* metabolism, enhances the growth of *T. denticola*. Oxygen tension is an important factor for periodontal pathogens, because many of these organisms are obligately anaerobic. The presence of aerobic, facultative anaerobic, or oxygen-tolerant species in dental plaque may be sufficient to deplete oxygen to levels that are consistent with growth of obligate anaerobes. Thus, aerobic growth of the strict anaerobe *P. gingivalis* was enabled by the presence of the relatively aerotolerant *F. nucleatum* (Bradshaw et al., 1997).

S. gordonii promotes the recruitment of *P. gingivalis* into biofilms *in vitro* through interactions mediated by the *P. gingivalis* major and minor fimbrial antigens (FimA and Mfa, respectively) (Daep et al., 2011; Simionato et al., 2006; Wright et al., 2013). The close proximity of cells enhances their capacity for metabolic exchange (Kolenbrander et al., 2010). In this pairing, the metabolite 4-aminobenzoate/*para*-amino benzoic acid (*p*ABA) has been shown to be a key nutrient for colonization by *P. gingivalis*. *S. gordonii* produces *p*ABA, a metabolite in the folate biosynthesis pathway, through the chorismate-binding enzyme Cbe. *S. gordonii* mutants lacking the gene encoding Cbe do not support the accumulation of *P. gingivalis* in biofilms. Exogenous *p*ABA results in up-regulation

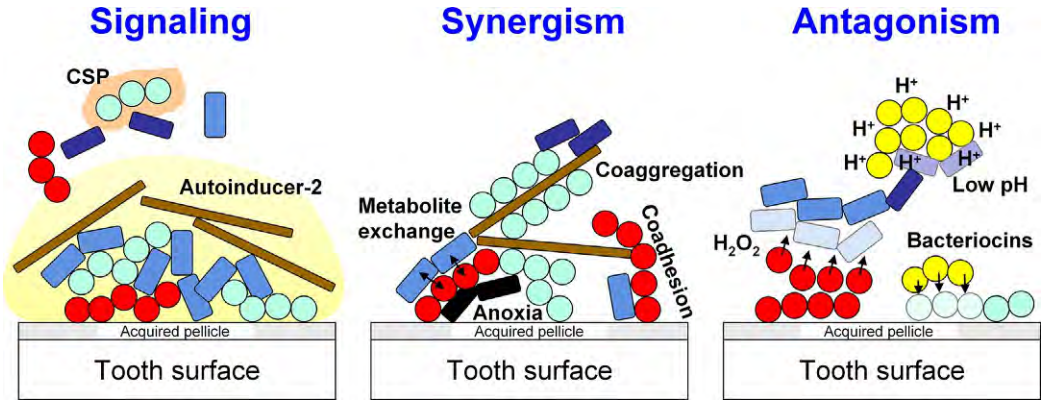

Fig. 4 Interbacterial interactions in dental plaque. Secreted signaling molecules such as competence-stimulating peptides (CSP) or autoinducer-2 accumulate around all bacterial cells in the multispecies community. The concentration of signaling molecules rises and falls in proportion to the density and diversity of bacterial cells. Various synergistic interactions have been described in oral biofilms. Exchange of metabolites between bacteria enables communities of cells to degrade substrates that cannot be utilized by individual species. Removal of oxygen by aerobic bacteria or facultative anaerobes creates pockets of anoxia that support growth of obligately anaerobic bacteria. The presence of specific receptors and adhesins on different bacteria results in coaggregation. If one partner also possesses adhesins for salivary pellicle receptors, then the coaggregated bacteria will attach to the surface in a process called coadhesion. Antagonistic interactions also occur between oral bacteria. Examples are the production of bacteriocins by *S. mutans*, and production of H_2O_2 by oral viridans streptococci. In addition, acidogenic bacteria create low-pH conditions that inhibit many other microorganisms.

of genes encoding both FimA and Mfa in *P. gingivalis* and hence promotes heterotypic biofilm formation with *S. gordonii*. Interestingly, in a murine infection model *p*ABA enhances the colonization and survival of *P. gingivalis*, but reduces virulence, indicating that this metabolite plays a complex role in regulating microbe-host interactions (Kuboniwa et al., 2017).

The ability to recycle nutrients is important for species growing in the presence of lactic acid-producing oral streptococci. Certain oral bacteria such as *Veillonella* spp. can use lactate as an energy source. In gnotobiotic rats, the presence of *S. mutans*, and in particular strains that produce large amounts of lactic acid, enhances colonization by *Veillonella alcalescens* (McBride and Van der Hoeven, 1981). *In vitro* studies on the relationship between *Veillonella atypica* and *S. gordonii* have revealed a novel signaling pathway by which *V. atypica* cells induce the upregulation of *amyB* gene expression and α-amylase enzyme activity in juxtaposed *S. gordonii* (Egland et al., 2004). This appears to be an active mechanism by which *V. atypica* releases a cue, possibly maltotriose or maltose-containing sugars, which increases the production of lactic acid from intra- or extracellular carbohydrates by *S. gordonii* and thus maximize the benefits from the interaction (Johnson et al., 2009).

Lactic acid is also a key nutrient for *A. actinomycetemcomitans* (Brown and Whiteley, 2007). However, hydrogen peroxide (H_2O_2) produced and excreted by many oral streptococci such as *S. gordonii* is potentially inhibitory to *A. actinomycetemcomitans*. It has been shown that when the *A. actinomycetemcomitans* catalase is overwhelmed and unable to tolerate the H_2O_2 from *S. gordonii*, *A. actinomycetemcomitans* instead uses the glycosidase enzyme Dispersin B to degrade the biofilm matrix in the local area and to move to a safe distance from *S. gordonii* cells (Stacy et al., 2014). In *S. gordonii* and many other oral streptococci, the majority of H_2O_2 produced is derived from the metabolism of pyruvate (Barnard and Stinson, 1996). Interestingly, *Streptococcus cristatus* strains (previously *Streptococcus oligofermentans* (Jensen et al., 2016)) produce H_2O_2 from the oxidation of lactic acid through the action of lactate oxidase, a product of the *lox* gene (Liu et al., 2012). The H_2O_2 produced by the oxidation of lactate inhibits the growth of the lactic acid tolerant species *S. mutans* (Liu et al., 2012; Tong et al., 2007; Tong et al., 2008).

H_2O_2 produced by oral streptococci is an important mediator of interspecies competition, at least *in vitro* (Redanz et al., 2018). As noted above, H_2O_2 is a substrate for salivary lactoperoxidase and it is not known exactly how this enzyme modulates the antimicrobial properties of streptococcal-produced H_2O_2 *in vivo*. Nevertheless, there is a correlation between increased numbers of *S. sanguinis* or other peroxidogenic streptococci in plaque and reduced levels of *S. mutans* or periodontopathogens such as *T. forsythia* or *A. actinomycetemcomitans* (Fakhruddin et al., 2018); Ren et al., 2017; Socransky et al., 1998). Although *S. mutans* strains on the whole do not produce large amounts of H_2O_2, this species has a different weapon in its armory: bactericidal peptides known as bacteriocins or mutacins (Merritt and Qi, 2012). Several bacteriocins from *S. mutans* have been described and production of these molecules is strain-dependent. Under conditions that support the synthesis of bacteriocins *in vitro*, *S. mutans* can compete effectively with neighboring bacteria including *S. sanguinis* (Kreth et al., 2008). However, it should be noted that both *S. gordonii* and *S. sanguinis* can inhibit bacteriocin production by *S. mutans* and a clear role for bacteriocins *in vivo* is yet to be demonstrated.

Cell–cell communication is a fundamental process governing the structure and organization of biofilm communities. A number of bacterial signaling systems have been described to date, and of these autoinducer-2 (AI-2) and peptide signals appear to be the most important in oral biofilms. AI-2 is the product of the *luxS* gene that is present in a variety of Gram-positive and Gram-negative oral bacteria (Pereira et al., 2013). Many aspects of AI-2 signaling remain to be elucidated. However, it is clear that this signal evokes a broad range of responses in many bacterial species. In *S. mutans*, for example, microarray analysis has revealed that almost one-third of the transcriptome (the total number of distinct mRNA sequences produced by the cell) is affected by AI-2 (Sztajer et al., 2008). AI-2 is required for biofilm formation by *A. actinomycetemcomitans* and is a key determinant of mutualistic biofilm formation by *S. oralis* and *A. naeslundii* (James et al., 2006; Rickard et al., 2006). Neither *A. naeslundii* nor *S. oralis* was able to grow in a glass flowcell in monoculture using saliva as the sole nutrient source. However, these two organisms formed a dual-species biofilm when coinoculated. A *luxS* mutant of *S. oralis* that did not produce AI-2 was unable to grow in monoculture or in coculture. Addition of chemically synthesized AI-2 restored growth in cocultures of *A. naeslundii* and the *S. oralis luxS* mutant, thereby giving AI-2 the status of a bona fide signaling molecule (Rickard et al., 2006).

Signaling with AI-2 is essentially species independent because many species produce and/or respond to the same signal. In contrast, small peptide signals produced by Gram-positive bacteria are species-specific cell density-dependent (quorum sensing) signals. These molecules were first shown to be involved in competence, a physiological state in which bacteria are able to take up DNA. It is now known that competence-stimulating peptides (CSP) of oral streptococci influence a number of physiological processes in addition to competence, including bacteriocin production by *S. mutans* and biofilm formation (Perry et al., 2009; Shanker and Federle, 2017). The combination of competence and bacteriocin production by *S. mutans* enables this organism to obtain DNA from neighboring *S. gordonii* cells in biofilms (Kreth et al., 2005). When the donor *S. gordonii* contained a shuttle plasmid, *S. mutans* cells that acquired DNA were able to maintain the plasmid during subsequent rounds of replication. Analysis of whole-genome sequences can reveal indicators of past events of horizontal gene transfer, the uptake and incorporation of DNA from a heterologous organism into the replicating DNA of a host. Such indicators include characteristic sequence patterns of mobile genetic elements including transposons or lysogenic bacteriophage, or the presence of DNA sequence "islands" containing a different GC content from the rest of the chromosome. Analysis of oral bacterial genome sequences provides strong evidence that "foreign" DNA is present in the genome sequences of many strains including *S. mutans* UA159, *P. gingivalis* W83, and *F. nucleatum* ATCC25586. The potential for DNA transfer between heterologous bacteria in oral biofilms is a concern because this could enhance the spread of antibiotic resistance between bacteria.

Cell-cell sensing may also occur in response to direct contact between cells. It has been demonstrated that *S. gordonii* senses coaggregation with *A. oris* and responds by changing the expression of 23 genes, including 9 genes involved in arginine metabolism

and transport (Jakubovics et al., 2008). It appears that this sensing depends on the action of a secreted *S. gordonii* protease, Challisin, which apparently cleaves proteins from the cell surface of *A. oris*, potentially releasing amino acids or peptides that can be uptaken by *S. gordonii* (Mohammed et al., 2018).

Methods of studying oral biofilms

The study of oral biofilms was first reported by Antonie van Leeuwenhoek, who in 1683 described oral bacteria ("very little living animalcules") sampled from plaque between his own teeth (Bardell, 1983). van Leeuwenhoek's prescient descriptions included coaggregating bacteria, and the fact that bacteria in dental biofilm are more resistant to vinegar treatment than the same bacteria suspended from a dispersed dental biofilm. To date, most knowledge of oral biofilms is based on analysis of the composition of dental biofilms sampled from healthy or diseased sites. Sampling is generally performed using dental instruments, paper points, or obtained from extracted teeth. Plaque samples can then be analyzed microscopically (as first performed by van Leeuwenhoek), by culturing techniques or, as has been performed more recently, by genomic approaches (see earlier text). Interactions between identified cultivable bacteria can be studied *in vitro* under planktonic or biofilm conditions. Biofilms can be grown in static biofilm models (usually wells of a 96-well plate), or under flow conditions such as those created in the disposable flow cell system described in Fig. 5. This model involves inoculating bacteria into a track constructed from a microscope slide, a coverslip, and silicone sealant (Kolenbrander et al., 1999). Medium such as diluted saliva is pumped through the flowcell continuously and the system is maintained at 37°C. If required, the flowcell can be run under anaerobic conditions. Biofilms in the flowcell can be stained non-disruptively and examined under the confocal laser scanning microscope. More recently, microfluidics systems have been used to provide tighter control over the shear forces and to reduce the requirements for large volumes of growth media or saliva (Nance et al., 2013).

An *in situ* approach for studying dental plaque is the retrievable enamel chip model system (Fig. 6). A volunteer wears an acrylic appliance on each side of the mandible. Each appliance contains three 3 × 3 × 1 mm enamel chips, as shown in the figure (Palmer Jr. et al., 2001). Enamel chips are prepared from extracted third molars of humans and sterilized. Thus, six replicates are possible for a time point. However, usually two time points are chosen, for example, 4 and 8 h of wear. The appliance is removed at the proper time and the enamel chips are retrieved, incubated with fluorescently conjugated specific antibodies or oligonucleotide probes, and processed for microscopy without disturbance to spatial relationships within the native biofilm. Use of the retrievable enamel chip model and confocal microscopy has confirmed the presence of coaggregation interactions in the development of initial oral communities (Palmer Jr. et al., 2003). Multispecies communities of streptococci and actinomyces developing *in situ* were shown to be composed of interdigitated mixed species, and not mono-species arranged side by side. Thus, evidence provided by the retrievable enamel chip model confirmed earlier cultivation-based observations that initial colonization is nonrandom and follows the sequential colonization of species. Many different *in situ* devices have been described to model different aspects of biofilm formation, including the accumulation of biofilms in protected regions that mimic the development of dental caries (Prada-Lopez et al., 2016).

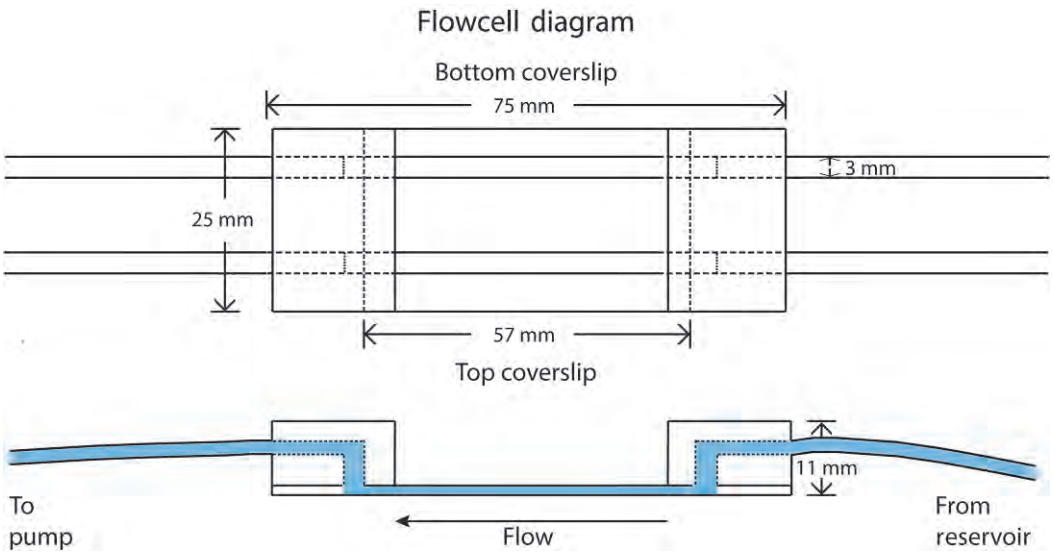

Fig. 5 Flowcell model for developing biofilms *in vitro*. Viewed from above (top diagram) and from the side (bottom diagram), the flowcell consists of two parallel tracks with a working volume of 250 μL per track. Each track is connected by tubing to a reservoir on the right and to a pump on the left. Saliva or culture media from the reservoir condition the glass surfaces before inoculation of oral bacterial species, which adhere to the conditioned surfaces. Saliva or media is pumped at about 200 μL min^{-1} to remove unbound cells and then continues to be pumped as a source of nutrient. Adherent cells and development of multispecies communities are monitored using fluorescent dyes to stain the cells and confocal microscopy to view the cells.

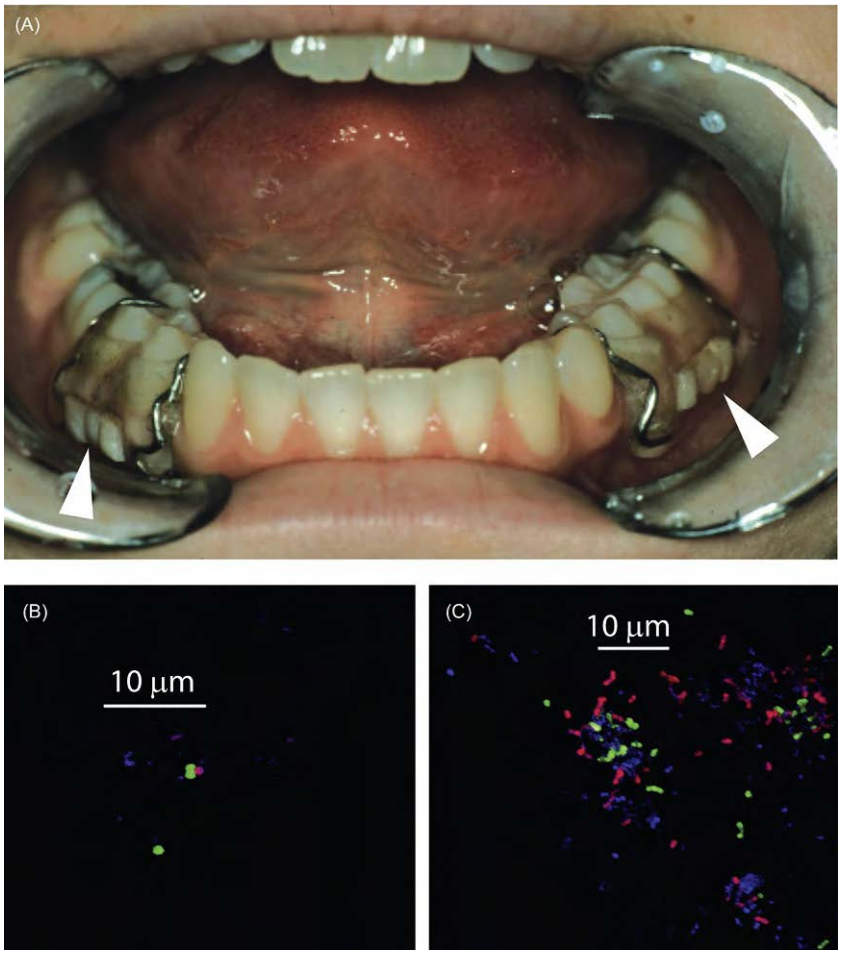

Fig. 6 Retrievable enamel chip for modeling dental plaque formation *in vivo* and multispecies bacterial communities on the enamel surface. (a) Two intraoral acrylic appliances are in place in a volunteer's mouth. The mandible carries both appliances, each containing a groove to accommodate, side-by-side, three small enamel chips prepared from sterilized extracted human teeth (each chip, 3 × 3 × 1 mm, arrowheads). At certain times (usually after 4 and 8 h of wear), an appliance is removed and the chips are stained and viewed as described in the legend to Fig. 4. (b) Confocal micrograph of 4-h-old plaque *in vivo* showing initial community composed of two species identified by reaction with fluorescently conjugated anti-*Streptococcus gordonii* DL1 (*green*; this organism bears a surface-exposed adhesin that recognizes receptor polysaccharides (RPS) on other streptococci) and anti-RPS (*red*). Only a few cells are stained with the general nucleic acid stain, Syto-59 (*blue*), indicating the sparse nature of initial colonization of enamel. (c) Confocal micrograph of 8-h-old plaque showing communities composed of at least three species, cells reactive with anti-*S. gordonii* DL1 (*green*), cells reactive with anti-RPS (*red*), and antibody-unreactive cells stained with Syto-59 (*blue*). Panels (b) and (c) show juxtaposition of different cell types forming multispecies communities and not mono-species colonies in developing dental plaque.

Oral Diseases

The microbial inhabitants of the oral cavity generally exist in a harmonious, commensal relationship with the host. Alteration of this relationship often follows a disturbance to the habitat and results in disease. Disturbances that can perturb the composition of the commensal, harmonious oral microflora and lead to dental disease include antibiotic intake, increase in carbohydrate consumption, or physiological changes such as alteration in the composition or flow rate of saliva, hormonal changes, and immunosuppression. The unexpected appearance of microbes at sites not accessible to them under normal conditions following tooth extraction or other trauma might also alter the host–microbe balance and initiate oral disease. The chronic presence of biofilms on tooth surfaces is often sufficient to initiate disease without additional factors.

Plaque-Related Diseases

Dental caries

Dental caries, or tooth decay, is the localized destruction of dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates. Studies on the etiology of dental caries have their roots in the early part of the twentieth century. Toward the end of the nineteenth century, the infectious agents of the major causes of death such as tuberculosis, cholera, and anthrax had been isolated. Identification of these deadly pathogens and the subsequent development of effective countermeasures were enabled by

advances in the formulation of growth media and the development of techniques for isolating and studying bacteria. W.D. Miller applied these techniques to study oral microbes and reported in 1890 that bacteria inhabiting the mouth were able to produce acid from fermentable carbohydrate when incubated at body temperature (Ruby et al., 2010). This work supported studies that had shown that acid was able to dissolve teeth. In the 1940s, R.M. Stephan discovered that following a glucose rinse, bacteria in dental plaque produced a rapid decrease in pH (Bowen, 2013). Return toward the pH baseline was slow. The total extent of exposure to acidic conditions is the primary determinant for whether demineralization of the tooth enamel will occur. However, in natural dental plaque, hydrogen ions are not uniformly distributed and there may be areas of differing pH within single biofilms (Bowen, 2013; Xiao et al., 2012).

Dental caries is associated with changes in the metabolism and composition of the oral microbiota at specific sites. Caries is a preventable chronic disease that progresses slowly in most people and is the primary cause for oral pain and tooth loss. Under acidic conditions, lesion formation occurs by dissolution of the tooth enamel and the transport of calcium and phosphate ions away into the surrounding environment. The initial stages of caries are reversible, and remineralization can occur, particularly in the presence of fluoride (Gao et al., 2016).

Sucrose has a pivotal role in caries etiology as it is the most cariogenic of all sugars (Sheiham, 1987). Molecular biology approaches enabled the identification of genes and metabolic pathways involved in sucrose metabolism by cariogenic bacteria (Bowen et al., 2018; Takahashi, 2015). The three properties that are distinctive characteristics of cariogenic bacteria are as follows: (1) the ability to rapidly import sugars. For this, cariogenic bacteria utilize high- and low-affinity transport systems that continue to operate at low pH (3.95–4.10); (2) acid production. An efficient glycolytic pathway rapidly produces a low pH plaque environment; and (3) aciduricity, particularly the ability to maintain the above activities under highly acidic conditions. Animal models have shown that abolishing any of the three properties impaired the ability of oral streptococci to colonize, persist, or generate caries. In addition, the ability of *S. mutans* to cause caries is associated with the capacity to produce extracellular polysaccharides (glucans and fructans) and intracellular polysaccharides (Busuioc et al., 2009; Koo et al., 2013). Glucans (glucose polymers) and fructans (fructose polymers) are synthesized from sucrose using extracellular glucosyltransferase and fructosyltransferase enzymes, respectively (Bowen et al., 2018). Glucans and fructans play an important role in *S. mutans* adhesion and accumulation on tooth surfaces and in the formation of the extracellular polysaccharide matrix, which is part of the structural integrity of dental biofilms. Glucans and fructans, together with intracellular polysaccharides generated from sucrose, provide an energy source for continued acid production once sources of dietary sugars become exhausted.

Caries-associated bacteria such as mutans streptococci and *Lactobacillus* spp. metabolize sugars to lactic acid. The decrease in surrounding pH inhibits metabolism and growth of many bacteria comprising the normal oral microflora. *S. mutans* is highly tolerant of low pH conditions (Buckley et al., 2014). The strongly acidic conditions facilitate the dominance of *S. mutans* in the bacterial community by restricting competition from neighboring plaque bacteria. This, in turn, increases tooth demineralization in a carbohydrate-dependent manner. *V. parvula*, a Gram-negative oral anaerobe, can potentially reduce the cariogenic potential of *S. mutans* by metabolizing the lactate produced by *S. mutans* to weaker acids, predominantly propionic acid. In addition, cariogenic acids can be neutralized by microbial degradation of arginine into basic end products, namely, urea or ammonia. The major mechanism for the production of ammonia from arginine is the arginine deiminase system, which is present in some oral streptococci such as *S. sanguinis* or *S. gordonii* (Nascimento et al., 2013). Urea produced from arginine or present in saliva secretions can also be catabolized by oral bacteria that express urease enzyme, such as *Actinomyces naeslundii* or *Streptococcus salivarius*, again producing the basic molecule ammonia (Barboza-Silva et al., 2005). High levels of arginolytic (arginine-degrading) and ureolytic (urea-degrading) enzymes in dental plaque correlate with reduced caries experience (Nascimento et al., 2009). The delivery of arginine, or of probiotic bacteria that express high levels of the arginine deiminase system, potentially may be an approach to combat localized acid production in oral biofilms and to control dental caries (Huang et al., 2016; Huang et al., 2018; Nascimento et al., 2013).

Polymicrobial nature of dental caries

In the past, the "specific plaque hypothesis" guided caries research in a search for a specific pathogen or pathogens involved in caries (Rosier et al., 2014). Currently, however, it is generally considered that the microbiology of dental caries is more complex, and that disease arises from an ecological shift from a health-associated community to a more acidogenic, and hence pathogenic, microbial community (Marsh and Zaura, 2017). The transition towards disease is mediated by changes in the local environment such as repeated cycles of low pH following frequent sugar uptake. There are multiple different types of health-associated community, and it is possible that some are more prone to undergoing the transition towards pathogenicity than others (Zaura et al., 2017) (Fig. 7). There are also several different types of cariogenic community, and many different species in addition to *S. mutans* have now been associated with caries including *Scardovia wiggsiae*, *Slackia exigua*, *Bifidobacterium* spp., *Granulicatella elegans* and many others (Fakhruddin et al., 2018). Thus, the elimination of a single species such as *S. mutans*, for example, with a vaccine, may not be completely effective in preventing dental caries in humans as this will simply make more room for one or more of the many acidogens remaining.

In developed and developing nations, the abundance of dental caries is highest in underprivileged groups, largely due to poor oral hygiene and lack of access to dental treatment facilities. In the developing world, malnutrition is an important factor that contributes to dental caries (Psoter et al., 2005). Malnutrition in infants and children leads to poorly calcified enamel that is highly susceptible to decay. In addition, saliva secretion rates and salivary concentrations of calcium, chloride, and protein are reduced in malnourished children.

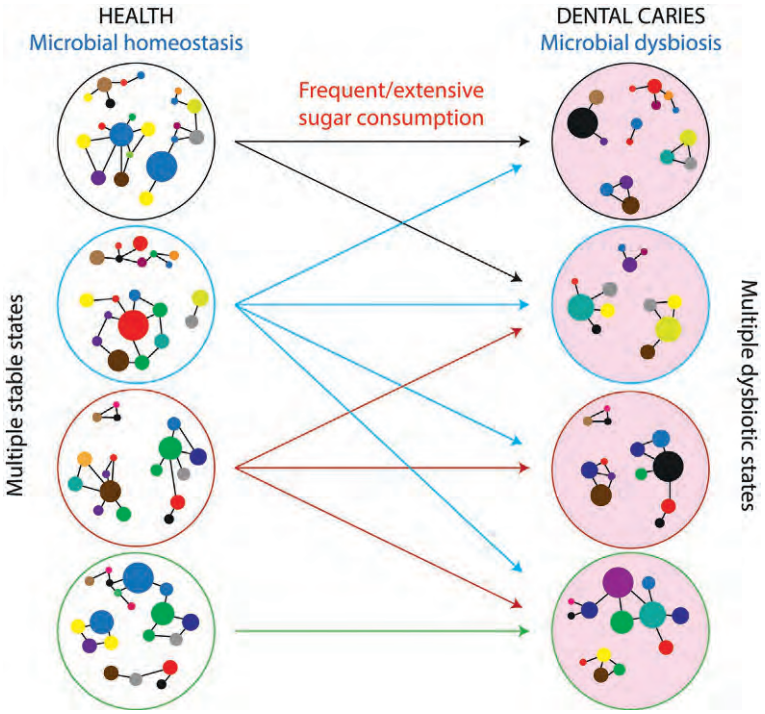

Fig. 7 Microbiological changes that lead to dental caries. In health, microbial communities in dental plaque can be described as cooccurrence networks. In this depiction, large circles represent the entire community, while smaller circles indicate individual species (OTUs). The size of the smaller circles indicates the relative abundance of the species. Different genera are shown as different colors. In some cases, multiple species of the same genus may be present. Connections between circles represent significant cooccurrence of species. Every individual will possess a unique microbial community that broadly matches one of the predominant cooccurrence networks. Environmental selection for acidogenic species, for example through the frequent and/or extensive intake of sugars, leads to changes in the relative abundance of different species, as well as losses and gains of species in the dental plaque microbial community. Overall, this results in microbial communities that are prone to generating significant quantities of cariogenic acids from carbohydrates (shown by pink shading). There are multiple dysbiotic states, each containing different combinations of acidogenic and aciduric species. It is not yet known whether specific health-associated networks are more prone to undergoing the transition to dysbiosis or whether there are correlations between specific health-associated networks and certain disease-associated networks. In this figure, one health-associated community is able to transition to any of the four dysbiotic states, whereas other communities can only transition to certain states.

The sites most susceptible to dental caries are fissures on the occlusal (biting) surface followed by approximal surfaces (areas between adjacent teeth) where the developing plaque is relatively protected from mechanical clearance by the tongue, salivary wash, and oral hygiene. In industrialized countries, a growing proportion of older adults retain their teeth. Gingival recession associated with old age exposes the susceptible cementum surface of the root to bacterial colonization. Exposed root dentine is especially vulnerable to demineralization by plaque acids because of its relatively low mineral content compared with enamel, and consequently there is a high incidence (approximately 60% of individuals aged 60 and older) of root surface caries in aging individuals. *Actinomyces* spp., in particular *A. naeslundii*, have been associated with root surface caries together with mutans streptococci and lactobacilli (Chalmers et al., 2015; Do et al., 2017; Takahashi and Nyvad, 2016). However, new molecular techniques for bacterial identification are reshaping our view of the organisms associated with dental diseases and have revealed that species such as *Streptococcus mutans*, *Olsenella profusa*, *Prevotella multisaccharivorax*, and *Lactobacillus crispatus* are also associated with root surface caries (Chen et al., 2015).

Periodontal diseases

Periodontal disease ("peri," around; -odontal from "odous," tooth) is a general term to describe a collection of bacteria-induced inflammatory conditions ranging from gingivitis, a transient gingival (gum) inflammation, to extensive destruction of the ligaments and of the alveolar bone supporting the teeth. Gingivitis usually initiates with bacterial colonization and the formation of a multi-species biofilm (dental plaque) on the tooth surface adjacent to the gingiva. The biofilm, consisted mostly of anaerobic Gram-negative bacteria and spirochetes, extends along the surface of the tooth roots and initiates irritation of the soft tissue supporting the tooth. Minor gingival inflammation is frequently accompanied by gingival bleeding. This inflammation is reversible. However, if left untreated, periodontitis will follow with the destruction of the collagenous attachment fibers of the periodontal ligament and of the alveolar bone supporting the teeth. Periodontitis is characterized by the loss of periodontal attachment due to the presence of a gap, or pocket formed between the tooth and its supporting tissues. The presence and extent of periodontal pockets (measured in millimetres using a periodontal probe) is the most common method for diagnosing and evaluating periodontitis (Savage et al., 2009).

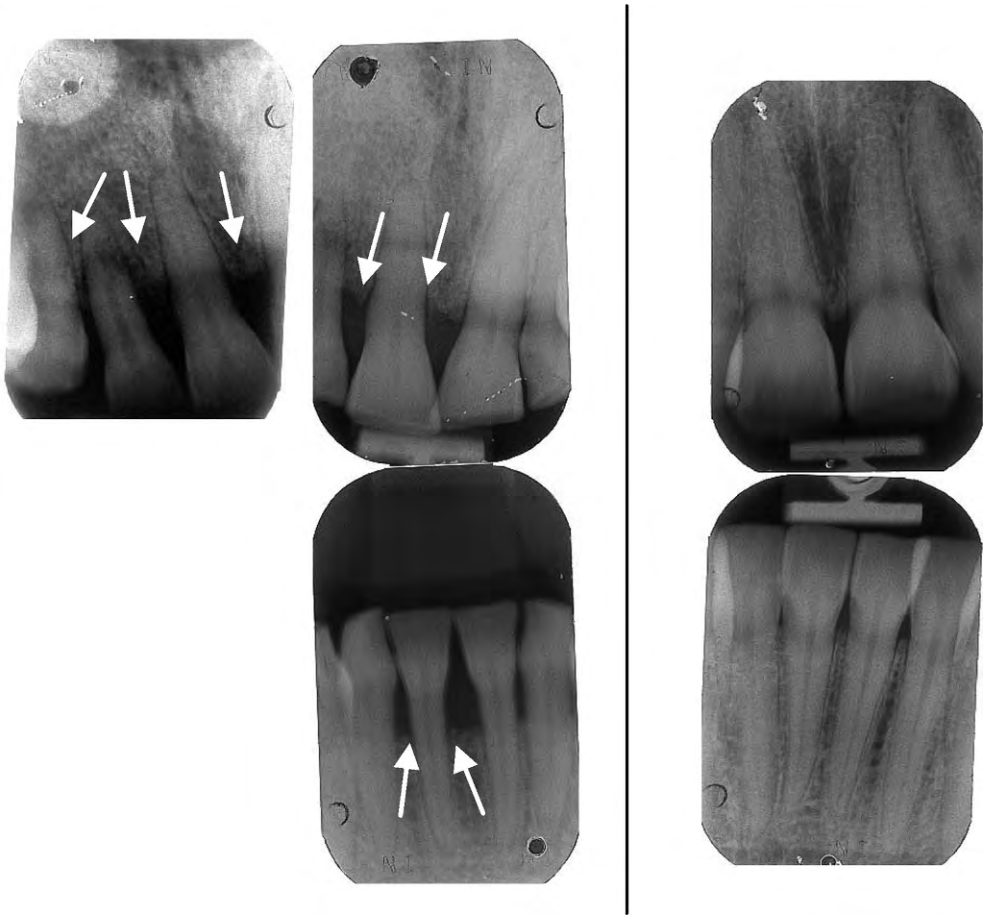

Fig. 8 Example of bone resorption in periodontitis associated with Down syndrome: radiograph shows loss of bone (indicated by arrows) between incisors of a 22-year old suffering from Down syndrome (*left*) compared with a 29-year-old healthy individual (*right*).

Untreated, periodontitis will lead to resorption of the alveolar bone (Fig. 8) and eventually to tooth loss. The progression of periodontitis is episodic, with active and inactive phases of tissue destruction, which reflects the opposing actions of bacterial challenges and host immune responses.

Periodontal diseases are currently divided into gingivitis (a reversible condition) and periodontitis (American Academy of Periodontology, 2015). Gingivitis is subclassified according to its source or inducing factor, for example, plaque, systemic factors, medications, malnutrition, systemic bacterial and viral diseases, fungal origin, genetic or traumatic. Periodontitis is classified according to the rate of disease progression, as chronic or aggressive. Gingivitis and periodontitis are subclassified by the distribution of lesions (localized or generalized). Periodontal diseases are highly prevalent. Gingivitis affects up to 90% of the worldwide population, whereas severe periodontal disease (pocket depth $\geq$6 mm) affects ~8% of adults aged over 30 years in the United States (Eke et al., 2018).

Although bacteria are essential for the initiation and progression of periodontitis, host factors play a major role in determining disease outcome (Hajishengallis, 2015). Hypo-responsiveness of the host's immunity associated with smoking, diabetes, or HIV infection, or with genetic disorders such as Down syndrome or impaired neutrophil function facilitate bacterial invasion of host tissues (Reynolds, 2014; Stabholz et al., 2010). However, hyper-responsiveness of certain immunological pathways results in a barrage of inflammatory mediators secreted by the host and is considered to be the main contributor for tissue destruction involved in chronic periodontal disease. Lipopolysaccharide extracted from periodontal pathogens such as *F. nucleatum* and *P. gingivalis* causes increased secretion of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and of the chemokine IL-8 by macrophage-like cells *in vitro* (Huang et al., 2004; Martinho et al., 2016). These immunomodulators are hypothesized to enhance tissue destruction and progression of periodontal diseases. IL-1 genotypes were associated with aggressive periodontitis. IL-1β, in particular, was associated with susceptibility to periodontitis, because of its ability to induce RANKL (receptor activator for nuclear factor κB ligand) (Belibasakis et al., 2011). RANKL is a cytokine belonging to the TNF family and is essential for the induction of osteoclastogenesis (bone resorption). Osteoblasts (mononucleate cells that are responsible for bone formation) and bone marrow stromal cells produce RANKL, and its signal is transduced by the specific receptor, RANK, which localizes on the cell surface of osteoclast progenitors that differentiate into osteoclasts that promote bone resorption.

In the periodontal pockets, the bacterial biofilm is in close contact with the epithelium. Bacteria-secreted enzymes and toxins break down the gingival lining, and neutrophils are recruited to the infected site and initiate an inflammatory process. Massive neutrophil recruitment and high levels of the proinflammatory cytokines interferon-γ, TNF-α, and IL-1β were found in the fluids of subcutaneous chambers (a model representing local infection involved in periodontitis) inserted into mice 2 h after challenge with *P. gingivalis* (Burns et al., 2006). Secretion of these proinflammatory cytokines in response to *P. gingivalis* was dependent on functionality of the Toll-like receptor 2 (TLR2) pattern recognition receptor.

Activated neutrophils also convert arachidonic acid to proinflammatory mediators prostaglandin E_2 and leukotriene B_4, which induce quintessential inflammatory symptoms such as heat, redness, swelling, and pain. By secreting proinflammatory cytokines and toxic agents such as oxidants and by phagocytosis of invading bacteria, the neutrophils usually restrain the infection, and inflammation subsides. If the infection persists because of lack of hygiene, leading to an overwhelming challenge to the host, or genetic vulnerability, the neutrophils continue to secrete proinflammatory agents. Recruitment of additional leukocytes including macrophages and lymphocytes accelerates tissue damage leading to a chronic inflammation.

Polymicrobial nature of periodontal disease

Periodontal disease involves complex interactions between the host, subgingival dental plaque and environmental factors such as smoking. As with dental caries, no single species is entirely responsible for periodontal disease, and disease results from a shift in the microbiota, known as dysbiosis, resulting in dental plaque that is enriched in Gram-negative, anaerobic and proteolytic bacteria. At least three species, *T. denticola*, *T. forsythia*, and *P. gingivalis*, have been commonly found together. On the basis of checkerboard DNA–DNA hybridization, a technique that allows the detection of 40 or more different species of bacteria in many samples simultaneously, these three species were found to commonly associate with one another and with periodontal disease (Socransky et al., 1998). These species have been termed the "red complex." Several studies have investigated the microbiome of periodontal disease using 16S rRNA gene sequencing. For example, in one study the genera *Provetella*, *Porphyromonas*, *Treponema* and *Fusobacterium* were enriched in deep periodontal pockets, whereas *Streptococcus*, *Actinomyces* and *Veillonella* were more abundant in health (Ge et al., 2013). Twenty-one "operational taxonomic units" (OTUs; essentially equivalent to species), including *T. forsythia*, *P. gingivalis* and *Filifactor alocis*, were found to be associated with periodontitis in a study of 30 periodontitis patients and 30 controls. Using checkerboard hybridization, eight taxa including *T. forsythia*, *Parvimonas micra* and *F. alocis* were elevated in saliva samples of periodontitis patients compared with controls (Belstrom et al., 2014).

Sequencing of the 16S rRNA gene has also been used to follow changes in the oral microbiome during the onset of gingivitis (Kistler et al., 2013). In this case, taxa associated with gingivitis onset included *Fusobacterium nucleatum* subsp. *polymorphum*, *Lachnospiraceae* [G-2] sp. HOT100, *Lautropia* sp. HOTA94, and *Prevotella oulorum*, whereas *Rothia dentocariosa* was associated with periodontal health.

Periodontitis initiates from infection by an oral biofilm composed of a diverse bacterial community. It has been suggested that certain species such as *P. gingivalis* may act as keystone pathogens to trigger disease (Hajishengallis et al., 2012). According to the "polymicrobial synergy and dysbiosis" model, these keystone pathogens are present in low numbers, but are critical for disease development (Hajishengallis and Lamont, 2012). They interact with existing colonizers to establish in the biofilm, and subsequently increase the virulence of the whole biofilm by disrupting immune surveillance of the host. Environmental factors such as smoking may render the host more susceptible to attack through modifying the microbial cells or the host immune response.

It is clear that many different combinations of species may be present in periodontally pathogenic biofilms. It is possible that these biofilms share common functions such as metabolic pathways, even if they differ in species composition. Metatranscriptomics has been employed to assess changes in gene expression during the transition from health to gingivitis (Nowicki et al., 2018). Pyrimidine, propanoate and butyrate metabolism were associated with gingivitis, while a range of pathways including carbon fixation in prokaryotes, the pentose phosphate pathway, and pyruvate metabolism, as well as porphyrin and chlorophyll metabolic gene products were associated with health. Genes associated with destructive pathways such as proteolysis and nucleolysis were upregulated in disease, which may reflect the ability of subgingival dental plaque to degrade the host tissue matrix. More studies of this kind on different stages of periodontitis may shed light on the key metabolites that are produced by the combined activities of the microorganisms in the biofilm and trigger host immune responses.

Candidiasis

Oral fungus infections are often called "diseases of the diseased" and some host predisposition usually occurs before the fungus can take hold. Likewise, cures for fungal infections necessitate alleviating the predisposing condition. *Candida albicans* is the most common fungus isolate from oral infections. In approximately half of the population, *C. albicans* is a part of the resident oral microflora and it is most commonly found on the tongue (Mun et al., 2016). Changes in the ecosystem, for example immunosuppression, utilization of broad-spectrum antibiotics, or trauma, lead to overgrowth resulting in infection (candidiasis). Oral *C. albicans* infections are generally associated with an increase in the mycelial (hyphal) form as opposed to the blastospore (yeast) form of the fungus. The virulence factors of *C. albicans* include adhesins that enable adhesion to epithelial cells and to acrylic used in dentures, and secreted proteinases and phospholipase that facilitate invasion of host tissues. A key factor in invasion of host tissues is a peptide toxin termed Candidalysin that is secreted into invasion pockets produced by hyphae, and accumulates to concentrations that cause lysis of host cells (Moyes et al., 2016). Oral candidiasis may appear in a number of chronic and acute forms. Treatment of oral candidiasis involves alleviating the predisposing condition and prescribing antifungal agents.

Extra-Oral Diseases

Oral bacteria are associated with a number of conditions that occur at body sites outside the oral cavity. Chronic inflammation associated with periodontal disease may have adverse consequences for general health. Bacteria can enter the bloodstream during dental surgery or, more frequently, during everyday activities such as toothbrushing or chewing food. These transient bacteremias are usually cleared quickly with no adverse effects. However, on occasion, this may be a route of inoculation for bacterial infections at distal sites. Bacteria in oral biofilms are also of concern for individuals with impaired lung function who require artificial ventilation. Lung infections are a common complication in this group of patients and oral bacteria have been implicated as a source of the infective agents.

Periodontitis-related conditions

Periodontitis and heart disease

Atherosclerosis involves elevated circulating lipids and arterial vessel lipid accumulation, combined with an ongoing inflammatory response. Atherosclerosis initiates by accumulation of excess low-density lipoprotein (LDL) on artery walls. The LDL goes through chemical modifications (lipid oxidation) that stimulate an inflammatory cascade in the intima (interior wall of the artery). Endothelial cells react with the modified LDL by displaying adhesion molecules that capture circulating monocytes and T cells, and by secreting chemokines that traffic the captured cells into the intima. Monocytes proliferate in the intima and mature into macrophages that, together with the recruited T cells, secrete proinflammatory cytokines. The macrophages display scavenger receptors and phagocytose the modified LDL. The intracellular lipid accumulation causes the macrophages to appear foamy under the microscope (foam cells). A yellow "fatty streak" (mix of foam and T cells) atherosclerotic lesion becomes visible. Inflammation continues to drive the growth of the atherosclerotic plaque and leads to the plaque becoming covered by a fibrous matrix formed by migrated smooth muscle cells. Inflammation is also hypothesized to make this cap vulnerable to rupture. Rupture will lead to the development of a blood clot over the break that will cause further narrowing of the blood vessel.

Local or distant ("echo effect") infections have been proposed to contribute to the inflammatory process in atherosclerosis by accelerating them or by enhancing the inflammatory properties of LDL (Hayashi et al., 2010). Although not conclusive, epidemiological studies support a link between periodontitis and atherosclerosis (Pinho et al., 2013; Tonetti and Van Dyke, 2013). In animal models, *P. gingivalis*, the oral pathogen most associated with chronic periodontitis, is capable of accelerating atheroma deposition (Fukasawa et al., 2012). Atherosclerosis acceleration, as well as periodontitis, in atherosclerosis-prone apolipoprotein E (ApoE)$^{-/-}$ mice was dependent on *P. gingivalis* fimbriae (Gibson 3rd et al., 2004). ApoE$^{-/-}$ mice challenged with a fimbria-deficient *P. gingivalis* mutant did not develop either periodontal disease or accelerated atherosclerosis. Further evidence that oral bacteria are involved in atherosclerosis has been provided by studies on human samples. *P. gingivalis* and *A. actinomycetemcomitans* were detected in human atherosclerotic plaque by molecular bacterial identification techniques (Kozarov et al., 2006).

Periodontitis and diabetes

It has been noted that diabetics have increased risk of periodontal diseases (Casanova et al., 2014; Holmstrup et al., 2017; Sanz et al., 2018). Human cell culture and animal studies support the concept that diabetes leads to increased inflammatory activity in the periodontal pocket (Polak and Shapira, 2018). Conversely, there is some evidence that treatment of periodontal disease has a positive effect on glycemic control of diabetes; possibly involving effects on insulin resistance (low responsiveness of cells to uptake blood sugar in response to insulin, and the hallmark of type 2 diabetes). There is evidence that inflammatory periodontal diseases increase insulin resistance (Martinez-Herrera et al., 2017). Overexpression of TNF-α in periodontal lesions has been hypothesized to promote insulin resistance, thereby exacerbating problems with glycemic control. Further research is needed to clarify this aspect of the relationship between periodontal diseases and diabetes.

Adverse pregnancy outcomes

Conflicting epidemiological data have been reported regarding the role that periodontal disease plays as a risk factor for preterm labor and low birth weight (Sanz and Kornman, 2013). Controversy also exists about the benefits of treating periodontitis during pregnancy. Cultivatable and noncultivatable species of putative periodontal pathogens have been detected in intrauterine infections that induced or were involved in preterm labor. Oral bacteria such as *F. nucleatum*, *P. gingivalis*, *F. alocis* and *Campylobacter rectus* have been isolated (often as pure cultures) from amniotic fluid, placenta, and chorioamniotic membranes of women delivering prematurely (Cobb et al., 2017). A possible mechanism linking *F. nucleatum* with preterm deliveries was supported by using a murine model, where *F. nucleatum* intravenously injected into pregnant mice and thus simulating transient bacteremia caused by periodontal infection, resulted in preterm deliveries (Han et al., 2004; Stockham et al., 2015). Fetal death was due to local infection of the fetoplacental unit rather than systemic effects of maternal bloodstream infection. The sequence of infection paralleled that in humans. The *F. nucleatum* infection initiated in the deciduas basalis of the placenta. This site is characterized by large venous sinuses where blood flow rate is slow and the shear force is low, which provides an opportunity for *F. nucleatum* to adhere and invade the endothelial cells. It was suggested that attachment to, followed by invasion of host cells is an important virulence mechanism for *F. nucleatum* infections of the placenta. A key adhesin involved in attachment and invasion was later identified as the *F. nucleatum* galactose-inhibitable adhesin Fap2 (Coppenhagen-Glazer et al., 2015). As the fetal death rate was significantly reduced in TLR4-deficient mice, a TLR4-mediated inflammation rather than the bacteria *per se*, was suggested as the cause of fetal loss (Liu et al., 2007). A synthetic TLR4 antagonist significantly reduced fusobacteria-induced

fetal death and may represent a promising approach of using anti-inflammatory agents for future treatment or prevention of bacteria-induced preterm births.

Infective endocarditis

Infective endocarditis (IE) is a rare but serious inflammation of the heart valves that is usually caused by bacteria. Despite advances in disease prediction, diagnosis, and treatment, mortality for this condition remains high at approximately 20% (Dunne et al., 2014). Oral streptococci along with *S. aureus* and *Staphylococcus epidermidis* are consistently identified as the most common causative agents of IE (Que and Moreillon, 2011). Less frequent isolates from IE lesions include Gram-negative organisms such as *A. actinomycetemcomitans* and *P. gingivalis* (van Winkelhoff and Slots, 1999). Culture-independent methods such as metagenomics have also detected oral bacteria in infective endocarditis lesions (Cheng et al., 2018). The process of IE involves translocation of bacteria to the site of infection, attachment to the endothelial lining, and bacterial growth. The ability of bacteria to bind platelets has also been suggested as a virulence mechanism because endocardial vegetations consist of bacterial cells encased in a fibrin matrix containing platelets and host proteins (Kerrigan et al., 2007). Virulence factors for IE include bacterial adhesins (e.g., EmaA of *A. actinomycetemcomitans*) (Tang et al., 2008), exopolysaccharides (glucans and fructans of *S. mutans*) (Taniguchi et al., 2010), and serine rich repeat proteins (GspB/Hsa) of *S. gordonii* and other oral streptococci (Bensing et al., 2018). A screen for IE virulence factors of *S. sanguinis* using signature-tagged mutagenesis identified several housekeeping genes involved in synthesis of the cell wall, amino acids, and nucleic acids (Paik et al., 2005; Turner et al., 2009).

Patients with preexisting heart conditions are at significantly increased risk of IE, and there is ongoing debate as to whether these individuals should receive antimicrobial prophylaxis in the form of antibiotics or chlorhexidine mouthrinse during dental treatment (Diz Dios, 2014). However, as there is a lack of compelling evidence that antimicrobial prophylaxis is effective in reducing the incidence of IE, and in light of concerns that overuse of antibiotics leads to resistance mechanisms in bacteria, the latest (2007) guidelines of the American Heart Association recommend antimicrobial prophylaxis during dental treatment for only a very small number of patients (Wilson et al., 2008).

Ventilation-associated pneumonia

Pneumonia affects between 9% and 27% of patients in US hospitals that rely on mechanical assistance for ventilation (Kalanuria et al., 2014). The etiology of ventilator-associated pneumonia (VAP) is complex, and may involve a number of opportunistic pathogens such as *S. aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *H. influenzae*. There is a growing body of evidence that the oral cavity is a route of entry for VAP pathogens. Using culture-independent techniques, respiratory pathogens *S. aureus* and *P. aeruginosa* were shown to invade dental plaque of approximately one third of patients following intubation and were present concurrently in both the lower respiratory tract and dental plaque of a number of VAP patients (Sands et al., 2017).

Strategies for Disease Control

Diagnosis of Dental Caries and Periodontitis

Visual and radiographic diagnoses of caries are in common use and are usually adequate. However, objective diagnostic tests with predictive value for the risk assessment of caries are greatly desired. The occurrence of mutans streptococci and lactobacilli in plaque correlates with their levels in saliva. On the basis of this correlation, commercial chair-side tests for semi-quantification of these indicator organisms in saliva samples have been introduced. These tests utilize user-friendly selective culture media for determining levels of mutans streptococci and lactobacilli in saliva and have exhibited some value in infants, toddlers, and in elderly subjects (Tanabe et al., 2006). However, since *S. mutans* and *Lactobacillus* spp. are not always strongly increased in caries, the sensitivity of this test for detecting all cases of caries will be limited. Including methods to detect other species such as *Scardovia wiggsiae* may improve the situation (Eriksson et al., 2017). Ultimately, longitudinal studies are necessary to determine the true value of these tests in predicting caries.

At present, microbiological sampling is not routinely used for diagnosing gingivitis or periodontitis. Gingivitis is identified by signs of gingival inflammation and by a bleeding response to a periodontal probe. Periodontitis is diagnosed when attachment loss (pockets of >3 mm) is found in addition to symptoms of gingivitis. Loss of alveolar bone associated with periodontitis can be detected using X-rays. Molecular methods for characterization of disease-associated microflora may one day be harnessed to develop more sophisticated techniques for diagnostic and prognostic evaluation of periodontal disease. However, the complexity of the microbial shifts in periodontitis make it unlikely that individual microbial biomarkers will ever be useful alone. Instead, it will be necessary to develop panels of markers that reflect multiple different disease states. Attempts have already been made using next-generation sequencing of the microbiome and machine learning to identify the 10 best biomarkers to use in combination (Szafranski et al., 2015).

Prevention and Control of Oral Microbial Diseases

Dental caries

The development of dental caries relies greatly on the frequency of sugar intake. If sugars are consumed continuously throughout the day, the pH stays below a critical level for extended times, and the tooth surfaces cannot regain lost mineral content

(remineralize). Therefore, control of sugar and particularly sucrose intake is a simple measure for controlling dental caries. In addition, regular mechanical removal of plaque by toothbrushing controls the number of bacteria in biofilms on teeth and limits the capacity for acid production from sucrose. Arginine also has potential to reduce caries since it is metabolized by dental plaque bacteria into alkali, thus preventing the acidification of dental plaque (Geraldeli et al., 2017). In addition, arginine appears to destabilize dental plaque, potentially reducing the overall microbial burden (Kolderman et al., 2015). Pit and fissure sealing in children and adolescents is also effective in lowering the bacterial burden at caries-susceptible sites, and this is an important preventative strategy for controlling dental caries (Ahovuo-Saloranta et al., 2017).

In the early twentieth century, Frederick S. McKay observed that people living in particular areas had mottled, stained teeth due to an ingredient of their domestic water supply. He also noticed that mottled teeth showed absence of decay. In 1931 this water ingredient was found to be fluoride (McKay, 1953). Fluoride, administered in drinking water, toothpaste, or topically by a professional, acts as a catalyst for the diffusion of calcium and phosphate into the tooth and remineralization of the crystalline structures in the lesion (Robinson, 2009). In addition, calcium fluorapatite is less soluble than calcium hydroxyapatite, and thus incorporation of fluoride makes the enamel more resistant to demineralization. Fluoride also has direct adverse effects on the microorganisms in dental plaque. By increasing membrane permeability to protons, fluoride effectively reduces acid tolerance of bacteria, including *S. mutans*. However, strains of *S. mutans* with resistance to fluoride have been isolated and it is possible that resistance will be selected in the population through the widespread use of fluoride, negating the beneficial effects of fluoride against cariogenic bacteria (Liao et al., 2017).

Periodontal disease

In otherwise healthy individuals, periodontal diseases are generally preventable by applying good daily oral hygiene. However, once periodontitis takes hold it is not possible to eliminate the disease. The common approach to halt progression of periodontal disease is mechanical plaque removal, known as debridement (Drisko, 2014). Smoothing, or debriding, the tooth root is often performed to facilitate daily hygiene by the patient, enhance healing, and retard plaque re-accumulation. Antibiotics may be administered systemically or locally via a sustained releasing device to control infection. Bone grafting followed by tooth transplant can be performed if extensive alveolar bone loss occurred resulting in tooth loss (Matarasso et al., 2015).

Innovative studies by Serhan and Van Dyke elucidated the proinflammatory importance of the neutrophil-derived leukotriene B_4 and prostaglandin E_2. While searching for endogenous anti-inflammatory compounds, they discovered lipoxins that derive from arachidonic acid, and resolvins and protectins that derive from omega-3 fatty acids (Serhan et al., 2008). These compounds rapidly halt and resolve inflammation. Preliminary results demonstrated that rabbits receiving topical application of lipoxins and resolvins were protected against periodontitis, compared to the placebo group, which developed severe periodontitis. Furthermore, transgenic rabbits expressing high levels of lipoxin in their blood were resistant to periodontitis-induced atherosclerosis, whereas wild-type rabbits developed periodontitis and atherosclerosis following oral infection with *P. gingivalis*. Lipoxins and resolvins can regenerate bone and show great potential for treating periodontal diseases in humans (Van Dyke et al., 2015). It is anticipated that advances in our understanding of microbial interactions in health- or disease-associated dental plaque will provide a basis for fundamental improvements to future dental care procedures.

Summary

Oral Microbiology is characterized by complexity. Biofilms on oral surfaces contain tens or hundreds of different species, all interacting with one another and contributing to the overall function of the system. The biofilm interacts with host tissues and with environmental factors such as dietary intake or inhalation of smoke. Oral biofilms are present in health, and evidence now indicates that species are relatively well-conserved between individuals, at least in comparison with other "microbiomes" on the body. Nevertheless, we each contain our own oral microbiota, and it is possible that some combinations of microorganisms are more prone to developing disease than others. Understanding the transition from health to disease is critical for the rational design of new strategies to enhance oral health. A great deal more work is needed before we can fully understand how and why oral disease occurs.

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Organic and Fatty Acid Production, Microbial[☆]

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Glossary

Eukaryotic organisms Organisms having one or more cells, with well-defined membrane-bound nuclei.
Filamentous fungi Fungi that grow as long, multicellular strands.
Genetic engineering Technique used to modify genetic information of microorganisms, reprogramming for a desired purpose (e.g., to produce a substance it would not naturally produce).
Global transcription machinery engineering Approach for reprogramming gene transcription to elicit cellular phenotypes important for technological applications.
Metabolic engineering Improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology.
Metabolomics Metabolite profiling, measuring the real outcome of the potential changes suggested by genomics or proteomics. It investigates regulation and metabolic fluxes in individual microorganisms.
Molar yield Gram dry weight of cells or product per mole of substrate (e.g., carbon source) utilized.
Oxidative TCA cycle The tricarboxylic acid (TCA) cycle, also known as the Krebs cycle or the citric acid cycle, is the major pathway for the complete oxidation of acetyl-CoA to CO_2 (catabolism) and the generation of energy in the form of ATP. Reactions of the TCA cycle also function in metabolic processes (anabolism) other than energy generation.
Primary metabolites Metabolites produced during and required for growth of the microorganism.
Prokaryotes Bacteria and blue green algae (cyanobacteria) with nuclear material not limited by membrane and DNA not organized in chromosomes.
Reductive TCA cycle Biochemical pathway including the carboxylation of pyruvic acid to oxaloacetic acid, which can be further converted to L-malic acid, fumaric acid, and/or succinic acid, dependent on the organism in use. The reactions are in the reverse direction of the oxidative TCA cycle and no energy is generated.
Submerged fermentation Production of different substances by growing producer microorganisms in depth in liquid culture.
Systems biology Systematic study of interactions in the organism using modeling, global analysis (or ome analysis), mapping of interactions between cellular components, and quantification of dynamic responses in living cells resulting in the quantitative description of the biological system under study.

Abbreviations

ARA	Arachidonic acid
DHA	Docosahexaenoic acid
EFA	Essential fatty acids
ESA	L-Trans-2,3-epoxysuccinic acid
GLA	γ-Lineolenic acid
GRAS	Generally recognized as safe
2-KGA	2-Keto-D-gluconic acid
5-KGA	5-Keto-D-gluconic acid
PHA	Polyhydroxyalkonate
PHB	3-Hydroxybutyric acid
PUFA	Polyunsaturated fatty acids
SCFA	Short chain fatty acids
SCO	Single-cell oil
TCA	Tricarboxylic acid

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Defining Statement

Organic acids are used as food acidulants and building blocks for other useful chemicals. Fatty acids were recently recognized for their beneficial health properties and a prospective energy source. The potential of various microorganisms to synthesize various organic and fatty acids will be highlighted with emphasis on the existing industrial large-scale production processes. The information has been updated and now includes data from public sources until June 2016.

Introduction

Organic and fatty acids are broadly distributed in nature and were used by humans in their natural forms since early ages. Presently, organic acids are used mostly as food acidulants and as building blocks for other useful chemicals of low and high molecular weight (polymers). Although high concentrations of acids are produced by various microorganisms, microbiological production process is important for only a few acids and an economic alternative to chemical synthesis. Within the world's fermentation market, organic and fatty acids constitute a significant portion, especially with the production of citric acid, L-lactic acid, acetic acid, gluconic acid, and itaconic acid (Table 1). Recent developments in the biotechnology field, environmental pressures, increased recognition of the positive contribution of specific fatty acids to health and prevention of chronic disease, steady increase in price of petrochemicals, integration of fermentation and agricultural products (e.g., corn), consumer awareness for natural instead of chemical food constituents (i.e., the use of natural L-malic acid in foods instead of the chemically produced DL-malic acid), and recent requirements for a specific isomer of the acid for biodegradable and natural polymer production (i.e., L- or D-lactic acid) may result in much improved economics for the implementation of the biological route for the industrial production of organic acids (Table 1).

This article describes the potential of various microorganisms to synthesize high amounts of organic and fatty acids. It emphasizes those acids that are produced in the industry by large-scale fermentation processes.

Organic Acids

Organic acids are intermediates or end products of cellular metabolism. Accumulation of various organic acids is the basis for a varied and extensive industry, often based on biological production methods. The majority of the organic acids discussed in this article are produced and excreted to the medium at high concentrations by filamentous fungi such as *Aspergillus* sp. (citric, L-malic, gluconic, itaconic, L-*trans*-2,3-epoxysuccinic, gallic, and kojic acid) and *Rhizopus* sp. (fumaric, L-lactic, and gallic acid). Most of these acids are intermediate metabolites in the primary biosynthetic metabolism and, therefore, do not accumulate under normal growth conditions (primary metabolites). Stress appears to be a common requirement for the unusually high production and accumulation of these acids, since under stress conditions most of the carbon source is used for acids production (metabolite) rather than increase

Table 1 Production of selected organic acids by chemical and biological routes[a]

Acid	*Annual production (t)*	*Chemical process*	*Biological process*	*Year*
Food acids				
Citric acid	1,600,000	−	+	2007
Acetic acid	10,000,000	+	+	2005
Lactic acid	240,000	−	+	2006
D-Gluconic	87,000[b]	−	+	2004
L-Tartaric	35,000	−	+	2003
Malic acid	60,000	+	+	2006
Ascorbic acid	110,000	+	(+)	2001
Fumaric acid	90,000	+	−	2008
Gallic acid	8,000	+	−	2010
Building blocks				
Polyhydroxyalkonates	50,000	−	+	2006
Itaconic	80,000	−	+	2009
Succinic acid	15,000	+	+	2003
Acrylic	4,200,000	+	−	2016
Adipic	2,760,000	+	−	2004
Propionic acid	176,000[c]		+	2003

[a]The estimated annual world production of the various acids is based on information in the published literature. The order of the acids is based on the volume produced by biological routes.

[b]The worldwide consumption of D-gluconic acid based on major industrial applications.

[c]Estimated consumption in the United States.

in biomass production (growth). Other acids (2-ketogluconic, 5-ketogluconic, acrylic, and adipic acid) are produced by bacteria under aerobic conditions, while succinic acid is produced by bacteria under anaerobic conditions. The formation of the latter acid is usually the way by which these bacteria regenerate NAD from the accumulated NADH, and thus the accumulation of succinic acid strictly parallels and is required for growth.

Food Acids

Citric Acid

Citric acid (2-hydroxy-1,2,3 propanetricarboxylic acid, $C_6H_8O_7$) (Fig. 1) is a white or colorless, odorless, crystalline solid. It is highly soluble in water, freely soluble in ethanol, and slightly soluble in ether. Citric acid has three carboxyl groups and, therefore, is a good buffer for pH control.

The discovery of citric acid has been credited to the eighth century alchemist Jabir Ibn Hayyan. The acid was first isolated in pure form in 1784 by Carl Wilhelm Scheele, who crystallized it from lemon juice.

Citric acid is found in almost all plants and in many microorganisms and animal tissues and fluids. It is a key element in the physiological oxidation of fats, proteins, and carbohydrates to carbon dioxide and water.

Citric acid, with very low toxicity, is mainly used as a flavoring agent and preservative in food and beverages, bestowing tartness to fruit and soft drinks. Citrate salts of different metals are used to deliver these metals, in a biologically available form, in food supplements. Citric acid is used in household cleaning chemicals and pharmaceuticals, for storage of blood, in tablets and ointments, and also in cosmetic preparations. It acts as a bacterial inhabitant and as an antioxidant.

The biosynthesis of citric acid has been well studied; however, all the reactions that lead to citric acid are still not fully understood. Citric acid is a primary metabolite formed in the tricarboxylic acid (TCA) cycle, discovered in 1937 by Hans Adolf Krebs (Fig. 2). It has been known for a long time that there is a condensation of a C4 moiety with a C2 moiety, both metabolites of pyruvic acid, to form the C6 molecule of citric acid. In high-glucose media the high yields obtained for citric acid are explained by two different reactions occurring with pyruvic acid. One of the two pyruvic acid molecules (C3), formed by the glycolytic pathway from glucose (C6), is converted to acetyl CoA (C2). The other molecule is carboxylated by a reaction catalyzed by pyruvate carboxylase, which is a part of the reductive direction of the TCA cycle and found to occur in the cytosol in *Aspergillus niger* (Fig. 3). The product of this reaction is oxaloacetic acid (C4). This acid is first reduced to malic acid (C4), and enters the mitochondria via a malate–citrate transporter, converted to malic acid, which together with acetyl CoA will form the citric acid. The enzyme performing this reaction, citrate synthase, is only present in the mitochondria.

The current world production of citric acid is estimated to be 1.6 million tonnes per year (Table 1), whereof 99% is produced by microbial fermentation. Several microbial species are able to accumulate citric acid during primary metabolism; both fungi, such as *A. niger*, *Aspergillus wentii*, *Penicillium luteum*, and *Trichoderma viride*, yeast like *Candida guilliermondii*, and *Saccharomycopsis lipolytica*, and bacteria such as *Arthrobacter paraffineus* and *Corynebacterium* sp. For production, *A. niger* is used (Fig. 4).

Modern fermentation technologies yield 95 kg of citric acid from 100 kg of sugar supplied, with final concentrations of over 200 g L^{-1}. The modifications that have led to the high producing industrial strains are well-kept secrets; however, mutant isolations by academic laboratories include high sugar tolerance and growth and acid production at low pH. This is an example where fermentation of sugar(s) is converted to an end product (citric acid) at very high efficiency. The medium for optimal production of citric acid should contain a sugar concentration of between 120 and 250 g L^{-1}, a nitrogen source (ammonium salts) over 2 g L^{-1}, a phosphate concentration between 0.2 and 1.0 g L^{-1}, Mn$<10^{-8}$ mol L^{-1}, Zn$<10^{-6}$ mol L^{-1} and Fe $<10^{-4}$ mol L^{-1}. The dissolved oxygen concentration should be >150 mbar, and the pH is 1.6–2.2.

As can be seen for other acids of the TCA cycle (fumaric and L-malic acid, see below), the high molar yield values obtained by the different filamentous fungi are due to the presence of a compartmentalized (in this case cytosolic) activity of a carboxylation enzymatic reaction, resulting in the fixation of carbon dioxide by the unique pyruvate carboxylase (in most eukaryotic cells located in the mitochondria) (Fig. 3).

Two methods are used by industry to manufacture citric acid, the submerged fermentation process (Fig. 5) and the surface method. Small amounts are made by solid-state fermentations, mainly in East Asia. The main industrial organism in use today is *A. niger*. Before the 1973 oil crises, use of yeast grown on *n*-alkanes under submerged conditions was also in use. The yeast most in use was *Yarrowia lipolytica* grown on straight chain paraffins (see also "Isocitric acid").

The surface method is still in use, being less labor- and energy-intensive and with much lower susceptibility to trace metal ions. The fermentation is carried out in aluminum trays, where spores are dispersed on the surface of the nutrient medium. The fungus forms a mat and yields reported are between 0.7 and 0.9 g g^{-1} of sugar after 1–2 weeks.

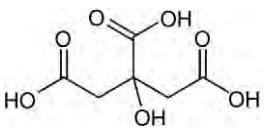

Fig. 1 Citric acid.

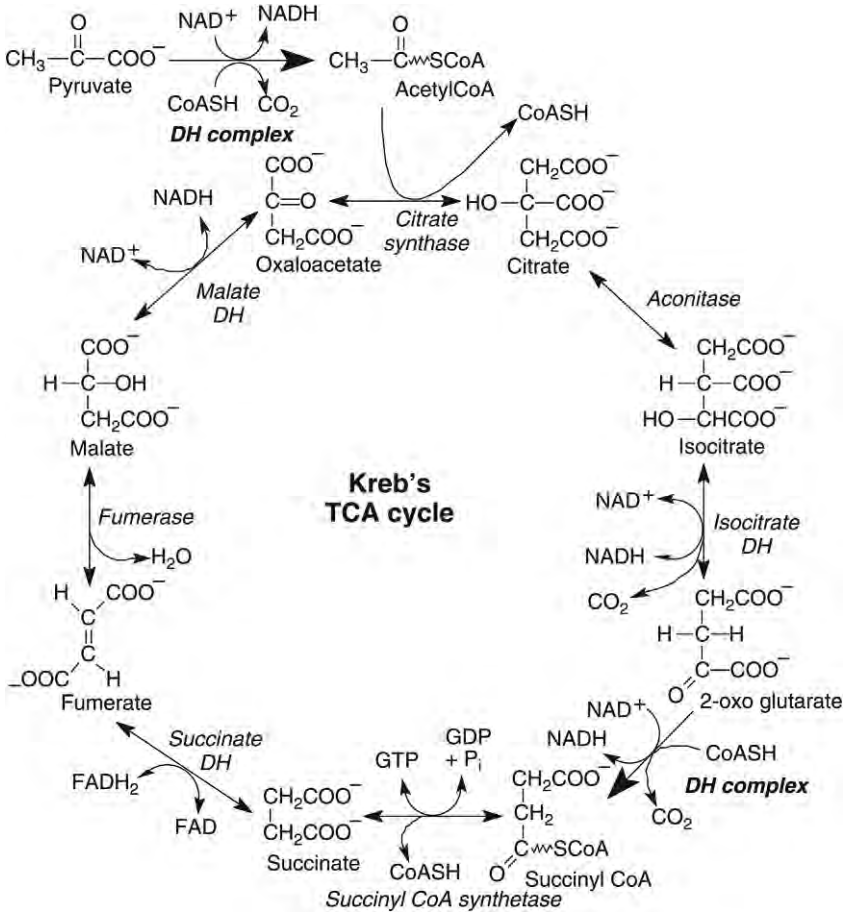

Fig. 2 The tricarboxylic acid (TCA) cycle (also Krebs cycle and citric acid cycle). With permission from Dr. Paltel, R., Humboldt State University, CA, USA.

The submerged fermentation has higher efficiency and is easier to automate. The carbon source, usually from renewable resources, needs to be analyzed for trace metals, and if present, these should be removed, to avoid inhibition of citric acid accumulation. Both aerated towers, where the air enters at the bottom of the vessel, and stirred-tank fermentors are in use by industry. The required growth mode is the formation of pellets made of the fungal mycelium, with a diameter of 0.2–0.5 mm, that sediment swiftly at harvest. The low pH of the process is critical for obtaining a high yield of citric acid and to avoid the potential accumulation of oxalic acid. The initial inoculum for vegetative growth is, in most cases, spores that require a pH above 5.0 in order to germinate. For production, the pH should be <2, which also reduces the risk for contamination.

The purification of citric acid from the fermentation broth starts with filtration with the help of filter aids to obtain good separation of the citric acid containing broth and the mycelium (Fig. 5). To avoid oxalic acid in the final product, lime (calcium hydroxide) is added at low pH and calcium oxalate precipitates and is separated from the liquid. Citric acid is then precipitated by increasing lime concentration and raising the temperature to 70–90°C and the pH to 7.2, followed by collection of calcium citrate using rotating filters. To obtain higher purity of citric acid, sulfuric acid is added, reprecipitating calcium sulfate. The citric acid is further treated with activated carbon, cation exchangers, and anion exchangers and finally crystallized either as citric acid or as citric acid monohydrate. The use of solvent extraction (aliphatic alcohols and ketones, amines and phosphines with hydrocarbons are mainly used) for citric acid purification has been introduced more recently and is also used by industry.

The recent sequencing of an *A. niger* strain has shed more light on the potential biochemical mechanisms involved in acid accumulation; however, it should be noted that the sequenced strain is not known to be a major producer of citric acid. Genes coding for several of the enzymes involved in the crucial formation of oxaloacetic acid were found. The identification of the various activities comprising citric acid metabolism and the implicated transporter genes provides the basis for a more complete understanding of the efficient accumulation of citric acid by *A. niger*.

In 2011 the global production reached 1.75 million tons per year and with an estimated increase of ~5% per year. There is an interest to use agro-industrial wastes as a source for carbon, instead of the more expensive sugars that have been the traditional carbon source in large-scale fermentations. There is also renewed interest in using the yeast *Y. lipolytica* for industrial manufacture mainly due to the wider substrate range since in addition to sugars, it can utilize alkanes, plant oils, starch hydrolysates and glycerol. There are no indications in the public domain that this yeast is used now in industry.

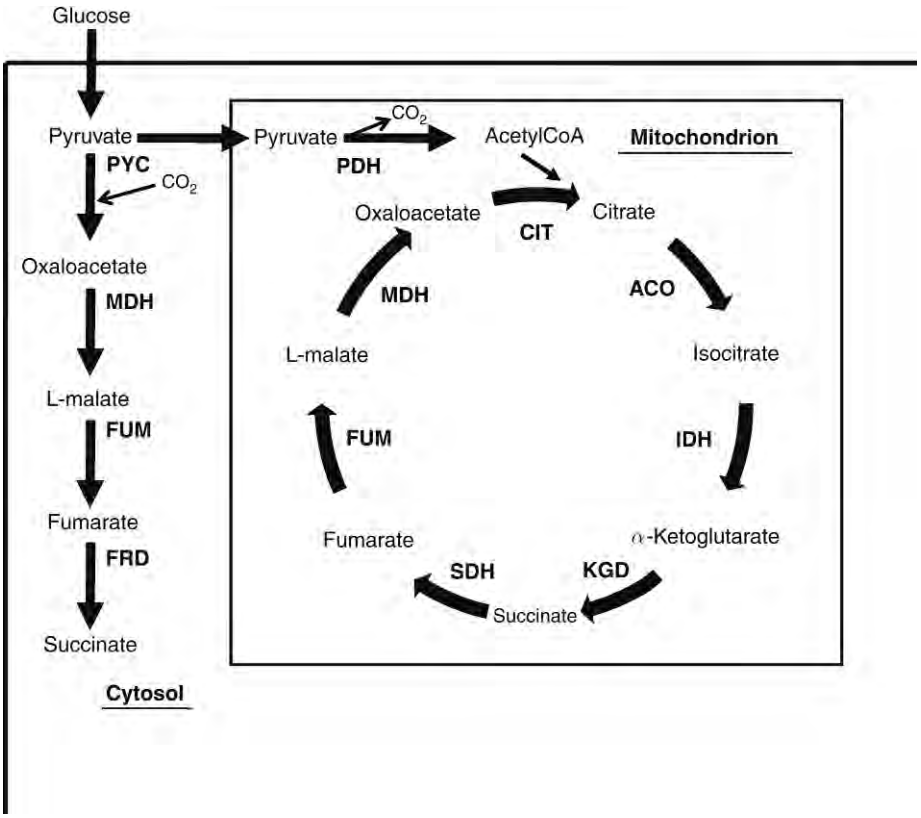

Fig. 3 The reductive reactions of the TCA cycle. The abbreviations of the enzymes are ACO, aconitase; CIT, citrate synthase; FUM, fumarase; FRD, fumarate reductase; IDH, isocitrate dehydrogenase; KGD-α, ketoglutarate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; PYC, pyruvate carboxylase.

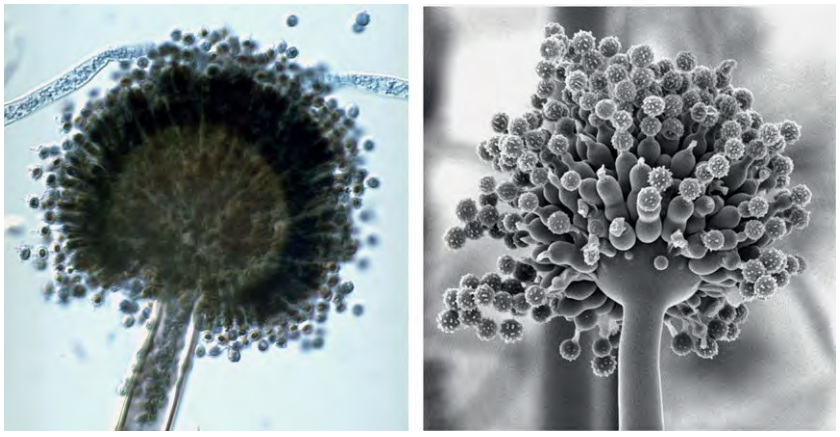

Fig. 4 *Aspergillus niger*. (Left panel) Slide culture, ×400. With permission of Mirhendi, H. Tehran University of Medical Sciences, Tehran, Iran. (Right panel) Scanning electron micrograph of asexual reproductive apparatus. Partially freeze-dried. Reproduced from Read, N.D., 1991. Low temperature scanning electron microscopy of fungi and fungus–plant interactions. In: Mendgen, K., Leseman, D.-E. (Eds.) Electron Microscopy of Plant Pathogens. Berlin: Springer Verlag, pp. 17–29. with permission from Springer Science and Business Media.

The biosyntesis of citric acid is still not completely understood. There are several control points from the entry of the sugar, its metabolism and transport outside of the cells that have been described. Among others it has been proposed that citrate accumulation is dependent on the activity of the NADP specific isocitrate dehydrogenase which was shown using mutants of isocitrate dehydrogenase in industrial strains of *A. niger*. The obligatory high concentration of metabolizable sugar necessary to obtain high titers of citric acid is probably due to the accumulation of fructose 2,6 biphosphate with a stimulating effect on glycolysis with concomitant inhibition of α ketoglutarate dehydrogenase, an enzyme further on in the oxidative TCA cycle. The limited activity of

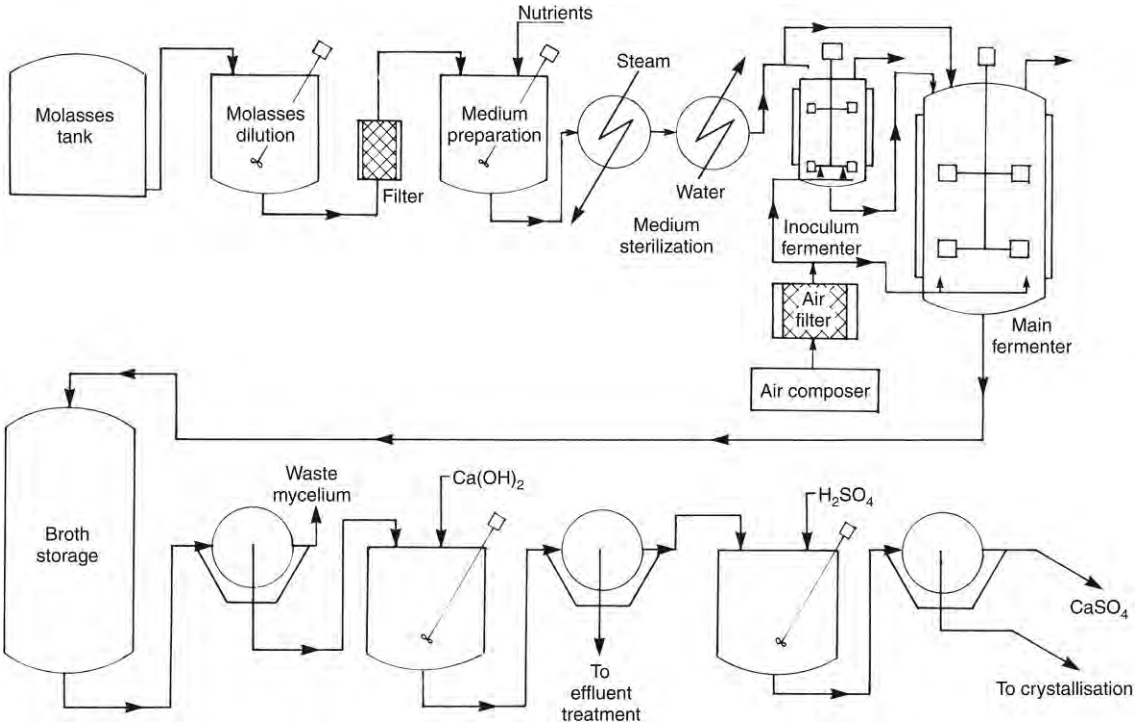

Fig. 5 Manufacturing of citric acid by a submerged process. Reproduced from Meers, J.L., Milsom, P.E., 1987. Organic acids and amino acids. In: Bu'lock, J., Kristiansen, B. (Eds.), Basic Biotechnology, first ed. London: Academic Press, pp. 359–383, with permission from Elsevier.

isocitrate dehydrogenase (by Mn^{2+} limitation) is another significant factor to achieve augmented citrate accumulation. Another important control point is the inhibition by high citrate of the enzyme phosphofructokinase which is reversed by the accumulation of high concentrations of ammonia, by degradation of intracellular nitrogenous macromolecules. Even though the conditions required for citrate accumulation are well documented, it is still not possible to assign the primary effect of a certain condition.

Gluconic Acid

Gluconic acid (2,3,4,5,6-pentahydroxy caproic acid, $C_6H_{12}O_7$) (Fig. 6) is a noncorrosive, nontoxic, mild organic acid with a brown clear appearance. It is very soluble in water and has a mild and refreshing taste. It is a good chelator at high pH, with better activity than commonly used chelators.

Gluconic acid was discovered in 1870 by Hlasiwetz and Habermann, when glucose was oxidized with chlorine. In 1922 it was isolated from a strain of *A. niger*. Later, other filamentous fungi, such as *Penicillium*, *Scopulariopsis*, *Gonatobotrys*, and *Gliocladium*, and also oxidative bacteria, such as strains of *Pseudomonas*, *Gluconobacter* (*Acetobacter*), *Moraxella*, *Micrococcus*, *Enterobacter*, and *Zymomonas* were found to produce gluconic acid. Already in the 1940s it was possible to obtain good yields of gluconic acid using *A. niger* by fermentation, neutralizing the accumulating acid with calcium carbonate.

The physiological functions of gluconic acid accumulation for these organisms are not clear; one possibility is its contribution to the competitiveness of the organism, removing glucose from the close environment. In the case of *P. expansum* (a phytopathogenic fungus), it was demonstrated that secreted gluconic acid contributed to the colonization and disease development of apple tissues by this fungus.

Gluconic acid is used in the manufacture of metal, leather, and food. It has been accredited with the capability of inhibiting bitterness in foods. Sodium gluconate is permitted in food and it has GRAS (generally recognized as safe) status. This salt is also utilized as a sequestering agent in many detergents, and added to cement to improve the hardening process.

The formation of gluconic acid is different from most other organic acids, since it is formed outside the cytoplasmic membrane, by the enzyme glucose oxidase. This enzyme has been shown to be localized in the cell wall, at least for fungi known to accumulate

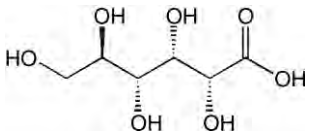

Fig. 6 Gluconic acid.

gluconic acid. Glucose in the medium is oxidized in a two-step reaction to gluconic acid; first glucose oxidase oxidizes β-D-glucopyranose to D-glucono-1,5 lactone with the formation of hydrogen peroxide, acted upon by catalase to form water and oxygen (Fig. 7). The hydrolysis of the lactone is spontaneous in aqueous solutions, but occurs six times faster with the enzyme gluconolactonase, resulting in gluconic acid (Fig. 7).

The main route of gluconic acid production has been by fermentation, mainly using *A. niger* in submerged fermentations. The two most important parameters of the fermentation is a high concentration of dissolved oxygen, used directly in the biosynthesis, and keeping pH 4.5–6.5, traditionally achieved by addition of calcium carbonate as the neutralizing agent. Best results are obtained with a high-glucose concentration (110–250 g L^{-1}), low concentrations of nitrogen and phosphorus (<20 mmol L^{-1}), and low concentrations of metal ions. Fermentations with almost quantitative yield (>90% on a molar basis) are completed in less than 24 h. The fact that the oxidation reactions occur outside of the cells allows for reuse of the mycelium up to 14 times. In the current industrial method, neutralization with NaOH allows for the use of higher initial sugar concentrations (up to 350 g L^{-1}) and the pH is then maintained close to 6.5. Recently, enzymatic processes have been introduced by conversion of glucose syrups with less formation of by-products and resulting in fewer problems in the downstream process.

There are alternative ways of gluconic acid production by chemical, electrochemical, and bioelectrochemical routes, but with lower yields than the fermentation processes. Bacteria, mainly *Gluconobacter oxydans*, are reported to be used by industry. Recently, a process based on *Acetobacter methanolicus* was developed. The process utilizes methanol as the carbon source for growth, with the addition of glucose for its conversion to gluconic acid.

It is still considered problematic to produce gluconic acid in an ecofriendly and cost-effective manner, mainly due to the multiple steps involved in the downstream processing, membrane methods are predicted to be optimal for purification. Very high yields of gluconic acid (1.05 g g^{-1}) with glucose solutions of 330 g L^{-1} have been obtained with dispersed growth of the fungus *A. niger* instead of the conventional pellets widely used in industrial production. The productivity is also increased since the fermentation time with dispersed growth is shortened.

L(+)-Tartaric Acid

L(+)-Tartaric acid (L-2R,3R-dihydrobutanedioic acid, $C_4H_6O_6$) (Fig. 8) derives its name from the medieval, alchemical term tartarus. It is a white, crystalline powder, odorless, and with an acidic taste. It is a strong organic acid, widely distributed in nature, and classified as a fruit acid (it is the most expensive fruit acid). The acid has two stereogenic atoms and it exists in three stereoisomeric forms – L(+), D(−), and the DL-racemic tartaric acid, which is distinct from the *meso*-tartaric acid. Although the dextrorotatory D(−)-isomer is the 'unnatural' form of the acid, its occurrence in small amounts in nature has been demonstrated. L-tartaric acid is present

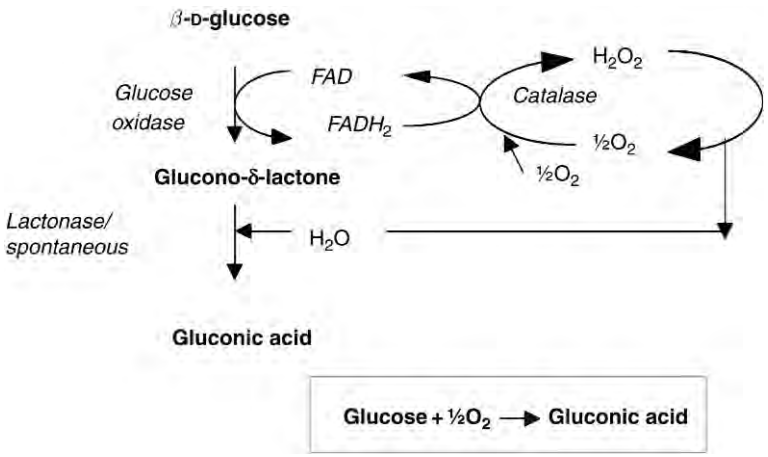

Fig. 7 Biosynthesis of gluconic acid. Reproduced from Ramachandran, S., Fontanille, P., Pandey, A., Larroche, C., 2006. Gluconic acid: Properties, applications and microbial production. Food Technology and Biotechnology 44, 185–195.

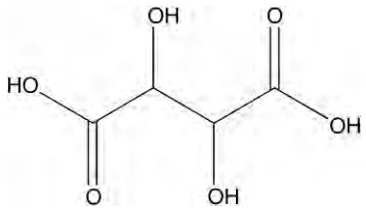

Fig. 8 L-Tartaric acid.

in its free form or combined with potassium, calcium, and magnesium. It is highly soluble in water, methanol, ethanol, and glycerol but is insoluble in chloroform.

Although tartaric acid was first isolated by the alchemist Jabir Ibn Hayyan, Carl Wilhelm Scheele is credited for its discovery in 1769.

L-Tartaric acid is present in the juices of various fruits, particularly in tamarinds, unripe grapes, and is one of the main acids in wine.

L-Tartaric acid is an extremely versatile acid and it is utilized in a wide range of industries. The largest single application for tartaric acid is as a raw material for the manufacturing of emulsifiers used for bread improvement. An important salt of tartaric acid, potassium hydrogen tartarate (or cream of tartar), has applications as an acidulant for baking powder and sugar confectionery. Tartaric acid is used in the food industry (in particular, in jams, fruit juices, pickles, soft drinks, etc.), in the production of various construction materials, and in certain medicines as an inert bulk substance, since it is not metabolized by the human body. The potassium sodium salt, called Rochelle salt, was the first compound used as a piezoelectric crystal. The acid is a useful raw material for the synthesis of other chiral molecules (e.g., L(+)-, D(−)-tartaric acids are used as chiral auxiliary reagents in the oxidation of alkenes to enantiomerically pure epoxides).

Tartaric acid can be produced by natural and synthetic routes. The natural route involves the recovery of the reddish precipitated salt, potassium bitartarate, from argol, the sediment in wine vats. The synthetic chemical route involves the production of the racemic mixture of tartaric acid from maleic anhydride.

Since the availability of L(+)-tartaric acid from wine is dependent on the climate, and because the chemical process results in the racemic mixture of the acid, attempts have been made to explore alternative biological ways of producing the high-cost tartaric acid. Several patents and reports suggest that various bacteria (such as *Corynebacterium* sp., *Rhodococcus* sp., *Alcaligenes levotartaricus*, *Acinetobacter tartaricus*, and *Pseudomonas agrobacterium*) show a high enzymatic activity of the stereo-specific *cis*-epoxysuccinic acid hydrolase to form L(+)-tartaric acid. Industrial processes based on these organisms are not known.

Currently, the industrial production of L(+) tartaric acid is mediated by transformation of *cis*-epoxysuccinic acid using various organisms that contain high activities of the enzyme *cis*-epoxysuccinate hydrolase (EC 3.3.2.3). The enzyme is convenient to use since it does not require cofactors, metals and is relatively stable. Until now there are no reports of a direct fermentation using sugars as substrates for the production of L(+) tartaric acid and it is still isolated from vinasses, the wastes of wine production or chemically produced.

Malic Acid

Malic acid (2-hydroxybutanedioic acid, $C_4H_6O_5$) (Fig. 9) is a white, odorless, crystalline solid. In contrast to other fruit acids, it is very hygroscopic and has a tendency to lump. Malic acid is a dicarboxylic acid and has an asymmetric carbon and occurs as L(the natural)- and D-isomers.

Malic acid was first described by Sheele who, in 1785, isolated this acid from unripe apples. The name malic is from the Latin for apple, malum.

Malic acid is found in other fruits such as grapes, watermelons, cherries, and in vegetables such as carrots and broccoli. This acid is mainly used in food applications including candy and beverages. It gives a tart taste, lowers the pH, has antimicrobial effects, and confers special blending and flavor-fixing properties. There are also nonfood applications such as use for metal cleaning and finishing, textile finishing, electroless plating, pharmaceuticals, infusions, and paints.

The biosynthesis of L-malic acid from carbohydrates (glucose) is similar to citric and fumaric acid, where the carboxylation of pyruvic acid leads to oxaloacetic acid that is reduced to L-malic acid (Fig. 3). The carboxylation reaction that leads to L-malic acid accumulation allows for a high acid molar yield (based on the glucose utilized).

World production of malic acid is estimated at around 60 000 t per year (Table 1), most of this is made by chemical hydration of maleic or fumaric acid, using high temperature (180–220°C) and high pressure (14–18 bar), yielding the racemic mixture of DL-malic acid.

Biological processes result in the L-isomer of the acid. In one process, species of *Brevibacterium flavum* or *B. ammoniagenes* with high fumarase (fumarate hydratase) activity are immobilized, in either polyacrylamide or κ-carrageenan, and are used to convert fumarate to L-malate. In another efficient process, the yeast *Saccharomyces cerevisiae* was amplified for the enzyme fumarase by cloning the single homologous nuclear gene downstream of a strong promoter. The overproducing strain can be used as free cells or can be immobilized (e.g., in agarose beads or microspheres); it converted fumarate to L-malate at a maximal rate of 65 mmol L^{-1} g^{-1} h^{-1}, with a yield of approximately 85%.

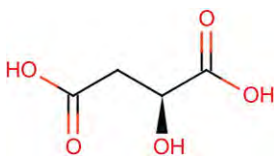

Fig. 9 L-Malic acid.

After optimization of the fermentation conditions, filamentous fungi, such as *Aspergillus flavus* using glucose, accumulate over 110 g L^{-1} of L-malate. Other species (e.g., *Aspergillus oryzae* and *A. niger*) have also been found to accumulate L-malic acid from glucose. Since these latter fungi are GRAS organisms, these are suitable for food applications.

The use of fermentation for the production of L-malic acid on an industrial scale has not been realized yet, even though non-aflatoxigenic strains, such as *Aspergillus oryzae* are able to produce 154 g L^{-1} with a productivity of 0.94 g L^{-1} h^{-1}. The utilization of non-conventional substrates is becoming crucial using low cost substrates instead of expensive sugars. One such substrate, which due to the high volume production of biodiesel, is glycerol. The fungus *Ustilago* is capable of growing as single cells when in its haploid form, lowering the energy demand to agitate filamentous organisms (*Aspergillus*). Concentrations reaching 200 g L^{-1} have been reported. Attempts to use synthetic metabolic engineering for accumulation of L-malic acid is still in its infancy and the yields are much below (molar yield of 60% from glucose) than those reported for whole cell fermentations (140% which is 69% of theoretical yield).

L-Ascorbic Acid (Vitamin C)

L-Ascorbic acid (3-keto-L-gulofuranolactone, vitamin C, $C_6H_8O_6$) (Fig. 10) is a colorless crystal, white or very pale yellow crystalline powder. It is sensitive and will decompose at prolonged exposure to light and air. Vitamin C is an essential nutrient for higher primates and its deficiency will cause scurvy, probably due to the stimulation, by vitamin C, of the oxidation enzymes (oxidases) involved in the formation of collagen. The gene encoding L-gulono-1,4-lactone oxidase is absent in these animals, and therefore an external source of vitamin C is necessary. L-Ascorbic acid is a strong antioxidant and it protects toward oxidative stress and has been implicated to protect tissues and hinder cardiovascular disease and cancer.

In 1928, Albert Szent Györgi discovered vitamin C (the third vitamin found, and given the third letter in the alphabet) and solved a long debate over the active ingredient with antiscurvy activity. The name ascorbic acid is derived from a scorbuticus meaning against scurvy. Vitamin C was the first vitamin to be artificially synthesized in 1935.

L-Ascorbic acid is found in citrus fruits such as lemon, lime, and orange and also in vegetables such as cabbage and processed cabbage (sauerkraut). Long before the isolation of L-ascorbic acid, it was known that including lemons and oranges in the diet prevented scurvy.

L-Ascorbic acid is used on a large scale (110,000 t in 2001, see Table 1) as an antioxidant in food, animal feed, beverages, pharmaceutical formulations, and cosmetic applications.

The manufacture of L-ascorbic acid is by the Reichstein–Grüssner synthetic pathway introduced in 1934 (Fig. 11). It is a chemical process with one step carried out by biocatalysis; conversion of D-sorbitol to L-sorbose by the bacterium *Gluconobacter suboxydans* (or by the formerly used *Acetobacter xylinum*). The yield of ascorbic acid from the starting material glucose is estimated to be around 50%. The process is energy consuming with high temperatures and pressures necessary for some steps of the process. Economic factors have given rise to tremendous interest for use of alternative processes.

There are several alternatives based on biological processes with 2-keto-L-gulonic acid (2-KLG) as the key intermediate. Potential use of the genera *Gluconobacter* (*Acetobacter*), *Ketogulonicigenium*, *Pseudomonas*, *Erwinia*, and *Corynebacterium* has been looked into furthermore. In the so-called two-stage process, the chemical reactions of the Reichstein–Grüssner process to produce di-acetone-ketogulonic acid are replaced by a second fermentation step, which results in the formation of 2-KLG (Fig. 12).

Another biological route, the 'one organism route', has great potential to become commercialized. Here glucose is converted to 2-KLG in one-step fermentation process, using *Erwinia* sp. (able to convert glucose efficiently to 2,5-diketo-D-gluconic acid) engineered with a gene encoding 2,5diketo-D-gluconic acid reductase from *Corynebacterium glutamicum*, resulting in more than 120 g L^{-1} of 2-KLG formed in 120 h of fermentation (Fig. 13). Although biotechnological processes are being exploited for the production of ascorbic acid and Reichstein–Grüssner intermediates, the future goal should be the total replacement of the chemical route by efficient biocatalytical systems for production, using cost-saving starting materials.

The formation by fermentation of 2-KLG is considered well established and with high efficiency using D-sorbitol as substrate, but the ascorbic acid is still not the final product. This fermentation is considered a bio catalytic functionalization of a specific substrate. The choice of carbon source is therefore limited to those with suitable stereochemistry. The two-step fermentation process is well established in China from the 1980s producing around 80% of the world market demand. The challenge, that has still not been resolved, which should result in commercial concentrations of vitamin C, is to convert an appropriate substrate directly to ascorbic acid. The biological conversion of the substrate to 2-KLG must followed by an effective bioconversion to ascorbic acid. One of the attempts has been to use the well-researched conventional yeast, *S. cerevisiae* with its well-developed resources for genetic engineering where three genes, GDP-D-mannose-3,5-epimerase, GDP-L-galactose-phosphorylase and

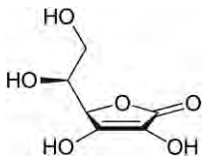

Fig. 10 L-Ascorbic acid.

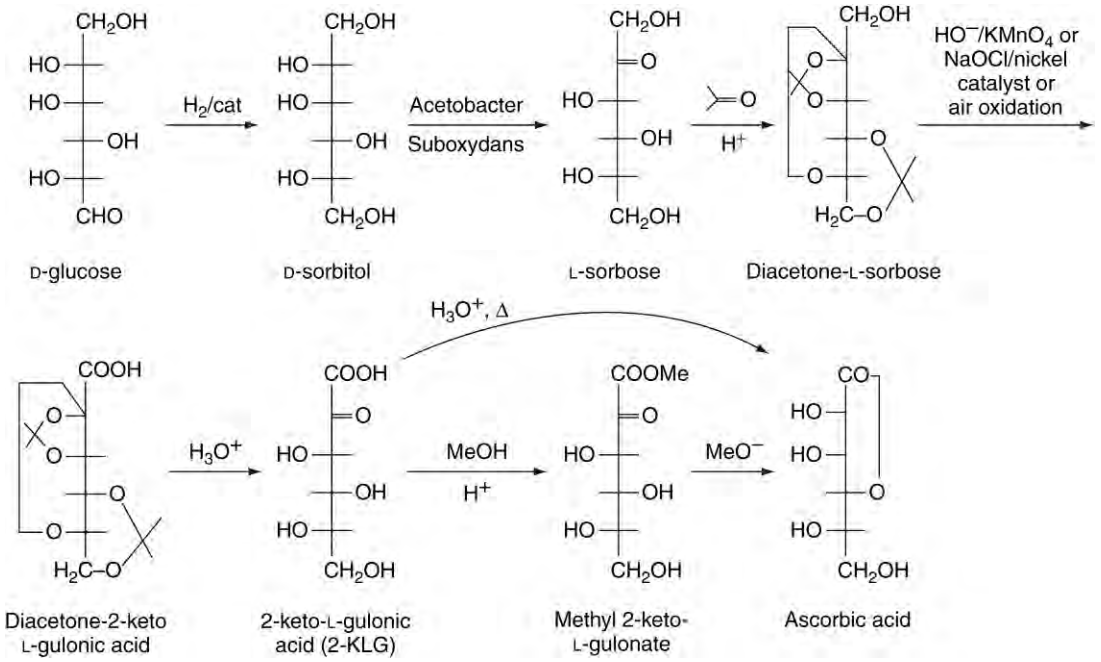

Fig. 11 The Reichstein–Grüssner process for the synthesis of L-ascorbic acid. Reproduced from Anderson, S., Berman Marks, C., Lazarus, R., *et al.,* 1985. Production of 2-keto-L-gulonate, an intermediate in L-ascorbate synthesis, by a genetically modified *Erwinia herbicola.* Science 230, 144–149, with permission from AAAS.

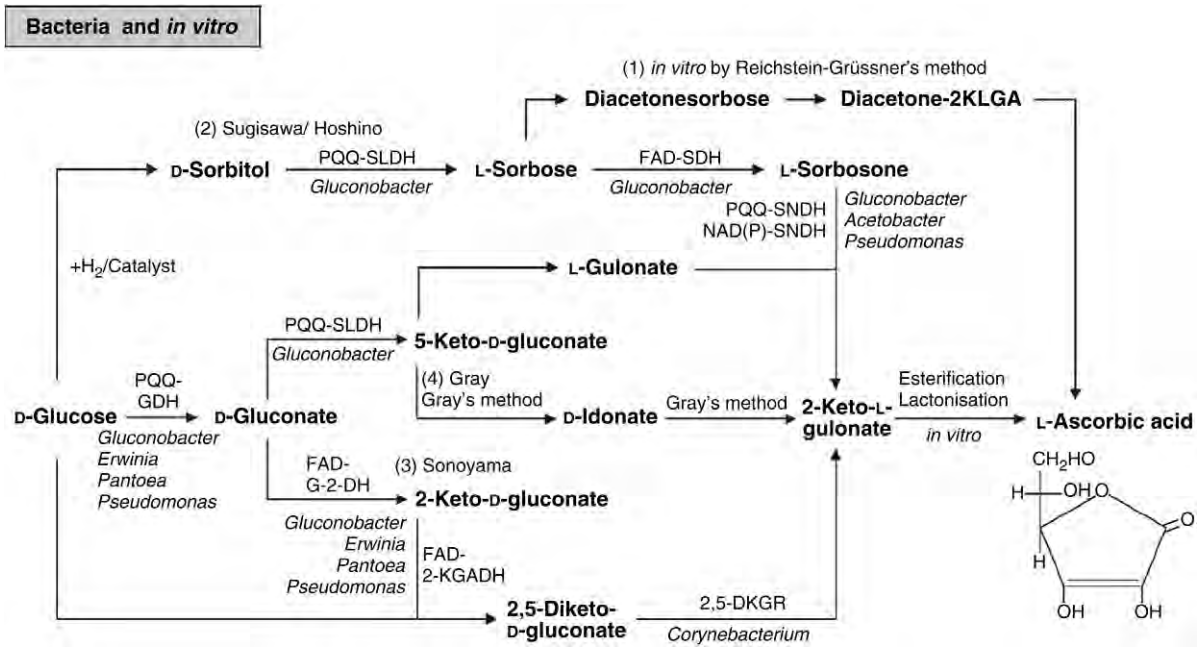

Fig. 12 Alternative processes for L-ascorbic acid production. Reproduced from Bremus, C., Herrman, U., Bringer-Meyer, S., Sahm, H., 2006. L-Ascorbic acid production. Journal of Biotechnology 124, 196–205, with permission from Elsevier.

L-galactose-1-phosphatase from plants should allow for the direct biological formation of L-ascorbic acid. However, the acid accumulates intracellularly at low concentrations. More research on precursor supply, possible compartmentalization and flux analysis to determine the rate limiting step(s) is to reach commercial production with the yeast. *Ketogulonicigenium vulgare* (formerly *Gluconobacter oxydans*) is used in industry to effectively provide the precursor 2-KLG at a concentration of 130 g L^{-1} from 150 g L^{-1} of sorbitol in 72 h.

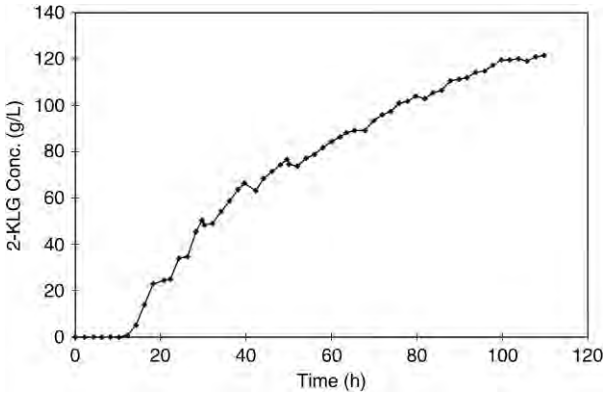

Fig. 13 Formation of 2-KLG from glucose in a single production host. Reproduced from Chotani, G., Dodge, T., Hsu, A., *et al.*, 2000. Commercial production of chemicals using pathway engineering. Biochimica et Biophysica Acta 1543, 434–455, with permission from Elsevier.

Fumaric Acid

Fumaric acid (2-butenedioic acid trans, $C_4H_4O_4$) (Fig. 14) derives its name from the fact that the acid is found in plants that belong to the genus *Fumaria*, a common European herb. Fumaric acid is the *trans*-isomer of symmetric, unsaturated dicarboxylic acid; the *cis*-isomer is maleic acid. It is produced as a colorless, crystalline powder with a fruit-like taste (a fruit acid), and it is a weak acid which forms diesters, has low solubility in water, and it undergoes additions across the double bond.

Fumaric and maleic acids were discovered in 1817 by Braconnet and independently by Vauquelin during dry distillation of malic acid.

Fumaric acid is widely used in the food industry as an acidulant because it is nontoxic and is the least expensive of the food-grade acids. Fumaric acid solubility in water is low (0.6 g per 100 g at 25°C), and, therefore, in order to increase its application in various foods a cold water-soluble (CWS) fumaric acid, which contains a wetting agent, for example 0.3% w/w dioctyl sodium sulfosuccinate, is used. Fumaric acid is used for the industrial preparation of L-malic acid catalyzed by the enzyme fumarase (see 'Malic acid') and L-aspartic acid, a component of aspartame, by the enzyme aspartase. Other industrial uses of fumaric acid are in jet printing inks, plastics surface coating and paper sizing, and as an intermediate in the preparation of unsaturated polyester and alkyd resins. Fumaric acid is used by the pharmaceutical industry to produce alexipharmic sodium dimercaptosuccinate and ferrous fumarate, as an optical bleaching agent, in formulations for alternative medicine or as fumaric acid esters monoethylfumarate and dimethylfumarate to treat psoriasis.

Fumaric acid does not accumulate under normal growth conditions of microorganisms and its synthesis is carried out through succinic acid by the mitochondrial oxidative TCA cycle (Fig. 2). Various species of the filamentous fungus *Rhizopus* (such as *Rhizopus nigricans* and *Rhizopus oryzae*) produce high concentrations of this acid, which is excreted to the medium. In *Rhizopus*, most of the fumaric acid is formed from pyruvic acid via a carboxylation reaction, yielding oxaloacetic acid, which is converted directly to L-malic acid and then to fumaric acid. These reactions, which are catalyzed by pyruvate carboxylase, malate dehydrogenase, and fumarase, respectively, are localized in the cytosol and are part of the reductive TCA cycle (Fig. 3), carried out under aerobic conditions. The production of fumaric acid from glucose through the reductive reactions of the TCA cycle does not provide net energy and is balanced for carbon, oxygen, and hydrogen. Therefore, part of the pyruvic acid must be utilized through the oxidative TCA cycle to provide energy, mainly for maintenance requirements. It should be noted that, in general, pyruvate carboxylase in eukaryotic organisms is localized in mitochondria, while in certain acid producing filamentous fungi (e.g., *Rhizopus* and *Aspergillus*), the enzyme is situated exclusively in the cytosol and in some cases in both compartments. The cytosolic localization of this enzyme appears to be important for the ability of these organisms to accumulate high concentrations of organic acids.

In the 1940s, fumaric acid was made by fermentation on a commercial scale (about 4000 t per year) using a strain of the fungus *Rhizopus arrhizus* (later named *R. oryzae*). It was the first submerged fermentation process with molds and served as a model for implementing and scaling up the techniques used for submerged fermentations. The biological production of fumaric acid was terminated when the chemical synthesis became economically more attractive. Fumaric acid is a symmetrical molecule having no isomers and, therefore, the biological process offers no specific advantage over the chemical process. Fumaric acid is presently

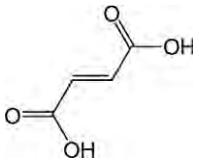

Fig. 14 Fumaric acid.

produced by a chemical process through the isomerization of maleic acid (or maleic anhydride), obtained from a catalytic vapor-phase oxidation of benzene or C_4 hydrocarbons.

The accumulation and excretion of fumaric acid from glucose by *R. oryzae* (about 100 g L^{-1} of fumaric acid) occurs under aerobic conditions in a high-glucose (an initial concentration of 120 g L^{-1}) medium containing a limited amount of nitrogen and a neutralizing agent ($CaCO_3$). Fumaric acid (molar yield of approximately 100%; moles acid produced per mole of glucose utilized×100), L-malic acid (15 mol%), and succinic acid (5 mol%) are the major acids formed during fermentation. C_4 acid (fumaric, L-malic, and succinic acid) molar yields of 120–145% are obtained after 4–5 days. The high fumaric acid molar yield and C_4 acid molar yield confirm that these acids are produced via a carboxylation reaction of pyruvic acid.

A low cost biological process for fumaric acid manufacture is still a challenge, partly due to the costs of downstream processing, with no low pH processes that are effective. Partly because of inherent technical problems involved in growing filamentous fungi on a large scale. Morphology is the main issue, where pellet growth is desired but not always attainable. Another main cost factor is the price of the substrate estimated to be 40–60% of the total and therefore unconventional C-sources have been and are tested, such as woodchips, dairy manure, crude glycerol (by-product of biodiesel formation) and corn straw and other lignocellulosic sources. Attempts of metabolic engineering has yet not resulted in either productivities or acid concentrations that can compete with the industrial chemical process still in use. Platform organisms, such as *S. cerevisiae* and *Escherichia coli* are being intensively studied to replace the filamentous and potentially pathogenic *Rhizopus* sp. The U.S. Government's Occupational Safety and Health Administration [OSHA] classifies *Rhizopus* species as an allergen and irritant and a cause of hypersensitivity pneumonitis and dermatitis. The concentrations accomplished are orders of magnitudes lower acid (5.6 g L^{-1} , 28.2 g L^{-2} respectively compared to 126 g L^{-1} for *Rhizopus*).

Isocitric Acid

Isocitric acid ((1R,2S)-1-hydroxypropane-1,2,3-tricarboxylate, $C_6H_8O_7$) (Fig. 15) is a TCA intermediate and an isomer of citric acid. It exists in four isomers and is a chiral molecule, and thus has two enantiomers of each of the isomers.

Isocitric acid (the (−)-D-threo-isomer) is synthesized by all cells as it is an intermediate of the TCA cycle (Fig. 2). Isocitric acid is synthesized from citric acid via the intermediate *cis*-aconitic acid by the enzyme aconitase (aconitate hydratase). Isocitric acid is a minor organic acid found in most fruit juices, especially in blackberries, youngberries, and boyberries, and in vegetables, especially in carrot. The determination of D-isocitric acid has become of importance in the analysis of fruit juices for the detection of illegal additives (adulteration). Since the quantities of citric (see "Citric acid") and isocitric acids are correlated in fruit juices, a too high ratio of citric to isocitric acid indicates the addition of citric acid.

Isocitric acid is mostly used in the food industry (food additive) as a food acidulant, and because of being a chiral molecule, isocitric acid is used in the pharmaceutical industry and as a potential raw material for synthesis of expensive chemicals.

Racemic isocitric acid was first prepared by Fittig and Miller in 1889 by the condensation of chloral with sodium succinate in the presence of acetic anhydride and by the subsequent hydrolysis of the resulting trichloromethylparaconic acid to isocitric acid; this is isolated as the lactone.

Formation of citric and isocitric acid by yeasts using various carbon sources has been the focus of investigations for the past four decades. Most of the research was directed for production of citric acid while isocitric acid was considered as an undesirable by-product. *Y. lipolytica*, several *Candida* species (such as *Candida oleophila*, *Candida parapsilosis*, *Candida brumptii*, *C. guilliermondii*, *Candida zeylanoides*, *Candida pulcherrima*, and *Candida chalmersi*), *Pichia polymorpha*, and *Hansenula pelliculosa* are examples of yeasts that are capable of producing different citric acid/isocitric acid ratios and quantities using various carbon sources, such as hexadecane, tetradecane, glucose, *n*-paraffins, calcium acetate, calcium butyrate, soybean oil, fish oil, canola oil, ethanol, and glycerol. Fermentation conditions, type of carbon source, and the organism utilized determine the amount of isocitric acid formed. It was reported that with *Y. lipolytica* grown on canola oil, up to about 75 g L^{-1} of isocitric acid and 110 g L^{-1} of citric acid were produced. It can be expected that an increase in isocitric acid synthesis, on the expense of citric acid, can be obtained in a mutant of *Y. lipolytica* having an amplified activity of aconitase. This strain could then be used more economically than the wild-type strain, for the commercial production of isocitric acid.

The complete genome sequence of *Y. lipolytica* has been determined and is opening up the potential for use of this yeast to produce specific acids (i.e., isocitric) at high productivity.

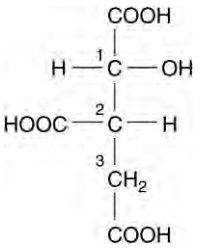

Fig. 15 Isocitric acid.

There is still a lot of interest to improve isocitric acid accumulation with as low as possible concentration of citric acid (the acid sells for a much higher price than the other acids reviewed). The purification of isocitric acid from the mixture is prohibitively expensive, and marketed isocitric acid is from *Sedum spectabile* (renamed *Hylotelephium spectabile)*. The optimization of cultivation conditions and manipulations of the concentrations of iron (co-factor of aconitate hydratase) and itaconic acid (inhibitor of isocitrate lyase) allowed for better ratios of the two acids in the yeast *Y. lipolytica*. The addition of itaconic acid has resulted in a dramatic change in the ratio of isocitric to citric acid to 6:1 in a mutant of *Y. lipolytica*.

Building Blocks

Polyhydroxyalkonates

Polyhydroxyalkonates (PHA) is a large group of polymers based on linear polyesters of 3, 4, 5 and 6-hydroxyacids (Fig. 16).

The molecular weight of PHA can be up to 2 million Daltons. All the monomers are enantiomerically pure and in the R-configuration. The PHA exhibit thermoplastic and elastomeric properties and are easily degraded to carbon dioxide and water. They are considered as good replacements for petroleum-derived polymers, in terms of physical–chemical characteristics and biodegradability.

In 1923, Lemoigne found that spore-forming bacilli make 3-hydroxybutyric acid. In 1927, after using chloroform extraction, he showed that a *Bacillus* sp. accumulated the polymer, which consists of the monomer of 3-hydroxybutyric acid (PHB), where PHB is a specific case of PHA. There are a multitude of possible monomers, of either 3–5 carbon atoms designed as short chain-length PHA or 6–14 carbon atoms, called medium chain-length PHA. A wide variety of bacteria, both Gram negative and Gram positive, have the capability to synthesize PHA. Among these are species of *Pseudomonas*, *Bacillus*, *Ralstonia*, *Aeromonas*, *Rhodobacter*, and even certain *Archaea*, such as *Haloferax sulfurifontis*. The main species in use by industry was initially designated as *Alcaligenes* sp., and today it is called *Cupriavidus* sp. The PHA function as energy storage compounds for the bacteria and are found as discrete cytoplasmic inclusions in the cells.

In the early 1970s, polyhydroxybutyrate was manufactured by the use of *Alcaligenes* sp., accumulating up to 70% of cell dry weight as polymer. However, this process had its problems such as that the product showed brittleness and poor mechanical characteristics. A copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate, BIOPOL, was developed and is used for medical devices such as bioengineered hearts, surgical sutures, meshes, and orthopedic fixtures. Another copolymer of 3-hydroxybutyrate and 3-hydroxyhexanoate is used in the production of nonwovens, binders, flexible packaging, synthetic paper, and medical devices. A recombinant *E. coli*, able to produce a mixture of 3-hydroxybutyrate and 3-hydroxyoctanoate to 90% of its cell dry weight in 24 h, has been developed.

The biosynthesis of PHA consists of three enzymes, PHA synthase, β kethothiolase, and β reductase, which are all found in producing microorganisms. Supply of monomers and polymerization are the two main steps in biosynthesis, and PHA formed is dependent on the carbon source used as well as on the different pathways (three) that are present in different microorganisms; pathway I where the carbon source (sugar) is the precursor, through the formation of acetyl-coA, pathway II where fatty acid degradation delivers the precursors, and pathway III where the polymer is formed through the route of fatty acid biosynthesis.

Attempts to produce PHAs on an industrial scale was initiated in the 1970s by Imperial Chemical Industries using the 1.5 million liter continuous fermentor initially used for production of single-cell protein. The two natural producers, *Ralstonia eutropha* and *Alcaligenes latus* are not ideal for large-scale production since they grow slowly and depolymerize the accumulated PHA and use it as a second energy source. The PHA biosynthetic pathway has been transferred to bacteria with robust central metabolic pathways and capable of using inexpensive carbon sources, engineered *E. coli* produced 80 g L^{-1} of polyhydroxybutyrate with a productivity of 2 g L^{-1} h^{-1}. One of the major producers is Telles, a joint venture between Archer Daniel Midlands and Metabolix Inc. producing a corn syrup-based PHA resin, Mirel, reported to have a production capacity of 50,000 t per year.

Itaconic Acid

Itaconic acid (methylenesuccinic acid, $C_5H_6O_4$) (Fig. 17) is a white colorless crystalline, hygroscopic powder soluble in water, ethanol, and acetone. It is an unsaturated diprotic acid, which derives its unique chemical properties from the conjugation of one of its two carboxylic acid groups with its methylene group.

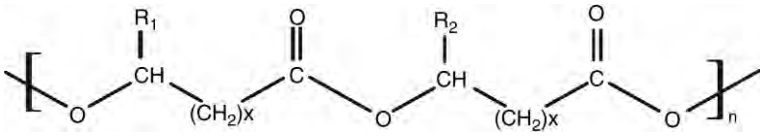

Fig. 16 General structure of polyhydroxyalkonates. Reproduced from Philip, S., Keshavarz, T., Roy, I., 2007. Polyhydroxyalkanoates: Biodegradable polymers with a range of applications. Journal of Chemical Technology and Biotechnology 82, 233–247, with permission from Society of Chemical Industry, granted by John Wiley & Sons Ltd on behalf of SCI.

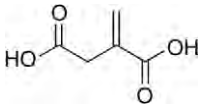

Fig. 17 Itaconic acid.

Itaconic acid was discovered by Baup in 1837 as a product of pyrolytic distillation of citric acid. The name itaconic was devised as an anagram of aconitic.

Itaconic acid is formed in fermentation of some sugars. In 1929, Kinoshita first showed the acid to be a metabolic product of *Aspergillus itaconicus*. A derivative of itaconic acid (*trans*-phenylitaconic acid) was isolated from another natural source (*Artemisia argyi*).

The biosynthetic pathway of itaconic acid from glucose is similar to that of citric acid, which occurs via the glycolytic pathway and anaplerotic formation of oxaloacetate by CO_2 fixation and via the TCA cycle (Fig. 2). Itaconic acid is formed by the cytosolic enzyme aconitate decarboxylase from *cis*-aconitic acid. Another biosynthetic pathway from pyruvate through citramalic acid, citraconic acid, and itartaric acid also results in itaconic acid (Fig. 18).

In contrast to several other organic acids (e.g., citric, isocitric, lactic, fumaric, and L-malic acid) itaconic acid is used exclusively in nonfood applications, especially in the polymer industry. Itaconic acid derivatives are used in medicine, cosmetics, lubricants, thickeners, and herbicides (e.g., substituted itaconic acid anilides).

Itaconic acid is produced solely by batch submerged fungal fermentation. *Aspergillus terreus* has been used from the 1940s in the fermentation process, which is similar to that of citric acid (see "Citric acid"), that is, it requires an excess of readily metabolizable sugar (glucose syrup, crude starch hydrolysates, and decationized molasses – up to 200 g L^{-1} sugar), continuous aeration, a low initial pH (between 3 and 5), sufficient nitrogen, high magnesium sulfate concentration (0.5%), low phosphate to limit biomass production, and a limitation in metal ions (zinc, copper, and iron). However, there exists one significant difference in that the sensitivity of this fungus to the formed acid, in contrast to *A. niger*, necessitates maintaining of the pH at 2.8–3.1 throughout the fermentation, in order to obtain high amounts of the acid. At present, the published production yield of itaconic acid is about 85% of theoretical, accompanied by-product concentrations of about 80 g L^{-1} during a cultivation at 39–42°C for 8–10 days. Recovery of itaconic acid is accomplished by first separating the fungal biomass by filtration followed by evaporation, treatment with active carbon, and crystallization and recrystallization. Actual markets for itaconic acid are currently limited because the fungal fermentation is carried out at a relatively high cost. New biotechnological approaches, such as published immobilization techniques, screening programs for other producing organisms (such as yeast), and genetic engineering of *A. terreus* (the annotated genome sequence of .*A terreus* strain NIH 2624 has been publicly released), or of *A. niger*, could lead to higher production of itaconic acid. Also, the use of alternative substrates may reduce costs and thus open the market for new and expanded applications of this acid.

The high production costs for itaconic acid is still an obstacle in its more widespread use in polymer chemistry, pharmacy and agriculture. Attempts to further optimize the natural producer *A. terreus* by investigating the fermentation conditions showed that high substrate concentrations and a low pH-value under phosphate-limited conditions allow for increase in accumulation of itaconic acid to 87 g L^{-1} after 7 days with 86% molar yield. Attempts to use metabolic engineering of the biosynthetic pathway have led to the understanding that the normal functionality of the TCA cycle results in low itaconic acid formation. The key enzyme for accumulation of itaconic acid is the *cis*-aconitate decarboxylase which requires a good precursor supply to allow for high accumulation. By the interference of the activity of pyruvate carboxylase by adding 10 mM L-aspartate, which also changed the growth morphology, it was possible to improve the itaconic acid yield by 8% and the final concentration by 60% compared to the

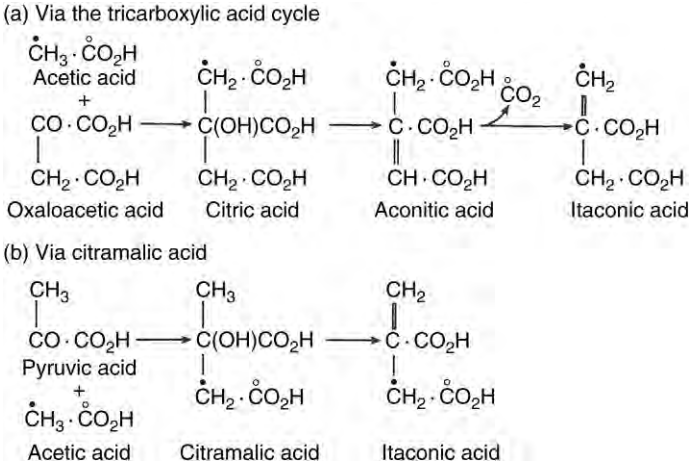

Fig. 18 Biosynthetic routes to itaconic acid. Reproduced from Winskill, N., 1983 Tricarboxylic acid cycle activity in relation to Itaconic acid biosynthesis by *Aspergillus terreus*. Journal of General Microbiology 129, 2877–2883, with permission from Society for General Microbiology.

control. With rigorous control of the manganese concentration in the medium, where the glucose solutions were put through an ion exchanger to lower the manganese concentration, it was possible to achieve an itaconic acid concentration of 131 g L^{-1} from 200 g L^{-1} glucose with *A. terreus*. This amount of acid is close to its solubility in water so growth at increased D-glucose concentrations of D-glucose would probably not allow for higher itaconic acid concentration.

Kojic Acid

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone, $C_6H_6O_4$) (Fig. 19) is a chelating organic acid freely soluble in water, ethanol, and acetone.

Kojic acid is a fungal metabolic product produced by a few species of *Aspergillus*, especially by *A. oryzae*, which has the Japanese common name koji. This acid is a by-product in the fermentation process of malting rice, for use in the manufacturing of sake, the Japanese rice wine.

Kojic acid was first isolated in 1907 by Saito from mycelia of *A. oryzae* grown on steamed rice. In 1912 Yabuta gave it the name kojic acid, and only in 1924 he deciphered the correct structure of the molecule of this acid.

Kojic acid is an inhibitor of growth of bacteria, fungi, and multiplication of viruses. It inhibits the catecholase activity of tyrosinase, which is rate-limiting, and an essential enzyme in the biosynthesis of the skin pigment melanin. Due to its properties as antibacterial, antioxidative, and color-protectant, kojic acid is used in the cosmetic industry, and in the food industry as a precursor for flavor enhancers (maltol and ethyl maltol), on cut fruits to prevent oxidative browning, antistaling of fruits and vegetables, and in sea food and meat to preserve pink and red colors. Kojic acid is consumed widely in the Japanese diet with the belief that it is of benefit to health (a functional food).

Kojic acid is produced chemically via pyranoid 3,2-enolones, or biologically by the direct fermentation of glucose by *A. oryzae* and *A. flavus*, where yield values of 70–90% of the acid are obtained, based on carbon source. When immobilized *A. oryzae* cells, entrapped in alginate gel beads, were used with glucose as the carbon source, kojic acid began to crystallize upon reaching a concentration of about 83 g L^{-1}. The carbon source can also be pyruvic acid, acetic acid, glycerol, and/or ethanol. The production using glucose by these filamentous fungi is very similar to that of citric acid and itaconic acid processes (i.e., at high sugar concentration and low phosphate concentration). By changing the fermentation parameters, such as stirring or aeration rate, some strains can be converted from citric acid or itaconic acid production to kojic acid production. This can be explained by the direct conversion of glucose, without splitting of the carbon chain, to kojic acid, as well as to citric and itaconic acids. By this proposed pathway, glucose is converted to D-gluconic acid by glucose dehydrogenase and D-gluconic acid is converted to kojic acid by gluconate dehydrogenase. However, direct evidence for the involvement of these enzymes in kojic acid biosynthesis has not been shown.

Research is still actively pursued to enable a biological process for kojic acid to become economical. There are attempts to use unconventional carbon sources (corn stover, cellulose) but pretreatment of the substrate is required and pentoses are inferior as C-source, fundamental drawbacks that increase the final cost. For a long time no information was available on genes responsible for kojic acid accumulation in *A. oryzae*, even though its sequence was known. Recently three genes have been isolated and described, one of them a transcription factor, all three genes are essential for the biosynthesis of kojic acid and its accumulation.

Succinic Acid

Succinic acid (butanedioic acid, $C_4H_6O_4$) (Fig. 20) is a symmetric, four-carbon, dicarboxylic acid, which forms colorless, odorless crystals with characteristic acid taste. The acid is soluble in water and slightly soluble in ethanol, ether, acetone, and glycerol.

Succinic acid (from Latin succinum, amber) was discovered in 1546 by Agricola by dry distillation (heating in vacuum) of amber.

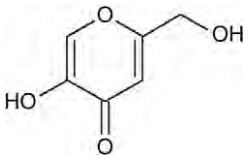

Fig. 19 Kojic acid.

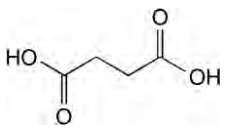

Fig. 20 Succinic acid.

Succinic acid is distributed widely through the natural world especially, in amber (3–8% by weight) and plant and animal tissues, and in microorganisms. It plays a significant role in intermediary metabolism (i.e., mainly in the TCA cycle (Fig. 2) and the glyoxylate pathway (Fig. 21).

Succinic acid is used for production of polymers, clothing fibers, plasticizers, solvents, paints, inks, food and feed additives, pharmaceuticals, perfumes, and an array of industrial and consumer products. Succinic acid is considered one of the main compounds of strategic importance as an intermediate for a multitude of applications, with the advantage that it could be produced from renewable raw materials

Succinic acid is synthesized as an intermediate of the TCA cycle (Fig. 2), operating under aerobic conditions, from α-ketoglutaric acid by α-ketoglutarate dehydrogenase. Succinic acid is an intermediate in the glyoxylate bypass (Fig. 21). Succinic acid is produced as an end product by the reductive TCA cycle (Fig. 3), under anaerobic conditions. While the biosynthesis of the acid through the first two aerobic pathways converts only four of the six carbons from glucose into the four-carbon succinic acid product, the anaerobic reductive TCA pathway produces two four-carbon acids for every glucose molecule utilized, due to the carboxylation reaction of phosphoenolpyruvate carboxylase. Therefore, anaerobic fermentations are preferred to aerobic fermentations for efficient and economical succinic acid production.

Succinic acid is produced mainly by the chemical catalytic hydrogenation of maleic or fumaric acids (about 15,000 t per year – see Table 1). Food-grade acid is probably produced through a fermentation and separation process. The market potential for products based on succinic acid (e.g., 1,4-butanediol, tetrahydrofuran, and adipic acid) is large, and, therefore, there is intensive ongoing research to develop an efficient biological route for its production.

Several biological routes to produce succinic acid are known but can as yet not compete with the chemical processes, mainly because the acid is a symmetrical molecule, similar to the fumaric acid production processes (see "Fumaric acid"). The first route is the bioconversion from fumarate to succinate, which is carried out by *Aerobacter aerogenes* strains, or by *E. coli* strains amplified for fumarate reductase. With the last bacterium, the bioconversion was shown to occur in the presence of glucose and under anaerobic conditions. A complete and rapid conversion of fumarate into succinate required high cell densities. It was found that the succinate molar yield (calculated from the utilized fumarate) was higher than 100%, suggesting that in addition to fumarate, glucose is converted to succinate. Production of succinic acid by fermentation from glucose was shown to occur by various anaerobic bacteria,

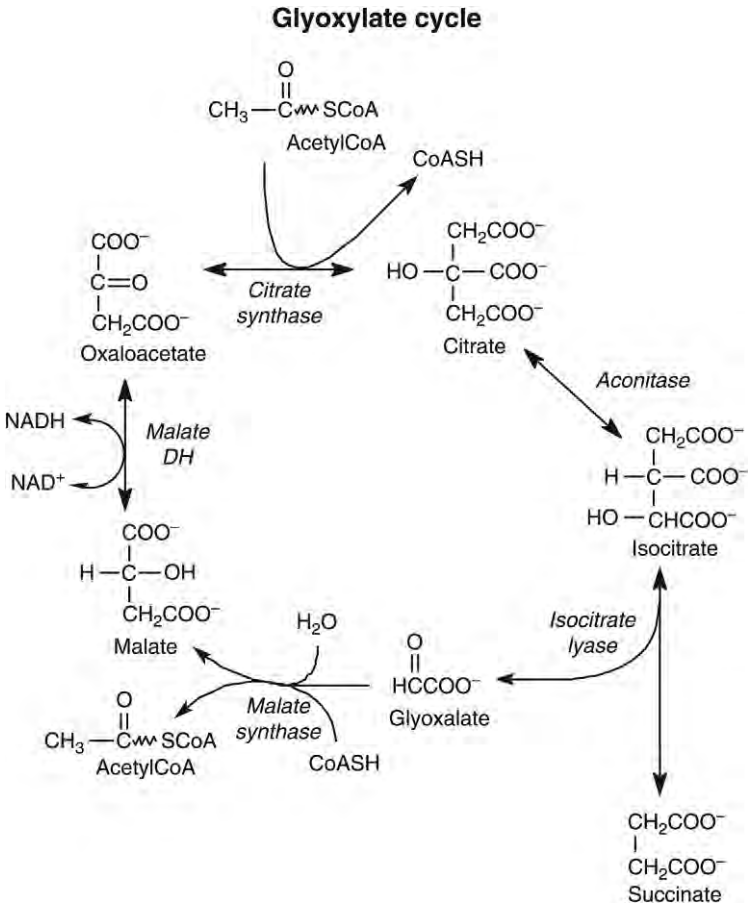

Fig. 21 The glyoxylate pathway. With permission from Dr. Paltel, R., Humboldt State University: CA, USA.

such as *E. coli*, *Enterococcus flavescens*, *Acinetobacter succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, and by the yeast *S. cerevisiae*. Succinic acid can be produced under aerobic conditions (see above) by *Fusarium* sp., *Aspergillus* sp., *Penicillium simplicissimum*, and *R. oryzae*, which excrete the acid but in rather low amounts and low yields. With *A. succinogenes* anaerobic fermentation, the succinic acid produced amounts to 110 g L^{-1}, with a yield value of 83–87% from glucose. Recent work that includes genetic engineering and immobilization techniques may improve the biological process to become competitive with the chemical process.

The industrial biomass derived production of succinic acid is well established as shown in Table 2 (reproduced with permission) where the estimates of production volumes for different companies are presented. Polybutylene succinate is one of the main predicted future products from succinate, to be used as an ecofriendly plastic, where the 1,4-butanediol is presently synthesized from acetylene and formaldehyde or from maleic anhydride. Succinic acid can also substitute for maleic anhydride as a precursor for the industrial production of butanediol , one of the large-scale chemicals that could then be derived by a biological process. The Myriant process (the largest producer listed in Table 2) as well as Reverdia use a genetically engineered yeast for succinic acid biosynthesis. In these yeasts, the reductive pathway of the TCA cycle for succinic acid accumulation have been amplified in the cytosol. In addition, since the yeasts are able to grow and produce at a lower pH (compared to the potential succinic acid producing bacteria), the free acid is formed. Accumulation of the free acid has a positive effect on the final manufacturing cost since the downstream process becomes much more straightforward. The BASF-Purac JV process is using *Bafisia succuniciproducens* with glycerol as the carbon source, whereas BioAmber initially used engineered *E. coli* but in their large-scale plants it was reported that a yeast is used. The Japanese company Ajinomoto has preferred to use the bacterium *Corynebaterium glutamicum* that was engineered to produce high succinic acid concentrations at microaerophilic conditions, with the resulting product being a salt. The reductive TCA cycle must be considered as more attractive since CO_2 is assimilated, considerably increasing the theoretical yield. It is a matter of finding the right organism with a natural or engineered reductive TCA capability and that does not produce significant amounts of byproducts and can produce succinic acid at a low pH.

5-Keto-D-Gluconic Acid

5-Keto-D-gluconic acid (5-KGA, $C_6H_{10}O_7$) (Fig. 22) was first obtained in 1940 by Stubbs and his colleagues, via a fermentation process.

The production from glucose of 5-KGA, and subsequently its conversion to 2-ketogluconic acid (2-KGA) (see "Ascorbic acid") (Fig. 23), is carried out by strains of the bacterium *G. oxydans*. Three different consecutive and membrane-bound periplasmic enzymes catalyze this conversion. The first enzyme is pyrroloquinoline quinine (PQQ)-dependent glucose dehydrogenase, which catalyzes the oxidation of glucose to D-gluconic acid. The second enzyme is a PQQ-dependent gluconate-5-dehydrogenase, which converts D-gluconic acid to 5-KGA. The third enzyme is a flavin-dependent gluconate-2-dehydrogenase, which converts 5-KGA to 2-KGA. This bacterium contains another set of these enzymes for the oxidation of glucose to the ketogluconic acids, which are soluble and NADP+-dependent enzymes. Since 2-KGA is an undesirable by-product in this fermentation, a mutant of *G. oxydans* was obtained in which the membrane-bound gluconate-2-dehydrogenase was inactivated. This mutant converted the available glucose almost completely (84%) into 5-KGA.

5-KGA can be converted effectively into L(+)-tartaric acid (see "L(+)-Tartaric acid"). The recent success in deciphering the genome of *G. oxydans* made it possible to develop a recombinant strain producing 5-KGA efficiently from glucose. This will enable the development of an economical biological process for tartaric acid, as compared to the existing process from argol, and thus increase its availability.

Table 2 Biosuccinic acid production capacity of commercial companies

Company	*Annual capacity (tans)*	*Plant location*	*Operational date*
BASF-Purac JV	50,000	TBA[a]	TBA[a]
BASF-PuracJV	25,000	Barcelona, Spain	2013
BioAmber-ARD	3,000	Pomacle, France	Full capacity by Q2 2012
BioAmber-Mitsui JV	65,000	TBA[a] (United States or Brazil)	TBA[a]
BioAmber-Mitsui JV	17,000	Sarnia, Ontario, Canada	2013
BioAmber-Mitsui JV	65,000	Thailand	2014
Myriant	13,600	Lake Providence, Louisiana	Q1 2013
Myriant: China National Bluestar	110,000	Nanjing China	TBA[a]
Myriant	77,110	Lake Providence, Louisiana	QI 2014
Myriant; Uhde (Owner and Operator)	500	Infraleuna site, Germany	H1 2012
Reverdia (DSM-Roquet te)	10,000	Cassano Spinola, Italy	H2 2012

Source: Reproduced from Kumar, R.V., Pakshirajan, K., Pugazhenti, G., 2016. Malic and succinic acid: potential C4 platform chemicals for polymer and biodegradable plastic production. In: Pakshirajan, K., Brar, S.K., Sarma, S.J. (Eds.) Platform Chemical Biorefinery, Future Green Chemistry. p. 174. With permission from Elsevier.

[a]TBA, to be announced.

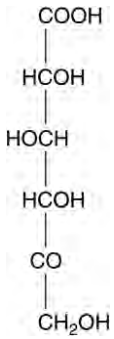

Fig. 22 5-Keto-D-gluconic acid (5-KGA).

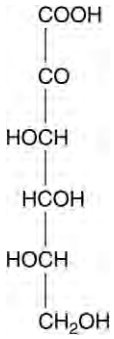

Fig. 23 2-Keto-D-gluconic acid (2-KGA).

2-Keto-D-Gluconic Acid

2-Keto-D-gluconic acid (2-KGA, $C_6H_{10}O_7$) (Fig. 23) is used commercially as an intermediate in the production of D-isoascorbic acid (a cheap isomer of L-ascorbic acid, which is absorbed in the body and has limited antiscorbutic activity). Its accumulation by different strains of *G. oxydans*, *Acetobacter pasteurianus*, *A. aceti*, *A. cerinus*, *Enterobacter intermedium*, and *Klebsiella aerogenes* has been described. 2-KGA can be produced at high concentrations with strains of the genus *Pseudomonas* and by *Serratia marcescens*. With the latter bacterium, a fermentation process was described that resulted in 95–100% of the theoretical yield of 2-KGA from glucose.

Acrylic Acid

Acrylic acid (2-propanoic acid, $C_3H_4O_2$), (Fig. 24) is the simplest unsaturated carboxylic acid. It has both a double bond and a carboxyl group linked to its C3, and these two functional groups enable its polymerization. In its pure form, acrylic acid is a clear, colorless liquid with a characteristic acrid odor. It is a major bulk chemical, being produced at over 3.4 million tonnes per year (2005, Table 1). It is miscible with water, alcohols, ethers, and chloroform. The esters and the salts of acrylic acid are collectively known as acrylates. Acrylates readily combine with themselves or other monomers (e.g., amides, acrylonitrile, vinyl, sterene, and butadiene) by reacting at their double bond, forming homopolymers (e.g., polyacrylic acid) or copolymers.

In 1843 Ferdinand Redtenbacher oxidized acrolein with aqueous silver oxide and isolated acrylic acid.

Acrylates are used in formulating paints and dispersions for paints, inks, and adhesives. Acrylates are used in manufacturing cleaning products, antioxidant agents, amphoteric surfactants, aqueous resins, and dispensions for textiles and papers. Methyl acrylate is used in making vitamin B1. Acrylate polymers have been successfully used in the manufacturing of hygienic products, detergents, and waste water treatment chemicals.

Acrylic acid is produced from propylene, a gaseous product of oil refineries, by a two-step gas-phase oxidation via carolein. This process has almost wholly replaced alternative technologies (i.e., acrylonitrile hydrolysis and the Reppe process). In the past decade, efforts have been made to use propane, which is more widely available and even lower priced than propylene, to produce acrylic acid.

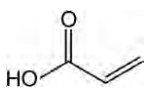

Fig. 24 Acrylic acid.

A combination of a sugar-based fermentation to produce an intermediate compound, which will then be converted by chemical catalysis to acrylic acid, provides multiple routes that may be feasible for the economical production of this acid (Fig. 25). Only one of the intermediates (e.g., β-alanine, methylcitric acid, methylmalonyl CoA, lactic, acid and 3-hydroxypropionic acid), lactic acid, is commercially available today.

Dehydration of lactic acid leads directly to acrylic acid. However, when chemical catalysts were used large amounts of side-products were formed. A biological conversion of lactic acid to acrylic acid was studied in *Clostridium propionicum*, but this process has not been implemented in industry. A possible pathway of interest is shown in Fig. 26, where 3-hydroxypropionic acid is produced at a theoretical yield of 100% from glucose and is then chemically converted in a cost-effective process to acrylic acid.

Another possibility is to use the enzyme nitrilase (produced by bacteria such as *Rhodococcus rhodochrous*, *Alcaligenes faecalis*, and *Klebsiella ozaenae*) to convert acetonitrile to acrylic acid. The highest accumulation of acrylic acid, using a periodic substrate feeding strategy, was 390 g L^{-1}, with almost 100% molar conversion yield.

An engineered *E. coli* strain is able to ferment glucose directly by combining the 3-hydroxypropionic acid pathway and a novel synthetic acrylic acid pathway, but toxicity of the product and low enzyme activities are still limiting the final concentration obtained (0.12 g L^{-1}).

Adipic Acid

Adipic acid (butane-1,4-dicarboxylic acid, $C_6H_{10}O_4$) (Fig. 27) is a white crystalline powder of C6-straight chain dicarboxylic acid, and is one of the most used chemicals in the world today (Table 1). It is slightly soluble in water and soluble in alcohol and acetone.

Adipic acid was first obtained by Dieterle and his colleagues in 1884 by oxidation of castor oil with nitric acid.

Adipic acid, despite its name (in Latin adipis is fat), is a product of the oxidative rancidity of fats (lipid peroxidation).

Adipic acid consumption is linked almost 90% to nylon (nylon-6.6) production by the polycondensation with 1,6-hexamethylenediamine. Adipic acid is used in manufacturing plasticizers, lubricant components, and polyester polyols for polyurethane systems. The acid and its derivatives are also used in the food industry (as gelling aid, acidulant, flavoring, leavening, and buffering reagents), and for the preparation of pesticides, dyes, textile treatment agents, fungicides, and pharmaceuticals (e.g., cephalosporin intermediates).

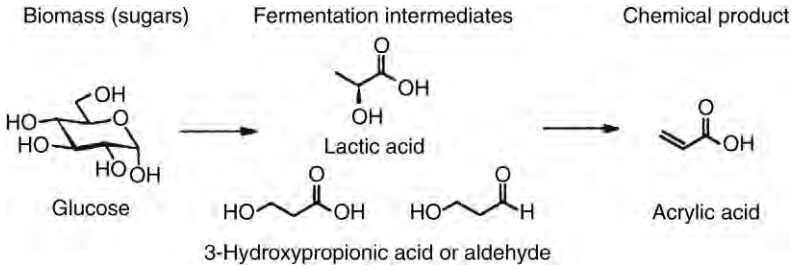

Fig. 25 Routes to acrylic acid from renewable carbon sources. With permission from Dr. Holladay, J., Pacific Northwest National Laboratory, WA, USA.

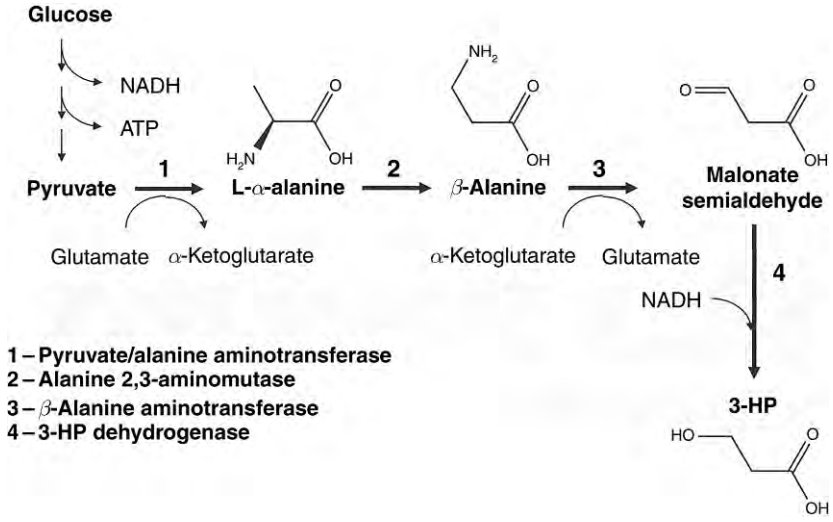

Fig. 26 Biosynthesis of 3-hydroxy propionic acid from glucose. With permission from Dr. Cameron, D., Koshla Ventures, CA, USA.

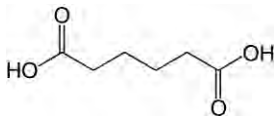

Fig. 27 Adipic acid.

Presently, almost all of the commercial adipic acid is produced from cyclohexane, obtained by the hydrogenation of benzene, through two sequential oxidation processes.

A plausible biological alternative can be the oxidation of cyclohexanol or cyclohexanone to adipic acid by strains of *Acinetobacter*, *Pseudomonas*, and *Xanthobacter*. The 14-kb gene cluster for cyclohexanol oxidation in Acinetobacter sp. contains genes encoding two alcohol dehydrogenases, an aldehyde dehydrogenase, a monooxygenase, a hydrolase, and a transcriptional regulator. A recombinant *E. coli* containing the 14-kb gene cluster was able to convert cyclohexanol to adipic acid. Theoretically, adipic acid can be produced from hexanoic acid (maybe by a *Pseudomonas* sp.), which is a product of anaerobic glucose fermentation by *Megasphaera elsdenii*. This bacterium metabolizes glucose or maltose into acetic, butyric, and hexanoic acids by serial condensations of C2 units produced from pyruvic acid. The yields of hexanoic acid are 5–8 g L^{-1} from 40 g L^{-1} glucose in a stirred batch fermentation. Other possibilities for the biological production of adipic acid include the production of minute amounts of the acid from aliphatic amines or diamines (e.g., dodecamethylenediamine) by *Nocardia* sp., and the conversion of straight chain monocarboxylic acids (e.g., myristic acid) into dicarboxylic acids including adipic acid by *Pichia carboniferous*. Both these biological processes are very inefficient and require substantial optimization to be competitive with the chemical process.

In the bacterium *Thermobifida fusca*, a robust thermophilic cellulolytic bacterium, it has been shown, that the use of the native occurring reversal of the adipate degradation pathway, resulted in accumulation of adipic acid with a final concentration of 2.23 g L^{-1} using glucose as the substrate. With a more natural C-source (corn cob) the final titer was 0.22 g L^{-1}. The engineering of *E. coli* for adipic acid production condensing acetyl-CoA and succinyl CoA was much less effective with a final concentration of only 0.0007 g L^{-1}.

L-*Trans*-2,3-Epoxysuccinic Acid

L-*trans*-2,3-Epoxysuccinic acid (ESA, $C_4H_4O_5$) (Fig. 28) is a rare natural acid and it was first isolated by Sakaguchi and his colleagues in 1939, from the culture broths of *Paecilomyces variotii* and *Talaromyces flavus*. Few other fungi (such as *A. fumigatus*, *A. clavatus*, *Monilia formosa*, and *Penicillium viniferum*) produce this compound.

ESA is hydrated to *meso*-tartaric acid by the enzyme fumarase from pig heart and by a hydrolase obtained from *Pseudomonas putida*. ESA can be used as a good starting material for the synthesis of optically active compounds such as specific single beta-lactam antibiotics. This epoxydicarboxylic acid can be converted into dialkyltin epoxysuccinates, which are important plasticizer stabilizers for polyvinyl chlorides as well as for cross-linkable epoxy-containing film-forming polyamides.

This acid may be regarded as an oxygenation product of fumaric acid (Fig. 14), whereby the oxygen molecule is incorporated to the double bond of fumaric acid to form the epoxide carbons. Optimization of the fermentation of *P. variotii* on glucose (12%, w/v) resulted in a 41% weight yield of ESA (or 61% of the theoretical 100% molar yield).

Gallic Acid

Gallic acid (3,4,5-trihydroxybenzoic acid, $C_7H_6O_5$) (Fig. 29) forms white, yellowish-white, or pale fawn-colored crystals of organic acid. It is soluble in alcohol and ether but its solubility in water is low. It melts at 250°C, and above this temperature gallic acid is

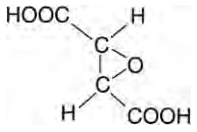

Fig. 28 L-*trans*-2,3-Epoxysuccinic acid.

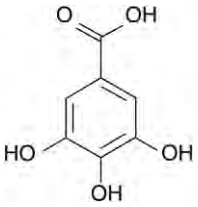

Fig. 29 Gallic acid.

converted into carbon dioxide and pyrogallol. The gallic acid molecule contains two functional groups, hydroxyl groups and a carboxylic acid group, and therefore two molecules of the acid can interreact to yield numerous esters and salts, including digallic acid.

Gallic Acid was Discovered by Carl Wilhelm Scheele in 1786.

Gallic acid is found in the leaves of bearberry, in pomegranate root bark, gallnuts, witch hazel, sumac, tea leaves, oak bark, and many other plants, both in its free state and as part of the tannin molecule.

Gallic acid is utilized in the ink industry, dye industry, food industry (antioxidants and preservatives), and, most importantly, in the pharmaceutical industry (as a raw material for the production of the hallucinogenic alkaloid, mescaline, and trimethoprim, a broad-spectrum antibiotic). Upon heating, gallic acid is converted to pyrogallol or 1,2,3-trihydroxybenzene, which are used in laboratories for absorbing oxygen, and in the production of azo dyes and photographic developers.

Gallic acid is produced chemically by the hydrolysis of tannic acid by sulfuric acid at 110–120°C.

Using the free and the immobilized filamentous fungi *R. oryzae* and *Aspergillus foetidus*, solid-state or submerged fermentation processes for the production of tannase (an inducible extracellular tannin acyl hydrolase) and the hydrolysis of tannic acid extracted from tara powder (a low-priced raw material), or tannin-rich substrates, were developed. Following the extraction of gallic acid (e.g., by diethyl ether), these processes resulted in good yield of gallic acid (up to about 95% of available tannic acid), with a minimum energy input and probably more economical, as compared to the conventional industrial acid hydrolysis process.

Fatty Acids

Fatty acids are monocarboxylic acids with a long hydrocarbon chain (having the general formula $C_nH_{2n}+1COOH$), usually linear, saturated or unsaturated acids, with an even number of carbon atoms. Most of the fatty acids in nature are combined, usually with glycerol, as triglyceride in fats and, thus, these are important components of lipids in plants, animals, and microorganisms. In this article, we will focus on two classes of fatty acids: short chain fatty acids (SCFA) and polyunsaturated fatty acids (PUFA), since these are, or may be, produced by microorganisms on an industrial scale.

Short Chain Fatty Acids

SCFA, also called volatile acids, are any of a large group of monobasic acids, especially those found in animal and vegetable fats and oils, with a chain length of 2–6 carbon atoms. The major SCFA are acetic, propionic, butyric, and valeric acids. These acids are the major end products normally produced by the bacterial fermentation of dietary carbohydrates and fiber polysaccharides in the colon. These acids are considered as improved dietary components that may have a significant role in protecting against diseases. Acetic, propionic, and *n*-butyric acids account for 83% of the SCFA produced. These acids can be used in fuel production, in addition to specific other uses for each of these acids (described below). The SCFA can be produced by an active and stable mixed culture of anaerobic bacteria (e.g., initially obtained from sewage sludge digestor effluent), which utilized H_2 and CO_2 (the major products from the gasification of a wide spectrum of fossil and renewable resources).

Butyric Acid

Butyric acid (*n*-butanoic acid, C4H8O2) (Fig. 30) is a carboxylic acid, which forms an oily colorless liquid that solidifies at 8°C and boils at 164°C. It is soluble in water, ethanol, and ether. Isobutyric acid (2-methylpropanoic acid) is an isomer of butyric acid and has similar chemical properties but different physical properties (i.e., it boils at 155°C).

Butyric acid was discovered in 1869 by Lieben and Rossi. In Latin, butyric acid means the acid of butter, as it was first discovered in rancid butter (butyric acid is hydrolyzed from the glyceride and causes a very unpleasant odor).

Butyric acid is a short chain saturated fatty acid found in the form of esters in animal fats and plant oils.

Butyric acid is utilized in the production of various butyrate esters (e.g., methyl butyrate), which have pleasant aromas and tastes and are used as additives in foods, perfumes, flavorings, varnishes, pharmaceuticals, and disinfectants. It is also used for the production of plastics, plasticizers, surfactants, and textile auxiliaries. Butyric acid and its derivatives (e.g., tributyrin) are also being considered as potential anticancer agents; the acid induces cytodifferentiation of a wide variety of neoplastic cells.

Butyric acid is synthesized under anaerobic conditions from acetyl CoA, obtained from the degradation of fatty acids, or from pyruvic acid via a unique reaction catalyzed by pyruvate-ferredoxin oxireductase, which forms acetyl CoA, CO_2, and H_2 (Fig. 31). Acetyl CoA is converted to butyryl CoA, which is then converted to butyric acid. Three molecules of ATP are produced per each

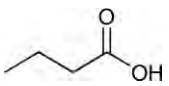

Fig. 30 Butyric acid.

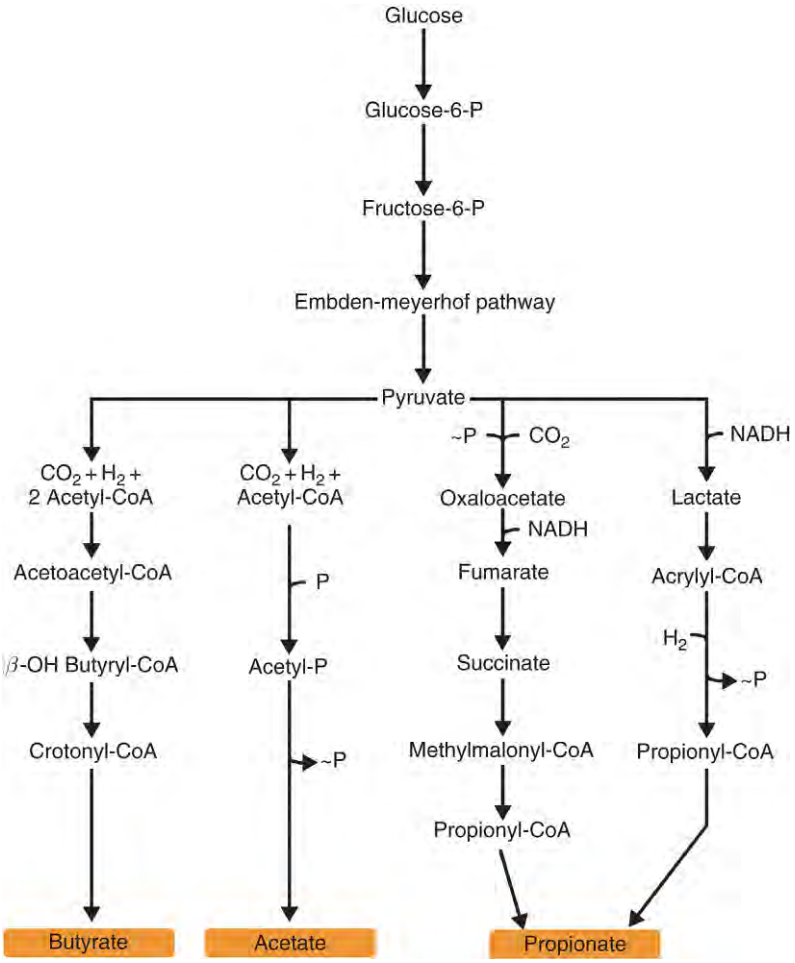

Fig. 31 Biosynthesis of acetic, butyric, and propionic acids from glucose.

molecule of utilized glucose during this anaerobic fermentation, as compared to 2 mol of ATP produced during the production of lactic acid and/or ethanol, and 4 mol of ATP produced during the anaerobic production of acetic acid.

Butyric acid can be prepared by the chemical oxidation of butyraldehyde which has been obtained from propylene by oxosynthesis. However, the butyric acid obtained this way cannot be regarded as a natural product and, therefore, is not used in the food industry. The acid can be extracted from butter (containing 2–4% of acid) but this method is too expensive. Butyric acid is produced as end product of fermentation of sugar by obligate anaerobic bacteria, first discovered in 1861 by Pasteur; *Clostridium butyricum* being the most prominent and main bacterium, but other organisms such as *C. kluyveri*, *C. beijerinckii*, *C. barkeri*, *C. acetobutylicum*, *C. thermobutyricum*, *C. thermopalmarium*, *C. pasteurianum*, *Butyribacterium* sp., *Sarcina* sp., *Megasphera* sp., *Fusobacterium nucleatum*, *Peptococcus asacelarolyticus*, *Butyrivibrio fibrisolvens*, *Pseudobutyrivibrio ruminis*, and *Eubacterium limosum* are also used. Optimal cultivation conditions are 35–37°C, an atmosphere of pure CO_2, N_2, and a pH range of 4.5–7.0. The *Clostridia* can utilize hexoses, several pentoses, and polysaccharides. Butyrate production is favorable in a fed-batch, glucose-limited, and slow-growing culture. It is also stimulated under phosphate and nitrogen limitation. Batch, fed-batch, or continuous processes combined with cell recycle or immobilization are applicable for these anaerobic fermentations. Inhibition of the fermentation caused by the butyric acid formed can be avoided by the on-line removal of the acid by various methods (e.g., distillation and evaporation, permeate electrodialysis, and adsorption). Butyric acid can also be removed by extraction, or by extraction combined with simultaneous stripping of the organic phase (liquid membrane) into the second aqueous phase (pertraction).

The amount estimated in 2013 for the volume of butyric acid produced is 80,000 t per year, made by chemical synthesis by oxidation of *n*-butyraldehyde, synthesized from propylene. To allow for an economical biological process efforts to use biomass (lignocellulose) both with mixed and axenic cultures (*Clostridium*) are under active investigation. All known fermentation set ups, ranging from batch and fed-batch to continuous fermentations with and without cell recycling, have been evaluated for butyrate production. The concentrations achieved are still too low to be competitive with the chemical synthesis. Use of immobilized adapted *Clostridium tyrobutyricum* in a fibrous bed reactor allowed for high concentrations of butyric acid to accumulate (86.9 g L^{-1}). There is still a need to further increase the butyrate tolerance in the prospective producing strains. Butanol, the alcohol derivative of butyric acid, is considered one of the future biomass fuels. Butyric acid may be used as a precursor for butanol manufacture since the

butyric acid tolerance is at least 5 times better in the producing organisms compared to the concentration of butanol that is tolerated by its producing organisms.

Propionic Acid

Propionic acid (propanoic acid, $C_3H_6O_2$) (Fig. 32) is a naturally occurring three-carbon carboxylic acid. In the pure state, it is a colorless, water soluble, and corrosive liquid with a sharp, somewhat unpleasant odor.

Propionic acid was first described in 1844 by Johann Gottlieb, who found it among the degradation products of sugar. Its name is derived from the Latin words protos and pion, which mean first and fat, respectively.

Propionic acid is used in the manufacture of herbicides, fine chemical intermediates, rubber chemicals, emulsions, and environmentally friendly solvents for coating formulations, artificial fruit flavors, pharmaceuticals, and modified synthetic cellulose fibers (e.g., cellulose acetate propionate). Propionic acid inhibits growth of mold and various bacteria and is used as a preservative for food (especially bread and other baked goods as its sodium or calcium salts), animal feed (directly or as its ammonium salt), and grains.

Propionic acid is biosynthesized as propionyl CoA, which is a product of the catabolism of fatty acids having odd numbers of carbon atoms, and of the degradation of some amino acids. Propionic acid bacteria (e.g., *Propionibacterium shermanii*) produce this acid as a product of their anaerobic metabolism of sugars. The initial catabolism of glucose to pyruvic acid follows glycolysis, and the NADH formed is oxidized via a pathway by which propionic acid is produced. Pyruvic acid accepts a carboxyl group from methylmalonyl CoA by a transcarboxylase reaction, leading to the formation of oxaloacetic acid and propionic acid (Fig. 31). *Propionibacterium* sp. (and other bacteria such as *C. propionicum*, *M. elsdenii*, and *Prevotella ruminicola*) also ferments lactic acid with the production of propionic acid, acetic acid, and CO_2 (Fig. 31).

Commercial production of propionic acid is mainly carried out via chemical synthesis from petroleum feedstocks by three process routes: ethylene hydroformylation/oxidation, carboxylation of ethylene with carbon monoxide and water, and direct oxidation of hydrocarbons. Propionic acid is also produced, in minor quantities, as a by-product of acetic acid manufacturing.

Fermentation is an attractive route to produce this acid from renewable sugars (such as glucose, xylose, maltose, sucrose, and lactose) by propionic acid bacteria (e.g., *P. shermanii* and *P. acidipropionici*). These bacteria play important roles in the dairy industry in the development of the characteristic flavors and holes (or eyes) production (by CO_2 evolution) in Swiss-type cheese. However, this fermentation process is inefficient and only barely competes with the chemical processes for the production of propionic acid. The fermentation parameters that affect the cost of the fermentation process are the low rates and the maximal amounts of the product, and the formation of undesirable by-products in addition to propionic acid. Thus, only small amounts of this acid are produced by fermentation of whey and are used as a natural product in foods to adhere to the labeling rules of natural foods. Since the genome sequence of *Propionibacterium freudenreichii shermanii* ATCC9614 is being almost completed, it may be conceivable that the fermentation route could become economically valuable. Propionic acid can also be obtained by oxidation of propanol with *G. oxydans*, in a fed-batch fermentation process, resulting in 56 g L^{-1} of the sodium propionate (the yields of the acid obtained are near the theoretical values) after 70 h. Since the bacterium cannot grow on propanol as a sole carbon source, but is able to convert it to propionic acid, glycerol was used as an assimilatable cosubstrate.

The biological production of propionic acid has not been realized due to the low productivity, yield and product purity obtained; even with metabolically engineered strains of *Propionibacterium* it cannot challenge the chemical process. The experiments described in the public space are at a small scale, either shake flasks or small laboratory fermentors. One report describes the use of a $10m^3$ vessel where 47.3 g L^{-1} propionic acid was obtained after 240 h in a glycerol fed-batch culture fermentation. Glycerol is considered an ideal substrate for the production of this acid since glycerol is more reduced than glucose and therefore beneficial in the production of reduced chemicals. Use of glycerol also reduces the accumulation of acetic acid a common by-product when glucose is used as a carbon source.

Valeric Acid

Valeric acid (pentanoic acid, $C_5H_{10}O_2$) (Fig. 33) is a straight, saturated chain, alkyl carboxylic acid. It is a colorless, oily liquid with a very unpleasant odor of stale cheese. The acid exists in four isomeric forms, one of which contains an asymmetric carbon atom and consequently occurs in two optically active modifications and one optically inactive modification.

Valeric acid is found naturally as free or as esters in the vegetable and animal kingdoms.

Valeric acid, and its esters, is mainly used in perfumes and cosmetics, as food additives because of the fruity flavor of the esters, and as plasticizers and pharmaceuticals.

Valeric acid can be extracted by boiling water or soda from the roots of *Angelica archangelica* and *Valeriana officinalis* (from which valeric acid gets the name). The acid can also be obtained by oxidizing fermentation amyl alcohol with chromic acid. The acid can be

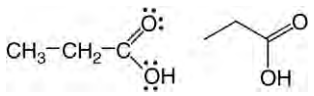

Fig. 32 Propionic acid.

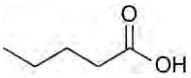

Fig. 33 Valeric acid.

formed by microorganisms during the anaerobic fermentation of CO_2 and H_2 (discussed above) and by the fermentation of other carbon sources, such as wastewater solids.

Polyunsaturated Fatty Acids

PUFA are fatty acids in which more than one double bond (generally separated by a single methylene group) exists within the representative molecule. The term PUFA is used only for acids with three up to six double bonds as those found in fish oil or brain tissue. These acids (also called essential fatty acids (EFAs)) are necessary fats that humans cannot synthesize, and must be obtained through their diet. There are two families of EFAs: omega-3 and omega-6 fatty acids, in which the position of the first double bond, from the terminal methyl group of the molecule, is indicated by the number following 'omega'.

There is a great variety of polyunsaturated fatty acids, and here we will discuss those that are of commercial interest and possible to produce by microorganisms, namely, arachidonic acid, $C_{20}H_{32}O_2$ (ARA) (Fig. 34); γ-lineolenic acid, $C_{18}H_{30}O_2$ (GLA) (Fig. 35); and docosahexaenoic acid, $C_{22}H_{32}O_2$ (DHA) (Fig. 36).

Microbial lipid production is of interest when the desired fatty acids are difficult to obtain from plant or animal oils. The formation of fatty acids for commercial purposes is called single-cell oil (SCO). Prokaryotes are known to form a variety of fatty acid structures including, branched chains, cyclopropane rings, and *trans*-double bonds, whereas eukaryotes only form straight chains with *cis*-double bonds. The fatty acids of interest (ARA, GLA, and DHA) are all straight chains containing solely *cis*-double bonds and with methylene interruption ($CH{=}CH{-}CH_2{-}CH{=}CH$). Most microorganisms do not accumulate triacylglycerol lipids and their main lipid is in the form of phospholipids. Microorganisms that do accumulate more than 20% of their dry weight as triacylglycerol lipids are called oleaginous and are of commercial interest. Fatty acids are important for both structure and storage, and also as precursors for a range of cell signaling molecules, involved in inflammatory responses, blood coagulation, and reproductive function. ARA and DHA are of particular interest, since they are common in neural tissues both of the eye and of the brain. They are both present in human breast milk and are added to infant formula in more than 60 countries, with beneficial effects on visual and intellectual development.

In 1985, the first commercial process growing the fungus *Mucor circinelloides* for SCO was started in Japan. The fungus was grown in stirred-tank fermentors followed by extraction of the oil and final refinement and purification. The product was rich in GLA (18–20% of the oil) and was produced for 6 years when the introduction of plant-derived 'borage oil' became a cheaper source of GLA. γ-Linolenic acid is now obtained primarily from the seed of the evening primrose plant.

In case the dietary requirement is for a single acid, this is best met by microbial oil, which has lead to renewed commercially successful production from 2000. Three organisms are in use for DHA production, *Crypthecodinium cohnii*, *Schizochyturium* sp., and *Ulkenia* sp., all grown by submerged fermentations. ARA was shown to be a major component of the oil from *Mortierella alpine*. Various strains of *M. alpina* are used in processes in Europe, China, and possibly also in Japan.

PUFA materials are introduced as dietary supplements for adults especially as DHA-rich oils, to help prevent coronary heart problems. There are also suggestions that DHA may be useful as a dietary supplement to prevent the onset of degenerative disorders such as Alzheimer's disease.

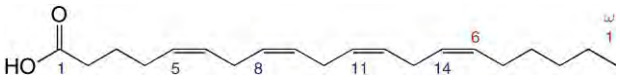

Fig. 34 Arachidonic acid (ARA).

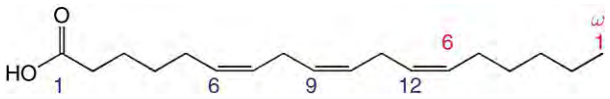

Fig. 35 γ-Linolenic acid (GLA).

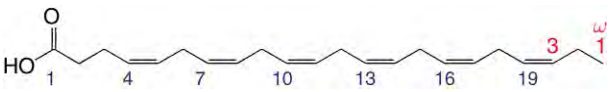

Fig. 36 Docosahexaenoic acid (DHA).

The commercial production of PUFA is profitable, with an estimated production volume of over 3000 t of DHA produced annually from either fungi or algae. Already in 1985–1990 gamma linolenic acid was produced with the filamentous fungi *Mucor circinellloides* by J&E Sturge in 220 m^3 fermentors. However, the fungal product was outcompeted in a price war by the plant derived Evening Primrose Oil. Some of the companies known to be involved in the production of PUFAs today are among others the Dutch company DSM using the Martek developed technology with a combination of DHA from the microalgae *C. cohnii* and ARA from the filamentous fungi *Mortierella alpina*. The mixture of the two acids inhibits the degradation of DHA that occurs if ARA is not present. CABIO a subsidiary of Cargill operating in China also utilizes a strain of *Mortierella alpina* and the Swiss company Lonza using the algae *Ulkenia*. At DuPont the yeast *Y. lipolytica* engineered with at least 15–20 genes is able to accumulate eicosapentaenoic acid that has obtained a GRAS status from the FDA.

Conclusions

As is evident from this article, there is a strong competition, based mainly on economical considerations, between the chemical and the fermentation industries to produce organic acids. At the present time, chemical processes, being more economical than the fermentation processes, are the existing industrial manufacturing processes not only for symmetrical molecules (e.g., fumaric acid and succinic acid) but also for asymmetrical molecules (e.g., malic acid). Unlocking the biochemical networks and the regulatory mechanisms of the producer strains, and implementing modern technologies of genetic and metabolic engineering (e.g., global transcription machinery engineering, systems biology) may provide an opportunity for their manipulation by amplifying or deleting critical steps in metabolism to improve acid production or to overproduce novel acids. In addition, the newly emerging information will allow the efficient production of acids (e.g., L-lactic and succinic acids) by a better suitable and specifically tailored microorganism (e.g., *A. niger*) under different production conditions (e.g., microaerophilic or aerobic instead of anaerobic conditions). These approaches may be a major breakthrough in the scientific methodology that can improve considerably the economy of industrial processes employing biological systems for the production of acids.

The use of metabolic engineering and omics technologies allow for fundamental changes in both natural producing organisms as with platform organisms. In some cases, it has been possible to show proof of concept where novel acids are produced by former non-producers. The challenge that remains is to optimize the conditions to allow for concentrations that can be competitive with the current chemical processes.

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Origin of Life, Theories of

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There are many facets to the problem of understanding life's origin and equally many ways to address it. The origin of life can be viewed from a variety of different standpoints: information theory (Yockey, 2005), RNA replication (Eigen and Schuster, 1977), meteorite impacts (Brack, 2009), physics (Smith and Morowitz, 2016), specific chemical synthesis (Powner et al., 2009), geochemistry (Martin and Russell, 2003), or entropy (Russell et al., 2013), to name a few. Common to all current theories for the origin of life is the view that some kinds of molecules, probably similar to nucleic acids, were able to replicate and undergo selection for the ability to replicate prior to the advent of fully fledged free living cells.

When it comes to addressing the origin of life as we know it, as opposed to considering forms of life that are imaginable, it is helpful to connect ideas on the origin of life with the biology of modern microbes. This constrains the thoughts on the topic from becoming too theoretical or otherwise decoupled from life (that which is to be explained). The properties of life and the nature of living things focus thoughts about how or where life might have been arisen. Understanding which forms of life among the many that we know are likely to be the most primitive is also important in understanding life's origin, because it helps to narrow down the many possibilities when it comes to trying to narrow the gap between spontaneous chemical processes on the early Earth and biological processes in microbes with primitive metabolism. In the literature on the origin of life, four attributes of life recurrently come up as central to the issue: compartmentation, information, replication, and energy.

Compartmentation

Because all life that we know is organized as cells, there is every reason to assume that the first forms of life were also cellular. It also means that in the time before there were fully fledged free-living cells, there were simpler entities that were not capable of independent replication in the wild (Gantí, 1975). Compartmentation is important for two reasons. First, whatever chemical synthesis one has in mind for the synthesis of life's building blocks, some kind of concentrating mechanism or barrier has to exist such that the products of synthesis do not diffuse off into the oceans (Kuhn, 1972). Second, for any form of evolution to take place among replicating molecules, the population of molecules has to be structured in some manner in order to permit selection to act. That is, selection for new populations of molecules with new and different properties, for example, a catalytic activity or efficiency of replication, cannot occur unless the molecular populations are distinct. In an unstructured population of molecules, the fastest replicators, also called parasites, will prevail (Branciamore et al., 2009). There are various thoughts about how early molecular systems might have been compartmentalized from the environment to permit concentration and selection, these include evaporation on land, freezing in ice (eutectic mixtures), or naturally forming inorganic compartments at hydrothermal vents (Martin and Russell, 2003).

Information

All forms of life have the universal genetic code. It is therefore reasonable to assume that a form of information processing that involves the modern genetic code was also present in the very first forms of life as well. That is consistent with the observation that the genetic code is one of the oldest and least modified traits of life as we know it (Wong, 1975). The genetic code is quite complex. It requires the accurate loading of coding amino acids onto tRNA, tRNA–mRNA interactions mediated by the ribosome (and ribosomal RNA). It is likely that earlier forms of the code were simpler than the modern code, using a two letter code, for example, rather than three letter triplets. The issue of how the code arose is an unresolved problem (Koonin and Novozhilov, 2009), although theories strive to link the origin of the code to metabolism by suggesting that the amino and oxygen moieties in the bases of the tRNA anticodon provided catalytic activity that synthesized the cognate amino acid on the tRNA acceptor stem, as opposed to linking preformed amino acids with tRNA (Copley et al., 2007).

Replication

Replication has long been viewed as one of the most important aspects at life's origin. For anything to evolve in a Darwinian sense, it must generate copies of itself that differ to some degree from the original (natural variation) and exhibit properties that would permit their differential success in a proliferation sense (natural selection). Since the pioneering work of Sol Spiegelman and Manfred Eigen with a viral enzyme called Qβ replicase, replication of RNA has stood in the foreground of thinking on the issue (Spiegelman, 1965; Eigen, 1971). RNA can be replicated (like DNA) and can exhibit catalytic activity (proteins) making it attractive as a precursor to both in evolution. Eigen's work in particular demonstrated the differential success of different RNA templates to replicate in vitro. That established the concept of natural variation and natural selection among molecules prior to the origin of variation and selection among cells. This aspect, in addition to replication plus catalysis, gave rise to the concept of evolution in an

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RNA world that preceded the evolution of cells (Gilbert, 1986). RNA evolution experiments are widely conducted under laboratory conditions (Lincoln and Joyce, 2009). However, on the early earth, the chemical environment was harsh. There were only rocks and water, there was the constant threat of diffusion into the ocean, UV light was abundant, as was constant meterorite impact and widespread volcanic activity. Such conditions have little if any similarity with modern laboratory conditions, a circumstance that acts as a kind of watershed in origin of life theories as concerns the role of RNA. Proponents of the RNA world point to the experimental accessibility of RNA replication as a model for early evolution, while geochemists point to the lack of known environmental conditions that would allow an RNA world to arise or evolve.

Part of the replication aspect of origins concerns speed. The fastest replicators will incorporate all environmentally available monomers into polymeric likenesses of themselves, thereby greedily consuming all available resources. Hence they will survive much better and dominate under molecular selection schemes than other sequence variants (stability to hydrolysis being equal among different sequences). But a fast replicating sequence variant might mutate so as to generate even faster variants, in which case they would outcompete the slower ancestor and follow an evolutionary trajectory toward the fastest replicating sequence. Once that locally optimal, fastest replicating sequence has evolved, mutational variants drift away from the optimum and generate slower replicating progeny while cluttering the sequence space (the sum of all possible sequence variants around the fastest replicator). That concept of a locally optimal replicator and its less optimal variants was developed by Eigen in the context of replication; such a replicator together with its variants is called a quasispecies (Eigen and Schuster, 1977).

Energy

Perhaps most essentially, all forms of life harness one or more sources of energy that are available in the environment. No form of replication or selection or evolution among either molecules or cells can take place without energy release hence energy supply, as stipulated by the second law of thermodynamics. As a consequence of that constraint, we can be rather certain that prior to the advent of biological cells, prebiotic chemical reactions had to occur spontaneously. All spontaneous chemical reactions entail, in turn, an overall exergonic reaction that supplies the free energy change needed for the reaction to take place (Thauer et al., 1977). This does not mean that every single reaction in prebiotic evolution had to be exergonic. Many reactions in modern metabolism are endergonic. But endergonic reactions are necessarily coupled to a main energy releasing reaction that the cell harnesses in order to drive metabolism and the life process forward. In modern metabolism, the most widespread form of coupling is through adenosine triphosphate (ATP). ATP hydrolysis releases energy, such that endergonic reactions can be coupled (enzymatically) to ATP hydrolysis such that the overall reaction is energetically downhill and will thus go forward (Sousa and Martin, 2014). There are other forms of energy currencies in metabolism besides ATP, these include thioesters (Goldford et al., 2017), acyl phosphates (Schönheit et al., 2016), and reduced ferredoxin, a small soluble iron–sulfur cluster containing protein that transfers low potential electrons in metabolism (Eck and Dayhoff, 1966). These currencies are synthesized in what biologists call energy metabolism (the main bioenergetic reaction of the cell), and are used to drive other reactions forward. At the origin of life, there must have been some sort of energy supply that drove the synthesis of organic compounds forward to a level where complex polymers (peptides and nucleic acids) were formed. Among the possible sources of environmental energy most often discussed are ultraviolet light, lightning, heat, and chemical energy (Kaufmann, 2009). If we look around among anaerobic chemoautotrophic microbes, only chemical energy is harnessed for energetic coupling.

As seen from the standpoint of biology, life is a side product or a byproduct of the main energy releasing (exergonic) reaction that serves to drive all other reactions in the cell forward. Put more succinctly, life is an exergonic chemical reaction. The synthesis of the very first chemical components of which cells are composed, often called the building blocks of life, must also have been the products of exergonic reactions, because the second law of thermodynamics stipulates that chemical reactions can only go forward if the overall reaction releases energy.

Molecular Networks

Living cells are side products of a main energy releasing reaction. Stated another way, metabolism generates life, not vice versa; the main flux of carbon, energy, and nutrients through the cells generates the substance and organization of cell mass. For example, acetogens channel 24 molecules of CO_2 through the cell as waste product (acetate) for every molecule of CO_2 that they incorporate into cell carbon (Daniel et al., 1990). One of the longstanding puzzles in the origin of metabolism is the emergence of biochemical networks. Recent progress on that topic has come from the investigation of autocatalytic networks (Hordijk and Steel, 2004; Hordijk et al., 2012), which have shown that the elements of a chemical network need not provide much catalysis for other steps in the network in order for flux through the system, hence net synthesis of the system itself, to increase. In the laboratory, chemical networks with oscillatory properties have been constructed, using thioesters as the chemical energy source (Semenov et al., 2016).

Two Main Theories for Carbon

As recently summarized elsewhere (Schönheit et al., 2016), a convenient structure to the problem is obtained if we sort theories on the origin of life into two categories based upon the assumptions they entail regarding the nature of carbon metabolism in the

earliest cells. By that criterion, theories about the earliest phases of evolution fall into two main categories: autotrophic origins versus heterotrophic origins. Theories for autotrophic origins posit that the first cells fulfilled their carbon needs from CO_2. Heterotrophic origin theories are based on the idea that the first cells lived from the fermentations of reduced organic compounds present in some kind of rich organic soup. The main contours of the two theories as they interface with biology can be summarized briefly.

Heterotrophic Origins

Heterotrophic origin theories are traditionally favored by chemists (Bada and Lazcano, 2008; Miller and Orgel, 1974; Orgel, 2008). Seen from a biological perspective, heterotrophic origin theories have two main drawbacks. First, they do not connect with the chemistry of modern cells. They start with cyanide (Levy et al., 1999), formamide (Saladino et al., 2012), or UV light-dependent (Ritson and Sutherland, 2013) condensations. The conditions underlying heterotrophic origin theories are sometimes viewed by geochemists as unlikely to ever have existed on early Earth (McCollom, 2013). Organic compounds delivered from space are seen as a source of carbon for the first cells in some heterotrophic origin theories, however, carbon from space is an unfermentable substrate and is furthermore chemically too heterogeneous to have served as a carbon source for the first cells (Schönheit et al., 2016).

Autotrophic Origins

Autotrophic origin theories, by contrast, generally operate with chemicals that undoubtedly did exist on the anaerobic early Earth: H_2, CO_2, N_2 and H_2S. Transition metals and transition metal sulfide (FeS and FeNiS) centers play a crucial role in autotrophic origin theories for several reasons. First, FeS and FeNiS centers serve as essential catalysts in the modern day core carbon and energy metabolism of anaerobic autotrophs, as such they provide direct links between the origin of life and modern cells (Eck and Dayhoff, 1966). Second, FeS and FeNiS minerals abound in anaerobic geological settings both today and on the early Earth (Wächtershäuser, 1992). Third FeS and FeNiS centers are naturally catalytic by virtue of their unfilled d and f electron orbitals, which can readily hybridize to generate metastable bonds with carbon and nitrogen. Under the theory of autotrophic origins the first cells satisfied their carbon needs from CO_2 (Schönheit et al., 2016).

Serpentinization

In recent years, geochemical processes in modern hydrothermal vents have been characterized that resemble biological processes germane to autotrophic origin theories. This development can perhaps be best summarized with the main keyword "serpentinization" (Russell et al., 2010). During serpentinization, water is drawn by the force of gravity into cracks in the Earth's crust. At depths of one to several kilometers and temperatures around 200°C, water reacts with Fe^{2+} in the iron–magnesium silicates that comprise the crust. Water is thereby reduced to generate molecular H_2. The effluent of hydrothermal systems can contain up to 10 vol% or more H_2 (Etiope et al., 2011). H_2 is a useful currency of chemical energy for many modern microbes, in particular for anaerobic autotrophs.

Geochemists have also discovered a modest variety of chemical reactions that are occurring naturally at hydrothermal vents today that entail the synthesis of methane (CH_4) and other small molecular weight carbon compounds such as formate and short chain hydrocarbons within hydrothermal systems (Schrenk et al., 2013; McDermott et al., 2015; McCollom, 2016). Methane can be present in the effluent of hydrothermal vents at up to 2 mmol kg^{-1} (Schrenk et al., 2013) and can constitute up to 87 vol% of the vent gas emission (Etiope et al., 2011), hydrocarbons in much lower amounts, formate has only been reported for some systems (Schrenk et al., 2013). The mechanism of methane synthesis in hydrothermal systems is still uncertain (McCollom, 2016). It might entail reduction of dissolved CO_2 or of rock-bound carbonate. Whether H_2 or reduced transition metal species in the crust are the source of electrons for CO_2 reduction is not known.

Some modern microbes called methanogens belonging to the domain archaea synthesize methane from H_2 and CO_2 or C_1 compounds (Thauer et al., 2008; Mayumi et al., 2013). To determine whether the methane produced in hydrothermal vents is of biological (methanogenesis) or of geochemical (serpentinization) origin, geochemists use stable isotope ratios that involve measuring the $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios of the respective compounds. Such measurements cannot be fully explained here; let it suffice to say that the $^{13}C/^{12}C$ and $^{2}H/^{1}H$ isotope signatures of methane produced by biological and geological processes can be distinguished, geochemically produced methane being typically enriched in the heavier carbon isotope and depleted in the heavier hydrogen isotope relative to biogenically produced methane (Etiope et al., 2011; Pedrera et al., 2016). In laboratory scale serpentinization reactions, acetate and formate synthesis has been reported (Miller et al., 2017).

Ancient Microbial Lineages

Various lines of evidence suggest that anaerobic autotrophs are the most ancient lineages among microbes known today. Modern anaerobic autotrophs thrive upon H_2, which is continuously generated in serpentinizing geological settings at activities of the order

of 10 mmol kg^{-1} or more via disequilibria driven by rock–water interactions in hydrothermal systems. At such high H_2 activities, and under strictly anaerobic conditions, the synthesis of cell mass from CO_2 is thermodynamically favorable. The core pathway of carbon and energy metabolism in anaerobic autotrophs that inhabit such hydrothermal and deep crust environments is the acetyl-coenzyme A (CoA) pathway, the most ancient of the six pathways of CO_2 fixation known (Fuchs, 2011) and the only one present in archaea and bacteria.

The observation that spontaneous exergonic organic syntheses from H_2 and CO_2 occur today at hydrothermal vents suggests that these processes are similar, if not homologous (Martin, 2012), to core energy-releasing reactions of carbon and energy metabolism in methanogens (Thauer et al., 2008) and acetogens (Schuchmann and Müller, 2014), which live from the reduction of CO_2 by H_2. Chemists are less taken by the similarity of the overall exergonic processes and tend to doubt in rather general terms that there are any traces of ancestral metabolic processes retained in any modern metabolic pathways (Orgel, 2008). However, the evidence is fairly solid that microbial communities have been thriving in hydrothermal vents for over 3.3 billion years (Westall et al., 2012). From the biological standpoint, there is no strong a priori reason to assume that the energy releasing reactions from which those ancient microbes harnessed energy were fundamentally different from those that modern anaerobes living in the same environment use (Martin and Sousa, 2016). From the geochemical standpoint, there is no clear indication that early biological processes were fundamentally different from those still existing today in anaerobic environments (Arndt and Nisbet, 2012). Investigations of microbial genomes support the view that the first cells lived from H_2 and CO_2 and that acetogens (bacteria) and methanogens (archaea) are among the most primitive prokaryotic lineages currently known (Weiss et al., 2016).

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Outer Membrane, Gram-Negative Bacteria

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Glossary

***β*-barrel** A protein-folding pattern in which an extensive β-sheet structure closes upon itself, producing a cylinder or a barrel structure.
Bile salts Amphiphilic derivative of cholesterol secreted in bile and functioning as detergents for the digestion of fat in the intestinal tract. Conjugated bile acids often have strongly acidic groups (for example from taurine).
Kdo An acidic, eight-carbon sugar 2-keto-3-deoxy-octonate or 3-deoxy-D-*manno*octulosonate.
Lipid bilayer An ordered two-layered arrangement of polar lipids so that their lipophilic portions associate in the center and their polar head groups face the outside.
Lipopolysaccharide (LPS) A complex polymer comprising the outer leaflet of the outer membrane bilayer. Its polysaccharide portion acts as a major antigen, and its lipid portion (lipid A) is responsible for its endotoxic activity.
Molecular chaperone A protein that helps the folding of nascent proteins by binding to unfolded or misfolded polypeptides.
Peptidoglycan (murein) An extensively cross-linked structure unique to bacteria, preventing the bursting of the cytoplasm by acting as a mechanically strong cage.
Periplasm The space between the cytoplasmic membrane and outer membrane, containing peptidoglycan, membrane-derived oligosaccharides (MDO) and many unique proteins.
Porin An outer membrane protein that allows the non-specific transmembrane diffusion of small, hydrophilic solutes.
R LPS An incomplete lipopolysaccharide containing only the lipid A and the core portion of the saccharide chain, devoid of the O chain. So named because it is produced by mutants that show a "rough" (R) colony morphology.
SecYEG A multiprotein export apparatus in bacteria, responsible for the transmembrane export of most proteins, including periplasmic and outer membrane proteins.
Sigma factor A subunit of bacterial RNA polymerase that recognizes a specific set of promoter sequences.
Toll-like receptor Transmembrane receptors on animal cell surface, which recognize various common components of microbial pathogens. So named because of its similarity to the Toll receptor in *Drosophila*.
Two-component system A signal transduction pathway widespread in bacteria, which consists of a histidine kinase sensor and a response regulator that becomes activated by the phosphorylation of its aspartate residue.

The Cell Envelope of Gram-Negative Bacteria

The cell envelope of gram-negative bacteria contains, in addition to an inner, cytoplasmic membrane, an outer membrane, a structure unique to organisms of this group. The space between the two membranes is the periplasm, where we find not only the peptidoglycan (murein) cell wall but also many unique proteins. The outer membrane acts as a protective barrier, limiting the access of noxious compounds present in the environment. The enteric bacteria, especially *Escherichia coli* and *Salmonella*, served as a paradigm for the studies of structure and function of the outer membrane.

Composition, Structure, and Functions of Outer Membrane

Lipids and Lipopolysaccharides

Outer membrane is distinctly different from the inner, or cytoplasmic membrane in terms of composition. The lipid bilayer domain, which is composed of mainly glycerophospholipids (phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin in the enteric bacteria) in the inner membrane, here contains in addition a characteristic component, lipopolysaccharide (LPS). LPS is composed of two domains – the lipophilic lipid A domain and the hydrophilic polysaccharide domain. A single lipid A domain contains 5–7 fatty acid residues, all saturated and usually containing a 3-hydroxyl group, in contrast to just two often unsaturated fatty acid residues found in phosphatidylethanolamine (Fig. 1) or phosphatidylglycerol. The lipid A head group is based on a phosphorylated glucosamine disaccharide, in contrast to the glycerol phosphate moiety in the glycerophospholipids. It is also connected to a polysaccharide structure, which protrudes into the external medium and plays a major role in the interaction of bacteria with the external world.

This article is an update of H. Nikaido, Outer Membrane, Gram-Negative Bacteria, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 439–452.

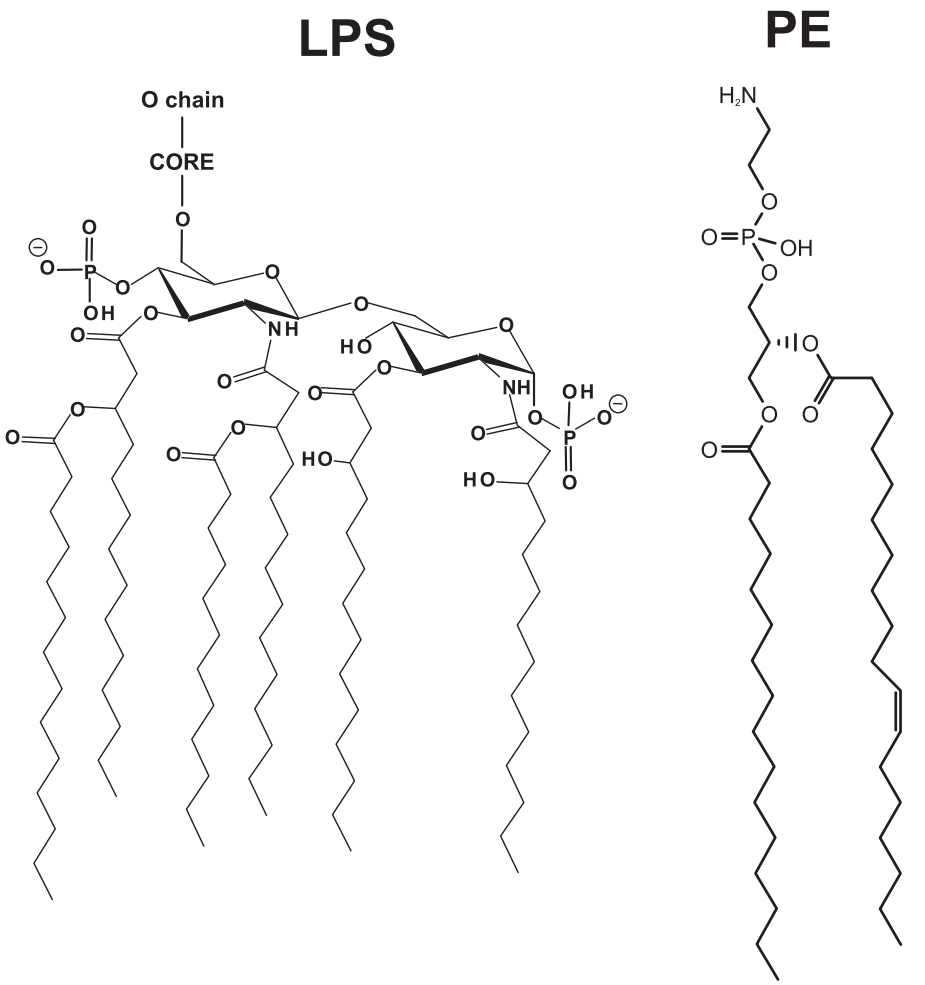

Fig. 1 Structure of lipid A and phosphatidylethanolamine. For the latter, the most abundant species in *E. coli*, containing palmitate (C16:0) and *cis*-vaccenate (C18:1) at 1- and 2-position, respectively, is shown.

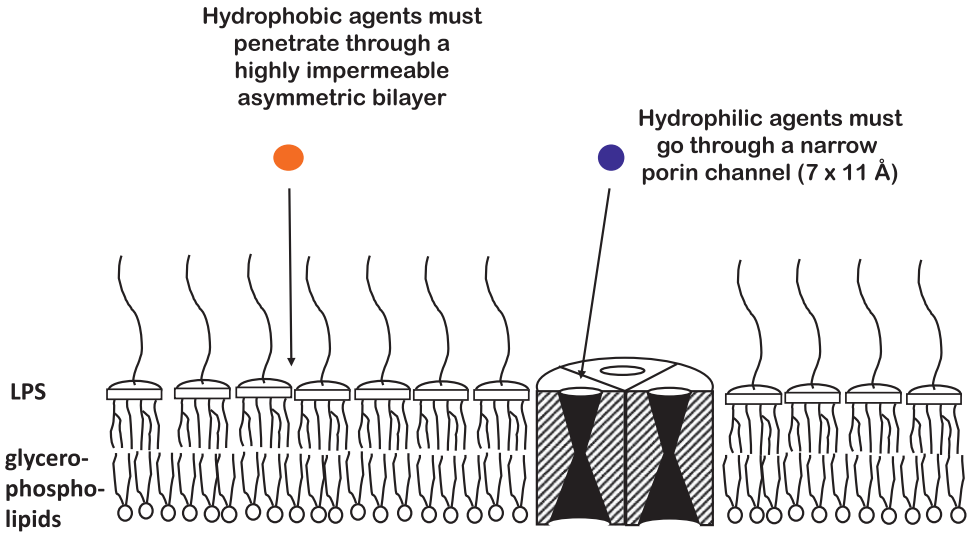

Fig. 2 A model of the outer membrane of enteric bacteria. In the bilayer domain, the outer leaflet is composed exclusively of lipopolysaccharides, the inner leaflet glycerophospholipids. A non-specific porin is also shown.

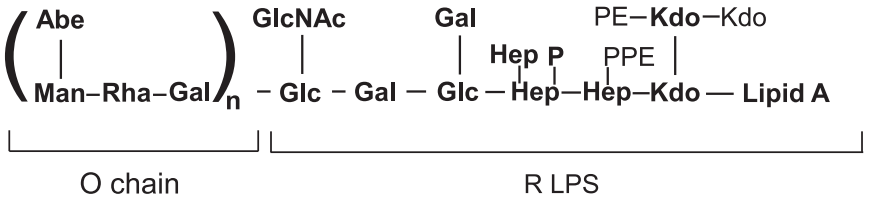

Fig. 3 Structure of the polysaccharide portion of LPS. The figure shows, as an example, the LPS of *Salmonella typhimurium* (*Salmonella enterica* serovar Typhimurium). In this species the O chain is composed of a repeating unit, containing a 3,6-dideoxyhexose, abequose. The more proximal part of the core may contain additional residues in non-stoichiometric amounts, shown in non-bold letters. Abbreviations: Abe, abequose; Rha, L-rhamnose; Man, D-mannose; Gal, D-galactose; Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine; Hep, L-*glycero*-D-*manno*heptose; Kdo, 3-deoxy-D-*manno*octulosonate; P, phosphate; (P)PE, (pyro)phosphorylethanolamine.

In *E. coli and Salmonella*, the entire outer leaflet of the bilayer appears to be composed of LPS, the phospholipids occupying only the inner leaflet (Fig. 2). This asymmetric structure produces an exceptionally impermeable bilayer. Lipid bilayers can usually be penetrated easily by lipophilic molecules, including various drugs, because these molecules can dissolve into the interior of the membrane, and redissolve in the aqueous phase on the other side of the membrane. However, the rate of such permeation is about two orders of magnitude slower across the outer membrane bilayer. This is caused by the very low fluidity of the interior of the LPS leaflet, which in turn is the result of the presence of only saturated fatty acids, of many fatty acid chains connected covalently to a single head group, and of the bridging, by divalent cations, of neighboring, negatively charged, LPS molecules (see below). Thus the LPS and the LPS-containing outer leaflet of the outer membrane play a major role in producing an effective permeability barrier against the entry of lipophilic compounds, including many antibiotics. Isoxazolylpenicillins (such as oxacillin), macrolides (such as erythromycin), and rifamycins show little activity against gram-negative bacteria yet show strong activity against gram-positive bacteria; this limited range is to a large extent due to the low permeability of the LPS leaflet.

The polysaccharide portion of LPS (Fig. 3) is usually divided into the "core", which is connected to lipid A, and "O side-chain", which in turn is connected to the core. The core is composed of a non-repeating oligosaccharide structure, which contains numerous acidic groups especially in its inner portion, close to the head group of lipid A. The glucosamine disaccharide head group also carries phosphate or pyrophosphate groups at both ends. Thus the inner portion of LPS core has a very high density of negative charges, which are usually neutralized by divalent cations, such as Mg^{2+} and Ca^{2+}, in living bacterial cells.

The O-chain is most often composed of an oligosaccharide repeat unit, which frequently contains unusual sugars like 3,6-dideoxyhexoses. Since this is the portion that is exposed to the external world, animal pathogens will induce the production of antibodies that bind this portion of LPS. If the repeating unit contains unusual sugars, this may help the bacteria to evade the antibodies prevailing in the population of higher vertebrates. The structure of O-chain has thus undergone extensive modifications during recent evolutionary history, so that serological analysis has shown the presence of more than 100 types of O chains (recognized as "O antigens") in *E. coli* alone.

Our body recognizes the lipid A domain of LPS as a characteristic common component of a pathogen, through the Toll-like receptor on the surface of many cells, and this results in the strong stimulation of specific immune response that follows. Thus this reaction is meant to help protect our body from infection, and some successful human pathogens are known to modify the lipid A structure in order to avoid the recognition by the host. Yet the recognition of lipid A domain by the Toll-like receptor can result in the overstimulation of many cells in our body, when many molecules of LPS are suddenly introduced, for example, by injection. Because of this reaction, LPS is also known as "endotoxin". Humans and rabbits are some of the most susceptible animals to endotoxin, and even 1 ng/kg body weight will cause significant increases in body temperature. A large amount of LPS is released into blood stream from dying bacteria, when patients suffering from sepsis (multiplication of bacteria in the blood stream) are treated by an effective antibiotic. This often causes severe, often fatal shock reactions (septic shock) in patients.

In addition to LPS, the outer membrane of the enteric bacteria contains enterobacterial common antigen (ECA). Unlike the O antigen polysaccharides, there is no variation in structure among strains or even species, and all ECA molecules are composed of polysaccharides of *N*-acetylaminomannuronic acid, *N*-acetylfucosamine, and *N*,*O*-diacetylglucosamine. In the common form of this component, the polysaccharide is anchored to the outer membrane through a covalent linkage to phosphatidic acid.

The inner leaflet of the outer membrane bilayer is composed of the common glycerophospholipids. However, the quantitative composition is different from that found in the inner membrane. Phosphatidylethanolamine occupies over 90% of the glycerophospholipids in the outer membrane of *Salmonella*.

Proteins

The outer membrane is high in its protein content. Several different classes of proteins are found.

Structural proteins

There is a need to anchor the outer membrane to the underlying peptidoglycan for stabilization (Fig. 4). In enteric bacteria, this function is mainly carried out by the murein lipoprotein (or Braun lipoprotein), which is a small periplasmic protein (7200 daltons) with a covalently linked lipid moiety (a diglyceride and a fatty acyl residue are connected to the sulfhydryl group and the

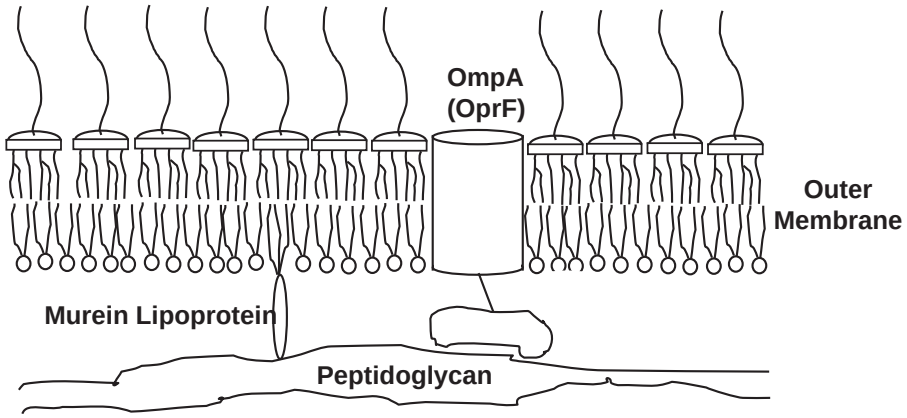

Fig. 4 Anchoring of outer membrane to the peptidoglycan layer.

amino group, respectively, of the N-terminal cysteine). This protein connects the two structures by becoming covalently linked to peptidoglycan at its C-terminus, and non-covalently linked to the outer membrane bilayer through its lipid extensions at its N-terminus.

In *Pseudomonas aeruginosa* and related bacteria, murein lipoprotein is absent. Instead, the outer membrane is connected to the peptidoglycan by OprF and its homologs. OprF is a homolog of *E. coli* OmpA, and most of this protein folds into a two-domain conformer, with its N-terminal 8-stranded β-barrel buried in the outer membrane, at the same time its C-terminal periplasmic domain non-covalently associating with the peptidoglycan (Fig. 4).

Porins

Bacterial cells must take up nutrients from the environment, in spite of the presence of the outer membrane permeability barrier. For this purpose, all gram-negative bacteria apparently synthesize a special class of outer membrane protein called porins, whose narrow internal channel allows the diffusion of only small, hydrophilic nutrients. In *E. coli*, porins, which are present in about 10^5 copies per cell, are the most abundant protein in the cell, in terms of mass. *E. coli* porin channel has a dimension of 0.7×1.0 nm at its narrowest point, and disaccharides barely pass through this channel. Larger compounds (as a rough yardstick larger than about 600 daltons) are essentially excluded. Interestingly, lipophilic molecules are strongly retarded in their passage through this channel; this is apparently because at the narrowest point of the channel (the "eyelet") the presence of acidic and basic amino acid residues on opposing walls (Fig. 5) creates a layer of highly ordered water molecules and passage of lipophilic solutes through this layer is energetically unfavorable as it has to disrupt this structure. Finally, *E. coli* porins favor the passage of cations over anions. It should be noted that these properties of the porin channels serve to prevent the entry of many toxic compounds, for example conjugated bile salts (lipophilic and anionic), perhaps the most important such compound for *E. coli* living in the intestinal tract of higher animals.

E. coli porins are trimers, each of which is composed of 16-stranded β-barrels (Fig. 5). *E. coli* produces three porins, OmpF, OmpC, and PhoE. There structures are very similar to each other, yet there are subtle differences especially within the channel.

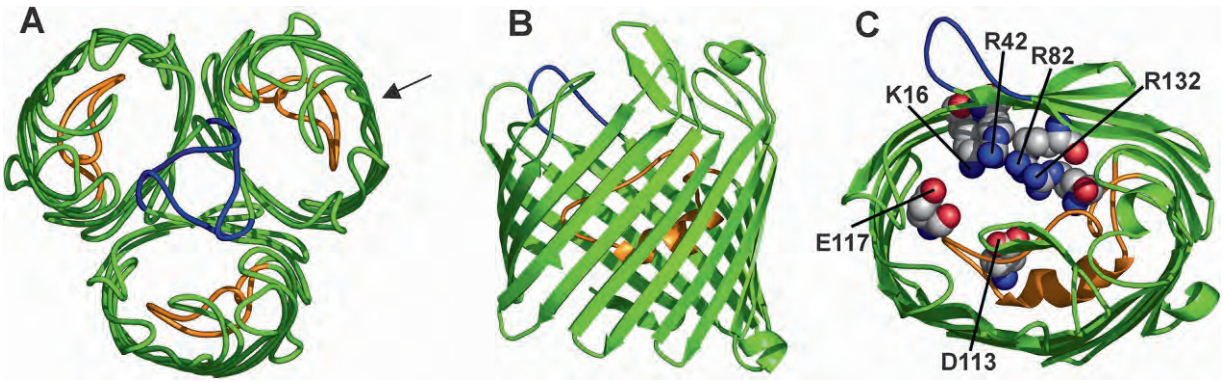

Fig. 5 X-ray crystal structure of *E. coli* OmpF porin. (A) View of the trimer from the top. Loop 2, colored blue, connects one protomer to the neighboring protomer. Loop 3, colored orange, folds back into the lumen of the β-barrel and narrows the diffusion channel. (B) View of the monomeric unit from the side. (C) View of the monomeric unit from the top, showing the "eyelet" or the constricted region of the channel. The eyelet is formed by acidic residues Glu117 and Asp113 from the Loop 3, and basic residues from the opposing barrel wall, Lys16, Arg42, Arg82, and Arg132, all shown in spherical models. This figure is from Nikaido, H., 2003. Molecular basis of bacterial outer membrane revisited. Microbiol. Mol. Biol. Rev. 67, 593–656.

In contrast to OmpF, PhoE favors the permeation of anions, and is expressed strongly under phosphorus starvation conditions presumably to enhance the uptake of phosphate and its esters from the environment. OmpC behaves as though its channel is slightly narrower than that of OmpF, and becomes a predominant porin when the ionic strength of the environment is close to, or over, that in the body fluids of higher animals. The predominance of OmpC in *E. coli* living in the intestinal tract will further enhance the exclusion of bile acids.

It should be emphasized that porins discriminate among various solutes on the basis of their global physicochemical properties, such as size, charge, and lipophilicity, and in this sense they show no specificity based on the exact fit between the protein and the ligand. Thus PhoE, for example, favors the diffusion of any anions, for example sulfate or anionic antibiotics, although such a behavior is not beneficial for the *E. coli* cells starved for phosphorus.

In recent years, electrophysiologists found that some antibiotics bind transiently to internal sites in porin channel, and proposals were made that one can calculate the diffusion rates of drugs from the kinetics of this binding. However, such an approach is flawed, because those drugs that show no tendency for binding do diffuse through porin channels quite rapidly. This "binding" (with a very low affinity) is evidently a laboratory artefact created by the very high resolution of modern electrophysiology.

Although trimeric porins are found universally in enteric bacteria, they are often absent in other groups of gram-negative bacteria. For example, *P. aeruginosa* and its relatives produce very impermeable outer membranes: they do not contain close homologs of OmpF/OmpC/PhoE, and the rate of penetration of hydrophilic solutes (such as cephalosporins) is almost two orders of magnitude slower than across the *E. coli* outer membrane. The major non-specific porin of these species is OprF, which is a homolog of *E. coli* OmpA and similarly acts as a structural protein anchoring the outer membrane to the peptidoglycan. The rate of penetration of solutes through the OprF channel is indeed nearly two orders of magnitude slower than through the OmpF channel, and this is explained by the fact that only a few percent of OprF protein folds to produce an open channel, although the channel itself is not narrow with an estimated diameter of about 2.0 nm.

Specific channels

It would be beneficial for *E. coli* to take up rapidly some solutes that are retarded or excluded by the trimeric porins. For *P. aeruginosa* with its inefficient general purpose porins, the need is acute to take up various types of nutrients more efficiently. For these purposes, many gram-negative bacteria produce specific diffusion channels. Since starch (amylose) is broken down to oligosaccharides of maltose series by pancreatic amylase in our intestinal tract, *E. coli* must take up these compounds efficiently, and for this purpose uses the LamB (lambda B, so named because it is used as the receptor by the *E. coli* bacteriophage lambda) channel, which served as a prototype of these specific channels.

LamB is also a trimeric protein composed of β-barrels. The barrel is slightly larger than in the porin, containing 18 strands in contrast to 16 in the OmpF homologs. However, the channel is narrower. The interior of the channel is exquisitely constructed to facilitate the passage of maltose oligosaccharides, with the succession of six aromatic amino acid residues constituting a "greasy slide" on which the sugars can slide past (Fig. 6). The channel has affinity to maltose and other higher oligosaccharides of this series,

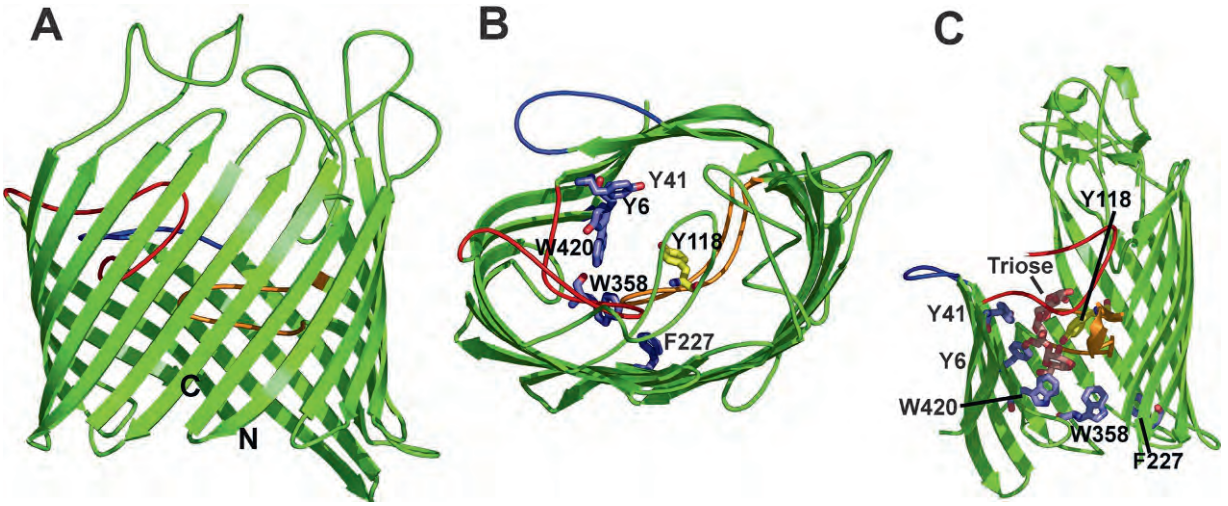

Fig. 6 X-ray crystallographic structure of a specific channel, *E. coli* LamB. (A) Side view of the monomeric unit composed of 18-stranded β-barrel. Loop 3 (orange) and loop 1 (red) between the transmembrane β-strands (shown as flat arrows) fold back deeply into the central channel of the β-barrel, and makes the channel narrower. Loop 2 (blue) folds outward and interacts with other protomers of the trimeric structure. (B) View of the monomeric unit from the top. The residues of the greasy slide (Tyr41, Tyr6, Trp420, Trp358, and Phe227) are shown as blue stick diagrams, and Tyr118, which constricts the channel from the other side, is shown as a yellow stick diagram. (C) View of the greasy slide and its interaction with maltotriose. This is a side view, with the front of the β-barrel cut out for a better view of the slide. The aromatic residues comprising the helically twisted slide and Tyr118 are shown as stick diagrams, colored as in panel B. The maltotriose molecule (Triose) is shown as a stick diagram colored in orange. The diagrams are based on PDB coordinate files 1MAL and 1MPN, and produced with PyMol. This figure is from Nikaido, H., 2003. Molecular basis of bacterial outer membrane revisited. Microbiol. Mol. Biol. Rev. 67, 593–656.

and this serves to accelerate the transmembrane diffusion of these sugars (up to maltohexaose, 990 daltons) when they are present in low concentrations. When they are present in nonphysiological, high concentrations, smaller oligosaccharides such as maltose can diffuse through the nonspecific porin channels as well, or even faster.

There are several other specific *E. coli* channels that have been studied in detail. These include the channels for sucrose (ScrY), arylglucosides (BglH), nucleosides (Tsx), and fatty acids (FadL). In *P. aeruginosa*, the channel protein OprD was studied because it plays a major role in the rapid influx of imipenem, an antibiotic that shows an exceptional activity against this organism, and appears to be a specific channel for basic amino acids and peptides. Interestingly, *P. aeruginosa* contains 19 homologs of this protein; probably each of these produces a specific diffusion channel for a specific type of nutrients needed by this organism, whose general-purpose porin, OprF, is very inefficient as described above.

TonB-dependent receptors

Gram-negative bacteria need to take up some large compounds that exist in extremely low concentrations in the environment. These include the iron-siderophore complexes for the uptake of iron, and vitamin B_{12}. *E. coli* for example produces specific outer membrane transporters for ferric enterobactin (726 daltons, the chelator produced by *E. coli* itself) (FepA), for ferrichrome (chelator produced mostly by fungus) (FhuA), for coprogen (FhuE), for ferric citrate (FecA), for dihydroxybenzoate-ferric complex (Fiu), for ferric complexes of similar catecholate-type compounds (Cir), and for vitamin B_{12} (1355 daltons) (BtuB). For the uptake of these compounds, even specific channels are not adequate, because diffusion through these channels is driven ultimately by the difference in concentrations across the membrane, which is vanishingly small for these compounds. They are, therefore, transported across the outer membrane by an active process, with the concomitant consumption of energy. Since there is no ATP in the periplasm or the outer membrane, the energy coupling is done by an ingenious process of using a periplasmic protein, TonB, to transduce the energy from the cytoplasmic membrane, as discussed in Section "Functional Complexes Involving Multiple Components of the Cell Envelope".

The outer membrane proteins involved are usually called receptors, because they show very strong affinity to the transported ligands, with the dissociation constants in the range of nanomolar. Since the ligands are present in such low concentrations, this high affinity is absolutely needed. X-ray crystallographic studies showed that the proteins have the basic construction of a β-barrel, with 22 transmembrane β-strands (Fig. 7). The proteins appear to exist as monomers, in contrast to the OmpF-type porin and the LamB protein. Furthermore, a large N-terminal portion is folded into the barrel, as though to "plug" the large central channel inside the β-barrel. Recent years have seen impressive progress in our understanding of the interaction between TonB and these receptors (see below), but we still do not know how the active transport of the ligands is achieved.

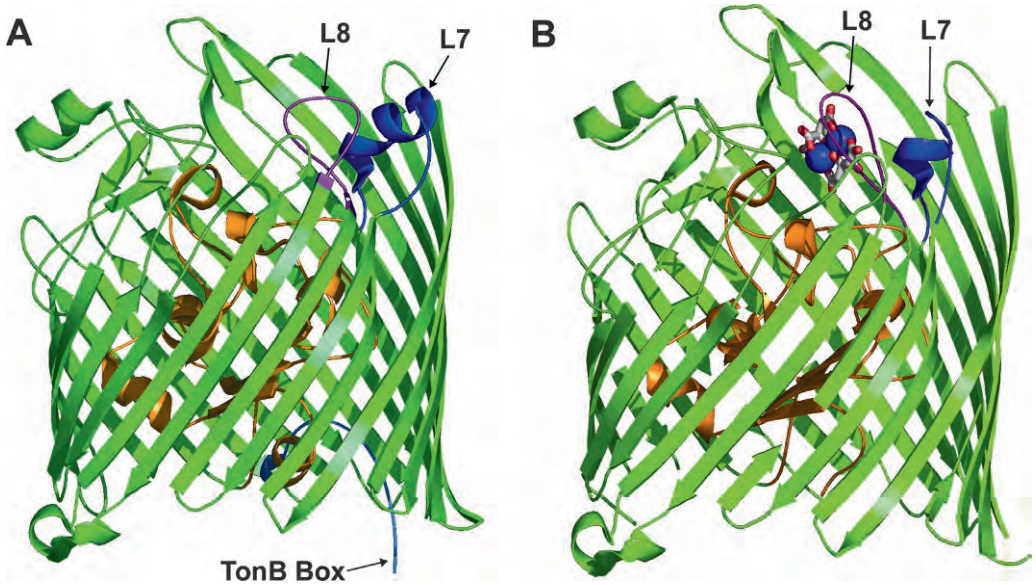

Fig. 7 X-ray crystallographic structure of the ferric citrate receptor, FecA. (A) Side view of the unliganded FecA. The N-terminal domain of this protein is inserted into the lumen of the large, 22-stranded β-barrel (green) as a "plug" (orange). Its N-terminal end contains the "TonB box" sequence, predicted, and now shown, to interact with the C-terminal part of the TonB protein. Loops 7 and 8 are shown in deep blue and mauve, respectively. (B) Liganded FecA. Binding of ferric citrate (shown with two large blue spheres indicating iron atoms and with stick models for citrate) produces movement of loops 7 and 8. The TonB box also becomes disordered and invisible. Based on PDB files 1KMO and 1KMP. This figure is from Nikaido, H., 2003. Molecular basis of bacterial outer membrane revisited. Microbiol. Mol. Biol. Rev. 67, 593–656.

Export channels

E. coli TolC is an outer membrane protein that exists as a symmetrical trimer. The trimer contains the 12-stranded β-barrel that functions as a channel traversing the outer membrane, but in this case each monomer contributes only four of the 12 transmembrane strands. Remarkably, the channel extends about 10 nm into the periplasm, this time as a bundle of 12 long α-helices. This arrangement allows the end of the periplasmic α-tunnel to become connected to the external end of the transporters in the inner membrane, producing a direct extrusion mechanism for substrates into the medium, bypassing the periplasm. Thus TolC and its homologs are essential components for some of the machineries that export proteins and drugs (see below).

A very different arrangement is also used in the export of various proteins into the medium. Here a rather large number (12–14) of intrinsic outer membrane proteins assemble to produce a large channel. This occurs in the type II secretion pathway, discussed below, and the protein here is called secretins. Similar large channels are also envisaged for the pathway leading to the export of P pili subunits, and here the proteins are called ushers.

Biosynthesis and Assembly of Outer Membrane Components

Biosynthesis of Component Macromolecules

The synthesis of macromolecules (LPS, outer membrane proteins) obviously takes place in the cytosol where ATP and other sources of energy are available. The biosynthesis of LPS is now understood in details. The synthesis of lipid A starts from an activated form of N-acetylglucosamine, UDP-N-acetylglucosamine (Fig. 8). In the first reaction, a C14 hydroxylated saturated fatty acid (3-OH myristic acid) residue is added to the 3-position of the sugar (step 1 in Fig. 8), followed by the deacetylation (step 2) and further addition of another 3-OH myristic acid residue on the now-deacetylated 2-amino group on the sugar (step 3). Two of these diacylated molecules are fused together, to produce a 1-phosphorylated tetraacylated disaccharide (step 4), which is then phosphorylated at the other end of the disaccharide (step 5), to produce a structure commonly called Lipid IV_A, which shows significant endotoxic activity. Just like the acylation reactions preceded the formation of disaccharide, the addition of the innermost sugar residues, two Kdo (ketodeoxyoctonate, or 3-deoxy-D-mannooctulosonic acid) residues of the core portion are added next (step 6), before the addition of two other fatty acid residues to complete the acylation reactions (steps 7 and 8).

The product of the series of reactions described above, $(Kdo)_2$-lipid A, then accepts various sugars of the core domain (Fig. 3) from nucleoside-sugar donor molecules. Since these nucleoside-sugars are components of the cytosol, these transfer reactions must occur at the inner surface of the cytoplasmic membrane. The final product of the synthesis up to this point, the complete R LPS (see glossary), is flipped across the cytoplasmic membrane by an ABC (ATP-binding cassette) transporter MsbA.

The biosynthesis of the outer part of the LPS polysaccharide, O chain, proceeds separately from that of the R LPS. Many O chains are composed of branched oligosaccharide repeat units, and such a unit is assembled again at the inner surface of the cytoplasmic membrane, by using nucleoside-sugar molecules as donors, on a large (C55) isoprenoid lipid carrier, undecaprenol phosphate,

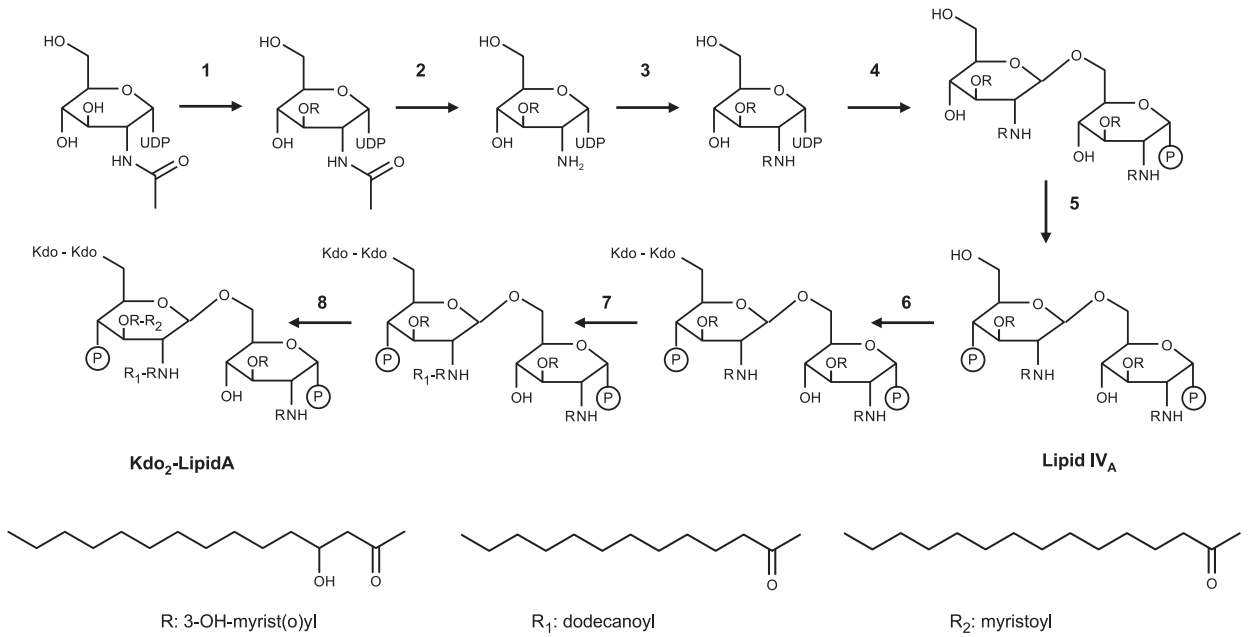

Fig. 8 Pathway of lipid A biosynthesis in *E. coli*. Note that acylation of the sugar occurs before the formation of the disaccharide head group, and that addition of two Kdo residues occurs before the completion of all acylation reactions. Thus the final product is $(Kdo)_2$-lipid A, and is not lipid A (which was defined as a product of partial acid hydrolysis of LPS). In other species of gram-negative bacteria, the acylation pattern of lipid A may be different from what is shown here.

bound non-covalently to the membrane. When the repeating unit assembly is complete, the oligosaccharide-lipid complex is apparently flipped over across the membrane by a transporter called Wzx. At the outer surface of the membrane, the repeating units are polymerized by an enzyme Wzy, and the completed O chain is finally transferred, at the periplasmic surface of the cytoplasmic membrane, to the nascent R LPS.

Some unbranched O chains of rather simpler structure are assembled again on the inner surface of the cytoplasmic membrane. However, in this case, the completely assembled, long, O chain is extruded by an ABC transporter across the cytoplasmic membrane, before its transfer to the R LPS presumably at the outer surface of the cytoplasmic membrane.

Outer membrane proteins are synthesized by ribosomes in the cytosol. They are then exported across the cytoplasmic membrane, apparently always utilizing the SecYEG pathway. How these proteins become assembled into the outer membrane is described in the next section.

Assembly

The components of the outer membrane, after their export across the cytoplasmic membrane, somehow travel across the periplasm, and are finally assembled in the outer membrane. After the export across the cytoplasmic membrane, all the processes must proceed without utilizing ATP. Some of the mechanisms involved are becoming elucidated in the last several years.

Assembly of outer membrane proteins

The export of lipoproteins is understood in most detail. After the apoprotein is exported in the unfolded state by the SecYEG machinery, the lipids are added. Since this is done on the periplasmic surface of the cytoplasmic membrane, where no ATP is available, the transfer is done by utilizing membrane phospholipids as donors. It was known for some time that some lipoproteins associate themselves with the inner leaflet of the outer membrane, while others remain anchored to the outer leaflet of the inner membrane, and furthermore that the fate of the lipoproteins is dependent on the nature of the second amino acid residue from the N-terminus after the cleavage of the leader sequence. If this residue is aspartate, the protein is retained in the inner membrane, and if it is a neutral residue such as serine, the protein is exported to the outer membrane.

Recent studies elucidated the molecular mechanism of this differential export process for lipoproteins. Thus lipoproteins become the substrates of an unusual ABC transporter, LolCDE, whose function is to "transport" them (with the utilization of ATP at the inner surface of the cytoplasmic membrane) to a periplasmic binding protein, LolA (Fig. 9). LolA-lipoprotein complex then reacts with the LolB protein, located at the inner surface of the membrane, and the lipoprotein is now delivered into the inner leaflet of the outer membrane. Those lipoproteins with aspartate as the second amino acid will escape the recognition by the LolCDE complex, and will thus remain anchored in the cytoplasmic membrane.

We have also gained much understanding on the mechanism of assembly of the integral outer membrane proteins. Unlike lipoproteins, which are inserted into the outer membrane only through their lipid groups, most integral outer membrane proteins traverse both leaflets of the membrane, and characteristically are composed of β-barrels. Since most intrinsic proteins of the cytoplasmic membrane are bundles of transmembrane α-helices, the unusual β-barrel conformation of the outer membrane proteins certainly suggests a unique mechanism of export and assembly for these proteins. An outer membrane protein, called

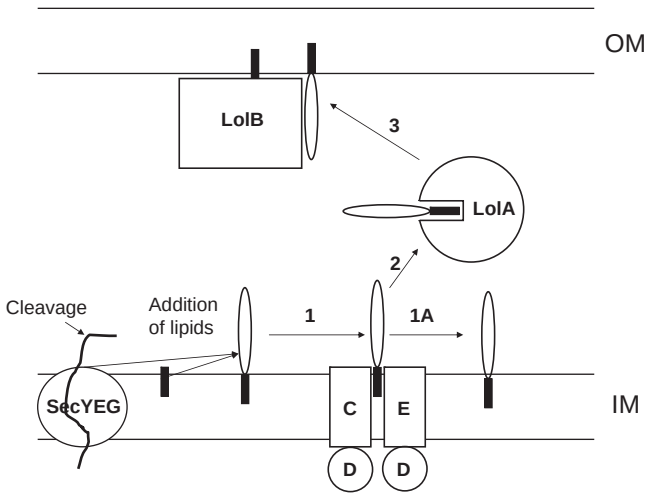

Fig. 9 Mechanism of lipoprotein export. After export through the SecYEG exporter, the signal sequence is cleaved, to expose the N-terminal cysteine of the mature protein. This residue is lipid-modified, and the lipoprotein interacts with the LolCED ABC transporter, with its ATPase units (LolD) (step 1). If the second amino acid residue is Asp, it is an inner membrane lipoprotein, and is released and stays in the inner membrane (step 1A). If it is a neutral amino acid, then the lipoprotein is actively transferred to a periplasmic binding protein, LolA (step 2), and finally interacts with the outer membrane receptor protein LolB (which itself is a lipoprotein), to be assembled into the inner leaflet of the outer membrane (step 3).

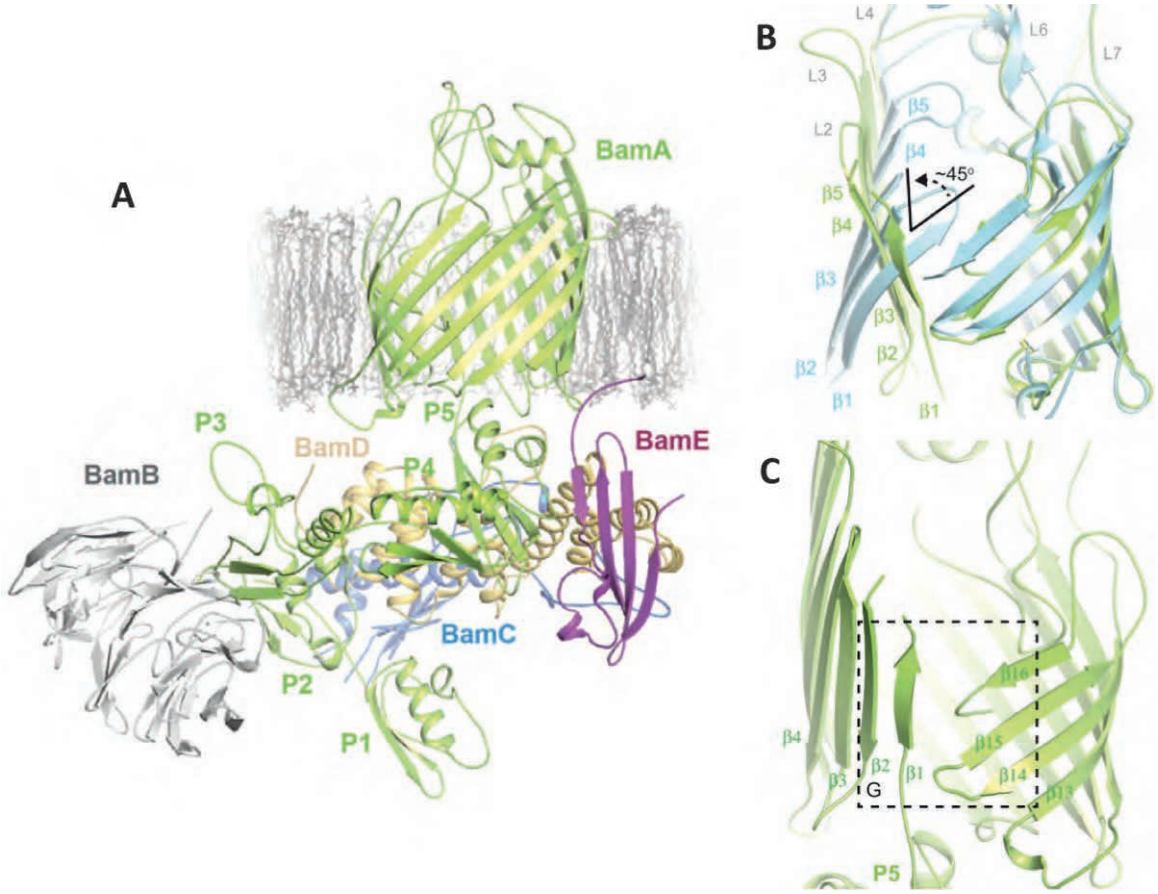

Fig. 10 BamA and associated proteins play a major role in the insertion of β-barrel proteins into outer membrane. (A) X-ray crystal structure of BamA-BamB-BamC-BamD-BamE complex. (B) Close-up of BamA β-barrel domain. (C) The first several β-strands of the barrel become reoriented upon binding of BamCDE to BamA, creating a lateral opening between β1 and β16. These figures are from Bakelar, J., Buchanan, S.K., Noinaj, N., 2016. The structure of the β-barrel assembly machinery complex. Science 351, 180–186.

Omp85 (or BamA), is at the center of this assembly process. In a cell depleted for BamA, outer membrane proteins accumulate in an unfolded form, and are not assembled into the outer membrane. BamA is also a β-barrel, but it has a long N-terminal extension, containing five repeated POTRA (polypeptide-transport-associated) domains that exist in the periplasm. The POTRA domains interact with four lipoproteins (BamB, BamC, BamD, and BamE) (Fig. 10(A)). It seems likely that new outer membrane proteins, exported into periplasm, become associated, in their unfolded state, with periplasmic chaperones such as SurA (a periplasmic chaperone that also has an activity in catalyzing the *cis-trans* interconversion of peptidyl-proline isomers) or Skp, and eventually enter the large cavity of BamA barrel. It is still unclear if the folding of the substrate protein into β-barrel mainly takes place during its association with POTRA domains, or within the central cavity of BamA barrel. However, it appears that the first several strands of BamA barrel undergo an unusually large reorientation upon the binding of BamCDE, so that a lateral opening is created for the export of folded substrate proteins into the outer membrane (Compare Fig. 10(C) with (B)). The presence of BamA homologs in the outer membranes of chloroplasts and mitochondria and their apparent role in the assembly of proteins in these plastid outer membranes support the important role for this protein.

The process of export and assembly of outer membrane proteins is thus complex, and errors are likely to occur at every step of the pathway. Thus the periplasm has a highly evolved mechanism for sensing such errors, and for correcting them (see Section "Periplasm" below).

Assembly of LPS

We have described how the complete LPS is assembled on the outer (periplasmic) surface of the cytoplasmic membrane. LPS, however, has still to cross the periplasm, become inserted into the outer membrane, and most importantly in the outer leaflet of this membrane. How this complex series of reactions takes place has been elucidated in recent years. An outer membrane protein, earlier called Imp (for increased membrane permeability), was known as an essential protein in *E. coli*, and its mutated version was known to produce *E. coli* cells with a "leaky" outer membrane. LPS is also essential for the survival of *E. coli*. In contrast, another species of gram-negative bacteria, *Neisseria meningitidis*, can survive without LPS. Imp is also not essential in this organism.

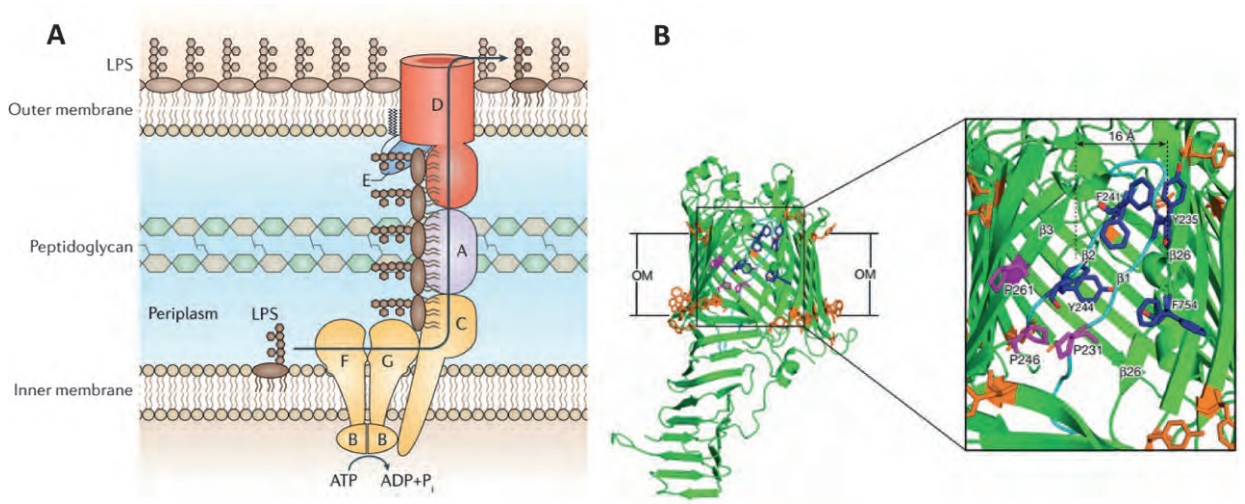

Fig. 11 Pathway of export of LPS to the outer membrane. (A) A schematic showing the pathway through Lpt proteins. A through G refer to LptA through LptG. Most likely more than one copy of LptA is involved in the transport, but only one copy is shown here for clarity. From Okuda, S., Sherman, D.J., Silhavy, T.J., Ruiz, N., Kahne, D., 2016. Lipopolysaccharide transport and assembly at the outer membrane: The PEZ model. Nat. Rev. Microbiol. 14, 337–345. (B) X-ray crystal structure of LptD. The external ring of aromatic amino acids that are characteristically located at the membrane-solvent boundary region is indicated in orange. The short and disordered β1 through β3 strands create crenellation in the β-barrel, through which LPS is thought to be moved directly into the outer leaflet of the outer membrane. From Qiao, S., Luo, Q., Zhao, Y., Zhang, X.C., Huang, Y., 2014. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. Nature 511 (7507), 108–111. http://dx.doi.org/10.1038/nature13484.

Inactivation of the *imp* gene in this organism showed that none of the newly synthesized lipopolysaccharide appeared on the cell surface, that is, in the outer leaflet of the outer membrane, suggesting the role of Imp in the correct assembly of lipopolysaccharide in its proper location.

Imp, now renamed Lpt(lipopolysaccharide transport)D, forms a huge β-barrel with 26 strands. In order to prevent the massive influx of large molecules from outside, it is plugged most of the time by LptE (see Fig. 11). LptD is the terminus of a long export route that is energized by an ABC transporter $LptFGB_2$ (Fig. 11(A)). ATP hydrolysis by this engine first drives newly assembled LPS to the inner membrane protein LptC. LptC, a few copies of periplasmic carrier LptA, and the periplasmic extension of LptD form a continuous structure that spans the thickness of periplasm (Fig. 11(A)). Moving newly made LPS along this export pathway (as in the delivery machine for PEZ candy) is the function of successive hydrolysis of ATP at the ABC transporter. Finally, once in the lumen of the LptD β-barrel, LPS is thought to be delivered laterally directly to the outer leaflet of the outer membrane, as the β-barrel here is crenellated with shorter and more unstable β-strands where the β3-to-β1 strands interact with the β26 strand (Fig. 11(B)).

Assembly of phospholipids

Phospholipids are synthesized in the inner leaflet of the inner membrane, and thus the first step for its export might be the flipping to the outer leaflet. Although MsbA was suggested to catalyze this reaction, in *Neisseria msbA* null mutants export and assemble phospholipids into the outer membrane, and thus MsbA cannot be essential for phospholipid export. Currently there is little information on the export pathway of phospholipids, which were shown to equilibrate rapidly between the outer and inner membranes. (Recently, a cardiolipin exporter PbgA was identified in *Salmonella*, although this system becomes activated only in animal tissues).

However, phospholipid molecules are expected to reach slowly the outer leaflet of the outer membrane, most likely through the spontaneous flipping process. This will destroy the permeation barrier that is dependent on the asymmetric construction of the outer membrane. Thus cells try to remove these phospholipid molecules from the outer leaflet. One mechanism is their hydrolysis by the outer membrane phospholipase OmpLA. The second mechanism is the use of outer leaflet phospholipid as the donor of acyl group to LPS, via the transferase PagP. The third system, Mla (maintenance of lipid asymmetry) resembles the Lol system (Fig. 9), and contains an outer membrane lipoprotein, a periplasmic protein, a putative binding protein associated with the inner membrane, as well as an ABC transporter that presumably energizes the active backward transport of phospholipids from the outer leaflet of the outer membrane all the way into the inner membrane. Interestingly, the inner-membrane-associated binding protein MlaD belongs to MCE family of proteins, known to be involved in the import of hydrophobic molecules, such as steroids, in mycobacteria.

Periplasm

The space between the outer and inner membranes contains the periplasm. This is not just an empty "space", and it contains many distinct components that carry out important functions. Obviously, this space contains peptidoglycan, but hints of its unique

function came from the discovery that several degradative enzymes of *E. coli*, for example ribonuclease and alkaline phosphatase, are found exclusively in the periplasm. Since these enzymes will destroy essential components of the cytosol if they existed in the cytoplasm, their compartmentalization within the periplasm, outside the cytosol, makes sense in terms of physiology.

One analysis on the basis of genome sequence of *E. coli* predicts about 370 periplasmic proteins, in comparison with only about 150 proteins to be located in the outer membrane. Another large class of periplasmic proteins includes ligand-binding proteins that form a part of the ABC (ATP-Binding Cassette) transporters, that import many compounds, including sugars, amino acids, peptides, inorganic ions such as phosphate and sulfate as well as metal cations, ferric-siderophore complexes, vitamin B12, and others. Another class includes proteins involved in electron transport. Now it is known that enzymes like formic hydrogenlyase and aerobic nitrate reductase, with their molybdate factor, are components of the periplasm. Although *E. coli* lacks the typical cytochrome c found in the corresponding space in mitochondria, there are several c-type cytochromes that function in the reduction of alternate electron acceptors. Yet other important class includes enzymes involved in peptidoglycan biosynthesis. β-Lactamase, which was apparently derived from one of these enzymes during evolution, plays a predominant role in raising the intrinsic level of resistance of gram-negative bacteria to penicillins and cephalosporins, the most important class of antimicrobial compounds. Finally, periplasm contains many proteins that assist in the folding of periplasmic and outer membrane proteins. These include proteins involved in the formation of disulfide bonds, such as DsbA, DsbB, DsbC, and others. The cytoplasm of gram-negative bacteria is a highly reducing environment, which contains hardly any disulfide-bond-containing proteins. In contrast, disulfide bonds are found commonly in periplasmic and outer membrane proteins, and their formation presumably helps in the folding of these proteins after their export, in an unfolded form, through the SecYEG export apparatus across the cytoplasmic membrane. Periplasm also contains many proteins that appear to function as molecular chaperones including Skp and SurA that have already been mentioned.

In addition, it is essential to have an intricate regulatory mechanism that senses the proper functioning of the protein folding and transport machinery. Periplasm in fact contains at least two major such signal transduction pathways. One of them, sigma E pathway, becomes activated when periplasmic or outer membrane proteins misfold at high temperature or under other conditions, and the activation of the alternative sigma factor E increases the transcription of many dozens of genes, many related to the folding of proteins in the periplasm, such as SurA, Skp, DsbC, and another peptidyl-prolyl isomerase, FkpA. At the same time, the synthesis of periplasmic proteases, such as DegP, is increased strongly, presumably to degrade misfolded proteins. The response also increases the transcription of many genes involved in lipid A synthesis, as well as the genes for the assembly of outer membrane proteins and lipopolysaccharides. The transcription of some outer membrane protein genes is on the other hand strongly repressed (*ompA* and *lamB* via small RNA *micA*; *ompC* and *ompX* via *rybB*). The sigma factor works with the core RNA polymerase in the cytosol, yet it has to be activated by signals in the periplasm. The mechanism is detailed in Fig. 12.

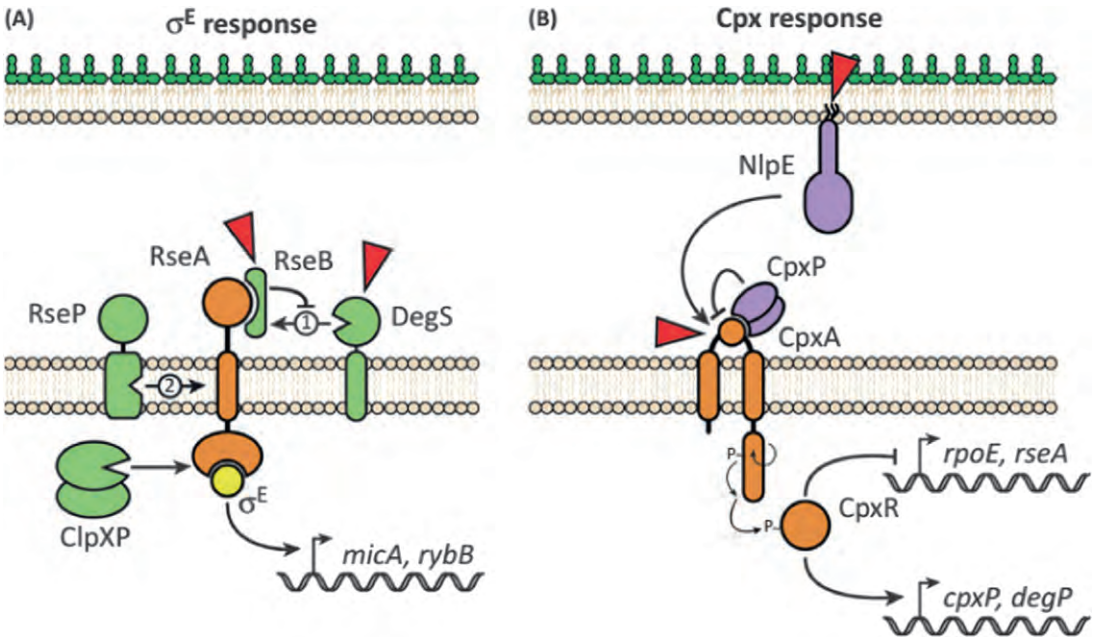

Fig. 12 Envelope stress responses. In σ^E response, the misfolded proteins in the periplasm binds to protease DegS and activates it. The periplasmic portion of the transmembrane protein RseA is normally protected by the bound RseB, but the latter becomes displaced by truncated LPS in the periplasm. The periplasmic and intramembranous portions of RseA are then degraded by DegS and RseP proteases, and the cytosolic portion of RseA is degraded mainly by ClpXP, finally releasing the alternative sigma factor σ^E. In the Cpx pathway, the sensory histidine kinase CpxA is activated by diverse conditions, leading to the phosphorylation of the response regulator CpxR, which also leads to the activation of the σ^E pathway. From Grabowicz, M., Silhavy, T.J., 2017. Envelope stress responses: An interconnected safety net. Trends Biochem. Sci. 43, 232–242.

The second signal transduction pathway, CpxAR, is a typical two-component system that uses an intrinsic inner membrane protein CpxA to sense the extracytosolic signal. It responds to the presence of misfolded proteins in the periplasm and the inner membrane (presumably caused by conditions like high pH, high salt concentration, and altered inner membrane lipid composition). It is also stimulated by the attachment of *E. coli* cells to a hydrophobic surface, via the outer membrane lipoprotein NlpE (Fig. 12). The activation of Cpx system stimulates the production of its own periplasmic inhibitor CpxP, preventing the runaway response. It also prevents the synthesis of OmpF and other outer membrane proteins through small RNA *micF*. There is also an interaction with the sigma E pathway.

The general physical environment of the periplasm requires some comment. It was shown by Stock et al. (1977) that the periplasm is in osmotic equilibrium with the cytosol. Cytosol is usually kept at a hyperosmotic state in comparison with the medium, so that the cytosol will not collapse and a moderate outward pressure is constantly present at the cytoplasmic membrane in a "balloon-in-a-cage" arrangement limited by the mechanically strong peptidoglycan. How then could the periplasm remain hyperosmotic in relation to the medium, in the presence of so many porin channels? Stock and coworkers found that interior-negative Donnan potential exists across the outer membrane, and that this results in the passive accumulation of cations in periplasm. Later studies led to the identification of the cause of this Donnan potential, the presence in the periplasm of "membrane-derived oligosaccharides" (MDO), a group of glucose oligosaccharides too large to escape through the porin channels and containing multiple negative charges. MDO is produced in large amounts when the osmotic activity of the medium is low, so that many cations can be concentrated in the periplasm. Protons, as cations, are also concentrated in the periplasm. Thus depending on the conditions, the periplasmic pH can be significantly lower than that of the medium, a fact that is not generally appreciated. The Donnan potential also affects the uptake of drugs across the outer membrane, favoring that of cationic drugs (especially polycationic ones such as aminoglycosides) and preventing to some extent the uptake of anionic compounds.

Functional Complexes Involving Multiple Components of the Cell Envelope

In the late 1960s, Manfred Bayer found by electron microscopy structures that seemed to connect the outer membrane with the inner membrane. This discovery was greeted with skepticism at that time, but recent genetic and biochemical studies showed many instances where physical interaction must occur between the components of the two membranes.

A prominent example is the bacterial flagella, whose "basal body" traverses the cytoplasmic membrane, peptidoglycan, and the outer membrane; we will not discuss flagella in detail, because this organelle is described in other chapters of this encyclopedia.

Another example of trans-envelope machinery contains the outer membrane receptors for iron-siderophore complexes and vitamin B_{12}. Bacteria need iron as a component of many enzymes and respiratory complexes. But the ferric ion found in aerobic environment occurs at an exceedingly low concentration because of its low solubility. Thus bacteria produce siderophores, or iron carriers, that bind the ferric ion strongly and move them into the cytoplasm. However, both iron-siderophore complexes (726 daltons for ferric enterobactin) and vitamin B_{12} (1355 daltons) are too large to pass through the porin channels of enteric bacteria. Thus these bacteria produce specific outer membrane receptor proteins that bind these compounds strongly; these receptors contain a large, 22-stranded β-barrel, which is occluded by an N-terminal "plug" domain. Transport of these substrates into the periplasm requires TonB, a periplasmic, elongated protein that is embedded in the cytoplasmic membrane at one end and appears to traverse the thickness of the entire periplasm, to interact with the outer membrane receptors. Near the N-terminus of the plug domain, there is a small structure called TonB box. The C-terminal domain of TonB apparently interacts strongly with the TonB box, by supplying one of the strands to complete a β-sheet structure. This process thus involves physical connection between the inner and outer membranes (Fig. 13). TonB-dependent transport requires proton-motive force and the inner-membrane-embedded proteins ExbB

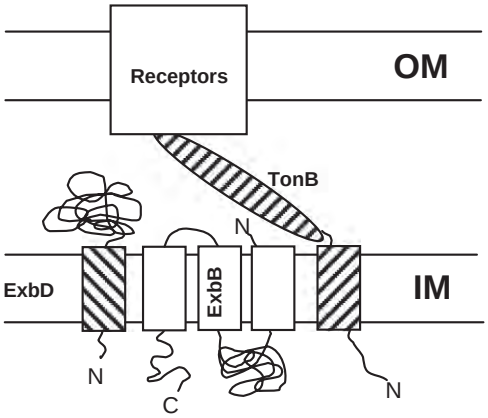

Fig. 13 Hypothetical model of TonB-dependent transport complex. TonB appears to have an N-terminal transmembrane helix, which may interact with at least two other intrinsic inner membrane proteins, ExbB and ExbD, needed for energy transduction. The other end of TonB has been hypothesized to interact with the TonB box of various receptors, and indeed cocrystals of receptors with TonB showed that this physical interaction occurs.

and ExbD. These latter proteins are homologous to the stator of flagellar motor, and proton-motive force was shown to result in the rotation of TonB protein, which presumably helps in locating the outer-membrane-embedded transporters and in facilitating the interaction of its C-terminal domain with the TonB Box.

Another example is a group of multidrug efflux pumps in gram-negative bacteria. These bacteria were found, since the mid-1990s, to pump out great many antibiotics and chemotherapeutic agents, often utilizing transporters of an astonishing degree of versatility. One class, called RND (resistance-nodulation-division) superfamily, is particularly important. They are ubiquitous and often show an extremely wide range of substrate specificity. For example, AcrB (so named for acridine resistance) pump of *E. coli*, which served as the prototype of these transporters, can pump out not only most of the commonly used antibacterials (including penicillins, cephalosporins, macrolides, rifampicin, fluoroquinolones), dyes, antiseptics, and detergents, but also even simple solvents. Furthermore, RND pumps are organized into multi-protein complexes that span both the inner and outer membranes (Fig. 14), together with an outer membrane channel (TolC in *E. coli*) and a periplasmic linker protein. This construction allows the complex to move drug molecules directly into the medium, a mechanism that is very advantageous in gram-negative bacteria. A simple, uncomplexed pump located in the inner membrane would only move drugs from the cytosol to the periplasm, and the drug molecules can diffuse into the cytosol spontaneously and rather easily, as drugs with cytosolic targets must have rather lipophilic structures to allow this influx process (Fig. 14). In contrast, the tripartite, cell-envelope-spanning structures of the RND pumps causes the movement of drugs from the periplasm into the external medium; thus the extruded drug molecule has to traverse the effective outer membrane barrier again, in order to come into the cell. It is well known that most of the naturally occurring antibiotics (for example penicillin G and erythromycin) and of the library of chemically synthesized antibacterial compounds (for example linezolid) exhibit activity only against gram-positive bacteria. This "intrinsic" resistance of gram-negative bacteria was often attributed to the barrier properties of the outer membrane. However, in many cases the inhibitors are actively pumped out by these ubiquitous multidrug efflux pumps, and the contribution of the pumps is at least as important as that of the outer membrane barrier.

Final examples of the structures spanning both membranes are machineries dedicated for extracellular secretion of proteins and other macromolecules. With gram-positive bacteria, protein secretion is only a matter of moving the protein across the cytoplasmic membrane. In contrast, with gram-negative bacteria, it becomes necessary to prevent the premature folding of the protein in the periplasm, and to push the protein through another membrane barrier, the outer membrane. Yet most bacteria, especially pathogens, have a need to secrete toxic proteins into the external space, and in some cases even into the host cells, a process requiring the breaching of yet another membrane barrier.

Several mechanisms have been devised for protein secretion in gram-negative bacteria (Fig. 15). As seen, most of these machineries involve protein assemblies that traverse the entire cell envelope, although there exist a few systems that are confined in the outer membrane (Type 5 secretion system that is autotransporter, chaperone-usher pathway used in the secretion of fimbriae subunits, and curli biogenesis system). In the simplest system, the type I secretion pathway, the proteins are pushed out across the

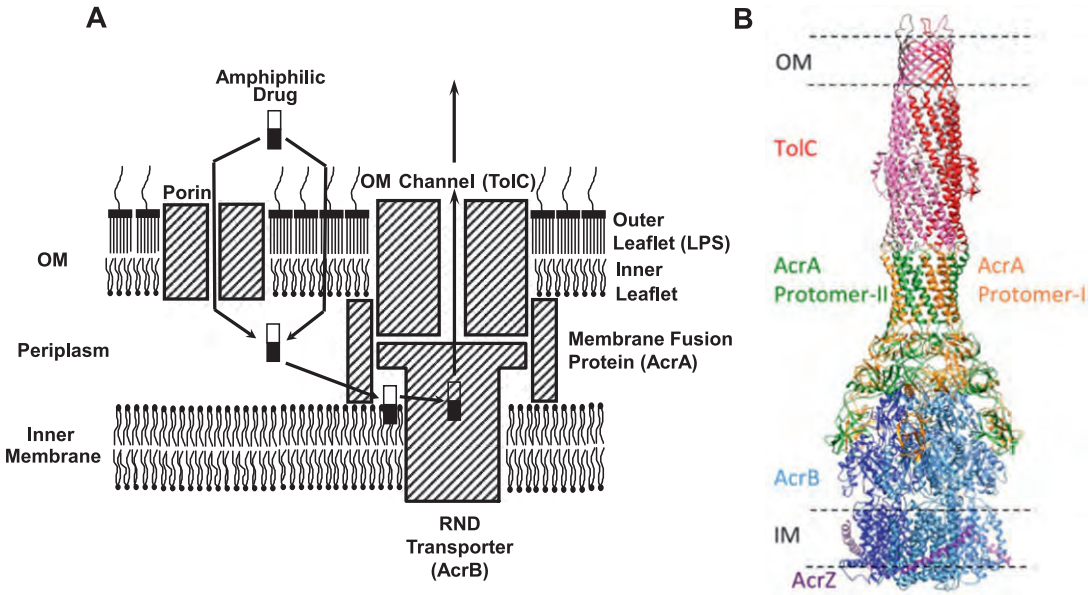

Fig. 14 AcrB-AcrA-TolC as an example of RND-type efflux pump complex. (A) A crude schematic depiction of the complex. Amphiphilic drugs slowly penetrate across the outer membrane, either through the porin channel or through the asymmetric bilayer, and upon reaching the periplasm are likely to become captured by the periplasmic domain of the transporter, to be expelled directly into the medium. (B) Cryo-EM structure of the entire complex (from Wang, Z., Fan, G., Hryc, C.F., *et al.*, 2017. An allosteric transport mechanism for the AcrAB-TolC multidrug efflux pump. eLife 29 (6), e24905. http://dx.doi.org/10.7554/eLife.24905). Component proteins are colored differently (TolC in red and pink; AcrA in orange and green, AcrB in blue, and AcrZ in purple. AcrZ is a recently discovered component that enhances the activity of this pump complex.

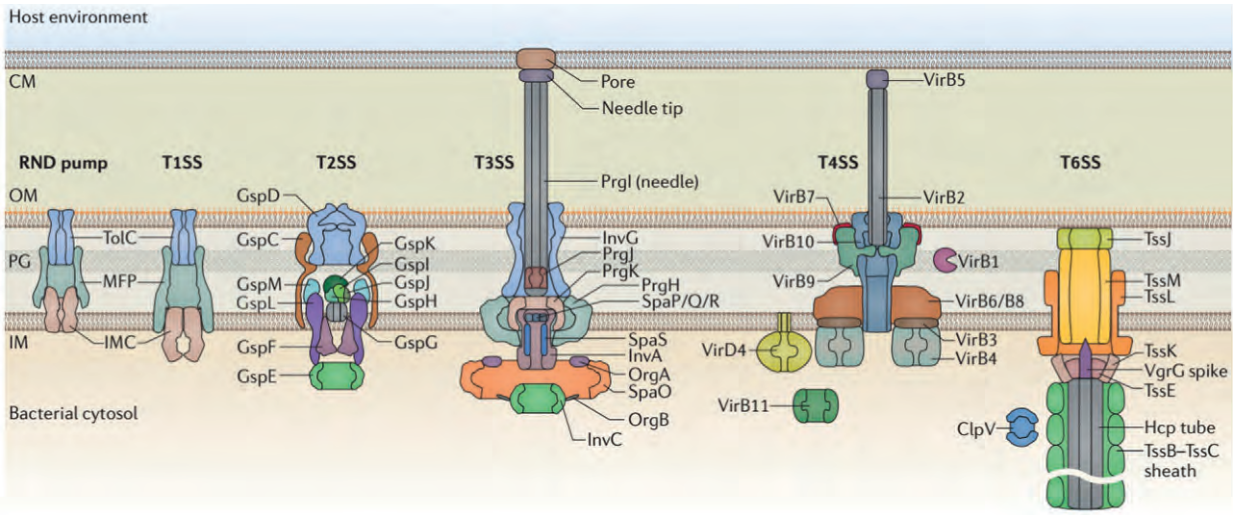

Fig. 15 Machineries for protein export in gram-negative bacteria. The figure shows the construction of protein secretion apparatus spanning the entire cell envelope, in a schematic manner. For the type 3 system, nomenclature for the *S. typhimurium* system is shown. The type 4 system shown is based on the nomenclature of the VirB/D system in *Agrobacterium tumefaciens*. The type 6 system nomenclature is those from enteroaggregative *E. coli* and *Vibrio cholerae*. This figure is from Costa, T.R.D., Felisberto-Rodrigues, C., Meir, A., *et al.*, 2015. Secretion systems in gram-negative bacteria: Structural and mechanistic insights. Nat. Rev. Microbiol. 13, 343–359.

cytoplasmic membrane by a transporter, a member of the ABC (ATP-Binding Cassette) superfamily. Although ABC transporters catalyze the active transport (both import and export) of a variety of substrates, both small and large, this system is unusual in the sense that the transporter exists as a multi-protein complex including the outer membrane channel protein TolC and a periplasmic linker protein, so that the exported protein can cross the entire cell envelope, without facing the aqueous environment of the periplasm. The system shown is utilized in the secretion, by *E. coli*, of hemolysin, a toxin. (An ABC system exporting drugs, MacAB, is coupled also to TolC using the periplasmic accessory protein MacA).

In the Type II secretion system (Fig. 15), the exported proteins include enzymes, such as pullulanase of *Klebsiella pneumoniae*. The substrate is exported across the cytoplasmic membrane usually by the SecYEG complex, but export across the outer membrane requires a complex machinery traversing both membranes, and is energized by a cytoplasmic ATPase GspE. Although the detailed mechanism is not yet clear, the overproduction of the major pseudopilin GspG (so called because of its homology to a class of pilins) in *E. coli* produces pili on the cell surface. Thus it is thought that the hydrolysis of ATP by GspE results in the polymerization of GspG (in a way similar to the polymerization of pilin by ATP hydrolysis), which produces a piston-like structure in the cavity, which would push the substrate protein through the outer membrane channel of GspD.

In the Type III secretion system (Fig. 15) again a complex assembly of many proteins are required. This system usually has a long, needle-like extension outside, and is often thought to "inject" proteins into the cytosol of the host cells. Indeed the genes coding for this system are often found in the "pathogenicity islands" on the bacterial chromosome, where many genes involved in the infection process are clustered together. Another fascinating aspect of this system is that many of its proteins are similar to the components of the basal bodies of bacterial flagella, suggesting that flagella and this secretion system have a common evolutionary origin.

Type IV secretion system is also complex and contains many proteins (Fig. 15). A prototype of this system is the VirB system, shown in the figure, that is responsible for the export of a piece of single-stranded DNA (and associated proteins) from the bacterium *Agrobacterium tumefaciens* into plant cells. (This system was used in the production of most of the genetically modified crop plants.) Interestingly, a very similar system is involved in the conjugational transfer of plasmid DNA from one bacterial cell to another.

Type VI secretion system often participates in competition between different species of bacteria, by injecting a toxic protein into cells of the competing bacterial species. It is evolutionarily related to the contractile sheath-tube assembly of bacteriophage tail, and is thus thought to function in a similar way, by the tail contraction leading to the injection of the toxic protein labeled VgrG spike in Fig. 15. After injection, the machinery is thought to become disassembled by the ClpV ATPase.

Mycobacterial Cell Envelope

Although mycobacteria are not gram-negative, their lipid-rich cell envelope appears to have an overall structure similar to the gram-negative envelope. This was suggested by the observation that the hydrocarbon chains of the cell wall lipids are in a state of very low fluidity, and are arranged in a direction perpendicular to the plane of cell surface (Fig. 16). However, unlike in the outer membrane, in mycobacteria the least permeable portion of the cell envelope or the outer membrane appears to correspond to its inner part,

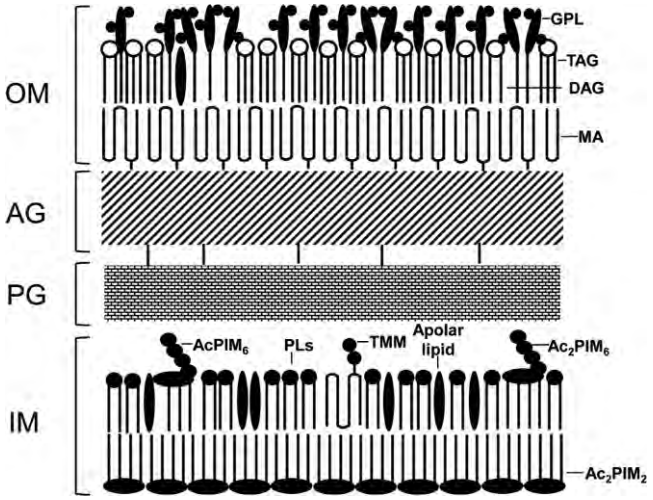

Fig. 16 A model of mycobacterial cell envelope, based on the quantitative analysis of outer and inner membrane lipids of *Mycobacterium smegmatis*. Reproduced from Bansal-Mutalik, R., Nikaido, H., 2014. Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. Proc. Nat. Acad. Sci. USA 111, 4958–4963. The envelope contains four layers, *i.e.*, OM (outer membrane), AG (arabinogalactan), PG (peptidoglycan), and IM, inner membrane. The inner leaflet of OM is composed of mycolic acid, a branched fatty acid with very long chains of uneven lengths (typically 24 and 60 carbons), covalently linked to AG, with its longer arm folded. The outer leaflet of OM is composed mostly of TAG (triacylglycerol), DAG (diacylglycerol), as well as GPL (glycopeptidolipid). IM is the conventional phospholipid (PL) bilayer, but we found that it contains a very high proportion of diacyl phosphatidylinositol dimannoside (Ac_2PIM_2), which was here assumed to comprise the entire inner leaflet. Since this phospholipid contains two additional fatty acid chains linked to the inositol and one of the mannose residues, it has four acyl chains linked to one head group, which may make mycobacterial IM bilayer less permeable than the usual phospholipid bilayer. IM contains also other (glycerol)phospholipids (PLs), diacyl phosphoinositol hexamannosides (Ac_2PIM_6), trehalose monomycolate (TMM), and apolar lipids.

where long, saturated hydrocarbon chains of mycolic acid residues are packed together. Indeed this portion of the cell wall seems to have a very low fluidity, and very low permeability for lipophilic molecules is expected for the mycobacterial cell wall. The mycobacterial cell envelope has an exceptionally low permeability also for hydrophilic solutes, even lower than the *P. aeruginosa* outer membrane in some species. Porins were identified in the envelope of fast-growing species such as *Mycobacterium smegmatis*, although they are very different proteins from gram-negative porins. The number and properties of these mycobacterial porins appear to explain the low permeability of the envelope.

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Overview of Plant Diseases☆

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Infectious diseases are caused by fungi, bacteria, viruses and nematodes. Fungi are organisms which have no chlorophyll, reproduce by sexual and asexual spores and typically possess mycelia or a mass of interwoven threads (hyphae). There are about 70,000 species living as parasites or saprophytes on other organisms or residues and more than 8000 species causing plant disease. The most serious and widespread fungal diseases of rose are powdery mildew caused by *Podosphaera pannosa* (Wallr.: Fr.) de Bary and black spot caused by *Diplocarpon rosae* Wolf.

Plant pathogenic bacteria are generally unicellular, nonspore-forming rods. There are about 80 species of bacteria which cause plant disease and many of them consist of numerous pathovars. The identification of bacteria can be very difficult; it involves special staining techniques, biochemical tests and serology. Pathogenic bacteria enter plants through insects or other wounds, through stomata, hydathodes, lenticels and flower nectaries. The most widespread bacterial disease of rose is crown gall caused by *Agrobacterium tumefaciens* (E. F. Sm. and Town.) Conn.

Viruses contain two major components: nucleic acid surrounded by a protein coat. There are over 850 described plant virus species. Some attack a large number of different plants and are of great economic importance: others are confined to a single host. Viruses are transmitted from plant to plant by insects, mites, fungi, nematodes, water, and by rubbing, abrasion or other mechanical means. Viruses are differentiated and identified by host specificity, physical properties, purification, electron microscopy, electrophoresis and serology. The most widespread viral disease of rose is rose mosaic. Rose mosaic is generally associated with isolates of *Prunus* necrotic ringspot virus (PNRSV) or with mixed infections with PNRSV, apple mosaic virus and *Arabis* mosaic virus.

Nematodes are unsegmented roundworms that inhabit fresh and salt water, decaying organic matter, soil, plants and animals throughout the world. More than 2000 species are parasitic on plants. Most plant nematodes are root parasites, but some live in stems, leaves or buds. Some cause galls and others produce general yellowing, stunting or dieback that is often ascribed to other causes such as nutrient imbalance.

Commercial production of plants free of disease requires frequent observations and intelligent strategic planning. Prevention is essential for good disease control. Although control can vary from partial to economic to perfect, the best disease control is when it is profitable to crop production. Even before the discovery of Bordeaux mixture, it was determined by trial and error that chemicals could be useful for pest control. For example, sulphur was known to avert pests by Homer in 1000 BC and was probably generally used by 1800. In 1824 Robertson recommended sulphur for control of powdery mildew of peach. Lime-sulphur found its way into disease control by 1906 and copper sulphate was recommended for roses in 1862.

These early observations and discoveries led to mixing lime and copper sulphate. This mixture was first used on grapes to deter pilfering in Médoc, France. However, Millardet in 1882 observed that the mixture (called Bordeaux mixture, after the location where it was first used) controlled the severe downy mildew disease of grapes. This discovery marked the true beginning of the modern era of chemicals to control plant disease. Bordeaux mixture is still used today; its greatest deterrent is a tendency to be phytotoxic. Sulphur, lime-sulphur and Bordeaux mixture were the major tools for disease control in ornamentals from 1900 to 1943.

The horizons for chemical controls were broadened measurably in 1934 with the announced development of alkyl dithiocarbamate fungicides. However, technical difficulties in development delayed by 10 years making them available to the public. Then in 1943 the dilemma was solved and nabam, zineb and maneb were introduced (the bisdithiocarbamate family of chemicals). These fungicides were broad-spectrum in control of fungal pathogens; production yields in agriculture increased 65% from 1901 to 1945 (a period of 44 years) and by 90% from 1945 to 1954 (a period of 9 years). The bisdithiocarbamates are still used today; however, their availability may be in peril due to the Food Quality Protection Act passed in 1996 by the US Congress.

Chemical soil fumigation, beginning with chloropicrin in 1948 and with methyl bromide in 1951, has been used in indoor and outdoor production of ornamental plants. Excellent control of soil-borne pathogens has been achieved with their use; however, alternatives are being sought due to toxicities associated with their use and their likely elimination for use by 2006.

The use of chemicals to control fungal, nematode and bacterial pathogens from 1945 to 1975 was exciting to plant pathologists and the industry since most disease could be controlled and better and more flowers grown and sold. Prior to 1945 disease control amounted to "putting out fires" and after 1945 it was preventive. Preventive disease control programmes could be designed and successfully performed with proper use of pesticides. Exceptions to this success story were systemic disease and viral-caused diseases. Systemic fungicides and viricides were difficult to develop since the genomes of pathogens and host plants shared some similarities; host and pathogen physiology shares some similar functional responses to some chemical compounds. Thus, pesticides that would control vascular wilts caused by fungi, bacteria and viral pathogens were also probably phytotoxic to the host. For example, prior to 1942 the control of *Verticillium* wilt of chrysanthemums and other vegetatively propagated ornamentals was dependent on selection of stock plants which were visually free from disease, on use of short terminal cuttings and the application of best preventive sprays

☆*Change History*: January 2017. R.K. Horst made edits to the text and updated the references.

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then available. This programme enabled the small grower to avoid disastrous losses, but few were ever free from *Verticillium*. However, during the 1940s and 1950s propagation became concentrated in the hands of a few specialists.

In 1942 A.W. Dimock showed that laboratory procedures for determining the presence or absence of pathogenic fungi and bacteria in the vascular tissues of plants had potential for commercial adaptation. The culture-index was enlarged to include virus-indexing, i.e., culture virus-indexing. The programme also included thermotherapy and meristemtip culture methods for obtaining plants free of virus when detection methods showed them to be infected. The success of the chrysanthemum programme led to similar culture virus-indexing programmes for carnations, geraniums, roses and orchids. These programmes are being used commercially for ornamentals on the European continent as well as in North and South America. These classic programmes have also been modified for use in agronomic crops. The employment of the culture virus-index programme has been so effective that many young growers do not recognize some of the diseases which in 1940 caused costly losses to every chrysanthemum grower in the business.

In 1961 a landmark book was published by Rachel Carson, entitled *Silent Spring*. This book had a major impact on the indiscriminate and successful use of pesticides. The main focus was on DDT and toxic environmental influences of its use. Public concerns raised by this book caught the attention of the US government which took action to establish the US Environmental Protection Agency (EPA) in 1970. The EPA's responsibility was to register and regulate the use of pesticides. These restrictions required exceptional planning in ornamental production.

During the 1980s there was increased interest in the reduced use of toxic pesticides in the environment. These concerns generated more interest in the use of integrated pest management (IPM). The main objective of IPM is to reduce the use of toxic chemicals which contaminate the environment and still control diseases. An important component of a successful IPM programme is the forecast of conditions which are favourable for disease epidemics and to spray only when necessary. IPM strategies which can be used include the following:

- Apply pesticides only when necessary.
- Make use of application methods that apply less pesticide or use a more efficient spray system.
- Use biocompatible chemicals as they become available.
- Use biological controls when available and when appropriate.
- Use cultural practices which are favourable to healthy plant growth.

A successful IPM programme depends on four basic techniques:

1. Scouting: regular and random visual observations provide early warning to disease problems.
2. Disease identification: the first and most important step is to identify the problem; misdiagnosis results in improper control.
3. Timing: improper timing of control measures will result in disease control failure; the control measure must be timed correctly to the stage of disease development.
4. Records: brief accurate records are a good tool for disease control decisions.

A grower's use of IPM strategies can reduce pesticide use by 50%. During the mid-1980s and into the 1990s there has been intense interest in the development and use of biological controls, genetically engineered disease-resistant plants, biocompatible chemicals and more efficient spray delivery systems. Biological controls have been developed from species of *Trichoderma*, *Streptomyces* and *Gliocladium* for control of fungal-caused diseases. Biocompatible chemicals are "soft" chemicals and/or "natural" substances that may bear profound antimicrobial properties and that exhibit low mammalian and environmental toxicities. Some examples are bicarbonates (used in baking), neem from the neem tree (*Melia azedarach*), horticultural oils and strobulins (from mushrooms). There has also been interest in improved efficiency in spray delivery systems, such as air-assisted aerosol generators and air-assisted electrostatic sprayers. Fungicides are dispersed effectively and at reduced dosages.

On 6 August 1996 the US Congress passed the Food Quality Protection Act. This Act requires EPA to reassess tolerances of all 9600 pesticides available to the industry. Thirty-three percent of these pesticides must be reevaluated by August 1999, 66% by 2002 and all of them by 2006. The impact this process will have on the ornamental industry will be great since it is likely that many commonly used pesticides will be eliminated for commercial use. Alternative disease control measures will become more important and in some instances disease control will be difficult. Growers and plant pathologists will need to develop creative disease control strategies to be successful in a very competitive ornamental market.

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P

Palaeontology, Microbial☆

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Glossary

Kerogen Carbonaceous organic matter in rocks, typically composed of interlinked polycyclic aromatic hydrocarbons.
Permineralization A process of fossilization that produces a mineral-infused inorganic-organic mix that can preserve physically robust structures such as organic-rich cell walls.
Phanerozoic The segment of geological time extending from the earliest appearance of shelly invertebrate animal fossils, ~550 million years ago, to the present.
Precambrian The segment of geological time extending from the formation of the planet, ~4500 million years ago, to the beginning of the Phanerozoic.
Stromatolite A laminated rock structure, commonly composed of carbonate minerals, formed by the microbially mediated accretion of laminae from the surface of an ancient seafloor or lake bottom.

Fossil Evidence of Ancient Microbes

Overview of the Fossil Record

As is shown in Fig. 1, geological time is divided into two major segments: the Precambrian Eon, the longer of the two segments that spans the earliest seven-eighths of Earth history and begins with the formation of the planet ~4500 million years ago; and the Phanerozoic Eon, a younger and much shorter segment that extends from the first appearance of shelly invertebrate animal fossils, ~550 million years ago, to the present. The science of paleontology is focused primarily on the fossil record of Phanerozoic, megascopic plants and animals. Because of their economic importance to the petroleum industry in the search for gas and oil, the fossil records of various groups of Phanerozoic microscopic protists (eg, foraminiferans, radiolarians and coccolithophorans) and of the spores and pollen of fossil plants have been intensively studied. But the Phanerozoic record of many other groups of microscopic fossils, most notably including that of fossil microbes, is largely undocumented.

In contrast to the Phanerozoic microbial fossil record, that of the earlier Precambrian is voluminous, known from hundreds of deposits and more than a thousand described taxa, showing that Precambrian microbes were abundant, ubiquitous, metabolically diverse, and biotically predominant. Nevertheless, this early fossil record, virtually all of which has become known since the mid-1960s, is far from complete. Due to the "geologic cycle," the repeated sequence of mountain-building, erosion, and deposition into sedimentary basins of eroded mineral grains thus produced, the average "lifetime" of a geological unit is ~200 million years. The rock record that has survived to the present therefore peters out with increasing geologic age (Fig. 1). Thus, although the fossil record of cellularly preserved microbes extends far into the Precambrian – throughout all of the Proterozoic (~550 to 2500 million years ago) and much of the Archean (>2500 million years ago) – in units older than ~2000 million years the known record of preserved microbes becomes increasingly sparse and patchy, and the history of the various fossil lineages becomes increasingly difficult to decipher.

Stromatolites

Stromatolites are laminated rock structures, most commonly composed of carbonate minerals (eg, calcite, $CaCO_3$), that formed by the microbially mediated accretion of laminae, layer upon layer, from the surface of an ancient seafloor or lake bottom. Their layered structure reflects the photosynthetic metabolism of the mat-building microorganisms. The thin (millimeter-thick) mats

☆*Change History*: April 2016. J. William Schopf modified the spelling of "Palaeontology" in title; added new Keywords and Glossary; made amendments and updates to text and "Further Reading" section; New sections of text added: III.C.2 Sulfuretum Fossils of Uncertain Systematic Position; IV.A.1 Cyanobacteria; IV. A.2 Sulfur-cycling Bacteria; revised Figure 1 and added Figure 13.

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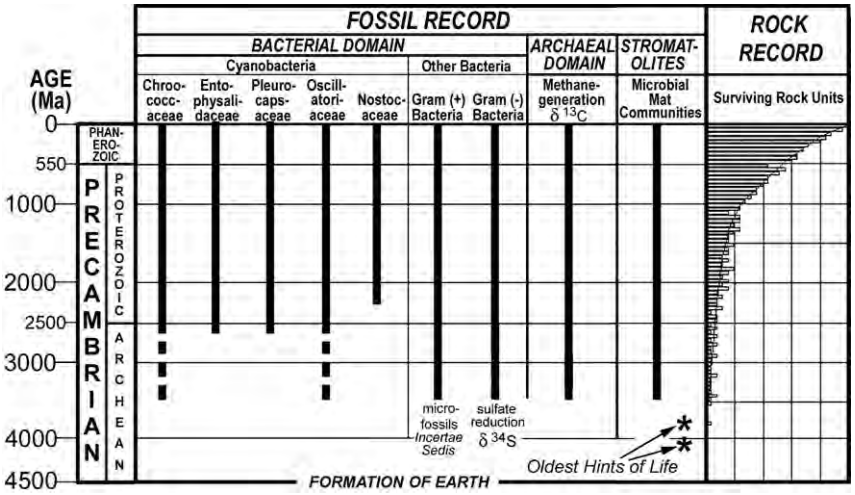

Fig. 1 The known fossil record of Bacteria, Archaea, microbially laminated stromatolites and surviving rock units over geological time.

formed as such microbes multiplied and spread across surfaces and were subsequently intermittently veneered by detrital or precipitated mineral grains that blocked sunlight. To maintain photosynthesis, mobile members of such communities, such as gliding cyanobacterial oscillatoriaceans, moved upward through the accumulated mineral matter to establish a new overlying microbial mat. The repeated accretion, compaction and subsequent lithification of such mats, their microbial community commonly augmented by an influx of non-mobile microbes (such as chroococcacean, entophysalidacean, and pleurocapsacean colonial cyanobacteria) can result in the formation of stromatolitic structures that range from small millimetric columns and pustular mounds to large, decimetric, bioherms. During diagenesis, the series of changes that lead to the lithification of such deposits, silica (SiO_2) or, more rarely, phosphate-containing minerals, can replace the initially deposited carbonate grains. If replacement occurs early, before the mat-building microorganisms decay and disintegrate, cellularly intact microbes can be preserved. However, the great majority of stromatolites, unchanged by such replacement, are devoid of fossil microbes: during diagenesis, carbonate grain growth crushes and obliterates the stromatolite-forming microbes, leaving only an amorphous thin coaly residuum of microbe-derived carbonaceous matter.

Cellular Fossils

Two principal processes preserve cellular microbial fossils: compression and permineralization. Compression-preserved microbes occur in fine-grained detrital sediments such as shales and siltstones, pressed and flattened along bedding planes as the sediment lithified. Such fossils are rare in sediments of the Phanerozoic, evidently due to the bioturbation of such deposits by invertebrate metazoans, but are well known from the Precambrian.

The microbial fossil record is best known from microorganisms preserved by permineralization. Of all modes of fossil preservation, this process (known also as "petrification") provides the most faithful representation of original biologic morphology. Such preservation, common for plants and fungi as well as fossilized microbes, results from the pervasion of mineral-charged solutions into cells during the early stages of diagenesis, prior to their disintegration. The permeating fluids infill micellar, intercellular, and intracellular spaces – replacing the watery milieu of their biomolecular components – to produce a mineral-infused inorganic–organic mix that can preserve physically robust structures such as organic-rich cell walls. As a result, both the organismal morphology and cellular structure of such fossils can be preserved in microscopic detail. Although permineralized microbes have been reported from a variety of matrices (eg, quartz, carbonate, apatite, and gypsum), the most common such matrix is quartz (silica), the primary mineral that comprises the rock-type known as chert or flint. Hundreds of microbe-preserving cherts are known from the Precambrian when silica was abundant in the world's oceans, well before the Phanerozoic appearance of silica-mineralized sponges, diatoms and radiolarians that today mediate the oceanic silica budget. Such cherts can contain exquisitely preserved fossil microbes.

Stromatolites: Lithified Microbial Mats

Carbonate microbial stromatolites occur today (Fig. 2A, B, D) that in shape, size, environmental setting and laminar structure are much like those known from the geological past (Fig. 2C–F). In Phanerozoic sediments, fossil stromatolites are rather uncommon, evidently due to the disruption of stromatolite-forming microbial mats by grazing and burrowing metazoans. But in shallow-water carbonate deposits throughout the Precambrian such structures are abundant and morphologically diverse, known earliest from rocks ~3500 million years old (Figs. 2F, 3 and 4). Their distribution over time parallels that of the fraction of the originally deposited Precambrian rock record that has survived to the present: stromatolites are common in younger-aged Precambrian

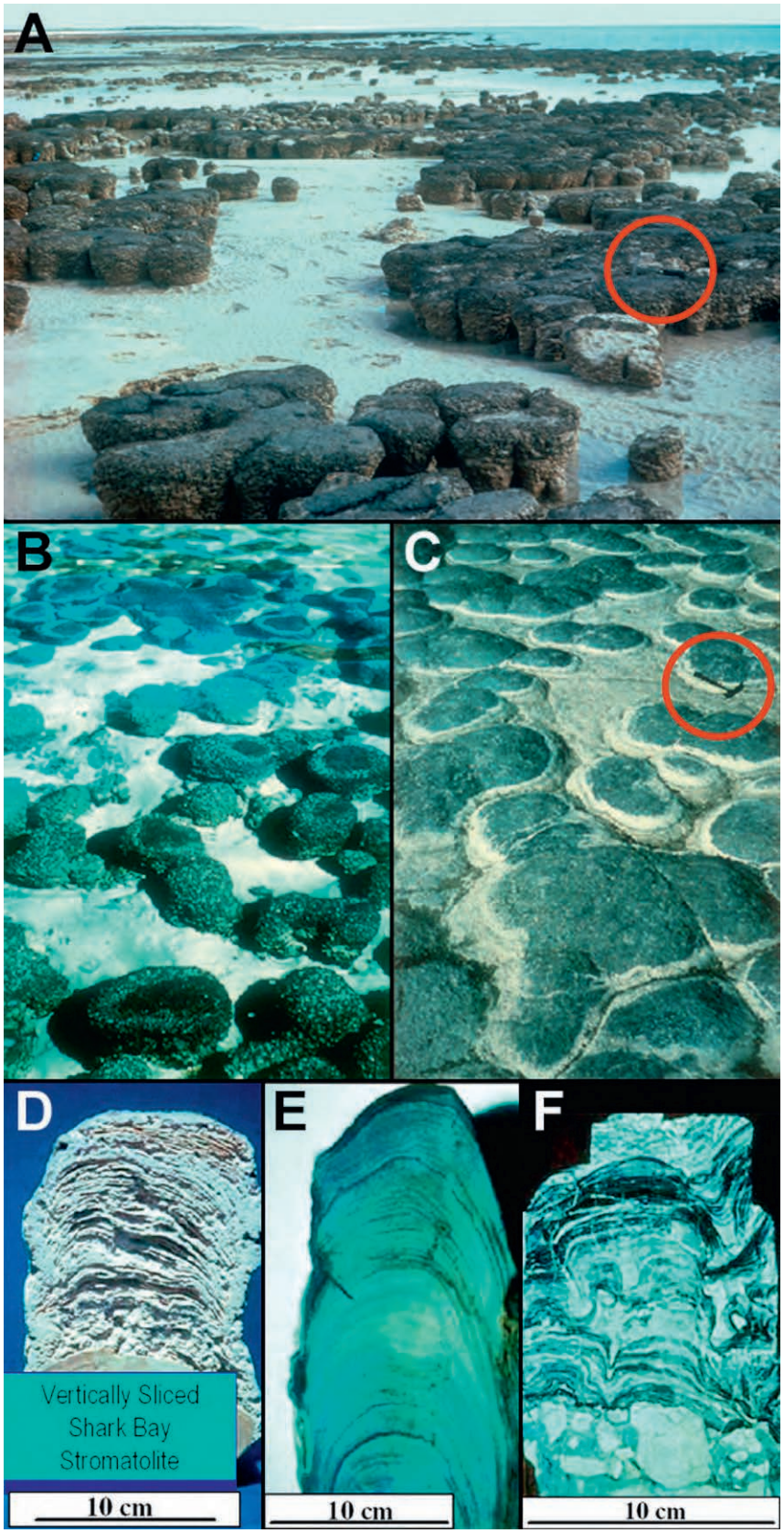

Fig. 2 Modern and fossil stromatolites. (A) Modern stromatolites at Shark Bay (Hamelin Pool), Western Australia. (B) Modern Shark Bay columnar and domical stromatolites for comparison with (C) fossil stromatolites from the ~2300-Ma-old Transvaal Dolomite, Cape Province, South Africa. (D through F) Modern and fossil vertically sliced columnar to domical stromatolites showing accretionary microbial lamination from Shark Bay (D), the ~1300-Ma-old Belt Supergroup of Montana (E), and the ~3350-Ma-old Fig Tree Group of the eastern Transvaal, South Africa. Scale for (A) and (C) shown by the geologic hammers enclosed by red circles.

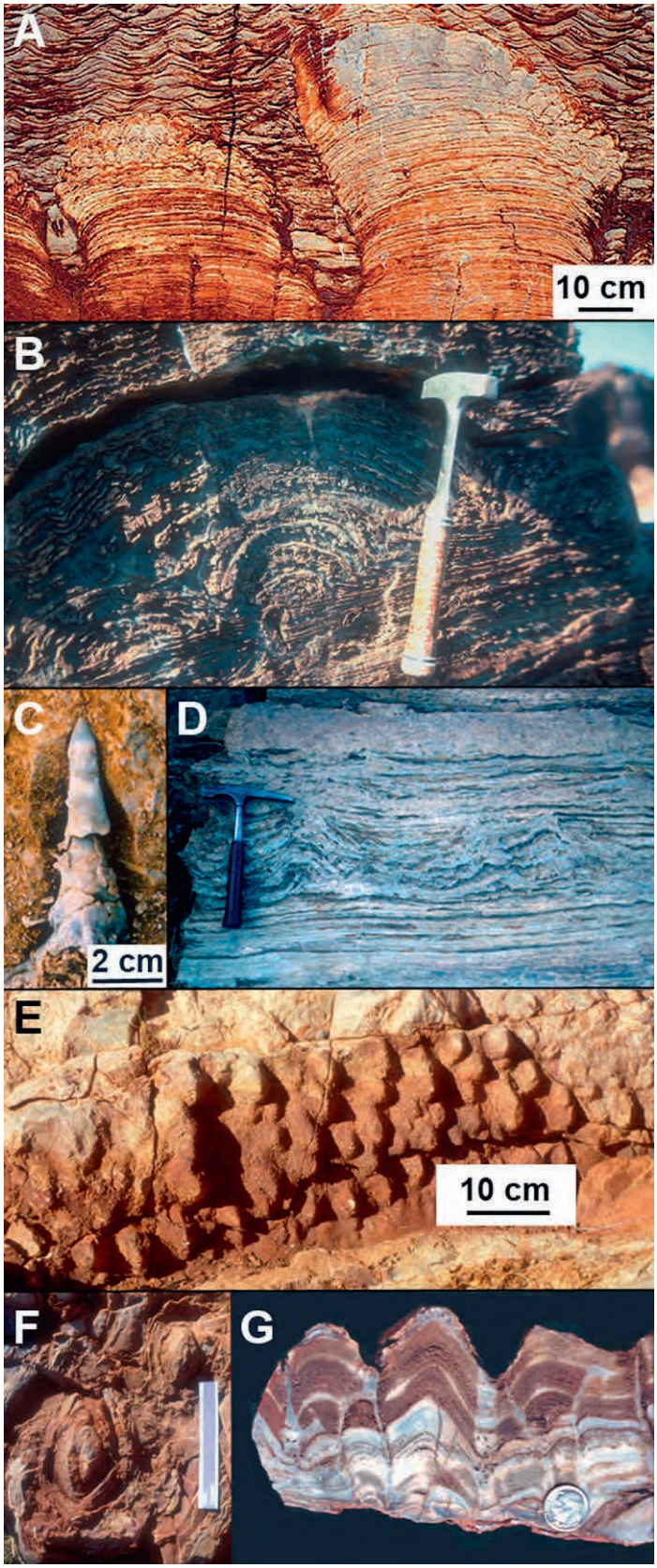

Fig. 3 Archean-age microbially laminated stromatolites. (A) Domical, pseudocolumnar and branching stromatolites, overlain by rippled sediments, and (B) a domical stromatolite, from the ~2723-Ma-old Tumbiana Formation, Fortescue Group, Western Australia. (C) Conical stromatolite, and (D) stratiform and conical stromatolites, from the ~2985-Ma-old Insuzi Group, South Africa. (E through G) Laterally linked conical stromatolites from the ~3388-Ma-old Strelley Pool Chert of Western Australia.

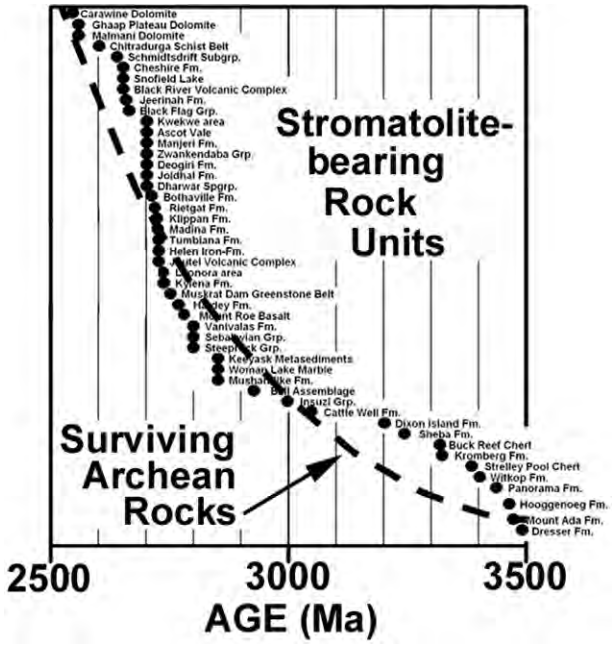

Fig. 4 Comparison of the temporal distribution of the 48 Archean-age stromatolite-bearing rock units now known (●) with that of Archean-aged rocks that have survived to the present (cf. Fig. 1).

deposits but become more rare as the rock record peters out with increasing age (Fig. 4). Such structures establish the presence of flourishing photosynthesis-based microbial communities, but because of their carbonate mineralogy they do not contain cellular fossils that might indicate whether the stromatolite-building photoautotrophs were oxygenic, like cyanobacteria, or anoxygenic, like photosynthetic bacteria.

Cellular Microbial Fossils

Taxonomy

Paleontological taxa

Although current microbial classifications are based primarily on biomolecular data (eg, rRNA-, genome-, and DNA base-compositions), for many modern microbial taxa such assignments are consistent with traditional morphology-based groupings. For example, cyanobacteria, the principal stromatolite-formers over geologic time, have been assigned on the basis of their pattern of structure and development to five "sections" (cf. morphology-defined taxonomic families) that for many genera mesh well with biochemically based classifications. Section I (cf. Chroococcaceae) is comprised of predominantly spheroidal, solitary and colonial unicellular cyanobacteria that reproduce by fission or by budding. Section II (cf. Pleurocapsaceae) consists of unicellular or pseudofilamentous forms that by multiple fission give rise to small daughter cells (baeocytes). Section III (cf. Oscillatoriaceae) encompasses uniseriate cyanobacterial filaments that lack cellular differentiation (eg, *Oscillatoria* and *Spirulina*). Section IV (cf. Nostocaceae) includes simple uniseriate filaments that exhibit cellular differentiation into akinetes and heterocysts. Section V (cf. Stigonemataceae) is composed of morphologically more complex filamentous cyanobacteria that exhibit true branching and heterocysts. Representatives of all of these groups are known from the fossil record, Sections I–IV dating from well into the Precambrian (Fig. 1) whereas representatives of Section V are known earliest from *Stigonema*-like fossils of ~400 million-year-old Rhynie Chert of Scotland. In general, Sections II, IV and V are consistent with biochemically based phylogenies whereas cyanobacteria included in Sections I and III do not appear to comprise monophyletic lineages. Nevertheless, because the biochemical components of microbes, like those of all living systems, are geochemically labile – converted over geological time to coaly kerogen, a complex mix of interlinked polycyclic aromatic hydrocarbons – the classification of such fossils is necessarily based on their morphology, not on their original biochemistry.

Identification of the major fossil types

As is shown in Fig. 1, four major categories of ancient microbes are known from the fossil record: (1) cyanobacteria; (2) fossils of uncertain systematic position (Bacteria *Incertae sedis*); (3) sulfur-cycling, including sulfate-reducing bacteria; and (4) methane-producing archaeans. Of these, the microbial fossils *Incertae sedis* are all regarded as members of the Bacterial rather than the Archaeal Domain, the uncertainty of their systematic position reflecting their morphological similarity both to cyanobacteria and to

members of other bacterial groups. The sulfur-cyclers and the methane-producers are known only or predominantly from isotopic evidence, not from morphologically preserved microscopic fossils. Of the four categories, cyanobacteria have the best documented fossil record, known from thousands of cellularly preserved specimens. Many such cyanobacterial fossils are essentially indistinguishable from their modern morphological counterparts in life cycle, behavior, and ecologic setting.

In comparison with other microbes, whether bacterial or archaeal, most cyanobacteria have somewhat larger cells and more complex morphology. Because of their light-requiring oxygenic photosynthetic metabolism, cyanobacteria occupy the uppermost surface of microbial mats, rather than lower regions of such biocoenoses where decay and cellular disintegration are prevalent. For this reason, cyanobacteria have a higher probability of becoming incorporated in the fossil record as cellularly intact specimens than do other prokaryotes, especially if they are preserved by permineralization during the early stages of sediment lithification. Of the various morphological components of cyanobacteria, extracellular sheaths and envelopes, initially composed largely of pentose sugar-containing carbohydrates and relatively resistant to degradation, are the most commonly preserved. Although physically robust and organic-rich cell walls are somewhat less commonly preserved, and in the cells of fossilized filamentous forms the originally peptidoglycan-containing lateral walls are much more commonly preserved than their peptidoglycan-deficient transverse walls. The organic components of the aqueous cytosol of such cells are never preserved intact. Along with their watery milieu, such organics are usually leached out of the cells during preservation, but small aggregates of intracellular carbonaceous remnants occur in some cyanobacterial fossils.

Cyanobacteria

Coccoidal and ellipsoidal fossils (Chroococcaceae, Entophysalidaceae, Pleurocapsaceae)

In Fig. 5 are shown photomicrographs that illustrate the morphological similarities of extant coccoidal and ellipsoidal cyanobacteria (Chrooccaceans, Entophysalidaceans, and Pleurocapsaceans; Fig. 5A, E, G, respectively) to their fossilized counterparts (Fig. 5B, F, H). Fig. 5C shows an image of a sheath-enclosed chroococcacean colony within a thin slice of rock, obtained by the use of confocal laser scanning microcopy (CLSM), for comparison with an optical image of the same specimen (Fig. 5D). Use of CLSM provides high-resolution three-dimensional images of such rock-embedded fossils unavailable from standard photomicrography. The morphological similarity of the fossil and modern cyanobacteria shown in Fig. 5 is typical of Chroococcaceans, Entophysalidaceans and Pleurocapsaceans generally, groups that have established fossil records dating from more than 2500 million years ago (Fig. 1).

Fig. 6 shows a fossil stalk-forming pleurocapsacean that provides evidence of its life cycle (inferred from specimens preserved at varying stages in its development), light-seeking "behavior" (evidenced by the orientation of its elongate stalks), and ecologic setting (documented by paleoenvironmental facies relationships). Like its modern morphological and ecological counterpart, *Cyanostylon* of the Bahamian tidal banks, this ancient pleurocapsacean colonized surfaces by producing laterally extensive pincushion-like aggregates. The virtually identical morphology, life cycle, behavior, and ecologic setting of this fossil pleurocapsacean and its living counterpart are typical of the coccoidal and ellipsoidal cyanobacteria known from the geologic record.

Filamentous taxa (Oscillatoriaceae, Nostocaceae)

Like coccoidal and ellipsoidal cyanobacteria, modern and fossil filamentous members of the group commonly exhibit essentially identical ecologic settings and organismal and cellular morphologies. Among the several extant families of filamentous cyanobacteria, stromatolite-building members of the Oscillatoriaceae have the most extensive fossil record, represented by diverse fossils in hundreds of ancient microbial communities (eg, Fig. 7). However, detailed studies of fossil oscillatoriaceans show that their optically discernable morphology (Fig. 7B, D, F, H) does not always accurately reflect their true structure. In modern oscillatoriaceans, cells divide by the centripetal invagination of partial septations that fuse in the center of a cell to produce transverse cell walls (Fig. 8). The lateral cell walls of such filaments are about twice the thickness of their transverse walls, and they contain rigidifying pepdidoglycans that are absent from partial septations and transverse walls except at the cell periphery. Because of these differences, lateral cell walls tend to be relatively well preserved in fossil specimens whereas transverse walls, and their precursor partial septations, are preserved only in part. A fossil oscillatoriacean that exhibits such differential preservation is illustrated in Fig. 9 in which an optical image of the specimen (Fig. 9C) is compared with images obtained by use of CLSM (Fig. 9D–H) and three-dimensional Raman imagery (Fig. 9I–K). The Raman images show that the biopolymers originally comprising the cell walls have been converted to carbonaceous kerogen. They and the CLSM images also document the presence of thin incompletely preserved partial septations, showing that this ancient oscillatoriacean divided by the same processes, and in a living state evidently exhibited the same wall structure composed of the same biochemicals, as extant members of the family. Data such as these establish that the fossil record of the Oscillatoriaceae extends deep into geological time (Fig. 1) and that such cyanobacteria have changed little in form, function and metabolic requirements over thousands of millions of years of evolutionary history.

In comparison with the fossil record of ocillatoriaceans, that of similarly filament-forming nostocaceans is poorly known. A prime characteristic of the Nostocaceae is the presence of intercalary heterocysts, thick-walled cells that protect from oxidation the nitrogenase enzyme complex used to fix atmospheric nitrogen. Such heterocysts develop when nostocaceans are deprived of other sources of usable nitrogen (viz., nitrate and ammonia). In stromatolitic microbial biocoenoses, however, bacterially generated ammonia is plentiful, so the nostocaceans of such communities, both fossil and modern, are not heterocystous (such differentiated cells being earliest known from *Stigonema*-like Section V fossils of the non-stromatolitic Devonian Rhynie Chert).

Nevertheless, based on organismal and cellular morphology, numerous ancient fossils have been assigned to the Nostocaceae. One such example is shown in Fig. 10B, compared with modern *Nostoc* (Fig. 10A). Fig. 10C–E shows images of a fossil akinete, a

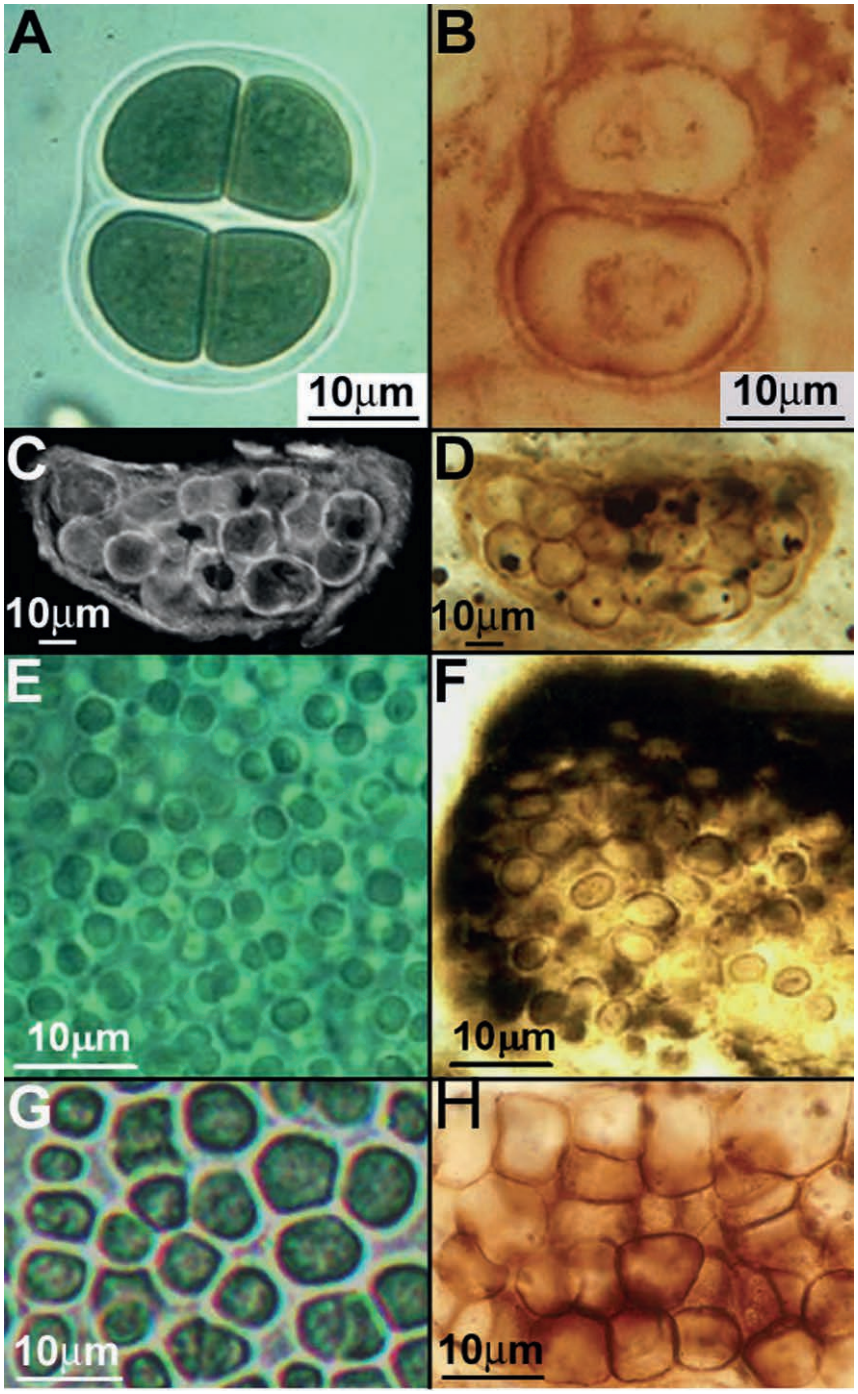

Fig. 5 Modern and fossil coccoidal and ellipsoidal cyanobacteria; all fossils are shown in petrographic thin sections of stromatolitic carbonaceous chert. (A) Modern *Gloeocapsa* sp. (Chroococcaceae) for comparison with (B) *Gloeodiniopsis uralicus* from the ~1500-Ma-old Satka Formation of Baskiria, Russia. (C) Confocal laser scanning micrograph and (D) optical photomicrograph of *Myxococcoides inornata*, an ensheathed colonial chroococcacean, from the ~650-Ma-old Chichkan Formation of southern Kazakhstan. (E) Modern *Entophysalis* sp. (Entophysalidaceae) for comparison with (F) *Eoentophysalis belcherenisis* from the ~2100-Ma-old Kasegalik Formation, Belcher Group, of North West Territories, Canada. (G) Modern *Pleurocapsa* sp. (PCC 7327, Pleurocapsaceae) for comparison with (H) *Paleopleurocapsa reniforma* from the ~650-Ma-old Chichkan Formation of southern Kazakhstan.

reproductive resting cell characteristic of nostocaceans that is known from geologic units dating to ~2100 million years ago. The temporal distribution of the Nostocaceae shown by such fossils fits with other lines of evidence. Geochemical data indicate that a stable oxygen-rich environment became established ~2300 million years ago, before which the nitrogenase-protecting heterocysts of akinete-producing nostocaceans would have been of little selective advantage. Further, rRNA phylogenies indicate that the Nostocaceae, like other heterocystous cyanobacterial families, originated in a burst of evolution well after the appearance of

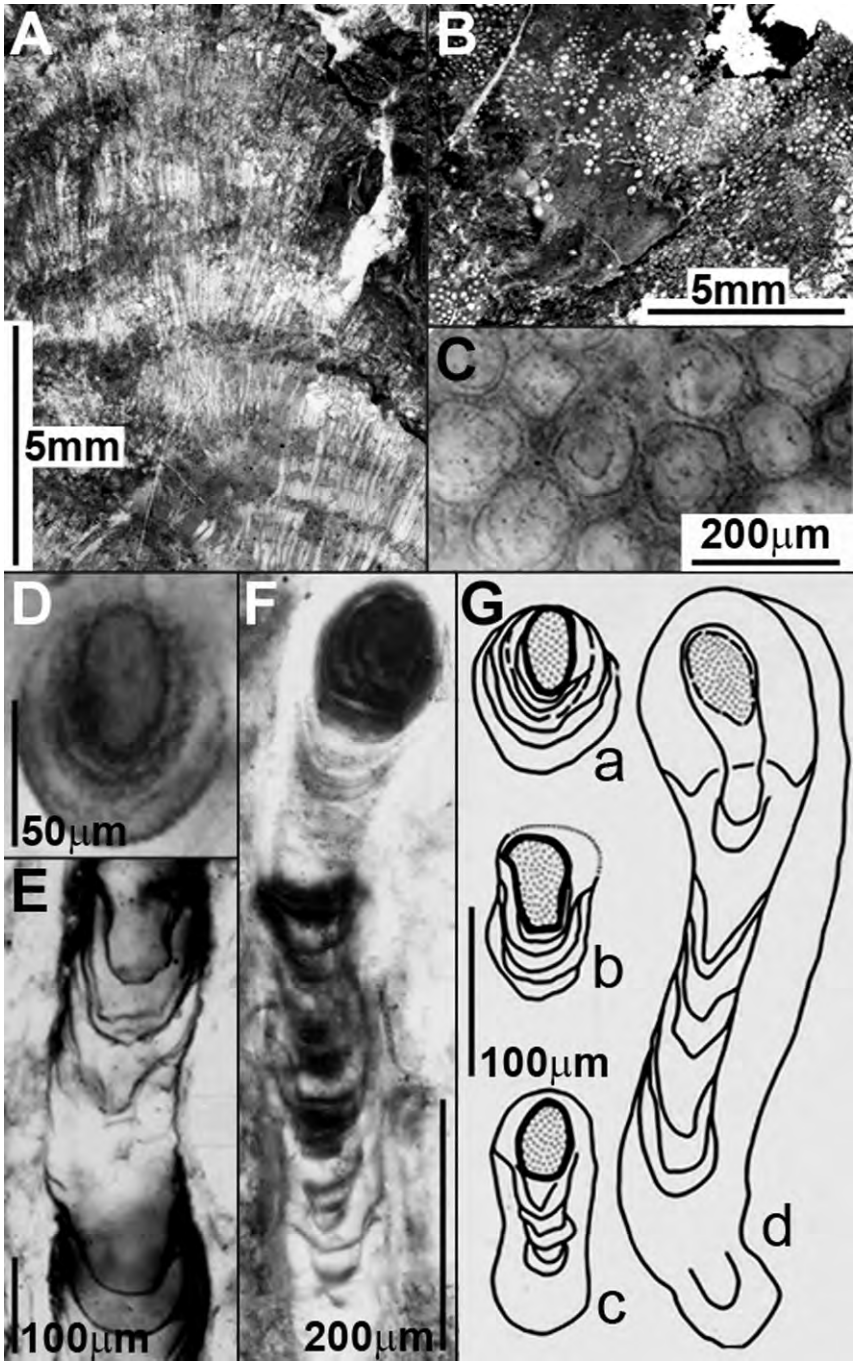

Fig. 6 Specimens of the colonial stalk-forming pleurocapsacean cyanobacterium *Polybessurus bipartitus* in petrographic thin sections of stromatolitic chert from the ~775-Ma-old River Wakefield Subgroup of South Australia. (A through C) Vertically oriented and radiating, pincushion-like originally mucilaginous extracellular stalks shown in longitudinal (A) and transverse sections (B and C). (D) A single, mucilage-embedded, ellipsoidal cell at the upper surface of the colony. (E and F) Fossilized asymmetrically laminated mucilaginous stalks, in (F) capped by the stalk-forming cell. (G) Interpretive drawings (based on tracings of photomicrographs) showing from (a) through (d) the ontogeny of stalk-formation.

coccoidal, ellipsoidal, and non-heterocystous filamentous oscillatoriaceans. Like other cyanobacteria, nostocaceans appear to have evolved little or not at all since their origination more than 2000 million years ago (Fig. 1).

Bacteria *Incertae Sedis*

Fossils regarded as Bacteria *Incertae sedis* – that is, fossil prokaryotes of the Bacterial Domain that cannot be firmly identified as members of any particular extant microbial group – are known throughout the geological record. Such remnants constitute the great majority of the fossils now known from Archean-age rocks. Because of geologic recycling, and the resulting decrease in the preserved

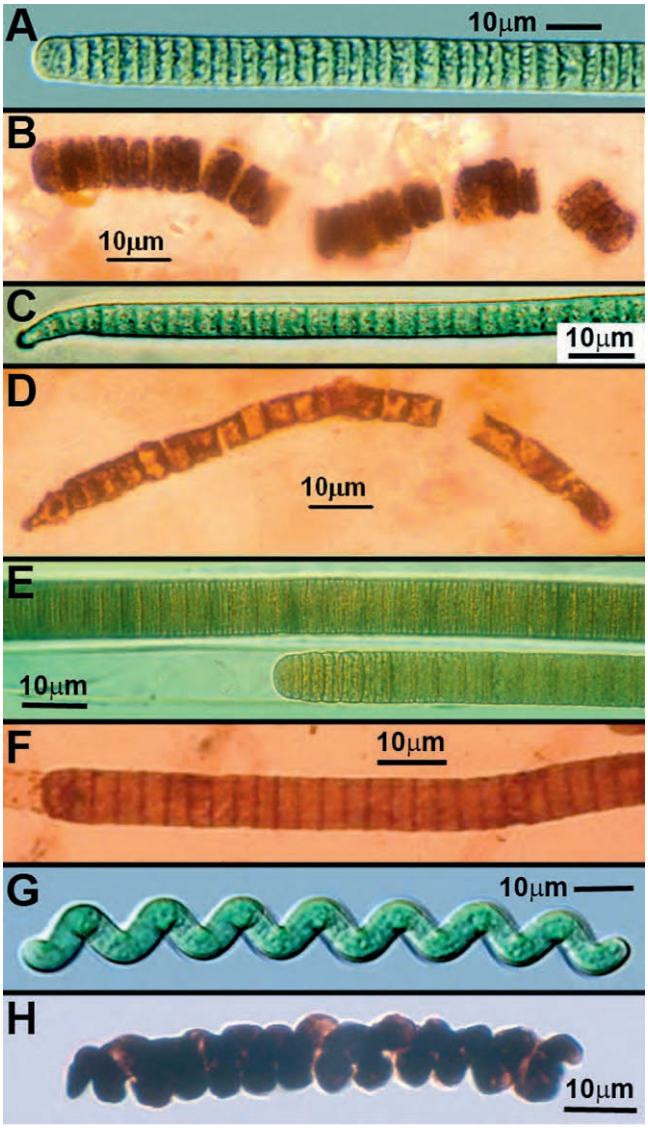

Fig. 7 Modern and fossil filamentous (oscillatoriacean) cyanobacteria; fossils in (B) and (D) are shown in petrographic thin sections of stromatolitic carbonaceous chert whereas those in (F) and (H) have been separated from their siltstone matrices by acid maceration. (A) Modern *Oscillatoria* sp. for comparison with (B) *Oscillatoriopsis breviconvexa* from the ~750-Ma-old Bitter Springs Formation of central Australia. (C) Modern *Oscillatoria amoena* for comparison with (D) *Cephalophytarion grande* from the ~750-Ma-old Bitter Springs Formation of central Australia. (E) Modern *Lyngbya* sp. for comparison with (F) *Paleolyngbya helva* from the ~950-Ma-old Lakhanda Formation, Siberia, Russia. (G) Modern *Spirulina* sp. for comparison with *Heliconema turukhania* from the ~850-Ma-old Miroedikha Formation, Siberia, Russia.

rock record with increasing geological age, the record of Archean fossils is sparse, reported from only some 20 Archean units, 2500 to 3500 million years in age, and represented by fewer than 10 fossil morphotypes. Of these Archean units, all but a few dates from the interval between 3200 and 3500 million years ago, well documenting the existence of microbe-level life at least this early in Earth history. For most of these ancient microbes, the uncertainty in their classification stems from their morphological similarity both to cyanobacteria and to non-cyanobacterial bacteria.

Representative fossils of uncertain systematic position

The specimens shown in Figs. 11 and 12 are representative of the Archean microscopic fossils now known. Rod-shaped fossils (Fig. 11A–F), small-diameter coccoids (Fig. 11G–R), and narrow filaments (Fig. 11S–Y) predominate, but larger-diameter uniseriate cellular microbes also occur (Fig. 12). Certain of the small-celled (Fig. 11A–F) and narrow filamentous forms (Fig. 11, S–Y) seem likely to be non-cyanobacterial bacteria, but others (Fig. 11G–R) have morphologies consistent with their assignment either to relatively large-sized non-cyanobacterial microbes, such as some sulfur-cycling bacteria, or to small-sized cyanobacteria. Similarly, the relatively large-diameter uniseriate trichomes shown in Fig. 12 are morphologically similar to extant and fossil cyanobacteria (eg, Fig. 7A–F) but are comparable also to large-celled sulfur-metabolizing bacteria (eg, *Thioploca* and *Beggiatoa*). A firm taxonomic

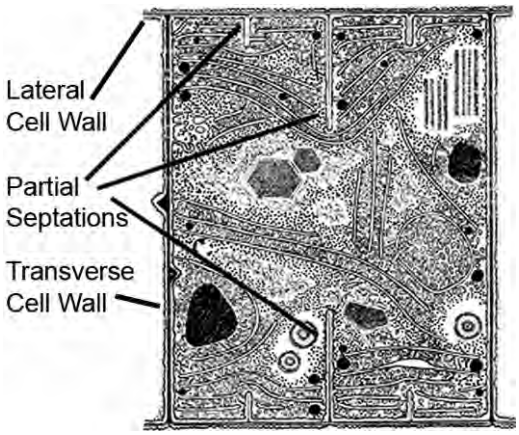

Fig. 8 Schematic drawing, based on transmission electron micrographs, of a medial section of a cell of a modern filamentous oscillatoriacean cyanobacterium showing the symmetrically distributed primary and secondary partial septations that with ingrowth give rise to new medial cells. Modified after Pankratz, H.C., Bowen, C.C., 1963., American Journal of Botany 50, 387–399.

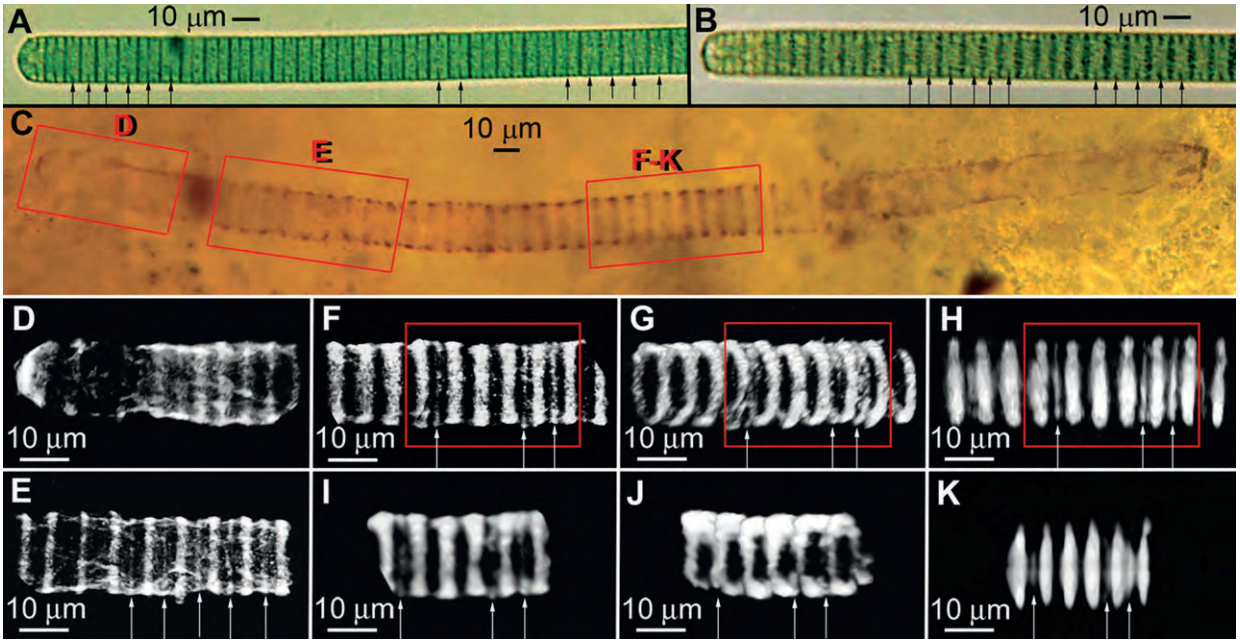

Fig. 9 Modern and fossil oscillatoriacean cyanobacteria showing partial septations; (A through C) show optical photomicrographs; (D through H) show confocal laser scanning micrographs; and (I through K) show three-dimensional Raman images (acquired in a spectral window centered in the kerogen "G" band at ~1605 cm^{-1} that establishes the carbonaceous composition of the fossil). (A and B) Two specimens of modern *Oscillatoria* sp. that exhibit well-defined partial septations (at arrows). (C) Optical image of the morphologically similar fossil *Oscillatoriopsis media*, in a petrographic thin section of stromatolitic chert from the ~650-Ma-old Chichkan Formation of southern Kazakhstan, in which the red rectangles denote the areas of the trichome shown in (D), (E), and (F through K). (D) The trichome terminus, showing its rounded end-cell and subtending disc-shaped medial cells. (E) A part of the trichome that exhibits well-defined partial septations (at arrows). (F through H) A second part of the trichome that exhibits partial septations (at arrows), in (F) showing the trichome as viewed from above its upper surface, in (G) with the image of the trichome tilted slightly to the right to show its interior, and in (H) with the image rotated to show the trichome as viewed from its side. (I) A Raman (chemical) image of the part of the specimen denoted by the red rectangle in (F), as viewed from above the trichome. (J) The part denoted in (G), titled slightly to the left; (K) The part denoted in (H), rotated to show the specimen from its side.

assignment of such fossils is not yet possible and, given their great age and the vagaries of preservation that preclude their assured linkage to modern microbes, they might be representatives of extinct lineages.

Sulfuretum fossils of uncertain systematic position

Sulfur-isotope data from sedimentary pyrite indicate that sulfate-reducing bacteria were extant ~3500 million years ago (Fig. 1). Cellularly preserved communities of such sulfuretum microbes are known from deep-sea sediments, 2300- and 1800 million-

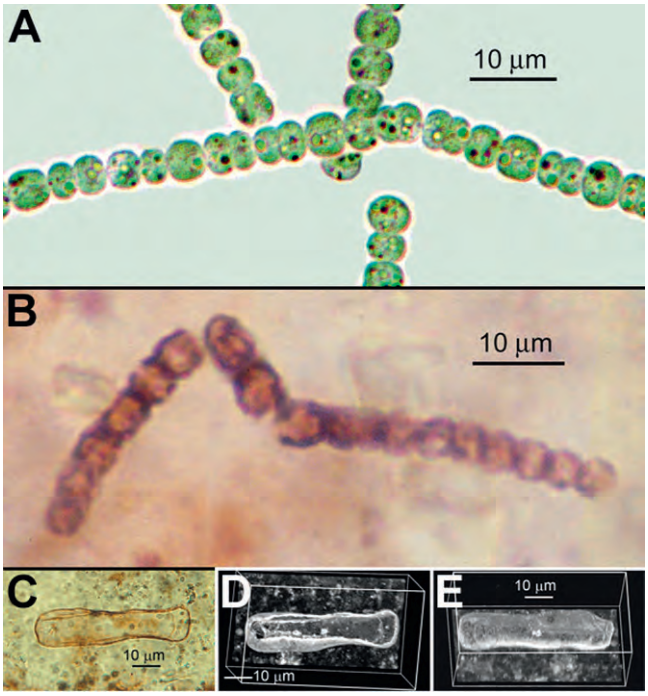

Fig. 10 Modern and fossil nostocacean cyanobacteria; (A through C) show optical photomicrographs; (D and E) show confocal laser scanning micrographs. (A) Modern *Nostoc* sp. (PCC 7936) for comparison with (B) *Veteronostocale amoenum* shown in a petrographic thin section of stromatolitic chert from the ~750-Ma-old Bitter Springs Formation of central Australia. (C through E) *Archaeoellipsoides longus*, a reproductive akinete characteristic of nostocacean cyanobacteria, from the ~650-Ma-old Chichkan Formation of southern Kazakhstan, in (C and D) shown as viewed from above the specimen, and (E) shown from its lower side.

year-old units of Western Australia, assemblages of both communities inhabiting similar sub-seafloor environments; both exhibiting distinctive "cobweb-like" microbial fabrics; both being composed of virtually identical microbial components; and both being notably similar to modern sulfur-cycling assemblages abundant in anoxic mud off the coast off South and Central America (Fig. 13). Diverse groups of extant bacteria have been shown to cycle sulfur, most prominently gram-negative Proteobacteria. The phylogenetic affinities of the sub-seafloor modern and fossil sulfur-cyclers (reported in 2007 and 2015, respectively) have yet to be determined.

Carbon- and sulfur-isotopic evidence of ancient microbes

The carbon isotopic record of photosynthesis, based on thousands of analyses, shows that Earth's ecology has been dominated by autotrophic primary producers since at least as early as ~3500 million years ago. Such data are consistent with carbon fixation by Rubisco (ribulose bisphospate carboxylase/oxygenase), the CO_2-capturing enzyme of cyanobacteria (as well as of photosynthetic protists and higher plants). But because of the mixing of carbonaceous matter from diverse sources that occurs during the deposition of sediments and the alteration of carbon isotopic compositions that can occur during geological metamorphism, these data do not indicate whether in early Earth history the dominant primary producers were cyanobacteria, anoxygenic photosynthetic bacteria, or anoxygenic chemosynthetic bacteria. Other carbon isotopic evidence, measured in carbonaceous matter so enriched C^{12} as to be plausibly derived only from CH_4-metabolizing methanotrophs, indicates that anaerobic methanogenic archaeans were present ~3500 million years ago (Fig. 1) and that such microbes were a major component of the ecosystem prior to the establishment of a stable oxygen-rich environment ~2300 million years ago. Similarly, isotopic analyses of sedimentary pyrite (FeS_2), enriched in S^{32} due to microbial activity, show that sulfate-reducing bacteria were also present in Earth's earliest biota (Fig. 1). The occurrence of C^{12}-rich graphitic carbon in the oldest sedimentary rocks now known, from Akilia Island off southwestern Greenland, suggests that CO_2-using autotrophs may have existed as early as ~3800 million years ago, and similarly C^{12}-rich graphite enclosed in ~4100-million-year-old zircon grains, redeposited in younger geological units in the Jack Hills region of southwestern Australia, suggests that microbial life may have existed earlier.

Fossil Evidence of Microbial History

IV.A Evolutionary Stasis

Numerous lines of evidence indicate that microbial communities composed of members of diverse microbial lineages have been extant since early in Earth history (Fig. 1). It seems rather remarkable that the great majority of cellularly preserved exceedingly

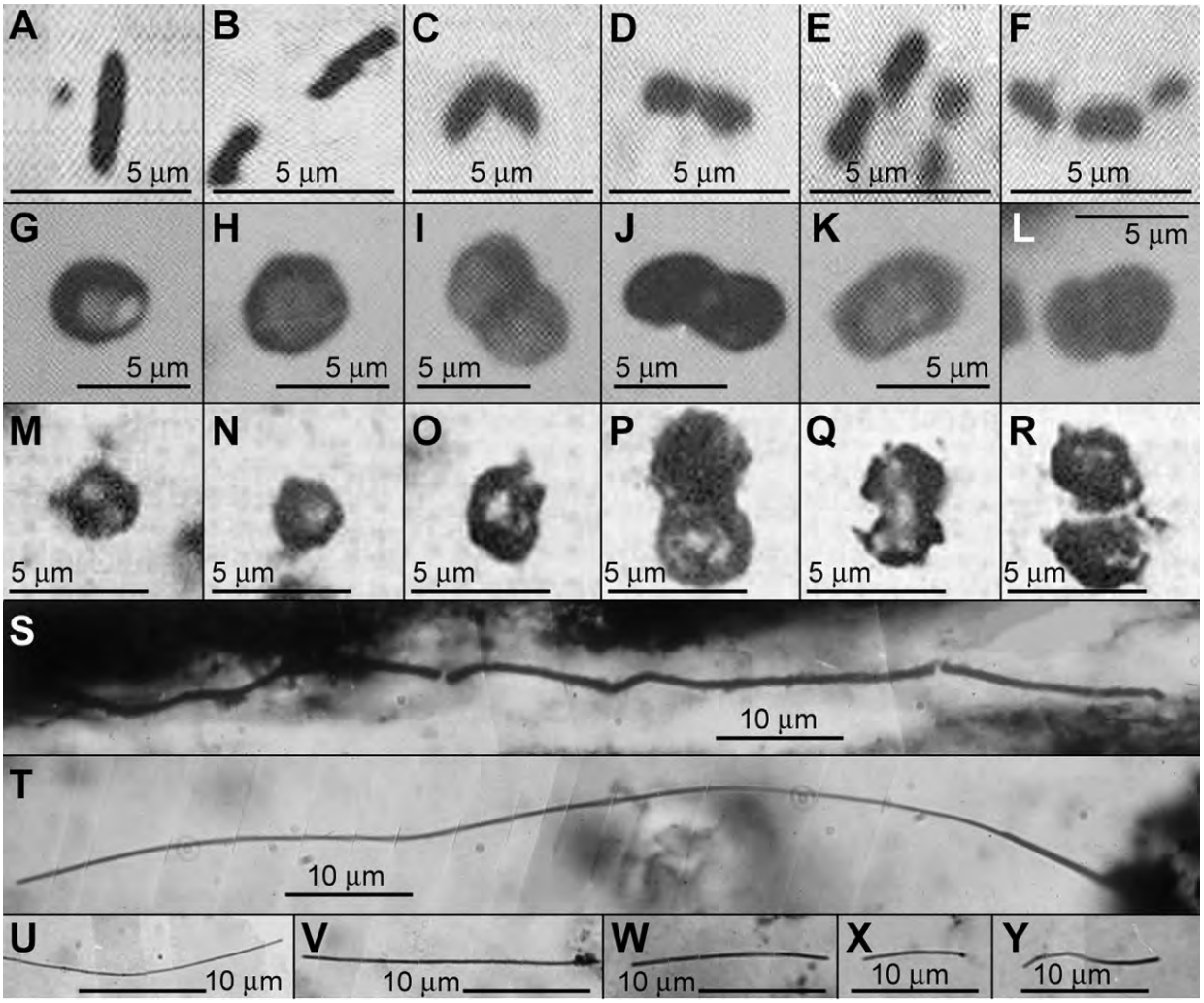

Fig. 11 Archean-age bacterial microfossils *Incertae sedis* (of uncertain systematic position), shown in petrographic thin sections. (A through F) Solitary and paired rod-shaped and (G through L) coccoidal unicells from the ~2600-Ma-old Monte Cristo Formation of the eastern Transvaal, South Africa. (M though R) Coccoidal unicells from the ~3260-Ma-old Swartkoppie Formation of Swaziland, South Africa, in (N) through (R) ordered in a sequence inferred to represent stages of cell division (Knoll, A.H., Barghoorn, E.S., 1977. Science 198, 396–398). (S) Narrow filamentous fossil from the ~3320-Ma-old Kromberg Formation of Swaziland, South Africa. (T though Y) Narrow filaments (*Archaeotrichion contortum*) from chert units of the ~3470-Ma-old Mount Ada Basalt of northwestern Western Australia.

ancient fossil microbes, hundreds or thousands of millions of years in age, can be related with confidence to members of still-extant lineages. However, such affinities seem unassailable, based not only on the organismal and cellular morphology of members of large populations but, additionally, on patterns of cell division and colony form; fossil-preserved evidence of light-seeking behavior (eg, stalk-growth in pleurocapsaceans, stromatolite-forming gliding motility in oscillatoriaceans); isotope-evidenced metabolic requirements; and geologic evidence of ecologic settings, with representatives of the fossil groups exhibiting the same community fabric and producing the same small-, medium-, and large-scale sedimentary structures as their modern counterparts in the same environments.

Nevertheless, a question arises: Why is it that such microbes, unlike later-evolved plants and animals, have exhibited such notably pervasive evolutionary stasis? A part of the answer is that such microbes are non-sexual, thereby lacking the genetic variability provided by the genetic recombination afforded by later-evolved eukaryotic sexuality. Is this the only explanation? Two microbial groups – cyanobacteria and sulfur-cycling bacteria – may provide an answer.

IV.A.1 Cyanobacteria

Perhaps most striking among such evidence of a lack of change over enormous segments of geological time is that showing the evolutionary stasis of cyanobacteria, the best documented of all ancient lineages. Such stasis may, or may not be characteristic of microbial lineages generally, but for cyanobacteria it is established firmly by the essentially identical morphologies, life cycles, behaviors, and ecologic settings of modern and fossil members of the group. Their early evolutionary success can be attributed to

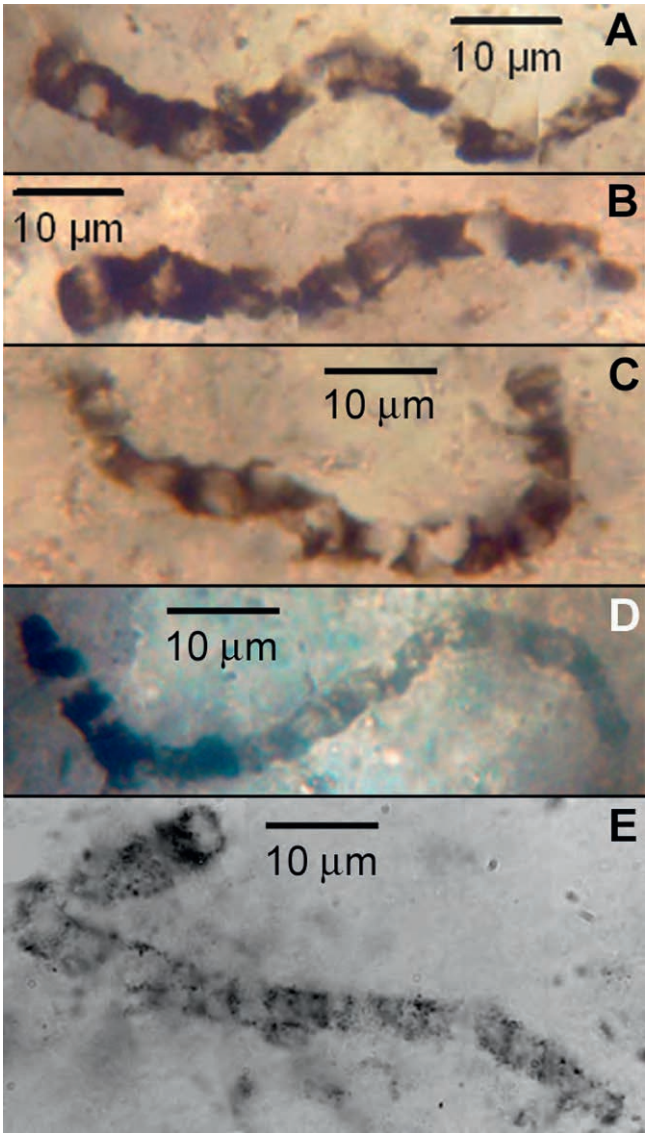

Fig. 12 Archean-age cyanobacterium-like microfossils *Incertae sedis* (of uncertain systematic position), shown in petrographic thin sections. (A-D) Four specimens of *Primaevifilum septatum* from a chert unit of the ~3465-Ma-old Apex Basalt of northwestern Western Australia. (E) A morphologically similar specimen of *P. septatum* from a chert unit of the ~3470-Ma-old Mount Ada Basalt of northwestern Western Australia.

their photosynthetic production of gaseous oxygen, a toxin to the earlier-evolved anaerobic photosynthetic bacteria with which they initially competed for photosynthetic space. Once established, their evolutionary stasis (their hypobradytelic rate of evolution) was presumably a result in part of their asexual reproduction and the lack of genetic variability it provides. But the prime source of such stasis seems almost certainly to be a result of their exceptional ecologic tolerance. Over their exceedingly long evolutionary history, cyanobacteria adapted to imperceptivity slow changes in the photic zone environment – from anoxic to oxygenic, UV-rich to UV-deficient, CO_2-rich to CO_2-deficient, short to increasingly longer daylengths and, perhaps, from relatively high ambient temperatures (~60°C) to that of the present-day Earth (~15°C). The genomes of cyanobacteria may thus encode a history of adaptation unparalleled by virtually any microbial lineage.

IV.A.2 Sulfur-cycling bacteria

The long-term evolutionary stasis of sulfur-cycling bacteria, while comparable to that of cyanobacteria, appears to differ in cause. Though members of both groups are non-sexual, genetic variability arising only from rare chance mutations, fossil evidence indicates that by 2300 million years ago sulfur-cycling sulfuretum bacteria occupied deep-sea mud. Thus, whereas coeval cyanobacteria inhabited an exceedingly slowly changing photic zone environment to which they incrementally adapted, sub-seafloor sulfuretum microbes inhabited a geologically "unchanging" environment: cold quiescent deep sea mud below the effects of wave

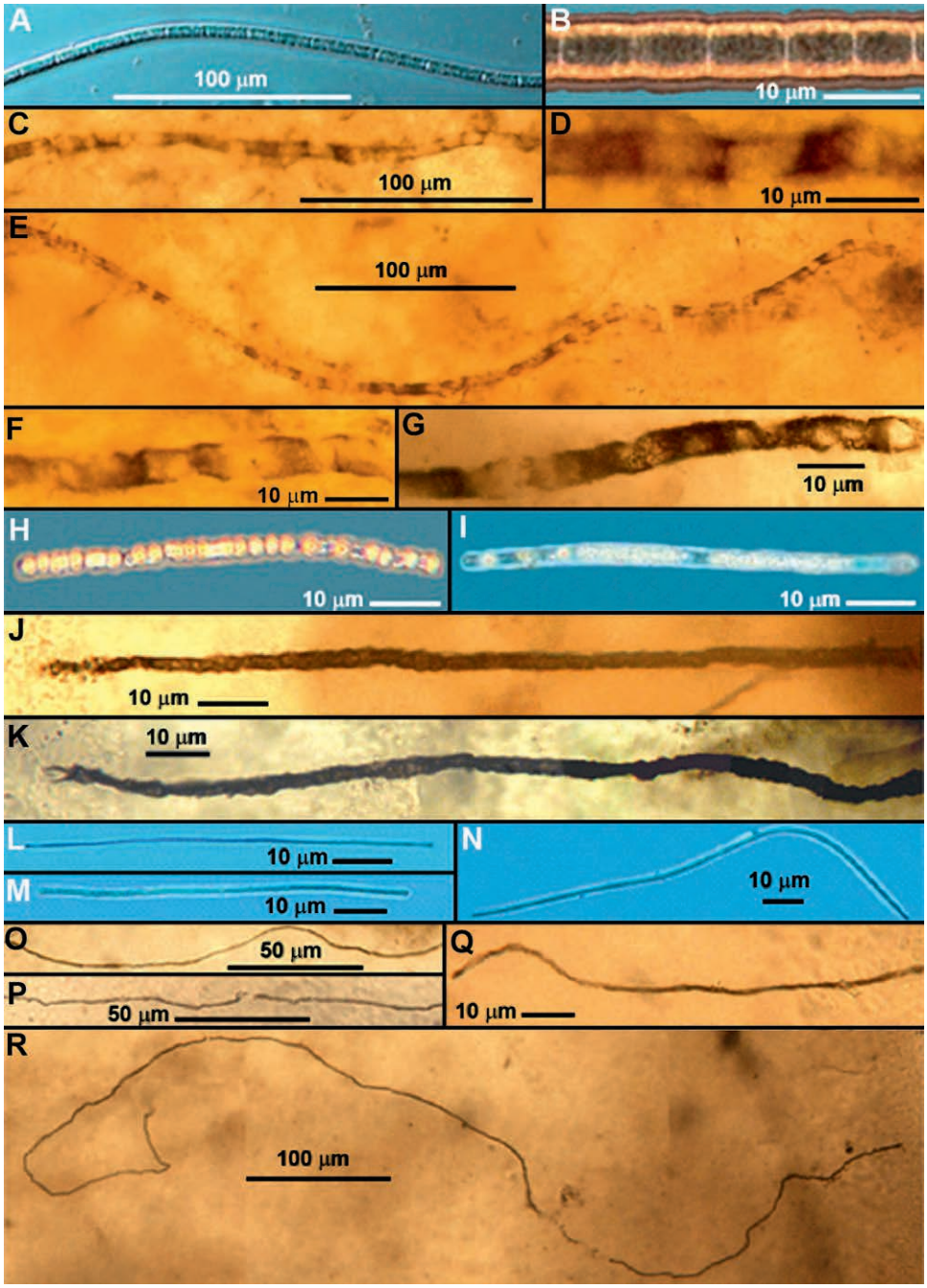

Fig. 13 Optical photomicrographs of modern and fossil sub-seafloor filamentous sulfur-cycling bacteria; all fossils are shown in petrographic thin sections of "cobweb-fabric" carbonaceous chert. (A, B) Modern large-diameter sulfuretum filaments from anoxic mud off the coast of Chile for comparison with similar fossil filaments from sub-seafloor sediments of the (C, D) ~2300-Ma-old Turee Creek Group Kazput Formation and the (E through G) ~1800-Ma-old Duck Creek Formation, both of Western Australia. (H, I) Modern sulfide-oxidizing bacteria from the Chilean sulfuretum, identifiable by their included sulfur granules, for comparison with similar filaments from the (J) ~2300-Ma-old Kazput Formation and the (K) ~1800-Ma-old Duck Creek Formation. (L through N) Modern small-celled narrow bacteria from the sub-seafloor Chilean sulfuretum for comparison with similar filaments from sulfuretums of the (O through Q) ~2300-Ma-old Kazput Formation and the (R) the ~1800-Ma-old Duck Creek Formation.

action in which there was no light, no day-night cycle, no oxygen and, before the much later emergence of burrowing metazoans, no churning of their enclosing sediments. Although such bacteria were no doubt affected by mutational changes, genetic drift, etc., would have moved their populations away from, not toward, the single adaptive peak of this "static fitness landscape." And because of lack of competition in this unchanging environment, they have exhibited long-term evolutionary stasis in their form, function and metabolic requirements. Such sulfur-cyclers have therefore been proposed as evidencing Evolution's Null Hypothesis: "If

evolution is a result of adaptation to a changing physical-biological environment; and if a changing physical-biological environment does not exist; then evolution will not occur."

Whether the marked evolutionary stasis of these two groups of fossils is typical of other ancient microbial lineages, such as methanogens, has yet to be determined.

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Paramecium Molecular Evolution

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Glossary

Cortex The cell surface, including the cilia and immediate underlying cytoplasm, extending a micrometer or so into the cell. The ciliate cortex includes the plasma membrane of the cell and a characteristic highly organized cytoskeletal and membranous array immediately under the membrane, all organized around the ciliary rows.

Macronucleus The large, highly polyploid (polycopy) nucleus that is functional during growth and proliferation in ciliate protozoa. This nucleus is transcriptionally active and its expression products determine most of the vegetative phenotype of the cell.

Micronucleus One or more small, usually diploid, nuclei in ciliate protozoa. This nucleus contains centric chromosomes, but the genes in them are not arranged to be capable of transcription, and the micronucleus contributes little to the vegetative phenotype. The micronuclei are a genetic reserve and become active only during nuclear reorganization.

Nuclear reorganization The process of rearrangement of the nuclei in ciliate protozoa, involving sexual events. The existing macronucleus is discarded, the micronuclei enter meiosis, and haploid products participate in nuclear fusion, making a new diploid zygotic nucleus. New macronuclei and micronuclei differentiate from mitotic products of the zygote.

Introduction

Paramecia clearly show the defining characters of the phylum of ciliophoran protozoa: nuclear dimorphism, the possession of two distinct types of differentiated nuclei in a single cell, and the cortex, an elaborate cytoskeletal and membranous array organized around the precisely organized rows of the few thousand cilia on the surface of each cell.

Cells of the genus *Paramecium* are very large, ranging in size from 100 to 300 μm in length and about 20–50 μm in width. In spite of this large cell size, the cell cycle time is typically short: as little as 5 h for small species at 27°C. Culturing of cells is easy, using simple plant extract media inoculated with gram-negative bacteria as food organisms.

A few dozen species of *Paramecium* have been described, all but one from freshwater habitats, around the world. Spores or resistant cysts have not been observed in the genus, so the worldwide distribution of the species is still a mystery. The species in the genus were originally defined and distinguished by classical morphological features. More recent analyses of some of the species indicate that a particular morphotype species could contain, in fact, a collection of genetically isolated groups, meeting the Mayr biological species criteria. The best-studied example of this is the *Paramecium aurelia* species group. From studies beginning in the 1950s, Sonneborn determined that the original morphotype *P. aurelia* was not one species, but contained at least 15 genetically isolated groups with nearly identical morphology. These groups, which he called syngens, were genetically isolated from one another by mating-type nonreactivity and/or hybrid inviability, and were thus distinguishable on that basis. After isozyme gel electrophoresis pattern work in the 1970s provided an independent objective means of distinguishing the syngens, Sonneborn elevated them to full species status. Hence, the original morphotype species *P. aurelia* no longer is valid – One must identify the particular mating type- and isozyme-defined species in the species group.

Cell Structure and Genetics

The two types of nuclei in paramecia are very different in morphology, molecular content, and function.

The small micronuclei are typically diploid, do not appear to be transcriptionally active, and contribute little to the phenotype of the vegetative cell. During mitosis or meiosis, the micronuclei show classical lower eukaryote nuclear behavior of chromosome condensation and formation of a spindle apparatus inside the nuclear envelope. The chromosomes are numerous (some 50–60 in *Paramecium tetraurelia*), very small, and metacentric. For these reasons, no karyotype has been characterized for any of the species. Depending on the species, a cell might possess 1, 2, or 4 or more micronuclei.

Transcriptional expression from the micronuclei is not possible, because the known coding sequences are not in an expression-competent state. The coding sequences of each gene are split by a series of non-coding sequences, known as internal intervening sequences (IES) that split the gene into a series of segments. IESs are short (26–883 bp), unique copy elements inserted into coding and non-coding regions of the micronuclear genome. There are several thousand identifiable in the micronuclear version of the genome. About 45,000 IES have been identified in the micronuclear genome. IES are flanked by TA dinucleotide boundaries – they

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are not introns, and do not carry typical intron sequence identities. The origin and significance of IES to the species is not understood. They could be 'parasitic' transposon sequences that have promiscuously propagated in the micronucleus.

The large macronucleus is polycopy (i.e., containing 800–1000 copies of each gene – the macronucleus of *P. tetraurelia* contains about 100 pgm of DNA), is transcriptionally active, and determines most of the phenotype of the cell. IES have typically been removed to generate the macronuclear version of the genome. Macronuclear division ('amitosis') involves no elaborate spindle apparatus, no chromosome condensation, and no centromeres. The dividing macronucleus elongates and partitions more or less in half.

The macronucleus is transcriptionally active during vegetative growth and proliferation of the cell, but is discarded during the sexual events known as nuclear reorganization. The micronuclei are the source of genomic material for sexual events, leading to the formation and differentiation of new macronuclei and micronuclei. The macronucleus does not contribute genomic material to reorganization. The parallels of this arrangement to animal germ and soma cell line differentiations are apparent, although in the ciliates, both types of nuclei are contained in a single cell, usually located next to each other.

Sexual reorganization of nuclei in paramecia involves an elaborate 'dance of the nuclei'. Using *P. tetraurelia* as an example, there are two mating types, O and E, and expression of the surface mating reactive proteins is induced by mild starvation. Conjugation can initiate after reactive cells of opposite mating type collide with, and adhere to, one another. After about 1.5 h of loose adhesion and interactions, cells will pair up ventrally into tightly adherent conjugants. Conjugant pair cells remain fused for about 6–7 h at 27°C, during which meiosis and fertilization are accomplished. Pairs then typically separate and complete the nuclear reorganization process individually. Members of the *P. aurelia* species group have a special additional option: if no conjugant partners are available, cells will instead invoke a self-fertilization pathway called autogamy.

With either conjugation or autogamy, nuclear reorganization begins with the fragmentation of the existing macronucleus and entry of the micronuclei into meiosis. All but one of the meiotic products in each cell degenerate, followed by mitosis of the remaining haploid nucleus in each conjugant partner. One of the two resulting haploid nuclei is reciprocally exchanged with the conjugation partner through a conjugation bridge. The two haploid nuclei then fuse to form a diploid fertilization nucleus (zygote nucleus) which then goes through two quick mitotic divisions. The two conjugating cells typically separate at about this time and complete nuclear reorganization individually. Of the four diploid nuclei produced, two differentiate into new micronuclei and two differentiate into new macronuclei.

During formation of the macronucleus from a mitotic product of the zygote nucleus, the chromosomes are fragmented, sequences identified as IES are identified and removed, and the remaining sequences are ligated together and telomerized to produce expression-competent genes located on acentric 'chromosomes' ranging from 50 to 1000 kbp in size. About 15% of the unique sequences in the micronuclear version of the genome are discarded when processed into the macronuclear version. These rearrangements convert the expression-incompetent version of the genes in the micronucleus into the expression-competent version found in the macronucleus. The resulting DNA segments are then replicated manyfold to produce the polycopy macronucleus. After fertilization, the process of macronuclear development takes about 12 h at 27°C.

In autogamy, two mitotic products of the surviving haploid nucleus fuse with each other and reorganization and development proceeds as described above. Conjugation produces two exconjugant cells with identical nuclear genetic content; autogamy produces a cell that is homozygous at all Mendelian loci.

Epigenetics

The genetics and epigenetics of paramecia have been studied for the past 70 years and have been used in developmental genetics studies of many different aspects of the cell. The Mendelian inheritance of the species allows controlled genetic crosses to be readily done, as well as the use of autogamy as the basis for mutant hunts in diverse expressions of the phenotype.

Paramecia also show a number of different fascinating, non-Mendelian, epigenetic inheritance phenomena. A few examples of these illustrate a mechanism by which an old macronucleus influences how the DNA of a developing new macronucleus is processed, potentially altering gene expression from that new macronucleus. Another intriguing phenomenon is the structural inheritance of alterations in the complex cellular cortex organization, by a process that could be considered analogous to the inheritance shown by prion proteins. All of these phenomena and processes are subjects of active investigation and analysis.

The mechanism of epigenetic determination of mating type expression in a macronucleus has been studied in some detail, and is instructive as to one general mechanism of the epigenetic heritability of a differentiated state. In paramecia, mating type is permanently determined as the new macronucleus develops during nuclear reorganization. Expression of either mating type is possible, but, in *P. tetraurelia* and some other species in the *P. aurelia* group, the mating type expressed by the existing macronucleus influences the choice of mating type developing in the new macronucleus, by a process of communication of information through the cytoplasm of the cell undergoing reorganization.

Central to mating type expression is the gene *mtA*. If the cell expresses *mtA*, it is mating type E; if *mtA* is not expressed, the cell is mating type O. After conjugation, the E partner typically differentiates as E when it builds its new macronuclei, and the O partner typically differentiates as O. The difference between the two is whether the *mtA* gene is processed in the developing new macronucleus into an expression-competent state or not. In mating type O cells, the promoter sequence of *mtA* is not present in the macronucleus, and so *mtA*, although present, is not transcribed. In mating type E cells, the promoter sequence is present, and *mtA* is expressed.

The process of mating type determination involves a small RNA molecule survey of the entire genomic sequences in the micronuclei, copied as the micronuclei progress through meiosis, and subsequent comparison of those sequences to small RNA

copies of the corresponding genomic sequences in the existing macronucleus. Sequences identified as micronuclear, but not found in the macronucleus, then migrate into the new developing macronucleus and are used to identify the IES to be removed and discarded from the differentiating genome. In mating type O cells, the promoter sequence of *mtA* is treated as an IES and removed from the new macronucleus; in mating type E cells, the promoter sequence is not identified as an IES and is located correctly to control expression of the coding sequence of *mtA*. Thus, a differentiated genetic state is communicated from the existing macronucleus into the genome of a newly developing macronucleus to replicate that state. This process involves an elaborate use of small RNA sequences from both the micronucleus and the existing macronucleus during reorganization.

Genetic studies have involved a wide range of cellular and developmental mechanisms and processes under both genetic and epigenetic control. Interested readers should refer to the general reviews cited by this article for more information and examples.

Molecular Genetics

The macronuclear genome of *P. tetraurelia* has been sequenced. The genome is unusually AT rich, containing only about 28% G+C content. Out of a total genome size of about 95 Mbp per haploid DNA set, an exceptionally high number of genes (nearly 40,000) have been identified by different estimation methods, indicating a very high density of genes per length of DNA (78% coding density). Only about half of those genes have recognizable similarities to genes from other organisms. The genes identified also contain many introns, most of them being quite small (25 bp average). RNA interference control of gene expression has been identified. Surveys of genomic diversity generated by point mutations indicates an exceptionally low rate of mutational change over time. This suggests that even the short intervening non-coding sequences in the macronuclear version of the genome are under intense selective pressure. These unique features of the paramecial genome, coupled with the unusual codon usage in gene expression (e.g., only one of the three possible termination codons (UGA) is actually used in paramecia; the other two (UAA and UGA) code for glutamine), are characteristic of the phylum Ciliophora and indicate that the ciliates diverged from the evolutionary lineages of other eukaryotes very early in the history of the planet.

Sequence information of the *P. tetraurelia* genome is available from online databases and is currently being used for both forward and reverse genetic/molecular searches, especially relating to the large collection of known Mendelian genes for the species.

Molecular Evolution

Large Scale

Subsequent to the sequencing of the genome of *P. tetraurelia,* analysis of genomic content indicated that the entire genome of the organism has been duplicated at least 3 times in the evolutionary history of the group. After each whole genomic duplication (WGD), there was an apparent extended period of genetic divergence between duplicated sequences, including many thousand sequences being deleted during this sorting-out period. The oldest genomic duplication appears to have predated the evolutionary divergence of *Paramecium* from the near-relative ciliate genus *Tetrahymena.* An intermediate duplication and loss event might be related to the major morphotype species divergences within the *Paramecium* genus. The latest duplication and gene loss event appears to have happened at about the time of species divergence within the *P. aurelia* species group, perhaps as much as 300 million years ago. The significance of these major genomic duplications and subsequent divergence and deletions of genes thus might be correlated with major evolutionary divergence and speciation events. The appearance of the IES in the micronuclear genome appear to have been before the first WGD, and they might contribute to genomic diversity by propagating by transposition and then decaying subsequent to their spread. These possible linkages between WGD, IES, and speciation deserves further study and analyses.

Within the *P. aurelia* Species Group

A more detailed molecular analysis of a limited number of nuclear and mitochondrial genes has been applied to representatives of the 15 members of the *P. aurelia* morphotype species group. It was expected that sequence differences for the selected genes among the different members of the group should reflect the degree of non-reactivity of mating among the species, but this relationship was not apparent. Some apparently closely related species that show some degree of mating cross-reactivity and mating are nevertheless very different in their sequence content for the genes surveyed, while other, more distant species with no mating reactivity show relatively little sequence diversity. These apparently paradoxical results indicate that much more analysis of the processes of mating-type determination and reactivity, versus evolutionary divergence in other parts of the genome, is necessary.

Conclusions

Although paramecia have been known since early days of light microscopy, and they have been intensively studied throughout the nineteenth and twentieth centuries, they still have many intriguing features that warrant further intensive studies well through this new century.

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Patenting of Microorganisms☆

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Glossary

Art A work exhibiting human creative skill or workmanship or its application.
Assignee Persons or businesses can acquire an ownership interest in a patent application by assignment from the inventor. The inventor granting the assignment is an assignor. The person or business receiving the assignment is the assignee.
Boolean search A patent search in which Boolean operators like conjunction (and), disjunction (or), and negation (not) are used along with keywords.
Budapest treaty An international treaty signed in Budapest, Hungary, on April 28, 1977, stipulating regulations for depositing microorganisms for patent purpose.
Compulsory license It provides that the owner of a patent or copyright licenses the use of their rights against payment either set by law or determined through some form of adjudication or arbitration.
Infringement The violation of an intellectual property law. Unauthorized use of a patent is an infringement.
Infringer A person/company indulging in patent infringement.
Intellectual property rights An intangible creation of the human mind, usually expressed or translated into a tangible form that is assigned certain rights of property. It is valuable because it provides ownership and exclusive rights to use, manufacture, reproduce, or promote a unique creation or idea.
Nonobviousness A person having ordinary skill in an art would not know how to solve the problem at which the invention is directed by using exactly the same mechanism of the claimed invention.
Novelty The quality of being new and unique.
Patentability Within the context of a national or multilateral body of law, an invention is patentable if it meets the relevant legal conditions. By extension, patentability also refers to the substantive conditions that must be met for a patent to be held valid.
Patent claims Usually present in the form of a series of numbered expressions, or more precisely noun phrases, following the description of the invention in a patent or patent application, and define, in technical terms, the extent of the protection conferred by a patent or by a patent application.
Prior art All information that has been disclosed to the public in any form anywhere in the world about an invention before the priority date of the claimed invention. It includes documentary sources such as publications and nondocumentary sources such as things known or used publicly.

Defining Statement

Patenting of microorganisms requires knowledge about intellectual property rights (IPR), patents, and the legalities of patenting in the home country and abroad by means of multilateral agreements like Trade-Related Aspects of Intellectual Property Rights (TRIPs), the Patent Cooperation Treaty (PCT), and the Budapest Treaty. The procedure for deposition of microorganisms intended for patenting into International Depositary Authorities (IDAs), the mode of operation of IDAs, and the responsibilities of the depositors are outlined. An outline of IDAs and their microbial diversity, patent databases, and strategic mining of patents has been provided.

Introduction

Nowadays, greater significance and coverage is given to intellectual property rights (IPR) and their protection. Intellectual property is a generic term that encompasses all expressions of human creativity. Ownership of intellectual property is usually expressed as "intellectual property rights." Intellectual property is divided into two categories: industrial property, which includes inventions (patents), trademarks, industrial designs, and geographic indications of source, and copyright, which includes literary and artistic works such as novels, poems, plays, films, musical works, and other artistic works such as drawings, paintings, photographs, sculptures, and architectural designs.

☆*Change History*: July 2016. S. Sekar and D. Kandavel updated Abstract, Glossary and Further Reading sections; added Keywords; revised Table 4 and 8 totally; Edits and updates were made throughout the article.

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Among intellectual properties, patents are extremely important. A patentable invention must be "novel, nonobvious, and have utility." A patent is a set of exclusive rights granted by a state to an inventor or his/her assignee for a fixed period of time in exchange for disclosure of the invention. Patents in the modern sense originated in 1474, when the Republic of Venice enacted a decree by which new and inventive devices, once they had been put into practice, had to be communicated to the Republic in order to obtain the right to prevent others from using them. In 1623 under King James I, England followed the Statute of Monopolies, which declared that patents could only be granted for "projects of new invention." During the reign of Queen Anne (1702–14), the lawyers of the English Court developed the requirement that a written description of the invention must be submitted. These developments, which were in place during the colonial period, formed the basis for modern English and US patent law. In the United States, during the colonial period and Articles of Confederation years (1778–89), several states adopted patent systems of their own. The first Congress adopted a patent act, in 1790, and the first patent was issued under this act on July 31, 1790.

Any patent has a definite period of life or existence. When the privilege time expires, the patented invention is free to be exploited by others. Patents promote next-generation researchers to further augment/refine the innovation. An invention is free to be exploited in the market if it is not patented. Patenting also prevents the duplication of research. Different kinds of patents exist for different protections. A product patent allows rights (including exclusive manufacturing and marketing rights) relating to the object, while a process patent relates to a means. A design patent protects the ornamental design, configuration, improved decorative appearance, or shape of an invention. This patent is appropriate when the basic product already exists in the marketplace and is not being improved upon in function but only in style; for example, designer eyeglass frames and original Coca-Cola bottles. A utility patent protects any new invention or functional improvements on existing inventions. This can be to a product, machine, a process, or even composition of matter. For example, going from light emitting diode (LED) technology to organic light emitting diode (OLED) would call for a new utility patent. In this case, the material of the light emitting diodes has changed from the synthetic material used in LEDs to organic material in OLEDs.

The System of Patenting

The Process of Patenting

In order to obtain a patent right one has to apply to his/her country's Patent Office and go through examinations to determine whether the application fulfills all the necessary requirements. Patents are technically national, given that the designated authority in each country grants patents in that country. But with many multilateral agreements, like Trade-Related Aspects of Intellectual Property Rights (TRIPs), the Patent Cooperation Treaty (PCT), and the Substantive Patent Law Treaty, patenting systems are increasingly being globalized. An international patent search/examination system mooted by the World Intellectual Property Organization (WIPO) may even bring a confluence of the decision-making processes in many countries. The PCT is an agreement for international cooperation in the field of patents. Through PCT, an inventor of a member country (contracting state) of PCT can ensure priority for his/her invention in all/any of the member countries simultaneously, without having to file separate applications in the countries of interest, by designating them in the PCT application.

The inventor, assignee, or legal representative of a deceased inventor or assignee may make an application either individually or jointly. The inventor is entitled to be mentioned in the patent if he applies for it. Two or more corporations as assignees may make an application jointly. The procedure for granting patents, the requirements placed on the patentee, and the extent of the exclusive rights vary widely between countries according to national laws and international agreements. The Patent Office, which examines all applications from around the world, takes precautionary measures before ultimately granting any patent right. These involve exchange of documents with the applicant to determine which claims, if any, are entitled to be patented. Even for inventions that are monumental, a patent right cannot be obtained naturally unless it is applied for. An application requires that one fill out the forms prescribed in the relevant ordinances and submit them to the respective Patent Office. There are two different legal procedures adopted by different patent offices for granting patent. They are as follows:

First-to-file system

In a first-to-file system, the right to the grant of a patent for a given invention lies with the first person to file a patent application for protection of that invention, regardless of the date of actual invention. Majority of patent office's use only this legal system. Prior to filing, publications on the intended patent should not be done. If the patent details are published, then this would amount to disclosure of information and the patent cannot be filed.

First-to-invent system

United States used a first-to-invent system, in which priority is established through a proceeding to determine which applicant actually invented the claimed invention first. In the first-to-invent system, a lengthy administrative proceeding (an interference proceeding) has to be conducted to determine who actually invented first, if another person also files for the same invention. Interference proceedings can take years to complete (even if there is no appeal to the US Court of Appeals for the Federal Circuit) and require extensive analysis. The America Invents Act signed on September 16, 2011, converted this system to newer "first-to-file" system for patent applications filed on or after March 16, 2013.

An application document submitted to the Patent Office will be checked to see whether it fulfills the necessary procedural and formal requirements. An invitation to correct the document will be made where necessary documents are missing or required

sections have not been filled in. The Patent Office will officially publish the content of an application after a specified period has elapsed from the date of filing. An examiner of the Patent Office, who will decide whether or not the claimed invention should be patented, will carry out an examination. The examiner first checks whether the application fulfills the requirements prescribed by law, that is, whether or not there are any reasons for refusal. These requirements include the following:

- Whether the claimed invention is based on a technical idea that utilizes a law of nature.
- Whether it has any industrial applicability.
- Whether the technical idea existed before the filing of the current application.
- Whether the claimed invention could have been easily invented by a person skilled in the art.
- Whether the claimed invention is liable to contravene public order and morality.
- Whether the descriptions in the specification conform exactly to the requirements for patentability.

If the examiner finds reasons for refusal, a notification to this effect is sent to the applicant. It is the sole responsibility of the patentee to see that someone else is not infringing upon his/her patents. It is the patentee's obligation to file a suit of infringement against the infringer. The patentee does not get any kind of money over the grant of the patent. However, when a patentee sells his/her patented invention or markets it, he gets money. TRIPs mandates a 20 year period of patent protection, which is applicable to the member signatories of the agreement.

Patent Cooperation Treaty

Patent Cooperation Treaty (PCT) is an international patent law treaty administered by the WIPO and concluded in 1970. It provides a unified procedure for filing patent applications to protect inventions in each of its Contracting States. As of July 2016, there were 150 Contracting States to the PCT. Any Contracting State that is a signatory to the Paris Convention for the Protection of Industrial Property can become a member of the PCT. A patent application filed under the PCT is called an international application or PCT application. Single filing of an international application is made at the Receiving National Office (RO) or directly at the International Bureau at the WIPO in Geneva. The applicant can be a national or resident of a Contracting State which is party to the European Patent Convention, the Harare Protocol on Patents and Industrial Designs (Harare Protocol), the revised Bangui Agreement Relating to the Creation of an African Intellectual Property Organization or the Eurasian Patent Convention, the international application may also be filed with the European Patent Office (EPO), the African Regional Industrial Property Organization (ARIPO), the African Intellectual Property Organization (OAPI) or the Eurasian Patent Office (EAPO), respectively. On filing a PCT application the applicants must designate the countries in which they wish to retain the option to file a patent application. There is a fee per country designated up to the first five and after that any number of further countries may be designated without fee. PCT has two phases, an international phase when they are international applications in the International Bureau, and a national phase when they are converted to national patent applications in the designated countries of interest. During the international phase, the designated International Searching Authority (ISA) (a Patent Office authorized by WIPO) conducts a patent search and an International Search Report (ISR) is provided within around 6 months of filing to assist the applicant in deciding whether or not to proceed with patent protection. It is optionally followed by a preliminary examination, performed by an International Preliminary Examining Authority (IPEA). Finally, the relevant national or regional authorities administer matters related to the examination of application (if provided by national law) and issuance of patent. The International Bureau also publishes the patent specification. The PCT is divided into two chapters. Chapter I requires that within 20 or 30 months of the earliest priority date (depending on whether or not the country concerned has adopted the most recent amendments to the PCT), the applicant must enter the national phase, that is, file patent applications in any one or more of the countries initially designated. Chapter II allows 30 months from the earliest priority date for entering the national phase and also requires a designated International Preliminary Examining Authority (authorized by WIPO to conduct international examinations) to conduct a non-binding substantive examination of the patent specification to determine whether it meets the requirements for patentability. It should be noted that certain designated offices have fixed time limits expiring even later than 30 months, or 20 months, as might be the case. The national phase is the second of the two main phases of the PCT procedure. The national phase starts only if the applicant files applications in each country of choice (the "designated office"); just the usual application is filed, either before the expiration of the time limit or together with an express request that it commences earlier. The applicant has sole responsibility for performing the act in due time. The consequences of failure to do so are fatal to the application in most designated states. In each such designated state, the international application has the effect of a national (or regional in the case of regional offices) application with regard to the international filing date and the decision to grant. The PCT does not lead to the grant of an "international patent," which does not exist.

Basis of Microbial Patenting

Patenting of microorganisms is not a new concept. In the brewing and baking industries, yeast has traditionally played an important part and patents for new types of yeast were granted in Belgium in 1833 and in Finland in 1843. Like any other patent, microbial patents should also satisfy novelty, nonobviousness, and utility requirements. In patent terms, "novel" means not previously available in the public domain. For example, a new microorganism (not previously known) isolated from a soil sample would be a potentially patentable invention. If the microorganism is claimed in the patent application in an "isolated" form, that form will be

novel over the previously known mixture of that microorganism with numerous other microorganisms in the soil. In addition, if the same microorganism has some practical use involving inventiveness, then it is eligible for patenting.

A product patent allows rights (including exclusive manufacturing and marketing rights) relating to the object, while a process patent relates to a means. A process patent allows the same products to be produced by different producers with different processes of production, whereas a product patent does not allow any firm except the patent holder to produce a particular product. Thus, it is easier to circumvent the process patent regime by inventing a different process to produce the same product whereas the only way to circumvent the product patent regime is to invent a new (differentiated) product in order to serve the consumers. In microorganisms, product and process patents are more applicable. An example of a product patent is the production of a new macrolide immunosuppressant by the microorganism *Actinoplanacete* sp. under novel fermentation conditions (USPTO 5,270,187: 1993). An example of a process patent is the process of production of Penicillin G (benzyl penicillin) in *Penicillium chrysogenum* by expression of the PCL (phenyl acetyl-CoA ligase) gene from *Pseudomonas putida*. PCL gene product converts phenyl acetic acid to phenyl acetyl-CoA and causes an increase in the production of Penicillin G (USPTO 6,251,655: 2001).

A new era was ushered in intellectual property when, in 1980, the US Supreme Court allowed the patenting of a living microorganism intended to soak up oil spills. Even though patent laws were originally framed keeping mechanical and chemical inventions in mind, patenting of life-forms was included under the same umbrella. From microorganisms, patent offices have marched on to grant exclusive monopolies for plants, animals, human cell lines, and even fragments of DNA. Generally, microbes or microorganisms are tiny living things, which are invisible to the naked eye. For the purposes of patent protection, the term microorganism often applies to other types of biological material including cell lines of plants or animals, and not excluding human genetic material. A patent coming under the microorganism category includes different biological material in different countries. For example, even seeds are accepted under the category of microorganisms in some culture collections. The term microorganism is an umbrella term that includes the following:

- Viruses.
- Bacteria, actinomycetes and mycoplasmas.
- Yeasts, filamentous fungi, and mushrooms.
- Protozoa and unicellular algae.
- Undeveloped animal or plant cells (cell lines and tissue cultures).
- Nematodes, embryos and hybridomas.
- Fused cells, transformants, and vectors utilized in genetic engineering (such as plasmids, phage).
- Oncogenes.
- DNA and RNA.

The European Union patents relating to Biotechnological Inventions embraces "biological material" instead of the term microorganism and it means any material containing genetic information and capable of reproducing itself or being reproduced in a biological system which are isolated from its natural environment or produced by means of a technical process even if it previously occurred in nature. Microorganisms are patentable, provided that the microorganisms intended to be patented are deposited in a designated culture collection known as International Depositary Authority (IDA). The status of IDA is conferred by WIPO under the Budapest Treaty to culture collections fulfilling the requirement criteria. The term "microorganism" is not defined in the Treaty; as such, it may be interpreted in a broad sense as depending on the applicability of the Treaty to microorganisms to be deposited under it. Thus, for example, tissue cultures and plasmids can be deposited under the terms of the Treaty, even though they are not microorganisms in the strict sense of the word. The range of materials accepted by different IDAs under the Budapest Treaty often includes the following:

- Cells, for example, bacteria, fungi, eukaryotic cell lines, and plant spores.
- Genetic vectors (such as plasmids, or bacteriophage, or viral vectors) containing a gene or DNA fragments.
- Organisms used for expression of a gene. Expression systems include bacterial, yeast, viral, plant, or animal cell cultures.
- Yeasts, algae, protozoa, eukaryotic cells, cell lines, hybridomas, viruses, plant tissue cells, spores, and hosts containing materials such as vectors, cell organelles, plasmids, DNA, RNA, genes, and chromosomes.

In many countries infectious agents are categorized in risk groups based on their relative risk. Depending on the country and/or organization, this classification system might take the following factors into consideration:

- Pathogenicity of the organism.
- Mode of transmission and host range.
- Availability of effective preventive measures (eg, vaccines).
- Availability of effective treatment (eg, antibiotics).
- Other factors.

Generally, IDAs do not accept microorganisms with a hazard categorization greater than WHO Classification Risk Group 2 (Table 1). In contrast to Risk Groups; Biosafety Levels (BSL) prescribes procedures and levels of containment for the particular microorganism or material (including Research Involving Recombinant or Synthetic Nucleic Acid Molecules). Similar to Risk Groups, BSL are graded from 1–4. BSL 1 commences with low hazard and progressively increases. Standard microbiological practices with no primary barriers can be performed under BSL–1; in BSL–2 in addition certain other precautions like using

Table 1 World Health Organization (WHO) classification of infectious microorganisms by biosafety levels

Risk Group 1 (No or low individual and community risk)
A microorganism that is unlikely to cause hazard to human, animal or environment.
Risk Group 2 (Moderate individual risk, low community risk)
A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock, or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.
Risk Group 3 (High individual risk, low community risk)
A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.
Risk Group 4 (High individual and community risk)
A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

personnel skilled in handling pathogenic agents, secluded laboratory, proper disposal of contaminated items, etc., are to be followed and it is suitable for work involving agents of moderate potential hazard to personnel and the environment; BSL–3 involves microbes which are potentially lethal via inhalation and the personnel are provided medical surveillance, offered relevant immunizations, use of biological safety cabinet, wearing solid-front protective clothing and drafting a safety operation protocol for procedures followed; BSL–4 is the highest level of biosafety precautions and is appropriate for work with agents that could easily be aerosol-transmitted within the laboratory and cause severe to fatal disease in humans, the laboratories are set up to be either cabinet laboratories or protective suit laboratories, wearing positive pressure suit and upon exit personnel must pass through a chemical shower for decontamination, then a room for removing the positive pressure suit and followed by a personal shower.

Legal Aspects of Microbial Patenting

WTO and TRIPs Compliance

World Trade Organization (WTO) is an organization for liberalizing trade across the globe and a forum for negotiating trade agreements and trade disputes among nations. It operates on the basis of certain trade rules negotiated and signed by the member governments. These documents provide the legal ground rules for international commerce. Following World War II, the victor nations sought to create institutions that would eliminate the causes of war. Their principles were to resolve or prevent war through the United Nations and to eliminate the economic causes of war by establishing three international economic institutions. The three institutions were the International Monetary Fund (IMF), the World Bank, and the International Trade Organization (ITO).

The US Congress did not object to the establishment of the World Bank and the IMF but refused to agree to the ITO on the grounds that it would cede too much sovereignty to an international body. Because the ITO was stillborn, the provisional agreement for the ITO, the General Agreement on Tariffs and Trade (GATT), became the agreement and the organization for establishing and enforcing, through dispute settlement, the international trade rules. The final and longest GATT round was the Uruguay Round, which lasted from 1986 to 1994 and led to the WTO's creation. As of now, there are 160 member nations. GATT mainly dealt with trade in goods, whereas WTO now deals with agriculture, textiles and clothing, banking, telecommunications, government purchases, industrial standards and product safety, food sanitation regulations, intellectual property, and much more.

The WTO's agreement on TRIPs, negotiated in the Uruguay Round, introduced intellectual property rules into the multilateral trading system for the first time. The WTO's TRIPs agreement is an attempt to narrow the gaps in the way these IPRs are protected around the world, and to bring them under common international rules. It establishes minimum levels of protection that each government has to give to the intellectual property of fellow WTO members. At the same time, additional time has been given to developing countries to fulfill their commitments. The TRIPs agreement says patent protection must be available for inventions for at least 20 years. Patent protection must be available for both products and processes, in almost all fields of technology. Governments can refuse to issue a patent for an invention if its commercial exploitation is prohibited for reasons of public order or morality. They can also exclude diagnostic, therapeutic, and surgical methods for the treatment of humans and animals, plants and animals (other than microorganisms), and biological processes for the production of plants or animals (other than non-biological and microbiological processes). However, any country excluding plant varieties from patent protection has to provide an effective *sui generis* system of protection. The agreement describes stipulates minimum rights of a patent owner. If a patent owner abuses his rights, for example, by failing to supply the product on the market then the governments can issue "compulsory licenses," allowing a competitor to produce the product or use the process under license. But this can only be done under certain conditions aimed at safeguarding the legitimate interests of the patent-holder. Nonpatentable inventions, in general, are given in Table 2.

There are several international intergovernmental organizations in the WTO, which have an observer status, to follow discussions therein on matters of direct interest to them. Some of them under the General Council are the Food and Agriculture Organization (FAO), the IMF, International Trade Center (ITC), the Organization for Economic Cooperation and Development (OECD), the United Nations (UN), the United Nations Conference on Trade and Development (UNCTAD), the World Bank, and WIPO. There is a separate council observing TRIPs. It includes FAO, the IMF, International Union for the Protection of New Varieties of Plants (UPOV), OECD, UN, UNCTAD, the World Bank, the World Customs Organization (WCO), and WIPO.

Table 2 Nonpatentable inventions

- An invention that is frivolous or that claims anything obviously contrary to well-established natural laws.
- An invention, the primary or intended use of which would be contrary to law, or morality, or injurious to public health, including to protect human, animal, or plant life or health, or to avoid serious prejudice to the environment.
- The mere discovery of a scientific principle or the formulation of an abstract theory. The mere discovery of any new property or new use for a known substance or of the mere use of a known process, machine or apparatus unless such known process results in a new product or employs at least one new reactant.
- A substance obtained by a mere admixture resulting only in the aggregation of the properties of the components thereof or a process for producing such substance.
- The mere arrangement or rearrangement or duplication of known devices, each functioning independently of one another in a known way.

WIPO and the Budapest Treaty

WIPO is a specialized self funding agency of the UN. The WIPO Convention of the UN established WIPO in 1967, with a mandate from its member states to promote the protection of intellectual property (IP) throughout the world through cooperation among states and in collaboration with other international organizations. WIPO's core tasks include developing international IP laws and standards, delivering global IP protection services, encouraging the use of IP for economic development, promoting better understanding of IP, and acting as a forum for debating various emerging issues. There are currently 188 member states of WIPO (as on July 15, 2016). WIPO administers 26 treaties dealing with IP protection, global protection system, and patent classification. Some of them are the Berne Convention, the Brussels Convention, the Madrid Agreement, the Marrakesh VIP Treaty, the Nairobi Treaty, the Paris Convention, the Patent Law Treaty, the Rome Convention, the Singapore Treaty on the Law of Trademarks, the Trademark Law Treaty, the Washington Treaty, the WIPO Copyright Treaty, the Budapest Treaty, the Hague Agreement, the Lisbon Agreement, the Madrid Protocol, the PCT, the Locarno Agreement, the Nice Agreement, the Strasbourg Agreement and the Vienna Agreement. The Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (hereinafter referred to as the Treaty) acts as a guide for depositing microorganisms for patenting purposes. The Treaty was concluded on April 28, 1977, and came into force on August 19, 1980. The regulations under the Budapest Treaty were modified in 1981 and in 2002. The Treaty is open to States party to the Paris Convention for the Protection of Industrial Property (1883). Disclosure of the invention is a requirement for the grant of patents. Normally, an invention is disclosed by means of a written description. Where an invention involves a microorganism or the use of a microorganism, disclosure is not possible in writing but can only be effected by the deposit of a sample of the microorganism with a specialized institution. Under the Treaty, a contracting state that allows or requires the deposit of microorganisms for the purposes of patent procedure must recognize, for such purposes, the deposit of a microorganism with any IDA, irrespective of whether such authority is on or outside the territory of the said state. What the Treaty calls an IDA is a scientific institution, typically a culture collection, which is capable of storing microorganisms. Such an institution acquires the status of IDA when the contracting state in the territory where it is located issues assurances to the Director General of WIPO to the effect that the said institution complies and will continue to comply with certain requirements of the Treaty. This treaty will save the depositor's money because deposition with one IDA is sufficient for patent application in any contracting states and it establishes a uniform system of deposit, recognition, and furnishing of samples of microorganisms.

Components of Microbial Patenting

Culture Deposition With IDA

Procedure for deposition

Most patent offices require the deposit to be made no later than the filing date of the relevant patent application (or the priority date if priority is being claimed from an earlier application). However, it is advisable to make the deposit as early as possible and not to leave it until just before the deadline. In total there are 14 forms, numbered BP/1 to BP/14, available under the Budapest Treaty that need to be presented when required. The International Bureau of WIPO has drawn them up. Some of the forms and their usage are given below:

(BP/4) – Receipt in the case of an original deposit.
(BP/5) – Receipt in the case of a new deposit.
(BP/6) – Receipt in the case of a transfer.
(BP/9) – The form for the viability statement.

These forms are called "international forms" and relate to the receipt and the viability statement. The form numbers BP/1, 2, 3, 7, 8, 10, 11, 13, and 14 are models for use by the respective IDAs. Form BP/12, which relates to the furnishing of samples to the legally entitled parties, is a form whose contents were fixed by the Assembly of the Budapest Union. The fee collected for storage covers the whole duration of storage and thus it is a one-time payment.

Obligations of IDA

The IDA has to have the expertise and facilities necessary to keep the microorganism viable and uncontaminated throughout the storage period required by the Treaty. If for any reason an IDA ceases to function as such, the Treaty provides for the microorganisms

deposited with it to be transferred to another IDA. When the microorganism has been accepted as an original or new deposit, the date of that original or new deposit, as the case may be, shall be the date on which the IDA received the microorganism. Except in the case of a conversion of a deposit made outside the Budapest Treaty under Rule 6.4(d), the date of deposit is held to be the date on which the IDA physically receives the microorganism, even though all procedural requirements for acceptance may not have been met on that date.

Rule 6.4(d) of the Treaty allows for a deposit originally made outside the provisions of the Treaty and before the culture collection became an IDA deposit to be converted to a deposit made under the Treaty. The requirements for converting an existing deposit into a "Budapest deposit" are essentially the same as those that must be met when making an original deposit under the Treaty, except that the microorganism itself, of course, will already have been sent and received. However, it must be realized that when a deposit is converted under Rule 6.4(d), for the purposes of the Treaty, the date of deposit is held to be the date on which the culture collection acquired IDA status, not the earlier date on which the collection physically received the microorganism. It is important that this "artificial" date of deposit be borne in mind in relation to the filing dates of patent applications referring to the deposited microorganism. Following an "understanding" reached by the Assembly of the Budapest Union (in 1981 and in 1990), the depositor may request that a deposit made with an IDA, but outside the scope of the Budapest Treaty, be converted into a Budapest Treaty deposit. Furthermore, according to such "understanding," the date recognized for the purposes of the Treaty as the date of deposit is determined by the applicable national law. In practice this means that whereas some industrial property offices may recognize the date on which the IDA received the microorganism as the date of deposit, others may recognize only the date of receipt of the request for conversion by the IDA. Depositors should bear this in mind and consider any effects it may have on patent applications or patents referring to the converted deposit. In American Type Culture Collection (ATCC), if a deposit has been released for distribution before converting it into a Budapest Treaty deposit, then there cannot be any restriction on its further distribution.

Having received and accepted a microorganism for deposit (or having converted an existing deposit), the IDA has to notify the depositor of this fact by issuing to him/her an official receipt for that deposit (Rule 7.1). The receipt must be made out on the BP/4 form. This form is one of the four "international forms," a model of which has been established by the Director General of WIPO and the Budapest Union Assembly (Rule 7.2(a)). Wherever the regulations specify the use of an "international form" by IDAs, such use is mandatory. After the depositor submits a frozen or freeze-dried sample, the IDA checks the viability of the deposited material. Other essential examinations are also carried out. If the results are good then the IDA preserves the material and a sample is returned to the depositor for verification of properties. A certificate of deposit and viability is provided along with an assigned patent deposit number. The deposit date is set as the date of receiving the viable material. If the results are bad, then the depositor has to supply a fresh deposit again. Any microorganism deposited with an IDA shall be stored by such authority, with all the care necessary to keep it viable and uncontaminated, for a period of at least 5 years after the most recent request for the furnishing of a sample of the deposited microorganism was received by the said authority and, in any case, for a period of at least 30 years after the date of the deposit. The depositary authority is obliged to maintain secrecy regarding the deposit and the nature of the deposited material. No international depositary authority shall give information to anyone whether a microorganism has been deposited with it under the Treaty. It shall not give any information to anyone concerning any microorganism deposited with it under the Treaty except to an authority, natural person or legal entity which is entitled to obtain a sample of the said microorganism under Rule 11 and subject to the same conditions as provided in that Rule. An IDA can refuse to accept a microorganism sent to it, if it is not the kind it accepts under the Treaty or if the IDA is unable to handle the microorganism sent.

Guidelines for the prospective depositor

In addition to making a deposit of the inventive material under the Budapest Treaty, the depositor is required to provide as much descriptive information about the characteristics of the material as is possible at the time of making the application. The type of information will vary with the nature of the material deposited and the invention, as outlined below:

Microorganisms, per se, should be provided with

- Species identification.
- Morphological details such as shape, size, stainability, and motility.
- Colony characteristics, for example, color, shape, size, swarming, and any distinguishing features in appearance.
- Metabolic characteristics including substrate requirements, products or by-products, and isozyme characteristics.
- Genetic characteristics such as specific genes or mutations or their variants (at either the nucleic acid or protein level).
- Plasmids and phage (if any) in the microorganism together with relevant genetic characterization.
- When a mixture of microorganisms is deposited, descriptions of the components of the mixture and at least one of the methods permitting the checking of their presence.

Viruses, plasmids, chromosomes, organelles, and so on, made as deposits within cells, should be provided with

- Sufficient information to enable the cells to be cultured.
- Description of the method of isolation of the virus, and so on, if it is necessary to repeat the invention.
- Any identifying genetic or morphological characterization.

Hybridomas should be provided with information on

- The antigen used for immunization.
- The cells used and method of fusion.
- The criteria used for selection of the clone.

Plant and animal cell lines should be provided with information on

- Clear description of the source of the tissue.
- Procedure used to immortalize the cell line.
- Growth factors and nutrients required in the culture medium.
- Relevant genetic characteristics such as any foreign genes or chromosomes.

The depositor should also provide an "identification reference," which may be a name, of course, but equally it may be merely a strain designation or even just a laboratory code number. Other than these, a depositor intending to submit a microorganism for deposition should have clear knowledge of the operation methodology of the intended IDA as well as the requirements of a depositor as per the Budapest Treaty before making a deposit (Table 3). It is very important that the depositor find out whether it is possible for him/her to cope with the cost, contracts, regulations, and other modalities of the IDAs. The kind of microorganisms accepted by different IDAs for patent procedures varies. In most of the IDAs across the globe, saprophytic forms are accepted while a few accept viral and other pathogenic forms. Only a few IDAs of the developed countries, like the ATCC, accept all kinds of microorganisms. After deposition, the culture cannot be reclaimed. The depositor should himself/herself keep samples of the culture for the same period of time; so that in case the culture is no longer available from the depositary authority, the depositor can replenish the stock. This deposit is known as New Deposit.

Global IDAs and microorganisms accepted for deposition

Each IDA has the freedom to decide the kinds of biological material it will accept. This mainly depends on the specific expertise of the collection and the equipment available. There are IDAs that accept a broad range of different kinds of biological material, whereas others restrict the kinds of biological material to one or two. Currently, there are 46 such authorities in 24 countries: seven in the United Kingdom, four in the Republic of Korea, three in China, Italy, United States of America and the Russian Federation, two each in Australia, India, Japan, Poland and Spain, and one each in Belgium, Bulgaria, Canada, Chile, the Czech Republic, Finland, France, Germany, Hungary, Latvia, Mexico, the Netherlands and Slovakia. IDAs vary in the kinds of microorganisms accepted for deposition. The National Collection of Yeast Cultures, United Kingdom, accepts only nonpathogenic yeasts for patent deposition. Similarly, Banco Nacional de Algas (BNA), Spain, accepts only algae for patent deposition. This is mainly due to the specialization and expertise of these IDAs in these organisms. But American Type Culture Collection (ATCC) accepts almost all kinds of microorganisms, except nematodes. Microorganisms are highly diverse with variations in metabolic requirements, culture requirements, and so on, and hence handling them requires experience, infrastructure, and detailed scientific knowledge about them. These act as the main factors in some IDAs, restricting the kind of microorganism accepted within their technical purview. Global IDAs and the kinds of microorganisms they currently accept are outlined in Table 4.

Table 3 Information to be known by the depositor prior to deposition

- The kinds of microorganisms accepted.
- Details that must be given in advance of deposition.
- The official language of communication.
- Any special transport and/or delivery arrangements.
- Requirements for furnishing a new deposit or converting a previous deposit into a patent deposit.
- The state in which the cultures (lyophilized, frozen, liquid suspension, agar slant, etc.) need to be submitted.
- The minimum number of replicates and minimum titer for each culture required.
- The time needed by the IDA to carry out viability tests.
- The manner in which IDA replenishes diminishing stocks.
- Information about the different kinds of contracts between the IDA and the depositors. Import and/or quarantine regulations.
- Forms that would be issued by the IDA as official notification.
- Whether the IDA advises third parties of the correct procedures to be followed in order to make a valid request.
- Whether the requesting party must meet any health and safety requirements.
- Whether the samples furnished by the IDA are from its own preparations or from those supplied by the depositor.
- The fees payable under and outside (eg, safe deposits) the Budapest Treaty provisions.
- Availability of detailed guidance booklet from the IDA.

Table 4 Global IDAs and the kinds of microorganisms accepted

Country	*IDA*	*Kinds of microorganisms accepted*
Australia (AU)	National Measurement Institute (NMI)	Bacteria (non-pathogenic), yeasts and fungi (non-pathogenic)
	Lady Mary Fairfax CellBank Australia (CBA)	Animal cell cultures, human cell cultures and hybridomas
Belgium (BE)	Belgian Co-ordinated Collections of Micro-organisms (BCCM)	Animal cell cultures, bacteria (pathogenic and non-pathogenic), fungi (pathogenic and non-pathogenic), human cell cultures, hybridomas, plasmids (either in hosts or not), RNA and yeasts (pathogenic and non-pathogenic)
Bulgaria (BG)	National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC)	Animal viruses, animal cell cultures, bacteria (non-pathogenic), fungi (non-pathogenic), hybridomas, plant viruses, plasmids (in hosts) and yeasts (non-pathogenic)
Canada (CA)	International Depositary Authority of Canada (IDAC)	Animal viruses, animal cell cultures, bacteria (pathogenic and non-pathogenic), bacteriophages, eukaryotic DNA, fungi (pathogenic and non-pathogenic), hybridomas, (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
Chile (CL)	Colección Chilena de Recursos Genéticos Microbianos (CChRGM)	Bacteria (non-pathogenic), fungi (non-pathogenic), molds, plasmids (in hosts) and yeasts (non-pathogenic)
China (CN)	China Center for Type Culture Collection (CCTCC)	Algae, animal viruses, animal cell cultures, bacteria (pathogenic and non-pathogenic), bacteriophages, eukaryotic DNA, fungi (pathogenic and non-pathogenic), human cell cultures, hybridomas, molds, mycoplasma, nematodes, oncogenes, plant cell cultures, plant viruses, plasmids (either in hosts or not in hosts), protozoa (non-parasitic), seeds, and yeasts (pathogenic and non-pathogenic)
	China General Microbiological Culture Collection Center (CGMCC)	Algae, animal viruses, animal cell cultures, bacteria (pathogenic and non-pathogenic), bacteriophages, fungi (pathogenic and non-pathogenic),mycoplasma, plant cell cultures, plant viruses, plasmids (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
	Guangdong Microbial Culture Collection Center (GDMCC)	Algae, bacteria (pathogenic and non-pathogenic), fungi (pathogenic and non-pathogenic), molds, plasmids (in hosts), seeds and yeasts (pathogenic and nonpathogenic)
Czech Republic (CZ)	Czech Collection of Microorganisms (CCM)	Bacteria (pathogenic and non-pathogenic), fungi (pathogenic and non-pathogenic), plasmids (in hosts) and yeasts (pathogenic and non-pathogenic)
Finland (FI)	VTT Culture Collection (VTTCC)	Bacteria (non-pathogenic), fungi (non-pathogenic) and yeasts (non-pathogenic)
France (FR)	Collection Nationale De Cultures De Microorganismes (CNCM)	Animal viruses, animal cell cultures, bacteria (pathogenic and non-pathogenic), fungi (pathogenic and non-pathogenic), human cell cultures, hybridomas, plasmids (in hosts) and yeasts (pathogenic and non-pathogenic)
Germany (DE)	Leibniz-Institute DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)	Animal cell cultures, bacteria (pathogenic and non-pathogenic), bacteriophages, fungi (pathogenic and non-pathogenic), human cell cultures, hybridomas, mycoplasma, plant cell cultures, plant viruses, plasmids (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
Hungary (HU)	National Collection of Agricultural and Industrial Microorganisms (NCAIM)	Bacteria (non-pathogenic), fungi (non-pathogenic), molds and yeasts (non-pathogenic)
India (IN)	Microbial Culture Collection (MCC)	Bacteria (pathogenic and non-pathogenic), fungi (pathogenic and non-pathogenic), plasmids (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
	Microbial and Gene Bank Type Culture Collection (MTCC)	Bacteria (pathogenic and non-pathogenic), bacteriophages, fungi (pathogenic and non-pathogenic), plasmids (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
Italy (IT)	Advanced Biotechnology Center (ABC)	Animal cell cultures, human cell cultures, and hybridomas
	Collection of Industrial Yeasts (DBVPG)	Fungi (non-pathogenic), and yeasts (non-pathogenic)
	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (IZSLER)	Animal viruses and Bacteria (pathogenic and non-pathogenic)
Japan (JP)	International Patent Organism Depositary (IPOD)	Algae, plant cell cultures, protozoa (non-parasitic) and seeds
	National Institute of Technology and Evaluation – Patent Microorganisms Depositary (NITE-NPMD)	Animal cell cultures, bacteria (pathogenic and non-pathogenic), bacteriophages, embryos, fungi (pathogenic and non-pathogenic), human cell cultures, hybridomas, plasmids (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
Latvia (LV)	Microbial Strain Collection of Latvia (MSCL)	Bacteria (pathogenic and non-pathogenic), fungi (pathogenic and non-pathogenic), plasmids (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
Mexico (MX)	Colección de Microorganismos del Centro Nacional de Recursos Genéticos (CM-CNRG)	Algae, animal viruses, animal cell cultures, bacteria (pathogenic and non-pathogenic), bacteriophages, embryos, eukaryotic DNA, fungi (pathogenic and non-pathogenic), human cell cultures, hybridomas, mycoplasma, nematodes, plant cell cultures, plant viruses, plasmids (either in hosts or not in hosts), protozoa (non-parasitic), RNA and yeasts (non-pathogenic)

Table 4 Continued

Country	*IDA*	*Kinds of microorganisms accepted*
Netherlands (NL)	Centraalbureau voor Schimmelcultures (CBS)	Bacteria (pathogenic and non-pathogenic), bacteriophages, fungi (pathogenic and non-pathogenic), plasmids (either in hosts or not in hosts) and yeasts (pathogenic and non-pathogenic)
Poland (PL)	Collection of Industrial Microorganisms (IAFB)	Bacteria (non-pathogenic), fungi (non-pathogenic) and yeasts (non-pathogenic)
	Polish Collection of Microorganisms (PCM)	Bacteria (pathogenic and non-pathogenic) and bacteriophages
Republic of Korea (KR)	Korean Agricultural Culture Collection (KACC)	Bacteria (non-pathogenic), fungi (non-pathogenic), plant viruses, plasmids (in hosts), seeds and yeasts (non-pathogenic)
	Korean Cell Research Foundation (KCLRF)	Animal cell cultures, eukaryotic DNA, human cell cultures, hybridomas, plant cell cultures and plasmids (either in hosts or not in hosts)
	Korean Collection for Type Cultures (KCTC)	Algae, animal viruses, animal cell cultures, bacteria (non-pathogenic), bacteriophages, embryos, eukaryotic DNA, fungi (non-pathogenic), human cell cultures, hybridomas, molds, murine embryos, plant cell cultures, plant viruses, plasmids (either in hosts or not in hosts), protozoa (non-parasitic), RNA, seeds and yeasts (pathogenic and non-pathogenic)
	Korean Culture Center of Microorganisms (KCCM)	Animal viruses, bacteria (non-pathogenic), bacteriophages, fungi (non-pathogenic), plant viruses, plasmids (either in hosts or not in hosts) and yeasts (non-pathogenic)
Russian Federation (RU)	Russian Collection of Microorganisms (VKM)	Bacteria (non-pathogenic), fungi (non-pathogenic) and yeasts (non-pathogenic)
	Russian National Collection of Industrial Microorganisms (VKPM)	Animal cell cultures, bacteria (non-pathogenic), bacteriophages, fungi (non-pathogenic), human cell cultures, hybridomas, plant cell cultures, plasmids (either in hosts or not in hosts) and yeasts (non-pathogenic)
	National Research Center of Antibiotics (NRCA)	Bacteria (non-pathogenic), fungi (non-pathogenic) and yeasts (non-pathogenic)
Slovakia (SK)	Culture Collection of Yeasts (CCY)	Yeasts (pathogenic and non-pathogenic)
Spain (ES)	Banco Español de Algas (BEA)	Algae
	Coleccion Espanola de Cultivos Tipo (CECT)	Bacteria (pathogenic and non-pathogenic), fungi (non-pathogenic), plasmids (either in hosts or not in hosts) and yeasts (non-pathogenic)
United Kingdom (GB)	Culture Collection of Algae and Protozoa (CCAP)	Algae and protozoa (non-parasitic)
	CABI Bioscience (International Mycological Institute – IMI)	Bacteria (non-pathogenic), fungi (non-pathogenic), nematodes and yeasts (non-pathogenic)
	European Collection of Cell Cultures (ECACC)	Animal viruses, animal cell cultures, eukaryotic DNA, human cell cultures and hybridomas
	National Collection of Type Cultures (NCTC)	Bacteria (pathogenic)
	National Collection of Yeast Cultures (NCYC)	Yeasts (non-pathogenic)
	National Collections of Industrial, Food and Marine Bacteria (NCIMB)	Bacteria (pathogenic and non-pathogenic), bacteriophages, plant cell cultures, plasmids (either in hosts or not in hosts), seeds and yeasts (non-pathogenic)
	National Institute of Biological Standards and Control (NIBSC)	Animal cell cultures and human cell cultures
Unites States of America (US)	Agricultural Research Service Culture Collection (NRRL)	Bacteria (non-pathogenic), fungi (non-pathogenic), molds, plasmids (in hosts) and yeasts (non-pathogenic)
	American Type Culture Collection (ATCC)	Algae, animal viruses, animal cell cultures, bacteria (pathogenic and non-pathogenic), bacteriophages, embryos, eukaryotic DNA, fungi (pathogenic and non-pathogenic), human cell cultures, hybridomas, molds, murine embryos, mycoplasma, oncogenes, plant cell cultures, plant viruses, plasmids (either in hosts or not in hosts), protozoa (parasitic, pathogenic, and non-parasitic), RNA, seeds and yeasts (pathogenic and non-pathogenic)
	Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA)	Algae, bacteria (non-pathogenic), bacteriophage and protozoa (non-parasitic)

Mining of Microbial Patents

Searching of Patents

The retrieval of patent information has been made easy by the Internet and now access to different patent databases is possible. Patent mining is the process of searching for meaningful trends, patterns, and relationships among patent records contained in patent databases. It helps to determine the possibility for acquiring a patent and checks of infringement chance. Patent searches are generally of two kinds: preliminary and conclusive or complete. Generally, inventors prefer to conduct a preliminary patent search themselves, but to improve the likelihood that our patent application will be allowed by the Patent Office, we must have a complete patent search conducted. With a complete patent search, a professional patent searcher will conduct an expanded search of issued patents and other relevant literature. The relevant utility patents and/or design patents and other literature will be carefully analyzed by a registered patent attorney to see if the invention is novel and nonobvious. Then the patent attorney

prepares a patentability opinion stating whether the invention is likely to receive a patent. Patent mapping and patent analytics are ways of visualizing patent mining results. There are several free and paid patent databases consisting of billions of patent documents. The databases for which patent search is free include the following major ones: USPTO, Espacenet (EPO), Indian Patent Advanced Search System (inPASS), Japan Patent Office (JPO), PatentScope (WIPO), China Patent Machine Translation system (CPMT), IP Australia and Google Patents. Some paid databases are PatentScout, Relecura, Innovation Q Plus, Thomson Innovation, PlanetPatent and CPA Global which also include inbuilt analysis tools. Patent search helps an inventor to determine if a particular invention is unique, to identify potential features for new product, to identify other possible uses for a new product, to determine independent inventors or companies currently or historically obtaining patents in a particular area, to find the patent(s) for a particular invention, to determine the state of the art in a particular area, to study the rate of innovation in a particular area, to identify potential licensees, to identify additional reference materials (journal articles, books, product literature) of use to those working in this area, and so on. Public databases have regional Patent data as per their geographic province and a recent attractive trend observed in certain IPOs is to include data of other Patent Offices available for searching. A global patent database search engine in future for member countries as a whole will improve and impact the patenting process more efficient and treaties should include suitable amendments to make this a reality. Earlier, the search fields were greatly assorted across different IPOs and now the search criteria and fields were mostly common among all IPOs. Patent search and analysis is carried out on the basis of a specific objective and can be classified as below:

Theme-based search

Theme searches provide an overview of patents related to our field of interest. These searches are helpful in detecting the recent trends in our technology area and in establishing the direction for research and development.

Patentability search (novelty search)

Patentability search is the first step in the patenting process. A patentability search surveys patents filed in each national intellectual property office to check whether there exist inventions similar to ours. If there is a plan to file an invention in other countries, this search is essential because foreign applications are cost intensive.

Keyword search

This search provides the information of patents retrieved by specific keywords including assignee, inventors, organism name, and product name.

Patent map (patent analysis)

Quantitative analysis based on statistical data of bibliographic information (country, assignee, International Patent Classification code, etc.). Qualitative analyses of core patents, technological road map, multifarious analysis, and so on, are also included.

Right-to-use searches

Right-to-use searches are conducted, prior to marketing a new product, to confirm that the new product does not infringe on an existing patent or potentially infringe on a patent application.

Public Databases

IP Australia

IP Australia is the Australian Government agency responsible for administering patents, trademarks, designs, and Plant Breeder's Rights. The Patent Enquiries system is known as AusPat and it enables one to access bibliographic and status information about Australian patents and patent applications filed since January 1979. In some cases, details of patents filed before that date are also available. An eDossier with access to a suite of documents relating to the prosecution of patent applications dating back to 2006 is Open to Public Inspection (OPI). Three levels of search are available and can be used based on the details available.

State intellectual property office of China

The former body of the State Intellectual Property Office (SIPO) is the Chinese Patent Office, founded in 1980. In 1998, when governmental bodies were undergoing reforms, the name of the Chinese Patent Office was changed to the State Intellectual Property Office, which is directly subordinated to the State Council. Now, the Patent Office is affiliated to the SIPO. As of 2015, it has collected patent records and abstracts from 104 countries (regions) or organizations, images from 103 countries (regions) or organizations, and texts from 33 countries (regions) or organizations. Among them, the number of documentation on Chinese patents has surpassed 15 million. Two databases on Invention and Utility Model are available to choose with.

Espacenet (European patent office, EPO)

The Espacenet provides a uniform application procedure for individual inventors and companies seeking patent protection in up to 39 European countries. It is the executive arm of the European Patent Organization and is supervised by the Administrative Council. Established in 1977 by the European Patent Convention (EPC) with the aim of creating a centralized patent application and grant system on behalf of all contracting states, the EPO's mission is to support innovation, competitiveness, and economic growth for the

benefit of the citizens of Europe. The EPO examines and grants "European patents," which, subject to formal requirements, then acquire the same status and influence as national patents under the national laws of such EPC contracting states as the applicant designates. In each contracting state for which it is granted, a European patent gives its proprietor the same rights as would be conferred by a national patent granted in that state. If its subject-matter is a process, protection is extended to products directly obtained by that process. Any infringement of a European patent is dealt with by national law. If the application discloses nucleotide or amino acid sequences (unbranched sequences of four or more amino acids or unbranched sequences of ten or more nucleotides), the description must contain a sequence listing complying with WIPO Standard ST.25 and presented as a separate part of the description. The sequence listing must be filed in electronic form, ie, in text format (.txt). EPO's free BiSSAP or PatentInsoftware, can be used for standardized sequence presentation. For full-text search in the worldwide collections Espacenet supports full-text (FT) search in English, French and German. Combisets, an ordered list of linked Cooperated Patent Classification (CPC) symbols created by patent examiners are now searchable as Smart search. All communications between our browser and Espacenet are encrypted. The users of the European patent system can look up procedural data on European patents and patent applications. The Register provides bibliographic data together with procedural data from the date of publication to the grant of the patent, plus any data concerning opposition and appeal proceedings. Internet access to the Register is free of charge. Further, data from the European Patent Register is published weekly in the European Patent Bulletin, where the bibliographic data relating to published European patent applications and granted European patents is arranged according to a number of reference criteria, including the International Patent Classification, European publication numbers and names of applicants/proprietors. Global Patent Index (GPI), an advanced tool for searching the EPO's worldwide bibliographic and legal status patent data is also available. With GPI one canget high-quality search results; save time when performing long and complex searches; re-use queries; identify technical and competitor trends; perform searches in a secure https environment.

Indian patent office

The Indian Patent Office is administered by the Office of the Controller General of Patents, Designs & Trademarks (CGPDTM). This is a subordinate office of the Indian Government and administers the Indian Law of Patents, Designs, and Trademarks. Free searches for awarded patents are available online. The official website provides public search systems for Indian patents, trademarks, and designs, and it also allows users to check the status of patents, trademarks, and geographical indications (GI). The databases on Indian Patent Information available are Ekaswa A, Ekaswa B, and Ekaswa C. The coverage areas of the databases are given below:

- Ekaswa A database – Patent applications filed in India as published in the issues of the Gazette of India (Part III, Section 2) from January 1995 to December 2004.
- Ekaswa B database – Patent applications notified for opposition in the Gazette of India (Part III, Section 2) published from January 1995 to December 2004.
- Ekaswa C Database – Patent applications published in the official Journal of Patent Office published from January 2005 onwards.

The access to the database is online through internet or can also subscribed as CD-ROM. The Indian Patent Office has a search facility named "Indian Patent Advanced Search System (InPass)" compared to the old Indian Patent Information Retrieval System (IPAIRS) search, with which full text searching is available for published applications as well as for granted patents jointly or separately. PCT publication number was also introduced as new search criteria. For the time being, the new InPASS is still running simultaneously with the old IPAIRS database.

Japan patent office (JPO)

Patent Abstracts of Japan (PAJ) is a database that has English abstracts of patent applications from 1976 to the present. The Japan Patent database contains full-text data of most patents issued from 1993. It also contains Optical Character Recognition (OCR) full-text data of most patents issued from 1986 to 1992. JPO Industrial Property Digital Library database contains Japanese patent information since 1885. Japan Platform for Patent Information (J-PlatPat) run by the National Center for Industrial Property Information and Training (INPIT) provides databases of publications of patent, utility model, industrial design and trademark. The legal status of each application can also be seen through this service. J-PlatPat provides around 100.3 million documents of patents, utility models, designs and trademarks and their relevant information that have been published since the end of the 19th century. Basic information in gazettes can also be retrieved and used in English, with the objectives of reinforcing the protection of rights overseas and as an international contribution.

Korean industrial property rights information service

It contains the entire Korean industrial property rights information since 1948 and an English abstract of Korean patents since 1979. Machine translations of Korean patent and utility model publications are available since 1983. Korean Intellectual Property Rights Information Service (KIPRIS) covers all Korean lP information of Korean IntellectualProperty Office (KIPO). KIPRIS is managed by Korea Institute of Patent Information (KIPI). Two types of patent searches are available. They are general search and advanced search. General search provides access to the information based on words, names, or numbers. Advanced search allows specifying the range of search for each field based on keywords, numbers, dates, and names, and/or a combination of these fields for quicker access to the information. KIPRIS provides access to bibliographic data and Korean full text. A fee-based service called K2E-PAT, is

available for users who wish to search the Korean full text using English keywords. Performing a search through the interface is free; however, attempting to open the machine translated text of individual search results will bring up a payment screen.

Russian agency for patents and trademarks

The Federal Institute of Industrial Property (FIPS) incorporated in the structure of the Federal Service for Intellectual Property (Rospatent) is inter alia in charge of issuing and dissemination of official publications of Rospatent. Russian Patent databases – RUPAT01, RUPAT02, RUPAT03, RUPAT04 and RUPAT_NEW contain full-text of Russian Patented Inventions (in Russian) from 1994 to current. Rupat_Old contain retrospective database of patent documents as facsimile from 1924 to 1993. RUPATABRU contain abstracts of Russian applications and patented inventions in Russian language from 1994 to current. RUPATABEN contain abstracts of Russian patented inventions in English for the same period. IMPIN contain full text of perspective inventions in Russian language from June 2015.

United Kingdom intellectual property office (UKIPO)

Online Patent Information and Document Inspection Service (IPSUM) allow checking the status and access information on UK patent applications. Patent status, up to date information on patents, access some documents from published patent applications, classifications and fields of search used and selected patent applications can be saved using IPSUM. Users can currently only search for patents by publication or application number. A small help box beside the search form describes the correct format for inputting the publication or application numbers. In the full record view of a patent document, users can view the document on Espacenet through a link provided above the document title.

United States patent and trademark office (USPTO)

The USPTO is a federal agency in the US Department of Commerce. Inventors are encouraged to search the USPTO's patent database to see if a patent has already been filed or granted that is similar to the searched patent. Patents may also be searched in the USPTO Patent Full-Text and Image Database (PatFT). The USPTO houses full text for patents issued from 1976 and PDF images for all patents from 1790. Global Patent Search Network (GPSN) enables users to search the full text of multiple international patent collections. The initial collection available is that of Chinese patent documentation from the State Intellectual Property Office (SIPO) of the People's Republic of China. The Patent Application Information Retrieval (PAIR) system helps to retrieve patent application status.

Patent scope search service of WIPO

The Patentscope database provides access to international Patent Cooperation Treaty (PCT) applications in full text format on the day of publication, as well as to patent documents of participating national and regional patent offices. The information may be searched by entering keywords, names of applicants, international patent classification and many other search criteria in multiple languages. Machine translations are available and default translations originate from Google Translate, but alternate machine translation tools such as Microsoft Translator are also available. A cross-lingual search query generating tool is available in PATENTSCOPE interface, termed as "Cross-Lingual Information Retrieval (CLIR) facility." This tool uses a number of cross-lingual dictionaries built in house by WIPO to generate keyword queries in other languages. The language dictionaries are built by using correlation methods between languages from WIPO's corpus of patent documents. The tool also uses a technology discipline filter to help ensure that the meaning of the desired synonym is clear to the system, disambiguating among polysemy. To further ensure that only desired meanings of a word are searched, the tool also generates a basic set of main IPC classes to be included in the search string. WIPO's International Cooperation on the Examination of Patents (ICE) service provides expert assistance, training, and access to collections of patent documents to developing countries – all free of charge. Through the Access to Specialized Patent Information (ASPI) program, patent offices and academic and research institutions in developing countries can receive free or low-cost access to sophisticated tools and services for retrieving and analyzing patent data. The WIPO Centralized Access to Search and Examination (CASE) system enables patent offices to securely share search and examination documentation related to patent applications, facilitating a more effective and efficient international examination process.

Google patents

Google Patents is a search engine from Google that indexes patents and patent applications from USPTO, EPO, WIPO, Deutsches Patentund Markenamt (DPMA), Canadian Intellectual Property Office (CIPO) and SIPO. These documents include the entire collection of granted patents and published patent applications from each database (which belong to the public domain). Presently this is provided as gratis service.

Private Databases

Thomson scientific IP services

Some of the major services offered by this group are enlisted here:

Westlaw IP – It offers the most comprehensive patent, trademark, copyright and trade-secret content and treatises globally, along with cases, court documents and filings, judge and examiner profiles and filing rules.

Derwent World Patents Index (DWPI) – It features global content in English and provides clear titles, abstracts summarizing the novelty of the invention, intellectual corrections to bibliographic errors and applies unique coding and indexing. It can also translate from 29 languages. The invention-based records allow seeing the full global protection for a single invention.

Thomson Innovation – It brings together a comprehensive international patent coverage with intellectual property (IP) analysis tools.

Thomson Data Analyzer – A desktop software which offers interface for managing and extracting business-critical insights from patent and scientific data within in-house or commercial databases. Users can analyze patent data and scientific literature from any structured database directly on their desktop.

IFI claims patent services

The CLAIMS Global Patent Database with more than 100 million patent data records aggregates patent information from over 50 national and international data sources. Data from different sources are normalized and merged into standardized records and available in a single proprietary ST.36-based XML format. New patent documents and updated records are provided. Legal status, US reassignments and referenced images are included.

PatentScout

PatentScout delivers critical patent data from a private, web-based platform. Enables everyone in the organization to conduct patent searches as needed, without the loss of privacy and search logging associated public tools. Free patent search tools can expose the conceived IP strategy to sniffing technology, competitors, and marketers. Searches performed from the organization using public tools are discoverable during litigation.

Relecura

Its patent search and analysis tool offers global patent coverage with translated and full-text data, curated data with high impact visualization and analytics, integrated patent and assignment search for IP commercialization, enterprise version to search internal, unpublished and patent documents, share patent portfolios along with Relecura analytics, auto-bucket patent portfolios with pre-defined criteria and customizable dashboards that can be shared with anyone.

Innovation Q plus

It is innovation discovery and analytics platform that indexes the full text of periodicals, conferences and Standards, which are cited in patents alongside a comprehensive, full text global patent database. The semantic search technology enables anyone to find valuable content that is buried deep in complex patent and technical documents, resulting in more accurate retrieval than a simple keyword search. It allows users to determine patent strengths and weaknesses, evaluate competition, make legal assessments, and leverage IP business intelligence to support critical portfolio management decisions. It eliminates the need to use Boolean syntax to inform retrieval and instead, the system uses a short phrase, a paragraph, or even an entire document to discover highly relevant results and gain insights. The Map tool creates a visual representation of results based on concepts and meanings. Visualizing document relationships lets users find whitespace in the marketplace, see what competitors are up to, and find more licensing and acquisition opportunities.

Planet patent

It examines US and other patents as well as non-patent prior arts. Over 100 million patents and applications from 100 countries were searched and details about collections, infringements, novelty, right-to-use/freedom to operate, state-of-the-art and validity are provided.

CPA global

Services offered include:

PatentScout – delivers patent data from a private, web-based platform without the loss of privacy and search logging associated with free, public tools.

PatentIQ – allows one to keep up-to-date with dynamic IP data. Whether sharing organization's IP status, competitive analysis or search results, the data is provided in an easily consumable format with PatentIQ. Many PatentIQ dashboards can be created and shared.

PatentGuard – a managed service that analyzes a patent portfolio to identify potential patent title issues, reviews the patent due diligence process, and reports measures to repair broken title chains where possible. This diligence software provides portfolio managers with a certified audit report of any existing issues to help assure the asset is properly protected.

Strategies for Efficient Patent Searching

Patent databases of several countries are available online, which can be consulted for patent searching. As an example the seven-step US Patent search strategy, which is based on US Patent Classification, has been outlined in Table 5. Inventors and

Table 5 Seven steps US patent search strategy

Classification
1. Brainstorm keywords related to the purpose, use and composition of the invention.
2. Look up the words in the Index to the US Patent Classification to find potential class/subclasses.
3. Verify the relevancy of the class/subclasses by using the Classification Schedule in the Manual of Classification.
4. Read the Classification Definitions to verify the scope of the subclasses and note "see also" references.
Access full-text
5. Search the Issued Patents and the Published Applications databases by "Current US Classification" and access full-text patents and published applications.
Review and references
6. Review the claims, specifications and drawings of documents retrieved for relevancy7. Check all references and note the "US Classification" and "Field of Search" areas for additional class/subclasses to search.

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entrepreneurs spend huge money to do a professional patent search. But, a prior knowledge about patent keywords and classifications will be helpful in devising a personal search strategy. The results can be analyzed using a private software tool and affirmed. There is a help section on the IPOs which gives detailed guideline regarding the search features and has to be gone through. The advantages and limitations of a search engine can be learned through experience. Before searching, it is advisable to check the date from which the data is available. In many search engines, an Advanced Search Page is given which can be used to improve the search quality. The preliminary step in a patent search is to frame the keywords. While framing keywords, terms directly identifying the search item and related terms should be enlisted. If the search term involves a group of organisms like cyanobacteria, the major generic keywords can be "Cyanobacteria," "blue-green Algae," "Cyanobacterium," and "blue-green Alga" (Table 6). A total of 155 patents were obtained using these four keywords in the USPTO database. Of these, 19 patents were overlaps, while the remaining 136 were unique patent hits. This approach of using generic keywords followed by the removal of overlapping patent hits is helpful in obtaining primary data. When a patent search was made using 84 genera of cyanobacterial as keywords, 156 new hits were obtained out of the total 295 hits obtained. When overlaps were removed, 98 new patents in addition to the earlier 136 patents were obtained. Information derived from these patents helped to identify five major areas of cyanobacterial utilization based on the USPTO database. Similar analysis for a bioproduct like "phycobiliproteins" present exclusively in cyanobacteria, cryptomonads, cyanelles, and rhodophytes could end up with 297 patents from global patent databases. The analysis further revealed patents existing on diverse applications of phycobiliproteins and globally patented production technologies. This ultimately helps to identify lacuna and suggests measures for improvement in the patenting of phycobiliproteins toward application and production technologies.

Even for differences in singular and plural forms for the same keyword the number of hits obtained may be different. Published applications are divided into many fields. Narrowing our search with a specific keyword in a specific field will result in a specific hit and can greatly decrease the likelihood of having extraneous documents returned. Some of the available fields are given in Table 7. While searching, Abstract, Claims and Title will yield specific hits. For additional searches "All fields" option can be chosen. When "All Fields" option is used, bulk hits are obtained due to the presence of both related patents and noise patents. Noise patents are patent hits that simply contain the keyword in the text but the patent is not related to the keyword. The noise patents can be excluded by analyzing the titles and contents of the patents to find any possible linkage and relevance to the major keyword. Such patents have to be compared with the patents obtained from the abstract- and claims-based search in order to identify any additional unique patents. This approach helps to obtain a maximum number of patent hits as well as to avoid missing of patent hits from the database.

Table 6 Major search keywords and the resultant hits from USPTO database for the taxonomic group Cyanobacteria

	Abstract- and claims-based search		*All fields search*
Major search keywords	*Number of hits obtained*	*Number of hits without overlaps to the other keywords*	*Number of hits obtained*
Cyanobacteria	68	68	907
"Blue-Green Algae"	44	37	624
Cyanobacterium	28	17	387
"Blue-Green Alga"	15	14	93
Total	155	136	2011

Source: From Sekar, S., Paulraj, P., 2006. Strategic mining of cyanobacterial patents from the USPTO patent database and analysis of their scope and implication. Journal of Applied Phycology 19, 277–292. With kind permission of Springer Science and Business Media.

Table 7 Important fields in a patent search

Some fields in a Patent search	*Explanation*
Abstract (ABST)	This field contains a brief summary of the published application.
Application Number (AN)	The application number of the patent. This is 15 characters long, for example, 96600006608137.
Applicant (AP)	It is the name of one or more persons or organizations that are registered as proprietor of a patent application. The applicant can also be the inventor.
Claim(s) (ACLM)	This field contains the text of the published application claims. Claims point out and distinctly claim the subject matter that the applicant regards as the invention and define the scope of the published application protection.
Inventor (TI)	It is the name of the person(s) who is/are registered on patent application as the inventor(s).
International Class (IP)	The International Classification(s) the patent has been placed under.
Patent Number (PN)	It is the unique number assigned by the respective Patent Office to a granted application filed by an inventor/applicant.
PCT Information (PCT)	This field contains PCT Information related to the published application.
Title (TI)	It is the title of the Patent.

Table 8 List of relevant web pages

Website	*URL*
Culture collections	
National Measurement Institute (NMI), Australia	http://www.measurement.gov.au/
Lady Mary Fairfax CellBank Australia (CBA)	http://www.cmri.org.au/
Belgian Coordinated Collections of Microorganisms (BCCM), Belgium	http://bccm.belspo.be/
National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC)	http://www.nbimcc.org/
International Depositary Authority of Canada (IDAC)	https://www.nml-lnm.gc.ca/
ColecciónChilena de RecursosGenéticosMicrobianos (CChRGM)	http://www.cchrgm.cl/
China Center for Type Culture Collection (CCTCC)	http://www.cctcc.org/
China General Microbiological Culture Collection Center (CGMCC)	http://www.cgmcc.net/english/
Guangdong Microbial Culture Collection Center (GDMCC)	http://www.gimcc.net/
Czech Collection of Microorganisms (CCM)	http://www.sci.muni.cz/
VTT Culture Collection (VTTCC)	http://culturecollection.vtt.fi/
Collection Nationale De Cultures De Microorganismes (CNCM), France	http://www.infect-era.eu/
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH German Collection of Microorganisms and Cell Cultures (DSMZ)	http://www.dsmz.de/
National Collection of Agricultural and Industrial Microorganisms (NCAIM)	http://www.infect-era.eu/
Microbial Culture Collection (MCC)	http://www.nccs.res.in/mcc/
Microbial and Gene Bank Type Culture Collection (MTCC)	http://mtcc.imtech.res.in/
Advanced Biotechnology Center (ABC)	http://www.abc.ae/
Collection of Industrial Yeasts (DBVPG)	http://www.dbvpg.unipg.it/
IstitutoZooprofilatticoSperimentaledellaLombardia e dell'Emilia Romagna "Bruno Ubertini" (IZSLER)	http://www.izsler.it/
International Patent Organism Depositary (IPOD), Japan	http://unit.aist.go.jp/ipod/cie/
NITE Patent Microorganisms Depositary (NPMD), Japan	http://www.nbrc.nite.go.jp/
Microbial Strain Collection of Latvia (MSCL)	http://mikro.daba.lv/
Colección de Microorganismos del Centro Nacional de RecursosGenéticos (CM-CNRG)	http://www.sagarpa.gob.mx/
CentraalbureauvoorSchimmelcultures (CBS)	http://www.cbs.knaw.nl/
Collection of Industrial Microorganisms (IAFB)	http://www.infect-era.eu/
Polish Collection of Microorganisms (PCM)	https://www.iitd.pan.wroc.pl/en/
Korean Agricultural Culture Collection (KACC)	http://www.acm-mrc.asia/m/kr_kacc/
Korean Cell Research Foundation (KCLRF)	http://cellbank.snu.ac.kr/english/
Korean Collection for Type Cultures (KCTC)	http://kctc.kribb.re.kr/
Korean Culture Center of Microorganisms (KCCM)	http://www.kccm.or.kr/
All Russian Collection of Microorganisms (VKM)	http://www.vkm.ru/
Russian National Collection of Industrial Microorganisms (VKPM)	http://eng.genetika.ru/
Culture Collection of Yeasts (CCY)	http://www.chem.sk/
BancoEspañol de Algas (BEA)	http://bea.marinebiotechnology.org/
Coleccion Espanola de CultivosTipo (CECT)	http://www.uv.es/cect
Culture Collection of Algae and Protozoa (CCAP), United Kingdom	http://www.ccap.ac.uk/
CAB International Bioscience (International Mycological Institute – IMI), United Kingdom	http://www.cabi.org/
European Collection of Cell Cultures (ECACC), United Kingdom	https://www.phe-culturecollections.org.uk/collections/ecacc.aspx

(*Continued*)

Table 8 Continued

Website	*URL*
National Collection of Type Cultures (NCTC), United Kingdom	https://www.phe-culturecollections.org.uk/collections/nctc.aspx
National Collection of Yeast Cultures (NCYC), United Kingdom	http://www.ncyc.co.uk/
National Collections of Industrial, Food and Marine Bacteria (NCIMB), United Kingdom	http://www.ncimb.com/
National Institute of Biological Standards and Control (NIBSC), United Kingdom	http://www.nibsc.org/
Agricultural Research Service Culture Collection (NRRL), United States	http://nrrl.ncaur.usda.gov/
American Type Culture Collection (ATCC), United States	http://www.atcc.org/
Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA)	https://ncma.bigelow.org/
Patent offices and public databases	
Australian Patent Office (IP Australia)	https://www.ipaustralia.gov.au/
European Patent Office (EPO)	https://www.epo.org/index.html
French Patent Office	http://bases-brevets.inpi.fr/en/
German Patent Office	http://www.dpma.de/english/
Indian Patent Office	http://www.ipindia.nic.in/
Japan Patent Office (JPO)	https://www.jpo.go.jp/
Korean Industrial property rights (KIPRIS)	http://eng.kipris.or.kr/ http://www.kipris.or.kr/enghome/
Patent scope, International Patent Search (WIPO)	http://www.wipo.int/patentscope/en/
Federal Service for Intellectual Property (Rospatent)	http://www.rupto.ru/start?lang=en
State Intellectual Property Office of China (SIPO)	http://english.sipo.gov.cn/
UK Intellectual Property Office	https://www.gov.uk/government/organisations/intellectual-property-office
United States Patent and Trademark Office (USPTO)	http://www.uspto.gov/
Private databases	
Thomson Scientific IP Services	http://ip.thomsonreuters.com/
IFI Claims Patent Services	http://www.ificlaims.com/
PatentScout	https://www.innography.com/
Relecura	https://www.relecura.com/
Innovation Q Plus	https://ip.com/innovationqplus/
PlanetPatent	http://www.planetpatent.com/
CPA Global	https://www.cpaglobal.com/
Organizations	
World Intellectual Property Organization (WIPO)	http://www.wipo.int/
World Trade Organization (WTO)	https://www.wto.org/
Important Pacts	
Budapest Treaty	http://www.wipo.int/treaties/en/registration/budapest/guide/
International Patent Classification	http://www.wipo.int/classifications/ipc/en/
Patent Cooperation Treaty (PCT)	http://www.wipo.int/pct/en/
Trade-Related Aspects of Intellectual Property Rights (TRIPs)	https://www.wto.org/english/tratop_e/trips_e/trips_e.htm
Draft Substantive Patent Law Treaty	http://www.wipo.int/patent-law/en/draft_splt.htm
Paris Convention for the Protection of Industrial Property	

Conclusion and List of Relevant Webpages

It is quite imperative that a microbiologist have thorough knowledge about IPR with special focus on patents, systems, and procedures in the home country and abroad as a prerequisite for microbial patenting. Further emphasis should be on the legality involved in microbial patenting; the art of efficient mining of patents, the procedures involved in culture deposition with IDA, and awareness on IDA status culture collections. A perusal of key websites of nodal agencies, organizations, culture collections, and patent databases (Table 8) helps a prospective patentee to achieve the goal. Research driven by such patent analysis is also of contemporary relevance.

Further Reading

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Sekar S, Karthikeyan S, and Iyappan P (2010) Trends in patenting and commercial utilisation of poultry farm excreta. *World's Poultry Science Journal* 66: 533–572.
Sekar S and Paulraj P (2006) Strategic mining of cyanobacterial patents from the USPTO patent database and analysis of their scope and implication. *Journal of Applied Phycology* 19: 277–292.

Pathogen Sensing: Toll-Like Receptors and NODs (Innate Immunity)

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Glossary

Danger-Associated Molecular Pattern (DAMP) Molecules released by stressed or dying cells that act as endogenous signals of danger and further enhance inflammation and/or cell-death signaling; also known as alarmins.

Inflammation The body's response to stimuli with the potential for harm (such as pathogens, external injuries, or chemicals) and an important component of the innate immune response. Inflammation is mediated by many different immune cells (including neutrophils, basophils, eosinophils, monocytes, and macrophages), which can release inflammatory mediators such as cytokines, chemokines, and lipids. These mediators can affect blood flow, vascular permeability, and fluid production and present as pain, heat, redness, and swelling of surrounding tissue.

Innate Immunity Early and non-specific host defense mechanisms against invading microbial agents. Composed of physical, chemical, and cellular defenses, especially protein cascades and phagocytic cells, that recognize pathogens by specific conserved features and subsequently initiate host defenses to destroy invading pathogens through mechanisms such as inflammation and complement.

Nod-like Receptor (NLR) Cytosolic innate immune receptors characterized by the presence of a nucleotide-binding and oligomerization domain (NOD) or neuronal apoptosis inhibitor protein (NAIP) class 2 transcription activator (C2TA) heterokaryon incompatibility (HET-E) and telomerase-associated protein 1 (TP1) (NACHT) oligomerization domain. This family of proteins has a wide range of functions including acting as sensors of intracellular microbes, in homeostatic tissue regulation, and as regulators of pro-inflammatory signaling mediators.

Pathogen-Associated Molecular Pattern (PAMP) A conserved molecular feature specific to classes of microbes that is discriminatory from the host self. PAMPs may be lipids, proteins, nucleic acid, or metabolites. Non-pathogenic microbial species display many of the same molecular patterns and are referred to as microbe-associated molecular patterns (MAMPs).

Pattern Recognition Receptor (PRR) A germline-encoded receptor capable of recognizing specific PAMPs. PRRs initiate signaling cascades that direct the innate immune response to best respond to the invading pathogen.

Toll-like Receptor (TLR) Membrane-associated PRRs present on/in innate immune cells that are capable of detecting MAMPs and PAMPs from mycobacteria, Gram-negative bacteria, Gram-positive bacteria, viruses, fungi, and parasites; may be present on the external cell membrane or endosomal membrane.

Abbreviations

AD	Activation domain
APC	Antigen presenting cell
ALR	Aim2-like receptor
ASC	Apoptosis-associated speck-like protein
ATP	Adenosine triphosphate
BIR	Baculovirus inhibitor-of-apoptosis repeats
CARD	Caspase activating and recruitment domain
CD	Cluster of differentiation
CLR	C-type lectin receptor
CpG	Cytosine-phosphate-guanosine deoxynucleic acid motif
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DD	Death domain
DNA	Deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
ER	Endoplasmic reticulum
FIIND	Function-to-find domain
GPI	Glycosylphosphatidylinositol
IFN	Type 1 interferon
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory transcription factor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat

LTA	Lipoteichoic acid
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MD-2	Myeloid differentiation factor 2
MyD88	Myeloid differentiation primary response gene 88
NAIPs	Neuronal apoptosis inhibitory proteins
NF-κB	Nuclear factor-kappa B
NLR	NOD-like receptor
NACHT	NAIP class 2 transcription activator (C2TA) heterokaryon incompatibility (HET-E) and telomerase-associated protein 1 (TP1)
NOD	Nucleotide-binding and oligomerization domain
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PYD	Pyrine domain
RLR	Rig-I-like receptor
RNA	Ribonucleic acid
SAR	Structure activity relationship
ssDNA	Single-stranded deoxyribonucleic acid
ssRNA	Single-stranded ribonucleic acid
TAB	TAK-1 binding protein
TIR	Toll/1L-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TRAF	Tumor necrosis factor receptor (TNF-R)-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing interferon-β

Introduction

Earth is predicted to be home to up to one trillion unique microbial species (Locey and Lennon, 2016). As such, mammals are constantly being assailed by innocuous and pathogenic bacteria, fungi, viruses, and parasites. Due to millennia of coevolution and selection of the innate and adaptive immune systems, most mammals are able to resist and overcome pathogenic infections while balancing symbiotic microbial colonization. The term "immunity" has roots in the Latin words *immunitas* and *immunis*, which in their original context in Ancient Rome referred to the legal concept of exemption. Although the concept of acquired immunity had been described several centuries earlier in China and elsewhere, it wasn't until the late 19th century that the phrase was formalized and used commonly in the field (Silverstein, 2001). It was during this time that several scientists began providing proof of classical immunity (that is, exposure to a non-lethal antigen that results in protection from subsequent exposure with a lethal pathogen). This included Edward Jenner's 18th century work on vaccination using the contents of cowpox pustules as a safe vaccine for smallpox and the 19th century observations of Pasteur, Koch, Ehrlich, and others for bacterial diseases (Silverstein, 2001; Willis, 1997; Smith, 2012; Blevins and Bronze, 2010; Bosch and Rosich, 2008). Despite the observed link between microbes and classical immunity, the phrase "immune system" didn't begin appearing in journals and articles until the mid-1960s, as the field of cellular immunology begin to develop. Originally, the immune system was referred to as the "lymphoid system". The cellular reference was eventually dropped and replaced with the term immune system (Silverstein, 2001).

Early definitions of the immune system described it as a set of cells that were unbiasedly involved in all immunological reactions, with emphasis on function and not mechanism (Silverstein, 2001). In the 1970s, our understanding of the complexity of the types of cells involved in immunity began to develop, and cells began to be broken into classes: killer cells, antigen-presenting cells, helper cells, suppressor cells, etc., (Silverstein, 2001). Today, we define the immune system as the group of organs, tissues, cells, and cell products (such as cytokines and antibodies) that discriminates between self and non-self to protect the body against infection and disease and to neutralize potentially pathogenic substances (Bellavite et al., 2005; Silverstein, 2001).

Although the concept of immunology has historically been linked to microbes, it wasn't until the late 20th century that our understanding of how our body senses individual classes of microbes began to fully develop. Charles Janeway was the first to propose the idea of host receptors that would specifically recognize microbial species, essentially laying the groundwork for what would become known as innate immunology. During his introduction to the Cold Spring Harbor Symposium in 1989, Janeway proposed that the immune system evolved to discriminate "noninfectious self from infectious non-self", essentially stating that the immune system evolved to defend against microbes and therefore the induction of an immune response depended on microbes (Janeway, 1989). He theorized that this level of defense would be an inherent mechanism dependent on invariant receptors on innate cells, as opposed to the highly diversified and selected receptors expressed on B and T cells of the adaptive immune system.

He also proposed that these receptors would be germline-encoded and would recognize specific microbial patterns. Part of this theory was drawn from the fact that members of invertebrate kingdoms appear to lack adaptive immune systems yet are still able to respond to pathogenic stimuli (Janeway, 1989; Silverstein, 2001). Based on these and other observations, he argued that a system that identifies self from non-self must have developed early in evolutionary history and would therefore be distinct from lymphocyte-mediated immunity (Janeway, 1989).

Today, we recognize the innate immune system as the first line of defense against invading microbes, providing protection from potential infection and disease. Innate immunity is primarily mediated by antigen-presenting cells (APCs) and phagocytic cells, including macrophages, dendritic cells, and granulocytes. Unlike adaptive immunity, which is characterized by generation of specific responses and immunological memory, innate immunity is rapid and considered relatively non-specific (Silverstein, 2001). Innate immune cells rely on recognition of pathogen-specific features that are highly evolutionarily conserved. These were termed pathogen-associated molecular patterns (PAMPs) and are composed of small molecular motifs that are characteristic to classes of microbes and discriminatory from the host self. Several germline-encoded receptors termed pattern recognition receptors (PRRs) are responsible for recognizing and responding to PAMPs, matching the description previously proposed by Janeway (Janeway, 1989). In addition to PAMPs, non-pathogenic microbial species can be discriminated by the innate immune system via microbe-associated molecular patterns (MAMPs). PRRs are localized on the external cell surface, within endosomes, or in the cytosol of host immune cells and respond to specific classes of PAMPs (Kawai and Akira, 2010; Takeuchi and Akira, 2010). In certain cases, PRRs may also interpret specific host factors as 'danger' signals, known as damage-associated molecular patterns (DAMPs). These could include host molecules present in atypical locations or in abnormal molecular configurations as a result of cell stress, infection, or inflammation (Kawai and Akira, 2010; Akira et al., 2006). Upon PAMP or DAMP recognition, PRRs initiate intracellular signaling cascades that result in proinflammatory and antimicrobial responses and alert the host to the presence of the microbe. PRR signaling pathways are unique but generally result in the activation of gene expression and subsequent synthesis of a wide range of molecules such as cytokines, chemokines, and immunoreceptors. Together, these receptors and molecules orchestrate the early innate response to infection (Akira et al., 2006; Kawai and Akira, 2010). In addition to initiating inflammation and other host defense responses, PRR activation and signaling can prime and coordinate antigen-specific adaptive immune responses, providing an important link between innate and adaptive immunity. Mammals possess distinct classes of PRRs that each recognize specific molecular signatures: Toll-like receptors (TLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), RIG-1-like receptors (RLRs,) and C-type lectin receptors (CLRs) (Kawai and Akira, 2010; Akira et al., 2006). Here, we focus on TLRs and NLRs. The other classes of receptors have been recently reviewed (Mathur et al., 2018; Shiokawa et al., 2017; Kawai and Akira, 2009). Additionally, we now appreciate the role of the immune system outside the context of microbial defense, such as in autoimmunity, though these concepts are outside the scope of this article.

Toll-like Receptors: A Historical Perspective

Of the mammalian PRRs, Toll-like receptors (TLRs) were the first to be identified and are the best characterized. TLRs are located on the cell surface or within endosomal compartments of immune cells and are crucial for host defense against microbes (Kawasaki and Kawai, 2014; Botos et al., 2011). The field of TLR research began in *Drosophila* with the identification of Toll, a protein with homology to a receptor for the inflammatory cytokine IL-1 called IL-1R type 1 (IL-1R1) (Gay and Keith, 1991). Subsequently, a plant protein (called the N protein), which conferred resistance to a Tobacco Mosaic Virus, was identified and characterized as having an amino-terminal domain similar to that of Toll and IL-1R1 (Whitham et al., 1994). This conserved domain was termed the Toll-IL-1 resistance (TIR) domain and was proposed to be involved in host defenses across disparate plant and animal kingdoms. Subsequent work by several groups, especially the Levine and Hoffmann groups, further identified downstream proteins and components linking Toll to innate immune activation in *Drosophila* (Broz and Monack, 2013; Ip et al., 1993; Steiner et al., 1981; Lemaitre et al., 1996; Rutschmann et al., 2000). However, it wasn't until 1997 that Ruslan Medzhitov, while in the Janeway laboratory, identified the first human homologue of the *Drosophila* Toll protein, termed hToll (currently TLR4) (Medzhitov et al., 1997). Upon recognition with its ligand, Medzhitov observed activation of a transcription factor called nuclear factor-κ B (NF-κB), leading to the up-regulation of genes involved in immune and inflammatory responses. This included the gene encoding CD80, a protein involved in T-cell co-stimulation, providing a crucial observation linking the innate and adaptive immune systems (Broz and Monack, 2013; Medzhitov et al., 1997). In 1998, four mammalian Toll homologues were described in addition to TLR4. Due to the similarity between Toll and these protein homologues (all of which contained conserved TIR domains), the proteins were termed Toll-like receptors (TLRs), a name which has since been adopted as standard nomenclature (Broz and Monack, 2013; Rock et al., 1998).

Research to identify the ligands recognized by individual TLRs focused on microbial products suspected to be involved with innate immune responses based on the previous studies in Drosophila (Broz and Monack, 2013). The initial search for PRR ligands focused largely on lipopolysaccharide (LPS), a major component of the outer leaflet of the outer membrane of Gram-negative bacteria. LPS was known to activate NF-κB and was therefore an obvious potential PAMP. Prior to the discovery of TLR4, the genetic basis for LPS recognition was identified in mice that had acquired a spontaneous mutation that conferred resistance to LPS toxicity (C3H/HeJ mice) (Heppner and Weiss, 1965; Sultzer, 1968). LPS resistance was mapped to a single autosomal gene named Lps^d (Qureshi et al., 1999; Watson et al., 1978; Coutinho and Meo, 1978). Subsequent to the discovery of TLR4, studies by Yang and colleagues showed that expression of a yet-unknown TLR made cells responsive to LPS preparations, providing evidence that TLRs were in fact PRRs (Yang et al., 1998). Initially, the LPS receptor was mid-identified as TLR2 due to high levels of contaminating

lipoproteins (which are potent activators of TLR2) in the experimental LPS preparations (Hirschfeld et al., 2000). However, positional cloning work by Bruce Beutler's group identified *Tlr4* as the genetic component responsible for LPS sensing and response (Poltorak et al., 1998). Subsequent work from the Malo and Akira groups also confirming that TLR4 is the signaling receptor for LPS, thereby showing that *TLr4* was in fact *Lps*^{d} (Qureshi et al., 1999; Takeuchi et al., 1999). By 2007, ten human TLRs and twelve murine TLRs had been identified (hToll was renamed TLR4) (Beutler, 2008). Work to characterize TLR ligands and modes of recognition is still ongoing. Knockout mouse lines, largely generated by Akira and colleagues, TLR crystal structures, and a better understanding of the structure of the lipid A component of LPS have been important resources for characterization of TLR sensing and signaling.

TLR Structure & Synthesis

Structurally, TLRs are type I integral transmembrane glycoproteins that possess an N-terminal ectodomain, a single transmembrane α-helix, and a C-terminal intracellular signaling domain, each of which are essential for its function (Botos et al., 2011; Beutler, 2008). The ectodomain is responsible for binding the associated PAMP and is characterized by a motif of leucine-rich repeats (LRRs), the number of which vary by TLR, and characteristic structures capping the N- and C-terminus (Fig. 1(A)). Also called the extracellular domain, this region is generally 550–800 amino acids in length, variably glycosylated, and exposed either on the cell surface or within endosomes (Wang et al., 2016; Botos et al., 2011). Crystal structures of human or murine TLR ectodomains report a horseshoe shape with PAMPs binding on the inner curvature of the horseshoe and ligand-induced dimerization occurring on the latter face of the receptor (Fig. 1(B); Jin et al., 2007; Kang et al., 2009; Liu et al., 2008; Park et al., 2009). The helical transmembrane domain typically contains a stretch of ~20 uncharged, mostly hydrophobic amino acids that integrate the receptor into the endosomal or plasma membrane. The intracellular signaling domain contains the ~200 amino acid TIR domain (Wang et al., 2016; Liu et al., 2008).

TLRs are synthesized in the endoplasmic reticulum and trafficked to the Golgi apparatus, where they are sorted and recruited to the cell surface or endosomal compartments (Kawasaki and Kawai, 2014). Proper intracellular localization of TLRs is critical for ligand recognition and prevention of autoimmunity, which could result from TLRs coming into contact with self-nucleic acids (Kawasaki and Kawai, 2014). In mammals, the transmembrane protein UNC93B1 controls intracellular trafficking from the ER to endosomes via interaction between its transmembrane region and the TLR transmembrane helix (Kim et al., 2008; Tabeta et al., 2006). TLRs that do not interact with UNC93B1 are trafficked directly to the cell surface (Botos et al., 2011; Kawasaki and Kawai, 2014). PRAT4A, an ER-resident protein, is also crucial for trafficking of TLR1, 2, 4, 7, and 9 (Takahashi et al., 2007). The heat-shock-related ER-resident protein gp96 serves as a general chaperone molecule for most TLRs, including intracellular TLR7

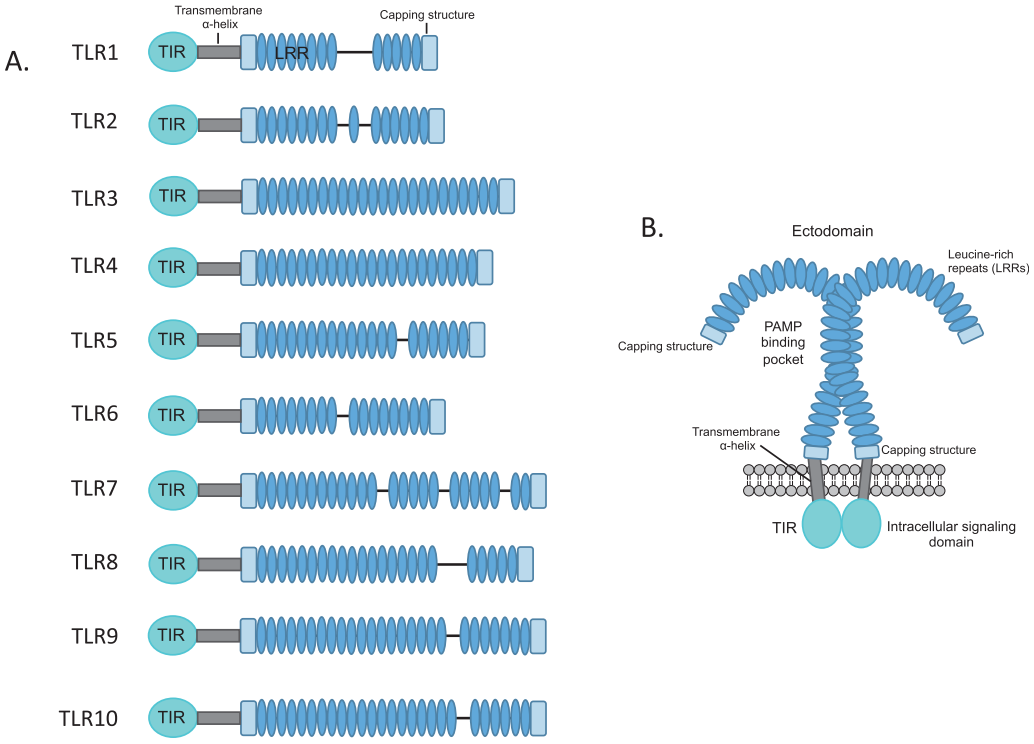

Fig. 1 TLR Structure. (A) Toll-like receptors are composed of leucine-rich repeats (LRRs), which vary in number based on the specific TLR. The LRRs are capped by characteristic structures and are connected to an intracellular TIR signaling domain via a transmembrane α-helix. (B) The TLR ectodomain, composed mainly of LRRs, forms a horseshoe-like shape, with PAMP ligands binding on the internal concave face. Ectodomains interact to for hetero- or homo-dimers, depending in the TLR. An α-helical transmembrane domain links the ectodomain to the intracellular, conserved TIR signaling domain.

and 9 and cell-surface-associated TLR1, 2, 4, and 5 (Yang et al., 2007). Endosomal-associated TLRs undergo specific proteolytic cleavage once they arrive in the endosome to produce the functional receptor capable of ligand recognition and signal initiation (Park et al., 2008; Ewald et al., 2011; Garcia-Cattaneo et al., 2012).

During PRR ligand studies and structural characterization, several co-receptors have been identified that are important for TLR function and activity. This includes myeloid differentiation factor 2 (MD-2), a 160 amino acid soluble protein that is glycosylated and binds to the concave, extracellular face of TLR4. MD-2 is required for recognition of LPS and therefore subsequent TLR4 function (Shimazu et al., 1999; Schromm et al., 2001; Park et al., 2009). Additional co-receptors are cluster of differentiation 14 (CD14) and cluster of differentiation 36 (CD36). CD14 is a glycoprotein of 375 amino acids that is found either soluble in the blood or linked to the membrane of myeloid cells via a glycosylphosphatidylinositol (GPI)-anchor and contributes to TLR2 and TLR4 ligand recognition (Zanoni et al., 2011; Baumann et al., 2010). Specifically, CD14 has been shown to bind LPS and deliver it into the TLR4/MD-2 complex. It can additionally extract LPS monomers from LPS-binding protein (LBP), which is a soluble protein that interacts with intact bacteria or LPS aggregates in the extracellular space (Kagan et al., 2008; Triantafilou and Triantafilou, 2002). CD36 has been shown to contribute to TLR2, TLR6, and TLR4 activation (Stewart et al., 2010; Sheedy et al., 2013). Models suggest that CD36 binds negatively charged lipo-ligands and transfers them to CD14, which then loads them into their respective TLRs. This has been proposed for ligands such as lipoteichoic acid (LTA, sensed by TLR2/6) and mycobacterial lipomannan (sensed by TLR1/2) (Jiménez-Dalmaroni et al., 2009).

TLRs are not analogous between systems, including across or within mammalian groups, as evidenced by the fact that humans possess ten TLRs (TLR1-TLR10) and mice possess twelve (TLR1-TLR9, TLR11-TLR13) (Kawasaki and Kawai, 2014). The TLR multigene family is composed of a variable number of genes that are species-specific (generally between 10 and 15 per species). However, comparative studies have shown that the TIR domains of TLRs are highly conserved across species, which is unsurprising due to their crucial role in signal transduction (Werling et al., 2009; Roach et al., 2005). Although all TLRs share the same common structural architecture, the specific TLRs that are present, what they recognize, and how they respond varies by species. The ectodomains, and specifically the face involved in ligand recognition, exhibit the highest level of variation between species, indicative of their function in recognizing PAMPs from multiple sources that are influenced by the microbial environment a particular species occupies (Werling et al., 2009). In some cases, homologous TLRs across species are proposed to have functional differences in what they sense. For example, bovine TLR1/TLR2 and chicken TLR2 type 2/TLR16, respond to both tri- and di-acylated peptides, whereas in other mammals, including mice and humans, these ligands are independently sensed by TLR1/2 and TLR2/6, respectively (Farhat et al., 2008). Additionally, homologous receptors across species may respond differently to the same ligand. This is the case for TLR4, in which specific *Pseudomonas aeruginosa* LPS structures differentially drive signaling in murine TLR4 versus human TLR4 and LPS from *Rhodobacter sphaeroides* is agonistic to TLR4 in horses and hamsters but antagonistic in humans and mice (Hajjar et al., 2002; Lien et al., 2000; Lohmann et al., 2007). LPS from some species, such as *E. coli*, activates TLR4 in a host-species-independent manner, while others, like that from *Salmonella enterica*, are recognized in a host-species-dependent manner (Hajjar et al., 2012; Lien et al., 2000; Lohmann et al., 2007; Werling et al., 2009). These inconsistencies are presumed to be due to differences in the variable regions in the TLR4 ectodomain and possibly differential associations of MD-2 with TLR4 (Werling et al., 2009; Hajjar et al., 2012).

The TLR Signaling Axes

TLR signaling is initiated upon binding and recognition of unique signature molecules, which are specific to the TLR (Table 1). Intracellular adaptor molecules are recruited to the TLR via the conserved and essential cytoplasmic TIR domain (Fig. 2; Werling et al., 2009; Kawasaki and Kawai, 2014). Cytoplasmic adaptor proteins involved in TLR signaling contain complementary TIR domains and include Myeloid differentiation primary response gene 88 (MyD88), TIR domain containing adaptor protein (TIRAP, also known as MyD88-adaptor-like or Mal), TIR-domain-containing adaptor-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM). Specific adaptors are differentially recruited to the TLRs after activation and influence the robustness of the signaling cascade (Werling et al., 2009; Kawasaki and Kawai, 2014).

TLR signaling can occur via two axes, mediated by MyD88 (the MyD88-dependent pathway) or TRIF (the MyD88-independent or TRIF pathway) (Fig. 2). The majority of TLRs signal exclusively through the MyD88-dependent pathway, with the exception of TLR3 which signals through the TRIF pathway and TLR4, which can signal through both pathways (Kawasaki and Kawai, 2014; Jiménez-Dalmaroni et al., 2016). Signaling is proposed to be initiated via conformational changes in the TLR ectodomain after ligand binding that bring the intracellular TIR domains in close proximity and allows for the recruitment of TIR-containing adaptor proteins. Additional adaptor proteins are required to facilitate signaling. TIRAP/Mal mediates interactions between MyD88 and TLR2, TLR4, and, in some cases, TLR9. TLR4 also requires an adaptor protein called TRAM when interacting with TRIF in the MyD88-independent signaling pathway (Kawasaki and Kawai, 2014; Jiménez-Dalmaroni et al., 2016).

After TLR activation, MyD88-depending signaling is initiated by the formation of the 'Myddosome' complex between MyD88, IRAK1, and IRAK4. IRAK4 activates IRAK1, which autophosphorylates at several sites and is released from the Myddosome (Kollewe et al., 2004; Jiang et al., 2002). IRAK1 then associates with TRAF6, a RING-domain E3 ubiquitin ligase, which acts in coordination with ubiquitin-conjugating enzyme UBC13 and UEV1A to polyubiquitinate the K63 residue of TRAF6 and the TAK1 protein kinase complex (Chen, 2012; Ajibade et al., 2013). TAK1 subsequently activates the IKK complex, composed of catalytic subunits IKKα and IKKβ and regulatory subunit NEMO (sometimes referred to as IKKγ). The activated IKK complex phosphorylates IkBα, the NF-kB inhibitory protein. Phosphorylated IkBα is degraded, freeing NF-kB to translocate to the nucleus and induce expression of pro-

Table 1 TLR localization and their associated PAMPs in human and mice

TLR – human	*TLR – mouse*	*Location*	*PAMP recognized*
1	1	Cell surface	See TLR2
2	2	Cell surface	Glycolipids, peptidoglycans, LTA, bacterial triacylated lipopeptides (TLR1/2) and diacylated lipopeptides (TLR2/6) (Kawai and Akira, 2010; Takeuchi et al., 1999; Dziarski and Gupta, 2000)
3	3	Endosome	Viral dsRNA, siRNA, self-RNAs from damaged cells (Zhang et al., 2007; Bernard et al., 2012; Takemura et al., 2014)
4	4	Cell surface/ endosome	Lipopolysaccharide (LPS)(Takeuchi et al., 1999; Dziarski and Gupta, 2000); mannuronic acid polymers, teichuronic acid, and viral proteins (Vaure and Liu, 2014)
5	5	Cell surface	Flagellin (Hayashi et al., 2001; Ramos et al., 2004)
6	6	Cell surface	See TLR2
7	7	Endosome	ssRNA from viruses, RNA from streptococcus B bacteria (cell-type specific) (Mancuso et al., 2009; Crozat and Beutler, 2004)
8	8	Endosome	Viral & bacterial RNA (Guiducci et al., 2013)
9	9	Endosome	Bacterial & viral DNA rich in unmethylated CpG-DNA motifs; heozoin (from *Plasmodium* infection) (Coban et al., 2010)
10	Pseudogene	Cell surface	*Listeria* ligands (TLR2/10), influenza A virus infection ligands (Regan et al., 2013; Lee et al., 2014)
Pseudogene	11	Endosome	Flagellin; components from *E. coli* UPEC and *Toxoplasma gondii* (Mathur et al., 2012; Yarovinsky et al., 2005); see also TLR12
None	12	Endosome	Homodimer or heterodimer with TLR11; profilin from *T. gondii* (Andrade et al., 2013; Koblansky et al., 2013; Broz and Monack, 2013)
None	13	Endosome	Bacterial 23S rRNA, vesicular stomatitis virus (Hidmark et al., 2012; Li and Chen, 2012; Oldenburg et al., 2012; Shi et al., 2011)

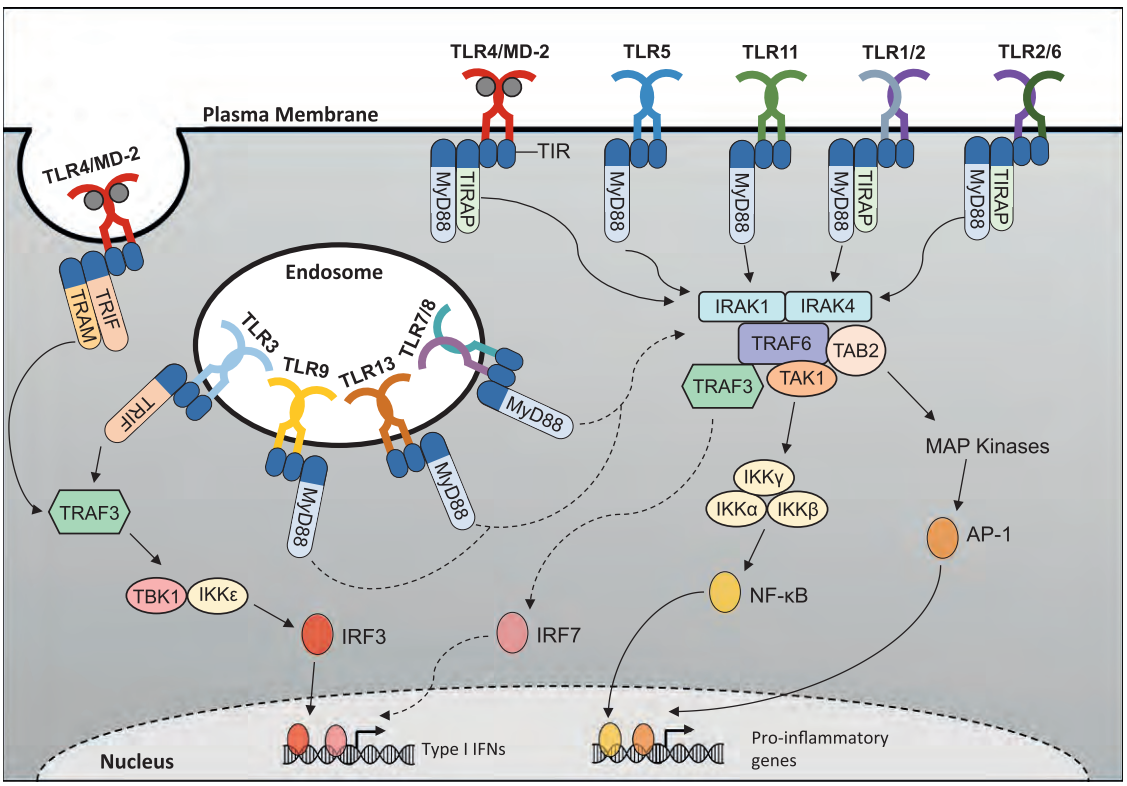

Fig. 2 TLR signaling. Ligand binding to a TLR is proposed to induce conformational changes that initiate intracellular signaling cascades. The specific signaling cascade and robustness of the response is influenced by the ligand, TLR, and adaptor molecules. Signaling can occur on the MyD88-dependent axis or the MyD88-independent/TRIF axis, with characteristic proteins involved in both pathways. The end result of both cascades is translocation of transcription factors into the nucleus, leading to up-regulation of specific genes that will aid in the host response to the initial microbial stimuli.

inflammatory genes. TAK1 activation additionally leads to activation of proteins in the MAPK family such as ERK1/2, JNK, and p38. This in turn activates transcription factors belonging to the AP-1 family or that are involved in regulation of the inflammatory response through stabilization of mRNAs (Kawasaki and Kawai, 2014; Akira et al., 2006; Takeuchi and Akira, 2010).

The second signaling axis is TRIF-dependent. Upon TLR activation, TRIF is recruited and interacts with TRAF3 and TRAF6. TRAF3 activation leads to recruitment of IKK-related kinases TBK1 and IKKi, in addition to the regulatory protein NEMO, resulting in phosphorylation of IRF3 (Kawasaki and Kawai, 2014; Akira et al., 2006; Takeuchi and Akira, 2010). Phosphorylated IRF3 forms a homodimer and translocates from the cytoplasm into the nucleus, where it promotes expression of type I IFN genes. Conversely, TRAF6 activation leads to the recruitment of the RIP-1 kinase, which recruits and actives the TAK1 complex. As with the MyD88-dependent pathway, TAK1 complex activation leads to activation of MAPKs and NF-κB and production of inflammatory cytokines (Kawasaki and Kawai, 2014; Akira et al., 2006; Takeuchi and Akira, 2010).

Surface-Associated TLRs

TLRs can be broadly categorized based on their cellular localization, either on the cell surface or within endosomes. It's important to note that TLRs do not remain static. Surface-expressed TLRs, such as TLR4, have been described to transit to the endosome and in some cases, endosomal TLRs may transit to the cell surface (Beutler, 2008). Cell surface-associated TLRs primarily recognize PAMPs that would be present outside of the host cell as a consequence of extracellular infection, such as microbial membrane components. Most TLRs function as homodimers, although some function as heterodimers, as is the case for TLR2 (Akira et al., 2006; Takeuchi and Akira, 2010). TLR2 recognizes the broadest range of PAMPs due to its heterodimerization with other TLRs, including TLR1, 6, and 10. TLR2 PAMPs include lipoproteins, peptidoglycans, lipoteichoic acid from Gram-positive bacteria, zymoxan, tGPI-mucin, and mannan (Table 1; Kawasaki and Kawai, 2014; Chandler and Ernst, 2017). Specifically, TLR1/2 recognizes triacylated lipoproteins (predominantly from Gram-negative bacteria) and TLR2/6 recognizes diacylated lipoproteins (predominantly from Gram-positive bacteria) (Chandler and Ernst, 2017; Kirschning and Schumann, 2002). While much research has been done to identify ligands sensed by TLR2, the second TLR partner required for such recognition is less well understood and is still an active area of research. TLR5 recognizes flagellum, which are protein-based appendages extruding from microbial cells that aid in movement, and has been specifically been described to respond to flagellum of pathogenic invasive bacteria in the intestinal epithelium (Hayashi et al., 2001; Ramos et al., 2004). TLR10 can sense influenza A virus infection in humans through an unknown mechanism and can pair with TLR3 to recognize specific extracellular ligands during listeria infection (Regan et al., 2013; Lee et al., 2014). In mice, TLR10 exists as a non-functional pseudogene (Roach et al., 2005).

TLR4 is perhaps the most well-studied TLR and primarily recognizes lipopolysaccharide (also known as endotoxin) from Gram-negative bacteria. Crystal structures of TLR4/MD-2 in complex with LPS suggest that up to five LPS acyl chains bind in a hydrophobic pocket in MD-2 with the sixth interacting with a hydrophobic patch on TLR4 itself (Park et al., 2009; Kim et al., 2007). Homodimers of TLR4/MD-2 recognize the lipid A portion of LPS in a structure-dependent manner and efficacy of recognition is variable between human and mouse cell lines (Scott et al., 2017; Hajjar et al., 2012). TLR4 has also been described to recognize mannuronic acid polymers, teichuronic acid, and viral proteins (Table 1; Vaure and Liu, 2014). TLR4 is unique because it can signal from the cell surface (through the MyD88-dependent pathway) and subsequently from endosomes (through the TRIF pathway) (Fig. 2). After ligand recognition and TLR4/MD-2 dimerization, TLR4 is endocytosed in a CD14-dependent manner. This process is termed 'inflammatory endocytosis' and is regulated by unique adaptors, GTPases, and calcium. Recent studies revealed that MD-2 is the key component allowing TLR4 to be selected for endocytosis by CD14 and previous work has shown that CD14 alone, but not TLR4 alone can be endocytosed in the presence of LPS. Endocytosis of TLR4 and subsequent TRIF signaling has also been observed to be differential between unique cell types (Kagan et al., 2008; Tan et al., 2015; Rajaiah et al., 2015).

Endosomal

Endosomal TLRs largely recognize microbial products that result from intracellular infection or that are taken into the cell via endocytosis, including bacterial- and viral-derived nucleic acids, in addition to self-nucleic acids in certain autoimmune disease conditions (Kawai and Akira, 2010; Kawasaki and Kawai, 2014). As obligate intracellular microbes, viruses are almost exclusively sensed by endosomal TLRs, with specific TLRs differentially sensing different viral classes. TLR3 senses double-stranded viral RNA (dsRNA, formed during the replication process of positive-strand RNA viruses), small interfering RNA (siRNA), and self-RNA produced from damaged host cells (Kawai and Akira, 2010; Kawasaki and Kawai, 2014). Crystal structures revealed that the phosphate backbone of the dsRNA, not the nitrogenous bases, are the feature recognized by TLR3 and that recognition involves the ectodomain LRR motif (Liu et al., 2008; Choe et al., 2005). TLR3 also has roles in the pathogenesis of influenza virus and encephalitis caused by West Nile virus and herpes simplex virus (Zhang et al., 2007; Bernard et al., 2012; Takemura et al., 2014). Endosomal TLRs may recognize both viral and bacterial-derived nucleic acid, as is the case for TLR7 and TLR8. Both form homodimers that primarily recognize viral-specific single stranded RNA (ssRNA), but can additionally recognize some bacterial RNA (Kawai and Akira, 2010; Kawasaki and Kawai, 2014). TLR7 is primarily expressed in plasmacytoid dendritic cells (pDCs) where it senses ssRNA, but when expressed in conventional dendritic cells (cDCs) it can recognize RNA from streptococcus B bacteria (Mancuso et al., 2009; Crozat and Beutler, 2004). In humans, TLR8 recognizes viral and bacterial RNA (Guiducci et al., 2013). Structural analysis of TLR8 suggests that it exists as a preformed dimer, which changes conformation upon ligand recognition and binding (Tanji et al., 2013). TLR9 recognizes DNA that is rich in unmethylated CpG-DNA motifs, which could be bacterial or viral in

origin and is notably distinct from 'self' nucleic acid. TLR9 has also been proposed to recognize a byproduct produced during *Plasmodium falciparum* infection called hemozoin (Coban et al., 2010).

Mice possess three TLRs that are not present in humans – TLR11, TLR12, and TLR13. TLR11 recognizes a component of uropathogenic *Escherichia coli* (UPEC; possible flagellin or a proteinaceous component) and profilin-like molecules originating from *Toxoplasma gondii* (Mathur et al., 2012; Yarovinsky et al., 2005). TLR12 is similar to TLR11 and is mostly expressed in myeloid cells where it senses profilin from *T. gondii*. TLR12 may function as a homodimer or a heterodimer with TLR11 (Koblansky et al., 2013; Andrade et al., 2013; Broz and Monack, 2013). TLR13 senses bacterial 23S rRNA and components of the vesicular stomatitis virus that have yet to be determined (Oldenburg et al., 2012; Hidmark et al., 2012; Shi et al., 2011).

Nod-Like Receptors: Cytosolic PRRs

Although TLRs were the first PRRs to be described, our knowledge about additional innate immune receptors has rapidly expanded in the past decade. Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) were first identified in plants where they were described to function in disease resistance against microbial pathogens (leading them to be called *R* genes) (Ogura et al., 2001; Chamaillard et al., 2003). As genome sequencing became more accessible, homologues of plant NLRs were subsequently identified in vertebrates and other organisms, including primitive organisms such as sea urchin (Shaw et al., 2008; Girardin et al., 2003). Today, we understand NLRs to be evolutionarily conserved soluble innate immune receptors that, like TLRs, serve as PRRs. Additionally, TLRs and NLRs can act synergistically or independently to promote stimuli-dependent innate immune responses (Kufer and Sansonetti, 2007; Shaw et al., 2008).

NLRs are multi-domain proteins with three conserved regions: a variable N-terminal effector domain; a central NOD (or NACHT) nucleotide-binding domain; and a C-terminal domain composed of a series of leucine rich repeats (LLRs) (Fig. 3(A)). NLRs are found in the cytosol and are therefore primarily responsible for surveying the intracellular environment (Stam et al., 2016; Yuen et al., 2014; Buckley and Rast, 2015; Wu et al., 2017b; Howe et al., 2016). Microbes capable of evading extracellular immunity and entering the cell across the plasma membrane or through escape from a phagosome should consequentially be detected by the cytosolic NLRs. Upon activation, the variable N-terminal domain interacts with downstream factors to initiate signaling cascades that result in inflammasome assembly, transcriptional activity, regulation of inflammatory responses, and pyroptosis. The distinct biological outcome of NLR activation is dependent on the N-terminal domain. As such, the NLRs are divided into four subfamilies based on their N-terminal domain: NLRP, NLRC, NLRA, and NLRB (Wilmanski et al., 2007). Humans possess 23 NLR family members, while mice have at least 34 *NLR* genes (Kufer and Sansonetti, 2007; Shaw et al., 2008).

NLR Signaling & the Inflammasome

In the absence of PAMP or DAMP signals, NLRs exist in the cytosol as auto-inhibited monomers (Fig. 3(B)). NLRs are capable of recognizing and binding specific PAMP/DAMPs through their C-terminal LRR domain, resulting in a conformational change that exposes the central NOD domain. Once exposed, the NOD domains of similar NLRs oligomerize and initiate the recruitment of accessory signaling proteins capable of propagating a wide range of responses. This most notably includes activation of the inflammasome and the MAPK signaling cascade (Zhong et al., 2013).

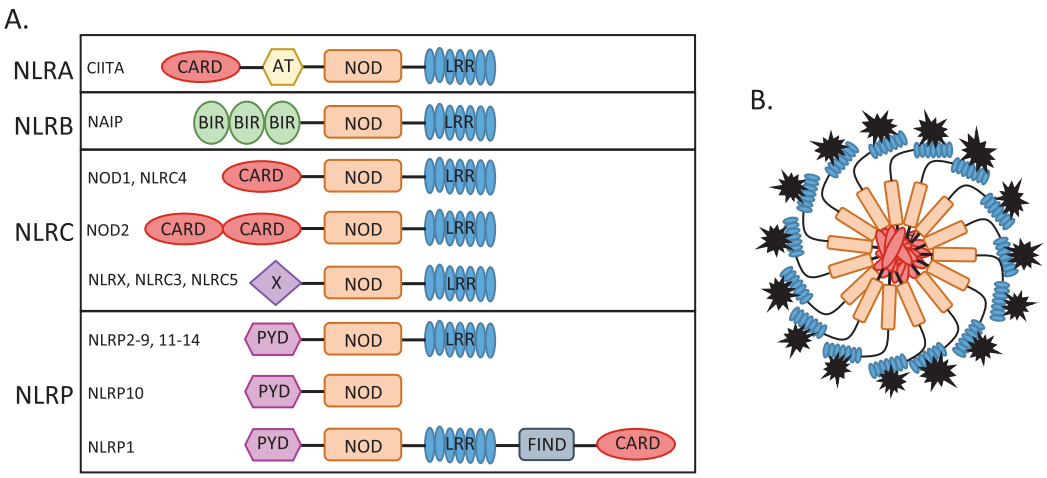

Fig. 3 NLR Structure. (A) NOD-like receptors each contain a NOD domain as well as different combinations of signaling domains, and leucine-rich repeats (LRRs). (B) An inflammasome (NLRC4 pictured) is formed when the LRR of an inflammasome NLR is bound by a microbial ligand (black starburst). Ligand binding causes the conformation of the protein to be altered so that there is a 90-degree angle between the NOD and LRR. This allows for circular oligomerization and brings the CARD domains, on either the NLR itself or inflammasome adaptor proteins, into close proximity. The CARD domains are then capable of activating caspase-1 and so initiation inflammasome-associated inflammation.

The inflammasome, candidly referred to as a 'wheel of death', is defined as a cytosolic oligomer of proteins that initiates the activation of caspase-1, subsequently leading to the release of pro-inflammatory mediators, including IL-1β and IL-18 (Martinon et al., 2002). Upon ligand binding, inflammasome formation commences with the oligomerization of the individual NLRs, which subsequently brings the N-terminal pyrin domains (PYDs) into close proximity with each other. Assembly of PYD recruits the apoptosis-associated speck-like protein (ASC), an adaptor protein that contains both a PYD and caspase-activating and recruitment domain (CARD) (Srinivasula et al., 2002). NLRs that contain a CARD domain, most notably the NLRC family and NLRP1, do not require ASC or other accessory scaffolding proteins (Fig. 3(A)). After oligomerization and ASC recruitment (if required), the CARD portion of pro-caspase-1 associates with the assembled CARD domains in the middle of the inflammasome. Pro-caspase-1 is then cleaved and activated caspase-1 is released from the signaling complex. Active caspase-1 subsequently cleaves zymogens of the pro-inflammatory IL-1 family of cytokines yielding bioactive IL-1β and IL-18, which can then be secreted (Fig. 4). Caspase-1 activation can ultimately lead to a specific form of cell death termed pyroptosis. Although the exact molecular mechanism that mediate caspase-1-dependent pyroptosis are not fully understood, studies have suggested that active caspase-1 monomers assemble in the plasma membrane forming a pore through which intracellular components are released into the environment (Ting et al., 2008). In addition to responding to intracellular pathogens, inflammasomes are necessary for the ordered destruction of tissue during fetal development and homeostatic cell death at environmental interfaces. Currently, the human NLR proteins that have been shown to form inflammasomes are NAIP, NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, and NLRC4 (Pétrilli et al., 2007). Inflammasomes are classified by the type of NLR they are composed of, resulting in three dominant types: NLRP3 inflammasome (also called NALP3), NLRP1 inflammasome (also called NALP1), and the NLRC4 inflammasome (also called IPAF) (Shaw et al., 2008).

NLRP Subfamily

The NLRP subfamily is characterized by the presence of a N-terminal pyrin domain (PYD), in addition to the NOD and LRR domains (Fig. 3(A)). The presence of a PYD means that this subfamily of cytosolic innate receptors can recruit ASC and other inflammasome-activating scaffold proteins, leading to strongly pro-inflammatory pyroptotic cell death. Humans possess fourteen NLRP proteins (Chavarría-Smith and Vance, 2015).

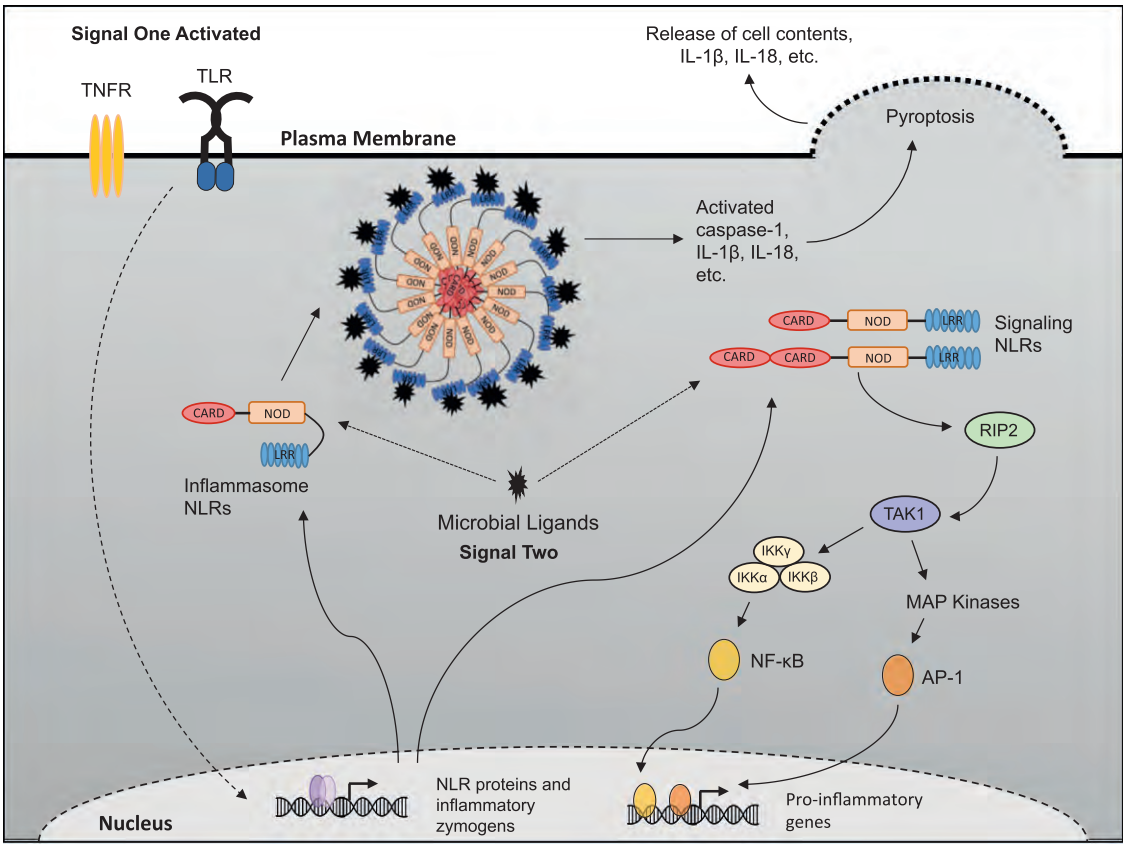

Fig. 4 NLR signaling. Transcription and translation of NLR proteins require activation of a 'signal one', most commonly through an extracellular TLR and/or TNF receptor. Once translated and transported to the cytosol, NLRs are activated by microbial ligands which bind to the LRR. Activated NLRs oligomerize form an inflammasome, initiate pro-inflammatory signaling, or regulate inflammatory pathways.

NLRP1 was the first protein described to lead to inflammasome formation, although at the time its activators were unknown. Subsequent research has shown that activators of NLRP1 include *Bacillus anthracis* lethal toxin, *Toxoplasma gondii*, muramyl dipeptide, and rapid ATP depletion (Ewald et al., 2014; Moayeri et al., 2012). In addition to PYD, NOD, and LRR, NLRP1 also contains a function-to-find domain (FIND) and a CARD domain, which enables it to recruit effector caspases without the requirement of an adaptor protein (Hiller et al., 2003). In mice, three NLRP1 paralogs have been identified: NLRP1a, NLRP1b, and NLRP1c. Interestingly, these proteins do not contain a PYD and high levels of expression have been linked to resistance to bacterial toxins (Sastalla et al., 2013).

NLRP2-9 and 11-14 all contain a PYD, NOD, LRR, and a small unique functional region that distinguishes them from each other (Fig. 3(A)). Although this category of NLRPs has similar functional domain organization, the resulting protein functions vary widely. Of these, NLRP3 is the most widely studied. NRLP3 is a strong negative regulator of pro-inflammatory signaling and is highly expressed in macrophages, forming an ASC-dependent pyroptosis-inducing inflammasome upon activation. Known NRLP3 inflammasome activators are components of cellular damage and dysregulated metabolic processes including extracellular ATP, potassium flux, reactive oxygen species, and crystalline uric acid (Stehlik and Reed, 2004). Traditional bacterial ligands have not been identified, although metabolic byproducts from bacterial growth or cell wall lysis are capable of activating the NLRP3 inflammasome (Martinon et al., 2004). The NLRP6 inflammasome, while less studied than NLRP3, is also activated by host and bacterial metabolites, but has been shown to be expressed at high levels in more specialized tissues. This includes the gut epithelia where it is thought to contribute to homeostatic cell death and tissue regeneration (Elinav et al., 2011; Anand et al., 2012; Chen et al., 2011).

NLRP2 is a regulator of NF-κB signaling and its functional expression is necessary for fertility, healthy maternal/fetal relationships, and embryo development (Fontalba et al., 2007). NLRP4 is also a negative regulator of immune signaling, thought to be involved in healthy fetal development and has been shown to directly interact with several infection-induced signaling molecules including TBK1 (involved in interferon production) and beclin1 (involved in autophagy) (Charoenthongtrakul et al., 2012; Jounai et al., 2011; Tian et al., 2009). Similarly, NLRP11 is found to regulate innate immune signaling by directly targeting the pro-inflammatory mediators TRAF6 (involved in TLR signaling) and MAVS (involved in interferon production) (Wu et al., 2017a; Qin et al., 2017). NLRP8, NLRP9, NLRP13, and NLRP14 have not been as widely investigated, but studies have shown that they have increased expression in reproductive tissue of mammals (Monk et al., 2017).

NLRP7 and NLRP12 form inflammasomes that are found to be activated in very specific instances of pathogenesis, as well as during fetal development as a means of tissue regulation (Murdoch et al., 2006). *Staphylococcus aureus*, *Listeria monocytogenes*, and bacterial acylated lipoproteins have been reported to activate the NLRP7 inflammasome (Radian et al., 2013). Aberrant signaling of NLRP7 has been associated with high incidences of inflammatory bowel disease, ulcerative colitis, chronic obstructive pulmonary disease (COPD), and recurrent pregnancy loss (Huang et al., 2013; Onoufriadis et al., 2018). NLRP12 is found to form inflammasomes when exposed to bacterial components from many species including *Yersinia pestis*, *Micobacterium tuberculosis*, and *Klebseilla pneumoniae* (Vladimer et al., 2012; Allen et al., 2013).

Additionally, it has been associated with murine neutrophil recruitment and maybe involved in regulation of the gut microbiota through the promotion of beneficial commensals (Hornick et al., 2018; Allen et al., 2012). Although specific mechanisms have not been elucidated, NLRP12 is classically described as a negative regulator of inflammatory signaling in immune cells (Arthur et al., 2010).

NLRP10 is unique because it is the only member of the NLRP subfamily that does not contain a LRR domain, yet still retains the characteristic PYD and NOD domains. NLRP10 is highly expressed in the epidermis of both mouse and human skin and contributes to the regulation of immune activation in the presence of commensals (Lachner et al., 2017). Furthermore, it is described as a negative regulator of ASC-dependent inflammasome signaling by inhibiting maturation of caspase-1 and IL-1β. Although the exact mechanism of inhibition has not been elucidated, it is thought that NLRP10 may interact with the PYD domain on the inflammasome scaffolding protein ASC. A role for the induction of adaptive immunity through dendritic cell migration and involvement in the NOD-1 signaling cascade have also been proposed for this unique NLRP protein (Eisenbarth et al., 2012; Lautz et al., 2012).

NLRC Subfamily

The NLRC subfamily of NLRs is characterized by the presence of an N-terminal CARD domain followed by the classic NOD and LRR domains (Fig. 3(A)). NOD1 and NOD2 are included in this category and are classic cytosolic PRRs responsible for responding to bacterial peptidoglycan fragments, DAMPs, and disturbances in homeostatic cellular processes, including actin cytoskeleton and endoplasmic reticulum formation (Keestra-Gounder and Tsolis, 2017; Byndloss et al., 2016). NOD1 is activated by the Gram-negative bacterial product γ-D-glutamyl-*meso*diaminopimelic acid (iE-DAP), while NOD2 is activated by muramyl dipeptide (MDP) present in both Gram-positive and Gram-negative bacteria. Additionally, viral particles have been described to activate both NOD1 and NOD2. NOD1 has an N-terminal CARD domain, followed by a NOD and LRR, whereas NOD2 has two N-terminal CARD domains. Despite the presence of CARD domains, activated NOD1 and NOD2 do not result in the formation of an inflammasome. Instead, they self-oligomerize after ligand stimulation and recruit an adaptor protein that activates TAK1. As in the TLR-signaling pathway, TAK1 subsequently activates NF-κB and MAPK signaling cascades, resulting in transcription factor translocation into the nucleus and up-regulation of pro-inflammatory mediators (Fig. 4; Kawai and Akira, 2009). Although these NLRC proteins do not directly result in pyroptotic cell death, they have both been found to contribute to activating autophagic processes through the pro-inflammatory signals they initiate (Kaparakis-Liaskos, 2015). As with all pro-inflammatory processes, unregulated NOD signaling

can lead to the formation of chronic inflammation syndromes. Strong evidence is emerging that genetic differences in the NOD2 protein are linked with the development of Crohn's disease (Caruso et al., 2014). Similar to NOD1, the NLRC4 inflammasome contains a CARD domain though its activation is ASC-independent. NLRC4 signaling is activated by bacterial flagellin and components of the type-3-secretion systems and results in the recruitment of caspase-1, which initiates inflammation and pyroptosis as previously described.

NLRC3, NLRX1, and NLRC5 all have unusual N-terminal signaling domains and therefore very different functions than the aforementioned NLRC family members. NLRC3 contains a CARD domain that does not activate caspase-1 and functions to negatively regulate inflammatory signaling pathways. This includes pathways induced by STING (a sensor of DNA) and NF-κB-activated signaling pathways (Allen, 2014; Zhang et al., 2014). NLRXI contains an N-terminal mitochondrial localization signal and similarity negatively regulates the host immune response to viral infection by promoting autophagy. Once activated, it is capable of directly binding the IKK complex to inhibit NFκB signaling or can interfere with the cytosolic Rig-I receptor, which dampens interferon responses to viral infection. NLRC5 contains a death domain at the N-terminus and has been shown to regulate components for the development of adaptive immunity, specifically MHC-I driven immunity, while dampening pro-inflammatory signaling (Benko et al., 2010). This NLR is found to be a strong trans-activator of the MHC-I locus, resulting in the recruitment of transcription factors to the MHC-I promoter region and allowing for increased expression of not only MHC-I, but also the proteins required to facilitate antigen presentation in the context of MHC-I (Kobayashi and van den Elsen, 2012; Neerincx et al., 2013; Meissner et al., 2012).

NLRA and NLRB

The NRLA subfamily is characterized by an N-terminal acidic transactivation domain (Fig. 3(A)) and contains only one known member: Class II major histocompatibility transactivator (CIITA). CIITA works as a trans-activator of MHC class II immunity by interacting with three main transcription factors (RFX5, NF-Y, and CREB) to promote the transcription of MHC II and related genes (Zika et al., 2003; Devaiah and Singer, 2013). CIITA tightly regulates MHC production through its combined acyltransferase and kinase activity. Mutations in CIITA have been linked with aberrant MHC expression-associated autoimmunity.

The NLRB subfamily includes the neuronal apoptosis inhibitory proteins (NAPIs), which possess LRR and NOD domains followed by three N-terminal baculoviral inhibitory repeat-like domains (BIR) (Fig. 3(A)). NAIPs have been shown to directly interact with bacterial PAMPs. In humans, there is a single expressed NAIP that is capable of detecting both bacterial type-3 secretion system and flagellum, whereas mice have seven paralogs of the human receptor that share similar ligands. Mouse NAIP1 and NAIP2 recognize components of the bacterial type-3 secretion system, and mouse NAIP5 and NAIP6 recognize flagellum. Subsequent to encountering PAMPs, these complexes interact with and activate the NLRP4 inflammasome, leading to caspase-1 activation and pyroptotic cell death (Reyes Ruiz et al., 2017; Zhao and Shao, 2015; Chavarría-Smith and Vance, 2015).

Perspectives

Due to their role in innate immunity, TLRs and NLRs are critical components for the early defense against pathogens and subsequent induction of adaptive immunity. As such, regulation of their expression and signaling is complex. Functional abnormalities of TLRs, NLRs, or their associated co-receptor and/or signaling molecules have been implicated in diseases ranging from autoimmunity to cancer and are worthy of a separate article. With improvements in characterization of PRR ligands and signaling mechanisms, our understanding of host defenses and disease has also developed. However, the minimal structural requirements leading to PRR activation and signaling still remains an active and needed area of research. In addition to initiating early responses to infection, signaling cascades initiated by these receptors induce key factors that link the innate and adaptive immune systems. Specifically, critical studies on receptor-ligand interactions and structure-activity relationships will help advance our understanding of microbial recognition and could pave the way for therapeutic development.

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Peptidoglycan (Murein)[☆]

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Glossary

Cell envelope Is the set of layers that delimit and wrap a bacterial cell, normally composed by the cytoplasmic membrane and cell wall (sacculus) plus an outer membrane in Gram-negative bacteria.
Muropeptide Each of the monomeric subunits of peptidoglycan.
Peptidoglycan Is a polymer consisting of sugars and amino acids. Murein (from the latin *murus*, meaning wall) is the particular form found in the bacterial cell walls; however, the generic term is most often used. Some Archaea present structurally similar components named pseudomurein.
Periplasm or periplasmic space Is the compartment between the cytoplasmic membrane and the outer membrane in Gram-negative bacteria.
Sacculus Also known as the cell wall, is the rigid layer of the bacterial cell envelope made of peptidoglycan and juxtaposed to the external side of the cytoplasmic membrane.
Turgor Is the osmotic pressure exerted by the cell contents against the cytoplasmic membrane as a consequence of the high intracellular concentration of metabolites and macromolecules.

Definition Statement

The peptidoglycan sacculus is a key structural component of the bacterial cell. Peptidoglycan metabolism is a complex process taking place in different cellular compartments. Present-day knowledge on the characteristics and central metabolic pathways of peptidoglycan metabolism is presented. The dynamic character of cell wall biology is given particular consideration.

Peptidoglycan is a fundamental component of the cell envelope of nearly all bacteria. It is a polymeric macromolecule made up of linear glycan chains cross-linked to each other by short peptide bridges. Peptidoglycan (or murein) is laid down on the outside of the cytoplasmic membrane as a covalently closed, netlike, macromolecular structure known as the cell wall or sacculus (small bag) (Figure 1). Preservation of cell integrity and determination of bacterial shape are the primary functions of the peptidoglycan. The netlike, covalently closed structure of the sacculus is particularly appropriate to maintain cell shape while standing the high (2 to more than 15 bar) turgor pressure exerted by the cytosol against the cytoplasmic membrane of the cell. Because the sacculus completely wraps the cell body, the cytoplasmic material has to adopt the shape of the relatively rigid sacculus (Figure 2). Therefore, variations in cell shape and size are constrained by a concurrent change of the sacculus itself. These characteristics together with the ability of isolated sacculi to accurately keep the shape of the original cell make the sacculus the bacterial equivalent to an exoskeleton (Figure 2). While bulk incorporation of new material is clearly necessary to enlarge the sacculus, the spatial distribution and temporal regulation of insertion events define the final shape of the bacterium.

The sacculus serves further important roles as a scaffold to support the external layers of the cell envelope (S-layers and the outer membrane (OM) of Gram-negative bacteria), in the biogenesis of supramolecular structures (export complexes and flagella), and in protein export in Gram-positive bacteria. Moreover, peptidoglycan is critically involved in developmental processes as the generation of resistance forms (spores) and appendages of different functions (prostheca).

Because peptidoglycan is exclusively found in bacteria and its biosynthetic pathway is both essential and highly conserved, it makes the ideal target for antibacterial drugs. Most of the commonly used antibiotics (β-lactams, glycopeptides, and fosfomycin) target enzymes involved in peptidoglycan biosynthesis.

A murein sacculus is present in all known bacteria with the remarkable exceptions of Mollicutes and the scrub typhus causative agent *Orientia tsutsugamushi*. Planctomycetes and Chlamydiae were also considered to lack a peptidoglycan sacculus until 2015 and 2013, respectively, when the application of new high-sensitivity methods demonstrated the presence of peptidoglycan in these organisms. The presence of some form of peptidoglycan in Chlamydiae was suspected because of the presence of most of the biosynthetic genes and because of their sensitivity to β-lactam antibiotics (the chlamydial anomaly). However, the biosynthetic route shows remarkable differences with other bacteria, in particular in the pathways for the synthesis of D-amino acids. The case of *O. tsutsugamushi* is also remarkable. This organism, formerly known as *Rickettsia tsutsugamushi*, has been separated from the genus *Rickettsia* mostly for the absence of a peptidoglycan sacculus, present in all other *Rickettsia*. Conversely, peptidoglycan layers entirely similar to bacterial sacculi are an essential structure in the photosynthetic organelles (cyanelles) of glaucophyte algae as *Cyanophora paradoxa*. This as well

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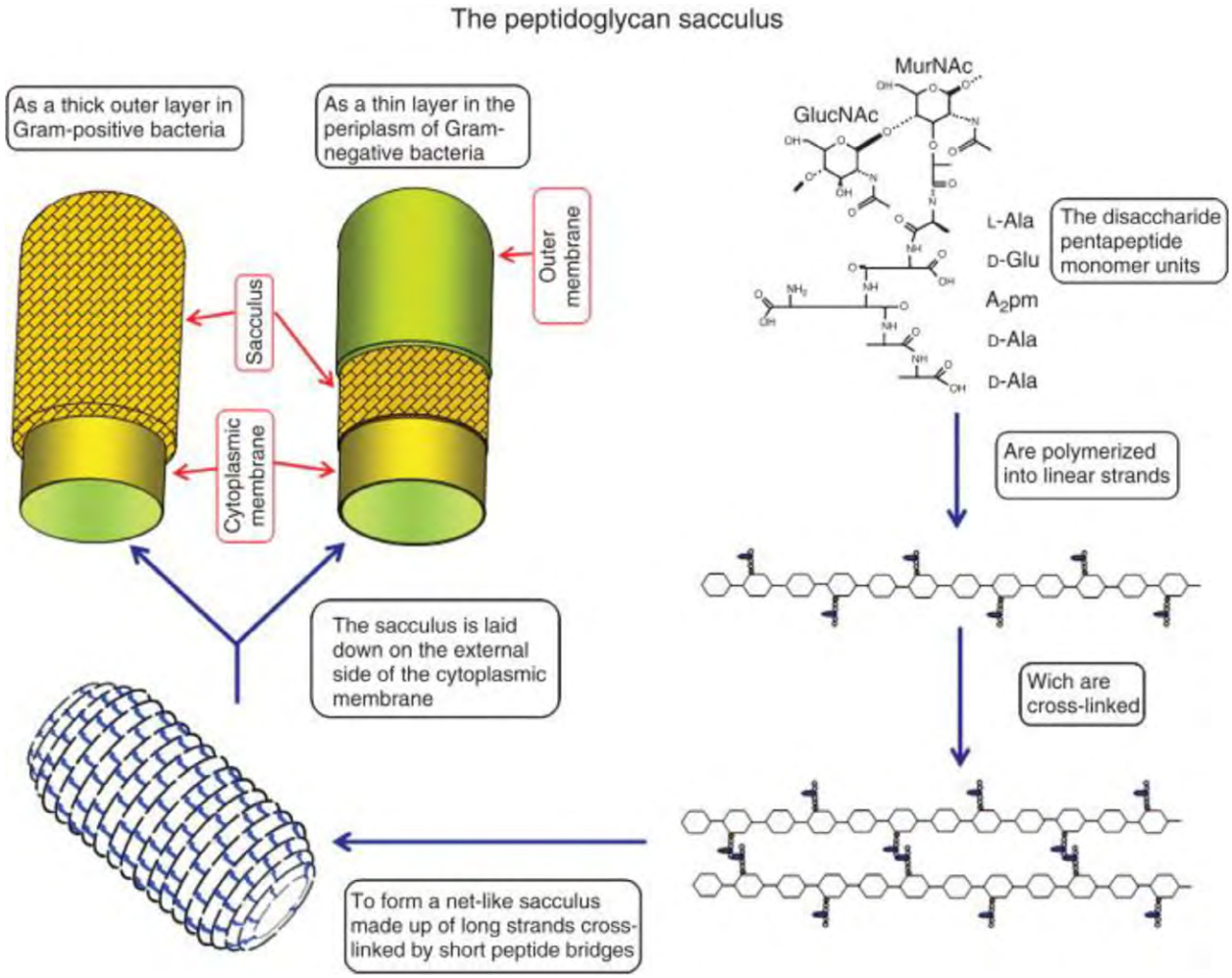

Figure 1 Basic structure and organization of the bacterial peptidoglycan sacculus.

as the recent identification of peptidoglycan biosynthesis genes in green plants (five genes in *Arabidopsis thaliana*) and mosses (nine genes in *Physcomitrella patens*) constitutes strong evidence supporting the endosymbiotic origin of eukaryotic organelles.

The exclusivity of peptidoglycan as a bacterial cell surface component has been successfully exploited by higher eukaryotes (plants and animals) as a means to recognize, and fight, bacterial infections. Indeed, peptidoglycan fragments (muropeptides) are recognized by specific 'peptidoglycan recognition proteins' (PGRPs), which are essential components of the innate immunity system in higher eukaryotes (with the exceptions of nematodes and plants) and play critical roles in antibacterial defense.

Because of peptidoglycan extracellular location and covalently closed nature, its biosynthesis represents a specially challenging problem to the bacterial cell. Precursors are synthesized in the cytoplasm but polymerization and attachment to the preexisting sacculus take place in the external environment, where there is no readily available source of metabolic energy. Besides, insertion of new precursors is not enough by itself to promote enlargement of the preexisting structure. Concomitant cleavage of chemical bonds in the sacculus is a strict requirement for growth. Therefore, both biosynthetic and degrading enzymes must work in concert for the sacculus to grow harmonically. In addition to the main biosynthetic pathway, rather complex by itself, peptidoglycan metabolism is further complicated by the nested intervention of pathways responsible for maturation, adaptive modifications, and recycling, not to mention species-specific pathways for the generation of resistance forms and/or specific appendages.

I will focus here on the more universal aspects of peptidoglycan composition, biosynthesis, and physical properties. Metabolism of the sacculus is intrinsically coupled with virtually all aspects of bacterial cell growth, division, development, and morphogenesis. However, treatment of such aspects in any detail would require a much more extensive and intensive treatment, out of the scope of this presentation.

Peptidoglycan Chemical Composition

Peptidoglycan composition and biosynthesis are fairly well established for a few model systems, but the base of knowledge is still relatively small. Not more than about 150 species have been studied to any detail and unexpected findings may indeed happen.

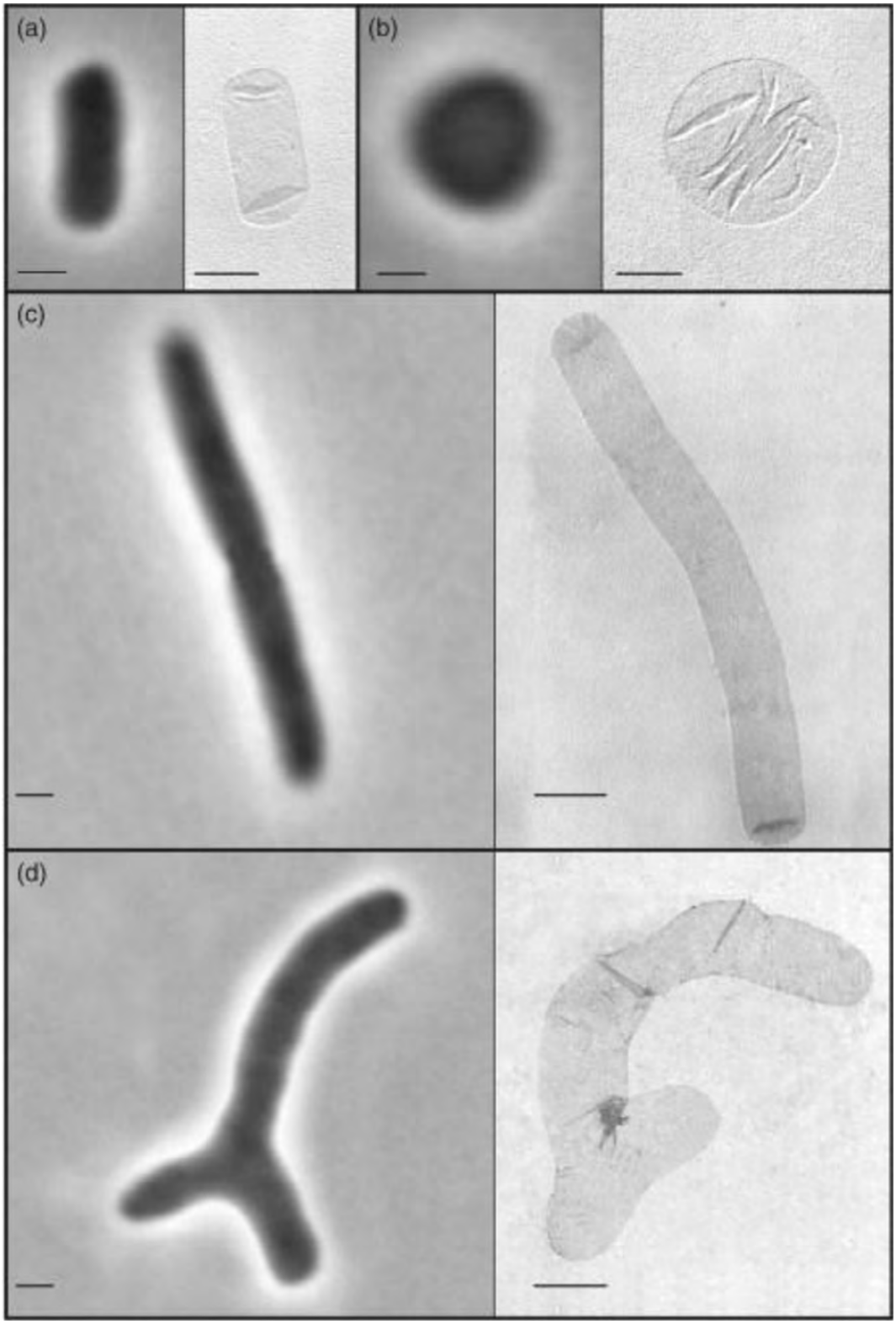

Figure 2 Peptidoglycan sacculi purified from shape mutants of *Escherichia coli*. Sacculi and whole cells of wild-type and mutant strains affected in cell morphology are compared to show how purified sacculi retain shape of the original cell. (a) Wild-type *E. coli* with normal rodlike shape, (b) spherical shape mutant affected in the *rodA* gene, (c) filament-shaped cells of a division thermosensitive *ftsI* mutant, (d) branched shaped cells from a *dacA* mutant grown in the presence of the cell division inhibitor aztreonam. Left panels are phase-contrast images of intact cells; right hand panels are TEM images of sacculi isolated from the corresponding strain. Images have been manipulated to approximate magnification of the phase-contrast and TEM images. The bars represent 1 μm. Notice the thinness of sacculi.

Traditionally, bacteria are divided into two main groups according to their response to the Gram stain: Gram-positive and Gram-negative. The distinction was established well before the discovery of peptidoglycan but reflects the basic dichotomy in the organization of the bacterial cell envelope. Gram-negative bacteria are characterized in structural terms by the presence of a thin peptidoglycan layer, surrounded by an asymmetrical OM made of an outer lipopolysaccharide leaflet and an inner phospholipid leaflet. The OM constitutes a permeability barrier to molecules of more than a few hundred dalton (a typical cutoff value is around 600 Da). As a result, the peptidoglycan layer is confined in a particular compartment defined by the OM and the cytoplasmic membrane, the periplasmic space (Figure 1). Conversely, Gram-positive bacteria display thick peptidoglycan layers, are devoid of OM, and have accessory polymers linked to the cell wall peptidoglycan (Figure 1). Gram-positive bacteria show a remarkable degree of chemical and structural variability in their cell walls, in contrast with the relatively homogenous nature of Gram-negative bacteria on these respects.

Peptidoglycan Basic Structure

The basic structural features of peptidoglycan are polymeric glycan strands cross-linked by short peptides. The canonical monomeric unit consists of the disaccharide N-acetylglucosamine (GlcNAc) (β-1 → 4) and N-acetylmuramic acid (MurNAc) substituted at the D-lactoyl group of MurNAc by a peptide stem with the sequence L-Ala-(γ)-D-Glu-*meso*A_2pm-D-Ala-D-Ala, known in short as the disaccharide pentapeptide. Successive monomers are linked to each other by (β-1 → 4) glycosidic bonds to form the glycan strands. Glycan strands become in turn cross-linked to each other through the stem peptides, normally between the amino acid at position four (invariably a D-Ala) in one strand and the dibasic amino acid at position three of the neighboring strand (Figure 1). Cross-linking can connect both amino acids either directly or through an intermediate short peptide (interpeptide bridge) that is added to the dibasic amino acid in the course of monomer unit biosynthesis. The monomeric subunits of peptidoglycan are commonly known as muropeptides.

The monomer is an unusual biomolecule characterized by the following features: (i) the presence of the rare MurNAc molecule, exclusively found in the bacterial sacculus and, oddly enough, in tissues of some gastropods; (ii) the presence of a (γ)-bonded D-Glu residue; (iii) the occurrence of L-D and D-D peptide bonds, never found in proteins; (iv) the existence of nonprotein amino acids (*meso*A_2pm, L-ornithine, and L-lanthionine); and (v) the presence of a C-terminal D-Ala-D-Ala dipeptide. These basic features are virtually universal. However, peptidoglycan from individual species normally presents modifications on this common theme, in both the glycan and the peptide moieties.

Structural Parameters of Peptidoglycan

The parameters more often used to describe the structural properties of peptidoglycan sacculi are cross-linkage and average glycan strand (chain) length.

Cross-linkage reflects the proportion of muropeptides that are covalently linked through peptide bonds bridging the respective stem peptides. Cross-linkage is normally expressed as the molar fraction of cross-linked with respect to total muropeptides in percentage. The higher the cross-linkage, the stronger, and stiffer, the peptidoglycan. Reported values show a large variability from species to species and also in response to environmental conditions (see section 'Postinsertional Metabolism of Peptidoglycan' in the succeeding text). Among Gram-positive bacteria, cross-linkage is habitually high, and values close to the theoretical 100% maximum have been reported (93% in *Staphylococcus aureus*). However, values for Gram-negative bacteria are much lower, ranging from 20% to 40% in most cases. This large difference is a consequence of the mostly monolayered arrangement of sacculi from Gram-negative bacteria. Geometric constraints limit the maximum theoretical cross-linkage to about 50% for a monolayer.

The length of the glycan strands is also a key structural parameter to understand the properties of the sacculus. Experimental determination of this parameter has been possible only in a very small number of cases. A major limitation comes from the fact that the length of glycan strands is not uniform but rather follows a very wide distribution. Precise data on the distribution of glycan strand lengths have only been obtained for *Escherichia coli*. In this organism, the average length is about 30 monomers, with a modal value of only 10 monomers. The distribution is very wide and strongly skewed towards the shorter length classes; nevertheless, about 30% of total muropeptides are in relatively long strands of more than 30 monomer units. In other bacteria, only an average value could be determined at best. The few data available indicate that peptidoglycan is predominantly made up of short glycan strands, with average lengths from 20 to 50 monomer units (*Helicobacter pylori*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Streptococcus faecium*). However, as found for *E. coli*, glycan strands considerably longer than average might contribute substantially to the sacculus structure. Anyhow, it is important to realize that because a monomer unit is about 1 nm in length, and most bacteria are about or over 1 μm in circumference, single glycan chains are by far too short to loop around the cell; therefore, a number of glycan strands have to be arranged 'head to tail' to encircle the cell.

Variations in the Glycan Moiety

The glycan backbone, which should consist exclusively of poly(GlcNAc–MurNAc), becomes invariably modified. Modification of the glycan moiety occurs in general at the late stages of synthesis, either associated to polymerization of the glycan strands or after insertion of new strands into the sacculus. The glycan strands of Gram-negative species have a residue of (1 → 6)-anhydro MurNAc as the 1-terminal saccharide, eliminating the reducing character that peptidoglycan should otherwise have. In Gram-positive

bacteria, covalent attachment of additional cell wall polymers, as teichoic acids, through phosphodiester bonds to GlcNAc or MurNAc is virtually universal.

Many species present secondary modifications in the glycan strands. O-acetylation, N-deacetylation, and N-glycolylation are the more common. These modifications often confer resistance against peptidoglycan-degrading enzymes, like the widespread muramidases (lysozyme), and modulate recognition of released muropeptides by the PGRPs of the host in pathogenic bacteria.

The most frequent modification of glycan chains is O-acetylation. This reaction, first detected in *Micrococcus luteus*, consists in the addition of an acetyl group to the C_6-OH of MurNAc residues, to form 2,6-*N*,*O*-diacetyl-MurNAc. O-acetylation occurs in both Gram-positive and Gram-negative species, including many important pathogens (*S. aureus*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *H. pylori*, etc.). O-acetylation occurs at the level of polymerized peptidoglycan by means of O-acetyltransferases. Two types of O-acetyltransferases have been described: OatA-type O-acetyltransferases are single integral membrane proteins that simultaneously perform transport of acetate through the cytoplasmic membrane and transfer onto the MurNAc residues; Pat-type O-acetyltransferases are composed of two proteins, one for acetate transport and one for transfer to MurNAc. O-acetylation renders peptidoglycan resistant to most known muramidases and contributes to virulence of pathogens as staphylococci. Besides, O-acetylation prevents lysozyme clearing of cell wall fragments from blood serum after bacterial infection and contributes to induction of rheumatoid arthritis in humans.

Elimination of the N-acetyl groups at position 2 of either amino sugar increased peptidoglycan resistance to lysozyme in several important pathogens as *Bacillus anthracis*, *Listeria monocytogenes*, and *S. pneumoniae*. In all cases, known N-deacetylation takes place on polymerized peptidoglycan and known deacetylases are, or are predicted to be, extracytoplasmic enzymes. Interestingly, some of the redundant N-deacetylases present in *B. cereus* and *Bacillus anthracis* influence cell shape and cell separation, but have no effect on lysozyme resistance.

The presence of glycolyl residues instead of acetate at the 2 amino group of MurNAc was first found in *Mycobacterium smegmatis*. Later, it was clearly stated that N-glycolylation was a hallmark of the Actinomycetales. Contrary to the modifications discussed earlier, N-glycolylation occurs at the stage of UDP-linked precursors in the biosynthesis of the monomeric subunit by the action of a monooxygenase in the presence of molecular oxygen and NADPH. As in the previous cases, impairment of the glycolylating enzyme results in hypersensitivity against lysozyme and β-lactam antibiotics.

Variations in the Sequence of the Stem Peptide

The stem peptide sequence shows a considerable degree of variability, but, in most cases, alterations can be viewed as conservative, in as much as the basic features are concerned. Variations in the stem peptide sequence are dictated either by the specificity of the biosynthetic enzymes or by postsynthetic modification of the otherwise standard sequence.

Replacement of the canonical amino acid L-Ala at position one is rare and only a few cases are known where Gly (*Mycobacterium leprae* and *Brevibacterium imperiale*) or L-Ser (*Butyribacterium rettgeri*) substitutes for the regular L-Ala.

In all species studied so far, the amino acid at position two is invariably D-Glu.

The third amino acid in the stem, usually a dibasic amino acid, shows the higher variability. Most often, A_2pm (most Gram-negative bacteria but also bacilli and mycobacteria) or L-Lys (most Gram-positive species) occupies the third position. However, in a number of species, this position is occupied by unusual dibasic amino acids as L,L-diaminopimelic acid (*Streptomyces albus*), L-ornithine (Spirochetes, *Thermus thermophilus*, and *Deinococcus radiodurans*), *meso*-lanthionine (*Fusobacterium*), or L-2,4-diaminobutyrate (*Corynebacterium aquaticum*). In a few instances, monoamino acids also take the third position as in *Corynebacterium poinsettia* (L-homoserine) and *Erysipelothrix rhusiopathiae* (L-Ala). Another interesting exception is the case of a few species where the third position is occupied by two amino acids as in *Bifidobacterium globosum* in which either L-Lys or D-lys can be at position three because of poor substrate specificity of the adding enzyme.

Amino acids at positions 4 and 5 consist almost invariably of D-Ala. The D-Ala-D-Ala terminal dipeptide is perhaps the most widely known peptidoglycan hallmark. However, a certain proportion of Gly is often found at these positions because of the lack of selectivity of D-Ala-D-Ala ligase, the enzyme making the dipeptide. The proportion of Gly is normally quite low ($\leq$1%) but in some species as *Caulobacter crescentus* can be as high as 19%. Undoubtedly, the most relevant alteration known has been recently identified in some strains of enterococci. In such strains, D-lactate or D-Ser substitutes for the D-Ala at position five. The modification is associated with development of high-level resistance against the antibiotic vancomycin. Vancomycin binds D-Ala-D-Ala with high affinity and blocks the late steps of peptidoglycan biosynthesis. However, the depsipeptide D-Ala-D-Lac is a very poor substrate for vancomycin, and therefore, substitution of D-Lac for D-Ala prevents antibiotic action. Actually, this mechanism of resistance is a serious clinical problem.

Secondary Modifications of the Stem Peptide

Other variations in the chemical composition of monomer peptide stem (amidation, acetylation, attachment of additional amino acids, etc.) occur at later stages by the modification of the otherwise regular monomeric subunit. These modifications more often affect amino acids at positions 2 and 3. Amidation of D-Glu and *meso*-A_2pm at the α and ε carboxyl groups, respectively, is quite common in Gram-positive bacteria. The membrane-bound form of the complete monomer is the substrate for amidation. Hydroxylation of D-Glu, *meso*-A_2pm, and L-Lys has been found in some species in direct relation with oxygenation levels. Addition of aminated compounds to the α-carboxyl group of D-Glu at position 2 has been documented in some organisms, as *M. luteus* (Gly)

and *Arthrobacter* (glycine amide) and also in peptidoglycan from *C. paradoxa* cyanelles (N-acetylputrescine). In many Gram-positive species, the third amino acid is often modified at the level of lipid I by addition of a short (1–7 amino acids), highly variable, peptide to the free amino group, which acts as an intermediate bridge in cross-linking reactions between peptide stems.

The peptide stem constitutes in addition the anchoring point for specific cell envelope proteins. Gram-negative enterobacteria often present a small-size lipoprotein (the 58-amino acid Braun's lipoprotein in *E. coli*) as the only known molecule covalently bound to murein. Murein-bound lipoprotein anchors the OM to the sacculus. The acylated N-terminal domain of the lipoprotein is buried into the phospholipid inner leaflet of the OM, while the ε-amino group of the C-terminal L-Lys becomes attached to the α-carboxyl group of *meso*-A_2pm, by means of a transpeptidation reaction, effectively bridging both cell envelope layers.

Gram-positive bacteria in general present a larger number of peptidoglycan-bound proteins, often involved in pathogenic processes. Binding is mediated by cytoplasmic membrane proteins denominated sortases. Sortases, exemplified by sortase A from *S. aureus*, recognize specific C-terminal sorting sequences in the exported proteins. The recognition event triggers a transpeptidation reaction between a peptide bond in the sorting sequence and the free amino group of a translocated molecule of lipid II (see section 'Synthesis of Lipid Intermediates'). Then, the protein-bound lipid II molecules enter the peptidoglycan polymerization pathway, pulling the proteins outward as the cell wall grows.

Biosynthesis of Peptidoglycan Monomer Units

Biosynthesis of peptidoglycan has been the matter of active research for more than 60 years. From these efforts, an overall view of the process thought to be valid for all bacteria has emerged.

Peptidoglycan biosynthesis is traditionally divided in two stages on the basis of the cell compartments where it takes place. The first stage concerns assembly of the monomer unit in the intracellular space by enzymes sitting in the cytoplasm or in the cytoplasmic membrane inner leaflet. The end product of the first stage is a membrane-anchored, lipid intermediate: disaccharide-pentapeptide pyrophosphate undecaprenol (lipid II) (Figure 3). The second stage addresses the extracellular polymerization steps that take place at the outer side of the cytoplasmic membrane and use the lipid intermediate as the initial substrate (Figure 4). Concomitantly with polymerization, nascent peptidoglycan is inserted into the macromolecular structure of the sacculus, in a way promoting further physical expansion of the preexisting structure and ensuring preservation of proper shape.

Assembly of the monomer can be subdivided into four stages: synthesis of UDP-GlcNAc, synthesis of UDP-MurNAc, sequential formation of UDP-MurNAc-peptides, and synthesis of the lipid-linked intermediates (Figure 3).

Synthesis of UDP-GlcNAc

Synthesis of UDP-GlcNAc occurs in four reactions. The first reaction is the conversion by the GlmS protein (glucosamine-6-phosphate synthase) of fructose 6-phosphate and glutamine into glucosamine-6-phosphate, which is isomerized to glucosamine-1-phosphate by GlmM protein (phosphoglucosamine mutase). Activity of GlmM is regulated by phosphorylation of a serine at the active site and seems to be the checkpoint in the regulation of UDP-GlcNAc synthesis. The final two steps are catalyzed sequentially by the bifunctional enzyme GlmU. The C-terminal domain of GlmU has glucosamine-1-phosphate acetyltransferase activity and catalyzes transfer of an acetyl group from acetyl CoA to the amino group of glucosamine-1-phosphate, while the N-terminal domain, endowed with N-acetylglucosamine-1-phosphate uridyltransferase activity, uridylates the product of the previous reaction in the presence of UTP, producing UDP-GlcNAc. The intracellular concentration of UDP-GlcNAc is the limiting factor for the subsequent steps in peptidoglycan biosynthesis.

Synthesis of UDP-MurNAc

UDP-MurNAc is synthesized from UDP-GlcNAc in a two-step process involving, first, transfer of enolpyruvate from phosphoenolpyruvate to the C_3-OH of a UDP-GlcNAc molecule by the MurA transferase (UDP-N-acetylglucosamine enolpyruvyl transferase) to yield UDP-GlcNAc-enolpyruvate and, second, reduction of enolpyruvate to D-lactate by the MurB reductase (UDP-N-acetylenolpyruvylglucosamine reductase) in the presence of NADPH to make UDP-MurNAc.

Addition of the Stem Peptide

Addition of the stem peptide is a sequential process in which L-Ala; D-Glu; a dibasic amino acid, most often *meso* A_2pm or L-Lys; and the dipeptide D-Ala-D-Ala are incorporated into UDP-MurNAc to produce UDP-MurNAc-pentapeptide. Amino acids are added stepwise by the highly specific synthases MurC (UDP-N-acetylmuramic acid:L-alanine ligase), MurD (UDP-N—Acetylmuramyl-L-alanine:D-glutamate ligase), MurE (UDP-N—Acetylmuramyl-L-alanyl-D-glutamate:*meso*-diaminopimelate ligase), MurF (UDP-N-acetylmuramy-L-alanyl-D-glutamyl-*meso*-diaminopimelate:D-alanyl-D-alanine ligase), and D-alanine:D-alanine ligase, which synthesizes the dipeptide D-Ala-D-Ala, the substrate for MurF. All these ligases are ATP-dependent and have a similar catalytic mechanism: activation of the C-terminal amino acid of the growing nucleotide precursor to an acyl phosphate intermediate at the expense of ATP, followed by a nucleophilic attack by the NH_2 group of the added amino acid leading to release of phosphate and formation

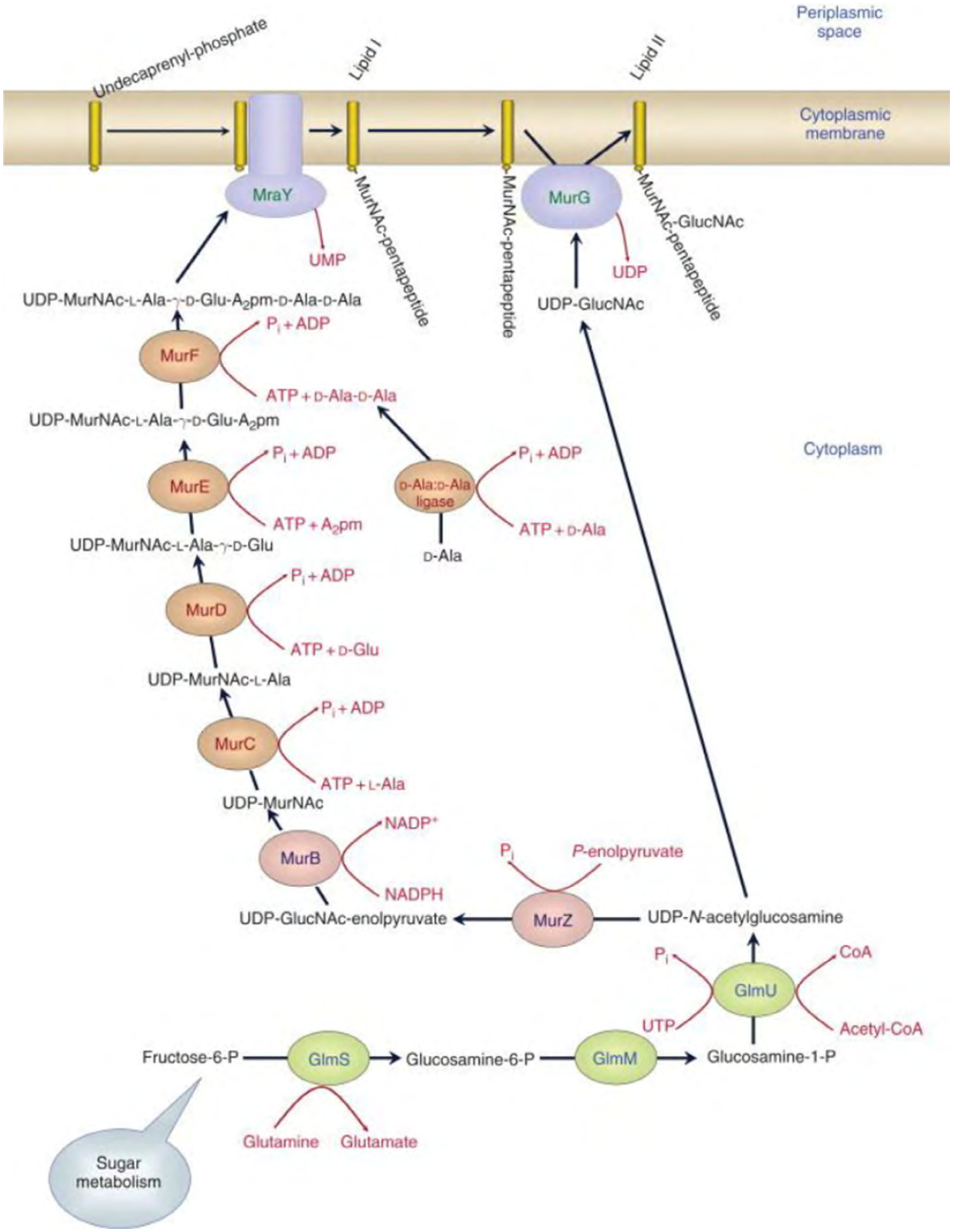

Figure 3 Schematic representation of peptidoglycan monomer unit biosynthesis. The individual stages, synthesis of N-acetylglucosamine, synthesis of N-acetylmuramic acid, addition of the stem peptide, and synthesis of lipid-linked precursors, are differentiated by colors. Cosubstrates required for each step are highlighted in red. Enzymes are indicated by their short names derived from the genetic *E. coli* nomenclature that corresponds to GlmS,glucosamine-6-phosphate synthase; GlmM, phosphoglucosamine mutase; GlmU, glucosamine-1-phosphate acetyltransferase plus N-acetylglucosamine-1-phosphate uridyltransferase; MurA, UDP-N-acetylglucosamine enolpyruvyl transferase; MurB, UDP-N-acetylenolpyruvylglucosamine reductase; MurC, UDP-N-acetylmuramic acid:L-alanine ligase; MurD, UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase; MurE, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate: *meso*-diaminopimelate ligase; MurF, UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-*meso*-diaminopimelate:D-alanyl-D-alanine ligase; MurG, UDP-N-acetylglucosamine:N-acetylmuramyl(pentapeptide)-P-P-undecaprenol-N-acetylglucosamine transferase; and MraY, phospho-N-acetylmuramyl-pentapeptide translocase.

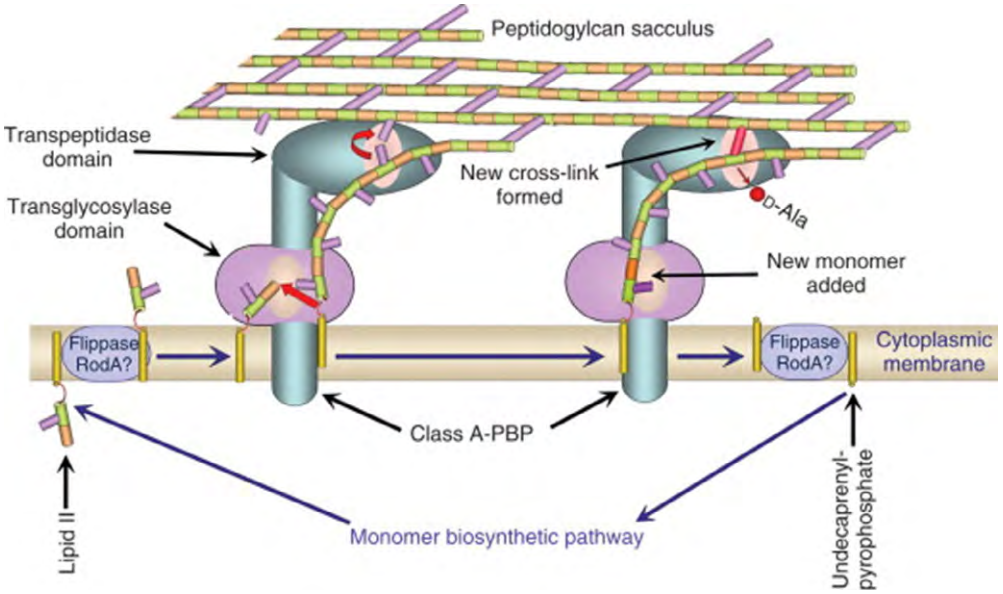

Figure 4 Schematic representation of peptidoglycan polymerization. In the first step, lipid II precursor molecules are transferred to the external side of the cytoplasmic membrane by the flippase MurJ. Once on the outside, a lipid II molecule interacts with the transglycosylase domain of a bifunctional class A high-molecular-weight PBP. A nascent glycan strand, already attached to the sacculus at the distal end, is transferred from undecaprenyl phosphate onto the C_4-OH of the lipid II molecule in the transglycosylase domain, causing the glycan strand to grow by one unit. A molecule of undecaprenyl pyrophosphate is also released and, upon transfer to the cytoplasmic side of the membrane, undergoes a new round of synthesis. Concomitantly, a stem peptide from the nascent chain and the second stem peptide in the preexisting sacculus find the right relative positioning at the transpeptidase domain active site and become cross-linked by the transfer of the peptide bond between the two terminal D-Ala residues of the stem peptide in the nascent glycan strand to the dibasic amino acid in the stem peptide at the sacculus, releasing a molecule of D-Ala. Iteration of the process leads to coordinated glycan strand linear polymerization and insertion of the nascent strands into the netlike structure of the sacculus.

of a new peptide bond. The specificity of both MurE and MurF is variable according to the dibasic amino acid present at position 3 of the stem peptide, a species-specific feature.

Synthesis of Lipid Intermediates

Attachment of the soluble precursors to the isoprenoid lipid undecaprenyl phosphate (bactoprenol) in the cytoplasmic membrane marks the beginning of the membrane-bound phase of peptidoglycan biosynthesis. The first reaction consists in the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to the membrane acceptor undecaprenyl phosphate to yield MurNAc-(pentapeptide)-pyrophosphoryl-undecaprenol (lipid I) and UMP. The reaction is catalyzed by the MraY translocase (phospho-N-acetylmuramyl-pentapeptide translocase), an integral membrane protein. In a consecutive reaction, the MurG transferase (UDP-N-acetylglucosamine:N-acetylmuramyl(pentapeptide)-P-P-undecaprenol-N-acetylglucosamine transferase) catalyzes the addition of a residue of GlcNAc from UDP-GlcNAc to the MurNAc moiety of a lipid I molecule through a (β-1$\rightarrow$4) glycosidic bond giving the completed lipid-linked monomer unit UDP-GlcNAc-(β-1$\rightarrow$4)-MurNAc(pentapeptide)-pyrophosphoryl-undecaprenol (lipid II). In *E. coli*, MurG is a peripheral membrane protein associated to the inner leaflet of the cytoplasmic membrane.

In growing cells, the proportion lipid I to UDP-MurNAc-pentapeptide is very low, in the 1% range, indicating that availability of undecaprenyl phosphate is a limiting factor for the membrane-bound steps of peptidoglycan biosynthesis. The scarcity of lipid I and lipid II (*c.*700 and 2000 molecules per cell in *E. coli*) implies a high turnover rate (about 1 s). This, in turn, constrains free diffusion of lipid precursors as an efficient way to sustain ongoing synthesis and supports the existence of associations with the transferases and later acting proteins in peptidoglycan-synthesizing complexes. Indeed, recent evidence gathered from *E. coli* supports an association of MurG with the synthetic complexes responsible for longitudinal growth of the sacculus.

Addition of Interpeptide Bridges

In many Gram-positive bacteria, cross-linking between peptidoglycan stem peptides occurs with the concourse of a short peptide, which is added to the dibasic amino acid at position 3. The size and composition of the interpeptide bridges are species-specific features. Size ranges from one to seven amino acids and composition includes Gly as well as D-amino acids (D-Ser, D-Glu, and D-Asx) and L-amino acids (L-Ala and L-Glu). The nature of the interpeptide bridges is the main cause of variability in peptidoglycan. However, knowledge of the enzymes involved (branching enzymes) is still limited. Synthesis of the interpeptide bridge occurs by

the sequential addition of amino acids to a pentapeptide precursor. The precursor substrate for branching enzymes is variable among species; for instance, in *S. aureus*, it is lipid II, but in other species, it can be lipid I and also UDP-MurNAc-pentapeptide. Furthermore, in some instances, the branching enzymes use more than one substrate (lipid II and UDP-MurNAc-pentapeptide in *Enterococcus faecalis*). Two different mechanisms coexist in dependence of the nature of the amino acid to be added. L-amino acids and Gly are activated as amino acyl-tRNAs and added to the precursor by the nonribosomal peptide-forming enzymes Fem transferases. D-amino acids are incorporated by enzymes of the ATP-grasp family. These enzymes activate the amino acids as acyl phosphates at the expense of ATP and catalyze ligation of the activated carboxyl to different chemical groups, in particular amino and imino nitrogens.

Polymerization of Peptidoglycan

The second stage in peptidoglycan synthesis is polymerization of monomer units and incorporation of the new polymeric product into the preexisting sacculus in such a fashion as to promote growth and preserve the correct cell shape.

The polymerization stages are performed by two different membrane-bound activities: glycosyltransferases that catalyze the formation of linear glycan polymers and peptidyl transferases (transpeptidases) that catalyze cross-linking of stem peptides. In growing bacteria, formation of glycan strands (transglycosylation) and cross-linking of stem peptides (transpeptidation) are continuous, tightly coupled reactions. However, detailed analysis of the process indicates that polymerization of the monomer proceeds by transglycosylation and precedes cross-linking of the nascent glycan strands by transpeptidation. It is important to realize that transpeptidation is responsible not only for the cross-linking of nascent material but also for its incorporation into the sacculus (Figure 4).

Translocation of Lipid-Linked Precursors

Polymerization of peptidoglycan is an extracellular process, but the immediate precursor lipid II is synthesized in the intracellular side of the cytoplasmic membrane. Therefore, the lipid II units must be translocated to the external side of the membrane (Figure 4). Lipid II translocation has been a long-standing controversial point, only recently settled by the demonstration of the protein MurJ, formerly known as MviN, as the lipid II flippase in *E. coli*. The experiments leading to the identification of MurJ as the flippase discard previous hypotheses in favor of the morphogenetic proteins RodA and/or FtsW. MurJ is a cytoplasmic membrane protein belonging to the multidrug/oligosaccharidyl-lipid/polysaccharide exporter superfamily of proteins. It has 14 transmembrane domains and a solvent-exposed cavity within its transmembrane region, which is essential for the flippase activity. As expected from its function, the MurJ protein is essential and its impairment leads to cell death. Whether MurJ-like mechanisms are unique and universal is still unknown, and other alternatives might be found.

Penicillin Binding Proteins: The Key Peptidoglycan Synthases

Penicillin binding proteins (PBPs) are a set of minor cytoplasmic membrane proteins ubiquitous in bacteria. PBPs are the specific targets for β-lactam antibiotics and critically involved in the late stages of peptidoglycan synthesis. PBPs are defined by their ability to covalently bind β-lactams yielding stable, enzymatically inactive, acyl–enzyme complexes. This property facilitated identification of PBPs by specific labeling with radioactive or fluorescent β-lactams. PBPs apparently share a common topology; they are anchored to the cytoplasmic membrane through a transmembrane domain, but the bulk of the protein is exposed to the external environment in close apposition to the sacculus, the PBP's insoluble substrate. Virtually every known bacterium possesses a set of PBPs, but number, size, abundance, and β-lactam binding spectrum are variable from species to species.

PBPs are habitually classified as high (>45 kDa)- and low (<45 kDa)-molecular-weight PBPs (HMW- and LMW-PBPs, respectively). The difference in molecular weight does indeed reflect a functional dichotomy: while HMW-PBPs have polymerase activity, LMW-PBPs are more often involved in cleavage of peptidoglycan peptide bonds and display endopeptidase and carboxypeptidase activities. The number of reactions that a single polypeptide is able to catalyze differentiates two classes of HMW-PBPs: the class A HMW-PBPs, which are bifunctional enzymes able to catalyze both polymerization of the glycan strands (transglycosylation) and cross-linking of the stem peptides (transpeptidation), and class B HMW-PBPs, which are monofunctional enzymes endowed with transpeptidase activity only.

Genetic analysis of HMW-PBPs in known bacterial genomes embodying more than 400 class A and 300 class B HMW-PBPs demonstrated a clear modular architecture. Both classes present a short cytoplasmic N-terminal segment, followed by a single transmembrane domain followed in class A enzymes by the transglycosylase module and in class B by a shorter module of unknown function, followed in turn by the C-terminal transpeptidase (peptidyl transferase) module responsible for cross-linking and β-lactam binding. The intermediate module in class B enzymes has been proposed to be involved in interactions with other membrane proteins. LMW-PBPs are monofunctional peptidyl transferases most often endowed with D,D-carboxypeptidase activity and with D,D-endopeptidase activity. As mentioned earlier, the active site of the peptidyl transferase is the target for β-lactam binding. Proteins of this group have an unusual architecture; the peptidyl transferase domain is encoded after a cleavable peptide signal, and the protein is anchored to the membrane through a C-terminal transmembrane domain or amphipathic helix.

Synthesis of Linear Peptidoglycan Polymers

Polymerization of peptidoglycan lipid II units is catalyzed by the bifunctional transglycosylase–transpeptidase class A HMW-PBPs. Many class A HMW-PBPs are known, but most of present-day knowledge derives from E. coli PBP1B, the paradigmatic peptidoglycan synthase.

The full-length enzymatically active PBP1B seems to be a dimer. The most widely accepted mechanism for peptidoglycan polymerization involves the repetitive addition of disaccharide-pentapeptide units from lipid II at the reducing end of a growing glycan chain attached to a second molecule of undecaprenyl pyrophosphate. In such a scheme, the undecaprenyl pyrophosphate-bound growing glycan chain acts as the glycosyl donor and is transferred to the C_4-OH of the GlcNAc molecule in a lipid II molecule, which becomes the glycosyl acceptor substrate. While the nascent glycan strand grows by a monomer unit each cycle, the reaction generates free undecaprenol pyrophosphate, which is transferred back to the cytoplasmic side of the membrane, hydrolyzed to undecaprenol phosphate, and reused for a new addition cycle (Figure 4).

The exact catalytic mechanism for transglycosylation is still uncertain. It is likely that the tranglycosylase domain of the PBP recognizes the sugar moieties of both the lipid II and a terminal section of the growing glycan chain, as is characteristic for other related glycosyl transferases. It has been proposed that the γ-COOH of an active site Glu residue (Glu_{223} in *E. coli* PBP1B) donates its proton to the phosphoester bond of the growing chain, to give an oxocarbenium cation, which then undergoes nucleophilic attack by the 4-OH of the GlcNAc in the lipid II molecule. Additional Asp and Glu residues in the active site (Asp_{234} and Glu_{290} in *E. coli* PBP1B) are likely involved in the stabilization of the oxocarbenium and activation of the C_4-OH group.

As mature peptidoglycan is devoid of any linkage to undecaprenyl phosphate, some enzymatic reaction must release nascent polymers from the membrane-anchoring undecaprenyl-phosphate moiety. Such enzyme(s) could play an important role in determining the length of the resulting peptidoglycan strands. However, there is no knowledge for any such enzyme so far. Release of the nascent glycan strand from undecaprenol pyrophosphate in Gram-negative bacteria results in generation of (1 → 6)-anhydro MurNAc, which becomes a tag for the glycan chain terminal muropeptide. Formation of (1 → 6)-anhydro MurNAc is thought to occur because transglycosylases may enter an idling state that results in an intramolecular glycosyl transfer of the nascent glycan strand onto the C_6-OH of the MurNAc residue, instead of the C_4-OH of a nearby lipid II molecule. A possible alternative is that glycan strands are polymerized in a continuous fashion and then are trimmed by the so-called lytic-transglycosylase enzymes able to split glycan polymers generating (1 → 6)-anhydro MurNAc (see section 'Brake to Make: Peptidoglycan Hydrolases'). The presence of (1 → 6)-anhydro MurNAc in the glycan strand terminal muropeptides is the reason for the nonreducing character of peptidoglycan in Gram-negative bacteria.

Class A HMW-PBPs clearly take the burden of lipid II polymerization. Nevertheless, membrane-bound monofunctional grlycosyl transferases (MGTs), able to polymerize Lipid II, have been identified and biochemically characterized in a number of unrelated bacteria as *M. luteus*, *E. coli*, *B. subtilis*, *Brucella abortus*, *Enterococcus faecalis*, *Haemophilus influenza*, *S. pneumonia*, and *S. aureus*. MGTs share considerable similarity with the glycosyl transferase domains of class A PBPs. In those species where it has been studied, MGTs often account for most of the lipid II glycosyl transferase activity in *in vitro* assays with cell extracts. MGTs are dispensable proteins, suggesting an auxiliary function. However, in the particular case of S. *aureus*, MGT exhibits a synthetic lethal phenotype with mutations in the tranglycosylase domain of PBP2, indicative of a certain degree of functional overlapping.

An intriguing aspect of peptidoglycan synthetic transglycosylases, indeed of most enzymes acting on macromolecular peptidoglycan, is their high redundancy. For instance, both *E. coli* and *S. pneumoniae* have at least four different enzymes each. It is believed that this multiplicity reflects a variety of functions. In fact, not all transglycosylases are essential. As an example, in *E. coli*, both the class A PBP1C and Mgl can be deleted without noticeable harm to the cell. However, either PBP1A or PBP1B must be present. It has been proposed that nonessential transglycosylases could be involved in localized reorganizations of the cell sacculus required for the assembly of envelope spanning complexes, such as flagella, and for specialized transport processes through the envelope.

Cross-Linking of Glycan Chains

In the last step, peptidoglycan strands synthesized by glycosyl transferases must be incorporated into the macromolecular, netlike structure of the sacculus. This is the role of the peptidyl transferase activities (D,D-transpeptidase) displayed by both class A and class B HMW-PBPs.

Transpeptidases cross-link stem peptides in the sacculus to the stem peptides in the nascent glycan strands, which, consequently, become part of the sacculus proper (Figure 4). The canonical D,D-transpeptidation reaction consists in the transfer of the D,D peptide bond linking the C-terminal D-Ala-D-Ala dipeptide of a disaccharide-pentapeptide unit in one strand, the donor muropeptide, onto the free NH_2 group of the dibasic amino acid at position 3 in the stem peptide of a second (acceptor) muropeptide in a nearby strand (Figure 4).

The transpeptidation reaction, the target for β-lactam antibiotics, has been analyzed in detail. The reaction proceeds in two steps. First, the transpeptidase domain binds the stem peptide -D-Ala-D-Ala dipeptide, and through a nucleophilic attack to the amide bond by the –OH of an active site Ser residue, an acyl–enzyme complex is formed. In a second step, the newly formed ester bond between the D-Ala at position 4 of the donor and the active site Ser is subjected to nucleophilic attack by the free NH_2– group of the dibasic amino acid in the acceptor stem peptide, resulting in the formation of a new amide bond bridging both stem peptides and regeneration of the –OH in the active site Ser. As a result of the transpeptidation reaction, the free NH_2 of the dibasic amino acid in the acceptor moiety of the resulting cross-linked peptide becomes substituted and unable to participate in further transpeptidation

reactions. Conversely, the equivalent group in the donor moiety remains free and capable to act as acceptor in a subsequent round of transpeptidation to yield higher-order cross-linked muropeptides. Indeed, tetramers have been demonstrated in *E. coli* and in a number of both Gram-negative and Gram-positive bacteria (*S. pneumoniae*) where they are particularly abundant.

As indicated before, in most Gram-positive bacteria, the stem peptide is modified by acylation with a short peptide of the non-α-NH_2 group of the dibasic amino acid. In these cases, the transpeptidation reaction proceeds as mentioned earlier except that the nucleophile in the second step of the reaction normally is the N-terminal NH_2 of the interpeptide bridge.

β-Lactam antibiotics react with D,D-transpeptidases, in general D,D-peptidyl transferases, because they mimic the natural donor substrate and are recognized by the donor site in the peptidyl transferase. The β-lactam bond substitutes for the D-Ala → D-Ala amide bond and acylates the active site serine residue yielding a stable penicilloyl–enzyme complex, which disables the enzyme.

With all likelihood, D,D-transpeptidation is the universal cross-linking reaction and the reason for the wide spectrum of β-lactam antibiotics. However, cross-linking by transfer of L,D peptide bonds has also been described in several bacteria. The dibasic amino acid at position 3 (A_2pm, L-Lys) can substitute for D-Ala at position 4 as the acyl donor in the transpeptidation reaction to yield a (3 → 3) L,D cross-link, instead of the regular (3 → 4) D,D cross-link produced by D,D-transpeptidases. In this case, the transferred peptide bond is of L,D conformation. The (3 → 3) L,D cross bridges were originally found in *Mycobacteria*. In some species as *M. smegmatis*, they account for as much as one-third of total cross bridges. In other bacteria, proportion is highly variable. In Gram-negative bacteria with (3 → 3) L,D cross bridges (often referred to as A_2pm → A_2pm cross bridges), the relative abundance is widely variable. In most cases, they account for below 15% of total cross bridges, but can represent up to 45% as in *Agrobacterium tumefaciens*. However, abundance of L,D cross bridges shows a substantial variability in response to environmental conditions, with a tendency to increase under stress conditions. At present, there is no clear understanding of the biochemistry of (3 → 3) L,D cross-links. It is generally accepted that synthesis occurs by a mechanism formally similar to D,D-transpeptidation, except for the nature of the donor peptide bond. Such a mechanism would be catalyzed by L,D-transpeptidases. In fact, L,D-transpeptidases have been described and characterized in some bacteria (*Enterococcus faecium*, *V. cholera*, and *E. coli*). L,D-transpeptidases are cysteine–peptidyl transferases unrelated to PBPs and consequently refractory to inhibition by most β-lactam antibiotics. Indeed, L,D-transpeptidases have been associated with β-lactam resistance. Computer modeling of L,D- and D,D-cross-linked muropeptides reveals substantial conformational differences, indicative of specific functionalities in the sacculus.

Class A versus Class B HMW-PBPs

As indicated before, both classes have D,D-transpeptidase activity, but only class A HMW-PBPs are bifunctional enzymes. Therefore, while class A HMW-PBPs are able to both polymerize lipid II into strands and incorporate the strands into the sacculus, class B HMW-PBPs are limited to the cross-linking of peptidoglycan strands necessarily fed to them by ancillary transglycosylases. However, in all bacteria where PBPs could be well studied, class B PBPs are essential and key enzymes for proper morphogenesis. For instance, PBP2 of *E. coli* is strictly required for longitudinal growth of the sacculus, and PBP3 is responsible for the synthesis of the septum when cells divide. Furthermore, it has been shown that PBP2 is by itself able to promote substantial growth of the sacculus under specific conditions. A possible interpretation of available information, by no means complete, is that class A enzymes provide bulk-synthetic capacity and class B enzymes direct the very final step of the process, fine-tuning incorporation of new material as to ensure proper shape is generated. Whether class A HMW-PBPs are feeders for class B or class B HMW-PBPs are associated with monofunctional glycosyl transferases is under discussion. However, the fact that at least in *E. coli*, MGT is dispensable but a class A enzyme must be active for the cells to grow favors class A and class B enzymes teaming together.

Brake to Make: Peptidoglycan Hydrolases

Enzymes capable of cleaving specific bonds in the sacculus are expected to play crucial roles to permit expansion of the whole structure. These enzymes are generally referred to as murein, or peptidoglycan, hydrolases. Some, but not all, cleave bonds that affect the physical continuity of peptidoglycan. Enzymes able to split cross-links or glycan strands can weaken the sacculus beyond a point when it is not more able to withstand the turgor pressure of the cell, and cell lysis would ensue. Therefore, murein hydrolases are dangerous enzymes for the cell, which must subject them to very stringent control. The capacity of some of them to cause cell lysis when control mechanisms are upset, by antibiotics, for instance, led to the designation of 'autolysins.' The number and variety of murein hydrolases are really staggering. Specific hydrolases exist for each and every peptide and glycosidic in peptidoglycan. Each bacterial species studied showed a wide repertory of hydrolases and very often a high degree of redundancy for at least some activities. To give a taste, in *E. coli*, not <5 different hydrolytic activities, represented by not <16 different proteins, have been described.

Peptidoglycan hydrolases can be roughly classified according to their substrate in peptidases and glycosylases. The former cleave bonds in the stem peptide and the later on the glycan strand.

An important group of peptidases comprises the D,D- and L,D-endopeptidases and carboxypeptidases, widespread and redundant in most bacteria. D,D-endopeptidase and D,D-carboxypeptidase activities are peptidyl transferases associated to low-molecular-weight PBPs. D,D-endopeptidases cut the cross bridges attaching nearby glycan strands to each other by cleaving the amide bond between the D-Ala at position 4 of the donor stem peptide and the dibasic amino acid on the acceptor stem peptide. Thus, a D,D-endopeptidase reverts the action of a D,D-transpeptidase. D,D-carboxypeptidases remove the C-terminal D-Ala from the stem peptides, which become shortened to tetrapeptides. Both sets of enzymes are generally dispensable but likely relevant for proper

morphogenesis. Impairment of their activity in *E. coli*, for instance, leads to severe morphological abnormalities as branching (Figure 2, panel D). The catalytic mechanism of these enzymes is similar to HMW-PBPs. In the case of D,D-transpeptidases, the donor amino acid is the D-Ala at position 4 in the donor side of the cross bridge, while for D,D-carboxypeptidases, it is the equivalent D-Ala in a non-cross-linked stem peptide. Upon the formation of the acyl–enzyme complex, the former acceptor moiety is released and the NH_2– group of the acceptor amino acid is regenerated. The ester bond between the donor peptide and the active site serine is then subjected to nucleophilic attack by a molecule of water releasing the free peptide and regenerating the active site amino acid. Because of their PBP character, most D,D-endopeptidases and carboxypeptidases are inhibited by β-lactams. However, in some bacteria as *E. coli*, penicillin-insensitive D,D-endopeptidases (MepA) have been also identified. The MepA-type endopeptidases are metallopeptidases completely unrelated to the penicillin-sensitive ones. L,D-endopeptidases and L,D-carboxypeptidases have similar activities to their D,D-counterparts on peptide bonds of L,D-stereochemistry and in most cases are believed to proceed through similar catalytic mechanisms.

A second very relevant set of murein peptidases is composed by the N-acetylmuramyl-L-alanine amidases. These enzymes cleave the amide bond between the lactyl group in MurNAc and the first amino acid (L-Ala) in the stem peptide. As a result of their activity, stem peptides are released from the peptidoglycan strands. Enzymes with this activity are quite heterogeneous in function and structure and still not fully characterized. Amidases are widespread and very often highly redundant. In *E. coli*, at least five molecular species present this activity. In some Gram-positive bacteria, amidases often present additional domains able to recognize, and bind to specific motifs in cell wall polymers, as the teichoic and lipoteichoic acids. Recognition of these domains might be required for enzyme activation as is the case for *S. pneumoniae* LytA, which has to recognize lipoteichoic acid choline to become active.

Among the glycosylases, the family of lytic transglycosylases is predominant. These enzymes catalyze the intramolecular transfer of a MurNAc (β-1 → 4) GlcNAc glycosidic bond in a peptidoglycan strand onto the 6-OH of the MurNAc residue, cleaving the glycan strands and generating (1 → 6)-anhydro MurNAc-containing muropeptides. Both endo- and exolytic transglycosylases have been identified. Exolytic enzymes are processive enzymes that degrade peptidoglycan strands from one end (GlcNAc) releasing a monomer at a time and shortening the strand by one unit. Endolytic enzymes randomly cleave MurNAc (β-1→4) GlcNAc glycosidic bonds in the glycan strand cutting it into a number of pieces.

N-acetylmuramidases (as lysozymes) and N-acetyl-glucosaminidases are the more frequent glycosyl hydrolases. The later are often predominant in some Gram-positive species, as *S. aureus*, where they are definitely involved in the morphogenetic pathway. However, both classes of enzymes seem to be mostly implicated in degradative processes as autolysis, peptidoglycan turnover, and even depredation (many bacteria release enzymes able to degrade peptidoglycan of niche-sharing species), than on biosynthetic pathways.

Physiology of Peptidoglycan Hydrolases

For the bacterial cell to grow, the sacculus has to expand accordingly. Because the sacculus is a covalently closed structure, just binding (cross-linking) new peptidoglycan strands to the sacculus is not enough to promote expansion. Indeed, it would only lead to thickening of the wall. To enlarge a closed net bag, meshes have to be cleaved, to permit insertion of new material between the existing nettings. However, the multiplicity of murein hydrolases as well as the high redundancy of many of them made investigation of their functionality a particularly frustrating business. It took more than 30 years of systematic work to identify the hydrolases responsible for peptidoglycan expansion in the model bacteria *E. coli*. Three different D,D-endopeptidases, MepS, MepH, and MepM (formerly Spr, YdhO, and YebA), turned out to be redundantly essential for the growth of the sacculus. In the absence of the three proteins cell wall growth is inhibited, and cell death ensues. Unexpectedly, impairment of these proteins results in inhibition of the insertion of new precursors into the sacculus, indicating the existence of a regulatory event, which impedes the activity of the synthases under such conditions. Interestingly, the three endopeptidases have quite different characteristics. MepS and MepH are both cysteine peptidases, but while MepS is an OM lipoprotein, MepH seems to be a periplasmic protein. MepM is a Zn^{2+}-dependent metallopeptidase anchored to the cytoplasmic membrane. The existence of different pathways to promote controlled opening of the peptidoglycan mesh looks likely. In *B. subtilis*, the essential L,D-endopeptidases CwlO and LytE could play a similar function. Both are cysteine peptidases, associated to the cell wall and required for cell growth, but active on L,D instead of D,D peptide bonds.

Participation of murein hydrolases in the elongation of the sacculus might have been hard to demonstrate, but their relevance for cell division has been undeniable for a long time. As a matter of fact, the most recurrently displayed phenotype in hydrolase mutants is a defective cell division. In both Gram-negative and Gram-positive bacteria, specific hydrolases (AmiA, AmiB, and AmiC in *E. coli*, LytA in *S. pneumoniae*, and Atl in *S. aureus*) are required for the resolution of the septal peptidoglycan. Impairment of the corresponding enzymes results in chained cells, which are unable to split their peptidoglycan septa to finish division. The cell division-specific hydrolases in *E. coli* are three N-acetylmuramyl-L-alanine amidases (AmiA, AmiB, and AmiC). All three are periplasmic proteins, and only AmpC shows a preferential location at the septum but exclusively in dividing cells. Amidases are potentially lethal hydrolases. To avoid the danger of autolysis, these proteins are activated exclusively at the division site by protein–protein interactions with the septum-associated proteins EnvC (activates AmiA and AmiB) and NlpD (activates AmiC). Similar mechanisms might be involved in the regulation of cell division associated hydrolases in other bacteria. Indeed, amidases are often associated to cell division in Gram-negative bacteria, and a factor (PcsB) similar to EnvC has been shown to play an important role in regulation of cell division in the Gram-positive *S. pneumonia*.

Postinsertional Metabolism of Peptidoglycan

The sequence of events schematically presented earlier ends up with the incorporation of new peptidoglycan into the sacculus and the corresponding expansion of the later. But this is by no means the end of peptidoglycan metabolism. As a matter of fact, the sacculus is subjected to a constant, and complex, reorganization. At least three processes have been documented in the *E. coli* model system: maturation, turnover, and growth-state adaptation.

Peptidoglycan Maturation

Investigation of the evolution of new peptidoglycan as it ages indicates that new and old mureins differ to a considerable extent at least in *E. coli*, *B. subtilis*, and *Lactococcus lactis*. In the *E. coli* case, maturation is characterized by the elimination of the D-Ala at position 5 of the stem peptides, by a notable increase in cross-linking and a reduction in the mean length of peptidoglycan strands. The enzymology of maturation is still poorly understood. Low-molecular-weight PBPs are for sure involved, but the molecular identity of many of the activities suspected to intervene in the process remains unknown.

Peptidoglycan Turnover and Recycling

The phenomenon of murein turnover, that is, shedding of peptidoglycan muropeptides from the sacculus in the course of normal growth, was first described in Gram-positive organisms (*B. megaterium*, *B. subtilis*, *L. monocytogenes*, *S. aureus*, *Lactobacillus acidophilus*, etc.). Because most Gram-positive bacteria lack a permeability barrier external to the sacculus, turnover products are normally released into the growth medium, making detection a relatively straightforward matter. Demonstration in Gram-negative bacteria (*E. coli*, *S. enterica*, *N. gonorrhoeae*, and *Neisseria subflava*) took more effort because turnover is often coupled to very efficient recycling of the released muropeptides, which are transported back to the cytoplasm and then enter the biosynthetic pathway. The magnitude of the turnover process is variable from species to species and may depend on growth conditions. In most instances, it is quite relevant. Between 20% and 50% of total peptidoglycan is turned over each generation in those species where it has been measured as *E. coli* (30–40%) *B. subtilis* (50%), *B. megaterium* (50%), *S. aureus* (25%), and *N. gonorrhoeae* (20–50%). These high turnover rates mean that peptidoglycan has to be synthesized and incorporated into the sacculus at a rate considerably higher than growth rate. Turnover is often considered a requirement for cell expansion and to be associated to damage assessment and defense mechanisms.

Turnover is an enzymatically catalyzed process mediated by peptidoglycan hydrolases. The main turnover products in many Gram-negative bacteria are (1→6)-anhydro MurNAc-containing monomeric muropeptides, the product of lytic transglycosylases. In Gram-positive bacteria, turnover products are more diverse. In some like *B. subtilis*, turnover is mediated by a single hydrolase, a MurNAc-L-alanine amidase, but often shed out products that are the result of a more complete degradation of muropeptides involving several enzymes.

Interestingly, peptidoglycan turnover is important for virulence in some pathogens as *N. gonorrhoeae* and *Bordetella pertussis*. Both species have intensive turnover and release considerable amounts of GlcNAc-(1→6)-anhydro MurNAc-L-Ala-D-Glu-A_2pm-D-Ala, into the extracellular environment. This muropeptide has a strong cytotoxic action on ciliated epithelia and is identical to the tracheal cytotoxin of *B. pertussis* and the peptidoglycan-derived cytotoxin of *N. gonorrhoeae*. Furthermore, peptidoglycan fragments have strong immunogenic and mitogenic activities influencing the course of infection.

Turnover products are often recycled in particular in Gram-negative bacteria where released muropeptides accumulate in the periplasmic space. Several recycling pathways apparently exist. In *E. coli*, the major route for recycling is via the transporter protein AmpG, a transmembrane protein acting as a specific permease for intact muropeptides. AmpG transports GlcNAc-(1→6)-anhydro MurNAc-L-Ala-D-Glu-A_2pm, the main turnover product in this bacteria, to the cytoplasm. There, it is further degraded by a β-N-acetyl-glucosaminidase, which splits the disaccharide releasing GlcNAc and (1→6)-anhydro MurNAc-L-Ala-D-Glu-A_2pm, and a MurNAc-L-alanine amidase (AmpD), which breaks up the later fragment into (1→6)-anhydro MurNAc and the stem tripeptide. AmpD has a strict requirement for (1→6) anhydro MurNAc and therefore is inactive against the cytoplasmic precursors present in the cytoplasm as UDP-MurNAc-pentapeptide. Interestingly, the tripeptide released by AmpD is not further degraded. Instead, it is directly hooked onto a molecule of UDP-MurNAc by the enzyme Mlp (UDP-N-acetylmuramate:L-Alanyl-D-glutamate-*meso*-diaminopimelate ligase) in the presence of ATP. Although both Mlp and MurC add amino acids to UDP-MurNAc, they could neither compensate for each other nor accept each other peptide substrates. Mlp paralogs have been identified in other bacteria (*Haemophilus influenzae*), indicating that similar pathways could be widespread in bacteria.

Peptidoglycan recycling has been recently associated with the induction of chromosomal β-lactamases of the AmpC type in enterobacteria. β-Lactamases are the major bacterial defense mechanism against β-lactams. Enterobacterial AmpCs are inducible enzymes, and induction requires an operational peptidoglycan recycling pathway. The presence of antibiotics causes increased intracellular levels of (1→6)-anhydro MurNAc-L-Ala-D-Glu-A_2pm, which binds to AmpR, a regulatory protein of the *amp* operon, and induces synthesis of β-lactamase. Therefore, turnover and recycling might also play a sensory function to detect cell wall-damaging agents.

Adaptive Modifications of Peptidoglycan

The composition and structure of peptidoglycan change in response to environmental variations, in particular during the transitions in growth phase. Information is still limited to a few systems (*E. coli*, *S. enterica*, *H. pylori*, and *B. subtilis*), but it may well be a widespread phenomenon. Transition into stationary phase is a complex adaptive process, which involves a global reorganization of cell metabolism and very often specific morphological changes. In *E. coli*, transition into stationary phase triggers a full set of modifications both in the characteristics of peptidoglycan itself and in the complement of enzymes involved in peptidoglycan metabolism, PBPs, and peptidoglycan hydrolases. Compared with exponentially growing cells, peptidoglycan from stationary-phase *E. coli* is about 50% more cross-linked, has close to double amount of bound lipoprotein, and is made up of, in average, 30% shorter glycan chains. These modifications affect total peptidoglycan and are thought to require activity of specific enzymes. Interestingly, *E. coli* cells in the so-called 'viable but nonculturable' state, a condition thought to be clinically relevant in pathogenic bacteria, display similar alterations in their peptidoglycan. In *B. subtilis* peptidoglycan, cross-linking is also higher in stationary-phase cells. Increased cross-linking seems to be a rather general feature of nongrowing cells and has been detected in all cases studied.

Stationary-phase cells often display specific modifications in both biosynthetic and hydrolytic peptidoglycan enzymes. Activation of specific hydrolases is in fact thought to be critical for the induction of the autolytic response of many bacteria, as *S. pneumoniae*, to prolonged starvation and other adverse conditions as the presence of antibiotics.

Stationary-phase *E. coli* cells retain a considerable peptidoglycan biosynthetic activity. This activity is apparently associated to turnover and could account for renovation of about 20% of the cell peptidoglycan per hour. Interestingly, the class B PBP2 seems to be the key synthase in this process. The relevance of stationary-phase synthesis has not been asserted yet; however, an involvement in cell survival seems most likely.

Adaptation of *V. cholerae* to the stress conditions associated to media exhaustion includes a particularly sophisticated mechanism to modulate peptidoglycan synthesis. When conditions deteriorate, *V. cholera* synthesizes a periplasmic, broad specificity amino acid racemase (BsrV) in response to the activation of the main stress regulator RpoS. Synthesis of BsrV results in the production of D-amino acids, mostly D-Met and D-Leu, which are secreted to the medium reaching concentrations in the millimolar range. The production of D-amino acids results in their incorporation into the peptidoglycan where they substitute for one of the D-Ala residues, more frequently the one at position 4. Incorporation of the D-amino acids leads to decreased peptidoglycan biosynthetic activity. Mutational impairment of D-amino acid production results in osmotically unstable cells with excess peptidoglycan. Incorporation at position 4 occurs by an D-amino acid exchange reaction catalyzed by the L,D-transpeptidases responsible for $(3 \rightarrow 3)$ L,D cross-linkage and results in the release of the D-Ala residue normally found at that position and a muropeptide with a terminal residue of the new D-amino acid. D-amino acid incorporation at position 5 either might be the consequence of misincorporation of the D-amino acid into peptidoglycan precursors or could be mediated by an exchange reaction as mentioned earlier but mediated by D,D-peptidyl transferases. In the later case, the enzymes would transfer the peptide bond between the two D-Ala residues to the amino group of the foreign D-amino acid, releasing D-Ala and a muropeptide with the foreign D-amino acid at position 5. Such a capability has indeed been demonstrated for the class A PBPs of *E. coli*. Production of D-amino acids is not restricted to *V. cholera* and has been demonstrated in a number of unrelated bacteria (*B. subtilis*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Streptomyces lividans*, and *Deinococcus radiodurans*). Furthermore, the demonstration of similar effects of D-amino acids in *B. subtilis* supports that comparable modulatory mechanisms might be relatively widespread.

Biophysical Properties of Peptidoglycan Sacculi

Thickness of Sacculi

As indicated before, bacteria fall in two broad categories, Gram-positive and Gram-negative, according to the organization of their cell envelopes. The former group displays a relatively thick peptidoglycan sacculus with covalently linked accessory polymers, while the later normally exhibits very thin sacculi devoid of accessory polymers. Precise measurement of the real thickness of sacculi has proved to be a rather complex task. Classical electron microscopy techniques had profound effects on the apparent thickness of sacculi in thin sections. Introduction of cryoelectron microscopy of frozen hydrated samples and atomic force microscopy recently led to a precise estimation of peptidoglycan thickness. In Gram-negative bacteria, results were unexpected because sacculi from *E. coli* and *Pseudomonas aeruginosa*, two species with identical peptidoglycan, gave different values. Cryoelectron microscopy of frozen hydrated cells gave peptidoglycan thickness values of 6.35 ± 0.53 nm for *E. coli* and 2.41 ± 0.54 for *P. aeruginosa*. Atomic force microscopy produced very close values, 6.0 ± 0.5 nm and 3 ± 0.5 nm for hydrated *E. coli* and *P. aeruginosa* sacculi, respectively. Concordance of both techniques is reassuring but clearly indicates that the three-dimensional organization of peptidoglycan in sacculi of Gram-negative bacteria is more variable than previously expected. Small-angle neutron scattering measurements indicate that in *E. coli*, the sacculus is not homogeneous in thickness; about 70% of total peptidoglycan is arranged in a monolayer, but up to 30% is in triple-layered domains. Unfortunately, the technique does not resolve the localization of the multilayered regions. Species-to-species variations in the abundance and distribution of multilayered regions could well explain the different apparent thicknesses in thin sections. In any case, these results seriously question the classical view of Gram-negative cell walls as a strict monolayer.

Sacculi from Gram-positive bacteria are undoubtedly multilayered and variable in thickness. Application of advanced techniques however revealed a bipartite organization of the cell wall. In all species studied, there is an inner zone of low density,

the inner wall zone, about 16 nm thick, apparently impoverish in polymeric materials, surrounded by the outer wall zone (OWZ) representing the polymeric peptidoglycan–teichoic acid complex. The OWZ is often in the 15–30 nm range but is quite variable.

Elasticity of Sacculi

Purified peptidoglycan sacculi behave like an elastic fabric. Available measurements indicate that surface of *E. coli* sacculi can reversibly expand and shrink by a factor of 3 without ruptures. Different approaches support the idea that sacculi are under dynamic stress in the living cell, expanding and shrinking in response to variations in turgor pressure. In the growing *E. coli* cell, sacculi appear to be stretched to about 140% of their surface at the relaxed (no turgor pressure) state.

The most interesting observation is the anisotropic character of elasticity in sacculi. Sacculi from rod cells are significantly more deformable along the longitudinal axis of the cell than in the transversal axis. Measurements in the atomic force microscope gave elastic moduli of $2.5 \text{ æ } 10^7$ and $4.5 \text{ æ } 10^7$ N m^{-2} in the respective directions (smaller elastic modulus means greater elasticity). These measurements are in agreement with the observation that changes in cell volume due to osmotic challenge are mainly due to elongation of the cells, while diameter remains nearly constant. In macromolecular peptidoglycan, peptide bridges can be stretched much further than the virtually inextensible glycan backbones. For this reason, elastic anisotropy is considered as a strong indication for a preferential orientation of the glycan strands perpendicular to the long axis of the cell. However, x-ray diffraction and infrared spectroscopy indicate that the sacculus is far from a regularly ordered structure. In fact, application of advanced electron cryotomography techniques permitted a direct visualization of peptidoglycan strands, roughly perpendicular to the longitudinal cell axis, encircling the cell in a disorganized hooplike distribution.

Permeability of Sacculi

Bacteria secrete numerous macromolecules that must necessarily cross the sacculus on their way to the external environment. Moreover, an assembly of supramolecular complexes spanning the cell envelope, as flagella or type III protein secretion systems, also have to deal with the presence of the sacculus. Therefore, it is relevant to know whether and how the structure of the sacculus, or the activity of particular enzymes, permits traffic and assembly of macromolecules.

Experimental data on the porosity of sacculi are scant and limited to *E. coli* and *B. subtilis*. Interestingly, pores in purified (relaxed) sacculi from both organisms are of similar average size, with estimated radii of 2.06 nm for *E. coli* and 2.12 nm for *B. subtilis*. This size limits free passage of globular proteins to molecular weights about 22–25 kDa in the relaxed state of peptidoglycan, which could go up to around 50 KDa for fully stretched sacculi. Bigger molecules could pass through the sacculus either in nonfolded or partially folded forms or through specialized mechanisms involving local peptidoglycan rearrangements. Assembly of supramolecular structures almost certainly requires such rearrangements. For instance, the flagellar rod has an average diameter of about 11 nm, much wider than peptidoglycan expected discontinuities. Participation of murein hydrolases in these rearrangements has been substantiated in a number of bacteria. In *C. crescentus*, the transglycosylase PleA is required for assembly of both pili and flagella at the cell pole. In *S. enterica* and *Rhodobacter sphaeroides*, flagellar proteins with muramidase activity (FlgJ) are required for flagellar assembly, which otherwise stalls at the M ring stage. Paralogs to hydrolytic enzyme genes have been found in operons coding for flagella, pili, and type III secretion systems, indicating that participation of peptidoglycan hydrolases in assembly processes might be a general mechanism.

Topological Heterogeneity in Sacculi

The existence in sacculi of peptidoglycan patches with different properties was first detected in *B. megaterium*. The polar caps of the rodlike cells were shown to turnover far more slowly than the lateral cell wall. More recently, it has been shown that the peptidoglycan at the polar caps of *E. coli* and *C. crescentus* sacculi is metabolically inert (IPG). That is, neither new precursors are inserted into these regions nor are they turned over. As a consequence, IPG has a very long life. As polar regions are the product of a previous septation event, IPG can be considered the final product of such a septation event. The mechanism underlying generation of IPG is unknown. Nevertheless, at least three proteins, PBP5, BolA, and FtsZ, have been shown to affect proper distribution of IPG in *E. coli*. All three proteins are involved in the morphogenetic pathway. Therefore, it seems that proper differentiation of IPG regions might be an important process for the cell. At present, no specific structural or chemical features have been found in IPG.

Concluding Remarks

The role of peptidoglycan as a reinforcement for the bacterial cell envelope was firmly established more than 65 years ago, and the basics of its structure and metabolism were worked out soon after. However, the full complexity of the bacterial sacculus became apparent only recently, hand in hand with the impressive advances in the field of bacterial division differentiation and morphogenesis. The structure once thought to be a rigid, and fundamentally inert structure, is showing a totally unexpected degree of complexity and dynamism at all imaginable levels. Furthermore, peptidoglycan is fast becoming an important player in unsuspected fields of bacterial biology, as pathogenesis, signaling, and ecology. Further research on the structural properties, biochemistry, and physiology of this fascinating macromolecule will undoubtedly help us to better understand bacterial life.

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Pesticides, Microbial[☆]

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Glossary

Antagonism A biological type of interaction between living organisms that has a detrimental effect on one or both partners.
Biological control A process derived from antagonistic interactions between a beneficial organism and a pathogen or parasite that result in the control of a disease or pest.
Biopesticide A formulated active ingredient of biological origin, generally composed of microbial cells, that is used to control plant diseases or pests.
Biosafety The property of an active microbial ingredient related to the lack of adverse effects in plants and animals, including humans.
Competitive exclusion An ecological process by which an organism excludes another organism from a habitat through more efficient use of nutrients and space colonization.
Fermentation The mass production of microorganisms by cultivating in solid or liquid culture media for industrial purposes.
Formulation A method of preparing biopesticides for commercial delivery that consists of the preservation of microbial cells to increase shelf-life and enhance field survival and efficacy.
Registration The administrative procedure by which an active ingredient is authorized for commercial use following specific legal regulations.

Defining Statement

Microbial biopesticides are products made from beneficial microorganisms that are used to control plant diseases. Many products composed of bacteriophage, bacteria, yeast, and fungi are marketed worldwide, and are used by spraying or drenching plants, by local application of sticks or tablets near the root system, by seed coating or root bacterization before transplanting, and by using helper insects for dispersion.

The Plant As a Microbial Habitat

Microorganisms have adapted to the plant environment because plants offer a wide diversity of habitats, including the phyllosphere (aerial plant part), rhizosphere (zone of influence of the root system), and endosphere (internal transport system). Interactions may be neutral, beneficial, or detrimental for either the microorganism or the plant. Beneficial interactions for plants are caused by symbiotic or nonsymbiotic bacteria and by mycorrhizae, a highly specialized type of fungi, and have applications in plant phytostimulation, biofertilization, and bioremediation. Pathogenic or detrimental interactions of microbes with plants involve viroids, viruses, bacteria, and fungi, and lead to infectious diseases affecting only the plant kingdom.

The microbiota associated with plants has been classically studied by cultivation-dependent methods, which are estimated to recover only 0.01–10% of the total population. However, based on molecular analysis, it has been estimated that there are from hundreds to thousands microbial species per gram of soil in the rhizosphere or plant material in the phyllosphere.

Healthy plants contain a diversity of commensal and saprophytic microorganisms that contribute to provide, as in animals, an environmental equilibrium as the initial barriers for entrance of pathogens (Fig. 1). Bacteria are abundant in plants because they enable rapid growth. The Proteobacteria usually dominate samples in rhizosphere, particularly those of the α and β classes. Other major groups include Actinobacteria, Firmicutes, Bacteroidetes, Planctomycetes, Verrucomicrobia and Acidobacteria. In the case of phyllosphere, the dominant bacterial phylum are also the Proteobacteria (the α and γ classes), with Bacteroidetes and Actinobacteria also commonly found. The predominant genera are represented by the Gram-negative *Pseudomonas* (*Pseudomonas fluorescens*), *Pantoea* (*Pantoea agglomerans*, synonymous with *Erwinia herbicola*), *Xanthomonas* (*Xanthomonas campestris*), *Flavobacterium*, *Micrococcus*, and in some plants or plant parts by lactic acid bacteria (*Lactobacillus* and *Leuconostoc*) or *Azospirillum* species. Plants are also colonized by Gram-positive spore formers, which are mainly represented by the *Bacillus* species that colonize the soil and root system together with actinomycetes. Yeasts such as *Aureobasidium*, *Sporobolomyces*, *Cryptococcus*, *Torulopsis*, *Saccharomyces*, *Kloeckera*, *Rhodotorula*, and *Candida* are part of the microbiota of leaves and develop

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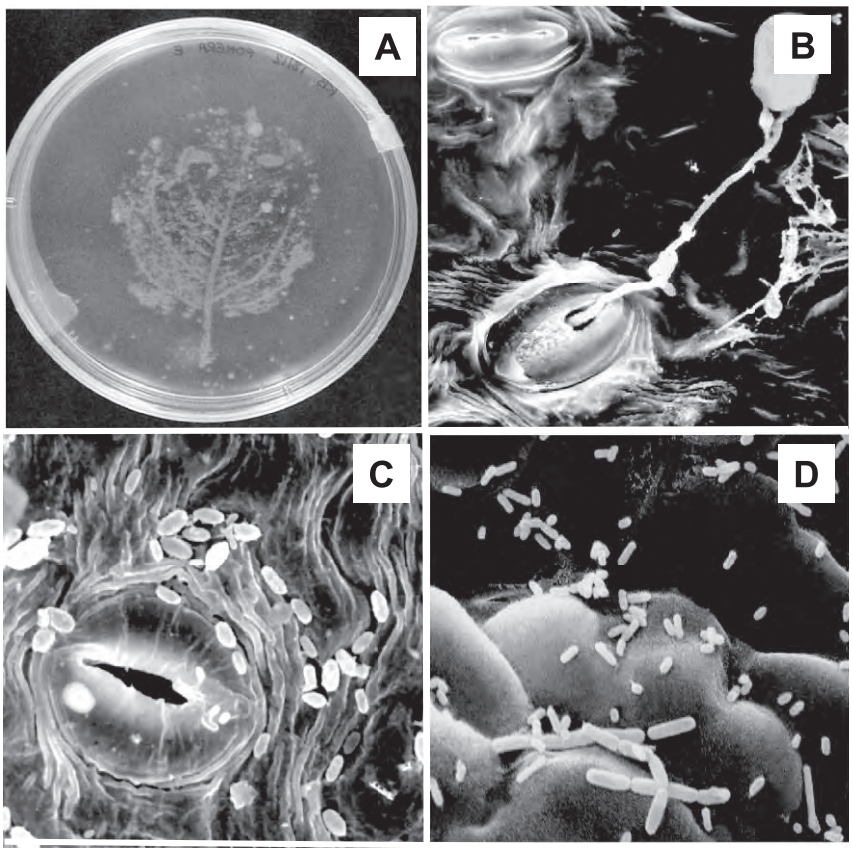

Fig. 1 Microorganisms interact with plant surfaces. (A) A print of a leaf on the surface of nutrient agar into a Petri plate reveals the presence of microbial colonies after several days of incubation; (B) infection of the stomata by a germinating spore of a pathogenic fungus; (C) colonization of the surrounding area of the stomata by bacteria; and (D) colonization of the hypanthia surface of a flower.

especially in fruits and flowers with high content of sugars. Different types of molds such as *Alternaria*, *Penicillium*, *Fusarium*, and *Aspergillus* can also be found. Several endophytic microorganisms also inhabit the phloem and xylem vessels of plants, and the intercellular apoplast and in dead or dying cells.

The composition and population levels of microorganisms in plants are determined by nutrient sources released by plant organs as root or leaf exudates or specific secretion systems as the nectaria in flowers. The degree of change in microbial composition is associated with the type of plant species or cultivar and varies according to the phenological state and organ (leaves, flowers, fruits, seeds, roots). Water availability on the aerial plant surface and temperature are environmental parameters that often exhibit strong fluctuations (daily, seasonal, hourly changes) and greatly affect microbial epiphytic life, as they determine growth rate, germination, taxis, and other essential processes for colonization of the plant surface. The root environment is more stable. The spatial distribution of the microbiota on the plant surface is not homogeneous because it depends strongly on suitable sites for colonization and growth. Microcolonies are often found in key environments like in the surrounding area of the stomata on the leaf surface, the nectaria in flowers, the calyx end in fruits, or the root tip.

Plant Disease and Biological Control Methods

Losses in crop production due to plant disease average from 13 to 15% worldwide and severely limit production, quality, and safety of food. Several thousands of diseases that are caused by 120 genera of fungi, 30 types of viruses, and 10 genera of bacteria have been described in plants.

Disease control is mainly achieved by treating crops with significant amounts of synthetic chemical pesticides. However, social and political concerns have influenced the practice of crop protection, which has been progressively reoriented toward a rational use of pesticides and toward a reduction in the number of registered active ingredients to those that are more selective, less toxic, and with a lower negative environmental impact. As a consequence, several countries have undertaken regulatory changes in pesticide registration requirements, given that consumer health and environmental preservation prevail over productive or economic considerations.

The therapeutic approach used in the past for plant protection is changing to a sustainable pest management approach. However, this change in crop protection methods is generating additional problems caused by a shortage of tools for control of

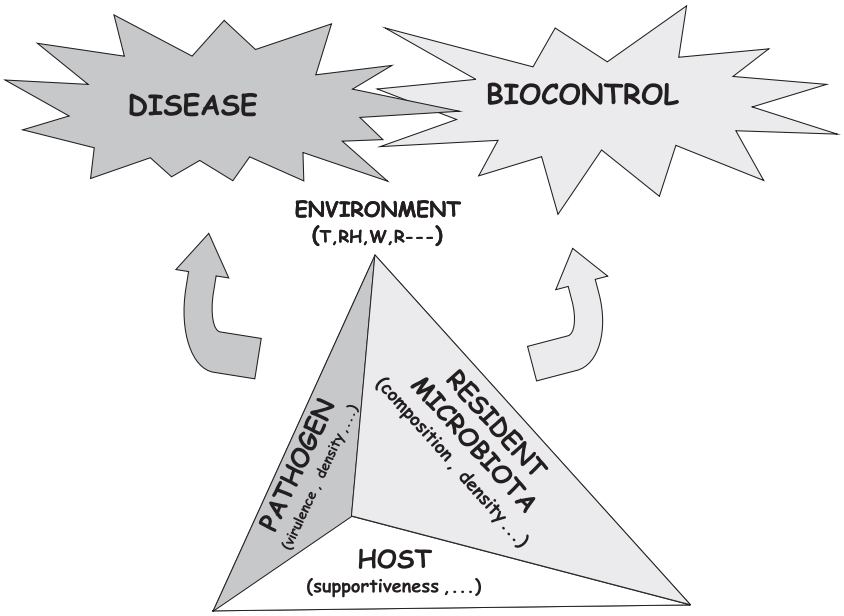

Fig. 2 The disease tetrahedron is composed of a susceptible host plant that can be infected by a virulent pathogen under suitable environmental conditions, and it is influenced by interactions with the natural microbiota. This four-level interaction may result either in the disease process in the plant host or in the biological control of the pathogen.

diseases in plants of economic importance. Fortunately, new methods of crop protection have emerged from traditional practices in agriculture and forestry such as the use of disease suppressive soils, crop rotation, soil solarization, and green manure or compost as organic amendments. Many of the benefits of these old techniques rely on naturally occurring microorganisms that exert a biological control on pests and diseases (Fig. 2).

Microorganisms Active in the Biological Control of Plant Pathogens and Their Mechanism of Action

Finding beneficial microorganisms to develop microbial pesticides is a trial-and-error process. The experience accumulated over several decades of research using different screening strategies indicates that many suitable sources come from disease-suppressive soils or healthy plants in epidemic areas affected by a given disease. However, success has also been obtained with nondirected search, that is finding microorganisms of interest from habitats far from the plant environment intended to be protected.

To be a biological control agent is not an attribute of a given genera or species of microorganisms, but it is generally restricted to the strain level. Usually, a few out of the thousands of strains that have been isolated result in a good candidate being developed as a commercial biopesticide. Many research projects have been successful and hundreds of strains of microorganisms have been reported as active in the control of plant pathogens, such as bacteria and fungi causing aerial or root diseases, or are effective against postharvest rot of products like fresh fruits and vegetables. Strains of biological control agents are distributed among Gram-negative bacteria of the families Rhizobiaceae, Pseudomonadaceae, and Enterobacteriaceae, and among Gram-positive bacteria such as Bacillaceae, Lactobacillaceae, Leuconostocaceae, and Streptomycetaceae. There are also representatives of yeasts and fungi within Ascomycota and Basidiomycota, and also in Oomycota. Finally, there are also hipovirulent pathogens with biocontrol ability like *Fusarium*, *Rhizoctonia*, *Sclerotinia*, and *Phytophthora*, mainly isolated from suppressive soils (Table 1).

Beneficial microorganisms able to control plant diseases can colonize or compete with the pathogens for nutrients and sites of interaction with the plant host, or exert antagonism through antimicrobial compounds, develop hyperparasitism or directly attach to the pathogen cells, interfere with pathogen signals, or induce resistance into the plant host. There are examples of strains that cover a single mechanism, or it is possible that a combination of several mechanisms converge within the same strain (Table 2; Figs. 3 and 4). Antibiosis against plant-pathogenic bacteria and fungi is very common among bacterial pesticides, for example, by the production of opines, a type of toxic derivative of amino acids, in *Agrobacterium radiobacter*, phenolic antifungal compounds in *Pseudomonas* species, antimicrobial peptides and polyenes in *Bacillus* and actinomycetes, or lytic enzymes in several yeast and fungi like *Candida* and *Trichoderma*. Competitive exclusion of the pathogen from sites of infection by better use of nutrients and colonization is also a common mechanism that can accompany other mechanisms. Several hyperparasites, especially abundant among the yeast and fungi biocontrol agents like *Coniothyrium*, *Gliocladium*, *Pichia*, *Ampelomyces*, *Streptomyces*, and *Trichoderma*, interact directly and degrade the fungal cell wall or spore envelope of pathogens. Some bacteria and fungi

Table 1 Groups of microorganisms that have strains of biocontrol agents of plant diseases

Group	*Family/Class*	*Genus*
Virus		
	–	Bacteriophages
		Weak strains plant viruses
Bacteria		
	Bacillaceae	*Bacillus*
	Paenibacillaceae	*Brevibacillus*
		Paenibacillus
	Cellulomonadaceae	*Cellulomonas*
	Enterobacteriaceae	*Enterobacter*
		Pantoea (Erwinia)
		Rahnella
		Serratia
	Lactobacillaceae	*Lactobacillus*
	Leuconostocaceae	*Leuconostoc*
	Pseudomonadaceae	*Burkholderia*
		Pseudomonas
	Rhizobiaceae	*Agrobacterium*
	Streptomycetaceae	*Streptomyces*
	Xanthomonadaceae	*Lysobacter*
Yeast		
	Ascomycota	*Aureobasidium*
		Candida
		Coniothyrium
		Debaryomyces
		Kloeckera
		Metschnikowia
		Pichia
		Saccharomyces
	Basidiomycota	*Cryptococcus*
		Pseudozyma
		Rhodotorula
		Sporobolomyces
Fungi (molds)		
	Ascomycota	*Ampelomyces*
		Aspergillus
		Chaetomium
		Epicoccum
		Gliocladium
		Muscodor
		Myrothecium
		Penicillium
		Trichoderma
		Ulocladium
		Verticillium
	Basidiomycota	*Phlebiopsis*
	Oomycota	*Pythium*

are able to induce defense responses in plants, by producing either elicitors (eg, cell wall components) or messenger molecules (eg, salicylic acid). Finally, a mechanism has emerged based on the finding that there are bacteria that can degrade chemical signal messengers necessary for quorum sensing (eg, acyl homoserine lactones) used by many plant-pathogenic bacteria to start the infection process in the host.

The basic and applied research on biological control agents has stimulated sequencing of their genomes. Some of the genomes that have been completely sequenced for various biological control strains are *Bacillus amyloliquefaciens* FZB42, Y2, and M27, *Bacillus pumilus* INR7, *Bacillus subtilis* 168 and XF-1, *Brevibacillus laterosporus* B9, *Pantoea vagans* C9-1 and *P. fluorescens* (*P. protegens*) Pf-5, CHA0 and Cab57. In the case of yeast strains, the genome sequence is available for *Debaryomyces hansenii* CBS767, *Cryptococcus flavescens* OH182.9-3C and *Pichia guilliermondii* and in the case of fungi *Pythium oligandrum* Po37, *Trichoderma gamsii* T6085, and *Trichoderma harzianum* T6776.

Table 2 Mechanisms of action in selected biocontrol agents of plant diseases

Microorganism and strain	*Target pathogen or disease*	*Mechanism involved*
Virus		
Bacteriophage	*Xanthomonas*, *Pseudomonas* and *Ralstonia* diseases	Lysis
Bacteria		
Agrobacterium radiobacter K84/K1026	*Agrobacterium tumefaciens*	Ab-opines
Bacillus amyloliquefaciens FZB42	*Fusarium oxysporum*	Ab-AMP, IR
Bacillus subtilis ATCC6633	*Pythium*	Ab-AMP
B. subtilis 6051	*Pseudomonas syringae*	Ab-AMP
B. subtilis GA1	*Botrytis*	Ab-AMP
B. subtilis UMAF6614	*Podosphaera fusca*	Ab-AMP
Pantoea agglomerans Eh318	Fire blight	CE, Ab-pseudopeptide
P. agglomerans EPS125/CPA-2	Postharvest fungi	CE
Pseudomonas chlororaphis PCL1391	*F. oxysporum*	Ab-PCA
P. chlororaphis MA342	*Pythium*, others	Ab-DDR, CE
Pseudomonas fluorescens A506	Fire blight and frost damage	CE, Ab
P. fluorescens Pf-5	*Pythium, Rhizoctonia*	Ab-DAPG, Plt, Prn
P. fluorescens Q2-87	*Gaeummanomyces graminis*	Ab-DAPG
P. fluorescens WCS417	*F. oxysporum*	IR
P. fluorescens SS101	*Phytophthora*	Ab-cAMP, IR
P. fluorescens EPS62	*Erwinia amylovora*	CE
P. syringae ESC10/ESC11 *Pantoea vagans* C9-1	Postharvest rot fungi Fire blight	Ab-Syr, CE Ab-pseudopeptide
Streptomyces griseoviridis K61	Soil-borne pathogens	CE, HP, Ab-polyenes
Fungi		
Ampelomyces quisqualis AQ10	Powdery mildew	HP
Aureobasidium pullulans CF10	Postharvest fungi, fire blight	CE, Ab
A. pullulans Ach1-1	Postharvest fungi	CE
Candida oleophila I-182/Q	Postharvest fungi	CE, Ab-lytic enzymes
Candida sake CPA-1	Postharvest fungi	CE
Coniothyrium minitans CON/M/91-08	Fungi	HP
Gliocladium catenulatum J1446	Soil-borne fungal pathogens	HP, Ab-lytic enzymes
Gymnopleurus virens GL21	Soil-borne fungal pathogens	HP, Ab-gliotoxin
Pichia guilliermondii 87	Fungi	CE, HP
Trichoderma harzianum T22, P1	Soil-borne pathogens	COM

Abbreviations: Ab, antibiosis; AMP, cyclic antimicrobial peptides; CE, competitive exclusion; COM, complex; DAPG, diacetyl phloroglucinol; DDR, didehydro-rhizoxin; HP, hyperparasitism; IR, induced resistance; PCA, phenylcarboxilic acid; Plt, pyoluteorin; Prn, pyrrolnitrin; SYR, syringomicin.

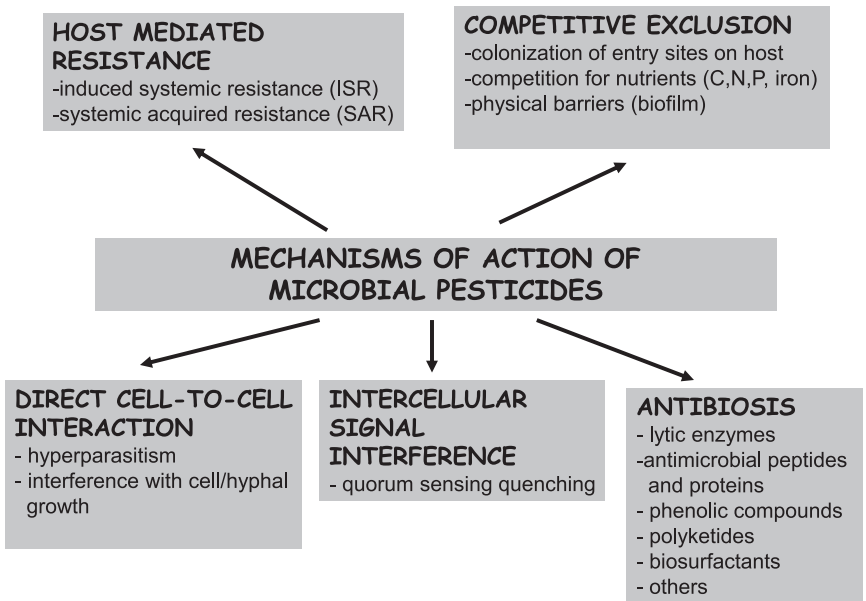

Fig. 3 An overview of the mechanisms of biological control of plant pathogens that have been demonstrated in beneficial microorganisms interacting with plants.

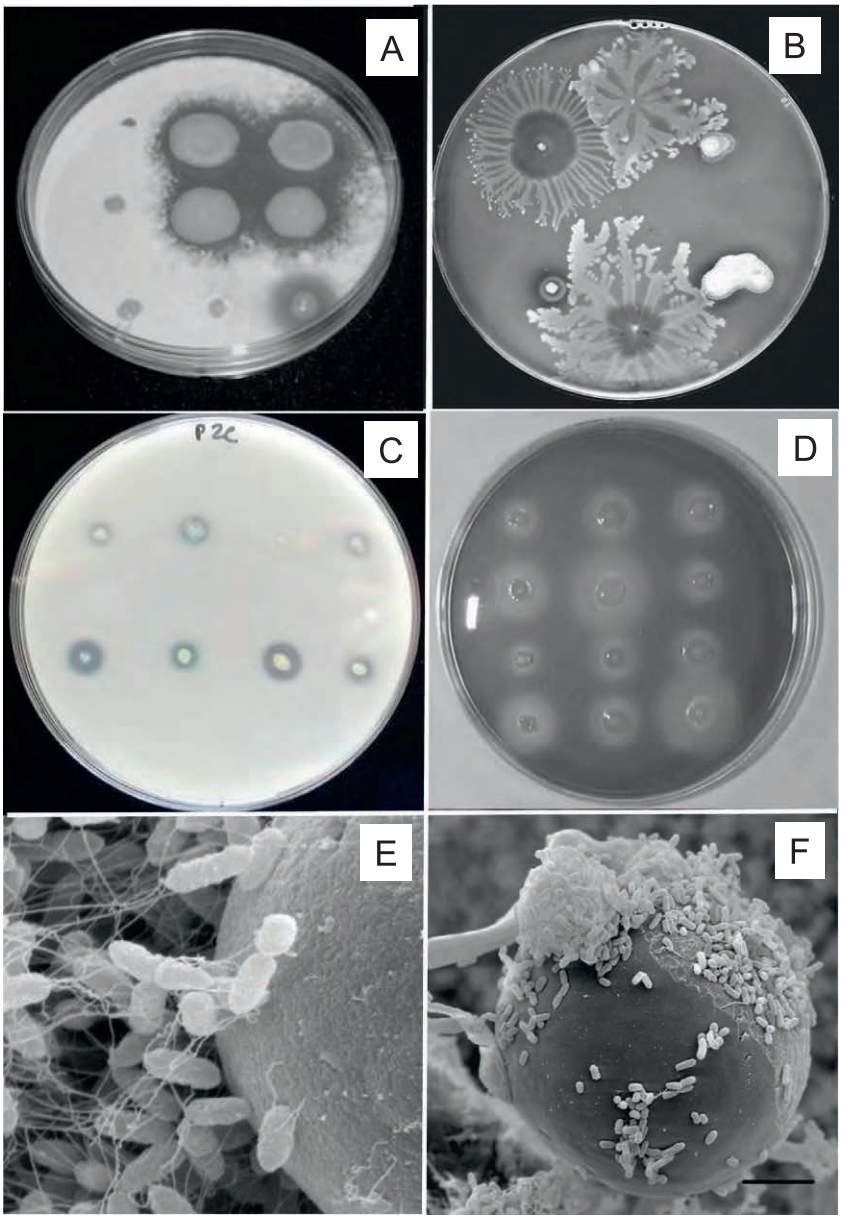

Fig. 4 Antagonism against plant pathogens can be revealed in vitro. (A) Halus of inhibition of confluent growth of a fungus produced by colonies of selected antagonistic bacteria and yeast. (B) Antibiosis is accompanied by swarming motility of the colonies of the antagonist bacteria against a plant-pathogenic bacterium. (C) Production of lytic enzymes that hydrolyze chitin, a component of the fungal cell wall that has been added to the agar plate. (D) Synthesis of siderophores around colonies that are involved in competition for iron. (E) and (F) Direct interference and hyperparasitism due to attachment of bacterial cells to the spores of fungi.

Discovery of Microbial Pesticides

The process of discovery and development of a microbial biopesticide consists of two stages. The first stage deals with the isolation of candidate microorganisms in pure culture, the identification and characterization of strains, and the measurement of efficacy in laboratory-controlled bioassays to finally perform pilot testing under greenhouse or field conditions. In the second stage, candidate microorganisms are cultivated at a large scale, preserved and formulated for industrial production, and submitted for toxicological and environmental impact studies to qualify for registration and authorization for commercial delivery (Fig. 5).

Finding bacteria, fungi, or virus able to interfere with the biological cycle of selected plant pathogens requires proper sampling to increase the probability of obtaining useful strains. For this reason, samples are taken at places or containing materials where there is evidence of beneficial microorganisms, such as in disease-suppressive soils or healthy plants or plant parts that survived the disease in epidemic areas.

The screening for antagonistic activity against the target pathogen is a critical step because the type of microorganism selected depends on the method used. Approaches based on host-mediated selective enrichment and phenotypic or molecular

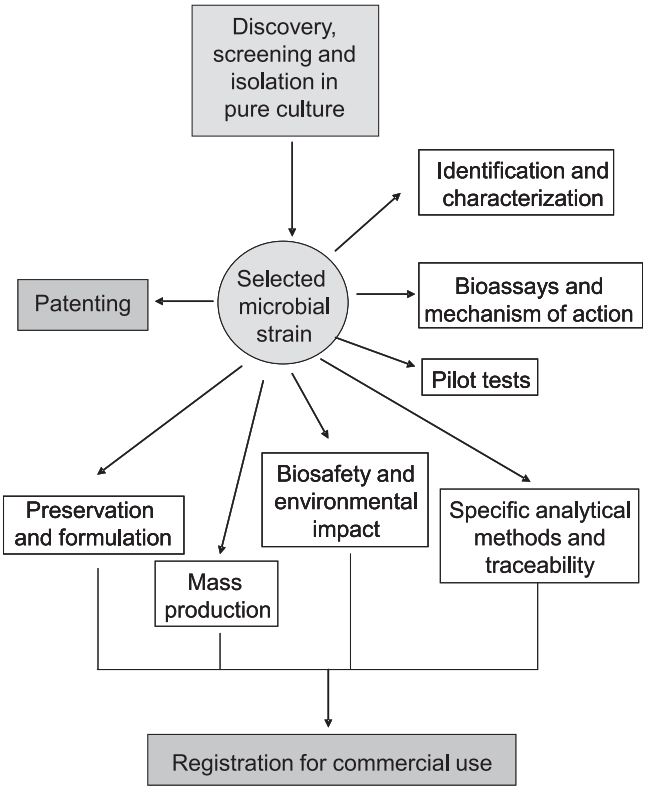

Fig. 5 Steps involved in the discovery of microbial pesticides that ends up with patent filing and registration and authorization for commercial delivery of formulated products.

marker-assisted selection have improved the isolation and development of new biocontrol agents. Usually, screening procedures are specific to a given mechanism of action, such as antibiosis, competition, or induction of plant defenses. Isolation in pure culture needs a suitable method of cultivation in synthetic media or of enrichment in a given biological system (cell cultures or trap organisms).

At this stage, several hundreds or thousands of isolates are collected, and after identification the strains are submitted for efficacy bioassays in a small-scale controlled laboratory environment using in vitro, ex vivo, or, in planta tests in three-partner model pathosystems (putative biological control agent, target pathogen, host plant) (Fig. 6). The knowledge of the mechanisms of action of a biological control agent and the information derived from partially or totally sequenced genomes can provide data on gene targets that can be used for high-throughput screening procedures. Modern techniques of assisted selection using phenotypic or genotypic markers contribute to improving the productivity of the screening stage. Advances in DNA arrays or screening of metagenomic libraries of cloned DNA can also contribute to developing potent screening methods. During the selection process, apart from efficacy and consistency of results between bioassays, additional criteria, such as the presentation of a low effective dose, the production of specific metabolites against the target pathogen, and tolerance to some pesticides commonly used in agriculture, are used to retain isolates.

Pilot tests are conducted under conditions as close as possible to the environment under which biological control will be practiced. During this stage, the biocontrol agent is confronted with several pathosystems to verify its action spectrum and under environmental conditions sufficiently diverse to guarantee a wide range of applicability. The high number of different conditions to be tested permit working with only those isolates that were selected as suitable candidates in the previous massive screening stage. Unfortunately, work on most biological control agents discovered concludes with this step because of a too narrow action spectrum, inconsistency of results between trials, or a low efficacy under field or practical conditions (Fig. 7).

The discovery of biopesticides based on microorganisms, if successful, is of economic interest because of the possibility of commercial exploitation of the technology. Several hundreds of patents exist involving strains of microorganisms with potential application as microbial pesticides, including fungicides, insecticides, herbicides, and plant growth stimulants.

Production and Formulation

For the commercial development of a microbial pesticide, the microorganism cells have to be produced at the industrial scale, preserved, and formulated for storage and delivery. Depending on the nature of the microorganism (bacteria, fungi, yeast, or

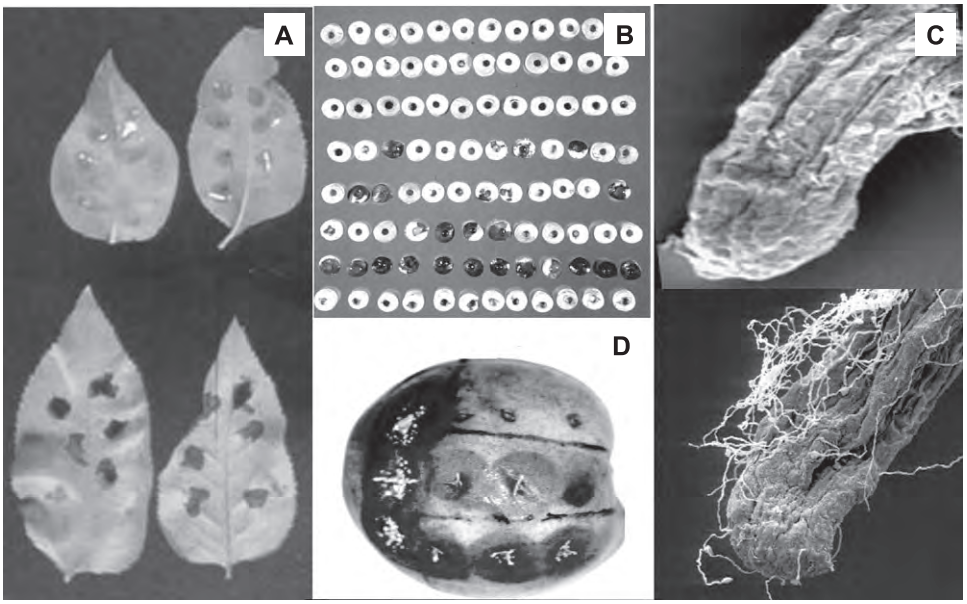

Fig. 6 Pathogen infection in a plant host is controlled by microbial pesticides. (A) Inhibition of brown spot infections on pear leaves that have been treated with an antagonistic bacterium (upper left panel) in comparison to a non-treated control (lower left panel). (B) Control of fire blight infections in immature pear disks by several strains of antagonistic bacteria (rows). (C) Inhibition of fungal infections in strawberry roots by antagonistic bacteria (upper right panel) in comparison to a non-treated control (lower right panel). (D) A pear fruit wounded, treated with antagonistic bacteria, and inoculated with spores of the blue mold shows strains that inhibit rot.

virus), the methods used for industrial scale-up are solid- or liquid-phase fermentation. Methods consist essentially of the technology used in the pharmaceutical and food industry. Bacteria and yeast are produced by liquid fermentation using bioreactors, but many fungi are fermented by using solid-state procedures. The objective is to obtain the highest yield possible using a culture medium with the lowest cost, often achieved using food-grade molasses, peptones, brewer's yeast extract, and protein hydrolysates for industrial use. After liquid fermentation the microbial cells are concentrated by filtration or centrifugation, and in solid fermentation the material is recovered and homogenized to get a concentrated liquid suspension of cells or spores. In cases in which the microorganism is not directly culturable in synthetic media (bacteriophage), industrial production consists of an initial step in which the host bacterial cells are produced and a second step of virus scale-up feeding on the previously produced bacteria. Concentrated suspensions obtained generally range from 10^8 to 10^{10} cfu mL^{-1}, depending on the nature of the microorganism (Fig. 8).

The storage and preservation of the microbial pesticide to increase its shelf-life is one of the main limiting factors for commercialization. The procedure involves stabilizing the viability of cells in a liquid-phase suspension maintained under refrigeration or frozen in the presence of cryoprotectant substances, or as a dehydrated product. Dehydration is the best from a practical point of view because it permits optimum storage conditions, handling, and distribution. Lyophilization is a method that can maintain better viability, but the cost is very high. Encapsulation of microbial cells is also used, in which the cells are mixed with a matrix-forming material such as gelatinized polysaccharides or emulsioned in a lipid material that is finally diluted and spray-dried to obtain the desired particles. At the industrial level and in order to obtain a low-cost product, the method preferred is spray or fluidized bed drying. However, spray drying produces a high loss of viability in some microorganisms (eg, Gram-negative bacteria) due to the thermal treatment that accompanies the process. Although many microbial pesticides are prepared as dried products, some are delivered as liquid suspensions of cells or spores. Irrespective of whether the final preparation is aqueous or dried, the active ingredient is formulated to obtain a marketable product. The formulations are composed of an active ingredient (microorganism cells or spores and sometimes culture components), carriers or inert materials used to support and deliver the active ingredient to a target, and adjuvants that promote and maintain the functionality of the active ingredient by including nutrients, chemical agents that filter ultraviolet light radiation, or osmoprotectant compounds that prevent damage from cell desiccation. However, care must be taken because certain components in the formulation can stimulate the pathogen and promote disease. In many cases, the technology for formulating biopesticides is similar to that used for formulating chemical pesticides and pharmaceutical products, and results in stable and active products for several months.

Liquid formulations contain around 10^7–10^9 cfu mL^{-1}, whereas powdered products have the active ingredient generally at a higher concentration (around 10^9–10^{10} cfu g^{-1} for bacteria and yeast), but can be lower in certain fungi. The method of application depends very much on the pathosystem to be controlled. Spraying or drenching plants is a common practice when using an inundative approach of control. However, inoculative or augmentative strategies consist of preparing sticks or

Fig. 7 Control of blue mold rot in apples (a), brown rot in peach (b) and potato root-rot disease (c) with antagonistic bacteria. Nontreated controls are shown on the left side and treated fruits on the right side.

immobilizing systems (alginate beads) and seed coating or bacterization of the root system in seedlings before transplanting. Inundative application methods are essentially the same as used for chemical pesticides. In this strategy, the dose of biocontrol agent has an important effect on the efficacy of control, because the latter depends on the relative amounts of pathogen and biocontrol agent. For many microbial pesticides, the median effective dose (ED50) is in the range of 10^6–10^7 cfu mL^{-1} for bacteria and 10^5–10^6 cfu mL^{-1} for fungi and yeast. The ratio between cells of the biocontrol agent and cells of the pathogen for optimum control has been used as a measure of efficiency, which ranges in value from one to several hundreds. The efficiency of biocontrol is also dependent on pathogen aggressiveness and host supportiveness. Therefore, to ensure covering a wide range of conditions of applicability in practice, a dose of around 10^8 cfu mL^{-1} is recommended for bacterial biocontrol agents and 10^7 for fungi or yeast. Accordingly, formulated products are diluted in nonchlorinated water to get the dose recommended.

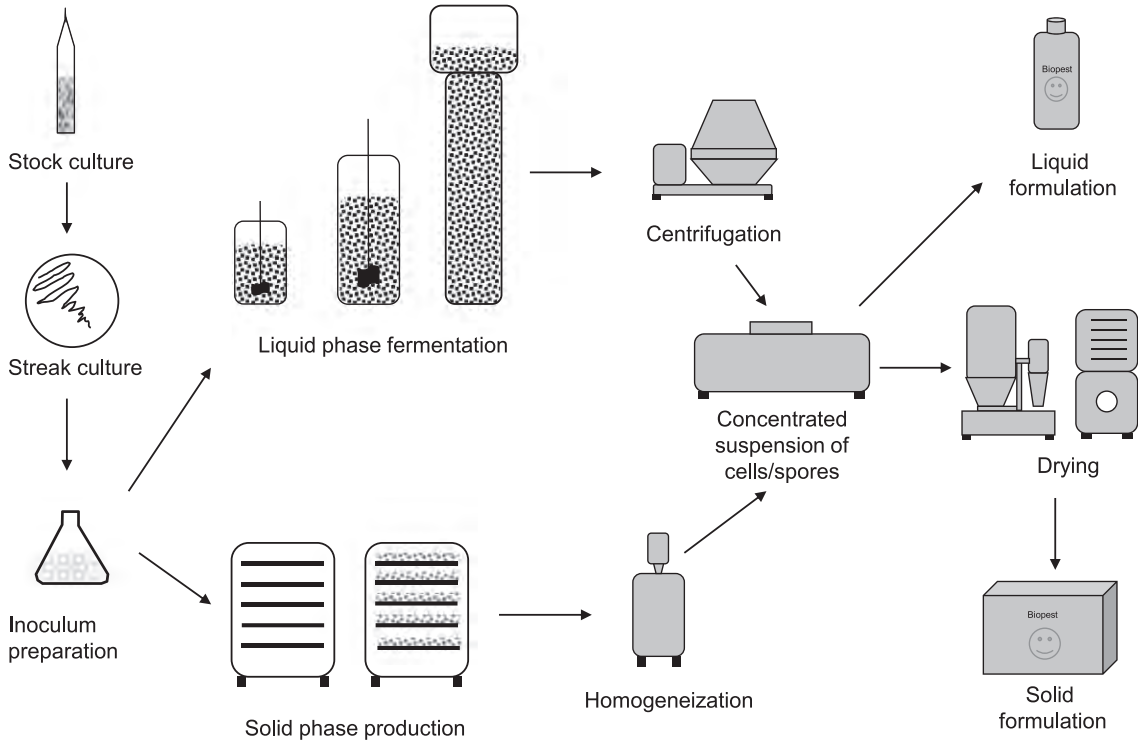

Fig. 8 Industrial production of microbial pesticides consists of scaled-up fermentation of a suitable strain, downstream processing of the biomass and supernatant of cultures, and formulation for commercial delivery.

Monitoring, Environmental Impact, and Biosafety

Specific analysis of the microorganism strain of the biopesticide is necessary for quality control during production and formulation and also to perform studies on traceability, residue analysis, and environmental impact. In field studies, the classical microbiological methods may not be suitable because they do not distinguish the strain that contains the microbial pesticide from the same species that could be present in the natural microbiota associated with plants. In certain cases, selecting an spontaneous mutant resistant to a suitable antibiotic for which it is rare to find resistance in nature is sufficient. However, genotypic markers are preferable because they are more stable and their expression does not depend on the type of culture media used for analysis. Specific genotypic markers can be found by DNA fingerprinting of the biocontrol agent strain, based on the amplification of gene sequences by PCR methods (RAPD, REP, ERIC, AFLP), or by digesting DNA with restriction enzymes (RFLP, PFGE). Genome sequencing is currently the best option due to the decrease in cost and the possibility to analyze several strains of the same species. The comparison of genome sequences and fingerprinting patterns of the biocontrol strain with those of a wide collection of representative strains of the same species can identify specific regions or bands that can be sequenced and characterized (SCAR fragments). The knowledge of the nucleotide sequence of SCAR fragments can aid in the design of primers for PCR analysis. Real-time PCR (quantitative PCR) is a powerful tool for developing methods of highly specific quantitative analyses of strains of the active ingredient in microbial pesticides under natural conditions.

Another important issue that has to be taken into consideration in microbial pesticides is biosafety. Biosafety is necessary to avoid nontarget effects on plants or animals, or on the environment, because the isolation of a given microorganism from plants is not a proof of its inocuity. Toxicological studies in mammals, including acute toxicity tests, are performed to guarantee safety to consumers and handlers of the microbial pesticide, especially if cases of opportunistic infections reported at the clinical level or if certain secondary metabolites of concern are produced. However, risk evaluation is not a simple task, because the risk of non-target effect by a given microorganism is estimated from the intrinsic toxicity–pathogenicity level, the degree of exposure, and the susceptibility of the possible receiver. Most microbial pesticide strains meet all biosafety rules. However, in a few cases, there are uncertainties regarding the potential risk, because human isolates reported in clinical opportunistic infections are not distinct from environmental isolates. There is scientific controversy regarding the use of strains of *Burkholderia cepacia*, *Pseudomonas putida*, and *P. agglomerans*, as biological control agents, which will need further studies of safety and toxicological risk. Interestingly, some of these microorganisms are part of the normal microbiota found in plants and are present in several plant-derived fresh products. These uncertainties are the reason for specific and differing regulations on risk classification of microorganisms among countries.

Registration and Commercialization

Approval of microbial pesticides for commercialization requires detailed dossiers accounting for scientific data on microorganism identity, biological properties, efficacy, specific analytical methods, residues, traceability, and potential adverse effects on human health and nontarget organisms. In the European Union, the registration and authorization of microbial pesticides as plant protection products depends on the Health and Food Safety Directorate, and is regulated by the Directive 2009/128/EC and the regulation 1107/2009/EC. In the United States, authorization is regulated by the Environmental Protection Agency. Furthermore, specific requirements may change when an authorization application considers the microorganism as a biocide or a plant enhancer. Therefore, the availability of commercial biopesticides depends on the specific regulations in each country, but several products that include bacteriophage, bacteria, yeasts, and fungi, which are active against a wide range of plant pathogens in very diverse crops and agroclimatic conditions, are marketed (Table 3).

Table 3 Some examples of commercial microbial pesticides

Active ingredient	*Commercial name*	*Target pathogen/disease*	*Host*
Agrobacterium radiobacter	Nogall, Galltrol-A	Crown gall	Fruit and nut trees, ornamentals
Ampelomyces quisqualis	AQ10	Powdery mildew	Apples, cucurbits, grapes, ornamentals, strawberries, tomatoes
Aspergillus flavus	AF36[a], Aflaguard, NRRL21882[a]	*A. flavus*	Cotton, peanut, corn
Aureobasidium pullulans	Blossom protect	Fire blight	Pome fruits
Bacillus licheniformis	Green releaf[a], 145F[a] Ecoguard	Several fungal diseases of seedlings	Ornamental plants, conifers and tree seedlings, ornamental turf
Bacillus mycoides	BmJ	*Cercospora beticola*	Sugar beet
Bacillus pumilus	Ballad, Yield shield[a], Sonata	Soil-borne fungi, powdery and downy mildew, early and late blight, brown spot,	Soybean, cucurbits, grapes, hops, vegetables, peanuts
Bacillus subtilis	Serenade, Rhapsody, Biopro[a], BS-F4, Promix, Subtilex, HiStick N/T, Companion, Kodiak[a], Clarity, Cease	Bacteria (fire blight) and fungal root diseases, *Alternaria*, *Aspergillus*, gray mold	Grape, hops, vegetables, peanuts, pome and stone fruits, soybean, alfalfa, cotton
B. subtilis var. amyloliquefaciens	Taegro, Tae-Technica[a], Double Nickel, Bacstar	Soil-borne fungal rots, Fire blight	Greenhouses shade and forest tree seedlings, ornamentals, and shrubs
Bacteriophage	Agriphage	Bacterial blight and spot	Tomato, pepper
Candida oleophila	Aspire[a], Nexy[a]	Postharvest fungal rot	Citrus and pome fruits
Coniothyrium minitans	Contans, Koni[a]	*Sclerotinia*	Vegetables, canola, oilseed rape, sunflower, soybean
Cryptococcus albidus	Yield plus	Postharvest fungal rot	Pome fruits
Gliocladium sp.	Gliomix	*Rhizoctonia solani*, *Pythium*	Vegetables and turf
Gliocladium catenulatum	Primastop[a], Prestop	Soil-borne fungal pathogens	Ornamental, vegetable, and tree crops
Gliocladium virens (Trichoderma virens)	Soilguard	Soil-borne root fungal diseases	Ornamentals and food crops in greenhouses and nurseries
Metschnikowia fructicola	Shemer	Fungal rot	Preharvest and postharvest fruits
Pantoea agglomerans	Bloomtime, Blossom bless,	Fire blight	Pome fruit trees, ornamentals
Pantoea vagans	Blightban C9-1[a]	Fire blight	Pome fruit trees, ornamentals
Phlebiopsis gigantea	Rotstop	*Heterobasidion annosus*	Coniferous trees
Pseudomonas aureofaciens	Spot-less[a]	Antracnosis	Turfgrass
Pseudomonas chlororaphis	AtEze[a], Cedomon, Cerall	Soil-borne fungi	Greenhouse crops and cereals
Pseudomonas fluorescens	Blightban A506	Fire blight, frost damage	Fruit trees, almond, potato and tomato crops
Pseudomonas syringae	Biosave10 LP, Biosave 11 LP, Esc-10[a]	Postharvest fungal rot	Pome fruits, citrus, cherries and potatoes
Pseudomonas sp	Proradix	Fungal and bacterial diseases	Potatoes
Pseudozyma flocculosa	Sporodex[a], Pseudozyma[a]	Powdery and downy mildew	Greenhouses roses and cucumber
Pythium oligandrum	Polyversum, DV74[a]	Soil-borne pathogenic fungi	Food crops, ornamentals and turf
Streptomyces griseoviridis	Mycostop	Root rot, gray mold and foot decay	Ornamental and vegetable crops
Streptomyces lydicus	Actinovate	Root rot, gray mold and damping-off	Greenhouse and nursery crops, turf

(*Continued*)

Table 3 Continued

Active ingredient	*Commercial name*	*Target pathogen/disease*	*Host*
Trichoderma asperellum and Trichoderma gamsii	Asperello, Biotam, Bioten, Radix, Remendier, Tenet	Fungal diseases	Greenhouse crops
Trichoderma harzianum	Binab T, Trichopel, T-22, Rootshield, PlantShield, Tusal, Trichodex[a], BiodermaH/Barrier	Root rot, gray mold and damping-off	Trees, shrubs, transplants, ornamentals, vegetables
Trichoderma polysporum	BinabT	Fungal infections through wounds	Ornamentals, shade, and forest trees
Trichoderma viride	Bioderma/Protector	Fungal diseases	Cotton, Cereals, Pulses, Vegetables, Oilseeds, Fruit
Ulocladium oudemansii	Botry-zen	Gray mold	Grape
Verticillium dahliae	Dutch Trig	Dutch elm disease	Elm

[a]Registered but currently not commercialized biopesticides.

Further Reading

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Phage Therapy

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A Bacterial Problem

Behind cardiovascular disease and cancer, infectious diseases are the third leading cause of human mortality. Civil engineering, vaccination, and antibiotic therapy, the trinity of antibacterial technologies, are directly correlated with a reduction in the worldwide impact of infectious diseases. But there is concern heading into the future about the continued effectiveness of these measures at lowering morbidity and mortality. First, the rise of antibiotic resistance has reached a tipping point. There are strains resistant to most if not all clinically prescribed antibiotics, including recently approved versions. The development of new antibiotics has not kept pace with the evolution of resistance. As a result, leading health organizations project that by 2050 millions of people will succumb to previously preventable infections, and at staggering costs.

Second, a flurry of successful vaccine approaches developed in the middle of the 20th century, mostly centered on the use of bacterial toxins to induce adaptive immunity, have made little progress in recent times. The "one-toxin, one-disease" paradigm may not be true for some bacterial species that demonstrate multiple virulence mechanisms and readily engage in the horizontal transfer of virulence genes. Thus, vaccines that demonstrate universal efficacy against multiple strains of the same species might not be feasible unless these strains share common virulence mechanisms.

Third, the recent emergence of the role of the microbiome in inflammation and chronic diseases, including those not historically thought of has having an infectious etiology, implies that even civil engineering approaches alone are unlikely to further reduce carriage of disease-inducing bacteria. Most of these microbes live in and on us, making sterilization all but impossible; and, many of them are adapted to the human immune system, thereby making vaccination more challenging.

These three facets, when considered as pieces of a larger problem, imply "outside the box" antibacterial approaches will be necessary to make further gains in the war on bacteria. One such approach is the use of phage, natural-born killers of bacteria, to destroy problematic strains. Phage may be the most diverse, and numerous, of any self-replicating entity on Planet Earth, representing an essentially unlimited supply. They too can quickly evolve and in clinically-relevant time frames. Thus, new strains of antibiotic resistant or virulent bacteria that emerge, perhaps strains where vaccination might not be realistic, can be killed with new strains of phage. Because phage can be targeted to a specific bacterial host, their use in "editing" the human microbiome to rid undesirable bacteria, either from the intestine or skin, may also prove useful in reducing bacterial burden in high risk individuals. Phage can (and are) used in agriculture and food processing to eliminate bacterial pathogens. Thus, they may spare the use of excess antibiotics, thereby decreasing resistance. The history of phage therapy is one of highs and lows. Only recently have researchers attempted to define the limits of these amazing antibacterial machines in models of infection.

What Is a Phage?

Any treatment of the topic of phage therapy should first begin by defining phages, their mechanisms of bacterial destruction, where we find them, and how they are isolated. If phage are to become commonplace in the worldwide clinical sector, not only will this knowledge be needed by healthcare personnel, the details of these properties will shape their regulation and use.

First and foremost, phage are replicating viruses that house nucleic acid in their proteinaceous head. This nucleic acid must be injected into the bacterial cytoplasm. There it encodes the proteins necessary to make new phage progeny. The progeny are released after the bacterial cell is lysed, a step induced by the phage proteins themselves. Phage that lyse bacteria immediately after the assembly of new phage in the cytoplasm are called lytic phage. Lytic phage are best exemplified by T4 (Fig. 1). The other component of the phage, the tail, is responsible for engaging the bacterial surface. This usually occurs via the binding of phage proteins at the end of the tail, called tail fibers, to specific receptors on the bacterial surface. After binding, the nucleic acid in the phage head is squeezed through the tail like a plunger pushing fluid through a syringe. The process of engaging the bacterium in a way that leads to injection of the nucleic acid into the bacterial cytoplasm is termed adsorption. The injected nucleic acid encodes all proteins needed to make new phage, including head and tail proteins, polymerases, and other accessory factors needed for assembly and enzymatic function of each phage-packaging step. After new phage are produced in the bacterial cytoplasm, specific protein holins and lysins, also encoded by the phage, begin to degrade the bacterial membrane and peptidoglycan, respectively, often referred to as "lysis from within." When considering phage therapy, the events that result in a production infection and death of the bacterial cell are critical.

Lytic phage contrast with what are termed lysogenic phage, best exemplified by lambda (λ). Following infection, lysogenic phages integrate their genome into the bacterial chromosome, a process called lysogeny. The phage genome then replicates along

*These authors contributed equally to this work.

with the bacterial genome every time the bacterium divides. The phage at this point is referred to as a prophage. The prophage can, under very specific conditions, excise its nucleic acid out of the bacterial genome, make phage progeny, and generate a lytic event to release the new phage. In general, lysogenic phage should be avoided in phage therapy, for reasons discussed below, but mainly because their infection too infrequently leads to lysis of the bacterial cell. Finally, it is possible for phage to induce bacterial cell lysis at high multiplicities of infection. The overproduction of lysins during multiple adsorption events leads to bacterial lysis without productive infection, a process termed "lysis from without."

Phage are everywhere. Estimates of their total abundance on Earth includes the number one with greater than 30 zeroes after it, making it the most numerous replicating entity on the planet. A general rule of thumb is that wherever bacteria are found, so too will phage. They are present in high concentrations in puddles of water, the soil, ponds and lakes, and even growing vegetation. They are in the air, being lifted by strong winds on dust particles. Even man-made objects can have phage on their surface, especially if they come into contact with people. Our own gastrointestinal tract is rich in phage; human stool, as a component of usually sewage, is often a source of new phage, especially those targeting human-adapted bacteria. Even treated tap water can have millions of phage in a single glass. The constant exposure to phage in all aspects of our life means that we are well adapted to them.

For many phage, their isolation is inexpensive and not technically challenging. This is one of their remarkable properties. What might amount to a few hundred dollars in material costs, a purified and highly active phage preparation can be attained in about a week's worth of time. The process begins by identifying a source. Not all sources contain the phage one might be interested in, so it is important to have an idea of the type of bacterium you want to infect. For example, bacterial pathogens that commonly infect the human gastrointestinal tract, such as shigella, salmonella or *Escherichia coli*, are common in human and animal stool, or the environment where human waste may run off. Thus, phage that efficiently kill these bacteria are highly abundant in these sources. A sample is usually collected, mixed with an appropriately buffered solution, and the bulk material pelleted. The resulting supernatant is often passed through a filter that will retain most sizable particles and cells but allow the smaller phage to pass through. This filtrate can next be added to an agar lawn of a test bacterium that is likely to support an infection (from the sewage example above, it may be *E. coli*), and the next day the plate is examined for plaques. A plaque is a circular clearing of the cloudy bacterial lawn in the area where the filtrate was applied. It suggests a phage in the filtrate lysed the bacteria on the plate, creating a zone of clearance, analogues to the same effect observed with antibiotics. Plaques can then be scraped into a fresh culture of growing bacteria which amplifies the number of phage progeny trillions of fold. These released phage can then be further purified using density gradient ultracentrifugation to yield high-titer preparations with efficient killing activity.

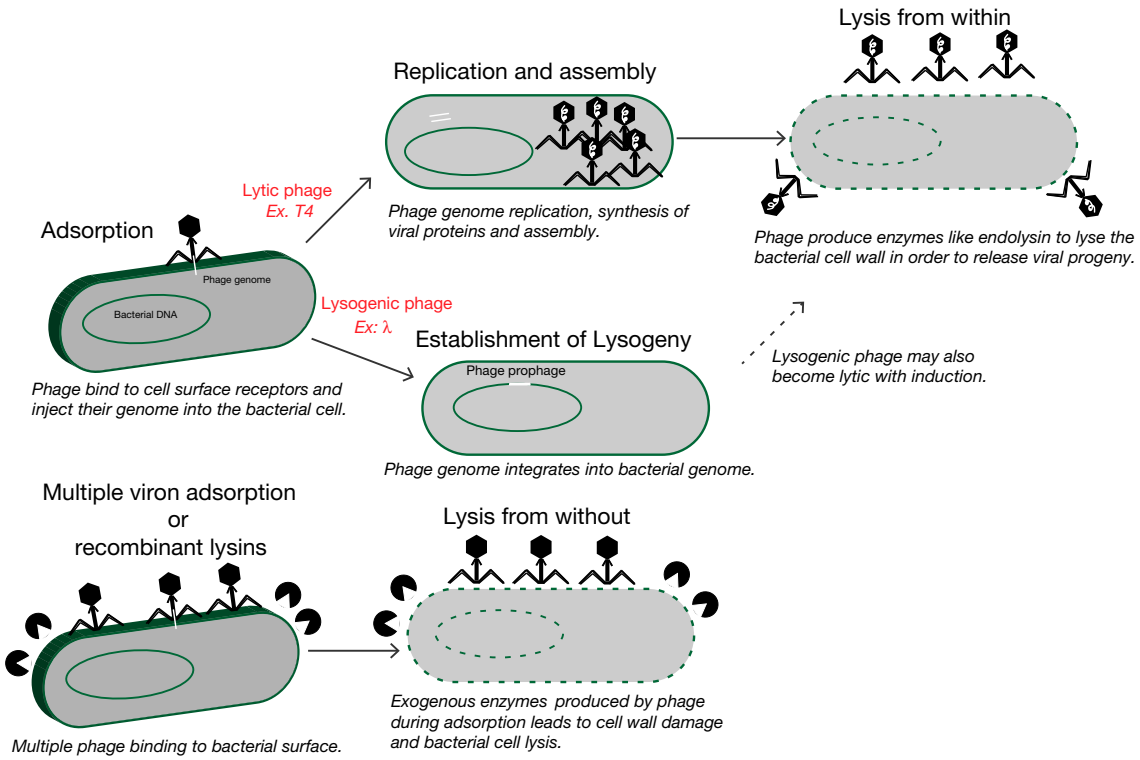

Fig. 1 *The phage life cycle.* The stages of phage infection are shown for both lytic and lysogenic phages. Phage products can also be used to lyse bacterial cells and themselves may constitute viable antimicrobials.

Phage Therapy: A History

(i) The Classical Years (1915–1942): Discovery and Early Hope

Phage therapy represents the therapeutic use of viruses to treat bacterial infections. Bacteriophages were likely first described in 1896 by British scientist Ernest Hankin. In Hanklin's published work in the *Annals of the Pasteur Institute*, he reported that some biologicals found in the Ganga and Yamuna rivers in India had the capacity to kill cholera-inducing bacteria. Whereas it is not known for sure if Hankin was describing phage (he did not isolate these biologicals), the idea that environmental sources contained antibacterial activity was enticing. Almost two decades later, William Twort (1915) and Félix d'Herelle (1917) independently discovered what we now know as bacteriophages. Twort attempted to follow Koch's postulates to isolate the antibacterial activity and demonstrate they maintained their bacterial killing ability in a new culture. D'Herelle, a French-Canadian microbiologist, isolated bacterial viruses from stool samples of patients who suffered from shigellosis. He demonstrated that these viruses were able to lyse Shigella in culture media, and coined the term "bacteria-eaters" or bacteriophages. Further, he treated shigellosis with phage in animal subjects, the first attempt at phage therapy. The discovery of phage spawned a new era of microbial research (Fig. 2).

D'Herelle continued his research into bacteriophages and traveled the globe popularizing these new findings. In 1919, a field trial using phages against *Salmonella gallinarum*, which causes avian typhosis, was performed in France. In the early 1920s, there were case reports of the administration of phage in the US to treat chronic furunculosis. The pharmaceutical company Eli Lilly developed a phage preparation to treat *Streptococcus* and *Bacilli* infections. D'Herelle's work led him to cofound with George Eliava an institute (which bears Eliava's name) in what is now the Democratic Republic of Georgia to carry out basic phage research and to use phage to treat bacterial infections in people. The Eliava Institute continues to this day to provide phage and treatments to thousands of patients from around the world. This period, circa 1915–1942, can be referred to as the classical period for phage therapy. During this time, phage were discovered, tested, and the beginning of their long-term development as antimicrobials, with the formation of companies around their distribution was underway. This would change quickly after the synthesis of penicillin in 1942.

The Silent Years (1943–2000): The Antibiotic Displacement and the Covet of the Curtain

On September 28, 1928, Alexander Fleming noticed the growth of *Staphylococcus* on a Petri dish was inhibited by what appeared to be a contaminating mold. Fourteen years would pass before the compound responsible for this inhibition, penicillin, was synthesized by Howard Florey and team and produced to scale to treat human patients. This remarkable discovery ushered in the era of antibiotic use, a golden age of medicine that largely continues to this day. The meteoritic rise of antibiotics all but eliminated the interest in the development of phage as clinically-used antibacterial agents, especially in the West. Major companies hitched their programs on the antibiotic train eventually yielding seven major classes of antibiotics encompassing greater than a hundred different compounds. Although largely discarded by the West as drugs, the rise in the biomedical research sector in the late 1940s in the United States and Europe fueled a strong interest in the use of phage as elegant model systems for the study of genetics, mutation, inheritability, and evolution. In fact, it was phage that were used to prove that DNA was the "transforming principle," a finding so revolutionary that many scientists adopted the use of phage in their laboratories. This work generated a wealth of information about the infectious and lysing properties of phage, and this knowledge helped to understand the limits and possibilities of phage therapy.

But in the East, phage therapy quietly pressed onward. The Eliava Institute in Tbilisi, Georgia continued to develop therapeutic phages, but perhaps because the knowledge and technology to make antibiotics was slower to spread to the East during the Cold War, phage therapy persisted in nearby countries that were a part of the Soviet bloc. In Poland, for example, the Hirszfeld Institute, which was formed in 1952, developed a strong record in the treatment of suppurative infections. To this day, Poland has an active program whereby patients can be evaluated to determine if they are good candidates for phage therapy, and, if so, can be treated

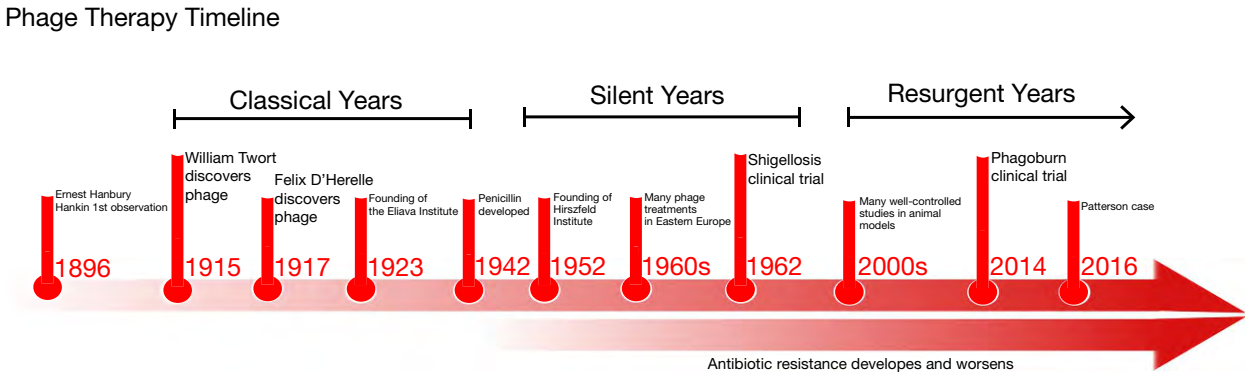

Fig. 2 *A timeline for phage therapy.* Phage therapy has progressed through three generalized eras characterized by discovery, quiescence, and revitalization. The current era, that of revitalization, is characterized by well-controlled studies in animal models of infection and an increase in the popular press reporting of some successful compassionate care cases in humans.

appropriately. Much of what we know of phage therapy in human patients comes from this period. There are numerous publications in either Russian or Polish journals. Most of the major themes discussed during the present time regarding phage therapy were to some degree also addressed during this period. This includes the use of oral phage to treat gastrointestinal infections, including shigellosis and salmonellosis. One study on this front, which occurred in the country of Georgia, stands out. More than 30,000 children were enrolled in a phage treatment trial to test the effect of a phage preparation directed against Shigella. Children on one side of the street were administered oral phage one time per week, while children on the other side were given the placebo. Weekly health checks were performed. The children that received phage were approximately three times less likely to demonstrate symptoms of dysentery.

Other phage preparations seemed to show efficacy in the treatment of localized or topical infections caused by *E. coli or Staphylococcus*, *Streptococcus*, and *Pseudomonas* spp. This included ulcers, surgical wounds, surface abscesses and other purulent infections. Yet other treatments focused on more invasive infections, including the lung, principally those caused by Staphylococcus and Pseudomonas spp. The comparison of phage therapy to antibiotic therapy was often performed, with phage in several cases apparently demonstrating superior efficacy; and, the combinatorial use of phage with antibiotics was reported to be more effective than antibiotics alone. Even human studies examining the production of neutralizing antibodies to phage was studied with overall a low incidence of neutralization reported. The language barrier, along with the fact that many trials and tests in these Eastern Bloc countries may not have been published, makes it difficult for the average Western scientist, let alone citizen, to critically evaluate the efficacy of phage therapy during this period. Nevertheless, there certainly is strong precedence for the use phage in treating human infections, an approach that continues to this day in this region.

The Resurgent Years (2000-Present): The Antibiotic Precipice and Renewed Hope

Within a year of the introduction of penicillin in 1942, bacterial strains resistant to the drug emerged. The generation of new antibiotics, along with entirely different structural classes of antibiotics distinct from penicillin in structure and mechanism of action, would continue to save countless lives. But as soon as a new class or version of antibiotics was heavily used, within a few years, resistance to that antibiotic would emerge. Resistance is now widespread, and new antibiotics to fill the gaps have not emerged at a pace sufficient to eliminate concern. Thus, in the last ~15 years or so, the conversation has once again shifted back to phage, a sort of second awakening for the idea of phage therapy. As a result, we are slowly learning more about the properties of phage as a dynamic treatment administered into a complex multiorgan being to treat bacterial infections, especially in the context of experimental animal models. With knowledge in this area expanding faster than ever, the well-controlled experiments that probably should (or would) have been done in d'Herelle's time are now just being formulated.

This third period in phage therapy is characterized by one important distinction: well-controlled studies in experimental models of infection. Efforts like this have generated a well-spring of useful information from everything from the breadth of possible bacterial infections treatable with phage to the parameters and variables that alter the experimental outcomes. These studies have addressed common nosocomial infections that have the potential to cause bacteremia, including the culprits *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, as well as infections of the urinary, respiratory, and gastrointestinal tract. Major routes of administration and various doses have been tested, most with phage belonging to the genera Myoviridae, Podoviridae, and Siphoviridae. What is clear from these studies is that the intensity of investigation using controlled models is increasing, and, generally, phage are demonstrated to be effective at reducing bacterial burdens and improving the health of test subjects. A summary of some of the more comprehensive of these studies is shown in Table 1. This work, in combination with the medical community being at the precipice of the antibiotic resistance cliff, has led to even more adventurous attempts to use phage in the treatment of human infections in the West. In Europe, a large multinational clinical trial to assess the efficacy of phage killing *P. aeruginosa* on burn wounds (phagoburn) represents an important step in the West's acceptance of such an approach. The recent first-in-human systemic and successful administration of phages since the time of d'Herelle in the United States to clear an *Acinetobacter baumannii* infection, the so called Patterson case (*Antimicro. Agents Chemother*. Oct 2017), is also evidence of the growing willingness to move this technology forward. In this case, phage were administered to a male patient with systemic *A. baumannii* who was undergoing organ failure. The patient recovered quickly after phage treatment and is currently resuming a normal life. This case received much media attention and highlighted the utility of phage when all other approaches had been exhausted.

Dynamic Properties of Phage as an Adaptable Antimicrobial

Killing Activity Is Genetically Encoded

Perhaps the single greatest virtue pathogenic bacteria possess is mutability. Among a population of billions, it is theoretically possible that a single bacterial cell might acquire a mutation that confers a survival advantage against the backdrop of a strong selective pressure; this cell can then divide and become the founder of a new population, the progeny having inherited the beneficial trait. Take resistance to antibiotics as an example. It takes years to screen, identify, characterize, and test a new experimental compound in clinical trials, yet once introduced into the clinic or agriculture, resistant strains can emerge almost instantly. The power of this mutability emanates from four main mutation-acquiring strategies, and bacterial strains capable of all four reign supreme in overcoming medical measures designed against them. We can call this four-way intersection the mutagenic tetrasect, being comprised of conjugation (exchange of DNA between two bacterial cells via direct contact), transformation (uptake of

exogenous DNA, usually plasmids, from the environment), transduction (acquisition of DNA via infection with phage), and finally de novo mutation (substitution, excision, or insertion of DNA, usually by stochastic processes). Not all bacteria perform all these functions, some preferring one mechanism over another as an agent of change. But many pathogens of humans, including those that constitute our own microbiome, harbor a vibrant mutagenic tetrasect. With the ability to mutate so readily via these four mechanisms, and the drug-making progress taking years, one wonders how chemically fixed compounds can keep pace with evolution driven by the tetrasect.

At the heart of the killing ability of phage are protein elements that bind and lyse bacteria. Since their assembly is encoded by nucleic acid, they can change rapidly. This inherent changeability stands in contrast to all currently approved chemical antibiotics whose structure is fixed and cannot adapt to new conditions or bacterial strains. Phage are perhaps the only other biological entity on the planet whose replication can keep pace with, even exceed, that of bacteria. This prodigious reproduction levels the arms race. Not only are there more phage types than bacteria, meaning scientists have a nearly limitless supply of existing environmental phages to choose from, but they can also be evolved at an accelerated pace under controlled laboratory conditions. A 3 mL culture of growing bacterial hosts can generate up to 10^{11} phages. Spontaneous mutants can arise in every cycle. Although the vast majority of

Table 1 List of some relevant animal infection studies where phage therapy has been efficacious

Animal model	*Phage (type)*	*Dose; route*	*Pathogen*	*Result*	*References*
Gastrointestinal infection (in mice)	M13 (genetically engineered with *H. pylori*-specific MAbs)	10^7 PFU; Oral	*Helicobacter pylori*	When premixed with infectious agent prevented bacterial growth in stomach	Cao et al. (2000)
Septicemia (in mice)	CK2, 153A-5, and 153A-7	10^8 PFU; I.V.	*Vibrio vulnificus*	Reduced disease, edema, and bacterial CFU in lesions and liver	Cerveny et al. (2002)
Bacteremia (in mice)	ENB-6	10^4–10^9 PFU; I.P.	*Enterococcus faecium*	With high dose, 100% survival	Biswas et al. (2002)
Wound infection (in rabbits)	LS2a	10^5–10^9 PFU; S.C.	*Staphylococcus aureus*	Lower bacterial CFU at infection site and abscess reduction	Wills et al. (2005)
Gastrointestinal infection (in broiler chickens)	NCTC 12669 and 12,671 (*Myoviridae*)	10^9–10^{10} PFU; Oral	*Campylobacter jejuni*	Reduction in caecal colonization (1–3 log)	Wagenaar et al. (2005)
Gastrointestinal infection (in mice)	SP15, 21 and 22	10^8–10^{10} PFU; Oral	*Escherichia coli 0157:H7*	With daily administration, reduction in *E. coli* in feces and gastrointestinal tract	Tanji et al. (2005)
Septicemia (in mice)	KPP10 (*Myoviridae*)	10^{10} PFU; Oral and I.P.	*Pseudomonas aeruginosa*	Increased survival rate with both routes of treatment	Watanabe et al. (2007)
Pulmonary infection (in mice)	BcepIL02	10^9–10^{10} PFU; I.N. and I.P.	*Burkholderia cenocepacia*	Reduction in bacterial density in lungs with I.P. route	Carmody et al. (2010)
Bacteremia (in mice)	A1122	10^9 PFU; I.P.	*Yersinia pestis*	Increased survival rate	Filippov et al. (2012)
Typhoid fever (in mice and chickens)	UAB_Phi20, 78 (*Podoviridae*) and Phi87 (*Myoviridae*)	10^{11} PFU; Oral	*Salmonella typhimurium*	Increased survival rate	Bardina et al. (2012)
Ulcers (in mice)	D29	10^8 PFU; S.C.	*Mycobacterium ulcerans*	Reduction in swelling, ulceration and bacterial burden	Trigo et al. (2013)
Gastrointestinal infection (in rabbits)	ATCC 51352-BI, B2, B3, B4 and B5	10^8 PFU; Oral	*Vibrio cholerae*	Reduction in fecal bacterial CFU and severity of diarrhea	Jaiswal et al. (2013)
Septicemia (in mice)	S13'	10^{10} PFU; I.P.	*Staphylococcus aureus*	Increased survival rate	Takemura-Uchiyama et al. (2014)
Pulmonary infection (in mice)	K56–2 and C6433	Up to 10^{11} PFU; aerosolized	*Burkholderia cepacia*	Reduction in bacterial load in lungs	Semler et al. (2014)
Pneumonia (in mice)	Phi1513 (*Siphoviridae*)	10^7 to 10^9 PFU; I.N.	*Klebsiella pneumonia*	Increased survival rate, decreased weight loss and reduction in bacterial burden	Cao et al. (2015)
Gastrointestinal colonization (in mice)	AL505_P1, P2, P3 (*Siphoviridae*, *Myoviridae* and *Podoviridae*)	10^5 to 10^7 PFU; Oral	*Escherichia coli UPEC*	Reduction in intestinal colonization	Galtier et al. (2016)
Gut-derived Bacteremia (in mice)	HP3 (*Myoviridae*)	10^9 PFU; I.P.	*Escherichia coli ExPEC*	Reduction in bacterial load in organs and disease	Green et al. (2017)
Pulmonary infection (in mice)	PAK_P1 (*Myoviridae*)	10^8 PFU; I.N.	*Pseudomonas aeruginosa*	Increased survival rate	Roach et al. (2017)

I.V. = Intravenous; I.P. = Intraperitoneal, S.C. = subcutaneous; I.N. = intranasal.

these mutants may not acquire new binding or lytic activity (in fact, most mutations are likely to be detrimental to the fitness of the phage), some novel mutations may enhance infectious ability. These mutations fall into three classes; (i) those that enhance binding to the bacterium, perhaps via mutation of the tail fibers, (ii) those that enhance postbinding infection (including adsorption, injection, production of phage proteins, replication and packaging), and (iii) those that enhance lysis. Designing clever ways to generate and select for mutations that enhance these properties could, conceivably, produce a more efficacious antimicrobial, and on the order of hours to days. There is no drug-making pipeline that exists today with such versatility.

Feasibility and Cost Are Low

One advantage phage may possess as an antimicrobial is the low technical know-how needed to find a phage that kills any given strain of bacteria, and at relatively low cost. In 2014, the estimated cost to bring a new drug to market was greater than 2 billion dollars and required approximately10 years of development. This is simply too costly and too long for new chemical antibiotics to be developed to combat the emergence of drug-resistant strains, which may arise in the clinical population on the order of weeks to months. Should a drug-resistant strain of bacteria arise in a hospital or health-care clinic, the diversity and evolvability of phage allows for an active phage to be isolated against that strain on a relatively short time scale. Several laboratories have demonstrated that phage can be isolated from the environment or evolved in real-time that are effective at killing pathogenic bacteria (even circulating pandemic strains) on the order of days or weeks. The cost in developing these phage preparations is in the hundreds to thousands of dollar range and, if production was streamlined, could even be lower. The technical knowledge to isolate, purify, and test most phage is low, and for the most part does not require expensive/complicated instrumentation. One might envision that a standardized pipeline of phage discovery, characterization, testing, and scalable production could be created that responds to each new drug-resistant threat as it emerges, either at the level of a company or local health-care institution.

Phage Cocktails and Adjuvants Can Overcome Resistance

The mechanism by which phage kill bacteria are distinct from the mechanism by which chemical antibiotics work. This means that resistance against antibiotics is unlikely to confer a selective advantage when faced with phage. Because of this, phage should have no barriers toward efficiently killing multidrug resistant bacteria. It does not, however, mean that bacteria will not develop resistance to phage. Bacteria and phage have been coevolving for quite possibly billions or more years. Phage resistance is common and well documented, even during the course of a single culture experiment or animal infection. The advantage of phage, unlike rigid chemical antibiotics, is that although resistance will be frequent, finding or adapting a counter phage to kill the phage-resistant strain can be readily achieved. Each phage may kill by a slightly different mechanism, or use different surface receptors for binding. It would be difficult for bacteria to overcome all the ways a phage can infect. There are also reports of bacteria becoming less fit and more easily killed by the immune system after they become resistant to phage. Indeed phage often use important surface proteins as receptors. Mutation or loss of these receptors may prevent a phage from binding, but it may come at a fitness cost to the bacterium.

There is also well documented evidence, in both humans and animal models, that phage used in combination with antibiotics is more effective than either alone. Since both use a different mechanism to kill, the likelihood of developing resistance simultaneously against both activities is lower than for each individually. Even more so, a cocktail of many unique phage, especially if they use different mechanisms to infect, will be very difficult to evolve resistance to on an appreciable timescale. A cocktail of phages plus an antibiotic might even be more effective. Since many physicians may be reluctant to exclude antibiotics from any treatment, the combinatorial use of phage and antibiotics may be a more favorable option for future phage therapy. In this case, phage may be considered a sort of "green" adjuvant to antibiotic action, a safe and environmentally friendly alternative that enhances the effectiveness of antibiotics. These phage adjuvants may also lower the effective dose of antibiotics, thereby decreasing the levels of resistance. Such a property may also bring previously unusable antibiotics back into the fold.

Targeted and "Self-Dosing"

One of the potential benefits of phage is that they are highly specific to the bacterium they infect. This means they will have low off-target effects since they do not infect eukaryotic cells. A major problem with antibiotics is that their nonspecific broad spectrum activity can also kill beneficial microbes that inhabit our gastrointestinal tract. Of course, the broad spectrum property is an advantage when a clinician does not yet know the species of bacterium that is infecting a patient; a sweeping approach is likely to be more successful. But clearing out protective species in the gut, which eliminates "colonization resistance" and allows pathogenic species to occupy previously occupied niches, can lead to serious infections. A classic example of this is the emergence of *Clostridium difficile* infections in the nosocomial setting. Phage are so selective that not only can they target a species of bacterium, they can also be strain-specific. Where knowledge of the type of bacterium that is infecting the patient is known, the use of phage would spare intestinal commensals while killing only the bacterial pathogen. Such an approach might reduce the incidence of nosocomial infections that are associated with wiping out the native intestinal flora. Of course, rapid diagnosis and typing of the infection is needed beforehand unless a broadly-acting cocktail is used that can target the most likely pathogens present during the infection.

Another virtue of phage is that they may be a "self-dosing" drug that will release new infections particles proportional to the density of the target bacterial host. This property has the virtue of amplifying the drug at the precise location where the bacteria are, thus concentrating the drug in a higher density at the site of infection that might otherwise not be achievable by any other delivery

method. Furthermore, once the target bacterial cells are killed, their density will decrease, and so will the concentration of phage. Should resistant cells arise at this point, it is possible mutant phage, having arisen on the spot, may re-infect, thereby further amplifying the response. Such activity may keep the bacterial population at a low density so that the immune system can adequately clear any remaining cells.

Guidelines for Phage Therapy

For all the realized and potential virtues of phage therapy, there are also substantial obstacles, each of which will need to be addressed if this approach is to gain wide-spread acceptance in the lay and medical community.

Safety

Phage are "generally regarded as safe" by the US Food and Drug Administration with at least one phage product approved for use to clear Listerial species from poultry. As discussed above, only compassionate care use of phage has been approved for human use in the US, largely through the Investigational New Drug (IND) process. Empirical evidence in animal models and the administration of phage to humans (mostly in the East, but cases in the West confirm) that even very high doses of purified phage do not yield adverse reactions. Working groups under the European Medicines Agency has formulated some useful guidelines pertaining to safety, and numerous academic researchers have written extensively about them. A comprehensive attempt to summarize these points is discussed below.

It is good practice to sequence any phages for human use. Whereas there is not a strong consensus about the type of genes a phage should possess before it can be used therapeutically, there is consensus on what genes it should not contain. A phage should not encode any elements that may confer resistance to antibiotics, known or suspected virulence factors (including protein toxins, adhesins, secretion systems, and nutrient transporters), and/or elements that promote the integration of phage DNA into bacterial or human cells. This implies that the phage will be free of lysogenic elements.

It would be helpful if there were some preclinical data available, perhaps from animal models, on the type of immune response the phage may produce. Commercially available assays can easily assess the complete cytokine response from a small sample of blood, and animal temperature can be readily measured with time. For long-term use of phage (>2 weeks), the production of neutralizing antibodies can be determined using assays already reported in the literature. Of course, these considerations are only applicable to phage that will enter systemic circulation. Phage used topically may be less restricted in adherence to these considerations.

Finally, currently phage can only be produced in a bacterial host. This means that products that might normally be toxic or induce inflammation in a human have the potential to contaminate the phage preparation. There are several density-dependent and organic-solvent based methods to remove these bacterial products, and they should be considered when readying phages for use. Post purification testing should include some knowledge about the endotoxin levels (lipopolysaccharide—can be measured by commercially available assays), exotoxin (if the bacterial host used to grow the phage is a pathogen—pyrogen tests in animals), and buffer and/or cryoprotectants used to purify or store the phage. It goes without saying that the preparation should be sterile.

Storage

Each phage preparation will require some standardization of its storage capacity, including temperature, buffer conditions, and how much potency loss is acceptable before new preparations need to be made. Considering that phage come from the environment and are thus exposed to extreme conditions, they may have the advantage of not requiring refrigeration or complex preserving formulations for their storage. Regardless of the conditions that are chosen, the half-life of the preparation will need to be determined, both during storage and once the seal is broken immediately prior to therapeutic use. This also implies that the bacterial test strain also be standardized so that loss of viability can be ruled out as a complicating factor in phage testing.

Phage Banks and Process Strategies

There currently is no formal or structured system in the West for phage therapy from diagnosis to treatment. There are several ways one may structure a phage treatment system, none of which are mutually exclusive. The first is the conventional way drugs are currently made, sold, and used. A company identifies an efficacious phage preparation, attains the intellectual property around this preparation, performs the quality control under good manufacturing practices, and sells based on need. The ever changing ability of phage make the acquisition of intellectual property and patentability surrounding phage use difficult. In addition, the streamlined production of antibiotics has a 75 plus year head-start. These obstacles make taking the plunge into the development of phage perceived as not economical. The current use of phage in people is very personalized. For investment in this area to be considered by the private sector, a more standardized application may be necessary.

The second approach is to generate a national repository of therapeutic phages that would be managed by subsidiary government organizations or subcontractors. There is precedence for this since several vaccines against uncommon but nonetheless serious infectious diseases are maintained in a type of national stockpile should their use be suddenly necessary. This approach

would require continuous investment of public resources for their maintenance, possibly offset in costs via their purchase when needed.

A third approach entails the development of individual phage treatment centers at medical institutions, be it hospitals, clinics, or allied academic and research units (such as medical schools). There may be a strong incentive for this model due to the overburdening liability associated with nosocomial infections. In addition to being deadly, bacterial sepsis and/or chronic bacterial infection is one of the most expensive in-patient diseases and a sizable fraction of these originate in the hospital while the patient is being treated for another ailment. There are well-documented instances of hospitals or clinics having high carriage of a particular multidrug resistant strain. It may even be localized to a ward or unit of an institution. Phage can be viewed as a type of intra-institutional infectious disease control mechanism. A bank of phages can be developed and maintained that is highly specific to the bacterial pathogens common to that institution. Upon plating or culture growth of the pathogen in question, hundreds to thousands of different phage preparations can be easily tested for lytic activity using automated methods, those phage pulled, quickly assembled into a cocktail, and subsequently administered to the infected patient. In addition to potentially being an in-house, low-cost solution to a much larger problem, the use of phage in this capacity may reduce the antibiotic use, encouraging good stewardship and decreasing resistance. Should resistance arise, one can go back to the original phage collection and make a new cocktail from either phage isolated from the environment or via their controlled evolution in the laboratory. An overview of these processes is shown in Fig. 3.

Treatment and Pharmacology

In people and in animal models of infection, phage have been applied topically, orally, rectally, subcutaneously, intraperitoneally, intramuscularly, and intravenously. In general, very few adverse reactions have been noted. Much like antibiotics, it seems sensible to apply the delivery method that is most likely to reach the intended bacterial target. Topical application might be useful for infections of the skin (example, diabetic foot ulcer) or wound dressings (burn victims) and may be less hindered by fears of delivering a virus systemically. Should harmful pathobionts be "edited" from their reservoir in the gastrointestinal microbiome, oral application of phage protected from the low pH of the stomach may be of value. The oral prophylactic use of phage to protect against diarrhea-causing bacteria (for example for travelers or children in unsanitary parts of the world) may also be useful. Clearly, systemic infections will need systemic delivery of phage.

Very little is understood about the half-life of different types of phages delivered via various routes. The relationship between phage size (which may affect its distribution and penetrance) and surface protein content (which may affect its ability to interact nonspecifically with host tissues or specifically with the host immune system). Long-lasting circulating phages have been identified by serially passaging phage in murine hosts, but the biological properties of phage that confer this longevity are still unexplored. The host factors that have the greatest impact on the efficacy of phage are also a rich ground for further research. Numerous reports clearly support a role for the host's immune system (either macrophages or antibody) in altering phage activity, but the effects of temperature, blood components including small molecules (salts, sugars, metals, etc.), xenobiotics (other drugs, ingested foods), and cells/complement etc., are not worked out. For example, some of the earliest phage literature invoked a significant role for some divalent metals (especially calcium and magnesium) in phage activity in culture, but the role of these factors during systemic action is unscrutinized. Phage may circulate to major organs and even can enter the central nervous system. They accumulate in the spleen and are readily excreted. But the long-term effects of phage on the human health, especially the immune response, will also need to

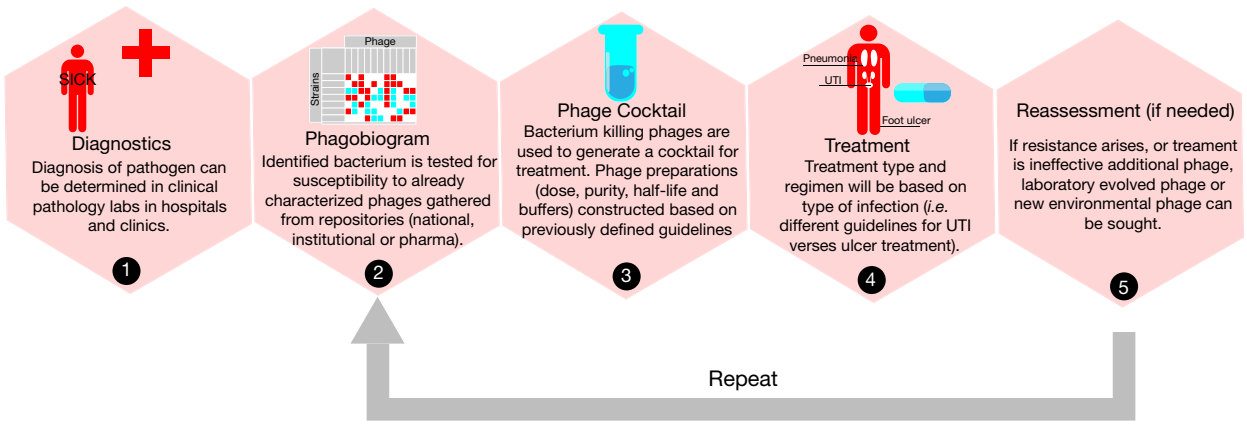

Fig. 3 *A pipeline primer for the use of phage to treat human infections.* Following the diagnosis and identification of the infecting bacterium, a single or a cocktail of phage are generated from a stored phage repository that are highly active against the target pathogen. Phage are propagated and administered according to defined criteria for that particular clinical infection and progress monitored. Should treatment not be effective or resistance emerge, a new series of phage are identified from the repository, or identified from the environment, or experimentally evolved in culture, and re-applied.

also be studied. To be fair, these questions also remain for many approved and commonly used drugs, but it certainly is clear that the pharmacology of phage after their administration is a giant black box waiting to be illuminated.

Engineered Phage or Phage Products

Advances in molecular biology, cloning, and technology have also facilitated the generation of phage preparations with enhanced features. The sequencing of phage genomes has allowed for the identification of closely related phages that differ in target specificity. Swapping the tail fiber genes of these related phages leads to lytic activity that is dependent on the specificity of the tail fiber, thereby demonstrating that the host range of phage can be either expanded or narrowed dependent on the constitution of their binding elements. Using this knowledge, others have generated phage engineering platforms, often using heterologous hosts such as yeast, as a synthetic system to generate phage with controlled targeting specificity. One might envision the day when an entire phage genome is constructed from a computer template, differing only in the elements that enhance activity or specificity, the nucleic acid synthesized, and phage produced in a host that lacks toxic elements.

There are numerous reports, and even some start-up biotechnology companies, centered around the use of phage products as antimicrobials. The hallmark feature of these include the use of phage lysins and/or holicins, as phage-independent bacterial killing proteins. After infection, phage often make a holicin to cleave the inner membrane so that a specific lysin can attack the intervening peptidoglycan. The cell then bursts, no longer able to withstand the osmotic pressure. Lysins contain numerous advantages over phage. These include that they; (i) are a protein and thus will not change during preparation or treatment, (ii) they are highly active at relatively low concentrations, (iii) are recombinant and thus can be enhanced or built with new modules simply by adding, removing, or mutating the gene that encodes them, and (iv) have demonstrated activity in several models of infection, including a *S. aureus* endocarditis model. Concerns with lysins include that the host may produce neutralizing antibodies (although evidence seems to suggest it does not occur frequently) and that they do induce an increase in several inflammatory cytokines (although the ramifications of this are not yet worked out).

Finally, the possibility of using noninfectious, nucleic-acid free phage, termed phagocins, has been raised. Phagocins appear to efficiently kill bacteria without lysis, which may reduce the release of endotoxin, and are stable because they lack any ability to mutate. The more defined and controlled nature of phagocins also means they might be more amenable to intellectual property claims. Future work will be needed to determine the level of efficacy and safety phagocins offer over phage or their lysins.

Development of Industry

One sign that phage therapy may have life is that companies have sprouted around the commercial use of phage. This includes PhagoBioderm, a biodegradable polymeric film that contains bacteriophages with demonstrated efficacy against ulcers that are typically resistant to antibiotic treatment. In the US, a company called Intralytix is undertaking phase I clinical trials for diabetic foot ulcers. A Portugal biotech company (Technophage) received approval in 2014 for a phase I trial using TP-201, a cocktail of bacteriophages for the treatment of chronic ulcers. Pherecydes Pharma, a France based company, has a product called Pneumophage in its pipeline that stands at the preclinical stage. This product is intended for the acute respiratory treatment of *P. aeruginosa*. A US based company (EnBiotix) developed an engineered phage EBX-003 in 2016 to treat prosthetic joint infection through the expression of a biofilm degrading enzyme.

Known Mechanisms of Phage Resistance

Bacteria may become resistant to phage in several ways (Fig. 4). One such mechanism includes the modification of cell surface receptors. This includes mutation or a variation in expression. Another mechanism of resistance to phage includes the digestion of phage nucleic acid via the production of endonucleases, including targeting nonmethylated foreign DNA. Finally, CRISPR Cas systems acquire phage DNA from previous infections and transcribe these elements into small interfering RNAs that guide nucleases the phage DNA.

Future Hurdles

Bacterial evolve at a pace that is greater than our ability to make new antibiotics. Phage are a promising option to turn the evolutionary table back on pathogenic bacteria since the adaptability of phage is just as great if not greater. Despite this potential and demonstrated efficacy in humans and animal models, three main challenges remain.

First, we must change our culture. Either we change the way we develop and approve new drugs, or we develop and approve new drugs that change. Whereas the first option is unlikely to occur for chemically-derived new drugs, the second will require an unconventional approval process. Entities that can change in real-time, which can be a great advantage in some cases, must be given some lee-way to do so. It is not just phage that would benefit from a different set of guidelines, CRISPR-based gene editing, gene therapy, and T cell treatments of cancer or viral infections also would fall under this umbrella. Like phage developed against a particular strain of bacteria in a certain clinical context, each of these approaches is specific for the problem (and sometimes patient), at hand. Basic parameters will need to be met (some discussed above are acceptable endotoxin levels, knowledge of

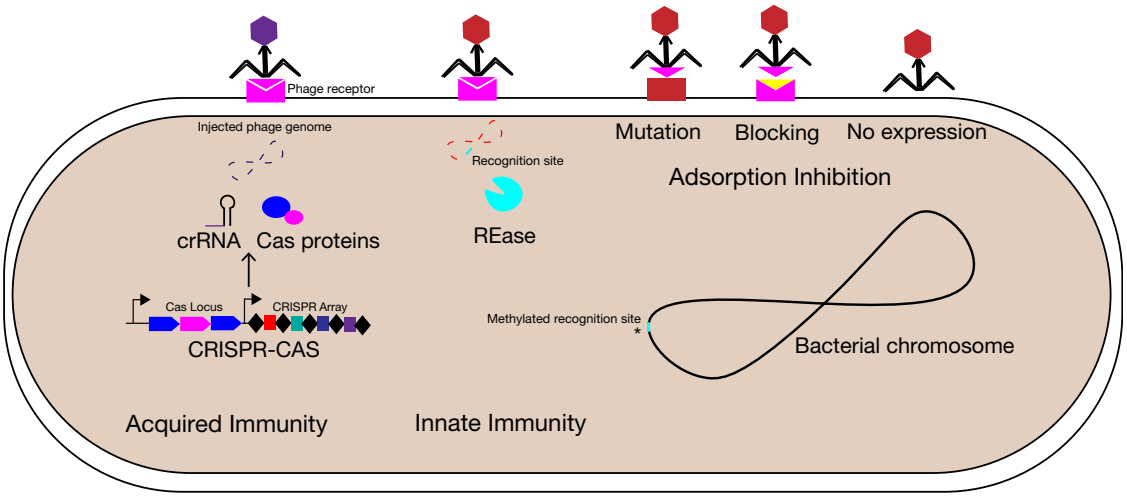

Fig. 4 *Resistance to phage*. Bacterial can acquire resistance to phage in a number of ways, including de novo mutation of receptors or other proteins needs for binding or nucleic acid injection, the digestion of phage nucleic acid, or via the use of CRISPR immunity systems.

neutralization, half-life, shelf-life, etc.), but these parameters will need universal consensus for each particular type of infection. Blanketing all phage therapy under one set of guidelines will not work, and there must be some way the process can be made profitable to encourage development and investment. This makes revising intellectual property guidelines an important part of the process. If not, there will need to be a basic understanding in our society that government dollars will be spent to maintain phage banks and pipelines for use by clinics in need.

Second, we must accelerate basic and clinical research into phage therapy. This includes determining the type of phages to study, the molecular mechanisms by which they kill and by which bacteria become resistant, their pharmacokinetic and storage properties (phagacology), the host factors that may affect efficacy, their short and long-term safety, and the leveraging of technology to genetically enhance these properties, either via whole virus or through the use of phage lysins or phagocins. Animal models that allow researchers to assess these properties should be agreed upon and standardized, preferably with results that translate to the human condition, and carefully controlled clinical studies in humans initiated. Funding for this work should be a priority for grant-issuing institutions and the public educated on the pros and cons of the approaches and research.

Finally, there must be investment, both in industry and the academic or private hospital system, in the infrastructure, expertise, and personnel needed to execute phage therapy as an accepted medical practice. Initial considerations were presented above but loosely include the support of phage banks, the rapid and automated ability to screen these banks for efficacious phage to pack into cocktails, and the wherewithal and confidence to administer clean and safe preparations for the infection at hand. Much like hospitals have invested in Diagnostic Microbiology labs to culture, type, and test the sensitivity to antibiotics in-house, so too should there be a similar commitment to the generation of a real-time drug capable of efficient clearance of multidrug resistant pathogenic bacteria.

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Phagocytes (Innate Immunity)

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Abbreviations

APC	antigen-presenting cell
cDC	classical or conventional DC
CGD	chronic granulomatous disease
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DC	dendritic cell
EAR	eosinophil-associated RNases
EPO	eosinophil peroxidase
GMPs	granulocyte/monocyte progenitors
HSCs	hematopoietic stem cells
MBP	major basic protein
MDP	macrophage and DC progenitor cell
MEPs	megakaryocyte/erythrocyte progenitors
moDC	monocyte-derived DC
MPO	myeloperoxidase
MPS	mononuclear phagocyte system
NET	neutrophil extracellular trap
NK	natural killer
pDC	plasmacytoid DC
PICD	phagocytosis-induced cell death
PMN	polymorphonuclear neutrophil
PRR	pattern recognition receptor
RBCs	red blood cells
ROS	reactive oxygen species
SOD	superoxide dismutase

Introduction

Phagocytes and phagocytosis were first described by Elie Metchnikoff in the late 1800s. He identified two groups of phagocytic cells during his studies of bacterial infection and named these cells macrophages and microphages. These cells could be differentiated further by staining methods developed by Paul Ehrlich and colleagues. Metchnikoff reported that the cells he named "microphages" were the same leukocytes Ehrlich described as polynuclear cells (later "cells with polymorphous nuclei"). These microphages are now known as granulocytes or polymorphonuclear leukocytes. Metchnikoff reported the ability of phagocytes to ingest invading microbes and thereby contribute to natural or innate immunity. Our understanding of phagocyte biology, including development, function, and turnover, has increased significantly since the early work by Metchnikoff and Ehrlich. Many of the most recent discoveries, especially those related to the origins and development of phagocytes, have been made possible by advances in technology and improved methodology. Inasmuch as information and detailed knowledge related to phagocyte biology is extensive, this article is not intended to provide a comprehensive discourse on phagocytes and/or phagocyte biology. Rather, it provides an overview of selected areas of phagocyte biology that can serve as a starting point for the interested reader.

Phagocyte Development

Hematopoiesis

Hematopoiesis is the formation and development of blood cells. A process named primitive hematopoiesis, which produces red blood cells (RBCs) and macrophages, occurs in the yolk sac early during embryogenesis. As embryos mature, hematopoietic stem cells (HSCs) are formed. All subsequent blood cells are derived from HSCs. This type of hematopoiesis is termed definitive hematopoiesis. Our understanding of this process, which includes development of phagocytic cells, such as granulocytes and

monocytes, has changed in recent years. According to the traditional model of hematopoiesis, the initial differentiation of pluripotent hematopoietic stem cells (HSCs) leads to two distinct progenitor stem cells – a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP). CMPs differentiate further into granulocyte/monocyte progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs), which in turn become mature myeloid cells (granulocytes and mononuclear phagocytes) and platelets or RBCs. CLPs differentiate into B cells, T cells, and natural killer (NK) cells. A key tenet of the traditional model is the stepwise maturation of bone marrow progenitor and precursor cells into specific types of leukocytes. However, recent studies have revised our understanding of hematopoiesis into a more dynamic model. For example, it is now known that HSCs are a heterogenous population of cells from a functional standpoint, and differentiation to mature blood cells occurs over a continuum rather than *via* distinct maturation steps, as per the traditional model of hematopoiesis. In any case, the development of each type of phagocyte is discussed in more detail below.

Granulocytes

Human granulocytes are a subset of myeloid cells that in circulation are comprised of $<1\%$ basophils, 2%–5% eosinophils, and $\geq 95\%$ neutrophils. Collectively, these cells are also known as polymorphonuclear leukocytes (Fig. 1). A fourth cell type, known as a mast cell, is functionally similar to a basophil (and is derived from a common basophil/mast cell precursor) but is a tissue-resident cell with a longer life-span by comparison. Mast cells, like basophils, contain effector proteins stored within cytoplasmic granules. Although the term "granulocyte" has been used historically for basophils, eosinophils, and neutrophils (cells in the bloodstream), an argument could readily be made to classify mast cells as granulocytes. Granulocytes and granulocyte precursors constitute $\sim$60% of all cells in bone marrow; 50%–70% of white cells in circulation are granulocytes. Thus, there is enormous production and turnover of granulocytes in humans – approximately 10^{11} granulocytes per day in a healthy human adult.

Neutrophils

Neutrophils, also known as polymorphonuclear neutrophils (PMNs), are the most prominent cellular component of the innate immune system. Their primary function is to ingest and kill invading bacteria and fungi. Neutrophils are terminally-differentiated cells and thus have a relatively short lifespan, which explains in part the need for high steady-state production of these cells, as mentioned above. In healthy adults, neutrophils develop in bone marrow for 5–6 days (excluding the time needed for differentiation of mitotic precursor cells) and are then released into the bloodstream. Neutrophils circulate in the bloodstream for ≤ 1 day, during which time they function as first-responder cells and can be recruited rapidly to sites of injury and/or infection. Neutrophils that are not recruited to the tissues by an inflammatory signal ultimately re-enter tissues and undergo apoptosis spontaneously (discussed below). The total lifespan of neutrophils is $\sim$9–10 days.

PMNs are readily identified by a characteristic multi-lobed nucleus and cytoplasmic granules (hence the term granulocyte) (Fig. 1). Key components of neutrophil microbicidal activity are contained with cytoplasmic granules (see below). There are at least three types of granules: azurophilic granules, specific granules, and gelatinase-containing granules. A fourth cytoplasmic organelle, the secretory vesicle, contains an array of membrane-bound receptors that can be readily mobilized to enrich the plasma membrane with cell surface receptors. These granule subsets form sequentially during neutrophil production (granulopoiesis) in bone marrow. The azurophilic granules, named originally because of their ability to be stained readily with the dye azure A, form at the promyelocyte stage of neutrophil development. Inasmuch as azurophilic granules are the first to form during granulopoiesis, they are also known as primary granules. The primary granules are the sole repository for myeloperoxidase (MPO) and are therefore peroxidase-positive granules. Specific granules form next (and are also known as secondary granules), followed by gelatinase-containing granules and secretory vesicles. These granules and vesicles lack MPO and can be classified as peroxidase-negative granules. Each type of granule subset contains a specific set of proteins in the membrane or lumen that is targeted to that organelle based upon timing of protein synthesis. Thus, neutrophil granule contents reflect the proteins being synthesized at that point during granulopoiesis.

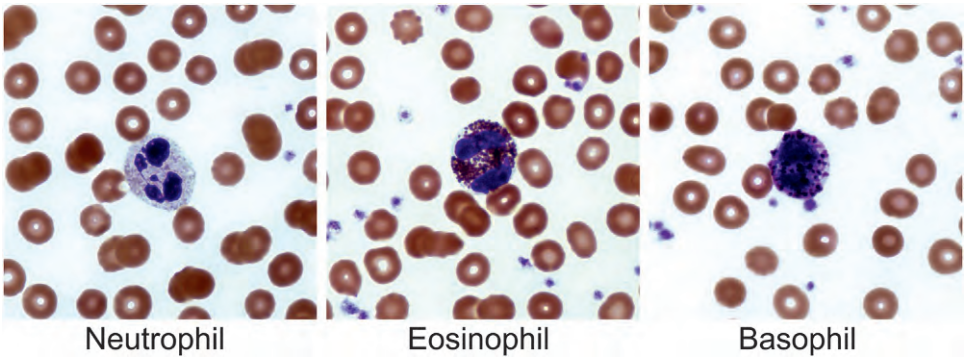

Fig. 1 Human granulocytes. Human blood cells were stained with a modified Wright-Giemsa stain and visualized on a light microscope at 100× magnification.

As neutrophils mature, the capacity for gene expression and protein synthesis decreases. Thus, microbicidal proteins and enzymes required to ingest and kill microbes need to be preformed and stored in granules and secretory vesicles, and mature neutrophils are fully equipped to carry out their role as frontline innate host defense cells. It is noteworthy that the stores of microbicidal components are not resynthesized, and new granules do not form in mature cells – a characteristic consistent with a terminally differentiated cell that has a short lifespan. That said, circulating neutrophils retain some capacity for gene expression and protein synthesis, which is needed to maintain functional capacity for extended periods (hours) and facilitate apoptosis and turnover.

Basophils, eosinophils, and mast cells

Basophils, eosinophils, and mast cells are widely known for their prominent roles in allergic responses. Basophils and eosinophils mature in bone marrow as granulocyte precursors and are then released into circulation as mature cells. By comparison, mast cell precursors are released into the bloodstream and ultimately migrate to tissues, where they differentiate into mature and fully functional cells.

Basophils and eosinophils are polymorphonuclear leukocytes that can be readily identified by distinct morphology on a microscope slide preparation, as described by Ehrlich. Indeed, granulocytes were named by their characteristic staining patterns with basic aniline dyes (basophils), acid aniline dyes such as eosin (eosinophils), or neutral dyes (neutrophils). Following stain with a modified Wright-Giemsa stain, basophils can be identified by their large cytoplasmic granules that stain dark purple, whereas eosinophils have a bilobed nuclei and large cytoplasmic granules that stain orange/red (Fig. 1). The granules of basophils and eosinophils are unique to each of these cell types and contain proteins that are key to their respective roles in immune and inflammatory responses (see below).

Eosinophils, like basophils, regulate immune and inflammatory responses by secreting effector proteins stored within cytoplasmic granules (see below). These secreted proteins contribute to the prominent role played by eosinophils in the response to allergens and in host defense against microorganisms, including parasites, viruses and bacteria. This antimicrobial activity is largely extracellular, although these cells can phagocytose bacteria to a limited extent (compared with neutrophils). In addition, eosinophils have the capacity to secrete a host of cytokines and chemokines, and they function as antigen-presenting cells. Eosinophils remain in circulation for ~1 day and then migrate into tissues.

Recent evidence suggests mast cells are derived from a common basophil/mast cell progenitor and thus share many functional similarities with basophils, such as a prominent role in allergic inflammation. Whereas basophils circulate as mature cells in the bloodstream, mature mast cells reside almost exclusively in tissues. Thus, only mast cell precursors are found in circulation. It is also notable that mast cells have a long lifespan (weeks or months) compared to other granulocytes such basophils (a few days). Mast cells are relatively abundant in skin and mucosal tissues, areas that are often exposed to invading microorganisms. As such, they are ideally positioned to coordinate innate and adaptive immunity, which is achieved by secretion of cytokines, chemokines, and effector proteins stored within mast cell granules. During activation, mast cells release numerous inflammatory mediators, including histamine (major contributor to allergic inflammation) and chemokines that recruit neutrophils.

Although basophils, eosinophils, and mast cells are myeloid cells, and eosinophils and mast cells can phagocytose bacteria, these cells are perhaps more widely considered as immune regulatory cells rather than phagocytes. Therefore, our discussion of the role of these cells in innate immunity is brief, and we refer the interested reader to review articles listed in the Further Reading section (*e.g.*, Travers and Rothenberg, 2015; Rosenberg *et al.*, 2013).

Mononuclear Phagocytes

Monocytes, macrophages, and dendritic cells (DCs) comprise a group of cells known as mononuclear phagocytes. These phagocytic leukocytes are important for innate immunity and can interact directly with invading microorganisms. In addition, they coordinate the acquired immune response, in part by producing chemokines and cytokines, and by functioning as antigen presenting cells. Therefore, these cells serve as a key bridge between innate and acquired immunity. Lastly, these cells contribute significantly to tissue and immune system homeostasis, largely by removing dead and dying host cells.

Our understanding of mononuclear phagocytes has evolved considerably since they were first described by Metchnikoff. It was Metchnikoff who first proposed a macrophage system, which was superseded by Ludwig Aschoff's reticuloendothelial system and then the mononuclear phagocyte system (MPS), as described by Ralph van Furth and colleagues. According to the original MPS, monocytes develop in bone marrow from mitotic precursor cells known as promonocytes, which in turn differentiate into all mononuclear phagocyte subsets. Based on this model, mature monocytes are released into circulation as terminally-differentiated end cells, which then enter tissues and differentiate to macrophages. Many of the basic tenets of the MPS remain today. For example, human monocytes can be differentiated into macrophages and specific dendritic cell (DC) subsets *in vitro*, a finding that supports the canonical MPS. However, the extent to which this occurs *in vivo*, especially during inflammatory states, remains incompletely determined. Studies in mice identified a common macrophage and DC progenitor cell (MDP) in bone marrow. These MDPs can develop into monocytes, macrophages, and dendritic cells *in vivo*, which is also largely consistent with the MPS theme. On the other hand, recent studies indicate that many of the long-lived (and self-renewing) tissue-resident phagocytes present in adults, including macrophages, dendritic cells, and Langerhans cells, arise from prenatal progenitor cells in the yolk sac or fetal liver during embryogenesis, and are thus not derived from monocyte precursors in bone marrow. That is, these tissue phagocytes are established before birth and maintained through adulthood. Although there have been many important discoveries in the area of mononuclear phagocyte development, this latter finding is arguably one of the most notable changes to the canonical MPS.

Monocytes

Human peripheral blood monocytes are typically identified and classified by the presence of a bean-shaped nucleus and the levels of specific surface receptors, such as CD14, CD16, CD11b, and CX3CR1 (fractalkine receptor). Mature monocytes comprise 5%–10% of the leukocytes in human blood ($\sim 2 \times 10^5$ cells/mL) and are in circulation for 1–2 days before migrating into tissues. Inasmuch as monocytes play a key role in the acute inflammatory response and innate immunity, they can be recruited readily from the bloodstream to sites of inflammation by multiple host or microbe-derived molecules. Monocytes in tissues function as phagocytic cells of the innate immune response, and much like neutrophils, ingest and kill invading microorganisms (see below). It is noteworthy that as tissue monocytes differentiate further into macrophages or DCs, they lose MPO-containing granules (lysosomes). Whether monocytes ultimately differentiate into specific macrophage or DC subsets is likely dictated by molecules present during the inflammatory response. Monocyte-derived tissue macrophages have significant biosynthetic capacity and can live for weeks to months in tissues.

Macrophages and DCs

Macrophages are phagocytes located in tissues throughout the body. These cells represent an integral component of innate and acquired immunity, and they contribute to tissue homeostasis. Macrophages established from yolk sac progenitors during embryogenesis (during primitive hematopoiesis) have the capacity to self-renew and are maintained independent of myeloid precursor cells in bone marrow. These macrophages include Kupffer cells of the liver, Langerhans cells in skin, and microglia in the brain. However, macrophages in some tissues (*e.g.*, skin, heart, and gut) are maintained by bone marrow precursor cells and are derived from monocytes. During specific conditions, such as inflammation and/or infection, there is a significant (and transient) increase in monocyte-derived macrophages. From a functional standpoint, macrophages have been traditionally categorized as belonging to M1 (classic) or M2 (alternative) phenotypes. An approximation of the level or type of macrophage activation is known as macrophage polarization. M1 macrophages are proinflammatory cells, whereas M2 macrophages are immunosuppressive and contribute to tissue repair and wound healing, although it is now acknowledged that this scheme is too simplistic to explain the complexity of macrophage phenotypes *in vivo*. Signals for macrophage activation or polarization include molecules produced by other host cells (*e.g.*, chemokines and cytokines), invading microbes (*e.g.*, lipopolysaccharide), and the host tissue environment. In short, macrophages ingest and kill microbes, serve as antigen-presenting cells (APCs), survey tissues, and remove debris and dead or dying host cells in a non-inflammatory manner. Each of these processes is important for maintaining the healthy host.

DCs are tissue-resident phagocytes that function primarily as APCs and immunoregulatory cells. They originate from DC precursors in bone marrow or from circulating monocytes. DCs can be classified into at least three distinct subsets – classical or conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DC (moDCs) – based largely upon the presence of specific cell surface receptors and morphology. cDCs arise from common dendritic cell precursors and are distributed in many tissues of the body. These cells have a lifespan of ~1 week and are replenished continually. The primary function of cDCs is to present antigen to T cells. Thus, they often migrate to tissues enriched with T cells (*e.g.*, lymph nodes and spleen). Compared with macrophages, cDCs have a much higher (~100-fold) capacity to stimulate naive T cells. pDCs also originate from bone marrow progenitor cells and are involved in the immune response to viral infection. moDCs are also known as inflammatory DCs and like cDCs, they present antigen to T cells. The extent to which each of these subsets functions as a phagocytic cell is incompletely determined, but phagocytosis has been best characterized in cDCs and moDCs.

Recruitment and Phagocytosis

Many of the fundamental mechanisms involved in recruitment, chemotaxis, and phagocytosis are shared among circulating phagocytic cells. Inasmuch as neutrophils are the most abundant phagocytic cell in circulation and in most acute inflammatory processes, and because these functions have been well-characterized in neutrophils, our discussion of recruitment and phagocytosis is focused largely on neutrophils. Notable exceptions for these processes in other phagocytes are indicated where appropriate.

Recruitment

Neutrophils are recruited rapidly from the bloodstream to sites of injury, infection, or other inflammatory process, and are typically among the first phagocytes to arrive at such sites. Monocytes are generally recruited later, after the peak of neutrophil recruitment, which is due at least in part to their role in removal of apoptotic neutrophils and tissue repair. Molecules known as chemoattractants are produced by host cells and invading microorganisms. Chemoattractants recruit phagocytes to infected or damaged tissues and include host chemokines, such as interleukin 8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) (previously known as GROα), or microbial products, such as *N*-formylated peptides. A chemokine named monocyte chemoattractant protein 1 (MCP1) or chemokine (C-C motif) ligand 2 (CCL2) is also important for recruitment of monocytes to sites of inflammation. The migration of phagocytes from the bloodstream into tissues is termed extravasation.

Circulating phagocytes detect low concentrations of chemoattracts, which in turn upregulates surface expression of receptors important for extravasation. In neutrophils, upregulation of surface receptors is caused by fusion of receptor-laden secretory vesicles with the plasma membrane. There can also be partial fusion of specific granules with the plasma membrane, which further prepares neutrophils for chemotaxis and microbicidal activity. At this point during recruitment, neutrophils are primed for enhanced

function but not fully activated. Priming is defined as the ability of a primary agonist to enhance specific neutrophil functions – *e.g.*, production of reactive oxygen species (ROS) – following exposure to a second agonist. In essence, priming prepares the neutrophil to be a more effective in the innate immune response. During the initial stages of extravasation, receptors on the neutrophil surface promote weak interactions with the vein endothelium, thereby allowing leukocytes to roll along the surface of postcapillary venules.

These interactions are mediated by selectin molecules: E-selectin (CD62E) on the surface of endothelial cells and L-selectin (CD62L) on the surface of leukocytes. Rolling is followed by firm adhesion, which is mediated by multiple receptors, including the β-integrins on leukocytes (*e.g.*, CD11b/CD18 or Mac-1) and intercellular adhesion molecules on endothelial cells (*e.g.*, intercellular adhesion molecule 1 or ICAM1). Adherent neutrophils then migrate or crawl along the capillary endothelium until they find a suitable cell-cell junction through which to transverse. This process is known as paracellular transendothelial cell migration and is used by the majority of phagocytes leaving the bloodstream. Alternatively, phagocytes can travel directly through endothelial cells, a process termed transcellular transendothelial cell migration. Phagocytes then cross the basement membrane and migrate toward increasing concentrations of chemoattractant (chemotaxis) until they reach the target site, *e.g.*, a site of infection, and/or come into direct contact with microbial invaders.

Phagocytosis

A primary function of phagocytic leukocytes is to ingest and kill microorganisms. Uptake of microbes is known as phagocytosis (Fig. 2), which is defined as the ingestion of objects or particles greater than 0.5 μm in diameter. Recognition and binding of microbial invaders by phagocytic cells, which can be considered an initial component of phagocytosis, is an important prerequisite

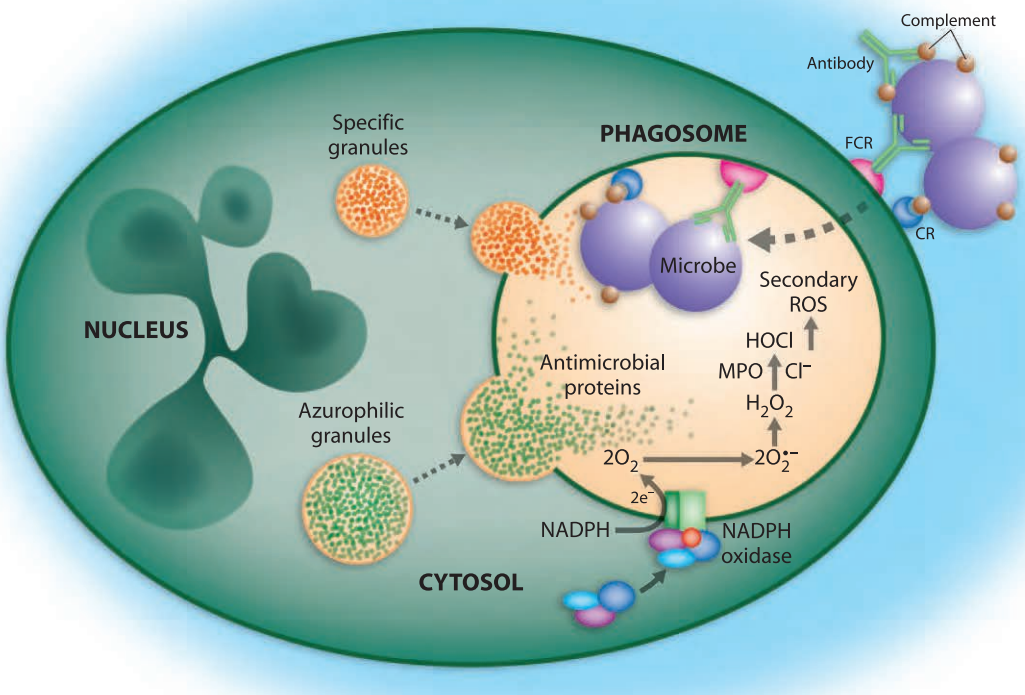

Fig. 2 Neutrophil phagocytosis and activation. Microbes opsonized with serum complement and antibody engage complement and antibody receptors (CRs and FcRs) present on the neutrophil surface (plasma membrane). Ligation of these receptors (and others) triggers phagocytosis, during which microbes are sequestered within a membrane-bound organelle known as a phagosome. Cytoplasmic granules enrich the newly forming phagosome with antimicrobial proteins, peptides and enzymes (*e.g.*, alpha-defensins, cathelicidins, and membrane components of NADPH oxidase). Concurrently, the NADPH-dependent oxidase assembles at the phagosome membrane and produces intralumenal superoxide. This superoxide dismutates to hydrogen peroxide, which in turn is converted to other ROS, including hypochlorous acid, a potent microbicide. The combination granule proteins and ROS are highly effective at killing bacteria and fungi. This figure was originally published in Quinn, M.T., Ammons, M.C., DeLeo, F.R., 2006. Clin. Sci. 111, 1–20. https://dx.doi.org/10.1042/CS20060059.

to ingestion *per se*. In general, recognition and binding of microbes is mediated by pattern recognition receptors (PRRs), such as the Toll-like receptors, and receptors for host proteins known as opsonins. Although ligation of PRRs primes neutrophils for enhanced functions, including phagocytosis and production of ROS, this binding alone is typically insufficient to trigger phagocytosis. Invading microorganisms are typically coated with host opsonins, such as serum complement proteins and/or antibodies, that recognize specific molecules on the microbe surface.

Ligation of receptors for host opsonins, most notably those specific for serum complement and antibody, promotes phagocytosis directly (Fig. 2). Thus, it is perhaps not surprising that phagocytes express multiple receptors for serum complement (CR1 or CD35, CR3 or CD11b/CD18, and CR4 or CD11c/CD18) and antibody (*e.g.*, FcγRI or CD64, FcγRII or CD32, FcγRIII or CD16, and FcαRI or CD89). The binding of microbes with surface-bound complement and/or antibody triggers phagocytosis, which is facilitated by actin polymerization and cytoskeletal remodeling. The signaling mechanisms involved in phagocytosis and cytoskeletal rearrangement have been well-studied but are beyond the scope of this article. As microbes are sequestered within a newly forming vacuole (phagosome), phagocytes become fully activated (as is the case with neutrophils), and microbes are in turn exposed to plethora of antimicrobial agents.

Microbicidal Activity

Neutrophils utilize an extraordinary array of microbicidal mechanisms to destroy and remove infectious agents, and these mechanisms can be generally classified as being oxygen-dependent and oxygen-independent.

Oxygen-Dependent Microbicidal Activity (NADPH Oxidase)

Phagocytes generate microbicidal ROS through the activation of a multi-protein enzyme complex known as the NADPH oxidase. The phagocyte NADPH oxidase is expressed in neutrophils, eosinophils, basophils, mast cells, monocytes, and macrophages and plays an essential role in host defense against pathogenic microorganisms. Note, however, that the NADPH oxidase is regulated differently in various types of phagocytes. For example, monocyte/macrophages and neutrophils express the same NADPH oxidase components; however, NADPH oxidase responses differ significantly in these phagocytes. Activated monocyte/macrophages exhibit a gradual increase in ROS production which peaks about 1 h after stimulation, whereas the response in neutrophils is very rapid, peaking in 2–10 min, depending on the stimulus. In addition, monocytes are capable of mounting an additional response after recovery from the initial response, which is typically not the case for neutrophils. Lastly, stimuli that activate the monocyte/macrophage NADPH oxidase do not necessarily activate the neutrophil NADPH oxidase. These differences likely reflect the distinct roles played by monocyte/macrophages and neutrophils in chronic versus acute inflammatory responses, respectively. Eosinophils, on the other hand, generate a substantially greater NADPH oxidase response than other phagocytes, which is directed to the extracellular environment rather than into a phagosome, which may be important for their ability to defend against parasitic helminths. Finally, basophils have been reported to lack a functional NADPH oxidase.

It is generally accepted that the core NADPH oxidase enzyme complex is composed of five oxidase-specific proteins ($p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$) and a small GTPase (Rac1/2) (Fig. 3). The nomenclature for the phagocyte oxidase-specific components includes the suffix *phox*, which refers to phagocyte oxidase. The one exception is $gp91^{phox}$, which is also known as NOX2. Overall, the *phox* proteins are highly conserved across species, confirming the absolute requirement for this system in

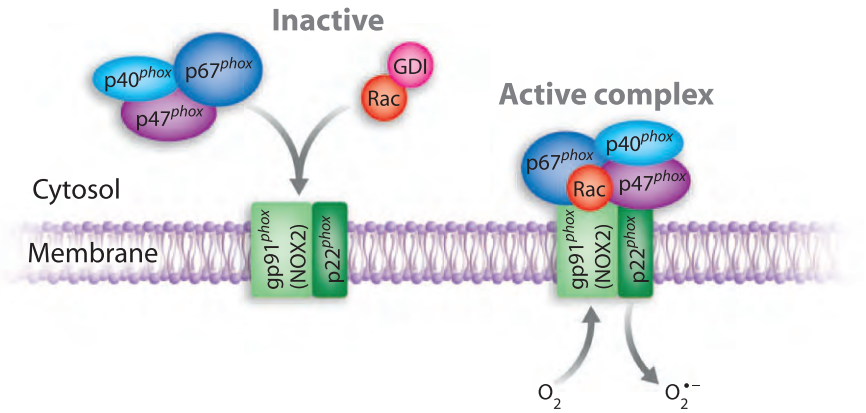

Fig. 3 Superoxide-generating NADPH-dependent oxidase. During phagocyte activation, cytosolic components of NADPH oxidase (p40 *phox*, p47*phox*, p67*phox*, and Rac) translocate to the plasma membrane or phagosome membrane and associate with the gp91 *phox*/p22 *phox* heterodimer known as flavocytochrome b_{558}. The assembled NADPH oxidase complex transfers electrons from NADPH in the cytosol (not shown) to molecular oxygen, thereby producing superoxide. See text for details. This figure was originally published in Quinn, M.T., Ammons, M.C., DeLeo, F.R., 2006. Clin. Sci. 111, 1–20. https://dx.doi.org/10.1042/CS20060059.

mammalian host defense. In resting cells, the NADPH oxidase proteins are segregated from each other (Fig. 3). Two of the proteins ($p22^{phox}$ and $gp91^{phox}$/NOX2) form a 1:1 heterodimer known as flavocytochrome b, which is an integral membrane protein present in specific granule and secretory granule membranes. Note that flavocytochrome b contains all redox components required for transmembrane electron transport (*i.e.*, hemes and flavin). However, flavocytochrome b does not function independently, thereby insuring against inappropriate activation. Rather, the other oxidase protein cofactors listed above, which are located in the cytosol of resting cells, are absolutely required for enzymatic activity, presumably to initiate or facilitate the electron transfer process. The phagocyte NADPH oxidase is activated when cells encounter a variety of soluble and particulate stimuli, including specific cytokines, chemokines at high concentrations, microbes and microbial products, viruses, and other foreign antigens.

Depending on the stimulus, NADPH oxidase assembly and activation can occur at the plasma membrane (*e.g.*, activation by soluble stimuli, such as cytokines or N-formyl peptides) or in the phagosomal membrane (*e.g.*, activation following phagocytosis of a pathogen or foreign antigen). During activation, flavocytochrome b becomes associated with the phagosome or plasma membrane through granule or vesicle fusion. Concurrently, the cytosolic oxidase proteins and GTPase participate in an intricate sequence of activating events, including phosphorylation and intramolecular protein:protein binding interactions that culminates in translocation and association of the cytosolic proteins with flavocytochrome b to form the active enzyme (Fig. 3).

The active NADPH oxidase enzyme catalyzes the transfer of electrons from cytosolic NADPH to O_2, resulting in the formation of extracellular or intraphagosomal superoxide anion ($O_2^{\bullet-}$). This results in a rapid and substantial increase in phagocyte O_2 consumption, which is known as the respiratory burst. Although high concentrations of $O_2^{\bullet-}$ can accumulate in phagosomes where the pH is low and can be directly toxic to some pathogens by virtue of the ability to oxidize iron-sulfur clusters present in bacterial enzymes, it is generally thought that $O_2^{\bullet-}$ represents the precursor to more powerful microbicidal oxidants that play more prominent roles in pathogen killing. Note, however, that the initial generation of $O_2^{\bullet-}$ is absolutely essential, as demonstrated by chronic granulomatous disease (CGD), which is a rare genetic disease resulting from defects in NADPH oxidase component proteins. The majority of CGD mutations that have been identified result in defective $gp91^{phox}$/NOX2, $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$ and result in absent or substantially reduced $O_2^{\bullet-}$ production. As a result, individuals with CGD experience severe, recurrent bacterial and fungal infections and can develop granulomas that form from accumulated phagocytes containing ingested bacteria. In addition to their impaired microbicidal function, CGD phagocytes exhibit abnormal apoptosis/turnover, which is also due to defective NADPH oxidase function.

At physiologic pH, $O_2^{\bullet-}$ is rapidly converted to hydrogen peroxide (H_2O_2) by spontaneous or enzymatic dismutation, which is catalyzed by superoxide dismutase (SOD). This abundant antioxidant enzyme plays an important role in protecting cells and tissues from excessive ROS. Additional antioxidant enzymes are also present to detoxify H_2O_2, including catalase and glutathione peroxidase. In addition to the production of ROS, phagocyte activation also results in the release of cytoplasmic granule contents into the phagosome, including high concentrations of myeloperoxidase (MPO). MPO is also found in monocytes, but its expression is normally lost when monocytes mature into tissue macrophages. On the other hand, MPO can be found in macrophages during certain disease states. Importantly, MPO utilizes H_2O_2 and chloride ions to form hypochlorous acid (HOCl), which is one of the most potent microbicidal oxidants and can kill a wide spectrum of pathogens, including bacteria, viruses, and fungi. $O_2^{\bullet-}$ can also be converted into hydroxyl radical ($HO^{\bullet}$) and singlet oxygen ($^1O_2{}^*$), which are potent oxidants, although their actual roles in phagocyte microbicidal activity *in vivo* are still being defined.

In addition to ROS, phagocytes also participate in the generation of reactive nitrogen species (RNS), including nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$). $NO^{\bullet}$ is also important in innate immunity and has been widely implicated in the inflammatory response. Although it was originally proposed that $NO^{\bullet}$ itself was directly cytotoxic, it is generally thought that the microbicidal effects of $NO^{\bullet}$ are due to the formation of $ONOO^-$, which results from the rapid reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$. In fact, $NO^{\bullet}$ can even out-compete SOD for $O_2^{\bullet-}$, making this one of the fastest known reactions. $OONO^-$ can decompose to nitrate (NO_3^-) at physiological pH or, in the presence of an oxidizable substrate, $ONOO^-$ decomposition also yields nitrite (NO_2^-), which can be converted into additional toxic metabolites that are microbicidal. Although it is clear that significant concentrations of $OONO^-$ can be generated in inflammatory tissues, the actual source of $NO^{\bullet}$ is still under investigation. Macrophages can generate $NO^{\bullet}$; however, the production of $OONO^-$ by human neutrophils *in vivo* has been a matter of debate, and it still remains controversial whether human phagocytes can actually generate enough $NO^{\bullet}$ to account for the observed $OONO^-$ levels. However, since $NO^{\bullet}$ is ubiquitously produced in the vascular system, it is also reasonable that $NO^{\bullet}$ derived from endothelial cells can react with phagocyte-generated $O_2^{\bullet-}$ at sites of inflammation, resulting in $ONOO^-$ formation. In any case, it is clear that $ONOO^-$ plays an important role in host defense and has also been implicated in the pathogenesis of various inflammatory diseases.

Oxygen-Independent Microbicidal Activity

Although significantly diminished in capacity, phagocytes from individuals with CGD are still able to kill a variety of microorganisms. Thus, it is clear that phagocytes also utilize an array of oxygen-independent microbicidal mechanisms. For example, neutrophils express a variety of antimicrobial peptides and enzymes that are stored in azurophil and specific granules and lysosomes so that they can be rapidly mobilized to the phagolysosome upon engulfment of a pathogen.

In addition to their high levels of MPO, neutrophil azurophil granules contain three neutral proteinases (cathepsin G, elastase, and proteinase 3), which can degrade microbial components, including bacterial cell walls. Azurophilic granules contain about 1/3 of the total neutrophil lysozyme, which is contained in both the azurophil and specific granules, can cleave linkages in the peptidoglycan of bacterial cell walls and can cooperate with complement in bacterial permeabilization. Azurophil granules also

contain several cationic proteins with microbicidal activity, including bactericidal/permeability-increasing protein (BPI), which is able to permeabilize and kill Gram-negative bacteria, and the α-defensins, which are small (3–4 kDa) cysteine-rich peptides that can permeabilize and kill a range of Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses.

Specific granules store unsaturated lactoferrin and lipocalin-2, which sequester iron away from bacteria in the phagosome to limit bacterial growth; transcobalamin II, which sequesters cyanocobalamin from bacteria; about 2/3 of the neutrophil lysozyme; collagenase, which cleaves type I collagen; phospholipase 2, which can degrade phospholipids; and hCAP-18, which is a cathelicidin that is cleaved by elastase to generate LL-37, a peptide that can kill Gram-negative and Gram-positive bacteria and may act synergistically with lactoferrin. Additionally, neutrophil specific granules contain a number of membrane proteins, such as flavocytochrome b, which are delivered to the plasma membrane or phagosomal membrane upon neutrophil activation, as described above.

Lysosomes contain acid hydrolases that are released into the phagolysosome after fusion of azurophil granules and are important for degrading a range of biomolecules necessary for bacterial growth. In addition, macrophages and neutrophils store cathelicidin-related antimicrobial peptides, which can damage and puncture microbial cell membranes. Note that neutrophils and macrophages utilize some common host defense mechanisms (*e.g.*, phagocytosis, NADPH oxidase, antimicrobial peptides), whereas other mechanisms are specific to only one type of phagocyte (*e.g.*, neutrophil granules). Thus, the phagocyte cell types have been adapted to their specific roles in host defense and work together to effectively eliminate pathogens.

Eosinophils are characterized by the presence of cytoplasmic crystalloid granules (also called secretory, specific, or secondary granules) that contain large stores of diverse, preformed cationic proteins, including eosinophil peroxidase (EPO), which is similar in function to MPO but catalyzes the formation of hypohalous acids with bromide and iodide (HOBr, HOI); major basic protein (MBP), which is involved in antiparasitic defense mechanisms as a cytotoxin for helminths and in immune hypersensitivity reactions; and eosinophil-associated RNases (EAR), which are RNase A-superfamily ribonucleases that have been proposed to play a role in defense against single-stranded RNA viruses. In addition, eosinophil granules contain a diversity of cytokines, chemokines, enzymes, growth factors, and receptors involved in innate immune defense processes. Interestingly, eosinophils mostly utilize selective secretion mechanisms to mobilize individual granule components (called piecemeal degranulation) rather than mass secretion of all contents at the same time, as appears to be the case with neutrophil granules. This allows eosinophils to respond appropriately to the stimulus or pathogen encountered.

Basophils are characterized by two types of granules (azurophil and specific granules), which represent stores of factors involved in the inflammatory response. As with eosinophils, basophil granule contents appear to be selectively mobilized by piecemeal degranulation. Basophil azurophil granules contain acid hydrolases and other enzymes. Basophil specific granules contain histamine, which is released in response to ligation of surface-associated FcεRI with IgE and increases capillary permeability to white blood cells and serum proteins; proteoglycans, such as heparin and chondroitin, which slow clotting and facilitate phagocyte recruitment; leukotrienes, such as leukotriene D_4, which also increases vascular permeability; and several cytokines. Notably, basophils secrete interleukin 4 (IL-4), which promotes T helper 2 (Th2) lymphocyte responses and is a key cytokine in the development of allergies and the production of immunoglobulin E (IgE).

Clearly, it is evident that phagocytes can utilize a wide array of forces to attack pathogens, and this represents a key component of the innate immune response. In addition, it is also clear that phagocytes require both oxygen-dependent and oxygen-independent microbicidal factors to be fully effective in their host defense functions. As seen in individuals with CGD, severe defects in oxygen-dependent microbicidal activity do compromise host defense; however, these individuals are still protected from a variety of pathogens, especially catalase-positive pathogens, through the implementation of oxygen-independent pathways. On the other hand, it has also been shown in protease-deficient animals that defects in oxygen-independent killing mechanisms also can also be detrimental to effective host defense.

Neutrophil Extracellular Traps (NETs)

Although the primary microbicidal mechanisms utilized by phagocytes are described above, more recent studies over the past 15 years have suggested an alternative defense mechanism whereby phagocytes trap and potentially kill microorganisms by forming neutrophil extracellular traps or NETs. They can be formed in response to infectious agents, inflammatory mediators, and/or under certain conditions, including non-specific osmotic cytolysis. NETs are composed of a web-like mesh of chromatin that is extruded from neutrophils undergoing NET formation in a process originally called NETosis. This mesh has been reported to be decorated with a variety of neutrophil proteins, including histones, MPO, elastase, cathepsin G, lactoferrin, and others. Thus, it appears that NETs can bind and sequester pathogens into a high local concentration of antimicrobial components in order to neutralize and kill these pathogens extracellularly but independent of phagocytic uptake. In addition to these microbicidal effects, NETs may serve as a physical barrier to prevent spread of the pathogens. It has also been proposed that NETs may keep potentially injurious granule proteases from diffusing away and damaging adjacent tissues.

NETosis requires ROS that are primarily produced by NADPH oxidase activation, although mitochondrial ROS production has been reported to substitute for the lack of an NADPH oxidase during basophil activation. In any case, ROS are required to trigger the activation and translocation of elastase from azurophil granules to the nucleus, where it degrades histones to disrupt chromatin packaging. This process is enhanced by MPO, which binds to nuclear DNA and works synergistically with elastase to decondense the chromatin, which is followed by rupture of the plasma membrane and extrusion of the DNA granule–protein complexes into the extracellular milieu to form NETs. Interestingly, this process does not seem to require MPO enzymatic activity, although the

mechanism is not clear. Finally, some stimuli, such as immune complexes, ionomycin and nicotine, and bacterial pore-forming toxins, trigger formation of NETs independent of ROS.

While NETs have been widely reported as an alternative mechanism for control and elimination of microbes, NETosis (as a cytolytic process) seems to be inconsistent with the high level of regulation exerted on neutrophil microbicidal functions because of the potential for host tissue damage resulting from inadvertent neutrophil lysis, release of cytotoxic agents, and post-lysis sequelae, such as inflammatory disorders. Indeed, NETs have been implicated in a number of inflammatory disorders, suggesting that neutrophil lysis and release of cytotoxic molecules may contribute pathological events in certain diseases.

Neutrophil Apoptosis and the Resolution of Inflammation

Many of the tissue-resident mononuclear phagocytes have an extended lifespan and/or self-renew, and the turnover rate for these cells is low. By comparison, there is enormous daily turnover of neutrophils ($\sim 10^{11}$ cells per day). During steady state or healthy conditions, neutrophils circulate in the bloodstream for a short period of time and then re-enter tissues. Inasmuch as neutrophils contain stores cytotoxic molecules that have potential to damage host tissues, it is critical that these cells are removed in a safe and timely manner. To that end, quiescent/unactivated neutrophils undergo constitutive or spontaneous apoptosis and are removed by macrophages. Importantly, as neutrophils undergo apoptosis, there is an accompanying decrease in their functional capacity (*e.g.*, reduced chemotaxis, phagocytosis, ROS production, and degranulation). This "shutdown" process likely limits unintended activation of cells marked for removal and/or helps prevent tissue damage should these cells lyse before removal. Uptake of apoptotic neutrophils by macrophages is termed efferocytosis and occurs in a non-inflammatory manner (Fig. 4(A)). These processes are integral to maintenance of host health and immune system homeostasis.

Phagocytosis-Induced Cell Death (PICD)

Neutrophil apoptosis and neutrophil turnover are altered during inflammatory processes, including following interaction with microbes. The short lifespan of neutrophils can be extended to some extent (a day or two during culture *in vitro*) by proinflammatory molecules, such as chemokines, cytokines, and some microbial products (*e.g.*, lipopolysaccharide). The ability of these molecules, many of which are priming agents, to delay the normal turnover of neutrophils is consistent with the increased host requirement for neutrophils during infection. On the other hand, phagocytosis alone can ultimately trigger neutrophil

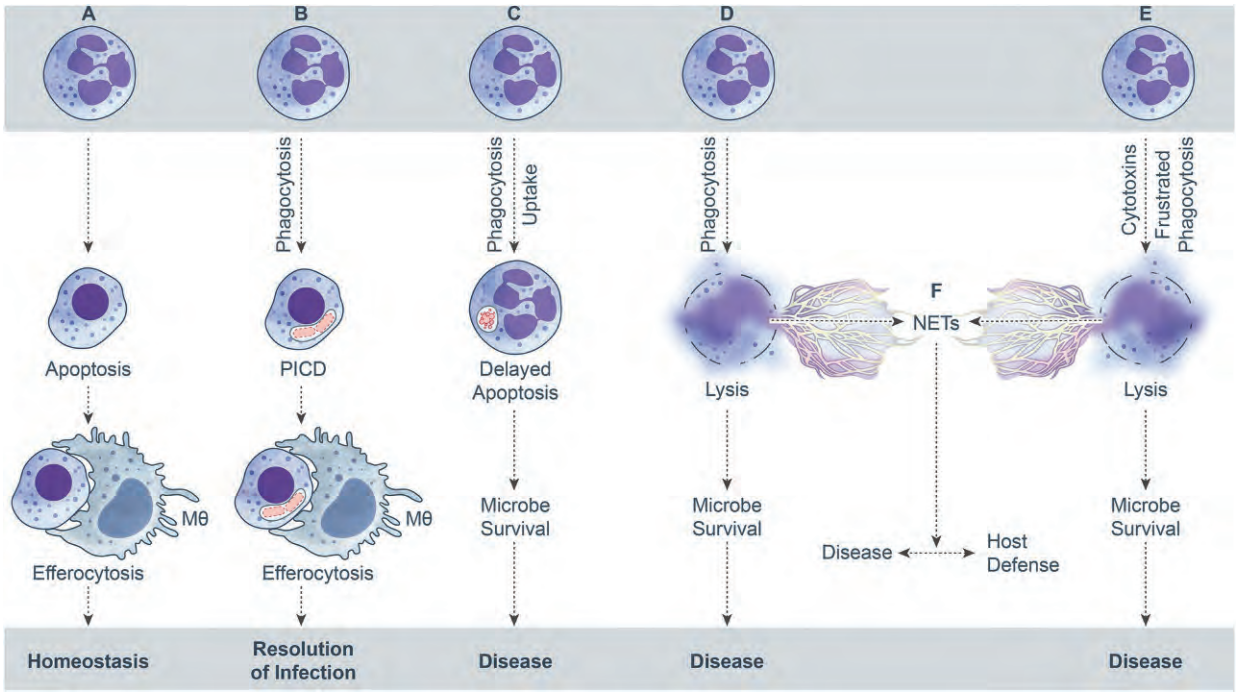

Fig. 4. Neutrophil turnover is influenced microorganisms. (A) Normal neutrophil turnover by spontaneous apoptosis and their removal by macrophages. (B) Neutrophil phagocytosis-induced cell death (PICD). (C) Bacteria-mediated delay in neutrophil apoptosis. (D) Neutrophil lysis after phagocytosis. (E) Lysis of neutrophil by pore-forming toxins. (F) Neutrophil extracellular traps can form during neutrophil lysis. This figure was originally published in Kobayashi, S.D., Malachowa, N., DeLeo, F.R., 2017. Front Cell Infect. Microbiol. 7, 159. https://dx.doi.org/10.3389/fcimb.2017.00159.

apoptosis – a process that has been named phagocytosis-induced cell death (PICD) (Fig. 4(B)). PICD occurs several hours following phagocytosis – after neutrophils have become effete – and is therefore a post-phagocytosis sequela.

PICD requires NADPH oxidase-derived ROS produced at relatively high levels, which can be achieved following phagocytosis of multiple target objects or microbes. For example, phagocytosis of one or two individual Gram-negative bacteria is insufficient to trigger PICD. Rather, induction of PICD *in vitro* often requires high bacteria-to-neutrophil ratios (*e.g.*, 5:1 or 10:1), ratios that likely exist only at the infection nidus. This is an important distinction, since neutrophils at sites of infection may exhaust all microbicidal capacity and thereby become effete, which explains the need for these cells to undergo PICD and be removed by macrophages. This "ROS threshold mechanism" thus limits PICD in neutrophils that retain microbicidal activity. Neutrophils from patients with CGD have impaired or absent PICD, underscoring the importance of NADPH oxidase-derived ROS in PICD. CGD patients often develop large granulomas (presumably the result of unresolved inflammation), a phenomenon consistent with a defect in the resolution of the inflammation response (*i.e.*, a defect in PICD).

Neutrophil PICD likely represents an important component of the healthy resolution of infection and the inflammatory response.

Microbial Pathogens and Altered Neutrophil Turnover

Although the vast majority of bacteria and fungi are eliminated readily by neutrophils, some microbes possess the means to circumvent destruction by neutrophils. In contrast to macrophages, which are often parasitized by intracellular microbes, few microbes survive within neutrophils. This phenomenon is explained in part by the increased microbicidal activity of neutrophils compared with macrophages and the short lifespan of neutrophils, which is not an optimal period of time for replication of intracellular pathogens. Nevertheless, *Anaplasma phagocytophilum* is an obligate intracellular bacterium that delays neutrophil apoptosis (and fails to trigger PICD) and replicates within an endosome like-vacuole. In doing so, *Anaplasma phagocytophilum* causes human granulocytic anaplasmosis.

Alternatively, some bacterial pathogens, such as *Staphylococcus aureus*, can cause neutrophil lysis after phagocytosis (Fig. 4(D)). Although most ingested *S. aureus* are killed by neutrophils, some of these bacteria survive and ultimately cause neutrophil lysis. This interesting cytolytic process, which occurs within a few hours after phagocytosis of *S. aureus*, may be an important means by which the pathogen disseminates during human infections. Thus, the ability of microbial pathogens to alter normal neutrophil apoptosis and turnover can result in human disease (Fig. 4).

Acknowledgments

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Phosphorus Dynamics in the Environment[☆]

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Glossary

Adsorption The physical adherence or bonding of organic and inorganic phosphate ions onto the surfaces of soil or mineral particles.
Assimilation (transitory immobilization) The process of incorporating nutrients into cellular biomass.
Authigenic Formed *in situ* rather than by having been transported or deposited in a location through secondary processes.
Chelation The reversible binding (complexation) of a ligand to a metal ion.
Decomposition The conversion of organic matter into simple inorganic compounds.
Diagenesis The process by which sediment undergoes chemical and physical changes during its lithification.
Eolian transport Advection of material via the atmosphere or wind.
Eutrophication Excessive nutrients in a body of water that causes a dense algal growth, followed by algal decomposition, anoxia, and impairment of the aquatic community.
Flux (phosphorus) Rate of flow of phosphorus per unit area, which has the dimensions [quantify]·[time]$^{-1}$·[area]$^{-1}$ (used for describing phosphorus turnover within a reservoir of flow from one reservoir to another.
Immobilization The process by which labile phosphorus is sequestered and removed from the environmental reservoir of bioavailable phosphorus for a period of time.
Inorganic phosphorus A class of chemical compounds that comprises those not existing in or derived immediately from living organisms (e.g., phosphate).
Mineral formation Processes in which anions react with cations in the environment to form insoluble precipitates.
Mineralization The conversion of organic matter into simple inorganic compounds by enzymes.
Mycorrhiza A symbiotic relationship between fungi and higher plants.
Nutrient limitation The cessation of photosynthetic biomass production in response to low availability of a physiologically important nutrient or nutrients.
Organic phosphorus A class of chemical compounds that comprises those existing in or derived from living organisms.
Orthophosphate Form of orthophosphoric acid (H_3PO_4) in which three protons have been lost (i.e., PO_4^{3-}).
Oxidation state The total number of electrons that an atom either gains or losses in order to form a chemical bond with another atom.
Phosphate Form of orthophosphoric acid (H_3PO_4); will be found as $H_2PO_4^-$ or HPO_4^- at pH 4–9, the pH range most common in nature.
Primary production The conversion of radiant or chemical energy into organic matter.
Productivity The rate at which radiant energy is used by producers to form organic substances as food for consumers.
Redox cycling Chemical reactions whereby the oxidation state of a molecule is either increased (oxidation) or decreased (reduction).
Soil solution The liquid phase of soil, surrounding mineral particles, from which plants and microbes draw nutrients.
Solubilization The conversion of insoluble compounds into soluble compounds.
Weathering Processes by which rocks, minerals, and soils are transformed through physical or chemical processes.

Defining Statement

This review includes a discussion of the global phosphorus cycle, including sources, sinks, and transport pathways of phosphorus in the environment, microbially-mediated transformations of phosphorus and their genetic regulation, and a discussion of microbial responses to anthropogenic changes to the phosphorus cycle.

[☆]*Change History*: April 2018. Adina Paytan, Barbara J. Cade-Menun and Benjamin Van Mooy updated the text and further readings to this entire article and added new Fig. 2 (Examples of phosphorus containing compounds associated with microbes). Specifically, two new authors were added (Cade-Menun and Van Mooy), and two new sections included ("Microbially-Mediated Processes", "Immobilization", "Oxidation-Reduction", and "Plant-Microbe Interactions").

This article is an update of K.R.M. Mackey, A. Paytan, Phosphorus Cycle, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 322–334.

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Introduction

Phosphorus is an essential nutrient for all living organisms, and the phosphorus cycle is an important link between Earth's living and non-living entities. The availability of phosphorus strongly influences primary production, the process by which photosynthetic organisms fix inorganic carbon into cellular biomass. Therefore, knowledge of the phosphorus cycle is critically important for understanding the global carbon budget and hence how biogeochemical cycles impact and are influenced by global climate.

The Global Significance of the Phosphorus Cycle

Natural assemblages of microbes have a critical role in the phosphorus cycle because they forge a link between phosphorus reservoirs in the living and non-living environment. Microbes facilitate the weathering, mineralization, and solubilization of non-bioavailable phosphorus sources, making phosphate available to microbial and plant communities and hence to higher trophic levels within the food web. However, microbes also contribute to immobilization of phosphorus, a process that diminishes bioavailable phosphorus by converting soluble forms to insoluble forms. The mechanisms of microbial involvement in these processes vary from passive (e.g., resulting from microbial metabolic byproducts) to highly regulated active contributions (e.g., the regulation of gene expression in response to environmental cues).

Microbially-mediated processes also link the phosphorus cycle to the carbon cycle and hence to global climate. During photosynthesis, photoautotrophs incorporate phosphorus and carbon at predictable ratios, with approximately 106 carbon atoms assimilated for every phosphorus atom for marine photoautotrophs. The phosphorus cycle therefore plays an important role in regulating primary productivity, the process in which radiant energy is used by primary producers to form organic substances as food for consumers, as in photosynthesis.

Because phosphorus is required for the synthesis of numerous biological compounds, its availability in the environment can limit the productivity of producers even when other nutrients are readily available. This fact carries important implications for the global carbon budget because the rate of incorporation (fixation) of carbon dioxide into photosynthetic biomass can be directly controlled by the availability of phosphorus; if phosphorus is not available, carbon dioxide fixation is halted. The intersection of the phosphorus and carbon cycles is of particular significance for global climate, which is affected by atmospheric carbon dioxide levels. Hence, through the growth of producers the phosphorus cycle contributes to the regulation of global climate.

In aquatic environments, photosynthetic microbes (i.e., phytoplankton) may comprise a substantial portion of the photosynthetic biomass and hence primary production. Marine and freshwater phytoplankton are characterized by extensive biodiversity and, as a group, inhabit an incredible number of different niches within aquatic environments. Phytoplankton, like other microbes, have strategies that enable them to adapt to changes in the amounts and forms of phosphorus available within their environment. For example, the production of phosphatase enzymes, which hydrolyze complex phosphorus compounds to simple, bioavailable inorganic phosphate, helps to mediate the mineralization of organic phosphorus compounds in surface waters, and allows cells to adapt when phosphate levels are low.

Recent estimates suggest that phytoplankton are responsible for as much as half of global carbon fixation, thereby contributing significantly to the regulation of Earth's climate. Phytoplankton productivity is strongly influenced by nutrient availability, and the nutrient that ultimately limits production depends on (1) the relative abundance of nutrients and (2) the nutritional requirements of the phytoplankton. With some exceptions, production in most lakes is limited by the availability of phosphorus, whereas limitation of primary production in the ocean has traditionally been attributed to nitrogen, another nutrient required by cells in large quantities. However, phosphorus differs from nitrogen in that the major source of phosphorus to natural aquatic environments is the weathering of minerals on land that are subsequently introduced into water bodies by fluvial and eolian sources. In contrast, microbially-mediated nitrogen fixation, in which bioavailable forms of nitrogen are generated from nitrogen gas in the atmosphere, is a major pathway by which phytoplankton gain access to nitrogen (in addition to continental weathering). Because there is no phosphorus input process analogous to nitrogen fixation, both marine and terrestrial productivity over geological time scales are considered to be a function of the supply rate of phosphorus from weathering and the rate at which phosphorus is recycled in soil or aquatic systems. Accordingly, the phosphorus cycle influences phytoplankton ecology, productivity, and carbon cycling in both marine and freshwater ecosystems as well as those of natural and agriculture vegetation systems on land.

Characterizing and Measuring Environmental Phosphorus Pools

Occurrence of elemental phosphorus in the environment is rare, given that it reacts readily with oxygen and combusts when exposed to oxygen; thus, phosphorus is typically found in nature bound to oxygen. Phosphorus has the chemical ability to transfer a 3s or p orbital electron to a d orbital, permitting a relatively large number of potential configurations of electrons around the nucleus of the atom. This renders the structures of phosphorus-containing molecules to be quite variable. These properties are likely responsible for the ubiquity and versatility of phosphorus-containing compounds in biological systems.

Because phosphorus exists in many different physical and chemical states in the environment, specific definitions are needed to clarify different parts of the phosphorus pool. Chemical names reflect the chemical composition of the phosphorus substance in question, whereas other classifications are based on methodological aspects of how the substance is measured. For example, "orthophosphate" is a chemical term that refers specifically to a phosphorus atom bound to four oxygen atoms, forming the

orthophosphate molecule (PO_4^{3-}); at pH 4–9, the range typically found in nature, this would exist as $H_2PO_4^-$ or HPO_4^{2-} (which are referred to as phosphate). In contrast the aquatic term "soluble reactive phosphorus," (SRP), is a methodological term referring to everything that gets measured when a phosphate assay is performed (such as the molybdate blue or ascorbic acid method, described below). The majority of measured SRP comprises phosphate but other forms of phosphorus may be included as well due to experimental inaccuracy. Therefore, while SRP tends to closely reflect the amount of phosphate in a sample, the values may not be identical.

The most common assay for measuring SRP is the ascorbic acid method, which is approved by the US Environmental Protection Agency for monitoring phosphate in environmental samples. In this method, ascorbic acid and ammonium molybdate react with dissolved phosphate in the sample, forming a blue compound that can be observed visually or determined spectrophotometrically. Assays for measuring total phosphorus are also based on the ascorbic acid method but begin with a step to transform all phosphorus in the sample to phosphate (typically through digestion by heating the sample in the presence of acid). Following digestion, the sample is analyzed by the ascorbic acid method.

When phosphorus is measured in water samples, distinguishing forms that are part of particulate matter from those that are in solution is often useful. Phosphorus is therefore classified as "soluble" or "particulate". Soluble phosphorus includes all forms of phosphorus that are distributed in solution and that pass through a filter with a given pore size (typically 0.45 μm), while the particulate fraction is the amount retained on the filter. Measurements of soluble phosphorus include both the "dissolved" and "colloidal" fractions. Dissolved phosphorus includes all forms that have entered a solute to form a homogenous solution. By contrast, colloidal forms include any tiny particles that are distributed evenly throughout the solution but that are not dissolved in solution. Soluble phosphorus is commonly reported because colloidal phosphorus particles are very small and differentiating between colloidal and dissolved phosphorus is methodologically difficult.

In soils, soil test phosphorus (STP) is a similar measurement to SRP, in that it measures labile or easily accessible phosphorus in the soil solution surrounding soil particles. There are several different methods to measure STP, each of which involves extracting a soil sample with an extractant, then measuring phosphate in solution with a colorimetric method such as the ascorbic acid method. The extractant will depend on soil pH, among other factors, and includes Bray P1 (dilute hydrochloric acid and ammonium fluoride), for low pH soils, and Olsen P (dilute sodium bicarbonate) for higher pH soils. Although STP is sometimes referred to as "available phosphorus", this is not correct; these tests are designed to predict a crop response to phosphate fertilizer. Other methods to estimate soil bioavailable phosphate include placing anion exchange resins into soil for set periods of time. Total soil phosphorus is determined by digestion, as previously described for water, as is total plant phosphorus. The phosphorus contained in living soil microbial biomass is determined in extracts before and after soil fumigation with chloroform; the chloroform will kill any living organisms, causing them to release phosphorus into the extract.

When living organisms assimilate phosphorus into their cells, the resulting phosphorus-containing compounds are collectively called "organic phosphorus." It must be stressed that this definition of the biologically-associated phosphorus as "organic phosphorus" is not identical to the chemical definition of organic compounds (e.g., containing carbon). For example, some intracellular biologically-synthesized compounds such as polyphosphate may not contain carbon bonding. The term organic phosphorus encompasses molecules within living cells as well as molecules that are liberated into the environment following decay of organisms. The major source of terrestrial organic phosphorus is plant material, which is released as vegetation decomposes, but microbial and animal sources also contribute significantly. In the marine environment, organic phosphorus comes from a variety of sources (e.g., plankton, fish excrement, advection from land, etc.), the relative contributions of which differ widely depending on location in the ocean. In contrast to organic forms, inorganic phosphorus compounds are not always directly of biogenic origin. Rather, they also include phosphate derived from the weathering of phosphorus-containing minerals, including soluble and particulate phosphate.

Although phosphate can be identified and quantified by simple colorimetric techniques, identifying and quantifying more complex phosphorus forms requires the use of advanced techniques and instruments, such as mass spectrometry or ^{31}P nuclear magnetic resonance (NMR) spectroscopy. These more complex phosphorus forms are discussed in detail in the section on Immobilization, below.

Phosphorus Sources, Sinks, and Transport Pathways

The phosphorus cycle encompasses numerous living and non-living environmental reservoirs and various transport pathways. In tracing the movement of phosphorus in the environment, the interplay between physical and biological processes becomes apparent. In addition to acting as reservoirs of phosphorus in the environment (as discussed in this section), microbes contribute to the transformation of phosphorus within other reservoirs such as in soil or aquatic environments (discussed below in Section "Microbially-Mediated Processes").

Within the earth's crust, the abundance of phosphorus is 0.10%–0.12% (on a weight basis), with the majority of phosphorus existing as inorganic phosphate minerals and phosphorus-containing organic compounds. A phosphate mineral is any mineral in which phosphate anion groups form tetrahedral complexes in association with cations, although arsenate (AsO_4^{3-}) and vanadanate (VO_4^{3-}) may also substitute into the crystalline structure. Apatite is the most abundant group of phosphate minerals, comprising hydroxyapatite, fluorapatite, and chlorapatite (Table 1). These three forms of apatite share nearly identical crystalline structures, but differ in their relative proportions of hydroxide, fluoride, and chloride, each being named for the anion that is most abundant in the mineral. Phosphate minerals generally form in the environment in magmatic processes or through precipitation from solution

Table 1 Phosphate minerals and their chemical compositions. Apatite is the general term for the three minerals hydroxylapatite, fluorapatite, and chlorapatite

Apatite	*$Ca_5(PO_4)_3(F,Cl,OH)$*
Hydroxylapatite	$Ca_5(PO_4)_3OH$
Fluorapatite	$Ca_5(PO_4)_3F$
Chlorapatite	$Ca_5(PO_4)_3Cl$
Frankolite	$Ca_{10-a-b}\,Na_aMg_b(PO_4)_{6-x}(CO_3)_{x-y-z}(CO_3F)_y(SO4)_zF_2$
Lazulite	$(Mg,Fe)Al_2(PO_4)_2(OH)_2$
Monazite	$(Ce,La,Y,Th)PO_4$
Pyromorphite	$Pb_5(PO_4)_3Cl$
Strengite	$FePO_4{\cdot}2H_2O$
Triphylite	$Li(Fe,Mn)PO_4$
Turquoise	$CuAl_6(PO_4)_4(OH)_8{\cdot}5H_2O$
Variscite	$AlPO_4{\cdot}2H_2O$
Vauxite	$FeAl_2(PO_4)_2(OH)_2{\cdot}6H_2O$
Vivianite	$Fe_3(PO_4)_2{\cdot}8H_2O$
Wavellite	$Al_3(PO_4)_2(OH)_3{\cdot}5H_2O$

(which may be microbially-mediated), and the chemical composition of the minerals depends on the ion or ions present in solution at the time of precipitation. For this reason, it is not uncommon for natural deposits of phosphate minerals to be heterogeneous, rather than composed of a single type of phosphate mineral. These natural deposits of phosphate minerals are collectively called "phosphorites" to reflect variations in their chemical compositions.

Soils and lake sediments are another terrestrial reservoir of phosphorus (Fig. 1). The phosphorus in recently formed soils will resemble the parent material on which the soil was formed, namely apatite and other phosphate minerals. Over time, the soil forming factors of climate (rainfall and temperature), topography and biota will transform phosphorus into other forms in conjunction with soil development. In warm, high-rainfall environments such as the tropics, cations will be lost with leaching

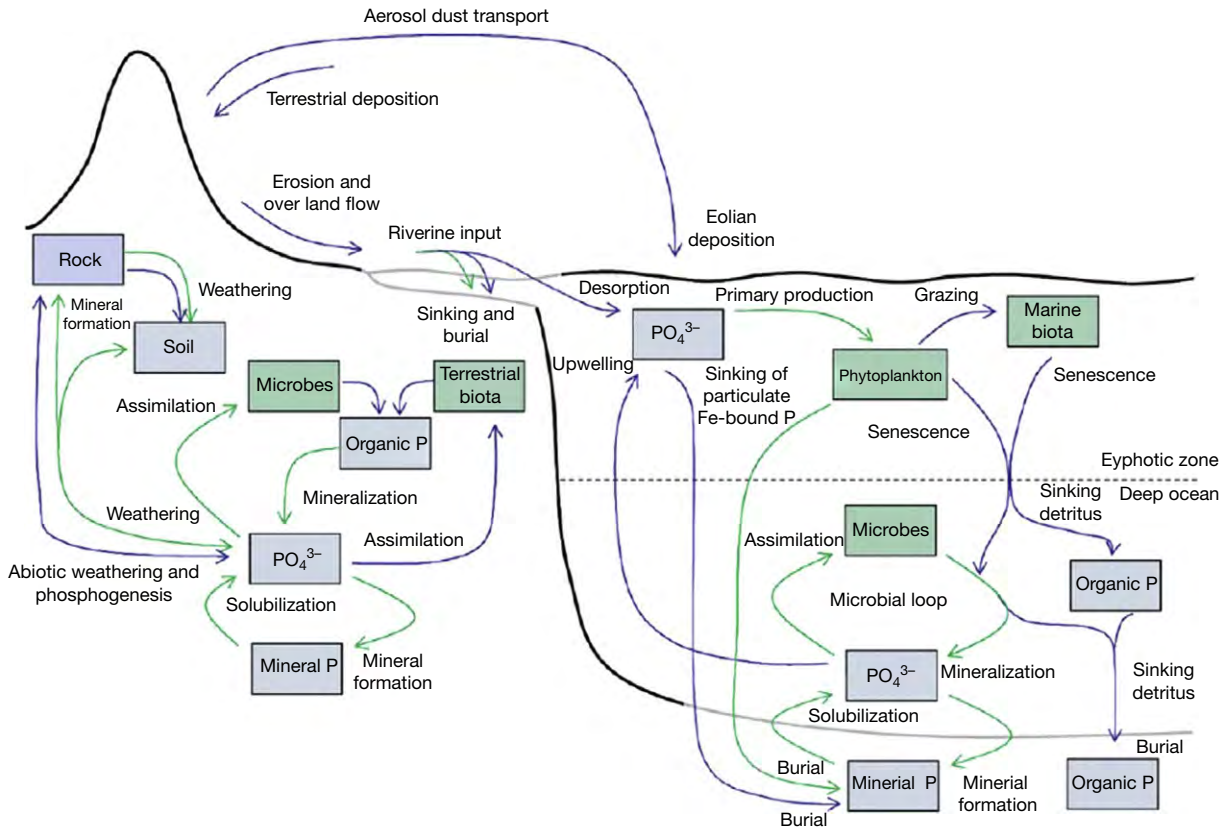

Fig. 1 Schematic diagram of the phosphorus cycle showing phosphorus reservoirs (living in green boxes; non-living in blue boxes), physical transport pathways (blue arrows), and microbially-mediated transformations (green arrows).

and the soil pH will drop. Phosphorus forms in these soils will shift from associations with calcium and magnesium to associations with iron and aluminum. Phosphorus in soil is chemically controlled by precipitation with cations such as calcium and magnesium (here termed 'mineral formation'), by adsorption (the physical adherence or bonding of organic and inorganic phosphate ions onto the surfaces of soil or mineral particles) onto iron or aluminum oxyhydroxides, and by microbially-mediated mineralization of organic phosphorus. Mineralization is discussed in detail in the Section "Microbially-Mediated Processes".

Plants and microbes remove phosphate from the soil solution surrounding mineral particles. This creates a gradient, and the soil solution phosphate is replenished by desorption from mineral surfaces, by dissolving precipitated phosphorus forms or by mineralizing organic phosphorus. Plants draw inorganic phosphorus from lower soil depths and convert it to organic forms. These will be deposited on the soil surface and mixed into the soil by organisms such as earthworms. Over time, phosphorus will become stratified in soil in undisturbed natural ecosystems, with higher concentration and more organic forms at the soil surface and lower concentrations and more inorganic forms with depth. Land use can alter the forms and distributions of phosphorus in soils. Soils managed as croplands may have phosphorus added as fertilizers and pesticides, and will have phosphorus removed from crops, while animals grazing in pastures add phosphorus as urine and feces and remove phosphorus as they eat plant material. Management practices such as tillage will redistribute phosphorus forms within the profile, while fire can convert organic phosphorus to inorganic phosphate.

Marine and lake sediments also represent an important phosphorus reservoir, but because the physical and chemical factors affecting marine sediment differ considerably from those on land, some processes controlling phosphorus dynamics in aquatic sediments are different than for soils, although many are the same. In sediments, phosphate can be present in insoluble inorganic phosphates minerals (such as phosphorites), which are relatively immobile. Phosphate can also be adsorbed onto iron or manganese oxyhydroxides. The adsorbed phosphate can regain mobility in response to changes in the redox potential at the sediment-water interface and thus is considered more mobile. As in soil, phosphorus in aquatic detrital organic matter can also become remobilized as decomposition progresses through microbially-mediated processes.

Biota (i.e., microbes, plants, and animals) serve as another reservoir of phosphorus in the environment, as they assimilate phosphorus within their cellular biomass. Biota can contribute significantly to environmental phosphorus levels; for example, microbial communities contribute 0.5%–7.5% of total phosphorus in grassland and pasture topsoil, and up to 26% in indigenous forests. Microbes are also responsible for generating the myriad of organic phosphorus compounds found throughout the environment. In particular, microbes and primary producers play an important role in providing nutrition, including phosphorus, to higher trophic levels by making it biologically available (bioavailable). Phosphorus assimilation is a microbially-mediated process discussed in the "Transitory immobilization" section below.

Phosphorus is transported within the environment through various mass transfer pathways. For example, rivers are important in the phosphorus cycle as both reservoirs and transport pathways. Phosphorus that has weathered from minerals and leached or eroded from soils enters rivers through a variety of vectors, including dissolved and particulate forms in water from overland flow and in groundwater, and particulates brought by wind. Approximately 95% of phosphorus in rivers is particulate, and approximately 40% of that is bound within organic compounds. Rivers influence the distribution of phosphorus in soils and lakes by contributing or removing phosphorus, and riverine input is the single largest source of phosphorus to the oceans.

A number of outcomes are possible for phosphorus entering the ocean. Much of the phosphorus entering from rivers is trapped in near-shore areas of the ocean, such as continental margins and estuaries, through immediate sedimentation and biological assimilation. The remaining phosphorus enters the dynamic surface ocean, also called the euphotic zone, in which nearly all bioavailable phosphorus is sequestered within biota through primary production. Upon death of the organisms, a fraction of the biologically-sequestered phosphorus sinks below the euphotic zone and most of it is regenerated into bioavailable forms like phosphate by heterotrophic organisms. This recycling is part of the so-called "microbial loop". Physical processes such as upwelling and deep convective mixing draw the deep water, which in most parts of the ocean is nutrient rich compared to the surface waters, back into the euphotic zone where up to 95% of it is re-used in primary production. The remainder is removed from the ocean reservoir through particulate sedimentation, mineral formation (which may be microbially-mediated), and scavenging by iron and manganese oxyhydroxides, all of which deposit phosphorus as a component of ocean sediment.

The phosphorus cycle differs from the cycles of other biologically-important elements, such as carbon, nitrogen, and sulfur, in that it lacks a significant gaseous component; nearly all phosphorus in the environment resides either in solid or aqueous forms. The one exception to this rule is the volatile compound phosphine (PH_3, also called phosphane), a colorless, poisonous gas formed in the environment from the breakdown of alkali metal or alkali earth metal phosphides with water. This process is poorly characterized and likely comprises various multistage chemical reactions. Microbially-mediated phosphine production can be a major source of the gas in engineered systems (e.g., sewage treatment facilities and constructed wastewater treatment wetlands) where organic phosphorus is abundant and reducing conditions are common, suggesting that microbes could also play a roll in phosphine formation in natural systems (although direct enzymatic production of phosphine has not yet been identified). While phosphorus can exist as phosphine, the gas does not persist in the environment due to rapid autoxidation, preventing significant accumulation of phosphine in the atmosphere. Phosphine is therefore a minor component of the environmental phosphorus pool.

The absence of a significant gaseous phase does not eliminate the atmosphere as an important reservoir in the phosphorus cycle. When weathering and erosion of soil generate inorganic and organic particulate phosphorus, wind transports some of the particles from their source to a new location. These particles can include mineral dust, pollen and plant debris, insect fragments, and organic

phosphorus bound to larger particles. This distribution of terrestrial particulate phosphorus, termed eolian deposition, plays an important role in delivering nutrients to the oceans. In ocean waters where nutrient levels are naturally low, such as in the open ocean gyres where riverine inputs do not extend and significant upwelling does not occur, eolian deposition may comprise a large portion of the nutrient flux that is available for primary production. The eolian phosphorus flux to the oceans is approximately 1×10^{12} g yr^{-1}, of which approximately half is organic and half is inorganic. The solubility, and therefore bioavailability, of the phosphorus in eolian particulate matter differs significantly depending on its source; however, estimates suggest that approximately 15%–50% is typically soluble.

Microbially-Mediated Processes

Immobilization

Immobilization refers to the process by which phosphorus is sequestered and removed from the environmental reservoir of labile phosphate for some time. Immobilization processes can generally be grouped into two categories. The first category, transitory immobilization or cellular assimilation, includes all processes that sequester phosphorus within living microbial cells and is rapidly reversible upon cell death, although some phosphorus may be incorporated into non-labile organic compounds. The second category, mineral formation, encompasses processes that generate phosphorus-containing minerals.

Transitory immobilization

Transitory immobilization, or assimilation, is an important mechanism of phosphorus sequestration in soil and aquatic environments. Within cells, phosphorus is incorporated in numerous essential biological-molecules and is required in larger quantities than many other elements. However, unlike other biologically important nutrients such as nitrogen and sulfur that must first undergo reduction prior to being incorporated into the cell, phosphorus remains oxidized before and after assimilation. Because mineralization of cellular material occurs rapidly following cell death, cellular assimilation of phosphorus into biological macromolecules leads to relatively short-term phosphorus retention in living cells where the duration is related to the characteristics of the microbial community in question, and to the nature of the compounds produced. Within the cellular reservoir, phosphorus is present as different compounds that serve various functions.

Complex phosphorus compounds are generally classified based on the bonding of phosphate (Fig. 2). Phosphomonoesters are compounds in which one phosphate group is linked to one carbon atom through an ester bond (through oxygen), phosphodiesters have one phosphate group linked to two separate carbon atoms, each through an ester bond, and phosphonates have one carbon-oxygen-phosphorus bond replaced by a direct carbon-phosphorus bond. Phosphoramidates contain a nitrogen-phosphorus bond in place of a carbon-oxygen-phosphorus bond; however, these are rare compared to other phosphorus compounds, and are poorly studied for microbes, and will not be discussed further in this article. Polyphosphates have phosphate groups linked by energy-rich phosphoanhydride bonds.

Phospholipids, lipids in which a phosphate group has replaced one of the fatty acid groups, are phosphodiesters. Lipids are generally hydrophobic; however, phospholipids are amphipathic, meaning that each molecule has a hydrophilic portion and a hydrophobic portion. The phosphate group is responsible for giving phospholipids their partially hydrophilic character, hence imparting a wide range of biochemical properties. In the cell, phospholipids are important in the formation of biological membranes and in some signal transduction pathways. The cell walls of gram-positive bacteria such as *Bacillus subtillis* and *Staphylococcus aureus* contain lipoteichoic acid (LTA), which contain long chains of ribitol phosphate or glycerol phosphate. Gram-positive bacteria cause many human infections (e.g., staph infections), which is linked to LTA. Phosphonolipids have phosphonates attached to lipids instead of phosphates. Phosphonolipids are wide-spread in nature; one example is 2-acyloxyethylphosphonate, which has been isolated from blooms of the aquatic cyanobacterium *Aphanizomenon flos-aquae*.

Nucleotides are biological compounds consisting of a pentose sugar, a purine or pyrimidine base, and one or more phosphate groups. Nucleotides are the structural subunits (monomers) of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and alternating bonds between the sugar and phosphate groups form the backbones of these nucleic acids. The phosphate groups form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings, imparting directionality to the molecule. In addition, nucleotides are present as several major cofactors in the cell (e.g., flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADP), etc.) that have important functions in cell signaling and metabolism.

Adenosine triphosphate (ATP) is a nucleotide that performs multiple functions of considerable importance to the cell and contains polyphosphate and phosphomonoester bonds. The primary function of ATP is to aid in intracellular energy transfer by storing energy that is generated during photosynthesis and respiration so that it can be used in other cellular processes that require energy (e.g., cell division and biosynthetic reactions). In the cell, phosphate can be assimilated in ATP through substrate-level phosphorylation, oxidative phosphorylation, photo-phosphorylation, and via the adenylate kinase reaction. ATP is also active in signal transduction pathways, where it can be used as a substrate for kinase enzymes in reactions that transfer phosphate groups to proteins and lipids, forming phosphoproteins and phospholipids respectively. Phosphorylation is an important and ubiquitous form of signal transduction in many organisms.

Inositol phosphates are phosphomonoesters and are complex molecules in which one to six phosphates are bonded with carbons on the inositol ring. Inositol phosphates with six phosphate groups, inositol hexaphosphates (IHP) are more common in

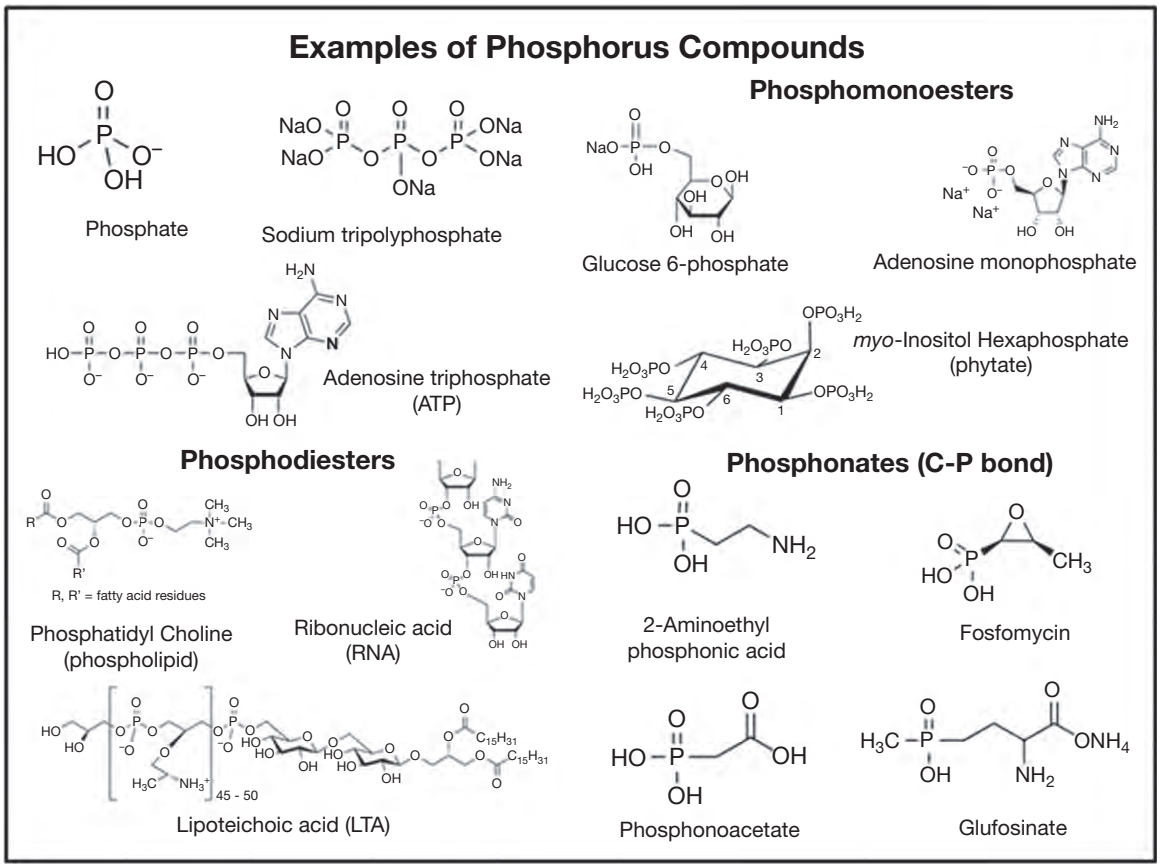

Fig. 2 Examples of phosphorus containing compounds associated with microbes.

nature than those with fewer phosphate groups, and the best-known and studied of these is the *myo*-IHP stereoisomer phytate. This compound is widely distributed in nature because it is a storage compound produced by higher plants, especially in seeds. Phytate is a highly reactive compound that forms stable complexes with a variety of mineral cations, and so can accumulate in soils. There is no evidence that phytate is synthesized by microbes. However, other IHP stereoisomers have been detected in soils and lake sediments, including *chiro*-, *scyllo*- and *neo*-IHP, and are thought to be produced by microbes by direct synthesis or by microbial transformation of phytate.

Other phosphomonoesters include intermediaries in many biochemical pathways, such as phosphoproteins and sugar phosphate such as phosphoenol pyruvate and glucose 6-phosphate, as well as mononucleotides such as adenosine monophosphate.

Phosphonates are widespread in terrestrial and aquatic environments. However, their role is poorly understood, but the C-P bond is thought to be more resistant to degradation than the more common ester bonds. In addition, some microbially-synthesized phosphonates have anti-microbial properties, providing protection. Some of these have been developed for commercial use. For example, some soil *Streptomyces* species produce glufosinate (phosphinothricin), which is used as a herbicide, while other soil *Streptomyces* species produce fosfomycin, which is used as a broad-spectrum antibiotic. In oceans, dissolved phosphonates have been shown to comprise up to a quarter of all high molecular weight dissolved organic phosphorus. Although the microbial sources of phosphonates are not fully understood, two genera of cyanobacteria and one species of *Thaumarchaeon* have been definitively implicated.

In addition to biological molecules like nucleic acids and phospholipids, which by weight consist primarily of carbon along with smaller portions of phosphorus, the intracellular accumulation of polyphosphate granules in microbial cells is an important type of transitory phosphorus immobilization. Polyphosphates form through the activity of polyphosphate kinase enzymes, and therefore represent a highly concentrated reservoir of phosphorus in the cell. The energy stored in the anhydride bonds can be used by the microbial cell during periods of starvation. Polyphosphates may also serve as ligands for metal cations such as calcium, aluminum, and manganese in some microbes; however, the extent and elemental stoichiometry of intracellular polyphosphate cation complexation varies among organisms. Short-chain polyphosphates, with only two phosphate groups, are called pyrophosphate.

Phosphorus immobilized within cells is considered transitory because many phosphorus compounds can rapidly reenter the reactive phosphate pool following cell death, through microbially-mediated mineralization processes. The immobilization of phosphorus via cellular assimilation is therefore relatively brief (i.e., within the lifetime of a cell) compared to other

processes in the phosphorus cycle, some of which occur over geological time scales. However, the strong adsorption to mineral surfaces in soils and sediments by some phosphorus compounds such as IHP stereoisomers and DNA will increase their immobilization.

Phosphate mineral formation

Phosphate mineral formation represents another phosphorus sink that is influenced by microbial activity; however, it encompasses processes other than cellular assimilation and short-term storage of phosphorus as biological molecules. In mineral formation (also called phosphogenesis), phosphate anions react with cations in the environment to form insoluble precipitates. Sediments that comprise significant amounts of phosphorus-containing minerals are called "phosphorites," and may contain apatite, francolite, and other phosphorus minerals. Mineral formation is generally an important mechanism of phosphorus sequestration in marine environments, where the high seawater calcium levels facilitate microbially-mediated formation of insoluble phosphorites. The sequestration of phosphorus by this process retains (immobilizes) phosphorus for longer periods of time than does transitory immobilization.

In order for an insoluble mineral to form, the concentration of the ions forming the mineral should be high enough such that super saturation is reached, and equilibrium of the precipitation reaction is shifted toward the product. In addition, the physical and chemical characteristics of the environment must be conducive to precipitation of that mineral based on its solubility characteristics, and factors such as pH, redox state, and concentrations of co-occurring ions all influence mineral precipitation. The mineral should also be stable in the environment for mineral formation to constitute a long-term sink for phosphorus.

Mineral formation occurs both authigenically and diagenetically in the environment. Authigenic mineral formation is the formation of insoluble precipitates (minerals) *in situ* rather than by having been transported or deposited in a location through secondary processes. In contrast, diagenetic mineral formation is the alteration of existing minerals by chemical changes occurring after the initial deposition of a mineral (i.e., during or after burial and lithification). The primary type of diagenetic phosphorus mineral formation in marine environments is the substitution of phosphate into calcium carbonate minerals such as calcite or aragonite. Microbes contribute to this process by mineralizing organic phosphorus to reactive phosphate, which then substitutes diagenetically into calcium carbonate.

In authigenic mineral formation, microbes may generate reactive phosphate from the mineralization of organic phosphorus sources, and the resulting localized high phosphate concentrations favor precipitation of phosphate minerals. In soils, microbes convert organic phosphorus to phosphate, increasing its concentration in the soil and promoting formation of stable minerals such as apatite. In productive areas of the ocean, microbial mineralization of detrital matter at the sediment-water interface generates reactive phosphate, some of which reacts with seawater calcium to form phosphorite. (Reactive phosphate that does not contribute to mineral formation is available for biological assimilation by benthic microbes or may be reintroduced to the euphotic zone by diffusion and upwelling for use by phytoplankton).

The accumulation of phosphorus in microbial cells during transitory immobilization also contributes to mineral formation by increasing the pool of phosphate that could react with cations to form minerals. This is particularly important in anoxic areas of the ocean and soils where phosphate levels are low. Under oxic conditions where phosphate is more abundant, luxury uptake and storage of phosphate as polyphosphate molecules occurs in some microbes (e.g., *Pseudomonas spp.*, *Actinobacter spp.*), as discussed above. Cells use the energy stored in polyphosphates to activate an alternative organic electron acceptor when conditions shift toward anoxia, freeing substantial levels of phosphate in the process. The sequestration and release of phosphate by the cell under oxic and anoxic conditions respectively represents a mechanism by which microbes contribute to mineral formation because it generates locally-elevated phosphate concentrations near the cells; these concentrations could be high enough to induce precipitation of minerals. Accumulation of phosphate within cells may also lead to phosphorus immobilization via mineral formation if phosphorus minerals are generated and stored within the cell. Intracellular formation of mineral apatite is an example of phosphorus immobilization that has been observed in some microbes (e.g., *Escherichia coli*, *Bacterionema matruchotii*) following incubation with calcium phosphate at a slightly basic pH. This process occurs in living and dead cells, suggesting that the locally-elevated phosphate concentrations within the cell help initiate apatite formation. Similarly, the formation of carbonate fluorapatite following cell death has been observed in Gram-negative rods, possibly pseudomonads, in coastal marine sediment, and is thought to be an important phosphorite formation process in locations where sedimentation rates are low.

Weathering

Rock material exposed to the atmosphere breaks down, or weathers, as the result of numerous environmental processes. Weathering processes are classified into two categories. In mechanical weathering, physical processes (including thermal expansion, pressure release, hydraulic action, salt crystal formation, freeze-thaw, and frost wedge events) cause deterioration of rock material without changing the chemical composition of the parent material. In contrast, chemical weathering causes deterioration by altering the chemical structure of the minerals from which the rock is made. Chemical weathering processes include dissolution, hydrolysis, hydration, and oxidation-reduction (redox) reactions. Living organisms can contribute to mechanical weathering by altering the microenvironments at the surface of the parent material (e.g., by increasing local humidity or forming bio-films on surfaces); however, most biological weathering processes are classified as chemical weathering because they chemically alter the composition of the parent rock material directly or indirectly. These biological weathering processes are also referred to as solubilization.

Solubilization

Inorganic phosphorus can occur in nature in soluble and insoluble forms. The solubility of the most abundant form of inorganic phosphorus, phosphate, is determined by the ambient pH and the cation to which it is bound as a mineral (e.g., K^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, and Al^{3+}). Microbially-mediated phosphorus solubilization plays an important role in the conversion of insoluble phosphorus minerals to soluble forms of phosphorus. Solubilization directly benefits the microbes that perform it by providing the bioavailable phosphorus needed for growth. Similarly, the process benefits other organisms (including other cells and higher plants) from the surplus of solubilized phosphorus.

Production of organic and inorganic acids is the primary mechanism of microbial phosphorus solubilization. In this process, biogenic acids interact with phosphorus minerals to form phosphates, thereby bringing phosphorus into solution. Chemoautotrophic bacteria (e.g., nitrifying bacteria and *Thiobacillus spp.*) generate inorganic acids (e.g., nitric and sulfuric acid) by oxidizing ammonium and sulfur respectively, and these acids can liberate soluble phosphorus from apatite, the most abundant phosphorus mineral. Production of organic acids occurs in numerous microbial taxa and can also contribute to the solubilization of phosphorus minerals. Many higher plants can also produce organic acids in their rhizosphere (the area adjacent to plant roots) to solubilize phosphorus. Many organic acids, such as citrate, oxalate, lactate and 2-ketogluconate, are chelating compounds. Chelation is the reversible binding (complexation) of a ligand to a metal ion. Chelators increase the solubility of insoluble phosphate mineral salts by complexing the metal cations, thereby making dissolution of the salt more energetically favorable.In addition to acid production, microbially-mediated redox reactions contribute to phosphorus solubilization through the reduction of iron oxyhydroxides and associated ferric (Fe^{+3}) phosphate (strengite). In this process, dissimilatory iron reduction of ferric phosphates liberates soluble ferrous (Fe^{+2}) iron as well as the phosphate associated with it. This occurs under reducing conditions, such as in flooded, anoxic soils and in some benthic (e.g., the sediment surface and some sub-surface layers) aquatic environments. In another redox process, hydrogen sulfide (H_2S) produced by sulfur-reducing bacteria reduces the ferric iron in iron phosphate ($FePO_4$) to ferrous iron. In this reaction iron sulfide and elemental sulfur are precipitated, and phosphate is generated.

Mineralization

Plant and animal detritus in the soil and in particulate and dissolved forms in aquatic environments comprise a large reservoir of organic phosphorus. However, because complex phosphorus forms such as those bound to carbon are generally unable to cross cell membranes, most of the organic phosphorus from the detrital pool is not directly available to many living organisms. To become bioavailable, phosphorus bound to organic material must be converted to phosphate by mineralization, which is accomplished through the activity of a number of enzymes, many of which are produced by microbes. Because this process makes nutrients available that would otherwise be sequestered in unavailable forms, mineralization provides a vital link between the detrital pool and living organisms. It is estimated that approximately 70%–80% of microbes can participate in phosphorus mineralization. As in many enzyme-catalyzed systems, mineralization is encouraged by higher levels of available substrate; however, high levels of inorganic phosphate (the product) do not necessarily impede the reaction, and mineralization will often occur even if an abundance of phosphate is present. Ambient conditions favoring phosphorus mineralization include those that also favor mineralization of other elements, such as optimal temperatures and pH values for the specific enzyme; in soil, adequate but not excessive soil moisture is also required.

Enzymes involved in mineralization comprise a diverse group of proteins, called phosphatases, with a broad range of substrates and substrate affinities, and varying conditions for optimal activity. In addition, phosphatases can either be constitutively expressed by an organism (produced all the time), or facultatively expressed under conditions of low phosphate (and in some cases, low carbon). The microbial phosphatases can be classified into general broad categories such as phosphonatases, phosphomonoesterases and phosphodiesterases, or into more specific categories such as nucleases or phytases, depending on the compounds they will hydrolyze. Phosphomonoesterases catalyze reactions with phosphomonoesters. The reaction involves the hydrolysis of the phosphorus-carbon bond, generating a free phosphate molecule and an alcohol as products. Phosphomonoesterases are further classified as “acid” or “alkaline” based on their optimal pH ranges for maximum catalytic activity. Because of their ubiquity and importance in phosphorus mineralization, phosphomonoesterases are often referred to simply as phosphatases in the scientific literature, rather than by their full name, and context is often necessary to determine which group is being referenced. Therefore, a phosphomonoesterase with a pH optimum near 8 might be referred to as an “alkaline phosphatase” rather than an “alkaline phosphomonoesterase”. In soils, both acid and alkaline phosphomonoesterase activities are usually measured, although the pH optima for these enzyme assays will vary with soil pH in some but not all soils.

Phosphodiesterases hydrolyze phosphodiesters. Cleaving of a diester proceeds similarly to the monoester reaction, with water added across the phosphorus-carbon bond yielding phosphate and an alcohol. Once a phosphodiester has undergone hydrolysis, the resulting alcohol phosphomonoester may undergo another hydrolysis step, catalyzed by a phosphomonoesterase. Specific examples of phosphodiesterases include depolymerizing nuclease enzymes such as deoxyribonuclease (DNAse) for DNA and ribonuclease (RNAse) for RNA.

Inositol hexaphosphates such as phytate are specifically hydrolyzed by phytases, of which there are four subclasses differing in pH optima, cofactor requirements, or mechanisms of action. Because it is found in seeds, phytate is a common part of human and animal diets. However, humans and many animal species do not produce phytases. The six phosphate groups of phytate allow it to

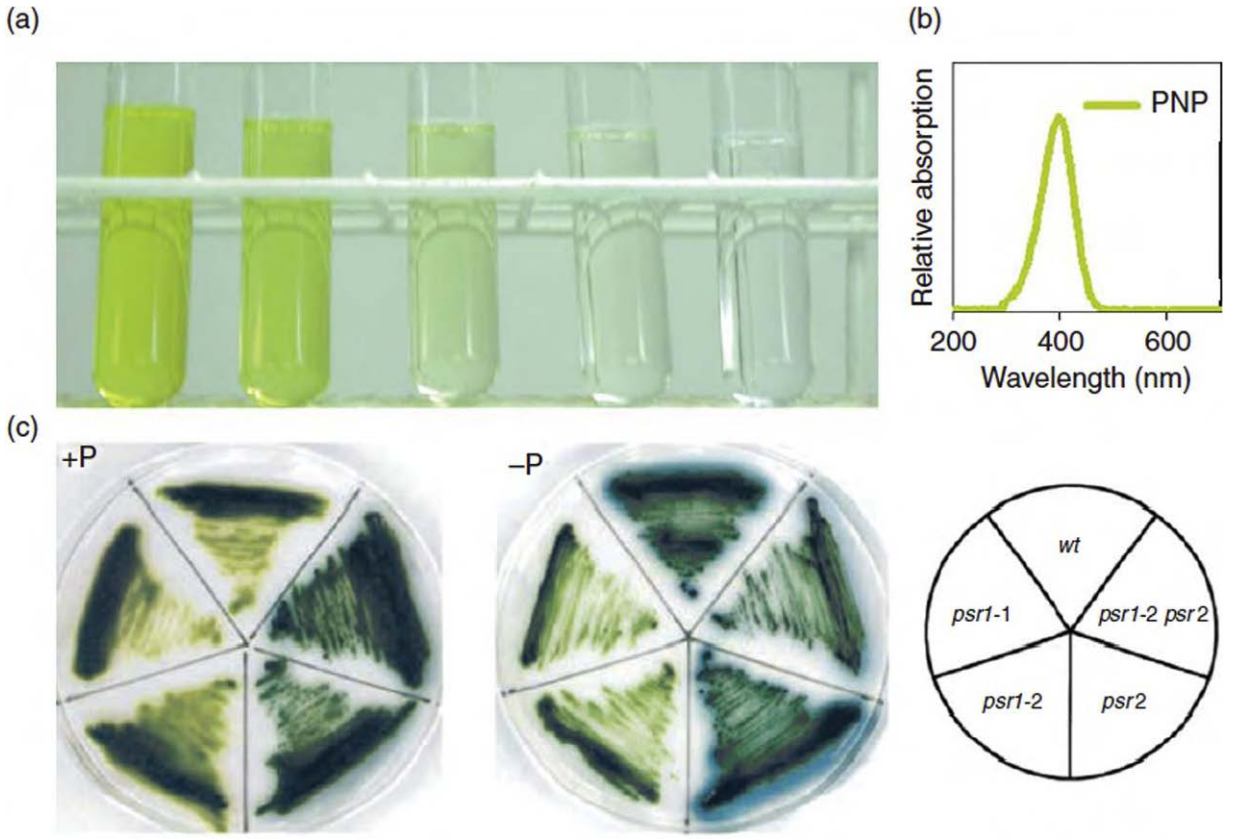

Fig. 3 (a) The *para*-nitrophenyl phosphate (PNP) assay for alkaline phosphatase activity produces a yellow color in the presence of the enzyme. (b) The PNP assay quantifies alkaline phosphatase activity based on the absorption of light at 380 nm. (c) The 5-bromo-4-chloro-3-indolyl-phosphate assay with *Chlamydomonas* algae under phosphate replete (left) and phosphate limited (middle) conditions shows phosphatase activity using the blue coloration formed around the cells when they express the enzyme. Key (right) identifies mutants used in the study (wt is wild type). The phosphatase of the wild type cells is induced in phosphate-free medium. Reproduced with permission from Shimogawara, K., Wykoff, D.D., Usuda, H., Grossman, A.R., 1999. *Chlamydomonas reinhardtii* mutants abnormal in their responses to phosphorus deprivation. Plant Physiology 120, 685–693. Copyright 1999 by the American Society of Plant Biologists.

sorb tightly to soils, and it can accumulate, especial when manure is applied to soils. Below pH 5, DNA can also sorb to soil minerals and accumulate because enzymatic mineralization will be restricted. A combination of organic acids to desorb phytate or DNA, followed by enzyme hydrolysis, will be required to release phosphate from these tightly sorbed molecules.

Because many phosphatase enzymes are synthesized in response to low environmental phosphate levels (i.e., when the cells experience phosphate limitation), phosphatase activity has been used extensively as a metric for determining the nutrient status of microbial communities in aquatic ecosystems. This is less common in soils, where microbes are generally more limited by carbon or nitrogen than phosphorus. The activity of phosphatase enzymes has been measured in many terrestrial, lake, and marine environments using a variety of methods, and numerous laboratory studies have also been conducted. In the environment, the bulk phosphatase activity of an entire microbial community is commonly measured by incubating soil, sediment, or water samples with a phosphate-bound substrate in which the hydrolytic product undergoes a color change that can be observed visually or spectrophotometrically, such as *para*-nitrophenyl phosphate (PNP) (Fig. 3(a) and (b)), phenolphthalein phosphate (PPP), glycerophosphate, and 5-bromo-4-chloro-3-indolyl-phosphate (Fig. 3(c)). In addition, measurements can be made fluorometrically for the substrates 3-o-methylfluoresein phosphate (MFP) and 4-methylumbellyferyl phosphate (MUP). Radiometric analyses can similarly be made using ^{32}P labeled glycerol phosphate (or an equivalent molecule). Chemical analysis of the hydrolytic products of glycerol phosphate and other bioenergetically important molecules has also been used to estimate phosphatase activity in bulk microbial populations.

A major drawback to measuring bulk phosphatase activity is that it provides limited information, if any, about which members of the microbial community experience phosphate limitation at the time of sampling. For aquatic samples, this can be addressed to some extent by size fractionating cells (as on a filter) prior to incubation with the substrate, thereby allowing phosphatase activity to be assigned to gross taxonomic classes of organisms. However, size fractionation introduces other obstacles for data interpretation, and results must be interpreted with care. For example, activity in different size fractions can be skewed by groups of bacteria that coalesce to form larger particles, although the individual cells are small and would otherwise be grouped with smaller size fractions. Studies with mixed populations of bacteria and green algae showed that 44% of the measured phosphatase activity was attributable

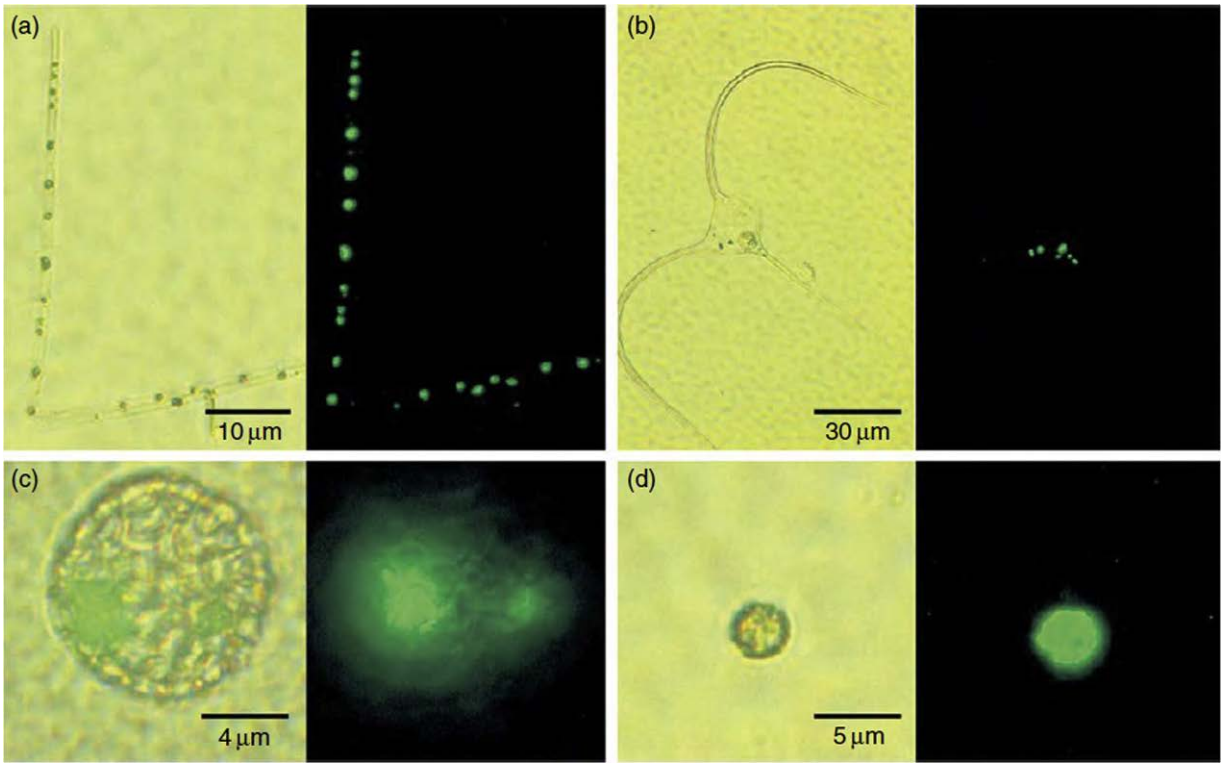

Fig. 4 Micrographs of ELF-97 labeled phytoplankton from the euphotic zone in the Gulf of Aqaba (a) *Trichodesmium sp.*, (b) *Ceratium sp.*, (c) coccolithophore, and (d) *Cyanothece sp.* For each pair, the left panel is a view under visible light and the right panel under UV illumination. ELF-97 labeled areas appear as bright areas under UV illumination and show the location of phosphatase enzymes on the cells. Reproduced with permission from Mackey, K.R.M., Labiosa, R.G., Calhoun, M., et al., 2007. Phosphorus availability, phytoplankton community dynamics, and taxon-specific phosphorus status in the Gulf of Aqaba, Red Sea. Limnology and Oceanography 52, 873–885. Copyright 2007 by the American Society of Limnology and Oceanography, Inc.

to aggregated groups of cells. Moreover, in the marine environment substantial phosphatase activity has been shown to persist for 3–6 weeks at 50% of initial levels in water samples filtered to remove particles. These observations suggest that phosphatases free in solution or bound to soluble organic material can contribute a significant amount of phosphatase activity, potentially leading to overestimates of cell-bound phosphatase activity in the small-cell size fraction. A difficulty common to both bulk and size fractionated samples is that phosphatases can persist for long periods of time without being bound to a living cell. It is not uncommon for microbial cells to retain phosphatase activity for months or years after being dried or preserved, including in dried soil samples, indicating that cell viability is not critical for maintaining phosphatase enzymes over these time periods, and dead cells may contribute to the overall phosphatase activity in a sample.

Several methods have been developed to overcome the limitations of bulk enzyme activity measurements by directly labeling cells when phosphatases are present. These methods allow phosphatase activity to be attributed to individual cells or taxa, allowing greater resolution of the phosphate status of organisms within a mixed community. In aquatic samples, direct cell staining with azo-dyes or precipitation of lead phosphate at the site of enzyme-mediated phosphate release have been used together with light microscopy to visualize phosphatase activity on individual cells. Similarly, enzyme-labeled fluorescence (ELF) labels individual cells with a fluorescent precipitate (ELF-97) following hydrolysis of the non-fluorescent substrate molecule (2-(5′-chloro-2′phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone) at the site of the enzyme (Fig. 4).

Oxidation-Reduction

Phosphorus can be found in a broad range of oxidation states. Like nitrogen, which occurs just above phosphorus in the periodic table of elements, the valence electrons of phosphorus allow −3, 0, +1, +3 and +5 oxidation states. However only the +5 oxidation state, i.e., phosphate, is thermodynamically predicted to remain stable in aqueous solutions (including soil solution). Consequently, inorganic phosphate is nearly always the dominant form of phosphorus in the ocean, lakes, sediments, and soils. The phosphorus-containing organic compounds that generally account for the vast majority of cellular phosphorus, including DNA, RNA, and phospholipids, also contain phosphorus in the +5 oxidation state. While the geochemistry and biochemistry of phosphorus generally reflects the chemical characteristics of phosphorus in the +5 oxidation state, there are pools of phosphorus at reduced oxidation states (−3, 0, +1 and +3) whose roles on Earth remain to be fully elucidated. The contribution of inorganic

reduced phosphorus species from geochemical sources, which include primarily metal phosphides produced via lightning strikes or meteorite strikes, is quite small. Thus, microbes are regarded as the primary sources of reduced phosphorus species. Phosphine (PH_3), which contains phosphorus in the -3 oxidation state, is a gas produced in environments where organic matter is actively degraded, such as sewage treatment plants or rice paddies. Atmospheric phosphine concentrations are much higher on land than over the open ocean. Phosphite ($HPO_3{}^{2-}$), which contains phosphorus in the $+3$ oxidation state, has been observed in low concentrations in soils, lakes, wetlands and rivers. Recent laboratory innovations are making phosphite and hypophosphite (H_2PO^{2-}; $+1$ oxidation state) analyses more accessible to investigators, and a growing body of observations suggests that these two reduced inorganic phosphorus species may be more prevalent than once thought. The biochemical mechanisms by which microorganisms produce reduced phosphorus species are poorly understood. Thermodynamic calculations suggest that the direct reduction of phosphate to phosphite is prohibitively endergonic in the presence of water. Thus, if this process is indeed mediated by microbes, then it would require highly specialized enzyme systems that have yet to be identified. By contrast, the direct oxidation of phosphite is highly exergonic, and dissimilatory phosphate oxidation has been tied to the microbial sulfate and nitrate reduction and possibly carbon dioxide fixation.

Plant-Microbe Interactions

Like all organisms, phosphorus is an essential nutrient for terrestrial plants. However, unlike aquatic environments where both phosphorus and organisms are mobile, terrestrial plants are fixed in place and can easily deplete the labile phosphate in the rhizosphere (the zone surrounding plant roots). To ensure a continued supply of labile phosphate, relationships between higher plants and rhizosphere microbes have developed. Carbon is the nutrient most limiting to many soil microbes; plants release carbon directly into the rhizosphere by exuding simple sugars or high molecular weight compounds, or indirectly by root turnover. Rhizosphere microbes in turn can produce organic acids to release adsorbed or chelated phosphate, phosphatases to mineralize organic phosphorus, or hormones to stimulate the growth of roots and root hairs. The majority of higher plants also form symbiotic relationships with fungi called mycorrhizas. These vary depending on the plant and fungal species. Arbuscular mycorrhizas are endomycorrhizas, where fungi colonize root cells and extend hyphae beyond the rhizosphere to increase phosphate uptake. In ectomycorrhizas, fungal hyphae form sheaths around plant roots and do not enter plant cells. The fungi involved in mycorrhizas extend hyphae into the soil to access phosphate beyond the reach of plant roots and root hairs and may also transform phosphorus by producing enzymes and organic acids.

Genetic Regulation of Microbially-Mediated Processes

Microbially-mediated processes, including those involved in the phosphorus cycle, are the outcome of numerous biological pathways occurring in concert across diverse microbial communities. Even cursory observations of natural microbial communities demonstrate that while microbially-mediated processes influence and change the environment, the environment likewise shapes the activity of microbes, in many cases by providing feedback that either inhibits or enhances the processes. (An example of this type of feedback is the synthesis of phosphomonoesterase enzymes, many of which are only present during periods of phosphate deprivation but not when phosphate is abundant in the environment). Likewise, microbially-mediated processes can also be controlled indirectly by secondary factors (other than phosphorus) that influence growth and metabolism, such as the availability of oxidized nitrogen or sulfur in some chemoautotrophic microbes.

These processes, which are manifest in the environment as the combined outcome of activities from a diverse microbial community, are in fact a result of genetic mediation within single cells. The regulation of genes in response to environmental stimuli determines how a cell will respond to its environment, including if and how it will contribute to microbially-mediated processes in the phosphorus cycle. To understand gene regulation in greater detail, highly sensitive genetic and molecular methods have been developed. Under laboratory conditions, these methods have elucidated pathways important in the immobilization (i.e., phosphorus assimilation into cell biomass) and mineralization (i.e., phosphatase production) of phosphorus, as well as countless other pathways and processes.

In *E. coli*, two major phosphate assimilation pathways have been identified. The phosphate transport system (Pit), which comprises a hydrogen-phosphate symport powered by proton motive force, is expressed constitutively and provides the cell with sufficient phosphorus for growth when phosphate concentrations in the media are not limiting. When media phosphate concentrations drop below a threshold concentration, the high affinity phosphate specific transport (Pst) system becomes engaged. This system has a 100-fold greater affinity for phosphate than Pit, enabling the cell to acquire phosphate from a limited reservoir. Uptake of phosphate through Pst is an ATP dependent process (e.g., requires energy input from the cell).

Pst is activated as part of the Pho regulon, a group of operons that is expressed when phosphate levels are low. Activation of the Pho regulon is initiated through phosphorylation of the PhoB cytoplasmic protein which, in its phosphorylated state, is a transcriptional activator of the operons within the Pho regulon. In addition to Pst, the Pho regulon also includes genes encoding alkaline phosphomonoesterase (PhoA), outer-membrane porin proteins that facilitate diffusion of phosphate into the periplasm (PhoE), and proteins for the uptake and processing of glycerol-3-phosphate (*ugp* operon) and phosphonates (*phn* operon).

Metabolism of glycerol 3-phosphate is an interesting strategy for heterotrophic microbes, such as *E. coli*, because it is a potential source of both phosphate and carbon for the cell. However, when grown under phosphate-deplete conditions and

expressing *ugp* genes, cells are only able to use glycerol 3-phosphate as a phosphate source, not as a carbon source. For cells to grow with glycerol 3-phosphate as the only phosphate source, another carbon source must also be provided. The *ugp* system is less efficient when internal cell phosphate levels are high and is no longer expressed if external phosphate levels increase above a threshold level. Another system that is not part of the Pho regulon, the *glp* transport system, is regulated by external and internal glycerol-3-phosphate levels rather than phosphate concentrations. Unlike in the *ugp* system, glycerol-3-phosphate acquired by the *glp* system can serve as the sole source of carbon and phosphate for the cell. Both *ugp* and *glp* systems facilitate direct cellular uptake of glycerol-3-phosphate; however, each is regulated by different internal and external cues (i.e., phosphate or glycerol-3-phosphate levels) and has a different nutritional strategy (i.e., supplying phosphate alone versus phosphate and carbon together).

These two systems are an example of how microbes, by developing multiple inter-related pathways, can contribute to microbially-mediated processes in the phosphorus cycle under a range of environmental and physiological conditions.

Experimental evidence shows that the phosphate assimilation pathways in other heterotrophic bacteria are similar to those of E. coli, and many microbes are known to have portions of the Pho regulon. In particular, the alkaline phosphomonoesterase gene (*phoA*) and homologues have been identified in numerous microbial taxa, and although the primary function of the protein remains the same, factors that influence its expression and activity vary from organism to organism. The diversity of organisms and environmental conditions in which this gene exist allow microbially-mediated mineralization of phosphorus to occur in nearly every environment where microbes are found. For example, photosynthetic cyanobacteria in the genus *Synechococcus*, which populate freshwater environments, coastal waters, and vast areas of the open ocean, have the *phoA* gene along with many of the other genes encoded in the Pho regulon, highlighting the global ubiquity of microbially-mediated processes in the phosphorus cycle.

Metagenomic techniques have the potential to significantly increase our understanding of phosphorus mineralization, as well as other aspects of phosphorus cycling, in terrestrial and aquatic ecosystems. These molecular-based techniques sequence extracted RNA and DNA to accurately identify and quantify a large number of microbial genes. In addition to better identification of the microbial community in a sample, metagenomics can identify the microbial genes coding for various phosphorus cycling processes, such as phytase or phosphonatase production. Metagenomics will only identify the presence and abundance of these genes, which can only give the potential gene activity; it does not indicate if these genes are actually active in a sample or ecosystem. However, these metagenomic results can be linked to measures of ecosystem processes, such as the enzyme activity measurements previously described, or to the presence of substrates such as phytate, identified and quantified by techniques such as ^{31}P-NMR spectroscopy.

Anthropogenic Alteration of the P Cycle: Eutrophication in Aquatic Ecosystems

As discussed above, microbes have an important role in nearly every aspect of the phosphorus cycle, and their activities help control the relative rates at which phosphorus is mobilized and immobilized within the environment. However, humans also influence the phosphorus cycle and alter the structure of microbial communities, sometimes causing devastating ecological consequences.

Post-industrial human activities, including deforestation, phosphorus mining, and agricultural practices, affect the phosphorus cycle by increasing the mobility of phosphorus in the environment and causing it to accumulate in soils and aquatic environments. Several factors contribute to the mobilization of phosphorus by these activities. Deforestation and mining expose phosphate (and other) minerals in rock and soil to the atmosphere, leading to increased rates of weathering and erosion. Some agricultural practices such as tillage can also increase soil erosion, while the localized elevation of phosphorus levels from application of fertilizers can be a large source of the anthropogenic phosphorus input into receiving water. Elevated soil phosphorus levels increase the amount of phosphorus in runoff and ultimately lead to the accumulation of phosphorus in lakes and estuaries. Livestock production for meat or dairy will produce manures containing high concentrations of phosphorus. When applied to soil as fertilizer at rates to meet plant nitrogen requirements, this will apply excess phosphorus that may be lost in runoff. Increasing human populations, both in cities and in rural areas, will increase phosphorus in human sewage that can enter water systems if not properly treated and disposed.

As a result of these practices, recent estimates suggest that the net storage of phosphorus in terrestrial and freshwater habitats has increased 75% over pre-industrial levels, and the total phosphorus flux to the ocean is 2-fold higher than pre-human levels. When phosphorus enters water bodies where phosphorus limits production, substantial changes in the microbial community occur. Reversal of phosphorus limitation leads to eutrophication, the rapid growth of bloom-forming phytoplankton, some of which are toxic or nuisance species (like *Pfiesteria sp.*) that are harmful to aquatic organisms and humans. As the bloom exhausts the supply of phosphorus, the phytoplankton senesce, sink to the bottom of the water body, and are decomposed by the heterotrophic microbial community. At depth, where light levels are low, photosynthetic phytoplankton are not able to balance the metabolic oxygen demands of the heterotrophs, and anoxia occurs in the bottom waters. Anoxia damages the benthic environment, leading to fish kills and harming benthic invertebrate communities. Loss of submerged aquatic vegetation, coral reef death, human shellfish poisoning, and a reduction in biodiversity are among the possible outcomes caused by microbial responses to the anthropogenic introduction of excess phosphorus to sensitive aquatic ecosystems. Eutrophication has been observed in many ecosystems, including fresh water lakes like Lake Erie, large estuaries like the Chesapeake Bay, and coastal areas like the hypoxic “dead zone” of the Gulf of Mexico.

Conclusion

Microbially-mediated processes in the phosphorus cycle forge a critical link between the geosphere and biosphere by assimilating phosphorus within biological molecules and contributing to chemical transformations of phosphorus in the environment. In addition to acting as living reservoirs of phosphorus, microbes also contribute to the transformation of phosphorus within other non-living reservoirs, such as rock, soils, rivers, lakes, and oceans. Microbially-mediated phosphorus transformation includes processes that increase the bioavailability of phosphorus in the environment, such as weathering, solubilization, and mineralization, as well as those that decrease its bioavailability, such as assimilation and mineral formation. These large-scale environmental processes are the outcome of numerous biological pathways occurring in concert across diverse environmental settings and microbial communities. Genetic diversity and finely-tuned regulation of gene expression allow microbes to adapt to harsh environments, and to contribute to the phosphorus cycle under numerous and diverse environmental conditions. Human alteration of the natural phosphorus cycle causes unintended consequences in microbial communities, and serious environmental, economic, esthetic, and human health problems are caused by microbial responses to the anthropogenic introduction of excess phosphorus to sensitive aquatic ecosystems.

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Phototaxis in Archaea and Bacteria

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Introduction

Scope of this article. Phototaxis is the process in which an organism alters its motility in response to light stimuli. This phenomenon is widespread in biology, encompassing complex multicellular organisms, unicellular eukaryotes, bacteria, and archaea. Here we will provide an overview of phototaxis in bacteria and archaea (prokaryotes). We will describe the process and mechanism of phototaxis using a small number of well-studied model systems to illustrate its general principles. In general, phototaxis involves a photosensory pigment (**photoreceptor**) that captures the light and converts it into a biological signal, a **signal transduction chain** (see "Sensory Transduction in Bacteria") leading to the **motility apparatus** of the cell, and a resulting change in motility of the organism.

Some light-induced changes in motility that fall under the general umbrella of phototaxis have been reported in chemotrophs. Here, we will focus on phototaxis in photosynthetic (in the broad sense of this term, involving both chlorophyll-driven photosynthesis and retinal-driven photosynthesis) bacteria and archaea, in which the majority of phototaxis responses have been reported and studied. It should be noted that photoreceptors have been identified in a large number of phototropic and chemotrophic microorganisms, but these proteins trigger a range of biological responses other than phototaxis, particularly biofilm formation. Various cases of photoregulation between motile and sessile states of prokaryotes have also been reported. This article will focus on phototaxis responses in phototrophic bacteria and archaea. The biological function of these phototaxis responses in photosynthetic prokaryotes is to accumulate in illuminated areas that allow photosynthetic activity while avoiding areas containing light of an intensity and spectral quality that can cause damage to the photosynthetic machinery (or other chromophoric components of the cell).

Helpful similarities and surprising differences exist between phototaxis in prokaryotes and chemotaxis in *E. coli*. Studies of phototaxis responses in phototrophic prokaryotes have greatly benefited from analogies with *E. coli* chemotaxis (see "Bacterial Chemotaxis: Conservation and Variation on a Theme"). These studies on chemotaxis focused on populations of free-swimming single planktonic cells. Core concepts used to understand both responses are as follows. In a constant, homogeneous environment, the swimming behavior of cells follows a **random walk**. Cell motion is caused by the rotation of **flagella,** or in the case of archaea: the **archaellum** (see "Archaellum, Bacterial Flagella", and "Swimming and Swarming Motility") attached to flagellar motors that are driven by the proton motive force. With a certain constant probability, a cell will reverse direction of the rotation of its flagellum (or flagella; there is considerable taxonomic variability in the number and placement of flagella on the cell), causing a change in the direction of swimming. When cells experience a change in signal input (either a change in chemoeffector concentration or light intensity), the probability of reversing the direction of flagellar rotation is altered (either increased or decreased), causing a change in motility of the cell. This (relatively) fast **excitation phase** of the response is followed by a (relatively) slow **adaptation phase**. As a result of this adaptation process, the initial value of the probability for reversing the direction of the flagellar motor is recovered. This mechanism causes cells to respond only to changes in light intensity or chemoeffector concentration, *i.e.*, cells effectively determine the time derivative of the external signal. As a result, when cells happen to move in a favorable direction (with respect to changes in light or chemoeffector concentration), the probability of changing swimming direction is reduced, while movement into an unfavorable direction increases the probability of changing swimming direction. This **biased random walk** causes cells in a spatial gradient to move towards favorable areas.

Key proteins and molecular processes that achieve this mechanism have been identified in studies of bacterial chemotaxis, particularly **methyl-accepting chemotaxis proteins** (MCPs) that serve as receptors for chemoeffectors, **CheA**, a kinase associated with MCPs that autophosphorylates its conserved His residue in response to changes in MCP conformation, and **CheY**, a diffusible protein that receives phosphoryl groups from CheA at a conserved Asp residue. Depending on its phosphorylation state, CheY binds to the flagellar motor to alter its probability of changing rotation direction. These protein phosphorylation events trigger the excitation phase of the response. The adaptation phase of the response is based on changing the methylation level of the MCP, which is performed by the methyl transferase **CheR** and the methyl esterase **CheB**. A range of variations on this theme has been discovered to govern the chemotaxis responses in different prokaryotes (See "Bacterial Chemotaxis: Conservation and Variation on a Theme"). A number of these components also occur in the phototaxis signal transduction chains in prokaryotes (see below).

Two basic mechanisms for light detection in prokaryotic phototaxis have been derived from extensive studies on a range of bacteria and archaea: (1) responses that are initiated by the bioenergetic consequences (changed in redox potential of components and/or changes in the transmembrane proton motive force) of photoexcitation of the photosynthetic machinery, and (2) responses that are initiated by the photoexcitation of dedicated photosensory receptors. The use of dedicated photoreceptors follows the conceptual framework provided by *E. coli* chemotaxis. Phototaxis responses mediated through the bioenergetic consequences of photoexcitation of the photosynthetic machinery are distinct from that case.

Recent progress in bacterial phototaxis has revealed two exciting aspects that move beyond the classical conceptual framework provided by *E. coli* chemotaxis, and are discussed below. First, a growing body of evidence indicates that single bacterial cells are able

to detect not only temporal changes in the intensity of light, but also the actual direction of the light beam. Since differences in chemoeffector concentration at the two poles of the bacterial cell are widely believed to be too small to be biologically useful, such directional sensing does not occur in prokaryotic chemotaxis. Second, in addition to phototaxis of free-swimming cells, entire colonies of bacteria can exhibit phototactic movement during social mobility, in which all cells in the colony engage in a coordinated pattern of motion.

Nomenclature of phototaxis responses. Different systems have been used in literature to describe phototaxis responses in microorganisms. One prevalent approach is based on the analogy between chemotaxis in *E. coli* and phototaxis in bacteria and archaea. In this approach, in analogy to molecules that act as attractants and repellents (chemoeffectors) in chemotaxis, light can attract microorganisms in **positive phototaxis**, while it repels microorganisms in **negative phototaxis**. The application of this approach is best fitted to cases of prokaryotic phototaxis where cells detect changes in light intensity as a function of time, corresponding to the detection of changes in chemical concentrations as a function of time in chemotaxis, as is the case for the archaeon *Halobacterium salinarum* and the proteobacterium *Halorhodospira halophila*, described below. It should be noted that in these cases the cells are not able to detect the direction of the light, only temporal changes in the intensity of the light. The other approach distinguishes four basic types of phototaxis: (a) the photophobic (fear of light) response, in which an increase in light intensity triggers a change in swimming direction, repelling the cells from the light; (b) the scotophobic (fear of darkness) response, in which a reduction in light intensity triggers a change in swimming direction; (c) photokinesis, in which cells alter the speed of their motility in response to change in light intensity; (d) true phototaxis, in which cells respond to the direction of the illumination (as opposed to temporal changes in light intensity or a spatial gradient in light intensity).

Model Systems for Bacterial and Archaeal Phototaxis

Much of our understanding of phototaxis in bacteria and archaea has been obtained through studies on a relatively small number of organisms, which can be viewed as model systems for prokaryotic phototaxis. Below we summarize understanding of phototaxis in four of these organisms (Table 1), including information on the photoreceptors and signal transduction pathway leading to the cellular motility machinery.

(1) ***Halobacterium salinarum***. The archaeon *H. salinarum* (previously called *Halobacterium halobium*) plays a prominent role in the field of prokaryotic phototaxis since the entire phototaxis process, starting from photoreceptor excitation, proceeding through an associated signal transduction chain to the flagellum, and the resulting cellular responses has been studied in considerable detail from a structural, genetic, biochemical, biophysical, and cellular perspective (Fig. 1(A)).

H. salinarum is an extremely halophilic organism that can grow both aerobically and (under conditions of illumination and reduced oxygen tension) photosynthetically. It swims using its archaellum, which morphologically appears as a flagellar bundle located at a single pole of the cell. It should be noted that evolutionarily the archaellum is closely related to **type IV pili** of Bacteria. The multiple flagellar motors in a cell rotate in unison in a single direction. In the absence of tactic stimuli, cells spontaneously reverse their swimming direction every 5–50 s. Cell tracking software to analyze the motion of populations of *H. salinarum* cells allows the identification of changes in the swimming direction of each individual cell in view of the light microscopy used. This approach has been applied extensively to quantify the background level of the reversal frequency, its transient increase upon a repellent response, and its suppression upon an attractant response. Upon exposure to a repellent light stimulus, the cell is triggered to reverse the direction of flagellar rotation and thus its swimming direction. *H. salinarum* exhibits both positive and negative phototactic responses.

The photosynthetic and phototactic capabilities of *H. salinarum* are based on four different retinylidene proteins, in which the light-sensitive cofactor is **retinal**. The discovery of the light-driven proton pump **bacteriorhodopsin** (BR) triggered extensive

Table 1 Phototaxis responses in four model organisms

Organism physiology/taxonomy	*Photoreceptor(s)/pigments*	*Motility system*	*Signaling pathway*	*Phototaxis responses*
Halobacterium salinarum Archaeon (Euryarchaeota) Extreme halophile	SRI, SRII/retinal BR/retinal	Free swimming archaellum (bundle at single cell pole)	HtrI/CheA/CheY pmf	Positive & negative
Rhodobacter sphaeroides α-Proteobacterium purple non-sulfur anoxygenic photosynthetic	Photosynthetic RC chlorophyll, carotenoids	Free swimming single flagellum on side of cell	? (involves redox signaling)	Mainly step-down
Halorhodospira halophila γ-Proteobacterium Extremely halophilic purple Sulfur anoxygenic photosynthetic	PYP/*p*-coumaric acid Photosynthetic RC Chlorophyll, Caroteniods	Free swimming single flagellum at each cell pole	?	Negative (PYP) Positive (RC)
Synechocystis sp. PCC6803 Cyanobacterium oxygenic photosynthesis unicellular, freshwater	PixJ, Cph2/bilin photosynthetic RC chlorophyll, carotenoids	Twitching by Type IV pili	?	True phototaxis in social (colony) motility true phototaxis in single cells by cell lensing

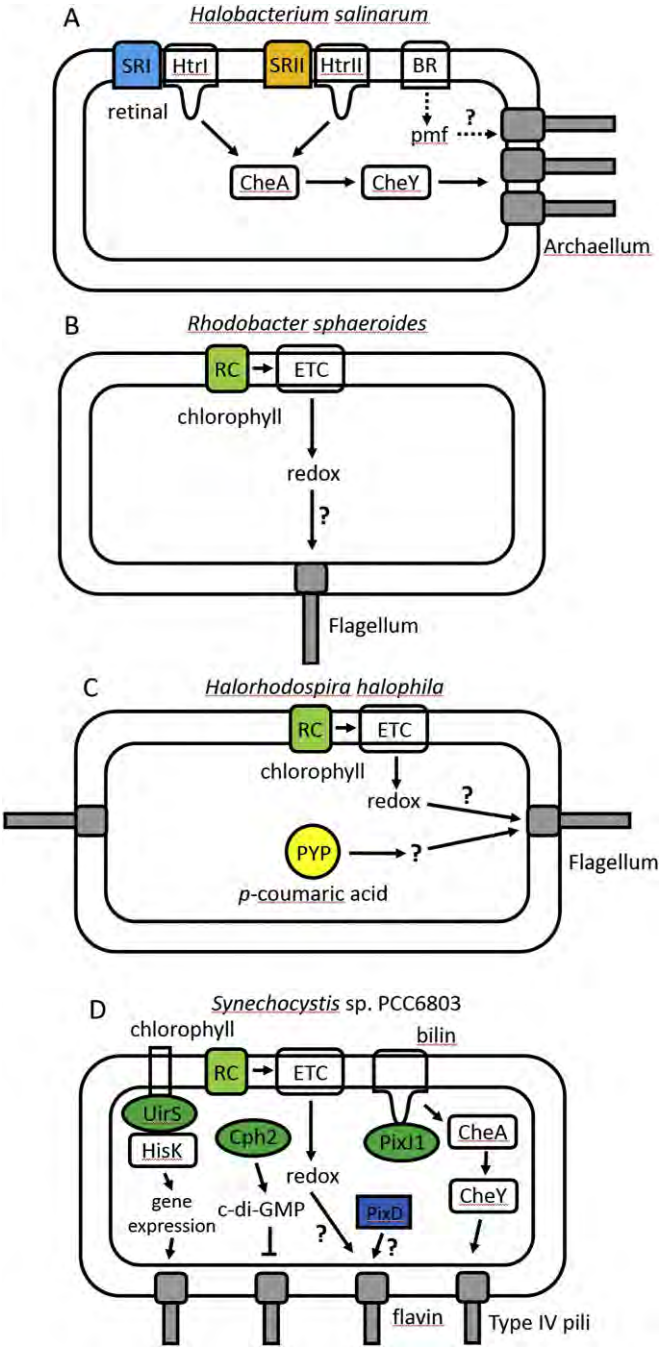

Fig. 1 Summary of phototaxis in four model systems. The motility system, photoreceptors (with chromophores), and signal transduction pathways are indicated for *Halobacterium salinarum* (A), *Rhodobacter sphaeroides* (B) *Halorhodospira halophila* (C), and *Synechocystis* sp. PCC6803 (D). Photoreceptors are indicated in color; these colors are the same for proteins containing the same chromophore, but are for illustrative purposes only and are not intended to reflect the absorbance spectra of the proteins.

studies of the photobiology of this microorganism, and this led to the discovery of three additional retinylidene proteins: the light-driven chloride pump halorhodopsin (HR) and two sensory rhodopsins (SRI and SRII) that cause distinct phototactic responses. SRII is also referred to as phoborhodopsin.

A cautionary note regarding nomenclature is that when BR was first discovered in *Halobacterium salinarum* (then called *Halobacterium halobium*), the Domain Archaea had not yet been recognized, and *Halobacterium* was taken to be a bacterium. Therefore, the newly discovered protein in this organism was called bacteriorhodopsin (not archeorhodopsin) based on its resemblance to animal rhodopsin. To complicate matters further, later a quite distinct bacteriorhodopsin-like protein was

discovered in actual bacteria (namely marine Proteobacteria). This protein, which functions as a light-driven proton pump and contributes significantly to planetary biological light harvesting, was named proteorhodopsin.

SRI has an absorbance maximum near 587 nm, while the absorbance maximum of SRII is near 487 nm. Photoexcitation of SRI triggers a positive phototaxis response towards photosynthetically active light, since BR has an absorbance maximum at 568 nm. Light absorbance by SRII causes a negative phototaxis response towards blue light. In addition, photoexcitation of S_{373}, the blue-shifted photocycle intermediate of SRI, triggers a negative phototaxis response. Thus, the single protein SRI is responsible both for positive phototaxis towards light around 590 nm and for negative phototaxis towards light around 370 nm. This constitutes a mechanism of color discrimination.

While most phototactic responses in *H. salinarum* are triggered by the two dedicated photoreceptors sensory rhodopsin I and II, mutants lacking these two photoreceptors still exhibit residual phototactic sensitivity. These are transmitted BR. These bacteriorhodopsin-based responses are likely mediated by a "protometer" that senses the transmembrane electrochemical gradient (**proton motive force** or pmf). The protein(s) constituting the protometer have not yet been identified.

While BR and HR are ion pumps that function autonomously in generating transmembrane ion gradients, the sensory rhodopsins function through interacting with an associated signal transduction chain. A seminal finding was the identification of **HtrI** for **Halobacterial transducer protein**, the signal transducer for SRI. Interestingly, HtrI is homologous to the highly studied methyl accepting chemotaxis proteins from *E. coli*. HtrI was first identified based on its reversible methylation during phototactic responses. Htr transducer proteins contain two transmembrane α-helices and a large intracellular domain that is active in downstream signaling to kinases and is methylated during signal adaptation. Each SR interacts with a dedicated Htr in the membrane, forming SRI-HtrI and SRII-HtrII signaling complexes. Photoexcitation of the SR-Htr complex alters interactions within the complex, such that a signal is relayed from the SR to the Htr. Interestingly in the absence of HtrI the SRI photoreceptor has transmembrane proton pumping activity. In the presence of HtrI this proton pumping activity is fully repressed. This observation suggests that the protein conformational change involved in transmembrane proton pumping is similar to that involved for signal relay from SRI to HtrI.

The signaling pathway linking the SR-Htr complexes to the archaeal flagellum also is very similar to that of *E. coli*, involving both protein phosphorylation and protein methylation. CheA and CheY proteins play key roles in the signal transduction chain downstream of Htr. While each of the two SRs is associated with a dedicated Htr protein, their signaling chains converge at the level of CheA.

A rich body of information is available on the structure and light-triggered molecular mechanism of these retinylidene proteins. All four proteins consist of a bundle of seven transmembrane α-helices (labeled A to G), and contain a covalently linked retinal chromophore embedded in this helical bundle. They share ~25% amino acid sequence identity, particularly in the retinal binding pocket. A wealth of structural data has been obtained on these proteins, including. X-ray structures of SRII at 2.1 Å resolution and SRII in complex with the transmembrane part of its signal transducer HrtII at 1.94 Å.

The ability of photoreceptor proteins to absorb light above 300 nm is based the involvement of protein-bound light-absorbing cofactors, or **chromophores**. All four archaeal rhodopsins contain all-*trans* retinal that is covalently attached to a highly conserved Lys side chain *via* a protonated Schiff base linkage. The protonated Schiff base is fully enclosed by the protein matrix, and thus represents a buried charge, which is stabilized by electrostatic interactions with nearby charged residues. In BR the counterion for the protonated Schiff base consists of two negatively charged side chains (Asp85 and Asp96) and one positively charged side chain (Arg82). A similar situation – but with significant modifications – is found in the other archaeal rhodopsins.

Photoexcitation of photoreceptors triggers a **photocycle** that results in the biological function of the protein. Such photocycles are initiated by a photochemical event, followed by a complex series of thermal transitions. In the halobacterial rhodopsins, light triggers the ultrafast all-*trans* to 13-*cis* photoisomerization of the retinal chromophore on a time scale of 0.5–5 ps. This initial process involves relatively small changes in the structure of the retinal, in which the strain of fitting the 13-*cis* chromophore in the all-*trans* binding pocket is distributed over a number of bond angles. This strain provides the driving force for a series of thermal reactions that trigger proton transfer reactions and the formation of the active **signaling state** of the photoreceptor. Finally, thermal recovery during the reformation of the initial state completes the photocycle. The role of light-triggered proton transfer events in photoreceptors for driving the conformational changes that initiate signaling is a recurring theme. This cyclic chain of thermal transitions is referred to as a photocycle. The photocycle of BR and HR is completed in ~10 ms, and results in the transmembrane translocation of a proton and chloride ion, respectively. In the SRs the photocycle is significantly slower: recovery of the initial state of the receptor requires ~1 s. During this photocycle the protein is converted from its initial receptor state to a signaling state that is active in relaying a signal into the associated downstream signal transduction chain. The ~100-fold slower photocycle of the SRs difference matches the sensory function of the SRs: the lifetimes signaling state(s) generated during their photocycle need to be sufficient for efficient relay of a signal into the attached signal transduction chain.

The proton transfer pathway involved in the deprotonation and reprotonation of the retinal Schiff base during the photocycle of the archaeal rhodopsins has received significant attention. An important example is provided by SRII, in which Aps73 is the acceptor for the Schiff base proton during the photocycle. The proton transfer from the Schiff base to Asp73 disrupts the active site salt bridge between these two residues, an event that is thought to be central to the receptor activation process in SRII. An important observation was made for the D73N mutant of SRII. In this mutant the salt bridge between the protonated Schiff base and residue 76 is already disrupted before photoexcitation of the photoreceptor. The D73N

mutation results in the **constitutive activation** of SRII. This mutation is highly reminiscent of the E113Q mutation in human rhodopsin, which also results in constitutive signaling. Thus, the light-triggered disruption of an active site salt bridge is a general mechanism for the activation of rhodopsin photoreceptors.

A set of important and unique experiments has allowed the *in vivo* identification of the signaling states of SRI and SRII. This work involved the *in vivo* reconstitution of retinal-deficient cells with various retinal analogs that differentially alter the various time constants in the SR photocycles. By relating the effects that these analogs have on the photocycle kinetics with changes in the phototactic sensitivity of the cells towards light, the functional signaling states could be identified. These experiments provide direct experimental verification for the validity of the concept of the signaling state in the living cell.

(2) ***Rhodobacter sphaeroides***. The purple non-sulfur anoxygenic photosynthetic α-Proteobacterium *Rhodobacter sphaeroides* (previously called *Rhodopseudomonas sphaeroides*) swims by means of a single flagellum located in the center of the long axis of the cell (not at the cell pole). This organism is important for the field of prokaryotic phototaxis because it exemplifies the role of the photosynthetic machinery in triggering phototaxis (Fig. 1(B)) and how more complex variants of the *E. coli* chemotaxis signaling components can be used to integrate a range of chemotactic and phototactic signals.

Movement of the cell is only mediated by flagellar rotation in the counterclockwise (CCW) direction. Cells switch (approximately every ∼10 s) between swimming and intermittent stops of approximately 1 s, during which the flagellum does not rotate. The resting flagellar resetting bias (probability of rotating CCW) in non-stimulated cells is quite high (near 0.85). As a result, *Rb. sphaeroides* responds significantly more strongly to negative stimuli than to positive stimuli. Therefore, phototaxis in *Rb. sphaeroides* is dominated by a step-down response, in which cells respond to a drop in photosynthetically active light by transiently stopping flagellar rotation. Upon a sudden drop in light intensity, cells stop moving within approximately 1 s, and subsequently resume normal motion within approximately 5 s. This response occurs when *R. sphaeroides* moves towards an area of lower light intensity, not when it swims into the direction of higher light intensity.

The photoreceptor for this positive phototaxis response is the photosynthetic machinery. This light detection mechanism therefore does not involve a dedicated photoreceptor protein. Instead, the pigments triggering these phototaxis responses are the bacteriochlorophyll and carotenoid molecules in the photosynthetic apparatus (the **photosynthetic reaction center** (RC) and the light harvesting complexes). The mechanism of phototaxis relayed *via* the photosynthetic machinery is important, since it occurs in various purple photosynthetic bacteria, including *Rhodobacter sphaeroides*, *Rhodocista centenaria* (formerly *Rhodospirillum centenum*), and *Halorhodospira halophila* (see below), allowing them to accumulate in areas illuminated with photosynthetically active light.

Illumination initiates photosynthetic electron transport, and a presumed redox sensor then relays the signal to the flagellar motor. Thus, at the heart of this type of phototaxis is a **redox sensing** mechanism, and the redox sensor is coupled to the photosynthetic machinery by the photosynthetic electron transfer chain. The identity of the proteins involved in this redox sensing mechanism has not yet been identified. The genome of *Rb. sphaeroides* encodes a rich complement of genes involved in chemotaxis responses (including nine transmembrane (MCPs) and four cytoplasmic MCPs, four CheA proteins, and six CheY proteins). Though an unknown mechanism, the redox signal relaying the phototaxis signal reaches this complex set of Che proteins, where it is integrated with chemotactic signals.

(3) ***Halorhodospira halophila***. The extremely halophilic purple sulfur anoxygenic photosynthetic γ-Proteobacterium *Halorhodospira halophila* (previously called *Ectothiorhodospira halophila*) exhibits both phototaxis towards photosynthetically active light triggered by the photosynthetic machinery and negative phototaxis towards potentially damaging blue light triggered by a dedicated photoreceptor: photoactive yellow protein (PYP) (Fig. 1(C)). *H. halophila* has two flagella, one on each of the tips of the cell and behaviorally its phototaxis responses resemble those of *H. salinarum*. PYP was identified as the photoreceptor for negative phototaxis in *H. halophila* based on the close resemblance of the wavelength dependence of this response matches the absorbance spectrum of PYP. However, because genetic tools have not yet been developed for this organism, the biological function of PYP remains to be confirmed. Its proposed function for negative phototaxis was strengthened by the absence of other photosensory proteins encoded in its genome. Studies on this organism are relevant because (1) they show how signals from the photosynthetic machinery and from a dedicated photoreceptor can be integrated, and because (2) PYP has developed into an important model system for the molecular mechanisms of photoreceptor activation and more generally for the biophysics of protein dynamics.

Upon its purification from *H. halophila*, the structural biology and biophysics of PYP has been studied extensively. The chromophore of PYP responsible for its yellow color is *p*-coumaric acid, covalently linked to a conserved Cys residue in the protein *via* a thioester bond. This represented the first time that *p*-coumaric acid was found as a protein cofactor, establishing PYP as a novel type of photoreceptor. The PYP photocycle is initiated by photoisomerization of this chromophore, and this event is followed by proton transfer and large protein conformational changes. Intramolecular proton transfer is a key part of converting light absorbance into large conformational changes in PYP, and this mechanism is likely to be of more general importance. The downstream components of the signal transduction chain associated with PYP remain to be identified.

The structure of the PYP from *H. halophila* has been determined at high resolution, revealing that PYP consists of a central 6-stranded β-sheet, flanked on both sides by α-helices. Interestingly, this structure is common to a large number of domains that are all involved in signaling and regulation. This family of proteins is referred to as **PAS domains**.

The PYP photocycle shows a striking resemblance to that of the archaeal sensory rhodopsins. This provides a striking example of convergent evolution, since the structure and chromophore of these two photosensory systems are entirely different.

While PYP is present in a number of Proteobacteria, it now also has been identified in organisms from the phyla Bacteroidetes and Spirochaeta. Studies on these PYP homologs have revealed considerable functional diversity. The PYP from *R. centenaria* (previously *R. centenum*) contains a PYP domain, a bacteriophytochrome module and a histidine kinase output domain with autokinase activity that is regulated by blue light.

(4) ***Synechocystis* sp. PCC6803**. Synechocystis sp. PCC6803 is a unicellular, freshwater cyanobacterium that can perform both phototrophic growth by oxygenic photosynthesis, heterotrophic growth by glycolysis and oxidative phosphorylation in the dark. It is a widely studied and genetically accessible model organism for oxygenic photosynthesis. Like all cyanobacteria it lacks flagella. However, it exhibits twitching motility (See "Non-Flagellar Bacterial Motility") that is driven by the retraction type IV pili. In this type of motility, cells move forward as these pili extend and attach to solid surfaces, followed by a retraction of the polymer back into the cell. In contrast to the archaellum and flagellum, which are driven by the transmembrane proton motive force, motility based on type IV pili is driven by ATP hydrolysis through cytosolic ATPases that are associated with the pili. Motility switches in *Synechocystis* likely involve the intracellular relocation of the motor ATPases. Gliding motility necessitates the added complexity of interactions between the pili and a variety of different surfaces. To enable their gliding motility, cells can secrete extracellular polysaccharides, which then forms "tracks" along the path of cellular movement. Such tracks aid in the phenomenon of **social mobility** because once a track is laid, other cells are likely to move along the same path. Therefore, movement by type IV pili along slime tracks is often involved in social mobility.

Synechocystis is capable of complex photoresponses. It can respond to a variety of light signals, depending on the color (spanning the range from UV to red), intensity, and direction of the light signal. Its photoresponses include photokinesis, true phototaxis, and concerted social movement of many cells (**community phototaxis**), in which an entire colony moves towards or away from a light source (Fig. 1(D)). Such phototactic movement of entire colonies has been referred to as community phototaxis.

While true phototaxis was reported for cyanobacteria, and for the motility of colonies of the purple photosynthetic bacterium *Rhodocista centenaria*, this ability to sense the direction of light remained controversial for many years. Where the light gradients over a single cell were considered too small to generate a biologically useful signal, social phototaxis could involve a light gradient across the entire colony. While *Synechocystis* phototaxis is enhanced for clusters of cells, individual cells retain phototactic capability, and – remarkably – also are able to detect the direction of light: true phototaxis in a single bacterial cell. Recently, the mechanism of this phototaxis response was found to be based on the phenomenon that the spherical cells of *Synechocystis* acts as a very effective microlens that focuses light at the edge of the cell, providing an appealing physical mechanism for such light detection. The true phototactic ability of single *Synechocystis* then presumably is enhanced in the community phototaxis responses of this organism.

The photoresponses of *Synechocystis* are triggered by an impressive array of photoreceptors, including three bilin-binding proteins and two flavin-binding proteins (the BLUF protein PixD, and possibly a Cry-DASH photoreceptor). In addition, light absorption by the oxygenic photosynthetic machinery of *Synechocystis* can contribute to its photosynthetic responses. Because of this complex photosensory machinery for phototaxis, it has proven challenging to unambiguously unravel if these proteins are directly involved as photoreceptors that control mobility or play a more indirect role in the light regulation of motility.

Cph2, PixJ, and UirS in *Synechocystis* belong to the phytochrome superfamily. In general, phytochromes exhibit photochromism between two stable light inter-convertable species that in the classical case have absorbance maxima in the red and far-red region. These photoreceptors contain a multi-domain photosensory module and a signal transmitter module. Their photochemistry is based on a linear tetrapyrrole chromophore that is attached to a Cys residue *via* a thioether bond. In plant phytochromes the photosensory module is formed by an N-terminal PAS domain, a central GAF domain, and a C-terminal PHY domain. However, bacterial phytochromes exhibit variations in this domain architecture, attachment site, and absorbance spectra.

The **Cph2** photoreceptor in *Synechocystis* is a complex multidomain protein that combines two photosensory domains that bind **bilin** chromophores and domains for the synthesis (GGDEF) and degradation (EAL) of the second messenger **c-di-GMP**. The N-terminal photoreceptor domain is formed by an unknotted GAF-PHY **phytochrome** that absorbs in the red/far-red region. The C-terminal **cyanobacteriochrome** module absorbs green/blue light. This protein provides light-dependent regulation of the cytosolic concentration of the second messenger c-di-GMP, which in turn controls cell motility. The light-induced increase in c-di-GMP inhibits type IV pilus-dependent motility, which switches the cell from a motile form to a non-motile form. Such light-triggered changes in lifestyle appear to be a more general phenomenon.

The membrane-bond **PixJ1** photoreceptor combines a bilin-binding cyanobacteriochrome domain, two transmembrane helices, and an MCP domain. It exhibits characteristic reversible photoconversion between blue and green absorbing states. The crystal structure of the complete PAS-GAF-PHY sensory module of Chp1 from *Synechocystis* showed that the PAS domain forms an unusual tongue-like protrusion that seals the chromophore from the solvent. Based on the role of MCPs in *E. coli* chemotaxis and *H. salinarum* phototaxis, it is likely that the signal from PixJ feeds into a CheA/CheY type signal transduction chain leading to the type IV pili machinery and triggering a phototaxis response.

The third bilin-binding photoreceptor involved in *Synechocystis* phototaxis is the multidomain protein **UirS**. It combines transmembrane regions with a cyanobacteriochrome, an ethylene-binding domain, and a classic **two-component system sensor kinase** (see "Sensory Transduction in Bacteria"). The protein acts as a UV-A sensor *via* its cyanobacteriochrome module, which contains a phycoviolobilin linked *via* two cysteine residues and photoconverts between a UV-absorbing state and a green-absorbing state. Its downstream effector UirR acts as a transcription factor that modulates gene expression of proteins affecting the phototaxis machinery, particularly its balance between positive and negative phototaxis.

PixD is a flavin-containing protein with an absorbance maximum near 443 nm that belongs to the BLUF family of photoreceptors first identified through AppA from *Rhodobacter sphaeroides*. If contains an FAD chromophore embedded in a unique fold. Disruption of the gene encoding this protein has the unexpected effect of altering the positive phototaxis response of *Synechocystis* towards 660 nm light into a negative phototaxis response. Since PixD does not absorb light in that region, it may not function as the direct photoreceptor for phototaxis responses triggered by blue light. Possibly, PixD could be a sensor of light intensity and of weak blue light. The crystal structure of PixD has been reported at 1.8 Å resolution, revealing that it is stacked into two pentameric rings in a complex also containing PixE. While this complex is stable in the dark, it dissociates into monomers upon blue light excitation. Exactly how this intriguing effect contributes to the photobiology of *Synechocystis* remains to be elucidated.

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Phototrophy and Phototrophs

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Introduction

Microorganisms are characterized on the basis of three important metabolic parameters: energy source, electron source, and carbon source for biomass production and growth. Phototrophs use light as an energy source, whereas chemotrophs derive energy from chemical sources. Lithotrophs and organotrophs obtain electrons for metabolic processes from inorganic or organic compounds, respectively. Finally, autotrophs or heterotrophs obtain the carbon required for biomass production from inorganic (C_i, CO_2 or bicarbonate or other C1 compounds like formate) or organic sources, respectively. These three parameters define four types of organisms: (1). Photolithoautotroph (e.g., cyanobacteria, purple sulfur bacteria, green sulfur bacteria); (2). Photoorganoheterotrophs (e.g., aerobic and anaerobic anoxygenic bacteria, heliobacteria); (3). Chemolithoautotrophs (e.g., hydrogen-oxidizing bacteria, sulfur-oxidizing bacteria, nitrifying bacteria, methanotrophs); (4). Chemoorganoheterotrophs (e.g., *Escherichia coli*, *Bacillus subtilis*, humans). This overview will consider microorganisms that use light as their energy source and will principally focus on those that use chlorophyll (Chl) for this purpose.

Two fundamentally different mechanisms for phototrophy occur among bacteria and archaea: retinalophototrophy and chlorophototrophy. Retinalophototrophs employ retinal-binding, rhodopsin-like proteins (e.g., bacteriorhodopsin, proteorhodopsin, halorhodopsin, or xanthorhodopsin) and light to produce stored potential energy in the form of ion gradients (either H^+, Na^+ or Cl^-) across the cytoplasmic membrane (Fig. 1(a) and (b)). Chlorophototrophs use Chl- or bacteriochlorophyll (BChl)-proteins to perform light-induced redox chemistry, i.e., photochemistry, which leads to oxidation of a (B)Chl molecule and the reduction of an electron acceptor, either a quinone or an Fe/S cluster. Subsequent dark electron transfer reactions can produce stable reductants (quinols, reduced ferredoxin, or NADPH) as well as proton motive force that can be used to produce ATP. All photosynthetic organisms are chlorophototrophs, but not all chlorophototrophs are photosynthetic; many require organic compounds and thus are photoorganoheterotrophs. Chlorophototrophs occur in only seven bacterial phyla (Table 1).

Although its evolutionary origins are still enthusiastically debated, most scientists agree that the evolution of photosynthesis was one of the seminal events in the history of biology. Photosynthesis allowed cells to couple a plentiful source of energy, the sun, to the production of anabolic energy requirements, namely ATP and reducing power – necessary for carbon dioxide fixation and life. The essential catalysts of photosynthesis are photochemical reaction centers (RCs), which can transduce the energy of photons into chemical potential energy. Photosynthesis allowed cells to produce ATP and strong reductants by processes that were independent of geochemically derived hydrogen or existing organic compounds, regardless of their source (geochemical or biological). Thus, photosynthesis ultimately fueled a massive increase in biomass production and simultaneously produced oxidizing agents that allowed life to diversify. Ultimately, these processes led to oxygenation of Earth's atmosphere when cyanobacteria evolved the capacity for oxygenic photosynthesis about 2.5 GYA. This article will briefly describe the two types of phototrophs and their occurrence among archaea and bacteria, with an emphasis on Chl-based phototrophy.

Retinalophototrophs

Retinalophototrophs are widely distributed among bacteria, archaea and even eukarya. As noted above, these organisms produce retinal-binding, rhodopsin-like proteins that are light-activated ion gates. Light causes the all-*trans*-retinal chromophore (Fig. 1(a)) to isomerize to 13-*cis*-retinal, and subsequent conformational changes of the rhodopsin and restoration of all-*trans*-retinal chromophore cause the directional release of an ion into the periplasm or the cytoplasm. Retinal-proteins can produce H^+ or Na^+ gradients that can be coupled to ATP synthesis or nutrient uptake, and halorhodopsin can translocate Cl^- ions into the cytoplasm to help halophiles achieve osmotic equilibrium with their environments. Because retinal-binding proteins do not produce a reductant, retinalophototrophs cannot directly couple light energy to CO_2 fixation.

The first retinal-binding protein to be characterized was bacteriorhodopsin of the halophilic archaeon *Halobacterium* sp. (Fig. 1(b)). Halobacteria are normally aerobes, but because oxygen has limited solubility in the hypersaline conditions under which these organisms thrive, aerobic respiration is often limited due to oxygen limitation. Under anoxic conditions, the cells induce the synthesis of very large amounts of bacteriorhodopsin, leading to so-called purple membrane patches in the cytoplasmic membranes of the cells. Large numbers of these bacteria can occur in salt evaporation ponds, and their numbers can become so high that the ponds adopt the coloration of the organisms (Fig. 1(c)). Bacteriorhodopsin has seven transmembrane alpha-helices that form a 4+3 bundle in the membranes of cells (Fig. 1(b)). The retinal chromophore is covalently bound in a Schiff-base linkage to lysine-216 of the protein, and it intercalates between the alpha-helices near the middle of the membrane. A multi-step photocycle is initiated upon absorption of a photon. All-*trans*-retinal initially and rapidly isomerizes to 13-*cis*-retinal, and relatively minor conformational changes in the protein lead to release of a proton near the periplasmic side of the membrane. Further conformational changes and shifting of hydrogen bonds among tightly bound water molecules and amino acid residues lead to

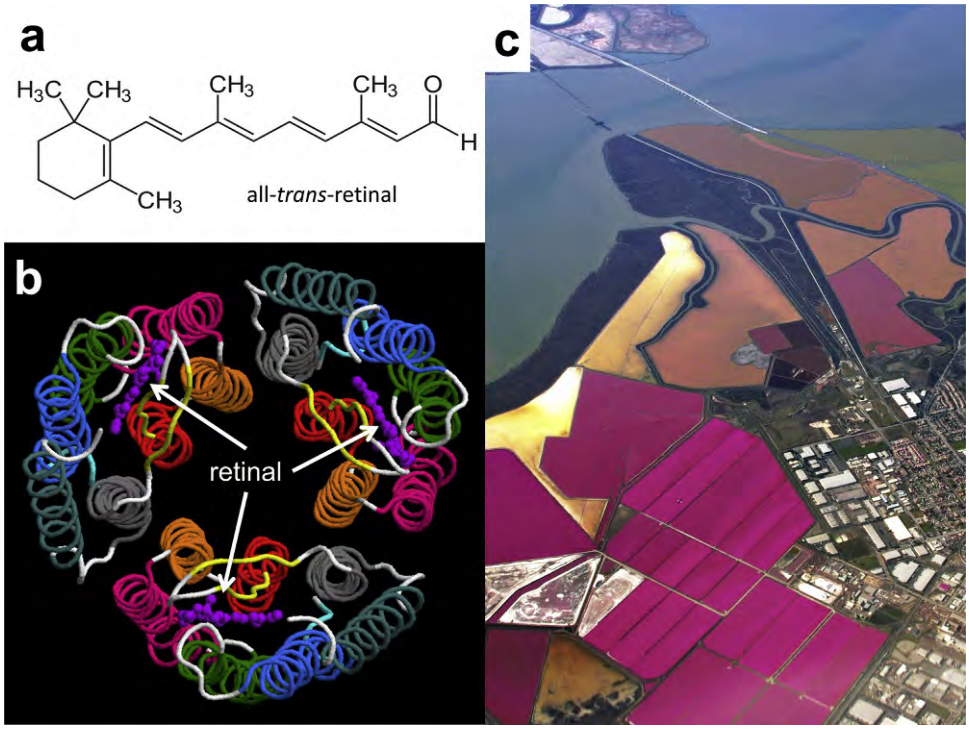

Fig. 1 The structural basis of retinalophototrophy and its appearance in Nature. (a). Structure of all-*trans*-retinal, the chromophore of all retinal proteins found in bacteria and archaea. (b). Structure of bacteriorhodopsin trimer from *H. salinarum*, showing the seven transmembrane α-helices arranged in a 4+3 bundle for each monomer and the covalently attached retinal chromophore (purple, arrows). The view is from the periplasmic surface side of the membrane. *Source*: Protein Data Bank entry: 1×0S. (c)**.** Ponds containing the halophilic archaeon, *Halobacterium salinarum*, and the eukaryotic green alga, *Dunaliella salina. H. salinarum* produces purple membrane and bacteriorhodopsin in Cargills's salt evaporation ponds in San Francisco Bay near Newark, CA. Purple-colored ponds have higher sodium chloride concentrations than the orange-colored ponds, which are orange due to beta-carotene produced by *D. salina.* (*Source*: Wikipedia Commons, dro!d.jpg).

reprotonation of key amino acid residues as well as the return of the retinal to the all-*trans* configuration. Thus, the absorption of one photon leads to the directional release of one proton to the periplasm. The resulting proton motive force can be used to support ATP synthesis via ATP synthase or can be coupled to nutrient uptake. By comparison to chlorophototrophs, which can have antenna complexes with 100′s to 1000′s of antenna pigments, a disadvantage of rhodopsins is that they usually carry a single retinal chromophore and thus have small absorption cross-sections. Xanthorhodopsin, found in a few bacteria, partly overcomes this limitation by binding an antenna carotenoid molecule, Salinixanthin, that can transfer excitation energy to the retinal chromophore and thus increase the light-harvesting capacity of such proteins.

One of the earliest success stories in metagenomics was the discovery that many marine proteobacteria contain genes for bacteriorhodopsin-like proteins. Heterologous expression of one such protein in *Escherichia coli* established that these proteorhodopsins are functional light-driven proton pumps. Further metagenomics surveys have established that rhodopsin-like proteins are very widely distributed in nature, and have further established that these proteins are laterally transferred among archaea and bacteria at high frequencies. This may be due to the relative simplicity of such proteins. If an organism already has the capacity to synthesize β-carotene, then the acquisition of only two additional genes, β-carotene 15,15′-monooxygenase and the gene encoding the rhodopsin-like protein, are all that are required to gain the ability to use light to produce an ion gradient. Intriguingly, in phototrophic mat communities, some (mostly) anoxygenic chlorophotorophs also have the capacity to produce retinal-binding proteins. Although the significance of this observation is not clear, it seems that some chlorophototrophs are also retinalophototrophs.

Chlorophototrophs

Reaction Centers and Antenna Complexes

Chlorophototrophs are defined by their ability to synthesize Chls or BChls and RCs. Two types of RCs occur with some variations: type-1 and type-2. All RCs have either homodimeric or heterodimeric core structures that bind the key electron transfer cofactors. A pair of Chls or BChls, the "special pair," acts as either the primary or secondary electron donor; upon illumination, the special pair is oxidized and an electron is transferred to a Chl-like molecule, (Chl *a*, 8^1-OH-Chl *a*, pheophytin (Pheo *a*), BPheo *a* or BPheo *b*) and ultimately to a secondary electron acceptor that is either a quinone or a [4Fe-4S] cluster. Type-1 RCs produce a weak oxidant (i.e., an

Table 1 Properties of the major taxa of chlorophototrophic bacteria

Group	Phylum	Carbon metabolism	CO_2 fixation pathway	(B)Chls	RC Type	LH	Oxygen	Genera # (#)[a]	Species # (#)[a]
Cyanobacteria[b] (>200[d])	*Cyanobacteria* 21[c] (>800[d])	Autotroph	CBB	Chl *a* (Chl *b*, *d*, *or f*)	PS I PS II	PBS, IsiA, Pcp	Aerobe	16[c]	
Purple sulfur bacteria	*Proteobacteria* (γ-class)	Autotroph/ Heterotroph	CBB	BChl *a or* BChl *b*	Type 2	LH1, LH2	Anaerobe	29	74
Purple non-sulfur bacteria	*Proteobacteria* (α- & β-class)	Heterotroph/ Autotroph	CBB	BChl *a* or BChl *b*	Type 2	LH1, LH2	Anaerobe	28	96
Aerobic anoxygenic phototrophic bacteria[e]	*Proteobacteria* (α-, β- & γ-class)	Heterotroph	None	BChl *a*, (Zn-BChl *a* (rarely))	Type 2	LH1	Aerobe	91	230
Chloracidobacterium thermophilum	*Acidobacteria*	Heterotroph	None	BChl *a*, BChl *c*, Zn-BChl *a'*, Chl *a*	Type 1 (Ho)	Csm	Aerobe	1	1
Gemmatimonas phototrophica	*Gemmatimonadetes*	Heterotroph	None	BChl *a*	Type 2	LH1	Aerobe	1	1
Heliobacteria	*Firmicutes*	Heterotroph	None	BChl *g* 8^1-OH-Chl *a*	Type 1 (Ho)	None	Anaerobe	3 (4)	11 (12)
Chlorophototrophic *Chlorobi*	*Chlorobi*	Autotroph/ Heterotroph	rTCA[f]	BChl *a*, Chl *a*, BChl *c*, *d*, *e*, or (*f*)[g]	Type 1 (Ho)	Csm	Anaerobe	4	20
Chlorophototrophic *Chloroflexi*	*Chloroflexi*	Heterotroph/ Autotroph/ Mixotroph	3-OH-PB; CBB[h]	BChl *a* and BChl *c* (or BChl *d*)	Type 2	Csm and/or LH1	Oxygen-tolerant	4 (8)	6 (10)

[a]Includes *Candidatus* genera/species.

[b]Cyanobacteria are the only oxygenic (oxygen-evolving) chlorophototrophs; all others are anoxygenic.

[c]Recognized species in LTP (Living Tree Project).

[d]Based on NCBI Taxonomy (current August 2017).

[e]This category includes all proteobacteria with the potential to produce PufLM RCs that are not traditional PSB or PNSB (e.g., methylotrophs, *Rhizobia*, *Agrobacterium*, etc.).

[f]None in "*Ca.* Thermochlorobacter aerophilum".

[g]Only in a mutant of *Chlorobaculum limnaeum* DSM1677T.

[h]*Oscillochloris trichoides*, Abbreviations: CBB; Calvin Benson Bassham cycle; rTCA; Reverse tricarboxylic acid cycle; 3-OH-PB; 3-hydroxypropionate bicycle; Chl; chlorophyll; BChl; Bacteriochlorophyll; PS; Photosystem; Ho; Homodimer; PBS; Phycobilisome; PCP, Prochlorophyte-type Chl *a/b*-binding Protein; LH; Membrane associated light-harvesting complex; Csm; Chlorosome.

oxidized Chl or BChl), and a strong reductant, a reduced [4Fe-4S] cluster. Type-2 RCs produce a strong oxidant (i.e., an oxidized Chl or BChl) and a weak reductant, a reduced quinone (quinol). Although there is no detectable sequence similarity for the polypeptides of type-1 and type-2 RCs, strong structural similarities indicate that these proteins evolved only once and that subsequent rounds of gene duplication and divergence led to the types of RCs that occur in modern chlorophototrophs.

Heliobacteria, *Chloracidobacterium thermophilum*, and chlorophototrophic members of the phylum *Chlorobi* have "homodimeric" type-1 RCs. The X-ray structure of the homodimeric RC of *Heliobacterium modesticaldum* was recently solved at 2.2-Å resolution. Surprisingly, the structure revealed that this "homodimeric" RC is actually a heterotetramer comprised of two PshA subunits, each of which have 11 transmembrane α–helices, and two PshX subunits. PshX has a single transmembrane α-helix and participates in binding two BChl *g* molecules and a carotenoid molecule. In total, the heliobacterial RC binds two carotenoids, 54 BChl *g* molecules, two 8^1-OH-Chl *a* molecules (primary acceptor), and four BChl *g'* molecules (two of which form the special pair and two of which are antenna pigments). Many but not all of the BChls occur in similar positions to Chls in the Photosystem I (PSI) RC of cyanobacteria, the second type of type-1 RC. Interestingly, the heliobacterial RC may not contain menaquinone, but the electron transfer chain terminates with an [4Fe-4S] cluster ligated between the two PshA subunits.

There are two families of type-2 RCs, both of which have heterodimeric structures. "Bacterial RCs" are found in chlorophototrophic members of the *Proteobacteria*, *Gemmatimonadetes*, and *Chloroflexi*, and Photosystem II (PSII) occurs in members of the *Cyanobacteria*. In anoxygenic bacteria, RCs are formed from two paralogous proteins, PufL and PufM; a third subunit, PufH, does not occur in members of the *Chloroflexi*. A fourth subunit, a membrane-bound tetraheme cytochrome *c*, is sometimes associated with these RCs. The PufL and PufM subunits each contain five transmembrane α–helices, which are structurally similar to the C-terminus of the core subunits of type-1 reaction centers. Type-2 bacterial RCs bind six pigment molecules – usually four BChl *a* (or sometimes four BChl *b*) and two BPheo *a* (or two BPheo *b*) molecules – one carotenoid molecule, and two quinones. The special pair transfers an electron sequentially to one of the two BPheo *a* molecules, to the first quinone (Q_A) and then to the second quinone (Q_B). Once

Q_B is doubly reduced and fully protonated, it leaves the RC and is reoxidized by ubiquinone:cytochrome *c* oxidoreductase; the reduced cytochrome *c* directly or indirectly reduces the oxidized special pair, completing a cycle that results in the production of protonmotive force for ATP production or other cellular work.

Cyanobacteria are unique among chlorophototrophic bacteria because they contain two types of RCs, PSI and PSII, which allow them to oxidize water. The core of PSI is formed by the PsaA and PsaB subunits, which are distant paralogs of PshH of heliobacteria. PSI is more complex than the homodimeric RCs of heliobacteria. PSI has 9 or 10 additional subunits and binds 96 Chl *a* molecules, 22 β–carotene molecules, two phylloquinones, and three [4Fe-4S] clusters. The core electron transfer chain comprises the special pair (a heterodimer of Chl *a* and Chl *a*′), four Chl *a* molecules, two phylloquinone molecules, and the F_X [4Fe-4S] cluster bound at the interface of the PsaA and PsaB subunits. PsaC, a small extrinsic, ferredoxin-like molecule, ligates two [4Fe-4S] clusters that form the terminal electron acceptors, F_A and F_B. PSI can reduce a soluble [2Fe-2S] ferredoxin or flavodoxin, which distributes electrons to ferredoxin:$NADP^+$ oxidoreductase and to other cellular processes that require electrons (e.g., nitrate or sulfate reduction).

Like PSI, cyanobacterial PSII is far more complex than the bacterial RC. As crystallized, PSII contains 19 or 20 subunits, 35 Chl *a*, two Pheo *a*, 11 carotenoids, and two plastoquinones in addition to the $Mn_4Ca_1O_5$ cluster that forms the active site for water oxidation. PsbA and PsbD, which form the core of the PSII RC, are distant paralogs of PufL and PufM, but phylogenetic analyses indicate that the core subunits of PSII arose from an independent gene duplication event. Until recently, no homodimeric type-2 RCs were known. However, the enzyme that catalyzes the four-electron oxidation of Chl *a* (or chlorophyllide *a*) to form Chl *f* in some terrestrial cyanobacteria is an homodimeric, type-2 photooxidoreductase that may have been an evolutionary predecessor to PSII.

The relatively small numbers of pigments associated with most RCs would not allow them to function efficiently at low and variable irradiance, and thus all chlorophototrophic bacteria except heliobacteria have extensive light-harvesting antenna complexes to increase substantially the number of chromophores associated with the RCs. Although RCs evolved once and then diversified, several independent solutions to the problem of light harvesting have evolved and are employed to optimize phototrophic metabolism. Antenna systems in chlorophototrophic bacteria are highly diverse, and only a few examples will be described here briefly. The light-harvesting complexes of *Proteobacteria* are ring-shaped, membrane-intrinsic, BChl-binding complexes comprised of two paralogous families of proteins with single transmembrane α–helices. Each subunit binds a single BChl molecule and the (αβ) protomer additionally binds one carotenoid molecule. The LH1 antenna complexes form ring-shaped arrays of about 15–17 protomers that surround the RC like a stockade. Many but not all *Proteobacteria* also produce LH2 antenna complexes that form ring-shaped octameric or nonameric complexes; the (αβ) protomers of LH2 each bind three BChls and one carotenoid. Because the ratio of LH2 to LH1 is not fixed, 200–300 BChl molecules can feed excitation energy to a single RC complex. LH2 complexes are not found in all chlorophototrophic *Proteobacteria*, *Gemmatimonas phototrophica* or in chlorophototrophic members of the *Chloroflexi*.

Green bacteria, which occur as members of the phyla *Chlorobi*, *Chloroflexi*, and *Acidobacteria*, have two defining properties: They synthesize BChl *c*, *d*, *e*, or *f*; and these special, self-assembling BChls form enormous, supramolecular, nanotubular arrays in light-harvesting antenna complexes known as chlorosomes. The characteristic green or brown colors of GSB result from the type of BChl present in chlorosomes (Fig. 2(i) and (j)). Green-colored strains have chlorosomes containing BChl *c* and/or *d*, and additionally contain chlorobactene and γ-carotene as major carotenoids (Fig. 2(i)). Brown-colored strains produce chlorosomes containing BChl *e* (or BChl *f*) and the bicyclic carotenoids, isorenieratene and/or β-isorenieratene (Fig. 2(j)). Self-aggregation of BChl *e* or *f* leads to a very strong absorption band at ~500–525 nm, which greatly enhances the ability of brown-colored GSB to absorb blue light. Brown-colored GSB are adapted to lower irradiances and are found at greater depths in aquatic environments than green-colored GSB. The chlorosomes of some members of the *Chlorobi* are the largest antenna structures known, and can contain up to 250,000 BChl molecules, along with substantial amounts of carotenoids and quinones. GSB cells contain up to 50 million BChl molecules, which collectively can account for ~30% of the cellular carbon. A protein-stabilized, lipid monolayer envelope surrounds the chlorosome. The major envelope protein is CsmA, a small, mostly α-helical protein that binds one BChl *a* molecule. CsmA forms dimers that assemble into a paracrystalline structure known as the baseplate in all chlorosomes. In members of the *Chlorobi* and *Acidobacteria*, CsmA interacts with another BChl *a*-binding protein, the trimeric Fenna-Matthews-Olson protein that binds eight BChl *a* molecules per monomer. The FMO protein in turn binds to the type-1 RCs in these organisms and ensures efficient energy transfer from the BChls of the chlorosome to the RCs. In chlorosome-containing members of the *Chloroflexi*, chlorosomes are associated with B808–866-RC complexes that are similar to LH1-RC complexes of purple bacteria, and *Roseiflexus* spp. have similar complexes.

Most chlorophototrophic members of the *Cyanobacteria* synthesize light-harvesting antenna proteins known as phycobiliproteins. Phycobiliproteins are a homologous family of brilliantly colored, water-soluble proteins that bind linear tetrapyrrole chromophores known as bilins. Together with assembly proteins known as linkers, phycobiliproteins assemble into supramolecular complexes known as phycobilisomes. Several structural classes of phycobiliprotein complexes are known. Typical cyanobacterial phycobilisomes can contain from ~500 to 1000 chromophores, which are usually associated primarily with PSII but can also transfer energy to PSI under some conditions. Phycobiliproteins absorb light in the visible range from about 500–650 nm that is poorly absorbed by Chl *a*; the phycobiliprotein content of PBS can be regulated by light wavelength in some organisms, allowing cells to acclimate to the wavelength composition of the incident light. Cyanobacteria can also synthesize integral membrane proteins that bind Chl *a* or Chl *a* and *b*; the IsiA protein can conditionally replace phycobiliproteins as antenna complexes under iron limitation. Other proteins paralogous to IsiA are the only light-harvesting proteins in some cyanobacteria that do not produce phycobiliproteins, such as *Prochlorococcus* spp. and *Prochlorothrix hollandica*. These Chl-protein complexes can associate with PSI, PSII, or both to improve light harvesting.

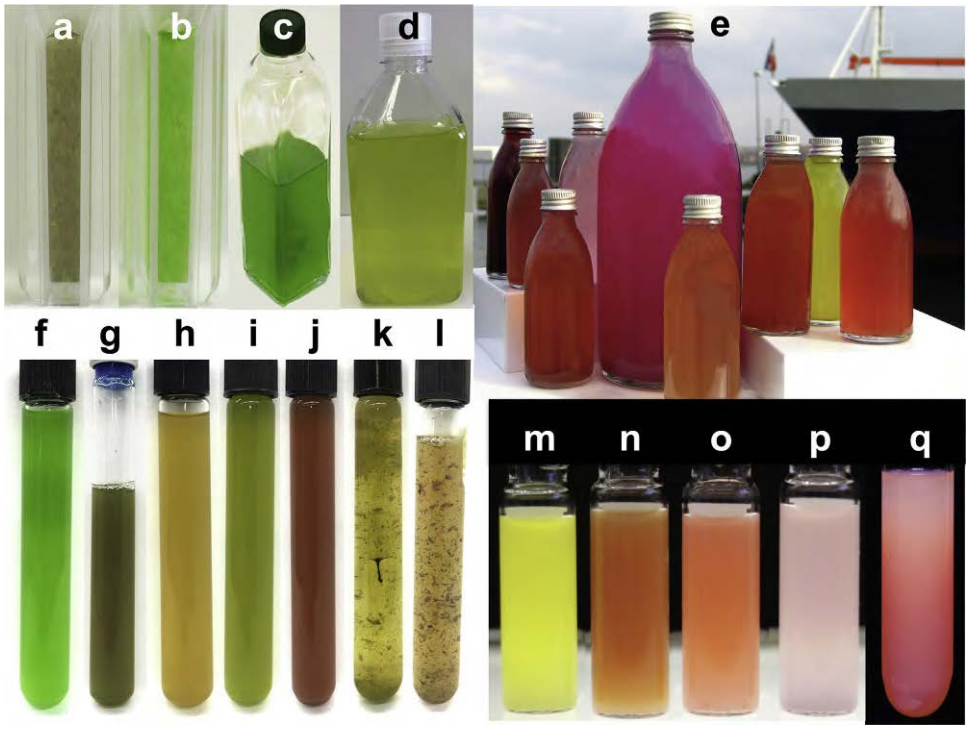

Fig. 2 The many colors of chlorophototrophic bacteria. (a, b) *Leptolyngbya* sp. JSC-1 (*Cyanobacteria*) grown in white light (a) or far-red light (b); (c) *Synechocystis* sp. PCC 6803 (*Cyanobacteria*); (d) *Prochlorococcus* sp. (*Cyanobacteria*); (e) a selection of purple bacteria (*Proteobacteria*) grown under anoxic, phototrophic conditions; (f) *Synechococcus* sp. PCC 7002 (*Cyanobacteria*); (g) BChl *g*-producing *Heliobacterium modesticaldum* ICE-1 (*Firmicutes*); (h) *Chloracidobacterium thermophilum* strain B^T (*Acidobacteria*); (i) green-colored, BChl *c*-producing *Chlorobaculum tepidum* (*Chlorobi*); (j) brown-colored, BChl *e*-producing *Chlorobaculum limnaeum* (*Chlorobi*); (k, l) green-colored and brown-colored *Chloroflexus* sp. (*Chloroflexi*), respectively, grown under anoxic conditions; (m-p) aerobic anoxygenic phototrophic bacteria (*Proteobacteria*): (m) *Sphingomonas* sp. AAP2; (n) *Erythrobacter longus*; (o) *Roseococcus thiosulfatophilus*; (p) *Roseobacter litoralis*; (q) *Gemmatimonas phototrophica* strain $AP64^T$ (*Gemmatimonadetes*). Source for images (m-q), Dr. Michal Koblížek, Center Algatech, Institute of Microbiology, The Czech Academy of Sciences.

Cyanobacteria: Oxyphotobacteria

Members of the phylum *Cyanobacteria*, first described by Gomont in 1829, are aerobic, Gram-negative, oxygen-evolving chlorophototrophs (formerly known as blue-green algae and now sometimes called *Oxyphotobacteria*) (Fig. 2(a-d) and (f)). Although numerous exceptions are known (see below), most cyanobacteria synthesize Chl *a* as sole Chl and have three major complexes for harvesting and transducing light energy into chemical potential energy: PSI, PSII, and PBS. Cyanobacteria reduce carbon dioxide to biomass via the Calvin-Benson-Bassham cycle, and many produce the enzyme nitrogenase that allows the reduction of dinitrogen to ammonia. Many cyanobacteria undergo complex developmental cycles and produce specialized cells for nitrogen fixation (heterocysts), dispersal (baeocytes, hormogonia), and/or survival (akinetes). An interesting exception to these general properties is "*Candidatus* Atelocyanobacterium thalassa," which includes photoheterotrophic cyanobacterial symbionts of prymnesiophyte algae. These marine symbionts have substantially reduced genomes, 1.44–1.48 Mbp in different strains, which have lost genes encoding PSII, ribulose 1,5-*bis*-phosphate carboxylase/oxygenase (RuBisCO) and enzymes of other metabolic pathways, but have retained genes for nitrogenase and PSI, leading to nitrogen-fixing, specialist cells with anoxygenic photoheterotrophic metabolism – "symbiont heterocysts."

No consistent taxonomic scheme that integrates molecular, morphological, and ecophysiological observations currently exists for cyanobacteria. However, using polyphasic methods, nine cyanobacterial clades have recently been described: *Gloeobacterales*, *Synechococcales*, *Oscillatoriales*, *Chroococcales*, *Pleuocapsales*, *Spirulinales*, *Rubidibacter/Halothece*, *Chroococcidiopsidales*, and *Nostocales*, and the CyanoDB database currently lists >270 "validly described" genera (see "Relevant Websites section"). Genomic data and polyphasic methods that combine both molecular and phenotypic criteria have increased the number of major groups of cyanobacteria beyond the five sections suggested by Rippka and coworkers in 1979. Currently, the NCBI Taxonomy database groups nearly 18,000 entries into 8 orders, 49 families, 213 genera and approximately 800 species (see "Relevant Websites section"). Some authors have estimated that there could be up to 8000 cyanobacterial species, although it is unclear what definition of "species" was used to produce such estimates. It is clear that the authors were not considering ecologically distinct species, i.e., ecotypes, which would increase such estimates by many orders of magnitude.

The 3.4-Mbp genome of *Synechocystis* sp. PCC 6803 (Fig. 2(c)) was completely sequenced in 1996, and at that time, it was only the third completely sequenced bacterial genome. More than 550 cyanobacterial genomes, including representatives of all major

taxonomic groups, have now been sequenced. Consistent with their ancient origin and their metabolic, structural, developmental and physiological diversity, cyanobacterial genomes are highly diverse. Their sizes (1.6–13 Mbp) and GC contents (~30%–69%) have ranges similar to those of all bacteria. The large number of available cyanobacterial genomes should allow comparative analyses that can identify genes and metabolic modules associated with specific processes (e.g., heterocyst differentiation) or a specific ecological niche. An example of the latter is illustrated by findings associated with ability to grow in far-red light.

Terrestrial cyanobacteria often grow in environments that are strongly enriched in light wavelengths greater than 700 nm (e.g., soil, or under plant canopies where light is strongly filtered by Chl *a*). Many of these cyanobacteria can acclimate to these conditions, a process known as far-red light photoacclimation (FaRLiP; Fig. 2(a) and (b)). Comparative genomic analyses of FaRLiP organisms have revealed a highly conserved cluster of 20 genes, 17 of which are paralogs of genes encoding core subunits of PSI, PSII and PBS that are expressed in white light. The FaRLiP gene cluster additionally encodes three regulatory proteins: RfpA, RfpB, and RfpC. When cells capable of FaRLiP are grown in far-red light, a knot-less phytochrome (RfpA) phosphorylates a phosphate-shuttle protein (RfpC), which activates the transcriptional activator (RfpB) and thereby the expression of the genes of the FaRLiP gene cluster. Upon acclimation, cells synthesize far-red light absorbing pigments, Chls *d* and *f*, as well as specialized forms of allophycocyanin that absorb far-red light and assemble into specialized PBS-like complexes. The extensive remodeling of the photosynthetic apparatus to accommodate these changes allows cells to grow in far-red light. One of the genes in the FaRLiP gene cluster, *chlF*, encodes a highly divergent paralog of the PSII core subunit, PsbA. ChlF is a photo-oxidoreductase that synthesizes Chl *f* from Chlide *a* or Chl *a*. Examples of cyanobacteria that can perform FaRLiP are found in all major taxonomic groups, but no extensive metagenomic survey to identify terrestrial cyanobacteria that can perform FaRLiP has yet been performed.

Marine *Synechococcus* spp. and especially *Prochlorococcus* spp. are numerically dominant organisms of Earth's oceans and are believed to be responsible for ~25% of total global carbon fixation (and thereby dioxygen production). *Prochlorococcus* spp. were first described in 1988, and interestingly, unlike most cyanobacteria, they employ divinyl-Chl *a* and divinyl-Chl *b* in their photosynthetic apparatus and lack PBS (Fig. 2(d)). *Prochlorococcus* spp. also dominate genomic databases, accounting for ~30% of the sequenced cyanobacterial genomes. Twelve major clades of *Prochlorococcus* spp. are known, but examples of only five have been cultivated. Comparative physiological studies have shown that each clade is adapted to different light, temperature, and nutrient conditions. Furthermore, quantitative PCR analyses have shown that temperature, light, nutrients and competitor densities shape the populations of these organisms in their natural habitats. Comparative genomics of low-light and high-light-adapted ecotypes of *Prochlorococcus* and *Synechococcus* spp. reveal differences in genome size as well as genomic islands possibly responsible for specific adaptations to niches.

High-light-adapted ecotypes of *Prochlorococcus* spp. have small genomes, ~1.62–1.78 Mbp, but low-light-adapted ecotypes have larger genomes, ~1.7–2.6 Mbp. Marine *Synechococcus* spp. genomes are still larger, 2.2–2.86 Mb, although these genome sizes are still smaller than average bacterial (3.69±1.96 Mbp) or cyanobacterial genomes (5.33±3.69 Mb). Sequence analyses of worldwide samples has revealed that 100's of distinctive, individual genotypes can coexist in a milliliter of seawater. Each genome type has a unique gene complement, which presumably confers selective advantages to the individuals of that population. Remarkably, the global pangenome for *Prochlorococcus* spp., ~80,000 genes, is 40–50-fold larger than the gene content of an individual cell. Cyanophages enhance *Prochlorococcus* spp. diversity by transferring genes among the various ecotypes and by affecting natural populations by lytic infection cycles. Finally, mathematical modeling studies suggest that *Prochlorococcus* spp. and the dominant, co-occurring heterotroph, *Pelagibacter ubique* (SAR11), have a coevolved mutualism that maximizes their collective metabolic rate and carbon recycling through complementary carbon excretion and uptake pathways.

Although the *Synechococcus* spp. that inhabit the microbial mats of alkaline siliceous hot springs in Yellowstone National Park are only distantly related to these organisms in the open ocean, an analogous distribution of complex ecotypes with different genotypes associated with specific niches is also observed across physical and chemical gradients in these mats. The *Synechococcus* spp. in these mats have also probably coevolved with preferred phototrophic and non-phototrophic partners to build stable communities of similar composition that can be observed in mat communities associated with alkaline hot spring world-wide. Recent studies suggest that the microbial mats of Mushroom and Octopus Springs contain more than 17 chlorophototrophic species, including representatives of all seven phyla known to contain chlorophototrophs.

The structure of the enzyme RNA polymerase has been studied extensively in numerous bacteria. In all bacteria studied the enzyme has a core consisting of four subunits, $\alpha_2\beta\beta'$, and a fifth protein, σ, needed for promoter recognition and the initiation of transcription. Careful examination of the structure of cyanobacterial RNA polymerase revealed that the core consists of five subunits. Simply, the β' subunit in cyanobacteria is divided into two subunits, β' and γ. Remarkably, consistent with the idea that plant chloroplasts evolved from cyanobacteria, the core of RNA polymerase in plant chloroplasts consists of the same collection of subunits that occur in cyanobacteria.

Proteobacteria: Purple Bacteria

Chlorophototrophic *Proteobacteria* were first described in the middle of the 19th century, and they now represent the most metabolically diverse group of anoxygenic chlorophototrophs. Members of this major group of organisms have contributed significantly to our knowledge of the biochemistry and molecular mechanisms of photosynthesis and its regulation, and they have further been used to elucidate the biochemistry of sulfur metabolism. Their members occur in the *Alpha-*, *Beta-*, and *Gammaproteobacteria*, and are currently assigned to 11 orders, 16 families, and more then 330 species. Based upon their capacities

to oxidize sulfide and to form microscopically visible sulfur (polysulfide) globules, Winogradsky and Molisch were the first to recognize two distinct groups, "*Thiorhodaceae*" and "*Athiorhodoceae*." Collectively described as "purple bacteria," these two traditional groups are commonly referred to as purple sulfur bacteria (PSB; 29 genera and 74 species) and purple non-sulfur bacteria (PNSB; 28 genera and 96 species), respectively. Most purple bacteria produce BChl and RCs under anoxic conditions and are thus facultatively photosynthetic, often being capable of other metabolic modes, including aerobic respiration. Cultures of purple bacteria range in color from yellowish green to pink to reddish purple to dark brown because of the synthesis of large amounts of carotenoids in addition to either BChl *a* or BChl *b* (see Fig. 2(e)). Purple bacteria produce type-2 RCs and at least one light-harvesting complex, LH1, although many synthesize one or more additional types of BChl-binding LH complexes (e.g., LH2 and/or LH3). Purple bacteria fix carbon via the Calvin-Benson-Bassham cycle, and many are also capable of reducing dinitrogen when grown under anoxic conditions. A third major group of related organisms, the aerobic anoxygenic phototrophic bacteria (AAPB), were discovered in Japanese coastal waters in 1979. Unlike PSB and PNSB, in which oxygen suppresses the synthesis of BChl and the chlorophototrophic apparatus, AAPB produce BChl *a*, type-2 RCs, and carotenoids under oxic conditions (Fig. 2(m-p)). Most AAPB are strictly aerobic chemoheterotrophs, which supplement their energy production with chlorophototrophic energy production under oligotrophic conditions. An alternative and broader term for AAPB is aerobic BChl-containing (ABC) bacteria, which includes methylotrophs, *Rhizobia*, as well as some anoxygenic chlorophototrophs from other phyla.

Chlorophototrophic proteobacteria are abundant in highly diverse habitats, including soils, freshwater and marine aquatic environments, hot springs, hypersaline springs, soda lakes, hydrothermal vents, activated sludge and waste-water treatment systems. In addition to their ability to thrive in the presence of different oxygen concentrations, they may exhibit preferences for acidic or alkaline conditions and tolerate temperatures from below freezing to about 60°C. PSB are members of the *Gammaproteobacteria* that utilize sulfide as the preferred electron source for carbon fixation, and they can be found in most environments where light and sulfide occur together. Two families, *Chromatiaceae* and *Ectothiorhodospiraceae*, are distinguished by the location of polysulfide/sulfur globule deposition. In members of the *Chromatiaceae*, sulfur globules are formed inside the cells, while in *Ectothiorhodospiraceae*, the sulfur globules are deposited extracellularly. PNSB are facultatively anaerobic, anoxyenic chlorophototrophic members of the *Alpha-* and *Betaproteobacteria*. In spite of their common name, most PNSB are capable of sulfide oxidation, but they tolerate sulfide levels (~0.5 mM) about 10-fold lower than PSB. In addition to reduced sulfur compounds and hydrogen used by most purple bacteria, some strains can use ferrous iron [Fe^{2+}], nitrite, or arsenite as electron donors for carbon fixation.

AAPB are a diverse group of aerobic chemoheterotrophs but are also anoxygenic chlorophototrophs that produce BChl under oxic conditions. *Roseobacter denitrificans* and *Erythrobacter longus* were the first organisms isolated from marine environments, and more than 80 species and a very large number of isolates are now known. The *Roseobacter* clade (*Alphaproteobacteria*, *Rhodobacteraceae*) is one of the most dominant clades in pelagic environments and can represent up to 30% of bacterioplankton communities. Overall, AAPB are the third most abundant chlorophototrophs in the ocean, and although members of the *Alphaproteobacteria* are most common, examples are now known among *Beta-* and *Gammaproteobacteria* as well. AAPB have been widely detected in other environments, including freshwater rivers and lakes. Numerous examples are also known for most extreme environments, including polar regions, hot springs, hypersaline springs, hydrothermal vents, and soils contaminated with toxic metal oxides. If one considers all organisms that have the potential to produce PufLM-containing type-2 RCs, then >500 genomes of potentially chlorophototrophic proteobacteria have now been sequenced. This currently includes representatives of about half of the described species of PSB and PNSB. These genomes characteristically have high GC contents (60%–74%) and are quite variable in size (~2.5–8.5 Mbp).

Roseobacter spp. are metabolically and physiologically versatile organisms that are also characterized by high genomic diversity. Although the type species, *Roseobacter litoralis*, is a chlorophototroph, chemoheterotrophic members are more numerous. AAPB synthesize BChl *a*, a variety of carotenoids, and type-2 RCs, and like other purple bacteria, have quite variable coloration (see Fig. 2 (e) and (m-p)). Unlike other purple bacteria, AAPB are obligate chemoheterotrophs, although it is likely that most fix some carbon via anaplerotic reactions. Marine *Roseobacter* spp. are environmentally important because some degrade dimethylsulfoniopropionate, which is a major source of the climatically active gas, dimethyl sulfide, in the atmosphere.

Chlorobi: Formerly Green Sulfur Bacteria (GSB)

Most chlorophototrophic members of the phylum *Chlorobi* are green sulfur bacteria (GSB). GSB are strictly anaerobic, anoxygenic Gram-negative bacteria that are exquisitely adapted to low-light environments. GSB occur in the anoxic waters of lakes, inland seas and other stratified aquatic habitats – where sulfide and light co-occur. They are also found in circum-neutral hot springs, microbial mats, and anoxic sediments, where sulfide and/or thiosulfate are present but light levels limit the growth of other chlorophototrophs. Remarkably, GSB have been found at the chemocline of the Black Sea and near black smokers on the floor of the Pacific Ocean, environments where it is estimated that individual BChl molecules absorb only ~1–10 photons per day. Because they have a very limited capacity to assimilate organic compounds, GSB are obligately autotrophic and obligately phototrophic. They oxidize reduced sulfur compounds or ferrous iron and use their homodimeric type-1 RCs to produce very low potential ferredoxins, which are used for Ci fixation by the reverse tricarboxylic acid (rTCA) cycle.

Because of its rapid growth on thiosulfate, its fully sequenced genome and its capacity for genetic modification by natural transformation or conjugation, *Chlorobaculum* (*Cba.*) *tepidum* (formerly *Chlorobium tepidum*) has become the laboratory model for GSB (Fig. 2(i)). Much of the current physiological and biochemical knowledge of GSB derives from studies of this organism. GSB

genomes are small (1.97–3.13 Mb) and have moderate GC contents (44%–57%). Although synteny of chromosomal genes has not been highly maintained, GSB share a conserved core genome that includes all genes necessary for their anoxygenic chlorophototrophic lifestyle by sulfur oxidation and CO_2 fixation. The loss of synteny may be due to an apparently high proportion of genes acquired by horizontal gene transfer (HGT), which has been estimated to be ~24% for *Cba. tepidum*. Phage-mediated transduction is another plausible mechanism for HGT, and might be responsible for exchange of the *sox* cluster for thiosulfate utilization among GSB. Surprisingly, however, no phages infecting *Chlorobi* have yet been isolated. Nevertheless, a recent metagenomic study provided strong evidence that a lytic DNA phage acted as a vector for phage-mediated HGT of genes for BChl *e* and isorenieratene biosynthesis in the natural GSB population in Lake Císo, a meromictic lake in Spain.

For nearly a century after their discovery in 1906 by Nadson, chlorophototrophic members of the phylum *Chlorobi* were considered to be synonymous with "green sulfur bacteria." After nearly a century of isolation and study, anaerobic species associated with only four genera had been cultivated: *Chlorobium*, *Chlorobaculum*, *Prosthecochloris* and *Chloroherpeton*. The discovery of "*Ca.* Thermochlorobacter aerophilum" by metagenomic analyses, and the establishment of enrichment cultures from several hot spring mats for the cultivation of this unusual organism, broke the dogma concerning the physiological conformity of GSB that was based on decades of cultivation-based studies. Although "*Ca*. T. aerophilum" has a phototrophic apparatus very similar to that of GSB, it lives in an environment where oxygen tensions can reach 800% of saturation during the day. It cannot oxidize sulfur compounds, cannot fix carbon or nitrogen, and requires all three branched-chain amino acids for growth. Unlike all strictly anaerobic GSB, it relies on oxygen for important metabolic reactions, including (B)Chl biosynthesis. Further genomics studies have revealed that at least some of the early diverging members of this phylum are metabolically versatile, aerobic chemoorganoheterotrophs (e.g., *Ignavibacterium album*, *Melioribacter roseus*, and NICIL-2, an uncultured representative of the OPB56 clade), which are closely related to some members of the phylum *Bacteroidetes*. Phylogenetic analyses of the genomes of these early diverging members of the *Chlorobi* indicate that GSB now are restricted to the family *Chlorobiaceae* (class *Chlorobea;* order *Chlorobiales*).

GSB form syntrophic associations with other types of bacteria. For example, sulfide-oxidizing GSB are frequently isolated together with sulfate- or sulfur-reducing bacteria; together, these organisms can cycle sulfide and sulfate and organic carbon and Ci, producing a stable association. During syntrophic growth of a *Prosthecochloris* sp. with sulfur-reducing *Geobacter sulfurreducens*, direct intracellular electron transfer occurs in a process known as "syntrophic anaerobic photosynthesis." *Prosthecochloris* sp. is independent of sulfide as an electron source and receives its electrons indirectly from the oxidation of organic matter by *G. sulfurreducens*. Although the partners are co-dependent in co-culture, the unknown mechanism(s) of electron transfer seems to be non-specific because the *Prosthecochloris* sp. strain is also able to receive electrons supplied by an electrode.

Phototrophic consortia are a particularly interesting type of symbiosis in which two types of bacteria combine to form a multicellular "holobiont" with quite distinctive properties. "Chlorochromatium aggregatum" and "Pelochromatium roseum" are examples in which the chlorophototrophic consortial partner can be either a green- or a brown-colored GSB, respectively, and the motile, heterotrophic partner is a betaproteobacterium from the candidate genus, *Symbiobacter*. Complete genome analysis of "Chlorochromatium aggregatum" suggests that this partnership may have arisen in spite of "chronic dysfunctionality," because the "*Ca*. Symbiobacter mobilis" requires oxygen for important metabolic functions, while its GSB partner, *Chlorobium chlorochromatium*, is exquisitely sensitive to oxygen, especially in the light. "*Ca*. S. mobilis" has a single polar flagellum, and can confer swimming motility as well as chemotaxis and phototaxis upon the GSB partner (Note: all members of the *Chlorobiaceae* lack flagella). "*Ca*. S. mobilis" can sense light and sulfide, neither of which it can use, allowing taxis to locate favorable growth conditions for the consortia and allowing consortia to outcompete free-living GSB with which they co-occur. In return, the GSB partner apparently provides fixed carbon, fixed nitrogen, hydrogen and vitamin B_{12} to its partner. "*Ca*. S. mobilis" lacks the genes for both known pathways for menaquinone biosynthesis, and it has an incomplete pathway for ubiquinone biosynthesis, yet its genome encodes electron transfer complexes that are known to require quinones as coenzymes for their function (e.g., type-1 NADH dehydrogenase, succinate dehydrogenase, and cytochrome *bd* quinol oxidase). *Chl. chlorochromatium*, which can be cultivated independently from its partner, clearly can synthesize menaquinone. Minimally, menaquinone must be provided to "*Ca*. S. mobilis," but bidirectional exchange of menaquinone and menaquinol would allow oxidation of menaquinol driven by photosynthetic C_i reduction, directly coupling the energy metabolism of "*Ca*. S. mobilis" to the photosynthetic productivity of its partner. The two cell types are extensively connected by numerous "periplasmic tubules," which suggests that the two organisms may share a common periplasmic space – and therefore share a common proton gradient for ATP synthesis.

Chloroflexi: Filamentous Anoxygenic Phototrophs (FAPs)

The phylum *Chloroflexi* currently comprises eight classes: *Anaerolineae*, *Ardenticatenia*, *Caldilineae*, *Chloroflexia*, *Dehalococcoidia*, *Ktedonobacteria*, *Thermoflexia*, and *Thermomicrobia*. Chlorophototrophic organisms occur in two classes: *Chloroflexia* and *Anaerolineae*. Most chlorophototrophs belong to the order *Chloroflexales*, which is further divided into two sub-orders: *Chloroflexineae* and *Roseiflexineae*. These two suborders include organisms colloquially called FAPs, filamentous anoxygenic phototrophic bacteria, which can often be differentiated by their color (*Chloroflexineae*: green; *Roseiflexineae*: reddish-brown) and by the presence (*Chloroflexineae*) or absence (*Roseiflexineae*) of chlorosomes. Because most do not require reduced sulfur compounds for growth, they were formerly called "green non-sulfur bacteria." Members of the *Chloroflexales* grow facultatively as aerobic chemoheterotrophs in the dark and grow as anaerobic photoheterotrophs in the light. Some members can grow photoautotrophically and/or photomixotrophically under anoxic conditions in light. Green-colored FAPs occur in two families, *Chloroflexaceae* and

Oscillochloridaceae. Cell suspensions are usually greenish-brown in color under anoxic conditions because the cells produce chlorosomes that contain BChl *c* with an *in-vivo* absorption maximum at ~740 nm (Fig. 2(k) and (l)); BChl *a* is additionally present (and sometimes BChl *d*). Unlike GSB, FAPs have type-2 RCs and lack FMO. Instead, a membrane-associated LH1-like complex with absorption bands at 808 and 866 nm surrounds the RC and functionally replaces FMO. All described *Chloroflexaceae*, *Chloroflexus* spp. and "*Candidatus* Chloranaerophilum corporosum," are thermophilic, unbranched, multicellular filamentous bacteria. They inhabit freshwater hot springs and grow well at ~55°C, and most exhibit gliding motility and flexing. All characterized *Chloroflexus* spp. have the genetic potential to fix CO_2 by the 3-hydroxypropionate bi-cycle, a pathway exclusively found in *Chloroflexi*. Most strains grow mixotrophically, but autotrophic growth has been demonstrated for *Cfl. aurantiacus* strains OK-70-fl (DSM 636) (with H_2) and *Chloroflexus* sp. MS-G (with H_2S).

The family *Oscillochloridaceae* is a second family of green-colored FAPs, which mostly includes *meso*philic filamentous, freshwater bacteria with gas vesicles and an outer sheath. Cultures of the type species, *Oscillochloris triochoides* DG-6^T, are green or yellow-green in color due to the presence of BChl *c*-containing chlorosomes and BChl *a*. In contrast to other FAPs, *O. trichoides* grows photolithoautotrophically by using the Calvin-Benson-Bassham cycle (reductive pentose-phosphate cycle).

A novel green FAP, provisionally named "*Candidatus* Chloranaerofilum corporosum," was recently found in a hot spring microbial mat in Yellowstone National Park. This novel FAP was first detected in metagenomic analyses of the orange-colored undermat and is a member of the suborder *Chloroflexineae*; however, it presently cannot be affiliated reliably with either of the two families of this suborder (<91% nucleotide identity). This FAP contains BChls *a* and *c* and grows at 52°C as long filaments of single cells, which have a relatively large diameter of ~2 μm and are separated by obvious septa. Suggesting the capacity for photoautotrophic or photomixotrophic growth, the partial genome contains genes encoding for enzymes of the 3-hydroxypropionate bi-cycle. However, autotrophic growth has not yet been tested, and the strain is currently grown photoheterotrophically.

Red FAPs of the genus *Roseiflexus* (family *Roseiflexaceae*) are the only described strains currently available (e.g., *Roseiflexus castenholzii*; other previously described strains (e.g., *Heliothrix oregonensis*) have been lost. Red FAPs are (moderately) thermophilic and have been found in slightly sulfidic (<0.5 mM sulfide) hot springs, where they form orange-, reddish- or pink-colored mats. Red FAPs synthesize BChl *a*, carotenoids, LH1, and type-2 RCs but lack BChl *c* and chlorosomes. *Roseiflexus* (*Rof.*) *castenholzii*, the only validly described species, was isolated from Nakabusa Hot Springs in Japan. It forms a distinct, dense reddish or pink layer underneath mats of *Cyanobacteria* and *Chloroflexus* spp. at temperatures of 45.5–68.5°C at pH 7.8–8.2. *Rof. castenholzii* grows photoheterotrophically under anoxic conditions in the light as well as chemotrophically under dark oxic conditions. Similar members of this genus have been observed or isolated from hot springs in Yellowstone National Park. Like *Rof. castenholzii*, the Yellowstone isolates contain BChl *a*, lack BChl *c* and chlorosomes, and grow photoheterotrophically or chemoheterotrophically under dark aerobic conditions. Although photoautotrophic growth has not been observed, the presence of genes encoding all enzymes of the 3-hydroxypropionate bi-cycle, as well as stable carbon isotopic compositions of FAP biomarkers, strongly suggest that photoautotrophic or photomixotrophic growth occurs *in situ*.

Red FAPs are no longer monophyletic because of the recent discovery of an uncultured organism that probably belongs to the class *Anaerolineae*. Metagenomic analyses of hot spring microbial mats from Yellowstone National Park initially suggested the existence of a chlorophototrophic member of the *Anaerolineae*. The metagenomic bin representing this organism contains *pufLM* genes encoding a type-2 RC as well as enzymes for the synthesis of BChl *a* but not BChl *c*. Very thin filaments (~0.2 μm in width) are observed in mat samples and enrichments with lengths of ~15–50 μm, which exhibit autofluorescence from BChl *a* but not BChl *c*. These filaments probably represent this novel phototroph, which has provisionally been named "*Candidatus* Roseilinea gracile." Interestingly, *Roseiflexus* sp. RS-1 as well as "*Ca*. Roseilinea gracile" contain rhodopsin genes with phylogenetic affiliation to light-driven, xanthorhodopsin-like H^+ pumps, indicating the possible co-existence of retinal-based phototrophy and chlorophototrophy in these FAPs. Finally, a poorly described member of the red FAPs is the type-isolate of Eikelboom morphotype 1851, *Kouleothrix aurantiaca*. On the basis of its 16S rRNA gene sequence as well as the presence of genes for chlorophototrophy, *K. aurantiaca* appears to be a new member of the red FAPs.

Firmicutes: Heliobacteriaceae

Gest and Favinger described the first chlorophototrophic member of the phylum *Firmicutes* in 1983. The serendipitous discovery resulted from an error in preparing a standard growth medium, which resulted in the enrichment and eventual isolation of a brownish-green, nitrogen-fixing, spore-forming rod, *Heliobacterium chlorum* (Fig. 2(g)). Heliobacteria characteristically produce "homodimeric" type-1 RCs that contain BChl *g*, which is extremely sensitive to oxygen, and 8^1-OH-Chl *a*, which acts as the primary electron acceptor in RCs. Heliobacterial cells lack intracytoplasmic membranes and do not produce a light-harvesting antenna system other than PshX, which is associated with the RCs.

Neutrophilic heliobacteria are common in diverse soils and rice paddies, where they may form symbiotic relationships with the roots of rice plants. Alkaliphilic species are known as well. The model organism for the group, *Heliobacterium modesticaldum*, was isolated from a hot spring microbial mat in Iceland, and similar organisms have been isolated from alkaline siliceous hot springs in Yellowstone National Park, WY, USA. The family *Heliobacteriaceae* currently includes only four genera and eleven species. Heliobacteria resemble clostridia and have a Gram-positive cell wall comprised of a thick layer of peptidoglycan but no outer membrane; they form heat-resistant endospores that remain viable during periods of environmental stress. Heliobacteria use electrons derived from organic compounds and light energy to produce strongly reducing ferredoxins that are used for nitrogen fixation. Consistent

with the photoheterotrophic lifestyle of all known members, the 3.08-Mbp genome of *H. modesticaldum* encodes enzymes of the incomplete rTCA cycle, but no complete Ci fixation pathway is present.

Acidobacteria: Chloracidobacterium thermophilum

Chloracidobacterium (*Cab.*) *thermophilum*, the first and currently the only chlorophototrophic member of the phylum *Acidobacteria*, was initially detected by metagenomic analyses of microbial mat communities associated with slightly alkaline, siliceous hot springs in Yellowstone National Park, WY, USA (Fig. 2(h)). Following the identification of a gene encoding an unusual core subunit of a homodimeric type-1 RC, a bacterial artificial chromosome fragment of the genome was identified that encoded an rRNA operon and the *pscA-pscB-fmoA* operon. Analyses of these sequences confirmed that the rRNA operon belonged to a member of the phylum *Acidobacteria*. An enrichment culture that contained *Cab. thermophilum* and two aerobic heterotrophs was initially obtained. Studies with this enrichment showed that *Cab. thermophilum* is an obligate chlorophototroph and allowed initial characterization of the organism, its photosynthetic apparatus, and its genome. *Cab. thermophilum* has two chromosomes of 2.68 and 1.01 Mbp. Based upon knowledge gained from the genome sequence and a metatranscriptomic analysis, classical microbiological and physiological methods led to the isolation of an axenic culture and a formal description of this novel organism.

Cab. thermophilum is the first oxygen-requiring, anoxygenic BChl-containing bacterium that contains a homodimeric, type-1 RC, chlorosomes, and the FMO protein, which previously were only known to occur in strictly anaerobic members of the GSB ("*Ca*. Thermochlorobacter aerophilum" is similar). BChl *c* and keto-carotenoids are the major light-harvesting pigments in the chlorosomes, whereas BChl *a*, Chl *a* and Zn-BChl a′, are found in the type-1 RCs. As in the RCs of GSB, Chl *a* apparently acts as the primary electron acceptor, which suggests that Zn-BChl *a*′ is possibly the primary donor of this RC. The *Cab. thermophilum* genome lacks key enzymes of all known carbon fixation pathways, yet bicarbonate is essential for growth; it presumably is employed in essential anaplerotic reactions, possibly the synthesis of 2-oxoglutarate from succinyl-CoA. As was inferred from the genome sequence and established through nutritional and biochemical studies, *Cab. thermophilum* requires all three branched-chain amino acids (which it can degrade but cannot synthesize), lysine, vitamin B_{12}, and a reduced sulfur source. *Cab. thermophilum* is unable to grow under anoxic conditions; it requires oxygen for essential anabolic reactions (e.g., for BChl and carotenoid biosynthesis as well as the synthesis of tyrosine from phenylalanine).

Survey studies by enrichment and cultivation, by sequencing the 16S rRNA gene, and by metagenomics show that *Cab. thermophilum* is found at temperatures from ~40–68°C at slightly alkaline pH around the world. Phylogenetic analyses of 16S rRNA genes from different locations strongly suggest that additional species may occur in the genus *Chloracidobacterium*. Physiological experiments with axenic strains of *Cab. thermophilum* from Mushroom Spring (Yellowstone National Park, WY, USA), as well as a 16S rRNA survey of another hot spring mat community, indicate that ecotypes with different adaptations to temperature and perhaps other parameters occur.

Gemmatimonadetes: Gemmatimonas Phototrophica

Using a high-throughput fluorescence screening approach, a novel chlorophototroph, *Gemmatimonas phototrophica*, from the bacterial phylum *Gemmatimonadetes* was initially isolated from a freshwater lake in the Gobi Desert in North China in 2011. This discovery extended the number of bacterial phyla containing chlorophototrophs from six to seven. *G. phototrophica* is an aerobic, anoxygenic photoheterotroph; it is a slow-growing, red-colored, mesophilic, oligotroph that is closely related to the aerobic chemoheterotroph, *Gemmatimonas aurantiaca* (Fig. 2(q)). *G. phototrophica* produces BChl *a* and fully functional type-2 RCs and LH1, but light energy is only used as an auxiliary energy source that supplements the chemoheterotrophic lifestyle of this organism. The 4.7-Mbp genome of *G. phototrophica* includes a 42.3-kb gene cluster encoding all of the genes required for chlorophototrophy. Phylogenetic analysis indicates that this gene cluster was obtained by horizontal gene transfer from a proteobacterium, the first clear evidence for transfer of chlorophototrophy between such distantly related taxa. Metagenomic analyses indicate that organisms related to *G. phototrophica* occur in diverse freshwater and terrestrial environments, where they can account for up to ~12% of the chlorophototrophic community.

Concluding Remarks

Retinalophototrophy is a relatively simple mechanism that allows microorganisms to supplement their ATP production when nutrient resources are limiting. If an organism can synthesize β–carotene, only two additional gene products are required, and thus horizontal gene transfer has broadly spread this capability among diverse bacteria and archaea. In contrast, chlorophototrophy can produce both reducing power and/or proton motive force that can be used to drive Ci fixation. Although more complex, some bacteria (e.g., AAPB) use chlorophototrophy merely as a mechanism to supplement ATP production under unfavorable growth conditions. Chlorophototrophic bacteria, predominantly cyanobacteria, are responsible for about half of the primary productivity on Earth. Thus, chlorophototrophs are foundational members of diverse microbial communities in both oxic and anoxic environments, and they play extremely important roles in globally important processes relating to major geochemical cycles, including carbon, nitrogen, and sulfur cycling. Many new chlorophototrophs have been identified and characterized through genomics and metagenomics in the past decade, and it is likely that more discoveries remain to be made.

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Phylogenetic Methods☆

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Glossary

Coevolution Interdependent evolution between organisms.
Distance tree Phylogeny created by converting alignment data into numerical distances and successively grouping taxa based on the smallest distances.
Gammaproteobacteria The gamma subclass of Gram-negative bacteria within the phylum Proteobacteria.
Homologue Gene sharing a common ancestry (see also orthologue and paralogue).
Horizontal gene transfer (HGT) Nonancestral movement of genes between organisms.
Housekeeping gene A gene whose product has a role in maintaining cellular function.
Likelihood tree Phylogeny based on statistical evaluation of alignment data and substitution matrix.
Long branch attraction (LBA) The erroneous clustering of fast evolving sequences due to shared character states that may not be the result of descent.
Orthologue Gene inherited vertically during speciation.
Paralogue A duplicated gene copy.
Substitution matrix A model of molecular sequence evolution that estimates the transition frequency of residues from one state to another.
Supermatrix Concatenation and alignment of multiple genes.
Supertree Consensus phylogeny of multiple independent gene genealogies.
Xenologue A gene inherited via horizontal gene transfer.

Abbreviations

Fn3	Fibronectin type III domain
HGT	Horizontal gene transfer
LBA	Long branch attraction
MCMC	Markov Chain Monte Carlo
ML	Maximum likelihood
NJ	Neighbor-joining
PP	Posterior probability
UPGMA	Unweighted pair group method with arithmetic mean

Defining Statement

This article examines the recent use of phylogenetic methods to infer relationships among bacteria, comparing results from single-gene and whole-genome analyses, and also from alignment-dependent and alignment-free approaches. The application of phylogenetic approaches for examining coevolution and horizontal gene transfer (HGT), and for establishing levels of diversification within protein superfamilies, are also examined.

Introduction

The ultimate goal of phylogenetics is to establish the evolutionary relatedness and relationships among genes, traits, or organisms. And although life has a single origin and only one true evolutionary history, the task of reconstructing this history can range from facile to formidable depending on the particular taxa or characters in question. Difficulties arise with phylogenetic reconstruction due to the fact that virtually all inferences about evolution and relationships are based on the study of contemporary organisms. It is rarely possible to fill in gaps – to verify how the record of life actually unfolded – by including archaic or extinct lineages.

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These problems are magnified in bacteria because they possess few visible traits and almost no fossil record. Moreover, bacteria are ancient and have diversified over billions of years and are subject to processes of horizontal gene transfer (HGT), which can potentially introduce any trait in any lineage and obscure traditional Darwinian patterns of descent, thereby making their history even more difficult to trace.

During the first hundred years of microbiology, bacteria were classified, distinguished, and grouped largely by their habitats, growth characteristics, biochemical attributes, and virulence potential. But during the latter half of the twentieth century, there was a pronounced shift toward the use of molecular genetic information, as gained through the analysis of protein and nucleic acid sequences. This provided three immediate advantages. First, comparative analyses became centered on universally distributed characters, which eliminated the situation whereby lineages are classed together due to their lack of specific defining feature(s). Second, in-depth knowledge of the physical mechanisms by which informational macromolecules can change has led to the development of robust models of sequence evolution. Third, the use of molecular sequences increased substantially the number of discrete characters by which microorganisms could be compared in that each nucleotide or amino acid residue can potentially serve as an informative trait.

Although the use of molecular sequences has undoubtedly revolutionized the study of microbial systematics and evolution, all inferences rely on two factors: the particular character(s) used for phylogenetic reconstruction and the algorithms used to infer relationships. As with phenotypic traits, molecular sequences are subject to character convergence as a result of chance events, natural selection, or horizontal transfer. Moreover, the vast amount of information obtained from a single sequence has often confined analyses to a single region of the genome, which may not be representative of the organism as a whole. Finally, phylogenetic methods have very distinct sets of underlying assumptions. Such problems stress the need to examine multiple genes and to infer statistically robust phylogenetic trees in order to recognize and understand any inconsistencies in the data.

In this article, we explain and examine the procedures by which microbial phylogenies have been and are being produced. We concentrate on the advantages and limitations of the various phylogenetic methods, and on the particular relationships evoked from varied types of genetic data, including full genome sequences, considering the phylogeny of Gammaproteobacteria. Due to the long-standing interest in its host-associated members, the Gammaproteobacteria have been the subject of concentrated genome sequencing efforts, resulting in a large amount of sequence information that makes this group particularly suitable for testing and evaluating methods of phylogenetic reconstruction.

Phylogenetic Analysis of Molecular Sequences

The use of molecular sequence data to infer relationships has been central to our understanding of evolution and evolutionary processes. Changes at the molecular level have been used to establish the phylogeny of many types of organisms. For microbes, in particular, where classic phenotypic markers are often ineffective or impractical for grouping bacterial isolates, molecular phylogenetic approaches have been revolutionary. Here, we outline the approaches involved in sequence-based phylogenetic reconstruction, covering the manner in which sequences are aligned, followed by the methods for translating alignments into a phylogeny.

Sequence Alignments

The prerequisite for producing an organismal phylogeny based on molecular sequences is a highly refined alignment of orthologous (vertically inherited) sequences that best approximates their true molecular evolution. Alignments can be generated with a variety of algorithms, most of which employ some form of substitution matrix to position homologous nucleotides or amino acid residues so as to maximize the number of identical or similar residues at a given site (column). The specific values in a substitution matrix provide the algorithm with the rate at which one character (nucleotide or amino acid) changes to an alternate state. Clustal, presently the most popular alignment software, uses the IUB and ClustalW1.6 matrices for DNA alignments, and the PAM, BLOSUM, and Gonnet matrices for amino acid alignments. To facilitate the alignment process, these algorithms can also introduce gaps at a frequency dictated by the particular gap opening and gap extension penalties, thereby reflecting biologically relevant insertions/deletions (indels) within the sequence. The introduction of gaps within a coding sequence, however, may sometimes be undesirable, as this is likely to infer insertions and deletions that may not be accurate representations of the actual molecular evolution of the sequences. This is particularly true of functional protein-coding sequences, in which the introduction of alignment gaps may result in nonsensical frameshifts.

When a sequence dataset is put into a residue-based alignment program, such as Clustal, the algorithm begins by creating a pairwise alignment of all sequences independently. The algorithm then calculates all distances between sequence pairs, creating a distance matrix that is subsequently translated into a guide tree (dendrogram). As its name suggests, this tree serves as a guide for beginning the multiple sequence alignment, with the most similar sequences being added first, followed by the progressive addition of more divergent sequences. This simple and relatively effective type of pairwise progressive sequence alignment approach has been used extensively in numerous phylogenetic studies, but is less effective for particular datasets. This has prompted the development of more improved alignment algorithms, such as MUSCLE, t-Coffee, POA, DIALIGN, SAGA, and MAFFT, which are efficient and accurate in recovering optimal alignments from various datasets, as established by comparison to BAliBASE (a database of manually refined reference alignments). Not all of these algorithms, however, are procedurally equivalent. Some, such as DIALIGN, utilize a segment-to-segment comparison whereby regions of sequence similarity are used as anchors around which the entire alignment is built, whereas others, such as MUSCLE, t-Coffee, and MAFFT, perform a series of progressive alignments that refine and optimize

during each iteration to ensure significantly higher alignment accuracy. The refined, progressive alignment offered by these approaches is essential for the accurate reconstruction of phylogenetic relationships.

Regardless of the program or algorithm used to generate the multiple sequence alignment, each alignment must be evaluated prior to its use for phylogenetic reconstruction. Sequence variation across an alignment may be heterogeneous, resulting in conserved regions being interspersed among highly variable regions. Since the alignment of hypervariable regions is often questionable, these portions are best masked or eliminated. This is particularly important because even small changes in the alignment will introduce noise that can potentially obscure the true phylogeny. As mentioned previously, it is also often necessary to optimize the alignment by adjusting gap opening and extension parameters and, in extreme cases, refining the alignment manually.

Phylogenetic Reconstruction

Once a reliable alignment is generated, a variety of tree-building methods can be utilized to transform the alignment data into a phylogenetic tree. These tree-building methods are categorized broadly into distance-based, parsimony, and likelihood approaches, each of which will be considered below (Table 1).

Distance trees

Distance-based approaches cluster representative taxa based on the number of nucleotide or amino acid substitutions between sequences. One of the original and simplest distance-based methods, developed for the generation of phenetic cladograms, was UPGMA (Unweighted Pair Group Method with Arithmetic Mean). UPGMA utilizes a successive clustering approach, whereby a matrix of all pairwise sequence similarities is generated, and the two taxa with the smallest distance are clustered first. The distance matrix is recalculated, considering the already clustered taxa as one, and the taxon with the next smallest distance is added to the tree. This is repeated until all taxa have been added, and a final phylogeny constructed. Because of this simplistic interpretation of the distance matrix and assumption of a constant evolutionary rate (molecular clock) for all sequences, UPGMA was prone to constructing unsupported phylograms. The Neighbor-Joining (NJ) method, which had a similar approach to taxonomic grouping as UPGMA, clusters closest sequences (neighbors) first and then recalculates the distances between neighbor pairs. Because of this, NJ does not suffer from the same limitations as UPGMA and has become the preferred distance-based method for phylogenetic reconstruction.

Distance-based phylogenetic reconstruction requires the calculation of the number of changes that have occurred in a given sequence; however, determining the exact number of substitutions between any pair of sequences, particularly highly diverged sequences, is virtually impossible. For example, it is often not clear whether the presence of different nucleotides at an aligned site

Table 1 Sorting through the phylogenetic methods applied to microbes

Approach	*Advantages*	*Disadvantages*
Single-gene	● Requires sequence information from only one locus	● Evolutionary history of individual loci may not be representative of entire genome/organism
UPGMA	● Rapid ● Highest accuracy when molecular rates are constant along lineages	● Assumes molecular clock ● Simple interpretation of distance matrix ● Always creates rooted trees ● Sequence information reduced to distances
Neighbor-joining	● Rapid ● Can be used with large datasets ● Produces reliable phylogenies with most datasets	● Sequence information reduced to distances ● Strongly influenced by substitution model ● Always produces only one possible tree
Maximum likelihood	● Produces robust phylogenies ● Dataset can utilize bootstrap	● Computationally intensive ● Not suitable for large datasets
Bayesian	● Rapid ● Suitable for larger datasets	● May be prone to creating unsupported phylogenies ● Does not always yield fully resolved phylogenies ● Uses more permissive posterior probability for topological support
Whole-genome	● Not as sensitive to evolutionary forces affecting individual genes	● Amount of phylogenetic signal unknown
Genome content	● Can be used to analyze many genomes	● Not suitable for reduced genomes ● Requires normalization to account for genome size variation ● Relies on accurate inference of homologues and/or orthologues ● No models available for evolution of the genetic repertoire
Gene order	● Can be used to analyze many genomes	● Computationally complex so scaling to multiple genomes difficult ● Genomic alignment difficult if additions, deletions, and inversions prevalent
Rearrangement/breakpoint distance	● Can be used to analyze many genomes	● Computationally complex so scaling to multiple genomes difficult
Alignment-free	● Rapid ● Simple to execute	● No models available for genome evolutionary dynamics ● May be subject to character convergence

occurred by a single or by multiple substitutions. Likewise, two sequences that are identical at a given position may not necessarily indicate the absence of a substitution, since it is possible that the sequence conservation at that site is due to an unobserved change followed by a reversion back to its original state.

This concept, known as 'multiple hits', requires correction during the estimation of pairwise distances. Numerous substitution models have been created to approximate multiple changes at the same site and are often incorporated into the calculation of pairwise distance/divergence between sequences. Substitution models for DNA may be simple, such as the Jukes–Cantor method, which assumes that any nucleotide has an equal probability of changing to any other (a single substitution rate for all nucleotides), or more complex, such as the General Time Reversible model, which assumes different rates depending on the particular nucleotide substitution. Similarly, substitution models for proteins, as discussed above, include the PAM, BLOSUM, and the more recently derived Jones–Taylor–Thornton matrices. The choice of substitution model can have significant impact on phylogenetic reconstruction, particularly when the true tree contains long branches separated by short internal branches. Several applications, such as MODEL TEST, have been developed to ascertain the best substitution model for a given set of sequences.

Likelihood trees

The application of likelihood methods to phylogenetic reconstruction has become increasingly popular over the last decade, due largely to their somewhat greater accuracy and consistency in recovering a correct phylogeny, and due to the significant increases in computational capacity and speed. Maximum likelihood (ML) and Bayesian approaches offer two distinct, but related, probabilistic approaches to determin the best phylogeny. ML approaches attempt to identify the tree topology that has the highest probability given the sequence data provided. This requires calculation of the log likelihoods for all possible tree topologies for a given dataset, with the tree having the highest likelihood being favored. Because all tree topologies must be sampled and evaluated with this approach, ML is computationally intensive and unsuitable for very large datasets.

The Bayesian approach to phylogenetic reconstruction is considered by many to be an ideal alternative to the ML approach. This method relies on Bayes' theorem, which takes into account the sequence data provided (likelihood) and the assumptions that can be made about the data (priors) to calculate a posterior probability (PP) for a given topology. Bayesian analyses utilize an algorithm known as Markov Chain Monte Carlo (MCMC), which explores and samples 'tree space' (the entire set of possible trees for a given dataset) and estimates the PP of each. The MCMC algorithm begins at multiple random locations in tree space, with each point (or chain) beginning to sample trees randomly. As each chain explores tree space, any tree sampled that has a higher PP than the previous tree serves as the starting point for the next sampling iteration, allowing the algorithm to sample those topologies with increasingly larger PP. Millions or hundreds of millions of generations are generally run with sampling frequency being at the discretion of the user. Ideally, all chains should converge on the same PP distribution, indicating that the trees with the highest PP distribution have been sampled; however, because tree space has many local maxima and minima, chains sometime become fixed at a local peak and incapable of crossing a valley toward a higher posterior distribution. A variant of the MCMC algorithm, called Metropolis-coupled MCMC (or MC^3), was developed to circumvent this problem.

Unlike distance-based approaches, the Bayesian approach does not produce a single tree; rather, it samples a series of probable tree topologies based on the dataset and priors. The entire tree set is first refined to eliminate the initially sampled suboptimal tree topologies and a consensus made of the remaining refined dataset. Because posterior probabilities have already been calculated during sampling, these are used to determine confidence values at each node on the tree, eliminating the need further access the statistical support of the topology by procedures such as the bootstrap or jackknife (see below).

Parsimony trees

Parsimony methods for phylogenetic reconstruction are premised on favoring the evolutionary path with the fewest number of changes. Unlike distance-based methods, parsimony relies on character-based inference of phylogenies, utilizing the sequence data in its original state rather than converting it to distances. One of the more popular parsimony methods is Maximum Parsimony, a tree-constructing approach that calculates the evolutionary steps for all likely tree topologies and presents the tree with the fewest evolutionary steps. Often, there are multiple, equally parsimonious trees, some having drastically different topologies than others. Much like the Bayesian approach, a consensus phylogeny may be built on this tree set, thereby combining all topologies into a single approximated tree topology.

Interpreting Phylogenetic Trees

There are several issues to consider when evaluating a phylogenetic tree. Of utmost importance is assessing the support and validity of the recovered topology. The support for the relative position of each taxon in a distance-based or parsimony-inferred phylogenetic tree is often evaluated with the parametric bootstrap or jackknife. The bootstrap is a resampling with replacement technique that effectively creates multiple datasets from the original sequence alignment and places data points in the process. Portions of the topology that are consistently recovered are given higher bootstrap values than those that vary between replicates. This approach is similar to that employed by the jackknife, except that instead of replacing sites, the jackknife approach deletes sites, that is, resamples without complete replacement. For Bayesian phylogenies, PP is used specifically as an assessment of confidence; however, this has been shown to be less conservative than the bootstrap or jackknife. While a bootstrap $>70\%$ is typically considered reasonable support for a node, a PP $>95\%$ is considered equivalent support in a Bayesian phylogeny. It should be noted that when attempting to evaluate the validity of a phylogeny, confidence values (bootstrap, jackknife, and PP) provide some

benchmark as to whether the sequence data support the observed branching pattern but need not corroborate the relationships within the tree.

The addition of new taxa, changes in the specific parameters used for generating the tree, or even the use of an alternate tree-building method, can result in a dramatically different phylogeny with equally high confidence values. Similarly, low support values do not necessarily suggest an incorrect topology, but are instead indicative of either inadequate or extremely high sequence variation. In the case of insufficient sequence variation, nucleotide sequences can provide substantially more informative variation over protein sequences, and in some cases, additional representative sequences are required. In cases of extremely high sequence variation, refining the sequence alignment and removing hypervariable regions can significantly enhance phylogenetic signal and increase bootstrap support. Alternatively, a slower evolving locus may have to be substituted to better resolve specific relationships. Irrespective of the circumstance, a disproportionate emphasis should not be placed on confidence values when attempting to validate the branching order in a recovered phylogeny.

Another issue that often emerges during phylogenetic reconstruction is the concept of long branch attraction (LBA). LBA is an artifact whereby two sequences are grouped together in a phylogenetic tree as a consequence of having a greater number of substitutions relative to others. Higher sequence variability increases the chance of sharing common states by chance, causing many tree-building approaches to misinterpret this as higher relatedness. Parsimony and distance-based methods are prone to LBA, while ML and Bayesian are, in theory, immune to such artifacts; however, there is some evidence that this can occur in these latter two methods under certain circumstances.

Most phylogenetic approaches assume a strictly bifurcating topology; however, this assumption may be particularly limiting when considering microorganisms, which may be prone to exchanging genetic material. Recombination (and HGT) may obscure the pattern of organismal descent and require alternative methods for reconstructing and testing phylogenetic trees. Split decomposition methods do not impose a strictly bifurcating topology; rather, trees may be network-like, with reticulations in the network reflecting recombination between taxa. Genes having undergone such horizontal exchange are generally not considered reliable for accurate phylogenetic reconstruction. Thus, when attempting to establish bacterial relationships, the choice of locus becomes critical to accurately recovering the correct evolutionary history for a given set of isolates.

The use of the 16S rDNA sequences has become the standard for bacterial identification and phylogenetic reconstruction due to its reduced likelihood for HGT, relatively slow rate of change, and predisposition to concerted evolution among the 16S operons within a single genome. But on account of the latter two characteristics, 16S sequences also pose severe limitations in resolving relationships between closely related lineages. To increase phylogenetic resolution, some studies have turned to bacterial 'housekeeping' genes for assessing bacterial relationships. By being both conserved among diverse bacterial lineages and under purifying selection (but containing rapidly evolving synonymous sites), these genes can provide the basis for determining relationships at several taxonomic levels. Protein coding genes are not immune to the effects of HGT, making explicit tests of recombination necessary for each locus prior to its inclusion in phylogenetic analyses.

In some cases, the phylogenetic signal associated with recombination/HGT within one locus can be diluted by the concatenation of multiple genes that are distributed around the bacterial chromosome. This concatenation approach is increasingly popular and has provided fairly robust species trees for a variety of different bacterial groups; however, there is substantial debate as to whether these genealogies reflect true species trees given the occurrence of HGT. Thus, with an increasing number of whole genome sequences becoming available, bacterial taxonomy has turned to nonsequence-based whole genome phylogenies, whereby genome composition, content, synteny, and other characteristics are harnessed to infer relationships in an attempt to circumvent several of the confounding factors that might otherwise affect single-gene trees.

Toward Resolving the Relationships Among the Gammaproteobacteria

The availability of numerous methods for establishing bacterial relationships has prompted intense comparative analyses that test the effectiveness, reliability, and consistency of these diverse phylogenetic approaches. The Gammaproteobacteria, comprising many sampled and well-characterized medically, agriculturally, and environmentally relevant bacteria, have been the subject of intense sequencing efforts and have served as the focus of such analyses.

In 2003 Lerat and colleagues attempted to resolve the relationships among gammaproteobacterial species by identifying the set of single copy (core) genes that could be used for phylogenetic inference. Orthologous sequences were extracted from each of 13 genomes (*Buchnera aphidicola* APS, *Escherichia coli* K12, *Haemophilus influenzae* RdKW20, *Pasteurella multocida* Pm70, *Pseudomonas aeruginosa* PAO1, *Salmonella enterica* sv. typhimurium LT2, *Vibrio cholerae* O1 bv. eltor str. N16961, *Wigglesworthia brevipalpis*, *Xanthomonas axonopodis* pv. citri str. 306, *Xanthomonas campestris* pv. campestris str. ATCC33913, *Yersinia pestis* CO92, and *Y. pestis* KIM) and the evolutionary history of each single-copy orthologue was examined. All except two of the 205 genealogies were found to be congruent, with the two exceptions exhibiting a single event of HGT. Compared to what is currently known about the relationships among these taxa based on 16S (Figure 1(a)), the resulting phylogeny was largely congruent (Figure 1(b)), illustrating that although HGT might complicate phylogenetic inferences, the selection of appropriate loci can help circumvent such problems. Despite the strong statistical support for this tree, Susko and colleagues, in 2006, suggested that perhaps 10% of the core gene genealogies had conflicting evolutionary histories, which could be indicative HGT, leading to their suggestion that the Gammaproteobacteria would be best represented as a network, rather than a traditional bifurcating tree.

The phylogeny of these 13 gammaproteobacterial genomes, as deduced by Lerat and colleagues currently serve as the benchmark for comparing evolutionary relationships deduced by other approaches that based on either different methods of phylogenetic

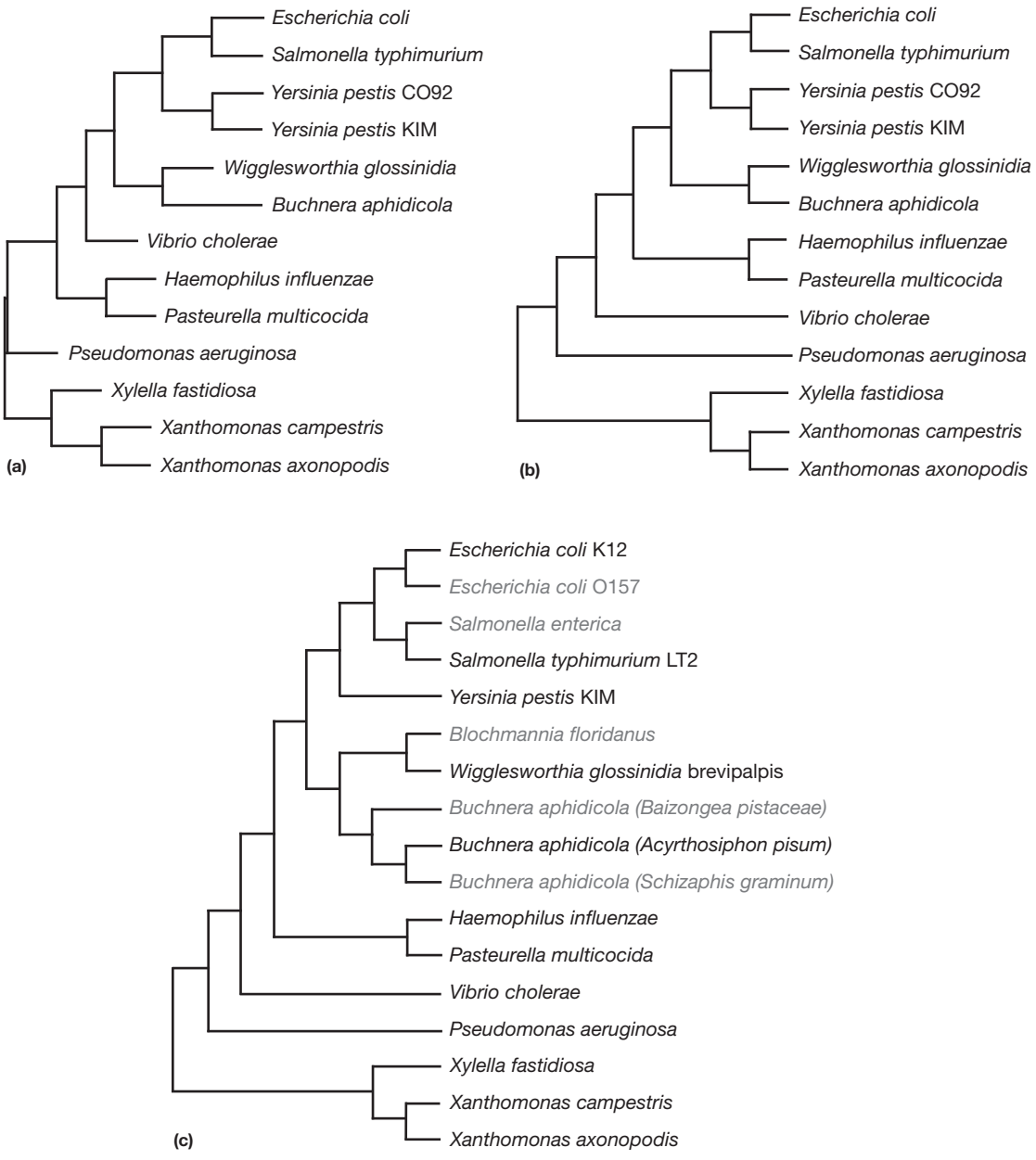

Figure 1 (a) Neighbor-Joining (NJ) phylogeny of 13 species from the Gamma subdivision of the Proteobacteria based on 16S rDNA. Reproduced from Luo YQ, Fu C, Zhang DY, and Lin K (2006) Overlapping genes as rare genomic markers: The phylogeny of [Gamma]-Proteobacteria as a case study. *Trends in Genetics* 22: 593–596. (b) NJ phylogeny of 13 species from the Gamma subdivision of the Proteobacteria generated by Lerat and colleagues, in 2003, using a 203-gene concatenated dataset. Reproduced from Lerat E, Daubin V, and Moran NA (2003) From gene trees to organismal phylogeny in prokaryotes: The case of the gamma-proteobacteria. *PLoS Biology* 1: 101–109. (c) Phylogeny recovered from supertree and supermatrix approaches of the Gammaproteobacteria. Reproduced from Comas I, Moya A, and Gonzalez-Candelas F (2007) From phylogenetics to phylogenomics: The evolutionary relationships of insect endosymbiotic gamma-proteobacteria as a test case. *Systematic Biology* 56: 1–16.

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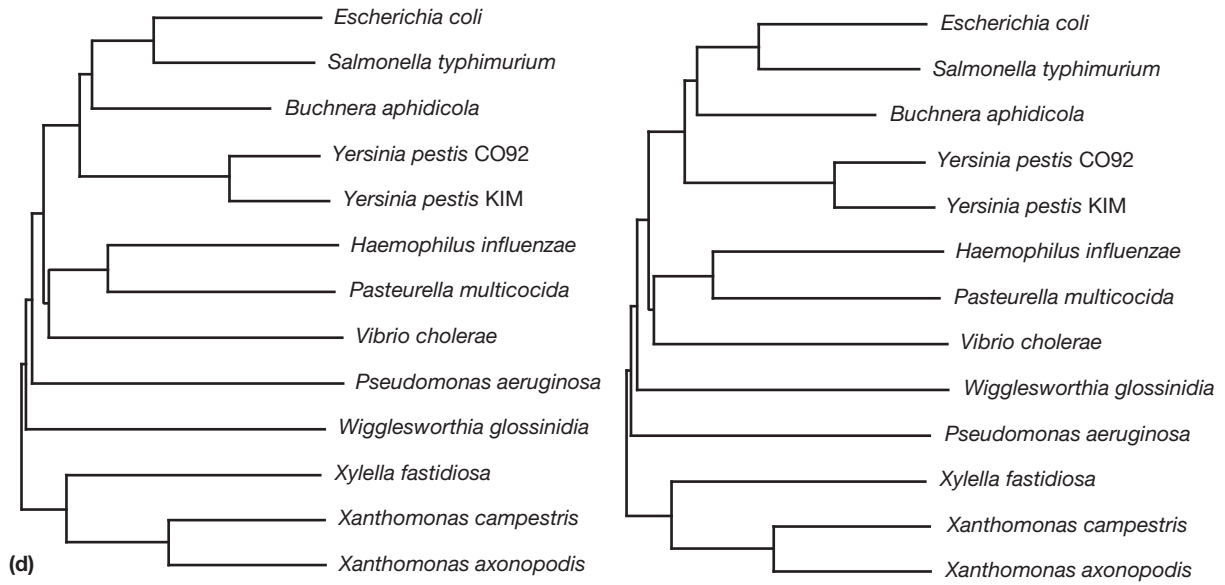

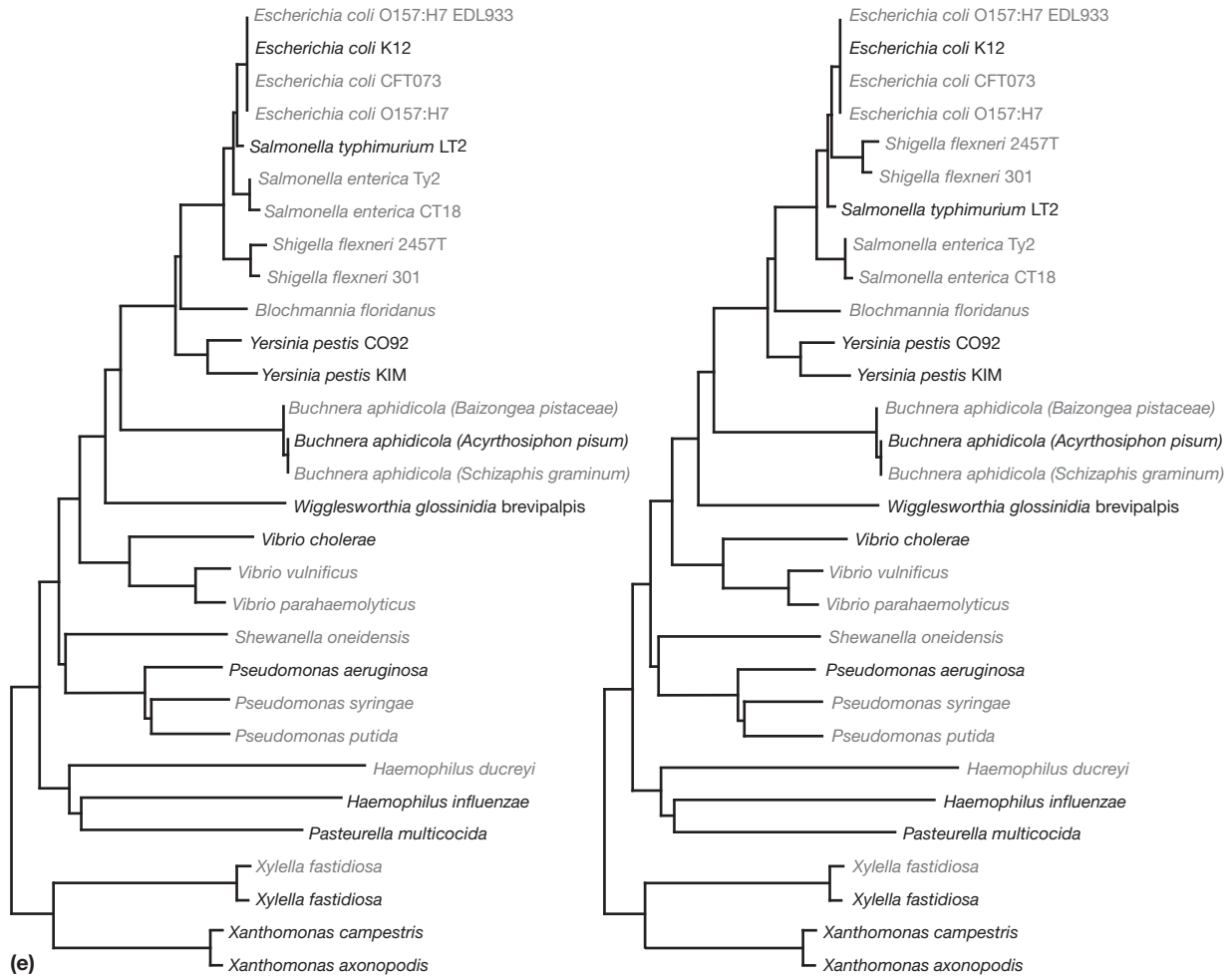

Figure 1—Cont'd (d) Phylogeny created from overlapping genes (whole-genome method), using both NJ (left) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (right) methods. In both phylogenies, *Wigglesworthia glossinidia* falls outside of its known position within the Enterobacteriaciae. Phylogenies reproduced from Luo YQ, Fu C, Zhang DY, and Lin K (2006) Overlapping genes as rare genomic markers: The phylogeny of [Gamma]-Proteobacteria as a case study. *Trends in Genetics* 22: 593–596. (e) Phylogeny of the Gammaproteobacteria using breakpoint distance (left) and inversion distance (right), as presented by Belda and colleagues (2007). Both phylogenies are largely congruent with that of Lerat and colleagues (2003), except the endosymbionts (*Buchnera* and *Wigglesworthia*), which are monophyletic in the Lerat and colleagues (2003) phylogeny. The breakpoint distance phylogeny also incorrectly places the salmonellae as a sister group to *Escherichia*, instead of the more closely related shigellae. Phylogenies reproduced from Belda E, Moya A, and Silva FJ (2005) Genome rearrangement distances and gene-order phylogeny in gamma-proteobacteria. *Molecular Biology and Evolution* 22: 1456–1467.

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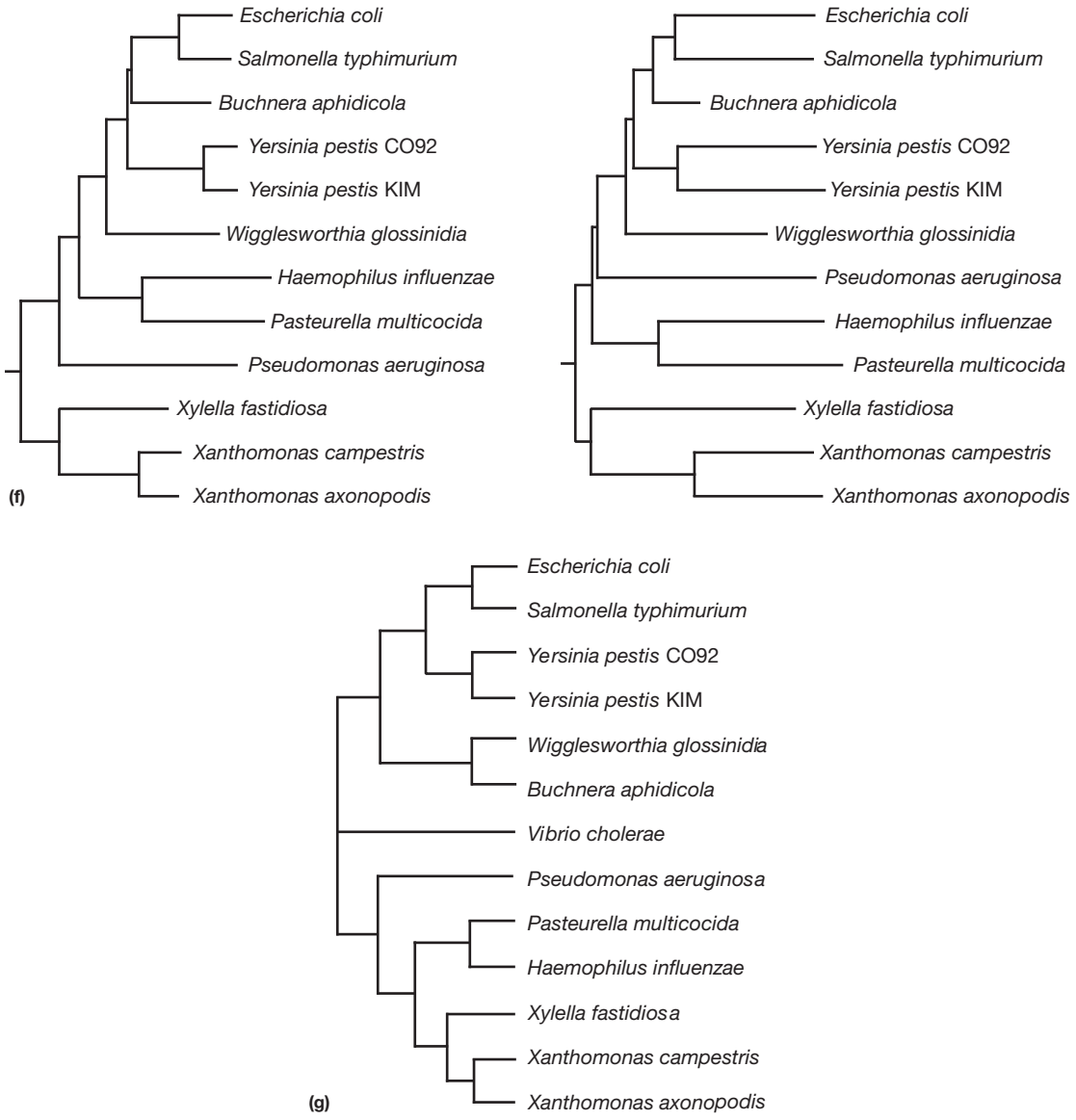

Figure 1—Cont'd (f) Phylogenies of the Grammaproteobacteria using breakpoint distance (left), and common/conserved intervals (right), which differ in the position of the opportunistic pathogen *Pseudomonas aeruginosa*. In both phylogenies, the endosymbionts *Buchnera aphidicola* and *Wigglesworthia glossinidia* are not resolved as a monophyletic group as in the phylogeny of Lerat and colleagues (2003), but do cluster within the Enterobacteriaceae. Reproduced from Blin et al. (2005) In: *Comparative Genomics*. RECOMB 2005 International Workshop, RCG 2005. Lecture Notes in Bioinformatics, vol. 3678, pp. 11–20. (g) Phylogenetic relationships among 13 species from the Gammaproteobacteria using whole-genome gene order data. Taxa not included in original study by Lerat and colleagues are in gray typeface.

reconstruction or alternate molecular characters (see below). For example, a recent study by Comas and colleagues, in 2007, examined 21 genomes, including the original 13 analyzed by Lerat and colleagues, and identified 579 orthologous genes that could be used for establishing phylogenetic relationships and creating a species tree. Two distinct approaches were used to evaluate phylogenetic signal: supermatrix (gene concatenation) and supertree (consensus of multiple tree topologies). Both approaches recovered a single supported topology, which was congruent to the phylogeny of Lerat and colleagues (see Figure 1(c)).

Whole-Genome and Sequence Alignment-Free Approaches

Up to this point, this article has been devoted to evolutionary inferences based on the alignment of the primary sequences of DNA or proteins. The availability of complete genomes has engendered a pronounced increase both in the number of sequences that can

be compared across taxa and in the resolution of phylogenetic trees. Analyses of the complete set of genes in genomes are usually thought to be more resistant to evolutionary processes or statistical artifacts that might affect phylogenetic reconstructions based on individual genes (Table 1).

In addition to the accuracy that might be gained through the incorporation of numerous genes, the availability of full genomes has also led to the development numerous methods that exploit characters other than nucleotide or amino acid substitutions to establish the relationships among organisms. But before describing these procedures, it should be noted that unlike the sequence alignment-based methods, there is rarely a robust, *a priori* model describing the evolution of the genomic characters on which these phylogenies are founded. Therefore, the results of such analyses are often compared to established gene genealogies before they are presumed to represent a true organismal phylogeny, and any similarities or incongruities help model the evolutionary dynamics of the particular genomic trait.

Several features can provide measures of similarity among genomes, which can then be transformed into a tree using a clustering algorithm, such as the NJ method or UPGMA. The use of whole-genome information to infer phylogenetic relationships (sometimes referred to as 'phylogenomics') has been the subject of two excellent and comprehensive reviews by Snel et al., 2005 and Delsuc et al., 2005. Below, we discuss briefly several of the whole-genome and the sequence alignment-free methods cited in these reviews, particularly those that address relationships among members of the Gammaproteobacteria, and then turn our attention to some recently developed procedures in this area.

Genome Content

Because full genome sequences provide the complete set of encoded genes (as well as knowledge of all genes that are absent), the earliest whole-genome approaches for inferring relationships were based on the proportion of shared genes among organisms. There are numerous mechanisms by which bacterial genomes can gain and lose genes; however, the rates and progression of such changes are not known and may therefore be highly variable across organisms. And as a metric, the fraction of genes by two genomes need not be reciprocal due to differences in genome size. As expected, larger genomes typically have higher numbers of genes and/or gene families in common, requiring either normalization of the similarity estimates by genome size or exclusion of microbes with highly reduced genomes.

Naturally, the observed similarity in genome repertoires will also be influenced by how shared genes are identified, and there is no single best method that can be applied to all genomes and all comparisons. Two basic approaches used include the 'homologue' method, which examines the taxonomic distribution of gene families or the representation of genomes in COGs, and the 'orthologue' method, which searches for the occurrence of 'true' orthologues, usually defined as sequences that are reciprocally the best matches in a pair of genomes. The latter is probably best applied to rather closely related organisms, since orthologues can be more reliably recognized.

Gene Order

The genes in a genome are arranged physically in an order that can be modified by additions, deletions, inversions, and translocations. Genetic maps revealed that despite differences in the particular set of genes harbored by an organism, overall gene order was highly conserved among related but divergent bacteria (e.g., *E. coli* and *S. enterica*); however, the earliest comparisons of fully sequenced genomes detected several local areas of synteny but little long-range conservation in gene order among species. Taken together, this suggests that gene arrangement contains some phylogenetic signal and could therefore be a useful feature for determining the evolutionary relationships among organisms. As additional genomes appeared, it became apparent that the degree of conservation of gene order reflected the evolutionary distance between organisms, indicating that genomes diverge principally by inversions that are symmetric with respect to the replication origin and terminus.

Due to the potentially large differences among genomes, early comparisons quantified gene order similarity by measuring the degree to which each pair of adjacent genes were retained as neighbors in another genome, a metric that auspiciously eliminated the need to initially align the corresponding genomes. Using this approach, gene order has usually been found to evolve more quickly than genome contents, which is perhaps not surprising given that changes to gene content (additions and deletions) and gene rearrangements (inversions and translocations) will both contribute to the disruption of gene adjacencies.

Kunisawa, in 2001, devised a method for reconstructing the relationships among organisms based on the physical order of conserved genes. In this method, the genome is scanned for pairs or clusters genes whose arrangements or orientations vary, and are phylogenetically informative across species. Genomes are grouped according to their shared gene arrangements with the changes in a particular gene order mapped parsimoniously to demarcate particular nodes along a phylogeny, and when applied to members of the several bacterial groups, the resulting topologies were consistent with the traditional sequence-based classifications. This method is similar in concept to analyses that use rare genomic changes as phylogenetically informative markers to denote evolutionary relatedness. Behind this approach is the notion that certain genetic events occur so infrequently that they are not subject to convergence or reversion and that organisms or genomes that possess the rare character share a more recent common ancestor than those that do not. Various types of genomic characters, including gene fissions or fusions, and the occurrence of specific insertions or deletions, have been used to define nodes in evolutionary trees, and whereas this approach is not based strictly on gene order, it relies on the presence or physical arrangement of particular genes or features within a genome.

A 2006 study by Luo and colleagues used the genome-wide occurrence of overlapping genes (i.e., adjacent genes whose coding regions overlap) as a way to determine the relationships among the Gammaproteobacteria. Because the dissociation of overlapping genes would probably have consequences on gene function, gene overlaps, once formed, are likely to be retained in that state in all descendent genomes containing those genes. Based on the presence of orthologous overlapping genes in 13 sequenced Gammaproteobacteria, this study computed the pairwise distance between genomes and reconstructed UPGMA and NJ phylogenies (Figure 1(d)) that differed from the canonical topology only in the placement of the two endosymbionts, *W. brevipalpis* and *B. aphidicola*. These insect endosymbionts have highly reduced genomes and therefore very few common gene pairs, making them less likely to be paired in analyses that are dependent on genome size and gene repertoires.

Rearrangement Distances and Gene Order (Breakpoint) Phylogenies

Although the phylogenies discussed in the previous section rely on the arrangement of genes or other characters within genomes to infer their similarity or relatedness, a complete genome sequence contains a substantial amount of information in addition to the sequence and occurrence of its constituent elements. Differences in gene order, expressed in terms of rearrangement distance – that is, the minimal number of events needed to transform one gene order into the other – reflect the evolutionary divergence between two genomes and can serve as a source of phylogenetic information. Unfortunately, phylogenetic methods based on gene order are computationally intensive and were previously restricted to the analysis of eukaryotic organelles, which have small and relatively constant gene sets. There have been two attempts to use complete genome sequences for reconstructing a gene order phylogeny for a bacterial genome. In 2005, Belda and colleagues circumvented the computational problems posed by variation in gene content (as resulting from duplications, deletions, and HGT), by extracting a common set of $\sim$250 orthologous genes from each of 30 sequenced Gammaproteobacteria. By reducing each of the analyzed genomes to a small set of common genes, the calculation of rearrangement distances between all pairs of organisms becomes computationally equivalent regardless of their actual genome sizes. Similarly in 2005 Blin and colleagues performed a genome-wide breakpoint analysis of 12 Gammaproteobacteria, but instead considered all genes common to a given pair of genomes when computing their rearrangement distance. The phylogenies presented by both studies (Figure 1(e) and 1(f)) were topologically congruent to that of Lerat and colleagues.

Since the Gammaproteobacteria contain thousands of gene families, we have recently implemented a method that reconstructs a phylogeny based on complete gene order information of the entire complement of genes in fully sequenced genomes. This work made use of all $\sim$3400 informative gene families (i.e., those present in more than two taxa) contained within 13 genomes and calculated the rearrangement distance between genomes by finding the most parsimonious sequence of three types of events – inversions, deletions, and insertions – that will transform the gene order of one genome into the other. The gene order tree derived computed from a normalized distance matrix by the NJ method differs from the sequence-based phylogeny in the placement of the clade containing *P. multocida–H. influenzae* (Figure 1(g)). This discrepancy matches that observed by Belda and colleagues and is consistently supported by jackknife resampling, which suggests that the source of the difference between the gene order and gene sequence phylogenies is biological, rather than methodological, and reflects distinctive patterns of genomic evolution in the affected lineages. Although all of the gene order trees are globally similar to the reference phylogeny, only in the present analysis are the two symbionts, *W. brevipalpis* and *B. aphidicola* grouped into a single clade.

Alignment-Free Methods

There have been several attempts to group bacteria and infer phylogenies using genome-wide properties that do not require the initial recognition, assignment or alignment of homologous characters. These alignment-free methods compute the distance between genomes based on such features as the frequencies of particular strings of nucleotides or amino acids but can also involve sequence motifs or protein folds and domains. Many of the genomic signatures applied in early studies, such as overall base composition, dinucleotide frequencies, and amino acid or codon usage patterns, contain only weak phylogenetic signals due to convergence and the lack of character complexity. However, there has been a substantial increase in the use and accuracy of such alignment-free methods because they are often computationally less intensive and offer a means to phylogenetically classify sequences, such as those generated in large-scale metagenomic surveys, in which homology assignment is impossible.

Other Applications of Phylogenetics

Functional Predictions

Phylogenetic analyses of all homologues within multiple completed genomes have the capacity to provide in-depth insight into the level of evolutionary and functional diversification within a particular gene family. This method relies on distinguishing sets of orthologous genes (inherited vertically through speciation) within a single phylogeny, with paralogous genes (acquired through gene duplication) representing new functional groups (Figure 2). By including functionally characterized reference proteins for each major monophyletic group, functional assignment of related orthologues can sometimes be inferred. It is notable that HGT can greatly complicate these types of analyses, as xenologous genes (genes acquired through horizontal transfer) may often be mistaken for orthologues or paralogues.

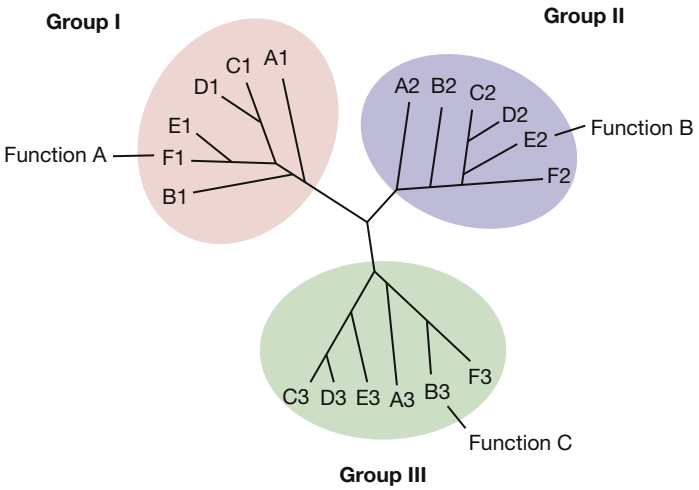

Figure 2 Phylogenetic inference of function involves generating a phylogeny of all homologues within single genomes (taxa A–F) and identifying orthologous groups (Group I, II, and III). Paralogues (A2 and A3 are paralogous to A1) may represent functionally distinct subgroups.

The use of such methods to understand protein evolution has increased substantially over the last several years, attributable to the dramatic increase in the availability of fully sequenced microbial genomes. For example, bacterial porins comprise a large family of proteins, which serve as channels through which solutes are exported out of the bacterial cell across the outer membrane. NmpC, OmpF, OmpC, and PhoE are well-characterized porin proteins that differ in solute specificity, but also exhibit sufficient evolutionary divergence and form clear monophyletic groups. A phylogenetic analysis of the porin family of proteins from the enteric bacteria, including both characterized and putative porins, resulted in the assignment of 30 putative porins to specific functional (solute-specific) groups. Two new porin groups also emerged – the OmpF2 and PhoE groups – suggesting additional diversification of specific orthologues, possibly through independent gene duplication events. Interestingly, the new PhoE group comprises proteins solely from species of *Yersinia*, and these may have evolved more recently via duplication due to porin antigenicity. Thus, the application of phylogenetic techniques has permitted a comprehensive analysis and characterization of the bacterial porins and the assignment of uncharacterized homologues to existing functional groups.

Phylogenetic approaches have also been used to identify evolutionarily and functionally relevant motifs within protein families, providing insight into both enzyme structure and biochemical mechanisms of action. La and colleagues, in 2005, conducted a sliding window analysis on a sequence alignment of the orthologues of the triosephosphate isomerase protein family to identify those residues that recover the overall family topology. Motifs that recover the complete sequence phylogeny are predicted to be functional sites and can subsequently be tested experimentally. In this study, predicted functional sites were tested by mapping them onto closely related protein structures, revealing that many corresponded to protein loops, active sites, and other structures, and illustrating the effectiveness of this phylogenetic approach in identifying residues responsible for protein structure and function.

Coevolution and HGT

Phylogenetic analyses have the potential to identify both uniformities in evolutionary patterns and deviations from expected patterns. For example, the comparison of evolutionary histories can identify cases of cospeciation, where long-term association is manifested in fully congruent phylogenies that denote organisms having very similar or identical evolutionary histories (Figure 3). Undoubtedly, one of the best studied cases of coevolution, identified using phylogenetic methods, is that of the aphid and its nutritional association with the bacterium *B. aphidicola*. *Buchnera* supplements the aphid diet of nutrient-deficient phloem sap with essential amino acids, including leucine and tryptophan, resulting in a long-standing, obligate association. Indeed, comparisons of the evolutionary history of *Buchnera*, as established by 16S rDNA phylogenies, with that of their aphid host species revealed phylogenetic congruence, illustrating the mutualistic association of *Buchnera* with the aphid prior to the divergence of extant aphid families. In an interesting twist, *B. aphidicola 16S* and *dnaN* gene genealogies were also congruent to those of the plasmid-encoded amino acid biosynthesis genes *trpEG* and *leuABC*, suggesting stable coexistence of plasmids with their *Buchnera* hosts. This sort of mutualistic association, involving a eukaryote, a bacterium, and it transmissible genetic elements is relatively unique, and serves to illustrate the power of phylogenetic analyses in establishing evolutionary mechanisms of coevolution.

Coevolutionary processes also appear to be maintaining the mutualistic relationships of bioluminescent *Vibrio fischeri* bacteria and squid. A comparison of molecular phylogenies of internal transcribed spacer and the cytochrome oxidase subunit I of the squid mitochondrial DNA to those obtained from the bacterial *gapA* gene revealed similar evolutionary histories, reinforcing maintenance of coevolutionary processes. Because the bacteria are environmentally acquired, this parallel evolution strongly implicates host-

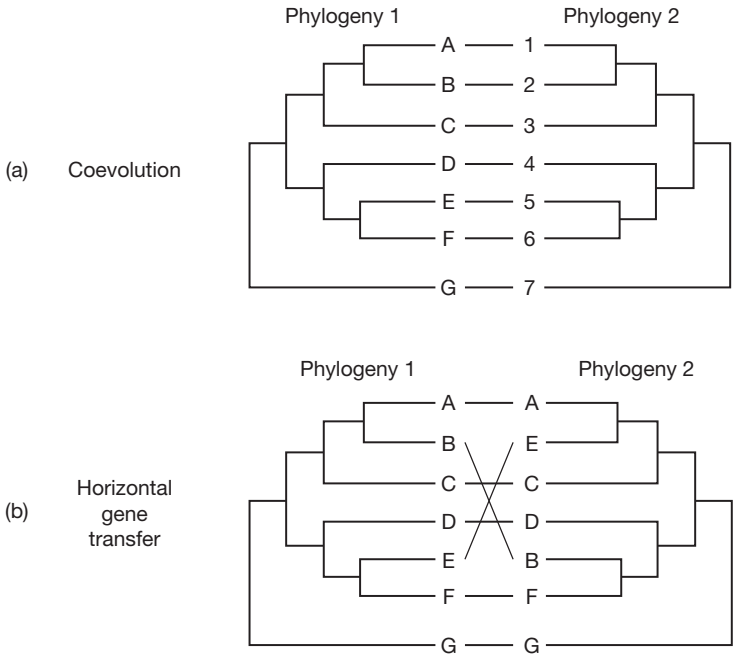

Figure 3 (a) Phylogenetic inference of coevolution, as resolved by comparing tree topologies. Congruent phylogenies are indicative of similar evolutionary histories for a given set of taxa (A–G, 1–7), and thus, cospeciation. (b) Incongruent tree topologies may indicate horizontal gene transfer (HGT) among organisms.

specific associations between symbiont partners. These phylogenetic inferences were further validated through competitive colonization assays, which illustrated that specific *V. fischeri* strains preferentially colonize their evolutionary counterparts.

Phylogenetic approaches have also been used to solve longstanding questions pertaining to the origins of new genes in the evolution of microbes and eukaryotes. The role of HGT in the evolution of bacteria has been well documented; however, the occurrence of HGT among eukaryotes, and its influence on the diversification of this domain, has remained a controversial topic in eukaryotic evolutionary biology. One early study by Bork and colleagues, in 1992, focusing on the evolution of the fibronectin type III domain (Fn3), reported its possible transfer to the bacteria. Although phylogenetic analyses revealed that the bacterial Fn3 proteins were monophyletic, a transfer event was favored due to the fact that (1) the bacterial sequences were more closely related to the animal sequences than expected by sequence divergence, (2) the Fn3 domain was represented sporadically in bacteria, and (3) it was not identified in either plants or fungi. The Fn3 domain, however, was later identified in a plant protein, suggesting that sampling bias was likely responsible for the seemingly limited distribution of the Fn3 domain in the original study.

With the sequencing and analysis of the human genome in 2001 came the report of a purported 223 cases of HGT between bacteria and vertebrates. Criteria for identifying these HGT events included a top BLAST hit to a bacterial gene and absence from a nonvertebrate genome. Of the 223 potentially acquired genes, only 28 had been confirmed to be present within the genome subsequently and served as the focus of a systematic phylogenetic analysis to determine the likelihood of HGT between bacteria and vertebrates. Of these 28 genes, 23 clustered with a nonvertebrate eukaryote positioned at the base of the tree (and were therefore not likely cases of HGT), whereas the remaining five cases were deemed ambiguous cases of HGT. Thus, although the vast majority of HGT cases reported in the original study were due to the absence of appropriate phylogenetic analyses; however, HGT between bacteria and vertebrates could not be discounted entirely. There was also some suggestion of bacterial contamination as a plausible explanation for the observed transfer events, which was later confirmed to be a contributing factor to the HGT events reported in the original study.

Phylogenetic approaches have been central to our ability to identify cases of horizontal transfer, even between distantly related organisms such as bacteria and eukaryotes. An increasing number of studies are employing phylogenetic and other methods to understand HGT and its impact on eukaryote evolution, with many studies providing evidence for HGT between the three domains of life.

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Phylum *Verrucomicrobia*

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Glossary

Gut microbiome The complex community of many bacterial species in the human intestine.
Kinesin Motor proteins that use energy released by ATP hydrolysis to move toward the (+) end of a microtubule, transporting vesicles or particles in the process.
Metagenome Genome sequences from an entire community of microbes in a natural habitat such as an environmental aquatic or soil habitat or within or on a biological organism and its tissues.
Metagenomics The technique of direct genome sequencing of an entire community of microbes in a natural habitat, bypassing the need to isolate and culture individual bacterial community members. The major aim is often to determine the structure and diversity of the species present in the community, but it can also be applied to retrieval of particular functional gene classes *e.g.*, for use in biotechnology and 'microbial prospecting'. In the community diversity version of such techniques, the sequences from a mixed metagenome have to be assembled and sorted ('binned') computationally for analysis of the structure of taxonomic phylum- or species-level diversity within the community.
Microtubules Cytoskeletal fibers typically found in cells of eukaryotes and in eukaryotes formed by polymerization of alpha- and beta- tubulin monomers which exhibit structural and functional polarity. In eukaryotes they are important components of cilia, flagella, the mitotic spindle, and other cellular structures. In eukaryotes they are 24 nm in diameter.
Mucin An important component of the mucus layer lining the human intestinal epithelium. Mucins are produced by goblet cells of the intestinal epithelium, and are highly glycosylated molecules also including serine, threonine and cysteine amino acids, and they form a transparent mucus layer on the intestinal tissue surface, containing in the colon a substantial bacterial community.
Prosthecae Rigid appendages of bacterial cells bounded by the cell wall, including stalk-like and hypha-like appendages of *Caulobacter*, *Asticacaulis* and *Hyphomicrobium* species in phylum *Proteobacteria*, projections of the cell surface in *Prosthecomicrobium* and *Ancalomicrobium* species in phylum *Proteobacteria*, and the stalk-like and wart-like projections of the cell surface in phylum *Verrucomicrobia* members *Prosthecobacter* species and *Verrucomicrobium spinosum*. They are projections of the cell cytoplasm and cell wall in such a way as to make the shape of the cell distinct from the classical rod or sphere shape of bacterial cells. Their contents are continuous with the cytoplasm of the rest of the cell.
PVC *Planctomycetes*-***Verrucomicrobia***-*Chlamydiae* superpylum grouping of phyla ***P****lantomycetes*, ***V****errucomicrobia* and ***C****hlamydiae* but also members of phylum *Lentisphaerae*, phylum *Omnitrophica* and possibly phyum *Poribacteria*. The phylum as a whole is grouped in a larger PVC superphylum with members of the distinct phyla *Plantomycetes*, *Chlamydiae*, *Lentisphaerae* and *Omnitrophica* (formerly phylum OP3), and the phylum *Kiritimatiellaeota* originally a subdivision of the *Verrucomicrobia* phylum. The member phyla cluster together in phylogenetic trees based on gene sequences such a 16S rRNA but some phyla also share at least one marker protein and some members of separate PVC phyla such as *Planctomycetes*, ***Verrucomicrobia*** and *Lentisphaerae* share cell structure features such as internal membrane development and probably membrane-bounded internal compartments.
Rhizosphere The interface between plant roots and soils, known to contain an enriched population of largely soil-derived microbial diversity, influenced by deposition of plant mucilage and root exudates.
Tubulin Globular cytoskeletal proteins that in eukaryotes have the ability to polymerize to form microtubules.
Verrucomicrobia A phylum-level group of the domain Bacteria named after one of the notable species described relatively early in the history of the discovery of the phylum, *Verrucomicrobium spinosum*. The phylum is a member of the *PVC* superphyum.

Introduction

The phylum *Verrucomicrobia* is an excellent example of the fascinating insights for microbiology to be gained from the study of diverse types of bacteria. The phylum was originally defined (Hedlund et al., 1997) on the basis of two genera, *Verrucomicrobium* and *Prosthecobacter*, and was only recognized by application of 16S rRNA gene sequencing (Albrecht et al., 1987; Hedlund et al., 1996) – *Prosthecobacter* had been originally suggested to be a close relative of *Caulobacter*, a member of phylum *Proteobacteria*, on the basis of morphological similarities (Staley et al., 1976). *Verrucomicrobium spinosum* was the first to be sequenced and a new bacterial division suggested as most suitable for its diverse phylogenetic position relative to other bacteria (Albrecht et al., 1987). At least 5 subdivisions of phylum *Verrucomicrobia*, comprising species forming closely related clusters within the 16S rRNA-based phylogenetic tree of the phylum, were initially recognized (mostly from analysis of uncultured sequences); one of these, subdivision 5, is distant and distinct enough to have been later proposed as a separate new bacterial phylum *Kiritimatiellaeota* (Spring et al., 2016),

and *Victivallis vadensis*, originally viewed as a member of a subdivision 7, is now known to be a member of separate phylum *Lentisphaerae* (Cho et al., 2004). The acidophilic methylotrophic verrucomicrobia (see below) have been referred to as members of a subdivision 6, and certainly form a distinct cluster within *Verrucomicrobia*. Verrucomicrobia have since their initial recognition contributed substantially to our knowledge of the range of diversity of bacterial cell biology, potential origin of proteins characteristic of eukaryotes, the significant role of these previously undetected bacteria in soil ecology, and the key roles such diverse bacteria may play in the human gut microbiome. The phylum *Verrucomicrobia* derives its name from the first cultured species isolated, *Verrucomicrobium spinosum* (Schlesner, 1987), so-called because of the resemblance of projections of the cell surface to human skin warts (*Verrucomicrobium* is derived from the Latin word *verruca* meaning a wart). These surface projections are now known to be true 'prosthecae' or projections of the cell wall including some cytoplasm (rather than external appendages).

The phylum *Verrucomicrobia* is now known to be related to several other phyla of the bacteria forming a cluster called a superphylum, including the phyla *Planctomycetes* and *Chlamydia*e as well as phylum *Lentisphaerae* and the 'Candidate phylum' *Omnitrophica* (Rinke et al., 2013), and possibly including other phyla of uncultured bacteria. The superphylum is known as the PVC superphylum (*Planctomycetes-Verrucomicrobia-Chlamydiae*) (Wagner and Horn, 2006; Fuerst, 2013). Phylogenetic and indel analysis supports the relationship of *Verrucomicrobia* and *Chlamydiae*, and at least one shared superphylum-specific signature protein is consistent with the grouping of phyla *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae* and *Omnitrophica* (Gupta et al., 2012; Lagkouvardos et al., 2014). The relationship of *Planctomycetes*and *Chlamydiae* was first recognized via shared features of 16S rRNA seen via oligonucleotide analysis (Weisburg et al., 1986). Consistent with the evolutionary relationship indicated by phylogenetic analysis, the group may also share some features of cell structure and biology which will be discussed below in more detail.

Our knowledge of the diversity of phylum *Verrucomicrobia* has been from very early in its history the result of a combination of study of cultured isolates and direct metagenomics studies of natural mixed microbial communities such as those in soil, resulting in clusters in phylogenetic analyses of rRNA sequences recognized as distinct subdivisions (Hugenholtz et al., 1998). After the initial discovery of verrucomicrobia members and the establishment of *Verrucomicrobia* as a bacterial division (Hedlund et al., 1997) which later became a phylum of the Bacteria (Hedlund, 2011b), five subdivisions were delineated (Hugenholtz et al., 1998) via 16S rRNA phylogenetics, comprising both cultured named species and many uncultivated sequences. Later, formal Class-, Order- and Family-level taxonomic categories (Hedlund, 2011b) were established for some Divisions such as subdivision 1 proposed as equivalent to the class *Verrucomicrobiae*, subdivision 2 (class *Spartobacteria*) (Sangwan et al., 2004) and subdivision 4 (class *Opitutae*) (Choo et al., 2007). Class *Verrucomicrobiae* now contains families *Rubritalaceae* and *Akkermansiaceae* as well as the original family *Verrucomicrobiaceae*, and interestingly these families seem to correspond to habitats – marine habitats for *Rubritalaceae*, animal intestines for *Akkermaniaceae* and freshwater for *Verrucomicrobiaceae* (Hedlund, 2011a). The subdivision system (sometimes 'subphylum' is used)- is also still widely referred to, since it can be used for clusters of sequences from culture-independent studies. However, members of subdivision 5 have recently been proposed as members of a new separate phylum, *Kiritimatiellaeota* (Spring et al., 2016). Some other verrucomicrobia members such as the methane-oxidizing acidophilic and thermophilic *Methyloacidiphilum* do not fit into the original subdivisions, and have been proposed as members of the new family *Methylacidiphilaceae*, (Op den Camp et al., 2009) but have also been referred to as subphylum 6 in the formal classification authority Bergey's Manual (Hedlund, 2011b).

Why are They Important?

The verrucomicrobia are an especially important group of bacteria from many perspectives. They have evolutionary and cell biology significance regarding their relationship to other bacterial phyla such as *Chlamydiae* and *Planctomycetes* with complex cell structure or biology, and the intriguing presence of close homologs to eukaryotic proteins such as tubulins. Some species display unusual projections of cell cytoplasm and wall called prosthecae, and may also contain membrane-bounded cell compartments unusual in Bacteria but present in the related phylum *Planctomycetes*. They are of immense significance to understanding of ecology, especially of the ecology of soil microbial communities and their function, since they appear to be a widespread and quantitatively significant component of soils throughout the planet. They may also play important roles in other natural ecosystems such as marine and fresh waters. Finally, certain species such as *Akkermansia muciniphila*, may play key roles in the microbial communities of animal guts, including humans, where understanding of such verrucomicrobia may prove of central importance to understanding diseases associated with the human intestine and the widespread and increasing modern problems of human obesity and diabetes. Initially thought to be mainly aerobic heterotrophs, the diversity of verrucomicrobia has yielded surprising new forms of physiology, such as the acidophilic and thermophilic methanotrophy of verrucomicrobia such as *Methyloacidiphilum* species, of significance in understanding bacterial methane oxidation and the full range of bacteria involved in global cycling of methane, an important greenhouse gas. Each of these aspects of the verrucomicrobia are dealt with in detail below.

Isolation, Culture and Physiology

The first representatives of the phylum *Verrucomicrobia* such as *Verrucomicrobium spinosum* and *Prosthecobacter* species such as *P. dejongeii* were isolated as aerobic chemoorganotrophs from aquatic habitats (Schlesner, 1987; Staley et al., 1976). Later studies systematically exploring soil bacteria expanded the named genera and species of cultured heterotrophic strains by use of very low

nutrient media and the replacement of potentially inhibitory agar as a gelling agent in solid media by polymers such as gellan (Janssen et al., 2002), and this approach was expanded to the use of polymeric carbon and energy sources such as xylan known to occur on soil and the use of both extended incubation times (*e.g.*, 1 month) and concentrated inocula (Sangwan et al., 2005). Interestingly, some verrucomicrobia such as *Brevifollis gellanilyticus* and *Luteolibacter gellanilyticus* actually appear capable of degrading the gellan polymer recommended for soil verrucomicrobia isolation (Otsuka et al., 2013; Pascual et al., 2017). Heterotrophic Verrucomicrobia have affinity for carbohydrate carbon and energy substrates, and sometimes a mixture of sugars (at concentration of 1 mM each) have also been used as substrates in isolation media (Janssen et al., 1997). Members of the family *Opitutaceae* in subdivision 4 such as *Opitutus terrae* from rice paddy soil were originally reported to be obligate anaerobes (Chin et al., 2001) but more detailed investigations indicate that *O. terrae* for example is facultatively anaerobic and can tolerate at least some oxygen (Tegtmeier et al., 2018). Both cultural and genome evidence indicate that a verrucomicrobia species within *Opitutaceae* (initially named *Diplosphaera colitermitumis* but later revised to *Geminisphaera colitermitum*) is microaerophilic, growing optimally between 2 and 8% oxygen, and is also able to fix nitrogen (Wertz et al., 2012). Anaerobic Isolates from rice paddy soil that later were shown to be related to the verrucomicrobia genus *Opitutus* were reported to possess 'ultramicrobacteria' cell sizes, with average cell volumes of only 0.03–0.04 μm^3 (cells are 0.35 μm wide compared to *V. spinosum* at 0.8–1.0 μm wide) (Janssen et al., 1997); the small cell size was not a feature due to starvation but was independent of substrate concentration used for culture. Such isolates seem to be in substantial numbers in their habitat (*e.g.*, $1.2–7.3\times10^5$ cells per g of dry soil), since a liquid dilution series followed by anaerobic agar tube dilution series was used for their isolation. It is of interest that at least one widely distributed uncultured soil verrucomicrobial bacterium, '*Candidatus* Udaeobacter copiosus' has a genome of only 2.8 Mbp, suggesting genome reduction associated with large population size combined with selection against metabolic versatility (Brewer et al., 2016). A number of members of distinct verrucomicrobial genera have been isolated from marine habitats (Choo et al., 2007) and may have some unusual cell wall features (see below) *e.g.*, a species such as *Coraliomargarita akajimensis* a member of subdivision 4 (Yoon et al., 2007c), and *Cerasicoccus arenae* from marine sand, proposed as representative of a family *Puniceicoccaceae* of marine species in the subdivision 4 Class *Opituta* (Yoon et al., 2007a). Simple media widely used in marine microbiology such as a diluted seawater version of marine agar 2216 were used for isolation of the marine verrucomicrobia (see for example for *Coraliomargarita akajimensis* (Yoon et al., 2007c)).

The important human and animal gut microbial community species *Akkermansia muciniphila* was isolated as an anaerobic mucin-degrader via dilution of faeces in anaerobic medium with gastric mucin as sole carbon source combined with purification via anaerobic soft agar subculture (Derrien et al., 2004).

Aerobic methane-oxidizing verrucomicrobial acidophilic thermophiles such as *Methylacidiphilum infernorum* and *Methylacidiphilum fumariolicum* were isolated from geothermal habitats such as methane-rich geothermally heated soil or mudpots near volcanic fumaroles (Dunfield et al., 2007; Pol et al., 2007), and these species grow optimally at pH 2.0–2.5 and 60°C and at a pH 0.8 and a temperature of 50°C respectively. Mesophiles have also been isolated from volcanic soil, such as *Methylacidimicrobium* spp., even more acid- tolerant than their thermophilic relatives; one species, *M. tartarophylax*, can grow even at pH 0.5 (van Teeseling et al., 2014). Interestingly, such isolates may have a streamlined genome (*e.g.*, 2.3 MBp in *M. infernorum* – (Hou et al., 2008)) with limited potential for gene expression regulation. The genuine autotrophic dependence of these methane oxidizers on CO_2 as sole carbon source is indicated by genomic and transcriptomic data; in *Methylacidiphilum fumariolicum* all genes required for a functional Calvin-Benson-Bassham (CBB) cycle are transcribed (Khadem et al., 2011). Methane is apparently used mainly as an energy source via its oxidation rather than as a carbon source (so activity of methanotrophs would be missed by methods depending on isotopically labeled methane). This organism also has a unique type of RuBisCo CO_2 fixation enzyme. The species is also capable of nitrogen fixation and possesses an extremely oxygen-sensitive nitrogenase (Khadem et al., 2010). *M. fumariolicum* can also oxidize hydrogen using carbon dioxide as a sole carbon source, so can be independent of methane as an energy source under some conditions, at least in oxygen concentrations less than 1.5% (Mohammadi et al., 2017). From direct studies of verrucomicrobia-dominated soil communities from an acidic geothermal field and data from a *Methylacidiphilum* isolate from those soils it appears that verrucomicrobial methanotrophs can use a strategy of mixotrophy to grow optimally in their natural geothermal methane- and hydrogen- rich habitats, by combining both methane- and hydrogen- oxidizing abilities, allowing expansion into wider niches than methane oxidation alone would allow (Carere et al., 2017).

Remarkably, growth of the volcanic mudpot verrucomicrobium *Methylacidiphilum fumariolicum* SolV is strictly dependent on the lanthanides, rare earth elements such as lanthanum, cerium, praseodymium and neodymium, which act as cofactors for methanol dehydrogenase, a key enzyme for methane oxidation (Pol et al., 2014).

Since methane is a potent greenhouse gas, and methanotrophic verrucomicrobia occupy unique and methane-rich habitats, it has been suggested that they could be an important contributor to reducing greenhouse gases contributing to global warming, since they can limit the amount of biologically or geochemically produced methane escaping into the atmosphere (Mohammadi et al., 2017) – this would imply that verrucomicrobial methanotroph ecology could be important to understand for global greenhouse gas modeling.

Genomes of verrucomicrobia have revealed potential for microbial sulfite/sulfate reduction ability, indicated by genes for dissimilatory sulfite reductases (DsrAB) which may include some of the earliest evolved such genes (Anantharaman et al., 2018).

Genomic data of verrucomicrobia and other members of the PVC superphylum is now extensive, and new databases available should ameliorate exploration of their genomes and proteomes for comparative studies and make understanding of their metabolome and cell biology-relevant genes more feasible (Bordin et al., 2018). The PVCBase database is a simple portal for published genomes of cultured verrucomicrobia (See Relevant Websites Section). Many more unpublished verrucomicrobia genomes are available in such genome databases as NCBI Genome (See Relevant Websites Section) or IMG – JGI's Integrated Microbial Genome and Microbiomes

(See Relevant Websites Section). At least draft genomes exist for named species *Verrucomicrobium spinosum* in class *Verrucomicrobiae* (see "Relevant Websites section"), *Akkermansia muciniphila* of class *Verrucomicrobiae* both as a cultured isolate (van Passel et al., 2011b) and from a whole genome assembly from uncultured cells from human faecal material (Caputo et al., 2015), *Terrimicrobium sacchariphilum* (Qiu et al., 2017) and the uncultured '*Candidatus* Udaeobacter copiosus' and *Chthoniobacter flavus* (Sangwan et al., 2004), all in Class *Spartobacteria* (Brewer et al., 2016), *Opitutae terrae* in Class *Opitutae* (van Passel et al., 2011a), *Pedosphaera parvula* of subdivision 3 (Kant et al., 2011), and *Methyloacidiphilum infernorum* representative of the acidophilic methanotroph 'subdivision 6' (Hou et al., 2008). Cultured named species vary from the 'streamlined' 2.3 Mbp and 2.66 MBp genomes of *Akkermansia muciniphila* and *Methyloacidiphilum infernorum* to the substantial 7.8 Mbp genome of *Chthoniobacter flavus*.

Culture-independent sequencing studies of metagenomes from natural microbial communities indicate that only a limited number of species existing in nature have been isolated in each subdivision or Class of *Verrucomicrobia*, *e.g.*, in soil, verrucomicrobia of class *Spartobacteria* appear especially significant but isolates do not represent the known phylogenetic breadth from culture-independent studies(Janssen, 2006), so there seems to be a vast wealth of verrucomicrobial species remaining to be cultured and described.

Distribution and Ecology Including Importance in the Soil Ecosystem

Verrucomicrobia are mostly free-living bacteria and can be found in freshwater, marine, soil and animal gut habitats. They appear to be globally distributed in both freshwater (Zwart et al., 1998) and seawater (Freitas et al., 2012). They are prevalent in temperate freshwater lakes in northern USA, for example, comprising the 4th most abundant phylum of the bacterial community; verrucomicrobia were relatively particle-associated, and lake habitats vary in the particular Class or subdivision of verrucomicrobia dominating (Chiang et al., 2018). In a study of two contrasting freshwater habitats, a eutrophic lake and a humic bog, analysis of verrucomicrobia via metagenomics suggested their potential role as polysaccharide degraders in freshwater, and also revealed possible biochemical distinctions between strains inhabiting the two habitats and different layers within a habitat (He et al., 2017). Eutrophic lake verrucomicrobia are rich in glycoside hydrolases while verrucomicrobia in the upper lake layers possess an unusual class of genes for synthesis of cytochrome c usually considered specific for phylum *Planctomycetes*, an observation of great interest in light of the evolutionary relations of verrucomicrobia within the PVC superphylum. Bog verrucomicrobia possessed genes for unusual porin-multiheme cytochrome c complexes with a potential extracellular electron transfer function. In the Arctic marine water column, verrucomicrobia comprise one of the most frequently detected bacterial community members based on culture-independent 16S rRNA gene clone libraries, and may be associated with genes for hydrolyzing polysaccharides such as laminarin, chondroitin sulfate and xylan (Cardman et al., 2014).

The wide distribution in soils has been extensively documented - they appear to be dominant in many soil bacterial communities across the globe, and a molecular ecology study of 16S rRNA sequences using many different soils from biomes in Antarctica, Europe, and the Americas, as well as soils collected from a montane coniferous forest using data collected from surface horizons indicate that verrucomicrobia average 23% of all bacterial sequences present in the microbial communities (Bergmann et al., 2011). Appreciating such quantitative significance depended on designing and applying PCR primers which are not biased against this phylum when soil microbial DNA is used as substrate (Bergmann et al., 2011). The class *Spartobacteria* (subdivision 2) verrucomicrobia appear especially important in soils, with some evidence also for significance in some soils of subdivision 1 (Kielak et al., 2010), and "Pedosphaera parvula" Ellin514 isolated from pasture soil (Sangwan et al., 2005) is one of the few cultured representatives of subdivision 3 of the phylum *Verrucomicrobia*. Complete genome sequences are available for two soil verrucomicrobial species, *Chthoniobacter flavus* from *Spartobacteria* subdivision 2 (Sangwan et al., 2004) and *Pedosphadra parvula* from subdivision 3 (Kant et al., 2011). Verrucomicrobia are particularly significant numerically in grassland and prairie soils, perhaps as one suggestion has it due to relationships of some spartobacteria with soil nematodes since "*Xiphinematobacter*" a member of the Class *Spartobacteria* is nematode-associated (Bergmann et al., 2011), but this hypothesis remains to be investigated in detail. 16S rRNA from and qPCR performed on microbial communities indicate that Verrucomicrobia can be significant members of plant root - associated rhizosphere microbial communities (Kielak et al., 2008). Some studies suggest differences in rhizosphere association can occur between different strains of subdivision 1 verrucomicrobia (da Rocha et al., 2011). Verrucomicrobia occur as apparent symbionts with animals such as nematodes (Vandekerckhove et al., 2002), within the nuclei of protists in termite guts (Sato et al., 2014) and within marine tunicates(Lopera et al., 2017). Symbionts belong to at least two different subdivisions, the Classes, *Spartobacteria* and the *Opitutae*.

Verrucomicrobia do not appear to be pathogenic, but there is one report that *Verrucomicrobium spinosum* at least increases mortality rates in invertebrate model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* (Sait et al., 2011), perhaps associated with the presence of a bacterial virulence factor Type III Secretion System(T3SS). The activity of *Akkermansia* is of relevance to susceptibility to effects of bacterial pathogens in the gut (at least in a germ-free mouse model) (Ganesh et al., 2013) as well as possible effects on protection against various intestinal and metabolic diseases described below.

Importance of Verrucomicrobial Genus *Akkermansia* for the Human Gut Microbiome and Related Disease

One species of the Verrucomicrobia, *Akkermansia muciniphila*, has been of very special interest and significance for understanding the microbial community of the human intestine, the gut microbiome. *Akkermansia* species occur in many different

animal species from pythons to horses as well as humans and model laboratory rodents (Derrien et al., 2017). As the species name *mucinphila* implies, this verrucomicrobial bacterium degrades mucin, an important component of the mucus layer lining the intestinal epithelium. Mucins are produced by goblet cells of the intestinal epithelium, and are highly glycosylated molecules also including serine, threonine and cysteine amino acids, and they form a transparent mucus layer on the intestinal tissue surface, containing in the colon a substantial bacterial community (Johansson et al., 2013). *Akkermansia muciniphila* is highly adapted to living from mucin degradation – for example it absolutely requires threonine for growth and has a variety of enzymes adapted for utilizing specialized sugars in mucin oligosaccharides such as sialidases and fucosidases and even sulfatases (Ottman et al., 2017a); it is also adapted to the low but definite oxygen concentration present in the mucus layer. Such adaptation suggests an advanced stage of coevolution between host animal and *Akkermansia* strains, and implies a potential functional significance of these verrucomicrobia for the host. This species may also be significant as a keystone species within the gut microbial community due to its supply of products of mucin degradation to other bacterial species (Chia et al., 2018). In mice, such a significance is suggested by correlations between increased abundance of *Akkermansia* in faeces of lean animals compared to the abundance in obese mice, and the observation that daily feeding of *Akkermansia* over a period of several weeks to mice with obesity induced by a high fat diet apparently reverses this induced obesity as well as improving epithelial integrity and related effects (Everard et al., 2013). The administration of *Akkermansia* to obese mice also results in an observed reduction in 'metabolic endotoxemia' via reduction of inflammatory lipopolysaccharides in the circulation, and in alleviation of insulin resistance (Everard et al., 2013; Shin et al., 2014) as well as cardiometabolic complications of obesity in mice such as arteriosclerosis (Li et al., 2016). At least conclusions from mice models regarding *Akkermansia* effects appear to be supported by replication from many different studies (Derrien et al., 2017).

There is also some data indicating that *Akkermansia* administration may form the basis for a protocol for obesity amelioration in human subjects (Sheng et al., 2018). Some data indicates the need for live *Akkermansia* cells to generate such effects in mice (Everard et al., 2013), but such protocols might eventually involve killed (pasteurized) cells or even purified *Akkermansia* proteins. Heat-killed cells of Akkermansia appear to have the same or even enhanced beneficial effects on high-fat-diet obese mice compared to effects of treatment with live cells (Plovier et al., 2017), and this is consistent with evidence that a protein associated with Type IV pili appendages in outer membranes of the cell wall may be the active component of *Akkermansia* cell effects in the mucin layer of the intestine. Outer membrane protein composition and especially the highly abundant pili-like protein Amuc_1100 of *A. muciniphila* assist host immunological homeostasis at the gut mucosa and improvement of the function of the gut barrier (Ottman et al., 2017b).

In summary, just one species of verrucomicrobia, *A. muciniphila*, may prove to be of dramatic significance to understanding human disease states of major importance to medicine.

Biotechnology

Verrucomicrobium spinosum possesses a tyrosinase with interesting properties of potential application in biotechnology (Fairhead and Thony-Meyer, 2010). There is an also an interesting connection of the applied features of this tyrosinase with the concept that verrucomicrobia may retain some eukaryote-like characteristics – the tyrosinase of *V. spinosum* shares a feature of eukaryotic tyrosinases in that a C-terminal extension is present, one absent from tyrosinase of bacteria such as *Streptomyces.* Recombinant tyrosinase derived from *V. spinosum* can be used for protein cross-linking and immobilization, and has potential uses in preparing protein aggregates useful for industrial biocatalysis. Tyrosinases catalyze oxidation of phenols and possible applications include removal of phenols, biosensor fabrication, and for a production of biocompatible adhesives with medical applications (Axambayeva et al., 2018). Recombinant *V. spinosum* tyrosinase has been used to modify tyrosine residues in proteins to produce strong molecular adhesives with DOPA (3,4-dioxyphenylalanine) residues in place of tyrosines (Axambayeva et al., 2018). Other enzymes of biotechnological potential from *V. spinosum* and other verrucomicrobial species may be also worth exploring. For example, a novel approach has been used to recover genomes of active polysaccharide degraders from natural, complex microbial assemblages, employing fluorescently labeled substrates with fluorescence-activated cell sorting and single cell genomics to analyze freshwater and coastal bacterioplankton for degraders of laminarin and xylan (Martinez-Garcia et al., 2012). Genomic sequencing of just five cells, representing the most predominant polysaccharide-active *Verrucomicrobia* phylotype found, revealed significant enrichment in genes encoding a wide spectrum of glycoside hydrolases, sulfatases, peptidases, carbohydrate lyases and esterases. The potential thus exists for exploitation of such enzymes via genome mining even from uncultured verrucomicrobia.

Another potential product of verrucomicrobia is biopharmaceuticals. Cytotoxic compounds of the mandelalide class have been found in the marine tunicate *Lissoclinum* sp., and there is evidence from megtagenomics that the compounds can be produced the verrucomicrobial symbionts in the tunicate (Weyna et al., 2015). The symbiont, designated "*Candidatus* Didemnitutus mandela," in a clade within the family *Opitutaceae*, possesses genes for synthesis of a type of trans-AT polyketides consistent with the mandelalide class (Lopera et al., 2017). The pathway appears functionally significant since the seven copies of the genes for mandelalide synthesis accounts for 19% of the symbiont's genome by length, suggesting functional importance for chemical defense of the host tunicate animal. A metagenomics study of a marine kelp forest microbial community also suggests that verrucomicrobia may be a rich source of potential natural products in the polyketide compound class (Vollmers et al., 2017).

Cell Biology and Significance for Evolution

The verrucomicrobia possess some very interesting features relevant to bacterial cell biology and potential evolutionary interconnections between some features of cell biology characteristic of eukaryotes and those characteristic of bacteria. Their close relationship to *Planctomycetes* as PVC superphylum members suggests that some features of the planctomycete cell plan involving internal membranes and potential membrane-bounded compartments within cells might also occur in verrucomicrobia.

Cell Structure

The first described verrucomicrobia, *Verrucomicrobium spinosum* and *Prosthecobacter* species, displayed prosthecae, projections of the cell cytoplasm and wall which had first been described in members of phylum *Proteobacteria* such as *Ancalomicrobium* and *Prosthecomicrobium*. In *V. spinosum* (Fig. 1(A) and (B)) these are randomly distributed finger-like extensions from which fimbriae appendages project (Schlesner, 1987), while in *Prosthecobacter* (Fig. 2(A) and (B)) the prostheca takes the form of a single stalk-like structure (Staley et al., 1976). Only some genera of verrucomicrobia display prosthecae – so far only *Verrucomicrobium* and *Prosthecobacter* among the approximately 30 cultured genera have been described as prosthecate. Presence of prosthecae detectable by phase contrast microscopy has been used in isolation of prosthecate verrucomicrobia via colony screening (Hedlund, 2011a).

Features of cell ultrastructure consistent with the simplest forms of cell compartment bounded by a major intracellular membrane are displayed via transmission electron microscopy of cells of several species representing three verrucomicrobial subdivisions, *Verrucomicrobium spinosum* (see Fig. 1(B)), *Prosthecobacter dejongeii* (see Fig. 2(B)) (*both subdivision 1, Verrucomicrobiae*)), *Chthoniobacter flavus* (*subdivision 2, Spartobacteria*), and strain Ellin514 (now classed "*Pedosphaera parvula*" in subdivision 3) (Lee et al., 2009). The form of compartmentation is similar to the simplest form of cell compartment found in some of the species of phylum *Planctomycetes* such as *Pirellula staleyi* and in a member of the phylum *Lentisphaera*. A similar form of compartmentation has been found in subdivision 3 member *Limisphaera ngatamarikiensis* (Anders et al., 2015), and is claimed for a marine member of subdivision 4, *Coraliomargerita akajimensis* (Pinos et al., 2016). Cells with this form of compartmentation have been proposed to

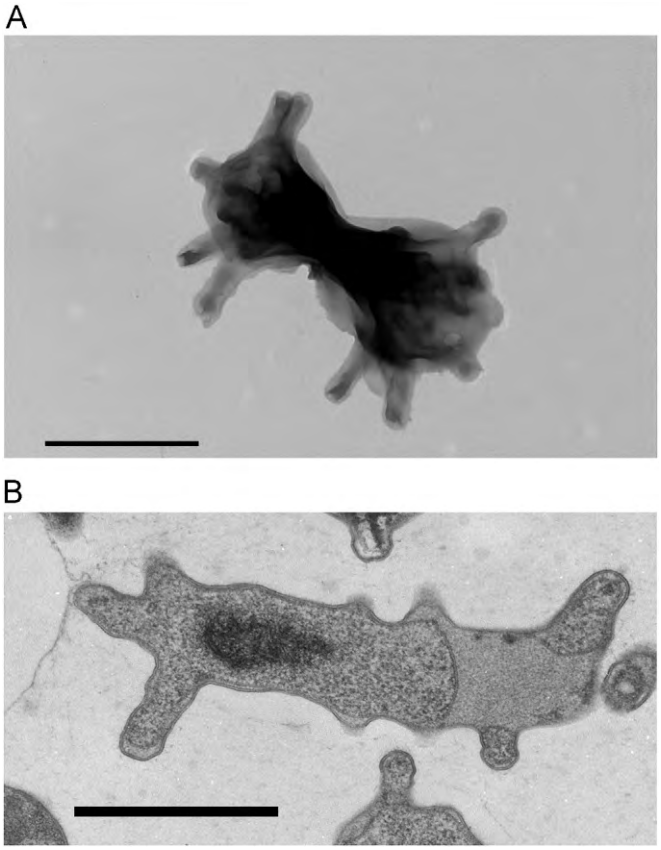

Fig. 1 (A) Transmission electron micrograph of negatively-stained cell of *Verrucomicrobium spinosum* showing many tube-like prosthecae projections on all regions of the cell surface. Bar marker 1 μm. Micrograph from Jeffrey Lee of the author's laboratory. (B) Transmission electron micrograph *Verrucomicrobium spinosum* sectioned cell (cryofixed via freeze-substitution method), showing major ribosome-containing cell compartments bounded by internal membrane, other ribosome-free regions, and the cytoplasmic and cell wall-bound nature of the prosthecae projections of the cell. Bar marker 1 μm. Micrograph from Jeffrey Lee of the author's laboratory.

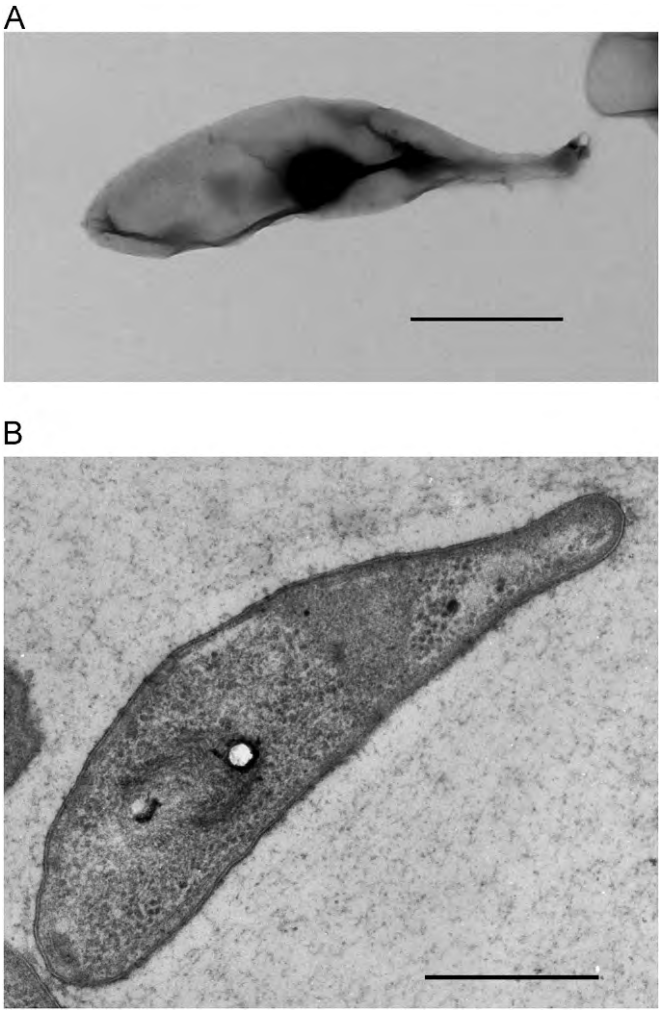

Fig. 2 (A) Transmission electron micrograph of negatively-stained cell of *Prosthecobacter dejongeii* showing single stalk-like prostheca projection on one pole of the cell only. Bar marker 1 μm. Micrograph from Jeffrey Lee of the author's laboratory. (B) Transmission electron micrograph *Prosthecobacter dejongeii* sectioned cell (cryofixed via freeze-substitution method) showing cytoplasmic nature of interior of the single stalk-like prostheca projection and cell compartmentation into major regions containing ribosomes and a less extensive ribosome-free region. Bar marker 0.5 μm. Micrograph from Jeffrey Lee of the author's laboratory.

possess a major membrane-bounded 'pirellulosome' compartment containing the nucleoid DNA and ribosomes, and an outer ribosome-free 'paryphoplasm', analogous to the cell regions in the planctomycete cell plan (Fuerst and Sagulenko, 2011, 2013). The closed nature of such compartments needs confirmation by cryoelectron tomography, but it is clear that there is a major internal membrane development in cells of some representative cultured verrucomicrobia.

Via electron cryotomography, remarkable external appendages have been observed on cells of *Prosthecobacter debontii*, ca. 20×50 nm in dimensions and having a complex disc- and multiple 'leg' structure, not observed previously in bacteria and of unknown function (Dobro et al., 2017).

Verrucomicrobia stain Gram-negative, and would be expected to possess cell wall characteristics of other Gram-negative bacteria including an outer membrane with lipolysaccharide (for which genomic evidence exists – (Speth et al., 2012)), and the classical and almost universal bacterial cell wall polymer peptidoglycan (PG), with diaminopimelic acid common in Gram-negative peptidoglycan as well as muramic acid. Verrucomicrobia originally discovered such as *Verrucomicrobium spinosum* were found to indeed possess peptidoglycan. However, there are some reports in newly described marine species in family *Puniceicoccaceae* within class *Opititutae* of the absence of peptidoglycan evidenced by the absence of muramic acid and diaminopimelic acid in wall analyses *e.g.*, *Pelagiococcus* species (Yoon et al., 2007b,d), *Cerasicoccus* spp. (Yoon et al., 2007a, 2010), and *Coraliomargerita akajimensis* (Yoon et al., 2007c). It has been noted however that at least *Coraliomargerita akajimensis* possesses genes consistent with peptidoglycan synthesis ability, and PG analysis of some other subdivision 4 *Opitutae* strains indicates that thin-layer chromatography is not suitable as an analysis method for detecting the low amounts of peptidoglycan in cells of some subdivision 4 strains (Rast et al., 2017); walls of marine *Puniceicoccaceae* had however been investigated with HPLC amino acid analysis as well as thin-layer chromatography (Yoon et al., 2007a). One reason for difficulty in detecting peptidoglycan in some species may be low proportional

amounts of peptidoglycan relative to cell mass, as indicated by the data on *Limisphaera ngatamarikiensis* where only trace amounts of peptidoglycan-specific meso-diaminopimelic acid were detected (Anders et al., 2015). Lipopolysaccharide and outer membrane proteins characteristic for Gram-negative bacterial walls have been found in the significant gut verrucomicrobium *Akkermansia muciniphila* (Ottman et al., 2016).

Symbiotic verrucomicrobia called epixenosomes found associated closely with the surfaces of the marine ciliate protozoan *Euplotidium* have a complex compartmented internal structure including an 'extrusive apparatus' which may be ejected under certain stimuli and which have been proposed to have a defensive function for the host protozoan against another predator ciliate (Petroni et al., 2000), and cytoskeletal features of evolutionary interest (see below).

Tubulins and Other Cytoskeletal Proteins

Via genome sequencing, two genes *btuba* and *btubb* with close similarity to eukaryotic tubulins were discovered in verrucomicrobia member *Prosthecobacter dejongeii*, and homologs confirmed in three other species of this genus, *P. debontii*, *P. fusiformis* and *P. vanneervenii* (Jenkins et al., 2002). The corresponding deduced tubulin proteins BtubA and BtubB had properties consistent with their homology to tubulins rather than to the common bacterial tubulin family FtsZ cell division protein. This is evolutionarily significant since tubulins are essential for formation in eukaryote cells of microtubules, cytoskeletal assemblies functioning in several important ways in providing motors for organelle transport within the cell and for movement of materials in mitotic segregation during cell division. BtubA and BtubB were shown to share 31%–35% and 34%–37% sequence identity with eukaryote α and β tubulins; BtubA and BtubB were shown to contain each of the nine motifs specific to eukaryotic tubulin but only two to three of the six signatures for bacterial FtsZ, indicating a closer relationship to tubulins than FtsZs. *Btuba* and *btubb* were adjacent to each other on the genome. Phylogenetic analysis in that study indicated that neither BtubA or BtubB clustered with tubulins of any specific extant eukaryotic clade, and that the bacterial tubulin sequences were always seen to branch more deeply than eukaryotic tubulins; so there was no indication from that analysis of likely horizontal gene transfer as an explanation for the eukaryotic-like tubulin occurrence in a bacterial species, and the alternative of close relationship with a shared tubulin-like ancestral protein was also considered in this initial study. Another species of *Prosthecobacter*, *P. fluviatilis*, does not (based only on attempted PCR amplification but using tubulin +ve *P. vanneervenii* as a positive control) appear to possess tubulins (Takeda et al., 2008), but does apparently possess a prosthecate stalk, which has been used to argue that the tubulin function in other *Prosthecobacter* spp. cannot be concerned with formation of prosthecae.

However, EM examination of cryo-fixed sectioned cells of the three other named *Prosthecobacter* species, *P. dejongeii*, *P. vanneervenii*, and *P. debontii*, in comparison with a mutant strain lacking *btubAB* genes, demonstrated tube-like structures in wild-type strains of *btubAB* positive species, localized to the prosthecate stalk or in the transition zone between stalk and cell body (Pilhofer et al., 2011). Cryo-fixation (as used for the EM preparation technique of freeze-substitution) is necessary to preserve such structures.

The more typical member of the tubulin family in Bacteria, ftsZ. has been found to co-occur with tubulins in *Prosthecobacter* species (Pilhofer et al., 2007b), and FtsZ alone has been found to occur in *Verrucomicrobium spinosum* (Yee et al., 2007). FtsZ was found in all three *Prosthecobacter* species where tubulins are found - *P. dejongeii*, *P. debontii and P. vanneervenii*. The co-occurrence of FtsZ was interpreted as support for horizontal gene transfer as the origin of tubulins (Pilhofer et al., 2007b), but phylogenetic analysis of FtsZ of *V. spinosum* suggested a deep branching position and ancient origin for that protein (Yee et al., 2007). Later more detailed studies of *Prosthecobacter* tubulin structure suggest that if HGT did occur from eukaryotes, it must have been a very ancient transfer, since the tubulin structure is compatible with an ancient form of the structure (Martin-Galiano et al., 2011). From considerations such as primitive assembly properties, it has been proposed that BtubA/B were acquired by *Prosthecobacter* in evolutionary time not long after an alpha- and beta-tubulin ancestor protein's duplication, possibly from a primitive eukaryotic cell via horizontal gene transfer (Martin-Galiano et al., 2011). Considerations of gene order surrounding the FtsZ gene and genes classically associated with FtsZ in other bacteria suggested that in verrucomicrobia a typical bacterial FtsZ-based cell division operates rather than one based on tubulin as in eukaryotic mitosis (Pilhofer et al., 2007b).

The BtubA/BtubB system has been subjected to considerable study regarding polymerization and other properties relevant to any comparison with eukaryotic tubulins and microtubule polymerization. It was first found that mixtures of BtubB and BtubA (purified from proteins cloned in *E.coli*) were capable of forming long protofilament bundles, 4–7 protofilaments wide (20–30 protofilaments in the three-dimensional bundle) (Sontag et al., 2005), and that BtubB possessed GTPase activity. Protofilament composition suggested that BtubA and BtubB alternate along the protofilament, and a cooperative assembly mechanism was suggested. Protofilaments formation BtubA/B does not need folding-assisting chaperonin proteins, unlike eukaryote tubulins (Schlieper et al., 2005) Crystallography studies of BtubA purified after cloning and expression in *E.coli* and of polymerized BtubA/B heterodimer demonstrated that the structure of BtubA/B is much closer to eukaryotic tubulins than to bacterial FtsZ and includes surface loops unique to tubulin, and also a C-terminal domain used in eukaryote tubulins for interactions with microtubule-associated proteins (Schlieper et al., 2005). Such eukaryote-adapted features, together with the largely bacterial nature of the *Prosthecobacter* genome, have been used to argue for an origin of verrucomicrobial tubulins via horizontal gene transfer from eukaryotes (Schlieper et al., 2005).

BtubA and BtubB do not seem to correspond exactly to the alpha and beta classes of eukaryote tubulin, rather they have mixed features of both classes in each, forming a mosaic of residues intermingling features of alpha and beta tubulins (Martin-Galiano et al., 2011). These features combined with the biochemical simplicity of the system have been used to support a scenario in which

bacterial tubulins originated from simple forms of tubulin genes that were transferred from a primitive eukaryotic cell rather than any modern lineage of eukaryotes, consistent with why the Btub sequences are mosaics of alpha and beta tubulins and why no existing eukaryotic donor is identifiable *e.g.*, BtubA and BtubB are divergent from any modern eukaryote lineage in phylogenetic trees of tubulins (Martin-Galiano et al., 2011). A phylogenetic analysis of *V.spinosum* FtsZ indicating deep-branching position is consistent with a deep-branching tubulin originating at a similar ancient time, with a divergence of FtsZ and tubulin from an ancient precursor (Yee et al., 2007). It may be useful to reexamine the phylogenetics of *Prosthecobacter* ftsZs as well as tubulins.

Four-stranded mini-microtubules have been generated by polymerization of cloned purified Btuba and BtubB and share many key structural features with eukaryotic microtubules. These features include a so-called structural 'M-loop' in protofilament protein facilitating contact between protofilaments, alternating BtubA and BtubB subunits, and a seam that breaks overall helical symmetry within the minimicrotubule (Deng et al., 2017).

By means of a special *in vitro* total internal reflection fluorescence microscopy technique, it has been demonstrated that bacterial minimicrotubules display so-called 'treadmilling' (simultaneous lengthening of one end together with shortening of the other end of the microtubule) and also display dynamic instability (switching between phases of growth and phases of shrinking), both distinguishing features of eukaryotic microtubule behaviour (Deng et al., 2017). In dynamic instability in eukaryote microtubules, microtubules switch between phases of growth (lengthening) and shrinking (shortening), and GTP hydrolysis closely follows incorporation of a tubulin dimer into a growing microtubule end (Borisy et al., 2016) Thus, these mini microtubules derived from *Prosthecobacter* tubulins seem to display functional as well as structural homologies with eukaryote microtubules, despite their relative simplicity.

The discovery of *Prosthecobacter* tubulins has led to deep investigation of these proteins both experimentally concerning their ability to form polymers and the properties of such polymers, and structurally regarding how similar the components critical for polymer formation by the Btub tubulins are to the analogous components of eukaryote tubulin structures. BtubA and BtubB have been claimed to form tubes containing 5 protofilaments and these can be demonstrated in three *Prosthecobacter* species inside cells imaged via cryoelectron microscopy (Fig. 3), apparently associated with the *Prosthecobacter* cell stalk region or connection of stalk with the rest of the cell (Pilhofer et al., 2011). Another study on BtubA/B polymerization *in vitro* found formation of 4-filament tubes (Deng et al., 2017).

In the initial study describing tubulins in *Prosthecobacter dejongeii*, it was also noted that a gene for a homolog of the light chain of the eukaryote protein kinesin, a microtubule motor protein, was the next open reading frame downstream of the BtubB tubulin gene in the genome (Jenkins et al., 2002). The Btub genes and this kinesin homolog gene form an operon the genomic environments of which have been argued to support the concept of horizontal gene transfer origin of the operon genes (Pilhofer et al., 2007a). The protein contains a TPR domain but has been proposed to differ in function from a eukaryote kinesin. That this *bklc* gene and its cognate protein Bklc (also called BtubC) (also known as "bacterial kinesin light chain") may be of functional significance to *Prosthecobacter* cell biology is indicated by the discovery that BtubC binds along BtubAB protofilaments at regular 8 nm intervals, inhibits BtubAB depolymerization or "mini microtubule catastrophe", and increases protofilament 'rescue' (Deng et al., 2017) and by the discovery that Bklc (BtubC) interacts specifically not only with Btub protofilaments but also with lipid

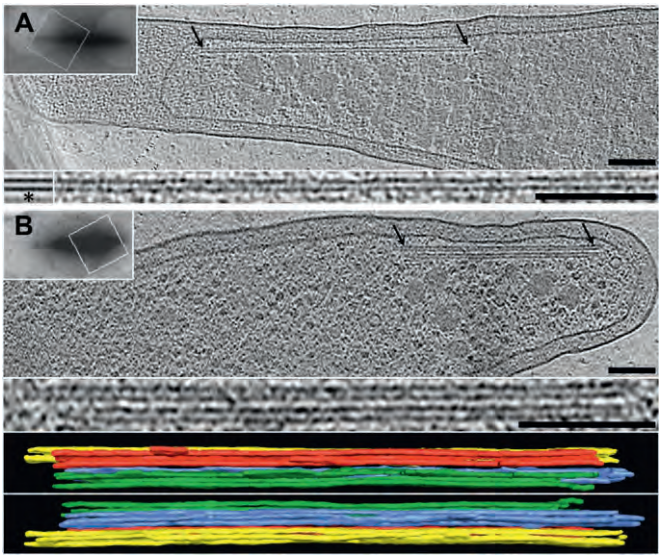

Fig. 3 Electron microscopy/cryotomography of cytoskeletal microtubule-like structures imaged in sliced frozen cells of *Prosthecobacter*. *Prosthecobacter vanneervenii* cells showing tube-like BtubA/B-candidate structures occurring (A) individually or (B) in a bundle, from 11-nm thick slices through cryotomograms. Arrows indicate cytoskeletal structures, which are also shown enlarged below. Asterisk in panel A identifies a sub-tomographic average. Upper-left insets show low-magnification overviews of the cells; rectangles indicate areas imaged in 3-D. Bottom: 3-D segmentation of the bundle of panel B shown from two views (four tubes are present). Scale bars are 100 nm. From Fig. 1 in Pilhofer et al., 2011.

vesicles,(Akendengue et al., 2017) and could thus play a role in anchoring BtubAB filaments to membrane protrusions in *Prosthecobacter*. In the eukaryote cytoskeleton cell biology kinesin is a protein linking vesicle-packaged cargo to microtubules, so a role for kinesin in binding to tubulin protofilaments could suggest a possible precursor stage in evolution of more advanced microtubule function (Akendengue et al., 2017). It is conceivable that tubulins of *Prosthecobacter* perform a function connected with cell division, membrane-attached chromosome segregation or transport of membranes or macromolecules inside the cell, if not with stalk formation as such (since stalks form in cells of *btubAB*-less *P. fluvialis*), but in any case these tubulins and their polymerized mini-microtubules appear to display *in vitro* properties remarkably analogous to those of eukaryotic tubules and their polymers.

Relevant to the possible more widespread occurrence of tubulins in verrucomicrobia is the discovery in verrucomicrobial epixenosome symbionts of protozoans (referred to above) of tubules which are sensitive to nocodazole, an inhibitor of eukaryotic tubulin polymerization, and which react positively with antibodies against eukaryotic tubulins (Petroni et al., 2000). There is not yet genome data indicating homology of any tubulin genes of epixenosomes to eukaryote tubulins. Wider occurrence of tubulins in distinct verrucomicrobial genera would be consistent with the concept of their presence in ancestral verrucomicrobia.

Membrane-Coat (MC) Proteins

Another feature analogous to eukaryotes present in verrucomicrobia is the occurrence of so-called membrane-coat proteins homologous to those in eukaryotes such as the clathrins involved in coated pit formation central to the eukaryote-characteristic process of macromolecule uptake called endocytosis (Santarella-Mellwig et al., 2010).

For example, there are 16, 14, and 9 MC-like protein genes respectively in the genomes of verrucomicrobia of 3 different subdivisions, *Verrucomicrobium spinosum* (subdivision 1) *Chthoniobacter flavus* (subdivision 2) and *Pedosphaera parvula* strain Ellin514 (subdivision 3) (Kant et al., 2011; Santarella-Mellwig et al., 2010). In addition to their occurrence in such verrucomicrobia, these homologs are also found in related PVC phyla *Planctomycetes* and *Lentisphaerae*, but appear to be quite rare among bacterial genomes as a whole (Santarella-Mellwig et al., 2010). The presence of such eukaryote-like proteins is consistent with the presence of internal membrane complexity and potential compartments in planctomycetes and verrucomicrobia, and may be correlated with an endocytosis-like ability to uptake proteins from the external milieu by the planctomycete *Gemmata obscuriglobus* (Lonhienne et al., 2010).

Conclusion/Summary

The phylum Verrucomicrobia is an excellent example of how a divergent group of bacteria can significantly enrich our understanding of the contributions of microorganisms to the ecosystem, to human health, and to understanding evolution of cell biology among divergent forms of cell organization. Before their discovery we had no knowledge of the contribution of this phylum to the gut microbiome, to the soil microbial community, or to the evolution of different forms of cytoskeletal proteins potentially linking ancestors of bacteria and eukaryotes. *Akkermansia muciniphila* may in the future form an important contribution to human medicine and public health, and *Prosthecobacter* species and their tubulin proteins may supply significant insights into the functioning of microtubules within even human cells. Understanding contributions of verrucomicrobia to plant and soil function is important for improved agriculture and understanding of global terrestrial ecosystems. And finally, understanding the full implications of the relationships of verrucomicrobia to related phyla such as *Planctomycetes* and the medically significant *Chlamydiae* may open up new fundamental fields of evolutionary microbiology relevant to understanding cell complexity and how diverse bacterial life cycles have evolved.

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Picoeukaryotes[☆]

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Glossary

Clone library Heterogeneous collection of cloned sequences (often 18S rDNA) derived from a complex assemblage of organisms.
Ecophysiology Physiological properties explaining the ecological adaptation of an organism.
Ecotypes Species varieties with indistinguishable morphology adapted to slightly different ecological niches. Genetic markers can be used to tell them apart.
Environmental molecular surveys Retrieval of genetic signatures from a complex microbial assemblage for diversity studies.
Epifluorescence microscopy Technique that allows observation by fluorescence of very small cells retained on a filter.
Fluorescent in situ hybridization (FISH) Microscopic method for detection of microbial cells by labeling them with a fluorescent probe that specifically labels ribosomes.
High throughput sequencing An emergent set of tools that can produce a huge amount of sequences from several samples at once.
Marine alveolates (MALV) Novel clades that belong to the eukaryote supergroup alveolates, detected in molecular surveys of marine picoplankton.
Marine stramenopiles (MAST) Novel clades that belong to the eukaryote supergroup stramenopiles, detected in molecular surveys of marine picoplankton.
Microbial loop Conceptual scheme stating that a fraction of primary production is released as dissolved organic matter (DOM) and channelized to higher trophic levels through heterotrophic bacteria and protists.
Phylogenetic clade Set of related sequences that originate from a single common ancestor.
Phylogenetics Study of the evolutionary relationships among a set of genes or organisms based on comparisons among a set of shared characters.
Picoplankton Heterogeneous assemblage of organisms $\leq$2–3 μm in size that live suspended in the water column.
Protists General term for eukaryotes not belonging to plants, animals, fungi, or macroalgae, generally single-celled organisms of sizes from 1 μm to more than 100 μm.
Single cell genomics Retrieval of the genome sequence from an individual microbial cell.
18S rDNA Gene encoding the RNA component of the small ribosomal subunit, and widely used to identify and classify organisms.

Abbreviations

ARISA	Automated ribosomal interspacer analysis
BAC	Bacterial artificial chromosomes
ciPCR	Culture-independent PCR
DGGE	Denaturing gradient gel electrophoresis
DOM	Dissolved organic matter
FISH	Fluorescent in situ hybridization
HNF	Heterotrophic nanoflagellates
HP	Heterotrophic picoeukaryotes
HPLC	High-performance liquid chromatography
HTS	High throughput sequencing
MALV	Marine alveolates
MAST	Marine stramenopiles
PNF	Phototrophic nanoflagellates
PP	Phototrophic picoeukaryotes
TEM	Transmission electron microscopy
T-RFLP	Terminal-restriction fragment length polymorphism

[☆]*Change History*: Ramon Massana updated the text throughout the article.

This article is an update of R. Massana, Picoeukaryotes, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 674–688.

Defining Statement

Microorganisms play fundamental roles in marine ecosystems, sustaining food webs and driving biogeochemical cycles. Unicellular eukaryotes smaller than 2–3 μm (picoeukaryotes) are recognized as important members of microbial assemblages in terms of both biomass and activity. They show a large functional and phylogenetic diversity and include many poorly or entirely uncharacterized taxa.

Introduction

What Are Marine Picoeukaryotes?

Marine picoeukaryotes are a heterogeneous assemblage of very small eukaryotic organisms. Although some examples of cultured picoeukaryotes exist, most have only been detected in the last decade using culture-independent sampling techniques and therefore have been poorly characterized. Nonetheless, we now know that picoeukaryotes are ubiquitous throughout the marine environment, occupying a wide variety of habitats. This includes distinct layers of the water column, each with its own dominant biogeochemical regime, marine sediments, and unique ecosystems such as hydrothermal vents.

Salinity is the main parameter that differentiates marine and freshwater habitats, and these habitats are for the most part populated by very different species. Little is yet known about picoeukaryotes in freshwater habitats. Therefore, this article will focus on planktonic marine picoeukaryotes, those that live suspended in the water column, and especially those living in the upper water column where photosynthesis occurs. Since most primary production in marine systems is due to photosynthesis by planktonic microorganisms (cells smaller than 200 μm), the microbial component plays a key role in marine food webs. Among these organisms, picoeukaryotes, many of which have only recently been discovered, are qualitatively and quantitatively important. Marine primary production accounts for roughly half of the Earth's production, indicating that the oceans and their microorganisms are crucial in sequestering inorganic carbon from the atmosphere and potentially in mitigating global change.

Picoeukaryotes have a typical eukaryotic cell structure in a miniaturized state. This includes the presence of a nucleus, an endomembrane system (endoplasmic reticulum, Golgi body, and vesicles), mitochondria, and in the case of photosynthetic picoeukaryotes, a chloroplast. Nonetheless, these cells are extremely small, the diameter ranging from 0.8 μm in the case of *Ostreococcus tauri*, the smallest known eukaryote, to an upper range of 2–3 μm. Due to this small size, picoeukaryotes are largely indistinguishable by light microscopy, the usual method for studying eukaryotic microbial diversity. In addition, few picoeukaryotes have been isolated and characterized.

In 1978 a scheme for classification of marine organisms according to size was delineated largely based on sieving technology. Microorganisms were operationally split into three categories: picoplankton (0.2–2 μm in cell diameter), nanoplankton (2–20 μm), and microplankton (20–200 μm). Initially, the picoplankton was thought to be almost exclusively made up of prokaryotes and the nanoplankton mostly of small single-celled eukaryotes. However, the existence and abundance of protists within the picoplankton size class was soon recognized. Today, the term picoeukaryotes is often used a bit loosely to include protists with a size up to 3 μm. Direct inspections of protist assemblages indicate that the 2 μm limit often falls in the middle of the size spectra and that a more coherent group is delimited using a 3 μm upper boundary.

Picoeukaryotes thus defined (protists smaller than 3 μm) are abundant in the marine plankton. They include diverse phototrophic and heterotrophic cells, and they play crucial roles as primary producers, bacterial grazers, and parasites. In recent years their diversity, abundance, and widespread distribution has begun to be recognized and they are attracting more attention.

Method-Driven History of Marine Picoeukaryotes

Microorganisms are invisible to the unaided human eye, so the history of their study is inevitably linked to the development of new methods for their observation and characterization (Fig. 1). The existence of very small cells in the marine plankton was known from the beginning of the 20th century by phytoplanktologists that inspected concentrated samples of seawater by light microscopy. By the middle of the century the first picoeukaryotes, such as the flagellated *Micromonas pusilla* (formerly, *Chromulina pusilla*), were obtained in pure culture. This and other cultured picoeukaryotes provided the basis for many early microscopic and physiological studies, leading to the description of new species. The easy cultivability of *M. pusilla* allowed the initial estimations of its abundance by the serial dilution method. This showed that it is widely distributed in the marine environment and can reach abundances as high as 10^4 cells mL^{-1}. Despite this particular success with cultured organisms, microbial ecologists soon became aware that the dominant microorganisms were often not easily cultured, so direct inspections of natural samples and culture-independent approaches were still much needed.

Electron microscopy, which had been used on cultured material since the 1950s, started to be applied to inspect natural protist assemblages during the 1970s. Transmission electron microscopy (TEM), which allows the inspection of intact specimens, revealed conspicuous features of nanoplanktonic protists. However, these studies rarely targeted picoeukaryotes. The latter were first detected by TEM in thin sections of centrifuged natural samples in 1982. *M. pusilla* and an unknown prasinophyte (later identified as *Bathycoccus prasinos*) were seen in many coastal and oceanic samples, sometimes being relatively abundant. These observations also

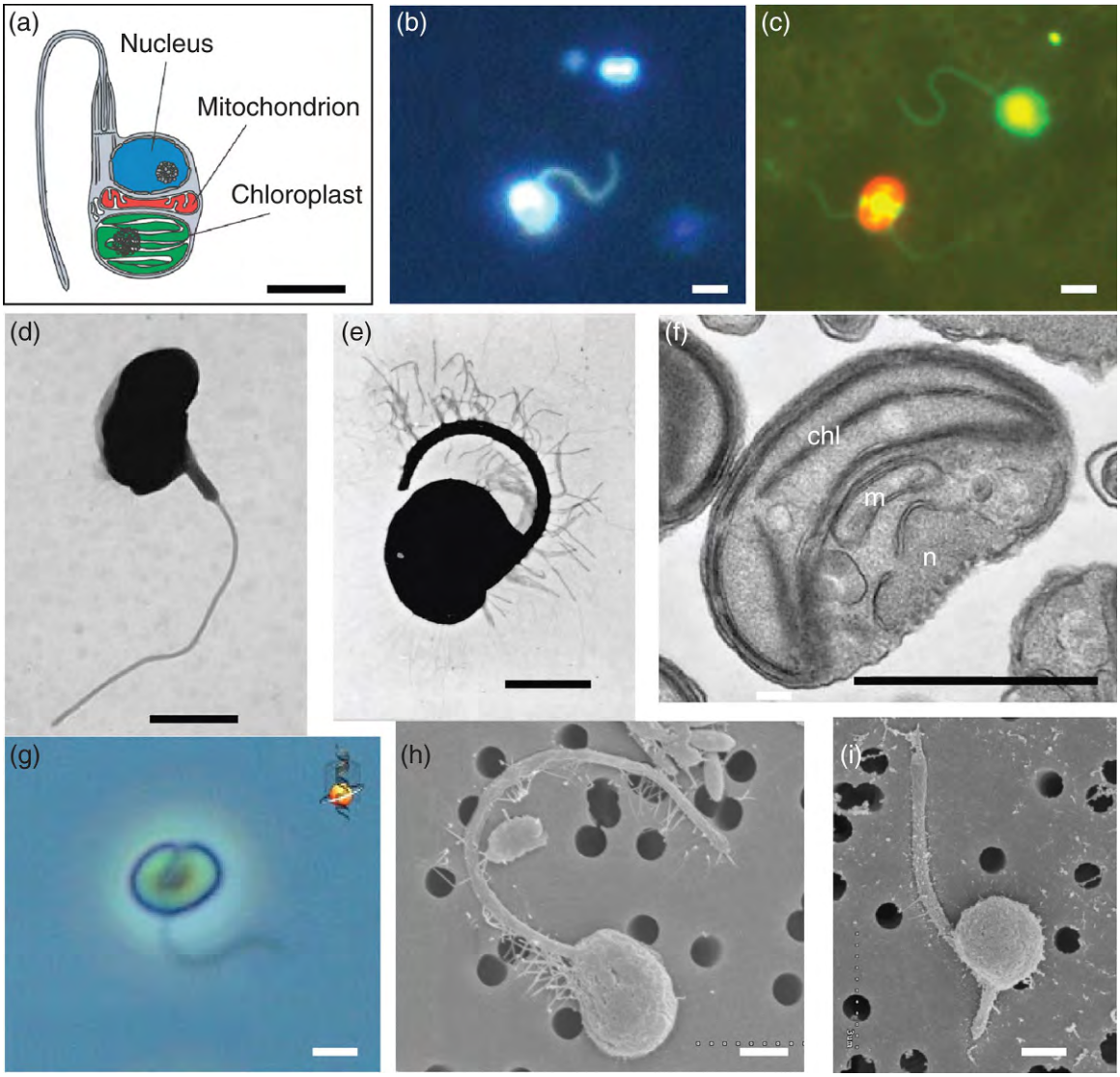

Fig. 1 Examples of marine picoeukaryotes. (a) Drawing of *Micromonas pusilla*. Reproduced from Slapeta, J., Lopez-Garcia, P., Moreira, D., 2006. Global dispersal and ancient cryptic species in the smallest marine eukaryotes. Molecular Biology and Evolution 23, 23–29. (b) Unidentified flagellate seen by epifluorescence under UV radiation after DAPI staining. (c) Unidentified phototrophic (left) and heterotrophic (right) flagellates seen by epifluorescence under blue light. Photo courtesy by Dolors Vaqué. (d) Stained whole mount of *M. pusilla*. Guillou, L., Eikrem, W., Chretiennot-Dinet, M.J.,et al., 2004. Diversity of picoplanktonic prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist 155, 193–214. (e) Stained whole mount of *Symbiomonas scintillans*. Reproduced from Guillou, L., Chrétiennot-Dinet, M.-J., Moon-Van der Staay, S.Y., Boulben, S., Vaulot, D., 1999. *S. scintillans* gen. and sp. nov. and *Picophagus flagellatus* gen. et sp. nov. (Heterekonta): Two new heterotrophic flagellates with picoplanktonic size. Protist 150, 383–398. (f) Thin section through *Ostreococcus tauri* with chloroplast (chl), mitochondrion (m), and nucleus (n). Guillou, L., Eikrem, W., Chretiennot-Dinet, M.J., et al., 2004. Diversity of picoplanktonic prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist 155, 193–214. (g) *Pelagomonas calceolata* by phase contrast. Reproduced from http://chloroplast.ocean.washington.edu/organisms/pelagomonas. (h) and (i) Unidentified flagellates by scanning electron microscopy (SEM). Scale bar=1 μm.

provided the first clue on mortality of picoeukaryotes, since cells often appeared infected with large viruses. Soon after this study, it was shown that a large fraction of marine primary production in offshore regions is due to picophytoplankton (cyanobacteria and picoeukaryotes).

The first accurate counts of marine picoplankton were obtained by epifluorescence microscopy in the early 1980s. This was based on the fluorescence (natural or stain induced) emitted by cells retained quantitatively on the surface of flat polycarbonate filters. Bacterial numbers revealed by this approach were orders of magnitude higher than deduced from previous cultivation-dependent techniques. Protists of different sizes were also evident at abundances in the thousands of cells per milliliter. In addition, phototrophic or heterotrophic protists, which play fundamentally different ecological roles, could be counted separately based on the presence or absence of chlorophyll autofluorescence. Epifluorescence microscopy still remains the method of choice (albeit

time-consuming) for counting heterotrophic picoeukaryotes (HP). Phototrophic picoeukaryotes (PP), on the other hand, can be more easily counted by flow cytometry, a technique imported from biomedicine based on the laser detection of single cells flowing through a small aperture. Flow cytometry was first applied to marine ecology in the 1980s, and was instrumental in the discovery of the most abundant phototroph on Earth, the cyanobacterium *Prochlorococcus*. This tool has been extensively applied to describe the global distribution of marine PP.

The 21st century started with a fair knowledge of the global abundance and distribution of marine picoeukaryotes in the sea and a sizeable collection of characterized cultures. However, there was a remarkable lack of knowledge about which species dominate natural assemblages, and whether or not the cultured strains represented relevant ecological models. This was because the techniques used for identification (culturing, electron microscopy) were not quantitative, and conversely the techniques used for quantification (epifluorescence microscopy, flow cytometry) did not permit species identification.

This changed radically with the advent of molecular tools, particularly culture-independent sampling of ribosomal RNA sequences (phylotypes) from selected environments. These provided exciting and novel results for marine bacteria and archaea during the 1990s, revealing whole new phyla and possibly even kingdoms of organisms. The first studies on the in situ diversity of marine picoeukaryotes by cloning and sequencing environmental 18S rRNA genes were published in 2001. Similar to studies of marine prokaryotes, these revealed new major groups of eukaryotes. Later, specific phylogenetic groups were targeted by fluorescent in situ hybridization (FISH), which allows for directly observing and quantifying natural cells in the environment. The FISH approach was particularly successful for organismal lineages seen in clone libraries but with no known close relative in culture. The use of molecular tools has revealed an unexpected diversity of protists, including the presence of novel groups, suggesting that marine picoeukaryotes represent a large reservoir of unexplored biodiversity. Recently, the implementation of HTS (high-throughput sequencing) tools on large sampling datasets, and on individual cells through SCG (single cell genomics), is providing a new impetus on studies of the ecological and evolutionary significance of picoeukaryotes.

Biology of Cultured Marine Picoeukaryotes

Cultured Strains

Marine picoeukaryotes have been isolated using standard methods, such as mineral media and light for phototrophic cells and rice (or yeast extract) enrichment media for heterotrophic cells, often inoculated with 2–3 μm filtered seawater. To describe a new species, isolates must be characterized by a set of complementary techniques. Optical microscopy reveals the cell shape and motility pattern. Electron microscopy uncovers the cell ultrastructure: architecture of mitochondria, chloroplast, and flagellar apparatus, number, size, and ornamentation of flagella, and presence of external structures. Molecular markers, mostly 18S rDNA but also other genes, allow for phylogenetic placement of new isolates. Biochemical markers, such as pigment analysis by high-performance liquid chromatography (HPLC), storage products, or fatty acid profiles also provide specific information. A subset of these techniques may be used to assign new isolates to a given species, but molecular markers are used most commonly because they are faster and easier to gather. Once formally described, strains are deposited in culture collections, such as the National Center for Marine Algae and Microbiota (formerly the CCMP) in the United States or the Roscoff Culture Collection in France.

Since the first culture of *M. pusilla* was isolated in 1952, there has been some success in isolating additional picoeukaryotes (Table 1). Most are phototrophic and belong to the green algae and the stramenopiles. Cultured green algal picoeukaryotes belong mostly to the class Mamiellophyceae (*Micromonas*, *Ostreococcus*, and *Bathycoccus*), although Pedinophyceae and Trebouxiophyceae also contain small cells. Stramenopile picoeukaryote cultures all belong to novel algal classes, such as Pelagophyceae (1993), Bolidophyceae (1999), and Pinguiophyceae (2002), described recently. The only example of a cultured picophytoeukaryote besides these two divisions is the cryptophyte *Hillea marina*, although this species is still not well characterized.

There are few HP in culture, and most belong to the chrysomonads or the bicosoecids. This scarcity probably reflects the difficulty of isolating and maintaining heterotrophic protists, which usually requires culturing with prey, typically bacteria in the case of phagotrophic picoeukaryotes. A promising approach involves trying to recreate natural conditions during the isolation, by directly adding previously concentrated natural bacteria. This approach has lead to the isolation of the rhizarian *Minorisa minuta*. In addition, there are parasitic protists that release very small heterotrophic cells as free-living dispersal zoospores and can only be maintained in culture with their specific host. For instance, some strains of the alveolates *Amoebophrya* and *Parvilucifera*, parasites of marine dinoflagellates, have zoospores as small as 3 μm.

In addition, some larger cultured species have a minimal cell dimension $\leq$3 μm. These are not strictly picoeukaryotes but would pass through the 3 μm pore size prefilter used to fractionate picoeukaryotes in environmental surveys. Phototrophic species of this size category belong to all classes with picoeukaryotes shown in Table 1 as well as Prymnesiophyceae and some additional stramenopiles (e.g., Bacillariophyceae, Dictyochopyceae, and Eustigmatophyceae). Some of these, such as the Bacillariophyceae (diatoms) and the Prymnesiophyceae, include very important marine phytoplankters. Additional heterotrophic species span the breadth of the eukaryotic tree including cercomonads (supergroup Rhizaria), kinetoplastids and jakobids (supergroup Excavates), choanoflagellates (supergroup Opisthokonts), and apusomonads (unclear affiliation).

Some picoeukaryotic species have been named based on direct observations of natural samples, enrichments, or temporary cultures. Examples are the heterotrophic flagellate *Pseudobodo minimum* (a bicosoecid 2.0 μm in size) and the green alga *Chlorella minima* (1.5–3 μm). Morphological descriptions can be accompanied by ultrastructural characters obtained by electron microscopy,

Table 1 Some examples of marine picoeukaryotes in culture, including the cell size (minimal and maximal length), trophic mode, and where the culture is deposited

Taxonomic group	*Species*	*Size (μm)*	*Trophic*	*Culture collection*
Green algae				
Pedinophyceae	*Marsupiomonas pelliculata*	3.0–3.0	P	PCC
	Resultor micron	1.5–2.5	P	SCCAP
Mamiellophyceae	*Bathycoccus prasinos*	1.5–2.5	P	RCC, NCMA
	Dolichomastix lepidota	2.5–2.5	P	
	Micromonas pusilla	1.0–3.0	P	RCC, NCMA
	Ostreococcus tauri	0.8–1.1	P	RCC, NCMA
Prasinophyceae	*Picocystis salinarum*	2.0–3.0	P	RCC, NCMA
Trebouxiophyceae	*Chlorella sp.*	2.0–3.0	P	RCC
	Picochlorum eukaryotum	3.0–3.0	P	RCC, NCMA
Cryptophyta				
Cryptophyceae	*Hillea marina*	2.0–2.5	P	
Stramenopiles				
Bicosoecida	*Caecitellus pseudoparvulus*	2.0–3.0	H	RCC
	Cafeteria roenbergensis	3.0–3.0	H	RCC
	Symbiomonas scintillans	1.2–1.5	H	RCC, NCMA
Bolidophyceae	*Bolidomonas pacifica*	1.0–1.7	P	RCC, NCMA
	Triparma laevis	2.0–3.0	H	RCC,
Chrysophyceae	*Picophagus flagellatus*	1.4–2.5	H	RCC, NCMA
Pelagophyceae	*Aureococcus anophagefferens*	1.5–2.0	P	RCC, NCMA
	Pelagomonas calceolata	2.0–3.0	P	RCC, NCMA
Pinguiophyceae	*Pinguiochrysis pyriformis*	1.0–3.0	P	RCC, MBIC
Rhizaria				
Chlorarachniophyceae	*Minorisa minuta*	1.0–3.0	H	

Abbreviations: H, heterotrophic; MBIC, Marine Biotechnology Institute Culture Collection; NCMA, National Center for Marine Algae and Microbiota; P, phototrophic; PCC, Plymouth Culture Collection; RCC, Roscoff Culture Collection; SCCAP, Scandinavian Culture Centre for Algae and Protozoa.

such as for the cercozoan *Phagomyxa odontellae* (a diatom parasite with zoospores of 3–4 × 2–3 μm) or the Parmales. The latter was an intriguing algal group, commonly observed in the sea thanks to their covering by siliceous plates. Although initially classified within the class Chrysophyceae, a few parmales species have been recently isolated and phylogenetically placed within the class Bolidophyceae.

Cellular Organization

Marine picoeukaryotes are miniaturized unicellular organisms that nonetheless retain all typical eukaryotic subcellular structures. They mostly divide asexually, a common feature in many protists, and a complete life cycle with the presence of a sexual phase is presumed, yet generally uncharacterized. The algal class with most cultured picoeukaryote species is the Mamiellophyceae. These cells have a single chloroplast (often with a starch granule) with chlorophyll b and prasinoxanthin as the main accessory pigments, one mitochondrion, and one Golgi body. The three most common cultured genera illustrate the variability within the class. *B. prasinos* lacks flagella and is covered by spider web-like organic scales. *M. pusilla* is naked, has a single flagellum with a short wide base and long thin distal end and a distinct characteristic swimming behavior. *O. tauri* is coccoid, nonmotile, and naked. For each of these, strains with indistinguishable ultrastructural features have been isolated from distant geographic sites. For *B. prasinos* these strains appear to be genetically similar, but there is a clear genetic structure among *M. pusilla* (at least five clades) and *O. tauri* (at least four clades) strains. It has been proposed that these clades can be viewed as ecotypes with specific adaptations to their environments (see later). The remaining picoeukaryotic green algal species show similar minimal cell structure but have different flagellar architecture, pigment signatures, and swimming behavior.

The second group with a significant number of cultured picoeukaryote strains is the stramenopiles. This is a vast and extremely diverse clade of autotrophic and heterotrophic taxa, most of which have two unequal flagella, the longer being covered by tripartite hairs that reverse the thrust from the flagellum. Photosynthetic stramenopiles have chlorophyll c as the main accessory pigment and the chloroplast is surrounded by an endoplasmid reticulum continuous with the outer nuclear membrane. Picostramenopiles have a simplified cell structure, with a single mitochondrion, one chloroplast, and one Golgi body. *P. calceolata* is covered by a thin organic theca and has only one flagellum with two rows of bipartite hairs, and even lacks the basal body of the second flagellum. Its main carotenoid is 19′-butanoxylofucoxanthin. *Bolidomonas pacifica* is naked and has two unequal flagella, the longer with tubular hairs similar to those of *P. calceolata*. It can swim vigorously, up to 1.5 mm per second, and contain fucoxanthin as the main carotenoid, like diatoms. Within the same class, *Triparma leavis* share some cell features and

pigment composition with *Bolidomonas* spp., but also differ considerably by virtue of being unmotile cells covered by silica plates. *Pinguiochrysis pyriformis* is coccoid, naked, lacks flagella, and produces large amounts of polyunsaturated omega-3 fatty acids. HP within the stramenopiles also have a simplified cell structure, with a nucleus, a single Golgi body, 1–2 mitochondria, and no chloroplast. *S. scintillans* is naked and has a single flagellum (and only one basal body) with two rows of tripartite tubular hairs. It contains several endosymbiotic bacteria located close to the nucleus. *P. flagellatus* is naked, has two unequal flagella, the longer with two rows of tripartite hairs, and swims energetically.

Physiological Parameters

Cultured picoeukaryotes provide necessary material not only for ultrastructural, biochemical, and molecular studies, but also for defining physiological properties. Some physiological parameters deal with how fast unicellular organisms use environmental resources, such as inorganic nutrients and light for phototrophs and prey for phagotrophs. For instance, the functional response of a phagotrophic protist relates the ingestion rate with prey availability: at low prey levels, the ingestion rate increases linearly with prey concentration; at medium levels, the rate still increases but not linearly; and at high levels, the rate reaches a maximum. This relationship can be described by several models. The most commonly used is analogous to the Michaelis–Menten equation for enzyme kinetics, which is based on the maximal ingestion rate (Um) and the prey concentration allowing half Um (Km). Both parameters are characteristic of a given species and have interesting ecological implications: Um gives the upper limit of a species grazing capacity and Km roughly indicates the prey concentration at which the species is adapted to live. The uptake of inorganic nutrients by a phototroph can be described by the same equation. The relationship between light and photosynthetic activity, on the other hand, uses a different model to incorporate the inhibitory effect of high irradiances.

The differential use of resources translates into different growth rates. In the case of phagotrophic protists, a similar relationship can be found between prey availability and growth rate, known as numerical response, again modeled by the Michaelis–Menten equation. A crucial parameter for phagotrophs is growth efficiency, the fraction of the food ingested that is converted to biomass. This relates ingestion rates and growth rates and has strong implications for respiration and nutrient remineralization. For phototrophic protists, growth rate is often the activity parameter measured to follow their relationship with inorganic nutrients and light. The plasticity with which protists change their reproductive rates according to the available resources is remarkable.

This simplistic view of resource–activity relationships becomes more complex when taking into account the properties of given resources, providing an additional set of more realistic species-specific physiological parameters. In photosynthetic protists, the quality of light and the chemical state of inorganic nutrients can be important. Accessory pigments can tune the cell to a given region of the light spectrum, whereas membrane transporters, genetically codified, determine the nutrient state that can be used. Heterotrophic protists can choose prey depending on size, phylogenetic composition, surface properties, and motility behavior. Finally, there are other species-specific responses related to environmental parameters, such as temperature. Altogether, physiological parameters for a given species may explain its competitive advantage and success in the environment. Conceptual models illustrating how variations in these parameters may induce similar species (or ecotypes of the same species) to occupy different ecological niches are shown in Fig. 2.

O. tauri represents a good example of ecotype differentiation. Twelve *O. tauri* strains are ultrastructurally indistinguishable but form distinct genetic clades using rDNA sequences. One clade is formed by strains isolated from the bottom of the photic zone (~100 m deep), which grow well at low irradiances but are inhibited at irradiances typical of surface waters. The clade formed by strains isolated from surface waters, on the other hand, represents a high-light-adapted ecotype only growing at surface irradiances. The low-light ecotypes possess additional photosynthetic pigments absent from high-light ecotypes. These different ecotypes, which together are able to exploit a wide range of light levels, might explain the success of *O. tauri* throughout the photic zone. Ecotype differentiation has also been observed in *M. pusilla*, where strains of one clade seem to be adapted to live in polar waters. It is plausible that ecotype diversity is a widespread phenomenon in the microbial world.

The Implications of Being Small

Cell size is the single trait that most influences the physiological and ecological properties of a given organism. Smaller cells, by virtue of their higher surface-to-volume ratio as compared to larger cells, are generally more efficient in resource acquisition and therefore may have higher specific metabolic rates. Very crudely, physiological rates are inversely proportional to body length, the so-called allometric relationship. Thus, picoeukaryotes would be the protists with the highest growth rates and better adapted to oligotrophic conditions. Picoeukaryotes live in an environment with low Reynolds numbers, where their motility is dominated by viscous forces and inertial forces are negligible. This implies that all movements have to be active and that cells do not sink passively. Finally, from an ecological perspective, cell size is the best indicator of the level an organism occupies in the trophic food web. Although there are many exceptions, phagotrophs eat organisms smaller than themselves with a general predator-to-prey ratio of 10:1 (length). So, picoeukaryotes would always be near the base of food webs, and their biomass would arrive at macroscopic trophic levels only after several trophic transfers.

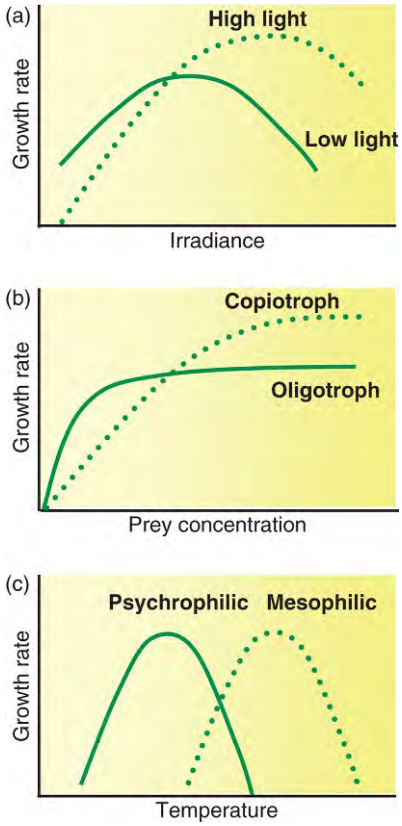

Fig. 2 Conceptual models illustrating how physiological responses induce ecological adaptation. (a) The low-adapted phototrophic ecotype grows better at low irradiances, and the high-adapted ecotype at high irradiances. (b) The oligotroph phagotropic ecotype grows better at low prey concentration and the copiotroph at high levels. (c) The psychrophilic ecotype grows better at cold temperatures and the mesophilic ecotype at warmer temperatures.

Picoeukaryotes in the Marine Environment

Bulk Abundance and Distribution

Very small protists are found in essentially all seawater samples inspected by epifluorescence microscopy. Since these protists were considered nanoplanktonic and flagellated, they are routinely referred to as phototrophic nanoflagellates (PNF) if they contain chlorophyll, and heterotrophic nanoflagellates (HNF) if they are colorless. However, few cells are larger than 5 μm and many are $\leq$2 μm. A recent effort in protist counting and sizing by epifluorescence in contrasting marine systems reveals that 84% of phototrophic protists (between 75 and 91% depending on the system), and 76% of heterotrophic protists (between 64 and 84%) are 3 μm or smaller. So, most PNF and HNF cells would qualify as picoeukaryotes in all marine habitats studied. It should be noted that, due to the absence of flagella in some picophytoeukaryotes, the terms PP and HP are probably more appropriate when referring to the epifluorescence counts of small protists.

There is an extensive database on epifluorescence counts of marine PP and HP. These operationally defined groups are ubiquitous throughout the photic zone at concentrations of thousands of cells per milliliter. PP are generally the most abundant picoeukaryotes and show a large variability, with cell counts typically increasing with the trophic status of the sample. Conversely, HP are several times less abundant than PP but vary only moderately across systems, generally less than one order of magnitude, and are often correlated with bacterial abundance. In coastal systems, typical ranges for PP are 1.1–8.5$\times10^3$ cells mL^{-1}, with episodic peaks well above 10^4 cells mL^{-1}, whereas HP concentrations typically vary between 0.6 and 3.1$\times10^3$ cells mL^{-1}. Cells are less abundant in offshore, more oligotrophic systems: typical PP concentration ranges from 1.0 to 3.3$\times10^3$ cells mL^{-1} and HP ranges from 0.6 to 1.5$\times10^3$ cells mL^{-1}. For example, in offshore Indian Ocean samples, average cell counts were 1.7$\times10^3$ cells mL^{-1} for PP and 0.45$\times10^3$ cells mL^{-1} for HP. The ubiquity, abundance, and constancy of picoeukaryotes suggest they are important players in the photic zone and that their growth and mortality rates are tightly coupled.

Understanding of the patterns of PP distribution has been considerably expanded by the semiautomatic counts provided by flow cytometry. Inspection of marine samples by this technique reveals an assemblage of photosynthetic picoeukaryotes, generally at thousands of cells per milliliter, yielding counts consistent with those of PP counted manually by epifluorescence. Flow cytometry is routinely used in oceanographic cruises and monitoring programs and allows direct comparisons of picophytoplankton groups.

Results indicate that PP generally covary with Synechococcus and dominate in coastal waters, whereas these protists are less abundant offshore where *Prochlorococcus* dominates. Flow cytometry has also been used to quantify HP cells, but the current approach provides data that is too noisy (large prokaryotes can be counted as HP), so this is not yet a standard tool in oceanography.

Photosynthesis does not occur below the photic zone, in the mesopelagic (200–1000 m deep) and bathypelagic (1000–4000 m) regions; so this extensive biome is largely devoid of photosynthetic protists. Nevertheless, bacterial production can occur in these deep waters based on sedimenting organic matter from the photic zone and on chemolithoautotrophic bacteria gathering their energy from reduced inorganic compounds. However, both bacterial abundance and production is still 1–2 order of magnitude lower here than at the surface, and the numbers of heterotrophic picoeukaryotes as seen by epifluorescence microscopy are also lower, ranging between 10 and 100 cells mL^{-1}.

Ecological Role of PP

It has long been known that microorganisms are responsible for most of the marine primary production. However, prior to 1983 we were unaware of the importance of picoplankton in this crucial process. Picophytoplankton generally dominate primary production in offshore, oligotrophic systems, and can also be important in coastal systems on a seasonal basis. For instance, picoplankton averaged 71% of photosynthetic biomass and 56% of primary production during four Atlantic Ocean cruises (50 degree N–50 degree S) crossing coastal, upwelling, and central oceanic regions. Picocyanobacteria, specifically *Synechococcus* in nutrient-rich and *Prochlorococcus* in oligotrophic regions, can reach abundances of up to 10^5 cells mL^{-1}, so they were initially thought more important in supporting food webs than picoeukaryotes. However, albeit less abundant, picoeukaryotes are larger (their biovolume can be 100 times that of picocyanobacteria), so they can contribute significantly to primary production. For example, a study in the central North Atlantic indicated that while only 10% of the surface picoplankton were eukaryotes, these contributed 61% of the biomass and 68% of the primary production.

The size spectrum of marine primary producers strongly influences the food web complexity and has crucial implications for transfer efficiency to higher trophic levels. Large cells such as diatoms and large dinoflagellates can be readily consumed by copepods and directly sustain fish larvae. However, primary production based on very small cells has to pass through several trophic steps (flagellates, ciliates) and a significant fraction of this organic matter is respired at these lower levels. Thus, even though picoeukaryotes appear to be optimal food for small predators, systems largely reliant on their production are less efficient in sustaining higher trophic levels. Other mechanisms, such as viral lysis, also potentially diminish trophic efficiency by causing direct remineralization of picoeukaryotic biomass. The role of viruses as mortality agents in picoeukaryotes (and other algae) remains poorly explored.

The magnitude of the ocean's biological pump (sequestration of atmospheric inorganic carbon to the deep ocean by biological export) is also considered to be heavily influenced by the cell size and species composition of phytoplankton. That is, larger cells are expected to contribute more to inorganic carbon sequestration. This is because larger cells, often with mineralized structures, can sediment directly (alone or in aggregates) or be readily consumed by copepods that excrete fecal pellets that sink out of the surface mixed layer. However, recent data suggest that picoplankton may also contribute to carbon export from the surface at a level proportional to its net primary production.

Ecological Role of HP

HP are mostly phagotrophs and are central in the microbial loop, a concept that has driven a fundamental revision of models of marine food webs. The microbial loop is based on the premise that there is a substantial supply of dissolved organic matter (DOM) created by phytoplankton exudation or inefficient zooplankton feeding that supports active bacterial production. These active bacteria are grazed upon by small protists that in turn are food for larger grazers. So, incorporated into the classical food web (phytoplankton, copepods), there is a microbial loop (DOM, small grazers, copepods) that potentially transfers energy from dissolved pools to higher trophic levels. The reality is closer to a web than a loop, since small grazers also consume picophytoplankton, often the dominant producers. The main grazers of bacteria and picophytoplankton are picoeukaryotes, as seen by microscopic inspections of protists with ingested bacteria and by size fractionation experiments showing that most bacterial grazing occurs in the fraction below 3 μm.

Grazing by HP represents an important mortality factor for bacterial assemblages over large oceanic scales. Other mortality agents like viruses may contribute in coastal systems but seem less important in oligotrophic regions. Mixotrophic protists, cells capable of both phagotrophy and photosynthesis, can contribute to half of bacterial mortality in both coastal and offshore systems. Bacterial grazing, by either heterotrophic or mixotrophic protists, is the first of a multistep food chain, during which bacterial production is mostly respired and inorganic nutrients bound to bacterial biomass (often enriched in P and N) are released. Thus, the main ecological roles of phagotrophic picoeukaryotes are controlling bacterial (and other picoplankton) abundances and remineralizing inorganic nutrients in the photic zone, allowing sustained primary production in oligotrophic systems.

HP may have other nutritional modes besides phagotrophy. Strictly osmotrophic protists are generally poor competitors to heterotrophic bacteria, which seem more efficient in using the diluted and refractory marine DOM. Nevertheless, there may be particular habitats (enriched coastal sites, the deep ocean) where eukaryotic osmotrophs may make a living. Also, the existence of

phagotrophic protists that can supplement their diet with DOM is plausible and ecologically relevant. Parasitism has long been known to occur in the sea, and there are many descriptions of parasites infecting protists (e.g., diatoms, dinoflagellates) and metazoans (e.g., copepods, crabs). These parasites always have a free-living dispersal form (zoospore), colorless and often very small, which would be considered as HP. Parasitism is fundamentally different from phagotrophy because the parasite is typically much smaller than the host, has a limited host range, and does not always kill its host. Given the high diversity of microbial assemblages and the dilute environment in which they live, parasitism was not considered as a major process in the marine plankton. However, recent environmental molecular surveys (see Section In Situ Phylogenetic Diversity) reveal many sequences of putatively parasitic protists retrieved from the picoplankton, even at the more oligotrophic stations studied. The ecological relevance of parasitism in coastal and offshore microbial assemblages is an open field for future research.

Molecular Tools to Study Picoeukaryote Ecology

Elusive View of In Situ Diversity by Nonmolecular Tools

Despite the ecological importance of picoeukaryotes as primary producers, bacterial grazers, and parasites, it is remarkable how little we knew about in situ diversity before the application of molecular tools. This is because picoeukaryotes cannot be identified by the techniques that provide accurate cell counts, such as epifluorescence microscopy or flow cytometry. Specific ultrastructural features can often be revealed by electron microscopy, but this cannot be applied routinely to all cells of an assemblage, although sometimes it has served to identify dominant species, such as *Micromonas* and *Bathycoccus*.

Culturing is an excellent approach to obtain biological models (see Section Biology of Cultured Marine Picoeukaryotes). However, many cells do not grow easily in the lab, so culturing provides a severely biased view of microbial diversity. In a few cases, there are dominant populations in the sea that are also easily cultured, so they can be counted by diluting and culturing, yielding a most probable number. An excellent example is the mamiellophyte *M. pusilla*, which is widely distributed in the sea in numbers ranging from 10^2 to 10^5 cells mL^{-1}, being more abundant in coastal areas but also found in the oligotrophic open sea.

Pigment analysis by HPLC yields information on the composition of marine phytoplankton, since each algal group has a specific pigment signature, and has often been used to compare samples during oceanographic cruises. When applied to samples prefiltered through 3 μm, it can provide a general view of picophytoplankton composition. Since the pigment profile derives from a complex assemblage, algorithms need to be used to infer the contribution of different algal classes to total chorophyll a (the only pigment common to all phytoplankters). However, translating pigment profiles to diversity depends on the pigment ratios generated in cultured species, which may not accurately represent natural populations, and even then only provides identification to an algal class, not to lower ranks such as genus or species.

Cloning and Sequencing Environmental Genes

Molecular tools were introduced in marine microbial ecology relatively recently. The most widely used gene for these studies is the SSU rDNA, which codes for the small subunit ribosomal RNA (16S rDNA in prokaryotes, 18S rDNA in eukaryotes). This gene has the distinct advantages that it codes for essentially the same function in all organisms, it has both highly conserved and variable regions, and its product (rRNA) is present in many copies in the ribosomes of all living cells. SSU rDNA has been used to delimit the three domains of life and to classify organisms within a given class, genus, and species. The basis of molecular protist ecology is identifying cells in situ by directly retrieving their rDNA. This is achieved by extracting DNA from marine picoplankton assemblages, amplifying the 18S rRNA genes by PCR, cloning and sequencing the PCR products, and comparing the sequences with SSU rDNA databases.

The first culture-independent PCR (ciPCR) studies on marine picoeukaryotes appeared in 2001. These showed that picoeukaryotes are extremely diverse, so the indistinguishable cells seen by epifluorescence hide phylogenetically different organisms. In fact, environmental picoeukaryote sequences are scattered throughout the eukaryotic tree (Fig. 3). Furthermore, while some of these sequences match well-known species, others form phylogenetic clades (sets of related sequences) that represent novel and unexplored diversity. Studies from widely separated sites often show similar phylogenetic clades, suggesting that few biogeographic barriers exist in the marine realm and similar protists thrive when conditions are similar. Besides 18S rDNA, other genes have been used for similar purposes, such as the chloroplast genes 16S rDNA, rbcL, and psbA. These target only the phytoplankton, and complement and expand the results obtained with the 18S rDNA.

The cloning and sequencing approach is critical to unveil novel microbial diversity but is severely limited in its ability to reveal the true community composition. First, samples are obtained by sequential filtration (through 3 and 0.2 μm pore size filters), and small cells can break during the process and be lost from the picoplanktonic sample. Organisms larger than 3 μm, on the other hand, can break into small fragments or squeeze through pores and be collected as picoplankton. This surely explains the finding of metazoan and ciliate sequences in picoplanktonic clone libraries. Second, some species can be more resistant to DNA extraction than others, depending on their cell structure and outer layers. Third, the required PCR step may bias the original gene abundance, both by primer mismatches and by preferential amplification in some groups. Finally, the rDNA copy number varies widely between eukaryotic species, from one to several thousands, and this surely influences clonal representation. Thus, 18S rDNA clone libraries are fundamental and informative but do not completely describe in situ protist diversity.

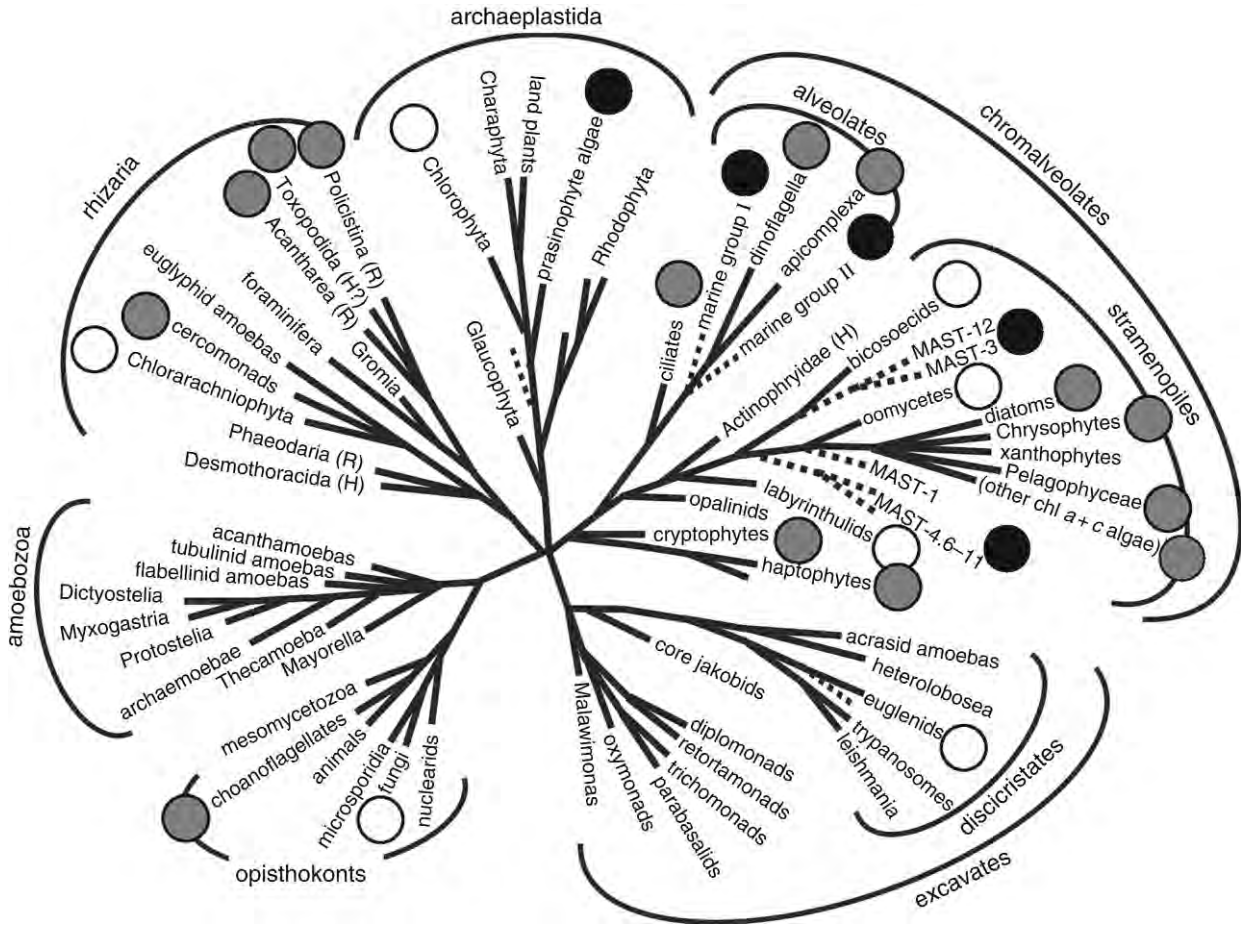

Fig. 3 Representation of eukaryotic phylogeny (modified from Baldauf, S.L., 2003. The deep roots of eukaryotes. Science 300, 1703–1706). Groups found in picoplankton 18S rDNA libraries are marked with a dot, colored depending its averaged clonal representation: black (>10%), gray (1–10%), and white (<1%).

Beyond Clone Libraries: FISH and Fingerprinting Techniques

After environmental sequencing has identified dominant phylogenetic groups, their abundance and distribution in the sea can be assessed by FISH, a technique that elegantly complements the rRNA approach. FISH also reveals critical features of novel phylogenetic clades such as cell size, thus confirming whether they are picoeukaryotes. During the FISH process, fixed microbial cells immobilized on a filter are hybridized with a taxon-specific 18S rDNA probe labeled with a fluorochrome. So, target cells fluoresce under epifluorescence microscopy. A crucial step in FISH is probe design, the selection of a short DNA sequence (around 20 nucleotides) specific for a given group, be it a species, genus, class, or domain. For probes targeting a novel clade, no culture is available in which to optimize hybridization conditions, so instead a natural sample containing these sequences can be used. Once the FISH protocol is optimized, it is relatively easy to apply, albeit time-consuming. FISH has been successfully used for several groups of marine picoeukaryotes, such as mamiellophytes, stramenopiles, and picozoa. It has provided data on their cell size and abundance, thus giving unambiguous estimates of their contribution to the bulk picoeukaryote assemblage. Presently, the FISH approach is mostly limited by the probes available, so major efforts in probe design and optimization are required.

Fingerprinting techniques are very popular in microbial ecology because they allow a fast comparison of the community composition among samples. They provide a characteristic fingerprint for each sample, typically a banding pattern where each band represents a specific taxa. The most commonly used techniques are denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP), and automated ribosomal interspacer analysis (ARISA). All start with a PCR amplification of rDNA genes from the assemblage in question using general primers. Individual sequences in this mixture are then separated by electrophoresis based on their melting domains (DGGE), the location of their first restriction site (T-RFLP), or the size of their ITS region (ARISA). Fingerprinting techniques are used to reveal spatio-temporal patterns of picoeukaryotic assemblages in the sea. Picoeukaryotic assemblages appear to have a relatively wide distribution, with large oceanic areas with similar composition, but they also show significant changes with depth, along onshore–offshore gradients and across frontal regions. Fingerprinting techniques are also very useful to follow the protist dynamics during manipulative experiments.

High-Throughput-Sequencing: A Tsunami of Sequences With Ecological Significance

The technology for massive parallel sequencing has appeared recently and has been applied to many scientific disciplines, including microbial ecology. HTS methods (initially 454 pyrosequencing and, subsequently, Illumina-based systems) allows obtaining thousands to millions of sequences from several samples in the same sequencing run. The big advantage of HTS tools in molecular diversity surveys is the huge number of reads obtained, obviating the time-consuming cloning step needed in Sanger sequencing. On the other hand, the main challenge of HTS is the bioinformatic analysis that is necessary to extract relevant and useful information from these large datasets, with the underlying concept being to group sequences with comparable phylogenetic meaning. So, individual reads are collapsed in OTUs (Operational Taxonomic Units) at a given level of similarity and one sequence is kept as representative of the OTU, which is then classified taxonomically. The resulting OTU table (number of reads of each OTU taxonomically classified in all samples analyzed) will serve to investigate the community structure and alpha diversity of a given sample, how this diversity changes among samples, what are the driving factors for these changes, and other particular aspects as the contribution of novel taxa to community structure or the magnitude and significance of the rare biosphere.

In Situ Phylogenetic Diversity

Overview of 18S rDNA Sequences From Marine Picoeukaryotic Communities

The phylogenetic diversity contained within marine picoeukaryotes is astonishing, as the majority of eukaryotic lineages are represented in 18S rDNA molecular surveys (Fig. 3). Some of the retrieved sequences are very similar to cultured picoeukaryotes (Fig. 4(a)). Others clearly affiliate within a given group but are distantly related to its cultured representatives, so likely represent new species or genera (Fig. 4(b)). Other sequences show a profoundly deep distance from all cultured organisms and can only be unambiguously affiliated to a supergroup, such as the marine alveolates (MALV)-I and -II (Fig. 3) and the marine stramenopiles or MAST (Fig. 4(c)). Finally, in a few cases the retrieved sequences form a novel high-rank taxonomic entity that cannot be assigned to any given supergroup, for example, the picozoa.

A summary of the clonal representation in 18S rDNA libraries published until 2008 from surface samples is shown in Table 2. These libraries derive from Atlantic, Pacific, Indian, and Southern oceans and Mediterranean and North seas (35 libraries and 2175 clones). The better-represented phylogenetic groups are alveolates (42.5% of clones), stramenopiles (22.8%), and chlorophytes (15.0%). Some groups are more abundant in coastal libraries (ciliates, MALV-II, cryptophyta, chlorophyta, and cercozoans), whereas others are more abundant in offshore libraries (MASTs, pelagophytes, prymnesiophytes, and radiolarians). Recent work using HTS approaches and using a large collection of samples from the open ocean (Tara Ocean project) or European coastal sites (BioMarKs project) are essentially confirming and expanding the information already gathered by clone libraries. The different levels of phylogenetic novelty shown in Fig. 4 are discussed below.

Relatively Well-Known Groups

Mamiellophytes show the best correspondence between molecular and culturing approaches. They are well represented in both approaches, and environmental sequences are often identical to that of cultured cells, particularly from *M. pusilla*, *O. tauri*, and *B. prasinos*. Using FISH probing, *M. pusilla* has been found to be very abundant in coastal systems (up to 10^5 cells mL^{-1}), supporting previous views from culturing, electron microscopy, and pigment analysis. It appears that a substantial fraction of picoeukaryotes in

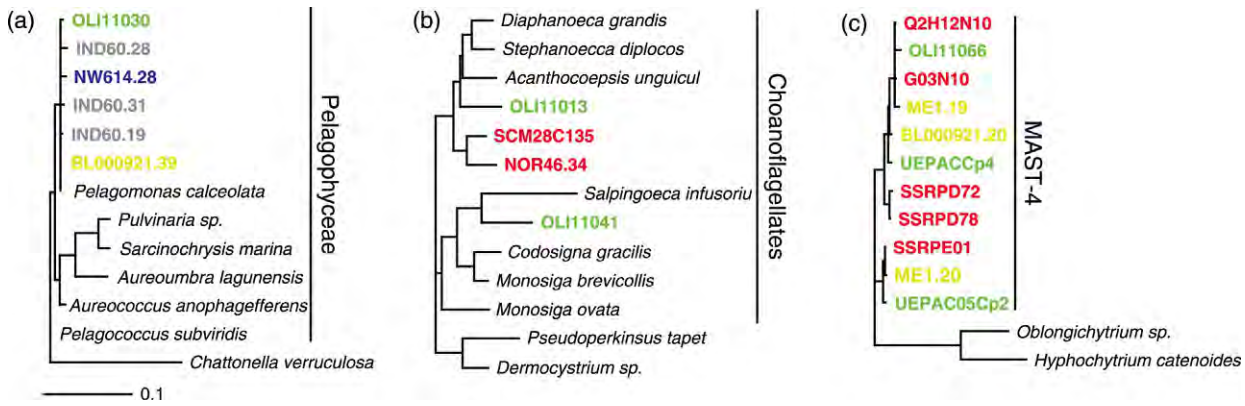

Fig. 4 Pelagophytes (a), choanoflagellates (b), and MAST-4 (c) phylogenetic trees with 18S rDNA sequences from the marine picoplankton and closest cultured representatives. Environmental sequences are colored depending on their origin: Atlantic (red), Pacific (green), Indian (gray), Arctic (blue), and Mediterranean (yellow).

Table 2 Clonal representation of phylogenetic groups in 18S rDNA libraries prepared from surface picoplankton from coastal (23 libraries and 1349 clones from Mediterranean, North Sea, English Channel, and Pacific Coast) and offshore (12 libraries and 826 clones from the Mediterranean Sea, Antarctica, and Indian, Pacific, and Atlantic oceans) systems

Chromalveolates		*% Total*	*% Coast*	*% Offshore*	*Tendency*
Alveolates	Ciliates	4.9	7.6	2.7	Coastal
	Dinoflagellates	5.3	6.1	4.7	
	MALV-I	14.6	13.8	15.2	
	MALV-II	17.6	22.1	14.1	Coastal
Stramenopiles	Chrysomonads	2.7	2.2	3.1	
	Diatoms	2.5	0.6	4.0	
	Dictyochales	1.1	1.5	0.9	
	MASTs	13.4	9.0	17.0	Open
	Pelagophytes	1.0	0.1	1.8	Open
Basal groups	Cryptophytes	2.4	3.6	1.4	Coastal
	Prymnesiophytes	4.5	1.2	7.2	Open
Archaeplastida	Chlorophyta	15.0	19.3	11.6	Coastal
Rhizaria	Cercozoans	2.6	4.8	0.9	Coastal
	Radiolarians	5.6	0.3	9.8	Open
Opisthokonts	Choanoflagellates	1.0	0.9	1.0	
Unassigned	Picozoa	0.9	1.2	0.7	
	Inserta sedis	0.7	0.5	0.9	Open
Remaining groups		4.0	5.3	3.0	

coastal systems are Mamiellophytes (50–90%), and these account for a lower percentage of cells (<20%) in offshore samples. Other picophytoplankton groups with cultured species are poorly represented in clone libraries: only 1% of the clones affiliate with pelagophytes, 0.5% with bolidophytes, and the pinguiophytes are seldom detected. Many pelagophyte sequences are nearly identical to *P. calceolata* (Fig. 4(a)). This species appears to be a widely distributed and significantly abundant marine picoplankton in open ocean systems, as supported by recent FISH probing and HPLC pigment analyses.

Prymnesiophytes are important marine algae and seem to contribute significantly to the picoplankton according to HPLC analysis. Also, many cultured prymnesiophytes have the smallest cell dimension $\leq$3 μm. Nevertheless, their contribution to clone libraries is moderate (4.5%), and the environmental sequences often form novel clades. FISH probing shows that prymnesiophytes are rather abundant in coastal and offshore samples (more than their clonal abundance would suggest), reaching up to 30% of the picoeukaryote assemblage. Other algal groups detected in clone libraries are the dinoflagellates (5.3%), important marine protists with phototrophic and heterotrophic species. Since the smallest dinoflagellates observed are around 5 μm, their molecular signal has been interpreted as filtration artifacts, although the presence of picodinoflagellates cannot be excluded. Similar concerns apply to cryptophytes (2.4% of clones) and diatoms (2.5% of clones), although the latter have very small centric and slim pennate species that would pass the 3 μm prefilter. A few sequences are close to the dictyochophyte *Florenciella parvula* and others affiliate distantly to the chlorarachniophytes.

Libraries show a large diversity of putatively heterotrophic protists at low clonal abundance. Some of these groups contain cultured heterotrophic pico- and nanoflagellates: apusomonads, bicosoecids, cercozoans, chrysomonads, choanoflagellates, katablepharids, and *Telonema*. Other groups are known to contain osmotrophic or parasitic forms, such as apicomplexa, fungi, labyrinthulids, oomycetes, and pirsonids. A significant number of sequences relate to ciliates (4.9%), important components of the nano- and microzooplankton but at least 10 μm in size, so their presence is most likely a filtration artifact. The chrysomonads also show a moderate clonal representation (2.7%) and contain sequences close to well-known heterotrophic flagellates, such as *Paraphysomonas*, as well as novel clades. The trophic mode of these novel chrysomonads is uncertain, but data obtained in plastid rDNA libraries suggest an unexpected importance of chrysomonads within the picophytoeukaryotes. Finally, a substantial fraction of clones affiliate with the radiolarians. This is surprising, since the radiolarian species known so far are rather large (typically between 30 and 300 μm) and most possess mineralized skeletons. They are virtually absent from coastal systems, and reach a significant clonal abundance in the surface of the open sea (9.8%). Moreover, the fraction of radiolarian sequences increases with depth in the water column, and can reach 20–30% of clones at the bottom of the photic zone and below. Marine radiolarian sequences are diverse and form at least five clades, two related to acanthareans, one to polycystinea, and two to taxopodida. Even though radiolarians can produce small swarmers during their life cycle, it is doubtful that the strong signal encountered derives exclusively from these swarmers, so the presence of radiolarian sequences within the picoplankton is still an unresolved enigma.

Marine Alveolates and Marine Stramenopiles

Environmental surveys have revealed novel clades that affiliate to a given eukaryotic supergroup but without a clear affiliation to any of its members. Among these, the MALV and MAST are particularly interesting because they appear in virtually all marine

surveys at high clonal abundance. MALV are divided into five main groups and two of them MALV-I (five clades) and MALV-II (16 clades) have a very high clonal abundance (14.4 and 17.6%, respectively). The first described MALV-II species was *Amoebophrya*, a dinoflagellate parasite, and additional parasite species have been recently published within both groups. So, it now appears that the whole assemblage might consist of parasites of marine organisms. The specific interaction with different hosts could explain their high level of genetic diversity. This opens new avenues for exploration of the role of parasitism as a trophic mechanism in the open sea. In coastal systems, MALV infection has been seen to control the blooms of dinoflagellate species.

MASTs form up to 18 independent clades at the basal part of the stramenopile tree, where all protists are heterotrophic, free-living flagellates, parasites, osmotrophs, or commensals. This suggests these MASTs are heterotrophs, which has been confirmed by FISH for several clades. On average, MAST sequences account for 13.4% of picoeukaryotic clones, and most of this signal is explained by clades MAST-1, -3, -4, and -7. These are colorless protists, with a size from 2 to 8 μm, able to grow in the dark and to ingest bacteria (Fig. 5). MASTs are widely distributed and account for a significant fraction of heterotrophic flagellates. For example, the very small MAST-4 protist (2–3 μm) is found in all samples except the polar ones, averages 130 cells mL^{-1}, and accounts for 9% of heterotrophic flagellates. This shows that still-uncultured groups can be dominant in marine picoeukaryote assemblages and that model cultured organisms might have limited use for understanding how marine ecosystems work.

Novel High-Rank Phylogenetic Groups

Early molecular surveys claimed the discovery of major novel groups at the highest taxonomic rank, in an effort to highlight the potential of this approach. However, it was soon recognized that some of these novel groups were artifacts due to the presence of undetected chimeras, misplacement of fast-evolving lineages, and incomplete representation of cultured strains. Nevertheless, it is clear that some novel high-rank groups are real and form robust and deep phylogenetic clades that cannot be assigned to any known eukaryotic supergroup. These are generally found at low clonal abundance, so probably are not very important ecologically. Instead, their interest resides in their evolutionary novelty. Perhaps the best example is the picozoa, a novel class that can be locally abundant, and for which a representative isolate was obtained in 2013. Another example would be the Rappemonads, described in 2011 in base of 16S rDNA plastid sequences and whose equivalence to the 18S is still unknown. Indeed, there may be many novel high-rank groups awaiting formal characterization.

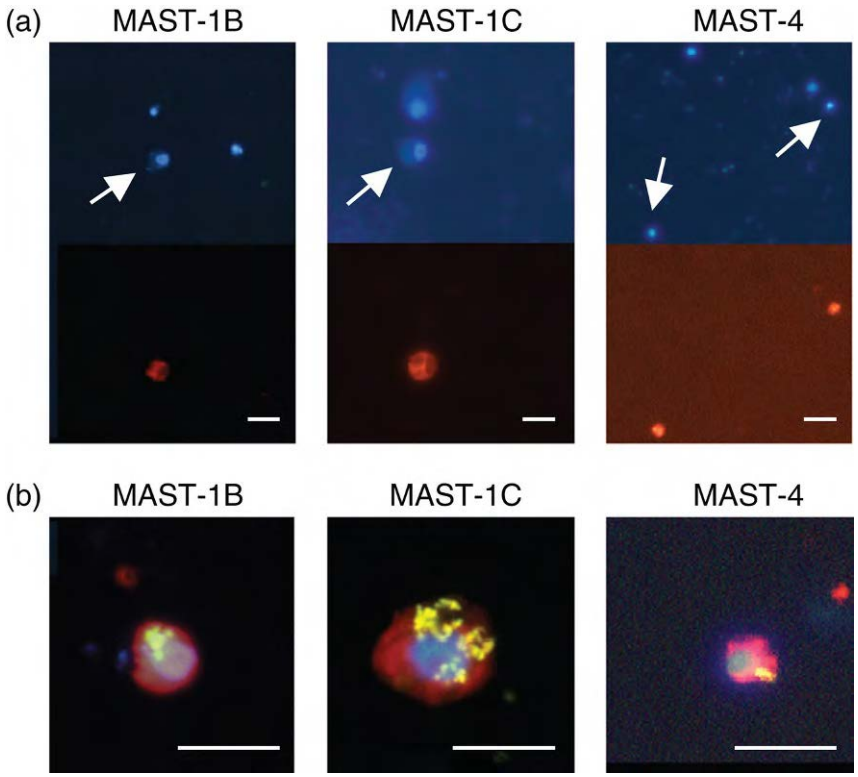

Fig. 5 Epifluorescence images of MAST-1B, MAST-1C, and MAST-4 cells. (a) Same microscopic field observed by UV (upper panels: DAPI-stained blue cells) and green light (lower panels: fluorescent in situ hybridization (FISH)-stained orange cells). MAST cells in the upper panels are indicated with a white arrow. (b) Combination of three images in several MAST cells: DAPI staining (blue nucleus), FISH (orange cytoplasm), and ingested FLBs (yellow spots). Scale bar=5 μm.

Biogeography

There is considerable debate about the extent of diversity and biogeographical distribution of microorganisms. It has been argued that given the huge population sizes and the potential for distant dispersal, most microbial species must be cosmopolitan and the total number of species must be relatively low. This is what is observed using the morphospecies concept to identify protists. However, this concept cannot account for cryptic species, the level of which may not be insignificant among protists, given that the same morphospecies can belong to genetically, reproductively, and ecologically isolated groups. Thus, the use of molecular tools to compare assemblages from distant systems provides more systematic and objective assessment of the extent of protist biogeography and diversity.

Clone libraries from widely separated picoeukaryote assemblages yield similar phylogenetic groups, suggesting that their overall community structure is comparable on a worldwide scale. Further, there appears to be no particular geographic separation as identical 18S rDNA sequences have been retrieved from distant oceans (Fig. 4). Altogether, we find little sign of geographic barriers for picoeukaryotes in the marine environment, and most groups seem to be roughly globally distributed. This supports the cosmopolitan view mentioned above, although finer resolution markers than 18S rDNA might be needed to detect locally adapted populations.

Global distribution has been used as an argument for low protist diversity as it should prevent speciation by geographic isolation or allopatry, the speciation mechanism considered most important. However, most taxonomic groups detected in environmental surveys show a large 18S rDNA sequence variability, thus disagreeing with the view of low protist diversity. The significance of this large intragroup rDNA diversity is presently not understood but can be relevant, since differences as small as 1% in the 18S rDNA might imply millions of years of evolutionary distance. It is also not clear how this large phylogenetic diversity of protists translates into functional diversity in the open sea or how this diversity is generated and maintained. One possibility is to view the seemingly homogeneous pelagic habitat as a continuum of environmental niches. Thus, there is a large genetic diversity of marine protists, which may have a global distribution, but the ecological implications of this large diversity remain to be investigated.

The Genomic Era

Genome Projects on Species of Picoeukaryotes

There has been an increasing effort in recent years to expand databases of genome sequences of microbial species in order to recover a good representation of the eukaryotic tree of life. Seminal projects used cultured species, which were needed to recover enough DNA for genome sequencing, and were often accompanied by parallel transcriptomic projects, i.e., the sequencing of total mRNA. Many of these genomes were derived from species with socio-economic importance like pathogens or parasites, but a number of free-living protists were also analyzed, such as the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, the mamiellophytes *Ostreococcus*, *Micromonas*, and *Bathycoccus*, the green algae *Chlamydomonas reinhardtii*, the ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila* and the choanoflagellate *Monosiga brevicollis*. Each genome was analyzed from a different evolutionary perspective, for example, searching for gene organization, speciation patterns, or the origin of multicellularity. Genomes can also reveal the gene basis of the ecological success. It is recognized that genomic data is pivotal for understanding the cellular function and evolutionary diversity of all living organisms.

The first picoeukaryote to be genome sequenced was *O. tauri*. This minute species has a 12.56 Mb haploid nuclear genome organized in 20 chromosomes. This genome is highly compacted, mainly due to the reduction in size of intergenic regions and the low copy numbers of most genes. The 8166 identified genes include all basic cell functions, such as photosynthesis, central metabolism, and cell–environment interaction. Genes encoding enzymes for C4-photosynthesis have been identified, which may help the cells adapt to the limiting CO_2 concentrations of phytoplankton blooms. There are genes for transport and assimilation of nitrate, ammonium, and urea, and the larger number of ammonium transporters as compared to nitrate transporters indicates that *O. tauri* could be a strong competitor for ammonium, which is uncommon in eukaryotic algae. *O. tauri* also has two chromosomes that differ structurally, being biased toward a lower GC content and a larger number of transposable elements. The first seems to have an alien origin, while the second could represent a sexual chromosome, preventing recombination with similar but not identical strains. Genes related to meiosis have been identified and are apparently functional, suggesting that this protist that usually reproduces asexually may also possess a sexual phase not yet observed but inferred from recombination signatures detected in hypervariable regions of the genomes. Thus, the genome of *O. tauri* follows prediction of compaction that might be driven by its specific lifestyle and ecology.

Genomic projects are no longer limited by the availability of relevant ecological models for culturing. Single cell genomics (SCG) is an emerging technology in biological sciences that has recently become feasible due to the improvements in single cell manipulation, whole genome amplification, high-throughput sequencing and bioinformatics. For the first time, these tools enable the sequencing of genomes of individual cells. SCG exploration of the plethora of ecologically dominant but elusive microbial eukaryotes offer tremendous promises to advance in multiple research fields, including ecology, evolution, and biotechnology. Applying SCG to microbial eukaryotes is still in its infancy, and is particularly challenging when compared to SCG on prokaryotes, because of target genomes of greater complexity (multiple chromosomes, introns, low codon density, high numbers of repeats, large gene paralogue families). Despite these difficulties, analyzing genomes of single picoeukaryote cells will be crucial to obtain the de novo genomes of uncultured lineages and to study the genome variability within natural populations.

Environmental Genomics or Metagenomics

A striking advance in microbial ecology has been the gathering of gene content of natural communities, an approach that is not culture-dependent nor hypothesis-driven. There were initially two main strategies depending on the size of the DNA being cloned. Large DNA fragments (40–200 kb) were cloned in bacterial artificial chromosomes (BAC) or fosmid vectors, and clones with these large inserts were screened before being completely sequenced. This provided complete operon information, and in the case of prokaryotes also linked phylogenetic and functional markers. Alternatively, small DNA fragments (3–5 kb) were cloned by routine methods and randomly sequenced at high throughput, which was known as shotgun sequencing. This provided a large amount of genetic data, but the short sequences obtained were difficult to assemble due to the large diversity of natural assemblages. Today, metagenomic studies are clearly benefiting from the application of HTS, which resulted in a much larger number of shorter sequences, but which also generates corresponding bioinformatic challenges. Metagenomic approaches have been particularly useful for marine bacteria and archaea, to identify previously unknown metabolic pathways, such as novel uses of light and reduced compounds to generate energy, and putatively novel enzymes, antibiotics, and signaling molecules. This approach has not yet been routinely applied to picoeukaryotes, which are prefiltered from most of the analyzed samples. The combination of single gene sequencing (typically the 18S rDNA), isolation in pure culture, single cell genomics and metagenomics offer new opportunities for a complete understanding of picoeukaryote performance in marine ecosystems.

Concluding Remarks

Picoeukaryotes are very small organisms ($\leq$3 μm in the maximal dimension) that are morphologically and structurally simple but with all components required for an independent life. It is fascinating to find all organelles necessary for cell metabolism, growth, and reproduction assembled in such a small package. The few model cultures available so far provide useful tools for ecophysiological and genomic studies. In the marine environment, picoeukaryotes are ubiquitous, account for a significant share of planktonic biomass, and play key ecological roles as primary producers, bacterial predators, and parasites of marine life. The true diversity contained in marine picoeukaryotes has been recently unveiled by the use of molecular tools, which have revealed a very high diversity within this assemblage and the presence of many novel eukaryotes uncharacterized and uncultured. This increase in diversity occurs at almost all possible phylogenetic scales: high-rank novel groups, novel clades within supergroups, and putatively new species, genera, families, and orders within known lineages. The challenge is to retrieve in culture these novel organisms and to determine their trophic role and ecological function. The implication of this large and novel eukaryotic diversity for biodiversity surveys and ecosystem functioning opens new avenues for future research.

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Relevant Websites

http://ncma.bigelow.org/— National Center for Marine Algae and Microbiota.
http://roscoff-culture-collection.org/— Roscoff Culture Collection.

Pigments, Microbial☆

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Glossary

Astaxanthin A reddish carotenoid pigment found in salmon, crustacean shells, and bird feathers (e.g., pink flamingos).
Bacteriochlorophyll A form of chlorophyll present in photosynthetic bacteria.
Carotenoids A group of yellow, orange, or red polyene pigments produced in plants and certain microorganisms. Divided into carotenes (hydrocarbons) and xanthophylls (oxygenated carotenes).
Color The sensation resulting from stimulation of the retina of the eye by light waves of certain lengths.
Melanins High molecular weight polymeric indole quinine pigments having brown or black colors.
Phenazines Compounds based on the tricyclic phenazine ring system, some of which are colored, and are secreted by some species of bacteria.
Pigment Any coloring matter in microbial, plant, or animal cells.

Abbreviations

ACNFP	Advisory Committee on Novel Foods and Processes
ADI	Acceptable daily intake
CWD	Cold water dispersible
DDGS	Distiller's dry grains with solubles
DXP	Deoxyxylulose phosphate
EFSA	European Food Safety Authority
GMP	Good manufacturing practices
GRAS	Generally recognized as safe
HDL	High-density lipoprotein
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LDL	Low-density lipoprotein
OTC	Over-the-counter
SCF	Scientific Committee on Food

Defining Statement

Microorganisms have been used for a long time for production of molecules as diverse as antibiotics, enzymes, vitamins, texturizing agents, and so on. In nature, pigmented bacteria, yeasts, or fungi are quite common, and the main functions of pigments in these living organisms are related to light. The industry is now able to produce some microbial pigments for applications in food, cosmetics, or textile.

Introduction

Nature is rich in colors (minerals, plants, microalgae, etc.), and pigment-producing microorganisms (fungi, yeasts, and bacteria) are quite common (Fig. 1). Among the molecules produced by microorganisms are carotenoids, melanins (Plonka and Grabacka, 2006), flavins, phenazines (Kerr, 2000), quinones, bacteriochlorophylls, and more specifically monascins, violacein, or indigo (Fig. 2; Dufossé, 2004). The success of any pigment produced by fermentation depends on its acceptability in the market, regulatory approval, and the size of the capital investment required to bring the product to market. A few years ago, some expressed doubts about the successful commercialization of fermentation-derived food grade or cosmetic grade pigments because of the high capital investment requirements for fermentation facilities and the extensive and lengthy toxicity studies required by regulatory agencies.

☆*Change History*: June 2016. Laurent Dufossé updated text and references throughout the article.

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Fig. 1 Spontaneous development of pigmented microorganisms at the surface of nutritive agar Petri dish.

Public perception of biotechnology-derived products also had to be taken into account. Nowadays some fermentative food grade pigments are on the market: *Monascus* pigments, astaxanthin from *Xanthophyllomyces dendrorhous* or *Paracoccus carotinifaciens*, Arpink Red (or Natural Red) from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, and β-carotene or lycopene from *Blakeslea trispora*. The successful marketing of pigments derived from algae or extracted from plants, both as a food color and a nutritional supplement, reflects the presence and importance of niche markets in which consumers are willing to pay a premium for "all natural ingredients."

Color plays a special role in the food we eat. For example, when confronted with an unattractive color, the consumer assumes that the food is poor or spoiled. On the other hand, products with atypical color – for example, green cheese or blue drink – in most cases, are rejected by the consumers. Typically, one associates colors with food items, such as cherry with red, lemon with yellow, or carrot with orange. Therefore, colors can serve as the primary identification of food and are also a protective measure against the consumption of spoiled food. Colors of foods create physiological and psychological expectations and attitudes that are developed by experience, tradition, education, and environment: "We inevitably eat with our eyes."

The controversial topic of "synthetic dyes in food" has been discussed for many years (Mapari et al., 2005). The scrutiny and negative assessment of synthetic food dyes by the modern consumer have given rise to a strong interest in natural coloring alternatives. Some companies decided to "color food with food," using mainly plant extracts or pigments from plants, for example, red from paprika, beetroots, berries, or tomato; yellow from saffron or marigold; orange from annatto; green from leafy vegetables.

Penetration of the fermentation-derived ingredients into the food and cosmetic industries is increasing year after year. Examples could be taken from the following fields: thickening or gelling agents (xanthan, curdlan, and gellan), flavor enhancers (yeast hydrolysate and monosodium glutamate), flavor compounds (gamma-decalactone and diacetyl, methyl-ketones), and acidulants (lactic acid and citric acid). Efforts have been made in order to reduce the production costs of fermentation pigments compared to those of synthetic pigments or pigments extracted from natural sources. Innovations will improve the economy of pigment production by isolating new or creating better microorganisms, by improving the processes. This article focuses on research works related to this field published over the past 10 years by private companies or academic laboratories, with an emphasis on pigments for food use. As recently described by our group, there is "a long way from the Petri dish to the market place," and thus to the product on store shelves.

Monascus Pigments – An Old Story for Asians

Monascus Pigment

Monascus is cultivated on solid medium in Asian countries to produce a red colorant named "Anka" used as a food ingredient. In a Chinese medical book on herbs published in the 1st century, this term "ang-kak" or "red mold rice" was first mentioned. Red mold rice has been used as a food colorant or spice in cooking. In 1884, a purple mold was isolated on potato and linseed cakes and was named *Monascus ruber*. This ascomycete was so named as it has only one polyspored ascus. Then in 1895 another strain was isolated from the red mold rice obtained from the market in Java, Indonesia. This fungus was named *Monascus purpureus*. Later, several other species were isolated around the world. *Monascus* is often encountered in oriental foods, especially in southern China, Japan, and Southeastern Asia. Currently, more than 50 patents have been issued in Japan, the United States, France, and Germany, concerning the use of *Monascus* pigments for food. Annual consumption of *Monascus* pigments in Japan moved from 100 t in 1981 to 600 t at the end of the 1990s and was valued at $1.5 million. New food applications, like the coloration of processed meats (sausage and hams) and marine products like fish paste, surimi, and tomato ketchup, were described.

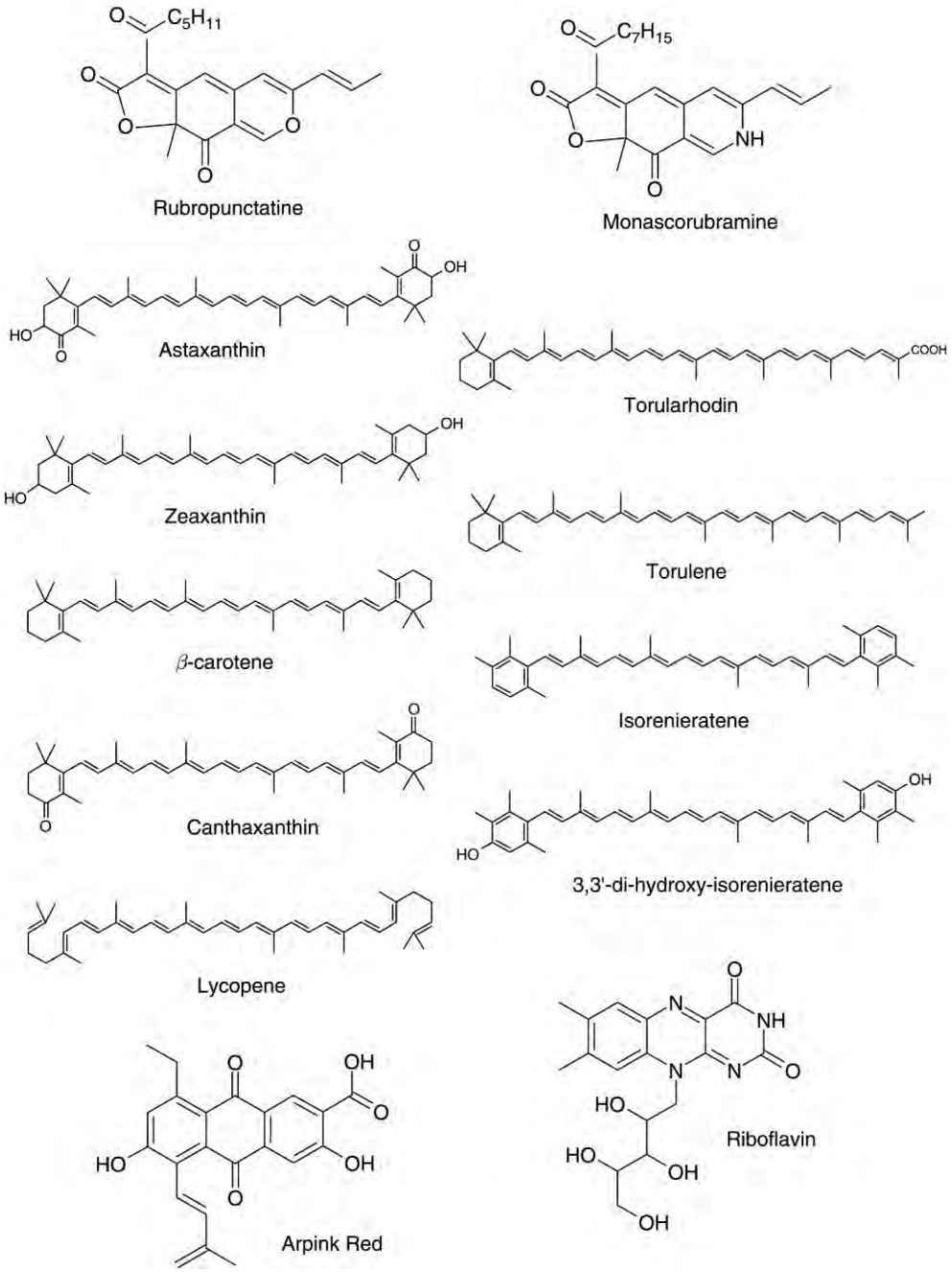

Fig. 2 Chemical formulae of some microbial food-grade pigments.

The Fungus *Monascus*

The genus *Monascus* was divided into three species: *pilosus*, *purpureus*, and *ruber*, which comprise the majority of the strains mainly isolated in oriental food.

The Fungal Metabolites

The main metabolites produced by *Monascus* are polyketides, which are formed by the condensation of one acetylcoA with one or more malonylcoA with a simultaneous decarboxylation as in the case of lipidic synthesis. They consist of the pigments, monacolins, and, under certain conditions, a mycotoxin.

Monascus pigments are a group of fungal polyketide metabolites called azaphilones, which have similar molecular structures as well as similar chemical properties. Two molecular structures of the *Monascus* pigments are shown on Fig. 2. Ankaflavine and monascine are yellow pigments, rubropunctatine and monascorubrine are orange, and rubropunctamine and monascorubramine are purple. The same color exists in two molecular structures differing in the length of the aliphatic chain. These pigments are produced mainly in the cell-bound state.

They have low water solubility, are sensitive to heat, unstable in the pH range of 2–10, and fade with light. A number of methods have been patented in order to make water-soluble pigments. The principle is the substitution of the replaceable oxygen in monascorubrine or rubropunctatine by nitrogen of the amino group of various compounds such as amino acids, peptides, and proteins, changing the color from orange to purple. *Monascus* pigments can be reduced, oxidized, and react with other products, especially amino acids, to form various derivative products sometimes called the complexed pigments. Glutamyl-monascorubrine and glutamyl-rubropunctatine were isolated from the broth of a submerged culture.

Stability of the pigments is affected by acidity, temperature, light, oxygen, water activity, and time. It was shown that these pigments added to sausages or canned pâté remained stable for 3 months' storage at 4°C, while their stability ranged from 92 to 98%. Thus, the main patents have focused on the solubilization, the stability and the extraction in solution of pigments. The pigments can easily react with amino group-containing compounds in the medium, such as proteins, amino acids, and nucleic acids, to form water-soluble pigments.

A series of hypocholesteremic agents has been isolated from *Monascus* and named monacolin J, K, and L. These polyketides were first isolated from cultures of *Penicillium citrinum* and they can inhibit specifically the enzyme controlling the rate of cholesterol biosynthesis. They are currently used in China in traditional and modern medicine.

Antibacterial properties of *Monascus* were first mentioned in 1977. The so-called monascidin A was effective against *Bacillus*, *Streptococcus*, and *Pseudomonas*. It was shown that this molecule was citrinin and its production by various *Monascus* species was studied using different culture media and conditions.

The Production in Various Modes of Cultures

Submerged cultures

Considerable contradiction exists in the published works as to the best carbon source for red pigment production in liquid cultures. Traditionally cultured on breads and rice, *Monascus* grows on every amylaceous substrate. *Monascus* grows quite well on starch, dextrines, glucose, maltose, and fructose. High production of pigments was achieved using glucose and maltose. The nitrogen source seems to have more importance than the carbon source and ammonium, and peptones as nitrogen sources gave superior growth and pigment concentrations compared to nitrate. The best results were obtained using glucose and histidine. The C/N ratio was also shown to be important: at a value close to 50 g g^{-1} growth would then be favored, while in the region of 7–9 g g^{-1}, pigmentation would be favored.

Solid-state cultures

The classical Chinese method consists in inoculating steamed rice grains spread on big trays with a strain of *Monascus anka* and incubating in an aerated and temperature-controlled room for 20 days.

In these types of cultures, moisture content, oxygen and carbon dioxide levels in the gas environment, as well as cereal medium composition, are the most important parameters to control.

Moisture content is a very important parameter. Red pigments were produced in plastic bags containing rice grains. It was observed that pigmentation occurred only at a relatively low initial moisture level (26–32%). Initial substrate moisture content regulated pigmentation as it was found that glucoamylase activity increased along with a rise in initial substrate moisture content. Therefore, at high moisture content, as high enzyme activity was produced, glucose was rapidly liberated in amounts (120 g L^{-1}), which inhibited pigmentation. The sugar was then transformed into ethanol.

As far as cultures on rice are concerned, an optimal pigmentation was found at an initial moisture content of 56%, while lower moisture contents led to a large decrease in pigment formation. Thus, it was confirmed that solid culture was superior to liquid culture for red pigment production by *M. purpureus*. This result has been attributed to the derepression of pigment synthesis in solid systems due to the diffusion of intracellular pigments into the surrounding solid matrix. In submerged culture, the pigments normally remain in the mycelium due to their low solubility in the usually acidic medium.

Levels of oxygen and carbon dioxide in the gas environment influence pigment production significantly while affecting growth to a lesser extent in solid state culture. With *M. purpureus* on rice, maximum pigment yields were observed at 0.5×105 Pa of oxygen partial pressure in closed pressure vessels. However, high carbon dioxide partial pressures progressively inhibited pigment production, with complete inhibition at 105 Pa. In a closed aeration system with a packed-bed fermentor, oxygen partial pressures ranging from 0.05 to 0.5×105 Pa at constant carbon dioxide partial pressures of 0.02×105 Pa gave high pigment yields with a maximum at 0.5×105 Pa of oxygen, whereas lower carbon dioxide partial pressures at constant oxygen partial pressures of 0.21×105 Pa gave higher pigment yields. Maximum oxygen uptake and carbon dioxide production rates were observed at 70–90 and 60–80 h respectively, depending on the gas environment. Respiratory quotients were close to 1.0 except at 0.05×105 Pa of oxygen and 0.02×105 Pa of carbon dioxide partial pressures.

When studying various cereal media, it was shown that the best results were obtained using "mantou" meal (yeast-fermented wheat meal).

Methods Developed to Avoid Mycotoxin Production

In order to identify chemically the so-called monascidin A discussed by some Chinese scientists in their papers as a component suitable for the preservation of food, it was isolated and chemical investigations using mass spectrometry and NMR were undertaken. Monascidin A was characterized as citrinin. Thus, in order to avoid the production of this toxin, various strains were screened in order to see if all were toxinogenic, and it was shown that in the species of *Monascus* available in public collections, nontoxinogenic strains were obtainable.

Another way to avoid the production of citrinin can be through controlling the biosynthesis of the metabolite. To control the biosynthesis, the metabolic pathway has to be investigated. The metabolic pathway is the same for citrinin and the pigment: the polyketide pathway in which condensation of acetates and malonates occurred. In the case of the pigment, there is at the end of the pathway an esterification of a fatty acid on the chromophore to obtain the colored molecules. Several modifications of the culture conditions are possible in order to increase the pigment production or reduce the citrinin one: addition of fatty acids and change of the nitrogen source. Adding fatty acids to the medium was effective in favoring the synthesis of pigment, but the citrinin production remained unchanged.

The final modification of the culture conditions was the replacement of glutamic acid by other amino acids. *M. ruber* was cultivated in a liquid medium containing glucose and various amino acids, and histidine was found to be the most effective nitrogen source regarding citrinin production inhibition. When the pathway of histidine assimilation was investigated, it was shown that during its catabolism, one molecule of hydrogen peroxide was produced per molecule of consumed histidine, and it is known that peroxidases can destroy citrinin in the presence of hydrogen peroxide. So, the production of citrinin can be avoided by control of the medium especially by the selection of a suitable amino acid, usually histidine.

Despite the enormous economic potential of *Monascus* pigment, it does not lead to a commercial exploitation in the Western world, mainly because of ignorance and also reluctance to change from food public agencies. Indeed these agencies do not approve *Monascus* pigments for use in the food industry, although they do appear to be non-toxic if correctly used. Thus, even though species of *Monascus* have been consumed in the Far East for many years, this does not help the pigment to gain approval in the European Union or the United States.

Using next-generation sequencing and optical mapping approaches, a 24.1-Mb complete genome of a *M. purpureus* YY-1 industrial strain has been described for the first time and this will allow huge improvements in the process in the coming years (Yang et al., 2015). It consists of eight chromosomes and 7491 genes. *M. purpureus* should belong to the Aspergillaceae, mainly comprising the genera *Monascus*, *Penicillium*, and *Aspergillus*. Phylogenetic analysis at the genome level provides the first comprehensive prediction of the biosynthetic pathway for *Monascus* pigments. Comparative genomic analyses demonstrated that the genome of *M. purpureus* is 13.6–40% smaller than that of closely related filamentous fungi and has undergone significant gene losses, most of which likely occurred during its specialized adaptation to starch-based foods. Some polyketide synthases (PKS) are expressed at high levels under high pigment yielding conditions. The citrinin PKS C6.123 has also been found in the genome, paving the way for research aiming at non-mycotoxin producing strains, if suppression of the citrinin gene does not change the ability of the strain to produce pigments, which seems to be feasible, as it seems that monascorubrin and citrinin are synthesized by two separate pathways, because, when the PKS gene responsible for synthesis of citrinin was disrupted, red pigment production from the fungus was not affected. Comparative transcriptome analysis revealed that carbon starvation stress, resulting from the use of relatively low-quality carbon sources, contributed to the high yield of pigments by suppressing central carbon metabolism and augmenting the acetyl-CoA pool. As for other pigments produced by biotechnology, the problem is to have enough carbon oriented in the correct pathway, i.e., the pigment pathway.

Monascus-Like Pigments From Non-Toxigenic Fungal Strains

Some species of *Talaromyces* secrete large amounts of red pigments. In the literature, this biosynthetic potential has been linked to species such as *Talaromyces purpurogenus*, *Talaromyces marneffei*, and *Talaromyces minioluteus* often known under their previous *Penicillium* names. However, since some of them do not exert enough stability for pigment production, then such species should be avoided for scale-up production.

Isolates identified as *T. purpurogenus* have been reported to be of industrial interest. They can produce extracellular enzymes and red pigments, but may also produce mycotoxins such as rubratoxin A and B and luteoskyrin in addition to extrolites that may be toxic following intraperitoneal (spiculisporic acid) and intravenal (rugulovasine A and B) injections in cats. Consequently, mycotoxin production may limit the use of isolates of a particular species in biotechnology, and some authors concluded that *T. purpurogenus* may thus not be recommended for industrial production of red pigments. *Talaromyces atroroseus* sp. nov. produces the azaphilone biosynthetic families mitorubrins (Frisvad et al., 2013) and *Monascus* like-pigments without being accompanied by mycotoxin synthesis (patent applications WO2012022765, US 20110250656). As it has been found for *Monascus*, these azaphilone pigments may react with amino groups containing compounds, to which reaction they owe their name, providing intense dark red colors. *Talaromyces albobiverticillius* isolated from Réunion island lagoon, Indian Ocean, is also under investigation.

Arpink Red From *Penicillium oxalicum* – Still a Strange and Unclear Case

Many patents from Ascolor s.r.o. (Czech Republic) relate to a new fungus strain having the properties to produce a red colorant that can be applied in the food and cosmetic industries (WO9950434, CZ285721, EP1070136, US6340586). The strain *P. oxalicum* var. Armeniaca CCM 8242, obtained from soil, produces a chromophore of the anthraquinone type (Figs. 1 and 3). Some strains of the same species are effective as biological control agents, for example, reduction of the incidence of *Fusarium* wilt of tomato under glasshouse and field conditions. Others have been described for production of a milk-clotting enzyme.

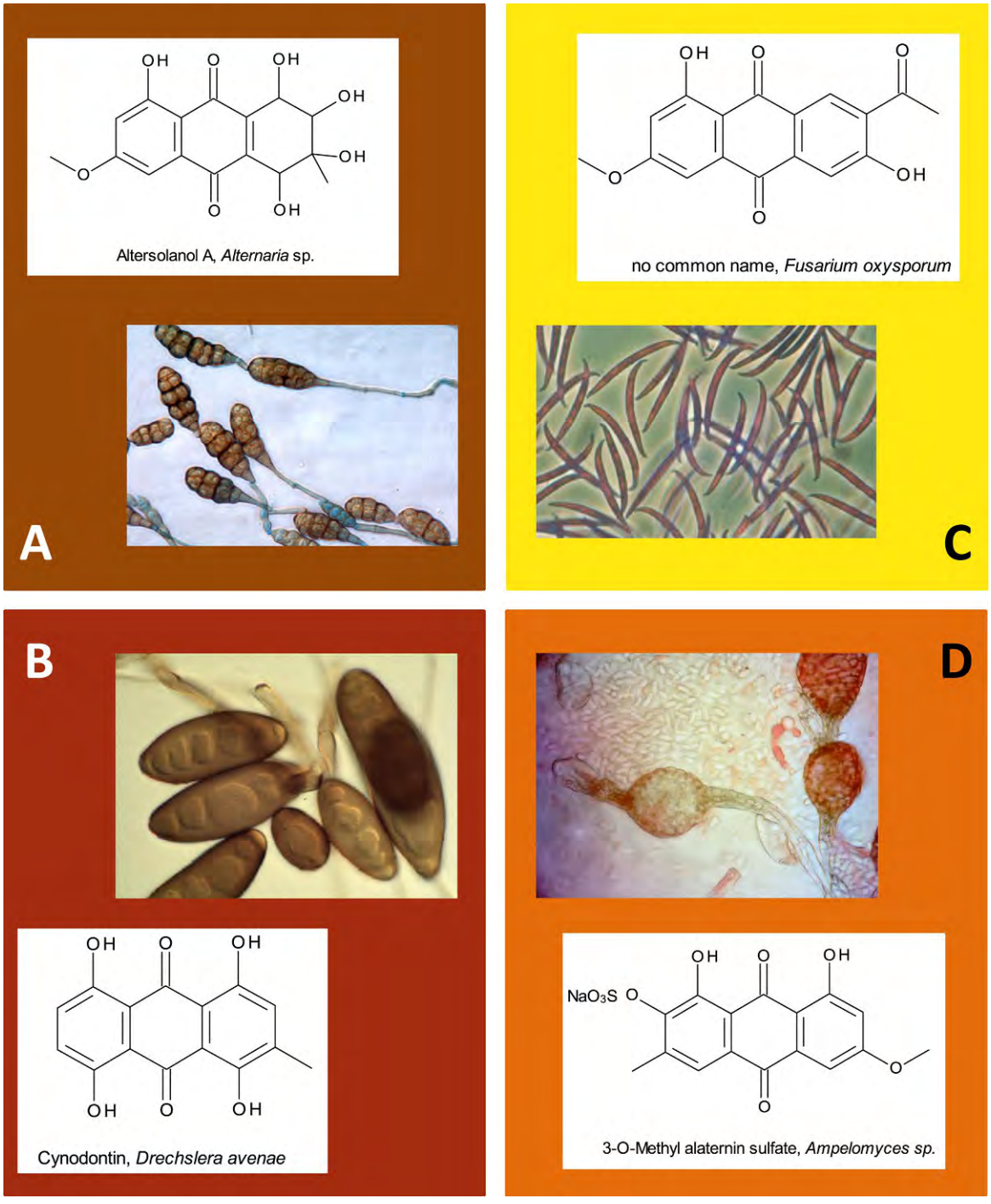

Fig. 3 Some anthraquinones from fungal origin (A) maroon, (B) bronze, (C) yellow, (D) red-orange (color of the box reflects color of the main pigment produced by the fungus).

The cultivation of the fungus in liquid broth requires carbohydrates (such as sucrose and molasses), nitrogen (corn extract and yeast autolysate or extract), zinc sulfate, and magnesium sulfate. The optimum conditions for performing the microbiological synthesis are pH value 5.6–6.2 and temperature 27–29°C.

On the second day of incubation a red colorant is released into the broth, increasing up to 1.5–2.0 g L^{-1} of broth after 3–4 days. After biosynthesis of the red colorant is completed, the liquid from the broth is filtered or centrifuged for being separated from the biomass. The liquid is then acidified to pH 3.0–2.5 to precipitate the colorant. The precipitate is dissolved in ethyl alcohol and filtered. Following removal of alcohol, the colorant in the crystalline form is obtained, that is, dark red powder.

The colorant gives a raspberry red color in aqueous solution, stable at pH over 3.5. Neutral solutions are stable even after 30 min of boiling and color shade does not change in relation with pH.

Many toxicological data are available on this red pigment: acute oral toxicity in mice 90-day subchronical toxicological study, acute dermal irritation/corrosion, acute eye irritation/corrosion, antitumor effectiveness, micronucleus test in mice, AMES test (*Salmonella typhimurium* reverse mutation assay), estimation of antibiotic activity, and results of estimation of five mycotoxins. A new patent on Arpink Red was filed in 2001 with claims of anticancer effects of the anthraquinone derivatives and applications within the food and pharmaceutical fields.

After evaluating all the materials provided by the company Ascolor s.r.o., the Codex Alimentarius Commission (Rotterdam meeting, 11–15 March 2002) made the following statement: "there will not be any objections to use the red coloring matter Arpink Red" in:

- meat products in the amount up to 100 mg kg^{-1},
- meat and meat product analogs in the amount up to 100 mg kg^{-1},
- nonalcoholic drinks in the amount up to 100 mg kg^{-1},
- alcoholic drinks in the amount up to 200 mg kg^{-1},
- milk products in the amount up to 150 mg kg^{-1},
- ice creams in the amount up to 150 mg kg^{-1},
- confectionery in the amount up to 300 mg kg^{-1}.

JECFA evaluation process is in progress and Arpink Red situation was discussed during the 63rd meeting of Joint FAO/WHO Expert Committee on Food Additives in Geneva, 8–17 June 2004. File is also under progress at the European Food Safety Authority (EFSA) under the reference EFSA-Q-2007-021.

Other Fungal Anthraquinones

Anthraquinones are widely spread in the kingdom of fungi (Fouillaud et al., 2016), and thus, the latter might serve as alternative sources being independent of agro-climatic conditions in contrast to plant and animal derived sources. For example, anthraquinones were found in *Aspergillus* sp., *Eurotium* sp., *Fusarium* sp., *Dreschlera* sp., *Penicillium* sp., *Emericella purpurea*, *Curvularia lunata*, *Mycosphaerella rubella*, *Microsporum* sp., etc.

Anthraquinones exhibit a broad range of biological activities, including bacteriostatic, fungicidal, antiviral, herbicidal, and insecticidal effects. Presumably, in fungi, these compounds are involved in interspecific interactions. For example, anthraquinones synthesized by endophytic fungi protect the host plant from insects or other microorganisms.

The present picture of fungal anthraquinones is quite complex, with a great variety of chemical structures, a huge number of factors or parameters which may have impact on the composition of quinoidal pigments biosynthesized by a particular species. Among them, for example, habitat, light, pH, temperature, O_2 transfer, liquid/solid media, culture medium, C and N sources, C:N ratio, presence of organic acids, mineral salts, and inoculum have been considered.

Today, research priority is laid on a small number of fungal anthraquinone-producing species meeting the following profile of requirements established during the identification of potentially safe fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale:

- fungus shall be non-pathogenic to humans,
- fungus shall be non-toxigenic under a broad range of production conditions,
- fungus shall be able to produce in liquid media.

Riboflavin – The Vitamin B2 But Also a Yellow Food Colorant

Riboflavin (vitamin B2) has a variety of applications as a yellow food colorant. Its use is permitted in most countries. Applications include dressings, sherbet, beverages, instant desserts, ice creams, tablets, and other products. Riboflavin has a special affinity for cereal-based products, but its use in these applications is somewhat limited due to its slight odor and naturally bitter taste. There are numerous microorganisms that produce riboflavin fermentatively. Riboflavin fermentation could be classified into three categories: weak overproducers (100 mg L^{-1} or less, e.g., *Clostridium acetobutylicum*), moderate overproducers

(up to 600 mg L^{-1}, e.g., yeasts such as *Candida guilliermundii* or *Debaryomyces subglobosus*), and strong overproducers (over 1 g L^{-1}, e.g., the fungi *Eremothecium ashbyii* and *A. gossypii*).

Current Carotenoid Production Using Microorganisms

Commercial processes are already in operation or under development for the production of carotenoids by molds, yeasts, and bacteria. The production of β-carotene by microorganisms, as well as by chemical synthesis or from plant extracts is well developed (Table 1; Britton et al., 2004), and several other carotenoids, notably lycopene, astaxanthin, zeaxanthin, and canthaxanthin are also of interest.

β-Carotene

β-Carotene from Blakeslea trispora

The source organism, *B. trispora*, is a commensal mold associated with tropical plants. The fungus exists in (+) and (−) mating types, of which the (+) type synthesizes trisporic acid, a metabolite of β-carotene. On mating the two types in a specific ratio, the (−) is stimulated by trisporic acid to synthesize large amounts of β-carotene. The mold has shown no pathogenicity or toxicity, in standard pathogenicity tests in mice, by analyses of extracts of several fermentation mashes for fungal toxins and by enzyme immunoassays of the final product, the β-carotene crystals, for four mycotoxins.

The production process proceeds essentially in two stages. Glucose and corn steep liquor can be used as carbon and nitrogen sources. Whey, a by-product of cheese manufacture, has also been considered, with strains adapted to metabolize lactose. In the initial fermentation process, seed cultures are produced from the original strain cultures and subsequently used in an aerobic submerged batch fermentation to produce a biomass rich in β-carotene. In the second stage, the recovery process, the biomass is isolated and transformed into a form suitable for isolating the β-carotene, which is extracted with ethyl acetate, suitably purified and concentrated, and the β-carotene crystallized. The final product is either used as crystalline β-carotene (purity 96%) or is formulated as a 30% suspension of micronized crystals in vegetable oil. The production process is subject to Good Manufacturing Practices (GMP) procedures, and adequate control of hygiene and raw materials. The biomass and the final crystalline product comply with an adequate chemical and microbiological specification and the final crystalline product also complies with the JECFA (Joint FAO/WHO Expert Committee on Food Additives) and EU specifications as set out in Directive 95/45/EC for coloring materials in food.

The first β-carotene product from *B. trispora* was launched in 1995 at the Food Ingredients Europe business meeting in London. Following the optimization of the fermentation process, many aspects had to be addressed many aspects before the product could be marketed:

- Presentation of the microorganism: A fungus isolated from a natural environment, not genetically modified; yield improvement achieved by classical genetics.
- Guidelines for labeling: Natural β-carotene; natural β-carotene from *B. trispora*; fermentative, natural β-carotene; natural β-carotene from a fermentative source.
- Lobbying from other β-carotene producers (nature-identical, mixed carotenes from palm oil, β-carotene from the microalgae *Dunaliella*): The EU Health and Consumer Protection Directorate General was asked to give an opinion on the safety of β-carotene from a dried biomass source, obtained from a fermentation process with *B. trispora*, for use as a coloring matter for foodstuffs.
- Safety of the fermentation-produced β-carotene: HPLC analysis, stability tests, and microbiological tests have shown that the β-carotene obtained by cofermentation of *B. trispora* complies with the EC specification for E 160 a(ii), also including the proportions of cis and trans isomers, and is free of mycotoxins or other toxic metabolites. Tests in vitro for gene mutations and chromosomal aberrations with the β-carotene produced by the manufacturer in the European Union showed it to be free of

Table 1 Microorganisms, a carotenoid source among many others. β-Carotene and β-carotene-containing preparations from various sources

Trademark	*Company*	*Origin*
AL CARC 9004	Diana naturals	Carrot
Altratene	Allied Industrial Corp.	Chemical synthesis
Betanat	Vitatene	*Blakeslea trispora*
Betatene	Cognis nutrition & health	*Dunaliella salina*
CaroPure	DSM	Chemical synthesis
CaroPure	DSM	*B. trispora*
Caroxan	Pot au Pin	Carrot
Lucarotin	BASF	Chemical synthesis
Mixed carotenoids	Global palm products	Palm oil (*Elaeis guineensis*)
Panaferd	JX Nippon Oil & Energy	*Paracoccus carotinifaciens*
Astaxanthin under development	Ajinomoto	*Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*)

Table 2 Isomers described in "β-carotene" from various sources

	Carotenoids			
Source	*All-trans β-carotene*	*Cis-β-carotene*	*α-Carotene*	*Other*
Fungus (*Blakeslea trispora*)	94	3.5	0	2.5
Chemical synthesis	98	2	0	0
Algae (*Dunaliella salina*)	67.4	32.6	0	0
Palm oil	34	27	30	9

genotoxic activity. In a 28-day feeding study in rats with the β-carotene manufactured in the European Union, no adverse findings were noted at a dose of 5% in the diet, the highest dose level used. In conclusion, evaluation of the source organism and the production process yielded no grounds to suppose that the final crystalline product, β-carotene, differs from the chemically synthesized β-carotene used as a food colorant. The final crystalline fermentation product has been shown to comply with the specification for β-carotene E 160 a(ii) listed in Directive 95/45/EC. The committee considers that "β-carotene produced by co-fermentation of *B. trispora* is equivalent to the chemically synthesized material used as food colorant and is therefore acceptable for use as a coloring agent for foodstuffs" (Table 2).

Today there are other industrial productions of β-carotene from *B. trispora* in Russia and Ukraine, and in León (Spain). The process has been developed to yield up to 30 mg of β-carotene per gram dry mass or about 17 g L^{-1}. *B. trispora* is now also used for the production of lycopene.

β-Carotene from Phycomyces blakesleeanus

Another mold, *P. blakesleeanus*, is a potential source for various chemicals including β-carotene. The carotene content of the wild-type grown under standard conditions is modest, about 0.05 mg per gram dry mass, but some mutants accumulate up to 10 mg g^{-1}. As for *B. trispora*, sexual stimulation of carotene biosynthesis is essential and can increase yields up to 35 mg g^{-1}. The most productive strains of *Phycomyces* achieve their full carotenogenic potential on solid substrates or in liquid media without agitation. *B. trispora* is more appropriate for production in usual fermenters.

β-Carotene from Mucor circinelloides

M. circinelloides wild type is yellow because it accumulates β-carotene as the main carotenoid. The basic features of carotenoid biosynthesis, including photoinduction, are similar in *Phycomyces* and *Mucor*. *M. circinelloides* responds to blue light by activating biosynthesis. Wild-type strains grown in darkness contain minimal amounts of β-carotene because of the low levels of transcription of the structural genes for carotenogenesis. When exposed to a light pulse, the level of transcription of these genes increases strongly, leading to high pigment concentrations. *M. circinelloides* is a dimorphic fungus that grows either as yeast cells or in a mycelium form, and research is now focused on yeast-like mutants that could be useful in a biotechnological production.

Applications of β-carotene

Research reports indicate that natural β-carotene possesses numerous benefits for the human body and consistently support the use of β-carotene as part of the human diet. The human body converts β-carotene to vitamin A via body tissues as opposed to the liver, hence avoiding a buildup of toxins in the liver. Vitamin A is essential for the human body in that it assists the body's immune system and helps battle eye diseases, such as cataracts and night blindness, various skin ailments such as acne, signs of aging, and various forms of cancer. β-Carotene has antioxidant qualities. Antioxidants help mediate the harmful effects of free radicals, which are implied in over 60 life-threatening diseases including various forms of cancer, coronary heart disease, premature aging, and arthritis. Additionally, the antioxidant qualities of β-carotene assist the body in suppressing the effects of premature aging caused by UV rays. β-Carotene is also added to numerous cosmetic and body-care products as a nonharmful colorant to improve the attractiveness of the product.

β-Carotene is one of the leading food colorants in the world. β-Carotene has been applied to a range of food and beverage products to improve their appearance to customers, including items such as margarine, cheese, fruit juices, baked goods, dairy products, canned goods, confectionary, and health condiments, to name just a few. β-Carotene is used in various pet foods as both a colorant and a precursor to vitamin A. β-Carotene can be applied to an array of animal foods designed for pets, including dogs, cats, fish, and birds. The antioxidant and precursory vitamin A properties increase the appeal and application of β-carotene in pet foods. Additionally, β-carotene is an important carotenoid that may assist in improving the color of birds, fish, and crustaceans, as well as improving the appearance of the pet food.

Lycopene

Lycopene is an intermediate in the biosynthesis of all dicyclic carotenoids, including β-carotene. In principle, therefore, blocking the cyclization reaction and the cyclase enzyme by mutation or inhibition will lead to the accumulation of lycopene. This strategy is employed for the commercial production of lycopene.

Lycopene from Blakeslea trispora

A process for lycopene production by *B. trispora* is now established, with the aim of marketing this product in Europe for use as a nutritional food ingredient and dietary supplement or a food color. Lycopene is an intermediate in the β-carotene biosynthetic pathway and microbial strains that accumulate lycopene are easy to obtain by mutagenesis, molecular biology, or use of inhibitors. In the commercial process imidazole or pyridine is added to the culture broth to inhibit the enzyme lycopene cyclase. The product, predominantly (all-trans)-lycopene (Table 3), is formulated into a 20 or 5% suspension in sunflower oil with α-tocopherol at 1% of the lycopene level. Also available is an α-tocopherol-containing 10 and 20% lycopene cold water dispersible (CWD) product. Lycopene oil suspension is intended for use as a food ingredient and in dietary supplements. The proposed level of use for lycopene in food supplements is 20 mg day^{-1}.

Approval for the use of lycopene from *B. trispora* was sought under regulation (EC) No 258/97 of the European Parliament and the Council concerning novel foods and novel food ingredients. The European Food Safety Authority was also asked to evaluate this product for use as a food color. It was stated in the application that it is likely that lycopene from *B. trispora* in food supplements would simply replace those supplements containing lycopene from other sources that are already being marketed so that overall consumption levels would not increase. Incorporation of lycopene into foods would result in additional intake.

The dossier first satisfied the United Kingdom Advisory Committee on Novel Foods and Processes (ACNFP) in 2004, but the panel from the European Food Safety Authority, in opinions adopted on 21 April and 5 October 2005, was unable to conclude whether the proposed use levels of lycopene from *B. trispora* would be safe.

Existing authorizations and evaluations on lycopene from various sources are quite numerous. Lycopene, extracted from tomatoes, is authorized as food coloring agent within the European Union (E160d) (Directive 94/36/EC) and the United States (CDR 21 73.295). This restricts the amount that can be added to foods. Lycopene was evaluated by the Scientific Committee on Food (SCF) in 1975 when it was unable to allocate an acceptable daily intake (ADI), but felt able to accept the use of lycopene prepared from natural foods by physical processes, without further investigations, as a coloring matter in food, provided that the amount consumed does not differ significantly from the amount consumed through the relevant foodstuffs. This opinion was reiterated by SCF in 1989. When JECFA evaluated lycopene from natural sources in 1977, they postponed a decision because of lack of data. In 1999 the SCF evaluated synthetic lycopene, but the available data were not sufficient to allow for an acceptance. The SCF concluded "The Committee is not able to allocate an ADI and considers its use in food is unacceptable at present." Synthetic lycopene is currently used as food ingredient but is not approved for coloring matters within the European Union and it is considered generally recognized as safe (GRAS) in the United States (GRAS notice No GRN 000119). In Australia and New Zealand, lycopene is permitted for use as a food color in processed foods in accordance with GMP under Schedule 3 of Standard 1.3.1 in the Food Standards Code. In Japan, tomato color, defined as "a substance composed mainly of lycopene obtained from tomato fruits," is permitted for use as a food additive under the Food Sanitation Law.

To summarize, the lycopene from *B. trispora* is considered by the EFSA Panel to be nutritionally equivalent to natural dietary lycopene, but further safety trials are necessary. While the toxicity data on lycopene from *B. trispora* and on lycopene from tomatoes do not give indications for concern, nevertheless these data are limited and do not allow an ADI to be established. The main concern is that the proposed use levels of lycopene from *B. trispora* as a food ingredient may result in a substantial increase in the daily intake of lycopene compared to the intakes solely from natural dietary sources.

Lycopene from Fusarium sporotrichioides

The fungus *F. sporotrichioides* has been genetically modified to manufacture lycopene from the cheap corn fiber material, the "leftovers" of making ethanol. Corn fiber is abundant (the US ethanol industry generates 4 million tons annually) and costs about five cents a pound. Distiller's dry grains with solubles (DDGS) could also be used as a substrate. Using a novel, general method for the sequential, directional cloning of multiple DNA sequences, the isoprenoid pathway of the fungus was redirected toward the synthesis of carotenoids. Strong promoter and terminator sequences from the fungus were added to carotenoid biosynthetic genes from the bacterium *Erwinia uredovora*, and the chimeric genes were assembled, introduced in the fungus, and expressed at levels comparable to those observed for endogenous biosynthetic genes. Cultures in laboratory flasks produced 0.5 mg lycopene per gram dry mass within 6 days and this is predicted to increase in the next few years.

Table 3 Comparison of the chemical compositions of synthetic lycopene, lycopene from tomatoes and lycopene from *Blakeslea trispora*

Lycopene	*Chemical synthesis (%)*	*From tomatoes (%)*	*From Blakeslea*
All-trans isomer	>70	94–96	≥90%
5-Cis isomer	<25	3–5	
9-Cis isomer	<1	0–1	1–5%
13-Cis isomer	<1	1	(Total of cis isomers)
Other cis isomers	<3	<1	

Astaxanthin

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is widely distributed in nature and is the principal pigment in crustaceans and salmonid fish. The carotenoid imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the market place. Since animals cannot synthesize carotenoids, the pigments must be supplemented in the feeds of farmed species. Salmon and trout farming is now a huge business and feeding studies have shown that astaxanthin is very effective as a flesh pigmenter. There are also reports of beneficial actions of astaxanthin for human health, so its use in supplements is of interest.

Astaxanthin from Xanthophyllomyces dendrorhous, *formerly* Phaffia rhodozyma

Among the few astaxanthin-producing microorganisms, *X. dendrorhous* is one of the best candidates for commercial production of (3R, 3′R)-astaxanthin. Many academic laboratories and several companies have developed processes suitable for industrial production.

Several reports have shown that constituents in the medium, among other environmental factors, affect astaxanthin production in this yeast. The effects of different nutrients on *X. dendrorhous* have generally been studied in media containing complex sources of nutrients such as peptone, malt, and yeast extracts. By-products from agriculture were also tested, such as molasses, enzymic wood hydrolysates, corn wet-milling coproducts, bagasse or raw sugarcane juice, date juice, and grape juice. Although such media are often convenient because they contain all nutrients, they suffer from the disadvantage of being undefined and sometimes variable in composition; this may mask important nutritional effects. For this reason, several studies have yielded results that are difficult to interpret in detail because of inadequate characterization of growth-limiting factors in the media. Thus, in order to elucidate the nature of nutritional effects as far as possible, chemically defined or synthetic media were used by some authors. In one study, 11 strains were assayed for their ability to utilize 99 different compounds as single carbon source. In a second study, carotenoid biosynthesis was increased at low ammonium or phosphate levels and stimulated by citrate. Factorial design and response surface methodology could be used to optimize the astaxanthin production. The optimal conditions stimulating the highest astaxanthin were 19.7°C temperature, 11.25 g L^{-1} carbon concentration, 6.0 pH, 5% inoculum, and 0.5 g L^{-1} nitrogen concentration. Under these conditions the astaxanthin content was 8.1 mg L^{-1}. Fermentation strategy also has an impact on growth and carotenoid production of *X. dendrorhous*, as shown with fed-batch (e.g., limiting substrate is fed without diluting the culture) or pH-stat (i.e., a system in which the feed is provided depending on the pH) cultures. The highest biomass obtained was 17.4 g L^{-1}.

A major drawback in the use of *X. dendrorhous* is that disruption of the cell wall of yeast biomass is required before addition to animal diet, to allow intestinal absorption of the pigment. Several chemical, physical, autolytic, and enzymic methods for cell wall disruption have been described, including a two-stage batch fermentation technique. The first stage was for "red yeast" cultivation. The second stage was the mixed fermentation of the yeast and *Bacillus circulans*, a bacterium with a high cell wall lytic activity.

Another starting point in optimization experiments is the generation of mutants, but metabolic engineering of the astaxanthin biosynthetic pathway is now attractive. It should be possible to manage carbon fluxes within the cell and resolve competition between enzymes such as phytoene desaturase and lycopene cyclase.

The case of *X. dendrorhous* (*P. rhodozyma*) is peculiar as hundreds of scientific papers and patents deal with astaxanthin production by this yeast but the process has not been economically efficient up to now. New patents are filed almost each year, with improvement in astaxanthin yield, for example, 3 mg g^{-1} dry matter in a US Patent. European Union recently decided to invest again in this yeast, with research programs leaded by Goethe University in Germany or the Royal University of London, United Kindom (PROCAR, ERA-IB-14-073 project).

Astaxanthin from Agrobacterium aurantiacum *and other bacteria*

Compared to the huge research effort devoted to *X. dendrorhous*, astaxanthin production by *A. aurantiacum* has been investigated to a lesser extent. The first description of astaxanthin biosynthesis in this bacterium was published in 1994. Astaxanthin is one of 10 carotenoids present. The biosynthetic pathway, the influence of growth conditions on carotenoid production, and the occurrence of astaxanthin glucoside were described in two subsequent papers, but commercial processes have not yet been developed.

Numerous screenings have been conducted with the aim of characterizing new biological sources of astaxanthin, and positive targets were isolated such as *P. carotinifaciens* or *Halobacterium salinarium*. The latter is particularly interesting because (1) the extreme NaCl concentrations (about 20%) used in the growth medium prevent contamination with other organisms, so no particular care has to be taken with sterilization; (2) NaCl concentrations under 15% induce bacterial lysis, so that no special cell breakage technique is necessary; and (3) pigments may be extracted directly with sunflower oil instead of organic solvents. This eliminates possible toxicity problems due to trace amounts of acetone or hexane and facilitates pigment assimilation by animals.

Advantages of astaxanthin over other carotenoids

Some of the advantages of astaxanthin over other carotenoids are

- more stable compared to other carotenoids,
- high antioxidant potential (10 times more than β-carotene and 500 times more than α-tocopherol),
- easily cross the blood–brain barrier,
- high tinctorial property.

Astaxanthin is a powerful bioactive antioxidant and has demonstrated efficacy in animal or human models of macular degeneration, a cause of blindness in a large population. It is also helpful in treating Alzheimer's and Parkinson's diseases, and is known to

offer protection against cancer. Astaxanthin has been shown to be a powerful quencher of singlet oxygen as evident from in vitro studies. Astaxanthin has stronger antioxidant activity, 10 times higher than β-carotene and more than 500 times more effective than α-tocopherol; astaxanthin has been proposed to be the super vitamin E. The antioxidant property has been demonstrated in a number of biological membranes, and astaxanthin has preventive effects against aflatoxin B1 carcinogenicity. Astaxanthin also has strong activity as an inhibitor of lipid peroxidation mediated by active forms of oxygen. The strong antioxidative activities of astaxanthin suggest its potential as photoprotectant against UV irradiation, and astaxanthin-containing preparations for prevention of light-induced aging of skin have been developed.

Mammals lack the ability to synthesize astaxanthin, or to convert dietary astaxanthin into vitamin A. Unlike β-carotene, astaxanthin has no provitamin activity in these animals. Astaxanthin has been shown in both in vitro and in a study with human subjects to be effective for the prevention of the oxidation of low-density protein, suggesting that it can be used to prevent arteriosclerosis, coronary artery disease, and ischemic brain development. A number of astaxanthin health products are under study. Studies on rats had shown no toxicity of astaxanthin preparations. Dietary administration of astaxanthin has proved to significantly inhibit carcinogenesis in the mouse urinary bladder, rat oral cavity, and rat colon. In addition it is reported to induce xenobiotic-metabolizing enzymes in rat liver. Astaxanthin has been shown to enhance in vitro antibody production by mouse spleen cells stimulated with sheep red blood cells and in human blood cells in vitro. Furthermore it has not exhibited any mutagenicity in in vitro study at doses up to 14.4 mg day^{-1} for 2 weeks. There was strong evidence to suggest that astaxanthin was shown to modulate the humoral and nonhumoral immune systems. It enhances the release of interleukin-1 and the tumor necrosis factor α in mice, to a greater extent than canthaxanthin or β-carotene and has the highest cytokinin-inducing activity. Astaxanthin has a significant enhancing action on the production of immunoglobulin A, M, and G and on T-helper cell antibody production, even when suboptimal amounts of antigen are present.

Astaxanthin for salmon and trout feeds

The predominant source of carotenoids for salmonids has been synthetic astaxanthin, which has been used for pigmentation for the last 20 years with FDA approval in 1996. Natural sources of astaxanthin for commercially raised salmonids have been utilized including processed crustacean waste from the krill, shrimp, crab, and crawfish. However, crustacean waste products contain high amounts of moisture, ash, and chitin. Another natural source, *P. rhodozyma* (*X. dendrorhous*) requires a large amount of feed for sufficient pigmentation leading to higher ash contents. The efficiency of utilization of dietary astaxanthin using microalgae for flesh pigmentation of Atlantic salmon and rainbow trout has been demonstrated. For salmon, astaxanthin is even considered as a vitamin essential for the proper development and survival of juveniles. In rainbow trout fed with algae up to 6% of the diet, no major effect on growth or mortalities was observed, and it was concluded to be a safe and effective source of pigment. Astaxanthin has been used to enhance the immune response of fish and shrimp for maximum survival and growth. Also, natural microalgal astaxanthin has shown superior bioefficacy over synthetic form. Full approval in Japan has been received as a pigment in feeds and foods; registration for approval is in progress for the United States, the European Community, and Canada. An amount of 25–100 ppm of carotenoids in the final feed has been considered to give desired pigmentation in various salmonid species. However, the livestock feed market for astaxanthin, presently small, will grow to a comparable size as synthetic ones, which is estimated to $185 million. The largest market for astaxanthin being aquaculture – making up 24% of total global fisheries – production is currently valued at $35 billion per annum and is expected to grow to $49 billion by 2010 (the total carotenoid market is planned to reach 1.53 billion USD by 2021).

Astaxanthin for humans

Limited studies have been carried out about dietary astaxanthin intake by humans. In a study, an astaxanthin-containing drink was used to protect low-density lipoprotein (LDL) from oxidation (astaxanthin was administered 3.6–14.4 mg day^{-1} over a period of 2 weeks). Progressively slowing down LDL oxidation with increasing doses of astaxanthin was observed, and no ill effects were reported. When 100 mg of synthetic astaxanthin in olive oil-containing meal was given to male volunteers, maximum plasma concentration of 1.24 mg L^{-1} astaxanthin was observed in the first 6 h postprandially. The relative concentration of total astaxanthin in high-density lipoprotein (HDL) decreases compared to the other lipoprotein fractions in the 72-h study. Based on a study conducted with 40 healthy volunteers, the effect of astaxanthin on mammalian muscle function was reported. Volunteers received one capsule of 4 mg astaxanthin each morning along with food. No significant difference was observed between the treatment and placebo group in any physical parameter measured. The effect of dietary astaxanthin on the health of humans as carried out by Aquasearch Inc. on 33 volunteers consuming daily 3.85 mg (low dose) and 19.25 mg (high dose) for a period of 29 days indicated no ill effects or toxicity due to the consumption of astaxanthin as analyzed by medical and clinical parameters. Other scientists suggested that astaxanthin could be useful for prevention and treatment of neuronal damage associated with age-related macular degeneration, and it may also be effective in treating ischemic reperfusion injury, Alzheimer's disease, Parkinson's disease, spinal cord injuries, and other types of central nervous system injuries. Astaxanthin was found to easily cross the blood–brain barrier and did not form crystals in the eye.

Zeaxanthin

Zeaxanthin (β,β-carotene-3,3′-diol) can be used, for example, as an additive in feeds for poultry to intensify the yellow color of the skin or to accentuate the color of the yolk of their eggs. It is also suitable for use as a colorant, for example, in the cosmetics and food industries, and as a health supplement that may help to prevent age-related macular degeneration.

In the mid-1960s, several marine bacteria were isolated that produced zeaxanthin. Cultures of a *Flavobacterium* sp. (ATCC 21588, classified under the accepted taxonomic standards of that time) in a defined nutrient medium containing glucose or sucrose, as carbon source, were able to produce up to 190 mg of zeaxanthin per liter, with a concentration of 16 mg g^{-1} dried cellular mass. One species, *Flavobacterium multivorum* (ATCC 55238), is currently under investigation in many studies.

Recently, a zeaxanthin-producing "*Flavobacterium*"was reclassified as a new *Paracoccus* species, *Paracoccus zeaxanthinifaciens* and earlier findings that IPP biosynthesis occurs exclusively via the mevalonate pathway were confirmed. A second strain, isolated in a mat from an atoll of French Polynesia, is peculiar as it also produce exopolysaccharides.

Sphingobacterium multivorum, the new name for *F. multivorum*, was recently shown to utilize the alternative deoxyxylulose phosphate (DXP) pathway. A strain was constructed for overproduction of zeaxanthin in industrial quantities.

As more bacteria are examined, the distribution of the mevalonate and DXP pathways will become better defined, thus facilitating the metabolic engineering of microorganisms with improved production of commercially important isoprenoid compounds including carotenoids.

Canthaxanthin

Canthaxanthin (β,β-carotene-4,4′-dione) has been used in aquafeed for many years in order to impart the desired flesh color in farmed salmonids. A *Bradyrhizobium* sp. strain was described as a canthaxanthin producer and the carotenoid gene cluster was fully sequenced. Interest in canthaxanthin is decreasing since the discovery of extreme overdosage, that is, the deposition of minute crystals in the eye, a fact leading to adverse media attention in the past, and some pressure to limit its use in aquafeeds.

A second bacterium under scrutiny for canthaxanthin production is *Haloferax alexandrinus*, which belongs to the extremely halophilic Archaea, chemo-organotrophic organisms that satisfy some of their energy requirements with light. Members of the family Halobacteriaceae are characterized by red-colored cells, the color in most cases being due to the presence of C50-carotenoids (especially bacterioruberins) as the major carotenoids. Some species have been reported to produce C40-carotenoids and ketocarotenoids as minor carotenoids. Recently, the biotechnological potential of these members of the Archaea has increased because of their unique features, which facilitate many industrial procedures. For example, no sterilization is required, because of the extremely high NaCl concentration used in the growth medium (contamination by other organisms is avoided). In addition, no cell-disrupting devices are required, as cells lyse spontaneously in freshwater. A 1 l scale cultivation of the cells in flask cultures (6 days) under nonaseptic conditions produced 3 g dry mass, containing 6 mg total carotenoid, and 2 mg canthaxanthin. Further experiments in a batch fermenter also demonstrated increases in the biomass concentration and carotenoid production.

A third example is *Gordonia jacobea* (CECT 5282), a Gram-positive, catalase-negative, G+C 61% bacterium that was isolated in routine air sampling during screening for microorganisms that produce pink colonies. Analysis of the carotenoid extracts by HPLC-MS revealed that the main pigment in the isolates is canthaxanthin. The low carotenoid content (0.2 mg g^{-1} dry mass) in the isolate does not support an industrial application, but after several rounds of mutations, a hyperpigmented mutant (MV-26) was isolated, which accumulated six times more canthaxanthin than the wild-type strain. Apart from their high pigment production, the advantages of mutants of this species from the industrial point of view are (1) the optimal temperature for growth and carotenogenesis is 30°C, which is usual in fermentors; (2) the use of glucose, an inexpensive carbon source, for optimal growth and pigmentation; and (3) the fact that >90% of the total pigments can be extracted directly with ethanol, a nontoxic solvent allowed for human and animal feed. Many other culture media were tested, giving canthaxanthin from 1 to 13.4 mg L^{-1}.

Torulene and Thorularhodin

Yeasts in the genus *Rhodotorula* synthesize carotenoids, mainly torulene (3′,4′-didehydro-β,ψ-carotene) and torularhodin (3′,4′-didehydro-β,ψ-caroten-16-oic acid), accompanied by very small amounts of β-carotene. Most of the research has focused on the species *Rhodotorula glutinis*, but some papers deal with other species such as *Rhodotorula gracilis*, *Rhodotorula rubra*, and *Rhodotorula graminis*.

Feed supplemented with a *Rhodotorula* cell mass has been found to be safe and non-toxic in animals. Its use in the nutrition of laying hens has also been documented. As the β-carotene content in wild strains of *R. glutinis* is low, efforts have been made to increase it through strain improvement, mutation, medium optimization and manipulation of culture conditions (temperature, pH, aeration, and C/N ratio). These studies mainly resulted in an increased yield of torulene and torularhodin, which are of minor interest, though some did succeed in increasing the β-carotene content up to about 70 mg L^{-1}.

Prospects for Carotenoid Production by Genetically Modified Microorganisms

Escherichia coli and Other Hosts

Metabolic engineering is, in a narrow sense, defined as the use of recombinant DNA techniques for the deliberate modification of metabolic networks in living cells to produce desirable chemicals with superior yield and productivity. The traditional assumption was that the most productive hosts would be microbes that naturally synthesize the desired chemicals, but microorganisms that have the ability to produce precursors of the desired chemicals with superior yield and productivity are also considered as suitable hosts.

As a starting point a large number (200) of genes and gene clusters coding for the enzymes of carotenoid biosynthesis have been isolated from various carotenogenic microorganisms and the functions of the genes have been elucidated. Then large range whole genome sequencing arrived and this number increased tremendously.

In bacteria such as *E. coli*, which cannot naturally synthesize carotenoids, carotenoid biosynthesis de novo has been achieved by the introduction of carotenogenic genes. *E. coli* does possess the ability for synthesize other isoprenoid compounds such as dolichols (sugar carrier lipids) and the respiratory quinones. It is thus feasible to direct the carbon flux for the biosynthesis of these isoprenoid compounds partially to the pathway for carotenoid production by the introduction of the carotenogenic genes. For example, plasmids carrying crt genes for the synthesis of lycopene, β-carotene, and zeaxanthin have been constructed and expressed in *E. coli*. Transformants accumulated 0.2–1.3 mg g^{-1} dry mass of lycopene, β-carotene, and zeaxanthin in the stationary phase. Several intermediates accumulated at the expense of the end product of the pathway. The use of shotgun library clones constructed with *E. coli* chromosomal DNA has revealed that genes not directly involved in the carotenoid biosynthesis pathway are important, such as *appY* that encodes transcriptional regulators related to anaerobic energy metabolism and can increase the lycopene production to 4.7 mg g^{-1} dry cell mass.

The most important task for biotechnology will be to identify rate-limiting steps or to eliminate regulatory mechanisms in order to enhance further the production of valuable carotenoids. Some stimulating effect has also been reported for *E. coli* strains that overexpress the DXP synthase gene from *Bacillus subtilis* or *Synechococcus leopoliensis*.

Optimization for high-yield carotenoid production should focus on several different aspects (Fig. 4) First, sufficient amounts of endogenous precursors (i.e., substrates for the reactions involved) should be available (e.g., by control of the pyruvate/glyceraldehyde 3-phosphate ratio, a yield of 25 mg lycopene per gram dry mass has been reported). Second, a balanced system of carotenogenic enzymes should be expressed, to enable efficient conversion of precursors without the formation of intermediate metabolite pools. Third, the correct plasmid combination is important to minimize the accumulation of intermediates and to increase the yield of the end product. Last, the host organism should exhibit an active central terpenoid pathway and possess a high storage capacity for carotenoids.

It has also been shown that the edible yeasts *Candida utilis* and *Saccharomyces cerevisiae* acquire the ability to produce carotenoids when the required carotenogenic genes are introduced (same result was observed for the oleaginous yeast *Yarrowia lipolytica*).

Directed Evolution and Combinatorial Biosynthesis

Directed evolution involves the use of rapid molecular manipulations to mutate the target DNA fragment, followed by a selection or screening process to isolate desirable mutants (Schmidt-Dannert et al., 2006). By various directed evolution protocols, several enzymes have been improved or optimized for a specific condition. Directed evolution was applied to geranylgeranyl diphosphate (GGPP) synthase (a rate-controlling enzyme) from *Archaeoglobus fulgidus* to enhance the production of carotenoids in metabolically engineered *E. coli*. The library of mutated genes that was created was transformed into an *E. coli* strain containing the reconstructed isoprenoid pathway and colonies were then color-screened for the increased conversion of glucose into astaxanthin or lycopene. Eight color-enhanced mutants were obtained from over 10,000 colonies. The production of lycopene was increased by about twofold.

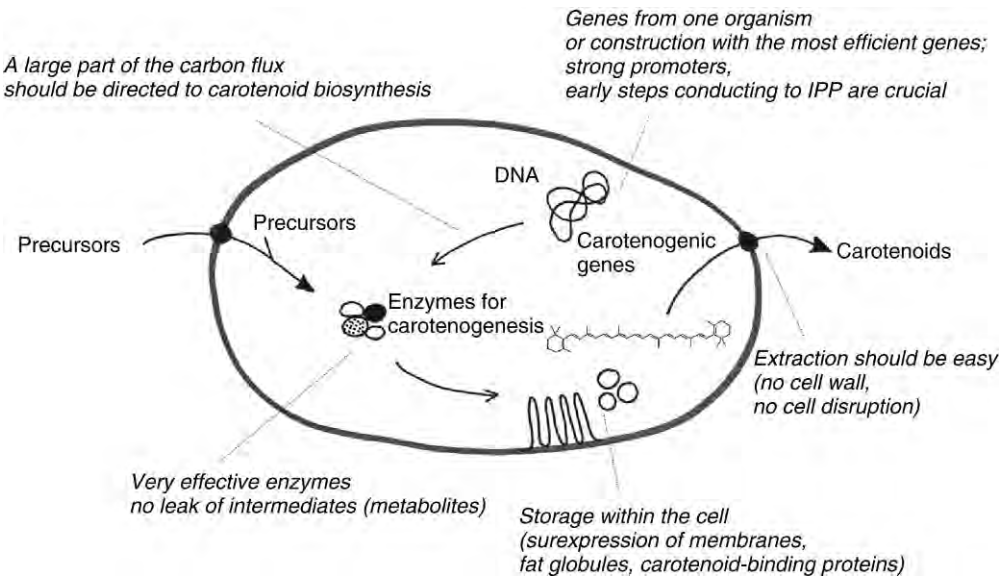

Fig. 4 Metabolic engineering of microbial carotenoid production.

A second example deals with the membrane-associated phytoene synthase, which appears to be the major point of control over product diversity. By engineering the phytoene synthase to accept longer diphosphate substrates, variants were produced that can make previously unknown C45 and C50 carotenoid backbones from the appropriate C20 and C25 isoprenyl diphosphate precursors. A C35 carotenoid backbone has also been biosynthesized. Various downstream enzymes (desaturases and cyclases) from the C30 and C40 carotenoid pathways were found to be functional on these unnatural susbtrates, leading to the production of a series of novel C35, C45, and C50 carotenoids. Thus, it appears that once a carotenoid backbone structure is created, downstream enzymes, either natural or engineered, such as desaturases, cyclases, hydroxylases, and cleavage enzymes, can accept the new substrate, and whole series of novel carotenoids can be produced.

A different approach to expand the recombinant production of known carotenoids and to synthesize new structures is to combine available biosynthetic genes and evolve new enzyme functions through random mutagenesis, recombination (DNA-shuffling), and selection. Prerequisites for this approach are that crt enzymes from different species can function cooperatively in a heterologous host and display enough promiscuity regarding the structure of their substrates. With a few exceptions, such as the zeaxanthin C(5,6) epoxidase gene, almost all cloned carotenoid biosynthetic genes are functionally expressed in *E. coli*. The success of functional color complementation in transgenic *E. coli* for the cloning of a number of carotenoid biosynthesis genes demonstrates that enzymes from phylogenetically distant species can assemble into a functional membrane-bound multienzyme complex through which carotenoid biosynthesis presumably takes place. The enzymes phytoene desaturase (CrtI) and lycopene cyclase (CrtY) have been targeted for evolution in vitro to achieve synthesis of novel carotenoids in *E. coli*. A variant enzyme, a desaturase chimera, efficiently catalyzed the extended desaturation of the linear C40 carotenoid pathway and introduced six rather than four double bonds into phytoene, to favor the production of the fully conjugated carotenoid (3,4,3′,4′-tetradehydro-ψ,ψ-carotene).

A related strategy is to combine carotenogenic genes from different bacteria that alone normally produce different end products and to express them in a simple *E. coli* host that carries the biosynthetic machinery for phytoene production. In conjunction with the four-step phytoene desaturase that yields lycopene, a five-step desaturase was used to produce 3,4-didehydro-ψ,ψ-carotene. Further diversification of the C40 skeleton using a 1,2-hydratase and a C-3,4 desaturase yielded a range of carotenoids, including 3,4-didehydro-1,2-dihydro-ψ,ψ-caroten-1-ol, 3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol, and 1′,2′-dihydro-β-ψ-caroten-1′-ol.

Concluding Comments

There are several advantages of microorganisms for the study of biosynthesis and function of pigments. Bacteria and fungi offer a tremendous resource in that they produce hundreds to thousands of various pigmented molecules. It is likely that many more natural pigments will be isolated from this biomass (Dufossé, 2016).

Synthetic pigments traditionally used by food or cosmetic processors continue to be utilized with success; however, with the increasing consumer preference for natural food additives, and the surge of the so-called Southampton study which started a worldwide ban on artificial colorants, natural colorants from plants is now a big business and most of the research efforts within the scientific field of colorants are conducted on natural ones. Regarding bacteria, yeast, or fungi (Table 4), despite common belief about the high production cost of fermentation pigments, two initiatives started in Europe these last decades: β-carotene from the filamentous fungi, *B. trispora* (produced by Gist-Brocades, now DSM; approved in 2000 by the EU Scientific Committee on Food Safety) and Arpink Red from *P. oxalicum* (manufactured by Ascolor now Natural Red) (Dufossé et al., 2014). These companies invested a lot of money as any combination of new source and/or new pigment drives a lot of experimental work, process optimization, toxicological studies, regulatory issues, and tremendous paper work. Time will tell whether investment was cost-effective. Another development under process is the production of lycopene using *B. trispora* by Vitatene, a subsidiary of Spanish penicillin firm Antibioticos. Exploration of fungal biodiversity is still going on, with special interest in water-soluble pigments. The case of *X. dendrorhous* (*P. rhodozyma*) is very peculiar as hundreds of scientific papers and patents deal with astaxanthin production using this yeast and the process has not been economically efficient up to now. Microorganisms could either be used for the biosynthesis of "niche" pigments not found in plants, such as aryl carotenoids. Carotenoids play an exceptional role in the fast-growing "over-the-counter (OTC) medicine" and "nutraceutical" sector. Among carotenoids under investigation for coloring or for biological properties, a small number are available from natural extracts or chemical synthesis. The list is rather short compared to the long list of 750 entries in the "Carotenoid Handbook." With imagination, biotechnology could be a solution for providing additional pigments including interesting aryl carotenoids. Isorenieratene (Φ,Φ-carotene), and its monohydroxy and dihydroxy derivatives, can be produced by bacteria, that is, *Brevibacterium linens*, *Streptomyces mediolani*, or *Mycobacterium aurum*.

Research projects mixing molecular biology and pigments were investigated all over the world, and it seems that current productions are not effective in terms of final yield. Nowadays, combinatorial genetic engineering is being addressed, based on an increasing number of known carotenogenic gene sequences. By combining genes, some authors were able to obtain more efficient biosynthesis, or new carotenoids, never described in nature, such as multihydroxylated ones, which could be very efficient as antioxidants.

Table 4 Microbial production of pigments (already in use as natural colorants or with high potential in this field)

Molecule	*Color*	*Microorganism*	*Status*
Ankaflavin	Yellow	*Monascus* sp. (fungus)	IP
Anthraquinone	Red	*Penicillium oxalicum* (fungus)	IP
Astaxanthin	Pink-red	*Xanthophyllomyces dendrorhous* (yeast), formerly *Phaffia rhodozyma*	DS
Astaxanthin	Pink-red	*Agrobacterium aurantiacum* (bacteria)	RP
Astaxanthin	Pink-red	*Paracoccus carotinifaciens* (bacteria)	RP
Canthaxanthin	Dark red	*Bradyrhizobium* sp. (bacteria)	RP
Canthaxanthin	Dark red	*Haloferax alexandrinus* (bacteria)	RP
Canthaxanthin	Dark red	*Gordonia jacobea* (bacteria)	DS
Lycopene	Red	*Blakeslea trispora* (fungus)	DS
Lycopene	Red	*Fusarium sporotrichioides* (fungus)	RP
Melanin	Black	*Saccharomyces neoformans* var. *nigricans* (yeast)	RP
Monascorubramin	Red	*Monascus* sp. (fungus)	IP
Naphtoquinone	Deep blood-red	*Cordyceps unilateralis* (fungus)	RP
Riboflavin	Yellow	*Ashbya gossypii* (fungus)	IP
Rubrolone	Red	*Streptomyces echinoruber* (bacteria)	DS
Rubropunctatin	Orange	*Monascus* sp. (fungus)	IP
Torularhodin	Orange-red	*Rhodotorula* sp. (yeast)	DS
Zeaxanthin	Yellow	*Flavobacterium* sp. (bacteria)	DS
Zeaxanthin	Yellow	*Paracoccus zeaxanthinifaciens* (bacteria)	RP
Zeaxanthin	Yellow	*Sphingobacterium multivorum* (bacteria)	RP
β-carotene	Yellow-orange	*B. trispora* (fungus)	IP
β-carotene	Yellow-orange	*F. sporotrichioides* (fungus)	RP
β-carotene	Yellow-orange	*Mucor circinelloides* (fungus)	DS
β-carotene	Yellow-orange	*Neurospora crassa* (fungus)	RP
β-carotene	Yellow-orange	*Phycomyces blakesleeanus* (fungus)	RP
Unknown	Red	*Penicillium purpurogenum* (fungus)	DS
Unknown	Red	*Paecilomyces sinclairii* (fungus)	RP

Abbreviation: DS, development stage; IP, industrial production; RP, research project.

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Pili, Fimbriae[☆]

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Glossary

Curli A class of thin, irregular, and highly aggregated adhesive surface fibers expressed by *Escherichia coli* and *Salmonella* spp.
Periplasmic chaperones A class of proteins localized within the periplasm of Gram-negative bacteria that facilitates the folding and assembly of pilus subunits, but which are not components of the final pilus structure.
Phase variation Reversible on and off switching of a bacterial phenotype, such as pilus expression.
Pilin Individual protein subunit of a pilus organelle (also known as a fimbrin). Immature pilins, containing leader signal sequences that direct the transport of pilins across the inner membrane of Gram-negative bacteria, are called propilins or prepilins.
***sec* secretion system** A system involving at least seven proteins that mediates translocation of proteins across biological membranes. Proteins are targeted for *sec*-dependent secretion by the presence of an amino acid signal sequence.
Sortase A transpeptidase that 'sorts' proteins containing the motif LPXTG into the cell wall fraction of Gram-positive bacteria by attachment to peptidoglycan.
Type II secretion system A system of protein secretion in which the substrates are already-folded proteins. Type II secretion systems share many features with type IV pilus assembly.
Type III secretion system A *sec*-independent secretion pathway, also known as T3SS. Numerous Gram-negative pathogens utilize T3SS to secrete and inject effector molecules into the cytosol of host eukaryotic cells.
Usher Oligomeric outer-membrane proteins that serve as assembly platforms for some types of pili. Usher proteins can also form channels through which nascent pili are extruded from bacteria.

Abbreviations

AAF	Aggregative adherence fimbria
BfpA	Bundle-forming pilus
CAP	Catabolite activator protein
CFA	Colonization factor I
CS1	Coli surface antigen 1
CTX	Cholera toxin phage
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
IncF1	F1 incompatibility
Lrp	Leucine-responsive regulatory protein
MR	Mannose-resistant
MS	Mannose-sensitive
T3SS	Type III secretion system
T4SS	Type IV secretion systems
TCP	Toxin coregulated pili
TcpA	Toxin coregulated pilus

Defining Statement

Pili, also known as fimbriae, are proteinaceous, filamentous polymeric organelles expressed on the surface of bacteria. They range from a few fractions of a micrometer to >20 μm in length and vary from <2 to 11 nm in diameter. Their functions include mediation of cell-to-cell interactions, motility, and DNA uptake.

Pili are composed of single or multiple types of protein subunits, called pilins or fimbrins, which are typically arranged in a helical fashion. Pilus architecture varies from thin, twisting thread-like fibers to thick, rigid rods with small axial holes. Thin pili with

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diameters of 2–3 nm, such as K88 and K99 pili, are sometimes referred to as 'fibrillae.' Even thinner fibers (<2 nm), which tend to coil up into a fuzzy adhesive mass on the bacterial surface, are referred to as thin aggregative pili or curli. High-resolution electron microscopy of P, type 1, and S pili of *Escherichia coli*, and *Haemophilus influenzae* pili has revealed that these structures are composite fibers, consisting of a thick pilus rod attached to a thin, short distally located tip fibrillum. Pili are often expressed peritrichously around individual bacteria, but some, such as type IV pili, can be localized to one pole of the bacterium.

Pili are produced by both Gram-negative and Gram-positive bacteria. The numerous types of pili have been ascribed diverse functions in the adaptation, survival, and spread of both pathogenic and commensal bacteria. Pili can act as receptors for bacteriophage, facilitate DNA uptake and transfer (conjugation), and, in at least type IV pili, function in cellular motility. The primary function of most pili, however, is to act as scaffolding for the presentation of specific adhesive moieties. Adhesive pilus subunits (adhesins) are often incorporated as minor components into the tips of pili, but major structural subunits can also function as adhesins. Adhesins can mediate the interaction of bacteria with each other, with inanimate surfaces, and with tissues and cells in susceptible host organisms. The colonization of host tissues by bacterial pathogens typically depends on a stereochemical fit between an adhesin and complementary receptor architecture. Interactions mediated by adhesive pili can facilitate the formation of bacterial communities such as biofilms and are often critical to the successful colonization of host organisms by both commensal and pathogenic bacteria.

Historical Perspective and Classification of Pili

Pili were first noted in early electron microscopic investigations as nonflagellar, filamentous appendages of bacteria. In 1955, Duguid designated these appendages 'fimbriae' (plural, from Latin for thread or fiber) and correlated their presence with the ability of *E. coli* to agglutinate red blood cells. Ten years later Brinton introduced the term 'pilus' (singular, from Latin for hair) to describe the fibrous structures (F pili) associated with the conjugative transfer of genetic material between bacteria. Since then 'pilus' has become a generic term used to describe all types of nonflagellar filamentous appendages, and it is used interchangeably with the term 'fimbria.'

Historically, pili have been named and grouped based on phenotypic traits, such as adhesive and antigenic properties, distribution among bacterial strains, and microscopic characterizations. In the pioneering work of Duguid and collegaues, pili expressed by different *E. coli* strains were distinguished on the basis of their ability to bind to and agglutinate red blood cells (hemagglutination) in a mannose-sensitive (MS) versus a mannose-resistant (MR) fashion. Pili mediating MS hemagglutination by *E. coli* were designated type 1, and these pili have since been shown to recognize mannose-containing glycoprotein receptors on host eukaryotic cells. Morphologically and functionally homologous type 1 are expressed by many different species of Enterobacteriaceae. Despite their similarities, however, type 1 expressed by the various members of the Enterobacteriaceae family are often antigenically and genetically divergent within their major structural subunits. In contrast to type 1, most other pili so far identified either are nonhemagglutinating or mediate MR hemagglutination. These pili are very diverse and possess a myriad of architectures and different receptor-binding specificities and functions.

Pili were first identified on Gram-positive bacteria in 1968 by electron microscopy of *Corynebacterium renale*. Other *Corynebacterium* spp., and a number of other Gram-positive bacteria that live in the human oral cavity, were also subsequently shown to express pili. In part because of limitations on molecular genetic analysis in Gram-positive bacteria, relatively little has been known about these structures until recently. Since 2005, it has been discovered that three clinically significant Gram-positive pathogens, *Streptococcus pneumoniae*, group A *Streptococcus*, and group B *Streptococcus*, all express pili. Because pili hold promise as vaccine candidates, we can expect an increase in research on pili in Gram-positive bacteria in coming years.

In the more than 50 years since the discovery and initial characterization of pili, substantial advances have been made in our understanding of the genetics, biochemistry, and structural and functional aspects of these organelles. A vast number of distinct pilus structures have been described and new types of pili continue to be identified. Pili are now known to be encoded by virtually all Gram-negative organisms and are some of the best-characterized colonization and virulence factors in bacteria. In this article, we have classified pili expressed by Gram-negative bacteria into six different groups according to the mechanisms by which they are assembled; pili expressed by Gram-positive bacteria are addressed in a separate section. This classification scheme is not all-inclusive, but provides a convenient means for discussing the diverse types of pili, their functions, structures, and assembly. Representatives of various pilus types assembled by the different pathways discussed in the following sections are listed in Table 1.

Chaperone/Usher Pathway

All pilins destined for assembly on the surface of Gram-negative bacteria must be translocated across the inner membrane, through the periplasm, and across the outer membrane. To accomplish these steps, various adhesive organelles in many different bacteria require two specialized assembly proteins: a periplasmic chaperone and an outer-membrane usher. Chaperone/usher assembly pathways are involved in the biogenesis of over 30 different structures, including composite pili, thin fibrillae, and nonfimbrial adhesins. Here, we will focus on the structure and assembly mechanisms of the prototypical P and type 1 pilus chaperone/usher systems.

Table 1 Pilus assembly pathways

Assembly pathway	*Structure*	*Assembly gene products*[a]	*Organism*	*Disease(s) associated with pilus expression*
Chaperone–usher pathway				
Thick, rigid pili	P pili	PapD/PapC	*E. coli*	Pyelonephritis/cystitis
	Prs pili	PrsD/PrsC	*E. coli*	Cystitis
	Type 1	FimC/FimD	*E. coli*	Cystitis
			Salmonella spp.	
			K. pneumoniae	
			X. fastidiosa	Plant diseases
	S pili	SfaE/SfaF	*E. coli*	UTI
				Newborn meningitis
	F1C pili	FocC/FocD	*E. coli*	Cystitis
	Hif pili	HifB/HifC	*H. influenzae*	Otitis media meningitis
	Haf pili	HafB/HafC	*H. influenzae* Biogroup *aegyptius*	Brazilian Purpuric Fever
	Types II and III pili	FimB/FimC	*B. pertussis*	Whooping cough
	MR/P pili	MrpD/MrpC	*P. mirabilis*	Nosocomial UTI
	PMF pili	PmfC/PmfD	*P. mirabilis*	Nosocomial UTI
	Long polar fimbriae	LpfB/LpfC	*S. enterica* ser. Typhimurium	Gastroenteritis
	Long polar fimbriae	LpfB/LpfC	*E. coli*	Diarrhea
	Pef pili	PefD/PefC	*S. enterica* ser. Typhimurium	Gastroenteritis
	Ambient-temperature fimbriae	AftB/AftC	*P. mirabilis*	UTI
	987P fimbriae	FasB/FasD	*E. coli*	Diarrhea in piglets
	REPEC fimbriae	RalE/RalD	*E. coli*	Diarrhea in rabbits
	AF/R1	AfrC/AfrB	*E. coli*	Diarrhea in rabbits
Thin, flexible pili	K99 pili	FaeE/FaeD	*E. coli*	Neonatal diarrhea in calves, lambs, piglets
	K88 pili	FanE/FanD	*E. coli*	Neonatal diarrhea in piglets
	F17 pili	F17D/F17papC	*E. coli*	Diarrhea
	F18 pili	FedC/FedB	*E. coli*	Diarrhea in piglets
	MR/K pili	MrkB/MrkC	*K. pneumoniae*	Pneumonia
	Acu pili	AcuD/AcuC	*Acinetobacter* spp. strain *BD413*	Opportunistic infections
Atypical structures	CS31A capsule-like protein	ClpE/ClpD	*E. coli*	Diarrhea
	Antigen CS6	CssC/CssD	*E. coli*	Diarrhea
	Myf fimbriae	MyfB/MyfC	*Y. enterolitica*	Enterocolitis
	PH 6 antigen	PsaB/PsaC	*Y. pestis*	Plague
	CS3 pili	CS3-1/CS3-2	*E. coli*	Diarrhea
	Envelope antigen F1	Caf1M/Caf1A	*Y. pestis*	Plague
	Nonfimbrial adhesins I	NfaE/NfaC	*E. coli*	UTI newborn meningitis
	SEF14 fimbriae	SefB/SefC	*S. enteritidis*	Gastroenteritis
	Agregative adherence fimbriae I	AggD/AggC	*E. coli*	Diarrhea
	AFA-III	AfaB/AfaC	*E. coli*	Pyelonephritis
Alternate chaperone pathway	CS1	CooB/CooC	*E. coli*	Diarrhea
	CS2 pili	CotB/CotC	*E. coli*	Diarrhea
	CS4 pili		*E. coli*	Diarrhea
	CS5 pili	CsfB/CsfC	*E. coli*	Diarrhea
	CS14 pili		*E. coli*	Diarrhea
	CS17 pili		*E. coli*	Diarrhea
	CS19 pili		*E. coli*	Diarrhea
	CFA/I pili	CfaA/CfaC	*E. coli*	Diarrhea
	Cable type II pili	CblB/CblC	*B. cepacia*	Opportunistic in cystic fibrosis patients
	Typhi colonization factor (Tcf)	SafB/SafC	*S. enterica* ser. Typhi	Typhoid

(*Continued*)

Table 1 Continued

Assembly pathway	*Structure*	*Assembly gene products*	*Organism*	*Disease(s) associated with pilus expression*
Type II secretion pathway	Type IVa pili	General secretion apparatus (main terminal branch) 14 to >20 proteins	*Neisseria* spp.	Gonorrhea, meningtitis
			P. aeruginosa	Opportunistic pathogen
			Moraxella spp.	Conjuntivitis, respiratoryinfections
			D. nodosus	Ovine footrot
			F. tularensis	Tularemia
			E. corrodens	Opportunistic pathogen
			L. pneumophila	Legionnaires' disease
			M. xanthus	Saprophyte
	Type IVa pili		*M. bovis*	Tuberculosis in cattle
			Azoarcus spp.	Denitrification and toluenemetabolism
			B. bacteriovorus	Bacterial pathogen
			X. fastidiosa	Plant diseases
			V. parahaemolyticus	Gastroenteritis
			V. vulnificus	Gastroenteritis
	Type IVb pili	General secretion apparatus (Main terminal branch) 14 to >20proteins	*E. coli*	Diarrhea
	Bundle forming pililongus		*E. coli*	Diarrhea
			E. coli	Diarrhea
	CFA/III		*E. coli*	
	R64 and CollB-P9 pili			
	Toxin coregulatedpili colonization factor(CfcA)		*V. cholera*	Cholera
			C. rodentium	Murine colonic hyperplasia
			S. enterica ser. Typhi	
	PilS pili			Typhoid
Conjugative pilus assembly (Type IV secretion) pathway	F pili (IncF1)IncN, IncP, IncW-encoded pili T pili	Type IV export apparatus, 12 to 16 proteins	*E. coli*	Antibiotic resistance
			E. coli	
			A. tumefaciens	Crown gall disease
			R. felis	Rickettsiosis
Extracellular Nucleation/ precipitation pathway	Curli	CsgG/CsgE/CsgF	*E. coli*	Sepsis
	Tafi (thin aggregativefimbrae)	AgfG/AgfE/AgfF	*S. enterica* ser. Typhimurium	Gastroenteritis
Type III secretion pathway	EspA pilus-likestructures	Type III secretion apparatus, ~20 proteins	*E. coli*	Diarrhea
	Hrp pili		*P. syringae*	Hypersensitive response (inresistant plants)
	Hrp pili		*E. amylovora*	Hypersensitive response
			X. campestris	Hypersensitive response
			R. solanacearum	Hypersensitive response
			Rhizobium spp.	Symbionts of legumes
			S. fredii	Symbionts of legumes
Pili in Gram-positive bacteria				
Sortases catalyzing transpeptidase reactions	SpaA-like pili	Sortases and pilins (SpaA, SpaB, SpaC, SpaD and SpaH)	*S. gordonii*	Dental plaque formation/ Endocarditis
			S. oralis	Dental plaque formation/ Endocarditis
			Corynebacterium spp.	Diphtheria/Oral pathogens
			Streptococcus spp.	Numerous diseases
			Actinomyces spp.	
Unknown			*A. photogonimos*	Rumen bacteria
			R. albus	
	M. tuberculosis pili (MTP)		*M. tuberculosis*	Tuberculosis

[a]Chaperone/usher for chaperone–usher and alternate chaperone pathway.

Molecular Architecture

P and type 1 are both composite structures consisting of a thin fibrillar tip joined end to end to a right-handed helical rod (Figure 1(a)–1(f)). Chromosomally located gene clusters that are organizationally and functionally homologous encode P and type 1 (Figure 1(g)–1(h)). P pili are encoded by the *pap* (pilus associated with pyelonephritis) gene cluster, whereas type 1 are encoded by the *fim* gene cluster. The P pilus tip is a 2-nm-wide structure composed of a distally located adhesin (PapG), a tip pilin (PapE), and adaptor pilins (PapF and PapK). The PapG adhesin binds to Galα(1–4)Gal moieties present in the globoseries (GbO4) of glycolipids found on the surface of erythrocytes and kidney cells. Consistent with this binding specificity, P pili are major virulence factors associated with pyelonephritis caused by uropathogenic *E. coli*. The minor pilin PapF is considered to join the PapG adhesin to the tip fibrillum, the bulk of which is made up of a polymer of PapE subunits. PapK is believed to terminate the growth of the PapE polymer and to join the tip structure to the rod. The pilus rod is composed of multiple PapA subunits joined end to end and then coiled into a right-handed, 6.8-nm-thick helical rod, having a pitch distance of 24.9 Å and 3.28 subunits per turn. The rod is terminated by a minor subunit, PapH, which may serve to anchor the pilus in the membrane.

Similar to the P pilus structure, the type 1 pilus has a short, 3-nm-wide fibrillar tip made up of the mannose-binding adhesin, FimH, and two additional pilins, FimG and FimF. The FimH adhesin mediates attachment to mannosylated receptors expressed on a wide variety of cell types and has been shown to be a significant virulence determinant for the development of cystitis. Like other pilus-associated adhesins, FimH has two distinct domains, a C-terminal pilin domain that links the adhesin to the pilus and an N-terminal receptor-binding or lectin domain (Figure 2). The adhesin domain of FimH is an elongated 11-stranded β-barrel with an overall jellyroll-like topology and a tip-localized mannose-binding pocket. The type 1 tip fibrillum is joined to a rod comprising

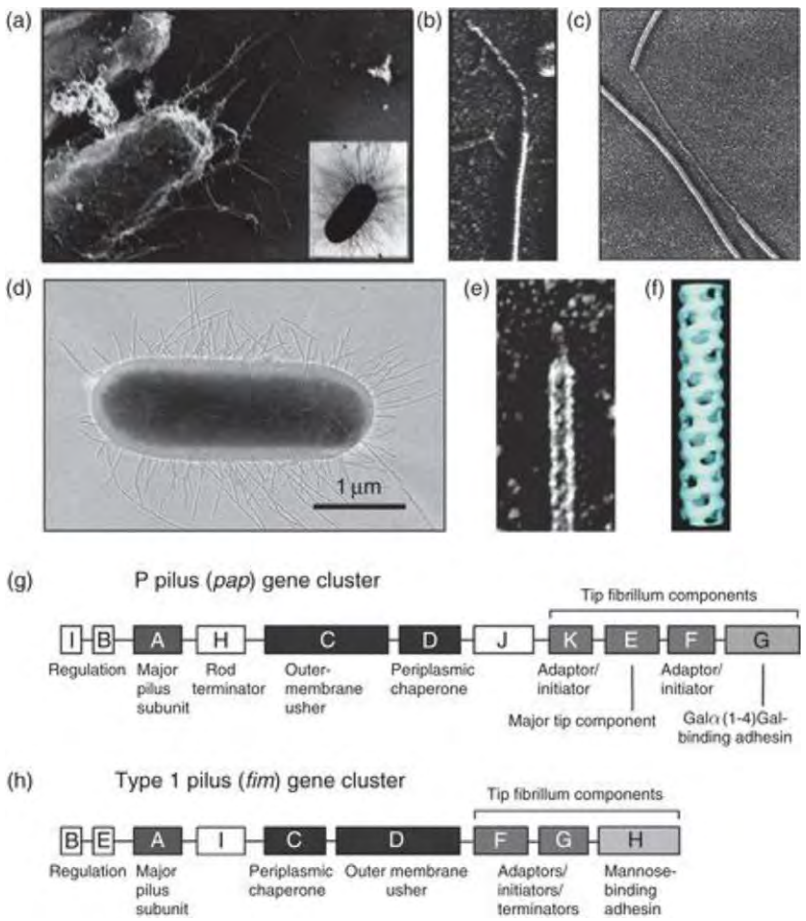

Figure 1 P and type 1 architecture and genetic organization. (a) High-resolution electron micrograph of *Escherichia coli* expressing P pili. The inset shows a negatively stained P piliated *E. coli* bacterium as seen by standard transmission electron microscopy. (b) The tip fibrillum structure of a P pilus. (c) P pili are able to unravel into linear fibers, a phenomenon that may help prevent their breakage under high shearing forces or other stresses. (d) *E. coli* expressing type 1. (e) A high-resolution view of the type 1 pilus tip fibrillum, and (f) a three-dimensional reconstruction of a type 1 pilus rod. The images shown are at different magnifications. The P (*pap*) and type 1 (*fim*) pilus gene clusters are depicted in (g) and (h), respectively. These gene clusters share organizational as well as functional homologies. Photos in (d) and (f) were provided by G. Capitani (Capitani G, Eidam O, Glockshuber R, and Grütter MG (2006). *Microbes and Infection* 8: 2284–2290) and S. Müller (Hahn E, Wild P, Hermanns U, Sebbel P, Glockshuber R, Häner M, Taschner N, Burkhard P, Aebi U, and Müller SA (2002). *Journal of Molecular Biology* 323: 845–857), respectively, with permission from Elsiever Ltd.

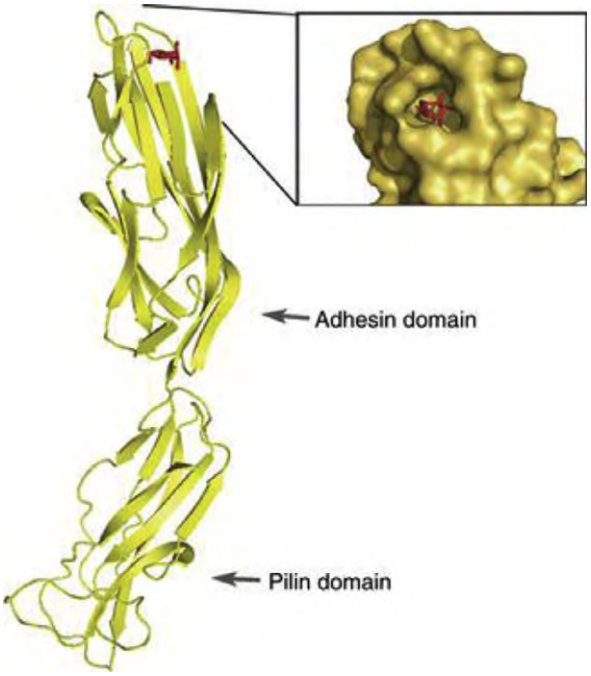

Figure 2 Structure of the type 1 pilus adhesin FimH. Shown on the left is a ribbon model of the FimH adhesin, consisting of two distinct domains. The pilin domain has an immunoglobulin-like fold and allows for incorporation of FimH into the pilus tip. The adhesin domain consists of an 11-stranded β-barrel with an interrupted jellyroll-like motif. This domain is about 50 Å in length and has a distally localized pocket that binds D-mannose (red). A surface model of the mannose-binding pocket of FimH, with bound D-mannose (red), is shown on the right.

predominantly of 500–3000 FimA subunits arranged in a 6- to 7-nm-diameter helix with a pitch distance of 23.1 Å and 3.4 subunits per turn. Both type 1 and P pilus rods have central axial holes with diameters of 2–2.5 and 1.5 Å, respectively. Despite architectural similarities, type 1 appear to be more rigid and prone to breaking than P pili.

In both P and type 1, the major pilin subunits comprising the rods are organized in a head-to-tail manner. Additional quaternary interactions between subunits in adjacent turns of the helical rod appear to stabilize the structure and may help drive the outward growth of the organelle during pilus assembly (see below). Disruption of the latter interactions by mechanical stress or by incubation in 50% glycerol can cause the pilus rod to reversibly unwind into a 2-nm-thick linear fiber similar in appearance to the tip fibrillum (Figure 1(c)). It has been proposed that the ability of the pilus rods to unwind allows them to support tension over a broader range of lengths. This may help P and type 1 better withstand stress, such as shearing forces from the bulk flow of fluid through the urinary tract, without breaking. Type 1 are able to helically unwind and thereby resist breakage under forces up to 60 pN, which is equivalent to the weight of about 60 red blood cells.

In addition to composite structures exemplified by P and type 1, chaperone–usher pathways also mediate assembly of thin fibrillae such as K88 and K99 pili and nonfimbrial adhesins. K88 and K99 pili are 2- to 4-nm-thick fibers that mediate adherence to receptors on intestinal cells. They are significant virulence factors expressed by enterotoxigenic *E. coli* (ETEC) strains that cause diarrheal diseases in livestock. These pili were given the 'K' designation after being mistakenly identified as K antigens in *E. coli*. In contrast to P and type 1, the adhesive properties of K88 and K99 pili are associated with the major pilus subunits. The receptor-binding epitopes on the individual major pilus subunits are exposed on the pilus surface and are available for multiple interactions with the host tissue. In general, pili with adhesive major subunits, such as K88 and K99 pili, are thin, flexible fibrillar structures. In comparison, pili with specialized adhesive tip structures, such as P and type 1, are relatively rigid and rod-like.

Assembly Model

The assembly of P pili by the chaperone/usher pathway is among the best understood of any pilus assembly pathway. PapD is the periplasmic chaperone and PapC is the outer-membrane usher for the P pilus system. These proteins are prototypical representatives of the periplasmic chaperone and outer-membrane usher protein families. Figure 3 presents the current model for pilus assembly by the chaperone/usher pathway, as depicted for P pili.

Periplasmic chaperones

The PapD chaperone, the outer-membrane PapC usher, and all of the P pilus structural subunits have typical signal sequences recognized by the *sec* (general secretion) system. The signal sequences are short, mostly hydrophobic N-terminal motifs that tag proteins for transport across the inner membrane by the *sec* system. This system includes several inner-membrane proteins (SecD-G,

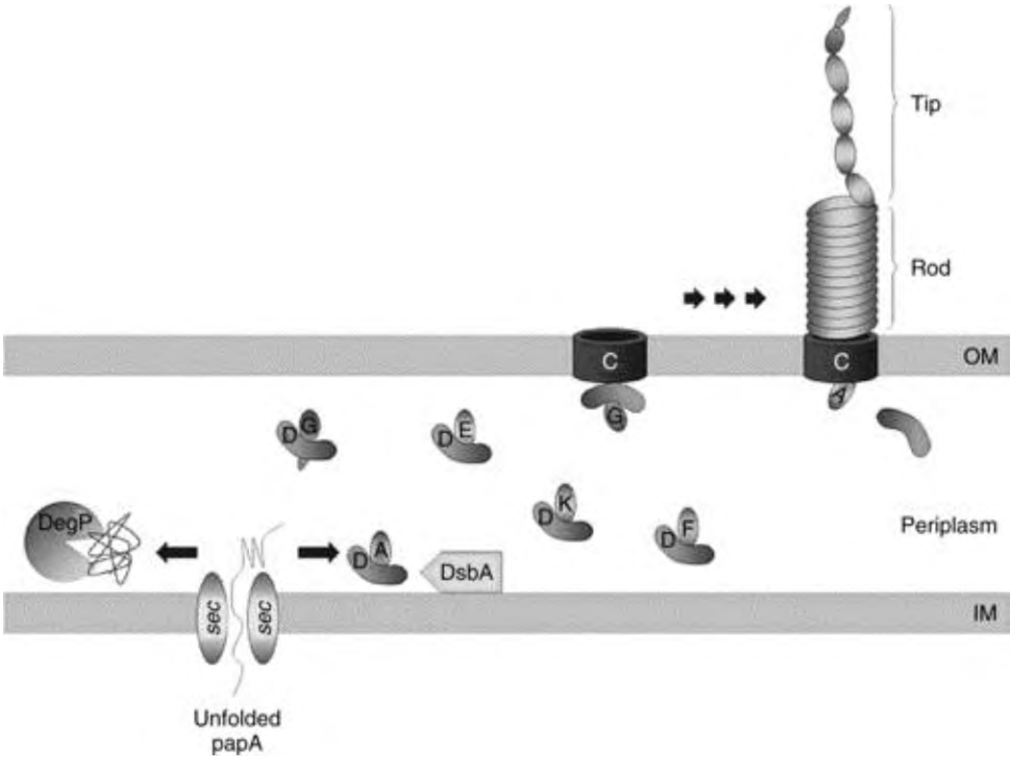

Figure 3 Model of P pilus assembly by the chaperone/usher pathway. Structural subunits for the pilus tip (PapG, PapF, PapE, and PapK) and for the pilus rod (PapA) are translocated across the inner membrane of *Escherichia coli* via the *sec* system. On the periplasmic side of the inner membrane they interact with the chaperone PapD, which facilitates the folding and release of the subunits from the inner membrane. DsbA is required for the proper folding of both the subunits and the PapD chaperone. In the absence of the PapD chaperone, pilus subunits aggregate and are degraded by the periplasmic protease DegP. Subunit–chaperone complexes are targeted to the outer-membrane usher, PapC, where the chaperone is released and subunit–subunit interactions occur. The PapC usher forms a 2-nm-wide pore through which the assembled pilus structure is extruded as a linear fiber across the outer membrane. Once on the exterior of the cell, the linear PapA polymer forms a thick, right-handed helical rod that is unable to slip back through the usher pore. The formation of the coiled PapA rod is thought to help drive the outward growth of the pilus. OM indicates outer membrane and IM indicates inner membrane.

SecY, and YajC), a cytoplasmic chaperone (SecB) that binds to presecretory target proteins, a cytoplasmic membrane-associated ATPase (SecA) that provides energy for transport, and a periplasmic signal peptidase. As the P pilus structural subunits emerge from the *sec* translocation machinery into the periplasm, PapD binds to each subunit, facilitating its release from the inner membrane. Each subunit forms an assembly competent, one-to-one complex with PapD. Proper folding of the subunits requires PapD and involves the action of the periplasmic disulfide bond isomerase DsbA. In the absence of PapD, subunits misfold, aggregate, and are subsequently degraded by the periplasmic protease DegP. The misfolding of P pilus subunits is sensed by the Cpx two-component system in which CpxA is an inner-membrane-bound sensor histidine kinase and CpxR is a DNA-binding response regulator. Activation of the Cpx system in response to misfolded pilin subunits or other types of periplasmic stress inhibits P pilus biosynthesis by downregulating pilin expression.

The three-dimensional crystal structures of both the PapD and FimC chaperones have been solved. Both chaperones consist of two Ig (immunoglobulin)-like domains, each consisting of seven β-sheets, oriented into a boomerang shape such that a subunit-binding cleft is created between the two domains. A conserved internal salt bridge is thought to maintain the two domains of the chaperone in the appropriate orientation. Using genetics, biochemistry, and crystallography, PapD was found to interact with pilus subunits in part by binding to a highly conserved motif present at the C-terminus of all subunits assembled by PapD-like chaperones. The finer details of how PapD-like chaperones interact with pilus subunits were unveiled by determining the crystal structures of the PapD–PapK and the FimC–FimH chaperone–subunit complexes (Figure 4). This work demonstrated that the PapD and FimC chaperones have similar interactions with their respective subunits. Only the PapD–PapK structure is considered here. PapK has a single domain consisting of an Ig fold that lacks the seventh (C-terminal) β-strand that is present in canonical Ig folds. The absence of this strand produces a deep groove along the surface of the pilin subunit and exposes its hydrophobic core. By an interaction known as donor strand complementation, a G_1 β-strand of the PapD chaperone occupies the groove in PapK and completes the Ig fold. This interaction shields the hydrophobic core of PapK and stabilizes immature pilus subunits within the periplasm. Interactions with PapD-like chaperones also accelerate the folding of pilin subunits about 100-fold, allowing for more rapid assembly of pili. The residues that make up the C-terminal groove formed by subunits and bound by PapD-like chaperones have been shown by mutagenesis and, more recently, by crystallographic studies to be involved in subunit–subunit interactions

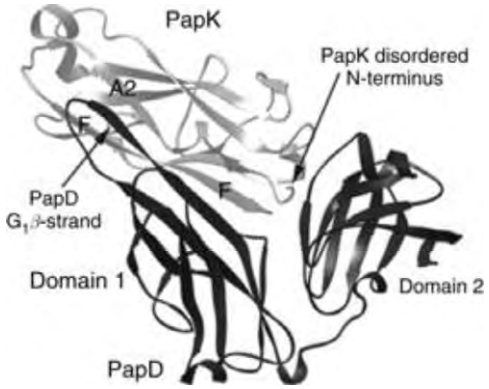

Figure 4 Ribbon model based on the crystal structure of the PapD periplasmic chaperone (in black) complexed with the PapK pilus subunit (in gray). The G_1 β-strand in domain 1 of PapD completes the Ig fold of the pilin, occupying the groove formed between the A2 and F strands of PapK. This interaction has been termed donor strand complementation. The eight N-terminal amino acids of PapK are disordered in the structure. These residues have been implicated in mediating subunit–subunit interactions within the mature pilus organelle. During pilus biogenesis, the N-terminal strand of a pilin can displace the G_1 β-strand of the chaperone and insert into the C-terminal groove of the neighboring subunit. The mature pilus thus consists of a linear array of canonical Ig domains, each of whose fold is completed by a strand from the neighboring subunit.

within the final pilus structure. Thus, in addition to stabilizing immature pilus subunits, the donor strand complementation interaction also caps one of the interactive surfaces of the subunit and prevents premature oligomerization and aggregation of pilus subunits within the periplasm.

Outer-membrane ushers

Once formed in the periplasm, chaperone–subunit complexes are targeted to the outer-membrane usher where the chaperone is released, exposing interactive surfaces on the subunits that facilitate their assembly into the pilus. Studies in the P and type 1 pilus systems have demonstrated that the adhesin–chaperone complexes, PapDG or FimCH, bind tightest and fastest to the usher and that the adhesins are the first subunits assembled into the pilus. Binding of the chaperone–adhesin complex induces a conformational change in the usher, possibly priming it for pilus assembly. Additional subunits are incorporated into the pilus depending, in part, upon the kinetics with which they are partitioned to the usher in complex with the chaperone. Conserved N-terminal regions, in addition to the conserved C-terminal motif of the pilus subunits, mediate subunit–subunit interactions within the mature pilus. Differences in the complementary surfaces in these conserved regions from one subunit to another may help dictate which of the subunits can be joined to one another during pilus assembly. Thus, the order of the subunits within the final pilus structure is determined by the specific contacts made between the different pilus subunits and also by the differential affinities of the various chaperone–subunit complexes for the usher.

In addition to acting as an assembly platform for the growing pilus, the usher protein appears to have additional roles in pilus biogenesis. High-resolution electron microscopy revealed that the PapC usher is assembled into an oligomeric 15-nm-diameter ring-shaped complex with a 2-nm-wide central pore. Each PapC monomer is composed predominantly of transmembrane β-sheets, typical of outer-membrane pore-forming proteins. The N-terminal domain of PapC binds chaperone-complexed subunits, whereas the C-terminal domain stabilizes the chaperone–subunit complexes and is involved in subsequent stages during pilus assembly. Oligomerization of the usher and subsequent pilus biogenesis are both initiated by interactions between the adhesin and the usher. During assembly of type 1, the FimD usher directs the receptor-binding domain of the FimH adhesin into the pore of the usher, whereas the pilin domain remains bound to the chaperone until displaced by the next incoming chaperone–subunit complex. After dissociating from the chaperone at the usher, subunits are incorporated into a growing pilus structure that is predicted to be extruded as a 1-subunit-thick linear fiber through the central pore of the usher complex. Packaging of the linear pilus fiber into a thicker, helical rod on the outside surface of the bacterium may provide a driving force for the translocation of the pilus across the outer membrane, possibly acting as a sort of ratcheting mechanism to force the pilus to grow outward. Combined with the targeting affinities of the chaperone–subunit complexes for the usher and the binding specificities of the subunits for each other, this may provide all the energy and specificity needed for the ordered assembly and translocation of pili across the outer membrane.

Alternate Chaperone Pathway

A variation of the chaperone/usher pilus assembly pathway has been identified in strains of ETEC and a few other pathogenic bacteria. ETEC are major pathogens associated with diarrheal diseases of travelers, infants, and young children. Strains of ETEC produce several types of uniquely assembled adhesive pili that are considered to be important mediators of bacterial colonization of the intestine. The best studied of these pili is coli surface antigen 1 (CS1), which appears to be composed predominantly of a major subunit, CooA, with a distally located minor component, CooD, that is required for ETEC adherence to host cells. Several CS1-like

pili have been identified and include CS2, CS4, CS14, CS17, CS19, CFA/I (colonization factor I, expressed by various ETEC strains), Tcf (expressed by *Salmonella enterica* serovar Typhi), and the cable type II pili of *Burkholderia cepacia*, an opportunistic pathogen of cystic fibrosis patients. Four linked genes, *CooA*, *CooB*, *CooC*, and *CooD*, are the only specific genes required for the synthesis of functional CS1. Electron microscopic examination reveals that the CS1-like pili are architecturally similar to P and type 1 assembled by the chaperone/usher pathway (Figure 5(a) and 5(b)), although none of the proteins involved in the biogenesis of CS1-like pili have any significant sequence homologies to those of any other pilus system.

The assembly of CS1-like pili is functionally similar to that of P pili but depends on a specialized set of periplasmic chaperones that are distinct from those of the chaperone/usher pathway described above. Therefore, this mode of pilus assembly is referred to as the alternate chaperone pathway. In the case of CS1 the chaperone CooB binds to and stabilizes the major and minor pilin subunits, CooA and CooD, which enter into the periplasm in a *sec*-dependent fashion (Figure 5(c)). Both CooA and CooD share a conserved sequence motif near their C-termini that may function as a chaperone recognition motif. One of the functions of CooB appears to be the delivery of the pilin subunits to an outer-membrane protein, CooC, which may function as a channel, or usher, for the assembly of pilus fibers. In addition to the pilin subunits, CooB also binds to and stabilizes CooC in the outer membrane. In the absence of the CooB chaperone, CooC and the pilin subunits are degraded. The minor subunit CooD localizes to the tip of the CS1 pilus and is required for secretion of the major pilus subunit CooA. Expression of CooD also seems to positively regulate the number of pili produced, in keeping with a model in which the positioning of a minor tip subunit (CooD in this case) by an outer-membrane usher complex is required to initiate assembly of the pilus shaft. As CS1-like pili do not appear to be related to those assembled by the chaperone/usher pathway, it has been suggested that these two pilus assembly systems arose independently through convergent evolution.

Type II Secretion Pathway for Type IV Pilus Assembly

Type IV pili are multifunctional, strong, and flexible filamentous structures expressed by a wide diversity of bacteria, including a number of animal and plant pathogens. These include *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Eikenella corrodens*, *Legionella pneumophila*, *Ralstonia solanacearum*, *Myxococcus xanthus*, *Francisella novicida*, *Francisella tularensis*, *Dichelobacter nodosus*, *Synechocystis* spp., *Moraxella* spp., and *Azoarcus* spp. Type IV pili can promote bacterial survival and pathogenesis by mediating bacterial interactions with animal, plant, and fungal cells, in some cases contributing to bacterial invasion of target host cells. In addition, these pili can modulate target cell specificity, function in DNA uptake, promote bacterial autoaggregation and biofilm formation, and act as receptors for bacteriophage. Type IV pili are also associated with a flagella-independent form of bacterial locomotion called twitching motility (also known as social motility in *Myxococcus* spp.) that allows for the lateral spread of bacteria across a surface.

Type IV pili are 6 nm in diameter and can extend up to several micrometers in length. These pili are typically assembled at one pole of the bacterium and can withstand forces up to 100 pN. Based on the length and amino acid sequence of the pilin subunits, type IV pili can be divided into two subclasses: type IVa and type IVb. Type IVa pilin subunits are usually composed of 145–160 amino acids. These subunits have several distinctive features, including a short (5–6 amino acids), positively charged leader

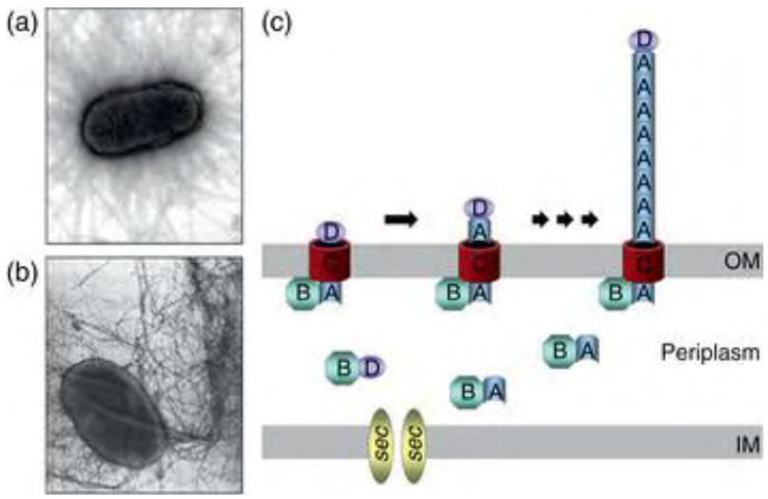

Figure 5 Pili assembled by the alternate chaperone pathway. Transmission electron micrographs show (a) CS2 pili (~2 nm thick) assembled by the alternate chaperone pathway in *Escherichia coli* (photo courtesy of Harry Sakellaris and June R. Scott) and (b) Cbl pili of *Burkholderia cepacia*. Reproduced from Sajjan US, Sun L, Goldstein R, and Forstner JF (1995). *Journal of Bacteriology* 177: 1030–1038, with permission from ASM press. (c) Assembly of CS1 from *E. coli* via the alternate chaperone pathway. The CooB chaperone forms periplasmic complexes with the main components of the pilus, CooA and CooD. CooB also appears to bind and stabilize the outer-membrane protein CooC during pilus assembly. CooC functions as an outer-membrane (OM) channel for passage of the pilin fiber.

sequence that is cleaved during assembly, *N*-methyl-phenylalanine as the first residue of the mature subunit, and a highly conserved, hydrophobic N-terminal domain that forms the core of the mature pilus.

Type IVb pili include the toxin coregulated pilus (TcpA) of *Vibrio cholera*, bundle-forming pilus (BfpA) of enteropathogenic *E. coli* (EPEC), longus (LngA) and CFA/III pili of ETEC, conjugative R64 thin pilus of *E. coli*, and colonization factor Citrobacter (CfcA) of *Citrobacter rodentium*. In contrast to type IVa pilins described above, the known type IVb pilins are somewhat larger (180–238 amino acids) and have a longer (15–30 amino acids) leader sequence. Also, in place of *N*-methyl-phenylalanine as the first amino acid in the mature pilus subunit, type IVb subunits have other methylated residues such as *N*-methyl-methionine for TcpA and *N*-methyl-leucine for BfpA. TCP, BFP, and longus pili form large polar bundles over 15 μm in length. In contrast, CFA/III pili are 1–10 μm long and are peritrichously expressed.

Atomic resolution structures of the type IVa pilins PilE (*N. gonorrhoeae*) and PilA (*P. aeruginosa* strains K and K122-4), as well as the type IVb pilin TcpA (*V. cholera*), have been solved (Figures 6 and 7). Based on these structures, all type IV pilin subunits are predicted to have a fairly similar structure consisting of a conserved N-terminal α-helical hydrophobic core surrounded by β-sheets and a hypervariable C-terminus. The core α-helix of type IV pilins, known as α1 (blue in Figure 6), is usually composed of 53 amino acids and is divided into a protruding hydrophobic N-terminal half (α1-N, residues 1–29) and an amphipathic C-terminal half (α1-C, residues 30–53). The hydrophobic face of α1-C is wrapped by antiparallel β-sheets to form a globular head domain, whereas the hydrophilic face is buried within the assembled pilus. This hydrophobic packing of the inner core of α-helices along with the flexibility of these helices may permit type IV pili to bend and adopt twisted, bundled conformations (Figure 7). Hydrogen bonds throughout the layer of β-sheets may provide much of the mechanical stability for the pilus.

Type IV pilin globular head domain (green in Figure 6) is flanked by two highly variable regions designated the αβ-loop (orange in Figure 6) and D-region (yellow in Figure 6). The αβ-loop functions as a linker between the α1 and the β-sheets, and facilitates pilin–pilin interactions before and after assembly. The D-region contains a conserved disulfide bridge, which is essential for pilus assembly. These hypervariable regions associate with the layer of β-sheets through only a few conserved interactions. Thus, they can be structurally pliant and accommodate extreme amino acid changes that lead to antigenic variation and altered binding specificities without disrupting the assembly of the pilus. The antigenic characteristics of type IV pili synthesized by *N. gonorrhoeae* can be modified extensively by a remarkable mechanism. This pathogen encodes more than 15 different silent pilin genes termed PilS that lack the invariant N-terminal domain present in PilE. By recombination of silent PilS genes with the PilE locus, a single neisserial strain can theoretically express more than 10 million PilE variants. This antigenic variation helps *N. gonorrhoeae* evade the host immune system, allowing the establishment and maintenance of infection.

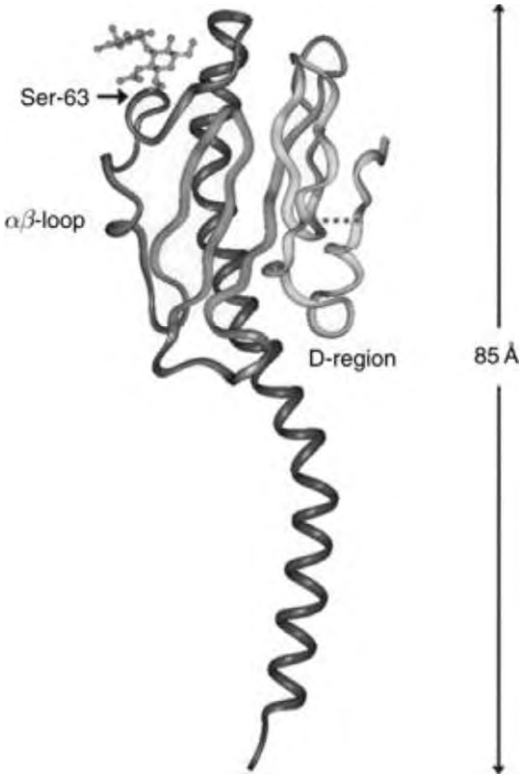

Figure 6 Ribbon model of the type IVa pilin, PilE, from *Neisseria gonorrhoeae*. Secondary structural elements include a hydrophobic core surrounded by β-sheets (green), a conserved N-terminal α-helical spine (blue) connected to a variable domain containing an αβ-loop (orange) with an O-linked disaccharide at Ser-63 and a disulfide-containing C-terminal D-region (yellow). The conserved disulfide bridge in the D-region is signified by a dotted line (magenta).

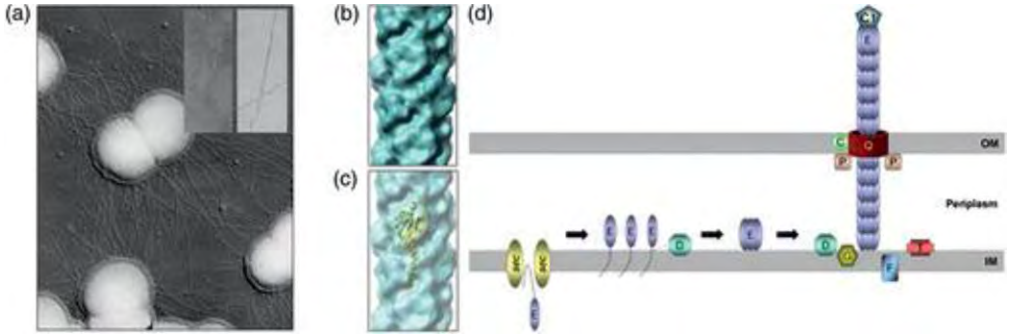

Figure 7 Type IV pili. (a) Scanning electron micrograph of *Neisseria gonorrhoeae* diplococci expressing peritrichous type IV pili. Insets show close-up views of the pili. (b) Three-dimensional reconstruction of type IV pilus rod, which is made up of repeating and overlapping PilE pilin subunits. (c) Localization of a single PilE subunit (yellow) within the pilus rod. Image courtesy of L. Craig and C. Brinton (Craig L, Volkmann N, Arvai AS, Pique ME, Yeager M, Egelman EH, and Tainer JA (2006). *Molecular Cell* 23: 651–662), with permission from Elsiever Ltd. (d) Model of type IV pilus assembly by *N. gonorrhoeae*. The PilE prepilin is translocated into the periplasm aided by the *sec* machinery. PilE is processed by the PilD signal peptidase, which cleaves the positively charged leader sequence from the N-terminus of the pilin subunit. An inner-membrane assembly complex then assembles the mature PilE subunit into a pilus fiber. PilQ mediates translocation of the pilus through the outer membrane, possibly with the assistance of other factors such as PilP. PilC associates with the pilus and may act as a restraining clip, preventing PilT-mediated retraction of the type IV pilus rod. The PilC1 adhesin, which appears to be incorporated at the tip of the growing pilus fiber, also seems to be required for translocation of the pilus through the outer membrane.

The biogenesis of type IV pili is substantially more complicated than pilus assembly by the chaperone/usher or alternate chaperone pathways. Most type IVa pili-encoding genes are located on the chromosome, whereas most type IVb pili are encoded by plasmids. The number of genes essential for type IV pilus biogenesis and function ranges from 14, for pili such as BFP, to over 20, for structures such as the type IVa pili of *N. gonorrhoeae*. In *P. aeruginosa*, it is estimated that about 0.5% of the bacterium's genome is involved in the synthesis and function of type IV pili. Among the various bacterial species expressing type IV pili, the genes encoding the type IV pilus structural components tend to be similar, whereas the regulatory components surrounding them are typically less conserved.

Type IV pilus biogenesis occurs in three steps: fiber formation, fiber stabilization, and surface localization of the intact organelle. In the case of *N. gonorrhoeae* pili, the process requires more than 14 assembly proteins and a type II secretion system-related multicomponent assembly complex. Components include a prepilin peptidase (PilD) that cleaves off the leader peptide from nascent pilin subunits; a polytopic inner-membrane protein that may act as a platform for pilus assembly; a hydrophilic nucleotide-binding protein located in the cytoplasm or associated with the cytoplasmic face of the inner membrane that may provide energy for pilus assembly; and an outer-membrane protein complex (PilQ) that forms a pore for passage of the pilus to the exterior of the bacterium.

The PilE/PilA propilin subunits are transported into the periplasm by the *sec* translocation machinery (Figure 7(d)). After translocation, the propilin subunits remain anchored in the inner membrane by their hydrophobic N-terminal α-helical domains, with their hydrophilic C-terminal heads oriented toward the periplasm. Removal of the positively charged propilin leader sequence by the PilD signal peptidase drives the hydrophobic stems of the pilin subunits to associate and form a pilus. An inner-membrane assembly complex made up of several proteins including PilD, PilF, PilG, and PilT aids in this process. The outer-membrane-associated PilC has been shown to stabilize the nascent pilus before translocation and prevent PilT-mediated retraction. The assembled pilus penetrates the outer membrane through an electrochemically gated oligomeric channel formed by PilQ. The ring-shaped oligomeric channel formed by PilQ has 12-fold symmetry and an internal diameter of 5–7 nm. Its assembly and localization is facilitated and stabilized by small lipoproteins, PilP in *N. gonorrhoea*, BfpG in EPEC, and Tgl in *M. xanthus*. The PilC1 adhesin associated with the tips of type IV pili in *N. gonorrhoea* may facilitate passage of the nascent pili through the PilQ pore.

One implication of this assembly model is that the N-terminal region of PilA/PilE resides in a continuous hydrophobic environment during both inner-membrane transport and pilus assembly. This may allow polymerization and, interestingly, depolymerization of the pilus to proceed with only minimal energy requirements. Type IV pili undergo rounds of extension and retraction by polymerization and depolymerization reactions. The nucleotide-binding protein PilB facilitates the polymerization and extension of pili, whereas PilT (a distant member of the AAA ATPase motor family) mediates rapid depolymerization and retraction, at rates up to 1500 pilin subunits per second. These rounds of extension and retraction are the basis of twitching motility, one of the functions of type IV pili. The current model for twitching motility can be compared to the usage of a grappling hook. It involves the extension of a pilus, the adhesion of the pilus tip to a surface, and then the retraction of the pilus to pull the cell forward. The capacity of type IV pili to retract may also provide a means for transforming DNA, which could potentially interact with the type IV pilus, to enter into the bacterial cell. Mutation in components of the type IV pilus PilT in *N. gonorrhoea* results in the loss of transformability. Naturally competent bacteria such as *Bacillus subtilis*, *H. influenzae*, and *S. pneumoniae* do not have type IV pili, but do have similar components that facilitate the transformation of naked DNA. The adhesive properties of type IV pili are, in general, determined by the major pilus subunit. Additional minor components, however, may associate with these pili and alter

their binding specificities. In the case of *Neisseria*, a tip-localized adhesin, PilC1, appears to mediate bacterial adherence to epithelial cells.

Many of the components involved in type IV pilus assembly share homology with proteins that are part of DNA uptake and protein secretion systems, collectively known as the main terminal branch of the general secretory (*sec*-dependent) pathway, or type II secretion. Type II secretion systems transport a variety of proteins, including toxins, proteases, cellulases, and lipases, from the periplasm to the extracellular space. The pullulanase secretion system in *Klebsiella oxytoca* is a prototypical type II secretion system. Transport of pullulanase (PulA, a starch-hydrolyzing lipoprotein) through the outer-membrane protein complex (PulD) involves four type IV pilin-like proteins, known as pseudopilins. Like type IV pilins, the pseudopilins are processed by a prepilin peptidase; the peptidase is even interchangeable between the two systems. The pseudopilins may assemble into a 'secretion tube' – a pilus-like structure that extends across the periplasm and facilitates PulA transfer to the outer membrane. Pseudopilin filaments have been observed upon overexpression of the pseudopilin genes, but have not yet been demonstrated to exist under normal conditions.

Another structure that is believed to have evolutionary relatedness to type IV pili is the archaeal flagellum. Archaeal flagella are about 10 nm in diameter, and, like bacterial flagella, rotate and switch directions to mediate motility. However, orthologues of bacterial flagellin motor proteins have not been found in any archaeal genome, and the mechanism of rotation and switching in archaea is still unknown. Archaeal flagellar subunits have no similarity to bacterial flagellins, but they are similar to type IV pilins. Like type IV pilins, archaeal flagellins require processing by a signal peptidase before assembly into a filament, and their assembly requires both a nucleotide-binding protein and a polytopic cytoplasmic membrane protein.

Conjugative Pilus Assembly Pathway

In Gram-negative bacteria, certain pili, collectively known as conjugative pili, facilitate the transfer of DNA among bacteria. These pili allow donor and recipient bacteria to make specific and stable intercellular contacts before DNA transfer is initiated. DNA is moved between cells through the mating pair formation system, which is related to the so-called type IV secretion systems (T4SS). Horizontal gene transfer, or conjugation, mediated by conjugative pili is inextricably associated with the spread of antibiotic resistance among bacterial pathogens. Conjugative pili are generally encoded by self-transmissible plasmids that are capable of passing a copy of their genes to a recipient bacterium. Closely related plasmids, with similar replication control systems, are unable to coexist within the same cell. This property has been termed 'incompatibility' and provides the primary basis for cataloguing conjugal plasmids and the pili that they encode. Thus far, in *E. coli* alone, over 25 incompatibility groups composed of well over 100 different plasmids have been defined. Plasmids within a particular incompatibility group usually encode conjugative pili with similar antigenic properties, sensitivities to pilus-specific phage, and morphologies.

Among the multitude of known incompatibility groups, three morphologically and functionally distinct types of conjugative pili have been defined: (1) rigid; (2) thick, flexible; and (3) thin, flexible pili. Rigid conjugative pili are 8- to 11-nm-wide structures that are usually specified by conjugal DNA transfer systems that function well only on solid surfaces. Thick, flexible pili, on the other hand, are 8- to 11-nm-wide structures that typically, but not always, promote conjugation on solid surfaces and in liquid media equally well. Conjugal DNA transfer promoted by rigid or thick, flexible pili can be enhanced, in some cases, by the presence of thin, flexible pili. These pili are similar in appearance to type IV pili and at least one member of the thin, flexible pilus group (the R64 thin pilus) has been identified at the molecular level as a type IVb pilus (as described above). Thin, flexible pili appear to function primarily in the stabilization of bacterial mating pairs, increasing the rate of DNA transfer. Conjugation does not occur in the presence of thin, flexible pili alone or in the absence of rigid or thick, flexible pili.

The most thoroughly studied conjugative pilus is the F pilus encoded by the self-transmissible, broad host range F (fertility) plasmid, a member of the F1 incompatibility (IncF1) group of plasmids borne by *E. coli*. The F pilus system is prototypical for numerous other conjugation systems, and F pilus biogenesis is distinct from type IV and other pilus assembly pathways. F pili are 8-nm-thick, flexible helical filaments that consists primarily, if not completely, of repeating 7.2 kDa (70 amino acid) TraA pilin subunits. Donor (F^+) cells typically express 1–3 F pili that are usually 1–2 μm long. Each F pilus possesses a 2-nm-wide central channel that is lined by basic, hydrophilic residues, which could potentially interact with negatively charged DNA or RNA molecules during conjugation. TraA is organized into pentameric, doughnut-like disks that are stacked within the pilus such that successive disks are translated 1.28 nm along the pilus axis and rotated 28.8° with respect to the lower disk. The TraA pilin has two hydrophobic domains located toward the center and at the C-terminus of the pilin. The hydrophobic domains are thought to extend as antiparallel α-helices from the central axis to the periphery of the pilus shaft. These domains are separated by a short, basic region that appears to form the hydrophilic wall of the central channel of the pilus. The N-terminal domain of TraA is predicted to face the exterior of the pilus. However, this domain is antigenically masked when the N-terminal residue of TraA is acetylated during maturation of the pilin (see below). This modification is common among all known F-like pilins and appears to cause the N-terminal domain to be tucked back into or along the pilus shaft. Acetylation is not essential for F pilus assembly or function, but does help prevent aggregation of F-like pili and affects the phage-binding characteristics of these organelles. Phage are also known to recognize the C-terminal hydrophobic domain of TraA. Although masked within the pilus shaft, the acetylated N-terminal domain of TraA appears to be exposed in unassembled pilin subunits and at the distal tips of pili. The F pilus tip is believed to initiate contact between donor and recipient cells during conjugation and serves as a receptor for F-specific filamentous phage. Alterations in the N-terminal sequence of pilin subunits provide the primary basis for the antigenic diversity observed among F-like pili expressed by plasmids F, R1-19, ColB2, pED208, and R100-1.

A number of genes encoded by the F plasmid *tra* operon are required for conjugation. TraA is synthesized as a 12.8 kDa (121 amino acids) cytoplasmic propilin, but is processed into a 7.2 kDa pilin form by host signal peptidase LepB in the periplasm. Pilin maturation gene products TraQ and TraX mediate the processing of the TraA pilin to its mature form. TraQ, a chaperone-like inner-membrane protein, escorts TraA into the inner membrane and helps position the TraA propilin for processing into mature pilin. In the absence of TraQ, the translocation of TraA is disrupted and most of the pilin subunits are degraded. After processing, the N-terminal residue (alanine) of TraA is acetylated by TraX, a polytopic inner-membrane protein. The core T4SS proteins, TraB, TraC, TraE, TraG, TraK, TraL, and TraV, are involved in both pilus assembly and DNA transport. Additional gene products (TraW, TraN TraU, TraF, TraH, TrbC, and TrbI) affect the assembly of TraA into the pilus filament. Most of these proteins appear to associate with either the inner or outer bacterial membrane and may constitute a pilus assembly complex that spans the periplasmic space.

The exact mechanism by which TraA is assembled into pili is not yet defined. Pools of up to ~100 000 mature TraA pilins accumulate in the inner membrane before pilus assembly, which appears to occur by addition of pilin subunits at the base of the growing pilus. Both hydrophobic domains of TraA span the inner membrane, with the hydrophilic region of TraA connecting them on the cytoplasmic side. Small clusters of TraA also accumulate in the outer membrane and these may function as intermediates in F pilus assembly and disassembly. Large regions of the TraA sequence have the propensity to assume both β-sheet and α-helical structures, although the α-helical conformation is known to predominate in assembled pili. It has been suggested that a shift between β-sheet and α-helical conformations drives pilus assembly and disassembly. F pilus assembly is energy dependent and the depletion of ATP levels by respiratory poisons such as cyanide results in F pilus depolymerization and retraction. It is possible that TraA is normally cycled between pili and periplasmic/inner-membrane pools by rounds of pilus outgrowth and subsequent retraction. During conjugation, F pilus retraction is believed to serve a stabilizing function by shortening the distance between bacterial mating pairs and allowing for more intimate contact.

Several components of the F pilus assembly machinery share significant homology with proteins encoded by other conjugative systems. These include proteins specified by broad host range plasmids in other incompatibility groups (such as IncN, IncP, and IncW) and many of the proteins encoded by the Ti (tumor inducing) or Ri (root inducing) plasmid-specific *vir* genes of the plant pathogens, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively. These bacteria elaborate 10-nm-wide promiscuous conjugative pili, called T pili, at one end of a cell that direct the interkingdom transfer of a specific genetic element, known as T-DNA, into plant and yeast cells. The introduction of T-DNA into plant cells induces plant tumor formation. The exact mechanism of T-DNA transfer is not known, but the T pilus is essential for T-DNA transfer. T pili may facilitate direct cell-to-cell contact for T-DNA transfer, possibly providing a conduit for passage of T-DNA. In addition, the T pilus could act as a sensor, allowing infecting bacteria to receive signals from the host plant. T pilus assembly by *A. tumefaciens* requires the expression of at least 12 *vir* gene products encoded by the Ti plasmid. VirB2 is the major, and possibly only, component of the T pilus and it is predicted to be structurally homologous to the F pilus subunit TraA. Unlike F pili, T pili do not retract, but instead wind together to form very compact coils, bringing the surface of bacterium and the host closer (Figure 8).

Other than possibly stabilizing donor–recipient interactions, it is not yet clear as to how F and T pili or any pilus structures function in conjugative DNA transfer processes. However, at least in F pili, substantial evidence suggests that pilus components or the pilus itself can serve as a specialized channel for the transmission of DNA and any accompanying pilot proteins across the donor and possibly the recipient cell membranes. In light of this possibility, it is interesting to note that many components of the conjugative pilus systems encoded by the IncF, IncN, IncP, and IncW plasmids and by the *vir* genes of *A. tumefaciens* are similar to the Ptl proteins responsible for the export of the multiple subunit toxin of *Bordetella pertussis*. Interestingly, in *Brucella suis* and *Bartonella henselae*, virB homologues are arranged in the same order as in the *A. tumefaciens virB* operon. Furthermore, these secretion systems seem to be distantly related to transport systems used by *L. pneumophila*, *Helicobacter pylori*, and *Rickettsia prowazekii* to inject virulence factors into host eukaryotic cells. Conjugative pilus systems such as those encoding F and T pili thus appear to be representative of a larger family of macromolecular transport systems. These so-called type IV secretion systems represent a major pathway for the transfer of both nucleic acid and proteins between cells. Understanding how conjugative pili help mediate the intercellular transfer of macromolecules remains a significant challenge.

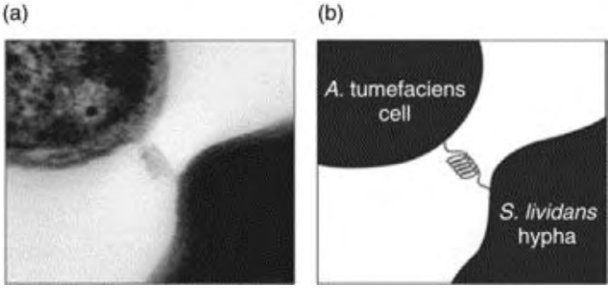

Figure 8 T pilus-mediated attachment of *Agrobacterium tumefaciens* to *Streptomyces lividans*. For clarity, a schematic representation of the electron micrograph showing a coiled T pilus linking the two microbes in (a) is shown in (b). Reproduced from Lai EM and Kado CI (2000). *Trends in Microbiology* 8: 361–369, with permission from Elsevier Ltd.

Extracellular Nucleation/Precipitation Pathway

Many strains of enteric bacteria, including *E. coli* and *Salmonella* species, produce a class of thin (<2 nm), irregular, and highly aggregated surface fibers known as curli (Figure 9(a) and 9(b)). Curli are described as amyloid-like fibers, and are composed mainly of extracellular matrix components, namely protein, cellulose, and an unknown polysaccharide. They are highly stable structures and extreme chemical treatments are required to depolymerize them. The major component of *E. coli* curli is a 15.3 kDa protein known as CsgA, which shares over 86% primary sequence similarity to its counterpart in *S. enterica* ser. Typhimurium, AgfA.

The formation of curli represents a departure from the other modes of pilus assembly discussed in the previous sections. Whereas structures exemplified by P, CS1, type IV, and F pili are assembled from the base, curli formation occurs on the outer surface of the bacterium by the precipitation of secreted soluble pilin subunits into thin fibers (Figure 9). In *E. coli*, the products of two divergently transcribed operons are required for curli assembly. The *csgBA* operon encodes the primary fiber-forming subunit, CsgA, which is secreted as an unpolymerized protein directly into the extracellular environment. The second protein encoded by the *csgBA* operon, CsgB, is proposed to induce polymerization of CsgA at the cell surface. In support of this model, by interbacterial complementation it has been demonstrated that a $CsgA^{+}CsgB$-donor strain can secrete CsgA subunits that can be assembled into curli on the surface of a $CsgA^{-}CsgB^{+}$-recipient strain. In the absence of CsgA, overexpressed CsgB is able to form short polymers on the bacterial cell surface.

The *csgDEFG* operon encodes a gene for a transcriptional activator of curli synthesis (CsgD), and three genes encoding putative assembly factors. One of these factors, CsgG, has recently been shown to be a lipoprotein that is localized to the outer membrane. In the absence of CsgG, curli assembly does not take place, and CsgA and CsgB are rapidly degraded. The precise role of CsgG is not known at this time. It has been suggested that CsgG might act as a chaperone that facilitates the secretion of the CsgA and CsgB and protects them from degradation within the periplasm. It is also possible that CsgG assembles into oligomeric, ring-shaped complexes that could function as a Csg-specific channel within the outer membrane. It has been reported that a strain lacking CsgE is defective in curli assembly, as the stability of both CsgA and CsgB in this mutant background is greatly reduced. Mutation of *csgF* does not affect CsgA secretion, but CsgA made by *csgF* mutants does not polymerize. Environmental factors such as very low salt concentrations, nutrient limitation, microaerophilic conditions, and temperatures below 30 °C favor maximal curli gene expression. RpoS, a stationary-phase sigma factor; OmpR/EnvZ, a two-component regulatory system; and IHF, a global regulatory protein all positively regulate curli expression. Two other two-component regulatory systems, CpxA/R and Rcs, negatively regulate curli expression. Interestingly, histone-like protein HN-S positively regulates curli expression in *S. enterica* ser. Typhimurium, but negatively regulates it in *E. coli*.

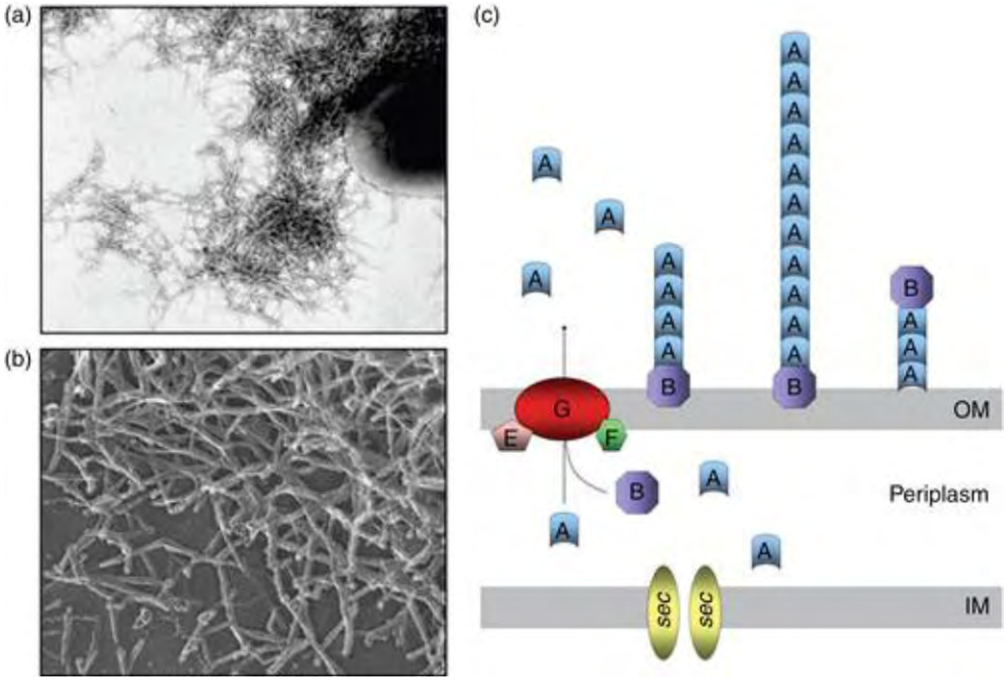

Figure 9 Curli assembly by an extracellular nucleation/precipitation pathway. (a) Electron micrographs of negatively stained *Escherichia coli* expressing curli. (b) High-resolution electron micrograph, obtained using a deep-etch technique, of purified curli. Photo courtesy of M. Chapman (Barnhart MM and Chapman MR (2006). *Annual Review of Microbiology* 60: 131–147), with permission from Annual Reviews. (c) Model of curli assembly by the extracellular nucleation/precipitation pathway. CsgA, the main component of curli from *E. coli*, is secreted across the outer membrane. CsgB serves to nucleate CsgA assembly. CsgG is an outer-membrane-localized lipoprotein that is required for the secretion of CsgA and CsgB, although its function is not yet entirely clear. CsgE and CsgF are periplasmic proteins that interact with CsgG at the outer membrane.

Curli promote bacterial adherence to host cells and tissues by binding to a variety of host proteins, including plasminogen, fibronectin, and human contact phase proteins. Interestingly, curli are recognized as a pathogen-associated molecular pattern by host Toll-like receptor 2, a component of the innate immune system that can recognize incoming pathogens and induce robust proinflammatory responses. Curli are also involved in bacterial colonization of inert surfaces, and have been implicated in cell aggregation and biofilm formation.

Type III Secretion Pathway

The various pilus assembly pathways described in the previous sections all rely on components of the *sec* machinery for the translocation of their respective pilus subunits across the inner membrane. Other types of pili are assembled by a *sec*-independent pathway known as the type III secretion system (T3SS). The T3SS is encoded by numerous Gram-negative pathogens and enables these bacteria to secrete and inject pathogenic effector molecules into the cytosol of eukaryotic host cells. About 20 gene products, most of which are inner-membrane proteins, comprise the T3SS. The components mediating type III secretion are conserved in pathogens as diverse as *Yersinia* and *Erwinia*, but the secreted effector proteins vary significantly between species. The T3SS apparatus, which appears to span the periplasmic space, resembles the basal body of a flagellum connected to a straight rod that extends across the outer membrane. Interestingly, all T3SS encode some components with homologies to proteins involved in flagellar assembly. The secretion of proteins by the T3SS is an ATP-dependent process that involves no distinct periplasmic intermediates. Type III-secreted proteins of EPEC, the nodule-forming strains of *Rhizobium* species and *Sinorhizobium fredii*, and several plant pathogens including *Pseudomonas syringae* pathovar tomato, *Erwinia amylovora*, *R. solanacearum*, and *Xanthomonas campestris* have been shown to assemble into pilus-like structures.

EPEC encodes four proteins, EspA, EspB, EspD, and Tir, which are secreted by a type III pathway. These proteins facilitate intimate contact between the pathogen and host intestinal cells and are required for the formation of specific (attaching and effacing) lesions. In 1998, Knutton and colleagues showed that one of these proteins, EspA, can assemble into 10- to 12-nm-thick peritrichously expressed pilus-like fibers that are organized into ~50-nm-wide bundles and extend up to 2 μm from the bacterial surface (Figure 10(a) and 10(b)). The assembly of these EspA filaments requires several other components of the T3SS, including an outer-membrane secretin called EscC, a type III translocator protein EspD, the T3SS ATPase EscN, and EscF, the major structural protein of the T3SS needle complex. Coiled-coil interactions of EspA polypeptides lead to the assembly of EspA filaments in a process similar to that of flagellar assembly from flagellin subunits. During the infection process, the EspA fibers appear to mediate contact between EPEC and the host cell surface before the establishment of more intimate bacterial attachment. The EspA fibers seem to assist the translocation of EspB and other effector molecules into host cells where they can subvert host signal transduction pathways.

In *P. syringae* and other plant pathogens, T3SS are encoded by *Hrp* and *Hcp* (hrp conserved) genes. *Hrp* stands for hypersensitive response and pathogenicity, reflective of these genes' roles in causing disease in susceptible plants and eliciting the hypersensitive response (a rapid, localized host cell death, which limits the spread of a pathogen) in resistant plants. HrpA from *P. syringae* and *E. amylovora*, HrpE from *X. campestris*, and HrpY from *R. solanacearum* have all been shown to be assembled into 6- to 8-nm-wide, 2-μm-long peritrichously expressed pili (Figure 10(c)). These Hrp pili subunits are small proteins (6–11 kDa) with significant variation in their amino acid sequences, but their assembled pili are remarkably consistent α-helix-rich structures. Assembly of Hrp pili contrasts against all other known pilus assembly systems in that subunits are added at the tip, rather than at the base, of the pilus. The Hrp secretion system is always found at Hrp pilus assembly sites, suggesting that the pili act as a conduit or guiding filament for the delivery of effector proteins into host cells. Hrp pili might also be in involved in the penetration of the host plant cell wall, perhaps by pore formation or cell wall modification during effector delivery.

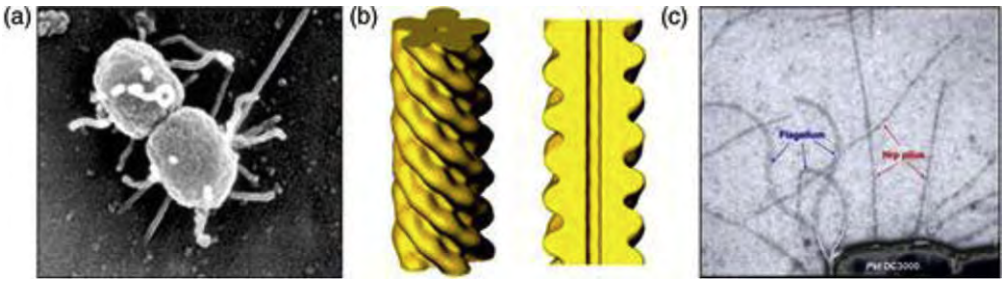

Figure 10 Pili assembled by a type III secretion pathway. (a) Scanning electron micrograph of EPEC elaborating ~50-nm-thick bundles of pili containing EspA, a protein exported by a type III secretion pathway. The individual 6- to 8-nm-thick pili comprising the bundles are not resolved in this micrograph. Reproduced from Knutton S, Rosenshine I, Pallen MJ, Nisan I, Neves BC, Bain C, Wolff C, Dougan G, and Frankel G (1998). *The EMBO Journal* 17: 2166–2176, with permission from Oxford University Press. (b) Three-dimensional reconstruction of EspA filaments showing a tilted side view (left) and cut-away side view (right) revealing a central channel. Reproduced from Daniell SJ, Kocsis E, Morris E, Knutton S, Booy FP, and Frankel G (2003). *MolecularMicrobiology* 49: 301–308, with permission from Blackwell Publishing. (c) Electron micrographs of negatively stained *Pseudomonas syringae* expressing Hrp pili (about 8 nm in diameter) and flagella (about 15 nm in diameter). Photo provided by S. Y. He. Reproduced from He SY and Jin Q (2003). *Current Opinion in Microbiology* 6: 15–19), with permission from Elsiever Ltd.

Pili in Gram-Positive Bacteria

Pili have been observed on a number of Gram-positive bacteria, but to date their study is less advanced than that of their Gram-negative counterparts. The best-defined Gram-positive pili are made by *Corynebacterium diphtheriae*, which carries at least three different types, designated SpaA, D, and H after their major subunits. SpaA is considered the prototypical Gram-positive pilus type, and most of the following information (and the model for pilus assembly in Figure 11) is based on findings from *C. diphtheriae* SpaA. It should be noted however that other *Corynebacterium* spp., *Actinomyces* spp., and *Streptococcus* spp. are believed to form pili in a similar manner. In addition, Gram-positive species *Arthrobacter photogonimos* and *Ruminococcus albus* are believed to assemble pili by a separate mechanism(s) that has not yet been elucidated.

Pili on Gram-positive bacteria differ from Gram-negative pili in several important ways. Most notably, while the subunits of Gram-negative pili are held together by noncovalent attachments, Gram-positive pilin subunits attach to each other, and to cell wall peptidoglycan, by covalent bonds. Sortases – transpeptidases named for their role in 'sorting' proteins to the cell wall fraction – are required for the formation of these covalent bonds. The SpaA, D, and H pili in *C. diphtheriae* are named for this requirement, where spa stands for sortase-mediated pilus assembly.

Like many Gram-negative pilins, Gram-positive pilus subunits carry N-terminal signal peptide sequences that target them for secretion through the cell membrane by the *sec* machinery. Spa-like pilins also carry crucial residues at the C-terminus: a cell wall-sorting pentapaptide (usually LPXTG), a hydrophobic region of 30–40 amino acids, and a positively charged tail. Upon translocation by the *sec* machinery, the pilin's hydrophobic region is inserted into the membrane, and held in place by the charged tail. Membrane-associated sortases then cleave the subunit's recognition peptide, leaving the C-terminus embedded in the membrane while the body of the pilin is attached to the enzyme to form an acyl-enzyme intermediate. Nucleophilic attack by an amino group – on either a peptidoglycan precursor (for joining to the cell wall) or another pilin subunit (for pilus growth) – releases the pilin from the sortase enzyme. This process is depicted in Figure 11.

Spa pili in *C. diphtheriae* are thin fiber-like structures, 2–6 nm in diameter and 0.2–3 μm long. Each pilus is composed primarily of its major subunit, although some also include a tip subunit and other minor subunits along the shaft that are not required for pilus synthesis or integrity. Major pilus subunits contain a conserved pilin motif, which is believed to mediate release of the previous pilin from its sortase by nucleophilic attack. The absence of this motif in tip subunit SpaC may account for its tip-only

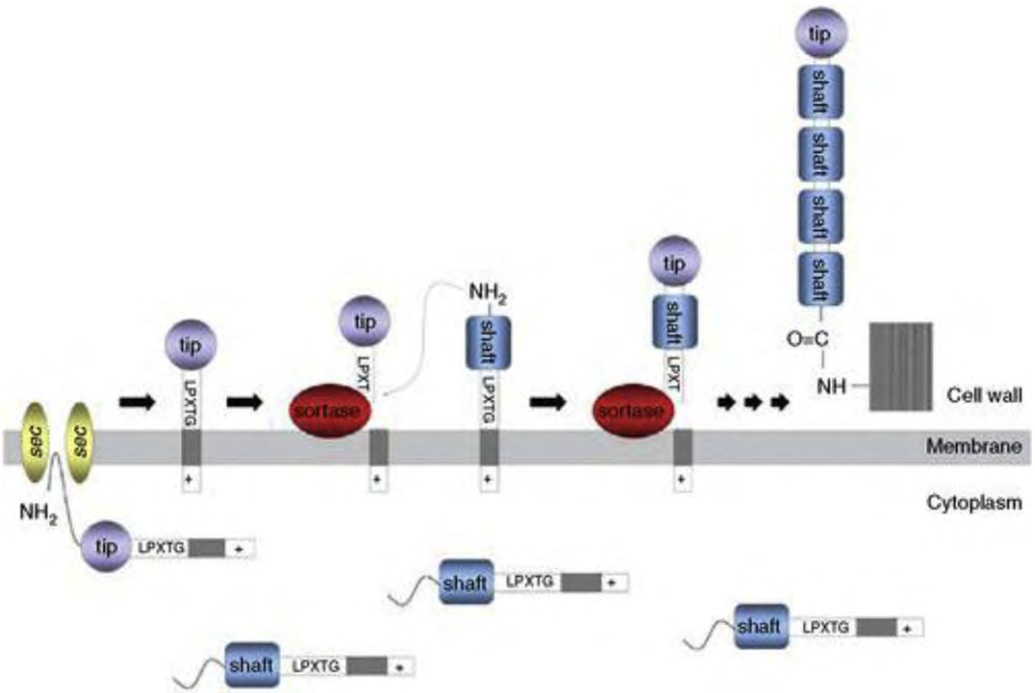

Figure 11 Generalized model of pilus assembly in Gram-positive bacteria. SpaA pili are composed of three types of pilins: SpaA, which forms most of the pilus shaft; SpaB, a minor subunit of unknown function not included in this schematic diagram; and SpaC, which is found at the pilus tip. Other Spa-like pili have similar components. The tip protein is probably the first pilin to be incorporated into a new pilus. Upon translocation by the *sec* machinery (mediated by an N-terminal secretion signal), a pilin's hydrophobic region is inserted into the membrane and held in place by a positively charged tail. A membrane-associated sortase then cleaves the subunit's recognition peptide (usually LPXTG), leaving the C-terminus embedded in the membrane while the main body of the pilin is attached to the enzyme to form an acyl-enzyme intermediate. Nucleophilic attack by an amino group on another pilin forms a peptide bond between subunits, leading to growth of the nascent pilus. The pilus is covalently attached to the cell wall through nucleophilic attack by an amino group on a peptidoglycan precursor, probably via a separate housekeeping sortase. Reproduced from Ton-That H and Schneewind O (2004). *Trends in Microbiology* 12: 228–234, with permission from Elsiever Ltd.

localization, as it is able to attach to another subunit at only one site rather than two. Minor subunit SpaB also lacks the pilin motif, but is found intermittently along the length of the pilus. The role and mechanism of incorporation for minor subunit SpaB have not yet been defined.

Each of the three Spa pilus types in *C. diphtheriae* has its own gene cluster, encoding one major subunit, two minor subunits, and one or two sortase-like transpeptidases. *C. diphtheriae* encodes six sortase (srt) homologues in all, five of which are within predicted pilus gene clusters. *SrtA*, which is adjacent to *SpaABC*, is required for assembly of SpaA pili. The SpaD pilus gene cluster includes both SrtB and SrtC, and either of these sortases is sufficient for SpaD pilus assembly. SrtF, which is not linked to a pilus, is likely a 'housekeeping' sortase, responsible for anchoring a variety of LPXTG motif-containing proteins in the cell wall. SrtF is also required for firm attachment of SpaA pili, indicating that its cell wall-anchoring activity may be important for pilins as well as for nonpilin proteins. Because SrtA seems to have limited efficiency in attaching pilins to the cell wall, it has been argued that the term sortase is a misnomer, and that SrtA should instead be referred to as a pilin polymerase. Further research will be required to establish the exact roles of the sortase-like transpeptidases in pilus assembly and attachment.

As in Gram-negative bacteria, pili play a vital role in mediating attachment of Gram-positive bacteria to target surfaces and tissues, and thus also in pathogenesis. The dental pathogen *Actinomyces naeslundii* uses two different types of pili: one promotes colonization by adhering to saliva-coated tooth enamel and the other mediates attachment and interactions with both mammalian cells and other bacteria, potentially promoting both infection and biofilm formation. *S. pneumoniae* lacking pili show impaired adherence to lung epithelial cells, and are less virulent than their wild-type counterparts in direct competition. Recent work in clinically important group A and group B *Streptococcus* strains has shown that pili can be effective vaccine candidates for Gram-positive pathogens. Given these clinical implications, one may expect an increase in research on Gram-positive bacterial pili in the coming years.

Regulation of Pilus Biogenesis

Pilus biogenesis, in general, is a tightly regulated process. Ideally, the costs in energy and other resources required for pilus assembly must be balanced with potential benefits that pilus expression might provide a bacterium. For example, by producing pili in a nutritionally poor environment, a bacterium will tax its available resources, but with pili the same bacterium may be able to gain access to a more favorable location. Pathogenic and other bacteria must also control pilus expression, in some cases, to avoid attachment to unfavorable sites (tissues) within their hosts. Furthermore, pathogenic bacteria may need to modulate pilus expression to escape detection by the host immune system. Whether or not a bacterium expresses pili is greatly affected by environmental factors. Changes in temperature, osmolarity, pH, oxygen tension, carbon source, and nutrient availability may either increase or decrease pilus expression. The presence of iron, aliphatic amino acids, and electron acceptors other than oxygen may also influence the expression of pili. A combination of these environmental cues can stimulate (or repress) pilus synthesis and alter the expression of a number of other factors, all of which can influence the tropism of bacteria for specific niches within the environment or within host organisms.

Environmental signals affect pilus biogenesis through global regulator proteins that can modify the transcription of pilus genes. Various global regulators have been identified and include H-NS, a DNA-binding histone-like protein that often mediates temperature regulation of pilus synthesis. H-NS appears to alter DNA topology and typically functions as a negative regulator. Regulation by carbon source can occur through the catabolite activator protein (CAP), whereas the leucine-responsive regulatory protein (Lrp) can modulate pilus expression in response to aliphatic amino acids. The CAP and Lrp regulators can control sets of pilus operons, enabling the expression of different types of pili to be coordinated and integrated with the metabolic state of the bacterial cells. In addition to these and other global regulators, specific regulator proteins encoded by genes within some pilus operons may also modulate pilus biogenesis. Multiple regulatory factors can act upon the same promoter region, switching pilus gene expression from on to off and vice versa. This on and off switching, known as phase variation, can also be modulated by the methylation status of a promoter region and by the inversion of sequence elements within a promoter. Extracytoplasmic and cytoplasmic stress response pathways also appear to be involved in regulation of the assembly and function of a large number of different pilus types. In keeping with the vast array of niches filled by bacteria and the different environments to which they must adapt, regulation of pilus biogenesis is highly complex, and may vary widely among strains.

Role of Pili in Disease Processes

The expression of pili can have substantial impact on the establishment and persistence of pathogenic bacteria within both plant and animal hosts. For many bacterial pathogens, adhesive pili play a key role in the colonization of host tissues. Uropathogenic *E. coli*, for example, require type 1 to effectively colonize the bladder epithelium. These pili attach to conserved, mannose-containing host receptors expressed by the bladder epithelium and help prevent the bacteria from being washed from the body with the flow of urine. Type 1 also mediate invasion of bladder cells, and can promote bacterial survival by modulating the host immune response. For example, P pili may serve a similar function in the kidneys, preventing the clearance of pyelonephritic *E. coli* from the upper urinary tract and allowing the establishment of an infection. Enteric pathogens produce a wide variety of adhesive pili that facilitate bacterial colonization of the intestinal tract. These include the K88, K99, and 987P pili made by ETEC strains, the long polar fimbria

and plasmid-encoded fimbria of *S. enterica* ser. Typhimurium, the subclass of Afa/Dr adhesins, including F1845, Dr and Afa-3 fimbriae of *E. coli*, and aggregative adherence fimbria (AAF) of enteroaggregative *E. coli*. In the small intestine, toxin coregulated pili (TCP) are essential for the attachment of *V. cholera* to gut epithelial cells. These pili also act as receptors for the cholera toxin phage (CTXΦ), a lysogenic phage that encodes the two subunits of the cholera toxin. This phage, with its encoded toxin, is transferred between *V. cholera* strains via interactions with TCP within the small intestine. Other pili also function in the acquisition of virulence factors. The uptake of DNA facilitated by type IV pili and DNA transfer directed by conjugative pili can provide pathogens with accessory genes, enabling them to synthesize a wider repertoire of virulence factors and giving them resistance to a greater number of antibiotics. Biofilm formation, which in some cases appears to require pili such as type 1, type IV, or curli, can also increase the resistance of bacteria to antibiotic treatments and may aid bacterial colonization of tissues and medical implants.

Pili are not necessarily static organelles, and dynamic alterations of pilus structures during the infection process may influence the pathogenicity of piliated bacteria. For example, electron microscopic studies of mouse bladders infected with type 1-piliated uropathogenic *E. coli* showed that the pili mediating bacterial adherence to the bladder epithelial cells were 10–20 times shorter than typical type 1. It is possible that the shorter type 1 observed are the result of pilus retraction, compact coiling, or breakage during the infection process. The shortening of pili may provide a means for reeling bacteria in toward their target host cells, allowing the bacteria to make intimate contact with the host after initial attachment at a distance. Within the gut, type IVb pili (BFP) promote autoaggregation and microcolony formation in EPEC strains, a phenomenon that facilitates the adherence of EPEC to the intestinal epithelium. After initial attachment, an energy-dependent conformational change in the quaternary structure of BFP appears to be needed for the further dispersal of EPEC over human intestinal cells and for the full virulence of this pathogen.

During the infection process, adhesive pili are often situated at the interface between host and pathogen where they can potentially mediate cross-talk between the two organisms. Pilus attachment to the receptor of a host eukaryotic cell may induce a number of host signal transduction pathways, potentially leading to cytoskeleton rearrangements, membrane ruffling, and pathogen internalization. For example, binding of the type IVa pili of *Neisseria* to host receptors (probably CD46) on target epithelial cells has been shown to stimulate the release of intracellular Ca^{2+} stores, a signal known to control a multitude of eukaryotic cellular responses. Likewise, the attachment of P pili to Galα(1–4)Gal-containing host receptors on target uroepithelial cells can trigger the intracellular release of ceramides, important second messenger molecules that are capable of activating a variety of protein kinases and phosphatases involved in signal transduction processes. The signals induced within uroepithelial cells on binding of P-piliated bacteria eventually result in the secretion of several immunoregulatory cytokines. Binding of type 1-piliated bacteria to mannosylated receptors on uroepithelial cells can similarly induce the release of cytokines, although apparently through different signaling pathways than those stimulated by P pilus binding. It has also been suggested that pili can transduce signals into bacterial cell, although this reported phenomenon requires further investigation and verification. Continued research into the biogenesis, structure, and function of pili not only promises to advance our basic understanding of the role of these organelles in pathogenic processes but may also aid the development of new-generation antimicrobial therapeutics and vaccines. Indeed, over the last decade, a tremendous amount of effort has been directed toward the development of pili-based vaccines and small molecule inhibitors called pilicides that can interfere with pilus assembly. At a time when the number of antibiotic-resistant bacterial strains is on the rise, effective pili-based vaccines and pilicides promise to be especially useful tools in combating bacterial infections.

Application of Pili in Immunology and Microbiology

During the recent couples of decades, the pili have been widely investigated for their application in many areas, including microbiology, immunology, vaccinology, and nanotechnology. Because of the repeated tertiary structure, pili are highly immunogenic. Because of the cell surface location, pili are used in tethering the bacteria. Here, we take the CFA/I fimbriae as an example to illustrate pili's application in the areas of immunology and immunoimmobilization.

ETEC causes both travelers' diarrhea and severe children's diseases in many endemic areas in the world. However, no licensed vaccines are available for human ETEC today. CFA/I fimbriae, a rigid fiber structure that is located on the ETEC surface, are an important virulence factor. At the primary stage of infection, ETEC needs to attach to the gut cells in the human small intestine to cause disease. The attachment of ETEC to epithelial cells is accomplished via CFA/I fimbriae. Therefore, by targeting the CFA/I fimbriae, the disease may be neutralized through the interaction between fimbrial antigen and the corresponding antibody because ETEC is an extracellular pathogen. The result in mouse model shows that two intramuscular (i.m.) immunizations with detoxed CFA/I fimbriae conferred 100% protection against intraperitoneal (i.p.) ETEC challenge, while a single dose conferred 88% protection. Challenge studies in humans also demonstrated that CFA/I fimbriae are protective antigens. Further, bactericidal assays show that ETEC is highly sensitive to anti-CFA/I sera, suggesting that parenteral immunization is critical for the effective protection. When CFA/I is expressed in a recombinant Salmonella vector, one dose of mucosal immunization can stimulate strong secretory immunoglobulin A (IgA) and serum IgG antibody responses to CFA/I fimbriae. In a word, both animal and human studies suggest that CFA/I fimbriae are a potent antigen which has the potential for generating a vaccine against ETEC. Of interest, the immune response of either purified CFA/I fimbriae or the Salmonella-carried CFA/I tends to exhibit a Th2-polarized profile. Due to the powerful mediation of the immune bias towards Th2-type immunity, CFA/I has been investigated for preventing and treating the autoimmune diseases via correcting the Th1-biased immunity to a Th2-biased immunity, such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). Studies such far demonstrated that autoimmune diseases can be successfully prevented and/or prohibited through immunizing the CFA/I in animal model.

Recently, microarrays using bacterial cells as sensors have been successfully applied in many diverse areas. After treating slide surface with anti-CFA/I antibodies in designated areas, ETEC cells are incubated on the whole slide surface. It is found that bacteria are successfully yet only tethered to the designated spots that contain the antibodies. The bacterial pattern can be easily monitored by an optical microscope. To further examine whether this technology can be extended to the diagnostic purpose, bacterial pathogens having different fimbriae are labelled with green, red, and blue flourecence, and then they are incubated with different anti-fimbria antibodies to attach the bacteria. The results suggest that all the fimbriae, including K88ab (F4), K88ac (F4), K99 (F5), 987P (F6), F41, and CFA/I, can be used for efficient immobilization of living E. coli. Furthermore, when immunoimmobilization is utilized in an antibody microarray immersed in a mixed culture of pathogens, rapid and simultaneous detection of multiple pathogens can be achieved <1 h. When compared with the previous approaches using antibodies against whole bacterial cells, the anti-pili antibody to immobilize bacteria significantly enhanced the immobilization efficacy with an elevated specificity. Certainly, the specificity may be further enhanced by using monoclonal anti-pili antibody. In conclusion, both specificity and the advantage of time efficiency should allow this technology to be utilized in hospital to speed up the diagnostic procedure, particularly in regions where bacterial pathogens are epidemic.

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Planctomycetes

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Introduction

The Planctomycetes form a fascinating phylum of Gram-negative (from now on referred to as "diderm") bacteria with some particular features. Taxonomically, they belong to the *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* (PVC) superphylum which also includes the *Lentisphaerae*, *Kirimatiellaeota* as well as some uncultured candidate phyla, such as the *Candidatus* Omnitrophica (previously known as OP3), the *Candidatus* Poribacteria and *Candidatus* division WWE2. The microscopic observation by Nándor Gimesi in 1924 of the characteristic rosette form of *Planctomyces bekefii* (Gr. adj. *planktos*, wandering, floating; Gr. masc. n. *mukês*, fungus; N.L. masc. n. *Planctomyces*, floating fungus) in which spherical structures were linked to numerous filiform appendages concentrated in a common center that resembles a planctonic fungus, is responsible for the incorrect inclusion of these organisms in the fungi and for the "mycetes" designation in the name of the phylum. After the initial observation of *P. bekefii* from Lake Ligymanyosi, Budapest, Hungary, Henrici and Johnson in 1935 also found similar stalked, budding bacteria in Lake Alexander, Minnesota U.S.A., which they named *Blastocaulis sphaerica*. After these two first observations, several limnological reports evidenced the presence of similar microorganisms in freshwater lakes giving rise to the description of novel morphotypes like *P. guttaeformis* and *P. stranskae*. Thought to be unculturable, it was only in the 1970s that *Pirellula staleyi* (initially designated *Pasteuria ramosa*) was isolated in pure culture. Since then and especially in the last decades, we have witnessed an increase in the isolation of novel strains in pure culture and in the description of new taxa (Table 1). One of the organisms that is still not cultivated in pure culture is the extremely slow growing ANaerobic AMMonium OXidizing (anammox) bacteria. This "missing lithothroph", *i.e.*, the organism capable of combining ammonium and nitrite directly into nitrogen gas anaerobically was discovered in 1999 in a wastewater treatment plant. The majority of the known Planctomycetes belong to the class *Planctomycetia* that include several orders and the "*Candidatus* Brocadiales" with still controversial positioning (Fig. 1, Table 1). In 2009 another class was described, the *Phycisphaerae* that contains the orders *Phycisphaerales* and *Tepidisphaerales* (Fig. 1, Table 1). In addition, environmental molecular studies have revealed that many non-described *Planctomycetes* exist.

Planctomycetal colonies are white, pink, orange or red pigmented. The spherical, ovoid or pear-shaped cells can display crater-like pits in the cell wall as well as different kind of hair and fimbria-like structures (Figs. 2 and 3). Planctomycetes have condensed DNA with a G+C content in the range of 50–73 mol% (the annamox Planctomycetes have G+C content around 40 mol%) (Table 1) and a 5S rRNA that is unusually short.

Members of the class *Planctomycetia* divide through polar budding (Fig. 2(B) and (C)) while all other Planctomycetes are thought to divide through binary fission. In addition, all Planctomycetes lack a recognizable homologue of the "**F**ilamentous **t**emperature-**s**ensitive mutant **Z**" (FtsZ) protein, fundamental for cell division in all other known bacteria. Some Planctomycetes have complex life cycles in which the daughter cell is motile (Fig. 2(C)). This cell swims until it attaches to a substrate by a holdfast structure or a stalk at the vegetative pole and becomes sessile. They are slow growing bacteria with doubling times that range from 6 h up to 1 month. Their characteristic quinone is menaquinone 6. As Planctomycetes are resistant to several classes of antibiotics like betalactams, and no evidence of the presence of peptidoglycan (PG) existed, they were initially thought to be devoid of PG. Only recently the presence of genes required for PG synthesis was demonstrated in planctomycetal genomes and two independent groups demonstrated its presence in the cell wall of different planctomycetes, in a wall structure consistent with a diderm organization.

Planctomycetes have a complex cell plan due to cytoplasmic membrane invaginations that form an extensive endomembrane network (Fig. 3(C)). For some time, this complexity was interpreted as intracellular compartmentalization with the presence of an intracytoplasmic membrane that divided the cytoplasm in two compartments, one containing the highly condensed DNA and rich in ribosomes. This confusion has been recently clarified and it is now widely accepted that Planctomycetes are variations of, and not exceptions to, the diderm cell plan. The only two membranes present in all Planctomycetes, with the exception of the anammox bacteria, are the cytoplasmic (inner) membrane and the outer membrane. Both are separated by the periplasm, where PG has been observed in some and it is likely to be present in most Planctomycetes. A particular group of planctomycetes, the anammox bacteria, has an intracellular compartment which is called the anammoxosome (Fig. 3(C)). This compartment is the location of the energy metabolism of the cell - the anaerobic ammonium oxidation (anammox), an important process in the biological nitrogen cycle.

Planctomycetes share with eukaryotes the presence of clathrin-like membrane coat proteins. These proteins are encoded in their genomes and might be involved in the building of their developed endomembrane system. The encoded proteins share structural features with the eukaryotic ones involved in membrane manipulation but no sequence relationship has been shown between the two groups of proteins.

In general, Planctomycetes possess relatively large genomes, in the range of 3.9–12.3 Mb in size, but the function for only 30%–55% of the proteins encoded in these genomes can be predicted. A characteristic shared by all sequenced planctomycetal

Table 1 Planctomycetes described, their affiliation, value of G+C content and habitat from where were isolated

Class	*Order*	*Species*	*G+C (mol%)*	*Reference*	*Habitat*
Planctomycetia	*Gemmatales*	*Fimbriiglobus ruber*	60.7	Kulichevskaya et al. (2017)	Acidic boreal *Sphagnum* peat bog
	Gemmatales	*Gemmata obscuriglobus*	64.4	Franzmann and Skerman (1984)	Freshwaters of Maroon Dam, south-east Queensland, Australia
	Gemmatales	*Gemmata massiliana*	64.07	Aghnatios et al. (2015)	Hospital waste water
	Gemmatales	*Telmatocola sphagniphila*	58.5–59.0	Kulichevskaya et al. (2012)	North European *Sphagnum* peat bogs Staroselsky moss and Obukhovskoye
	Gemmatales	*Zavarzinella formosa*	62.5	Kulichevskaya et al. (2009)	Acidic *Sphagnum*-dominated northern wetlands, Russia
	Isosphaerales	*Aquisphaera giovannonii*	70.0	Bondoso et al. (2011)	Freshwater sediments
	Isosphaerales	*Isosphaera pallida*	62	Giovannoni et al. (1987)	Kah-nee-tah hot springs, Oregon
	Isosphaerales	*Paludisphaera borealis*	66	Kulichevskaya et al. (2016)	Boreal *Sphagnum* peat bog and a forested tundra wetland
	Isosphaerales	*Singulisphaera acidiphila*	59.9	Kulichevskaya et al. (2008)	Acidic *Sphagnum*-dominated northern wetlands, Russia
	Pirellulales	*Blastopirellula marina / Pirellula marina/ Pirella marina*	57	Schlesner (1994); Schlesner et al. (2004)	Brackish water, Baltic Sea
	Pirellulales	*Bythoypirellula goksoyri*	52.8	Storesund and Øvreås (2013)	Iron-hydroxide deposits from the Arctic Mid Ocean Ridge (AMOR)
	Pirellulales	*Mariniblastus fucicola*	54.2	Lage et al. (2017)	Microbial biofilm of the marine macroalga *Fucus spiralis* sampled in Carreço, northen of Portugal
	Pirellulales	*Pirellula staleyi /Pirella staleyi/Planctomyces staleyi*	57	Starr et al. (1983)/Schlesner and Hirsch (1984, 1987)	Freshwater Lake Lansing MI, USA
	Pirellulales	*Rhodopirellula baltica (Pirellula sp.* SH 1^T*)*	55	Schlesner et al. (2004)	Kiel Fjord, part of the Baltic Sea
	Pirellulales	*Rhodopirellula caenicola*	57.5	Yoon et al. (2015)	Iron sand in depth of 30 cm from the surface collected at Murohama Beach, Kamaishi, Iwate, Japan
	Pirellulales	*Rhodopirellula lusitana*	54.6	Bondoso et al. (2014)	Biofilm of macroalgae Portugal
	Pirellulales	*Rhodopirellula rosea*	53.0	Roh et al. (2013)	Dead ark clam *Scapharca broughtonii,* Gangjin Bay in the south coastal region of Korea
	Pirellulales	*Rhodopirellula rubra*	56.1	Bondoso et al. (2014)	Biofilm of macroalgae Portugal
	Pirellulales	*Roseimaritima ulvae*	57.0 ± 0.6	Bondoso et al. (2015)	Biofilm of macroalgae Portugal
	Pirellulales	*Rubripirellula obstinata*	56.1 ± 0.2	Bondoso et al. (2015)	Biofilm of macroalgae Portugal
	Pirellulales	*Thermogutta hypogea*	57.3	Slobodkina et al. (2015)	Deep gold mine, South Africa
	Pirellulales	*Thermogutta terrifontis*	66.6	Slobodkina et al. (2015)	Hot spring, Kunashir Island, Russia
	Pirellulales	*Thermostilla marina*	58.5	Slobodkina et al. (2016)	A shallow submarine hydrothermal vent, Vulcano Island, Italy
	Planctomycetales	*Fuerstia marisgermanicae*	55.9	Kohn et al. (2016)	Crustacean shell, German Wadden Sea
	Planctomycetales	*Gimesia maris (Planctomyces maris)*	50.5	Bauld and Staley (1976, 1980), Scheuner et al. (2014)	Shallow waters at Puget Sound, Washington, USA
	Planctomycetales	*Planctomicrobium piriforme*	59.0	Kulichevskaya et al. (2015)	From a littoral wetland of a boreal lake
	Planctomycetales		53.24		Schrevenpark, Lake Mondsee, a 'cattle manure' (all near Kiel, Germany)

(*Continued*)

Table 1 Continued

Class	Order	Species	G+C (mol%)	Reference	Habitat
		Planctopirus limnophila (Planctomyces limnophilus)		Hirsch and Müller (1985), Scheuner et al. (2014)	
	Planctomycetales	*Rubinisphaera brasiliensis (Planctomyces brasiliensis)*	56.4	Schlesner (1989), Scheuner et al. (2014)	The salt pit Lagoa Vermelha, Brazil
	Planctomycetales	*Schlesneria paludicola*	56.3	Kulichevskaya et al. (2007)	*Sphagnum*-dominated boreal wetlands, northern Russia
Brocadiae	Brocadiales	*Candidatus* Anammoximicrobium moscowii	[a]	Khramenkov et al. (2013)	Moscow River silt, Russia
		Candidatus Brocadia anammoxidans	[a]	Strous et al. (1999)	Fluidized bed reactor
		Candidatus Kuenenia stuttgartiensis	[a]	Schmid et al. (2000)	Wastewater treatment plant in Stuttgart, Germany
		Candidatus Scalindua sorokinii	[a]	Kuypers et al. (2003)	Water column of Black Sea
		Candidatus Scalindua brodae	[a]	Schmid et al. (2003)	Rotating Biological Contactor 1, The Pitsea Landfill Site wastewater treatment plant, UK
		Candidatus Scalindua wagneri	[a]	Schmid et al. (2003)	Rotating Biological Contactor 1, The Pitsea Landfill Site wastewater treatment plant, UK
		Candidatus Anammoxoglobus propionicus	[a]	Kartal et al. (2007)	Batch reactors
		Candidatus Jettenia asiatica	[a]	Quan et al. (2008)	Granular sludge anammox reactor
		Candidatus Jettenia caeni	[a]	Ali et al. (2014)	Activated sludge collected from a wastewater treatment plant operated in the Kumamoto city, Japan
		Candidatus Jettenia moscovienalis	[a]	Nikolaev et al. (2015)	Activated sludge of a semi-industrial bioreactor treating digested sludge of the Kuryanovo wastewater treatment plant, Moscow, Russia
		Candidatus Brocadia sinica	[a]	Hu et al. (2010)	Sludge samples from nitrogen removal reactors from China
		Candidatus Brocadia fulgida	[a]	Kartal et al. (2008)	Batch reactors
		Candidatus Brocadia caroliniensis	[a]	Vanotti et al. (2011)	Livestock manure sludge at the USDA-ARS laboratory in Florence, South Carolina
Phycisphaerae	*Phycisphaerales*	*Phycisphaera mikurensis*	73	Fukunaga et al. (2009)	Macroalgae *Porphyra* sp., Mikura Island, Japan
	Phycisphaerales	*Algisphaera agarilytica*	63.0	Yoon et al. (2014)	Green coloured marine alga (*Cladophora* sp.) collected at the rocky shore of Sado Island, Niigata, Japan
	Tepidisphaerales	*Tepidisphaera mucosa*	53.0	Kovaleva et al. (2015)	Terrestrial hot springs in Baikal Lake region and Kamchatka, Russia

[a]All known anammox bacteria have GC contents close to 40%.

genomes is the presence of "giant genes" (more than 5 kb) most probably encoding structural proteins, non-ribosomal peptide synthetase or polyketide synthase. Various specific molecular markers of planctomycetes have been described, which include conserved signature inserts or deletions or conserved signature proteins in ABC transporter protein, cobyrinic acid a,c-diamide synthase (protein of cobalamin biosynthesis), and Stage V sporulation protein G, SpoVG.

The ubiquity and broad phylogenetic diversity of Planctomycetes have been revealed by culture-dependent and –independent studies in the last decades. Based essentially on the analysis of the 16S rRNA gene, their presence has been detected in aquatic (marine and freshwater), terrestrial, extreme and polluted environments and in association with many organisms. This myriad of different habitats occupied by diverse planctomycetes is indicative of a great metabolic versatility that accommodates aerobic chemoheterotrophs and anaerobic chemolithoautotrophs. Many Planctomycetes are mesophilic or neutrophilic organisms. They are environmentally relevant in the global biogeochemical cycles namely carbon, nitrogen and sulfur. Planctomycetes are known for

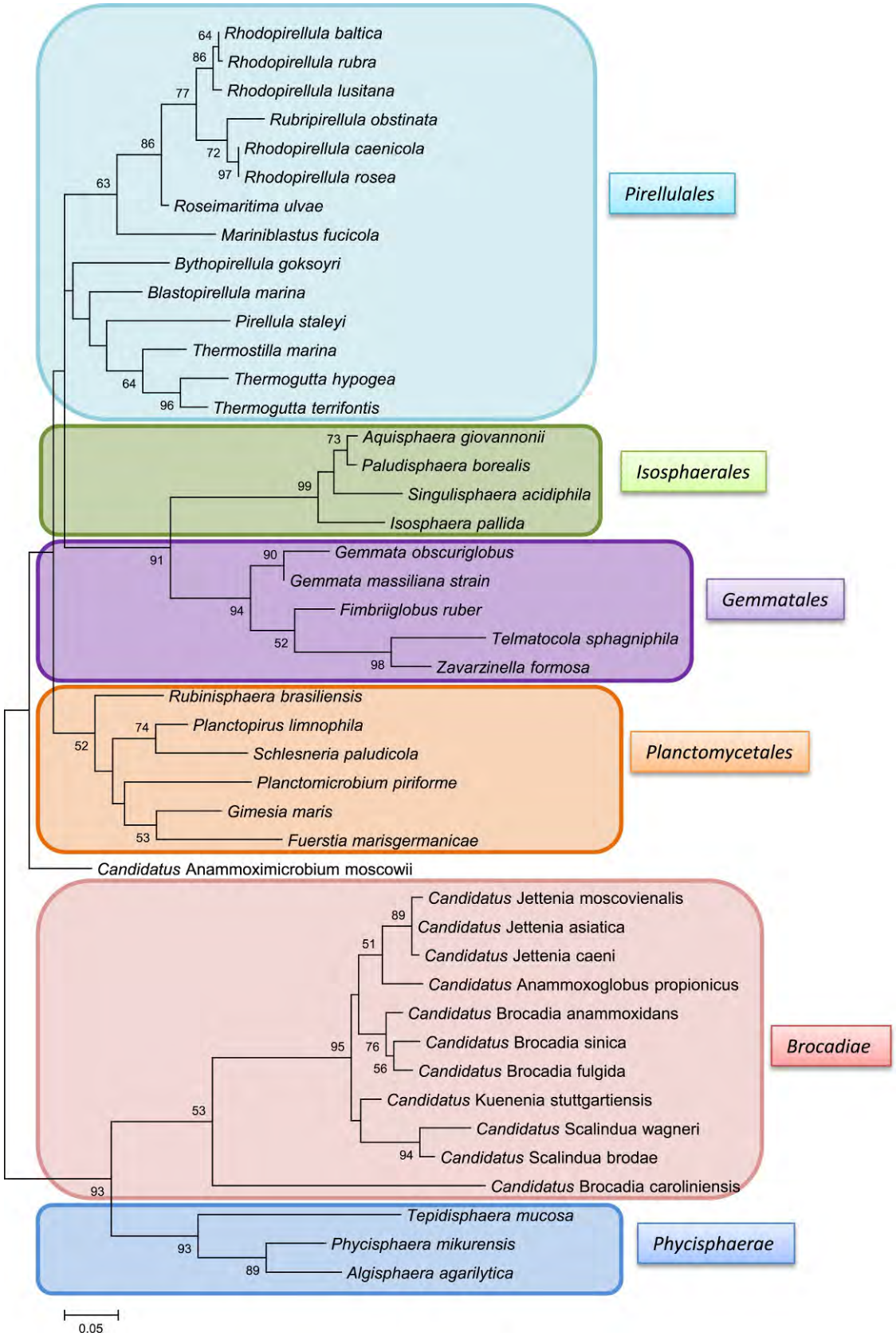

Fig. 1 A 16S rRNA gene based tree of the phylogenetic position of selected Planctomycetes species.

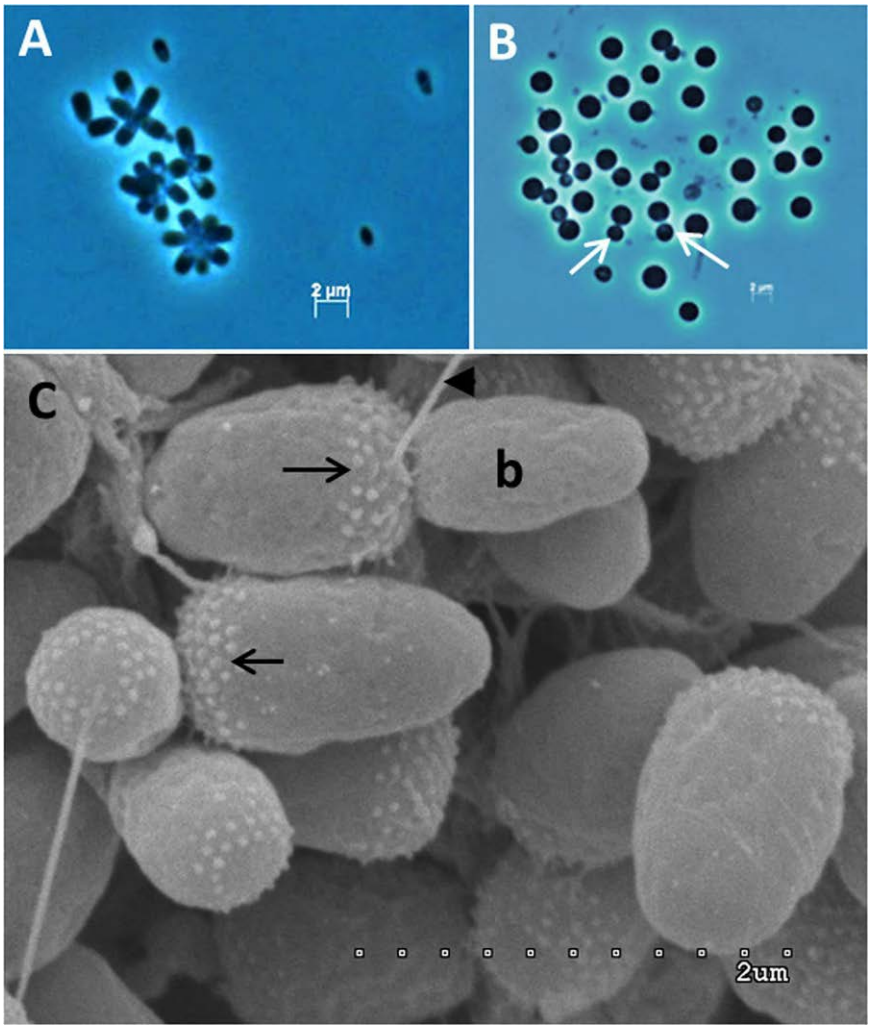

Fig. 2 (A) Pear-shaped cells of *Rhodopirellula rubra* forming rosettes; (B) Spherical cells of *Aquisphaera giovannonii*, some of which are in the process of budding; (C) Scanning electron microscopy of *Roseimaritima ulvae* cells evidencing a forming bud (b) with the flagella (head of arrow) and the place of insertion of the fimbriae (the crateriform pits – arrow) in the apical pole of the cells.

their ability to metabolize *N*-acetyl-D-glucosamine as the only carbon and nitrogen source which coupled to the resistance to antibiotics are elements used for planctomycete isolation.

Biotechnologically, Planctomycetes are used for the anaerobic oxidation of ammonium in wastewater treatment plants and their potential as antimicrobial or anticancer agents has recently started to be explored. They share a complex life cycle and large genome size with Actinobacteria and Myxobacteria (both well-known producers of bioactive molecules) and possess a promising genomic repertoire of secondary metabolites.

The in-depth study of planctomycetal genomes coupled to the great effort being made to enlarge the number of sequenced Planctomycetes is providing relevant information on their unique cellular biology, structure, physiology and metabolism, evolutionary aspects and the roles played in the environment. Recently, genetic tools were developed for some Planctomycetes, which associated with the information generated by genome analyses and the increase of available new strains in pure cultures will allow a better understanding of this peculiar group of bacteria.

Particularities of Cell Structure

One of the most distinctive features of planctomycetes is their extensive and dynamic endomembrane system. Indeed, whereas most diderm bacteria have outer- and cytoplasmic membranes without major features, their organization in most members of the phylum *Planctomycetes* present modifications. Most importantly, the cytoplasmic membrane invaginates, sometimes quite extensively, towards the cytoplasm (Fig. 3(C)). This distinctive feature is more or less developed in different species of Planctomycetes. The situation in the anammox bacteria is similar to a complete enclosure, and isolation of this central region from the rest of the

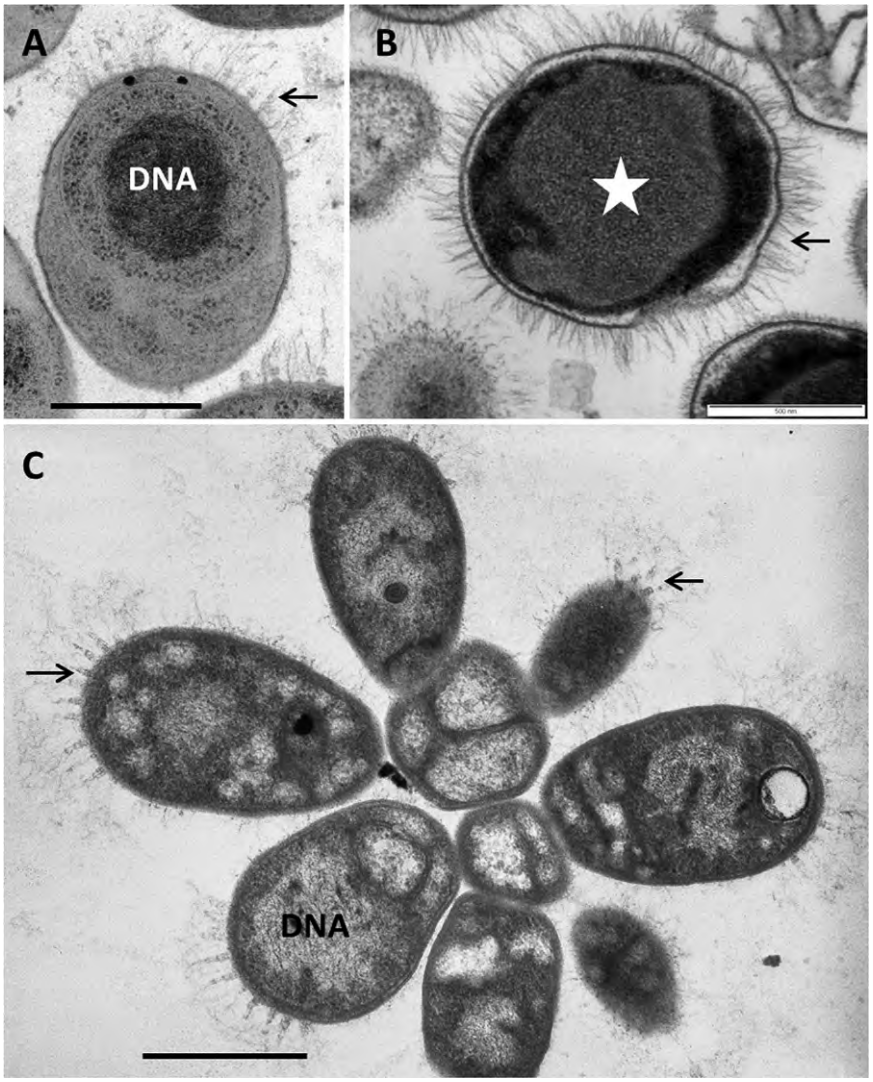

Fig. 3 Transmission electron microscopy of (A) *Rhodopirellula rubra*, (B) the anammox *Candidatus* Scalindua and (C) a rosette of *Rhodopirellula lusitana*. The cells have condensed DNA, many ribosomes in the cytoplasm and fimbriae or pili on the apical pole of the cell (A and C) or surrounding the cell (B). In B is visible the anammoxosome (*). In C is visible in some cells the endomembrane network. Bar – 500 nm.

periplasm, with a possible separation from the cytoplasmic membrane, represents possibly one of the very few bacterial organelles (Fig. 3(B)) (see below). *Planctopirus limnophila* contains one to a few major invaginations of the cytoplasmic membrane towards the inside of the cell. Eventually, the membranes of *Gemmata obscuriglobus* show a complex and extensive network of thinner invaginations of the cytoplasmic membrane. Some of these might even appear to surround the genetic material. However, a complete isolation of the DNA has been ruled out. Another type of *G. obscuriglobus* cells show an increased periplasmic volume filled with vesicles. Similar vesicles are also present in the periplasm of other Planctomycetes such as *I. pallida* and *Rhodopirellula lusitana* (Fig. 3(C)). In *G. obscuriglobus*, these vesicles have been shown to be in membrane continuity and to form a network of tubules and vesicles. The functions associated to these membrane organizations are still unknown. In *G. obscuriglobus* and in *P. limnophila*, external compounds internalization before their internal degradation has been reported associated to this membrane networks. At least in *G. obscuriglobus*, this membrane network is in close contact with proteins showing structural (but no sequence) similarity with eukaryotic proteins involved in the formation of proteic cages around bend membranes, such as the nucleopore complex or coated vesicles. In addition to the Planctomycetes, these proteins are also found in Verrucomicrobia and Bacteroidetes.The function of these proteins in bacteria is still to be defined. It is also unknown if they are homologous to the eukaryotic ones or if they have reached similar structural features by convergence.

As far as we know, the anammox bacteria are the only Planctomycetes that have a separate intracellular compartment with a specific cellular function. This anammoxosome compartment constitutes up to 60% of the cell volume (Fig. 3(B)). This compartment harbours all the (heme-containing) enzymes involved in the energy metabolism of the cell and it is hypothesized to conserve energy by the use of a proton motive force across the anammoxosome membrane. This is a highly curved, and at times extensively

invaginated, bilayer membrane. The curvature of the anammoxosome membrane might be linked to its function in energy conservation by enlarging the membrane surface area and thus the metabolic rate. Inside the anammoxosome, two structures stand out: Iron-containing electron dense particles (16–25 nm diameter) of unknown function and long tubular structures that are or contain the hydroxylamine oxidase HOX protein (kustc 1061) which is hypothesized to provide an important hydroxylamine detoxification system to the cell (see below). The anammoxosome is surrounded by the cytoplasmic compartment, which contains a condensed nucleoid that is always attached to the outside of the anammoxosome, and the periplasm. Unlike previously believed, the periplasm contains PG and most likely also an asymmetrical bilayer outer membrane typical of diderm bacteria. Finally, the outermost layer of the cell is mostly constituted by a heavily glycosylated protein surface layer (S-layer).

Anammox bacteria contain unique membrane lipids called ladderanes. They consist of (three or five) linearly concatenated cyclobutane rings bound to a polar head group by ester or ether bonds with no known preference for ester or ether binding in distinctive ladderane lipids. All membranes of the anammox cell contain ladderane lipids but they are especially abundant in the anammoxosome membrane. Their role might be in rendering the membrane less permeable and more rigid. It is hypothesized that the bacteria need a less permeable membrane to limit the passive diffusion of valuable intermediates and protons or toxic intermediates. In addition to ladderane lipids, the membranes also contain hopanoid lipids that might serve in maintaining the optimal equilibrium between membrane fluidity and rigidity.

Cell Division

Another of the fascinating features of Planctomycetes is their mode of division. Most bacteria divide by binary fission which involves lateral growth of the cell, followed by its pinching at mid-cell and division at the equatorial plane.

A similar phenomenon is observed in the orders *Phycisphaerales* and "*Candidatus* Brocadiales" which includes the anammox bacteria. Unlike most of the other Planctomycetes, anammox bacteria divide by binary fission. In the anammox, the first sign of cell division is the appearance of a putative cell division ring in an unusual location: the periplasm. Next, the cell slightly invaginates, doubles in size and finally constricts to separate the two daughter cells like in any other binary fission dividing bacteria. During the process of cell division, the anammoxosome is equally divided between the two daughter cells. However, a particularity is that anammox bacteria, like other Planctomycetes, lack some of the canonical and essential cell division genes such as FtsZ.

All other planctomycetes divide by a mode that appears more related to budding (Fig. 2(C)). Intermediary phenotypes have also been reported as in the genus *Fuerstia* where dividing cells are interconnected by a tubular like structure. It will certainly be of interest to discover novel species of planctomycetes with diverse modes of division.

Planctomycetes are however not the only bacteria that do not divide by binary fission. Where Planctomycetes are unique is that all of them are dividing without the otherwise bacterial ubiquitous protein FtsZ. Bacterial division without FtsZ remains one of the last mysteries of molecular microbiology and evolutionary cell biology. In other bacteria, FtsZ orchestrates the process of cell division, forms a ring in the cytoplasm, attached to the cytoplasmic membrane at midcell and serves as a scaffold for other cell division proteins. How cell division is organized in the FtsZ-less binary fission or budding Planctomycetes is entirely unknown and an interesting topic for future research. In addition to FtsZ, the proteomes of Planctomycetes also lack various other division-associated proteins like MurC, AmiA, RodZ or FtsA. Deciphering the modes of division of planctomycete bacteria is thus of extreme relevance not only for planctomycetal molecular biology and for microbiology but also for the field of evolutionary cell biology.

Physiology and Metabolism of Anammox

Anammox bacteria perform anaerobic ammonium oxidation in three consecutive reactions: the reduction of nitrite to nitric oxide, the condensation of nitric oxide and ammonium to the "rocket fuel" hydrazine, and the oxidation of hydrazine to dinitrogen gas (Fig. 4). The oxidation of hydrazine releases four electrons that are proposed to be transferred to a membrane-bound cytochrome bc1 complex. Cytochrome c-type proteins might serve as intermediate electron carriers, also facilitating the shuttling of electrons to the reduction of nitrite (one electron) and the synthesis of hydrazine (three electrons). The electron transport could facilitate the translocation of protons by the bc1 complex across the anammoxosome membrane, giving rise to a proton motive force. This proton motive force could then be used by membrane bound ATPases to produce ATP in the cytoplasm. Some of the electrons from hydrazine oxidation are proposed to be shuttled towards carbon dioxide reduction for carbon fixation through the Wood–Ljungdahl pathway. To replenish these electrons, some of the nitrite is oxidized to nitrate. Finally, it is proposed that anammox bacteria have a detoxification system that keeps inhibitory compounds like hydroxylamine at low concentrations. This might be done through the very abundant cytochrome c-containing protein kustc1061 which can convert hydroxylamine to nitric oxide.

For a long time, it was though that anammox bacteria were specialists only capable of converting ammonium and nitrite into dinitrogen gas. In fact, anammox bacteria might be considered as generalists as they are also capable of converting some organic as well as other inorganic compounds. Few examples; certain anammox species can perform formate, acetate, propionate and methylamines oxidation, nitrate respiration (using iron as electron donor), and organic acid oxidation (using nitrate as electron acceptor – DNRA (dissimilatory nitrate reduction to ammonium)).

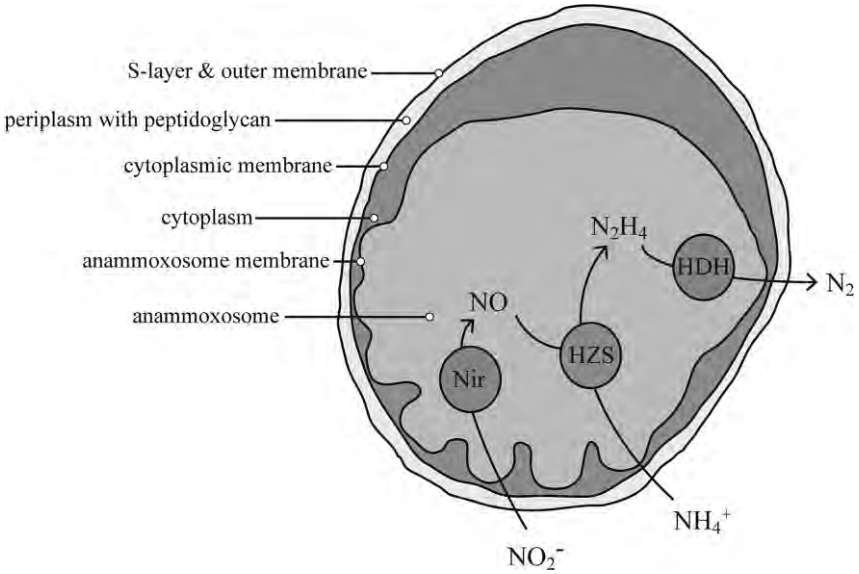

Fig. 4 Simplified model of the anammox cell plan and energy metabolism. The anaerobic ammonium oxidation takes place inside the anammoxosome compartment and proceeds in three consecutive steps: The reduction of nitrite to nitric oxide by a nitrite reductase (Nir), the condensation of nitric oxide and ammonium to hydrazine by hydrazine synthase (HZS) and the oxidation of hydrazine to dintrigen gas by hydrazine dehydrogenase (HDH).

Anammox bacteria are slow growing; their doubling time ranges from one to several weeks depending on the growth conditions. Therefore, anammox bacteria cannot be cultivated using standard microbiological techniques such as plating. Instead, they are cultivated in bioreactor systems where they can reach an enrichment level ranging between 74% (sequencing batch reactor – flocular biomass) and 98% (membrane bioreactor – planktonic cells).

Genomes

Since 2003, year of the publication of the genome of *Rhodopirellula baltica* SH1, several planctomycete genomes have been released (Table 2) and various aspects of the biology of the Planctomycetes disclosed. The majority of the sequenced Planctomycetes have big genomes comparatively to other bacteria (over 5 Mb and up to 12 Mb) although some smaller sizes have been found in the class *Phycisphaerae*. Comparatively to the genomes of other phyla, the function for a lower number of the proteins in the genomes could be predicted, only about half or less, which represents a relevant gap in the functional understanding of Planctomycetes.

The genome of *R. baltica* revealed that this species has the conventional pathways for an heterotrophic bacteria (glycolysis, the Kreb cycle and oxidative phosphorylation) but also several other metabolic capacities like the pentose-phosphate cycle (an alternative to glycolysis), the genes for heterolactic acid fermentation and for the interconversion of C1 compounds plus an unexpected high number of sulfatases which were related to the utilization of sulfated glycopolymers produced essentially by macroalgae and thus abundant in marine environments. *R. baltica* has the capacity to synthesize all amino acids and possesses genes for nitrate transport and nitrate to nitrite reduction. The glyoxylate bypass and the Entner–Doudoroff pathway were missing. The presence of genes related to the biosynthesis of PG and of the outer membrane was already indicative of a diderm nature for planctomycetes cell wall as posteriorly demonstrated. The big size of its genome cannot be justified by multicopy genes because only 25.4% are multicopy genes in the genome of *R. baltica*, a lower value comparatively to *Bacillus subtilis* (30%) or *Escherichia coli* K-12 (29%).

Based on comparative genomics, it was observed that the Planctomycetes have the three major regulatory systems involved in transmembrane signaling typical of bacteria: one-component systems (1CSs), two-component systems (2CSs) and extracytoplasmic function sigma factors (ECFs). In general a direct correlation between the absolute numbers of signaling proteins and genome size exists. The common bacterial ratio of about four times between 1CSs and 2CSs was observed but these components differ from other bacteria in several aspects. Furthermore, planctomycetes are especially rich in extracytoplasmic function sigma factors (ECFs). ECFs control genes that regulate cell envelope related processes and are important in conferring resistance to agents that threaten the integrity of the cell envelope. Regarding planctomycetal chemotaxis, great differences were observed among eight planctomycetal genomes analyzed. While some Planctomycetes possess a high number of chemotaxis systems, others showed limited resources especially *R. baltica* that completely missed any chemotaxis machinery. Planctomycetes with a motile swarmer cell in their life cycle possess the genes for a functional flagellum but the non-motile strains only possess a restricted flagellar assembly.

Some planctomycetal genomes display an enlarged carbohydrate-active enzyme (CAZyme) repertoire as it is the case of *Paludisphaera borealis* and *Thermogutta terrifontis* that possess an extensive glycolytic potential. The evolutionary origin of the enzymes

Table 2 Planctomycetal genomes >93% complete. Plasmids are indicated in brackets

	Species	Size [Mbp]	GC [%]	Scaffolds	Protein-coding genes	Hypothetical proteins [%]
Planctomycetia						
Pirellulales	*Blastopirellula marina* SH 106	6.7	57.0	3	5406	56.0
	Mariniblastus fucicola FC18	6.5	53.4	57	5082	54.3
	Pirellula staleyi ATCC 27377	6.2	57.5	1	4705	55.3
	Rhodopirellula baltica SH1	7.1	55.4	1	5465	56.3
	Rhodopirellula europaea SH398	7.4	55.8	208	5803	57.3
	Rhodopirellula islandica K833	7.4	57.2	55	5557	56.4
	Rhodopirellula maiorica SM1	8.9	54.7	1132	7306	62.5
	Rhodopirellula rubra SWK7	8.8	56.0	141	6543	57.2
	Rhodopirellula sallentina SM41	8.2	56.7	491	6214	57.8
	Roseimaritima ulvae UC8	8.1	59.1	103	5761	55.2
	Rubinisphaera brasiliensis IFAM 1448	6	56.4	1	4824	53.5
	Rubripirellula obstinata LF1	6.6	54.1	307	5206	58.3
	Thermogutta terrifontis R1	4.8	57.3	1	3586	50.0
Planctomycetales	*Fuerstia marisgermanicae* NH11	8.9	55.9	1	6645	58.6
	Gimesia maris	7.8	50.5	125	6001	55.2
	Planctomicrobium piriforme P3	6.3	58.8	41	5050	55.7
	Planctopirus limnophila Mü 290	5.4 (0.04)	53.7	2	4275	54.2
	Schlesneria paludicola MPL7	8.7	55.7	23	6867	58.9
Isosphaerales	*Isosphaera pallida* IS1B	5.4 (0.06)	62.5	2	3761	48.5
	Paludisphaera borealis PX4	7.49 (0.11+0.04)	66.3	3	5855	53.9
	Singulisphaera acidiphila MOB10	9.6 (0.05+0.04+0.03)	62.2	4	7542	57.2
Gemmatales	*Fimbriiglobus ruber* SP5	12.4	64.2	20	10,434	68.9
	Gemmata obscuriglobus UQM 2246	9.2	67.2	922	7819	66.2
	Gemmata massiliana IIL30	9.3	64.0	22	8022	62.3
	Zavarzinella formosa A10	10.1	59.1	105	8662	67.2
Phycisphaerae, Phycisphaerales	*Phycisphaera mikurensis* NRBC 102666	3.9	73.2	2	3110	46.3

present in *P. borealis* seems to have been acquired by horizontal gene transfer (HGT) from other phyla, such as *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Verrucomicrobia* and *Acidobacteria* and not from the two phyla with great hydrolytic potential, the *Firmicutes* and *Bacteroidetes*. Planctomycetes associated to macroalgae also showed evidence for extensive HGT from the *Bacteroidetes* and *Proteobacteria*. In fact, planctomycete genomes possess genomic islands, which are horizontally transmitted gene clusters, and high number of transposable elements. Some strains also possess clustered regularly interspaced short palindromic repeats (CRISPR) that are essential in the adaptive immunity, playing a key role in the bacterial defense against invading genetic material like bacteriophages and conjugative plasmids.

The genome of several Planctomycetes have the metabolic pathways for degrading diverse toxic compounds, including ethylbenzene, aminobenzoate, naphthalene, chloroalkane, polycyclic aromatic hydrocarbon, toluene, bisphenol, benzoate, chlorocyclohexane and chlorobenzene.

Another peculiarity of planctomycete genomes is the presence of giant genes (with a size >5 kb) which could be coding for surface proteins, non-ribosomal peptide synthetases or polyketide synthases.

Recent analysis of TARA Oceans metagenomes revealed complete set of genes for nitrogen fixation in metagenome-assembled genomes (MAGs) from two Planctomycetes inhabiting the surface of the open ocean. Besides Planctomycetes, seven Proteobacteria MAGs also contained genes for nitrogen fixation. This is the first genomic evidence of putative of non-cyanobacterial heterotrophic bacterial diazotrophs (HBDs) in the surface of the open ocean. These HBDs were surprisingly abundant in the Pacific Ocean and the Atlantic Ocean northwest. One of the Planctomycetes MAGs with 88% identity to *Algisphaera agarilytica* represents a new order.

Ecophysiology

Planctomycetes inhabit a wide range of niches which include terrestrial, aquatic, extreme and polluted habitats. Indeed, since the discovery of members of planctomycetes in Australian soil in 1992, planctomycetes have been found widespread and abundant in different kinds of soil around the globe, such as cultivated, pasture, forest, tundra, sandy loam and thermal soils. They first have been overlooked due to a mismatch in a popular bacterium-specific 16S rRNA gene amplification primers. But their presence and quantity have been extensively detected by culture independent methods like fluorescence *in situ* hybridization, clone libraries, denaturing gradient gel electrophoresis (DGGE) and, more recently, by metagenomic approaches. Even though they appear many

times in relative low abundances, in some environments they contribute substantially to the native population as it is the case on the surface of macroalgae, in acidic *Sphagnum* peat bogs, in marine snow and in some sediments. For example, planctomycetes represent a major part of the bacterial population in mangrove wetlands, agricultural fields and lichen-dominated sub-arctic ecosystems in Siberia. They were also found in salty sandy costal soils in China, chinese leek rhizosphere and grazed pasture soil.

Surprisingly, planctomycetes were found to be very abundant in aridic regions of China as well (23.7% of the bacterial community), while their abundance decreased along salinity gradients in comparable habitats. Another striking enrichment of planctomycetes can be observed in the active layer above permafrost soils for example on the Tibetan Plateau or in moist acidic tundra soil. This fits to the observation of planctomycetes at all stages of fennoscandian boreal forest development where they can account for up to 21% of the bacterial community. They were found to be associated with decomposing dead wood in natural temperate forests, indicating that they might be involved in the breakdown of complex carbon compounds in terrestrial habitats. Planctomycetes are also significantly enriched in the rhizosphere from an Amazon rainforest compared to the surrounding soil. Regarding taxonomy, 16S rRNA planctomycete sequences detected in soils are affiliated to various genera of the family *Planctomycetaceae*, such as *Pirellula*, *Planctomyces*, *Isosphaera* and *Gemmata* but also to non-described independent clusters.

Planctomycetes dwell in all sorts of limnic and marine habitats. For example 8.2% of the bacterial community in rivers of the upper Amazon basin are planctomycetes. Furthermore, they were detected in Hindu Kush caves, in a cold subseafloor aquifer and in rain collected groundwater.

Their diversity is grouped in two clusters distantly related to *Pirellula*, *Planctomyces* and *Gemmata* but highly related to 16S rRNA gene sequences obtained from freshwater environments worldwide. This evidences the existence of globally distributed freshwater clusters of planctomycetes. In the hypereutrophic freshwater Lake Taihu, China, planctomycetes were the dominant non-cyanobacteria phylum (36% of the sequences) in the microbial community associated to carpet-like cyanobacterial blooms. In the low temperature and high altitude of two moraine lakes in Mount Everest, planctomycetes were present as 2% of water bacterial community. In two perialpine lakes with different eutrophication status, the oligotrophic Lake Annecy and the mesotrophic Lake Bourget, planctomycetes are more diversified and with less temporal variations in the hypolimnia comparatively to the epilimnia.

In lotic environments, planctomycetes were found in river snow (lotic microbial aggregates) in the river Elbe, Germany, in a relative abundance of 2% but more abundant in spring, summer and autumn and in hyporheic zone within sediments in three different streams in Montana, United States. Furthermore, in the polluted river Elbe and its tributary Spittelwasser, the biofilm community presented planctomycetes abundance peaks (7%–10%) in summer and 2% in winter that also varied in composition.

In extreme aquatic habitats like the South African saltpans with a pH of 8.0 and a salinity of 12.8%, planctomycetes form a major part of the bacterial community. A well-diversified community is present in the anaerobic, sulfide-saturated sediments of a *meso*phlic spring (Zodletone Spring) in southwestern Oklahoma as assessed by the analysis of the 16S rRNA gene sequences. The diversity obtained was related to the class *Planctomycetia*, to the *Cand*. class WPS-1 and to a deeply branching clade of Planctomycetes. Many of the clones were related to the ones reported from anaerobic environments. Moreover, two novel strains related to the *Pirellula-Rhodopirellula-Blastopirellula* clade were isolated and are able to reduce elemental sulfur to sulfide under anaerobic conditions and form acids from sugars. The acidic *Sphagnum* peat bogs with a pH between 3.9 and 4.5 harbour a rich community of recently described planctomycetes with densities of 1.1×10^7 cells g^{-1} peat. Further studies showed the presence of planctomycetes in nine different acidic or mildly acidic wetlands of Nothern Russia. In some of these sites their abundance decreased sharply with depth but in two sites they present two abundance peaks in the depth profile: one in the uppermost oxic layer and another, with anoxic conditions, at a depth of 30 cm below the water table level. The upper peak was dominated by members of *Isosphaera* and *Singulishpaera* and the anoxic one with *Zavarzinella* and *Pirellula*-like representatives. In both zones, members of the *Cand*. Omnitrophica were detected. Another acidic environment also colonized by planctomycetes is the acidic El Coquito hot spring in the colombian Andes.

Like other planctomycetes, anammox bacteria have been detected in diverse habitats including marine oxygen-minimum zones and sediments, freshwater lakes and rivers, peat soil, fish intestinal tract systems and anaerobic and oxygen-limited wastewater treatment plants. Studies in different oxygen minimum zones indicated that anammox bacteria are key players responsible for the nitrogen loss from these systems. Since oxygen minimum zones are estimated to be responsible for up to 50% of the nitrogen loss from the ocean, anammox bacteria play a major role in the nitrogen cycle on a global scale.

In addition, Planctomycetes can live in association with other organisms. In particular, microbial mats are natural enrichments of planctomycetes. For example, up to 21% of the bacterial community composition in microbial mats of the hypersaline Laguna Tebenquiche (Chile) are planctomycetes. They were identified in mats from an iron-rich thermal spring in South Africa, in mats at Little Hot Creek, USA, and in black mats in a hypersaline lake in Chile. In the latter particular habitat, even 42% of the bacterial community were planctomycetes. A special case of microbial mats are stromatolites and up to 15% of the stromatolite forming bacteria was found to be planctomycetes. Even in Antarctic marine biofilms on artificial surfaces up to 21% of the bacterial community were planctomyctes. Noteworthy, in most microbial mats and in stromatolites in particular, cyanobacteria are the most abundant bacteria. Thus, there is a striking co-occurrence of cyanobacteria and planctomycetes that was already known for cyanobacterial blooms.

Planctomycetes are particularly abundant in certain biofilms and in eukaryote-associated microbiomes. For example, planctomycetes were found to be enriched in the particle associated fraction (7%) compared to the surrounding water (1%) in samples from the Mediterranean Sea. In Wadden Sea sediments, Planctomycetes can account for up to 7% of the bacterial community on

individual sand grains, while they form on average 5.7% of the bacterial community. On marine macroscopic detrital aggregates known as marine snow 22% of the bacteria can be Planctomycetes. They are thought to degrade compounds such as sulfated polysaccharides in these particles that sink to the deep sea. Consequently, Planctomycetes can dominate deep-sea sediments, for example in the Gulf of Mexico.

Planctomycetes were described to be associated to eukaryotes. A study comparing human gut microbiota from Senegalese versus French individuals showed higher prevalence and diversity of planctomycetes (1.8% versus 0.4%, p=0.05) in individuals from Senegal. 16S rRNA gene sequences related to *Gemmata*, *Planctomyces*, *Isosphaera*, *Pirellula*, *Blastopirellula*, *Schlesneria*, *Singulispaera* and *Phycisphaera* were found. Planctomycetes were identified in fecal samples of several other mammals like wild gorilla, bat and pigs. When mice were fed with a simulated fungal diet, planctomycetes were detected in the gut, while they were absent in mice on a regular diet. In other trophic levels, planctomycetes were found (1) in the gut of different species of penguins (up to 11%); (2) in the gastrointestinal track of the common carp (*Cyprinus carpio*) with a percentage of 12% where they might be involved in polysaccharide degradation; (3) in the whole gut of individuals of the damselfish and cardinalfish where an 80-fold enrichment of *Pirellulaceae* occurred between the microbiome of pre- and post-settlement larvae; (4) in the hepatopancreas (midgut gland) of the giant tiger prawn *Penaeus monodon* and in commercially important white shrimp (*Litopenaeus vannamei*) where 2.9% of the intestinal microbiota were planctomycetes and their abundance changed significantly at different culture stages; (5) as part of the coral *Montastraea cavernosa* microbiome and in association with the deep-sea octocoral *Paramuricea placomus* where even 10% of the bacterial community were planctomycetes; (6) in Baikalian fresh-water sponges; (7) in the microbiome (about 10%) of the calcifying benthic foraminifera *Amphistegina lobifera* of the Great Barrier Reef in Australia where it remains unclear whether this is or is not a symbiotic relationship; (8) in the lightless habitats of the deep-sea cold seeps dwell chitin tube forming worms (*Escarpia* sp.) and Planctomycetes form the most abundant OTU in such chitin tubes, which are extreme natural enrichments for Planctomycetes as they are barely detected in the surrounding water or sediment samples; (9) in macroinvertebrates: the hindgut of soil-feeding termites *Cubitermes* spp. and the midgut of humivorous larvae of the cetoiid beetle *Pachnoda ephippiata*. In the termites *Cubitermes* spp. planctomycetes are extremely abundant with cell densities of 2.6×10^{9} cells ml^{-1}, representing one-third of the FISH bacterial counts in the alkaline hindgut. The Planctomycetes diversity forms three major clusters that are novel, deeply-branching lineages. The role played by planctomycetes in the gastrointestinal tract of the various organisms is unknown but may be related to the breakdown of products released by other bacteria like polysaccharides. The anammox planctomycetes present in the carp gut might be involved in the nitrogen cycle.

However, thus far, the best studied habitat where planctomycetes seem to play a significant role are micro- and macroalgae. Macroalgae are the dominant habitat-forming organisms on temperate rocky marine coastlines. Within mid-latitude belts, they frequently form kelp forests that provide shelter and a source of nutrition for marine mammals, fish, crabs, sea urchins, mollusks, other algae and epibiota. For example, planctomycetes could account for 51%–53% of the bacterial biofilm cells on surfaces of the kelp *Laminaria hyperborea* from Norway where they are part of a stable community over time. In addition, a broad association of planctomycetes and 12 macroalgae from the north coast of Portugal was shown through the isolation of 138 isolates in pure culture. During isolation, colonies of planctomycetes grew on the surface of portions of the macroalgae, evidencing a clear nutritional role of the macroalgae and the existence of an intimate relationship between both organisms. A novel planctomycete from the surface of the macroalga *Porphyra* sp., *Phycisphaera mikurensis* that belongs to a new class, the *Phycisphaerae* was also described. Afterwards, many other studies also confirmed the existence of this association and several new taxa were described: *Roseimaritima ulvae*, *Rubripirellula obstinata*, *Mariniblastus fucícola*, *R. lusitana*, *R. rubra*, *A. agarilytica*. Besides the new described biodiversity, macroalgae harbour representatives of *R. baltica*, *B. marina*, *Planctomyces*, the Pir4 lineage, the uncultured class OM190 and other deep branching unidentified groups. Some OTUs seem to be specifically associated with particular macroalgae. Several studies suggest that the planctomycetal-containing microbiome is host specific and probably determined by nutritional molecules provided by the algae and the set of sulfatases inherent to each planctomycete species. However, the planctomycetal association with macroalgae is not fully understood. Planctomycetes dominated the biofilms on the surface of *Laminaria hyperborean* with up to 55.7% abundance, while the biofilm composition was subject to seasonal variations. One hypothesis is that the role of planctomycetal carbon remineralization is key for the ecology of the kelp forest.

Given the slow growth of Planctomycetes -some species have doubling times of up to one month- their high abundance in such important habitats as kelp, that are packed with alluring carbon sources in contrast to the largely oligotrophic surrounding water, appears counter intuitive. Most other heterotrophs that dwell in such ecological niches divide much faster (for example 1.2–6.3 h for the *Alphaproteobacteria Roseobacter* sp.) and should therefore outcompete planctomycetes. Thus, the phototroph-planctomycetal allelopathic interactions might involve the production of various secondary metabolites that might act as antimicrobials (defense against other, faster growing, heterotrophic bacteria) or algicidals (to destroy algae namely diatoms or cyanobacteria for scavenging). In addition, climate change might lead to an increase in planctomycetal abundance on macroalgae: if *Caulerpa taxifolia* was incubated under higher pCO_2, some planctomycetes were ten times more abundant than under normal pCO_2 and accounted for 5% of the bacterial community an effect that was observed in soil as well. Besides macroalgae, the planctomycetal abundance was found to correlate in two following years with a *Centrales* diatom bloom in the German North Sea and with *Skeletonema* diatoms in the Baltic Sea. In *meso*cosm experiments it was shown that a higher abundance of planctomycetes can correlate with environmental changes, which seems to be a more general feature in particular if pCO_2 levels are increased to simulate climate change.

However, the planctomycetal association with phototrophs is not limited to marine algae. For example, a correlation of planctomycetal abundance with chlorophyll a concentrations in a shallow mesoeutrophic lake in Italy, pointed towards a role of planctomycetes in the degradation of algal biomass. A study in the eutrophic Lake Taihu (China) demonstrated that planctomycetes

were the dominant members of the microbial community attached to *Microcystis* cyanobacteria during an intensive bloom and their abundance increased to 22% of the bacterial community during such blooms. More planctomycetal sequences were found on the surface of the freshwater macrophyte *Nuphar lutea* compared with the surrounding water, while the species composition in this natural enrichment differed from marine phototrophs such as algae. Planctomycetes were shown to be involved in phytoplankton bloom utilization in a fast-flowing, river-dominated estuary as well.

One might conclude that planctomycetes have a striking relationship with phototrophs as such while evidence point towards their main role in degradation of such phototrophs. This relationship might not be limited to aquatic habitats since for example 10.61% of the bacterial microbiome on infected citrus plants were planctomycetes as well.

Taken together, planctomycetes seem to be associated with particles, surfaces, microbial mats, biofilms and eukaryotes. However, abundance alone is no measure for environmental importance and a deeper understanding of the planctomycetal physiology is required to understand their ecological role in the environment. The current knowledge points towards a role of planctomycetes in degrading complex polysaccharides a role that fungi fulfill in terrestrial habitats. However, other than initially speculated, planctomycetes can be dominant in terrestrial habitats as well and might even be associated with fungi given the weak evidence that they might digest fungal polysaccharides in mice intestine. A field tempting to explore.

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Plant Pathogens, Minor (Phytoplasmas)☆

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Glossary

Aster yellows phytoplasmas (AY) Phytoplasmas that belong to the 16SrI group within the '*Candidatus* Phytoplasma' genus.
Aster yellows phytoplasma strain witches' broom (AY-WB) A single phytoplasma strain belonging to '*Ca*. P. asteris' subgroup 16SrI-A that infects many plant hosts and is transmitted by the leafhopper *Macrosteles quadrilineatus* (Forbes). The strain was isolated from infected lettuce plants collected from fields in Ohio, USA. The genome of AY-WB has been fully sequenced.
Hemiptera An order of insects consisting of about 80,000 insect species that have sucking mouthparts with a rostrum capable of piercing tissues to suck out liquids. Within the order Hemiptera, three groups of phloem-feeding insects can transmit phytoplasmas from plant to plant. These groups are leafhoppers (Cicadellidae), planthoppers (Fulgoroidea), and psyllids (Psyllidae).
Mollicutes A class of bacteria with reduced genomes and no cell walls that forms one clade within the phylogeny of the Gram-positive eubacteria.
Mycoplasmas Human and animal pathogenic bacteria that belong to the order Mycoplasmatales within the class *Mollicutes*. All mycoplasmas use the UGA codon for tryptophan.
Onion yellows phytoplasma strain M (OY-M) A single phytoplasma strain of '*Ca*. P. asteris' subgroup 16SrI-B that is a pathogen of onions and is transmitted by the leafhopper *Macrosteles striifrons* and several other leafhopper species. OY-M was obtained from infected plant tissues in Japan.
Phloem Living tissue of plants that transports nutrients, including carbohydrates such as sucrose and glucose, to all parts of the plant. Phloem consists of two cell types: sieve cells and companion cells. The sieve cells form a tube in which individual cells are connected by sieve plates that have small pores. Sieve cells are live cells with various cell organelles, but don't have nuclei. They depend on companion cells for survival. Phytoplasmas are mainly located inside the cytoplasm of the live phloem sieve cells.
Phytoplasmas Insect-transmitted plant pathogens that form a monophyletic group within the class *Mollicutes*. Phytoplasmas and acholeplasmas share a common ancestor and use the standard genetic code in which three codons are used as stop codons. Phytoplasmas were previously named mycoplasma-like organisms (MLOs). Recently, they were assigned to the genus '*Ca*. Phytoplasma'.
Spiroplasmas Bacteria with no cell wall with predominantly helical morphologies associated with various invertebrate hosts in pathogenic and commensal interactions. Spiroplasmas belong to the family Spiroplasmataceae of the order Entomoplasmatales within the class *Mollicutes*. Spiroplasmas use one of the three stop codons, the UGA codon, for tryptophan. Three spiroplasma species are insect-transmitted plant pathogens and share host ranges with phytoplasmas. These are citrus stubborn spiroplasma (*Spiroplasma citri*), corn stunt spiroplasma (*Spiroplasma kunkelii*), and *Spiroplasma phoeniceum*.

Abbreviations

AMP	antigenic membrane protein
AYP	aster yellows phytoplasma
AY-W	Baster yellows phytoplasma strain witches' broom
BYDV	barley yellow dwarf virus
CSS	corn stunt spiroplasma
dnaB	DNA helicase
dnaG	DNA primase
ELISA	enzyme-linked immunosorbent assay
hflB	Zn-dependent protease
himA	DNA-binding protein HU
IDP	immunodominant membrane protein
IRPC	MInternational Research Program on Comparative Mycoplasmology
MalE	maltose-binding protein
MAMP	microbe-associated molecular pattern

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MBSP	maize bushy stunt phytoplasma
MLO	mycoplasma-like organism
MR	maize redness
MRFV	*maize rayado fino virus*
NLS	nuclear localization signal
ORF	open reading frame
OY-M	onion yellows phytoplasma strain M
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PMU	potential mobile unit
PTS	phosphotransferase transport system
qPCR	quantitative PCR
RFLP	restriction fragment length polymorphism
SBP	solute-binding protein
sigF	sigma factor
sigma(H)	stress response sigma factor
SP	signal peptide
ssb	single-stranded DNA-binding protein
SVM	sequence-variable mosaic
tmk	thymidylate kinase
tra5	transposase
YFP	yellow fluorescent protein

Introduction

Phytoplasmas are economically important plant pathogens that are transmitted by phloem-feeding insects. These bacteria replicate intracellularly in their plant and insect hosts. Phytoplasmas have probably evolved from a Gram-positive *Clostridium*-like ancestor through genome reductions. The cells of these bacteria are small, averaging ~500 nm in diameter, and are surrounded by a single membrane and no cell wall. Phytoplasma detection has been difficult, because they are still difficult to culture and are frequently present in low numbers. Phytoplasma diagnostics have greatly improved with the accumulation of sequence information of primarily 16S ribosomal DNA genes. The complete genome sequences of five phytoplasma strains provided insights into the metabolic capabilities of phytoplasmas. Even though phytoplasma genomes are small, some of them have multiple copies of units up to approximately 20 kb in length that bear similarities to replicative composite transposons. Several efforts of scientists have focused on the functional characterization of phytoplasma virulence factors. Phytoplasmas use the Sec-dependent protein translocation system for export of virulence factors. Virulence factors characterized so far are cell surface proteins involved in insect transmission and secreted proteins of which some target plant cell nuclei and manipulate plant and insect processes.

Phytoplasmas form a monophyletic group within the class *Mollicutes*. First thought to be viruses, they are recognized as cell wall free pleomorphic bacteria (Fig. 1). They have small genomes averaging ~750 kb. Close to a 1,000 phytoplasmas have been detected in hundreds of monocot and dicot plant species all over the world. They commonly induce dramatic symptoms in plants, for example, witches' broom (clustering of branches due to the loss of plant apical dominance) and phyllody (floral organs that are changed to the condition of leaves) combined with generalized yellowing. The phytoplasmas are transmitted by sap-feeding insects

Fig. 1 Electron microscopy picture of phytoplasmas in a sieve tube of gladiolus showing virescence and phyllody in the inflorescence. Different sizes and shapes of the organisms are present (magnification X 8,000).

and invade and replicate efficiently in their plant and insect hosts. They thus represent a rare example of pathogens with a trans-kingdom habitat consisting in plant and animal hosts.

Evolutionary History

Phytoplasmas belong to the class *Mollicutes* of which members form one clade within the phylogenetic tree of the phylum *Firmicutes* (Fig. 2). This phylum embraces Gram-positive eubacteria with low GC versus AT ratios in their genome sequences, such as *Bacillus*, *Lactobacillus*, *Clostridium*, and *Streptococcus* spp., *Mollicutes* are believed to have diverged from a Gram-positive *Clostridium*-like bacterium through extensive genome reduction events. Mollicute cells can be flask-like, oval, and round-shaped with sizes ranging between 80 and 800 nm in diameter.

Within the class *Mollicutes*, all phytoplasmas are placed in a single monophyletic group that has common ancestry with the acholeplasmas (Fig. 2). Together, the phytoplasmas and acholeplasmas belong to the order Acholeplasmatales. Members of this order diverged early from the other mollicutes that are grouped into three other orders: the Mycoplasmatales (mycoplasmas), Entomoplasmatales (spiroplasmas and mesoplasmas), and Anaeroplasmatales (anaeroplasmasa and asteroplasmas) (Fig. 2). Their phylogeny is consistent with important metabolic characteristics of the mollicutes. For example, the phytoplasmas and acholeplasmas use the standard genetic code in which three codons are used as stop codons, whereas the mycoplasmas, spiroplasmas, and mesoplasmas use one of these stop codons, the UGA codon, for tryptophan (Fig. 2). The majority of the described mycoplasmas are pathogens of humans and livestock, but they are also present in natural faunas (Fig. 2). Spiroplasmas are most often associated with

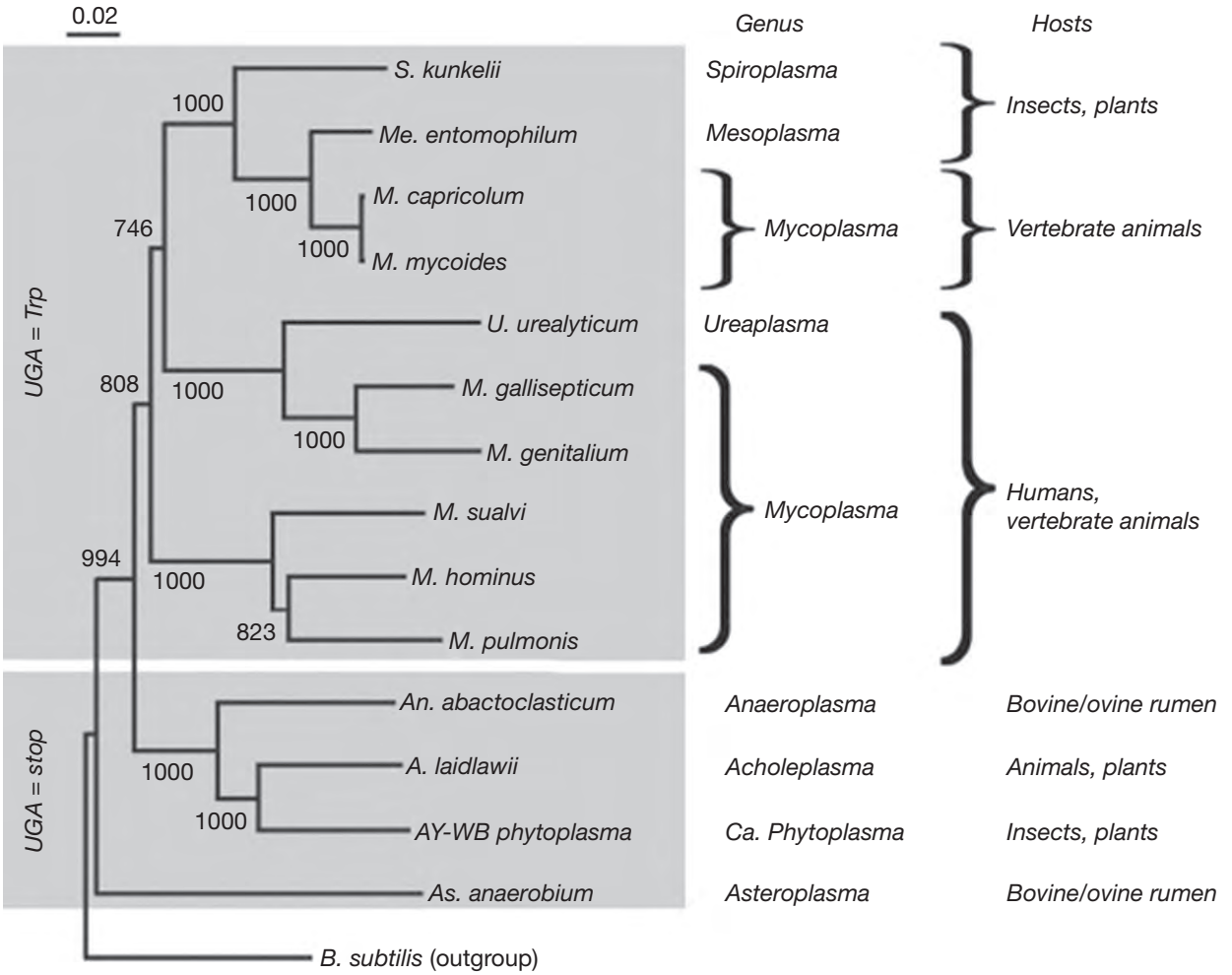

Fig. 2 Taxonomy and other characteristics of members of the class *Mollicutes*. The phylogenic tree was constructed based on 16S ribosomal DNA (rDNA) sequences. Sequence alignments were generated in Clustal X (1.83), and the phylogenic tree was constructed using the Neighbor Joining method. Confidence values out of 1000 bootstrap trials are indicated at the branches. The *Bacillus subtilis* 16S rDNA sequence was selected as outgroup. Genus names and host range of members of each genus are indicated. Some mollicutes use a standard genetic code with three stop codons, whereas other mollicutes use one of these codons, the UGA codon, as tryptophan, as indicated in the phylogenic tree.

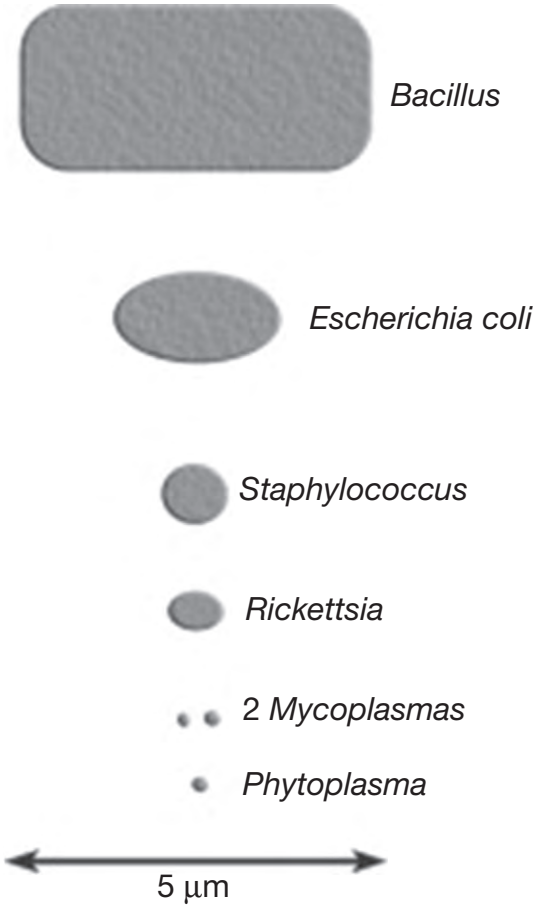

Fig. 3 Size comparisons of some eubacteria sizes. Phytoplasmas have the same size as mycoplasmas.

insects and crustaceans as obligate or facultative symbionts in pathogenic or beneficial interactions (Fig. 2). Unlike most other mollicutes, the spiroplasmas have helical cell morphologies in addition to the flask-like, oval, and round shapes. Three *Spiroplasma* spp. are also insect-transmitted plant pathogens. These are *Spiroplasma citri*, *S. kunkelii*, and *S. phoeniceum*. *S. citri* and *S. kunkelii* are transmitted by leafhopper species, whereas the vector of *S. phoeniceum* has not yet been identified. These *Spiroplasma* spp. can share plant and insect host ranges with phytoplasmas.

Before 1967, the disease now associated with the presence of phytoplasmas were thought to be caused by viruses. Indeed, phytoplasmas are much smaller than most other bacterial plant pathogens (Fig. 3) and they can pass through fine sterilization filters used for capturing bacteria. Furthermore, phytoplasmas are not easily cultured and are insect-transmitted. In 1967, Japanese scientists discovered by chance the presence of organisms that looked like mycoplasmas in the phloem tissues of plants that were associated with yellows and witches' broom symptoms. Since then, the name mycoplasma-like organisms (MLOs) was adopted and used to refer to the mycoplasmas found in plants. To distinguish the plant-associated mycoplasmas from the phylogenetically distinct plant infecting mycoplasmas, the trivial name phytoplasma was proposed at the Tenth Congress of the International Organization of Mycoplasmology held in Bordeaux (France) in 1994.

Thanks to molecular tools, phytoplasmas were initially classified into groups based on restriction fragment length polymorphism (RFLP) patterns of polymerase chain reaction (PCR) products of 16S rDNA sequences. To date 33 groups (16SrI, II, III, etc.) have been identified. In these 16Sr groups, phytoplasmas were further divided into subgroups (16SrI-A, I-B, I-C, etc.). Later on full 16S rDNA sequences and subsequent phylogenetic analyses have provided further evidence for phytoplasma diversity. In 2004, the Phytoplasma/Spiroplasma working team within the International Research Program on Comparative Mycoplasmology (IRPCM) published the '*Candidatus* Phytoplasma' species for strains with a 16S rRNA gene sequence that has lower than 97.5% similarity to that of any previously described '*Ca*. Phytoplasma' species. 43 '*Ca*. Phytoplasma' species have been described so far.

Ecology

Phytoplasmas need plants and insects for their dispersal in nature (Fig. 4). In plants, phytoplasmas are mainly located in the phloem sieve cells that transport sugars and other nutrients to plant sink tissues that need these sugars and nutrients for growth.

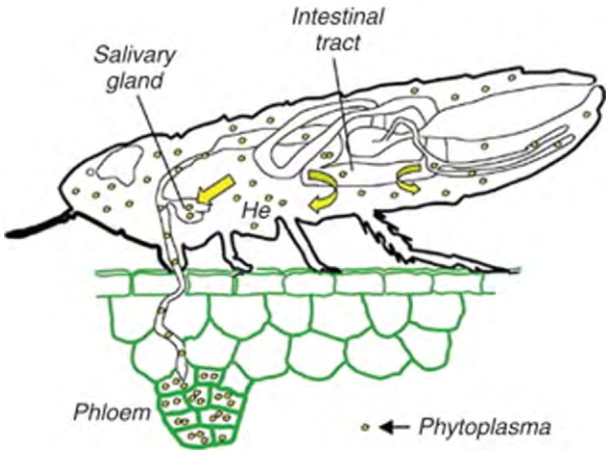

Fig. 4 Schematic representation of the route of phytoplasma movement between a plant and a leafhopper vector. Phytoplasmas (yellow dots) are limited to the phloem in plants. A phloem-feeding leafhopper acquires phytoplasmas from the plant phloem. Phytoplasmas that are ingested with plant sap arrive in the intestinal tract and invade (arrows) epithelial and muscle cells of the intestinal tract. Phytoplasmas enter the hemolymph (He), and from there invade (arrow) the salivary glands. They are then introduced back into the phloem tissue of host plants along with the saliva during feeding of leafhoppers.

Fig. 5 Plant of *Callistephus chinensis* (China aster) showing symptoms of virescence and phyllody associated with the presence of phytoplasmas in their sieve tubes.

Phytoplasmas spread throughout the plant by moving through the pores of the sieve plates that connect the phloem sieve tubes. Insect vectors of phytoplasmas are mainly phloem-feeding leafhoppers (Cicadellidae), planthoppers (Fulgoroidea), and psyllids (Psyllidae) of the order Hemiptera. Once acquired by their insect vectors, phytoplasmas invade their guts and salivary glands and many other tissues where they can accumulate in great numbers. They have to reach the saliva for subsequent introduction into the plant phloem during insect feeding. The latent period, that is the time between initial acquisition of the phytoplasmas by the insect vector from plants and the ability of the insect to introduce phytoplasmas back into the plants, can vary between 7 and 80 days. In plants, symptoms can develop in ~7 days after introduction of the phytoplasma by the insect vector, but it can take much longer (6–24 months), depending on phytoplasma - plant species combinations.

Phytoplasmas have a broad plant host range, which mainly depends on the plant feeding range of their insect vectors. The more than 100 members of the 16SrI ribosomal group ('*Candidatus* Phytoplasma asteris'-related strains), collectively known as aster yellows phytoplasmas (AY) (Fig. 5) are vectored by at least 30 often polyphagous insect species and, as a consequence, are capable of infecting over 300 species in 38 botanical families. A single AY strain can infect diverse plants. For example, the witches' broom

strain (AY-WB) can be transmitted by the generalist *Macrosteles quadrilineatus* (Forbes) to China aster (*Callistephus chinensis* Nees), lettuce (*Lactuca sativa* L.), *Nicotiana benthamiana* L., tomato (*Solanum lycopersicon* Mill), *Arabidopsis thaliana* L., and maize (*Zea mays* L.).

Overall, crops susceptible to AY include but are not restricted to lettuce, celery, escarole, endive, carrot, tomato, rape, cabbage, broccoli, kale, turnip, radish, onion, strawberries, flax, and potato. Susceptible ornamentals include aster, marigold, chrysanthemum, *Coreopsis*, *Primula* species, *Catharanthus*, *Delphinium*, *Gladiolus*, and *Hydrangea*. AY-susceptible woody fruit crops and ornamentals include grapevines, peach trees, blueberry, apricots and poplar trees. Wild plants and weeds that frequently border crop production fields, such as clover, plantain, purple coneflowers, *Ranunculus* spp., dandelions, parrot's feather, amaranth, false daisy, sowthistle, and Flora's paint brush, are also AY hosts. The broad range of plant hosts of phytoplasmas, along with generalist insect vectors, makes phytoplasma outbreaks unpredictable. Furthermore, because of the long incubation periods of phytoplasmas in plants and insects, outbreaks are often detected too late, that is, close to harvesting of the crops and after dispersion of the phytoplasmas by the insect vectors. This makes it harder to control or manage the disease cycle.

Economic Importance

The presence of phytoplasmas is associated with dramatic losses in many annual crop production areas. Yield losses of 60%–80% are common in leaf lettuces and carrots in years when AY phytoplasmas are prevalent. These phytoplasmas are also common in wheat, barley, and oats in some geographic areas of the world. They represent an important threat also for ornamental plants such as gladiolus and other bulbous species in which the phytoplasma presence changes the phenotype (Fig. 6). Disease symptoms induced by AY and the aphid-transmitted barley yellow dwarf virus (BYDV) are similar in small grains. A survey conducted from 2003 to 2005 in the USA states of Minnesota and North Dakota revealed that up to 49% of the production fields tested positive for AY compared to only a maximum of 5% BYDV-positive fields. Phytoplasmas are prevalent in other grain crops, such as maize. Maize bushy stunt (MBS) causes major yield losses of maize in Mexico and South America, where maize is grown throughout the year. Furthermore, in Serbia, Romania, and Bulgaria maize redness (MR) was a disease present for 60 years. Recent outbreaks of MR resulted in 40%–90% yield loss and were associated with the presence of "stolbur" phytoplasma ('*Ca*. P. solani') within the last 15 years. Moreover at least three different phytoplasmas were found associated with potato purple top disease that has been devastating the potato production industries of USA and Mexico. Thus, annual field crops frequently encounter outbreaks of phytoplasma diseases.

Phytoplasmas cause particularly devastating losses in high-value production crops. "Flavescence dorée" (ribosomal subgroups 16SrV-C/-D) and "bois noir" (ribosomal subgroup 16SrXII-A) phytoplasmas are a severe threat to vineyards of major grapevine growing areas in Europe affecting up to 80%–100% of the plants when in epidemic phases (Fig. 7). *Rubus* stunt ('*Ca*. P. rubi') phytoplasma is widespread in cultivated and wild blackberries (*Rubus* spp.); European stone fruit yellows ('*Ca*. P. prunorum'), apple proliferation ('*Ca*. P. mali'), and pear decline ('*Ca*. P. pyri'), which all belong to the apple proliferation (16SrX) group, are also prevalent in fruit orchrds of most European countries. In America, stone fruit trees and grapevines are infected by phytoplasmas classified in the 16SrIII group ('*Ca*. P. pruni'). A final example is that of the phytoplasmas associated with coconut lethal yellowing disease that enclose phytoplasmas in the 16SrIV group in America and phytoplasmas of the 16SrXXII group ('*Ca*. P. palmicola') in western Africa (Fig. 7). These phytoplasmas have devastated coconut plantations resulting in serious losses to the national economies in countries that, in many cases, utilize coconut as a primary crop for farming, tourism, and export.

Fig. 6 Gladioulus inflorescence showing virescence (top) compared with a normal inflorescence (bottom): the presence of phytoplasmas modify the anthocian metabolism and the flower production is therefore without commercial value and represents source of phytoplasma infection.

Fig. 7 Symptoms of grapevine yellows in leaves (left) associated with phytoplasma presence represented by reddening of the leaf lamina and therefore reduced plant production. On the right symptoms of lethal yellowing in palm represented by progressive yellowing of the external fronds that will eventually fall down leaving a bare trunk.

Phytoplasmas are also associated with diseases in ornamental and native plants and trees. Phormium yellow leaf phytoplasma, which is a '*Ca*. P. australiense'-related strain (16SrXII-B subgroup), is associated with a disease of *Phormium tenax* (New Zealand flax) that is a native plant in New Zealand and Norfolk Island. This plant has played an important role in the cultural history and (export) industry of New Zealand as its long fibers were used for a broad variety of products, including, for example, clothing. Unfortunately, in the last century, the phytoplasma disease resulted in a massive collapse of the *P. tenax* fiber industry in New Zealand. More recently, evidence was provided that phormium yellow leaf phytoplasma has spread to other plant species in New Zealand, including the native cabbage tree (*Cordyline australis*). Usually the phytoplasma infection induces yellowing and the falling of leaves, leading to the loss of the cabbage tree within 3–12 months. Similar phytoplasmas were also found in strawberries (strawberry lethal yellows) in New Zealand, and in grapevines (grapevine yellows) and papaya (papaya dieback) in Australia. In Europe and North America, trees are frequently destroyed by phytoplasmas. Elm yellows and witches' broom phytoplasmas greatly reduced elm (*Ulmus* spp.) plantations in urban and forest areas. Many of the elms surviging to severe epidemic of Dutch elm disease actually died for phytoplasma infections. Ash yellows phytoplasma ('*Ca*. P. fraxini') induces slow growth, progressive dieback of stems, leaf necrosis, yellowing, and witches' broom symptoms in ash trees (*Fraxinus* spp.). In several cases, 30% mortality rates of ash trees due to phytoplasma infections have been reported mainly in USA.

There is a general concern that phytoplasma disease incidences will increase in the future. An increase in phytoplasma epidemics is expected in more intensive farming in which crops may be grown the whole year round. For example, outbreaks of corn stunting agents, including MBS phytoplasma, corn stunt spiroplasma (CSS, *S. kunkelii*), and *Maize rayado fino virus* (MRFV), are prevalent in regions of Brazil, Argentina, and Mexico where maize is grown in summers and winters. This is because the leafhopper *Dalbulus maidis* (Cicadellidae), which uses maize as the main feeding and developmental host and is the most important vector of MBS, CSS, and MRFV in these regions, can reproduce all year round, resulting in massive population sizes of this leafhopper. In contrast, in regions where maize is grown once a year, only few *D. maidis* survive and, consequently, incidences of these infections are less frequent.

Global warming is likely to result in greater phytoplasma outbreaks in the temperate regions of the world. Leafhoppers generally do not survive for long periods in areas with below-freezing temperatures, but several species can migrate for long distances. For example, the leafhopper *M. quadrilineatus* (Cicadellidae) does not survive the snow and below-freezing temperatures of northern America, but in spring it migrates north from southern regions where temperatures generally remain above 0 C in winter.

M. quadrilineatus is the vector of many AY phytoplasmas. Temperate regions in Europe and northern America that have experienced warmer winters in the past years have simultaneously experienced more problems with leafhopper-transmitted phytoplasmas in various crops such as ornamental flowers, and trees. It should be noted that insect vector populations may also have increased because of a decrease in pesticide use in recent years.

The presence of phytoplasmas in diseased and asymptomatic plants is probably underreported to a great extent. The major reason is that phytoplasma detection is not easy. Phytoplasmas were only cultured *in vitro* recently and some technical problems with these approaches persist, they also are often present in low numbers, especially in plants that are in a dormant stage of their life cycle. Enzyme-linked immunosorbent assay (ELISA)-based techniques are not sufficiently specific in most cases. However phytoplasma diagnostics have greatly improved since the 1990s. Molecular techniques and phytoplasma genome sequences became available allowing the development of sensitive and specific PCR techniques. PCR combined with RFLP analyses and sequencing enabled phytoplasma identification in the preliminary '*Candidatus*' taxon or at the ribosomal group and subgroup level. Through these methodologies, the huge diversity of phytoplasmas, their ability to infect a broad range of plant species and the presence of two or more phytoplasmas in one diseased plant species were discovered. It also became apparent that plant species can harbor phytoplasmas but are asymptomatic. Quantitative PCR (qPCR) and bioimaging techniques to quantify phytoplasma concentration in plants and insects have been developed. These techniques are important to obtain a more fundamental understanding of phytoplasma growth curves in plants and insects, which in turn, will help to determine differential susceptibility of plant species and varieties to the phytoplasma infections.

Association With Plants

In plant hosts, the mature sieve tubes of the phloem normally contain the highest concentration of phytoplasmas (Fig. 1). In addition, intracellular phytoplasmas with various morphologies, some probably caused by budding or multiplying, were also found inside the cytoplasm of immature phloem element. Furthermore, phytoplasmas have been detected in the cytoplasm of phloem parenchyma cells adjacent to sieve elements, inside parenchyma cells in or close to the vascular system of *Cascuta* shoots, and in embryos of coconut. Phytoplasmas were detected in seed and in seedlings from infected mother plants of a number of species such as maize, tomato, alfalfa, sesame, winter oilseed rape, and there is also some evidence that phytoplasmas can be transmitted to not only next-generation plants but also, only in the case of some tomato variety, to second generation plants. These phytoplasma plants are usually asymptomatic.

AY-WB is usually abundant in phloem tissue of sink areas, such as young shoots and roots, consistent with the development of typical symptoms (witches' broom) in these young tissues. Some phytoplasmas (e.g., MBS) induce severe phloem necrosis in their host plants, indicating that these plants react to the phytoplasma infection. However, many phytoplasmas, including AY-WB, do not induce phloem necrosis but accumulate in high numbers in phloem elements. As has been shown for several other bacterial plant pathogens, it is likely that phytoplasmas produce a series of virulence proteins that suppress plant responses.

Phytoplasmas generally have a negative impact on the fitness of their plant hosts. Plants are frequently stunted and may not produce normal flowers, fruits, or seeds. In contrast, phytoplasmas may have a positive effect on their insect vectors. For example, the longevity and number of offspring of the aster leafhoppers (*M. quadrilineatus*) can significantly increase when AY-infected, as compared to healthy China aster, lettuce, carrots (*Daucus carota* L.), and periwinkle [*Catharanthus roseus* (G) Don]. In addition, some leafhopper species of the genus *Dalbulus* that carry MBS phytoplasmas live longer when deprived of their main plant-feeding host compared to leafhoppers that do not carry them. Phytoplasmas can also manipulate plants to become new hosts for leafhoppers that normally do not use these plants as hosts. For example, the leafhopper *D. maidis*, which is a maize specialist, can feed and survive on AY-infected but not on healthy lettuce and China aster plants. In fact, the survival rates of *D. maidis* on AY-infected China aster plants and healthy maize plants are comparable. Similar findings were reported for nine leafhopper species on AY-infected celery (*Apium graveolens* L.) and China aster. Thus, phytoplasmas have a remarkable effect on the interactions between insects and plants, that is, they may convert plants from being nonhosts into hosts or better hosts for some phloem-feeding insects.

The mechanism by which phytoplasmas convert plants into more attractive hosts for insects is not yet known. One explanation for this conversion is that phytoplasma infection induces the production of additional younger green/yellow plant tissues (witches' broom, phyllody, virescence, and yellowing) that is more attractive to leafhoppers, which prefer young green/yellow tissues for feeding as well as for egg laying. It is also possible that phytoplasmas are involved in the down regulation of insect-specific defense responses in plants to attract maladapted insects and improve insect fitness on these plants. For example, phytoplasmas may be able to down regulate general defense responses to insects, such as the jasmonic acid signaling pathway that is involved in defense responses to leafhoppers.

Varieties of some plant species have some degree of resistance or are to some extent tolerant of the phytoplasma presence. However, phytoplasma resistance genes have not yet been cloned and characterized. Unlike the majority of bacterial plant pathogens that are extracellular and use specialized secretion systems for delivering virulence factors into plant cells, phytoplasmas are phloem-limited intracellular pathogens of plants and release their virulence factors directly into the plant cell cytoplasm. Hence, plant resistance mechanisms to phytoplasmas will most likely different from those of other bacterial pathogens.

Association With Insects

When acquired by the insect vectors from plants (Fig. 4), phytoplasmas have to attach them selves to the membranes of midgut epithelial cells, on or between microvilli, and initiate the mudgut colonization. Spiroplasmas and mycoplasmas have specific tip structures that are primarily responsible for attachment to cell membranes between microvilli. Such tip structures have not been identified in phytoplasmas, but it is likely that phytoplasmas use a comparable mechanism for attachment. At least one membrane protein, the antigenic membrane protein (AMP), important for insect invasion of onion yellows phytoplasmas strain M (OY-M), has been identified.

Phytoplasmas can accumulate in large numbers outside the basal lamina of these epithelial cells in the hemolymph, hemocytes, and particularly in muscle fibers and tracheae that form the outer layer of the midgut. Phytoplasmas, *S. citri* and *S. kunkelii* accumulate at similar locations in their leafhopper vectors. There is evidence that *S. kunkelii* ruptures the extracellular matrix of the basal lamina of the midgut, possibly with specific enzymes, for entering the hemolymph. The hemolymph provides a vehicle for these mollicutes to reach other tissues, including the salivary glands.

Insect salivary glands can get heavily infected with AY-WB and MBS. Individual phytoplasma cells appear to reside directly, and probably multiply, in the cytoplasm of salivary gland cells sometimes close to the nucleus. Like spiroplasmas, phytoplasmas probably enter the canaliculi at the center of secretory cells, before reaching the main salivary duct that leads to the stylet's salivary canal. They are then introduced into the plant phloem elements along with the insect salivary secretions during feeding (Fig. 4).

Metabolism

All members of the class *Mollicutes* are resulting from severe genome reductions. The genome of the mollicute *Mycoplasma genitalium* was the first to be sequenced to completion because it has one of the smallest genomes (580 kb) for a living organism. *M. genitalium* and many other mycoplasmas lack many anabolic pathways, including those for synthesis of amino acid, purines and pyrimidines, and fatty acids. In addition, even though the glycolytic pathway for catabolic metabolism is present, mycoplasmas generally lack tricarboxylic acid cycle and electron transport systems. Spiroplasmas have maintained more metabolic genes. For example, they still have the majority of the amino acid synthesis pathways, and more cytoskeleton genes that play a role in the formation of helical cell shapes typical to most spiroplasmas. Spiroplasmas have genome size ranging from 760 to 2,220 kb. Mycoplasmas and spiroplasmas also have phosphotransferase transport systems (PTSs) for import of glucose, trehalose, and fructose to feed the glycolytic pathway.

The complete genome sequences of two phytoplasmas (OY-M and AY-WB) revealed that phytoplasma genomes are even more reduced than those of mycoplasmas and spiroplasmas. AY-WB and OY-M lack PTSs for the import of sugars essential for glycolysis. AY-WB and OY-M also lack F-type ATP synthases. This is in contrast to mycoplasmas and ureaplasmas that have these ATP synthase complexes, including the A, B, and C subunits for the transmembrane channel and the five-subunit (alpha, beta, gamma, delta, and epsilon) catalytic core for ATP synthesis. Phytoplasmas have a rudimentary set of genes involved in SOS response and the standard recombination pathway, including only, *recU*, *ssb*, *polA*, *gyrA*, and *gyrB*.

The phytoplasma metabolism appears different from that of mycoplasmas and spiroplasmas in several ways. Even though phytoplasmas do not have PTS systems to feed the glycolysis pathway, they seems to possess ABC transporters for the import of maltose, including a maltose-binding protein (MalE) that may have affinity to maltose, trehalose, sucrose, and palatinose. Affinity of MalE to trehalose is likely, as trehalose is a major sugar in the insect hemolymph. However, the fate of these sugars after import is not clear, because enzymes required for the conversion of these sugars to glucose-6-phosphate for glycolysis were not found in the phytoplasma genomes. Furthermore, the sucrose phosphorylase gene, which is important for sucrose degradation, is fragmented in the OY-M phytoplasma genome and is completely absent from the AY-WB phytoplasma genome.

Phytoplasmas and mycoplasmas apparently use different routes for the generation of pyruvate. Phytoplasmas have malate/citrate–sodium symporter genes for import of L-malate or related molecules such as oxaloacetate, and a NAD-specific malic enzyme (EC 1.1.1.38) for generating pyruvate. In contrast, mycoplasmas and spiroplasmas predominantly use L-lactate dehydrogenase (EC 1.1.1.27) for converting L-lactate into pyruvate. Thus, phytoplasmas may use malate/oxaloacetate as carbon sources.

Unlike mycoplasmas, phytoplasmas appear to be capable of biosynthesis of at least some of their own membrane phospholipids. The genomes of AY-WB, OY-M, and Western X-disease phytoplasma contain the *pssA* and *psd* genes encoding CDP-diacylglycerol-serine-*O*-phosphatidyltransferase (EC 2.7.8.8) and phosphatidylserine decarboxylase (EC 4.1.1.65), respectively. Both are part of the phosphatidylethanolamine pathway. Furthermore, the AY-WB and OY-M genomes contain a candidate *pmt* gene for phospholipid *N*-methyltransferase that is involved in phosphatidylcholine synthesis in conjunction with PssA and Psd. Phosphatidylcholine is a phospholipid that is a major constituent of cell membranes. AY-WB and OY-M also have all enzymes that link the glycolysis pathway to the glycerolipid pathway and an ABC transporter gene, *phnL*, involved in lipoprotein release.

Repeat-Rich Genomes

Even though phytoplasma genomes are small, they contain many repeated sequences. The AY-WB and OY-M genomes both contain long repeating units of DNA. Of the 671 predicted open reading frames (ORFs) on the AY-WB chromosome, 191 (28%) ORFs covering 97,374 bp (13.8%) are present as multiple copies. Of these 191 ORFs, 134 ORFs (20%) are contained in clusters named

potential mobile units (PMUs) or sequence variable mosaic (SMV). The studied PMUs consist of ORFs in a conserved order that all point in the same direction on the AY-WB chromosome. The ORFs that have similarities to sequences of other organisms are sigma factor (*sigF*), single-stranded DNA-binding protein (*ssb*), DNA-binding protein HU (*himA*), Zn-dependent protease (*hflB*), thymidylate kinase (*tmk*), DNA helicase (*dnaB*), DNA primase (*dnaG*), and transposase (*tra5*). One or more ORFs with unknown functions, of which some are predicted to encode membrane-targeted proteins, are present in three locations on PMUs, that is, between *himA* and *hflB*, *hflB* and *tmk*, and *dnaG* and *tra5*. The AY-WB genome contains three apparent full-length PMUs (PMU1, 2, and 3) and many PMU-like sequences that appear to be degenerated versions of PMUs based on the presence of partial ORFs and deletion of PMU sections. The repeated presence of PMUs, their gene contents, including genes for recombination (*tra5*, *ssb*, *himA*) and replication (*dnaG*, *dnaB*), and their conserved gene orders suggest that the PMUs are replicative composite transposons. Clusters that contain one or more PMU ORFs were also found in other phytoplasma genomes in addition to the AY-WB and OY-M phytoplasmas, and were named sequence-variable mosaics (SVMs).

The *tra5* gene encodes a full-length ORFAB fusion transposase of the IS*3* family insertion sequences. In most bacteria, the IS*3* ORFs A and B are two separate ORFs in which the protein product of ORFA suppresses transposition activity, and the ORFAB fusion, which encodes the active transposase, is produced only in the event of a frameshift. The separation of ORFs A and B is important to prevent too frequent transposition events and subsequent genome alterations. In contrast, the majority of PMU *tra5* genes of AY-WB encode full-length ORFAB-fused transposases, suggesting that these transposons are continuously active. However, most *tra5* ORFs are preceded by a conserved sequence of 395 bp, indicating that *tra5* expression is regulated. This conserved 395 bp region coincides with the presence of conserved ~330 bp repeats that flank PMU1, and are present in PMU3 and other PMU-like regions. The function of these ~330 bp repeats is unclear. One possibility is that they mediate assembly of both ends of PMUs to enable transposition or PMU replication in the presence of the transposase.

PMUs apparently have had a major role in the phytoplasma genome evolution. The AY-WB genome is approximately 150 kb smaller than the OY-M genome, primarily due to fewer PMU sequences. In addition, alignment of the AY-WB and OY-M genomes revealed that regions containing PMUs in both genomes had undergone various rearrangements, whereas regions without PMUs had not. Indeed, generally large repeated DNAs have a destabilizing effect on a bacterial genome since they can recombine to generate deletions, inversions, and duplications of genome segments. The PMUs themselves apparently undergo rapid degeneration as evidenced by the presence of PMU-like regions carrying truncated copies of PMU genes and lacking various segments of full-length PMUs. There are also large differences in genome sizes and compositions in other members of '*Ca*. P. asteris', ranging from 660 to 1,130 kb and consisting of several fragments of 500 kb and larger. It is possible that PMUs are capable of splitting a single chromosome into two smaller chromosomes. The phytoplasmas sequenced so far have an incomplete set of genes involved in DNA recombination, including the lack of a full-length *recA*. The absence of these genes is likely to increase the stability of repeat-rich genomes.

Virulence

Several phytoplasma potential virulence factors have been characterized in recent years. It is likely that membrane proteins located on the exterior of the phytoplasma cell and secreted phytoplasma proteins play important roles in the interactions of phytoplasmas with plant and insect host cells. Indeed, one surface-exposed membrane protein, named Amp, appears to be involved in determining the transmissibility of OY phytoplasma by specific insect vectors. This protein interacts with insect microfilament complexes of the leafhopper intestinal tracts *in vitro* and *in vivo*. However, the OY Amp binds to microfilament complexes of only those leafhopper vectors that can transmit OY and not of leafhoppers that cannot transmit OY. Thus, Amp appears to determine insect specificity by mediating interactions of phytoplasmas with the first barrier to leafhopper infection, the insect gut.

The Amp of OY and other phytoplasmas constitute one type of a subset of proteins that are named immunodominant membrane proteins (IDPs). The IDPs make up a major portion of the exterior cell surface of most phytoplasmas. IDPs of many phytoplasmas have been characterized and have been classified into three major types. These are Amp, IDP, and immunodominant membrane protein A (IDPA). These three protein types are not orthologous to each other, but may have roles comparable to that of OY Amp in determining interactions with the insect host. This is consistent with the observation that IDPs are apparently undergoing positive selection. The sequence similarities among members of each type of different phytoplasmas are usually lower than sequences located adjacent to the genes encoding IDPs. Also, the portions of the IDPs that are exposed toward the surfaces of phytoplasmas that are likely to be in direct contact with host components are more divergent compared to portions of IDPs that are inside the phytoplasma membranes that are generally not in direct contact with host components. Thus, the surface-exposed portions of the IDPs seem to have accumulated changes in sequence in order to adapt to the host environment (i.e., positive selection).

Surface-exposed proteins of spiroplasmas are also involved in insect transmission. Phytoplasmas and spiroplasmas follow a similar route through their insect vectors, that is, they have to invade and escape the gut and salivary glands. Spiralin is a 26 kDa lipoprotein that is the most abundant protein on the cell surfaces of spiroplasmas, and it binds insect glycoproteins. A *S. citri* mutant with a disrupted spiralin gene multiplies at high titers in leafhopper vectors and plants, but the leafhopper transmission of this mutant is 100 times less efficient than of wild-type *S. citri*.

Phytoplasmas appear to predominantly secrete proteins via the Sec-dependent translocation system. So far, researchers have focused on the characterization of virulence (effector) proteins of Gram-negative bacteria. These bacteria generally remain

extracellular and use specialized secretion systems, such as the type 3 and type 4 secretion systems, for injecting effector proteins into host cells. The effector proteins introduced via these specialized secretion systems are involved in the suppression of plant defense mechanisms that plants trigger upon recognition of microbe /pathogen-associated molecular patterns (MAMPs/PAMPs) and avirulence proteins of the Gram-negative bacteria. Compared to Gram-negative bacteria, phytoplasmas have a quite different biology. First, they are located mainly intracellularly and can deliver proteins directly into the cell cytoplasm through the Sec-dependent pathway without the need of "injection needles" generated by type 3 and type 4 secretion systems. Furthermore, because phytoplasmas are more related to Gram-positive bacteria and lack an outer cell wall, they do not carry many of the MAMPs/PAMPs known to trigger the defense mechanisms to Gram-negative bacteria in plants.

Phytoplasmas have a functional Sec-dependent protein secretion system. The three genes for SecA, SecY, and SecE required for successful translocation of proteins across the membrane are present in the OY-M and AY-WB genomes. Furthermore, the immunodominant protein Amp is a substrate for the phytoplasma Sec system. Amp has an N-terminal signal peptide (SP) sequence that is recognized by the Sec system and is cleaved upon secretion, as in other bacteria. The SPs of mollicute proteins carry obvious similarities in composition compared to those of other bacteria. Thus, computer algorithms that can predict the presence of SPs at the N-termini of proteins, such as SP and pSORT, are useful for the mining of phytoplasma genomes for other secreted proteins. Indeed, among the 695 proteins identified in the AY-WB genome, 76 proteins contain SPs. Of these 76, 20 proteins contained a SP and additional transmembrane domains as predicted by TMHMM2.0. Protein AY-WB_599 that has similarity to OY Amp was in the list of 20 proteins. The remaining 56 proteins contained only SPs and no additional transmembrane domains, and are therefore likely secreted into the extracellular environment of AY-WB. This list of 56 proteins contains the five solute-binding proteins (SBPs) ArtI (AYWB_263), DdpA (AYWB_529), NlpA (AYWB_588), ZnuA (AYWB_624), and MalE (AYWB_667). The presence of AY-WB Amp and SBPs in the list of 76 proteins validates the SP predictions by the computer algorithms.

SBPs are frequently involved in bacterial virulence. For example, the SBP Sc76 of another insect-transmitted mollicute pathogen, S. *citri*, is important for infection of the salivary glands and transmission by the leafhopper *Circulifer haematoceps* (Cicadellidae). Many SBPs, including Sc76, are lipoproteins that remain attached to the bacterial membrane through a lipid anchor at the first conserved cysteine residue of the mature peptide. None of the five AY-WB SBPs and 51 other predicted secreted proteins have a conserved cysteine residue at the +1 position. Hence, the 56 secreted AY-WB proteins (SAPs) are likely released in the extracellular environment of AY-WB upon secretion, and therefore are putative effector proteins involved in the manipulation of plant and insect cells.

Additional independent evidence that the AY-WB SAPs are effector proteins was obtained. Two phytoplasma effectors, SAP11 and SAP30, contain eukaryotic nuclear localization signals (NLSs) that are functional in plant cells. Yellow fluorescent protein (YFP) fusions of SAP11 and SAP30 accumulated in nuclei of *N. benthamiana* cells, whereas YFP fusions of SAP11 mutants in which various portions of the NLS domain were deleted distributed throughout the plant cells. In *N. benthamiana*, nuclear localization of YFP-SAP11 requires a host factor, importin α, that is required for import of proteins into plant nuclei, including virulence proteins of plant pathogens. In immunofluorescence microscopy experiments with a specific antibody to SAP11, it was shown that SAP11 also accumulates in nuclei of AY-WB-infected China aster cells. Thus, SAP11 and perhaps also SAP30 are likely virulence factors involved in the manipulation of plant processes.

The unstable genomes of phytoplasma allow for relatively quick selection of spontaneous mutants. OY-M and NIM (OY-NIM) that were maintained in plants by insect transmission and grafting, respectively, gave rise to the non-insect-transmissible line OY-NIM. Comparison of the plasmids of OY-M and OY-NIM revealed that OY-NIM plasmid lacks two ORFs that are present on the OY-M plasmid. Thus, plasmids are also involved in insect transmission of phytoplasmas.

Conclusions

Phytoplasmas are economically important plant pathogens that affect annual and perennial crops, bushes and fruit trees, ornamental trees, and natural floras worldwide. All phytoplasmas are transmitted by phloem-feeding insects, mostly leafhoppers, planthoppers, and psyllids. Phytoplasmas have a unique biology among bacteria, because they need plants and insects for survival in nature and they can effectively multiply in both hosts. Furthermore, they replicate intracellularly in plants and insects. Phytoplasmas have evolved, along with other mollicutes, from a Gram-positive *Clostridium*-like ancestor through genome reductions and loss of outer cell wall. The cells of these bacteria are small but pleomorphic, averaging ~500 nm in diameter, and are surrounded only by a membrane.

Phytoplasma detection has been difficult they are frequently present in low amounts, particularly in dormant plants. Phytoplasma diagnostics have greatly improved with the availability of PCR methods and the accumulation of phytoplasma sequence information. The recent achievement on phytoplasma isolation from infected plants looks promising for further improvement of knowledge and diagnostic tools that will allow a focused management of phytoplasma associated diseases. Phytoplasma classification, now consisting in 43 '*Candidatus*' species and 33 ribosomal groups, as well as huge number of subgroups, will also benefit future progress.

Phytoplasmas have small genomes lacking major known metabolic pathways. The complete genome sequences of two phytoplasmas provided some insights into the anabolic and catabolic pathways utilized by phytoplasmas. Recently three other phytoplasmas were fully sequenced a '*Ca*. P. mali' strain, representing one of the few prokaryotes with a linear chromosome, and two strains of '*Ca*. P. australiense' that share several features with those already studied. These sequences revealed that the metabolic

requirements of phytoplasma seems to be different from the related mycoplasmas and spiroplasmas, albeit some phytoplasmas and spiroplasmas share the same plant and insect hosts. Even though phytoplasma genomes are small, they are repeat-rich. The repeats are clustered into large units, named PMUs and SVMs, which have had a major influence on phytoplasma genome evolution. These repeats are likely responsible for the large differences in sizes and compositions among phytoplasma genomes. The gene content and repeated presence of PMUs in the phytoplasma genomes suggest that they are replicative composite transposons.

More recent efforts of scientists have focused on the characterization of phytoplasma virulence factors. Proteins expressed by plasmids and abundant cells surface proteins are virulence factors involved in insect transmission. Phytoplasmas use the Sec-dependent protein translocation system for secretion of virulence factors, including effector proteins, into plant and insect hosts. Several effector proteins of the AY-WB phytoplasma target nuclei of plant cells and are likely involved in the manipulation of plant processes. Extending the concept of effectors to the phytoplasmas points to novel research strategies for unraveling pathogenicity mechanisms of these fascinating pathogens.

Preliminary evidence that phytoplasmas can be grown in cell free media in a suitable and flexible medium has been reported. This is an important development to facilitate the study of the phytoplasma biology. Further studies in progress aim to validate this result and to confirm that, despite the reduced genome size and the tight relationship with their hosts, phytoplasmas seems to retain an independent metabolism.

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Plastics: Colonization and Degradation

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Plastics, a Diversity of Synthetic Polymers

Plastic is defined as "material that contains, as an essential ingredient, one or more organic polymeric substances of large molecular weight, is solid in its finished state, and, at some stage in its manufacture or processing into finished articles, can be shaped by flow" (ASTM standard D883). While all plastics are polymers - meaning that they are repeated units of monomers - many polymers are not plastic (e.g., cellulose, starch etc.). Likewise, plastic can be synthesized from both petrochemical (fossil fuel-based) or biological (corn, soybean) stock sources, the later earning the label "bio-based". An example of this is polyethylene produced from corn made into drink bottles. These terms sometimes cause confusion in the literature because the term "bio-based" refers to the raw materials used to manufacture the plastic, and is not synonymous with "biodegradable", referring to "end of life" characteristics.

While plastic is sometimes considered a monolithic entity, one need only refer to resin identification codes on commonly discarded plastic consumer items to note the diversity of polymers in use. There are in fact hundreds of plastics commercially produced with the most common being polyethylene (high density HDPE - resin code 2; low density LDPE - 4); polypropylene (PP - 5); polyethylene terephthalate (PET - 1); polyvinyl chloride (PVC - 3); and polystyrene (PS - 6). Code 7 refers to "other" plastics and includes acrylic, nylon, polycarbonate (PC) and polylactic acid (PLA) (Table 1). Nearly all plastics contain additives such as plasticizers or colorants and other organic and inorganic compounds that impart the desired final characteristics in the polymer. The ability of plastics to be reshaped categorizes them further into thermoplastics and thermosets. Thermoplastics include HDPE, LDPE, PP, PET, PS and PVC that can be reheated and reformed while thermosets (e.g., polyurethane, epoxy, silicone and melamine to name a few) undergo chemical modifications when heated that prevent reheating and re-molding. Although the first "plastics" were invented in the early 20th century (bakelite, a phenol formaldehyde thermoset), it wasn't until the end of World War II that mass production of plastics began. According to PlasticsEurope, in 2016 the world production of plastics (excluding PET fibers, PA fibers, PP fibers and polyacryls-fibers) was 335 million metric tons, an increase of 13 million metric tons from 2015. Asia leads production in plastics materials at 50% of the total, with Europe at 19% and NAFTA countries (USA, Canada, and Mexico) at 18%.

Plastics have recently received much attention in the scientific and popular press due to a growing awareness of plastic litter in the ocean concentrated in so-called "Garbage Patches" or open ocean accumulation zones. Once thought to be restricted to the five ocean gyres - concentrations of plastic are also being described in the Polar regions and the Mediterranean. Most of the plastic that accumulates in accumulation zones is confetti-sized (<5 mm) and small pieces dominate visible plastic marine debris (PMD) numerically, but macroplastic (>5 mm) still accounts for the largest volume of plastic debris floating in our oceans. An estimated 8 million tons of plastic garbage enters the ocean each year, but estimates of total global floating plastic based on existing data are all

Table 1 Common petroleum and bio-based plastics and their behavior in aquatic environments

Plastic	*Abbr.*	*Resin ID code*	*Production*	*Origin(s)*	*Behavior in aquatic environment*
Polyethylene terephthalate	PET	1	Synthetic	Petroleum or bio-based	Sinks
High-density polyethylene	HDPE	2	Synthetic	Petroleum or bio-based	Floats
Polyvinyl chloride	PVC	3	Synthetic	Petroleum	Sinks
Low-density polyethylene	LDPE	4	Synthetic	Petroleum or bio-based	Floats
Polypropylene	PP	5	Synthetic	Petroleum	Floats
Polystyrene	PS	6	Synthetic	Petroleum	Sinks
Expanded polystyrene	PS	6	Synthetic	Petroleum	Floats
Biodegradable polyhydroxyalkanoate (PHA) aliphatic polyester derivatives					
Poly-3-hydroxybutyrate	PHB	7	Biological	Bio-based	Sinks
Poly-hydroxyvalerate	PHV	7	Biological	Bio-based	Sinks
Poly-hydrohexanoate	PHH	7	Biological	Bio-based	Sinks
Other biodegradable aliphatic polyesters					
Polyglycolic acid	PGA	7	Synthetic	Petroleum or bio-based	Sinks
Polylactic acid	PLA	7	Synthetic	Petroleum or bio-based	Sinks
Polybutylene succinate	PBS	7	Synthetic	Petroleum	Sinks
Polybutylene succinate adipate	PBSA	7	Synthetic	Petroleum	Sinks
Polycaprolactone	PCL	7	Synthetic	Petroleum	Sinks
Biodegradable polybutylene terephthalate (PBT) aromatic polyester derivatives					
Polybutylene succinate terephthalate	PBST	7	Synthetic	Petroleum	Sinks
Polybutylene adipate terephthalate	PBAT	7	Synthetic	Petroleum	Sinks
Polytetramethylene adipate terephthalate	PTMAT	7	Synthetic	Petroleum	Sinks

far lower than this annual input, so an important question is where is the missing plastic? Many plastics are less dense than water and float, though others, including most bio-based plastics are more dense than water and sink (Table 1). Because it is inexpensive and versatile, plastic use continues to increase, and its durability allows it to persist in the environment, presenting a threat to wildlife and polluting marine ecosystems. Mostly as a result of public pressure, there are new laws coming into effect that will ban the use of plastic microbeads in some consumer products and plastic bag bans are becoming more widespread world-wide. China will no longer accept foreign plastic waste for recycling; so many nations must rethink how to handle their plastic waste infrastructure.

Living in the Plastisphere: Colonization

The problem of PMD in the ocean gained media attention due to entanglement of and ingestion by large charismatic megafauna such as marine mammals, sea turtles, and birds, as well as reports on the accumulation zone in the North Pacific, what was called the "Great Pacific Garbage Patch". In the last five years, however, examination of the impact of plastic pollution on the entire marine ecosystem has increased dramatically. This includes examining the impacts and consequences of plastic litter at a scale relevant to microbial life - in the realm of microplastics that are defined as pieces of plastic that are <5 mm in size. Microplastics, whether primary particles that constitute pre-production pellets and microbeads employed in cosmetics or industry, or secondary fragmented remnants of larger items, has earned center stage in the environmental science arena. The estimated trillions of microplastic pieces in the ocean represents a new, anthropogenic substrate, and the thin layer of life that forms on the surface of this plastic - often referred to as the Plastisphere - is rapidly becoming an active focus of environmental microbiology research (Fig. 1). The Plastisphere is home to all three domains of microbial life, as well as many multicellular organisms. The discovery of the Plastisphere in the ocean has prompted scientists working in freshwater environments to carry out parallel investigations, confirming the ubiquity of microbial assemblages associated with plastic in all aquatic environments.

Colonization of surfaces in natural and anthropogenic environments (i.e., biofouling) has been a topic of investigation for decades and the field even has a dedicated journal. Much of this research is aimed at trying to figure out how to prevent surfaces from serving as a habitat for microbial and macrobial life on structures such as boat hulls, buoys and ballast water tanks, but there is also a lot of research in biomedical (dental and medical) and food science applications covering bioadhesion and biofilm formation.

In the marine environment, microbial colonization of plastic was first confirmed via culturing methods and later via Scanning Electron Microscopy (SEM) imaging in the mid-70′s when bacteria and diatoms were identified alongside hydroids on microplastic and macroplastic in open ocean and coastal environments. Several decades later, incubation experiments in combination with molecular methods employing DNA sequencing examined succession on plastic and other abiotic substrates *in situ* and provided the first glimpse of the taxonomic composition of microbial communities that colonized plastic surfaces directly submerged in the marine environment. While the sophistication of the different culture-independent methods has matured over time, this incubation

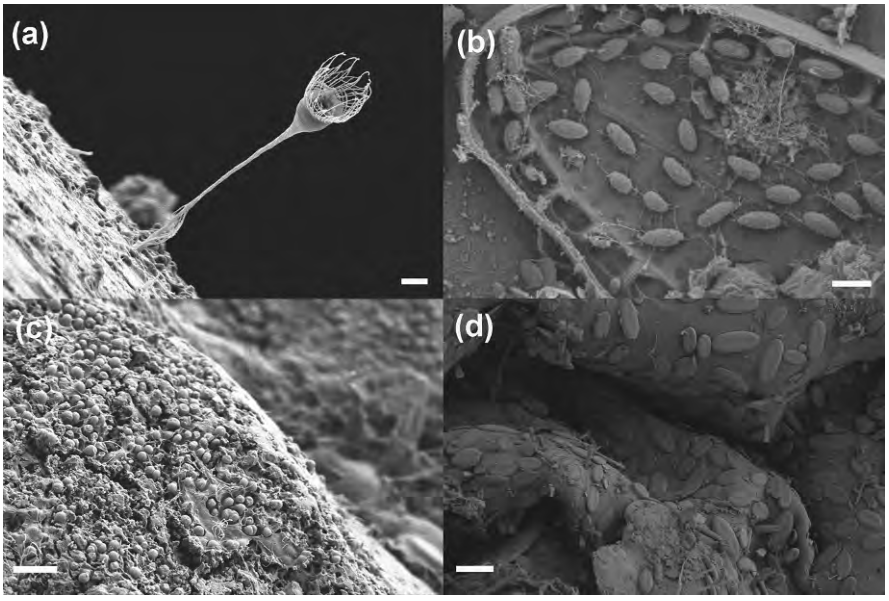

Fig. 1 Examples of Plastisphere members include: (a) heterotrophic flagellated protists such as choanoflagellates scale bar=2 μm; (b) secondary bacterial colonizers of an empty diatom frustule, scale bar=1 μm; (c) a field of "pit-forming" microbes appearing to be engaged in plastic degradation of the polymer surface via excretion of extracellular enzymes or metabolic products that etch the plastic surface, scale bar=10 μm; and (d) diatoms growing in the crevices of a piece of microplastic, scale bar=20 μm.

Table 2 Examples of *in situ* colonization experiments in marine environments

Location	*Plastic substrate*	*Size*	*Duration*	*Study*
Atlantic	PE	Macro (20×28 cm)	3 weeks	Lobelle and Cuncliffe, 2011. Mar. Poll. Bull. 62, 197–200.
	PET	Macro (bottles)	6 weeks	Oberbeckmann *et al.*, 2014. FEMS Microbiol. Ecol. 90, 478–492.
	HDPE, PP, PS, PET	Micro (5 mm)	Weeks - months	Amaral-Zettler *et al.*, 2015. Front. Ecol. Environ. 13, 541–546.
	PE	Macro (4×5 cm)	Weeks - months	ES&T, 51, 7350–7360.
	LDPE	Micro (5×5×1 mm)	0, 6 h, & 1, 2, 4, 7, 14 days	Harrison *et al.*, 2014. BMC Microbiology, 14:232.
	PET	Macro (bottles)	Winter, spring, summer	Oberbeckmann *et al.*, 2016. PLOS ONE, 11.
	PVA, PEG, PU	Macro (30×30)	24 and 72 h	Dang and Lovell, 2000. Appl. Environ. Microbiol. 66, 467–475.
	PC	Macro (25 mm circles)	2, 4, 6, 10, 20 days	Jones *et al.*, 2006. Microbial Ecology, 53, 153–162.
Mediterranean Sea	PS	Macro (5×5 cm) (10×10 cm)	2 weeks	Briand *et al.*, 2012. Biofouling, 28, 453–463.
	PE, Mater-Bi	Macro (15.5×5.4 cm)	15 and 33 days	Eich *et al.*, 2015. PLOS ONE, 10.
Pacific	PVC, PMMA	Macro (30×30 cm)	24 and 72 h	Dang and Lovell, 2008. Appl. Environ. Microbiol. 74, 52–60.
	PS	Micro (5 mm)	Weeks	Amaral-Zettler *et al.*, 2015. Front. Ecol. Environ. 13, 541–546.
	Acryl	Macro (170×100×1.5 mm)	3, 9, 24, 36 h	Lee *et al.*, 2008. J. Microbiol. 46, 174–182.
Australia	PET	Macro (1×1 cm)	6 months	Webb *et al.*, 2009. Microbes Environ. 24, 39.
Caribbean	PS, HDPE, PP	Micro (5 mm)	Weeks - months	Amaral-Zettler *et al.*, 2015. Front. Ecol. Environ. 13, 541–546.

Source: Abbreviations: PE: polyethylene, PET: polyethylene terephthalate: HDPE: high-density polyethylene, PP: polypropylene, LDPE: low-density polyethylene, PVA: polyvinyl alcohol, PEG: polyethylene glycol, PU: polyurethane, PC: polycarbonate, PS: polystyrene, PVC: polyvinyl chloride, PMMA: polymethylmethacrylate.

approach has allowed microbiologists to observe changes in community succession and their relation to polymer type, duration, season, geographic location and environment. Such molecular ecology studies have been published and described for a variety of plastic types and are summarized in Table 2.

From these efforts emerge some general observations that can characterize the state of knowledge of colonization of plastics in the marine environment: (1) There is a clear succession that occurs on plastic materials that are introduced into the marine environment that involves shifts in dominance of major groups of bacteria and eukaryotes (particularly photosynthetic bacteria and photosynthetic eukaryotes) over time scales ranging from hours to months; (2) Colonization studies that employ molecular approaches are limited by the available biomass that colonizes the surfaces, especially early in the process (this is also a limitation for studying biofilm communities on very small pieces of microplastic in general); (3) Many factors influence the observed results including size of the plastic piece used in the incubation experiment, its composition, the form of the control substrate, surface texture or degree of rugosity, geographic location, physico-chemical conditions, and season. For example animal larvae and microinvertebrates can settle on cm-sized fragments of plastic but would be less inclined to do so on micron and mm-sized fragments and this can influence biofilm composition. Other variables in the natural environment include the exposure to UV and mechanical action (including ingestion and defecation by marine organisms), the extent to which the plastics have been submerged in the marine environment (i.e. time), and the location in the water column or sediments where the plastics have resided (i.e. photic zone vs. mid-waters vs. sediments). One important observation in the wild is the importance of biofilms and biofouling communities in determining the fate of floating plastics in the ocean. A significant amount of plastics that float (especially PE and PP) have been detected in sediments. They are probably transported by marine snow and fecal pellets, and in addition, biofilm formation can increase the density of PMD. All of these mechanisms have been suggested as a reason for the presence of plastics that float in oceanic compartments below the surface, but the timeframes of this flux into the sediments is not well understood. All of these uncertainties make it difficult to extrapolate the results of experiments to the fate of microplastics and macroplastics in the natural environment.

Fomite Potential in the Plastisphere

One of the first descriptive studies employing microscope images of algae in the Mediterranean unveiled an important discovery: macroplastics could carry harmful algal blooming species such as the Paralytic Shellfish Poisoning (PSP)-producing dinoflagellate *Alexandrium taylori*. Subsequent studies have expanded the list of possible nuisance microalgae bloomers attached to PMD, to include the diatom *Ceratoneis closterium* that secretes nuisance mucilage that affects fishing and tourism.

One of the first studies to employ high-throughput DNA sequencing methodologies to characterize plastisphere communities in the open ocean made another important discovery: relatively high abundances (up to 24%) of the total microbial community on a piece of PMD in the North Atlantic was *Vibrio* bacteria, implicating plastic as a possible fomite transporting potential pathogens in marine environments. Subsequent studies employing culture-dependent techniques in the North and Baltic Seas isolated several species of *Vibrio* on 13% of their collected microplastics, including potential human pathogens such as *V. parahaemolyticus* responsible for gastrointestinal disease in humans. An unknown aspect of the fomite potential of plastics is the source of the harmful microbes: whether these pathogenic or HAB hitchhikers attach to plastic in coastal areas and then become swept up in ocean currents to potentially cross entire ocean basins. The change in biofilm communities attached to microplastics as they shift from freshwater sources (rivers and streams) to more marine estuaries and ultimately the open sea is an understudied aspect of Plastisphere research that warrants further investigation.

Future Outlook

The challenges associated with reconstructing the natural history of the Plastisphere community over time and space based on studies that characterize stages of colonization point to the following future research needs (Fig. 2): (1) A better understanding of the role of microbes in ingesting PMD in the nano and micron-sized fractions and how this may get transferred up the food web via ingestion by animals: that is, the connection between the microbial food web and higher trophic levels with respect to PMD; (2) The role of biofilms on the mobilization of persistent organic pollutants and metals that adsorb to the surfaces or additives in plastic and their transfer to higher trophic levels; (3) The turn-over rate of organisms living in the Plastisphere and how long they can survive in transit (particularly with respect to potential pathogens and HABs); (4) The role of viruses in the Plastisphere and their transfer to new environments via microplastics; and, (5) The extent to which biofouling via biofilm development is responsible for the sinking of otherwise positively buoyant plastics in the water column and to what extent they contribute to the missing plastic in the ocean.

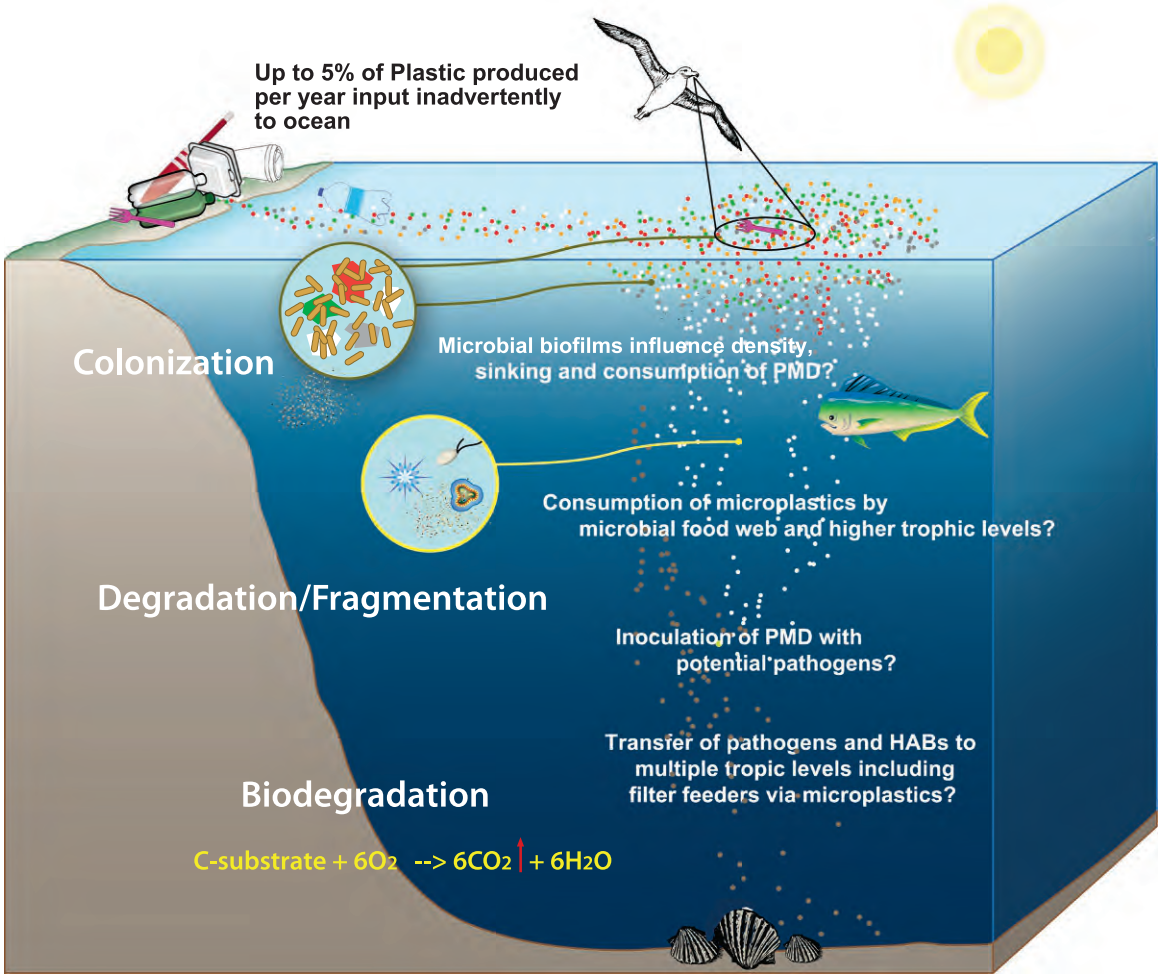

Fig. 2 A schematic diagram showing microbial interactions with PMD and possible impacts on different ocean compartments and at different tropic levels.

Degradation or Biodegradation?

The topic of degradation of plastics in the environment is a timely one that has mobilized the microbiology community, particularly in light of recent interest in applying biotechnological solutions to the growing environmental problem of plastic pollution. The focus of this section will be microbial plastic biodegradation with the important caveat that the terms degradation and biodegradation are not interchangeable. The term biodegradation refers to the complete breakdown of plastic into CO_2, H_2O, and biomass in aerobic settings and CO_2, CH_4, and biomass in anaerobic settings, in a reasonable timeframe (i.e. >90% carbon converted to CO_2 within 180 days in an industrial composting facility; at least 30% carbon converted to CO_2 within 180 days at 30°C in marine environments). Industrial composting facilities maintain a controlled biodegradation environment, but plastic in the ocean ends up on many different uncontrolled environments, and very few of them correspond to the 30°C standard for marine environments. This point is very important since, as noted in a recent European Commission Report, "in theory, almost all materials ultimately may biodegrade, even in the open environment though some will do so only after sometimes hundreds of years or more". As a result of differing interpretations of these separate concepts, several peer-reviewed articles mention microbial "biodegradation" when they are really describing microbial degradation. Weight loss, carbonyl index, biofilm formation, and oxygen evolution are sometimes used to quantify biodegradation, but only CO_2 production is considered proof of direct biodegradation measurement according to standards organizations such as ASTM, CEN and ISO.

Fragmentation in the marine environment almost certainly is dominated by physical mechanisms including weathering due to UV radiation, mechanical, thermal, and chemical action. Microbial biodegradation and degradation in most marine environments is a much slower process. UV and mechanical weathering often precede microbial action on polymer surfaces, but once formed, microbial biofilms are thought to shield polymers from UV penetration, thereby preventing further polymer chain scission and abiotic degradation. Among early colonizers of plastic surfaces are cyanobacteria and eukaryotic phototrophs such as diatoms that may serve to oxidize the surface of plastic making the polymers more susceptible to other types of degradation. In addition to microbial adhesion and growth on plastic that may contribute directly to the surface degradation, the action of predators chewing plastic contributes to mechanical breakdown. There is evidence that the formation of a microbial biofilm contributes to the settlement of marine larvae and that microbes and larvae both make the plastic smell and taste like food for higher trophic levels. Accelerated fragmentation was initially considered beneficial to speed breakdown of plastic in the environment, but with recognition that plastic is interacting with organisms at all levels of the food web, the distinction between fragmentation and biodegradation has become more important. Plastics that contain pro-oxidant additives (PACs) have recently come under fire in the European Union due to unknown environmental impacts associated with their fragmentation in the marine environment. It is sometimes difficult to determine, even in the peer-reviewed literature, what form of plastic is being employed in research projects, and this can complicate interpretation and comparison of different studies.

The most convincing measures of environmental biodegradation follow methods that have been vetted by standards organizations such as ASTM, ISO and CEN. The ASTM standard D7081 (water) and the test method ASTM 7991 (sediment) rely on respirometry (evolved CO_2) to measure biodegradation of plastic in the marine environment. The microbiological testing that led to the development of ASTM D7081 involved a three-tiered approach and a defined consortium of microbes including bacterial species within *Alteromonas, Xanthomonas, Bacillus, Pseudomonas,* and the Actinomycetes. This standard also allows for the use of a natural seawater inoculum instead of a defined microbial consortium. A seawater inoculum is what is most frequently employed in testing nowadays. As of 2018, however, ASTM D7081 is under revision and requires reinstatement. Some of the concerns regarding this standard test method is that it doesn't take into account the range of temperatures and other physico-chemical conditions encountered in the marine environment (for example, the current tests are done at 30°C in the dark). It must be emphasized that the goal of biodegradation standards is to determine the inherent biodegradability of a given plastic polymer, not to provide a license for littering. Even certification agencies like Vinçotte in Belgium with their "OK-Marine" do not allow products that are commonly littered to carry this label since they acknowledge that the marine environment is not an appropriate disposal environment for plastics and none of these standards are designed to unintentionally imply that plastic released into the environment is acceptable if the standard is met.

Bio-based plastics that have been shown to biodegrade in the natural environment include: polyhydroxyalkanoates (PHA) and their derivatives: poly-3-hydroxybutyrate (PHB), poly-hydroxyvalerate (PHV), as well as the poly(α-hydroxy acids): polylactic acid (PLA) and polyglycolic acid (PGA). PHA, PHB, and PHV are examples of bio-based plastics that are microbial in origin. PLA and PGA can be synthesized from bio-derived monomers or petroleum sources so are examples of biodegradable plastics that are not always bio-based. Additional petroleum-based aliphatic polyesters that are biodegradable include polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), and polycaprolactone (PCL), as well as modified derivatives of polyethylene terephthalate (PET) and polybutylene terephthalate (PBT), aromatic polyesters such as polybutylene succinate terephthalate (PBST), polybutylene adipate terephthalate (PBAT), and polytetramethylene adipate terephthalate (PTMAT). Many of these polymers are used in the medical industry for drug delivery, but others have a variety of applications beyond the scope of this review. Several biodegradable plastics are better recognized by their commercial names such as Mirel (PHA) - Metabolix, EcoPLA (PLA) - NatureWorks, EcoFlex (PTMAT) - BASF, and Mater-Bi - Novamont. In many cases, polymer blends (with bio-based, biodegradable and non-biodegradable components) are created that possess the desired characteristics, so methods exist that help determine the so-called biogenic carbon present in a given plastic.

Genomics and transcriptomics are informing the mechanisms of plastic biodegradation with PHA being one of the best-studied examples. Genes such as b-ketothiolase (*PhaA*), acetoacetyl-coenzyme A reductase (*PhaB*), PHB polymerase (*PhaC*), and PHB depolymerase (*PhaZ*) are involved in both the synthesis and degradation of PHB in *Rhizobium eutropha* while *Pseudomonas putida* is known to possess PHA depolymerase. Since PHAs are biopolymers that microbes can both synthesize and degrade, it is important to understand that there are intracellular and extracellular PHA depolymerases. The bacterial PHA-degrading phenotype involves secretion of extracellular depolymerases, but also successful biodegradation depends on the degree of crystallinity of the bioplastics as well. Some bacteria possess multiple depolymerases capable of degrading a suite of different types of PHAs. More than 300 microbial species can produce PHAs but there are likely many more. Well-studied species include *Alcaligenes latus, Chromatium vinosum, Cupriavidus necator, Bacillus megaterium, Pseudomonas putida* and *P. lemoignei,* and archaeon *Haloferax mediterranei*. Likewise, there are many bacteria and fungi capable of biodegrading PHAs and there are likely protists yet to be characterized that do as well. As a result, some argue that currently this class of bio-based plastics are the only truly biodegradable ones in the marine environment.

Future Outlook

The role of microbes in degradation of plastics in the environment cannot be denied, but their abilities to completely biodegrade polymers is generally overstated. The recent reports of plastic metabolizing microbes in various industrial settings, and pit-formers on the surface of open-ocean microplastics lead some to make false claims of a microbial solution to a global problem. In this and other examples, partial degradation of plastics in any environment may lead to more harm than good as the microbial food web incorporates these small pieces of recalcitrant polymers up the food chain where they can biomagnify. While microbes can address many biotechnological challenges - they are not the final solution to all problems, particularly that of marine litter. Recent discoveries of microbe-wielding mealworms capable of consuming polystyrene and PET-degrading bacteria need to be tempered with alternative solutions including those that address appropriate plastic uses and global waste management infrastructures that currently allow too much plastic to enter our oceans.

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Polyomaviruses and Papillomaviruses☆

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Glossary

***Cis*-acting element** A specific DNA sequence which is present within the promoter or enhancer elements of genes, to which transcription factors bind and regulate the expression of genes on the same chromosome.
Coding region A portion of DNA that is transcribed into mRNA and translated into proteins.
Coding region A portion of DNA that is transcribed into mRNA and translated into proteins.
Oncogenic Causing or tending to cause the formation and development of tumors.
Papillomavirus A group of small DNA viruses that cause papillomas in various animals and is responsible for human genital warts.
Polyomavirus A DNA tumor virus with a very small genome that infects animals and humans.
Regulatory element A promoter, enhancer or other segment of DNA where regulatory proteins such as transcription factors bind preferentially and control gene expression.
Regulatory region A region of a chromosome that controls gene expression.
Replication The action or process of duplication of DNA.
TATA box DNA sequence found in the promoter region of most genes in eukaryotes, to which basal transcription factors bind and initiate the process of transcription by RNA polymerase.
Transcription A process by which genetic information on a strand of DNA is used to synthesize a strand of complementary RNA.
Transcription factor A protein that binds to specific segments of DNA using the DNA binding domain.

Abbreviations

AP-1	Activating protein-1
BKV	BK virus
BPV-1	Bovine papillomavirus-1
CMV	Cytomegalovirus
CNS	Central nervous system
CR1	Conserved region-1
CR2	Conserved region-2
E1	Early protin-1
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
GRS	GC-rich element
HHV-6	Human herpesvirus-6
HPV	Human papillomavirus
HPyV12	Human polyomavirus-12
HPyV6	Human polyomavirus-6
HPyV7	Human polyomavirus-7
HPyV9	Human polyomavirus-9
IE2	Immediate-early transactivator 2
JCV	JC virus
KIPyV	Karolinska Institute polyomavirus
LCR	Long control region
LT-Ag	Large T antigen
MAP	Mitogen activated protein
MBP	Myelin basic protein

☆*Change History:* July 2014. M Safak updated Defining statement and Table 1, modify all figures, and added six new references for further reading and delete some references from list.

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MCPyV	Merkel cell carcinoma-Associated polyomavirus
mRNA	Messenger RNA
MT-Ag	Middle T antigen
MuLV	Murine leukemia virus
MWPyV	Malawi human polyomavirus
MXPyV	Mexico X human polyomavirus
NF-1	Nuclear factor-1
NF-□B	Nuclear factor kappa B binding element
NLS	Nuclear localization signal
ORF	Open reading frame
Ori	Origin of DNA replication
Penta	Pentanucleotide element
PLP	Proteolipid protein
PML	Progressive multifocal leukoencephalophathy
PNS	Peripheral nervous sytem
PVAN	Polyomavirus-associated nephrophathy
Sm t-Ag	Small t antigen
SV40	Simian virus 40
TAR	Transactivation response element
TBP	TATA binding protein
TSPyV	Trichodysplasia spinulosa-associated polyomavirus
VLP	Virus like structures
WUPyV	Washington University polyomavirues

Polyomaviruses

Classification

Polyomaviruses are classified within the polyomaviridae family and are comprised of small tumor viruses that infect a number of species, including human, mouse, monkey, hamster, baboon, rabbit, and bird (Table 1). Their genomes are a single molecule of covalently closed, super helical double stranded DNA that replicates in the nucleus (Figure 1, *a typical genomic organization of the polyomaviruses is exemplified by the JC virus genome*). All family members form capsids but in similar size. Capsids are mainly composed of three capsid proteins, VP1, VP2, and VP3 although production of a fourth capsid protein has been recently reported

Table 1 Polyomaviruses were isolated from different species, including humans, monkey, mouse, hamster, baboon, rabbit and bird

Virus	*Host species*
Simian virus 40 (SV40)	Rhesus Monkey
Polyomavirus (PyV)	Mouse
JC virus (JCV)	Human
BK virus (BKV)	Human
KI virus (KIPyV)	Human
WU virus (WUPyV)	Human
MCPyV	Human
HPyV6	Human
HPyV7	Human
HPyV9	Human
HPyV10	Human
MWPyV	Human
MXPyV	Human
HPyV12	Human
K papovavirus KPV)	Mouse
Hamster papovavirus (HaPV)	Hamster
Stump-tailed macaque virus (STMV)	Stamp-tailed macaque
Lymphotropic papovavirus (LPV)	African green monkey
Budgerigor fledgling disease virus	Bird
Simian agent 12 (SA12)	Baboon
Rabbit kidney vacuolating virus (RKV)	Rabbit

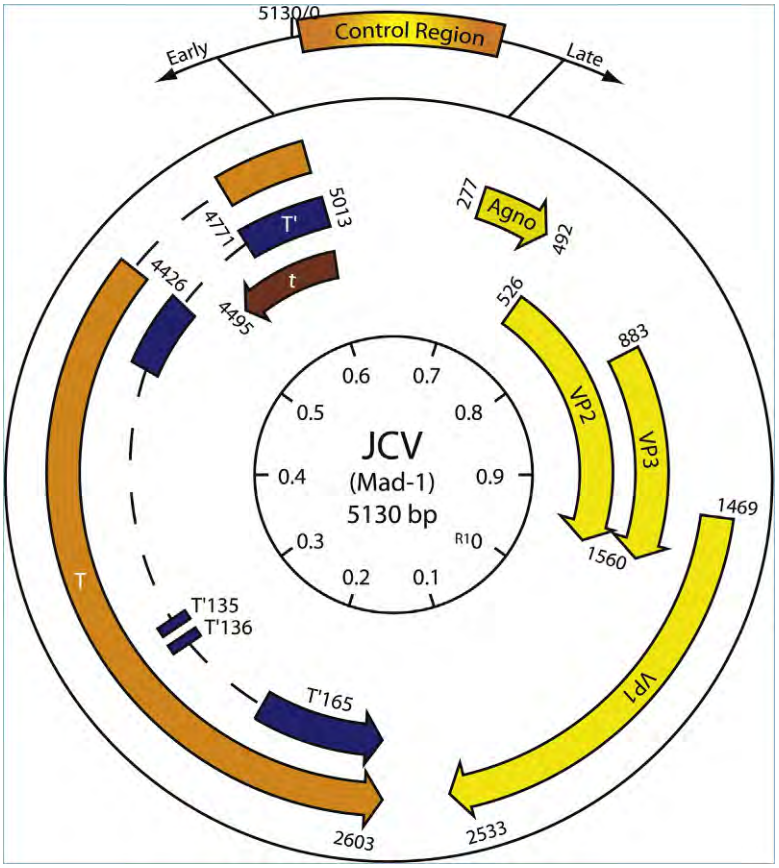

Figure 1 JCV genome shows a bidirectional expression pattern. Early coding region encodes regulatory proteins, LT-Ag, Sm t-Ag and T′ proteins (T'_{135}, T'_{136} and T'_{165}). Late coding region encodes regulatory (agnoprotein) and structural proteins (VP1, VP2, and VP3). Control region contains origin of DNA replication (ori) and promoter/enhancer elements for early and late promoters.

within the family for SV40. Their genome is about 5000 base pairs and exhibits a similar genomic organization and regions within their genomes are highly conserved, suggesting that family members are descended from a common ancestor. Despite the high genomic similarity, most of the family members display a narrow host range and do not productively infect other species (Table 1). In addition, some family members induce tumors in experimental animals, including mice, hamsters and monkeys.

Among the polyomavirus family, polyoma (referred as mouse polyomavirus here in) was the first family member to be discovered. Ludwig Gross was studying the transmission of murine leukemia virus in mice and extracts from infected animals could be used to transmit leukemia. It was surprising that some of the animals developed salivary gland tumors rather than leukemia. Further studies by Gross demonstrated that two agents, the one that caused salivary gland tumors and the other that caused mouse leukemia could be separated on the basis of differences in their filtration, sedimentation ability (the murine leukemia virus (MuLV) turned out to be larger in size) and heat inactivation properties (MuLV can be inactivated completely at 65 °C whereas the agent that cause salivary gland tumors was insensitive to heat inactivation at the same temperature). The virus that caused salivary gland tumors was then isolated and named mouse polyomavirus because of its characteristics to cause a variety of tumors in different tissues in new born mice. Adult mice, however, do not usually support the formation of tumors when infected with the same virus.

SV40 is among the viruses that were identified through screening of secondary rhesus monkey kidney cells, which were being used for production of polio vaccine. SV40 normally does not cause cytopathic effect on infected cells. However, when African green monkey kidney cells were infected with extracts from the rhesus kidney cell cultures, the cells produced a pronounce cyctopathic effect, which led the investigators to further study the cause. It turned out that that cytopathic effect was caused by viral infection, namely SV40. Contamination of a considerable amount of polio vaccine stock vials with live SV40, inoculation of some of those in the general public during the late 1950s and early 1960s raised a concern among the scientific community, because the oncogenic potential of this virus had been demonstrated in experimental animals, for example, in new born hamsters. It was thought that it may also do so in humans. Studies were intensified to determine the risk factor of SV40 to be a causative agent for induction of tumors in humans but such efforts did not show a significant association with the increased rates of ependymomas and osteosarcomas or mesatheliomas or brain tumors. The detection of SV40 LT-Ag genome in these cancer types in general population, however, have let to the renewed interest in the possibility that SV40 may be involved in the induction of some human tumors.

Human polyomaviruses, JC virus (JCV) and BK virus (BKV) were the first to be discovered in 1971. JCV was isolated from a brain tissue of a PML patient by overlaying infected extracts over the primary cultures derived from human fetal brain. The virus was then

successfully cultivated from those long-term cultures. This also demonstrated the first direct evidence that a neurotropic virus was associated with the development of PML. In the same year, a virus similar in structure was isolated from the urine of a patient who underwent a renal transplantation. Both viruses were named with the initials of donors, JCV for a patient with PML and BKV for a patient, who underwent renal transplantation. JC virus causes a fatal demyelinating disease of the central nervous system (CNS), which is known as progressive multifocal encephalopathy. BK virus can cause organ loss in renal transplant recipients, which is the medical condition, described as 'the polyomavirus-associated nephropathy' (PVN).

Eleven new human members of polyomavirus family have recently been discovered; one was isolated from a human respiratory tract samples by PCR amplification and named KI polyomavirus (KIPyV); and the other was isolated from a patient with symptoms of acute respiratory tract infection and named WU virus (WUV). Follow up studies do not so far conclusively support an evidence for the association between infections with WU and KI polyomaviruses and respiratory tract diseases. Another polyomavirus was isolated from the merkel cell carcinoma (a rare neuroendocrine skin tumor) by pyrosequencing of the cDNA made from the host transcripts followed by bioinformatic subtraction analysis of the host sequences and was named merkel cell carcinoma-associated polyomavirus (MCPyV). The other one was isolated from 'Trichodysplasia Spinulosa' samples by rolling circle amplification of the viral genome with specific primers followed by DNA sequence analysis and was named Trichodysplasia Spinulosa-associated polyomavirus (TSPyV). The rest of the recently discovered human polyomaviruses, including HPyV6, HPyV7, HPyV9, HPyV10, MWPyV, MXPyV, and HPyV12 were isolated from either serum of a patient or stool or skin samples but were not yet definitively linked to with a particular human disease.

The oncogenic potential of polyomaviruses is one of the major driving forces behind the further study of the biology of these viruses. In addition, the small genome size and easy growth in culture made these viruses (particularly SV40) as suitable model systems to examine many fundamental questions in eukaryotic molecular biology. Many major discoveries have been made in this sense using these viruses, including the structure of supercoiled DNA, the identification of eukaryotic origins of DNA replication, discovery of alternative splicing, organization of promoters and enhancers and their involvement in gene expression and understanding of the mechanisms of negative and positive regulation of gene expression. Studies of the biology of these viruses also led to discoveries related to the cell cycle regulation, oncogenesis and tumor suppressor genes. In this article, we devote a special attention to the biology of human polyomavirus JCV and simian virus SV40.

Virion Structure

Polyomavirus family members all form capsids, which are composed of three viral proteins, VP1, VP2, and VP3. The viral genome, which is a double stranded circular DNA, complexed with cellular histones, H2A, H2B, H3, and H4 in the form of chromatin is surrounded by capsid proteins. It is estimated that outer shell of the capsids are made up of VP1, organized into 72 pentamers (in the case of SV40), and each pentamer associates with a single VP2 and VP3. VP1 shows the ability form pentamers when produced in either *Escherichia coli* or baculovirus systems. These pentamers form virion like structures in the absence of the other two-capsid proteins (VP2 and VP3). It appears that these pentamers forms flexible structures, which allow all capsid proteins and the viral genome to fit together and make an icosohedral capsid structure. Calcium ions are required for the stability of pentamer–pentamer interactions. There are no disulfide bonds formed within the pentamer subunits, but they can form between adjacent pentamers, which is evident from the fact that reducing agents are required for disassembling virus particles. The sequences of minor capsid proteins, VP2 and VP3 completely overlap and these two proteins form the second layer in the polyomavirus capsid shell. The C-terminus region of the minor capsid proteins is most conserved within the polyomavirus family and this is the region that interacts with the N-terminus of VP1. When the capsid proteins are produced in the cytoplasm, they are transported into the nucleus where the encapsidation of the virions takes place. Although the process by which viral DNA is encapsidated into the virions is totally unknown, there are reports suggesting that viral late regulatory protein, agnoprotein, may play a major role, directly or indirectly in the process by regulating the interaction of capsid proteins with viral DNA.

Genomic Organization

The genome of all polyomaviruses is composed of bidirectional regulatory elements (Figures 1 and 2(a) and 2(b)) and coding regions. The regulatory region of each genome contains the origin of DNA replication and promoter/enhancer elements (Figure 2). Due to space restrictions, only the genomes of JCV and SV40 have been described in detail here, but others were either briefly mentioned or referred to the further readings. In the case of JCV, the regulatory region exhibits hypervariability in its sequence composition. That is, although two 98-bp tandem repeats are a characteristic of Mad-1 strain of JCV, there are considerable deviations from this characterization in other strains of JCV. For example, the regulatory region of Mad-4 strain contains two tandem repeats which are only 84-bp in length, whereas that of archetype strain contains only one 98-bp repeat with two insertions, 23-bp and 66-bp. JCV archetype regulatory region is thought to be more adaptive to a latent infection and, upon reactivation, virus undergoes a deletion/duplication process within its regulatory region, through which apparently gains the ability to become a more virulent virus to infect oligodentrocytes.

In the case of SV40, the regulatory region also contains repeated DNA elements, which wor as enhancers for the viral gene expression. The two 72 base-pair repeats and the three GC-rich 21 base-pair repeats are the main characteristics of the SV40 regulatory region. These DNA elements are located at the late site of origin of the DNA replication with respect to late coding region

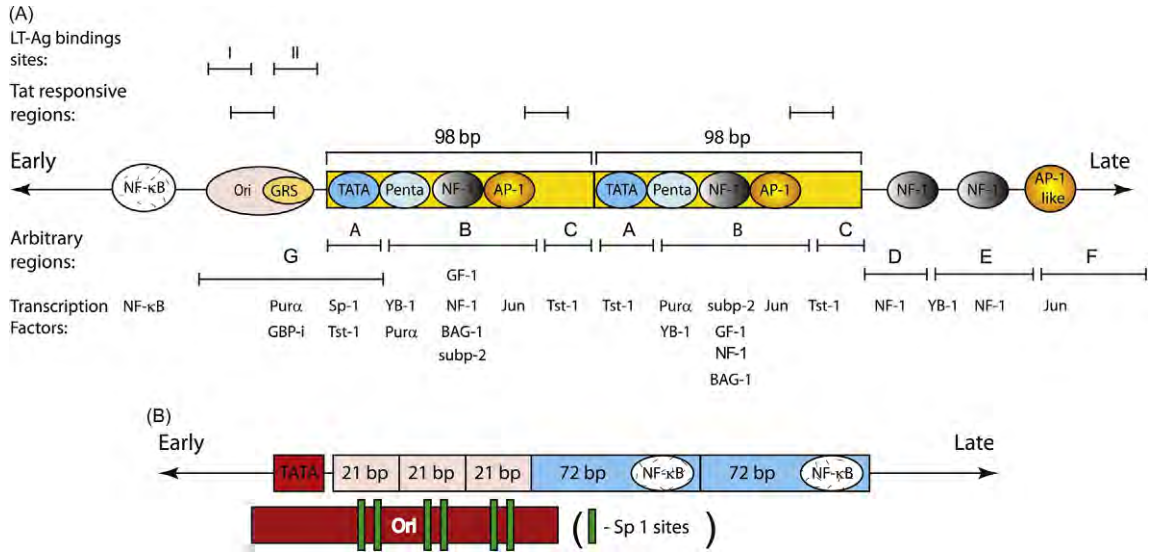

Figure 2 Promoter and enhancer elements of the control region of JCV and SV40. (a) Control region of JCV contains origin of DNA replication (ori) and promoter/enhancer elements. Two-98-bp tandem repeats is a characteristic of Mad-1 strain of JCV. *Cis*-acting elements including nuclear factor kappa B binding element (NF-κB), pentanucleotide element (Penta), nuclear factor 1 binding element (NF-1), GC-rich element (GRS), activating protein 1 binding element (AP-1) serve as binding sites for many transcription factors. Transcription factors that have been shown to bind the promoter/enhancer elements are shown at the lower part of the figure. Approximate location of LT-Ag antigen binding sites and Tat responsive elements are indicated. A portion of the control region was subdivided into arbitrary sub regions, including A, B, C, D, E, F and G to define more easily the locations of the different *cis*-acting element. (b) Schematic representation of SV40 control region. Twenty-one base pair (21 bp) and 72 bp repeats are the characteristics of SV40 control region. As shown for JCV control region (panel A), SV40 control region also contains the origin of viral DNA replication and many *cis*-acting elements, which serve as target sites for many transcription factors, including NF-κB and Sp1.

SV40 (Figure 2(b)). The bidirectional nature of the regulatory elements of the polyomaviruses may provide an advantage to each virus to efficiently transcribe their genes, but the mechanism of which is currently unknown.

The coding region of polyomaviruses has two directions, early and late, for transcription. Transcription extends directionally from initiation sites near the origin to produce the early and late messenger RNAs (mRNAs). They are transcribed from the opposite strands of the viral genome. The early region of JCV, for example, encodes only regulatory proteins including LT-Ag, Sm t-Ag and T′ proteins (T′135, T′136, and T′165). Similarly, the early region of SV40 encodes two main regulatory proteins, LT-Ag and Sm t-Ag. In addition, this region of SV40 also encodes a 17KT protein. Moreover, Polyoma and closely related viruses encode three T antigens, LT-Ag, middle T (MT-Ag) and Sm t-Ag. All of the T antigens of each polyomavirus, at least JCV, BKV, SV40 and polyoma, share N-terminal sequences but contain different C-terminal regions. The pre-mRNA encoded from the early region of each virus is alternatively spliced to produce the mRNA that encodes these proteins.

The LT-Ag of all polyomaviruses is the main regulatory protein of polyomaviruses (Figure 3). It is absolutely required for the replication of the viral genome. It also transactivates the late promoter and auto regulates its own promoter (early promoter). Sm t-Ag on the other hand, although the precise function of it is unknown, also play regulatory roles in the viral replication cycle. Along with the LT-Ag, it pushes the cells into the S-phase of cell cycle, during which all DNA viruses including polyomaviruses replicate their DNA. The function of the T′ proteins (T′135, T′136, and T′165) in the viral life cycle, which are specifically transcribed from the early genome of JCV, is unknown, although recent evidence suggests that they may have roles in cell transformation. The MT-Ag of polyoma appears to behave much like Sm t-Ag during the viral replication cycle. That is, it does not participate directly in the replication of viral DNA as LT-Ag does, but rather it contributes to the conditioning of the infected cells by activating some of the genes that are required for S-phase entry.

Contrary to the early region, the late region of JCV, BKV and SV40 encodes a mixture of structural (capsids, VP1, VP2 and VP3) and regulatory (agnoprotein) proteins (Figure1). The mouse polyomavirus, however, only encodes structural, not regulatory proteins from its late coding region (VP1, VP2 and VP3). Alternative splicing of the late common pre-mRNA also produces the mRNAs for the late gene products. VP2 and VP3 sequences overlap but VP3 sequences are a subset of VP2 sequences (Figure 1). The late region of monkey (SV40) and human (JCV and BKV) polyomaviruses encodes a small, basic regulatory protein, called agnoprotein. This small protein is encoded by the leader region of some species of the late viral mRNA and accumulates mostly around the perinuclear region. Null mutants of this protein in JCV and SV40 result in fewer progeny virions and PKC-phosphorylation mutants of this protein exhibit even more severe phenotypes in JCV, which are totally defective in viral life cycle. In other words, the virus with phosphorylation mutants of this protein is unable to continue the viral life cycle due to empty capsid formation, suggesting that the agnoprotein is actively involved in JCV virion biogenesis. The same is perhaps true for SV40 and BKV. The other polyomaviruses, including KIPyV, WUPyV, MCPyV TSPyV HPyV6, HPyV7, HPyV9, HPyV10, MWPyV, MXPyV

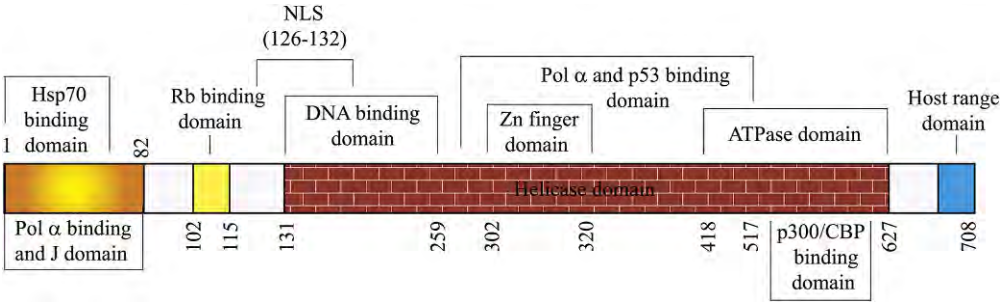

Figure 3 SV40 large T-antigen contains several functional domains. They include the binding regions to polymerase α-primase (Polα), tumor suppressor proteins Rb and p53, human heat shock protein 70 (hsc70) and coactivators p300 and CBP. ATPase activity domain, DNA binding domain, nuclear localization signal (NLS) domain, helicase domain, Zn finger domain, host range domain, and J domain are also depicted.

and HPyV12 do not contain an open reading frame for the agnoprotein and replicate in tissue culture in vitro well suggesting that that if the agnoprotein is provided in trans, these viruses may replicate sufficiently in appropriate cell culture systems. JCV agnoprotein was recently found to form highly stable dimers and oligomers in vitro and in vivo through its amphipathic α-helix domain located between amino acids 23 and 39. The α-helix domain of the protein plays critical roles in function and stability of the protein, which can be exploited for drug discovery purposes against JCV-induced progressive multifocal leukoencephalopathy (PML).

As opposed to the regulatory region, the coding regions of JCV show a significant sequence homology to BKV and SV40 (70–80%). The sequences within the regulatory region of each virus are considerably divergent from one another. This divergence mostly determines the tissue or species-specific replication of each virus. In other words, the regulatory region is mainly responsible for the tissue-specific expression of each virus, although LT-Ag, at least in the case of JCV, is also known to contribute to this unique expression. Moreover, the tissue-specific cellular factors also appear to be critical for the unique expression of each viral promoter. For example, *in vivo* and *in vitro* transcription assays as well as cell-fusion experiments have clearly indicated that there are positively and negatively acting transcription factors responsible for the expression of the JCV genome in glial and non-glial cells respectively. For instance, it was shown that the JCV early promoter expressed well in glial cells. However, when glial cells are fused with fibroblasts to form heterokaryons, early gene expression is significantly reduced in heterokaryons, suggesting that there are positively and negatively acting factors in glial and in non-glial cells respectively.

Structural organization of the regulatory region

The regulatory region of polyomaviruses exhibits the most divergent region among the family members and is responsible for the tissue specific expression of each family member, although LT-Ag also contributes to this specificity. The promoter/enhancer elements and origin of DNA replication are the common elements present with the regulatory region of each the family member, however, the structural configuration of these elements exhibits significant divergence from one strain to another one within each member or one member to another within each family. For example, the Mad-1 strain of JCV contains two 98-bp repeats within its control region, which is a prototypic property of this strain. Other strains of JCV, such as the archetype strain and Mad-1 strain, however, show significant divergence from this property. The regulatory region of the Mad-1 strain of JCV and that of SV40 are schematically shown in Figure 2. Origin of the DNA replication is the only region in the regulatory region of each family member, at least between JCV, SV40, and BKV, which shows substantial sequence homology among the family members. Other regions are quite divergent between these three viruses. Each repeat of the regulatory region of the Mad-1 strain of JCV contains a TATA box located in the early side of the individual repeats and first TATA box with respect to origin of DNA replication appears to be involved in the positioning of the transcription start sites for viral early genes. The second TATA box, however, does not have a similar function for the viral late genes. The other enhancer elements including penta, AP-1 and NF-1 follows there after TATA box. They are important DNA elements for the regulation of JCV promoters. These elements are targets for several transcription factors and contribute to tissue tropism. The major *cis*-acting elements and transcription factors that bind to those regions are illustrated in Figure 2.

Enhancer elements and cellular transcription factors involved in viral regulation

Many cell type-specific and ubiquitous factors are involved in the expression of the polyomavirus early and late promoters. These factors directly or indirectly interact with the enhancer elements present in the regulatory region and regulate viral gene expression. For the simplicity of the description of those factors, we focus on those, which are involved in JCV regulation. LT-Ag and agnoprotein are two regulatory proteins, which play critical roles in the regulation of JCV promoters. LT-Ag is the initiator of JCV DNA replication. It binds to the origin of DNA replication in double hexamer configuration, unwinds it, and the replication fork proceeds in bidirectional manner in the presence of the cellular factors that are required for this process. With respect to its role in transcription, studies also showed that while LT-Ag autoregulates its own transcription from the viral early promoter, it significantly activates the late promoter, resulting in the expression of agnoprotein and viral capsid proteins. In addition to LT-Ag and

agnoprotein, Sm t-Ag of polyomaviruses is also involved in viral regulation. Although the precise function of it is unknown, at least in JCV case, a stop codon mutation at Ser90 was observed to negatively but significantly affect the virus yield.

There are multiple enhancer elements present in the regulatory region of polyomaviruses. A close inspection of regulatory regions of JCV, for example, revealed the presence of multiple enhancer elements, which are targets of many cellular transcription factors (Figure 2), which include, NF-κB, GRS, penta, NF-1, AP-1, and 'AP-1 like' motifs. In addition to those, Tat responsive elements and LT-Ag binding sites are also present. The entire regulatory region of JCV, for the sake of the simplicity of the description of JCV regulatory elements and transcription factors that targets those sites, is divided into arbitrary regions including A, B, C, D, E, F, and G (Figure 2). NF-κB element, which is located at the early site of the promoter with respect to the origin of DNA replication is a target of stress inducible transcription factor, NF-κB. This factor represents a large family of transcription factors, which are inducible in response to a wide variety of extracellular stimuli including cytokines, phorbol esters, and viral infection. Two constitutively expressed subunits of NF-κB, p50 and p52, activate transcription from D domain, while another subunit of NF-κB, p65, regulates the transcription of JCV through NF-κB motif itself. A 23-bp element, which is present within the regulatory regions of many JCV variants, including archetype and Philadelphia-1 strain of JCV is also targeted by NF-κB family members. The GRS sequences, which are present within the G region of the viral promoter, serve as binding sites for another inducible cellular factor GBP-i, which suppresses viral late promoter activity in reporter promoter assays. The A and C regions contain binding sites for a member of well-characterized tissue-specific and developmentally regulated POU family of transcription factors, for example, Tst-1. This factor regulates JCV transcription from viral early and late promoters. It also interacts with the JCV LT-Ag and synergistically activates the viral early and late promoters. GC-rich sequences which are located within pentanucletide region of JCV appears to be a target for a ubiquitously expressed transcription factor, Sp-1, which was found to activate gene expression from the viral early promoter. Another important regulatory element present in the control region of JCV is 'nuclear factor 1' (NF-1) binding sites, which are scattered within the regulatory region including arbitrary sites, B, D, and E. NF-1 is involved in both regulation of JCV transcription and replication. B region contains target sites for several factors, including GF-1, YB-1, Purα, and AP-1. GF-1, a human homologue of the murine Sμbp-2 protein, transactivates viral early and late promoters. Two well-characterized cellular transcription factors, YB-1 and Purα, regulate viral gene expression and replication through interaction with LT-Ag. An additional binding site for YB-1 is also present in the JCV regulatory region, which is localized to arbitrary region E. BAG-1, a ubiquitously expressed cellular factor, interacts with Bcl-1 and regulates JCV promoters through the NF-1 binding site. Another inducible factor that interacts with B region is c-Jun, which is a member of the AP-1 family of transcription factors. The family members are known as the immediate early inducible protooncogenes and are critical for the expression of many cellular and viral genes. C-Jun functionally interacts with LT-Ag and is phosphorylated during the JCV life cycle in an infection cycle-dependent manner. Besides the above-described cellular and viral regulatory proteins, the JCV regulatory region is subject to cross-regulation by the regulatory proteins that are encoded by other viruses including immediate-early transactivator 2 (IE2) from cytomegalovirus (CMV) and Tat from HIV-1

Life Cycle

The infection cycle of polyomaviruses starts with the attachment of the viral particles to the cell surface receptors (Figure 4). The serotonin receptor $5HT_{2A}$ as well as α(2–6)-linked sialic acid were shown to be critical for the attachment of JCV to the cell surface. Gangliosides play an important role in the attachment of both SV40 and mouse Polyomavirus on the cell surface. BKV, on the other hand, appears to use α(2,3)-linked sialic acid and gangliosides (GD1b and GT1b) as cell surface receptors for attachment. The attached polyomavirus particles enter the cells through either caveolae-mediated (BKV and SV40) or clathrin-mediated (JCV) or cell type specific (Polyoma) endocytosis pathways. Figure 4 highlights the chain of events step by step during the life cycle of a polyomavirus. Following internalization, the viral particle is transported to the endoplasmic reticulum, at least in the case of SV40, for uncoating of the capsid and viral DNA is transported into the nucleus by an unknown mechanism. The next step in the viral infection cycle is the expression of the viral early genome in the nucleus. Viral transcripts undergo splicing events before they are transported back to the cytoplasm for translation. Translation of early transcripts results in the production of viral early regulatory proteins. In the case of JCV, this results in the production of large T antigen (LT-Ag), small t antigen (Sm t-Ag) and T′ proteins (T′135, T′136 and T′165). LT-Ag is then transported back to nucleus to initiate viral DNA replication and to trans-activate the viral late promoter for the production of regulatory agnoprotein and three structural capsid proteins, VP1, VP2 and VP3 (Figure 1). Upon translation, capsid proteins are transported to the nucleus and the encapsidation process starts. It appears that the capsid proteins are sequentially added to and arranged on the viral genome to package the viral DNA into the capsids. The final step in the viral life cycle is to release the virions from infected cells. Although the mechanism of this process is not known, it is likely that they are released upon the lysis of the infected cells.

Cross-Regulation of JCV Promoters and JCV Reactivation

Impaired cellular immunity is a predisposing condition for the reactivation of JCV and the onset of PML. Lymphoproliferative disorders used to be the predominant underlying factor for the reactivation of JCV from latency. In recent years, however, due to the AIDS epidemic, this trend has changed and a strong association of PML with AIDS has been established. The statistical data in this regard suggest that 5–10% of AIDS patients eventually develop PML. However, there is a discrepancy between the degrees of the reactivation of JCV in AIDS patients versus in those with other immunosupressive conditions. In other words, JCV is reactivated relatively more often in immmunosuppressed AIDS patients than in the cases of other immunosuppressive states, including in those

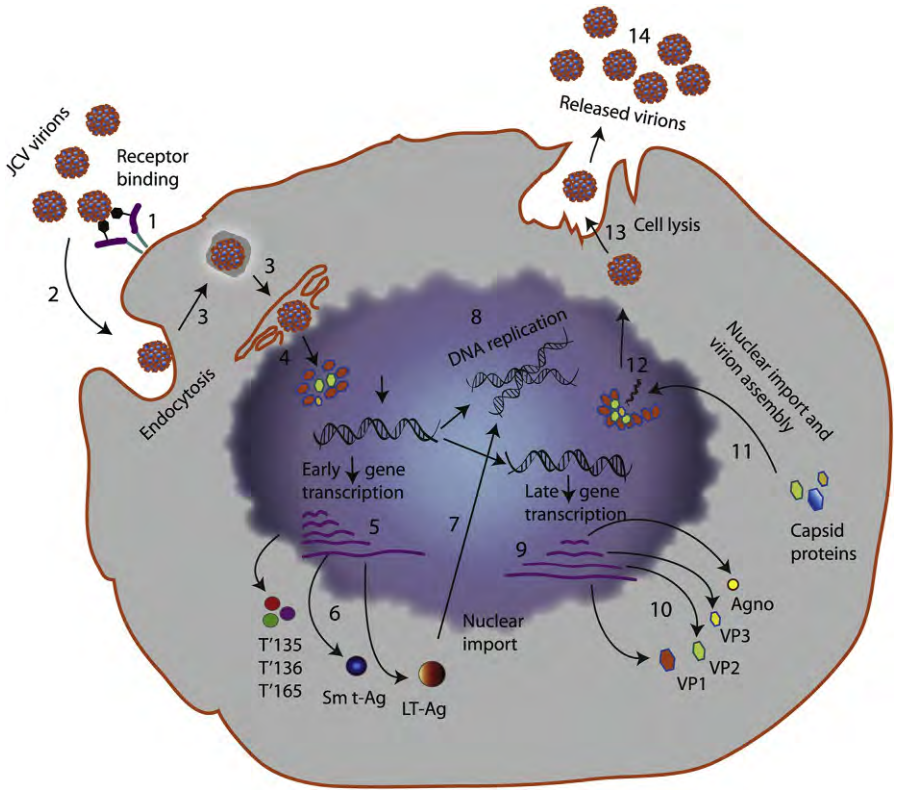

Figure 4 Schematic representation of JCV life cycle. Sequential events that take place during the JCV life cycle are indicated by numbers as follow: (1) adsorption of virus to the cell surface receptors; (2) entry by clathrin-dependent endocytosis; (3) transport to the endoplasmic reticulum (ER); (4) uncoating either in ER or in the nucleus; (5) transcription of early coding region; (6) translation to produce early regulatory proteins, LT-Ag, Sm t-Ag and T′ proteins (T'_{135}, T'_{136} and T'_{165}); (7) Nuclear localization of LT-Ag; (8) replication of viral genome; (9) transcription of viral late genome; (10) translation of viral late transcript to produce agnoprotein and capsids (VP1, VP2 and VP3); (11) Nuclear localization of capsids; (12) assembly of viral progeny in the nucleus; (13) release of virions from infected cells; (14) released virions.

with hematological malignancy, solid tumors and organ transplantation, all of which have a long period of immunosuppressive states. This suggests that there is something special about the HIV infection cases and JCV reactivation, perhaps the degree and duration of immunosuppression in HIV infection may not be the only factor for the development of PML. It could be that HIV infection affects many arms of the host defense system as opposed to one or two seen in other immunosuppressive states. In addition, other alterations including the deficiencies in chemotaxis, monocyte-dependent T-cell proliferation, Fc-receptor function, C3 receptor-mediated clearance, and oxidative burst response greatly contribute to the suppressive conditions of the immune system in AIDS patients and this in turn may facilitate JCV reactivation. As a result, it is tempting to suggest that a combination of the conditions mentioned above may have a substantial contributing effect than single case on the reactivation of JCV.

In addition to the immunosuppressive conditions, viral regulatory proteins, produced by viruses other than JCV may have a direct or indirect impact on JCV reactivation and regulation. For example, HIV regulatory protein Tat (a potent transactivator of HIV long terminal repeats) may have such a potential effect. Tat mediates its transregulatory activity through a specific RNA sequence located in the leader of all HIV-1 RNAs, called transactivation response elements (TAR). Several critical G residues within the TAR element are required for its function. Similar sequences, called Tat response elements, are also present in the regulatory region of JCV. Tat also induces transcription from the JCV late promoter through these sequences. The critical G residues required for the function of HIV-TAR are also conserved in the JCV-TAR element and play an important role in Tat-mediated activation of the JCV late promoter. Thus, the TAR homologue may participate directly in the overall activation of the JCV late promoter. Tat may also influence JCV gene expression indirectly through the activation of inducible transcription factors and cytokines, among which NF-κB and c-Jun/AP-1 were shown to activate the JCV promoters. Taken together, the regulatory factors induced by HIV Tat or Tat-induced cytokines may directly or indirectly play regulatory roles in activation of JCV promoters.

In addition to JCV, there are several other human viruses that establish persistent infection in different tissues and organs of the human body including those such as BKV, cytomegalovirus (CMV) and human herpesvirus-6 (HHV-6). Some of the regulatory proteins produced by these viruses may also cross-regulate JCV as Tat does. CMV is, for example, highly prevalent in the human population and infects any organ of its host. It is commonly reactivated in renal transplantation and bone marrow patients. It is also

reactivated after renal transplantation in AIDS patients in the kidney, lymphoid organs, stromal cells and CD 34-positive bone marrow progenitor cells. It is reported that CMV infection positively affects the level of JCV DNA replication and transcription. JCV is known to infect only glial cells but this restricted cell specificity can be overcome if the immediate-early transactivator 2 (IE2) protein of CMV is provided to the JCV infected cells. For instance, fibroblasts can be permissive to JCV replication if this protein is present in the JCV-infected cells

The presence of HHV-6, another ubiquitous human virus, is reported in oligodentrocytes within PML lesions suggesting that JCV and HHV6 may have a common host cell. This virus establishes a life-long infection in different organs, including the urinogenital tract, the brain, the liver, the lung, and peripheral blood cells. There appears to be a high correlation between polyomavirus infection and HHV6 infection and this co-infection may have an impact on JCV gene regulation and perhaps in the reactivation process.

JCV and BKV have been known to concomitantly infect human tissues, such as the kidneys and the urinogenital tract. Additionally, both viruses were also detected in the peripheral blood cells of both healthy and immunocompromised individuals. These viruses encode very similar regulatory proteins, including LT-Ag, Sm t-Ag and agnoprotein. These proteins may well cross-regulate the replication and gene expression of each virus and may contribute to the reactivation of each virus.

Polyomavirus-Associated Diseases

Progressive multifocal leukoencephalopathy

PML is a fatal demyelinating disease of the central nervous system (CNS), which results from the lytic infection of the oligodentrocytes by the human polyomavirus, JCV. Oligodentrocytes produce the myelin sheets, which rape around the axons of neurons to facilitate the transmission of the neuronal current. The infection of oligodentrocytes by JCV indirectly causes the death of neurons in the white matter in CNS, because neurons depend on the support provided by oligodentrocytes for their survival. Subsequently, the loss of both oligodentrocytes and neurons in the CNS results in small plaque formation in the infected areas of the CNS. These areas, as the disease progresses, coalesce and form the characteristics of PML lesions resulting in the death of the infected individual. PML mostly develops in individuals with underlying immunosuppressive conditions, including lymphoproliferative diseases, AIDS and Hodgkin's lymphoma, in a small number of cases, it may also affect individuals with no underlying disease. PML used to be a rare complication of middle-aged and elderly patients with lymphoproliferative diseases before the AIDS epidemic. However, it is now a commonly encountered disease of the CNS particularly in HIV patients. This increase suggests that HIV infection may directly or indirectly influence the reactivation or progression of CV infection. This is supported by recent reports, indicating that the incidence of PML in HIV-sera positive patients reached up to 5%, compared to that of 0.8% before the AIDS epidemic. Furthermore, there has been an incidence of reactivation of JCV in Crohn's disease and Multiple Sclerosis patients, who underwent treatment with both interferon $\beta 1\alpha$ and natalizumab, a monoclonal antibody that selectively blocks $\alpha 4$ integrins. These incidences suggest that any immunosuppressive treatment of patients would be a risk factor for the reactivation of JCV and progression of PML.

Polyomavirus-associated nephropathy (PVAN)

JCV and BKV are the two members of the polyomavirus family that naturally infect humans. Sequences of monkey polyomavirus, SV40 were also found in different human tumors, which suggests that it may also infect humans. In kidney transplant recipients, the replication level of BKV significantly increases, when those recipients undergo immunosuppressive therapies. This leads to the lytic destruction of infected cells in the kidneys and compromises the function of the organ. This medical condition is described as 'the polyomavirus-associated nephropathy (PVAN)' and is a serious problem in 4–9% of the kidney transplant recipients.

Cell Transformation and Tumor Induction by Polyomaviruses

Many polyomaviruses including JCV, BKV, SV40, and mouse polyomavirus are known to induce a variety of tumors in experimental animals. Many also have the ability to induce neoplastic cell transformation in tissue culture. After its isolation, JCV for example was demonstrated to induce tumors in experimental animals in tissues of neuronal origin, but the type of tumor that is induced by JCV depends on the type and the age of the animal and the site of virus inoculation. For example, subcutaneous and intracerebral inoculation of JCV Mad-1 strain into newborn Syrian hamsters resulted in the formation of several types tumors, including medulloblastomas, glioblastomas, neuroblastomas in a majority of the animals (more than 80%). In contrast, intraocular inoculation of the same group of animals with the same strain of JCV resulted in abdominal neuroblastomas in several locations of the body. It was also observed that these tumors were found to be metastasized to the bone marrow, lymph node and liver in these animals.

It is interesting that JCV is the only polyomavirus that induces tumors in nonhuman primates, like monkeys. The other members of the polyomavirus family, including BKV and SV40 do not cause tumors in those animals. To evaluate a case that resembles PML in humans, owl and squirrel monkeys were inoculated with? subcutaneously, intraperitoneally, and intracerebrally. One owl monkey developed a malignant cerebral tumor similar to astrocytoma that resembles the one seen in humans, after 16 months of inoculation. Another animal developed a malignant neuroblastoma after 25 months of inoculation.

Transgenic mouse models were also used to evaluate an acute demyelination case, that is observed in PML patients. Transgenic animals were created using the promoter and coding regions for JCV LT-Ag. The phenotypes of these transgenic animals showed a mild to severe tremor phenotype and further evaluation of the animals revealed hypo- and dys-myelination in the CNS but not in

the peripheral nervous system (PNS) suggesting that expression of LT-Ag only affects the myelin formation in CNS rather than in PNS. Microscopic evaluation of the myelin sheet around the axons revealed a low level of the layer of myelin formation although the expression level of proteolipid protein (PLP), myelin basic protein (MBP) and myelin associated glycoprotein genes appeared to be normal at the RNA level. Their respective protein levels, however, were reduced. This suggests that LT-Ag influences the rate of the translation of mRNA for those genes rather than their transcription.

JCV genome has also been detected in a variety of human tumors, which raises a possibility of the involvement of JCV LT-Ag in tumor formation in humans. In fact, E. P. Richardson, who first described PML in 1961, reported an incidental case of the detection of an oligodendroglioma in a patient with concomitant occurrence of chronic lymphatic leukemia and PML. Similarly, another case, where a patient with a long history of immunodeficiency syndrome with PML, was described, in which numerous foci of anaplastic astrocytes were observed. Electron microscopy studies revealed the presence of viral particles in the demyelinating lesions in both oligodentrocytes and astrocytes within PML foci, but not in the neoplastic astrocytes. The presence of dysplastic ganglion-like cells in a patient with PML was also recently described, where a large number of dysplastic or dysmorphic gangloan-like cells were detected in the cerebral cortex, which showed properties of neurons. The expression of JCV LT-Ag was detected in those cells but not the viral capsid proteins.

JCV has been shown to be associated with human brain tumors in the absence of PML lesions. The detection of JCV DNA in the brain tumors of an immunocompetent patient with a pleomorphic xanthoastrocytoma was reported. The detection of JCV DNA as well as the expression of LT-Ag in tumor tissue from an immunocompetent HIV-negative patient with oligoastrocytoma was also reported. These two cases demonstrated the association of JCV in brain tumors of immunocompetent non-PML patients. These findings further prompted attempts to establish the association of JCV with different types of brain tumors in humans. Analysis of multiple brain tumors for the detection of JCV genome revealed that 76.9% of astrocytomas, 83.3% of ependymomas, 80% of pilocytic astrocytomas, 62.5% of oligoastrocytomas, 57.1% of oligodendrogliomas and 66% of anaplastic oligodendrogliomas, contained JCV early gene sequence. Furthermore, JCV genomic DNA has also been detected in tumor tissues that originate from non-neural origin. For example, the JCV genome was detected in solid non-neural tumors including colorectal cancers.

Oncogenicity of BKV was also demonstrated in experimental animals including young or newborn mice, rats and hamsters. The route of inoculation mostly determines what types of tumors will be induced by this virus. For example, it is highly oncogenic when inoculated intracerebrally or intravenously, but it weakly induces tumors when inoculated subcutaneously. BKV induces tumors in hamsters in a variety of tumors including neuroblastoma, ependymoma, pineal gland tumors, fibrosarcoma, esteosarcoma, and tumors of pancreatic islets. Rats inoculated with BKV developed osteosarcoma, fibrosarcoma, nephroblastoma, liposarcoma, and glioma. In a similar setting, mice, however, developed only choroids plexus papilloma.

During the late 1970s, detection of BKV DNA in a variety of human tumors and tumor cell lines prompted researchers to further investigate the possible association of BKV with the human tumors. It appears that BKV exhibits a specific oncogenic tropism for the ependymal tissue, endocrine pancreas, and osteosarcomas in rodents. This finding led investigators to primarily focus on the characterization of such tumors in humans for the detection of the BKV genome. Genome detection studies showed that some brain and some pancreatic islet tumors were found to contain the BKV genome in a free, episomal state.

Thefirst detection of the SV40 genome in a metastatic melanoma patient in 1974 linked the association of SV40 with human cancers. The viral genome was isolated from a lung metastasis and the expression of LT-Ag and capsid protein was detected in different organs, including lung, liver and muscle to which metastasis has occurred but not in normal tissue. Since then numerous reports have indicated a possible link between SV40 and human tumors. The SV40 genome and the expression of LT-Ag were detected by different methods, including DNA hybridization, PCR, DNA sequencing and immunofluorescence techniques in mesotheliomas, brain tumors and other human tumors and nontumor tissues including AIDS-related lymphomas, osteosarcomas, peripheral blood cells, kidney tissue from pediatric renal transplant patients and non-Hodgkin's lymphomas.

The mechanism of tumor induction by polyomaviruses is not known, but the tumorigenic protein, LT-Ag, is known to play a major role in this process. This protein was shown to target major cell cycle regulators including retinoblastoma gene products, pRb and tumor suppressor protein p53. This targeting inhibits the functions of these two key cell cycle regulators and perhaps other regulators as well. It should be mentioned here that as opposed to JCV, BKV and SV40 LT-Ag, polyomavirus LT-Ag does not target tumor suppressor p53, suggesting that it has alternative targets in the cell during the cell transformation.

Another mechanism by which human polyomavirus (BKV and JCV) and others including SV40 and mouse polyomavirus LT-Ag may cause cell transformation through the induction of a number of chromosomal structural alterations characterized by the breaks, gaps, dicentric and ring chromosomes, deletions, duplications and translocations. While the molecular mechanism of this clastogenic effect of LT-Ag on host DNA is unknown, it is thought that it may achieve this activity through the interference of the activity of topoisomerase I or through its helicase activity in which it may induce chromosomal damage while unwinding the strands of cellular DNA. This may result in disturbances of the crucial functions of the genes that are important for the maintenance of genomic stability. These important genes may include oncogenes, tumor suppressor genes and DNA repair genes.

In recent years, more direct evidence has been recently presented with respect to the association of a polyomavirus with a specific human tumor type, Merkel cell carcinoma, a rare neuroendocrine skin tumor. H. Feng et al., has first demonstrated the presence of a polyomvirus like sequences in merkel cell carcinoma samples by a digital transcriptome subtraction method. cDNA from the host cell transcripts were subjected to pyrosequencing followed by subtracting out the host sequences from the sequencing data by bioinformatic analysis. Data showed that viral sequences are integrated into the host genome and in some instances either the helicase domain of viral large T antigen or some VP1 sequences are deleted from the viral genome. Later studies showed that expression of large T antigen is required for the maintenance of oncogenic state of the merkel cell carcinoma cells.

Another human polyomavirus associated with a particular human disease is the 'Trichodysplasia Spinulosa'(TS)-associated virus (TSPyV). TSP is a rare skin disease in ummunosuppressed patients who manifest papules, spines and alopecia in their facial areas. For the discovery of this virus, samples collected from the specules were subjected to DNA extraction and rolling circle amplification with specific primers followed by DNA sequencing and bioinformatics analysis. Results showed a circular DNA genome similar to that of a polyomavirus. The association of TSPyV with TS disease is now demonstrated to be highly likely.

Papillomaviruses

Classification

Papillomaviruses are composed of a group of small DNA viruses that cause papillomas (warts) in a variety of higher vertebrates, some of which also cause malignant tumors in animals and humans. In particular, human papillomavirus (HPV) genotypes, HPV-16 and HPV-18 induce cervical cancer in women and other epithelial tumors. G. Ciuffo first recognized the viral cause of the human warts more than 93 years ago, through the demonstration of the transmission of common warts using filtrated cell extracts. R. E. Shope first recognized the rabbit papillomavirus in 1933 as the causative agent of cutaneous papillomatosis in cottontail rabbits. Research in the papillomavirus field remained slow over the years because it was not possible to propagate the virus in tissue culture systems until the viral genome was cloned during the late 1970s. This opened up new frontiers in papillomavirus research and cloning of the individual genes of the papillomavirus has provided many opportunities to initiate a systematic mutational analysis of the individual genes out of their context as well as in their background.

Papillomaviruses were initially classified in the papovavirus family along with the polyomaviruses due to the fact that both groups of viruses share common properties, including having a small and double stranded DNA; replicating in the nucleus. They used to be divided into two genera: the papillomavirus and the polyomavirus (mouse polyomavirus and simian virus 40 (SV40), based on the particles size of each group. That is, papillomavirus is about 53–55 nm and polyomavirus measures about 42–45 nm in diameter size. However, studies of the each group's genome showed that there are fundamental differences between these two viral groups. The papillomavirus genome is about 8 kb and that of polyomavirus is about 5 kb. In addition, the papillomaviruses have more open reading frames for their structural and regulatory proteins than polyomaviruses (compare genomic organization of polyomavirus in Figure 2 with that of papillomavirus in Figure 5. More interestingly, all papillomavirus genes are transcribed from the same strand of the genome, whereas the polyomavirus genome is bidirectional, at least in the case of JCV, BKV, SV40 and mouse polyomavirus. These genomic differences let these two viral groups to be recognized as the distinct virus families.

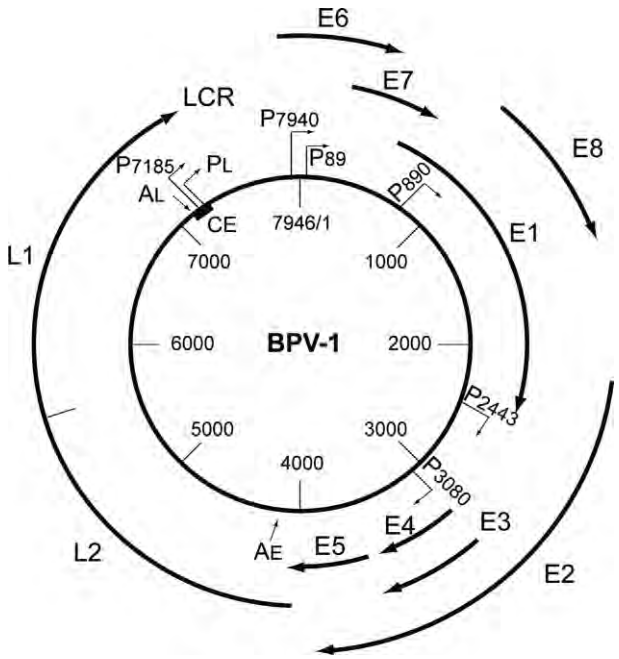

Figure 5 Circular map of bovine papillomavirus-1 (BPV-1). Approximate locations of the early (E) and late (L) coding regions are depicted. Transcription occurs in the clockwise direction and only one strand of the genome is transcribed. Approximate location of the promoters for early coding regions is indicated as Pn. The promoter for late genes is located between nucleotides 7214 and 7256. The control region is designated as LCR (long control region), which contains the origin of DNA replication (nucleotides 7911–22) and constitutive transcriptional enhancer element (CE). Polyadenylation signal sites for early (A_E, nucleotide 4202) and late (A_L, nucleotide 7175) genes are also depicted.

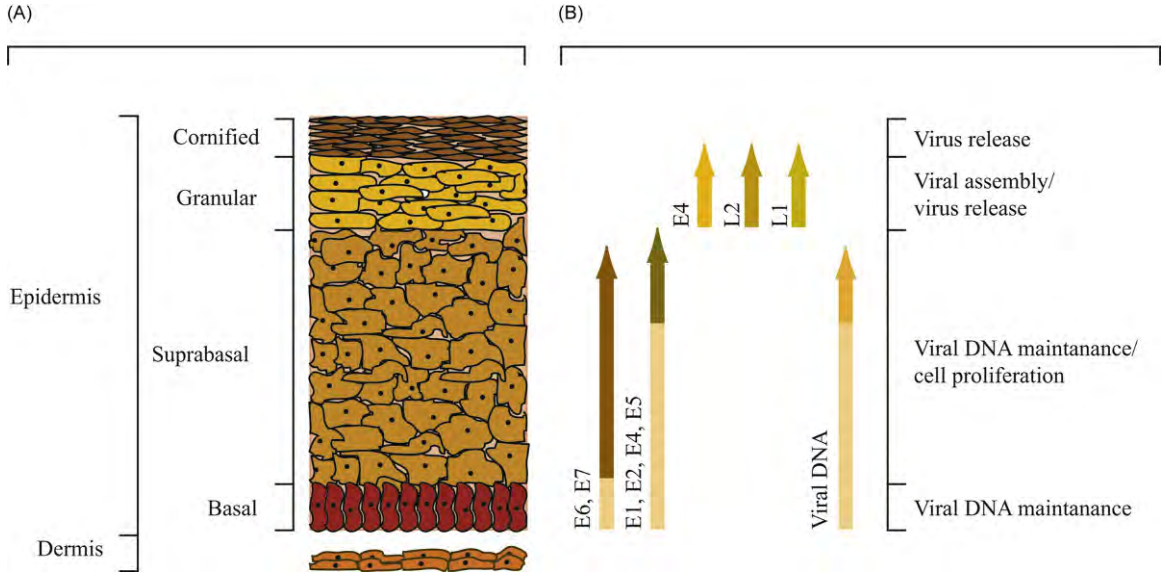

Figure 6 Expression pattern of human papillomavirus (HPV) in infected epithelial cells. (a) Schematic representation of undifferentiated and differentiated epithelium, containing basal, suprabasal, granular and cornified layers. (b) Different expression levels of HPV genes in undifferentiated aand differentiated layers of epithelium are depicted. Expression levels of HPV genes are tightly linked to the differentiation stages of epithelium. E1, E2, E5, E6, and E7 are expressed early during the differentiation. Early gene E4 and late genes (L1 and L2) are expressed during the final stages of differentiation. Viral particles are assembled and released upon cell lysis. The level of viral DNA replication mostly parallels to the early gene expression pattern as depicted.

Papillomaviruses are widespread in nature primarily infecting higher eukaryotes, including humans, cattle, rabbit, horse, dog, sheep, elk, deer, nonhuman primates, harvest mouse and multi-mammate mouse. Some avian species are also infected by the papillomaviruses, including the parrot and chaffinch. This family of viruses displays a highly species-specific infection mode. That is, they do not cause a productive infection in a second species. Most papillomaviruses even present a specific cellular tropism and their life cycle is tightly regulated by the differentiation stages of the epithelial cells that they infect (Figure 6).

To date, more than 150 different human papillomavirus (HPV) types have been completely sequenced. This number is only over 60 for animal papillomaviruses. It is possible that there are still more human and animal papillomavirus types to be discovered. Initially, the genome of HPVs was classified using liquid hybridization techniques under stringent re-association conditions but now classification is based on the nucleotide sequence homologies of specific regions of the genomes. For example, if a newly identified virus has more than 90% sequence homology to the previously identified ones in E6, E7 and L1 open reading frames (ORFs), it is considered to be as a new subtype or a variant, otherwise it is called a new genotype of HPV.

Virion Structure

Like the polyomavirus, the papillomaviruses are small, non-enveloped icosahedral DNA viruses with about 8 kb genome (Figure 5). A virion consists of 72 pentameric capsomers, arranged on a $T=7$ surface lattice. Both the major capsid protein (L1, about 55 kd) representing about 80% of the total viral protein and a minor protein (L2, about 70kd) form the shell of the viral capsid. L1 alone or L1 and L2 together can form virus-like structures (VLPs) in expression systems. L1 plays a major role in infectivity of the virus. The capsomers exist in two states within the capsids; one makes the contacts with six neighbors or the other with five neighbors.

Genome Organization

Genomic organization of all papillomaviruses is remarkably similar. That is, all contain a non-coding region (about 1 kb) and a coding region (Figure 5). The non-coding region is also designated as the viral regulatory region and contains the origin of DNA replication and the promoter/enhancer elements that serve as binding sites for many transcription factors. One of the remarkable characteristics of all papillomavirus genomes is the fact that only one of the strands of the genome serves as template for transcription. A typical papillomavirus genome contains 10 open reading frames (ORFs), some of which encode regulatory proteins and some structural (capsid) proteins. The degree of the expression levels of an ORF is dependent upon the differentiation state of the infected cells. That is, the early region of the viral genome is mostly expressed during initial stages of infection cycle and during the non-productive infection cycle of the virus. This region encodes only regulatory proteins, including those that are necessary for initiation of viral DNA replication and maintenance of the viral genome in infected cells. The late ORFs, however, encode viral structural proteins and are expressed only in productively infected cells (Figure 6).

Life Cycle

Papillomaviruses have a remarkable life cycle. The expression levels of ORFs of the viral genome are tightly regulated by differentiation states of the infected squamous epithelial cells. In other words, the infection stages (early and late) are tightly linked to the differentiation stage of the epithelial cells (Figure 6). The productive infection cycle of the virus can be divided into early and late stages. Initial infection takes place at the basal epithelial cells, which are the only cells in the squamous epithelium that are capable of dividing (Figure 6). The infection cycle starts with the attachment of the viral capsids to the cell surface receptors. α6-integrin plays an important role in this process, although it is not an authentic receptor for viral entry. The heterodimeric forms (α6β4 or α6β1) of the integrins appear to serve better binding sites for viral attachment than individual ones and are also good candidates for being a receptor for papillomavirus entry. The virus also enters the cells in the absence of these molecules, suggesting that this complex is not 100% necessary for viral entry. Papillomaviruses can also bind to heparin, cell surface glycosaminoglycans on human keratinocytes, which suggests that they may also play a supportive role in viral entry. Attachment of the virus is followed by internalization as shown in polyomavirus case, in Figure 3, and by the transport of the virus to the nucleus. It is not known where the uncoating process takes place. However, it is likely that, as is the case for polyomaviruses at least in the case of SV40, papillomaviruses may use the protein folding machinery in the endoplasmic reticulum (ER) for the initial uncoating of the viral particles. The treatment of infected cells with cytochalisin B and paclitaxel (Taxol) inhibits transport of the virions, suggesting that microtubules and microfilaments play a role in viral transport.

Transcription and replication of papillomaviruses are very complex processes. The virus uses multiple promoters and alternative splice patterns in different cells during the transcription and splicing processes, suggesting that the virus has the ability to adapt itself to different cell environments for its productive cycle. The differential promoter usage of the virus appears to result from the support of cell-type specific transcription factors. In other words, cell-type specific transcription factors make the expression of different viral promoters and splice patterns possible. This could be one of the explanations why papillomavirus requires differentiated patterns of the epithelial cells during its life cycle. The expression of the viral genome is tightly regulated and is dependent on the differentiation process of the epithelial layers (Figure 6). The virus infects the basal cells of the epithelium and the low level of viral early protein expression (E6 and E7) and that of viral DNA replication occurs in these cells. Then, the virus infects the subrabasal cells, where, in addition to E6 and E7, E1, E2; E4 and E5 proteins are continuously produced. The viral genome is also amplified at relatively high levels in these cells. Vegetative replication of the viral genome takes place at the terminally differentiated epithelial cells, where the viral capsid proteins, L1 and L2, are expressed, virions are assembled, and finally the infected cells are lysed (Figure 6).

Viral Proteins

The papillomavirus genome as mentioned previously encodes regulatory and structural proteins. Early protein-1 (E1) is a regulatory protein and required for the replication and maintenance of the viral genome. E2, a major transcriptional regulator of the viral genome, is involved in viral DNA replication and transcription. It activates viral genome through interaction of the E2-responsive elements located with the viral regulatory region. E2 protein is relatively conserved among the papillomaviruses and contains two major domains; sequence-specific DNA binding domain and dimerization domain, which is located in the carboxyl-terminal domain of the protein. It also contains a transactivation domain, located towards the amino-terminal half of the protein. Mutations within the E2 coding region have deleterious effects on viral transcription, replication and virus-induced transformations, namely those event are disrupted. The function of E4 protein is not clear, although it has the ability to disrupt the cytokeratin networks. It is also involved in the splicing of viral transcripts. It is an early protein but is expressed during the late vegetative state of the viral infection cycle, suggesting that it may play a role in viral encapsidation process. E5 protein is another regulatory protein, and is indirectly involved in the promotion of cell growth, because it activates the epidermal growth factor receptor in infected cells. It has also the ability to inhibit the expression of one of the cell cycle inhibitors, p21, through which it may also affect the cell cycle control. HPV encodes two major oncoproteins, E6 and E7. They are also involved in maintenance of the viral genome in an episomal state, during the productive infection cycle. E7 has been suggested to play a role in disruption of keratinocyte differentiation process. L1 and L2 are the major and minor capsid proteins of the papillomavirus respectively.

Transforming Ability of Papillomaviruses and Their Oncoproteins

Although bovine papillomavirus-1 (BPV-1) has been intensively used to investigate the transforming ability of papillomaviruses in tissue culture, due to their clinical importance, we focus our efforts on the oncogenic properties of human papillomaviruses (HPVs). High-risk HPVs, HPV-16 and HPV-18, are capable of transforming primary rodent cells, primary human fibroblasts and keratinocytes, but low-risk HPVs, HPV-6 and HPV-11 are not able to do so. HPV-16 and HPV-18 alone do not transform primary rat fibroblasts, however, E7 protein of those viruses can do so by cooperating with activated ras gene.

HPV E5 protein

E5 oncoprotein is highly conserved among the papillomaviruses that display an oncogenic property. It shows a growth stimulatory effect on cells and localizes to the golgi apparatus, endosomes and to some extent to the cell membranes. It is associated with the activation of the epidermal growth factor (EGF) receptor and mitogen activated protein (MAP) kinase pathways. It binds to a 16 kd

subunit of the vacuolar ATPase and can inhibit the acidification of endosomes. A controversy surrounding its nature is that it is not expressed in most HPV-positive cancer cells, suggesting that if the E5 gene is involved in cell proliferation, it presumably functions in benign papillomas and not in the cancers. It may also be involved in the initial stages of the cell transformation rather than later stages. This would be another explanation for not being expressed in cancer cells and yet has the ability to induce growth stimulatory pathways.

HPV E6 oncoprotein

E6 oncoprotein provides a helper function to E7 to transform cells and is a more potent protein with respect to cellular transformation. For example, E7 is sufficient for the transformation of established rodent cells such as NIH 3 T3 but the combination of E6 and E7 can readily extend the life span of human keratinocytes and can lead to the outgrowth of immortalized clones. It is a 150 amino acid long protein with four C–X–X–C motifs, which are believed to bind to Zinc.

All oncoproteins evolved mechanisms to disrupt the normal cell cycle regulation, through targeting of the cell cycle regulators including p53, a tumor suppressor protein that is activated in response to the DNA damage and genotoxic stress. It mediates the arrest of the cells at the G1 phase of the cell cycle until DNA damage is repaired. If the damage is unrepairable, it induces apoptotic pathways to eliminate the damaged cells. It causes the G1 arrest by inducing the p21 cyclin-dependent kinase inhibitor encoded by the WAF1 gene. Like the SV40 LT-Ag and the Ade5E1B 55 kd protein, the E6 oncoprotein of HPV can complex with p53. However, unlike SV40 LT-Ag and Ade5 E1B 55 kd protein, the interaction of E6 with p53 leads to the degradation of the protein rather than stabilization of it. In both cases, in practical terms, the function of p53 is greatly impaired, which leads to genomic instability and finally to cell transformation.

E6 can also have p53-independent functions with respect to cell transformation. For example, a mutant of E6 oncoprotein cannot bind p53 but can promote colony formation on soft agar, suggesting a p53-independent function of E6 oncoprotein during cell transformation. E6 can also induce telomerase activity when expressed in the cell, through which it may contribute to cell transformation by a p53-independent pathway(s). Telomerases are known to be highly active in transformed cells. In addition, E6 targets the cellular proteins like those that are involved in negative growth regulation and mediates their degradation by ubiquitination, through which it may also contribute to the cell transformation. One such target of E6 is the PD2-domain containing proteins.

HPV E7 oncoprotein

E7 is another regulatory protein of papillomaviruses. It is a small nuclear protein of 100 amino acids. It shows functional similarity to Ad 12S E1A protein in that it activates the Ad E2 promoter, induces DNA replication in quiescent cells and cooperates with an activated ras oncogene for transformation of rodent cells. It appears that the functional similarity between these two proteins stems from the fact that it shares important amino acid sequence similarity with the portions of Ad E1A protein (Figure 7) and SV40 LT-Ag. Those shared sequences are important for their function with respect to targeting cellular regulatory proteins, including retinoblastoma protein, pRb and pRb-related proteins, p107 and p130. pRb family of proteins are a group of regulatory proteins critically involved in the G/S phase transition. They are active in their dephosphorylated state, targeting the S phase-specific transcription factors, namely the E2F family of proteins. In other words, they tightly bind to the E2F family of proteins in their derphosphorylated state and prevent their transcriptional activity, through which they arrest the cells at the G1 state of the cell cycle. Upon hyperphosphorylation by the cyclin-dependent kinase complexes,the pRb family of proteins releases E2F and subsequently free E2F binds to the promoter regions of S phase-specific genes and activates their transcription. Finally, cells enter S phase for DNA replication. As is

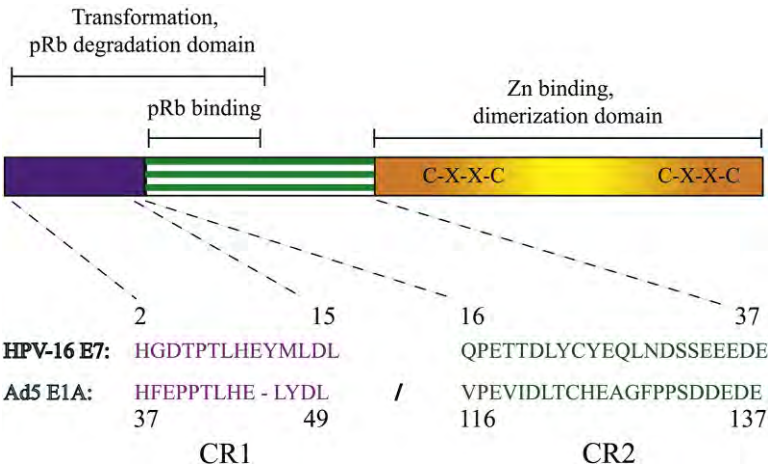

Figure 7 Schematic structure of HPV E7 oncoprotein. HPV E7 and Ad5 E1A proteins show sequence similarity between portions of conserved region 1 (CR1) and conserved region 2 (CR2). CR2 of HPV contains the casein kinase II phosphorylation site and the pRb binding site. The approximate location of the pRb binding domain, pRb degradation domain and Zn binding domain are also depicted.

case for the targeting and degradation of p53 by HPV E6 oncoprotein, E7 protein binds to pRb and induces its degradation, through which it largely contributes cell transformation. However, genetic studies showed that the targeting of the pocket proteins by E7 oncoprotein is not sufficient to account for its immortalization and transformation functions. This suggests that E7 appears to have additional cellular targets that are involved in E7-mediated cell transformation. Such targets may include TATA binding protein (TBP), activating protein-1 (AP-1), F actin and Forkhead transcription factor, but the physiological relevance of this targeting by E7 is not clear.

E7 oncoprotein also targets other regulatory proteins, involved in cell cycle regulation, including cell cycle-dependent kinase inhibitors, p27 and p21. p27 is involved in mediating cellular growth inhibition by transforming growth factor β (TGF-β) in keratinocytes. This activity of E7 may also contribute to the transforming activity of E7 in that E7 may override indirectly TGF-β associated growth arrest and eventually have a positive effect on cell transformation. p21 is a cyclin-dependent kinase inhibitor and inhibits the kinase activity of cdk4/cyclin E complex. E7 inhibits the inhibitory function of p21 and thereby contributes to the unscheduled S phase entry and cell cycle progression. The E7 oncogene appears also to induce abnormal centrosome duplication, the mechanism of which is unknown. Such an abnormality is known to lead to genomic instability, which is one of the processes that is very often encountered in cancer cells. Such an activity of E7 is therefore possibly functionally relevant to the HPV-mediated cell transformation.

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Polysaccharides, Microbial[☆]

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Glossary

Capsular polysaccharide (CPS) An exocellular polysaccharide secreted in such a large amount that it encapsulates a microorganism, facilitating adhesion and helping screen from immune response. Sometimes a distinction is made with more loosely attached polysaccharides 'released polysaccharide' (RPS).
Epimerase An enzyme that converts mannuronic acid residues to guluronic acid residues in alginates.
Intrinsic viscosity, [η] (mL g^{-1}) A measure of a particle's conformation and volume occupancy in solution.
Mark–Houwink 'a' parameter A measure of the conformation of a macromolecule, and determined from measurements of solution viscosity and molecular weight. Limits are 0 (sphere), 1.8 (rod), with values 0.5–0.8 for a flexible coil-like molecule.
Molecular weight (Da or g mol^{-1}) Popular terminology for 'molar mass' – the mass of 1 mol (6.023×10^{23}) molecules.
Molecular weight – number average, Mn (Da or g mol^{-1}) In a mixture of macromolecules Mn is the molecular weight averaged over the number concentrations of each species. It can be determined by techniques such as osmotic pressure, size exclusion chromatography coupled to multiangle light scattering (SEC-MALLs) or analytical ultracentrifugation.
Molecular weight – weight average, Mw (Da or g mol^{-1}) In a mixture of macromolecules Mw is the molecular weight averaged over the weight concentrations of each species. It can be determined by techniques such as size exclusion chromatography coupled to multiangle light scattering (SEC-MALLs) or analytical ultracentrifugation.
Non-Newtonian fluid A fluid in which viscosity changes with applied strain, an example of which is shear thinning.
Persistence length, Lp (nm) A measure of the flexibility of a linear macromolecule ranging from ~2 nm for a very flexible molecule to ~200 nm for very stiff.
Polydispersity index The ratio of the weight average molecular weight to the number average molecular weight, Mw/Mn. Minimum value=1.

Nomenclature

CPS	capsular polysaccharide
GDP	guanosine diphosphate
GRAS	generally regarded as safe
HA	hyaluronic acid
RPS	released polysaccharide

Defining Statement

Microbial polysaccharides are used for food, pharmaceutical, and medical applications: this wide range of usefulness derives from the great diversity in structural and functional properties. We consider the structure, properties, extraction, production, modification, and applications of commercially available microbial polysaccharides including xanthan, xylinan, gellan, curdlan, pullulan, dextran, scleroglucan, schizophyllan, and cyanobacterial polysaccharides.

Introduction

Over the last two decades there has been an expanding interest in polysaccharides produced extracellularly by microorganisms for food, pharmaceutical, and medical use, including vaccines. The wide range of their usefulness derives from the great diversity in structural and functional properties even though they are built up from very similar building blocks: the pyranose or furanose carbohydrate ring structure. One can have rod-shaped molecules like schizophyllan and scleroglucan from the fungi *Schizophyllan commue* and *Sclerotium rolfsii*, respectively, linear random coil type structures like pullulan from the fungus *Aureobasidium pullulans*, and intermediate structures such as alginates from the bacterium *Azotobacter vinelandii*. One can have highly electronegative or

[☆]*Change History*: December 2016. Shi Lei and Tian Feng Edmund added new references; updated throughout the text, revised all figures and introduced new ones.

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'polyanionic' molecules like xanthan secreted by *Xanthomonas campestris* or neutral polysaccharides like dextrans from the bacteria *Leuconostoc mesenteroides*.

Polysaccharides made by microorganisms are secreted from the cell to form a layer over the surface of the organism, often of substantial depth in comparison with the cell dimensions (Fig. 1). Because of their position they are characterized as exopolysaccharides, to distinguish them from any polysaccharides that might be found within the cell. The functions are thought to be mainly protective, either as a general physical barrier preventing access of harmful substances, or more specific as a way of binding and neutralizing bacteriophage. In appropriate environments they may prevent dehydration.

They may also prevent phagocytosis by other microorganisms or the cells of the immune system. The capsular polysaccharides (CPSs) are often highly immunogenic, and may have evolved their unusual diversity as a way of avoiding antibody responses: advantage of this feature can be taken in the development of vaccines. They also have a role in adhesion and penetration of the host. In the case of plants this involves interaction with polysaccharide structures in the cell walls and there are possibilities of specific interactions. Plant lectins (glycoproteins) that have specific binding properties with respect to carbohydrate structures may play a part in this and in the general defense of plants against bacterial infection.

Some secreted polysaccharides can be involved in pathogenicity. *Pseudomonas aeruginosa*, commonly found in respiratory tract infections, produces alginate which contributes to blockage in the respiratory tract, and leads to further infection, and similar blockages of phloem in plants have also been described. However, the secreted polysaccharides themselves present virtually no known toxicity problems and many are harvestable at low cost in large quantities, making them attractive for biotechnological use.

Microbial products are very important in all aspects of polysaccharide biotechnology. They range from bulk materials such as xanthan, priced at about US$10 per kg, used mainly in food and personal care consumption and oil industry, to cyclic dextrans, valued at about US$30 per kg and used in high-value applications in research and pharmaceuticals.

Certain microbes are known to produce nearly all the major plant polysaccharides such as glucans, alginate-like materials, and even cellulose – as well as the complex bacteria-specific materials. Genetic manipulation of bacteria has been studied for longer and is in general much easier than for higher organisms, so that they are an obvious target for both manipulation of biosynthetic pathways and the expression of heterologous genes to produce especially desirable enzymes. Polysaccharides are not of course under the direct control of genetic material in the way that enzymes are, and must be approached indirectly by manipulating the biosynthetic or degradation pathways by way of the enzymes responsible: there is currently great scope for this approach. In addition, since there are now fermentation processes involving the large-scale growth of bacteria, all the advances in this field can be achieved by biotechnology.

There are also limitations. It is unlikely that large-scale fermentation could ever compete with existing processes for pectin manufacture, for example, since processors currently use waste by-products from other processes as their raw material. This is quite apart from the inherent costs of large-scale fermenters in comparison with simple extraction processes. It follows that bacterial products must have particularly useful properties in order to justify their probable greater cost.

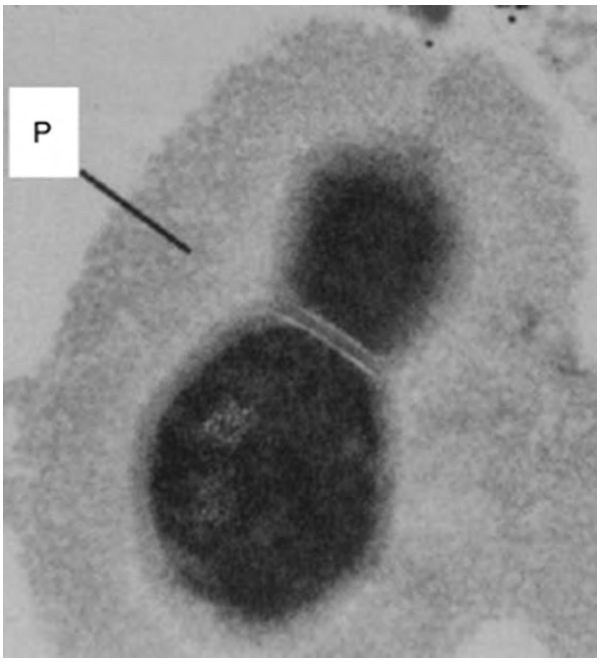


Fig. 1 Extracellular or 'capsular' polysaccharide layer (labeled P) from *Streptococcus pneumoniae*. Adapted from Skov-Sørensen, U.B., Blom, J., Birch-Andersen, A., Henrichsen, J., 1988. Ultrastructural localization of capsules, cell wall polysaccharide, cell wall proteins and F antigen in pneumococci. Infection and Immunity 56, 1890–1896. Reproduced with permission from the American Society for Microbiology.

This article looks at the production, properties, and biomodifications where appropriate of a selected range of important microbial polysaccharides because it is impossible to cover each substance comprehensively. Instead, we focus on xanthan as our main example because of its commercial importance but also consider xanthan-like exopolysaccharides such as gellan, XM6, curdlan, xylinan, and the increasingly important group of cyanobacterial polysaccharides – also xanthan-like – as well as pullulan, dextrans, scleroglucan, schizophyllan, microbial hyaluronic acid (HA), and bacterial alginates as representative examples. We complete our review with a look at the use and potential further use of exopolysaccharides as vaccines against serious diseases such as meningitis in infants.

Xanthan

Structure

Xanthan (or, as commonly called, xanthan gum) at US$10 per kg falls in a similar price range as other gums and exudates and has similar functional properties. It is made by the organism *Xanthomonas campestris*, strain NRRL B-1459 (which in its existence in the wild is responsible for cabbage blight) grown largely on glucose, itself derived from maize starch, which it converts with high efficiency (80%) to the xanthan gum structure. Typically, for a fermentation product, the raw material costs are a small part of the total, which are mainly to do with recovering the gum from the culture medium. Worldwide consumption of xanthan in 2014 was estimated between 150,000 and 160,000 metric tons, produced by companies in Europe (Jungbunzlauer), China (Deosen, Fufeng, Jianlong, Meihua), and United States (CPKelco, ADM, Cargill, DuPont/Danisco)). It has a β(1→4)-linked glucan main chain with alternating residues substituted on the 3-position with a trisaccharide chain containing two mannose and one glucuronic acid residue (Fig. 2). It is thus a charged polymer. Some of the mannose residues may also carry acetyl groups. It is useful because it forms relatively rigid rod-like structures in solution at ambient temperatures, though they convert to the random configuration on heating. These rods are able to align themselves, rather like agarose and the κ- and ι-carrageenans, with the unsubstituted regions of galactomannans, such as guar and its derivatives and locust bean gum, to produce fairly rigid mixed gels with applications in food manufacture.

The primary structure (as worked out by sequential degradation and methylation analysis) consists of a repeating unit of β-D-glucose:

$$\rightarrow 4)\beta - D - Glcp(1 \rightarrow \ldots\ldots \rightarrow 4)\beta - D - Glcp(1 \rightarrow$$

and in this respect it resembles cellulose, with the important difference that with the substitution at C3 (i.e., the third position on the carbon ring) every alternate Glcp residue is substituted by a negatively charged trisaccharide involving mannose and acetylated mannose:

$$\beta - D - Manp(1 \rightarrow 4)\beta - D - GlcpA(1 \rightarrow 2)\alpha - D - Manp - 6 - OAcetyl(1 \rightarrow 3)$$

with some of the terminal β-D-Manp residues substituted at positions C4 and C6 by pyruvate $CH_3.CO.COO^-$. Thus xanthan (except under highly acidic conditions) is, like alginate, a highly charged polyanion. Fig. 2(a) shows the Haworth projection of a (pyruvalated) alternate repeat section of xanthan.

It is not only charge repulsion effects that convey on xanthan an extended rigid rod conformation. X-ray fiber diffraction studies have shown the presence of some helical structure although there is still uncertainty as to whether this helix is a coaxial duplex or just a single chain one, with the projecting trisaccharides folded back along the axis of the helix giving a chain rigidity effectively similar to that of a double helix. Using a combination of electron microscopy (contour chain length, L), and light scattering (weight average molecular weight, Mw), Stokke and coworkers evaluated a mass per unit length, M_L, of 1950±250 g mol^{-1} nm^{-1}. From X-ray fiber diffraction studies, a corresponding value of 950 g mol^{-1} nm^{-1} was obtained, and a duplex or double-helical structure was inferred for the native structure. High temperatures reversibly melt this helix to give a more random structure.

Production Process

The primary laboratory and commercial fermentation medium – as has been known for over 50 years – for *X. campestris* growth and xanthan production is a phosphate-buffered (pH ~7) broth containing D-glucose (30 g L^{-1}) (or sucrose, starch, hydrolyzed starch), NH_4Cl, $MgSO_4$, trace salts with 5 g L^{-1} casein (or soybean) hydrolysate, and the fermentation process takes place aerobically at a temperature of ~28°C. Xanthan production is further stimulated by the presence of pyruvic, succinic, or other organic acids. The xanthan produced in this way is very similar to the naturally produced xanthan made by the microbes living on a cabbage. In the commercial process the oxygen uptake from the broth is controlled to a rate of 1 mmol L^{-1} min^{-1}. Treated in this way the bacterium is an extremely efficient enzyme minifactory converting >70% of the substrate (D-glucose or related) to polymeric xanthan. Following precipitation, xanthan fibers are separated by filtration or centrifugation, dried, milled, and packed (Fig. 3). Used isopropanol, which is collected from the solid-liquid separation step and from the drying process, is distilled in a rectification column and reused. Xanthan production is an energy-intensive process, especially power for the agitation of the fermenters, cooling, and rectification contribute to a great extent to variable production costs.

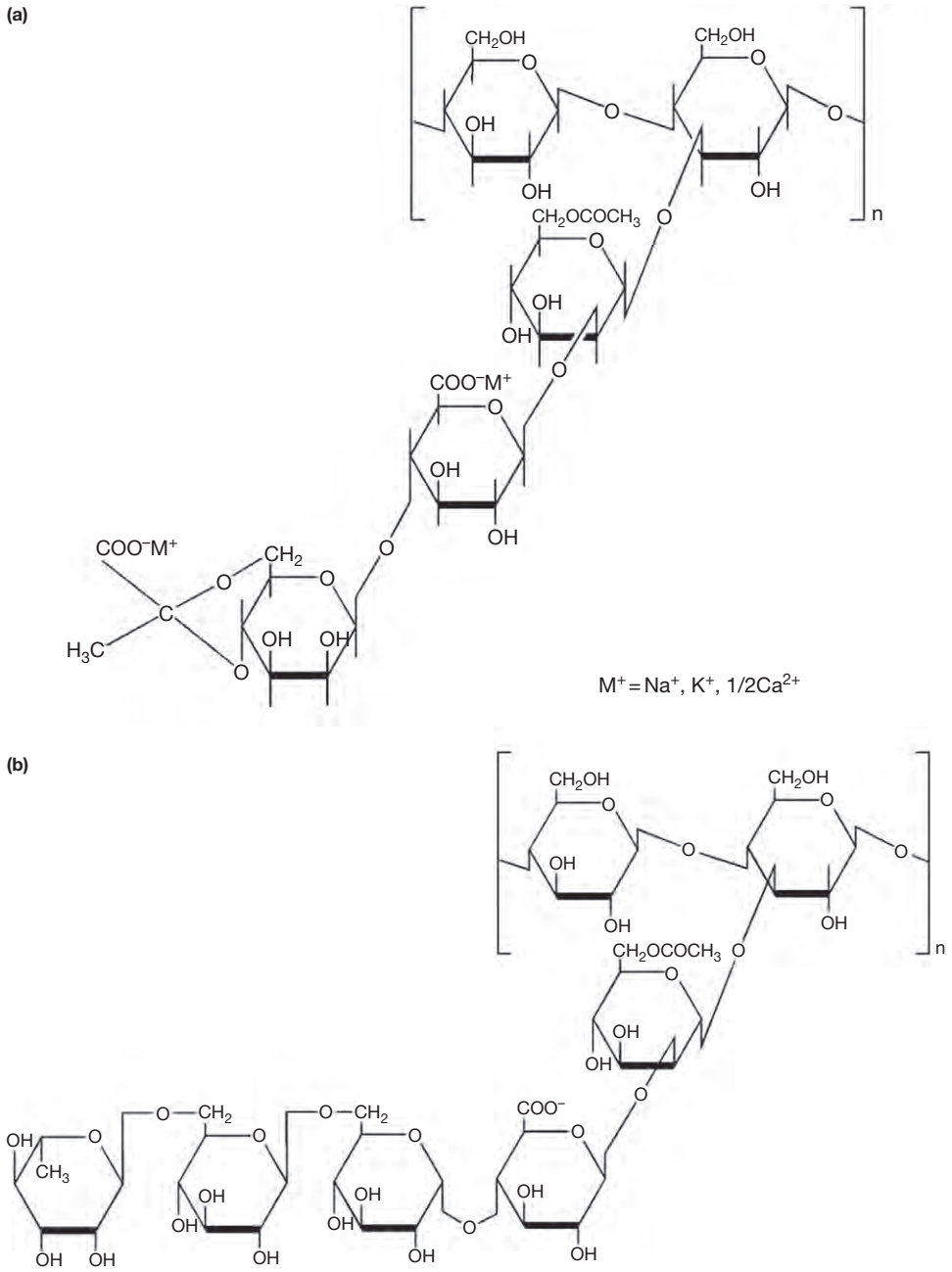


Fig. 2 (a) Pentasaccharide repeating unit of xanthan and binding sites for cations (M^+). (b) Xylinan (acetan) repeat unit showing a pentasaccharide side chain.

The biosynthetic process by the bacterium worked out by Sutherland and later confirmed by others follows the same basic pattern proposed for other microbial polysaccharides: (1) substrate uptake; (2) substrate metabolism; (3) polymerization (4) modification and extrusion, and involves lipid carriers (although there is still uncertainty over their precise role and how the whole process is controlled).

Xanthan biosynthesis is an energy intensive process with a high number of enzymes which are either directly or indirectly involved in the synthesis. Glucose is mainly metabolized via the Entner-Doudoroff pathway and the citric acid cycle to build up biomass and produce energy rich intermediates. Fig. 4 shows a model for the synthesis of xanthan in *X. campestris*. It is assumed that the first steps are the assembly of the pentasaccharide unit which are subsequently polymerized and exported. The genes which are directly involved in the xanthan synthesis are located on the gum-operon, a cluster of 12 genes, named *gumB*, *gumC* to *gumM*.

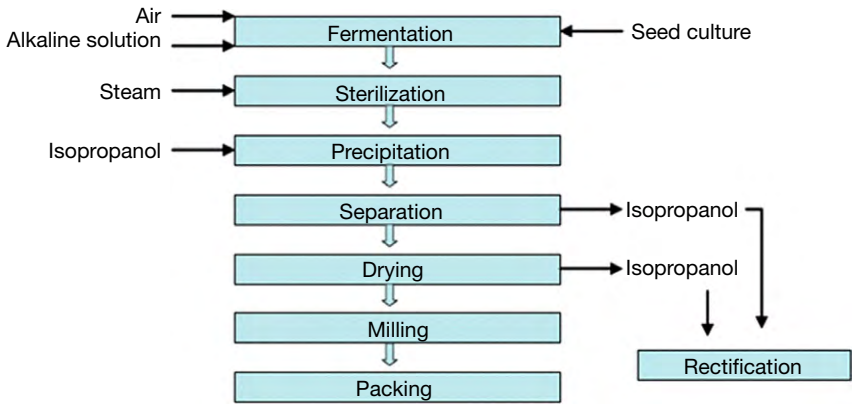


Fig. 3 Industrial production process of xanthan.

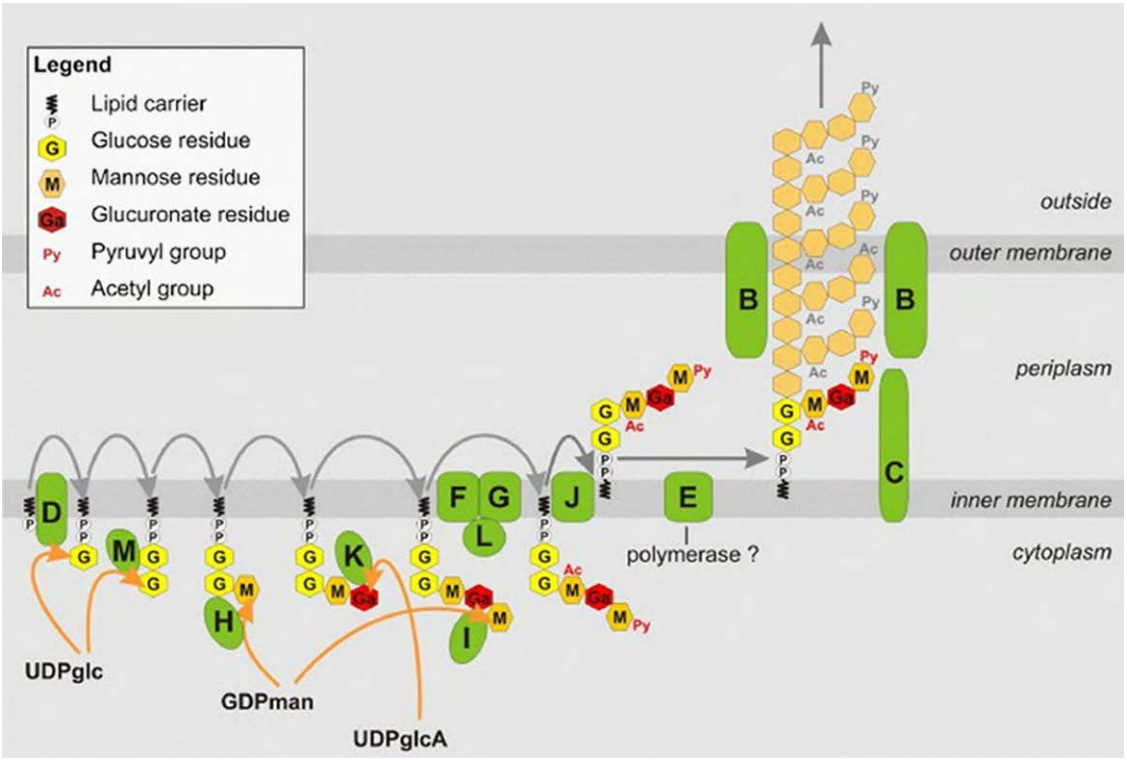


Fig. 4 A model for the synthesis of xanthan at the cell membrane of *X. campestris* (reproduced with permission from Vorhölter F.-J., *et al.*, 2008. Journal of Biotechnology 134, 33–45). The letters in the green fields represent the enzymes involved in xanthan production.

Properties

These molecules are extremely large, for example, the weight average Mw of keltrol xanthan has been found by ultracentrifuge techniques to be $\sim 6 \times 10^6$ g mol^{-1}, and stiff, with a molecular persistence length Lp of ~150 nm (the practical limits for linear molecules are ~2 nm for a very flexible, or 'random coil' structure, and ~200 nm for a rigid rod structure). Xanthan gives very viscous solutions (intrinsic viscosities, [η], approximately several thousand milliliters per gram) and indeed is one of the largest of the aqueous soluble polysaccharides. The very high viscosity at low concentrations (e.g., at 5 mg mL^{-1} a viscosity of ~1000 cP has been observed at room temperature) makes it ideal as a thickening and suspending agent.

Xanthan is a tasteless, white to cream-colored free-flowing powder soluble in hot and cold water, but insoluble in most organic solvents. Besides the above described high viscosity at low concentrations, xanthan still has many valuable properties compared to

other hydrocolloids: 1) highly pseudoplastic over a broad shear range; 2) good thermal stability; 3) good stability over a broad pH spectrum; 4) resistant to degradation by most enzymes; 5) soluble and stable in salt solutions; 6) synergistic behavior with galactomannans.

It is these unique physicochemical properties that make xanthan be widely used in food, animal feed, personal care, pharmaceutical, and industrial fields. For example, the industrial importance of xanthan is based on its ability to control the rheology of water based systems.

Modification

Modifications of xanthan usually occur at three different stages of the production process: 1) modifications at the molecular structure; 2) modifications during downstream processing; 3) end-product modifications. The most common modifications at the structure of xanthan are variations of the acetate or pyruvate content.

The key sites for modification are the first and terminal mannose residues on the trisaccharide side chains (particularly extent of acetylation of the first and pyruvalation of the latter) and the helical backbone formed by noncovalent interactions with galactomannans: these interactions also appear to be affected by the substitutions in the side chains. Insofar as the trisaccharide side chains are concerned there are two approaches for alteration or control: one is changing the physiological conditions of fermentation; the other is the use of different pathovars or strains of *X. campestris*. The pathovars pv *phaseoli* and *oryzae* yield virtually acetyl-free and pyruvate-free xanthan, respectively.

The other approach has been to look at the genetics of the enzymes controlling the biosynthetic pathway and to attempt to produce and isolate in sufficient quantity genetic mutants deficient or defective in one or more of these enzymes to give 'polytetramer' (i.e., lacking in the terminal mannosyl or pyruvalated mannose group) and 'polytrimer' (lacking in addition the adjacent glucuronic acid residue). Although yields of 50% polytetramer have been found, corresponding attempts for polytrimer and other xanthan variants have thus far been disappointing.

There has been considerable interest in improving the weak gelation characteristics of xanthan by inclusion of galactomannans such as locust bean gum and gum into mixtures. As with carrageenan and agarose, excellent synergistic interaction occurs between xanthan and the galactomannans such as locust bean gum or guar. Xanthan gels are very weak and transient but made in the presence of guar or locust bean gum give stronger gels with an optimum mixing ratio ~50:50 by weight. Deacetylating the xanthan side chains seems to enhance these synergistic interactions.

Uses of Xanthan

Xanthan was approved as food grade by the US Food and Drug Administration nearly 30 years ago. This makes it not only attractive as a food product but also useful in packaging material in contact with food and also for use in pharmaceutical and biomedical applications that involve ingestion. Its uses chiefly derive from its solubility in hot and cold water and its very high thickening and suspending potential, which in turn derives from the very high viscosity of its suspensions. Despite its high viscosity, xanthan suspensions exhibit high shear thinning, which means they also flow easily (i.e., good pourability). For food applications, xanthan suspensions have high acid stability besides high viscosity and thickening and suspending ability. This makes it highly popular in sauces, syrups and toppings, and salad dressings. In drinks, the addition of xanthan together with carboxymethylcellulose adds body to the liquid and assists with uniform distribution of fruit pulp. It is also used to add body to dairy products. The high freeze–thaw stability of xanthan suspensions makes it particularly attractive for the frozen food industry. The high suspending and stabilizing properties are also taken advantage of by the animal feed industry for transporting liquid feeds with added vitamins and other supplements, which would otherwise sediment out during transport or with storage time. It has also been suggested as an additive to fruit drinks to reduce tooth decay. For pharmaceuticals, xanthan has recently been added to the list of hydrophilic matrix carriers, along with chitosan, cellulose ethers, modified starches, and scleroglucan. Tablets containing 5% xanthan gum under low shear conditions were shown to enable the successfully controlled release of acetaminophen into stomach fluid and tablets containing 20% xanthan successfully carried a high loading (50%) of the drug theophylline. The high suspension stability is made use of in pharmaceutical cream formulations and in barium sulfate preparations. This high cream stability is also taken advantage of by the cosmetic industry, including toothpaste technology, facilitating the suspension of ingredients (high viscosity) and the easy brushing onto and off the teeth (high shear thinning). Uniform pigment dispersal along with other ingredients and long-time stability make xanthan a good base for shampoos. The high suspension stability of xanthan makes it an ideal base for suspending adhesive agents for wallpapers. Just to reinforce the diversity of application, the suspension-stabilizing property of xanthans makes it ideal for producing sharp prints from dyes, with a minimum risk of running in textiles and (in conjunction with guar) carpets.

In summary, due to its extraordinary properties as stabilizer and thickener, xanthan is extensively used in the food, personal care and pharmaceutical industry. In addition, xanthan is applied in various industrial applications and for oil drilling. In all of these areas xanthan is recognized as an excellent stabilizer and useful processing aid. Table 1 lists the functions of xanthan in various applications.

Table 1 Some examples of xanthan applications

Application	*Usage in (%)*	*Function*
Food		
Salad dressings	0.1–0.5	Provides easy pourability and good cling; suspends spices
Bakery products, gluten free bakery	0.05–0.3	Binds water; improves texture and moisture retention; suspends fruits and nuts
Beverages	0.05–0.2	Enhances mouthfeel; suspends fruit pulp
Instant products	0.05–0.2	Contributes body; quick viscosity build up in cold and hot water
Prepared food	0.1–0.3	Stabilizes; avoids syneresis
Soups, sauces and gravies	0.05–0.5	Provides good temperature stability; prevents separation; improves texture
Frozen food	0.05–0.2	Provides good freeze/thaw stability; contributes smooth texture
Dairy products	0.05–0.2	Inhibits syneresis; stabilizes emulsions
Toppings	0.05–0.3	Stabilizes foams and emulsions; provides good flow and cling
Meat products	0.2–0.5	Binds water; inhibits syneresis
Low-calorie products	0.1–0.5	Improves texture; stabilizes
Personal care		
Toothpaste	0.7–1.0	Provides easy pumpability and excellent flow properties; gives good stand on the brush
Creams and lotions	0.2–0.5	Stabilizes emulsions; gives creamy consistency
Shampoo	0.2–0.5	Controls rheology; suspends insolubles
Industrial		
Agricultural chemicals	0.1–0.3	Suspends active ingredients; controls drift and cling
Cleaners	0.2–0.7	Provides good pH-stability; extends contact time
Polishes	0.2–0.7	Suspends abrasive components
Water based paints	0.1–0.3	Controls rheology; stabilizes pigments; inhibits drip off
Textile and carpet printing	0.2–0.5	Improves processing; controls color migration
Adhesives	0.1–0.3	Controls rheology and penetration
Paper industry	0.1–0.2	Acts as suspension aid and rheology control
Ceramic glazes	0.3–0.5	Suspends solids effectively
Oil drilling	0.1–0.4	Controls rheology; provides good stability against salt, temperature and shear
Enhanced oil recovery	0.05–0.2	Functions as mobility control agent
Animal feed		
Liquid milk replacers	0.05–0.2	Stabilizes water-insoluble ingredients
Pet food	0.1–0.4	Prevents syneresis; contributes body to gravy
Pharmaceuticals		
Suspensions and emulsions	0.1–0.5	Provides excellent stability and good flow
Tablets	1.0–3.0	Retards drug release
Lozenges	0.3–1.0	Prolongs contact time of active ingredients

Xylinan (Acetan)

Structure

Xylinan (or acetan) is a complex anionic exopolysaccharide produced by the Gram-negative bacterium *Acetobacter xylinum*. The structural repeat unit is similar to that of xanthan and consists of a cellulosic backbone (. . . →4) β-D-Glcp(1→ . . .) where alternate glucopyranose residues are substituted at the 3-position with a pentasaccharide side chain (Fig. 2(b)):

$$\alpha - \mathrm{L} - \mathrm{Rhap} - (1 \rightarrow 6) - \beta - \mathrm{D} - \mathrm{Glcp} - (1 \rightarrow 6) - \alpha - \mathrm{D} - \mathrm{Glcp} - (1 \rightarrow 4) - \beta - \mathrm{D} - \mathrm{GlcAp} - (1 \rightarrow 2) - \alpha - \mathrm{D} - \mathrm{Manp} - (1 \rightarrow 3)$$

The branched glucopyranose residues together with the mannopyranose residue are partially O-acetylated at the 6-position. The pentasaccharide side chain protects the cellulosic backbone from enzymatic degradation by cellulases.

Analysis from X-ray diffraction and atomic force microscopy show that xylinan adopts a helical structure in both the solid and solution phases. Hydrodynamic characterization using sedimentation velocity in the analytical ultracentrifuge, viscometry, and multiangle laser light scattering (MALLs) have shown that double-stranded helices are predominant although single- and multi-stranded chains are also present. Xylinan chains upon heating undergo thermoreversible helix (ordered state)–coil (disordered state) transition.

Extraction and Production

Xylinan is extracted as a water-soluble by-product during the commercial production of bacterial cellulose by *A. xylinum*.

Properties

These molecules are extremely large and a weight average Mw of $\sim 2.5 \times 10^6$ g mol^{-1} has been found by ultracentrifuge and light scattering techniques. Furthermore, the double-helical structure results in a stiff macromolecular chain with a molecular persistence length Lp of ~100 nm and a mass per unit length M_L of ~2500 g mol^{-1} nm^{-1}, resulting in viscous solutions, [η], approximately several thousand milliliter per gram. A Mark–Houwink 'a' parameter of 0.90 was found, which is consistent with a rigid conformation. Intermolecular binding has been demonstrated between xylinan and the industrially important glucomannan (konjac gum) and galactomannan (carob gum), resulting in the formation of synergistic gels (or increased viscosity at lower concentrations).

Modification

Chemical modification of xylinan has been through deacetylation of the 6-linked O-acetyl groups. It has been shown that acetylation does not prevent helix formation but it does reduce the synergistic interaction with glucomannans or galactomannans.

Xylinan synthesis involves several genes including *aceA*, *aceB*, *aceC* and it has recently been shown that chemical mutagenesis of the native bacterium results in a strain that produces a polysaccharide in which the side chain is a tetrasaccharide. Furthermore, this novel xylinan variant has higher viscosity. A further novel polysaccharide with a trisaccharide side chain was designed by deactivating the *aceP* gene.

Uses

Xylinan is used in the food industry as a viscosifier and gelling agent. It is also a component in nata, a confectionery popular in Japan and the Philippines.

Gellan, XM6, and Curdlan

Inspired by the example of xanthan, other bacterial polysaccharides with useful properties have been developed, for example, gellan, the result of a systematic search for a polysaccharide having the required properties, followed by the identification of the organism.

Gellan is obtained from cultures of *Pseudomonas elodea* found growing on the elodea plant. It is isolated by ethanol precipitation from the culture medium, and may be partially deacetylated by alkali treatment. It has a linear structure with a repeat unit of a tetrasaccharide (Fig. 5), each with one carboxyl group and in the native state one acetyl group. It is therefore sensitive to calcium levels but has some rheological properties similar to those of xanthan, which has a similar charge density and adopts a double-helical conformation in solution. The double-helix molecular weight is reported to be $\sim 5 \times 10^6$, with an intrinsic viscosity of ~3500 mL g^{-1}.

It was clearly intended to be a xanthan competitor, though before permission for food use was obtained it was promoted as an agar substitute, particularly for use in growth media. Gels produced on untreated gellans are weak and rubbery although deacylation produces hard, brittle gels, and after prior removal of all multivalent cations and addition of Ca^{2+} ions, gellan forms gels in rather the same way as with alginates. It is one of many film-forming polysaccharides being considered for use in implants for insulin in the treatment of diabetes.

The XM6 repeat unit structure is also shown in Fig. 5 and is a polysaccharide made by an *Enterobacter* discovered at Edinburgh University. It lacks acetyl groups but is classed along with xanthan and gellan, and can be induced to gel by adding calcium ions. It is included here as a representative of many similar bacterial polysaccharides all of which have interesting and potentially useful rheological properties, although it is not commercially available, and as far as we are aware has not been approved for food use, and probably never will.

Curdlan is one of two polysaccharides produced by *Alcaligenes faecalis* with a repeat unit of three β(1→3)-linked D-glucose residues and unlike the examples above is not charged. Curdlan suspensions gel on heating, apparently irreversibly. There are a number of potential applications for it in the food industry, mostly as a replacer for existing gums, and it is in use in Japan in some products. Novel products based on its heat set ability may also appear.

Cyanobacterial Polysaccharides

Another group of polysaccharides with reputed xanthan-like properties are the exopolysaccharides from Cyanobacteria – blue-green algae – known to produce large amounts of these substances.

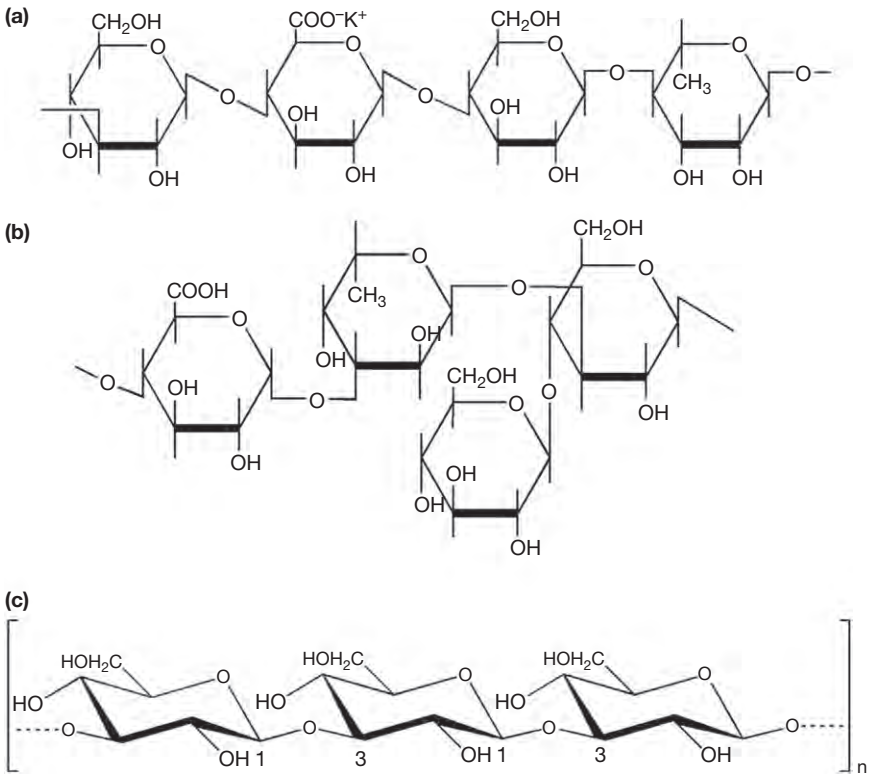


Fig. 5 Repeat units, gellan in deacetylated form (a), the XM6 polysaccharide (b), and curdlan (c).

Structure

Like xanthan, the cyanobacterial polysaccharides are relatively complex containing on average 6–10 different saccharide residues, with the neutral sugars xylose, arabinose, fucose, galactose, rhamnose, mannose, glucose, and uronic acids, with glucose normally being the dominant residue. Charged groups such as sulfate and pyruvate together with hydrophobic groups, for example, acetates have also been reported. All the cyanobacterial polysaccharides studied so far have been branched structures.

Extraction and Production

Due to their diversity, cyanobacteria are found growing under a wide variety of conditions. Nitrogen seems to have a negative effect on polysaccharide production whereas temperature, salinity, illumination, and exposure to UV radiation can have either positive or negative effects on exopolysaccharide production in cyanobacteria. It is worth noting that optimal exopolysaccharide production does not necessarily coincide with those for cell growth. The daily production of cyanobacterial polysaccharides from different sources is up to 2 g L^{-1}, which is small in comparison to xanthan production, which can be up to 10 g L^{-1}. The general process of extraction, production and characterization of microalgal and cyanobacterial exopolysaccharides is shown in Fig. 6.

Properties

Cyanobacterial exopolysaccharides have been shown (Fig. 7) to exhibit xanthan-like shear-thinning properties. Polysaccharide molecular weights have been measured in the range of $1–2\times10^6$ g mol^{-1} and 'gross' macromolecular conformations have been estimated to be a random coil for the exopolysaccharide from *Cyanospira capsulata* (CC-EPS), a rigid rod type polysaccharide from *Aphanothece halophytica* GR02 (AH-EPS), or intermediate between random coil and rigid rod for the polysaccharide from *Anabaena* sp. ATCC 33047.

Uses

The importance of cyanobacterial polysaccharides of biological soil crusts (BSCs) in sand surface stabilization and soil nutrient retention has been long acknowledged. These exopolysaccharides are of potential biotechnological importance as conditioners of

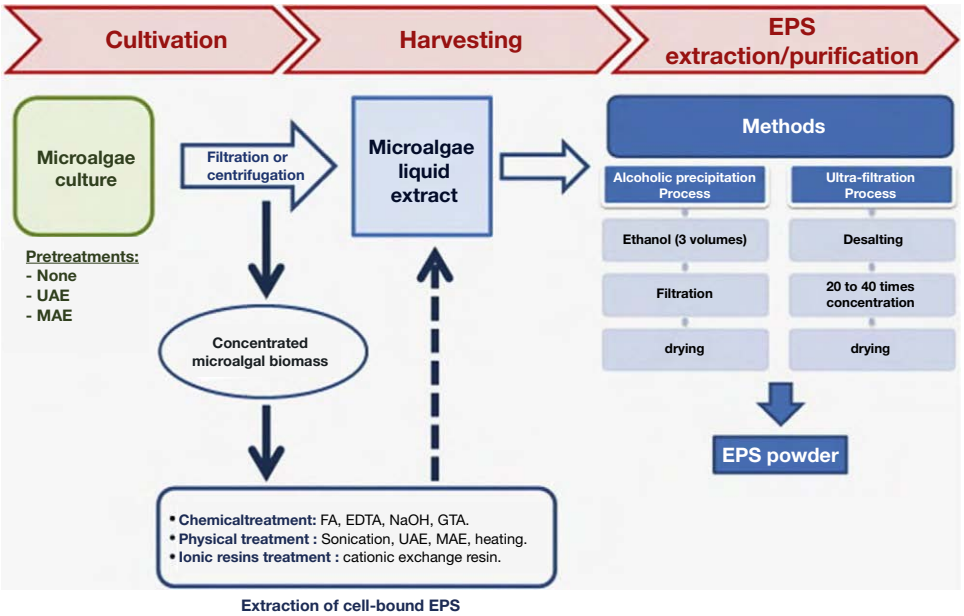


Fig. 6 Flowchart describing the main processes from the production to the extraction and purification of microalgae exopolysaccharide. (reproduced with permission from Delattre C., *et al.*, 2016. Biotechnology Advances 34, 1159–1179). FA: formaldehyde; EDTA: ethylene diamine tetraacetic acid; GTA: glutaraldehyde; UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction.

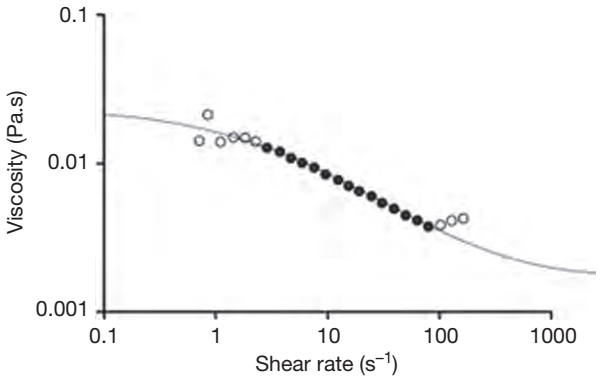


Fig. 7 Viscosity and fitting for the shear-thinning cyanobacterial exopolysaccharide from *Aphanothece halophytica* GR02 (AH-EPS) at 0.6 g L^{-1} measured at 25°C. Circles: points not used for fitting. Square: points used for fitting. Line: fitted curve. From Morris, G.A., Li, P., Puaud, M., *et al.*, 2001. Hydrodynamic characterization of the exopolysaccharides from the halophilic cyanobacteria *Aphanothece halophytica* GR02 A comparison with xanthan. Carbohydrate Polymers 44, 261–268.

soils as they improve water-holding capacity; as emulsifiers, viscosifiers, medicines, bioflocculants; and for heavy metal removal. A pectin-like polysaccharide that is able to absorb metal cations has also been described.

Pullulan

Pullulan is an α-glucan made up from maltotriose units linked by α(1→6) bonds (Fig. 8). It is obtained from *Aureobasidium pullulans* and is hydrolyzed by pullulanase to yield maltotriose. It is not attacked by digestive enzymes of the human gut, and is used to form films. Production is now substantial, and has found particular application in formulating snack foods and as a packaging film. It is slowly digested in humans.

It is water soluble, with molecular weights in the range 5000–900,000 g mol^{-1}, with straight unbranched chains, and behaves as a very flexible molecule, with properties of a 'random-coil' (with a molecular persistence length Lp of approximately 2 nm) depending on the combination of sedimentation coefficient and intrinsic viscosity measurements. It has been proposed as a 'standard polysaccharide' because its behavior resembles random coil behavior, and it is readily obtainable in a very reproducible

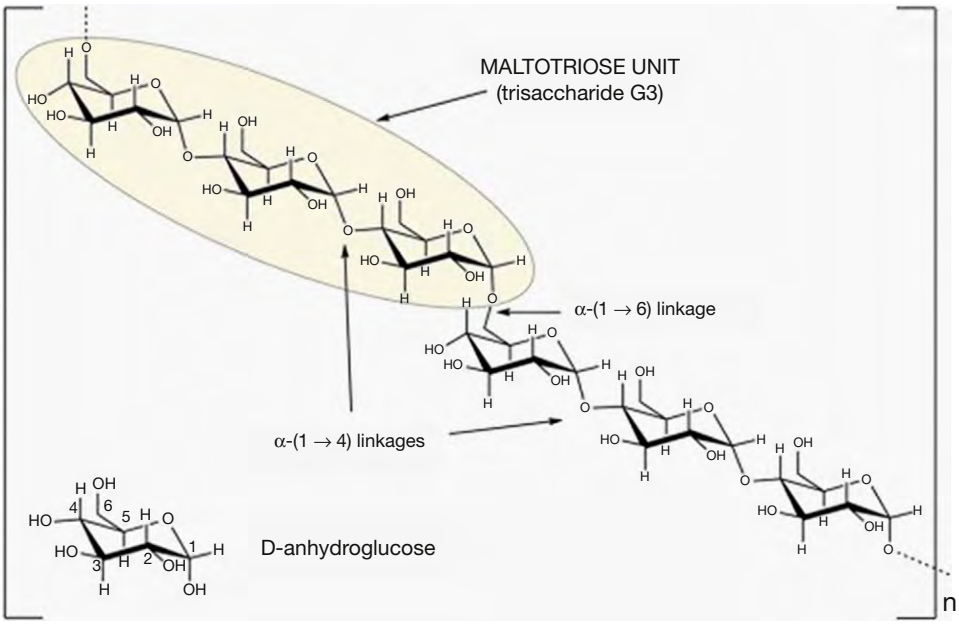


Fig. 8 Structure of pullulan and its repeating unit maltotriose (reproduced with permission from Farris, S., *et al.*, 2014. Journal of Applied Polymer Science 131, 1–12). The basic monomer (D-anhydroglucose) is also depicted, with the position of each carbon atom indicated by a number.

form so that it can be used for comparative tests with other polysaccharides. The idea is presumably that deviation from pullulan type behavior must imply a more complex structure. It might also be used to standardize instruments. The solutions in water are liable to bacterial attack, with rapid degradation of the molecular weight, and preservatives such as azide may be needed.

Dextrans

Bacterial dextrans are produced in substantial quantities by *Leuconostoc mesenteroides* and are familiar to laboratory workers as the basis for cross-linked dextran beads used in gel filtration columns. They are mostly α(1→6)D-glucopyranosyl polymers with molecular weights up to $\sim 1 \times 10^6$ g mol^{-1} more or less branched through 1→2, 1→3, or 1→4 links. In most cases the length of the side chains is short, and branched residues vary between 5% and 33%. The major commercial dextran is about 95% 1→6 linked and 5% 1→3 linked (Fig. 9), and is made from selected strains of *Leuconostoc*. After ethanol precipitation from the culture medium, acid hydrolysis is used to reduce the overall molecular weight, though fungal dextranases can also be used. The product with an average molecular weight of about 60,000 g mol^{-1} is used in medicine as a blood extender, while fractions of defined molecular weights (e.g., the Pharmacia T-series, e.g., T-500 Dextran, which stands for dextran of weight average molecular weight 500,000 g mol^{-1}) are familiar in laboratories and to some extent, like the pullulan P-series, serve as polysaccharide standards in molecular weight calibrations. "Blue dextran" is a well-known marker for the void volume in gel filtration studies. Dextrans are also used as part of incompatible phase separation systems, usually with polyethylene glycol. Their solubility in both aqueous and non-aqueous media renders them good membrane formers using electrospinning techniques.

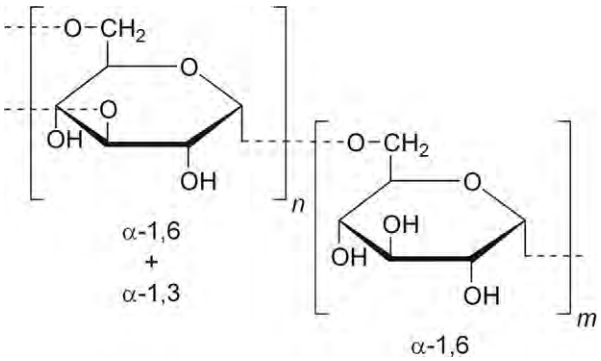


Fig. 9 Chemical structure of the major commercial dextran, which is about 95% 1→6 linked and 5% 1→3 linked.

Dextrans have found very wide application in laboratory work because they are particularly free from positive interactions with proteins. The interactions can be almost entirely characterized as co-exclusion. This has found application, like in the case of pullulans, in calibrating gel filtration media, but cross-linked dextran gels show other effects. For example, they swell and shrink in a way related to the osmotic pressure of the solvent system, and can be used to make miniature osmometers.

Scleroglucan and Schizophyllan

A consideration of microbial polysaccharides would not be complete without at least a brief consideration of two fungal polysaccharides that are attracting increasing commercial interest: the weak-gelling scleroglucan and schizophyllan systems. They are both large, neutral polysaccharides of weight average molecular weights, Mw, (as largely established by light scattering techniques) ~500,000 g mol^{-1}, with a greater diversity being reported for scleroglucan. X-ray diffraction studies indicate that they exist as hydrogen-bond-stabilized triple helices, stabilized by hydrogen bonds with resultant extrarigid rod-like properties in solution; they have virtually the largest persistence lengths known for polysaccharides: ~150 nm for schizophyllan and 200 nm for scleroglucan. The existence of the triple helix for scleroglucan has been further supported by electron microscopy/light scattering measurements of the mass per unit length, M_L=2100±200 g mol^{-1} nm^{-1}, along similar lines to that which supported the duplex model for xanthan as discussed above. The Mark–Houwink viscosity 'a' parameter (a measure of how the intrinsic viscosity of a substance changes with molecular weight and a parameter that reflects the conformation of a macromolecule) of 1.7 for schizophyllans of Mw <500,000 g mol^{-1} is again the highest known for a polysaccharide and is on the rigid rod limit, and these molecules have been used to flocculate small colloidal particles. For chains of Mw >500,000 g mol^{-1} the 'a' parameter falls to ~1.2 and corresponds to slightly more flexibility as the polymer length increases.

Chemically also they are very similar, with a backbone of repeating β(1→3)-linked glucose residues:

$$\ldots \rightarrow 3)\beta - \mathrm{D} - \mathrm{Glcp}(1 \rightarrow \ldots$$

In scleroglucan every third residue has a β(1→6)-linked D-glucose branch that protrudes from the triple helix (Fig. 10). Using electron microscopy, Stokke and coworkers have demonstrated that certain denaturation–renaturation treatments cause the formation of interesting ring structures or 'macrocycles'. In common with other branched glucans they appear to stimulate an immune response against tumor cells, and, particularly scleroglucans, have been considered for use in cosmetics (as part of skin and hair products), for application in pesticides (to assist binding to foliage), and, along with xanthan and other polysaccharides, for binding water and providing high heat stability in oil well drilling fluids.

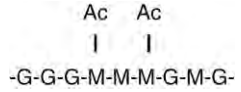
Bacterial Alginates

Structure and Production

Alginate is now known to be a whole family of linear copolymers containing blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. The blocks are composed of consecutive G residues (GGGGGG), consecutive M residues (MMMMMM), and alternating M and G residues (GMGMGM) (Fig. 11). Alginates extracted from different sources differ in M and G contents as well as the length of each block, and more than 200 different alginates are currently being manufactured. The G-block content of *Laminaria hyperborea* stems is 60%, and for other commercially available alginates is in the range of 14.0–31.0%.

Alginates are copolymers of the residues guluronic acid (abbreviated as GulA or just G) and mannuronic acid (ManA or M), (Fig. 11) and hence under normal solution conditions (except in acidic environments) these molecules are highly charged polyanionic polyelectrolytes. Although commercial alginates derive largely from algal sources there is a large potential for producing tailor-made alginates from bacterial sources, especially if advantage is taken of the genetic tools for controlling the production of the enzymes that are responsible for the synthesis and epimerization (conversion of D-mannuronic (M) to L-guluronic residues (G)) of the polymeric alginate chain (Fig. 12). There appears to be considerable structural diversity (poly-M, poly-G, and poly-MG residues) and our understanding of the genes and the enzyme gene products is much greater for bacterial alginate production compared to the case for seaweed.

The main alginate-producing bacteria that have been studied are *Pseudomonas aeruginosa* and *Azotobacter vinelandii*. *P. aeruginosa* has been the subject of particular attention because of its association with respiratory disease and is found in patients suffering from cystic fibrosis. *A. vinelandii* appears to be the most promising in terms of industrial production because of its stable output of alginate. *P. aeruginosa* alginate has no poly-G residues, (and hence has a low G content) whereas *A. vinelandii* can, like seaweed alginate, possess all three block sequences (poly-M, poly-G, and poly-MG residues). However, all bacterial alginates are O-acetylated to varying degrees:



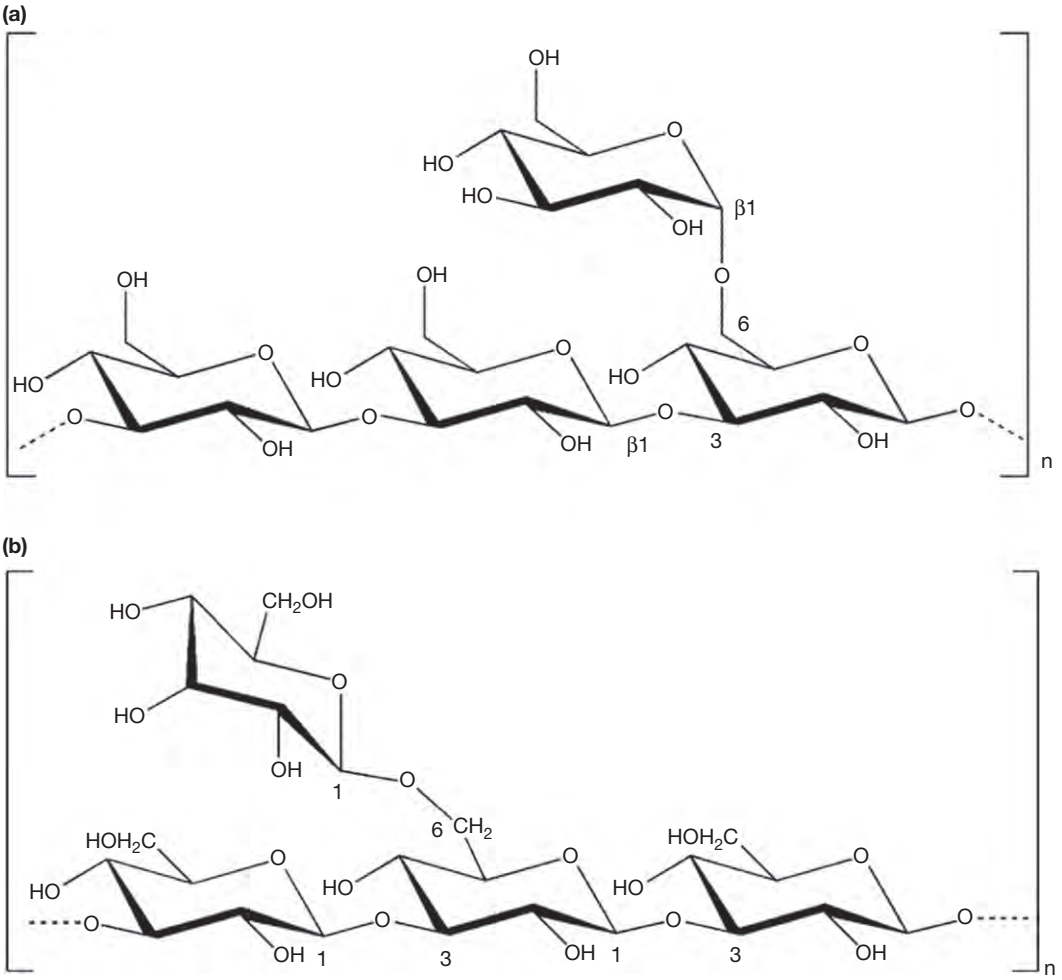


Fig. 10 Schematic representation of the repeating unit of scleroglucan (a) and schizophyllan (b).

Structural Enzymes

Starting from fructose-6-phosphate the following enzymes are involved: hexokinase; phosphomannose isomerase; D-mannose-1-phosphate guanyl transferase; GDP (guanosine diphosphate)-mannose dehydrogenase; transferase; acetyl transferase; and mannuronan C-5-epimerase. Schemes involving these have been worked out for *A. vinelandii* and *P. aeruginosa*. Key structural genes have been identified in a gene cluster for *P. aeruginosa* with algG encoding for the epimerase and algF the acetylase (Fig. 13).

Possibilities

It may be possible to treat alginate with epimerases to increase the poly-G content, producing a stiffer polymer. In principle this can be done in vivo by the incorporation and expression of genes from a plasmid in an alginate-producing bacterium (Fig. 14). The degree of acetylation is another control point: acetylated mannuronic acids cannot be converted by epimerases from M→G.

Uses of Alginate

Alginate has demonstrated great utility and potential as a biomaterial for many biomedical applications, particularly in the areas of wound healing, drug delivery, in vitro cell culture, and tissue engineering. The most attractive features of alginate for these applications include biocompatibility, mild gelation conditions, and simple modifications to prepare alginate derivatives with new properties. Alginate has a track record of safe clinical uses as a wound healing dressing material and pharmaceutical component, and has been safely implanted in a variety of applications, including islet transplantation for treatment of type 1

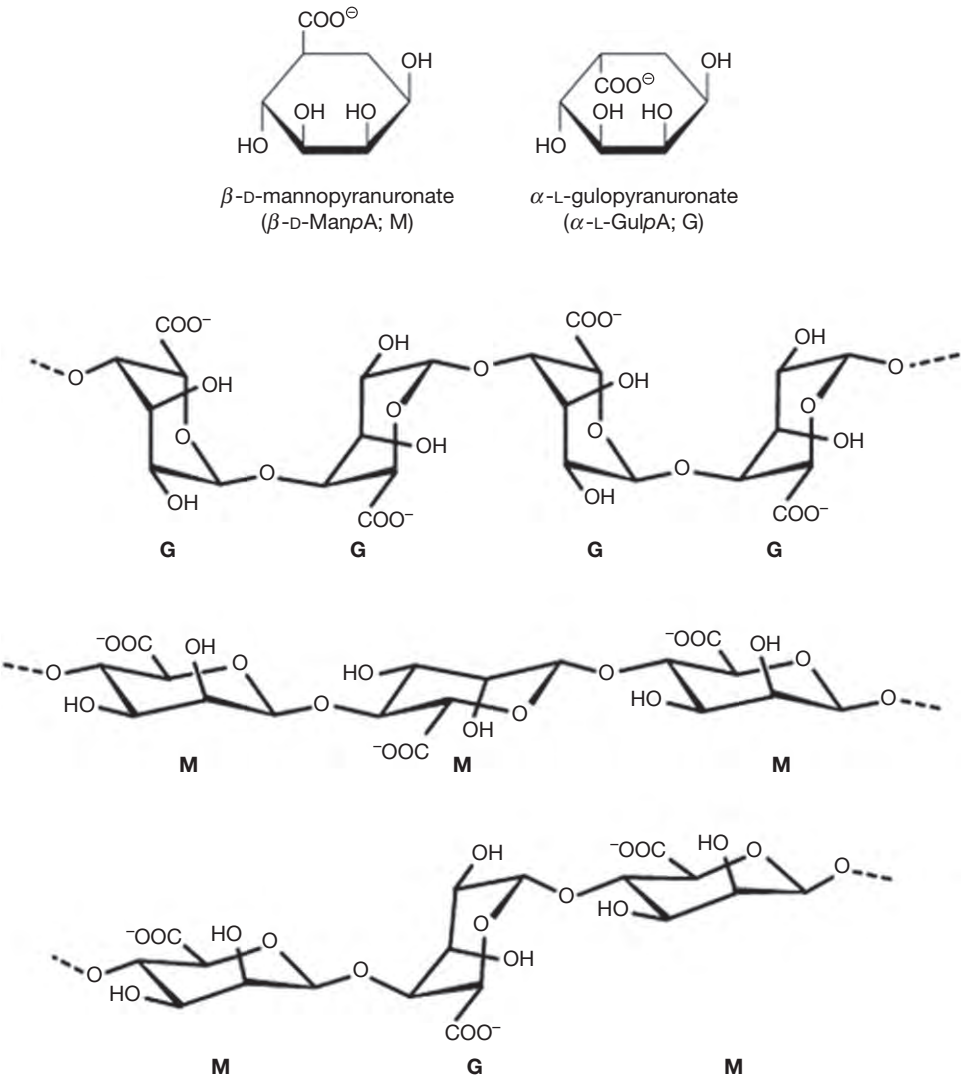


Fig. 11 Guluronic and mannuronic acid residues of alginates. Chemical structures of G-block, M-block, and alternating block in alginate (reproduced with permission from Lee, K., *et al.*, 2012. Progress in Polymer Science 37, 106–126).

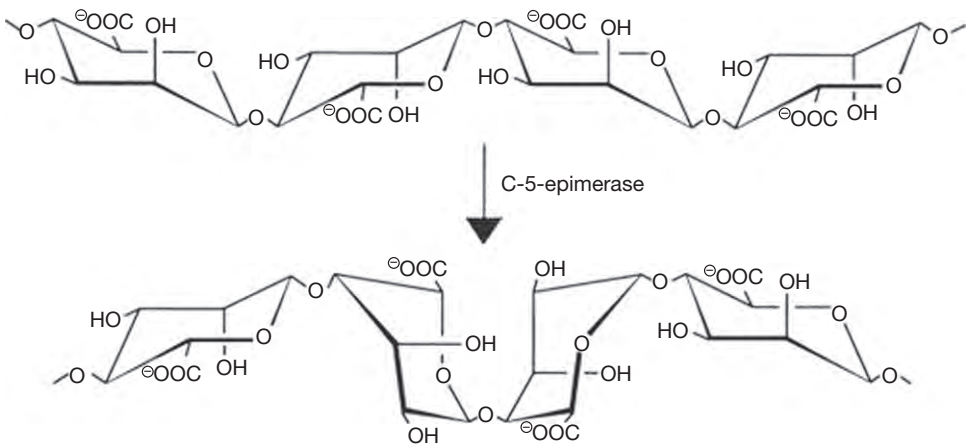


Fig. 12 Action of epimerases. Reproduced from Skjåk-Braek, G., Espevik, T., 1996. Application of alginate gels in biotechnology and biomedicine. Carbohydrates in Europe 14, 19–25, with permission from the Carbohydrate Research Foundation.

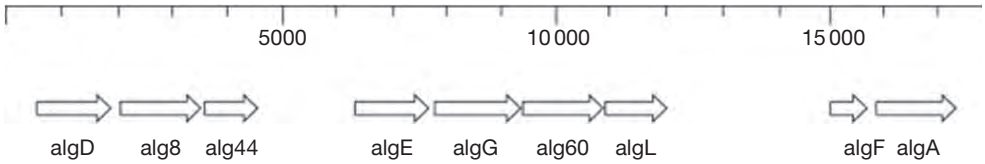


Fig. 13 Gene cluster organization encoding most of the alginate structural enzyme genes in *Pseudomonas aeruginosa*. Scale is in base pairs. Reproduced from Ertesvag, H., Valla, S., Skjak-Braek, G., 1996. Genetics and biosynthesis of alginates. Carbohydrates in Europe 14, 14–18 and May, T.B., Chakrabarty, A.M., 1994. *Pseudomonas aeruginosa*: Genes and enzymes of alginate synthesis. Trends in Microbiology 2, 151–157, with permission from the Carbohydrate Research Foundation.

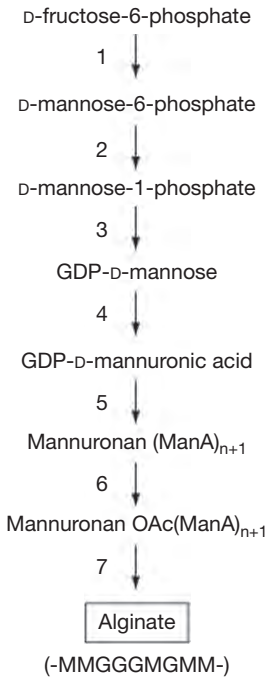


Fig. 14 Alginate biosynthesis in *Azotobacter vinelandii* and the principal enzymes involved. 1, hexokinase; 2, phosphomannose isomerise; 3, D-mannose-1-phosphate guanyl transferase; 4, guanosine phosphate mannose dehydrogenase; 5, transferase (n is the number of uronic acid residues in the polymer chain, i.e., the degree of polymerization); 6, acetyl transferase; 7, mannuronan C-5 epimerase. Reproduced with permission, from Pindar, D.F., Bucke, C., 1975. The biosynthesis of alginic acid by *Azotobacter vinelandii*. The Biochemical Journal 152, 617–622. © the Biochemical Society.

diabetes and chondrocyte transplantation for treatment of urinary incontinence and vesicoureteral reflux. A chemically modified alginate has also been widely used as a carrier to promote periodontal regeneration.

Bacterial Hyaluronic Acid

The commercial value of HA far exceeds that of other microbial extracellular polysaccharides. The HA industry is worth an estimated US$10 billion per year and medical grade HA sells at US$100,000 per kg. Potentially the most interesting of the glycosaminoglycans, this biopolymer is found in the joints, skin, vitreous humor of the eyes, and umbilical cord of vertebrates; it is also produced by *Staphylococcus* and some *Streptococci*. The HA layer may enable virulent stains of *Streptococci* to attack immune systems of higher organisms unrecognized.

Structure

HA is a linear polymer consisting of alternating β-(1→4) N-acetyl-D-glucosamine (GlcNAc) and β-(1→3) D-glucuronic acid (GlcUA), resulting in stiff chains due to strong interchain hydrogen bonding and water bridges between acetamido and carboxylate groups (Fig. 15). HA therefore adopts a conformation between that of a random coil and a rigid rod in dilute solution.

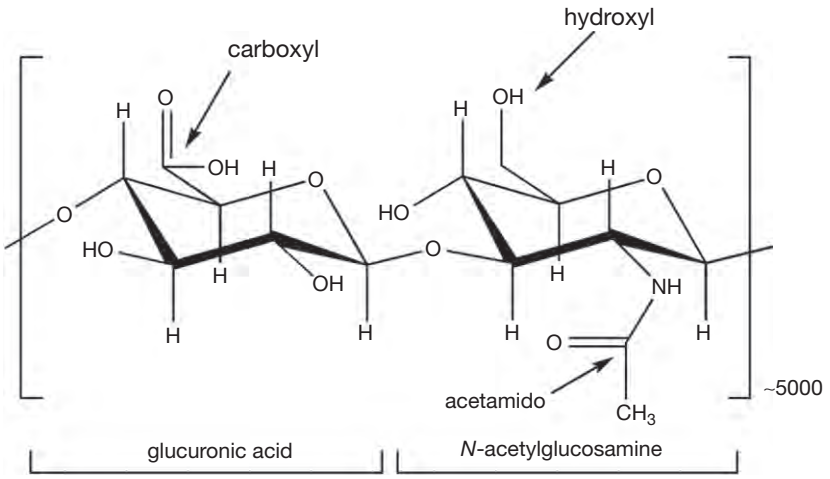


Fig. 15 Structure of disaccharide repeating unit of HA.

Extraction and Production

Industrial manufacturing of HA is based on two main processes, the extraction from animal tissues and microbial fermentation using bacterial strains. Both technologies produce polydisperse high molecular weight HA for biomedical and cosmetic applications. In the past decade a new technology emerged using isolated HA synthase to catalyze the polymerization of the UDP-sugar monomers (UDP: Uridine diphosphate). This novel enzymatic technology for HA synthesis is very versatile allowing producing both high M_W HA and HA oligosaccharides with defined chain length and low polydispersity. Production of monodisperse HA oligosaccharides on milligram-scale using two single-action mutants of the *Pasteurella multocida* HA synthase was demonstrated, but large scale production was not achieved yet. A comparative analysis of established and emerging technologies for high M_W HA production is listed in Table 2.

HA is commercially extracted from rooster combs or by microbial fermentation by *Streptococcus* bacteria (e.g., *Streptococcus zooepidemicus*). The fermentation medium typically includes yeast, glutamine, mineral salts (sodium chloride, potassium hydrogen phosphate, and magnesium sulfate), and glucose.

HA quality, in terms of productivity and molecular weight, has been studied under various conditions: temperature; pH; agitation speed; and aeration rate. These experiments demonstrated that under non-optimal growth conditions HA is produced in larger amounts and is of higher molecular weight. It should be noted that HA production greater that ~5–10 g L^{-1} is impractical due to the high viscosity of the medium.

Table 2 Comparison of technologies for production of HA.

Name	*Advantages*	*Disadvantages*
Extraction from animal materials	Well-established technology Available raw material at low costs Product with very high M_W up to 20 MDa Natural product	Variation in product quality Risk of polymer degradation Risk of contamination with protein, nucleic acids, and viruses Extensive purification needed Low yield
Microbial fermentation	Mature technology High yield Product with high M_W (1–4 MDa)	Use of genetically modified organism (GMO) Risk of contamination with bacterial endotoxins, proteins, nucleic acids, and heavy metals
Enzymatic synthesis	Versatile technology Control of the MW of the products. Products up to 0.55–2.5 MDa and also defined oligosaccharides can be obtained No risks of contamination Constant product quality	Emerging technology in development stage Technological and economic viability must still be demonstrated

Properties

Due to its high molar mass of $\sim 1–3 \times 10^6$ g mol^{-1} and semiflexible coil conformation, HA behaves as a non-Newtonian solution even at low concentrations and the critical coil overlap concentration c^* has been reported to be molecular weight dependent where $c^* \times Mw \approx 2800$ g^2 mL^{-1} mol^{-1} and therefore we enter the semidilute regime at approximately 1.5 g L^{-1} and the concentrated regime (independent of molecular weight) at >15 g L^{-1}. HA molecules of high molecular weight have also been shown to have mucoadhesive and anti-inflammatory effects and patients require less frequent injections of higher molecular weight material for arthritis treatment by viscosupplementation, which is important in patient's acceptance of a treatment.

Modification

Chemically modified HAs are available commercially, examples of which include HA benzyl esters (Fidia farmaceutici, Albano Terme, Italy) and divinyl sulfone cross-linked HA (Genzyme, Cambridge, United States). Internally cross-linked (or autocross-linked) HA molecules have also been prepared. HA nanofibers for wound healing are also commercially available (CPN, Dolní Dobrouč, Czech Republic). Modification in terms of molecular weight and molecular weight distributions has also been achieved by means of changes to the growth medium.

Genetic modification of the non-hyaluronic-acid-producing bacteria is an important development in bacterial HA production. The introduction of the *hasA* gene into *Escherichia coli* or *Bacillus subtilus* results in the production of HA and although there is no improvement in quality (in terms of molecular weight and yield) any possible contaminations by streptococcal exo- and endotoxins are eliminated and HA produced by *B. subtilus* has received GRAS (generally regarded as safe) status and should be released in the market in the near future.

Uses

HA is used extensively in the medical, cosmetic, and food industries and its applications have been reviewed recently. The most important industrial applications include the following (Table 3):

1. Viscosurgery – protects delicate tissues during surgical interventions, for example, ophthalmological surgery.
2. Viscosupplementation – supplements tissue fluids, for example, the replacement of synovial fluid in arthritis. Higher molecular weight material requires fewer injections.
3. Viscoaugmentation – used widely in cosmetic surgery to fill tissue spaces.
4. Viscoseparation – prevention of connective tissue surface adhesion after injury or surgery, thereby causing less scarring.
5. Drug delivery – HA microspheres for controlled drug delivery.

Although not strictly speaking HA, the high molecular weight HA-like bacterial exopolysaccharide from *Vibrio diabolicus*, a bacterium from deep-sea hydrothermal vents, shows great potential in bone healing.

Polysaccharide Vaccines

No survey of microbial polysaccharides would be complete without reference to the use of polysaccharides in the production of vaccines against serious diseases. Certain types of pathogenic bacteria such as from *Streptococcus pneumoniae*, *Neisseria meningitidis* (Meningococcus), and *Haemophilus influenzae* type B, besides producing harmful or dangerous toxins also produce high molecular weight CPSs, which themselves appear harmless. They do though help the bacterium establish an infection and help hide cell surface components from immune recognition and complement activation. Purified extracts of polysaccharides may themselves be

Table 3 Biomedical and pharmacological applications of HA

Area	*Application*	*Example*
Biomedical	Orthopedic surgery	Arthritis
	Rheumatology	Osteoarthritis
	Ophthalmology	Eye surgery
	Otolaryngology	Treatment of vocal fold
	Dermatology and plastic surgery	Dermal filler
	Wound healing and dressing	Diabetic ulcer, skin burns
	Tissue engineering	
Pharmacological	Drug delivery	

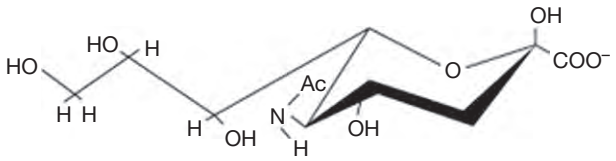


Fig. 16 N-acetyl neuraminic acid.

immunogenic and can be used at least in principle to produce immunity against the organism that is producing them. As a result, vaccines against some of these organisms are now available.

The polysaccharides themselves consist of repeat sequences of saccharide residues. One residue that appears frequently is N-acetyl neuraminic acid (Fig. 16), a type of 'sialic acid'.

This residue is common on the surface of membrane glycoproteins and also on mucosal surfaces, so considerable care has to be taken against possible autoimmunity problems. A more serious problem with polysaccharide vaccines is that their effects are not generally long lasting: the repeat sequences produce a T-cell-independent IgM rather than IgG response in infants with little immunological memory effect and are ineffective for infants <2 years. To counter this there has been a lot of recent work on the development of conjugate vaccines where the polysaccharides – or repeat sequences from them – are covalently attached via a linker to an appropriate protein carrier usually based on a bacterial toxoid from diphtheria or tetanus (Fig. 17).

Such conjugates have been successful in enhancing IgG antibody production and stimulating T-cell-dependent immunity against diseases such as meningitis. Two recent articles from the National Institute of Biological Standards in London provide an excellent description of current developments in this area.

In generating these vaccines important issues have to be addressed about the reproducibility of preparations with regard to not only antigenicity and chemical purity but also physical properties including molecular weight and molecular weight distribution limits, and in this regard the use of techniques – with correctly established operating procedures – like SEC-MALLs, analytical ultracentrifugation, viscometry for physical characterization, and NMR and GC mass spectroscopy for chemical characterizations will become increasingly important. This highlights the increasing difference in characterization requirements for a microbial polysaccharide for food use, such as xanthan, and those for biomedical pharmaceutical use, with vaccines as the outstanding example.

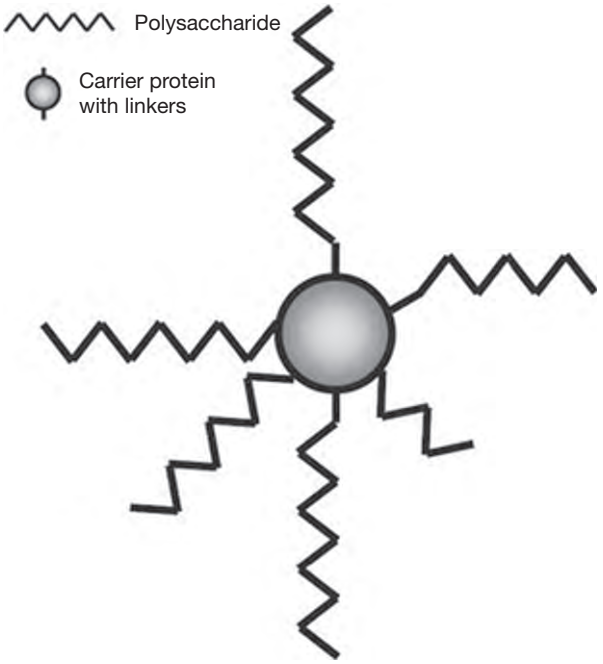


Fig. 17 Schematic conjugate vaccine showing capsular polysaccharide (CPS) fragments coupled to an appropriate carrier protein. Reproduced from Ward, J., Lieberman, J.M., Cochi, S.L., 1994. Haemophilus influenza vaccines. In: Plotkin, S.A., Mortimer, E.A. (Eds.), Vaccines, Philadelphia: Saunders & Co, pp. 337–386 with permission from W.B. Saunders & Co.

Conclusion

The microbial production of polysaccharides is providing a valuable and cost-effective supply of valuable materials for a wide range – potential wide range – of uses, including food and pharmaceuticals because of the absence of or virtually no known toxicity problems. The relative simplicity of bacterial genetics as compared to that for higher organisms renders the manipulation of the production system much easier as we have seen for alginates. Furthermore, the virtual lack of toxicity is providing hope in the development of effective conjugate vaccines against serious disease, provided, issues concerning autoimmunity and product reproducibility are properly taken care of.

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Relevant Website

http://www.surgery.org/ —TheAmerican Society for Aesthetic Plastic Surgery.

Posttranscriptional Regulation☆

TM Henkin, The Ohio State University, Columbus, OH, United States

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Glossary

Antiterminator RNA element that prevents the activity of an attenuator.
Attenuator Terminator located in the leader region of a transcriptional unit, used to regulate expression of downstream genes.
Autogenous regulation Regulation of a gene by the product encoded by that gene.
***cis*-Acting element** Element that affects only a region with which it is physically connected (e.g., a DNA site).
Coupling Coordination of cellular processes, for example, transcription and translation.
Leader peptide Short peptide encoded in the leader region of a transcriptional unit.
Leader region Segment of a gene between the transcription initiation site and the start of the coding sequence.
Leader RNA Segment of an RNA that is upstream of the first coding sequence.
Ribosome binding site (RBS) Translation initiation signal, comprised of SD plus start codon (usually AUG, GUG, or UUG).
Shine–Dalgarno sequence (SD) Binding site for the 30S ribosome on an mRNA for translation initiation.
Terminator Signal for RNA polymerase to stop transcription and release the DNA template and the nascent transcript.
***trans*-Acting factor** Factor or substance that can act at a distance within a cell (e.g., a protein or RNA that can diffuse to a different site).
Transcription elongation complex RNAP as it moves along the DNA template, after leaving the promoter site.

Abbreviations

5′ UTR	5′ Untranslated region
PNPase	Polynucleotide phosphorylase
RBS	Ribosome binding site
RF2	Release factor 2
RNAP	RNA polymerase
SAM	*S*-Adenosylmethionine
SD	Shine–Dalgarno sequence
sRNAs	Small regulatory RNAs
$tRNA^{Trp}$	Tryptophanyl-tRNA

Defining Statement

Posttranscriptional gene expression events that occur after RNA polymerase (RNAP) leaves its promoter site offer many opportunities for regulation in bacteria. This article reviews some of these mechanisms, including modulation of premature termination of transcription, mRNA degradation, and translation initiation, and also illustrates each class with specific examples.

Introduction

Regulation of gene expression at the level of transcription initiation is of obvious importance in all biological systems. Recognition of the promoter site on the DNA by RNA polymerase (RNAP), melting of the DNA, synthesis of the first few nucleotides of the transcript, and escape from the promoter site all represent important steps at which transcription initiation can be controlled. However, it has become increasingly obvious that events that occur after RNAP leaves its promoter site offer many additional opportunities for regulation, and mechanisms that affect these events are considered to be posttranscriptional regulatory events, although certain of these mechanisms affect transcript levels. Premature termination of transcription results in failure to synthesize the complete transcript. Modulation of transcript degradation, which can begin before the transcript has been fully synthesized, can have a major effect on expression of the encoded genes. The efficiency with which an mRNA is translated can dramatically affect the

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synthesis of the encoded protein product and can also affect the rate of transcript degradation. This article reviews mechanisms of gene regulation that occur after transcription initiation in bacteria. Specific examples are described to illustrate each class of event.

Regulation of Premature Termination of Transcription

One of the most common mechanisms of posttranscriptional gene regulation involves positioning of a transcription termination signal (or terminator) in the region between the promoter and the start of the regulated coding sequence. This region is commonly called the leader region in bacteria, and corresponds to the 5′ untranslated region (5′ UTR) in eukaryotic cells; because this region can be translated in bacteria to yield a peptide product, as described below, leader region is a more general term. Termination signals located within leader regions are called attenuators, as their role is to attenuate synthesis of the full-length transcript. Modulation of the activity of the terminator element in response to an appropriate physiological signal therefore can be used to determine the amount of full-length transcript made and the level of expression of the gene(s) that are downstream of the attenuation site. A wide variety of regulatory mechanisms of this type have been uncovered. Many of these mechanisms depend on effects on the folding of the nascent RNA transcript, while others affect the ability of the transcriptional machinery to recognize termination signals.

Termination Mechanisms

Two types of transcriptional terminators have been described in bacteria. Detailed biochemical analyses have been carried out primarily in *Escherichia coli*, but it appears that the basic features of terminators, and therefore their utilization as a regulatory target, are conserved in many groups of bacteria. Both types of termination events require features of the nascent RNA transcript that emerges from the elongating RNAP that the terminator is attempting to halt. The most common class of terminator elements are called intrinsic (or factor-independent) terminators. These termination signals are defined biochemically by their activity in a purified in vitro transcription system in the absence of any cellular factors other than RNAP. Intrinsic terminators are comprised of a G + C-rich RNA helix, usually 7–8 nt in length, immediately followed by a stretch of U residues in the nascent transcript. Positioning of RNAP so that the U residues form the RNA–DNA hybrid within the enzyme, and the RNA helix abuts the distal portion of the enzyme, results in destabilization of the transcription complex and termination. The role of the helix has been proposed to involve pushing forward on RNAP to remove the 3′ end of the transcript from the active site of the enzyme, while the U–A RNA–DNA hybrid is likely to assist in complex destabilization. Regulation of the activity of intrinsic terminators often occurs by alternate folding of the nascent RNA transcript, so that the region necessary for formation of the 5′ side of the terminator helix is instead sequestered in an alternate structure, termed an antiterminator. Intrinsic terminators can also be affected by increasing the processivity of RNAP, which reduces the sensitivity of the transcription complex to destabilization, at least in part because RNAP passes through the termination site before the RNA helix can exert its destabilizing effect.

The second class of bacterial termination signals are designated Rho-dependent (or factor-dependent) terminators, based on the requirement for an additional protein (Rho factor) for termination activity in vitro. Rho binds to the nascent RNA transcript, moves along the RNA until it encounters RNAP (usually at a pause site), and promotes destabilization of the transcription complex, probably by removal of the 3′ end of the transcript from the active site of the enzyme. Binding sites for Rho on the RNA, designated *rut* (Rho utilization) sites, are poorly conserved, but are generally unstructured, with overrepresentation of C and U residues and underrepresentation of G residues. Regulation of Rho-dependent termination usually occurs by sequestration of the *rut* site on the RNA to prevent Rho binding or by modification of the transcription complex to increase processivity of RNAP, interfering with the ability of Rho to reach the transcription elongation complex.

Regulation of Termination by Modulation of Nascent Transcript Structure

As noted above, the activity of intrinsic terminators is dependent on folding of the nascent transcript as it emerges from the RNA exit channel of RNAP into a helix that plays a crucial role in destabilization of the transcription elongation complex. Formation of the helix therefore represents an effective target for regulation of termination activity. A variety of factors can interact with the nascent RNA to determine whether it folds into the terminator helix, in most cases by determining whether the terminator helix or a competing antiterminator helix is formed. The relative stabilities of the terminator and antiterminator helices play a major role in determining the sensitivity of the RNA to the interacting factors, and whether binding of the factor results in attenuation of transcription or increased synthesis of the full-length transcript. Interacting factors that have been demonstrated to affect leader region terminator activities include translating ribosomes, RNA-binding proteins, *trans*-acting RNAs, and small molecules.

Leader peptide attenuation systems

The classic model for regulation of nascent transcript structure to control termination involves the presence of a short peptide coding sequence within the 5′ region of the transcript. The processivity of the ribosome during translation of this peptide coding sequence modulates the structure of the RNA, therefore affecting both pausing of the transcription elongation complex from which this nascent RNA is emerging, and the folding of this transcript into competing terminator and antiterminator elements. Processivity of the ribosome can in turn be affected by the sequence of the nascent peptide itself. For example, in systems like the *E. coli trp* operon, which encodes gene products involved in tryptophan biosynthesis, the peptide coding sequence includes tandem tryptophan

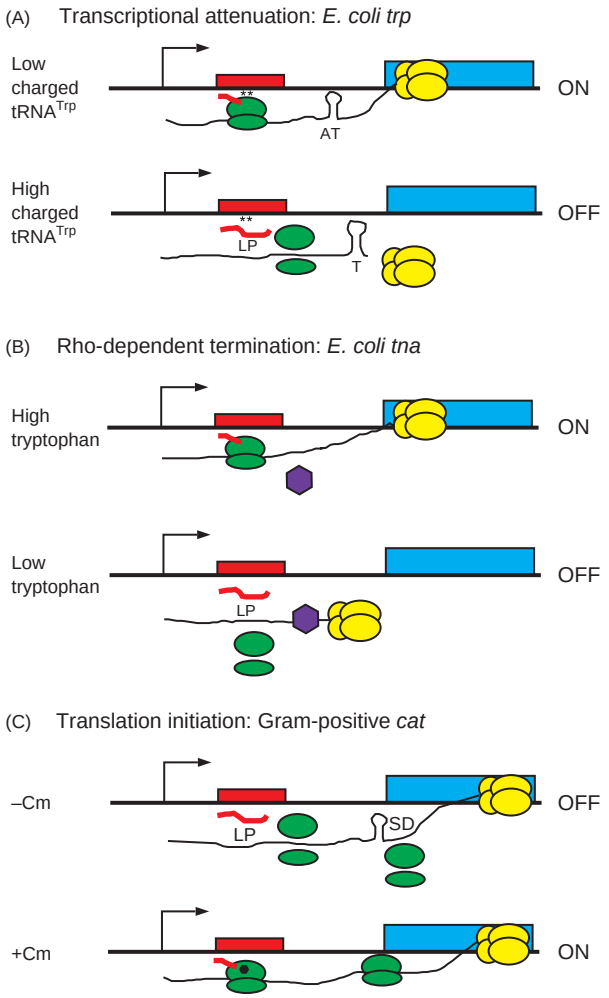

Fig. 1 Leader peptide-mediated effects on gene expression. (A) Transcriptional attenuation in the *E. coli trp* operon. Translation of the leader peptide coding sequence (*red* box), which includes two tandem tryptophan codons (**), results in stalling of the ribosome when availability of charged $tRNA^{Trp}$ is low; this allows the nascent RNA to form into an antiterminator structure (AT) that permits RNAP (*yellow* ovals) to proceed through the attenuator site, and transcribe the downstream gene (*blue* box). High availability of charged $tRNA^{Trp}$ causes efficient leader peptide (LP, *red* line) translation, and the movement of the ribosome (*green* ovals) causes folding of the leader RNA into the terminator helix (T); this prevents synthesis of the full-length transcript, and expression of the downstream genes is repressed. (B) Regulation of Rho-dependent transcription termination in the *E. coli tna* operon. The ribosome translating the leader peptide coding sequence stalls in the presence of high tryptophan, preventing binding of Rho (*purple* hexagon) to the nascent RNA; this results in expression of the downstream genes. When tryptophan is low, the ribosome completes synthesis of the leader peptide (*red* line) and releases the RNA, which allows access of Rho and Rho-dependent termination. (C) Chloramphenicol-dependent translational control of the *cat* gene. In the absence of chloramphenicol, the leader peptide coding sequence is efficiently translated, and the SD sequence of the downstream cat gene is sequestered in an inhibitory structure. Low concentrations of chloramphenicol cause the ribosome translating the leader peptide to stall, which unfolds the mRNA structure and allows access of a second ribosome to the SD of the downstream gene.

codons (Fig. 1A). The presence of these tryptophan codons renders translation of the leader peptide coding region sensitive to the availability of charged tryptophanyl-tRNA ($tRNA^{Trp}$). Reduced abundance of tryptophan results in a reduction of charged $tRNA^{Trp}$, causing the translating ribosome to stall as it attempts to translate the tandem tryptophan codons. The stalled ribosome allows the unoccupied RNA ahead of it to fold into an antiterminator element, which prevents formation of the more stable terminator helix, therefore resulting in readthrough of the leader region transcription terminator site (or attenuator), and synthesis of the full-length transcript. The presence of a large pool of charged $tRNA^{Trp}$ results in efficient translation of the leader peptide coding sequence. Rapid progress of the ribosome through the leader peptide coding region prevents formation of the antiterminator, and allows formation of the terminator helix and attenuation of transcription. Synthesis of the full-length transcript, and therefore expression of the tryptophan biosynthesis genes, therefore occurs only when cells are limited for charged $tRNA^{Trp}$, which signals a requirement for increased tryptophan biosynthetic activity. This type of mechanism is easily converted to allow recognition of a different charged tRNA by changing the sequence of the leader peptide itself, so that the tryptophan codons in the nascent RNA are replaced with clusters of codons specifying a new amino acid (e.g., the leader peptide of the histidine biosynthetic operon contains multiple histidine codons).

This type of transcription termination control mechanism is often superimposed on a second level of regulation. For example, initiation of transcription of the *E. coli trp* operon is regulated by the TrpR DNA-binding protein, which blocks binding of RNAP to the promoter region and represses transcription initiation when tryptophan levels are high. The combination of the two regulatory mechanisms allows sensing of both free tryptophan (by TrpR) and charged $tRNA^{Trp}$ (by the ribosome that is translating the leader peptide coding sequence). This results in a highly sensitive regulatory response that prevents wasteful expression of *trp* operon genes when tryptophan is abundant and also ensures an adequate supply of charged $tRNA^{Trp}$ for efficient protein synthesis.

Leader peptide transcription attenuation mechanisms are dependent on the tight coupling of transcription and translation in bacteria, where binding of the 30S ribosomal subunit to an RNA transcript can occur as soon as the RBS emerges from the transcription elongation complex. Translation of the leader peptide coding sequence must be coordinated with pausing of RNAP during synthesis of the leader region of the transcript to allow a response to ribosome positioning before RNAP escapes from the attenuator region or terminates transcription. In the *E. coli trp* system, a specific signal in the leader RNA causes RNAP to pause, which allows translation of the leader peptide to initiate; the translating ribosome releases RNAP from the pause site, so that transcription and translation now proceed in tandem unless the ribosome is stalled by low abundance of charged $tRNA^{Trp}$.

Coupling of transcription and translation is also crucial to the *E. coli pyrB1* attenuation mechanism. This operon encodes products involved in biosynthesis of pyrimidine nucleotides, and direct sensing of NTP abundance by the transcription elongation complex is used to repress expression when pools of these nucleotides are high. Pausing of RNAP during transcription of segments of the leader region containing runs of C and U residues is triggered by limitation for pyrimidine nucleotides. This pause allows time for initiation of translation of a leader peptide coding sequence, and positioning of the translating ribosome on the nascent RNA prevents formation of the terminator helix. When pyrimidine nucleotides are abundant, rapid progress of RNAP through the pause sites before the ribosome can bind and occlude the terminator allows termination, and repression of *pyr* operon expression, to occur. As in the *E. coli trp* system, the position of a translating ribosome relative to the transcribing RNAP determines the structure of the nascent RNA, and therefore regulates whether transcription is attenuated within the leader region. In the *pyrB1* system, RNAP serves as the molecular sensor for the effector molecule (pyrimidine nucleotides), whereas in the *E. coli trp* system, the translating ribosome senses charged $tRNA^{Trp}$; in both cases, the molecular sensors directly monitor their biochemical substrates while carrying out their normal biological reactions.

Regulation of RNA structure by RNA-binding proteins

A number of systems have been described in which binding of a regulatory protein to a leader RNA affects formation of an intrinsic terminator. These proteins can act negatively to stimulate termination (i.e., the terminator is inactive in the absence of the protein, binding of which promotes termination) or positively to increase readthrough of the attenuation site (i.e., the terminator is active in the absence of the protein, binding of which prevents termination and allows synthesis of the full-length transcript). The best characterized system in which a protein promotes terminator formation is the *Bacillus subtilis trp* operon, which encodes gene products involved in tryptophan biosynthesis (Fig. 2A). The *trp* leader RNA terminator helix is relatively unstable, and its formation is prevented by folding of the RNA into the more stable antiterminator structure, which includes residues necessary for terminator helix formation. Binding of the regulatory protein, designated TRAP, sequesters a region of the RNA that participates in formation of the antiterminator element and allows the less stable terminator helix to form, which causes termination. The RNA-binding activity of TRAP requires association with tryptophan, which serves as a corepressor. Termination therefore occurs when tryptophan is abundant, as described above for the *E. coli trp* operon, but in this case the molecular mechanism for sensing tryptophan availability involves binding of tryptophan to the regulatory protein rather than monitoring of charged $tRNA^{Trp}$ during leader peptide translation. Charging of $tRNA^{Trp}$ is sensed indirectly in *B. subtilis* through a second regulatory protein, designated anti-TRAP, which is expressed in response to a decrease in charged $tRNA^{Trp}$ (via the T-box mechanism; see below) and antagonizes TRAP activity, allowing increased *trp* operon expression. This dual measurement of both free tryptophan and charged $tRNA^{Trp}$ is similar to that observed in *E. coli*, although different molecular mechanisms for sensing the effector molecules are employed.

Whereas the *E. coli trp* and *pyr* operons use leader peptide attenuation regulatory mechanisms, the *B. subtilis trp* and *pyr* operons both use an RNA-binding protein to control transcription termination. The *B. subtilis pyr* system is similar to the TRAP system, but has an added complexity in that the PyrR regulatory protein (in the presence of pyrimidine nucleotides as corepressor) binds to and stabilizes a leader RNA element that sequesters sequences necessary for formation of the antiterminator. Binding of PyrR prevents antiterminator formation and allows the less stable terminator helix to form. The PyrR-binding site therefore serves as an "anti-antiterminator element," because it antagonizes the antiterminator element. This differs from the TRAP system, in that TRAP directly sequesters sequences involved in formation of the antiterminator without stabilizing an alternate RNA element. The reliance of both the *E. coli trp* and *pyrB* mechanisms on coupling of transcription and translation, while the analogous mechanisms in *B. subtilis* instead use an RNA-binding protein to modulate expression, may reflect differences in the efficiency of coordinating transcription and translation in these two organisms (and by extension, their close relatives).

RNA-binding proteins can also promote antitermination. Systems of this type utilize leader RNAs in which the terminator helix is more stable than the competing antiterminator, so that termination is the default state that occurs in the absence of protein binding. Binding of the protein to the RNA stabilizes the antiterminator element, which sequesters sequences that would otherwise participate in formation of the terminator helix. An example of this type of system is provided by the *E. coli bgl* operon, which is involved in utilization of β-glucoside sugars (Fig. 2B). The BglG protein acts as the regulatory protein, and its

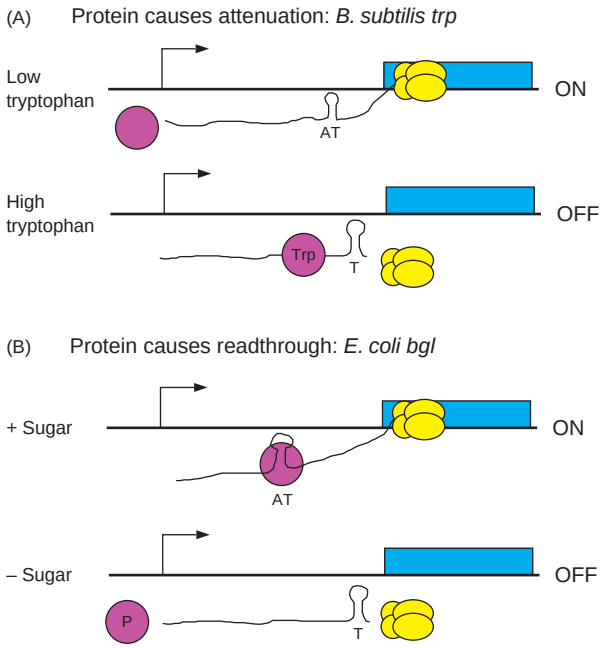

Fig. 2 Regulation of transcription attenuation by RNA-binding proteins. (A) Protein-dependent attenuation in the *B. subtilis trp* operon. When tryptophan levels are low, the *trp* operon leader RNA folds into an antiterminator element (AT) that allows RNAP (*yellow* ovals) to proceed past the attenuation site and transcribe the downstream gene (*blue* box). High tryptophan allows TRAP protein–tryptophan complex (*pink* circle) to bind to the leader RNA; TRAP sequesters sequences necessary for antiterminator formation, which allows formation of the terminator helix (T) and repression of downstream gene expression. (B) Protein-dependent antitermination in the *E. coli bgl* operon. In the presence of the substrate sugar, the BglG RNA-binding protein (*pink* circle) binds to and stabilizes the antiterminator element (AT), which allows transcription to continue past the termination site. In the absence of the substrate sugar, BglG protein is phosphorylated (P) and unable to bind the RNA; the terminator helix (T) forms, and transcription of the downstream gene is repressed.

RNA-binding activity is induced when the substrate sugar is present. The RNA target of the BglG protein includes residues necessary for formation of the terminator helix, so that BglG binding prevents termination and allows expression of downstream genes involved in sugar utilization. Unlike the TRAP or PyrR proteins, BglG does not directly bind the effector molecule but is instead dephosphorylated by BglF, the transporter for the sugar substrate, when the sugar is present. Dephosphorylation of BglG by BglF allows BglG to dimerize, which is required for RNA-binding activity. BglF therefore serves as the sensor of the effector molecule, and transmits information about effector availability to BglG via phosphorylation, in a manner analogous to that used by two-component regulatory systems in which phosphotransfer between a sensor kinase and a response regulator allows transmission of a regulatory signal from one protein to another. Several sugar utilization operons in *B. subtilis* are regulated by mechanisms similar to the *E. coli bgl* system.

Control of termination by RNA–RNA interactions

The ability of RNA molecules to base-pair with other RNA molecules provides another mechanism by which the structure of a leader transcript can be modulated, and RNA–RNA interactions have been exploited in a variety of regulatory systems. While most *trans*-acting regulatory RNAs in bacteria affect translation or stability of the target mRNA, binding of a *trans*-acting RNA to the nascent RNA can also affect the ability of the targeted region of the transcript to fold into terminator or antiterminator structures, thereby affecting attenuation or readthrough.

The best-characterized example of this type of mechanism is the T-box system, which is widely used in Firmicutes to control genes involved in amino acid metabolism, including aminoacyl-tRNA synthetase, amino acid biosynthesis, and transporter genes; as noted above, this mechanism also regulates synthesis of the anti-TRAP regulatory protein. Genes regulated by the T-box mechanism exhibit a complex set of conserved structural and primary sequence elements in their leader regions, which include a terminator and competing antiterminator. Embedded at a specific position within this array of conserved elements is a single codon that matches the anticodon of a tRNA that belongs to the amino acid class relevant to the gene product encoded in the coding sequence(s) located downstream of the terminator. For example, the *B. subtilis tyrS* gene, encoding tyrosyl-tRNA synthetase, contains a UAC tyrosine codon at the appropriate position. Binding of the matching uncharged tRNA (e.g., $tRNA^{Tyr}$ for *tyrS*) to the leader RNA, which is determined by base-pairing of the leader region codon with the anticodon of the tRNA, is required to stabilize the antiterminator element by pairing of the four unpaired residues at the 3′ end of the tRNA with residues located in a bulge within the antiterminator (Fig. 3A). The interaction of the appropriate uncharged tRNA with the nascent leader

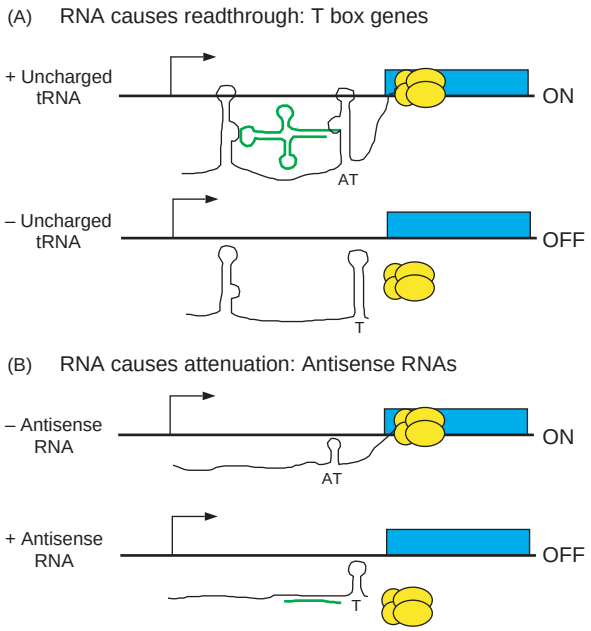

Fig. 3 Regulation of transcription attenuation by *trans*-acting RNAs. (A) tRNA-dependent antitermination in the T-box system. When levels of a specific uncharged tRNA are high, the tRNA (*green* cloverleaf) binds to the nascent transcript of the gene that senses that tRNA; binding of the tRNA stabilizes an antiterminator element (AT), which allows RNAP (*yellow* ovals) to transcribe the downstream gene (*blue* box). When levels of the specific uncharged tRNA are low (i.e., the tRNA is predominantly in the charged state), the leader RNA folds into the terminator helix (T), and transcription of the downstream gene is repressed. (B) RNA-dependent attenuation by antisense RNAs. In the absence of the antisense RNA, an antiterminator element (AT) forms, and RNAP proceeds through the attenuator site and transcribes the downstream gene. Binding of the antisense RNA (*green* line) sequesters sequences necessary for antiterminator formation, and causes formation of the terminator helix (T) and repression of downstream gene expression.

transcript therefore prevents formation of the terminator helix, and results in increased transcription of the downstream genes. The cognate charged tRNA (e.g., $tRNA^{Tyr}$ aminoacylated with tyrosine) can also interact with the codon region, but is unable to stabilize the antiterminator because of the presence of the amino acid at the 3′ end of the tRNA. Codon–anticodon pairing is therefore responsible for selection of a particular tRNA species as the effector for a particular transcriptional unit, and pairing between the tRNA acceptor end and the antiterminator is responsible for discrimination between the uncharged and charged forms of that tRNA. The ability of the cognate charged tRNA to interact with the codon but not the antiterminator results in inhibition of binding of the effector uncharged tRNA, so that the true physiological signal is the relative amounts of uncharged and charged species of a specific tRNA. The cell therefore modulates expression of genes involved in converting a particular tRNA class from its uncharged to its charged state, in response to the substrate–product ratio.

The tRNA–leader RNA interaction and tRNA-directed antitermination have been demonstrated in vitro using purified components, which indicates that no factors other than the leader RNA itself are necessary for specific recognition of the cognate uncharged tRNA. The T-box system therefore operates as a riboswitch, in which binding of a specific ligand results in an RNA structural change that regulates expression of a downstream gene (see below). The T-box riboswitch, like systems that utilize leader peptide translation (e.g., *E. coli trp*), monitors the charging of a specific tRNA class to control leader RNA structure; however, while leader peptide transcription attenuation systems monitor only availability of the appropriate charged tRNA, by using a translating ribosome, the T-box system monitors both charged and uncharged tRNA charging, by using direct binding of the tRNA to the nascent RNA transcript.

Several systems have been described in which binding of a *trans*-acting small RNA is proposed to modulate leader RNA structure to promote transcription termination. These systems, which have been analyzed primarily in plasmids, utilize *cis*-encoded antisense RNAs that bind to their target transcripts to, in some way, facilitate formation of the terminator helix, usually by destabilization of a competing antiterminator element (Fig. 3B). Mechanisms of this type often utilize more extensive base-pairing interactions between the regulatory RNA and its target. This role of small RNAs, while so far applicable only to *cis*-encoded RNAs, adds to the diversity of regulatory mechanisms that noncoding RNAs can affect in bacteria (see below).

Metabolite-binding riboswitch RNAs

A number of systems have recently been described in which binding of a small molecule to the nascent RNA transcript results in an RNA structural rearrangement that determines whether the transcript folds into an intrinsic terminator helix or a competing antiterminator structure. Like the T-box system described above, systems of this type have been called riboswitches. In most of these systems, the leader RNA includes an element that serves as an effector-binding "aptamer" domain, which in isolation can

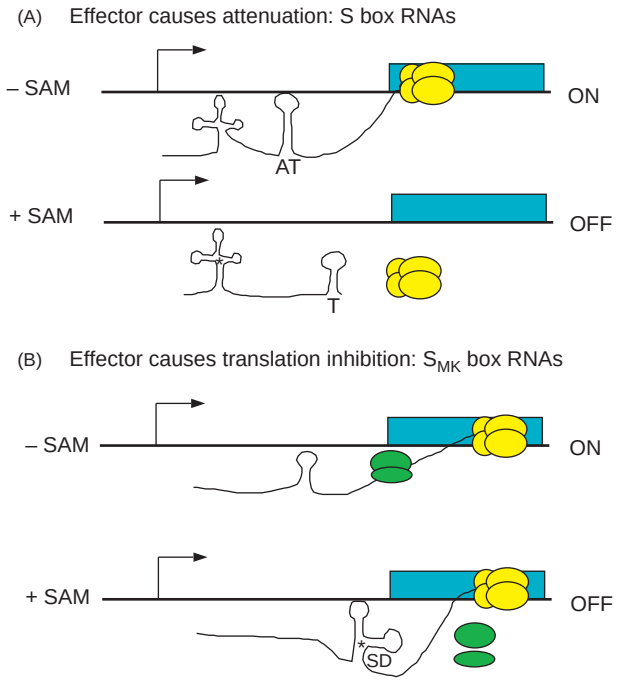

Fig. 4 Regulation of transcription attenuation or translation initiation by riboswitch RNAs. (A) SAM-dependent transcription attenuation in the S-box RNAs. In the absence of SAM, the antiterminator element (AT) forms in the leader RNA, allowing RNAP (*yellow* ovals) to proceed through the termination site and transcribe the downstream gene (*blue* box). In the presence of SAM (*), an RNA structural rearrangement causes formation of the terminator helix (T), resulting in attenuation of transcription. (B) SAM-dependent inhibition of translation initiation in the S_{MK}-box RNAs. In the absence of SAM, the SD region of the transcript is available for binding of the ribosome (*green* ovals), and translation of the downstream gene occurs. Binding of SAM (*) to the leader RNA causes a structural rearrangement that results in sequestration of the SD region, preventing ribosome binding and downstream gene expression.

specifically recognize the cognate ligand. Binding of the ligand usually sequesters a segment of RNA that would otherwise participate in forming the antiterminator element (Fig. 4A). The ligand-binding domain therefore serves as an anti-antiterminator that prevents formation of the antiterminator, and therefore allows termination. Metabolite-binding riboswitches are generally used to regulate genes involved in uptake or biosynthesis of the signal molecule, and represent a type of feedback repression, where accumulation of high amounts of the ligand represses expression of genes that encode proteins involved in increasing the intracellular concentration of that ligand. For example, *S*-adenosyl methionine (SAM) binds to the S-box riboswitch to repress transcription of genes involved in biosynthesis and uptake of SAM and methionine (which is a substrate for SAM synthesis). A few systems show an opposite arrangement, whereby binding of the ligand results in formation of the antiterminator, and promotes synthesis of the full-length transcript; in systems of this type, the ligand generally is a substrate of the regulated pathway. The metabolite binding riboswitch RNAs exhibit highly specific recognition of their cognate effector molecules, which include vitamins, cofactors, nucleotides, amino acids, and metal ions, as well as an affinity for their target molecule appropriate to the physiological concentration of the effector. As in the T-box system, the effector-dependent RNA conformational change and transcription termination response can be reproduced in vitro in the absence of other cellular factors, indicating that the RNA transcript encodes all features necessary for the regulatory mechanism.

Reiterative transcription

As noted above in reference to the *E. coli pyrB1* operon, NTP abundance can affect the processivity of RNAP. The *B. subtilis pyrG* gene represents a system in which limitation for an NTP not only affects pausing but also results in nontemplated addition of residues to the transcript, in a process termed reiterative transcription. In this system, the transcript initiates with three G residues, followed by a C residue. When CTP is abundant, transcription proceeds through this region, and terminates at an intrinsic terminator prior to the start of the *pyrG* coding region. Limitation for CTP results in stalling of RNAP during synthesis of the 5′ region of the transcript, and RNAP stalling causes slippage relative to the template DNA and incorporation of a series of extra G residues into the transcript. RNAP eventually escapes from this site and continues transcription into the leader region. The resulting transcript now contains a longer run of G residues that are capable of pairing with C and U residues normally found on the 5′ side of the terminator helix. This pairing causes formation of an antiterminator element that is not directly encoded in the DNA template, and arises only from the reiterative transcription event. Reiterative addition of G residues, triggered by starvation of RNAP for CTP, therefore promotes synthesis of the full-length transcript, and *pyrG* expression, when cells are limited for pyrimidine nucleotides. This mechanism, like that of the *E. coli pyrB* operon, utilizes the transcription

complex itself to monitor availability of its NTP substrates, but the mechanistic consequences of specific NTP limitation differ in the two systems.

Regulation of Termination by Modulation of Transcription Complex Activity

All the systems described above involve changes in the structure of the nascent transcript, which arise as the result of interaction of other factors with the RNA, or through changes in the RNA sequence that occur during transcription. Another major class of transcription termination control systems does not utilize structural changes in the nascent RNA but instead involves changes in the behavior of the transcription machinery itself. These systems can modulate the processivity of RNAP or the ability of the termination machinery (i.e., Rho factor) to access the transcription elongation complex.

Protein-dependent changes in transcription complex processivity

The classic example of a system in which binding of a protein to a nascent transcript causes the transcription elongation complex to ignore downstream termination sites is provided by the bacteriophage lambda N-mediated antitermination mechanism. The phage-encoded N protein is responsible for the transition between the earliest stage of phage gene expression (during which only N and another regulatory protein, Cro, are synthesized) and the next stage of gene expression. N binds to specific sites (designated *nut* sites, for N utilization) on transcripts initiating at both the leftward (p_L) and rightward (p_R) major promoters. Once bound to these sites, N nucleates assembly of a complex of host-encoded proteins (the Nus factors, for N usage substance) that interact with RNAP (Fig. 5A). NusA protein binds first, and is sufficient for antitermination at sites close to the *nut* site; formation of a stable complex requires the addition of the remaining factors (NusB, NusG, and ribosomal protein S10). Assembly of the complete complex results in a more processive transcription elongation complex with reduced pausing and reduced termination activity at both Rho-dependent and intrinsic terminators, and this complex remains intact during transcription of long segments of DNA distal to the modification site. This effect has been termed processive antitermination because it allows readthrough of multiple termination sites that are encountered in succession as the transcription complex moves along the DNA.

Bacteriophage lambda uses a second protein-dependent antitermination mechanism to promote the transition to late gene expression, mediated by the phage-encoded Q protein. In contrast to N, Q is a DNA-binding protein that interacts with the nontemplate strand of the DNA in a transcription complex paused just downstream from the late gene promoter. Q-directed antitermination also differs from the N system in that only a single host-encoded factor, the NusA protein, is required. Both the N and the Q systems are conserved in related lambdoid phage.

The lambda N antitermination system also serves as the target of a protein-directed termination system, in which a related phage, designated HK022, encodes a protein (Nun) that is related to N but interferes with N-mediated antitermination, causing failure of the N system and abrogation of the lambda lytic cycle. Nun binds to the *nut* sites on the nascent lambda transcripts and, in complex with the same Nus factors that are utilized by N to promote antitermination, instead promotes transcriptional arrest and termination. It is important to note that Nun does not inhibit N-mediated antitermination only by competing with N for binding to *nut* sites, but instead directly promotes termination at sites immediately downstream from the *nut* sites even in the absence of N. The biological role of the Nun system appears to be to promote HK022 propagation in cells coinfected with lambda, and HK022 utilizes a different class of antitermination mechanism to carry out its own life cycle (see below).

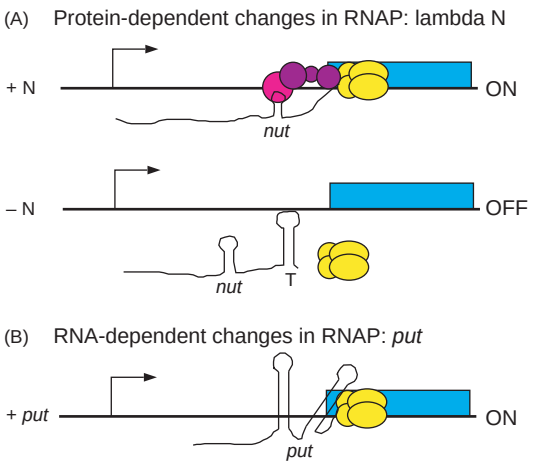

Fig. 5 Changes in processivity of the transcription elongation complex. (A) In the bacteriophage lambda N system, N protein (*pink* circle) binds to the *nut* site on the nascent transcript and recruits additional factors (*purple* circles) that together modify RNAP (*yellow* ovals) to a complex that is resistant to transcription termination signals; this allows transcription of downstream genes (*blue* box). In the absence of N, the *nut* site is unoccupied and RNAP stops at termination signals that prevent downstream gene expression. (B) Bacteriophage HK022 uses the *cis*-acting *put* RNA element to modulate the processivity of RNAP, which results in readthrough of termination sites and expression of downstream genes. As the *put* site is always present in the RNA, its antitermination activity appears to be constitutive.

A different mode of protein-dependent modulation of the transcription elongation complex is represented by the RfaH system, which is found in a number of enteric bacteria. RfaH protein interacts with the nontemplate strand of the DNA on the surface of a transcription elongation complex paused at specific *cis*-acting sequences, designated *ops* sites. RfaH, a structural homolog of NusG, remains stably associated with the transcription elongation complex, and increases its processivity in a manner similar to the N and Q systems. This results in readthrough of potential termination sites in the downstream DNA, and thereby stimulates synthesis of longer transcripts and expression of the downstream coding sequences. The RfaH system differs from the phage-encoded systems in that no cellular factors other than RfaH are required. RfaH-dependent antitermination is important for a number of processes involved in virulence in *E. coli* and its relatives.

RNA-dependent changes in transcription complex processivity

As noted above, phage HK022 utilizes the Nun protein not to mediate antitermination during expression of its own genome, but rather to prevent N-mediated antitermination by a coinfecting lambda phage. As for other lambdoid phage, antitermination is required for HK022 to advance into the lytic cycle, but in this case *cis*-encoded elements within the nascent transcript are responsible for readthrough of the early termination sites. Transcription originates from two divergent promoters (as described for lambda), and RNA elements (designated *put* sites) located downstream from the two promoters act in *cis* to promote readthrough of downstream termination sites and allow expression of genes located distal to the terminators (Fig. 5B). The *put* sites are each comprised of two stem-loop elements, both of which are required for antitermination activity. These RNA elements are sufficient to promote antitermination in the absence of any HK022-encoded protein factors, and no host-encoded factors are required, in contrast to the lambda system. The *put*-encoded RNA elements interact directly with the β′-subunit of RNAP, and specific mutations in β′ block *put*-mediated antitermination (and HK022 growth) without obvious effects on *E. coli* transcription or lambda growth. HK022 *put*-dependent antitermination appears to be constitutively active, as the *put* sites are always present in the nascent transcripts; this differs from the lambda N system, which allows regulation of downstream gene expression in response to N availability. Although the detailed mechanism of *put*-mediated antitermination remains to be elucidated, it appears that its function is similar to that of N protein in that it promotes both reduced pausing by the transcription elongation complex and processive antitermination.

Interference with Rho binding

Rho protein is the key factor responsible for mediating Rho-dependent transcription termination, and the requirement for interaction of Rho with the nascent transcript at *rut* sites provides a target for regulation. Binding of other factors to the transcript can affect the ability of Rho to access its binding sites on the RNA. The clearest example of a regulatory mechanism of this type is provided by the *E. coli tna* operon, encoding tryptophanase, which is involved in the utilization of tryptophan as a source of carbon and nitrogen. The tryptophanase coding region is preceded by a leader region that includes both a Rho-dependent termination site and a short peptide coding region (Fig. 1B). When tryptophan levels are low (conditions under which *tna* operon expression is repressed), translation of the leader peptide coding sequence proceeds normally, and the ribosome and nascent peptide are released. Under these conditions, the Rho-dependent terminator in the leader region is active, and transcription is attenuated. When tryptophan levels are high, signaling a need for *tna* operon expression, a ribosome translating the *tnaC* region in the nascent transcript stalls and occludes the *rut* site within the leader region; Rho binding is prevented, and synthesis of the full-length transcript occurs, which allows production of tryptophanase. Tryptophan-dependent stalling of the ribosome during *tnaC* translation depends on specific features of the *tnaC*-encoded peptide, which in the presence of free tryptophan interacts in *cis* within the exit channel of the translating ribosome to inhibit peptidyl transferase activity, thereby preventing translocation. The requirement for free tryptophan to promote stalling of the ribosome–nascent peptide complex provides the sensitivity to tryptophan concentration necessary for an appropriate physiological response, since degradation of tryptophan is advantageous only when tryptophan is highly abundant.

Recent studies have demonstrated that metabolite-binding riboswitches, which usually operate by effects on intrinsic terminators or translation initiation, also can affect Rho-dependent termination. An example is provided by the *E. coli ribB* operon, which is involved in riboflavin biosynthesis and is repressed by flavinmononucleotide (FMN). Regulation was proposed to occur via sequestration of the SD region of the transcript; however, in vitro studies revealed that FMN stimulates the activity of a terminator, only in the presence of Rho protein. The importance of Rho in this regulatory mechanism is supported by the reduction of repression (and termination) in vivo in the presence of bicyclomycin, a Rho inhibitor. The mechanism by which FMN binding and the resulting RNA structural rearrangement interferes with Rho activity remains to be determined.

Rho-dependent termination can also be regulated by the abundance of Rho protein itself. This sensitivity to Rho availability is utilized in autogenous regulation of *rho* gene expression. The *rho* gene contains several Rho-dependent termination sites, and synthesis of the full-length transcript is repressed when the cellular concentration of Rho protein is high. This represents a classical autorepression response that makes use of the biological function of the regulated protein to control its own synthesis.

Effects of Premature Termination on Upstream Gene Expression

While termination in a leader region is normally considered to control expression of coding sequences located downstream of the termination site, by determining whether the transcript includes those downstream regions, it is also possible for termination mechanisms to affect expression of genes located upstream of the termination site, in a process termed retroregulation. The classical example of this type of effect is observed in bacteriophage lambda, where N-mediated antitermination not only promotes

transcription of genes downstream from the termination sites but also results in downregulation of the *int* gene, which is located upstream of one of the N-regulated termination sites. The *int* gene encodes the integrase necessary for integration of the lambda genome into the host chromosome, and its repression when N is active is a component of the switch between the lysogenic and lytic cycles. In the absence of N, the *int* transcript terminates at the intrinsic terminator site located at the end of the *int* coding sequence. N-mediated readthrough of this termination site results in synthesis of an extended transcript that now includes a new element (designated *sib*) that is sensitive to cleavage by the endoribonuclease RNase III. RNA cleavage at the *sib* site triggers the degradation of the mRNA region 5′ to the sib site by exonucleolytic degradation from the 3′ end released by RNase III, which results in decreased *int* mRNA levels. N-mediated antitermination therefore causes decreased stability of the *int* transcript, despite the fact that *int* is encoded upstream of the termination site targeted by N. It is likely that this type of regulatory effect occurs in other polycistronic transcripts in which the regulated terminator is located between coding sequences, especially since the presence of the helix of an intrinsic terminator often stabilizes transcripts by inhibiting access of the RNA degradation machinery (see below). Extension of the transcript by antitermination separates the 5′ coding region from the terminator element, which can provide additional targets for endoribonuclease cleavage and RNA destruction.

Regulation of mRNA Stability

The net amount of an mRNA transcript in a cell at any given time arises from the combined effects of transcript synthesis and transcript destruction. Changes in the stability of a particular RNA can have as great an effect on expression of the encoded gene product(s) as changes in the synthesis rate of that RNA. RNA degradation in *E. coli* occurs through the combined activity of a series of 3′–5′ exonucleases, and endonucleases that cleave within the RNA to provide new 3′ ends for attack by the exonucleases. Structure at the 3′ end of a transcript, as occurs when the 3′ end of the RNA is generated by an intrinsic terminator, inhibits access of the 3′–5′ exonucleases. Polyadenylation of the transcript by poly(A) polymerase (the product of the *pcnB* gene) provides a site for 3′–5′ exonuclease recruitment. While no 5′–3′ exonuclease activity has been found in *E. coli*, this activity is present in other bacteria, including *B. subtilis*. In either case, elements at the 5′ end of the transcript have been shown to affect mRNA stability, by affecting recruitment and activity of ribonucleases that attack there or at other sites on the RNA. The presence of the 5′ triphosphate (from initiation) also reduces ribonuclease susceptibility, and removal of the triphosphate by RNA pyrophosphohydrolase (RppH) can serve as a triggering event for degradation. Modulation of transcript stability can occur by binding of factors and/or structural rearrangements that affect accessibility of the RNA to ribonucleases and/or RppH. As noted above, premature termination of transcription can determine whether the transcript includes a binding site for a ribonuclease. Translation and mRNA degradation are also intimately intertwined, as binding and processivity of a ribosome influence the susceptibility of the mRNA to ribonucleases.

Mechanisms of mRNA Degradation

In *E. coli*, the endonuclease RNase E is the major initiator of degradation of full-length transcripts that contain intrinsic terminators at their 3′ ends. RNase E nucleates assembly of a complex, designated the RNA degradosome, that includes polynucleotide phosphorylase (PNPase, a 3′–5′ exoribonuclease), an ATP-dependent RNA helicase (RhlB), and enolase (a metabolic enzyme that may serve as a scaffold); poly(A) polymerase is also associated with the complex. RNase E binding requires several unpaired nucleotides at the 5′ end of the mRNA, and exhibits a preference for RNAs with a 5′ monophosphate (generated by other cleavage reactions or by removal of the 5′ triphosphate that is normally found at transcription initiation sites). Other cellular endonucleases can participate in further cleaving the RNA products, and polyadenylation, which is stimulated by endonuclease cleavage, plays a major role especially in degradation of RNAs with structural elements at the 3′ end. As noted above, *B. subtilis* contains additional ribonucleases (RNase J1/J2) with 5′–3′ exonucleolytic activity that may be especially important for degradation of RNAs that are protected from 3′ to 5′ exonucleases.

Regulation of Transcript Stability by Interactions With the mRNA Target

The crucial role of events at the 5′ end of the mRNA for initiation of the degradation process provides a target for regulation by modulation of 5′ end structure or accessibility. Sequences throughout the mRNA also can provide targets for endonucleases, and blocking the accessibility of these cleavage sites, through specific binding or by occupancy of the mRNA by translating ribosomes, can have major effects on the stability of the mRNA.

Regulation of mRNA degradation by RNA-binding proteins

A number of systems have been described in which binding of a protein to a transcript has a major effect on the lifetime of the transcript in the cell, and therefore on the expression of genes encoded within that transcript. This effect can be direct (by binding of the protein at a position that overlaps a site for recognition by a ribonuclease, which results in occlusion of the RNase binding site and stabilization of the transcript) or indirect (by interfering with translation initiation, thereby reducing protection of the mRNA by translating ribosomes; see below). The *E. coli* RNase E protein provides a clear example of a case where binding of a protein has been shown to directly affect mRNA stability (Fig. 6A). RNase E represses its own synthesis by binding to a complex structural

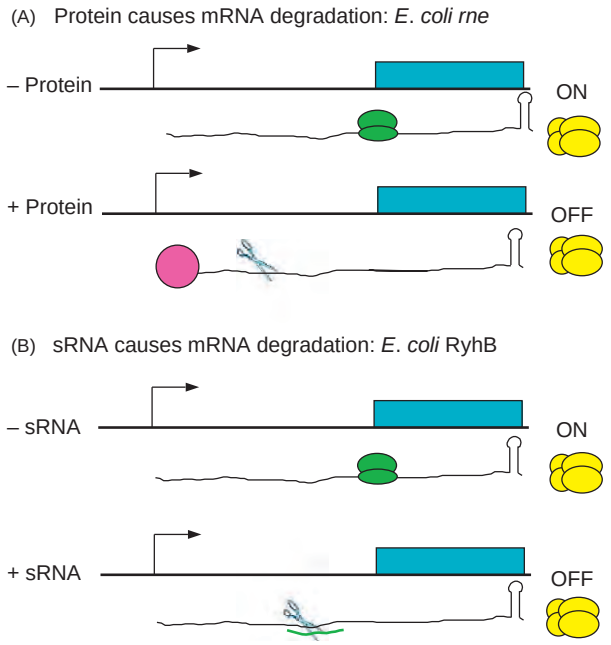

Fig. 6 Regulation of mRNA degradation. (A) Protein-dependent RNA degradation in the *E. coli rne* gene. When levels of the regulatory protein (RNase E, the product of the *rne* gene) are low, the RNA transcript is stable and efficiently translated by ribosomes (*green* ovals). High abundance of RNase E (*pink* circle) results in binding to the mRNA and increased degradation (*scissors*). (B) sRNA-directed mRNA degradation. In the absence of the sRNA, the target mRNA is stable and is efficiently translated. Binding of the sRNA to the mRNA by partial complementarity results in cleavage and rapid degradation of both the sRNA and its mRNA target.

element in the 5′ UTR of its mRNA; this binding results in cleavage and subsequent destruction of the mRNA, and reduced RNase E synthesis. This autoregulatory mechanism takes advantage of the function of the gene product to maintain appropriate cellular levels.

RNA-binding proteins also can cause destabilization of a transcript by promoting binding of ribonucleases. An example of this type of mechanism is provided by the CsrA protein of *E. coli*, which controls a variety of carbon metabolism genes by affecting either mRNA degradation or translation initiation. CsrA binds to a helical element at the 5′ end of its target mRNAs, and CsrA activity is controlled by two regulatory RNAs (CsrB and CsrC) that contain an array of CsrA binding sites and therefore titrate CsrA away from its normal targets. A second regulatory protein, CsrD, controls the abundance of the CsrB and CsrC regulatory RNAs. Binding of CsrD to the CsrB and CsrC RNAs results in their rapid degradation, mediated in part by RNase E. CsrD therefore acts as a specificity factor that directs degradation only of its designated CsrB and CsrC RNA targets. This complex system allows rapid changes in CsrA activity, and therefore in target gene expression. The related Rsm system regulates genes important for virulence in a number of pathogenic species.

RNA-mediated changes in RNA stability

As noted above, the CsrB and CsrC regulatory RNAs indirectly affect mRNA stability by controlling the availability of the CsrA regulatory protein, which in turn affects mRNA stability or translation of other transcripts. A growing number of small regulatory RNAs (sRNAs) have been identified that affect the stability of other mRNAs, without their effect being directed by a regulatory protein (Fig. 6B). These RNAs directly promote mRNA degradation (often with concomitant self-degradation) or interfere with translation initiation and therefore indirectly affect mRNA stability via the absence of translating ribosomes. Regulatory RNAs of this class (unlike CsrB and CsrC) interact with their targets by base-pairing, and may be encoded in *cis* (from the opposite strand of the same DNA region as the target) or in *trans* (from some other location in the chromosome). *cis*-Encoded regulatory RNAs are completely complementary to their targets (i.e., they are antisense RNAs), while *trans*-encoded regulatory RNAs are partially complementary and often have multiple targets within the cell. In most cases, target RNA destabilization by *cis*-encoded sRNAs requires RNase III, which cleaves long double-stranded RNA regions, while RNA degradation by *trans*-encoded sRNAs has in several cases been shown to involve RNase E. For example, the *E. coli* RyhB sRNA, which is induced in response to limitation for iron, promotes degradation of a set of mRNAs encoding proteins that bind iron, allowing more efficient utilization of limiting supplies (Fig. 6B). The activity of *trans*-encoded sRNAs (like RyhB) often depends on the RNA-binding protein Hfq, which acts as an RNA chaperone to enhance the RNA–RNA interaction, at least in part by modulating RNA folding to promote base-pairing between the two RNA partners. Both *cis*-encoded and *trans*-encoded sRNAs are usually degraded in concert with their target RNAs. Although destabilization of the target mRNA is the most common effect of sRNAs, the GadY sRNA in *E. coli*, which regulates genes involved in the response to low pH, stabilizes its mRNA targets and therefore increases expression.

Global Changes in the RNA Degradation Machinery

There is increasing evidence for modulation of degradosome composition and activity, especially in response to cellular stress. RNase E activity can be inhibited by two proteins, designated RraA and RraB, which cause alterations in the stability of many cellular mRNAs. Exposure to cold shock in *E. coli* also results in alteration of the degradosome. Starvation for amino acids activates several toxin–antitoxin systems that stimulate global degradation of ribosome-associated mRNAs, liberating resources for adaptation to the starvation state. Other general effects on translation affect overall mRNA stability via effects on ribosomal occupancy. It seems likely that a large variety of regulatory events may target RNA degradation, given the complexity of the process and its importance to the cell.

Regulation of Translation

Translation of an mRNA provides another opportunity for regulation of gene expression. In bacteria, translation can begin as soon as the RBS has appeared within the 5′ region of the transcript emerging from the transcription elongation complex. Transcription and translation are usually temporally coupled, and the efficiency with which an mRNA is recognized by the translational machinery is variable and depends on primary sequence and the presence of structural elements. Translational elongation can also be modulated by elements within the RNA or *trans*-acting factors that influence ribosome processivity. Translational efficiency can in turn affect the stability of the mRNA by affecting accessibility to endoribonucleases, as noted above.

Regulation of Translation Initiation

In bacteria, translation initiation usually involves initial binding of the 30S ribosomal subunit, initiation factors, and initiator tRNA to the RBS, which is comprised of the Shine–Dalgarno (SD) sequence (a 5-nt sequence with the consensus GGAGG that is complementary to a region at the 3′ end of 16S rRNA within the 30S subunit) and the AUG initiation codon that specifies binding of the initiator tRNA; GUG and UUG codons are also recognized, albeit at slightly lower efficiency. Translation initiation in bacteria differs from that in eukaryotic cells in that multiple translation initiation regions can be present on a single transcript, which allows the existence of polycistronic mRNAs from which multiple coding sequences can be independently translated. Binding of the translation initiation complex requires that the RBS be accessible, with no structure in the immediate region. Modulation of transcript structure in the RBS region therefore provides an opportunity for regulation that can be effective both while transcription is proceeding and after transcription has been completed.

Regulation of translation initiation by RNA-binding proteins

A number of systems have been described in which binding of a protein to the RBS region of an mRNA causes inhibition of translation initiation. As noted above, this inhibition may result not only in decreased translation of the mRNA but also in decreased mRNA stability, as a decrease in ribosome occupancy often causes more rapid degradation of the transcript. This type of mechanism is clearly illustrated in ribosomal protein genes in *E. coli*, where binding of one ribosomal protein encoded in a ribosomal protein gene operon results in sequestration of the RBS for the first gene in the operon, and decreased translation of the first coding sequence (Fig. 7A). Expression of the downstream genes in that operon is also repressed by a combination of translational and mRNA destabilization effects. Reduced translation of the downstream genes is likely to be due to translational coupling, where availability of the downstream RBS is dependent on unfolding of the mRNA by a ribosome translating the upstream coding sequence; interference with translation of the upstream region allows the transcript to fold into a structure in which the downstream RBS is sequestered. Inhibition of translation also can result in transcriptional polarity, where cryptic Rho-dependent transcription termination sites are activated by the absence of translating ribosomes that would normally occlude these sites from Rho binding. Together, these effects result in low expression of all downstream coding sequences, mediated by direct translational inhibition of only the first coding sequence.

Regulation of translation initiation by sRNAs

Accessibility of an RBS can readily be modulated by binding of a regulatory RNA, which can be encoded in *cis* (in which case the sRNA is perfectly complementary to its mRNA target) or in *trans* (in which case complementarity is usually imperfect, and Hfq protein is often used to enhance binding). Sequestration of the RBS region represents the simplest regulatory action of sRNAs, as binding of the sRNA to the RBS is sufficient to prevent ribosome binding, and recruitment of RNases by formation of a specific RNase binding site is unnecessary (although transcript destabilization is a likely indirect outcome of translational repression). There are many examples of sRNAs that repress translation, including a variety of plasmid replication control systems (which usually involve *cis*-encoded sRNAs), and the *trans*-encoded OxyS RNA, which is involved in the oxidative stress response in *E. coli* (Fig. 7B). More rarely, translation initiation can be induced by an sRNA, as is the case for induction of expression of the *rpoS* gene, which encodes the major stationary phase sigma factor in *E. coli*, by the DsrA sRNA. The *rpoS* mRNA contains a self-complementary region that encompasses the RBS, so that binding of the ribosome is blocked in the native transcript by an inhibitory helix. Binding of the DsrA sRNA to a region that includes the 5′ portion of this inhibitory helix results in unfolding of the helix, and liberation of the RBS

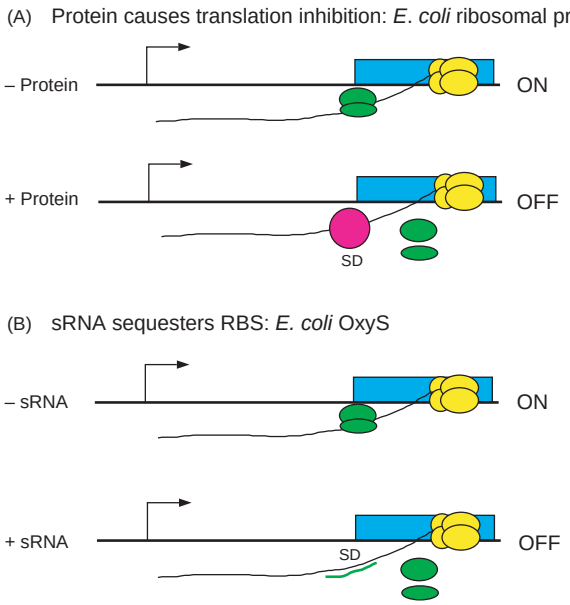

Fig. 7 Inhibition of translation initiation by RNA-binding proteins or sRNAs. (A) Sequestration of the SD region in *E. coli* ribosomal protein genes. When the levels of the regulatory protein are low, the SD region of the target gene (*blue* box) is available for binding by the ribosome (*green* ovals), and translation occurs. When levels of the regulatory protein are high, the protein (*pink* circle) binds to an RNA element that overlaps the SD region, and ribosome binding is inhibited. (B) Inhibition of translation initiation by an sRNA. In the absence of the sRNA, the SD region of the target gene (*blue* box) is available, which allows binding of the ribosome and translation initiation. Binding of the sRNA (*green* line) sequesters the SD region and prevents ribosome binding.

region so that translation initiation can occur. As noted above, effects on translation initiation also result in changes in mRNA stability, amplifying the translational effect.

Regulation of translation initiation by RNA structural rearrangements

Most of the classes of regulatory events in which RNA structural rearrangements are used to control gene expression at the level of premature termination of transcription (discussed above) can also be used to regulate at the level of translation initiation. The major difference between transcription attenuation systems and translation initiation systems is that in the latter systems the crucial regulatory helix acts to sequester the RBS of the regulated gene, rather than serving as the helix of an intrinsic terminator. For example, the processivity of the ribosome during leader peptide translation in systems like the *E. coli trp* operon determines whether a terminator or competing antiterminator helix forms in the nascent transcript, as described above. In an analogous translational control system, like that of the Gram-positive *erm* and *cat* genes (which encode resistance to erythromycin and chloramphenicol, respectively), leader peptide translation modulates whether the RBS of the downstream coding sequence is sequestered in a helix that prevents translation initiation (Fig. 1C). Expression of these genes is induced by exposure to subinhibitory concentrations of the antibiotic, which promotes stalling of the ribosome during leader peptide translation. The stalled ribosome sequesters a region of the transcript that would otherwise form an inhibitory helix that occludes the RBS of the downstream resistance gene, allowing translation of the downstream gene to commence. The stalled ribosome also stabilizes the downstream region of the mRNA, by a mechanism that may include blocking the activity of the 5′–3′ exonuclease found in this group of organisms. In both the antibiotic resistance genes and systems like the *E. coli trp* operon, the translating ribosome serves as the sensor for the regulatory signal (the antibiotic or charged $tRNA^{Trp}$, respectively); a major difference is that transcription termination control systems like the *E. coli trp* operon require precise coupling between transcription and translation, so that the transcription elongation complex can respond to the translational signal as it reaches the termination site, while translational control systems like *erm* and *cat* can exert their effect either while transcription is taking place or after transcript synthesis is complete.

Riboswitch systems also can operate at the levels of premature transcription termination or translation initiation, and in several systems the same effector binding domain is used for both regulatory mechanisms. The major difference between transcription termination and translational riboswitch systems is the position of the final inhibitory helix, the formation of which is determined by effector binding. In transcription attenuation systems, this helix is usually located at least 30 nt upstream of the start of the regulated coding sequence, and includes a run of U residues in the transcript that together with the helix serve as the intrinsic termination signal. In contrast, the inhibitory helix in translational control systems lacks the U residues and is positioned to include the RBS (or at least the SD sequence) in the 3′ side of the helix, so that helix formation occludes the RBS and prevents translation initiation. There are also examples where binding of the effector (e.g., the uncharged tRNA in T-box genes) stabilizes a helix that includes sequences that would otherwise base-pair with the SD (i.e., the "anti-SD" sequence), so that effector binding results in increased accessibility of the RBS and increased translation. In most riboswitch systems, the effector binding domain is separate

from the regulatory domain, which allows an easy transition between transcription termination control and translational control. An exception to this is the SAM-responsive S_{MK} box, which regulates SAM synthetase genes in lactic acid bacteria; in this case, the SD–ASD pairing region is an intrinsic component of the SAM-binding element (Fig. 4B).

RNA thermosensors represent the simplest class of RNA-based systems for regulation of translation initiation. In these RNAs, the RBS is sequestered into a helical structure that is stable under normal growth temperatures. An increase in temperature results in unfolding of the helical structure, and accessibility of the RBS to binding of the 30S ribosomal subunit. Regulatory elements of this type are obviously well suited to genes that are involved in cellular responses to increased temperature, and are found in certain heat shock genes including the *E. coli rpoH* gene, which encodes the heat shock sigma factor, sigma H. The *rpoH* mRNA is present in the cell during normal growth conditions, but translation is inhibited by the thermosensor element. A sudden temperature increase melts the inhibitory helix. This results in a rapid increase in sigma H protein levels, which triggers the general heat shock response by directing transcription of other heat shock genes. Restoration of the normal growth temperature allows the *rpoH* transcript to refold into the inactive helical structure, blocking further synthesis of the heat shock sigma factor and facilitating a return to steady-state conditions. Similar RNA thermosensors have been identified in heat shock genes of other bacteria, and have also been shown to regulate the *Listeria prfA* gene, which encodes a key regulator of virulence gene expression. In this case, the temperature change is used by the organism to sense movement into the host, which signals a requirement for induction of the virulence response.

Repression of translation initiation by alterations in the translational machinery

The *E. coli infC* gene, encoding translation initiation factor IF3, provides an interesting example of use of the function of a protein in an autogenous regulatory mechanism. IF3 plays a role in discrimination of authentic translational start codons. The initiation codon for *infC* itself is a highly unusual AUU codon, which is poorly recognized by initiation complexes containing IF3. Therefore, if IF3 is present in saturating amounts, translation of the *infC* gene is inhibited, and no additional IF3 is made. A reduction in IF3 abundance is signaled by the accumulation of initiation complexes that lack IF3. These defective complexes fail to discriminate against the *infC* mRNA, which results in increased IF3 synthesis until the complexes are again saturated. This regulatory mechanism is absolutely dependent on the presence of the AUU codon, which is found in *infC* genes of many bacteria, suggesting that the regulatory mechanism is conserved.

Regulation of Translation Elongation or Termination

The shift from translation initiation to elongation requires addition of the 50S ribosomal subunit, and loss of initiation factors. Once this step has occurred, the translating ribosome becomes highly processive, and is generally effective at displacing RNA structural elements or bound proteins. As noted above, translating ribosomes can affect the stability of the transcript by preventing access of ribonucleases, or the structure of the mRNA, which can affect transcription termination or translation initiation for downstream coding sequences.

Codon bias effects

Processivity of the ribosome during translation is dependent on the supply of charged tRNA entering the A site of the elongation complex. The presence of a codon for which the corresponding charged tRNA is at low abundance can cause a transient pause in translation; this is the basis for leader peptide transcription attenuation systems, as described above. Not surprisingly, codons for which the corresponding tRNAs are not abundant in a given organism are not heavily used in that organism. These rare codons can generally reduce translational efficiency, and bias against rare codons has been noted in highly expressed genes, such as those encoding ribosomal proteins. The presence of rare codons also can be used in regulation of gene expression. The best-characterized example of this is provided by the *Streptomyces coelicolor bldA* gene, which encodes a tRNA specific for the UUA leucine codon. This codon is rarely used in the *Streptomyces* genome, with a strong bias for genes involved in developmental processes. The *bldA*-encoded tRNA is dispensable for normal growth, but a *bldA* mutant is defective in secondary metabolism and sporulation because of the presence of the corresponding UUA codons in the genes required for these processes. Regulation of *bldA* expression can therefore affect a variety of genes containing UUA codons, resulting in a shift in the gene expression pattern; this effect can be further amplified if *bldA*-regulated genes include genes that encode regulatory proteins that affect expression of other groups of genes.

Programmed frameshifting

While translating ribosomes are usually resistant to structural features in the mRNA, certain RNA structural elements can promote ribosome pausing; elements of this type are important for alterations in normal coding, such as programmed frameshifting and insertion of unusual amino acids like selenocysteine. Frameshifting occurs when a ribosome shifts from its standard triplet decoding of an mRNA to a movement by 1 nt either forward (+1 frameshift) or backward (−1 frameshift). The ribosome then resumes translation, and decodes a different set of triplets on the same mRNA. Programmed frameshifting is a genetically encoded phenomenon that directs ribosomal frameshifting at a specific site, and in a specific direction. It can promote synthesis of multiple products from the same mRNA, as in the *E. coli dnaX* gene, which encodes two subunits of DNA polymerase, one of which is derived from a shift in reading frame that is dependent on both a structural element and a primary sequence element that promotes pausing of the elongating ribosome. A frameshift event is also used in autoregulation of the *E. coli prfB* gene, which encodes a translation termination factor, ribosome release factor 2 (RF2). The *prfB* gene is highly unusual in that it contains a UGA nonsense codon early in the coding sequence. Translation termination at UGA codons employs RF2, and efficient termination at this site (and failure to

synthesize additional RF2) occurs when cellular levels of RF2 are high. A decrease in RF2 abundance results in ribosome stalling at the UGA codon; this stall increases the probability of a shift of the ribosome into an alternate reading frame, which allows synthesis of the full-length RF2 protein. Like a variety of other autogenous control systems, *prfB* autoregulation makes direct use of the function of the RF2 protein in translation termination to modulate expression of its gene.

Conclusion

The variety of posttranscription initiation gene regulation events that occur in bacterial systems demonstrates the versatility of the gene expression machinery. Each step in gene expression represents a potential target for regulation, and the investigation of multiple gene systems in a broad range of experimental organisms broadens our appreciation of what remains to be discovered.

Further Reading

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Protozoa☆

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Glossary

Benthos The substrate at the bottom of the sea or fresh waters. Most benthic protozoa live in the spaces between sediment particles.
Commensal Of a protozoon – one that is loosely but not obligately associated with another organism (e.g., the ciliates attached to the external surfaces of crustacean zooplankton).
Endosymbiont An organism living in a long-term association inside a host organism for their mutual benefit (e.g., the endosymbiotic methanogenic bacteria that live inside anaerobic protozoa and utilize waste H_2 produced by the host).
Eukaryote An organism with membrane-bounded nuclei in its cells. Protozoa are unicellular eukaryotes.
Heterotrophic Mode of nutrition in which carbon is obtained from the organic compounds made by autotrophic organisms.
Phagotroph An organism that ingests solid food particles (e.g., bacteria).
Planktonic Living in the water column of lakes or the sea.

Protozoa are microscopic eukaryotic organisms. Each protozoon typically exists as a single, independent cell, and all free-living protozoa fit to the definition of phagotrophic microbial eukaryotes. In some species, independent cells unite to form colonies (e.g., colonial choanoflagellates) (Lee et al., 2000). There has never been unanimous agreement as to where the boundaries of the protozoa should lie, largely because of the extraordinary diversity of their lifestyles (Margulis et al., 1990; Finlay and Esteban, 1998; Weisse, 2017). Many species cause disease (e.g., malaria): Others thrive as commensals in the digestive tracts of ruminants and wood-eating insects, and many plant-like flagellates (e.g., *Euglena*) have at various times been referred to as protozoa, algae, or plants. Here, the authors are concerned principally with free-living protozoa, and the definition of this group is not taxonomic but one that is based on its key function in the natural environment: Protozoa are capable of phagotrophy – the ability to capture and ingest food particles (Fenchel, 1987). Many also have functional chloroplasts or endosymbiotic algae, and the resulting consortia are known as "mixotrophs" because they are capable of both phagotrophy and phototrophy (Esteban et al., 2010). Like most microorganisms, protozoa have very large population sizes, and they are the most abundant group of phagotrophic organisms in the biosphere. Biodiversity at the level of protozoa has characteristics that are not shared by macroscopic animals and plants. Most protozoan species are probably globally ubiquitous, so the global number of species is relatively small. A significant proportion of local protozoan species richness, at any moment in time, is rare or cryptic and awaiting the arrival of environmental conditions suitable for growth and reproduction.

Functional Roles

All of the important functional roles of free-living protozoa derive from their small size. The smallest flagellates are 2–4 μm and most are <20 μm, most amebae are <50 μm, and most ciliates are <200 μm. Exceptionally, some ameboid protozoa, such as the radiolarians and foraminiferans, and the agglutinated foraminiferans of the deep-sea benthos, may reach 2 mm or more, especially if they have spiny extensions. Because protozoa are so small, most suitable prey items are other, smaller microbes. Protozoa are the principal consumers of the immense natural resource of bacteria and other microorganisms, and because they have population growth rates that are similar to those of the microbes on which they feed (doubling times on the order of one to several days), they are usually able to control microbial abundance. Flagellated protozoa can probably consume all bacterial production in the plankton. In the benthos, protozoa overlap in their niche requirements with nematodes, rotifers, tardigrades, turbellarians, and gastrotrichs, but because of their great abundance protozoa are indeed quantitatively the most important grazers in the freshwater and marine (including deep-sea) benthos. Also, just as microbes achieve astronomical abundance on a global scale, so too are the protozoa that graze on them represented by species populations with proportionately smaller but still unimaginably large global abundances.

Protozoan grazing on microorganisms also stimulates activity of the whole microbial community, in both oxic and anoxic environments. The process involved is not fully understood, although it may operate by increasing the rate of turnover of essential nutrients that would otherwise remain locked up in the bacterial biomass. The net effect is that grazing by protozoa stimulates the rate of decomposition of organic matter (Fenchel, 1987, 2008; Finlay et al., 1997).

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The variety of shapes, sizes, and relative abundances of microbial food items has driven the evolution of a comprehensive suite of methods to capture them and a considerable diversification of protozoan morphologies. In general, the size of a protozoon relative to its prey dictates the most efficient food-capturing mechanism. Where the predator is typically much larger than its prey, filter-feeding prevails, and where the size difference is less the protozoon is more likely to be a raptorial feeder – One that seeks out relatively large individual food items. Thus, the planktonic choanoflagellate feeding on a dilute suspension of tiny bacteria 20 times smaller than itself does so with a very fine filter, and the ciliate feeding on dinoflagellates half of its size seeks out and captures each one individually. There are, of course, many exceptions, such as the marine heterotrophic dinoflagellates that use a feeding veil to trap and digest diatoms much larger than themselves.

The third main feeding type in protozoa is termed "diffusion feeding." This is particularly common in planktonic ameboid protozoa (radiolarians, foraminiferans, and heliozoans) and in the suctorian ciliates. It works when prey items collide with the sticky spines, tentacles, or axopods that radiate from the protozoon. Unlike the other two main modes of feeding, the protozoon simply waits for the arrival of its prey, much as a spider waits for an insect to be snared in its web.

Thus, there is a close link between protozoan morphology (especially of the food-capturing organelles) and the way in which a protozoon functions as a grazer. Therefore, when the authors classify the free-living protozoa into broad morphological groups, they are simultaneously allocated to broad functional groups. The three broadest morphological–functional groups are the ameboid, the flagellated, and the ciliated protozoa, and each has its own strengths as a phagotroph. Representatives of all three may feed on the same type of microbes in the same place (e.g., in an aquatic sediment), but they will differ in the mechanics and efficiency of capture of any particular food particle. A filter-feeding flagellate will have a relatively large filter area, a high volume-specific clearance and competitive superiority over filter-feeding ciliates when grazing on planktonic bacteria. A helioflagellate and a suctorian ciliate will both practice diffusion feeding, but the former will be adapted for snaring bacteria, whereas a diffusion-feeding suctorian will specialize in trapping flagellates and ciliates.

Many protozoa are microaerophilic: They seek out habitats with a low level of dissolved oxygen that is just sufficient to drive their aerobic respiration and low enough to exclude metazoan competitors and predators. Microaerobic habitats are common in aquatic sediments and in oceanic oxygen minimum zones. These are zones in which the raw materials for microbial growth arrive from opposite directions (e.g., where oxygen and light arriving from above meet carbon dioxide and sulfide from below) and in which there is therefore an elevated abundance of microbial food. Therefore, microaerophily is an adaptive behavior: It brings protozoa into contact with high abundances of microbial food. It also stimulates the growth of nutritional symbionts such as sulfide-oxidizing bacteria and endosymbiotic algae. Many microaerophilic protozoa are also temporary anaerobes, but unlike the true anaerobes that live permanently in the absence of oxygen, their metabolism is fundamentally aerobic. The true anaerobes – Those that complete their entire life cycle in the absence of oxygen – Live principally in aquatic sediments. There are many species, but none is ever abundant. Most use hydrogen-evolving fermentations for energy generation, and the hydrogen is used by anaerobic bacteria, especially endosymbiotic methanogens. Thus, methane is released from these protozoan consortia. The anaerobic protozoa are probably the only phagotrophic organisms capable of living permanently in the absence of dissolved oxygen (Fenchel and Finlay, 1995) (with perhaps the exception of species of the metazoan phylum *Loricifera* - see Danovaro et al., 2010).

The real diversity of symbiotic associations involving protozoa is insufficiently known. In some cases, complex interactive behaviors have evolved between the partners. In the marine, sand-dwelling ciliate *Kentrophoros*, the entire dorsal surface of the ciliate is a coat of sulfide-oxidizing bacteria that can grow only in the narrow layer within the sediment where oxygen and sulfide overlap. The ciliate host's innate microaerobic behavior enables it to seek out the habitat that the symbiotic bacteria need for growth. The ciliate then invaginates its dorsal surface and digests the bacteria because it does not have a mouth, and this is its only source of nutrition.

It is clear that many of these symbiotic consortia involving protozoa represent tightly integrated functional units; indeed, the symbionts may be almost as deeply embedded functionally in the consortium as the protozoon's other organelles. Two points must be noted. First, it is the combined phenotype of the consortium, rather than that of any individual consortium partners, on which natural selection will operate, and there are examples of how the fitness of a protozoon in a particular habitat can be improved by the acquisition of endosymbionts (e.g., the algal symbionts of ciliates living in the metalimnia of freshwater lakes). Second, the biodiversity of protozoa, when quantified simply in terms of protozoan species richness, will fail to take account of the large supplementary microbial diversity with which the protozoa are necessarily associated.

Therefore, the diversity of free-living protozoa may be classified into broad morphological–functional groups: ameboid, flagellated, and ciliated protozoa. Almost any free-living protozoon can be placed without difficulty in one of these groups. It must be stressed, however, that these groups are not concordant with any system of classification of protozoa published in recent years; nor are they in most cases aligned with the independent lineages that are emerging in the molecular phylogenies (e.g., those based on sequence variation in ribosomal RNAs) which reflect the main episodes in the history of eukaryotic evolution. Heterotrophic flagellate groups, such as the diplomonads and trichomonads, appear in the early emerging lineages, but other flagellates (e.g., choanoflagellates, chrysomonads, dinoflagellates, and haptomonads) are classified within recently diverging lineages. The ameboid protozoa too are scattered across many lineages. The naked amebae without mitochondria (the pelobionts) diverge early and close to the diplomonads, whereas the vahlkampfiids, slime molds, and various other groups of naked and testate amebae appear in other independent lineages that are evolutionarily quite distant from each other. The morphological–functional group of the ciliates is the only one which remains intact, as a monophyletic group, in current molecular phylogenies.

The process of distilling the vast quantity of molecular information generated in recent years has generated some entirely new phyletic assemblages, including the stramenopiles, a group containing organisms as morphologically and functionally dissimilar as

chrysomonad flagellates and diatoms, and the alveolates – A group that embraces the dinoflagellates, the ciliates, and a large group of exclusively intracellular parasites (the apicomplexans) (Adl et al., 2012). In the next section, the authors focus on the broad morphological–functional groups of free-living protozoa, define what is meant by species, and quantify the species within these groups.

The Nature of Protozoan Species

There is no universal agreement on what constitutes a protozoan species. The most widely used concept is the morphospecies because it is relatively easy to discriminate a great diversity of protozoa using body form alone. This concept is especially useful because morphology is closely related to the ecological function. In many free-living protozoa, the structure of the feeding apparatus and the size and shape of the cell determine how the protozoon functions in the natural environment; therefore, the cell form largely determines the ecological niche that the protozoon occupies. Discriminating species on the basis of form might then be equivalent to discriminating them according to the ecological niches they occupy. We could say that if a protozoon looks the same in different places, then it is the same in different places. However, there are problems with such a simple concept. It is known that a morphospecies can be composed of ecologically distinct populations, such as those adapted for maximum growth rate at different temperatures. Therefore, although it is possible to collect apparently identical representatives of a morphospecies in different corners of the world, and one might be encouraged by the belief that they were filling exactly the same niche in these different places, in reality they could be phenotypically quite different. The same problem applies with respect to genetic differences. It is possible to find genetic differences, especially sequence differences in ribosomal RNAs, in morphologically identical isolates collected from different places. The significance of these genetic differences is not known; nor is it known if genetic and phenotypic divergence are correlated. It appears, however, that there is no correlation between genetic divergence and geographic distance.

Protozoa spend most of their time as asexual organisms, but some do reproduce sexually, if only periodically, and in some well-studied cases (e.g., the ciliate genera *Tetrahymena* and *Paramecium*) morphologically indistinguishable biological species (reproductively isolated gene pools, also known as sibling species) have been studied thoroughly. Different sibling species within a morphospecies may be genetically identical to each other or extremely divergent (at least with respect to ribosomal RNAs), and there is no apparent correlation between genetic isolation and genetic divergence. Again, there is no good evidence for biogeography of protozoan sibling species, and members of the same sibling species can be found on different continents. It is possible that sibling species carry unique phenotypic traits that equip each species for a particular niche, but this has not been demonstrated. The real problem in using a biological species concept for protozoa is that it is not practical for all but a few easily cultivated protozoa. The majority of protozoan species have never been cultivated, and neither the frequency nor the character of their sexual behavior (if any) are known or are ever likely to be known. Most people who study protozoa in the natural environment use the morphospecies concept because it is practical, it embodies the close link between form and function, and it is the morphospecies that fills the niche, if not always the minutest niches, that can be discerned by the human observer. The morphospecies may contain much genetic variation and be capable of expressing a wealth of phenotypic variation, but it is the best tool that is available for ordering the diversity that lies within the protozoa.

One problem that is particularly acute when dealing with organisms the size of protozoa is that the quality of one's perception of morphology is very definitely a function of the tools that are available (Mitchell and Meisterfeld, 2005). Thus, better tools (e.g., quality microscopes) enable the discrimination of finer detail. The logical extension of this is that even individual protozoa in a population might be discriminated one from the other and could therefore be referred to as separate morphospecies. Modern optics have undoubtedly lent impetus to the business of describing many new species, some of which are undoubtedly legitimate, whereas many have been established on morphological criteria that are trivial or have no conceivable functional significance.

An additional problem with the morphospecies is that it is often difficult to decide exactly where a species begins and ends. Although most observations are made of the vast number of individuals that cluster around the central tendency of any population, the individuals at the tail ends of the distributions abut and often overlap those of other species. Morphological variation then appears to be continuous across many species. Examples of this have been described: In some of the large spongiose spumellarian radiolarians, the major features (e.g., skeletal and cytoplasmic morphology) that are used to discriminate species intergrade to such an extent that it is not possible to unambiguously ascribe individual radiolarians to nominal species.

However, the evolutionary process in radiolarians and other protozoa probably works in the same way as it does for other organisms such that it maintains phenotypically discrete species in niche space that is essentially continuously variable. The phenotypic traits that sustain these discrete species in protozoa are presumably quite diverse in character and possibly not totally comprehensible. Among the more accessible of these traits are the morphological characters that the authors use to separate species. However, the likely limitation of these is that they enable us to perceive only the fairly coarsely resolved, sometimes overlapping entities that we call morphospecies.

Protozoa and Ecosystem Function

Protozoa are abundant. One gram of soil typically contains 103–107 naked amebae, 105 planktonic foraminiferans can often exist beneath 1 m^2 of oceanic water, and almost every milliliter of fresh water or sea-water on the planet supports at least 100

heterotrophic flagellates. When expressed in global terms, these numbers are very large, and an inevitable consequence of the persistence of such a large number of very small organisms is that migration rates will be relatively high. It follows that rates of speciation and extinction must be low, as will the consequent global number of species. It also follows that free-living protozoa are unlikely to have biogeographies, and endemic species probably do not exist. The authors might expect that the local diversity of protozoa would account for a significant proportion of global diversity, even if at any moment in time much of this diversity is represented by rare or inactive individuals (e.g., cysts awaiting the arrival of suitable conditions). There is indeed good evidence for the global distribution of protozoan species, including the morphologically distinctive flagellate *Rhynchomonas nasuta* that has been found in most aquatic and terrestrial environments worldwide; marine foraminiferans and ciliates found living in slightly salty water of desert oases, hundreds of kilometers from marine coasts; the same radiolarian species living in high northern and southern oceanic latitudes; the same pond-dwelling ciliates living in Australia and northern Europe; and the cosmopolitan distribution of the same species of agglutinated foraminiferans in the deep-sea benthos. In general, protozoan morphospecies are ubiquitous and apparently cosmopolitan if the habitats to which they are adapted are distributed in different parts of the world (Finlay, 1998, 2002). In accordance with this, the global number of protozoan species is indeed relatively modest (Table 1), and the number of species that can be retrieved from a local area (e.g., a pond), in both active form and from a passive state is a significant proportion (usually at least 10% for various morphological-functional groups) of the global total. This fact may not be obvious from short-term ecological sampling programs because only a limited number of microbial niches are available at any moment in time.

Protozoa and other microorganisms have other special properties. Microbial activities interact strongly with physical and chemical factors in the natural aquatic environment (e.g., light transmission or the concentrations of essential nutrients) to create a continuous turnover of microbial niches. These niches are quickly filled from the locally available diversity of rare and dormant microbes, and the activities of the latter create further reciprocal interactions. Therefore, the diversity of active protozoan species in a pond, at any moment in time, is the result of preceding reciprocal interactions involving many biological and nonbiological factors, and the biodiversity of protozoa and other microbes is an integral part of ecosystem functions such as carbon fixation and nutrient cycling (Finlay et al., 1997).

Table 1 Estimates of global species richness of extant free-living protozoa[a]

			Marine	*Nonmarine*	*Total*
Ameboid protozoa	Slime molds	Dictyostelids	–	60	60
		Myxomycetes	–	550	550
	Rhizopod amebae	Naked	180	220	400
		Testate	–	200	200
	Foraminiferans	Planktonic	40	–	40
		Benthic, inshore	4000[b]	–	4000
		Benthic, deep sea	250[b]	–	250
	Actinopod amebae	Acantharians	150	–	150
		Radiolarians, solitary	750	–	750
		Radiolarians, colonial	50	–	50
		Heliozonas	–	120	120
Flagellated protozoa	Excluding heterotrophic dinoflagellates[c]	Marine plankton	420	–	420
		Marine benthos	330	–	330
		Freshwater and soil	–	350	350
	Heterotrophic dinoflagellates		900	110	1010[d]
	Other mixotrophic flagellates		–	–	150[e]
Ciliated protozoa			1400	1660	3060
Total					11,890

[a]Compiled from numerous published and unpublished sources. The more problematic estimates are highlighted.

[b]There is considerable uncertainty attached to these estimates. The figure of 4000 is generally accepted as a working figure for extant species but is probably inflated by synonyms, especially those of shallow-water benthic species. There is no firm information for species richness of deep-sea benthic foraminiferans (Gooday et al., 1998). The estimate of 250 is approximately double the typical figure for local richness of species, most of which may be cosmopolitan, but there are probably many undescribed deep-sea soft-shelled foraminiferans that have in the past been ignored by geologists.

[c]The total given here for heterotrophic flagellates is 1100 species. This includes some synonyms and mixotrophs. It is believed by some that the real global total is closer to 3000 species.

[d]Assuming there are 1800 marine and 220 freshwater species, and that 50% of these are heterotrophs.

[e]This estimate is simply derived by doubling some recent estimates. Note that these species may be only temporarily phagotrophic.

Biological Diversity and Global Species Richness

Ameboid Protozoa

Slime molds may be regarded as protozoa because they spend most of their active lives as amebae or as naked ameboid (and often macroscopic) plasmodia (Fig. 1). They have been known by many names, including mycetozoa ('fungus animals'), because although they never develop hyphae they produce fruiting bodies (sporangia) supported on cellulose-rich stalks. There are two large groups – The cellular slime molds (the dictyostelids, e.g., *Dictyostelium*) and the acellular slime molds (e.g., *Physarum*). Two smaller groups are the acrasids (e.g., *Acrasis*) and the protostelids (e.g., *Cavostelium*). The dictyostelids are typically phagotrophic amebae, but when starved they aggregate, form a migrating slug, and then produce a fruiting body from which spores are released. These later germinate to produce amebae. In the acellular slime molds (*Physarum*), individual amebae (sometimes thousands of them) with or without flagella coalesce to form a distinctive (often brightly colored) multinucleate plasmodium which gives rise to fruiting bodies. Slime molds are common in damp forest soils, tree bark, and dead or dying wood, and abundance and local species richness are greater in deciduous than in coniferous forests. There is extreme patchiness in the distribution of dictyostelid species in forest soils, and coexistence of multiple species in the same small soil samples is rare. Many slime molds species are believed to be cosmopolitan. The phagotrophic ameboid stages of slime molds may be quantitatively important grazers of bacteria, fungi, and the

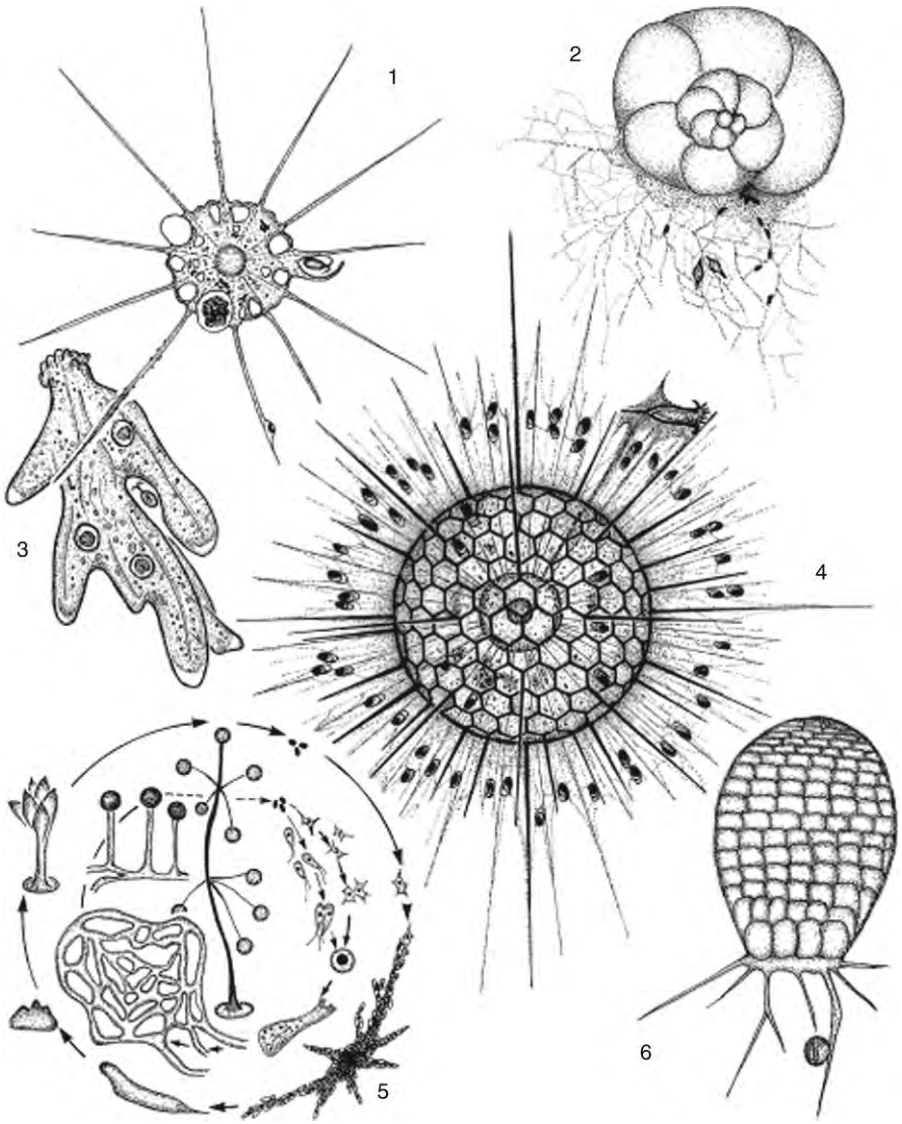

Fig. 1 A selection from the variety of form and function in ameboid protozoa and slime molds. (1) an actinophryid heliozoon (*Actinophrys*; diameter of about 0.1 mm) ingesting a flagellate; (2) a benthic foraminiferan (*Rotalia*) trapping diatoms and bacteria in its reticulopodial net; (3) a naked ameba (Ameba) using pseudopodia to trap a flagellate; (4) a polycystine radiolarian (*Heliosphaera*; spherical body ~0.3 mm) with symbiotic dinoflagellates and an entrapped tintinnid ciliate; (5) polymorphic life cycles of dictyostelid slime molds (e.g., *Polysphondylium*; outer circle) and acellular slime molds (e.g., *Physarum*; inner circle); (6) testate ameba (*Assulina*; ~0.08 mm) with its prey (an algal cell) caught on a sticky filopod.

other primary decomposers of organic matter in soil. Other ecological interactions of slime molds may be at least as complex as their life cycles (e.g., the migrating slugs of dictyostelids appear capable of repelling grazing nematodes).

Rhizopod amebae use pseudopodia for locomotion and feeding. There are two large groups (Fig. 1) – the "naked amebae" (e.g., *Acanthamoeba*, *Vannella*, *Ameba*, and *Vampyrella*) and the shelled "testate amebae" (e.g., *Arcella*, *Nebela*, and *Euglypha*). Most rhizopod amebae feed nonselectively by engulfing diatoms and other algae, unicellular and filamentous cyanobacteria, detritus, and bacteria. However, there are notable variants: *Vampyrella* dissolves a hole in the cell wall of a green alga or desmid and then enters through the hole to digest the cytoplasm of the prey. Some are opportunistic pathogens of man (e.g., in the genera *Acanthamoeba* and *Naegleria*).

Naked amebae occur in the plankton, but these species are usually very small (<10 μm) and typically associated with suspended flocs (e.g., marine snow). However, it is likely that all rhizopod amebae need to be attached to a surface if they are to feed, and much larger numbers are found in aquatic sediments. Amebae are particularly abundant in soils (103–107 per gram dry weight), where together with heterotrophic flagellates they probably control bacterial abundance. A few testate amebae can be planktonic (e.g., *Difflugia*), but most live in soils and in *Sphagnum* bogs.The prey size of testates must be limited by the shell aperture size.

Naked and testate amebae offer different challenges when attempting to identify species. The naked forms are distinguished by the forms of their bodies and pseudopods in locomotion, but many species constantly change shape and the absence of fixed characters often makes it difficult to identify them without resorting to an analysis of ultrastructure. The testates construct tests which have species-specific architectures, but these are often modified according to the nutritional status and local environmental factors, such that the variety of tests described is extraordinarily large and the task of deciding where one nominal species begins and the other ends is at least as difficult as it is for the naked amebae. One practical solution to the problem of identifying living naked amebae in natural samples is to allocate them to one of the several broad morphotypes, e.g., those that extend lobose pseudopodia, slug-like forms with eruptive or noneruptive locomotion, and fan-shaped forms. No clear relationship has been established between morphology and ecological function in naked amebae, although it is likely that the limax (slug-like) amebae are predominantly benthic species.

Some naked amebae (especially those that form extensive anastomosing plasmodial networks) resemble hybrids of shell-free foraminiferans and slime molds. Some can reach several centimeters in diameter. They are typically very slow moving and apparently perfectly adapted for grazing extensive areas of soil surface (*Leptomyxa*) or diatom mats in the marine benthos (*Corallomyxa*). Some free-living naked amebae are anaerobic and do not have mitochondria. As a group, these are referred to as pelobionts, rhizomastigids, archamoebae, or karyoblasteans (e.g., *Mastigamoeba*, *Mastigella*, and *Pelomyxa*). They are relatively common in organically enriched anoxic freshwater and marine sediments. The very large (up to several millimeters in diameter) *Pelomyxa* supports several types of endosymbiotic bacteria, including methanogens, and probably lives exclusively in freshwater sediments. It appears to ingest any organic particles with which it makes contact. It can be locally abundant and the dominant grazer in some anaerobic lake sediments. Most species of naked amebae have been described from fresh waters and soil, and most marine species have been collected from the inshore areas. It is not clear to what extent freshwater species can also live in seawater. Some genera (e.g., *Mayorella* and *Acanthamoeba*) are well represented in both environments.

Foraminiferans live in marine or brackish water environments, and most have calcareous shells (Fig. 1). Cytoplasm emerges through the aperture and pores in the shell to form a repeatedly branching and anastomosing reticulopodial net in which prey are trapped (Lee et al., 2000). The spinose planktonic species (e.g., *Globigerinoides ruber*) have calcareous spines, and familiar Cretaceous chalk deposits (e.g., the white cliffs of Dover, UK) consist largely of the sedimented shells of these species together with the coccoliths of haptomonad flagellates. Some naked amebae that produce similar pseudopodial networks live in fresh waters (e.g., *Biomyxa*) or soil (e.g., *Reticulomyxa*).

The shells of planktonic foraminiferans are generally 0.1–1 mm in diameter, but the pseudopodial network can reach 10 mm and the spines may increase the size to more than 20 mm, making them larger than many marine metazoans. Planktonic species are open-water organisms. They are most abundant in tropical and subtropical waters, in which there is typically one per liter (or $\sim$10 5 m^{-2}, depth integrated to 150 m). Each species tolerates only small variations in temperature and salinity, and distinct assemblages of species are associated with five biogeographical provinces – the surface water masses of equivalent temperature on either side of the equator (polar, subpolar, temperate, subtropical, and tropical). However, species in these assemblages are not isolated from each other: Polar species have been recorded in upwelling zones at 10° N, and species characteristic of high latitudes are also found in the tropics, but in very deep and cool waters.

A typical planktonic foraminiferan produces a mass of reticulopodia (the "halo") that radiates outward from the shell. This is used to snare prey and to support algal symbionts. Spinose species appear to prefer zooplankton prey, and nonspinose species (e.g., *Globorotalia menardii*) prefer phytoplankton. These preferences may affect the local distribution of species: In the Red Sea north of 20° N, the dominating spinose species appear to reflect the greater local abundance of zooplankton.

Many planktonic foraminiferans, especially the spinose species (e.g., *G. ruber*), contain algal symbionts that are carried along the spines by cytoplasmic streaming. There are probably only two types of symbionts in planktonic species: the dinoflagellate *Gymnodinium beii*, which is morphologically identical in many host species, and a small yellow–green (chrysomonad-like) symbiont. The symbionts are protected from digestion by the host. The carnivorous foraminiferan *Hastigerina pelagica* envelopes itself in a cytoplasmic bubble capsule which is probably a flotation device but is also used for trapping and digesting prey. *H. pelagica* does not have symbionts but commensal dino flagellates (*Pyrocystis* spp.) that live in the capsule. The role of symbionts and commensals is unclear. High rates of photosynthesis are maintained within the consortium, the symbionts probably transfer significant amounts of fixed carbon to the host, their photosynthetic activity may be necessary for calcification of the host's shell, and the symbionts may use the host's (nitrogenous) waste.

Benthic foraminifera have a greater diversity of symbiotic partners, including diatoms (*Nitzschia*), dinoflagellates (*Symbiodinium microadriaticum*), red algae, and chlorophytes (*Chlamydomonas*). Some also sequester chloroplasts from ingested algae. Although many benthic foraminiferan species have been described, information on their life cycles and ecology is available for only about 20 shallow-water species (e.g., *Allogromia*, *Rosalina*, *Spiroloculina*, and *Sorites*). These typically drag themselves slowly over surfaces, continually extending reticulopodia to trap prey (diatoms, algae, and bacteria). The abundance of benthic species is correlated with local biological productivity. In the fertile Mississippi Delta, numbers may reach several thousands in an area of 10 cm^2. Close to sewage outfalls, they are typically very abundant but low in species diversity. The spatial distribution of all benthic species is very patchy. This may be related to asexual reproduction (known only for benthic species) and the consequential limited range of migration of offspring from the parent. Benthic foraminiferans become relatively more important with increasing water depth, possibly due to a competitive advantage they have over metazoans in their ability to rapidly exploit seasonally deposited phytodetritus. In oxygen minimum zones along the continental margins of tropical oceans, they can, because of their relatively small size, tolerate low oxygen conditions better than can metazoans. Also, certain taxa (e.g., *Bolivina* and *Uvigerina*) seem to be adapted for life in oxygen-depleted waters.

Foraminiferans in the deep-sea benthos are often abundant (typically hundreds per 10 cm^2) and diverse (species richness may exceed that of nematodes), and they are regarded as major components of the fauna in this zone (Gooday et al., 1998). Because they consume bacteria and detritus, they probably act as a link in the food chain to higher trophic levels in the benthos. Very small species (30–63 μm) are common, but the dominant fauna >500 μm in central oceanic regions (and particularly at depths greater than the carbonate dissolution depth of ~4000 m) are large agglutinated foraminiferans, especially the komokiaceans, which have complex anastomosing networks of tubules. In the benthos underlying the oligotrophic waters of the central North Pacific, the biovolume of komokiacean tests may greatly exceed the biovolume of metazoans. However, the amount of protozoan biomass in komokiacean tests is relatively small, and it may be even less than that of the metazoans that also occupy the test. *Edgertonia floccula* consists of a network of branching agglutinated chambers buried in a large (2–8 mm) inorganic mudball that it shares with cohabiting metazoans. This foraminiferan is one of the most abundant meiobenthic animals in some areas of the abyssal northeast Atlantic. Other deep-sea agglutinated foraminifera (e.g., *Bathysiphon* and *Hyperammina*) may even reach several centimeters in length, and some species stand vertically in the sediment and project into the overlying water, in which they presumably feed. Many of the common deep-sea foraminiferan species are probably cosmopolitan in their distribution.

The xenophyophoreans are another deep-sea group about which relatively little is known. They live in agglutinated, often fragile tests (typically 1–10 cm in length) and they too probably feed on sedimented detritus. Abundances of hundreds per square meter have been reported. The multinucleate protoplasm is enclosed within a labyrinthine organic tube encrusted with foreign particles. The organism also contains numerous crystals of barium sulfate, which have no obvious function. Growth is episodic, with intervening periods of months of inactivity. They have no confirmed fossil record, although they often use the sedimented shells of the planktonic foraminiferan *Globigerina* to strengthen their tests (as in *Homogammina maculosa*).

Foraminiferan fossils are useful for biostratigraphy because (1) their sedimented remains are abundant (e.g., in the "*Globigerina* ooze" which covers much of the abyssal plain beyond the continental shelves), (2) individual shells are identifiable to species level, and (3) their remains allow determination of sea surface paleotemperature. Seawater and calcite differ in their $^{18}O/^{16}O$ ratio. This difference increases with temperature, so the ratio is used to estimate the water temperature at the time the calcite was deposited in the fossil foraminiferan shell. This estimate can then be compared with the established correlation between the temperature of water mass and the extant species it typically supports. Much of this work is supported financially by the oil exploration industry.

Actinopod amebae all have axopods, which are stiffened ray-like pseudopodia that radiate from the cell body (Fig. 1). They also have a peripheral network of filopodia, which are long, thin, sticky pseudopodia used for trapping food organisms that make chance contact. There are three principal groups: (1) Radiolarians, consisting of the polycystines with perforated spiny lattice skeletons of silica and the phaeodareans with skeletons of hollow tubes and spines; (2) acantharians with skeletal spines of strontium sulfate radiating from the center of the organism; and (3) heliozoans, which often lack an internal mineral skeleton (although some species produce skeletal spicules which are either organic or siliceous) and superficially resemble polycystine radiolarians. All actinopod protozoa, apart from a large number of heliozoans, are exclusively planktonic in deep oceanic water.

Radiolarians (Fig. 1) and acantharians range in size from ~30 μm to several millimeters in diameter. Colonial radiolarians (e.g., *Collozoum longiforme*), which occur as gelatinous cylinders with diameters of several centimeters, can reach 3 m in length. Typical food items include diatoms, tintinnids and other ciliates, crustacean larvae, and other zooplankton, all of which are trapped in the peripheral network of axopods and filopodia. The smallest species may eat bacteria (Anderson, 1983; Suzuki, 2015).

Dinoflagellate (*Symbiodinium*), chrysomonad, and prasinomonad symbionts are associated with many polycystine radiolaria. They probably contribute to the host carbon metabolism because the consortium can last many weeks without exogenous food. The symbionts are carried about in the host's cytoplasmic streaming, gathering in the peripheral filopodia at dawn and withdrawing inside the host shell at sunset. The phaeodareans that live near the ocean floor do not have algal symbionts. Radiolarians are found at all water depths down to ~8000 m, although they are most abundant and diverse between 200 and 2000 m (Anderson, 1983). No benthic species are known. Like planktonic foraminiferans, each radiolarian species inhabits an oceanic water mass that lies within a particular temperature range. Moreover, because of the immense depths to which living radiolarians sink, those species living in surface waters at high latitudes are also found in the tropics but in very deep and cool waters. As a consequence, the species assemblages found in surface waters give a misleading impression of radiolarian biogeography. It is believed that radiolarians may be transported in very deep oceanic waters between high northern and high southern latitudes.

Radiolarian abundance and productivity increase in warmer waters. Abundance may be almost nil in the surface waters of the Antarctic, up to 50 m^{-3} in the Caribbean and Gulf Stream, and up to several thousand per m^3 in the Gulf of Mexico. Colonial radiolarians are much less abundant. In the Sargasso they are considered abundant if represented by more than one colony per 100 m^3. Acantharians can reach relatively high abundances (up to 25–35 l^{-1}) and they are often the most abundant of the planktonic shelled amebae, but relatively little is known about living organisms because as soon as they are collected the fragile cell membrane breaks and the strontium sulfate skeleton dissolves. Radiolarians are important stratigraphic tools, and skeletal morphology is the main characteristic used to identify species; hence, much information is available on the diversity of radiolarian skeletons.

The heliozoans are predominantly a freshwater group. They resemble functionally the radiolarians, especially with respect to their diffusion feeding. They span a wide size range. Some (e.g., *Actinosphaerium*) can be >1 mm, but most species are ~40 μm (*Actinophrys*). Depending on their size, different species feed with little apparent selectivity on algae, flagellates, ciliates, and rotifers. Small heliozoan species account for most of the biomass of ameboid protozoa in the freshwater plankton. Most, however, are attached to or loosely associated with sediment and other submerged surfaces (Page and Siemensma, 1991).

Flagellated Protozoa

There is a little consensus on how to classify the flagellates, and they are here divided into broad functional groups (Fig. 2). Heterotrophic flagellates are ecologically very important because they are abundant (there are seldom less than 1000 ml^{-1}, even in the plankton) and because their grazing activities are largely responsible for controlling the abundance of bacteria in aquatic environments. In some taxonomic groups, all species are exclusively heterotrophic (e.g., choanoflagellates and bodonids); others contain many mixotrophs (e.g., the euglenids and chrysomonads), whereas the haptomonads and cryptomonads are dominated by phototrophs and only a minority are capable of phagotrophy. In the past 30 years, a large diversity of heterotrophic flagellates has been discovered. Most of these are choanoflagellates, chrysomonads, euglenids, or bodonids. Some of the more easily recognized species (e.g., *Rhyncomonas nasuta*) have been recorded from a wide range of habitat types in marine, freshwater, and terrestrial environments (Patterson and Larsen, 1991; Lee and Patterson, 1998).

Many heterotrophic flagellates are anaerobes, including intestinal parasites of man (e.g., *Giardia intestinalis*), but free-living diplomonads (*Hexamita* and *Trepomonas*) and *Retortamonas* are relatively common bacteria feeders in organically enriched anoxic

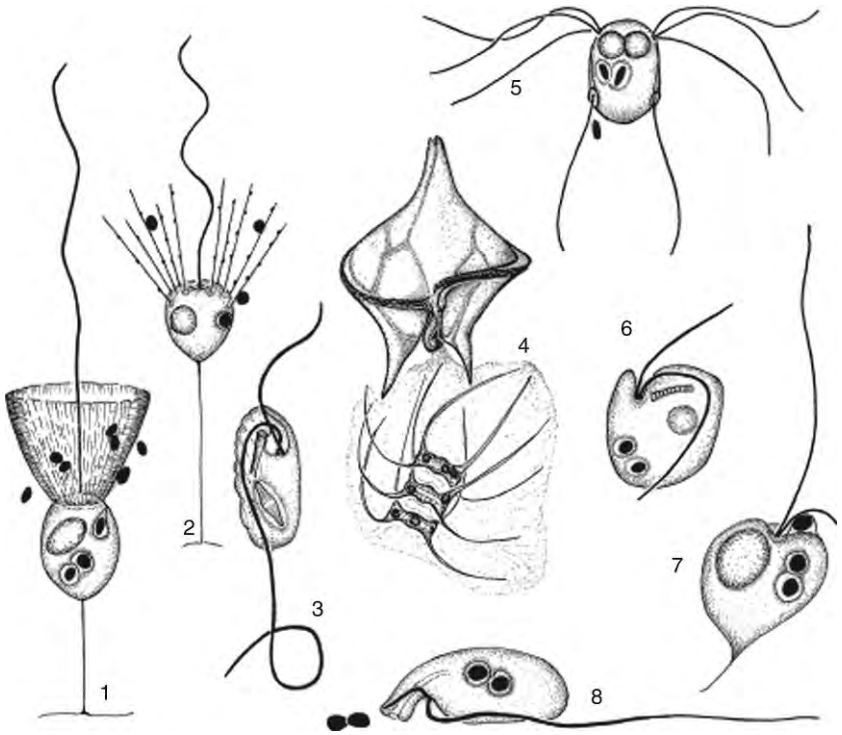

Fig. 2 A selection from the variety of form and function in flagellated protozoa. (1) A choanoflagellate with bacterial food trapped on the external surface of the collar filter (*Monosiga*; body+collar ~0.015 mm); (2) a bacteria-feeding helioflagellate (*Pteridomonas*; body 0.007 mm); (3) a phagotrophic euglenid (*Dinema*; ~0.03 mm) with an ingested diatom; (4) a marine heterotrophic dinoflagellate (*Protoperidinium*; ~0.06 mm) with a diatom trapped in its feeding veil; (5) a free-living, bacteria-feeding, anaerobic diplomonad (*Hexamita*; body ~0.008 mm); (6) a small heterotrophic (bacteria-feeding) cryptomonad flagellate (*Goniomonas*; ~0.008 mm); (7) a heterotrophic, typically bacteria-feeding, chrysomonad flagellate (*Spumella*: body ~0.008 mm); (8) a bacteria-feeding bodonid (*Rhynchomonas*; body ~0.005 mm).

waters and sediments. Some anaerobic flagellates have hydrogenosomes, the anaerobic derivatives of mitochondria. Almost all of these are endosymbionts or internal parasites of animals. They are also known as parabasalia and include many of medical or economic importance, e.g., the human parasite *Trichomonas vaginalis* and *Trichomitopsis*, which degrades cellulose in the hindgut of termites and other wood-eating insects and also supports endosymbiotic methane-producing bacteria (termites may be responsible for a globally significant flux of methane to the atmosphere) (Fenchel and Finlay, 1995).

Most choanoflagellates are small (<10 μm), with a feeding filter that forms a collar around the single anterior flagellum. The flagellum creates the water current that flows through the filter. Choanoflagellates are exclusively phagotrophic and either solitary (e.g., *Monosiga*) or colonial (e.g., *Sphaeroeca*). They are important grazers of the smallest suspended bacteria, especially in the marine plankton (e.g., *Salpingoeca*, *Stephanoeca*, and *Parvicorbicula*).

Euglenids usually have two flagella which emerge from a small anterior pocket. This is a large group of planktonic and benthic protozoa, common in both fresh and marine waters. Species associated with surfaces typically keep one trailing flagellum in contact with the substrate, whereas the other pulls the cell forward. Many species are green and phototrophic (e.g., *Euglena*), but there is a great diversity of phagotrophs (e.g., *Astasia*, *Petalomonas*, *Entosiphon*, and *Notosolenus*). These are especially common in marine shallow-water sediments, in which they graze on bacteria. All bodonids are small flagellates with characteristic heterodynamic flagella (e.g., *Bodo* and *Rhynchomonas*). They are almost always found in organically polluted (even anoxic) waters or soils that are rich in bacteria, on which they feed. Many closely related ("kinetoplastid") flagellates are parasites: *Ichthyobodo* causes costiasis in freshwater fish and the "trypanosomatids" are important parasites of man (*Trypanosoma brucei* causes sleeping sickness). In heterokont flagellates, each organism has two flagella – one hairy and one smooth, with the former creating the water current that brings mainly bacterial food to the (filter-feeding) flagellate. They include the bicosoecids that live in a lorica (*Bicosoeca*) or are naked (*Cafeteria* and *Pseudobodo*), that may be stalked or colonial, and which resemble the mixotrophic loricate chrysomonads (e.g., *Dinobryon*).

In the phagotrophic chrysomonads (e.g., *Paraphysomonas* and *Spumella*), the chloroplast is essentially vestigial (and the cell is colorless). Functional types range from heterotrophs (e.g., *Paraphysomonas*) to mixotrophs (e.g., *Dinobryon* and *Uroglena*) and those that are predominantly phototrophs (e.g., *Synura* and *Mallomonas*). Chrysomonads are the most abundant heterotrophic flagellates in the freshwater plankton. The helioflagellates (also referred to as "pedinellid stramenopiles," e.g., *Actinomonas*, *Pteridomonas*, and *Ciliophrys*) are filter or diffusion feeders that superficially resemble heliozoa or choanoflagellates.

The haptomonads are typically photosynthetic, biflagellated organisms. They include the coccolithophorids and the prymnesiophytes, and some (e.g., *Emiliania*) are often abundant in the marine euphotic zone. They have an additional flagellar appendage known as the haptonema. In the marine *Chrysochromulina*, this is used in food uptake, but in *Prymnesium*, which has a very short haptonema, ingestion of food (dinoflagellates, chrysomonads, and green algae) is by means of a posterior pseudopodium. Prey organisms may be immobilized by toxins prior to ingestion (toxic bloom-forming species of *Prymnesium* are responsible for fish kills). The scale and importance of phagotrophy in freshwater haptomonads is unclear.

Most cryptomonads are yellow–brown and phototrophic, with two flagella of similar length (e.g., *Cryptomonas*). They are especially common in fresh waters. Heterotrophic species either lack plastids (*Goniomonas*) or have a plastid that is devoid of photosynthetic pigments (*Chilomonas*). Colorless species can be relatively abundant, especially when associated with benthic detritus.

The heteromitids and cyathobodonids represent a varied assemblage of small, poorly studied flagellates (e.g., *Apusomonas*, *Heteromita*, *Cercomonas*, *Cyathobodo*, and *Kathablepharis*), some of which (e.g., *Cercomonas*) produce pseudopodia to ingest bacteria. Others secrete a stalk (*Cyathobodo*) to attach the cell to the substrate. Many are common in soil. Others feed mainly on bacteria in the freshwater plankton (e.g., *Kathablepharis*, which also feeds on small algae) and on sediment surfaces. The percolozoans are a diverse collection of bacteria-feeding organisms, including ameboflagellates (e.g., *Naegleria*), the filter-feeding flagellate *Percolomonas*, and anaerobic flagellates with hydrogenosomes and endosymbiotic methanogens (*Psalteriomonas*).

Dinoflagellates are best known as a large group of photosynthetic flagellates and for the ability of some species to cause "toxic blooms" and "red tides" (*Protogonyaulax* causes paralytic shellfish poisoning). However, about half of all known marine species lack chloroplasts. Some of these (in the genera *Gymnodinium* and *Amphidinium*) feed on cryptomonads and sequester their chloroplasts, thus transforming themselves into mixotrophs. Moreover, many typically photosynthetic species can become mixotrophs through their ability to ingest particulate food. The nonphotosynthetic and mixotrophic species are referred to collectively as heterotrophic dinoflagellates. These can be quantitatively important in marine food webs, especially as consumers of diatoms. Some species are benthic, but little is known about these. The diversity of heterotrophic species in fresh waters is much less, and it is known mainly from the genera *Katodinium*, *Peridinium*, *Gymnodinium*, and *Ceratium*. This may be due in part to the relative rarity in fresh waters of large, chain-forming diatoms – a typical food item of many marine dinoflagellates. These large food items are trapped and digested externally in a pseudopodial feeding veil or pallium. This explains why it has often been difficult to recognize ingested organisms within dinoflagellates. Other heterotrophic dinoflagellates use a feeding tube known as a peduncle. The common freshwater dinoflagellate *Peridiniopsis berolinensis* uses such a tube to ingest the fluid contents from injured and dying protists and small metazoans.

The number of marine dinoflagellate species with a recorded capacity for phagotrophy is increasing rapidly. These include thecate species (e.g., the peridinioids) previously considered to be incapable of phagotrophy. It is widely believed that most dinoflagellates – at least in marine environments – may be capable of phagotrophy. Heterotrophic dinoflagellates (e.g., *Oblea*, *Polykrikos*, *Heterocapsa*, and *Protoperidinium*) can dominate the protozoan biomass in coastal and oceanic waters, in which they feed

on bacteria, flagellates, diatoms, other dinoflagellates, and ciliates. Their biomass can be approximately the same as that of ciliates, and the two groups may compete with each other for food (although the size range of dinoflagellates is slightly greater – from 3 or 4 μm in *Gymnodinium simplex* to 2 mm in *Noctiluca*, with the majority in the size range 20–200 μm). Also, most planktonic ciliates are incapable of ingesting the large diatoms that are consumed by dinoflagellates.

Almost all stony and stinging corals (Cnidaria) in shallow tropical waters harbor photosynthetic dinoflagellates (especially *Symbiodinium*) as symbionts. These consume carbon dioxide and bicarbonate. This promotes calcium deposition in the external skeletons of their hosts and may enhance the rate with which coral reefs are built. In the marine plankton, outbursts of parasitic dinoflagellates are sometimes associated with the collapse of zooplankton populations. The parasite *Ichthyodinium chabelardi* destroys the eggs of sardines, *Blastodinium* sp. in the copepod gut castrates its host, and *Gonyaulux catenella* (a dinoflagellate that can cause red tides) is parasitized by another dinoflagellate (*Amoebophrya ceratii*). The fossil record of dinoflagellate cysts extends over the past 220 million years, and they have a role as biostratigraphic tools.

Dinoflagellates in the marine plankton exhibit latitudinal cosmopolitanism, such that a morphospecies occurs in a circumglobal belt within fairly broad latitudinal limits which correspond to a specific temperature range in the surface waters. The recorded species richness is greatest in tropical marine waters.

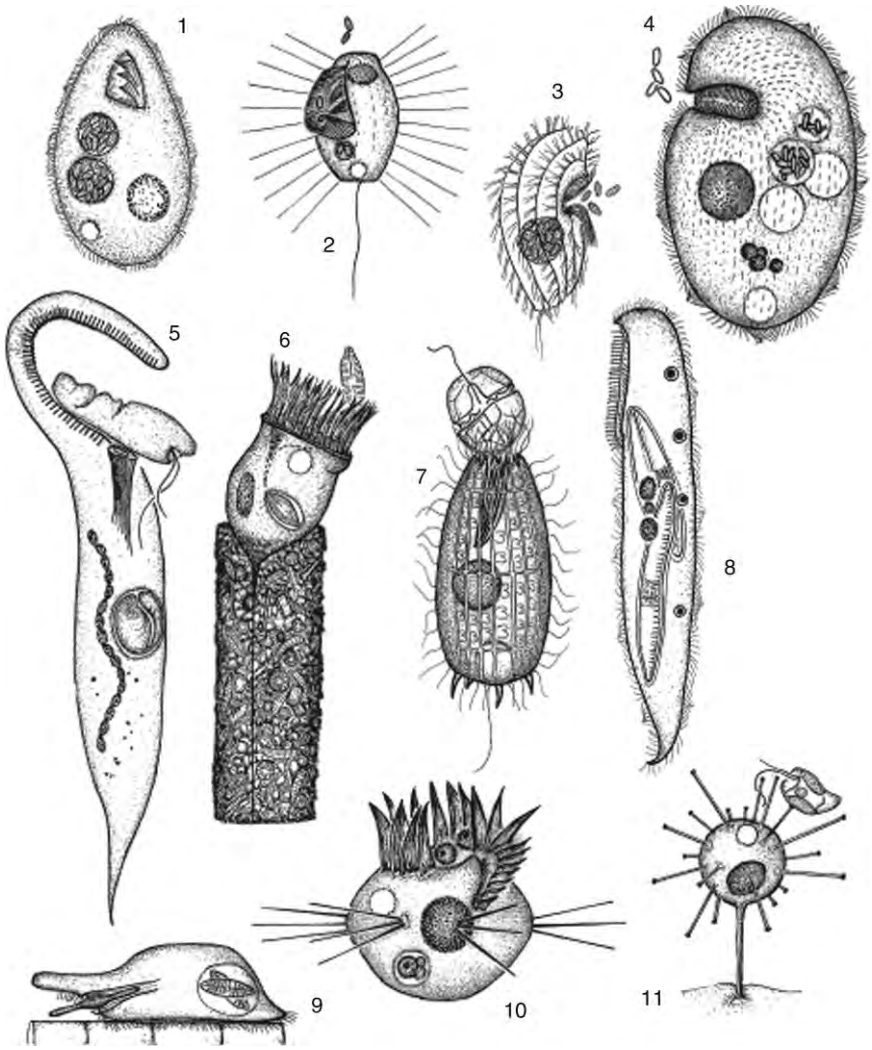

Fig. 3 A selection from the variety of form and function in ciliated protozoa. (1) A bacteria-feeding tetrahymenid (*Tetrahymena*; length ~0.05 mm); (2) a small scuticociliate (*Cyclidium*; length ~0.02 mm) filter feeding on bacteria; (3) a bacteria-feeding colpodid ciliate (*Colpoda*; length ~0.02 mm) from soil; (4) an anaerobic, typically benthic plagiopylid feeding on bacteria (*Plagiopyla*; length ~0.1 mm); (5) a large haptorid (*Dileptus*; length ~0.4 mm) with toxicysts used to kill motile prey (flagellates and other ciliates) prior to ingestion; (6) a planktonic, loricate, diatom-feeding tintinnid (*Tintinnopsis*; length ~0.1 mm); (7) a gymnostome ciliate (*Coleps*; length ~0.07 mm) about to ingest a dinoflagellate; (8) a diatom-feeding karyorelictid (*Remanella*; length ~0.12 mm) typical of the marine interstitial; (9) a "hoover-feeding" cyrtophorid (*Chilodonella*; length ~0.04 mm) ingesting a diatom; (10) a planktonic oligotrich (*Halteria*; diameter ~0.04 mm) filter feeding on small algae; (11) a "diffusion-feeding" suctorian (*Podophrya*; diameter ~0.04 mm) with a flagellate trapped on a feeding tentacle.

Ciliated Protozoa

This diverse and distinctive group uses cilia for locomotion and feeding (Fig. 3). They demonstrate a considerable adaptive radiation of feeding mechanisms and cell morphologies (e.g., the ribbon-shaped forms *Trachelorapbis* and *Geleia* adapted for life in the marine interstitial) (Fenchel, 1987; Lynn, 2008). The smallest species tend to feed on bacteria-sized particles and the larger species on unicellular algae, filamentous cyanobacteria, other protozoa, and even rotifers and other microzooplankton. The raptorial feeders (e.g., *Prorodon*) use a simple mouth to catch diatoms, dinoflagellates, and other large food items individually; some (e.g., *Lacrymaria*) kill motile prey, whereas others (e.g., *Chilodonella*) "hoover" diatoms and other elongate food particles from surfaces. The filter feeders (e.g., *Cyclidium*) use fine-mesh filters to sieve suspended bacteria, and some of these ciliates (e.g., *Paramecium* and *Tetrahymena*) thrive in habitats with very high bacterial concentrations. Other ciliates (e.g., *Pleuronema* and *Tintinnopsis*) use coarser filters to collect small algae. In many ciliates (e.g., *Oxytricha*, *Aspidisca*, and *Strombidium*), a row of membranelles generates a water current and acts as a relatively coarse feeding filter, and some of these ciliates (e.g., *Euplotes*) feed most efficiently if they are raised on cirri (fused cilia). Many ciliates are typically sessile and aligned perpendicular to the substrate (e.g., *Stentor* and *Vorticella*). Diffusion feeders (e.g., *Podophrya* and other Suctoria) catch swimming prey (usually other protozoa) that collide with their sticky tentacles.

More is probably known about the biodiversity and ecology of free-living ciliates than any other protozoan group. There are several reasons for this, including the distinctive and immediately recognizable ciliate morphology and swimming behavior and the relative ease with which many species can be cultured. They are a group of predominantly free-living protozoa. A few are parasites (e.g., *Ichthyophthirius* infects fish and there is one human endoparasite of minor importance – *Balantidium coli*), but most species are either free-living or harmless commensals of aquatic invertebrates (the ciliate epifauna of marine crustaceans is particularly diverse). Free-living species are known from all natural aquatic habitats in which temperatures are $<45^{\circ}C$, including oceanic sinking detritus, freshwater and marine sediments, anaerobic municipal landfill sites, and sewage treatment plants. They are abundant (up to $\sim$106 ml^{-1}) in activated sludge plants, in which they consume bacteria and also flocculate bacteria and other suspended particulate matter. These activities aid the clarification of the effluent and the formation of sludge. About a dozen ciliate species are frequently recorded in used-water treatment plants, although more than 200 ciliate species have been recorded. Ciliates also have a role as indicators of the level of organic pollution in river water (Corliss, 1979, 2000; Lynn, 2008).

Most ciliates are in the size range 0.02–2 mm, so they are generally larger than the hetero trophic flagellates and other nanoplankton (0.002–0.02 mm) on which many of them feed. Planktonic ciliates are relatively abundant (1–100 ml^{-1}) and important grazers of nanoplankton in marine and fresh waters. They are probably key grazers within the microbial loop which is responsible for the rapid remineralization of organic matter in the water column. The diet of planktonic metazoans includes ciliates, although the quantitative significance of this link is unclear. Benthic ciliates are often abundant (>1000 ml^{-1}) and usually the most important grazers in freshwater sediments (especially in lakes) and marine sandy sediments of inshore waters.

Many ciliate species harbor prokaryotic or eukaryotic symbionts. At the oxic–anoxic boundary in the water column of lakes, most ciliates may harbor sufficient endosymbiotic algae (*Chlorella*) to render the consortia capable of net photosynthesis in dim light. The marine interstitial ciliate *Kentrophoros* carries ectosymbiotic chemolitho trophic sulfide-oxidizing bacteria, and most anaerobic ciliate species harbor methanogenic bacteria that act as a sink for (potentially inhibitory) H_2 produced by the ciliate. The endosymbiotic nonsulfur purple bacteria in the anaerobic ciliate *Strombidium purpureum* use waste H_2 from the ciliate as a reductant for photosynthesis. Most marine anaerobic ciliates carry ectosymbiotic sulfate-reducing bacteria (Fenchel and Finlay, 1995)

Many ciliate species are microaerobic, and they seek out the (microbe-rich) oxic–anoxic boundary in sediment or the water column. Some of these species are facultative anaerobes (in the genera *Cyclidium*, *Euplotes*, *Strombidium*, and *Paranophrys*). There are approximately 70 known species of anaerobic free-living ciliate species, mainly in the genera *Metopus*, *Caenomorpha*, *Saprodinium*, *Epalxella*, *Trimyema*, and *Plagiopyla*. It is likely that all these contain hydrogenosomes.

Anaerobic ciliates also live as endocommensals in the enlarged forestomach (rumen) of ruminants and in the cecum of other mammals with postgastric fermentation. It is unlikely that any of these ciliates (e.g., in the genera *Dasytricha*, *Entodinium*, and *Polyplaston*) are capable of a free-living existence. Rumen ciliates are typically extremely abundant (105–106 ml^{-1} of rumen liquor). They consume bacteria and microscopic fragments of grass and other plant material. Some species are capable of degrading cellulose and other structural carbohydrates, and the endosymbiotic methanogenic bacteria in some species contribute to the methane emitted from the ruminants. The economic significance of such large numbers of ciliates living in the rumen is unclear.

Ciliates also live in soil, in which all species are probably capable of producing desiccation-resistant cysts (e.g., *Colpoda*). The abundance of active ciliates in soil is extremely variable and reflects repeated cycles of cyst formation and excystment in response to fluctuating physical factors such as the level of soil moisture. Many species found in soil are frequently found in other (predominantly freshwater) habitats, although only about 200 ciliate species have been described from soil.

Others

The following are often referred to as protozoa. Because they are endocommensals or parasites, or incapable of phagotrophy, they do not fall within the definition of protozoa used here (i.e., free-living unicellular phagotrophs). They are mentioned briefly for the sake of completeness but are excluded from Table 1.

Chytridiomycetes, oomycetes, and hyphochytriomycetes have the typical fungal characteristics of saprotrophic (absorptive) nutrition and cell walls in the vegetative state. Some are economically important (the oomycete *Phytophthora infestans* causes potato

blight). The plasmodiophorids are cell wall-free endoparasitic slime molds (*Plasmodiophora brassicae* causes club root disease in cabbages). The labyrinthulids (e.g., the "slime net" *Labyrinthula*) feed saprotropically, especially on marine algae, and form complex branching colonies of spindle-shape cells that move through slime channels. All five groups reproduce by means of unicellular flagellated zoospores (hence, they are also known as "zoosporic fungi").

The opalinids superficially resemble ciliates, but all are mouthless endocommensals living in the hindgut of amphibians and some fish (Lee et al., 2000). The microsporidians are a large group (~800 species) of intracellular parasites of animals and other protozoa. They extrude a tubular filament through which the spore is injected into the host cytoplasm. They are believed to be highly derived fungi rather than early diverging eukaryotes. The haplosporidians are a small group of parasites of aquatic invertebrates, notably marine mollusks, and the parabasalans (anaerobic flagellates with hydrogenosomes, e.g., *Trichomonas*) are all parasites, with one or two possible exceptions (*Pseudotrichomonas*).

The organisms now referred to as apicomplexans used to be an important component of the sporozoans. All are intracellular parasites of animals, and many cause life-threatening diseases of man. They share the distinctive morphological feature of an apical complex at certain stages in the polymorphic life cycle. The degree of host specificity of many apicomplexans is not known. They include the gregarines that infect insects, marine polychaetes, and other invertebrates (e.g., *Monocystis* in earthworms), and also the "coccidian" parasites including *Toxoplasma gondii* (the domestic cat is the final host, but intermediate infection in the human can cross the placenta) and about 1050 species of *Eimeria* infecting mammals, chickens, and other farmed birds. *Cryptosporidium* is an apicomplexan, and eight species are known that infect the digestive and respiratory systems of vertebrates. *Cryptosporidium parvum* infects humans and cattle, and waterborne transmission of oocysts is thought to complete the fecal–oral cycle. Infection can cause life-threatening diarrhea in immunocompromised humans. *Cryptosporidium* may be sufficiently different from other coccidians to warrant creation of the new higher taxon "cryptosporidia." *Plasmodium*, the causative agent of malaria, is an apicomplexan, as is *Pneumocystis*, which is an enigmatic parasite that can persist in the human lung for long periods. *Pneumocystis carinii* (currently renamed as *Pneumocystis jiroveci*) causes *Pneumocystis* pneumonia in HIV patients. In the early 1970s, the organism was considered to be a fungus; in the early 1980s it was considered to be an apicomplexan, and by the late 1980s it was again considered to be a fungus, possibly an ascomycete. Confusingly, it can resemble *Cryptosporidium* when viewed with the light microscope. There are approximately 4000 nominal species of apicomplexans.

The Myxozoans are a large group (~1200 species) of parasites, of which the myxosporidians are important parasites of farmed fish (*Myxobolus cerebralis* causes "torsion disease" in trout). During their life cycle they show both protozoan and metazoan characters. Recent gene sequence data (1818S rDNA rDNA) indicate that myxozoans are grossly modified and simplified metazoans of the phylum Cnidaria (Siddall et al., 1995; Okabmura et al., 2015).

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Quinolones☆

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Glossary

Cleaved complexes Quinolone-topoisomerase-DNA complexes that contain broken DNA covalently attached to a topoisomerase.

DNA gyrase The enzyme responsible for introducing negative supercoils into DNA. The genes encoding gyrase are *gyrA* and *gyrB*.

DNA topoisomerase IV The enzyme responsible for decatenating (separating) interlinked circles of DNA at the end of a round of replication; also relaxes DNA. The genes encoding topoisomerase IV are *parC* and *parE*.

Minimal inhibitory concentration (MIC) The lowest concentration of an antimicrobial that blocks the growth of a given bacterial strain or isolate, generally when 10^4 to 10^5 bacteria are tested.

Mutant prevention concentration (MPC) The concentration of an antimicrobial that blocks the growth of the least susceptible resistant mutant subpopulation within a large bacterial population; approximated at no colony recovered when 10^{10} bacteria are applied to drug-containing agar plates.

Reactive oxygen species (ROS) Toxic molecules, such as superoxide, hydrogen peroxide, and hydroxyl radical, that participate in bacterial self-destruction following treatment with lethal concentrations of quinolones.

Introduction

The quinolone antibacterials have evolved into a diverse group of compounds that target two essential bacterial enzymes, DNA gyrase and DNA topoisomerase IV. While most of the compounds are true quinolones, the group also includes naphthyridines (nalidixic acid, gemifloxacin) and quinazolinediones. Quinolone development was initially driven by the need to broaden the spectrum of susceptible pathogens, since the prototype compound, nalidixic acid, is active only with a few Gram-negative bacteria. Its replacement, norfloxacin, is still used clinically due to good activity and pharmacokinetic properties. Ciprofloxacin succeeded norfloxacin by substitution of a cyclopropyl group at the N-1 position, which improved both lethal activity and the number of susceptible species. Indeed, ciprofloxacin remains the most effective quinolone with Gram-negative bacteria; however, activity with Gram-positive organisms is marginal. Adding a halogen or methoxy moiety to position C-8 and a variety of changes in the C-7 ring system improved Gram-positive activity. Compounds with these changes are sparfloxacin, moxifloxacin, and gatifloxacin. Other important compounds, such as levofloxacin and gemifloxacin, developed along different pathways. Keeping track of the various quinolone groupings can be important, since they have different mechanistic properties.

As quinolone activity improved, use of the compounds increased markedly, and resistance developed. Resistance recently entered a new phase with the emergence of plasmid-mediated resistance. The present challenge is to find ways to overcome resistance, an effort that likely involves understanding how quinolones poison bacterial cells. It may also involve changing the focus of antimicrobial therapy from curing disease to restricting resistance, a change that would require using higher doses. The present article seeks to provide an understanding of quinolone action and resistance as a way toward preserving the utility of the compounds.

Understanding quinolone action focuses on topoisomerase mechanism. The two enzyme targets of the quinolones, DNA gyrase and DNA topoisomerase IV, act by passing one region of duplex DNA through another using a strand cleavage-rejoining

☆*Change History:* August 2014. X Zhao, M Malik, Y Hong, L Li, and K Drlica, have made changes in Quinolone mechanism and resistance is an active area of research; consequently, the previous version of this article was completely rewritten. New results include the involvement of reactive oxygen species in quinolone-mediated cell death, the discovery of a second quinolone-binding mode in cleaved complexes, and the finding that quinolone resistance is due largely to loss of a drug-magnesium-enzyme bridge when mutations occur in GyrA or ParC. The mutant selection window hypothesis continues to serve as a framework for describing the selective enrichment of resistant mutants, and quinolone dosing continues to place drug concentrations inside the selection window.

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mechanism. During that process the drugs trap a reaction intermediate as a drug–enzyme–DNA complex. The resulting complexes block DNA replication rapidly (within minutes) and inhibit bacterial growth. At elevated drug concentrations, cell death can be observed within 30 min to an hour, depending on the quinolone and bacterial species. Resistance results from a variety of factors that include reduction of intracellular drug concentration and binding to the target proteins. The genetic changes underlying resistance arise in three general ways: through spontaneous mutation, through the mutagenic SOS response, and through plasmid-mediated mobilization of protective chromosomal genes. Resistant bacterial subpopulations are then selectively enriched by suboptimal quinolone treatment, and the resulting resistant clones disseminate. We begin by describing the drug-enzyme complexes that form on DNA.

Cleaved Complexes

As a part of their reaction mechanism, gyrase and topoisomerase IV introduce a pair of staggered, single-strand breaks into DNA and become covalently linked to the 5′ ends of the broken DNA. Quinolones bind to the enzyme-DNA complexes, probably before DNA cleavage occurs, and by intercalating into DNA they prevent the enzymes from religating the broken DNA ends. The resulting ternary complexes, called cleaved complexes due to the presence of DNA breaks, block the movement of DNA and RNA polymerases. Treatment of cleaved complexes with protein denaturants, such as sodium dodecyl sulfate (SDS), releases the DNA ends from protein-mediated constraint, and broken DNA is readily observed. Since the topoisomerases have access to most regions of the chromosome, quinolone treatment creates DNA break-containing complexes throughout the chromosome.

An important feature of complex formation is reversibility, which can be observed as resealing of broken DNA following removal of quinolone, mild thermal treatment, or addition of the chelating agent EDTA. Chromosome supercoiling is not relaxed by quinolone treatment. Thus, the ends of the broken DNA must be constrained in the complexes. That constraint, which facilitates DNA resealing, prevents massive DNA relaxation when DNA exits from the protein at the conclusion of the strand passage reaction.

The reversible nature of complex formation emphasizes that cleaved complexes are not by themselves lethal. Indeed, complex formation correlates with bacteriostatic activity, commonly measured as minimal inhibitory concentration or efficiency of plating. In both of these assays the quinolone is present in the medium used to score growth. We emphasize this point, because such assays are sometimes mistakenly thought to reflect killing of cells. To measure lethal action, the cells are challenged with drug and then plated under non-selective conditions. Since separation of challenge and colony scoring is generally not performed with genetic procedures such as transduction, the term ‘conditional-lethal mutant’ is often a misnomer.

Cleaved complexes have been crystallized, and their structures have been analyzed by X-ray crystallography. Consequently, we now know that a pair of quinolone molecules intercalate into broken DNA such that the C-7 ring system faces the GyrB/ParE subunits. The carboxyl end of the compounds extend to helix-4 of GyrA/ParC (gyrase is composed of two subunit types, GyrA and GyrB, which in topoisomerase IV are called ParC and ParE). The amino acid substitutions most often associated with clinical resistance are located in helix-4. Resistance is explained by changes in amino acids at positions 83 and 87 (*Escherichia coli* GyrA numbering system) that disrupt a magnesium-water bridge formed between the quinolone C-3-carboxyl, C-4-keto, and gyrase or topoisomerase IV. Disruption destabilizes drug binding. Quinazolinediones, which lack the 3-carboxyl group, cannot form the magnesium bridge, and their activity is unaffected by substitutions at positions 83 and 87. Unfortunately, the diones are less active than their cognate quinolones; consequently diones do not yet substitute for fluoroquinolones. Current work aims to find new ways to stabilize cleaved complexes formed with diones and to reduce crossover activity of diones with human topoisomerase II.

A second drug–enzyme interaction has been identified by crosslinking between the C-7 ring of quinolones to GyrA-81, which is far from the GyrB interaction site seen in the crystal structures. This second interaction can be explained in two ways that have not been distinguished. One involves an inversion of drug binding orientation shown in the crystal structure such that the C-7 ring system extends to helix-4 of GyrA/ParC. A second postulates the existence of an additional drug-binding site. Examination of the surface of GyrA/ParC reveals a potential binding pocket that bridges the interface between the GyrA (ParC) subunits. This second binding site is potentially important, because it might allow additional drug molecules to bind to the complexes and help explain why rapid killing requires higher drug concentrations than inhibition of growth.

Chromosome Fragmentation, ROS, and Cell Death

Since even a few chromosomal DNA breaks can be lethal and since quinolone-topoisomerase complexes that contain broken DNA are distributed throughout the *E. coli* chromosome, we considered the possibility that lethal chromosome fragmentation arises from the release of DNA breaks constrained in the complexes. Constraint was initially observed as maintenance of DNA supercoils in nucleoids extracted from cells treated with concentrations of quinolone that block DNA synthesis and trap broken DNA in ternary complexes. When quinolone concentrations were increased to levels that rapidly kill cells, nucleoids lost the ability to maintain supercoils, even in the presence of ethidium bromide, an intercalating dye that would otherwise introduce supercoils. Since supercoiling in nucleoids is partitioned into many topologically independent domains, the inability to maintain supercoiling is

most readily explained by widespread chromosome fragmentation. When inhibition of protein synthesis blocks quinolone-mediated lethality, it also blocks quinolone-mediated loss of supercoiling. Thus, supercoiling assays provide a correlation between chromosome fragmentation and cell death.

Chromosome fragmentation has also been measured more directly. One method involves treating *E. coli* with quinolone and then measuring cell lysate viscosity. Cell lysates, when prepared by gentle methods, can exhibit little viscosity; subsequent heating unfolds the nucleoids and causes lysates to become very viscous. If chromosomes are fragmented by quinolone action, lysates fail to become viscous when heated. Another assay involves sedimentation of DNA into sucrose density-gradients: broken DNA sediments slowly.

Both viscosity and sedimentation measurements indicate that nalidixic acid causes chromosome fragmentation having kinetics similar to cell death and much slower than inhibition of DNA replication. Additional correlations are revealed by inhibition of protein synthesis, which blocks both killing by nalidixic acid and chromosome fragmentation. However, inhibitors of protein synthesis do not interfere with cleaved complex formation because cells co-treated with chloramphenicol and nalidixic acid still reveal DNA breaks upon treatment with sodium dodecyl sulfate (SDS). Therefore, complex formation and cell death are distinct processes in which cell death correlates with chromosome fragmentation.

How nalidixic and oxolinic acids cause chromosome fragmentation is not known. The inhibtion of nalidixic acid-mediated cell death by chloramphenicol, an inhibitor of protein synthesis, suggests that a suicide factor is involved. Such a factor is likely to be short-lived, since the lethal action of nalidixic acid is rapidly blocked by addition of chloramphenicol even after killing by quinolone has progressed for 45 min.

Surprisingly, cell death due to treatment with nalidixic and oxolinic acids does not arise directly from chromosome fragmentation. Instead, the fragmentation, which we postulate is the initial lesion, stimulates a cascade of reactive oxygen species (ROS; commonly studied species are superoxide, hydrogen peroxide, and hydroxyl radical). That explains why the lethal effect of quinolones is increased by a deficiency in KatG, an enzyme that converts hydrogen peroxide to water. Moreover, quinolone lethality is blocked by agents expected to reduce the accumulation of hydroxyl radical (a highly toxic ROS) and by passing an anaerobic gas mixture through *E. coli* cultures.

Not all quinolones behave like nalidixic and oxolinic acids. For example, cell death caused by norfloxacin is also blocked by chloramphenicol, but at high concentration norfloxacin kills cells during anaerobic growth (low, but aerobically lethal concentrations do not kill anaerobically). Norfloxacin also kills cells resuspended in saline, while nalidixic acid does not. Thus norfloxacin action and nalidixic acid action are distinct. More potent quinolones, such as ciprofloxacin and gatifloxacin, fragment chromosomes and kill cells in the presence of protein synthesis inhibitors. Some compounds, such as PD161144 (a C-8 methoxy experimental fluoroquinolone), do not require ROS to kill cells, and in some bacterial species ROS accelerate cell death rather than increase the fraction of cells killed. To explain these diverse effects of quinolone structure, we postulate the existence of two pathways by which quinolones kill cells. Some compounds kill by both pathways, with the relative contribution of each pathway dependent on drug structure and concentration. In the next section we speculate about quinolones-mediated killing in the absence of protein synthesis.

Before proceeding, we note that several challenges to involvement of ROS in quinolone-mediated lethality have led to refinements that firmly establish the phenomenon. The challenges are instructive because they reveal the complexity of the phenomenon and the importance of focusing assays sharply on the lethal response to quinolone treatment (factors acting before lesion formation, such as drug uptake and efflux, are largely irrelevant to the response).

Cleaved Complex Destabilization and Cell Death

Although incubation of reaction mixtures containing plasmid DNA, gyrase, and quinolone has never revealed DNA breaks without addition of SDS, we have seen chromosome fragmentation when nucleoids are used as substrates for complexes formed with purified gyrase and gatifloxacin, a very active fluoroquinolone. Nalidixic acid failed to exhibit this behavior unless the gyrase in the reaction was a mutant form that allows the drug to kill *E. coli* in the presence of chloramphenicol. This GyrA variant contained an amino acid substitution that was expected to weaken the GyrA-GyrA interaction at the interface of the two subunits in gyrase. Since a weakened dimer interaction could promote gyrase subunit dissociation and release of DNA breaks, we speculated that chromosome fragmentation can derive from cleaved complex destabilization. It is not known why cleaved complex destabilization is not observed with plasmid DNA as a substrate.

Several other observations also fit with the cleaved complex destabilization hypothesis. One emerged from studies of quinolone-stimulated illegitimate recombination, a phenomenon most easily explained by gyrase subunit dissociation-reassociation. A second concerns a GyrB mutation that allows oxolinic acid to kill *E. coli* in the presence of chloramphenicol. Other observations distinguish the two lethal pathways. For example, the complex destabilization pathway is sensitive to the *lexA-3* mutation, while the ROS pathway is not. In another example, fluoroquinolone PD161144 kills *E. coli* in the presence of chloramphenicol, agents that interfere with hydroxyl radical accumulation, and during anaerobic growth, none of which occur with oxolinic acid. Thus, there is little doubt that a distinct pathway occurs, but the underlying mechanism is far from established.

The connection between cleaved complex destabilization and chloramphenicol-insensitive killing allows conclusions to be drawn about effects of quinolone structure. For example, the N-1 cyclopropyl group appears to be important for complex

destabilization, since this group is the only difference between ciprofloxacin, a compound that kills in the presence of chloramphenicol, and norfloxacin, a compound that does not. The C-8-methoxy group is also important, since it increases chloramphenicol-insensitive killing by fluoroquinolones. A third factor is the structure of the C-7 moiety. With mycobacteria, the large C-7 ring system of moxifloxacin increases chloramphenicol-insensitive lethality relative to that of gatifloxacin, another C-8-methoxy compound with a small C-7 ring. With *E. coli*, the C-7 N-ethyl piperazine of PD161144 confers maximal activity. This effect of quinolone structure on ternary complex destabilization can be explained if the N-1 cyclopropyl and C-8 methoxy groups enhance binding to helix-4 while the C-7 rings, pointing across the GyrA-GyrA dimer interface, destabilize the dimer interaction. The subunit bridging model derived from C-7 ring-GyrA-81 crosslinking (see last paragraph of section 'Cleaved Complexes') could allow such an interaction.

Genes Involved in Fluoroquinolone Resistance

Clinical fluoroquinolone resistance arises in a step-wise fashion in which each mutation increases the probability that a given strain will acquire another mutation. The gradual hill-climb to resistance can begin with mutations that only moderately lower susceptibility or with high-level target mutations that can confer resistance in a single step. Continued quinolone exposure selects additional mutations. Below we briefly describe the major types of resistance.

Permeability-Based Resistance

Gram-negative bacteria have two cell envelope membranes. In enterobacteria, the outer membrane is composed of two layers, an outer surface of lipopolysaccharide and an inner phospholipid bilayer. Underlying the phospholipid is a peptidoglycan network. Proteins called porins serve as channels for certain hydrophilic molecules; decreased quinolone susceptibility can correlate with a deficiency in particular porin protein. The highly charged lipopolysaccharide probably serves as a permeability barrier to hydrophobic quinolones, since alterations in that layer can also affect quinolone susceptibility.

In *E. coli*, membrane-associated quinolone resistance sometimes arises following selection for resistance to chloramphenicol or tetracycline. This phenomenon is explained in part by the multiple antibiotic resistance (Mar) system affecting expression of the OmpF (outer membrane protein F) porin. Mutation of *marA* increases expression of *micF*, a gene encoding an RNA that post-transcriptionally decreases *ompF* mRNA. Therefore, upregulation of MarA reduces the amount of OmpF and thereby uptake of quinolone. The influence of *marA* on quinolone uptake is not restricted to OmpF, since susceptibility of *marA* mutants is lower than when *ompF* is simply deleted and since deletion of *micF* in a *marA* mutant fails to restore wild-type susceptibility. Thus a complex regulatory network probably governs entry of particular compounds into the cell.

Efflux-Based Fluoroquinolone Resistance

Fluoroquinolone resistance due to efflux generally arises from up-regulation of genes involved in removal of toxic agents from cells. Gram-negative bacteria contain two types of efflux system. One type is composed of at least three proteins, a transporter located in the cytoplasmic membrane, an outer membrane channel, and a periplasmic linker. The transporter proteins fall into two general groups. One is called the major facilitator superfamily (MFS), which is characterized by 12–14 transmembrane helices and uses proton motive force for energy. MFS members export nalidixic acid. The other family is called RND (resistance-nodulation-cell-division) superfamily. Proteins in this group contain 12 transmembrane helices plus two large periplasmic domains that are absent in the MF family. RND systems also use proton motive force to export fluoroquinolones and other antimicrobial agents.

The *E. coli* genome has 29 potential efflux pumps, nine of which are known to export drugs when expressed at high level. The AcrAB-TolC system is among the better characterized. This three-component system crosses both the inner and outer membranes. The inner membrane protein, AcrB, is a transporter of the RND type. It exports many agents including fluoroquinolones, tetracycline, β-lactams, chloramphenicol, erythromycin, rifampicin, disinfectants, dyes, and organic solvents. The TolC protein, which is anchored in the outer membrane, forms a long channel that spans both the outer membrane and the periplasmic space. AcrA is a lipoprotein located in the periplasm that is thought to coordinate AcrB and TolC activity. Strains deficient in the AcrAB proteins are hypersusceptible to quinolones. Conversely, fluoroquinolone-resistant efflux mutants often overproduce the periplasmic protein AcrA, which lowers the accumulation of ciprofloxacin and ethidium bromide. It also decreases susceptibility to tetracycline, ampicillin, and chloramphenicol.

Two global regulatory systems, the *marRAB* and *soxRS* systems, are involved in expression of the AcrAB-TolC system. The MarA (multiple antibiotic resistance A) and SoxS proteins are positive regulators; MarR and SoxR are repressors that down-regulate *marA and soxS*, respectively. Fluoroquinolone-resistance mutations sometimes map in the repressor genes and sometimes in the operators of the *mar* or *sox* operons. The two positive regulators, MarA and SoxS, also down-regulate OmpF, which, as pointed out above, is an outer membrane protein that facilitates fluoroquinolone uptake. Thus increased efflux can be coupled to decreased permeability.

Among Gram-positive bacteria, two principal efflux systems have been described, both of which fall in the MF family. One is called the Bmr (Bacillus multidrug resistance)-NorA (norfloxacin resistance) group; the other is termed the QacA (quaternary ammonium compound export) group. Some staphylococci also produce the Smr (small multidrug resistance) pump, an unusually small transporter that contains only four transmembrane helices.

The ability to efflux fluoroquinolones is associated with a propensity to acquire additional fluoroquinolone resistance determinants. In the case of an inhibitor of efflux or with efflux-deficient strains, recovery of resistant mutants decreases, sometimes by orders of magnitude. Conversely, fluoroquinolone-resistant efflux mutations are associated with an increase in the recovery of additional fluoroquinolone-resistance determinants. These effects are likely to arise from changes in intracellular drug concentration, since mutant recovery for many species depends strongly on fluoroquinolone concentration, as discussed below.

Qnr

Examination of plasmids for quinolone resistance led to the discovery of a protein family called Qnr. Qnr proteins can profoundly influence cleaved complex stability and interfere with quinolone action. When gene probes are used to screen clinical isolates for members of the Qnr family, about 8% (6/78) of *E. coli* isolates of Chinese origin are found to contain plasmid-borne *qnrA*. A similar result was later found with *E. coli* and *K. pneumoniae* isolates from the United States. These results pose a new challenge for maintaining the quinolones as effective antibacterial agents.

Target-Enzyme-Based Resistance

As pointed out in previous sections, gyrase and topoisomerase IV are the intracellular targets of the quinolones; mutations in the gyrase genes (*gyrA*, *gyrB*) and topoisomerase IV genes (*parC*, *parE*) lower susceptibility. For both *gyrA* and *parC*, resistance mutations map within a narrow stretch of nucleotides termed the quinolone-resistance-determining region (QRDR), which in *E. coli gyrA* encodes amino acids 51 through 106 (in *S. aureus*, mutations that lower susceptibility are distributed over a larger region). Resistance mutations mapping in *gyrB* and *parE* often reduce susceptibility by only moderate amounts, and they tend to be acquired after *gyrA* and *parC* alleles are fixed in the population. Most clinically resistant isolates contain a mutation in either *gyrA*, *parC*, or both. Those mutations generally alter amino acids at *E. coli* GyrA positions 83 and 87 as pointed out above.

Quinolone Concentration and Acquisition of Resistance

The quinolones are widely known as inducers of the mutagenic SOS response. The importance of this property was emphasized when an *E. coli* infection of mouse thigh was treated with quinolone: many more resistant mutants were recovered when the infecting strain was wild-type than when it carried a *lexA* mutation that prevents induction of the SOS regulon.

Quinolone concentration is expected to have two effects on the induction of resistance. First, it must be high enough to induce the SOS response. Second, it must not be too high, since cells will be killed before mutant gyrase replaces susceptible gyrase or before other quinolone-resistance mutations are fixed in the population. Thus, a threshold concentration should exist above which induced mutations are not fixed. The traditional approach for quinolone dosing has been to use MIC as a threshold (for fluoroquinolones, values of MIC are often only slightly higher than values of minimal bactericidal concentration (MBC), making it likely that drug concentrations above MIC kill susceptible cells and reduce the size of the bacterial population). To accommodate fluctuating drug concentrations during treatment, concentration is expressed by the pharmacokinetic term AUC (area under the time-concentration curve), often obtained by administering drug to human volunteers. Combining AUC with bacterial susceptibility (MIC) produces a term (AUC/MIC) that estimates drug exposure in the host. AUC/MIC is then measured and related empirically to favorable clinical outcome, which is assumed to also restrict the acquisition of resistance.

Traditional pharmacodynamic approaches for restricting mutants, popularly expressed by the phrase 'dead bugs don't mutate,' do not consider mutant subpopulations that are present prior to initiation of therapy: quinolone concentrations that kill susceptible cells do not necessarily kill mutant subpopulations and can allow those mutants to amplify if concentrations fall with a specific range called the mutant selection window. The boundaries of the range were defined experimentally by measuring the recovery of mycobacterial mutants from quinolone-containing agar. As drug concentration is increased, the number of colony-forming units recovered drops sharply, levels to a broad plateau, and then drops sharply a second time. The sharp drop at low concentration occurs when the growth of wild-type cells is inhibited. This concentration, which is approximated by MIC, defines the lower boundary of the selection window, since little selective pressure exists at drug concentrations below such a boundary. The second sharp drop defines the upper boundary of the window. The upper boundary, which is the concentration that blocks growth of the least susceptible, single-step mutant, is called the mutant prevention concentration (MPC). For growth above that concentration, susceptible bacterial cells must acquire at least two resistance mutations concurrently, an event that is expected to occur rarely.

Examination of *M. smegmatis* mutants selected inside the window reveals that low concentrations of fluoroquinolone select nongyrase mutations, of which about 60% exhibit cross-resistance to ampicillin and chloramphenicol. As fluoroquinolone concentration is increased, these variants decrease in prevalence and *gyrA* mutants increase. At high fluoroquinolone concentration, colonies arise only from the GyrA variant that has the highest MIC. Eventually the MPC is exceeded, and colony recovery occurs only very rarely. *Streptococcus pneumoniae* exhibits a similar phenomenon: non-topoisomerase mutants are obtained by challenge with low fluoroquinolone concentration, and increasing the drug concentration shifts mutant recovery to either GyrA or ParC variants,

depending on the particular fluoroquinolone being examined. Thus low doses or marginally effective derivatives are more likely than high doses or highly effective compounds to enrich pre-existing and induced mutants that can then serve as populations for induction of even higher resistance. However, maintaining drug concentration above the selection window, above MPC, should restrict the selective amplification of resistant mutant subpopulations.

Once a target (gyrase or topoisomerase IV) mutant has been enriched, a change occurs in the relationship between fluoroquinolone concentration and the recovery of additional resistance mutations. For example, with *Streptococcus pneumoniae*, the steep dependency of mutant recovery on drug concentration shifts to a broad plateau with an elevated value of MPC (the window widens). With *Haemophilus influenzae*, each successive mutation raises the selection window (the window increases its overlap with drug concentrations achievable in the patient). Both phenomena increase the ease with which additional resistance mutations can be acquired. Thus controlling resistance becomes more difficult as resistance mutations accumulate in bacterial populations.

Plasmid-Mediated Resistance

Fluoroquinolone resistance genes carried by plasmids pose a serious threat to quinolone efficacy for two reasons. First, plasmids can carry genes for resistance to multiple antibiotics, which means that fluoroquinolone resistance can be acquired through the use of other antimicrobial classes. Second, plasmids can enter a bacterial population at a much higher frequency than spontaneous resistance mutations. That makes cells carrying plasmid-mediated resistance a larger fraction of the bulk population and more likely to become the dominant class when fluoroquinolones are applied.

The best-studied family of plasmid-mediated fluoroquinolone-resistance genes is called *qnr*. Five major family groups, termed *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*, have been identified, and some family groups have many members (seven forms of *qnrA* and 42 forms of *qnrB* have been found). Thus, the *qnr* determinants are highly diverse. Surveys that revealed Qnr diversity also show that Qnr-expressing plasmids are globally distributed and that they occur in a wide variety of bacterial species. Since plasmids carrying a QnrA determinant often mediate resistance to aminoglycosides, β-lactams, chloramphenicol, and sulfonamides, Qnr is likely to be a major contributor to antimicrobial resistance in general.

A second type of plasmid-borne resistance gene expresses the quinolone-acetylating enzyme Aac (6′)-Ib-cr. This factor lowers the activity of compounds, such as ciprofloxacin and norfloxacin, by about 4 fold. The geographical distribution of *aac (6′)-Ib-cr* has not been studied as thoroughly as that of *qnr*. Nevertheless, it is clear that drug-modification-based resistance is widely distributed. As with *qnr*, *aac (6′)-lb-cr*-containing plasmids are found in a variety of Enterobacteriaceae, and the prevalence of *aac (6′)-lb-cr* can be substantial. Since *aac (6′)-Ib-cr* is part of an integron cassette, it is likely to move readily among plasmids. Indeed, fluoroquinolone-resistance plasmids are conjugative and tend to have a broad host range.

Plasmid-mediated quinolone resistance also involves efflux pumps. The QepA efflux pump increases MIC about 10-fold for hydrophilic fluoroquinolones, such as norfloxacin and ciprofloxacin. So far, the prevalence of QepA-mediated resistance in humans is low. Another *E. coli* efflux system, *oqxAB*, has recently been found in food animals in China and Denmark, and plasmid-mediated efflux has been reported in *S. aureus*. Such reports are likely to increase now that laboratories are looking for efflux determinants.

Plasmid-mediated resistance appears to derive from mobilization of chromosomal genes followed by enrichment due to environmental contamination with quinolones. The presence of integrons in conjugative plasmids is known to mobilize other chromosomal genes; thus, a potential mechanism is available. Since some aquatic bacteria contain chromosomal genes for *qnrA* and *qnrS*, the idea has developed that gene mobilization occurred within these bacteria. The selective pressure needed to amplify the resistant, plasmid-bearing strains could easily result from massive use of quinolones and contamination of aquatic environments. It is reasonable to assume that resistance genes then move from the aquatic environment to food animals and eventually to humans.

Concluding Remarks

One of the main clinical problems with quinolones is the development of bacterial resistance. Resistance genes are now being disseminated by plasmids, and the compounds themselves induce resistance through the mutagenic SOS response. Moreover, resistance arises spontaneously, producing mutant subpopulations that can be selectively amplified by traditional treatment strategies. Since many different resistance alleles exist and since resistance develops in a gradual, step-wise manner, many paths can be taken to reach clinical resistance. It is possible to adjust drug concentrations to force bacteria to acquire two or more mutations concurrently for growth. Such mutant-restricting concentrations are likely to be lower for compounds that kill resistant mutants. Thus, new quinolones, and indeed all new antimicrobials, should be developed to be highly lethal with resistant mutants.

Although it is possible to define doses that will restrict the emergence of resistance, changing treatment protocols to protect existing quinolones is difficult. Part of the problem is that mutant-restricting doses are higher than usually needed to cure disease, and they may increase the frequency of toxic side-effects. Consequently, the mutant selection window hypothesis will not be applied until medical philosophy changes from seeking cure to restricting resistance.

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Quorum-Sensing in Bacteria

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Glossary

AHLs Acylhomoserine lactones, quorum signal molecules produced by many Gram-negative bacteria.
autoinducer Molecule produced by a cell that causes self-regulation of pathways within the producing cell above a threshold concentration.
biofilm A community of bacteria associated with a surface.
bioluminescence The production of visible light by living organisms.
biomining The utilization of microorganisms to extract substances from rocks and minerals.
competence The ability of a cell to uptake and utilize DNA from outside sources.
conjugation The physical transfer of DNA from one organism to another.
lantibiotic Antibiotic peptides that contain the amino acid lanthionine.
mucinase Enzyme that degrades the glycoprotein material mucin.
orphan regulator Transcriptional regulators that are not part of a dedicated signal synthase/response system but can interact with the same signal.
synthase A class of enzymes that catalyze biosynthetic reactions of many compounds including quorum signals.

Abbreviations

AHL	Acylhomoserine Lactone
AIP	autoinducing peptide
CSP	competence-stimulating peptide
CT	cholera toxin
DPD	4,5-dihydroxy-2,3-pentanedione
EHEC	enterohemorrhagic *E. coli*
EPS	exopolysaccharide
GBAP	gelatinase biosynthesis-activating pheromone
HHQ	4-hydroxyl-2-heptyl-quinoline
LEE	locus of enterocyte effacement
PQS	Pseudomonas Quinolone Signal
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SRH	S-ribosylhomocysteine
TCP	toxin coregulated pilus

Defining Statement

Bacteria utilize numerous mechanisms to monitor and adapt to their external environment. One such mechanism involves the ability to 'count' their local population numbers. Once a specific number of cells, or quorum, is reached, bacteria are able to modify their group behavior through a mechanism known as quorum sensing.

Introduction

Bacterial growth and survival is dependent upon the ability of an organism to sense its environmental conditions and respond to external stimuli. Stimulus can come from a variety of sources including the nutrients available for growth, the presence of secondary metabolites, and the presence of other microorganisms. In the case of nutrients and secondary metabolites, sensor and regulatory proteins exist that affect a change in gene expression as these compounds change concentration. These changes in expression can

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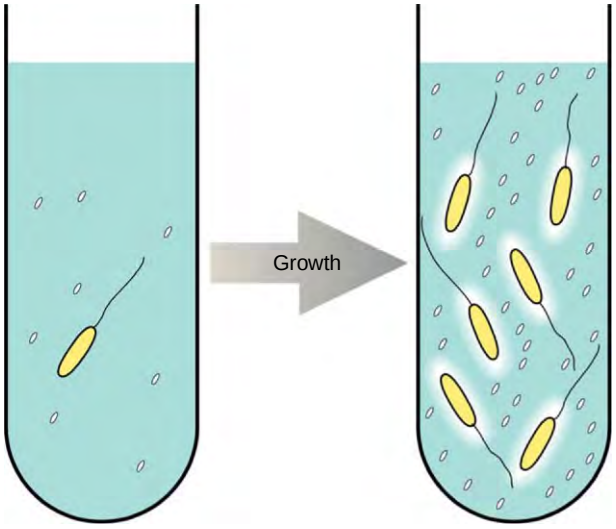

Figure 1 Cell density-dependent bioluminescence. As cells multiply, autoinducer concentrations increase. At a critical concentration, they induce synthesis of bioluminescence genes and subsequent light production.

cause bacteria to synthesize new proteins for catabolism in the case of diauxic growth, can lead to the production of extracellular enzymes to liberate nutrients from the environment in the case of starvation, or can cause changes in motility, chemotaxis, or metabolism that allow the bacteria to avoid or eliminate toxic concentrations of a secondary metabolite. In addition to these basic stimulus–response events, bacteria have evolved a mechanism to 'count' the local cell population. This process is known as quorum sensing and is mediated by the bacterium's ability to produce and recognize soluble factors known as quorum signals.

The ability of bacteria to utilize extracellular signals to modify their behavior in a cell density-dependent manner was first described in the early 1970s by Kenneth Nealson, Terry Platt, Woodland Hastings, and Anatol Eberhard. These researchers discovered that the bioluminescent bacterium *Vibrio fischeri* only produced light when bacterial cell numbers were high (Figure 1). They also demonstrated that culture supernatants from high cell density cultures were able to stimulate light production in low cell density cultures. This phenomenon was deemed autoinduction and provided the first clues that bacteria were able to utilize a soluble extracellular signal to monitor population density.

Subsequent work characterized the autoinducer of *V. fischeri* and the genes responsible for its synthesis and detection by the cell. As autoinducer-related genes were studied, it was discovered that many other Gram-negative bacterium contained the genes necessary for autoinducer synthesis and detection. It was demonstrated that autoinducer sensing was dependent upon a critical number of cells in a defined volume. This threshold density of cells was first referred to as a quorum by Clay Fuqua, Stephen Winans, and Peter Greenberg in 1994, and they proposed the term *quorum sensing* to describe this event.

Quorum sensing has rapidly expanded as a field of study, and the discovery of new quorum signaling bacteria as well as new types of quorum signals is increasing at an ever growing rate. Quorum signals in Gram-negative species such as *V. fischeri* were the first to be studied at the genetic and chemical levels; however, a significant amount of work has subsequently been performed with Gram-positive species. It is notable that quorum signal-dependent behavior was observed in Gram-positive bacteria long before the discovery of autoinducers or quorum sensing.

Quorum Signaling in Gram-Positive Bacteria

Although the molecular mechanism was unknown, the physiological effects of quorum signaling were known for a considerably long time in some Gram-positive species. Early work in bacterial genetics determined that some bacterial species were able to participate in horizontal gene transfer through natural competence. Several pioneering studies were done with organisms such as *Streptococcus pneumoniae* well before it was known that the process was quorum signal-dependent. Gram-positive species typically utilize small polypeptide signals as quorum signals. Some of these polypeptides undergo significant modifications to become a soluble extracellular signal (Figure 2).

Streptococcus pneumoniae ComC

S. pneumoniae is a common causative agent of pneumonia, otitis media, meningitis, and several other diseases. Genetic transformation studies were carried out as early as 1928 in this organism by Frederick Griffith. Subsequent work by Avery, McCarty, and MacLeod in 1944 demonstrated that genetic transformation in *S. pneumoniae* was due to uptake of extracellular DNA. Competency

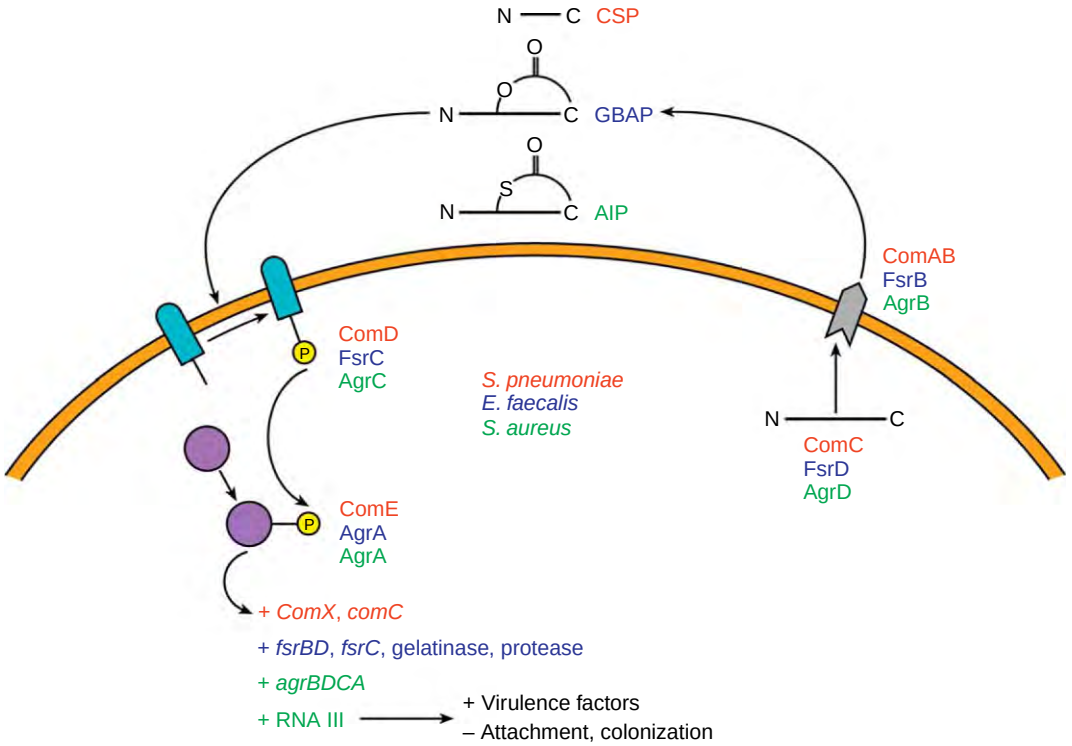

Figure 2 Quorum sensing peptides of Streptococcus pneumoniae, Enterococcus faecalis, and Staphylococcus aureus. Polypeptides ComC, FsrD, and AgrD are modified and exported out of the cell by the ComAB, FsrB, or AgrB transporters. Peptide autoinducers bind to the sensor kinases ComD, FsrC, or AgrC on the cell surface and initiate a phosphorelay to a cytoplasmic response regulator ComE or AgrA, which upregulates cell density-dependent gene expression.

in *S. pneumoniae* is regulated by several factors but is primarily controlled by the competence stimulating peptide (CSP) quorum signal encoded by the gene *comC*.

Genetic competence in *S. pneumoniae* occurs during exponential phase and is initiated by CSP as cell density increases. The 17-amino acid CSP is produced by posttranslational modification of the 41-amino acid precursor peptide ComC and is exported from the cell by the ComAB ABC transporter. When extracellular CSP reaches a threshold concentration due to increasing cell number, it is recognized by the sensor histidine kinase ComD. ComD autophosphorylates and subsequently transfers its phosphate group to the response regulator

ComE. Phosphorylated ComE activates transcription of the alternative sigma factor ComX, which goes on to induce expression of multiple genes involved in DNA uptake and recombination (Figure 2).

This peptide-induced, two-component signal transduction system is typical for Gram-positive quorum signaling circuits and was among the first to be characterized. Induction of competency at high cell density allows *S. pneumoniae* to sample and incorporate genetic material from its environmental counterparts. Surprisingly, *S. pneumoniae* competency permits the organism to take up DNA irrespective of its sequence or host origin. Such behavior gives *S. pneumoniae* the ability to adapt to selective pressures by taking up genes from neighboring cells that may have acquired genes or mutations that allow for enhanced fitness in a specific growth environment.

Enterococcus faecalis FsrB/D

E. faecalis is an opportunistic pathogen that causes endocardial, urinary tract, epidermal, and septic infections in clinical environments. *E. faecalis* is resistant to many common antibiotics and has recently acquired resistance to more modern antibiotic types making it a dangerous human pathogen. *E. faecalis* quorum sensing uses a cyclic lactone-modified peptide signal known as the gelatinase biosynthesis-activating pheromone (GBAP). GBAP is an 11-residue, circular polypeptide closed by a lactone moiety not seen in other Gram-positive-produced cyclic peptides. GBAP is not produced by all *E. faecalis* strains, but its production has been observed in all strains that produce the virulence factor gelatinase.

Originally, the *E. faecalis* GBAP signal was thought to be derived from the C-terminal region of the 212-amino acid FsrB protein. Recent studies have shown that a previously unknown gene, *fsrD*, exists in frame with *fsrB*, and GBAP is derived from FsrD by proteolytic activity of the FsrB protein. The FsrB protein was originally thought to autocatalyze by simultaneously forming and modifying GBAP while exporting it out of the cell. This new data show that the N-terminal region of FsrB is necessary for modification of the previously unknown FsrD peptide to produce the mature GBAP signal.

GBAP is produced maximally at the end of exponential phase in *E. faecalis*. When GBAP concentration reaches a density-dependent threshold, it induces autophosphorylation of the sensor kinase FsrC followed by phosphotransfer to the AgrA protein.

Phosphorylated AgrA is a response regulator that promotes expression of *fsrBD*, *fsrC*, and the virulence factors gelatinase and serine protease (Figure 2). Recent transcriptome analyses of *fsrBD* mutants have shown indirect regulation of genes involved in biofilm formation, surface protein synthesis, and carbon source uptake, as well as direct regulation of an uncharacterized open reading frame. Interestingly, GBAP production is critical for successful colonization in an animal model. It is hypothesized that density-dependent regulation of gelatinase and serine protease by GBAP at the onset of stationary phase allows *E. faecalis* to liberate nutrient sources in the host as they become limiting.

Staphylococcus aureus AgrD

S. aureus is a human pathogen that causes toxic shock syndrome, food poisoning, and epidermal and endocardial infections. The prevalence of antibiotic-resistant *S. aureus* strains has placed it among the most commonly encountered nosocomial infections. *S. aureus* uses the accessory gene regulation (*agr*) system to regulate numerous genes involved in virulence and colonization in a densitydependent fashion via an autoinducing peptide (AIP) signal.

AIP is a thiolactone containing octapeptide ring derived from the 46-amino acid protein AgrD. To form AIP, AgrD is proteolyzed, modified by addition of a thiolactone moiety and exported from the cell by AgrB. Extracellular AIP is detected by the AgrC sensor histidine kinase, which autophosphorylates when bound to AIP. Phospho-AgrC transfers its phosphate group to the response regulator AgrA. The AgrC-AgrA two-component sensor kinase system upregulates the *agrBDCA* operon, establishing a positive feedback loop at sufficient extracellular AIP concentrations. AgrA also induces transcription of the regulatory RNA, RNAIII. RNAIII is an untranslated RNA that upregulates many *S. aureus* virulence factors including toxins and extracellular proteases while down-regulating low-density genes involved in attachment and colonization (Figure 2). Thus, AIP signaling provides *S. aureus* with the ability for transition from colonization and survival at low cell density to pathogenesis and nutrient acquisition at high cell density. This behavior has been hypothesized to allow *S. aureus* to establish a colonization site in the host before virulence factor activity stimulates the host-immune response.

The *S. aureus* quorum sensing and response pathway is very similar to the one found in *E. faecalis*; however, there are notable differences including an increased number of AIP-regulated genes as well as strain specificity of the AIP signal. *S. aureus* AIP has been shown to modulate expression of a larger number of genes, a feat accomplished by induction of RNAIII, and other transcriptional regulators. AIP signaling has also been shown to be strain-specific. There are four known *agrD* specificity groups in *S. aureus* strains. AIP from a single specificity group has been shown to inhibit the AgrC sensor kinase of other groups. Thus, *S. aureus* has acquired a way to compete against other strains of its own species in an infection site when an individual strain reaches a threshold cell density. Therefore, the first strain in a single specificity group to achieve autoinduction of the *agr* pathway will induce its own virulence factor production and block quorum signaling activity in other strains. Quorum sensing systems similar to AIP are present in other *Staphylococcus* species, and these systems exhibit group strain competition as well.

Bacillus subtilis ComX and Phr Peptides

B. subtilis is a soil bacterium that can undergo spore formation or display genetic competence upon entry into stationary phase. Roughly 10% of *B. subtilis* cells entering stationary phase become competent. Competency in this situation is thought to aid *B. subtilis* in DNA repair and contribute to the inheritance of genetic material from *B. subtilis* strains that have successfully grown to high cell density. The decision to become competent or to sporulate is influenced by many factors in a complex regulatory pathway through which quorum signals play a significant part.

The ComX peptide is a 10-amino acid linear peptide derived from a 55-amino acid precursor product of the *comX* gene. The original ComX polypeptide is cleaved, modified, and exported out of the cell by the ComQ protein. Extracellular ComX is sensed by the ComP sensor kinase. Above threshold concentrations of extracellular ComX, ComP phosphorylates the response regulator ComA, which induces the *comS* gene. ComS blocks proteolysis of the transcriptional activator ComK, which upregulates the production of many genes that stimulate competency in the host cell (Figure 3).

A second quorum signaling pathway utilizes multiple proteinaceous pheromone (Phr) signals in a complex regulatory circuit (Figure 3). Phr precursors are proteolyzed and exported out of the cell as linear pentapeptides. Extracellular Phr is taken up into the cell by an ABC oligopeptide transporter. Multiple Phr genes exist and serve to antagonize the activity of several Rap phosphatase enzymes, which regulate many steps along the sporulation and competence pathways. For example, extracellular PhrA enters *B. subtilis* and binds directly to the RapA regulatory phosphatase either preventing it from binding or disassociating it from bound Spo0Fphosphate. RapA bound to the intermediate response regulator Spo0F-phosphate prevents a phosphorelay cascade to the global response regulator Spo0A, thereby preventing sporulation. Thus, increased PhrA levels are one of many factors that allow sporulation to begin by interrupting Spo0F inhibition of Spo0A. In addition to PhrA, there are several other Phr-type signals that are recognized by *B. subtilis* at the end of exponential phase and help direct the organism to specialize in sporulation or competence.

Lactococcus lactis Nisin

L. lactis is a fermentative bacterium commonly used in the dairy industry to produce buttermilk and cheese. *L. lactis* is classified as a lactic acid bacterium as it produces large amounts of lactate upon fermentation of sugars found in dairy products. *L. lactis* produces

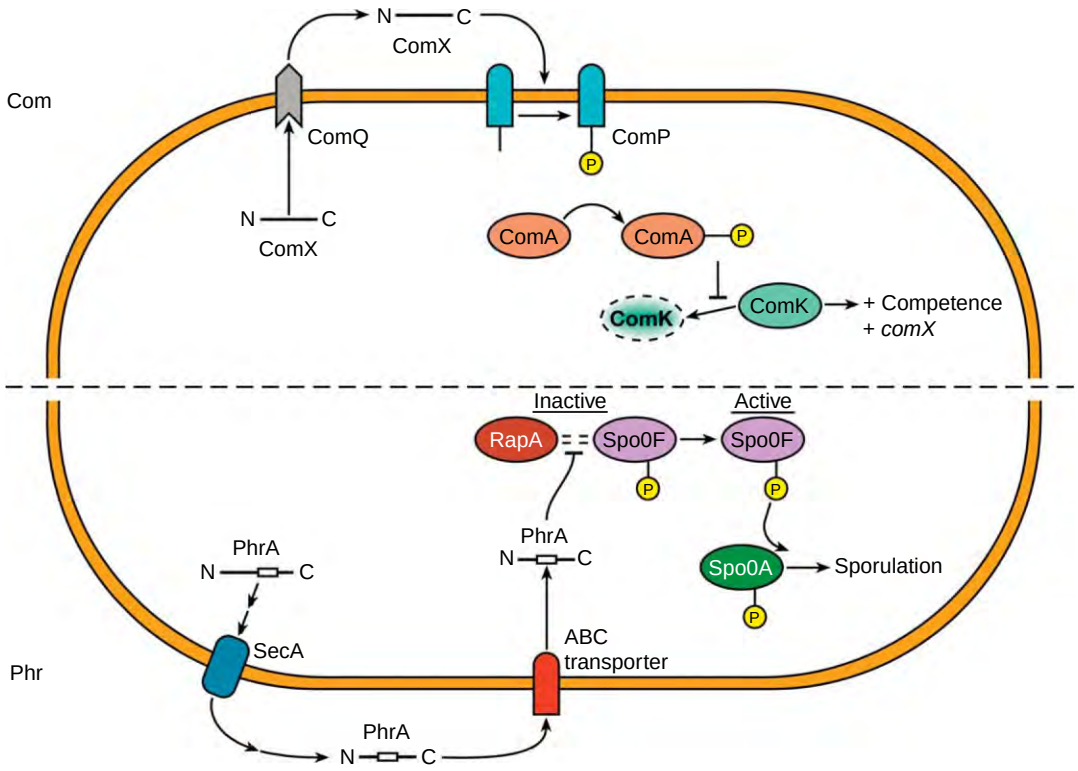

Figure 3 Quorum sensing pathways in Bacillus subtilis. In the Com system (upper panel), the ComX protein is cleaved, modified, and exported out of the cell by the ComQ transporter. Mature ComX interacts with the ComP sensor kinase that phosphorylates ComA. ComA-phosphate blocks the turnover of the transcriptional regulator ComK, which induces comX and competence gene expression. The Phr system (lower panel) utilizes the PhrA protein, which is cleaved and exported out of the cell using the Sec transport system. Mature PhrA returns to the cytoplasm via an oligopeptide ABC transporter and interferes with RapA binding to Spo0F-phosphate. Spo0F-phosphate can then phosphorylate the response regulator Spo0A, which induces sporulation genes.

the antimicrobial peptide nisin, which was discovered in 1928 due to its ability to inhibit growth of other lactic acid bacteria. In 1988, nisin was approved by the FDA for use as a preservative agent in the food industry.

Nisin is a Class I bacteriocin or lantibiotic characterized by its small size and the inclusion of the amino acids lanthionine and -methyllanthionine as well as other dehydrated amino acids. Nisin is bactericidal and forms pores in bacterial membranes. This activity is especially effective against other Gram-positive bacteria where cell membrane perforations immediately cause a loss of membrane integrity.

Despite its role as an antimicrobial agent, nisin also serves as a density-dependent signal for *L. lactis*. Nisin is a highly modified 34-amino acid product of the 57-amino acid polypeptide NisA. NisA is modified and exported out of the cell by the NisBCT membrane-associated complex. Upon leaving the cell, modified NisA is cleaved to form nisin by the extracellular NisP protease. Damage of the parent cell membrane by nisin is prevented by the production of several proteins that block nisin activity at the cell surface. Extracellular nisin concentration is sensed by the NisK sensor kinase, which phosphorylates and activates the NisR response regulator. Nisin production is maximal during early stationary phase growth. NisR promotes expression of the *nisA* gene as well as genes that encode for proteins that block nisin activity at the cell surface, allowing for a positive feedback loop to upregulate nisin production as *L. lactis* enters stationary phase. Interestingly, the production of nisin demonstrates the ability of a compound to act as both a quorum signal and an antimicrobial agent. This type of dual function by an extracellular compound provides *L. lactis* with an efficient and powerful quorum signaling system that might provide a notable competitive advantage in polymicrobial environments.

Acylhomoserine Lactone (AHL) Quorum Signals in Gram-Negative Bacteria

The distribution of Gram-negative and Gram-positive bacterial species throughout the world is highly similar. Both types of organisms inhabit nearly every known niche on the planet and are involved in biogeochemical mineral cycles, biochemical degradation, disease, and the production of a staggering number of extracellular small molecules. While Gram-positive species have largely evolved to utilize polypeptide quorum signals that depend on cell-surface receptors, Gram-negative bacteria often utilize soluble signals known as AHLs. Many AHLs are able to pass through the lipid bilayer and are therefore able to interact with cytoplasmic regulatory proteins; thus, AHLs do not rely on the phosphorelay cascades that Gram-positive quorum sensing

pathways commonly use. Both types of signals are effective due to their stability and solubility in environments colonized by the organisms that synthesize them.

V. fischeri LuxI/R

As mentioned previously, quorum signaling was discovered in the luminescent marine bacteria *V. fischeri* and *Vibrio harveyi*. In the early 1970s, researchers observed that supernatants from stationary phase cultures could be added to cells at low density and trigger light production. This suggested the presence of an autoinducing compound made in later phases of *V. fischeri* and *V. harveyi* growth. Further study determined that the factor in spent medium was relatively species-specific and dependent on cell density rather than the nutritional status of the cells. In 1981, the *V. fischeri* autoinducer was purified and determined to be the AHL 3-oxo-hexanoyl-HSL ($3OC_6$-HSL). A variety of AHL compounds have been characterized from other organisms and are collectively referred to as autoinducer-1 (AI-1).

V. fischeri can be found free-swimming and can also participate in a symbiotic relationship with the Hawaiian bobtail squid *Euprymna scolopes*. *V. fischeri* colonizes a cavity on the squid host known as the light organ. *V. fischeri* receives nutrients from the host in the light organ and emits light as its population density and AI-1 concentrations increase. Luminescence from the light organ of *E. scolopes* is thought to help the squid evade predation by masking the organisms' shadow in shallow water. *V. fischeri* has also been observed to enter symbiotic relationships with other marine organisms such as the fish *Monocentris japonicus* where a *V. fischeri*-dependent light organ is used to attract a potential mate.

The *V. fischeri* LuxI protein synthesizes 3-oxo-C6-HSL from *S*-adenosylmethionine (SAM) and acylated acyl carrier protein. Once synthesized, $3OC_6$-HSL freely diffuses across the membranes and out of the cell. At a particular density, a critical concentration of AI-1 is reached that stimulates AI-1 to interact with and activate the response regulator LuxR. Activated LuxR promotes transcription of the *luxR* gene as well as the *luxICDABEG* operon, which serves to produce light and generate more AI-1 (Figure 4). Thus, a positive feedback loop develops at sufficient cell densities, which allows for a substantial increase in light production. AI-1 exists in many Gram-negative species and is thought to function as an intraspecies-specific signal.

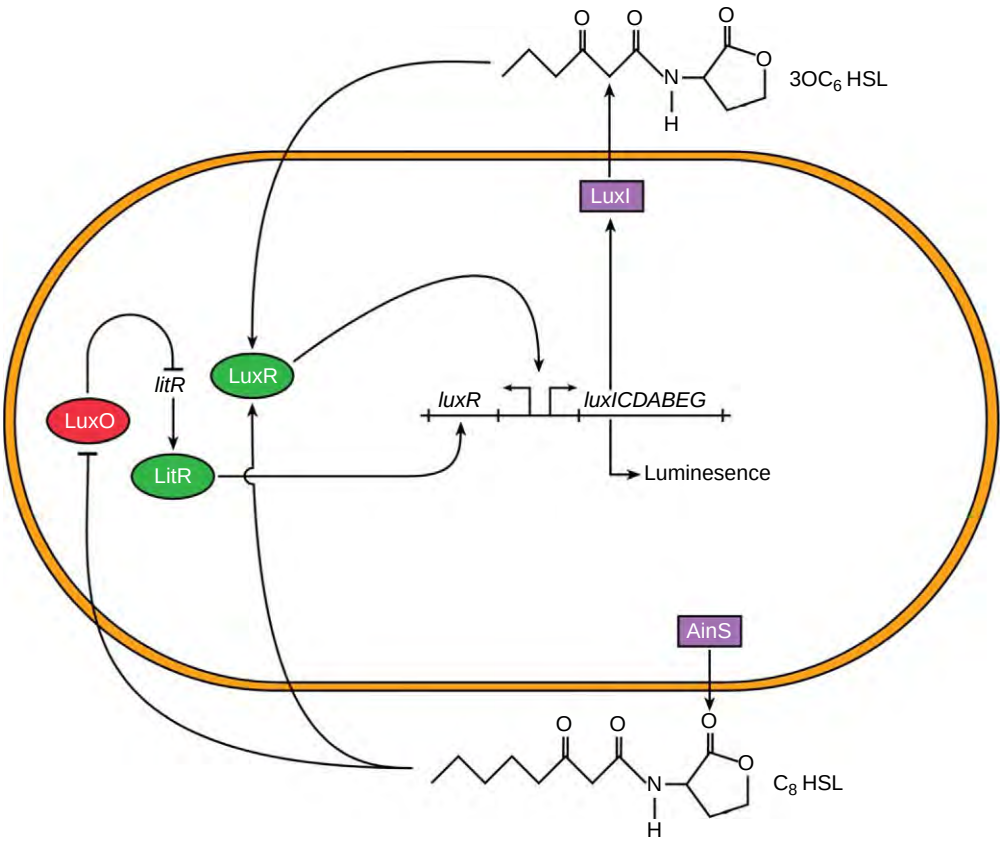

Figure 4 Quorum sensing in *Vibrio fischeri*. The LuxI-produced $3OC_6$-HSL diffuses out of the cell until it reaches sufficient concentration to induce the LuxR response regulator. $3OC_6$-HSL-LuxR induces expression of the *luxR* and the *luxICDABEG* genes, which increase synthesis of $3OC_6$-HSL as well as proteins involved in bioluminescence. At low cell density, C_8-HSL, produced by the AinS gene, provides initial activation of LuxR by direct activation as well as by inhibiting LuxO, preventing the *litR* gene product from inducing the *luxR* gene, thereby increasing levels of *luxR* transcript in the cell.

Recently, a second AHL quorum signal has been identified in *V. fischeri* and has been shown to function as part of a regulatory network in combination with $3OC_6$-HSL and a third signal, the furanosyl borate diester signal AI-2 (which will be discussed later). This AHL signal is *N*-octanoyl HSL (C_8-HSL) and is produced by AinS (Figure 4). C_8-HSL regulates luminescence at low culture densities by relieving negative regulation of the LuxR protein due to inactivation of the transcriptional regulator LuxO. Inactivation of LuxO allows upregulation of *litR*, whose gene product serves to activate *luxR* transcription. C8-HSL also interacts with LuxR directly, allowing for initial activation of the *luxICDABEG* operon.

Pseudomonas aeruginosa Las/Rhl AI-1 System

P. aeruginosa is a Gram-negative bacterium commonly isolated from soil environments, which is frequently used as a model organism to study quorum sensing. *P. aeruginosa* is an opportunistic pathogen that can cause an array of infections in immunocompromised individuals, most notably those inflicted with the heritable disease cystic fibrosis. Many of the genes necessary for infection, nutrient acquisition, virulence factor production, and biofilm growth are regulated by the concentrations of two AHL signals produced by LasI and RhlI. Due to its effects on virulence factor production, interference with quorum signaling in *P. aeruginosa* has been proposed as a therapeutic strategy. This novel approach to antimicrobial therapy in a notoriously antibiotic-resistant organism makes further study of quorum sensing in *P. aeruginosa* increasingly important.

The LasI protein synthesizes the signal *N*-(3-oxododecanoyl)-L-HSL ($3OC_{12}$-HSL). $3OC_{12}$-HSL interacts with the LasR response regulator, which upregulates the expression of multiple genes involved in virulence such as the *lasB* protease as well as the *lasI* gene itself, establishing $3OC_{12}$-HSL as an autoinducer. While $3OC_{12}$-HSL is diffusible, it can also partition into the cell membrane and has been shown to be exported by efflux pumps in *P. aeruginosa*.

The LasR-$3OC_{12}$-HSL complex serves to upregulate a second AHL-dependent system by inducing expression of *rhlR* (Figure 5). RhlI synthesizes *N*-butyryl-L-HSL (C_4-HSL), which interacts with the response regulator RhlR to regulate numerous genes including those involved in secondary metabolite production, as well as the *rhlI* gene that establishes a second AHL autoinduction loop. C_4-HSL-dependent regulation overlaps with $3OC_{12}$-HSL-mediated regulation in the control of several genes such as

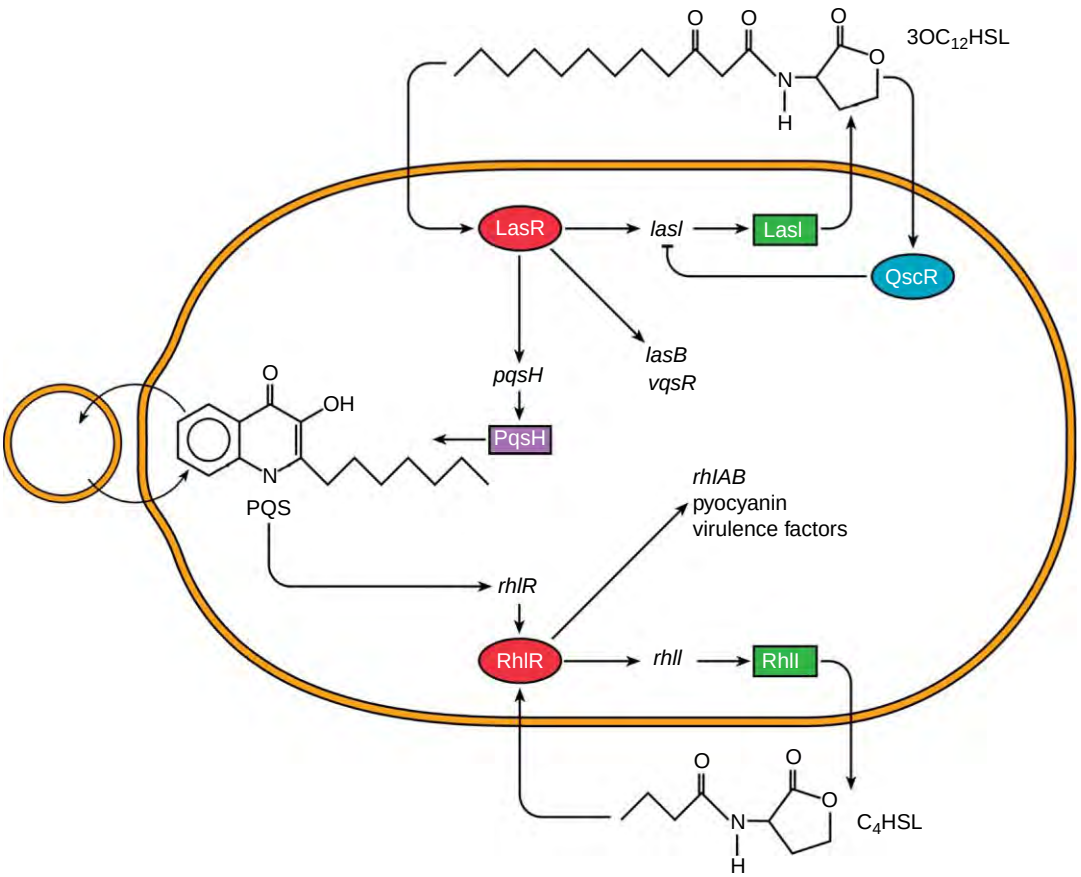

Figure 5 Quorum sensing in Pseudomonas aeruginosa. $3OC_{12}$-HSL produced by the LasI synthase interacts with the LasR transcriptional regulator, which upregulates lasI, lasB, vqsR, and pqsH. $3OC_{12}$-HSL also interacts with the orphan transcriptional regulator QscR to repress lasI expression. PqsH produces the quinolone signal PQS that is transported between cells via outer membrane vesicles. PQS induces expression of the rhlR gene whose product induces expression of the rhlI synthase gene as well as several other virulence factors when induced by the RhlI-produced C_4-HSL signal.

the extracellular protease encoded by the *lasB* gene. It is notable that $3OC_{12}$-HSL inhibits the interaction of C_4-HSL with its response regulator RhlR while inducing expression of the *rhlR* gene. This interaction serves to maintain levels of C_4-HSL low until RhlR concentration increases to levels sufficient to interact with the low levels of C_4-HSL produced in late exponential phase. Thus, the activation of the C_4-HSL–RhlI feedback loop becomes functional only after LasR-$3OC_{12}$-HSLdependent regulation has occurred.

Both quorum signals in *P. aeruginosa* ultimately serve to regulate the expression of over 300 genes involved in diverse pathways. Such regulation is not achieved by the LuxR-like LasR and RhlR response regulators alone. For example, LasR induces expression of the *vqsR* gene, whose product upregulates genes involved in virulence factor production and quorum signaling. Apart from downstream regulators induced by LasR or RhlR, 'orphan' LuxR-type regulators that do not have a cognate LuxI-type produced AHL signal have been discovered. QscR is a LuxR-type response regulator that binds the promoters of several genes in the presence of the LasIgenerated signal $3OC_{12}$-HSL. Apart from these interactions, QscR has been shown, at least indirectly, to repress *lasI* expression (and was in fact named for this activity, quorum signaling control repressor). The manner in which QscR represses *lasI* expression is unclear but has been hypothesized to involve heteromultimerization of QscR and LasR. Another hypothesis is that QscR binding to $3OC_{12}$-HSL prevents this AHL from coming in contact with its cognate regulator LasR. Interestingly, QscR has also been shown to be more promiscuous in AHL binding than either LasR or RhlR, suggesting a role in interspecies AHL sensing. Ultimately the HSL-dependent quorum signaling network combined with orphan response regulators contribute a great deal to global gene regulation in *P. aeruginosa*.

Agrobacterium tumefaciens *Tra System*

A. tumefaciens is a Gram-negative soil bacterium, which is the causative agent of crown gall disease in plants. Some *A. tumefaciens* strains carry a Ti (tumor-inducing) plasmid that contains the LuxI/R-type quorum signaling genes *traI* and *traR*. The Ti plasmid also carries genes that encode for specific plant hormones. After conjugation of the Ti plasmid from *A. tumefaciens* to the host nucleus, plants synthesize these hormones that then stimulate cell proliferation and tumor formation.

Free-swimming *A. tumefaciens* move throughout the soil by flagellar motility and search for susceptible hosts by chemotaxis toward metabolic intermediates released from wounded plants. Upon contacting the host, *A. tumefaciens* produces cellulose fibrils as well as attachment proteins to anchor it to host tissue. After attachment, *A. tumefaciens vir* (virulence) genes are induced by host-derived compounds that in turn activate synthesis of a secretion apparatus that directs translocation of the Ti plasmid from *A. tumefaciens* into the host. Ti plasmid-encoded hormones then cause cell proliferation and tumor formation. The Ti plasmid also contains genes whose products direct synthesis of opines. Opines are specialized amino acids that *A. tumefaciens* uses as a growth substrate. There are at least two known classes of opineencoding genes, octapine and nopaline, and *A. tumefaciens* strains or pathovars can be classified by which genes are present on the Ti plasmid. These plasmid-borne genes not only direct host synthesis of opines, but contain opine catabolism genes, allowing *A. tumefaciens* to use opines as a source of carbon and energy.

A. tumefaciens uses the TraI/R quorum sensing system to regulate conjugation of the Ti plasmid between *A. tumefaciens* strains. The *traI/R* quorum signaling genes are similar to the *luxI/R* genes from *V. fischeri*, but there are key differences in regulation. In octapine-utilizing pathovars, the *A. tumefaciens* transcriptional activator OccR is induced by the presence of the opine octapine. OccR positively regulates expression of *traI*, whose product synthesizes the *N*-(3-oxo-octanoyl)-LHSL ($3OC_8$-HSL) quorum signal. In nopaline-utilizing pathovars, the opine agrocinopine inactivates the *traR* repressor AccR. This interaction also increases expression of *traI* in the cell (Figure 6).

Increased *traI* expression in the cell ultimately leads to an increase in $3OC_8$-HSL. As $3OC_8$-HSL reaches a critical threshold, it induces *traR* expression. When TraR is bound to $3OC_8$-HSL, it induces *traI*, establishing an autofeedback loop. Activated TraR also induces expression of the *trb* operon, which produces the mating pore between two *A. tumefaciens* cells; the *tra* operon, which helps mobilize the Ti plasmid; and the *traM* gene. TraM inactivates TraR, thus providing a means for *A. tumefaciens* to downregulate the quorum signal-dependent synthesis of the conjugation apparatus after it has begun. The *A. tumefaciens* TraI/R system, while similar to the LuxI/R system of *V. fischeri*, has an additional level of regulation dependent on host opine production ensuring that quorum signal-controlled induction of conjugation does not occur prematurely outside of a compatible host. This system serves as an elegant example of interspecies-dependent cues having regulatory input in bacterial signaling pathways.

Pantoea stewartii EsaI/EsaR System

The Gram-negative bacterium *P. stewartii* is the causative agent of Stewart's bacterial wilt and leaf blight in sweet corn and maize. *P. stewartii* is spread by infected populations of the corn beetle, *Chaetocnema pulicaria* that introduces the bacterium into the xylem and intercellular leaf spaces of the plant during feeding. Infection by *P. stewartii* results in wilting due to colonization of the xylem and formation of water-soaked lesions due to bacterial growth within young leaves. Buildup of large amounts of exopolysaccharide (EPS) results in vascular occlusion of plant tissue. EPS secreted by *P. stewartii* is the principal virulence factor and is part of a multistep invasion process.

P. stewartii secretes EPS in a cell density-dependent fashion. Biosynthesis of EPS is encoded by the *cps* gene cluster, while regulation of EPS synthesis is mediated partly by the EsaI/R quorum signaling system. The EsaI/R system also controls the Hrp (hypersensitivity and pathogenicity) regulon. *P. stewartii* requires the Hrp type III secretion system for infection of the intercellular leaf spaces and formation of the characteristic watersoaked lesions.

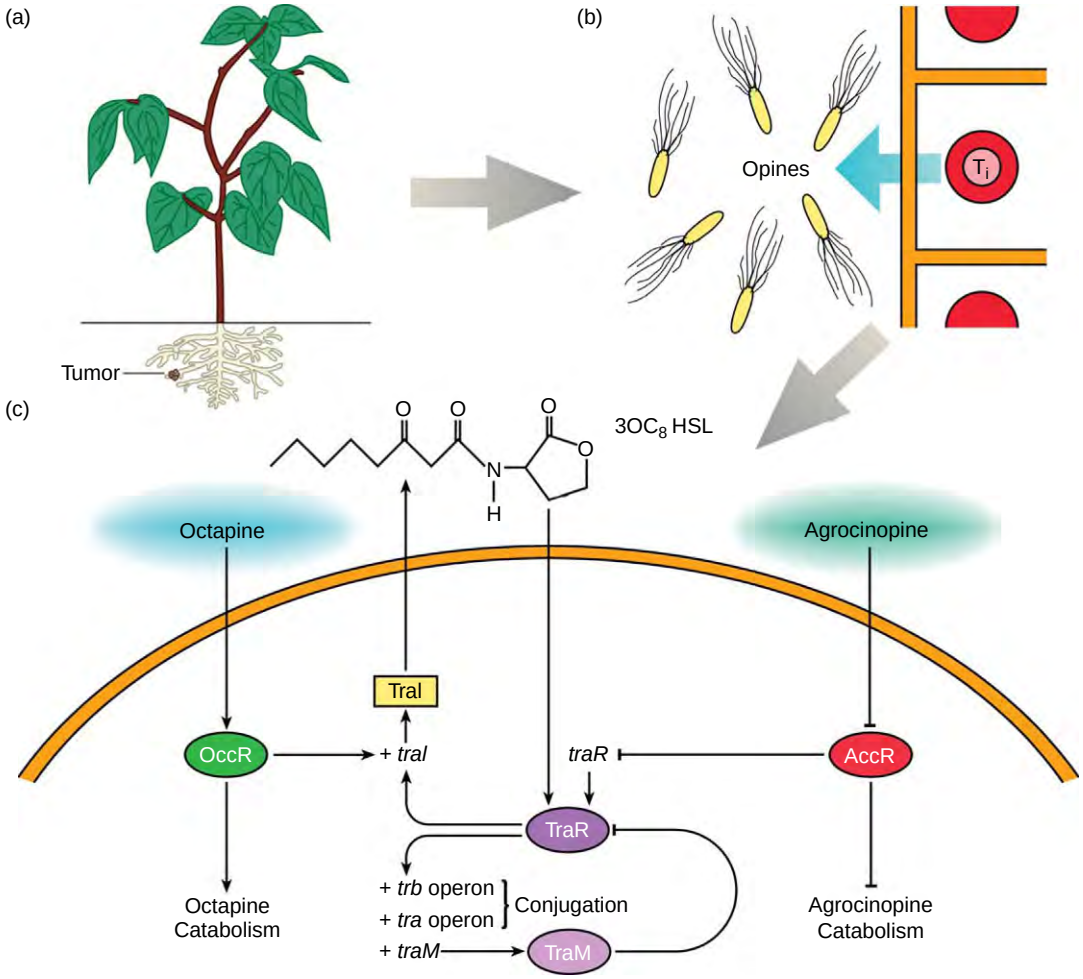

Figure 6 Quorum sensing in Agrobacterium tumefaciens. (a) A plant infected with A. tumefaciens displaying a characteristic tumor. (b) Inside the tumor, proliferating host cells that carry the Ti plasmid produce an opine nutrient for A. tumefaciens. (c) Octapine-type opines interact with the OccR transcriptional regulator causing it to upregulate octapine catabolism genes as well as induce tral expression. Tral synthesizes the 3OC8-HSL signal that interacts with the TraR protein, causing it to further induce tral expression and the trb and tra operons utilized in conjugation. 3OC8-HSL-TraR also induces the traM gene, whose product inhibits TraR activity.

The *cps* genes are regulated by the Rcs (*r*egulator of *c*apsule *s*ynthesis) two-component signal transduction system that detects environmental signals. The Rcs system is composed of the RcsB cytoplasmic response regulator and the RcsC transmembrane sensory protein. It is thought that for complete induction of the *cps* genes, another protein (RcsA) may be required. Also, in the absence of AHLs, EsaR negatively regulates the *cps* genes.

Interestingly, the AHL synthase gene *esaI* is constitutively expressed and is not autoregulated by *esaR* like many other *luxI*/*R*-type genes, while *esaR* is autorepressed by the EsaR protein. EsaI synthesizes 3-oxo-C_6HSL and small amounts of 3-oxo-C_8HSL. Mutants in *esaI* do not produce AHLs or EPS and are avirulent. In contrast, *esaR* and *esaI*/*R* double mutants demonstrate a constitutive hypermucoidy phenotype and are less virulent than the wild type. The hypermucoidy mutants of *P. stewartii* also appear impaired in attachment compared to wild-type *P. stewartii*. This indicates the importance of quorum control of pathogenicity factors in *P. stewartii* as production of EPS at an incorrect location and phase of infection renders the cells avirulent and unable to colonize the host.

Acidithiobacillus ferrooxidans AfeI/R

A. ferrooxidans is a Gram-negative acidophilic chemolithotrophic bacterium that is commonly found in multispecies biofilms on mineral surfaces such as pyrite and elemental sulfur in rock and soil. *A. ferrooxidans* catalyzes the oxidation of iron and sulfur yielding sulfuric acid. It is a causative agent of acid mine drainage, which can lead to groundwater contamination. The ability of *A. ferrooxidans* to dissolve mineral structures can also be used in 'biomining' where acid solubilization of rocks and soil can release minerals such as copper and gold that are used in industrial applications. This process, also known as bioleaching, is a slower yet more energy efficient and environmentally containable process as opposed to traditional smelting of ores to release rare metals.

To date, AHL quorum signaling in *A. ferrooxidans* is relatively poorly understood. Recent studies have determined that *A. ferrooxidans* produces nine different medium to long-chain (C8–C16) AHL signals containing an even number of carbons. The AHL signals also contain either oxo or hydroxyl modifications at the third carbon of the molecule. At this time, only one AHL synthesis gene, *afeI*, has been identified and characterized, but another open reading frame has been hypothesized to be an AHL synthase due to its sequence homology to the AHL synthase *hdtS* from *Pseudomonas fluorescens*.

AfeI is similar to LuxI of *V. fischeri* and produces five different AHLs when expressed in an AHL-lacking *Escherichia coli* strain. Immediately downstream of the *afeI* gene in *A. ferrooxidans* is the *luxR* homologue *afeR*. Transcriptional studies show that *afeI* and *afeR* transcripts are present and *afeI* transcript levels increase relative to increases in AHL concentration. Downstream regulatory targets for AfeR in *A. ferrooxidans* have not been fully determined, but it has been shown that *afeI* transcripts are strongly induced during phosphate-limiting conditions. It has also been shown that there are differential increases of specific AHL molecules during growth in sulfate, thiosulfate, or iron-containing medium. This data suggest that the type of AHL signal produced may be influenced by the growth substrate as well as cell density. The fact that *A. ferrooxidans* is often found in multispecies biofilms and produces a variety of AHL types could also suggest a role for *A. ferrooxidans* in modulating density-dependent expression of other species in response to growth substrates *in situ*.

The Study of AHL-Dependent Signal Pathways Demonstrated the Presence of Another Quorum Signal AI-2

V. harveyi and the LuxS-Produced Signal AI-2

Previously we discussed the discovery of quorum signaling in the luminescent marine bacteria *V. fischeri*. Further work with *V. fischeri* identified and characterized the LuxI/R regulatory circuit and the AI-1 HSL signal. However, determining the nature of the densitydependent signal in the closely related luminescent bacterium *V. harveyi* yielded different results. *V. harveyi* luminescence is density-dependent, inducible by spent culture medium, and is dependent upon a $3OHC_4$-HSL. Despite these similarities, genes homologous to *luxI* and *luxR* were not found on the *V. harveyi* chromosome.

Mutational analysis determined that *V. harveyi* $3OHC_4$-HSL was produced by the AHL synthase proteins LuxL and LuxM. It is not clear which protein is the actual synthase, but the presence of both is necessary for $3OHC_4$-HSL production. Neither gene has significant sequence homology to *V. fischeri luxI* but appear to carry out similar reactions to synthesize $3OH\text{-}C_4$-HSL. It was also found that a *luxN* gene encoded for a sensor kinase protein, which was necessary to sense extracellular AHLs. This mechanism is reminiscent of Gram-positive AIP sensors. LuxN was later shown to autophosphorylate at low $3OHC_4$HSL concentrations and transfer phosphate to the LuxO response regulator via the intermediate LuxU. LuxO-phosphate represses expression of the *luxCDABE* luminescence operon, ensuring that light production is turned off at low AHL concentrations. Knockout mutants in either the *luxLM* synthesis genes or the *luxN* sensor kinase gene did not completely abrogate density-dependent expression of luminescence genes. This suggested the presence of a second quorum signaling system in *V. harveyi*.

The same mutational study that characterized the *luxL*, *luxM*, and *luxN* genes also showed that deletions in the sensor kinase *luxQ* and periplasmic binding protein *luxP* were necessary for density-dependent luminescence independent of the $3OHC_4$-HSL signal. These proteins were shown to be part of a second sensory mechanism in *V. harveyi.* Reporter strains were created using the *luxP* and *luxQ* genes to detect a second signal. Initial biochemical characterizations revealed that a second signal could activate the *luxQP* reporter strains, and this signal was named AI-2. Surprisingly, AI-2 is produced by several other species of bacteria and the *lux*S gene responsible for AI-2 synthesis was identified in *V. harveyi*, *E. coli*, and *Salmonella typhimurium* shortly after.

LuxS synthesizes AI-2 as part of a detoxification reaction in the activated methyl cycle. Methyl transfer reactions are vital in bacterial metabolism and are dependent on the methyl donor SAM. SAM is converted into the toxic *S*adenosylhomocysteine (SAH) molecule and can be detoxified by two different mechanisms. The first mechanism utilizes the SAH hydrolase gene, which hydrolyzes SAH into the SAM precursors adenosine and homocysteine. Organisms that do not produce AI-2, such as *P. aeruginosa*, utilize this pathway. The second mechanism utilizes the Pfs enzyme, which hydrolyzes SAH into adenosine and *S*-ribosylhomocysteine (SRH). Homocysteine is removed from SRH leaving 4,5-dihydroxy-2,3-pentanedione (DPD) in a reaction catalyzed by the LuxS enzyme; DPD spontaneously cyclizes to form the furanosyl diester AI-2. Recent work has shown that AI-2 in *V. harveyi* incorporates boric acid into its structure, the first known biological use of this molecule. To date, it does not appear that boron-containing AI-2 signals are used outside of the *Vibrio* genus and that AI-2 molecules lacking boron function as signals in other bacteria.

As mentioned above, AI-2 is recognized by the sensor kinase LuxQ via the periplasmic AI-2-binding protein LuxP. When a threshold concentration of AI-2 is reached, LuxQ changes from a kinase to a phosphatase, removing the phosphate from its downstream target LuxU. As LuxU is dephosphorylated, it removes phosphate from the LuxO protein. Unphosphorylated LuxO loses the ability to repress the *luxCDABE* operon, and luminescence genes are then activated by the transcriptional activator LuxR, which has no homology to the *V. fischeri* LuxR regulator. Thus, the *V. harveyi* quorum-signaling pathway utilizes the input of two separate density-dependent signals that act on the central regulator LuxO. This pathway is similar to the *Vibrio cholerae* quorum-signaling pathway described in Figure 7.

It is notable that AI-2 synthesis is directly linked to a key metabolic pathway in organisms that carry the *luxS* gene. Because of this link, it is hypothesized that LuxS production is an indicator of the metabolic status of the organism as well as its population density. This could be similar to the differential AI-1-type signal production seen in *A. ferrooxidans*. It should also be mentioned that only two genomes sequenced to date contain genes for both SAH detoxification mechanisms, and this phenomenon may indicate a point of divergence in bacterial evolution.

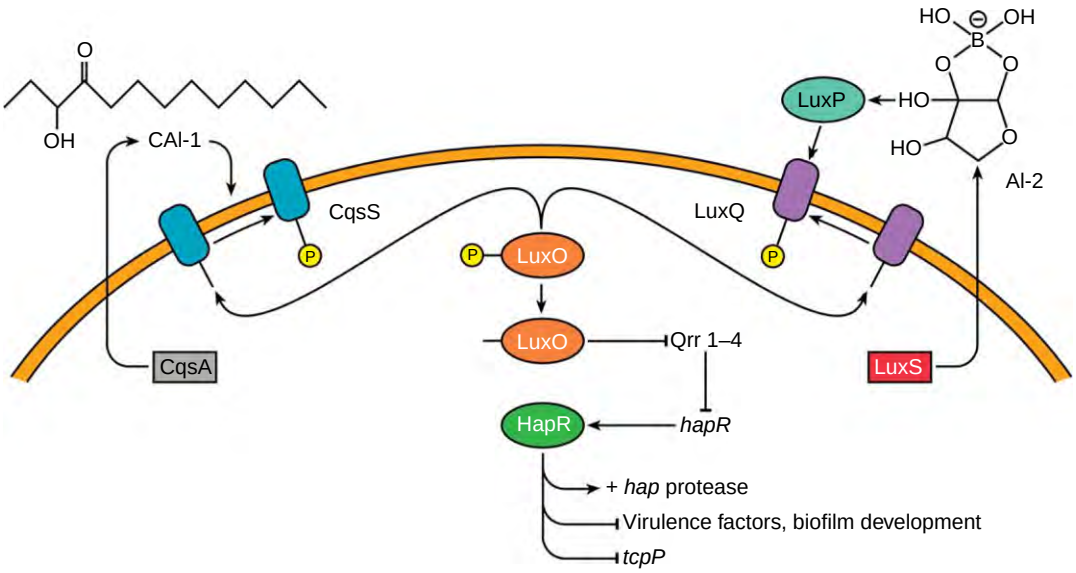

Figure 7 Quorum sensing in Vibrio cholerae. CqsA and LuxS produce CAI-1 and AI-2, respectively. CAI-1 interacts with the CqsS sensor phosphatase, and AI-2 interacts with the LuxQ sensor phosphatase via the AI-2-binding protein LuxP. Both phosphatases remove phosphate from the LuxO protein. Dephosphorylated LuxO is inactive and no longer induces expression of the small RNAs Qrr1–4, which served to repress hapR expression. Translation of HapR induces expression of the hapR protease gene and represses expression of genes involved in virulence and biofilm formation.

AI-2 and *V. cholerae*

V. cholerae is a free-swimming marine bacterium and is the causative agent of cholera in humans. *V. cholerae* is commonly found attached to the surface of zooplankton in the ocean. Cholera outbreaks can be associated with zooplankton blooms near human populations. During the course of disease, *V. cholerae* is ingested and survives the low pH of the stomach to colonize the host small intestine. During colonization, *V. cholerae* uses motility and mucinase to penetrate the mucus layer of the intestine and gain access to the underlying epithelial cell layer.

Currently, *V. cholerae* strain El Tor is the cause of a global cholera pandemic in underdeveloped nations. *V. cholerae* El Tor contains the HapR regulator that represses expression of virulence factors such as the cholera toxin (CT) and the cholera toxin coregulated pilus (TCP). TCP and CT have been shown to be critical for *V. cholerae* host colonization in animal models. The HapR regulator is acted upon by quorum signals in *V. cholerae* El Tor and links the quorum signaling pathway to virulence factor production. Previous pandemic strains of *V. cholerae* contain mutations within the *hapR* gene and are not considered to be responsive to quorum signaling.

V. cholerae contains a quorum sensing system that is similar to *V. harveyi*. However, one of the autoinducer signals as well as the genes regulated by quorum signaling are different in *V. cholerae*. In *V. cholerae*, the AI-2 signal and the recently characterized signal (*S*)-3-hydroxytridecane-4-one (CAI-1) are recognized by the sensor kinases LuxQ and CqsS, respectively (Figure 7). In low cell density conditions, LuxQ and CqsS act as kinases that phosphorylate LuxU, which then transfers its phosphate to LuxO much like the pathway in *V. harveyi*. Phosphorylated LuxO induces the expression of four small regulatory RNAs (sRNAs) known as Qrr1–4. These sRNAs interact with the sRNA chaperone protein Hfq and induce expression of an uncharacterized regulatory gene (vca0939) as well as destabilize the mRNA for *hapR*, the master quorum signaling regulator. When *hapR* is repressed, virulence genes are upregulated, due in part to removal of HapR repression of the *tcpP* gene, which encodes the signaling protein TcpP that induces expression of the ToxT regulator. ToxT induces expression of the virulence factors TCP and CT, so in conditions of low cell density and autoinducer concentrations, virulence and biofilm development genes are upregulated. TCP allows for cell–cell aggregation of *V. cholerae* as well as attachment to the host. CT activity causes epithelial cells to excrete large amounts of water, ultimately causing the host to rapidly expel its intestinal contents, one of the hallmark symptoms of a *V. cholerae* infection.

At high cell densities, LuxQ and CqsS act as phosphatase in the same fashion as LuxQ and LuxN in *V. harveyi*. LuxQ and CqsS ultimately cause the dephosphorylation of LuxO, which causes expression of the Qrr sRNAs to be decreased. As Qrr sRNA expression decreases, *hapR* is upregulated. HapR blocks expression of virulence factors and genes involved in biofilm development. HapR also induces expression of the *hap* gene, which encodes for a protease that aids *V. cholera* in release from a colonized area. In *V. cholerae*, density-dependent signals provide the cell with a method to change cell behavior from a colonizing, scavenging lifestyle at low density, to a motile, migratory lifestyle at high cell density. Interestingly, this change in behavior occurs at a time when pathogenic effects of *V. cholerae* are inducing the host to expel large amounts of intestinal contents, thus allowing the freshly liberated *V. cholerae* to return to a free-swimming lifestyle. It is also interesting that quorum signal-utilizing pathogens such as *S. aureus* and *P. aeruginosa* upregulate virulence factors at high density to form persistent infections, whereas *V. cholerae* downregulates virulence and rapidly leaves the host once high population density has been achieved.

E. coli, *S. typhimurium*, and AI-2

The Gram-negative enteric bacteria *E. coli* and *S. typhimurium* both grow in the lower intestine of mammals. While certain *E. coli* strains are pathogenic, many strains are part of the normal flora. *S. typhimurium* is the causative agent of typhoid fever and, while closely related to *E. coli*, is often a more transient inhabitant of the gastrointestinal tract. Both of these bacteria produce AI-2 using the LuxS enzyme, with maximum production occurring at mid-exponential phase growth. Surprisingly, extracellular AI-2 concentrations drop rapidly as either organism enters stationary phase. This suggests a mechanism for AI-2 sequestration or degradation.

AI-2 has been shown to regulate several genes in both *E. coli* and *S. typhimurium*. These genes include the *lsrACDBFGE* operon, the *lsrK* kinase gene, and *lsrR* whose product represses the *lsr* operon. In both organisms, AI-2 is produced during growth and accumulates outside of the cell. When threshold AI-2 concentrations are reached, AI-2 is imported by the LsrABCD ABC transporter. After import into the cell, AI-2 is phosphorylated by the LsrK kinase and catabolized by the LsrE, LsrF, and LsrG proteins. The products of phospho-AI-2 catabolism have not been characterized at the time of this writing. While the import and catabolism of AI-2 by these organisms is regulated in a density-dependent fashion, it is unclear if these organisms utilize AI-2 to direct group activities. The simplest explanation is that AI-2 is produced as a metabolite during SAH detoxification in the activated methyl cycle and is taken as backup to be utilized as a carbon source later in the growth phase.

Despite the possibility that *E. coli* and *S. typhimurium* may utilize AI-2 only as a metabolite, production of AI-2 by these bacteria may affect gene expression of other AI-2 responsive bacteria in polymicrobial environments. In a 2005 *Nature* paper, Karina Xavier and Bonnie Bassler demonstrated that when *V. harveyi* or *V. cholera* were grown in coculture with *E. coli*, they were subject to interference of AI-2-mediated signaling due to the ability of *E. coli* to respond to higher AI-2 concentrations. As *E. coli* cell density increased, it was able to remove AI-2 from the culture and reduce expression of AI-2-dependent genes in either *Vibrio* species. At low cell densities, premature induction of AI-2-regulated genes was observed in *V. harveyi*, as the AI-2 produced by *E. coli* increased the AI-2 concentration that *V. harveyi* would normally encounter at that cell density. It is apparent from these studies that AI-2 signal turnover can have substantial effects on multispecies interaction even if AI-2 does not appear to have a strong role in quorum-signaldependent behavior in a single species.

luxS-Dependent Interactions in Human Oral Bacteria

The human oral cavity is populated by over 300 different bacterial species. There are several distinct niches within the oral cavity (tooth enamel surface, epithelial cell surface, and the subgingival space), all of which can be colonized by bacteria growing in multispecies biofilms. Many species isolated from the oral cavity contain the *luxS* gene, and in several cases, density-dependent AI-2-mediated behavior has been observed in single and multispecies cultures.

Streptococcus mutans is a common inhabitant of the oral cavity and is the causative agent of dental caries. Merritt and Shi in a 2003 *Infection and Immunity* paper demonstrated that biofilm formation by *S. mutans* was altered in a *luxS* mutant. The *S. mutans luxS* mutant formed a biofilm with denser cell aggregates that was more resistant to detergent and antibiotics. While growth rate and acid production did not appear to be altered in the *luxS* mutant, it was not determined whether extracellular AI-2 concentration or the effects of impeded SAH detoxification were responsible for the mutant phenotype.

Direct AI-2-dependent interaction in oral bacteria was reported in 2003 by McNab and Lamont in the *Journal of Bacteriology*. The researchers demonstrated that *Streptococcus gordonii* and *Porphyromonas gingivalis* formed significantly dense biofilm structures if either organism contained a functional *luxS* gene. Simultaneous *luxS* mutations in both species led to diminished biofilm formation, suggesting that *luxS*-dependent AI-2 formation from one organism can complement the ability of the other to form more fully developed multispecies biofilms.

This phenomenon is similar to another described in a 2004 *Proceedings of the National Academy of Sciences* paper by Egland and Kolenbrander. In that study, *S. gordonii* was shown to upregulate production of amylase only when grown in close proximity to *Veillonella atypica*. This interaction was hypothesized to be dependent on an increase in local concentration of a diffusible signal when the bacteria grow in close proximity to one another. This data suggest that signal production by *S. gordonii* alone is insufficient to upregulate amylase under the conditions tested, and the presence of another signal-producing organism is also necessary. AI-2 is hypothesized to be the diffusible signal in this system, but this has not been unequivocally shown. Both of these cases demonstrate that extracellular signal concentrations are critical to modulate behavior in each organism. This is notable because in the case of *S. mutans* and other literature discussing *luxS*-dependent signaling events, it is often unclear if the phenotypes observed are due to actual AI-2-dependent signaling, or due to metabolic effects caused by an impediment of SAH detoxification.

AI-2-dependent quorum signaling has also been observed in the opportunistic pathogen *Aggregatibacter actinomycetemcomitans*. LuxS activity or exogenous AI-2 addition to cultures have been shown to cause differential expression of iron uptake genes as well as leukotoxin production. AI-2 production by *A. actinomycetemcomitans* has also been shown to complement *luxS*-dependent gene expression in a *P. gingivalis luxS* mutant when cocultured. A 2007 *Infection and Immunity* paper by Shao and Demuth demonstrated that *A. actinomycetemcomitans* is impaired for biofilm production in a *luxS* mutant. Subsequent complementation of *luxS* by *P. aeruginosa sahH* seems to restore the ability to detoxify SAH in the activated methyl cycle, but exogenously added AI-2 was necessary to restore the biofilm phenotype. While *sahH* transcripts were present in the complemented strain, it is not clear if SahH was produced and able to detoxify SAH. Despite the unknown status of SahH activity, this data strongly suggest that AI-2 serves as a signal for community behavior in *A. actinomycetemcomitans*, and not just a metabolite produced in the activated methyl cycle.

AI-2 signaling is responsible for density-dependent regulation of genes in several well-characterized systems. At this time, over 153 separate eubacterial genomes contain a *luxS*-like sequence according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. It is evident that in the case of *Vibrio* species and several inhabitants of the mammalian oral cavity, AI-2-dependent signaling plays a critical part of these organisms' lifestyle. However in many organisms, the presence of a *luxS* gene does not correlate to densitydependent signaling behavior. The 'other' function of LuxS as part of the activated methyl cycle is an important component of metabolism, and it cannot be assumed at this time that phenotypic changes in *luxS* knockouts are exclusively due to AI-2 signaling and not disruption of SAH detoxification.

Other Types of Quorum Signals

In addition to AI-1, AI-2, and peptide autoinducer signals, there are many other types of quorum signals utilized by bacteria. We are only beginning to understand the molecular aspects of these systems, and in some cases the structure of the signal is not known.

The AI-3/Epinephrine Quorum Signaling System

E. coli is a common inhabitant of the human lower intestine. Recently, strains have evolved that carry multiple virulence factors giving rise to enterohemorrhagic *E. coli* (EHEC) strains such as *E. coli* O157:H7. EHEC strains are notorious for their ability to cause hemorrhagic colitis and hemolyticuremic syndrome. As EHEC colonizes the lower intestine, it forms lesions on host epithelial tissue and produces a Shigatype toxin. A majority of the genes involved in attachment, lesion formation, and virulence exist on the EHEC chromosome in a pathogenicity island known as the locus of enterocyte effacement (LEE).

Expression of *LEE* genes is regulated by factors present in the native *E. coli* chromosome as well as factors encoded on LEE itself. In addition to these, some *LEE* genes appear to be regulated in a cell density-dependent fashion. Preliminary studies of a *luxS* EHEC mutant showed changes in LEE gene expression, indicating that AI-2 may play a role in regulation. However, the addition of exogenous AI-2 to the *luxS* mutant did not complement LEE gene regulation. This data suggested that LEE gene regulation was subject to metabolic changes observed in the *luxS* mutant. It was then shown that LEE gene regulation could be complemented by concentrated extracts from *luxS* mutant supernatants, and that an unidentified compound chemically different from AI-2 was synthesized at a low rate in the *luxS* mutant. LEE gene expression phenotypes were also restored in a *luxS* mutant strain that was complemented with the SAH hydrolase enzyme from *P. aeruginosa*. This experiment determined that homocysteine formation by either the SAH hydrolase enzyme or LuxS restored LEE gene expression. This suggested the presence of an inducer deemed, AI-3, whose biosynthesis utilizes precursors from the activated methyl cycle.

Other studies indicated that LEE gene expression could be influenced by epinephrine and norepinephrine hormones from the host, and it was hypothesized that AI-3 might resemble epinephrine/norepinephrine due to similarities in chemical properties. At this time, the structure of AI-3 is still unclear; however, the addition of exogenous epinephrine/norepinephrine induces similar sets of genes when compared to addition of impure AI-3 extracts. It has also been shown that AI-3 and epinephrine induce *LEE* genes as well as other targets and are dependent on the sensor histidine kinase QseC. EHEC are likely to come in contact with epinephrine/norepinephrine in the lower intestine during colonization as norepinephrine is produced by neurons in the enteric nervous system and epinephrine is secreted as a systemic response to host stress during EHEC disease progression. The discovery of QseC as a receptor for both a hostproduced hormone and a bacterially produced quorum signal is astounding and contains many implications for future discoveries in quorum signaling as well as evolution between the bacterium and the host.

Bradyrhizobium japonicum Bradyoxetin Signal

Bradyoxetin is produced by the nitrogen-fixing soil bacterium *B. japonicum*. Genes involved in biosynthesis are unknown at this time, although the biosynthetic pathway is likely similar to that of the siderophore mugenic acid. Similar to siderophores, bradyoxetin production is regulated by Fe^{3+} concentration as well as cell density with bradyoxetin produced maximally at high cell densities or at low Fe^{3+} concentrations. At high cell densities, bradyoxetin appears to upregulate its own synthesis as well as the response regulators *nolA* and *nodD2*, whose products repress the expression of *nod* genes necessary for symbiotic root nodule formation in legumes. This system is yet another instance of quorum signal production being controlled by population density as well as the metabolic state of the organism.

Pseudomonas aeruginosa Pseudomonas Quinolone Signal (PQS)

In addition to the LasI- and RhlI-produced AI-1 AHL signals, *P. aeruginosa* synthesizes a quinolone compound PQS important for quorum signaling gene expression. PQS was discovered in 1999 by Everett Pesci, and its chemical structure is 2-heptyl-3-hydroxy-4-quinolone (Figure 5). PQS is synthesized from anthranilate via the *phnAB* and *pqsABCDE* gene products, which produce the precursor 4-hydroxy-2-heptyl-quinoline (HHQ). HHQ is converted into PQS by the PqsH protein.

PQS functions as part of the AI-1-mediated quorum signaling network in *P. aeruginosa*. Activated LasR (bound to $3OC_{12}$-HSL) can upregulate *pqsH*, ultimately increasing PQS concentration in a $3OC_{12}$-HSL-dependent manner. As PQS concentration increases,

it induces *rhlI* expression in a *rhlR*-dependent manner. PQS negative mutants lack the ability to produce many virulence factors that are also absent in *rhlR* mutants. Thus, PQS serves as a link between the LasI/R and RhlI/R quorum signaling systems (Figure 5).

PQS is also interesting due to its hydrophobic nature. The hydrophobicity of PQS suggests that it would be a poorly diffusible signal in aqueous environments. Recently, it has been discovered by our lab that PQS is transported by outer membrane vesicles. This phenomenon allows PQS to be moved by a water-soluble carrier that may also serve to protect the signal from extracellular degradation by other organisms. Another interesting property of PQS is that its production appears to be restricted to *P. aeruginosa* (at least to this point), while its precursor HHQ is synthesized by many other Gramnegative bacteria, notably *Burkholderia*, *Pseudomonas*, and *Alteromonas* species. HHQ has also been shown to act as a signal in *P. aeruginosa* because of its release and uptake by neighboring cells and subsequent conversion into PQS. Thus, the production of 2-alkyl-4-quinolone compounds by these organisms suggests another class of quorum signaling molecules.

Ralstonia solanacearum 3-Hydroxypalmitic Acid Methyl Ester (3-OH PAME)

R. solanacearum is a Gram-negative plant pathogen that causes wilting in many different plant species. *R. solanacearum* infects plants at the root and over several days enters the xylem where it travels to the aerial portions of the plant. *R. solanacearum* colonizes the xylem and expresses several extracellular and intracellular virulence factors. A primary virulence factor in wilting disease is the production of copious amounts of EPS that physically blocks fluid transport through the xylem of the plant, thereby leading to wilting. This disease is very similar to the previously mentioned Stewart's wilt disease caused by *P. stewartii*.

R. solanacearum virulence factor production is ultimately controlled by the concentration of the quorum signal 3-OH PAME (Figure 8). 3-OH PAME is produced by the PhcB protein and released from the cell. When 3-OH PAME reaches a threshold concentration, it interacts with the sensor kinase PhcS, which then transfers a phosphate group to inactive the PhcR protein. PhcR inactivation allows the regulator PhcA to activate. PhcA ultimately controls the expression of many virulence genes in *R. solanacearum* as well as a LuxI/R like signaling system encoded by the *solI/R* genes.

3-OH PAME is significant among quorum signals because it is a volatile compound. Volatility of a quorum signal has positive and negative consequences in that it allows rapid signaling over greater distances than an aqueous signal; however, the signal can also diffuse away from receptive cell populations before a threshold concentration is reached. It is interesting that a volatile signal appears to function sufficiently inside plant tissues, which are highly heterogeneous in terms of fluid and gas density. It is also notable that *R. solanacearum* appears to use multiple quorum signal cascades to regulate virulence gene expression much like *P. aeruginosa*.

Extracellular Effectors of Quorum Signaling Systems

Quorum signals are produced by many diverse bacterial species in many different environments. The ability to utilize signals to direct efficient and timely changes in gene expression almost certainly provides that species with a strong competitive advantage for

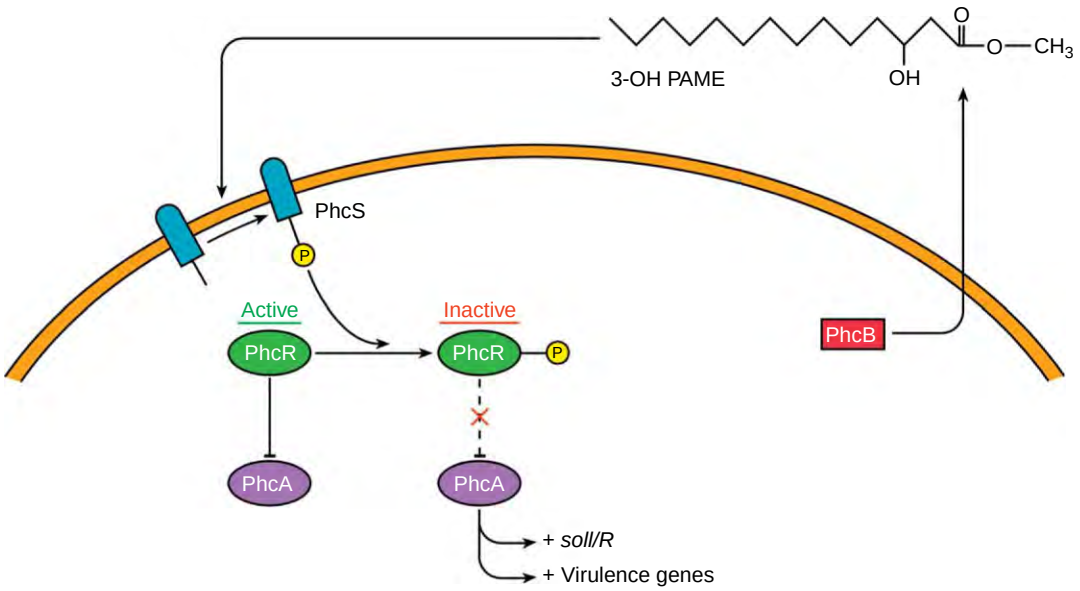

Figure 8 Quorum sensing in Ralstonia solanacearum. PhcB synthesizes the volatile signal 3-OH PAME. 3-OH PAME interacts with the PhcS sensor kinase to phosphorylate the PhcR regulatory protein. PhcR-phosphate is unable to block activity of the PhcA transcriptional regulator that induces expression of virulence genes as well as the *solI/R*-dependent AHL quorum sensing system.

nutrient acquisition and defense. Likewise, a competitor or host would gain tremendous benefit if it were able to interfere with a pathogen or competitors' quorum-sensing system. The evolutionary 'arms race' between quorum signaling and signal interference by other species could be millions of years old, yet was just recently revealed by researchers.

In 1996, Michael Givskov and Staffan Kjelleberg and colleagues described the production of AHL mimics by the marine seaweed *Delisea pulchra*. *D. pulchra* produces several halogenated furanone molecules that structurally resemble AHL signals. Furanones were shown to inhibit quorum signal-dependent swarming motility of *Serratia liquefaciens* as well as several marine bacteria. AHL reporter systems in *E. coli* as well as *V. harveyi* were shown to be inhibited by exogenous addition of furanones. Further work on *D. pulchra* furanones showed that they disrupt quorum signaling by binding to LuxR-type proteins in a competitive fashion with the native AHL signal. In the case of *D. pulchra*, furanone-dependent quorum inhibition could protect the plant from colonization by pathogenic bacteria that regulate motility, biofilm, or virulence gene expression through quorum-signaling systems.

A similar phenomenon was observed in the unicellular eukaryotic alga *Chlamydomonas reinhardtii*, which also produces quorum signal mimics. *C. reinhardtii* grows in soil and freshwater environments where it likely encounters many bacterial species that utilize quorum signaling. It was shown that *C. reinhardtii* produced at least 12 uncharacterized compounds that stimulated the LasR and CepR regulators in *E. coli* reporter strains. Interestingly, none of the compounds stimulated LuxR or AhyR regulators in similar reporter strains, suggesting that the putative AHL mimics produced by *C. reinhardtii* are receptor-specific. The chemical properties of the *C. reinhardtii*-produced mimics suggest that they are AHL-like in nature, but subsequent characterization of the compounds revealed that they are not known AHLs. Despite their dissimilar structure, *C. reinhardtii* AHL mimics were shown to increase the production of many AHL-controlled genes to levels observed with the native AHL; however, some proteins were found to be downregulated by the AHL mimics. The full impact of these results is still unclear, but demonstrates that *C. reinhardtii* likely has significant effects on bacterial quorum signaling in the natural environment.

Recent research by several groups has identified eubacterial and eukaryotic enzymes that degrade AHLs. There are many incidences of production of these enzymes in bacteria, and it appears that one function may be to degrade the bacteria's own AHL signal in order to downregulate the quorum signal response after its desired effects are achieved. Another function of these enzymes may be to degrade AHLs from competing organisms as a means of interfering with their quorum signaling systems. The observation that some enzymes produced by AHLsynthesizing bacteria cannot degrade their own AHLs, yet degrade those produced by other organisms, supports the idea of competitive interference between species.

In a 2004 *Proceedings of the National Academy of Sciences* paper, Carlene Chun and E. Peter Greenberg demonstrated that human airway epithelial cells were able to degrade the $3OC_{12}$-HSL from *P. aeruginosa*. As mentioned previously, *P. aeruginosa* can cause respiratory infections in humans. During the course of infection, airway epithelial cells are likely to come in contact with colonizing *P. aeruginosa*, and the ability of the host to interfere with quorum signaling may be of great importance in preventing infection. Surprisingly, this study showed that the degradation of $3OC_{12}$-HSL was contact-dependent and that degradation did not occur in culture supernatants from the airway cells. It was also shown that degradation was specific for $3OC_{12}$-HSL, C_{12}-HSL, and C_6-HSL. No degradation of C_4-HSL or $3OC_6$-HSL was observed. The AHL degradation phenotype varied widely among cell types tested but seemed to occur most frequently in cell lines that were derived from epithelial cells that are most likely to encounter AHL-producing organisms. These data suggest that humans, and potentially other mammals, have evolved mechanisms to specifically defend against AHL-producing bacteria.

Quorum Signal Interaction with the Host

Eukaryotic hosts utilize both quorum signal-degrading enzymes and mimics to interfere with bacterial quorum signaling. Some organisms produce these substances throughout their life cycle, while others produce them in response to bacterial quorum signals. The ability of eukaryotic organisms to sense and respond to quorum signals is fascinating and further emphasizes the affect that prokaryotes may have on the evolution of eukaryotes and vice versa. We will describe a few representatives from the growing number of cases of eukaryotic responses to bacterially produced quorum signals.

The legume *Medicago truncatula* senses and responds to AHL signals produced by *Sinorhizobium meliloti* and *P. aeruginosa*. Many *M. truncatula* proteins are regulated in a similar manner when exposed to AHLs from either bacterial species; however, some proteins are differentially expressed in response to AHLs from one species compared to the other. This suggests that *M. truncatula* has a conserved response to AHLs but also has the ability to discriminate between AHL signals from these bacteria. AHL signals were found to differentially regulate over 150 proteins in *M. truncatula*, the most notable of which were involved in primary metabolic pathways, stress response, and enzymes used in quorum signal mimic production. At this time, it is unclear how these responses specifically benefit *M. truncatula* or how they affect *S. meliloti* or *P. aeruginosa*, but it is apparent that both organisms stimulate a significant change in behavior of the plant.

In 2006, Elmus Beale and Kendra Rumbaugh demonstrated that the soil nematode *Caenorhabditis elegans* is able to sense AHL signals from bacteria. *C. elegans* was shown to preferentially move toward AHL-producing organisms as opposed to AHL-lacking species. This AHL-dependent chemotaxis is thought to aid *C. elegans* in locating bacteria as a source of food. Another surprising observation was that *C. elegans* would move toward *P. aeruginosa* despite the fact that *P. aeruginosa* kills *C. elegans*. After exposure to *P. aeruginosa*, surviving *C. elegans* would display avoidant behavior toward AHL-producing organisms including nonharmful species. This work provides an elegant example of a eukaryote behavioral response to bacterial quorum signals. Another study by the same lab described the ability of *P. aeruginosa* AHLs to enter and modulate expression in mammalian cells; thus demonstrating

that AHLs are able to cross the host cell membrane and stimulate host gene expression. These changes in gene expression are thought to occur through AHL stimulation of host nuclear hormone receptors. Further work demonstrated that *P. aeruginosa* AHLs can induce inflammation, immunosupression, and apoptosis in immortalized cells as well as primary cell cultures. Many of these events are hallmarks of tissue exposed to *P. aeruginosa* during infection, but it remains to be determined if the responses seen during infection are actually due to *in vivo* AHL signaling or the myriad of other virulence factors *P. aeruginosa* produces.

Diffusion Sensing, Efficiency Sensing, and the Future of Quorum Signaling Systems

In 2002, Rosemary Redfield at the University of British Columbia proposed that quorum sensing may be a property of what she referred to as diffusion sensing. Before her proposal of this concept, quorum signaling had been widely accepted as a cell density-dependent signal that affected gene expression of a population, with the consequence of directing a population to exhibit behavior that affected the group as a whole. The concept of diffusion sensing proposes that individual bacteria sense autoinducer concentrations and subsequent changes in gene expression direct a response that is intended for the individual cell, not the population as a whole. She proposed that evolutionary selection for quorum sensing behavior was selected for by the benefit given to an individual cell that has the ability to use a signal to gather information on properties of the local environment.

While the concept of diffusion sensing is interesting, it must be considered that any receptor-based system should be induced when ligand concentration exceeds that of the receptors' affinity for it. This condition can be achieved either by increased production of signal, decreased mass transfer rate, or decreased local volume around the cell. In quorum signaling, we ascribe that signal-dependent behavior is due to an increase in population and subsequent increase in signal. In many instances of quorum signaling systems, it may indeed be a product of diffusion sensing by the cell. However, observations of autoinducer-producing organisms in some characterized systems seem to indicate behavioral changes that are more beneficial to a group of cells as opposed to an individual. These changes include densitydependent luminescence as well as conjugation, which serve little purpose when local cell numbers are low.

Hense *et al*. in a 2007 *Nature* review attempted to unify the gap between diffusion and quorum sensing and propose the novel concept of efficiency sensing. Efficiency sensing suggests that cells produce signaling compounds to 'test' the population density, volume, and mass transfer rate of their environment. If signal concentration increases, then induction of energetically expensive, signal-dependent pathways occurs. This hypothesis takes into account that signal concentration can be affected by local concentration of cell density as well as the mass transfer rates and the volume of the local environment. Hense and colleagues propose that efficiency sensing provides a means for individual as well as community advantages of diffusible signals to overlap and provide evidence through mathematical models that indicate that spatial distribution of cells may be more important than density.

It is possible that diffusion sensing behavior in individuals may be the primary means of positive selection for organisms that have quorum signaling systems of either low complexity or are newer aspects in the organism's evolutionary history. Thus as bacteria adapt and evolve, diffusion sensing behavior can be recruited into true quorum signaling behavior and exert a community benefit on a population of cells.

What's Next for Quorum Signaling?

Research in quorum signaling initially uncovered a few specific cases that demonstrated density-dependent signaling and its impact on a single response in the signaling organism. Phenomena such as luminescence and regulation of competence were largely thought to be the single process in the cell directed by a diffusible signal, and much initial research focused on utilization of a specific signal and its interaction with a single receptor or transcriptional regulator to direct regulation of just a few related genes or operons. The discovery of orphan regulators in *P. aeruginosa* and other species and the convergence of multiple types of signals involved in gene expression in *V. harveyi*, *V. cholerae*, *P. aeruginosa*, *R. solanacearum*, and *B. subtilis* (to name a few) demonstrate that many organisms utilize quorum signals in increasingly complex pathways. Further research into orphan regulators and how bacteria integrate multiple quorum signal pathways may reveal an even greater role for quorum signaling in bacteria than was previously considered.

There are ever increasing observations of interspecies quorum signal interactions between bacteria as well as interdomain interactions between bacteria and eukaryotic hosts. These studies demonstrate that prokaryotes and eukaryotes may utilize signals not only as a means to detect signal producers, but as a way to defend against them *in situ*. These adaptations shed light on possible therapeutic strategies for infections caused by quorum signal-producing bacteria. The discovery of quorum signal interference, especially in plants, may be useful in developing new variants of commercial crops that are more resistant to bacterial infections.

A poorly understood aspect of quorum signaling is that some microorganisms utilize quorum signals not only as a strict density-dependent signal but also as a way of monitoring and reporting the nutrient status of the environment. PQS signals in *P. aeruginosa* have been shown to be differentially produced in different environmental concentrations of aromatic amino acids. Also, the types of quorum signals produced by *A. ferrooxidans* vary depending on the substrates available to the organism. These phenomena as well as the production of AI-2 and its dependency on the metabolic status of the cell indicate that quorum signal production may serve functions aside from monitoring cell density.

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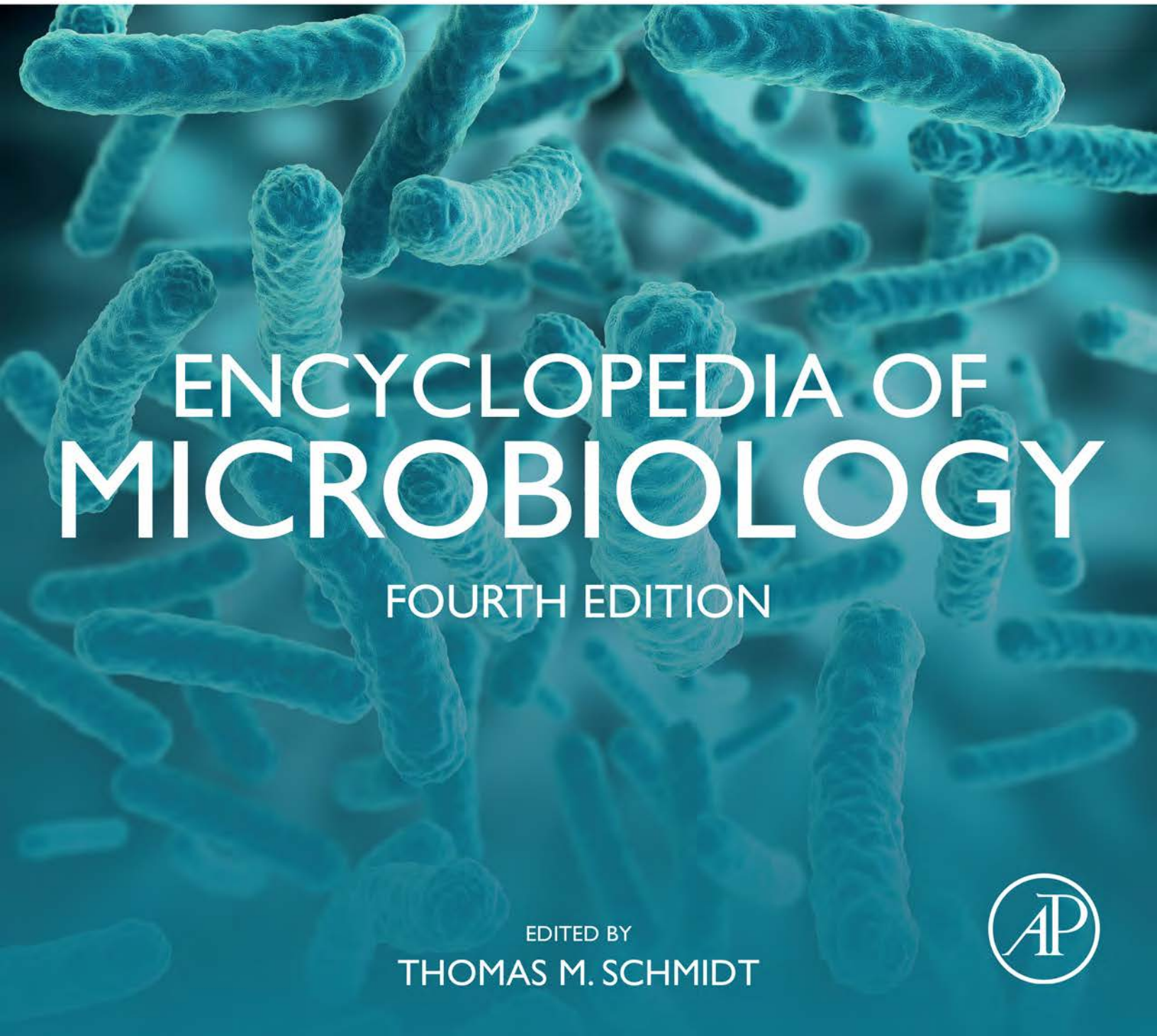

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Thomas (Tom) Schmidt is a microbial physiologist and ecologist who studies diverse microbes and microbial communities. Tom received a PhD from The Ohio State University and conducted postdoctoral research at Scripps Institute of Oceanography and Indiana University. He spent much of his career studying the ecology of microbes in soil that are responsible for the exchange of greenhouse gases with the atmosphere. More recently, he joined the University of Michigan and focused his research on the human microbiome. His joint appointment in the Departments of Internal Medicine and Ecology & Evolutionary Biology reflects his expertise in applying ecological and evolutionary principles to understand the functioning of complex microbial communities.

Tom is a fellow of the American Academy for Microbiology and was director of the Marine Biological Laboratory's Microbial Diversity Course in Woods Hole. Through that course and in his laboratory, he has helped numerous scientists incorporate molecular approaches into traditional strategies for studying the microbial world. He currently teaches a university course that merges his research and teaching goals by engaging students in a coordinated study of the effects of diet on the gut microbiome, and he directs a graduate program that combines laboratory sciences and modeling. Tom was a section editor for previous editions of the *Encyclopedia of Microbiology* and was delighted to assume the role as editor-in-chief to help bring the microbial world to an expanding audience.

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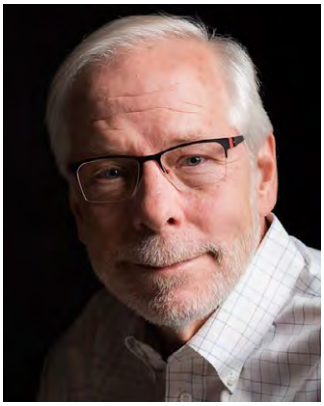
Bianca Brahamsha is a research microbiologist at Scripps Institution of Oceanography at the University of California, San Diego. She was a biology and history major at Goucher College, earned her PhD in microbiology at Cornell University, and carried out postdoctoral work in molecular genetics as an American Cancer Society fellow at the University of Chicago. Her research interests have centered on bacterial motility, the genetics and physiology of marine cyanobacteria, and on the interactions between cyanobacteria and their eukaryotic predators.

James (Jim) Brown was born in Atlanta, Georgia, and lived in Dade City (Florida), Bloomington (Indiana), Lima (Peru), and Muncie (Indiana) while growing up. From the beginning, he had an intense interest in nature, including anything found in the woods, rivers, beach or ocean that were always nearby.

Jim attended Ball State University beginning in 1976. A single lecture on microbial diversity in a general microbiology class, followed by the announcement of the discovery of the Archaea (George Fox and Carl Woese, 1977), sparked his lasting interest in microbiology. This led to undergraduate research examining *Beggiatoa* in a sulfur spring in French Lick, Indiana. After receiving his BS in biology in 1980, he joined the graduate program in microbiology at Miami University, where he worked on plant tissue culture mRNAs with Prof. Ronald Treick. After obtaining an MS degree in 1982, he moved to the MCD Biology Program at The Ohio State University to work on the molecular biology of methanogenic Archaea in the Department of Microbiology with Prof. John Reeve. While there, Jim worked on polyadenylation of mRNAs, RNA polymerase, and promoters in Archaea, and received his PhD in 1988. Jim then went to Indiana University for 5 years of postdoctoral work in Prof. Norman Pace's lab on the comparative analysis of the structure of a bacterial ribozyme, RNase P.

In January of 1994, Jim started as an assistant professor in the Department of Microbiology and moved to North Carolina State University. Research in Jim's lab focused on the comparative sequence and biochemical analysis of RNA, and in particular RNase P in Archaea. Students in the Brown lab developed a high-resolution model for the structure of this RNA, how it changed over the diversification of the Archaea, the protein subunits associated with the RNA, and how they contribute to the function of the holoenzyme. Jim developed, teaches, and wrote the textbook for a senior-level undergraduate lab course in microbial diversity. Jim is now Professor Emeritus in the Department of Biological Sciences at NC State University.

Jim has a son and two daughters, and is married to Melanie Lee-Brown, professor of biology and director of Research and Creative Endeavors at Guilford College. For fun, Jim enjoys skin and scuba diving, driving (i.e., working on) his 1968 Lotus Super Seven, and restoring vintage racing bicycles. Jim and Melanie have been renovating an abandoned fishing lodge on North Caicos Island (Turks and Caicos Islands, BWI), which will be reopened soon as a bed and breakfast.



Larry Forney is a university distinguished professor and a member of the American Academy of Microbiology with academic appointments in the Department of Biological Sciences and Bioinformatics and Computational Biology at the University of Idaho. Dr. Forney is an evolutionary ecologist who conducts research on the complex array of factors that influence the function, composition, structure, and temporal dynamics of bacterial communities in a wide array of habitats. In recent years he has largely focused on the community ecology of the human vagina across a woman's lifetime with an eye on understanding how changes in community structure and function affect a woman's risk to bacterial vaginosis, sexually transmitted infections, and pre-term birth, and how host factors shape these communities. His research extends to understanding the mutation-selection processes that govern the occurrence and persistence of genetic diversity of bacterial populations in spatially structured environments such as microbial biofilms and porous media. These studies have shown that spatial structure creates conditions in which mutation frequencies are high and selective sweeps are protracted, which leads to extraordinary within species diversity that increases the resilience of populations to environmental changes.

Robert Haselkorn was the F. L. Pritzker distinguished service professor of molecular genetics and cell biology at the University of Chicago, retiring several years ago. He was an undergraduate at Princeton, a graduate student at Harvard, and a postdoctorate in Cambridge, England. He started his teaching career at Chicago in 1961 in biophysics, extending later to microbiology, biochemistry, and chemistry. His research interests have centered on heterocyst differentiation in nitrogen-fixing cyanobacteria, in bacterial genomics, and in the enzyme acetyl-CoA carboxylase in plants, parasites, and people. He is a member of the National Academy of Sciences, a fellow of the American Academy of Arts and Sciences, and a member of the American Philosophical Society. Among other external activities he was a founder and adviser for 20 years to the International Center for Genetic Engineering and Biotechnology, located in Italy and India. For the past 15 years Haselkorn and his wife Margot have been working on selection and supporting a speaker for the Haselkorn Lecture at the University of Chicago, an award they have endowed. Many of the Haselkorn Lecturers have been Nobel Prize winners or will be soon.

Jennie C. Hunter-Cevera received her PhD from Rutgers University in 1978 (microbial physiology and biochemistry), an MS in microbial ecology in 1972, and a BS in biology from West Virginia University in 1970. She is the founder of Hunter and Associates, a consulting firm focusing on finding integrative solutions to complex problems involving sustainability in the life sciences arena. From July of 2009 to August 2012, she was the executive vice president of Discovery and Analytical Sciences (DAS), Corporate Development and Government Relations. She has 22 years of experience in the pharmaceutical and biotechnology industries (E. R. Squibb and Sons, Cetus Corporation, GeoBiotics, and Universal Foods). She was the co-founder of The Biotic Network and BlueSky Laboratory that contracted with biotechnology and pharmaceutical companies on basic and applied natural product research. Dr. Hunter-Cevera was an

employee for 5 years with the Department of Energy as the head of the Lawrence Berkeley National Laboratory's Center for Environmental Biotechnology. She worked in academia for 10 years as the president of the University of Maryland Biotechnology Institute and 2 years as the Interim Provost of Mount St. Mary's University. She also served as project manager for UC Davis's CIFAR (Center for Industrial Food and Agricultural Research). She has published many papers and chapters, and served as senior editor of the *Journal of Industrial Microbiology and Biotechnology* for 10 years. She is a co-section head of microbiology for Faculty of 1000. She holds 15 patents and specializes in areas of screen design for the discovery of natural compounds in the area of human therapeutics, biodefense, sustainable agriculture, bioremediation, and biocatalysis for industrial processes in the food and clothing industries.

While in Maryland, she served as a member of the Executive Committee for Governor Erhlich's transition team, his Committee on science, technology, engineering, and maths, and was the Technology Representative for Governor Glendenning on the Southern Governor's Association, and she served on the Technology Economic Development Corporation, TEDCO (Board of Directors for 4 years and was Chair for 2 years). Dr. Hunter-Cevera also served on the Advisory Board for the Maryland Industrial Partnerships, the MDBio's Board of Directors, and the MDBio Foundation for 10 years and on the BioIT Coalition for 5 years. She served as an Entremed Board Member for 10 years and served on the Executive Committee for 2 years. She was a board member for Patients with Power, a software company that designs programs for cancer patients to make the best decisions for their treatments. Jennie also served as acting secretary for Maryland's Higher Education Commission in 2015.

She is a member of several professional societies and has served as president of the Society for Industrial Microbiology, the International Marine Biotechnology Association, and the United States Federation of Culture Collections. Dr. Hunter has served on many national committees and commissions and was Chair of the National Research Council's Committee on Large Scale Production of Biofuels from Algae. She also chaired two other NRC Committees: Standing Committee on DOD's Translational Medicine and the DOE's Genome to Life for Biofuels.

She is the recipient of several awards and honors including Maryland's Top 50 Influential People (2007, 2009) and Maryland's Top 100 Women (2003, 2006, 2009), American Society for Microbiology's Porter Award for distinguished research in microbial systematics and taxonomy, elected as a SIM fellow, a member of the ASM Academy of Microbiology, and an AAAS fellow. She is also a WVU Distinguished Alumni Awardee and Nath lecturer and served as an entrepreneurial coach for the UNC Executive MBA program. She was awarded an Honorary Doctorate from West Virginia University, May 2013, and the Rutgers Cook College Dennis M. Fenton Distinguished Graduate Alumni Award in 2014.

She currently serves on the International Advisory Council for Brazil's Fundação Dom Cabral which is a world-class Brazilian business school that develops strategic thinking skills of executives, entrepreneurs, and public-sector managers. In addition, Jennie served 6 years on the Edison Awards Steering Committee and currently serves on the Edison Awards Advisory Board.

Stanley Maloy is a professor of microbiology and associate vice president for research and innovation at San Diego State University. Stanley's research has focused on bacterial and phage genetics and physiology, evolution of infectious diseases, and development of antimicrobials and vaccines. In addition, he has consulted with large and small companies in multiple sectors, played a role in starting several Biotech companies, and served leadership roles in multiple start-up companies.

Dr. McCormick earned her PhD in microbiology in the topic area of intestinal ecology, and completed postdoctoral training at Harvard Medical School. She remained on the faculty of Harvard Medical School where she was an associate professor of pediatric gastroenterology, and director of research for the Mucosal Immunology and Biology Research Center at Massachusetts General Hospital. In 2008, she joined the University of Massachusetts Medical School where she is professor and vice chair of the Department of Microbiology and Physiological Systems. Dr. McCormick is also the founding executive director of the University of Massachusetts Center for Microbiome Research, which she established in 2014. Dr. McCormick is one of the original pioneers in the field now known as cellular microbiology. Her work provided the first evidence that epithelial cells in response to pathogen contact orchestrate a pro-inflammatory program, which recruits inflammatory cells. Dr. McCormick has since identified new, previously unidentified and unexpected virulence mechanisms that are key to the inflammatory response, leading to both novel biological principles of host–microbe interactions and therapeutic intervention strategies for the treatment inflammatory bowel disease, and cancer. Her work continues to identify novel ways in which microbes interact with the intestinal epithelium, publishing over 100 original research papers and opinion pieces in this area. Dr. McCormick is an elected fellow of the American Academy of Microbiology, is on the Board of Editors for *Gastroenterology*, and *Gut Microbes*, and serves as a member of four Editorial Review Boards.

Dr. Mobley received his BS degree in biology from Emory University in 1975 and his PhD degree in microbiology and immunology from University of Louisville in 1981. He conducted postdoctoral training in biological chemistry and then bacterial genetics in the Center for Vaccine Development at the University of Maryland School of Medicine. He served on the faculty at the University of Maryland School of Medicine from 1984 until 2004 in the Division of Infectious Diseases (1984–97) and then the Department of Microbiology and Immunology (1997–2004) where he led the graduate program. During that time, he held a joint appointment in the Department of Biochemistry and Molecular Biology and trained graduate students in that program. In 2004, Mobley moved to the University of Michigan to chair the Department of Microbiology and Immunology and was installed as the inaugural Frederick G. Novy Collegiate Professor of Microbiology and Immunology.

Dr. Mobley's research interests focus on the molecular mechanisms of bacterial pathogenesis and on the fundamental basic research that will lay the groundwork for future therapeutics and vaccines. His lab studies virulence mechanisms of uropathogenic *Escherichia coli* and *Proteus mirabilis* that cause uncomplicated and complicated urinary tract infections, respectively, and, in the recent past, *Helicobacter pylori* that causes gastritis and peptic ulcer disease. For *E. coli* and *Proteus mirabilis*, his lab is focused on identifying surface-exposed proteins that are both synthesized by the bacteria during a urinary tract infection and conserved among uropathogenic *E. coli and Proteus* strains. Using these conserved antigens, his lab is determining the efficacy of candidate proteins as components of a multivalent subunit vaccine to protect against urinary tract infection.

Dr. Mobley is a fellow of the American Academy of Microbiology and a fellow of the American Association for the Advancement of Science and a member and past president of the Association of Medical School Microbiology & Immunology Chairs. He was the recipient of the inaugural University of Michigan Postdoctoral Association Excellence in Mentorship Award in 2012. He is a member of the editorial review boards of *Infection and Immunity and Microbiology Spectrum* and has served as a study section member for the National Institutes of Health. Dr. Mobley was awarded and named a distinguished university professor in 2015. This is the University's most prestigious professorship established to recognize senior faculty with exceptional scholarly achievements, national and international reputations for academic excellence, and superior records of teaching, mentoring, and service.

Dr. Mobley has published 255 peer-reviewed articles which have been cited in the literature over 18,000 times as of June 2019, as well as 49 book chapters and 5 books. He has trained 29 PhD students and 38 postdoctoral fellows, and has delivered 232 invited lectures in 21 countries.

Carlos Pedrós-Alió graduated in biology at the Autonomous University of Barcelona and got his PhD in bacteriology at the University of Wisconsin–Madison. After a postdoctoral stay at the Autonomous University he became an assistant professor of microbiology. He moved to the Marine Sciences Institute (CSIC, Barcelona) in 1989 where he was a research professor since 2000. In 2016 he moved to the National Center for Biotechnology (CSIC, Madrid). Dr. Pedrós-Alió's interest is to understand the ecology of aquatic microorganisms. Around 2005 he started to use genomics as a tool to generate hypotheses that could later be tested experimentally. He also likes to study extreme environments such as hypersaline systems, thermal springs, or polar waters. Another interest is in finding the mechanisms maintaining a large number of rare bacteria in aquatic ecosystems. He is also interested in outreach, relationships between art and science, biology of spirituality, fiction writing, and birding.

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SUBJECT CLASSIFICATION

HISTORICAL

AIDS, Historical
Cholera, Historical
Historical Plague
Historical Smallpox
History of Microbiology
History of Virology
Methods, Philosophy of
Spontaneous Generation
Syphilis, Historical
Typhoid, Historical
Typhus Fevers and Other Rickettsial Diseases, Historical

MICROBIAL DIVERSITY

Acidobacteria
Amitochondriate Protists (Diplomonads, Parabasalids and Oxymonads)
Amoebozoa
Aquificae
Archaea – An Introduction
Aspergillus: A Multifaceted Genus
Bacillus Thuringiensis: Mechanisms and Use
Bacteriophage: Overview
Brochothrix thermosphacta
Caulobacter
Chlamydia
Chloroflexi
Clostridia
Cyanobacteria
Dictyostelium
Dinoflagellates
Escherichia coli
Green Algae: Chlorophyta and Streptophyta
Haemophilus Influenzae
Halophilic Archaea
Helicobacter pylori
Legionella and *Bartonella*
Leishmania
Listeria monocytogenes
Mollicutes
Nanoarchaeota
Phylum *Verrucomicrobia*
Picoeukaryotes
Planctomycetes
Plant Pathogens, Minor (Phytoplasmas)
Protozoa
Rhizobia
Spirochetes
Staphylococcus

Streptococcus Pneumoniae Evolving – Impact of Antibiotics and Vaccines
Streptomyces
Trypanosomes
Viroids/Virusoids

PHYSIOLOGY AND GENOMICS

Archaellum
Autotrophic CO2 Metabolism
Bacterial and Archaeal Cell Membranes
Bacterial and Archaeal Cell Structure
Bacterial Bioluminescence
Bacterial Cell Cycles and Division
Bacterial Chemotaxis: Conservation and Variation on a Theme
Bacterial Development
Bacterial Flagella
Bioluminescence in Eukaryotic Microbes
Chromosome Replication and Segregation
Circadian Rhythmicity in Prokaryotes
Conjugation, Bacterial
CRISPR–Cas9
Crystalline Cell Surface Layers (S-Layers)
Energy Transduction Processes
Fundamentals of Metabolic Systems Biology
Gene Transfer Agents
Genetically Modified Organisms: Guidelines and Regulations for Research
Glycogen Biosynthesis
Horizontal Gene Transfer: Uptake of Extracellular DNA by Bacteria
Intracellular Structures of Prokaryotes: Inclusions, Compartments, and Assemblages
Iron Metabolism
Lipid Biosynthesis
Magnetotaxis
Methanogenesis
Methylation and other Modifications of Nucleic Acids and Proteins
Microbial Solute Transporters
Nitrogen Assimilation in Bacteria
No Bones About It: The Bacterial Cytoskeleton
Nonflagellar Bacterial Motility
Outer Membrane, Gram-Negative Bacteria
Peptidoglycan (Murein)
Phototaxis in Archaea and Bacteria
Phototrophy and Phototrophs
Pili, Fimbriae
Posttranscriptional Regulation
Regulation of Carbon Assimilation in Bacteria
Regulation of Replication Origin Firing
Regulatory RNAs
Restriction-Modification Systems
RNA Processing
Sensory Transduction in Bacteria
Single-Particle Cryo-Electron Microscopy
Stress, Bacterial: General and Specific
Stress Responses: Heat
Swimming and Swarming Motility
The Bacterial Glycome: From Monomers to Complex Carbohydrate Polymers
The Social Evolution of Bacterial Quorum Sensing
Transcription Regulation in Bacteria
Transduction: The Transfer of Host DNA by Bacteriophages
Translational Control and Fidelity

ECOLOGY AND EVOLUTION

Adaptations of Microorganisms to Low Nutrient Environments: Managing Life in the Oligotrophic Ocean
Adaptive Radiation in Microbes
Algal Blooms
Bacteriophage Ecology
Ecology of Rare Microorganisms
Ecology, Microbial
Endophytic Microbes
Evolutionary Theory and Experiments With Microorganisms
Extremophiles and Acidic Environments
Extremophiles: Cold Environments
Extremophiles: Dry Environments (Including Cryptoendoliths)
Extremophiles: Hot Environments
Extremophiles: Hypersaline Environments
Freshwater Habitats
Genomes From Uncultivated Microorganisms
Intracellular Symbionts and Parasites
Marine Deep Biosphere
Microbial Biofilms
Microbial Cycling of Methane
Microbial Ecology of the Rumen
Microbial Mats: Impact on Geology
Mixotrophy Among Freshwater and Marine Protists
Models in Microbial Ecology
Nitrogen Cycle
Origin of Life, Theories of
Overview of Plant Diseases
Palaeontology, Microbial
Paramecium Molecular Evolution
Phosphorus Dynamics in the Environment
Plastics: Colonization and Degradation
Quorum-Sensing in Bacteria
Rhizosphere
Secondary and Tertiary Endosymbiosis
Sediment Habitats, Including Watery
The Evolutionary Ecology of Microbes
Virus Evolution

PATHOGENESIS AND IMMUNOLOGY

Adhesins During Infection
Airborne Infectious Microorganisms
Antibiotic Resistance
Antifungal Agents
Antigenic Variation in Bacterial Pathogens
Antiviral Agents
Arboviruses
Bacterial Iron Acquisition Strategies
Biofilms and Disease: A Persistent Threat
Capsules and Secreted Extracellular Polysaccharides
Chlamydophila pneumoniae, A Pathogen Causing More Than Pneumonia
Commensal to Pathogen Transition of *Candida albicans*
Complement
Cutaneous Fungal Infections
Diagnostic Microbiology
Emerging and Reemerging Infectious Diseases
Entamoeba Histolytica: Biology and Host Immunity
Enteric Viruses
Epidemiological Concepts and Historical Examples

Exotoxins
First Principles of Clinical Microbiology: Collection, Handling, and Diagnostics
Fungal Biofilms
Fungal Infections, Systemic
Gastrointestinal Microbiology in the Normal Host
Gut Microbiota in Human Health and Diseases
Hemorrhagic Fever Viruses
Hepatitis Viruses
Herpesviruses
HIV and Retroviruses
How Microbial Pathogens Subvert Host Innate Immune Defenses
Human Fungal Infections
Lipopolysaccharides (Endotoxins)
Medically Relevant *Mycoplasmas and Ureaplasma*
Microbial Agents to Treat Cancer
Microbiology of the Cystic Fibrosis Airway
Mycotoxins
Oral Microbiology
Pathogen Sensing: Toll-Like Receptors and NODs (Innate Immunity)
Phage Therapy
Phagocytes (Innate Immunity)
Polyomaviruses and Papillomaviruses
Quinolones
Rabies
Respiratory Viruses
Retroviruses
RNA Viruses: Plant Pathogenic
Role of B Cells and Antibodies in Controlling Bacterial Pathogens
Sexually Transmitted Diseases
Toxoplasmosis
Unusual Infectious Agents

TECHNOLOGICAL ADVANCES AND APPLIED MICROBIOLOGY

Amino Acid Production
Amylases
Antimicrobial Susceptibility Testing
Bacterial Targeting of Tumors
Bacteriophages and Rapid Detection of Bacterial Pathogens: A Novel Approach
Beer/Brewing
Biocides
Biodeterioration – Including Cultural Heritage
Biological Warfare
Bioreactors
Biosensors
Bioterrorism
Continuous Cultures (Chemostats)
Corrosion, Microbial
Directed Evolution
DNA Cloning Strategies
Drinking Water
Drinking Water Microbiology
Foodborne Pathogen Detection, Using Rapid Technologies
Fungal Extracellular Vesicles
Genome Sequence Databases: Annotation
Genome Sequence Databases: Sequencing and Assembly
Genome Sequence Databases: Types of Data and Bioinformatic Tools
Genome Sequencing of Microbes
Heavy Metal Pollutants: Environmental and Biotechnological Aspects
Industrial Biotechnology (Overview)

Industrial Production of Glycosaminoglycans
Infectious Waste Management
Insecticidal Toxins from *Photorhabdus and Xenorhabdus*
Metal Extraction and Biomining
Microbial Adhesion
Microbial Forensics
Microbiology of Fermented Dairy Products
Municipal Water Treatment
Organic and Fatty Acid Production, Microbial
Patenting of Microorganisms
Pesticides, Microbial
Phylogenetic Methods
Pigments, Microbial
Polysaccharides, Microbial
Solvent (Acetone–Butanol: AB) Production
Teaching Resources, Microbiology
Technology Advances in Medical Microbiology
Trehalose: A Crucial Molecule in the Physiology of Fungi
Type Culture Collections their Databases
Vitamins and Vitamin-Like Compounds: Microbial Production
Water Treatment, Industrial
Wine
Xylanases

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PREFACE

As with previous editions of the *Encyclopedia of Microbiology*, the 4th edition takes on the challenge of providing a contemporary overview of microbes—the most abundant and diverse forms of life on Earth. These single-celled organisms were the first life forms to inhabit Earth and they transformed the planet and its atmosphere. They continue to maintain Earth's atmosphere, drive essential processes in terrestrial and aquatic ecosystems, and form intimate relationships with all plants and animals. Microbes can reproduce by doubling every 20 minutes or can remain dormant for many thousands of years. They colonize every known habitat on Earth, accounting for approximately half of the living biomass on our planet. Microbes also cause the most devastating diseases known to humans and are winning the antibiotic war we wage against them. Yet, we cannot live without microbes as symbionts of humans and drivers of Earth's biosphere.

Despite the central roles of microbes, we are only just becoming aware of our limited knowledge of interactions among microbes, other organisms, and the environment. Molecular techniques continue to lead a revolution in our understanding of microbial diversity and function, with the new "big data" sciences of genomics, transcriptomics, proteomics, and metabolomics now being applied to communities of microbes. These techniques provide the ability to identify and study complex communities of microbes in diverse environments including the human gut, forest soil, water-treatment biofilters, and the open ocean.

Given the remarkable physiological and phylogenetic diversity of microbes, their capacity for rapid evolution, and the pivotal role of host-associated and environmental microbiomes, it is difficult, if not impossible, to identify contemporary questions in biology that are not influenced by microbes. It is also increasingly difficult to assemble an encyclopedia that provides comprehensive coverage of the vast universe of microbes. Despite these challenges, we have engaged the expertise of scientists around the globe to provide an exceptional overview of and perspective on the microbial world. This edition of the *Encyclopedia of Microbiology* will help readers develop a framework for understanding microbes and will provide references directing readers to the primary literature that is needed for a more thorough appreciation of specific topics.

We have included a number of articles which provide a historical perspective of microbiology that is focused on disease—one of humanity's earliest acknowledgment of microbes. These help frame more contemporary issues in microbiology that have moved far beyond the relatively small collection of microbes that cause disease. Rather than simply alphabetize topics as in traditional encyclopedias, articles in the 4th edition are also listed under the following themes in a Subject Classification: Historical, Microbial Diversity, Physiology and Genomics, Ecology and Evolution, Pathogenesis and Immunology, and Technological Advances and Applied Microbiology. We hope this helps readers navigate the work more effectively.

On behalf of an insightful and collegial editorial team, I hope that you emerge from your forays into the *Encyclopedia of Microbiology* with some of the awe that we share for the elegance and power of the microbial world.

Thomas M. Schmidt
Editor-in-Chief

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INTRODUCTION: MICROBIAL DIVERSITY

It is what we think we know already that often prevents us from learning.

—Claude Bernard

One of the difficult lessons for any professional scientist is that, as much as we know, as good as our tools and questions are, our knowledge is crude and far smaller than we imagined when we were students. Particularly troublesome is that most fundamental aspects of various scientific fields are simply not very well understood. The physicist wonders what, exactly, "time" is, how space is structured, and why the Planck Constant is $6.62607004 \times 10^{-14}$ m^2 kg/s instead of any other number you might care to choose. Chemists argue about the nature of a covalent bond, or whether or not transition states exist in reality. Biologists are at a loss to describe how life originated, or even what exactly life (in a general sense) actually is. In the most important book of modern times, "On the Origin of Species," Charles Darwin clearly (but not concisely) deconstructs the idea of the biological "species," and despite what many of us learned in school, our view of the "species" even for multicellular sexually reproducing creatures remains murky. The modern reader of "On the Origin of Species" might be forgiven for concluding that the reason for this is that it is the actual *biology* of species, and by extension "life," our origin, etc., rather than our concepts of them, that are fundamentally disordered.

The subject of this section of the Encyclopedia is Microbial Diversity. What does this mean? What *is* "microbial diversity?"

Let us start with "microbial." Literally speaking, the term "microbial" means small living things, usually understood to mean life too small to see with the unaided eye. The scientific field of microbiology, however, mostly emerged from our need to solve crucial health dangers created by a tiny fraction of microbes. As a result, "microbiology," and so "microbes," refer to Bacteria (and in modern times the Archaea), fungi, a scattering of problematic single-celled or small multicellular eukaryotes, and the physiological systems of the human body that manages our interactions with these creatures. This sells the microbial world very short. For 85% of the history of our planet, life was *entirely* microbial, and it remains *predominantly* microbial. In the words of Stephen J. Gould, "*We live now in the 'Age of Bacteria.' Our planet has always been in the 'Age of Bacteria,' ever since the first fossils—bacteria, of course—were entombed in rocks more than 3 billion years ago. On any possible, reasonable or fair criterion, bacteria are—and always have been—the dominant forms of life on Earth.*" Another view of this comes from the phylogenetic perspective. If you scrutinize any objective/quantitative "Tree of Life," based on molecular sequence analysis or any other criterion of choice, you will quickly discover that nonmicrobial life is limited to the tips of a small number of otherwise un-noteworthy branches. Only from our self-centered anthropocentric viewpoint is microbiology distinguishable from biology as a whole.

And what of "diversity?" Living things and their morphology, structure, physiology, ecology, internal mechanisms, behavior, interactions, etc., are far more diverse than most of us have been led to believe. For example, if you think you know how mitosis works, look it up in *Giardia* and be amazed. The notion of what constitutes a "gene" in the kinetoplast of *Trypanosoma* requires you to throw almost everything you think you know about molecular genetics away. Then have a look through the genome of the archaeon *Nanoarchaeum.* These are just the tips of icebergs; these and many more can be found within the chapters that follow. And yet . . . all of this diversity rests over an almost entirely uniform biochemistry that speaks to the shared ancestry of all living things on Earth. How, then, do we assess the "diversity" of living things? Historically, this meant comparing the morphology of different living things; this was the origin of Linnaean taxonomy and, to be fair, is very useful for organisms on the human size scale. More recently molecular phylogenetics has allowed us to more clearly understand how creatures are related, and draw an objective and quantitative graph of these

relationships; the molecular "Tree of Life" is a more useful roadmap of biological diversity. But it is clear that an even more sophisticated view of biological diversity is required, for example to describe how horizontal gene transfer impacts "diversity," and many of these questions emerge from microbiology. Perhaps the most important fundamental question in microbiology today is "What is a microbial species?" In other words, how do we conceive of a "species" in organisms that do not reproduce sexually? This is not a trivial or parochial question; remember that the concept of biological evolution, and so the foundation of the science of biology, emerged directly from exploration of what a "species" is and how it originates, in plants and animals. But this was over 150 years ago. Broadening our view of microbial diversity, how it is structured and its history and origins, is the key to new and deeper biological insight.

And so, as is usual in science, attempt to answer a simple question that raises many more questions and provides few answers. What *is* microbial diversity? I dunno, but let us have a look. . ..

James Brown

INTRODUCTION: PHYSIOLOGY AND GENOMICS

The first two editions of EOM were edited entirely by Dr. Joshua Lederberg. The third edition was also planned largely by Dr. Lederberg, but he died during that phase, in 2008, and was replaced by Dr. Moselio Schaechter, promoted from the ranks of associate editors. Dr. Thomas Schmidt was also an associate editor for that edition and moved up to editor-in-chief for the fourth edition.

The time that has passed from the first to the fourth edition has seen remarkable advances in understanding the variety of microbes. Complete genome sequences were determined for 18 microbes by October 1998 and published in the second edition in 2000. By 2019, a single company had determined the complete annotated genome sequences of several thousands of microbes. Comparative genomics, including the ancillary "omics," as well as the application of systems biology to reconstruct metabolic networks, have provided new insights into many aspects of microbial physiology and genetics. The advent of cryo EM and of single particle cryo EM has revolutionized how we understand the structure of cells and macromolecules. For this fourth edition, we were guided by the splendid organization of the third and additional areas in which considerable advances have been made. The fourth edition now includes articles describing additional features of microbiology: cryo-electron microscopy, CRISPR/*cas*, and metabolic systems biology, as examples of completely new methods to study microbes, circadian rhythms as an important aspect of the physiology of at least one group of microbes, and new developments in our understanding of the many ways in which bacteria and archaea move. We have also made use of updates of chapters that were included in the third edition.

Bianca Brahamsha
Robert Haselkorn

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INTRODUCTION: ECOLOGY AND EVOLUTION

Evolution

Seven articles deal with different aspects of evolution. Two articles analyze the remains of the past evolution of microbes in the fossil record. Three other look at theories, mechanisms, and experimental procedures. Two more examine specific aspects of the evolution of viruses and of a ciliate. Finally, one article bridges ecology and evolution looking at the emerging field of evolutionary ecology of microbes.

Ecology

The ecology section contains a few general articles about what microbial ecology is about, and the particularities of the ecology of viruses and rare microorganisms.

The remaining articles can be placed in two complementary approaches: habitat oriented or process oriented. In the first approach there is a lot of attention devoted to extreme environments. These environments are usually exclusively microbial and show the capacity of microbes to deal with really difficult conditions. Besides, the relative simplicity of such ecosystems allows somewhat easier manipulations than in "normal" ecosystems. Several of the latter are also examined in articles ranging from the deep ocean to plastics. Special attention is given to animal and plant habitats and how microbes interact with their hosts.

The process-oriented approach shows, on the one hand, adaptive traits of microorganisms such as the ability to live in diluted environments or to use chemotaxis to find the optimal conditions. One promising and novel avenue to study adaptations is the analysis of the genomes of uncultured microorganisms. On the other hand, several articles review processes relevant at the ecosystem level, from feeding strategies such as myxotrophy to element cycles such as methane formation and oxidation and, finally, to the use of models in microbial ecology.

We hope this collection of articles provides an overview of the exciting times that microbial ecology is going through, with many new discoveries appearing every few years, and a fascinating and invisible world to be explored.

Carlos Pedrós-Alió
Larry Forney

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INTRODUCTION: PATHOGENESIS AND IMMUNOLOGY

This section of the encyclopedia focuses on the relatively narrow slice of all microbes that infect living hosts and how the hosts combat these microbes using innate and adaptive immunity. In addition, we examine how antimicrobial agents including unique metabolites and even bacteriophages have been enlisted to combat these infections. This section takes a broad look at infectious agents ranging from prions, viroids, and satellites to the more traditional etiologic agents like bacteria, viruses, fungi, and selected parasites. Also addressed are epidemiologic and diagnostic techniques used to identify and track the spread of infectious microbes including the most advanced diagnostic methods used by the clinical microbiology laboratory. A closer look at pathogenic mechanisms includes groups of virulence factors such as adhesins, iron acquisition, exotoxins of bacteria and fungi, and bacterial endotoxin (lipopolysaccharide). Strategies adopted by bacteria and fungi to colonize implanted foreign bodies such as catheters are also discussed. Plus, infections of the gastrointestinal tract, lung, oral cavity, and the skin are examined. In addition to pathogenic microbes, some articles examine the protective effect of the microbiome and commensal organisms. With respect to immunity this section also focuses on the interplay of the host immune system with pathogenic microbes covering evasion immune strategies in humoral and cellular immunity as well as innate immunology and the complement immune system.

Beth McCormick
Harry Mobley

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INTRODUCTION: TECHNOLOGICAL ADVANCES AND APPLIED MICROBIOLOGY

This section focuses on the development and refinement of the tools commonly used for both basic and applied microbiology. The articles in this section are written by authors who personally have worked on developing and fine-tuning technology for research in microbiology. Many of the products and processes described have multiple uses in a variety of disciplines such as health, the environment, food and agriculture, and industrial biocatalysis. The advances made in sequencing, protein analysis, and bioinformatics have given microbiologists new insights into old problems. These advances have also enabled the rapid identification of microorganisms in studies of biowarfare, infectious disease, and pollution of our water sources. At the same time the integration of mathematics and physics with biology and chemistry has provided instrumentation that enables a greater depth of analysis than ever before. Never in the history of microbiology have so many technological advances been developed and commercialized for use by scientists. Microbiologists today are equipped with improved "tools" within the toolbox to push the boundaries of not only elucidating the relationship of microbes to humans, plants and animals but also moving basic research much faster from the bench to development of new products and processes that benefit society and the planet. The renaissance of applied microbiology powered by technological advances has been both exciting and promising in all fields of study.

Jennie C Hunter-Cevera
Stanley Maloy

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Rabies☆

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Glossary

Acinus A minute terminal lobule in the salivary glands.
Apoptosis A process by which cells undergo a natural programmed cell death or physiological cell death in response to diverse stimuli.
Encephalomyelitis Acute demyelinating disease caused by virus without invoking an immunopathologic mechanism.
Epitopes Structural sites on proteins that bind antibodies and T-cell receptors.
Fixed rabies virus Laboratory strains that grow in tissue culture at a predictable rate, with predictable biological properties, and regularly kill inoculated animals within a predictable period.
Guillain–Barré syndrome An acute inflammatory demyelinating polyradiculoneuropathy that in the majority of cases follows a 'virus-like' illness.
Hydrophobia The unique characteristic of rabies involving both severe thirst, accompanied by a desire to drink and at the same time intense fear of water, which is regarded as the most specific manifestation of clinical rabies in humans.
Leptomeninges The two innermost layers of the meninges; cerebrospinal fluid circulates between these innermost layers.
Lyssavirus Rabies virus and variant viruses known as 'rabies-related' viruses, which share with rabies virus the unique capability to produce a rabies-like encephalomyelitis.
Negri body Cytoplasmic matrix composed of rabies viral nucleocapsid proteins.
Nodes of Ranvier Tiny gaps formed between myelin sheaths that wrap and insulate axons, which themselves (the gaps) are not insulated allowing them to generate electrical activity and pass nutrients and waste into and out of neurons.
Rhabdoviridae Taxonomic family of rhabdoviruses in the order Mononegavirales that distinguishes itself as identifying with bacillus or 'rod- or bullet-shaped' virus particles, including Vesicular Stomatitis virus as the prototypical member of the *Vesiculovirus* genus and Rabies virus as the prototypical member of the *Lyssavirus* genus.
Street rabies virus Virus isolated from natural infection that has not been serially passaged in animals or tissue culture.
Superantigen A class of proteins that bind class II major histocompatibility proteins and stimulate powerful non-specific proliferation of T-cells bearing particular Vβ sequences as part of their αβ antigen receptor.
Viropexis Entry of virus particles into cells by engulfment, followed by fusion of membrane-bound vesicles with lysosomes.
Xoplasmic transport Bi-directional (anterograde or retrograde) flow of proteins, particles, and organelles along the length of the neuronal cell's axon or nerve track to reach intraneuronal destinations.

Abbreviations

ABLV	Australian bat lyssavirus
ACIP	Immunization Practices Advisory Committee
AGP	Antigenome promoter
ARAV	Aravan
BBLV	Bokeloh bat lyssavirus
BSR	A clone of BHK (baby hamster kidney) cells
CCV	Cell culture-based vaccine
CD	Cytoplasmic domain
CD44	Cell determinant 44 (a cell marker protein)

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CNS	Central nervous system
cRNA	Complementary (antigenome) RNA
CRU	Cellular receptor unit
CSF	cerebrospinal fluid
CTL	Cytotoxic lymphocyte
CVS	Challenge virus standard
DFA	Direct fluorescent antibody
DNA	Deoxyribonucleic acid
DUVV	Duvenhage virus
EBLV-1	European bat lyssavirus type 1
ED	Ectodomain
EM	Electron microscope
ER	Endoplasmic reticulum
ERA	Evelyn–Robitnicki–Abelseth
ERIG	Equine rabies immunoglobulin
GP	Genome promoter
HDCV	Human diploid cell vaccine
HEP	High egg passage
HRIG	Human rabies immunoglobulin
hsp70	Heat-shock protein 70
IB	Inclusion body
ID	Intradermal(ly)
IFA	Indirect fluorescent antibody
IFN	Interferon
IKOV	Ikoma lyssavirus
IM	Intramuscular(ly)
IRKV	Irkut
IU	International unit
KHUV	Khujand
LBV	Lagos bat virus
LC8	Light chain 8
Le	Leader
MAb	Monoclonal antibody
MIT	Mouse inoculation test
MOKV	Mokola virus
Mr	Relative molecular mass
mRNA	Messenger ribonucleic acid
nAChR	Nicotinic acetylcholine receptor
NC	Nucleocapsid
NCAM	Neural cell adhesion molecule
NTR	Neurotrophin receptor
nts	Nucleotides
NTV	Nerve tissue vaccine
ORF	Open reading frame
PCECV	Purified chick embryo cell vaccine
PEP	Postexposure prophylaxis
PET	Postexposure treatment
PNS	Peripheral nervous system
PrEP	Preexposure prophylaxis
PRP	Partners for rabies prevention
RABV	Rabies virus
RIFFIT	Rapid fluorescent focus inhibition test
RIG	Rabies immunoglobulin
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rNTPs	Ribonucleoside triphosphates
RT-PCR	Reverse-transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
SHIBV	Shimoni bat virus

TM	Transmembrane
Tr	Trailer
TUNEL	Terminal deoxynucleotide transferase dUTP nick end labeling
VAP	Viral attachment protein
VNA	Virus-neutralizing antibody
vRNA	Viral ribonucleic acid or genome RNA
VSV	Vesicular stomatitis virus
WCBV	West Caucasian bat virus

Defining Statement

Rabies is an acute neurologic disease caused by a lyssavirus (of the Rhabdoviridae family) that attacks the central nervous system of all warm-blooded animals and humans and is normally a fatal viral infection. The first written record of the disease dates to twenty-third century BC, and in fourth century BC Plato used the word 'Lyssa' to describe the 'erotic passion' or 'madness' (viciousness) associated with the disease at the time. Outbreaks of rabies in Europe were rare until the Middle Ages, when rabies epizootics occurring in dogs, rabid wolves, and foxes were reported in many countries. By the 1700s, rabies was common throughout Europe, particularly in central Europe, and was beginning to be reported in the New World.

Rabies virus (RABV) is a highly neurotropic virus in infected animals and humans. Virus infection in humans causes alternating symptoms of depression and agitation and almost invariably leads to extreme types of violent behavior and death. Hydrophobia is a unique characteristic of rabies in humans. RABV is transmitted to humans from wild and domestic animals, through bites and deposition of virus-laden saliva. The predominant animal reservoir for RABV continues to be in dogs, which causes a sizable clinical problem for humans, especially in those countries, mainly of Asia and Africa and to a lesser extent in Central and South America, where canine rabies is largely uncontrolled.

Rabies and Rabies-Related Viruses

Rabies virus (RABV) is the prototype virus of the genus *Lyssavirus* in the family Rhabdoviridae. RABV, which is distributed worldwide, is a highly neurotropic virus in infected animals and humans, invariably causing a fatal encephalomyelitis, commonly referred to as rabies. Until 1956, RABV was considered the only virus associated with the oldest known disease, recognized in ancient times (as early as the twenty-third century BC) as 'mad dog' disease. Variants of RABV, known as 'rabies-related' or non-RABV lyssaviruses, have since been identified after the initial discovery of two rabies-related viruses originating from Africa: Lagos bat virus (LBV) and Mokola virus (MOKV) in 1956 and 1968, respectively. Rabies-related viruses share with RABV the unique ability to elicit the clinical disease of rabies, producing a rabies-like encephalomyelitis, and also share other RABV structural characteristics. There are now fourteen recognized lyssaviruses, 12 of these have been differentiated into three phylogroups based on phylogenetic analyses. RABV (formerly identified as genotype 1) belongs to phylogroup I along with Duvenhage virus (DUVV) (genotype 4), European bat lyssavirus, type 1 (EBLV-1) (genotype 5), and type 2 (EBLV-2) (genotype 6), and Australian bat lyssavirus (ABLV) (genotype 7). Three putative species, Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), and Bokeloh bat lyssavirus (BBLV) the newest of species to be characterized are considered independent species of phylogroup I. Phylogroup II includes LBV (genotype 2), MOKV (genotype 3), and Shimoni bat virus (SHIBV), the newest of species to be characterized and considered an independent species within phylogroup II. West Caucasian bat virus (WCBV) and the Ikoma lyssavirus (IKOV), isolated from an African civet, which do not cross-react serologically with any members of the two phylogroups, could tentatively belong to a third phylogroup (phylogroup III). Phylogenetic analyses suggest that all lyssaviruses originate from a precursor bat virus.

Virus Structure

RABV and the non-RABV lyssaviruses belong to the class of RNA viruses that contain a linear, single-strand, negative-sense (noninfectious) RNA genome that is approximately 12 kb or 12 000 nucleotides (nts) in length (Mr 4.2×10^6). The viral genome (2–3% of the virion weight) forms the backbone of the tightly coiled helical RNP (ribonucleoprotein/RNA plus protein) core, which extends along the longitudinal axis of the bullet-shaped virus particle (Figure 1). The RNA genome contains five genes that code for the five structural proteins of the virion, which are designated N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), and L (RNA transcriptase/polymerase or large protein). The five genes are organized in the genome in order, starting at the 3′ end, N, P, M, G, and L. The first four genes are separated by short (di- or penta-) intergenic nt stretches (N–P, P–M, and M–G) and a longer 19–28-nt sequence between the last L gene and the G gene (Figure 2). The full nucleotide sequence of the RNA genome includes a noncoding leader (Le) sequence at the 3′ terminus and a noncoding trailer (Tr) sequence at the 5′ end. These short (58–70 nt) sequences provide specific *cis*-acting signals (a specific nucleotide sequence 'acting within' the genome RNA) to initiate specific functions in genome RNA transcription and replication.

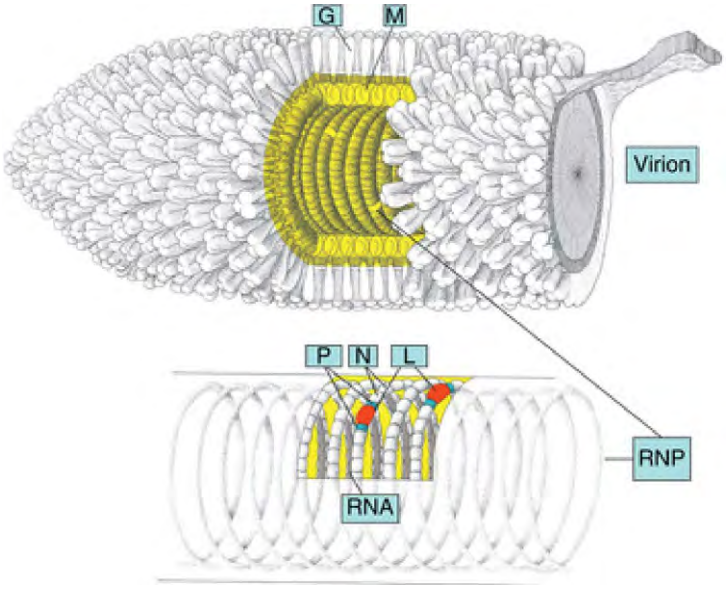

Figure 1 Schematic representation of the rabies virion. The drawing shows the internal ribonucleoprotein (RNP) core consisting of the single-strand, negative-sense genome RNA encapsidated with nucleocapsid protein (N), the virion-associated RNA polymerase (L), and polymerase cofactor phosphoprotein (P). The RNP core in association with the matrix protein (M) is condensed into the typical bullet-shaped particle that is characteristic of rhabdoviruses. A lipid bilayer envelope (or membrane) in which the surface trimeric glycoprotein (G) spikes are anchored surrounds the RNP-M structure. The membrane 'tail' depicted in the drawing represents the trailing piece of envelope that is frequently observed attached to the virus as it buds from the plasma membrane of the infected cell when viewed under the electron microscope (EM). Reproduced from Wunner, W. H., Larson, J. K., Dietzschold, B., Smith, C. L. (1988). The molecular biology of rabies viruses. *Review of Infectious Diseases* 10(Suppl. 4): S771–S784, with permission.

Figure 2 Organization of the rabies virus (RABV) RNA genome. The nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large RNA-polymerase protein (L) genes (orange) are separated by four intergenic nucleotide sequences and flanked by the leader (Le) RNA and trailer (Tr) RNA sequences (green) at the 3′ and 5′ termini, respectively. Numbers shown above the five genes and below represent nucleotides per gene and in the Le, Tr, and intergenic nucleotide sequences, of the RABV Evelyn–Robitnicki–Abelseth (ERA) strain. Reproduced with modification from Wunner, W. H. (2007). Rabies virus. In: Jackson, A. C., Wunner, W. H. (eds.) *Rabies*, 2nd edn., pp. 23–68. San Diego, CA: Elsevier, Academic Press; From Wunner and Conzelmann (2013) Rabies virus. In: Jackson, A.C. (ed.) *Rabies*, 3rd edn., pp. 17–60. San Diego, CA: Elsevier, Academic Press, with permission.

The five structural proteins of RABV (as well as non-RABV lyssaviruses) comprise 67–74% of the virion by weight. The three viral proteins associated with the viral RNP core include the N (the most abundant protein in the RNP core) and the noncatalytic polymerase-associated P and the catalytic L components of the RNA polymerase complex. All three proteins are involved in the RNA polymerase activity of the virion, which generates monocistronic mRNA transcripts of each of the viral protein genes and a full-length complementary (plus-sense) transcript of the viral genome that serves as a template to produce a progeny (negative-sense) RNA genome. The remaining two structural proteins of RABV, G and M, are associated with the lipid bilayer envelope that surrounds the RNP core. M is a small-size nonglycosylated protein that lines the viral envelope forming an inner leaflet between the envelope and the RNP core. G is the only glycoprotein. It forms the trimeric spikes on the outer surface of the viral envelope. The mature viral G is glycosylated with branched-chain oligosaccharides, which account for 10–12% of the total mass of the protein. There are approximately 450 trimeric spikes distributed on the outer surface of each virion. Other host-derived minor protein components such as actin and heat shock proteins of the hsp70 type, CD44 and CD99-related glycoprotein (VAP21), are also found in rabies virions. The role of these proteins is unclear; it is possible that some of them act to chaperone the viral proteins during synthesis and are incorporated into virions after binding to and assisting in G folding. Cytoskeletal proteins normally expressed on the host cell surface may also be incorporated into virions as a consequence of their proximal location and function in virus budding. Some of these proteins ensure intracellular transport of viral RNP along the pathogenetic pathway of the virus. A lipoprotein bilayer that forms the viral envelope around the helical RNP core is therefore a mixture of viral and host proteins and lipids, which constitute 20–26% of the viral envelope.

RABV particles or virions are best described as bacilliform or bullet-shaped with one end rounded (hemispherical) and the other end flattened (planar) (see Figure 1). The physical appearance of this rigid rod-like, bullet-shaped virion was first described from images seen using the electron microscope (EM). The average length of standard-size, infectious virions is 180 nm (130–250 nm) and the average diameter is 75 nm (60–110 nm). Besides standard-size virions, other shorter, often cone-shaped virions are sometimes observed. These are defective virions that have genomes approximately one-third to one-half the size of the full-length genome of standard-size virions. These shorter genomes are the result of internal nucleotide sequence deletions that occur during virus replication, particularly in virus-infected cell cultures.

Viral Protein Function

The nucleoprotein (N; 450 amino acids) is the most abundant of the viral proteins produced in RABV-infected cells. The model for rhabdovirus genome transcription proposes that the viral RNA polymerase will produce the greatest number of transcripts (mRNA copies of a gene) from the gene closest to the 3′ end of the RNA genome. A gene that is closer to the 3′ end of the genome is transcribed more frequently than the next gene and so on along the genome. One of the primary functions of N is to 'encapsidate' the nascent (newly synthesized) viral RNA that is made during virus replication, which protects it from ribonucleases that degrade naked single-stranded RNA in the cell. The most conserved amino acids of N stabilize certain key protein-RNA interactions. N is also the major protein of cytoplasmic inclusion bodies (Negri bodies) that are prominent as morphologically distinct lesions found in infected neurons of the diseased brain ('rabies pathogenesis and histopathology'). Although the amino acid sequence of N is the most conserved of the viral proteins among the lyssaviruses, the noted amino acid differences provide unique, genotype-specific epitopes on the N making it a useful protein for comparative antigenic analysis and genetic typing based on its nucleotide sequence in diagnostic and phylogenetic analyses. Determination of a stretch of nucleotide sequence is a commonly applied method for comparing a collection of lyssaviruses and allowing by sequence alignment a direct comparison of virus isolates to reference sequences leading to the phylogenetic classification of a particular virus.

N is the second most extensively analyzed of the RABV proteins (after G) with respect to its antigenic and immunogenic structure and function. The RNP of RABV induces protective immunity against peripheral challenge, with lethal RABV (infection by a virulent form of RABV) in animals due to dominant B- and T-cell-specific epitopes (amino acid regions) on N. Three antigenic epitopes (antibody binding sites) on N have been mapped to amino acids 358–367 (antigenic site I) and three linear epitopes (antigenic site IV) have been mapped to two independent regions, amino acids 359–366 and 375–385. The apparent paradox that N appears to have two independent antigenic sites (I and IV) recognized by antibodies that do not compete with each other for binding to the N antigen suggests that the two overlapping epitopes are recognized independently on different forms of N. The paradox was resolved when it was discovered that N, which is diffusely distributed in the cytoplasm and available to encapsidate RNA, represents one form and the N that is associated with cytoplasmic inclusion bodies represents the other form. N is also the major target for T-helper (Th) cells that cross-react among rabies and rabies-related lyssaviruses. N has other properties that enable it to function as an exogenous viral 'superantigen', and is perhaps the only viral superantigen that has been identified in humans. Some of the properties include its potent activation of peripheral blood lymphocytes in vaccinated humans and its ability to produce a more rapid and heightened virus-neutralizing antibody (VNA) response upon injection of inactivated rabies vaccines. N is also capable of inducing early T-cell activation steps and expansion and mobilization of $CD4^+$ Vβ8T-cells to trigger and support production of VNA and bind to HLA class II antigens expressed on the surface of cells.

Phosphoprotein (P; 297 amino acids), which is derived from the second gene (next after the N gene) in the RABV and non-RABV lyssavirus genome, is a multifunctional protein and a key component and regulatory protein of the virion-associated RNA polymerase complex in viral genome replication. In the cell, one of the functions of P is to act as a chaperone specifically directing N to encapsidate viral RNA, by interacting with newly synthesized (nascent) soluble N (N°) to maintain N° in a competent form for RNA encapsidation, preventing N° from polymerizing (self-aggregating) and nonspecifically binding to cellular RNA. P also plays a pivotal role as a noncatalytic cofactor in the transcription and replication of the viral genome. It serves to stabilize L and place the polymerase complex on the RNA template, which L alone is unable to do. These two functions of P are accomplished via domains of P that are specific for each of these two proteins. P is present in the infected cell in a form that is distinguishable from its presence in the virion and these forms can be differentiated by their degree of hypo- to hyperphosphorylation when resolved by electrophoresis in sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Phosphate groups are added to RABV P by two distinct protein kinases, one of which phosphorylates the protein at serine (S)63 and S64 in the N-terminal portion of the molecule altering the migration of P in SDS-PAGE. The other kinase, which appears to be selectively packaged in mature virions, does not alter the migration of P in SDS-PAGE. Other protein–protein interactions that involve RABV P are equally important, such as its interaction with cellular proteins that influence or help regulate virus tropism and cell-to-cell spread. Dynein light chain 8 (LC8) is a cellular protein, part of cytoplasmic dynein and the myosin V microtubule-associated motor protein complex that facilitates the transport of RABV through neurons. Other interactions include the inhibition of innate immune responses, such as the interferon (IFN) response, that typically prohibit productive virus replication.

The rabies virion-associated RNA polymerase or large protein (L; 2142 amino acids) is the catalytic component of the polymerase complex of RABV. It is encoded in the fifth or most distal gene from the 3′ end of the RNA genome, which accounts for more than half, 54%, of the viral genome-coding potential. Along with noncatalytic P, L is responsible for the majority of enzymatic activities involved in viral RNA transcription and replication. The virion-associated RNA polymerase has the unique function at the start of infection to initiate the primary transcription of the genome RNA after the RNP core is released into the

cytoplasm of the infected cell. The transcripts, which include the 3′ Le RNA and monocistronic mRNAs of the N, P, M, G, and L genes, are initiated, elongated, and in the case of mRNAs cotranscriptionally modified to include 5′ capping, methylation, and 3′ polyadenylation. These enzymatic activities that are assigned to L are identified with but not precisely mapped within one (block III) of four blocks of highly conserved amino acid sequences distributed noncontiguously in L. Block III, the catalytic domain, between amino acid residues 530 and 1177 in the central part of the primary sequence of L, consists of four motifs (A–D) that represent regions of highest similarity and are thought to constitute the polymerase module of L. These structurally conserved motifs are engaged in the transcriptional activity of L that requires binding of substrate ribonucleoside triphosphates (rNTPs), polyadenylation, and protein kinase activity for specific phosphorylation of the P in transcriptional activation. At least two other consensus sequences bind ATP that is utilized in the essential activities of L.

The matrix protein (M; 202 amino acids) of RABV and the non-RABV lyssaviruses is derived from the third gene in the RNA genome. It is the smallest of the virion proteins. There are two isoforms of M: a major form, Mα, which accounts for 70–75% of total M in lyssa virions, and a minor form, Mβ, which accounts for the rest of M in the virion, but only 10–15% of total M in the cell. M plays a critical role in the morphogenesis of lyssa virions as it binds to and condenses the nascent RNP complex (nascent virus core) into a tightly coiled, helical RNP-M structure, forming a sheath around the RNP core. This structure, which assumes a 'bullet' shape, is referred to as the virus 'skeleton.' At the time that M binds to the RNP core, it also interacts with the host plasma membrane at the marginal region of the cytoplasm where it initiates the virus budding process from the plasma membrane. The virus budding process appears to be triggered by a proline-rich motif (PPEY), referred to as a 'late-budding domain' (L-domain), located within the highly conserved 14-amino acid sequence near the N-terminus of the RABV M. Similar proline-rich motifs identified in other types of budding viruses are also associated with virus budding from the plasma membrane. While the release of virus particles from the plasma membrane of infected cells requires the M of the RNP-M skeleton structure, the efficiency of virus budding is greatly enhanced by the interaction of the skeleton structure with the viral envelope glycoprotein (G).

The glycoprotein (G; 505 amino acids) of RABV, like non-RABV lyssaviruses, is translated from mRNA that is transcribed from the fourth gene in the RNA genome. The G gene encodes a 524-amino acid protein, which includes a signal peptide of 24 amino acids at the N-terminus that is posttranslationally cleaved before the mature 505-amino acid G is integrated into the envelope of the virion. G is a type I glycoprotein, which indicates that the protein is anchored in the membrane envelope by a transmembrane (TM) domain located near the C-terminus of the protein. The short C-terminal portion (22 amino acids) of G that remains beneath the membrane (toward the cytoplasm) is the cytoplasmic domain (CD) and the large N-terminal portion (439 amino acids) of the mature G, the ectodomain (ED), extends outward from the membrane surface of the infected cell and released virion. The CD of G interacts with M of the skeleton particle to complete virion assembly and enables efficient release of virions. During posttranslational modification (glycosylation) of G in the endoplasmic reticulum (ER) and Golgi apparatus, three molecules of G form trimers (aggregates of nascent G molecules presumably held together at the TM domain), which later become the trimeric spikes on the surface of the virus. The ED of each molecule in the trimeric spike is responsible for virus binding to cell surface receptors and the entry of virus into cells in the early phase of the RABV life cycle and for induction of anti-rabies VNA, when engaging the host's defense against the virus.

Glycosylation of RABV G is important for its expression on the membrane surface and proper function. Complex carbohydrates (oligosaccharides) are linked to asparagine residues in the tripeptide sequence (sequon) asparagine-X-serine or asparagine-X-threonine, where X is any amino acid except proline. While RABV G can have one, two, or three (or sometimes four) sequons (N-glycan sites) per molecule, not all sites are occupied on all molecules, which depends on various factors that influence efficiency of glycosylation. At least one site (N319 in RABV G) is required, however, and it is a sequon that is highly conserved in all the lyssavirus genotypes and other rhabdoviruses such as vesicular stomatitis virus (VSV). The sugar residues of N-glycan are presynthesized in the cell and transferred from a lipid precursor by the enzyme oligosaccharyltransferase as a precursor core oligosaccharide unit (Glc3Man9GlcNAc2) to the specific sequons of the nascent G molecule, as the protein is processed in the lumen of the ER and Golgi apparatus. There the high-mannose core oligosaccharide unit is trimmed with the help of the chaperone calnexin, which assists in the correct folding of G to achieve its biological activity, stability, and antigenicity.

A key biological role of G is its ability to facilitate virus entry into host cells after targeting the binding of the virus to its receptor(s) on host cells. Acting as a fusion protein, the lyssavirus G enables the virus to become internalized as it triggers a low pH-dependent fusion with the plasma membrane and endosomal membranes to enter cellular endosomes. During this process, G assumes at least three structurally distinct 'conformational' states. The G on the virion surface assumes the 'native' state prior to the virus binding to the cellular receptor. After the virus attaches to the receptor and is internalized, the G on the virion surface is 'activated' to a hydrophobic state, enabling it to interact with the hydrophobic endosomal membrane. After entering the endosomal compartment and its low-pH environment, the fusion capacity of G is further activated via a major conformational change in the G that exposes the fusion domain, which interacts with and destabilizes one or both of the participating membranes. Once activated to fuse with endosomal vesicles, the viral uncoating process begins; at pH 6.2–6.3, G assumes a reversible 'fusion-inactive' conformation, which makes the G monomer appear longer than the 'native' form and assume selective antigenic distinctions and be highly sensitive to cellular proteases. RABV spreads from cell to cell in two ways. It either buds from one infected cell into the culture medium (*in vitro*) or extracellular space (*in vivo*) to infect another cell by attaching to the next cell's surface receptor or spreads directly from cell to cell without budding into the culture medium or extracellular space. The precise method of direct cell-to-cell spread of virus *in vitro* and *in vivo* has not been determined. But the fact that the virus can enter a cell without first attaching to the cell surface receptor in cell culture (direct cell-to-cell spread) points to the importance of the pH-dependent and pH-independent fusion functions of G in virus spread.

Another important biological role of G is its ability to define the pathogenicity of the virus and determine the neuroinvasive pathway the virus takes to reach the central nervous system (CNS) and the brain, and cause a lethal infection. The RABV and non-RABV lyssavirus strains are defined by their 'neurotropism' as having a pathogenic (virulent) or nonpathogenic (avirulent) phenotype. The virulent virus causes a lethal infection, while the avirulent virus does not cause a lethal disease. The virulent and avirulent phenotypes are often defined by a single amino acid substitution in G. The position most often associated with a single amino acid substitution causing the change from virulent to avirulent phenotype is the position 333 of the 505-amino acid sequence. Normally, arginine occupies position 333 in the G of the virulent phenotype, while glutamine or lysine occupies the same position in the G of the avirulent phenotype. Although substitution of other amino acids at position 333 can make this transition in phenotype happen, there are still other identifiable factors that can play a role in the pathogenicity of RABV and non-RABV lyssaviruses. These may include the transcription activity of L, the length of the long intergenic nucleotide sequence between the protein-coding or open reading frame (ORF) regions of the G and L genes, and optimal protein–protein (e.g., G–M) interactions. Nevertheless, at least with 'fixed' strains of pathogenic and avirulent RABVs that distinguish themselves by having an arginine and glutamine at position 333 of their G, respectively, all else being the same, it has been shown that the two phenotypes invade the CNS from a peripheral site at different rates. It is clear that not only does the G allow entry into the nervous system by selecting the appropriate receptor it facilitates and enhances retrograde axonal transport of the virus to the CNS. By selecting different neuronal pathways to the brain from a peripheral site of inoculation, it is also clear that the pathogenic virus reaches the brain faster than the avirulent phenotype. Still, other studies have found that the two virus phenotypes showed no difference in the rate of speed and pathway of spread to the CNS. So, it remains controversial and requires further investigation into whether the viruses, depending on their amino acid constituents in the G and phenotype, use different receptor sites on the cell surface or use the same receptor sites with different efficiencies in determining the neuronal pathways for the viruses.

Finally, RABV G is highly immunogenic and of major importance for the induction of VNA and T-cell-mediated immunity, two of the host's adaptive immune responses against virus infection. G induces VNAs that recognize both conformational and linear antigenic epitopes and stimulates helper as well as cytotoxic T-cell activity. The various epitopes that bind VNAs delineate a total of eight antigenic sites that have been physically mapped (located) on the ED of G. Epitopes that bind T-cells have also been mapped on G. To visualize and understand the precise topography of the antibody and T-cell binding sites on the G, it will be necessary to study the three-dimensional structure of the antigen (derived from G crystals). So far, this has denied investigators, who have made numerous attempts to crystallize the RABV G for X-ray diffraction analysis, any success.

Viral Genome Structure

The linear single-strand negative-sense RNA (vRNA) of lyssavrus genomes of about 12 kb in length includes the 5 genes that appear in a strictly conserved order 3′-N-P-M-G-L-5′ flanked by the short 3′- and 5′- terminal regulatory extragenic regions known as leader (Le) and trailer (Tr) regions. These two extragenic regions are terminated by unmodified 3′-hydroxyl (3′-OH) and 5′-triphosphate (5′-PPP) ends, respectively (Figure 2). The Le region, acting as a genetic promoter (GP), initiates transcription of five monocistronic mRNAs from the genes of the vRNA genome as well as the synthesis of full-length antigenome RNA (cRNA). The 3′-end of the cRNA functions as the antigenome promoter (AGP) that directs synthesis of full-length N-encapsidated vRNAs representing synthesis of progeny vRNAs in viral genome replication. Much as recently been learned from X-ray diffraction studies of crystal ring structures of helical N-viral RNA (N-vRNA) or RNP complexes containing as few as 10 N molecules how the genome vRNA is encapsidated by N. One should not lose site of the role that P plays in directing N encapsidation of vRNA (see description of P above) and that an N-P complex is involved in vRNA encapsidation process. The vRNA in these structures is sequestered in a cavity at the interface between the N- and C-terminal lobes of N that seem to 'clamp down' onto the bound RNA. The complete occlusion of the vRNA by N (N-vRNA) explains the excellent protection of the vRNA against high salt, attack by RNases, and silencing by siRNAs. During transcription and replication of the viral genome RNA, the tight clamping of the N-vRNA interaction opens exclusively and transiently to allow the polymerase complex access to the vRNA. Easiest access to the N-vRNA genome would be at the 3′-end of the genome as well as the 3′-end of the antigenome (N-cRNA) to allow transcription and replication of the downstream copying of successive genes to occur.

Gene Transcription and Genome Replication

The Le RNA and mRNAs of the five genes are sequentially transcribed off the N-vRNA genome according to the widely accepted stop/start model of transcription for all single-strand (non-segmented) negative-sense RNA viruses. The first transcript initiated by the 3′-terminal GP is the Le RNA, however recent studies suggest that transcription of the downstream protein-encoding genes starting with the N gene can proceed independent of Le RNA synthesis. Directing the activity of the viral RNA polymerase, acting as a transcriptase, are conserved stop/start signals separated by non-transcribed intergenic sequences at the gene borders that control the observed transcription. A short run of mostly 7 U residues signal the polymerase to stop transcription while the polymerase continues to move back and forth on the N-vRNA template to synthesize a 50–150 nt-long poly(A) tail that is added onto the mRNA. Without fully separating but purposely dissociating from the N-vRNA template at the gene borders, it is thought that the polymerase releases the newly synthesized polyadenylated mRNA and re-associates with the template to initiate transcription of the next downstream gene at the restart signal without transcribing the border signals between sequential genes. Since the polymerase enters exclusively at the 3′-end of the genome and dissociates from the genome at every gene junction, upstream mRNAs are produced more abundantly than

downstream mRNAs. This stop/restart process of the polymerase as it travels along the genome represents an efficient method of attenuation of transcription creating a differential accumulation of transcription products for translation. The mRNAs typically have a 5′-terminal 7-methylguanylate cap structure (m^7G) for translation initiation and a short 5′-noncoding region of 20–30 nts, as well as a longer 3′-noncoding region of 100–500 nts prior to the poly(A) tail. A particularly long 3′-noncoding region exists in the RABV G mRNA corresponding to the long intergenic region in the RABV genome between the G and L genes (initially described as a pseudogene). Differences in the 3′-noncoding region of genes mostly account for the differences in the overall length of lyssavirus genomes. The mRNAs produced are efficiently translated by host cell protein synthesis machinery into the viral proteins. Replication of the negative-sense (-strand) genome requires synthesis of the full-length positive-sense (-strand) antigenome (cRNA) or replicative intermediate. The cRNA is produced by the viral polymerase, acting as a replicase ignoring the stop/start signals that delineate the mRNAs. Synthesis of the cRNA starts at the 3′-end of the N-vRNA by primer-independent synthesis to produce the 5′-end of the cRNA antigenome (complementary to the vRNA) including a sequence corresponding to that of the short leader RNA. Like the N-vRNA, the newly formed positive-strand cRNA is encapsidated by N (N-cRNA) as the cRNA sequence is elongated in the 5–3′ direction. The resulting antigenome cRNA by definition has a 3′-end that is identical in the first 11 nts to that of the genome vRNA and can efficiently direct the synthesis of abundant amounts of progeny genome N-vRNAs from the N-cRNA template. Since progeny genome vRNAs makes up 98% of full-length RNAs in virus-infected cells, it is obvious that the AGP is much more active in directing genome replication than GP is in directing full-length genome transcription.

Virus Life Cycle

Following the bite of a rabid animal, infectious virus in the saliva is introduced into the bite wound and enters susceptible cells in the muscle or skin at the wound site and the virus's life cycle begins. In the virus-infected cell the virus begins to multiply (replicate) to produce more (progeny) virus. RABV and non-RABV lyssaviruses maintain their life cycles in selected (targeted) cells of the infected host. After the virus enters the first cell and replicates, the progeny virions bud from the cell and spread to other susceptible cells. As more progeny virions are produced, more and more (the growth can be exponential) infectious particles populate the extracellular space (*in vivo*) or enter the medium of cell culture (*in vitro*). Typically, RABV does not enter or circulate in blood. The sequence of events in the RABV life cycle can be divided into three phases. In the first, or early phase, the virus particle either attaches to the surface of a targeted or susceptible host cell via a cell surface receptor or enters the cell via direct virus fusion (externally) with the plasma membrane through coated pits (viropexis) and subsequently (internally) with endosomal membranes of the cell. During the endosomal fusion process (called endocytosis), virus particles are stripped (uncoated) of their viral envelope components and the helical RNP is released into the cytoplasm. When the virus attaches to the surface of a target cell via a 'specific receptor,' it most likely binds to a receptor molecule or cellular receptor unit (CRU) that leads to or permits direct virus entry into the cell. Various 'receptor' molecules have been implicated in binding RABV to cells in culture including lipid, gangliosides, carbohydrates, and proteins of the plasma membrane, but none have been proven to be 'specific' receptors. However, three specific *in vivo* CRUs appear to correlate with the defined neurotropism of the virus. The nicotinic acetylcholine receptor (nAChR), the first identified receptor for RABV found at neuromuscular junctions, where RABV can also be found colocalized *in situ*, is regarded as a likely candidate for a receptor for the virus. It was thought that RABV binding to AChRs would localize and concentrate the virus on postsynaptic cells at neuromuscular junctions facilitating uptake and transfer of virus to peripheral neurons and ultimately the CNS. Another possibility is the neural cell adhesion molecule (NCAM) CD56, a cell adhesion glycoprotein, which is found on the cell surface of RABV-susceptible cell lines (*in vitro*) as well as *in vivo*. Blocking the NCAM receptor with the physiological inhibitor heparin sulfate or specific anti-NCAM receptor polyclonal or monoclonal antibodies, which inhibited RABV infection of susceptible cells expressing NCAM receptor helped identify this receptor molecule as a cell surface receptor for RABV. A third possibility is the low-affinity p75 neurotrophin receptor (p75NTR), a nerve growth factor receptor expressed on the surface of cultured BSR cells. Whether p75NTR is an obligatory receptor for RABV has been questioned since the challenge virus standard (CVS), a fixed laboratory strain of RABV infects mice that lack p75NTR. Whatever receptor is present on the cell surface *in vivo*, it seems the virus is not limited to choosing only one type of receptor in order to initiate the virus life cycle in the infected animal or human.

In the second or middle phase of the RABV life cycle, after the virus is internalized by endocytosis in the cell, the tightly coiled transcriptionally 'frozen' viral RNP core, which is released into the cytoplasm, relaxes to form a loosely coiled RNP helix to facilitate N-vRNA genome transcription and replication in the cytoplasm. The viral proteins are synthesized from the viral mRNA transcripts. Four of the mRNAs, N-, P-, M-, and L-mRNA, are translated on free ribosomes in the cytoplasm and the G-mRNA is translated on ribosomes bound to the ER (referred to as the rough ER). Before the nascent G is completely synthesized, it enters cotranslationally into the lumen of the ER where the protein is folded with the help of molecular chaperones and undergoes modification at the asparagine-X-serine or -threonine sequons by core glycosylation and N-glycan processing, that is, trimming of high-mannose triglucosylated oligosaccharides in the rough ER and Golgi stacks to form the 'complex type' N-linked monoglucosylated oligosaccharide of the mature G molecule. After final processing of the N-linked carbohydrate side chains in the Golgi apparatus, viral G trimers are formed and appear on the plasma membrane surface of the infected cell. While protein synthesis is happening, the incoming genome vRNA is transcribed into full-length positive-strand cRNA (antigenome RNA or replicative intermediate), which in turn is copied into full-length negative-strand progeny genome RNA or vRNA (Figure 3).

In the late phase of the RABV life cycle, once sufficient pools of vRNA and viral N, P, and L proteins have accumulated, vRNA is encapsidated by N (directed by the N–P complex) and L is added and nascent rabies viral RNP complexes are formed. 'Skeletons' of

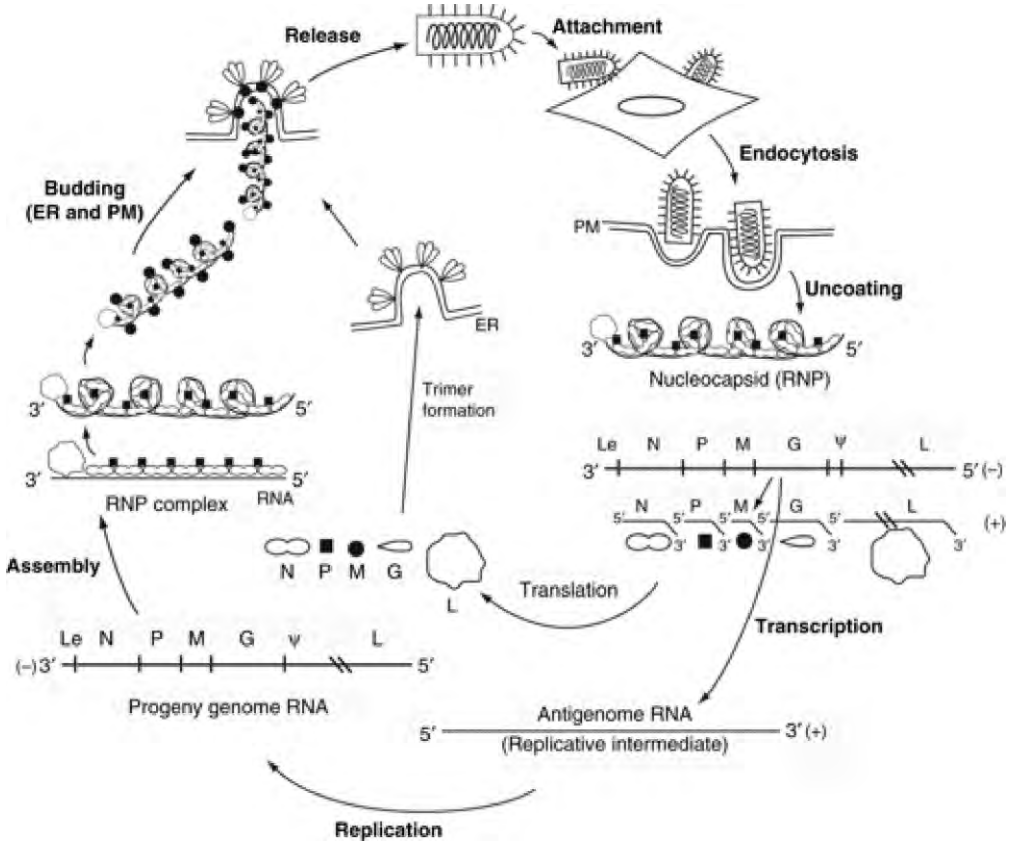

Figure 3 Rabies virus (RABV) life cycle in the cell. Virus enters the cell following attachment through coated pits (viropexis) or via cell surface receptors, mediated by the viral glycoprotein (G) fusing with the cellular membrane (endocytosis). After internalization, the viral G mediates low pH-dependent fusion with the endosomal membrane and the virus is uncoated, releasing the helical nucleocapsid (NC or N-vRNA) of the ribonucleoprotein (RNP) core. The five structural genes (N, P, M, G, and L) of the genome RNA (vRNA) in the NC are transcribed into five positive (+) strand monocistronic messenger RNAs and a full-length (+) strand (antigenome) cRNA (replicative intermediate). The antigenome cRNA serves as the template for replication of progeny genome (−) strand vRNA. The proteins N, P, M, and L are synthesized from their respective mRNAs on the free ribosomes in the cytoplasm and G is synthesized from the G-mRNA on ribosomes bound to endoplasmic reticulum (rough ER). Some of the N–P molecular complexes produce cytoplasmic inclusion bodies (Negri bodies) *in vivo* and some N–P complexes encapsidate the (+) strand and (−) strand viral RNAs. After progeny genome RNA is encapsidated by the N–P protein complex, and L protein is incorporated to form progeny RNP (both full-length standard and shorter defective) structures, the M protein binds to the RNP and condenses the RNP into the 'skeleton' structures. The skeleton structures interact with the trimeric G protein structures anchored in the plasma membrane and assemble into virus particles that bud from the plasma membrane of the infected cell into adjacent extracellular or interstitial space. Reproduced from Wunner, W. H. (2007) Rabies virus. In: Jackson, A. C., Wunner, W. H. (eds.) *Rabies*, 2nd edn., pp. 23–68. San Diego, CA: Elsevier, Academic Press, with permission.

virus particles and finally mature virus particles are assembled with the addition of M and G, respectively, to the RNP complexes. During RABV morphogenesis, an amorphous, intracytoplasmic ground substance or 'matrix', known as inclusion bodies (IBs), long known as Negri bodies commonly found in brain tissue as well as tissue culture, is formed. The formation of IBs comprising filamentous matrix substance (generally N–P complexes), which precedes virus particle formation in neurons of the infected brain, is often a hallmark for the diagnosis of rabies. The association of M with the newly formed transcriptionally active RNP selectively inhibits further transcription and replication of the viral genome and localizes the RNP at the cellular membrane where the glycosylated trimeric G is concentrated and M interacts with G. Finally, M begins to alter the structure of the RNP by condensing it into a tightly coiled 'skeleton-like' (RNP + M) form in which the polymerase activity is 'frozen' in place. Thereby, M plays an important role by covering the RNP and giving the virus particle its 'bullet' shape. In the final stages of virus assembly the mature virion acquires its lipid envelope derived from the host cell's plasma membrane, with the trimeric G spikes embedded in it as the virion buds through the membrane.

Epidemiology of Animal and Human Rabies

The natural course of RABV transmission is from animal to animal and hence it is principally a zoonotic disease. On the occasions that RABV spreads to humans it is primarily the consequence of an attack by an aggressive infected animal biting the human and transmitting the virus present in saliva, which comes from the animal's salivary glands. The reservoir for all RABV and non-RABV

lyssaviruses is the animal species pool that circulates the various lyssavirus species. But not all populations of terrestrial carnivores serving as reservoir hosts for rabies are affected proportionately. Transmission of RABV and the non-RABV lyssaviruses is usually among animals of the same species with cross-species transmission to other mammals usually depending upon direct interactions with infected individuals of the reservoir host species. Such virus spillover from one host species to another can and has led to viral evolution and adaptation in a new host species over broad timescales. Bat lyssavirus variants, for example, sporadically spill over to infect wild and domestic animals. New host species, such as red foxes on Prince Edward Island, Canada, and Nova Scotia, Canada, and striped skunks in Arizona, have sustained transmission of bat variants of RABV within terrestrial carnivore populations until possible natural extinction. Extinction can occur because of disappearance of the virus or the virus reaching undetectable levels within the local species population, or by vaccination control programs as has occurred with skunk rabies in Arizona. Initial epidemics of rabies in animals are typically the largest in a series of epidemics that emerge over time. As 'wildlife' rabies enters into a new region to infect previously naive populations, a series of successively smaller epidemics may occur at increasing frequency. This has occurred with red fox rabies in regions of Europe and with a raccoon-adapted variant of RABV in regions of the USA. In the mid-1970s, the raccoon-adapted variant was introduced into the mid-Atlantic region of the United States as a consequence of transporting raccoons unsuspected of incubating rabies from a couple of southeastern states for the purpose of restocking dwindling local populations. These animals were moved, apparently while incubating the raccoon-adapted RABV variant, and when placed among naïve animals in state parks and game reserves soon spread the virus to other raccoons that over time and distances transported the virus northward to Maine and Ontario, Canada and westward to Ohio, creating the most expansive outbreak of animal rabies so far recorded. Typically, regions of higher population densities of wildlife species suffer greatest population depression due to rabies transmission and the highest incidence of disease during the initial epidemic. In the United States, raccoons have been the wild animal species most frequently diagnosed as rabid since the early 1990s, whereas skunks and foxes were the most frequently reported rabid cases during previous decades, 1960–90s and 1950–60s, respectively (Figure 4). Rabies among skunks in North America has a long history, since the 1800s, as a source of human rabies. Currently, two RABV variants that circulate within specific wildlife species circulate among skunks in the central United States; one of these, the North Central Skunk variant, also occurs in central and western Canada, and the other RABV variant circulates among skunks in California. Periodic epizootics (periods of increased disease activity) or epidemics of rabies in skunk populations that occur in other states tend to drive the cyclic variations in skunk population numbers (Figure 5).

Bats are perhaps the most unique group of wildlife mammals to transmit RABV and non-RABV lyssaviruses to carnivores and humans. Currently, there are 12 uniquely identifiable lyssavurus species and a further two recently described viruses that appear to cause a rabies-like pathogenesis in humans and terrestrial carnivores. Only two of these lyssaviruses, MOKV and IKOV, have not yet been detected in bat species. Among the many families, subfamilies and genera of bats that comprise the order Chiroptera, it is the vampire bat that preferentially attacks livestock and transmits rabies sporadically to cattle. Observations linking vampire bats to rabies epidemics among terrestrial animals, particularly in South and Central America, causing paralytic rabies in cattle, the preferential target species of vampire bats, go back more than 90 years. Occasionally, vampire bats bite and infect humans, particularly in many South and Central American countries, but other chiropteran hosts historically associate more with

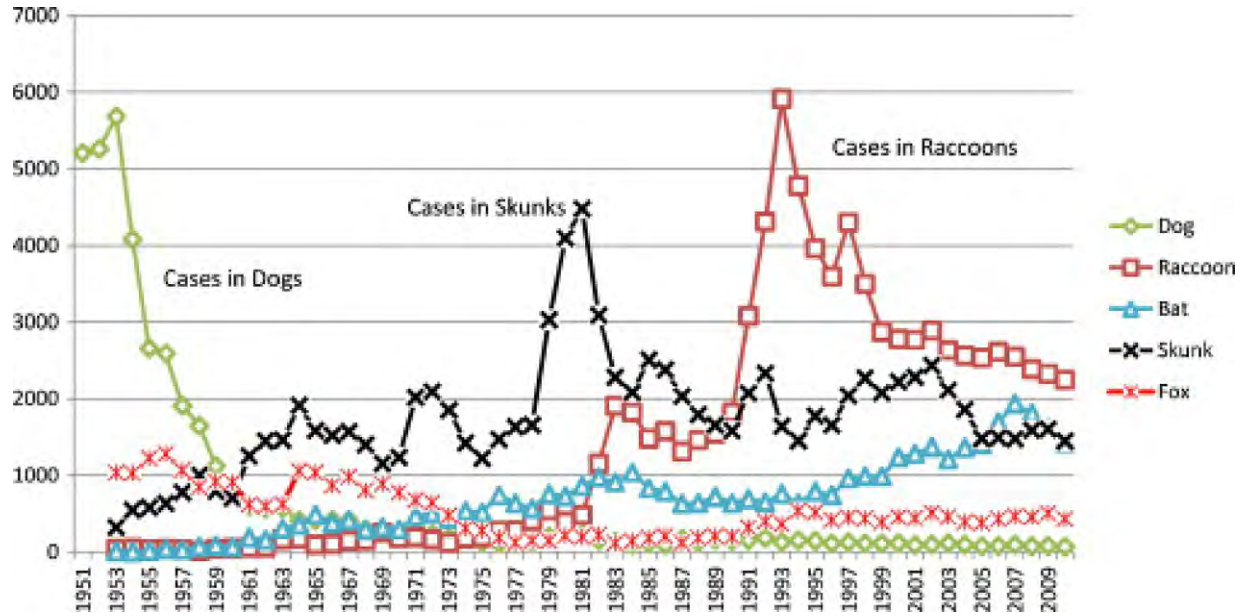

Figure 4 Rabies among wildlife animal species in the United States, 1951–2010. The number of reported cases of rabies among selected wildlife animals in the United States, 1951–2010, as reported through national surveillance (data obtained from annual CDC surveillance reports). Reproduced from Hanlon, C. A., Childs, J. E. (2013). Epidemiology. In: Jackson, A. C. (ed.) *Rabies*, 3rd edn., pp. 61–121. San Diego, CA: Elsevier, Academic Press, with permission.

Figure 5 Distribution of rabies virus (RABV) variants in the United States. Common species of terrestrial carnivores among wildlife serving as reservoir hosts. The geographic occurrence of two skunk rabies variants is represented as Northcentral Skunk and Southcentral Skunk. In addition to this distribution of RABV variants is the reservoir present in species of bats, which covers the entire continental United States. Reproduced from Childs, J. E., Real, L. A. (2007). Epidemiology. In: Jackson, A. C., Wunner, W. H. (eds.) *Rabies*, 2nd edn., pp. 123–199. San Diego, CA: Elsevier, Academic Press, with permission.

transmission of rabies to animal and human populations. Clinically, RABV transmitted by vampire bats to cattle, humans, and other bats cause 'paralytic' disease and virtually never 'furious' rabies ('Human rabies'). Paralytic rabies in cattle starts with signs of posterior unequal coordination followed in later stages with anorexia, bellowing, weakness in the hind legs, and other signs of brain damage. Finally, ascending paralysis, temporary cessation of breathing, and death occur. Humans can experience similar clinical signs and fatal outcome because of contact with infectious saliva from the bite or by inhalation of aerosolized saliva of a rabid vampire bat. It is not known whether some specific property or properties of the RABV variant circulating among vampire bats might be responsible for this rather oblique presentation of the disease. Bats are the second most common transmitters of rabies to humans. Human rabies resulting from insectivorous bat species, particularly in the New World, did not appear until the 1950s. The first human case to be particularly identified with an insectivorous bat occurred in 1953, in Florida, and since then the number of rabid bats reported in the United States has increased dramatically. The association of classical RABV with insectivorous bat species across the New World over the past 60 years as been as diverse as there are different insectivorous bat species that appear to serve as virus reservoirs for lyssaviruses in the New World compared to that of the Old World. This diversity of RABV variants that has emerged through natural infections of insectivorous bats has manifested itself in different clinical signs of the disease, i.e., aggression, weakness, and anorexia with relatively short morbidity periods (4–20 days). With highly sophisticated genetic analyses of the different lyssaviruses isolated from insectivorous bats, it has been possible to differentiate the virus variants that associate with bats, as well as terrestrial hosts such as red fox, skunks and raccoons, so that the virus variant can now be correlated with a particular bat species and probably the country where the transmission occurred. Human infection caused by RABV variants from insectivorous or suspected insectivorous bats has been increasing in North America (at least 69 cases identified between 1950 and 2010) now that rabies in the domestic dog population has been eliminated.

The dog is the most common animal to transmit rabies to humans as well as to other carnivores in all regions of the world in which public health concern is highest. When dog rabies is reduced by effective disease control measures, such as mass vaccination of both domestic and stray dogs, human rabies cases also decline. Implementation of control programs and policies for bat rabies, on the contrary, has not been possible, largely because regimens for administration of vaccines in bats have not been adequately developed, or have been much less widely implemented. In contrast to RABV variants found predominantly in wildlife in North America (the United States and Canada) and Western Europe, the greatest dissemination of RABV in many locations in the world has been by dogs. The domestic dog continues to be the major reservoir species for RABV throughout Africa and Asia, and to a lesser extent in South America and Latin America. The similarity of canine RABV isolates from South America, Africa, Eastern Europe, and parts of Asia suggests that rabies in dogs arose from a common source, perhaps from a single origin in the Palearctic region that includes Europe, the Middle East, and northern Africa. Canine rabies was imported from Europe to the New World in the eighteenth century during the time of the Spanish dominion in southern parts of North America and in Mexico as early as 1709. Outbreaks of dog rabies in South America followed early in the nineteenth century in Peru (1803) and Argentina (1806). Later, epidemics of canine rabies came in 5- to 6-year cycles in South America. Feral, stray, and neighborhood dogs contribute to free-ranging populations in both developed and developing countries. Interestingly, young dogs, frequently less than 1 year of age, which have not received a complete course of rabies vaccination, contribute in a major way to rabies outbreaks, particularly during major mass vaccination campaigns in canine rabies endemic regions.

The greatest number (>99%) of human rabies deaths result from infection by RABV maintained by domestic dogs and transmitted by dogs that bite humans and these occur mostly in tropical areas in developing countries of the world, where accurate estimates of human rabies deaths are impossible to obtain by surveillance methods. Regional laboratories are also inadequate or nonexistent for systematic detection and laboratory confirmation of human or animal rabies cases. Recent estimates by the World

Health Organization (WHO), nevertheless, suggest that the highest incidence of human rabies deaths is 55 000 annually in Africa and Asia from being bitten by RABV-infected dogs. The global public health burden and cost of canine rabies in Africa and Asia alone, based on direct medical expenses and indirect costs borne by patients seeking postexposure vaccine treatment (PET), amounts to roughly US$196 million/year and US$289 million/year, respectively. Worldwide each year an estimated 7 million people bitten by suspect rabid dogs increases the high demand for expensive postexposure prophylaxis (PEP) and PET.

Control and Prevention of Rabies in Domestic and Wildlife Animals

Control and prevention of rabies in domestic animals has been most successful in countries that advocate strong veterinary and public health programs and have the cooperation of people who take their responsibility for animal pets seriously. Nationwide vaccination programs together with rules or laws to enforce the elimination of stray dogs and cats and use of quarantines in rabies-free countries have done the most to keep rabies from spreading. To be effective, these measures as well as the oral immunization approach for wild animals must reduce the number of unvaccinated animals to a level at which rabies transmission is interrupted and this level must then be maintained.

Preventing the spread of rabies to susceptible animals and humans continues to be a major battle for biologists and public health officials throughout the world. Persistence of multiple variants of RABV and non-RABV lyssaviruses in wildlife animal reservoirs presents a continuing challenge to those dealing with medical, veterinary, and wildlife management. To control rabies at its source is to control the disease in wildlife vector species, and this may be the most challenging and difficult task of all. Many techniques have been tried over the past three decades, beginning with the control of rabies in foxes in Western Europe using the concept of oral vaccination and baits carrying vaccine to immunize free-ranging animals. Almost every species of mammal that acts as a vector for the disease requires a unique approach depending on many factors, including density of wildlife populations, restriction of movement, food sources, and functions of the species to prevent a particular species from becoming infected. On the contrary, the impact that vaccines have had on protecting domestic animals, causing a dramatic reduction in the incidence of canine and human rabies, clearly holds great promise for the prevention and ultimate control of rabies in wild animals.

The main transmitters of wildlife rabies throughout the world are the wild carnivores. These animals are highly susceptible to rabies: they excrete virus in the saliva and live in high-density populations, and the long incubation period of the virus ensures maintenance of the infection. The most significant terrestrial wild carnivores transmitting rabies in the world are the red fox (*Vulpes vulpes*) in Central and Western Europe and the red fox and raccoon dog (*Nyctereutes procyonoides*) in Eastern Europe. Foxes (*V. vulpes* and *Urocyon cinereoargenteus*), skunks (predominantly *Mephitis mephitis*), and raccoons (*Procyon lotor*) are the major animal vectors in various parts of North America, and wolves, stray dogs, mongooses (*Herpestes auropunctatus*), meerkats, and polar foxes in other areas and countries of Africa, Asia, and Central and South America. Establishing well-organized and well-managed programs with extensive national and international cooperation among veterinary and public health personnel who share as a common goal the control of rabies in wild animals, from local eradication to inhibition of spread into uninfected areas, will undoubtedly be more difficult than controlling rabies in domestic animals. Several European and North American team efforts, nevertheless, have made significant progress in evaluating the efficacy and safety of vaccines and vaccine delivery systems for oral immunization of wild carnivores. The progress is such that it can be said that the oral immunization method can be an effective and practical approach for the elimination of enzootic rabies in terrestrial animals, one species at a time.

The Blueprint for Rabies Prevention and Control

Experts in rabies management worldwide formed the 'Partners for Rabies Prevention' (PRP) initiative and developed a 'blueprint' to provide government officials and their public health professionals, veterinarians, scientists, medical doctors, and other experts in diagnostics, virology, epidemiology, ecology, immunology, animal welfare, outreach health educators and communicators with advice in the form of a comprehensive set of guidelines and the necessary encouragement and support to develop their own national canine rabies elimination programs. This online document of operating procedures is designed to help governments plan, implement, evaluate, and sustain canine rabies elimination programs as described in 'The Blueprint for Rabies Prevention and Control' (available at http://www.rabiesblueprint.com). Information to help governments focus their planning, implementation, evaluation, and ability to sustain canine rabies elimination programs, in particular, is contained in one section of the blueprint (www.canine rabiesblueprint.org). The Canine Rabies Blueprint, which was established in 2010, is a powerful toolkit in simple language that is freely accessible to a wide range of users. This type of 'one health' approach to canine rabies control and elimination is expected to grow in interest and have considerable impact on local endemic canine rabies in high-risk countries.

Human Rabies

Clinical Signs and Symptoms

Humans infected with RABV develop classical encephalomyelitis, often referred to as 'furious' or 'encephalitic' rabies, in approximately 80% of the cases and the rest (20%) exhibit a paralytic form of the disease. Rabies in humans usually develops 20–90 days after

exposure, although occasionally the disease develops after only a few days and rare cases have occurred after a year or more following exposure. The clinical illness in humans, which follows such a variable and sometimes prolonged nonsymptomatic incubation period, usually begins with a prodromal phase, which may last 2–10 days. At this point the symptoms are almost entirely nonspecific, comprising fever, malaise, anorexia, nausea, vomiting, diarrhea, sore throat, cough, myalgia, and headache. After the prodromal period, signs of nervous system involvement develop, leading to an acute neurological phase, including intermittent hyperactivity marked by agitation lasting 1–5 min, occurring spontaneously or triggered by a variety of tactile, auditory, and visual stimuli, disorientation, hallucinations, seizures, bizarre behavior, nuchal stiffness, and paralysis. Other abnormalities include muscle fasciculation (particularly near the site of the exposure), hyperventilation, and hypersalivation. Hydrophobia, a term often used to describe rabies involving both severe thirst and fear of water, is regarded as a unique characteristic and most specific manifestation of rabies in humans, and appears to develop in 50–80% of patients with encephalitic or furious human rabies. The mental status of the patient gradually deteriorates during a period of 2–12 days until the onset of coma, which may last for hours or months, or an abrupt death due to cardiac or respiratory arrest. With intensive medical support, cardiac and respiratory arrest may not occur, and the patient may experience rare recovery. The incubation period (from exposure to onset of clinical signs of disease) in rabies has a greater length and variability than in most other infectious diseases, which can cause considerable stress to the patient. Severe facial bites and multiple bites to other areas of the body tend to have shorter incubation periods. In cases of long incubation periods, it is often difficult for the patient to recall a bite exposure, particularly if the bite was from an insectivorous bat, or the patient becomes unintelligible from the disease. From cases of untreated humans who developed rabies following known animal bites, the highest number (50–80%) occurred from bites to the head, the next highest number (15–40%) from bites to the hand or arm, and the fewest (3–10%) from bites to the leg. The risk from scratches or contamination of minor wounds with saliva is about 0.1%.

In paralytic (or 'dumb') rabies, muscle weakness develops early in the course of the disease. The paralysis may start in the bitten extremity and spread to other extremities, but is not necessarily related to the anatomical site of the animal bite (exposure). Consequently, rabies is often misdiagnosed in this clinical form, especially if there is no history of animal exposure. At the onset of this clinical form of rabies, patients are alert with a normal mental status. They may have local pain, paresthesias, or pruritus at the bite site even though sensory perception upon examination may be normal. Muscle fasciculations may be present and facial muscles become weak bilaterally. The clinical picture may be confused with the Guillain–Barré syndrome. Unlike Guillain–Barré syndrome, urinary incontinence is common and pain and sensory disturbances may occur in paralytic rabies.

Other lyssaviruses, in addition to RABV, have also caused rabies. Only LBV, which was first isolated from fructivorous bats in Nigeria, has not been associated with human rabies. Four other lyssaviruses recently isolated from bats, ARAV and KHUV, which were isolated from bats in Central Asia, and IRKV and WCBV, which were isolated from bats in Russia, have also not been associated with human disease. MOKV, which was isolated from shrews in Nigeria, was associated with meningoencephalitis, without the typical features of brainstem involvement seen in classical rabies, in a 6-year-old girl who died in Nigeria in 1971 after a 6-day illness. Another patient, a 3 1/2-year-old girl from Nigeria, who presented with a sudden onset of fever and convulsions, made a complete recovery. In this case, MOKV was isolated from her cerebrospinal fluid (CSF), although it is questionable whether cross-contamination of her CSF might have occurred since the shrew isolate of MOKV was handled in the same laboratory. In 1970, a 31-year-old man from rural South Africa developed an illness that was indistinguishable from rabies. The patient's illness was characterized with fever, excessive sweating, hydrophobia, and spasms of his face, arms, and torso. He was confused, irritable, and periodically aggressive. He died 5 days after the onset of illness. The man had been bitten on the lip by a bat 4 weeks earlier. The virus that was isolated from the patient's brain after death was DUVV. In 1985, two cases of rabies-like illness occurred in Europe; one was an 11-year-old girl from Belgorod, Russia, who was bitten on the lip by an unidentified bat, and the other was a 30-year-old man, who was a zoologist from Finland. Both died with signs indistinguishable from that of rabies. The virus isolated from the girl was called *Yuli virus* and classified as EBLV-1, and the virus isolated from the man who died 23 days after the onset of illness was like other enzootic European bat RABV isolates and was classified as EBLV-2. In 1996, a 39-year-old woman from Australia died after a 20-day illness. She sustained numerous scratches to her left arm over 4 weeks while she cared for fruit bats and apparently had been bitten by an insectivorous bat prior to the onset of her illness. Her illness was marked by progressive left arm weakness that later developed into progressive limb and facial weakness with reduced tendon reflexes and fluctuations in her level of consciousness prior to her death. The virus she was infected with was identified by the reverse-transcription polymerase chain reaction (RT-PCR) assay, perhaps the most frequently applied test presently available, and found to be identical to ABLV, which had been identified in flying foxes (fruit-eating bats). This patient had rabies that typically involved the brainstem and quickly progressed to diffuse brain involvement. Two years later, in 1998, a 37-year-old woman from Queensland, Australia, experienced fever and paresthesia in her left hand and pain in her left shoulder, along with a sore throat and difficulty in swallowing for 5 days before she was admitted to hospital. She had pharyngeal spasms, evidence of autonomic instability, and progressive neurologic deterioration before she died 19 days after onset of her illness.

Rabies Diagnosis

An estimated 7–10 billion diagnostic tests of all types for rabies are performed in the laboratory each year in the United States alone, most frequently for the postmortem examination of animals that have either bitten a person or in some other way potentially caused human exposure to the disease. Evidence of RABV infection in a contact animal (and sometimes in an exposed person subjected to an antemortem diagnosis of rabies), based on a positive diagnostic test, prompts administration of rabies PET and PEP to the exposed person and any other persons who had direct contact with the exposed person, respectively. A skin biopsy from the

nape of the neck that includes several nerve-innervated hair follicles is often the best sample for a human antemortem diagnosis of rabies using a direct fluorescent antibody (DFA) test or RT-PCR assay. Fresh saliva is another sample source for RT-PCR assay, while serum and CSF are suitable for the detection of anti-rabies antibodies that can be assayed in a rapid fluorescent focus inhibition test (RFFIT). Often, a positive diagnosis of RABV in animals, as in the observation of rabies viral antigen in the brainstem at the level of the medulla, pons, or midbrain, also initiates the appropriate management of exposed domestic animals, including booster immunization of previously immunized animals and euthanasia or quarantine of unvaccinated animals.

While various types of diagnostic tests may be performed, diagnosis of rabies based upon clinical presentation alone may be difficult to discern. Clinical symptoms or signs of human rabies often occur long after exposure to the virus and can be difficult to distinguish from encephalitis caused by other viral infections, such as poliovirus or canine distemper virus in humans, or may be confused with Guillain–Barré syndrome. RABV infection can be inferred in some cases from indirect evidence, such as the demonstration of RABV-neutralizing antibody in the serum of an unvaccinated individual or antibody in CSF. Specific histopathologic changes in postmortem examination, namely the presence of Negri body inclusions in neurons of the CNS, can provide microscopic evidence of RABV infection, but only with a low degree of sensitivity. A more specific indication of RABV infection from a postmortem examination of humans suspected of having rabies is the direct visualization of RABV particles in brain tissue and CSF or saliva using EM. Similarly, specific indications of rabies can be used to determine the treatment of humans based on examination of brain specimens taken from contact animals suspected of having rabies after they have been euthanized by any humane method that does not damage the head. Other indications can be made in postmortem tissue (from animal or human) prepared as a smear, impression, or section or from CSF or cultured cell monolayer infected with the suspected virus. These are detection of the viral proteins (antigens) by DFA and indirect fluorescent antibody (IFA) tests and viral genome RNA using genetic hybridization probes and RT-PCR techniques. Immunoglobulin (or viral antigen-specific monoclonal antibody (MAb)) molecules labeled by covalent chemical attachment of a fluorescent tag to produce a fluorescent-MAb conjugate permit visualization and localization of the target protein in tissue preparations or saliva. These viral antigen-specific antibody diagnostic reagents, which interact specifically with the desired viral antigens (targeting specific epitopes on the proteins) in the DFA and IFA tests, provide a more definitive diagnosis of rabies infection in the contact animal and exposed human. The most common confirmatory test is cultivation of virus by inoculation of laboratory (test) animals or cell culture. The mouse inoculation test (MIT) is a sensitive and reliable procedure to confirm the presence of virus in pieces of the brain that test positive by the DFA or IFA methods. Direct detection of the viral genome or virus-specific mRNAs by hybridization with nucleic acid probes applied to tissue sections (*in situ* hybridization) requires close attention to the specificity of the probe sequences that are used to anneal with its viral positive or negative RNA strand target sequence. Since no commercial sources of RABV-specific probes are available, all probes must be developed for the laboratory, and special attention must therefore be paid to the diversity of lyssavirus strains that might be present in the specimen.

Rabies Pathogenesis and Histopathology

The pathogenetic process of rabies usually begins with the introduction of virus-laden saliva into a bite or scratch wound inflicted by a rabid animal. The incubation period after the bite can be extremely variable depending on the relative susceptibility of the species, the location of the wound (to the head vs. extremities), and the depth and location of virus deposit in the wound. In humans, the incubation period is usually 20–90 days, more generally 15 days to 1 year, but cases have been described recently in which the extremely sophisticated, highly sensitive, and precise antigenic (using MAbs) and genetic (employing the RT-PCR technique) diagnostic methods established that the incubation period could last several years. Other routes of infection have included intranasal (in animals that sniff) and oral routes (among bats that spray in high-density cave dwellings) as well as corneal transplants (in human cases). Because the neural routes from mucosal membranes and the eye to the CNS are relatively short, the virus has almost direct access to the brain from these tissues. RABV in natural infections does not establish a viremia.

Perceptions of what happens to the virus at the site of entry *in vivo* and the events that precede the transit of virus toward the CNS (the 'neural phase') are still vague. One puzzling aspect of preneural RABV infection is the sequestering of virus at the site of inoculation. Macrophages *in vitro* can be persistently infected with RABV, but it has not been demonstrated in animal models that macrophages *in vivo* sequester RABV. Evidence of viral antigen accumulating in striated muscle in experimental animals after intramuscular inoculation of RABV has suggested that muscle becomes a potential initial site of virus replication although this has not been observed in humans. The 'preneural phase' of virus infection in muscle is usually confined to a small number of myocytes close to the inoculation site and produces few infectious particles. Any shedding of virus into extracellular spaces around myocytes, of course, makes this virus vulnerable to the PET with anti-rabies antibody and vaccine.

Exposed sensory nerve endings of neuromuscular or neurotendinal spindles deep in muscle are the most likely sites of virus entry into the peripheral nervous system after virus replication in muscle. The primary site of virus attachment might actually be nerve endings leading directly to neuronal infection in badly torn muscles of a bite wound. Similarly, the many sensory nerve endings of epithelial and subepithelial tissues exposed by superficial abrasions of skin and mucous membranes may also serve as primary sites of virus entry. The entry of RABV into peripheral nerves begins the 'neural' or 'neuronal' phase of infection, which is characterized by the centripetal movement of virus exclusively within axons to the CNS. Schwann cells and perineural elements do not appear to be infected.

Virus migration from peripheral sites on the body to the brain is suspected to occur passively in the axoplasm of peripheral motor and perhaps sensory nerves (centripetal passage to the brain) at the estimated rate of retrograde axoplasmic transport (3 mm h^{-1} or between 50 and 100 mm day^{-1}) in peripheral nerves. Evidence that the RABV P (phosphoprotein) of the RNP complex interacts with LC8, a component of both cytoplasmic myosin V and dynein, which are involved in actin-based transport (important in early steps of

viral entry) and microtubule-based transport in neurons, respectively, has led to speculation that the RABV P–dynein interaction may be fundamentally important in axonal transport of RABV. Other mechanisms of virus transport may facilitate its spread (e.g., movement of virus across cell-to-cell junctions, including synaptic junctions). Observations of virus budding on axonal membranes and virus particles accumulating intra-axonally at nodes of Ranvier as the virus progresses to the brain support the possibility of other mechanisms of virus transport. The ascending paralysis seen in many infected animals and humans reflects the progressive infection of dorsal root ganglia of the CNS. The virus ascent toward the brain has been confirmed by sequential immunofluorescence studies. The ability of virus to spread to the CNS from a peripheral site (its neuroinvasiveness) is an important component of the 'neurovirulence' characteristic of the virus. Studies have shown that while the spread of pathogenic (virulent) and nonpathogenic (avirulent) viruses *in vitro* (spreading from cell to cell in cell culture) reflects their ability to spread *in vivo*, the pathogenic virus spreads more rapidly in the CNS and infects more neurons than the avirulent virus *in vivo*. Once RABV infection enters the brain, the virus spreads rapidly along neuroanatomical pathways, spreading within the CNS as in the peripheral nerves.

Inflammatory lesions caused by perivascular infiltration of lymphocytes, macrophage, and occasionally polymorphonuclear cells are the most common and frequently noted histological signs of change in the spinal cord and brainstem of animals and humans infected with RABV. The ganglionic changes observed in rabies, although not exclusively confined to rabies, could serve as diagnostic lesions for rabies, because they are not encountered in diseases that must be considered in the differential diagnosis of rabies. The morphologically distinct lesions that are significant in rabies diagnosis are the specific eosinophilic inclusion bodies (Negri bodies) found within neurons of peripheral and basal ganglia of the spinal cord, in the pons and medulla of the brainstem, in the cerebral cortex and thalamus and Purkinje cells of cerebellum, and in ganglion cells of Ammon's horn of the hippocampus. Signs of active transport of virus in the brain include replication in the neurons, budding from the plasma membrane, and release into tissue interstitial spaces. The virus appears to infect susceptible cells by viropexis or direct cell-to-cell transmission. Once delivered in the extracellular spaces of the brain, the virus can presumably spread by passive transport over relatively long distances through intercellular spaces large enough to pass virus by interstitial fluid movement. Virus that is transmitted directly from one nerve cell to a contiguous nerve cell could also spread and result in the same long-distance movement because of the many long, intertwined neuronal processes that connect in the brain.

Surprisingly, despite the severe neurological signs of clinical rabies (Human rabies) the neuropathological findings are generally mild (see below). This suggests that RABV-infected neurons become dysfunctional without developing major morphological changes or positive terminal deoxynucleotide transferase dUTP nick-end-labeling (TUNEL) staining, indicating fragmented cellular DNA that is normally associated with apoptosis. If true, what is the fundamental underlying basis for the proposed neuronal dysfunction, if not apoptosis, that would account for the severe clinical neurological signs observed in rabies? One hypothesis has been that apoptotic cell death in the CNS of humans and in experimental animals in which apoptotic changes have been examined is caused by RABV infection. After finding evidence of oligonucleosomal DNA fragmentation typical of apoptosis and that cell death was concomitant with expression of the rabies viral G, an (unexpected) inverse relationship was found between the pathogenicity of the virus (comparing the virulent phenotype with the avirulent phenotype) and apoptosis. In other words, apoptosis may be a protective rather than a pathogenic mechanism in RABV infections both *in vitro* (studies with cell culture) and *in vivo*. The less pathogenic (avirulent) viruses induce more apoptosis than the more pathogenic (virulent) viruses *in vitro* as well as *in vivo*, using peripheral routes of inoculation. In RABV infection the mechanisms involved in the ensuing cell death or survival of neurons, both *in vitro* and in animal models using different viral strains and routes of inoculation, are clearly complex.

Once the virus begins to spread from the brain in the final phase of rabies infection, the tropism of infectious virus changes and the virus is delivered to a variety of extraneural and nonneural tissues. The centrifugal (outward) spread of virus generally occurs via the same axoplasmic transport routes that were used for the centripetal (inward) passage of virus inward to the CNS. The spread of RABV in the absence of CNS infection is virtually nonexistent. Among the most heavily infected tissues following propagation of virus in the CNS are the end organs in oral and nasal cavities, and the head and neck. Infection may be found in free sensory nerve endings of tactile hair follicles or in epithelial cells of hair follicles in the skin. To take a skin biopsy from the nape of the neck is one of the best diagnostic methods of confirming an antemortem diagnosis of rabies in humans ('Human rabies') (see Rabies Diagnosis). The fact that rabies antigen is occasionally detectable in skin biopsy and in corneal impressions is evidence of its nerve-mediated dissemination.

Infection of the salivary glands by dissemination of virus from the CNS is extremely important in the life cycle of the virus because it is required for effective transmission of disease. Virus titers in the salivary glands often exceed those in the brain. In some animal species, the salivary glands become infected before the signs of CNS infection become apparent, and at the height of salivary gland infection, large amounts of fluorescent viral antigen may be detected in individual acinar cells, in cell clusters, and occasionally in the entire acinus.

Presently, the molecular basis of RABV pathogenesis is linked to surface G, which also plays an important role in the initiation of RABV infections as the viral attachment protein that interacts with the cell surface receptor. Through selection of neutralization-resistant variants of RABV (using virus-neutralizing MAbs against G) that lost their virulence phenotype and by identification of single amino acid changes in G, the pathogenicity of the virus appears to correlate with the presence of an epitope on G within antigenic site III. The amino acid substitution in the mutant that renders the virus nonpathogenic for adult mice was located at position 333 of RABV G where arginine is normally found. Isoleucine at position 333 in the fixed RABV Evelyn–Robitnicki–Abelseth (ERA) strain variant and glutamine or glycine in the fixed rabies CVS strain variants replaced arginine at position 333 of RABV G in the respective pathogenic parent viruses. Several other fixed RABV strains including attenuated viruses that were not selected by MAbs but resisted neutralization by specific MAbs (194-2 and 248-8), such as the Flury high egg passage (HEP) and Kelev strains,

also substituted isoleucine, glutamine, or glycine for arginine 333. Other discrete amino acid substitutions for arginine 333 include methionine and serine. The biological relevance of arginine at position 333 of RABV G or its influence on the pathogenic mechanism responsible for the restricted neurotropism of RABV infection is not completely understood. Experiments have suggested that the neural routes of entry to the CNS and the rate of virus spread may differ depending on the amino acid at position 333, but as yet the complete story has not been told.

Many of the dominant pathologic features of RABV infection were first described in the early 1870s to early 1990s. With the introduction of EM and immunohistochemistry, pathology studies have continued to provide key insights into our understanding of rabies. Although the clinical outcome of RABV encephalomyelitis might suggest severe degrees of destruction, the histopathological changes observed in the CNS are typically relatively mild. Some inflammatory cell infiltration of the leptomeninges usually occurs in human rabies cases. Infiltrates are composed of lymphocytes and monocytes, small numbers of plasma cells, and a predominance of neutrophils when inflammation is intense. Perivascular 'cuffing' is seen predominantly in gray matter, especially in the brainstem and spinal cord, marked with infiltrates of lymphocytes and monocytes. The development of neuronophagia (accumulations of activated microglia/macrophage in the process of phagocytosing degenerating and/or dying neurons) is typical in many rabies cases. Microscopic accumulations of proliferating microglia/monocytes known as Babes nodules (first described by Babes in 1892) can be observed in gray matter of the medulla oblongata in human rabies cases. However, because Babes nodules can be present in other viral encephalitides and other infectious disorders they are less reliable for rabies diagnosis. On the other hand, Negri bodies, described in the 1903 by Adelchi Negri are more of a classical hallmark of cytological features in rabies. Stained with hematoxylin- and eosin, they appear as dense, well-defined, oval or round (typically 2–10 μm in diameter, but can be 0.5–27 μm) eosinophilic intracytoplasmic inclusions (Figure 6). Although found in 50–90% of human rabies infections, especially in hippocampal neurons and cerebella Purkinje cells, their significance in the pathogenesis of rabies is not as obvious. The ultrastructure of Negri bodies includes randomly orientated viral NC (source of RABV N for antigen diagnosis) resembling large aggregates of granulo-filamentous matrix material occasionally interspersed with bullet-shaped virus particles giving the bodies an overall granular, electron dense manifestation. They are usually found in areas of the CNS where there is little inflammation, and less frequently seen in degenerating neurons and/or in association with inflammatory foci.

Histopathology in peripheral nerves as virus spreads through the peripheral nervous system (PNS) is associated with inflammatory, reactive, and degenerative changes in structural components of the PNS. Changes in sensory and autonomic ganglia as well as a neuronopathy (degeneration and loss of sensory and autonomic neurons) leading to degeneration of spinal and peripheral nerve fibers and routes have particular diagnostic utility.

Rabies Vaccine Prophylaxis

First Line of Protection Against Rabies – Preexposure Prophylaxis

Rabies is a preventable disease in humans if the right precautions are taken to ensure pre-exposure prophylaxis (PrEP). Those who consider themselves to be at increased risk of possible exposure to rabid animals, especially unvaccinated free-roaming dogs should

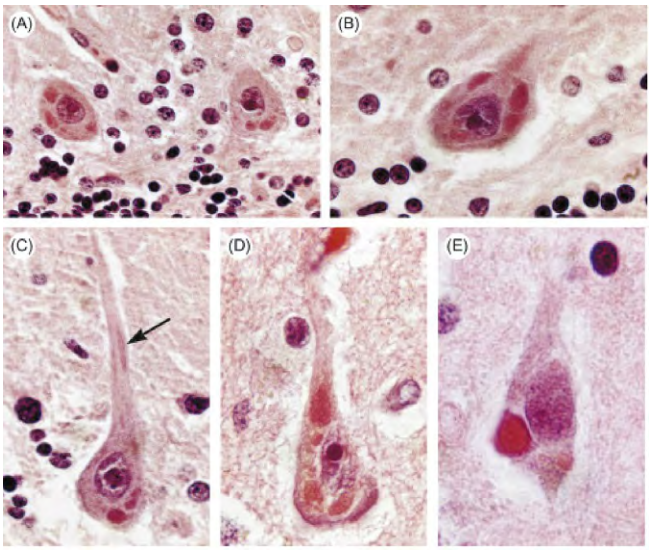

Figure 6 Negri bodies stained with hematoxylin and eosin (HE) in perikarya of (A-C) cerebellar Purkinje cells and (D, E) pyramidal neurons in the cerebral cortex of human rabies cases. The arrow in (D) indicates a Negri body in an apical dendrite. (magnifications, A × 315, B × 460, C × 550, D × 730, E × 865). Reproduced from Rossiter, J. P., Jackson, A. C. (2013). Pathology. In: Jackson, A. C. (ed.) *Rabies*, 3rd edn., pp. 351–408. San Diego, CA: Elsevier, Academic Press, with permission.

be administered PrEP vaccination. Veterinarians and animal handlers because of their frequent and often unsuspecting contacts with domestic as well as wildlife animals, and those persons who by the nature of their work, hobbies (e.g., caving) or travels to rabies endemic areas put themselves at high risk of exposure and infection, particularly where access to timely PEP is not an option (e.g., no medical clinics) should receive the full regimen of inoculations. PrEP consists of a series of three doses of vaccine, given either intramuscularly (IM) or intradermally (ID) on each of 3 days; 0, 3, and either day 21 or 28. Antibody titers of 0.5 international units (IU)/ml or higher are considered to be protective against rabies. A single booster dose of vaccine would be necessary should the titer fall below this minimum titer.

First Line of Defense After Exposure to Rabies – Postexposure Prophylaxis

Since the incubation period may be variable and often sufficiently long following the peripheral inoculation of RABV from an animal bite, depending on the location and severity of the bite exposure, it is possible to neutralize the virus at the wound site with anti-rabies antibody before the virus enters and spreads along neuronal pathways to the CNS. According to WHO recommendations, PEP must be administered to every patient that has been exposed to rabies. Immediately or as soon as possible after an exposure to a potentially rabid animal, it is essential to start PET and PEP by first cleaning the wound using proper wound cleansing procedures as a first line of defense against RABV infection. It is important in this procedure to inactivate as much as possible of the RABV that may have entered the wound. Proper wound cleansing involves thorough washing of the wound or mucous membrane for at least 15 min using soap, water, detergent, povidone iodine, or another antiviral substance. If soap and detergent are not available the wound should be flushed with copious amounts of water. After the wound has been thoroughly cleansed, rabies immunoglobulin (RIG) (virus neutralizing antibody) must be injected into and around the bite (wound) or mucous membrane sites to neutralize virus deposited with saliva from the animal's bite. Two types of RIG are administered to patients globally; human rabies immunoglobulin (HRIG) is used more frequently in industrialized countries whereas equine rabies immunoglobulin (ERIG) is more frequently administered to patients in developing countries. To administer the RIG properly, the full dose of 20 IU kg^{-1} of body weight of HRIG and 40 IU kg^{-1} of body weight of ERIG must first be injected directly into as well as around the wound site in as many places as anatomically feasible. Any HRIG or ERIG that is left over after infiltrating the wound sites should be administered deep intramuscularly at a site that is distant from the vaccine inoculation site so as not to interfere with (neutralize) the vaccine dose that is also given at this time for prophylaxis (infection prevention).

Rabies vaccines available in developing countries for PEP are mostly nerve tissue vaccines (NTVs) produced from the brain tissue of infected sheep or mice although these are gradually being replaced by more immunogenic and less reactogenic cell culture-based rabies vaccines (CCVs), particularly in large cities. The problem with the modern cell culture vaccines is they cost more to produce, limiting their use to those at the highest risk of RABV infection, who ironically are often among the poorest of society. The postexposure vaccination regimens recommended for CCVs use either IM or ID routes of inoculation of which the latter is an option for reducing the dose administered and the cost of treatment. The first dose of vaccine is given with RIG administered in and around the wound site. It is important to note that there is sufficient data to insure that when used in conjunction with prompt appropriate wound care along with RIG, the postexposure vaccination regimens recommended by the global WHO Expert Committee on rabies can prevent death from rabies. There are two IM and two ID postexposure vaccination regimens recommended for use with cell culture rabies vaccines. In the United States, the Advisory Committee on Immunization Practices (ACIP) recommends a four-dose rabies vaccination regimen with human diploid cell vaccine (HDCV) or purified chick embryo cell vaccine (PCECV) administered IM on days 0 (as soon as possible after exposure), 3, 7, and 14 instead of the Essen regimen, which stipulates five IM doses, previously recommended for use in North America. The Essen regimen is also used in Europe and in those parts of Asia and Africa where ID administration of rabies vaccines is not yet approved. The Essen regimen specifies one full dose IM on days 0, 3, 7, 14, and 28. The Zagreb regimen, which specifies two doses IM on day 0 (one in each deltoid) and one additional dose on days 7 and 21, reduces the number of doses required from five to four, and, importantly, it reduces the number of visits to get the rabies vaccine from five to three. The Zegreb regimen is used in some European countries.

The WHO-recommended ID vaccination regimens, which use reduced doses of vaccine, were developed to reduce the cost of expensive CCVs that were introduced to replace NTVs in Asia. Thailand was the first country to begin using ID regimens to administer CCVs. The Thai Red Cross or '2-2-2-0-2' regimen and the Eight-site or '8-0-4-0-1-1' regimen are two ID regimens currently recommended for use by the WHO, the Thai Red Cross regimen being the most widely used ID vaccination regimen. The updated '2-2-2-0-2' regimen is administered with 0.1 ml doses of vaccine ID over the deltoid region on days 0, 3, 7, and 28. In the '8-0-4-0-1-1' regimen, vaccine is administered ID in a 0.1 ml dose at eight different sites (upper arms, lateral thighs, suprascapular region, and lower quadrant of abdomen) on day 0, another 0.1 ml dose of vaccine at four different sites (each upper arm and each lateral thigh) on day 7, and another 0.1 ml dose of vaccine in the upper arm on days 28 and 90. It is important to note that all of the PEP regimens have been developed with the administration of RIG to provide initial passive immunity.

Second Line of Defense: Establishing Active Adaptive Immunity

Because RABV is strictly neuroinvasive and little viral antigen is present in tissue in the early stages of the disease, the humoral response to viral antigens in the infected host is negligible until later in the course of infection, usually after clinical signs appear, and remains low until the terminal phase of the disease. High levels of antibody appear in serum and in CSF only at death and in cases where illness is naturally or artificially prolonged. In the rare cases of chronic and abortive RABV infections, virus-specific antibodies have been

detected in the serum and CSF, and in the brain of animals. VNA is produced in response to the massive amount of viral antigen (G in particular) that is generated through widespread infection of the CNS and made accessible to the host's reticuloendothelial system.

The critically important cell-mediated immune response to RABV infection is perhaps the most puzzling of the host's total immune response to RABV infection. T-helper lymphocytes, which play an essential role in supporting B-cell production of antibodies, and cytotoxic lymphocytes (CTLs), which function independently in cell-mediated viral clearance, are both key responses in the cell-mediated immunity derived from viral antigens. However, in spite of their importance in viral clearance, T-lymphocytes appear to be suppressed in animals infected by pathogenic 'street' viruses. As a result of virus-induced immunosuppression, the disease increases in severity and mortality rises. The strongest indications that T-lymphocytes play a role in immune protection against RABV infection comes from studies in which athymic (nude) mice died when infected with the attenuated Flury HEP strain that normally causes nonlethal infection of the CNS. Normal mice, similarly infected, show no signs of the disease. Likewise, when normal mice infected with Flury HEP virus are treated with the immunosuppressive agent, cyclophosphamide or antithymocyte serum, the infection becomes as lethal as street virus (mortality in groups of mice increases to 50% and occasionally to as high as 100%). Mice that survive avirulent virus infection develop CTLs, whereas the mice treated with immunosuppressants and mice infected with virulent virus fail to develop CTLs. Further evidence for the protective role of CTLs has come from adoptive transfer experiments in which G-specific CTL clones were observed to protect mice against RABV infection. Since the function of CTLs is to destroy target cells that display virus-induced changes on their surfaces, it is clear that a strong CTL response is essential in mice that survive RABV infection. It is clear from these studies and others that both B- and T-cells play an important role in virus clearance.

Unlike the response to virulent RABV infection, immunization with attenuated live and inactivated RABV vaccines induces humoral and cell-mediated immune responses that develop to functional levels in 7–10 days and persist for a year or more. Vaccine-induced antibody and T-cell-mediated responses in animals and humans, known as adaptive immune responses, are regarded as key and are of paramount importance in the prophylatic protection of animals and humans. Also, acute infections caused by live virulent RABV strains, as well as the less virulent strains, injected into a peripheral site (e.g., the hind leg of an animal), that progress to and within the spinal cord and the brain are accompanied by the production of the inflammatory cytokines IL-1, TNF-α, INF-β, and IL-6, as well as chemokines such as CCL-5 and CXCL-10. This indicates that the nervous system can sense RABV entry and mount a reactive innate, as well as an adaptive immune response. An adaptive innate immune response is helpful in PEP and PET if, as proposed, chemokines and inflammatory cytokines attract lymphocytes that are activated in the periphery. It is to be emphasized that even with a relatively rapid adaptive immune response of 7–10 days, the requirement in PET for the administration of RIG is critical. RIG is the fraction of anti-rabies serum produced in immunized humans (HRIG) or equines (ERIG), which provides immediate passive immunity. It is administered with proven effectiveness over the initial few days and even weeks after RABV exposure in humans. RIG must be administered to humans exposed to RABV as a passive immune treatment in order to provide immediate access to VNAs until the patient's immune system can begin to produce its own antibody. The importance of antibodies in controlling the spread of RABV infections is clear when one considers that antibodies are capable of effectively neutralizing virus that is present in intercellular spaces or in body fluids. Antibodies may bind to virus expressed on the cell surface, allowing complement- or antibody-dependent cellular cytotoxicity to mediate killing of infected cells.

Antibodies, however, are also implicated in an immunopathologic role in regard to the 'early death' phenomenon, which is associated with rabies in animals and possibly humans. If animals have low levels of virus- or vaccine-induced rabies-specific antibody at the time they are challenged with RABV, they frequently die earlier than those without antibodies. In an analogous situation, where humans appear to develop clinical rabies with shorter incubation periods as a result of postexposure immunization, compared with individuals who were exposed to rabies but not immunized, the early death phenomenon may also apply. Inasmuch as the mechanism of early death may resemble the immune cytolysis observed in RABV-infected cells treated with antiserum and complement *in vitro*, the immunopathologic effect of antibody would be to accelerate the pathogenesis of the virus.

The efficacy of postexposure immunization and long-term effects of vaccine-induced prophylaxis against rabies seems also to be linked to the stimulation of a strong CTL response. Induction of CTLs and other effector T-cells during infection with live attenuated RABV vaccine strains and in response to immunization with inactivated virus is consistent with the observation that T-lymphocytes, and CTLs in particular, are essential for protection against a lethal dose of RABV. There is no evidence that early death is associated with rabies-specific T-cells or does any other form of immunopathology appear to be mediated through rabies immune T-cells.

For further information regarding practical approaches toward rabies prevention, including the rationale for PrEP and PEP, treatment of wounds, vaccines and vaccine dosage, booster vaccination, and serologic testing, the reader is referred to the recommendations of the ACIP.

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Regulation of Carbon Assimilation in Bacteria☆

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Glossary

Anaplerosis Process that replenishes TCA cycle intermediates that were lost for anabolic purposes.
Autotrophic organism One that manufactures organic compounds from inorganic compounds using energy from sunlight or from chemical reactions; for example, oxidation of H_2S, NH_3, or Fe^{2+}.
Carbon catabolite repression (CCR) Process in which glucose or other rapidly metabolizable carbon sources prevent the uptake and/or utilization of other carbon sources.
Catabolite activator protein (CAP) Also known as cAMP receptor protein (CRP). Transcription factor of enteric bacteria that, in complex with cAMP, is required for the transcription of most catabolite-repressed genes.
Catabolite control protein (CcpA) Transcription factor of Firmicutes that, in complex with P-Ser46-HPr, is responsible for repression of most catabolite-controlled genes.
Central metabolism The combined pathways of glycolysis, namely, the Embden–Meyerhof–Parnas pathway, the Entner–Doudoroff pathway, the pentose phosphate pathway, and the TCA cycle. Their role is to generate energy (ATP), to reduce power (NADH and NADPH), and to act as the precursor compounds for the biosynthesis of all other cellular constituents.
Diauxie The process by which bacteria supplied with more than one carbon source uses one carbon source before the others and then induces the enzymes necessary for the use of the second carbon source.
Inducer exclusion Process in which glucose or other rapidly metabolized carbon sources prevent the uptake of alternative sugars and hence prevent the induction of the genes necessary for the metabolism of the alternative sugar. Inducer exclusion is one aspect of CCR.
Phosphotransferase system (PTS) System of proteins for the simultaneous uptake and phosphorylation of sugars. A phosphate derived from phospho-*enol*-pyruvate (PEP) is passed via a series of proteins and is attached to the sugar as it traverses the internal membrane. Proteins of the PTS are involved in inducer exclusion and catabolite repression in both Gram-positive and Gram-negative organisms.
Phototrophic (or photosynthetic) organism One that manufactures organic compounds using energy from sunlight, carbon dioxide, and water (cf. autotrophic organism).
PTS regulation domain (PRD) A domain subject to phosphorylation by either the EIIB domain of a specific PTS transporter or HPr. It is found in transcriptional activators or antiterminators, mostly in Firmicutes.
Regulon A group of genes possibly dispersed on the bacterial chromosome, but which are controlled by the same factor or signal.

Abbreviations

α-CTD	C-terminal domain of the alpha subunit of RNAP
α-NTD	N-terminal domain of the alpha subunit of RNAP
ABC	ATP-binding cassette
AC	Adenylate cyclase
AR1	Activating region I
CAP	Catabolite activator protein (=CRP)
CCA	Carbon catabolite activation
CcpA	Catabolite control protein
CCR	Carbon catabolite repression

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Crc	Catabolite repression control
cre	Catabolite response element
CRP	cAMP receptor protein (=CAP)
crr	Carbohydrate repression resistant
ED	Entner–Doudoroff pathway
EI	Enzyme I
EMP	Embden–Meyerhof–Parnas pathway
PEP	Phospho-*enol*-pyruvate
PQQ	Pyrroloquinoline-quinone dependent
PRD	PTS regulation domain
PTS	Phosphotransferase system
RAT	Ribonucleotide antitermination targets
RNAP	RNA polymerase
UAS	Upstream activating site

Defining Statement

Bacteria must adapt to the nutrients available in their environmental niche. One important regulatory mechanism to optimize carbon use is carbon catabolite repression. Different strategies adopted by bacteria to optimize their use of available carbon sources are described.

Introduction

The Need to Regulate

Like all living organisms, bacteria have to adapt to their environment. Their major concern is to find the nutrients that they need to survive. A source of assimilable carbon is a primary requisite and, except for phototrophic (photosynthetic) bacteria or other autotrophs, this means some form of organic carbon compound. The ability of bacteria to use an incredible range of carbon sources is indicative of the equally wide range of habitats where they are found. Their ability to inhabit a particular environment is reflected in the cohort of genes that are present in their genomes for taking up and degrading the variety of compounds likely to be available in a particular location. These range from the nutrient-rich environments of lactic acid bacteria to the low-nutrient aqueous environments frequented by *Caulobacter crescentus* and ocean-dwelling bacteria. There is a rough correlation between genome size and metabolic capacity. Lactic acid bacteria, which are endogenous to food-related habitats, or pathogens and symbionts living in close connection with their host cells, have slimmed down their genomes, whereas bacteria adapted to more diverse or desertlike conditions have retained all their biosynthetic capacities plus diverse catabolic pathways for a wide range of potential nutrients.

Most experimental studies have been performed in the completely artificial environment of the Erlenmeyer flask, supplied with high concentrations of a single carbon source or a defined mix of nutrients during exponential growth. Bacteria in nature will rarely find themselves in a richly supplied medium and, at best, pass between times of relatively high levels of nutrients to a stage when all has been used up and the bacteria must either go searching for a new supply or wait until more food comes their way. This has led to the "feast or famine" concept. In the last decades, attempts have been made to mimic a more natural state by using carbon limitation in chemostats and long-term growth experiments (Ferenci, 2008).

It has long been known that not all carbon sources are equal, and some are certainly more equal than others; that is to say, one carbon source is used preferentially before others. In general, this equates to a rapidly metabolizable carbon source being used first. This observation poses another question of why some carbon sources are used more rapidly than others. The general consensus (dogma) is that uptake is the rate-limiting step. Limitation at the level of uptake could be due to the amount of the transporter available, the activity of the transporter for a specific substrate, or the supply of energy to energize the transport system. However, other intracellular steps, such as the amount or activity of an enzyme needed to metabolize a compound or the supply of an intermediate that is required for its metabolism, for example, ATP or NADH, could be limiting. It is frequently observed that overproduction of one key enzyme within a pathway does not increase flux through the pathway because something else is limiting. Metabolic flux analysis has addressed the problem of flux control during glycolysis, and for *Escherichia coli* the rate of use of ATP, by the anabolic pathways, appeared to be the rate-limiting step. Artificially increasing ATP hydrolysis, by overexpressing the soluble part of ATPase (encoded by *atpAGD*), increased the flux through glycolysis. However, in a similar study on *Lactococcus lactis*, control by ATP consumption was not observed, implying that in this organism there is no excess glycolytic capacity (Koebmann et al., 2002a,b). Getting the most out of available carbon sources is a major concern of the biotechnological industries, and much effort is being put into identifying and overcoming the bottlenecks in biosynthetic production routes and improving yields from starting compounds, for example, for the synthesis of GlcNAc (Liu et al., 2016; Gu et al., 2017) or scyllo-inositol in *Bacillus subtilis* (Tanaka et al., 2017).

Diauxie and the Hierarchy of Carbon Use

The classic experiment showing the preferential use of one sugar over another is the phenomenon of diauxic growth, as described by Jacques Monod. *E. coli* supplied with a mixture of glucose and lactose completely uses the glucose first, and it then effectively stops growing (lag time) while the genes for uptake and degradation of lactose are induced before the bacteria resume growth, at a slower rate, using lactose as the carbon source.

The ability of glucose to inhibit the use of other carbon sources in enteric bacteria was called the "glucose effect" and is now known to be an aspect of the general phenomenon of carbon catabolite repression (CCR). This is a term that covers a range of physiological phenomena that enable bacteria to make a hierarchical choice between different sources of carbon. CCR exists in all bacteria, but the form it takes varies between different species. Generally, glucose is the preferred carbon source but this is not a universal rule. For example, the nitrogen-fixing *Azotobacter vinelandii* uses acetate or galactose before glucose and in *Pseudomonas putida* succinate produces stronger CCR than glucose (Rojo, 2010). CCR has been most extensively studied in two model organisms: *E. coli* for Gram-negative bacteria and *B. subtilis* for Gram-positive species (Görke and Stülke, 2008). In these two organisms, the molecular mechanisms are quite different, and although they are representative of bacterial species closely related to themselves, it is clear that there are alternative, completely different, mechanisms operational in other bacteria. Studies on carbon control in other microorganisms, particularly those with biotechnological or pharmaceutical applications, such as *Streptomyces coelicolor* or *Corynebacterium glutamicum*, are revealing a very diverse set of mechanisms. Even the dogma of hierarchical choice of nutrients is not sacrosanct because *C. glutamicum* prefers to use several carbon sources simultaneously. Both experimental and theoretical considerations show that, depending on conditions, *E. coli* can use several sugars or other carbon sources simultaneously, following a hierarchy generally reflecting the growth rates of individual compounds, and provided that they are not exerting strong catabolite repression (Aidelberg et al., 2014; Hermsen et al., 2015). Improved techniques of analysis of individual cells have shown that the gene induction response within individual cells to external sugars is not homogeneous and that there can be "all-or-none" and "graded" responses that are possible depending on the carbon source (Afroz et al., 2014; Rao and Koirala, 2014). Similar considerations can explain the existence of so-called bistable states within isogenic bacterial populations (Kotte et al., 2014), and these considerations led to the concept of "microbial bet-hedging," which posits that a phenotypically mixed population of bacteria might be better prepared to adapt to and survive within rapidly changing environments (Grimbergen et al., 2015).

Global Versus Specific Regulation

The proteins and genes responsible for CCR are the prime examples of global regulators in bacteria. They control a large number of genes in a coordinated manner, albeit with a certain level of flexibility depending on the individual genes under regulation. It has been estimated that 50% of the genes in *E. coli* are regulated by one of the seven global regulators (Martinez-Antonio and Collado-Vides, 2003) and, of these seven, catabolite activator protein (CAP; also called CRP, cAMP receptor protein) is by far the most important with more than 200 direct targets. Expression of catabolite-regulated genes in *E. coli* usually requires transcriptional activation by the cAMP/CAP complex. The magnitude of the activation by cAMP/CAP of different genes varies considerably and depends on the promoter and the sequence and location of the CAP-binding site on the target DNA. The global reduction in cAMP/CAP activation when a preferred carbon source (e.g., glucose in the case of *E. coli*) is present, means that none of the other carbon utilization systems is expressed. However, in the absence of glucose, the bacteria do not want to express their full repertoire of all other carbon utilization operons, which can amount to many hundreds of genes. So, superimposed on the generalized response to catabolite regulation, there are specialized regulatory mechanisms dedicated to individual carbon sources. In the case of the diauxie experiments of Monod, the presence of glucose prevented the expression of genes for most other carbon utilization systems, but when the glucose had been used up, the presence of lactose meant that only the genes of the *lac* operon were expressed and not those for use of other carbon sources like maltose or arabinose.

Subsequent experiments, however, showed that this idea is too simplistic. The bacterial response to changing energy supplies is not just on/off but is more measured. Global transcriptome analysis of RNA from bacteria grown in the presence of different carbon sources, producing a wide range of growth rates from 0.97 h^{-1} (glucose) to 0.13 h^{-1} (proline), has shown that, as the quality of the carbon source declines and the growth rate decreases, bacteria gradually upregulate a wide range of genes to broaden their metabolic potential and scavenge for alternate carbon sources (Liu et al., 2005). Similarly, a transcriptome analysis of cells undergoing diauxie showed that a range of stress-related genes (controlled by σ^S, the stationary phase sigma factor, encoded by *rpoS*) and stringent response genes (controlled by ppGpp) are also activated (Traxler et al., 2006). Current model-based approaches of gene expression analysis have shown that both specific (transcription factor) and global (including CAP/cAMP) regulatory mechanisms are active during steady-state conditions but that the global physiological controls are crucial for transitions between steady states (Berthoumieux et al., 2013; Gerosa et al., 2013).

Sensing and Signaling

The general rule is that genes required for use of a particular carbon source are only expressed at a high level when the compound is available to be assimilated. This requires a signal transduction pathway to sense the external presence of the compound and turn on

the expression of the required genes. This type of regulation is nearly always transcriptional, implicating transcription factors acting either positively (activators) or negatively (repressors), of which the classic examples in *E. coli* are the lactose and galactose repressors and the maltose and arabinose activators. A sugar outside the cell enters at a low level, generating a signal that is detected inside the bacteria, indicating the presence of the carbon source outside. The signal is generally a small molecule related to the pathway under control (allolactose, galactose, maltotriose, and arabinose in the four examples listed). The signal starts to induce the genes necessary for the transport of the sugar, thus amplifying the intracellular signal and the level of induction. There are some alternative methods of signaling an available nutrient. Some signaling is due to phosphorylation cascades, as in the case of two-component regulators. Detection of a signal outside the bacteria provokes the autophosphorylation of a histidine sensor kinase, which in turn phosphorylates and activates a second protein, a response regulator, which is generally a positive transcription factor. There are a few examples of two-component systems regulating the use of a carbon supply; for example, the uptake of hexose phosphates in *E. coli* by the UhpT transporter is controlled by the UhpABC-encoded two-component system, but generally this type of regulation is associated with stress responses. In bacteria, the phosphotransferase system (PTS), a major sugar uptake system, is responsible for many signaling phosphorylation reactions (Deutscher et al., 2014a; Galinier and Deutscher, 2017), which are described in the text that follows (see "Catabolite Repression in Gram-Negative Bacteria: *E. coli*" and "Catabolite Repression in Firmicutes: *B. subtilis*" sections). A slight variation to the rule that regulation of sugar use is at the level of transcription initiation is the case of some PTS sugar operons of *B. subtilis*, whose induction is at the level of transcription elongation involving a transcription antitermination mechanism (see "Transcriptional antitermination of PTS operons" section).

These sugar-specific factors all work in collaboration with the global regulators (cAMP/CAP in *E. coli*) so that together there is a combination of positive induction superimposed on relief of catabolite repression to allow the expression of the genes at the required time. The details of each regulated system are unique. Transcription factors, even those belonging to well-described families, all have their own individual characteristics. Common variations are the number and position of operator sites involved and the need for other DNA-binding proteins producing complex nucleoprotein structures in either the activated or the repressed state. For example, repression of both the *gal* and *lac* operons in *E. coli* requires DNA loop formation between Lac or Gal repressors bound to three (in the case of *lac*) or two (in the case of *gal*) operators. In the case of the *galETK* operon, the histone-like protein, HU, is also required for formation of the *gal* repression loop (Kar and Adhya, 2001).

Since the pioneering studies of Jacob and Monod on the *lac* operon in the 1950s and 1960s, people have been dissecting in more and more detail the mechanisms of regulation inherent in the use of numerous carbon sources, especially in *E. coli* and *B. subtilis*, but also in other more exotic bacteria. The 21st century launched the genomic era, which revolutionized the study of bacterial carbon use. The sequences of thousands of bacterial chromosomes allow comparative genomics to (try to) predict what carbon sources are likely to be used and in some cases identify gene functions and potential regulatory mechanisms, for example (Yang et al., 2006; Plata et al., 2012). It should be noted, however, that structural genes for enzymes are much better conserved than either their gene organization or the intergenic regulatory sequences. Regulatory patterns are only conserved in closely related organisms. Adenylate cyclases (AC) and CAP homologues are found in a wide range of bacteria, but the erstwhile paradigm of catabolite repression by cAMP/CAP only exists in the γ-proteobacterial lineage.

In addition to genomic sequencing, the other "-omic" studies of transcriptome (microarrays and RNA sequencing), proteome and metabolome, have allowed studies of the global response of bacteria to a wide range of nutrients, stimuli, and mutations. Exploiting the data from these studies has led to the large advances in the fields of flux analysis and systems biology to understand the flow of metabolites and generalized coordination of cell growth (Kochanowski et al., 2013a; Chubukov et al., 2014). These studies have shown that the number of genes in a regulon, that is, the family of genes coregulated by a transcription factor, is often higher than previously anticipated. Moreover, they have confirmed that the genes of the central carbon pathways in bacteria, the housekeeping genes of glycolysis, pentose-phosphate and TCA cycle are not just constitutively expressed but are also subject to complex regulation. Comparative studies in different bacteria have revealed that, even if the same basic pathways exist, their relative use can be very different from species to species (Fuhrer et al., 2005; Tannler et al., 2008a).

Posttranscriptional Regulation of Carbon Use

Regulation involving sRNA

Although it remains true that the switching on or off of a gene or an operon at the level of transcription initiation remains the most economical method of controlling expression in response to large changes in environmental conditions, like the detection of a new carbon supply, there is increasing evidence of many other layers of regulation that are superimposed to coordinate bacterial cell metabolism. These "posttranscriptional" mechanisms include translational regulation and mRNA degradation and particular control via sRNAs (Görke and Vogel, 2008; Bobrovskyy and Vanderpool, 2013; Bobrovskyy et al., 2015). For example, under conditions of excess glucose-6P (phospho-sugar stress), the sRNA, SgrS, is induced, and by binding in the region of the translational initiation site it inhibits translation of *ptsG*, encoding the major glucose transporter in *E. coli*. This results in the RNase E-dependent degradation of the *ptsG* mRNA (see "The SgrS/SgrT Novel sRNA/small protein pair" section).

The Spot42 sRNA was one of the first sRNAs identified. Initially it was found to be responsible for the discoordinate expression of *galK* in the *galETK* operon, and now microarray analysis has identified numerous carbon-use-related targets. Since these targets are mostly activated by cAMP/CAP, whereas expression of the Spot42 RNA is repressed by cAMP/CAP, this

creates a system for modulating their expression in response to catabolite repression (Beisel and Storz, 2011). On the other hand, expression of the CyaR sRNA is activated by cAMP. Microarrays identified diverse targets that are negatively regulated by CyaR, including outer membrane proteins. These are thus indirectly repressed by cAMP/CAP and represent another manner of tuning carbon metabolism to outside stimuli (De Lay and Gottesman, 2009). The Csr (carbon storage regulator) system includes the CsrA RNA-binding protein and two "decoy" sRNAs: CsrB and CsrC. CsrA was initially implicated in repressing the glycogen synthesis gene and coordinating the shift between glycolytic and gluconeogenesis carbon use. It has now been shown to have multiple roles including in biofilm formation and pathogenicity (Romeo et al., 2013) (see "Other Global Regulators of Carbon Use" section). sRNA seem to play a similarly important role in the carbon metabolism of *B. subtilis* and other Gram-positive bacteria (Mars et al., 2016).

Allosteric regulation

As in the case of transcriptional regulation, controls via sRNAs affect the cellular *levels* of enzymes. In addition, protein modifications (phosphorylation, acetylation) or protein–protein or protein-small-molecule interactions can affect the *activity* of enzymes or regulators (Deutscher et al., 2014a). The last few years have seen significant developments in the techniques of metabolomics, that is, the ability to accurately measure the intracellular concentrations of a large number of metabolites within the cell. Improvements in mass spectrometry resolution and sampling techniques mean that the number of compounds identified and the time taken to identify them have improved considerably (Bennett et al., 2009; Zamboni et al., 2015; de Raad et al., 2016). These have allowed the comparison of the pathways of carbon metabolism under many different conditions. The use of isotope labeling, such as ^{13}C-isotopologue profiling (Eisenreich et al., 2010), has allowed real-time analysis of bacterial cultures undergoing a nutrient or environmental switch (Link et al., 2015).

It has long been known that many of the enzymes of glycolysis are allosteric, that is, their activity changes in response to the binding of a compound at a site other than the active site. Allosteric enzymes, such as phosphofructokinase, have been extensively studied in vitro, but visualizing allosteric effects in vivo are extremely rare. However, the application of rapid sampling metabolomics and advanced mathematical modeling have demonstrated the consequences of an allosteric transition in vivo and have identified potentially new allosteric regulations (Xu et al., 2012; Link et al., 2013). The current consensus of opinion is that transcriptional regulation allows (long-term) adaption to changing environmental conditions, whereas the posttranslational and allosteric regulations are (short-term) fixes to maintain flux balance (Kochanowski et al., 2015). Together, the cell has integrated these multiple controls to allow balanced growth. A major aim of the systems biology approach is to describe this complex regulation in terms of mathematical models of glycolysis or even cellular metabolism (O'Brien et al., 2013; Link et al., 2014; Kremling et al., 2015; Huan et al., 2017).

Carbon Assimilation Pathways: Central Carbon Metabolism

Carbon assimilation can be divided into two levels: peripheral and central. The peripheral steps transform diverse and varied carbon sources into phosphorylated intermediates, which can then be channeled into one of the major routes for the dissimilation of carbon. These central routes are the three interconnecting pathways of glycolysis (EMP = Embden–Meyerhof–Parnas pathway), the Entner–Doudoroff (ED) pathway, and the pentose phosphate pathway, which all feed carbon into the TCA (Krebs) cycle (Fig. 1 and Table 1). There are, of course, exceptions, for example, lactic acid bacteria, which do not contain a complete TCA cycle, can normally only use carbohydrates via fermentation. The enzymes of central metabolism are historically some of the best studied. Together, these reactions generate energy (ATP), reducing power (NADH and NADPH), and, directly or indirectly, the precursor molecules for all the biosynthetic reactions. The 13 intermediates that are drawn away from the central pathways of carbon metabolism are glucose-6P (to be used to make sugar nucleotides), fructose-6P (for amino sugars), pentose phosphates (for various vitamins and cofactors, purine and pyrimidine nucleotides, histidine, and tryptophan), sedoheptulose phosphate (for heptoses in lipopolysaccharides), erythrose phosphate (for aromatic amino acids and some vitamins and cofactors), dihydroxyacetone phosphate (for nicotinamide coenzymes and phospholipids), 3-phosphoglycerate (for serine family amino acids, tryptophan, and purine nucleotides), phospho-*enol*-pyruvate (for various vitamins, cofactors, aromatic amino acids and peptidoglycan), pyruvate (pyruvate family amino acids), acetyl-CoA (for fatty acids, peptidoglycan, and leucine), oxaloacetate (aspartate family amino acids and pyrimidine nicotinamide coenzymes), 2-ketoglutarate (glutamate family amino acids, polyamines, and heme derivatives), and succinyl-CoA (for lysine family amino acids and heme derivatives).

Glycolysis, Entner–Doudoroff, and Pentose Phosphate Pathways

In glycolysis, six-carbon sugars are split into three-carbon compounds and then are converted into PEP (phospho-*enol*-pyruvate), pyruvate, and acetyl-CoA, generating ATP and NADH (Fig. 1). Glycolysis (EMP pathway) was considered the trunk route of sugar metabolism because it is the predominate route in the model organisms *E. coli* and *B. subtilis*. However, the relative importance of the EMP and ED pathways and the oxidative branch of the pentose phosphate pathway varies in different organisms. Quantitation of intracellular fluxes during growth on glucose of seven bacterial species showed that the relative use of the different pathways of central metabolism varied considerably (Fuhrer et al., 2005). In the ED route, glucose is converted to gluconate-6P using the

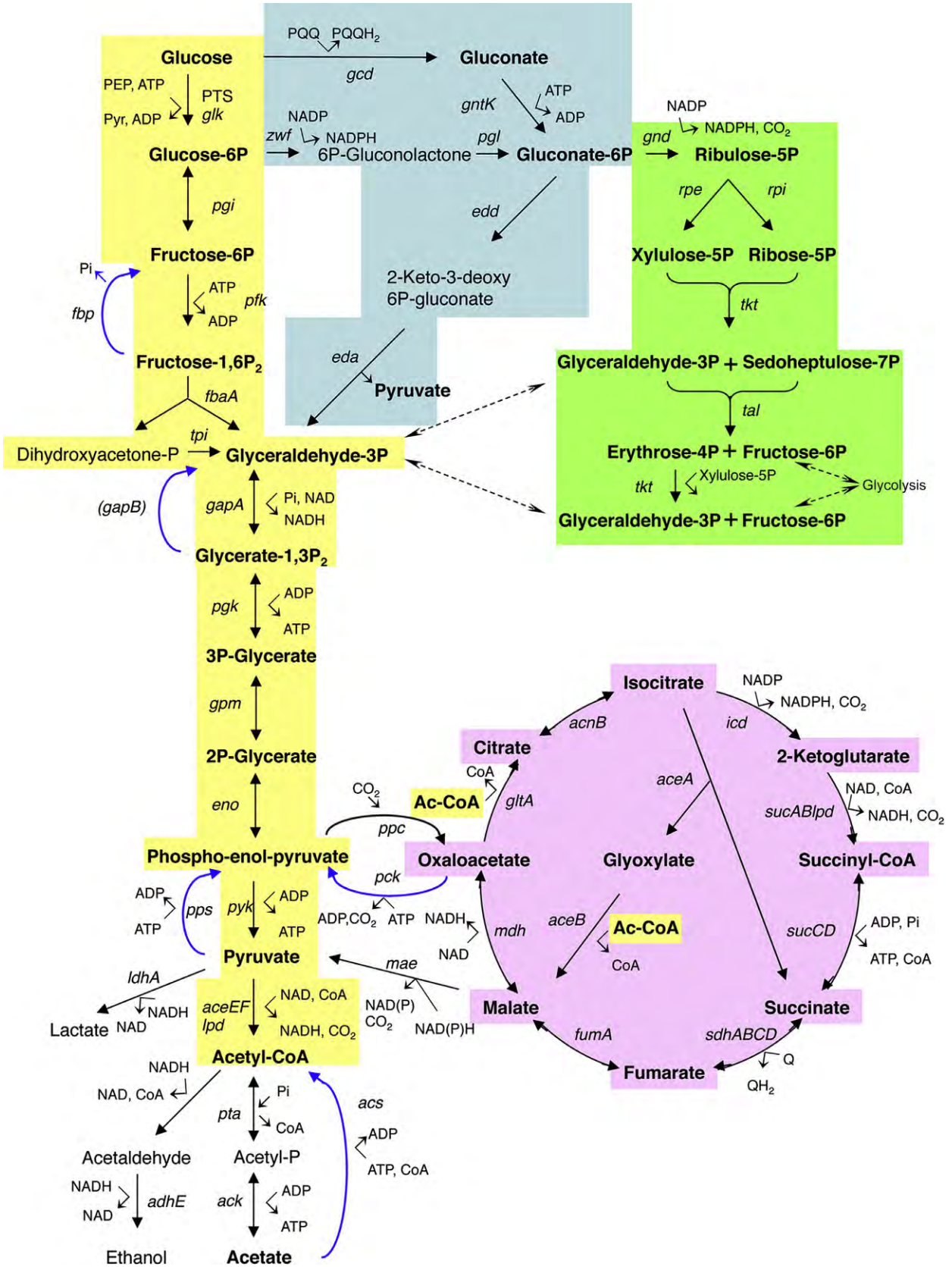

Fig. 1 Pathways of central carbon metabolism. Routes of glucose use via glycolysis (*shaded yellow*), Entner–Doudoroff (*blue*), pentose phosphate pathway (*green*), and TCA cycle (*pink*) are shown. Reactions specific to gluconeogenesis are shown with *blue arrows*. The enzymes encoded by the different genes are listed in Table 1. The pathways shown are those of *E. coli*, and certain specific differences existing in *B. subtilis* are indicated in Table 1. The *gapB* gene for gluconeogenic glyceraldehyde-3P dehydrogenase is only present in *B. subtilis*. The *ppc* gene does not exist in *B. subtilis*, anaplerosis occurs via *pycA,* encoding pyruvate carboxylase, which forms oxaloacetate from pyruvate.

Table 1 The genes and enzymes of central carbon metabolism

Gene name		
E. coli	B. subtilis	*Enzyme*
Glycolysis		
glk	*glcK*	Glucokinase
pgi		Phosphoglucose isomerase
pfk	*pfkA*	Phosphofructose kinase
fbaA		Fructose-1,6 aldolase
tpi		Triose phosphate isomerase
gapA		Glyceraldehyde-3P dehydrogenase
pgk		Phosphoglycerate kinase
gpmA	*pgm*	Phosphoglycerate mutase
eno		Enolase
pyk		Pyruvate kinase
aceEF lpd	*pdhABCD*	Pyruvate dehydrogenase
Entner-Doudoroff pathway		
gcd	–	Glucose dehydrogenase (PQQ[a])
gntK		Gluconokinase
zwf		Glucose-6P dehydrogenase
pgl (*ybhE*)	*ykjB*[b]	Phosphogluconolactonase
edd	–	Phosphogluconate dehydratase
eda	(*kdgA*)[c]	2-Keto-3-deoxygluconate-6P aldolase
Pentose phosphate pathway		
gnd	*gndA* (*yqjI*)[d]	Gluconate-6P dehydrogenase
rpe		Ribulose-5P epimerase
rpi	*ywlF*[e]	Ribulose-5P isomerase
tkt		Transketolase
tal	*ywjH*[e]	Transaldolase
TCA cycle and glyoxylate shunt		
ppc	–	PEP carboxylase
–	*pycA*	Pyruvate carboxylase
gltA	*citZ* (*citA*)	Citrate synthase
acnB	*citB*	Aconitase
icd	*citC*	Isocitrate dehydrogenase
sucAB lpd	*odhABpdhD*	2-Ketoglutarate dehydrogenase
sucCD		Succinyl-CoA synthetase (succinate thiokinase)
sdnCDAB	*sdhCAB*	Succinate dehydrogenase
fumA	*citG*	Fumarase
mdh	*citH*	Malate dehydrogenase
maeB maeA (*sfcA*)	*ytsJ*[f]	Malic enzyme
aceA	–	Isocitrate lyase
aceB	–	Malate synthase
aceK	–	Isocitrate dehydrogenase kinase/phosphatase
Gluconeogenesis and growth on acetate		
pps		PEP synthetase
fbp		Fructose bisphosphatase
pck	*pckA*	PEP carboxykinase
acs	*acsA*	Acetyl-CoA synthetase
–	*gapB*	Glyceraldehyde-3P dehydrogenase
Overflow metabolism		
pta		Phosphotransacetylase
ack	*ackA*	Acetate kinase
ldhA		Lactate dehydrogenase
adhE	*adhA*	Alcohol dehydrogenase

The gene names for the enzymes in *E. coli* are given in the first column. When different, the *B. subtilis* name is given in the second column. A *dash* indicates that the enzyme is not found in either of the bacteria. Isozymes exist for many genes, which are active under different conditions, for example, aerobic, anaerobic, oxidative stress, stationary phase, etc. The name of the gene encoding the predominant enzyme active during aerobic exponential growth is given.

[a] *PQQ*, Pyrroloquinoline-quinone dependent.

[b] Plata et al., 2012.

[c] Part of the *kdgRKAT* operon.

[d] Other paralogues (GntZ, YqeC) exist, but YqjI (renamed GndA) is the major enzyme required for growth on glucose and gluconate (Zamboni et al., 2004).

[e] Indicates putative identification based on homology.

[f] YtsJ (NADPH-dependent) is required for growth on malate. Three other paralogous genes (NADH-dependent) encoding malic enzymes also exist in *B. subtilis maeA, malS, mleA* (Lerondel et al., 2006).

enzymes Glc6P dehydrogenase (*zwf*) and phosphogluconolactonase (*pgl*) and then dehydrated to 2-keto-3-deoxygluconate (*edd*), which is cleaved into glyceraldehyde-3P and pyruvate (*eda*). In *E. coli*, sugar acids such as gluconate and galacturonate, derived from mammalian and plant tissues, are processed via the ED pathway. The ED route is absent in *B. subtilis* but in many other bacteria it is the major route for glucose metabolism. In some bacteria, such as the xenobiotic-degrading *Pseudomonades*, the ethanol-producing *Zymomonas mobilis*, and the human pathogen *Neisseria meningitides*, there is no *pfk* gene for phosphofructose kinase, a key enzyme of glycolysis, implying that glucose is processed through the ED pathway. It has been hypothesized, based on phylogenetic analysis, that the ED route is the original route to degrade glucose (Romano and Conway, 1996; Flamholz et al., 2013). The oxidative part of the pentose phosphate pathway uses the same first two enzymes (*zwf* and *pgl*) as the ED route does in forming gluconate-6P, which is then decarboxylated to ribulose-5P, generating NADPH. A series of transketolase and transaldolase reactions produces sugar phosphate precursors (for the biosynthesis of aromatic amino acids, vitamins, and nucleotides) and ultimately glyceraldehyde-3P and fructose-6P (Fig. 1). A recent metabolic flux analysis of glucose use by *P. putida* has confirmed the dominance of the ED route but also has shown that *P. putida* processes 10% of glucose via the pentose-phosphate route and that a significant part of the glyceraldehyde-3P generated is recycled back to hexose-P (gluconeogenesis) via the upper part of the EMP pathway (the EDEMP route). This is proposed to facilitate maintaining a correct NADPH/NADP balance (Nikel et al., 2015) (see "Balancing the Redox Potential, NADPH/NADH" section).

Glycolysis and Gluconeogenesis

During rapid growth on glycolytic sugars such as glucose, *E. coli* or *B. subtilis* generate most of the energy they need by substrate-level phosphorylation. A part of the carbon flux is directed into the TCA cycle to generate 2-ketoglutarate and oxaloacetate (anaplerosis), while the excess carbon is excreted as acetate (a process normally called *overflow metabolism*). When all the glucose is used, the acetate can be reabsorbed and used to generate acetyl-CoA. This has been called the "acetate switch," when the bacteria switches its enzymatic machinery from excreting acetate to using acetate for growth (Wolfe, 2005). Acetate and other carbon sources that generate acetyl-CoA feed into the TCA cycle so that for bacteria growing on acetate or dicarboxylic acids, the TCA cycle is used to generate energy. A part of the carbon supply must return backward up the glycolysis pathway, in the process called gluconeogenesis, to produce the sugar phosphates required for the synthesis of sugar nucleotides and other precursors to cell-wall and membrane components (El-Mansi et al., 2006).

The majority of the reactions of glycolysis are reversible, but some are irreversible, and specialized enzymes exist to catalyze the reverse gluconeogenic reactions (Fig. 1, *blue arrows*). Phosphofructokinase (*pfk*) is a classic allosteric enzyme, inhibited by PEP and activated by nucleoside diphosphates (ADP), thus signaling the need for more or less energy generation. Fructose bisphosphatase (*fbp*) performs the reverse reaction; it is inhibited by AMP, activated by PEP, and is active during growth on gluconeogenic carbon sources. Other enzyme pairs performing reverse reactions are pyruvate kinase (*pyk*) generating pyruvate in glycolysis and PEP synthetase (*pps*) synthesizing PEP during gluconeogenesis. In *B. subtilis*, the glyceraldehyde-3P dehydrogenase reaction is also catalyzed by two enzymes, encoded by *gapA*, using NAD as the cofactor, for the glycolytic direction and *gapB* (cofactor NADP) for the gluconeogenic step (Fillinger et al., 2000). In *E. coli*, the *gapA* enzyme functions in both directions. A gene for a highly homologous enzyme exists in *E. coli* and was initially suspected to be another phosphorylating glyceraldehyde-3P dehydrogenase because it is located in an operon with two other glycolytic enzymes, *pyk* and *fbaA*. However, it was shown to be a nonphosphorylating erythrose-4P dehydrogenase (*epd*), using NADP as reductant, and to be part of the biosynthetic pathway toward chorismate.

In both *E. coli* and *B. subtilis*, control mechanisms exist to direct the carbon flow in the glycolytic or gluconeogenic direction. In *B. subtilis*, the genes for the lower steps of glycolysis *gapA*, *pgk*, *tpi*, *pgm*, *eno* are arranged in an operon controlled by the product of the first gene of the operon, *cggR* (*c*entral *g*lycolytic *g*enes *r*epressor). Transcriptome analysis suggested that CggR has only one target and is a dedicated regulator of the *gapA* operon. Its primary inducer is Fru1,6P (Doan and Aymerich, 2003). The *B. subtilis gapA* operon is also controlled by CcpA, the global catabolite regulator in Gram-positive bacteria (see "Catabolite Repression and Regulation by CcpA and the *cre* Element" section). The expression of the *B. subtilis* genes specific for gluconeogenesis, *gapB*, and *pckA*, is very low in the presence of glucose but is high during growth on succinate. Repression is due to the DNA-binding protein, CcpN (*c*atabolite *c*ontrol *p*rotein of gluco*n*eogenic genes). GapA is also regulated indirectly by CcpN under gluconeogenic conditions. The dual function sRNA, SR1, encodes a small peptide, SR1P, which binds the GapA protein and results in a stabilization of the *gapA* mRNA by an as-yet unknown mechanism. The expression of the SR1 RNA is repressed by CcpN, and so SR1P is only active under gluconeogenic conditions (Gimpel and Brantl, 2016). The loss of *ccpN* impairs growth on glucose since the high levels of PEP carboxykinase (PckA) provoke a futile cycle through pyruvate kinase (Pyk) and pyruvate carboxylase (PycA) (Tannler et al., 2008b).

In *E. coli*, the expression of several genes of glycolysis has been shown to increase during growth on glucose, for example, *gapA*, *pgk*, and *fbaA*. The FruR repressor of the fructose PTS, in addition to repressing the *fruBKA* operon 20-fold, more weakly represses the *epd pgk fbaA* operon and other catabolic genes such as *pfk*, *edd*, *eda*, and *ptsH* (part of the PTS, see "The Bacterial Phosphotransferase System" section). FruR also acts as an activator of the *pps* gene (encoding PEP synthetase), some other genes of gluconeogenesis (*pckA*, *fbp*), and the TCA cycle genes (*aceBA*, *icd*). Genomic selex experiments have further increased the number of binding sites (Shimada et al., 2011b). The binding of FruR is inhibited by fructose phosphates, especially Fru1P, but also Fru1,6P, which signals the activity of the glycolytic pathway (Kochanowski et al., 2013b). FruR thus plays a pleiotropic role in directing the carbon flow in response to metabolic signals, and it was renamed Cra (catabolite repressor activator) to reflect

this function. Allosteric regulation also plays a role in directing the flux by the opposing reactions of phosphofructokinase (*pfk*), inhibited by PEP and fructose 1,6-bisphosphatase (*fbp*), activated by PEP. As mentioned earlier, the extensive data generated by high-throughput metabolomics and transcriptomics studies are providing compelling evidence for the importance of these and other posttranslational regulatory mechanisms in maintaining a balanced microbial metabolism (Chubukov et al., 2014; Kochanowski et al., 2015).

The PEP–Pyruvate–Oxaloacetate Node

As in the case of the glycolytic pathway, different sets of enzymes control the direction of the carbon flow between glycolysis and the TCA cycle, which has been called the PEP–pyruvate–oxaloacetate node (Sauer and Eikmanns, 2005). PEP carboxylase (*ppc*) catalyzes the anaplerotic formation of oxaloacetate from PEP and carbonate, whereas PEP carboxykinase (*pck*) carries out the reverse reaction, conversion of oxaloacetate to PEP (Fig. 1). Bacteria with *ppc* mutants require a TCA cycle intermediate (succinate) for growth on sugars to replenish the TCA cycle. PEP carboxylase is a tetrameric allosteric enzyme with many effectors including acetyl-CoA, Fru1,6P, GTP, and long-chain fatty acids (positive), and aspartate and malate (negative). *B. subtilis* does not possess a PEP carboxylase but replenishes the TCA cycle from glycolysis by forming oxaloacetate using pyruvate carboxylase (*pycA*). Pyruvate carboxylase is activated by acetyl-CoA. An exchange between TCA cycle and glycolysis can also occur at the level of pyruvate and malate, catalyzed by so-called malic enzymes, of which several isozymes exist: two in *E. coli* and four in *B. subtilis*. They differ in their cofactor requirements, NAD or NADP. In *E. coli*, both enzymes are active during growth on dicarboxylic acids. In *B. subtilis*, the NADP-linked enzyme (YtsJ) is particularly required for growth on malate (Lerondel et al., 2006). This is most likely related to maintaining the correct NADPH balance. The expression of the operon for the pyruvate dehydrogenase complex (*aceEFlpd*), which transforms pyruvate into acetyl-CoA, is repressed by the pyruvate-sensing repressor, PdhR, a GntR family repressor. PdhR also controls some genes of the respiratory electron transfer system in *E. coli* (Ogasawara et al., 2007), whereas in *B. subtilis*, it is the stringent control mechanism dependent on the ATP/GTP ratio that controls expression of the *pdhABCD* operon, as well as several other glycolytic enzymes (Tojo et al., 2010).

The TCA Cycle and Glyoxylate Shunt

The TCA cycle completes the oxidation of acetyl-CoA produced by glycolysis and provides intermediates for biosynthesis of aspartate, glutamate, and their derivatives. In fact, the TCA cycle rarely runs as a cycle but mostly as two arms producing succinyl-CoA and 2-ketoglutarate from oxaloacetate. During growth on acetate or fatty acids, it runs as two cycles including the glyoxylate shunt (El-Mansi et al., 2006).

The glyoxylate shunt represents a bifurcation of the pool of isocitrate directly toward malate and succinate rather than via 2-ketoglutarate, because the latter route involves two irreversible decarboxylations and hence depletion of the TCA intermediates (Fig. 1). The direction of carbon flow from isocitrate to glyoxylate or to 2-ketoglutarate is decided by the relative activities of isocitrate dehydrogenase (*icd*) and isocitrate lyase (*aceA*). The activity of isocitrate dehydrogenase is strongly downregulated by reversible phosphorylation carried out by the Icd kinase/phosphatase enzyme (IcdK/P), encoded by *aceK*, which was the first ATP-dependent protein kinase identified in bacteria (Cozzone and El-Mansi, 2005). This is the third gene of the inducible *aceBAK* operon, where *aceB* and *aceA* encode the two enzymes of the glyoxylate shunt. All three genes are induced by growth on acetate and repressed by growth on glucose, coordinating a simultaneous decrease in Icd activity and an increase in the glyoxylate shunt or vice versa. A specialized repressor, IclR, whose inducing signal appears to be PEP rather than acetate, controls the operon (Molina-Henares et al., 2006).

The genes of the TCA cycle are controlled by several global regulatory circuits. They are subject to CCR, repressed during growth on glucose, and their expression is dependent on the cAMP/CAP complex in *E. coli* (see "cAMP/CAP Regulation of Transcription Initiation" section). Isozymes for several of the enzymes exist and are subject to different regulations, for example, *fumA* and *acnB* encode the major aerobic fumarase and aconitase activities, whereas *fumC* and *acnA* are expressed under conditions of oxidative or iron stress from an *rpoS*-dependent promoter [*rpoS* encodes σ^S (σ^{38}), the stationary phase/stress-related sigma factor]. Similarly, there are two genes for transketolase; *tktA*, encoding the major isozyme in exponential growth, is repressed in stationary phase when *tktB* is induced. RpoS is implicated in both regulations. The ArcA/ArcB redox-sensing two-component system is responsible for the repression of the TCA cycle genes in anaerobic and microaerobic conditions, but mutations in *arcA* also increase the flux through the TCA cycle during aerobic growth on glucose (Perrenoud and Sauer, 2005).

Succinate dehydrogenase (*sdhCDAB*) is the only membrane-bound member of the TCA cycle and produces a reduced quinone to be used by the respiratory chain. It contains a $[4Fe–4S]^{2+}$ cluster. Aconitase (*acnA* and *B*) and fumarase (*fumA* and *B*) are also Fe–S enzymes. The Fur protein (iron-specific repressor) was known to positively control the expression of all three enzymes such that *fur* mutants do not grow on succinate. The explanation for this unexpected behavior came with the discovery of the RhyB sRNA. Synthesis of the RhyB RNA is under negative control by Fur so that it is only synthesized when intracellular iron is low. The sRNA, by binding to the *sdhCD* mRNA, initiates RNase E-dependent degradation of the *sdh* mRNA, thus lowering the intracellular content of succinate dehydrogenase (and other nonessential iron proteins) and liberating iron when iron is scarce (Massé et al., 2007). In addition, scarcity of iron is thought to destroy the Fe–S clusters in aconitases. Apo-aconitases, from both bacteria and vertebrates, are mRNA-binding proteins and have been shown to repress

the translation of their own mRNA, thus constituting a double regulation to conserve precious iron supplies (Hantke, 2002). A similar iron-sparing response is found in *B. subtilis*, where Fur represses expression of a sRNA, FrsA, with global effects on TCA cycle function (Smaldone et al., 2012).

In *B. subtilis*, CcpC, a LysR-type regulator, represses the *citZCH* operon (encoding citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase) and *citB* (aconitase) and its own gene (Sonenshein, 2007). The genes of the glyoxylate shunt are missing in *B. subtilis* and in some related species. These bacteria cannot grow on acetate as the sole carbon source. Modified TCA cycles are also found in some other bacteria, such as the γ-aminobutyric acid shunt in the cyanobacteria, *Synechocystis* (Xiong et al., 2014).

Balancing the Redox Potential, NADPH/NADH

A general rule is that catabolic enzymes are coupled to NAD reduction, whereas reactions forming part of anabolic pathways use NADP. Thus the *B. subtilis* GapA enzyme uses NAD while the gluconeogenic enzyme, GapB, uses NADP. The major routes for generating the anabolic reductant NADPH were thought to be via the pentose phosphate pathway and isocitrate dehydrogenase, but in *E. coli*, a membrane-bound proton-translocating transhydrogenase (PntAB) accounts for at least one-third of the cellular NADPH generated from NADH during growth on glucose. A second soluble transhydrogenase (UdhA) seems dedicated to the reverse reaction, generating NADH from NADPH under conditions when NADPH is high (growth on acetate or on sugars metabolized via the pentose phosphate pathway). The expression and/or activity of these enzymes must be carefully controlled to eliminate a futile cycle dissipating NADPH (Haverkorn van Rijsewijk et al., 2016). As the two enzymes have only been detected in enterobacteria, alternative mechanisms must exist in other bacteria to balance their redox potential. In *B. subtilis*, two transhydrogenation cycles, constituted by NADH- and NADPH-dependent isozymes (GapA, GapB and Malic enzymes, MalS, YtsJ), could be responsible for redox balancing (Ruhl et al., 2012).

Uptake of Carbon Sources From the Medium

Sugar-specific utilization operons generally encode two types of enzymes: those involved in the transport of (usually) hydrophilic carbohydrates across the inner membrane and those encoding the enzymes that transform the more exotic starting materials into one of the compounds of the central carbon degradation pathway. For some (larger) carbon sources, a specific outer membrane porin is also required (e.g., LamB for maltose and maltooligosaccharides, or ChiP for chitobiose and chitotriose). The transporters belong to many protein families and are of many types, including H^+ symporters (e.g., LacY where lactose uptake is associated with proton uptake), ATP-binding cassette (ABC)-type transporters (e.g., MalFGK for maltose uptake), and facilitated diffusion (e.g., GlpF, for glycerol uptake). These systems transport the sugars unchanged so that inside the cell they must be phosphorylated by specific kinases.

Another important route is the phosphotransferase system (PTS), which is widely distributed in bacteria. In this group translocation mechanism, sugar transport across the inner membrane is associated with the concomitant phosphorylation of the sugar as it enters the cytoplasm. The phosphate is derived from PEP and passes via a protein phosphorylation cascade to the incoming sugar (Fig. 2). The proteins of the phosphotransferase system are intimately involved in CCR in both Gram-positive and Gram-negative bacteria, although the mechanisms of their action are fundamentally different. Thus we will first describe the PTS as a sugar transporter and then deal with CCR in Gram-negative and in Gram-positive bacteria (Deutscher et al., 2006). In many cases, the transport proteins are intrinsic parts of the machinery for the regulation of their own expression (Vastermark and Saier Jr., 2014), as in the case of PtsG by Mlc in *E. coli*, as discussed later.

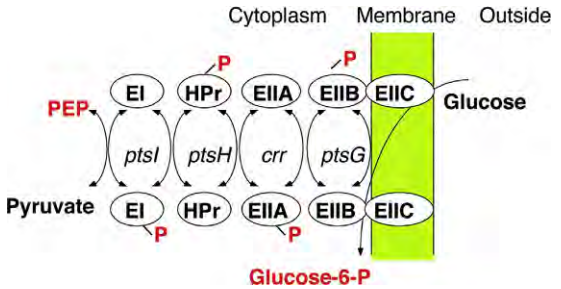

Fig. 2 The glucose PTS in *Escherichia coli*. The phosphate from PEP is passed via a series of cytoplasmic proteins EI (*ptsI*), HPr (*ptsH*), and $EIIA^{Glc}$ (*crr*) to the glucose-specific transporter $EIICB^{Glc}$ (*ptsG*). Passage of glucose across the membrane via the $EIIC^{Glc}$ domain results in its simultaneous phosphorylation by $EIIB^{Glc}$ to give cytoplasmic Glc6P.

The Bacterial Phosphotransferase System

The basic PTS consists of two cytoplasmic proteins called Enzyme I (EI, gene *ptsI*) and HPr (*ptsH*) that are common to nearly all sugars and which pass a phosphate derived from PEP to the sugar-specific transporters, called Enzymes II (EII) (Fig. 2). The EIIs are multidomain proteins where the three domains, EIIA, EIIB, and EIIC, can be found on one, two, three, or, occasionally, four polypeptide chains. EIIA and EIIB are soluble cytoplasmic proteins, whereas EIIC is an integral membrane protein that serves as the passage for the sugar through the membrane.

EI autophosphorylates on a histidine residue and then passes the phosphate to the small protein HPr. HPr distributes phosphates to the EIIA domain of any of the sugar-specific EII transporters within the cell. From EIIA, the phosphate is transferred to EIIB, another soluble domain, but this one is associated with the cytoplasmic side of the inner membrane. From EIIB, the phosphate is transferred to the sugar after it has been transported by the EIIC domain across the cytoplasmic membrane by a so-called "elevator" mechanism (McCoy et al., 2016). An alternative "rigid body rotation" mechanism has been proposed for the ascorbate PTS transporter (Luo et al., 2015). The phosphate is carried by a histidine residue in EI, HPr, and EIIA but usually by a cysteine in most EIIB domains. In the resting state, that is, in the absence of a PTS sugar, the PTS proteins are predominately phosphorylated. It should be noted that these phosphate bonds are relatively labile because they are of high and similar-standard free energies, so that the phosphates can be easily passed between different EIIs via the HPr protein.

For the glucose PTS of *E. coli* (Fig. 2), $EIIC^{Glc}$ and $EIIB^{Glc}$ are present on the same polypeptide, encoded by the gene *ptsG*. The $EIIA^{Glc}$ protein is a separate polypeptide, encoded by the gene *crr* (for *c*arbohydrate *r*epression *r*esistant), a name that reflects the primordial role of $EIIA^{Glc}$ in CCR in *E. coli* (see "Catabolite Repression in Gram-Negative Bacteria: *E. coli*" section). In fact, the gene *crr* is part of the *ptsHIcrr* operon, encoding the common components of the PTS, whereas *ptsG* is expressed as a single gene elsewhere on the chromosome. The PTS proteins are modular proteins par excellence. The structural genes can be recognized in bacterial genomes by homology-scoring programs, and the EIIs have been divided into several subfamilies, but the arrangement of their domains both within the polypeptides and at the genetic level can vary enormously (Barabote and Saier, 2005). For example, in *E. coli*, the *nagE* gene (with domain order $EIICBA^{Nag}$) encodes the *N*-acetylglucosamine (GlcNAc) transporter whereas the homologous β-glucoside transporter, encoded by *bglF*, has the domains arranged as $EIIBCA^{Bgl}$. The fructose PTS in *E. coli* does not use HPr but has a specialized fusion protein, FPr, including an HPr domain and an $EIIA^{Fru}$ domain. Other unusual fusion proteins exist between both PTS and non-PTS domains, as in the nontransporting dihydroxyacetone kinase system (Gutknecht et al., 2001). Although PTS proteins can be easily identified by sequence homology, it is not usually obvious to predict their sugar substrates. A ground-breaking multiplex mutagenesis protocol (MUGENT) in *Vibrio cholerae* generated a strain lacking 13 PTS operons and enabled assignment of transported sugars to individual transporters (Hayes et al., 2017). The genes for the EII transporter are often associated in operons with genes encoding the enzymes required for further metabolism of their substrate and the transcription factor controlling their expression.

Catabolite Repression in Gram-Negative Bacteria: *E. coli*

The key molecule in CCR in *E. coli* is the $EIIA^{Glc}$ protein. At least two regulatory circuits are in action during CCR by glucose. First, glucose reduces the concentration of cAMP/CAP and so decreases the transcription of target genes, and second, it causes inducer exclusion by preventing the uptake of other carbon sources, and hence prevents induction of the genes required for their metabolism (Deutscher et al., 2006). Both activities depend on $EIIA^{Glc}$, but their relative contributions to CCR have been the subject of much debate (e.g., Inada et al., 1996; Crasnier-Mednansky, 2008; Görke and Stülke, 2008). There have been many attempts to produce mathematical models of CCR in *E. coli* (e.g., Kremling et al., 2015).

Inducer Exclusion

Mutations in EI or HPr prevent the use of both PTS and non-PTS carbohydrates. Mutations called *crr* (carbohydrate repression resistant) were isolated as suppressors, which restored growth on a range of non-PTS sugars. The *crr* gene, in which the suppressors were mapped, encodes the EIIA component of the glucose PTS. As such, it can exist in two forms: phosphorylated and nonphosphorylated. These two forms were shown to have complementary roles in CCR. The nonphosphorylated form of EIIA, the form expected to predominate when the bacteria are growing on glucose, was shown to interact with and inhibit the activity of numerous transport systems for non-PTS sugars such as lactose, melibiose, raffinose, maltose, and also glycerol kinase (Fig. 3). Inhibition of the basal level of these transporters in the bacteria is responsible for the phenomenon of "inducer exclusion." Inhibiting the activity of the transporters for different carbon sources prevents the uptake of these alternative substrates and the induction of the genes required for their transport and metabolism. It has been reported that $EIIA^{Glc}$ interacts strongly with transporters only when their specific substrates are present, thus preventing the wasteful sequestration of $EIIA^{Glc}$ to unused transporters. The interaction between $EIIA^{Glc}$ and many of its targets has been demonstrated in vitro. The structures of $EIIA^{Glc}$ in complex with GlpK and MalK (Chen et al., 2013) have been determined. On the other hand, a novel interaction involving dephosphorylated HPr (rather than $EIIA^{Glc}$) and the mannitol repressor, MtlR, determines the preference of glucose over mannitol (another PTS sugar) in *E. coli* (Choe et al., 2017).

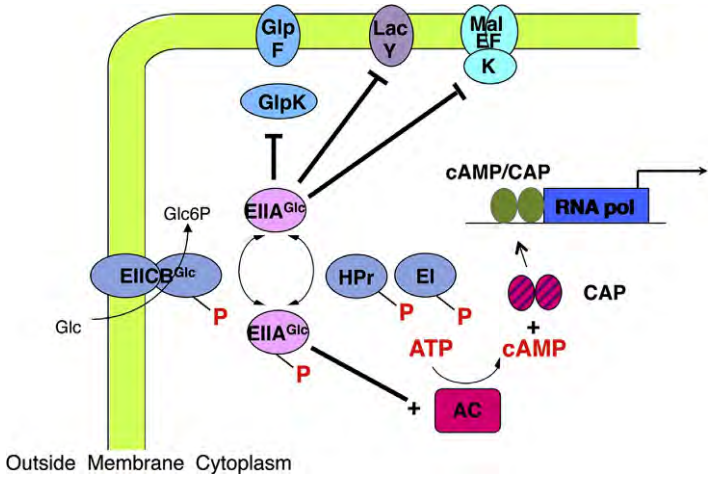

Fig. 3 Carbon catabolite repression in enteric bacteria: *E. coli*. Transport of glucose by the PTS and EIICB^{Glc} (PtsG) produces predominately unphosphorylated EIIA^{Glc}. The nonphosphorylated form of EIIA^{Glc} leads to inducer exclusion because it inhibits the transport activity of non-PTS transporters like LacY and MalEFK and the glycerol kinase enzyme, GlpK. In the absence of glucose, EIIA^{Glc} is mostly phosphorylated and in this form it allosterically activates the enzyme AC, thus increasing the concentration of cAMP, which forms a complex with CAP. Higher concentrations of cAMP/CAP allow RNA polymerase to bind and transcribe genes for use of alternative carbon sources, in the presence of their specific inducing signal.

Activation of AC

EIIA^{Glc} is also largely implicated in changing the functional concentration of the global activator cAMP/CAP. When available glucose is exhausted, EIIA^{Glc} becomes predominately phosphorylated and cAMP concentrations increase manyfold. This increase has been shown to be in the majority due to an increase in the activity of the enzyme adenylate cyclase (AC, encoded by the gene *cya*). Phosphorylated EIIA^{Glc} stimulates the enzymatic activity of AC 50-fold (Fig. 3). Deletion of the 48C-terminal amino acids from AC prevented this allosteric activation of cyclase activity without affecting the intrinsic basal activity. A small transcriptional effect of EIIA^{Glc}-P on *cya* gene expression has been observed (fourfold), but the major regulation is via the activity of the enzyme. Although the in vivo evidence was strong, it was only much later that the stimulation of AC activity by purified EIIA^{Glc} could be demonstrated in vitro. It required the addition of a crude *E. coli* extract, indicating that some other, so-far unidentified, factor is also required (Park et al., 2006). More recently, 2-ketoglutarate (2KG) and α-ketoacids in general have been implicated in inhibiting AC in vivo. Since 2KG is a key compound for amino acid biosynthesis and an indicator of the nitrogen status of bacteria, this important observation provides a way to coordinate carbon use with both the carbon and nitrogen requirements of the cell's proteome (in particular the ribosomal translational machinery) rather than just to the growth rate or carbon supply (You et al., 2013). This hypothesis led to the idea that α-ketoacids are the key catabolites linking CCR and carbon and nitrogen availability (Rabinowitz and Silhavy, 2013).

It has long been known that glucose is not the only sugar that produces catabolite repression in *E. coli*. Growth on other PTS sugars producing fast growth (e.g., *N*-acetylglucosamine or mannitol) also provokes dephosphorylation of EIIA^{Glc} via the reequilibration of phosphates between the different PTS components. In addition, certain non-PTS sugars, including glucose-6P, gluconate, and lactose, can exert repression on bacteria growing on carbon sources that are not as good (Hogema et al., 1997). The addition of these compounds to bacteria growing on lactate affects cAMP/CAP levels and provokes at least a partial dephosphorylation of EIIA^{Glc}. The level of dephosphorylation of EIIA^{Glc} seemed to correlate with the decrease in the PEP/pyruvate ratio, implying that products from the far end of the glycolysis route are involved in the sensing. It remains to be determined how transport of non-PTS sugars affects the PEP/pyruvate ratio (Hogema et al., 1998; Bettenbrock et al., 2007).

The *crp* and *cya* genes are also subject to multiple levels of regulation. *Crp* is autoregulated by cAMP/CAP, both negatively (at physiologically relevant cAMP concentrations), and positively (at very high concentrations), whereas *cya* is repressed by cAMP/CAP. How these controls are integrated with the EIIA^{Glc} regulation and whether other mechanisms are also implicated is not known. Although under extensive study since the discovery of cAMP in the 1960s, the molecular mechanisms governing the physiological control of cAMP/CAP levels inside the cell are still far from clear. Despite the presence of two cAMP phosphodiesterases, cytoplasmic CpdA and periplasmic CpdB, the majority of the cAMP synthesized by *E. coli* is found in the external medium. There are other unanswered questions. For example, why does Glc6P produce much stronger levels of repression than glucose (Hogema et al., 1997)?

cAMP/CAP Regulation of Transcription Initiation

Despite our still incomplete understanding concerning the physiology of cAMP/CAP, the molecular mechanisms of how cAMP/CAP activates transcription by RNA polymerase have been described in exquisite detail. CAP is undoubtedly the best-understood transcriptional activator (reviewed in Lawson et al., 2004). The crystal structures of the free protein and its co-complex with DNA are known, thus defining its interaction with the DNA operators. CAP operators are found in two distinct locations relative to the

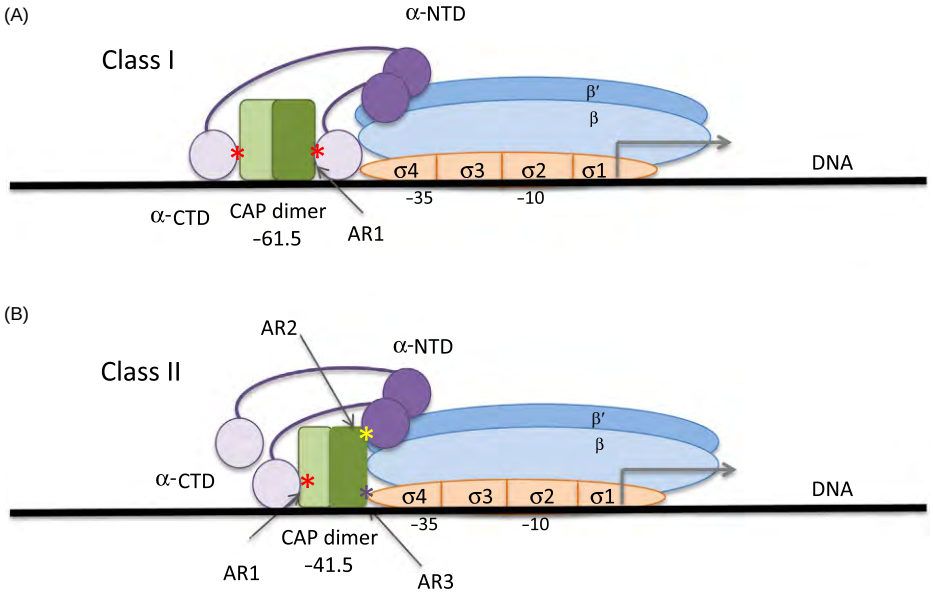

Fig. 4 Transcription activation by cAMP/CAP. (A) Activation at Class I CAP-dependent promoters. Ternary complex of CAP and RNA polymerase (RNAP) at a Class I CAP-dependent promoter having the DNA site for CAP centered at position −61.5 relative to the transcription start site. Transcription activation involves interaction between activating region 1 (AR1) on the downstream subunit of CAP (*dark green*) and one C-terminal domain of an α protomer (α-CTD) (*violet*) indicated by a *red star*. The AR1-α-CTD interaction facilitates the binding of α-CTD to the DNA segment downstream of region 4 of σ (*orange*). The second α-CTD protomer can also contact CAP via AR1 and interact nonspecifically with upstream DNA. (B) Activation at Class II CAP-dependent promoters with the CAP site at −41.5. Transcription activation involves three sets of CAP-RNAP interactions: interaction between AR1 of the upstream subunit of CAP (*light green*) and one α-CTD (*violet*), an interaction that facilitates binding of α-CTD to the DNA segment immediately upstream of CAP (*red star*); interaction between AR2 of the downstream subunit of CAP (*dark green*) and α-NTD (*purple*) (*yellow star*); and interaction between AR3 of the downstream subunit of CAP (*dark green*) and region 4 of σ (*purple star*). The second α-CTD protomer can interact nonspecifically with upstream DNA.

promoter. At so-called Class I CAP-dependent sites, the center of the CAP operator is positioned at about −61.5 or −72.5 upstream of the +1 transcription start site (Fig. 4A). At this position, the surface-exposed group of seven amino acids, called activating region I (AR1), on the downstream subunit of CAP makes a precise contact with a region in the C-terminal domain of the alpha subunit (α-CTD) of RNA polymerase (RNAP). α-CTD is attached to the N-terminal domain of the alpha subunit (α-NTD) by a long and flexible linker, allowing α-CTD to adopt various positions at the promoter. At Class I promoters it is probably tucked in between CAP and RNAP and in contact with the DNA and also in contact with the sigma subunit of RNA polymerase. It is presumed that the role of CAP (and transcription factors in general) is to provide an additional point of anchorage to increase the affinity of RNAP for the promoter; this is the classic recruitment model of transcription activation as originally proposed by Ptashne and Gann (1997).

At Class II CAP-dependent promoters, the CAP operator is centered at about −41.5 bp from the transcription start site and is nestled adjacent to the RNAP (Fig. 4B). At this position, at least three types of contact between RNAP and CAP have been detected. ARI is still active but this time it is the upstream subunit of CAP, which is in contact with α-CTD, now located on the DNA on the distal side of CAP. Two other activating regions on CAP have been demonstrated. AR2 on the downstream subunit interacts with amino acids from α-NTD, whereas AR3 contacts region 4 of σ^{70}. Contacts between CAP and RNAP at these two positions are thought to activate transcription by a postrecruitment mechanism, facilitating isomerization of the closed complex to yield the open RNAP–DNA complex (Lee et al., 2012).

The *crp* regulon is one of the most extensive and best characterized. More than 50 targets were identified by classical studies of individually regulated genes. Transcriptome studies of both glucose-grown bacteria and *crp* mutants expanded this list to at least 200 genes (Gosset et al., 2004). Glucose affected even more genes, both positively and negatively, and this is partly due to indirect effects. Precise identification of sites on the *E. coli* chromosome that bind CAP has been achieved by chromatin immunoprecipitation and microarrays (ChIP on chip analysis) (Grainger et al., 2005). This identified 68 targets of which only 29 corresponded to previously identified sites. An alternative technique using transcripts synthesized in vitro yielded nearly 200 targets of which about 40 were previously known (Zheng et al., 2004), whereas a genomic Selex approach predicted many more (Shimada et al., 2011a). These experiments have thus confirmed the vast extent of the *crp* regulon and provided valuable information for the study of the previously unidentified target genes.

Other Global Regulators of Carbon Use

As explained earlier, a specific regulator controls the genes for use of a particular sugar. Two of the repressors of PTS operons in *E. coli* have acquired a more global function. It is perhaps significant that they are the repressors for two of the most abundant sugars:

glucose (Mlc) and fructose (FruR, renamed Cra). The role of FruR in directing the flow of carbon in either the glycolytic or the gluconeogenic direction has been described previously (see "Glycolysis and Gluconeogenesis" section).

Mlc: The glucose PTS repressor

Mlc has been shown to repress several genes related to glucose use in *E. coli*. This includes *ptsG*, encoding the major glucose transporter, *ptsHI*, encoding the central components of the PTS, and *manXYZ*, encoding a second transporter capable of transporting glucose and other hexoses. All genes so far identified as being repressed by Mlc are also controlled by CAP, so that growth on glucose has two opposing effects: it relieves repression by Mlc but at the same time lowers cAMP/CAP concentrations, provoking catabolite repression. In the case of *ptsG* and *ptsH*, expression is greater during growth on glucose than on glycerol, but at other genes, like *manXYZ*, it is catabolite repression that dominates. It should be noted that even expression of *ptsG* requires a functional cAMP/CAP complex. There is no expression of *ptsG* in a *cya* mutant (i.e., in the complete absence of functional cAMP/CAP) but the *ptsG* promoter can work with the reduced level of cAMP/CAP present in the cell during growth on glucose.

The PTS is remarkable for the large number of regulatory functions exerted by its constituent proteins involving protein–protein interactions (Galinier and Deutscher, 2017), and PtsG is another example. Induction of Mlc-repressed genes is not due to a small molecule signal, as in the case of most prokaryotic transcription factors, but results from Mlc sequestration to membrane-bound PtsG. Mlc only interacts with dephosphorylated PtsG, which is the predominant form when PtsG is actively transporting glucose (Plumbridge, 2002). Sites on both Mlc and $EIIB^{Glc}$ that prevent this interaction have been identified by mutagenesis (Seitz et al., 2003; Pennetier et al., 2008), and a cocrystal structure of Mlc and $EIIB^{Glc}$ is available (Nam et al., 2008). The membrane location is an essential part of the inactivation of Mlc; the soluble $EIIB^{Glc}$ domain is not sufficient even though it binds Mlc. The exact role of the membrane is still unknown. It has been suggested that contact with the membrane reduces Mlc flexibility so that it can no longer bind DNA (Nam et al., 2008).

The SgrS/SgrT novel sRNA/small protein pair

The *ptsG* gene is also subject to posttranscriptional regulation. The *ptsG* mRNA is rapidly degraded during conditions of phosphosugar stress (e.g., in a *pgi* mutant growing on glucose) (Morita et al., 2003). The sugar stress initiates the synthesis of a transcriptional activator (SgrR) that promotes the synthesis of an sRNA, SgrS (*s*ugar-*r*elated *s*RNA). SgrS, in the presence of Hfq, binds to the *ptsG* mRNA and prevents its translation, which in turn initiates *ptsG* mRNA degradation by RNase E (Bobrovskyy et al., 2015). The logic of this posttranscriptional regulation is to turn down expression of PtsG and to prevent the accumulation of toxic sugar phosphates. In fact, the SgrS RNA promotes a second and even more rapid response because, unlike most other sRNAs, it is a dual function sRNA that also encodes a small polypeptide, SgrT, which inhibits PtsG transport activity (Kosfeld and Jahreis, 2012; Lloyd et al., 2017). Other targets for SgrS have been identified, including repression of an alternative glucose transporter encoded by the mannose PTS (*manXYZ*) and activation of a sugar phosphatase (*yigL*) facilitating the removal of the stressful sugar phosphates (Bobrovskyy et al., 2015; Papenfort and Vanderpool, 2015).

The carbon storage regulator, CsrA

Another regulatory system employing sRNA and affecting carbon use is the RNA-binding protein, CsrA, and its two sRNA "decoy" regulators, CsrB and CsrC. CsrA represses translation of the *glgC* mRNA for glycogen biosynthesis and represses or activates several key enzymes of central carbon metabolism. It thus participates in the switch from glycolysis to gluconeogenesis in stationary phase. The impact of CsrA during growth on glucose has been investigated using a multilevel approach (flux analysis, metabolomics, transcription, and enzyme assays), which has demonstrated that the main CsrA function is regulation of the upper glycolysis pathway and pinpointed *pfk*, encoding phosphofructokinase, as its major target (Morin et al., 2016). CsrB and CsrC are noncoding RNA carrying many copies of the CsrA RNA-binding site, which titrate CsrA. An increase in CsrB and CsrC in stationary phase results in the sequestration of CsrA by the sRNA and expression of CsrA-repressed genes. Expression of *csrB* and *csrC* is activated by the BarA/UvrY two-component system and repressed by cAMP/CAP (Pannuri et al., 2016). The levels of CsrB and CsrC are controlled by the CsrD protein, which targets the sRNAs to RNase E for degradation. CsrD has been identified as yet another target of $EIIA^{Glc}$. CsrD is activated by interaction with dephosphorylated $EIIA^{Glc}$, which decreases CsrB and CsrC levels in response to glucose transport; this interaction fine tunes CsrA regulation to PTS activity (Leng et al., 2016; Perez-Morales and Bustamante, 2016). CsrA homologues exist in numerous bacteria and have been implicated in motility, virulence, biofilm formation, and quorum sensing (Romeo et al., 2013).

The nitrogen PTS

In addition to the sugar-transporting PTS, there is a series of paralogous PTS proteins, equivalents of EI, HPr, and EIIA that are not involved in sugar uptake, in *E. coli* and a variety of other Gram-negative bacteria (Peterkofsky et al., 2006). The three proteins coded by *ptsP* (EI^{Ntr}), *ptsO* (NPr), and *ptsN* ($EIIA^{Ntr}$) lack any equivalent of the sugar-specific EII transporters and are proposed to have purely regulatory functions. Because *ptsO* and *ptsN* are usually localized with the *rpoN* gene, encoding σ^{54}, the nitrogen-specific sigma factor, they were termed the nitrogen or Ntr PTS and it was postulated that they have a role in coordinating the use of different carbon and nitrogen sources. The EI^{Ntr} protein has an additional N-terminal GAF domain that is not present in the sugar PTS homologue. GAF domains are found in a range of regulatory proteins and are implicated in signal perception for a variety of small molecules (including cyclic dinucleotides and 2-ketoglutarate). The presence of this conserved domain in the EI^{Ntr} proteins is

consistent with the proposed pleiotropic regulatory functions attributed to the Ntr PTS ranging from growth defects on certain carbon sources to leucine sensitivity and regulation of intracellular K^+ levels (Pflüger-Grau and Görke, 2010).

It is now well documented that $EIIA^{Ntr}$ controls K^+ uptake by two mechanisms. First, dephosphorylated $EIIA^{Ntr}$ interacts with and inhibits the activity of the low-affinity Trk K^+ transporter (Lee et al., 2007) and at the same time activates the expression of the high affinity KdpFABC transporter by stimulating the sensor kinase KdpD of the KdpDE two-component system in response to K^+ availability (Lüttmann et al., 2009). As K^+ levels affect the selectivity of RNA polymerase loaded with different sigma factors, K^+ homeostasis effects could explain many of the observed phenotypes of *ptsN* mutants (Lee et al., 2007; Huergo and Dixon, 2015). Two-component systems seem to be a preferred target of $EIIA^{Ntr}$-mediated regulation. In addition to KdpDE, $EIIA^{Ntr}$ was also found to interact with the two-component systems PhoR/PhoB, which regulates phosphate metabolism (Lüttmann et al., 2012) and SsrA/SsrB of *Salmonella enteric* serovar Typhimurium, which controls expression of numerous genes located within pathogenicity island 2 of this bacterium (Choi et al., 2010).

The level of phosphorylation of $EIIA^{Ntr}$ depends on the glutamine/2-ketoglutarate ratio: high 2-ketoglutarate to glutamine is the hallmark of N-starvation and stimulates EI^{Ntr} autophosphorylation (Lee et al., 2013). On the contrary 2-ketoglutarate was found to inhibit EI of the classic PTS (Doucette et al., 2011). Cross phosphorylation between the carbon PTS and the Ntr PTS was also observed. So by all these criteria, Ntr PTS is part of the machinery linking carbon utilization to nitrogen regulation (Pflüger-Grau and de Lorenzo, 2014).

Further evidence of the involvement of the Ntr PTS in carbon–nitrogen metabolism comes from the fact that the *yhbJ* gene often co-localizes and is cotranscribed with the *ptsO*, *ptsN* genes. Bacteria with mutations in *yhbJ* accumulate the enzyme Glucosamine-6P (GlcN6P) synthase (GlmS). GlcN6P is the first intermediate in the production of the essential aminosugar-containing components of the cell wall, peptidoglycan, and lipopolysaccharides. The YhbJ protein is necessary for the feedback regulation of *glmS* (Kalamorz et al., 2007) via the two small RNA (GlmZ and GlmY), which act hierarchically to activate *glmS* translation. GlmZ activates *glmS* translation but is normally prevented by the YhbJ protein, renamed RapZ for *R*Nase *a*daptor *p*rotein for sRNA GlmZ. RapZ targets GlmZ to RNAse E and prevents its stabilizing *glmS* mRNA. Low GlcN6P levels somehow increase GlmY sRNA levels, which act as a "decoy," binding preferentially to RapZ and allowing GlmZ to increase *glmS* translation (Gopel et al., 2014; Bobrovskyy et al., 2015). To add yet another complexity to GlmS regulation, phosphorylated $EIIA^{Ntr}$ has been shown to interact with GlmS and inhibit its activity, presumably in response to low glutamine levels to limit its diversion to glucosamine production (Yoo et al., 2016).

Many other proteobacteria possess homologues of the Ntr PTS proteins (EI^{Ntr}, HPr, and EIIA), often without possessing a complete carbon PTS. The Ntr PTS has been well examined in *P. putida* (see "Pseudomonades" section). *P. putida* has a total of only five PTS proteins, a specialized fructose PTS *fruBA* (where *fruA* encodes EI:HPr:$EIIA^{Fru}$ and *fruB* $EIIBC^{Fru}$), plus the three proteins of the Ntr PTS, *ptsO*, *ptsN*, and *ptsP* (Velazquez et al., 2007). Both *ptsN* and *ptsO* mutations affect glucose repression of the Pu promoter of the σ^{54}-dependent xylene utilization operon. A proteome study showed that *ptsN* affected the expression of a large number of genes not just those that are glucose-repressed or σ^{54}-dependent, implying it has wide regulatory functions (Pflüger-Grau and de Lorenzo, 2014).

Catabolite Repression in Firmicutes: *B. subtilis*

In low GC-content Gram-positive bacteria, as in Gram-negative bacteria, there are several distinct pathways contributing to the preferential use of glucose over other carbon sources. The key molecule in this case is the HPr protein, the small (9 kDa) protein of the central PTS. HPr from Gram-positives, in addition to being phosphorylated by EI of the PTS on His15, can be phosphorylated at a second regulatory site, Ser46, by a specific serine kinase. The same enzyme is also responsible for the dephosphorylation of P-Ser46-HPr and so is called HPr kinase/phosphorylase (HprK/P gene *hprK*) (Mijakovic et al., 2002). HPr can be phosphorylated on His15 and Ser46 at the same time, and so four forms of HPr exist in the cell: HPr, P-His15-HPr, P-Ser46-HPr, and the doubly phosphorylated form (Fig. 5). The kinase activity is stimulated by Fru1,6P, whereas the phosphorylase activity is stimulated by inorganic phosphate (P_i). The dephosphorylation activity is not hydrolytic, but phosphorolytic, using P_i and producing pyrophosphate. A gene for a pyrophosphatase is encoded in the *hprK* operon. The presence of P-Ser46 on HPr reduces the rate of the PTS phosphorylation on His15 and the P-His15 inhibits the kinase phosphorylation on Ser46. Although the Ser46 residue exists in Gram-negative HPr, the surrounding amino acids are not conserved in enteric bacteria, which lack HprK/P, and the HprK/P of firmicutes do not phosphorylate *E. coli* HPr (Deutscher et al., 2014a).

P-Ser46-HPr and Inducer Exclusion

The relative population of the four forms of HPr depends on the growth conditions. The kinase activity of HprK/P is high during growth on rapidly metabolized carbon sources, when Fru1,6P concentrations are high. P-Ser46-HPr and the doubly phosphorylated form predominate during rapid growth on glucose with only a small percentage in the single P-His15 form, capable of transferring phosphates to the incoming sugar. Considering the strong inhibitory effect of Ser46-P on PTS-mediated phosphorylation, it would appear that the 5%–10% of HPr, which is not phosphorylated on Ser46, must be responsible for and sufficient for the rapid growth on glucose. Indeed, strains with mutations preventing Ser46 phosphorylation grow more slowly on glucose, which could be due to too much glucose uptake. Moreover, in these strains, inducer exclusion was

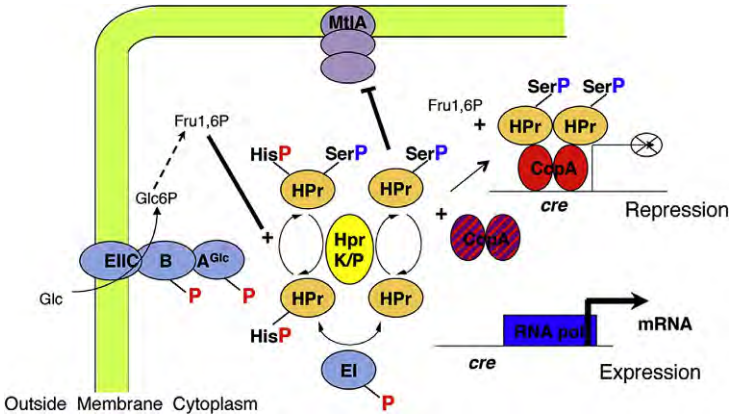

Fig. 5 Carbon catabolite repression in Firmicutes: *Bacillus subtilis*. Transport of glucose by the PTS and EIICBA^{Glc} (PtsG) produces internal Glc6P, which is converted by glycolysis to Fru1,6P. Fru1,6P activates the kinase activity of HprK/P producing P-Ser46-HPr and P-Ser46,P-His15-HPr. P-Ser46-HPr interacts with the transcription factor CcpA and increases its DNA-binding affinity. The P-Ser46-HPr:CcpA complex binds to *cre* sites and inhibits transcription. P-His15-HPr levels are low and the phosphate from His15-HPr is preferentially directed to EIIA^{Glc}. Other PTS transporters such as EIICBA^{Mtl} (MtlA) are inactive due to a lack of phosphates from HPr and the sugar-specific PTS components. In the absence of glucose transport when concentrations of Fru1,6P are low, HprK/P dephosphorylates Ser46 of HPr and the P-Ser46-HPr:CcpA complex detaches from *cre* sites. Operons encoding proteins for use of other carbon sources are transcribed in the presence of the appropriate specific signal (not shown). P in *red* denotes the high-energy PTS phosphates attached to histidine or cysteine residues. P in *blue* are the result of HPr kinase acting on HPr Ser46.

eliminated and simultaneous uptake of glucose and another sugar (mannitol) was observed. On the other hand, mutations that specifically eliminate the phosphorylase activity of HprK/P grow very slowly on glucose and all PTS sugars, because all the HPr is now in the P-Ser46 form and is unable to supply phosphates to PTS sugars. Thus, inducer exclusion of other PTS sugars by glucose is thought to be because of competition for P-His15-HPr (Deutscher et al., 2006). Inducer exclusion of non-PTS sugars is also observed in firmicutes (e.g., maltose in *L. casei* and *L. lactis*; ribose in *L. lactis*) and requires P-Ser46-HPr. P-Ser46-HPr inhibits the maltodextrin-stimulated ATPase activity of MalE1, one subunit of the ABC transporter for maltose (MalE1F1G1K1) (Monedero et al., 2008; Homburg et al., 2017). The phenomenon of inducer expulsion has also been described in *B. subtilis* and in some other bacteria. After precharging bacteria with a (nonmetabolizable) sugar, the addition of glucose causes the first sugar to be dephosphorylated and secreted into the medium. The molecular mechanism for this phenomenon is not known; experimental evidence suggests it does not require P-Ser46-HPr.

Catabolite Repression and Regulation by CcpA and the *cre* Element

AC and CAP are absent from most Gram-positive bacteria and inhibition of expression of non-PTS sugars by glucose is due to P-Ser46-HPr regulation of the CcpA repressor. In addition, a few genes are better expressed in glucose in a process called carbon catabolite activation (CCA), for example, acetate kinase (*ackA*) and phosphotransacetylase (*pta*), which allow the bacteria to get rid of excess metabolites produced by glycolysis. The CcpA protein is a LacI-type repressor, which binds to *cis*-acting sites called *cre* (*c*atabolite *r*esponse *e*lement) in the vicinity of the transcription start sites of catabolite-regulated genes. Mutations in either the *cre* site or in *ccpA* in many cases reduced or eliminated catabolite repression or catabolite activation (Fig. 5). The locations of the *cre* sites vary greatly in different operons from upstream of the promoter (for those subject to activation), to covering the promoter or transcription start site, to near the translational start site, or even within the translated region. *Cre* sites near the transcription-initiation site inhibit RNAP binding. The mechanism of CcpA inhibition by binding at the sites within the transcribed region has not been investigated but is presumably due to a "road block" mechanism inhibiting the elongating RNAP (Deutscher et al., 2006).

As in the case of CAP, several hundred genes have been identified as true or potential CcpA targets by genomic screens for *cre* sites and transcriptome and proteome analyses of wild-type and mutant strains grown with and without glucose as well as by conventional studies on individual genes. P-Ser46-HPr is an essential cofactor in the repression of most CcpA-regulated genes. The transcriptomes for the *ccpA* mutant and a strain carrying the HPr Ser46Ala mutant, (which cannot be phosphorylated by HprK/P) are very similar (Lorca et al., 2005). The two strains have similar phenotypes because the CcpA protein alone has only low affinity for its DNA targets and requires P-Ser46-HPr as a corepressor, although a few (e.g., *amyE* in *B. subtilis*) can be repressed by CcpA alone (Fig. 5). The P-Ser46-HPr concentration is highest during growth on glucose, which is the condition for maximum repression. Interestingly, these studies also revealed that CcpA controls several nitrogen-related genes (e.g., *sigL* encoding σ^{54}).

The structure of the P-Ser46-HPr:CcpA:*cre* DNA co-crystal shows that P-Ser46-HPr binds to the N-terminal subdomain of the dimerization and effector-binding domain of CcpA. The phosphate is locked into a small pocket on CcpA, and a comparison with

the structure of free (apo) CcpA reveals how the interaction with Ser46-P provokes both local and global structural changes in the subdomains. The so-called hinge helices are formed, they contact the center of the quasipalindromic *cre* site and kink the DNA and widen the minor groove, analogous to their function in LacI and PurR-operator structures. This facilitates contact between the *cre* and the helix-turn-helix motif. On the other hand, His15 of HPr is in contact with Arg296 of CcpA so that P-His15 is not compatible with HPr binding to CcpA, thus explaining the discrimination against formation of P-His15-HPr:CcpA (Schumacher et al., 2004). Glc6P and Fru1,6P are reported to stimulate catabolite repression at some loci, and they bind in the groove between the two subdomains of the effector/dimerization domain, thus bolstering subdomain movements produced by P-Ser46-HPr binding (Schumacher et al., 2007).

A protein called Crh (*c*atabolite *r*epression *H*Pr) with 45% identity to HPr, is found in Bacilli and close relatives. It is also phosphorylated by HprK/P and can regulate CcpA binding. His15 is absent in Crh, which has no function in PTS sugar transport. *Crh* mutations have no effect in a strain with a wild-type HPr, but Crh can partially replace HPr function at some operators in strains carrying the HPr Ser46Ala mutation. A much lower cellular level of Crh compared to HPr might account for its limited effect in vivo. Unphosphorylated Crh, but not HPr, inhibits the activity of methylglyoxal synthase, the enzyme that initiates the methylglyoxal bypass of glycolysis under carbon overflow conditions (Landmann et al., 2011).

PTS Regulation of PRD-Containing Proteins

Although *B. subtilis* does regulate some catabolic genes and operons for use in different carbon sources by classical repressor–operator interactions, which are sensitive to a small metabolite cofactor (e.g., XylR, which controls the use of xylose, or RbsR, which controls the use of ribose), a large number of transcription regulators carry a sequence element called PRD, which stands for PTS regulation domain, which is subject to both EIIB- and HPr-dependent phosphorylations. These phosphorylations antagonistically affect, positively or negatively, the activity of the regulator. Two major types of proteins carry this domain, which exist in pairs on each protein. One class is transcriptional activators, like the LevR and MtlR proteins, and the other is transcriptional antiterminators (Stülke et al., 1998; Deutscher et al., 2014a).

Transcriptional antitermination of PTS operons

Transcriptional antitermination is a common mechanism of regulating gene expression in *B. subtilis*. A relatively long leader region is transcribed upstream of the structural gene. This mRNA can fold into alternative structures, including one resembling a classical Rho-independent transcription terminator (GC-rich stem loop followed by a series of U) and another structure destroying the terminator, the antiterminator. Various RNA-binding effectors bind to the antiterminator and stabilize it and promote read-through of the terminator and hence expression of the downstream genes. The known types of effectors include uncharged tRNA, in the case of aminoacyl-tRNA synthetase operons, or SAM, in the case of methionine/cysteine biosynthetic genes (in this case SAM binding enhances termination).

In the case of the PTS antiterminators, the structured leader RNA region is called a RAT (*r*ibonucleotide *a*ntitermination *t*argets). In *B. subtilis*, they are found upstream of PTS operons for transport and use of glucose (*ptsGHI*), sucrose (*sacPA* and *sacX*), aryl β-glucosides (*bglPA*), as well as *bglS* (an endoglucanase) and *sacB* (a levansucrase). A specific antitermination protein controls each operon: LicT controls *bglPH* and *bglS*, SacY controls *sacB* and *sacX*, SacT controls *sacPA*, and GlcT controls the *ptsGHI* operon. The β-glucosidase operon in *E. coli* (*bglFB*) is also regulated by a similar mechanism using the antiterminator BglG, and was one of the first studied in detail. The RAT-binding antitermination protein is usually encoded by the first gene of the operon, separated from the genes for the metabolic functions by a terminator. The RAT-binding antitermination proteins carry an RNA-binding domain (called CAT for *coa*nti*t*erminator) at their N-terminal, and two PRD domains. Each PRD domain carries two phosphorylatable histidine residues; PRD1 is preferentially phosphorylated by the sugar-specific EIIB and PRD2 by EI and HPr. The relative importance of phosphorylations at the two sites of the two PRDs varies with the different antiterminators, but the basic principle is the same. Each antitermination protein is able to undergo two types of antagonistic phosphorylations, one of which enhances the antitermination activity and the other suppresses it (Fig. 6A) (Deutscher et al., 2014a; Galinier and Deutscher, 2017).

In the absence of its particular carbon source, the EIIB domain should be predominately phosphorylated. P-EIIB is able to transfer its phosphate to the PRD1 domain of the corresponding RAT-binding antitermination protein. With PRD1 phosphorylated, the antitermination protein is inactive and cannot bind to the RAT. The loss of this phosphate, retransferred back to the EIIB domain when the corresponding transporter is actively transporting its sugar, activates the antitermination function and allows it to bind to the RAT, preventing formation of the terminator so that the downstream genes are expressed. For several operons, mutations that knock out EIIB are sufficient to produce constitutive expression of the operon, in the absence of the inducing sugar.

Some antitermination proteins are active in the absence of all phosphorylations, for example, SacY and GlcT. Thus, in *ptsH* or *ptsI* mutants, where all PTS phosphorylation is abolished, *sacB* and *ptsG* are constitutively expressed. Other antiterminators require phosphorylation by HPr on the PRD2 domain. This is the case for SacT and LicT and also for BglG from *E. coli*. Phosphorylation of PRD2 is responsible for the residual CCR of some antiterminator-regulated PTS operons in a *ccpA* strain. The level of P-His15-HPr during uptake of a rapidly metabolizable carbon source is low, since most HPr is phosphorylated on Ser46 and is unavailable for sugar phosphorylation (see "P-Ser46-HPr and Inducer Exclusion" section). All the P-His-HPr is thus engaged in phosphorylating EIIA and the incoming preferred sugar and PRD2 remains unphosphorylated and in its inactive form.

In both PRDs, there are two conserved histidines and both can be phosphorylated; one seems to be the major target for initial phosphorylation in each PRD but the second site plays a greater or lesser role as shown by mutagenesis studies. Both intradomain

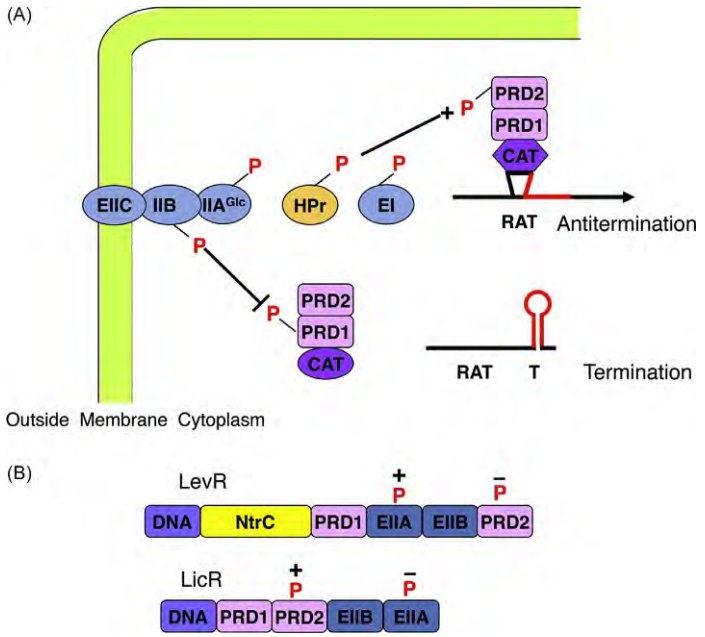

Fig. 6 PRD-containing proteins. (A) Antitermination by PRD-containing antiterminators. In the absence of its specific sugar substrate, EIIB is predominately in its phosphorylated form. It can transfer a phosphate to the PRD1 domain of the antiterminator, which prevents it from binding to the RAT operator within the leader of the mRNA. The terminator stem-loop structure can form so that transcription termination occurs. In the presence of the specific sugar substrate, PRD1 is nonphosphorylated but PRD2 can be phosphorylated by P-His15-HPr, which activates the RNA-binding function of the antiterminator. The N-terminal CAT domain of the activated antiterminator binds the RAT sequence and prevents formation of the terminator, so that transcription can occur. (B) Domain organization of PRD-containing transcriptional activators. In LevR the N-terminal DNA-binding domain is followed by an NtrC-like domain and the two PRD domains are interspersed by EIIB and EIIA domains. In LicR, the domain order is different. The domains subject to negatively controlling phosphorylation by EIIB and positively controlling phosphorylation by HPr are indicated by + and − signs above the P.

and interdomain as well as interprotein phosphate transfers are possible and complicate the analysis of the role of individual His residues in each antitermination protein (Deutscher et al., 2006). Crystal structures of native LicT and a constitutively active form (where the phosphorylatable histidines are replaced by aspartates that mimic the phosphorylated state) indicate that phosphorylation produces a massive structural rearrangement of PRD2 relative to PRD1. The aspartate residues are buried in the middle of the dimer and the movement of PRD1 changes the interface between PRD1 and the RNA-binding CAT domain, presumably producing the RNA-binding conformation (van Tilbeurgh and Declerck, 2001).

PTS regulation of PRD-containing transcriptional activators

PRD-containing activators are generally large multidomain proteins with an N-terminal DNA-binding domain and two PRD domains interspersed with EIIA and EIIB domains (Fig. 6B). There are two classes known. The best characterized of the "NtrC" class is LevR, which regulates the *levDEFGsacC* operon encoding a fructose/mannose PTS and an extracellular levanase. The *lev* operon is expressed from a σ^{54} promoter, which correlates with the presence of an NtrC-like domain within LevR. LevR binds to an upstream activating site (UAS) and activates expression of the *lev* operon in response to fructose. As in the case of the PRD antitermination proteins, it is subject to antagonistic phosphorylations. Phosphorylation of PRD2 by the EIIB component of the LevPTS (LevE) inhibits its activity, while phosphorylation by HPr has a positive effect, but unexpectedly occurs on the EIIA domain rather than PRD1. The second class of activators, for example, LicR or MtlR, have an N-terminal DeoR-type DNA-binding transcription activation domain followed by two PRD domains and then EIIB and EIIA domains. Once again, multiple phosphorylations by HPr and EIIB are responsible for positive and negative regulation of the activators depending on the presence of the transported sugar (Deutscher et al., 2014a; Galinier and Deutscher, 2017). Unlike other PRD-containing transcription activators, *B. subtilis* MtlR, which controls the expression of the *mtlAFD* operon for transport and catabolism of mannitol, is inactivated by phosphorylation by P-EIIA^{Mtl} rather than P-EIIB^{Mtl}. In addition, MtlR needs to interact with the EIIB domain of the mannitol-specific PTS permease MtlA. Similar to *E. coli* Mlc, MtlR is therefore sequestered to the cytosolic membrane. However, while membrane sequestration of Mlc leads to its inactivation, MtlR is activated by interaction with the membrane (Bouraoui et al., 2013).

PTS regulation of glycerol kinase

The use of glycerol is also regulated by PTS-mediated phosphorylation in *B. subtilis*. The phosphorylation target is not a transcription factor but an enzyme, the glycerol kinase, encoded by *glpK*. PEP-dependent phosphorylation of GlpK by HPr on a histidine residue

increases the kinase activity about 10-fold, thus enhancing the use of glycerol and the formation of glycerol-3P. The presence of glucose promotes the dephosphorylation of GlpK (via a reversal of the reaction with HPr) and exerts a form of catabolite repression by reducing the activity of GlpK and hence the concentration of Gly3P. Gly3P is the inducing signal for the GlpP antiterminator protein that controls the *glpFK* operon, encoding the glycerol facilitator transport protein and the kinase, so that the end result is that in the presence of glucose the *glpFK* operon is not induced. However, in the absence of glucose or another preferred PTS carbon source and the presence of glycerol, GlpK is phosphorylated by P-His15-HPr enhancing its activity and thus increasing Gly3P concentrations, so that the *glpFK* operon is derepressed (Darbon et al., 2002).

Consequences of Carbon Metabolism on Virulence

Certain bacteria have developed the capacity to survive and proliferate within animals or plants either as disease-causing pathogens or as symbionts or probiotes. To gain this capacity, pathogenes have acquired specific genes, which are frequently organized in so-called pathogenicity islands and which are absent from closely related nonpathogenic species. The foodborne pathogen *Listeria monocytogenes* and the closely related nonpathogenic *Listeria innocua* are well-studied examples of a pathogen/nonpathogen pair. Only *L. monocytogenes* possesses a set of virulence genes (allowing the bacteria to cross the intestinal barrier, invade host cells, and escape phagosomes, etc.) controlled by the virulence gene activator PrfA, a CAP-like protein (de las Heras et al., 2011). *L. monocytogenes* and *L. innocua* are soil bacteria able to use a wide variety of carbon sources. Nevertheless, *L. monocytogenes* contains several carbohydrate transport systems absent from *L. innocua*. Some of them were found to be important for the switch from the saprophytic lifestyle to virulence and therefore for intracellular proliferation, such as the H^+/hexose phosphate symporter UhpT or the presumed 2-keto-3-deoxygluconate-specific PTS permease, Lmo1971 (Deutscher et al., 2014b). These observations led to the idea that pathogens developed the capacity to survive within animals and plants in order to gain access to a broader range of carbon sources (Rohmer et al., 2011) and that detecting specific carbon sources in certain host tissues can enhance virulence.

Given the importance of carbon source availability for bacterial growth, it is not surprising that mutations affecting carbon metabolism have an impact on the virulence of numerous pathogens. In the majority of cases, these are general effects mainly related to slower growth and/or reduced fitness. In some pathogens, however, carbon sources can exert specific effects on the virulence of the bacterium. In *Streptococcus pyogenes*, the presence of glucose was found to stimulate the expression of virulence genes controlled by the transcription regulator Mga. The glucose effect seems to be mediated via two mechanisms. First, CcpA, the general catabolite repressor in firmicutes, which is activated during growth on glucose (see "Catabolite Repression and Regulation by CcpA and the *cre* Element" section), binds to *mga* promoter and enhances the expression of the gene encoding the virulence gene regulator (Almengor et al., 2007). Second, Mga has been shown to become phosphorylated by PEP and the PTS components EI and HPr. Phosphorylation of Mga probably occurs when glucose and other efficiently utilized PTS substrates are absent, a condition during which PTS proteins are mostly phosphorylated. Phosphorylation of Mga was reported to strongly inhibit in vitro expression of the Mga-regulated virulence gene *emm* (Hondorp et al., 2013).

In contrast to the virulence-stimulating effect of glucose in *S. pyogenes*, the presence of glucose was found to inhibit the virulence of *Clostridium difficile*. This pathogen is a major cause of nosocomial diseases associated with antibiotic therapy. The presence of glucose lowers the production of toxin A and B of *C. difficile* and therefore its virulence. In this pathogen, the glucose effect is also mediated via CcpA, which was found to bind upstream from the toxin-encoding genes *tcdA* and *tcdB*, although not to targets resembling known *cre* sites. In addition, formation of P-Ser45-HPr is apparently not required for the binding of CcpA to these targets, because an *hprK* mutant still exhibited glucose repression of the toxin genes. Binding of fructose-1,6-bisphosphate, the intracellular concentration of which increases during growth on glucose, seems to be sufficient for CcpA activation (Antunes et al., 2011).

Repression of virulence gene expression by various PTS-transported carbon sources, such as glucose, cellobiose, mannose and fructose, was also observed for *L. monocytogenes* (Milenbachs et al., 1997). The presence of these carbohydrates inhibits the activity of the virulence gene activator PrfA, however, in *L. monocytogenes* the repressive effect of carbohydrates on virulence genes is CcpA-independent, even though CcpA plays its usual role in repression of catabolic genes. Instead, PTS proteins, such as the EIIAB component of the glucose/mannose-specific PTS and P-Ser46-HPr, were suspected to play a role in *L. monocytogenes* virulence gene repression. Specific mutations in *prfA* were identified, which render PrfA highly active and no longer repressible by carbohydrates (Herro et al., 2005; Mertins et al., 2007). It is tempting to assume that these mutations, which were called *prfA**, might cause structural changes similar to those caused by effector binding. An interesting aspect of PrfA regulation is the observation that glucose concentrations as low as 2.5 mM strongly inhibit PrfA activity in strains grown in a minimal medium. In human blood, glucose is present at concentrations between 5 and 7 mM. Nevertheless, when *L. monocytogenes* was grown in blood, its virulence genes were strongly expressed (Toledo-Arana et al., 2009). It is not yet understood how PrfA escapes the repressive effect of glucose in blood.

Carbon Regulation and Catabolite Repression in Other Bacteria

Original mechanisms regulating carbon use and catabolite repression have been uncovered in certain bacteria, and a few examples are discussed here to demonstrate the versatility of regulatory mechanisms in bacteria.

Pseudomonades

Pseudomonades are free-living Gram-negative bacteria of which the best studied are *P. putida* and *P. aeruginosa*. They thrive in a variety of habitats, both terrestrial and aquatic. *P. aeruginosa* is an opportunist pathogen that infects the lungs and is a major agent of nosocomial infections. *Pseudomonades* are metabolically versatile and can degrade a wide range of organic and aromatic compounds especially derived from degrading plant material. Complex aromatic compounds are degraded to simple intermediates that feed into one of four chromosomally encoded central aromatic pathways (homogentisate, catechol, protocatechuate, and phenylacetate), which are responsible for cleaving of the aromatic rings (Jimenez et al., 2002). There is only one PTS sugar, fructose, but there is a complete Ntr PTS (see "The nitrogen PTS" section). Mutations in *ptsN* have several effects suggesting a role in maintaining carbon/nitrogen balance and also CCR of xylene utilization (Pflüger-Grau and de Lorenzo, 2014). Glucose is taken up via an ABC transporter, converted to gluconate-6P, and degraded predominately via the ED pathway (Nikel et al., 2015). Glucose represses the use of some hydrocarbon-degrading pathways, but succinate, some organic acids, or complex media like LB exert the strongest repression. A close CAP homologue, Vfr, exists, but cAMP levels are constant and although a *vfr* mutation affected the level of about 60 genes, and is a global regulator with pleiotropic effects, including the use of some amino acids and organic acids as carbon or nitrogen sources, CCR by succinate was not affected (Rojo, 2010).

Crc (catabolite repression control) protein was identified as a major regulator of carbon metabolism (Rojo, 2010). It has been implicated in the repression of many catabolic genes, but the pattern of repression is complex, depending on the promoter studied, the carbon source, and especially whether studies are in minimal or rich media. A metabolic-footprint analysis implied that Crc is involved in, but is not solely responsible for, the hierarchical use of carbon sources present in LB (La Rosa et al., 2016). Crc inhibits the translation of transcription factors like AlkS and BenR, which are the direct activators of the alkane and benzoate degradation pathways. Crc control of these catabolic operons is thus indirect, by keeping the levels of the direct activators low. Moreover, by preventing the expression of the first genes in a degradation pathway, Crc prevents the synthesis of the intermediates that act as inducers for subsequent steps in the pathway, and so the whole pathway is repressed (Rojo, 2010).

The Crc protein controls gene expression posttranscriptionally and was thought to function as an RNA-binding translational repressor; an adenine-rich consensus sequence (CA) was identified upstream of target genes. However experiments have confirmed that Crc does not bind RNA alone and the RNA-binding function is supplied by Hfq, the ubiquitous RNA chaperone (Milojevic et al., 2013; Moreno et al., 2015). Mutations in *hfq*, as in *crc*, relieve CCR but as Hfq binds a wide range of sRNA and mRNA, it has more dramatic consequences on cell growth. Hfq is in excess compared to Crc, and how Crc targets specific sites is still being investigated.

The level of the Crc protein varies only slightly between repressing and nonrepressing conditions. Its activity is controlled by "decoy" sRNA, CrcZ (in *P. aeruginosa*) and CrcY and CrcZ (in *P. putida*), which have multiple adenine-rich Crc consensus-binding sites and are proposed to antagonize Crc binding to its mRNAs targets by acting as decoys (Moreno et al., 2007, 2012; Sonnleitner et al., 2009). How Hfq fits into this scenario is only now being addressed. Expression of CrcZ is controlled by a two-component system, CbrA/CbrB, and RpoN. The Crc/CrcZ protein/sRNA pair resembles the well studied CsrA/CsrB,CsrC system of *E. coli* controlled by the BarA/UvrY two component system (Romeo et al., 2013). The Csr system is required for efficient switching between glycolytic and gluconeogenic carbon sources and experiments also assign a role to Crc in integrating gluconeogenic and glycolytic metabolism in Pseudomonades (La Rosa et al., 2015).

Specific mechanisms exist that control the expression of the novel Pseudomonas genes for the degradation of alkanes, phenol, and toluene/xylene. These are mostly plasmid-encoded, for example, the TOL plasmid carries genes for the conversion of toluene/xylene to benzoate/methylbenzoate, the "upper" operon, which is controlled by XylR. XylR is an NtrC-like activator that binds to a UAS and activates a σ^{54}-controlled promoter. A second "meta" operon encodes genes, which convert the benzoates to pyruvate and acetate. It is controlled by XylS, which is an AraC-like activator. These genes are subject to multilevel catabolite regulation by many other carbon sources including succinate and glucose via the ED intermediates gluconate-6P and 2-keto 3-deoxy gluconate-6P. Other specific and global factors implicated in their control include the Ntr PTS, two two-component systems, NtrCB and CbrAB, ppGpp, and others (Rojo, 2010). Pseudomonas have great potential in biodegradation of toxic chemicals but their utility requires precise management of their overall metabolism (Nikel et al., 2014).

Streptomyces coelicolor

Streptomycetes are aerobic, spore-forming, soil bacteria of the species actinomycetes (high-GC Gram-positives) that are important sources of secondary metabolites including antibiotics. They can use a wide range of carbon sources including insoluble materials found in soil, such as lignocellulose and chitin, and they produce a range of biotechnologically useful enzymes, like α-amylases, xylanases, and chitinases. *S. coelicolor*, the best studied, has a PTS system that is apparently dedicated to the use of *N*-acetylglucosamine (GlcNAc). GlcNAc is generated by the action of secreted chitinases on the chitin derived from fungal walls and insect cuticles in the soil. The genes for the cytoplasmic components of the PTS, *ptsH*, *ptsI*, and *crr*, as well as the GlcNAc-specific $EIICB^{Nag}$, are induced by GlcNAc and are controlled by a FadR family transcription factor called DasR, which also controls chitobiose uptake. DasR was originally characterized as affecting the formation of the aerial mycelium and spores. High GlcNAc concentrations prevent *S. coelicolor* from entering the developmental pathway leading to sporulating hyphae (Rigali et al., 2008).

Readily assimilable carbon sources are used preferentially and in a hierarchical fashion. Homologues of the *crp* and *cya* genes exist, but mutants are affected in germination and not catabolite repression. CCR is absolutely dependent on the glycolytic enzyme glucose kinase, GlkA, so that *glkA* mutants are no longer glucose repressible. The glucokinase activity per se is not sufficient for CCR since heterologous glucokinases can restore growth on glucose without restoring CCR, and *glkA* mutants are defective in CCR by carbon sources like galactose or glycerol, which are not metabolized via glucose kinase. The protein is constitutively synthesized, but its activity is sugar-source dependent, being high during growth on glucose and low in mannitol-grown bacteria. A complex between glucose permease (GlcP) and Glk has been suggested (van Wezel et al., 2007). Despite much effort, the exact mechanism of CCR by Glk is still elusive. One hypothesis is that it acts on the specific transcription factors of the regulated operons (Romero-Rodriguez et al., 2017). Glucose kinase has been implicated in CCR in several related bacteria, for example, *Mycobacterium smegmatis*, and in the less-related *Staphylococcus xylosus*, which also has a CcpA-mediated system.

Corynebacterium glutamicum

Corynebacteria are aerobic, rod-shaped, nonsporulating, high-GC Gram-positive soil bacteria. *C. glutamicum* has worldwide importance for the biotechnological production of amino acids, initially for glutamate and lysine (both derived from TCA cycle intermediates), but it is now also used as a biocatalyst for a range of commodity chemicals (Vertes et al., 2012). As a consequence, the central metabolism of *C. glutamicum* has been well investigated. Novel regulatory mechanisms controlling TCA cycle gene expression have been described. For example, AcnR is a TetR-family–specific repressor of aconitase. Iron homeostasis is achieved by the combined action of two repressors DtxR and RipA, which control the amount of succinate dehydrogenase and aconitase, which are both Fe–S proteins, in response to available iron. In *E. coli*, a similar iron control is achieved via Fur and the sRNA RyhB (see "The TCA Cycle and Glyoxylate Shunt" section). An opposing activator and repressor pair, RamA and RamB (*r*egulator of *a*cetate *m*etabolism), controls genes for the use of acetate as a carbon source (*ack*, *pta*, *aceB*, *aceA*). In addition, the GlxR protein, a CAP homologue, represses the genes of the glyoxylate shunt, *aceBA*. Both bioinformatics and binding studies have confirmed that GlxR is a global regulator in *C. glutamicum* but, unlike CAP, GlxR is predominately a repressor (Bott, 2007; Schröder and Tauch, 2010). GlxR is sensitive to cAMP, which is reported to be high during exponential growth on glucose but low during growth on acetate, unlike *E. coli*. The level of cAMP is controlled by the action of a phosphodiesterase, encoded by *cpdA*, the deletion of which produced severe growth defects on all carbon sources tested (Schulte et al., 2017).

C. glutamicum also has an original posttranscriptional regulatory mechanism controlling the TCA enzyme, 2-ketoglutarate (2-oxoglutarate) dehydrogenase (ODHC). The small protein, OdhI, inhibits ODHC. OdhI undergoes phosphorylation by a threonine kinase, PknG, and a second kinase, in response to glutamine availability. The relative levels of phospho-OdhI and PknG determine the flux through ODHC and hence the yield of glutamate from the action of glutamate dehydrogenase on 2-ketoglutarate.

The metabolism of *C. glutamicum* is unusual in that co-utilization of several carbon sources occurs, for example, glucose with acetate, lactate, pyruvate, or fructose. On the other hand, the use of ethanol as a carbon source is repressed by glucose, even though the *adhA* gene is controlled by the RamA and RamB regulators that are responsible for control of the genes for acetate use. Co-utilization of glucose and gluconate has been shown to be a result of two functionally redundant GntR-specific repressors of the gluconate operon, acting as activators of the *ptsG* enzyme encoding the glucose transporter. The presence of gluconate causes a simultaneous decrease in PtsG levels and glucose transport and an increase in gluconate uptake. There are three PTS transporters: for glucose (*ptsG*), sucrose (*ptsS*), and fructose (*ptsF*). The genes for the central components *ptsHI* are in an operon with *ptsF* and SugR, a DeoR-type repressor, represses the expression of all five genes during growth on gluconeogenic carbon sources. SugR also represses several glycolytic genes and is reportedly sensitive to several hexose-phosphate inducing–signals. SugR along with RamA, RamB, and GlxR constitute the top of the hierarchy of transcriptional regulators in *C. glutamicum* (Schröder and Tauch, 2010; Teramoto et al., 2011).

Lactic Acid Bacteria and Bifidobacteria

Lactic acid bacteria (LAB) comprise a group of low-GC, acid-tolerant, Gram-positive bacteria, which have been exploited for centuries for the preservation of food and drinks, because the lactic acid they produce lowers the pH sufficiently to prevent the action of other spoilage agents. They carry out either homolactic (e.g., *Lactococcus* and some Lactobacilli) or heterolactic (e.g., *Leuconostoc*) fermentations. Considering the size reduction that has occurred in LAB genomes, typical of bacteria habiting nutrient-rich environments, genes for sugar utilization, including PTS transport, are predominant in the genomes (Kowalczyk and Bardowski, 2007; Monedero et al., 2007). Lactose is transported by a PTS and is metabolized via glycolysis to lactate during homofermentation. In heterofermentation, some glucose is metabolized via the pentose phosphate pathway (Fig. 1) producing CO_2, acetic acid, and/or ethanol in addition to lactic acid. Glucose transported by the PTS is the preferred sugar and produces CCR via CcpA and HPr as described for *B. subtilis* (see "Catabolite Repression and Regulation by CcpA and the *cre* Element" section). The lowering of the pH through the generation of lactic acid affects carbon metabolism and increases glucose consumption mostly through changes in enzyme activities in *L. lactis*. Analysis of the PTS and other transporters has been performed in commercially important *Lactobacillales*, for example, *Oenococcus oeni* (which is primarily responsible for malolactic fermentation in wine), and has shown significant variations between strains (Jamal et al., 2013). Specific pathways for the utilization of human milk-associated glycans have been identified (Bidart et al., 2014). Current research applies the -omics techniques and systems biology to explore and exploit the metabolic potential of these important industrial workhorse bacteria (Kok et al., 2017). *Lactobacillus* are also considered important probiotic organisms.

Bifidobacteria are anaerobic commensals of the intestinal tract and are among the first bacteria to colonize the infant gastrointestinal tract. They are particularly adapted to metabolize the complex oligosaccharides of milk (Garrido et al., 2013), but they are also able to degrade a wide range of other carbon sources including many mono- and disaccharides (e.g., lactose) and complex carbohydrates derived from indigestible plant oligosaccharides of our diet, like pectin and including the prebiotic xylo-, galacto- and fructo-oligosaccharides, which makes them the most important probiotic organism (Turroni et al., 2010; Hidalgo-Cantabrana et al., 2017). They are heterofermentative, nonsporulating Gram-positive rods, now classed as Actinomycetes (rather than lactic acid bacteria). They lack the reductive half of the TCA (Krebs) cycle. Hexoses are degraded via an unusual version of the pentose phosphate pathway (or bifido shunt) in which an original enzyme, fructose-6P phosphoketolase (*xfp*), converts Fru6P to erythrose-4P and acetyl-phosphate. The latter is converted to acetate with the production of a mole of ATP. The erythrose-4P reacts with another molecule of Fru6P to start the pathway of sequential transketolase, transaldolase reactions of the pentose phosphate pathway (Pokusaeva et al., 2011).

There are a large number of ABC-type transporters for a variety of sugars and oligosaccharides. The genes for a PTS transporter for glucose (*ptsG*) as well as *ptsH* and *ptsI* are found in *B. longum* but are only weakly expressed. A gene for a glucose permease (*glcP*) appears to encode the major glucose uptake system. *ptsG* and *glcP* are transcribed divergently and are separated by a putative RAT-controlled antiterminator. Lactose is used preferentially over glucose and represses expression of *glcP* (Parche et al., 2006). An unusual fructose-specific PTS, which forms fructose-6-P and not fructose-1-P, is present in *Bifidobacterium breve* (Maze et al., 2007). Although a lot of LacI-type transcriptional regulators have been identified on the chromosome, suggesting substrate specific regulation of the various transporters and glycohydrolases, few experimental data are available (Pokusaeva et al., 2011). Genome sequencing of individual subspecies, together with metagenomic analysis, confirm the wide range of carbohydrate metabolizing capabilities of these social gut inhabitants (Milani et al., 2015).

Conclusions

Control of carbon use involves numerous regulatory circuits operating at various levels. The enormous range of carbon sources available to bacteria must be specifically converted into simple sugar phosphates, which can then enter one of the ubiquitous pathways of central carbon metabolism. These central pathways consist of about 100 key enzymes that catalyze the formation of the energy and reducing power, as well as the precursors and cofactors necessary for bacterial growth. Major advances in our understanding of bacterial metabolism have come from the application of the large-scale genomics, transcriptome, and metabolomics techniques (Buescher et al., 2012; Nicolas et al., 2012). Together, metabolomics and flux analyses have revolutionized the analysis of metabolism in a cellular setting and identified certain original pathways and regulatory strategies. A high correlation between metabolic flux analysis data and mRNA levels from transcriptome studies suggests that carbon flux is primarily controlled at the transcriptional level. However, superimposed on this base is metabolic control, due to the large number of allosteric or otherwise regulated enzymes. Rapid kinetic experiments have demonstrated that transcriptional regulation is not sufficient to explain the rapid changes in flux observed when the carbon source is changed (Chubukov et al., 2013; Kochanowski et al., 2015; Link et al., 2015). Moreover, metabolic pathways are robust. A block in one pathway causes a local rerouting of the flux.

Under the banner of posttranscriptional regulators of carbon metabolism, we have to include several sRNAs, which have specific roles in controlling carbon utilization, for example, Spot42, SgrS (Bobrovskyy et al., 2015). In addition, there are now several "decoy" sRNAs, such as CsrB, CsrC in *E. coli*, or CrcZ, CrcY in *P. putida*, which act by titrating away RNA-binding protein regulators from their targets (Gopel and Görke, 2014).

A few key metabolites have been identified as important signaling molecules in different bacteria. For example, the glycolytic intermediate Fru1,6P is used to signal high glycolytic flux rates in numerous situations and has been proposed as the intracellular flux sensor (Kochanowski et al., 2013b). It is a key molecule to signal CCR in low-GC Gram-positive bacteria since it activates the kinase function of HprK/P and is a corepressor of the complex between CcpA and P-Ser46-HPr to repress catabolite-regulated operons. It is implicated in the switch from use of the PTS and glycolytic sugars to gluconeogenesis, since it is an effector for CggR in *B. subtilis*, FruR (Cra) in *E. coli*, and SugR in *C. glutamicum*. It is also an allosteric effector for several enzymes, including pyruvate kinase, PEP carboxylase, and glycerol kinase from *E. coli*. Another common metabolite that has emerged as a global regulator is 2-ketoglutarate (2KG). It is both a member of the TCA cycle and the substrate for glutamine biosynthesis, straddling the C–N metabolic pathways. The ratio of glutamine to 2KG is the classic signal of the nitrogen balance of the bacteria. Independently, 2KG has been shown to coordinate carbon and nitrogen utilization in other ways; it inhibits EI of sugar PTS, affects the autophosphorylation of EI^{Ntr}, and inhibits AC (Huergo and Dixon, 2015).

Synthesis of secondary metabolites is an important biotechnological application of microbes, and a lot of genetic engineering is devoted to improving yields. The cheapest carbon sources for industrial scale cultures are often a mixture of lignocellulose-derived sugars, mostly glucose and xylose. However the preferential use of glucose from the mix generates CCR with two bad consequences: acetate production and its associated acidification of the medium, which inhibits growth and, when coupled with inducer exclusion, means that the other sugar components of the feeder sugar mix are not used and are thus wasted. A lot of effort has been invested in trying to reduce the production of acetate, and some simple solutions can help considerably (e.g., the elimination of the glucose PTS in *E. coli* so that uptake is via less efficient routes generating less CCR). Intensive genetic engineering of carbon use is a major area of research in the field of microbial cell factories. For example, bacteria are being tailored to enhance production of many commercially important compounds. (Papagianni, 2012; Nikel et al., 2014; Gu et al., 2017). The advent of new-generation sequencing

techniques and large-scale metagenomic projects of bacteria from diverse ecosystems, ranging from terrestrial to aquatic and including animal symbionts has massively increased the database of bacterial genomes. Data mining these sequences shows genes and enzymes for potential polysaccharide utilization systems for a multitude of presumed, predicted, or unknown carbon compounds (Grondin et al., 2017). These genome sequences represent a rich resource for future biotechnology applications.

The emerging picture of the importance of the gut microbiota for health is fueling the analysis of metabolic pathways within different classes of the gut's inhabitants. There are clear indications of variations of the microbiome with diet, thus with available carbon sources. The variation in composition of the microbiome associated with various pathologies, especially diabetes and inflammatory bowel disease, is well documented but it is not clear whether the change in bacterial populations (dysbiosis) is a cause or an effect of the pathology (Koropatkin et al., 2012). One little observation that might relativize all the studies on *E. coli* is that the Enterobacteriacae are only minor constituents of mammalian guts, which are dominated by Bacteriodetes and Firmicutes.

Note Added in Proof April 2019

This review was completed in the summer of 2017. Since then work on the control of carbon assimilation has continued its inexorable shift from test tube to high-through-put analyses generating data sets requiring rigorous analysis to extract the salient take-home messages. A few recent highlights are described here.

Rapidly improving analytical techniques, coupled with new approaches to mathematical modelling and data analysis have re-visited concepts that were already well established.

As an example of confirming what we thought we knew already; RNAseq and chromatin immunoprecipitation (ChIP) of cultures grown on different sugar and organic acid carbon sources identified an enlarged Cra regulon. Systems analysis of the data within the framework of a genome scale metabolic model of *E. coli* confirmed Cra and CAP are responsible for the majority of transcriptional regulation occurring in central carbon metabolism but in addition it placed Cra in the forefront of carbon metabolism in the case of non-hexose carbon sources (Kim et al., 2018). The importance of CRP and Cra was underscored when the transcriptional activity of central carbon metabolism genes was measured via transcriptional fusions with GFP (green fluorescent protein). 70% of variance over 26 environmental conditions could be assigned to "global" growth-rate- dependent regulation. The remaining "specific" transcriptional regulation was mostly correlated with the regulatory metabolites cAMP, fructose-1,6P_2 and fructose-1P—precisely the signalling molecules for CAP and Cra (Kochanowski et al., 2017). We can also mention here a new facet of CRP regulation; a lysine residue, part of AR2 (Fig. 4), can be acetylated in vivo and this modification has the potential to affect transcriptional activation by CAP (Davis et al., 2018).

Advances in metabolomics, and in particular the speed and resolution of mass spectrometric identification of compounds coupled with fast kinetic studies in cells have allowed rapid progress on the dynamics of sugar metabolism e.g. the switch from glycolytic to gluconeogenic carbon sources. The switch depends upon both transcriptional regulation and allosteric regulation and interestingly *E. coli* and *B. subtilis* adopt different strategies (Buffing et al., 2018). An alternative coarse-grained modelling approach to analyse growth transitions based on principles of flux balance was able to adequately reproduce many experimental data e.g. for diauxie (Erickson et al., 2017). Interestingly this paper and several others are coming to the conclusion that the proteome (which in large part is constituted by ribosomes and the translational apparatus) is in excess of that required for balanced growth but that this apparent wastefulness of resources maximizes the cell's flexibility to adapt rapidly to changing environmental situations e.g. between feast and famine or oxidative stress (Mori et al., 2017; Christodoulou et al., 2018). In the later case allosteric regulation was deduced to keep the excess enzymatic capacity quiescent. There is increasing interest in metabolic heterogeneity within clonal populations of cells (Takhaveev and Heinemann, 2018). A comprehensive understanding of these processes in bacteria will require application of single-cell-based techniques and especially MS-based metabolomics which, at the moment, are more easily applicable to larger yeast and other eukaryotic cells than bacteria (Duncan et al., 2019).

Another emerging area of study are "interactomes"; after protein-DNA and protein-protein interactions, interest has turned to protein-metabolite interactions (Diether and Sauer, 2017). By data-mining all available databases, Reznik *et al.*, (2017) extracted all the known small molecules interacting with all listed *E. coli* proteins and their binding constants, and incorporated them into a mathematical model of *E. coli* metabolism. Together with published experimental data on metabolite concentrations they able to deduce how small molecules can perform both as substrates and regulatory molecules (Reznik et al., 2017).

Concerning the post-transcriptional CCR in Pseudomonas; more details are emerging of the how Crc/Hfq regulate translation (Sonnleitner et al., 2018). A cryoEM structure has shown a complex of two molecules of Crc sandwiched between two Hfq hexamers bound to RNA targets (Pei et al., 2019). Hfq also provides a regulatory link between iron and carbon metabolism under low CCR conditions, where Crc is mostly inactive, since it is sequestered by the sRNA, CrcY and CrcZ (Sanchez-Hevia et al., 2018). The CrcC/CrcY,Z system is also important in other members of the Pseudomonadaceae family. For example it is responsible for the preferential use of acetate over glucose in *Azoterbacter vinelandii* (Martinez-Valenzuela et al., 2018). In general there are more and more examples of sRNA affecting use of sugars and CCR post-transcriptionally (Durica-Mitic et al., 2018).

Finally there are still new mechanisms of CCR to be discovered. In *Camplylobacter jejeuni*, which cannot grow on sugars but metabolises amino acids and organic acids via the TCA cycle, serine is the preferred carbon source. First experiments, exploring how serine reduces use of other carbon sources, hint at a role of succinate in monitoring the intracellular carbon status (van der Stel et al., 2018). How this is achieved remains to be explored.

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Regulation of Replication Origin Firing

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Replication Origin Firing: Assembly of Biological Switches at the Right Place and the Right Time During the Cell Cycle

There are few events during the life of a cell that are more important than initiating new rounds of chromosome replication. This is the step at which cells make the commitment to reproduce, sanctioning the time-consuming process of genome duplication as well as the subsequent events required to successfully divide. Proper regulation of the initiation step is essential to eliminate the threat of genome instability due to over or under-replication of chromosomal DNA. For this reason, sophisticated and sometimes redundant mechanisms exist to ensure that new DNA synthesis is not only tightly coupled to the cell cycle, but cannot be re-activated until the next scheduled round of chromosome duplication.

Mechanistically, the initiation step (origin firing) is best described as the assembly and activation of biological switches that ensure the proper positioning of the DNA copying machinery on the genome. Each switch is assembled and disassembled in an orderly fashion using a variety of specialized initiator proteins and a region of chromosomal DNA, termed a replication origin. New genomic DNA synthesis ensues bi-directionally from each fired origin, and the number of replication origins vary according to genome size, reviewed in Leonard and Méchali (2013). In bacteria, the small circular double-stranded genomes, averaging about 5 million base pairs (bp), carry a singular origin termed *oriC*, and once initiated, bidirectional DNA synthesis can usually be completed within about 40 min, although some bacteria require more time. Budding yeast genomes (12 million bp) are replicated within a similar time frame, but the number of replication origins increases to about 700 (and all available origins do not fire every cell cycle). Complete duplication of a metazoan genome (average size of 3 billion bp), can be accomplished in 6–8 h, but DNA synthesis is triggered from tens of thousands of replication origins. Of course, triggering DNA replication from high numbers of eukaryotic origins must be controlled with the same stringency as the single *oriC* found in bacteria.

Protein components of the firing mechanism interact with replication origins to assemble two essential complexes: 1) origin recognition complexes (ORC) that identify origins for use during the cell cycle, and 2) pre-replication complexes (pre-RC) that load, and, when necessary, activate replicative DNA helicases (the DNA unwinding activity required for replication fork movement) on origin DNA. Despite many structural similarities among initiator proteins and DNA helicases, the mechanics of origin recognition and helicase loading are fundamentally different in bacteria, archaea and eukaryotes.

Key differences also exist in the mechanisms that regulate origin firing and will be described below. In bacteria, these mechanisms are focused primarily on setting the available intracellular levels and accessibility of the active initiator protein (DnaA-ATP) needed for origin unwinding and helicase recruitment. Initiator protein availability and helicase recruitment are also cell cycle-regulated in archaea. In eukaryotes, critical regulation is shifted to the activation of pre-loaded helicases by cell cycle-dependent kinase activities. A comparison of origin recognition and helicase loading, as well as regulation of these stages in bacteria, archaea, and eukaryotes are described below.

Overview of Replication Origins

Replication origins are remarkably diverse and there are obvious differences in the level of nucleotide sequence specificity when comparing prokaryotes and eukaryotes. Bacterial origins encode nucleotide sequence motifs that serve as protein recognition sites and sequences that are regions for DNA bending and DNA unwinding. In most eukaryotes, there are fewer requirements for specific nucleotide sequences, and the lack of sequence specificity is an obvious advantage for organisms whose chromosomes carry thousands of replication origins.

Replication Origins of Bacteria

In most bacteria, *oriC* is distinguished from other regions of the chromosome by two prominent features: 1) multiple recognition sites for the principal initiator protein, DnaA, based on the consensus sequence motif [5′-TT(A/T)TNCACA-3′] (Speck et al., 1997), and 2) a region of high A-T richness, termed the DNA unwinding element (DUE), where single-stranded DNA becomes available for helicase loading (Kowalski and Eddy, 1989). In *E. coli*, the DUE contains three 13 mer repeats with consensus sequence 5′-GATCTnTTnTTTT-3′ (Bramhill and Kornberg, 1988), although the nucleotide sequences of DUEs can be quite variable among different bacterial types, as can their relative locations within *oriC* (Zawilak-Pawlik et al., 2005; Leonard and Méchali, 2013). Based on an extensive bacterial origin database, it is clear that the numbers and spacing of DnaA recognition sites are also remarkably diverse, suggesting there are many ways to assemble DnaA and *oriC* into triggering complexes (Gao et al., 2013), see Relevant Website section and Fig. 1.

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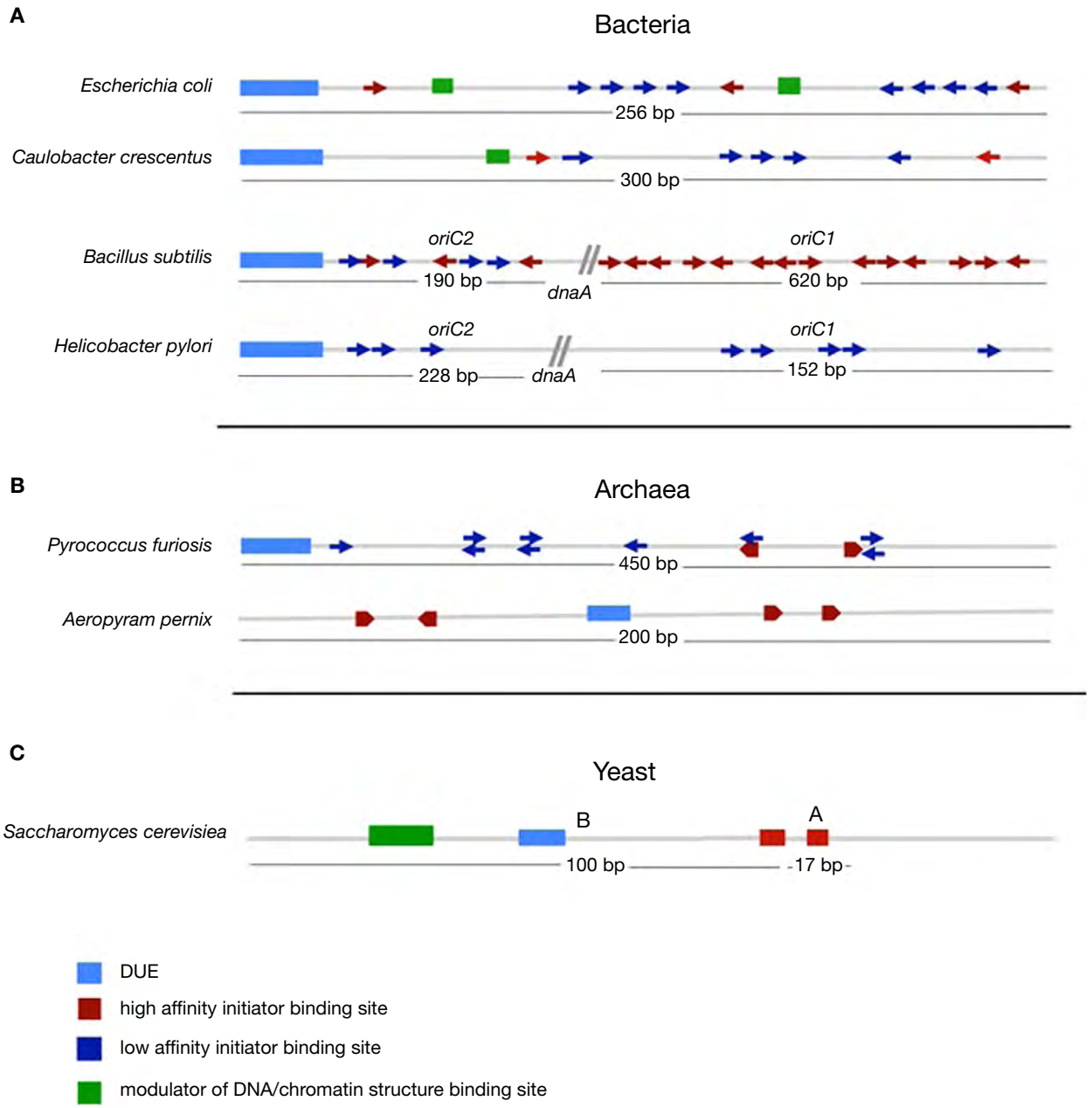

Fig. 1 Structure of replication origins in three domains of life. (A) Schematic maps of several bacterial replication origins. Relative locations of the binding sites for DnaA are shown. Binding sites with consensus sequence are indicated by red arrows, and sites that deviate from consensus by one or more bases are indicated by blue arrows. The direction of the arrows indicates the likely orientation of the arginine finger in the AAA+ domain. The region where the origins unwind (DUE) is shown by light blue boxes. Binding sites for DNA bending proteins are indicated by green boxes. (B) Schematic maps of two archaeal replication origins. Red arrows indicate the ORB Orc1/Cdc6 binding sites and blue arrows show the mini-ORB initiator binding sites. Blue boxes show the region of origin unwinding. (C) Schematic map of the *Saccharomyces cerevisiae* ARS replication origin. Relative locations the A and B1 sequences required for ORC binding (red boxes), as well as the B2 sequence where helicase is loaded and the origin is unwound (blue box), and the B3 sequence (green box) where the ABF1 chromatin modifier binds, are shown.

In addition to perfect consensus DnaA recognition motifs, some origins contain DnaA recognition sites with mismatches from consensus at one or more nucleotide locations (Fig. 1; Charbon and Lobner-Olesen, 2011; Taylor et al., 2011; Leonard and Méchali, 2013). These altered sites display reduced affinity for DnaA and occupation of these sites requires cooperative DnaA interactions, reviewed in Leonard and Grimwade (2015). In *E. coli*, a subset of low affinity sites show binding preference for the active initiator DnaA-ATP (over DnaA-ADP) and impart a requirement for DnaA-ATP in higher order pre-RC assemblies (Rozgaja et al., 2011) (see Fig. 2 and below).

Not all origins harbor their multiple DnaA boxes in single stretch of DNA. Bacterial origins from *Helicobacter* (Donczew et al., 2012), *Mycoplasma* (Cordova et al., 2002; Lartigue et al., 2003) and *Bacillus* (Moriya et al., 1999) have spatially separated clusters of DnaA recognition sites (Fig. 1). DnaA molecules bound at each cluster might interact if the DNA becomes bent during pre-RC assembly.

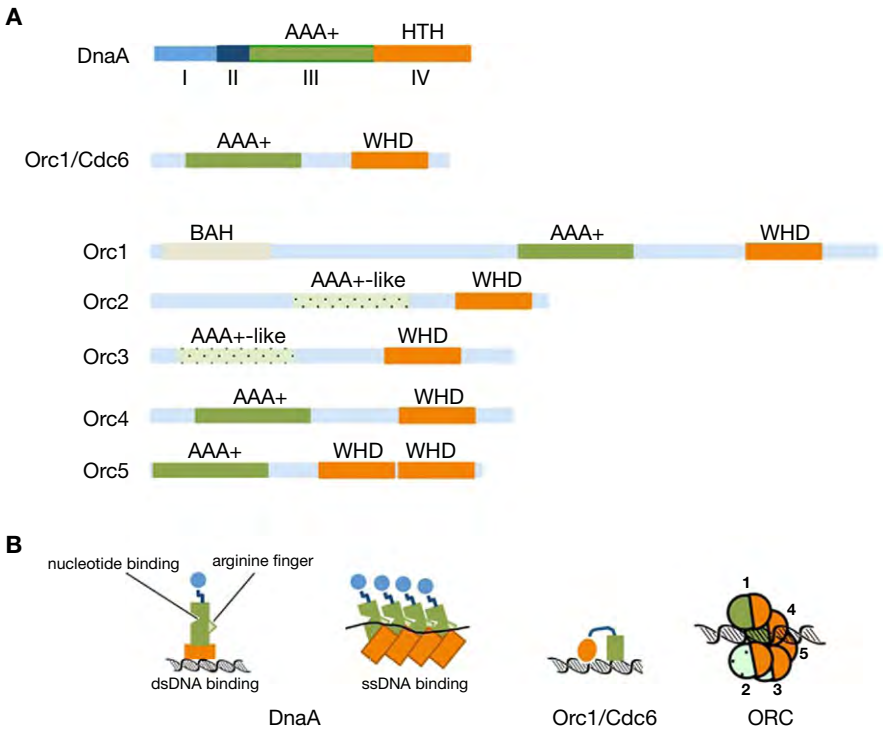

Fig. 2 Bacterial, archaeal, and eukaryotic initiator proteins. (A) Schematic of domains in initiator proteins, showing AAA+ domains (green) and motifs shared by DNA binding proteins (orange). HTH, helix-turn-helix; WHD, winged helix domain. (B) Cartoons of DNA binding modes and possible oligomeric structures are also shown for each domain of life. The position of each Orc protein is indicated in the DNA-bound ORC.

Replication Origins of Archaea

Studies of archaeal origin DNA reveal a mixture of attributes found in both bacteria and eukaryotes, reviewed in Costa et al. (2013), O'Donnell et al. (2013), Kelman and Kelman (2014), and Ausiannikava and Allers (2017). Although most small circular archaeal genomes carry one replication origin, several genera carry multiple origins, although origin number does not correlate with genome size (Robinson and Bell, 2005; Ausiannikava and Allers, 2017). Archaeal origins are usually located adjacent to the gene that encodes for the initiator protein, Orc1/Cdc6 (named for homology to both eukaryotic proteins, see below). When multiple origin-initiator gene combinations are present, each origin appears to respond to its associated initiator (Samson et al., 2013). In some cases, Orc1/Cdc6 genes are not associated with an origin, see Ausiannikava and Allers (2017).

Many archaeal replication origins show some similarity to bacterial *oriC*, having distinctive AT-rich DUEs as well as clustered Orc1/Cdc6 recognition site motifs, termed Origin Recognition Boxes (ORBs), reviewed in Leonard and Méchali (2013) and Wu et al. (2014) (Fig. 1). Most ORBs are 22–35 bp with dyad symmetry and are usually mapped at two or more locations within an origin. ORBs often contain a conserved 13-bp core sequence called the mini-ORB [5′-(T/C)TNCANNNGAA(A/C)-3′], and in some archaeal origins, mini-ORBS exist as multiple (7−15) direct repeats (Capaldi and Berger, 2004; Matsunaga et al., 2010). As is the case for bacteria, the number, orientation, and spacing of initiator recognition sites vary among archaeal genera (Leonard and Méchali, 2013; Fig. 1).

Eukaryotic Replication Origins

DNA replication origins were first defined by their ability to confer autonomous replication to plasmids in *Saccharomyces cerevisiae* (Stinchcomb et al., 1979; Deshpande and Newlon, 1992). These origins, termed Autonomous Replication Sequences (ARS), unlike the origins of higher eukaryotes, all contain a specific consensus sequence motif (autonomous consensus sequences) [5′-(A/T)TTTA(T/C)(A/G)TTT(A/T)-3′] (Stinchcomb et al., 1979; Fig. 1). Adjacent to the ACS lies the B domain, which contains three subdomains (Marahrens and Stillman, 1992; Rao et al., 1994). Closest to the ACS, B1 is important for ORC binding (Rao et al., 1994; Rowley et al., 1995). B2 interacts with DNA helicase (in eukaryotes this is a hetero-hexamer of Minichromosome maintenance proteins, Mcm 2–7) (Wilmes and Bell, 2002). The more distant B3 is a recognition site for ARS Binding Factor 1 (Abf1) (Marahrens and Stillman, 1992), a transcription factor that mildly stimulates origin activity, probably by modulating chromatin structure and nucleosome assembly (Ganapathi et al., 2011).

Large numbers of mapped eukaryotic replication origin sequences are now compiled in a database (Gao et al., 2012), and it is clear that origins of yeast strains other than *S. cerevisiae* and metazoans display little sequence specificity with the exception of CpG

islands, GC-rich regions, and G-quadruplexes (Cayrou et al., 2011, 2012, 2015; Valton et al., 2014). The exact role of these features in origin activity remains unclear.

Overview of Initiator Proteins and Origin Recognition Complexes (ORC)

Protein initiators from all domains of life share similar structural and functional features, although there are obvious mechanistic differences in the way origin firing is accomplished. All initiators carry domains for DNA binding and, as members of the AAA+ (Adenosine triphosphatases Associated with various cellular Activities) family of proteins, also carry domains able to bind and hydrolyze ATP (Iyer et al., 2004; Duncker et al., 2009). AAA+ domains often play a role in initiator oligomerization and the assembly of higher order structures (short oligomers, helical arrays, and cracked rings), see review by Duderstadt and Berger (2008). The coupling of initiator activity to ATP binding and hydrolysis creates a convenient on-off switch that can be used to prevent over-replication.

Initiator proteins must interact with replication origins in reproducible ways during every cell cycle, and ORC are unquestionably important for both origin identification and correct step-wise assembly of pre-replication complexes. Despite their pivotal roles, striking differences exist among bacterial, archaeal and eukaryotic ORC. Eukaryotic initiators assemble into a highly conserved hetero-hexameric ORC prior to interacting with origin DNA using little or no nucleotide sequence specificity (Bell and Stillman, 1992; Quintana and Dutta, 1999). ORC remains bound to DNA throughout the cell cycle, but during late M/early G1 phase it recruits DNA helicase (Mcm 2–7); see reviews in DePamphilis (2005) and Li and Stillman (2012). In contrast, assembly of the bacterial equivalent of ORC is determined by high affinity initiator (DnaA) interactions with recognition site motifs encoded into the nucleotide sequence of *oriC* (Miller et al., 2009). Archaeal ORC is the most enigmatic version since origin DNA interactions with the archaeal initiator, Orc1/Cdc6, have aspects of both eukaryotes and bacteria (Wigley, 2009; Bell, 2012). Rather than forming a complex, in many archaea each Orc1/Cdc6 may act as both ORC and helicase loader combined.

The Bacterial Initiator DnaA and bORC

All bacterial origin firing depends on higher order assemblies of the DnaA protein initiator, but each *oriC* recognition site is occupied by a DnaA monomer (Speck et al., 1997; Weigel et al., 1997). DnaA's C-terminal domain (IV) with helix-turn-helix structure (Erzberger et al., 2002) is responsible for double-stranded DNA binding (Schaper and Messer, 1995), and signature amino acids of DnaA make base-specific contacts (Fujikawa et al., 2003; Fig. 2). DnaA is active in the ATP-bound state (Sekimizu et al., 1987) and the amino acids required for ATP-binding and hydrolysis are localized within an internal domain (III) (Fig. 2). Proteins that interact with DnaA usually contact the N-terminus (domain I) (Simmons et al., 2003), located on the end of a flexible stalk (domain II) whose length varies among bacterial types (Messer et al., 1999; Zawilak-Pawlik et al., 2005; Fig. 2).

Both ATP and ADP forms of DnaA are capable of self-oligomerization mediated by domain I-domain I interactions (Simmons et al., 2003; Zawilak-Pawlik et al., 2017). DnaA-ATP also self-oligomerizes when an arginine finger in one molecule's domain III contacts the ATP bound to an adjacent protomer, combined with additional domain III contacts (Erzberger et al., 2006; Fig. 2). The DNA-binding domain (domain IV) of DnaA-ATP also folds up toward domain III, helping to stabilize the intermolecular interactions, leading to the formation of a compact helical filament (Duderstadt et al., 2011). Compact DnaA-ATP filaments bind single-stranded DNA through a central channel formed by domains III and IV, and are reported to melt short DNA duplexes, presumably by stretching the helix (Duderstadt et al., 2011). However, the compact helical filament has very low affinity for double-stranded DNA (Duderstadt et al., 2011) and if a DnaA oligomer is formed on double-stranded DNA during pre-RC assembly, it must have an alternative structure that is not yet characterized.

DnaA does not readily form into complexes in the absence of DNA (Duderstadt et al., 2010), and for this reason, a true structural equivalent of eukaryotic ORC (see below) does not exist. However, it is possible to define the bacterial ORC (bORC) as the DnaA-*oriC* complex that, like the eukaryotic ORC, persists during extended periods of the cell cycle and serves as a scaffold for the recruitment of additional proteins that lead to origin DNA unwinding and helicase loading (Miller et al., 2009). Surprisingly, on native supercoiled DNA, the DUE of *oriC* spontaneously unwinds in the absence of associated protein (Kowalski and Eddy, 1989), and the bORC constrains *oriC* DNA to prevent unwinding until a complete pre-RC is assembled (Kaur et al., 2014). In *E. coli*, bORC comprises DnaA occupying the available three high affinity DnaA recognition sites (R1, R2, and R4) (Kd in the range of 4–20 nM) (Miller et al., 2009; Fig. 3). Since these recognition sites in *E. coli* are widely separated, DnaA-DnaA interactions within ORC are likely to form DNA loops. Clustered high affinity DnaA recognition sites are found within most bacterial *oriCs* (Gao et al., 2013) and these sites are also expected to be occupied in a similar fashion to *E. coli*, unless the availability of DnaA were extremely limiting. Each DnaA molecule in ORC is able to recruit additional DnaA molecules and nucleate the formation of DnaA oligomers during pre-RC assembly, see below Miller et al. (2009) and Rozgaja et al. (2011).

Eukaryotic ORC

The proteins comprising the heteromeric eukaryotic ORC (Fig. 2) are well-studied and the ORC structure was recently determined, see for example, Chen et al. (2008) and Bleichert et al. (2015). Although five members share attributes of AAA+ proteins, only Orc1 and Orc5 bind to ATP, and only ORC1 has intrinsic ATPase activity mediated by contact with an arginine finger (Klemm et al., 1997;

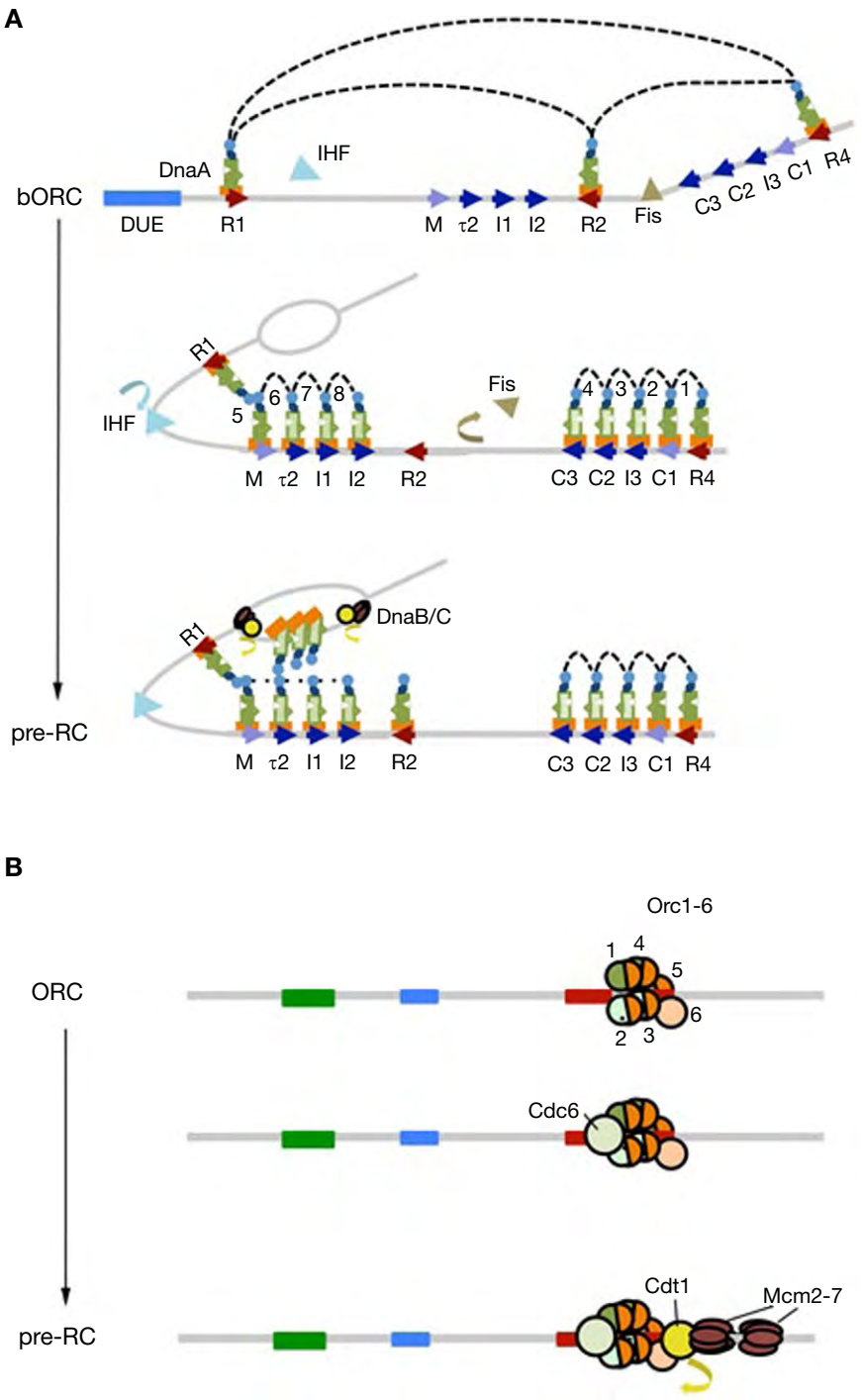

Fig. 3 Multistep assembly of nucleoprotein complexes at replication origins. (A) Formation of pre-RC in *E. coli*. *E. coli oriC* is maintained throughout most of the cell cycle with DnaA (either nucleotide form) bound to R1, R2 and R4, with Fis binding adjacent to R2. Dotted lines indicate possible interaction between DnaA molecules. Immediately before DNA replication initiates, the remaining, lower affinity sites are filled in a specific order, indicated by numbers; this step requires DnaA-ATP (DnaA-ATP is shown by lighter green domain III; low affinity DnaA-ATP sites shown by dark blue arrows, low affinity sites without preference shown as light blue arrows). As DnaA binds, Fis is displaced, IHF binds between R1 and R5, and the DNA strands in the DUE separate. The single-stranded DNA region is stabilized by binding of oligomeric DnaA-ATP filaments, which assist in loading the DnaB/C (helicase/helicase loader) complex. DnaC must leave the pre-RC for helicase activation. (B) Formation of pre-RC in budding yeast. ORC is bound throughout the cell cycle at the A domain and B1 domains. Orc1–5 proteins encircle the DNA, while Orc6 binds to Orc5 and Orc3. In G1, when CDK levels are low, bound ORC recruits Cdc6, which binds in the gap between Orc1 and Orc2. After Cdc6 binds, Cdt1/Mcm2–7 proteins are recruited, and the Mcm2–7 complex (helicase) is loaded onto double-stranded DNA in the B2 domain, forming the pre-RC. Cdt1 is displaced prior to helicase activation.

Neuwald et al., 1999; Iyer et al., 2004). Orc1-5 proteins have winged helix domains that are typical for DNA binding proteins (Speck et al., 2005; Duncker et al., 2009; Fig. 2). Orc6 lacks any homology to the other ORC components, does not bind to DNA and is composed of tandem cyclin-box folds similar to transcription factor IIB (Balasov et al., 2007). Orc6 is not necessary for ORC–ARS binding, but is required for early stages of pre-RC assembly (Chen et al., 2007).

Eukaryotic ORC are dynamic complexes that are assembled in stages: Orc2/Orc3 interacts with Orc4/5, Orc1 is then added to Orc2/Orc3 to complete a ring in the order Orc1-Orc4-Orc5-Orc3-Orc2, and Orc6 then binds directly to Orc3 and Orc5 (Vashee et al., 2001; Chen et al., 2008). Orc1's ATPase requires the conserved arginine residue from Orc4 for catalytic activity, see Bleichert et al. (2015). ORC is initially inactive, but an ATPase-dependent conformational change produces a gapped ring that permits the DNA binding elements in ORC's central channel to encircle DNA (Bleichert et al., 2015). The gap (between ORC1 and ORC2) must be filled by the interaction of Cdc6 to begin pre-RC assembly (Sun et al., 2012). Orc1, Orc4, and Orc6 in higher eukaryotes also carry auxiliary domains for DNA/chromatin interactions (e.g., the BAH domain of Orc1) (Noguchi et al., 2006; Müller et al., 2010).

The Archaeal Initiator Orc1/Cdc6

Although the replication origins of archaea carry distinct initiator recognition sites, similar to bacteria, their initiator protein, Orc1/Cdc6 is clearly more similar to eukaryotic initiators, reviewed in Wigley (2009), Bell (2012), Kelman and Kelman (2014), and Ausiannikava and Allers (2017). Despite structural similarities, archaeal Orc1/Cdc6 interacts with double-stranded DNA differently than ORC, using both N-terminal AAA+ and C-terminal winged- helix domains (Dueber et al., 2007, 2011; Gaudier et al., 2007; Fig. 2). This unique bipartite interaction makes few base-specific contacts, but produces local distortions in origin DNA. Like DnaA, Orc1/Cdc6 is active in the ATP-bound form, but unlike DnaA, ATP does not appear to regulate interactions with origin DNA (see below). In some archaea, Orc1/Cdc6 binding to proximal miniORBs is cooperative (Capaldi and Berger, 2004; Grainge et al., 2006) suggesting that, oligomeric ORC or pre-RC structures may be formed that are analogous to those observed for DnaA. However, there remains no clear consensus regarding the ability of Orc1/Cdc6 to bind cooperatively or persist on origins as an ORC-like complex.

Assembly of Pre-RC for Loading/Activation of DNA Helicase

Loading DNA helicase onto the replication origins of both eukaryotes and bacteria requires building a pre-RC by adding proteins to ORC, but there are noteworthy differences in the way this is accomplished, see recent reviews by Bell and Kaguni (2013), Yao and O'Donnell (2016), and Parker et al. (2017). In bacteria, there are three steps: 1) pre-RC are assembled by progressively adding DnaA molecules to *oriC* DNA, guided by recognition sites, 2) the DUE is unwound by the assembled pre-RC, and 3) pre-RC associated DnaA, with assistance from specialized helicase loader proteins position the helicase onto the available single-strands. In eukaryotes, inactive DNA helicase (Mcm2–7) complexes are loaded (with the assistance of several loading factors) onto ORC-associated, double-stranded origin DNA in late M or G1 stages of the cell cycle (referred to as origin licensing). The Mcm complex is then activated prior to the onset of S phase by association with additional protein regulators, some of which must be phosphorylated by regulatory kinases. Origin DNA unwinding is not detected until after helicase activation. The archaeal DNA helicases resemble those in eukaryotes, and archaea also contain analogs of many of the eukaryotic helicase activating proteins The order of DNA unwinding and helicase loading in archaea remains uncertain and is probably type-specific.

DNA Unwinding and Helicase Loading in Bacteria

Although the mechanism responsible *oriC* unwinding remains uncertain, the bacterial pre-RC is, generally speaking, a DNA stress-inducing machine that introduces bends, kinks and less well defined topological changes within *oriC*. Despite the lack of mechanistic details, it is clear that precise timing of replication onset requires orderly and reproducible addition of DnaA during pre-RC assembly. To achieve this, DnaA additions are guided by *oriC*- encoded recognition sites (Leonard and Grimwade, 2011; Rozgaja et al., 2011; Fig. 3). In *E. coli*, these are low affinity (Kd >200 nM) and become occupied sequentially during pre-RC assembly (Schaper and Messer, 1995; Messer et al., 1999; Rozgaja et al., 2011).

Low affinity site arrays are found in the gap regions between two high affinity recognition sites (R1-R2 or R2-R4) (Rozgaja et al., 2011; Figs. 1 and 3). Within an individual array, all sites face in the same direction and are separated by two base pairs (Rozgaja et al., 2011; Figs. 1 and 3). Most importantly, three of the low affinity sites in each array bind DnaA-ATP preferentially, coupling pre-RC assembly to the availability of this form McGarry et al. (2004).

Filling the arrayed recognition sites requires assistance from a DnaA molecule residing at proximal high affinity site, and this nucleation step utilizes DnaA's domain I (Schaper and Messer, 1995; Miller et al., 2009). Thus, pre-RC assembly order is determined by proximity of high and low affinity recognition sites with right side sub-complexes preceding those on the left (Rozgaja et al., 2011; Fig. 3). Each bound DnaA molecule bends double-stranded DNA (Schaper and Messer, 1995), and although the DnaA oligomer assembled is not well characterized, the position of DnaA boxes is likely to increase torsional stress for DUE unwinding.

Although a likely common feature of bacterial *oriCs* (Charbon and Lobner-Olesen, 2011; Leonard and Méchali, 2013), low affinity DnaA recognition sites remain difficult to identify by nucleotide sequence alone and some bacterial origins may lack these sites. As an alternative, closely spaced high affinity recognition sites may be used for cases where DnaA synthesis rates are very low or

availability is restricted. It is also important to not rule out the role of DNA topology in the ability of DnaA to access some recognition sites in select bacterial types, and supercoiling dependent DnaA binding in *oriC* is reported in *Helicobacter pylori* (Donczew et al., 2014).

As noted above, DnaA-ATP interacts with single-stranded DNA as a compact filament, which is likely to enhance unwinding and stabilize the single-stranded DNA (Duderstadt et al., 2010). Once *oriC* is unwound, oligomers can extend from DnaA residing at a DUE-proximal recognition site (Duderstadt et al., 2010; Richardson et al., 2016). As is the case for all DnaA oligomers assembled on double-stranded *oriC* DNA, specialized arrays of nucleotide motifs, termed trio sequences (3′-GAT-5′, 3′-AAT-5′ and 3′-GAA-5′), specify single-stranded DNA interactions in a wide range of bacterial types (Richardson et al., 2016).

Bendable DNA regions are often located in bacterial replication origins and DNA bending is known to be regulated by accessory DNA bending proteins, such a IHF or Fis, see Swinger and Rice (2004) and Gimenes et al. (2008). By moving high and low DnaA recognition sites into or out of proximity, these proteins can dynamically promote or prevent the assembly of the pre-RC (Fig. 3). This is the case for *E. coli* where Fis-catalyzed DNA bending in the right half of *oriC* represses pre-RC assembly (Ryan et al., 2004). As the available levels of DnaA rise in the cell, Fis is displaced by DnaA, and a second bending protein, IHF, gains access to a recognition site in the left half of *oriC* (Ryan et al., 2004). The resulting bend brings a high and low affinity recognition site into proximity (R1 and R5M) as a nucleation switch for the subsequent stage of low affinity DnaA site filling (left array) and *oriC* unwinding (Rozgaja et al., 2011; Ozaki et al., 2012). Switches of this kind add precision to the triggering mechanism during the cell cycle and set the levels of DnaA necessary to fire origins within a narrow time window. DNA bending switches may also modulate *oriC* firing by utilizing bending proteins whose synthesis is coupled to growth rate or environmental cues.

The replicative helicase in *E. coli* is a homo-hexameric protein (DnaB), and is complexed with a specialized loading protein, termed DnaC, before it is recruited to the unwound origin by the pre-RC, reviewed in Bell and Kaguni (2013) (Fig. 3). DnaA then loads one DnaB-C complex on each of the separated strands. Domains I and III of DnaA are involved in the helicase loading step (Seitz et al., 2000; Abe et al., 2007). Although the exact details of this step may vary among bacterial types, the DnaC molecule of the DnaB-DnaC complex is proposed to act as a molecular adapter by interacting with DnaA-ATP at or near the end of an oligomeric filament extending into the DUE (Mott et al., 2008). Helicase loading must be performed on both the top and bottom strands of the DUE, suggesting different DnaA molecules within the oligomer are responsible, see Bleichert et al. (2017). Once loaded, helicase activation requires the dissociation of DnaC, and this step may not occur until the loaded helicase interacts with the replicative primase in the presence of ATP and other ribonucleotides (Chodavarapu et al., 2016). DnaC is not a universally-used protein and in Gram positive bacteria the helicase (termed DnaC) is loaded by two proteins (DnaD and DnaB) (Smits et al., 2011). In some bacteria, DnaA alone may be capable of loading helicase (Robinson et al., 2010).

Helicase Loading and Activation in Eukaryotes and Archaea

The intricate choreography of helicase loading and activation in eukaryotes is described in several excellent reviews (Bell and Kaguni, 2013; Tognetti et al., 2015; Parker et al., 2017), and will only be summarized here (see Fig. 3). The licensing step requires two regulatory factors, Cdc6 and Cdt1, which are sequentially recruited to the origin by ATP-bound ORC. Cdc6 is an ORC remodeling factor that stabilizes the ring-like ORC on origin DNA and generates new binding sites for the recruitment of Cdt1 (Speck et al., 2005; Sun et al., 2012). Cdt1 is the Mcm2–7 loader, acting in a similar fashion to DnaC in *E. coli*. When associated with Mcm2–7, Cdt1 is recruited to ORC through interactions with Orc6 (Chen et al., 2007). Loading of the Mcm2–7 requires ATP hydrolysis of Cdc6 which allows DNA to gain access to a central channel in the Mcm2–7 ring due to alterations in protein conformation associated with the release of Cdt1 (Samel et al., 2014). An initial Mcm2–7 complex is loaded on the winged helix domain face of ORC, followed by a second Mcm2–7 complex in a head to head configuration (Evrin et al., 2014). Bound Cdc6 inhibits helicase activity and must also be displaced by ATP hydrolysis (Chang et al., 2015).

The post-loading events required for helicase activation require a large number of protein factors. At the onset of S phase, two kinase activities: the Dbf4-dependent Cdc7 kinase (DDK) and S phase cyclin-dependent kinase (CDK) phosphorylate Mcm2–7 which allows Mcm2–7 to interact with the helicase activating factor Cdc45 (complexed with additional non-catalytic chaperone factors that differ somewhat between yeasts and metazoans) (Petojevic et al., 2015). Since helicase loading can only take place when CDK levels are low, further loading is blocked during this stage, ensuring once only triggering during the cell cycle. CDK is also required to assemble a second activating complex known as GINS (Go Ichi, Ni San) (Takayama et al., 2003). GINS is a tetramer that assembles into a higher order complex, also with a variety of non-catalytic chaperones, prior to interacting with Mcm-Cdc45 (Gambus et al., 2006). The resulting large protein complex of Cdc45-Mcm2–7-GINS, referred to as CMG (Moyer et al., 2006), is the DNA unwinding-proficient, activated version of the eukaryotic replicative helicase. It remains unclear why so many proteins are necessary to activate eukaryotic helicases, but cells may benefit by having many targets for checkpoint regulation or a variety of chaperones to accommodate many chromatin configurations.

The archaeal Mcm complex is a homo-hexamer with significant structural similarities with eukaryotic Mcm 2–7 (Chong et al., 2000), and there are also archaeal homologs of GINS and Cdc45 suggesting that archaea must share the eukaryotic activation scheme (Marinsek et al., 2006; Xu et al., 2016). Unlike eukaryotes, archaeal Cdc45 (two copies) and GINS form an extremely stable complex before binding to the N terminal domains of Mcm to produce an activated DNA helicase (Xu et al., 2016). The archaeal helicase loading step also deviates from both bacteria and eukaryotes since there are no analogs of either DnaC or Cdt1, see Tye (2000). No loader protein appears to be needed and Orc1/Cdc6 is able to perform the role of Cdt1 in helicase recruitment. ATP-bound Orc1/Cdc6 is required for proper helicase loading with Mcm interacting directly with the ATPase

domain (Samson et al., 2016). Thus, a one to one relationship exists between each Orc1/Cdc6 and Mcm for recruitment. The placement of ORB sites on either site of the DUE, is consistent with directional helicase loading on each of the available single strands of DNA (Samson et al., 2013; Fig. 1).

Regulating Origin Triggering During the Cell Cycle

Origin triggering is regulated by diverse mechanisms, some of which are specifically tuned to cells with either few or many replication origins on their genomes. In bacteria, the triggering mechanism must accommodate rapid growth rates wherein new rounds of chromosome replication must be triggered before previous rounds are completed. For this reason, regulation is based almost exclusively on controlling the availability of active initiator DnaA-ATP during each cell division cycle. In eukaryotes, the availability of ORC proteins and the helicase activation step are dependent on cell cycle-coupled kinase activities. Chromatin state also appears to play an important role in the regulation of origin firing. In archaea, regulation appears to focus primarily on the availability and ATP-bound state of Orc1/Cdc6.

Bacteria

There is little evidence for cell cycle-specific gene expression or kinase activity involvement in the triggering of most bacterial replication origins, but triggering does depend on achieving the appropriate threshold level of DnaA-ATP available to interact with *oriC*. Although DnaA-ATP levels in bacteria are due predominantly to new synthesis, the levels and availability of DnaA-ATP at *oriC* fluctuates during the cell cycle (Kurokawa et al., 1999), due to post-triggering mechanisms that are coupled to the movement of replication forks. These include: 1) DnaA-ATP inactivation by hydrolysis, 2) titration of DnaA away from *oriC* by newly replicated recognition sites located around the genome, 3) blocking DnaA access to *oriC*, and 4) reactivating inactive DnaA-ADP into active DnaA-ATP (Fig. 4), although there is considerable variety among bacterial types. In *E. coli*, conversion of DnaA-ATP to DnaA-ADP is accomplished primarily by interactions with a replication fork-associated protein, Hda, which stimulates the hydrolytic activity of bound DnaA in a process termed Regulatory inactivation of DnaA (RIDA) (Katayama and Sekimizu, 1999; Keyamura and Katayama, 2011). However, post-triggering mechanisms to stimulate DnaA-ATP hydrolysis are not effective in all bacteria (Smits et al., 2011; Bonilla and Grossman, 2012), and consistent with this idea, Hda is not a widely-conserved protein.

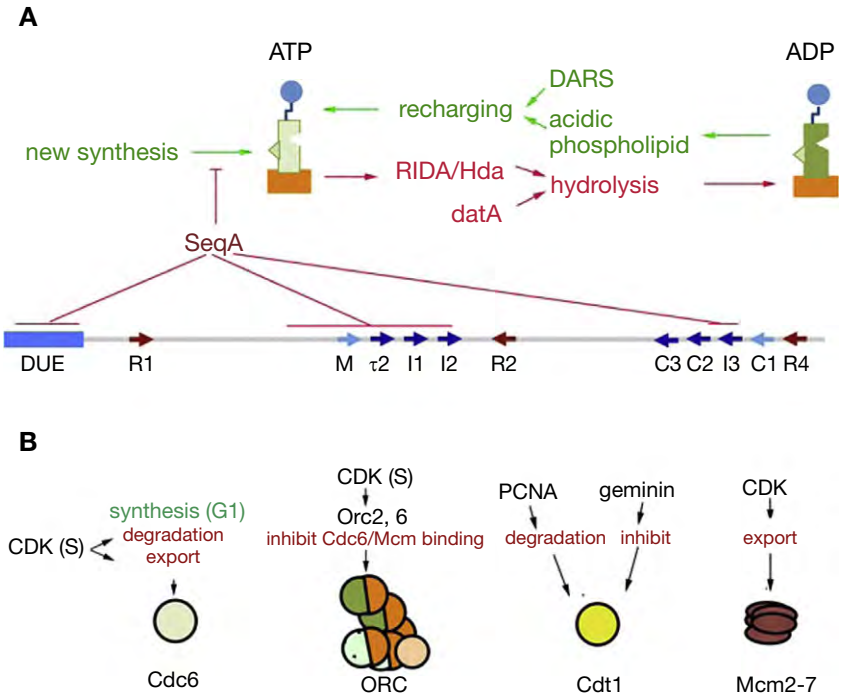

Fig. 4 Regulatory mechanisms ensuring that replication origins fire only once per cell cycle. (A) In *E. coli*, levels of DnaA-ATP are regulated to control origin firing. Mechanisms that increase DnaA-ATP levels are in green, and mechanisms that decrease DnaA-ATP levels are in red (see text for details). DnaA-ATP access to *oriC* is also regulated by the blocking/anti-cooperativity factor SeqA, which prevents DnaA from re-binding to low affinity sites immediately after initiation. (B) In eukaryotes, several mechanisms regulate the levels and/or activity of proteins involved in eukaryotic replication origin firing (see text for details). Mechanisms that increase activity/levels are shown in green, and mechanisms that lower activity/levels are shown in red.

DnaA may be titrated away from *oriC* by individual DnaA recognition sites dispersed over the chromosome or by localized clusters at one or more positions on the genome (Roth and Messer, 1998; Christensen et al., 1999). In *E. coli*, the *datA* locus, distanced from *oriC* by over 450,000 bp (Ogawa et al., 2002) titrates any DnaA-ATP displaced from *oriC* into a complex that hydrolyzes DnaA-ATP by an Hda-independent mechanism (Kasho et al., 2017). Similar regulation exists in *B. subtilis* where clusters of DnaA recognition sites are also found at eight intergenic regions on the Bacillus chromosome (Ishikawa et al., 2007). The protein YabA, found in several Gram-positive bacteria (Noirot-Gros et al., 2006), tethers DnaA to replication forks and reduces its availability to *oriC* (Schenk et al., 2017).

Factors exist in many bacterial types that have the ability to block DnaA accessibility or affect its cooperative binding/oligomerization during the cell cycle. In *E. coli*, low affinity recognition sites are blocked for about 1/3 of the cell cycle (Campbell and Kleckner, 1990) following each new round of DNA synthesis by the sequestration protein, SeqA (Lu et al., 1994; Slater et al., 1995), which recognizes and binds to newly-replicated hemimethylated GATC sites found in high numbers within *oriC* and in the dnaA promoter. High affinity recognition sites in *oriC* are not sequestered and can remain occupied throughout the cell cycle to immediately reset the bacterial ORC (Nievera et al., 2006). Origin binding of the blocking factor CtrA in *Caulobacter* is also coupled to DNA methylation state (Quon et al., 1998). Other blocking/anti-cooperativity factors, may be independent of methylation, such as AdpA in *Streptomyces* (Wolański et al., 2012), MtrA in *Mycobacteria* (Rajagopalan et al., 2010), HP1021 in *Helicobacter* (Donczew et al., 2015), as well as SirA (Rahn-Lee et al., 2011), Soj (Scholefield et al., 2011) and DnaD (Bonilla and Grossman, 2012), Spo0A (Boonstra et al., 2013), and YabA (Soufo et al., 2008; Scholefield and Murray, 2013) in *Bacillus*.

In rapidly growing *E. coli*, new rounds of DNA replication must be triggered prior to the completion of previous rounds to ensure each daughter inherits the correct genome content. Under rapid growth, there may be insufficient new DnaA-ATP synthesis to allow for proper initiation timing at all copies of *oriC*. A DnaA-ADP recharging system, dependent on two chromosomal loci termed DnaA recharging sequences (DARS) raises the levels of DnaA-ATP (Fujimitsu et al., 2009). DARS sites contain a specific arrangement of DnaA recognition sites, but the mechanism for recharging remains unclear. DnaA also interacts with the acidic phospholipids of the cytoplasmic membrane in *E. coli* (Crooke, 2001; Regev et al., 2012) and like DARS, membranes can recharge DnaA-ADP (Garner and Crooke, 1996).

Eukaryotes and Archaea

Besides the CDK and DDK-dependent regulation required for helicase activation described previously, there is clear evidence that multiple mechanisms ensure once-*per*-cycle origin triggering in eukaryotic cells, reviewed in Arias and Walter (2007), Drury and Diffley (2009), and Siddiqui et al. (2013). Although many of the regulatory details remain obscure and considerable diversity exists among eukaryotic cell types, CDK-dependent prevention of re-replication is accomplished at both the origin licensing as well as origin firing steps by component destruction, blockage of complex assembly, and removal (export) of components from the nucleus (Fig. 4).

In most eukaryotes, the Cdc6 helicase loader is limiting during the cell cycle and synthesis is tightly restricted to late G1, to ensure that no origin is retriggered prematurely, see Arias and Walter (2007). To prevent relicensing, ubiquitin mediated degradation of Cdc6 is activated by S-phase CDK phosphorylation and phosphorylated Cdc6 is also transported out of the nucleus, see Drury and Diffley (2009). CDK-dependent phosphorylation of ORC (ORC2 and 6) also inhibits ORC/Cdc6/Mcm2–7 establishment to ensure once per cell cycle replication (Chen and Bell, 2011; Fernández-Cid et al., 2013; Frigola et al., 2013). CDK phosphorylates any Mcm2–7 not loaded onto origin DNA resulting in its export from the nucleus (Labib et al., 1999; Nguyen et al., 2000).

In metazoans, licensing is blocked by a CDK-independent protein factor geminin, which binds to and inhibits Cdt1 activity (Diffley, 2004; Caillat and Perrakis, 2012). Geminin is degraded during mitosis (McGarry and Kirschner, 1998; Li and Blow, 2004). Cdt1, as well as several other DNA replication/repair proteins, are also degraded by an S phase-coupled mechanism that is dependent on chromatin-bound PCNA, the processivity clamp for DNA polymerase delta (Arias and Walter, 2006; Havens and Walter, 2009; Coleman et al., 2015). Cdt1 ubiquitination precedes proteolysis and requires that a specialized motif found in Cdt1 interact directly with PCNA to recruit ubiquitin ligase (Havens and Walter, 2009).

Recent studies suggest chromatin structure plays a dynamic role in determining which origins will be triggered during any given eukaryotic cell cycle, reviewed in Cayrou et al. (2012), Méchali et al. (2013), Rhind and Gilbert (2013). Dynamic nucleosome positioning can enhance origin activity in an open chromatin state or occlude origins and block access to pre-RC factors (Kurat et al., 2017; Rodriguez et al., 2017). A permissive chromatin environment is a major characteristic of active transcription promoters and a correlation exists between transcription activity and enrichment for DNA replication origins (Cayrou et al., 2015; Sequeira-Mendes and Gutierrez, 2015; Miotto et al., 2016). However, interactions of pre-RC proteins with adjacent nucleosomes may also be important (Azmi et al., 2017). For example, ORC interactions with chromatin are regulated by the BAH domain of the ORC1 subunit in *S. cerevisiae* and in human cells (Noguchi et al., 2006; Müller et al., 2010). Histone modification also plays a role in the choice of DNA replication origins and in the regulation of their activation (Aggarwal and Calvi, 2004). At a higher level of chromosomal organization, the timing of replication domains strongly correlates with long-range chromatin interactions (Pope and Gilbert, 2013; Pope et al., 2014) and DNA replication origins may be clustered within open chromatin or chromatin loops (MacAlpine et al., 2010; Ryba et al., 2010).

Since archaea are not known to carry genes for cyclin kinases or Cdt1, cell cycle regulation must be accomplished by either changing the levels or activities of Orc1/Cdc6 and/or Mcm proteins (Akita et al., 2010; Lindås and Bernander, 2013; Samson et al., 2016), but this is not a simple issue to resolve due to the diversity of archaeal types. For example, the levels of Orc1/Cdc6 are reported to fluctuate during the cell-cycle in *Sulfolobus solfataricus* and *Sulfolobus islandicus* (Robinson et al., 2004; Samson et al.,

2016), but not in *Sulfolobus acidocaldarius*, or *Pyrococcus abyssi* where the protein is abundant and remains associated with origins (Matsunaga et al., 2007; Duggin et al., 2008).

In order to recruit DNA helicase, Orc1/Cdc6 must be bound to ATP (Samson et al., 2016). The ATPase domain of Orc1/Cdc6 interacts with the winged helix domain of the Mcm complex, and spontaneous ATP hydrolysis prohibits re-replication by blocking further helicase recruitment (Samson et al., 2016). A once-*per*-cycle switch of this type may require deactivation/reactivation factors, but these are not yet identified.

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Relevant Website

http://tubic.tju.edu.cn/doric — DoriC: An updated database of bacterial and archaeal replication origins.

Regulatory RNAs☆

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Glossary

aa Amino acid.
Aptamer Part of an riboswitch, RNA sequence that can bind a small ligand.
Antisense RNA RNA that is transcribed in opposite direction to its target RNA and interacts with its target by basepairing.
bp Base pair.
CDS Coding sequence of mRNA.
FMN Flavin mononucleotide.
FRET Fluorescence resonance energy transfer.
Hfq Host factor 1 for replication of phage Qβ. Abundant heat-stable RNA chaperone found in 50% of all sequenced bacterial species.
Inc Incompatibility group. Two plasmids are incompatible, when they cannot coexist in the same cell without selective pressure, because they share the same replication control elements.
kD Kilodalton.
nt Nucleotide.
ORF Open reading frame.
Riboswitch Sensory RNA, part of the 5′ untranslated leader region of an mRNA which can adopt two mutually exclusive conformations depending on the presence or absence of a small ligand molecule.
RNAP RNA polymerase.
RNA pseudoknot A single RNA molecule consisting of ≥ 2 helical segments connected by ≥ 1 single-stranded segment that often results in a complex tertiary structure.
RNase III Double-stranded RNA (dsRNA) specific endoribonuclease.
RNA thermometer Sensory RNA, part of the 5′ UTR of an mRNA, which can respond to temperature changes by adopting an open or closed conformation.
Ribozyme Catalytic RNA. Currently, nine natural ribozymes are known.
SAM *S*-adenosyl methionine.
SD Shine-Dalgarno sequence; equivalent to the ribosome binding site.
sRNA Small RNA, term used for bacterial RNAs, which are mainly untranslated and in size range between 50 and 500 nt.
TA module Chromosomally encoded toxin–antitoxin system.
TMP Thiamine monophosphate.
TPP Thiamine pyrophosphate.
Transcription attenuation Mechanism that includes two alternatively folded structures in the 5′ leader region of an mRNA, a terminator structure characterized by a rho-independent terminator, yielding a truncated RNA that terminates upstream of the SD sequence, and a read-through structure, that allows transcription of a full-length mRNA. Attenuation can be achieved by various means, for example, binding of an antisense RNA, prevention of binding of an aminoacylated tRNA, translation of a leader peptide, binding of a modified or unmodified protein etc.
Two-component system Regulatory system composed of a sensor histidine kinase and a response regulator that can be phosphorylated at asparagine residues.
UTR Untranslated region of mRNA.

Defining Statement

Small RNAs in microorganisms can be divided into cis- and trans-encoded base pairing sRNAs, sRNAs acting via protein binding, sensory RNAs, and RNAs with specific functions like tmRNA, RNase P, 4.5S RNA, and CRISPR RNAs. The article summarizes our current knowledge on the biochemical properties of these RNAs, their mechanisms of action, and their biological functions.

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Introduction

Small RNAs (sRNAs) have become increasingly recognized as critical gene expression regulators—both in prokaryotes and in eukaryotes. Already in the 1970s, about 10 small abundant RNAs were found in *Escherichia coli* using a two-dimensional gel electrophoretic approach. However, the function of most of these RNAs remained elusive long after their discovery. Since 2001, systematic computer-based searches and more recently, RNA-seq studies, have revealed that bacteria encode a surprisingly vast number of unannotated non-coding RNAs. In many cases, this can be upwards of 10–20% of the entire genome. By 2012, approximately 140 sRNAs have been identified in *E. coli* with an additional >160 putative sRNAs predicted computationally or detected via RNA-seq. Only a fraction of these have experimentally validated functions, indicating the difficulties that still exist to unravel their mechanisms of action. For species outside of the enteric bacteria, the number of characterized sRNAs drops precipitously. Thus, our current mechanistic understanding of sRNA biology is heavily biased for sRNAs produced in the enteric bacteria, principally *E. coli* and *Salmonella*. However, with the tremendous growth in the number of prokaryotic RNA-seq studies, a much more detailed picture of sRNAs is slowly emerging and new discoveries are increasingly being reported from species other than the traditional model organisms. In recent years, it has also become increasingly evident that sRNAs play a central role in virulence factor regulation for both plant and animal pathogens, which has greatly increased the general awareness and interest in sRNA biology.

Principally, small RNAs can be divided into two major groups: The first group is comprised of antisense RNAs that regulate gene expression by complementary base pairing. Among these antisense RNAs, we can distinguish between cis- and trans-encoded RNAs (Fig. 1). Cis-encoded RNAs contain regions of perfect complementary to their target RNAs, and consequently, form perfect duplexes with them. By contrast, trans-encoded RNAs are only partially complementary to their targets and typically form imperfect partial duplexes with usually multiple target RNAs. In both cases, the interaction between antisense RNA and target mRNA results in post-transcriptional inhibition or activation of target gene expression. The second group of sRNAs comprises RNAs that regulate gene expression either by binding to proteins or serving as "sponges" to titrate regulatory RNAs from their targets. In addition to these two major groups of RNAs, other prokaryotic small RNAs with diverse functions have been discovered and investigated over the past 30 years. Among them are tmRNA acting in trans-translation for increased translational efficiency, the RNase P ribozyme required for 5′ end processing of tRNA precursors, the 4.5S RNA component of the prokaryotic signal recognition particle, and CRISPR RNAs that confer adaptive immunity to phages and foreign genetic elements.

Small Regulatory RNAs

Location and Sources of Riboregulatory Elements

Initially identified on extra-chromosomal and mobile accessory genetic elements like plasmids, transposons, and bacteriophages, sRNAs are also encoded on the chromosomes of bacteria. Considering that the majority of bacteria reside in surface-attached communities (biofilms) that are notorious for their high rates of horizontal gene transfer, the actual pool of accessible sRNAs is dynamic and likely extends beyond what is chromosomally-encoded by an individual organism. Overall, most characterized sRNAs are non-coding, expressed from independent promoters, and have specific transcription terminator sequences. A recent survey comparing over 900 validated bacterial sRNAs and 23,000 intergenic regions (IGRs) indicated that sRNAs occur more frequently in IGRs. sRNA-encoding IGRs tend to be longer with a higher degree of conservation compared to the average IGRs. IGRs might also be

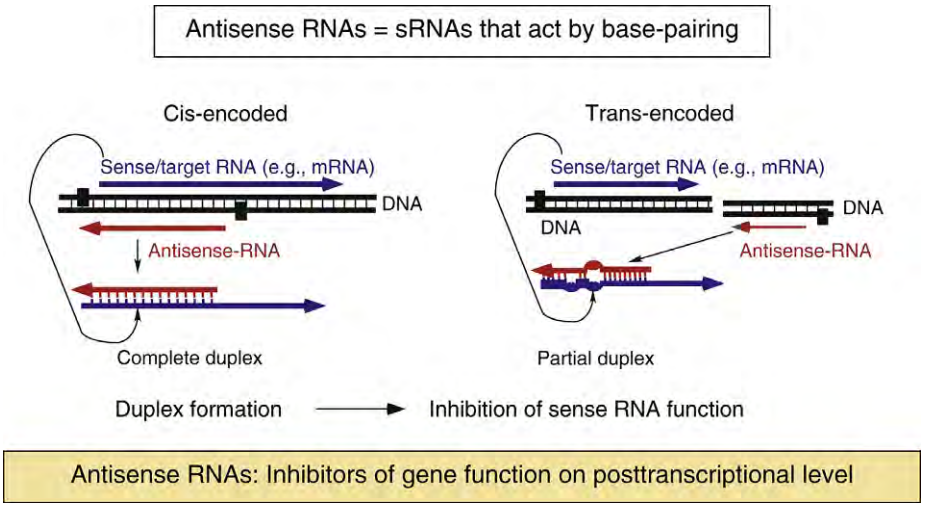

Fig. 1 Overview of cis- and trans-encoded antisense RNAs. Antisense RNAs are drawn in *red*, sense RNAs in *blue*. *Black* rectangles denote promoters.

the preferred breeding ground for the acquisition and/or evolution of new sRNAs, since mutations in IGRs tend to have less pronounced effects on overall cellular fitness. sRNAs can also be generated through RNase-dependent processing of larger transcripts, particularly at the 5′ or 3′ ends of transcripts. Furthermore, 5′ untranslated regions (UTRs) of mRNAs can act as regulatory RNA as well, further extending the repertoire of riboregulatory elements. Dual-function sRNAs encode both a functional riboregulatory sRNA and a peptide/protein. The recent discovery of riboregulatory protein-encoding mRNAs has revealed that our functional understanding of mRNA is likely oversimplified and demonstrates yet another bacterial mechanism of riboregulatory gene expression control. Specific examples are given throughout the text.

Cis-Encoded sRNAs

The majority of cis-encoded antisense RNAs (asRNAs) were found in so-called accessory genetic elements (i.e., plasmids, phages, and transposons). Among the first of these RNAs were discovered in 1981 in the *E. coli* plasmids ColE1 and R1 where they regulate replication, and hence, control plasmid copy numbers. Subsequently, in a wide variety of plasmids, phages, and transposons, cis-encoded antisense RNAs were found and intensively characterized over the past 35 years. In plasmids, antisense RNAs regulate replication, maintenance, and segregational stability. In phages, they have a fine-tuning function in the decision between lysis and lysogeny. In transposons, they control transposition frequency. Meanwhile, an increasing number of chromosomally encoded cis-acting sRNAs have been characterized as well. Among them are a variety of type I toxin-antitoxin (TA) modules, such as the *txpA*/RatA TA module from *Bacillus subtilis* and the *symE*/SymR TA module from *E. coli*. Cis-encoded asRNAs also regulate a variety of genetic pathways, such as the IsrR sRNA of *Synechococcus* that regulates the production of the photosynthesis component IsiA or the GadY sRNA of *E. coli* involved in the acid stress response via regulation of GadX and GadW synthesis (Fig. 2A). Table 1 provides a sampling of the diverse cis-encoded antisense RNAs for which targets have been identified.

Mechanisms of action

Thus far, the majority of antisense RNA interactions result in post-transcriptional inhibition of target RNA function, but a variety of activating mechanisms have been found as well. Characterized regulatory mechanisms employed by cis-encoded antisense RNAs are discussed below and summarized schematically in Fig. 2A.

Transcription attenuation

This mechanism was first discovered for the replication control of the staphylococcal plasmid pT181 (in 1989) and later (in 1993) for the streptococcal plasmids pIP501 and pAMβ1, belonging to the Inc18 family. Transcription attenuation has been only detected for one antisense RNA in Gram negative bacteria, in a virulence plasmid of *Shigella flexneri*. The nascent *rep* mRNA from these plasmids can adopt two mutually exclusive conformations. Upon binding of the antisense RNA, a terminator stem-loop is induced in the nascent *rep* mRNA, and consequently, transcription is terminated prematurely upstream of the *rep* ribosome binding site (RBS) preventing Rep protein synthesis and ultimately, replication. If the nascent *rep* RNA escapes antisense RNA binding, it can refold by complementary base pairing into an alternative structure that prevents terminator formation and allows transcriptional read-through. This results in Rep protein synthesis and consequently, replication (see Fig. 2A). The antisense RNA binds and exerts its inhibitory effect only during a short window of time and without a helper protein. RNAIII of plasmid pIP501 is composed of 4 stem-loops L1 to L4, of which the first two are dispensable both in vivo and in vitro. Loops L3 and L4 must both interact simultaneously with the complementary loops L1 and L2 of RNAII (*repR* mRNA) to yield efficient inhibition. The inhibition rate constant of RNAIII has been determined to be $1 \times 10^6\ M^{-1}s^{-1}$, whereas the pairing rate-constant was 10-fold lower, indicating that inhibition occurs faster than stable duplex formation between RNAIII and its target, RNAII. Structure probing of the complex between RNAII and RNAIII of pIP501 showed that it is not a complete duplex. The intracellular concentrations of both RNAII and RNAIII have been determined to be 1–2 μM and 50nM, respectively, ensuring a large excess of the regulator over its target. Normally, antisense RNAs that regulate plasmid copy numbers are short-lived with half-lives of 1–2 min. to quickly correct fortuitous copy number fluctuations. However, RNAIII is unusually long-lived with a half-life of ∼30 min. Consequently, a second control element called CopR is needed for proper control. The dimeric CopR protein binds asymmetrically at its two consecutive sites in the major groove of the DNA upstream of the *repR* promoter pII and represses *repR* transcription ∼10-fold. CopR also prevents convergent transcription from the sense and antisense promoters, thereby indirectly increasing transcription at the antisense promoter pIII. The concerted action of both control elements is required for efficient replication control.

Both the pIP501 and the pT181 systems work similarly and have been studied biochemically in terms of secondary structures of sense and antisense RNAs, binding kinetics, and sequence requirements for efficient inhibition (see below).

Translation inhibition

The inhibition of target mRNA translation by occlusion of its RBS is a commonly employed mechanism of cis-encoded sRNAs. In the plasmid pLS1, RNAII is complementary to the *repB*-RBS and directly inhibits ribosomal loading. The same mechanism is used by the FinP antisense RNA that blocks the RBS of *traJ*, an activator of conjugal transfer operons in the F and R1 plasmids. Duplex formation between the FinP sRNA and *traJ* mRNA is catalyzed by the FinO protein, which prolongs FinP half-life by protecting it against RNase E degradation (Fig. 2A). Additionally, the Sok and RNA I sRNAs (plasmids R1, pAD1) and RNA-OUT (IS10) use translational inhibition. In the former two cases, toxin synthesis from their respective plasmid addiction modules is inhibited, whereas in the latter, transposase expression is regulated. In the replication control systems of plasmid R1 and the IncB/IncIα

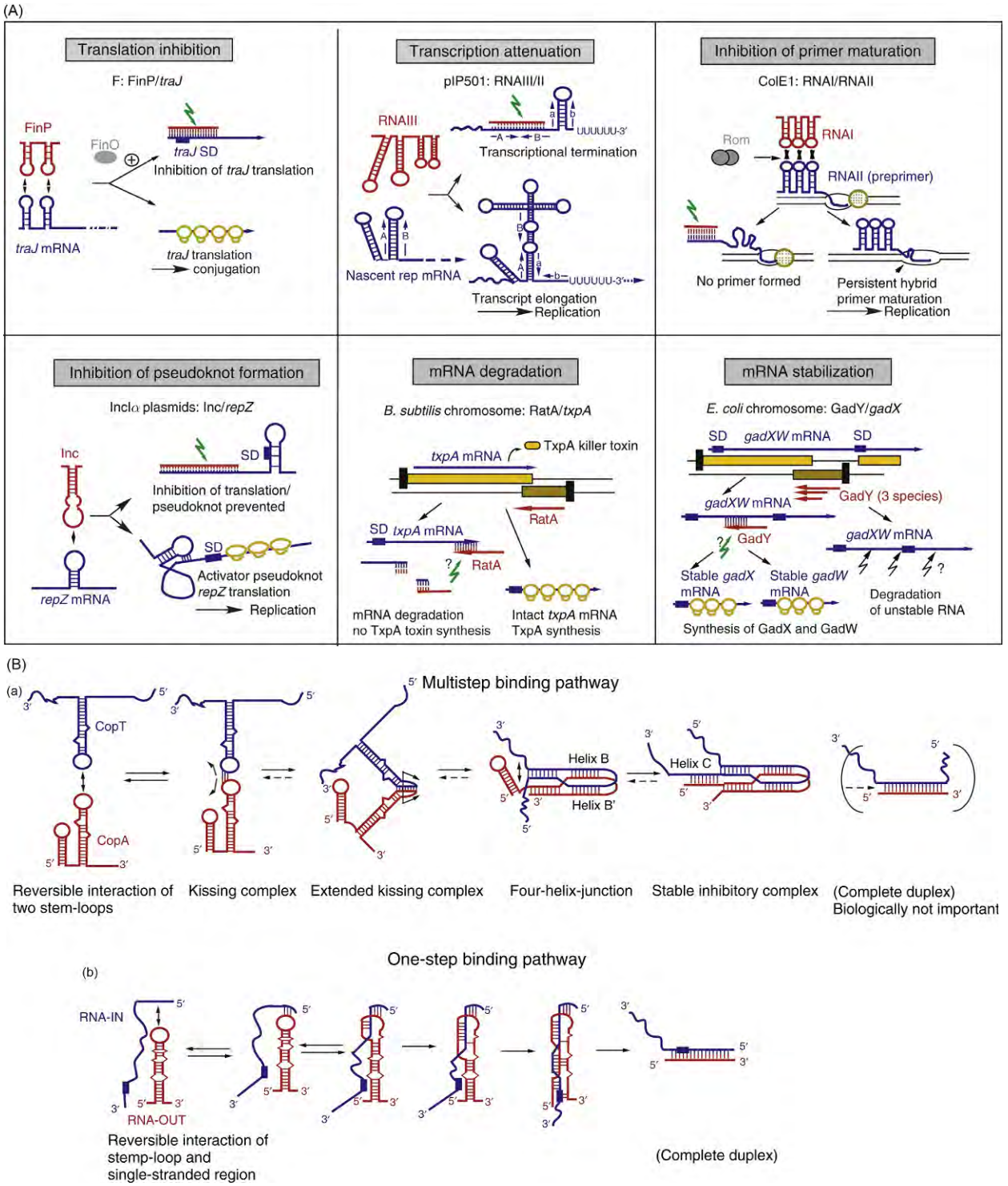

Fig. 2 Overview of regulatory mechanisms employed by cis-encoded antisense RNAs. (A) Antisense RNAs are drawn in *red*, sense RNAs in *blue*. *Black rectangles* denote promoters, *yellow* and *brown boxes* sense and antisense RNA genes. *Brown dotted circle*, DNA polymerase I; *yellow symbols*, ribosomes. *Green arrows* indicate action of RNase III, black arrows the putative action of other RNases. Details are described in the text. One example for each mechanism is given (?, not yet experimentally confirmed). The upper part of the figure is based on Brantl, 2002. (B) Binding pathways of antisense/sense RNAs. Antisense RNAs are shown in *red*, sense RNAs in blue. (1) The multi-step binding pathway of the sense/antisense RNA system CopT/CopA regulating replication of plasmid R1. (2) The one-step binding pathway of the sense/antisense RNA system RNA-IN/RNA-OUT regulating transposition of IS10. The initial contact occurs between the first 3G9s or C9s at the 59 end of RNA-IN and the complementary bases of RNA-OUT followed by an extension of stable pairing through the loop domain and the stem domain of RNA-OUT. Reproduced from Brantl (2007) Regulatory mechanisms employed by cis-encoded antisense RNAs. *Current Opinion in Microbiology* 10(2): 102–109.

Table 1 Examples of cis-encoded antisense RNAs

Antisense RNA/ target RNA	*Length of As RNA*	*Location*	*Biological function*	*Mechanism of action*	*Peculiarity*
Plasmid encoded RNAs		*Plasmid*			
RNAI/RNAII	108 nt	ColE1	Replication control	Inhibition of primer maturation	Rom protein; promotes kissing
RNAI/rep	115 nt	ColE2 and relatives	Replication control	Translation inhibition	
CopA/CopT	90 nt	R1 and other IncFII relatives	Replication control	Translation inhibition	Leader peptide; translat. coupling
RNAIII/RNAII	136 nt	pIP501 and *Inc18* relatives	Replication control	Transcription attenuation	Unusually stable antisense RNA
RNAI, II/repC	84 nt, 141 nt	pT181	Replication control	Transcription attenuation	2 antisense RNAs
Inc/repZ	71 nt	IncIαIncB relatives	Replication control	Inhibition of translation and pseudoknot formation	Leader peptide
RNAII/rep	50 nt	pLS1	Replication control	Translation inhibition	
RNAI/repA	82 nt	pSK41	Replication control	Translation inhibition	
Incα/repC	54–57 nt	pTiR10 relatives	Replication control	Transcription attenuation*	
Incα/repC	67 nt	pRmeGR4	Replication control	mRNA stability*; transcriptional interference*	
Sok/hok	64 nt	R1	Segregational stability	Translation inhibition	Activity in absence of gene locus
RNAII/RNAI	64 nt	pAD1	Segregational stability	Translation inhibition	2 complementary regions involved in complex
FinP/traJ	79 nt	F, R1 and relatives	Conjugation control	Translation inhibition	FinO protein stabilizes duplex
Qa, mD/prgX		pCF10, pAD1	Conjugation control	Transcription attenuation*	Pheromone represses Qa/mD
RNAα/fatA,B	650 nt	pJM1	Iron transport regulation	mRNA stability*	Regulation by chromosomal Fur protein, RNAα stabilized by iron
Transposon encoded RNAs		*Transposon*			
RNA-OUT/RNA-IN		IS10/Tn10	Transposition	Translation inhibition	
RNA-C		IS30	Transposition	Inhibition of translation elongation*	
Phage encoded RNAs		*Phage*			
OOP/cII	77 nt	I	Switch lysis/ lysogeny	mRNA stability	
C4, CI/icd-ant	77 nt	P1, P4	Switch lysis/ lysogeny	Transcription termination	
Sar/arc-ant	68 nt	P22	Switch lysis/ lysogeny	Translation inhibition	
Sas/sieB-esc	105 nt	P22	Superinfection override	Switch of translation start site	
T_{ant}/T1	151 nt	ΦH	Switch lysis/ lysogeny	RNA processing	Archaebacterial
Chromosomally encoded RNAs		*Species*			
P3 RNA/glnA	43 nt	*C. acetobutylicum*	Glutamine synthetase	Translation inhibition[a]	
Sof/gef		*E. coli*	Toxin/antitoxin system	Translation inhibition[a]	
Isf/sulA	350 nt	*E. coli*	SOS response?	?	
RdlD/ldrD	66 nt	*E. coli*/relatives	Toxin/antitoxin system	mRNA stability[a]	
GadY/gadXW	105/90/59 nt	*E. coli*	Acid response regulation	mRNA stabilization	3 GadY species; different length
RatA/txpA	222 nt	*B. subtilis*	Antitoxin/toxin	mRNA degradation	75 nt overlap
IsrR/isiA	176 nt	*Synechocystis spec.*	Photosynthesis component	mRNA degradation[a]	Regulation by iron stress
SymR/symE	77 nt	*E. coli*	Antitoxin/toxin	Translation inhibition	SOS induced
RyjB/sgcA	90 nt	*E. coli*		?	

Table 1 Continued

Antisense RNA/ target RNA	*Length of As RNA*	*Location*	*Biological function*	*Mechanism of action*	*Peculiarity*
			Phosphotransferase II component		
RyeA, B/pphA	100, 275 nt	*E. coli*	Phosphatase I	?	
srSM/fst-Sm	68 nt	*S. mutans*	Antitoxin/toxin	Inhibition of toxin translation	Persister cell formation

For plasmid encoded RNAs, the antisense RNA of best studied example is given.

?, no mechanism proposed.

[a]Mechanism proposed but not experimentally substantiated.

plasmids, the sRNAs CopA and Inc inhibit translation of a leader peptide, which is required for efficient Rep translation via translational coupling. A variety of chromosomally encoded type I toxin-antitoxin (TA) systems also utilize various mechanisms of translation inhibition to modulate the production of toxins. Both type I and type III TA modules utilize cis-encoded sRNAs as antitoxins. However, only type I systems act post-transcriptionally upon toxin mRNAs, whereas type III antitoxins act post-translationally by binding directly to toxin proteins. For type I systems, toxin mRNA translation can be either directly repressed or indirectly via the modulation of toxin mRNA stability. The former mechanism is employed by the *symE*/SymR TA module of *E. coli*. SymR is a 77-nt sRNA transcribed from the opposite DNA strand as the SymE toxin. The +1 site of SymR is located within the 2nd codon of the *symE* ORF. Thus, its region of complementarity encompasses both the *symE* translation initiation codon and its RBS, resulting in potent translation inhibition when the mRNA/sRNA molecules are duplexed. A similar mechanism is observed in the *fst-Sm*/srSm TA module of *Streptococcus mutans*. However, in this case, the region of complementarity does not include the RBS of *fst-Sm* toxin mRNA, rather the hybrid duplex begins 7-nt downstream of the RBS to sequester the translation initiation codon. Thus, it is not always necessary to directly occlude the RBS to efficiently inhibit mRNA translation. A similar result has also been demonstrated for the *B. subtilis bsrG*/SR4 type I TA system. In this case, the bifunctional antitoxin SR4 binds to the 3′ region of the toxin mRNA promoting its degradation, in addition to inhibiting toxin translation by introducing a structural change far upstream near the *bsrG* RBS.

Inhibition of primer maturation

Replication via a plasmid-encoded primer (known as RNAII) has thus far only been observed for ColE1 plasmids and its relatives. These plasmids are among the few known antisense RNA regulated plasmids, which also do not encode their own replication initiator proteins, and therefore, rely solely on host proteins. To initiate plasmid replication, a persistent RNAII/DNA hybrid must be formed at the plasmid origin and this hybrid is itself dependent upon RNAII first forming specific secondary and tertiary structures during RNAII synthesis. If RNAII is able to adopt its mature structure, it can bind at the plasmid origin, be further extended by DNA polymerase I, and then processed by RNase H to yield the final primer required for DNA replication. Conversely, to inhibit plasmid replication, duplex formation with the RNAII pre-primer and the plasmid-encoded antisense RNA called RNAI occurs to prevent RNAII primer maturation. The kissing complex between RNAI and RNAII is stabilized by the plasmid-encoded Rom protein, but can also occur spontaneously in the absence of Rom. It is worth noting that ColE1-derived cloning vectors like pUC18/19 replicate at greatly increased copy numbers (200–300 copies/cell), due to a mutation in RNAI that impairs its inhibitory function (Fig. 2A).

Inhibition of an activator RNA pseudoknot

Rep protein production in IncB, IncIα, IncK, IncL/M plasmids requires a long-distance activator RNA pseudoknot. As in R1, a leader peptide ORF, *repY*, must be translated to promote the formation of an activator pseudoknot structure instead of an alternate mRNA structure that would otherwise occlude the *rep* RBS. The corresponding antisense RNAs have two functions, as they prevent leader peptide translation, and consequently, also prevent activator pseudoknot formation (Fig. 2A).

Promotion of mRNA degradation or processing

For many years, only a few antisense RNAs were known to influence mRNA stability, among them the λ-OOP RNA that facilitates RNase III-dependent decay of the *cII* mRNA as well as RNAα expressed from plasmid pJM1 that affects the stability of both *fatA* and *fatB*-mRNA in *Vibrio anguillarum*. For the IsrR sRNA involved in the iron stress response of *Synechocystis* 6803, IsrR-mediated inhibition of its target mRNA *isiA* occurs through a codegradation mechanism. Under normal growth conditions, IsrR remains highly abundant in the cell and will trigger the degradation of duplexed *isiA* mRNA. However, during iron deplete conditions, *isiA* transcription is induced, causing its accumulation in the cell. Once *isiA* abundance exceeds that of the inhibitory sRNA IsrR, *isiA* mRNA can be translated to yield the iron stress-induced protein A (IsiA). Likewise, a number of type I TA systems from *B. subtilis*

have also been shown to utilize an antisense RNA-mediated target mRNA degradation mechanism. For example, the aforementioned RatA and *txpA* TA module encodes >100 bp region of complementarity within the 3′ portion of both molecules that supports duplex formation and serves as a substrate for RNase III and RNase Y cleavages. The degradation of duplexed *txpA* mRNA prevents its translation and the subsequent accumulation of toxin within the cell. A variation of this theme was observed for the *B. subtilis bsrG*/SR4 TA module. Here, 3′ complementarity between the SR4 antitoxin and *bsrG* mRNA similarly creates a substrate for RNase III activity. However, duplex formation serves the additional function of remodeling a *bsrG* secondary structure to sequester its RBS, thus further suppressing translation of the transcript. Duplex formation with cis-encoded antisense RNAs can trigger stabilizing RNA processing events as well. The *E. coli* GadY/*gadX* system is an example. The *gadX* gene is the first gene of the *gadXW* operon and encodes a transcription regulator involved in the glutamate-dependent acid resistance system. In addition, *gadX* mRNA also possesses 3′ complementarity with the GadY sRNA that is directly responsible for increasing *gadX* mRNA levels by increasing its stability due to processing via RNase III. Duplex formation between GadY and *gadX* mRNA creates a substrate for RNase III cleavage of the *gadXW* transcript resulting in two products that are more stable than the full-length transcript.

Binding kinetics, binding pathway, and requirement of RNase III

For many systems, antisense/sense RNA binding pathways have been studied in detail and binding kinetics measured. Analyses of pairing rate constants have usually yielded values of $\sim 10^6\ M^{-1}\ s^{-1}$. The initial contact between antisense and sense RNA can either occur between two complementary loops (many replication control systems) (Fig. 2 B) or between a loop and a single-stranded region (e.g., RNA-IN/RNA-OUT of IS10, *hok*/Sok of plasmid R1) (Fig. 2 C). In the first example, simple helix progression in both directions is topologically impossible due to accumulating torsional stress. Therefore, loop-loop initiating systems require a subsequent interaction at a distal site to remodel its secondary structure and circumvent this limitation. Regardless of whether a one-step or multi-step pathway is utilized (see Fig-. 2B and C), the final result of the interaction is a complete duplex that is often degraded by the double-strand specific RNase III, which requires a $\geq$20 bp duplex (two helical turns) as a minimal substrate (shown for CopA/CopT, RNA-OUT/RNA-IN and *hok*/Sok). However, despite the fact that cis-encoded antisense RNAs are fully complementary to their targets, the formation of complete duplexes is sometimes too slow to account for the observed biological effects. Instead, many antisense RNAs mediate inhibition by forming complexes that involve limited numbers of base pairs with their targets. As has been demonstrated for R1, ColE1, pIP501, and the IncB/IncIα type plasmids, full duplex formation is not required for control. For the replication control of R1, a partially paired binding intermediate is sufficient for inhibition in vivo. Similarly, in vitro analyses of two antisense RNA-mediated transcription attenuation systems of Gram-positive bacteria, pT181 and pIP501 also yielded partial duplexes. In pIP501, complexes between the complementary loop pairs of sense and antisense RNA form and progress into the stems, but the spacer between the two stem-loops remains single-stranded and is unimportant for inhibition.

For the replication control system of plasmid R1, the binding pathway between the CopA sRNA and its complementary target within the *repA* mRNA (called CopT) has been elucidated in detail (Fig. 2B). Binding initiates with an unstable loop-loop interaction (kissing complex) that is converted into an extended kissing complex. Later, a single-stranded region is required to overcome the torsional stress created upon the unidirectional progression of this loop-loop interaction. Next, a binding intermediate is formed, which contains a four-helical junction. This intermediate is transformed into a stable inhibitory complex, which is only a partial duplex and is slowly converted into a stable duplex cleaved by RNase III. This step-wise binding pathway and four-helix junction is conserved in the IncIα and related plasmids as well.

In contrast to R1 and IS10, in λ OOP RNA, RNase III cleavage was found to be necessary for control. This is probably because the mechanism exerted by λ OOP RNA is principally mRNA degradation, whereas in the other systems, translation is inhibited before degradation occurs.

Pervasive antisense transcription

Despite the limited number of chromosomally derived cis-encoded asRNAs with known regulatory functions, data from numerous transcriptomic studies have demonstrated that most bacteria produce a surprisingly large number of antisense transcripts. The abundance of asRNAs seems to vary widely among species, but thus far, it appears that Gram positive organisms produce a greater abundance compared to the enteric species. It is currently unclear whether the majority of these asRNAs have *bona fide* riboregulatory functions (like those described previously) or whether they serve some other purpose, if any at all. In a comparative study of *E. coli* and *S. enterica*, it was found that both of these closely related organisms produce similar abundances of asRNA. However, only about 14% of these asRNA transcripts appeared to be conserved between them and there was no obvious conservation of promoter elements, even among strains of the same species. This contrasted sharply with a concurrent comparison of protein-encoding genes, leading to the suggestion that most asRNAs are likely nonfunctional. Another comparative genomic analysis of a more diverse collection of species further observed that the abundance of asRNAs in a species correlates exponentially with its genomic A+T content. This result argues in favor of a transcriptional noise hypothesis that stipulates most asRNAs are transcribed due to the creation of spurious promoters having sequences similar to the sigma 70 (σ^{70}) consensus −10 (TANAAT). However, studies of double stranded RNA (dsRNA) in both *Staphylococcus aureus* and *E. coli* seem to support an opposite conclusion. In *S. aureus*, it was estimated that 75% of sense RNAs from annotated genes are subject to processing from RNase III due to their interaction with asRNAs. In *E. coli*, specific enrichment of dsRNA yielded 316 potentially functional asRNAs that are also subject to processing from RNase III. In both cases, it appears that asRNA plays a significant role in shaping global gene expression patterns, particularly via the action of RNase III. Thus, it is currently difficult to reconcile the lack of evolutionary conservation among asRNA with their observed function as global regulators of gene expression.

Trans-Encoded sRNAs

The first chromosomally derived trans-encoded RNA, MicF, was detected in 1984. This 93 nt sRNA forms a 20 bp imperfect RNA duplex with the translation initiation region of the *ompF* mRNA encoding a porin of the *E. coli* outer membrane. By the mid 1990s, only a handful of trans-encoded sRNAs were known, such as Spot 42, DsrA, and OxyS. However, it is now known that most, if not all, bacteria produce a tremendous number of trans-encoded sRNAs. Many of the *E. coli* sRNA genes are conserved in closely related pathogens, such as *Salmonella* and *Yersinia* species, indicating they are likely functional regulators. A large number of these characterized sRNAs regulate porins of the *E. coli* outer membrane. Many other sRNAs still await target identification and functional characterization. In Gram positive bacteria, a smaller, but increasing number of sRNAs has been characterized and a much larger number of putative sRNAs has been predicted computationally or detected via RNA-seq. Table 2 gives an overview of characterized trans-encoded sRNAs.

Biological functions

Trans–encoded sRNAs have been implicated in a wide variety of regulatory pathways. Often, sRNAs appear to function in the fine-tuning of gene regulation, which is reflected by a frequent lack of severe phenotypes upon their deletion or overexpression. However, trans–encoded sRNAs typically control diverse regulons and exhibit functional redundancies. In these instances, the full influence of sRNA regulation upon a particular pathway may only be obvious after mutating multiple sRNAs within the same cell. An example of such redundancy is found among the 4 Qrr sRNAs of *V. cholerae*, which together play a central role in regulating quorum sensing in this species. Some of the better-characterized sRNAs from enteric bacteria regulate pathways such as iron transport and storage (RyhB), membrane composition (MicA, MicC, MicF, RseX, OmrA/B, RybB, InvR), carbohydrate utilization (Spot 42), stress response to phosphorylated carbohydrates (SgrS), and stationary phase regulation (OxyS, DsrA, RprA). In some instances, a single sRNA regulates a set of mRNAs involved in the same metabolic pathway. One example is RyhB of *E. coli* that regulates at least 6 target mRNAs, *sodB*, *sdhD*, *acnA*, *fumA*, *bfr*, *ftn* and *shiA*, encoding proteins required for iron transport and storage. Similarly, two σ^E-controlled sRNAs of *Salmonella*, MicA and RybB, destabilize various outer membrane porin mRNAs as part of the envelope stress response. While MicA selectively facilitates the decay of *ompA* mRNA, RybB was found to accelerate the decay of at least 8 major outer membrane porin mRNAs, *ompA*, *ompF*, *ompS*, *ompC*, *ompN*, *ompW*, *ompD*, and *fadL*. In other cases, one mRNA can be the target of several sRNAs that inhibit or activate its expression under different environmental conditions. One such example is *rpoS* mRNA encoding the *E. coli* stationary phase sigma factor σ^S. At low temperatures, DsrA sRNA activates translation of *rpoS* mRNA, whereas under conditions of oxidative stress, RpoS levels are down-regulated by OxyS. A third sRNA, RprA, activates *rpoS* mRNA translation under conditions of osmotic shock and cell surface stress. A fourth *rpoS*-activating sRNA, ArcZ, is repressed by the aerobic/anaerobic-sensing ArcA-ArcB TCS under anaerobic conditions.

Mechanisms employed by trans-encoded antisense RNAs

Currently, translation inhibition is the most frequently observed regulatory mechanism employed by trans-encoded sRNAs and there are three different ways sRNAs can achieve this: (i) by the direct occlusion of an RBS, (ii) by the induction of an mRNA secondary structure that occludes the RBS, or (iii) by blocking a ribosome standby site required for efficient translation (Fig. 3). Interestingly, in some cases translational inhibition is accompanied by mRNA degradation, whereas in others, no mRNA degradation has been observed. In some instances, trans-encoded sRNAs can specifically target an mRNA for degradation, which ultimately prevents translation as well. In a minority of characterized examples, trans-encoded sRNAs are known to activate translation of their target mRNAs. In such cases, sRNA base pairing either promotes access to an RBS or ribosome standby site or it directly inhibits RNase cleavage of an mRNA. Common mechanisms employed by trans-encoded sRNAs are discussed below and summarized in Fig. 3.

Translation inhibition

Translation inhibition by direct occlusion of the RBS Direct RBS occlusion by sRNA base pairing is the most frequently observed mechanism of translation inhibition, shared by both cis- and trans-encoded antisense RNAs. Examples of trans-encoded sRNAs using this mechanism include OxyS, Spot42, MicA, MicC, MicF, RyhB, and SgrS. For this mechanism, sRNA base pairing overlaps with the target RBS and/or its adjacent 5′ or 3′ regions. For MicA and MicF, their complementary regions include the *ompA* and *ompF* RBSs, respectively, whereas for MicC, the two complementary regions within *ompC* are 6 and 16 bp immediately upstream of the RBS. In the case of OxyS, two interacting regions were found, 7 bp overlapping the target *fhlA* RBS and a 9 bp interaction about 25 nt downstream of the AUG start codon. For SgrS, a 23 bp discontinuous stretch of complementarity was found to overlap the RBS and start codon of *ptsG* mRNA. However, mutational analyses revealed that only 6 bp around the RBS are crucial for regulation.

Translation inhibition by induction of structural changes downstream from the RBS SR1 from *B. subtilis* is one of the few sRNAs known to employ this mechanism. SR1 interacts with *ahrC* mRNA encoding a transcriptional activator of the arginine catabolic operon. Both RNAs share seven complementary regions in the 3′ regions of both SR1 and *ahrC* mRNA. The distal complementary region (called Region G) is located ~100 nt downstream from the RBS of *ahrC* mRNA. Binding of SR1 induces structural alterations in all seven complementary regions as well as immediately downstream of the *ahrC* RBS and upstream of region G. These changes in secondary structure effectively inhibit translation initiation as demonstrated by toeprinting analysis. Thus, base pairing interactions far from the RBS can still potentially prevent ribosome assembly at the RBS.

Table 2 Examples of trans-encoded antisense RNAs

sRNA (length)	*Target RNA(s)*	*Biological function*	*Mechanism of action*	*Control of expression*
Escherichia coli				
MicA (72 nt)	*ompA*	Membrane composition	Translation inhibition	σ^E, stationary phase
MicC (109 nt)	*ompC*	Membrane composition	and mRNA degradation	Low temperature
MicF (93 nt)	*ompF*	Membrane composition	Translation inhibition	High temperature, salt, HU, H-NS, Lrp, OmpR, SoxS, MarA, Rob
RseX (≈90 nt)	*ompA, ompC*	Membrane composition		?
OmrA/OmrB (88/82 nt)	*cirA, fecA, fepA, ompT*	Iron transport, protease		High osmolarity, OmpR
IpeX (167 nt, on prophage)	*ompC, ompF*	Membrane composition	mRNA degradation?	Phage-encoded porin gene
RybB (78 nt)	*ompC, ompW*	Membrane composition		σ^E
RyhB (90 nt)	*sodB, sdhD, acnA, fumA, bfr, ftn, shiA*	Iron metabolism, shikimate permease	Translation inhibition and mRNA degradation, translation activation	Fur, iron
Spot42 (109 nt)	*galK*	Galactose utilization	Translation inhibition	cAMP-CAP, glucose
SgrS (227 nt)	*ptsG*	Glucose transport	Translation inhibition (mRNA degradation)	SgrR, phosphorylated, carbohydrate stress
DsrA (87 nt)	*rpoS*	Stationary phase	Translation activation	Low temperature, osmotic shock, LeuO?
	hn-S	Stationary phase	Translation inhibition	
RprA (106 nt)	*rpoS*	Stationary phase	Translation activation	Osmotic shock, cell surface stress, RcsB
OxyS (109 nt)	*rpoS*	Stationary phase	Sequestration of Hfq	Oxidative stress
	fhlA	Formate metabolism	Translation inhibition	
GcvB (130 nt)	*oppA, dppA*	Peptide transport	Post-transcriptional control	GcvA, GcvR, glycine excess
IstR-1 (75 nt)	*tisB*	Antitoxin	Translation inhibition by competing with standby ribosomes	–
Salmonella typhimurium				
MicA (72 nt)	*ompA, X*	Membrane composition	Translation inhibition?	σ^E
RybB (78 nt)	*ompC, D, F, N, S, W*	Membrane composition	Translational inhibition? mRNA degradation	σ^E
InvR (80 nt)	*ompD*	Membrane composition		HilD
SdsR (100 nt)	*ompD*	Stress control	Post-transcriptional repression	σ^S
RydC (64 nt)	*csgD*	Biofilm formation, curli production	Prevention of translation initiation	
Pseudomonas aeruginosa				
PrrF1/PrrF2/PrrH (110 nt, 110 nt, 325 nt)	*sodB, sdhD, bfr*	Iron metabolism	Translation inhibition and mRNA degradation	
RgsA (122 nt)	*fis, acpP*	Swarming motility, pyocyanin synthesis, oxidative stress response	Post-transcriptional level	RpoS, exponential to stationary phase
PhrS (212 nt)	*pgsR*	Quorum sensing	Facilitates ribosome access	
NrsZ (processed form 60 nt)	*rhlA*	Nitrogen dependent modulation of swarming	Liberation of RBS	NtrB/C TCS, RpoN
EsrA (132 nt)	*algC*	Host infection/colonization	Translational inhibition	Stationary phase
Chlamydia trachomatis				
IhtA (120 nt)	*hctA*	Histone homologue, RB to EB differentiation	Translational inhibition	?
Bacillus subtilis				
SR1 (205 nt)	*ahrC*	Arginine catabolism	Translation inhibition by inducing structural changes downstream of RBS	CcpN, glucose
RoxS(RsaE) (115 nt)	*ppnKB, sucCD*	Oxidative stress	Translation inhibition	Increased NO levels
RnaC (126 nt)	*abrB*	Generates diversity in growth speed	Post-transcriptional	Exponential growth phase
Staphylococcus aureus				

Table 2 Continued

sRNA (length)	*Target RNA(s)*	*Biological function*	*Mechanism of action*	*Control of expression*
RNAIII (514 nt)	*hla*	Hemolysin synthesis	Translation activation	
AgrC, AgrA,	*spa, rot, sa1000, sa2353, coa*	Host–pathogen interaction	Translation inhibition and mRNA degradation by RNaseIII	Stationary phase
SprA (202 nt)	*SA2216-ORF*	ABC transporter?	Post-translational?	Strain-specific
SprX (150 nt)	*spoVG*	Glycopeptide resistance	Inhibition of ribosomal loading	
SprD (142 nt)	*sbi*	Immune evasion	Inhibition of ribosomal loading	Stationary phase
RsaA (139 nt)	*mgrA*	Bacterial persistence	Inhibition of ribosomal loading	Late-exponential phase
Streptococcus pyogenes				
pel RNA (459 nt)	*speB, emm, sic, nga*	Cysteine protease, M- and -related proteins	Post-transcriptional, transcriptional control	Stationary phase, conditioned media
fasX (205 nt)	*ska*	Thrombolytic streptokinase	Post-transcriptional, increased	Dissemination
	prtF1 & *prtF2*	Fibronectin binding	Post-transcriptional, decreased	Dissemination
	fctA	Pili, adhesion	Post-transcriptional, decreased	Dissemination
Vibrio cholerae/ harveyi				
Qrr1-4 (96, 108, 107, 107 nt)	*hapR*	Quorum sensing	mRNA destabilization	LuxO-P, σ^{54}
VqmR (151 nt)	*vpsT*	Biofilm formation	Translation repression	VqmA
TarA (91 nt)	*ptsG*	Virulence	Inhibition of ribosomal loading	ToxT, colonization
TfoR (102 nt)	*tfoX*	Natural competence	Translational activation	Activated by chitin
VrrA	*vrp*	Stress survival	Ribosome modulation	Alternative sigma factor E (σE), stationary phase

Translation inhibition by regulation of a ribosome standby site or a translational enhancer A well-characterized example of this mechanism involves the regulation of *E. coli tisB* mRNA by the sRNA IstR1. A ribosome standby site has been found for *tisB* located approximately 100 nt upstream of the *tisB* RBS and this site is required for efficient translation of the TisB toxin. The antisense RNA, IstR-1, is complementary to this site and competes with standby ribosomes for binding. The IstR-1/*mRNA* interaction generates a cleavage site for RNase III, which results in a 5′ truncated *tisB* mRNA that cannot be translated. A variation on this theme has been found for the *E. coli* GcvB sRNA involved in the regulation of amino acid uptake genes. GcvB contains U/G-rich regions that are crucial for binding to upstream ACA trinucleotide translational enhancer elements required for the efficient translation of downstream RBSs. By antagonizing these elements, GcvB can potently inhibit the translation of multiple target mRNAs containing ACA enhancers, such as *yifK* and *dppA*.

Combined translation inhibition and mRNA degradation Frequently, translation inhibition by a trans-encoded sRNA is found to be accompanied by degradation of the target mRNA by one or more RNases. In some cases, RNase degradation is essential for effective inhibition, as with RNAIII of *S. aureus*. RNAIII is an unusually long sRNA (514nt) composed of 14 stem-loops and two long-distance interactions that define independent structural domains. Among these, three hairpin-loops carry C-rich sequences complementary to multiple RBSs. RNAIII is also an example of a peptide-encoding dual-function sRNA (see below) and can both activate translation of some targets like *hla* and inhibit translation of others, such as *spa*, *sa*1000, and *rot* (Fig. 3). Both *spa* and *sa*1000 mRNAs are targeted by a single hairpin loop of RNAIII, whereas *rot* is targeted by two redundant hairpin loops. One loop-loop contact occurs within the *rot* RBS and the other with another stem-loop. Despite the slight differences in target mRNA binding, the subsequent RNAIII inhibitory mechanism is the same: the formation of RNAIII/mRNA hybrid duplexes, inhibition of ribosome binding, and degradation of duplexed RNA by RNase III (Fig. 3). It should also be noted that as a general rule, untranslated mRNAs are less stable than actively translated transcripts, since ribosomes tend to exclude access to endogenous RNase cleavage sites. Thus, translation inhibition and mRNA degradation are frequently coupled events, though not exclusively.

Translation activation For some mRNAs, Shine-Dalgarno (SD) sequences are sequestered within double-stranded secondary structures, which prevent ribosome access and inhibit translation of the transcript. An sRNA can promote the melting of such a structure by forming a hybrid duplex to one strand of the mRNA, thus liberating its complementary strand and RBS. For the aforementioned RNAIII from *S. aureus*, its ability to activate translation of the *hla* mRNA encoding α-hemolysin is due to the remodeling of an endogenous *hla* secondary structure that normally sequesters its RBS within the stem of a hairpin. RNAIII interacts with the anti-SD sequence located in *hla* mRNA to remodel this hairpin and free the RBS for ribosomal loading. A similar mechanism has been demonstrated for DsrA from *E. coli*, which promotes translation of *rpoS* mRNA encoding the stationary phase sigma factor σ^S. Under normal growth conditions, the *rpoS* RBS is sequestered within ~100 nt hairpin that inhibits its

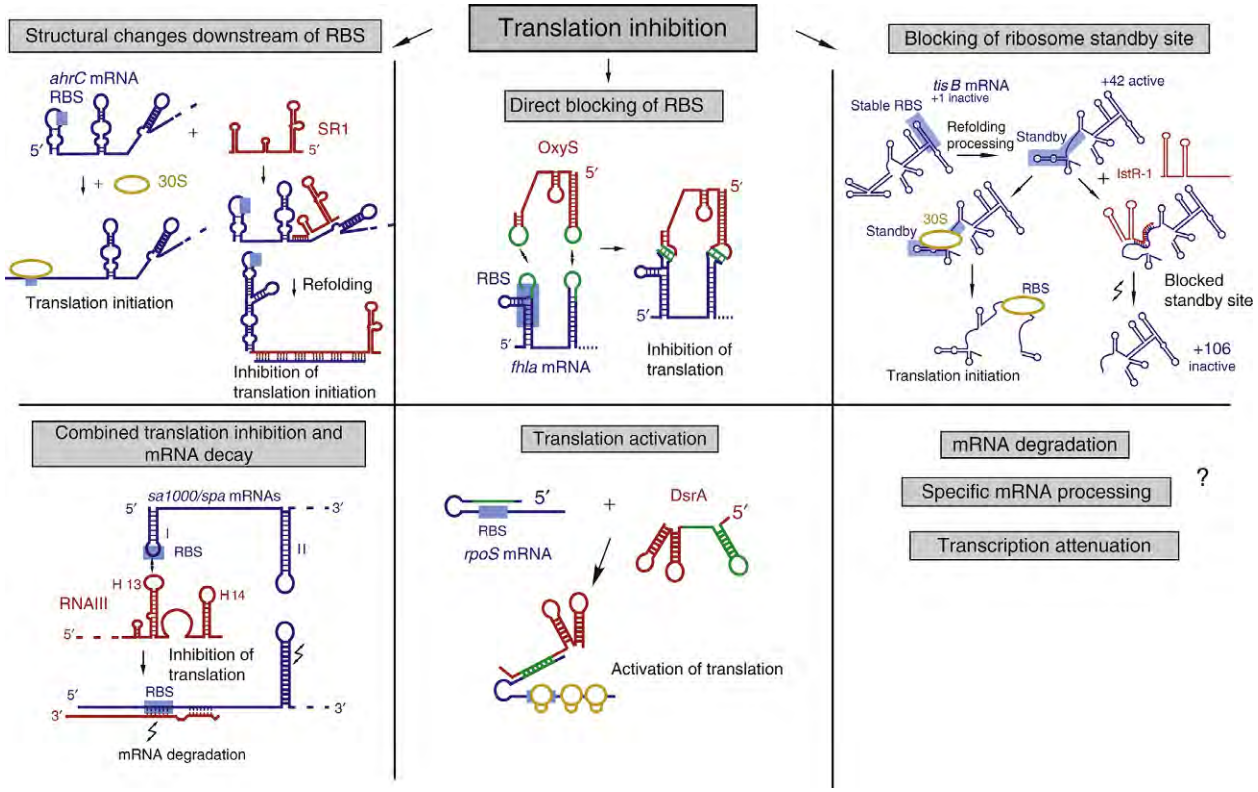

Fig. 3 Overview of regulatory mechanisms employed by trans-encoded antisense RNAs. Antisense RNAs are drawn in *red*, sense RNAs in *blue*. *Green*, regions complementary between sense and antisense RNA, *light blue*, ribosome binding sites. *Black arrows* denote RNase III action. *Yellow symbols* indicate ribosomes.

translation initiation. However, under conditions where DsrA is active, such as low temperature, it can bind to the anti-SD to free the RBS for translation. In contrast to the RNAIII example, the interaction between DsrA and *rpoS* also requires the RNA chaperone Hfq (see below) to catalyze duplex formation.

Dual-function sRNAs and mRNAs While sRNAs are generally regarded as non-coding RNAs, it is now evident that a subset of trans-encoded sRNAs encode actively translated ORFs. These molecules are referred to as dual-function sRNAs and examples can be found in both Gram positive and Gram negative bacteria. The antisense riboregulatory functions of dual-function sRNAs are identical to the aforementioned examples of typical sRNAs (i.e., translation activation or repression, target mRNA stabilization or destabilization, etc.). The distinguishing feature of dual-function sRNAs is simply the presence of their encoded proteins. Thus far, all characterized dual-function sRNAs have been demonstrated to encode peptides ranging in size from 26–53 amino acids, and of these, only several have known functions. Interestingly, these peptides may or may not function within the same pathways targeted by their encoding sRNAs. For example, RNAIII from *S. aureus* was the first recognized dual-function sRNA. The *hld* coding sequence (CDS) within RNAIII encodes the 26 amino acid δ-toxin, which seems to primarily function in the lysis of host cell membranes. The δ-toxin is not known to directly affect any of the pathways targeted by RNAIII. In fact, base pairing interactions between RNAIII and one of its target mRNAs (*hla*) obscures the RBS of *hld*, suggesting that riboregulation by RNAIII and the production of δ-toxin are likely to be uncoupled events. In contrast, the SgrS dual-function sRNA from *E. coli* encodes the 43 amino acid peptide SgrT, which functions independently in the same glucose-phosphate stress response pathway as SgrS. Production of SgrS is triggered by a toxic accumulation of phosphorylated carbohydrates within the cytoplasm. To mitigate this stress, SgrS represses the translation of the *ptsG* and *manXYZ* mRNAs encoding carbohydrate transporters, whereas the SgrT peptide inhibits carbohydrate uptake through preexisting carbohydrate transporter complexes. Another example is SR1 from *Bacillus subtilis*. In addition to its base-pairing function in arginine catabolism (see above) SR1 encodes a small, 39 aa peptide designated SR1P. SR1P binds to GapA, one of the two *B. subtilis* glyceraldehyde-3P-dehydrogenases that participate in glycolysis. In 2016, it was shown that GapA has a moonlighting function in RNA degradation by forming a complex with RNase J1 and the main *B. subtilis* endoribonuclease RNase Y. Interestingly, SR1P modulates the moonlighting activity of GapA. By promoting the interaction between GapA and RNase J1, it can also regulate the activity of RNase J1.

The existence of dual-function sRNAs indicates that riboregulatory RNAs are fully capable of serving as templates for translation like typical protein-encoding mRNAs. Such a function is not unique to prokaryotes, as a variety of riboregulatory RNAs in eukaryotes also encode functional peptides, such as the *ENOD*40 RNA from the legume *Medicago truncatula*. Perhaps not

surprisingly, examples of riboregulatory protein-encoding mRNAs also have been reported, which suggests that mRNAs in general should be considered as potential riboregulatory molecules. In the Gram positive organism *Streptococcus mutans*, the *irvA* gene encodes a putative transcription regulator that was originally hypothesized to control the expression of a target gene called *gbpC*. Surprisingly, the 5′ UTR of *irvA* and not its encoded protein was determined to be the principal regulator of *gbpC* gene expression. In this case, the *irvA* 5′ UTR was shown to bind to the CDS of *gbpC* mRNA and block access to an endogenous RNase cleavage site, thereby preventing *gbpC* degradation and stimulating GbpC protein production. The *irvA* 5′ UTR was also demonstrated to be functionally modular, since its riboregulation of *gbpC* remained fully intact when the UTR was fused to the CDS of green fluorescent protein (GFP). This result implies that other mRNAs could still have a wild-type riboregulatory function even if the CDS has been mutated. In addition, there are a variety of examples of mRNAs that yield non-coding riboregulatory sRNAs through RNA processing events or via the regulation of transcription termination. For example, both the CpxQ and RybD sRNAs from *S. typhimurium* as well as the MicX sRNA from *Vibrio cholerae* are all produced from RNase E cleavages within the 3′ UTRs of mRNAs. For the CpxQ sRNA of *Salmonella*, its parent mRNA *cpxP* encodes a stress chaperone that functions in tandem with CpxQ to reduce the abundance of misfolded proteins in the inner membrane during episodes of membrane stress. While the CpxP protein chaperone is required for degrading misfolded proteins, the CpxQ sRNA post-transcriptionally inhibits the production of a variety of inner membrane proteins to prevent further accumulation of misfolded proteins in the membrane. The regulation of transcription termination is another mechanism to generate sRNAs from mRNAs. In *Listeria monocytogenes*, two S-adenosylmethonine (SAM) riboswitches (SreA and SreB) located in the 5′ UTRs of the mRNAs *lmo2230* and *lmo0049* both terminate the transcription of their respective mRNAs in the presence of SAM. Upon termination, the riboswitches are able to serve as sRNAs to reduce the abundance of the *prfA* mRNA, which encodes a critical regulator of virulence genes. This mechanism provides a key regulatory link between the metabolic state of the cell and the production of virulence factors. Transcriptional read-through is an alternative transcriptional mechanism capable of producing riboregulatory sRNAs from protein-encoding mRNAs. In the Gifsy-1 prophage found in species of *Salmonella*, the IsrK sRNA is located immediately upstream of a two-gene operon encoding the ORF45 and AnrP proteins. The transcripts from this operon can exist in two forms. A rho-independent terminator located between IsrK and the downstream orf45 and *anrP* CDSs creates the short transcript, which is comprised solely of the IsrK sRNA. However, a subset of transcripts can read-through this terminator structure to produce a long transcript that includes both CDSs. Interestingly, the long transcript is translationally silenced by its own endogenous secondary structure and is only able to yield appreciable quantities of protein when directly activated by the IsrK sRNA. Thus, IsrK is riboregulatory for its own mRNA. Given the limited number of examples of dual-function sRNAs and mRNAs, it is still unclear whether translated riboregulatory RNAs are the exception or the rule. However, the fact that such activity can already be observed among a broad diversity of prokaryotes and eukaryotes, it seems likely that mRNA will eventually be recognized as a much more sophisticated molecule than is currently appreciated. It should also be noted that alternative functions of mRNAs are not solely limited to riboregulation. The VegT gene in the model organism *Xenopus laevis* encodes a transcription factor that plays a key role in mesendoderm patterning in developing embryos. In addition to its protein-encoding function, VegT mRNA integrates into the cytokeratin cytoskeleton of Xenopus oocytes to provide structural support that is both essential for the integrity of the cytoskeleton and for proper germline development. Thus, translated RNAs are likely to function via a wide variety of currently unrecognized mechanisms that may or may not participate in the same pathways or functions as their encoded proteins.

Role of Hfq

One important hallmark of many trans-encoded antisense RNAs from *E. coli* is their ability to bind the RNA chaperone Hfq. In 1968, Hfq was identified in *E. coli* as a host factor required for the replication of bacteriophage Qβ. It is present in half of all sequenced bacterial species. Several bacteria such as *Bacillus anthracis* and *Ralstonia metallidurans* even encode two Hfq proteins. At least one archaeon, *Methanococcus jannaschii*, contains a protein that is related to Hfq. Hfq comprises between 70 and 110 amino acids and forms homohexamers. It is an abundant protein, which is present in up to 60,000 monomers/*E. coli* cell in the stationary phase. The major fraction is associated with the ribosomes, whereas a minor Hfq fraction appears to be associated with the nucleoid. Hfq was shown to bind to AU-rich sequences in single-stranded regions generally flanked by one or two stem-loops. The Hfq homohexamer is very similar to the eukaryotic Sm and Sm-like proteins involved in splicing as well as the archaeal Lsm proteins (Fig. 4A and B). It forms a toroid quaternary structure with an outer diameter of ~70 Å and a thickness of 25 Å. The central pore is 8–12 Å wide. The N-terminal α helix is followed by 5 β strands that form a tightly bent sheet (Fig. 4C). Hfq has two primary RNA binding sites for sRNAs and mRNAs, a proximal and a distal (Fig. 4D). The co-crystal structure of the *S. aureus* Hfq bound to an AU_5G oligo shows how the RNA expands and fills the central, basic pore on the 'proximal side' of the hexamer and binds in a circular manner with the exception of the 3′ G located at the entrance of the pore (Fig. 4D). Each of the six potential AU nucleotide-binding pockets is composed of residues from two adjacent subunits. The repetition of identical binding pockets on the Hfq hexamer suggests that the binding surface can accommodate more than just a single target, which is particularly important for catalyzing sRNA-mRNA base pairing interactions. Recent studies of the *E. coli* Hfq indicate that it has two binding mechanisms for sRNAs. sRNAs binding by the first mechanism (termed Class I), depend upon sRNA interactions with the proximal and rim sites of Hfq. These sRNAs exhibit rapid turnover and regulate mRNAs containing ARN repeats (A = adenine, R = purine, and N = any nucleotide), which target the mRNAs to the distal face of Hfq. Class II sRNAs primarily interact with the proximal and distal faces of Hfq, while their target mRNAs contain UA-motifs that interact with the rim of Hfq. Class II sRNA interactions tend to result in target regulation in the absence of substantial degradation. Thus, Class II sRNAs have a higher intrinsic stability compared to those of Class I.

In *E. coli*, inactivation of the *hfq* gene causes pleiotropic effects, such as reduction of growth rate, UV sensitivity, and increased cell length. Hfq is involved in mRNA stability, mRNA polyadenylation, and translation. The importance of Hfq is further underscored

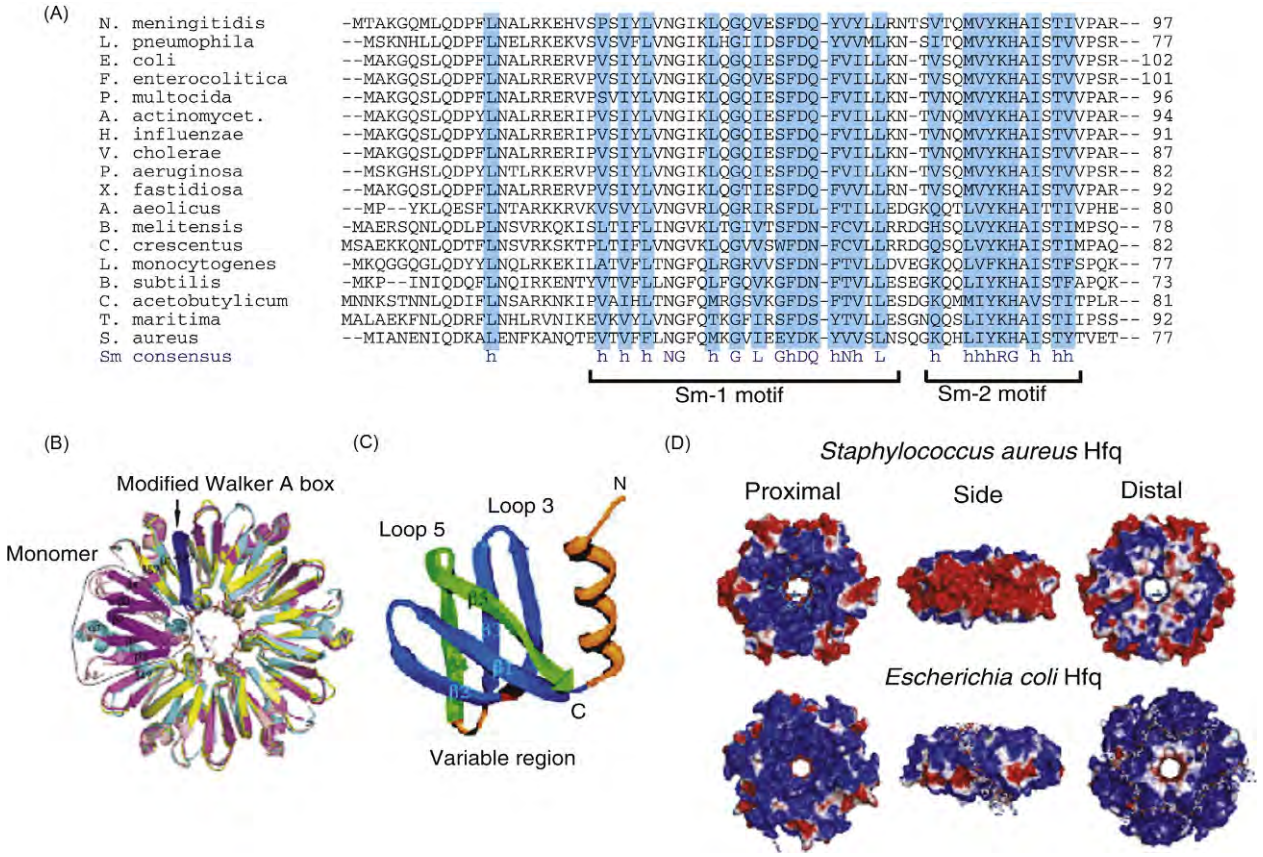

Fig. 4 The RNA chaperone Hfq. (A) Amino acid sequence alignment of a subset of bacterial Hfq proteins. The Sm consensus based on 80 Sm and Sm-related proteins is shown in *blue* at the bottom. The Sm1- and Sm2-consensus positions are highlighted in *light blue*. h, bulky hydrophobic residue (L, I, M, V, F, Y, or W). Based on Møller et al., 2002. (B) *Overlay of the structures of four bacterial Hfq proteins.* The Hfq structures of *P. aeruginosa*, *E. coli*, *S. aureus* without and *S. aureus* with RNA are depicted as ribbons and *colored cyan*, *salmon*, *yellow* and *magenta*, respectively. The view is into the 'proximal' face. The bound RNA, AU_5G, is shown as solid sticks with carbon, nitrogen, oxygen and phosphorous atoms colored with *white*, *blue*, *red* and *orange*, respectively. The dashed line envelops one monomer of the hexameric protein with the β sheets colored *magenta* and the single α helix in *cyan*. The secondary structures are labeled for this monomer, as are the β strands β4' and β5', which form the majority of inter-subunit interface. The 'modified Walker A box' (see text) is labeled on one subunit and shown in *blue*. (C). *Ribbon diagram of an Hfq subunit.* The Sm-1 motif is colored *blue* and the Sm2 motif is *green*. Regions outside the two motifs, that is, the N-terminal a-helix and the variable region, are colored orange. (D). *Electrostatic potential energy surfaces of the known and proposed RNA binding sites of the S. aureus and E. coli Hfq proteins.* Views of the proximal, side and distal electrostatic surfaces of the *S. aureus* Hfq and the *E. coli* Hfq are shown. *Blue*, electropositive; *red*, electronegative. The side view of *E. coli* Hfq includes a plausible RNA binding cleft that would enable A_{27} to bind to both proximal and distal sites. The view of the distal site of the *E. coli* Hfq shows a possible binding site for A_{18} (i.e., polyA tail). The bound RNAs are shown as solid sticks with carbon, nitrogen, oxygen and phosphorous atoms colored *white*, *blue*, *red* and *orange*, respectively. Part (B) and (D) taken from Brennan, R. G. and Link, T. M. (2007). Hfq structure, function and ligand binding. *Current Opinion in Microbiology* **10**, 125–133.

by its multiple roles in virulence, bacteriocin production, and nitrogen fixation. It has been reported to have a relatively weak ATPase activity, for which a 'modified Walker A box' (Fig. 4B) could be responsible.

Interestingly, Hfq was shown to interact with the 30S ribosomal subunit, RNase E, PNP, PAPI, and the ribosomal protein S1. The C-terminal scaffold domain of RNase E, which was shown to participate in RyhB-mediated degradation of target mRNAs, acts as an Hfq binding site. The Hfq-PNP-PAPI complex is involved in regulating the polyadenylation of full-length mRNAs that contain Rho-independent transcription terminators. Hfq could destabilize the terminator stems, which are then direct targets of PAPI activity, and later subject to degradation by PNP and RNase II. Hfq interacts with RNA polymerase in an S1-dependent manner.

Many trans-encoded sRNAs, such as DsrA, Spot42, and RyhB, require Hfq for their stability. In other cases, such as OsyS/*fhlA*, Spot42/*galK*, RyhB/*sodB*, MicA/*ompA*, Hfq was shown to promote the interaction between the sRNAs and their targets. A FRET study on the intermolecular base pairing between DsrA and *rpoS* mRNA demonstrated that Hfq accelerates strand exchange and subsequent annealing between DsrA and *rpoS* mRNA to increase exposure of the *rpoS* RBS (see also Fig. 3). Expression of the *E. coli hfq* gene is under the control of at least three σ^{32} and four σ^{70} promoters and is highly expressed overall. Furthermore, Hfq autoregulates its own expression by binding to two sites on the *hfq* mRNA, leading to translation inhibition.

For the sRNAs of Gram positive bacteria, the putative function of Hfq has remained largely elusive, and for many species like the lactic acid bacteria, obvious Hfq orthologs are not even encoded. Some characterized sRNAs are known to bind Hfq (RNAIII of *S. aureus*, LhrA and LhrC from *Listeria monocytogenes*, and SR1 of *B. subtilis*), but thus far, there has only been one example where Hfq

could be assigned an in vivo function. It was shown to stabilize LhrA and promote the LhrA/target-mRNA interaction. By contrast, for SR1 from *B. subtilis* and RNAIII from *S. aureus*, Hfq is not required for target interactions. Accordingly, Hfq is not highly expressed in *S. aureus*. It cannot be excluded that in the majority of Hfq-encoding Gram positive bacteria, Hfq is needed for other, still unidentified functions. It is also conceivable that other RNA binding proteins might fulfill the role of Hfq in these bacteria, especially for those that do not encode Hfq. One candidate is CsrA (see below) that was recently shown to be bound and sequestered by the trans-encoded base-pairing sRNA McaS in *E. coli*.

Differences between cis- and trans-encoded antisense RNAs

Cis-encoded RNAs have regions that are fully complementary to their targets over a large nucleotide stretches and can, therefore, spontaneously form stable duplexes with their target RNAs. Consequently, RNA chaperones like Hfq are generally less important to promote their target interactions. Exceptions seem to be Tn10/IS10 and Tn5. For IS10, Hfq was demonstrated to bind and restructure RNA-IN and RNA-OUT to facilitate duplex formation and inhibit transposase translation by binding to the RBS. Similarly, for the plasmids ColE1 and R1/F, plasmid-encoded RNA binding proteins (Rom and FinO, respectively) were shown to further enhance duplex formation. In contrast to most cis-encoded sRNAs, a large number of trans-encoded antisense RNAs have been demonstrated to require Hfq either for protection from RNase degradation or for duplex formation. Presumably, this is because sRNA-target interactions are only partially complementary and likely have less favorable binding kinetics. Another major difference between cis- and trans-encoded sRNAs is the diversity of regulation exhibited by individual sRNAs. Each cis-encoded antisense RNA uses a single defined mechanism of action for an individual target. On the contrary, trans-encoded sRNAs can simultaneously employ multiple mechanisms for multiple target mRNAs. For instance, RNAIII, DsrA, and RyhB inhibit the translation of some targets, yet activate translation of others. Conceivably, a single trans-encoded sRNA might be able to use three or four different mechanisms to regulate various targets.

Trans-Encoded sRNAs That Act via Protein Binding

In contrast to the trans-encoded sRNAs that exert their function by base-pairing interactions with target mRNAs, another class of sRNA employs protein binding as its mechanism of action. Two principal classes of protein-binding sRNAs can be distinguished: 6S RNA that binds RNA polymerase and sRNAs that sequester small proteins inhibiting the translation of target mRNAs. In both cases, RNA binding reversibly inhibits the target protein and results in global changes in gene expression. A potential third class of sRNAs binds to and modulates the activity of enzymes. Thus far, this mechanism has proven to be quite rare and is exemplified by Rcd of the *E. coli* ColE1 plasmid. (Table 3)

6S RNA

6S RNA was discovered in the late 1960s and for more than 30 years its function remained enigmatic. By 2005, it was recognized to be ubiquitous among bacteria. To date, more than 100 putative 6S RNA homologues have been identified in diverse bacteria and many have been confirmed experimentally. In *B. subtilis*, two differentially expressed 6S RNA species exist during growth. 6S RNA has a characteristic secondary structure consisting of a central region with a largely single-stranded internal loop flanked by two long irregular double-stranded stem regions interrupted by small bulges. This secondary structure resembles a partially single-stranded DNA bubble and has led to a hypothesis for the potential function of 6S RNA (Fig. 5). 6S RNA forms a stable complex with RNA polymerase and was assumed to act as an open promoter DNA mimic that interferes with the formation of transcription initiation complexes. Its levels increase ~10-fold to about 10,000 molecules during stationary phase and it interacts preferentially with RNA polymerase holoenzymes containing the exponential phase sigma factor σ^{70}. Based on these observations, it was proposed that 6S RNA participates in shifting global gene expression from exponential to stationary phase. However, there is reason to believe that its

Table 3 Examples of trans-encoded sRNAs acting via protein binding

sRNA (length)	*Interacting protein*	*Biological function*	*Mechanism*	*Control*
Escherichia coli				
CsrB (366 nt)	CsrA (61 aa)	Glycogen biosynthesis	Protein sequestration	BarA/UvrY
CsrC (245 nt)		Biofilm production		
6S RNA (200 nt)	σ^{70}-RNAP, σ^{S}-RNAP	Stationary phase regulation	Protein sequestration	Stationary phase
Rcd (70 nt)	Tryptophanase (TnpA)	Stable plasmid maintenance	Activation of enzyme	
Vibrio cholera				
CsrB/CsrC/CsrD	CsrA	Virulence	Protein sequestration	GacS/GacA
Erwinia carotovora				
RsmB	RsmA (61 aa)	Proteases, pectinases, etc.	Protein sequestration	GacS/GacA
Pseudomonas fluorescens				
RsmZ/RsmY (118 nt), RsmX	RsmA (61 aa)	Secondary metabolism	Protein sequestration	GacS/GacA
Pseudomonas aeruginosa				
RsmZ/RsmB	RsmA	Secondary metabolism	Protein sequestration	

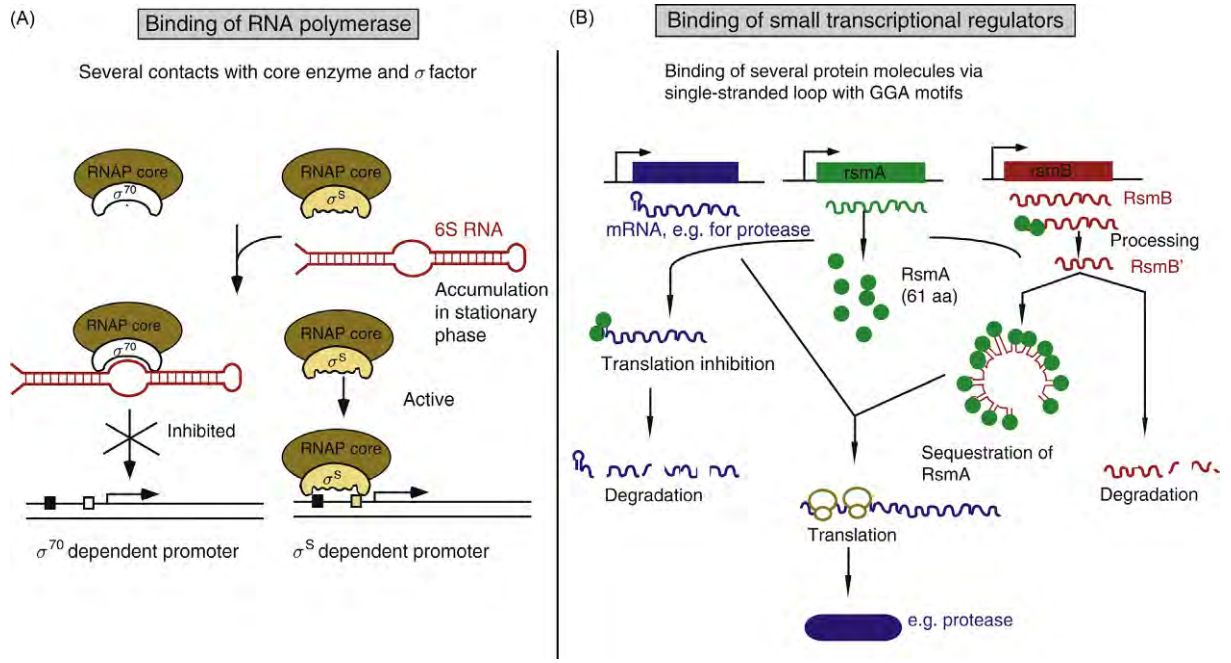

Fig. 5 Overview of sRNAs acting by protein binding. *Red*, 6S RNA, *brown*, RNA polymerase core, *yellow* and *white* symbols denote σ^{70} and σ^{S}, *Black* and *white* rectangles indicate −35 and −10 boxes, respectively. (A) *Mechanism of 6S RNA action.* (B) *Mechanism of action of sRNAs binding translational regulators.* The RsmA-RsmB regulatory system of *Erwinia carotovora ssp. carotovora.* RsmB primary RNA is subsequently processed yielding a 259 nt long functional RNA (RsmB′). Free RsmA protein induces degradation of target mRNA. Binding of RsmA protein to RsmB′ depletes the pool of free RsmA thereby inhibiting target mRNA degradation. The binding of RsmA to RsmB' also protects the RsmB′ transcript from degradation.

actual function is more complex. 6S RNA is still expressed and functional during exponential phase and its effect upon gene expression is not uniform with some genes affected, while others are not. 6S RNA can also act as a template for the production of small de novo transcripts, which suggests another aspect of 6S RNA function. 14–22nt transcripts (pRNA) were observed when the 6S RNA concentration was high, whereas 170nt transcripts were observed when RNA polymerase (RNAP) concentrations were high. In *B. subtilis*, newly synthesized pRNAs form stable duplexes with 6S RNA, which induce structural rearrangements *in cis* and trigger 6S-1 RNA release from RNA polymerase upon outgrowth from stationary phase, delaying sporulation. Both the generation and the role of the longer transcripts are still unclear. Likewise, the physiological function of 6S RNA is still the subject of debate. While 6S RNA is widely conserved among bacteria, 6S RNA mutants grow normally in laboratory conditions, indicating its function is dispensable under certain growth conditions. However, 6S RNA mutants do exhibit decreased survival during long-term nutrient deprivation.

Small RNAs that act by sequestration of translational regulators

In 1997, it was shown that a small untranslated RNA, CsrB from *E. coli*, does not act via base pairing to an mRNA target, but exerts its function by binding to the small protein CsrA. CsrA is a repressor of stationary phase processes, such as gluconeogenesis and biofilm production, and an activator of glycolysis, motility, and acetate metabolism. For example, CsrA binds to the 5′ UTR of the *glgCAP* transcript, where it blocks translation and causes rapid mRNA degradation. In contrast, the activation of gene expression by CsrA involves binding to the 5′ UTR to prevent the degradation of transcripts, such as the master operon for flagella biosynthesis *flhDC*. For *flhDC*, CsrA prevents 5′-end dependent RNase E cleavage by binding to two sites within the 5′ UTR. Furthermore, CsrA represses ribosome binding to *pgaABCD* mRNA, which is involved in biofilm formation. In addition, it also remodels the *pgaABCD* 5′ UTR to unveil *rut* sites for terminator Rho binding, resulting in premature transcription termination. Highly conserved CsrA orthologues are found in diverse eubacteria and regulate virulence factors of plant and animal pathogens. The CsrA/CsrB ribonucleoprotein complex comprises 18 CsrA protein subunits and a single CsrB sRNA. Binding of CsrA to CsrB or target mRNAs involves the same CAGGAUG imperfect repeat sequence that can be found primarily in the loops of the CsrB hairpins. In 2003, a second RNA, CsrC, has been found that acts by binding 9 CsrB molecules. Null mutations of either CsrB or CsrC cause a modest increase in abundance for the other sRNA. The consensus binding motif of the *E. coli* CsrA protein has been identified as 5′-RUACARGGAUGU-3′ (R = purine), which naturally forms a stem-loop with an important GGA located within the loop. The GGA sequence can also be found in both CsrB and CsrC as well as CsrA-regulated mRNAs. This motif is recognized on the related RsmY and RsmZ sRNAs from *Pseudomonas fluorescens* as well. These two sRNAs regulate secondary metabolism and biocontrol traits in *P. fluorescens*, such as antifungal metabolites and extracellular enzymes, and are under the control of the two-component GacS/GacA system. An *rsmY/rsmZ* double mutant is strongly impaired in the synthesis of extracellular enzymes, whereas single mutants do not exhibit significant defects.

A similar pathway is present in *Erwinia carotovora*, where the GacS/GacA system promotes expression of the *rsmB* gene (Fig. 5). Homologous elements are also found in *S. typhimurium* where they control the expression of genes related to invasiveness.

A small RNA that acts by binding tryptophanase

The *cer* site is required for the resolution of ColE1 plasmid dimers by the site-specific Xer-cer recombination system composed of four proteins, XerC, XerD, ArgR, and PepA. The resolution of plasmid multimers must be achieved before the cell divides, as this is the only time when a plasmid may be lost. Upon induction by plasmid multimers, the untranslated Rcd RNA is transcribed from a promoter within the *cer* site. In 2007, it was discovered that Rcd acts by binding the enzyme tryptophanase (TnpA), increasing its affinity for tryptophan and stimulating indole production by rapidly dividing cells (low density cultures). Indole serves as an intracellular signal molecule by acting as an ionophor that modulate the membrane potential to delocalize FtsZ, FtsA, and MinD, and thus inhibit formation of the divisome. The resulting delay in cell division then gives the plasmid time to convert multimers into monomers.

sRNAs that act as RNA sponges

The sequestration of the translational regulator CsrA by the small untranslated RNA CsrB as well as the association of the 6S RNA with the RNAP/σ^{70} holoenzyme during the stationary phase are prime examples of how sRNAs are able to direct the regulatory function of a protein away from its original target. A similar observation has been made with RNAs that sequester heterologous sRNAs, thus excluding them from their regulatory target interactions. Such RNAs have been referred to as RNA sponges, RNA traps, competing endogenous RNAs, or RNA decoys. The basic principle is that a regulatory sRNA can be competed away from its intended mRNA target by forming duplexes with sponge RNAs. Consequently, a previously repressed mRNA can once again become available for translation. An sRNA sponge mechanism has been demonstrated in the regulation of the enterobacterial chitoporin ChiP protein required for the transport of chito-oligosaccharides across the outer membrane. Under non-inducing conditions, translation of the *chiP* mRNA is repressed by base-pairing between the ChiX sRNA and the 5′ UTR of *chiP* mRNA leading to RNaseE-dependent degradation of *chiP* mRNA. However, a sponge mechanism is activated upon chitobiose induction of the chitobiose-specific phosphotransferase (PTS) system operon (*chbBCARFG*). The intercistronic sequence between *chbB–chbC* provides an alternative hybridization side for the ChiX sRNA, sequestering ChiX and relieving the translational repression of *chiP*, eventually allowing for maximal chito-oligosaccharide utilization. Another novel regulation has recently been linked to the 3′ external transcribed spacer (ETS) of the tRNA *leuZ*. This sRNA functions by sponging the pool of RyhB and RybB sRNAs transcribed as a result of leaky basal expression from their strong promoters. RyhB and RybB are known to be involved in TCA cycle remodeling, iron homeostasis, envelope stress response, and antibiotic resistance, which highlights their global influence upon bacterial metabolism and stress adaptation. It is intriguing to speculate that the 3′ ETS will likely have a similar functional role in other species, since the 3′ ETS of *leuZ* exhibits global sequence conservation among the various Enterobacteriaceae. Furthermore, the 3′ ETS of certain pre-tRNAs seem to be even more highly conserved than the 3′ ETS of *leuZ*, suggesting other uncharacterized regulatory functions likely exist. Another example involves a target mRNA-derived sponge of the sRNA GcvB, which is also widely conserved among Enterobacteriaceae. The decay of *gltlJKL* mRNA encoding an amino acid ABC transporter produces a stable RNA fragment designated SroC that base-pairs with GcvB causing its degradation by RNase E. This alleviates GcvB-mediated repression of mRNAs encoding amino acid-related transport and metabolic genes. Interestingly, as *gltIJKL* mRNA is itself a GcvB target, the SroC sponge can both activate its own production in cis as well as activate many trans-encoded sRNAs in the same pathway.

Sensory RNAs

Only recently was it demonstrated how RNAs can directly sense environmental conditions and, thus, play a central role in the genetic control of metabolic processes. In the majority of cases, such sensory RNA elements are located in the 5′ UTRs of mRNAs where they form complex secondary structures controlling the expression of downstream genes via signal induced conformational changes. Such signals can either be of physical origin (RNA thermometers) or of chemical origin (riboswitches).

RNA Thermometer

RNA thermometers (RNATs) are RNA structures that gradually change their conformation in response to temperature. A conformational arrangement of the RNA structure is able to detect variations as low as 1°C. Most, but not all characterized RNA thermometers are located in 5′ UTRs and sequester RBSs by intramolecular base pairing at low temperatures. The temperature-dependent refolding of mRNA secondary structures results in a gradual melting of the double-stranded portion of the inhibitory stem loop(s), thus allowing ribosome access and translation initiation (Fig. 6A). The majority of RNA thermometers participate in the control of heat-shock and cold-shock responses and several regulate virulence gene expression in pathogenic bacteria.

Some of the best-characterized RNA thermometers include the *rpoH* thermometer as well as the ROSE and FourU elements (Fig. 6B), which are involved in the control of heat-shock and virulence gene expression. Expression of the *E. coli* heat-shock sigma factor *rpoH* (σ^{32}) is regulated at the transcriptional, post-transcriptional, and post-translational levels. Transcription is regulated by σ^{32} itself. Translation of *rpoH* is regulated by an RNA thermometer that is composed of two stem-loop segments (I and II in Fig. 6B),

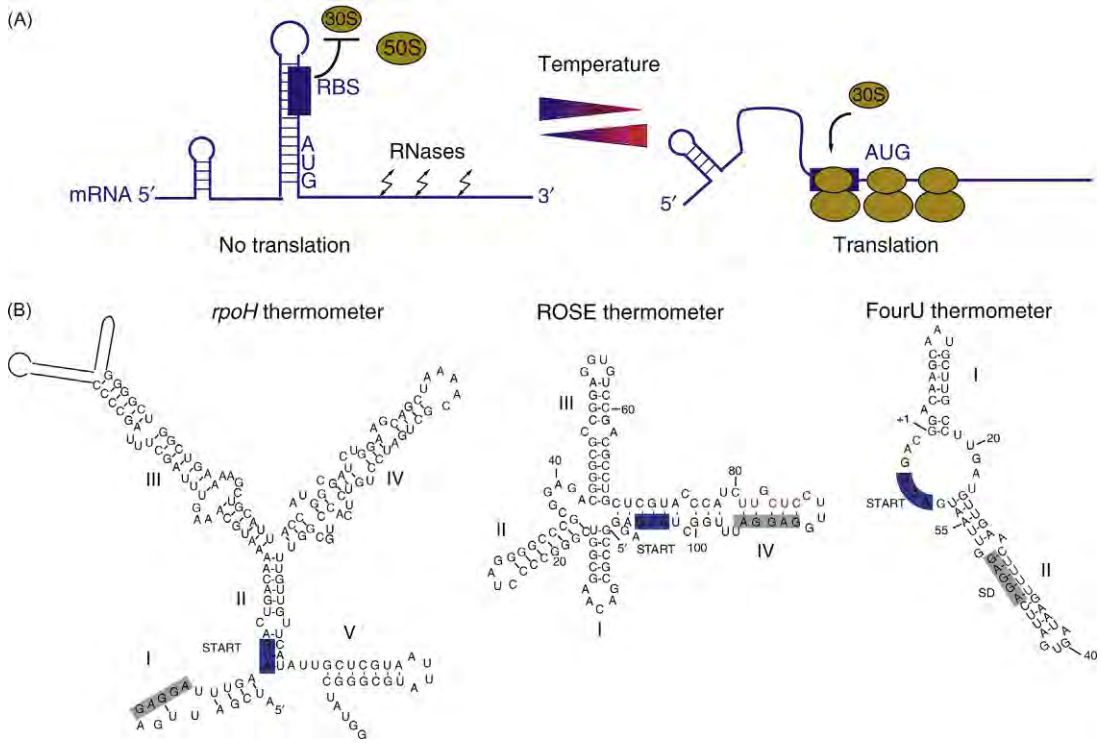

Fig. 6 RNA thermometer. *(A). Regulatory principle of an RNA thermometer.* This principle has been postulated for heat shock and virulence genes. RBS, ribosome binding site, AUG, start codon. 30S and 50S symbolize the ribosomal subunits. Temperature changes are indicated by a color change within triangles (by *blue* = cooler, and *red* = warmer). (B). *Secondary structures of three representative RNA thermometers* Right: FourU thermometer from the 5′ UTR of the *agsA* gene of *S. typhimurium*. The secondary structure was confirmed experimentally.

which extend into the *rpoH* CDS. Under heat shock conditions, stem-loop I melts allowing ribosomes to access the RBS. Interestingly, the other stem-loops in the CDS also contribute to the thermometer function, since a minimal *rpoH* thermometer composed only of stem-loops I, II, and, V required additional mutations in stem II to maintain heat inducibility. While the *rpoH* thermometer is found only in *E. coli* and its relatives, another RNA thermometer, the ROSE element (Repression Of heat Shock gene Expression) - originally discovered in *Bradyrhizobium* - is widespread in different alpha and gamma proteobacteria. Located in the 5′ UTR of genes for small heat-shock proteins, ROSE elements consist of two to four stem loops ranging from 60 to >100 nt. The 3′ proximal stem loop comprises the RBS and the AUG start codon (Fig. 6B). In all known cases, a highly conserved G opposite of the RBS has been found to be important for heat induction of the downstream genes. Mismatches and a mixture of canonical and non-canonical base pairs in the RNA thermometer ensures that the reversible melting process occurs within the physiological temperature range between 25°C and 42°C. Another type of RNA thermometer, designated FourU, has been discovered in the 5′ UTR of the *S. enterica agsA* gene, encoding a small heat-shock protein. It is composed of only two stem-loops, I and II, the latter of which contains a sequestered RBS that base pairs with 4 U residues in the anti-SD (Fig. 6B). Additional FourU thermometers have been predicted upstream of the *Yersinia pestis lcrF*, *S. aureus groES*, and *Brucella melitensis dnaJ* genes.

Riboswitches

The majority of riboswitches are located in 5′ UTRs and regulate in cis the transcription or translation of downstream open reading frames. Riboswitches can detect a variety of signals including vitamins, divalent metals, purines, amino acids, phosphorylated carbohydrates, and charged tRNAs (Fig. 7A and B). They are comprised of an aptamer (i.e., ligand receptor) characterized by a consensus sequence determining its substrate specificity and an output (expression platform) region. Binding of a small molecule results in a conformational switch that alters gene expression by one of three possible mechanisms: transcription attenuation, inhibition of translation initiation, or mRNA cleavage.

The FMN riboswitch is a prominent regulator of riboflavin biosynthesis that responds to cellular pools of FMN (flavin mononucleotide), which is an end product of the riboflavin synthetic pathway. In many species, this riboswitch acts by transcription attenuation, due to the binding of FMN to the 5′ UTR of nascent mRNA. In the FMN riboswitch found in the *ribDEAHT* operon of *B. subtilis*, FMN binds to a specific element in the riboswitch called the RFN element, inducing a change in secondary structure that remodels an endogenous antiterminator structure in favor of a transcriptional terminator. However, when FMN concentrations in the cell are low, the riboswitch fails to bind FMN and the 5′ UTR adopts an antiterminator structure allowing transcriptional

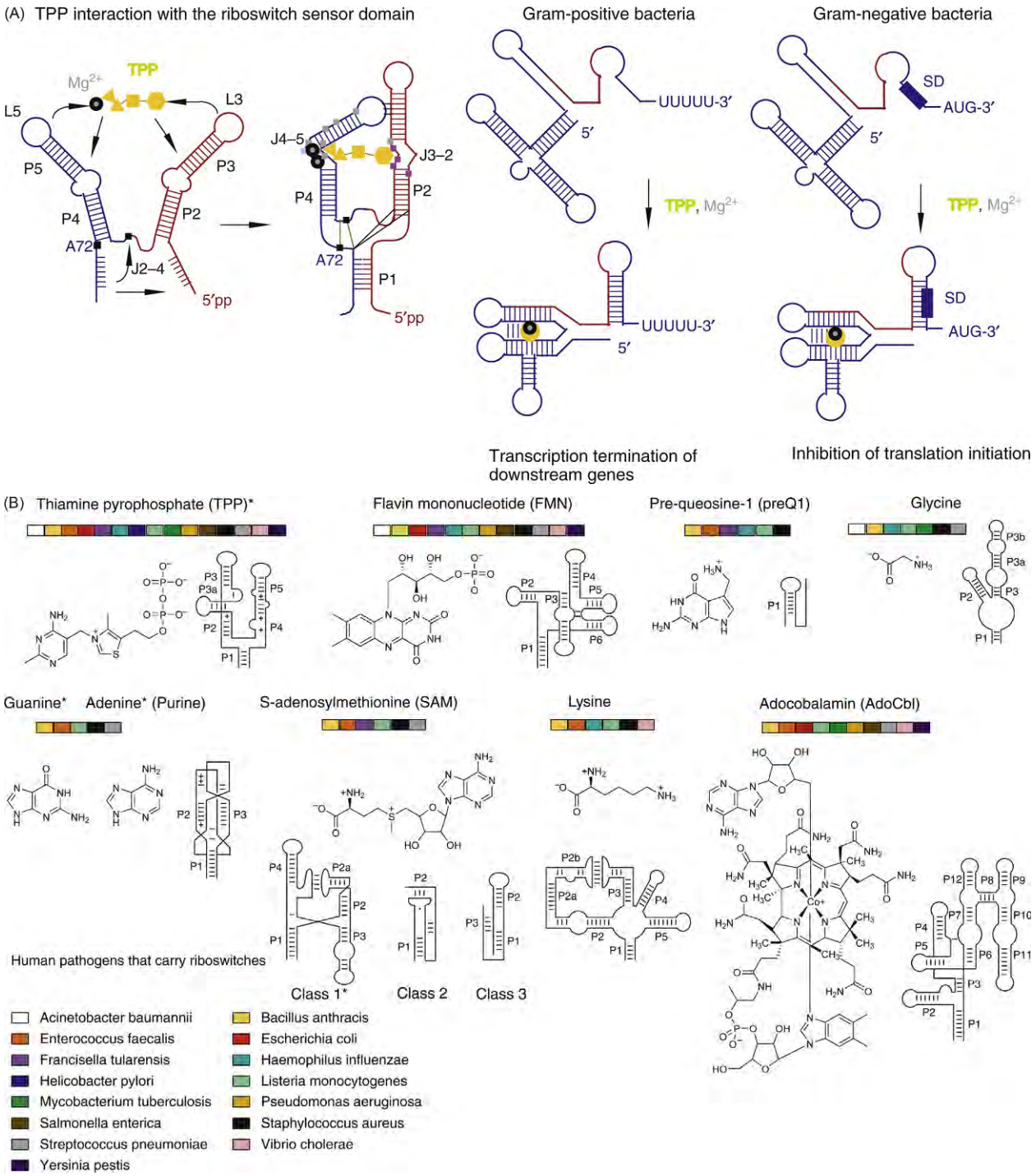

Fig. 7 Riboswitches. (A). The thiamine-pyrophosphate sensing riboswitch. Depending on the expression platform, the TPP riboswitch acts as a transcription terminator (middle) or as a suppressor of translation initiation (right). The contacts between TPP and the riboswitch sensor domain are summarized on the left side. The TPP binding pocket is formed by two parallel helices: a pyrophosphate binding helix (*blue*, comprising P4, J4-5, P5 and L5) and a pyrimidine binding helix (*red*, comprising P2, J3-2, P3 and L3). P1 is only formed in the presence of TPP. TPP is shown in *yellow*, Mg^{2+} in *grey*, nonstandard basepairs are in *green*, and hydrogen bonds are shown by *black lines*. *Light blue* (G78) and *magenta boxes* (G40, G42, A43 and G19) symbolize bases contacted by the pyrophosphate and the pyrimidine moiety of TPP, respectively. *Grey boxes* indicate the binding sites for the four Mg^{2+} ions. Only the two Mg^{2+} that are involved in the pyrophosphate contact with J4-5 are shown as *grey*/*black* circles. The other Mg^{2+} ions are required for folding. TPP binding stabilizes the three-way junction with A72, thus helping the sensor domain to sequester the segment that would otherwise participate in the alternative structure of the expression platform. (B) *Overview of known riboswitch classes and their occurrence in selected pathogenic bacteria.* The secondary structure models of the receptor domains of 11 riboswitch classes and the chemical structures of the metabolites they bind. The expected protonation states at physiological conditions are shown for each functional group. Three different structural classes of riboswitches have been reported that recognize SAM (*, riboswitch class for which a representative 3D structure has been published). Colored rectangles denote the occurrence of the riboswitch classes in the selected pathogenic bacteria, which are listed below. Thereby, no distinction has been made between the three classes of SAM riboswitches.

read-through. This mechanism is mainly found in low G + C Gram positive bacteria. An FMN riboswitch is also found in the *ribB* gene within the *E. coli* riboflavin biosynthetic locus. In contrast to the *B. subtilis* FMN riboswitch, the *E. coli* version functions largely through a translational control mechanism that involves sequestration of the *ribB* RBS in the FMN-bound state (Fig. 7B). This regulatory mechanism occurs in addition to a weaker antitermination mechanism that proceeds in an analogous fashion to that described for *B. subtilis*. The added layer of translational control was speculated to have evolved to improve upon the sensitivity of the riboswitch, given its relatively modest level of transcription attenuation (~2-fold reduction in gene expression).

Riboswitches that bind FMN, thiamine pyrophosphate (TPP), SAM, vitamin B_{12}, lysine, and guanine have all been found to inhibit gene expression for a wide variety of genes encoding metabolic enzymes. In contrast, the adenine and the glycine riboswitches both work as activators of gene expression via disruption of transcription terminators. The glycine riboswitch is particularly unusual, as it contains two aptamer domains. For sulfur and methionine metabolism, three different riboswitch classes have been identified that contain either an S-box, an S_{MK} box, or a SAM-II box. Fig. 7B gives an overview of various characterized metabolite-sensing riboswitches and their occurrence in a number of pathogenic bacteria. The TPP riboswitch is the most ubiquitous and it is the only one found in eukaryotes, fungi, and plants, where it is involved in intron splicing and mRNA processing/stability.

The 3D structures of several riboswitches have been solved, such as the TPP, adenine, guanine, and SAM riboswitches. The thiamine riboswitch structure revealed how polyanionic RNA is able to both bind a molecule with a negatively charged pyrophosphate group as well as discriminate between TPP and TMP. The structure of the THI-box resembles a tuning fork in which the prongs are formed by two parallel stacking helices (P2, bulge J3-2, loop L3) and (P4, bulge J4-5, and loop L5) arranged via a central three-way junction with helix P1 (Fig. 7A, left). Each of the parallel helices binds to one part of TPP. The pyrimidine part of TPP is bound by bulge J 3-2 located in the pyrimidine sensor helix P2-P3 that contains the conserved sequence UGAGA. The pyrophosphate group of TPP is recognized by the J4-5 internal loop present in the pyrophosphate-sensor helix P4/P5, due to an interaction with two Mg^{2+} ions and their corresponding coordinated water molecules. The conformational switch induced by TPP binding is due to TPP forcing the parallel disposition of the pyrimidine and pyrophosphate sensor helices, leading to a kink at the J2-4 junction. This triggers the formation of the P1 helix as well as the formation of a terminator stem or sequestration of the RBS. When the TPP concentration decreases, helix P1 adopts an alternate conformation that forms an anti-terminator hairpin or frees the RBS from sequestration. The purine and SAM binding riboswitches show similar architectures, consisting of three and four helical junctions, respectively.

The first metal ion-sensing riboswitch (Mg^{2+}) was discovered in 2006. This riboswitch is located in the 5′ UTR of the *mgtA* gene of *S. typhimurium* and can fold into two structures depending upon the cytoplasmic concentration of Mg^{2+}. The secondary structure of the riboswitch determines the fate of transcription complexes elongating into the coding region of *mgtA*. It also forms an additional layer of transcriptional control beyond that of the PhoP/PhoQ Mg^{2+} sensory system. The Mg^{2+}-bound state induces premature termination of *mgtA* transcription, or in some cases, transcription pausing followed by RNA polymerase release from the template. At sub-micromolar Mg^{2+} concentrations (i.e., low Mg^{+2}), the unbound riboswitch adopts an alternate conformation that permits transcriptional read-through, leading to MgtA synthesis. Metalloregulatory riboswitch RNAs are widespread in bacteria and are able to bind divalent metal ions such as Co^{2+}, Mn^{2+}, and Ni^{2+}. In addition, an ion-responsive riboswitch able to detect fluoride has been described in Gram positives and Gram negatives and has been demonstrated to aid in the response to toxic levels of heavy metals.

Other Small RNAs With Diverse Functions

In addition to the large class of regulatory antisense RNAs and riboswitches, a few other non-coding regulatory RNAs with diverse functions exist in bacteria. Among them, are the tmRNA involved in translation quality control, the RNaseP ribozyme required for pre-tRNA processing, the 4.5S RNA portion of the signal recognition particle, and the CRISPR-CAS systems involved in bacterial immunity (Fig. 9). These RNAs act in concert with proteins and will be briefly discussed below.

tmRNA

The 363 nt transfer-messenger RNA (tmRNA) performs translational surveillance and ribosome rescue in all eubacteria by recognizing aberrant mRNA molecules lacking stop codons. The tmRNA, also known as 10Sa RNA or SsrA RNA, is unique because it combines the properties of both a tRNA and mRNA in a single molecule, ensuring that stalled ribosomes complete the translation cycle and are recycled for subsequent use. The tmRNA associates with small protein B (SmpB), elongation factor Tu (EF-Tu), and ribosomal protein S1 to form an active ribonucleoprotein complex. tmRNA and SmpB are essential in several species and involved in pathogenesis in species like *S. enterica* and *Yersinia pseudotuberculosis*. Although tmRNA was discovered in 1978, it was only in 1994 that it was recognized its 5′ and 3′ ends could fold into a structure resembling the $tRNA^{Ala}$ acceptor stem and T–Ψ–C stem loop (Fig. 8A), while it was also shown to bind alanine in vitro. In 1995, it was found that a heterologous protein expressed in *E. coli* yielded incomplete proteins of varying size sharing the same 11-aa C-terminal extension, AANDENYALAA. The last 10 of these amino acids corresponded to a portion of a short ORF within the *ssrA* gene encoding tmRNA, whereas the peptide tag was missing in an *ssrA* deletion strain. Initially, the tmRNA acts as a tRNA and is aminoacylated with alanine. With the help of EF-Tu and the SmpB C-terminal tail, the aminoacylated $tmRNA^{Ala}$ is delivered to stalled ribosomes on aberrant mRNAs lacking stop codons.

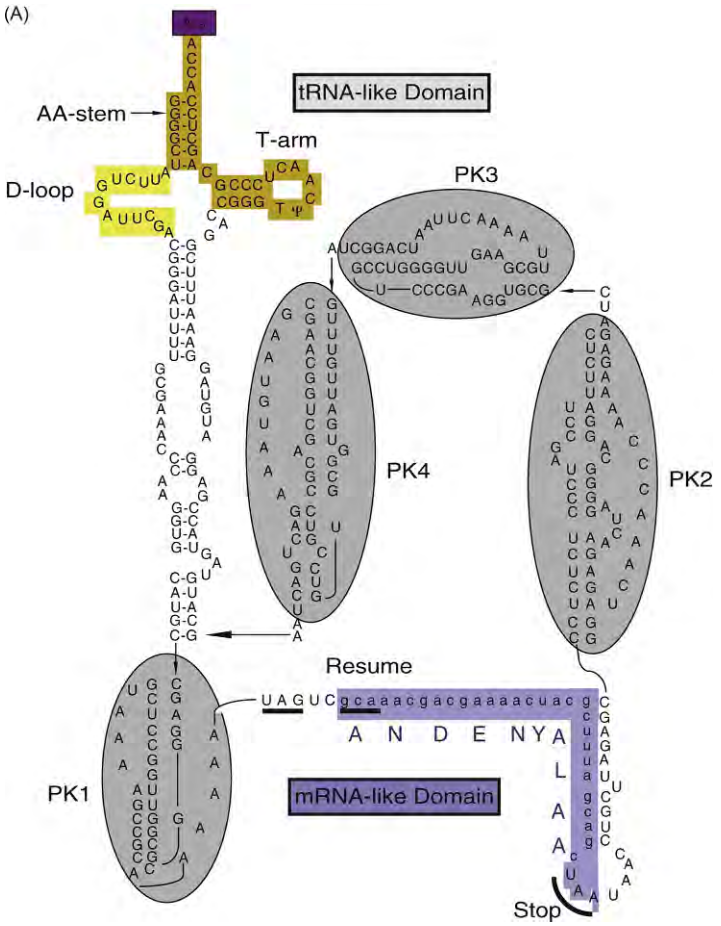

Fig. 8 tmRNA. (A) Proposed secondary structure of *E. coli* tmRNA. The tRNA- and mRNA–like properties are highlighted, including amino acid acceptor stem (AA-stem with G-U wobble base pair), the D-loop and T-arm. The mRNA domain is shown in *purple* with the conserved UAG and GCA resume signals, the peptide tag coding and amino acid sequence and the UAA stop codon. PK1 to PK4 represent the four pseudoknots drawn in *grey*. Based on Dulebohn et al., 2007. *(Continued)*

The ribosome accepts the $tmRNA^{Ala}$ and transfers the nascent peptide followed by the releases of the aberrant mRNA transcript from the ribosome. RNase R will subsequently degrade the mutant mRNA. Next, the tmRNA functions as an mRNA template and the ribosome resumes translation of a short 10-codon ORF encoded by the tmRNA. The tmRNA ORF encodes the SsrA peptide tag as well as a stop codon to complete translation of the defective protein. The encoded C-terminal SsrA tag targets the protein for proteolysis by cellular proteases. As a last step, the tmRNA and the ribosomal subunits are recycled for additional cycles of translation (Fig. 8B). Although tmRNAs are ubiquitous and use the same mechanism of action, the hydrophobic tags they encode differ in length between different bacterial species.

RNase P

Ribonuclease P (RNase P) is an endoribonuclease responsible for the maturation of the 5′ end of tRNA in almost all known organisms. Bacterial RNase P is a ribonucleoprotein complex consisting of a large RNA subunit ranging from 340 to 420 nt as well as a small protein subunit (RnpA). The RNA portion of RNase P is a ribozyme that is responsible for the ribonuclease catalytic activity and requires Mg^{2+} for catalysis and proper folding. In vitro, the RNA component alone can process RNA, albeit with reduced efficiency, but the RnpA protein component is essential for in vivo function. RNA secondary structure comparisons have identified three distinct types of prokaryotic RNase P molecules: the most-widely distributed are class A (for ancestral), class B (for *Bacillus*) types, and class C (for *Chloroflexi*), which is the least common and seems to be intermediate between classes A and B. The core structure of bacterial RNase P contains two domains, named C- and S-domains. While the C-domain catalyzes pre-tRNA cleavage, the S-domain and RnpA provide substrate affinity/specificity, recognizing both the D- and TψC-domains of pre-tRNA.

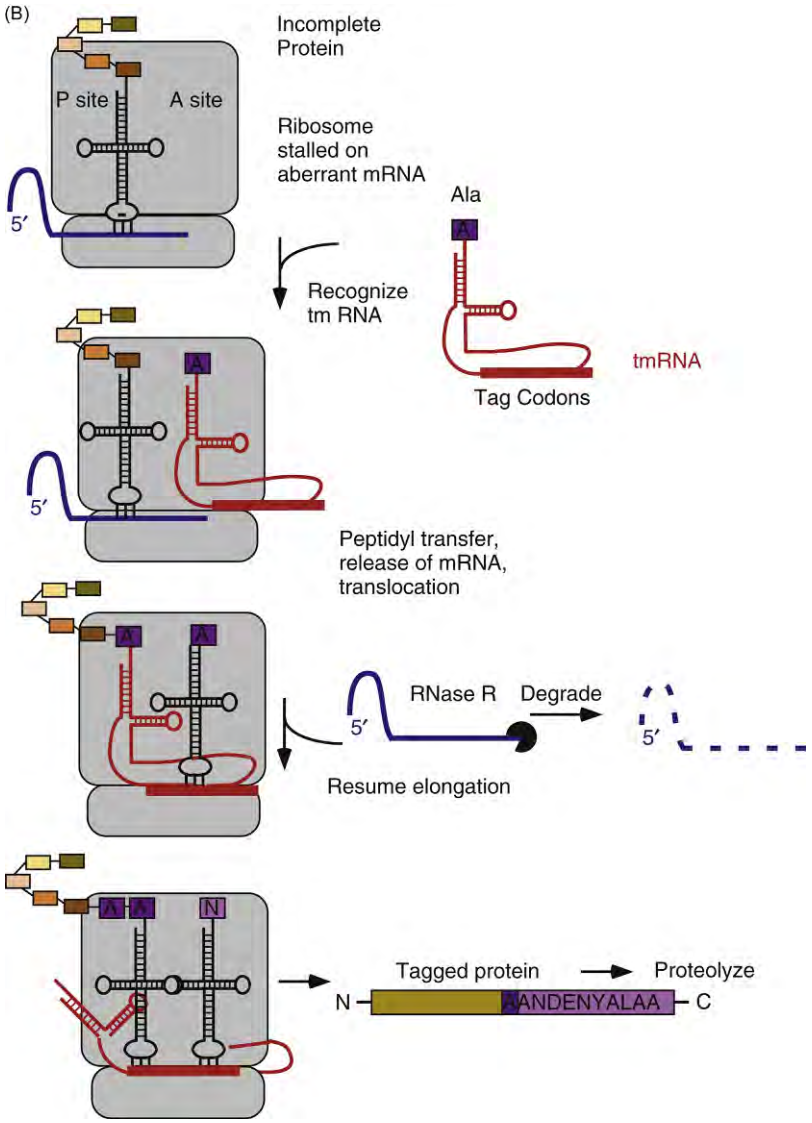

Fig. 8, Cont'd (B) *Trans-translation mechanism.* Ribosomes are drawn in *grey*, mRNA in *blue*, tRNA in *black* and tmRNA in *red*. Colored boxes symbolize the nascent peptide chain. Amino acids are shown in the 1-letter code. For explanations, see text.

4.5S RNA of the Signal Recognition Particle Pathway

Protein targeting to bacterial plasma membranes for secretion can follow several pathways. Among them is the co-translational pathway, which combines the targeting and translocation process into a coupled step with ongoing peptide synthesis by the ribosome. The universally conserved ribonucleoprotein complex SRP (signal recognition particle) plays an essential part in the co-translational pathway by identifying ribosomes with exposed signal peptides that are subsequently targeted to the protein-translocating channel SecYEG via FtsY. In prokaryotes, the complex consists of the small GTPase Ffh and the 4.5S RNA. The 4.5S RNA plays an essential role in every aspect of the translocation process from the recognition of the signal peptide, the interaction with the ribosome, as well as the efficient targeting and translocation process.

CRISPR-CAS Role in Bacterial Immunity Against Heterologous DNA and Alternative Post-Transcriptional Gene Regulation

The ecological pressure of bacteriophages and other mobile genetic elements able to infect bacterial cells has led to a remarkable small RNA-dependent adaptation mechanism conferring heritable immunity against heterologous DNA. The CRISPR (clustered, regularly interspaced, short, palindromic repeats) - CAS system (CRISPR-associated) is found in about 40% of all sequenced bacterial genomes and in the majority of sequenced Archaea. It provides sequence-specific recognition of foreign genetic elements

with subsequent cleavage and inactivation of the target sequence. The central genomic component is the CRISPR locus encoding small targeting CRISPR or crRNA templates that associate with CAS proteins. The crRNA-CAS complex is able to survey the cytoplasm and hybridize to complementary sequences present in invading DNA. Nucleolytic activity of the CAS protein results in target cleavage, thus inactivating potentially harmful foreign DNA. The CRISPR locus consists of alternating spacer- and repeat-sequence arrangements. Each spacer sequence represents a match to a DNA segment of a bacteriophage or conjugative plasmid previously encountered by the cell or its ancestors. In *Streptococcus pyogenes*, the CRISPR-CAS locus encodes 4 *cas* genes (*cas*9, *cas*1, *cas*2 and *csn*2) as well as a small trans-activating RNA called tracrRNA. Immediately downstream of the *cas* genes, a variable, strain-specific collection of CRISPR repeats and intervening spacers comprise the CRISPR locus. In the initial step of adaptation, immunological memory is acquired through the stable integration of heterologous proto-spacer DNA that is identified and generated by the Cas1-Cas2 complex in the CRISPR locus. During the expression and maturation stage, the CRISPR locus is expressed as a single precursor-crRNA containing all of the spacers and repeats. During maturation, a ribonucleoprotein complex is formed with the tracrRNA, crRNA, RNase III, and RNA-guided DNA endonuclease Cas9. The tracrRNA is complementary to the CRISPR repeats found in the crRNA precursor and forms double-stranded (ds)RNA that can be cleaved by RNase III, releasing each crRNA spacer from the precursor. Cas9 and the individual crRNA spacers remain associated in one ribonucleoprotein complex. During the interference stage, this complex is able to scan DNA for the presence of a Protospacer Adjacent Motif or PAM (nucleotides 5′-NGG-3′ in *S. pyogenes*), which is found directly downstream of the target sequence. If the crRNA spacer exhibits sufficient homology to the target sequence found upstream of the PAM, Cas9 will cleave between the small crRNA spacer and the target sequences leading to the degradation of the target nucleic acid, thus providing immunity against the invading DNA (Fig. 9).

The regulatory role of the CRISPR-Cas system is not restricted to bacterial immunity against heterologous DNA. Immune evasion by the Gram-negative intracellular pathogen *Francisella novicida* has been linked to a unique small RNA located between the *cas* genes and the crRNA array, named scaRNA (small, CRISPR-Cas-associated RNA). When present in the phagolysosome of macrophages, an environment inflicting substantial envelope stress, expression of the CRISPR-Cas system is enhanced. An RNA-protein complex is formed containing Cas9, tracrRNA, and scaRNA that is capable of targeting the endogenous bacterial lipoprotein (BLP) mRNA. scaRNA and tracrRNA form a dsRNA, which is believed to stabilize the tracrRNA to facilitate its interaction with the BLP transcript. As a result, the amount of BLP mRNA is drastically reduced, possibly through either Cas9 cleavage or the recruitment of another cellular RNase. Since BLP is a substrate for TLR2 activation, its reduced abundance decreases pro-inflammatory cytokine production and promotes innate immune evasion. In addition, another CRISPR-CAS associated small RNA, *nilD* found in the bacterium *Xenorhabdus nematophila* has been demonstrated to play a role in the mutualistic colonization of host nematodes,

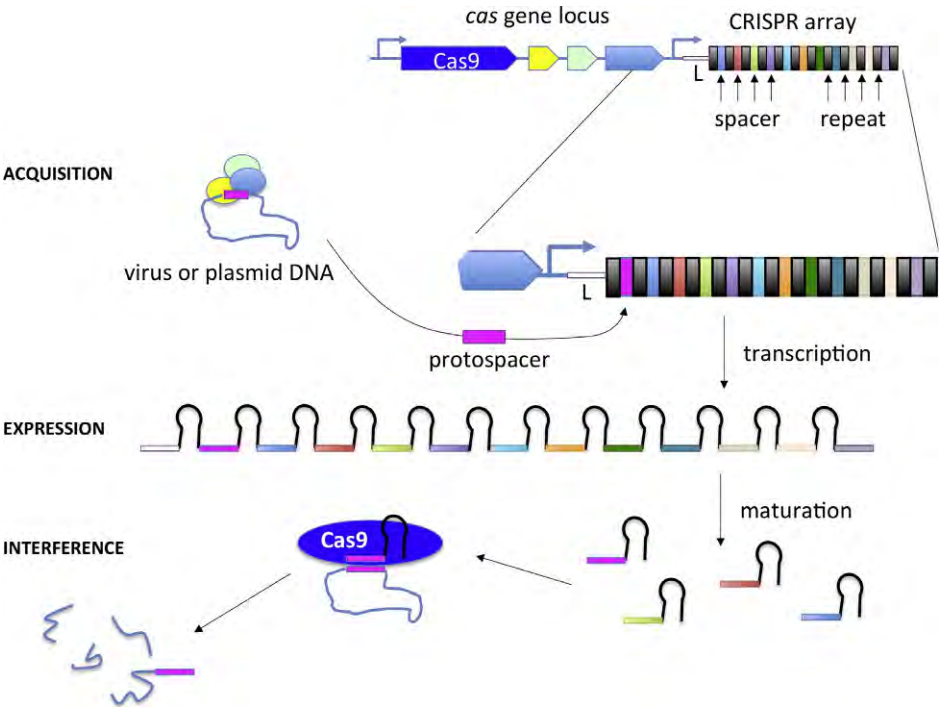

Fig. 9 CRISPR-CAS. General overview of the prokaryotic adaptive CRISPR-CAS immune system. In the acquisition step, a short fragment of heterologous DNA (protospacer) processed from the chromosome of a virus or plasmid DNA by the CAS proteins is acquired and integrated into the host CRISPR locus right upstream of the leader (L). The CRISPR locus is comprised of short direct repeat sequences (black blocks) that separate similarly sized spacer sequences (colored blocks). During the expression step, the CRISPR locus is transcribed as a single RNA and subsequently processed to mature crRNAs specific for different sequences. In the silencing step, the crRNA-Cas9 protein complex recognizes foreign DNA through base-pairing of the crRNA with the target sequence. The heterologous DNA is subsequently destroyed, thus providing immunity against the invading DNA.

presumably through its ability to regulate *X. nematophila* gene expression. Thus, there is accumulating evidence that CRISPR-CAS systems play critical regulatory functions in the cell in addition to their better-characterized role in immunity against foreign DNA.

Do RNA Modifications Play a Role in sRNA Regulatory Functions?

Recently, there has been an increasing interest in the role of RNA modifications in determining RNA functionality. Over 140 different modifications have been described thus far and it is conceivable that post-transcriptional regulation is influenced by the modification of either target mRNAs or regulatory sRNAs. While bacterial mRNA modifications have been known for some time and efforts have been made to detect transcriptome-wide RNA modifications, there is still little known about the occurrence and consequences of sRNA modifications. A global transcriptomic survey of N 6-methyladenosine (m^6A), one of the most common RNA modifications, identified 15 modified sRNAs in *E. coli* and 2 sRNAs in *P. aeruginosa*. Presumably, additional modified sRNAs still await discovery in both species, as sRNA expression is context-dependent and only one RNA modification was covered in the study. Therefore several questions remain to be answered: i) How widely distributed are sRNA modifications in prokaryotes? ii) What types of modifications are present? iii) Is a particular sRNA uniquely associated with a certain type of modification, or can this vary depending upon environmental conditions? iv) Are sRNA modifications dynamic or static? And most-importantly, v) What are the regulatory implications of sRNA modifications? This is currently a newly developing area of regulatory RNA research that will presumably reveal a variety of new facets to our mechanistic understanding of regulatory RNAs in the coming years.

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Respiratory Viruses

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Abbreviations

ARDS	Acute respiratory distress syndrome
HAdV	Human adenovirus
HBoV	Human bocavirus
HCoV	Human coronavirus
HMPV	Human metapneumovirus
HRSV	Human respiratory syncytial virus
HRV	Human rhinovirus
ICU	Intensive care unit
LRTI	Lower respiratory tract infection
RT-PCR	Reverse transcription polymerase chain reaction
SARS	Severe acute respiratory syndrome
URTI	Upper respiratory tract infection
WHO	World Health Organization

Introduction

Respiratory viruses are the leading cause of disease in humans, worldwide. While severe morbidity from respiratory virus infection occurs mostly in children, there is risk to healthy adults, the aged and immunocompromised as well. A wide range of virus families are able to infect and cause disease through the respiratory tract, and generally there is little treatment available other than supportive care. There is a critical need for understanding pathogenesis of infection and the development of therapeutics for this wide-ranging category of viruses.

Most common respiratory viruses cause mild cold symptoms in healthy individuals, resulting in significant loss of productivity. The recently emerged severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) demonstrate the ability for zoonotic emergence. Owing to the ability to spread via the respiratory route, newly emerging pathogens such as these have the potential for pandemic spread. By increasing our knowledge of these pathogens, we will be able to develop approaches to protect against the common cold and more severe, potentially pandemic, reparatory diseases.

Respiratory Syncytial Virus

The Virus

Human respiratory syncytial virus (HRSV) was first isolated from chimpanzees in 1956, and subsequently from infants suffering severe lower respiratory tract disease the following year. The virus is now recognized as a leading cause of childhood respiratory tract infection across the globe. By age 3, nearly all children will have been infected by the virus. HRSV is a member of the *Orthopneumovirus* genus within the family *Pneumoviridae* of the *Mononegavirales* order. Viruses within the *Mononegavirales* order have a nonsegmented, negative sense, single stranded RNA genome (Table 1). The HRSV genome is approximately 15.2 kilobases in size, comprising 10 genes, which encode 11 proteins (for a schematic view of the genome organization, see Fig. 1). HRSV virions are pleomorphic particles, with either spherical or filamentous forms, around 120–200 nm in size. Virions are encased in a host-derived lipid bilayer, from which three viral proteins protrude; F, G and SH. Within the virion, the genome is bound to the viral nucleoprotein (N) in a ribonuclear protein (RNP) complex. Similar to other negative sense RNA viruses, HRSV particles carry an RNA-dependent RNA-polymerase (L). The virion also contains the phosphoprotein (P) and the matrix protein (M2-1).

HRSV has two glycoproteins involved in cellular entry, F and G. G is responsible for receptor binding, but infection is possible in the absence of this protein, albeit it at lower efficiency. F is a type I fusion protein and is essential for infection. The precise cellular receptor for HRSV has been debated, with heparan sulfate been suggested for many years. More recent work has suggested that this receptor was an artifact of working with immortalized cell lines, and that the receptor on more physiologically relevant human airway epithelial cells is the chemokine receptor CX3CR1, which is found exclusively on the apical surface of

Table 1 A comparative table of the major features of the viruses discussed in this article

Virus	*Family*	*Genome type*	*Genome size*	*Virion structure*	*Major clinical disease*
RSV	*Pneumoviridae*	−ssRNA	15.2 kb	120–200 nm, enveloped	Bronchiolitis, wheezing
HMPV	*Paramyxoviridae*	−ssRNA	13 kb	150–200 nm, enveloped	URT symptoms
HPIV	*Paramyxoviridae*	−ssRNA	15 kb	150–200 nm, enveloped	Croup, bronchiolitis
HRV	*Picornaviridae*	+ssRNA	6–7 kb	30 nm, nonenveloped	URT symptoms
LP-HCoV	*Coronaviridae*	+ssRNA	27–32 kb	120 nm, enveloped	URT symptoms
SARS-CoV & MERS-CoV	*Coronaviridae*	+ssRNA	27–32 kb	120 nm, enveloped	LRT, ARDS
AdV	*Adenoviridae*	dsDNA	35 kb	90 nm, nonenveloped	URT, conjunctivitis, gastroenteritis, myocarditis

RSV, respiratory syncytial virus; *HMPV*, human metapneumovirus; *HPIV*, human parainfluenza virus; *LP-HCoV*, low pathogenicity coronaviruses (HCoV-299E, -OC43, -NL63, -HKU1); *SARS-CoV*, severe acute respiratory syndrome coronavirus; *MERS-CoV*, Middle East respiratory syndrome coronavirus; *AdV*, adenovirus; *URT*, upper respiratory tract; *LRT*, lower respiratory tract; *ARDS*, acute respiratory distress syndrome.

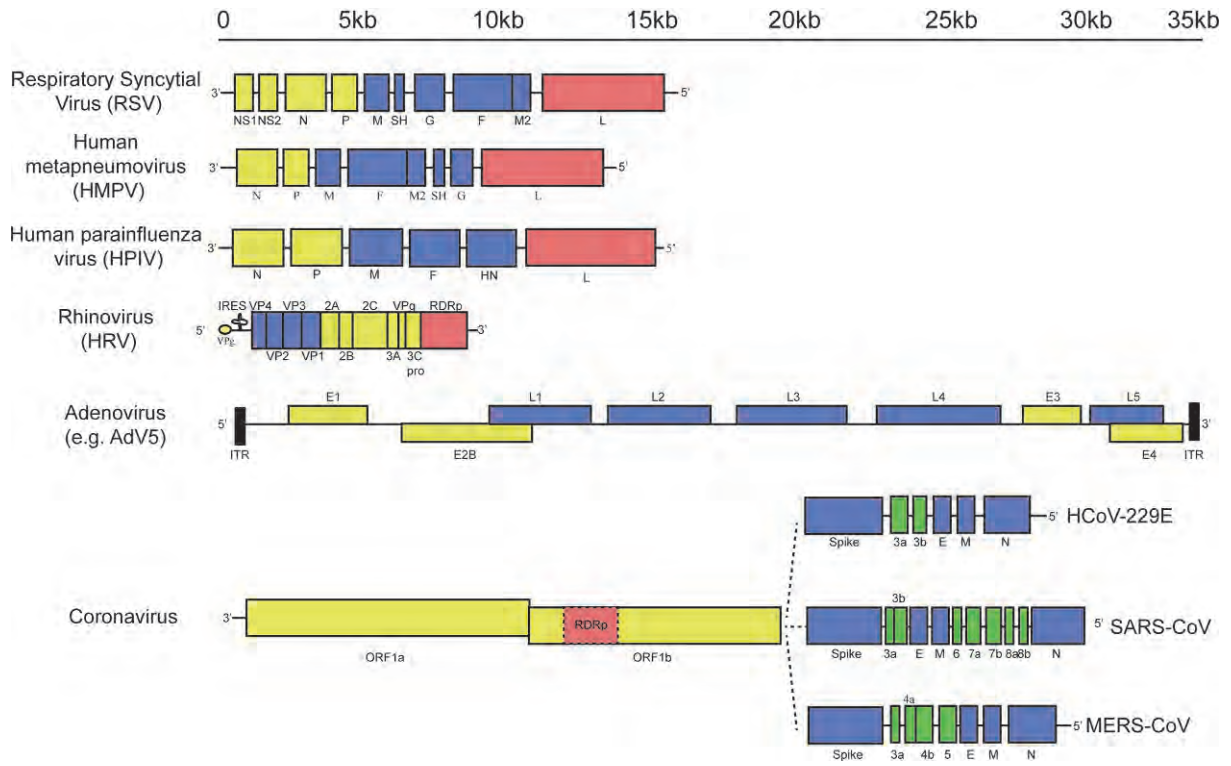

Fig. 1 Schematic representation of genomes of respiratory viruses. The genomes of the discussed respiratory viruses are displayed with approximate size of the viral gene features. The genome features of each virus are color coded with red for viral polymerase, blue for structural/envelope associated proteins, and yellow for replication factors and viral genome associated proteins. For coronavirus genomes, the 5′ two-thirds are similar however the 3′ one-third is unique to each family, as noted. The green gene products denote virus specific genes unique to each coronavirus species with HCoV-229E as an example of the seasonal coronavirus family. *ITR*, inverted terminal repeats; *IRES*, internal ribosome entry site; *RDRP*, RNA-dependent RNA polymerase.

these cells. The precise trigger for F-mediated membrane fusion is unknown, but is pH-independent because when F is expressed at the plasma membrane of cells it can cause syncytia formation at neutral pH. This syncytia formation is characteristic of HRSV infection and is from where the virus' name is derived. HRSV is generally thought to fuse with the plasma membrane, although there have also been suggestions that the virus is internalized through macropinocytosis prior to fusion with an endosomal membrane. Once membrane fusion is completed the viral genome is deposited to the cytosol. The negative sense RNA genome is transcribed by the actions of the L, P, N and M2-1, giving a set of mRNAs to direct the synthesis of viral proteins. The viral mRNAs are produced in a gradient with decreasing amounts of transcript to the 3′ end of the genome. The genes transcribed from the 5′ end at the highest abundance are the nonstructural proteins NS1 and NS2 that function to suppress the innate immune response of the infected cell. Over time, the polymerase complex switches from producing mRNA transcripts to producing full-length copies of the viral genome, the antigenome. This switch is in part regulated by the M2-2 protein. The antigenome is used as a template to direct replication of the negative sense genome that associates with N to form the RNP complex. Progeny virions

assemble at the plasma membrane through an interaction of M with the RNP complex and the viral glycoproteins, prior to budding and release. In polarized epithelial cells virion release is directed toward the apical plasma membrane (for a schematic view of the life cycle, see Fig. 2).

Along with directing viral entry into cells, F and G are the main proteins presented to the immune system. HRSV is divided into two serotypes, group A and B, largely based on differences in the G protein, and the differing antibody responses this produces. Within these serotypes there are various genotypes. Group A has 11 genotypes, GA1–7, NA1, NA2, SAA1 and ON1, while group B has 23 genotypes, GB1–4, SAB1–4, URU1, URU2, BA1–12 and THB. Both serotypes and multiple genotypes have been found to co-circulate, although often one serotype will be dominant in a given season. It is thought this genetic and serological diversity is a major reason for the abundance of re-infection throughout life and lack of prolonged immunity.

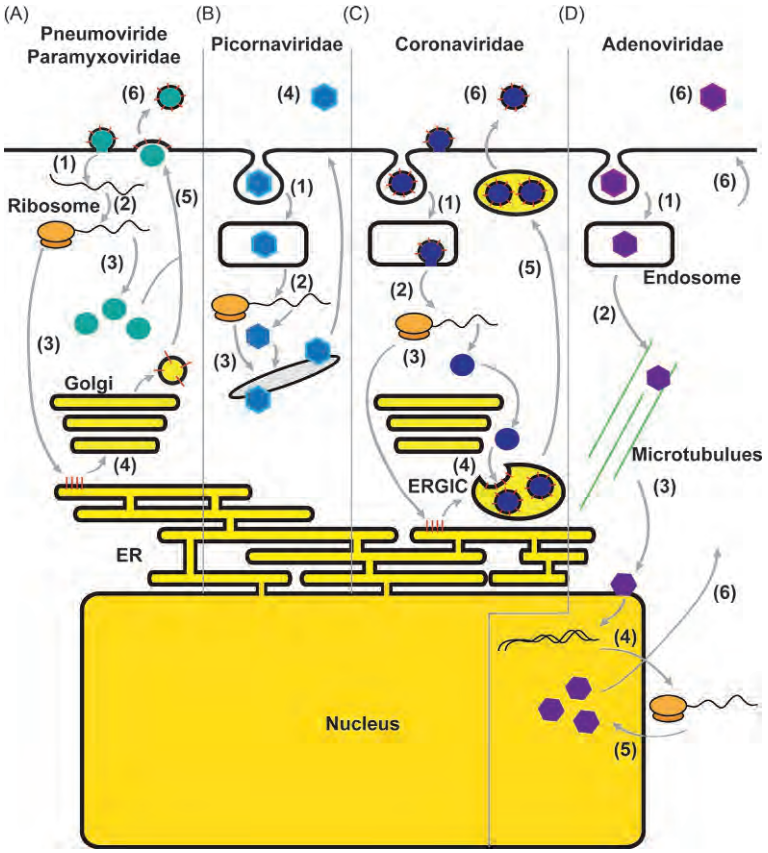

Fig. 2 A schematic view of the life cycle of the four major viral families that cause respiratory diseases. (A) *Paramyxoviridae* and *Pneumoviridae* have very similar replicative life cycles and illustrated is a view of the HRSV replication life cycle. (1) HRSV attaches to cell surface receptors and has pH-independent fusion. It is generally thought that HRSV fusion occurs at the cell surface, but may also be within endosomes. Some members of the *Paramyxoviridae* have receptor-mediated endocytic uptake and fuse at internal membrane sites (as is depicted in the *Coronaviridae* life cycle). (2) Following release of the negative sense RNA genome to the cytosol, positive sense RNA is produced for protein production and genome replication. (3) Viral proteins are produced, with soluble proteins in the cytosol and the membrane interacting glycoproteins being synthesized at the endoplasmic reticulum (ER). (4) Glycoproteins are trafficked from the ER to the plasma membrane. (5) Viral structural proteins and genomic material associate with glycoproteins at the plasma membrane. (6) Nascent virions bud from the plasma membrane. (B) (1) *Picornaviridae*, such as HRV, bind to cell surface receptors and trigger endocytic uptake. (2) Virions are delivered to endosomes and release their genomes into the cytosol. (3) The positive sense RNA is used to produce viral proteins. Not depicted, the positive sense RNA is used as a template to produce an antigenome which is used for replication of genomic RNA. (3) New viral particles are formed on intracellular double membrane compartments. (4) Viral particles are released from the cell through lysis. (C) (1) *Coronaviridae* are internalized to cells through receptor-mediated endocytosis or have direct fusion at the plasma membrane following proteolytic cleavage of the spike glycoprotein. (2) Cathepsins in acidified endosomal compartments cleave spike to promote membrane fusion to release genomic material to the cytosol. (3) New viral proteins are produced, and the viral genome is replicated similarly to other positive sense RNA viruses. (4) Soluble viral structural proteins and the glycoproteins assemble new virions on membranes in the ER-Golgi intermediate compartment (ERGIC). The new virions that are produced bud into these vesicles. (5) Virus containing vesicles leave the cell through the exocytic pathway to release virus to the extracellular space (6). (D) *Adenoviridae* are taken into cells through endocytic mechanisms. (2) Virus particles are released from the endosome and a partially uncoated virus associates with the microtubule network through dynein motors to traffic to the nucleus. (3) Final viral uncoating occurs at nuclear pore complexes. (4) Double stranded DNA is released into the nucleus and treated similarly to cellular DNA to produce new viral proteins. (5) Viral particles assemble in the nucleus and are released by cell lysis (6).

Epidemiology

HRSV is the leading cause of lower respiratory tract infection (LRTI) in children across the globe. Moreover, pneumonia is the leading cause of mortality in children with HSRV being the most common single cause. Estimates suggest that each year there are over 30 million HRSV infections and over 60,000 childhood deaths worldwide. Approximately 99% of HRSV-associated deaths are in developing countries. HRSV has annual seasonal outbreaks in winter/early spring in temperate regions and during the rainy seasons of tropical regions. The peak of spread in temperate climates is during mid-December until early February. Along with changing climactic conditions and the influence this has on behavior (e.g., increased indoor crowing), protective antibodies may influence the seasonality of HRSV infection. It has been suggested there is a 6 month waning period of protective antibodies, leading to susceptibility come the following season.

It is estimated that around 30% of infants infected with HRSV require medical attention, with 2%–3% of these cases needing hospitalization. Bronchiolitis is the most common cause of hospitalization. Bronchiolitis occurs in roughly 10% of newborns within the first year of life, with between 60% and 90% of cases being due to HRSV infection.

HRSV is predominantly transmitted through close contact, either in the form of large aerosol particles, or through contaminated surfaces and fomites. The virus is stable on hands for half an hour or more, and up to 5 h on surfaces. Following initial contamination, there is usually a 4 to 6 day incubation period, although this can be between 2 and 8 days.

Pathogenesis and Clinical Features

The clinical manifestation of HRSV infection ranges from mild upper respiratory tract infection (URTI), often with otitis media, to more severe LRTI. Approximately a third of patients infected with HRSV will progress to LRTI. The most common presentation of LRTI in infants is bronchiolitis, but pneumonia and croup are also seen. The virus replicates in epithelial cells throughout the respiratory tract causing necrosis of ciliated cells, syncytia formation, peribronchiolar inflammation and impairment of secretion, all of which play a role in airway obstruction and lung hyperinflation, typical of bronchiolitis. HRSV infection is particularly severe in infants because their under-developed lungs are unable to compensate for the damage induced by viral replication and the inflammatory response. Re-infection usually displays reduced severity of disease. In healthy adults, infection usually causes mild, nonspecific features of viral URTI, such as coryza, sore throat, fever and malaise. In the elderly, immunocompromised or those with underlying health conditions, the symptoms are often more severe.

Many factors associated with risk of infant hospitalization have been found such as preterm birth, chronic lung disease, congenital heart disease, low birth weight, acquired or congenital immunodeficiency along with others. However, around half of infants hospitalized by HRSV infection are otherwise healthy. To date, very few protective factors have been found, although breast feeding, and the level of maternally derived antibodies correlate with a decreased likelihood of hospitalization. There is debate over whether there are any viral determinants of disease severity. Various studies suggest that serotype A viruses are more virulent, while others suggest serotype B causes more severe disease. Other studies show no particular impact of viral serotype on disease severity. However, it is established that a higher viral load is associated with increased risk of severe disease; a higher viral load on day 3 of hospitalization was found to be associated with an increased risk of admittance to the intensive care unit (ICU) and respiratory failure.

The immune response has been found to play a role in disease severity. A skew toward a Th2 cytokine response leads to more severe disease. This appears to have been responsible for the failure of a HRSV vaccine trialed in the 1960s. The formalin-inactivated vaccine designed to provide protection actually exacerbated the symptoms of circulating HRSV infection with 80% of recipients to be hospitalized, resulting in two deaths. One suggestion for the enhanced severity was that the vaccine promoted a Th2 response upon subsequent natural infection, whereas a primary infection by circulating HRSV and subsequent re-infection would be skewed toward producing a Th1 response.

Finally, there have been suggestions that HRSV hospitalization is associated with long-term sequelae. Infants hospitalized by HRSV infection have been suggested to have an increased likelihood of developing persistent wheezing, exacerbations of asthma and in some cases develop inflammatory airway disease.

Diagnosis

Nasopharyngeal aspirates, nasal washings, swabs and lower respiratory tract samples can all be used for viral detection. From these samples, virus can be recovered from various immortalized cell lines. Most commonly used are HEp-2, although other epithelial derived cells such as HeLa and A549 can also recover virus. All culture methods have a 3 to 7 day turnaround time so are not suitable for diagnostics.

Rapid antigen detection tests (RADT) have been developed such as enzyme-linked immunosorbent assays (ELISA) and chromatographic immunoassays (CIA). These are both easy to use and have a rapid (under 10 min) turnaround time making them applicable at the point of care. Immunofluorescence assays can also be used from sloughed respiratory cells collected from a patient. This approach is slower than the RADTs but has a higher degree of sensitivity.

Overall, the most common, and sensitive, diagnostic tool is reverse transcription polymerase chain reaction (RT-PCR). Using real time methods, RT-PCR can be used to generate quantitative results to assess viral load as well as detect the virus. As discussed

previously, high viral load is associated with increased disease severity, so assessing viral load may be beneficial to judge likelihood of severe disease developing.

Management and Control

Currently, the majority of HRSV infection treatment is supportive. Most national recommendations emphasize the need for appropriate delivery of supplemental oxygen and hydration, in cases requiring treatment. The treatment of bronchiolitis is more extensive, but again supportive. Bronchodilators can be used such as alpha- and beta-adrenergics, anticholinergics and nebulized epinephrine, however, there is no conclusive evidence that these have a positive impact on disease outcome. Aspiration of respiratory secretions, or mechanical ventilation can be used to prevent hypoxemia, and in the most severe cases, extracorporeal membrane oxygenation can be used.

Ribavirin is currently approved for treatment of HRSV infection, but there is limited evidence for efficacy with meta-analyses suggesting that the investigations performed have lacked appropriate sample sizes to draw firm conclusions. The lack of conclusive evidence regarding efficacy, coupled with the side effects associated with ribavirin treatment means the drug is only administered to infants at the highest risk of severe disease. Ribavirin use in adults is off-license and limited to severely immunocompromised patients.

Passive immunization with humanized monoclonal antibodies is currently the most effective means of control for HRSV infection but its use is limited due to cost. Palivizumab is a monoclonal antibody (mAb) that targets the F protein of HRSV to block infection. The mAb is administered prophylactically by monthly intramuscular injection through the HRSV season in infants at highest risk of severe disease (5 monthly injections). A second generation mAb, Motavizumab, was developed but found to be no more efficacious than Palivizumab. Other companies have worked on the development of mAb therapy; Suptavumab went to a phase III clinical trial but was discontinued when primary end point measures were not met, and MEDI8897 is currently undergoing clinical development. Additionally, the approach of using nanobodies to target the F protein is under clinical development.

Vaccination would be the gold-standard for prophylactic protection, and has been long sought after, although hampered by the initial attempts. As discussed previously, in the 1960s a formalin-inactivated vaccine against HRSV was trialed in infants, but discontinued as it was found to enhance disease severity. This outcome has limited the development of an appropriate HRSV vaccine ever since. Other factors compound the issues of vaccine development; the vaccine would need to be administered at a very young age when the immune system is poorly developed, maternal antibodies may interfere, natural infection does not provide durable immunity, and it is unethical to test the vaccine efficacy in the appropriate target population of newborns/infants. However, much work is going into the development of an HRSV vaccine with four categories of vaccine being investigated; live-attenuated vaccines, subunit vaccines, vector-based vaccines and particulate vaccines.

In addition to work toward developing a vaccine to target HRSV, much effort is being placed in the development of antiviral compounds. Many different small molecules targeting various aspects of the viral life cycle are under development. Many fusion inhibitors are being trialed, along with compounds that target the G attachment protein, the L polymerase function (nucleoside and nonnucleoside inhibitors), the viral mRNA guanylylation activity of the L protein and inhibitors of the N protein.

In summary, currently, the majority of treatment for severe HRSV infection is supportive toward the respiratory symptoms caused by viral infection. Monoclonal antibody therapy appears to be effective, but its use is limited by cost and the need for monthly injections, making it unviable in the developing countries where 99% of HRSV-associated deaths occur. Various antiviral compounds and vaccine strategies are being trialed, but at the time of this writing, none have been approved for use in humans.

Human Metapneumovirus

The Virus

Human metapneumovirus (HMPV) is a negative sense, single stranded RNA virus of the *Pneumoviridae* subfamily within the *Paramyxoviridae*. It was first isolated in 2001 in the Netherlands through analysis of samples from children with respiratory tract infection of unknown etiology. HMPV is now recognized as the second most common cause of LRTIs in children after HRSV, which is in the same viral order (*Mononegavirales*). Retrospective studies have found HMPV to be present in the human population for at least 50 years. The virus can be divided into two serotypes, A and B, based on alleles of the surface proteins.

The approximately 200 nm diameter viral particles of HMPV are enveloped and infect epithelial cells in the nose and lungs (Table 1). Overall, replication of HMPV is similar to that previously described for HRSV. Infection proceeds through the attachment of glycoprotein (G) to heparan sulfate and other glycosylated proteins. The fusion (F) protein then interacts either with integrins or a currently unknown receptor to induce endocytosis. The F protein is activated by low pH in endosomal compartments to promote fusion between viral and cellular membranes for release of genetic material to the cytosol. The negative sense RNA genome is then used to direct production of new viral particles, which bud from the plasma membrane, as described previously for HRSV.

Epidemiology

HMPV is associated with approximately 20% of respiratory tract infections in children worldwide. Serologic surveys show that by 5–10 years of age, between 90% and 100% of children have been infected by HMPV. The median age of initial HMPV infection is close to 12 months of age, older than HRSV.

HMPV infections mostly occur in late winter and early spring, generally following the influenza and HRSV season. Infection causes mild URTI in healthy children and adults, however in the immunocompromised and elderly, severe symptoms are commonly observed. Despite the seroprevalence of HMPV in young children, re-infection is common due to either insufficient antibody generation or infection with a different serotype.

Pathogenesis and Clinical Features

The clinical features of HMPV infection are similar to those presented during an HRSV infection, causing mild upper, or severe lower respiratory tract infections. Studies in humans have found diffuse alveolar damage, soughing of epithelial cells, eosinophilic cytoplasmic inclusions, syncytia and hyaline membrane formation. Common symptoms are fever, cough, tachypnea, dyspnea, hypoxia, rhinitis and sore throat. It has been reported that approximately 50% of infants present with otitis media. Hospitalization from HMPV infection is most common in infants aged 6–12 months, with bronchiolitis and pneumonia being the most frequent causes. Higher viral load is significantly associated with disease severity. Infection inhibits the immune response which can delay clearance of the virus from the respiratory tract and is probably the reason for low level neutralizing antibody development.

Diagnosis

Detection of HMPV is by RT-PCR from nasal or throat swabs. Serum from patients can also be tested. Virus from nasal secretions can be grown in cell culture and immunofluorescence used for detection.

Management and Control

Virus transmission is through respiratory secretions and contaminated surfaces. As such, spread can be limited by general hygiene countermeasures. No treatments are licensed although ribavirin has been used in severe cases. No clinical trials have been performed to examine the efficacy of ribavirin in humans, but it has been shown to be effective in vitro and in mouse models of infection. As previously discussed, monoclonal antibody therapy is used for HRSV infection and therefore may have potential for protection against HMPV infection, although this is at an early stage of development. Monoclonal antibodies have been shown to be protective in mouse, hamster and cotton rat models of infection, but have yet to be thoroughly examined in humans. Fusion inhibitors and RNAi approaches are also being explored. There is no HMPV vaccine, although similarly to HRSV, multiple approaches are being explored. Currently, palliative care is recommended to allow for clearance of the virus and recovery from the respiratory inflammation resulting from infection. Oxygen therapy, corticosteroids and mechanical ventilation can all be used for symptomatic treatment.

Human Parainfluenza Virus

The Virus

Human parainfluenza viruses (HPIV) are nonsegmented, negative sense, single stranded RNA viruses of the *Paramyxovirus* genus (like HMPV). HPIV was discovered in the 1950s as the cause of LRTI in children that were not the result of the already known influenza virus. Based on genetic and antigenic diversity, HPIV is divided into four types, HPIV-1, 2, 3 and 4, with HPIV-4 having subtypes a and b. These types are further subdivided into the respiroviruses, HPIV-1 and HPIV-3, and the rubulaviruses, HPIV-2 and HPIV-4.

The genome of HPIV is a single, negative sense strand of RNA of approximately 15 kilobases in a pleomorphic enveloped virion that ranges from 150 to 200 nm in diameter (Table 1). The virion envelope contains a glycoprotein that has both hemagglutinin and neuraminidase enzymatic activities (HN protein). HN is involved in entry of the virion via attachment of the hemagglutinin to sialic acids on the surface of permissive cells. Entry for HPIV occurs at the plasma membrane when the fusion protein (F) is cleaved by surface proteases causing a conformational change that promotes fusion of the viral envelope and plasma membrane. The viral genome, wrapped in a nucleocapsid, is released into the cytoplasm following the membrane fusion reaction and replication proceeds as described for HRSV.

Epidemiology

HPIV infections occur worldwide in young children with risk factors being malnutrition, vitamin deficiency, and respiratory irritants. Of the 5 million respiratory tract infections in the United States each year, HPIV is responsible for over one-third of

cases. Serologic surveys have found that by the age of 6–10 years, most children are seropositive for HPIV, demonstrating the widespread pathogenicity of this virus family. HPIV-1 to 3 are more prevalent than HPIV-4. Little is known about HPIV-4 since it is not often isolated from patients. HPIV-1 is strongly linked to croup with approximately 50% of cases being caused by the virus. Other severe symptoms include bronchiolitis, pneumonia and wheezing. HPIV-3 is second only to HRSV as a cause of pneumonia and bronchiolitis in young children.

HPIV-1 and HPIV-2 outbreaks occur in opposite years with each peaking in a year that the other wanes. The reason for this is unknown. Biennial fall outbreaks are common for both viruses with infections in children under age 5 years. Limited symptomatic infection is found in adults. HPIV-3 infections generally occur during the spring and summer months but can be found at low levels through the year. Of the HPIV viruses, HPIV-3 is most often associated with morbidity and mortality. HPIV-4 is detected at low levels throughout the year. The viruses are spread by fomites and large droplets with minimal persistence on solid surfaces.

Pathogenesis and Clinical Features

HPIV infection occurs primarily in children and is associated with upper and lower respiratory tract infections. Symptoms range from mild common cold with fever, to more severe disease such as croup, bronchiolitis and pneumonia. Classic "flu-like" symptoms are rarely seen in young children but are more common in adult infection. Croup presents with a hoarse barking cough and inspiratory stridor. This is the key clinical diagnostic phenotype for HPIV infection. Croup is the most common cause of infant hospitalization, with HPIV-1 and HPIV-2 most frequently the causative agents, although HPIV-3 has also been found. Bronchiolitis is also common in HPIV infection, especially during the first year of life. Immunocompromised children are at the greatest risk of severe HPIV infection, and in this population, it can be fatal. Minor risks of neurologic disease, apnea and bradycardia have been reported for HPIV infection. HPIV re-infection can occur throughout life with the elderly and immunocompromised at the greatest risk.

Diagnosis

Virus can be found in respiratory secretions, but the major diagnosis is from the bark-like cough and the stridor observed by chest x-ray where a "steeple sign" can be seen due to narrowing of the subglottic airway. RT-PCR is a common molecular diagnostic after infection of cell monolayers to amplify virus.

Management and Control

No treatments are licensed although acetaminophen or ibuprofen are often used for treating fever. For croup, supportive care is available including taking the child outside into cool air, having them breathe steamy air from a shower and calming then down to slow their breathing. Additionally, corticosteroids and nebulizers can be used to treat respiratory symptoms.

Rhinovirus

The Virus

Human rhinoviruses (HRV) are in the *Enterovirus* genus of the *Picornaviridae* family and cause common cold symptoms. They are the most commonly isolated respiratory virus from people with mild URTI, responsible for between a half and two-thirds of all colds. HRV is considered one of the most common human infectious agents worldwide.

Rhinoviruses have very broad genetic diversity, with over 150 different strains identified by either serology or sequencing. The phylogeny can be divided into HRV-A and HRV-B, which are the most common strains found in humans, and HRV-C, which is less common but is linked to more severe disease. HRV causes a lytic infection of the epithelial cells lining the airways with increased infection in the upper versus lower airways. This segregation is thought to be due the virus having better replication kinetics at 34°C compared to 37°C, with the nasal epithelium being at a lower temperature compared to deeper parts of the airways.

HRVs are single stranded positive sense RNA viruses with a genome size between 7 and 8 kilobases (for a schematic view of the genome organization, see Fig. 1). HRV virions are small, approximately 30 nm diameter, nonenveloped viruses (Table 1). Viral particles have an icosahedral capsid structure consisting of 60 copies each of VP-1, 2, 3 and 4. The viral particles are stable for days on surfaces and resistant to many common disinfectants.

Receptor usage by HRV depends on the subtype; minor group HRV-A bind to low-density lipoprotein receptor (LDLR) family members, the major group HRV-A and HRV-B bind to intracellular adhesion molecule 1 (ICAM-1), and HRV-C binds cadherin-related family member 3 (CDHR3). HRV is taken up from the cell surface by receptor-mediated endocytosis and a conformational change occurs in the capsid upon exposure to low pH to promote pore formation and release of genomic material into the cytosol. The RNA genome has a 5′ internal ribosomal entry site (IRES) that initiates translation of the viral RNA. The genome is translated as a single polypeptide that is subsequently cleaved by host and virally encoded proteases to yield 11 viral proteins required for

replication. Newly transcribed viral RNAs can either be used for protein production or packaged into new virions for release (for a schematic view of the life cycle, see Fig. 2).

Epidemiology

HRV causes common colds around the world and humans are the only host of the virus. Adults average between 2 and 3 colds per year, with more than half of these due to HRV infection. Children typically suffer a higher frequency of colds, with estimates being up to 12 infections per year. HRV transmission is seen year-round, but cases of HRV-A and -B are seen to have two peak periods in April/May and September/October. There is an association of incidence with children returning to school. HRV-C cases appear to peak in winter months.

Virus is transmitted by droplets or fomites with close contact generally needed for cross infection between individuals. Viral particles can survive for several hours on skin which can facilitate human-to-human transmission. The virus is highly infectious, with only a small infectious dose needed to initiate disease in a human. The incubation period for infection is less than 2 days and usually requires 7–10 days for clearance.

Pathogenesis and Clinical Features

HRV infection is via the aerosol route with infection primarily occurring in the nasal or oral mucosa and eye conjunctiva. The primary site of viral replication is in airway epithelial cells. HRV infection can disrupt epithelial barrier function which can, in turn, allow translocation of pathogens across this barrier to cause disease complications. The host response to HRV is swift with an increase in systemic chemokines and cytokines that limit virus spread but causes clinical symptoms. Infection is usually mild and self-limiting, although more severe disease such as pneumonia has been seen in the elderly and immunocompromised.

HRV symptoms start with sore throat and runny nose and can develop to cough, sneezing and congestion. Other symptoms can include fatigue, body aches and loss of appetite. Patients can have several colds a year due to the wide spectrum of serotypes in the environment and the limited immune response to each strain that is produced during infection. HRV is also associated with asthma exacerbations in addition to causing common colds.

Diagnosis

HRV diagnosis is by clinical symptoms and by RT-PCR of nasal secretions. Some clinical microbiology laboratories will grow samples from nasal swabs in cell culture for analysis. The vast genetic differences between the large number of isolates makes ELISA analysis difficult due to the variability in surface antigen alleles present in the environment. Usually RT-PCR is targeted to conserved regions of the genome for diagnosis in hospitals.

Management and Control

There are no licensed therapeutics and only supportive care is given for HRV infection. Many antiviral drugs have been proposed, however, all lack potency in humans. For instance, zinc has been tried as an over the counter therapeutic but with limited success. Vaccine development is problematic due to the high variability of surface proteins, although current efforts are directed to regions of conservation between many of the serotypes. Future work on prophylaxis is ongoing.

Coronaviruses

The Viruses

Coronaviruses are part of the *Coronaviridae* family in the *Nidovirales* order that contain the largest viral RNA genomes currently known. The first two human coronaviruses (HCoV) were identified in the 1960s and are known as HCoV-229E and -OC43. These two viruses, and coronaviruses in general, were given relatively little attention until the severe acute respiratory syndrome (SARS)-CoV outbreak in 2003. Subsequently, three further HCoVs were identified. HCoV-NL63 was found in a child suffering bronchiolitis in the Netherlands, while HCoV-HKU1 was discovered in adult cases of pneumonia in Hong Kong in 2005. Finally, an outbreak of Middle East respiratory syndrome (MERS)-CoV was first identified in 2012. These six HCoVs can be broadly divided into two groups; HCoV-229E, -OC43, -NL63 and -HKU1 can be described as low pathogenicity (LP) HCoVs that typically cause mild respiratory infection. While SARS- and MERS-CoV can be considered highly pathogenic HCoVs. The unifying virology of these pathogens will be considered first, then they will be divided when discussing their epidemiology, pathogenesis, clinical features, diagnosis, management and control.

The *Coronaviridae* family can be broken into two subfamilies, the Coronavirinae and Torovirinae. The Coronavirinae has four groups, alpha, beta, gamma, and delta. HCoV-229E and -NL63 are alpha-coronaviruses, while the other four HCoVs are beta-coronaviruses (Table 1). The family of viruses have positive sense RNA genomes ranging from 27 to 32 kilobases (kb) (for a

schematic view of the genome organization, see Fig. 1). Coronavirus virions are spherical with an approximate diameter of 125 nm and are wrapped in a host-derived lipid envelope. The virions have large protrusions from the surface of the spike (S) protein which gives a crown-like appearance, leading to the naming of coronaviruses (corona deriving from the Latin for crown). Along with S, the viral envelope also contains matrix (M) and envelope (E) proteins. M is the most abundant protein in the virion and is thought to be responsible for the shape. The expression of M and E in a cell can produce a viral-like particle (VLP). Within the enveloped virion, the viral RNA genome is wrapped in the nucleocapsid (N) protein. All coronaviruses have these four structural proteins, but some beta-coronaviruses, including HCoV-OC43, also have a hemagglutinin esterase (HE) in the lipid envelope.

The S protein of coronaviruses is usually responsible for receptor binding and is solely responsible for membrane fusion for cellular entry. HCoVs use different cell surface receptors: HCoV-229E binds aminopeptidase N (APN), HCoV-NL63 and SARS-CoV bind angiotensin converting enzyme 2 (ACE2), HCoV-OC43 binds 9-*O*-acylated sialic acids through HE, MERS-CoV binds dipeptidyl peptidase 4 (DPP4) and the HCoV-HKU1 receptor is yet to be identified. Unifying features of these receptors are that they are expressed throughout the human airway and are found on the apical surface of cells. S, a type I membrane fusion protein, requires proteolytic cleavage to activate the fusion reaction. The precise and most physiologically relevant site of membrane fusion is debated. HCoVs have been found to fuse both at the plasma membrane following cleavage by TMPRSS2, or within acidified endosomes following cleavage by cathepsins. Data appear to suggest that both routes of entry can be used, but that at least for clinical isolates of the LP-HCoVs there may be more efficient infection through cleavage by TMPRSS2, although whether fusion occurs at the plasma membrane or within endosomal compartments is unclear.

Once viral and cellular membranes fuse, the viral genome can enter the cytosol. Since the genome is positive sense, there is direct translation to produce viral proteins. ORF1a and ORF1b are translated to express the polyproteins pp1a and pp1ab. These polyproteins contain nonstructural proteins (nsp), with pp1a comprising nsp1–11 and pp1ab additionally having nsp12–16. Within these polyproteins there are two proteases, papain-like protease (PLpro) and main protease (Mpro) that release the nsps by proteolytic cleavage. Liberated nsps then regulate production of subgenomic RNAs that encode the structural and accessory genes, and controls replication of the viral genome, both of which occur through negative sense RNA intermediates.

After translation from subgenomic RNA, the structural proteins E, M and S insert to the endoplasmic reticulum membrane. Subsequently, these proteins are trafficked to the endosomal reticulum-Golgi intermediate compartment (ERGIC) where virion assembly occurs. M is the main driver of virion assembly as it is responsible for interaction with the E protein to form VLPs and interacts with N for encapsulation of the viral genome. Following the interaction of the four structural proteins and the viral genome, there is inward budding of cellular membrane to the ERGIC to produce a virion. These virions then exit the cell through the Golgi exocytic pathway and fusion of a cellular vesicle at the plasma membrane. In polarized cells, such as those in the airways, there is preferential but not exclusive, release of progeny viral particles at the apical plasma membrane (for a schematic view of the life cycle, see Fig. 2).

Low Pathogenicity Coronaviruses

Epidemiology

The four LP-HCoVs are considered to be the second most frequent causes of the common cold behind rhinoviruses. These viruses display seasonality with spread occurring in winter and spring months in temperate regions, although summer activity has also been observed. During the season, LP-HCoVs can be responsible for anywhere between 1% and 35% of acute respiratory infections. Seroprevalence levels are seen to raise through age 5 years. By adulthood, there is about 40% seroprevalence for LP-HCoVs, in the United States. Epidemiological studies of these four viruses have often found cases of co-infection with other respiratory viruses, yet the relevance of this to clinical outcome has yet to be thoroughly examined.

Pathogenesis and Clinical Features

Studying the pathogenesis of LP-HCoVs has been hindered by a lack of small animal models that support replication and/or reproduce disease. The pathogenesis of HCoV-229E has been examined in volunteers that were experimentally infected and subject to ultrastructural studies. This work demonstrated that viral infection causes epithelial cell damage, ciliary loss and cytolysis beginning at 3 days postinfection. Virus is seen to be shed for approximately 5 days, starting 48 h after initial infection, coincidentally with symptom onset. Symptoms associated with LP-HCoVs are similar to those of other respiratory pathogens; cough, sputum, rhinorrhea, tachypnea, sore throat and occasionally fever. Disease severity can be worse in the young, elderly and those with underlying health conditions, but usually infection is self-limiting and clears within a week. HCoV-NL63 has been associated with cases of croup; a study in Germany determined that up to 45% of children under 3 years of age infected with HCoV-NL63 developed croup.

Diagnosis

The most often used technique for diagnosis of LP-HCoVs is RT-PCR. It is also possible to isolate virus in cell culture, and serological assays can be used.

Management and Control

LP-HCoV infections are usually self-limiting and often not associated with severe disease. As a result, treatment of infection is usually supportive care and symptomatic relief. There are currently no direct acting antivirals against LP-HCoVs, nor any vaccines.

Highly Pathogenic Coronaviruses—SARS-CoV

Epidemiology

The SARS-CoV outbreak began in the Guangdong province of China in November 2002. However, the outbreak was not fully appreciated until early 2003 when a single infected patient traveled from China to stay at Hotel Metropole in Hong Kong. This patient, staying on the 9th floor, managed to infect over 10 people, who subsequently spread the virus throughout Asia and Canada. One of these patients traveled to Hanoi, Vietnam and was responsible for a hospital-based outbreak of 38 cases. It was from here that the highly contagious nature of this novel pathogen was reported to the WHO by Carlo Urbani, who himself became infected and died from the virus in March 2003. Within weeks of the transmission out of Hong Kong, SARS-CoV had spread to 27 countries and infected thousands of people, with about a third being healthcare workers. The virus responsible for SARS was identified in April 2003. By July 2003, the outbreak was halted through public health measures, although a handful of additional cases were detected in December 2003 to January 2004. In a little over a year, SARS-CoV infected 8098 people and was responsible for 774 deaths, with a predicted economic impact of over 30 billion USD in the Far East alone. No cases of SARS-CoV have been reported since.

SARS-CoV emerged through zoonotic transmission from the natural reservoir host, horseshoe bats. The outbreak was however facilitated by an intermediate, amplification host, of palm civets which were kept in live animal markets in China. These animals appear to be incidental hosts since serological analyses failed to detect antibodies in animals in the wild or in breeding facilities. The virus initially transmitted from animals into the human population and was found to then adapt to facilitate efficient human–human spread. Many surveillance studies have since been performed, and multiple SARS-like viruses have been found in bats, suggesting the potential for re-emergence of a similar pathogen.

Human–human transmission occurs through aerosol droplets or contaminated surfaces and fomites. It has been shown that the virus can persist for up to 2 days on environmental surfaces. Many cases were nosocomial as the majority of virus shedding only occurs after the onset of illness (around 5 days postinfection). Approximately 33%–42% of SARS cases were in healthcare works, while 22%–39% of cases were through transmission between family members. The R0 for the virus is between 2 and 4, but measures to limit spread were highly effective and brought this down to around 0.4 to quell the outbreak.

Pathogenesis and Clinical Features

The incubation period for SARS-CoV infection is typically between 4 and 6 days. Initial presentation is general flu-like symptoms such as fever, myalgia, and lethargy, with respiratory symptoms such as cough and sore throat developing between 2 and 7 days later. The infection then progresses with more severe respiratory symptoms such as shortness of breath, pneumonia and acute respiratory distress syndrome (ARDS). Infection causes diffuse alveolar damage, lung edema, hyaline membrane and syncytium formation. Approximately half of SARS cases resulted in hypoxemia around 9 days after onset of symptoms. Many of these patients required admittance to the ICU and mechanical ventilation. A higher viral load was found to be associated with poorer clinical outcome.

Patients display widespread immune cell infiltration to the lungs. Immunopathology plays a major role in the more severe forms of SARS-CoV infection; indeed, ARDS is heavily associated with upregulation of proinflammatory cytokines and chemokines. The immunopathology of the disease is best evidenced by the fact that the most severe symptoms occur as virus titer begins to decline.

In addition to respiratory disease, SARS-CoV infection was associated with gastrointestinal symptoms such as diarrhea and vomiting. The precise cause for this is unclear and there is no evidence of fecal–oral transmission of the virus. Virus was also detected in the urine of up to 30% of patients.

The median age of SARS patients was under 45, but symptoms were typically more severe in the elderly. The case fatality for SARS-CoV infection was much higher in older patients with 43% of patients over 60 dying from infection, compared to 13% of patients under 60. About 10%–30% of patients had comorbidities associated with infection and the mortality rate in this population was 46%.

Diagnosis

Samples for diagnosis can be recovered from respiratory secretions, feces and urine. There is a higher viral load in the lower respiratory tract, so samples from that area are more reliable, but more invasive and difficult to obtain. RT-PCR, along with serological assays were used to confirm SARS-CoV infection. Virus can be isolated with Vero E6 cells, and indeed this was the approached used to determine that SARS was caused by a novel coronavirus.

Management and Control

The SARS-CoV outbreak was halted by public health intervention, not through any direct acting antiviral or vaccine. Measures such as quarantine in negative pressure rooms, handwashing, disinfection of surfaces, appropriate personal protective equipment and avoidance of contact with bodily fluid all helped to block spread of infection.

Much of the care given to SARS patients was supportive to mitigate issues of hypoxemia and ARDS. In numerous cases ribavirin along with pegylated interferon was used, although this intervention was not thoroughly tested, and retrospective studies have shown that the efficacy is debatable. Corticosteroids and thymosins were also used, but again, no thorough clinical trials were performed, and the role any of these interventions played in disease outcome remains unclear. However, convalescent patient plasma use did seem to be associated with a reduced frequency of poor outcomes when given before day 14 of infection.

To date, there are no approved direct acting antivirals against SARS-CoV. However, many compounds are currently under investigation for treating MERS-CoV infection with many of these being shown to also be effective against SARS-CoV (discussed further in the following section). Protease inhibitors did appear to have some degree of efficacy during the SARS-CoV outbreak. A number of patients received lopinavir, ritonavir or a combination of both. These drugs are licensed for use as HIV-1 protease inhibitors and are part of highly active antiretroviral therapy. Again, the use of these compounds did not undergo rigorous clinical investigation, but when used in combination with ribavirin, with or without corticosteroids, a reduction in viral load was detected and symptoms were found to subside, with disease progression being milder.

Similar to antiviral drug therapy, there is no approved vaccine against SARS-CoV infection. Multiple approaches were undertaken, however, none were successfully trialed in humans. With the emergence of MERS-CoV, there has been a renewed push for countermeasures against HCoV infection with some approaches looking to produce broadly acting vaccines. However, to date these studies are at early stages.

Highly Pathogenic Coronaviruses—MERS-CoV

Epidemiology

In June 2012, around 10 years after the emergence of SARS-CoV, a novel coronavirus was identified in the Middle East when a 60-year-old man died of acute pneumonia and renal failure in Saudi Arabia. MERS-CoV was isolated from the sputum of this patient by scientists at Erasmus Medical Center in the Netherlands and was initially called HCoV-EMC, before being re-named MERS-CoV. Retrospective studies determined that the virus had been responsible for the hospitalization of 11 patients in Jordan in April 2012. In contrast to SARS-CoV, there has been no explosive spread of MERS-CoV. The largest outbreak occurred in Spring 2014 which saw around 500 hospital acquired cases in Saudi Arabia. May 2015 also saw a large outbreak in South Korea initiated by a patient that had traveled to the Middle East. This outbreak saw 186 infections across 16 hospitals with 36 deaths. The epidemic was halted largely through quarantine measures, with around 17,000 people being isolated.

While MERS-CoV has reached 27 different countries, the vast majority of cases are confined to the Middle East. Again, in contrast to SARS-CoV, MERS-CoV continues to sporadically cause disease and in early 2018 there have been over 2100 cases, responsible for around 750 deaths. MERS-CoV therefore has a significantly higher case fatality ratio than SARS-CoV and has caused fewer cases over a longer period of time.

Similar to SARS-CoV, MERS-CoV has close ancestors in bats. MERS-CoV is also found extensively in dromedary camels in the Middle East, Northern and Eastern Africa. It is estimated that in certain areas, the seroprevalence of MERS-CoV antibodies in dromedary camels may be as high as 90%. The ubiquity of MERS-CoV in camels is most likely responsible for the continued, sporadic, emergence of cases due to close contact between humans and camels, particularly in the Middle East. It is worth noting that MERS-CoV is found extensively in camels in regions of Africa, yet there have been very few reported cases of human infection on this continent. There may be various reasons for this such as reduced surveillance, differences in the interaction between humans and camels, or the spread of a virus with less virulence. In addition to camels, MERS-CoV has been found in sheep, goats, cows, water buffalo and alpaca, suggesting the virus could become more widespread.

MERS-CoV does not seem to have adapted to spread from human-to-human effectively, unlike SARS-CoV. The majority of MERS-CoV cases are through zoonotic events. Most human-to-human transmission of MERS-CoV has been nosocomial, with estimates suggesting between 62% and 79% of cases in a hospital setting, compared to 12%–21% between family members. This nosocomial transmission likely relates to the fact that there is only significant virus shedding following the onset of symptoms. Poor human-to-human transmission of the virus is reflected in the fact the R0 value is currently estimated to be around 0.7.

Of all MERS cases to date, around 66% have been in males. However, there does not seem to be any sex-based differences in the case fatality ratio. A likely explanation may be cultural and as a result of the relative levels of interaction with the animal reservoirs of the virus. The median age of MERS patients is about 50 years, older than seen for SARS cases. Age appears to be associated with an increased risk of infection. The clearest risk factors are underlying comorbidities. Diabetes is the leading comorbidity, seen in 68% of cases, but chronic renal disease (49%), obesity (34%), chorionic cardiac disease (28%) and other lung diseases are also highly associated. Approximately 75% of MERS patients have underlying conditions, and the case fatality ratio in this population is close to 60%.

Pathogenesis and Clinical Features

MERS-CoV infection causes a spectrum of disease, from mild respiratory symptoms, usually confined to the upper respiratory tract, to more severe disease impacting the lower airways. The virus infects cells throughout the lungs via the receptor DPP4, which is found on type I and II pneumocytes, nonciliated bronchial epithelial cells, endothelial cells and some hematopoietic cells. The receptor is found at lower abundance in the upper respiratory tract, leading to higher viral load in the lower airways. DPP4 is also found in the kidneys, intestine, liver, thymus and bone marrow.

The incubation period for the virus is 2–14 days with the median being 5–7 days. For patients that suffer severe disease, there is usually a 4-day period of symptoms before hospitalization. MERS initially starts with flu-like symptoms, but then rapidly progresses to pneumonia and ARDS in severe cases (a more rapid progression than seen in SARS patients). Similar to SARS-CoV infection, there can also be gastrointestinal involvement with diarrhea, abdominal pain and vomiting often seen.

The full extent of the pathogenesis of MERS-CoV infection is still being established, and at the time of writing, only two postmortem examinations have been performed. The first of these was a full autopsy of a 45-year-old male who died in Abu Dhabi in April 2014. Prior to hospitalization, this patient had a 4-day history of fever, rhinorrhea and productive cough and was diagnosed with bronchitis. He developed a persistent cough and shortness of breath and was diagnosed with pneumonia before being admitted to the ICU. His condition worsened, he developed kidney injury and renal failure and died 3 days later. An autopsy was performed 10 days postmortem. The patient had clear signs of pleural effusion, pericardial effusion, abdominal effusion, edematous and consolidated lungs and generalized congestion. Additionally, there was diffuse alveolar damage, denuding of bronchiolar epithelium, hyaline membrane formation, alveolar fibrin deposits, type II pneumocyte hyperplasia, syncytia formation and immune cell infiltration to the lungs. Extensive damage to the kidney was also seen, but there was no evidence of virus dissemination to extra-pulmonary sites suggesting other mechanisms for this damage. The lung pathology seen in this patient was similar to that seen in SARS patients.

The second postmortem examination was of a 33-year-old male who died in Riyadh in September 2015. This patient was additionally suffering from T-cell lymphoma, which had previously been treated with chemotherapy and radiotherapy. He had been admitted to hospital in Riyadh in May 2015 for further chemotherapy and possible stem cell transplantation. The patient initially presented with cellulitis and sepsis in July. His condition worsened as he developed thrombocytopenia and neutropenia along with a fever and productive cough with chest infiltrates revealed by X-ray. At this stage the patient's sputum suggested bacterial pneumonia and was negative for MERS-CoV. But 20 days later, the patient tested positive for MERS-CoV and with increasing hypoxemia was admitted to the ICU and given mechanical ventilation. The patient died 4 days later from refractory shock and hypoxemia. Tissue samples were collected within 45 min of death, but no full autopsy was performed. Samples demonstrated severe acute hemorrhagic pneumonia, diffuse alveolar damage and immune infiltrates, and many other similarities to the previously described autopsy. Electron microscopy examination revealed the presence of virus particles in pneumocytes, alveolar macrophages, renal tubular epithelial cells and macrophages infiltrating skeletal muscles. In contrast to the previous case, there was evidence of systemic spread of MERS-CoV. However, this patient may be an atypical example of infection owing to potential exacerbation of symptoms as a result of the immunosuppression from lymphoma and chemotherapy.

Currently, a full understanding of the pathogenesis of MERS is limited by the lack of human postmortem examination and the ongoing development of appropriate animal models.

Diagnosis

The WHO have developed guidelines for the diagnosis of MERS-CoV infection. These state that sampling should be from the lower respiratory tract during the acute phase of illness and use RT-PCR to detect the presence of two parts of the MERS-CoV genome; a region upstream of the E gene (UpE) and in the ORF1ab gene. Serological tests can also be used to test for MERS-CoV infection with ELISA, immunofluorescence, and microneutralization assays all employed.

Management and Control

Currently, the best forms of control for MERS-CoV infection are public health interventions. Quarantine methods, appropriate personal protective equipment, avoiding contact with bodily fluids, and general hygiene measures can all reduce spread of infection. Moreover, limiting exposure to the animal reservoir may aid in reducing infection.

There is currently no approved vaccine against MERS-CoV, although much research is ongoing. Inactivated, live-attenuated, vector, subunit, DNA and recombinant vaccine strategies are all being explored. Along with vaccine approaches in humans, camel vaccinations are being developed to look to reduce zoonotic transmission of the virus.

Along with vaccination approaches, immunotherapy measures are being explored. Passive immunotherapy with convalescent patient plasma has been tested, although there is currently a lack of surviving patients with high antibody titers to test this approach effectively. Neutralizing antibodies targeting the spike protein of MERS-CoV are also being examined but have yet to be fully trialed in humans. Along with antibodies targeting the spike protein of the virus, monoclonal antibodies targeting the DPP4 receptor are also being examined.

There are also no approved direct acting antivirals. However, many avenues are being explored to inhibit various aspects of MERS-CoV infection. Lopinavir and ritonavir have been used in MERS patients alongside interferon and ribavirin, however, as in

SARS patients, the efficacy remains unclear. Other protease inhibitors that were initially examined as anti-SARS-CoV compounds are being explored but have yet to be fully evaluated in vivo. Repurposing of currently approved drugs is a common theme among the currently examined antiviral compounds. Antimalarials such as chloroquine, amodiaquine, or mefloquine have all been found to have antiviral activity against SARS- and MERS-CoV. A widely used antidiarrheal agent, loperamide, was found to be inhibitory to coronavirus replication through in vitro screening. Cyclophilin inhibitors currently used as immunosuppressive agents can inhibit replication as can various kinase inhibitors in use for cancer treatment. However, most of these compounds have only been tested in vitro or have limited in vivo examination. Nucleoside analogs are being explored to inhibit the RNA-dependent RNA-polymerase such as GS-57434 and BCX4430.

Overall, the best approach for control of MERS-CoV is public health measures to limit infection. There are no approved therapies or vaccines, but extensive research is taking place to move toward such approaches. Much of this work is also looking for broad-activity of any compound to not only protect against MERS-CoV infection, but also any novel coronavirus that may emerge in the future.

Adenoviruses

The Viruses

Adenoviruses are a large family of double strand DNA viruses. There are seven different species, A–G, with over 50 distinct serotypes. The viruses are in the *Mastadenovirus* genus, family *Adenoviridae*. The first identification of an adenovirus came in 1953 when a transmissible agent was found in adenoid tissue, giving rise to the name.

Adenovirus virions are nonenveloped, have icosahedral symmetry and are around 90 nm in size. The viral capsid is made up for 252 capsomere proteins that are divided into three types, hexons (240 copies), penton bases and penton fibers (12 base/fiber copies) (Table 1). Each vertex of the icosahedral virus has a penton base/fiber protruding from it. These three proteins are all exposed to the immune system and are the targets for neutralizing antibodies. The genome inside the virion is linear, nonsegmented double stranded DNA of between 26 and 46 kilobase pairs (for a schematic view of the genome organization, see Fig. 1).

Virus entry to cells and uncoating to release the genome is a complex, multistep process. Initially, the virus binds to a cellular receptor through the penton fiber. Two receptors are used depending on the viral species, with most human adenoviruses (HAdV) using the coxsackie B and adenovirus receptor (CAR). HAdV species B utilizes CD46 instead. Certain strains of HAdV have also been suggested to use desmogelin-2, sialic acids or GD1a glycan as receptor molecules.

Following initial interaction with surface receptors, the virus binds to a second receptor in the integrin family of proteins, in particular αV integrin, through the penton base. Binding to the two receptors begins the uncoating process. Interaction between penton fibers and CAR triggers drift of the virus along the plasma membrane in an actomyosin-2 dependent fashion. This drift exerts a pulling force on the virus which, if also attached to relatively immobile integrins will start to exert pressure on the virion. As such, penton fibers begin to shed from the virus at the cell surface.

Along with promoting the initial stages of virus disassembly, interaction with integrins triggers an endocytic response of the cell, causing uptake of virions either in a clathrin-dependent or macropinocytic manner. In the endosomal system, further rearrangement of the capsid occurs to allow release of protein VI. HAdV protein VI is responsible for rupturing endosomes to allow for release of the partially disassembled virion into the cytosol.

Once in the cytosol, HAdV associates with the microtubule network through the dynein motor protein and is trafficked to the nucleus. The remaining capsid proteins then interact with the nuclear pore complex and the joint interaction between microtubule motors, nuclear porins and viral capsid proteins leads to the final stages of uncoating to deposit the double stranded DNA genome into the nucleus.

The HAdV genome is treated similarly to cellular DNA and transcribed to give mRNAs that are translated to produce the viral proteins. Transcription/translation occurs in early and late phases. Early gene expression produces proteins that are nonstructural and promote viral replication. For instance, E1A and E1B which bind to Rb and p53, respectively, promote progression to the cell cycle to allow co-option of cellular DNA replication machinery for replication of the viral DNA. These early genes also play roles in expression of the late genes such as the virally encoded DNA polymerase. In the late phase, structural proteins are produced to assemble new virions. Assembly occurs in the nucleus where the viral DNA is encased in new capsid. Progeny virions are released through cell lysis (for a schematic view of the life cycle, see Fig. 2). However, it is also possible for HAdV to enter into a latent life cycle; this life cycle was how adenovirus was first discovered in adenoid tissue.

Epidemiology

HAdVs cause a spectrum of disease depending on the species but respiratory infections are among the most common, especially in children under 5 years. It is estimated that globally, about 5%–7% of pediatric respiratory tract infection are from HAdV. Most of these infections are mild and self-limiting causing general common cold symptoms. However, there can be more severe disease such as pneumonia, bronchitis and croup. Along with respiratory infection, HAdV infection can cause conjunctivitis, gastroenteritis, hepatitis and myocarditis.

Of the various species of adenovirus, B, C and E have been found to infect the respiratory tract. Infections by respiratory HAdVs occurs globally and do not appear to show seasonality. When outbreaks do occur, they are often in small populations such as in

hospitals and particularly in military installations. Most infections occur in young children or immunocompromised adults, but there have been severe outbreaks in healthy adults. The reasons for the spectrum of disease severity are not fully clear, but it is hypothesized that it may be down to emergence of novel strains of HAdV. For instance, 2007 saw the emergence of a novel HAdV-14 that initially spread in US military personnel and then in civilian populations that caused pneumonia and ARDS. Antibody responses to HAdV infections have been shown to be effective and long lived, suggesting that the emergence of strains that can avoid preexisting immunity may indeed play a role in more severe infections.

Respiratory HAdVs are transmitted in aerosol form through coughs and sneezes and through contaminated surfaces. Adenoviruses have a high degree of environmental stability, they are unperturbed across a pH range of 5–9 and are resistant to isopropyl alcohol, ether, and chloroform. HAdV can survive on surfaces for weeks at room temperature and even longer at colder temperature.

Pathogenesis and Clinical Features

Respiratory tract infection by HAdV typically causes mild, self-limiting, common cold symptoms. The most common manifestations of HAdV infection are cough, chest pain, headache, malaise, pharyngeal symptoms and fever. The virus has a lytic life cycle, so causes damage to the respiratory tract through cell death and can cause infiltration of immune cells. More severe forms of disease can occur, particularly in the young or immunocompromised that are associated with pneumonia or bronchitis. Children under the age of 5 years can often develop fever, pharyngitis and otitis media, which can occur in up to 30% of cases.

Patients are seen to develop a neutralizing antibody and T-cell immune response to HAdV infection that protects in a type-specific manner. However, because of the various species of HAdV, re-infection is common. Owing to the immune response many re-infections can be asymptomatic, and indeed, up to 50% of HAdV infections are thought to be asymptomatic due to the combination of latency and the immune response.

Diagnosis

Virus can be found in respiratory, ocular and ear secretions, but there is a need for clinical correlation when virus is detected because asymptomatic shedding is common. Virus can be grown out of samples using various cell lines such as HEp-2, HeLa and A549 and can be easily detected through the cytopathic effect infection causes. PCR is the most sensitive method for detection, but again requires clinical correlation due to asymptomatic HAdV infection. Point of care immunochromatography tests are also available.

Management and Control

Owing to the usually mild and self-limiting nature of HAdV infection, there is little impetus for intervention. No treatments are currently licensed, although cidofovir has been suggested to have therapeutic potential in pediatric patients. A live-attenuated oral vaccine is licensed for use in US military recruits attending basic training, but this is not available to the public. General countermeasures of hygiene and surface disinfection with virucidal agents are routinely used to limit spread.

Human Bocavirus

The Virus

Human bocavirus (HBoV) was first identified in 2005 by screening of nasopharyngeal aspirates for novel viruses causing respiratory disease. A random PCR amplification strategy followed by sequencing and bioinformatics identified a previously unknown member of the *Parvoviridae* that became known as human bocavirus. The name derives from there being genetic similarities to two already defined parvoviruses, bovine parvovirus 1 and canine minute virus. Since the initial identification, three further subtypes of HBoV have been found in stool samples (HBoV2, 3 and 4).

HBoV is a member of the *Parvoviridae* which are small single stranded DNA viruses. Parvoviruses are among the smallest known viruses both in terms of genome, around 5–5.5 kilobases, and particle size, ranging from 18 to 26 nm (Table 1). Virions are nonenveloped and made up of 60 capsid proteins. The HBoV genome contains three ORFs (ORF1–3). There are four nonstructural proteins encoded from ORF1, NS1–4, and an additional nonstructural protein, NP1 encoded from ORF2. The final ORF encodes the three capsid proteins, VP1–3.

The precise infectious life cycle of HBoV has yet to be fully characterized. It has been suggested that sialic acids act as the receptor at the cell surface, however, the virus has proven hard to grow in many cell lines which could suggest other receptor requirements. Uptake from the cell surface is thought to be through a clathrin-mediated endocytosis. HBoV escape from the endosomal system is dependent on a phospholipase A2 domain in the VP1 protein, although the precise mechanism of escape is unclear. Replication occurs in the nucleus, and similar to other parvoviruses, HBoV requires many cellular DNA replication components. The virus can be released from cells through a lytic process, but can also be persistent in an episomal form, the determinants of these life cycles is unclear.

Epidemiology

HBoV has been found to have a worldwide distribution, with infection occurring year-round, although there does appear to be more cases in winter and spring months. A large meta-analysis of epidemiological studies conducted between 2005 and 2016 suggested that HBoV is found in about 6% of respiratory infection cases. This study also showed that HBoV is often detected in conjunction with other respiratory viruses, with over 50% of cases being co-infection. This high degree of co-infection has caused some suggestion that HBoV may not cause disease itself and just be present in a certain percentage of the human population. However, HBoV has been detected in cases of acute respiratory infection where no other causative agents could be found.

The greatest disease burden for HBoV appears to be in children between 6 and 24 months of age. Studies suggest that HBoV can be found in up to 20% of children suffering acute respiratory infection. The virus has also been associated with community acquired pneumonia in young children.

There are some limitations to the current knowledge of HBoV epidemiology. Virus has been found to shed for up to 12 months after acute infection. Thus, detection may not necessarily indicate a causative link to disease. Moreover, it has been suggested that the routine use of DNA and PCR to detect HBoV may not be appropriate due to prolonged shedding and persistence. An alternative approach of using RT-PCR to look for a spliced form of the capsid mRNA that is only present during active replication. Using this approach, HBoV was still associated with community acquired pneumonia in young children, but at much lower levels than when using DNA; approximately 10% of cases were suggested to be HBoV positive by DNA, compared to 2% by mRNA.

Pathogenesis and Clinical Features

The pathogenesis of HBoV infection is not well understood owing to the lack of an animal model and difficulties of in vitro study, although new culture systems are being developed. The understanding of HBoV pathogenesis is also clouded by the fact that the virus has a high prevalence of detection in patients infected with other viruses known to cause respiratory infection.

Studies in human airway epithelial cell culture models have suggested that HBoV infection is highly disruptive. HBoV was found to perturb tight-junction barriers, cause a thinning of epithelium, a loss of cilia and epithelial cell hypertrophy. HBoV has also been found to cause cell death, with both apoptotic and pyroptotic mechanisms suggested. It could therefore be speculated that HBoV infection in humans may disrupt the respiratory tract epithelia and cause an inflammatory response which could contribute to pathogenesis.

Symptoms associated with HBoV range from mild common cold to more severe disease. The most commonly reported symptoms in clinical studies are cough, fever, rhinorrhea, asthma exacerbation, bronchiolitis, acute wheezing and pneumonia. Viremia has also been observed in patients and viral genetic material has been detected in urine and stool. HBoV has been suggested to be associated with gastrointestinal symptoms such as nausea, vomiting and diarrhea.

Diagnosis

HBoV is most commonly detected by PCR. Virus can be detected either from respiratory secretions or stool samples. Patients have also tested positive for HBoV specific antibodies and ELISA tests can be performed.

Management and Control

The study of HBoV is largely in its infancy and there is still some debate about whether the virus is an etiologic agent of acute respiratory infection. As such, there are no antivirals or vaccine candidates against the virus.

Additional Viruses With Respiratory Transmission

Measles Virus

Measles virus (MV) is in the *Morbillivirus* genus, family *Paramyxoviridae*. Similar to the other Paramyxoviruses discussed in this article, measles has a nonsegmented, negative sense, single stranded RNA genome, which is roughly 16 kilobases in length. This genome encodes six structural proteins: nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H) and large (L). In addition, there are two nonstructural proteins, V and C, which are encoded within the P gene. MV particles are spherical and roughly 150–300 nm in diameter. Virions are enveloped and have the F and H proteins protruding on the surface that regulate cell entry. Inside the virion, the RNA genome is bound by the N protein which associates with the RNA-dependent RNA-polymerase L, and the cofactor P, as a ribonuclear protein complex. This complex is surrounded by the M protein.

Cell entry of MV is regulated by the H and F proteins. H is responsible for receptor binding. Wild type MV bind to CD150/SLAM that is found on the surface of B and T lymphocytes, immature thymocytes, monocytes and dendritic cells. In contrast, the attenuated form of MV used for vaccination uses CD46 that is found ubiquitously on nucleated cells. MV can also utilize nectin-4. Following receptor engagement, fusion of the viral envelope and cellular membranes is mediated by the F proteins and occurs at neutral pH. The genome is then replicated in a similar fashion to that described previously for other paramyxoviruses.

MV is a highly contagious pathogen, with an R0 value of between 12 and 18. Indeed, it is estimated that in an immunologically naïve population, 90% of people in close contact with an infected individual will contract the virus. MV has been shown to spread by respiratory droplets and aerosolized particles, but infects immune cells resident in the lungs, rather than respiratory epithelial cells, as a result of the tropism imposed by CD150/SLAM usage. Symptoms typically develop 9–12 days following exposure. Initial symptoms present with a fever, cough, conjunctivitis and coryza. Over time, further, highly characteristic, systemic symptoms appear such as Koplik's spots in the mouth and an erythematous, maculopapular rash. Further complications can occur such as diarrhea, blindness, encephalitis and pneumonia. After spreading through the blood stream in lymphocytic cells, MV returns to, and infects, airway epithelial cells from the basolateral side through nectin-4, allowing for respiratory shedding and onward transmission.

A highly effective, live-attenuated vaccine is available. The vaccine was originally developed by John Enders in 1963 and is now routinely given to children before the age of 18 months, along with vaccines against mumps and rubella (MMR). Immunity to measles virus is lifelong and the virus is on a target list for eradication by the WHO.

Enterovirus-D68

Enterovirus-D68 (EV-D68) was first identified in California in 1962 from children suffering severe respiratory tract infections and pneumonia. The virus is in the *Picornaviridae* family. Like other picornaviruses, EV-68 virions are small at around 18–30 nm, made up of four structural proteins and are nonenveloped. The genome is approximately 7.5 kilobases and produces 11 proteins that are released from a single polyprotein.

EV-D68 appears to require sialic acid for infection, and in vitro studies have suggested that sialic acid binding causes virus uncoating. ICAM-5/telencephalin has also been suggested as a receptor. Once inside cells, EV-D68 replicates similarly to other positive sense RNA viruses, and particularly the already discussed rhinovirus, another member of the *Picornaviridae*.

From initial identification in 1962, until the early 2000s, EV-D68 infection was considered rare. However, in recent years there have been large scale outbreaks of the virus, especially in the United States which has seen epidemics in 2014 and 2017. Infection is typically associated with respiratory symptoms, either mild common cold, or more severe pneumonia. For a period, EV-D68 was referred to as rhinovirus 87 by some. In contrast to many other enteroviruses, EV-D68 is sensitive to low pH and therefore does not effectively infect through the gastrointestinal tract, leaving the respiratory tract as the primary site of infection. Similar to rhinovirus, EV-D68 has been found to replicate better at lower temperatures. In addition to respiratory symptoms, EV-D68 has been associated with cases of acute flaccid myelitis, similarly to poliovirus, suggesting the virus may be capable of infection at sites other than the lungs.

To date, there is no effective antiviral therapy or vaccine against EV-D68 and care is supportive and symptomatic.

Enterovirus 71

Enterovirus 71 (EV71) is another *Picornavirus*. EV71 was also identified in California, a number of years after EV-D68 in 1969. EV71 utilizes P-selectin glycoprotein ligand-1 and scavenger receptor class B member 2 as receptors for infection. EV71 is best known for causing hand-foot-mouth disease, largely in the Asia-Pacific region, and in some cases severe neurological symptoms. However, the virus is also associated with respiratory disease such as pharyngitis, bronchiolitis, croup and pneumonia, mostly in young children. As with most other viruses discussed in this article, there is currently no treatment or antiviral therapy for EV71, but research is ongoing in the area.

Conclusion

Respiratory viruses are the most frequent causes of human disease worldwide. Each year, this broad group of pathogens is responsible for a huge number of deaths and economic loss through days of sickness. Moreover, owing to the nature of respiratory transmission, this category of viruses has the potential for wide-ranging spread. SARS-CoV, a virus that reached 27 countries within a matter of weeks, highlights the explosive outbreak potential of respiratory pathogens. Emergence of novel respiratory viruses carries with it the fear of pandemic disease spread. To date there is generally a lack of antiviral therapy or vaccination against any of these viruses, further fueling fears for far reaching spread. By developing a deeper understanding of how these viruses replicate, cause disease and transmit, we will be able to be prepared for, and counter, the next great pandemic respiratory virus.

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Restriction-Modification Systems

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Introduction

Restriction-modification (R-M) systems are exclusive to unicellular organisms and are ubiquitous among bacteria. They are one of the primary defense mechanisms against bacteriophage infection and other invading DNA elements. That the R-M systems constitute prokaryotic arsenals against invading genomes was appreciated by the discoveries of Arber and co-workers as early as 1962. Typical R-M systems comprise two distinct enzymatic activities: (i) a restriction endonuclease (REase) activity that cleaves phosphodiester bonds of the DNA backbone and (ii) a methyltransferase (MTase) activity that catalyzes the addition of a methyl group from the donor S-adenosyl methionine (Adomet) to DNA adenine or cytosine. In every R-M system, both enzyme activities recognize the same specific nucleotide sequence (termed the recognition site). Methylation of host genome recognition sites prevents restriction and thus provides the R-M system with an intrinsic basis for distinguishing self from non-self; each system is hardwired for surveillance of a particular recognition sequence. With the ability of the gene pair to target the incoming DNA for cleavage and protect self-DNA from being degraded, R-Ms can be considered as primitive immune systems. With the rapid strides made in our understanding of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas interference, they appear to fit in as bacterial adaptive immune systems in accordance with their mode of functioning. The functionally diverse R-M enzymes also provide excellent model systems to study protein-nucleic acid interactions and to engineer new specificities.

When the phenomenon of R-M was discovered more than six decades ago, little did the researchers imagine their wide-spread occurrence, diversity in their structure, function, and mechanisms, or their utility as molecular scissors for genetic engineering. It is evident that R-M systems represent one of the largest groups of enzymes, some having very high turnover while others catalyze only one round of reaction. Diversity is seen in their subunit composition, metal ion requirement for binding and/or cleavage, cleavage characteristics, mode of DNA binding, and catalytic mechanism. The accumulation of a large body of literature on these various aspects on R-M points to how the field has evolved over the years.

R-M Systems as Engines of Diversity

R-M systems are broadly classified as Type I, Type II, Type III, and Type IV based on their subunit structure, oligomeric status, recognition sequence, cleavage characteristics, cofactor requirements, mode of action, and use of a modified or unmodified substrate (Table 1). The REase can be a very simple endonuclease, as in the case of Type II systems, or a more complex, multi-subunit molecular machine, exemplified by Type I and Type III enzymes. Type I R-M systems are comprised of subunits that exhibit three functions: host specificity determinants (Hsd) - restriction (HsdR), modification (HsdM), and specificity (HsdS). The Type I systems form two configurations of these three subunits: (i) a holoenzyme with heteropentamer organization ($HsdR_2M_2S$) or its dimer that exerts both

Table 1 Properties and cleavage characteristics of four types of restriction modification systems (REBASE: http://rebase.neb.com/rebase/rebase.html)

Type	*Examples*	*Subunits*		*Recognition/cleavage*	*Co-factor(s)*	*No. of REases – cloned (putative)*[a]
I	EcoKI EcoBI	R_2M_2S/ M_2S	Multifunctional protein with restriction, modification and specificity subunits	Asymmetric and bipartite recognition site. Cleavage remote (1000–10,000 bp) from recognition site	Mg^{2+}, ATP, Adomet	115 (19,191)
II	EcoRI	R_2 and M	Separate restriction and modification.	Cleavage within or near the recognition sequence	Divalent metal ion	4260 (29,209)
	EcoRV		Many subtypes (see Table 2)			
III	EcoP1 EcoP15	$R_{1-2}M_2$	Heterodimer with M and R subunits	Cleavage at a short distance (~25bp) from recognition site. Two non- palindromic sites in opposite direction	Mg^{2+}, ATP, Adomet	22 (7509)
IV	McrBC McrA	R	One or two subunits	Target methylated/modified DNA	Mg^{2+}	19 (11,681)

[a]*Source*: REBASE as of April-29-2018.
R – Restriction, M – Methylation, S – Specificity subunit, Adomet – S-Adenosyl methionine, REase – Restriction endonuclease.

Table 2 Subtypes of Type II REases (REBASE: http://rebase.neb.com/rebase/rebase.html)

		Example		
Subtype[a]	*Defining feature*	*REase*	*Recognition sequence*[b]	*No. of REases*[c] *(including putative)*
A	Recognizes asymmetric sequence	BbvCI	CCTCAGC (−5/−2)	–
B	Cleaves both sides of recognition site	BcgI	(10/12)CGAN$_6$TGC(12/10)	28
C	Combined R and M in one polypeptide	GsuI	CTGGAG(16/14)	–
E	Two targets (one effector, one cleaved)	EcoRII	/CCWGG	12
F	Coordinated cleavage of two sites	SgrAI	CR/CCGGYG	8
G	Cleavage effected by Adomet	BseRI	GAGGAG(10/8)	12,162
H	Similar to Type I gene structure	BcgI	(10/12)CGAN$_6$TGC(12/10)	3
M	Methylated target	DpnI	GA/TC	796
P	Palindromic recognition site and cleavage	KpnI	GGATC/C	13,872
S	Asymmetric recognition and cleavage	FokI	GGATG (9/13)	3070
T	Heterodimeric R proteins	BslI	CCN$_5$/N$_2$	–

[a]Subtypes are not mutually exclusive. For example FokI is of subtype A and S.

[b]Cleavage position is marked by/and number denotes bases after recognition site on top and bottom strand respectively.

[c]*Source*: REBASE as of Apr-29-2018.

Adomet – S-Adenosyl methionine, REase – Restriction endonuclease, – denotes they are included in other subtypes.

the REase and MTase activities, and (ii) a HsdM$_2$S alone that has methylation activity. Sequence specificity in Type I systems is achieved by HsdS, which contains two target recognition domains that recognizes sequences on both DNA strands. These enzymes recognize asymmetric bipartite sequences comprising a 6- to 8-bp nonspecific spacer sequence flanked by 5′ trinucleotide- and 3′ tetra- or pentanucleotide-specific sequences. Type I enzymes cleave DNA 1000–7000 bp away from the recognition sequence in a reaction requiring ATP, Mg^{2+} and Adomet. Various models for the mode of cleavage have been proposed- (a) enzyme tracking along the DNA, forming ever larger loops until the cleavage site is reached (b) the requirement of at least two recognition sites wherein two enzyme molecules bind and the translocating enzymes are drawn together, to collide and trigger DNA cleavage.

Type II R-M systems consist of two separate polypeptides (REase and MTase) that act independently of each other. Generally, Type II REases are homodimeric or homotetrameric; they cleave DNA at or near their recognition site. Due to their enormous diversity, Type II systems are further classified (Table 2) into 11 non-mutually exclusive subgroups. The four abundant subtypes include Type IIP (recognize and cleave palindrome sequences), Type IIS (recognize asymmetric recognition sequences and cleave away from the recognition site), Type IIG (Adomet effects cleavage activity) and Type IIM (cleaves methylated recognition sequences). Among them, Type IIP enzymes are the best studied, as they are the primary tools for genetic engineering.

Type III R-M systems are encoded by two genes, *mod* and *res*. The R-M enzyme is composed of two subunits of MTase (Mod) and a REase subunit (Res) having an ATPase and an endonuclease domain. Type III REases cut DNA 25–27 bp away from the recognition site and show a strong preference for cleavage where there are two copies of their asymmetric un-methylated recognition sites in an indirectly repeated, head to head orientation within a distance up to 3.5 kb. DNA cleavage occurs at one of the two asymmetric sites, 25 or 26 nt downstream in the top strand and 27 or 28 nt downstream from the recognition site in the bottom strand. Interestingly, after cleavage, the enzyme remains bound to the still intact recognition site. The dimeric Mod (M_2) or the trimeric Res-Mod (RM_2) complex catalyzes hemi-methylation of the DNA at 4–6 bp asymmetric recognition sites. Type III restriction enzymes must communicate between a pair of distant sites in order to cleave a DNA substrate. A variety of biophysical, biochemical and single-molecule studies were carried out over the last decade on the Type III RM enzymes, EcoP1I and EcoP15I. The basis of long-distance communication for which a low-level ATP hydrolysis is required has not been adequately defined. Several models for DNA cleavage have been proposed, driven by either active DNA translocation, passive three-dimensional diffusion or both DNA looping and limited translocation.

Type IV systems recognize modified DNA with low sequence selectivity; they exhibit separation of DNA binding and cleavage into different domains on the same protein, or even into different polypeptide chains. Finding genes encoding RglA and RglB (restriction of glucoseless phage) that restrict non-glucosylated hydroxymethylcytosine in T-even phages led to the discovery of a new class of R-M system. The *rgl* genes were renamed *mcr*A and *mcr*BC (modified cytosine restriction) and were classified as Type IV modification-dependent REases. *Escherichia coli* K12 strains code for at least three REases selectively directed against DNA containing modified bases. These enzymes are encoded by *mcr*A, *mcr*BC, and *mrr*. REases that cleave DNA containing modifications such as ^{m5}C, ^{hm5}C, ^{ghm5}C, ^{m6}A, ^{m4}C and more recently phosphorothioate DNA are loosely grouped as Type IV REases.

While the REases exhibit a wide diversity with respect to a number of characteristics, MTases are primarily of three types: two involve catalyzing methyl group transfer to the amino groups of adenine (N^6-methyladenine – ^{m6}A) and cytosine (N^4-methylcytosine -^{m4}C), and a third type catalyzes methyl group transfer to the cytosine pyrimidine ring (5-methylcytosine - ^{m5}C). While all three types of methylation have been reported to be catalyzed by Type II R-M systems, so far only ^{m6}A catalysis have been observed in Type I and Type III R-Ms. Recent advances in the application of single-molecule real-time DNA sequencing to analyse DNA methylation have revealed for the first time comprehensive pictures of the genome-wide distribution of methylation sites (methylome) in various bacteria.

R-M systems often spread by horizontal gene transfer between microbes. In such a scenario, to facilitate timely expression of MTase before REase, regulatory elements of the R-M systems control REase expression in an intricate fashion through gene organization, *cis*-acting elements and *trans*-acting factors. Typically, the *trans*-acting transcription factor C protein acts as repressor that is transcribed prior to REase gene expression, whereas the MTase is transcribed from a separate promoter. The two promoters of the R-M system are found arranged in either a convergent or in a divergent fashion. This arrangement ensures that the REase gene is repressed and the C protein is auto-regulated.

Sequence analysis of a large number of bacterial and archaeal genomes has identified R-M systems in a majority. More than 90% of the sequenced genomes possess at least one R-M system with a direct correlation of their occurrence to the size of the genome. R-M systems are evenly distributed in bacteria and archaeal genomes; so far 15,601 REases are found in 3623 bacterial genomes and 495 REase genes in 153 archaeal genomes. R-M systems of archaea usually function at higher temperatures. *Helicobacter pylori*, *Neisseria species*, and *Campylobacter species* are exceptions in that they have an unusually large numbers of R-M systems relative to their genome size. This feature may reflect the evolution of additional cellular functions (see below). Analysis of the genome sequences of more than 75 *H. pylori* strains identified an extraordinary number of genes (~45) having homology to R-M genes in other bacterial species. In addition all *H. pylori* strains possess their own unique complement of active R-M systems. Some strains even encode members of all four types of R-M systems. An overview about known and predicted R-M genes for many *H. pylori* strains can be found in the REBASE database (see "Relevant Website section").

Structure, Mechanism, and Organizational Diversity of R-M Systems

Although Type I enzymes were discovered first and now all four kinds are abundant in microbes, the number of Type II enzymes exceeds all others (Table 1). While Type II enzymes are by far the simplest, they are highly diverse with respect to their cleavage characteristics, reaction mechanism, active sites and several other features. Since the enzymes serve as tools for recombinant DNA technology, they are well studied, including structure determination and engineering new specificities. So far, five types of catalytic folds have been found: PD-(D/E)XK, PLD, HNH, GIY-YIG, and HALF-PIPE (Table 3). Many structurally characterized REases belong to the PD-(D/E)xK superfamily, which is, the most common and well-studied group of REases. The HNH superfamily of REases is the second largest group of enzymes; it is characterized by the presence of a ββα-me finger fold. PD-(D/E)xK, HNH, and GIY–YIG catalytic motifs are widespread among DNA transaction enzymes, including mobile intron-encoded homing endonucleases and sequence non-specific nucleases. Crystallographic studies of R.BfiI revealed a three-dimensional fold that is different from the above examples. This enzyme belongs to the PLD superfamily, which includes enzymes of phospholipid metabolism, nucleases, as well as ORFs of unknown function in viruses and pathogenic bacteria. These enzymes are characterized by the invariant sequence motif, $H(X)K(X)_4D$. Crystal structure, mutagenic analysis, and *in silico* modeling of a Type II REase, R.PabI, revealed that the protein adopts a novel fold referred as a 'HALF-PIPE' domain. The PLD and HALF-PIPE domain-containing REases are metal-ion independent enzymes.

The hydrolysis of the phosphodiester bond consists of three major steps: generation of the attacking nucleophile, transition state formation-stabilization, and protonation of the 3′ hydroxyl group. These reaction steps are similar to those of DNA transaction enzymes that synthesize nucleic acids in which the phosphoryl group is transferred to a growing phosphoryl ester. The general mechanism for the phosphodiester bond hydrolysis/formation in nucleic acids has been shown to be the S_N2 type, which involves an overall inversion of the stereochemical configuration of the tetrahedral phosphorous atom. Divalent metal ions play a major role at one or more steps during phosphodiester bond hydrolysis. Different metal ion mechanisms have been proposed for a variety of REases, involving one, two or three metal ions. In contrast to the S_N2 mechanism of PD-(D/E)xK, HNH and GIY–YIG family of REases, the PLD and HALF-PIPE REases seem to utilize ping-pong and β-elimination reaction chemistry respectively, for DNA cleavage. More structural studies are required to establish the catalytic mechanism for these enzymes.

Engineering Specificity: Chimeric and Synthetic Enzymes

In spite of having a large repertoire of sequence specificities, altering the specificity of REases (especially Type II), as well as engineering specificities, has been a major research goal. Such studies were facilitated by the detailed characterization of a number of

Table 3 Active site and mechanism of DNA cleavage in REases

Superfamily[a]	*Example*	*Divalent cation*	*DNA cleavage*	*Base flipping*	*Active site core*	*Chemistry*
PD-(D/E)XK	EcoRI	Yes	phosphodiester	Yes (EcI18kI, EcoRII)	αββββαβ	S_N2
HNH	KpnI	Yes (diverse)	phosphodiester	ND	ββα	S_N2
PLD	BfiI	No	phosphodiester	ND	α/β fold	Ping-Pong
GIY-YIG	Hpy88I	Yes (diverse)	phosphodiester	ND	α/β fold	S_N2
HALF-PIPE	PabI	No	N-glycosidic	Yes (PabI)	α/β half barrel	β-elimination

[a]Superfamily is named after important residues in the active site except in the case of PLD (phohpholipase D) and HALF-PIPE (see text).
ND – not determined, REase – Restriction endonuclease.

enzymes and the availability of structural information. The approaches involved were random mutagenesis followed by *in vivo* selection strategies to generate changed specificity. In parallel, site-directed mutagenesis approaches were employed to rationally design new specificities relying on the structural information about the REase- DNA complexes to generate different contacts. Other efforts included strategies to lengthen the recognition sequences and generate hetero-dimeric enzymes. While a number of attempts provided interesting insights and some success in generating highly specific nickases, an engineered, efficient REase with a new sequence specificity has, so far, not met with success.

An alternate approach, to generate chimeric endonucleases having a longer recognition sequence, was conceived to target large eukaryotic genomes. Type II REases having typical recognition sites of 4–8 bp would cut DNA once every 4096–65,536 bases. For genome engineering, enzymes that recognize sequences of 16–18 bp in length are needed to make a unique targeted double-strand break (DSB). Work on FokI, a Type IIS REase showed that it possesses separate DNA recognition and endonuclease activity domains. The bipartite nature of the FokI endonuclease suggested that it might be feasible to construct chimeric nucleases having novel sequence-specificities by linking other naturally occurring DNA-binding proteins to the cleavage domain of FokI endonuclease. This proved to be the case. Chandrasegaran and co-workers constructed the first 'chimeric' REase by linking the Ubx homeodomain from *Drosophila* to the FokI REase cleavage domain. They then created other novel site-specific endonucleases by linking different DNA-binding motifs, namely the helix-turn-helix motif, the zinc finger motif and the basic helix-loop-helix protein containing a leucine zipper motif. Since, engineered nucleases can further be altered experimentally, this foundational work established the potential for inducing targeted recombination in a variety of organisms and ushered in the era of genome engineering.

Taking a cue from this approach, Transcription Activator-Like Effector Nucleases (TALENs) were developed that were fusions between transcription factors and REases. TALENs comprise a nonspecific DNA-cleaving nuclease fused to a DNA-binding domain that can be easily engineered so that these nucleases can target essentially any sequence. The capability to quickly and efficiently alter genes using TALENs promises to have a profound impact on biological research and to yield potential therapeutic strategies for genetic diseases.

Bacteria and archaea have evolved an adaptive defense mechanism that uses a CRISPR-Cas system to degrade complementary sequences present within invading viral and plasmid sequences. The type II CRISPR/Cas system (the simplest of the three involving one Cas9 and two RNA components) relies on integration of foreign DNA fragments into CRISPR loci. Upon transcription and processing, these inserts produce short CRISPR RNAs (crRNAs), which then anneal to a *trans*-activating crRNA (tracrRNA), enabling CRISPR-associated (Cas) proteins to direct sequence-specific degradation of the foreign DNA. To generate a nuclease with a given 20 bp specificity, fusion of a specific 20 bp sequence followed by 3 nucleotides NGG (PAM or Proto-spacer Adjacent Motif) with the tracrRNA directs the Cas9 to that specific locus. As the Cas9 enzyme utilizes two different active sites (HNH and RuvC) for generating double strand breaks, variations in Cas9 involving one active site mutated are also used. In addition, variations involving Cas9 enzymes having different PAM sites, such as Cpf1 which recognizes TTTV (V – A/C/G), are also used. The easily programmable RNA-guided nucleases from CRISPR-Cas systems are regarded as the most reliable tools for genome editing and engineering.

Arms Race-Evolutionary Pressure for Phages and Bacteria Evolve New Anti-Restriction Systems and REase Specificity

The rudimentary mechanism for self and non-self discrimination has a built-in vulnerability to host mimicry evasion paradigms. Further, exogenous DNA often bypasses restriction if it arrives pre-methylated (for example, through modification in a neighboring cell possessing the same MTase). The methylated foreign DNA is tolerated as a self-component and recognized by the host MTase during subsequent rounds of replication. Therefore, an invading genome can propagate freely in spite of REase surveillance once it manages to achieve methylation.

Evolution of new R-M specificities and the ways in which invading genomes escape or counter host defense strategies have been the subject of detailed investigation in a few model systems. Study of interactions between *E. coli* and coliphages and encounters of *Bacillus subtilis* with a number of its phages have provided instructive examples of the "war" between the host and the infecting viral particles. Table 4 provides a summary of various anti-restriction strategies employed by phages. The mechanisms range from mutational alterations in the enzyme recognition sites, a variety of DNA modifications, overcoming enzyme action by avoiding the cleavage, countering REase action by encoding neutralizing proteins, DNA substrate mimicry, degradation of essential co-factors needed for enzyme activity and many less understood mechanisms, underscoring the success of phages in outnumbering bacteria in nature (Table 4). On the bacterial part, although they appear to be on the losing side, a number of strategies have either evolved or been acquired through horizontal gene transfer. Table 5 provides a summary of the various nuclease-dependent anti-phage strategies. Thus in many bacteria, in addition to chromosomally encoded R-M systems, plasmid and phage encoded R-M systems have been acquired. Notably, even the complex Type I enzymes are transmitted by plasmids. Many of the plasmid-encoded enzyme modules fit into the description of addiction modules, as their "selfish behavior" has been demonstrated. Thus, it is no surprise that many bacteria exhibit more than one kind of R-M system (Type I, II) while some even possess several systems of the same group, strengthening their armory against invasion.

On yet another front, several strategies are found in nature to evolve new R-M specificity. Genetic recombination within *hsdS* genes, transposition, slippage synthesis, and mutational events are some of the mechanisms that generate new enzyme specificities. Evolution of modification-dependent REase systems (Type 4) is yet another formidable strategy developed by bacteria against phages that had evolved the ability to by-pass restriction by modification of their genomes. Further, a number of REases do not

Table 4 Bacteriophage anti-restriction strategies

Phage strategy	*Examples*	*Remarks/characteristics*
Phages encoding MTases	Phages SPR, Ψ3T, δI1, and SPβ that infect Bacillus subtilis	Acquired from host-encoded MTases
Complex genome modification	Glycosylation (Phage T_{even}), hydroxymethylation (Phage T_{even}), Acetamidation. Mommifcation by transfer of acetamide group to N^6 position of adenine in phage Mu	Repurposing of non-nucleic acid enzymes
Depletion of co-factor	Adomet hydrolase (phage T3)	Targets Type I R-M system
Loss of R-M recognition sites	Under-representation of CCGG, GGCC and CGCG in phage PZA	Palindrome sequence avoidance is the oldest outcome of selection in phages
Exploitation of cleavage mechanism of Type III R-Ms	Strand bias of EcoP1I sites in phage T7 genome	Type III methylase utilizes the same mechanism of single-stranded asymmetric host methylation to protect genome
Genome level changes	Single stranded DNA genome to avoid REases	Broad anti-restriction strategy against REases
Hyper-activation of MTase	ral gene (phage I) activates MTase of Type I R-M	Ral induces conformational change in hsdMS
Hacking bacilli cell architecture	Phages T4, T7, KVP40, P1, and A1122 localize to poles	Not supported by experimental evidence

REase – Restriction endonuclease, MTase – Methyltransferase, R-M – Restriction modification systems, Adomet – S-Adenosyl methionine.

Table 5 Anti-phage mechanisms involving degradation of phage genomes

Bacterial strategy	*Example*	*Targeted phage anti-restriction strategy*
Modification specific REases	Type IV REases such as McrBC	Targets phage acquisition of genome modification mechanisms
Altering R-M specificity	*Lactobacillus lactis* alters specificity by recombination of two HsdS subunits	Counters the decrease in specific restriction sites
Type II REase DNA cleavage promiscuity	Ability to recognize and cleave recognition site differing in single base	Counters the decrease in specific restriction sites
Phase variation	*Streptomyces coelicolor* undergoes phase variation to limit Phage fC31 population	High frequency on or off switching mechanism to prevent phages from developing anti-restriction strategy
Antisense mechanism	*Rhodobacter sphaeroides* argonaute protein targets plasmid-encoded genes	Broad strategy against foreign plasmids
CRISPR-Cas system	Widely distributed in bacteria and archaea	Provides acquired resistance mechanism

REase – Restriction endonuclease, R-M – Restriction modification systems, CRISPR – Clustered regularly interspaced short palindromic repeats.

appear to be exquisitely sequence-specific, as originally described. Recent studies reveal that many of the Type II enzymes exhibit promiscuous activities. Promiscuity in REases provides an upper hand to the organism to restrict incoming DNA even if the phages have evolved the strategy of palindromic avoidance (i.e., mutation in canonical recognition sites). Above all, a number of diverse CRISPR-Cas systems have evolved at a later stage after the initial escape and success of the phages against the host REases. However, strikingly, novel hitherto unexpected anti-CRISPR mechanisms are also being discovered. Thus, there is a continuum of evolving mechanisms, both on the host front and with the invading genomes.

R-M Systems and Phase Variation

Phase variation is a process by which bacteria undergo frequent and reversible genotypic changes in specific genes that impart phenotypic changes in organisms. Phase variation has been shown to help pathogenic bacteria in colonization and evasion of host immunity. The main mechanism of phase variation involves insertion or deletion of polyG or polyC repeat tracts in ORFs during genome replication. While phase variation is typically associated with genes encoding surface antigens, several host-adapted bacterial pathogens have MTases (*mod* gene), belonging to Type III R–M systems, that contain single tandem repeats that are prone to phase variation.

In every case studied to date, phase-variable ON/OFF switching of the Type III DNA MTase is mediated by a simple sequence repeats that results in differential regulation of multiple genes. Currently, the well characterized "phase-varions" and *mod* genes include *mod*A in non-typeable *Haemophilus influenzae* (NTHi), *Neisseria meningitidis*, and *Neisseria gonorrhoeae; mod*B in *N. meningitidis* and *N. gonorrhoeae*, *mod*D in *N. meningitides*, *mod*H in *H. pylori*, and *mod*M in *Moraxella catarrhalis*. The list of phase-variable DNA MTases controlling phase varions is expanding, with many new systems characterized within the past few years

(Table 6). The identification of a variety of phase-variable MTases that switch their expression *via* distinct mechanisms, implies that phase varions have evolved independently in different species and suggests that this type of variable epigenetic regulation provides a strong selective advantage. The random and reversible switching of phase-variable DNA MTase leads to multiple distinct phenotypes in a population that is subject to periodic selection and counter selection in different environments.

R-M Systems are More Than a Bacterial Defense Strategy

Perhaps only a few groups of enzymes have manifested such a large diversity in number and variation as is seen in R-M systems. However, the estimate that there are 10^{21} phages against 10^{19} bacterial species underscores the fact that invading genomes may have been more successful in their evasive strategies against host defense mechanisms. Now it is becoming increasingly clear that the large diversity may have also culminated in evolving other roles for these versatile enzymes given that the defense provided by them is not fool-proof. Further, many of the REases do not appear to have high turnover. If some of the early biochemical studies were to be

Table 6 Phase variable DNA MTases

R-M system	*Repeat motif*	*Organisms*	*R-M Genes*
Type I	poly-G repeats	*Neisseria gonorrhoeae*	NgoAV (hsdSngoAV), hsdS
	GACGA	*Haemophilus influenzae*	hsdM
	Unknown	*Streptococcus pneumoniae, Mycoplasma pulmonis*	***Streptococcus pneumoniae***: SpnD39III (SpnD39IIIA-FP), ***Mycoplasma pulmonis***: hsd1 and hsd2 loci
Type II	poly-G repeats	*Campylobacter jejuni*	*cj0031*
	poly-A repeats	*Helicob acter pylori*	HpyAIV
Type III	poly-C repeats	*Helicob acter pylori*	*mod* (HP1407), JHP1296 (mod), JHP1297 (res)
	poly-G repeats	*Helicob acter pylori:J99, 26695,HPAG1 Helicobacter acinonychis:Sheeba*	***Helicobacter pylori***: *modH* (formerly *modC*), JHP1411 (mod), JHP1410 (res), HP1522 (mod), HP1521 (res), HP1369, HP137, HP1371, HPAG1_1393/HPAG1_1394 (1314/1314, HP1315/1316, ***Helicobacter acinonychis***: Hac0007/0008 (modC4), Hac0009
	(AG)8	*Mycoplasma pulmonis*: UAB CTIP *Mycoplasma hyopneumoniae* :J2	***Mycoplasma pulmonis***: MYPU3950 (res), MYPU3960 (mod), MYPU3970 (mod), MYPU3980 (mod), MYPU4800 (mod), MYPU4790 (res) ***Mycoplasma hyopneumoniae***: MHJ0382 (mod), MHJ0383 (mod), MHJ0384 (res)
	(GA)6–18 repeats	*Mycoplasma hyopneumoniae*: str. J, *Mycoplama pulmonis*: str. UAB CTIP	***Mycoplasma hyopneumoniae***: MHJ0423 (mod), MHJ0382/0383, MHJ0384 ***Mycoplama pulmonis***: MYPU480, MYPU479, MYPU3960/3970/3980, MYPU3950
	CACAG repeats	*Pasteurella haemolytica*: *A1 Haemophilus ducreyi*: 35000HP *Streptococcus thermophilus*: CNRZ1066 *Mannheimia haemolytica*: str. A1	***Pasteurella haemolytica***: *mod, AF060119 (mod, res)* ***Haemophilus ducreyi***: *HD1690 (res), NT014296, NT01426* ***Streptococcus thermophilus***: Str0885 (mod), Str0884 (res)
	CCGAA repeats	*Neisseria meningitidis, Neisseria lactamica, Neisseria mucosa, Neisseria cinerea, Neisseria polysaccharea*	*modD*
	CAAC repeats	*Moraxella catarrhalis*: ATCC 23246	*modM, AY049056 (mod, res; McaRI), AY049057 (mod, res; McaRII)*
	(AGTC)32	*Haemophilus influenzae* Rd (serotype d)	HI1056/HI1058 *(modAI)*, HI1055 *(res)*
	(AGTC/AGCC)n	*Haemophilus influenzae str. Rd, Neisseria meningitidis, Neisseria gonorrhoeae*	***Haem ophilus influenzae***: *modA1-m odA10*, *modA13 -modA17, (HI1056/58;HI1055/54)* ***Others***: modA
	(AGCC)3-n repeats	*Haemophilus influenzae*: 86-028NP (non-typeable), R2866 (non-typeable), R2846 (non-typeable), 10810 (serotype b) *Neisseria meningitidis*: Z2491 (serogroup A), MC58 (serogroup B), str. Z2491, str. FAM18 (serogroup C) *Neisseria lactamica*: str. ST-640 *Neisseria gonorrhoeae* : FA1090	***Haemophilus influenzae***: NTHI1216 (res), modA10, m odA4, m odA2, res, ***Neisseria meningitidis***: NMA1589/NMA1590 (modA12), NMA1591 (res) NMB1375 (modA11), NMB1376 (res), NMC1310 (modA12), NMC1311 (res) ***Neisseria lactamica***: m odA7, res, ***Neisseria gonorrhoeae***: NGO0641 (modA13), NGO0640 (res)
	(CCCAA)3-n repeats	*Neisseria meningitides:str. MC58, str. Z2491, str. FAM18, Z2491 (serogroup A), MC58 (serogroup B), FAM18 (serogroup C) Neisseria gonorrhoeae*: *str. FA 1090*	***Neisseria meningitides***: NMA1467 (modB2); NMA1466 (res), NMB1261 (modB1), NMB1260 (res), NMC1194 (modB2), NMC1193 (res) ***Neisseria gonorrhoeae***: NGO0545 (modB1), NGO0546 (res)
	(CCCAA/TCCAA) 24	*Neisseria lactamica ST-640*	*modB3; res*
	CCCAA or GCCAA repeats	*Neisseria gonorrhoeae, Neisseria m eningitidis*	*modB (ngoAXmod)*
Type IV	poly-G repeats	*Escherichia coli*	mrr

Table 7 Biological functions of R-M systems

Function	*Characteristic*	*Mechanism*	*Examples*
Phage restriction	Serves as host defense mechanism against phages	Foreign DNA cleavage	All R-M systems
Selfish genes	To promote survival and frequency of R-M systems	Post-segregational killing	Bsp6I, EcoRI, EcoRII, EcoRV, PaeR7I, PvuII, SsoII
Stabilization of genomic islands	To stabilize neighboring genomic regions	Post-segregational killing	Not supported by evidence
Programmed cell death	Stationary phase induced cell death for long time survival	Promiscuous activity and lower methylation	KpnI
Bacterial autoimmunity	Stochastic self restriction shaping adaptive evolution	Occasional cleavage of self DNA	EcoRI
Role in nutrition	Re-allocation of host resources to the virus	Degradation of host DNA	Chlorella and Marseille virus
Maintenance of species identity	Immigration controllers	Restriction of horizontal transfer of DNA	Type I and Type IV R-Ms of *Staphylococcus aureus*
Recombination/Genome rearrangements	Enhancement of recombination by REases	Foreign DNA restriction generates recombination compatible products	EcoKI REase
Genetic variation	C-T variations	Deamination of 5-methyl cytosine to thymine	HpaII MTase
Adenine methylation	Altered gene expression caused by epigenetic modification	Effects transcription	m^6A MTases
Virulence	Effects virulence gene expression	Variable	R-M systems of *H. pylori*

R-M – Restriction modification, REase – Restriction endonuclease, MTase – Methyltransferase.

taken as pointers, Type I enzymes may not turn over at all. Many Type III enzymes also appear to function in a similar fashion, although, after the initial cleavage, the DNA could be the target of cellular exonucleases. The biological fitness for a bacterium having an active R-M system has been debated. Restriction site avoidance and expending too much energy for DNA hydrolysis in addition to complex organization escalate the cost/benefit ratio. Moreover in bacteria such as *H. pylori*, many putative R-M systems appear to be non-functional. The inadequacy of cellular defense manifested by R-M systems may have led to the evolution of CRISPR- Cas systems to better counter the invading genomes. These observations, taken together, suggest the possibility that R-M systems indeed may participate in other cellular functions. Indeed, now it is apparent that they participate in a variety of processes, such as providing nutrition, cellular apoptosis, maintenance of species identity, genomic rearrangement and recombination, auto-immunity, selfish behavior, maintenance of genomic islands, and possibly other yet unidentified roles (Table 7). What is not clear is whether these functions are moonlighting functions or in some cases, the R-M system may have ancestrally possessed these abilities even before becoming a *bona fide* R-M entity. Often some of these additional properties may be crucial for the bacteria to survive under extreme stress conditions or circumstances not conducive for normal growth.

Concluding Remarks and Future Considerations

In addition to the information explosion and conceptual break-throughs summarized above, R-M systems have long been integral tools of modern biological research. Engineering novel REase specificities and development of Zinc-finger nucleases have allowed TALENS to pave the way for genome engineering. With the discovery of CRISPR-Cas systems, there has been a quantum leap in new technologies in the area of gene targeting, genome engineering and allied fields. Little did one realize that these adaptive immune strategies could be exploited in modern biological research in such a short time. Moreover, conventional R-M tools along with emerging tools based on CRISPRs, offer many opportunities in the area of synthetic biology. In nature, the arms race will continue and new mechanisms will be discovered, thereby providing opportunities for new technologies to emerge. Admittedly, among the vast diversity of R-M systems, a select minority has been subjected to intense examination. With the explosion of genomic data, more varieties are likely to be discovered. Meanwhile the large pool available provides opportunities to construct hybrid enzymes and engineer specificities tailored for specific purposes. It is apparent that CRISPR-Cas technologies will be further refined. Newer and much improved efficiency can be achieved with shorter Cas 9 (natural or engineered) proteins. Given the enormous pool, it is probable that other moonlighting functions of R-M systems would be uncovered. Cas genes could be engaged in other cellular functions as well. Further, surveillance provided by both R–M and CRISPR–Cas systems can be programmed for selective incorporation of genes that would reduce the risks of parasitism and immunopathology. They can also be possibly engineered to induce regulated programmed cell death in treatment strategies. Such an approach would be useful in dealing with microbial infections.

Although R-M systems and CRISPRs form a formidable combination of self-defense against infecting phages, they can be antagonistic to each other in certain situations. There could be some natural barriers for the acquisition of additional R-M or CRISPR-Cas systems. For example, although natural transformation is not obstructed normally by a resident R-M system in

Pneumococcus, resident CRISPRs form a barrier against the process and affect virulence. Such studies will be invaluable in understanding genome dynamics and evolution in bacteria. Among the interesting future experiments will be the construction of a synthetic genome having representatives of all 4 types of R-M systems as well as different kinds of CRISPR-Cas to evaluate the efficiency of such "super bacteria" against a few phages that have developed elaborate anti-restriction and anti-CRISPR strategies. Further, the *Helicobacter*, *Neisseria and Bacillus* genera are unlikely to be the only examples carrying a large number of R-M systems. Search in the genome databases, which have been expanding, is likely to reveal newer genomes with a bagful of R-M systems.

In summary, the fundamental research and understanding of R-M systems and CRISPRs is far from over. The current knowledge base provides a platform to develop newer, improved technologies, while understanding the characteristics of the new enzymes. Sequencing of more bacterial and phage genomes is likely to reveal more surprises. These efforts will provide better insights into bacterial and viral genome dynamics, interplay, and evolution that will open up avenues for developing new tools for synthetic biology approaches. The emergent information on R-M systems, anti-restriction mechanisms, and CRISPRs emphasize the fast paced evolutionary dynamics of the microbial world.

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Relevant Website

http://rebase.neb.com/rebase/rebase.html—REBASE.

Retroviruses☆

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Glossary

Endogenous retroviruses DNA forms of retroviral DNA that are vertically transmitted; they are present in the genomic DNA. They represent infection of retroviruses into the germline during evolution of a species. Most eukaryotes (including humans) carry multiple copies of endogenous retroviral DNA.

Exogenous retroviruses Retroviruses that replicate by passage from one cell to another – typical infectious retrovirues. They are not endogenous retroviruses.

Polyprotein Initial viral translation products contain the peptide sequences for multiple viral proteins. These are referred to as polyproteins. Nomenclature for polyproteins includes the molecular mass (in Kd) as well as the gene encoding the polyprotein – for example $Pr65^{gag}$. Polyproteins are cleaved into the mature proteins by viral or cellular proteases.

Retrovirus Enveloped RNA-containing viruses that carry the enzyme reverse transcriptase in their virions. Synthesis of viral DNA from the viral RNA and integration of the viral DNA into the host cell chromosomes are characteristic features of these viruses.

Classification

Retroviruses of higher eukaryotes (family *Retroviridae*) are classified by the International Committee on Taxonomy of Viruses (ICTV) based on their phylogeny and molecular biology into two subfamilies, orthoretroviruses (*orthoretroviranae*) and spumaviruses (*spumaretroviranae*) (Table 1). They have also been termed simple retroviruses if their genomes contain only the standard three viral genes (*gag*, *pol* and *env*); complex retroviruses carry additional viral genes. Within the orthoretroviruses there are six genera, five of which are associated with development of cancers. Alpharetroviruses are simple retroviruses native to birds (e.g. avian leukosis virus of chickens), and they were the first retroviruses discovered – predominantly in chickens, where they cause leukemias and sarcomas. Betaretroviruses have been found in several different species, including mice (murine mammary tumor virus [MMTV]), monkeys (Mason-Pfizer monkey virus [MPMV]) and sheep (jaagsiekte sheep retrovirus [JSRV]); they cause carcinomas of the breast (MMTV) and lung (JSRV). Gammaretroviruses are simple viruses that have been found in mice, cats, birds and gibbon-apes (e.g. murine leukemia virus [MuLV], reticuloendotheliosis virus [REV], feline leukemia virus [FeLV] and gibbon-ape leukemia virus [GaLV] respectively). They cause different kinds of leukemia. Deltaretroviruses are complex retroviruses associated with development of lymphoid leukemias in humans and cattle (human T-cell leukemia virus [HTLV] and bovine leukemia virus [BLV] respectively). Epsilonretroviruses are native to fish (e.g. walleye dermal sarcoma virus [WDSV]), and they have transduced cellular genes such as cyclins; they cause sarcomas and epithelial tumors. Lentiviruses are complex retroviruses native to several species including sheep, goats, old world monkeys and humans (e.g. maedi/visna virus of sheep [MVV] and human immunodeficiency virus [HIV]). Lentiviruses are associated with development of immunodeficiency and neurologic symptoms – the long latency for disease development gave these viruses the name 'lenti'. The spumavirus subfamily contains one genus, foamy viruses (e.g. simian foamy virus) which are complex retroviruses characterized by strong cytopathic effects when they infect cells in culture (intense cytoplasmic vacuolization leading to a 'foamy' appearance). However, they are not associated with disease. Foamy viruses differ from other retroviruses with regard to several features of their replication, most notably the fact that virions contain reverse-transcribed viral DNA.

Structure

Retroviruses are enveloped RNA-containing viruses with single-stranded RNA as their genetic material (Figure 1). The viral RNA resembles a cellular messenger RNA in that it is capped at the 5′ end and polyadenylated at the 3′ end. It is positive-stranded, in that the viral RNA is the same polarity as viral mRNA. There are two identical copies of viral RNA in each virion, and they are held together by hydrogen-bonding in a parallel fashion near their 5′ ends (the 'dimer linkage'). Retroviral genomes range from 7–12 kb in length. All retroviral genomes contain three genes: *gag* (encoding the proteins of viral core or capsid), *pol* (encoding the viral enzymes) and *env* (encoding the viral envelope proteins).

☆*Change History:* July 2014: H Fan updated the text and further readings to this entire article

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Table 1 Retrovirus classification.

Subfamily	*Genus*	*Representative species*	*Host species*	*Diseases*	*Extra genes (beyond gag-pol-env)*
Orthoretrovirinae	Alpharetrovirus	Avian leukosis virus (ALV)	Chicken	B-lymphoma	
		Rous sarcoma virus (RSV)	Chicken	Sarcoma	
	Betaretrovirus	Murine mammary tumor virus (MMTV)	Mouse	Breast cancer	Sag, Rem
		Mason-Pfizer monkey virus (MPMV)	Monkey		
		Jaagsiekte sheep retrovirus (JSRV)	Sheep	Lung cancer	Rej
	Gammaretrovirus	Murine leukemia virus (MuLV)	Mouse	Leukemias	
		Feline leukemia virus (FeLV)	Cat	Leukemia	
		Gibbon-ape leukemia virus (GaLV)	Gibbon	Leukemia	
		Reticuloendotheliosis virus (REV)	Chicken	Immunosuppression, lymphomas	
		Koala retrovirus (KoRV)	Koala	Leukemia	
	Deltaretrovirus	Human T-cell leukemia virus (HTLV-I and -II)	Human	T-cell leukemia HAM/TSP (HTLV-I)	Tax, Rex, others
		Simian T-cell leukemia virus (STLV)	Old World monkeys		Tax, Rex, others
		Bovine leukemia virus (BLV)	Cow	B-cell leukemia	Tax, Rex, others
	Epsilonretrovirus	Walleye dermal sarcoma virus (WDSV)	Walleye pike	Dermal sarcoma	Orf A (retroviral cyclin), Orf B, Orf C
	Lentivirus	Maedi/visna	Sheep	Neurodegeneration, pulmonary disease	Tat, Rev, Vif
		Human immunodeficiency virus (HIV-1 and −2)	Human	Immunodeficiency Neurologic disease	Tat, Rev, Nef, Vif, Vpu, Vpr
		Simian immunodeficiency viruses (SIVs)	Old World monkeys	Immunodeficiency (nonnative hosts)	Tat, Rev, Nef, Vif, Vpr, Vpx
		Feline immunodeficiency virus (FIV)	Cat	Immunodeficiency	Tat, Rev
		Equine infectious anemia virus (EAIV)	Horse	Anemia	Tat, Rev, S2
		Caprine arthritis encephalitis virus (CAEV)	Goat	Arthritis encephalitis	Tat, Rev, Vif
Spumavirinae	Spumavirus	Simian foamy viruses	Nonhuman primates		Tas, Bet
		Foamy viruses of nonprimates	Cow Horse Cat		Tas, Bet

Retroviral envelopes consist of viral protein embedded in cell-derived lipid bilayer. The viral envelope proteins consist of SU and TM. These proteins are glycosylated, predominantly with N-linked carbohydrates. SU (surface, ~70 – 120 Kd depending on virus) is on the surface of the virus particle and it contains the receptor binding domain (RBD) that is responsible for specific attachment of the virus to a susceptible cell at the beginning of infection cycle. The attachment of the virus particle to a cell is mediated by binding of the SU RBD to a receptor molecule on the surface of the infected cell. Different retroviruses have evolved to recognize different cell surface molecules as receptors, and the types of cells or tissues that can be infected in vivo is restricted to those cells/tissues that express the receptor. SU is anchored to the virus particle by interaction (typically disulfide bonds) with the transmembrane protein (TM, ~15 – 45 Kd); TM spans the lipid bilayer of the envelope. The external domain (ectodomain) of TM contains sequences involved in formation of envelope multimers (trimers), as well as sequences that mediate fusion of viral and cellular membranes during infection. The C-terminus of TM contains a short stretch of polypeptide that is on the interior of the viral envelope (or in the cytoplasm of the infected cell). These residues may interact with viral Gag protein to localize Gag to areas of the infected cell membrane where virus particle formation occurs. The envelope proteins are initially translated as one combined polypeptide (polyprotein), with SU at the amino terminus and TM at the carboxyterminus. Cleavage is carried out by a cellular protease (typically furin) during passage through the RER.

The viral core is a condensed structure consisting of viral core proteins, viral enzymes, the genomic RNAs and cellular tRNAs. The viral core proteins consist of matrix (MA, lying under the envelope ~10-15 Kd), major capsid (CA, the structural protein of the viral core particle, ~25-30 Kd) and nucleocapsid (NC, that binds and compacts viral RNA, ~10 Kd). There are approximately 1000 of each core protein in a retrovirus particle. In addition depending on the virus, other proteins are also encoded by *gag* and are present in virions (e.g. p12 of MuLV and p6 and p1 of HIV). When retroviral cores are first assembled, they have spherical symmetry; in their mature condensed forms, some cores are condensed and roughly spherical, while mature lentiviral cores are conical. The fundamental viral capsid structure consists of hexagonal arrays of CA protein, with a smaller number of pentagonal arrays. However retroviral capsids do not have uniform structures such as icosahedrons.

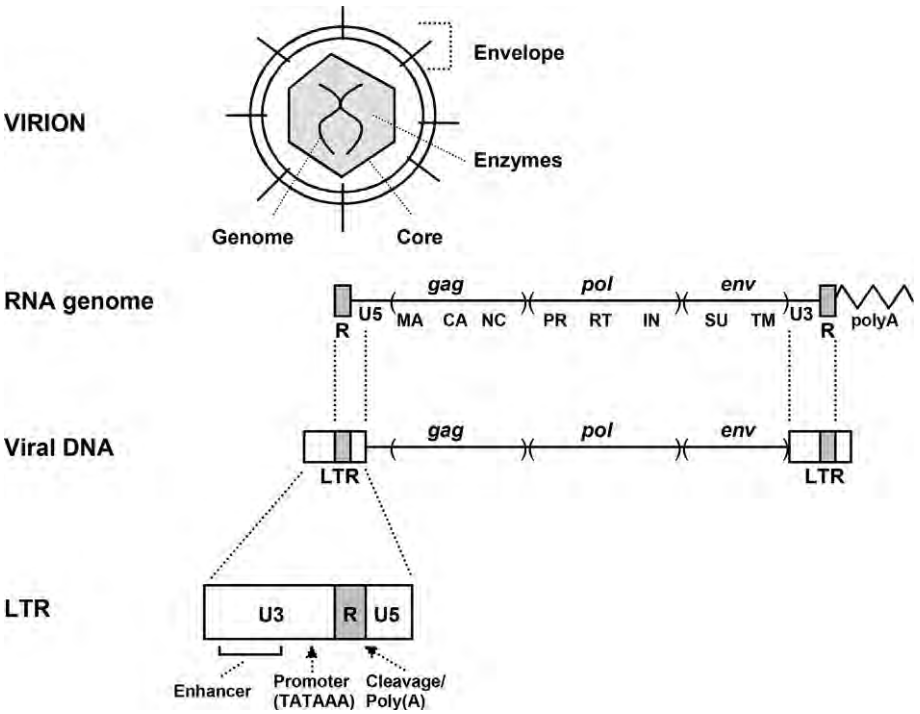

Figure 1 Retrovirus structure. Schematic structure of a retrovirus virion is shown at the top. The organization of genomic RNA (from 5' to 3') is shown for a simple retrovirus, with the locations of the different viral genes and coding regions indicated. Reverse transcribed viral DNA is shown relative to viral RNA, with the long terminal repeats (LTRs). The regions of the viral RNA encoding different portions of the LTR are indicated (dotted lines). An LTR is shown in greater detail at the bottom, with the enhancer, promoter, and cleavage/poly adenylation signals.

The viral enzymes consist of reverse transcriptase (RT, ~80 – 120 Kd), integrase (IN, ~30-40 Kd) and protease (PR ~10-12 Kd). Depending on the virus, the RT enzyme consists of monomers (MuLV) or heterodimers in which one subunit lacks C-terminal sequences (e.g. the p66/55 complex of HIV RT). There are approximately 50–100 copies of the viral enzymes in a virus particle. They carry out essential steps in retroviral replication, as described below. The *gag* and pol genes also are initially translated as polyproteins, followed by proteolytic cleavage during maturation to give the final proteins in the virus particles. Proteolytic cleavage of the *pol* polyprotein is required for enzymatic activities of RT, IN and PR.

For complex retroviruses, the additional genes carry out various functions. One common type of additional gene encodes a transcriptional transactivator that stimulates transcription from the viral LTR. Such transactivating proteins include HIV Tat, HTLV-I Tax and foamy virus Bel-1. Another common additional retroviral gene encodes a factor that facilitates nuclear export of unspliced viral RNA from the nucleus to the cytoplasm and/or efficiency of translation (e.g. HIV Rev, HTLV-I Rex, MMTV Rem and JSRV Rej). Other proteins encoded by complex retroviruses counteract host resistance mechanisms to retroviral infection (e.g. HIV-1 Vif and VpU – see below).

Retroviral replication

The retroviral replication cycle is described in this section (Figure 2). The first step in retroviral replication is binding of a virion to the surface of the target cell. This is mediated by a specific interaction between the RBD of the SU protein and receptor molecule on the surface of the target cell. Retroviruses have evolved to recognize a wide variety of cell surface proteins as receptors. These include multiple pass membrane spanning proteins that function as transporters (e.g. amino acid and phosphate transporters for different MuLVs), single-pass membrane-spanning proteins (e.g. a member of the TNF receptor family for subgroup B avian leukosis viruses) and proteins that are on the cell surface (e.g. the CD4 protein for HIV). The tissue distribution of virus receptor molecules determines the cells or tissues that the virus can potentially infect. For instance, the CD4 receptor for HIV-1 is predominantly present on T-helper lymphocytes, macrophages and dendritic cells; thus these cells are the primary targets for infection in vivo. In the case of HIV-1 and related simian lentiviruses, successful infection requires interaction with both CD4 as well as a co-receptor (the chemokine receptors CCR5 for CXCR4 for HIV-1).

Once the virus has bound to the cell, the viral core enters the cytoplasm (viral entry). For some retroviruses, this may occur at the cell surface by fusion between the viral envelope and the cellular plasma membrane. For other viruses, receptor-mediated

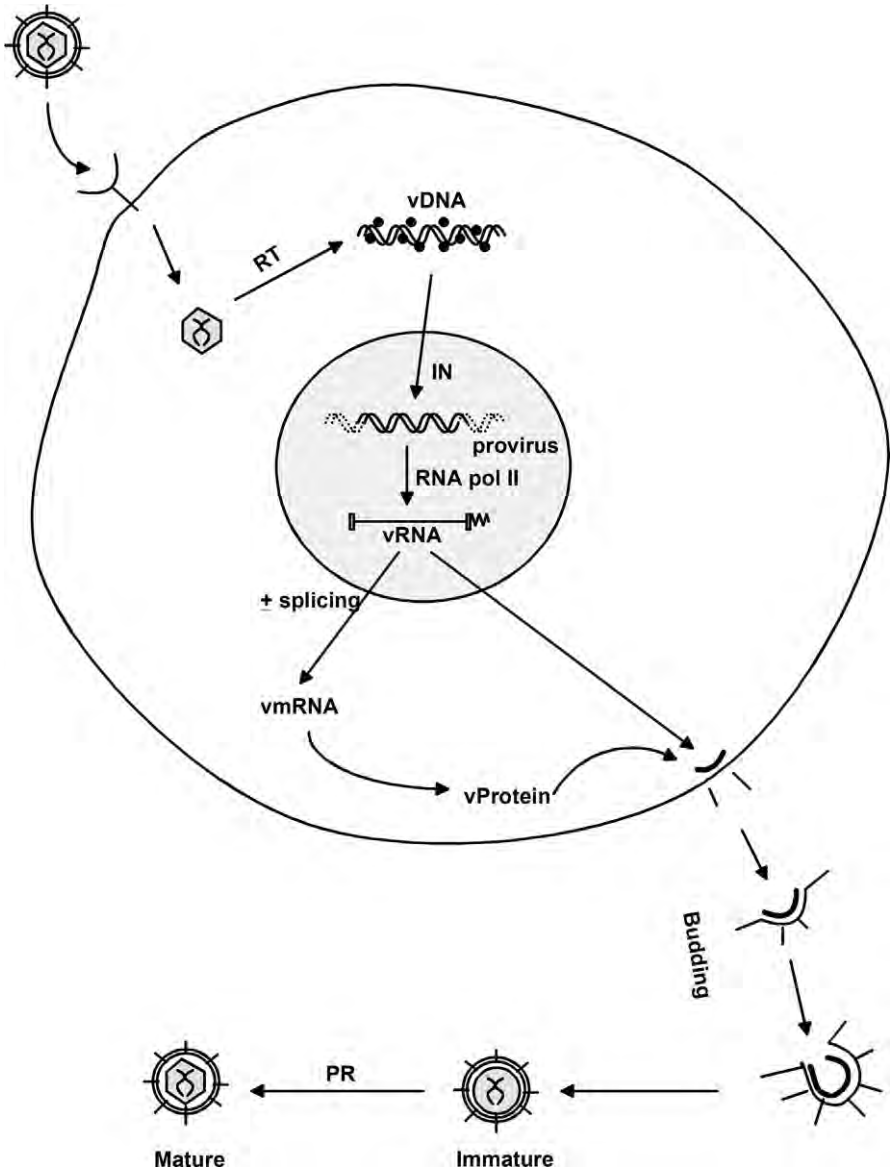

Figure 2 The retrovirus replication cycle. A cell undergoing infection is shown; the nucleus is shaded. Infection begins at the upper left, with binding of a virion to a receptor on the cell surface. Different steps in the infection process are described in the text. They culminate in release of immature virions by budding from the cell surface. Maturation involves cleavage of viral polyproteins by viral protease.

endocytosis of the entire virion may occur, followed by fusion within the lysozome of the viral envelope with the lysozomal envelope. The membrane fusion is mediated by the TM protein, which undergoes a major conformational shift that brings the cellular and viral lipid bilayers into such close proximity that they fuse. Membrane fusion leads to release of the core into the cytoplasm. The ectodomain of the TM protein mediates the membrane fusion.

Once the viral core is released into the cytoplasm, the enzyme reverse transcriptase in the core is activated. RT uses the viral RNA as a template to synthesize linear double-stranded viral DNA. RT has three enzymatic activities that are all important for synthesis of viral DNA: 1) RNA-dependent DNA polymerase (used in synthesis of the first [−] strand of DNA, resulting in an RNA-DNA hybrid), 2) DNA-dependent DNA synthesis (used in synthesis of the second [+] DNA strand), and 3) RNAseH that degrades RNA from an RNA-DNA hybrid. For initiation of synthesis of the first strand of viral DNA, RT requires a polynucleotide primer with a free 3′ hydroxyl hydrogen-bonded to the viral RNA. The primers for retroviral reverse transcription are cellular tRNAs; different retroviruses employ different tRNAs. After reverse transcription, the resulting viral DNA is longer than the template RNA by carrying two copies of the same sequences (long terminal repeats or LTRs) at either end (Figure 1). Viral LTRs can be subdivided into three regions: the U3 regions are encoded in viral RNA uniquely at the 3′ end, the U5 regions are encoded in viral RNA uniquely at the 5′ end, and the

R regions are encoded in repeated sequences at either ends of the viral RNA. After reverse transcription, the viral DNA remains complexed with viral core proteins, most notably integrase.

After the viral DNA is reverse transcribed in the cytoplasm, it and its associated proteins (the pre-integration complex) enter the nucleus. For simple retroviruses such as MuLV, this process is dependent upon passage of infected cells through mitosis and the accompanying breakdown of the nuclear envelope. On the other hand, lentiviruses are able to transport their pre-integration complexes across the nuclear membrane via virus-encoded proteins (Vpr, IN and potentially MA proteins); thus these viruses can infect non-dividing cells. Once the pre-integration complex is in the nucleus, the viral DNA is inserted into host chromosomal DNA through the action of the viral integrase. During integration the two ends of the viral DNA are held together by the IN protein (multimer), and insertion is a concerted reaction where both ends of the DNA are integrated simultaneously. As a result of integration, two viral DNA nucleotides are lost at either end, and a short stretch of adjoining cellular DNA sequences (4–13 nucleotides depending on the virus) are duplicated at either side of the integrated viral DNA. This reflects the way that IN protein cleaves the host and viral DNAs during the integration reaction – 'staggered cuts' in both cases, followed by ligation of one strand of viral DNA to one strand of host chromosomal DNA. Cellular DNA repair enzymes then complete the process by removing unpaired nucleotides and filling in single-stranded regions, resulting in the viral nucleotide deletions and cellular nucleotide duplications. The integrated form of viral DNA is termed the provirus, and it resembles the linear reverse transcribed viral DNA. Integration of viral DNA into the host chromosomal DNA occurs at multiple (virtually random) sites. Within the context of these multiple insertion sites, for some retroviruses there is a bias for integration near the start sites of transcription (e.g. MuLV), for others there is a bias for integration generally within genes (e.g. HIV) and for others there is no bias for insertion sites (e.g. avian leukosis virus and MMTV).

Once integrated into the host DNA, the retroviral provirus is transcribed into viral RNA. Transcription is carried out by host cell machinery, including RNA polymerase II, the enzyme responsible for transcription of cellular mRNA precursors, as well as host enhancer-binding proteins. The retroviral provirus functions as a single transcription unit, with transcriptional control sequences contained within the LTRs. The U3 region contains a promoter (i.e. TATAAA at -30 bp) and upstream enhancer elements; transcription initiates at the U3-R boundary in the upstream LTR. The 3′ end of the RNA is determined by a cleavage/polyadenylation signal in the R region of the LTR which leads to cleavage/polyadenylation of the RNA at the R-U5 boundary in the downstream LTR. The resulting transcript (capped at the 5′ end and polyadenylated at the 3′ end) is identical to genomic viral RNA present in the virion.

Some retroviruses encode viral transactivators that activate transcription for the viral LTR, including HTLV-I Tax and HIV Tat. The mechanisms of action are different, with HTLV-I Tax binding to cellular transcription factors (e.g. CREB) and potentiating the CREB binding to enhancer sequences in the LTR. In contrast, Tat binds to a 5′ stem-loop in the 5′ end of the viral transcript, which leads to recruitment of Cyclin T/CDK9 and associated proteins. This complex phosphorylates the C-terminal tail of RNA polymerase II, which enhances processivity of the enzyme and production of full-length viral transcripts. In the absence of Tat, transcription stalls after formation of the 5′ stem-loop. Some retroviruses also carry additional internal promoters (e.g. MMTV and foamy viruses) or promoters on the opposite strand (HTLV-I).

The newly transcribed viral RNA in the nucleus is exported to the cytoplasm along two pathways. In one pathway, viral RNA with and without mRNA splicing is transported to the cytoplasm to function as mRNA. The viral mRNAs are translated by host cell ribosomes and translation factors. There are two forms of viral mRNA for simple retroviruses: unspliced RNA that is translated into Gag and Pol proteins, and a singly spliced mRNA that is translated into Env proteins (Figure 3). As mentioned above, each of the

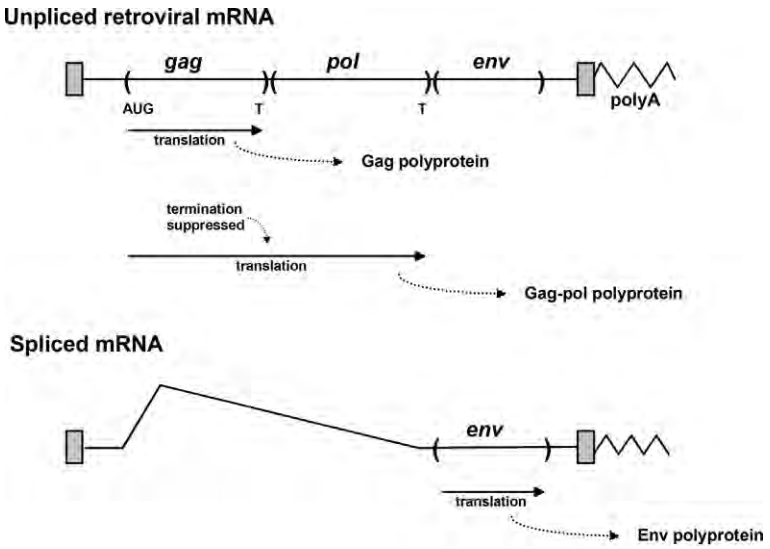

Figure 3 Retroviral mRNAs. All retroviruses use two forms of viral mRNA: unspliced RNA is translated into Gag and Gag-pol polyproteins, and singly spliced RNA is translated in Env polyprotein. The positions of initation codon (AUG) and termination codons (T) are shown on the unspliced viral RNA.

viral genes is translated as a single polypeptide. For unspliced viral mRNA, translation is initiated at the 5′ end of the *gag* gene and the majority of translation products are terminated at 3′ end of *gag* to give a Gag polyprotein (e.g. $Pr65^{gag}$ for MuLV). Infrequently, (5–10% of the time) translation does not terminate at the end of the *gag* gene, and it continues into the *pol* gene, resulting in a Gag-Pol fusion polyprotein ($Pr180^{gag\text{-}pol}$ for MuLV). For MuLVs, this results from translational suppression where the *amber* termination codon at the C-terminus of *gag* is suppressed by insertion of a glutamine into the growing polypeptide by a naturally occurring glutamyl suppressor tRNA. For other retroviruses, an RNA pseudo-knot in the vicinity of the Gag termination codon results in a -1 frameshift by the ribosome into the *pol* reading frame without release of the growing poypeptide chain. Cellular mRNAs typically require splicing for efficient export from the nucleus, and retroviruses have evolved mechanisms to facilitate export of unspliced viral mRNA to the cytoplasm. For complex retroviruses, virus-encoded proteins (e.g. HIV Rev) bind to the unspliced RNA and facilitate its nuclear export by interacting with cellular proteins involved in nuclear export (i.e. Crm-1). Simple retroviruses (e.g. MPMV, RSV) tend to contain constitutive cytoplasmic transport elements in their RNAs; these elements bind host cell factors (e.g. TAP/NXF1) that facilitate nuclear export of the unspliced viral RNA.

Once the viral polyproteins are translated, they combine with other unspliced viral RNA (from the second pathway) to form virus particles that bud from the cell surface. Core particles are assembled by Gag-Gag interactions among Gag (and Gag-pol) polyproteins, viral RNA may function as a scaffold in this process, through the RNA-binding capacity of the NC domain. Incorporation of viral RNA into particles requires specific sequences in the viral RNA (packaging site or Ψ sequences), typically located near the 5′ end. If the Ψ sequences are deleted from a retroviral RNA, it will not be packaged. However, if Ψ + viral RNA is not available, cells expressing viral proteins will still assemble particles, incorporating cellular RNAs instead. For most retroviruses, assembly of virus particles and budding occurs at the plasma membrane. Interactions between the cytoplasmic tail of envelope TM protein and the amino terminal MA domain of Gag polyprotein serves to localize Env protein the region of virus budding. For some viruses (e.g. HIV), budding takes place in plasma membrane regions enriched in cholesterol (lipid rafts).

The assembly of retroviral particles can be visualized by transmission electron microscopy, typified by partially assembled crescent-shaped 'C-type' particles at the cell surface. However betaretroviruses assemble intact core particles intracellularly (B-type particles) at a perinuclear region (the microtubule organizing region), after which the cores are transported to the plasma membrane for budding. Another pattern of budding for retroviral particles is into intracisternal membranes (A-type particles). Recently, progress has been made in understanding the retroviral assembly process. In particular, cellular proteins involved in trafficking of protein cargoes between different cellular compartments (e.g. the Escort complexes) have been implicated. They interact with 'L -domains' present on the viral Gag proteins during these processes. Viral L-domains contain amino acid motifs such as PTAP and YPDL which serve as the binding sites for components of the Escrt complexes (e.g. Tsg101 binding of the HIV-1 Gag PTAP motif). Another feature of this assembly is that the Gag proteins become modified by the addition of mono-ubiquitin as a result of their interactions with the cellular proteins, some of which are ubiquitin ligases.

When retroviral particles are initially budded from the cell, they are immature. In particular, the Gag and Gag-Pol polyproteins have not been cleaved, and the immature cores consist of spherically symmetric arrays (hexagonal lattices) of Gag polyproteins along with Gag-pol polyproteins. Virion maturation is mediated by self-cleavage and activation of the viral protease (PR) from the Gag-Pol polyprotein; PR then cleaves the Gag and Gag-Pol polyproteins into the final mature proteins. Retroviral proteases are site-specific aspartyl proteases. PR cleavage of the Gag and Gag-Pol polyproteins into the mature virion proteins results in rearrangement of the core structure into a hexagonal lattice of mature CA protein hexamers containing twelve CA pentamers (that allow closure of the CA lattice into spheres or cones). This results in a condensed mature core that does not fill the entire volume of the original immature particle; ca. half of the Gag protein is not retained in the mature core. Particles with mature cores are infectious. PR cleavage of the Gag-pol polyprotein also results in activation of the RT and IN enzymatic activities. Gag and Gag-pol cleavage is essential for retroviral infectivity; HIV-1 antiviral drugs of the protease inhibitor class inhibit this process.

For most retroviruses, infection does not result in death (lysis) of the cell. Infected cells typically will continually produce virus particles. The fact that retrovirus-infected cells are not lysed can lead to oncogenic transformation of the infected cells and development of tumors. One exception to the general rule is HIV-1, which kills infected T-helper lymphocytes (but not infected macrophages or dendritic cells). The killing of T-helper lymphocytes by HIV-1 is important for the development of AIDS.

Retroviruses and disease

Cancer

Retroviruses first received attention because they can cause cancer. The first discovered retroviruses were those of chickens that cause leukemias (avian myeloblastosis virus) and sarcomas (Rous sarcoma virus, RSV). Oncogenic viruses have subsequently been discovered that infect a variety of higher eukaryotes including man. Oncogenic retroviruses can be divided into two categories, acute transforming retroviruses (Table 2) and non-acute retroviruses. They differ in their mechanisms of oncogenesis.

Acute transforming retroviruses cause tumors quite rapidly in experimental animals (mean times of disease in weeks-months), and they can frequently alter the growth properties of cells in culture (transformation). The altered growth properties include many properties associated with tumor cells, such as loss of contact inhibition, the ability to grow in suspension, and reduced requirements for growth factors. The common feature of acute transforming retroviruses is that they carry oncogenes. These viral

Table 2 Representative acute transforming retroviruses and their oncogenes.

Virus	*Host species*	*Disease*	*Viral oncogene*	*Proto-oncogene*	*Normal proto-oncogene function*
Rous sarcoma virus (RSV)	Chicken	Sarcoma	v-src	c-src	Tyrosine-specific protein kinase
MC29	Chicken	Myeloid leukemia	v-myc	c-myc	DNA binding protein/transcription factor
Avian erythroblastosis virus (AEV)	Chicken	Erythroid leukemia	v-erbA	c-erbA/thyroid hormone receptor	Hormone-dependent DNA binding protein
			v-erbB	c-erbB/EGFR	Epidermal growth factor receptor tyrosine kinase
E26	Chicken	Erythroid leukemia	v-ets	Ets-1	DNA binding protein/transcription factor
Avian myeloblastosis virus (AMV)	Chicken	Myeloid leukemia	v-myb	c-myb	DNA binding protein/transcription factor
Reticuloendotheliosis virus -T (REV-T)	Turkey	Reticuloendo-theliosis	v-rel	c-rel	NF KappaB subunit
S17 sarcoma virus	Chicken	Sarcoma	v-jun	c-jun	DNA binding protein/AP-1 transcription factor
Harvey murine sarcoma virus	Mouse	Sarcoma	$v\text{-}ras^{H}$	H-ras	G-protein involved in signal transduction
Kirsten murine sarcoma virus	Mouse	Sarcoma	$v\text{-}ras^{K}$	K-ras	G-protein involved in signal transduction
Murine sarcoma virus 3611	Mouse	Sarcoma	v-raf	c-raf	Serine/threonine protein kinase involved in signal transduction (MEKK)
Abelson murine leukemia virus	Mouse	B-lymphoma	v-abl	c-abl	Tyrosine protein kinase
AKT-8 murine leukemia virus	Mouse	T-lymphoma	v-Akt	Akt	Serine/threonine protein kinase in the PI3K signaling pathway
FBJ osteosarcoma virus	Mouse	Osteosarcoma	v-fos	c-fos	Component of AP-1 transcription factor (with c-Jun)
Feline sarcoma virus (McDonough strain)	Cat	Sarcoma	v-fms	c-fms/CSF-1R	Receptor tyrosine kinase; receptor for CSF-1

oncogenes (see Table 2) result from capture into the viral genome of normal cellular genes (proto-oncogenes). Viral oncogene proteins differ from the normal cellular proto-oncogene proteins. Depending upon the virus, the differences may be single amino acids substitutions (e.g. the *v-ras* proteins), truncation of regulatory sequences (e.g. *v-erbB*), or fusion with viral proteins sequences (e.g. Gag-v-Abl fusion protein encoded by Ab-MuLV). The viral oncogene proteins stimulate cell growth and division in an unregulated manner, unlike their cellular counterparts. Moreover, the viral oncogenes are under transcriptional control of the viral LTR, which leads to high level constitutive expression of the viral oncogene.

During the retroviral oncogene capture process, viral sequences are often lost. As a result, most acute transforming retroviruses are actually replication-defective, since they cannot encode some or all of the viral structural proteins. In order for them to propagate, co-infection with a related replication-competent retroviruses is required – these viruses are referred to as helper viruses. Infectious stocks of acute transforming viruses will consist of mixtures of an acute transforming retrovirus and a helper virus – sometimes identified as an 'associated' virus (e.g. Rous-associated virus [RAV] in Rous sarcoma virus stocks). In a co-infected cell, the helper virus will provide the structural proteins that package acute transforming retrovirus genomes into particles. Infection of such stocks at low multiplicity can yield transformed 'non-producer' cells that are only contain a genome for the acute transforming virus. These cells do not produce virus particles because they cannot encode the viral structural proteins, but they are transformed due to expression of the viral oncogene. Reinfection of transformed non-producer cells with a replication-competent virus can lead to production of infectious acute transforming retrovirus again.

Study of acute transforming retroviruses, their oncogenes and the corresponding cellular proto-oncogenes was key in uncovering basic processes in the molecular biology of cancer. In particular, many cellular proto-oncogenes originally discovered as cellular counterparts of viral oncogenes were later found to be activated in human tumors not induced by retroviruses. In addition, many cellular genes important in signal transduction, normal cell growth regulation and cancer were first discovered as the cellular proto-oncogene counterparts of retroviral oncogenes, for example *c-myc*, the *ras* genes, *c-raf*, *c-fos* and *c-jun* (Table 2). In general the normal function of proto-oncogenes is to stimulate cell growth or division in response to physiological signals. Signaling from normal cell proto-oncogenes is tightly regulated and transient.

One exception to the nature of retroviral oncogenes is found in jaagsiekte sheep retrovirus (JSRV, a betaretrovirus), the causative agent of a transmissible lung cancer in sheep (ovine pulmonary adenocarcinoma). JSRV is an acute transforming retrovirus in that induces lung tumors rapidly and it can transform cells in culture. Unlike for other acute transforming retroviruses, the viral oncogene is actually the viral envelope protein and not a captured version of a cellular proto-oncogene. The C-terminus ('cytoplasmic tail') of the TM protein is crucial for transformation, although other domains may be important as well. Expression of the JSRV Env protein alone is sufficient to induce tumors in several animal models.

Oncogenic **non-acute retroviruses** are typical replication-competent viruses lacking oncogenes. Nevertheless, they can induce tumors, although with longer latency than acute transforming retroviruses (months-years). A common feature of tumors induced by non-acute retroviruses is the presence of proviral DNAs inserted into the same chromosomal loci in different independently arising tumors (common insertion sites or CISs). This is noteworthy because of the random insertion of retroviral proviruses into host cell chromatin. The CISs represent selection in the tumors for cells that have sustained a viral integration at that particular site. The CISs have been found to contain proto-oncogenes or other genes whose normal functions are to positively signal cell growth and division. Proviral insertion leads to constitutive over-expression of the cellular proto-oncogene, which drives unregulated growth of the tumor cell. Multiple proto-oncogenes may be activated by a given retrovirus, and the spectrum of activated proto-oncogenes is characteristic of the particular retrovirus-tumor combination. An online database for proto-oncogenes activated in tumors induced by MuLVs is available (see Relevant Websites).

The insertional activation of proto-oncogenes occurs by two mechanisms (Figure 4). First, transcriptional readthrough can occur from the provirus into the proto-oncogene, often from initiation in the downstream LTR. This is referred to as the promoter insertion mechanism, and was first described for activation of the *c-myc* proto-oncogene in B-lymphomas induced by avian leukosis virus in chickens. Promoter insertion results in a fusion transcript between viral sequences and the proto-oncogene. This places the proto-oncogene under transcriptional control of the viral LTR, which leads to constitutive high level expression. In some cases, promoter insertion may occur within the introns of a proto-oncogene, leading to expression of a fusion transcript that encodes a truncated version of the proto-oncogene protein. In the second mechanism of LTR activation, enhancer sequences in the viral LTR activate the endogenous promoter of the proto-oncogene (enhancer activation). In this case, the proviral DNA may be inserted in the opposite transcriptional orientation from the proto-oncogene, or it may be situated downstream from the proto-oncogene. In enhancer activation, the proviruses may be inserted over a greater range of distances (up to 50–100 Kb) from the proto- oncogene. In tumors with LTR enhancer activation, fusion transcripts containing viral and proto-oncogene sequences are not observed; there is simply over-expression of the normal proto-oncogene transcript.

The slower rate of tumor induction by non-acute retroviruses can be rationalized. Since viral DNA insertion is essentially random in infected cells, the probability of a provirus integrating next to a proto-oncogene in a given infected cell is low. Multiple rounds of infection in an animal will be required before one cell sustains a proviral insertion in the vicinity of a critical proto-oncogene, in a configuration appropriate for activation. Once this occurs, then that cell will have enhanced replicative potential that can ultimately lead to outgrowth of a tumor.

Studies of CISs in retrovirus-induced tumors have led to the discovery of new proto-oncogenes. In some cases, some of these newly discovered genes have been found to be activated in non-virus induced tumors, including those from humans. Thus study of non-acute retrovirus-induced tumors has been a useful method of cancer gene discovery. Proto-oncogenes identified in this way include the Pim-1 kinase discovered in tumors induced by Moloney MuLV, and the Wnt-1 signaling proteins discovered in MMTV-induced tumors. Recently, high throughput techniques have been developed to increase the efficiency of proto-oncogene discovery, for instance in MuLV-induced leukemias. Inverse PCR (iPCR) is used to rapidly clone multiple proviruses (including adjacent cell DNA) from collections of tumors induced by an MuLV. The adjacent cell DNAs from the iPCR clones are sequenced and aligned with the mouse genome, which allows location of each provirus in the genome. Bioinformatics is then used to identify chromosal loci where more than one provirus has integrated in the tumor collection. Currently retroviral

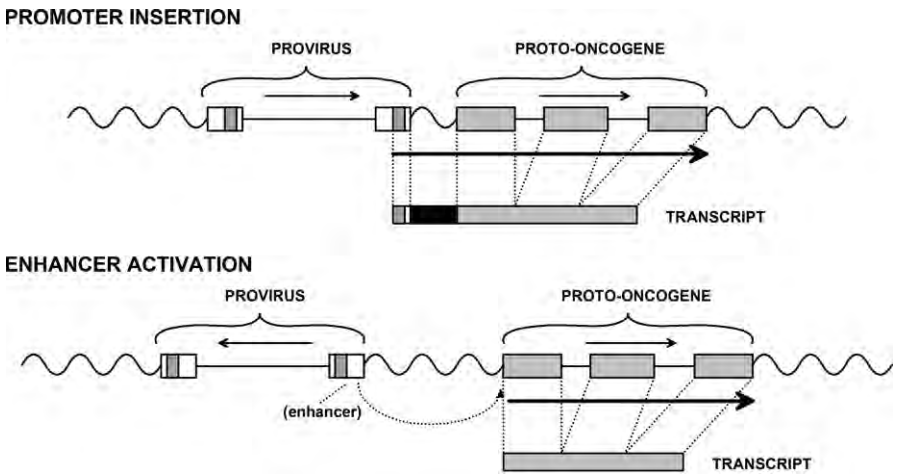

Figure 4 Oncogenesis by nonacute retroviruses. Transcriptional activation of cellular proto-oncogenes in tumors results from insertion of a retroviral provirus in the vicinity of the proto-oncogene. In promoter insertion, readthrough transcription from the viral LTR into the proto-oncogene leads to production of a fusion transcript. In enhancer activation the viral LTR enhancer activates over-expression from the proto-oncogene's own promoter. The over-expressed proto-oncogene transcripts are shown.

integration site analysis has been facilitated by high throughput DNA sequencing (deep sequencing) of virus-induced tumor DNAs.

For non-acute retroviruses, the viral LTRs play an important role in the disease specificity and efficiency of tumorigenesis. For instance, the Friend and Moloney strains of MuLV induce erythroid and T-lymphoid leukemias respectively. Exchange of LTRs between the two viruses is sufficient to reciprocally alter the disease specificities. In fact, exchange of just the U3 enhancer sequences between the two viruses is sufficient to change disease specificity. This can be understood in light of the importance of the LTR in proto-oncogene activation. In order for a non-acute retrovirus to induce oncogenic transformation of a cell, its LTR must be active in that cell type in order to activate an adjacent proto-oncogene. Indeed, the M-MuLV LTR is more active in T-lymphoid cells, while the F-MuLV LTR is more active in erythroid cells. It is interesting that any given non-acute retrovirus may activate a particular proto-oncogene or subset of proto-oncogenes when it induces tumors. This may reflect the relative importance of a particular proto-oncogene for development of tumors of a particular cell type, as well as the ability of enhancer sequences in a particular LTR to activate that proto-oncogene.

When tumors develop, multiple genetic and biochemical changes are necessary to convert a normal cell into the final tumor cell. This is also true for retrovirus-induced tumors. In tumors with one proto-oncogene activated by proviral insertion, presumably other non-viral secondary events occurred. In some cases the second events may also be induced by the retrovirus. For instance, more than one proto-oncogene may be activated by proviral insertion in the same tumor. Another example is Abelson MuLV, an acute transforming retrovirus carrying the *v-abl* oncogene that induces rapid B-lymphomas in mice. In order for Ab-MuLV to be tumorigenic, it requires M-MuLV as a helper, while other MuLV helper viruses are not effective. Ab-MuLV-induced tumors not only contain and express the *v-abl* oncogene, but they also have insertional activation of a proto-oncogene (*ahi-1* or *ahi-2*) by the helper virus (M-MuLV).

Immunodeficiency

Several retroviruses can induce immunodeficiency, most notably the lentiviruses HIV and related simian immunodeficiency viruses (SIVs). The SIVs are native to African old world primates, and in general they do not cause significant disease in their native species. However, when they infect other species, they can cause immunodeficiency. For instance, SIV_{mac} is actually native to African mangabeys, but when it infects Asian rhesus macaques it causes acquired immunodeficiency (simian AIDS). Likewise, HIV-1 is native to chimpanzees and HIV-2 is native to sootey mangabeys; both of these viruses have relatively recently entered the human population and they both cause AIDS while they have little or no effects on their native host species. HIV and AIDS is covered in more detail in another entry in this encyclopedia but some key points will be mentioned here. HIV-1 is the predominant cause of the worldwide AIDS epidemic, while HIV-2 induces AIDS less efficiently and is largely confined to Africa. Current estimates are that approximately 35 million people were infected by HIV worldwide at the beginning of 2012, with more than 90% of the infections in the developing world. Without treatment, most HIV-infected individuals will progress to clinical AIDS (severe immunodeficiency) with a latency of 10–15 years. Symptoms of AIDS include opportunistic infections (e.g. pneumocystis pneumonia [PP]), cancers (e.g. Kaposi's sarcoma [KS]) and neurological problems (e.g. dementia). The clinical definition of AIDS is HIV infection and one AIDS-defining illness (e.g. PP or KS) or CD4 T-cell counts below 200 mm^{-3} (normal CD4 counts are typically >1000 mm^{-3}).

As mentioned above, HIV is a complex retrovirus with six additional genes (*tat*, *rev*, *nef*, *vpr*, *vif* and *vpu*). The CD4 receptor for HIV is present on T-helper lymphocytes, macrophages and dendritic cells, so these are the cells that can be infected *in vivo*. Infection of T-helper lymphocytes results in cell killing, while infected macrophages and dendritic cells are not killed. During the initial stages of infection, there is typically substantial loss of T-helper lymphocytes. The initial infection may be accompanied by flu-like symptoms, or it may be asymptomatic. Development of cell-mediated and humoral immune responses limits the initial infection, after which levels of circulating virus (viral loads) substantially decrease and T-helper (CD4) counts recover. The infected individual then enters an asymptomatic period (typically 8–10 years) where there are relatively low viral loads and relatively normal T-helper counts. However the infection has not been eliminated, as low levels of virus infection persist, and individuals continually produce virus-specific antibodies (the common diagnostic test for infection). Ultimately, the immune system is unable to control the viral infection, and rising viral loads result in progressive loss of T-helper lymphocytes. Since T-helper lymphocytes are essential for function of both cell-mediated immunity and humoral immunity, loss of T-helper lymphocytes leads to failure of both arms of the adaptive immune system. This results in the appearance of the symptomatic opportunistic infections and cancers.

One of the reasons that infected individuals cannot eliminate HIV infection is that the virus can establish latency – in latently infected cells the viral genetic information is present but virus is not produced. Latently infected cells can evade the immune system since they do not express viral proteins. Latently infected cells include macrophages and T-lymphocytes and they persist in infected individuals for many years. At later times they may be re-activated to produce virus that can spread. Another mechanism by which HIV evades host defenses is rapid and progressive mutation in infected individuals, particularly in the *env* gene. This mutation is largely driven by viral evasion of host immune adaptive responses (antibodies and cytotoxic T-lymphocytes) to the virus. In individuals being treated with anti-viral drugs, the high virus mutation rate also leads to development of drug-resistant virus. Temporal variation of HIV is also seen in infected individuals. The initial virus transmitted generally is one that uses the CCR5 co-receptor. With time, the virus will mutate so that it is more cyopathic (killing T-helper lymphocytes efficiently), which will often be accompanied by a switch to usage of the CXCR4 co-receptor.

Antiviral drugs targeted at HIV have substantially improved the clinical outlook for infected individuals. So far, antivirals targeted at RT, protease and integrase have been developed. RT inhibitors are divided into two classes, nucleoside reverse transcriptase inhibitors [NRTIs] and non-nucleoside reverse transcriptase inhibitors [NNRTIs]. NRTIs (typified by AZT) are chain-terminating nucleoside analogs that are incorporated by RT into viral DNA during reverse transcription; once incoporated, an NRTI cannot be further extended which kills the growing DNA molecule. The antiviral selectivity of NRTIs is due to the fact they are relatively inefficiently incorporated by host cell DNA polymerases. NNRTIs (e.g. nevirapine) inhibit reverse transcription by binding to RT and blocking its enzymatic activity. Additional drugs that block the initial entry events for HIV (fusion and co-receptor binding) have been developed and are available.

Prior to development of different classes of inhibitors, treatment with single HIV antivirals (NRTIs) proved to be of limited benefit. Monotherapy of infected individuals resulted in initial suppression of viral loads (with improvements in immune system function), but the effects were transient. Emergence of drug-resistant virus occurred quite quickly, with resulting decline in immune function. The development of additional classes of antivirals (notably protease inhibitors) paved the way for combination therapies. Combination therapies with several antiviral drugs are highly effective at reducing viral loads and improving the immune systems of individuals with AIDS. There are multiple combinations that are effective (some available as once-daily pills), and they always include inhibitors of more than one class. The reduction in viral loads can be prolonged, resulting in clinical improvement or even recovery in individuals with AIDS. However despite these very positive effects, combination therapies have limitations. The therapies must be taken continually since the virus has not been eliminated. Development of drug resistance is a major limiting factor, even for combination therapies. Clinical management of HIV-infected individuals includes monitoring for appearance of drug resistant virus and finding new combinations of drugs that control it. A second limitation is that many of the HIV antiviral drugs have side effects; this is particularly important because they must be taken continually to control the virus. Side effects include anemia, neuropathies, pancreatitis, diabetes, cardiac disease and lipodystrophy. The antiviral drugs are also expensive – beyond the means of many infected individuals in the developing world. For these reasons there is tremendous interest and effort in developing an effective preventative HIV vaccine. However efforts so far have not been successful.

In addition to HIV, other retroviruses also induce immunodeficiency in animals and they have been studied for insights into HIV pathogenesis. The most frequently studied animal retroviruses that cause immunodeficiency are simian lentiviruses (SIVs) infected into non-native primate species (e.g. SIV_{mac} into rhesus macaques). Simian retroviruses of the betaretrovirus class (simian retrovirus Type-D) also induce immunodeficiency in rhesus macaques. In addition, a murine retrovirus complex also induces murine AIDS (MAIDS). The MAIDS complex consists of an MuLV as well as a replication-defective MuLV derivative. The replication-defective component is responsible for the development of MAIDS. The mechanism of immunodeficiency is distinct from that associated with HIV infection, with the virus actually inducing proliferation of B-lymphocytes.

Neurologic disease

Several retroviruses also induce neurologic symptoms. HIV-infected individuals frequently develop such symptoms in the central nervous system (dementias) and in peripheral nerves (neuropathies). The dementias are associated with loss of neurons in the brain (spongiform encephalopathies) but the neurons themselves do not appear to be targets for HIV infection. Infection is observed in supporting cells, most notably microglial cells. Thus the mechanism of neuropathogenesis by HIV appears to be indirect. Some strains of MuLV also induce neurologic symptoms such as hind limb paralysis (in particular those derived from wild mouse ecotropic MuLV). These neuropathic strains of MuLV also induce spongiform encephalopathies of the brain or spinal cord. Similar to the situation with HIV, neuropathic MuLVs do not appear to infect neurons, so the pathogenesis likely results indirectly from infection of other cells.

The human retrovirus HTLV-I, which induces T-cell leukemia (see below) also induces a demyelinating disease known as HTLV-associated myelopathy or tropical spastic paraparesis (HAM/TSP). The symptoms resemble multiple sclerosis, and the disease may be immune-mediated.

Retroviruses of humans

While early research on retroviruses involved studies of animal retroviruses, several retroviruses of humans have been discovered, some of which cause disease. The first human retrovirus discovered was HTLV-I, which causes adult T-cell leukemia (ATL) and HAM/TSP. This virus is a member of the deltaretrovirus family, and it encodes several additional proteins from different reading frames in the *X* region. These include the transcriptional transactivator Tax and the Rex regulatory protein. Leukemogenesis by HTLV-I is relatively inefficient, with decades elapsing between infection and development of the leukemia. The Tax protein is likely involved in the process, since it can immortalize primary T-lymphocytes and transform fibroblasts in culture. Tax can transactivate cellular promoters in addition to the viral LTR, including the promoters for interleukin-2 (IL-2, T-cell growth factor) and the IL-2 receptor; the activation of these cellular genes may be involved in tumorigenesis. Simultaneous expression and IL-2 and IL-2R in a lymphocyte can lead to an autocrine loop in whcih there is continual signaling for cell division. On the other hand, many HTLV-induced ATL tumor cells actually do not express Tax, so Tax may be involved in establishment but not maintenance of the tumor. A more recently discovered protein is encoded by the opposite strand of HTLV-I, HBZ. This is a DNA binding protein with a B-zip

motif that can affect transcription of both HTLV and cellular genes negatively or positively. Moreover it is expressed in all ATL cells, which suggests that it is required for maintenance of HTLV-induced tumors. A virus related to HTLV-I, HTLV-II has also been isolated. HTLV-II has not been causally linked with any neoplasms, although it was first isolated from hairy cell leukemia cells (a form of T-cell leukemia). HTLV-II infection is present in indigenous populations in North and South America, while HTLV-I infection is predominantly in Africa, the Caribbean and Japan.

The most important human retroviruses in terms of disease are HIV (HIV-1 and HIV-2). These viruses cause human AIDS, and as described above they represent relatively recent movement of infection from African primates into humans.

Recently another retrovirus was reported to be associated with human prostate cancer (PC) and chronic fatigue syndrome (CFS), xenotropic murine leukemia virus-related virus (XMRV). XMRV was originally suggested to be a human retrovirus present in prostate cancers from patients with an inherited predisposition to PC. Subsequently XMRV also was reported to be detectable in tissues from CFS patients. However a number of follow-up studies did not confirm the presence of XMRV in either of these diseases. Subsequently XMRV was found to have arisen by activation and recombination of endogenous murine leukemia viruses during passage of a human PC tumor through immunocompromised (nude) mice. XMRV could replicate in the human PC tumor cells growing in the nude mouse, but it was not present in the original human tumor. The detection of XMRV sequences in human tumors or CFS tissues resulted from laboratory contaminations, coupled with the highly sensitive PCR-based detection techniques employed.

Several reports have suggested that a virus closely related to MMTV is present in human breast cancer or tissues from biliary cirrhosis patients. These reports also are controversial, since other investigators have not confirmed them.

Host restriction factors for retroviruses

Due to the potential deleterious effects of exogenous retroviruses and their endogenous counterparts (ERVs, see below), many species have evolved restriction systems that limit exogenous retrovirus infection or transposition by retrotransposons. The initial infection step can be blocked by expression of an endogenous (genetically transmitted) envelope protein that binds to the receptor, blocking infection by an incoming retrovirus (viral interference). Certain chicken and mouse strains express such envelope proteins that block incoming retroviral infection. There are also host restriction systems that block viral infection intracellularly. The first intracellular restriction identified was the Fv-1 system for MuLVs in mice. There are two alleles for *Fv-1*, *Fv-1*^{n} and *Fv-1*^{b}; likewise, MuLVs can be classified as N-tropic or B-tropic. *Fv-1*$^{n/n}$ mice (or cells derived from them) are permissive for replication by N-tropic but not B-tropic MuLVs, while B-tropc but not N-tropic MuLVs can replicate in *Fv-1*$^{b/b}$ mice. Resistance is dominant, that is *Fv-1*$^{n/b}$ mice are resistant to both N- and B-tropic MuLVs. Fv-1 restriction occurs at an early post-entry step in viral replication – between reverse transcription and integration of viral DNA. The Fv-1 protein appears to be distantly related to a retroviral gag protein and it may interfere with viral replication by interacting with or mimicking MuLV Gag protein.

Another early post-entry restriction system is the TRIM proteins – proteins containing a tripartite motif consisting of a RING domain, a B Box-2 and a coiled-coil domain. This restriction was first identified in studies of HIV-1 replication. Cells of old world primates (e.g. rhesus macaques) are poorly permissive for HIV-1 infection, and it was found that they express a species-specific restriction factor, TRIM5α – a TRIM protein containing a C-terminal extension with a B30.2 domain. The B30.2 domain binds viral capsid protein; the species specificity of different TRIM5α's is determined by this binding. Rhesus TRIM5α restricts HIV-1 replication at an early step, similar to the stage for FV-1 restriction. Restriction may result from TRIM5α-mediated degradation of viral cores, through a ubiquitin ligase activity on the protein. Species-specific restriction by TRIM proteins affects more retroviruses than HIV-1. For instance human TRIM5α restricts intracellular replication of N-tropic MuLV, and TRIM5 proteins from African green monkeys and cattle restrict HIV-1, HIV-2, SIV_{mac} and N-tropic MuLV. Like Fv-1, TRIM5α restriction can be abrogated by high levels of viral Gag protein.

Another interesting retroviral restriction system is the APOBEC3 proteins. APOBEC proteins are cytidine deaminases that deaminate cytosine residues to uracils in DNA or RNA. If incorporated into retroviral particles, certain APOBEC proteins can induce cytidine deamination in the first strand of reverse transcribed DNA, leading to G→A mutations in the viral genome. This restriction was first discovered by studies of HIV-1 and its *vif* gene. In *vif*-negative HIV-1, one APOBEC protein (APOBEC3G) is incorporated into virions as particles are assembled. Upon infection into the next cell, cytidine deamination of viral DNA during reverse transcription leads to extensive G→A mutations and blockage of viral replication. APOBEC3G can also block the overall rate of viral DNA synthesis, in addition to inducing G→A mutations. The Vif protein counteracts this restriction by binding to APOBEC3G protein in the initially infected cell and triggering proteosomal degradation by recruitment of a ubiquitin ligase. Thus virus particles produced from a Vif-expressing infected cell are free of APOBEC3G. The APOBEC restriction also affects other viruses. For instance human APOBEC3G also restricts MuLV infection in human cells, and murine APOBEC3 restricts replication of HIV-1 in mouse cells. For some situations (e.g. murine APOBEC3 restriction of Moloney MuLV in mouse cells), restriction does not involve mutation of viral DNA. For MuLVs, a novel form of Gag protein (glycosylated Gag) counter-acts the effects of murine APOBEC3 in mice. It is interesting that multiple APOBEC genes are present in many species; for instance mice have one APOBEC3 gene, while humans have seven (APOBEC3A – H). One hypothesis is that this resulted from evolutionary selection for resistance to infection by retroviruses and other viruses, as well as retrotransposition of endogenous retroelements. *In vitro* assays indicate that human

APOBEC3A and –B reduce retrotransposition of IAP retroelements in human cells, and murine APOBEC3 reduces retrotransposition of MusD elements in mouse cells. Moreover, all primate APOBEC proteins restrict retrotransposition of ubiquitous non-LTR retroelements (e.g. LINE and ALU elements).

The restriction factor BST2/tetherin functions at a late step in viral replication – release of virus particles from the infected cell. BST2/tetherin was discovered by studies on HIV-1, where it was found to be the target of the viral protein Vpu. BST2/tetherin is a component of the innate immune system, and it is inducible by interferon. When Vpu negative HIV-1 is infected into cells, budded virus particles are retrained (tethered) to the cell surface by interactions with BST2/tetherin. Vpu expression results in proteosomal degradation of BST2/tetherin or its direction away from the cell surface so that virions can be released from the infected cell. Some other retroviruses utilize other viral proteins to counteract BST2/tetherin, including SIV and HIV-2 that use the Nef and Env proteins respectively.

The most recently identified host restriction factor is SAMHD1, identified through studies on HIV-1. SAMHD1 is a deoxynucleotide hydrolase that hydrolyzes dNTPs and reduces the intracellular pools of these DNA precursors. This protein is also a component of the innate immune system and inducible by interferon. SAMHD1 is expressed in various hematopoietic cells, and it restricts viral replication in non-dividing myeloid cells and T-cells, predominantly by reducing their dNTP levels. It does not restrict replication in dividing cells, where dNTP levels are higher. SAMHD1 is counteracted by the Vpx protein encoded by HIV-2 and SIVs; interestingly HIV-1 does not encode a Vpx or other proteins that counteract SAMHD1.

Endogenous retroviruses

Most eukaroytic organisms carry retroviral DNA in their genomes as stably inherited elements. These elements are referred to as endogenous retroviruses (ERVs) and they represent retroviral infection of germ cells during evolution of a species. Once a germ cell is infected by a retrovirus, all progeny will inherit the proviral DNA. The 'endogenization' of retroviruses has occurred throughout evolution, with some ERVs entering the genomes relatively early during speciation (e.g. entry of some human ERVs [HERVs] in common ancestors to humans and great apes) while others have entered the germ lines relatively recently (e.g. entry of other HERVs after separation of humans from chimpanzees). Indeed, endogenization of retroviruses is presently occurring in some species (e.g. at high levels in the koala). In addition to the ERVs, there are also related genetic elements that resemble retroviruses except they lack coding sequences for an env protein. These elements, LTR retrotransposons, assemble particles and re-insert reverse transcribed DNA into the genome without an extracellular phase (retrotransposition). Over evolutionary time scales, multiple copies of endogenous retroviruses or LTR retrotransposons have accumulated in the genomes of most eukaryotes; once inserted, there is no molecular mechanism for removal. Indeed, it is estimated that approximately 8% of the human genome is made up of ERVs and LTR retrotransposons. This includes 'solo LTRs' that result from excision of the ERV coding sequences by homologous recombination between the two LTRs.

Insertion of retroviral proviruses into the host genome can be detrimental to the host organism if the provirus insertion disrupts a gene or leads to activation of a proto-oncogene. Thus there has been genetic selection against replication-competent ERVs that could continually reinfect the host; most ERV genomes carry mutations and/or deletions. However, for some species there are ERV genomes that can encode full-length proteins, and some species carry replication-competent ERV proviruses (e.g. mice). There are no replication-competent HERVs in the human genome.

Endogenous retroviruses have several biological effects on their host species. In some cases, endogenous retroviruses can prevent infection by related exogenous retroviruses. A common mechanism is expression by an ERV envelope protein (see above). This has been observed for restriction of alpharetroviruses in chickens. For instance C/A chickens express an endogenous envelope protein for subgroup A retroviruses; these cells are resistant to infection by exogenous subgroup A viruses that use the same receptor. In mice, strains carrying the *Fv-4* gene are expressing the Env protein of an endogenous MuLV that renders them resistant to infection. ERVs can also interact genetically with related exogenous retroviruses when they infect the cell. For instance, when exogenous MuLVs infect mice, recombinant MuLVs containing a new *env* gene appear, MCF recombinants; MCFs result from recombination with an endogenous MuLV. MCF recombinants have an expanded host range in that they can infect cells of both mouse and non-mouse origin (polytropic); this reflects the host range of the Env protein encoded by the endogenous MuLV donors (polytropic or modified polytropic ERVs). MCF recombinants have been implicated in the development of MuLV-induced leukemia. It is noteworthy that the polytropic and modified polytropic ERV proviruses are themselves replication-defective, even though they can donate a functional *env* gene by recombination. Finally in some cases, ERV's have affected expression of a cellular gene by insertion into it. For instance, the *dilute* coat color mutation in mice results from insertion of an MuLV-type ERV.

The most dramatic biological effects of ERVs is the role of ERV envelope proteins in normal embryonic development. During early human development, extra-fetal embryonic cells fuse to make syncytial trophoblasts. The fusion is mediated by the proteins sincytin-1 and 2. Cloning and sequencing of these genes revealed that they are actually endogenous retroviral envelope proteins encoded by HERV-W class proviruses. It is striking that in mice and sheep ERV envelope proteins also have been co-opted by the host to mediate syncytial trophoblast fusion. However different ERVs have been employed for each of these different species. Thus in at least three independent cases the host has taken advantage of the membrane fusing capacity of endogenous retroviral envelope proteins and used them to optimize fusion of host cells during embryogenesis. Presumably a less efficient mechanism independent of ERV Env proteins is the default mechanism for syncytial trophoblast fusion in all mammals, but enlisting fusion by ERV env proteins facilitates this process.

Retroviral vectors

Retroviruses have been developed as gene expression vectors. Retroviral vectors have been used in the laboratory to obtain efficient expression of genes in cells, and also to knock down of expression of specific genes by introduction of interfering RNAs (e.g. small hairpin or shRNAs). In addition, they are being developed as clinical gene therapy vectors where introduction of genes into patients may be used to treat different diseases. Retroviral vectors take advantage of several aspects of the retroviral lifecycle, most notably that integration of proviral DNA into the host genome is a step in the replication process. As a result, these vectors offer the prospects of prolonged high level expression of the target gene in the vectored cell. Retroviral vectors based on gammaretroviruses (e.g. MuLVs), alpharetroviruses (e.g. spleen necrosis virus) and lentiviruses (e.g. HIV-1) have been developed. A typical retroviral vector DNA plasmid is generated by inserting the target gene into a retroviral provirus by molecular cloning. While some retroviral sequences may be eliminated during the cloning (e.g. those encoding the viral structural proteins), the essential retroviral sequences maintained in the vector plasmid are the LTRs and the RNA packaging signal (Ψ). This vector DNA is then co-transfected into cells along with helper expression plasmids for the viral structural proteins; the mRNAs from the latter plasmids lack the Ψ sequences so they themselves cannot be incorporated into retroviral particles. Alternatively, the vector DNA may be transfected into a retroviral vector 'packaging cell' that permanently expresses viral structural proteins from Ψ-negative mRNAs. In either case, the transfected cells will produce retroviral particles that contain only RNAs encoded by the vector plasmid ('helper-free'). The resulting vector particles will infect susceptible cells, reverse transcribe the vector DNA and integrate it into the chromosomal DNA where it can be stably expressed. However, since the retroviral vectors do not express the viral structural proteins, they will not assemble infectious particles that spread the vector infection. Introduction of genes into cells by way of retroviral vector infection is termed transduction.

Variations of the basic retroviral vector design have been developed that address practical and theoretical concerns. Some retroviral vectors rely on the viral LTR as the promoter for the target gene, in which case the transcriptional specificity is dictated by that of the viral LTR. To increase expression in other kinds of cells, substitutions by LTRs with different transcriptional specificities has been employed. Alternatively, an internal cellular or viral promoter is inserted within the vector to drive target gene expression independent of the LTR. Expression of the target gene protein (or expression of two target genes from the same promoter) can be enhanced by insertion of an internal ribosome entry site (IRES) upstream of the target gene coding sequences. Another challenge has been that integrated retroviral vector DNAs tend to undergo transcriptional silencing over time (e.g. through DNA methylation). Approaches to this problem have been to include cellular transcriptional insulator elements in the vectors, or to use lentiviral vectors where there may be less silencing. The original retroviral vectors employed retroviral envelope proteins on the vector particles for cell entry. Thus the host and tissue specificities of the vectors were determined by the Env proteins; moreover, retroviral envelope proteins are dissociated from viral particles relatively easily during purification or concentration. It is possible to use the heterologous G-protein from vesicular stomatis virus (VSV, a rhabdovirus) to form pseudotypes in which the vector particles contain retroviral Gag and Pol proteins, but with VSV-G protein in the envelope. These pseudotypes have the advantages that VSV-G has a broad host range, and it is tightly anchored by way of a glyco-phophatidyl inositol (GPI) linkage into the lipid bilayer of the viral envelope. As a result infectious vector particles can be efficiently concentrated by ultracentrifugation. In contrast, ultracentrifugation strips many retroviral Env proteins from the particles.

Other aspects of retroviral vector design address safety concerns. One concern is that recombination between retroviral vector and helper plasmid DNAs or RNAs could result in recovery of replication-competent retroviruses (RCRs). To reduce the likelihood of RCR formation, modern vector systems generally employ at least three different plasmids to express the vector genome and viral structural proteins. Thus formation of an RCR would require three or more recombination events. In practice, in clinical experiments all retroviral vector stocks are tested for the absence of RCRs by sensitive biological assays prior to use. The second safety concern is that introduction of retroviral vectors into patients could lead to induction of cancers by insertional activation of proto-oncogenes (see above). Recent clinical trials have indicated that this is a real concern in at least one setting (see below). One approach is self-inactivating (SIN) vectors. In these vectors, the vector plasmid has a complete upstream LTR, but deletion of the promoter and enhancer sequences in the U3 region of downstream LTR; target gene expression is driven by an internal heterologous promoter (Fig 5). In the transfected vector-producing cell, the vector genome transcription is driven by the wild-type upstream LTR. However, in the transduced cell, reverse transcription will result in the integrated vector provirus containing U3-deleted (and inactive) LTRs in both upstream and downstream positions. The transgene will be expressed from the internal promoter, but the likelihood of activation of adjacent proto-oncogenes will be reduced since there are no active LTRs present.

Retroviral vectors have been tested in human clinical trials for several medical applications. One application is to use retroviral vectors to restore genes to individuals with single-gene defects such as hemophilia, adenosine deaminase deficiency (SCID), and common gamma chain deficiency (X-linked SCID). In some cases target cells (e.g. hematopoietic progenitors) are isolated from the patient and subjected to vector transduction *ex vivo*. Alternatively, infectious vectors are infected directly into target tissues. In some cases, positive clinical results have been obtained, most notably correction of the severe combined immunodeficiences (SCIDs) associated with inherited adenosine deaminase and common gamma chain deficiency. However, in the treated X-linked SCID patients (treated with a first-generation MuLV-based vector), a significant percentage developed T-cell leukemia resulting from insertional activation of a proto-oncogene (LMO2). While this had been a theoretical possibility, the actual occurrence of leukemias in at least one clinical setting demonstrated the importance of this concern. In another more recent trial, gene therapy

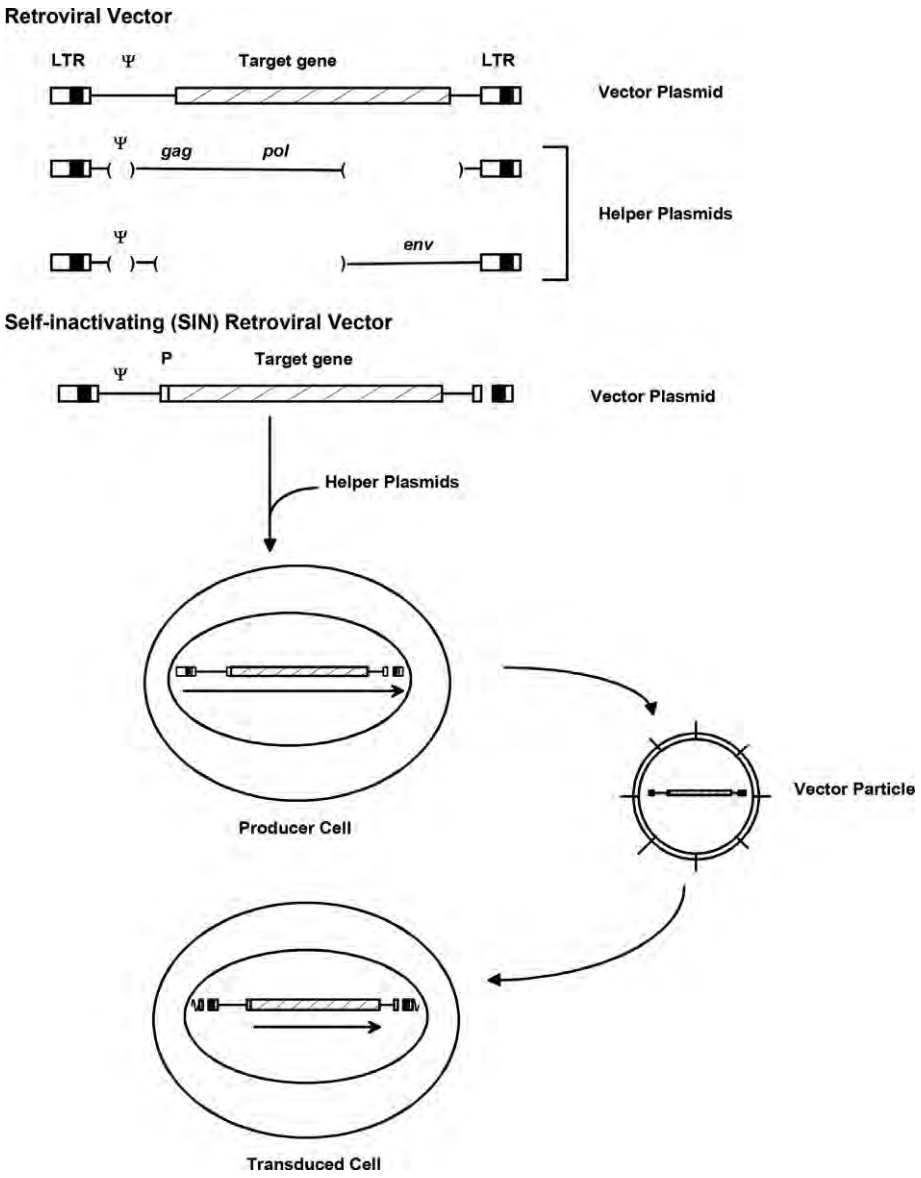

Figure 5 Retroviral vectors. Components to generate a typical retroviral vector are shown at the top. They include a plasmid encoding the retroviral vector as well as helper plasmids encoding the viral structural proteins from mRNAs that cannot be packaged since they lack the RNA packaging signal (Ψ). Vector particles are generated by transient co-transfection of vector and helper plasmids, or by transfection of vector plasmid into cells that stably express the helper plasmids (packaging cells). A self-inactivating (SIN) vector is shown at the bottom, in which the U3 promoter/enhancer sequences are deleted from the downstream LTR of the vector plasmid. Transient transfection into a producer cell yields vector particles with deleted U3 regions in their RNA; after infection into a target cell (transduced cell), the resulting provirus will contain deletions in both LTRs. Transcription of the target gene will be driven by an internal promoter.

of a beta-thalassemia patient with a lentiviral vector expressing beta-globin resulted in clinical benefit. In this patient there is a major clone of modified cells, which shows over-expression of the HMGA2 gene and protein. HMGA2 has been associated with malignancies in some cancers, but so far this patient has shown no evidence of malignancy.

Another clinical application of retroviral vectors has been to use them to express antigens or immunostimulatory molecules in cancer cells with the goal of stimulating a host immune response to the tumors – cancer vaccines.

Further Reading

The definitive reference book for retroviruses is Coffin et al. (1997). It contains comprehensive extensively cited chapters for most topics. For more recent updates, chapters on retroviruses in Fields Virology (Knipe & Howley, 2013) are valuable.

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Rhizobia☆

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Glossary

Bacteroids Nitrogen-fixing rhizobia in the central zone of a legume nodule.
Classical rhizobia Bacteria of the genera Rhizobium, Bradyrhizobium, Sinorhizobium (Ensifer), Mesorhizobium, and Azorhizobium.
Infection thread The tubular structure through which rhizobia invade the developing legume nodule.
Legumes Members of the Leguminoseae, the third largest family of flowering plants, traditionally distinguished by their fruit morphology (seed pods) and divided into the subfamilies Caesalpinioideae, Mimosoideae, and Papilionoideae.
Nitrogen fixation The reduction of molecular nitrogen (N2) to ammonia (NH3).
Nitrogenase The enzyme system that catalyzes the reduction of N2 to ammonia within prokaryotic organisms.
Nodule The organ on legume roots, and sometimes stems, induced and invaded by rhizobia.
Nod factor A lipochitooligosaccharide molecule that incites nodule formation and root hair curling on susceptible legumes.
Plasmid Bacterial DNA molecules that are smaller than the chromosome(s), ranging in size from 1000 to 2,000,000 nucleotides, and generally dispensable for basal bacterial growth.
Symbiont One of the partner organisms in a symbiosis.
Symbiosis The intimate association of two organisms of different species, classically divided into three categories: mutualism (both organisms benefit), commensalism (one organism benefits), and parasitism (one organism benefits and the other is harmed).

Defining Statement

Rhizobia are diverse Gram-negative members of the Proteobacteria that fix nitrogen inside root and stem nodules, which they incite on leguminous plants. All are facultative symbionts, and as a group they are successful soil bacteria. They have diverse metabolic capabilities, but most often couple versatile heterotrophy with aerobic, microaerobic, and anaerobic respiration. As rhizobia are metabolically versatile, they are widely used as plant growth promoters of multiple legumes and cereals. Rhizobia utilizes C4 acids in preference to sugars and the sugar utilization is reserved as long as C4 acids are present. This is apparent as a diauxie when rhizobia are grown in the presence of a sugar and a C4 acid together. Several reports have shown succinate to repress the enzymes required in transport and utilization of sugars, sugar alcohols, hydrocarbons, etc. This mechanism is termed as Succinate Mediated Catabolite Repression (SMCR).

Nitrogen Fixation in Legumes and its Global Significance

Nitrogen fixation is the reduction of atmospheric nitrogen (N2) to ammonia (NH3). The nitrogen atoms in N2 are triply bonded to each other, and this molecule is very inert chemically. Abiotic chemical conversions of N2 either cannot occur (are disfavored energetically) or occur at exceedingly low rates under normal ambient conditions. Only rare intense bursts of energy such as lightning provide sufficient activation energy and highly reactive molecules that allow formation of other compounds from N2. Even with suitable catalysts, high temperatures and pressures are required to form ammonia from N2 (as in the industrial Haber–Bosch process first commercialized about a century ago). In this context, biological nitrogen fixation is truly remarkable. It provides almost the only natural entry into living systems from the huge reservoir of nitrogen in the atmosphere.

Nitrogen fixation occurs only in prokaryotes. The minority of prokaryotes capable of it are termed diazotrophs, and rhizobia are the best-known symbiotic diazotrophs. In the rhizobia–legume symbioses, this reaction occurs within nodules, organs that develop only in the presence of rhizobia (Fig. 1). Nodules may be determinate or indeterminate. Determinate nodules are found on certain tribes of tropical legumes like soybean, common bean, and on some temperate legumes such as Lotus. As determinate nodules lose meristematic activity shortly after initiation, growth is due to cell expansion resulting in mature spherical shaped nodules.

☆*Change History*: February 2017. Bhagya Iyer and Shalini Rajkumar updated the sections Abstract, Defining statement, Nitrogen fixing in legumes and its global significance, Phylogeny and taxonomy, Rhizobial host ranges, Deviation from the foregoing model, Rhizobial genes and components required in symbiosis, Rhizobial genomes and genetics, Other properties of the rhizobia and Notable traits found in some rhizobia. Replaced Figures 1, 3 and 4, Updated Table 1 and added Tables 2, 3 and 4.

This article is an update of K.D. Noel, Rhizobia, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 261–277.

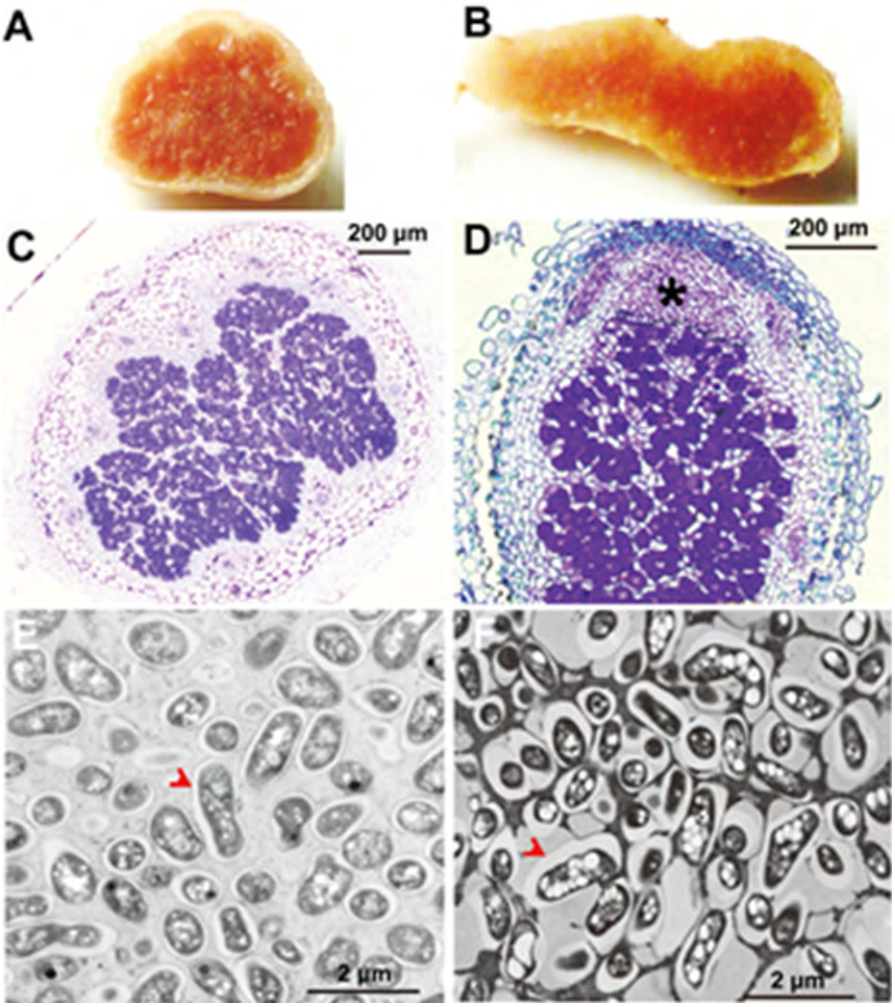

Fig. 1 Nodule sections of *Vigna unguiculata* and *Leucaena leucocephala*. (A–B) Nitrogen fixing nodules of *V. unguiculata* (21 days post inoculation, A) and *L. leucocephala* (35 days post inoculation, B). (C–D) Semithin sections of nodules from *V. unguiculata* (C) and *L. leucocephala* (D), the asterisk indicates the meristem zone of indeterminate nodules of *L. leucocephala* (D). (E–F) Ultrathin sections of nodules from *V. unguiculata* (E) and *L. leucocephala* (F), red arrowheads indicate the peribacteroid membrane; and PHB granules are seen as electron-transparent droplets in the cytoplasm of bacteroids. ©: 2013 Li *et al.*

Indeterminate nodules are found in the majority of legumes from all three sub-families and are termed "indeterminate" because they maintain an active apical meristem which produces new cells for growth over the life of the nodule. This results in formation of cylindrically shaped nodule, which may be extensively branched. Indeterminate nodules manifest zones demarcating different stages of development/symbiosis, as they are actively growing. On some water-tolerant legumes, nodules develop on stems, but much more commonly they appear on roots. Other important symbiotic diazotrophs include *Frankia*, an actinomycete genus whose species incite root nodules on at least 23 genera of plants in eight families of the rosid I clade of the dicotyledonous angiosperms (Fig. 2), and cyanobacteria, which form nitrogen-fixing symbioses with lower plants and fungi (in lichens). Some animals, such as those which feed on wood, also enter into symbiosis with diazotrophs.

The relationship between rhizobia and legumes is the best-known, best-understood mutualistic symbiosis between a prokaryote and a eukaryote. It is clear that the legumes benefit as they are allowed vigorous growth in nitrogen-deficient soil. This symbiosis is undoubtedly a major factor in the great success of legumes as a plant family. In contrast, some scientists have questioned whether the bacteria benefit or, at least, how they benefit. Nevertheless, the increased soil population of specific rhizobia after the cultivation of a compatible legume crop seems an obvious indicator that the symbiosis provides a reproductive benefit that should promote evolution of the species.

After photosynthesis, N fixation is considered as the second most important process influencing primary productivity and is the basis of all life on earth. Annually, approximately 2.5×10^{11} kg NH_3 is fixed from the atmosphere through BNF (by legumes and cyanobacteria) and approximately 8×10^{10} kg NH_3 are manufactured by ammonia industry. Further, lightning may also contribute approximately 1×10^{10} kg NH3/year worldwide. Currently, approximately 2 tonnes of industrially fixed N is needed as fertilizer for crop production to equal the effects of 1 tonne of N biologically fixed by legume crops. Therefore, biologically fixed N influences the global nitrogen cycle substantially less than industrially fixed N. In order to keep up with the demands of food, world population has been increasingly relying on nitrogen fertilizers. World demand for total fertilizer nutrients grew at

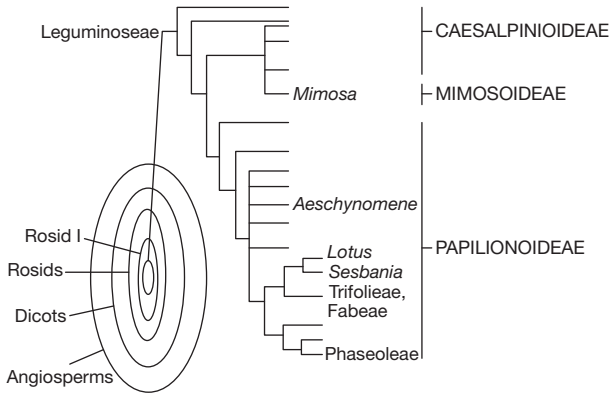

Fig. 2 Gross outlines of legume classification for the purposes of this article. At the bottom left the concentric ovals indicate first that the legumes belong to the Rosid I clade of the Rosids. The Rosids in turn are a clade within the Eudicots ('dicots') of the angiosperms, the flowering plants. Genus *Parasponia* is in Rosid I outside of the legume family. The branchings on the right side are meant to give a rough sense of the divergence among the various groups of legumes specifically mentioned in the text. Four genera (in italics), three tribes, and the three subfamilies (in capital letters) are indicated. © 2008 Dale Noel. Published by Elsevier Inc. All rights reserved.

2% per annum between 2011 and 2015; the demand for N is forecast to grow annually by 1.7% globally and by 2.6% in south Asia. Globally, BNF in natural terrestrial ecosystems contributes about 107 Tg of N (1 Tg=1 million tonnes) while marine N fixation contributes 121 Tg of N each year. BNF induced via agricultural crop cultivation adds 33 Tg per year. Symbiotic BNF by *Rhizobium* associated with seed legumes adds around 10 Tg, leguminous cover crops add about 12 Tg/yr, non *Rhizobium* N fixing species 4 Tg/yr, cyanobacteria in wet rice fields 4–6 Tg and endophytic N fixing organisms in sugarcane- 1–3 Tg. This makes the total terrestrial nitrogen fixation around 140 Tg N/year. It is estimated that cultivation induced BNF may have risen to 40 Tg/yr due to increase in soybean and meat production. Soybean is the dominant crop legume representing 50% of the global crop legume and fixes 16.4 Tg N annually, representing 77% of the N fixed by the crop legumes. Haber Bosch process can fix about 3 times as much N relative to cultivation induced BNF. About 100 Tg N per year of ammonia produced via Haber Bosch process in 1995, about 86% was used to make fertilizers. The industrial fixation of N is increasing each year with the setting up of more plants and it reached to 121 Tg by 2006 with the worldwide manufacture of N fertilizer at 105 Tg. Predicament with man-made fertilizers is that they are used in excess to achieve maximum crop yields which leaches into water sources, creating problems in inland aquatic ecosystems and sometimes massive plumes of eutrophic pollution. Hence, a transition toward agricultural diversification using legume-based crop rotations may be highly desirable, as it may limit the intensity of ammoniacal fertilization with the input of less reactive organic N.

Rhizobia and legumes are important in agriculture and related commerce. Certain legumes are very important agricultural crops, as forage, animal feedstock, food directly consumed by humans, and commodities processed into a large number of end products. Legumes are estimated to supply about 20% of worldwide food protein. Soils deficient in the appropriate rhizobia can be inoculated with that specific rhizobia to enhance nodulation and the overall yield of a legume crop. The Global Agricultural Inoculants Market is perched to grow at a CAGR of around 9.9% over the next decade to reach approximately $ 710.16 million by 2025. Sometimes, an objective of planting legumes is to improve the N content of the soil for the benefit of other crops. In this respect, it is important to realize that growing legume plants do not leak appreciable amounts of fixed N into the soil. Soil N is materially improved by cultivated legumes only when the legume plants and seeds are plowed into the soil, rather than harvested or eaten directly by livestock.

Phylogeny and Taxonomy

Taxonomy and phylogeny of bacteria is a dynamic topic; any current classification scheme is subject to future revision. Most of the rhizobial species in the alpha-proteobacteria class of phylum proteobacteria in Rhizobiacae family are in either the *Rhizobium*, *Mesorhizobium*, *Ensifer*, or *Bradyrhizobium* genera. However, recent research has exposed many other rhizobial species in addition to already existing ones which may have occurred through lateral gene transfer of symbiotic genes. Genera of rhizobia are currently divided into the groups shown in Table 1.

The term 'rhizobia' defines a group of bacteria based on one complex property, the inciting of legume nodules inside which nitrogen is fixed. The rationale for considering these bacteria as a group is based on the great interest in and the importance of this property, rather than believing that they all belong to one monophyletic evolutionary group. On the contrary, rhizobia are polyphyletic and much more diverse than what had been supposed even a decade ago. The history of the ongoing revisions in their classification, and the accompanying consternation among nontaxonomists, illustrates the overall revolution in bacterial taxonomy within the past three decades. However, the realization that rhizobia did not belong collectively to a single, simple taxon had emerged, though gradually, much earlier.

Table 1 Classification of rhizobia

Proteobacteria Division	*Genus*	*Species Number*	*Species*	*Representative hosts*
Alpha	*Rhizobium*	48	*Alamii*	Arabidopsis
			alkalisoli	Caragana
			azibense	Phaseolus
			calliandre	Calliandra
			cauense	Kummerowia
			cellulosilyticum	Populus
			endophyticum	Phaseolus
			etli	bean
			fabae	Vicia
			freirei	Phaseolus
			galegae	Galega
			gallicum	Phaseolus
			giardinii	Phaseolus
			grahamii	Dalea
			hainanense	Tropical legume
			halophytocola	Rosa
			herbae	Herb legumes
			huautlense	Sesbania
			indigoferae	Indigofera, Kummerowia
			jaguaris	Jaguar
			laguerreae	Vicia
			leguminosarum	Pea, clover, bean
			leucaenae	Leucaenae
			loessense	Astragalus, Lezpedeza
			lusitanum	Phaseolus
			mayense	Calliandra
			mesoamericanum	Phaseolus, Siratro, Cowpea, Mimosa
			mesosinicum	Albizia, Kummerowia, Dalbergia
			miluonense	Lezpedeza
			mongolense	Medicago
			multihospitium	Lotus, Astragalus, Oxytropis
			oryzae	Oryza
			paranaense	Phaseolus
			petrolearium	Oil contaminated soil
			phaseoli	Phaseolus
			pisi	Pea
			tibeticum	Trigonella
			sophorae	Sophora
			sophoriradicis	Sophora
			sphaerophysae	Sphaerophysa
			sullae	Hedysarum
			taibaishanense	Kummerowia
			tropici	Bean, leucaena
			tubonense	Oxytropis
			undicola	Neptunia
			vallis	Phaseolus, Mimosa, Indigofera
			vignae	Vigna
			yanglingense	Arid and semi arid regions of china
	Bradyrhizobium	9	*canariense*	Genistoid legumes
			cytisi	Cytisus
			elkanii	soyabean
			iriomotense	Eantada
			japonicum	Soyabean, cowpea
			jicamae	Pachyrhizus
			liaoningense	Glycine
			pachyrhizi	Pachyrhizus
			yuanmingense	Lespedeza, Vigna
	Sinorhizobium (Ensifer)	16	*abri*	Sesbania, Abrus
			americanum	Acacia

Table 1 Continued

Proteobacteria Division	*Genus*	*Species Number*	*Species*	*Representative hosts*
			arboris	Acacia, Prosopis
			fredii	Soyabean, cowpea
			indiaense	Sesbania, Abrus
			kostiensis	Kosti region, Sudan
			kummerowiae	Kummerowia
			medicae	Medicago
			meliloti	Alfalfa
			mexicanus	Acacia
			morelense	Leucaena
			adhaerens	
			grammaticus	Lotus, Argyrolobium and Medicago
			saheli	Sesbania
			sojae	Glycine max
			terangae	Sesbania
	Azorhizobium	2	*caulinodans*	Sesbania
			doebereinerae	Sesbania
	Mesorhizobium	20	*Abyssinicae*	Woody legume
			albiziae	Albizia
			alhagi	Alhagi
			amorphae	Amorpha
			australicum	Biserrula
			camelthorni	Alhagi
			caraganae	Caragana
			chaoense	Prosopis
			ciceri	Chickpea
			erdmanii	
			gobiense	Wild legumes
			hawassense	Woody legume
			huakii	
			jarvisii	
			loti	Lotus sp.
			mediterraneum	Chickpea
			metallidurans	Anthyllis
			opportunistum	Biserrula
			plurifarium	Acacia
			qingshengii	Astragalus
			robiniae	Robinia
			sangaii	Astragalus
			shangrilense	Caragana
			shonense	Woody legume
			silamurunense	Astragalus
			septentrionale	Astragalus
			tamadayense	Anagyris
			tarimense	Wild legumes
			temperatum	Astragalus
			tianshanense	Chickpea
	Allorhizobium	1	*undicola*	Neptunia
	Methylobacterium	1	*nodulans*	Crotalaria sp.
	Devosia	1	*neptuniae*	Neptunia
	Ochrobactrum	2	*cytisi*	Cytisus
			lupini	Lupinus
	Phyllobacterium	3	*trifolii*	Trifolium and Lupinus
			ifriquiyense	Astragalus
			leguminum	Argyrolobium
	Shinella	1	*kummerowiae*	Kummerowia
	Microvirga	3	*lupini*	Lupinus
			lotononidis	Listia
			zambiensis	

(*Continued*)

Table 1 Continued

Proteobacteria Division	Genus	*Species* Number	Species	Representative hosts
	Devosia	1	*neptuniae*	
Beta	*Burkholderia*	6	*caribensis*	
			mimosarum	Mimosa
			nodosa	Mimosa
			phymatum	Mimosa
			sabiae	Mimosa
			tuberum	Cyclopia
	Cupriavidus (Ralstonia)	2	taiwanensis	Mimosa

The early history of studying root nodules climaxed in 1888 when Beijerinck became the first to isolate a bacterium capable of nodulating a legume, naming the isolate *Bacillus radicicola*. Frank in 1889 decided that all bacteria isolated from legume nodules would be assigned the name *Rhizobium leguminosarum*. The type strain for the genus and species was isolated from pea nodules. Subsequently, after E.B. Fred and colleagues in 1932 proposed the cross-inoculation concept, the taxonomy of the genus *Rhizobium* was, for many years, based on the host specificity of nodulation. Cross inoculation is a test for whether an isolate from one plant species incites nodules on another plant species. The taxonomic concept was that the rhizobia fell into groups defined according to the plants nodulated by each group. This concept was very useful in agriculture, but, gradually, it was appreciated that this classification scheme and the cross-inoculation concept often were very poor predictors of the true relatedness and diversity of these bacteria. The first departure from this taxonomy was based on growth rate in culture ('fast growers' vs. 'slow growers'), with the slow growers being assigned to a new genus, *Bradyrhizobium*.

Since the 1980s, rhizobial taxonomy has been based on nucleotide sequences, mainly of the DNA encoding ribosomal RNA, mirroring the efforts of bacteriologists in general to create a taxonomy reflective of true phylogenetic relatedness. Recently, the availability of total genomic sequences has supported the basic genetic relatedness deduced in this way.

By 2006, 48 species of rhizobia had been classified among six genera whose names include the root word 'rhizobium'. By 2016, this number increased to 125 species of rhizobia classified among fifteen genera (Table 1). In addition, at least 11 Proteobacterial species outside these more 'classical' genera are capable of eliciting nitrogen-fixing nodules on legumes. These latter species are found in nine genera in the alpha division of the Proteobacteria and two genera in the beta division of the Proteobacteria (Table 1). Discovery of legume-nodulating bacteria among the beta Proteobacteria is the most recent and the most dramatic indication of the breadth of distribution of this property among diverse bacteria. The current taxonomy predicts what physiological characterizations have borne out: aside from entering into symbiosis with legumes, the different groups of rhizobia exhibit great diversity in other properties.

The dynamic nature of current bacterial taxonomy is illustrated by the case of *Sinorhizobium meliloti*. This species has a long history of study and was one of the first species to be included within '*Rhizobium*' by the cross-inoculation concept. About two decades ago, it became apparent that this species did not belong to a genus whose type species was *R. leguminosarum*. Because of its close genetic relationship with the type species of the *genus Sinorhizobium*, *S. fredii*, this well-known bacterium was renamed *S. meliloti*. Very recently, it has been discovered that this genus should include bacteria previously assigned to the genus *Ensifer*. Members of the genus *Sinorhizobium* have undergone another name change to *Ensifer*, as *Ensifer* as a genus name preceded the naming of *Sinorhizobium*.

It is likely that some of the organisms now classified as *Bradyrhizobium* and *Rhizobium* will eventually be reclassified under different genus names. Both are very diverse genera according to the current norm in bacterial classification. They include rhizobia more closely related to bacteria in different genera than to other rhizobia within the genus to which they are presently assigned. Likewise, certain species currently assigned to other genera probably should be moved to *Rhizobium* or *Bradyrhizobium*, if progress toward a rational taxonomy is to be continued. A controversial example, which has implications for certain species grouped within the venerable genus *Agrobacterium* and some currently assigned to *Rhizobium*, involves the single species of the genus currently named *Allorhizobium*. In Table 1 this latter genus name is in parentheses because this species and possibly several closely related *Rhizobium* species are likely to undergo reclassification.

Rhizobial Host Ranges

Legumes are a very successful family of plants, known in botany as the Fabaceae or Leguminosae. In traditional taxonomy it is the third largest family within the angiosperms (flowering plants), with more than 700 genera and 20,000 species. This family is divided into three subfamilies: Caesalpinioideae, Mimosoideae, and Papilionoideae (Fig. 2). About 20% of the known legume species have been studied to determine whether they nodulate. All three subfamilies have members that form nitrogen-fixing nodules, but most of the well-known symbioses are in the Papilionoideae. It should be noted that there are legumes that, despite extensive

examination in some cases, have never been documented as nodulating. In surveys among the Caesalpinioideae, 10% of the genera were observed to nodulate. Nodulation is much more common among Mimosoideae (about 80% of the genera surveyed) and Papilionoideae (about 90% of the genera surveyed).

Historically, great attention has been placed on the host range of the rhizobia, and any description of a species generally has included its known host range. Some rhizobia have very narrow host ranges, composed of only one or a few closely related legume species, whereas others have much broader host ranges. One *Sinorhizobium (Ensifer)* strain, NGR234, can nodulate legumes belonging to at least 112 genera that include representatives of all three subfamilies.

Host range is a complex issue with many caveats. Obviously any statement regarding host range is limited by the number of potential hosts that have been tested. In addition, it should be strictly qualified according to the symbiotic parameters that have been observed. A strain may incite nodules, but may not provide nitrogen fixation. Hence, the host range of nitrogen fixation ('effective' symbiosis) is narrower than the host range of nodulation.

Within a rhizobial species, different strains can have clearly different host ranges. For instance, some strains *of R. leguminosarum* nodulate pea ('*biovar*' *viciae*), whereas others nodulate clover (*bv. trifolii*) or bean (*bv. phaseoli)*. These bacteria are all assigned to the same species because of the close homologies of their chromosomes, whereas, in this species, host range is specified by genes on plasmids. A given strain can be converted from one host range to another by replacing one plasmid with another. This was one type of observation that persuaded geneticists that cross inoculation was not a proper way to define species. Adding further complication is that these genetically defined host ranges overlap to differing degrees. Certain *bv. viciae* strains elicit some degree of nodulation on beans, and the quality of the nodulation can depend on the variety or race of the bean plant. The converse is also true. There are variant individuals within a host species that resist nodulation by a rhizobial strain that does well on most other races, cultivars, and varieties of that host. This type of incompatibility can be strain-dependent and sometimes has been traced to presence or absence of specific genes in the rhizobial strain. However, rhizobial host range can be modified as transfer of a plasmid carrying *Rhizobium meliloti* host genes to *R. leguminosarum* bv. *viciae* enabled *R. leguminosarum* bv. *viciae* to infect and nodulate alfalfa, the normal host of *R. meliloti* but stringly inhibited infection and nodulation in normal vetch, its normal host.

Given that rhizobial taxonomy is now based on divergence of ubiquitous chromosomal genes, such as those for ribosomal RNA, and that the genetics of symbiotic host specificity allows variation of host range among the strains of one species, one might ask whether there are any correlations between rhizobial taxonomic groups (species and genera) and host taxonomic groups. Table 1 implies that there are such correlations, and, indeed there are many inconclusive overall trends of this sort. For example, among legumes in the genus *Mimosa* (in the subfamily Mimosoideae), a majority of the reported nodule isolates belong to the beta Proteobacteria. Nevertheless, some of the beta Proteobacterial rhizobia that do well on Mimosa were isolated first from legumes in the subfamily Papilionoideae. Moreover, Bradyrhizobia also are commonly isolated from Mimosa. Therefore, this correlation is not a tight one. Another generalization is that, among highly diverged 'temperate' legume tribes within the Papilionoideae (including Trifolieae and Fabeae among others), 'fast-growing' rhizobia of the alpha Proteobacteria predominate. However, such rhizobia are also found as symbionts elsewhere among the legume family. Particular species have been observed to deviate from the general trend shown by the other species within a genus or tribe of rhizobia or legumes. For example, among isolates of *Phaseolus vulgaris* (common bean) nodules, fast growers predominate, but for other genera of tribe Phaseoleae and even other species of the genus *Phaseolus*, Bradyrhizobia usually predominate.

A related evolutionary question is whether symbiotic organisms, particularly in mutualisms, evolve toward high specificity for each other as a way to maximize the efficiency of interaction. There is no convincing evidence to support this possibility as the overall tendency among the rhizobia. However, there are instances in which the host genus and the bacterial species are mainly restricted to each other. One well-documented example is the legume genus *Trifolium* (clover) and R. *leguminosarum bv. trifolii*. Within this host–bacterial group, there is further restriction in that some of the bacterial strains nodulate and fix nitrogen only on certain *Trifolium* species. Does such specificity provide selective advantage, or is it an evolutionary accident? Judging by whether specialists give better nitrogen fixation than generalists, the overall evidence is mixed. Although the most promiscuous rhizobial strain, NGR234, is a poor symbiont on many of its hosts, there are many instances in which, for example, a *Bradyrhizobium* strain is very efficient on several disparate hosts.

Parasponia in the Ulmaceae (elm) family is the only known nonlegume genus that is nodulated by bacteria that also nodulate legumes. Strains within several different groups of rhizobia, including the aforementioned *Sinorhizobium sp.* strain NGR234, have been found to nodulate this host, but its most effective known symbioses are with certain bacteria currently classified within the genus *Bradyrhizobium.* It is striking how often members of this latter genus, or closely related bacteria, are found as the dominant symbionts of legumes in the Caesalpinioideae and Mimosoideae, as well as in the diverse taxa of the Papilionoideae.

Development of the Symbiosis

As a general rule, legumes develop nodules in nature only in the presence of the appropriate rhizobia. There is now a basic understanding at the cellular and molecular levels regarding how nodules develop. The paradigm described in the following sections has been established by studies with a relatively few legume–rhizobia combinations, those involving certain agronomically important legumes and some closely related model legumes. All are from the subfamily Papilionoideae and fall into just four of the many diverse tribes within this subfamily. They include soybeans, common beans, and cowpea in the tropical tribe Phaseoleae and

alfalfa, clover, and peas in the temperate tribes Trifolieae and Fabeae (Fig. 2). The model organisms are *Medicago truncatula* in the same genus as alfalfa in the Trifolieae and *Lotus japonicus* within a separately-diverged tribe, the Loteae. These hosts exhibit what is sometimes called the root hair-infection type of nodule development, which is observed in the majority of legume–rhizobia symbioses. After presenting a summary of this type of development, deviations shall be discussed.

Development Involving Nod Factors and Root Hair Infection

Formation of the Nodule Primordium

Legume roots and germinating seedlings exude flavonoid compounds that induce specific rhizobia in the vicinity to produce and secrete a glycolipid, the Nod factor (Fig. 3). This rhizobial compound then elicits two important responses in the plant: the curling of root hairs as they develop and the triggering of cell division in root cortex cells to form the nodule primordium. The primordium further generates cells that differentiate to become the nodule. In the Phaseoleae, this cell division initiates in the outer cortex. In the Trifolieae and Fabeae, it begins within the inner cortex near the root pericycle. In the latter cases, the cell division generates a permanent meristem that leads to elongated indeterminate nodules, whereas the former leads to transient meristematic activity and determinate nodules (Fig. 1). Lotus nodule development has elements of these two extremes; the primordium develops deeply in the cortex but the meristem is transient and determinate nodules result.

Recent progress with the plant genetics and cell biology of nodule formation has provided insight into the plant response to Nod factors. The earliest responses that have been documented are a rapid calcium flux into the root hair cell and the resulting fluxes of other ions that cause plasma membrane depolarization. About 10–15 min later, dramatic oscillations in cytosolic calcium, called calcium spiking, have been ordered into a developmental pathway according to the relative time at which the proteins specified by the genes appear to act. The first genes in the pathway encode presumptive Nod-factor receptors whose amino acid sequences align with those of a family of proteins that bind molecules resembling the oligochitin backbone of the Nod factors. Mutations in these genes block all responses to Nod factors including root hair curling. Mutations in some of the other genes affect actions downstream in the pathway; they block the calcium spiking completely or partially. Further downstream in the pathway is a nuclear calcium/calmodulin-dependent kinase. Null mutants in this protein block further progress to nodule primordium. More interesting are mutants in which this protein is constitutively active and that form nodule primordia in the absence of Nod factor. Further downstream in the pathway is a cytokinin receptor with cytokinin-dependent kinase activity. Mutants in which this protein is constitutively active also form nodule primordia in the absence of Nod factor. Hence, plant genetics suggests that formation of nodule primordia is a series of events involving at first mainly calcium fluxes as secondary signals to activate downstream protein phosphorylation events and, later, activation of further protein phosphorylation by the important plant hormone cytokinin. How this pathway connects with regulation of transcription factors and the proteins that carry out visible organogenesis remains to be elucidated. These latter proteins include nodulins, proteins that are highly upregulated in nodules. A large number of such proteins have been identified.

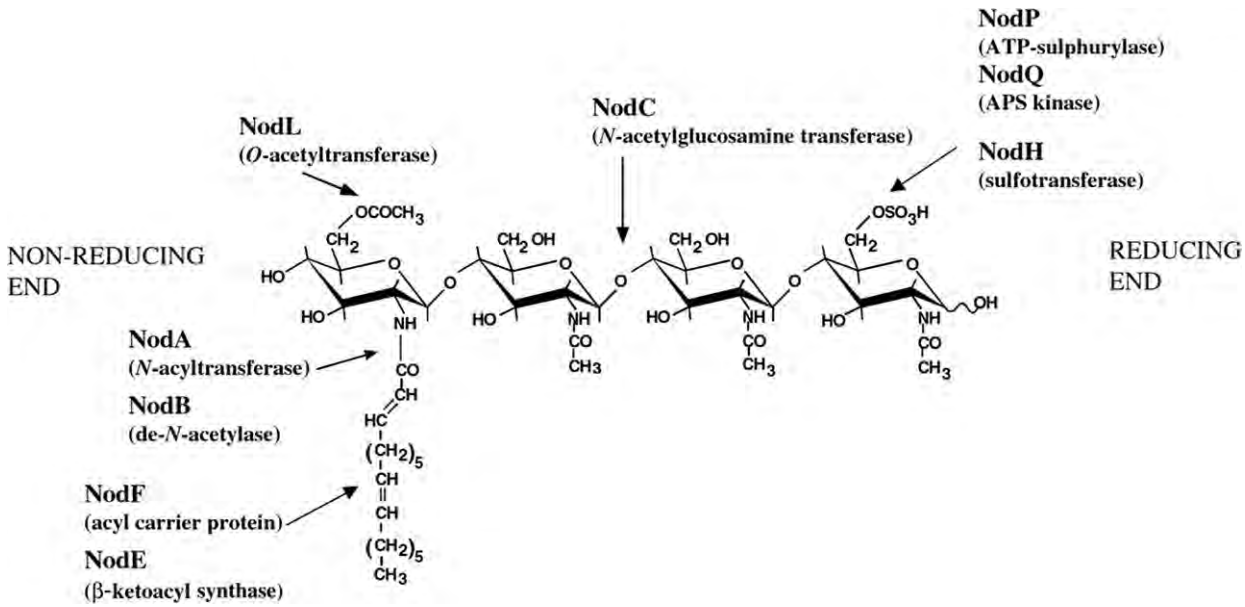

Fig. 3 *S. meliloti* NF structure and Nod protein function. Each Nod protein is encoded by an equivalently named *nod* gene. NodA, NodB and NodC are common to all rhizobia. The remaining Nod proteins are responsible for the modifications of NF that confer activity on selected legume species. NodF and NodE are required for the synthesis of the unsaturated fatty acid whereas NodL is required for the attachment of an O-acetyl group at the non reducing end.

Infection

Coincident with this organogenesis is the second major developmental program, the infection process whereby the rhizobia enter the developing nodule. Nod factor is believed to potentiate this second process by causing the development of curled, and often otherwise deformed, root hairs. Generally, the infection starts from a tightly curled portion of the root hair or where a curled root hair is folded against the main body of the root hair cell. From a colony of rhizobia seemingly trapped within the folds of the root hair, there develops a narrow tubular structure known as the infection thread. At the core of the infection thread are rhizobial cells in linear files. Surrounding the bacterial colony is a tubular plant wall. This wall is itself surrounded by a tubular plant plasma membrane, which at all times and locations separates the bacteria from the plant cytoplasm through which the infection thread develops. At the growing tip of the infection thread, one end of the leading bacterium is in close proximity to the plant membrane. Everywhere else the bacterial colony is surrounded by the plant wall. Fig. 4 shows infection thread development in *M. truncatula* root hairs.

Growth of the infection thread is thought to derive basically from two influencing factors: bacterial growth and growth of the surrounding plant membrane. According to this idea, bacterial cell fission and enlargement provide the basic driving force for the process. Observations indicate that the bacteria divide only near the growing tip of the infection thread, whereas the rest of the bacterial colony is enmeshed in a matrix of polymers deriving from both the plant and the bacteria. This enmeshed portion of the colony provides a solid anchor for the push from the dividing bacterial cells. As the bacterial colony pushes into a plant cell, the plant plasma membrane invaginates to surround the bacteria. The narrow tubular nature of its subsequent growth hypothetically arises from membrane vesicles in the plant cytosol fusing only at the tip of the infection thread membrane. Not only membrane lipids and proteins, but also the material and enzyme machinery for extending the thread wall inside the growing membrane are delivered by these vesicles. The shape and dimensions achieved in synthesizing this tubular wall are the third, and the permanent, influencing factor on the size and orientation of the infection thread.

Formation of the Bacteroid Zone and Nodule Maturation

The infection thread branches and spreads the infection in all three dimensions. As the infection thread penetrates deeper into the layers of cells in the developing nodule, the last stage of infection begins: bacterial release into certain plant cells that become filled with rhizobia. During this endocytosis at the unwalled tips of infection threads (or from enlarged unwalled areas known as infection droplets) the bacteria get surrounded with plasma membrane from the plant. The bacteria differentiate into nitrogen-fixing cells known as bacteroids, and the surrounding plant membrane becomes the specialized peribacteroid membrane. The

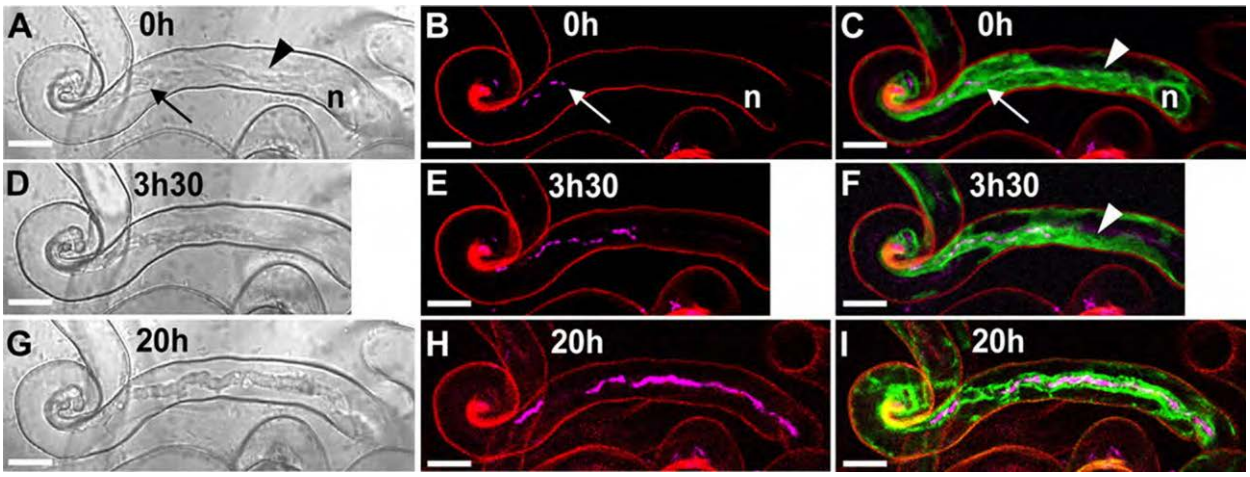

Fig. 4 Cytoarchitecture associated with IT development in *M. truncatula* root hairs. Three successive stages of IT growth in a root hair of the supernodulating *M. truncatula* mutant sunn expressing the 35S-GFP-HDEL transgene (4 d postinoculation). Bright-field images are shown on the left (A, D, and G). The corresponding confocal images (z axis projections of serial optical sections) showing the cCFP fluorescence of the rhizobia (magenta) are presented in B, E, and H, and combined with the ERtargeted GFP-HDEL (green) in C, F, and I. Cell wall autofluorescence in the confocal images is in red. A to C, At this early stage of IT development, there are only a very few bacteria in the thread, and several gaps are clearly visible within the file (B). The broad ER-rich cytoplasmic bridge (A and C, arrowheads) connecting the nucleus (n) with the elongating IT (arrow) is characteristic of actively growing ITs (C). Note that the IT tip is embedded in the dense, ER-rich cytoplasm and therefore difficult to visualize in the bright-field image. D to F, 3.5 h later, the IT has progressed within the root hair (D), and the rhizobia multiplied within the thread (E). Gaps are again visible within the single file of bacteria. The more mature section of the IT is surrounded by a layer of cytoplasm, whereas the newly synthesized region is embedded in the cytoplasmic bridge (F, arrowhead). Note that the root hair nucleus has moved down the shaft of the root hair and is no longer visible in the image frame. G to I, 20 h later, the IT has grown further down the hair toward the base of the cell. The bright-field image (G) shows the clear outline and uneven surface of the typical mature IT, and the confocal image (I) reveals that this fully differentiated IT is still surrounded by a thin layer of ER-labeled cytoplasm. Thicker stretches of the colonizing bacterial file (H) suggest that there is a doubling in certain regions (H) and, even at this late stage, bacteria-free gaps are still present along the file. Bars=10 mm. Copyright © 2008 American Society of Plant Biologists.

resulting cellular units consisting of bacteroids and the surrounding peribacteroid membranes are often termed symbiosomes. Depending on the host, a symbiosome contains one or several bacteroids. Up to 10,000 bacteroids are densely packed within one infected cell.

Bacteroids are generally rod-shaped. However, they may take on different shapes as determined by the plant host. Bradyrhizobial bacteroids in peanut nodules are spherical, whereas the same strain on cowpea forms rod-shaped bacteroids. Clover and pea bacteroids have pleomorphic or club shape, and they are very sensitive to osmotic stress. It is this type of bacteroid that has led to the erroneous generalization that bacteroids are terminal cell forms incapable of reproduction. On the contrary, bacteroids from many plants very efficiently form colonies on appropriate agar media.

In the nodules of the Phaseoleae, Fabeae, and Trifolieae, not all of the cells in the infected region of the maturing nodule are filled with bacteria. The others, the uninfected cells, differentiate to carry out steps in the nitrogen assimilation pathway that are downstream of the initial steps carried out within the infected cells. The bacteria-filled cells assume a distinctly cooperative metabolism and chemical environment that allows nitrogen fixation in the bacteria, as well as assimilation of the fixed nitrogen transported from the bacteria on the plant side. This physiology is outlined in the section titled 'Physiology of rhizobial symbiotic nitrogen fixation' below.

Integration and Regulation of Nodule Development

Rhizobial mutants defective in infection have revealed that nodule differentiation depends on infection. The degree to which this is true varies from one host to another. Some hosts (e.g., pea) form almost no nodule tissue unless infection is sustained beyond the root hair. Others (e.g., alfalfa or bean) can undergo organogenesis in response to noninfective rhizobial mutants, but exhibit anatomy and cytological differentiation that are quite distinct or incomplete compared to normal nodule development. Researchers working with some of these plants (e.g., bean and Lotus spp.) refer to the resulting structures as pseudonodules to emphasize the underdevelopment and deviation from normal development. Although pseudonodules result from infections on bean that do not progress into cell layers below the root hair, almost normal anatomy results if infection stops only a few cell layers further, well short of the stage at which bacteria are released from infection threads.

Nodule development is controlled by the plant according to at least two factors. One factor is whether the legume senses nitrogen limitation. Both development of nodules and feeding of the rhizobia to allow nitrogen fixation are energy-intensive processes. Perhaps because of the energy cost, the plant does not enter into symbiosis if soil has an excess of fixed nitrogen. Plants also repress nodule formation on some root sites according to whether other nodules are already forming nearby on the root. Nodule development generally initiates only in immature portions of the root where root hairs are just emerging. Nodules developing in older portions of the root will suppress nodule formation in younger portions. This is sometimes called autoregulation of nodule development. Autoregulation leads to optimal spacing of nodules that prevents wasteful unchecked nodule formation, but at a certain distance allows further nodulation as the plant grows and its need for nitrogen increases.

Deviations From the Foregoing Model

Infection Not in Root Hairs; Crack Entry

Some legumes do not have root hairs but still nodulate. Even those that do form root hairs may not have infection via root hairs. One alternate mechanism is that rhizobia enter at cracks in the root epidermis at the base of lateral root emergence. This process is sometimes referred to as crack entry. On certain legumes, the bacteria enter at other types of wounds, cracks, or, on some plants, by penetration between the cells of initially intact epidermal surfaces. *Azorhizobium caulinodans* ORS5721 is a microsymbiont of the water tolerant tropical legume *Sesbania rostrata* which forms N_2 fixing nodules on the roots and stems of the host legume via crack entry. These root nodules are formed at the curled root hair under well-aerated conditions whereas they occur at the base of lateral roots under hydroponic conditions. During crack entry, bacteria proliferate either in the epidermal fissures at the lateral root base or at the adventitious root primordia on the stems. Cortical infection pockets are formed by Nod factor-dependent local cell death induction and subsequent colonization of bacteria. From the infection pockets, infection threads guide bacteria towards the cells in nodule primordia for symbiotic uptake.

No Symbiosomes; Fixation Threads

The formation of symbiosomes represents a key step in the evolution of legume nodule symbiosis, as symbiosomes facilitate metabolite exchange between the two symbionts. Symbiosome formation does not occur in nodules formed on legume species that form a primitive symbiosis (e.g., *Andira* spp., many species belonging to the *Fabaceae* subfamily *Caesalpinoideae*) and *Parasponia* spp. (the only nonlegume species that can establish a symbiosis with rhizobia). *Rhizobium* bacteria in these species are not released into the nodule host cells but remain in infection threads, called fixation threads, which are enclosed by a cell-wall-like structure. Rhizobia fixes atmospheric nitrogen within these fixation threads. As nodules mature, the infection threads enlarge greatly within plant cells inside the nodule cortex, allowing bacterial proliferation inside this engorged thread. In this state, nitrogen fixation

occurs within the bacteria, and the fixed nitrogen presumably is transferred into the surrounding cytoplasm. Such structures are known as fixation threads.

Infections Between Cell Walls, Not as Threads That Penetrate Plant Cells

In some legumes, infection threads that travel through plant cells are not observed. Instead, the infection is channeled between plant cells until the point at which bacteria enter cells in large numbers within either symbiosomes or fixation threads. Some crack-entry infections are of this type; but, on some plants, infections may start between cells and later develop true infection threads. In perhaps 25% of all nodulating legumes, infections are not observed in root hairs, and true infection threads do not develop.

All Plant Cells in the Central Infected Zone Fill With Bacteroids

In the paradigm above, it is considered very important for nitrogen assimilation by the plant that development give rise to specialized uninfected cells in the central, nitrogen-fixing zone of the nodule. Such cells are thought to channel the nitrogen-containing carbon compounds to the vasculature so that the nitrogen can be transported to the shoot. In plants that use ureide compounds for this purpose (soybeans, beans, and cowpea), the final steps of synthesizing the transport compounds occur in these specialized uninfected cells. However, this division of labor is not found uniformly in all legumes; there are tribes of legumes in which all the cells in the central nitrogen-fixing zone are infected.

Nod Factor Not Needed

Existence of alternative Nod factor (NF)-independent symbiosis between legumes and rhizobia was first recognized in two *Aeschynomene* species (*A. indica* and *A. evenia)*, a genus in the Papilionoideae nodulated by photosynthetic bradyrhizobia (BTAi1 and ORS278) deficient in the canonical *nodABC* genes. Another Bradyrhizobial strain that nodulates these two species does have nod genes. However, mutation of its nod genes does not prevent nodulation of these plants, whereas nod mutations do prevent its nodulation of certain other species of Aeschynomene. These latter species cannot be nodulated by strains BTAi1 and ORS278. It is very interesting that these strains lack identifiable nod genes, but thus far the lack of this requirement in these particular symbioses cannot be correlated with any marked difference in the nodulation process. Aeschynomene species support infection by crack entry, without true infection threads, but rhizobial nod mutants on other hosts with these characteristics have failed to nodulate. The fact that Nod factors are not involved in the *Aeschynomene-Bradyrhizobium* spp. interaction suggests existence of alternative molecular dialoguesin the legume family. Recent research indicated that CCaMK (*Ca^{2+}/Calmodulin-Dependent Kinase)*, SYMRK (*Symbiosis Receptor Kinase)* and HK1 (Histidine Kinase 1) are obligatory for efficient nodulation in *Aeschynomene evenia.* CCaMK and SYMRK are recruited in Nod factor-independent symbiosis and hence conserved in all vascular plant endosymbioses described so far.

Stem Nodules

Certain water-tolerant legumes such as *Aeschynomene* and *Sesbania* (Fig. 2) form nodules on stems and on roots. The processes of nodule formation on the stems in these two plants exhibit many differences, but in both cases infections start where adventitious roots emerge from the stem, in a variation of the theme of crack entry. The two exceptional *Aeschynomene* species (*A. indica* and *A. evenia)* do not require *nod* genes of the rhizobia for either stem or root nodulation. However, *Sesbania rostrata* does not nodulate on stems or roots if its rhizobial partner lacks *nod* genes. Interestingly, under some conditions on this latter plant, root nodules also start by infections in curled root hairs. The same bacterium (*Azorhizobium caulinodans*) is accepted by the plant under all these variants of nodulation, although a wide variety of other rhizobia may also nodulate the roots.

Parasponia

Nodulation of the nonlegume genus *Parasponia* by rhizobia has many of the above 'deviant' properties. The rhizobia enters between epidermal cells and, as the bacteria penetrate deeper, true infection threads may form. Nitrogen fixation occurs in fixation threads. One additional property distinguishes *Parasponia* nodules from all known legume nodules. In the mature nodule, the infected cells, those that harbor the large fixation threads, are in the periphery (the nodule cortex) and vasculature develops in the center as it does in roots. This is the reverse of one property that appears to be true of all legume nodules: the infected nitrogen-fixing cells are central, with the vasculature being peripheral. In this respect, *Parasponia* nodule development is like that of the nodules of hosts of the actinorhizal bacterial genus *Frankia*. The presence of nod genes has been confirmed in rhizobia that infect *Parasponia* in all cases examined for this property. When the *nod* genes of one of these strains were mutated, it could not nodulate Parasponia or its legume hosts siratro and cowpea.

In all of these cases, it is the plant that determines these differences in nodule development; the differences assort with membership in different legume taxonomic groups. In contrast, a given rhizobial strain (usually a *Bradyrhizobium* strain) can nodulate and fix nitrogen on plants showing different modes of nodule development and infection, for example, crack-entry on

peanut and root hair infection on cowpea. Whether the set of bacterial genes and components required is any different on these different hosts is for the most part undetermined.

Although the issues of infection threads and fixation threads have been addressed in recent sampling of the Caesalpinioideae, the issues of root hair infection and the requirement for Nod factor have hardly been addressed at all. It is important to do so, to assess whether or not the evolution of Nod factor was central to the early evolution of the symbiosis. Many of the variations in nodule development appear to be exclusively controlled by the plant, but Nod factor obviously required evolution involving the bacteria. Similarly, it would be revealing to investigate the requirements for bacterial polysaccharides (see 'Rhizobial genes and components required in symbiosis' and 'Other properties of the rhizobia') in the symbioses of the Caesalpinioideae. In general, the theory of nodule development would benefit from investigating mutants of the rhizobia that form symbioses with properties that deviate from those of the paradigm described above.

Physiology of Rhizobial Symbiotic Nitrogen Fixation

Nitrogen fixation in legume nodules occurs within the bacteria inside specialized plant cells in the central region of mature nodules. Where they are carrying out nitrogen fixation, rhizobia are known as bacteroids, a term that has already been introduced in connection with nodule development. Functioning bacteroids must have the enzyme machinery for carrying out nitrogen fixation, encoded by *nif* genes, and the specialized catabolic and respiratory components that supply energy and electrons for the nitrogen fixation reaction, encoded by *fix* genes. In addition, they must have a compatible cell surface and transport systems for the exchange of metabolites that characterize the symbiosis. Once again, it should be emphasized that what is known is mainly from studies of the important agricultural legumes in the Papilionoideae.

The enzyme dinitrogenase catalyzes the ultimate reaction of nitrogen fixation:

$$N_2 + 8H^+ + 8 \text{ electrons} \rightarrow 2NH_3 + H_2$$

The electrons are delivered at high energy potential to dinitrogenase from internal reductants by a second enzyme known as dinitrogenase reductase in a reaction that requires the hydrolysis of two ATP to two ADP and two phosphates for each electron transferred. Dinitrogenase and dinitrogenase reductase traditionally are considered as a complex known as nitrogenase. Although other nitrogenases called alternative nitrogenases exist in nature, to the extent surveyed, all nitrogen fixation in rhizobia is by the classical, and most widespread, dinitrogenase containing molybdenum. The immediate source of electrons for this reaction in at least some rhizobia appears to be a ferredoxin. Although the exact connection of this ferredoxin to the rest of bacteroid metabolism is still not clear, electrons for nitrogen fixation are provided by oxidation of dicarboxylates from the plant, and the energy (ATP) is generated by aerobic respiration (Fig. 5).

Oxygen Concentration, Microaerobic Metabolism, and Bacteroid Regulation

As nodule organogenesis and infection reach the mature stage at which nitrogen fixation begins, a marked gradient in oxygen concentration is generated across the nodule. In the central region where nitrogen fixation occurs, the infected cells' free oxygen concentration is 5–30 n mol l^{-1}, whereas a typical saturating aerobic concentration is 250 μmol l^{-1}. One factor that causes this extreme decrease in oxygen is the high population of rhizobia in the central zone of the nodule. Rhizobia are obligate respirers and use oxygen in preference to other terminal electron acceptors. Another factor appears to be the creation of oxygen diffusion barriers as the nodule differentiates, documented to exist particularly in the determinate nodules of the Phaseoleae. The anatomy responsible for attenuating oxygen diffusion appears to be an intercellular layer in the apoplastic spaces in the periphery of the nodule cortex. Importantly, this layer appears to be subject to regulation that somehow changes the underlying physical attributes according to physiological cues. A third important factor is leghemoglobin, an oxygen-binding plant protein present in high concentrations in the infected cells of the central nodule zone. Up to 30% of the soluble plant protein of a nodule is leghemoglobin, which is responsible for the characteristic red color of active nodules. As an oxygen-binding protein, it buffers the free oxygen to a low concentration consistent with its dissociation constant. At the same time, like myoglobin in animal muscle cells, leghemoglobin facilitates oxygen diffusion at a high rate within the complete consumption of this oxygen by rhizobial respiration prevents poisoning of nitrogenase, both enzymes of which are irreversibly inhibited by oxygen.

Because the concentration of free oxygen in the bacteroid environment is low, aerobic respiration by bacteroids requires a cytochrome oxidase with very high affinity for oxygen, the cbb3 enzyme that is found in many Proteobacteria. It is encoded by genes named *fixNOPQ* in rhizobia. Another essential component in this symbiotic microaerobic respiratory chain is cytochrome bc1, whereas cytochrome bc1 is not required by rhizobia for aerobic growth ex planta in media and conditions commonly used.

The low oxygen concentration provides the cue for regulation of fix and nif genes in the nodule, mainly through its effects on activity and synthesis of central regulatory proteins such as NifA, FixLJ, and others. Indeed, unlike for bacteria that fix nitrogen nonsymbiotically, nitrogen status within bacteroids appears to be largely irrelevant in regulating symbiotic nitrogen fixation. The logic of symbiotic regulation is developmental. Nitrogenase and other enzyme systems for bacteroid metabolism are induced when

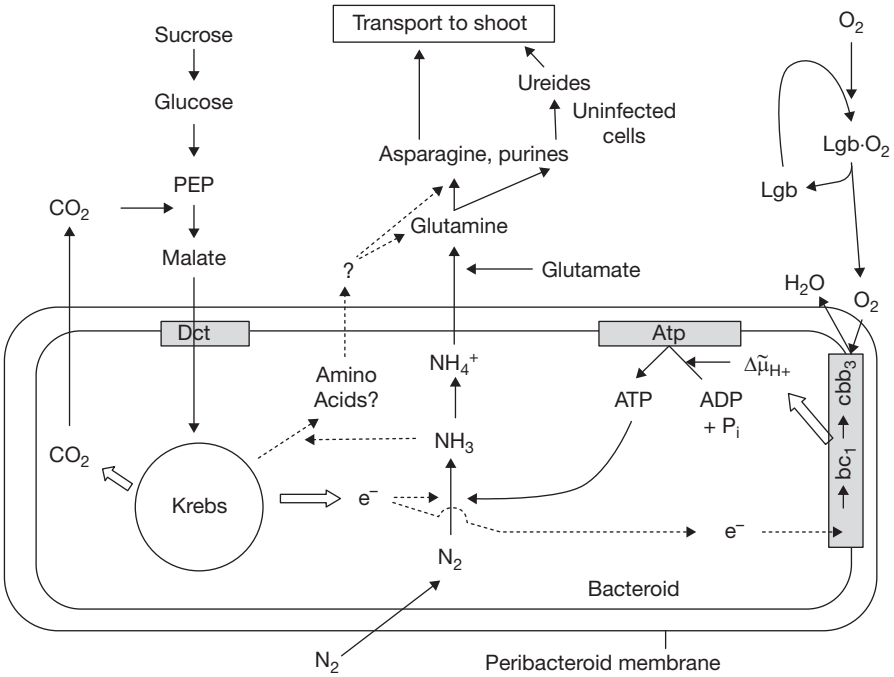

Fig. 5 Coordination of plant and bacteroid metabolism. The leftmost third of this diagram depicts carbon metabolism. Sucrose in the infected plant cell is catabolized to malate, which enters the bacteroid via the Dct transport system and is oxidized by the Krebs cycle and ancillary reactions to yield CO2 and 'electrons' (e−), mainly in the form of NADH. The middle third of the diagram depicts nitrogen fixation and assimilation. In some manner the electrons from catabolism reduce ferredoxins that in turn donate the electrons to nitrogenase for the reduction of N2 to NH3. Most of the fixed nitrogen may be excreted from the bacteroid as ammonium (NH4+), but some may be incorporated into organic compounds diverted from the Krebs cycle to form amino acids that are transported to the plant cytosol (question marks in the diagram). The plant assimilates the nitrogen into nitrogen-rich carbon molecules such as asparagine and ureides (allantoin and allantoic acid) for transport in the xylem to the shoot. ATP for nitrogen fixation is generated by microaerobic respiration (rightmost third of the diagram). It is presumed that electrons enter the electron transport chain mainly as NADH from the Krebs cycle that reduces ubiquinone, whose reduction of oxygen is mediated by the bc1 and cbb3 respiratory complexes. Leghemoglobin (Lgb) in the plant cytosol maintains low free oxygen concentrations but mediates high oxygen flux to the bacteroids, where the high affinity of the cbb3 oxidase pulls oxygen flux toward the bacteroid membrane. The electrochemical gradient of protons ($\Delta\mu H+$) generated by the electron transport chain drives ATP synthesis via the bacteroid ATP synthase (Atp). © 2008 Dale Noel. Published by Elsevier Inc. All rights reserved.

development and infection have provided the optimum environment and plant components for nitrogen fixation by the bacteria and assimilation by the plant. Oxygen concentration is a simple cue for signaling that the proper stage has been achieved.

Regulation by low oxygen implies that these bacteroid metabolic pathways actually might not be unique to bacteroids, and that they can be induced ex planta under low oxygen. In general, this is indeed the case. Because the degree of induction of a particular *nif* or *fix* gene cluster often is much higher in the nodule than under microaerobic conditions ex planta, it is possible that additional factors are at play in the nodule.

Bacteroid Carbon Metabolism, Host Contributions, and Nitrogen Assimilation

The plant supplies the bacteroids with carbon compounds, whose oxidation in the bacteria releases electrons for the reduction of nitrogen and makes possible the microaerobic respiration that generates ATP needed to drive nitrogen fixation. In as much as rhizobia generally can use glucose as carbon source, it might be supposed that the sucrose provided to roots from shoot photosynthesis would be simply broken down to glucose for the bacteroids. Such is not the case, however. Instead, glucose and fructose in the nodules are catabolized to phosphoenolpyruvate and then carboxylated to form dicarboxylic acids. Bacteroid proliferation and nitrogen fixation are highly dependent on the ability of bacteroids to utilize dicarboxylic acids of the Krebs cycle (malate and succinate). This was suspected first based on physiological studies that tested the availability of different compounds to the bacteroids and their ability to utilize these and other potential carbon sources. Confirmation came with the isolation of rhizobial mutants defective in transport of dicarboxylates (Dct mutants). Such mutants typically fail in nodule development at the point of bacterial release from infection threads; as a consequence, the central zone of the nodule where nitrogen fixation should occur is very sparsely infected by such mutants.

Another carbon compound that is relatively abundant inside legume nodules is inositol. However, rhizobial mutants unable to utilize inositol do not show the severe defects of Dct mutants. Another property of bacteroids, nonessential for nitrogen fixation but perhaps evolutionarily important, is the tendency to store excess carbon as polyhydroxybutyrate or glycogen or both. Production of

these storage compounds begins in the infection thread and may provide ready fuel for the energy-demanding period of rapid bacteroid proliferation.

Nitrogenase activity produces ammonium. It is generally accepted that a significant portion of the ammonium is transported directly out of the bacteroid. However, there are many indirect indications that some portion of the fixed nitrogen first is incorporated into a carbon compound within the bacteroid and transported as an amino acid into the plant cytoplasm (Fig. 5). In the mature nodule, the bacteroids are not growing or reproducing. Hence they have little need for fixed nitrogen. Therefore, whether transported as ammonium or as amino acid, almost all of the nitrogen fixed by the bacteroids is ultimately assimilated by the plant to provide for the biosynthesis of nitrogen compounds needed during plant growth. This assimilation results in glutamine as an early product within the plant cells harboring bacteroids (Fig. 5). Generally, the nitrogen is passed from glutamine to other metabolites, resulting in a few abundant N-rich compounds, such as asparagine and ureides, for transport of the nitrogen via the xylem.

Just as the plant controls whether nodules will form, it also can inhibit nitrogen fixation by the bacteroids in response to sudden influxes of fixed nitrogen (e.g., nitrate) in the root environment. It appears that the underlying mechanism starves the bacteria of carbon or oxygen or both. This inhibition is reversible. For at least a week the inhibited nodules and their bacteroids retain full capacity such that, once the exogenous fixed nitrogen is used up or removed, nitrogen fixation activity is rapidly restored to levels observed before inhibition.

Rhizobial Genes and Components Required in Symbiosis

Nodulation

Aside from recently discovered exceptions mentioned earlier, the capability to incite nodules on host legumes has been shown to depend on rhizobial Nod factors (Fig. 3), whose key role in triggering nodule development have been delineated already. The production of these glycolipids (lipooligochitosaccharides) is specified by rhizobial *nod*, *nol*, and *noe* genes (collectively referred to as nod genes). Genes *nodA*, *nodB*, and *nodC* specify the Nod factor basic structure – the N-acetylglucosamine oligomer (*nodC*) and the fatty acyl adduct (*nodA* and *nodB*) (Fig. 3). *nodIJ* genes are generally found in nod clusters as well; they are presumed to effect transport of the Nod factors out of the rhizobial cell.

Other nod genes provide strain-dependent features to Nod-factor structure (Fig. 3) that are presumed to contribute to host specificity of nodulation, efficiency of eliciting nodule initiation, and perhaps refinement of multiple roles of the Nod factor in promoting nodule development. For instance, different alleles of the *nodE* gene specify different unsaturated fatty acids attached as the lipid on the Nod factor. The *nodH* gene of *S. meliloti* specifies a sulfation of the Nod factor that is required for nodulation of *Medicago* species (including alfalfa). Mutation of the *nodL* gene of *R. leguminosarum* bv. *viciae* strains strongly decreases its nodulation of some of its normal hosts but has little effect on others.

Summed over all rhizobial strains, the number of genes associated with *nod* genes and shown (or suggested) to play an accessory role in nodulation is impressive, having run the entire alphabet on nod, nol, and almost on noe. As many as half of these genes may not affect Nod factor structure, but instead affect nodulation in other ways. The best characterized is *nodO*, which encodes a protein postulated to form cation-selective pores in the plant plasma membrane and thereby complement the plant response to Nod factors. It is dispensable for nodulation by wild-type *R. leguminosarum* bv. *viceae* in the laboratory, but is essential in strains that are mutated in *nodE*.

Transcription of the common and accessory *nod* genes is controlled by *nodD* genes. NodD proteins bind to nucleotide sequences called 'Nod boxes' upstream of the transcription start site of the various nod operons. In the presence of the flavonoids of the rhizobial host, NodD activates transcription. Certain other exuded plant compounds, aside from flavonoids, also have been shown to trigger nod induction.

The *nod* genes help to explain the host range of a rhizobial strain. The first factor is the combination of the rhizobial *nodD* and plant flavonoids; generally, they must be compatible to induce the other nod genes. The second factor is the Nod factor structure. A given plant generally responds only to a certain spectrum of structures. For example, modifications of Nod factor by previously mentioned genes *nodE*, *nodH*, and *nodL* can have important effects on the way in which legumes are nodulated by a given strain. In a given strain a mixture of Nod-factor structures is generally observed. This mixture can be relatively simple (e.g., in *S. meliloti*) or extremely complex (e.g., in *Sinorhizobium* spp. NGR234, whose plethora of Nod factors contributes to its broad host range). Interestingly, it is often found that the best known host of a strain does not require what appears to be a distinctive modification.

Even though Nod factor is essential for nodule formation on almost all legumes studied, it is important to realize that additional factors can be required to elicit observable nodule tissue. For instance, pea roots form hardly any nodule tissue if an *R. leguminosarum* strain is incapable of exopolysaccharide production. Soybean's response to a *Bradyrhizobium japonicum* mutant lacking lipopolysaccharide (LPS) O-antigen is similarly restricted. In this respect, polysaccharides and perhaps other molecules besides the Nod factor are crucial in determining the host range.

Homologous *nod* genes are found in the rhizobia of all known taxa, including those in the beta Proteobacteria. According to phylogeny based on rRNA sequences, the two strains without nod genes are located deeply within an outlying branch of the Bradyrhizobia. Whether or not nod was central to the earliest legume nodulation in evolution, it is obviously a hugely successful invention as deduced from its near ubiquity among studied rhizobia. Yet it seems to be confined to rhizobia (i.e., to legume nodulation). The presumptive coevolution of Nod factors, plant receptors, and downstream plant responses are subjects currently

Table 2 Nod genes and their predicted function

Genes	*Predicted functions*
nodA	Acyltransferase, nodulation protein
nodB	Nodulation chitooligosaccharide deacetylase
nodC	N-acetylglucosaminyltransferase
nodD	Transcriptional regulator, nodulation protein
nodE	Nodulation beta keto acyl ACP synthase
nodF	Nodulation acyl carrier protein
nodG	Nodulation protein, 3-ketoacyl-(acyl carrier protein) reductase
nodH	Sulfotransferase
nodI	Nodulation ATP binding protein
nodJ	Membrane transport nodulation protein
nodL	Putative nodulation protein
nodM	Glutamine-fructose-6 phosphate trasnaminase nodulation protein
nodO	Calcium ion binding nodulation protein
nodP	Nodulation protein, sulfate adenylate transferase, subunit 2
nodQ	Nodulation protein, sulfate adenylate transferase, subunit 1
nodS	Methyltransferase involved in Nod factor biosynthesis, nodulation protein
nodT	Putative exported nodulation protein
nodX	Putative nodulation protein, probable sugar acetylase
nodZ	Nodulation protein, fucosyltransferase
nolK	GDP-L- Fucose synthetase
nolL	Acetyltransferase
nolO	Nodulation protein
nolR	Transcriptional regulator
nolT	Nodulation protein
nolU	Nodulation protein
nolV	Nodulation protein
nolW	Nodulation protein
nolX	Nodulation protein
noeA	Host specific nodulation protein
noeB	Host specific nodulation protein
noeC	Nodulation protein
noeK	Phosphomannomutase
noeL	GDP-D-Mannose dehydratse
nopC	Nodulation outer protein C
nopA	Nodulation outerprotein A

becoming amenable to research and may have implications for engineering other plants to enter into this type of symbiosis. Genes involved in the nodulation process with their predicted functions are enlisted in Table 2.

Infection

Infection ofcourse requires growing rhizobia capable of reproduction. Any mutation that prevents growth in the rhizosphere or infection thread will arrest infection. Bacterial auxotrophs may have problems on some symbiotic combinations but not on others. Presumably, this relates to whether the nutrients are available in sufficient quantities to the bacterium in the environment of the infection. It also may depend on the particular transport systems available and the pathways for scavenging biosynthetic end products, intermediates, and derivative compounds. On almost all hosts, rhizobial purine auxotrophs fail early in infection. Whether this arises from a special role for the purine biosynthesis pathway in symbiosis has not been established.

In most symbioses that have been studied, a bacterial-surface polysaccharide, often more than one type, is required for successful completion of the infection process. Examples include LPS O-antigens, cyclic glucans, acidic exopolysaccharides (EPS), and 'K-type' capsular polysaccharides. None of these is individually required at any particular stage in all symbioses.

Over the years of studying the agricultural legumes, a generalization has emerged regarding whether EPS is required in symbiosis. The Phaseoleae do not seem to require them, whereas infection of the Trifolieae and Fabeae generally are very limited unless the bacterium is capable of producing its major acidic EPS. The structural basis for this requirement is best understood for the S. meliloti–alfalfa symbiosis. The main EPS of S. meliloti is known as succinoglycan, the bulk of which is produced by the bacterium ex planta as long polymers of a repeat unit composed of eight sugars modified on some residues by acetyl, pyruvyl, and succinyl groups. However, in symbiosis, the active form is believed to be the succinylated oligomers (monomers, dimers, and trimers) of the octasaccharide repeat unit. Perhaps it acts as a diffusible signal compound but its exact role is unknown. One clue might be that high production of either a different EPS (galactoglycan) or a K-type capsular polysaccharide can substitute for succinoglycan on some

hosts of S. meliloti. Many investigators speculate that EPS and other polysaccharides somehow suppress host-defense responses that otherwise would arrest rhizobial infection.

On legumes in all of these tribes, *Rhizobium* and *Bradyrhizobium* symbionts are required to produce LPS O-antigen. On hosts in the Trifolieae and Fabeae, this requirement occurs late in the infection process, whereas on the Phaseoleae the requirement comes about very early. O-antigen-containing LPS from *A. caulinodans* is proposed to act as a diffusible signal in nodule development on Sesbania. In several of the symbioses, the LPS has been shown to undergo changes during the interaction with the plant. In some cases, the same plant compounds that trigger nod induction also trigger changes in LPS. *Sinorhizobium fredii* strains may undergo the greatest change. Strain NGR234 produces an entirely new O-antigen in response to a nod inducer. As proposed for EPS, LPS O-antigens possibly suppress host defenses. The phenotypes of deficient mutants suggest that they also may serve as ligands for plant receptors in sustaining infection and/or for endocytotic release at the end of infection.

There has been limited study of the requirement for these polysaccharides on hosts highly diverged from the agricultural Papilionoideae legumes. Requirement of both LPS O-antigen and EPS in the infection of some legumes in the Mimosoideae have been inferred from singular mutants of certain rhizobial symbionts.

Nitrogen Fixation and Oxygen-Responsive Regulation

Rhizobial *nif* genes are those that are homologous to genes required for synthesis and function of nitrogenase in nonsymbiotic bacteria. For instance, *nifKD* genes encode the protein subunits of dinitrogenase, and *nifH* encodes the polypeptide of nitrogenase reductase. *nifBEN* genes specify the proteins needed to produce the iron–molybdenum cofactor of dinitrogenase. In addition, as in all Proteobacterial nitrogen-fixing bacteria, the regulatory gene *nifA* is invariably present.

fix genes are additional genes needed for symbiotic nitrogen fixation. For instance, the cbb3 cytochrome oxidase that has high affinity for oxygen is encoded by genes *fixNOQP*. This operon is generally followed on the DNA by the *fixGHIS* operon, whose function has not been established in detail but is believed to be in the transport of copper and perhaps its insertion into the cbb3 enzyme. Other fix genes generally present are *fixABCX*, whose function still has not been established. One or more of the regulatory genes *fixLJ* and *fixK* usually are present as well.

Although usually not termed *fix* genes, the *dct* genes fit the definition of *fix* genes noted above. They are needed for uptake of most of the carbon supplied by the plant, which is in the form of Krebs-cycle dicarboxylates, mainly succinate and malate (Fig. 5). All rhizobia in which they have been mutated become deficient in nitrogen fixation (as well as bacteroid proliferation). Also of utmost importance are the genes for the enzymes of the Krebs cycle and associated reactions needed to catabolize the carbon supplied by the plant. Interestingly, in certain rhizobia, some of the enzymes of the Krebs cycle can be eliminated by mutation without severely affecting nitrogen fixation. One explanation is that pathway shunts can bypass a given step(s). Even though the enzyme for this step is normally present and active, this metabolic plasticity compensates if the activity is lost. Such plasticity may have evolved to allow rapid adaptation to changing conditions of carbon and oxygen supply. Another explanation is that not all steps in the Krebs cycle are needed if ammonium is incorporated into one of the metabolites and then exported into the plant cytoplasm (Fig. 5).

It has been stated already that symbiotic nitrogen fixation is regulated according to oxygen concentration. In all systems the NifA protein is a positive regulator that activates the transcription of other *nif* genes. In classical rhizobia this protein is inhibited directly by oxygen. Transcription of the *nifA* gene also is controlled according to oxygen concentration. In *Sinorhizobium*, this control is exerted via proteins FixL and FixJ. FixL is an oxygen sensor that under low oxygen activates FixJ, which is a transcriptional activator of *nifA* and certain other genes. In *Bradyrhizobium*, the corresponding control is via RegS and RegR, which respond to respiratory electron flow to activate transcription of *nifA*. In both *Bradyrhizobium* and *Sinorhizobium*, the other fix genes are controlled by FixK protein; transcription of *fixK* is controlled according to oxygen concentration, through regulation by FixL and FixJ (Table 3).

Rhizobial Genomes and Genetics

Genomes

The total nucleotide sequences of multiple rhizobial genomes have been determined till date along with their complete plasmid profile. The known whole genomes represent four genera of the 'classical' rhizobia: Rhizobium (*R. leguminosarum* 3841 and *R. etli* CFN42), *Sinorhizobium* (*S. meliloti* 1021), *Mesorhizobium* (*M. loti* MAFF303099), and *Bradyrhizobium* (*B. japonicum* 110 and the photosynthetic Bradyrhizobial strains BTAi1 and ORS278 mentioned earlier). All of these rhizobial strains have larger than average bacterial genomes, ranging from 6.5 to 9.1 Mb (1 Mb=106 nucleotides). Genome information of the members of four representative rhizobial genera are depicted in Table 4.

Plasmids

Plasmids are bacterial DNA molecules that are smaller than the chromosome(s). Generally, they are dispensable for bacterial growth at least under some conditions. Instead, they typically encode properties that allow growth or otherwise give the bacteria selective advantages under niche-specific conditions. In some rhizobia (e.g., *Rhizobium* and *Sinorhizobium* species) *nod*, *nif*, and *fix* genes are

Table 3 Nif and fix genes and their predicted functions

Genes	*Predicted function*
nifA	Nif- specific regulatory protein, transcriptional activator
nifB	Fe - Mo cofactor biosynthesis protein
nifD	Nitrogenase molybdenum -iron protein, alpha chain
nifE	Nitrogenase molybdenum cofactor synthesis protein
nifH	Nitrogenase protein
nifK	Nitrogenase molybdenum - iron protein, beta chain
nifN	Nitrogenase Fe - Momolybdenum cofactor biosynthesis protein
nifQ	Nitrogen fixation protein, molybdenum - iron binding
nifR	Nitrogen regulation protein
nifS	Nitrogenase metallocluster biosynthesis protein, Cysteine desulfurase
nifU	Iron - sulphur cluster scaffold protein
nifW	Nitrogenase stabilizer
nifV	Homocitrate synthase
nifX	Iron - molybdenum cluster binding protein protein
nifZ	Nitrogen fixation protein
fixA	Nitrogen fixation protein, electron transfer flavo protein beta chain
fixB	Nitrogen fixation protein, electron transfer flavoprotein alpha chain
fixC	Nitrogen fixation protein, oxidoreductase
fixG	Iron sulphur binding domain
fixH	Nitrogen fixation cation transport protein
fixI	Transmembrane copper transport ATPase protein
fixJ	Two component nitrogen fixation regulatory protein
fixK	Ferrodoxin like protein, FNR/CRP transcriptional regulator
fixL	Two component nitrogen fixation oxygen regulated sensory histidine kinase
fixN	Cytochrome c oxidase
fixO	Cytochrome c oxidase
fixP	Cytochrome c oxidase membrane anchored subunit
fixQ	Cbb3 Cytochrome oxidase
fixR	Short chain dehydrogenase, oxidoreductase
fixS	Nitrogen fixation protein
fixU	Nitrogen fixation protein
fixX	Ferredoxin like protein

Table 4 Genome statistics of representative rhizobia

Rhizobia	*M. loti MAFF303099*	*B. japonicum USDA110*	*S. meliloti 1021*	*R. etli CFN42*	*R. leg bv. viviae 3841*	*Bradyrhizobium sp. BTAi1*	*Bradyrhizobium sp. ORS278*
Total size (bp)	7596,297	9105,828	6691,694	6530,229	7751,309	8493,513	7456,587
Total genes	7343	8374	6287	6022	7342	7694	6783
Chromosome size (bp)	7036,071	9105,828	3654,135	4381,608	5057,142	8264,687	7456,587
Chromosome genes	6814	8374	3425	4094	4797	7466	6783
Plasmid size (bp)	351,911 2,08,315	–	1683,333 1354,226	642,517 505,334 371,225 250,948 194,229 184,338	870,021 684,202 488,135 352,782 151,546 147,463	228,826	–
Plasmid genes	320 209	–	1569 1293	567 455 336 232 175 163	790 644 470 312 187 142	228	–

found on a plasmid termed the symbiotic plasmid. This plasmid is distinct in each strain, having a different size and different additional genes that have no apparent role in symbiosis. These other genes generally outnumber the genes devoted to the symbiosis. In *Bradyrhizobium* and *Mesorhizobium* species, the *nod*, *nif*, and *fix* genes are found on the chromosome.

In some of the rhizobia, a significant portion of the genome is contained on plasmids. Plasmids larger than 1 Mb arbitrarily are termed megaplasmids. *S. meliloti* has two of them, which account for almost half of its 6.7 Mb genome. Plasmid A is 1.35 Mb and plasmid B is 1.68 Mb. These plasmids are larger than the entire genomes of many obligately symbiotic bacteria and even some free-living bacteria. Plasmid A is the typical symbiotic plasmid with *nod*, *nif* and *fix* genes, whereas plasmid B has genes for exopolysaccharides required in the symbioses of this species.

In the two *Rhizobium* strains whose entire genomic nucleotide sequences have been determined, the plasmids are smaller, but there are more of them. Strain *R. leguminosarum* 3841 has 6 plasmids which account for about 40% of the 7.8 Mb genome. Strain *R. etli* CFN42 also has six plasmids that account for about one third of the 6.5 Mb total genome. In both of these strains, one of the plasmids is a typical symbiotic plasmid carrying the *nod*, *nif*, and *fix* genes, as well as genes not required in the symbiosis.

Duplicated Genes

The classical rhizobia have a high incidence of duplicated genes, genes that are not merely homologous, but sometimes have near identity of nucleotide sequence. This creates a problem in analyzing gene function by mutation. Often mutating a gene has no effect because there is an isofunctional duplicated gene (paralog) compensating for its loss. Potentially, a new function can evolve in one of the paralogs of each set of duplicated genes. Therefore, rhizobia appear to have a very rich potential for evolution of new functions. Whether they are atypical in this respect among bacteria is not clear, but they were among the first bacteria in which a high rate of duplications was recognized and studied in detail.

Mobile Genetic Elements

One explanation for the diversity of the rhizobia is that genes for symbiosis can be transferred from bacteria of one species to those of another species. Studies of genetic sequences strongly support this notion. Emerging examples are the rhizobia belonging to the beta Proteobacteria. Studies so far indicate that their nod and fix genes have been acquired more than once in evolution and from different sources within the rhizobia of the alpha Proteobacteria. Although sequence divergence indicates that these events happened long ago, studies among the rhizobia of the alpha Proteobacteria document that genetic transfers of clusters of symbiotic genes are occurring at readily detectable rates all the time in the soil. Most studies have documented transfers among strains in the same species, but transfers between genera have been reported as well. With the modern techniques of microbial ecology, it is possible to detect and isolate 'nonsymbiotic' rhizobia, rhizobia whose genomes belong to a particular rhizobial species, but lack *nod*, *nif* and *fix* genes. Moreover, it has been possible to demonstrate that within a given period of time, such strains introduced into the soil had picked up symbiotic gene clusters from rhizobia that were indigenous or also introduced into the soil at the same or different times. Such transfers have been demonstrated in the laboratory as well.

In retrospect, these observations are not at all surprising. Rhizobia harbor types of mobile genetic elements that are known to foster such transfers in other bacteria, and, indeed, they provide some of the best case studies for such genetic mobility in nature. Although the emphasis in rhizobia is almost always on symbiotic properties, such exchanges between rhizobia and other bacteria are not limited to symbiotic gene clusters. It is very common for bacteria to acquire in this way genes that allow growth in special niches that are not always encountered by the species. The spread of pathogenesis, antibiotic resistance, and utilization of aromatic carbon compounds are notable examples found in other bacteria. The common transfer agents and mechanisms include conjugative plasmids, conjugative symbiosis islands, specialized transduction of genes on proviruses, and insertion elements that border genetic islands and translocate them to new DNA. Studies of the rhizobia have demonstrated at least three of these mechanisms: mobilization via plasmids, symbiosis islands, and translocations mediated by insertion elements. In the genera containing symbiotic gene clusters on plasmids (e.g., *Rhizobium, Ensifer (Sinorhizobium)*), plasmid-mediated conjugation appears to be a main mechanism of the spread of symbiotic determinants and changes in host specificity. Even when the symbiotic plasmid is not a conjugative plasmid, it is mobilized by another indigenous plasmid that is conjugative. Examples include the changes in biovars among *R. leguminosarum* mentioned in the section titled 'Rhizobial host ranges'. In *Bradyrhizobium* and *Mesorhizobium*, symbiotic genes are instead found on the chromosome in genetic clusters known as symbiotic islands. They can be recognized in *Bradyrhizobium* strains but in most cases the conjugative transfer elements that moved them into the strains have become inactive during evolution. However, in *Mesorhizobium loti* the transfer functions are still active. This species has been a model for demonstrating the transfer of symbiotic islands in both the field and the lab.

Genetic Analysis of Rhizobia

Rhizobia have many natural agents for achieving genetic change. They have the conjugative elements noted above, and, like other forms of life, rhizobia are afflicted by viruses and insertion elements. These agents have been very important in shaping the genomes of present-day strains, but, except for a few viruses, they have not been exploited as tools for genetic analysis. Instead most such tools

were developed for use in other Proteobacteria and then adapted for use in rhizobia. One example has been the extremely useful transposon Tn5, which is the workhorse mutagen for obtaining null mutations in the initial study of a particular rhizobial property. In rhizobia, Tn5 inserts itself in a relatively random manner at sites throughout the genome, generally at only one location per mutant strain. The resulting insertions are very stable (rarely revert), and, very importantly, Tn5 and its man-made derivatives carry various antibiotic-resistance genes that tag genes for selecting a mutation with no other selectable phenotype in matings and cloning. Broad- and narrow-host conjugative plasmids isolated from other bacteria also have been key genetic tools. They work as vectors to shuttle DNA between *Escherichia coli* (or sometimes other bacteria) and rhizobia. The narrow-host plasmids can be transferred to rhizobia, but do not replicate in them. They serve to introduce transposons or to force molecular recombination between transferred rhizobial DNA and resident DNA in the recipient rhizobial genome. This has allowed replacement of resident wild-type DNA with mutant alleles constructed in vitro. The broad-host plasmids do replicate in rhizobia and allow multiple copies of introduced DNA to be maintained in the recipient rhizobia for testing genetic complementation. Such plasmids can be used to transfer DNA between rhizobia as well. In studies of some rhizobia, notably *S. meliloti* and *R. leguminosarum*, generalized transducing phage (viruses) have been developed for this same purpose.

Other Properties of the Rhizobia

Common Traits

Generalizations about rhizobia can be made, with the caveat that there will be exceptions among a group this diverse. All characterized rhizobia are Gram-negative rods that are catalase- and 'oxidase'-positive. They have flagellar motility and exhibit chemotaxis. All of the classical rhizobia can carry out hexose catabolism by the Entner–Duodoroff pathway. Gluconeogenesis, pentose phosphate and glyoxylate pathway do exists while Embden Meyerhof Parnas pathway seems to be virtually absent with *Bradyrhizobium japonicum* being an exception. In keeping with the catabolism needed in symbiosis, all have complete Krebs cycles and associated anaplerotic reactions so that organic acids can be used for growth.

Considered obligate respirers, all can use oxygen, and most also use at least nitrate as the terminal electron acceptor. Rhizobia grow well microaerobically. A key enzyme for this growth, the cbb3 cytochrome oxidase, is an example of a function encoded by fix genes important in symbiosis but also induced under microaerobic conditions that rhizobia may encounter in the soil (and fresh water environments) in the absence of the plant. The cbb3 oxidase is widely distributed among related Proteobacteria that are not known to form symbioses with eukaryotes, for instance, the purple phototrophs.

A point to be emphasized is that rhizobial populations persist indefinitely in the soil as part of the overall bacterial population, and they do so regardless of the presence of the host plant. Therefore, they have capabilities that allow growth in this environment. Like 'pseudomonad' soil bacteria, individual rhizobial strains that have been studied in the laboratory are extremely versatile heterotrophs, able to use a plethora of carbon sources, including sugars, organic acids, amino acids, and phenolics. Total genomic nucleotide sequences bear this out. The genomes are consistently large relative to the average bacterium and have a wealth of annotated genes devoted to diverse catabolic pathways and transmembrane transport systems. They utilize diverse nitrogen sources as well, including molecular nitrogen (mainly in symbiosis), ammonia, nitrate, nitrite, and amino acids. Aside from versatile heterotrophy, they all seem proficient at biosynthesis of amino acids, nucleotides, and other small molecules needed for growth. Certain vitamins, in contrast, greatly stimulate rhizobial growth. Hence, they can be grown in the laboratory on a wide variety of simple de fined minimal media with one carbon source, a nitrogen source, salts, and certain vitamins. They also do well on defined and undefined rich media. How well they do depends on the genus and the composition of the particular medium. In general, the classical rhizobia require a relatively high calcium concentration for growth.

Rhizobia are commonly obtained as clonal isolates from nodules by requiring colony formation on agar containing yeast extract and mannitol. As a link to older literature that categorized rhizobia according to growth rate, the 'fast growers' on this medium include the classical genera *Rhizobium* and *Sinorhizobium*. *Bradyrhizobium* strains are 'slow growers,' and *Mesorhizobium* strains are said to exhibit intermediate growth rates. If there are legume symbionts that do not grow on yeast extract–mannitol medium, they would have been missed in most studies because of the presumption that legume isolates will grow on this medium.

Polysaccharides

Historically, there has been great interest in the surface polysaccharides produced by the rhizobia, because of the likelihood that interactions between these bacteria and legume cells, as well as other biological entities, involve molecules on the rhizobial surfaces. However, the full spectrum of polysaccharides produced by any rhizobial strain has never been identified completely. Genome annotations indicate a staggering number of gene clusters apparently devoted to polysaccharides. Long before their genetics were known, certain polysaccharides, including many EPSs that are important in symbiosis, were studied biochemically because they were the most abundant of a particular class of polysaccharides. In other cases, a polysaccharide was first studied as a result of mutations that caused a deficiency in symbiosis, which was traced to a deficiency in that polysaccharide. Genes for LPS O-antigens, cyclic glucans, and some capsular polysaccharides have been discovered in this way. The functions of these polysaccharides, aside from requirements in symbiosis, have, for the most part, undergone little scrutiny. Hypothetical roles include protection against desiccation, environmental toxins, and plant defenses. They may help evade viruses, but there are cases where viruses have evolved to use them as receptors for the first step in infection. Most divisive among the proposed EPS functions is its role as a signaling

molecule triggering plant developmental response and as a determinant of host specificity. There is a strong proposition that production of a variety of symbiotically active polysaccharides permits rhizobial strains to adapt to changing environmental conditions as well as to interact efficiently with legumes.

Rhizobia, particularly in the genus *Rhizobium*, produce impressive amounts of exopolysaccharide on carbon-rich media such as yeast-mannitol. Within a few days after appearing on inverted agar plates containing this medium, colonies will drip copious slime onto the Petri dish cover even if stored at 4°C. Outside the symbiosis, EPSs are regulated according to exogenous carbon/nitrogen ratios; that is, excess carbon leads to high EPS production. Much of the total production occurs after the net growth of a culture or colony has ceased. The structures of several rhizobial EPSs have been determined. Each genus makes a structurally distinct major EPS, whereas within a genus, the most abundant EPS in all species may be very similar. Most common rhizobial EPS is succinoglycan (EPS I) produced by several *S. meliloti* strains composed of octasaccharide repeating units containing one galactose and seven glucose residues (in molar ratio 1:7). These are joined by β-1,3, β-1,4 and β-1,6 glycosidic linkages. Single repeating unit is adorned by acetyl, pyruvyl and succinyl groups. *S. meliloti* has the ability to produce another distinctive exopolysaccharide termed galactoglucan or EPS II. EPS II is synthesized only under phosphate starvation or when mutation in one of the regulatory genes, either *mucR* or *expR* occurs. It differs significantly in structure from EPS I as it is a polymer of disaccharide repeating unit composed of an acetylated glucose and one pyruvylated galactose coupled by α-1,3 and β-1,3 glycosidic bonds. EPS I and EPS II produced by the wild-type *S. meliloti* strain 1021 are symbiotically active exopolysaccharides. *Rhizobium leguminosarum* strains, have the same octasaccharide conserved repeating unit of EPS, composed of glucose, glucuronic acid and galactose (in the molar ratio of 5:2:1) even though they included into different biovars (i.e., *trifolii* and *viciae*) and nodulate different plant hosts. Nevertheless, certain strains of *R. leguminosarum* secrete EPS, the repeating unit of which differs in sugar content and the length of the side chain. In *R. trifolii* 4S, the EPS subunit with seven sugars lacking terminal galactose from the side chain has been described while in *Rhizobium leguminosarum* bv. *viciae* 248 the repeating unit with additional glucuronic acid in the side chain has been reported. Nevertheless, all the EPSs mentioned above are similar by means of possessing identical backbones and the same β-1,6 linked glucosyl residue starting the side chain. The pattern of non-sugar decoration with acetyl, pyruvyl and hydroxybutanoyl residues was found to be different for several *R. leguminosarum* strains.

exo/*exs* or *pss* genes governing exopolysaccharides biosynthesis form large clusters located either on the chromosome or on megaplasmids. Such proteins are transferases which assembles EPS repeating units, synthesizes nucleotide sugar precursors and enzymes involved in adding non sugar moieties to EPS. in addition to polymerization and export of the growing EPS chain onto the cell surface. EPS biosynthesis represents a multi-step process which depends on activity of a protein complex localized both in the inner and outer membrane. Specific glycosyltransferases function in transferring precursors and nucleotide diphospho-sugars to growing polysaccharide chain attached to an acceptor. Undecaprenol diphosphate was identified as a sugar acceptor in the case of most heteropolysaccharides. The repeating unit is formed at the inner leaflet of the cytoplasmic membrane while the individual repeating units gets polymerized at the periplasmic face of the inner membrane after they have been flipped across the IM by flippase. Polymerization is thought to be coupled to export of the growing polymer to the cell surface and engages Wzy-like polymerase and Wzc-like inner membrane-periplasmic auxiliary protein (MPA) with an ABC module which controls chain length of the growing heteropolymer. Outer membrane auxiliary protein (OMA) facilitates the growing polysaccharide to reach the cell surface by forming a channel in the outer membrane, thereby completing the process of translocation. Effective EPS translocation is the result of physical association of the proteins localized in both membranes.

EPS synthesis and regulation of EPS I produced by *S. meliloti* has been studied in detail while data concerning synthesis of acidic exopolysaccharide in *R. leguminosarum* are more patchy. EPS biosynthesis in *Rhizobium* is a highly complex process regulated at both transcriptional and posttranslational levels and influenced by multiple environmental conditions. Extensive genetic studies have resulted in identification of several regulators like *exoR*, *exoS*, *mucR*, *expR*, *syrM* and *exoD* mapped on the chromosome and *exoX*, *exsB* and *expG* genes on the second megaplasmid, pSymB regulating EPS I and EPS II synthesis in *S. meliloti*.

Notable Traits Found in Some Rhizobia

In view of the phylogenetic diversity of these bacteria, it is not surprising that various rhizobial species have capabilities that appear to be uncharacteristic of the majority. For instance, *A. caulinodans* and *Burkholderia phymatum* can grow ex planta with N2 fixation as the sole nitrogen source. Several other rhizobia have been shown to induce *nif* genes and even to produce functional nitrogenase, but indefinite growth dependent solely on nitrogen fixation has not been observed otherwise. The impact in nature of this nonsymbiotic nitrogen fixation is unclear, but is probably trivial compared with the fixation by these bacteria inside legume nodules.

Many rhizobia appear to harbor ribulose bisphosphate carboxylase and perhaps other enzymes of the Calvin cycle. A few have been demonstrated to grow autotrophically, particularly with hydrogen as the lithotrophic energy source. As mentioned above, there are also certain Bradyrhizobia that appear to be capable of phototrophy. *Methylobacterium nodulans* is described to be the only rhizobial species capable of growth on certain one-carbon compounds including methanol, although it is not clear how many legume nodule isolates have been tested for this property.

S. meliloti survives remarkably well under very dry conditions, an exceptional property that is exploited in using it as a commercial inoculant of alfalfa. This property may be shared among other *Sinorhizobium (Ensifer)* species. The survival is not due

to spore formation. None of the rhizobia is known to form spores, and other classical rhizobia used in agriculture must be carefully maintained at certain moisture levels to assure sufficient viability at the time of inoculation of designated crops. The basis of this property in *S. meliloti* is under active investigation in parallel with long-term projects aimed at increasing the desiccation tolerance of other agronomically important rhizobia.

Rhizobia are not particularly acid tolerant. *S. meliloti* is among the most acid-sensitive, showing growth inhibition at pH values below 6. Among the most acid tolerant are *Rhizobium tropici* and some Bradyrhizobia, which survive at pH 4.5.

Succinate Mediated Catabolite Repression

Rhizobia may be free living and symbiotic rhizobia in nature with the capability to utilize variety of carbon compounds thereby raising the aspect of catabolite repression control. Preliminary studies on R. meliloti have showed that it can utilize wide range of sugars and TCA cycle intermediates and the presence of succinate can inhibit sugar uptake and metabolism by limiting α-glucosidase, β-glucosidase and β-galactosidase activity. Glucose catabolism enzymes in *Rhizobium sp.* and *Bradyrhizobium sp.* are inducible in nature with succinate repressing the glucose catabolic enzymes in a cAMP independent manner. *S. meliloti* 2011 does not show diauxie when grown on lactose plus other sugars like maltose, sucrose or glucose which was however apparent when cells were grown on succinate along with sugars like lactose, maltose and cellobiose. Addition of succinate to the culture growing in lactose, abruptly decreased β-galactosidase and β-glucosidase activities. Presence of succinate influence the levels of ED and EMP pathway enzymes in many rhizobia. Hence, it can be concluded that by repressing the uptake and utilization of other non-preferential carbon sources, dicarboxylates like succinate may influence multiple metabolic functions of *Rhizobium sp.* This repression has been termed as Succinate mediated catabolite repression (SMCR).

Plant Growth Promotion

Multiple reports have shown various direct and indirect mechanisms of plant growth promotion by rhizobia with the ability to fix N being the most exploited one. Efficiency of N fixed by legumes varies, depending on the host genotype, efficiency of rhizobia harboring the host and soil and climatic factors. Reported quantum of N fixation ranged from 126 to 319 kg N ha^{-1} and 33–643 kg N ha^{-1} in groundnut and soybean respectively with 77–92 kg N ha^{-1} in pigeon pea, 25–100 kg N ha^{-1} in cowpea, 71–74 kg N ha^{-1} in green gram and 125–143 kg N ha^{-1} in black gram. In addition to legumes, symbiotic N contribution is also reported to benefit the cereal crops with a relative yield increase of 11%–353% in as maize, rice, wheat and sorghum Rhizobia can be indisputably used as inoculants for enhanced N fixation as studies have demonstrated their predominance in nodules for 5–15 years after initial inoculation. Such studies confirm that they are effective colonizers persisting in soil for many years even in the absence of their host.

Besides nitrogen, phosphorus (P) is an important plant growth limiting nutrient existing in both inorganic (bound, fixed, or labile) and organic (bound) forms in soil. Multiple factors limit their availability for plant growth. Soil microbes like *R. leguminosarum*, *R. meliloti*, *M. mediterraneum*, *Bradyrhizobium sp.* and *B. japonicum* are reported to help in P release to the plants. Organic acids secreted by such bacteria acts on inorganic phosphorous. For instance, 2-ketogluconic acid production with a phosphate-solubilizing ability has been identified in *R. leguminosarum* and *R. meliloti*. Importance of this P solubilizing capacity in enhancing plant growth by M. mediterraneum has been established in chickpea and barley plants.

Siderophores are water soluble and are of two types viz. extracellular and intracellular and forms stable complex with heavy metals such as Al, Cd, Cu etc. Rhizobial species, such as *R. meliloti*, *R. tropici*, *R. leguminosarum bv. viciae*, *R. leguminosarum bv. trifolii*, *R. leguminosarum bv. phaseoli*, *S. meliloti* and *Bradyrhizobium sp.* are known to produce siderophores.

Phytohormones are the substances stimulating plant growth at lower/equal to micromolar concentrations which include indole-3-acetic (IAA) acid (auxin), cytokinins, gibberellins and abscisic acid. IAA is the primary phytohormone which accelerates plant growth and development by improving root/shoot growth and seedling vigor. 80% of bacteria isolated from the rhizosphere can produce IAA with the salient ones being *A. caulinodans*, *B. japonicum*, *B. elkanii*, *M. loti*, *R. japonicum*, *R. leguminosarum*, *R. lupine*, *R. meliloti*, *R. phaseoli*, *R. trifolii* and *Sinorhizobium sp*. 60-fold increase in IAA was observed in the nodules of vetch roots, on inoculation of *R. leguminosarum bv. viciae*. Another phytohormone cytokinin stimulates plant cell division and in some instances root development and root hair formation as well. 90% of rhizospheric microorganisms are capable of releasing cytokinins. *Rhizobium* strains are also reported as the potent producers of cytokinins. Gibberellins, plant hormone responsible for stem elongation and leaf expansion is shown to be produced by *Rhizobium* and *S. meliloti* while *Rhizobium sp*. and *B. japonicum* had been reported to produce abscisic acid.

Rhizobia also has the ability to uptake ACC thereby converting it into a-ketobutyrate and NH3 which is used as carbon and nitrogen source. On inoculation of rhizobia producing ACC deaminase, plant ethylene levels get lowered resulting in longer roots and providing relief from stresses such as heavy metals, pathogens, drought, radiation and salinity. Rhizobial strains like *R. leguminosarum bv. viciae*, *R. japonicum*, *R. gallicum*, *B. japonicum*, *B. elkani*, *M. loti* and *S. meliloti* had been known to produce ACC deaminase.

Several rhizobial strains are reported to have biocontrol properties including competition for nutrients, production of antibiotics, siderophores and cell wall degrading enzymes including production of metabolites like HCN, phenazines, pyrrolnitrin,

viscoinamide and tensin. *R. leguminosarum bv. trifolii*, *R. leguminosarum bv. viciae*, *S. meliloti* and *B. japonicum* have been reported to secrete antibiotics and cell-wall degrading enzymes that can inhibit the phytopathogens.

Population Biology

The most basic issues in microbial ecology are to recognize the diversity and to enumerate the population under study. These are challenging objectives with all bacteria. With regard to rhizobia, one difficulty already mentioned is that rhizobia in the soil are in a dynamic genetic situation whereby they lose and pick up gene clusters that confer composite properties such as the one that we use to define them, the ability to incite nodules and fix nitrogen in association with legumes. Traditionally, soil rhizobia have been counted by using legume hosts as a means of selecting them from the notoriously diverse microbiota of the soil. Most probable numbers are calculated from the incidence of nodules arising from more and more dilute extracts of a particular sample of soil. This method is a reasonable measure of the most competitive rhizobia capable of nodulating the host that is used to trap them. It misses completely all those rhizobia that cannot do so, for example, rhizobia for other hosts and the aforementioned strains that have lost symbiotic capability. This is only one of many challenges in making absolute enumerations and enumerations relative to other bacteria (which themselves are subject to similar caveats). Therefore, the advent of new technologies, chiefly based on genetic probes, for identifying and enumerating bacteria in natural populations is revolutionizing the study of the ecology of rhizobia, just as it is of other bacteria. With these cautionary realities having been stated, it has been estimated through traditional sampling of soils that *Rhizobium*, *Sinorhizobium*, and *Bradyrhizobium* represent between 0.1% and 8% of the total population in bulk and rhizosphere soil. In view of the tremendous variety of bacteria that may inhabit the soil, even the lower of these numbers is significant. As might be expected, studies show that one of the most important factors determining the relative populations of rhizobia is whether a legume host has been cultivated in that soil. Generally, repeated cultivation of a particular legume will greatly enhance the populations of bacteria capable of nodulating it, relative to other rhizobia.

Therefore, rhizobia effectively compete with other bacteria in sustaining their soil populations. Also interesting in a basic sense, and very important to the inoculant industry, is that rhizobia of course compete with one another for the habitat of the legume nodule. In a given field setting, there are 'dominant' strains, strains that appear to inhabit the nodules of the particular host in higher numbers than other strains. An example is the so-called serogroup 123 of *B. japonicum* that competes well with inoculants and other indigenous strains for nodulation of soybean in the Midwestern region of the United States. The mechanisms involved in this important property are not yet elucidated. They are likely to be multiple and complex, involving not only innate differences in the early interactions with the host legume, but also coping with the particular and varying soil conditions, such as pH, viruses, antibiotics, predation, temperature, desiccation, and available nutrients.

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Rhizosphere☆

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Glossary

Biocontrol microorganisms Microorganisms that benefit the host plant by suppressing plant pathogenesis. They accomplish biocontrol by various mechanisms, for example, antibiosis, hyperparasitism, nutrient competition, and activation of induced systemic disease resistance in plants. Serves as an example indicating the benefits of enhanced plant growth due to PGPR bacteria (see below), or of microbial communities defeating plant pathogens.
Bioremediation Natural degradation of xenobiotic compounds that pollute the environment. In terrestrial habitats, bioremediation is enhanced in plant rhizospheres due to the increased availability of nutrients in this environment that fuel the microbial metabolism required to degrade the pollutant.
CMEIAS Center for Microbial Ecology Image Analysis System; advanced software tools of bioimage informatics designed to strengthen microscopy-based methods for understanding microbial ecology, especially the microbial colonization of roots.
Endophytes Microorganisms living within healthy plants, especially abundant in the interior of mature roots grown in soil but several can also colonize aerial parts.
Exudates Various root-derived, low molecular weight, carbon-rich, and energy-yielding compounds, for example, organic acids, released from the plant root into the surrounding environment where they enrich and are catabolized by microorganisms.
Plant growth-promotive rhizobacteria (PGPR) Root-colonizing bacteria that benefit the growth and development of the host plant by various means, including nitrogen fixation, phosphate solubilization, phytohormone production that enhances root development and increases nutrient acquisition efficiency, biological control of soil-borne root-infecting pathogens, and induction of systemic plant disease resistance.
Rhizoplane The root epidermal surface at the plant–soil interface that is colonized by rhizosphere microorganisms.
Rhizosphere The region of soil immediately influenced by living roots, where exudates are deposited to stimulate microbial metabolism and productivity.

Abbreviations

AHLs	*N*-acyl homoserine lactone
CLSM	Confocal laser scanning microscopy
CMEIAS	Center for Microbial Ecology Image Analysis System
Gfp	Green fluorescent protein
HHPR	Human health-promoting rhizobacteria
IFM	Immunofluorescence microscopy
PGPR	Plant growth-promoting rhizobacteria
Rfp	Red fluorescent protein
R:S	Rhizosphere-to-soil ratio
SIP	Stable isotope probing

Defining Statement

The rhizosphere includes living plant roots and closely associated soil where deposited exudates stimulate microbial activities that significantly influence plant health and disease, and therefore is immensely important to terrestrial life on Earth. Activities within the rhizosphere impact on the equilibria of microbial immobilization and mineralization of plant nutrients that

☆*Change History*: August 2016. Frank B. Dazzo improved the text throughout the article; updated Fig. 2; and added data for Table 2, new references, and a list of Relevant Websites. A. Garoutte introduced a description of metatranscriptomics applied to rhizosphere research. May 2018. Anton Hartmann revised and updated portions of the article and added new references accordingly.

This article is an update of F.B. Dazzo, S. Ganter, Rhizosphere, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 335–349.

profoundly influence their cycling in soil, on plant growth and development, and on the susceptibility and resistance of plants to infectious diseases.

Historical Aspects of the Rhizosphere Concept

In 1904, Lorenz Hiltner coined the term "rhizosphere", which he defined as the "soil compartment influenced by the root". His thorough investigations showed that microorganisms heavily populate the soil surrounding plant roots, the plant surface and root interior. Root exudates support different microbial communities, and plant roots and their associated microflora can significantly influence each other's activity and development. For instance, some of the microbes whose abundance and metabolic activity are stimulated in the rhizosphere are welcomed friends that can significantly promote the growth of the plant, whereas other uninvited guests are foes that capitalize on root exudates by proliferating in the rhizosphere where they harm the plant. Hiltner also reported clear microscopic evidences that healthy plant roots are colonized inside by bacterial cells. These endophytic bacteria had been first reported by the French botanist Galippe in 1887. In analogy to the "mycorrhiza" (fungal root symbionts), he called the root associated bacterial community "bacteriorhiza". In 1891, Hiltner and his academic teacher F. Nobbe had launched the first patent of an effective rhizobial inoculant for legumes, which they named "nitragin". Hiltner's key findings, as summarized by Hartmann et al. catalyzed the beginning of a greater than century-old era of rhizosphere microbiological research that enjoys an abundance of basic and applied scientific discoveries. Currently, plants and their associated microbes are recognized as metaorganisms or holobionts, in which roots and the rhizosphere are important organs to contact and explore the vast microbial reservoir of soils.

General Characteristics of the Rhizosphere

Nutrient Enrichment in the Rhizosphere

Most root-free soils are nutritionally oligotrophic, and therefore ~80% of the microbes there by and large persist on a low-calorie diet, with the major limitation to their productivity being the low availability of energy-yielding organic nutrients. The rhizosphere includes the root and surrounding soil influenced by living plant roots (Fig. 1). Rhizosphere soil contains significantly increased supplies of organic matter that include readily utilizable organic nutrients as soluble exudates, and also polymeric carbon substances like polysaccharides and mucus substances on the root surface. This nutritional enrichment results in more intense microbial metabolism and productivity in the rhizosphere than what is supported in bulk soil, with accompanying changes in respiration, gas exchange, nutrient and moisture availability. Plant roots can also be colonized by symbiotic fungi forming ecto- and endomycorrhizal structures. The "mycorrhizosphere" is also colonized by diverse bacterial communities – a part of it has mycorrhizal helper functions.

Measurements of the Rhizosphere Effect

Early studies on the microbial ecology of the rhizosphere measured the degree of enrichment with respect to culturable rhizosphere microorganisms and the various environmental factors that influence it. A rhizosphere-to-soil ratio (R:S) was introduced to define the magnitude of the rhizosphere effect. This metric is typically computed as the parameter (e.g., density or activity of microbes) measured in rhizosphere soil divided by the value of the same metric in nearby nonrhizosphere control soil uninfluenced by roots. Thus, root-associated enrichments would produce R:S values >1, whereas those parameters that are unaffected or suppressed by roots would have R:S values equal to or less than 1, respectively. Typical R:S values based on microbial culturability vary among plant species, soil horizon, and microbial physiological groupings, as indicated in Table 1. In contrast to A horizon soils enriched with organic matter, B horizon soils containing very low organic matter content supports microbial activity with typically higher R:S ratios. In addition, low carbon soils like dunes or desert sandy soils have extremely high R:S ratios. High R:S ratios for protozoa reflect the increased abundance of their bacterial prey in this environment, and this higher bacteriovory activity accounts for a significant microbial loop that accelerates mineral nutrient cycling in the rhizosphere, thereby benefiting plant nutrition overall. A high R:S ratio of the denitrifier functional guild reflects this environment's increased abundance of organic matter, lower pO_2

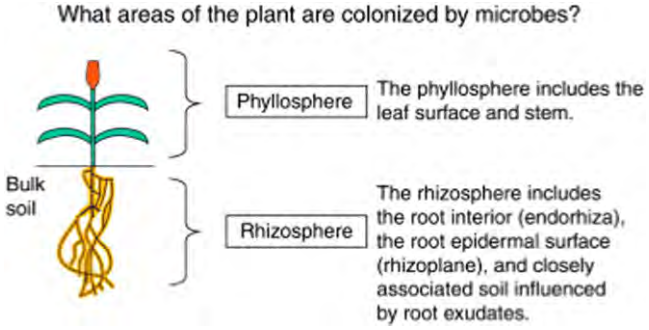

Fig. 1 Areas of plants colonized by microorganisms.

Table 1 Classical analysis data of the rhizosphere effect based on measurements of the influence of various plant species on the major microbial groups and functional guilds in rhizosphere soil[a]

Microbial group or functional guild	*Plant*	*Rhizosphere effect (R:S ratio)*[b]
Bacteria	Red clover	24
Bacteria	Maize	3
Bacteria	Yellow birch, A horizon	15.1
Bacteria	Yellow birch, B horizon	57.8
Bacteria	Mangles	120
Protozoa	Mangles	3–23
Filamentous fungi	Wheat	6
Microalgae	Wheat	11
Cellulose-degrading bacteria	Wheat	5
Nitrifying bacteria	Wheat	1
Ammonifying bacteria	Wheat	50–125
Denitrifying bacteria	Wheat	90–1260

[a]Data are based on viable plate counts and derived from studies by Katznelson and coworkers.
[b]The R:S is the metric that defines the magnitude of the rhizosphere effect on its associated microbial community and is typically computed as the value of the parameter measured (e.g., microbial abundance, diversity, or activity) in rhizosphere soil divided by its corresponding value in nearby nonrhizosphere control soil. Thus, root-associated enhancements would produce R:S values >1, whereas those parameters that are unaffected or suppressed by roots would have R:S values equal to or less than 1, respectively.

due to the higher biological oxygen demand that accompanies degradation of organic matter and depletion of oxygen by microbial aerobic respiration, and the utilization of NO_3^- (if available) as the microbes' second best choice of alternate electron acceptor to support ATP-yielding respiration.

Evidence for a lower in situ pO_2 in the rhizosphere is supported by manometric measurements of total oxygen uptake in rhizosphere versus nonrhizosphere soil that produce R:S values ranging from 2 to 5, depending on the plant species. This increased indicator of aerobic respiration reflects the combined influence of readily oxidizable nutrient enrichments and the large community of metabolically active, O_2-utilizing rhizosphere microorganisms.

Microbial Diversity and Nutritional Requirements in the Rhizosphere

Derived from neighboring soil, the rhizosphere harbors a vast diversity of prokaryotic and eukaryotic microorganisms, including bacteria, archaea, fungi, protozoa, green algae and diatoms as well as viruses of these host organisms. Most studies of rhizosphere microbial ecology focus on the bacterial and fungal (especially mycorrhizal) components, although some new research focuses on the involvement of archaea in soil, the occurrence and functionality of viral populations, pathogenic repression, nitrogen transformations (including nitrogen fixation, denitrification and ammonium oxidation), and the association of green algae with desert plants. Early studies indicated that the large diversity of culturable bacteria in the rhizosphere is typically dominated by Gram-negative, rod-shaped pseudomonads with nutritional requirements satisfied by one or more amino acids or organic acids. In contrast, the large diversity of culturable bacteria in nonrhizosphere bulk soil is typically dominated by Gram-positive, *Arthrobacter*-like pleomorphic forms and *Acidobacteria* with complex nutritional requirements satisfied by hot-water extractable nutrients in soil organic matter, but not by simple sugars, amino acids, or organic acids. Using the metagenomics approach with high throughput sequencing and informatics tools (see below), nearly the whole diversity of microbial communities is readily accessible, which allows differentiating much better between rhizosphere microbial communities of different plants in different soil environments.

Culturability and Fitness Characteristics of Rhizosphere Microbes

Populations of rhizosphere bacteria increase rapidly and are most competitive when organic nutrient inputs are high (as in the rhizosphere), but decline dramatically when they become limiting. These characteristics are typical of zymogenous, copiotrophic R strategists. In contrast, typical nonrhizosphere soil bacteria are classified as autochthonous, oligotrophic K strategists that exhibit the opposite behavior of successful competition and perseverance with low mortality rates in habitats with low nutrient concentrations (as in bulk soil) because of their superior high-affinity nutrient transport systems, ability to convert soil organic matter into microbial biomass and to synthesize/utilize storage reserve polymers that enable extended starvation survival in the absence of abundant external nutrient apportionments.

The zymogenous nature of typical rhizosphere microorganisms like *Pseudomonas* is also reflected in the significantly higher proportion of the resident community that can be cultured in the laboratory. Interestingly, 40%–70% of the total rhizosphere bacterial community is culturable, whereas only 0.1%–1.0% of the total bacterial community in nonrhizosphere bulk soil is typically culturable. Thus, this "plate count anomaly" poses a more severe challenge when attempting to culture bacteria from bulk soil compared to rhizosphere soil, and therefore the rhizosphere represents an accessible reservoir of culturable microorganisms with high phylogenetic and metabolic diversity. This significantly higher proportion of the rhizosphere community being culturable

likely reflects microorganisms sampled while in a more metabolically active physiological state, that is, when they have faster growth rates and less stringent nutritional and physiological growth requirements that can be satisfied by culture media formulations and cultivation conditions in the laboratory.

Studies using mutant analysis of rhizobacterial *Pseudomonas* and *Bacillus* spp. indicate that their high competence in ability to colonize tomato rhizospheres involves several physiological systems, including chemotaxis, pili-mediated twitching motility, lipopolysaccharide O-antigen biosynthesis, metabolism of vitamins/nucleotides/organic acids, cell autoaggregation, diverse nutrient uptake and utilization pathways, protein secretion, high affinity iron siderophore production and various antibiotic synthesis, lipopeptides. Other studies using *Pantoea agglomerans and Rhizobium leguminosarum* have added production of acidic exopolysaccharides and cellulose microfibrils, in situ biofilm or microcolony formation, and cell surface adhesins to this list of important fitness determinants of bacterial competence in rhizosphere colonization.

Root Exudation and Rhizodeposition in the Rhizosphere

The high metabolic activity and productivity of the microbial community in the rhizosphere is mainly supported by carbon inputs from live roots that are delivered into that environment in various forms, including water-soluble compounds in root exudates, dead epidermal root cells, root cap cells, and mucilage polymers that slough off the root to lubricate its tip during its penetration and geotropic growth through soil.

Classic studies by Rovira and colleagues analyzed root exudates of plants grown "axenically" (without associated microorganisms). These studies identified various low molecular weight organic compounds including numerous sugars, amino acids, vitamins, organic acids, nucleotides, enzymes, and several phenolic compounds in root exudates. The spectrum and abundance of the excreted plant metabolites varied among different species and ages of plants, and this information helped to solidify the concept that the rhizosphere effect is primarily a consequence of increased microbial metabolic/physiological activity made possible by nutrient enrichments through root exudation of carbon-rich and energy-yielding compounds into the soil environment surrounding roots.

All of the carbon lost from plant roots is termed "rhizodeposition," and in addition to the above types, includes respiratory CO_2 and other organic volatiles like ethylene. Much effort has been expended to measure rhizodeposition and identify factors that influence its magnitude. Its values range widely in different experimental systems, reaching as high as 50% of net primary production in some ecosystems. For optimized lab studies, typically, $^{14}CO_2$ is provided to the photosynthetically active aboveground plant system, and the amount of ^{14}C-labeled photosynthates exuded from roots is measured. These studies show that rhizodeposits of amino acids and carbohydrates are higher when roots are grown in a porous matrix of soil particles (rather than in hydroponics), and the proportion of ^{14}C-labeled rhizodeposits is significantly higher (18%–25% vs. 4%–10%) when plant roots are grown in unsterilized soil (with an active rhizosphere microbial community) rather than in sterilized soil. Other studies show that extracellular metabolites of rhizosphere-competent microorganisms like *Pseudomonas* spp. and *Fusarium oxysporum* can significantly increase carbon exudation from plant roots. Important implications of these rhizodeposition studies are that plants release significant amounts of newly fixed photosynthates through their roots without assimilating them into plant biomass, and that rhizosphere microorganisms can significantly increase – even double – the amount of fixed carbon lost through root exudation. Plants are even able to respond to pathogen uptake with the exudation of specific metabolites controlling the attacking pathogen by a disease-induced microbial assemblance, as summarized by Berendsen et al. In general, there are ample evidences that plants drive the selection of rhizosphere microbes from the soil microbial community.

Rhizodeposition does not occur uniformly along the plant root. Various approaches have been used to locate the major sites of nutrient exudation, including ninhydrin staining of filter paper imprints of growing roots to locate concentrated sites of amino acid exudation, localized chemotropism of amino acid-requiring auxotrophs of *Neurospora crassa* fungi on roots, and localization of genetically engineered LacZ or Lux reporter strains of rhizobacteria inoculated on plant roots. All of these methods revealed that the major sites of root exudation are located in the elongation region of the root just above the meristem and quiescent center, and at the base of emerged lateral root branches.

The Volume and Spatial Scale of the Rhizosphere

The volume of the rhizosphere that extends out from the root cylinder into surrounding soil is a dynamic function of the rate of plant rhizodeposition, the diffusion of nutrients influenced by their size/solubility/charge within the soil matrix, absorption to and mechanical impedance of the soil matrix, and the extent of uptake and utilization of the rhizodeposits by the rhizosphere community. The radial distance of the rhizosphere has been examined by measuring the concentration gradient of carbon rhizodeposits and the density of colony-forming units and individual bacterial cells in soil sampled at various distances from the root surface. These studies indicate that the rhizosphere effect is typically most intense at the root surface (called the "rhizoplane") and its gradient extends outward a few millimeters to little more than a centimeter within the soil. Factors that influence this gradient of intensity and spatial scale of the rhizosphere effect include the species and growth stage of the plant, its root architecture and density, the light intensity and duration of the photoperiod affecting the synthesis of photosynthates, the texture/depth/fertility/and moisture status of the soil, and the types/abundance/and activities of the microbes, just to name a few. Fig. 2 schematically illustrates how root architecture influences the volume of different plant rhizospheres, indicating that its volume is much larger when produced by plant root systems that are fibrous and highly branched.

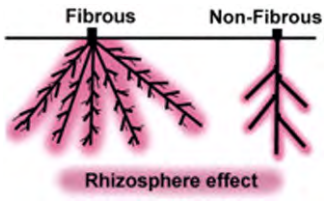

Fig. 2 Schematic drawing illustrating how the root architecture influences the volume of the rhizosphere.

Rhizospheres of neighboring plants can occupy significant volumes that even overlap when their branched root densities are sufficiently high. For example, 30%–40% of the top 10 cm of A horizon soil lies within 1 mm of a wheat root at its dough stage in development. Considering the high root density of lawn turfgrass, all the soil to depths of its longest roots is rhizosphere.

Direct Microscopy of Microbial Colonization of Roots

Early Microscopy Studies

Starkey, Krasilnikov, Rovira, Bowen, and Foster conducted pioneering studies that utilized microscopy to examine the rhizosphere effect directly. A clever way to examine the microbes inhabiting this habitat in situ utilized a seedling contact slide modification of the Rossi–Cholodny buried slide technique (Fig. 3). Also, roots grown in soil were gently removed, washed, processed and examined by brightfield light microscopy, and by transmission/scanning electron microscopy to examine the rhizoplane microorganisms. These studies verified earlier findings obtained by cultivation methods, showing the increased abundance of microbes in soil surrounding roots, their cooperative growth into microcolonies, ultrastructural details of the rhizosphere soil fabric, preliminary descriptions of the spatial distributions of the microbes on the rhizoplane, direct demonstration of a mucilage layer on the root surface that sometimes embeds microorganisms within it, bacteria surrounded by abundant fibrillar capsules (made during carbon excess) whose periphery is covered with a particulate layer of protective clay envelopes, increased abundance of bacterial colonization in grooves between adjacent epidermal cells where exudation is predictably elevated, local sites of eroded epidermal walls allowing microbial penetration, and direct evidence of biological control via "hyperparasitism" (beneficial microorganisms directly attacking and killing other microbial pathogens of plants). Examination of root cross sections of soil-grown plants showed that their internal root cortex naturally supports large populations of microorganisms without the plant expressing symptoms of disease. This benign (often beneficial) associated community of microorganisms inside the root is termed the "endorhizosphere," and the organisms are called "endophytes" or "internal root colonists."

Fig. 4 is a color fluorescence micrograph of a microbial assemblage within rhizosphere soil surrounding a white clover seedling root. This image acquired by conventional epifluorescence microscopy shows the clustered distribution of the morphologically diverse unicellular and filamentous bacteria in close association with reddish-brown autofluorescent particles of organic matter, but its image quality is reduced because many foreground objects lie outside its plane of focus.

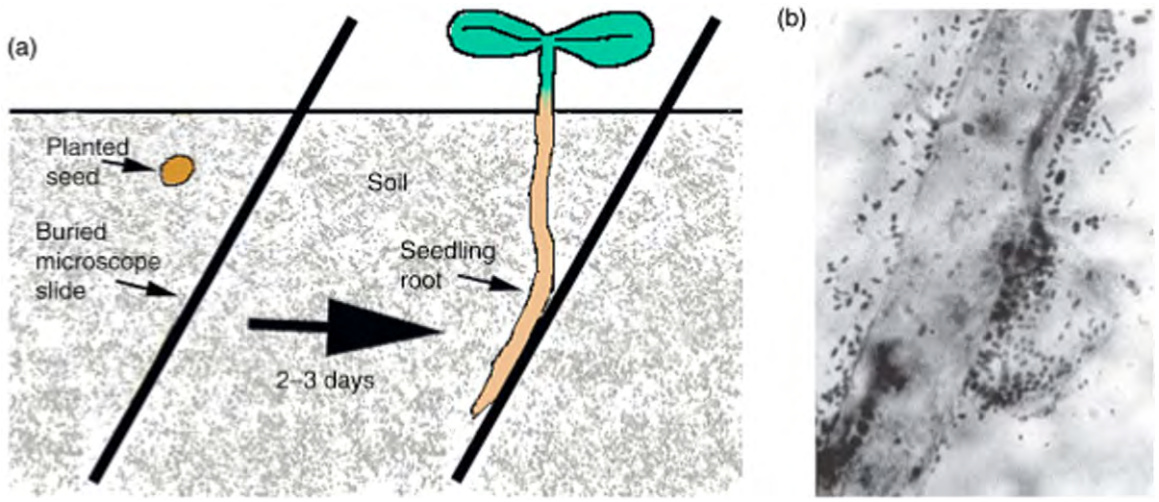

Fig. 3 (a) Schematic diagram illustrating the experimental design of the seedling contact slide method to examine the rhizosphere effect by direct microscopy. (b) Direct microscopy of the abundant, localized colonization of seedling root tissue by rhizosphere microorganisms on a seedling contact slide. The sample was stained with aniline blue and photographed using brightfield light microscopy.

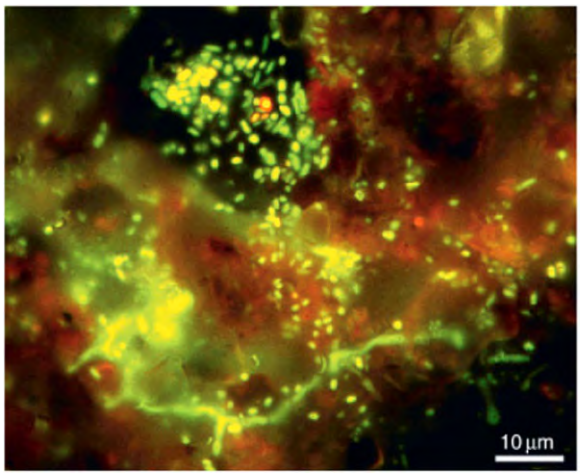

Fig. 4 Color fluorescence micrograph of a microbial assemblage within rhizosphere soil surrounding a white clover seedling root. The sample was treated with acridine orange (a vital nucleic acid stain), then rinsed with sodium pyrophosphate solution and examined by conventional epifluorescence microscopy.

Improvements in Direct Microscopy Using CLSM

The most significant advancement in the use of fluorescence microscopy to examine root-associated microbes was the development of confocal laser scanning microscopy (CLSM). The confocal imaging system has an optical design utilizing pinhole apertures at the laser light source and at the detection of the object's image, thus eliminating the stray and out-of-focus light that interferes with the formation of the object's image. This ability to use only signals from the focused plane eliminates a major limitation of the conventional fluorescence microscope when examining plant tissue, soil particles, and organic debris that emits a significant amount of objectionable background autofluorescence and/or absorbs the fluorescent dye itself. Because the light from outside of the plane of focus is not included when the confocal image is formed, the 2-D (x,y) image becomes an accurate optodigital thin section with a thickness that can approach the theoretical 0.2 μm resolution of the light microscope. Also, by digitizing a sequential series of 2-D images acquired at incremental depths while focusing through the specimen in the third (z) dimension (called the "Z series"), an all-inclusive flattened stack or a 3-D reconstructed digital image can be produced, optimally rotated, and quantitatively analyzed. Dazzo and Hartmann independently and simultaneously first reported the successful use and benefits of CLSM to examine the colonization of roots by the associated bioactive microbial community (Fig. 5(a) and (b)), or by a specific inoculated organism using a homologous fluorescent antibody.

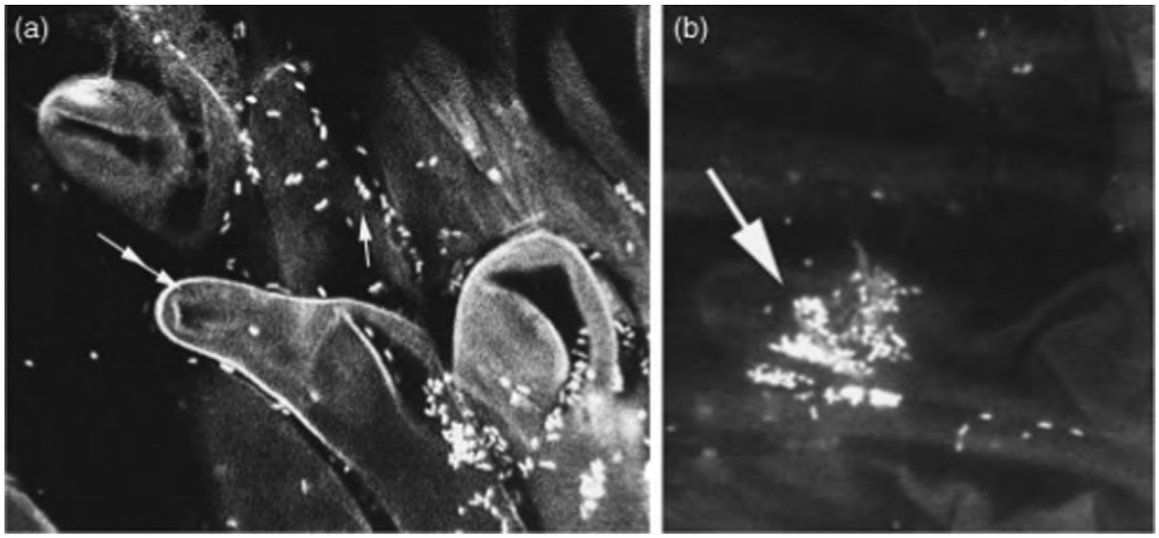

Fig. 5 Use of confocal laser scanning microscopy (CLSM) to detect rhizoplane microorganisms colonized on roots grown in soil. White clover seedlings were grown in soil for 2 days, cleared free of rhizosphere soil using optimized methods described in the book COST 631: Handbook of Methods in Rhizosphere Research, then stained with acridine orange vital dye, washed, mounted with an anti-fading reagent (e.g., Vectashield, Citifluor) to inhibit photobleaching of fluorescent dyes, and examined by epifluorescence CLSM. The image (a) historically introduced CLSM technology to visualize bioactive rhizosphere microorganisms in situ, and the image (b) shows direct evidence of bacterial population growth and formation of a microcolony biofilm (arrow) developed on the rhizoplane. Reproduced from Dazzo, F.B., 2004. Applications of quantitative microscopy in studies of plant surface microbiology. In: Varma, A., Abbott, L., Werner, D., Hampp, R. (Eds.), Plant Surface Microbiology. Germany: Springer-Verlag, pp. 503–550, with permission from Springer-Verlag.

Fig. 6 Binary montage image depicting the spatial distribution of microorganisms colonized on the rhizoplane of a white clover seedling after 2 days of growth in soil. Note the discontinuous spatial density of microcolonies that is greater on older regions of the root.

By utilizing these CLSM technologies, one can map the distribution of rhizoplane microbial communities that, when analyzed, can reveal their colonization behavior on seedling roots grown in soil. Fig. 6 shows a typical density map, illustrating that the spatial distribution of microorganisms on the rhizoplane is discontinuous and spatially discrete, being almost completely devoid of microbes at the root tip while actively growing in soil, and individual cells just above the root tip, some of which eventually develop into microcolonies further up older portions of the root. Generally, microbes reach population densities that cover not more than ~20% of the rhizoplane substratum of roots that are actively growing in soil. During early stages of microbial colonization, discrete regions of the rhizoplane environment are favorable for growth of individual microbial cells into localized microcolony biofilms with in situ population generation times as short as 2–3 h.

A Case Study: *Rhizobium* Colonization of Rice Roots

Rhizobium is the well-known nitrogen-fixing root nodule bacterial symbiont of legume plants. Interestingly, this soil microorganism also develops natural, invasive and sometimes beneficial endophytic associations with major cereal crops like rice, corn and wheat. This alternate ecological niche of *Rhizobium* can be exploited in sustainable agriculture in the form of efficient biofertilizers that are inoculated on the cereal plant host at planting and can significantly promote its growth, while reducing its need for chemical fertilizer applications to achieve maximum grain yield. Figs. 7 and 8 illustrate stages of the colonization and endophytic infection of rice roots by *Rhizobium* using CLSM, scanning and transmission electron microscopy, respectively. The dynamic infection process of this association begins with their epiphytic colonization of the rhizoplane (especially collars surrounding the base of lateral roots and at junctions between epidermal cells), then localized pit erosion of root cell walls mediated by *Rhizobium* cell-bound cellulases, portals of crack entry into void spaces created by natural wounds at lateral root emergence and fissures between epidermal cells, endophytic colonization of dead cortical cells within the root interior, and ascending migration of *Rhizobium* cells into above-ground tissues.

CMEIAS Image Analysis of Microbial Colonization Behavior

Computer-assisted microscopy and digital image analysis can significantly enhance the quantitative assessment of microbial colonization on roots by allowing one to extract, store, retrieve, and electronically transmit numerical information regarding selected and pertinent image features. Image analysis of bacterial cells can provide useful information on the morphological diversity, local abundance, and patterns of spatial distribution reflecting their nutritional apportionment, allometric relationships of metabolic ecophysiology, and colonization behavior involving cell–cell interactions between microbial community members. A system of computer-assisted microscopy has been developed by the Center for Microbial Ecology at Michigan State University to assist in such assessments. The Center for Microbial Ecology Image Analysis System (CMEIAS; see "Relevant Websites section") is a semiautomated software analysis tool of bioimage informatics that uses computer vision and pattern recognition techniques in conjunction with microscopy to extract quantitative information from digital images of microorganisms, allowing culture-independent, object analysis and classification of individual cells, microbial populations and complex microbial communities. Quantitative in situ studies of microbial biogeography on rhizoplanes are greatly facilitated using this bioimage informatics software, especially to define the real world spatial scale of quorum sensing at single-cell resolution, niche overlap and competition among populations, and the landscape ecology of root colonization by microcolony biofilm communities.

As an example of using computer-assisted microscopy to examine the autecological biogeography of specific organisms colonized on root surfaces, CMEIAS image analysis was performed on Fig. 9 containing 136 fluorescent antibody-labeled bacterial cells of *Rhizobium leguminosarum* bv. trifolii on the white clover rhizoplane. In approximately 400 ms of computing time, CMEIAS extracted several measurement features from these foreground objects, and reported the data (Table 2) that defined their sizes, local spatial abundance and pattern of distribution providing insights on their colonization behavior and ecophysiology on the root surface.

Of particular ecological importance are the image analysis data needed to perform nearest neighbor distance-based and quadrat-lattice based spatial pattern analyzes, and geostatistical analyzes on autocorrelated spatial interactions among the bacteria. These attributes define indices of spatial association, dispersion and mathematical modeling that predict bacterial colonization behavior in situ. The spatial randomness index, computed from the image area, average and standard deviations of the first nearest neighbor distances (Table 2), provides quantitative assessments (values <1.0) indicating that the bacteria in this Fig. 9 landscape image exhibit a clustered pattern of spatial distribution. Other analyzes of spatial aggregation are also included because the use of only a single index may be misleading. This indication of cells having an aggregated pattern on the root surface is confirmed quantitatively by five other plot-based spatial distribution indices (variance:mean ratio, Morista's dispersion index, negative binomial K, Lloyd's mean crowding index, and Lloyd's patchiness index; Table 2) computed from the CMEIAS data. Geostatistical analysis shows that

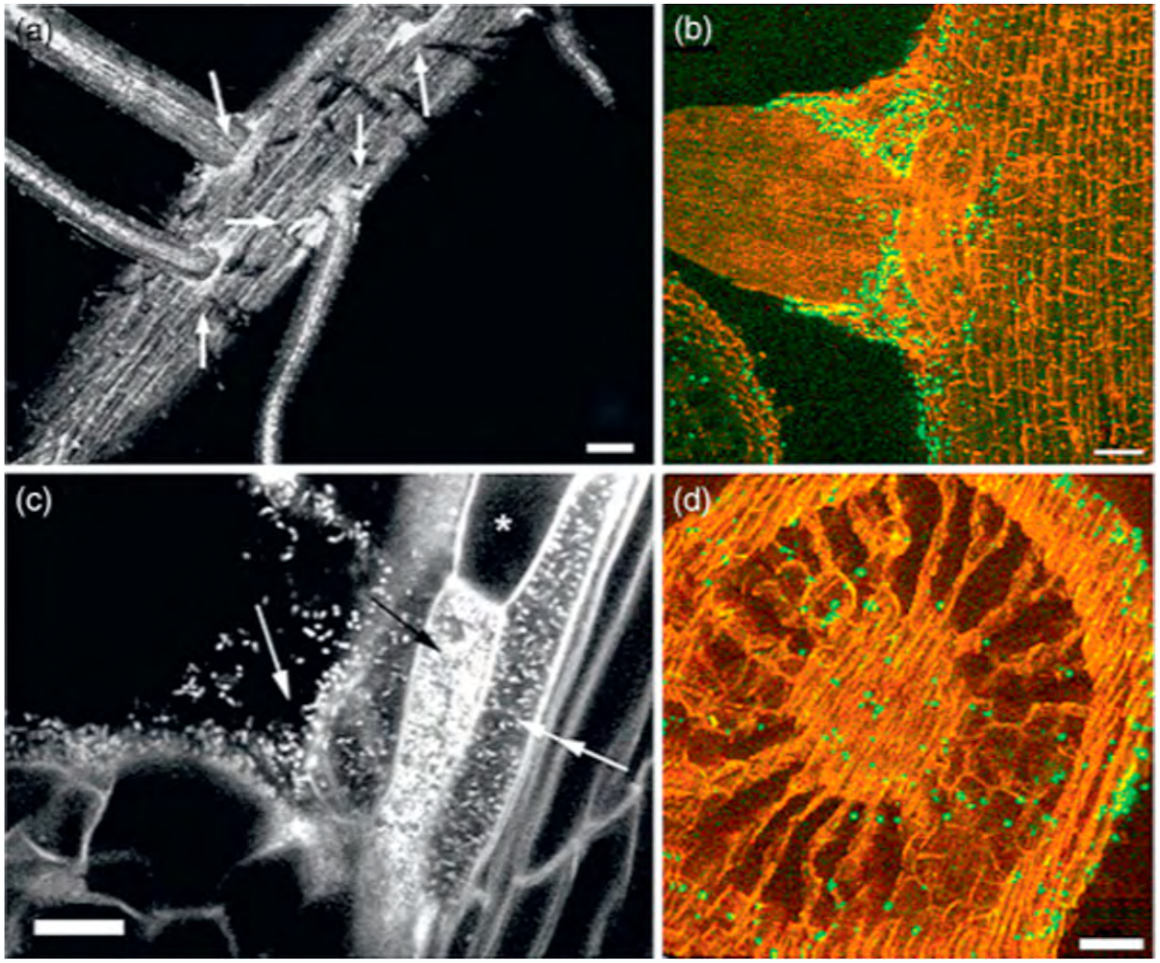

Fig. 7 Epifluorescence confocal laser scanning microscopy showing colonization of the rhizoplane and internal root structures of rice by rhizobia. (a) and (b) Preferential colonization of a dense collar of rhizoplane rhizobia (arrows) surrounding the base of emerged lateral roots. (c) and (d) Endophytic rhizobial colonization of underlying, lysed root cortical cells and the vascular cylinder of rice roots. Rhizobia are stained with acridine orange in (a) and (c) and are an engineered reporter expressing the green fluorescent protein in (b) and (d). Images (a) and (c) are reproduced from Reddy, P.M., Ladha, J.K., So, R., et al., 1997. Rhizobial communication with rice: Induction of phenotypic changes, mode of invasion and extent of colonization in roots. Plant and Soil 194, 81–98, with permission from Springer. Images (b) and (d) are reproduced from Chi, F., Shen, S.H., Cheng, H.P., et al., 2005. Ascending migration of endophytic rhizobia from roots to leaves inside rice plants and assessment of their benefits to the growth physiology of rice. Applied and Environmental Microbiology 71, 7271–7278, with permission from the American Society for Microbiology.

this clustered behavior is spatially autocorrelated, with an intensity of influence that extends from individual bacterial cells out to an effective radial distance sufficiently large enough to include all their neighboring cells in this image of the rhizoplane landscape. Aggregated spatial patterns of distribution arise from positive, cooperative colonization behaviors of the bacteria in situ, benefitting each other's ability to actively growing into microcolony biofilms on the root surface. This occurs frequently at fissure grooves between adjacent epidermal cells just below and within the root hair region where localized root exudation is high. This category of colonization behavior differs from a random colonization pattern (occurs infrequently on roots when there are no spatially explicit events affecting colonization), and is diametrically different from a uniform spatial pattern of colonization that arises from negative conflicting cell–cell interactions during colonization, resulting in their maximally dispersed, self-avoiding distributions.

Further CMEIAS analysis shows that this spatially structured distribution among cells also exhibits fractal geometry, which reflects complex (often anisotropic) patterns and ecophysiological processes occurring within natural microbial landscapes, including the scale-dependent heterogeneities of their biomass distribution and cell–cell interactions in the biofilm architecture, all driven by the benefits that populations gain by their optimal spatial positioning to maximize their apportionment, accessibility and efficiency in utilization of allocated nutrient resources *in situ* within their local environment.

This clustered colonization strategy is further indicated by the multimodal (rather than normal) frequency distribution of the first and second nearest neighbor distances (Fig. 10(a)), and by the clustered abundance of short separation distances between individual cells revealed in the 2-D scatterplot of the first versus second nearest neighbor distance (Fig. 10(b)).

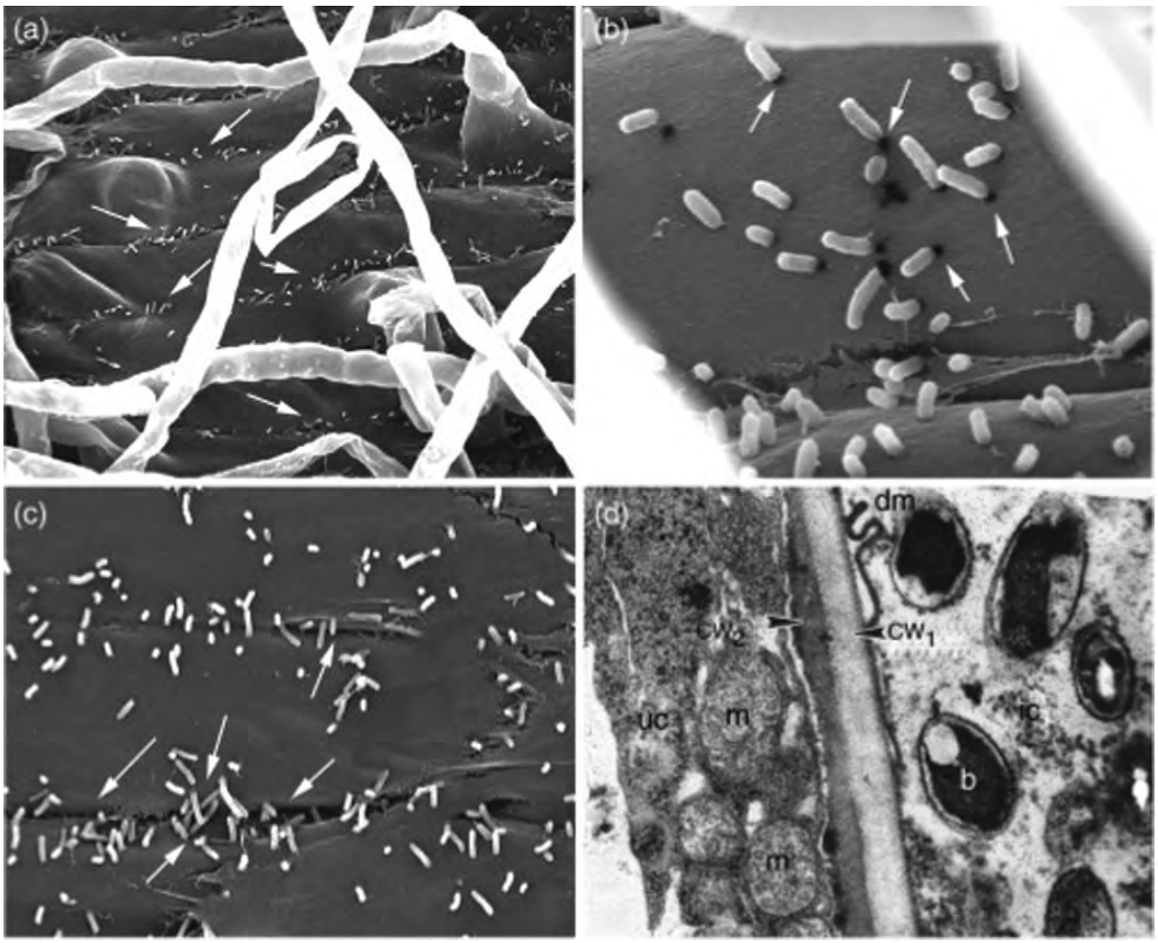

Fig. 8 Scanning (a)–(c) and transmission (d) electron microscopy of the colonization and infection of rice by endophytic rhizobia. Images illustrate (a) colonization at epidermal cell junctions (arrows); (b) epidermal cells with walls containing localized eroded pits (arrows) made by cell-bound cellulase activity of the attached rhizobia; (c) crack entry mode of infection of the rice root at fissure grooves between epidermal cells (arrows); and (d) endophytic colonization of rhizobia within a dead cortical cell adjacent to an uninfected host cell with intact membranous organelles. Reproduced from Yanni, Y.G., Rizk, R.Y., Abd El-Fattah, F.K., et al., 2001. The beneficial plant growth-promoting association of *Rhizobium leguminosarum* bv. trifolii with rice roots. Australian Journal of Plant Physiology 28, 845–870, with permission from CSIRO.

Microscopy of Genetically Marked Bacteria on the Rhizoplane

Several approaches of microscopy can be used to detect and measure colonization of the rhizosphere and/or rhizoplane by specific microbes. For instance, immunofluorescence microscopy (IFM) can be used to track inoculant strains of bacteria in the environment if strain-specific antibodies are available (e.g., Fig. 9). However, chemical inhibiting factors in soil and nonspecific absorption of the fluorescent antibody by the root can interfere with the IFM approach, and gelatin pretreatment of samples can help to suppress these problems. Alternative approaches using genetic engineering have also been developed to avoid such problems. Genetic markers encoding fluorescent proteins are inserted into their genome, which when expressed can be excited by light of specific wavelengths to make the cell autofluorescence. Commonly used genetic markers encode for a green fluorescent protein (Gfp) and red fluorescent protein (Rfp). Such techniques have been applied to study the distribution of bacterial populations colonized on roots (Fig. 11(a)). By using different markers and specific fluorescence optics, one can track root colonization by multiple bacterial populations in the same environment (Fig. 11(b)).

Two major concerns arise when applying this autofluorescent genetic marker approach to study bacterial colonization in situ. One is whether the metabolic burden of added biosynthesis to constitutively produce the autofluorescent protein influences the colonization behavior of the recombinant strain in ways that differ from its parental wild-type strain. The other is whether the recombinant cell stably maintains the genetic marker during its population growth within the environment. Such markers were originally introduced into the organism using plasmid constructs to ensure fast adaptations in their environment. But in this case, the antibiotic whose resistance is conferred by the plasmid must be present within the environment in order to impose the selective pressure needed to maintain the plasmid inside the bacteria and its replication in succeeding generations. Such selection pressure may severely disturb other members of its associated microbial community in the rhizosphere environment. Nowadays,

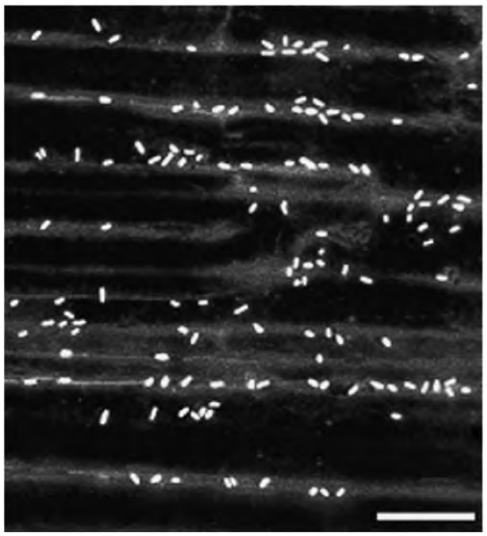

Fig. 9 Epifluorescence micrograph of immunofluorescent cells of *Rhizobium leguminosarum* bv. trifolii 0403 colonized on the root surface (especially at junctions between epidermal cells) below the root hair region of a white clover seedling. Scale=20.0 μm. Reproduced from Dazzo, F., Schmid, M., Hartmann, A., 2007. Immunofluorescence microscopy and fluorescence in situ hybridization combined with CMEIAS and other image analysis tools for soil- and plant-associated microbial autecology. In: Hurst, C., Crawford, R., Garland, J., et al. (Eds.), Manual of environmental microbiology, third ed. Washington, DC: American Society for Microbiology Press, pp. 595–792, with permission from the American Society for Microbiology.

Table 2 CMEIAS image analysis of the abundance and spatial distribution of immunofluorescent bacteria on the white clover root epidermis shown in the image of Fig. 9

Spatial ecology parameter	*Value obtained*
Image area analyzed	10,434 μm^2
Bacterial cell count	136
% Rhizoplane substratum coverage	2.7%
Total microbial biovolume	259.0 μm^3
Total microbial biomass carbon	51.8 pg
Total microbial biosurface area	1126.6 μm^2
Spatial density	13,034 cells mm^{-2}
Fractal dimension Index	1.724
1st nearest neighbor distance (mean±SD)	3.9±2.4 μm
2nd nearest neighbor distance (mean±SD)	5.7±2.9 μm
Spatial randomness index	0.880 (clustered)
Quadrat-based variance:mean ratio	1.796 (clustered)
Morista's dispersion index	1.996 (clustered)
Negative binomial K index	0.950 (clustered)
Lloyd's mean crowding index	1.595 (clustered)
Lloyd's patchiness index	1.995 (clustered)
Effective range autocorrelated cluster behavior	86.7 μm (includes all cells)

Source: Dazzo, F., Schmid, M., Hartmann, A., 2007. Immunofluorescence microscopy and fluorescence in situ hybridization combined with CMEIAS and other image analysis tools for soil- and plant-associated microbial autecology. In: Hurst, C., Crawford, R., Garland, J., et al. (Eds.), Manual of Environmental Microbiology, third ed. Washington, DC: American Society for Microbiology Press, pp. 712–733.

these autofluorescent genetic markers are stably inserted into the bacterial chromosome to ensure transfer to each subsequent generation without the need for selective pressure. If such a marker is connected to a specific gene of interest, it can be used to display gene expression activity as a reporter construct in situ as described next.

A Case Study: CMEIAS In Situ Analysis of Quorum Sensing by Rhizobacteria

Many bacteria modulate their colonization and occupation of ecological niches in natural environments like the rhizosphere using specific cell-to-cell-communication systems based on elaboration of low molecular weight, extracellular signal molecules that allow

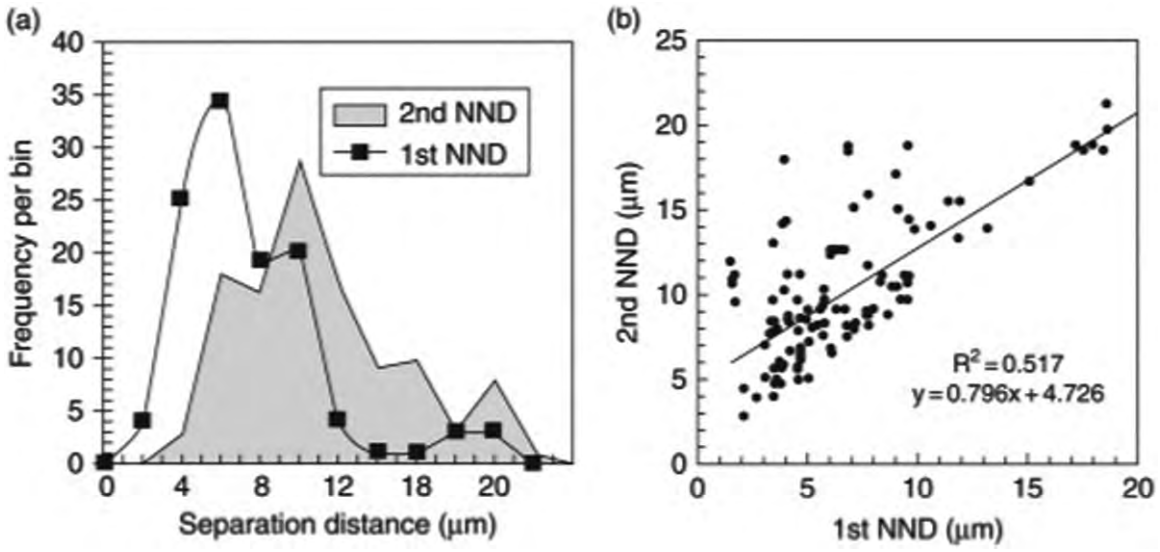

Fig. 10 CMEIAS point-pattern spatial distribution analyzes of the first and second nearest neighbor distances separating each individual bacterium in Fig. 9. (a) Frequency distribution histograms and (b) 2-D scatter plots. Reproduced from Dazzo, F.B., 2004. Applications of quantitative microscopy in studies of plant surface microbiology. In: Varma, A., Abbott, L., Werner, D., Hampp, R. (Eds.), Plant Surface Microbiology. Germany: Springer-Verlag, pp. 503–550, with permission from Springer-Verlag.

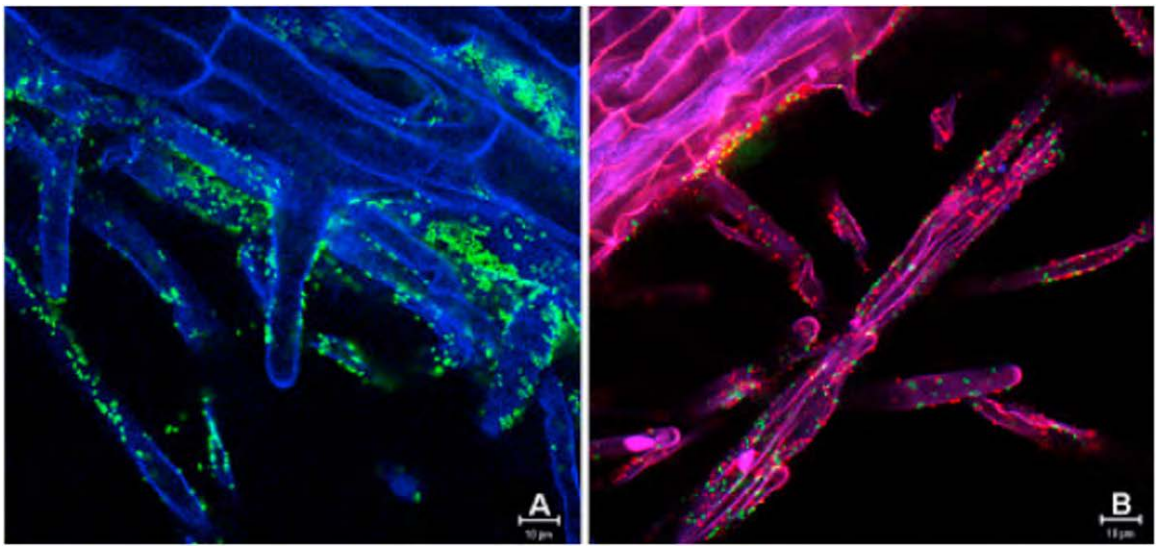

Fig. 11 (a) Distribution of bacterial colonization on tomato roots by *Serratia liquefaciens* cells chromosomally tagged with the *gfp* gene encoding an autofluorescent green fluorescent protein. (b) Use of dual (green fluorescent protein and red fluorescent protein) gene markers in *S. liquefaciens* and *Pseudomonas putida*, respectively, to visualize their colonized distributions within the same tomato rhizoplane landscape.

the bacteria to perceive neighboring microbes while colonizing the habitat. The signal molecules are commonly oligopeptides for Gram-positive bacteria and *N*-acyl homoserine lactone (AHLs) for Gram-negative bacteria.

The cell-to-cell communication system for Gram-negative bacteria is better understood. Above threshold concentrations, AHLs activate expression of certain bacterial genes to occupy their ecological niches, for example, biofilm development, production of virulence factors or antibiotics, and persistence and viability on colonized host surfaces. AHL-mediated cell-to-cell cross-talk can also be effective across species borders within microbial communities and even between prokaryotes and their eukaryotic plant hosts. Since the bacterial traits activated by AHLs can be beneficial or harmful to hosts, identification of the attributes that govern cell-to-cell communication is very important and has major practical application in the design and implementation of synthetic antimicrobial strategies that expressly target microbial biofilms, plant–microbe interactions in soil, and in aquatic environments.

It is generally thought that high bacterial population densities are needed to exceed the threshold level of AHL concentrations required to activate genes and their physiological functions. Hence, this specific type of microbial communication has become known as "quorum sensing," functioning primarily as sensors of high population density, thus optimizing the expression of functions that are most beneficial when simultaneously performed by dense populations. This prevailing view is commonly based on measurements of gene expression within populations in liquid suspension or when grown into large biofilms on artificial substrata (e.g., pellicle in unshaken broth cultural media or on plastic surfaces). During planktonic growth, dispersion and dilution of the signaling molecules are much faster and further than their diffusion rates within biofilm matrices. For instance, spatial patterns of population dispersion and active growth within discontinuous domains are major ecological determinants that govern microbial colonization of host surfaces, especially plants, and during early stages of biofilm development. One could theoretically predict that individual bacteria should benefit significantly if their sensory system is able to respond efficiently to small, local concentrations of the signal molecule since that would communicate information about competitive and/or cooperative activities by a few local neighbors before they grow to high population densities in the cell's immediate microenvironment. Core principles of bacterial autoinducer systems have recently been summarized by Hense and Schuster, which provide the general theoretical basis for an evolutionary concept of auto-inducing quorum sensing systems.

The combined technologies of fluorescent reporter strain constructions, confocal microscopy, computer-assisted digital image analysis, and geostatistical modeling for single-cell resolution experiments have been used to examine the *in situ* spatial scale and local population size requirements for AHL-mediated cell-to-cell communication during bacterial colonization of roots. These studies showed that this type of cellular communication occurs in the rhizosphere and can be accomplished by individual bacterial cells that are separated from each other and from high population densities by long-range distances (Fig. 12(a)–(c)). Reporter rhizobacteria were able to conduct AHL-mediated cell-to-cell communication on roots with a minimum quorum of two cells (no requirements for high population density) and an *in situ* separation "calling distance" of up to 78 μm (equivalent to two people talking to each other while standing ~130 m apart!). Also, individual bacteria in small clusters (2–3 cells) communicated with each other when separated from dense populations by even longer distances. Thus, during colonization of plant roots, a single bacterium is able to produce sufficient AHL signal molecules to communicate with another single bacterial cell neighbor even when they are separated apart by long distances. Geostatistical modeling of local spatial densities predicts that in situ AHL-mediated cell-to-cell communication is governed more by the spatial proximity of cells within AHL gradients than by a quorum requirement of high population density (Fig. 12(c)).

Nucleic Acid-Based Technologies in Rhizosphere Studies

Ribosomal RNA Gene-Based Fingerprint Techniques

The discovery by Carl Woese in 1977 that the 16S ribosomal nucleic acid sequence can be used as identification criteria for microorganisms has revolutionized the characterization of microbial communities in environmental studies. Nowadays, many important and even cutting edge techniques use the combination of conservative and variable regions in the ribosomal gene sequence analysis to reveal taxonomic relations between organisms or similarities and differences in microbial community

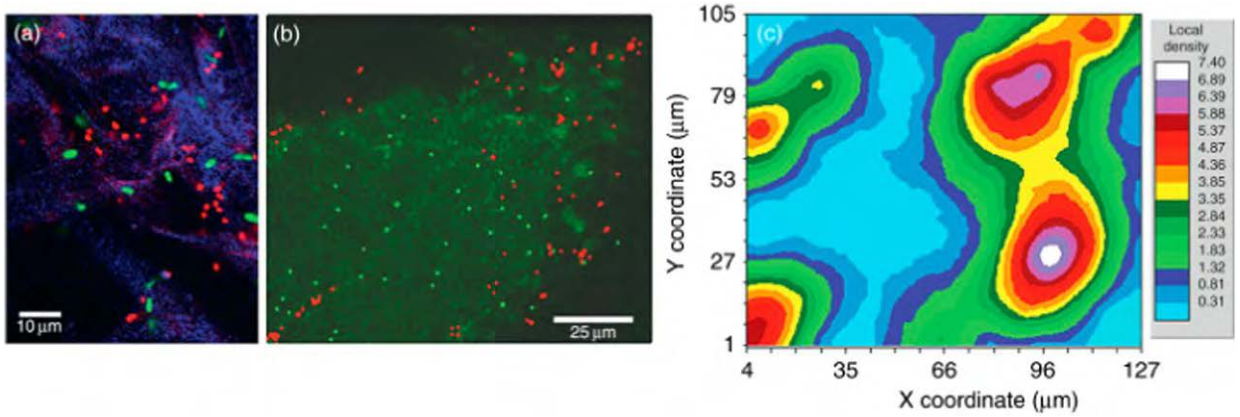

Fig. 12 *In situ* distribution of individual fluorescent *Pseudomonas putida* cells conducting *N*-acyl homoserine lactone (AHL)-mediated cell-to-cell communication on tomato roots. (a) Confocal laser scanning micrograph of the root surface colonized by the red fluorescent IsoF source strain that produces AHLs, and the green fluorescent F117 (pKR-C12) reporter sensor strain activated by extracellular AHLs from neighboring source cells. Bar scales are indicated. (b) Posted locations of red AHL source and green AHL-sensor cells colonized on a root tip, and (c) continuous spatial interpolation map of the predicted gradients of AHL signal molecules in the same region of the root as indicated in (b), based on a CMEIAS-supported geostatistical block kriging analysis of the spatial distribution of each red (AHL-producing) cell weighted by its local spatial density (cells per quadrat subsample). Reproduced from Gantner, S., Schmid, M., Dürr, C., et al., 2006. In situ spatial scale of calling distances and population density-independent *N*-acyl homoserine lactone mediated communication by rhizobacteria colonized on plant roots. Federation of European Microbiological Sciences Microbiology Ecology 56, 188–194, with permission from FEMS Mic Ecol.

structure. Nucleic acid-based fingerprint techniques based on ribosomal gene sequence identification or detection methods allow one to track microbial community changes in environments quickly and without dependence on culturability. Refinements of this approach have made it more sensitive and specific, improving the detection of less-dominant bacteria, and recently, extending its use to include eukaryotic microorganisms within the same community sample. However, issues of their accuracy are raised since these techniques most often address only partial gene sequences, cannot guarantee that the total unbiased community is sampled (Gram-negatives are easier to lyse than Gram-positives or spores), are quantitatively affected by variations in genomic copy number among species, and will also include protected extracellular DNA sampled after cells have already naturally lysed.

In general, 16S rDNA gene analysis for prokaryotes and the corresponding 28S rDNA gene analysis for eukaryotes have revealed important taxonomic and environmental relationships of organisms in the rhizosphere. For instance, studies have documented the diversity of the nonculturable portion of the rhizosphere community and the strong impact environmental pressure plays in forming those microbial community structures. Consistent with early findings based on culturing techniques, gene-based sequencing studies especially targeting the 16S ribosomal RNA gene of prokaryotes indicate that the λ-proteobacteria group (of which pseudomonads are representative) embody, on average, ~35% of all bacterial cell clones isolated from rhizosphere samples of many plant species, and their numerical dominance in the rhizosphere of some plant species extends up to ~65% of all bacterial clones analyzed. Many microbial specialists occupying similar or identical ecological niches show closer relations to the rhizosphere community than to other members of their taxonomical orders. Such findings raise the question of the extent to which the taxonomical nomenclature and phylogeny related to the origin and evolution of microbial life can help to explain their real ecological functions in situ.

Array Technology – Functional Gene Arrays

Nanotechnologies have created the ability to differentiate thousands of gene sequences spotted on polymer-coated microchips, allowing for the simultaneous search for gene sequence similarity to environmentally extracted community DNA. Today, several biotechnology companies and research groups provide DNA chips for bacterial community identification. Functional gene arrays can potentially reveal the diversity of various known metabolic pathways potentially utilized by the rhizosphere microbial communities.

A Case Study: Functional Gene Arrays and rDNA Sequencing

The effects of global climate changes are influencing our daily life in an increasing scale. However, many changes in the biosphere remain unnoticed. The rhizosphere community of microbes contains potentially useful indicators of environmental changes, as they are responsive to changes in root exudations of plants, which can be perturbed by environmental changes affecting plant metabolism before variations in growth patterns are discerned. A combination of ribosomal DNA-based techniques and functional gene arrays has addressed this environmentally important question. Community analysis using terminal restriction fragment length polymorphism (a 16S ribosomal RNA-based fingerprint technique) revealed strong shifts in populations of certain microbial ribotypes in the rhizospheres of grassland plants treated with nitrogen fertilizer and CO_2 enrichments to simulate global climate changes as compared to nontreated controls. These population shifts resulting from the environmental perturbations were grass cultivar- and treatment-dependent, and revealed strong connections between the belowground microbial rhizosphere community and the aboveground plant host effects. Functional gene arrays further characterized the potential functional diversity in the microbial rhizosphere community. These results showed that different plants select and define their own functional rhizosphere communities, and that simulated climate changes have a strong impact on the diversity selected within those microbial communities, which in turn reflects on the physiological conditions of the plants. Indeed, despite their small spatial scale (from the human perspective), some rhizosphere processes impact on large-scale ecosystem responses to various drivers of global change, for example, rising atmospheric CO_2 concentration.

In related studies addressing impacts of global climate change, the successful colonization of the rhizosphere by species of *Burkholderia* and *Pseudomonas* spp. was significantly influenced by elevated CO_2, whereas species of *Bacillus* and actinomycetes (representing 2 groups of bacteria that are more dominant in bulk soil rather than rhizosphere) were not. These findings clearly indicate physiological differences between the lifestyles of rhizosphere-competent bacteria and other microbial groups not well adapted to that plant root-modified soil environment.

Use of Stable Isotope Probing to Examine Bioremediation Processes

Another modern technique used to characterize microbial community activity is the stable isotope probing (SIP) technology, which identifies and tracks microorganisms that are actively utilizing certain specialized compounds in the environment of interest. This method utilizes a labeled substrate containing heavy, stable isotopes (e.g., ^{13}C, ^{15}N, ^{34}S) harboring an additional neutron in their atomic structure. Microbial communities can metabolize this labeled substrate qualitatively (not necessarily quantitatively) the same way as unlabeled substrates, thereby incorporating the heavy labeled atoms into their DNA, RNA, or other metabolic products, which are then physically separated from the corresponding unlabeled polymer by density gradient ultracentrifugation. This technique allows one to study specific bacterial groups selected by the substrate they can utilize in situ.

Using this approach, one can introduce ^{13}C-labeled xenobiotic compounds into the rhizosphere and identify organisms in that microbial community that are potentially important in degrading them, based on their utilization of the labeled substrate. This is an

important tool being used to identify organisms involved in bioremediation of environmental pollutants. In terrestrial habitats, bioremediation is enhanced in the rhizosphere due to the increased availability of nutrients that fuel the metabolism of microbial communities responsible for this activity. This SIP molecular tool provides the opportunity to explore more in-depth connections of the microbial communities involved in bioremediation and will likely reveal many more details about rhizosphere microbial ecology in the future.

Omics Technology Introduces New Opportunities in Rhizosphere Microbial Community Ecology Research

Advances in Next Generation high throughput sequencing technologies provide various new ways to investigate microbial communities in the rhizosphere without the use of culture-based techniques. Generally speaking, metagenomics is used to sequence DNA present in an environmental sample. Metagenomic techniques can be targeted to a specific gene of interest such as the 16S rDNA gene for taxonomic diversity analysis or another gene of interest such as *nifH* to identify variability within the portion of the community capable of nitrogen fixation. Metagenomic techniques can also be untargeted, often called "shotgun metagenomics," where DNA is randomly sequenced from the environmental sample.

Gene-targeted and shotgun metagenomics suffer many of the same drawbacks as discussed above in "Ribosomal RNA Gene-Based Fingerprint Techniques." Since shotgun metagenomics randomly sequences environmental DNA, the metabolic state of the microbes sampled is unknown and the sampled DNA can originate from dead or dormant microbes as well as living active microbes. Therefore metagenomics represents the functional potential of the microbial community. Additionally, short reads produced from illumina sequencing (currently the most popular sequencing platform) need to be computationally assembled and annotated to adequately determine the function of the DNA sequence. Sequence annotation is dependent upon matching the sequenced DNA fragment to already isolated and sequenced microbe genomes, therefore – omics technologies are still very much reliant on information already gained by culturing and laboratory based techniques. As a consequence, annotations of sequencing data are often incomplete, as sequences of some genes are not represented in the current databases. Sequences can also be annotated as "hypothetical protein" indicating there is no currently known function for the gene sequence. Nowadays, this latter category commonly represents 30%–50% of the bacterial genomes. Despite these drawbacks, metagenomes provide a wealth of information regarding the metabolic potential of the microbes in the environmental sample.

Another – omics method, called metatranscriptomics, specifically targets the sequencing of mRNA transcripts within an environmental sample. This technology removes one of the limitations of metagenomics since mRNA is only produced by active microbes and is short-lived within the cell, thereby offering a glimpse into the likely functional activities of the microbial community occurring at the time of sampling. Because of its instability, mRNA in meta-transcriptome samples will degrade quickly if not preserved via RNase-inhibiting solutions or frozen at -80°C. Technically challenging drawbacks of meta-transcriptomics include short read assembly and sequence annotation issues as described above, and the need to remove rRNA noise in the environmental sample as much as possible before sequencing since it can represent $\geq$90% of the total extracted RNA.

A Case Study: Functional Gene Expression in the Rhizosphere

Yergeau et al. conducted a meta-transcriptomics study comparing the expression patterns of upregulated genes in bulk soil and willow rhizosphere soil. The study found that the rhizosphere samples were preferentially enriched for several categories of functional gene expressions related to plant–microbe interactions. *First*, the rhizosphere was enriched in expression of genes related to carbon uptake and utilization, especially the utilization of organic acids as a major component of root exudate, and also degradation of oligosaccharides and histidine. *Second*, the willow rhizosphere was enriched for upregulated expression of genes conferring antibiotic resistance, suggesting that active microbial resistances to inhibitory antibiotics present within the rhizosphere are important traits of stress fitness for successful colonization of that habitat. *Third*, the rhizosphere was enriched for upregulated expression of genes related to quorum sensing and biofilm formation. This indicates that microbial cells are communicating with one another while colonizing the rhizosphere. Finally, upregulated expression of many genes related to the nitrogen cycle were also enriched in the rhizosphere. These genes include ammonia monooxygenase, nitrogenase, and genes related to nitrate reduction, while bulk soil showed enrichment for genes related to denitrification. Those data show that the rhizosphere microbial communities are fixing nitrogen and converting it to a form that would be eventually usable by the plant, whereas the bulk soil microbial community is actively respiring nitrate as an anoxic alternate electron acceptor.

That study illustrates the power of meta-transcriptomics to identify upregulated genes involved in the colonization fitness of different microbial communities, the diversity of various known metabolic pathways currently being utilized by the rhizosphere communities, and the induction of stress resistance and signaling events based on their transcriptional activity. Importantly, that study confirmed many earlier laboratory-based findings of plant–microbe interactions conducted on microbial populations.

Importance of the Rhizosphere to Plant Growth

All of the physical, chemical, and biological factors or conditions of soil that influence the physiology of plant growth are expressed through the rhizosphere and its associated microbial communities. Thus, the rhizosphere is the business end of soil where major,

important soil–plant relationships occur. The outcomes of ecological interactions between rhizosphere microorganisms and higher plants embrace the full range of beneficial, neutral, or harmful associations, including commensalisms (one partner benefits), mutualisms (both partners benefit), amensalisms (one partner impedes the success of the other without being affected positively or negatively by the presence of the other), and pathogenic consequences (one partner benefits by harming the other). This section presents brief examples on negative and positive effects of such interactions.

Negative Effects of Microbes on Plants

Hiltner's view of the rhizosphere as an important habitat for microbes supporting plant growth and health as well as causing plant disease is still completely valid. Both prokaryotic and eukaryotic rhizosphere colonizers can harm their plant host. Some microorganisms successfully compete with plants for nutrients in soil, thereby immobilizing and limiting their availability sufficiently to inhibit plant growth. Some pathogens are necrotrophs that kill the plant hosts and then proliferate while degrading them (e.g., *Erwinia carotovora* degrades the plant cell wall structure to enter karat roots and causes a root rotting disease). Certain eukaryotic microorganisms are major pathogens of plants. For example, the filamentous oomycete, *Pythium* spp. causes a devastating root rot disease of significant agricultural importance. This pathogen invades the roots (particularly of seedlings) of many plant species, eventually killing the plant hosts by sealing off their nutrient and water transport systems for its own use. Such plant pathogens cause enormous economic damage to agricultural productions every year and therefore are of special interests in rhizosphere research. Understanding the mechanisms and characteristics of soil-borne, root-infecting plant pathogens is a major task in plant-associated research to prevent diseases and increase agricultural productivity.

Positive Effects of Microbes on Plants

Many microbial associations can significantly benefit plants, thereby attracting special interest in various fields of microbial ecology, especially in aspects of agricultural sustainability when it involves an important crop plant. Bacteria that benefit plant growth while colonizing their roots are called plant growth-promotive rhizobacteria (PGPR). PGPR use numerous direct and indirect mechanisms to promote plant growth. One common example is the ability of PGPR to supply the plant with essential nutrients that are otherwise limiting in soil. Examples include nitrogen-fixing bacteria (called diazotrophs) that reduce dinitrogen gas into ammonia and provide this form of fixed nitrogen to supplement the plant's nitrogen nutrition. The root nodule symbiosis between rhizobia and legumes is the classic example that uses this mechanism directly to promote the growth of the legume plant host, particularly when grown in soils limited in available combined nitrogen. Another example in this direct benefit category is the mycorrhizal fungus, which colonizes roots of 95% of all vascular plants. Their external hyphae extend from the mycorrhizal root out into the soil where they efficiently access nutrients, especially phosphorus, and transport them into the plant roots, thereby significantly impacting on plant mineral nutrition. The beneficial association of mycorrhizal fungi with plants also provides protection against soil-borne root infecting plant pathogens. Mycorrhizal helper bacteria (MHB) are colonizing the surface and even the interior of mycorrhizal hyphae and spores as summarized by Frey-Klett et al. In addition to mycorrhizal fungi, also members of the Sebacinales order of fungal kingdom, like *Piriformospora indica*, readily colonize roots of many crop plants leading to increased growth, salt resistance and pathogen response as reviewed by Varma et al. Examples of the indirect benefit category involve increased bioavailability and enhanced acquisition of mineral nutrients include PGPR like *Azospirillum* or *Rhizobium* that stimulate elongation and branching of root growth (see section "A Case Study: *Rhizobium* Colonization of Rice Roots"). The resultant improved root architecture increases the plant's ability to access and exploit nutrient reserves in soil much more efficiently. Other examples using indirect mechanisms in this category include PGPR that solubilize soil complexes of inorganic and organic phosphates and iron that are otherwise largely insoluble in the rhizosphere and hence have limited availability to the plant. Crop plants can also benefit from the nitrogen fixation of root associated, mostly endophytic diazotrophs, like *Azoarcus* spp. in rice and *Gluconacetobacter diazotrophicus* and other diazotrophs in sugarcane.

Another major way that rhizosphere microorganisms improve plant health is mediated by their biocontrol against plant diseases. A direct benefit in this category includes the microbial stimulation of plant defense responses that induce systemic disease resistances in plants, enabling them to successfully defeat plant pathogenic attacks. Examples were revealed in studies using *Serratia liquefaciens* and *Rhizobium leguminosarum* that secrete bacterial quorum sensing molecules of the AHL-type inducing a systemic disease resistance reaction in tomato and *Arabidopsis* plants, respectively. In these cases, the induced systemic resistance system is primed leading to "immunization" of the plant that protects it against pathogen attack, as recently reviewed by Schikora et al. In the case of *Bacillus amyloliquefaciens*, the production of lipopeptides and other antibiotically active substances lead also to the induction of the plant's innate immune system resulting in improved defense against *Rhizoctonia solani*. Other beneficial microbe–plant interactions involve biocontrol mechanisms that act directly on the invasive pathogen itself rather than on the plant host. Examples include hyperparasitism whereby the PGPR aggressively attacks and kills the pathogen thereby reducing its inoculum potential, production of siderophores that restrict ferric (Fe^{3+}) availability and uptake by the pathogen, production of antimicrobial antibiotics that inhibit the pathogen's growth physiology thereby diminishing their virulence, and development of dense hyphal mantle sheaths of ectomycorrhizal filaments surrounding rootlets that impose physical barriers to block pathogen ingress.

Closing Remarks

The plant rhizosphere harbors a diverse reservoir of culturable and non-culturable microorganisms that can be exploited to benefit mankind. Many rhizosphere microbes benefit crop production, reducing the dependence on chemical fertilizers to achieve high productive yields. Others protect plants from the ravages of pathogens and the diseases they cause. Utilization of these beneficial examples is fully consistent with sustainable agriculture, where the goal is to utilize natural processes that promote the crop's output without irreparably damaging the natural resource base where the crop can be grown. Finally, the rhizosphere studies contribute to the greater understanding of plant microbiomes and plant holobionts. These metagomic approaches are now even integrated with agronomic and environmental aspects into the holistic "phytobiome concept" of sustainable crop production.

Among the many recent discoveries in rhizosphere research is the ominous finding that certain potential human pathogenic microorganisms are also successful colonizers inhabiting this nutrient-enriched plant soil environment, and this ecology poses potential public health hazards for the people (both producers and consumers) who encounter them. Furthermore, there are also several examples reporting that some rhizosphere bacteria have probiotic abilities. However, care has to be taken, because it is quite challenging to clearly separate beneficial from possibly health threatening microbes. Much more experience and in depth genomic, in situ and functional studies of these organisms are necessary to reach the required safety level.

Acknowledgments

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RNA Processing☆

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Glossary

Distributive Describing an enzyme that releases its substrate after each catalytic cycle.
Endoribonuclease Enzyme that cuts RNA internally.
Exoribonuclease Enzyme that chews RNA one nucleotide at a time from either the 5′ or 3′ end.
Homolog(ous) Having the same origin.
Intergenic region Sequence between two open reading frames.
Paralog(ous) Having the same origin, but in a single organism.
Processive Describing an enzyme capable of performing many catalytic cycles on the same molecule before releasing it.
Primary transcript Transcript first synthesized by RNA polymerase from point of transcription initiation to point of transcription termination.
Ribonuclease Enzyme that cuts or chews RNA.
RNA processing/maturation Conversion of precursor RNA to shorter, functional and usually more stable species by ribonucleases.
Shine-Dalgarno Bacterial ribosome binding site, complementary to 3′ end of 16S rRNA, named after its discoverers.

Defining Statement

RNAs are synthesized as so-called primary transcripts extending from the point of transcription initiation by RNA polymerase to the point of transcription termination. In some cases, the primary transcript is the final functional molecule. In others, the primary transcript is "processed" to a shorter, more stable functional species by ribonucleases (RNases).

Introduction

While primary messenger RNA (mRNA) transcripts can typically be bound by ribosomes and used directly for translation, stable RNAs such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), are almost always processed from the primary transcript into shorter functional molecules. RNases remove sequences from the 5′ or 3′ end of stable RNA precursors, usually both. As we will see later, some mRNAs are also processed, that is, the major translated species found in the cell in vivo is not the primary transcript. The classic case is where an mRNA encodes more than one protein and is processed between cistrons. This can have important consequences for the stoichiometry of the different proteins produced from a single operon. At the outset, therefore, it is useful to distinguish the term "RNA processing" (or maturation), which is used to describe RNase-catalyzed events leading to the generation of a functional RNA, from "RNA degradation," used to describe its decay. The first generally leads to an increase in RNA half-life relative to the primary transcript and, the second, to a decrease. In this article, we first describe some specific examples of mRNA processing and then focus for the remainder of the review on the maturation of stable RNAs. The vast majority of the work we describe will be from studies in *Escherichia coli* and *Bacillus subtilis*, the best-studied examples of the Gram-negative and Gram-positive bacteria, respectively. We emphasize some of the historical examples that served as paradigms for the different types of processing events. New examples are regularly discovered that give further weight to these pioneering studies.

The Players

Ribonucleases come in two flavors; endoribonucleases, which cut or cleave RNAs internally, and exoribonucleases, which remove nucleotides one at a time from either the 3′ or 5′ end. The former are typically symbolized as a pair of scissors, while exoribonucleases are usually represented by a "Pacman" symbol. Exoribonucleases can be either processive, remaining attached to the same RNA molecule for many rounds of catalysis, or distributive, releasing the substrate with each nucleotide removed. They can also be

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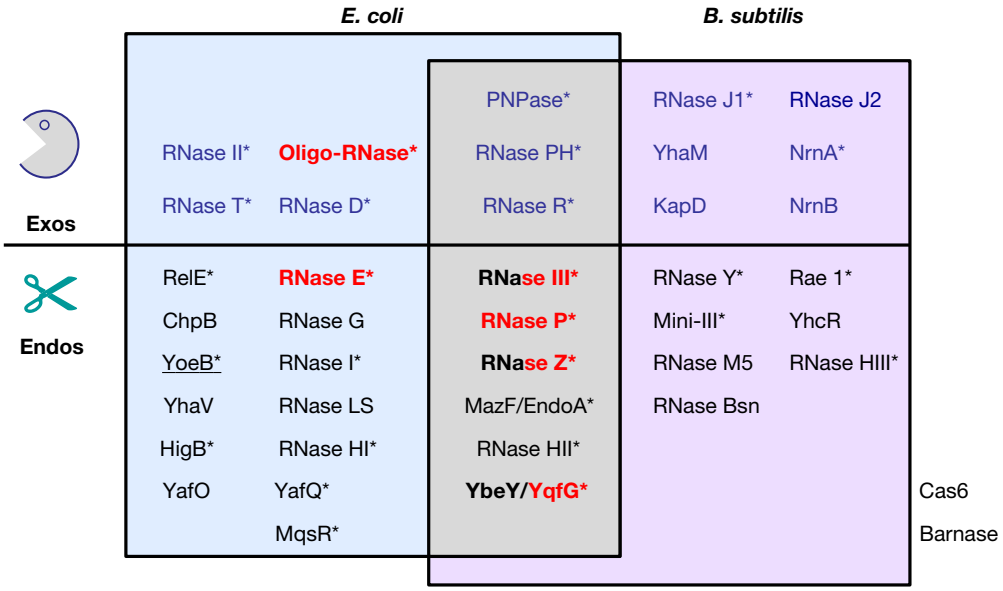

Fig. 1 Venn diagram of ribonucleases identified in bacteria. Exoribonucleases (Exos) are above the horizontal dividing line, mostly in *blue type* and represented by a "Pacman" symbol. Endoribonucleases (Endos) are below the dividing line, mostly in *black type* and represented by a scissors symbol. Enzymes unique to *E. coli* are in the *blue square* on the left, those unique to *B. subtilis* are in the *pink square* to the right, and those in common are in the *gray square* in the center. Essential enzymes are in *bold red type*. Shared enzymes that are essential to *B. subtilis*, but not *E. coli* are in *black type* on the *E. coli* side of the Venn diagram, and in *red type* on the *B. subtilis* side. RNase J1 and J2 are in *gray type* as endonucleases as the relevance of this activity in vivo is under question. Only two bacterial enzymes have so far been identified that are not present in either *E. coli* or *B. subtilis*, Barnase, found in some Gram-positive species, and Cas6, part of the CRISPR defense mechanism.

either hydrolytic, consuming a molecule of water upon the breaking of each phosphodiester bond, or phosphorolytic, using inorganic phosphate instead. Most RNases are hydrolases. Not surprisingly, bacterial RNases have been studied most extensively in *E. coli*. In recent years, however, significant progress has been made in *B. subtilis*, where 22 RNases have now been identified (vs. 26 in *E. coli*) and it is clear that most of the enzymes are different from those of *E. coli* (only 9 are in common). While *E. coli* appears to only possess 3′ exoribonucleases, for example, exonucleases of either orientation (5′ or 3′) can be found in *B. subtilis* (as in eukaryotes). A Venn diagram showing the full set of currently known RNases of *E. coli* and *B. subtilis* is shown in Fig. 1. Many of these enzymes have specialized functions in RNA processing, which we will describe as we go along. The three-dimensional structures of many of these enzymes are known, permitting dissection of their activity mechanisms at the molecular level (indicated by an asterisk in Fig. 1).

Messenger RNA Processing

Ribonuclease processing of mRNAs leading to an increase in stability over the primary transcript has been described in a number of cases in both Gram-negative and Gram-positive bacteria. In many of the examples described in the literature, an endonucleolytic cleavage event occurs in the intergenic region between two open-reading frames encoded by the same RNA, maintaining the translational integrity of each fragment. In many cases, however, the upstream and downstream products of the cleavage reaction have different chemical and functional half-lives. Such mechanisms are typically exploited to produce different stoichiometric amounts of different products of the same operon. A regulator of an operon, for example, is often required in much lower quantities than the enzymes or structural components of the cognate pathway and thus the RNA fragment encoding a regulatory protein is typically destabilized by this kind of processing event, while the remaining fragment remains relatively resistant to RNases.

Messenger RNA Processing in *E. coli*

RNase E is the primary enzyme involved in intergenic RNA processing in *E. coli*. This is without doubt the key endoribonuclease of *E. coli*, and plays a role in mRNA processing and decay, and in stable RNA maturation. It is a large (1061 amino acids), essential protein that has an N-terminal catalytic domain and an intrinsically disordered C-terminal domain permitting both association with the inner membrane and interactions with other proteins, such as the RNA helicase RhlB, the 3′ exoribonuclease

polynucleotide phosphorylase (PNPase), and the glycolytic enzyme enolase. This complex is known as the degradosome and similar RNase E-based degradosomes have been found in many organisms, with some interesting variations in the associated proteins. Although the importance of the degradosome in *E. coli* RNA decay is generally accepted, its role in RNA processing is less clear. Only the catalytic domain is necessary for the maturation of 5S rRNA and the RNase P RNA subunit, for example. The structure of the catalytic domain of RNase E has been solved in complex with RNA (pdb 2C0B). *E. coli* also possesses a nonessential paralog of the catalytic domain of RNase E, known as RNase G. Both enzymes have overlapping (although nonidentical) cleavage site specificities, that is to say, AU-rich single stranded regions.

Other enzymes, such as RNase III and RNase P, have been implicated in intergenic cleavages of a small number of specific mRNAs in *E. coli* and *Salmonella*. These enzymes play a more important role in rRNA and tRNA maturation and will be described in more detail later.

The rpsU *dnaG* rpoD *operon*

One of the first described examples of intergenic processing of mRNA in *E. coli* was the *rpsU dnaG rpoD* operon, encoding ribosomal protein S21, the DNA primase DnaG and the sigma-70 subunit of RNA polymerase, respectively (Fig. 2A). Primary transcripts of this operon are transcribed from tandem promoters and extend to either a weak transcription terminator located between *rpsU* and *dnaG* or a strong terminator at the 3′ end of the operon. The long primary transcripts are cleaved between the *dnaG* and *rpoD* open reading frames by RNase E, leading to rapid degradation of the DnaG-encoding upstream portion and a stable downstream fragment encoding sigma-70. The terminator hairpin between *rpsU* and *dnaG* presumably protects the S21-encoding RNA from attack by 3′ exoribonucleases degrading the *dnaG* RNA fragment. The particular processing and degradation pattern of this operon leads to the production of an estimated 50,000 molecules of S21, 50 molecules of DNA primase and 3000 copies of sigma-70 per cell.

The pap *operon*

The *pap* locus of uropathogenic *E. coli* strains encodes 11 proteins involved in pilin formation. Transcription of the rightward operon begins at the *papB* promoter and is attenuated at a terminator hairpin downstream of the second cistron *papA* (Fig. 2B). The *papBA* primary transcript is processed between the two open reading frames by RNase E, and the upstream fragment, encoding the regulator of the operon PapB, is rapidly degraded, while the downstream portion, encoding the major pilin protein PapA, is stable. The primary transcript has a half-life of 2.5 min while that of the PapA encoding fragment is 27 min, accounting in large part for the relative proportions of the regulatory and structural proteins of this operon.

Many other examples of intergenic processing have been found in *E. coli*, too numerous to describe here. The principle is always the same; endonucleolytic cleavage followed by selective degradation of the upstream fragment by 3′ exonucleases, while the downstream fragment remains relatively stable.

Bacteriophage mRNA Processing

Bacteriophage mRNAs tend to be highly processed. In some cases, the processing event does not lead to an increase in mRNA stability but rather to an increase in translation. The *N* gene of phage lambda and the *0.3* gene of phage T7 are two such examples.

The lambda N *gene*

The *N* gene of lambda is the first gene transcribed from the P_L promoter of the leftward operon. Transcription of the *nutL* sequence between the P_L promoter and the *N* gene leads to the assembly of an antitermination complex on RNA polymerase, that includes the *nut* RNA itself, the N protein and at least four host factors NusA, NusB, NusG and ribosomal protein S10 (Fig. 3A). This antitermination complex is critical for lambda development, as it allows RNA polymerase to read through multiple transcription terminators in this operon. The proximity of the N protein in the antitermination complex to its own Shine Dalgarno (SD) also causes steric inhibition of ribosome binding and hence auto-repression at the translational level. This translational auto-repression can be relieved (about 200-fold) by cleavage of a hairpin structure between *nutL* and the ribosome binding site by the double-strand nuclease RNase III, freeing up the SD element for ribosome binding. Lambda development is therefore highly sensitive to the RNase III activity of its host. As we will see below RNase III is occupied primarily in rRNA maturation in *E. coli*. Thus, changes in growth conditions, which affect rRNA synthesis, would be predicted to temporarily alter the amount of free RNase III in the cell and have a knock-on effect on lambda growth.

The phage T7 0.3 *gene*

Bacteriophage T7 early mRNAs are quite stable, with half-lives in the 10–20 min range. A 7 kb precursor transcript, made by the host RNA polymerase soon after infection, is processed in five places by RNase III (Fig. 3B). In most cases, RNase III processing occurs on the distal side of hairpin structures, creating recessed 3′ ends that are resistant to 3′ exoribonuclease action, likely accounting for the high degree of early transcript stability. The *0.3* gene is the first gene of the early T7 operon and it plays a role in relieving host DNA restriction. Like the lambda *N* gene, the *0.3* gene transcript possesses a stem-loop structure in its 5′ untranslated region. This hairpin is also cleaved on its distal side by RNase III. The stability of the downstream *0.3* portion of the transcript is unaffected by processing, however. Like lambda *N*, processing rather appears to influence the accessibility of the SD sequence to ribosome binding.

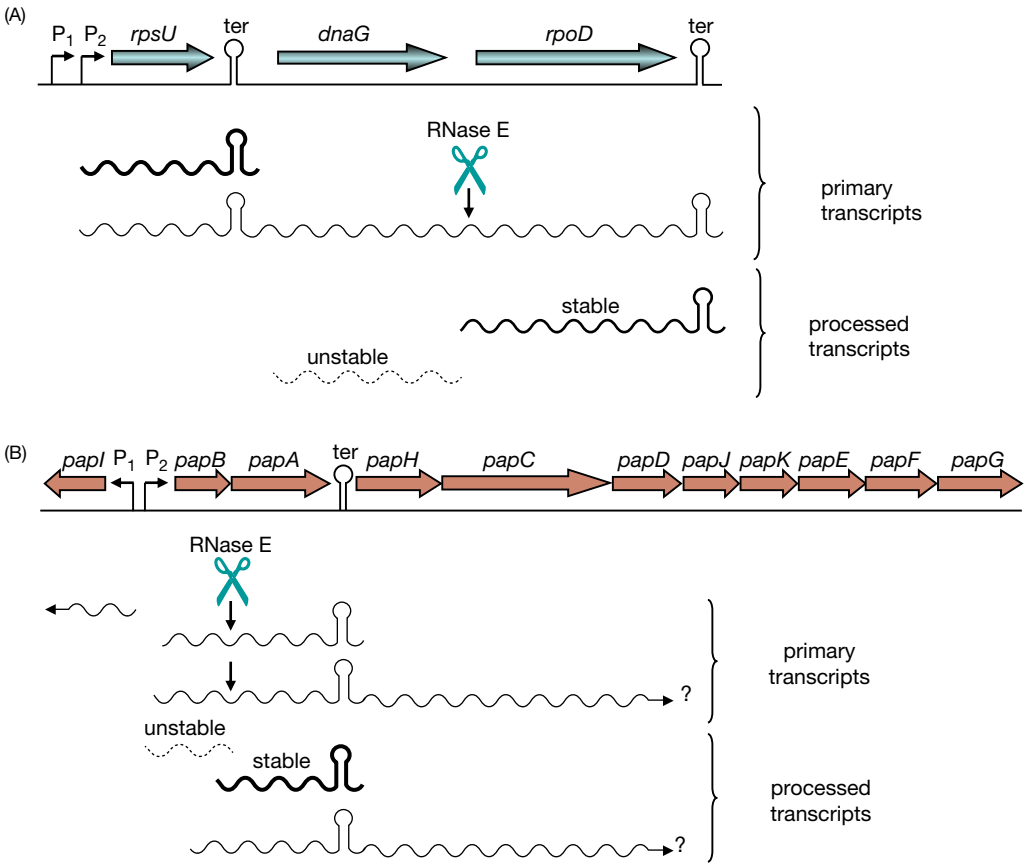

Fig. 2 Processing of mRNA in *E. coli*. (A) Organization of the *rpsU dnaG rpoD* operon and processing of its primary transcripts. Operon promoters are indicated as P_1 and P_2. Open reading frames and their orientation are indicated by *light blue arrows*. Rho-independent transcription terminators (ter) are also shown. Transcripts are indicated by *wavy lines* and amounts detected in vivo are roughly proportional to the thickness of the lines. The site of RNase E cleavage in the intergenic region between *dnaG* and *rpoD* is represented by a *blue scissors*. (B) Organization of the *pap* locus of uropathogenic *E. coli* and processing of its primary transcripts. Promoters are indicated as P1 and P2. Other symbols and conventions are as in (A). The 3′ end of the long rightward *pap* transcript is unknown and indicated by a *question mark*.

Retroregulation of the lambda int *gene*

The lambda *int* gene, encoding the integrase protein, provides an example where processing leads to a decrease rather than an increase in mRNA stability. One can justify considering this a processing pathway rather than a simple RNA decay mechanism on the grounds that targeted degradation of the *int* mRNA is part of a regulatory switch that determines whether the phage will enter the lysogenic or lytic development cycle. The *int* gene of lambda is transcribed from either the P_I or P_L promoter, depending on the stage of the phage life-cycle, but only the P_I transcript is translated. The P_I transcript traverses the *int* gene and ends at a Rho-independent terminator immediately downstream. The P_L transcript, as we saw earlier, is synthesized by terminator-resistant RNA polymerase, and extends further downstream. The longer read-through transcript can fold into a hairpin structure called *sib*, which is a site for endonucleolytic processing by RNase III (Fig. 3C). Cleavage at this site provides access to the mRNA by the 3′ exoribonuclease PNPase (pdb 1E3P) and the *int* transcript is rapidly degraded. In this mode, the phage does not synthesize the integrase protein and remains in the lytic cycle. This phenomenon has been called retroregulation because the regulatory elements are located downstream of the target gene.

Messenger RNA Processing in *B. subtilis*

Examples of mRNA processing have also been seen in *B. subtilis*. Interestingly, many Gram-positive bacteria do not possess RNase E or RNase G homologs, with the *Clostridia* and the *Actinobacteria* being notable exceptions. Although there has been evidence for some time of an RNase *E*-like activity in *B. subtilis*, the identity of this enzyme remained elusive until recently. Although RNases J1 and J2 were originally proposed to fulfill this role, there is now a general consensus that a different enzyme, RNase Y, is the functional analog of RNase E in *B. subtilis*, despite a complete lack of homology between the two enzymes. RNase Y has similar cleavage specificity to RNase E and is found associated with the membrane through its N-terminal trans-membrane domain like its protobacterial analog.

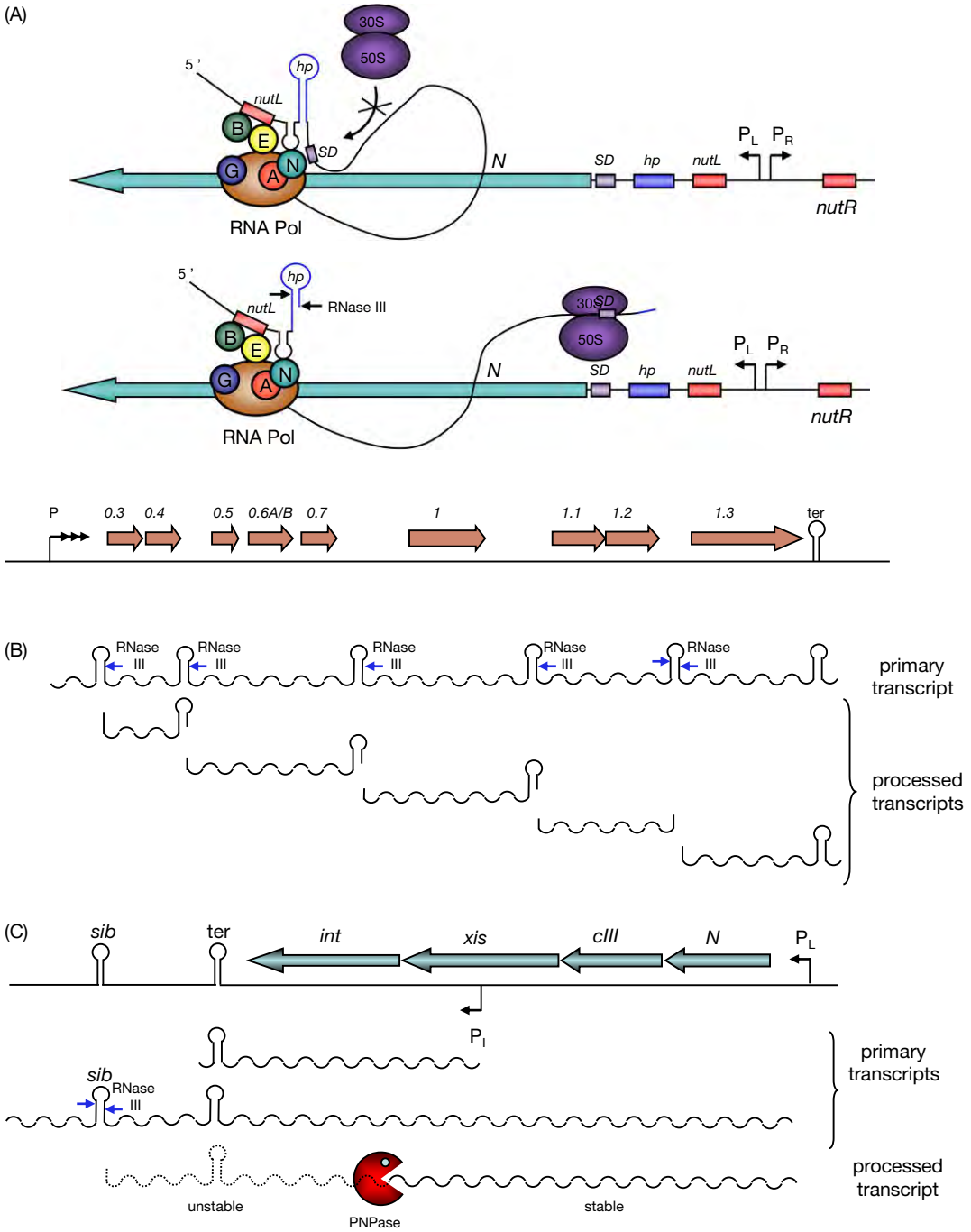

Fig. 3 Processing of phage mRNA. (A) Transcriptional and translational control of the lambda *N* gene. The top panel shows the assembly of the antitermination complex on the leftward operon of lambda, transcribed from promoter P_L. A similar complex is formed during transcription of the rightward operon from promoter P_R. The *N* open reading frame is indicated by a *leftward oriented arrow*. The positions of *nutL*, RNase III hairpin (hp) and Shine-Dalgarno (SD) elements are indicated. Transcription of *nutL* by RNA polymerase (RNA Pol) leads to the formation of an antitermination complex, containing the *nutL* RNA itself, N protein, and host factors NusA, NusB, NusG and NusE (=ribosomal protein S10), represented by the letters A, B, G and E, respectively. The 5′ end of the *N* transcript (represented by a *curved black line*) remains attached to RNA polymerase through the interaction with *nutL*. In this conformation, ribosomes do not have access to the SD element of the N coding sequence. The lower panel shows cleavage of the hairpin (hp) by RNase III, freeing up the SD element for ribosome binding and translation of *N*. (B) Organization of the phage T7 early operon and processing of its primary transcript. The operon's three promoters (P) are indicated by the *triple headed arrow*. Open reading frames and their orientation are indicated by *brown arrows*. The operon's Rho-independent transcription terminator (ter) is also shown. Transcripts are indicated by *wavy lines* and sites of RNase III cleavage in five intergenic regions are indicated with *blue arrows*. (C) Organization of the phage lambda leftward operon and retroregulation of *int* gene expression. For description, see text. Promoters are indicated as P_I and P_L. Open reading frames and their orientation are indicated by *light blue arrows*. The transcription terminator (ter) and site of RNase III cleavage (sib) are indicated. PNPase is represented by a red "Pacman" symbol.

RNase Y has been proposed to be a scaffold for a degradosome-like network (DLN) in *B. subtilis* involving PNPase, an RNA helicase, CshA, and glycolytic enzymes. It has also been shown to interact with three proteins, YlbF, YmcA and YaaT, to form a separate complex, known as the Y-complex. Although RNase Y has mostly been shown to be involved in mRNA turnover, a number of RNase Y-dependent cleavages have been discovered that can be considered as processing events. Recent data suggest that the Y-complex might be specifically involved in RNase Y processing events allowing differential abundance of mRNA fragments.

In addition to RNase Y, *B. subtilis* has another major RNA enzyme involved in RNA processing and decay, the 5′-exoribonuclease RNase J1. While neither RNase J1 nor RNase Y are essential on their own, a double mutant lacking RNase Y and RNase J1 is inviable, as is a double mutant lacking RNase J1 and the 3′-exoribonuclease PNPase. RNase J1 forms a complex with its paralog RNase J2, with RNase J1 providing most of the 5′-exonucleolytic activity. Although both enzymes have endonucleolytic activity at high concentrations in vitro, the in vivo relevance of this additional activity is not clear. *B. subtilis* RNase J1 is a member of the β-CASP family of metallo-β-lactamases and its structure has been solved (pdb 3ZQ4). Genome analysis has revealed that most bacterial phyla contain at least two of three major ribonucleases: RNases E/G, J, or Y.

The thrS *mRNA*

The threonyl-tRNA synthetase gene of *B. subtilis thrS* is one of about 20 so-called T-box genes in this organism, regulated by a tRNA-dependent antitermination mechanism. Genes regulated by this mechanism are involved in amino acid metabolism and are sensitive to the ratio of uncharged to charged levels of the cognate tRNA. Hence, expression of the *B. subtilis thrS* gene is induced in response to threonine starvation. Binding of uncharged $tRNA^{Thr}$ to the *thrS* leader sequence stabilizes an antiterminator structure in the mRNA at the expense of a mutually exclusive transcription terminator upstream of the *thrS* coding sequence. Under threonine starvation conditions, the major 5′ end found in vivo corresponds to a processing event that occurs nine nucleotides (nts) upstream of the leader terminator hairpin (Fig. 4A). The cleaved transcript is about fivefold more stable than the primary transcript, presumably due to the presence of a hairpin structure protecting each extremity from further degradation by exonucleases. At least five other T-box mRNAs are processed in a similar manner just upstream of the leader terminator and thus this appears to be a general mechanism to amplify the antitermination effect for this family of genes in *B. subtilis*. Although cleavage at this site was originally attributed to the endonucleolytic activity of RNase J1/J2 based on in vitro studies, it was subsequently shown that RNase Y is a more likely candidate for this cleavage in vivo. Indeed, the leader regions of at least five of the six T-box mRNAs known to be processed upstream of their terminators accumulate in strains depleted of RNase Y, but not in strains depleted of RNase J1.

The hbs *gene*

Evidence for mRNA processing by RNase J1, exploiting its 5′ exoribonuclease activity, has also been seen in *B. subtilis*. The *hbs* gene, is transcribed from two promoters, about 210 and 180 nts upstream of the open reading frame encoding the histone-like protein HU. A third major transcript is also observed in vivo, whose 5′ end maps about 20 nts upstream of the *hbs* start codon. Although originally thought to represent transcription from yet another promoter, the 5′ end of this stable transcript is in fact generated by 5′-exoribonuclease digestion of the *hbs* mRNA by the RNase J1/J2 complex until it bumps up against ribosomes initiating translation at its very strong Shine-Dalgarno (SD) sequence. Increasing the strength of the *hbs* SD increases the level of this mRNA species in the cell, while weakening only slightly it causes this transcript to disappear completely (Fig. 4B). The 5′ ends of the two primary *hbs* transcripts are highly protected by secondary structure. RNase Y cleaves in the 5′-UTR sequence, allowing RNase J1/J2 access to the mRNA just upstream of the ribosome. Such a pathway potentially provides a built-in destruction mechanism for the *hbs* RNAs once it has surpassed its usefulness for translation, as access to the coding sequence is no longer protected by ribosomes from 5′-exonucleolytic attack. A similar example of processing and RNA protection by ribosomes has been identified for the *cryIIIA* mRNA, encoding the insecticidal crystal toxin of *B. thuringiensis*, expressed in *B. subtilis*. In this case a strong SD-like sequence with no associated start codon, called the stab-SD, binds a 30S ribosomal subunit and blocks RNase J1/J2 progression, stabilizing the mRNA for many kilobases downstream.

Intergenic mRNA processing

Several cases of intergenic processing have been described in *B. subtilis*, similar to those described for the *rpsU dnaG rpoD* and *papBA* operons of *E. coli* above. The best known examples are those of the *gap* operon, encoding six genes involved in the interconversion of triose phosphates of the glycolytic pathway (Fig. 4C), and the *dnaK* heat shock operon (Fig. 4D). In both of these cases, processing occurs between the first and second ORFs of the operon. The RNA fragment containing the first ORF, encoding the regulator of the operon is quickly degraded, while the downstream fragment is relatively stable. The differential stability of the upstream and downstream cleavage products can account for the differences in stoichiometry between the regulator and other proteins of the operon. RNase Y has been shown to be responsible for the cleavage in the *gapA* operon mRNA. This processing event was recently shown to require the Y-complex. Interestingly, the *cggR* (*gapR*)-*gapA* operon of *S. aureus* is similarly matured by RNase Y and requires YlbF, one of the Y-complex proteins, suggesting that the function of the Y-complex is conserved among the Firmicutes.

Stable RNA Processing

Ribosomal RNA Processing

In *E. coli* and *B. subtilis*, ribosomal RNAs are transcribed from *rrn* operons containing the genes for 16S, 23S and 5S rRNA, in that order, sometimes interspersed with tRNAs (Fig. 5A). Although this is the most commonly encountered scenario throughout the

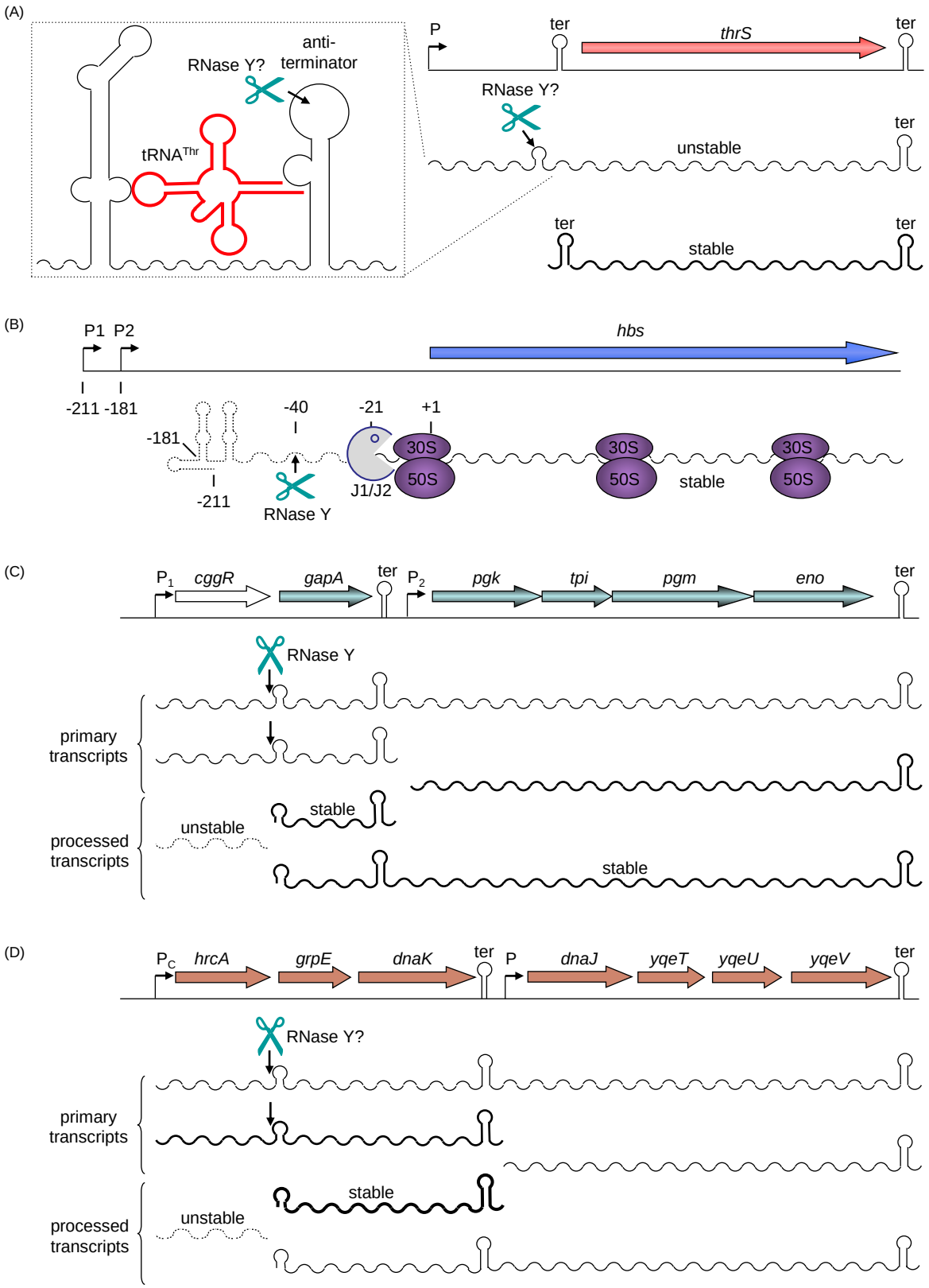

Fig. 4 Processing of mRNA in *B. subtilis*. (A) Antitermination and processing of the *thrS* transcript. The *left panel* shows binding of $tRNA^{Thr}$ (*red* cloverleaf structure) to the *thrS* leader mRNA, stabilizing the antiterminator hairpin. The position of suspected RNase Y cleavage is indicated by a *blue scissors*. The right panel shows the transcriptional organization of the *thrS* gene, with promoter (P) and terminators (ter). Transcripts are indicated by *wavy lines*, with the thickness of the line roughly proportional transcript abundance in vivo. (B) Organization of the *B. subtilis hbs* gene and processing of its transcript. The 5′ exoribonuclease activity of the RNase J1/J2 complex is represented by a "Pacman" symbol. The RNase Y cleavage is indicated by a scissors symbol. Ribosomal subunits are represented by purple ovals. Nucleotide coordinates are given above the *hbs* transcript, indicated by a *wavy line*. (C) Organization of the *gap* operon and processing of its primary transcripts. Symbols and conventions are the same as in previous figures. RNase J1 is the prime candidate for cleavage at the site indicated by the *scissors symbol*. (D) Organization of the *dnaK* heat-shock operon and processing of its primary transcripts. Symbols and conventions are the same as in previous figures. RNase J1 is also the prime candidate for cleavage at the site indicated by the *scissors symbol*.

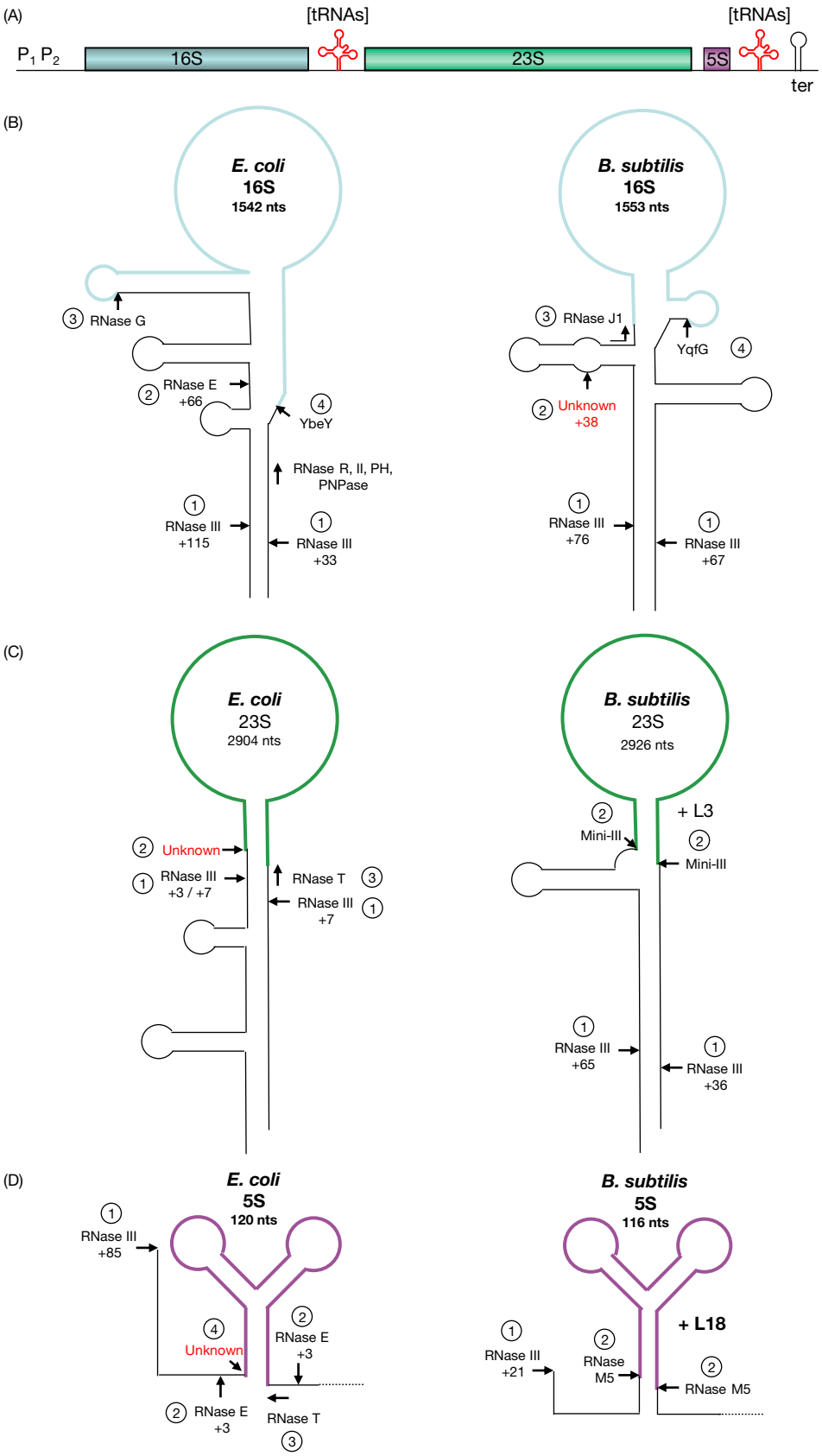

Fig. 5 Processing of rRNA in *E. coli* and *B. subtilis*. (A) General organization of rRNA operons. Transcription begins at tandem promoters (P_1 and P_2) and ends at the transcription terminator (ter). Transfer RNA genes [tRNAs] sometimes occur in the spacer regions between the 16S and 23S rRNA genes or downstream of the 5S gene. (B) Processing of 16S rRNA in *E. coli* (left) and *B. subtilis* (right). The mature 16S sequence is in *light blue*, precursor sequences in *black*. The number of extra nts remaining following processing at each site is indicated below the enzyme in question. Unknown enzymes are indicated in *red* type. Endonucleolytic cleavages are represented by *black arrows* perpendicular to the transcript; exonucleolytic digestion is represented by *black arrows* parallel to the transcript. Although the different cleavage steps are numbered, the order of cleavages is not obligatory for 16S rRNA maturation in either organism. Adapted from Britton et al. (2007). *Molecular Microbiology* **63**,127–138. (C) Processing of 23S rRNA in *E. coli* (left) and *B. subtilis* (right). The mature 23S sequence is in *green*, precursor sequences in *black*. Mini-III processing of *B. subtilis* 23S rRNA requires r-protein L3. Symbols and conventions are as in (A). In *E. coli*, RNase III cleavage is an obligatory first step for generation of mature 5′ and 3′ ends. The order of steps 2 and 3 is not known. In *B. subtilis*, accurate maturation can occur in the absence of RNase III. (D) Processing of 5S rRNA in *E. coli* (left) and *B. subtilis* (right). The mature 5S sequence is in *mauve*, precursor sequences in *black*. RNase M5 processing of *B. subtilis* 23S rRNA requires r-protein L18. Symbols and conventions are as in (A). As for 16S rRNA, prior cleavage by RNase III is not obligatory in either organism.

bacterial kingdom, there are scattered cases where the 16S and 23S genes are not cotranscribed. The *rrn* operons of *E. coli* and *B. subtilis* are transcribed as long 30S precursor RNAs from tandem promoters upstream of the operon. The 5′ and 3′ flanking sequences of both 16S and 23S rRNA can base pair to form long "processing stalks" which are substrates for RNase III. RNase III is the founder member of a large family of double-stranded RNases including Dicer, well known for its role in posttranscriptional gene silencing in eukaryotes. RNase III consists of an N-terminal catalytic domain and a C-terminal double-stranded RNA binding domain (dsRBD) and forms dimers (pdb 1RC7). It generally cleaves on both strands of long double-stranded helices to produce RNAs with a recessed 5′ end and a 2-nt 3′-overhang. Particular sequence and structure contexts can cause it to cleave on just one of the two strands, however. In *E. coli*, RNase III cleavage of the 30S rRNA precursor is known to occur as it is being transcribed and leads to separation of the 16S, 23S and 5S rRNA species. Processing by RNase III is not absolutely necessary for the production of functional rRNAs in either of these two organisms, however. The RNase III-generated processing intermediates are whittled down to their mature sizes by other enzymes, in many cases subsequent to subunit assembly or even translation initiation. With one notable exception (see below), the enzymes responsible for the late steps of rRNA maturation in *B. subtilis* have proven to be different to those in *E. coli*. In fact, in most cases, the enzymes involved are unique to each species.

16S rRNA maturation

The mature 5′ side of *E. coli* 16S rRNA is generated by the cooperative action of RNase E and RNase G (Fig. 5B). RNase E first cleaves 66 nts from the mature 5′ end and this facilitates the final endonucleolytic cleavage by RNase G, presumably by permitting some structural rearrangement of the RNA. These reactions occur after ribosome assembly.

In *B. subtilis*, 16S rRNA 5′ maturation is catalyzed by the 5′-exoribonuclease activity of RNase J1, presumably in complex with RNase J2, since this is the primary form of both enzymes found in vivo. Some feature close to the 5′ end of 16S rRNA in the assembled ribosome, for example, a bound ribosomal protein, is required to tell RNase J1 where to stop. A processing intermediate, generated by an unknown RNase, accumulates 38 nts from the mature 5′ end in *B. subtilis* cells lacking RNase J1.

Two different pathways have been proposed for the maturation of the 3′ end of 16S rRNA in *E. coli.* One involves a new enzyme called YbeY (pdb 1XM5). Although a mutation in the *ybeY* gene was shown to affect processing of all three rRNAs, it was proposed that YbeY specifically catalyzes the endonucleolytic processing of the 3′ end of 16S rRNA. Another group has more recently proposed that 3′-maturation can also be catalyzed by a number of redundant 3′-exoribonucleases including PNPase, RNase PH, RNase R and RNase II. One of these enzymes, RNase R, has also been shown to perform exonucleolytic trimming of 16S precursors in the psychrophilic γ-Proteobacterium *Pseudomonas syringae*. Recently, it was shown that the 3′ processing pathway of 16S rRNA in *B. subtilis* involves the YqfG enzyme, an ortholog of *E. coli* YbeY. Indeed, the *B. subtilis yqfG* gene can complement the *E. coli ybeY* deletion mutant for 16S rRNA processing. In contrast to *E. coli* YbeY, YqfG is essential in *B. subtilis*, but the lethality of the *ygfG* gene knockout can be suppressed by also deleting the gene encoding RNase R (*rnr*). This suggests that, unlike the proteobacterial RNase R, which can act in an alternative processing pathway for 16S rRNA 3′-maturation, RNase R in *B. subtilis* acts mainly in a quality control pathway to rapidly degrade 16S rRNA species that YqfG has failed to process. In the absence of RNase R, 16S rRNA species with 3′-extensions, which would be expected to be highly inefficient in translation, accumulate to sufficient levels to allow enough translation for cell viability.

23S rRNA maturation

Although accurate processing of *E. coli* 16S rRNA still occurs in the absence of RNase III, maturation of 23S rRNA fails completely. Thus, RNase III processing is an obligatory first step for 23S rRNA maturation in this organism. The 23S precursor RNA can still be assembled into functional ribosomes, however. RNase III cleavage of the 23S precursor produces an intermediate species with three to seven extra nucleotides on the 5′ end and seven to nine extra nucleotides on the 3′ end (Fig. 5C). The enzyme responsible for the subsequent generation of the mature 5′ end remains unknown, while the 3′ end is matured by the distributive 3′-exoribonuclease RNase T. RNase T (pdb 2F96) is an important enzyme in *E. coli* that can degrade to within one or two nucleotides of double-stranded stems. It is a member of the DEDD family of exoribonucleases that includes oligoribonuclease, which degrades small oligonucleotides in the 2–5 nt range, and RNase D, which is involved in tRNA maturation (see below). The final maturation steps of both the 5′ and 3′ end of *E. coli* 23S rRNA are most efficient under conditions of protein synthesis, suggesting that some structural rearrangement occurs at the onset of translation that allows better access to the two extremities.

Maturation of 23S rRNA in *B. subtilis* follows a different pathway than *E. coli*. In this case, the base-paired 5′ and 3′ ends of 23S rRNA are matured by an enzyme homologous to the catalytic domain of RNase III, and lacking the dsRBD, called Mini-RNase III (pdb 4OUN). Endonucleolytic cleavage by Mini-III on both sides of the processing stalk to directly yield 5′- and 3′-mature 23S rRNA requires prior binding of the large subunit RNA by the ribosomal protein L3. This provides a potential quality control check for correct ribosome assembly before proceeding with the final maturation step. Interestingly, this L3 quality control step is also used in the maturation of the large subunit RNA in *S. cerevisaie*, but with a different processing enzyme. In *B. subtilis*, prior cleavage of the large 23S processing stalk by RNase III is not necessary for Mini III action, and an alternative back-up pathway exists in Mini-III deletion mutants (*ΔmrnC*) that results in slightly different 5′ and 3′ ends. This pathway is catalyzed by exoribonucleases: RNase J1 at the 5′ end and RNases PH and YhaM at the 3′ end, but its physiological relevance is unknown. It may be the vestige of an older pathway that can also be found in some proteobacteria such as *Sinorhizobium meliloti*.

Some variations on the pathways of 23S rRNA processing can be seen in other organisms. In some species, including *Salmonella*, the 23S rRNA contains short intervening sequences that require removal by RNase III processing. In these cases, the "23S" rRNA is assembled into 50S subunits as fragments. In *Deinococcus radiodurans*, maturation of the 3′ end of 23S rRNA is catalyzed principally by RNase PH and RNase II.

5S rRNA maturation

RNase III processing of 23S rRNA releases the 5S rRNA precursor in both *E. coli* and *B. subtilis* and defines the 5′ end of the naturally occurring precursor species. The 3′ end is quite variable, however, and depends on the distance to the terminator hairpin or downstream tRNA for each of the individual *rrn* operons (there are 7 of these in *E. coli* and 10 in *B. subtilis*). Once again, these two organisms have found very different strategies for the final maturation steps of an rRNA. In *E. coli*, RNase E cleaves in a single-stranded region on either side of the 5S rRNA, producing an intermediate species with three extra nucleotides at each end (Fig. 5D). The 3′ exonuclease RNase T catalyzes the final maturation of the 3′ end, while the enzyme that removes the 3 nts from the 5′ end is still unidentified. In *P. syringae*, the 3′ maturation reaction is carried out by RNase R at cold temperatures.

B. subtilis, on the other hand, uses the double-stranded maturase RNase M5, cleaving once on each strand and producing the mature 5S rRNA in a single endonucleolytic step. RNase M5 is a small protein (20.5 kDa) that belongs to the Toprim domain family of enzymes, including the topoisomerases and the primase DnaG. It is essentially confined to the low G + C Gram-positive bacteria. Cleavage requires the binding of ribosomal protein L18 to 5S rRNA, presumably to fold it correctly for processing. Thus, like 23S rRNA, final processing of 5S rRNA is subjected to a quality control step. Strains lacking RNase M5 can no longer mature 5S rRNA, but yet are viable despite the presence of long (up to 165 nts) 5S rRNA precursors in their translating ribosomes.

Transfer RNA Processing

Transfer RNA genes are found in many different contexts in bacteria, as single transcription units, cotranscribed with other tRNAs, or cotranscribed with other components of the translational apparatus, such as ribosomal RNAs or elongation factors. In *E. coli*, all tRNAs have the so-called CCA motif encoded by their genes. This is the single-stranded motif found at the 3′ end of all tRNAs to which the cognate amino acid is attached. In *B. subtilis*, about one third of tRNA genes lack the CCA motif and in these cases the CCA is added posttranscriptionally by an enzyme called nucleotidyl transferase. The proportion of tRNA genes with a CCA motif in any particular organism is extremely variable throughout the bacterial kingdom, ranging from all to none and everything in between. As we shall see this plays an important role in determining which of two possible tRNA maturation pathways is used for the 3′ processing of a particular tRNA.

All tRNAs, even those that occur as single transcription units, are synthesized as primary transcripts bearing extraneous sequences at both their 5′ and 3′ ends. The 5′ extensions are universally removed by the endoribonuclease RNase P (Fig. 6). RNase P is a fascinating enzyme consisting of a catalytic RNA subunit and a protein subunit, both of which are essential. The 13 kDa protein subunit has been proposed to play multiple roles, the most important of which appears to be to increase the affinity of the 350–400 nt RNA subunit for the precursor tRNA, by changing its conformation near the tRNA binding site. The crystal structure of RNase P holoenzyme bound to tRNA has been solved (pdb 3Q1Q). Two different protein-only forms of RNase P have also been identified, one in some bacteria, notably the *Aquificaceae* (HARP), and the archaea, and one found only in eukaryotes (PRORP), but the ribozyme form is by far the most dominant form found in nature.

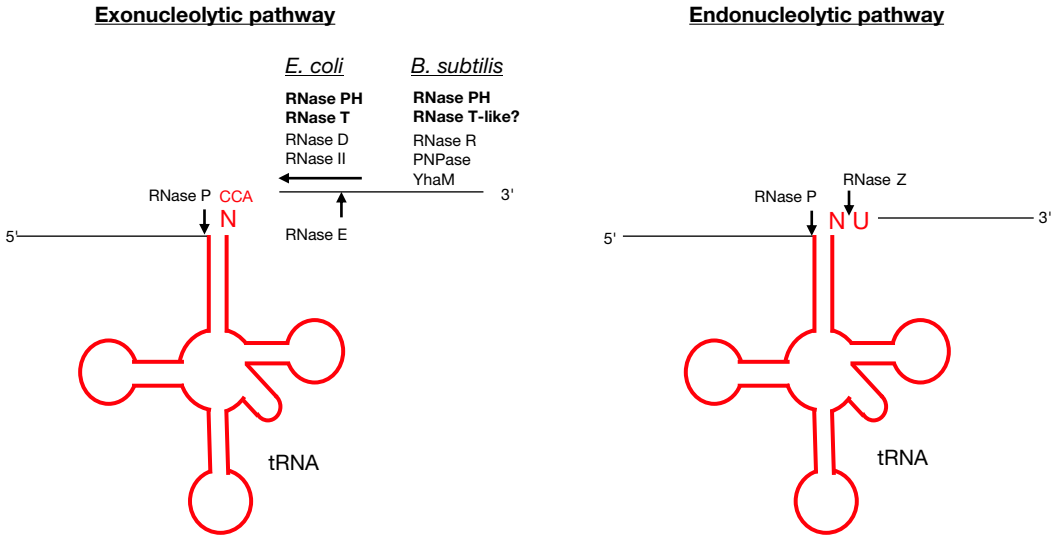

Fig. 6 Processing pathways of tRNAs in *E. coli* and *B. subtilis*. (A) Exonucleolytic pathway. The mature tRNA is represented by a *red cloverleaf structure*, precursor sequences in *black*. Endonucleolytic cleavages are represented by *black arrows* perpendicular to the transcript; exonucleolytic digestion is represented by *black arrows* parallel to the transcript. The key 3′ exonucleases are in *bold type*. In *E. coli*, extremities accessible to the 3′ exonucleases are often generated by prior cleavage by RNase E. An RNase T-like enzyme remains to be identified in *B. subtilis*. (B) Endonucleolytic pathway. The "mature" tRNA is represented by a *red cloverleaf structure*, although this requires further addition of the CCA motif by nucleotidyl transferase, with the discriminator base (N) and the downstream uracil (U) residue indicated. Symbols and conventions are as in (A). Adapted from Redko et al. (2007). *Nature Reviews Microbiology* **5**, 278–286.

Maturation of the 3′ end of tRNAs can occur via a primarily exonucleolytic or endonucleolytic pathway, depending mainly on the presence or absence of a CCA motif in the precursor species. In *B. subtilis*, an enzyme called RNase Z endonucleolytically cleaves those tRNA precursors lacking a CCA motif. RNase Z is a member of the Zn-dependent metallo-β-lactamase family of enzymes, like RNase J. The structure of RNase Z has been solved in complex with tRNA (pdb 2FK6). RNase Z generally cleaves tRNA precursors just downstream of the discriminator nucleotide, the unpaired nucleotide (labeled N in Fig. 6) at the 3′ end of the acceptor stem. Most CCA-less *B. subtilis* tRNAs have a naturally occurring uracil (U)-residue immediately downstream of the cleavage site and RNase Z activity is stimulated >200-fold by the presence of this residue, explaining why this class of tRNAs is a preferred substrate for RNase Z. In fact, the U-residue also determines the site of initial cleavage. For the few CCA-less tRNA precursors where the U-residue is one or two nucleotides further downstream of the discriminator base, endonucleolytic cleavage occurs just 5′ to the U-residue and then the enzyme chews back to the discriminator base in 3′-exonucleolytic mode. Like many of the ribonucleases of the β-lactamase family, RNase Z has both *endo-* and exonuclease activity.

The 3′-exonucleolytic tRNA maturation pathway was first discovered in *E. coli*. Here, any of at least four redundant 3′ exonucleases, RNase PH, RNase T, RNase D and RNase II can perform the task, with the first two distributive enzymes being the most important. Should any of these RNases degrade into the CCA motif present in *E. coli* tRNA precursors, the damage can be repaired by nucleotidyl transferase. RNase PH (pdb 1OYP) is primarily involved in maturation of small stable RNAs and is a member of the PDX family of phosphorolytic 3′-exonucleases that includes PNPase. Homologs of RNase PH form the hexameric core of the eukaryotic exosome that plays an important role in both mRNA degradation and stable RNA maturation. RNase D (pdb 1YT3) is a distributive enzyme, also dedicated to small stable RNA 3′-maturation in *E. coli* and a member of the same family of RNases as RNase T. A homolog of RNase D is also associated with the yeast nuclear exosome. Finally, RNase II (pdb 2IX1) is processive 3′-exoribonuclease that plays a more important role in mRNA degradation. In the absence of these four redundant 3′ exonucleases, sufficient tRNA 3′ maturation can be performed by RNase Z (also known as RNase BN) in 3′-exonuclease mode to maintain viability in *E. coli*. RNase Z progression is halted by the C residues of the CCA motif.

Of the four enzymes implicated in the *E. coli* 3′-exonucleolytic tRNA maturation pathway, only RNase PH is found in *B. subtilis*. *B. subtilis* strains lacking RNase PH accumulate precursors of those tRNAs with an encoded CCA motif. These precursors are 1–4 nts longer than the mature tRNA species, indicating that RNase PH plays a key role in the removal of the last few precursor nucleotides from the 3′ end. RNase PH null mutants still produce large quantities of accurately matured CCA-containing tRNAs, indicating that other enzymes can perform the same function. Indeed, *B. subtilis* strains lacking from one to four of its known 3′-exoribonucleases, RNase PH, PNPase, RNase R and YhaM accumulate progressively longer tRNA precursors, suggesting that, as in *E. coli*, multiple redundant RNases can perform the maturation reaction. PNPase is a processive phosphorolytic enzyme, related to RNase PH, whose principal role is mRNA degradation. RNase R is a processive hydrolytic 3′-exonuclease, with a much better ability to degrade through RNA secondary structures than either PNPase, or its cousin RNase II. It is known to play a major role in rRNA degradation and, more recently, in the decay of structured portions of mRNAs. The primary RNA substrates of YhaM are unknown and this enzyme is also thought to play an important role as a DNase in *B. subtilis*. In the absence of other 3′-exoribonucleases, YhaM can clearly provide some back-up, however, as we saw for 23S rRNA. At least one other enzyme, likely to have an RNase T-like function, remains to be identified in the 3′-exonucleolytic tRNA maturation pathway in *B. subtilis*, since a significant portion of all CCA-containing tRNAs are still correctly processed at their 3′ ends in strains lacking these four enzymes.

Processing of Other Stable RNAs

A number of small stable RNAs with specific properties have been identified in *E. coli* and *B. subtilis*. These too are synthesized as precursors requiring maturation by RNases. Identification of the RNases involved underscores the idea that the maturation pathways of stable RNAs are generally very different in these two organisms.

4.5S/scRNA Maturation

The 4.5S RNA of *E. coli* and its equivalent in *B. subtilis*, called small cytoplasmic RNA (scRNA), are part of the signal recognition particle (SRP) whose function is to direct proteins bearing an N-terminal export sequence to the plasma membrane as they emerge from the ribosome. This RNA is synthesized as a precursor with 5′ and 3′ extensions in both *E. coli* and *B. subtilis*. In *E. coli*, 4.5S RNA is matured on the 5′ side by RNase P and on the 3′ side by exonucleolytic trimming. As for tRNA 3′-maturation, several of the 3′-exonucleases appear capable of contributing to this process, with RNase T playing the most important role (Fig. 7A).

B. subtilis has two possible pathways of scRNA maturation. The first step of both pathways is catalyzed by RNase III on the upstream side of a long double stranded region, to produce the mature 5′ end directly. The 3′ end is then generated either by a second, less efficient cleavage by RNase III on the 3′ side of the stem, 4 nts downstream of the mature 3′ end, or by endonucleolytic cleavage by RNase Y 12 nts downstream of the mature 3′ end. In both cases, the final maturation step is catalyzed by 3′ exonucleolytic trimming by redundant exonucleases, with RNase PH playing the predominant role. Although the cleavage by RNase Y is shown in a double-stranded region in Fig. 7A, it is thought that the maturation of the 5′ side by RNase III permits rapid degradation of the fragment of RNA base-pairing with the RNase Y recognition site. Thus, these steps are likely to precede RNase Y cleavage.

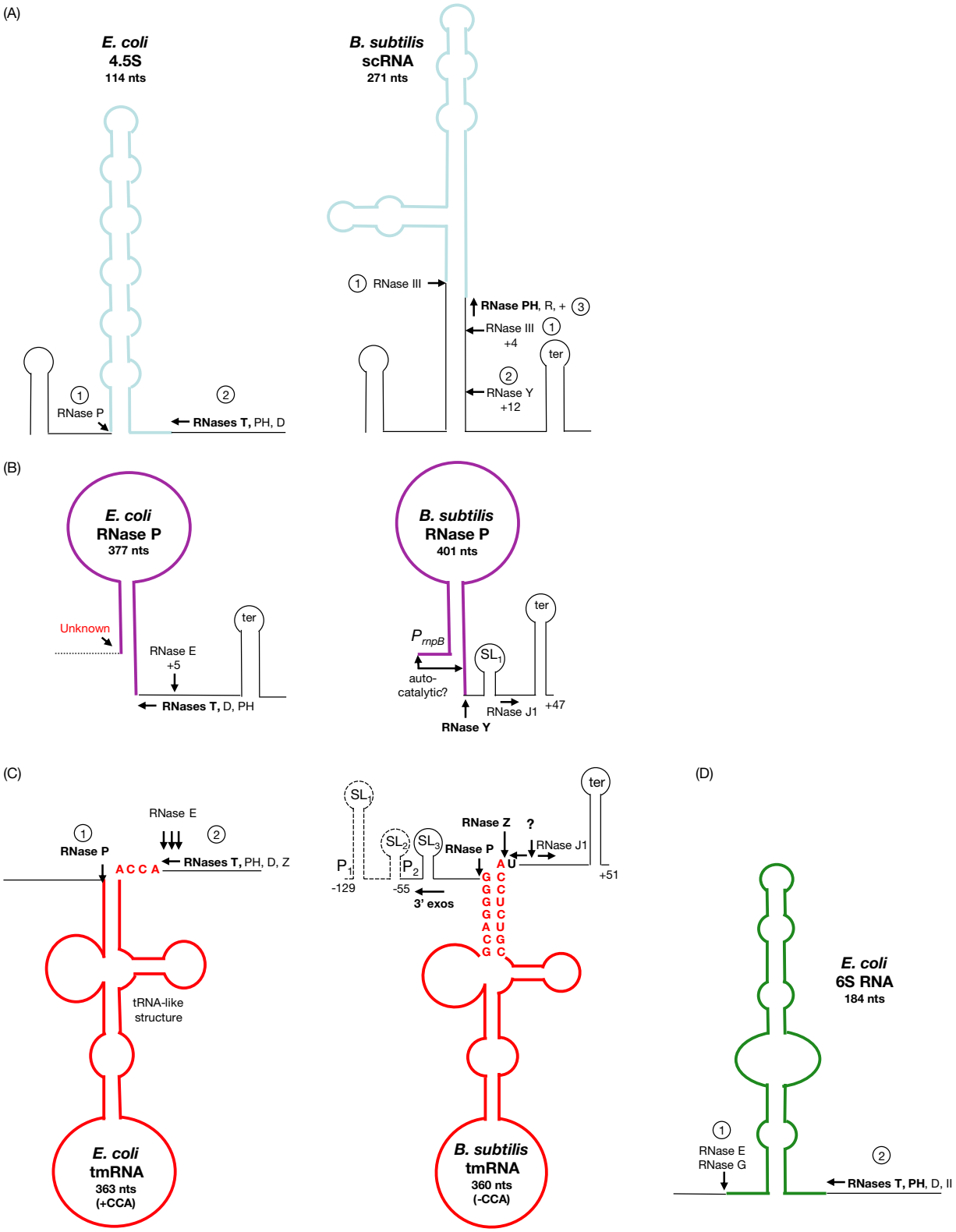

Fig. 7 Processing of various small stable RNAs in *E. coli* and *B. subtilis*. (A) Processing of 4.5S/scRNA. The mature RNA is in *light blue*, precursor sequences in *black*. Endonucleolytic cleavages are represented by *black arrows* at an angle to the transcript; exonucleolytic digestion is represented by *black arrows* parallel to the transcript. The key 3′ exonucleases are in *bold type*. (B) Processing of RNase P RNA. The mature RNA is in *mauve*, precursor sequences in *black*. Symbols and conventions are as in (A). Although most RNase P RNA is synthesized with a mature 5′ end in *E. coli*, some precursor can be produced by read-through transcription from upstream genes (represented by a *dotted line*). The details of the processing pathway of these transcripts are unknown, but involves RNase E in *E.coli* and RNase Y in *B. subtilis*. (C) Processing of tmRNA. The mature RNA is in *red*, precursor sequences in *black*. Symbols and conventions are as in (A). (D) Processing of 6S RNA in *E. coli*. The mature RNA is in *green*, precursor sequences in *black*. Symbols and conventions are as in (A).

Maturation of the RNA subunit of RNase P

The RNA subunit of RNase P (also known as P-RNA or M1 RNA) is transcribed from the *rnpB* gene as a precursor molecule. The main promoter of the *rnpB* gene in *E. coli* produces a P-RNA that does not require further maturation at the 5′ end. Longer RNAs resulting from read-through transcription from upstream genes accumulate in RNase E mutants, however, and there is some debate about whether these RNAs can produce functional P-RNA. Maturation of the 3′ side of *E. coli* P-RNA requires cleavage by RNase E a few nts downstream, followed by exonucleolytic trimming. RNase T plays the most important role among the 3′-exonucleases of overlapping function (Fig. 7B).

B. subtilis P-RNA maturation, on the other hand, has been proposed to be autocatalytic. Indeed, accurate maturation of the 5′ end of *B. subtilis* P-RNA can be efficiently catalyzed in vitro by the RNase P holoenzyme. It has also been proposed that RNase P processes its own 3′, in this case producing a P-RNA 4 nts shorter than the mature species identified in vivo. While autocatalytic maturation of the 3′ end is still possible, about 80% of RNase P RNA is in its precursor form in cells lacking RNase Y, strongly suggesting this is the major 3′ processing enzyme in vivo.

Maturation of tmRNA

The transfer-messenger RNA (tmRNA or 10Sa RNA) plays an important role in the rescue and recycling of ribosomes stalled on truncated mRNAs. Its tRNA-like structure can be aminoacylated and inserted into the ribosome A-site. A conformational change then occurs on the ribosome that causes it to switch templates from the truncated mRNA to the tmRNA. The tmRNA encodes a small peptide tag that is added to the truncated protein, targeting it for proteolysis. Maturation of tmRNA has been characterized in *E. coli* (Fig. 7C). Not surprisingly, for a molecule whose 5′ and 3′ extremities come together to form a tRNA-like structure, maturation of tmRNA employs the same pathway as *E. coli* tRNAs. RNase P removes the 5′ leader sequence, while the 3′ exoribonucleases perform the 3′ reaction. RNase R, which copurifies with tmRNA, has also been shown to play a role in 3′ processing under conditions of cold growth. In *B. subtilis*, the tmRNA processing pathway is also very similar to that of its tRNAs. RNase P is responsible for processing at the 5′ end, and since the tmRNA gene lacks the appropriate CCA motif for aminoacylation, RNase Z processes the 3′ end. The CCA-motif is added subsequently to create a functional tmRNA, presumably by nucleotidyl transferase. Like the vast majority of tRNAs lacking an encoded CCA motif in *B. subtilis*, the tmRNA precursor also has a U-residue immediately downstream of the discriminator base to stimulate RNase Z cleavage.

Maturation of 6S RNA

The 6S RNA binds RNA polymerase and modulates sigma-70 holoenzyme activity in response to growth phase. It is thought to function as a mimic of the transcription bubble, thus inhibiting RNA polymerase activity. In *E. coli* 6S RNA is synthesized as a precursor molecule from two different promoters and has both 5′ and 3′ extensions. Transcription from the distal promoter P_2 is dependent on both sigma-70 and the stationary phase factor sigma-S, whereas the proximal promoter P_1 is sigma-70 dependent only. The long precursor is cleaved exclusively by RNase E to generate the mature 5′ end of 6S RNA, while the short precursor can be processed by either RNase E or RNase G. Thus the biogenesis pathway of 6S is an interesting interplay between sigma factors and endoribonucleases. Maturation of the 3′ side occurs via 3′ exonucleolytic trimming, with RNases T and PH playing predominant roles.

The 6S RNA processing pathway has not yet been reported for *B. subtilis*.

Role of Precursors in Quality Control?

Why synthesize stable RNAs as precursors, rather than directly as mature species? Although it would be feasible for cells to synthesize RNAs directly with mature 5′ ends, simply by adjusting the point of transcription initiation (and this indeed is the case for *E. coli* RNase P RNA), direct production of the mature 3′ end is more difficult. This is because the transcription termination signals are transcribed into RNA, making at least one processing step, that is, removal of the terminator, almost unavoidable. It is also possible, however, to imagine that synthesis of stable RNAs as precursor molecules provides cells with a quality control option, by leaving "access-ramps" in place for exonucleases to degrade these RNAs should they prove to be nonfunctional. This idea is supported by the fact that the final steps of rRNA maturation generally only occur once subunit assembly is completed and, for some reactions, not until after the ribosome has initiated translation. Quality control mechanisms involving RNase R have been shown to exist in *E. coli* for rRNAs and tRNAs and these mechanisms rely on a re-synthesis of an exonuclease access-ramp by poly(A) polymerase. A similar phenomenon is seen with the role of RNase R in degrading unprocessed 16S rRNAs in *B. subtilis*. Moreover, in *B. subtilis*, RNase J1 can degrade 16S rRNA completely in 5′-exonuclease mode unless it is stopped by some feature of the assembled 30S subunit. This phenomenon could be, and likely is, exploited by the cell as a quality control mechanism for 30S subunit assembly, afforded by a 5′-extension. The intervention of ribosomal proteins L3 and L18 in 23S and 5S rRNA maturation is thought to play similar role in the quality control of these rRNAs.

Future Orientations

Although the majority of the processing reactions of stable RNAs have now been characterized in *E. coli* and *B. subtilis* there are still a few loose ends. The 6S processing pathway and the enzyme that gives access to RNase J1 for the maturation of the 5′ end of 16S

rRNA remain to be identified in *B. subtilis*, as do the enzymes that catalyze the maturation of the 5′ side of 23S and 5S rRNA in *E. coli*. The future of RNA processing probably lies outside of these two organisms, however. Many were taken by surprise by the extent of the differences between *E. coli* and *B. subtilis* maturation enzymes and pathways, despite their evolutionary distance (estimates vary between 1 and 3 billion years, greater than that of plants and animals). Clearly, other highly divergent model organisms, a representative of the archaea, for example, and perhaps a representatives of the cyanobacteria, require intense study before we can claim to have a global understanding of RNA processing throughout the prokaryotes.

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RNA Viruses: Plant Pathogenic

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Glossary

Ambisense RNA A viral single-stranded RNA genome or genome segment that contains protein coding sequences in the positive sense for some genes but in the negative sense for others. The positive sequences are not used directly as mRNA but are only expressed after sub-genomic RNA transcription.
Metagenomics Sequencing and analysis of DNA and RNA molecules present in the environment (for example, field-collected samples from viral hosts, vectors, or other materials). An important advantage is that this analysis does not depend upon isolation/culturing/purification of viruses/microorganisms.
Phloem In vascular plants, the conductive tissue responsible for transport of organic substances. Phloem consists of living cells and is the major conduit for systemic movement of viruses.
Plasmodesmata (sing. plasmodesma) Channels of cytoplasm existing between adjacent plant cells. Plasmodesmata penetrate the cell wall, allowing transfer of substances (including viruses or viral nucleic acids) between cells. Almost all plant cells, including those in the phloem, are interlinked in this way: A network sometimes referred to as the 'symplasm'.
Replicase A viral RNA-dependent RNA polymerase (RdRP) encoded by a virus, usually with additional enzyme activity (e.g., helicase, methyltransferase), and normally complexed with host factors (membranes, proteins). The replicase has the main function of generating new genomic RNA molecules but may also function as a transcriptase for synthesis of sub-genomic viral RNAs. The replicase should not be confused with RNA-directed RNA polymerase (RDR) enzymes that are encoded by a host cell, which synthesize short RNA molecules and are components of the RNA silencing mechanism.
RNA silencing A mechanism, which is highly conserved between plants and animals, that is important for antiviral defense, regulation of endogenous gene expression and control of transposons. RNA silencing comprises a number of different pathways that share related or overlapping components and that is guided by small (s)RNAs of 20–25 nucleotides in length. sRNAs bear high sequence homology to their target loci, which confers specificity on the silencing process. As a means of counter-defense, many plant viruses encode proteins that suppress RNA silencing.
Sense In the context of single-stranded RNA viruses, positive-sense genomes are those that can act directly as mRNA. However, given the constraints of the eukaryotic protein synthesis apparatus additional strategies may be employed to express genes that are not proximal to the 5′ end of the genomic RNA molecule. Negative-sense RNA viruses express their proteins by synthesis of mRNAs that are complementary to the sequence of the encapsidated genomic RNA(s).
Sub-genomic RNA These RNA molecules are produced as a result of viral RdRP activity. They are shorter than full-length genomic RNAs but their nucleotide sequences are identical to a part of the viral genomic RNA. It is not always possible to ascribe a function to a sub-genomic RNA. However, many are known to function as mRNAs for the expression of viral proteins that cannot be synthesized using the genomic RNA as a translation template.
Systemic and local infection In a local infection the virus either remains in the directly inoculated cells or exhibits limited spread between cells in the inoculated organ(s) of the plant. Systemic infection occurs when the virus enters the vascular tissue and spreads (typically *via* the phloem) to tissues that were not directly inoculated with the virus.
Virion An individual, complete virus particle. Virions typically consists of a shell (capsid) of protein and/or lipid surrounding the viral genomic nucleic acid molecule(s).

Defining Statement

The article covers the genome organization, the replication and gene expression strategies of double-stranded and single-stranded (positive and negative sense) RNA viruses infecting higher plants. An overview of the mechanisms by which these viruses induce disease and suppress host resistance is also included.

Introduction

About 80% of the viruses known to infect flowering plants (angiosperms) have RNA genomes. Viruses with genomes consisting of single-stranded (ss) positive sense RNA constitute the single largest group of plant RNA viruses (c. 65%). The remainder have double-stranded (ds) or negative-sense ss RNA genomes (<5% and c. 10%, respectively) (Table 1). The discovery of new plant-

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Table 1 Principal groups of RNA viruses infecting higher plants[a]

Genome	*Family*	*Genus*	*Type Species*
dsRNA	*Endornaviridae*[b]	*Endornavirus*	*Vicia faba endornavirus*
	Partitiviridae[b]	*Alphacryptovirus*	*White clover cryptic virus 1*
		Betacryptovirus	*Atkinsonella hypoxylon virus*
		Deltapartitivirus	*Pepper cryptic virus 1*
	Reoviridae[c]	*Fijivirus*	*Fiji disease virus*
		Phytoreovirus	*Wound tumor virus*
		Oryzavirus	*Rice ragged stunt virus*
(-) ssRNA	*Bunyaviridae*[c]	*Tospovirus*	*Tomato spotted wilt virus*
	Rhabdoviridae[c]	*Cytorhabdovirus*	*Lettuce necrotic yellows virus*
		Nucleorhabdovirus	*Potato yellow dwarf virus*
	Unassigned	*Emaravirus*	*European mountain ash ringspot-associated virus*
		Ophiovirus	*Citrus psorosis virus*
		Tenuivirus	*Rice stripe virus*
		Varicosavirus	*Lettuce big-vein virus*
(+) ssRNA	*Alphaflexiviridae*[b]	*Allexivirus*	*Shallot virus X*
		Botrexivirus	*Botrytis virus X*
		Lolavirus	*Lolium latent virus*
		Mandarivirus	*Indian citrus ringspot virus*
		Platypuvirus	*Donkey orchid symptomless virus*
		Potexvirus	*Potato virus X*
		Sclerodarnavirus	*Sclerotinia sclerotiorum debilitation-associated RNA virus*
	Betaflexiviridae		
	Quinvirinae[d]	*Carlavirus*	*Carnation latent virus*
		Foveavirus	*Apple stem pitting virus*
		Robigovirus	*Cherry rusty mottle-associated virus*
	Trivirinae[d]	*Capillovirus*	*Apple stem grooving virus*
		Chordovirus	*Carrot Ch virus 1*
		Citrivirus	*Citrus leaf blotch virus*
		Divavirus	*Diuris virus A*
		Prunevirus	*Apricot vein clearing associated virus*
		Tepovirus	*Potato virus T*
		Trichovirus	*Apple chlorotic leaf spot virus*
		Vitivirus	*Grapevine virus A*
	Bromoviridae	*Bromomovirus*	*Brome mosaic virus*
		Alfamovirus	*Alfalfa mosaic virus*
		Anulavirus	*Pelargonium zonate spot virus*
		Cucumovirus	*Cucumber mosaic virus*
		Ilarvirus	*Tobacco streak virus*
		Oleavirus	*Olive latent virus 2*
	Closteroviridae	*Closterovirus*	*Beet yellows virus*
		Ampelovirus	*Grapevine leafroll-associated virus 3*
		Crinivirus	*Lettuce infectious yellows virus*
		Velavirus	*Grapevine leafroll-associated virus 7*
	Luteoviridae	*Luteovirus*	*Barley yellow dwarf virus*
		Enamovirus	*Pea enation mosaic virus-1*
		Polerovirus	*Potato leafroll virus*
	Potyviridae	*Potyvirus*	*Potato virus Y*
		Brambyvirus	*Blackberry virus Y*
		Bymovirus	*Barley yellow mosaic virus*
		Ipomovirus	*Sweet potato mild mottle virus*
		Macluravirus	*Maclura mosaic virus*
		Poacevirus	*Triticum mosaic virus*
		Rymovirus	*Ryegrass mosaic virus*
		Tritimovirus	*Wheat streak mosaic virus*
	Secoviridae	*Cheravirus*	*Cherry rasp leaf virus*
		Sadwavirus	*Satsuma dwarf virus*
		Sequivirus	*Parsnip yellow fleck virus*
		Torradovirus	*Tomato torrado virus*
		Waikavirus	*Rice tungro spherical virus*
	Comovirinae[e]	*Comovirus*	*Cowpea mosaic virus*

(*Continued*)

Table 1 Continued

Genome	*Family*	*Genus*	*Type Species*
		Fabavirus	*Broad bean wilt virus 1*
		Nepovirus	*Tobacco ringspot virus*
	Tombusviridae	*Tombusvirus*	*Tomato bushy stunt virus*
		Alphacarmovirus	*Carnation mottle virus*
		Alphanecrovirus	*Tobacco necrosis virus A*
		Aureusvirus	*Pothos latent virus*
		Avenavirus	*Oat chlorotic stunt virus*
		Betacarmovirus	*Turnip crinkle virus*
		Betanecrovirus	*Tobacco necrosis virus D*
		Dianthovirus	*Carnation ringspot virus*
		Gallantivirus	*Galinsoga mosaic virus*
		Gammacarmovirus	*Melon necrotic spot virus*
		Macanavirus	*Furcraea necrotic streak virus*
		Machlomovirus	*Maize chlorotic mottle virus*
		Panicovirus	*Panicum mosaic virus*
		Pelarspovirus	*Pelargonium line pattern virus*
		Umbravirus	*Carrot mottle virus*
		Zeavirus	*Maize necrotic streak virus*
	Tymoviridae	*Tymovirus*	*Turnip yellow mosaic virus*
		Maculavirus	*Grapevine fleck virus*
		Marafivirus	*Maize rayado fino virus*
	Virgaviridae	*Furovirus*	*Soil-borne wheat mosaic virus*
		Goravirus	*Gentian ovary ringspot virus*
		Hordeivirus	*Barley stripe mosaic virus*
		Pecluvirus	*Peanut clump virus*
		Pomovirus	*Potato mop top virus*
		Tobamovirus	*Tobacco mosaic virus*
		Tobravirus	*Tobacco rattle virus*
	Unassigned	*Albetovirus*	*Tobacco albetovirus 1*
		Aumaivirus	*Maize aumaivirus 1*
		Benyvirus	*Beet necrotic yellow vein virus*
		Higrevirus	*Hibiscus green spot virus 2*
		Idaeovirus	*Raspberry bushy dwarf virus*
		Ourmiavirus	*Ourmia melon virus*
		Papanivirus	*Panicum papanivirus 1*
		Polemovirus	*Poinsettia latent virus*
		Sobemovirus	*Southern bean mosaic virus*
		Virtovirus	*Tobacco virtovirus 1*
			(satellite tobacco mosaic virus)

[a]Collated from information in Hull (2014), The Universal Virus database of the International Committee on Taxonomy of Viruses, the Association of Applied Biologists Descriptions of Plant Viruses and Viral Zone. See *Further Reading*.
[b]Includes fungus- as well as plant-infecting member species.
[c]Plant-infecting members also able to infect insect vectors.
[d]Sub-family of Family *Betaflexiviridae*.
[e]Sub-family of Family *Secoviridae*.

infecting viruses, as with viruses in general, is accelerating due to the application of advanced nucleic acid sequencing techniques to environmental samples, an approach called 'viral metagenomics'. Currently there are no known plant retroviruses. In addition to true RNA viruses, plants can also be infected by viroids, which are infectious agents consisting of small (240–380 nucleotide), circular ssRNA molecules that encode no proteins and are entirely dependent on the host for replication. For up to date information on the taxonomy of plant-infecting RNA viruses, which is changing constantly, and their relationships to other virus types, the reader is referred to the Universal Virus database of the International Committee on Taxonomy of Viruses, the ViralZone website and the Association of Applied Biologists Descriptions of Plant Viruses (websites listed under *Further Reading*).

General Characteristics of Plant-Infecting RNA Viruses

History

Viruses were discovered first in plants and the 'first' virus, named tobacco mosaic virus (TMV) by Beijerinck in 1898, is a positive-sense ssRNA virus that has been used extensively for over a century as a model for the investigation of many important

concepts in modern biology. These concepts include the elucidation of the genetic code, the mechanisms of protein synthesis, and the mechanisms of self-assembly of biological macromolecular structures. More recently, TMV and other plant viruses have been engineered for use as gene expression vectors in plants or as reaction vessels, substrates and scaffolds for nanotechnology.

Transmission

Over 70% of plant viruses are transmitted (vectored) by invertebrates. Insects with probing/sucking mouthparts (stylets) such as aphids and close relatives (whiteflies, leafhoppers, planthoppers, etc.) vector the most viruses, with a small number being vectored by chewing insects, such as beetles, or other arthropods such as mites and ticks. Dagger-type nematodes (those feeding on plants using a stylet) belonging to the order *Dorylaimida* transmit members of two genera of positive-sense RNA viruses: *Nepovirus* and *Tobravirus*. A number of positive-sense RNA viruses are transmitted on the zoospores (motile spores) of soil-living organisms often referred to as 'fungi' that are, strictly speaking *Plasmodiophoromycetes*. Zoospores of *Olpidium* species transmit viruses with icosahedral particles (for example *Tobacco necrosis virus* and *Satellite tobacco necrosis virus*, STNV), while those of *Polymyxa* species transmit rod-shaped viruses such as *Soilborne wheat mosaic virus* or *Beet necrotic yellow vein virus*. Some RNA viruses [e.g., TMV, *Potato virus X* (PVX)] and viroids have no known vectors but are known to be mechanically transmissible (i.e., entering the plant through wounds and abrasions). These agents, as well as those with known biological vectors, can in many instances also be transmitted through plant material via grafts or as a result of infestation with dodder (*Cuscuta* spp.: A parasitic plant). In certain cases, vertical (inter-generational) transmission can occur through seed or pollen.

Broadly, there are two types of interaction between viruses and their biological vectors. Viruses may be taken up internally by the vector (circulative transmission) or carried on the exterior of the vector, for example on the mouthparts of invertebrate vectors (non-circulative transmission). The majority of insect-vectored positive-sense RNA viruses are transmitted in a non-circulative fashion and the key viral gene product facilitating binding of virus particles to the vector mouthparts is the viral coat or 'capsid' protein (CP). The CP is the sole determinant of transmission for many viruses, for example *Cucumber mosaic virus* (CMV). However, certain viruses encode proteins called helper factors that are not required for virus particle assembly but that are required to bind the virus particle to the vector. The helper component/protease (HC-Pro) proteins encoded by potyviruses bind a specific three amino acid domain on the viral CP and act as a bridge to link the particle to the stylet of the aphid vector.

During circulative transmission of viruses by insects virions are ingested, cross the gut wall into the hemolymph (circulatory fluid), and are transported into the salivary glands. No plant positive-sense RNA viruses are known to replicate in the cells of their insect vectors but several negative-sense and dsRNA viruses do. Aphids and closely-related insects possess specialized cells that harbor bacterial endosymbionts (*Buchnera* species). These bacteria release symbionin, a homolog of the *Escherichia coli* chaperonin, GroEL. Symbionin interacts with and protects the particles of circulatively transmitted viruses, allowing the safe passage from gut to salivary gland of viruses that cannot replicate in the insect. This allows transmission of circulatively transmitted positive-sense RNA viruses, for example the luteoviruses and poleroviruses.

Spread Through Host Plants

In contrast to viruses infecting many other types of organism, plant viruses do not need to physically break out of one host cell in order to invade the next. This is because most cells within a plant are linked symplastically by a local network of intercellular cytoplasmic connections (plasmodesmata). Plasmodesmata are dynamic, structures with the ability to regulate the selective cell-to-cell trafficking of various small and large molecules. This local system of cell-to-cell connections has links with the cells of the phloem. The phloem is a network of living vascular cells that conduct sugars and other more complex molecules, including RNA and protein molecules, over longer distances through the plant. Thus, in principle, the virus has access to nearly all parts of a host plant.

Cell-to-cell movement through plasmodesmata is relatively slow and is mediated in most cases by viral movement proteins. Movement proteins fall into two broad types. The first type, exemplified by the tobacco mosaic virus (TMV) movement protein, facilitates cell-to-cell transfer of viral nucleic acids by interacting with host factors to increase the permeability of the plasmodesmata and by unfolding the secondary structure of the viral nucleic acid. The second type of movement protein, exemplified by that of *Cowpea mosaic virus* (CPMV), forms tubules. These tubules obliterate the internal plasmodesmal structure and are wide enough to allow passage of entire viral particles. In some cases (for example closteroviruses and potyviruses) it appears that protein components of the virion or non-structural viral proteins associated with virus particles *in vivo* can facilitate the binding of virus particles to plasmodesmata. Subsequently viral RNA is transferred by a process in which ribosomes of the receiving cell translate viral RNA into protein and co-translationally pull the RNA in through the plasmodesmata. For viroids, which encode no proteins, the secondary structure of the viroid RNA in some way facilitates movement through plasmodesmata.

Double-Stranded RNA Viruses

In plants, the dsRNA viruses that are infectious and which cause recognizable diseases are members of the *Reoviridae*, which possess segmented genomes (10 or 12 dsRNA components). The other known plant-infecting dsRNA viruses are the 'cryptoviruses' and endogenous RNA viruses (*Endornaviridae*). Cryptoviruses belong to genera of the *Partitiviridae*, which have genomes consisting of

two dsRNA segments (Table 1). The cryptoviruses are transmitted vertically (i.e., through pollen/seed) and the majority having no other known mode of transmission. Virtually none of the cryptoviruses are known to induce disease in their host plants. Endornaviruses are also horizontally transmitted but have monopartite dsRNA genomes. Their genomic dsRNA molecules are associated with the viral RNA-dependent RNA polymerase (RdRP) but are not encapsidated in virions.

The Family *Reoviridae* contains a large number of viruses infecting vertebrates, insects, and plants. There are three genera of plant-infecting reoviruses. Members of the genus *Phytoreovirus* (for example *Wound tumor virus*: WTV) have genomes consisting of 12 dsRNA segments and most are vectored by leafhopper insects. WTV was the first plant virus demonstrated to be able to replicate in the cells of its insect vector. Viruses in the genus *Fijivirus* (Type species: *Fiji disease virus*, a pathogen of sugarcane) and *Oryzavirus* (e.g., *Rice ragged stunt virus*) possess 10 segment genomes and are transmitted, respectively, by planthoppers and leafhoppers.

Reovirus virions are double-layered isometric particles each containing a complete set of genome segments. The inner shell or 'core' contains the RdRP required for genome replication and the transcription of viral mRNAs. Replication occurs in the cytoplasm of the host cell (plant or insect) with new genomic dsRNA being formed within the cores of nascent virus particles. The ends of each genome segment contain virus-specific and segment-specific sequence tags that ensure that each new virion contains the appropriate complement of 10 or 12 genomic dsRNA segments.

Negative-Sense Single-Stranded RNA Viruses

Most known plant-infecting viruses possessing negative-sense linear ssRNA belong to the families *Rhabdoviridae* and *Bunyaviridae*. Both of these families include viruses infecting vertebrates. Other plant-infecting negative-sense RNA virus genera include *Tenuivirus* (type species: *Rice stripe virus*), *Ophiovirus* (type species: *Citrus psorosis virus*) and *Varicosavirus* (type species: *Lettuce big-vein virus*) among others (Table 1).

Rhabdoviruses have the largest virions among viruses infecting higher plants. Rhabdovirus virions are bullet-shaped or bacilliform particles and contain one genomic RNA molecule, between 11 and 13 kilobases in length, which is associated with a protein, N, to form a nucleocapsid core. A lipid membrane envelops this core, together with several other viral proteins, including the viral RdRP. The genomic RNA molecules of plant-infecting rhabdoviruses are distinguished from those unable to infect plants by encoding a cell-to-cell movement protein in addition to the five proteins required for replication and assembly. The negative-stranded RNA can function as a template for a full-length complementary copy that serves as an intermediate in replication and for the transcription of subgenomic mRNAs. Rhabdovirus replication occurs in either the nucleus (nucleorhabdoviruses e.g., *Sonchus yellow net virus*) or cytoplasm (cytorhabdoviruses e.g., *Lettuce necrotic yellows virus*) of the host cell. Plant-infecting rhabdoviruses can replicate in the cells of their vector insects, which may be aphids, planthoppers or leafhoppers. In general, rhabdoviruses infecting monocotyledonous hosts are transmitted only by planthoppers and leafhoppers.

Tospoviruses, tenuiviruses, ophioviruses and varicosaviruses possess segmented ssRNA genomes. Some of the genome segments of tospoviruses and tenuiviruses are ambisense. That is, these RNAs encode more than one protein by having one negative-sense and one positive-sense open reading frame. However, neither protein can be expressed directly using the genomic RNA as a translation template and protein synthesis occurs from subgenomic RNAs. Tospoviruses and tenuiviruses initiate transcription of their mRNAs by 'cap snatching', which is the removal of cap structures and up to 20 nucleotides from molecules of cellular mRNAs, or even the RNAs of other viruses, to use as primers of RNA-directed RNA synthesis. In the tospovirus life cycle the level of the N (nucleocapsid) protein regulates the switchover in L protein (RdRP) activity from mRNA transcription to replication of the genomic RNAs.

Tospoviruses are plant-infecting members of the family *Bunyaviridae*, which also includes a larger number of vertebrate-infecting viruses. The type species of the *Tospovirus* genus is *Tomato spotted wilt virus* (TSWV), which has a large host range (in excess of 600 plant species) and is transmitted by thrips, in which it also replicates. The TSWV genome comprises three ssRNAs: one negative-sense and two ambisense RNAs. TSWV virions contain one copy of each of the three genomic RNAs complexed with N protein and L proteins contained within a spherical lipid envelope. These plant-infecting bunyaviruses are distinguished from their vertebrate-infecting relatives by encoding a tubule-forming movement protein, NS_M.

Positive Sense Single-Stranded RNA Viruses

The RNA genomes of these viruses contain open reading frames in the sense orientation and consequently can serve directly as mRNAs for synthesis of at least some of the proteins they encode. In many cases, RNA purified from virions is infectious and can be used to program *in vitro* protein synthesis systems. Typically, replicase protein(s) can be synthesized by translation of the genomic RNA on host ribosomes. Consequently, the virions of positive sense RNA viruses do not contain viral RdRP. Generally, the virions of plant-infecting positive sense RNA viruses are constructed more simply than those of dsRNA and negative-sense ssRNA viruses, or compared with many positive sense RNA viruses infecting non-plant hosts. The majority of plant-infecting positive sense RNA viruses possess virions with shells formed from one or two types of CP molecule, with simple geometries based on the rod or icosahedron.

About two-thirds of the known species of plant infecting positive-sense RNA viruses are classified into seven families, with the remainder unassigned (Table 1). Most of these viruses encode a small number of polypeptides (typically between four and eleven). However, satellite viruses encode only one protein (the CP). Satellite viruses are hyper-parasitic upon another, unrelated virus

(the helper) for replication. Satellite viruses should not be confused with satellite RNAs, which are sub-viral, hyper-parasitic RNA molecules that are dependent on a helper virus for replication and encapsidation, and often do not possess any protein coding information. The presence of satellite viruses and satellite RNAs can modify the symptoms induced by a helper virus and affect the helper's titer in host tissue.

Genomic RNA molecules range in length from 1240 (e.g., STNV) to 20,000 nucleotides (e.g., the *Closterovirus Citrus tristeza virus*). The genomes of many plant-infecting positive sense RNA viruses are multipartite, comprising two or more mono- or multi-cistronic RNA segments. However, in contrast to negative sense ssRNA and dsRNA viruses with segmented genomes, which encapsidate an entire set of genomic RNA molecules in the same virion, the genome segments of positive sense RNA viruses are encapsidated in separate particles.

Viral RNA molecules possess various virus-specific 'non-coding' nucleotide sequences or structural features that enable particular viruses to replicate or express their genes. These sequences and structural features may act as promoter sequences for synthesis of full-length or subgenomic RNAs; they may facilitate the interaction of viral RNA with the cellular protein synthesis machinery, or they may have additional functions, such as promoting the interaction of viral RNA with CP during virion assembly. Structural features in these viral RNAs arise by folding and internal hybridization of complementary nucleotide sequences or by the covalent attachment of additional chemical moieties following transcription.

Virus-specific structural elements in the RNA of positive sense RNA viruses include tRNA-like structures occurring at the end of the 3′ untranslated region (3′-UTR) of some viruses (e.g., species in the genera *Tobamovirus*, *Cucumovirus*, *Bromovirus*). These 3′-tRNA structures can be aminoacylated with an amino acid specific to the virus but their role appears to be in translation of viral RNA. tRNA-like structures and other complex folding arrangements occur when nucleotide sequences on the loop of a stem-loop base-pair with complementary sequences outside the loop, forming pseudoknots. These can play roles in the regulation of viral gene expression and RNA synthesis.

The 5′-UTRs perform an important role in facilitating translation of viral RNA by the host cell ribosomes and may contain sequences that enhance translation of viral RNA, presumably to out-compete cellular mRNAs for access to the translation machinery. One of the best-studied translational enhancers is the Ω sequence of TMV RNA: a 68 nucleotide long leader containing a 25 nucleotide poly (CAA) sequence that enhances the initiation step of translation. *In vitro* studies indicate that enhancement of translation is mediated by binding of the heat shock protein HSP101 and the initiation factor eIF4F to Ω. However, it is unclear if the HSP101 is an absolute requirement for enhancement *in planta*. 5′-UTRs with similar translational enhancer properties have also been identified for PVX and alfalfa mosaic virus.

Post-transcriptional modifications of viral RNA include the addition of a 7-methyl guanosine triphosphate cap structure to the 5′ terminus of the molecule. This simulates a modification common to the majority of cellular mRNAs in a eukaryotic host and increases the translational efficiency and stability of the viral RNA. Viruses with capped RNAs often encode a replicase protein with methyltransferase (RNA capping) activity (e.g., species in the genera *Tobamovirus*, *Cucumovirus*, *Bromovirus*). The genomic RNAs of certain viruses, for example the potyviruses, are modified at the 5′ end by the covalent addition of a virus-encoded protein called the VPg (virus protein, genome-linked). The VPg can fulfill a similar role to the cap in the initiation of translation by interaction with host translation factors, particularly variants of eukaryotic initiation factors (eIF) 4E and eIF(iso)4E. Following virion formation, VPg projects from the end of these filamentous virus particles allowing interactions with HC-Pro (the helper factor in aphid transmission) and, potentially, the plasmodesmal pores to initiate cell-to-cell movement. Potyviruses are also an example of a virus type that is modified on the 3′ end of its genomic RNA by the addition of poly(A) tracts. These are a feature of the 3′-termini of most cellular mRNAs. These enhance the efficiency of translation and in viruses influence replication and RNA stability.

Replication of Positive Sense RNA Viruses

Composition and Assembly of the Replicase Complex

All known plant-infecting RNA viruses, with the exception of satellite viruses, encode one or more enzymes that function in the synthesis of viral RNA. Replication of positive-sense RNA viruses is carried out in the cytoplasm of the host cell by a replicase complex, which possesses RdRP activity. Replicase complexes also possess helicase activity, in order to separate nascent RNA strands from template RNA and may also possess other, virus-specific, activities (e.g., methyltransferase activity for addition of 5′cap structures to nascent RNA).

Positive-sense RNA virus replicase complexes contain proteins encoded by the virus and the host cell. Active preparations of the CMV and *Brome mosaic virus* (BMV) replicase complexes have been purified and both preparations contained two virus-encoded proteins and one host protein. The nomenclature used to describe the proteins encoded by BMV and CMV is very similar and in both cases these proteins are called 1a (c. 110 kDa proteins possessing methyltransferase and helicase activities) and 2a (c. 90 kDa proteins with RdRP activity). The CMV complex was found to contain a host protein (c. 50 kDa) of unknown identity and the BMV complex is associated with a protein related to a subunit of the translation factor eIF3. A membrane-associated preparation of the TMV replicase was found to contain the two TMV-coded replicase proteins. The 126 kDa (methyltransferase and helicase) and 183 kDa (methyltransferase, helicase and RdRP) proteins are associated in the viral replicase complex as heterodimers. At least one host-coded protein of unknown identity has been found in replicase extracts.

The replicase complexes of positive-sense RNA viruses assemble on the host cell's intracellular membranes. Ishikawa and colleagues (Tokyo) identified and analyzed mutant lines of the plant *Arabidopsis thaliana* that were unable to support the replication

of TMV. They identified the protein products of the genes that had been mutated in some of these lines. These proteins, called TOM ('tobamovirus multiplication') 1, 2A, 2B and 3 all proved to be transmembrane proteins that interact with each other and/or the helicase domain common to the TMV 126 and 183 kDa replicase proteins, functioning to anchor the replicase complex to the ER and/or the vacuolar membrane of the host cell.

Some of the most revealing insights on the interactions of viral replicase complexes with host cell membranes and other host factors have emerged from the work of P. Ahlquist and colleagues (Madison, Wisconsin) and of P. Nagy (Kentucky). These groups have made extensive use of yeast, a highly developed genetic model organism, to identify cellular genes required for viral replication. Mutagenesis of transformed yeast lines expressing the viral replicase proteins and harboring replicons (replicating RNA molecules) derived from BMV or tombusviruses revealed that virus replication is dependent on the action of multiple cellular genes. For example, proper association of viral replicase proteins with host cell membranes is sensitive to membrane composition and thus mutations in yeast genes encoding specific lipid biosynthetic enzymes (e.g., Δ9 fatty acid desaturase) inhibited replication of a BMV-derived replicon.

Further work using transformed yeast cells showed that the BMV replicase complex induces lipid synthesis and remodels the membrane to form simple-to-complex invaginations with an open 'neck' to the cytoplasm in which viral RNA synthesis takes place. The complexity of these invaginations is controlled by the relative proportions of the BMV 1a and 2a proteins (Fig. 1). Ahlquist has speculated that the synthesis of nascent viral RNA in these membrane invaginations by positive-sense RNA viruses is a functional analogue of RNA synthesis in nascent virions or virion cores by negative-sense and dsRNA viruses. The enclosure of RNA synthetic activity in open membrane vesicles or in virions provides benefits including: the concentration of replication proteins, RNA templates and substrates; the linkage of successive steps in replication by keeping replicase components and RNA intermediates in close proximity, and the sheltering of template RNA, nascent viral RNA, and replication intermediates from the RNA silencing machinery of the host (see 'Mechanisms of pathogenesis').

The primary function of the replicase complex is to generate identical copies of the full-length genomic RNA (Fig. 2). The RdRP component of the viral replicase complex recognizes the sequences and/or their folded secondary structures (see 'Positive sense single-stranded RNA viruses') at the 3′ end of the genomic RNA (plus-sense) as a promoter.

In general, for plant-infecting positive sense RNA viruses, initiation of RNA polymerization occurs *de novo*, without any need for an oligonucleotide or a protein primer. However, some purified RdRPs can utilize a short primer *in vitro*. The helicase activity of the complex unwinds and separates the template and nascent RNA strands. The RdRP can use either the released plus-sense genomic RNA to synthesize more minus-sense RNA or it can use the minus-sense RNA as template for synthesis of plus-sense viral RNA (Fig. 2). The replication process is biased in that much more positive sense than minus sense RNA is made. Current thinking is that this bias is controlled by inhibitory 'silencer' sequences in the positive strand, not by the relative strength of the minus and plus sense promoters.

For some viruses the replicase has a secondary role, which is in the transcription of shorter than full-length subgenomic RNA molecules that may function as mRNAs. For most plant-infecting positive sense RNA viruses the synthesis of subgenomic RNA probably occurs by initiation of RNA synthesis at an internal promoter sequence on the full-length minus-sense RNA replication intermediate (Fig. 2). Examples of viruses using this approach to subgenomic RNA synthesis include BMV, CMV and TMV. For certain other plant-infecting positive sense RNA viruses, templates for subgenomic RNA synthesis are formed by premature

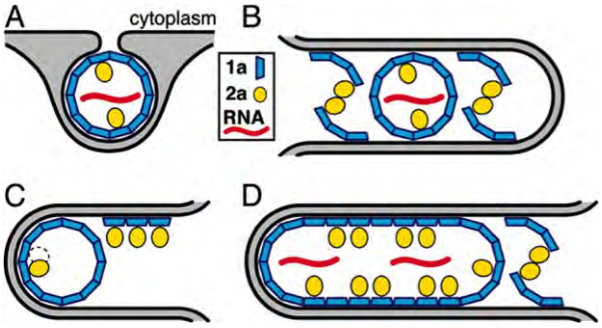

Fig. 1 *Models for rearrangements of host cell intracellular membrane by the replication proteins 1a and 2a of Brome mosaic virus* Infection of cells with positive-sense RNA viruses is often accompanied by the re-shaping of intracellular membranes. Based on experiments with BMV, Ahlquist and colleagues argue that the formation of vesicles provides an environment conducive to efficient replication and protection against host defenses. In this diagram bold lines and gray shading represent ER membranes and ER lumen, respectively. (A) Invagination of a spherule (50 nm in diameter) into the outer perinuclear ER membrane by 1a. Formation of a membrane-enveloped, capsid-like shell occurs by 1a-mediated recruitment of 2a (the RNA polymerase) and viral RNA. (B) High 2a concentrations may promote zippering together of double-membrane layers by 1a–2a complexes. Replication factor 1a might participate as monomers or multimers (hexamers or pentamers). Self-interaction of 2a might be direct or mediated by RNA or 2a-interacting host proteins. (C) High $2a^{pol}$ concentrations may block 1a–1a curvature needed to form spherules by steric hindrance between 2a factors bound to adjacent 1a factors (left side), tending to restrict 1a to planar lattices (right side). (D) Between the stacked double-membrane layers, extended planar lattices of 1a with bound 2a might provide a local replication complex environment similar to spherule interiors and may be closed by occasional regions of 1a curvature. Adapted from Schwartz, M., Chen, J., Lee, W.M., Janda, M., Ahlquist, P., 2004. Alternate, virus-induced membrane rearrangements support positive-strand RNA virus genome replication. Proceedings of the National Academy of Sciences of the United States of America 101, 11263–11268.

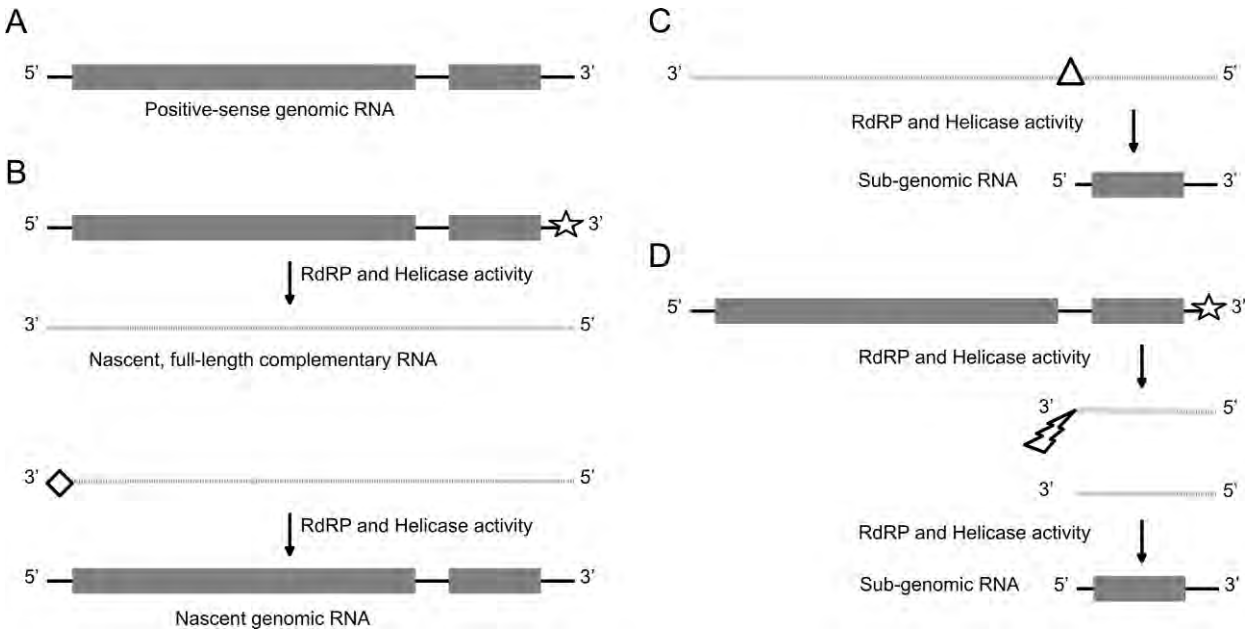

Fig. 2 *Replication and transcription of sub-genomic RNAs by positive-sense RNA viruses* Panel. (A) depicts the genome of an archetypal plant-infecting positive-sense RNA virus with two open reading frames shown for a replicase protein (solid gray box) and the coat protein (hatched box). The replication of full-length genomic RNA. (B) The genomic RNA serves as the template for synthesis of a full-length complementary-sense RNA molecule. The 5′–3′ synthesis of the complementary RNA by the RNA-dependent RNA polymerase (RdRP) activity of the viral replicase initiates following recognition of a promoter sequence at the 3′ terminus of the genomic RNA (indicated by a star). The helicase activity of the replicase separates the nascent strand from the template RNA. The full-length complementary RNA is the template for synthesis of new full-length genomic RNA molecules, following recognition by the replicase of a promoter of positive-sense RNA synthesis at the 3′ terminus (indicated by a diamond). Among plant-infecting positive-sense RNA viruses that express subgenomic RNAs to serve as mRNAs for nested open reading frames (such as that encoding the coat protein in this example), the majority transcribe them by initiation of positive-sense RNA synthesis at an internal promoter sequence (indicated with a triangle) within the full-length complementary RNA. (C) In some cases, transcription of sub-genomic RNA utilizes a less-than-full-length complementary-sense RNA generated by premature termination of copying of the genomic RNA. (D) Premature termination (indicated by a lightning bolt) is usually facilitated by interactions between viral RNA sequences on the same strand (*cis*-interactions) or on separate strands (*trans*-interactions) of virus-derived RNA.

termination of minus-strand synthesis (Fig. 2). Premature termination at a specific point on the minus strand is fostered by interactions between sequences within the same RNA strand (*cis* interactions: suggested for subgenomic RNA synthesis by PVX and *Tomato bushy stunt virus*) or by interactions between different RNA segments of multipartite viruses (*trans* interactions: e.g. the *Dianthovirus Red clover necrotic mosaic virus*).

Viral Gene Expression

Virtually all mRNAs in eukaryotic cells are monocistronic i.e. they encode a single polypeptide. For the majority of cellular mRNAs translation begins with the attachment of the 40S ribosomal subunit to the cap structure. This 'scans' in a 5′ to 3′ direction until it reaches an AUG codon. In eukaryotic cells, the likelihood of translation initiating at any given AUG depends on the sequences immediately flanking it. For example, a sequence context that makes an AUG a 'strong' translational start is 5′-. . .AXCA*AUG*G. . .3′, where X is a purine and the translational start is italicized. At the translational start 80S ribosome assembly is completed and protein synthesis commences. Protein synthesis ceases once the ribosome reaches a termination codon. Although circularization of eukaryotic mRNAs allows ribosome subunits to be recycled back to the initiation codon (see 'Interactions of viral mRNAs with the cellular translational apparatus'), re-initiation of translation from downstream ORFs will not normally occur and any sort of internal initiation of translation is extremely rare. The characteristics of the eukaryotic translation system pose significant challenges to viruses, which often encode multiple proteins on the same RNA molecule. Viruses have evolved gene expression strategies, used in various combinations, to evade or exploit the constraints of the cellular translation machinery.

Strategies Used to Overcome the Constraints of Eukaryotic Translation

Around half of the known plant-infecting viruses possess multiple genome segments. The division of genomes in this way can give directly translatable RNAs with an open reading frame adjacent to the 5′ end of the RNA (many positive sense RNA viruses e.g., BMV, CMV) or provide a template for a monocistronic mRNA (e.g., reoviruses). The generation of subgenomic RNAs

provides a means of expressing open reading frames that would otherwise be in an unsuitable context for translation due to orientation (e.g., tospoviruses, reoviruses) or placement within a positive sense RNA molecule downstream of a 5′-proximal open reading frame. For example, the RNAs 3 of BMV and CMV can be used directly as translation templates for synthesis of their respective movement proteins. However, in both viruses the open reading frame for CP, which is also on RNA3, is downstream of the movement protein open reading frame, and translation of CP occurs from a subgenomic RNA on which the CP open reading frame is proximal to the 5′ end.

Some viruses avoid the problem with re-initiation of translation by generating two versions of a protein, one shorter protein initiated from the 5′ proximal start codon, or a longer fusion protein in which the shorter protein constitutes the N-terminal domain. This can be achieved either by inhibiting the termination of the synthesis of the shorter protein ('read-through'), or by shifting the frame of translation before a stop codon is reached ('frame-shift'). TMV offers the best-studied example of read-through translation. The 5′-proximal open reading frame on the genomic RNA of TMV encodes the 126 kDa replicase protein. In up to 10% of translation rounds, the ribosome fails to detect the UAG stop codon of this open reading frame and elongation of the nascent polypeptide continues in-frame to yield the 183 kDa replicase protein. The effect is permitted by the existence of 'suppressor' tRNAs: tRNAs that can misread a stop codon. In tobacco there are two tyrosine tRNAs that possess the anticodon sequence G-pseudouridine-A and which can suppress termination of synthesis of the 126 kDa protein. However, the nucleotide sequence immediately flanking the UAG codon, i.e. its sequence context, determines the efficiency of read-through and mutations in the flanking sequences decrease the rate at which read-through occurs.

Frame-shifting allows a ribosome to avoid a stop codon at the end of an open reading frame by sliding forward or back by one nucleotide. The switch into the +1 or −1 reading frame allows further translational elongation and generation of a longer fusion protein. As with read-through, translation proceeds normally and without shifting in the majority (90%–95%) of ribosome passes and sequence context of the shift site is important in determining the rate at which frame-shifting occurs. The shift site itself is sometimes termed a 'slippery' site and its shifting activity is further modified by the folding of adjacent RNA sequences into stem-loop and pseudoknot structures. Examples of plant-infecting RNA viruses using frame-shifting include BYDV (−1 shift) and the *Closterovirus Beet yellows virus* (+1 shift). Both of these viruses utilize frame-shifting in translation of replicase proteins.

Viruses belonging to species within the *Potyviridae*, *Comoviridae* and a number of other virus taxons synthesize polyproteins from monocistronic genomic RNAs. A polyprotein is a precursor polypeptide that is broken down by selective proteolysis to yield the mature viral structural proteins (constituents of the virion) and non-structural proteins. The majority of the *Potyviridae* are monopartite RNA viruses that encode a single polyprotein, while viruses within the *Comoviridae*, which have two genomic RNAs, give rise to two polyproteins. Self-processing of the polyprotein has been studied in detail in species of the genus *Potyvirus* (e.g., *Potato virus Y*, *Tobacco etch virus*). Potyviral RNA contains a single open reading frame that is translated to yield an approximately 330 kDa polyprotein. This is processed by three proteinase active sites located within the nascent polypeptide to yield the majority of mature viral proteins. The potyviral proteinases are: The P1 serine proteinase that cleaves only *in cis*; the HC-Pro, which has cysteine proteinase activity but cleaves only *in cis*, and the 27K Pro, which is a serine proteinase that can carry out *cis* and *trans* cleavage.

Recently, bioinformatic analysis of collected sequences of the *Potyviridae* and deep sequencing analyses of the products of viral RNA synthesis in plants infected with these viruses revealed an additional form of gene expression for (+) sense RNA viruses. During (+) strand synthesis from the (-) strand template, the potyviral RdRP slips at specific sequence to yield a small number of molecules that are a variant of the viral RNA that can act as the translation template for a potyviral movement protein. Sweet potato – Infecting potyviridae also employ transcriptional slippage to generate the template for translation of a suppressor of RNA silencing.

Other, less common strategies include leaky scanning in which two potential translation start sites occur proximal to the 5′ end of an RNA, where the first AUG is in a usable but not particularly strong sequence context, allowing a proportion of scanning 40 S ribosomal subunits to pass by and detect a downstream AUG. These potential sites may be in-frame with each other. An example of this is in the translation of alternative in-frame polyproteins from the shorter of the two genomic RNAs of CPMV. In some cases two out-of-frame AUG codons generate overlapping ORFs, allowing a single RNA molecule to act as a mRNA for two distinct polypeptides, a strategy utilized by BYDV for proteins encoded by its open reading frames 3–4. Internal ribosome entry sites (IRESs) are sequences within the RNA molecule that allow binding of ribosomes without scanning and play an important part in allowing certain animal viruses, notably the picornaviruses, to evade the constraints of ribosomal scanning. However, apart from reports of potential IRESs in certain strains of TMV, and in small number of other examples (the *Carmovirus Hibiscus chlorotic ringspot virus* and the *Nepovirus Blackcurrant reversion virus*) internal initiation does not appear to play a widespread role in gene expression among plant-infecting RNA viruses.

Interactions of Viral mRNAs With the Cellular Translational Apparatus

Eukaryotic mRNA is normally translated in a circularized form (Fig. 3). Sub-components of the eIF4 complex form a bridge between the 5′ cap structure of the mRNA and poly(A) binding protein (PABP) bound to the 3′ tail. This circularization enhances the efficiency of translation by re-cycling 40S ribosomal subunits back to the 5′end of the mRNA. There are viruses with genomic RNAs that possess 5′ cap structures and 3′ tails (a small number of positive sense RNA viruses e.g., *Beet necrotic yellow vein virus*) or which give rise to subgenomic mRNAs that possess these features (e.g., dsRNA and negative sense RNA viruses). These RNAs effectively mimic cellular mRNAs and can circularize during translation. However, many plant-infecting positive sense RNA viruses lack a 5′ cap and most do not possess a 3′ poly(A) tail.

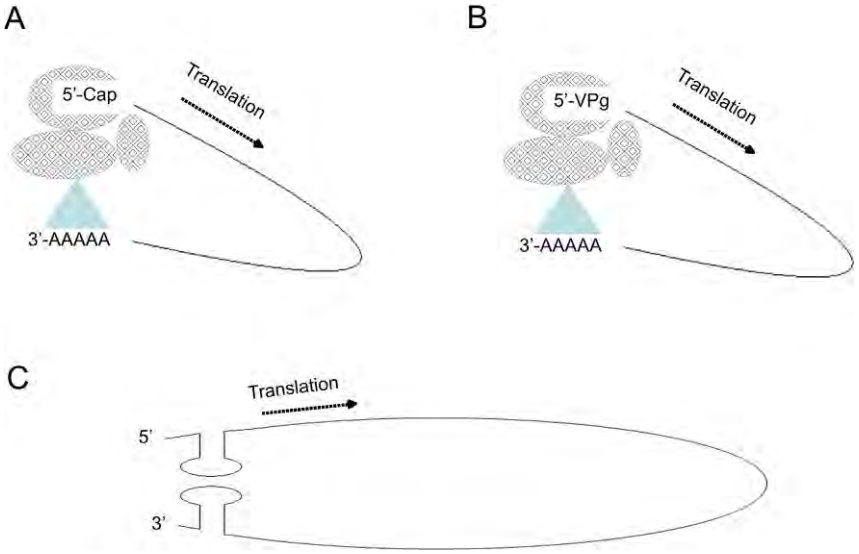

Fig. 3 Interactions of positive-sense RNA viruses with the translation machinery of the plant cell Cellular mRNAs undergoing translation can be circularized due to interactions between the 3′ poly(A) tail with poly(A)-binding protein (PABP: Indicated by a triangle) and between PABP and subunits of eukaryotic initiation factor 4 (eIF4: Indicated by ovals) bound to the cap structure 5′ terminus of the mRNA. (A) This arrangement enhances the rate of protein synthesis by facilitating recycling of 40S ribosomal subunits to the 5′end of the mRNA. Viral RNAs can exploit this circularized mode by possessing 5′ cap structures or 3′ poly(A) tails, or by possessing other features that allow interaction with host components. For example, the VPg (viral protein genome-linked) of potyviruses, and members of several other groups of plant-infecting viruses, interact with certain eIF4 subunits. (B) However, viruses with RNAs that lack any ability to interact with eIF4 or PABP (e.g., *Barley yellow dwarf virus*) can enhance the rate of cap- and poly(A)-independent translation through circularization by self-interaction between complementary sequences on stem loops proximal to the 5′–3′ termini (C).

Translatable viral RNA molecules that lack a 5′ cap or 3′ poly(A) tail possess various combinations of structural features to allow them to associate with PABP and the eIF4 complex and become circularized during translation. Viral RNAs lacking both structures can be translated efficiently due to the presence of translational enhancer sequences within the 5′- and 3′-UTRs. In the example of the *Luteovirus Barley yellow dwarf virus* (BYDV) W.A. Miller and colleagues (Iowa State University) showed that these translational enhancer sequences fold, forming so-called kissing stem-loops. Base-pairing between complementary RNA sequences in the loops allows circularization of the RNA molecule (Fig. 3). For viral genomic and subgenomic RNAs with 3′-tRNA like structures, such as TMV, pseudoknot structures within or immediately upstream of the tRNA-like structure can substitute for a 3′ poly(A) tail. The binding of host factors such as HSP101 may aid in bridge formation between the upstream pseudoknots and the cap structure and Ω sequence at the 5′ end of the RNA molecule but work in mutant arabidopsis plants suggests that this interaction may not be vital for a successful infection. Around a third of species of plant-infecting positive sense RNA viruses encode VPg proteins. As mentioned in the section titled 'Positive-sense single-stranded RNA viruses', a VPg can functionally substitute for a 5′ cap structure by binding to subunits of eIF4, allowing bridging to occur to the PABP bound to the viral RNA 3′ poly(A) tail (e.g., potyviruses: Fig. 3).

Mechanisms of Pathogenesis

Models of Pathogenesis: Competition for Host Resources *Versus* Specific Virus-Host Interactions

Plant RNA viruses elicit a diverse range of disease symptoms that vary dramatically between viruses and between virus strains. In the context of agriculture and horticulture, viruses are responsible for around 30% of the pathogen-induced diseases that cause significant losses in crop yield or quality. Their impact on crop production is likely to increase over time as around 45% of novel or emerging diseases appear to have a viral etiology.

In most plant–virus interactions in which the host is susceptible to infection, death of the host does not occur. Given that plant viruses can move from cell to cell through plasmodesmata (see 'Spread through host plants'), there would be no advantage to the virus in inducing lysis of host cells. The occurrence of host cell death is often an indication of resistance, or attempted resistance, to the virus.

It might be assumed that viral disease symptoms occur because a virus competes for and to some extent exhausts the resources of its host cells. However, in the majority of examples this model proved to be overly simplistic. For many virus-plant interactions the pathogenicity of a particular virus strain depends much more upon the qualities of one or more specific viral protein or nucleic acid sequences rather than upon any correlation with the quantity of virus that is produced during infection. Thus, mild and severe strains of the same virus often achieve the same titer in host tissues.

In recent years, major advances have been made in the identification of viral and host factors involved in pathogenesis. For example, it has been found that suppression of host defense mechanisms, particularly of those based on RNA silencing, is important

for the success of infection. RNA silencing is a mechanism, universal among eukaryotes, in which small RNAs direct the sequence-specific cleavage of target RNAs. One of the functions of this homology-based destruction of RNA is to contribute to host resistance to viruses by limiting or preventing the accumulation of viral RNAs (see 'Viral counter-defense: RNA silencing and its suppression by viruses'). Recent advances have shown that it is the dynamic interplay between host RNA silencing pathways and viral counter-defense factors that engender many common viral disease symptoms in plants.

Cytological Changes

Electron microscopy reveals striking changes in cellular ultrastructure resulting from virus infection. These include changes in cellular membrane morphology associated with assembly of positive-sense RNA virus replication complexes (see 'Composition and assembly of the replicase complex'). One of the first characterized examples of this was the interaction of the *Turnip yellow mosaic virus* (TYMV) replication machinery with the outer membrane of infected cell chloroplasts. In TYMV-infected cells the chloroplasts swell, aggregate, and adopt a cup-shaped form. Two TYMV proteins are involved in these processes, the 66–140 kDa proteins. Both are targeted to the chloroplast membrane. The 140 kDa protein induces chloroplast aggregation and also induces invagination of the outer chloroplast membrane to form small peripheral vesicles, which are the sites of TYMV replication (see 'Composition and assembly of the replicase complex').

Virus-infected cells may contain inclusions of virus-derived material, sometimes termed 'viroplasms'. Inclusions may be crystalline, quasicrystalline, or amorphous in appearance. They may result from the accumulation of nascent virions in the host cell. Some inclusions are aggregates of viral proteins. For example, the potyviruses use a polyprotein strategy to express most of their proteins ('Strategies used to overcome the constraints of eukaryotic translation'). As a result, certain viral proteins are produced in excess of need. The surplus proteins form nuclear inclusion bodies, composed of the nuclear inclusion (NI) proteins NIa and NIb, and cytoplasmic bodies, formed from aggregated cylindrical inclusion (CI) protein. Cytoplasmic bodies sometimes display 'pinwheel' morphology. Other inclusion bodies may represent the sites of active replication associated with nascent virions in the cytoplasm or nucleus (e.g., cyto- and nucleorhadoviruses, respectively) or virion core structures (e.g., reoviruses).

Macroscopic Symptoms: Mosaics and Vein Clearing Symptoms

Among the most common, visible virus symptoms on the younger, developing leaves of systemically-infected plants is the generation of mosaic or stripe patterns (Fig. 4). These patterns are formed from discrete regions of chlorotic (yellow or light green) tissue surrounding or adjacent to healthy-appearing areas of darker green tissue, which are sometimes called dark green islands (DGIs). This patterning takes the form of a mosaic on dicotyledonous hosts or stripes on monocotyledonous hosts and reflects the respective patterns of cell division and expansion in these two groups of angiosperms.

The cells within DGIs exhibit cytology and photosynthetic capacity comparable to that of leaves of uninfected plants. Importantly, they contain much less virus than chlorotic areas and resist reinfection with the same or closely related viruses but not with unrelated viruses. Several lines of evidence indicate that DGI formation and maintenance is controlled by localized RNA silencing (for description of RNA silencing, see 'Viral counter-defense: RNA silencing and its suppression by viruses'). During infection of *Nicotiana benthamiana* with the potyvirus *Tamarillo mosaic virus* (TaMV), virus-specific transcripts were undetectable in DGIs. Prior systemic infection of plants with an asymptomatic, unrelated heterologous viral vector harboring a region of the TaMV genome, followed by superinfection with TaMV, resulted in reduced titers of both viruses in the DGIs. DGI formation in TMV-infected tobacco can be suppressed by a viral RNA silencing suppressor and by silencing of RNA-directed RNA polymerase 1 (RDR1), a component of the host RNA silencing machinery. Finally, high levels of short interfering RNAs (siRNAs) complementary to the infecting virus were detected in DGIs.

Fig. 4 Symptoms of TMV in tobacco (*Nicotiana tabacum*) leaves Non-inoculated, systemic leaves of mock-inoculated and TMV (strain YSI/1) inoculated *N. tabacum* plants. Note mosaic patterning and vein-clearing in the infected leaves. Photographs supplied by S. F. Fu (University of Cambridge, UK).

In systemically-infected leaves of dicotyledonous hosts the cells bordering the veins become light green, yellow, or even white. This effect is known as vein clearing and often occurs before the establishment of mosaic symptoms. The precise cause of vein clearing is not fully understood and may differ between virus-host combinations. In her classic observations of virus-infected sugar beet, K. Esau noted that in plants infected with beet mosaic virus, the cells surrounding veins enlarge and their chloroplasts break down resulting in translucency. However, W. Dawson has observed that vein clearing induced by TMV occurs without chloroplast degradation and is seen only between 25°C and 40°C, seemingly due to host signaling since the phenomenon precedes viral invasion. The 'chlorotic' appearance of veins in TMV-infected tobacco is thus an optical illusion produced by a change in size and translucency of the surrounding cells.

Changes in Plant Metabolism

Virus infection can cause dramatic changes in photosynthesis, respiration, and the translocation of carbohydrates and other substances around the host plant. Chlorosis in virus-infected leaves can result from damage to chloroplasts resulting from inhibition of photosynthetic activity. Studies of TMV-infected tobacco plants by several laboratories suggest that chlorosis in these plants is caused by the uptake of viral CP by chloroplasts. R.N. Beachy's laboratory showed that the CP accumulates in the thylakoid membrane, where it disrupts photosystem II (the water-splitting reaction of photosynthesis), resulting in severe free radical damage to the organelle. Using isolated chloroplasts for *in vitro* studies, M. Zaitlin's laboratory showed that uptake of the CP did not exploit the host's normal pathways for protein import. Rather, it was a property of the CP, when it was assembled into multimeric disks (an intermediate structure in TMV virion assembly), that allowed it to be taken up spontaneously into the chloroplasts and subsequently into the thylakoid membrane. Work from this group, and the group of K. Lehto, demonstrated that a single amino acid in the CP determines the efficiency of its uptake into chloroplasts and the relative severity of chlorosis caused by different TMV strains.

Changes in photosynthesis, respiration and carbohydrate translocation often cause localized accumulation and depletion of starch, the main storage carbohydrate of most plants. A. Maule and colleagues showed by detailed examination of virus infection sites that this and other metabolic changes wrought by virus infection result from highly complex, dynamic and apparently organized changes in host gene expression, enzyme activity and physiology (e.g., photosynthetic activity) within cells in and around the infection site. However, it is still unclear what, if any, importance these changes in metabolism have in determining the outcome of a plant-virus interaction.

In plants, carbohydrate, typically in the form of sucrose, is translocated through the phloem elements. Translocation of this fixed carbon is vectorial, with sucrose moving from carbon source tissues to carbon sink tissues. Examples of carbon source tissues are photosynthetically active leaves or storage tissues undergoing mobilization of reserves (e.g., during seed germination), whereas carbon sinks include immature or rapidly growing tissues and storage organs during the development of seeds, tubers, and so on. The partitioning of carbohydrate between plant organs is a delicate and dynamic equilibrium that alters subtly as plant development moves from germination to vegetative growth, and on to senescence and flowering. Disruption of this process by virus infection results in deregulation of sink – source relationships and is caused primarily because of the effects of movement proteins (see 'Spread through host plants'). Studies with transgenic potato and tobacco plants expressing the TMV movement protein demonstrated that by enhancing the gating of plasmodesmata, fine control of carbohydrate partitioning was abolished resulting in inhibition of development in younger tissues and storage organs.

Disruption of Phytohormone Signaling

Virus infection can disrupt signaling mediated by plant growth substances, auxins and cytokinins, altering plant growth and development and causing striking symptoms including stunting and a wide range of developmental abnormalities. We now know that in some instances this can be caused indirectly, for example, by the interference with the regulation of turnover of specific mRNAs (e.g., those encoding auxin response factors: ARFs) by microRNA (miRNA) (see 'Viral counter-defense: RNA silencing and its suppression by viruses'). J. Culver and colleagues (University of Maryland) demonstrated a direct effect of a viral protein on hormone signaling. The phytohormone auxin is involved in a broad range of developmental processes and disruption of auxin signaling results in strong developmental phenotypes. In yeast two-hybrid studies, the TMV 126/183 kDa replicase protein was identified as an interactor with a number of auxin/indole acetic acid proteins (Aux/IAAs). Aux/IAAs bind to and inhibit the activity of ARFs, which are transcription factors that activate auxin-responsive genes. By binding Aux/IAAs, the TMV replicase protein deregulates auxin-responsive gene expression, inducing developmental abnormalities. A mutant TMV strain encoding a replicase protein defective in the Aux/IAA interaction induced milder symptoms, while silencing of the plant gene for the interacting Aux/IAAs caused uninfected plants to phenocopy some of the developmental symptoms seen in TMV-infected plants.

Viral Counter-Defense: RNA Silencing and its Suppression by Viruses

RNA silencing describes a number of interrelated mechanisms that regulate host gene expression, control transposons, and target the RNAs of invading viruses for degradation. The mechanisms are highly conserved between plants and animals. For detailed explanations, readers are directed to recent reviews (see Further Reading). In the case of host genes, silencing is effected by DNA

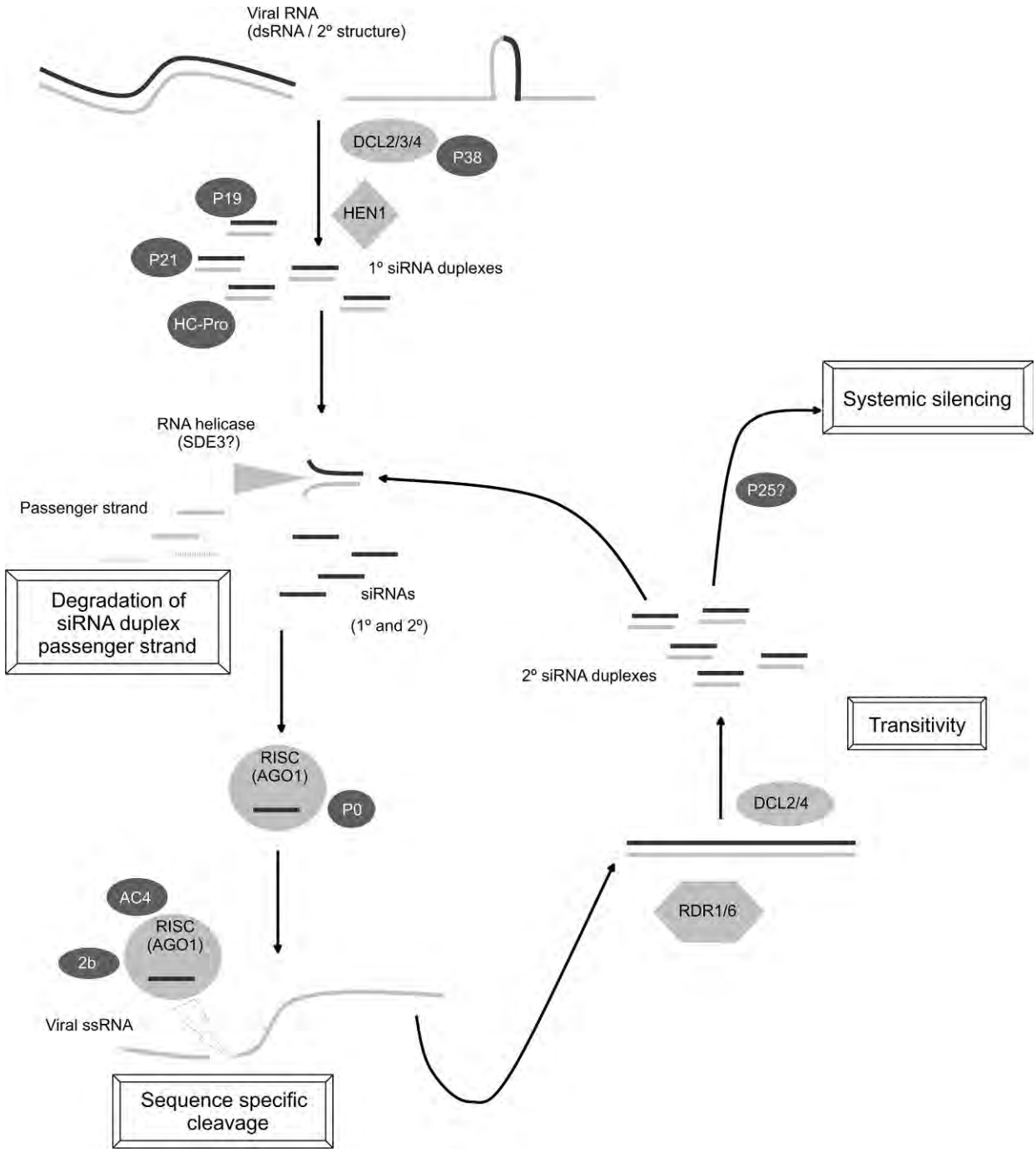

Fig. 5 Simplified schematic illustrating siRNA-mediated RNA silencing against viruses RNA silencing is triggered by dsRNA formed during viral replication, as a consequence of viral gene expression or due to secondary structure. A number of viral silencing suppressors are depicted by dark gray ovals containing white lettering, at probable or confirmed sites of action (see *Further Reading*). Host-encoded components of the silencing machinery are depicted by light gray shapes containing black lettering. dsRNA is cleaved by DCLs 2, 3 and 4, yielding primary siRNA duplexes. The viral silencing suppressors p19 (encoded by tombusviruses), HC-Pro (encoded by potyviruses) and p21 (encoded by closteroviruses) interfere with silencing by binding siRNAs, whilst p38 (encoded by carmoviruses) is thought to suppress silencing by inhibiting siRNA production by DCL enzymes. Primary siRNA duplexes are methylated by HEN1 and bind to a RISC, whereupon the passenger strand is degraded, yielding a primary siRNA (or guide strand). The primary siRNA guides sequence-specific degradation of complementary (target viral) RNAs by an AGO protein, which is the only identified member of the RISC in plants. The viral silencing suppressor P0 (encoded by poleroviruses) operates by targeting AGO proteins for degradation, whilst the 2b protein suppresses silencing by directly binding the RISC. In the transitivity phase of silencing, the products of target viral RNA cleavage are used as templates by host RDRs 1 and 6 to synthesize dsRNA. The dsRNA is cleaved by DCLs 2 and 4 to generate secondary siRNA duplexes that are derived from regions beyond the primary cleavage site. RISC the conducts further sequence-specific degradation of the original target viral RNA, guided by the secondary siRNAs from the secondary siRNA duplexes. Adapted from Lewsey, M., Palukaitis, P., Carr, J.P., 2008. Plant-virus interactions: Defence and counter-defence. In: Parker, J., (Ed.), Molecular Aspects of Plant Disease Resistance. Oxford: Blackwell.

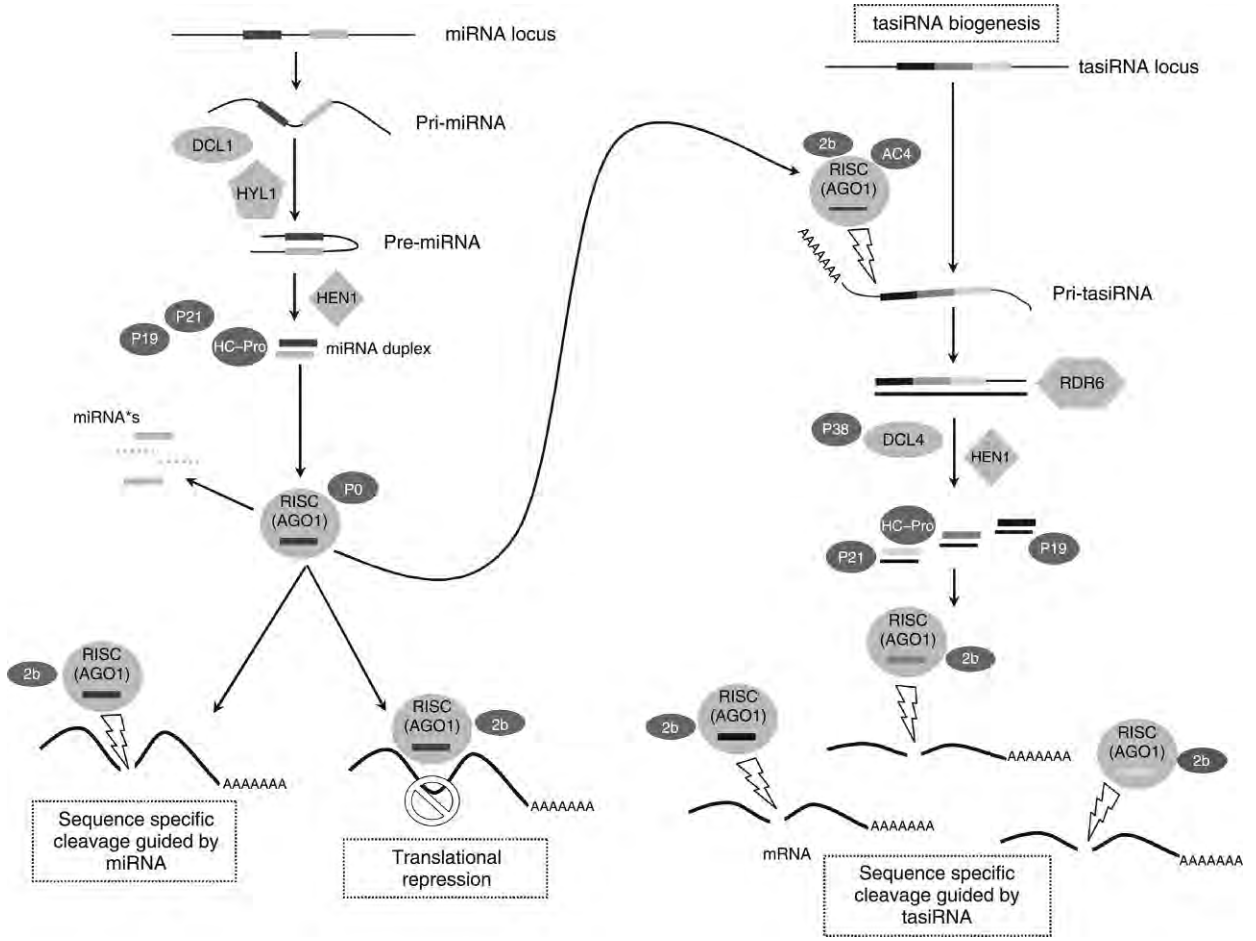

Fig. 6 Simplified schematic illustrating silencing of endogenous transcripts by miRNAs and tasiRNAs Silencing mediated by miRNAs commences with transcription of a pri-miRNA from an endogenous *MIR* gene. A number of viral silencing suppressors are depicted by dark gray ovals containing white lettering, at probable or confirmed sites of action (see *Further Reading*). Host-encoded components of the silencing machinery are depicted by light gray shapes containing black lettering. Contained within the pri-miRNA is a miRNA sequence (thick dark gray line). The pri-miRNA folds back and is processed by the enzymes DCL1 and HYL1, which produces the smaller pre-miRNA. Cleavage of the pre-miRNA yields a miRNA duplex, containing the miRNA and its complementary miRNA*. The viral silencing suppressors p19 (tombusviruses), HC-Pro (potyviruses) and p21 (closteroviruses) interfere with miRNA-mediated silencing by binding miRNAs. The miRNA duplex is incorporated into a RISC, one component of which is an AGO protein, and the complementary miRNA* is degraded leaving the mature, single stranded miRNA. The mature miRNA targets the RISC against endogenous mRNAs in a sequence-specific manner, causing them to be cleaved or translationally repressed. As with siRNA-mediated silencing, the viral silencing suppressors P0 (poleroviruses) and 2b (cucumoviruses) target the AGO proteins (see legend to Fig. 6) in order to suppress miRNA-mediated silencing, and likewise tasiRNA-mediated silencing. Finally, miRNAs also target RISC against complementary, non-coding pritasi-RNA transcripts. Pritasi-RNA transcripts are transcribed from endogenous *TAS* genes and their targeting by RISC initiates dsRNA synthesis by RDR6 using the pritasi-RNA as a template. Phased cleavage is conducted by DCL4, which may be inhibited by the viral silencing suppressor p38 (carmoviruses), generating tasiRNA duplexes that are methylated by HEN1. tasiRNAs may also be targeted by the viral silencing suppressors p19, HC-Pro and p21. These duplexes are taken up into a RISC and the passenger strand is degraded, leaving the mature tasiRNAs. These are complementary to endogenous RNA and target them for endonucleolytic cleavage by the RISC. Adapted from Lewsey, M., Palukaitis, P, Carr, J.P., 2008. Plant-virus interactions: Defence and counter-defence. In: Parker, J., (Ed.), Molecular Aspects of Plant Disease Resistance. Oxford: Blackwell.

methylation of genetic loci, translational inhibition, and degradation of mRNAs, whereas RNA viruses appear to be silenced only by degradation (Figs. 5 and 6).

RNA silencing is guided by two major classes of small RNAs (sRNAs), termed miRNAs and siRNAs that are 20–25 nucleotides in length. sRNAs bear high sequence homology to their target loci, which confers specificity on the silencing process. Host RNAs are targeted by miRNA-mediated silencing and also by transitive (ta)siRNA-mediated silencing, while RNA viruses are thought to be targeted only by siRNA-mediated silencing.

The biogenesis and activity of miRNAs, tasiRNAs, and virus-derived siRNAs involve a number of conserved steps. *MIR* genes, which encode miRNAs, are transcribed and form a partially dsRNA structure. This is processed by ribonuclease III-like enzymes of the DICER-like (DCL) family into a 20–25 nt duplex. The duplex then binds to an RNA-induced silencing complex (RISC), which includes a ribonuclease H-like protein of the ARGONAUTE (AGO) family. Upon binding to the RISC one strand of the duplex, the 'passenger' strand, is degraded, leaving the single-stranded 'guide' strand, or mature miRNA. The miRNA directs the AGO protein to

cleave endogenous transcripts in a sequence-specific manner. In addition to degrading mRNAs by endonucleolytic cleavage, AGOs may in some cases inhibit their translation.

miRNAs may also direct cleavage of the transcripts of *TAS* genes, which encode tasiRNAs. Using the cleaved *TAS* transcripts as templates, dsRNA is generated by cellular RDR enzymes. These transcripts are processed into sRNA duplexes by the activity of DCL enzymes. The duplexes bind to a RISC and the passenger strand is degraded, leaving the single-stranded, mature tasiRNA. The mature tasiRNA directs the endonucleolytic cleavage activity of AGO against mRNAs containing complementary sequences.

Virus-derived siRNAs are generated by a mechanism related to that which generates miRNAs and tasiRNAs. RNAs of invading viruses are initially cleaved into sRNA duplexes by DCL enzymes. Cleavage may occur because of the formation of dsRNA during viral replication, recognition of aberrant features of viral RNA (e.g., secondary structural features), or abnormally high expression levels of the viral RNA. The virus-derived sRNA duplexes bind to a RISC, which degrades the passenger strand. Mature virus-derived siRNAs direct sequence-specific degradation of viral RNA by the RISC. There is also a systemic signaling phase of RNA silencing that is triggered by RNA viruses and which initiates silencing-based resistance against the virus in distant, uninfected parts of the plant. Although assumed to involve mobile RNA, the exact nature of this signaling is not resolved.

The importance of RNA silencing in antiviral defense is indicated by the fact that most, if not all, plant viruses have evolved proteins that inhibit RNA silencing to some extent. These viral silencing suppressors differ from each other in many respects including the point, or points, at which they exert their inhibitory activity on the RNA silencing pathways (Figs. 5 and 6). The 2b protein, the silencing suppressor protein encoded by cucumoviruses, inhibits antiviral silencing by binding ds sRNA molecules. It also binds to certain AGO proteins, which is important in disease symptom induction and in viral manipulation of host-insect (e.g., vector) interactions. Crystal structures have been obtained for two tombusviral p19 silencing suppressor proteins. P19 operates by binding 21 nt siRNAs in a size-selective manner, sequestering them and preventing their incorporation into a RISC. Many other viral silencing suppressors also appear to act, at least in part, through sequestration of sRNAs, for example the potyviral P1/HC-Pro or the carmoviral p21 protein, while the carmovirus p38 (which is also the viral coat protein) inhibits sRNA-mediated dicer action. Strikingly different approaches to the disruption of RNA silencing is are demonstrated by the P0 silencing suppressor proteins of poleroviruses and the RNAse III type silencing suppressors of criniviruses. P0 suppressors act as F-box proteins that promote ubiquitination and consequent proteasome-mediated destruction of AGO1 (Figs. 5 and 6). The RNAse III type silencing suppressors degrade ds sRNAs.

RNA Silencing and Pathogenesis

The first indications that inhibition of RNA silencing pathways may cause symptom induction came from studies where viral silencing suppressor proteins were expressed constitutively in transgenic *A. thaliana*. These transgenes induced phenotypes that were reminiscent of, though not identical to, virus symptoms. It was found that these symptom-like phenotypes were caused by disruption of the miRNA-mediated silencing pathway, which is crucial in the regulation of plant development. As discussed previously, viral silencing suppressor proteins inhibit various steps in the silencing pathway (see 'Viral counter-defense: RNA silencing and its suppression by viruses'). In some cases disruption of miRNA-mediated silencing may perhaps be incidental, resulting from targeting of components of the miRNA pathway that are shared with the antiviral siRNA-mediated silencing pathway. However, in the case of the CMV 2b silencing suppressor protein its ability to interact with component(s) of the miRNA pathway and induce strong symptoms varies considerably between strains. This suggests that some silencing suppressors can selectively target the miRNA pathway. It remains to be seen what advantage or disadvantage this might have for the virus.

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Role of B Cells and Antibodies in Controlling Bacterial Pathogens

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As the workhorses of our internal defense system, we owe an enormous debt to antibodies. Responding to external threats such as disease-causing microorganisms, protein antibody molecules are released by specialized immune cells into the blood stream to disarm the threat. Their mode of attack is target recognition: millions of different molecules can be created, each with a different binding site, which can identify distinctive proteins on their specifically assigned foe with pinpoint accuracy. From Anatomy of a Killer. NobelPrize.org.

Perspective

Antibodies are the primary means by which the immune system eliminates bacterial pathogens from the human body. Antibodies clear bacteria that have gained access to circulation or normally sterile tissues, such as the liver and heart. Antibodies also protect the mucosal or "wet" surfaces of the human body, such as the lungs, oral cavity, urogenital tract, and the gastrointestinal tract, which are constantly exposed to a spectrum of opportunistic and pathogenic microorganisms.

When a host encounters a pathogen for the first time, there is an initial lag before the first wave of antibodies arrive on the scene. It is the job of the innate immune system to keep the invading bacteria at bay, while antibody-producing cells or "B cells" are enlisted and dispatched to the site of infection. The initial sortie consists of IgM antibodies. Although IgM antibodies are relatively low specificity and low affinity, they can resolve infections by working in concert with the innate immune system to lyse and engulf invading pathogens.

The second offensive, which appears 5–7 days after the start of an infection, is led by IgG or IgA antibodies, depending on the anatomical compartment. IgG antibodies predominate in the systemic compartment (e.g., blood, liver, spleen, heart), while IgA antibodies are the principal antibody type in mucosal secretions (e.g., saliva, intestinal mucus). The invading bacteria are soon confronted by swarms of IgG and IgA antibodies that fasten themselves to surfaces and appendages, such as flagella.

Antibody-coated bacteria have little chance of escape. In the systemic compartment, IgG-coated microorganisms are engulfed by professional phagocytic cells like neutrophils and macrophages. Or they are attacked by complement, a cascade of serum proteins that form pores in bacterial cell walls. In either case, IgG-coated pathogens are destroyed. In mucosal compartments, IgA-coated bacteria become entrapped in mucus and are unable to gain a foothold on host tissues. Consequently, IgA-bacteria immune complexes are eliminated from the body through normal physiologic processes such as peristalsis, mucociliary action, and coughing.

The job of B cells is not over when an acute infection is cleared. A subset of B cells known as plasma cells (effectively antibody "factories") takes up residence in peripheral tissues and bone marrow and secretes pathogen-specific IgG or IgA for months or even years. These so-called long-lived plasma cells provide a continuous supply of pathogen-specific antibodies in blood and mucosal secretions, positioned to intercept a pathogen upon its return. The term "antibody titer" refers to the levels of a given pathogen-specific antibody in a particular body fluid.

Another subset of B cells, called memory cells, patrols central and peripheral tissues, on the look-out for return visitors. Memory B cells remain quiescent until they reencounter a pathogen-derived product known as an antigen. At this point, some of the memory B cells will differentiate into plasma cells to further increase levels of circulating antibody, while others will reenter lymph nodes and undergo additional rounds of antibody gene editing through a process known as somatic hypermutation (SHM), yielding new swarms of antibodies with greater affinities for their targets. Through repeated rounds of SHM, B cells give rise to a broad and essentially infinite diversity of antibodies capable of adapting to the ever-evolving onslaught of microbial invaders.

The goal of this article is to provide an introduction to how antibodies and B cells function in immunity to bacterial pathogens. Considering the sheer number of bacteria that cause disease in humans, it is not possible to address them all in a single article. Therefore, for the sake of illustration, the discussion will be limited to the nine bacterial pathogens (and their respective toxins, when appropriate) for which there are currently approved vaccines in the United States (Table 1). For a detailed description of these vaccines and their targets, the reader is referred to The Pink Book, published by the Centers for Disease Control and Prevention (CDC). All nine of the vaccines work by stimulating pathogen-specific antibodies that target exotoxins, promote complement-dependent killing, mediate opsonophagocytosis, and/or prevent colonization of mucosal epithelial surfaces.

Antibodies, B Cells, and the Nobel Prize

The discovery of the inner workings of antibodies and B cells has gone hand-in-hand with the pursuit of vaccines to combat some of the most significant killers in public health. In the early 20th century, diphtheria caused more than 200,000 cases and greater than 15,000 deaths per year in the United States alone. Today, almost a century after implementation of a universally administered vaccine, diphtheria is essentially eradicated in the United States. The etiological agent of diphtheria is the aerobic gram-positive

bacterium, *Corynebacterium diphtheriae*, which colonizes the human nasopharynx and secretes an extraordinarily potent and fast-acting toxin called diphtheria toxin. For physicians and scientists in the late 1800s, it was a mystery how an infection seemingly localized to the throat could be so deadly. Equally mysterious was how the immune system works to combat infectious agents like diphtheria.

It was Emil Adolph von Behring (1854–1917) and Kitasato Shibasaburo (1853–1931) who first identified a soluble substance in the blood of convalescent animals that when injected into naïve animals imparted protection against diphtheria. The protective substance, termed "antitoxin," established the concept of passive immunity and revolutionized therapies for diphtheria at the turn of the 20th century. In 1901, the first Nobel Prize in Physiology or Medicine was awarded to Emil von Behring "for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths." Within a matter of years, public health laboratories, including the New York State Department of Health, were generating diphtheria antitoxin in horses and using it statewide in children to treat disease. An antitoxin therapy against tetanus toxin soon followed.

Of course, we now know that antibodies are responsible for the soluble "antitoxin" activity described by Emil von Behring and Kitasato Shibasaburo and characterized by other early immunologists, including Paul Ehrlich. These early discoveries are documented in the articles of "Microbe Hunters," Paul de Kruif's 1926 book that profiles the pioneers of immunology and microbiology.

A more thorough understanding of the biochemical nature of antibodies and the cells involved antibody production emerged in the early 20th century, as documented in a brief historical review of B cells by the preeminent immunologist, Dr. Max Cooper. Milestones in the field of antibodies were acknowledged by the Nobel Prize committee. For example, the 1919 Nobel Prize in Medicine or Physiology was awarded to the Belgian microbiologist Jules Bordet for the discovery of complement proteins that promote bacterial killing in conjunction with antibodies. The Australian born immunologist, Sir Frank Macfarlane Burnet, received the 1960 Nobel Prize in Physiology or Medicine for the clonal selection theory, which essentially argued that an antibody of a given specificity arises from a single B cell clone that expands in the presence of its cognate antigen (i.e., an antibody's target). The discovery that antibodies arise as a result of self-imposed genetic engineering through processes known as VDJ recombination and SHM occurred in the 1970s. In 1972, Gerald M Edelman and Rodney R Porter shared the Nobel prize for the elucidation of the structure and function of antibody molecules at the protein level. A little more than a decade later, the 1984 Nobel Prize was awarded to Niels K Jerne, Georges J.F. Kohler, and César Milstein for the ". . ..discovery of the principle for the production of monoclonal antibodies," a technology that revolutionized the field of immunology and related disciplines and has given rise to modern drugs to treat breast cancer, leukemia, and other diseases. In 1987, Dr. Susumu Tonegawa was awarded a Nobel prize for elucidating how B cells are able to generate antibody diversity. Finally, the 2018 Nobel prize in Chemistry was awarded to the American microbiologist, George P. Smith, and the British immunologist, Sir Gregory P. Winter, for the ability to genetically manipulate antibodies in bacteria and bacteriophages, which has impacted biotechnology industry and fields of cancer therapy.

Antibody-Structure and Function

Today, antibodies are arguably some of the most well studied molecules in biology. In mice and humans, there are five isotypes: IgM, IgG, IgA, IgE, and IgD. Because IgE primarily functions in allergic responses and the role of IgD in immunity is still very much an active area of research, these two isotypes will not be discussed further. Rather, the focus of this article will be on IgM, IgG, and IgA. It should be underscored that the terms "immunoglobulins" and "antibodies" are frequently used interchangeably. However, there is an operational distinction: the term immunoglobulin refers to the bulk fraction of all antibodies precipitated from serum; the term antibody refers to a subset of immunoglobulins directed against a specific target or antigenic determinant (antigen).

Table 1 Role of antibodies in currently approved vaccines for bacterial pathogens.

Vaccine	*Pathogen*	*Antibody mode(s) of action*
Anthrax	*Bacillus anthracis*	Neutralize anthrax toxin
DT	*Corynebacterium diphtheriae*	Neutralize diphtheria toxin
Tetanus	*Clostridium tetani*	Neutralize tetanus toxin
aP	*Bordetella pertussis*	Neutralize pertussis toxin Inhibit bacterial adhesion to respiratory tract
Cholera	*Vibrio cholerae*	Inhibit bacterial colonization of intestine
Hib	*Haemophilus influenzae*	Complement-dependent SBA Opsonophagocytosis (OPA)
Meningococcus	*Neisseria meningitidis*	Complement-dependent SBA
Pneumococcus	*Streptococcus pneumoniae*	Opsonophagocytosis (OPA)
Typhoid	*Salmonella enterica* spp. Typhi	Opsonophagocytosis (OPA)

IgG Antibodies

IgG is often considered the prototypic antibody isotype. IgG consists of two identical polypeptides of ~50,000 MW, known as the heavy chains (H), and two identical polypeptides of ~25,000 MW known as the light chains (L). Each heavy chain is paired covalently to a light chain in a H-L configuration. The two H chains are covalently linked to each other via a disulfide bond so that a single IgG molecule consists of HL + HL pair. Structurally, the IgG molecule is typically compared to the letter "Y" with each of the antibody's arms extending upward to engage with its cognate antigen. In reality, however, the arms have a degree of freedom due to a hinge region, such that at any given time an antibody may resemble something between a "Y" and a "T." The upward facing arms are referred to as the Fab regions and constitute the antigen binding elements. The tail or stalk is known as the Fc region. If you imagine an IgG molecule bound to the surface of a bacterium, the Fab elements would be associated with the pathogen, while the Fc region would be projecting outward.

Although the Fc elements are not interacting with the pathogen per se, they are essential for pathogen killing. Specifically, Fc elements are recognized by two components of the innate immune system: the complement system and phagocytic cells like macrophages and polymorphonuclear cells (PMN). The complement system is a collection of more than two dozen serum proteins that work in concert to promote bacterial cell lysis through the formation of pores in bacterial cell walls. Phagocytic cells recognize and engulf antibody-coated bacteria through surface expressed Fc receptors (FcR). Fc receptors specific for IgG are referred to as FcγR. Thus, bacterial pathogens coated with IgG are either killed through complement-mediated lysis or cleared by phagocytic cells that express Fc-receptors.

IgG's two identical Fab elements involved in binding to antigens (e.g., flagella, lipopolysaccharide, toxins) and a single Fc domain that serves as a molecular adaptor recognized by complement and phagocytes, can be separated from each other by proteolytic digestion. Treatment of IgG with papain, a cysteine protease from the papaya plant, cleaves IgG at its hinge region and liberates the two monovalent Fab elements, which are referred to as Fab fragments, from the Fc element. Treatment of IgG with the acid protease, pepsin, also clips IgG at its hinge and releases the stalk, but in this case the two Fab elements remain joined to each in a molecular form known as $F(ab)_2$. Thus, Fab fragments are monovalent (i.e., one antigen binding site) molecules, while whole IgG molecules and $F(ab)_2$ fragments are bivalent (i.e., two antigen binding sites).

IgG-Antigen Interactions

The interaction of a given IgG antibody (Ab) with its antigenic determinant (Ag) is non-covalent and reversible. As such, the interaction is essentially a bimolecular reaction where Ab + Ag <−> Ab-Ag. The strength of an antibody's interaction with its antigen is driven by a net negative change in the Gibbs free energy, as a result of changes in enthalpy (heat) and entropy (structure). The rate of association (k_a) is expressed as $M^{-1}\,s^{-1}$ and the rate of dissociation (k_d) is expressed as s^{-1}. The equilibrium constant or K_D (M) is defined as free [Ab] [Ag]/[Ab-Ag]. K_D is the most commonly used reference point when citing an antibody's strength.

Strictly speaking, an antibody's affinity for its target is derived by measuring the binding kinetics of monovalent Fab fragments with its antigen using techniques like surface plasmon resonance (SPR). Fab binding affinities (K_D) range from weak (micromolar; 10^{-4}–10^{-6}) to very strong (picomolar; 10^{-10}–10^{-12}), with most falling within the nanomolar range (10^{-7}–10^{-9}). In practice, however, avidity may be a more appropriate measure of an antibody's strength than affinity, since avidity takes into account the fact that IgG is bivalent and may be able to engage two antigens simultaneously, thereby affecting the overall stability of that antibody-antigen interaction. In simple terms, avidity is equivalent to a person holding onto a vertical pull-up bar with both hands. If one hand comes off the bar temporarily, the other hand is still holding tight. Together, however, two hands make for a more stable grasp of the bar than just one hand. Likewise, antibody avidity is a measure of the strength of an interaction when both Fab arms can engage their targets simultaneously, which occurs frequently with highly repetitive bacterial surface antigens like LPS and capsular polysaccharides (CPS).

IgM and IgA Antibodies

IgM and IgA antibodies share the basic architecture of IgG but differ in several important respects. The most significant difference is valency. IgM is secreted by B cells as a pentamer in which five IgM molecules are linked via their Fc tailpieces to form a rosette-like structure with 10 Fab elements facing outwards. This unique arrangement is consequential. IgM antibodies, which are the first to arrive on the scene of a new infection, tend to have low binding affinities for their targets. However, what IgM antibodies lack in affinity, they make up for in avidity. The 10 Fab elements enable IgM to hold tight to polymeric antigens like LPS, CPS and flagella. In addition, IgM's Fc region is an extremely potent activator of the complement system. These two properties (i.e., high-avidity binding and potent complement activation) enables IgM to keep bacterial invaders at bay until IgG reinforcements arrive on the scene.

IgA is the predominant antibody type in most mucosal compartments. In the gut, for example, IgA-producing B cells (plasma cells) outnumber IgG and IgM plasma cells by approximately 10 to 1. IgA is generally produced as a dimer in which two IgA monomers are joined via their Fc tailpieces by a small protein called J chain. IgA is therefore tetravalent. Unlike IgG and IgM, IgA cannot activate the complement system. Rather, IgA functions in mucosal secretions like saliva, intestinal fluids and breast milk where serum components like complement are absent.

Transport of IgM and IgA Into Mucosal Secretions

It is the polymeric nature of both IgA (dimer) and IgM (pentamer) that permits their transport into the secretions that bathe the alimentary tract, the upper respiratory tract, and the upper female genital tract. IgA and IgM are also actively transported by mammary epithelial cells into colostrum and breast milk. The process of antibody transport from inside to outside is most clearly exemplified in the gastrointestinal tract, where a single layer of rectangularly shaped epithelial cells are joined side-by-side to form a barrier that prevents the diffusion of macromolecules and particles (including viruses and bacteria) from the lumen ("outside") to lamina propria ("inside"). Dimeric IgA and pentameric IgM antibodies produced by B cells in the lamina propria are recognized by the so-called polymeric immunoglobulin receptor (pIgR) located on the underside (basolateral) of epithelial cells. The pIgR captures IgA and IgM and shuttles them intracellularly across epithelial cells, a process known as transcytosis. Once on the apical face (outside), the pIgR is proteolytically clipped and a fragment of the receptor, known as secretory component (SC), still bound to IgA or IgM, is released into the intestinal lumen. IgA and IgM complexed with SC are known as secretory IgA (SIgA) and secretory IgM (SIgM), respectively. SC is a large, heavily glycosylated protein that wraps around IgA and IgM's Fc elements, thereby protecting them from the harsh environment of mucosal secretions. The pIgR does not transport IgG, although there are other mechanisms by which small amounts of IgG can gain entry into mucosal secretions.

Evolution of Antibody Responses

As alluded to above, microbial pathogens that successfully breach the body's natural defenses and gain access to normally sterile environments like the blood or any of the visceral organs will soon come under attack by swarms of antibodies. The first wave consists of low-affinity, high-avidity IgM antibodies capable of triggering the complement system and bacterial lysis. The second wave consists of high-affinity IgG antibodies, which also recruit complement and phagocytic cells to sites of infection. If the infection originates in a mucosal compartment, then the second wave of antibodies consists mainly of IgA, rather than IgG. The generation of long-lived IgG or IgA secreting plasma cells in response to a particular infection guarantees a steady reserve of circulating antibodies should the microbial invaders return. As further reinforcements, memory B cells are poised to proliferate and produce new swarms of IgG and IgA antibodies on demand. But how do antibody responses originate in the first place and what drives the formation of plasma cells and memory cells? These are central concepts in immunology and well beyond the scope of this article. However, it is important to appreciate three basic tenets.

Tenet 1: Antibody Affinities Evolve With Time

The first tenet is that antibodies evolve during the course of an active infection from low- to high-affinity and tailor their specificity to available targets on the surface of the invading pathogen. B cells originate in the bone marrow and migrate into the periphery where they develop into so-called naïve mature B cells that express IgM (and IgD) on their surfaces. When naive mature B cells "recognize" a foreign antigen the will secrete large amounts of IgM for immediate use in combatting an infection. Alternatively, they will enter the germinal centers and undergo switching to IgG- or IgA-positive B cells.

Broadly speaking, the conversion from IgM-positive to IgG-positive (or IgA-positive) B cells occurs in two stages. Class switch recombination (CSR) is a process where the DNA encoding the IgM Fc region is swapped for IgG or IgA Fc region. The choice between IgG and IgA Fc elements is dictated by the local environment. SHM is the process whereby the DNA encoding the antibody variable regions (V_H and V_L) are edited to generate a better "fit" with its antigenic determinant. This process is also referred to as affinity maturation, since the antibody's affinity for its target can increase by orders of magnitude as a result of SHM. Affinity maturation requires help from a number of other immune cell types, most notably follicular dendritic cells and T cells.

Tenet 2: Evolution is Driven SHM and Clonal Selection

The second tenet is that the process of SHM gives rise to an infinite number of antibodies against virtually any microbial protein or carbohydrate target. As defined earlier above, SHM is a process in which the DNA encoding an antibody's variable regions (V_H and V_L) are edited in an effort to improve overall binding affinity to its target. To be clear, any given B cell will produce antibodies of only one specificity (i.e., B cell "x" produces antibody "x," while B cell "y" produces antibody "y"). Therefore, the collective diversity of the antibody response to a given pathogen is dictated by the ensuing number of B cells that arise following infection. The term B cell "repertoire" refers to the total diversity of B cell specificities. However, B cell clones can give rise to progeny that carry minor variations in their variable regions that alter target specificity. B cell clone "x" can give rise to subclones x.1, x.2, x.3. which bind the same or nearly the same epitope but with slightly different affinities. The constant release of new generations of antibodies capable of saturating a given target has given rise to the concept of an antibody "swarm."

Tenet 3: B Cell Memory Guarantees Long-Term Immunity

The third tenant is that upon re-exposure to a pathogen, the recall antibody response is faster, more specific, and of higher magnitude than the first exposure, due to the existence of memory B cells. The preeminent immunologist Antonio Lanzavecchia

refers to memory B cells as the "...repository of an immune experience of an individual." In other words, memory B cells are the equivalent of an antibody hard drive that can be accessed on demand upon re-encounter with a pathogen. Concurrent with the formation of memory B cells are long-lived IgG or IgA plasma cells that can sustain circulating antibody levels over a period of years or even decades. In fact, modern day vaccines are predicated on the ability of a surrogate antigen or attenuated pathogen to elicit a memory B cell response capable of responding to an actual infection over a period of years or even decades.

In summary, a bacterial infection in a mucosal (e.g., lung, gut) or systemic (e.g., liver, kidney) compartment will elicit a two-phased antibody response (IgM followed by IgG and/or IgA) that will continue to mature or evolve until the pathogen is cleared. Long lived plasma cells will maintain circulating antibody titers for years to ward off a recurrent infection, while memory B cells will lie in waiting until called into action.

Antibody Function: Finding a Pathogen's Achilles' Heel

Just as the proverbial well-placed arrow to Achilles' heel led to the death of the mythic Greek warrior, antibodies eliminate bacterial pathogens by targeting sites of microbial vulnerability. To fully appreciate the shrewd nature of the immune system, it is important to recall the strategies used by pathogens to cause disease: exotoxins, evasion of innate defenses, tissue colonization, cellular invasion, and proliferation in the blood stream. B cells respond in kind to each of these insults by releasing their equivalent of poison arrows.

Bacterial Toxins, Virulence Factors, and Surface Polysaccharides as Antibody Targets

Without question, exotoxins are the most potent virulence factors associated with bacterial pathogens. Two prime examples are diphtheria toxin produced by *C. diphtheriae* and cholera toxin secreted by *V. cholerae* (Table 1). Diphtheria toxin is released by *C. diphtheriae* after the bacterium has colonized the human upper respiratory tract, while cholera toxin is produced when *V. cholerae* has set up shop in the small intestine. Diphtheria toxin kills mammalian cells by inhibition of protein synthesis as a result of ADP-ribosylation of elongation factor 2 (EF-2). Cholera toxin causes severe watery diarrhea by perturbing cyclic AMP levels in intestinal epithelial cells as a consequence of ADP-ribosylation of small G proteins. Strains lacking these toxins are essentially avirulent, attesting to the singular contributions of cholera and diphtheria toxins to disease. Three other pathogens listed in Table 1 produce exotoxins; *B. anthracis* (anthrax toxin), *C. tetani* (tetanus toxin) and *B. pertussis* (pertussis toxin).

Bacterial pathogens also express an array of other virulence factors involved in attachment to and invasion of mammalian hosts. Surface appendages like fimbriae and pili enable bacterial adherence to specific cell types. For example, both *B. pertussis* and *V. cholerae* express adhesins required for colonization of human epithelial cells. Other pathogens express macromolecular "syringes" or secretion systems on their surfaces that inject "effector" proteins into mammalian cells that facilitate bacterial entry into intracellular compartments. In fact, *Salmonella* species, including *S. enterica* serovar Typhi, employs two different so-called type-three secretion systems (T3SS) during infection of the gastrointestinal tract.

LPS, capsular polysaccharides (CPS), and exopolysaccharides (EPS) are classified as virulence factors by virtue of their ability to serve as shields against components of the innate immune system. The importance of CPS in pathogenesis is best exemplified by *H. influenzae*, a leading cause of bacterial meningitis in young children (Table 1). *H. influenzae* is a small, nonmotile Gram-negative bacterium that adheres to epithelial cells and mucins in the human upper respiratory tract. In some individuals, the bacterium becomes invasive and breaches epithelial defenses and surmounts the innate immune system to gain access to the blood stream. The invasive phenotype and ability to evade phagocytosis is associated with a specific CPS consisting of polyribosyl-ribitol-phosphate (PRP). Strains of *H. influenzae* that lack this specific CPS are associated with invasive disease much less frequently. Three other pathogens listed in Table 1 rely on capsule production to evade innate immunity and replicate within the human host: *S. pneumoniae*, *N. meningitidis*, and *S.* Typhi.

As a rule, immune responses to microbial pathogens, such as those listed in Table 1, are complex and involve antibodies targeting multiple different bacterial-associated antigens. However, when a single virulence factor like a toxin is central to disease manifestation, the most important antibody response may be against a single target. The list below demonstrates the strategies used by antibodies to exclude and/or clear bacterial pathogens from systemic and mucosal compartments.

Direct Neutralization of Toxins and Other Virulence Factors

The current anthrax, diphtheria, pertussis, and tetanus vaccines listed in Table 1 are designed specifically to elicit anti-toxin antibody responses. The primary mode by which antibodies neutralize toxins is by blocking attachment to host cell receptors. Antibodies that recognize epitopes on the binding subunits of anthrax, diphtheria, pertussis, and tetanus toxins have been described. However, toxin-neutralizing antibodies do more than just interfere with receptor binding events. In the case of anthrax, there are antibodies that prevent toxin oligomerization, pore formation, proteolytic cleavage, and subunit assembly. In fact, Raxibacumab is an FDA approved human IgG monoclonal antibody against anthrax toxin protective antigen (PA) designed as a pre-exposure prophylaxis in the event of anthrax being used in bioterrorism. Raxibacumab doesn't interfere with PA from binding to receptors on target cells, but it does prevent PA from associating with its toxic components, lethal factor and edema factor. Thus, antibodies are capable of interfering with essentially all the steps in the intoxication process.

Antibodies can also "neutralize" virulence factors associated with bacterial attachment and invasion of mammalian cells, as best exemplified by the acellular pertussis (aP) vaccine (Table 1). Whooping cough is caused by the Gram-negative bacterium, *Bordetella pertussis*, which colonizes the human upper respiratory tract where it secretes pertussis toxin. The aP vaccine consists of a collection of recombinant proteins that stimulates antibodies against pertussis toxin, plus several of the fimbriae and adhesins involved in bacterial attachment to the respiratory epithelium. Thus, antibodies against fimbriae and adhesins interfere with bacterial attachment to mucosal surfaces of the airway, while anti-toxin antibodies neutralize pertussis toxin that gains access tissues and circulation.

Activation of the Classical Complement Pathway

Simply stated, the complement system is a collection of more than two dozen serum proteins that function as a bridge between the innate and adaptive immune systems. The so-called classical complement pathway is initiated when two or more IgM or IgG antibodies are deposited in close proximity to each other on the surface of a pathogen. Clusters of IgM or IgG are recognized by the complement component, C1. C1 has an affinity for the Fc elements of IgM and IgG (namely IgG1, IgG2, and IgG3 subclasses), but not IgA. IgM, by virtue of its pentameric star-like structure, is particularly effective at recruiting C1. Once bound, C1 recruits a cascade of additional complement proteins to the bacterial cell surface, resulting in the assembly of the membrane attack complex (MAC). The MAC is a molecular channel or pore in the bacterial cell wall that perturbs cellular osmolarity and results in cell death by lysis.

Antibody-dependent, complement-mediated killing is a significant biological activity associated with the *H. influenzae* and *N. meningitidis* vaccines (Table 1). The *H. influenza* vaccine consists of the type B capsular polysaccharide (PRP) chemically linked to a carrier protein like the non-toxic variant of diphtheria toxin CRM 197 or inactivated tetanus toxin. The *N. meningitidis* vaccines in the United States consist of polysaccharide protein conjugates and various combinations of recombinant protein surface antigens. In all cases, vaccination stimulates serum IgG of antibodies capable of decorating (opsonizing) the surfaces of *H. influenzae* and *N. meningitidis* and subsequently activating complement-mediated killing. In fact, efficacy of *N. meningitidis* vaccines correlates with how well antibodies from a vaccinated individual perform in a standardized serum bactericidal assay (SBA) (McIntosh et al., 2015).

Killing by Opsonophagocytosis

Opsonophagocytosis is the engulfment, by macrophages and other phagocytic cells like neutrophils, of bacteria opsonized with antibodies and/or complement proteins. Thus, microbial cells in the blood or within a specific tissue that become coated with IgG are soon recognized by patrolling macrophages and neutrophils. Recognition occurs through specific surface-expressed FcγRs on the phagocytes. When multiple FcγRs are engaged simultaneously, it cues the phagocyte to envelope its prey and confine it within an intracellular compartment known as the phagosome. The phagosome is a dead-end for most microbial pathogens. Within minutes, the encased bacteria are exposed to reactive oxygen species, acidic pH and a barrage of degradative enzymes.

Opsonophagocytosis is a primary means by which antibodies promote the clearance of *S. pneumoniae* and accounts for the biological activity associated with the successful pneumococcal vaccines in widespread use around the globe (Table 1). In fact, one measure of vaccine efficacy is the opsonophagocytosis killing (OPK) assay, which involves the engulfment of *S. pneumoniae* by human neutrophils in the presence of serum from a vaccinated individual. Antibodies also coordinate opsonophagocytosis in the clearance of *H. influenzae* and *S.* Typhi.

Secretory IgA Paralyze and Entrap Bacteria in Mucosal Secretions

In mucosal compartments, secretory IgA (SIgA) plays by different rules. As noted earlier in the article, IgA is actively transported by the pIgR into intestinal secretions in the form of SIgA. SIgA does not activate complement, nor does it promote opsonophagocytosis. Instead, SIgA functions by a process known as immune exclusion in which pathogens are aggregated by antibodies and entrapped in mucus. Immune complexes between SIgA and microbial invaders are not only restricted in their ability to diffuse through mucus but can also be cleared from the body through normal physiologic processes such as cilia movement in the respiratory tract and peristalsis in the intestinal tract.

The concept of immune exclusion is best exemplified in the gut where pathogenic bacteria like *V. cholerae* set up shop and cause the disease cholera (Table 1). The bacteria employ flagellar-based motility to penetrate intestinal mucus and then deploy pili to secure themselves onto epithelial cells. Local production of cholera toxin results in a deadly bout of watery diarrhea that can kill an otherwise healthy adult within a matter of hours. Thus, the battle between host (antibodies) and microbe occurs in the intestinal lumen. As it turns out, antibodies against LPS are associated with immunity to *V. cholerae* infection, presumably because they are able to entrap the bacteria in mucus well before *V. cholerae* can gain a foothold on the epithelium. In contrast, anti-cholera toxin antibodies fail to protect against disease because the toxin is released only when the bacterium is in intimate contact with host epithelial cells, where there is little time or space for antibodies to intercept their target.

Anti-LPS SIgA likely functions by more than one mechanism. On the one hand, they are highly effective at promoting bacterial agglutination to the point where "rafts" of bacteria are so large that they are visible to the naked eye when the assay is performed in a

test tube. Experimentally, agglutination has also been observed in the intestinal lumen, so there is credence for immune exclusion play a role in vivo. On the other hand, recent evidence has demonstrated that anti-LPS IgA antibodies are potent inhibitors of *V. cholerae* motility. In fact, certain IgA preparations have been shown to completely paralyze *V. cholerae* within just a matter of minutes. This has led to the hypothesis that SIgA might work at two levels: paralyze, then handcuff. The whole cell-killed and live attenuated cholera vaccines in current use are administered to children and adults by the oral route as a means to stimulate *V. cholerae*-specific SIgA in the intestinal tract.

Additional Modes of Antibody Function

There are certainly under-explored or yet to be discovered mechanisms by which antibodies function in clearing bacterial pathogens, beyond complement activation, opsonophagocytosis, and immune exclusion (Lu et al., 2018). For example, there are reports describing monoclonal IgM and IgG antibodies against outer surface proteins of *Borrelia hermsii* (relapsing fever) and *B. burgdorferi* (Lyme disease) that cause direct bacterial lysis, independent of complement. There are also examples of antibodies that playing a central role in immunity against obligate intracellular pathogens like *Ehrlichia chaffeensis*, the causative agent of tick-borne disease ehrlichiosis. The recent discovery of an intracellular Fc receptor known as TRIM-21 has already challenged the conventional notions about antibody function. Finally, in the past several years has it become apparent that subtle differences in Fc glycosylation patterns on human IgG can have profound effects on antibody functionality.

Back to the Future With Monoclonal Antibodies (MAbs)

With the global crisis in antibiotic resistance, there is a desperate need to find alternative measures to control bacterial pathogens. As we look forward, new generations of vaccines are an obvious avenue to pursue. But an equally exciting prospect is the use of recombinant monoclonal antibodies (MAbs) in prophylactic and therapeutic applications. Historically, MAbs have been generated from mice; B cells from immunized animals are immortalized by hybridoma technology and then isolated as individual clones the secrete antibodies of a single specificity. Hence, the term "monoclonal antibody." In the past decade, however, it has become technologically feasible to generate MAbs from humans, using recombinant antibody technology as described by Corti and Lanzavecchia (2014). Basically, the genes encoding the variable regions of antibodies of interest are cloned and expressed in mammalian cell lines. In the case of HIV, Ebola and other viruses, recombinant antibodies have been shown to afford short term immunity. It is not inconceivable that MAbs will be used as a supplement or even as a replacement for antibiotics for some hard to treat bacterial infections. In a back to the future-like scenario, the use of high tech, recombinant "antitoxins" may soon be routine in the clinic.

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Secondary and Tertiary Endosymbiosis☆

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Glossary

Apicomplexans Parasitic protists belonging to the supergroup Alveolata, many of which possess a remnant, nonphotosynthetic plastid of secondary endosymbiotic origin.
Apicoplast Remnant secondary plastid of apicomplexans.
Chlorarachniophytes Marine amoeboflagellate algae with plastids of green algal origin. Together with cryptophytes, noteworthy for their possession of a nucleomorph.
Chloroplast Photosynthetic chlorophyll-containing eukaryotic organelle of endosymbiotic origin, sometimes used to specifically refer to the photosynthetic organelle of green algae and their land plant descendants.
Chromerids Marine unicellular algae with chlorophyll *a*-pigmented plastids of secondary endosymbiotic origin. Related to apicomplexans and dinoflagellates.
Cryptophytes (cryptomonads) Marine and freshwater unicellular algal lineage; most possess a secondary plastid of red algal ancestry. Together with chlorarachniophyte algae, noteworthy in that they possess a nucleomorph.
Dinoflagellates Ecologically significant lineage of microbial eukaryotes, comprising photosynthetic and nonphotosynthetic species. Diverse in the pigmentation and evolutionary origin of their plastids.
Endosymbiosis The process by which a free-living cell (prokaryotic or eukaryotic) becomes a permanent resident inside another cell.
Euglenids Abundant and well-studied algal lineage with a plastid of green algal origin (chlorophyll *a* and *b* pigments).
Glaucophytes (glaucocystophytes) Freshwater algal lineage containing a chlorophyll *a*-pigmented plastid of primary endosymbiotic origin. Unlike other plastids, glaucophyte plastids possess a carboxysome and a layer of peptidoglycan between the inner and outer plastid membranes, presumably inherited from their cyanobacterial progenitors.
Green algae Algal lineage from which land plants evolved. Plastids contain chlorophylls *a* and *b*.
Haptophytes Abundant and ecologically significant marine and freshwater algae, many of which possess a haptonema (a flagellum-like structure) and scales made of calcium carbonate called coccoliths.
Nucleomorph Remnant nucleus of secondary endosymbiotic origin, found in cryptophyte and chlorarachniophyte algae between the inner and outer pairs of plastid membranes.
Plastid Organelle of cyanobacterial ancestry residing within plant and algal cells. The term encompasses both photosynthetic and nonphotosynthetic forms.
Primary endosymbiosis The engulfment and retention of a photosynthetic prokaryote by a heterotrophic eukaryote. Gives rise to a primary plastid.
Red algae (rhodophytes) Unicellular and multicellular group of algae with chlorophyll *a*-containing plastids surrounded by two membranes.
Secondary endosymbiosis Acquisition of a primary plastid-containing alga by a nonphotosynthetic eukaryote. Gives rise to a secondary plastid surrounded by three or more membranes.
Stramenopiles (heterokonts) Diverse assemblage of photosynthetic and nonphotosynthetic unicellular and multicellular eukaryotes. Photosynthetic members possess chlorophyll *a*+*c*-containing plastids.

☆*Change History*: August 2016. C.J. Grisdale and J.M. Archibald added the Sections "Tertiary Endosymbiosis" and "Integration and Gene Transfer in Tertiary Endosymbiosis," updated the text in all sections, Table 1 and added references to "Further Reading."

This article is an update of J.M. Archibald, Secondary Endosymbiosis, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 438–446.

Abbreviations

ER	Endoplasmic reticulum
EST	Expressed sequence tag
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
TIC	Translocons of the inner chloroplast membrane
TOC	Translocons of the outer chloroplast membrane

Defining Statement

Secondary endosymbiosis is the uptake and retention of a eukaryotic alga by a nonphotosynthetic host, a process that has given rise to a significant fraction of algal biodiversity. This article summarizes current knowledge of the pattern and process of secondary endosymbiosis from the perspective of cell biology, genetics, and evolution. Higher-order "tertiary" endosymbioses are also considered.

The Origin of Eukaryotic Photosynthesis

Primary Endosymbiosis

Endosymbiosis has had a profound impact on the evolution and diversification of eukaryotes. Mitochondria and plastids, the energy-generating organelles of modern-day eukaryotes, evolved from free-living prokaryotes that were taken up by eukaryotic hosts and transformed into permanent subcellular compartments. In extant cells, these organelles now function as the sites of respiration and oxygenic photosynthesis, respectively. Unlike the endosymbiotic origin of mitochondria, which appears to have occurred in the common ancestor of all known eukaryotes, the endosymbiosis that gave rise to plastids occurred after the deepest divergences in eukaryotic evolution had taken place. This pivotal event paved the way for the evolution of a diverse array of algal lineages and for the spread of plastids between unrelated groups of eukaryotes by "secondary" (ie, eukaryote–eukaryote) endosymbiosis.

Photosynthesis is widespread in bacteria, but oxygenic photosynthesis occurs only in the cyanobacteria, a lineage of unicellular and colony-forming prokaryotes once referred to as blue-green algae. Among bacteria, only cyanobacteria possess both photosystems I and II. This allows them to use light energy to split water and produce ATP, NADPH, and glucose, liberating oxygen in the process. These products, together with carbon dioxide, are then converted to carbohydrate via the Calvin cycle. The fundamental similarities of cyanobacterial photosynthesis and that of plastids, which also possess photosystems I and II, argue strongly that eukaryotes derived their plastids from a harnessed cyanobacterium, in what is generally referred to as a "primary" endosymbiotic event (Fig. 1(a)).

The evidence supporting a cyanobacterial ancestry for plastids is overwhelmingly strong and is based on a large amount of biochemical, ultrastructural, and molecular phylogenetic data. As discussed below, three eukaryotic lineages – the green algae (and land plants), red algae, and glaucophyte algae – possess primary plastids (Table 1), indicating that they arose directly from this landmark event.

One of the hallmark features of plastids is their membranes. Many bacteria have two membranes, with a layer of peptidoglycan in between. Based on what is understood about the process of phagocytosis in eukaryotes, one would expect plastids to be surrounded by three membranes, two from the cyanobacterial endosymbiont and a third, outermost membrane derived from the phagosomal membrane of the heterotrophic eukaryote (Fig. 1(a)). However, primary plastids possess only two membranes, both of which appear to be cyanobacterial in origin (the plastids of glaucophyte algae also possess a peptidoglycan layer (Fig. 1(b))). One possible explanation is that the engulfed cyanobacterium "escaped" the confines of the host cell phagocytic vacuole and took up residence in its cytoplasm. Regardless, it is reasonable to assume that the photosynthetic abilities of the endosymbiont were of great benefit to the eukaryotic host and provided a strong selective advantage to those cells that happened to retain their endosymbionts for progressively longer periods of time. The two cells became increasingly dependent on one another and, eventually, the cyanobacterial endosymbiont evolved into a fully integrated component of the host.

Endosymbiotic gene transfer and plastid protein import

While the genomes of modern-day cyanobacteria range from ~2 to 12 Mbp in size and encode >1000 genes, the largest and most gene-rich plastid genomes are only ~0.5 Mbp (eg, the green alga *Floydiella*) and possess at most ~250 genes (eg, the red alga *Porphyra*). Roughly half of this massive genome reduction involved the elimination of genes no longer essential for intracellular life. The remainder involved the transfer of genes from the endosymbiont to the host nuclear genome, a process known as endosymbiotic gene transfer (Fig. 1(a)). Based on the similarities in gene content of plastid genomes of red, green, and glaucophyte algae, much of the plastid-to-nucleus gene transfer probably occurred early in the evolution of photosynthetic eukaryotes, in a common ancestor shared by all primary plastid-containing algae. However, this has been an ongoing process throughout the evolution of photosynthetic eukaryotes and probably still occurs in at least some lineages. Indeed, examination of plant nuclear genomes reveals

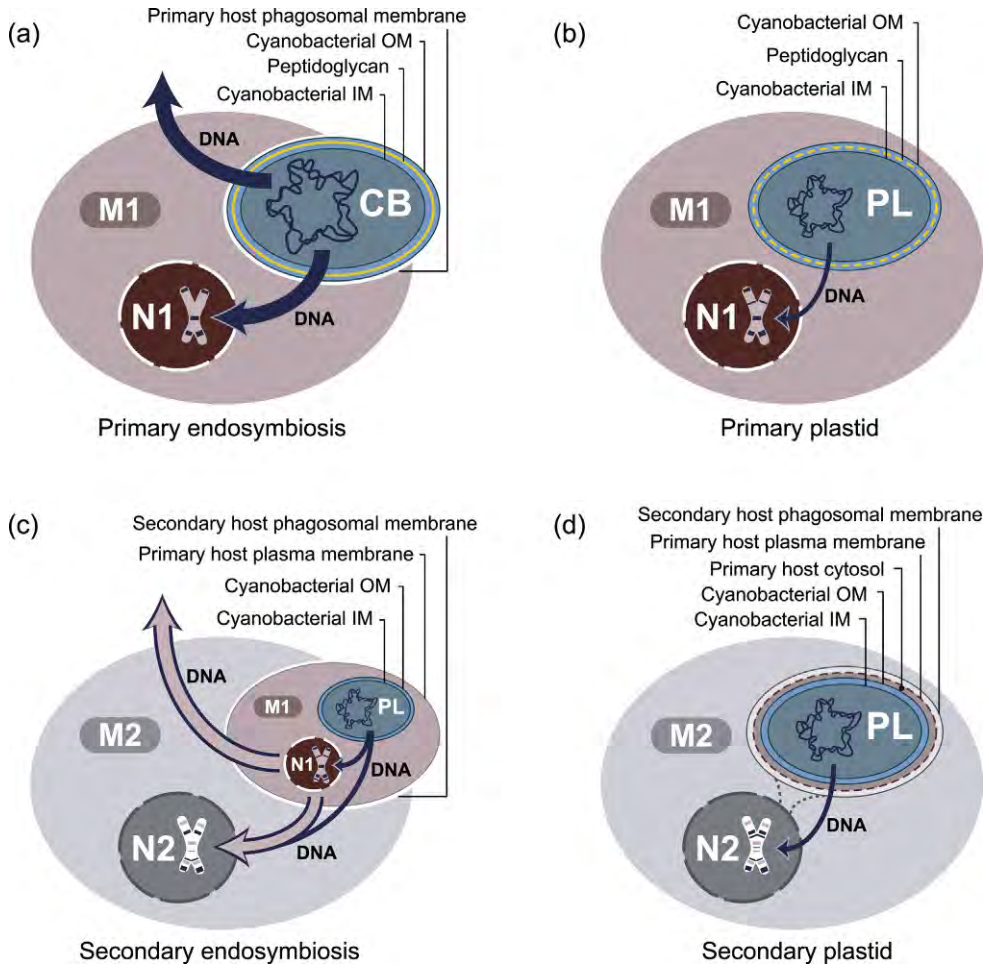

Fig. 1 Plastid evolution by primary and secondary endosymbiosis. (a) Diagram depicting the process of primary endosymbiosis in which a nonphotosynthetic eukaryote engulfs a cyanobacterium. The process involves extensive loss of DNA from the cyanobacterial genome as well as transfer of cyanobacterial genes to the nuclear genome of the host eukaryote. (b) Primary plastid-containing cell with two membranes surrounding its plastid, both of which are cyanobacterial in nature. The plastids of glaucophyte algae retain a layer of peptidoglycan between the two membranes. (c) Diagram shows the process of secondary endosymbiosis, whereby a primary plastid-containing alga is taken up by a heterotrophic eukaryote. Secondary endosymbiosis involves the large-scale movement of cyanobacterial and eukaryotic DNA from the primary host nucleus (N1) to the secondary host nucleus (N2), as well as DNA loss. DNA transfers from the plastid to the primary nucleus or directly to the secondary host nucleus are also possible. (d) Secondary plastid-containing eukaryote with three or four membranes surrounding the plastid (the primary host plasma membrane is believed to have been lost in euglenids and some dinoflagellates). The space between the inner and outer pairs of membranes corresponds to the remnant cytosol of the primary plastid-containing alga and, in cryptophytes and chlorarachniophytes, still harbors the primary host nucleus. The outermost plastid membrane in cryptophytes, haptophytes, and stramenopiles is contiguous with the nuclear envelope and endoplasmic reticulum (ER). Movement of DNA involving the mitochondrion has been omitted for simplicity. Abbreviations: CB, cyanobacterium; IM, inner membrane; M, mitochondrion; N, nucleus; OM, outer membrane; PL, plastid.

that they are littered with numerous and often large (albeit presumably nonfunctional) fragments of plastid DNA. Experimental evidence has shown that endosymbiotic gene transfer (EGT) of plastid genes in *Tobacco* plants is possible via direct DNA transfer, resulting in functional expression of the gene in the nuclear genome.

An early requirement for the functional transfer of endosymbiotic genes to the host nucleus was the establishment of a protein-targeting apparatus capable of directing the products of these nuclear-encoded genes back to their organelle of origin. Genomic and proteomic analyses of primary plastid-containing organisms have revealed that ~1000 proteins function in the chloroplast. Most of these are now nucleus-encoded and targeted to the organelle posttranslationally. This targeting is accomplished by an N-terminal "transit peptide" extension of ~20–150 amino acids present on nearly all plastid-targeted proteins. These extensions are poorly conserved at the primary sequence level but possess a net positive charge and a hydrophobic N-terminus.

Transit peptides are recognized by receptors in the plastid membrane and the preprotein is then pulled into the plastid by a pair of translocation machineries. These are referred to as TIC and TOC, for translocons of the inner and outer chloroplast membranes, respectively. Once inside the plastid, the transit peptide is cleaved from the preprotein to yield the mature protein product. Genomic comparisons have revealed that the plastid import machineries of red and green algae are predominantly cyanobacterial in nature and quite similar to one another, suggesting that they were established in their common ancestor (less is known about protein

Table 1 Distribution of primary and secondary plastids and their basic characteristics

Organism	*Putative origin*	*Cellular location*	*Membranes*	*Pigmentation*
Glaucophytes	1°	Cytosol	2[a]	Chl *a* phycobiliproteins
Green algae+land plants	1°	Cytosol	2	Chl *a*+*b*
Euglenids	2° (Green)	Cytosol	3	Chl *a*+*b*
Chlorarachniophytes[b]	2° (Green)	Cytosol	4	Chl *a*+*b*
Red algae	1°	Cytosol	2	Chl *a* phycobiliproteins
Cryptophytes[b]	2° (Red)	RER lumen[c]	4	Chl *a*+*c* phycobiliproteins alloxanthin
Haptophytes	2° (Red)	RER lumen[c]	4	Chl *a*+*c*
Stramenopiles	2° (Red)	RER lumen[c]	4	Chl *a*+*c*
Dinoflagellates[d]	2° (Red)	Cytosol[e]	2–3[d]	Chl *a*+*c* Peridinin
Apicomplexans	2° (Red)	Cytosol[e]	4[f]	None (non-photosynthetic)
Chromerids[g]	2° (Red)	?	4	Chl *a*

Abbreviations: Chl, chlorophyll; RER, rough endoplasmic reticulum.

[a]Glaucophyte plastids possesses a layer of peptidoglycan between the inner and outer membranes.

[b]The cryptophytes and chlorarachniophytes are unusual in that the nucleus of their red and green algal endosymbionts persists in highly degenerate form called a nucleomorph. In both lineages, the nucleomorph is located in the space between the inner and outer pairs of plastid membranes, which is derived from the remnant cytosol of the primary algal host cell and sometimes referred to as the periplastid space.

[c]In cryptophytes, haptophytes and most stramenopiles (heterokonts), the outermost plastid membrane is continuous with the endomembrane system of the host cell. The plastid thus physically resides within the lumen of the rough endoplasmic reticulum, an arrangement sometimes referred to as the chloroplast endoplasmic reticulum.

[d]Dinoflagellates are exceptionally diverse in terms of the type of plastid they possess. Among photosynthetic dinoflagellates, the chlorophyll *a*+*c* and peridinin-pigmented plastid is most common. Plastid membrane number varies depending on plastid type (see main text).

[e]The plastids in these lineages are not physically connected to the host cell endoplasmic reticulum, although they are surrounded by additional membranes and thus enveloped by a region of endomembrane lumen of unknown origin.

[f]The general consensus is that 4 membranes surround the apicomplexan plastid, though some studies suggest that this number can vary.

[g]*Chromera velia* and *Vitrella brassicaformis* are newly discovered photosynthetic organisms with 4-membrane plastids whose genes most closely ally them with apicomplexans and peridinin plastid-containing dinoflagellates.

targeting to the plastids of glaucophytes, though it appears to be similar). While the TIC–TOC-based import pathway appears to be the predominant system for plastid protein import, it should be noted that protein targeting in the absence of canonical N-terminal signals is also possible. Exactly how this occurs is at present unclear.

While the nuclear genomes of plants and algae harbor hundreds or even thousands of genes encoding proteins that service the plastid, a significant fraction of the cyanobacteria-derived genes residing in the nuclear genomes of modern-day photosynthetic eukaryotes appear to have nothing to do with photosynthesis. For example, the plastid proteome of *Arabidopsis* has approximately 1500 curated proteins, while it is estimated that 4300–4500 nuclear genes may have been acquired from the ancestor of the plastid. Approximately 3000 nuclear genes have predicted chloroplast targeting signals, while a significant proportion (10–15%) of known plastid proteins lack identifiable targeting signals. The exact numbers are still being debated and analyses of other primary plastid-containing eukaryotes, such as the green alga *Chlamydomonas reinhardtii*, the red alga *Cyanidioschyzon merolae*, and the glaucophyte *Cyanophora paradoxa*, have yielded slightly lower estimates. Nevertheless, the cyanobacterial progenitor of the plastid clearly had a major impact on the nuclear genome of the ancestral eukaryotic phototroph, donating hundreds of genes whose products took on roles in a wide range of cellular processes unrelated to photosynthesis.

Secondary Endosymbiosis

Canonical plastids appear to have evolved only once in the history of life. That is, all plastids appear to trace to a single endosymbiotic event. However, primary plastid-containing organisms account for only a fraction of the known diversity of photosynthetic eukaryotes. A wealth of data clearly indicates that on multiple occasions plastids have spread from one eukaryote to another by a process known as "secondary endosymbiosis." These events gave rise to a diverse set of environmentally, economically, and medically significant algal lineages. Secondary endosymbiosis (Fig. 1(c)) occurs when a primary plastid-containing alga is taken up by a nonphotosynthetic eukaryote and transformed into a "secondary" or "complex" plastid (Fig. 1(d)).

The process of secondary endosymbiosis is complicated by the fact that the bulk of plastid-targeted proteins are encoded in the primary host nuclear genome. Thus secondary endosymbiosis is not just the acquisition of a plastid by a new host. In order for a secondary plastid to be stably maintained and inherited, hundreds of nuclear genes must be transferred from the primary host nucleus to the secondary host nucleus. Comparative genomic analyses have shed light on the genetic processes that accompany this transformation. One of the most important advances has been the discovery that the nucleus of the algal endosymbiont still exists in some secondary plastid-bearing lineages (albeit in a miniaturized form). Among other things, this proves beyond all doubt that secondary endosymbiosis of plastids has occurred on multiple occasions.

Diversity of secondary plastid-containing algae

At the most fundamental level, secondary plastids are of two basic types: those derived from green algal endosymbionts and those that evolved from red algae. Two evolutionarily distinct lineages, the euglenids and chlorarachniophytes, possess "green" secondary plastids (Table 1). The euglenids are a relatively well-studied group of marine and freshwater algae, which include the laboratory specimen *Euglena*. In contrast, the chlorarachniophytes are a rare and poorly understood group of amoeboflagellate algae that together with the cryptophytes are unique in that they still possess the nucleus of their algal endosymbiont, referred to as a "nucleomorph." Four membranes surround the chlorarachniophyte plastid, whereas the euglenid plastid has three membranes (Table 1).

The evidence supporting a green algal ancestry for the plastids of both chlorarachniophytes and euglenids comes primarily from their shared possession of chlorophyll *b* (as do green algae and land plants) and from molecular phylogenies inferred from plastid-encoded genes. In the case of chlorarachniophytes, this has also been confirmed by analysis of nucleomorph genes. While the euglenid and chlorarachniophyte plastids are clearly of green algal origin, molecular phylogenies have thus far not revealed the closest free-living relatives of their endosymbionts within the green algal/land plant radiation.

A much larger number of eukaryotic algae harbor plastids believed to be of red algal origin. Perhaps best known are the stramenopiles (or heterokonts), which include unicellular forms such as diatoms and multicellular groups such as the giant kelps. Stramenopile plastids are surrounded by four membranes and contain chlorophyll *a*+*c* and the accessory pigment fucoxanthin (Table 1). The haptophytes are another well-known and ecologically significant algal lineage with "red" secondary plastids. Other groups include the cryptophytes, a relatively abundant group of marine and freshwater algae that possess a nucleomorph.

Red algae-derived plastids are also found in alveolates such as the dinoflagellates, notorious bloom formers that are also well known for their ability to "steal" the plastids of other secondary plastid-containing algae by tertiary endosymbiosis (see below), as well as the apicomplexans, a secondarily nonphotosynthetic group of parasites that include the malarial parasite *Plasmodium*. The most recently discovered red secondary plastid-containing lineage is Chromerida, containing the species *Chromera velia* and *Vitrella brassicaformis*. Recent genomic analysis of these species provides support for the hypothesis that chromerids are specifically related to apicomplexans and dinoflagellates. Chromerids are unicellular phototrophs with four-membrane, chlorophyll *a*-containing plastids (Table 1). As will be discussed below, the evolutionary history of red secondary plastids is complex and controversial. There are currently many unanswered questions regarding when these plastids were acquired and how they are related to one another.

Gene transfer and protein import in secondary plastids

As is the case for primary endosymbiosis, endosymbiotic gene transfer and gene loss play an important role in the establishment of secondary plastids. The plastid genomes of a variety of secondary plastid-containing algae have been sequenced and, as expected, their coding capacity is similar to that of the red or green algal plastids from which they evolved. This suggests that at the time of engulfment, most of the cyanobacteria-derived genes were already located in the nuclear genome of the primary alga. In secondary endosymbiosis, these genes must again be transferred en masse, this time from the primary host nucleus to that of the secondary host (Fig. 1(c)). Genes can also be transferred directly from the plastid to the secondary host nuclear genome.

In most secondary plastid-containing algae the process of gene transfer has gone to completion and the endosymbiont nucleus has disappeared. Even in cryptophytes and chlorarachniophytes, where a nucleomorph persists, its coding capacity is extremely limited, indicating that most of the genes present in the primary algal nucleus have relocated to the secondary host nucleus. A unique aspect of secondary endosymbiosis is the potential for transfer of eukaryotic genes from the primary algal nucleus to the secondary host nucleus. The functions of many of these genes presumably overlap with those already present in the nuclear genome of the secondary host, and they would be prone to degradation and loss. However, they also have the potential to acquire novel functions in their new genomic and cellular context or to replace their secondary host nuclear counterparts. The frequency and significance of "eukaryote–eukaryote" endosymbiotic gene transfer is currently poorly understood.

Unlike primary plastids, which are surrounded by two membranes, most secondary plastids are characterized by the presence of three or four membranes (Table 1), the outermost of which is thought to be derived from the phagosomal membrane of the secondary host (Fig. 1(c) and (d)). This fact has important ramifications for the process of protein targeting. Thus, a critical stage in the evolution of a secondary plastid is the establishment of a second import pathway – in addition to the transit peptide-based system already used by the engulfed algal cell – capable of targeting the products of plastid-derived genes now encoded in the new host nuclear genome to their compartment of origin. Such proteins are characterized by the presence of a bipartite N-terminal extension consisting of a signal peptide as well as a transit peptide. The exact nature of the targeting process varies depending on the membrane topology of the plastid in question.

In cryptophytes, haptophytes, and stramenopiles, whose plastids are surrounded by four membranes, the outermost membrane is continuous with the nuclear envelope and endoplasmic reticulum (ER) (Fig. 1(d)), and plastid preproteins are first inserted cotranslationally into the ER lumen. The signal peptide is cleaved by signal peptidase and the transit peptide is then used to divert the preprotein from the secondary host cell's normal secretion pathway and direct it across the remaining plastid membranes. In organisms whose plastids do not physically reside within the host cell's endomembrane system, such as chlorarachniophytes and euglenids (Table 1), bipartite N-terminal extensions are also used, with plastid proteins presumably being shuttled from the ER lumen to the plastid by vesicular transport. In some cases, host nucleus-encoded proteins must be targeted to the residual cytosol of the engulfed algal cell (the periplastidal space) but not to the plastid itself. In these instances, specific sequence characteristics of the N-terminal end of the transit peptide appear to determine whether or not the protein will be translocated across the remaining plastid membranes.

Number of secondary endosymbiotic events

Determining the number of endosymbioses that have spawned the known diversity of secondary plastid-containing algae has proven challenging. Given that secondary plastids are either of green or red algal origin, a minimum of two endosymbioses must be inferred. However, as many as seven independent events, involving multiple green and red algal endosymbionts, have also been proposed. This discrepancy is based on different interpretations of the evolutionary significance of nonphotosynthetic relatives of the major secondary plastid-containing lineages described above. For example, in addition to possessing multiple photosynthetic groups, the stramenopiles also include many nonphotosynthetic (and plastid-lacking) lineages such as the oomycete plant pathogens. In the case of dinoflagellates, only ~50% of known species are actually photosynthetic, and the most basal lineage of cryptophytes, the genus *Goniomonas*, is an extremely diverse, heterotrophic group whose members do not harbor a plastid. In addition, the chlorarachniophytes and euglenids are evolutionarily deeply embedded within lineages of predominantly non-photosynthetic groups (Rhizaria and Euglenozoa, respectively).

This "patchy" distribution can thus be explained by invoking either a separate, recent, endosymbiotic event for each secondary plastid-containing lineage, or fewer, more ancient, secondary endosymbioses coupled with extensive plastid loss. Distinguishing between these two possibilities relies on our ability to accurately infer whether or not the phylogenies of plastid and host nuclear genes agree with one another and are consistent with all other available data, such as plastid pigmentation, ultrastructure, and biochemistry. Significant advances have been made in this area, though many uncertainties remain and the topic is actively debated in the primary literature.

Regarding the origin(s) of photosynthesis in chlorarachniophytes and euglenids, current data are most consistent with the idea that their green algal plastids are the product of two separate secondary endosymbioses. Single-locus phylogenies of plastid and nuclear genes typically provide little information about the evolutionary placement of these two lineages relative to one another, but the picture emerging from analyses of concatenated plastid- and nucleus-encoded proteins is that two evolutionarily distinct green algal endosymbionts were taken up independently by the nonphotosynthetic ancestors of the two groups.

Discerning the evolutionary history of photosynthesis in lineages harboring red algae-derived plastids is somewhat more complicated. At one extreme, the "chromalveolate" hypothesis posits a single, ancient secondary endosymbiosis in an ancestor of cryptophytes, haptophytes and stramenopiles (chromists), and dinoflagellates, apicomplexans and ciliates (alveolates). This demands secondary plastid loss in all plastid-lacking lineages specifically related to these groups (eg, ciliates, oomycetes). Alternatively, a separate secondary endosymbiosis could have given rise to the plastids in each of these groups, eliminating the need to invoke plastid loss. Still another possibility is tertiary endosymbiosis, which occurs when a secondary plastid-containing alga is engulfed by a heterotrophic eukaryote. As discussed below, although well known to have occurred in dinoflagellates, the extent to which red algal-derived secondary plastids have spread by tertiary endosymbiosis is unknown.

A variety of data have been brought to bear on the question of congruence between host cell and plastid phylogenies, with mixed results. Plastid gene phylogenies typically do not strongly favor either hypothesis, although such analyses are often hampered by limited taxonomic sampling and artifacts associated with the highly divergent sequences that are found in some of these plastid genomes. Interesting early evidence consistent with the chromalveolate hypothesis came from nuclear-encoded plastid-targeted proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Eukaryotic phototrophs typically possess two nucleus-encoded GAPDH isoforms, one noncyanobacterial protein functioning in the cytosol, and a second cyanobacteria-derived protein targeted to the plastid. In chromists, dinoflagellates, and alveolates, the plastid-targeted GAPDH protein is not cyanobacterial in origin, but is instead the product of a duplicated version of the cytosol-localized protein. This phenomenon, referred to as "endosymbiotic gene replacement," is potentially an important source of rare genomic characters that can shed light on deep evolutionary relationships. However, it has been difficult to rule out the possibility that such genes have evolved in a nonvertical fashion (ie, have been horizontally transferred between unrelated species).

With the increasing availability of complete genome sequences and large expressed sequence tag (EST) and RNA-Seq transcriptome data sets, it is now possible to analyze large data sets of concatenated protein sequences in an effort to better resolve the interrelationships among "chromalveolate" taxa. To date, such analyses have provided support for a close relationship between some, but not all, chromalveolate lineages. Until recently, phylogenetic evidence supported a specific relationship between cryptophytes and haptophytes, but the evolutionary connection between these two groups and stramenopiles was tenuous, despite the results of plastid gene phylogenies and their similar plastid membrane topologies. Recent multi-gene phylogenies have provided mounting evidence for the grouping of SAR (stramenopiles, alveolates, and rhizaria), to the exclusion of cryptophytes and haptophytes. Interestingly, this puts the secondary green plastid-containing lineage Chlorarachniophyta together with two diverse lineages, some of the members of which have secondary red algal plastids. This has implications for the evolution of complex plastids, as additional organelle losses must be invoked if a red algal-derived plastid was present in the common ancestor of SAR.

The similarities in plastid morphology, plastid gene sequences, and plastid protein import machinery suggest that red algal-type complex plastids arose only once during eukaryotic evolution. In its current state, however, the tree of eukaryotes based on nuclear loci has haptophytes and the plastid-lacking, heterotrophic centrohelids branching together as sister group to SAR, while cryptophytes branch within Archaeplastida. If this topology is correct, it rules out the possibility that the single red algal secondary endosymbiosis occurred in the common ancestor of all of these groups. Instead, one or more cryptic tertiary endosymbioses must have occurred to spread red plastids amongst "chromalveolate" taxa. One possibility is that a red alga was taken up by an ancestor of today's cryptophytes in a secondary endosymbiotic event, followed by spread to haptophytes, stramenopiles, and alveolates by

tertiary and perhaps even quaternary events. Determining the exact sequence of evolutionary events leading to the pattern of red plastid-containing lineages is confounded by the extremely ancient nature of the endosymbioses involved. However, recent evidence does not support the hypothesis of a single, ancient endosymbiotic event in the ancestor of all red plastid-containing lineages, leaving open new possibilities for future work.

Inferring the minimum number of endosymbioses necessary to explain the diversity of secondary plastid-bearing eukaryotes is desirable from the perspective of understanding the processes of gene transfer and protein import. As summarized above, secondary endosymbiosis is very complex at the genetic and biochemical level: each event requires (1) the relocation of hundreds of plastid-derived genes from the primary host nucleus to the secondary host nucleus; (2) the acquisition of a functional signal peptide by each of these genes/proteins; and (3) the adaptation of the existing signal peptide secretion system such that plastid proteins containing bipartite N-terminal leader sequences can be targeted to the plastid.

Conversely, plastid loss would also seem to be difficult. Critics of the chromalveolate hypothesis point out that, in addition to photosynthesis, the plastids of plants and algae are the site of essential cellular processes such as the biosynthesis of isoprenoids and fatty acids, which explains why nonphotosynthetic organisms such as the malaria parasite *Plasmodium* retain a vestigial form of the organelle (the apicoplast). However, evidence for outright plastid loss in the apicomplexan *Cryptosporidium* has come from the presence of cyanobacteria-derived genes of apparent plastid origin in its nuclear genome. In addition, organisms previously thought to be plastid lacking, such as the dinoflagellate-like genus *Perkinsus*, have been found to harbor a remnant plastid. Finally, the discovery of organisms such as *C. velia* and *V. brassicaformis*, unicellular phototrophs that appear to be the closest known relatives of the apicoplexan parasites, has revealed how gene content changes during the transition from a free-living photosynthetic cell to an obligate intracellular parasite. Evidence suggests that gene gains were limited during this transition, while gene losses were frequent during the transition from a free-living alga to an apicomplexan ancestor, and further lineage-specific losses occurred during the diversification of apicomplexans. There is also evidence of proteins present in chromerid genomes that were co-opted for parasitic processes in apicomplexans. In sum, there is a steady stream of new molecular and ultrastructural data to account for when considering the evolutionary history of red algal-type complex plastids.

Nucleomorphs and Their Genomes

As mentioned above, the cryptophytes and chlorarachniophytes are of special interest with respect to the study of secondary endosymbiosis. This is because they are the only two lineages in which the nucleus of the engulfed algal cell still exists inside the new host. These remnant nuclei (nucleomorphs) lie within the residual cytosolic compartment of the alga, which is located between the outer plastid membrane and the primary algal outer membrane (Fig. 1(d)). The nucleomorph genomes of cryptophytes and chlorarachniophytes have been completely sequenced and are surprisingly similar in basic structure and composition. This is intriguing given that they evolved independently of one another (the cryptophyte plastid is derived from a red alga, while the chlorarachniophyte plastid has a green algal ancestry). Analysis of nucleomorph genome sequences has provided fascinating insights into the consequences of nuclear genome reduction and compaction.

The first nucleomorph genome to be analyzed in detail was that of the model cryptophyte *Guillardia theta*. The complete *G. theta* genome comprises three small chromosomes totaling a mere 551 kbp. Each of the three chromosomes is capped with unusual telomeres ([AG7]AAG6A)11) and near-identical subtelomeric ribosomal DNA (rDNA) repeats. As is often the case with genomes of endosymbiotic origin, the G. theta genome is compositionally biased (~75% AT in single-copy regions, ~55% in the repeats) and its gene density is high (~1 kbp/gene). Of 465 protein-coding genes, only 30 encode plastid-targeted proteins, counter to initial speculation that nucleomorph genomes would be enriched in genes related to photosynthesis and plastid-related processes. Instead, the bulk of the *G. theta* genome encodes proteins involved in core eukaryotic processes such as transcription, translation, and protein folding/degradation. These proteins presumably function to maintain the nucleomorph genome, express its "housekeeping" genes, and enable it to produce the few proteins that it continues to target to the plastid. The *G. theta* gene repertoire is in fact far from complete and it is already missing numerous genes for essential cellular processes such as DNA replication as well as important structural proteins (eg, actin). Sequencing of the *G. theta* nuclear genome revealed ~2400 proteins that were predicted to function in the nucleomorph or remnant algal cytosol, and that are targeted post-translationally. This explains the whereabouts of many essential genes lacking from the nucleomorph genome.

The second cryptophyte nucleomorph genome to be sequenced was that of *Hemiselmis andersenii*, an organism quite distantly related to *G. theta*. At 572 kbp, the genome is similar in size and coding capacity to that of *G. theta* but possesses a number of interesting differences. The *H. andersenii* nucleomorph telomeres are (GA17)4–7, very different from those of *G. theta*, and only three of the six chromosome ends possess intact rDNA operons, the other three harboring stand-alone 5S rDNA loci. Most unexpected is the complete absence of spliceosomal introns and genes for splicing RNA and proteins in the *H. andersenii* genome. This contrasts with the *G. theta* genome, which possesses 17 small introns and encodes a variety of proteins that make up the spliceosome, including the splicing "platform" prp8. Together with a few highly reduced microsporidian parasite genomes, the *H. andersenii* genome represents a rare instance of complete intron loss in a nuclear genome, presumably as a result of intense evolutionary pressure to reduce genome size.

A further two cryptophyte nucleomorph genomes were published in 2010 and 2012. *Chroomonas mesostigmatica* and the non-photosynthetic *Cryptomonas paramecium* contain the largest (703 kbp) and smallest (486 kbp) cryptophyte nucleomorph genomes, respectively. Despite the differences in size, their genome architecture and total gene number are in line with one another and the

two previously described cryptophyte nucleomorphs. The relatively large *C. mesostigmatica* nucleomorph genome contains 32–61 more genes than the other three cryptophyte nucleomorphs. It also has slightly longer proteins, with an average of 357aa versus 289aa, 312aa and 338aa in the other three genomes. However, gene number and length do not account for the discrepancy in nucleomorph genome sizes. The *C. mesostigmatica* genome contains many short sequence repeats as well as multi-copy genes, highly unusual for a nucleomorph genome. The single largest contributing factor to cryptophyte nucleomorph size variation seems to be non-coding DNA content, with significantly longer intergenic spacers in *C. mesostigmatica*. The increased intergenic sizes have further implications for this genome. *C. mesostigmatica* seems to have a lower degree of synteny shared with the other three species than they do with one another, possibly as a result of more frequent recombination facilitated by larger non-coding regions. Some of these syntenic breaks coincide with the position of protein degradation genes in the other species, suggesting a recombination-mediated gene loss mechanism. The presence of small ORFs in syntenic positions to full-size genes in the other species is evidence of ongoing genome decay and gene loss in cryptophyte nucleomorphs.

Patterns of nucleomorph gene content variation in cryptophytes provide insight into the reductive processes that take place during endosymbiosis. Evidence of specific biochemical pathways being uniquely lost in individual species can be found. For example, many of the 24 genes missing from the non-photosynthetic species *C. paramecium* have plastid-related functions. Similarly, the intron-devoid *H. andersenii* genome lacks seven genes whose functions are associated with splicing. Surprisingly, the relatively large *C. mesostigmatica* nucleomorph seems to have completely lost an important protein degradation pathway, as all proteasome subunit genes are missing, a trait shared with the Chlorarachniophyte *Bigelowiella natan*. Until more host nuclear genomes are sequenced we cannot rule out the possibility of nucleomorph to host gene transfer, however, current evidence does not support this. Interestingly, the exact same set of 31 plastid-associated proteins is found in the three photosynthetic cryptophyte nucleomorph genomes, with a subset being present in *C. paramecium*. This suggests that these plastid proteins may not be capable of being transferred to the host nucleus, and that this reduced set was likely in-place prior to the diversification of modern-day cryptophytes. The presence of these plastid proteins likely explains, at least in part, the reason for nucleomorph persistence in cryptophytes (see below).

The nucleomorph genome of the chlorarachniophyte *B. natans* is 373 kbp in size, significantly smaller than that of any known cryptophyte nucleomorph genome. Like the cryptophyte nucleomorphs, the *B. natans* genome consists of three small, AT-rich chromosomes, each with subtelomeric rDNA operons. Remarkably, the nucleomorph genomes of cryptophytes and chlorarachniophytes have converged upon the same karyotype and basic chromosome structure despite being derived from different algal endosymbionts, each of which presumably possessed dozens of nuclear chromosomes. The significance of this convergence is unknown. The *B. natans* nucleomorph encodes 293 protein genes, only 17 of which are involved in photosynthesis. Unlike the situation in cryptophytes, introns are abundant in the nucleomorph genes of *B. natans*, with an average of ~3 introns/gene. The introns themselves are, however, greatly reduced in size, being between 18 and 21 bp in size, the smallest spliceosomal introns known. Overall, the functional distribution of nucleomorph genes in *B. natans* is similar to that seen in cryptophytes with the exception of genes involved in RNA metabolism, for which *B. natans* is somewhat enriched. While the *B. natans* host nuclear genome has acquired ~1000 endosymbiont nuclear genes whose products are posttranslationally targeted, this is substantially fewer than in *G. theta*. Relative to *B. natans*, the *G. theta* nuclear genome appears to be enriched in genes for PPC and nucleomorph-targeted proteins functioning in protein degradation, RNA degradation, protein folding, and metabolic enzymes. On the other hand, *B. natans* encodes more nucleomorph-targeted spliceosome components, in line with the presence of many introns in the *B. natans* nucleomorph genome itself.

Three additional chlorarachniophyte nucleomorph genomes have been sequenced in recent years. This provided the data necessary for comparative genomics of green algal derived endosymbiont nuclei. As expected, the nucleomorph genomes of *Lotharella oceanica*, *Lotharella vacuolata*, and *Amorphochlora amoebiformis* are composed of three chromosomes and have GC contents of 25–35%. *L. oceanica* has the largest chlorarachniophyte nucleomorph genome at 612 kbp, with *L. vacuolata* a distant second at 432 kbp. The nucleomorph genome of *A. amoebiformis* is 374 kbp, nearly identical in size to that of *B. natans* (373 kbp). Similar to cryptophytes, nucleomorph genome size variation among chlorarachniophytes correlates with the length of intergenic spacers. However, frequent gene duplication is the main reason for larger genome sizes in *Lotharella* species. Several duplicated genes are even shared between *L. vacuolata* and *L. oceanica*, consistent with gene duplications having occurred prior to the diversification of *Lotharella* species. In addition, a large sequence consisting of 45 ORFs was found within the subtelomeric repeats of *L. oceanica* but not in *L. vacuolata*, explaining much of the genome size difference between the two closely related species. While nucleomorph subtelomeric rDNA operons are highly conserved, it appears that an inversion has reversed the orientation of the *B. natans* rDNA operons relative to those in other chlorarachniophyte nucleomorph genomes.

The number of non-redundant protein-coding genes in the four sequenced chlorarachniophyte nucleomorphs ranges from 288 to 338. However, gene duplications have had a large effect on the total number of genes in *Lotharella* species, with 596 genes in *L. oceanica*, 319 in *L. vacuolata*, 300 in *A. amoebiformis*, and 288 in *B. natans*. There is an 86% overlap in genes with predicted functions between the chlorarachniophyte nucleomorphs, more than the proportion shared among cryptophyte nucleomorphs. Also, the four chlorarachniophyte nucleomorph genomes share the same set of 17 plastid-associated proteins. This suggests that the reduced set of plastid genes was present prior to diversification of the lineage, in line with the situation in cryptophytes. There is evidence of a small number of lineage specific gene losses in chlorarachniophyte nucleomorphs, and no evidence of recent nucleomorph-to-nucleus gene transfer. There are also many lineage specific hypothetical nucleomorph genes. These genes have nevertheless been found in syntenic positions to genes of known function in other species, raising the possibility that they have simply diverged beyond the point where their functions can be predicted. Finally, although a high level of synteny is typical of

nucleomorphs and other reduced genomes, there appears to be smaller syntenic blocks in chlorarachniophyte nucleomorphs than in cryptophytes, suggesting higher recombination rates. This is especially true in *Lotharella* species that contain more duplicated regions than nucleomorphs in other species.

One of the most fundamental, and unanswered, questions regarding nucleomorph evolution is why they exist at all. By definition the process of secondary endosymbiosis includes a "transition period" during which the nucleus of the algal endosymbiont resides within its residual cytosolic compartment and continues to service its plastid. However, other than cryptophytes and chlorarachniophytes, the nucleomorphs of all other secondary plastid-containing lineages have completely disappeared, having transferred all of their essential genes to the secondary host nucleus. There are two main possibilities for the persistence of nucleomorphs. First, it is possible that not enough time has elapsed for nucleomorph-to-host-nucleus gene transfer to go to completion and the nucleomorph genomes (and nucleomorphs) of cryptophytes and chlorarachniophytes will eventually disappear. Alternatively, there may be barriers to the successful transfer of the remaining genes in the cryptophyte and chlorarachniophyte nucleomorph genomes, as has been proposed to explain the persistence of mitochondrial and plastid genomes.

There are currently not enough data to determine with confidence which of these two possibilities is most likely, though the comparison of the gene content of the nucleomorph genomes of the cryptophyte *G. theta* and the chlorarachniophyte *B. natans* is consistent with the former scenario. There is very little overlap in the suite of plastid proteins encoded in cryptophyte and chlorarachniophyte nucleomorph genomes, suggesting that nucleomorph to host nucleus gene transfer could essentially be a random process. The presence of the same set of plastid proteins within each of these lineages is most likely the result of strong reductive processes early in the evolution of the host–endosymbiont relationship, prior to the diversification of each lineage.

The completion of several cryptophyte and chloraraphniophyte nucleomorph genomes has provided insight into the patterns of gene content variation among nucleomorphs. The core set of housekeeping genes is quite well conserved, with the majority of gene content diversity involving hypothetical genes. Some lineage-specific losses of core genes have been found, most notably in cryptophytes. Interestingly, recent analysis of the nuclear genomes of *G. theta* and *B. natans* showed no evidence of recent EGT from the plastid or nucleomorph to the host nuclear genome. Several cases of mitochondrion-to-nucleus transfer were found in *G. theta* and *B. natans* (involving short DNA fragments), evidence that EGT can still occur in these organisms. The apparent absence of recent plastid and nucleomorph EGTs can be explained by the "limited transfer window" hypothesis, which posits that there is a greater chance of EGT from multi-copy organelles because lysis of a single-copy organelle would presumably be lethal. Importantly, *G. theta* and *B. natans* each contain a single plastid–nucleomorph complex per cell, whereas multiple or reticulated mitochondria are present in the cytosol, making mitochondrial lysis and DNA transfer possible. While cryptophytes have lost some genes involved in core eukaryotic processes, the functional significance of these losses is unclear, and it would seem that nucleomorph reduction is nearing an endpoint, at least in species with a single nucleomorph per cell. Additional nucleomorph and nuclear genome sequence data from diverse cryptophytes and chlorarachniophytes will be needed to more rigorously test the limited transfer window hypothesis for the persistence of nucleomorphs.

Tertiary Endosymbiosis

Algae with secondarily-derived plastids are the result of eukaryote–eukaryote fusions that lead to increased genetic and morphological complexity. These cells are unusual in containing genes from two phylogenetically distinct eukaryotes and two prokaryotes. However, even more complex symbiotic mergers exist. In serial secondary events, a primary alga is taken up and replaces the current secondary plastid of the cell. This is known to have occurred in certain dinoflagellates. Tertiary endosymbiosis is the uptake of a secondary plastid-containing alga by a heterotrophic host. In order for the tertiary plastid to be maintained and inherited, genes must be transferred from the secondary host to the tertiary host nucleus. Many of these genes are likely of cyanobacterial origin, and have taken a convoluted evolutionary path from a bacterial genome through two eukaryotic intermediates, to finally end up in the tertiary host nucleus. The degree of endosymbiont streamlining and level of integration with the host varies greatly in different tertiary and serial secondary endosymbioses, as will be discussed below.

Dinoflagellates are notorious for their frequent loss and uptake of endosymbionts. The types of endosymbiotic events known within this lineage include: secondary or cryptic tertiary endosymbiosis of a red alga or red algal-derived secondary plastid (as in peridinin plastids), serial secondary endosymbiosis of a green alga, and at least three tertiary endosymbioses involving haptophytes, diatoms, and cryptophytes. With the exception of nucleomorphs, secondary endosymbionts typically lose most cellular and genetic features, leaving just the plastid with one or two additional membranes. However, in the more recent tertiary events the endosymbiont is remarkably unreduced. In so-called "dinotoms," the diatom endosymbionts retain their nuclear genomes and mitochondria. This is an extremely rare case of a cell containing mitochondria of two distinct origins. On the other hand, green algal and haptophyte-derived endosymbionts found in other dinoflagellate lineages appear to have lost their nuclei and mitochondria.

Integration and gene transfer in tertiary endosymbiosis

Sequencing of multiple dinoflagellate peridinin plastid genomes has revealed several unusual and unique features. Dinoflagellate plastids contain fewer than 20 genes, encoded individually or in small numbers on plasmid-like circular chromosomes. This is in contrast to most plant and algal plastid genomes that have a single circular chromosome encoding 60–250 genes. Therefore, more plastid genes have been transferred to the dinoflagellate host nucleus than in other algal lineages. In the chromerid algae, close photosynthetic relatives of dinoflagellates and apicomplexans, we see evidence of more typical plastid genome architecture, suggesting that the ancestral peridinin plastid looked like other red and green algal derived plastids. In addition, dinoflagellate

plastid sequences undergo transcript processing in the form RNA editing and polyU addition. The unconventional nature of dinoflagellate peridinin plastids creates a unique situation which allows comparisons with subsequent endosymbionts.

Tertiary endosymbiotic events typically involve the replacement of a secondary plastid, adding an additional level of complexity in terms of plastid protein redundancy. Whether the secondary plastid is retained during the transition to a tertiary or serial secondary derived plastid, or is lost prior to the uptake of the "replacement" organelle, is unknown. However, there is some evidence of dinoflagellate peridinin plastid proteins with predicted targeting to haptophyte and green algal-derived plastids. This mixing of proteins from different sources suggests the evolutionary co-existence of the two organelles during plastid replacement. Proteins of peridinin plastid origin have also been found in dinotom host genomes. However, homologous copies in the diatom endosymbiont nuclear genome appear to encode proteins functioning in the plastid, while those encoded in the host nucleus lack targeting signals. This provides further evidence that dinotom tertiary endosymbionts are not as tightly integrated with their hosts as are the haptophyte and green algal endosymbionts.

Dinoflagellate peridinin plastid genes undergo RNA editing and polyU tail addition, processes not typically associated with plastids. Intriguingly, haptophyte-derived tertiary plastid transcripts also show these types of processing, while free-living haptophyte secondary plastids do not. On the other hand, no evidence of transcript processing has been found in dinotom and serial secondary green algal plastids. This suggests that the level of endosymbiont integration may be affected by the use of processing pathways associated with the original peridinin plastid. Also, there is evidence of a haptophyte tertiary plastid gene located on a minicircle chromosome, similar to the genome architecture found in other dinoflagellate plastids. Perhaps transcript processing provides a means of compensating for high mutation rates and changes in genome structure that would otherwise disrupt essential gene expression. This may even contribute to more frequent permanent establishment of endosymbionts, thus helping to explain the prevalence of plastid replacements in the dinoflagellate lineage. Many of the evolutionary steps involved in tertiary endosymbiosis are still unclear, but the study of dinoflagellate endosymbionts that exhibit differing levels of integration has begun to provide some answers.

Secondary endosymbiosis has had a significant impact on the evolution of eukaryotes, in particular the spread of photosynthetic capabilities to a huge diversity of organisms. Recent genomic and transcriptomic analyses have provided important insight into the integration between endosymbionts and their hosts. However, much of what happens between the initial association of two eukaryotes and the end product – a fully integrated plastid – remains a mystery. More recent serial secondary and tertiary events in dinoflagellates are just starting to reveal what sorts of endosymbiotic intermediates are possible, with a wide range in the level of genetic and cellular integration being seen. The clear cases of tertiary endosymbiosis observed in dinoflagellates also raise the possibility that one or more cryptic tertiary endosymbioses have taken place during eukaryotic evolution, and are responsible for the present discrepancy between host cell phylogeny and plastid evolution. Despite substantial progress in the age of comparative genomics, there is still much to learn about the origin and spread of red algal-derived plastids across the eukaryotic tree.

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Sediment Habitats, Including Watery[☆]

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Glossary

Cable bacteria Multicellular filamentous forms of bacteria that are capable of long range electron transport between the sulfide-rich zoner and the oxic zone in sediments.
Diffusion The process whereby molecules tend to distribute themselves to uniform concentration. This often dominates sedimentary habitats, where the environment is physically stabilized, and the only input of oxygen is via diffusion from oxygen-rich environments above the sediments.
Extracellular electron transport (EET) The process whereby some bacteria and archaea can oxidize and/or reduce insoluble redox-active components outside the cell wall, allowing long-distant electron transport to otherwise unavailable compounds or cells.
Layered microbial community (LMC) The community organization often seen in sediments, in which electron acceptors, such as oxygen, are consumed, and then other electron acceptors (nitrate, manganese, iron, sulfate, carbon dioxide) are used in sequence, resulting in microbial (and chemical) layers due to the activities of microbes present.
Macrofauna Eukaryotic multicellular organisms that are found in many sediments. These organisms take advantage of the energy-rich sediment environment, feeding on the prokaryotic biota. They also impact the physically stabilized sediments by pumping oxygen-rich water through the sediments, thus disrupting the diffusion-dependent layers and gradients.
Pore water The water found in the spaces between sediment particles. Pore water is the most commonly used parameter to judge the chemical reactions occurring in sedimentary niches.
Sediment A water-saturated, physically stabilized environment that obtains input (and grows) via the addition of material from above. In sediments located in shallow water, input can also occur via the activity of photosynthetic microbes within the sedimentary niche.

Abbreviations

C_{org}	organic carbon
EET	extracellular electron transport
LMC	layered microbial community
PAR	photosynthetically active radiation
SRB	sulfate-reducing bacteria
SRR	sulfate reduction rate

Defining Statement

The sedimentary niche is one of the largest ecosystem on Earth, harboring ~4PgC and 3×10^{29} cells (Kallmeyer et al., 2012). The processes that occur in these water-saturated, physically stabilized niches often lead to the formation of chemical gradients or layers, which can be used to infer modern and/or ancient sedimentary microbial processes.

Introduction

What is Sediment?

For our purposes, active sedimentary environments are defined as the underlying matrix of aquatic environments in which sedimentary (depositional) processes occur, and in which some form of deposit (sediment) accumulates with time via input of particles from the overlying water. Thus, the entire ocean bottom, excluding rocky outcrops and the active wave-breaking zone, in which sediments seldom permanently accumulate, is a sedimentary environment, as are virtually all lake bottoms. Rivers can

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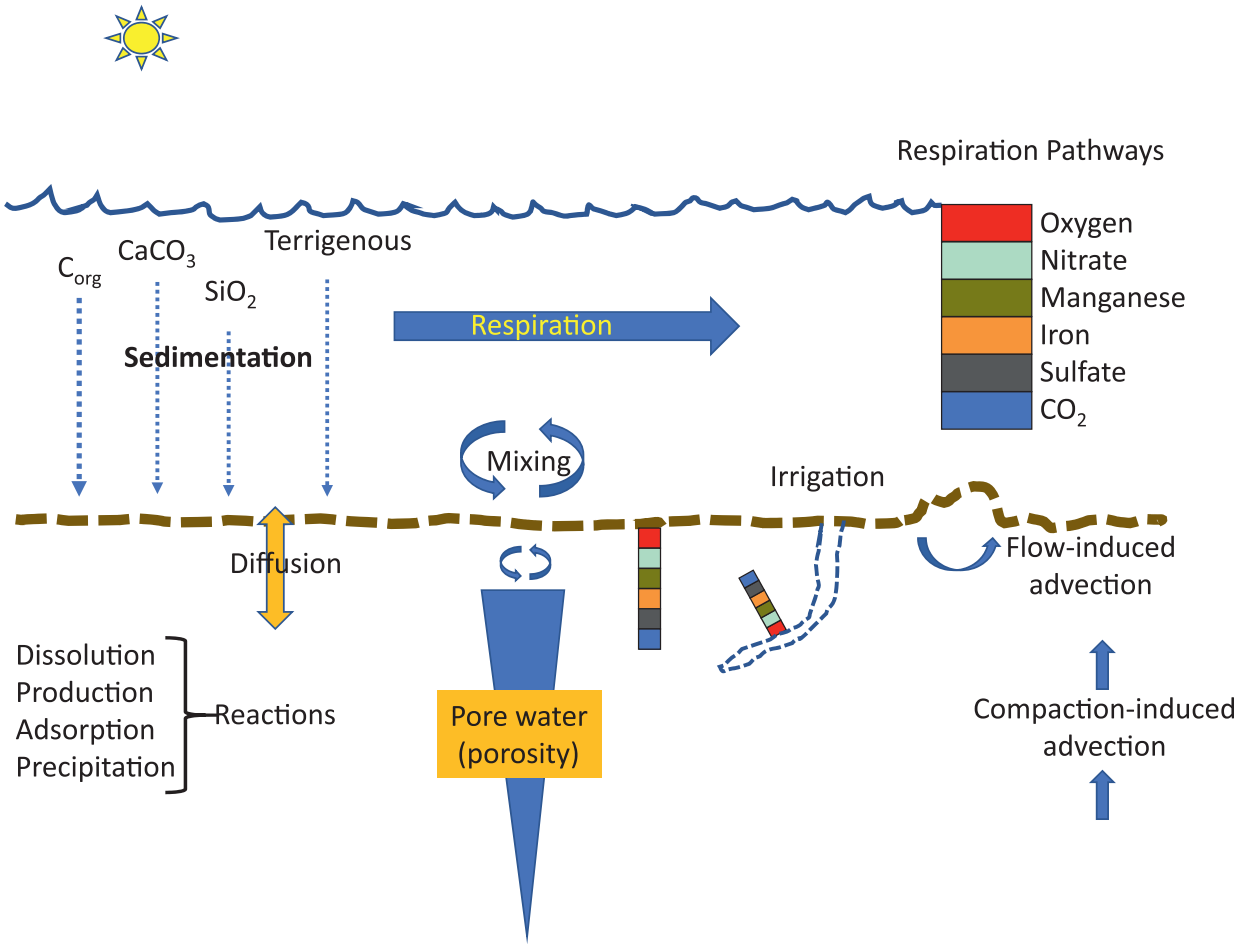

Fig. 1 Some of the fundamentals of the sedimentary niche. While simple in comparison to complex soil environments, the sediments have a number of important properties that define them. Both organics and inorganics are constantly removed to the sediment water interface (SWI) by sedimentation. Organics are removed by respiration. In well mixed waters this is oxic respiration, but if poorly mixed, oxygen is consumed and anoxic respiration prevails, as shown. In the sediments, with limited mixing, anoxic respiration prevails, and electron acceptors are primarily supplied by addition from above. If irrigation by eukaryotic worms occurs, gradients of oxidants can be seen emanating outwards from the worm tubes.

accumulate sediments, but depending on the dynamics of the river, these deposits may be short-lived and difficult to predict and/or define. Not all sediments are alike, of course, and their variations are subject to several key factors: (1) the chemical nature of the overlying water; (2) the impact of light (photosynthetically active radiation or PAR) on the sediment surface; (3) the type and amount of organic input to the sediments; and (4) the type and amount of inorganic input to the sediments (Fig. 1).

One of the important properties of sediments relates to the fact that the environment is physically stabilized: That is, there is minimal mixing or convection, and chemical transport to and from the niche is dominated by diffusion. For example, if the oxygen is consumed within a given sediment, then it must be supplied from above by diffusion, and this interplay is extremely important in defining the chemistry and microbiology of the sedimentary niche.

Where are Sediments Found?

Sediments are found at the bottom of virtually every body of water: streams, rivers, lakes, and throughout the ocean. Even in the absence of any obvious sedimentary source, the mean global dust fallout from the atmosphere alone can generate sediment accumulation rates of one or more millimeters per kiloyear. Thus, any body of water on this planet will accumulate sediments, and as material falls through water, it can accumulate a variety of 'hitchhikers' including prokaryotic and eukaryotic cells, as well as organic and inorganic materials (metals, minerals, clays, nutrients, etc.).

What are Sediments Composed of?

Dust is the source of ~35% of sediment accumulating in the global ocean: Such dust-dominated sediments are known as red clay deposits, and they are abundant in the deepest ocean and sites furthest removed from continental land masses. In contrast, about

50% of oceanic sediments are termed biogenic (SiO_2 or $CaCO_3$) insofar as the dominant sediment particles are mineral grains formed via precipitation (biomineralization) by either diatoms and/or radiolarians (SiO_2), or coccolithophores and/or foraminifera ($CaCO_3$). These mineral grains, like dust particles, settle through the water column and accumulate an array of 'hitchhikers', much like an elevator headed down a skyscraper. Lake sediments and some nearshore oceanic sediments tend to be dominated by particles either carried by rivers or eroded along the shore. These sediments, termed terrigenous, tend to vary considerably both in time and in space, are subjected to mass movement during storm events, and are prone to move offshore and downslope via current dispersion and turbidity flow. When viewed from afar, sediments seem to be slowly and constantly accumulating, but, in fact, the distribution of sediment types and amounts has varied considerably throughout geologic time, revealing past changes in tectonic activities, climate, and global (especially oceanic) productivity.

The Diversity of Sediments

The Importance of Overlying Water

As opposed to soils, which often alternate between wet and dry states, sediments are constantly wet, and are commonly described by the water that overlays them: marine sediments and lacustrine or freshwater sediments. Superimposed on the marine/lacustrine division is the issue of light. Sediments in shallow water can differ drastically from those in deeper water because of the impact of photosynthesis that can occur in the sediments themselves: Deepwater sediments are nearly always the recipients of photosynthate (photosynthetically produced organic carbon (C_{org})) from above, rather than internal primary production. Since photosynthesis can also be a source of oxygen, the shallow water sediments are prone to being oxic in the daytime, and exhibiting strong day/night differences in terms of redox chemistry, something not seen in deepwater sediments.

Of course, not all lake sediments are overlain by 'fresh' water – their chemistries may range widely in pH, salinity, and nutrient chemistry, and in each case, this leads to a different set of conditions in the sediments – conditions that alter the microbial ecology of the sediment basin. Examples of such environments include alkaline lakes such as Walker Lake, NV, alkaline hypersaline lakes such as Mono Lake, CA, and hypersaline lakes such as the Great Salt Lake, UT, and the Salton Sea in the Southern California Desert.

In general, lacustrine sediments receive higher levels of organic input than do marine sediments, and as a result they become anoxic rather quickly (within centimeters or less of the sediment water interface). In many cases, because lakes are often poorly mixed, the bottom water overlying the sediments can itself be anoxic, leading to sediments lacking macrofauna or other eukaryotes.

Marine Sediments

Marine sediments, with a few notable exceptions, are characterized by a sediment–water interface that includes oxic water overlying sediments that at some level become anoxic. This is because oceanic circulation constantly supplies the deep ocean with oxygen-rich water from the high latitudes, and keeps the deep ocean water often more oxygen-rich than the near-surface waters. Notable exceptions to this 'rule' include: (1) the Black Sea, which is isolated from oceanic circulation, and is thus anoxic for most of its depth; (2) marine basins, such as the Cariaco Trench, which is also isolated from deepwater flow and mixing due to the presence of a structural sill that inhibits mixing and results in the water becoming anaerobic far above the sediment–water interface; and fjords in which a freshwater layer forms over marine water due to riverine flow coupled with a sill at the fjord entrance. Other, smaller anoxic (lacking oxygen) basins include Saanich Inlet, Canada; Gotland Deep, Baltic Sea, and the Orca Basin, Gulf of Mexico.

As a rule, marine sediments are dominated by the sulfur cycle, due to the fact that seawater usually has $\sim$28 mmol l^{-1} sulfate, which, after oxygen is depleted, becomes the major electron acceptor. Thus, marine sediments are notorious for sulfide production and for internal sulfur cycling (reduction–oxidation–reduction, etc.). Except in cases of very high input of organic matter, sulfate depletion does not occur, and thus, biological methane production in marine sediments is usually not a major process.

Lacustrine Sediments

Lacustrine (i.e., having to do with lakes) sediments vary widely with regard to the oxidation state of the overlying water. In many lakes the waters are well mixed, and very ocean-like in terms of being permanently oxic, while others range from being seasonally stratified (and thus having anoxic bottom waters for some portion of the year) to the so-called meromictic (permanently stratified) lakes, with bottom water that never sees oxygen in abundance. In addition to this rather simple delineation are those sediments beneath lakes that may have chemistries more reminiscent of oceanic environments, such as the Salton Sea, or evaporitic lakes, where salinity can be quite high, and sulfate concentrations that are similar to, or even higher than, those found in the ocean.

In marked contrast to marine sediments, where sulfate reduction dominates, lake waters are typically very low in sulfate, and lacustrine sediments are usually dominated by methane production. Sulfate concentration in most lacustrine sediments is in the tens of micromolar range (0.1%–1% of the level in seawater), and when it is depleted, carbon dioxide becomes the major electron acceptor, and methane is the major product. Thus, in terms of sediments as microbial habitats, one might regard sulfate concentration as one of the key variables regulating the microbial metabolism and biogeochemical cycling.

Sampling and Description of Sediments

Pore Water

Pore water is what it implies: the interstitial water occupying the spaces between sediment particles (Fig. 1). Pore water chemistry is commonly the parameter that is measured to characterize a sedimentary environment in terms of the microbial ecology of these environments, and is arguably the single most important property of sediments to measure. Pore waters can be obtained by sectioning and centrifuging sediment core samples, by whole-core squeezing, by sucking water out using Rhizon fileters, or by allowing diffusion into chambers called 'peepers' that are allowed to equilibrate with the sedimentary environment. Sectioning and squeezing are usually performed in an anaerobic glove box to avoid effects of oxygen on sensitive components like sulfide or ferrous iron, but even with this precaution, results can be quite variable. Squeezing subcored sediment and expressing the pore waters directly into syringes minimize handling, and often yield much more reproducible results with regard to non-surface-reactive compounds. Peepers, while yielding high quality data, often need to be equilibrated for long periods, not compatible with many field expeditions. Another approach to obtaining pore water chemistry profiles is that of the use of microelectrodes, which directly measure the concentrations (activities) of certain species. This method allows for very high spatial resolution, rapid data acquisition, and high reproducibility. However, the electrodes are solute specific, often very expensive, and can be quite fragile.

Porosity and Tortuosity

Interlinked with the concept of pore water nutrient chemistry are two other key sediment properties: porosity and tortuosity. The porosity is a measure of sediment water content, defined as the volume of pore space per unit volume of total sediment. Typical surface ocean or lake sediments will have a porosity exceeding 80% (0.80); coarse-grained sediment will have a lower porosity than fine-grained sediment (as clays tend to stand on edge and create large porous volumes); and sediment porosity will decrease with depth due to compaction. Tortuosity describes the path a molecule or any other solute must take in order to move from point A to B within a sediment column. Because sediment grains come in the way, the distance between A and B becomes a tortuous path with a length greater than the straight line between points. The ability for grains to compact and hence move 'down' relative to a static water column provides a mechanism for microbes associated with certain mineral horizons to be translocated within the sediment column – this process is termed pore water advection.

Sediment Properties

Redox Budgets of Sediments

Sediments accumulate as material arrives at the floor of an aqueous body carrying a load of C_{org} associated with the inorganic matter. The source of the C_{org} can be productivity within the water or it can be transported in from the continents by erosional processes. Insofar as there is no known sterile body of water on the earth's surface (albeit some ponds subjected to extreme metal loading may be near to being sterile), there will always be this association of adsorbed C_{org} and microbes with sediments.

Sediments can also carry a certain oxidizing potential, most often as particulate iron oxyhydroxides (FeOOH) but also manganese oxides (MnO_2). Terrigenous-dominated sediments have the largest quantity of this solid-phase oxidant, typically 4–7 wt% Fe, whereas Mn might constitute up to 0.1% of sediment mass. While there is no 'typical' C_{org} content of sedimenting particles because this parameter will depend on the productivity of the surface ocean or lake, coastal ocean sediments settle to the seafloor with a C_{org} content of 5–10 wt% and many deep-sea sediments settle to the seafloor carrying 3–5 wt% C_{org}. As an example of an electron budget for sediments, assume that particulate material falling to the coastal ocean seafloor is 50% terrigenous and 50% biogenic – the terrigenous material carries 5 wt% Fe as FeOOH and the sedimenting material contains 10 wt % C_{org} (all reasonable values). Such a sediment will be carrying an oxidizing potential (electron acceptors) in deficit of the total reducible load by 2 orders of magnitude! As illustrated here, the reduced load of settling particles is typically much greater than the oxidizing potential of the solid phase, hence the importance of dissolved oxidants and their utilization by heterotrophic prokaryotes in sediment diagenesis.

As indicated by the abovementioned example, an important part of the sedimentary redox budget relates to the dissolved oxidants in the overlying and interstitial spaces. Most sediments are overlain by oxic water, providing ~200–400 μmol l^{-1} oxygen (depending on salinity and temperature), and minor amounts of nitrate and CO_2. In sediments of high organic content (and high microbial activity), CO_2 and other oxidants are generated in situ, further complicating the matter. The major source of oxidizing power in sediments originates from solutes within the overlying water column. Sulfate (SO_4^{2-}) is perhaps the defining redox (and microbiological) difference between marine and freshwater sediments. It is the major oxidant in marine systems, being present in oceanic waters at a concentration of ~28 mmol l^{-1} (100 × the concentration of oxygen and 4 times the number of electrons to be accepted makes sulfate 400 times more potent than oxygen as an oxidant in marine waters), yet often at only ~50 μmol l^{-1} in freshwater systems. Thus, the chemical reduction of a variety of sulfur species (sulfate, elemental sulfur, thiosulfate) dominates marine sediments, and is comparatively absent in freshwater sediments, which tend to be dominated by methane production as the final redox activity. The end-member of sulfate reduction, H_2S (S^{2-}) can strongly impact the cycling of both iron and manganese through the formation of insoluble iron sulphides, on one hand, and the reduction of Mn oxides (MnO_2) to soluble Mn^{2+}.

Microbial Input to Sediments

Particles arrive at the sediment–water interface carrying a microbial community. Counts of bacteria on sedimenting particles in the ocean yield a wide range in values (1×10^5–1×10^{10}) bacteria per gram resulting in upper surface layers of sediments usually harboring about 10^7–10^9 microbes per gram of sediment. Typically, the bacterial density on sedimenting particles exceeds the bacterial density within sediments. However, the biomass of these particle-associated microbes is much less than the biomass of organic matter associated with the particles, with the prokaryotic community contributing typically 3% or less of the total C_{org} burden.

Sediments as Habitats for Microbes

Sediment Properties Relevant to Microbial Ecology

From the point of view of microbial ecology, sedimentary environments are inextricably linked by the facts that they are (1) continuously wet and (2) physically stabilized – that is, the dynamics of the sedimentary environments are defined by diffusion and advection, and not by convection and turbulence more characteristic of the water column. While sediments and soils are often considered to be similar, from a microbiological perspective, they are in fact quite different. Soils are subject to constant changes in wetting and drying, often with extreme dehydration being part of the niche description. In addition, soils lack the constant deposition of new material from above that is so characteristic of sedimentary systems, which are built from above. While diagenetic changes occur within sediments as a result of microbial activity, the primary inputs of material and energy are supplied from above. Soils, on the contrary, are strongly impacted by inputs from within – usually supplied by roots of plants, or decaying plant matter. To some extent, then, sediments can be viewed as much simpler and more predictable environments, once the key variables noted earlier have been characterized (Table 1).

Perhaps one of the most relevant properties of sediments, and one that greatly increases their diversity (and makes them somewhat unpredictable), is that they are composed of particles (clays, minerals, detritus, etc.), and these particles are excellent habitats for microbes and the places where biofilms form. Thus, the biomass in sediments often exhibits a rather nonuniform distribution of microbial biomass, with some particles being covered with thick biofilms, and others being rather devoid of microbes. This nonrandom distribution must surely be of great importance in influencing the way(s) that microbes interact with the geochemistry of the sedimentary niche, and represents one of the great intellectual challenges to understanding the sedimentary niche.

Microbial Abundance in Sediments

Surficial sediments (those near the sediment–water interface) are typically rich in microbes present because of the energy available in the sediments. As mentioned earlier, typical surface sediment numbers have resident prokaryotes that reach numbers on the order of 10^9 per cm^3 of sediment. Yet these are sediments located near organic C inputs, such as coastal environments. Sediments located far from the continents and beneath low productivity surface waters (like the central South Pacific) have on the order of 10^6 cells per cm^3.

Table 1 Comparison of major properties of sediment and soil environments

Sediments	*Soils*
	Input from within: plant roots (photosynthesis and nutrient transport), decaying cellular material (e.g., roots)
Input primarily from above via sedimentation: clay particles, minerals, and microbial biomass	Some accumulation via wind-blown particles (dust and minerals)
Permanently wet – no airspaces	Alternately wet and dry cycles
Electron acceptors rapidly used up	In dry periods, airspaces are present over all oxic soils
Anoxic zone usually shallow	
Because of anoxia, eukaryotes are rare	Because of airspaces, abundance of eukaryotes (nematodes, protists, and fungi)
Dominated by bacteria and archaea	
Primarily controlled by diffusion	Primarily controlled by mixing (plant roots, fungi, nematodes, small eukaryotes, and water flow during wet periods)
Eukaryotes not common except at surface	
Characterized by chemical layers due to consumption of nutrients and production of products (at rates faster than they can diffuse)	Layers are rare, though occasionally present due to wind-blown events (e.g., clay particles)
	During wet periods, anoxia is common, and layers can form
Gradients that form tend to be simple, 2-dimensional gradients, unless disrupted by bioturbation	Gradients can form around particles, and can be seen as complex 3-D gradients
	Much less predictable

One can imagine that such bacteria- and organic-rich environments lead to a rapid depletion of oxygen, and this in fact is a nearly universal feature of sediments. Depending on organic matter input, the so-called oxic–anoxic interface will be present as a result of aerobic activity, rendering the remainder of the subsurface an anoxic environment. Thermodynamic 'logic' then prevails, and the other available oxidants are consumed in order, with each oxidant providing a measurable chemical layer as it is consumed.

Generation of Chemical Gradients by Microbial Activities

Dr. Robert Berner of Yale University was the first to describe the succession of chemical reactions that occur when various common oxidants react with C_{org} in sediment. The succession is based on the free energy derived from each reaction; the oxidants are those commonly found in aqueous solutions or associated with sediment solid phases. The succession of reactions that are seen in pore water profiles is the result of microbial metabolism leading to the accumulation of products, or the consumption of reactants at rates greater than they can be removed or supplied by diffusion. The succession is readily seen in pore water chemistry, and it occurs as different oxidants, used in the free energy succession, are sequentially depleted (see Fig. 1). The free energy paradigm applies to freshwater sediments as well, with the major difference relating to the paucity of sulfate in freshwaters. Hence, sulfate reduction is a minor process in freshwater sediments, where the transition to methane production occurs more rapidly than in seawater.

The cascade of oxidants involves both reactants and products of C_{org} respiration. For example, the oxidation of organic matter with oxygen yields both CO_2 (which can serve as an oxidant in some reactions) and nitrate, as organic NH_3 is oxidized. These product oxidants are also utilized in the cascade sequence of free energy such that even in sediments underlying water containing no nitrate, nitrate can be one of the oxidants utilized by sedimentary heterotrophs.

The succession of geochemical profiles implies that sedimentary bacteria adhere strictly to the 'rules' of thermodynamic energetics. The structure of geochemical profiles obtained from pore water analyses generally supports this argument. However, one misguided assumption associated with the layered microbial community (LMC) paradigm is that oxygen consumers 'out-compete' nitrate consumers, for example. It has not been demonstrated that competition is involved with the structure of geochemical profiles or bacterial communities. While there is a clear trend in declining bacterial numbers with depth in the sediments, the association of bacterial turnover rates and geochemical zonation has not been explicitly shown.

A recent discovery also shows how the 'energy cascade' model is subject to exceptions. Cable bacteria have been shown to shuttle electrons between zones of sulfide abundance to zones of oxygen. This typically occurs in sediments over a vertical span of several centimeters (although it is not limited to the vertical orientation). This electron shuttle allows reduction of dissolved oxygen in near-surface sediments (Fig. 2). Typically, in high sulfide sediments (those undergoing rapid rates of sulfate reduction) the sulfide can diffuse until it reaches the zone of oxygen diffusing downward. But cable bacterial will bridge the sulfide-rich zone with the oxygen-plentiful zone thereby leaving a gap in the sediments where there is the absence of both oxygen and sulfide. Further, this reaction drives low pH in sulfidic pore waters and high pH near the sediment-water interface. The electron shuttling is thus a microbial mechanism that actually geo-engineers the sediment column.

Chemical Profiles in Sediments (Layered Microbial Communities)

What Is a Layered Microbial Community?

Perhaps the key indicator of an active sedimentary environment is the presence of distinct layers like those described earlier – geochemists call these chemical profiles, while geobiologists prefer to call them LMCs, or layered microbial communities. It is a semantic issue on one hand, and an important intellectual issue on the other. What is measured is pore water chemistry, and the chemical gradients are clearly seen – what is responsible for them is the activity of the resident microbes, feasting on the energy available at these interfaces, and providing the chemical catalysis for their very existence. That is, dead or sterile sediments have chemical profiles that are dictated by diffusion between end-member boundary conditions. Sediments with life often show chemical gradients that overrule diffusion as life will involve consumption or production at rates greater than diffusion, and by these activities, modify the diffusive profiles. The interplay between metabolic activity and the physics of the sedimentary environment leads to the establishment of metabolic layers, and these layers can then be used to establish the processes that lead to their establishment. The definition of a metabolic layer does not preclude any other geochemical reaction from occurring within that layer given the right circumstances – it is simply defining the predominant geochemical activity for the given environmental setting.

The chemical and biological consequences of the physical nature of sediments have to do with the solubility and movement of key nutrients through this diffusion-limited environment. Perhaps the key nutrient is oxygen, the solubility of which ranges from about 200–400 $\mu mol\ l^{-1}$ depending on temperature and salinity. Thus, one usually sees oxygen depletion in sedimentary systems, depletion that can occur within a millimeter or less of the sediment–water interface (in eutrophic (highly productive) lakes or marine sediments), to many meters in deep-sea sediments underlying the oligotrophic (minimally productive) ocean gyres. Oxygen limitation, of course, leads to the use of other electron acceptors, including nitrate, metal oxides, sulfate, and finally CO_2. Each of these electron acceptors is used in its thermodynamic 'order', and its removal (or the appearance of its reduced product) can be used to characterize the microbial activity of the sediment system, as shown in Fig. 2.

The bottom line in terms of defining this environment is that the physical and geochemical conditions set the boundaries for the system; these boundaries define to some degree the chemical and biological consequences on the environment – the processes that

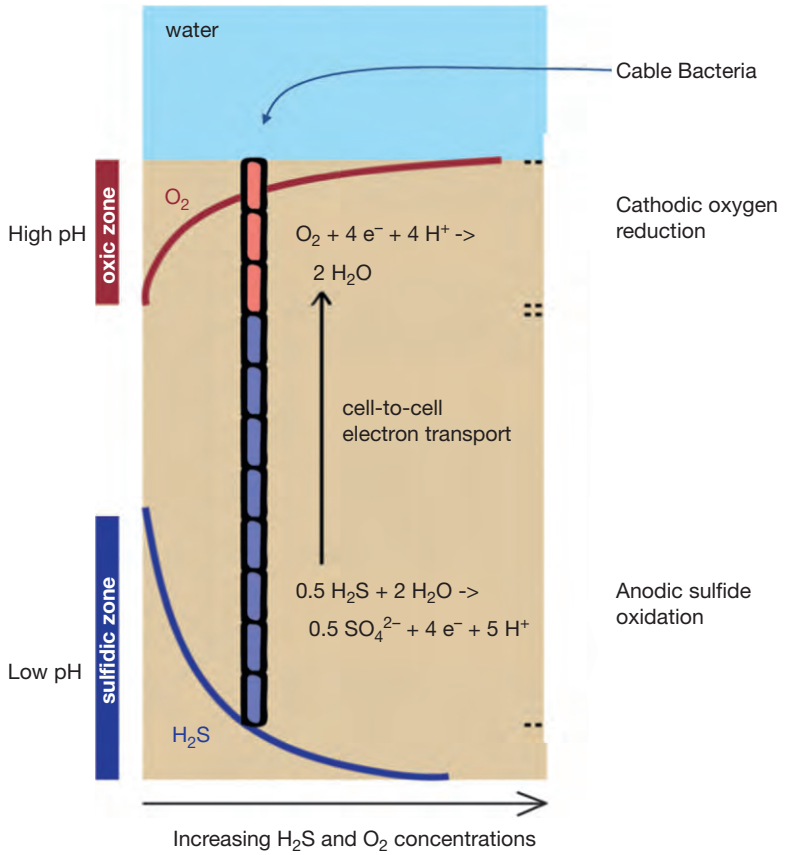

Fig. 2 Diagrammatic view of the cable bacteria. These bacteria, many of which appear to be affiliated with the genus *Desulfobulbaceae* in the delta Proteobacteria, form conductive multicellular filaments that allow bacteria deep in the suboxic zone to oxidize sulfide using oxygen from millimeters or even centimeters above for a review and perspective.

will occur can be predicted with some certainty – thus, the biological consequences can be predicted in terms of process. Prediction in terms of species is much less certain: For some of the processes, like oxygen depletion and nitrate reduction, many taxonomic groups are capable of the catalysis, while for others, like sulfate and CO_2 reduction, the taxa that can be expected are much more well defined (Fig. 3).

The Rates of Sediment/Solute Transport Processes

The microbial community associated with sediment solid grains is likely dependent on the movement of solutes to bring various nutrients and energy-yielding chemicals to their habitat. However, it is possible that the sediment grain to which they attach is replete with all the necessary food items. Solid grains including C_{org}, N_{org}, P_{org}, P_{inorg}, and various trace metals are commonly found adsorbed to these and other particles. The oxidants manganese oxide and iron oxide are solids themselves, and are excellent scavengers of trace metals, as well as being found associated with other solid particles. Thus, the sediment grain itself can provide all of life's essential nutrients and the ultimate source of these solid nutrients is the sedimenting particle. As energy-yielding reactions occur, for example, manganese and iron oxide reduction, some of the solid particles (e.g., manganese and iron oxides) can be solubilized, leading to the release of trace metals and other adsorbed nutrients. Such reactions can lead to movement within sediments of layers of metal oxides and the nutrients with which they are associated. Chemical thermodynamic theory predicts that the most easily reduced oxides will be utilized and depleted first, followed by other more crystalline and less reactive oxides. Thus, manganese oxides, which are very easily reduced, will almost always be reduced (solubilized) prior to iron oxides in sedimentary environments, leading to a separation of these two metals in space.

Because sediments represent a record of time, the oldest sediments are deeper within the column than shallower sediments, and because metal oxide depletion occurs close to the sediment–water interface, as particles are buried, the abundance of easily reducible iron or manganese oxides declines. Reduced manganese and iron are soluble and will diffuse away from regions of high concentration. The consequence of this process is that manganese and iron oxides get diagenetically recycled or regenerated when these solutes come in contact with molecular oxygen or nitrate. Alternatively, the reduced manganese and iron may be sequestered as minerals. The reoxidation generally occurs near the sediment–water interface. Thus, a cycle of manganese and iron reduction and oxidation is very common in sediments overlain by water containing oxygen or nitrate.

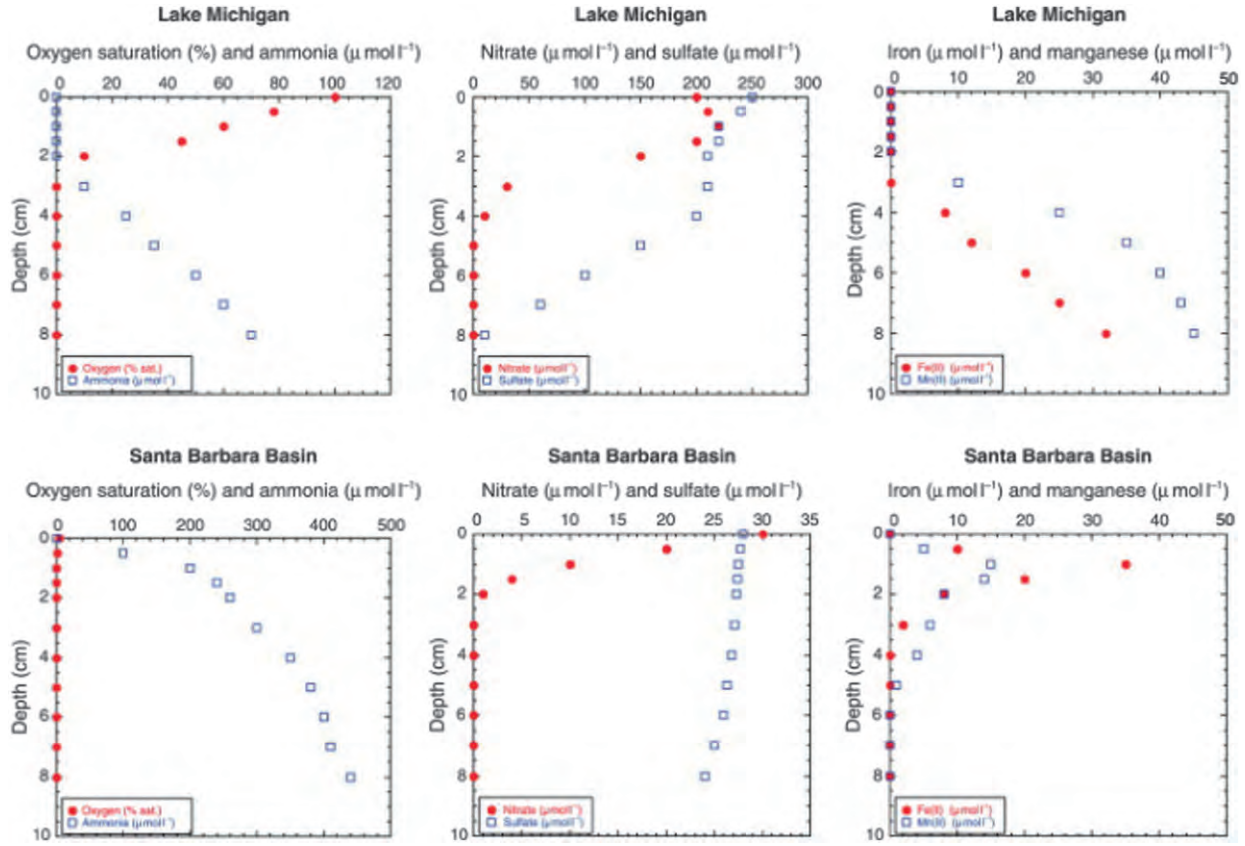

Fig. 3 Comparison of freshwater and marine pore water profiles from Lake Michigan (Nealson Laboratory) and Santa Barbara Basin (Berelson Laboratory). Of particular note is the lack of oxygen in Santa Barbara Basin sediments, the rapid removal of Fe(II) and Mn(II) in these sediments and the high concentration of ammonia within these marine sediments. The production of ammonia in Lake Michigan sediments is linked to denitrification, sulfate reduction, and metal reduction.

The reoxidation of reduced metals generates a surplus of solid-phase oxidants near the sediment–water interface. However, the process of bioturbation, the physical mixing of sediments by the activity of macrofauna, will carry oxidized Fe and Mn particles downward into the deeper sediment column where solute oxidants are absent. Hence, not only are Fe and Mn recycled and renewed when they become oxidized from the soluble reduced form, but they are also injected back into zones that are free of oxidants by sediment mixing. In sediments subjected to bioturbation, there will always be a race between microbes adapting to their particular redox environment before mixing carries them into a new and different redox zone.

Microbial environments also change within sediment habitats because there is a slow transport of pore water due to the compression of sediments as burial progresses. Upon initial settling, sediments are relatively uncompacted – the porosity can approach 0.95 or greater. As sediments become buried, compaction of the grains reduces the porosity. By 10 cm the porosity may be reduced by 10%, by 10 m the porosity is often reduced by 25%–40%. This effectively creates an upward flow of pore water, relative to the compacting sediment (the water is incompressible; the grains settle and compact within this medium, giving the mathematical impression of advective 'flow' toward the sediment–water interface). This advective process, termed pore water advection, will juxtapose pore water microbial communities with sediments other than those with which they were originally associated. In shallow coastal regions and some lakes, the rate of sediment accumulation is fast and the upward advection of pore water can be on the order of centimeter per year. However, in most of the ocean this rate is very slow, much lesser than a millimeter per year.

Because sediment pore water is generally not subject to turbulent mixing, the most ubiquitous transport mechanism for solutes moving through sediments is molecular diffusion. In the absence of any chemical reaction, diffusion would homogenize the concentration of all solutes throughout the sediment column. And in the vast majority of ocean sediments that are absent of bioirrigation or other mass transport processes, diffusion limits the rate at which solutes can move through pore fluids. An added complication in a pore water–sediment environment is that diffusion cannot take a straight path between high and low concentration. Sediment grains effectively block the most direct diffusive path length; hence, the term tortuosity describes how far from linear the path length has become.

The fact that virtually all sediment pore water solute profiles of the biologically relevant chemicals show curvature, maxima, and minima and approach zero is evidence that there are reactions occurring within the sediments. As diffusion will generate a linear

gradient between two fixed boundary conditions, the curvature in a solute profile is easily transformed into an understanding and quantification of reaction rates. The production or consumption of a chemical between two sediment horizons is defined by the flux across each boundary. If more solute is entering the sediment zone (between the two defined horizons) than is leaving the zone, there is net consumption occurring between these horizons. If more solute is leaving a sediment zone than is entering, there is net production occurring within that zone. The rate at which a solute will pass through a sediment horizon (a plane) via diffusion is defined by Fick's first law:

$$J = \frac{dC}{dz} D\phi$$

where J is the flux (moles cm^{-2} s^{-1}), C the concentration in pore water, z the depth in sediment, D the compound's molecular diffusivity (corrected for the tortuosity and considered a physical constant for a given ion, given temperature and salinity), and ϕ the sediment porosity. The difference in flux between two sediment horizons defines the reaction rate. Production or consumption is defined as

$$R = \frac{J_1 - J_2}{z}$$

where R is production or consumption (moles cm^{-3} s^{-1}), J the flux across horizon 1 or 2, and z the distance between horizons 1 and 2.

The ability to translate geochemical gradients into an understanding of fluxes and reaction rates (production and consumption) is extremely important to understanding microbial activity in sediments. A rate measurement made from diffusive flux calculations at two sedimentary horizons will, of course, only be as good as the quality of the gradient data and the assumption that diffusion is the major transport mechanism. Yet these types of data and the diffusive assumption are remarkably robust, and form the basis for much of our thinking about sedimentary dynamics.

It can be informative to compare bulk sediment reaction rate calculations, derived and used by geochemists, to microbial respiration rates as determined by microbiologists. There are numerous estimates of the rates of oxygen consumption in sediments, but to our knowledge, there are no published microbial oxygen respiration rates normalized to a per cell basis. To this end, our own laboratories have made some bacterial oxygen consumption measurements and found that within a factor of 3, microbes during growth phase can consume oxygen at a rate of 50×10^{-10} nmol CFU^{-1} s^{-1}. Given this rate of oxygen consumption per cell and applying a rate of 0.05 nmol cm^{-3} s^{-1} for bulk sediment oxygen consumption (a high rate indicative of eutrophic coastal environments) suggest that 1×10^7 cells cm^{-3} in growth phase could account for the measured oxygen consumption. This might represent 1%–10% of the cells living in a sediment community, or imply that few, if any, of the cells are in the so-called growth phase that we so often study in the laboratory.

There are also many published values for sulfate reduction rates (SRRs) in sediments, generally established using a tracer spike of radiolabeled ^{35}S, applied to recently collected bulk sediments, but in this case, in opposition to the situation with oxygen, there are many published estimates of SRRs per cell, generally measured on pure cultures in the laboratory. The range of SRR per cell is very broad, as are rates measured in the field, but given published values of SRR within bulk sediments, and a range of 3 orders of magnitude in cellular SRRs, it is possible that sulfate-reducing bacteria (SRB) make up a substantial fraction (>10%) of the total sediment inventory.

The two abovementioned examples demonstrate that the connection between cellular activity and bulk sediment activity is not well understood. One outstanding question is how many sedimentary microbes are undergoing exponential growth, and another is whether there are a few superconsumers or if all sedimentary microbes are tuned to respire within a certain range. It is certainly true that sedimentary microbial systems dominated by C_{org} respiration are much more complex than pure cultures examined during optimal growth conditions.

Can Microbial Populations be Predicted From Chemical Layers?

One of the key questions that arises from the study of sedimentary environments is that of whether or not it is possible to predict the nature of the microbial populations by the profiles that are present. We propose that it is to some extent possible to predict the general nature of the reactions that must have taken place to account for the observed profiles. However, that is probably as far as it goes. There is a tremendous redundancy in nearly every process – a redundancy that precludes the specification of which phylotypes should be expected to be present. Thus, historically, one has had to settle with knowing that oxygen utilizers, denitrifiers, iron and manganese reducers, and so on must be present. Add to this the fact that the taxonomic diversity of microbial sediment communities is far greater than the few of the sediment organisms that have been cultivated, and the situation has looked impossibly complex for decades.

For example, as the geochemists would identify the 'zone of manganese reduction' by a profile of manganese as shown in Fig. 2, microbiologists would call this the zone of manganese reducer activity. However, there are literally hundreds of different microbial species capable of manganese reduction, thus relegating one to specifying processes rather than names of species, or even genera.

The other part of the predictive process is that most of the processes being studied are in fact cyclical. The oxygen diffusing down into the sediments acts not only as the electron acceptor for heterotrophic metabolism, but as the oxidant of many

inorganics, regenerating them for further oxidative activity in the zones of reduction. Thus, near every reductive interface, as described by the geochemists, is an oxidative one, in which the electron acceptors are regenerated, almost always via the microbial activity(ies). As with the reductive activities discussed earlier, these activities can be specified, but the microbes responsible for them have been very difficult to identify and have traditionally only be designated by metabolic group.

To this end, the rapidly moving fields of molecular taxonomy, genomics, metagenomics, and metatranscriptomics have made it possible to identify and quantify the members of sediment microbial populations (ref), identify the gene content of different sediments, assemble the genomes of the microbes that are present, and even pinpoint which genes are being induced by which organisms. While it is not yet possible for one to link microbes and their activities to the geochemical patterns seen in sediments, the future looks bright in terms of teasing apart the microbial interactions that are occurring within this ecosystem using a combination of metagenomics, genome assembly and analysis, and metatranscriptomics coupled with stable isotope probing and metabolomics approaches.

Complications to the usual view of LMCs have been generated with the discovery of the so-called cable bacteria by the laboratory of Lars-Peter Nielsen and colleagues. These bacteria, now known in marine sediments around the world, form multicellular filaments that allow the transfer of electrons from hydrogen sulfide, across several redox zones to the oxygen zone, sometimes far away from the source of reductant, as illustrated in Fig. 2. They also represent a phenomenon that is now well described operationally, but which is not yet understood mechanistically.

For the microbial ecologist, then, the sedimentary environment remains a great challenge in terms of understanding the diversity and interactions that occur as one proceeds downward through the LMCs.

Sediments as a driving force for discoveries in microbiology

During the past decades, it has become apparent that careful geochemical analysis of sediments reveals microbiological processes that must be occurring to explain the observed profiles. One of the most distinguishing features of life is its ability to catalyze reactions that, while thermodynamically favorable, are kinetically constrained due to large activation energies. Indeed is the invention of specific protein catalysts (enzymes) is key to the success of life, allowing low-temperature conversion of many different energy sources. To this end, as noted below, it is notable that some major discoveries in microbial metabolism were discovered in attempts to explain sedimentary data in which the rates of geochemical processes were far too rapid to be explained by chemistry alone.

Anaerobic methane oxidation

In the late 1970s and early 1980s geochemical data from two different laboratories noted the disappearance of methane at depth in marine sediments, far below the oxic/anoxic interface (Goldberg & Barnes; Rheeburgh), a process that at the time was deemed impossible by microbiologists. The geochemical data, however, were convincing, and after considerable searching, two laboratories reported the existence of microbial enrichments that catalyzed anaerobic methane oxidation (Delong & Orphan; Jorgensen & Boetius), leading eventually to the identification of the ANME group of Archaea capable of catalyzing anaerobic oxidation of methane in concert (symbiosis) with sulfate reducing bacteria.

Manganese reduction/extracellular electron transport

In the early 1980s geochemical flux data from Oneida Lake in New York revealed rates of manganese oxide reduction that were orders of magnitude too fast to be explained by the chemistry of the lake sediments (Moore). A similar situation was reported for iron flux from sediment incubations from the Potomac river (Lovley). For both iron and manganese oxides, reduction by direct electron transfer was deemed to be impossible, as the metal oxides are insoluble and thus not available for direct reduction. As with the anaerobic methane oxidation, however, the process was revealed by the geochemical data, and enrichment cultures for iron and manganese reducers revealed two different groups of metal reducers, Shewanell (Myers and Nealson, 1988) and Geobacter (Lovley and Phillips, 1988), both of which proved to be capable of transporting electrons to solid substrates outside the cell via a process now termed extracellular electron transport, or EET.

In both of the examples noted above, considerable variation on the original “theme” has been seen, and major changes in the view of microbial physiology have occurred in the search for answers to geochemical anomalies.

Complications Introduced by Activities of the Macrofauna

Sediments that are mixed by macrofaunal activities (bioturbation) show complexities in microbial distribution due to the introduction of oxygen (and other oxidants) at depth due to burrowing, pumping of water, and other activities of multicellular macrofauna of a variety of types (Fig. 1). Despite these activities, however, which tend to mix the upper surface of the sediments, one sees that bacteria distribute themselves along geochemical gradients and, in fact, are often responsible for the maintenance of these gradients although sometimes pore water chemistry and microbial community structure are uncoupled by this process. Sediments that do not undergo physical mixing provide excellent systems for the calibration of bacterial community activity, gradient formation, and community evolution: Mixed sediments provide sites for understanding the same processes in a much more complex setting of 3-dimensional diffusion gradients, perhaps more reminiscent of soil environments.

Intersection of Chemical Gradients

A fascinating feature of sediment communities relates to the places in which chemical gradients of different types intersect with one another, such as can be seen the sulfate/methane horizon in deep-sea cores. At such interfaces abundant microbes are seen, and the process of anaerobic methane oxidation, which occurs seldom if at all elsewhere, is abundant. The advantage of living near a redox boundary is that the energy-yielding reaction space increases: For example, oxygen utilization in the respiration of C_{org} requires a microbial community capable of breaking C_{org} into oxidizable-sized compounds and it requires that the respiring microbes 'find' both the oxidizable compound and the oxygen simultaneously. The presence of oxygen in contact with ammonia, diffusing up from deeper in the sediment column, provides a fixed location where both energy-yielding compounds coexist. In fact, the constancy of geochemical gradients and redox boundaries within the sediment column is evidence that sedimentary microbes adapt to and will aid in the perpetuation of these gradients and boundaries.

Sometimes the net free energy yield of a coupled set of geochemical reactions is sufficient to support a sedimentary microbial community even when some of the 'players' are working in a free energy deficit. An example of this is where sulfate is used in the oxidation of methane, a process known to be catalyzed by a consortium of archael methanotrophs and SRB. It has been determined that while the SRB capture significant energy from this coupled reaction, using H_2 as an intermediate, there must be something 'in it' for the archaea for their role in this coupled reaction costs them energy.

The further one looks into sedimentary bacterial activities, the more one sees that a simple thermodynamic explanation (i.e., the free energy paradigm) is a rule that is commonly broken. Under eutrophic and low oxygen water columns lie sediments containing little or no oxygen. In these sediments live bacteria that can sequester nitrate from the overlying water column and transport that nitrate within the sediments to zones where sulfide is present in high quantities. Under diffusive conditions, nitrate would not penetrate to the depth of high sulfide, yet these bacteria, for example, *Beggiatoa* and *Thioploca*, are capable of bypassing the diffusive zone and injecting nitrate into sediments rich in sulfide, thereby oxidizing the sulfide to S^0 or SO_4^{2-}. An unsolved enigma is that the greater free energy yield would be obtained if nitrate were to react with C_{org} compounds. Sedimentary bacteria find ways to utilize unusual combinations of reduced and oxidized compounds, not only those in association with free solutes but also those in the solid phase. This is, like the cable bacteria, yet another example of exceptions to the LMC rule.

Recent findings have revealed a surprisingly high diversity in marine sediments of microbes capable of using solid-state electron donors, including poised electrodes as energy sources (see references to Rowe and Lam), further complicating the energetic situation in sediments, especially those that may be lacking in abundant organic carbon (see below).

Organic-Poor Sedimentary Environments

Recent studies of deep-sea sediments underlying low productivity oceanic zones have revealed that a major part of the deep sea (perhaps 20% or more) consists of sediments that are so organic poor that the numbers of microbes are far less than those usually encountered in near-shore and productive environments. For example, Steve D'Hondt, Bo Jørgensen, and their colleagues, in studies of sediments in the South Pacific gyre area, have found surface sediments that typically harbor only 10^3–10^5 bacteria per gram of sediment – orders of magnitude below those found in other more nutrient-rich horizons. Predictably, the chemical gradients typical of more active zones are absent here. Life becomes 'cryptic' in terms of using geochemical profiles to define it. The nature of the microbes in such environments is curious – they appear to be alive, but nondividing or extremely slowly dividing, with estimates of doubling times extending to thousands of years.

Subsurface Sedimentary Microbiology

In a situation similar to that seen in nutrient-poor sediments, the deep subsurface of ocean sediments appears to harbor a low-density (10^5 per gram) population that is dividing at very low rates (again, perhaps thousands of years), This so-called starving majority as it has been called by Bo Jørgensen constitutes one of the current great mysteries in microbial ecology, and it is tempting to speculate that it may be similar to environments of early Earth, when C_{org} may have been much less abundant, and organisms might have utilized mechanisms for survival not often needed on the nutrient and energy-rich surface of our planet. The radiolytic dissociation of water and production of hydrogen gas may be an energy source to these nutrient poor environments, as may be the reduced iron and sulfur-containing solid minerals.

Paleontological Properties of Sediments: The Rock Record

As noted earlier, diagenesis is the process of sediment alteration, a process that begins as soon as the sedimentary material arrives, and continues as long as energy is flowing through the system. Superimposed on this are the paleontological consequences of sedimentation. As diagenesis occurs, a number of biosignatures can be deposited as records left to be examined in ancient sediments – records of early metabolism, even in the absence of living biomass, or even cell structures. Biosignatures can consist of molecules indicative of certain taxa and the processes they catalyze (i.e., markers indicative of photosynthetic activity, particularly of oxygenic photosynthesis), as well as stable isotopes of carbon, sulfur, and/or nitrogen that can be fractionated by biological

systems. Such molecular or isotopic fossils are often the only records available in the ancient sediments, and even these are often altered beyond recognition by diagenesis and metamorphosis (cooking and alteration!) of the sediments. However, it is these ancient sediments and their geobiological records that provide hints as to the earliest metabolism(s) on Earth, and even to the first detectable signs of life on our planet.

Sediments in Shallow Water, and the Impact of Light

Almost everything discussed in this article has dealt with the sedimentary environment in the deeper and dark zones of oceans and lakes. Of course, along the continental margin, and in many lakes, sunlight can penetrate the overlying water, adding new complexity to the sedimentary environment. Now the environment becomes even more distinctly layered, often with oxygen-producing cyanobacteria at the surface, and other layers of anoxygenic photosynthetic bacteria below. The oxygen penetration depth can change dramatically between day and night, with oxygen production by the cyanobacteria during the day often pushing the oxic zone to several centimeters, and oxygen consumption by the heterotrophs leading to anoxia, even at the surface at night. This being said, however, the general features of the environment still apply: It is a permanently wet, diffusion-controlled niche that is strongly impacted by physical (porosity, tortuosity, etc.) and chemical (salinity, anion content, etc.) factors.

Conclusions

In closing, it is appropriate to remind ourselves of the general properties of the sedimentary niche, which is perhaps the largest single niche available to life on the planet.

(1) Sediments are not soils – they accumulate biomass by sedimentation from above, and are constantly water saturated.
(2) Sediments are physically stabilized, and therefore solute distributions are primarily controlled by diffusion – nutrients must diffuse from above (or sometimes below), and this diffusion controls the overall character of the niche.
(3) Sediments can (and often do) have zones of bioturbation, a type of mixing imposed by eukaryotic multicelled organisms that pump water and nutrients into the sediments, as well as physically mixing the environment.
(4) Sediments typically become anoxic due to respiration of the microbial population removing the oxygen at a rate faster than it can be supplied by diffusion, thus leading to widespread anoxic sediment distribution.
(5) Other oxidants (nitrate, manganese oxides, iron oxides, sulfate, and CO_2) are then used up via respiration, leading to chemical layering of sediments, processes that result in LMC formation.
(6) Pore water analyses reveal the existence and nature of the LMCs in sediments, and allow one to specify to some degree the nature of the processes occurring in a given sedimentary habitat.
(7) Sediments in the photic zone take on new properties due to the daily cycles of photosynthesis and oxygen consumption, and are further complicated by the input of organic matter directly to the environment by photosynthesis. However, they are still permanently wet, diffusion-controlled systems, subject to the same rules as other sediments.
(8) Sediments are in general excellent habitats for prokaryotes, with 10^9 per gram or more cells being common. The sedimentary niche may harbor up to 50% of the biomass on our planet, and interact strongly with the biogeochemical cycles of nearly every major biologically active element.

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Sensory Transduction in Bacteria

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Glossary

Cache Sensor domain found in calcium channel and chemotaxis proteins.
EAL domain Conserved protein domain, usually with *c*-di-GMP-specific phosphodiesterase activity, named after its conserved Glu-Ala-Leu sequence motif.
HAMP domain Conserved cytoplasmic linker domain, named after histidine kinases, adenylate cyclases, methyl carrier proteins and protein phosphatases, where it was originally described.
HD-GYP domain Conserved protein domain, usually with *c*-di-GMP-specific phosphodiesterase activity, named after its two conserved sequence motifs, His-Asp and Gly-Tyr-Phe.
GAF domain Conserved ligand-binding protein domain, named after cGMP phosphodiesterases, adenylate cyclases, and FhlA proteins, where it was originally described.
GGDEF domain Conserved protein domain, usually with diguanylate cyclase (*c*-di-GMP synthetase) activity, named after its conserved Gly-Gly-Asp/Glu-Glu-Phe sequence motif.
MASE Membrane-associated sensor domain.
PAS domain Conserved ligand-binding protein domain, named after period circadian protein (Per), Aryl hydrocarbon receptor nuclear translocator (ARNT), and single-minded protein (Sim) proteins, where it was originally described.
Phosphorylation Transfer of the phosphoryl group $-PO_3^{2-}$ (the phosphate group $-O\text{-}PO_3^{2-}$ without an oxygen atom), for example, from ATP to various acceptors.
Protein domain A discrete structural unit of proteins that can be found in different contexts. Most domains range in size from 50 to 300 amino acid residues.
Protein motif A group of amino acid residues, conserved among several different proteins.
REC domain Receiver domain, a conserved protein domain that is similar to the CheY chemotaxis protein and contains a conserved Asp residue that can be reversibly phosphorylated by histidine kinases or artificial phosphoryl donors.

General Principles of Signal Transduction

The ability to sense changes in the environment and respond to them is a key property of all living cells. In principle, the level of a cellular metabolite could be regulated by simple feedback mechanism with a single transcriptional regulator. For example, a simple system consisting of a cellobiose-binding transcriptional regulator and a cellobiose-degrading enzyme would be sufficient for controlling the level of cellobiose in the cell. Increased levels of cellobiose would shift a larger proportion of the transcriptional regulator into the active (DNA-binding) form, which would lead to increased expression of the cellobiose-degrading enzyme that would eventually lead to the decrease in cellobiose level. Decreased levels of cellobiose would cause dissociation of the sugar from the transcriptional regulator, gradual decrease in the expression of cellobiose-degrading enzyme and, as a result, stabilization of the cellobiose level.

Such simple schemes allow the cell to respond to the changing levels of a cellular metabolite and are used—with modifications—by a variety of regulatory systems. However, this kind of regulation is not highly sophisticated and will not allow coordinated regulation of complex metabolic pathways. Rather than measure the levels of nutrients in the cytoplasm, it is strategically more efficient for the cell to monitor the environmental levels of these nutrients. That would make it possible, even at relatively low levels of certain nutrients outside the cell, to induce the respective transport systems and import these nutrients. This is exactly the strategy utilized by many bacteria. For example, uptake of glucose-6-phosphate and several other sugar phosphates by *Escherichia coli* is induced by micromolar levels of extracellular glucose-6-phosphate, which are 1000 times less than the concentration of this glycolytic intermediate in the cell. It also makes sense to detect antibiotics and other toxins in the external milieu before such compounds damage the cell. The question then becomes how to detect an extracellular signal and transmit the information into the cell. Summing up, bacterial signal transduction systems, as opposed to feedback mechanisms, are usually utilized to:

- Detect extracellular signals of a chemical or physical nature, particularly weak ones
- Detect antibiotics, toxins, and other hazardous substances before they irreversibly damage the cell

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- Regulate complex cellular processes. The complexity of the cellular organization calls for highly coordinated response(s) to environmental challenges, making it necessary for the signal transduction machinery to
  Constantly monitor a variety of extracellular conditions (pH, osmotic stress, redox level, nutrient levels, presence of neighbors), and
  Organize defined, yet confined, hierarchical responses at appropriate cellular locations.

Detection of environmental signals is usually carried out by transmembrane proteins that are composed of an extra-cytoplasmic (periplasmic or extracellular) sensory domain, usually located at the N-terminus of the protein, one or several transmembrane segments, and a cytoplasmic effector domain (Fig. 1). The cytoplasmic domain typically (1) occupies the C-terminal part of the protein, (2) serves as a signal-transducing module, and (3) is enzymatically active as a dimer (or multimer). It is believed that signal reception (i.e., ligand binding) by the extracellular domain causes a conformational change that favors dimerization of the effector domains enabling their enzymatic activity. The mechanisms of the propagation of the signal across the membrane are still not yet fully elucidated and subject of research and debate.

The environmental signals monitored by the bacterial cell vary in nature, intensity and over time and may include changes in temperature, pressure, light intensity, pH, salt concentration, changes in the levels of nutrients in the surrounding medium, levels of oxygen and reactive oxygen species, levels of other terminal electron acceptors, CO_2, CO, NO, and other dissolved gases, proximity of other cells, and possibly many other parameters. Accordingly, cellular responses can occur at several levels, such as individual genes and operons, the whole-cell level (chemotaxis, phototaxis, sporulation), and the level of multicellular communication (e.g., biofilm formation). The regulation of gene expression can occur at the transcriptional, posttranscriptional (e.g., changes in mRNA stability), translational, and posttranslational (e.g., modulation of enzyme activity) levels. For this reason, the signal transduction machineries comprise mechanisms of communication not just between the sensory and the response modules, but also between different and independent modules.

Types of Bacterial Receptors and Signaling Pathways

Public Resources on Signal Transduction

Our current knowledge of signal transduction comes from a combination of genetic, physiological, and biochemical studies, primarily on model organisms such as *E. coli*, *Bacillus subtilis*, *Caulobacter crescentus*, *Myxococcus xanthus*, *Pseudomonas aeruginosa*, *Synechocystis* sp., and *Halobacterium halobium*, from bioinformatic analyses of signal transduction proteins, and from structural characterization of some of these proteins. Comparative analysis of protein sequences played a key role in the identification of the receiver (phosphoacceptor) domain in several different response regulators, which paved the way to the discovery of two-component signal transduction. In the past several years, availability of complete genome sequences led to a dramatic expansion of the list of organisms that could be used for comparing signal transduction proteins. Even more importantly, analysis of genome sequences allowed an accounting of all signal transduction proteins encoded in any given genome. This analysis revealed the presence of previously unknown receptor proteins even in such favorite model organisms as *E. coli* and *B. subtilis*. It also showed that the complexity of signal transduction systems encoded in these two organisms is rather modest, compared with the signaling repertoire of, for example, *P. aeruginosa* or *Synechocystis* sp., not to mention the enormous expansion of signaling genes in the genomes of *M. xanthus* or *Streptomyces* spp.

The diversity and complexity of the bacterial signal transduction machinery make a systematic description of its components in any given organism a difficult task (for *E. coli*, such a description is provided at the end of this article). Furthermore, it is hard—if at all possible—to make generalizations for the signaling systems across the entire bacterial world. Most of the current work is devoted to characterizing and comparing individual sensory pathways in selected bacteria and lacks a "grand view." This has led to creation of public databases that attempt to collect information on the whole spectrum of signal transduction systems in various organisms. Several such databases are freely available online (Table 1). Unfortunately, several popular databases have been recently discontinued, and some others have not been updated for a long time. Nevertheless, these web sites still offer helpful insight into the organization of the signal transduction machinery in certain bacteria.

The microbial signal transduction (MiST) database, which used to offer information on the signal transduction proteins from all completely sequenced bacterial and archaeal genomes, identified by their domain architecture, is currently unavailable but is expected to be back online in the near future. The P2CS database lists predicted two-component signal transduction proteins from all completely sequenced bacterial and archaeal genomes. It also provides manually curated annotations of the major classes of response regulators. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a comprehensive genomic database on all completely sequenced bacterial, archaeal, and eukaryal genomes. One of its sections provides a graphical representation of all bacterial two-component systems. Kinases in Genomes (KinG) database provides a comprehensive listing of Ser/Thr/Tyr kinases and related proteins encoded in various bacterial, archaeal, and eukaryal genomes. Finally, Signaling Protein Census and Response Regulator Census, two web sites maintained by one of the authors, provide counts of each class of signal transduction protein and response regulator in complete genomes from more than 500 bacterial and archaeal species.

In addition, signal transduction pathways for individual model organisms are often well covered in organism-specific databases, such as EcoCyc (https://ecocyc.org/, part of the vast BioCyc database collection) for *E. coli*, SubtiWiki (http://subtiwiki.uni-goettingen.de/) for *B. subtilis*, *Pseudomonas* Genome Database (http://www.pseudomonas.com/) for various pseudomonads, and CauloBrowser (http://www.caulobrowser.org) for *C. crescentus*. It is worth checking these databases before undertaking any genome analysis project: there is a good chance that these databases already contain all the answers.

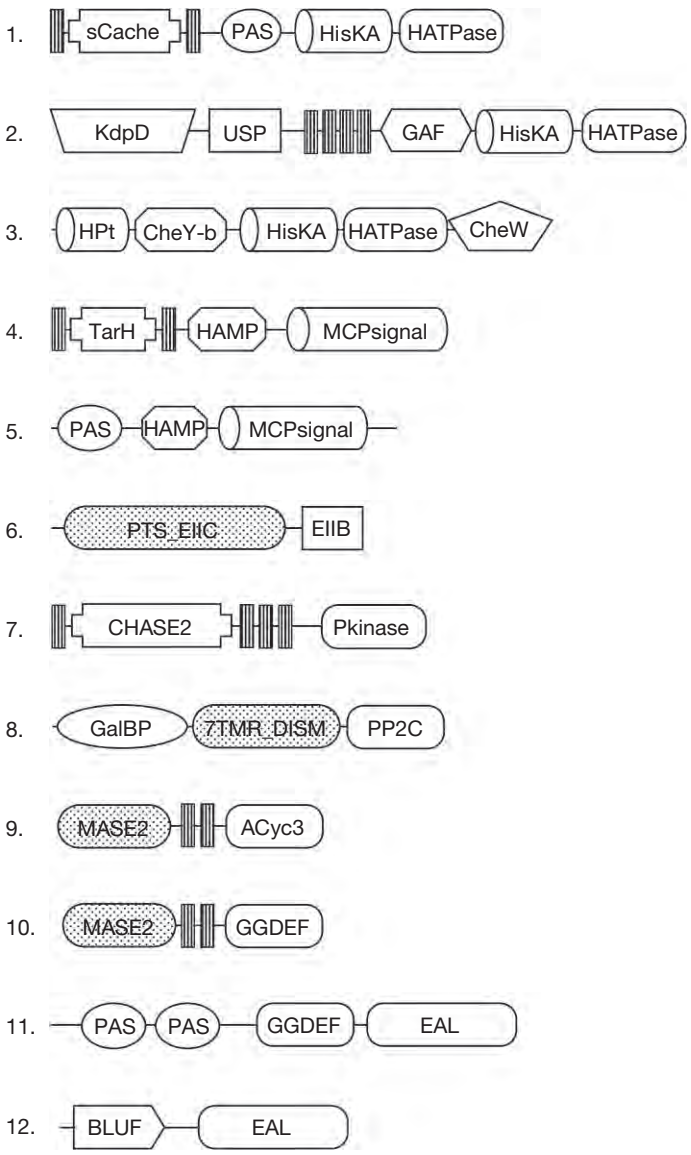

Fig. 1 Domain architectures of some bacterial receptors. (1) Escherichia coli citrate-sensing histidine kinase DpiB (UniProt accession number P77510, InterPro entry https://www.ebi.ac.uk/interpro/protein/P77510). (2) *E. coli* turgor-sensing histidine kinase KdpD (P21865). (3) *E. coli* chemotaxis histidine kinase CheA (P07363). (4) *E. coli* serine chemotaxis receptor Tsr (P02942). (5) *E. coli* redox chemotaxis receptor Aer (P50466). (6) *E. coli* PTS system glucose-specific EIICB component PtsG (P69786). (7) Anabaena variabilis serine/threonine protein kinase with Chase2 sensor domain (Q3M3S5). (8) Leptospira interrogans serine phosphatase RsbU, regulator of sigma subunit (Q8F087). (9) *Pseudomonas aeruginosa* adenylate cyclase CyaB (Q9HZ23). (10) *E. coli* diguanylate cyclase AdrA (P0AAP1). (11) *E. coli* c-di-GMP phosphodiesterase Dos (P76129). (12) *E. coli* c-di-GMP phosphodiesterase BluF (P75990). Domain names are as Tables 3 and 4; striped boxes indicate individual transmembrane segments; dotted shapes indicate integral membrane domains, containing multiple transmembrane segments. Pfam entries for other domains are as follows: USP, PF00582; CheW, PF01584; TarH, PF02203. Domain sizes are not necessarily drawn to scale.

Signal Transduction Pathways

Historically, experimental studies initially focused on two classes of membrane-bound receptor proteins, sensory histidine kinases and methyl-accepting chemotaxis proteins (MCPs). Subsequent analyses of microbial genomes, as well as experimental studies, revealed several additional classes of bacterial receptors, which include Ser/Thr/Tyr protein kinases and protein phosphatases, membrane components of the sugar:phosphotransferase system (PTS), adenylate cyclases, diguanylate cyclases and c-di-GMP-specific phosphodiesterases, and diadenylate cyclases (Table 2). It must be noted that, in addition to transmembrane sensors, there exist cytoplasmic versions of these receptors that appear to sense intracellular cues but use the same signal transduction machinery to propagate the signal and trigger the cellular response. Such cytoplasmic forms have been found for all kinds of receptors listed in Table 2 except for the membrane components of the PTS.

Table 1 Electronic resources on bacterial signal transduction

Name and URL	*Comments*[a]
Specialized databases of signal transduction proteins	
Microbial Signal Transduction (MiST)	Predicted signal transduction proteins from all completely sequenced bacterial and archaeal genomes
Kyoto Encyclopedia of Genes and Genomes (KEGG), http://www.genome.ad.jp/kegg/pathway/ko/ko02020.html	Graphical representation of two-component systems encoded in all completely sequenced bacterial and archaeal genomes
P2CS (Prokaryotic 2-Component Systems), http://www.p2cs.org/	Classification of histidine kinases and their cognate response regulators
Kinases in Genomes (KinG), http://king.mbu.iisc.ernet.in/	Ser/Thr/Tyr kinases encoded in completely sequenced genomes of bacteria, archaea, and eukaryotes
Signaling Protein Census, http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html	A listing of signal transduction proteins encoded in completely sequenced genomes of representative bacterial species
Response Regulator Census, http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html	A listing of response regulators encoded in completely sequenced genomes of 896 bacterial and archaeal species
Protein family and domain databases	
Protein families database (Pfam), http://pfam.xfam.org/	An extensive collection of protein domains, includes many signal transduction domains
Simple modular architecture research tool (SMART), http://smart.embl.de/	Protein domain database with special emphasis on bacterial and eukaryotic signal transduction domains
Integrated protein family databases	
Conserved Domain Database (CDD), http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml	Protein domain database with curated domain alignments that are based on known 3D structures
Integrated database of protein families, domains, and functional sites (InterPro), http://www.ebi.ac.uk/interpro/	An integrated database that combines results from several protein domain and sequence motif databases and complements them with convenient search tools

[a]Detailed descriptions of these databases with up-to-date references are available at the respective websites.

Table 2 Principal classes of bacterial and archaeal receptors

Receptor type	*Regulated functions*	*Signaling mechanism*	*Phylogenetic distribution*
Histidine kinase	Regulation of transcription, other signaling systems	Phosphorylation of the receiver domain in various proteins	Bacteria, archaea
Methyl-accepting chemotaxis protein	Chemotaxis	Interaction with chemotaxis-specific histidine kinase CheA	Bacteria, archaea
Ser/Thr/Tyr protein kinase	Regulation of transcription, enzyme activity	Phosphorylation of Ser, Thr, or Tyr residues in target proteins	Bacteria, archaea
Ser/Thr/Tyr protein phosphatase	Same as above	Dephosphorylation of Ser, Thr, or Tyr residues in protein kinases or other proteins	Bacteria, archaea
Membrane components of the sugar: phosphotransferase system (PTS)	Sugar transport, chemotaxis	Binding of $EIIA^{Glc}$ and/or EI to chemotaxis proteins, adenylate cyclase, membrane transporters	Bacteria
Adenylate cyclase	Global regulation of transcription	Synthesis of cAMP	Bacteria
Diguanylate cyclase	Regulation of motility, cell division, protein and polysaccharide secretion	Synthesis of c-di-GMP	Bacteria
c-di-GMP-specific phosphodiesterase	Same as above	Hydrolysis of c-di-GMP	Bacteria
Diadenylate cyclase	Sensing of DNA integrity, cell wall stress	Synthesis of c-di-GMP	Bacteria, archaea

The signaling pathways utilized by various bacterial receptors are shown on Fig. 2. Signaling by histidine kinases is usually referred to as two-component signal transduction, as it includes phosphoryl transfer between two separate proteins, a histidine kinase and a response regulator. Two-component signal transduction pathways are extremely diverse but always include the following common steps: (1) ATP-dependent phosphorylation of a conserved His residue in the kinase; (2) transfer of the phosphoryl residue to a conserved Asp residue in the receiver (REC) domain of the response regulator; (3) conformational change in the response regulator that alters its interaction with the target(s). The majority of response regulators serve as transcriptional regulators and bind to the chromosomal DNA, but there are response regulators with alternative targets (see below).

Chemotaxis signaling, which starts from MCPs, is a special case of two-component signal transduction that involves a specialized histidine kinase CheA, which directly interacts with MCPs, and a separate response regulator CheY that represents a stand-alone REC domain without output domains. Regulation of flagellar motility is based on the interaction of the phosphorylated form of CheY (CheY∼P) with the FliM protein at the base of the flagellum, which affects FliM, FliN, and FliG proteins and changes the direction

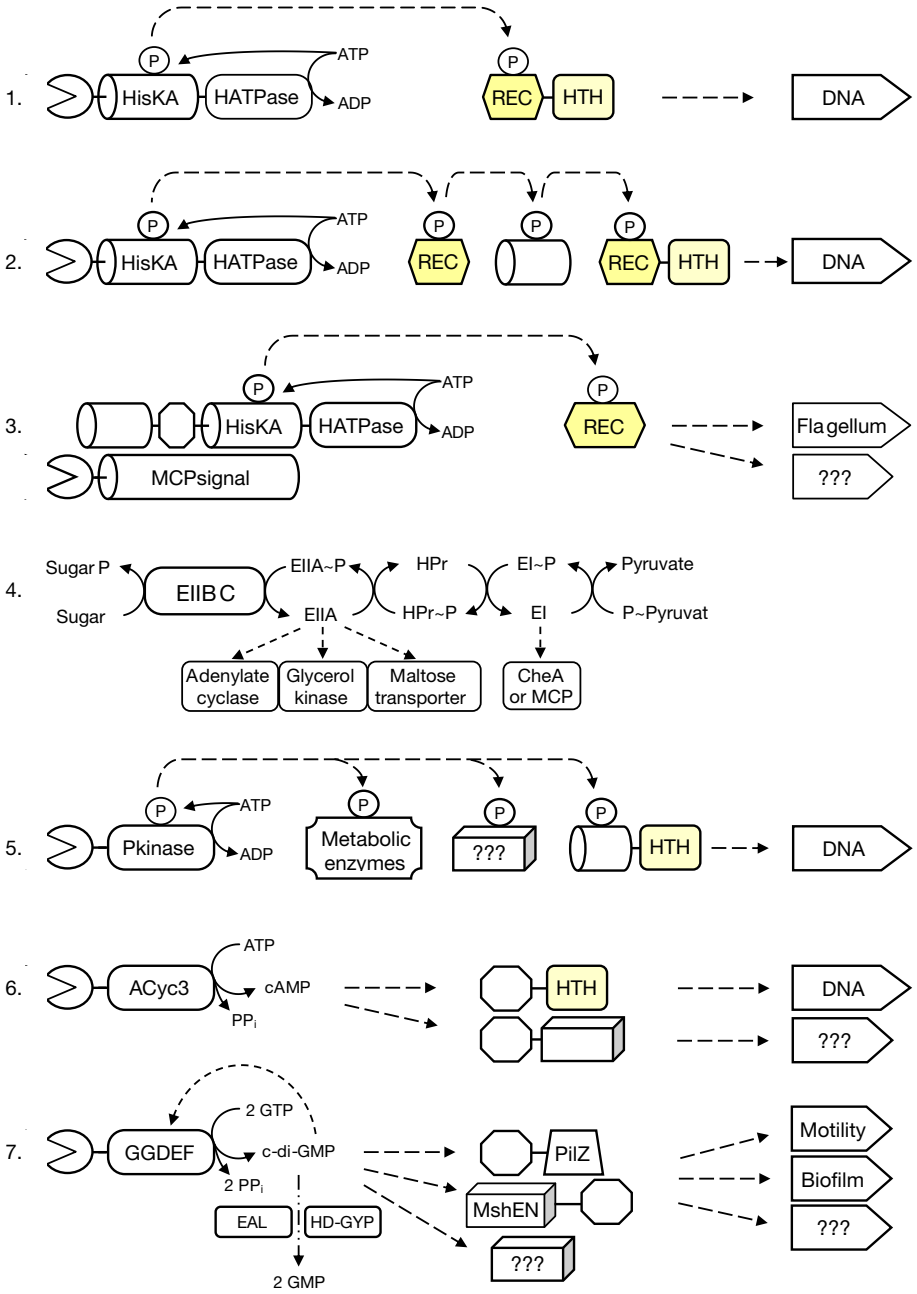

Fig. 2 Signal transduction pathways of the principal classes of bacterial receptors. (1) Transcriptional regulation by two-component signal transduction systems usually includes a sensor histidine kinase and response regulator that consists of the phosphoryl-accepting receiver (REC) domain and a DNA-binding helix-turn-helix (HTH) domain. (2) Signal transduction in the *Bacillus subtilis* sporulation machinery includes a histidine kinase (KinA, KinB, KinC, KinD, or KinE), response regulator (Spo0F) with a stand-alone REC domain, intermediate phosphorelay component (Spo0B) and transcriptional response regulator Spo0A that consists of REC and Spo0A-specific HTH domains. (3) Signal transduction in the chemotactic machinery starts from a sensor (methyl-accepting chemotaxis protein, MCP) that interacts with the histidine kinase CheA, which phosphorylates CheY. Phosphorylated CheY interacts with the FliM protein at the base of the flagellum and with still unknown targets in other systems. (4) In *E. coli*, signal transduction in the phospho*enol*pyruvate-dependent sugar:phosphotransferase system (PTS) involves protein–protein interactions involving the nonphosphorylated forms of PTS components EI and EIIA. EIIA interacts with adenylate cyclase, glycerol kinase, maltose transporter and some other proteins, while EI can interact with the chemotaxis histidine kinase CheA. In *B. subtilis*, the nonphosphorylated form of EI interacts with one of the MCPs. (5) Signal transduction from Ser/Thr/Tyr protein kinases involves direct or indirect phosphorylation of various (mostly unknown) targets. Known targets of Ser/Thr/Tyr protein phosphorylation include metabolic (e.g., glycolytic) enzymes and transcriptional regulators. (6) Signal transduction from sensor adenylate cyclases involves interaction of the cAMP with specific cNMP-binding domains, found in the transcriptional regulator CRP and in a few other proteins. The cAMP-CRP complex binds to the chromosomal DNA and activates transcription from a variety of bacterial promotors. There are also other targets of cAMP action. (7) Signal transduction from sensor diguanylate cyclases involves interaction of c-di-GMP with proteins that contain PilZ, MshEN or other c-di-GMP-binding domains. c-di-GMP hydrolysis is catalyzed by c-di-GMP-specific phosphodiesterases, containing either EAL or HD-GYP domains. In addition, c-di-GMP serves as an allosteric regulator of its own production. Domain names are as in Tables 3 and 4. Domain architectures are simplified for clarity and domain sizes are not drawn to scale.

of flagellar rotation. In archaea, whose flagella (archaella) are unrelated to the bacterial ones, there is no FliM homolog and Che∼P binds to the archaeal-specific adaptor CheF. In bacteria that move by type IV pili or utilize other nonflagellar means of motility, such as gliding, the targets for CheY∼P binding remain obscure but likely include specific motor proteins. Recent studies revealed that signaling components of certain MCPs reach beyond chemotaxis. For example, in *M. xanthus*, an MCP has been shown to participate in regulation of developmental gene expression, whereas in *P. aeruginosa*, methyl-accepting protein WspA turned out to be part of a chemosensory system that regulates biofilm formation.

Components of the PEP-dependent sugar:phosphotransferase system (PTS) participate in uptake of sugars, coupled with their phosphorylation, and are rarely considered to be part of a signal transduction machinery. Yet, two members of the PTS phosphorelay play key roles in signal transduction. The phosphorylation level of the PTS enzyme I (EI) directly affects the chemotaxis machinery, whereas the phosphorylation level of the enzyme IIA^{Glc} ($EIIA^{Glc}$) modulates the activity of certain membrane transporters and such enzymes as the adenylate cyclase, at least in *Enterobacteriaceae*.

Ser/Thr/Tyr protein kinases phosphorylate Ser, Thr, and/or Tyr residues in various cellular proteins. Analyses of cell-wide protein phosphorylation revealed a wide variety of phosphopeptides coming from numerous cellular proteins. However, only a small fraction of them appear to be true targets of regulation by Ser/Thr/Tyr protein kinases. These include transcriptional regulators (such as actinobacterial AfsR) and metabolic enzymes (e.g., phosphofructokinase). Ser/Thr/Tyr protein phosphatases reverse the effect of Ser/Thr/Tyr protein kinases by dephosphorylating these target proteins or, in some cases, the protein kinases themselves. Most of these enzymes are in the cytoplasm and only a few are membrane-bound.

Adenylate cyclases convert ATP to cyclic adenosine 3′,5′-monophosphate (cAMP), a key cellular second messenger. There are several different classes of adenylate cyclases, of which only class III enzymes have been shown to function as transmembrane receptors. Cyclic AMP participates in at least two different signaling pathways, both mediated by its interaction with a specific (cNMP-binding) domain of the target proteins. In bacteria, cAMP typically binds to a cAMP receptor protein (CRP, also known as catabolite activator protein, CAP). Cyclic AMP binding triggers a conformational change in CRP that increases its affinity to regulatory DNA sequences and allows it to interact with the RNA polymerase and activate transcription from certain weak promotors. This way, activation of adenylate cyclase(s) stimulates transcription of certain genes (operons) that are poorly expressed in the absence of the cAMP-CRP complex. This regulatory mechanism is sometimes referred to as catabolite repression, although the classical catabolite repression (or glucose effect) is a sum of several independently acting mechanisms. The second pathway of cAMP-mediated signaling is an allosteric regulation of the activity of certain target proteins. In bacteria, it can be mediated by cAMP-binding to a GAF domain of multi-domain signal-transduction proteins. In eukaryotes, this pathway involves cAMP binding to specialized CRP-like binding domains on protein kinases, calcium channels, and nucleotide exchange factors for small GTPases.

Diguanylate cyclases produce another second messenger, 3′,5′-cyclic diguanylic acid (c-di-GMP), which regulates a variety of functions related to the cell surface such as motility, secretion of proteins and polysaccharides, biofilm formation, and production of virulence factors. Some of the c-di-GMP functions are mediated by its binding to dedicated receptors, such as PilZ and MshEN domains, while other functions are regulated through other binding proteins, including diguanylate cyclases themselves and their inactivated variants. Two different types of c-di-GMP-specific phosphodiesterases which catalyze c-di-GMP hydrolysis contain either EAL or HD-GYP domains; their inactivated variants may also function as c-di-GMP-binding protein.

Importantly, these signaling pathways are not entirely separate. In many organisms, there exists a cross-talk between components of different signaling pathways. For example, a significant number of response regulators have adenylate or diguanylate cyclases or c-di-GMP-specific phosphodiesterases as their output (effector) domains, which allows histidine kinase-mediated pathways to affect the cAMP or c-di-GMP levels.

Structural Organization of Signal Transduction Proteins

A key aspect of the bacterial signal transduction machinery is its modular structure. Many signal transduction proteins consist of several distinct protein domains that can be shuffled almost like Lego blocks, resulting in an amazing diversity of proteins.

Sensory Domains

Sensory (signal input) domains of bacterial receptors are diverse and reflect the multiplicity of the signals they perceive. Nevertheless, sequence similarities between sensory domains of different receptors from distantly related organisms allows identification of distinct domain families. Members of the same domain family typically recognize the same—or closely related—ligands. Therefore, characterization of a sensory domain in one organism can be used as a basis for functional annotation of all proteins that contain the same domain. Some sensory domains have a rather narrow ligand specificity, for example, the citrate-binding domain found in the citrate-sensing histidine kinase CitA (DpiB) of *E. coli*. Other sensory domains, such as the periplasmic domain of the *E. coli* dicarboxylate sensor DcuS, which is similar to CitA, bind a number of related molecules such as several C4-dicarboxylates and citrate. For many other domains, the potential ligands—and hence the nature of the sensed molecules—remain unknown. Their potential participation in environmental sensing is deduced primarily from their presence at the N-termini of likely transmembrane receptors.

While most characterized sensory domains are extracellular (periplasmic in Gram-negative bacteria), sequence analyses also identified a number of cytoplasmic and membrane-embedded sensors without extracellular sensing domains (Table 3). Many

Table 3 Examples of bacterial sensory domains

Domain name	*Length, aa*	*Pfam entry*[a]	*Structure*[b]	*Ligand specificity*
Periplasmic (extracellular)				
SBP_bac_3	~220	PF00497	1lag	Amino acids
NIT	~250	PF08376	4akk	Nitrate, nitrite
PASTA	~55	PF03793	1pyy	β-Lactams
PhoQ_sensor	~180	PF08918	1yax	Ca^{2+}
dCache	~240	PF02743, PF08269, PF14827	4jgo	Citrate, C4-dicarboxylates, other small molecules
sCache	~150	PF16736, PF17200, PF17201, PF17202, PF17203	4exo	pH, Small molecules
CHASE	~220	PF03924	3t4l	Unknown
CHASE2	~240	PF05226	n/a	Unknown
CHASE3	~130	PF05227	3va9	Unknown
CHASE4	~250	PF05228	n/a	Unknown
Membrane-embedded				
5TMR-LYT	~90	PF07694	5TM	Murein derivatives (?)[c]
7TMR-DISM	~200	PF07695	7TM	Unknown
MHYT	~200	PF03707	6TM	NO
MASE1	~280	PF05231	11TM	Redox status
MASE2	~170	PF05230	5TM	Unknown
MASE3	~160	n/a	7TM	Unknown
Cytoplasmic				
PAS	~100	PF00989	1wa9	Heme, flavin, others
GAF	~150	PF01590	1f5m	cGMP, other small molecules
BLUF	~90	PF04940	1x0p	FAD
Globin sensor	~150	PF00042	1or4	Heme (O_2, CO, NO)
HNOB	~170	PF07700	1xbn	Heme (NO)
Phytochrome	~180	PF00360	1u4h	Tetrapyrrole

[a]Pfam database (http://pfam.xfam.org/) provides short descriptions of these domains and links to the underlying literature. As an example, the entry page for the domain PF00497 can be found at http://pfam.xfam.org/family/PF00497.

[b]Protein DataBank (PDB, http://www.rcsb.org/pdb/) entry, where available, or the number of transmembrane segments; n/a, not available. Additional structure entries may be listed in the respective Pfam entries. References for the structural entries can be found on the PDB web site (e.g., https://www.rcsb.org/structure/1lag) on the NCBI web site (e.g., https://www.ncbi.nlm.nih.gov/Structure/pdb/1lag).

[c]The question mark signifies unverified prediction.

sensory domains are detected in more than one receptor type (see below), which is how they often have been identified as sensory domains in the first place. Indeed, if the same periplasmic or membrane-embedded domain is found in two or more different classes of receptors, for example, in histidine kinases and diguanylate cyclases, there is little doubt that this domain serves as a sensor involved in recognition of some ligand(s).

Unfortunately, identification of sensory domains through sequence analysis rarely gives insights into the functions of these domains. Even when such insight is possible, the predicted function must be considered tentative until experimentally verified. For example, *Vibrio cholerae* protein VC0303 (GenBank accession number AAF93477) belongs to a large family of histidine kinases that contain membrane-embedded N-terminal domains, which are followed by PAS, histidine kinase, and REC domains. The N-terminal membrane domain of VC0303 homologs consists of 12 predicted transmembrane α-helices and is similar to Na^+/proline and Na^+/pantothenate symporters (PutP and PanF, respectively). This observation has led to the suggestion that these proteins serve as sensors for the Na^+ gradient. This suggestion has not been experimentally verified, and members of this family are currently annotated in the protein databases either as sensor histidine kinases (which is correct) or as Na^+/proline symporters (which is most likely incorrect).

Although the majority of signal transduction pathways respond to environmental cues and employ extracellular (periplasmic) sensor domains, a substantial fraction of bacterial receptors are cytoplasmic without detectable membrane segments. The enzymatic activities of such receptors, which include the *E. coli* nitrate sensor NtrB and *B. subtilis* sporulation regulators KinA, KinB, and KinC, are apparently modulated by intracellular, rather than extracellular, cues. Indeed, many of them contain N-terminal cytoplasmic sensor domains, such as PAS, GAF, or globin sensor domain. The first two of these domains, PAS and GAF, have similar structures, characterized by ligand-binding pocket that can accommodate various small molecules as ligands such as heme, flavin (PAS), cAMP, cGMP (GAF), and others. PAS and GAF domains are often found on the cytoplasmically-located fragments of multidomain bacterial receptors that also contain extracellular sensory domains. For the globin sensor domain, heme is the only known ligand. Heme-containing domains can be sensors of oxygen, carbon monoxide, and NO. In each case, binding of oxygen, CO or NO triggers a significant conformational change that affects interaction of the sensor domain with downstream domains. Thus, the signal generated by oxygen, CO or NO binding is transmitted to the C-terminally located effector domains. This allows PAS-containing

receptors to serve as sensors for the cellular energy state (e.g., *B. subtilis* sporulation-regulating histidine kinase KinA), its redox state (*E. coli* aerotaxis receptor Aer) or oxygen level (*E. coli* c-di-GMP phosphodiesterase Dos). Many receptors display multiple sensor domains, for example, *B. subtilis* KinA with three PAS domains, whose combined mode of action and hierarchy, if any, are unknown.

Of note, not every N-terminal periplasmic or integral membrane domain of a bacterial sensor protein is necessarily involved in signaling. For example, the histidine kinase UhpB, which regulates transport and metabolism of glucose-6-phosphate and related sugars, has a membrane-embedded N-terminal domain that does not bind extracellular glucose-6-phosphate. Instead, this domain interacts with UhpC protein, which is closely related to sugar phosphate transport proteins but actually works as the true sensor of glucose-6-phosphate in the UhpB-UhpC complex. In the *E. coli* histidine kinase KdpD, which regulates membrane transport of K^+ ions, the function of the membrane segment appears to be limited to anchoring the enzyme molecule in the membrane and ensuring proper interaction between its domains in the cytoplasm, the N-terminal turgor-sensing domain and the C-terminal histidine kinase domain. Thus, for many putative sensory domains, the current functional prediction should be considered at best an educated guess. The nature of the signal(s) that they are sensing needs to be explored in the future.

Structural Organization of Two-component Signal Transduction Systems

Histidine kinases

Histidine kinases are the most numerous and most diverse cellular receptors that control the greatest variety of responses. Almost all of the diversity of histidine kinases arises from the sensory (signal input) domains which can be periplasmic, membrane-embedded or cytoplasmic. A single histidine kinase may contain several sensory domains, for example a periplasmic ligand-binding domain and one or more PAS domains in the cytoplasm (Fig. 2). By contrast, cytoplasmic signal transduction modules of histidine kinases are rather uniform. Analysis of sequence similarities between different histidine kinases by Parkinson and Kofoid in 1992 revealed five conserved sequence motifs, referred to as H, N, G1, F, and G2 boxes. The first of them corresponds to the sequence motif around the conserved phosphoryl-accepting histidine residue in the dimerization and histidine phosphotransfer domain (DHp or HisKA domain in Pfam, Table 4), other boxes correspond to the residues in the ATPase domain (HATPase_c domain in Pfam, see Table 4) that are involved in ATP binding. These motifs are widely used in descriptions of histidine kinases. However, the diagnostic value of these motifs is somewhat restricted by discovery of proteins that lack one or more of these boxes but nevertheless function as histidine kinases, such as *P. aeruginosa* AlgZ/FimS, a sensor protein controlling alginate biosynthesis regulator AlgR, or *Clostridium perfringens* VirS, a sensor protein for the virulence regulator VirR. Cytoplasmic modules of histidine kinases are usually homodimers with each monomer containing both HisKA and HATPase domains (sometimes there are one or more additional domains). The HATPase domain binds ATP and transfers its γ-phosphate to a conserved histidine residue in the H-box of the HisKA domain, either on the same or the other protein chain in the homodimer (cis- or trans-phosphorylation, respectively). The catalytic HATPase domain is always located C-terminally of the HisKA domain.

Several variants of HisKA domains exist, which, despite having similar structures, share limited sequence similarity. The Pfam database currently assigns the HisKA domain the status of a domain group, or a clan, and divides it into four separate domain families, HisKA (PF00512), HisKA_2 (PF07568), HisKA_3 (PF07730), and HWE_HK (PF07536).

In addition to the sensor, HisKA, and HATPase domains, certain histidine kinases contain additional distinct domains. In transmembrane receptors, extracellular (or more precisely, extracytoplasmic) sensor domains are connected to the intracellular signal transduction modules by one or more transmembrane segments and, sometimes, the cytoplasmic helical linker (HAMP) module (Table 4). In addition, some histidine kinases, referred to as hybrid histidine kinases, contain an additional C-terminal phosphoacceptor (receiver, REC) domain with a conserved aspartate residue which is also a part of the signal transduction phosphorelay. Another phosphoacceptor protein domain, histidine phosphotransfer (HPt) domain, has a four-helix-bundle structure, similar to that of HisKA, and similarly contains a conserved histidine residue. The HPt domain can be found at the N-terminus of the protein (e.g., in the chemotaxis histidine kinase CheA), in the middle of it, or at its very C-terminus, see Fig. 2 for examples.

Response regulators

Response regulators of the two-component signal transduction systems are diverse proteins that share the common phosphoacceptor REC domain (often referred to as CheY or CheY-like domain, after its best-known representative). This domain is enzymatically active: it catalyzes both the phosphotransfer from the histidine residue of the HisKA domain to its own conserved aspartate residue, and also its own dephosphorylation. The ratio of these activities of the REC domain of each specific response regulator determines the half-life of the phosphorylated form (CheY∼P or, more generally, REC∼P) and hence, the fraction of the response regulator molecules that are in the active (phosphorylated) conformation at any given time.

While about 85% of response regulators combine the REC domain with some kind of a signal output domain, approximately 15% represent stand-alone REC domains. This group includes two well-studied proteins, the chemotaxis response regulator CheY of *E. coli* and *S. enterica* and the sporulation regulator Spo0F of *B. subtilis*. Despite obvious sequence and structural similarities and participation in similar phosphorylation-dephosphorylation cycles, these two proteins have strikingly different modes of action. Signal transduction through the first of them, CheY, relies exclusively on protein–protein interactions. Phosphorylation shifts the CheY molecule into a conformation with increased affinity to its target molecule FliM in the flagellar basal body. Thus, phosphoryled CheY shifts the equilibrium of the two forms of FliM resulting in a reversal of flagellar rotation, which is reflected in altered motility patterns. In contrast, Spo0F accepts the phosphoryl group from the sporulation histidine kinase KinA and, in turn, serves as

Table 4 Properties of the signal transduction domains

Signaling system, domain name	*Length, aa*	*Pfam entry*[a]	*Structure*[b]	*Protein name, function, comments*
Two-component signal transduction				
HATPase	~120	PF02518, PF14501	1bxd	Histidine kinase ATPase domain, phosphorylates the HisKA domain.
HisKA	~120	PF00512, PF06580, PF07536, PF07568, PF07730	1joy	Histidine kinase phosphoacceptor domain, may have a phosphatase activity toward P~REC. Upon dimerization, forms a four-helix bundle. In Pfam, divided into several distinct variants: HisKA, HisKA_2, HisKA_3, and HWE_HK
HAMP	~50	PF00672	2asw	Signal transfer domain. Upon dimerization, forms a four-helix bundle
HPt	~100	PF01627	1tqg	Histidine-containing phosphotransfer domain. Upon dimerization, forms a four-helix bundle
REC	~120	PF00072	2che	Phosphoacceptor domain, dephosphorylates HisKA domains and transfers phosphate to its own Asp residue
Chemotaxis				
MCP signal	~350	PF00015	1qu7	Methyl-accepting chemotaxis protein (MCP), dimerization domain. Upon dimerization, forms a four-helix bundle. Can be reversibly methylated on Glu residues
HAMP	~50	PF00672	2asw	Signal transfer domain. Upon dimerization, forms a four-helix bundle
H_kinase_dim	~67	PF02895	1b3q	Helical bundle domain in chemotaxis histidine kinase CheA
CheW	~140	PF01584		MCP-interacting protein or domain in CheA and CheV-type response regulators
CheB_methylest	~180	PF01339	1chd	MCP-glutamate methylesterase, demethylates the MCPs
CheR	~190	PF01739	1af7	SAdoMet:MCP methyltransferase, methylates the MCPs
Sugar:phosphotransferase system (PTS)				
EI	~570	PF05524 + PF00391 + PF02896	2hwg	PTS enzyme I, catalyzes phosphoenolpyruvate-dependent autophosphorylation and phosphoryl transfer to HPr
PTS-HPr	~90	PF00381	1ptf	Phosphocarrier protein domain
PTS_EIIA_1	~170	PF00358	1gld	PTS enzyme II A protein or domain, accepts phosphoryl residue from HPr and transfers it to EIIB
PTS_EIIB	~80	PF00367	1iba	PTS enzyme II B domain, accepts phosphoryl residue from EIIA and serves as a donor for the sugar substrate
PTS_EIIC	~320	PF02378	3qnq	PTS enzyme II C domain, catalyzes phosphoryl transfer from EIIB to the sugar substrate
Ser/Thr/Tyr protein phosphorylation				
Pkinase	~250	PF00069, PF01163, PF03109	1jwh	Ser/Thr/Tyr protein kinase, autophosphorylates at the expense of ATP and phosphorylates Ser, Thr, or Tyr residues in various target proteins
PP2C	~250	PF00481, PF07228, PF13672	1a6q	Ser/Thr/Tyr protein phosphatase, dehosphorylates Ser, Thr, and/or Tyr residues in protein kinases and other target proteins
cAMP-mediated signaling				
Adenylate_cycl	~600	PF01295	N/A	Adenylate cyclase class I, found in gamma- and deltaproteobacteria, produces cAMP from ATP
Guanylate_cyc	~190	PF00211	1y11	Adenylate cyclase class III, produces cAMP from ATP
cNMPbind	~90	PF00027	1hw5	cAMP/cGMP binding domain
CRP_HTH		PF00325	1hw5	Interaction with the target sites on the DNA
c-di-GMP-mediated signaling				
GGDEF	~180	PF01590	1w25	Diguanylate cyclase, catalyzes c-di-GMP synthesis, inactivated domains serve as allosteric regulators
EAL	~250	PF00563	2bas	c-di-GMP-specific phosphodiesterase, hydrolyzes c-di-GMP, inactivated domains serve as allosteric regulators
HD-GYP	~180	PF01966 + PF13487	4r8z	c-di-GMP-specific phosphodiesterase, hydrolyzes c-di-GMP
PilZ	~100	PF07238	1ywu	c-di-GMP receptor domain
MshEN	~60	PF05157	5htl	c-di-GMP receptor domain
c-di-AMP-mediated signaling				
DisA_N	~120	PF02457	3c1y	Diadenylate cyclase, synthesizes *c*-di-AMP
DHH, DHHA1	~180	PF01368 + PF02272	5xsn	*c*-di-AMP-specific phosphodiesterase, hydrolyzes *c*-di-AMP
7TMR-HD	~220	PF07698	4s1b	*c*-di-AMP-specific phosphodiesterase, hydrolyzes *c*-di-AMP
CdAMP_rec	~110	PF06153	4rww	*c*-di-AMP receptor domain
TrkA_C	~70	PF02080	4xtt	*c*-di-AMP receptor domain

[a]Pfam database (http://pfam.xfam.org/) provides short descriptions of these domains and links to the underlying literature. As an example, the entry page for the domain PF02518 can be found at http://pfam.xfam.org/family/PF02518. Alternative Pfam entries for related domains are separated by a comma. The plus sign indicates several Pfam domains that together form a protein entry.

[b]Protein DataBank (PDB, http://www.rcsb.org/pdb/) entry; *N/A*, not available. Additional structure entries may be listed in the respective Pfam entries. References for the structural entries can be found on the PDB web site (e.g., https://www.rcsb.org/structure/1bxd) on the NCBI web site (e.g., https://www.ncbi.nlm.nih.gov/Structure/pdb/1bxd).

a phosphoryl donor for Spo0B, which further transfers the phosphate to the sporulation response regulator Spo0A. Given the existence of phosphatases that specifically dephosphorylate Spo0F∼P, Spo0F appears to serve as a sporulation checkpoint. According to the Response Regulator Census (see Table 1), many bacterial genomes encode multiple response regulators, among them many stand-alone REC domains (for example, there are 42 such genes in *M. xanthus* and 38 in *Solibacter usitatus*). In many archaea, stand-alone REC domains comprise the majority of response regulators. It is likely that some of them, like CheY, transmit the signal through protein–protein interactions, whereas others, like Spo0F, participate in phosphorelays.

With the exception of members of the CheY/Spo0F protein family, all other response regulators are two-domain (or even three-domain) proteins that combine the REC domain with a signal output domain, which is usually located at the C-terminus of the polypeptide chain. Most of these proteins are transcriptional regulators that activate or repress transcription of specific target genes. Accordingly, the most common output domains bind DNA or RNA, although some response regulators have enzymatic or ligand-binding output domains.

The most common DNA-binding response regulators belong to the OmpR/PhoB family and have a winged helix-turn-helix (wHTH) DNA-binding domain (Table 5). The second in abundance is the NarL/FixJ family of response regulators, which have a typical helix-turn-helix (HTH) DNA-binding domain. Less common DNA-binding response regulators contain DNA-binding domains of the Fis type (NtrC and ActR/PrrA families), AraC type (YesN family), LytTR type (LytR/AgrA family), or Spo0A_C type (Spo0A family), among others. There are families of response regulators that are found just in one or two instances in the current databases. In each case, phosphorylation of the REC domain causes changes in its conformation that lead to the formation of a interactive surface (the α4-β5-α5 interface) and also favors its dimerization. Specifically, phosphorylation of the Asp residue (Asp57 in CheY of *E. coli*) causes reorientation of a conserved Ser/Thr residue (Thr87 in CheY of *E. coli*) that forms a hydrogen bond with a phosphate oxygen. In addition, reorientation of a Phe/Tyr residue (Tyr106 in CheY of *E. coli*) positions it between the active site and the α4-β5-α5 interface. Dimerization of response regulators is a key mechanism of the transcriptional regulation by two-component systems, as these dimers have a high affinity to the tandem (or palindromic) transcriptional regulator binding sites on the chromosome.

Within each family of response regulators, the signal specificity is determined by the interaction of the REC domains with their cognate histidine kinases and of the HTH domains with recognition sequences on the DNA. As a result, transcriptional regulators with dramatically different biological functions (e.g., OmpR and PhoB) can possess rather similar sequences. Therefore, sequence-based assignments of biological function are often unreliable, despite confident recognition of the DNA-binding output domains in most response regulators.

Table 5 Signal output domains in bacterial response regulators[a]

Regulator family	*Output domain*	*Length, aa*	*Pfam entry*[a]	*Structure*[b]	*% Total*[c]	*Protein function*
Stand-alone						
CheY/Spo0F	None	∼120	PF00072	2che	16.8%	Protein–protein interaction, phosphorelay
DNA-binding						
OmpR/PhoB	wHTH	∼120	PF00486	1bl0	29.6%	Transcriptional regulation
NarL/FixJ	HTH	∼120	PF00196	1rnl	16.5%	Transcriptional regulation
LytR/AgrA	LytTR	∼110	PF04397	3bs1	2.9%	Transcriptional regulation
ActR/PrrA	Fis (HTH_8)	∼50	PF02954	3fis	1.1%	Transcriptional regulation
AraC/YesN	HTH_AraC	∼140	PF00165	1bl0	0.7%	Transcriptional regulation
PhyR	RpoE	∼140	PB13556 PF08281	1or7	0.4%	Transcriptional regulation
Spo0A	Spo0A_C	∼120	PF08769	1fc3	0.2%	Transcriptional regulation
GlnL	YcbB	∼140	PF08664	n/a	0.2%	Transcriptional regulation
Three-domain DNA-binding						
NtrC	AAA, Fis (HTH_8)	∼230 ∼50	PF00158 + PF02954	1ojl, 3fis	9.7%	Transcriptional regulation
SARP	HTH, BTAD	∼220	PF00486 + PF03704	2fez	<0.1%	Transcriptional regulation
RNA-binding						
AmiR/NasR	ANTAR	∼55	PF03861	1qo0	1.0%	Regulation of transcription (anti)termination
CsrA	CsrA	∼60	PF02599	1vpz	<0.1%	Regulation of translation and mRNA decay
Enzymatic						
CheB	CheB	∼180	PF01339	1chd	2.6%	Methylesterase
FliY	CheC	∼200	PF04509	1squ	0.2%	Asp∼P phosphatase
Protein-binding						
CheV	CheW	∼140	PF01584	1k0s	1.4%	Protein–protein interaction
HDOD	HDOD	∼190	PF08668	1vqr	0.2%	Inactivated phosphodiesterase, unknown function

[a]This listing does not include GGDEF, EAL, HD-GYP, AC3, or PP2C output domains, listed in Table 4.

[b]Protein DataBank entry, if available; *n/a*, not available.

[c]Fraction of the response regulators from this family among 27327 response regulators encoded in complete genomes of 896 prokaryotic species, see Response Regulator Census, http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html.

In a significant fraction of response regulators, the output domains have an intrinsic enzymatic activity, such as the methylesterase domain in the chemotaxis response regulators of the CheB family. These output domains are typically the same as in environmental receptors: adenylate and diguanylate cyclases, *c*-di-GMP-specific phosphodiesterases, PPC2-type Ser/Thr protein phosphatases, and even histidine kinases. These are discussed in detail below. It is important to note, however, that such response regulators put other signaling mechanisms under the control of the two-component signal transduction. Indeed, a response regulator with an adenylate cyclase output domain may control cellular cAMP levels irrespective of the status of receptor adenylate cyclases. This could also be the case for control of c-di-GMP. The case of Ser/Thr protein phosphorylation is even more illuminating. Response regulators with the PPC2-type protein phosphatase domain far outnumber those with a protein kinase domain. The two-component signaling system apparently controls the level of Ser/Thr/Tyr protein phosphorylation primarily by regulating the rate of their dephosphorylation.

Certain response regulators consist of more than two domains. In transcriptional regulators of the NtrC family, the N-terminal REC domain and the C-terminal DNA-binding, Fis-like domain are separated by a central AAA-type ATP-binding domain, whose ATPase activity is required for precise DNA-binding. The response regulator VieA from *Vibrio cholerae* contains two output domains, a c-di-GMP-specific phosphodiesterase (EAL) and a DNA-binding HTH domain. In that case, the role of the HTH domain remains to be established.

In summary, bacterial response regulators contain a variety of output domains that put the histidine kinases at the top of signaling hierarchy, allowing the cell to control its metabolism and behavior in response to a wide array of conditions.

Alternative Signal Transduction Systems

Methyl-accepting chemotaxis proteins

Methyl-accepting chemotaxis proteins (MCPs) contain a characteristic signal transduction domain (Pfam domain PF00015) that consists of two long antiparallel α-helices, connected via a hairpin at their base. This hairpin serves as a distinct signaling module and contains a characteristic, highly conserved sequence motif, which directly interacts with the chemotaxis histidine kinase CheA, modulating its activity. Thus, in the MCP-CheA assembly, the whole MCP can be considered a sensor domain of CheA. Despite giving the MCP its name, methylation itself does not seem to be a necessary part of the signal transduction mechanism. Rather, methylation (by CheR) and demethylation (by CheB) change the charge distribution along the α-helices and affect the packing of these α-helices against each other, which, in turn, is reflected in the interaction between MCP and CheA. This adds yet another regulatory circuit to the chemotaxis signaling machinery which allows the cells to carry out sensory adaptation after a chemotactic response.

Phosphotransferase system

The phospho*enol*pyruvate-dependent sugar:phosphotransferase system (PTS) catalyzes uptake of certain sugars, coupling substrate transport with phosphorylation. In addition to its transport function, the PTS is an important component of the signaling machinery that controls chemotaxis to its sugar substrates. Like histidine kinases, PTS proteins are phosphorylated on histidine residues. However, in contrast to the ATP-His-Asp or ATP-His-Asp-His-Asp phosphorelay, typical for the two-component signaling, the PTS phosphorelay starts from phosphoenolpyruvate (PEP) and only proceeds via His residues, at least until the final step. The high free energy of PEP hydrolysis ensures that in the absence of their carbohydrate substrates all PTS components are phosphorylated. The limiting step in the phosphorelay appears to be PEP-dependent autophosphorylation of the first component, EI. Therefore, in the presence of carbohydrates, phosphoryl flow through the PTS components occurs at a higher rate than rephosphorylation of EI by PEP. As a result, EI, HPr and EIIA components become partly dephosphorylated. This serves as a signal both for the chemotaxis machinery and for the *E. coli* adenylate cyclase. Although any direct interaction between PTS components and MCP or CheA remains to be demonstrated, the data suggest that unphosphorylated EI might interact with one or more MCPs (in *B. subtilis*) or with CheA (in *E. coli*), modulating the CheA activity and, hence, the cellular level of CheY∼P. The second mechanism of signal transduction from the PTS involves $EIIA^{Glc}$. This protein has been shown to interact with the adenylate cyclase and other targets, including lactose permease, typically inhibiting their activities. Interaction of $EIIA^{Glc}$ with the adenylate cyclase inhibits cAMP production and decreases transcription of cAMP-dependent (so-called catabolite sensitive) operons. This effect is often referred to as catabolite repression (see below). Interaction of $EIIA^{Glc}$ with lactose permease inhibits lactose uptake, diminishing the cellular levels of allolactose, the inducer of the lactose operon. This effect is referred to as inducer exclusion. These two effects of $EIIA^{Glc}$ combine to prevent utilization of certain sugars (such as lactose in *E. coli*) in the presence of a preferred sugar (such as glucose in *E. coli*). Inducer exclusion can be observed in many PTS-encoding organisms that lack the adenylate cyclase, such as *B. subtilis*. In some organisms, PTS-mediated signaling includes additional control mechanisms, such as reversible phosphorylation of the HPr protein on its Ser residue, which affects the ability of this protein to participate in the PTS phosphorelay.

Ser/Thr/Tyr protein kinases and protein phosphatases

Reversible protein phosphorylation on serine, threonine, or tyrosine residues is a key regulatory mechanism in eukaryotic cells. In the past several years, Ser/Thr/Tyr protein kinases have been recognized in a variety of bacterial and archaeal cells as well but nevertheless are often referred to as "eukaryotic-type" protein kinases. Ser/Thr/Tyr protein kinases are encoded in multiple copies in many bacteria, particularly in *Actinobacteria* and *Cyanobacteria*; the genomes of *M. xanthus* and *Sorangium cellulosum* encode more

than 100 of them, almost as many as histidine kinases. In many archaea, Ser/Thr/Tyr protein kinases appear to be the principal type of receptors. Despite their abundance, Ser/Thr/Tyr protein kinases are still poorly studied; only a handful of their targets have been recognized and the importance of this kind of regulation often gets overlooked. A recent survey of phosphorylated proteins in *B. subtilis* suggested that most glycolytic enzymes, as well as several enzymes of the tricarboxylic acid cycle and the pentose phosphate pathway, are subject to phosphorylation on serine or threonine residues and, theoretically, could be subject of regulation by Ser/Thr/Tyr protein kinases. Given that *B. subtilis* genome encodes only four Ser/Thr/Tyr protein kinases, they might have rather wide substrate specificity.

Bacterial Ser/Thr/Tyr protein phosphatases are primarily of the PP2C-type. Cellular targets for most of them remain unknown, although some of them have been shown to reverse the action of their cognate Ser/Thr/Tyr protein kinases, possibly by dephosphorylating the kinases themselves.

Adenylate cyclases

Adenylate (adenylyl) cyclases, the enzymes that produce cAMP from ATP, exist in several unrelated variants, referred to as classes. Traditionally, the enzyme from *E. coli* is considered class I adenylate cyclase. This protein appears to be associated with the cell membrane but does not seem to directly sense any environmental signals. However, its activity is modulated by the $EIIA^{Glc}$ component of the glucose-specific phosphotransferase system (PTS). The phosphorylated form of $EIIA^{Glc}$ appears to activate adenylate cyclase, whereas the dephosphorylated form, which accumulates in the presence of extracellular glucose, does not activate adenylate cyclase and might even inhibit it. Thus, in the presence of glucose or other PTS sugars, adenylate cyclase activity decreases, leading to a drop in cAMP level. This is one of the mechanisms contributing to the phenomenon of catabolite repression.

Bacterial receptor-type adenylate cyclases belong to the so-called class III and were first described in 1996 in the cyanobacterium *Spirulina* (current name *Arthrospira*) *platensis*. Analysis of the gene sequence predicted that its product contains a signal peptide, a periplasmic sensor domain, a membrane-spanning domain and an adenylate cyclase-like catalytic domain. A similar gene from another cyanobacterium, *Anabaena* sp. PCC7120, was shown to complement a *cya*$^{-}$ mutant of *E. coli*, indicating that it encoded an active adenylate cyclase domain. Receptor-type adenylate cyclases were later found to play regulatory roles in a variety of bacteria, including *Mycobacterium tuberculosis*. Some of them are located in the cytoplasm, while others have extracytoplasmic or membrane-embedded sensor domains. One of them, the *P. aeruginosa* protein CyaB (PA3217), proved to be a key regulator of the expression of virulence factors, contributing to mouse infection by this bacterium. Ligands for these receptor adenylate cyclases have not yet been identified.

In eukaryotes, many class III adenylate cyclases possess limited substrate specificity and partially accept GTP as a substrate producing cGMP, a second messenger on its own that controls the activities of protein kinases, calcium channels, and other proteins. Similar enzymes, predominantly producing cGMP, have been reported in several bacteria, with cGMP apparently used as a second messenger for such developmental processes as cyst formation in *Rhodospirillum centenum* and biofilm formation in *Xanthomonas campestris*. The issue of bacterial cGMP signaling remains highly controversial, in particular because the intracellular concentration of ATP far exceeds that of GTP.

Diguanylate cyclases and c-di-GMP phosphodiesterases

A widespread group of bacterial membrane receptors includes proteins with GGDEF, EAL, and HD-GYP signal transduction domains that synthesize and hydrolyze the second messenger c-di-GMP. These proteins have been initially recognized as sensor proteins through computational analysis of bacterial genomes. Subsequent experimental studies revealed their role in regulating biofilm formation, development of flagellar apparatus, and a variety of other processes.

The GGDEF domain, so named after a conserved Gly-Gly-Asp/Glu-Glu-Phe sequence motif, is a diguanylate cyclase that synthesizes c-di-GMP from two molecules of GTP. This enzyme only works as a dimer with each monomer binding a GTP molecule (see Table 4). Hydrolysis of c-di-GMP is carried out by two different classes of c-di-GMP-specific phosphodiesterases, referred to as the EAL and HD-GYP domains, also named after their conserved sequence motifs. The HD-GYP domain (HD_5 in Pfam) is a member of the widespread HD phosphohydrolase domain family that contains an additional C-terminal subdomain.

Almost a half of GGDEF domains and two-thirds of all EAL domains are found in fused proteins that contain both GGDEF and EAL domains. The overall activity of these fusion proteins can be either synthesis or hydrolysis of c-di-GMP, as has been observed when these proteins were first characterized in 1998 by M. Benziman and colleagues. It appears that in many such fusion proteins, at least one of the domains is enzymatically inactive and serves as an allosteric regulator of the other one. In some cases, however, both domains appear to be active. While many GGDEF, EAL, and HD-GYP domains comprise the signal transduction modules of membrane-anchored environmental sensors, others are found in cytoplasmic proteins, including many response regulators.

Diadenylate cyclases

Cyclic diadenosine 3′-5′ monophosphate (c-di-GMP) is a recent addition to the list of bacterial second messengers. It was originally discovered as a molecule that signals the DNA integrity in *B. subtilis*, an important checkpoint before the cell enters the sporulation process. In non-spore-forming *Listeria* spp. and *Staphylococcus* spp., synthesis of c-di-GMP served as a signal of the cell wall stress and was essential for cell viability. Cyclic di-AMP appears to regulate the osmotic pressure inside the bacterial cell through its control of potassium ion transport. The c-di-GMP-producing diadenylate cyclase DisA_N domain is encoded in a variety of bacteria and archaea, suggesting that c-di-GMP might be the most widespread second messenger in the prokaryotic world.

Interaction of Signal Transduction Pathways

As mentioned above, different classes of bacterial receptors often contain similar sensory domains. This phenomenon is often observed between different groups of organisms. Thus, histidine kinases with CHASE4 sensor domain are found mostly in archaea, whereas diguanylate cyclases with CHASE4 domains are found exclusively in bacteria (see Table 6). This suggests that different organisms employ different signaling pathways and, ultimately, different mechanisms to respond to similar environmental cues. For example, *Thiomicrospira denitrificans* sensing extracellular nitrate by a NIT sensor histidine kinase (Table 6) would generate an intracellular response, transcriptional or otherwise, modulated by its cognate response regulator. *Shewanella amazonensis*, which encodes a NIT-coupled MCP, would respond to nitrate by chemotaxis, whereas *Chromobacterium violaceum*, encoding a NIT-coupled diguanylate cyclase, most likely reacts to nitrate by adjusting its motility speed and/or polysaccharide secretion. Thus, the diversity of signal transduction pathways ensures that different organisms respond to similar—or even identical—environmental signals with differentiated cellular responses. These responses may be particularly complex in free-living organisms with large genomes, which encode a large number of signal transduction proteins. In such organisms, diverse receptors with common sensory domains often coexist, allowing a sophisticated multilevel response to environmental cues. For example, the genome of *Anabaena* sp. PCC 7120 encodes 13 proteins with the periplasmic CHASE2 domain, including four histidine kinases (All1178, All3347, All5309, and Alr5151), two Ser/Thr/Tyr protein kinases (All4838 and Alr1869), an adenylate cyclase (All1118), and a diguanylate cyclase (All1219). Although the environmental signal sensed by the CHASE2 domain has not been identified, there are certain indications that it might be osmolarity or a related parameter.

Another mechanism of cross-talk between signaling pathways involves response regulators with enzymatic output domains. For example, signals sensed by the *P. aeruginosa* methyl-accepting protein WspA are transmitted through the hybrid histidine kinase WspE to the response regulator WspR that has a diguanylate cyclase (GGDEF) output domain and regulates biofilm formation. This way, signals from receptors such as MCPs or histidine kinases may end regulating a variety of diverse processes.

In addition, genome analysis reveals numerous cases of signaling proteins that combine different kinds of signal transduction domains, for example, an adenylate cyclase with a Ser/Thr/Tyr protein kinase. Such proteins are difficult to annotate, let alone figure out their cellular functions. Nevertheless, preservation of such multidomain proteins through millions of years of evolution indicates that they do have a function, at least in some environmental conditions. It is reasonable to suggest that the extreme diversity of signal transduction proteins, generated through numerous combinations of core protein domains, reflects different paths of adaptation to their environment.

How Bacteria See the World

Despite many years of studies, our understanding of signal transduction pathways is still limited, even in the best-studied model organisms such as *E. coli* and *B. subtilis*. We still lack the complete understanding which environmental parameters these organisms sense, let alone why they choose these parameters and not others. However, the availability of complete genomes provides us with an opportunity to list the components of the signaling network and start analyzing and modeling their respective contribution to the regulation of gene expression and cellular behavior. Back in 1999, James Hoch and colleagues published a review on two-component signal transduction in *B. subtilis* (see Further Reading) entitled "How one organism sees its world." In the same fashion, it could be instructive to look at the genome of *E. coli* and see what receptors it uses to monitor the environment and the intracellular milieu. To the best of our knowledge, the signal transduction machinery of *E. coli* includes the following components:

- 30 histidine kinases with 34 response regulators
- 23 membrane components of the sugar PTS
- 12 diguanylate cyclases
- 10 c-di-GMP phosphodiesterases
- 7 proteins with both GGDEF and EAL domains
- 5 methyl-accepting chemotaxis proteins
- 2 predicted Ser/Thr protein kinases
- 1 class I adenylate cyclase

How much do we know about their functions? The signals sensed by each of the five MCPs have been experimentally determined:

- Tsr—serine
- Tar—aspartate, maltose
- Trg—ribose, galactose
- Tap—dipeptides, pyrimidine
- Aer—redox state of the respiratory chain

The last of these MCPs, Aer, is obviously important for sensing the presence of usable terminal electron acceptors, reflecting the choice between a respiratory and fermentative metabolism. The ligands sensed by the other four MCPs include amino acids, peptides, and sugars. However, it is hard to say why evolution has chosen these particular ligands over other amino acids and sugars.

Table 6 Diversity of the sensor–signal transduction domain combinations in bacterial receptors[a]

Domain name	*HisK*	*MCP signal*	*Pkinase*	*PP2C*	*Adenylate cyclase*	*GGDEF*	*GGDEF-EAL*	*EAL*	*HD-GYP*	*Other*
Extracellular										
SBP_bac_3	GSU2755	Taci_0133	SCO4911	EAY27325	–	SO_3700	PP_0386	CPE0914	TM_1170	SACE_4896
NIT	Tmden_1926	Sama_1713	EGD40999	–	*CG5719*	CV_3252	SO_0141	–	–	Bcen_5756
dCache	Gura_1422	PA4309	RB7058	DGl_2922	Lpg1322	Ddes_2370	VV0195	–	VVA0286	DVU_2637
sCache	Abu_0508	TM1428	azo0898	SU9_03106	DBW_1366	VVA0051	XCC2274	–	SYN_02721	SSEG_11338
CHASE	MXAN_6941	KIL41837	AKF10553	BAF29743	AAA33164	VVA0456	VV0017	–	AKF10553	AMC36677
CHASE2	PA4036	–	Ava_4764	Daro_1993	Lpg1739	DR_1633	Bll6124	Saro_2326	Daro_1897	Ialb_0285
CHASE3	DRA0205	GSU0683	–	FRAAL1878	BAA22996	GSU1937	All4897	–	–	Desku_3511
CHASE4	RL3120	Acpl_2764	–	AFZ04336	Sce0574	PA0847	GSU2511	–	Taci_1598	Gura_0265
Membrane-embedded										
5TMR-LYT	VV0692	Cbc4_1648	–	Swol_1793	–	DR_1090	SO_1500	–	Galf_0773	Jann_0472
7TMR-DISM	Gura_0744	TP0040	EDM76192	LBJ_0248	LBJ_0018	SO_1570	Bll1502	EAT12480	–	LBJ_2951
MHYT	Sden_3722	KYC37504	–	AFZ12724	Ppl_00592	Bcen_1563	PA1727	CEG22834	–	SPO2400
MASE1	MXAN_6855	–	–	Mmcs_3900	EIJ43105	Slr1798	PA1181	AHJ77119	Dde_0263	DVU3106
MASE2	Hch_06299	–	–	KOY85373	PA3217	STM0385	–	–	–	OAI51911
Cytoplasmic										
PAS	PA5124	Aer_Ecoli	*AAC27293*	BSU34110	Tery_3993	Lpg0155	BCE_0696	Arth_1906	DVU0129	PtsP_Ecoli
GAF	Ava_1719	GSU1704	Arth_0192	LB112	LA2834	SO_3759	Mflv_4377	–	VV2051	ABB34328

[a]The domain names are as in Tables 3 and 4. Each cell contains an example of a protein with the specified domain combination, listed by its gene name (locus tag) or accession number in the NCBI protein database. The names in italics indicate domain combinations found so far only in eukaryotes. Dashes indicate domain combinations not found in the NCBI protein database as of 31 December 2017.

For example, why serine and aspartate are more important for chemotaxis than glutamine and asparagine, which link carbon and nitrogen metabolism? Likewise, why maltose and galactose and not glucose or fructose? A partial answer to the second question is given by the listing of substrates of the sugar PTS, which serves as an MCP-independent mechanism of regulating chemotaxis towards sugars:

- FruA, FrvB, FrwC, HrsA, YpdG—fructose
- AscF, CelB—cellobiose (β-D-Glu-D-Glu)
- MtlA, CmtA—mannitol
- GatC, SgcC—galactitol
- AgaC/D, AgaW—*N*-acetylgalactosamine
- NagE—*N*-acetylglucosamine
- ManX/Y—mannose
- SrlA—sorbitol
- PtsG—glucose
- MalX—maltose (α-D-Glu-D-Glu)
- TreB—trehalose (α-D-Glu-D-Glu)
- GlvC—α-glucosides
- BglF—β-glucosides
- SgaB—ascorbate
- YfeV—*N*-acetylmuramic acid

Thus, *E. coli* carries in its genome genes encoding chemotaxis receptors for almost any commonly found monosaccharide and several disaccharides. Whether these genes are constitutively expressed at sufficient levels to contribute to the cell behavior remains an open question. It appears that at least for some of the PTS receptor genes need to be induced by the corresponding sugar.

Regarding transcriptional regulation, signals for 28 out of 30 histidine kinases have been characterized. These signals (ligands) are as follows:

- CheA—chemotaxis, perceives signals from MCPs
- AtoS—acetoacetate, histamine, spermidine
- BaeS, BasS, CpxA, EvgS, RcsC, RscD—envelope stress
- EnvZ, KdpD—osmotic stress, K^+ gradient
- PhoM, PhoQ, PhoR—phosphate (and/or Ca^{2+}, Mg^{2+})
- NarQ, NarX—nitrite/nitrate
- CusS, ZraS—heavy metals (Cu^+/Ag^+, Zn^{2+}/Pb^{2+})
- ArcB, BarA—redox stress, O_2, H_2O_2
- CitA—citrate
- DcuS—fumarate, C4-dicarboxylates
- UhpB—glucose-6-phosphate
- GlnL—glutamine
- TorS—trimethylamine oxide
- QseC—quorum sensing
- BtsS (formerly YehU)—amino acids, peptids
- GlrK (formerly YfhK)—epinephrine, sulfate
- YpdA—pyruvate

The signals for the remaining two histidine kinases, RstB and YedV, remain unknown. RstB is apparently activated by stress but the exact effector has not been identified. YedV, along with CusS, participates in copper homeostasis but binding of Cu^+ ions to the protein remains to be demonstrated. It is remarkable how many histidine kinases are sensing either envelope and osmotic stress or redox state of the cell and the availability of terminal electron acceptors. The fact that these histidine kinases coexist in the same cell suggests a certain degree of sophistication in their interactions, seen, for example, in the complex division of functions between NarQ and NarX. In most cases, however, the hierarchy between different sensors, if any, remains unknown.

Our current knowledge of the functions of 29 *E. coli* proteins with GGDEF and/or EAL domains that function as diguanylate cyclases and/or c-di-GMP-specific phosphodiesterases is more limited. The sensed ligand, oxygen (and potentially CO and NO), has been established for two of them, "direct oxygen sensors" DosP (formerly YddU) and DosC (formerly YddV). The diguanylate cyclase DgcZ (YdeH) senses zinc ions and BluF (YcgF) senses blue light. Five c-di-GMP phoshodiesterases, PdeB PdeC, PdeG, PdeN, and PdeT, contain the CSS-motif sensor domain that can exist in dithiol and disulfide forms and appears to sense the redox status of the periplasm. The phosphodiesterase PdeF (YfgF), which has the MASE1 periplasmic sensor domain, senses aspartate, and three other *E. coli* enzymes, diguanylate cyclases DgcE and DgcF and the phosphodiesterase PdeA, which all contain the same MASE1 sensor domain, could be predicted to sense aspartate as well (and/or a closely related molecule). The N-terminal GAF domain of the DgcP likely regulates the c-di-GMP synthase activity of its downstream GGDEF domain by binding cAMP or a similar ligand. Several other GGDEF and/or EAL domain proteins, such as DgcC (AdrA), DgcM, PdeR, and CsrD, have been shown to regulate, respectively,

cellulose biosynthesis, production of curli fimbriae, and carbon storage. RflP (YdiV) and PdeH (YhjH) regulate flagellar motility. For other GGDEF and/or EAL domain proteins (YcdT, YeaI, YeaJ, YedQ, YfiN, YhjK, YliF, YahA, and YliE), neither the sensed signal nor the regulated process are known at this time, although their overexpression leads to the expected changes in c-di-GMP levels, which affect motility and biofilm formation.

The situation with the two predicted Ser/Thr/Tyr protein kinases of *E. coli* is not much better. Although these proteins have been identified as members of the Ser/Thr protein kinase superfamily and have all the key active site residues intact, neither of them has been actually shown to function as a Ser/Thr protein kinase. One of them, UbiB, is required for a hydroxylation step in ubiquinone biosynthesis and was initially thought to function as 2-octaprenylphenol hydroxylase. However, this enzymatic activity has not been experimentally demonstrated. Instead, this activity has been demonstrated in a different protein, VisC, renamed UbiI. Thus, UbiB is probably not an enzyme of ubiquinone biosynthesis but a Ser/Thr protein kinase that regulates this process. The functions of the second predicted Ser/Thr protein kinase, YegI, remain unknown.

Summing up, there remain major puzzles even in signal transduction pathways of *E. coli*. For most other free-living bacteria, as well as for most archaea, understanding the signal transduction mechanisms and pathways will remain a challenge for years to come.

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Sexually Transmitted Diseases☆

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Glossary

Adnexa In this context, usually refers to the fallopian tubes and ovaries.
Ectopic pregnancy A pregnancy that occurs outside of the uterus, such as in a fallopian tube or ovary, often resulting in extensive damage to the affected organ.
Endometritis Inflammation of the inner lining of the uterus.
Epidemiology The study of the factors determining the frequency and distribution of disease in a community, and the relationships among those factors.
Epididymitis Sometimes painful inflammation of the epididymis, often resulting from infection.
Incidence The rate at which new cases of a disease occur in a community over a given time period.
Lymphangitis Inflammation of the lymph nodes and associated lymphatic ducts that occurs as a result of infection at a distal site.
Orchitis Testicular inflammation.
PID Pelvic inflammatory disease (PID) is a term referring to endometritis, an infection with inflammation in the fallopian tubes, tubo-ovarian abscess, peritonitis, or any combination of these findings.
Prevalence The total number of cases of a given disease in a community at a given time.

Abbreviations

AIDS	acquired immunodeficiency syndrome
CSF	cerebrospinal fluid
DFA	direct fluorescent antibody
DGI	disseminated gonococcal infection
EIA	enzyme immunoassay
FDA	Food and Drug Administration
HIV	human immunodeficiency virus
HPV	human papilloma virus
LGV	lymphogranuloma venereum
LOS	lipooligosaccharide
MSM	men who have sex with men
NAAT	nucleic acid amplification tests
NGU	nongonococcal urethritis
P&S	primary and secondary
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
RPR	rapid plasma reagin
STD	sexually transmitted diseases
TMA	transcription-mediated amplification
TNF	tumor necrosis factor
VDRL	Venereal Diseases Research Laboratory
WHO	World Health Organization

☆*Change History:* August 2014. KG Ghanem and TC Quinn have updated all epidemiology sections; gonorrhea diagnosis; gonorrhea treatment; chlamydia diagnosis; chlamydia treatment.

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Defining Statement

The term 'Sexually transmitted diseases (STDs)' refers to a large number of distinct infections, which are typically transmitted through sexual contact and result in a wide variety of illnesses. This article summarizes some important epidemiological trends among the more common STDs, and reviews the clinical syndromes, diagnosis, treatment, and pathogenesis of some select STDs.

Introduction

Over the past three decades, there have been tremendous advances in our understanding of sexually transmitted diseases (STDs). There has been recognition of new pathogens, new clinical syndromes, emerging antimicrobial resistance, the increasing importance of viral pathogens, and a better understanding of the epidemiology of many STDs, and progress has been made in the field of prevention including vaccine development. There are approximately 50 distinct clinical syndromes associated with more than 30 different sexually transmissible organisms. Table 1 summarizes some of the most important pathogens, the associated clinical syndromes, and potential complications.

Even apart from the HIV epidemic, STDs constitute a significant public health problem worldwide, despite improvements in diagnosis, treatment, and prevention strategies. STDs are seen in virtually every society, though developing nations and underserved populations experience the greatest burden of disease. According to 2008 World Health Organization (WHO) estimates, there were nearly 500 million new cases worldwide of curable STDs in adults aged 15–49 years. The global prevalence of infection with the agents of common viral STDs, such as genital herpes simplex virus, genital human papillomavirus, hepatitis B, and HIV, is estimated to be in the billions, as many individuals are infected with more than one of these pathogens. According to the WHO, in developing countries STDs and their complications comprise the top five disease categories for adults seeking medical care. In the United States, the Centers For Disease Control And Prevention (CDC) estimates that there are approximately 20 million new cases of STDs annually, nearly half of them occurring among young people aged 15–24 years. Of reportable infectious diseases in the United States, chlamydia and gonorrhea remain the top two most common diseases reported to the CDC. According to CDC surveillance data, there were 107.5 cases of gonorrhea per 100 000 people and 456.7 cases of chlamydia per 100 000 people in 2012. Both of these rates represented an increase from the previous year.

STDs are recognized as a source of tremendous social, health, and economic costs worldwide. There are many factors contributing to the disease costs exacted by STDs. Challenges to the control of STDs include limitations on the availability and quality of medical care, public education regarding safer sexual practices, access to condoms and other methods of prevention, convenient and affordable methods of diagnosis and treatment, the emergence of antibiotic resistance, and the social stigma associated with STDs. Furthermore, STDs are commonly asymptomatic in both women and men. All of these factors can result in a delay or complete lack of appropriate treatment, with implications for ongoing transmission of infection to sexual partners.

The largest share of the disease burden from STDs is a result of the complications and sequelae that may follow initial infection. When left untreated, infections can migrate upward from the lower reproductive tract and result in pelvic inflammatory disease (PID), chronic pelvic pain, infertility, ectopic pregnancy and tubo-ovarian abscess in women, and prostatitis, epididymitis, or orchitis in men. It has been estimated that the economic cost of PID alone exceeds $2 billion per year in the United States. In 2010, direct medical costs associated with STDs in the United States were estimated at up to $16 billion annually. Untreated infections in pregnant women may lead to fetal loss, stillbirths, low birth weight, and eye, lung, or neurologic damage in a newborn. The viral STDs are typically incurable and result in chronic disease, often associated with significant morbidity and mortality. The most obvious example of this is HIV infection and AIDS. Another example is human papilloma virus (HPV), some types of which are known to cause cervical cancer in women. Cervical cancer is the second leading cause of female cancer mortality worldwide.

In general, the major STDs pose a greater threat to women's health than to men's, for a number of reasons. Women are more likely to get an STD from a man than the other way round because STDs are generally more efficiently transmitted from men to women. Women are more likely to be asymptomatic than men, and thus women may be less likely to seek timely medical attention. STDs are often more difficult to accurately diagnose in women than in men because symptoms are less specific and diagnostic tests are less sensitive. Finally, the potential complications of STDs are more common and more serious in women.

Studies of the epidemiology of STDs have revealed several risk factors consistently associated with the acquisition of STDs. These risk factors appear to apply to most, if not all, populations. Risk factors include multiple sexual partners, urban residence, single marital status, and young age. The risk of STDs for young adults and adolescents has not been fully appreciated by the public and by policy makers. STD prevention efforts in young people have remained controversial in the United States despite the fact that approximately 3 million teenagers acquire an STD each year. The CDC estimates that one in four young women in the United States between the ages of 14 and 19 is infected with at least one of the most common STDs, namely HPV, chlamydia, or trichomoniasis. Young adults and adolescents are at greatest risk for acquiring an STD for a number of reasons, related to patterns of sexual behavior and other behaviors associated with STDs, such as substance abuse. There is some evidence that biological factors may also play a role in young women. Thus, any successful STD prevention program needs to specifically include this population, and multiple groups including the CDC recommend annual screening for chlamydia and gonorrhea at a minimum in all sexually active women under 26 years of age, whether or not they report symptoms. With the development of an effective

Table 1 Major sexually transmitted organisms and associated diseases

Agent	*Disease or syndrome*
Bacteria	
Neisseria gonorrhoeae	Urethritis, epididymitis, prostatitis, proctitis, bartholinitis, cervicitis, conjunctivitis, pharyngitis, PID and related sequelae (infertility and ectopic pregnancy), perihepatitis, complications of pregnancy (e.g., chorioamnionitis, premature rupture of membranes, premature delivery, and postpartum endometritis), DGI associated with bacteremia, endocarditis, arthritis
Chlamydia trachomatis	Urethritis, epididymitis, prostatitis, proctitis, bartholinitis, cervicitis, conjunctivitis, pharyngitis, PID and related sequelae (infertility, ectopic pregnancy), perihepatitis, complications of pregnancy (e.g., chorioamnionitis, premature rupture of membranes, premature delivery, and postpartum endometritis), LGV, proctocolitis (serovars L1, L2, or L3)[a]; Reiter's syndrome, infant pneumonia
Treponema pallidum	Syphilis[a] (see text for further discussion)
Haemophilus ducreyi	Chancroid[a]
Klebsiella granulomatis	Granuloma inguinale/donovanosis[a]
Mycoplasma hominis	Postpartum endometritis
Mycoplasma genitalium	Urethritis, cervicitis, endometritis, PID
Ureaplasma urealyticum	Urethritis and postpartum endometritis
Gardnerella vaginalis, *Mycoplasma hominis*, and anaerobic bacteria (*Mobiluncus* sp. and *Prevotella* sp.)	Bacterial vaginosis and intraamniotic infection syndrome in pregnancy
Group B β-hemolytic streptococci[b]	Neonatal sepsis and neonatal meningitis
Viruses	
HSV-1 and HSV-2	Primary and recurrent genital herpes, urethritis[a], proctitis, and meningitis and encephalitis in infants
HBV and HCV	Acute, chronic, or fulminant hepatitis, with associated immune complex phenomena and long-term sequelae, including cirrhosis and hepatocellular carcinoma in chronic active infection
CMV	Congenital infection: gross birth defects and infant mortality, cognitive impairment (e.g., mental retardation and 8th nerve deafness), heterophile-negative infectious mononucleosis, potential multiorgan disease in the immunosuppressed host
HPV	Condyloma acuminata/genital warts and respiratory tract warts in infants (recurrent respiratory tract papillomatosis), HPV types 6 and 11 most commonly; cervical cancer and other anogenital cancers, most commonly associated with HPV types 16 and 18
MCV	Genital molluscum contagiosum
Human immunodeficiency virus (HIV-1 and HIV-2)	AIDS and related complications
Human T-lymphotropic virus type 1	T-cell leukemia/lymphoma and tropical spastic paraparesis
Protozoa	
Trichomonas vaginalis	Vaginitis and urethritis
Fungi	
Candida albicans	Vulvovaginitis and balanitis
Ectoparasites	
Phthirus pubis	Pubic lice infestation
Sarcoptes scabiei	Scabies

AIDS, acquired immunodeficiency syndrome; CMV, cytomegalovirus; DGI, disseminated gonococcal infection; HBV, hepatitis B virus; HCV, hepatitis B virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; HSV, herpes simplex virus; LGV, lymphogranuloma venereum; MCV, molluscum contagiosum virus.

[a] These infections are responsible for the syndrome known as genital ulcer disease.

[b] Group B β-hemolytic streptococcus is not believed to be sexually transmitted *per se*, but vaginal colonization is associated with recent sexual activity, more frequent sexual activity, and with multiple partners.

vaccine against clinically important types of human papillomavirus, the CDC now recommends that all individuals between the ages of 11 and 26 years be vaccinated.

Another factor associated with the acquisition of an STD is a history of prior STD infection. It is estimated that 40% of the annual incidence of chlamydial and gonococcal infections in the United States occur in adolescents who have had these infections previously, and thus repeated screening is important in this population. Similarly, simultaneous infection with more than one STD is not uncommon. In fact, several studies have found that up to 25% of individuals with gonorrhea are also infected with *Chlamydia trachomatis*. On the basis of these data, treatment recommendations include antibiotics that are effective against both organisms.

The control of STDs has become an even higher public health priority in the setting of the HIV epidemic. STDs and HIV share many behavioral risk factors, and thus efforts at educating individuals to eliminate high-risk behaviors and adopt safer sexual practices may help reduce the incidence of both STDs and HIV. In addition, many studies have clearly documented that both ulcerative and nonulcerative STDs increase the risk of HIV transmission. Thus, an individual with both HIV and another STD is more likely to transmit HIV to a sexual partner, as compared with an HIV-infected individual without another STD. Similarly, an HIV-

uninfected individual with another STD is more likely to get HIV from an HIV-infected sexual partner than if he or she did not have an STD at the time of sexual exposure. In a study from Malawi by Cohen and colleagues, urethritis, particularly that due to gonorrhea, was associated with eightfold higher concentrations of HIV-1 RNA in semen than in HIV-infected men without urethritis. The association between STDs and HIV is further strengthened by the findings of an STD treatment study in Tanzania by Grosskurth and colleagues, where communities provided with improved STD diagnosis and treatment experienced a substantial reduction in the incidence of HIV, as a result of a decrease in sexual transmission.

Fortunately, many recent advances have been made in the field of STDs. Intensive research efforts have yielded improvements in the clinical recognition, microbiologic diagnosis, treatment, and prevention of STDs. Epidemiologic data have helped to identify high-risk populations, and thus prevention programs and resources can be appropriately targeted. New clinical algorithms have aided in the early recognition of PID, urethritis, cervicitis, and vaginal infections. Newer diagnostic assays are more rapid, sensitive, and specific for diagnosis of diseases caused by specific pathogens. The development of nucleic acid amplification tests (NAATs) for some STDs has enabled health care providers to quickly, easily, and accurately screen large numbers of individuals for certain STDs, even in the absence of symptoms, with little discomfort or inconvenience to the individual. This has made it possible to identify and treat those individuals who may be more likely to transmit infection or to develop complications, because they would not be prompted to seek health care in the absence of symptoms. Finally, newer antibiotics ensure the possibility of effective treatment of many STDs, including those that have become resistant to older therapeutic agents.

There have been several important developments in the area of STD prevention. The Food and Drug Administration (FDA) has licensed two vaccines effective against clinically important types of HPV. HPV infection is the most common STD in the United States, with an estimated 79 million people currently infected and approximately 14 million new infections each year. As many as half of these infections occur in individuals aged 15–24 years. The development of a vaccine has been an especially important advance, because although many of these infections are asymptomatic and transient, about 10% of women develop persistent infection. Chronic infection with certain types of HPV can lead to cervical cancer, which is one of the most common cancers in women worldwide. Cervical cancer disproportionately affects women of lower socioeconomic status, who are often without medical insurance or regular access to health care; hence, the development of an effective vaccine has great implications for cancer prevention in women in underserved populations. Vaccination is also effective at preventing hepatitis A and B infections, both of which can be transmitted sexually. Hepatitis B is frequently transmitted sexually, and hepatitis B vaccination is recommended for all individuals not previously infected or vaccinated who are being evaluated for an STD. The hepatitis B vaccine is also now routinely recommended as part of childhood vaccinations. Hepatitis A vaccine is recommended for men who have sex with men (MSM) and illicit drug users. Efforts to develop effective vaccines against herpes simplex virus 2 and HIV have thus far been unsuccessful, but are ongoing. An emphasis on consistent condom use remains a mainstay in efforts to prevent most STDs; however, data suggest that concomitant use of the spermicide nonoxynol-9 might be associated with increased HIV transmission.

Unfortunately, there have been several troubling trends in the field of STDs as well, particularly with regard to emerging drug resistance among several STDs. In recent years the prevalence of antibiotic resistance among isolates of *Neisseria gonorrhoeae* has been increasing and becoming more widespread in the United States. As of April 2007, fluoroquinolones, once a mainstay of treatment for gonorrhea, are no longer recommended by the CDC for the treatment of gonococcal infections and associated complications including PID. In 2004, several cases of treatment failure among individuals with syphilis who were treated with azithromycin were reported and resistance to azithromycin was identified in the United States. Currently, the CDC recommends that gonorrhea be treated with two drugs: an injectable cephalosporin and either oral azithromycin or oral doxycycline.

The STDs discussed in this article have been selected because they are among the more common STDs. In addition, they are illustrative of important variations in epidemiology, diagnosis, treatment, prevention, and pathogenesis, concepts that are central to our understanding of STDs. Additional information regarding '*Chlamydia*', 'Polymaviruses and Papillomaviruses', 'herpesviruses', and 'Syphilis, historical' can be found in related articles.

Gonorrhea

Epidemiology

Despite previous declines in new cases of gonorrhea, it remains the second most commonly reported infectious disease in the United States. The greatest number of cases reported to the CDC was 1 013 436 in 1978. Over the following years, there was an approximately 74% decline in reported cases, reaching an all-time low in 2009. More recently the number of cases has increased slightly in the United States. A 9.6% increase was observed from 2011–2012 according to the CDC, with 334 826 reported cases of gonococcal infections. These numbers most likely underestimate the true prevalence of gonorrhea in the United States, given that many health care workers do not comply with reporting guidelines and many patients are treated empirically without confirmatory diagnostic testing because of highly suggestive symptoms or known exposure. It is believed that approximately twice as many new infections occur each year as are reported. Estimates of disease burden attributable to gonorrhea are even less reliable in many developing countries. Available data suggest, however, that gonorrhea remains relatively common in the developing world as it does in the United States. According to the WHO, the global estimated incidence of gonorrhea is 106 million infected people annually.

The overall decline in cases since 1976 has been suggested to be due, in part, to a national control program and to the HIV epidemic and the subsequent decrease in high-risk behaviors seen in many segments of the population. An increase in condom use, screening of asymptomatic individuals at risk for gonorrhea, and contact tracing used to identify individuals who are at risk for

gonorrhea given a known exposure to an infected individual are also factors believed to have contributed to the decline in gonorrhea cases. In the United States, however, the decline in gonorrhea cases has not been observed consistently throughout the population. The most striking decline in gonorrhea had been among MSM and white men and women. In contrast, smaller declines have been observed among urban, minority segments of the population, primarily African American and Hispanic. Greater declines have been observed among older age groups as compared with the young. High rates still persist, however, particularly among adolescents and young adults and among racial minorities. In 2012, the rate among African Americans was 462 cases per 100 000 population- almost 15 times higher than among whites. Between 2008 and 2012, gonorrhea rates decreased 15.5% among African Americans but increased 62% among American Indians/Alaska Natives, 34% among Native Hawaiians/Other Pacific Islanders, 23% among whites, and 19% among Hispanics.

Several factors have been shown to correlate with an increased risk of gonorrhea. Gonorrhea is more common among African Americans. According to the CDC, in 2012 the rate of gonococcal infections was 15 times higher for African Americans than for whites. This may be due in part to relatively limited access to quality health care and poverty, in addition to higher prevalence rates in this population; however, some of this disparity may also be a result of greater attendance of minority individuals at public health clinics, where reporting of cases is much better than in other health care settings. Young age is also a risk factor for gonorrhea, as it is for most STDs, and in fact half of all new sexually transmitted infections occur in young people 15–24 years of age. According to 2012 CDC surveillance data, the overall highest rate of gonococcal infections occurred among individuals 20–24 years of age. Young women seem to be at particularly high risk for gonorrhea. In 2012, women aged 15–19 and 20–24 years had the highest rates of gonococcal infection, that is, 521.2 and 578.5 cases per 100 000 people, respectively. This may be due in part to patterns of sexual behavior, but there may also be a biologic basis to this observation in that young women have larger areas of cervical ectropion (columnar epithelial cells on the ectocervix) that provide a greater surface area susceptible to infection than in older women. Low socioeconomic status, early onset of sexual activity, multiple sexual partners, unmarried marital status, and sexual activity associated with illicit drug use and previous infection are also well-documented risk factors for gonorrhea. Annual screening for gonorrhea is recommended for high-risk men and women. Factors that increase risk include age younger than 25 years, having previous gonorrhea infection or other STDs, having new or multiple sex partners, using condoms inconsistently, working in the commercial sex industry, using drugs, or living in communities with a high prevalence of disease.

Clinical Manifestations

The term *gonorrhea* is often assumed to refer to an illness characterized by urethral discharge in men or vaginal discharge in women. Infections caused by *N. gonorrhoeae* are in fact typically limited to superficial mucosal surfaces lined by columnar or cuboidal, nonkeratinized epithelial cells, such as those sites noted above. Infection of these mucosal surfaces is usually accompanied by a marked inflammatory response that results in the production of copious, purulent discharge. However, *N. gonorrhoeae* can infect several anatomic sites, including the cervix, urethra, rectum, oropharynx, and conjunctiva, and is associated with a wide spectrum of disease. An individual with gonorrhea may have no symptoms at all, may have localized symptomatic disease, may have localized complicated disease, or may be very ill with disseminated infection and bacteremia. Of men and women who report sexual exposure to partners with gonorrhea, urethral infection in men is asymptomatic up to 50% of the time and cervical infection in these women is asymptomatic up to 70% of the time. Pharyngeal and rectal infections secondary to gonorrhea are typically asymptomatic in up to 90% of cases. Individuals who develop disseminated gonococcal infection (DGI) commonly have asymptomatic mucosal infections, detected only by diagnostic testing. It is unknown whether strains that cause DGI are more likely to cause asymptomatic mucosal infection or if individuals who lack symptoms simply do not seek medical treatment before the infection eventually spreads.

Gonococcal infections in women

The most common form of uncomplicated gonorrhea in women is cervicitis. The vagina is usually not infected because it is lined by a squamous epithelium. The urethra is colonized in 70–90% of infected women. In women who have undergone a hysterectomy, the urethra is the most common site of infection. The Skene's glands (paraurethral glands) and Bartholin glands are also commonly infected in the setting of endocervical infection with *N. gonorrhoeae*. Symptoms typically appear within 10 days of infection, although the incubation period can vary. The symptoms associated with endocervical infection may include vaginal discharge, genital itching, intermenstrual bleeding, unusually heavy menstrual bleeding, or painful urination. An infected woman may have all, none, or any combination of these symptoms; and symptoms may range from mild to severe. Physical examination may be normal but may reveal a purulent discharge from the cervix, redness and swelling of the cervix, and easily induced cervical bleeding. These signs and symptoms are not specific for gonorrhea, however. Thus, evaluation of sexually active women suspected of having gonorrhea must include testing for *C. trachomatis*, *Candida albicans*, *Trichomonas vaginalis*, bacterial vaginosis, and other STDs.

PID is the most serious complication of *N. gonorrhoeae* infection in women, affecting approximately 10–20% of all women with acute infection. Gonococcal PID results from the spread of the organism into the upper genital tract. Symptoms of PID can include fever, unilateral or bilateral lower abdominal pain, pain associated with sexual intercourse, abnormal menses or intermenstrual bleeding, or other complaints associated with an intraabdominal infection. These symptoms may be severe or mild. Findings on physical exam include abdominal or uterine tenderness, adnexal tenderness, cervical motion tenderness, and occasionally an adnexal mass or fullness that may be suggestive of a tubo-ovarian abscess. PID can be caused by a number of other infectious agents,

and treatment of PID thus includes broad-spectrum antibiotics targeting chlamydia, gonorrhea, and sometimes, anaerobic bacteria. PID is a significant complication not only because of the acute manifestations but also because of the long-term sequelae, including infertility, ectopic pregnancy, and chronic pelvic pain. Approximately 10% of women will become infertile after a single episode of PID. For those women who remain fertile, there is a tenfold increase in the risk of ectopic pregnancy. The manifestations of gonorrhea in pregnant women are similar to those in nonpregnant women, although PID is less common. Pregnant women with genital gonorrhea are at significant risk for spontaneous abortion, premature rupture of membranes, premature delivery, and acute chorioamnionitis, as well as for transmitting gonorrhea to their newborns during delivery. Neonates infected with gonorrhea can suffer eye and pharyngeal involvement, as well as other complications. Consequently, all newborn infants' conjunctivae are treated prophylactically with topical antibiotics to prevent ocular infection.

Gonococcal infections in men

For symptomatic men, the most common manifestation of infection with *N. gonorrhoeae* is urethritis. Infected men often develop a urethral discharge that can range from scant and clear to copious and purulent. Acute infection is usually accompanied by dysuria (painful urination), which develops after the onset of discharge, and may be the sole manifestation. Symptoms typically develop 2–5 days after exposure to an infected sexual partner, although there can be a delay of over 2 weeks. Untreated acute infection can result in complications, including acute epididymitis, acute and chronic prostatitis, and rarely cellulitis, penile lymphangitis, or periurethral abscess. The most common complication is acute epididymitis, which presents with gradually worsening unilateral scrotal pain. Examination of a man with epididymitis may reveal previously unnoticed urethral discharge as well as tenderness and swelling of the posterior of the scrotum. In young men under the age of 40 years, coinfection with other STDs, particularly *C. trachomatis*, is as common as it is for their female counterparts. Thus, treatment of gonococcal urethritis and associated complications should target both organisms. In older men, structural abnormalities of the urinary tract predispose to infections caused by Gram-negative bacteria and other urinary tract organisms.

Other gonococcal infections

Anorectal gonorrhea is uncommon in heterosexual men, but it is seen more frequently in homosexual men and in approximately 35–50% of women with gonococcal infection of the endocervix. Receptive anal intercourse is the principal mode of transmission in homosexual men, whereas rectal infections in women more commonly arise from local contamination by cervicovaginal secretions. Most men and women with anorectal gonorrhea are asymptomatic; however, some will develop symptoms of proctitis, including severe rectal pain, tenesmus, rectal discharge, and constipation. Other symptoms may include anal itching, painless purulent discharge, or a small amount of rectal bleeding. The vast majority of gonococcal pharyngeal infections are asymptomatic; however, these cases may rarely be associated with acute pharyngitis, tonsillitis, fever, or lymph node enlargement in the neck. Extragenital infections may occur in the absence of genital infections. Several recent studies suggest that a majority of gonococcal infections would be missed in MSM not screened at extragenital sites. In women, up to 30% of gonococcal infections would be missed if extragenital screening were not performed (but the number needed to screen to detect a case of extragenital gonorrhea is much higher in women than in MSM). Consequently, the CDC recommends that all sexually active MSM be screened at all sites of exposure. *N. gonorrhoeae* infections of the eye occur most commonly in newborns, termed ophthalmia neonatorum, as a result of exposure to infected maternal secretions during delivery. These infections can result in corneal scarring or perforation of the cornea, and thus prompt diagnosis and treatment are crucial. Fortunately, the use of silver nitrate or erythromycin eye drops at birth is effective in preventing gonococcal ophthalmia neonatorum. Adults can also acquire gonococcal ocular infection through auto-inoculation, and develop keratoconjunctivitis as a result.

DGI

DGI occurs in approximately 1–2% of individuals with untreated gonorrhea as a result of the spread of the organism from typically asymptomatic mucosal infections of the pharynx, cervix, urethra, or rectum into the bloodstream. DGI is more common in women than in men, and bacteremia probably begins 7–30 days after initial infection. DGI is often referred to as an arthritis/dermatitis syndrome, given the usual findings of an asymmetric polyarthritis and associated rash. The joint manifestations in DGI may begin with painful joints, which progress to frank arthritis later in the course of disease. Approximately 30–40% of patients with DGI will present with overt arthritis, usually involving the wrist, metacarpophalangeal, ankle, or knee joints, although any joint may be involved. Joint fluid cultures are often negative except when the fluid has a high white blood cell concentration, usually more than 40 000 white blood cells per cubic millimeter. Overall, only 20–30% of joint fluid cultures from patients with DGI will be positive for the organism. The rash associated with DGI is composed of a small number (fewer than 30) of tender, necrotic pustules on a reddened base, usually located on the distal extremities. The skin lesions may, however, appear as macules, papules, petechiae, bullae, or ecchymoses. Despite the fact that DGI represents a bloodstream infection, patients often do not clinically appear particularly ill. Blood cultures, like joint fluid cultures, are only positive in 20–30% of patients with DGI. As previously noted, the mucosal infections in DGI are usually asymptomatic. Thus, the diagnosis of DGI can be difficult to make. When DGI is suspected, it is very important to test all potential mucosal sites, in addition to blood and joint fluid, for *N. gonorrhoeae*, to help confirm the diagnosis. Rarely, DGI can be further complicated by seeding of the meninges or the heart valves, resulting in potentially life-threatening gonococcal meningitis or endocarditis. DGI can also occur in newborns of infected mothers, when exposed to secretions during vaginal delivery. The illness in neonates can be similar to that in adults, with associated meningitis and arthritis. Gonococcal disease in newborns usually manifests 2–5 days after birth.

Diagnosis

Gram stain and culture are inexpensive methods for the detection of *N. gonorrhoeae*, although newer nonculture techniques, particularly NAATs, are proving to be more convenient and more sensitive. Specimens for both Gram stain and culture must be obtained directly from the urethra or cervix with a special swab. For the detection of gonococcal urethritis in symptomatic men, a Gram stain of an appropriately obtained specimen can provide rapid and accurate results. A Gram stain is considered positive for gonococcus if Gram-negative diplococci are seen within polymorphonuclear leukocytes. Gram stain results are more difficult to interpret in women and on rectal and pharyngeal specimens because of the presence of other Gram-negative organisms. Isolation of *N. gonorrhoeae* by culture is necessary for antibiotic resistance testing. Optimal recovery of *N. gonorrhoeae* requires the direct inoculation of the specimen onto selective media, followed by incubation at 35–37 °C under 5–7% carbon dioxide. The most commonly used selective media include modified Thayer-Martin, Martin-Lewis, and New York City media. These media contain antimicrobial substances to suppress the overgrowth of other microorganisms that can obscure the presence of the small colonies typical of *N. gonorrhoeae*. *N. gonorrhoeae* can survive for 5–7 h in special transport media, permitting clinics without the necessary laboratory capabilities to transport clinical specimens to nearby laboratories better able to process the specimens appropriately.

Nonculture techniques detect gonococcal antigens, gonococcal enzyme activity, or gonococcal genetic material. One test uses a single-stranded DNA probe to detect gonococcal ribosomal RNA, and it is fairly sensitive and highly specific for gonorrhea. DNA probes have been approved by the FDA for the diagnosis of *N. gonorrhoeae* from urethral and endocervical specimens. In most studies, compared with culture, DNA probes have similar accuracy. NAATs are the most commonly used diagnostic tests. They amplify *N. gonorrhoeae* DNA or RNA using polymerase chain reaction (PCR), transcription-mediated amplification (TMA), or strand displacement amplification. These tests tend to be more sensitive and can provide results more rapidly, within hours, compared with culture, but they are more expensive. Another advantage of NAATs is that they can be performed on different specimen types. In women, the CDC recommends that NAATs be run preferentially on vaginal swabs (either clinician-collected or self-collected). Urine, endocervical, and liquid Pap medium are acceptable alternate specimens for NAATs. In men, the CDC recommends that NAATs be run preferentially on first-catch urine. A urethral swab is an acceptable alternate specimen. Despite not being FDA-cleared for diagnosing rectal or pharyngeal infections, the CDC currently recommends NAATs as the preferred diagnostic modality for these infections because of their enhanced sensitivity compared with culture. Most commercial laboratories have conducted in-house validation studies on NAATs using extragenital specimens. Hence, these tests are routinely available. Currently, there are no acceptable serologic tests for the diagnosis of gonorrhea.

Treatment

For several decades, penicillin was the drug of choice for the treatment of gonococcal infections. By the late 1980s, however, penicillin resistance had become widespread, and penicillin was no longer a reliable choice for effective therapy. Antibiotic resistance may be plasmid-mediated or chromosomally mediated, and some strains of *N. gonorrhoeae* have become resistant to most antibiotics. In 1990, the CDC initiated the Gonococcal Isolate Surveillance Project to monitor trends in antibiotic resistance among gonococcal isolates. Between 1990 and 2001, the rate of fluoroquinolone-resistant isolates remained less than 1%. Subsequently, the rate of resistance increased steadily each year, and by 2007, the CDC no longer recommended the use of fluoroquinolones for the treatment of gonococcal infections and associated complications including PID. As a result, only one class of drugs, cephalosporins, remained available for the treatment of gonorrhea. At that time, the CDC recommended the use of intramuscular ceftriaxone or oral cefixime to treat uncomplicated gonorrhea. Within a short period of time, the MICs to cefixime were noted to be increasing. The CDC increased the recommended dosage of ceftriaxone. Then, in 2012, the CDC recommended that a combination of two drugs be used to treat gonorrhea- even if concomitant infection with chlamydia were ruled out. The only first-line regimen currently recommended by the CDC is the combination of intramuscular ceftriaxone with either oral azithromycin or doxycycline. Cefixime, in combination with either azithromycin or doxycycline is an alternate regimen that should not be used unless the preferred regimen is not feasible. For those with a cephalosporin allergy, the only recommended alternate option is high-dose azithromycin. If alternate regimens are used to treat gonorrhea, the CDC recommends that a test of cure be performed one to two weeks after therapy to ensure eradication of the infection. Specific treatment recommendations have been published by the CDC most recently in the *Sexually Transmitted Diseases Treatment Guidelines, 2010* (Centers for Disease Control and Prevention. Sexually Transmitted Diseases Treatment Guidelines, 2010. MMWR 2010;59(No. RR-12) and can also be found at the CDC's website, http://www.cdc.gov/. The most common cause for treatment failure is repeated exposure to an untreated sexual partner. Given the prevalence of asymptomatic gonorrhea, treatment of all sexual partners from within the previous 60 days is recommended, regardless of whether they have symptoms suggestive of infection. If the patient's most recent sexual encounter was more than 60 days before the onset of symptoms or diagnosis, then the patient's most recent partner should be treated. All persons treated for gonorrhea should be re-tested in 3 months because reinfection rates are very high.

Organism and Pathogenesis

N. gonorrhoeae belongs to the family Neisseriaceae, which includes several nonpathogenic species and the well-recognized pathogen *Neisseria meningitidis*. *N. gonorrhoeae* is a fastidious Gram-negative diplococcus, 0.6–1.0 μm in diameter, with complex growth requirements described earlier. *In vivo*, gonococci grow under relatively anaerobic conditions and are capable of growing under

strictly anaerobic conditions *in vitro*. *N. gonorrhoeae* produces high levels of catalase and produces an oxidase. Because oxidase production is easy to detect in the laboratory, oxidase-positive Gram-negative diplococci that are obtained from appropriate clinical specimens, and that grow on selective media, are presumed to be *N. gonorrhoeae* in most cases. *N. gonorrhoeae* can be distinguished from other *Neisseria* species on the basis of sugar fermentation patterns. *N. gonorrhoeae* utilize only glucose, pyruvate, and lactate as their carbon source. Other *Neisseria* species, *Kingella dentrificans*, *Moraxella* species, and *Branhamella catarrhalis* can be mistaken for *N. gonorrhoeae*, particularly given their similar appearance on Gram stain. Such errors can have serious repercussions for patients and their health care providers, given the social and medicolegal implications of being diagnosed with an STD.

The structure, molecular biology, and pathogenesis of *N. gonorrhoeae* have been studied extensively, yet many processes remain elusive. The cell wall is made up of pili, a capsule, several characteristic outer membrane proteins referred to as protein I, Opa protein (opacity-related proteins or protein II), protein III (Rmp), iron-regulating proteins, lipooligosaccharides (LOS), H.8 (Lip), and other components that can vary with growth conditions. The pili are hair-like filamentous appendages that extend from the cell surface. Pili are an important virulence factor. Pili, in concert with protein II, enable gonococci to attach to the microvilli of host nonciliated columnar epithelial cells. Protein I is the most prominent outer membrane protein and functions as a porin, providing a hydrophilic channel through the outer membrane. Protein I triggers endocytosis of gonococci by the host mucosal cell. Following endocytosis, the membrane of the mucosal cell retracts around the organism, forming a membrane-bound vacuole, which is transported to the base of the cell, where gonococci are subsequently released into the subepithelial tissue. Unlike most of the other outer membrane proteins, protein I is always expressed, and it is useful in identifying individual strains of gonococci because each strain produces a single antigenically stable protein I. Protein I also appears to inhibit neutrophil function, and thus may limit the ability of the host to respond to infection.

In addition to enhancing the adhesion of gonococci to mucosal cells, Opa/protein II appears to mediate infection by enhancing gonococcal cell-to-cell adhesion, which may result in a more infectious unit. It specifically appears to enhance adhesion to conjunctival cells, epithelial cells, and neutrophils. Protein III (Rmp) appears to be able to block bactericidal antibodies produced by the host directed at other surface proteins acting as antigens, specifically protein I and LOS. LOS is similar to the lipopolysaccharide component characteristic of the cell wall of all Gram-negative bacteria, except that it lacks long, hydrophilic, neutral polysaccharides. LOS appears to mediate most of the damage to host tissues. LOS is the primary target of host antibodies, and it regulates complement activation on the cell surface of gonococci in addition to prompting the release of enzymes, such as proteases and phospholipases. Evidence suggests that gonococcal LOS stimulates the production of tumor necrosis factor (TNF) in fallopian tube organ cultures, and in other settings, the inhibition of TNF has been shown to limit tissue damage. LOS also appears to be capable of molecular mimicry as demonstrated by the similarity of terminal LOS to host glycolipids, and thus hinders the ability of the host to mount an effective host immune response. Pili, protein II, and LOS also exhibit extensive intra- and interstrain antigenic variations, as well as phase variation in pili and protein II expressions. Such variation likely represents a mechanism by which gonococci can escape the host immune response.

Chlamydia

Epidemiology

Chlamydia is the leading bacterial cause of STDs worldwide. Chlamydia, caused by *C. trachomatis*, is the most commonly reported STD in the United States There are an estimated 2.9 million new cases in the United States each year, at an estimated cost of $2.4 billion, with an estimated $10 billion annually associated with the treatment of PID. Chlamydia became a reportable disease in several states in 1986, and for years thereafter the number of reported cases increased steadily, likely as a result of improved diagnostic techniques, screening practices, and reporting in more states. Chlamydia became the most common reportable disease in the United States in 1995, when 477 638 cases were reported. In 2012 that number had risen to 1 422 976. As with other STDs, these numbers most likely underestimate the actual burden of disease as data on the true incidence and prevalence of chlamydia are limited by a number of factors. Chlamydial infections are often asymptomatic, and in screening study of women attending a family planning clinic, 70% of the women found to have genital infections secondary to chlamydia had neither clinical findings nor symptoms of infection. Thus, a significant number of cases likely go unrecognized by the patient or health care provider. When symptoms are present, individuals are often treated empirically without diagnostic confirmation, and thus these cases of chlamydia are never reported.

Several studies in a variety of settings, utilizing different diagnostic screening strategies, have provided additional estimates of the prevalence of genital chlamydial infections in the United States. Approximately 3–5% of asymptomatic men and women seen in general medical settings are infected with chlamydia, whereas women and men screened in STD clinics have a prevalence of 15–20%. More recent studies using highly sensitive diagnostic assays reported even higher prevalence data. In one study, Burstein and colleagues demonstrated that the prevalence of chlamydial genital infections among adolescent females screened in family planning, STD, and school-based clinics in Baltimore, Maryland, was 29.1%. The majority of infected girls were asymptomatic. In a study in which 13 204 new female army recruits were screened, the prevalence was 9.2%. Interestingly, there was wide geographic variation in the prevalence of chlamydial infection according to the state of origin of the recruit, ranging from more than 15% in women from some southern states to 5% in other states, including Washington and Oregon, where aggressive public health initiatives have been in place since the late 1980s, aimed at reducing the number of chlamydial infections. In a similar study of 2245 male army recruits, the prevalence of chlamydial urethritis was 5.3% overall, with similar geographic variation by state. Of the men infected with chlamydia, over 85% were asymptomatic.

Specific risk factors for chlamydial infection have been consistently identified in these studies. The single most important risk factor for chlamydial infections in women is young age Among women, the 20–24 year age group had a rate of 3695.5 per 100 000 while the 15–19 year group had a rate of 3291.5 per 100 000. The respective rates in men were 1350.4 and 744.8 per 1 000 000, respectively. Studies of reinfection have shown that up to 20–30% of female adolescents had evidence of another chlamydial infection within 6 months of the initial infection, and it is recommended that women be retested for chlamydia 3 months after treatment. Other risk factors associated with chlamydial infection mirror those associated with other STDs and include multiple sex partners, a recent new sex partner, inconsistent condom use, and nonwhite race. Although chlamydia is common among all races in the United States, African American women are disproportionately affected. In 2012, according to the CDC, the rate of reported chlamydia infections per 100 000 African Americans females was 1229.4, more than 7 times that of whites (179.6 per 100 000) and more than twice that of Hispanic females (380.3 per 100 000). Another important risk factor for chlamydia is the diagnosis of gonorrhea. The CDC recommends annual screening of all sexually active women under the age of 26 years, whether or not symptoms or other risk factors appear to be present. Data from a study in a managed care setting showed that chlamydia screening and treatment can reduce the incidence of PID by more than 50%. Increased screening and treatment efforts would also most likely reduce other adverse health consequences such as infertility.

Chlamydia is also a problem for pregnant women and their newborns. Prevalence studies in pregnant women using relatively insensitive screening techniques yielded prevalence rates of 5–7%, although prevalence rates of 21–25% have been identified in inner-city and Native American populations. Of infants born to untreated infected mothers, approximately 60–70% can become infected with chlamydia, a significant proportion of which go on to develop pneumonia and ocular complications (chlamydial 'ophthalmia neonatorum') as a result. Given the results of these studies, some areas of the United States have instituted broad-based screening and treatment programs, and the prevalence of chlamydial infections in these areas has declined dramatically. In the Pacific Northwest of the United States, screening for chlamydia in patients attending family planning clinics began in 1988 and in STD clinics in 1993. The prevalence of chlamydia in this region declined from 10–12% in the late 1980s to 4–5% in 1995. One challenge to these efforts is the relatively long incubation period of chlamydia of 1–3 weeks. Infected individuals may not develop symptoms for a prolonged period of time, if at all, and thus may remain sexually active while infectious. The efficiency with which chlamydia is transmitted between sexual partners is not well understood. Studies evaluating the sexual partners of infected individuals demonstrated an infection rate of 85% among female contacts of men with chlamydial urethritis. Traditionally, researchers suspected that men were more likely to transmit chlamydia to women than the other way round, but more recent data from studies using highly sensitive diagnostic techniques suggest that transmission may be equal in both directions.

From a global perspective, however, perhaps the greatest burden of disease caused by *C. trachomatis* is trachoma, which affects the inner upper eyelid and is one of the most common infectious causes of blindness. According to the WHO, in some parts of the developing world 90% of the population is infected. The disease is especially common in poorer, rural communities. Here, the infection is not sexually transmitted but tends to be a communicable disease among family members and is exacerbated by limited access to water and adequate personal hygiene.

Clinical Manifestations

There are several subtypes of *C. trachomatis*, the trachoma and lymphogranuloma venereum (LGV) subtypes being the only human pathogens. LGV is caused by *C. trachomatis* serovars L_1, L_2, or L_3, and are transmitted through sexual contact. Infection with one of these serovars is typically associated with painful unilateral lymphadenopathy and sometimes ulceration and abscess formation. Rectal infections in MSM can result in severe proctocolitis with chronic sequelae if untreated, including colorectal fistulas and strictures. In recent years a resurgence of LGV proctitis has been observed in western Europe and the U.S. associated with LGV serovar L_2.

Genital infections caused by the D-K serovars of *C. trachomatis* have many of the same characteristics as those caused by *N. gonorrhoeae*. Both organisms preferentially infect columnar epithelial cells and do not appear to be capable of growth in squamous cells. Thus, like gonorrhea, chlamydia infects the urethra, with extension to the epididymis and possibly the prostrate gland in men, the squamocolumnar junction of the endocervix in women, with the potential for spreading to the endometrium and upper genital tract, and the rectum. *C. trachomatis* can also cause conjunctivitis. In contrast to gonorrhea, however, chlamydial infections generally have a longer incubation time, are associated with a less robust inflammatory response, and are more likely to be asymptomatic. Unlike gonorrhea, chlamydia does not disseminate to cause a systemic infection like DGI. Unfortunately, there are no unique signs or symptoms to help make the diagnosis of *C. trachomatis* infection of the genital tract. The associated symptoms and physical findings, if present at all, can also result from other infections or complications, and therefore one has to have a high index of suspicion to diagnose a chlamydial infection.

Chlamydial infections in women

In women, *C. trachomatis* most commonly infects the cervix, although approximately 50% of infected women will have simultaneous infection of the urethra. Chlamydial infection of the cervix typically results in cervicitis with a mucopurulent discharge, present in only 20–40% of infected women. On examination, there is sometimes visible swelling and easily inducible bleeding in a zone of ectopy, an area with a relative increase in exposed columnar cells at the squamocolumnar junction. As previously noted, however, in studies in which women were screened for chlamydia, 70% or more of infected women did not have any abnormal clinical findings. Women with cervical ectopy have a higher prevalence of chlamydia than those without ectopy. Cervical ectopy is

normally present in 60–80% of sexually active adolescent females, and declines with age. This finding may in part explain the higher prevalence of chlamydia among adolescent females. Oral contraceptives are also associated with cervical ectopy, and this may account for the increased risk of chlamydia, and gonorrhea, for women on oral contraceptives. Chlamydial urethritis has also been well documented in women and is estimated to occur without concomitant cervical infection about 25% of the time. The prevalence of isolated urethral infection increases with age. Chlamydial urethritis may be associated with painful and frequent urination, although the majority of women with urethritis do not have symptoms. Nonetheless, *C. trachomatis*, as well as *N. gonorrhoeae*, should be considered as a possible diagnosis in sexually active women with urinary symptoms if there is evidence of coexisting cervicitis or if other STD risk factors are present.

The most serious form of *C. trachomatis* infection is upper genital tract disease. Approximately 10% of women with cervical chlamydia infection will develop symptomatic PID. There is likely an even larger proportion of infected women who develop asymptomatic upper tract disease. All of these women are at increased risk for infertility and ectopic pregnancy as a result of tubal inflammation and scarring. The clinical manifestations of PID secondary to chlamydia are similar to those of PID caused by gonorrhea, and are discussed more completely in section 'Gonococcal infections in women'. As with other sites of infection, chlamydial infection of the upper tract is clinically milder and more likely to be asymptomatic than infections caused by gonorrhea or anaerobic bacteria, despite ongoing tubal damage. Like gonorrhea, chlamydia can cause perihepatitis, with or following the development of PID, referred to as the Fitz-Hugh–Curtis syndrome. Women who are infected with chlamydia in the first trimester of pregnancy are at increased risk of postpartum endometritis following vaginal delivery.

Chlamydial infections in men

More common than gonococcal urethritis is the entity known as nongonococcal urethritis (NGU). A diagnosis of NGU is made when *N. gonorrhoeae* cannot be identified in urethral specimens from a man with urethritis. *C. trachomatis* is believed to be the causative organism in 25–35% of cases of NGU, with a lower prevalence among older men. Other organisms include *Mycoplasma genitalium*, *T. vaginalis*, and herpes simplex virus. NGU is often characterized by the presence of inflammatory cells in the urine and a urethral discharge that is usually less purulent and relatively scant as compared with the urethral discharge associated with gonorrhea. Infected men may experience painful or frequent urination. However, the symptoms are variable enough that one cannot reliably distinguish NGU from gonococcal urethritis clinically. If a Gram stain of the urethral specimen shows a large number of inflammatory cells (specifically polymorphonuclear cells) without intracellular diplococci, then a presumptive diagnosis of NGU is made. A diagnosis of chlamydial urethritis requires specific diagnostic testing.

Chlamydial infections of the lower genital tract in men may also become complicated. *C. trachomatis* is the most common cause of epididymitis in sexually active young men, accounting for approximately 70% of cases. Epididymitis results from the spread of an untreated chlamydial urethral infection to the upper genital tract, and it is characterized by progressive unilateral scrotal pain and swelling. Chlamydial urethritis has also been associated with the subsequent development of Reiter's syndrome. Reiter's syndrome consists of urethritis, conjunctivitis, arthritis, and characteristic skin lesions. Some studies have found that 80% of men with Reiter's syndrome have evidence of prior or ongoing chlamydial infection. This syndrome is more common is men with the HLA-B27 haplotype. These patients may also develop arthritis or tenosynovitis, without the other features of Reiter's syndrome, in association with infection with *C. trachomatis*, and usually improve following treatment for chlamydia.

Other chlamydial infections in adults

Both men and women can develop proctitis as a result of rectal infection with *C. trachomatis*. Rectal infections are usually asymptomatic, but if proctitis develops patients may experience rectal pain, bleeding, and a mucous discharge. Adults can also develop an acute follicular conjunctivitis as a result of autoinoculation of the eye with infected genital secretions. Conjunctivitis can develop within 1–3 weeks and does not typically result in permanent damage or vision loss.

Chlamydial infections in neonates

Neonates exposed to *C. trachomatis* during vaginal delivery are at risk for developing conjunctivitis and pneumonia. Nasopharyngeal infection is also common. Conjunctivitis develops within 5–21 days and is characterized by copious purulent discharge and redness of the eye. As in adults, it is usually self-limited and does not result in permanent eye damage or vision loss. Pneumonia caused by chlamydia can be life-threatening, however. The incubation period for chlamydial pneumonia in neonates is 2–12 weeks. Infected infants often have a history of conjunctivitis and present with rhinitis, rapid breathing, and a characteristic cough in the absence of fever. There is evidence to suggest that infants who have chlamydial pneumonia frequently go on to develop asthma or obstructive airway disease, even after appropriate treatment of the initial pneumonia.

Diagnosis

Because *C. trachomatis* is an obligate intracellular organism, cell culture has traditionally been the gold standard for the diagnosis of chlamydia. Cyclohexamide-treated McCoy cells are the most commonly used cell culture system. Cell culture is often impractical, however, because of the high level of technical expertise needed, as well as the strict requirements for specimen collection and transport, and high cost. Samples of discharge or secretions containing only inflammatory cells are not adequate. Instead, carefully collected samples of cervical or urethral columnar cells are necessary. Specimens must then be refrigerated in special transport media and inoculated onto cell culture plates within 24 h. When done correctly, cell culture has a sensitivity of

80%. Because of these limitations, several nonculture techniques have been developed, including a direct fluorescent antibody (DFA) test, enzyme immunoassay (EIA), and direct DNA probes. Although these tests may be relatively inexpensive and more convenient in some settings, they still require careful specimen collection and must be processed by an experienced technician. The DFA test is slightly more sensitive than the EIA when performed properly, with a sensitivity of 80–85% and a specificity of 99% relative to culture. The EIA is only 60–80% sensitive when compared with culture. The performance of direct DNA probes is comparable with that of DFA.

The development of NAATs to detect amplified chlamydial DNA or RNA has markedly improved diagnosis. These techniques utilize PCR or TMA. Perhaps the greatest benefit of NAATs is that they can be performed on vaginal, cervical, first-catch urine, urethral, rectal, and liquid-based Pap samples. The preferred specimens are similar to those for gonorrhea testing (see above). NAATs are highly sensitive and 99–100% specific. The CDC recommends rectal screening for *C. trachomatis* in all MSM who report rectal exposures, but not pharyngeal testing because the prevalence is low. NAATs testing will detect both D-K and L1-L3 serotypes of C. trachomatis but will not distinguish the two serotypes. Additional testing (multiplex PCR or sequencing) must be obtained to reliably distinguish between D-K and LGV serotypes. It is important to distinguish between these two serotypes because treatment regimens differ. Serologic tests are not appropriate in the diagnosis of uncomplicated genital tract infections, primarily because of the high prevalence of antibodies to *C. trachomatis* in sexually active adults. High antibody titers may be seen in patients with complicated upper tract disease, and thus support the diagnosis of chlamydial PID or epididymitis. However, sexually active patients with PID or epididymitis should be treated for chlamydia regardless of the results of serologic testing. Serologic testing may be used to diagnose LGV, but these tests are not standardized for LGV proctitis.

Treatment

Macrolides, tetracyclines, and quinolones, with the exception of ciprofloxacin, are all effective against chlamydia. There has not been any convincing evidence of emerging antibiotic resistance, as has been the case for gonorrhea. Currently, the treatment of choice for uncomplicated chlamydial infections of the lower genital tract is 1 g of oral azithromycin. Alternatively, a 1-week course of doxycycline can be used. Although azithromycin is more expensive than doxycycline, cost-effectiveness analyses demonstrate that this treatment option is a cost-effective choice because of the improvement in compliance with single-dose therapy. Infected patients and their partners should abstain from sexual activity until they have completed a course of antibiotic therapy, to prevent the transmission of chlamydia before the infection has been indicated. If single-dose therapy is given, individuals should abstain for 7 days following treatment, and all women treated for chlamydia should be rescreened 3–4 months after therapy because of the high prevalence of *C. trachomatis* infections among women with a history of recent infection.

Because tetracyclines and quinolones are not acceptable for use in pregnant women or infants, azithromycin or erythromycin should be used. The effectiveness of erythromycin may be reduced compared with azithromycin due to compliance issues, and therefore patients treated with erythromycin should be retested for chlamydia 2 weeks after the completion of therapy (or 3 weeks if using an amplification test). Conjunctivitis should be treated with oral antibiotics, not topical therapy, because of the risk of infection at other sites. The treatment of PID should routinely include antibiotics effective against chlamydia. All sexual partners within the previous 60 days should also be treated for chlamydia and gonorrhea in a timely manner. If the patient's last sexual encounter was more than 60 days before the onset of symptoms or diagnosis, then the patient's most recent partner should be treated. Specific treatment recommendations have been published by the CDC most recently in the *Sexually Transmitted Diseases Treatment Guidelines, 2010* (Centers for Disease Control and Prevention. Sexually Transmitted Diseases Treatment Guidelines, 2010. MMWR 2010;59(No. RR-12) and can also be found at the CDC's website, http://www.cdc.gov/.

Organism and Pathogenesis

C. trachomatis is one of four species within the genus *Chlamydia*. Among the others, *C. pneumoniae* and *C. psittaci* are also human pathogens. The fourth, *C. pecorum*, is a pathogen of mammals but is not believed to cause disease in humans. A detailed review of *Chlamydia* species and their pathogenesis is found in a related article.

Syphilis

Epidemiology

Syphilis is a complex and fascinating disease, caused by infection with the bacterium *Treponema pallidum*. The disease is systemic, potentially affecting nearly every organ in the body and so mimics many other disease processes. Syphilis is characterized by several different stages, including primary, secondary, tertiary, and latent stages, and it can persist for decades if untreated. Syphilis is distributed worldwide and is particularly a problem in the developing world, where it is the leading cause of genital ulcer disease. Syphilis is highly infectious, but easily curable if treated during its early stages. Before the development of penicillin in the 1940s, syphilis was a big public health problem in the United States. At the beginning of World War II, the US Public Health Service estimated that approximately 2.5% of all Americans were infected with syphilis. With the advent of penicillin, however, the prevalence of primary and secondary (P&S) cases declined by about 93%. Fortunately, penicillin remains fully effective against syphilis, with no evidence of the emergence of penicillin resistance.

Nonetheless, syphilis remains a significant public health concern. According to the CDC, beginning in the mid-1980s there was a marked and progressive resurgence of P&S syphilis, the most infectious stages of the disease, in the United States that peaked in 1990, with a total of 55 132 reported cases of P&S syphilis, representing a 20.2% increase over the previous year. The reasons for these trends are unclear. Of concern was the dramatic increase in cocaine use in the United States during the mid- to late 1980s. Cocaine use has been associated with high-risk sexual behaviors. Also of concern have been declines in the resources allocated for public health programs involved in the control of syphilis over the previous two decades. During the 1980s, the majority of cases were initially observed in MSM, and subsequently in intravenous drug-using individuals. In the 1980s, more than 40% of infected men reported other men as their sexual contacts. With the onset of the AIDS epidemic and the associated changes observed in sexual behavior among MSM, the proportion of syphilis cases attributable to this segment of the population declined dramatically. Subsequently, mirroring trends in the HIV epidemic, a growing proportion of syphilis cases were seen in inner-city, heterosexual, drug-using populations. Throughout the 1990s, however, there was a steady decline in reported cases of P&S syphilis in the United States, reaching an all-time low in 2000. Since then, however, there has been a resurgence of cases such that between 2000 and 2013, there was a doubling of the number of cases of primary and secondary syphilis. In 2013, 91% of the cases were diagnosed in men-83% among MSM. Estimates suggest that over half of all men diagnosed with primary and secondary syphilis are co-infected with HIV. Compared to a rate of 9.8 cases per 100 000 men, the rates in women are 0.9 per 100,000, a stable rate after an increase noted between 2005 and 2008. The trend among men, particularly MSM, is troubling, especially given that syphilis, like many other STDs, facilitates the spread of HIV by increasing the likelihood of transmission of the virus between individuals during a sexual encounter. It is recommended that all high-risk groups be screened for syphilis on a routine basis: at least annually for HIV-infected persons and sexually active MSM, and every 3–6 months for higher-risk MSM 9e.g. those who report sexual encounters with anonymous sex partners or those who use drugs in conjunction with sex).

As observed with many other STDs, there remain marked discrepancies in the proportion of cases among different racial or ethnic groups. According to the CDC, in 2012 the rate of P&S syphilis among African Americans was 16.4 per 100 000, 6 times that among whites; however, this marks a significant decline from 1999 when the rate among African Americans was 29 times that of white Americans. It is important to recognize though that although this change is a result of a decline in rates among African Americans, it also reflects a significant increase in rates of P&S syphilis among white men. Biases in reporting explain some of the discrepancies in rates among different racial or ethnic groups, but cannot explain them completely. Other more fundamental risk factors for STDs that are also associated with race or ethnicity, such as socioeconomic status, access to quality health care and health education, and others, may also play an important role. Interestingly, syphilis rates do not follow the same age distribution as many other STDs, with the highest numbers of cases among women 20–24 years of age, and among men 20–29 years of age. In 2006, the highest rates among men had been in the 35–39 year age group.

Clinical Manifestations

Syphilis is unique among STDs in its tendency to spread throughout the body via the bloodstream, resulting in the involvement of multiple distant organs, including the central nervous system, bones, arteries, skin, and other sites. Because it is a systemic disease, with sometimes nonspecific signs and symptoms, syphilis can mimic many other disease processes, and patients may be easily misdiagnosed if syphilis is not considered and ruled out by appropriate diagnostic testing. Transmission usually occurs as a result of sexual contact with an infected individual possessing the mucosal lesions typical of P&S syphilis. Syphilis can also be transmitted via contact with infectious lesions seen in the mouth or on the skin in secondary syphilis, or it may be transmitted transplacentally from the mother to the fetus during any stage of the infection, resulting in congenital syphilis. Infected mothers can also transmit syphilis to their newborns through breast-feeding.

T. pallidum begins to multiply at its site of entry, and after an incubation period of 10–90 days, a papule appears at the infected site. The papule eventually converts into the painless superficial ulcer, known as a chancre, characteristic of primary syphilis. Chancres are highly contagious lesions, harboring a high concentration of *T. pallidum*. During this period, infected individuals often develop local lymph node enlargement, but often this sign goes completely unnoticed. After a period of 2–6 weeks, the chancre usually heals spontaneously. Subsequent spread via the bloodstream and multiplication of the organism in other organs results in findings characteristic of secondary syphilis. The signs and symptoms of secondary syphilis usually do not appear until after a 2–24-week period after the resolution of the primary stage, during which the infected individual may be completely asymptomatic. Individuals with secondary syphilis typically develop a rash but often experience other symptoms, such as low-grade fever, malaise, headache, sore throat, generalized lymph node enlargement, and muscle aches. Patients may also develop bone inflammation and hepatitis, both of which are usually asymptomatic. The rash associated with secondary syphilis usually begins with a mild, transient macular rash, which often goes unnoticed. This quickly evolves into an itchy, diffuse, symmetric, raised rash, which involves the entire trunk and extremities, classically including the palms of the hands and the soles of the feet. Other typical lesions, known as condylomata lata, are large, raised white or grayish patches that occur in warm, moist areas, such as the mouth, vagina, or rectum. These skin lesions contain a high concentration of organisms and are highly contagious. Hair loss as well as changes in skin pigmentation of the skin can occur. As seen in primary syphilis, the signs and symptoms of secondary syphilis usually resolve spontaneously after a 2–6-week period, even without treatment.

About 25% of individuals with P&S syphilis may experience multiple relapses of the secondary stage, usually within the first year of infection. After the secondary stage, untreated patients with P&S syphilis become asymptomatic and enter a stage referred to as latent syphilis, for which there are no signs or symptoms at all, and a diagnosis of syphilis can only be made by serologic screening.

Latent syphilis can persist for decades. This stage is arbitrarily divided into early-latent and late-latent stages. By definition, patients with early-latent syphilis are asymptomatic and have been infected for less than 1 year, based on previously negative serologic tests. Those with late-latent syphilis are asymptomatic and have been infected for more than 1 year. The term 'early syphilis' refers to those individuals who, within the preceding year, had a documented seroconversion, unequivocal symptoms of P&S, or a sex partner with documented primary, secondary, or early-latent syphilis. Individuals with early syphilis are believed to be infectious and can thus transmit syphilis to their partners during the primary, secondary, and early-latent stages. If the duration of latent syphilis infection cannot be documented for certain, then patients are assumed to have late-latent syphilis for the purposes of treatment.

One-half of patients who develop latent syphilis will continue to have serologic evidence of infection but will never develop late complications of the disease. The other half may remain asymptomatic for decades but will ultimately develop late manifestations of syphilis if untreated. Individuals with late-latent syphilis are immune to reinfection with *T. pallidum*. This latter group of individuals, if untreated, will eventually experience disease progression and develop what is referred to as tertiary syphilis. Individuals with tertiary syphilis may have involvement of their skin, bones, central nervous system, heart, and arteries, as well as other sites. At this stage, syphilis may be characterized by the presence of destructive granulomatous lesions known as gummas. Gummas may be found in the bones and skin, but they may also be found in other organs, including rarely the heart and digestive tract. The pathogenesis of gumma formation is not known, but gummas usually result in extensive damage to surrounding tissues. Tertiary syphilis involvement of the central nervous system and cardiovascular system may also result from direct invasion by the organism, without gumma formation. Cardiovascular involvement is thought to result from the multiplication of the organism in the aorta and proximal coronary arteries. The associated arteritis can lead to severe aortic valve insufficiency, aneurysm formation, and myocardial infarction. The neurologic effects of tertiary syphilis take two forms. Meningovascular syphilis results from the involvement of the meninges, the tissues surrounding the brain. The parenchymatous form affects the brain, referred to as general paresis, and spinal cord, known as tabes dorsalis, directly. The neurologic manifestations of tertiary syphilis may include meningitis, stroke, dementia, cranial nerve deficits, sensory disturbances, and paralysis. Approximately 80% of deaths from syphilis are attributable to cardiovascular involvement and the remaining result from neurologic disease. Fortunately, tertiary syphilis has become extremely rare since the introduction of penicillin.

Congenital syphilis is a potentially devastating disease, resulting from transplacental transmission of syphilis from an infected mother to her fetus. Approximately 50% of infected fetuses are spontaneously aborted or are stillborn. Those that survive to delivery may exhibit a wide range of signs and symptoms of syphilis. Newborns with symptoms before the age of 2 years have early congenital syphilis, and they develop skin lesions, mucous membrane involvement often with copious secretions, bone involvement, severe anemia, and enlargement of the liver and spleen. Children with late congenital syphilis may not have symptoms until after the age of 2 years, at which time they may develop interstitial keratitis with subsequent blindness, tooth deformities, deafness, neurosyphilis, cardiovascular involvement, and skeletal deformities. Pregnant women who receive prenatal care are routinely screened for syphilis given the terrible consequences of infection for the newborn.

Individuals with syphilis and concurrent HIV infection appear to have a similar experience with regard to P&S syphilis, with one notable exception. Numerous case series have documented rapid progression from early syphilis to neurosyphilis in patients infected with both HIV and syphilis. Many of these patients develop early neurosyphilis, characterized by the clinical manifestations of meningitis, cranial nerve deficits, including hearing loss and blindness, and even stroke, often in the context of therapeutic failure with conventional doses of penicillin for P&S syphilis.

Diagnosis

The most sensitive and specific test for the diagnosis of primary syphilis is the identification of the organism on dark-field microscopy, when performed by an experienced observer on an adequate sample of fluid obtained from the surface of a chancre. *T. pallidum* has a characteristic corkscrew appearance on dark-field microscopy, and false-positive results do not occur in experienced hands. Serologic tests can be useful in the diagnosis of primary syphilis, although false-negative results occur 10–20% of the time. The rapid plasma reagin (RPR) and the Venereal Diseases Research Laboratory (VDRL) are referred to as 'nontreponemal tests' because they do not measure antibodies specific to treponemal components. As a result, these assays may occasionally be positive in other disease states and are less specific than treponemal tests. These assays are also more likely than treponemal tests to be negative in primary syphilis. The RPR titer does, however, correlate with disease activity and often becomes negative after adequate treatment, although it may remain positive at a low titer throughout one's life. Thus, even when the diagnosis of syphilis is made by other means, it is useful to obtain an RPR to monitor subsequent disease activity. Tests such as the fluorescent treponemal antibody absorbed or the microhemagglutination assay for antibody to *T. pallidum* are referred to as treponemal tests. These assays are more likely to be positive in primary syphilis than the RPR or VDRL and remain positive for life, but they are not useful in monitoring disease activity. Because these older assays are specific for syphilis, but are more costly and labor intensive, they are not used for screening but rather to confirm a diagnosis of syphilis when an individual has a positive RPR or VDRL. Newly developed treponemal antibody tests, the treponemal enzyme immunoassays (EIA) and the chemoilluminescent assay (CIA) are automated tests that are now being used by many laboratories as the initial screening tests for syphilis. If positive, the laboratory automatically obtains a reflex RPR. If the RPR is also positive, clinicians should stage the infection based on history and treat accordingly. If the RPR is negative following a positive intial EIA or CIA, the laboratory should run a second different treponemal test to confirm that the EIA or CIA is a true positive. If the second treponemal test is positive, clinicians must obtain a careful history to determine the next appropriate step. Causes of positive treponemal tests with a negative RPR include old treated syphilis, old untreated syphilis (RPR

titers may become negative over time even in the absence of therapy), or early syphilis (treponemal tests may become positive before the non-treponemal tests in early syphilis).

Secondary syphilis can be diagnosed by dark field examination of material taken from skin lesions of individuals with secondary syphilis. Serologic assays are much more reliable in diagnosing secondary syphilis compared with primary syphilis, however, and are the preferred method of diagnosis in this setting. The RPR is always positive in secondary syphilis, usually with a high titer. The treponemal tests are also always positive in secondary syphilis, and may be used to confirm that a positive RPR is in fact due to syphilis. The RPR is then used to monitor the response to therapy. In rare cases, non-treponemal tests may be falsely negative in secondary syphilis due to the prozone reaction. Dilution of the serum sample is t he preferred approach if a prozone reaction is suspected.

Because individuals with latent syphilis are asymptomatic, latent syphilis is usually diagnosed by serological tests. A diagnosis of latent syphilis is easily made if an individual recalls a recent history of a chancre or skin rash for which he or she did not seek medical care and is currently asymptomatic. Unfortunately, most asymptomatic individuals with positive serologies are unable to provide a diagnostic medical history. For public health purposes, these individuals are assumed to have late latent syphilis and are thus treated accordingly. Most local public health departments keep a record of all positive syphilis serologies and attempt to maintain records on treatment and subsequent response to therapy. If a current RPR titer is positive at fourfold lower than previously documented titers (e.g., from 1:16–1:4), then the individual has likely had an appropriate response to prior therapy.

A diagnosis of neurosyphilis is easily made when there are neurologic findings in the setting of positive serologies and abnormal CSF. However, the presence of only two of these three criteria is usually sufficient to mandate treatment for neurosyphilis. The CSF in patients with neurosyphilis may exhibit any combination of the following findings, including an elevated opening pressure, an elevated white blood cell count, an elevated protein concentration, or a positive CSF VDRL. A positive CSF VDRL is diagnostic of neurosyphilis, but this test is not sensitive and is often negative in neurosyphilis. Stroke, dementia, and other neurologic abnormalities are more frequently due to etiologies other than syphilis, but may coincidentally occur in the presence of positive serology. Currently, the CDC recommends that clinicians perform a CSF examination in patients with serological evidence of syphilis and (1) neurological signs or symptoms (2) other signs or symptoms of tertiary syphilis (3) in those whose serological titers do not respond to therapy and in whom reinfection is ruled out. Although HIV-infected persons with syphilis appear to be at greater risk for neurosyphilis, it is not known whether a CSF examination among asymptomatic HIV-infected persons improves long-term outcomes. As such, there are no definitive recommendations that can be made about the utility of CSF examination in these patients. In the absence of gummas, diagnosis of cardiovascular disease from tertiary syphilis is usually made on clinical grounds in the presence of positive serology or a history of syphilis and characteristic findings on angiography. Other forms of tertiary syphilis are usually diagnosed on the basis of the identification of a gumma.

Treatment

Parenteral penicillin G is the preferred treatment for all stages of syphilis. The duration of therapy depends on the stage of syphilis. HIV may alter the response to therapy, and treatment failures have been reported in HIV-infected individuals but the current recommendations recommend similar regimens in HIV-infected and uninfected persons. Specific treatment recommendations have been published by the CDC most recently in the *Sexually Transmitted Diseases Treatment Guidelines, 2010* (Centers for Disease Control and Prevention. Sexually Transmitted Diseases Treatment Guidelines, 2010. MMWR 2010;59(No. RR-12) and can also be found at the CDC's website, http://www.cdc.gov/.

About one-third of patients with primary syphilis and two-thirds of patients with secondary syphilis develop an unusual reaction to treatment known as the Jarisch–Herxheimer reaction. This reaction typically consists of fever, chills, headache, worsening rash, and even hypotension in severe cases. The onset is usually within the first 4 h after the initiation of therapy, and the reaction resolves within 24 h. It is believed to result from the release of treponemal components, resulting in an endotoxin-like reaction. The Jarisch–Herxheimer reaction may be confused with an allergic reaction to penicillin but is not an indication for discontinuing therapy. Instead, treatment should continue and the patient should be treated with nonsteroidal anti-inflammatory medication, in addition to other supportive measures as indicated. Sexual partners of index cases should be sought out and treated based on the index patient's stage of infection.

Organism and Pathogenesis

T. pallidum is one of several treponemal species associated with human disease. Pathogenic species belonging to the genus *Treponema* are responsible for yaws, endemic syphilis, pinta, periodontal disease, as well as venereal syphilis discussed here. *T. pallidum* is an obligate human parasite, with no animal or environmental reservoirs. It is a helical corkscrew-shaped Gram-negative bacterium, 6–20 μm in length and 0.10–0.18 μm in diameter, placing it below the resolution of light microscopy. The unstained organism is best visualized by dark field or phase-contrast microscopy. *T. pallidum* generally stains poorly with many dyes but can be visualized using silver impregnation techniques. Like other Gram-negative bacteria, *T. pallidum* has an outer membrane, an inner membrane, and a thin cell wall consisting of peptidoglycan. The outer membrane is unusual, however, in that it lacks lipopolysaccharide, thus rendering it more susceptible to damage resulting from physical disruption or detergent use during handling. The outer membrane also contains relatively few proteins, which may account for the limited antibody response seen with this pathogen. *T. pallidum* is also unusual in that it has flagella, located in between the inner and outer membranes, which are

responsible for its characteristic motility, consisting of rapid rotation along its longitudinal axis, as well as bending and flexing. This motility is believed to play an important role in the organism's ability to invade and disseminate.

T. pallidum is a fastidious organism and has not been successfully cultured *in vitro*, and thus diagnosis depends on direct visualization and serologic testing. For study purposes, viable organisms can, however, be maintained for several weeks in tissue cell culture. The organism appears to be microaerophilic and is very sensitive to environmental conditions, being easily inactivated by mild temperature fluctuations, desiccation, and chemical agents including most disinfectants. These characteristics have hampered the study of the organism. Recently, the complete genome of *T. pallidum* was successfully sequenced. The genome consists of 1 138 006 bp containing 1041 predicted coding sequences. The information gained from sequencing has helped to elucidate systems for DNA replication, transcription, translation, and repair, as well as possible virulence factors and metabolic processes. Potential virulence factors include a family of 12 membrane proteins and several putative hemolysins. Catabolic and biosynthetic capabilities appear to be limited. Glucose is the main carbon and energy source for *T. pallidum*, although pyruvate appears to be an alternative.

The pathogenesis of *T. pallidum* is not well understood. Following penetration of mucosal surfaces, *T. pallidum* appears to adhere to host cells and then begins multiplication. The organism has been shown to adhere to a variety of cell types, perhaps through an interaction with fibronectin and host cell receptors. After multiplication, the organisms disseminate via the bloodstream and invade distant organs, aided by their unusual motility. Lesion resolution is thought to be the result of cell-mediated immunity, involving phagocytosis by macrophages activated by lymphokines and opsonic antibodies, released from antigen-specific sensitized T and B cells. Despite the destruction of millions of organisms, some persist and eventually cause the later stages seen in syphilis. There are several possible mechanisms by which the organism escapes the host immune response, probably the most important of which is the paucity of outer membrane proteins that would ordinarily provide ample targets for the immune response. In addition, *T. pallidum* may also have some surface components that inhibit complement-mediated killing. Secondary syphilis results from the dissemination and further multiplication of persistent organisms. Some manifestations may also result from immune complex deposition, especially with regard to the skin and kidney involvement seen at this stage. After a period of latency, persisting organisms may cause damage to the central nervous system, the cardiovascular system, and other organs in patients with tertiary syphilis. Here, much of the damage seen is likely due to the associated immunological response of the host.

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Single-Particle Cryo-Electron Microscopy

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Abbreviations

3D	Three-dimensional
CCD	Charge-coupled device
CMOS	Complementary metal oxide semiconductor
cryo-EM	Cryo-electron microscopy
CTF	Contrast transfer function
DQE	Detective quantum efficiency
DW	Dose-weighted
EM	Electron microscopy
NMR	Nuclear magnetic resonance

Perspectives and Overview of Single Particle Cryo-Electron Microscopy

Macromolecules, as they interact in the cell, drive the processes that constitute life in bacteria, plants and animals. They function as architectural elements, tracks, transporters, catalysts, receptors, channels, and storage compartments. They provide mechanical support, generate movement, catalyze thousands of essential reactions, and control growth, to name some of their roles in the cell. The function of a macromolecule as it interacts with others is constituted by its unique three-dimensional structure and its propensity to undergo conformational changes in a highly specific way. Many macromolecules or supramolecular assemblies are considered "molecular machines" as they contain moving domains and perform work cycles in a highly processive way. Biophysical methods to determine their three-dimensional structure include X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy (cryo-EM). To date, most of the structures in the public database by far (~140,000) have been determined by X-ray crystallography. This method requires the macromolecules to be packed into highly-ordered 3D-crystals, which rarely exist in nature. Obtaining such highly organized crystals, however, is a major challenge for many macromolecules. Single-particle cryo-EM is rapidly becoming a preferred method for structure determination because of the ability of the technique to image molecules without the need for crystallization. These include large, multi-component, and flexible structures, molecule-ligand combinations in multiple stoichiometries, as well as many membrane proteins.

The single-particle approach to visualization is based on the assumption that a given molecule exists in numerous "copies" randomly oriented in the sample, and its images can be interpreted mathematically as a collection of projections of a rigid body (or several distinct bodies) in different orientations. In single-particle cryo-EM, specifically, the EM grid is prepared by pipetting the sample containing the molecules onto the grid, blotting off excess liquid, and plunging the grid rapidly into a cryogen at liquid-nitrogen temperature. Under those conditions, water is transformed into vitreous ice, which closely resembles the liquid state, without expansion of volume characteristic for crystalline ice. The EM grid so prepared is then imaged at liquid-nitrogen temperature in the transmission electron microscope, yielding a data set of projection images. Using a variety of program packages, one or several three-dimensional images (often referred to as "reconstructions" or "density maps") are reconstructed from these projections, providing the basis for modeling the structure of the molecule. Depending on the resolution of the reconstruction, modeling entails the use of existing structures in the database ("flexible fitting") or sequence-directed fitting of structural elements (amino acids for proteins, nucleotides for RNA). Structural details at resolutions in the range 2–4 Å have now been demonstrated for an increasing number of biological macromolecules. Notably, even individual water molecules and ions at the expected coordinating distances can be observed within the density maps.

Principle of Cryo-EM of Biological Molecules

For electrons to travel in the column of the electron microscope, a high vacuum must be maintained. Unlike most samples in the Materials Science field, biological molecules usually are hydrated. The difficulties of imaging them were initially overcome by using

a technique termed *negative staining*, where heavy-metal salt is added to the solution of molecules, and the sample is air-dried on the grid. A layer of heavy-metal salt forms around each molecule, preventing its collapse and, at the same time, providing strong contrast through stain exclusion. However, the molecule is dehydrated and deformed in the process, and interior details of the structure are not visualized.

An efficient way to overcome the problem of vacuum incompatibility and preservation of native structure is the frozen-hydrated specimen preparation technique, developed by Jacques Dubochet and colleagues. In this method, the biological molecules are embedded in a thin layer of ice that is kept at a temperature where evaporation is negligible. The term "cryo-electron microscopy," abbreviated cryo-EM, has been coined to describe this preparation and imaging method. It is synonymous with the less often used term "electron cryomicroscopy," also abbreviated "cryo-EM."

Cryo-EM Specimen Preparation

The basic specimen preparation technique involves an apparatus that rapidly plunges the grid into liquid ethane held at a temperature in the range of −170 to −180°C (Fig. 1). Under these conditions, vitreous ice is formed, and the formation of crystallized ice, deleterious for the molecule, is prevented. First, a droplet of the liquid sample (2–3 μL) containing the molecules is applied to the EM grid that is mounted on tweezers fastened to a vertically-mounted rod. Excess liquid (∼99%) is blotted off such that only a thin liquid film remains (ideally in the range of 800–1000 Å). Next, the EM grid is plunged into liquid ethane through the release of the gravity-operated rod. After the plunge, the grid is first transferred into a storage box containing liquid nitrogen, from which it is later transferred to a cryo-specimen holder, to a cartridge, or an autogrid, depending on the type of electron microscope. In all these transfers, ideally, the grid is always kept at the temperature of liquid nitrogen. The grid is finally inserted into the EM.

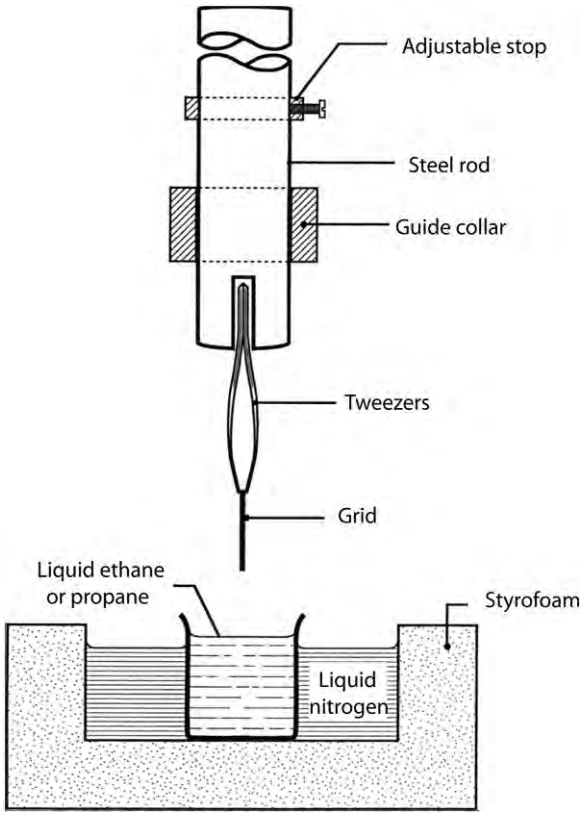

Fig. 1 Schematic diagram of a freeze-plunge apparatus. The EM grid, on which the specimen is suspended within a thin water layer, is held at the tip of tweezers, which are in turn mounted on a steel rod that is held vertically, in a position to fall under gravity. The thickness of the water layer is controlled by blotting off excess sample prior to plunging. As the rod is released, the grid is rapidly plunged into a bath of liquid ethane or propane at liquid-nitrogen temperature. Owing to the rapid decrease in temperature, the water turns into vitreous ice, which has properties akin to those of liquid water. Most importantly, no crystals are formed, and a disruption of the molecule is avoided.

The correct thickness of the ice in which the molecules are suspended is critical for obtaining interpretable images. With increasing thickness the proportion of inelastic to elastic scattering increases. Since only the former carries useful information, the thickness is restricted to less than 2000 Å, beyond which the sample essentially turns electron-opaque. On the other hand, a thickness close to, or smaller than, the size of the biological molecule will lead to the formation of damaged, partially "freeze-dried" samples, and images bearing little resemblance to the molecule. The main parameters determining the thickness of the vitreous ice layer are blotting time, temperature, humidity, and the length of time the blotted sample is exposed to these conditions before the fast plunge.

The purification of biomolecules, especially membrane proteins, often poses problems such as limited solubility of the protein in water, limited purification yield per batch, and the difficulty to closely match the physiological environment. Due to these problems the grid preparation often results in unproductive sessions on the electron microscope. Technology is now under development that allows smaller amounts of sample to be used to prepare the cryo-EM grid, and to use the large surface area of the grid for trying out multiple buffer conditions. Along these lines, two promising techniques have been developed, both involving robotic hardware: (1) Spotiton and (2) cryoWriter. Both of these techniques are automated, require no blotting, and only need pico- to nano-liter amounts of volume to prepare each grid.

Time-Resolved Cryo-EM Specimen Preparation

In time-resolved EM studies, a biological reaction is stopped at one or multiple time points by fast-freezing. Biologically relevant fast reactions, on the time scale of tens of milliseconds, can be studied by cryo-EM, because the rapid-freezing step itself takes merely a fraction of a millisecond. However, imaging snapshots of such fast reactions require a means of mixing, reacting and depositing the product on the grid in a fast, controlled way. In the mixing-spraying method developed by Lu *et al.* mixing and reacting are achieved in a monolithic silicon chip, a microfluidic device with two inlets for solutions to be mixed and one spray outlet which sprays the reaction product onto the grid with the help of inert driving gas (Fig. 2). While the time for mixing itself is fixed and in the range of 0.5 ms for all chips, the mean reaction time varies for different chips and is defined by the length of a

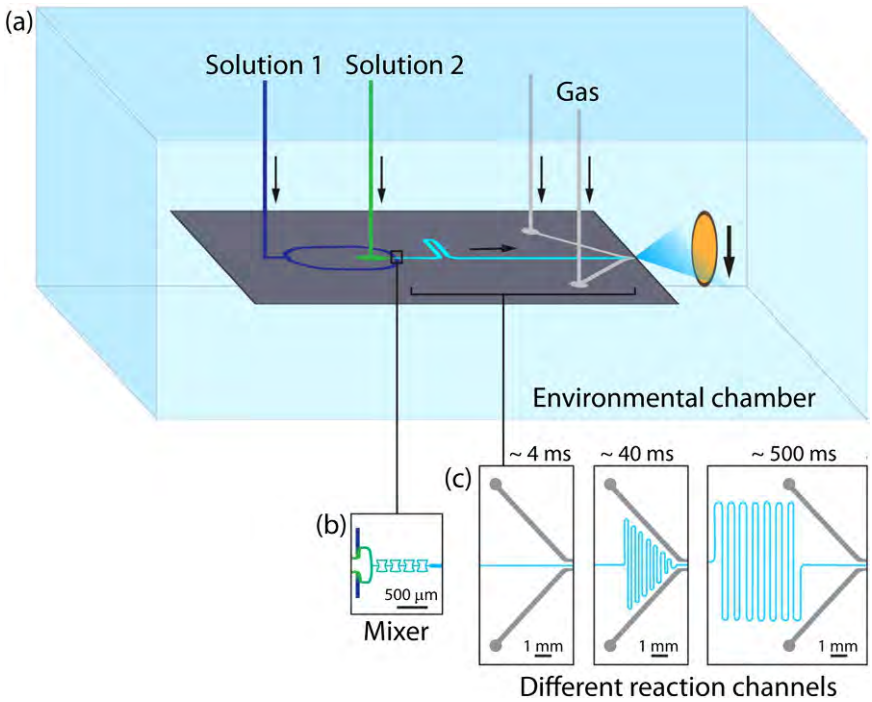

Fig. 2 Experimental setup of the mixing-spraying method and the design of the mixing-spraying chip. (a) Experimental setup. Two solutions (1 and 2) are injected into the mixing-spraying chip, mixed and reacted for a short, defined time. The mixture solution meets compressed nitrogen gas (Gas) and is sprayed into a plume of droplets, which hit the EM grid as it is plunged into the cryogen. The resulting grid is covered with thin blobs of vitreous ice in which the molecules are trapped. (b) Design of the mixer. The two solutions which enter with a total flow rate of 6 µl/s are mixed completely within 0.5 ms in the four tandem butterfly-shaped mixer channels. (c) Design of the reaction channels with different lengths. The length of the reaction channel is varied to achieve different reaction times (from 4 to 1000 ms) on different chips.

meandering or spiraling reaction chamber, which in the present design can be varied in the whole range from 4 to 1000 ms. The combined time of flight for the spray and the plunging of the grid adds another 5–40 ms, depending on the geometry of the apparatus.

Unlike the conventional pipetting-blotting-plunging technique, the spraying-plunging method allows the sample to be deposited on the grid in a controlled way. Controlling parameters are driving gas pressure and distance between the spray nozzle and the grid. With a series of microfluidic chips at different reactions times, all short-lived intermediates on the reaction pathway, along with the structures of the initial components and the end products, can be visualized. In the first biological application of this technique, short-lived intermediates during the processes of translation initiation and termination have been visualized. Future developments in the time-resolved method include reduction of sample consumption, shortening of the minimum effective reaction time, and optimization of droplet size and distribution on the EM grid.

Imaging in the Electron Microscope

Electron microscopes use electrons to image the sample. As negatively charged particles, they are repelled by electrons surrounding the nucleus of atoms. This interaction causes the incoming electrons to scatter. There are three possible outcomes. If the electrons pass through in between atoms and do not interact with them at all, they are called unscattered electrons. The second outcome is a scattering event without dissipation of energy ("elastic scattering"), such that a coherent relationship is maintained between scattered and un-scattered beam, the origin of productive, high-resolution image formation. The third outcome, in contrast, is a scattering event accompanied by energy loss ("inelastic scattering"), leading to an incoherent relationship between scattered and un-scattered beams and making no productive contribution to the image.

Elastically scattered electrons undergo a phase shift by $\pi/2$. Further phase shifts are introduced by the wave aberrations of the lens – mainly spherical aberration – and by operator-controlled defocussing. The size of these additional phase shifts is dependent on the scattering angle. In bright-field imaging, the normal imaging mode, phase contrast is created by the interference between scattered and unscattered electrons. Phase contrast is alternately positive and negative in successive zones of the Fourier transform of the image, with bands of zero contrast in between, as described by the (phase) contrast transfer function (CTF). In addition, a small contribution to the total contrast comes from amplitude contrast.

The CTF is a function of the spatial frequency, or the radius in the Fourier transform of the bright field image, and it depends on two parameters: the spherical aberration constant of the objective lens, and the defocus setting. Without correction for the effects of the CTF, neither the images nor the 3D reconstruction derived from them will be faithful representations of the object (Fig. 3). One of the problems of bright-field imaging, reflected by the shape of the CTF, is the fact that low spatial frequencies of the object's Fourier transform, representing the overall contrast of the particle, are strongly suppressed. This affects the ease with which small particles can be located in the micrographs by particle-picking software.

For the purpose of CTF correction, a series of images are taken in the EM with different defocus settings, so as to cover the entire Fourier domain up to the band limit (the spatial frequency limit up to which information is present) without any gaps. The information from this "defocus series" is then merged in Fourier space using a weighting function termed *Wiener filter*.

In instruments equipped with a *phase plate*, an extra $\pi/2$ phase shift is introduced in the path of the primary beam, resulting in constructive interference between the primary beam and elastically scattered electrons starting at low spatial frequencies. As a result, particles stand out strongly in the image from the background, and even small molecules are easily picked by automated software. The Volta phase plate (Thermo-Fischer, Waltham, MA, United States), the only commercially available device, comprises a thin ($\sim$10 nm) amorphous carbon film constantly heated to $\sim$250°C. The phase shift is created "on-the-fly" by the interaction of the central diffraction beam with the film. However, exposure to the electron beam changes the properties of the carbon film, changing the phase shift over time and requiring periodic adjustments.

The main limitation to directly visualizing high-resolution details of individual biological molecules, as compared to conventional electron microscopy in the Materials Science field, is the damage inflicted by the electrons at the large dosages required to define the image statistically. Thus radiation damage limits the information in electron micrographs of biological specimens. Electron diffraction measurements at the beginning of the 1970s by Robert M. Glaeser and coworkers showed the electron diffraction patterns of 2D crystals formed by L-valine to disappear after exposure to as little as 6 $e^-/Å^2$. Crystals of the less sensitive adenosine ceased to diffract at an exposure of about 60 $e^-/Å^2$, still much lower than the "normal" conditions for taking an exposure.

There are three categories of damage caused by inelastic scattering, the destructive interaction of electrons with frozen-hydrated specimens: (1) primary interactions: excitation, ionization or displacement of an atom; (2) secondary effects: further chemical reactions caused by the secondary electrons and free radicals in the specimen, for example, bond breakage or cross-linking; and (3) tertiary effects: damage from temperature rise, electrostatic charging and morphological change. Cooling to low temperature increases the biological material's resistance to radiation damage. The reason for the reduction of damage is that free radicals produced by ionization during electron irradiation are trapped under these conditions, preventing, or at least reducing, the visible damage to the structure. Even with this provision, it is necessary to reduce the electron dose to a range below $\sim$30 electrons/$Å^2$, resulting in so-called *shot noise* superimposed on the signal. This noise is effectively eliminated as a result of averaging, by using large numbers of projections of the molecule in computing the reconstruction.

Energy filters are devices whose purpose is to block electrons that have suffered energy loss ("inelastic scattering"), and therefore do not contribute in a coherent way to the image of the molecule, but rather to the background. In thick (> 0.25 μm) specimens, contributions by inelastic scattering to the image are strong, and may make the image uninterpretable. There are two types of energy filter that have been developed for TEM, "in-column" and "post-column". The in-column filter has the spectrometer built into the path of the electrons in the column. The post-column energy filter is a "magnetic prism" mounted right below the place where the final image is formed in normal EM. The main application of energy filters is in electron tomography of cell sections, which are at the crucial thickness range where inelastic scattering is starting to overwhelm the image.

Recording Media

For decades, the only recording medium for EM was the photographic film. As digital processing of micrographs started, the direct recording of images in the EM using a fluorescent screen with fiber-optic coupling to a charge-coupled device (CCD) became an attractive alternative, despite the shortcomings of this recording medium. These shortcomings were limited resolution or small field size, and relatively poor detective quantum efficiency (DQE), a measure of the efficiency of the detector to record quantums of radiation. Recently, in 2012, the most significant advance in recording has been made by the introduction of direct electron detection cameras. These utilize a complementary metal oxide semiconductor (CMOS) chip with a direct detection device (DDD) sensor, and have a performance far superior to film and CCD cameras.

In direct electron detection cameras, the capture of multiple frames is necessary to avoid saturation of electron counting. A high internal frame rate of 50 per second (Falcon 3EC, Thermofisher) to 1500 per second (K3, Gatan) is used so that in each frame the individual primary electron events can be identified and counted. The capture of multiple frames also offers two advantages. First,

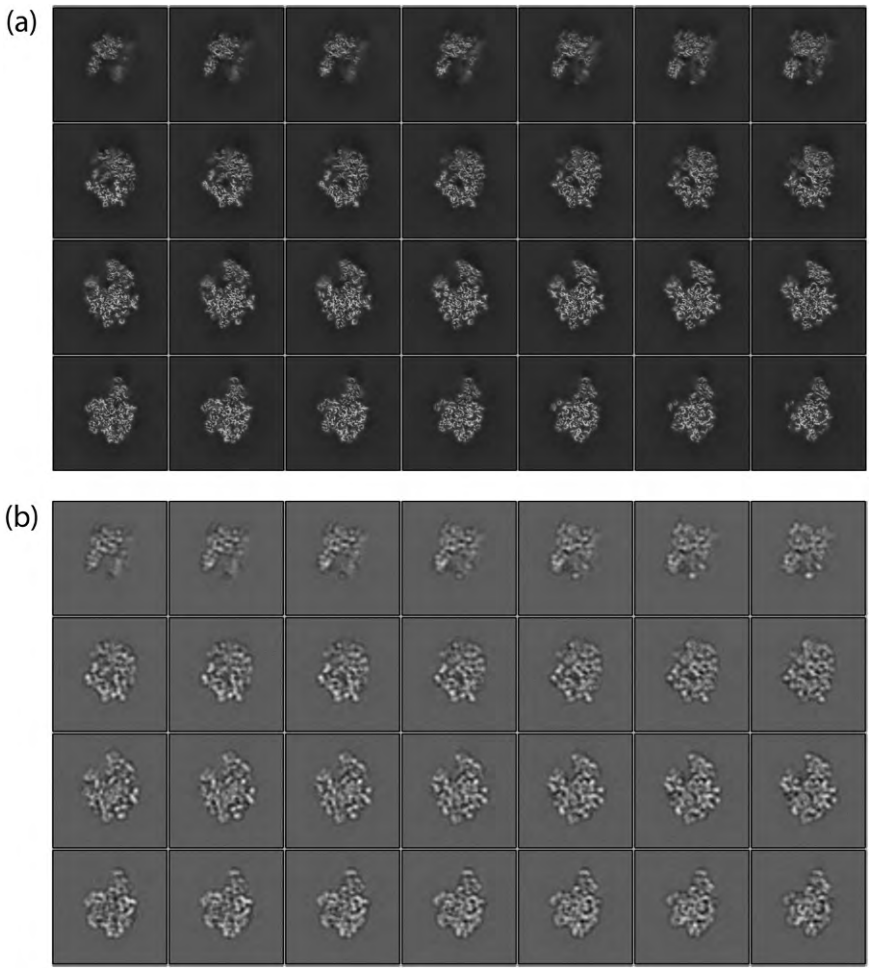

Fig. 3 Effect of the CTF. Ribosome reconstruction with (a) and without correction for the effect of the CTF (b).

by selective use of a subset of frames for reconstruction, one can minimize radiation damage to the specimen. Secondly, stage drift and beam-induced motion of particles in vitreous ice can be readily compensated. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM.

Image Processing

Raw images of the sample recorded do not allow us to get immediate insights, for three reasons: (1) they are extremely noisy as a consequence of the low dose required to curb radiation damage to the molecule, (2) the information about the molecule is scrambled on account of the CTF, and (3) they depict only two-dimensional projections of the structure. To obtain noise-free unscrambled three-dimensional information for the sample, we need to do an extensive amount of image processing. There are numerous steps of image processing that bring us from the raw data to the finished product, the three-dimensional image (Fig. 4).

The first step is to align frames recorded in movie mode to remove, at least partially, beam-induced motion. Algorithms for correcting beam-induced motion have been developed by many groups, for example unblur, Motioncor2 and optic flow. The most commonly used algorithm for drift correction of the movies recorded from the Gatan K2 camera is Motioncor2. Here, the frames are divided into patches, and drift within each patch is separately determined. The reason to do so is for correction of anisotropic local motion. The molecule has multiple modes of movement, shifting in the image projection plane (X-Y plane), shifting up and down along the Z-axis, and rotation. Then all frames are summed with or without radiation-damage weighting. As mentioned in the previous section, recording in movie mode allows the effective radiation damage to be minimized in the three-dimensional image. High-resolution features (i.e., the conformation of amino acid side chains) decay faster than low-resolution features (i.e., backbone structure of a protein). This means that for a given dose, low-resolution information is still present in the later frames, but high-resolution information is lost.

The next step is to estimate the CTF. Accurate estimation of the CTF and properly correcting for it is extremely important for the quality of the high-resolution reconstruction. The principle for estimation of the contrast transfer function is to maximize the cross-correlation of a simulated power spectrum based on the estimated CTF with the power spectrum of the experimental image. Many different programs perform this task. The most popular ones are CTFFIND3, CTFFIND4, GCTF and Warp.

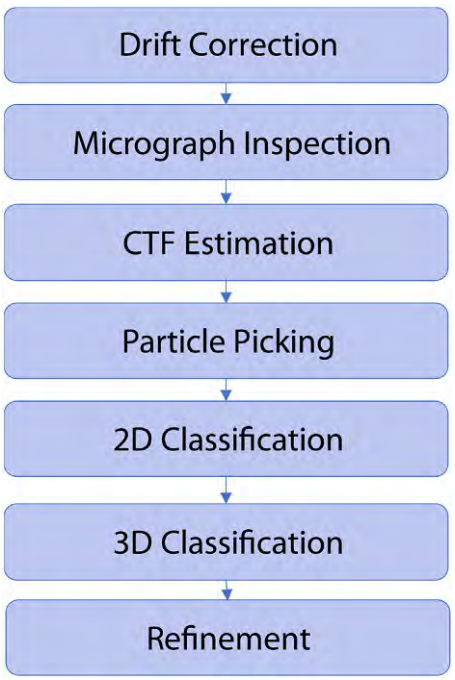

Fig. 4 Cryo-EM single particle data processing work flow. Microscope beam-induced drifts are corrected by dividing the frames into patches and calculating each drift. All frames are summed with or without radiation damage-weighting. The micrographs are visually inspected to remove those with crystalline ice and large drift, etc. Next, the CTF of each micrograph is estimated. Particles are manually picked to create references. Auto-picking utilizes the references to automatically pick particles for all micrographs. The picked particles are extracted for further classification. The extracted particles are sorted into classes to remove classes with noisy or contaminated particles and any possible "false picks". Using a 3D reference map, the "good" classes are assigned to different 3D classes to marginalize orientation and class assignments of particle images. The accuracy of orientation is further optimized in 3D refinement.

The third step is to obtain the coordinates for projection images of molecules (particles) in each image. This step is usually referred to as *particle picking*. Over the years, many stand-alone software or modules of larger packages have been developed to pick particles semi-automatically or automatically. Various techniques including cross-correlation, variance mapping, intensity comparisons, texture-based methods, segmentation/edge detection have been explored and used in this step. Neural networks and deep learning are in increasing use for automatic particle picking.

No particle picking program is perfect. Each one has its own merits and drawbacks. Common issues are that too many false particles are picked or that too many true positives are missed. Part of the problem is that the molecules whose projections are picked as "particle" may be partially damaged due to radiation damage or damage resulting from interaction with the air-water interface. As a consequence, the delineation between "false" and "true" particles is blurred in practice.

In the following steps, individual particle images are extracted from the micrographs based on the coordinates determined in the particle picking step. Ideally, each particle image contains the particle in its center, and some background around the particle. Sometimes the particles are too crowded in the micrograph, and in that case an image based on the selection by particle picking may contain more than one particle. To remove the false picks, two-dimensional (2D) classification is applied to the extracted images. In the 2D classification step, all the extract particles are aligned and sorted iteratively into different classes representing different projections of the 3D object. In this classification, false picks are readily recognized as outliers as they lack common features and fail to form high-resolution class averages with high numbers of class members.

After the particles in the "good" classes have been pooled together, a strategy for 3D reconstruction has to be determined based on the amount of prior knowledge about the molecule structure. Either a 3D reference map is already available, which might be a low-resolution map of the same or a similar sample, or a reference map has to be first created by some kind of bootstrap reconstruction technique.

In the latter case, there are several ways to obtain a 3D reference. One of these is the random-conical tilt method. In this method, one identifies a subset of particles with closely matching orientation on the grid. Two images are taken of the same field of particles, one at zero tilt angle, and one at a high tilt angle. With this collection geometry, the orientation of each particle of the subset can be determined, and a provisional reconstruction can be built that can serve as a reference for subsequent refinement. Another method uses a set of 2D class averages to build up a 3D map employing the technique of common lines. A third method starts with a small set of class-averages and builds a set of 3D maps by an iterative procedure.

A complication arises from the fact that samples are often heterogeneous. Molecules may possess compositional or conformational heterogeneity, or both. In this case, images need to first be sorted into homogeneous classes in a step termed 3D classification. In this step, using a maximum-likelihood approach, probability distributions for both orientation and class assignments for each particle image are determined, and multiple 3D reconstructions are refined at the same time. In the first iteration, all particles are first assigned randomly to each class and then refined against the low-resolution 3D reference. For a few iterations, global search is performed with an angular sampling rate of 15° or 7.5°. In these coarse beginning steps, large compositional or conformational differences are immediately distinguished. Because the actual number classes for a given specimen is unknown, one may need to determine it by trial and error, by repeating the 3D classification with different numbers of classes. If there are still small residual compositional or conformational differences within each class, indicated by a blurred region in the 3D reconstruction, finer classification – focusing only on the blurred region with smaller angular degrees 3.75 or 1.8 degrees – will be needed.

Orientation angles of all particles are further improved in accuracy in the refinement step, run for each homogenous class separately. All particle images, assumed to represent exactly the same object from different angles, are iteratively aligned to the reference. After each iteration, the reference is updated and the angular sampling rate is decreased (from 7.5 down to 0.45 degrees or smaller), resulting in improved resolution. For resolution estimation, the particle set is split into half sets that are refined independently. Thus, at each iteration of the refinement, the resolution can be estimated from a comparison of the running half set reconstructions in Fourier space. To this end, the Fourier Shell Correlation (FSC) is commonly used. For truly independent half set reconstructions, FSC = 0.143 is an accepted resolution criterion.

Several comprehensive software packages exist with specialization in electron microscopy, among these CISTEM, EMAN, FREALIGN, IMAGIC, JSPR, RELION, SPIDER, THUNDER, and XMIPP. Each of these is a more or less tightly integrated collection of modules, designed to perform the entire processing job. However, there is an increasing trend toward the use of modules from multiple platforms, mixing and matching these as they have different strengths and weaknesses in different areas of the methodology. APPION and SCIPION are examples of platforms designed for this purpose. Both provide transparent interconversions between the different file formats and angle conventions used in the various packages.

Structure Interpretation

Some medium- or low-resolution 3D maps represent complexes formed by molecules of known atomic structure. For interpretation of such 3D maps, we dock the known structures into the maps. Integration of the knowledge gained from other techniques such as cross-linking or mass spectroscopy is becoming increasingly important in the interpretation of medium- or low-resolution 3D maps.

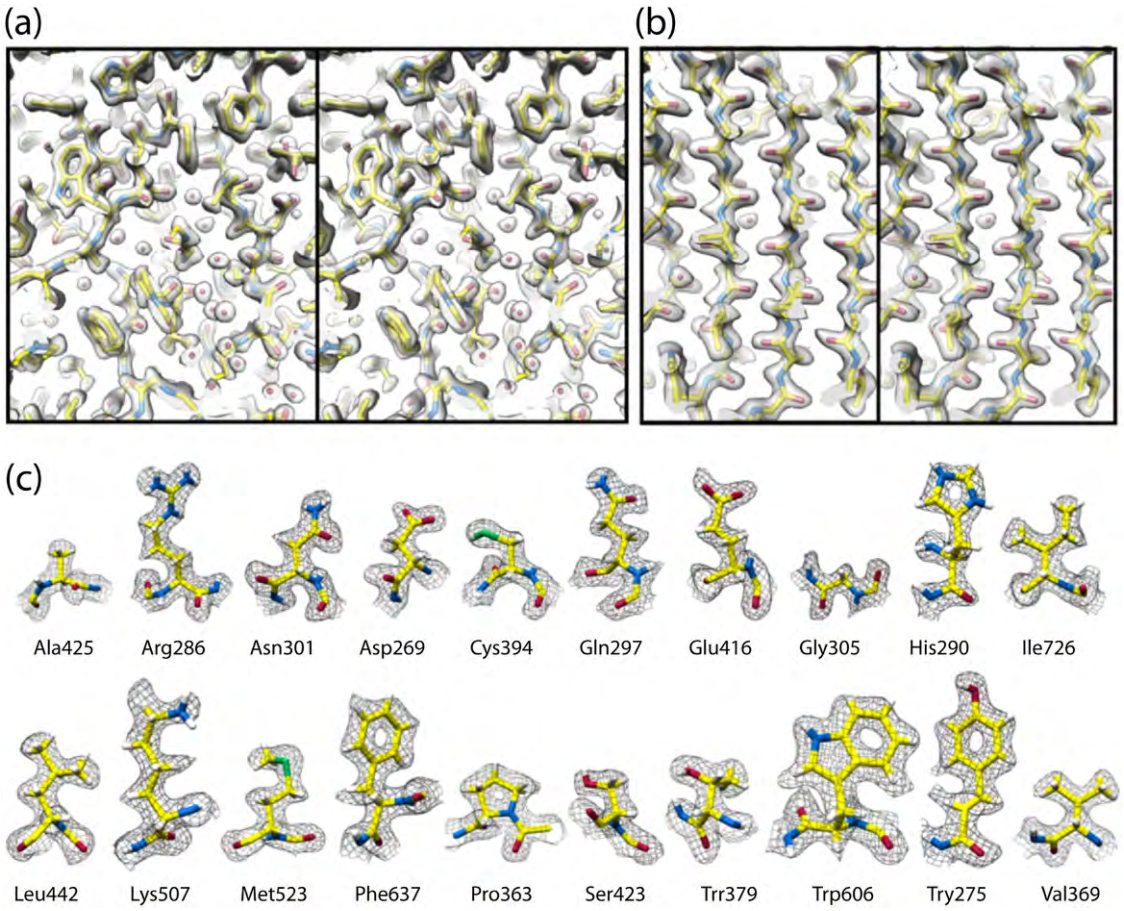

Fig. 5 High-resolution features in a cryo-EM single particle reconstruction of AAV2L336C at 1.86 Â resolution. (a) Stereo view of a slice through the map and model containing both amino-acid residues and water molecules. (b) Stereo view of a slice through a beta sheet. (c) Map densities for each of the 20 types of amino-acid residues. The amino acid residues are shown as stick representation and colored according to atom type: C = yellow, O = red, N = blue, S = green inside either a translucent solid density ((a), (b)) or black mesh density map (c). H = white atoms are displayed for (c). Figure reproduced with permission from Tan, Y.Z., Aiyer, S., Mietzsch, M., *et al.*, 2018. Sub-2 Å Ewald curvature corrected structure of an AAV2 capsid variant. Nature Communications 9, 3628.

However, if the 3D map approaches a resolution of 3.5 Å or better, *de novo* model building becomes possible. Segmentation is usually the first challenge in interpreting a 3D density map de novo. Because a 3D map often contains multiple subunits, segmentation is necessary to divide the 3D map into single polypeptide or nucleic acid chains (Fig. 5). At high resolution, the identity of each amino acid in a protein is readily distinguishable based on the shape of the side chain. Likewise, in RNA or DNA, the identity of the bases can be inferred from their characteristic signatures. Many model building tools are now available that originated from model building techniques in X-ray crystallography, such as Phenix and Coot. Some tools are for automatic model building, such as phenix autobuild, Buccaneer and Rosetta structure prediction.

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Solvent (Acetone–Butanol: AB) Production☆

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Abbreviations

AB	Acetone–butanol (or solvent)
ABE	Acetone–butanol–ethanol (or solvent)
ATP	Adenosine-5′-triphosphate
BOD	Biological oxygen demand
BSH	Barley straw hydrolysate
CF	Corn fiber
CFH	Corn fiber hydrolysate
CS	Corn stover
CSH	Corn stover hydrolysate
CSTR	Continuous stirred tank reactor
DDGS	Dry distillers' grains and solubles
DOW	Domestic organic waste
EMP	Embden–Meyerhof-pathway
FW	Food waste
HMF	Hydroxymethyl furfural
IBE	Isopropanol, butanol, ethanol
LCS	Liquefied corn starch
MW	Municipal waste
PEP	Phosphoenolpyruvate
PTS	Phosphotransferase system
SDSM	Spray dried soy molasses
SLCS	Saccharified liquefied corn starch
SSB	Sweet sorghum bagasse
WS	Wheat straw
WSH	Wheat straw hydrolysate
WWI and WWII	World War I and World War II

Defining Statement

Production of butanol (acetone–butanol–ethanol (ABE)) from agricultural products employing novel technologies. The article details production of AB from various novel substrates, in various types of reactors, and using a number of product recovery technologies. In this article, a brief description of various cultures that are available for butanol production has been given.

Introduction

Butanol (acetone–butanol–ethanol, ABE or AB or solvents) production is one of the oldest fermentation processes employed for commercial production of a chemical to benefit mankind. Production of butanol was discovered by Pasteur in 1861. Its production by fermentation comes second to ethanol in importance and history as there were commercial plants that were operational during World War I (WWI) and WWII. It should be noted that after the WWII (between the 1950s and 1960s), butanol fermentation could not compete with petrochemically derived butanol due to the development of petrochemical industries. The last biobased butanol plant ceased operations in the early 1980s in South Africa due to a shortage of molasses, the feedstock for this fermentation, brought on by drought, and the high cost of recovery. Butanol can be produced by fermentation employing a number of microorganisms

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such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, *Clostridium saccharobutylicum*, and genetically altered *Escherichia coli*. The typical ratio of ABE in the final product is usually 3:6:1 with maximum concentration of total solvents (ABE) being 20 g L^{-1} when using traditional strains and traditional batch fermentation process. Recovery of butanol from such a dilute product is cost intensive when recovered by distillation (traditional technology).

Currently, butanol is produced using either the oxo process from propylene (with H_2 and CO over a rhodium catalyst) or the aldol process starting from acetaldehyde. Acetone (a co-product of the butanol fermentation), is produced either by the cumene hydroxide process, or the catalytic dehydration of isopropanol. These chemical synthetic routes have proven to be economically superior compared to the fermentation based processes. Since 1990, production of butanol in the United States has been constant (1.20×10^9 kg $year^{-1}$), while worldwide production has fluctuated slightly. The worldwide annual production of acetone and butanol has been of the order of 2.1×10^9 and 2.5×10^9 kg, respectively. Butanol has numerous applications as an automotive fuel and in the chemical industry. When used as an automotive fuel butanol contributes to clean air by reducing emissions such as unburned hydrocarbons in the tail pipe exhaust. Butanol possesses superior fuel properties compared to ethanol. As compared to ethanol's research and motor octane values of 111 and 92, these values for butanol are 113 and 94, respectively. The heat of vaporization for butanol is 141.3 k cal kg^{-1} as compared to 204.1 k cal kg^{-1} for ethanol. In addition, the high boiling point (118°C) and lower vapor pressure for butanol should enhance cold starting. There are various other properties that make butanol a superior fuel. It has 30% higher energy content per liter as compared to ethanol, is less miscible with water, and more miscible with gasoline and diesel fuel.

Recently, there has been renewed interest in fermentation derived butanol both as a potential fuel and as a chemical feedstock. Continuously rising and/or fluctuating prices and increased dependence on foreign fuel have been major driving factors for the increased interest in butanol as a motor fuel replacement. Increased oil prices in 1973 resulted in a revival of research activities on a number of fermentations, including ethanol and butanol, with a long range view toward becoming less dependent on foreign oil. As a consequence, research was focused on developing technologies to produce fuels and chemicals from readily available and abundant renewable agricultural resources. As a result some degree of success was achieved in numerous countries when bioconversion programs involving alcohol fuel production were examined. Brazil had introduced its ethanol production program in 1977 followed by the United States. Soon after this low oil prices were restored. However, constant conflicts in the oil supply regions and rising oil prices have reminded the world that alternate energy sources be sought for future energy independence. With this view the United States has initiated various energy initiatives including replacing 30% of transportation fuel by ethanol by 2030. In 2006 approximately 14.62×10^9 kg (4.7 billion gallons) of ethanol was produced from corn (in the United States) which is approximately 3.5% of total fuel consumption (413.7×10^9 kg or 133 billion gallons) in the United States. In 2015, annual production of ethanol from corn increased to 46.0×10^9 kg (14.8 billion gallons). It is anticipated that up to 65.3×10^9 kg (21 billion gallons) (15%) of ethanol can be produced annually from corn without affecting food and feed supply. Further increases in ethanol production would likely require the use of cellulosic biomass such as corn fiber, corn stover, wheat straw, or energy crops like switchgrass and miscanthus.

Intensive research efforts were focused on this fermentation during the early 1980s with the overall objective to make butanol fermentation economically competitive. As a result of research intensification, a number of problems associated with this fermentation were identified including cost ineffective recovery of butanol by traditional recovery technology (distillation), low butanol concentration in batch reactor (<20 g L^{-1}), and low productivity (<0.50 g L^{-1} h^{-1}) due to butanol inhibition. An account of some of these problems and their possible solutions has been documented in literature. Because of this research, highly productive continuous reactor systems and butanol recovery technologies were developed not only to recover butanol economically but to relieve butanol inhibition in the reactor systems. This gave birth to "integrated" technologies where production of butanol was combined with product recovery. Combination of production and recovery technologies resulted in the use of higher amount of substrates, improvement in productivities, and achieving a more concentrated product stream to be presented for further recovery and purification. These research and development activities were aimed at use of substrates such as whey permeate (from dairy industry) and glucose (from wet milling corn industry).

In the author's opinion, research activities slowed during 1990s due to the reduction or stabilization of gasoline prices. The years 2002–07 once again witnessed sharp increases in gasoline prices which prompted a considerable increase in research activities in this fermentation, in particular bioconversion of economically available lignocellulosic biomass to butanol. Substrates such as corn fiber, wheat straw, barley straw, corn stover and sweet sorghum bagasse (SSB) have been used to produce butanol. Additionally, waste streams such as food waste have also been used. Although productive reactors were not used for these applications, some recovery technologies have been successfully integrated for production of butanol from some of these agricultural residues. In this article, it is the author's aim to give latest information to the reader on production of these biofuels, in particular butanol, and various process technologies such as recovery of butanol using adsorption, pervaporation, liquid–liquid extraction, and gas stripping. Process integration is a fascinating technology which allows use of concentrated sugar solutions, thus reducing process streams and economizing production of butanol from agricultural residues, and wastes or by-product streams. As a result of recent technological developments in butanol fermentation, and recovery, Dupont (United States) and British Petroleum (United Kingdom), have been working on the commercial development of butanol or isobutanol production processes from biomass. Other ongoing efforts relating to the commercial production of butanol from biobased substrates include those of Green Biologics (1-butanol or n-butanol), and Cobalt Technologies (1-butanol). It is noteworthy that China has emerged as a large scale producer of butanol from biobased materials such as corn (Cathay Industrial Biotech). Currently, Dow, Exxon Mobile, BASF, and Eastman Chemical Companies produce butanol on a large scale by chemical synthesis.

Problems of AB Fermentation

Traditionally AB is produced in batch reactors. A typical batch fermentation system is initiated with 60 g L^{-1} substrate, usually glucose, cornstarch, molasses, or whey permeate. During the course of fermentation (36–72 h) this sugar is converted to <20 g L^{-1} ABE, resulting in a productivity of 0.5–0.6 g L^{-1} h^{-1} and a yield of 0.30. Such a low product concentration is due to product (butanol) toxicity. Inhibition begins at a butanol concentration of as low as 10 g L^{-1} and stops fermentation at 20 g L^{-1} total ABE. As a result batch AB fermentation suffers from low productivity (0.5–0.6 g L^{-1} h^{-1}) as compared to ethanol (usually 2–3 g L^{-1} h^{-1}), and results in the use of dilute sugar solution (60 g L^{-1} as compared to approximately 150 g L^{-1} for ethanol), and low yield. In ethanol fermentation, a product concentration as high as 130 g L^{-1} can easily (can be higher than that) be achieved. Additionally, recovery of AB from dilute product stream requires a large amount of energy for product separation. The author is not aware if water can be recycled from this process in commercial scale plants as all such plants are located in China. However, if water can be recycled, it would be a significant economic gain. The recycling of water was considered in some of our economic evaluations. For these reasons (low AB concentration in the broth, low productivity, low yield, lack of water recycle, and high recovery costs), economic viability of commercial butanol fermentation has been a challenge.

Traditional Substrates

Sugarcane Molasses

Cane molasses was successfully used in the commercial production of butanol in South Africa (National Chemical Products (NCP) Germiston, South Africa) until the early 1980s. Unfortunately, fermentation of butanol was terminated due to molasses shortage caused by severe drought. Molasses contains approximately 50% sucrose which can be hydrolyzed to glucose and fructose by butanol producing cultures. This substrate is easy to handle as it requires only dilution prior to fermentation. There are a number of molasses types which can be used including beet, blackstrap, and invert (high-test) molasses.

Whey Permeate

Whey permeate is a byproduct of cheese making industry which contains 44–50 g L^{-1} lactose, that makes it a suitable substrate for AB fermentation. The substrate (lactose) content in whey permeate varies from process to process. For other fermentations such as ethanol it is an unsuitable substrate (unless the medium is supplemented with additional sugar) because of its relatively low sugar content. However, whey permeate has numerous applications in the fermentation industry which have been documented in the literature. Various types of whey permeates have been used for the production of butanol using *C. acetobutylicum* P262 (the industrial strain) suggesting that it is a good substrate for this fermentation. However, in a batch reactor productivity of the order of 0.1 g L^{-1} h^{-1} is obtained. It should be noted that this substrate is a rich source of minerals and hence further supplementation with minerals to the fermentation medium is not required. Extensive studies have been reported on the use of this substrate for ABE fermentation process which included development of immobilized cell reactors and product recovery systems.

Soy Molasses

Spray dried soy molasses (SDSM) is a by-product of the soy processing industry and contains 746 g kg^{-1} sugar of which 434 g kg^{-1} is fermentable. The fermentable sugars that are present in soy molasses include glucose, sucrose, fructose, and galactose while nonfermentable sugars include pinitol, raffinose, verbascose, melibiose and stachyose. Soy molasses has been used to produce ABE using *C. beijerinckii* BA101. The culture was able to utilize fermentable sugars and produced 10.7 g L^{-1} ABE from 80 g L^{-1} SDSM. However ABE up to 22.8 g L^{-1} was produced when 80 g L^{-1} SDSM was supplemented with 25.3 g L^{-1} glucose suggesting that 80 g L^{-1} SDSM was deficient in fermentable sugars and that was the reason for poor (10.7 g L^{-1}) ABE production using SDSM alone as a carbon source. Further increase in SDSM concentration was not beneficial for growth or ABE production due to presence of excessive amount of salt in this substrate. It has been determined that *C. beijerinckii* BA101 was not able to ferment raffinose, pinitol, melibiose, verbascose, and stachyose due to its inability to hydrolyze the α-1-6 glycosidic bond present in these sugars. In order to be able to use these nonfermentable sugars a strain should be developed with a capability of hydrolyzing α-1-6 glycosidic bonds. Development of such a strain will result in the more complete utilization of sugars present in SDSM. Alternately, soy molasses should be supplemented with isoamylase or hydrolyzed using dilute/mild acid treatment.

Potatoes

Since clostridia possess strong amylolytic activity, substrates containing starch have been considered for this fermentation. These substrates include corn, rye, cassava, and potatoes. Potatoes and potato wastes are economically viable substrates. Although it has been suggested that this substrate has viscosity problems, both hydrolyzed and unhydrolysed potatoes have been successfully used for butanol production using *C. acetobutylicum* or *C. beijerinckii* strains. Using unhydrolyzed potatoes, an initial potato starch concentration of 45 to 48 g L^{-1} was used in the fermentation thereby producing up to 12 g L^{-1} ABE. Potato starch hydrolysate

obtained with α-amylase and amylo-glucosidase prior to the fermentation resulted in the production of 11.4 and 10.4 g L^{-1} total ABE, respectively. This suggested that hydrolysis prior to fermentation was not limiting the fermentation and the culture was able to hydrolyze potato starch to glucose efficiently during the fermentation.

Substrates Based on Grains

As stated above solventogenic clostridia can hydrolyze starch and produce ABE without a need for separate hydrolysis process. Hence corn, millet, wheat, rice and tapioca have been used as substrates for this fermentation. Butanol can also be produced both by using corn co-product from: (1) corn dry grind and (2) wet milling processes. During the dry grind process corn fiber and germ are not typically removed before fermentation. At the end of fermentation (after starch utilization during fermentation) corn fiber and other insoluble solids are removed by centrifugation, dried and sold as cattle feed. The dried solids are known as "Distillers Dry Grains and Solubles" or DDGS. On the contrary, during the wet milling process, corn fiber and germ are removed prior to fermentation. In this process cornstarch can be converted to any of the three products (liquefied cornstarch, glucose sirup, or glucose) each of which is fermentable by *C. beijerinckii* to produce butanol. It should be noted that often corn refineries add sodium metabisulfite during the wet milling operation as a corn kernel softening agent and preservative to the liquefied corn starch (LCS). The presence of sodium metabisulfite may interfere with the direct fermentation of the liquefied corn starch. However, glucose sirup or glucose does not contain any such fermentation inhibitors.

Cassava and Jerusalem artichokes have also been investigated as potential substrates for butanol fermentation. It has been reported that cassava alone was not a suitable substrate, but could be used successfully when supplemented with 20% corn meal or other sources of organic nitrogen. Jerusalem artichoke is a potential agricultural crop that contains carbohydrates that can be used for AB production. The carbohydrates present in the tuber occur mainly in the form of short oligomeric fructans which have an inulinic structure and must be hydrolyzed by acid or inulinase prior to AB fermentation. Also supplementation of the hydrolysate with corn or soy meal was reported to be necessary. The use of Jerusalem artichoke has also been investigated as a part of French research program on the production of fuel extenders from biomass. Hydrolysates were prepared using enzyme treatment with inulinase and, apart from ammonia, no nutritional supplements were added to the fermentation medium. After a period of 36 h, 23–24 g L^{-1} of solvents was obtained.

Liquefied Corn Starch

Liquefied corn starch (LCS) is a potential industrial substrate that can be used for AB production. It is a product of corn wet-milling process and is composed of 35–40% dry solids as reducing sugars and dextrins. In order to avoid microbial contamination, sodium metabisulfite, a microbial inhibitor, is added to this substrate. In one of the studies on the use of this substrate for butanol production, concentrations of glucose, reducing sugars, and sodium metabisulfite were measured to be 5.58±0.26, 337.5±2.12 and 0.71±0.10 g L^{-1}, respectively. It has been reported that this concentration of sodium metabisulfate is inhibitory for growth of *C. beijerinckii* BA101. Hence to reduce the inhibitory effect of this chemical on cell growth, it was diluted seven times. This resulted in the production of comparable (compared to glucose) amount of ABE in a batch reactor. This substrate was also saccharified (saccharified liquefied corn starch, SLCS) prior to feeding to a fed-batch reactor integrated with product recovery.

Novel Substrates

The high cost of substrates including molasses, whey permeate, corn, and starchy roots has been identified as a major factor affecting the economic viability of butanol production by fermentation. In order to produce butanol cost competitively, use of more economic substrates must be evaluated. These substrates include agricultural residues and energy crops such as corn stover, corn fiber, switchgrass, miscanthus, and wheat, barley and rice straws. It should be noted that butanol producing cultures are able to utilize a wide variety of carbohydrates (including hexoses, pentoses, and cellobiose) such as sucrose, glucose, fructose, mannose, lactose, dextrin, starch, xylose, and arabinose. The use of these carbohydrates by butanol producing cultures illustrates the potential to ferment sugars derived from hydrolysates of agricultural residues to butanol.

Corn Fiber

Recently, it was demonstrated that corn fiber (CF) could be used to produce butanol. Corn fiber was hydrolyzed using dilute (0.5%, v/v) sulfuric acid at 121°C for 1 h followed by cooling to 45°C and adjusting pH to 4.5. The mixture was then treated with enzymes (cellulase and cellobiase) for 72 h under agitation (150 rpm). Following hydrolysis, the pH of the mixture was raised to 6.5 followed by fermentation to produce butanol. The culture was unable to produce more than 1.7 g L^{-1} ABE, due to inhibition caused by salts (neutralization product) and other inhibitory products formed during hydrolysis. However, the hydrolyzate was successfully fermented after removal of these inhibitors. The inhibitors were removed partly by overliming the hydrolyzate and partly by treating it with a nonionic polymeric adsorbent resin (Amberlite XAD-4).

Wheat Straw

Attempts have been made to produce butanol from wheat straw (WS) hydrolyzate. WS was hydrolyzed using dilute (1%, v/v) sulfuric acid at 121°C for 1 h followed by cooling to 45°C and adjusting pH to 5.0 using 10 M KOH solution. The mixture was then hydrolyzed with enzymes (cellulase, xylanase, and β-glucosidase) for 72 h followed by filter sterilization. The hydrolyzate (pH raised to 6.8) was then fermented to butanol using *C. beijerinckii* P260. In these studies no inhibition due to salts or inhibitory products was observed. Rather fermentation was more rapid than control batch fermentation where glucose was used as a substrate. These studies demonstrated that hydrolyzates of some of the agricultural residues can be fermented to butanol with little inhibition. In addition to the novel substrates mentioned in this section (Section V) of the article, substrate such as distillers' dry grain solubles (DDGS) has been explored at the University of Illinois (Urbana, IL, United States) for butanol production using a number of clostridial strains including *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum* and *Clostridium butylicum*.

The composition of WS on dry basis is presented in Table 1.

Barley Straw

Although barley straw appears to be similar to wheat straw, its hydrolysate's fermentation characteristics were found to be different than wheat straw hydrolyzate. Prior to detoxification, barley straw hydrolyzate (BSH) did not produce more than 7.09 g L^{-1} ABE resulting in a poor ABE productivity of 0.10 g L^{-1} h^{-1}, which were improved to 26.64 g L^{-1}, and 0.39 g L^{-1} h^{-1}, respectively, upon detoxification using overliming technique. However, wheat straw hydrolyzate was successfully fermented to butanol without any detoxification and the culture produced 25.00 g L^{-1} total ABE. These results suggest that BSH can be fermented, however, it has to be done at the expense of additional, overliming, detoxification process step.

Corn Stover

Unlike wheat straw and barley straw hydrolysates, corn stover hydrolysates (CSH) were found to be difficult to ferment to butanol. In a CSH solution, the culture showed no cell growth and no fermentation prior to the removal of inhibitors by overliming. Upon inhibitor removal, the culture produced 26.27 g L^{-1} ABE with a productivity of 0.31 g L^{-1} h^{-1}. In this fermentation an ABE yield of 0.44 was obtained. Compared to the other substrates, CS hydrolyzate was relatively difficult to ferment.

Food Waste

Food waste (FW) is a substantial pollution problem in the United States and many other countries in the world, in particular when it is used to incinerate or landfill. In the United States alone more than 33 million (33×10^6) tons of food waste was generated in 2012, which included discarded food by food processing industries, restaurants, and consumers. It is assumed that approximately 33 million tons is generated each year. There are several major advantages with the use of FW for butanol production: (1) it contains significant amount of sugars or carbohydrates that can be used for this fermentation; (2) it does not require pretreatment as opposed to cellulosic feedstocks; (3) it contains proteins, fatty acids, and minerals that can be used as nutrients for cell growth and fermentation by the culture; (4) its availability is expected to be at much lower cost than cellulosic biomass; and, (5) it addresses environmental pollution problem. Keeping these advantages in view, we have successfully produced butanol from this substrate.

Municipal Waste/Domestic Organic Waste

Both Municipal Waste (MW) and Domestic Organic Waste (DOW) can be used as feedstocks for butanol production with similar kind of advantages listed in the above Section V(E). In a report on production of butanol from DOW, it was mentioned that this

Table 1 Composition of wheat straw on dry solid basis

Component	*Dry solids (%, w/w)*
Cellulose	48.6±0.3
Hemicellulose	27.7±0.1
Lignin	8.2±0.9
Acid detergent fiber	58.9±0.0
Neutral detergent fiber	86.6±0.1
Crude protein	3.5±0.1
Crude fat	0.5±0.0
Crude fiber	45.9±0.2

Source: Printed with permission from Elsevier. Saha, B.C., Iten, L.B., Cotta, M.A., *et al.*, 2005. Dilute acid pretreatment enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry 40, 3693–3700.

feedstock contained 25.1 g glucose, 8.4 g xylose, and 5.8 g other monosaccharides per 100 g dry matter. DOW was used to produce ABE and IBE (isopropanol, butanol, ethanol) with some degree of success. It is emphasized that every source of carbohydrates should be used to produce butanol.

There are other substrates that have been used for this fermentation including industrial wastes (starch based packing peanuts), orchard wastes such as apple drops and apple pomace, and sugarcane factory wastewater. Due to space limitation, details of these feedstocks are not presented in this article.

Hydrolysis Inhibitors of Agricultural Residues

As mentioned in the corn fiber section, dilute acid (0.5%, v/v) hydrolysis of this substrate resulted in poor fermentation of the hydolysate. In order to ferment CF hydrolyzate (CFH) to ABE, the CFH was treated with molecular sieve to remove fermentation inhibitors. Upon treatment, the CFH was fermented successfully, suggesting that inhibitors prevented fermentation of CFH. In another study, attempts were made to quantify effect of inhibitors such as salts, furfural, hydroxymethyl furfural (HMF), syringealdhyde, and acetic, ferulic, ρ-coumaric, and glucuronic acid on cell growth and product formation. Furfural, HMF, and acetic acid (as acetate) did not inhibit fermentation at the concentrations that were attempted. On the contrary when 0.3 g L^{-1} each of ferulic, and ρ-coumaric acids were added to the fermentation medium cell growth and ABE production decreased significantly. Fermentation with added furfural, and HMF was found stimulating to the fermentation. A comparison with CFH, CSH and WSH (wheat straw hydrolysate) demonstrated that generation of inhibitors is substrate specific. It was noticed that fermentation of WSH was vigorous and no inhibition was observed.

Microbial Cultures and Culture Development

A number of strains that produce significant amounts of solvents from different carbohydrates have been reported in the literature. These cultures include *C. acetobutylicum* P262 (renamed as *C. saccharobutylicum*), *C. acetobutylicum* ATCC 824, *C. acetobutylicum* NRRL B643, *C. acetobutylicum* B18, *C. beijerinckii* P260, *C. beijerinckii* BA101, *C. beijerinckii* LMD 27.6, *C. butylicum*, *Clostridium aurantibutyricum*, and *Clostridium tetanomorphum*. *C. saccharobutylicum* P262 and *C. beijerinckii* P260 were industrial strains that were used for commercial solvent production in South Africa until early 1980s. These butanol producing strains are distinguished based on the ratio and type of solvents production. *C. butylicum* produces isopropanol rather than acetone while *C. aurantibutyricum* produces all three solvents including acetone, isopropanol, and butanol. *C. tetanomorphum* produces equal amounts of butanol and ethanol and does not produce acetone or isopropanol.

It should be noted that significant research efforts have focused on developing or genetically improving butanol producing cultures. A group in Germany isolated strains that produced up to 29.1 g L^{-1} total solvents. Attempts were also made to improve *C. acetobutylicum* ATCC 824 to produce more solvents than the parental strain. The new strain was able to produce up to 30 g L^{-1} total solvents. *C. beijerinckii* BA101 was developed from *C. beijerinckii* 8052 and the newly developed strain has been reported to produce up to 25.6–33 g L^{-1} total ABE. *C. beijerinckii* P260 has been reported to produce 23.1–28.2 g L^{-1} total ABE.

Biochemistry and Metabolic Pathways

A brief description of biochemical pathways involved in AB production from corn has been described in literature. During solvent production, the culture passes through three phases known as cell growth, acidogenic (acetic and butyric acid production) followed by solventogenic (assimilation of acids and conversion into acetone and butanol). In the early stages of acidogenic phase cell growth also occurs. During acidogenic phase hydrogen and a large amount of ATP are produced. During the acidogenic phase the pH of the culture medium drops from an initial pH of 6.8 to approximately 5.0. As solventogenesis begins, pH starts to rise from 5.0 to 6.8–7.0. In the solventogenic phase hydrogen and ATP production decreases. Carbon dioxide gas is produced during both acidogenic and solventogenic phases (two moles are produced from each mole of glucose metabolized to pyruvate; CO_2 production in the solventogenic phase is higher as an additional mole is produced for every mole of acetone produced). It has been suggested that the switch to solvent production is an adaptive response of the cell to the medium pH resulting from acid production. It has been reported that clostridia that are able to utilize cellulose produce little or no ABE.

In solventogenic clostridia, the uptake of carbohydrates occurs through a phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS). This mechanism involves uptake and simultaneous phosphorylation of carbohydrates. In the case of glucose, it is converted to glucose-6-phosphate which is subsequently metabolized to pyruvate via the Embden–Meyerhof–Parnas (EMP) pathway. Fructose is converted to fructose-1-phosphate and enters the EMP pathway upon conversion to fructose 1,6-diphosphate. As the culture is able to utilize pentose sugars such as xylose and arabinose, D-xylose is converted to D-xylulose by the xylose isomerase enzyme and the metabolism proceeds by a phosphorylation reaction. The reaction is catalyzed by xylulose kinase which results in the formation of D-xylulose-5-phosphate. The pentose phosphate pathway utilizes enzymes transaldolase and transketolase to convert D-xylulose-5-phosphate to glyceraldehyde-3-phosphate and fructose-6-phosphate. The glyceraldehyde-3-phosphate and fructose-6-phosphate enter the EMP pathway resulting to the formation of pyruvate. The solventogenic clostridia

have advantage over other cultures such as yeasts used for ethanolic fermentation as the former (clostridia) can utilize both hexoses and pentoses derived from lignocellulosic biomass. Table 2 shows conversion of various sugars to pyruvate which is then converted to acids and/or ABE by EMP pathway.

Fig. 1 shows a simplified biochemical pathway for AB production. During acidogenic phase 3.25 mol of ATP are formed per mol of glucose. The enzymes phosphate acetyltransferase and acetate kinase convert acetyl-CoA to acetate and similarly phosphate butyltransferase and butyrate kinase convert butyryl-CoA to butyrate. The acetic and butyric acids produced during fermentation may be freely permeable to the cell membrane and these acids equilibrate the internal and external (in fermentation broth) pH. Both pH reduction and accumulation of acetate and butyrate have been associated with triggering solventogenesis.

During solventogenic phase ATP production is reduced to 2 mol per mol of glucose. In this phase butyric and acetic acids are reassimilated by the action of acetoacetyl-CoA:acetate/butyrate:CoA transferase. This enzyme catalyzes the reaction that transfers CoA from acetoacetyl-CoA to either acetate or butyrate. Acetate is converted to acetyl-CoA, followed by conversion to acetone, butanol, or ethanol. Butyrate is converted to butyryl-CoA followed by conversion into butanol. This happens as there is no metabolic pathway to regenerate acetyl-CoA from butyryl-CoA. As a result of removal of CoA from acetoacetyl-CoA, acetoacetate is produced, which is transformed directly into acetone and CO_2 by acetoacetate decarboxylase.

In acidogenic and solventogenic pathways a series of reactions occur that produce butyryl-CoA from acetyl-CoA. The enzyme thiolase condenses two molecules of acetyl-CoA into one molecule of acetoacetyl-CoA. Acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA by hydroxybutyryl-CoA dehydrogenase. This results in the formation of crotonyl-CoA (by dehydration) and is catalyzed by crotonase. The carbon–carbon double bond in crotonyl-CoA is reduced with NADH to produce butyryl-CoA. The last reaction is catalyzed by butyryl-CoA dehydrogenase.

The accumulation of butyric and acetic acids and butanol in the fermentation broth is toxic to the culture which causes cell death. As a result of shift to solventogenesis, fermentation is extended. However butanol produced reaches toxic levels. The presence of butanol in the membrane increases membrane fluidity and destabilizes the membrane and membrane-associated processes.

Use of Concentrated Sugar Solutions

As stated in Section III of this article, in a typical batch fermentation system sugar concentration in excess to 60 g L^{-1} is rarely used due to inhibition caused by butanol. However attempts have been made to use concentrated sugar solutions. In a batch system *C. acetobutylicum* P262 (renamed as *C. saccharobutylicum* P262) was grown in a medium containing 200 to 227 g L^{-1} lactose (in whey permeate medium). In a similar approach *C. beijerinckii* BA101 was grown in a medium containing 159–165 g L^{-1} glucose. In another report on the use of concentrated sugar solution, *C. beijerinckii* P260 was able to grow and produce AB in solution containing 200 g L^{-1} glucose. Hence, it has been found that sugar tolerance is culture specific. Although, these cultures can grow in concentrated sugar solutions, they cannot utilize all the sugar present in the system. Other substrates where concentrated sugar solutions have been used include LCS, SLCS, WSH, BSH, and CSH (supplemented with glucose). The reader is advised to read Section X(B) and learn how complete sugar utilization has been possible in this fermentation. Table 3 shows sugar tolerance of various butanol producing cultures.

Table 2 Carbohydrates that can be used by solventogenic clostridia for ABE production

A. Glucose
Glucose→glucose-6-phosphate→pyruvate
B. Fructose
Fructose→fructose-1-phosphate→fructose-1,6-biphosphate→pyruvate
C. D-Xylose
D-Xylose→D-xylulose→D-xylulose-5-phosphate→glyceraldehyde-3-phosphate+fructose-6-phosphate→pyruvate
D. Agricultural biomass
Agricultural biomass→hexoses+pentoses (upon hydrolysis). Then steps A and C follow
E. Cellobiose
Cellobiose→glucose. Then step A follows
F. Sucrose
Sucrose→glucose+fructose (upon hydrolysis). Then steps A and B follow
G. Starch
Starch→glucose. Then step A follows
H. Inulin
Inulin→fructose. Then step B follows
I. Dextrin
Dextrin→glucose. Then step A follows

Note: Hydrolysis of agricultural biomass (D) to hexose and pentose sugars requires pretreatment with acid/alkali and hydrolysis by enzymes. Inulin (H) requires hydrolysis by inulinase. Solventogenic *Clostridia* cannot hydrolyze these substrates efficiently.

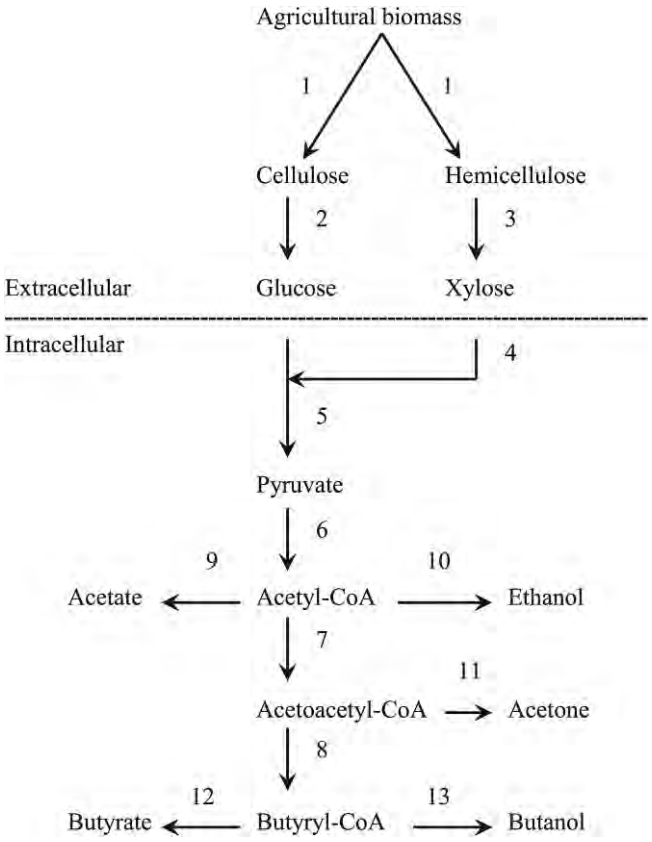

Fig. 1 Simplified metabolism of polysaccharides by solventogenic *clostridia.* Symbols: (1) pretreatment of agricultural residue; (2) cellulose hydrolysis; (3) hemicellulose hydrolysis; (4) xylose uptake and subsequent breakdown via the transketolase–transaldolase sequence; (5) glucose uptake by the phosphotransferase system (PTS) and conversion to pyruvate by the Embden–Meyerhof–Parnas (EMP) pathway; (6) pyruvate–ferrodoxin-oxidoreductase; (7) thiolase; (8) 3-hydroxybutryl-CoA dehydrogenase, cronotase and butyl-CoA dehydrogenase; (9) phosphate acetyltransferase and acetate kinase; (10) acetaldehyde dehydrogenase and ethanol dehydrogenase; (11) acetoacetyl-coA:acetate/butyrate:coA transferase and acetoacetate decarboxylase; (12) phosphate butyltransferase and butyrate kinase; (13) butyraldehyde dehydrogenase and butanol dehydrogenase. Printed with permission from CRC Press, Taylor & Francis Group. Ezeji, T.C., Qureshi, N., Karcher, P., Blaschek, H.P., 2006. Production of butanol from corn. In: Minteer, S. (Ed), Alcoholic Fuels. Boca Raton, FL: CRC Press, Taylor & Francis Group, pp. 99–122 (Fig. 6.1).

Table 3 Production of AB from concentrated sugar solutions using various butanol producing cultures in batch reactors

Culture	*Initial sugar (g L^{-1})*		*Residual sugar (g L^{-1})*
Clostridium acetobutylicum P262	lactose	227	0
Clostridium beijerinckii P260	glucose	200	0
C. beijerinckii BA101	glucose	159–165	0
C. acetobutylicum ATCC824	glucose	60	(Fed-batch system)

Bioreactors for Fermentation

Bioreactors play an important role in fermentation industry that impact production cost. However, their use depends on a particular application. A variety of reactors have been used for the production of chemicals such as AB. These reactors include batch, continuous stirred tank (suspended cell), compartmentalized (immobilized cell), fed-batch, fluidized bed (immobilized cell) and membrane cell recycle reactors. The details of these reactors are presented in the individual sections below.

Batch Reactors

Traditionally, batch reactors are used in fermentation industry to produce chemicals such as butanol and ethanol. In a batch process, the reactor is charged with a substrate solution (60–80 g L^{-1}) followed by sterilization, cooling, and inoculation with actively growing culture. The fermentation is run for a specified duration depending upon the culture and the substrate used. For butanol

fermentation process, the reactor is run from 36 to 72 h and during this time period approximately 20–33 g L^{-1} total ABE (ratio 3:6:1) is produced depending upon the culture used. The ABE concentration in the fermentation broth is limited due to butanol inhibition to the cell. At the end of fermentation, cell mass is removed by centrifugation or settling. The cell mass so recovered is sold as cattle feed. During fermentation, temperature is controlled at 35–37°C and pH at approximately 5.0. After removal of cell mass, the fermentation beer is sent for solvent removal which is usually by distillation. Fig. 2 shows a typical batch fermentation profile using cornstarch as a substrate.

During medium cooling, nitrogen or carbon dioxide is swept across the surface to keep the medium anaerobic. After inoculation, the medium is sparged with these gases to mix the inoculum. Depending on the size of the final fermentor, the seed may have to be transferred several times before it is ready for the production fermentor. Various substrates (outlined in Sections IV and V) can be used to produce butanol. It should be noted that some substrates may require processing prior to fermentation known as "upstream processing" such as dilution, hydrolysis, concentration, centrifugation, or filtration, etc.

During the 1940s and 1950s, production of butanol (from corn) on an industrial scale (Terre Haute, IN and Peoria, IL; both in the United States) was carried out using large fermentors ranging in capacity from 200,000 to 800,000 L. The industrial process used 8–10% corn mash, which was cooked for 90 min at 130–133°C. Corn contains approximately 70% (dry weight basis) starch. The use of molasses offers a number of advantages over using corn including the presence of essential vitamins and micronutrients. In industrial processes beet, invert, and blackstrap molasses were diluted to give a fermentation sugar concentration of 50 to 75 g L^{-1}, most commonly 60 g L^{-1}. The molasses solution was sterilized at 107 to 120°C for 15 to 60 min followed by adding organic and inorganic nitrogen, phosphorus, and buffering chemicals. The yield of solvent using *C. acetobutylicum* was usually low at 0.29–0.33. Distillation has been the method of choice to recover butanol, however, during the last three decades a number of alternative techniques have been investigated for the economical recovery of butanol which will be discussed in the Recovery Section XI.

Batch Process With Concentrated Sugar Solutions

Due to the butanol toxicity, the initial substrate concentration in a batch reactor is limited to <80 g L^{-1} (usually 60 g L^{-1}). A substrate concentration in excess of this results in a high residual substrate, thus resulting in inefficient sugar utilization and increased biological oxygen demand (BOD) load for wastewater treatment. However, recent developments in simultaneous recovery of AB have made it possible to use concentrated sugar solutions for this fermentation. During the fermentation, the toxic products such as butanol are removed simultaneously, thus relieving inhibition which results in the utilization of more substrate. The details of the various recovery techniques have been given in the Recovery Section XI. Employing butanol removal techniques, sugar solutions containing 161.7 g L^{-1} glucose (*C. beijerinckii* BA101) and 227 g L^{-1} lactose (*C. saccharobutylicum* P262) have been successfully fermented. Use of concentrated glucose and lactose solutions has resulted in the production of 76 and 137 g L^{-1} ABE, respectively. In such fermentations, less acids are produced, thus improving the ABE yield. In another process, 200 g L^{-1} lactose was successfully fermented in a batch reactor of *C. saccharobutylicum* P262 when integrated with product recovery by gas stripping. This system resulted in the production of 70 g L^{-1} ABE with a productivity of 0.32 g L^{-1} h^{-1} as compared to 0.07 g L^{-1} h^{-1} in the control batch reactor. Studies reported in this section demonstrated that a fermentation medium containing over three times the sugar concentration as compared to a batch reactor can be successfully fermented when integrated with product removal techniques. Sugars were used to exhaustion in these fermentations (Table 3).

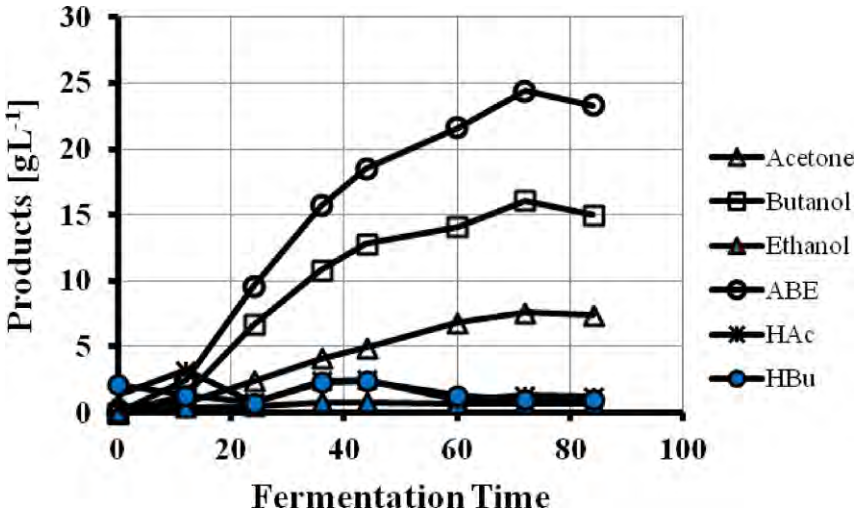

Fig. 2 Production of AB from 60 g L^{-1} cornstarch in a batch reactor of *Clostridium beijerinckii* BA101. Printed with permission from Taylor & Francis. Ezeji, T.C., Qureshi, N., Karcher, P., Blaschek, H.P., 2006. Production of butanol from corn. In: Minteer, S. (Ed), Alcoholic Fuels. Boca Raton, FL: CRC Press, Taylor & Francis Group, pp. 99–122 (Fig. 6.2). HAc, acetic acid; HBu, Butyric acid.

Fed-Batch Reactors

Fed-batch reactors are usually employed when the substrate is toxic to the culture. In such a case, the reactor is initiated in a batch mode with a low substrate concentration (usually 60–100 g L^{-1}) and low fermentation medium volume, usually less than half the volume of the fermentor. Upon inoculation the culture initiates utilizing substrate. As the substrate is used by the culture, a fresh feed solution containing concentrated sugar is added to the reactor at a rate that is equal to utilization of substrate. Both the liquid volume and product concentration increase with time. As the culture volume reaches near 70% of the reactor volume, the fermentation is stopped and product is harvested. This kind of system is applied to the production of chemicals that are not so toxic to the culture. An example of this process is bioconversion of glucose to 2,3-butanediol. Using fed-batch technique, 2,3-butanediol in excess of 100 g L^{-1} can accumulate in the fermentation broth.

Since butanol is toxic to *C. acetobutylicum* or *C. beijerinckii* cells, the fed-batch fermentation technique cannot be applied in this case unless one of the novel product recovery techniques is applied for simultaneous recovery of butanol. In a number of studies this technique has been applied successfully to the ABE fermentation. In a fed-batch fermentation up to 500 g glucose in 1 L culture volume (500 g L^{-1}) was used as compared to 60 g L^{-1} in a control batch process.

In a study, a substrate of whey permeate (lactose 350 g L^{-1}) was used to produce butanol in a fed-batch reactor of *C. acetobutylicum* P262. In this process, four different techniques of butanol separation were compared including liquid–liquid extraction, perstraction, gas stripping, and pervaporation. In the four processes, 23.8, 57.8, 69.1 and 42.0 g L^{-1} ABE were produced, respectively. ABE yield of 0.35, 0.37, 0.38, and 0.34 were obtained, respectively. Overall, application of these four techniques suggested that fed-batch fermentation technique can be applied to the ABE fermentation provided ABE is removed from the culture broth simultaneously.

In another study, ABE was produced in a fed-batch fermentation of *C. acetobutylicum* (ATCC 824) and the solvents were recovered using a silicone–silicalite synthesized pervaporation membrane. The reactor was fed with a concentrated (700 g L^{-1}) glucose solution. In this system, 155 g L^{-1} ABE was produced with an average yield of 0.31–0.35 and productivities ranging from 0.13 to 0.26 g L^{-1} h^{-1}. Using the fed-batch technique, fermentation of more sugars (2–3 times) was possible when novel product removal techniques were applied to the fermentation process. For further details on product separation the reader is advised to read "Novel product recovery technologies" (Section XI).

Continuous Bioreactors

Batch reactors often result in low productivity due to down time or the time required for harvesting, cleaning, re-filling, sterilization, cooling and then growing culture again for the next batch. In a batch reactor each time the culture is inoculated, cell growth is slow. The time required that the culture takes to grow is known as lag time. A continuous reactor does not encounter these problems as a bioreactor inoculated once may run for a long time. As a result of elimination of down time, reactor productivity is improved. There are three types of continuous bioreactors that can be used for butanol production. These reactors include suspended cell, immobilized cell, and cell recycle continuous bioreactors.

Suspended cell continuous bioreactors

The continuous culture technique may be used to improve reactor productivity and study the physiology of the culture in steady state. For continuous fermentation of butanol a number of studies have been carried out. Laboratory studies have demonstrated that due to production of fluctuating levels of solvents and complexity of butanol fermentation, the use of a single stage continuous reactor does not seem to be practical at the industrial scale. In continuous culture, a serious problem exists in that solvent production may not be stable for long time periods and ultimately declines over time, with a concomitant increase in acid production. In a single stage continuous system high reactor productivity may be obtained, however this occurs at the expense of low product concentration in the effluent when compared to that achieved in a batch process. In a single stage continuous reactor 15.9 g L^{-1} total solvents was produced at a dilution rate of 0.1 h^{-1} resulting in a productivity of 1.5 g L^{-1} h^{-1}. In order to improve the productivity, the dilution rate was increased to 0.22 h^{-1}, thus improving productivity to 2.55 g L^{-1} h^{-1}. However the product concentration decreased to 12 g L^{-1}. In a more recent study, a continuous fermentation was run in order to study the effect of fermentation gases and dilution rate on solvent production. In this system a productivity of 0.58 g L^{-1} h^{-1} was obtained at a dilution rate of 0.07 h^{-1}, thus producing 8.3 g L^{-1} total solvents. In order to improve reactor productivity a single stage spin filter perfusion bioreactor has also been used. In the perfusion bioreactor a maximum productivity of 1.14 g L^{-1} h^{-1} was obtained, however, solvent concentration fluctuated over time.

Two or more multistage continuous fermentation systems have been investigated to reduce fluctuations and increase solvent concentration in the product stream. This is done by allowing growth, acid production and solvent production to occur in separate bioreactors. In a two stage continuous system a solvent concentration of 18.2 g L^{-1} was reported using *C. acetobutylicum* DSM 1731 which is comparable to the solvent concentration in a batch reactor. This type of multistage reactor system (7–11 fermentors in series) was successfully tested at the pilot plant and full scale plant level in Russia (then Soviet Union).

Immobilized cell bioreactors

Batch, fed-batch, and suspended cell continuous reactors result in low reactor productivity due to low cell concentrations caused by butanol toxicity. In a batch reactor a cell concentration <4 g L^{-1} is achieved. In a butanol batch process, reactor productivity is

limited to less than 0.50 g L^{-1} h^{-1} due to a number of reasons including low cell concentration, down time, and product inhibition. In order to increase reactor productivity, cell concentration inside the reactor should be increased. It should also be noted that suspended cell reactors cannot be operated at high flow rate due to cell washout. Hence methods to retain high cell concentration inside the reactor and still operate the reactor at high flow rates, have been developed. These methods are known as "cell immobilization" and "cell recycle". By using cell immobilization method, cell concentration in excess 50–70 g L^{-1} can be achieved in the reactor. Such reactors can be operated at high flow rates with no cell washout. Cell recycle method will be discussed later.

Cell immobilization can be grouped in three different categories, namely adsorption, entrapment, and covalent bond formation. Adsorption is a simple technique where cells adsorb onto surfaces of various materials such as wood shavings, bonechar, glass wool, clay brick, etc. Adsorption is a natural process, and this technique does not require any chemicals for attaching cells to the surfaces. Often adsorption process is carried out inside the reactor. The reactor is packed with adsorbent followed by inoculation with the culture and feeding the reactor with a nutrient solution. Packed bed reactors show quicker growth than fluidized bed reactors.

Entrapment is another technique where cells are enclosed in gel layers such as calcium-alginate, poly-acryl-amide, or carrageenan. In order to immobilize cells by entrapment, cells are grown in batch reactors followed by harvesting them by centrifugation, mixing with a gel suspension/solution followed by passing through a needle to form gel beads. The gel beads containing cells may require further treatment such as hardening or polymerization. The beads thus formed are either packed in a reactor or suspended in a continuous stirred tank reactor (CSTR) for chemical production. Immobilization by covalent bond formation require chemicals such as glutaraldehyde to bond cells to the support of choice. It should be noted that some of the chemicals that are used to bond the cells may be toxic to the culture.

Increased reactor productivity results in the reduction of process vessel size and capital cost thus improving process economics. In an attempt to improve the reactor productivity use of immobilization techniques such as entrapment and adsorption has been investigated for this fermentation. While entrapment resulted in low productivities, adsorption was able to offer productivities as high as 4.5–6.5 g L^{-1} h^{-1}. Fig. 3 shows an electron micrograph of adsorbed cells of *C. acetobutylicum* P262 on bonechar. The cells were heavily adsorbed onto the support and formed as many as 36 single cell layers. In an attempt to explore use of clay bricks as an adsorption support, cells of *C. beijerinckii* BA101 were adsorbed onto this support with a reactor productivity of 15.8 g L^{-1} h^{-1}. In another approach a group from the Ohio State University (Columbus, OH, United States) immobilized cells of *C. acetobutylicum* on a fibrous support which was used in a continuous reactor to produce ABE. This system resulted in a maximum reactor productivity of 4.6 g L^{-1} h^{-1}.

Cell recycle membrane reactors

As described above, microbial cells are immobilized using one of the three techniques called adsorption, entrapment, and covalent bond formation. In all three cases the culture may experience diffusion limitations including substrate, product, and nutrients. In substrate diffusion limitation, the inner most cells may not receive carbon source required for its energy needs thus resulting in death of these cells. This will reduce amount of active cells that take part in the reaction. Starvation or diffusion limitation may inactivate a significant amount of cells thus resulting in reducing reactor productivity. At the same time product diffusion limitation may not allow diffusion of toxic products from the cell surroundings thus affecting cell viability. Both of these problems can result in significantly reduced productivity.

Similarly as cell immobilization increases cell concentration inside the bioreactor, cell recycle membranes are other means of increasing cell population inside the reactor. Unlike immobilized cell particles, the cells remain suspended in the liquid medium. Membrane is used as a means of preventing the cells from going with the out flow. Using membrane cell recycle reactors, cell concentrations as high as 100 g L^{-1} can be achieved inside the bioreactor. In these reactors there is no nutrient limitation to the cells as opposed to the immobilized cell reactors. Using this approach, reactor productivities up to 6.5 g L^{-1} h^{-1} (as compared to $<$0.5 g L^{-1} h^{-1} in batch fermentation) have been achieved in the butanol fermentation. In a similar approach a single stage spin filter perfusion bioreactor was used which resulted in a maximum productivity of 1.14 g L^{-1} h^{-1}, however, the ABE concentration fluctuated over time. Fig. 4 shows schematic diagrams of various reactor types that have been used for butanol production.

Novel Product Recovery Technologies

Adsorption-Based Technologies

Adsorption is a simple technique to separate solvents by adsorbing onto a number of adsorbents known as silicalite, resins (XAD-2, XAD-4, XAD-7, XAD-8, XAD-16), bone charcoal, activated charcoal, bonopore, and polyvinylpyridine. In order to separate butanol, the adsorbent and fermentation beer are brought in contact where the solvents are selectively adsorbed followed by recovering the product by heat treatment (of the adsorbent). The adsorption process occurs adequately at room temperature. The adsorption capacity of different adsorbents varies from 48 to 206 mg g^{-1} adsorbent. It has been reported that use of silicalite is better than other adsorbents as butanol from dilute solutions (5 g L^{-1}) can be concentrated to approximately 790 g L^{-1}. A number of investigators have used actual fermentation broth to separate butanol or ABE. In one of the investigations a comparison was made between total ABE produced and total sugar utilized in the control and integrated fermentation. In a control batch fermentation 13.5 g ABE was produced from 43.8 g sugar, while in the integrated batch fermentation 23.2 g of ABE was produced from 73.3 g sugar. These studies were extended to a repeated fed-batch fermentation system in which 1198.5 g sugar was used and 387.3 g ABE was produced. A schematic diagram of adsorption is shown in Fig. 5(A).

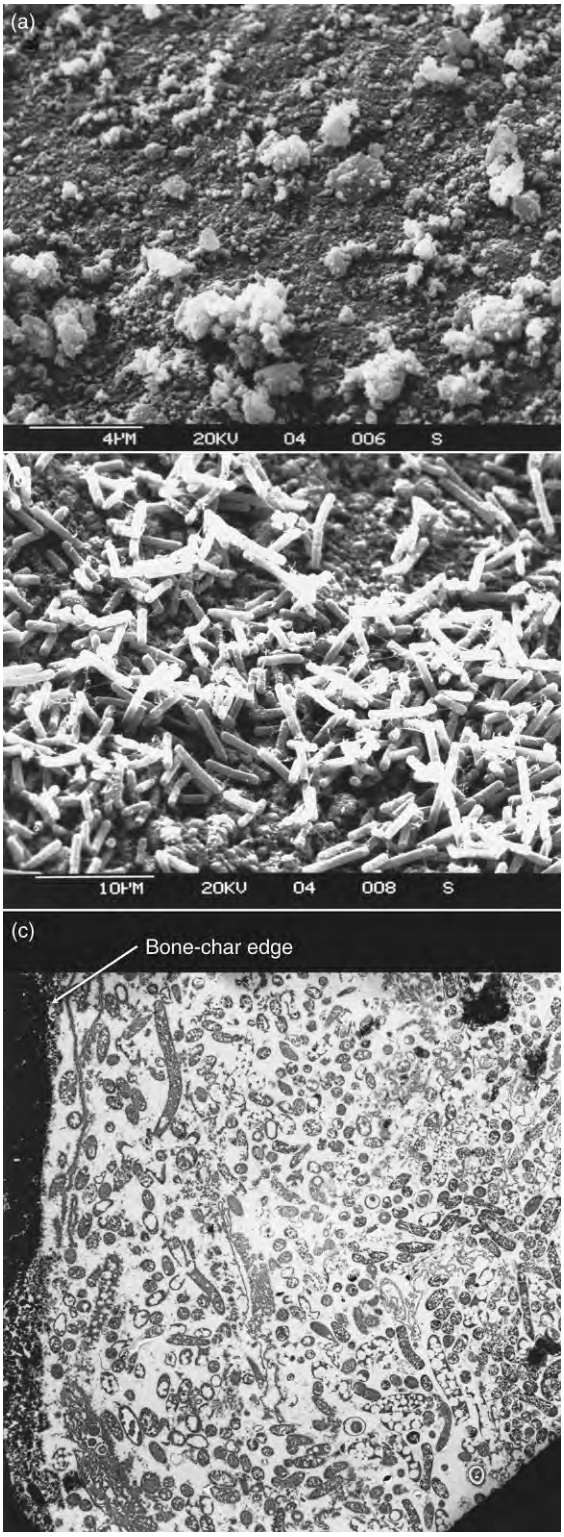

Fig. 3 Scanning electron micrographs of adsorbed cells of *Clostridium acetobutylicum* P262 onto bonechar. (a) Bonechar surface (magnification 5500); (b) adsorbed cells onto bonechar (magnification 2200); (c) transmission electron micrograph of adsorbed cells (magnification 2300). Similar Figs. 3(b) and (c) with different magnification were published previously in the following article: Qureshi, N., Paterson, A.H.J., Maddox, I.S., 1988. Model for continuous production of solvents from whey permeate in a packed bed reactor using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Applied Microbiology and Biotechnology 29, 323–328. Printed with permission from Springer, Germany. These figures were also published in: Qureshi, N., Annous, B.A., Ezeji, T.C., Karcher, P., Maddox, I.S., 2005. Biofilm reactors for industrial bioconversion processes: Employing potential of enhanced reaction rates. Microbial Cell Factories 4, 24.

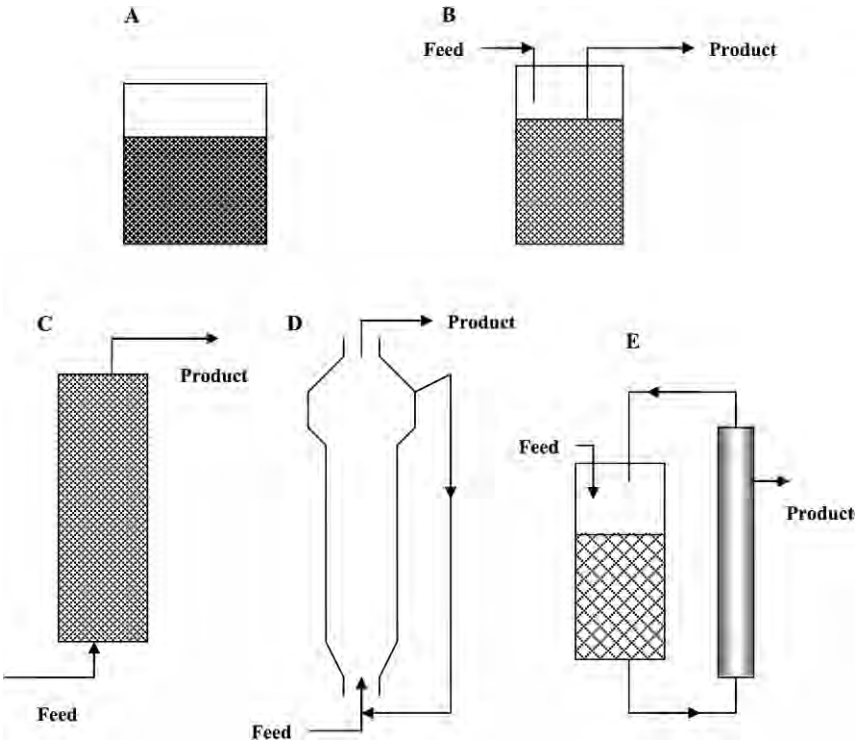

Fig. 4 Schematic diagrams of various reactor types used to produce AB. (A) Batch reactor; (B) continuous stirred tank reactor (CSTR); (C) immobilized packed bed reactor; (D) fluidized bed reactor; (E) membrane cell recycle reactor.

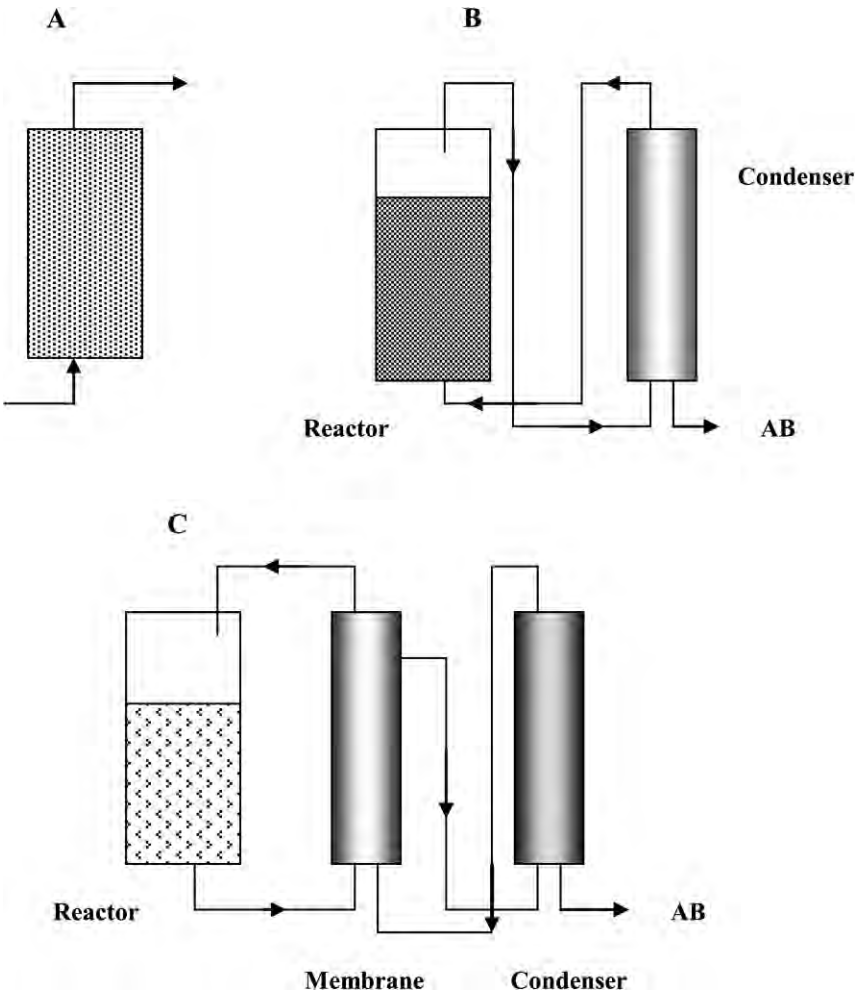

Fig. 5 Schematic diagrams of some of the product recovery processes. (A) Adsorption (butanol containing stream is passed through the column (flow bottom to top) and effluent low (or free of ABE) in ABE is obtained at the top); (B) Recovery by gas stripping; (C) Recovery by pervaporation using a selective membrane.

Recovery Based on Extraction and Perstration

Extraction or Liquid–liquid extraction has been considered to be an important technique for the recovery of ABE from fermentation broths. A water-insoluble organic extractant is mixed with the fermentation broth where butanol being more soluble in the organic phase than in the aqueous (fermentation broth) phase is extracted. Therefore, butanol selectively concentrates in the organic phase. Because the extractant and fermentation broth are immiscible, the extractant can easily be separated from the fermentation broth by phase separation after butanol extraction. It should be noted that liquid–liquid extraction is able to remove fermentation products without removing substrates, water, or nutrients.

In order to enhance substrate utilization and improve productivity, liquid–liquid extraction must be integrated with the butanol fermentation, in a manner that simultaneous fermentation and butanol removal from the fermentation broth is achieved. The choice of extractant is important because an extractant with low partition coefficient will not be efficient in the recovery of butanol and a toxic extractant will inhibit cell growth and fermentation or kill the bacterial cells. Unfortunately, most extractants with high partition coefficient are toxic to the clostridia. However, the extractant of choice among researchers has been oleyl alcohol because it is seen as a non-toxic good extractant.

During extraction, a number of problems such as emulsion formation, accumulation of bacterial cell mass at the organic–aqueous interphase, toxicity to the culture, and loss of extractant during phase separation are encountered. In order to solve these problems, a selective membrane is placed in between aqueous phase and organic phase. The membrane contactor provides surface area where the two immiscible phases can exchange the butanol. Since there is no direct contact between the two phases, extractant toxicity, phase dispersion, emulsion and rag layer formation are drastically reduced or eliminated. In such a system, butanol should diffuse preferentially across the membrane, while other components such as medium compositions, microbial cells, and fermentation intermediates (acetic and butyric acids) should be retained in the aqueous phase. The total mass transport of butanol from the fermentation broth to the organic side depends on the rate of diffusion of butanol across the membrane. The membrane does, however, present a physical barrier that can limit the rate of solvent extraction.

Recovery Using N_2 or Fermentation Gases (CO_2 and H_2)

Gas stripping is a simple technique that can be applied for recovering solvents (ABE) from model solution or the fermentation broth. Oxygen free nitrogen or fermentation gases (CO_2 and H_2: produced during solvent fermentation) are bubbled through the fermentation broth followed by cooling the gas (or gases) in a condenser. As the gas is bubbled through the broth in the fermentor, it captures ABE which is condensed in the condenser followed by collection in a receiver. Once the solvents are condensed, the gas is recycled back to the fermentor to capture more ABE. This process continues until fermentation stops due to lack of sugar (as a result of utilization of all the sugar). In some cases a separate stripper can be used to strip off solvents followed by recycling the stripper effluent that is low in ABE. Fig. 5(B) shows a typical schematic diagram of solvent removal by gas stripping.

Gas stripping has been successfully applied to remove solvents from batch, fed-batch, fluidized bed, and continuous reactors. In addition to removal of solvents, a concentrated sugar solution was fed to the reactors to reduce the volume of process streams and economize the butanol fermentation process. The reader is referred to Section X(B) (Batch process with concentrated sugar solutions) and Section X(C) (Fed-batch fermentation) of this article where concentrated sugar solutions were successfully fermented in combination with product recovery using this technique. In these processes, the reactor productivities, and product yield were also improved. Additional advantages of gas stripping included achieving a high ABE concentration in the condensed stream for further recovery and the requirement for no membrane or chemicals for the recovery process.

Membrane Based Recovery Such as Pervaporation

Pervaporation is a simple technique that allows selective removal of volatiles from model solutions/fermentation broths using a membrane. The volatile organic component (AB in this case) diffuses through the membrane as a vapor followed by recovery by condensation. During this process, a phase change occurs from liquid to vapor. Since it is a selective removal process, the desired component requires a heat of vaporization at the feed temperature. In pervaporation, the effectiveness of separation of a volatile is measured by two parameters called selectivity (a measure of selective removal of volatile) and flux (the rate at which an organic volatile passes through the membrane per m^2 membrane area). A schematic diagram of the pervaporation process is shown in Fig. 5 (C). Application of pervaporation to batch butanol fermentation has been described by numerous investigators. Pervaporation has also been applied for the removal of butanol from the fermentation broth in fed-batch reactors. In the fed-batch reactors concentrated sugar solutions have been used to reduce the volume of process streams. It is interesting to note that acids did not diffuse through some of the membranes.

In an attempt to improve membrane selectivity, the two techniques known as liquid–liquid extraction and pervaporation were combined to recover butanol. The extraction solvent used for this process was oleyl alcohol which formed a thin liquid layer (known as a thin membrane) on a microporous 25 μm thick polypropylene flat sheet. The oleyl alcohol got impregnated into the sheet pores. In this combination of liquid–solid membrane, oleyl alcohol dissolved butanol relatively quickly followed by diffusion through the polypropylene membrane. The advantage of combining the liquid (oleyl alcohol) and solid membrane was that a high butanol selectivity (180) was achieved in comparison to a low selectivity (10–15) when using polypropylene film alone. It should be noted that a membrane of selectivity 180 would concentrate 5 g L^{-1} feed butanol to 475 g L^{-1} butanol in a single pass, while a membrane of selectivity 15 would concentrate the same feed to 70 g L^{-1} butanol. A product containing 475 g L^{-1} butanol is 6.79 times more concentrated than 70 g L^{-1}. It was estimated that if this solid–liquid pervaporation membrane with a selectivity of 180

were used for butanol separation, the energy requirement would be only 10% of that required in a conventional distillation. Unfortunately, the membrane was not stable as the oleyl alcohol that formed a thin film and was impregnated into the polypropylene film pores diffused out of the membrane during the recovery process.

In order to develop a stable and high selective membrane, two techniques known as adsorption and pervaporation were employed. It is known that adsorption of butanol onto silicalite and molecular sieves is a quick and selective process. Hence a membrane was synthesized in which silicalite was included into a silicone membrane. By combining this, butanol selectivity was improved from 40 (silicone membrane) to 209. The membrane so developed was called silicalite–silicone membrane and the performance of this membrane was found to be stable with a working life of 3 years. This membrane was used to separate butanol from both butanol model solutions and fermentation broths. A comparison of various pervaporation membranes suggested that this membrane may be superior to other pervaporation membranes used for butanol separation. Some of the details of use of this system with product recovery have been given in Section X(C) (Fed-batch fermentation) where 155 g L^{-1} ABE was produced in the integrated fermentation and product recovery process as compared to <20 g L^{-1} in a batch process. This membrane was so efficient that a butanol concentration up to 700 g L^{-1} was achieved in the permeate. It should be noted that a butanol solution above 78 g L^{-1} separates into two phases known as organic phase and aqueous phase. The organic phase (top layer containing over 700 g L^{-1} butanol) can be separated from the aqueous phase easily by decanting off. The bottom phase that contains 78 g L^{-1} butanol can be recycled to the pervaporation separation unit to concentrate it further.

While a significant amount of progress has been made to separate butanol from fermentation broth, cost of membrane still remains a prohibitive factor. A number of years ago it was found that the cost of a silicone membrane was \$75 m^{-2} with butanol selectivity of 30–40. At that stage it was projected that a silicalite membrane would be several times expensive than the silicone membrane. It should also be noted that butanol flux through a silicone membrane is of the order of 10–12 g^{-2} h^{-1} at a feed butanol concentration of 8–10 g L^{-1}. Such a low flux would require a huge pervaporation section thus adding to the capital cost of the process. Another factor that is important to the process is lifespan of a pervaporation membrane. The author of this article has an extensive experience in pervaporation. It is anticipated that the working life of a pervaporation membrane may not exceed 3 years.

Vacuum Recovery

Recovery of ABE by applying vacuum to the reactor is one of the more simple recovery techniques. In this process, the volatiles boil at fermentation temperature, thus generating vapors of the desirable volatile products or components. Then, these vapors are sent to a condenser where they are condensed followed by removing them as liquid. In comparison to pervaporation where a membrane and vacuum are required, this process eliminates the need for the membrane. Recovery of alcohols by this process was applied to remove ABE from the actual fermentation broth of *C. beijerinckii* P260. This process is also characterized by two factors known as selectivity of volatiles, and the rate of recovery (g L^{-1} h^{-1}). The rate of recovery by vacuum can be as high as 10–15 g L^{-1} h^{-1}, which is 25–40 times that of recovery rate by gas stripping, usually 0.35 g L^{-1} h^{-1}. With such a high rate of recovery, this technique can also be integrated with the highly productive reactors where productivities of the order of 4.5–17 g L^{-1} h^{-1} are obtained.

Liquid CO_2 Extraction

Liquid CO_2 extraction is another technique that can be used to remove ABE from fermentation broth. Since CO_2 is inexpensive, its use for the extraction of butanol/ABE from a model butanol and ABE solutions was explored. The recovery was performed at 35°C and 10.34 MPa pressure and at these conditions a high separation efficiency (selectivity) was obtained from a feed solution containing 20 g L^{-1} butanol. Although, an economic evaluation of butanol recovery using this technique has not been performed, it is anticipated that this can be a potential technique for this process.

A comparison of various product recovery systems are listed in Table 4.

Use of Novel Substrates in Combination With Novel Technologies (Efficient Pretreatment, Hydrolysis, Fermentation, and Product Recovery Technologies)

In our economic studies, it was identified that substrate cost is one of the most important factors that influence butanol price significantly, thus indicating that more economical substrates be used for this valuable fermentation. As a result of this indication,

Table 4 A comparison of AB production using various product recovery systems

Recovery system	*Reactor system*	*Cumulative ABE production (g L^{-1})*	*Sugar used (g L^{-1})*
None (control)	Batch	20–33	60–80
Adsorption	Fed-batch	387.3	1198.5
Extraction	Fed-batch	23.8	68.6
Pervaporation	Fed-batch	165.1	307.2
Gas stripping	Fed-batch	233.4	500.0
	Continuous	460.0	1163.0

and numerous others, it has been primary focus of our laboratory (United States Department of Agriculture (USDA), National Center for Agricultural Utilization Research (NCAUR), Peoria, IL, United States) to use agricultural residues such as corn fiber, corn fiber xylan, wheat, and barley straws, corn stover, sweet sorghum bagasse (SSB) and energy crops such as switchgrass, for the production of biofuels such as ethanol, butanol and other chemicals. The reader is advised that fermentation of these substrates is a challenging task as they require pretreatment and hydrolysis prior to fermentation. During pretreatment, inhibitors are often formed that inhibit cell growth and fermentation. Studies on CFH fermentation to butanol revealed that CFH contained inhibitors that inhibited cell growth of *C. beijerinckii* BA101 and fermentation. Fermentation of treated CFH with XAD resin to remove fermentation inhibitors was partially successful.

Next, we investigated use of WS to produce ABE. As stated above pretreatment and hydrolysis of WS to simple sugars is essential prior to producing ABE. In our studies WS was hydrolyzed to simple sugars (hexoses and pentoses) using dilute acid pretreatment and enzymatic hydrolysis. The hydrolysate so obtained was successfully used to produce ABE without any treatment (to remove inhibitors) of the hydrolysate to remove fermentation inhibitors. Rather the hydrolysate was fermented vigorously and there were no signs of inhibition caused by hydrolysis inhibitors. These studies (hydrolysis and fermentation) were conducted in separate reactors (hydrolysis, fermentation, and recovery). Furthermore, WS pretreatment, hydrolysis, fermentation, and product recovery were combined in a single step and the studies were successfully completed.

These studies were also applied to the production of butanol from corn stover (CS). Of the 86 g L^{-1} CS, over 97% of the sugars were released during the hydrolysis process and fermented completely to butanol (ABE). During the pretreatment and hydrolysis, acetic acid was also released from the hemicellulose, which was also converted to AB. The reader is advised that butanol producing cultures can utilize both hexose and pentose sugars obtained from the hydrolysates. The studies on production of solvents from cellulosic and hemicellulosic sugars were performed in a single reactor using *C. beijerinckii* P260. In this system the four technologies namely pretreatment, hydrolysis, fermentation, and product recovery were combined into a single process. These studies represent a major breakthrough on the conversion of cellulosic biomass to butanol. Furthermore, a process of simultaneous saccharification (starch to glucose), fermentation, and recovery was applied to food waste. In the above processes ABE were simultaneously removed by applying vacuum to the reactor. Followed by ABE removal as vapors, these solvents were recovered as liquid by condensation in a condenser.

Solution to the AB Problems: Where Are We Now?

It can be stated that solutions to some of the problems associated with butanol fermentation have been investigated. In early 1980s reactor productivities higher than 0.20–0.50 g L^{-1} h^{-1} were not possible. Development of continuous immobilized cell reactors and cell recycle reactors have resulted in much higher reactor productivities (4.5–15.8 g L^{-1} h^{-1}) which would reduce the size and operational cost of the reactor substantially. Additionally, solvent yield has been improved as a result of combining production and recovery. In these systems, acids (acetic and butyric acids: reaction intermediates or metabolic by-products) cannot leave the reactor until they are converted into the final product. In traditional systems, ABE yield of 0.30–0.33 have been reported while in continuous integrated reactors yields of the order of 0.40–0.42 have been achieved.

With the advancement in technology, it has become possible to produce solvents from agricultural residues such as wheat straw, barley straw, corn fiber, corn stover, food waste, SSB and domestic waste. Production of solvents from LCS, SLCS, and whey permeate has also been successful. It should be noted that some years ago use of some of these substrates was not considered possible. Use of these substrates, novel hydrolytic enzymes, and combination of pretreatment, hydrolysis, fermentation, and recovery technologies has made this fermentation attractive as compared to butanol obtained from petrochemicals. Also application of in-situ product recovery technologies has allowed the use of concentrated sugar solutions. Sugar solutions containing sugar at 250–700 g L^{-1} have been used in fed-batch reactors successfully. This has resulted in reduced size of various streams associated with solvent production.

Economic Scenarios

Economics of Butanol Production

During the last two decades a number of economic studies have been performed on the production of butanol from corn, whey permeate, molasses, and wheat straw. In these studies it has been determined that distillative recovery of butanol from fermentation broth is not economical as compared to butanol derived from petrochemicals (current route). It has also been identified that new developments in process technology (energy efficient pretreatment of cellulosic feedstocks, continuous reactor design, product recovery, use of concentrated sugar solutions for fermentation, recycle of water, use of H_2 as an energy source produced during fermentation, credit for CO_2 and waste biomass) for butanol production from renewable substrates would allow for a significant reduction in the price of butanol. The price of butanol derived from corn also depends upon the co-products credit which is significant.

To bring fermentatively derived butanol closer to commercialization and compete with petrochemically derived butanol, it is suggested that further research be focused on the development of superior cultures (as compared to the existing strains: *C. beijerinckii* BA101, *C. acetobutylicum* PJC4BK, and *C. beijerinckii* P260). These cultures produce total ABE on the order of 20–33 g L^{-1}. Further improvements in ABE yield, which is 0.40–0.42 when using *C. beijerinckii* BA101 or *C. beijerinckii* P260, should also be examined.

Other cultures have been reported to result in a product yield of approximately 0.30. Material balance suggests that approximately 53% of carbon is lost as CO_2, indicating that only 47% of the carbon (from substrate) is directed for the product conversion.

Another problem with the butanol fermentation is the inability of these cultures to ferment hydrolysates of corn fiber, corn stover, barley straw, sugarcane bagasse, and switchgrass without inhibitor removal. In order to meet these challenges, new strains capable of utilizing agricultural biomass hydrolysates should be developed. Alternately, economic methods capable of removing inhibitors from the hydrolysates should be developed or novel pretreatment methods that do not produce inhibitors should be developed. It should be noted that use of wheat straw and corn stover has been a major breakthrough in the fermentation of cellulosic biomass to butanol. It is anticipated that hydrolyzed corn stalks, rice husk, and some of the energy crops such as switchgrass and miscanthus would be utilized without any inhibitor removal. In case of WS and CS simultaneous saccharification, fermentation, and recovery have been possible.

Conclusions

The production of butanol via the fermentation route is a relatively complicated process because the solventogenic clostridia are obligate anaerobes and the fermentation product (butanol) is toxic to the producing cultures. The possibility of incorporating in-line product recovery processes such as liquid–liquid extraction, perstraction, pervaporation, gas stripping, vacuum fermentation, and liquid CO_2 extraction, has generated a significant amount of interest. Simultaneous butanol fermentation and recovery has dramatically improved the productivity of butanol production from corn, wheat straw and corn stover (pretreatment, hydrolysis, fermentation, and recovery in case of WS and CS). By employing in-line recovery systems during butanol fermentation, substrate inhibition (due to high substrate concentration) and butanol toxicity to the culture are drastically reduced. Given that butanol is an

Fig. 6 A photograph of a fluidized bed reactor that was used to produce AB from whey permeate using *Clostridium acetobutylicum* P262. This picture was previously published in: Qureshi, N., Annous, B.A., Ezeji, T.C., Karcher, P., Maddox, I.S., 2005. Biofilm reactors for industrial bioconversion processes: Employing potential of enhanced reaction rates. Microbial Cell Factories 4, 24. The reactor was started with bonechar particles shown in Fig. 7(a). During operation of the reactor cell mass grew around the bonechar particles and the biofilm particles became highly active as shown in Fig. 7(b).

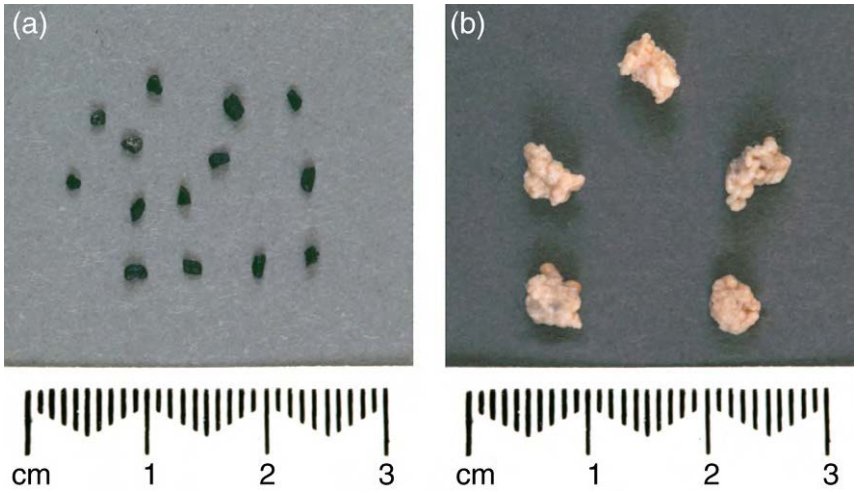

Fig. 7 Bioparticles of *Clostridium acetobutylicum* P262 used to produce AB in a fluidized bed reactor. (a) Bonechar particles before growth; (b) Bonechar particles after biofilm growth. This picture was previously published in: Qureshi, N., Annous, B.A., Ezeji, T.C., Karcher, P., Maddox, I.S., 2005. Biofilm reactors for industrial bioconversion processes: Employing potential of enhanced reaction rates. Microbial Cell Factories 4, 24 (Fig. 4).

excellent potential fuel and the United States is rich in biomass, butanol production from corn and agricultural biomass has a promising future. As it is seen at this stage, the technology of butanol production from agricultural substrates such as WS, BS, CS, SSB, LCS, and SLCS appears to be ready for scale-up; however this also depends upon the fluctuations in crude oil prices. It should be noted that over last many years reactor productivity has been improved significantly. Fluidized bed reactors offer high productivity (Fig. 6) and can be integrated with product recovery. In these reactors size of cell-mass particles was increased by many fold (Fig. 7), thus improving reactor productivity dramatically. Novel advancements such as pretreatment, simultaneous hydrolysis, fermentation, and product recovery technologies (adsorption, gas stripping, pervaporation to some extent, and vacuum fermentation) appear to be ready for this challenging fermentation.

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Acknowledgments

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Spirochetes☆

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Glossary

Chemotaxis The movement along a chemical concentration gradient either toward or away from a chemical stimulus.
Commensal An organism participating in a relationship in which that species derives benefit while the other is unaffected.
Microbiome The entourage of associated microflora in a host.
Parasite An organism participating in a relationship in which that species derives benefit while the other is harmed.
Pathogenesis The process by which a disease occurs.
Saprophyte An organism that grows on and derives its nourishment from dead or decaying organic matter.
Symbiont An organism participating in a relationship in which both species derive benefit.

Overview

The spirochetes form one of the major Phyla of the Kingdom of Eubacteria. The depth of the spirochetal branch of the bacterial tree of life is indicated by the fact that Phylum *Spirochaetes* has a single Class, and a single Order. As shown in Fig. 1, the Order *Spirochaetales* is separated into three families, *Spirochaetaceae*, *Serpulinaceae*, and *Leptospiraceae*. The first family, Spirochaetaceae, includes a complex group of organisms that have adapted to diverse niches. At one extreme, there are a large number of free-living *Spirochaeta* organisms that can be cultivated from virtually any moist, nutrient rich environment. At the other extreme is the obligate parasite, *Treponema pallidum*, which relies on the activities of a single animal host, man, for its survival and dissemination. In between these two extremes are the commensal, parasitic, and symbiotic organisms with life cycles involving insects, animals, or both. The second family, *Serpulinaceae*, contains a single Genus, *Brachyspira*, and a more narrowly focused life-style involving residence in the lower intestinal tracts of animals. The third family, *Leptospiraceae*, includes both environmental saprophytes (e.g., *Leptospira biflexa*) and animal parasites (e.g., *Leptospira interrogans*) that cycle between bodies of fresh water and their preferred reservoir host.

Comparison of genome sizes indicates that life outside the host is much more genetically challenging than a life of host dependence. Free-living organisms such as *Spirochaeta aurantia* and *L. biflexa* have relatively large genomes relative to *E. coli* (Fig. 2). In contrast, adaptation of spirochetes to a commensal or parasitic lifestyle has resulted in genomic contraction. For example, while *L. borgpetersenii* and *L. interrogans* evolved from a common ancestor that had the ability to survive both in nature and in the mammalian host, *L. borgpetersenii* has become an obligate parasite of cattle that requires direct transmission for animal to animal. As a result, the *L. borgpetersenii* genome has become 16% smaller and remains in a process of decay with 12% of its genes as nonfunctional pseudogenes. *Treponema denticola* has an intermediate-sized genome, perhaps related to the fact that although it is found only in animal hosts, it competes for nutrients in the complex oral microbial community. The *Borrelia* spp. and *T. pallidum* have the smallest genomes, with chromosomes only 1 Mb in size, which is consistent with their host dependence and lack of a free-living phase of their life cycle.

Spirochetes are defined by their unique morphology and rotational motility. Most spirochetes are helical coils—the one exception being *Borrelia burgdorferi*, which is actually a flat-wave. Spirochetes are expert swimmers that are entertaining to watch by dark-field microscopy. In low viscosity liquids, spirochetes appear to spin in place. Increasing the viscosity by addition of methylcellulose allows spirochetes to bore through the medium at a high rate of speed. The observer is quickly led to an understanding of how their screw-like movements would impart invasive properties to spirochetal pathogens. The organs of motility are flagella anchored near each end of the cell. Spirochete flagella are sometimes referred to as "endoflagella" because they are subsurface structures, wrapping around the protoplasmic cell cylinder, as shown in Fig. 3, instead of extending out beyond the surface of the cell as in all other flagellated bacteria. In at least one case, spirochete flagella also determine cell shape. *B. burgdorferi* mutants lacking the FlaB flagellar filament protein are rod-shaped rather than wave. Spirochetes differ in flagellar number and length. *Leptospira* have a single flagellum at each end of the cell that extends only a short distance along the length of the cell. In contrast, *Cristispira* spp. have bundles of over a 100 flagella at each end.

Chemotaxis allows bacteria to swim toward attractants such as nutrients and away from repellants by controlling the rotational direction of the flagellar motor. Flagella can rotate in a clockwise or counterclockwise direction. Sensory proteins called methyl-

☆*Change History*: May 2019, F Yang updated Figures 1, 2, 9, and 11, and updated the Borreliae and Leptospira pathogenesis sections. XF Yang and DA Haake updated figures.

This article is an update of D.A. Haake, Spirochetes, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 278–292.

Phylum *Spirochaetes*

Class *Spirochaetes*

Order *Spirochaetales*

Family *Spirochaetaceae*
Genus *Spirochaeta*
aurantia, etc. (free-living spirochetes)
Genus *Borrelia*
burgdorferi, etc. (Lyme Disease)
recurrentis (Louse Bome Relapsing Fever)
hermsii, etc. (Tick Borne Relapsing Fever)
Genus *Brevinema*
andersonii (spirochetosis of rodents)
Genus *Cristispira*
pectinis, etc. (shellfish symbionts)
Genus *Spironema*
culicis (mosquito isolate)
Genus *Treponema*
pallidum (Syphilis, Yaws, Bejel)
carateum (Pinta)
denticola, etc. (oral treponemes, Periodontitis)
phagedenis, etc. (Papillomatous Digital Dermatitis)
bryantii, etc. (intestinal treponemes of mammals)
primitia, etc. (termite gut treponemes)

Family *Serpulinaceae*
Genus *Brachyspira*
hyodysenteriae (Swine Dysentery)
pilosicoli, etc. (Intestinal Spirochetosis)
innocens, etc. (Intestinal commensals)

Family *Leptospiraceae*
Genus *Leptospira*
interrogans, etc (Leptospirosis)
licerasiae, *etc* (intermediates)
biflexa, etc. (nonpathogenic)
Genus Leptonema
illini (nonpathogenic)
Genus Turneriella
parva (nonpathogenic)

Fig. 1 Taxonomic organization of the spirochetes. Three families of spirochetes have been defined. Family Spirochaetaceae includes the free-living Spirochaeta spp., the parasitic Borrelia, and the commensal, parasitic, and symbiotic Treponema spp. Family Serpulinaceae are bacteria that colonize the lower intestinal tracts of mammals. Family Leptospiraceae includes both free-living nonpathogens and organisms that are able to invade animal reservoir hosts.

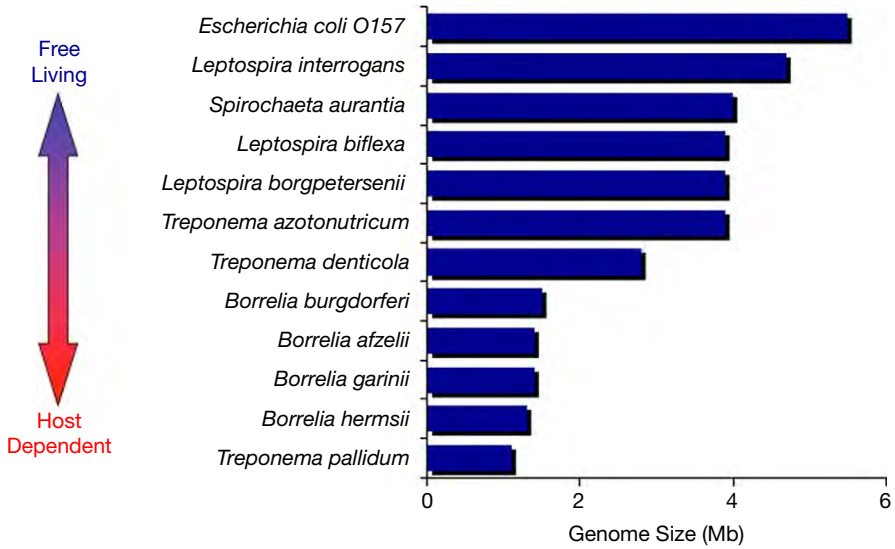

Fig. 2 Comparative genome sizes of spirochetes. Free living spirochetes including Leptospira and Spirochaeta spp. have genomes that rival the size of E. coli. Host dependent spirochetes such as T. pallidum and Borrelia spp. have some of the smallest known genome sizes. Treponemes that live in the complex environments of the oral cavity (T. denticola) and termite gut (T. azotonutricum) have intermediate-sized genomes.

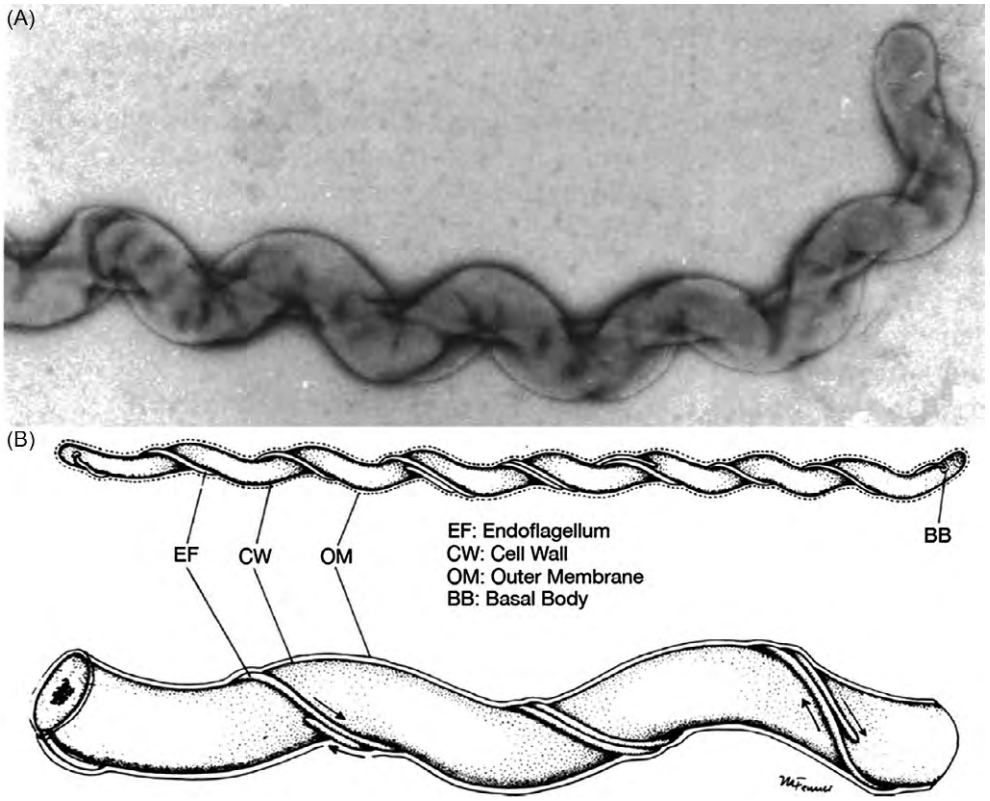

Fig. 3 Spirochetal architecture. Spirochetes share a unique structure and motility strategy in which the endoflagella are inserted at opposite poles and wrap around the protoplasmic cylinder. (A) Electron micrograph of Leptospira showing a single endoflagellum at one end of the cell. (B) Schematic diagram showing endoflagellar location relative to the outer membrane and cell wall. Reproduced from Holt, S. M. (1978). Anatomy and chemistry of spirochetes. Microbiological Reviews 42, 114–160.

accepting chemotaxis proteins (MCPs) control the directional switch in the flagellar motor. Clockwise rotation causes a cell to tumble (stop), counterclockwise rotation causes a cell to run (go). Spirochetes are unique in having flagella at each end. Effective spirochete movement involves flagellar rotation in the counterclockwise direction at the leading end and in the clockwise rotation at the trailing end. If the flagella at alternate ends are rotating in the same direction, spirochetes will flex in place rather than spin. It is not known how spirochetes coordinate the flagella at their alternate ends, or how MCPs orient spirochete movement. In any case, spirochetes are clearly adept at chemotaxis. For example, *B. burgdorferi* are able to find their way into a capillary tube containing *N*-acetyl glucosamine, a sugar required for cell wall biosynthesis. Sensory proteins are not only used for chemotaxis but also for regulation of gene expression. Spirochetes vary widely in terms of the number of sensory proteins they have. As mentioned previously, life outside the host is challenging and "extroverts" (organisms with a free-living stage) have far more sensory proteins than "introverts" that never leave the host. For example, *L. interrogans*, which lives both inside and outside a mammalian host has 10 times the number of sensory proteins as *T. pallidum*, an obligate human parasite. Fig. 4 shows the correlation between life-style and numbers of sensory proteins.

The unique spirochetal architecture has both gram-negative and gram-positive features. Like gram-negative bacteria, spirochetes are "diderms," or double-membrane bacteria. However, the spirochetal outer membrane is much more fluid and labile than the outer membrane of gram-negative organisms. In typical enteric gram-negative bacteria, the outer membrane is supported by, and closely associated with, the underlying peptidoglycan cell wall. In contrast, the spirochetal cell wall is more closely associated with the inner, or cytoplasmic membrane than the outer membrane (Fig. 5), a feature of gram-positive bacteria. Another important difference between the outer membranes of most Gram-negative bacteria and those of treponemes and borreliae is a lack of lipopolysaccharide (LPS). Leptospires have LPS, but there are significant structural differences between leptospiral and *E. coli* LPS such that human toll-like receptor 4 is unable to bind to leptospiral LPS. The lack of recognizable LPS allows spirochetes to function as "stealth pathogens" that are able to invade and persist in the bloodstream and tissues of the body without detection by the early warning system of innate immunity.

Protein export pathways of spirochetes resemble those of other bacteria. The Sec pathway for exporting proteins with signal peptides across the cytoplasmic membrane is conserved. Genes encoding enzymes that process signal peptides are present in spirochete genomes but their specificities are clearly unique because prediction algorithms such as Psort and LipoP frequently do not apply to spirochetal signal peptides. Computer recognition of signal peptides of spirochetal lipoproteins requires development of spirochete-specific approaches such as the SpLip algorithm. Upon reaching the periplasmic face of the cytoplasmic membrane,

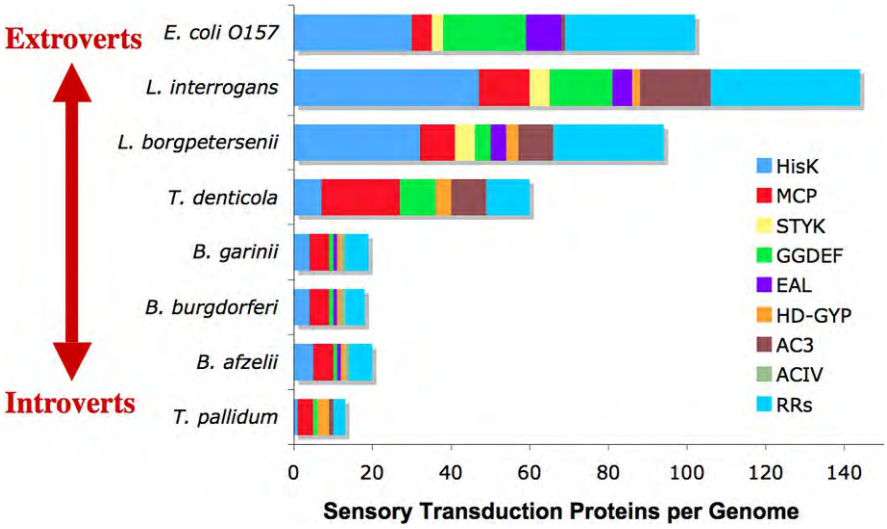

Fig. 4 Comparison of spirochetal life styles and sensory transduction genes. Spirochetes have a wide variety of sensory transduction genes including histidine kinase sensors (HisK), methyl-accepting chemotaxis proteins (MCP), etc. (see Galperin, BMC Microbiology 2005; 5:35 for further definitions). Spirochete "extroverts" that live outside the host have a much greater number of sensory transduction proteins per genome than host dependent "introverts."

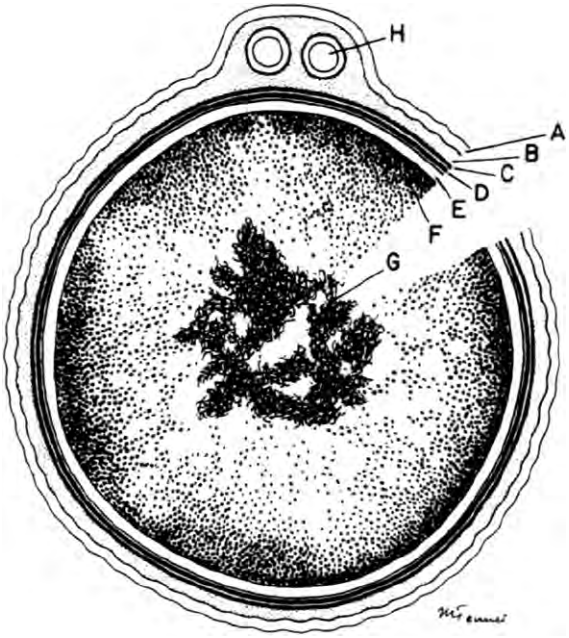

Fig. 5 Spirochete cross-section. Elements of spirochetal architecture include (A) outer membrane; (B) Periplasm; (C and D) Peptidoglycan cell wall; (E) Cytoplasmic membrane; (F) Cytoplasm; (G) Nuclear material; and (H) Endoflagella. Note that the endoflagella are subsurface structures and that the cell wall is more closely associated with the cytoplasmic membrane than the outer membrane. Reproduced from Holt, S. M. (1978). Anatomy and chemistry of spirochetes. Microbiological Reviews 42, 114–160.

spirochetal lipoproteins are shuttled to the outer membrane via the Lol pathway. Here again, rules that apply for *E. coli* lipoproteins have been altered for spirochetal lipoproteins such that retention of spirochetal lipoproteins in the cytoplasmic membrane involves negatively charged amino acids after the amino-terminal cysteine and export to the outer membrane is by default. Membrane fractionation and ultrastructure studies demonstrate three types of spirochetal outer membrane proteins: transmembrane porin-like molecules, lipoproteins, and peripheral (nonintegral) membrane proteins. All spirochetes have Omp85 (also known as BamA) and TamB homologs for assembly and insertion of outer membrane proteins (Omps). Transmembrane Omps are required for transport functions and both transmembrane and surface lipoprotein Omps have been shown to be involved in host-pathogen interactions.

Treponema

The Genus *Treponema* includes a broad diversity of parasitic and commensal species, most of which exist in complex bacterial communities. A notable exception is highly invasive obligate human pathogen, *Treponema pallidum*, subspecies *pallidum*, the agent of syphilis. *T. pallidum*, syphilis, and its history are covered of separate chapters in this *Reference Module*. In passing, it should be mentioned that *T. pallidum* has two other subspecies and a sister species that cause nonvenereal skin infections of humans (see Fig. 1). *T. pallidum* subspecies *pertenue* causes yaws, *T. pallidum* subspecies *endemicum* causes endemic syphilis (bejel), and *T. carateum* causes pinta. Another member of this species group is *T. paraluiscuniculi*, the agent of venereal spirochetosis of rabbits, which has an overall genome sequence similarity of 98.6–99.3% with *T. pallidum* spp. *pallidum*. It should also be mentioned that a number of additional *Treponema* species have been isolated from the intestinal tracts of animals including cows (*T. bryantii* and *T. saccharophilum*) and pigs (*T. succinifaciens*). In this section, we will cover the oral treponemes, the organisms that cause papillomatous digital dermatits of cattle, and the termite gut treponemes involved in the digestion of cellulose.

Oral Treponemes

Oral treponemes were some of the first bacteria described in the writings and drawings of Antonie van Leeuwenhoek, the Father of Microbiology. In 1676, when examining a dental plaque from the mouth of an old man, van Leeuwenhoek found "an unbelievably great company of living animalcules, a-swimming more nimbly than any I had ever seen up to this time. The biggest sort. . .bent their body into curves in going forwards. . ." Today, anyone with a dark-field microscope can repeat van Leeuwenhoek's experiment. If the sample is taken from the periodontal space (located between the tooth and the gum) of a patient with gum disease, it is likely that spirochetes will be observed to be the predominant bacterial forms. A remarkable diversity of oral treponeme morphologies is present in the mouth, with a broad variety of diameters, lengths, wavelengths, amplitudes, and numbers of endoflagellae. Sizes range from 0.1 to 0.4 μm in diameter and 5–20 μm in length.

The microbial community of the mouth, referred to as the oral "microbiome," is estimated to include upwards of 500 different bacterial species. Given the complex environment in which they live, it is not surprising that it has been relatively difficult to isolate and cultivate oral treponemes. Like most bacteria that live in the periodontal space, most oral treponemes are strict anaerobes. However, some treponemes, such as *T. denticola*, can tolerate low concentrations of oxygen. Treponemes are intrinsically resistant to rifampin, which makes it possible to use rifampin-containing culture medium to exclude other bacteria and select for treponemes. More than 10 species of oral treponemes have been isolated, allowing more detailed studies of their morphologies and metabolic requirements. More detailed enumeration of oral treponeme diversity has become available through microbiome studies. In one comparison of healthy and periodontitis subjects, five novel treponemal species were found for every one that had been cultivated. Nearly 25% (49/215) of the new oral bacterial species discovered were treponemes! Based on these molecular studies, 10 phylogenetic groups of human oral treponemes in two clusters have been defined (Fig. 6). One of these clusters also contains treponemes associated with bovine digital dermatitis (see below), while the other cluster contains intestinal treponemes of cattle and swine.

Several lines of evidence implicate treponemes as oral pathogens. Although treponemes can be found in small numbers in the mouths of healthy individuals, their numbers and diversity are strongly correlated with the severity of chronic and aggressive forms of periodontitis and their numbers are diminished with clinical treatment. Two species that have been associated with periodontitis are *Treponema denticola* and *Treponema lecithinolyticum*. Both gingivitis and periodontitis are extremely common inflammatory gum diseases. The distinction is that while gingivitis is reversible, periodontitis involves erosion of the dental ligament that attaches the tooth to the supporting bone at the base of the periodontal pocket (Fig. 7). Peridontitis affects 50% of the US population over 30 years of age and is the leading cause of tooth loss. While treponemes are typically found at the base of the periodontal pocket in association with other pathogenic organisms, such as *Porphyromonas gingivalis* and *Tannerella forsythia*, immunofluorescence microscopy shows that the treponemes the most invasive organisms, typically invading the epithelial cells at the leading edge of invasion process. Treponemes also appear to be involved in endodontal (root canal) infections; *Treponema maltophilum* DNA was detected in 50% of root canal samples using 16S rDNA-based PCR methods.

Several pathogenetic mechanisms have been identified by which oral treponemes cause disease. By virtue of their motility, chemotaxis, and narrow diameter, spirochetes are able to slip between epithelial cells and invade the subepithelial layers of the gum tissue. Although treponemes do not make Gram-negative lipopolysaccharides, they do elaborate a variety of glycolipids and lipoproteins that stimulate innate inflammatory pathways. *T. denticola* expresses a serine protease, dentilisin, that digests host extracellular matrix proteins including fibronectin, laminin, and fibrinogen. Dentilisin activates host matrix metalloproteinases, and together with dentilisin these enzymes serve to alter and eventually degrade the barriers that prevent invasion by other periodontal bacteria. Exposure to *T. denticola* causes cytoskeletal rearrangements that disrupt normal host cells functions. These cytotoxic effects are probably caused by *T. denticola* release of Msp, the major sheath protein. Msp is a porin-like molecule that appears to insert into host cell membranes and trigger intracellular calcium fluxes, which are thought to damage epithelial cell barriers and impair clearance of invaded bacteria by polymorphonuclear leukocytes.

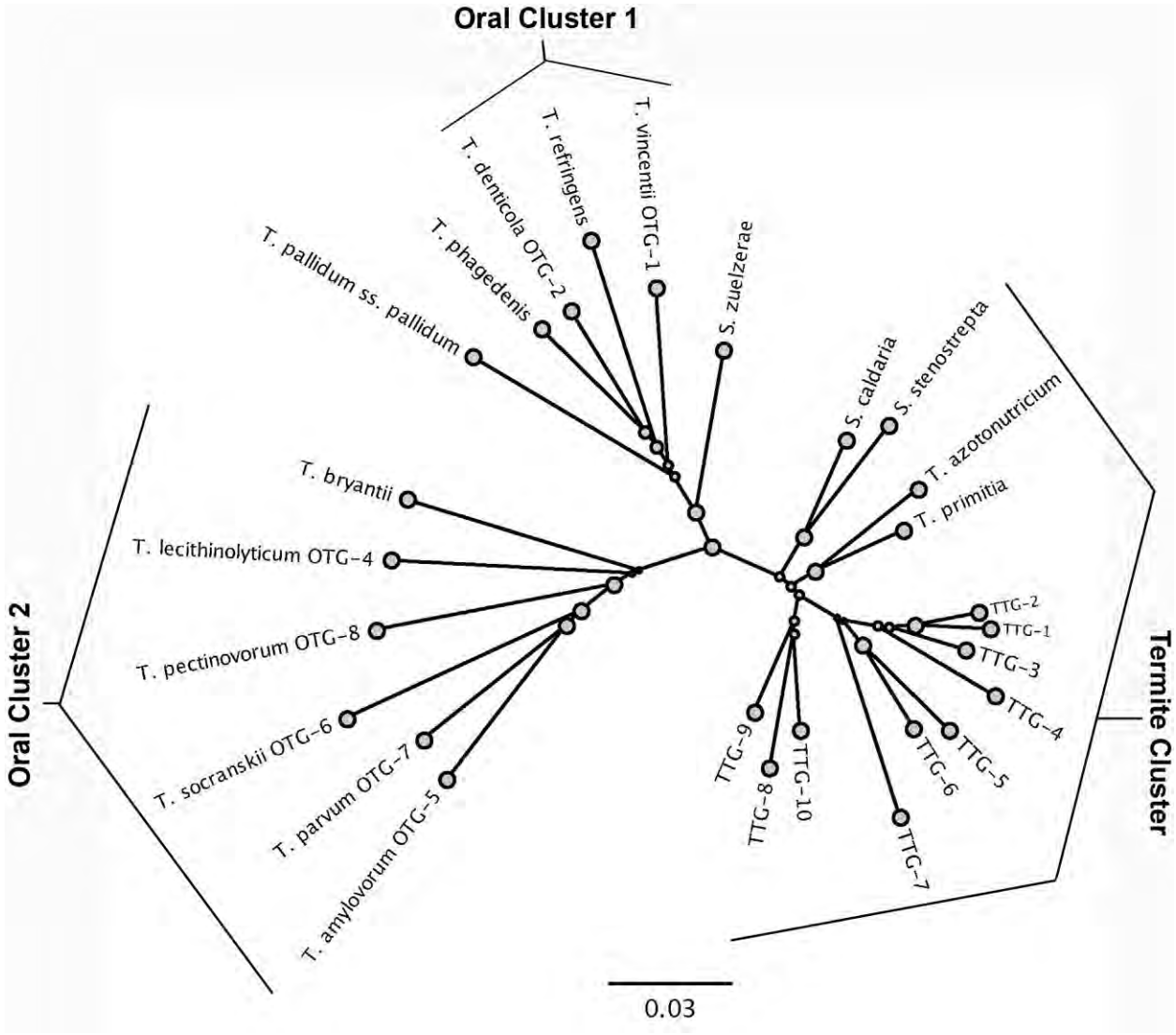

Fig. 6 Phylogenetic tree of the treponemes. Comparison of treponeme 16S rRNA sequences shows segregation into three relatedness clusters: Two clusters of oral treponemes and a cluster of termite gut treponemes. Note that T. pallidum, the agent of syphilis is related to the first cluster of oral treponemes.

Papillomatous Digital Dermatitis Treponemes

Papillomatous digital dermatitis, or PDD, is a polymicrobial infection of the soft tissue adjacent to the hoofs of cattle. Affected animals have painful ulcers referred to as heel warts or footwarts. Since it was first described in the 1970s, PDD has spread throughout the world, including most herds in the United States, and is now the leading cause of lameness in dairy cattle. The spread of PDD is related to industrial-scale dairy practices where cattle are continuously kept in barns or feedlots on moist surfaces and not allowed to graze. Cultures of PDD lesions show a mixed population of anaerobic bacteria including a number of spirochetes that are closely related to oral treponemes such as *T. denticola*, *T. medium*, and *T. vincentii* found in Treponema cluster 1 (Fig. 6). Immunofluorescence studies of biopsies of PDD lesions reveal invasion of treponemes into the soft tissues of foot, not unlike the invasion of treponemes observed in the periodontium of the mouth.

Termite Gut Treponemes

Diversity

Although the volume of the termite hindgut can be as small as 1 μL, the diversity of treponemal phylotypes and morphotypes within a single termite is staggering. Microscopy of the termite hindgut contents reveals treponemes ranging from 0.1 to 1 μm in diameter and 3–100 μm in length, with a variety of cell wavelengths and amplitudes (Fig. 8). Some of the larger forms can have 100

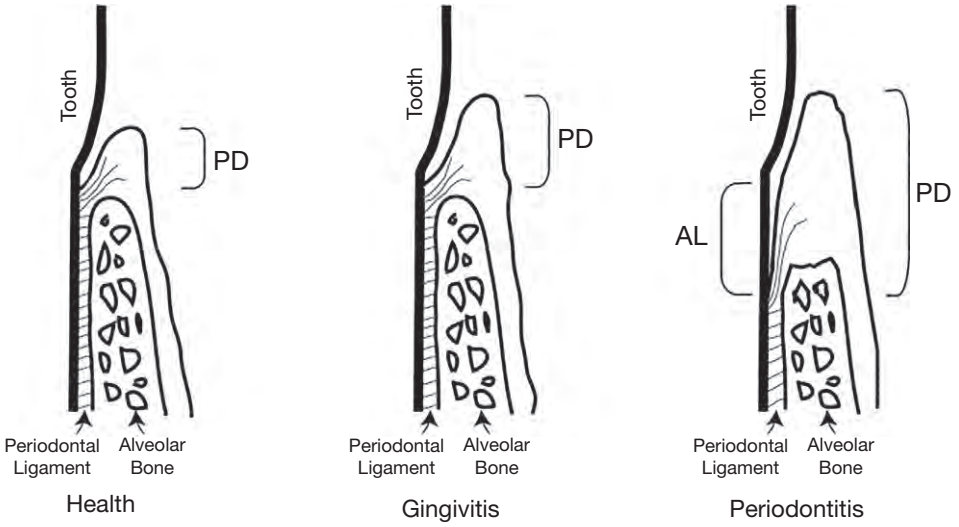

Fig. 7 Schematic representation of health, gingivitis and periodontitis. The periodontal pocket depth (PD) is increased in gingivitis due to tissue swelling associated with inflammation. In periodontitis the PD is further increased due to the loss of the tissue attachment to the root of the tooth (AL: attachment loss). Periodontitis is further characterized by loss of supporting alveolar bone. Treponemes are typically found at the base of the periodontal pocket. Reproduced from Kinder-Haake, S. et al. (2006). Periodontal diseases, In: Lamont et al. (eds) Oral Microbiology & Immunology. ISBN-13: 9781555812621.

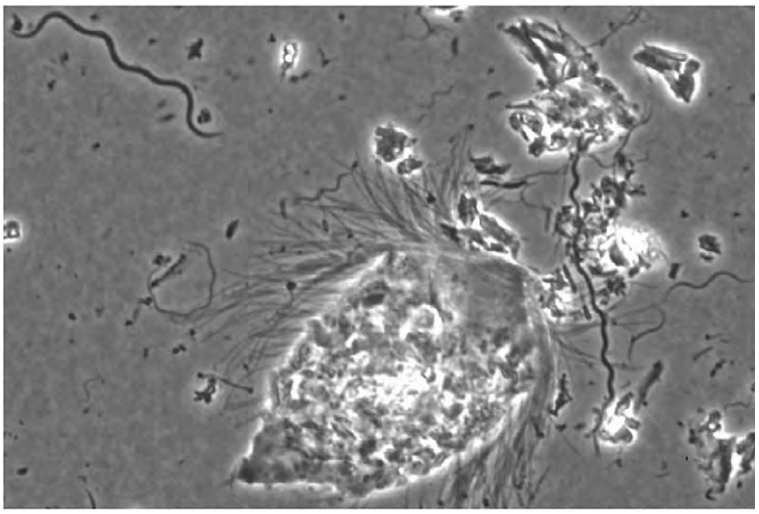

Fig. 8 Phase contrast micrograph of termite gut treponemes. A variety of treponeme sizes and morphologies are present in the termite gut. Note the treponemal appendages attached to the hypermastigote protozoan, Trichonympha agilis. Reproduced from Breznak, J. A. (2006). In Radolf, J. D. and Lukehart, S. A. (eds.) Pathogenic Treponema: Molecular and Cellular Biology. ISBN: 1-904455-10-7.

or more periplasmic flagella. Many termite gut treponemes are individual cells, while others are ectosymbionts of protozoa, functioning as their motility organelles. Recent metagenomic analysis of the total hindgut microbiota of arboreal wood-feeding *Nasutitermes* termites revealed that 68% of the genetic material was treponemal in origin. As many as a hundred *Treponema* species can be present within the digestive tract of a single termite species. These termite treponemes form a distinct cluster within the treponeme phylogenetic tree (Fig. 6). In addition to several named species, 10 termite *Treponema* groups have been defined. The *Spirochaeta* species, *S. stenostrepta* and *S. caldaria*, were isolated from water as free-living organisms and named before their 16S sequences were known, but fall within the termite treponeme cluster and should be considered *Treponema* species likely to have been released from animals or insects.

Metabolism

Termite gut treponemes are symbionts, benefitting their termite hosts by contributing to the digestion of woody plant material. Like most host-dependent spirochetes, termite gut treponemes were difficult to isolate. Eventually, the first termite gut treponemes to be grown in pure culture were isolated from the California dampwood termite, *Zootermopsis angusticollis*, and assigned to the new

species, *T. primitia*. *T. primitia* is an anaerobe, and was grown in sealed containers. In the process of working with the *T. primitia* cultures, it was discovered that a vacuum had developed in the headspace of the *T. primitia* cultures. This observation led to the finding that *T. primitia* could convert H_2 and CO_2 gases to acetate. Acetate was known to be a major source of energy and carbon for termites. The treponemes were found to reduce single carbon CO_2 to two-carbon acetate molecules via a well-known acetyl-CoA pathway, thus providing nutrients to the termite that would otherwise have escaped in a gaseous form.

Treponemes were subsequently found to benefit termite metabolism in other ways. Cellulose is high in energy but relatively poor in nitrogen required for formation of amino acids. A second species, the aptly named *T. azotonutricum*, was found to be able to convert significant amounts of atmospheric N_2 to ammonium using its unique dinitrogenase reductase activity. Until recently, it was not known to what extent termite gut treponemes participated in other aspects of wood polysaccharide digestion. Termite treponeme enzymes have now been shown to cleave aromatic ring compounds of lignan, the most difficult component of wood to metabolize. Eventually, it is hoped that the highly evolved digestive abilities of termite treponemes can be harnessed to convert woody biomass to green energy.

Borreliae

Morphology and Metabolism

Borrelia spp. are separated into two large genetic groups: the relapsing fever (RF) borreliae and the Lyme disease (LD)-related borreliae. This chapter covers the RF borreliae. The LD borreliae are covered in a separate chapter of this *Reference Module*. Borreliae vary from 8 to 30 μm in length and 0.2–0.5 μm in width, with the RF borreliae tending to be shorter and wider than the LD-related borreliae. Borreliae exhibit the unique rotational motility of spirochetes powered by endoflagella. The RF borreliae have 15–30 endoflagella while the LD-related borreliae have 7–11 endoflagella. Unlike the endoflagella of other spirochetes, the endoflagellae of borreliae lack sheaths. The other distinguishing morphological characteristic of the borreliae is the lack of cytoplasmic tubules.

Borreliae are obligate parasites with a life cycle that alternates between arthropod vectors and mammalian hosts. Despite their host dependence and small genome size, many *Borrelia* spp. have been cultivated using nutritionally rich media, similar to tissue culture media, including many amino acids, vitamins, and rabbit serum. Unlike other bacteria, borreliae do not require iron and lack most of the iron-dependent enzymes in their genome. Glucose is required and is metabolized via the Embden-Meyerhof glycolytic pathway, but borreliae lack TCA cycle, and ATP production solely relies on the substrate phosphorylation. Borreliae also lack the pyruvate dehydrogenase complex to synthesize acetyl-coA form pyruvate, rather using acetate to produce the essential acetyl-coA with the AckA-Pta pathway. Borreliae require exogenous *N*-acetylglucosamine for cell wall synthesis, presumably because this chitin component is constitutively available in ticks. In addition, borreliae have the glycerol uptake and metabolic pathway to utilize glycerol as an alternative carbon source, which appears to be important for borreliae to survive in ticks. Relapsing fever borreliae have an extra gene that is absent in Lyme disease borreliae, *glpQ*. *glpQ* encodes glycerophosphodiester phosphodiesterase that allows hydrolysis of deacylated phospholipids to glycerol-3-phosphate, which may contribute to the high cell density of RF borreliae but not LD borreliae in patients' peripheral blood. Components in commercial Bovine serum albumin (BSA) fraction V is provided as a source of long-chain fatty acids for membrane biosynthesis. The bane of borrelial researchers is the variable ability of different lots of BSA (as well as rabbit serum) to support borrelial growth. Although borreliae make superoxide dismutase but lack catalase, they are oxygen sensitive and can only tolerate low levels of oxygen. One possible explanation for lot to lot variability of BSA is that polyunsaturated fatty acids supplied by certain lots of BSA appear to be the target of reactive oxygen species (ROS), resulting in damage to borrelial membranes.

Epidemiology and Phylogeny

The borrelial life cycle involves alternating parasitism of arthropod vectors and mammalian hosts. The aptly named *Borrelia recurrentis* is the only one of the RF Borrelia transmitted by the human body louse (*Pediculus humanus*) and is historically the most important of the RF borreliae. Numerous plagues of RF have been recorded, going back at least as far as the time of Hippocrates. Associations with war, famine, and displaced populations resulting in poverty and overcrowding were well known, but the specific association with body lice was not recognized until 1907. The 20th century witnessed devastating epidemics of louse-borne RF (LBRF). In the aftermath of the Russian revolution, there were 13 million cases of LBRF in Russia and Eastern Europe, resulting in 5 million deaths. Because humans are the only mammalian host of LBRF and its vector, outbreaks can be effectively aborted by treatment of clothes and bed linins with insecticides or simply by heating to at least 55 °C (130 °F) for 5 min. LBRF has been eradicated everywhere except for isolated areas of Ethiopia and neighboring countries involved in war (Sudan, Eritrea, and Somalia).

Aside from *B. recurrentis*, all other RF borreliae are transmitted by ticks and have nonhuman animal host reservoirs. These tick-borne forms of RF are considered endemic zoonoses and are found worldwide. Most (but not all!) borreliae causing tick-borne relapsing fever (TBRF) are transmitted by the *Argasidae* family of soft-body ticks, while the LD-related borreliae are transmitted by the *Ixodidae* family of hard-body ticks. Because of the close relationship between TBRF borreliae species and their tick vectors, the names of the *Borrelia* species derive from the species of tick vectors that transmit them: *B. hermsii* is transmitted by *Ornithodoros hermsi*, *B. parkeri* is transmitted by *Ornithodoros parkeri*.

The distinction between different types of ticks is important because their different feeding strategies dictate the circumstances under which humans are likely to encounter the borreliae they carry. Soft-body ticks are nocturnal feeders that seek out sleeping animals by following carbon dioxide and temperature gradients. Although large in size, they have a painless bite and their soft bodies have a distensible stomach that allows them to feed rapidly (15–90 min), drop off, and disappear before being recognized. The large blood meal allows soft body ticks to live for up to 15 years between feedings, while retaining viable borreliae in their midgut. Ornithodoros species of soft body ticks may transmit borreliae to their progeny, a process referred to as "transovarial transmission." The frequency of transovarial transmission of borreliae to tick progeny varies greatly between tick species. The frequency of transovarial transmission is high in *O. turicata*, low in *O. hermsi*, and does not occur in *O. parkeri*.

Hard-body ticks seek out their blood meal during the day and feed for longer periods of time (typically days). The smaller stomach size also requires more frequent feedings. Blood meals are required for a hard-body tick to mature from larvae to nymph, from nymph to adult, and then for the adult to reproduce. Hard-body ticks are mentioned here because there are two notable exceptions to the rule that TBRF Borrliea are transmitted by soft-body ticks: *B. miyamotoi* is transmitted by *Ixodes* species (wood ticks), and *B. lonestari* is transmitted by *Amblyomma americanum* (the lone star tick). In north America, the prevalence of *B. miyamotoi* in nymphal and adult *I. scapularis* ticks is 10-fold lower than that of the Lyme disease spirochetes. However, *B. miyamotoi* spirochetes are also present in host-seeking larval ticks as *B. miyamotoi* can infect ticks transovarially from adult ticks to their offspring, whereas *B. burgdorferi* must be acquired by larval ticks during a blood meal.

16S rRNA sequences of RF *Borrelia* spp. separate phylogenetically into three relatedness groups: Old World RF borreliae, New World TBRF borreliae, and the *B. hermsii*/*B. anserina* group (Fig. 9). *B. hermsii* is the most common agent of human TBRF in North America and is endemic to the coniferous forests of the western United States and southern British Columbia from 3000 to 9000 ft. in elevation. The incidence of TBRF peaks in July and August when vacationers visit rustic cabins in mountainous locations that are inaccessible during the winter. *B. hermsii* achieves high blood densities for prolonged periods of time in pine squirrels (*Tamiasciurus* spp.), which serves to facilitate transmission to other ticks. Chipmunks and some rodents may also become infected but with lower blood densities and for shorter periods of time than in pine squirrels. Two distinct genomic groups of *B. hermsii* have been described based on sequencing the intergenic spacer region between the 16S rRNA and *ileT* tRNA genes. The distribution of the two *B. hermsii* genomic groups overlap geographically, indicating that migratory animals, such as birds, may play a role in dissemination. *B. hermsii* has been found in the bloodstream of a dead owl, and is phylogenetically related to *B. anserina*, the agent of avian spirochetosis (Fig. 9).

Other New World TBRF Borrelia differ from *B. hermsii* in their geographical distribution. *B. turicatae* occurs in the southwestern United States and northern Mexico. TBRF outbreak of *B. turicatae* infection has been reported in Texas. *B. parkeri* isolates from ticks in the coastal regions of California and Baja California have been implicated as a cause of human disease, but the evidence is circumstantial. Recently, the 16S sequence of a related *Borrelia* species was obtained from the argasid bat tick, *Carios kelleyi*, from an attic in Iowa. There is the potential for human disease given the close phylogenetic relationship with human pathogens, the

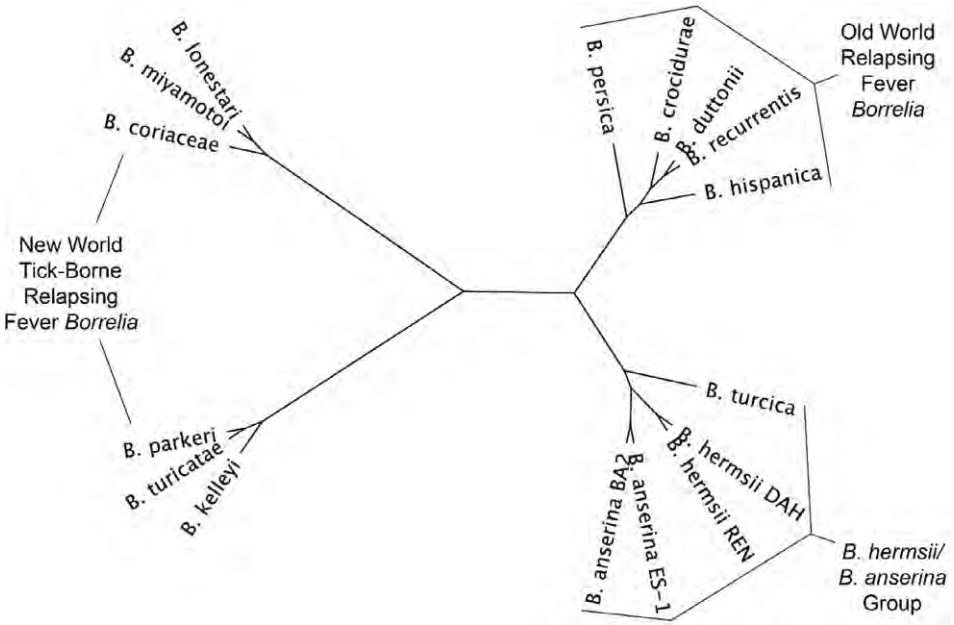

Fig. 9 Phylogenetic tree of the relapsing fever borreliae. 16S rRNA sequences of Old World Borrelia spp., including B. recurrentis, the agent of louse-borne relapsing fever, cluster in the lower right section of the tree. Sequences of New World Borrelia spp. cluster in the upper section of the tree. Sequences from B. hermsii and the bird-associated B. anserina cluster in the lower left section of the tree.

cohabitation of *C. kelleyi* in homes and the willingness of *C. kelleyi* to feed on humans. *B. coriaceae* is transmitted by soft-body Ornithodoros ticks and its reservoir in North America is the black-tailed deer. Human infection with *B. coriaceae* has not been described, but it is thought to cause abortion in cattle. *B. mazzottii* and *B. venezuelensis* have been described in Central and South America, but their 16S rRNA sequences and relatedness to other New World TBRF borrelia are unknown.

Some New World TBRF species are transmitted by hard-body ticks. Like *B. burgdorferi*, *B. miyamotoi* is found in Ixodes ticks and *Peromyscus leucopus*, the white-footed mouse. *B. lonestari* is carried by *Amblyomma americanum*, the lone star tick, which is widely distributed in North America and known to transmit ehrlichiosis and tularemia. The ability of *B. lonestari* to infect humans is unknown. Among Old World TBRF borrelia, *B. duttonii* is the species that is genetically most similar to *B. recurrentis*, and they probably share a common ancestor. *B. duttonii* and the related species, *B. crocidurae*, are transmitted by soft-body ticks and are important causes of TBRF in Sub-Saharan Africa. A number of other Old World TBRF Borrelia have been described in the Middle East, the Caucasus, and Central Asia, but 16S rRNA sequences are available for only a couple of these species: *B. persica* and *B. hispanica*, found in Israel and Spain, respectively.

Molecular Pathogenesis and Disease

The molecular mechanisms of antigenic variation that are the hallmark of relapsing fever have been best described in *B. hermsii*. In the tick, the major *B. hermsii* surface protein is the variable tick protein, which is required for transmission to the mammalian host during tick feeding. In response to temperature changes during the blood meal, *B. hermsii* switches expression to the variable major protein (vmp) locus located on the expression plasmid. As the bacteria begin to reach high densities in the bloodstream of the infected animal, the host mounts an antibody response to the protein encoded by the gene in the vmp expression locus. Clearance of bacteria by vmp-specific antibody is eventually followed by emergence of bacteria that have undergone a recombinational event on the expression plasmid involving insertion of an alternate gene from the repertoire of vmp genes. It had long been observed that there was a bias toward a patterned sequence of vmp gene insertion events. An explanation for the pattern was provided by the upstream homology sequence (UHS) and downstream homology sequence (DHS) of the variable genes. The probability of a subsequent gene being inserted into the variable protein expression locus was related to the homology of its UHS with the gene currently in the locus and the distance from the end of the new gene to its DHS.

A programmed succession of surface proteins enables RF borreliae to repeatedly emerge at high levels in the bloodstream (Fig. 10). The ability to repeatedly emerge into the bloodstream is advantageous to the bacteria, because it favors acquisition by bloodfeeding arthropods. However, such a high density of bacteria is very hazardous to their animal host, because it evokes such an intense immune response to the foreign antigens. Bouts of LBRF and TBRF differ in their intensity and in the number of relapses. LBRF tends to recur less often, but the episodes are much more severe, with a mortality rate of 4–40%. After a typical incubation period of 7 days, patients experience sudden onset of fever, rigors, headache, muscle pain, and lethargy. In LBRF, most patients have liver and spleen enlargement, while cough and symptoms of meningitis are common. Nerve palsies, paralysis, seizures, and coma may occur in severe cases. The most common causes of death in LBRF are arrhythmias of the heart, brain hemorrhage, and liver failure. LBRF during pregnancy frequently results in miscarriage. The mortality rate in TBRF is typically much lower: 2–5%. Nevertheless, TBRF due to *B. turicate*, *B. duttonii*, and *B. crocidurae* are frequently associated with debilitating neurologic symptoms not seen with other forms of TBRF.

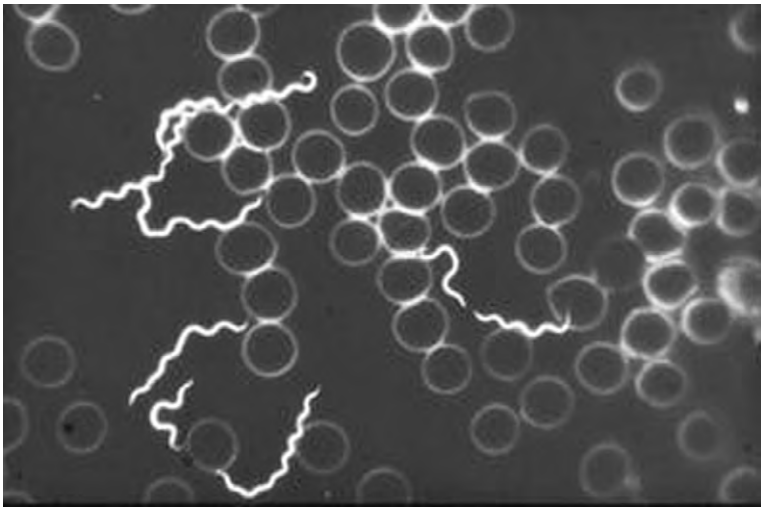

Fig. 10 Dark-field micrograph of blood containing relapsing fever borreliae. Variation in surface antigens enables relapsing fever borreliae to reach high levels in the bloodstream, often achieving densities as high as 10^6–10^7 bacteria per milliliter. Spirochetes appear as bright wavy forms while the red blood cells appear as fainter circles.

RF Borrelia are susceptible to a broad range of antibiotics. LBRF can be successfully treated with a single dose of tetracycline. Beta-lactam antibiotics such as penicillin are typically avoided in LBRF because they may result in the sudden lysis of large amounts of bacterial antigens, which can precipitate the Jarisch-Herxheimer reaction, a paradoxical worsening of symptoms with severe chills, fever, and potentially life-threatening shock. Patients should be observed for 2 h after initiation of antibiotics in case there is a need for resuscitation with intravenous fluids.

Brachyspira

Another major grouping within the order *Spirochaetales* are the *Brachyspira*, which are intestinal spirochetes classified within the family, *Serpulinaceae*. *Brachyspira* are large, loosely coiled spirochetes ranging in size from 2 to 13 μm in length, and 0.2–0.4 μm in width. *Brachyspira* are able to grow under strict anaerobic conditions, but small amounts of oxygen can increase growth efficiency. The *nox* gene, encoding NADH oxidase, is required for oxygen tolerance. Inactivation of the *nox* gene increases oxygen sensitivity 100-fold. *Brachyspira* are cultivated anaerobically on blood agar at 37 °C and selective media are typically used for primary isolation of organisms from stool specimens.

The *Brachyspira* have undergone a series of changes in nomenclature. The isolation of the swine dysentery agent was originally described in the early 1970s and referred to as "*Treponema hyodysenteriae*." In the 1990s, DNA-DNA hybridization and partial 16S sequence data indicated that the "*T. hyodysenteriae*" organism had little genetic relatedness to the treponemes and was assigned its own genus, *Serpula*, which was quickly reclassified *Serpulina* to avoid confusion with a previously named fungal genus. However, *Serpulina* eventually gave way to *Brachyspira* when it was realized that *S. hyodysenteriae* was related to *Brachyspira aalborgi*, which had been isolated from humans with intestinal spirochetosis in Aalborg, Denmark in the early 1980s. The prefix Brachy-, deriving from the Greek word for "short," was used as a descriptive term because the Danish isolates were only 2–6 μm in length.

B. hyodysenteriae is an important worldwide problem for the pig industry. Outbreaks with mortality rates of up to 50% occur in naïve herds. The infection is a true dysentery, causing inflammatory and hemorrhagic disease of the colon. *B. hyodysenteriae* is beta hemolytic on sheep blood agar and hemolysins are thought to be important virulence factors. The organism is difficult, but not impossible, to eradicate from farms. In Scandinavia, where the use of antibiotics is strictly controlled, few herds are infected by *B. hyodysenteriae*. In most countries, antibiotic supplementation of feed is used to suppress the *B. hyodysenteriae* problem, and infection rates are often over 30%. However, antibiotic resistance is growing and new strategies for prevention and control of *B. hyodysenteriae* infection are urgently needed.

The genus *Brachyspira* is now populated with a number of commensal and pathogenic species, which have been isolated from the intestinal tracts of a variety of animal hosts. Species with predilections for pigs, humans, and birds are clustered on a phylogenetic tree from their 16S sequences (Fig. 11). "*B. suanatina*" is the name proposed for an organism that is related to, but genetically distinct

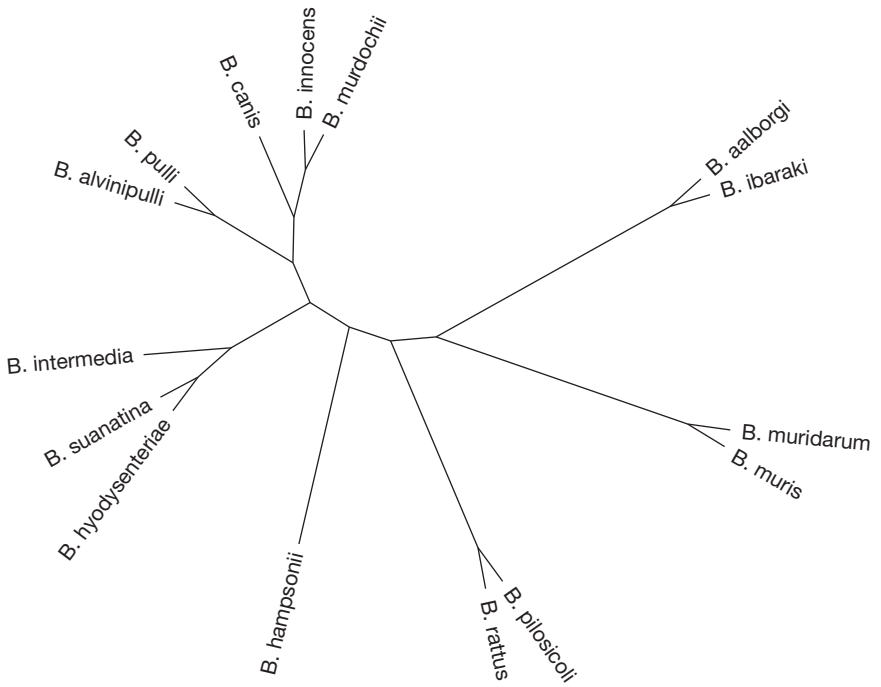

Fig. 11 Phylogenetic tree of the Brachyspira. Relatedness tree of 16S rRNA sequences of Brachyspira spp., including B. hyodysenteriae, the agent of swine dysentery. B. aalborgi and B. pilosicoli cause intestinal spirochetosis in humans in developed and developing countries, respectively.

from, *B. hyodysenteriae* by 16S rRNA sequence analysis. *B. suanatina* has been isolated both from pigs and from mallard ducks, is beta hemolytic and can cause disease in experimentally infected pigs. *B. intermedia* is a third pig isolate found in the same genetic cluster with *B. hyodysenteriae* and *B. suanatina* and may cause disease under certain circumstances. Nondysenteric porcine diarrhea due to intestinal spirochetosis has been linked to *B. pilosicoli*, which has also been associated with disease in chickens and humans (see below). *B. innocens* and *B. murdochii* are considered to be commensals occasionally isolated from healthy pigs.

Brachyspira species are also important in the poultry industry. Diarrhea and egg production problems in chickens have been attributed to *B. alvinipulli*, *B. intermedia*, and *B. pilosicoli*. As in pigs, *B. innocens* and *B. murdochii*, and a third species, *B. pulli* appear to be nonpathogenic for chickens. Chronic watery diarrhea due to human intestinal spirochetosis (HIS) has been linked to two species, *B. aalborgi* and *B. pilosicoli*, with the latter being associated with intestinal disease in pigs and chickens. High prevalence rates of *B. pilosicoli* carriage have been found in aboriginal populations living in poor sanitary conditions with high levels of animal exposure. In contrast, *B. aalborgi* occurs more frequently in developed countries, typically in AIDS patients with chronic diarrhea. The pathogenic potential of *Brachyspira* spp. for humans is controversial. Biopsies show palisades of brachyspires lining the surface of colonic epithelial cells, which is likely to impair function (Fig. 12). *B. pilosicoli* is associated with watery diarrhea and has been isolated from the bloodstream of sick patients.

The genome sequences of several *Brachyspira* species have now been elucidated. Large numbers of proteases and hemolysins were identified in the *B. hyodysenteriae* genome as potential virulence factors. A surprising discovery was the finding of an *rfb* gene cluster on the 36 Kb circular plasmid of *B. hyodysenteriae*. Among *B. hyodysenteriae* strains, plasmid loss is associated with virulence attenuation. The most abundant outer membrane proteins of *B. hyodysenteriae* are the Vsp proteins, which are encoded by 7–10 paralogous genes per genome. Lack of significant sequence variability of vsp genes suggests that these genes are not used as a mechanism for antigenic variability. In fact, multiple Vsps are transcribed simultaneously and expressed as high molecular mass protein complexes. Vsps and other outer membrane proteins expressed during infection are of great interest as potential vaccines and serodiagnostic antigens.

Leptospira

Morphology and Metabolism

"*Leptospira*" derives from the Greek *leptos* (thin) and Latin *spira* (coiled). Aptly named, the leptospires are among the thinnest bacteria known: a mere 0.1 μm in diameter and 6–12 μm in length (Fig. 13). Leptospires are right-handed helices, with 18 or more coils per cell, frequently forming hooks at one or both ends. Hooks at both ends gave rise to the species name *L. biflexa*, and a hook at one end was thought to look like a question mark, leading to the name *L. interrogans*. The hooks are due to a single endoflagellum at each end of the cell. In liquids, viable leptospires are continuously in motion. In semi-solid (0.2% agarose) conditions, leptospires can be observed by darkfield microscopy to be remain motionless for periods of time, with occassional corkscrew-like movements. This resting state may in part explain the ability of leptospires to persist in the environment. Most leptospires are able to remain motile for months in distilled water, and their survival can be significantly prolonged by addition of a substrate such as agarose.

Several different leptospiral growth media have been developed. The standard culture medium is Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, which provides long-chain fatty acids in the form of tween (polysorbate) as an energy and carbon source, several divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+}), iron, and vitamins (thiamin and cobalamin). EMJH medium contains bovine serum albumin (BSA), which is thought to function by preventing fatty acid oxidation, which is toxic for

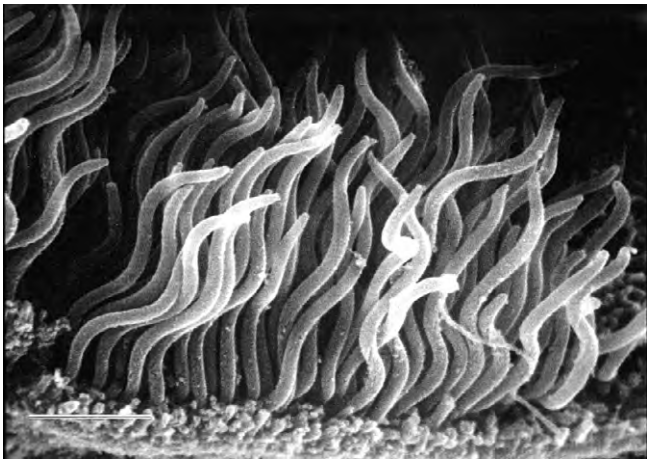

Fig. 12 Scanning electron micrograph showing palisades of brachyspires exhibiting end-on attachment to the luminal surface of colonic epithelial cells. Marker bar = 2 μm. Reproduced from Hampson, D. J. and Stanton, T. B. (eds.) (1997). Intestinal Spirochaetes in Domestic Animals and Humans. ISBN: 0-85199-140-8.

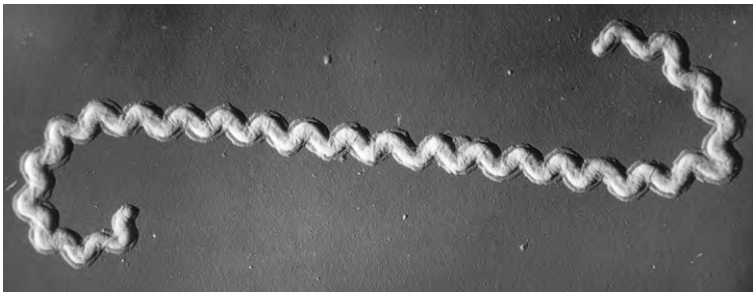

Fig. 13 Transmission electron micrograph of leptospiral cell showing characteristic helical morphology and a single endoflagellum at each end of the cell. Magnification c. 30,000 ×. Shadowed electron micrograph obtained by Annabella Chang and used with permission from Ben Adler, Microbiology Department, Monash University, Australia.

spirochetes, and by providing additional trace nutrients. BSA is expensive and batch-to-batch variability in its ability to support leptospiral growth is a major problem. Serum is not required for EMJH but is often added to promote growth. Another problem with BSA-containing media is that due to prion concerns many countries, including the United States, require any bovine products such as BSA to be autoclaved prior to import. A non-BSA containing leptospiral medium that can be used as an alternative transport medium is modified Kortoff medium, which consists of peptone, salts, and 8–10% heat-inactivated slightly hemolyzed rabbit serum. The optimum growth temperature is 30 °C.

Phylogeny

A number of different *Leptospira* species have been described with a range of pathogenic potentials (Fig. 14). On one end of the spectrum are the nonpathogenic saprophytic species, including *L. biflexa* and *L. wolbachii*, which are unable to cause infection. On the other end of the spectrum are the pathogens, including *L. interrogans*, which are able to produce lethal infection in a variety of mammals, including man. Species with intermediate pathogenicity, such as *L. fainei*, can be isolated from clinical specimens, but cause minimal or no disease. Leptospires can also be classified serologically, and over 250 different named serovars have been described. Serovars are classified into one of 28 different serogroups on the basis of antigenic cross-reactivity. Serovar specificity appears to be driven by the carbohydrate structure of lipopolysaccharide side-chains, a dominant antigen on the leptospiral surface.

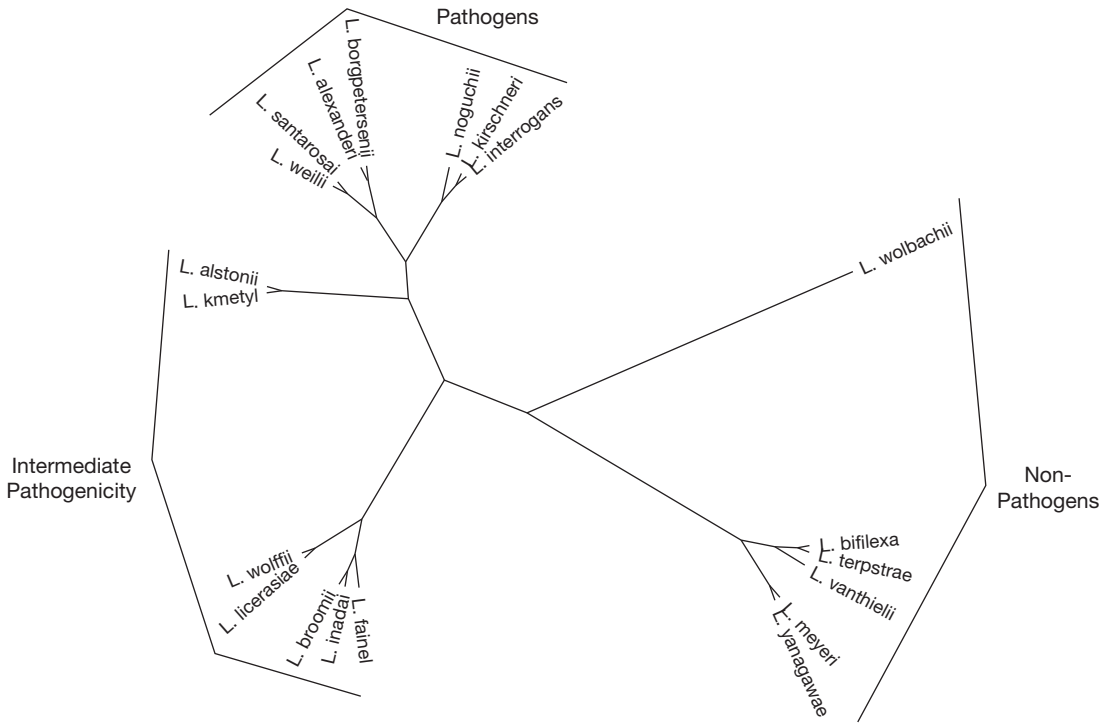

Fig. 14 Phylogenetic tree of the Leptospira. Comparison of leptospiral 16S rRNA sequences shows segregation into three relatedness clusters: Nonpathogens (lower left), pathogens (lower right) and organisms with intermediate pathogenicity (upper section).

There is limited correlation between the genetic and serologic classification systems, with serologically identical strains occurring in multiple species.

The phylogenetic and antigenic diversity of *Leptospira* spp. reflects their ability to adapt to a variety of different environmental niches. Leptospires have been isolated from most animal species (including reptiles and amphibians) and natural bodies of fresh water wherever the effort has been made. Presumably, leptospires represent an ancient branch of the bacterial family tree that has coevolved with vertebrates. In reservoir host animals, leptospires have developed a unique commensalist strategy. Organisms with the capacity to infect animals typically reside in the lumen of the proximal renal tubule and are shed into the environment in the urine. The fluid within the proximal tubule is a nutritionally rich filtrate of serum and is yet an immunologically protected site; kidney sections of infected rats show little or no inflammatory reaction surrounding infected tubules. By not subjecting their host to any detrimental effects, this arrangement effectively affords leptospires a "free ride" for the life of the animal host. The life-cycle of the organism is completed when organisms released into the environment encounter a new host through adhesion, vascular invasion, and dissemination to the kidney.

Genome sequences provide insight into differences between the various leptospiral lifestyles. The free-living saprophytic nonpathogen, *L. biflexa*, has a genome size of 3.96 Mb, with a relatively high coding density and an abundance of signal transduction genes enabling it to respond to the unpredictable environmental stresses found outside the host. In contrast, *L. interrogans* has a biphasic lifestyle and seems equally at home in the aquatic environment and in the mammalian host. The 63% of *L. interrogans* genes shared with *L. biflexa* consist of essential housekeeping genes and genes important in survival outside the host. The other 37% of *L. interrogans* genes are presumed to be important for life within the mammalian host. On the other end of the spectrum is *L. borgpetersenii*, which has evolved into an obligate parasite of cattle. Infection occurs through direct contact with carrier animals, *L. borgpetersenii* has limited survival outside the host and is difficult to culture. This host dependence is reflected in the erosion of the *L. borgpetersenii* genome; many of its genes are lost or inactivated by mutations or transposon insertions. *L. borgpetersenii* has only about half the signal transduction genes that *L. biflexa* has, confirming the "locked-in" nature of its host dependency.

Pathogenesis

To acquire the ability to invade and colonize the mammalian host, *L. interrogans* has acquired a large array of novel genes. Some of these pathogen-specific genes are known to encode outer membrane proteins such as the porin, OmpL1, and a number of lipoproteins, some of which are involved in host-pathogen interactions. An essential host-pathogen interaction that distinguishes leptospiral pathogens from saprophytes is serum resistance. Leptospiral serum resistance is mediated, at least in part, by LenA, an outer membrane lipoprotein found exclusively in leptospiral pathogens. LenA binds Factor H, a complement regulatory protein that prevents the alternative pathway of complement from damaging host cell membranes. Leptospires (and other spirochetes) coat their surfaces with Factor H to avoid the bactericidal effects of complement.

Pathogenic leptospires coat their surfaces with additional host factors using proteins belonging to the Lig (leptospira immunoglobulin-like repeat) family. Leptospiral pathogens, but not the saprophytes, have between 1 and 3 Lig proteins. Ligs are very large (112–220 kD) proteins containing a series of 12–13 immunoglobulin-like repeats, some of which mediate high-affinity binding to multiple host proteins, including fibronectin and fibrinogen. Interactions with host proteins are facilitated by induction of Lig expression in response to levels of osmolarity (300 mOsm) found in host tissues. Lig expression by leptospires grown in EMJH, which has low osmolarity (67 mOsm), is poor. Addition of salt (or any other osmotically active molecule) to EMJH medium rapidly induces Lig expression. As a second regulatory mechanism, Lig expression is posttranscriptionally regulated by temperature. Temperature-sensitive RNA stem-loop structures sequester the *lig* ribosome binding site and start codon preventing translation. In these ways, leptospires in low osmolarity, low temperature aquatic environments are saved the metabolic expense of expressing Lig proteins until they are needed for colonization of a warm-blooded host.

Acquisition of virulence genes was essential in the evolution of leptospires from free-living to pathogenic organisms. Genes appear to have been horizontally transferred from a variety of sources. For example, the gene encoding the major outer membrane lipoprotein, LipL32, is highly conserved among leptospiral pathogens, does not occur in the nonpathogens, and appears to have been acquired by a gene insertion event. LipL32 has recently been shown to be primarily subsurface, exposed on the periplasmic side of the outer membrane. The function of LipL32 is unknown and does not appear be required for virulence; an *L. interrogans lipL32* mutant was found to retain the ability to cause acute infection in hamsters and chronic infection in rats. Horizontal genetic transfer also occurs between leptospiral pathogens; 20% of *ompL1* genes are mosaics containing fragments of multiple leptospiral lineages. However, permissiveness for gene acquisition is a double-edged sword—the genomes of leptospiral pathogens have much higher numbers of insertion sequence (IS) elements than the nonpathogens. The IS elements contain transposon genes that, once they infect the genome, mediate IS element proliferation and gene disruption. IS elements appear to be a major mechanism of genome erosion in *L. borgpetersenii*. Transposons are now being put to good use in leptospiral research—leptospiral pathogens had been much more difficult to transform than the nonpathogens, which had been a major impediment in leptospiral pathogenesis research. Now, however, the mariner transposon has been found to be useful for manipulating the genome of leptospiral pathogens—hundreds of single-gene knock out mutations have been generated in *L. interrogans*. Mutants with disrupted LPS, outer membrane protein, regulatory, and motility genes have been found to have reduced virulence for hamsters, providing new insights into leptospiral virulence.

Epidemiology and Disease

Leptospirosis epidemiology has traditionally been carried out by serotyping isolates or examining the serologic response of infected patients. However, serologic approaches are fraught with problems, including the frequent observation that patients' antibody responses may not be specific for the infecting serovar. Genetic tools provide more accurate molecular approaches for tracking the epidemiology of leptospirosis. Genome sequencing technology is replacing 16S sequencing, DNA-DNA hybridization, and multi-locus sequence typing for leptospiral species and strain identification. Approaches such as these reveal that rat-associated strains of *L. interrogans* are frequently the cause of leptospirosis outbreaks in urban settings. Rats are found wherever people live and wherever the studies have been done, urban rats are found to have a high leptospirosis carriage rate in their kidneys. The prevalence of leptospiral carriage among the ubiquitous *Rattus norvegicus* is probably the reason that leptospirosis is the most widespread zoonosis. Leptospirosis occurs less frequently in westernized countries because housing standards are effective at excluding rats from human living spaces. However, in developing countries with poor housing standards, leptospirosis outbreaks occur regularly in urban settings after heavy rainfall and flooding.

Leptospirosis infections range in severity from self-limited flu-like illness to multiorgan system failure and death. After an incubation period of 5–14 days, there is the onset of fever, myalgia, headache, abdominal pain, nausea, and vomiting. In tropical regions where leptospirosis typically occurs, these early nonspecific symptoms can be confused with dengue fever or malaria. When observed, conjunctival suffusion (scleral redness without discharge) can be a distinguishing sign of leptospirosis. During this initial septicemic phase, spirochetes can be recovered from the blood and spinal fluid. Formation of agglutinating antibody leads to clearance of organisms and, in milder cases, a temporary resolution of symptoms. However, a second, immune phase of the disease may follow, with milder fever, headache, and vomiting. In more severe infections, the initial phase progresses rapidly to jaundice and renal failure, known as Weil's syndrome, with a mortality rate of 10%. The renal failure due to leptospirosis is a unique form of kidney dysfunction associated with high urine output and low serum potassium levels. At this stage, complications can be avoided with replacement of fluids and electrolytes. If, on the other hand, dehydration occurs and renal failure ensues, access to peritoneal or hemodialysis is essential for survival. Certain strains of *L. interrogans* cause acute lung involvement with shortness of breath due to airspace hemorrhage and a much higher mortality rate of 50%.

Pathogenic leptospires are highly susceptible to common antibiotics including doxycycline and ampicillin and it is likely that antibiotic therapy given at the first signs of infection would significantly reduce morbidity and mortality. However, currently available diagnostic tests have relatively low sensitivity during early infection and patient populations at highest risk typically have poor access to medical care. Recent studies show that most patients with early infection have antibodies to the Lig proteins. What is needed is a diagnostic test that is portable, easy to use, does not require electricity, and has a long shelf-life at room temperature. Whole-cell vaccines are used widely in domestic animals, including dogs, pigs, and cattle. A similar vaccine has been found to be effective in humans, but is generally not available because of concerns regarding side-effects and a relatively short duration of immunity. A preventative approach for adventure travelers participating in water sports in areas with a history of leptospirosis is weekly doxycycline, which has been shown to be effective in US soldiers undergoing jungle training in Panama. Doxycycline is not appropriate for children or pregnant women and may cause photosensitivity or gastrointestinal side-effects. An alternative approach recommended by some travel-experts is weekly azithromycin, which has a better safety profile, but has not been rigorously tested for efficacy.

Conclusions

Spirochetes are widely distributed in nature as free-living bacteria, as metabolic symbionts of insects, and as commensals and parasites of animals. *Spirochaeta* spp. isolated from natural bodies of water are related by 16S rRNA sequence analysis to treponemes found in the oral cavity and in the digestive tracts of termites. *Borrelia* spp. also have the ability to colonize the digestive tracts of insects, in this case ticks and lice, which serve as vectors for transmission to animal host reservoirs. *Brachyspira* spp. colonize digestive tracts of animals either as commensals or parasites. *Leptospira* spp. exist as free-living organisms or cycle between the aquatic environment and animal host reservoirs via their renal tubules. The diversity of spirochete life-styles demonstrates the functional versatility of their unique morphology and mechanism of motility.

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Spontaneous Generation

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Glossary

abiogenesis Term used in 1870 by Huxley to mean life arising from a combination of inorganic starting materials. Since that time, has come to be used more widely to indicate origin of life from any nonliving matter.
archebiosis Term coined by Bastian in 1870 to mean life arising from strictly inorganic starting materials.
biogenesis Term used in 1870 by Huxley to mean life arising only from other living things, the opposite of which he termed abiogenesis. The term seems to have been first coined by Bastian earlier that year, but to mean exactly the opposite: spontaneous generation. It is Huxley's usage that became famous and remains the definition of this term today.
heterogenesis Nineteenth century term used to mean the origin of living things from organic materials, for example, from infusions of plant or animal matter, or from decaying tissue in a diseased or dying organism. Thought to be the source of tumors, parasitic worms, microorganisms from putrefaction, as these organized themselves from the smallest microscopically visible particles (molecules) of organic tissues. Note that many supporters of heterogenesis might not support the more extreme position of archebiosis/abiogenesis from inorganic matter.
molecules Term used from the writings of Buffon (c.1750) onward to indicate the tiniest (bacteria-sized) microscopically visible particles of organic matter in blood, tissue, infusions, and so on. This term was used by histologists, pathologists, and cytologists until the 1870s, obviously in a way quite different from the chemists' use of the term that developed through the same time period. Important examples include the 'active molecules' of Robert Brown (1828–29), the cellforming cytoblastema 'molecules' of Theodor Schwann (1839), and the histological 'molecules' of John Hughes Bennett (1840–75). Many thought clumping together of these units was the basis for heterogenesis.
pleomorphism The doctrine that microorganisms did not come in distinct, stable Linnean species but rather were highly variable in shape and metabolic capabilities in response to changes in their environment. Many believed this to be true of the bacteria, especially in Britain and Germany through the 1880s. More extreme versions held that bacteria, yeasts, molds, and algae were all interconvertible stages in the life cycle of a single organism. Heterogenesis can be seen as the next logical step, including the transition from nonliving organic matter to the simplest bacterial forms as but one more stage in that fluid transformability.

Introduction

The idea that living things can originate from nonliving materials, spontaneous generation, has a long history, inseparably intertwined until about 1880 with the development of microbiology as a science. No less an authority than Aristotle claimed that cases of spontaneous generation could be observed in nature, and his support was important in establishing the idea for many centuries. Beginning around the time of the Scientific Revolution of the seventeenth century, however, the doctrine of spontaneous generation was increasingly challenged and became the subject of numerous episodes of controversy. As combatants tried to answer one another's criticisms, the new breakthroughs in technique and in experimental design that were developed served as some of the most important foundations upon which a science of microbiology could be built. As just one example, the development of a sterile technique as well as procedures to sterilize glassware and growth media all grew directly out of experiments trying to prove or disprove the possibility of spontaneous generation of microorganisms.

In seeking to answer a question as basic as how life originates, theory played a role as important or more so than technique or experiment. From the first, the doctrine of spontaneous generation was seen to be fraught with religious implications. If life could originate spontaneously from lifeless matter, the position of philosophical materialism, then a Creator God was irrelevant. If spontaneous generation occurred in present times, this was at odds with a single original Creation as told in the Bible. However, for those interested in a naturalistic worldview, such as supporters of Darwinian evolution, there was also a potential conflict. The doctrine of evolution was based on a profound philosophical assumption of continuity in nature, that is, that there were no sudden unbridgeable gaps between similar living forms, which would require supernatural intervention. Furthermore, Darwin's theory implied that the vast diversity of living things had come from one or at most a few original ancestral organisms, and these must have originated somehow. For many, then, to believe in evolution and a completely naturalistic worldview required the belief that no unbridgeable gap occurred between living matter and nonliving matter and that living organisms must have been capable of arising from nonlife, at least once on the early earth. Those with this view, for example, Henry Charlton Bastian,

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challenged hypocrisy on the part of those who supported Darwin but were unwilling to believe in the necessity of spontaneous generation.

Still another important source of disagreement, even among scientists who claimed not to care about religious implications, was fundamental epistemological assumptions about the nature of life. Some believed very deeply that all living things must reproduce by 'seeds' or 'germs', by analogy with the large number of organisms for which this process had been observed. So fundamental was this belief that, even in cases where microscopic life appeared in tubes of fluid boiled for hours, those scientists concluded that either the tubes must have leaked after boiling or there must be some kind of structures produced by microorganisms that were capable of withstanding previously unheard of temperatures, even though nobody had ever seen such structures. Their opponents, as late as the 1870s, believed that the most clearly observed and reliably established fact known about living things was that they were totally unable to survive the boiling temperature of water for more than a few minutes. From this equally tenable premise, they concluded that spontaneous generation was a less strained explanation of organisms in boiled infusions than the *ad hoc* invoking of 'spores' that nobody had ever experimentally demonstrated with totally unprecedented heat-resisting abilities. Thus, before ever carrying out an experiment, both sides in the spontaneous generation controversies were often begging the question at issue. Clearly, these philosophical issues were crucial points showing how experimenters could disagree because they were talking past one another, more than because of differences in the experiments themselves. The doctrine of spontaneous generation rose and fell in popularity repeatedly at different times in different countries in the past several centuries, and to tell the story of spontaneous generation controversies only as a series of battles about 'dueling experiments' would be to misunderstand much or even most of what the controversies were really about.

Early History

From antiquity it had been maintained that frogs, eels, mice, and numerous worms and insects, especially parasitic worms living inside animals, could arise by spontaneous generation, mostly heterogenesis. With Leeuwenhoek's discovery of microorganisms, many naturalists assumed that these too could arise without parents. Indeed, as microbes seemed exceedingly simple, and bacteria simplest of all, it was believed that they were the most likely to be organizable from nonliving materials. Francesco Redi, natural philosopher to the same Tuscan court that was patron to Galileo, carried out some famous experiments in 1668 that investigated specifically the origin of insects. Redi's experiments showing that maggots come from fly eggs, not from rotting meat, usually led off histories of spontaneous generation debates. It was commonly believed until that time that the appearance of maggots in rotting meat was a clear example of spontaneous generation. Redi placed samples of many different types of meat and fish in glass jars. One set of jars was open to the air and soon swarmed with maggots. The other set was covered with fine muslin cloth. Redi saw that, while maggots never appeared in the meat of those jars, flies crawled about on the cloth and sometimes laid eggs there. Those eggs were seen to hatch into maggots, disproving spontaneous generation as their origin. Though Redi himself continued to believe that some insects, such as gall flies, did arise by spontaneous generation, many naturalists assumed from this time onward that spontaneous generation, if it occurred, only did so among parasitic worms and microorganisms.

Of course, working at the time of the Scientific Revolution, Redi did participate at a time when experiment came to the fore as an activity ever afterward central in natural philosophy. Spontaneous generation debates, like all other natural philosophic questions, featured experiments more and more prominently from the late seventeenth century onward. Just as 'natural history' or 'natural philosophy' historically did not mean what is now called 'science' until fairly recently, neither did 'experiment' always mean what it means now. Many natural philosophers in the seventeenth century were interested in the power of the experimental method, but were just as interested in public demonstrations of 'experiments' as a way of convincing audiences, for example, prospective patrons, that their enterprise was qualitatively different from the book-dominated natural philosophy of the past, and from the subjective and often bloody religious disputes that had traumatized European life for over a century. Recent studies have looked closely at Redi's career, especially his relationship with the Medici Grand Dukes, his principal patrons. One such study concludes that Redi's public demonstration of experiments was a form of court entertainment in which the final arbiter of the meaning and success of the outcome was the Grand Duke. Thus there was a world of difference between Redi's procedures and what would by the nineteenth century be called 'controlled experimentation by a biologist'. He set high value on repeated observation and demonstration, saying, "I do not put much faith in matters not made clear to me by experiment." Paradoxically, however, he suggested elsewhere that he experimented "in order to make myself more certain of that of which I am already most certain." For Redi, then, especially in his primary role as an early modern courtier, 'experiment' meant something quite different from what we understand by that term today, particularly with regard to the role of 'preconceived expectations'. The paradox results only if we assume in a naively ahistorical way that what Redi called 'experiment' should be assumed to have the same meaning that term came to have after 'natural philosophy' had been transformed into 'science'.

Once we gain this richer sense of what experiment meant in the historical context in which Redi lived and worked, we may begin to question the validity of an ahistorical narrative that uncritically compares his work with experiments performed by Pasteur, Tyndall, and their antagonists two centuries later, merely because of superficial similarity in 'the use of controls'. Perhaps there are more similarities in the meaning of 'experiment' in such different historical settings, but this is an open question that can only be answered by looking at the full context in which each investigator had to operate, including such questions as the existing system of patronage upon which each depended to support work on scientific pursuits. Such points of comparison will be developed further when discussing Pasteur's work.

The Needham/Spallanzani Controversy

Eighty years after Redi, in 1748, another series of experiments by the Irish priest John Turberville Needham was published, and this time was widely believed to support the spontaneous generation of microorganisms. Needham soon collaborated with the French aristocrat Comte de Buffon. And over the next several decades the work of Buffon and Needham was opposed by many, including Charles Bonnet and Lazzaro Spallanzani.

Needham's first experiments found that, when mutton gravy was stoppered and sealed with mastic in glass tubes and heated to boiling for a time, the fluid afterward teemed with microorganisms (protists). Buffon and Needham later reported seeing tiny particles (in the size range of bacteria) that they called 'organic molecules', which came from disintegrating organic material and moved very actively. These, they said, clumped together to form the larger animalcules (protists). They also claimed that sealing and boiling proved that the molecules did not originate from "insects or eggs floating in the atmosphere." Most naturalists considered this, if true, to be a case of spontaneous generation.

Spallanzani, an Italian cleric, carried out experiments challenging these claims and first published his results in 1765. His opposition was based on two main arguments. First, he said that with his own microscope he never saw anything like the 'organic molecules' and therefore charged that Buffon and Needham were only seeing what they wanted to see, because Buffon's philosophy of nature had predicted that such particles must exist. This claim was widely repeated. Second, he said that when he boiled infusions similar to Needham's, he did not find any living microbes in them, as long as he sealed the tubes by melting the glass shut in a flame before boiling and continued the boiling for at least an hour. Thus, Spallanzani claimed that the microorganisms in Needham's infusions must indeed have gotten in, despite Needham's precautions, from the atmosphere after boiling.

Needham responded that boiling for as much as an hour would clearly be so severe a treatment as to deprive the air in the tubes of its power to generate or support life. Later experiments showing that indeed such treatment decreased or consumed the oxygen content in the tubes seemed to support Needham's claim. Thus the experimental outcome was underdetermined by the experiments themselves, so the controversy was able to continue through the 1780s.

A popular notion of experiment has it that 'proper science' can only occur when the scientist approaches his experiment with no 'preconceived ideas' about the outcome, and intends to "let the chips fall where they may." In many histories of the spontaneous generation debates, the 'losers' are often accused of having been biased beforehand by their belief in a 'vital force' or some similar overarching philosophy, while Redi, Spallanzani, Pasteur, and Tyndall epitomize the openminded investigator. A closely related claim has been that Spallanzani, Pasteur, and others were often able to defeat their foes by virtue of possessing better instruments, especially superior-quality microscopes. Thus, as the story has been told since the early nineteenth century, the Comte de Buffon and his collaborator Needham in the 1740s were portrayed as 'armchair philosophers' who cooked up a doctrine of 'organic molecules', a vital 'plastic force', and spontaneous generation; and they had such an inferior microscope that they were able to interpret whatever fuzzy images they saw as supporting the fuzzy ideas they wanted the data to confirm. Victorian biologist T. H. Huxley's version established one of his most famous cliche´s by describing Buffon and Needham's work as an object lesson for generations of young scientists:

> Such as it was, I think it [Buffon and Needham's doctrine] will appear ... to be a most ingenious and suggestive speculation. But the great tragedy of Science – the slaying of a beautiful hypothesis by an ugly fact – which is so constantly being enacted under the eyes of philosophers, was played almost immediately, for the benefit of Buffon and Needham.
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> Huxley (1870) Biogenesis and Abiogenesis. In: *Discourses Biological and Geological, Essays*, New York: Appleton, 1925, pp. 232–274, 239

Huxley argued, based perhaps on Pasteur's similar claim, that Buffon and Needham had a poor microscope and their opponent Spallanzani had a much more objective outlook and understanding of the method of controlled experiment.

Though seldom emphasized, it is very important to note that spontaneous generation was seen to be directly supportive of two other broader doctrines: epigenesis and materialism. Epigenesis is the doctrine that all the parts of an embryo are assembled gradually, having at first been nonexistent. Epigenesis was opposed in the seventeenth and eighteenth centuries by the doctrine of preformation, which claimed that all embryonic organs existed inside the sperm or the egg in miniature from even before fertilization and only needed to grow, unfold, and expand as gestation proceeded. This implied logically that all the generations of, for example, humanity must have been contained, each within the previous one like Russian dolls, all the way back to the eggs in Eve's ovaries or the sperm of Adam. (The overwhelming majority of naturalists writing during this period were Christian.) Since Buffon and Needham's description of the origin of protist microbes suggested their organization from originally homogeneous 'molecules', this seemed a blow for preformationists. Buffon indeed ridiculed preformation theory, insisting that the microscope showed no trace of these successively enclosed generations within the sperm or eggs of animals. Spallanzani eventually came down on the side of preformation and rejected Needham's theory. We would probably admit today that, since his microscope could not have shown him little preformed homunculi inside eggs or sperm cells, Spallanzani, too, must have been willing to go some way in believing in things he could not verify and was thus also operating in an atmosphere of 'philosophizing'.

More than that, if Buffon and Needham's observations were correct, they showed that matter contained within itself all the properties necessary to organize into life. This was the basic tenet of philosophical materialism, a doctrine profoundly at odds with Christianity or any kind of Deism. Voltaire, among others, feared that Needham's claims would support atheism and materialism. It was thought by many that this theory implied that life could originate by a chance, random combination of substances. This was

so contrary to mainstream religious beliefs, and to a natural philosophy still very much in the service of demonstrating the existence of a beneficent Creator, that it generated heated opposition. Further, this led to spontaneous generation becoming strongly associated, beginning around 1750–70, with atheism, materialism, and political radicalism. (Since this chance combination of chemicals has become such a crucial element of modern views of the origin of life, such as the famous 1953 Urey–Miller experiment, it is interesting that Buffon and Needham are not celebrated as thinkers far ahead of the religious biases of their time. Voltaire seems to have been one of those who most actively spread the opinion that Needham and Buffon were poor scientists, though this was based on seriously misreading Needham.)

Only recently for the first time has close study been applied to the actual technical evidence on what kind of microscopes were being used by Spallanzani and by Needham. This work has shown that Buffon and Needham's results cannot be successfully explained by assuming that they worked with poor instruments, nor that their work was sloppy or biased in advance by *a priori* theorizing, despite the fact that these explanations are ubiquitous among previous accounts of the controversy. Indeed, a close review of the evidence on the actual experiments suggests that the experiments of Needham and Buffon were careful, high-quality work with quite a bit of sophisticated hypothesis formation and testing.

Many of the contemporary critics of Buffon and Needham, including Spallanzani in the 1760s, had originated this claim of *a priori* bias, at least partly because they assumed the pair to have worked with a British Cuff compound microscope, common at that time (Figure 1). This type of device had a maximum magnification of only about 100×, and even at that low power the image it produced suffered from severe chromatic and spherical aberration, as indeed was a problem with all compound microscopes prior to the achromatic lenses not perfected until 60 years later. By careful analysis of the original publications, however, it has been shown that the instrument used by Needham was actually a high-quality single lens microscope of the Wilson screw-barrel design, capable of at least 400 × magnification with outstanding resolution, that is, not plagued by the aberrations of compound scopes (Figure 2). This was similar to the instrument with which Robert Brown discovered the cell nucleus in plant cells and Brownian movement in pollen particles in the 1820s and 1830s. In fact, Buffon and Needham's observations anticipate those that led to the discovery of Brownian movement, their 'organic molecules' being what Brown called 'active molecules'. The simple microscope employed in the famous experiments by Spallanzani was a variant of the Lyonnet aquatic microscope. Spallanzani's instrument was

Figure 1 Plate from Buffon's Histoire naturelle depicting an eighteenth century scientist using the compound Cuff microscope. Perhaps because of the illustration, many assumed that Buffon and Needham had used this instrument to make their observations, when in fact they had used the much superior Wilson screw-barrel design (see Figure 2). Compound microscopes of that era suffered from severe chromatic and spherical aberration, and the Cuff microscope had a maximum magnification of only about 100. Courtesy of Beinecke Rare Book and Manuscripts Library, Yale University.

Figure 2 Single-lens Wilson screw-barrel microscope used by Buffon to make his observations in support of his interpretation of the theory of spontaneous generation. In this theory, smallbacterium-sized particles, which Buffon called 'organic molecules', clumped together to form living 'animalcules'. Courtesy of Beinecke Rare Book and Manuscripts Library, Yale University.

incapable of the short focal length, high-resolution work permitted by the Wilson screw-barrel design, so that he could not even see the bacteria-sized 'organic molecules' under dispute. Thus the truth of this controversy appears to be that Spallanzani was the technically handicapped participant, though exactly the opposite story has become universal. Buffon and Needham's discovery of 'organic molecules' was not the work of imagination but an actual discovery of active molecules and of Brownian movement, some 80 years before Brown. This explains why Buffon and Needham refused to back down from their original observations despite accusations of atheism and decades of controversy. Furthermore, both parties seem to have had plenty of *a priori* philosophical commitments at stake, and nobody approached as explosive a subject as materialism with a completely open mind.

Worms, Molecules, and Evolution

The appearance of parasitic worms within animals was long seen, until perhaps the 1840s, as the strongest single piece of evidence supporting spontaneous generation claims. The complex life cycles of most of these parasites involved intermediate life cycle stages that had to live in other host animals. Thus, egg-feeding experiments would not have been able to show eggs from one animal directly producing worms in another. Only in the 1840s and 1850s, with the working out of the complex life cycles and intermediate hosts, did it finally become unambiguously clear that the worms reproduced via eggs.

Despite the denunciation of Buffon and Needham, a great many pathologists, histologists, and, later, cytologists continued to observe bacteria-sized, actively moving particles in blood, tissues, and infusions. These particles continued to be called 'molecules' by life scientists. They were using the term, obviously, in a way quite different from the chemists' use of the term that developed through the same time period. Important examples include the 'active molecules' of Robert Brown (1828–29), the cellforming cytoblastema 'molecules' of Theodor Schwann (1839), and the histological 'molecules' of John Hughes Bennett (1840–75). Many thought clumping together of these units was the basis for heterogenesis, and that this furthermore supported epigenesis as the correct explanation of embryonic development. Especially vocal in this belief were Lorenz Oken, Karl Burdach, and other German

biologists of the *Naturphilosophie* school. (It should be emphasized that neither Brown nor Schwann themselves interpreted their 'molecules' in this way, and Schwann did some significant experiments early in his career that seemed to disprove certain cases of spontaneous generation.) In Britain, both Charles Darwin and Richard Owen worked in private on the molecule theory during the 1830s, as did William Addison in the 1840s. More importantly, the British histologist Hughes Bennett was one of the first professors to teach a physiology course in a university and medical school, and he taught from 1840 until almost 1870. His theory of 'molecules' as the essential units from which cells formed, explicitly embracing heterogenesis by the late 1860s, was thus widely influential among British scientific and medical circles for over a generation.

J. B. Lamarck, the early French evolutionist, had made spontaneous generation of microorganisms an integral part of his evolution theory from its beginning in 1800. Lamarckian supporters were numerous and widespread, though often associated with radical politics, as selforganization and development of matter was seen as a crucial scientific underpinning for democratic, bottom-up organizing political theories. A few such Lamarckians did rise to university teaching posts, however, and notable among them was the outspoken British comparative anatomy professor Robert Grant. Though considered not quite respectable in Victorian intellectual circles, his teaching of evolution and spontaneous generation continued from the 1820s through his death in 1874, and thus gave those ideas a presence in British thought decades before *On the Origin of Species* appeared. Charles Darwin, for instance, was one of Grant's earliest students and prote´ge´s (though Darwin later felt he had to avoid any contact with the less-than-respectable Grant when he was developing his own evolution theory in secret for 20 years). Grant and other Lamarckians had established in the public mind a strong link between evolutionary theories and spontaneous generation.

The Role of Louis Pasteur

As Louis Pasteur's experiments on spontaneous generation, particularly his 'swan-necked flasks', are among the most famous of all, the Pasteur–Pouchet debate of the 1860s in France deserves close attention. It is also worth noting that the Historical Introduction to Pasteur's famous 1862 Memoir on the subject has served as the model upon which almost all subsequent histories of the controversy were based for over a 100 years . This has led to the casting of the controversy as a series of what I call 'dueling experiments', and has often stripped it of its crucial philosophical context. Pasteur also chose to leave out an entire chapter of the story: the argument that parasitic worms must have arisen by spontaneous generation. So dominant has Pasteur's master narrative been that it is only with historical detective work in the early 1970s that this omission of Pasteur's was finally restored to its place of importance in the story.

Between 1860 and 1862 Pasteur carried out a lengthy series of experiments focusing exclusively on the development of microbes in previously boiled infusions, in response to the experiments in favor of heterogenesis published by Felix Pouchet. With great ingenuity, Pasteur designed numerous experiments that would allow the boiled infusions to come in contact with air that itself had not been altered by the heat, to answer Needham's objection of a century before. Air was led through dense gun cotton filters, for example, before coming in contact with the infusions. The filters were then dissolved and the solid particles trapped from the air were examined microscopically. Pasteur saw some spherical bodies that he assumed were the 'germs' of microbes, since in those infusions microbial life rarely developed. Perhaps most famous of Pasteur's experiments was a series of flasks which, after being filled with infusion, had their necks heated in a flame then drawn out into long, curved shapes, for example, like a 'swan-neck' (Figure 3). Air could enter through the long neck, but dust particles settled in the dip and could not come in contact with the flask's contents. Again, if these flasks were boiled then cooled, rarely did any microbial growth ever appear in them. However, if the neck was broken off and dust allowed to enter the flask, microbial growth in the infusion soon followed. Pasteur concluded that this proved microbes or their germs were carried on dust particles, so the exclusion of dust followed by no growth meant the disproof of spontaneous generation.

As the swan-necked flasks have in our time become textbook icons for exemplary experimental practice, it is difficult for us to reconstruct the historical period when, even after Pasteur's famous public lectures on his experiments, a significant number of scientists remained unconvinced. In particular Pasteur claimed to have sweepingly proven that germs must be the source of growth in previously boiled infusions, but his opponents pointed out that what he had shown could be read to be only that dust was a necessary ingredient for spontaneous generation with the yeast/sugar water infusions that he used. Recent historians have also pointed out, as did Pouchet and other supporters of spontaneous generation at the time, that Pasteur never replicated some of Pouchet's most convincing experiments: those involving boiled hay infusions. (It was only recognized a decade later that the hay bacillus, *Bacillus subtilis*, produces heatresistant endospores.) If Pasteur had tried his famous swan-necked flask method with a hay infusion, it has been suggested that the debate might have ended quite differently on this point alone.

Since even in this most famous of textbook cases, it still seems the experimental evidence at the time was not as finally conclusive as Pasteur declared it to be, why did the French Academy of Sciences award the victory to Pasteur and declare the spontaneous generation controversy settled once and for all? Spontaneous generation was still just as politically and religiously charged a subject in the French Second Empire of Louis Napoleon as it had been 100 years earlier in Buffon and Needham's time. Furthermore, while spontaneous generation had in the past tended to be associated with the doctrine of transmutation of species, the latter doctrine was currently undergoing a particularly heated wave of notoriety in the wake of the recent publication of Darwin's *On the Origin of Species*. The first French translation, by Cle´mence Royer, of the *Origin* had just appeared in 1862 and was prefaced by a long diatribe by Royer against the Catholic Church. The Church was, however, on closer terms than ever with the conservative government, since Louis Napoleon's coup of 1851. In this environment, since the Academy of Sciences was a state-supported institution, Darwinian

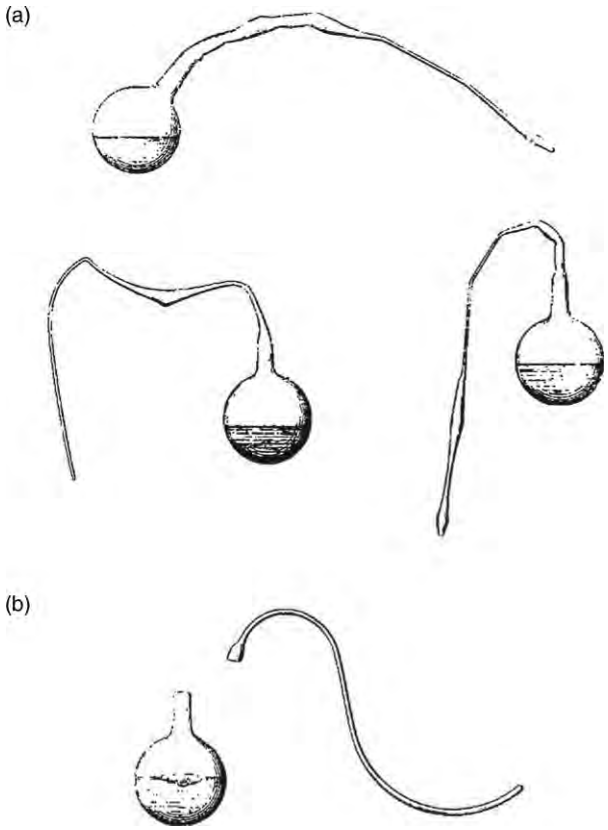

Figure 3 Pasteur's illustrations of his 'swan-necked flasks'. Courtesy of the American Society for Microbiology Press.

evolution was regarded in France as a politico-theological doctrine allied with forces that threatened the Church and State. In addition, spontaneous generation was seen as directly undermining belief in a providential Creator, so that the outcome of the Pasteur–Pouchet debate carried implications of enormous importance to the power structure of the Second Empire. Even foreign savants observing the controversy at the time, including Britain's premier anatomist Richard Owen and Germany's Friedrich Lange, noted that opposition to spontaneous generation seemed intimately tied to support for the forces of conservatism and the established Church.

The force of Pasteur's experimental genius has been surpassed historically only by his rhetorical skill. For, while he broached all these controversial subjects in a famous 1864 Sorbonne lecture on spontaneous generation, yet he succeeded in portraying himself (and science done at its best) as able to remain entirely aloof from such matters, saying that he had approached the question in a totally unbiased manner, equally ready to accept or reject the existence of spontaneous generation, based solely on the outcome of the experiments. While Pasteur was truly an experimental genius compared to Pouchet, the crucial problem with this "let the chips fall where they may" portrayal of himself is that it is not true. By this time Pasteur had been dominated for some years already by his own preconceived notions (derived from his work on crystalline asymmetry) of life as dependent upon a Cosmic Asymmetric Force. He had done numerous experiments in private, trying to produce living conditions in the laboratory by application of light, electricity, and magnetism to experimental setups, and he was convinced that Pouchet was wrong, not so much because spontaneous generation was somehow impossible, but because Pouchet was not aware of the importance of asymmetric forces to the problem of life, and was not approaching the experiments by that route. Furthermore, Pasteur was fully aware that all of his important scientific patrons were entrenched in the conservative regime, and he was constantly trying to cultivate those contacts, particularly with the Emperor and Empress personally. Thus it is no surprise that during all the years of the Second Empire he kept silent about his own attempts to simulate or create life in the laboratory and concentrated in public on refuting the work of Pouchet, which he knew to be very unpalatable to the Academy and the State.

The British Debate of the 1870s: Darwin, Spontaneous Generation, and the Germ Theory of Disease

Charles Darwin himself had remarkably ambivalent feelings about spontaneous generation. In the first edition of *On the Origin of Species* he appears to have carefully avoided the topic through almost the entire book. Then in a single throwaway line near the very end, he upset many of his supporters by adopting this language:

> Therefore I should infer from analogy that probably all the organic beings which have ever lived on this earth have descended from some one primordial form, *into which life was first breathed*.
>
> Darwin, *On the Origin of Species*, London: John Murray, 1859, p. 484, italics mine

In the second edition, released just 7 weeks later on 31 December 1859, Darwin made it "... breathed by the Creator." Darwin received much criticism, from both supporters and opponents of evolution, about dissembling in this passage. In the 1862 third edition he simply dropped the entire phrase after "one primordial form" and left it that way through all remaining editions of the *Origin* in his lifetime, essentially taking a public position of 'no comment' on the implications of his theory for the origin of life on Earth. In private, to close confidantes, he said, "I have long regretted that I truckled to public opinion, and used the Pentateuchal term of creation, by which I really meant 'appeared' by some wholly unknown process. It is mere rubbish, thinking at present of the origin of life; one might as well think of the origin of matter." He thought Pasteur's experiments much more convincing than Pouchet's. But to others Darwin said it would be a great thing if spontaneous generation could be proven, as it would undergird his theory in an important way. His reluctance to discuss the matter in 1859 seems to have become genuine ambivalence on the topic.

On the British scientific scene in the 1860s and 1870s the most entrenched advocates of spontaneous generation and those most resistant to the germ theory of disease were the medical community. At that time, despite Pasteur's suggestive research on silkworm diseases, British doctors strongly favored a theory of contagious disease as analogous to fermentation via chemical catalysis, a theory first promulgated by Justus Liebig in the early 1840s. This was in part due to a brief but highly publicized theory of 1849 that cholera was caused by a fungus. When the supposed cholera fungus was soon discovered to be a common mold contaminant, many British medical men who might have had leanings toward a germ theory felt that it had been discredited. Thus, when Pasteur's claims of the mid-1860s reached Britain, most British doctors were skeptical that living microbes were the sources of contagion, and they continued to favor a chemical poison, viewing the microbes as some kind of product or concomitant of the disease process rather than its cause. Thus, when the physicist John Tyndall gave a famous lecture on "Dust and Disease" in January 1870, arguing that doctors must accept the germ theory of Pasteur in order to have any really scientific approach to disease, many British doctors took offence. Tyndall was known for his brash public persona and as a general spokesman for science in London society circles. In addition to his quite haughty tone, he was trained as a physicist and certainly had no clinical experience of disease or of the role of patients' constitutions in causing their susceptibility to be so highly varied. So it was not surprising that doctors accused Tyndall of being an interloper in biology and medicine, an area where his opinions had no weight. They thought he was totally unaware of the cholera fungus hoax of 1849 and of other important technical reasons for medical skepticism about an over-simplified germ theory. Tyndall's version of the germ theory denied any role to the 'constitution': it compared patients to so many identical tubes of infusion. If germladen dust particles fell into the patient, he would get sick, Tyndall said. Those in hospitals or towns with epidemics who did not get sick were those who such particles, or 'germ clouds', passed by.

Chief among the medical professionals who opposed Tyndall was Henry Charlton Bastian, professor of pathological anatomy at London's University College Medical School. Bastian was an avowed supporter of Darwin's and Spencer's writings on evolution, and did much experimental work to try to show that microorganisms could arise by spontaneous generation, or biogenesis as he at first called it. Bastian, like Alfred Russel Wallace and many others interested in natural selection, thought at that time that Darwin's theory required spontaneous generation to explain in a nonmiraculous way where the original common ancestor of all species came from. Wallace wrote excitedly to Darwin and in a review in *Nature* in August 1872 claimed that Bastian's findings could help Darwinian evolution out of the time crunch presented by William Thomson's (later Lord Kelvin) challenge: that the age of the earth – a few tens of millions of years at most, by his physics calculations – was utterly inadequate to allow the hundreds of millions of years of evolution. Darwin's gradualist theory required to get from single-celled creatures to complex vertebrates by a single, unbroken chain of descent. Darwin was tempted but still ambivalent. By this time he wrote to T. H. Huxley and J. D. Hooker that he certainly believed that life at first emerged from nonlife by natural processes in 'some warm little pond'. However, Bastian's idea of spontaneous generation going on all the time, everywhere, up to the present meant many different possible lines of descent with new ones beginning constantly, a model similar to Lamarck's. Darwin was still deeply committed intellectually to a single branching tree of descent, which would require only one (or a small handful) progenitor(s) created by an original emergence of life entirely confined to the very earliest period of life on Earth. He followed closely the debates over Bastian's experiments, as did all Darwinians who had come to know Bastian as one of their own, indeed one of their brightest rising young stars in experimental science.

Bastian also thought that bacteria in diseased patients resulted from spontaneous generation, as by-products of the disease process. Between 1870 and 1875 he had published the results of hundreds of experiments in which he showed that bacteria could be found in tubes of various infusions boiled for periods varying from a few minutes up to an hour. Attempting to refute Bastian's work from 1875 to 1877, Tyndall devised an ingenious dust-free cabinet in which to carry out the experiments (Figure 4).

Tyndall's close friend T. H. Huxley was at first interested in Bastian's experiments, but soon concluded that the pathologist must be mistaken in his conclusions, especially given Huxley's belief at this time in pleomorphism. He declared that Bastian "had gotten out of his tubes exactly what he put into them," that is, organisms must have gotten into the tubes as contaminants. Both Huxley and Tyndall were also evolutionists, but found it much easier to believe in living things able to survive boiling somehow (or, even more believable, that Bastian was a sloppy experimenter) than to believe that organisms as complex as protozoa could come from anything other than the 'seeds' or 'germs' of other like organisms. They lobbied widely among scientific colleagues to convince others that Bastian was at best a poor experimenter and at worst a fraud and a cheat. As mentioned above, Bastian, for his part, found it much more difficult to believe that life could survive the boiling temperature for more than a minute or two, than to believe (especially since he saw it as a logical necessity of continuity in nature and of evolutionary science) that the transition in stages from

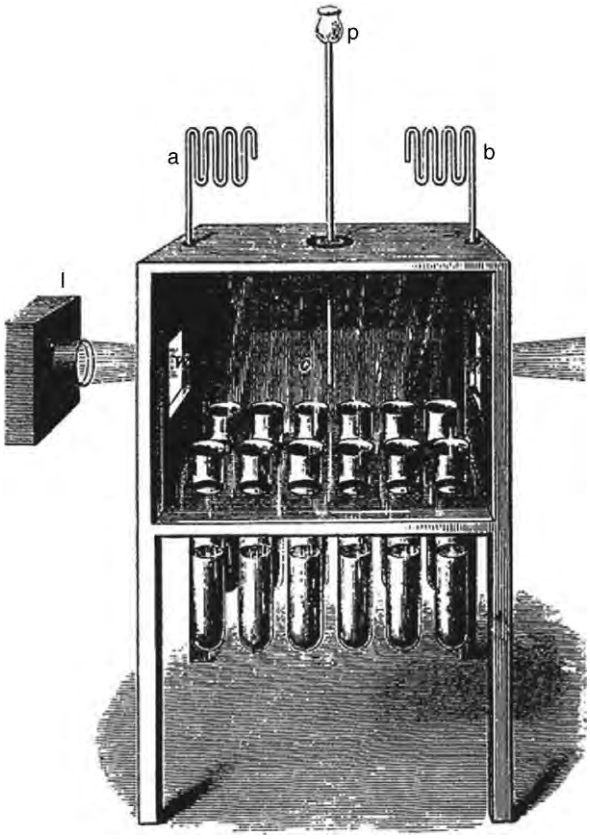

Figure 4 Tyndall's dust-proof chamber for carrying out spontaneous generation experiments. Courtesy of the Royal Institution, London.

nonliving to living matter should be possible. A noted physiologist, John Burdon Sanderson, observed Bastian's technique carefully and reported that it was up to high scientific standards.

Huxley, in a famous address to the British Association for Advancement of Science meeting at Liverpool in September 1870, moved to gain the rhetorical upper hand in the debate by defining the terms. He defined 'biogenesis' to mean life only from other life. The opposite belief he termed 'abiogenesis' and began to argue that it very well might be possible, even probable, but only in the conditions of the primitive earth. This convenient dualistic terminology was rapidly picked up by contemporaries and propagated in textbooks. Given this, it is more than a little ironic that Huxley had hijacked the term 'biogenesis' from Bastian, who was using it up until that time to mean exactly the opposite, that is, spontaneous generation (After this, Bastian substituted the term archebiosis, but it never caught on as Huxley's terms did.). Also important, it is from this time that all discussion of the origin of life question began to be pushed more and more into the distant past. Only after Huxley's talk, for example, did Darwin advance the argument that if spontaneous generation had occurred in the earth's distant past, it would no longer be possible after the evolution of the first heterotrophs, since they would consume any organic molecules that formed before those could assemble into a new organism.

Those opposed to both evolution and spontaneous generation had a simpler solution. The physicist William Thomson, Lord Kelvin, for example, responded to both Huxley and Bastian in his own Presidential Address to the BAAS in 1871. He suggested that the germs of life might have originally been brought to earth from another world via a meteorite. To those demanding a completely naturalistic origin of life, of course, this merely pushed the question back a step to some other planet.

Beginning in the autumn of 1876, Tyndall began to have difficulty with the experiments carried out in his dust-free cabinet. Infusions that had been sterilized by only 5 minutes' boiling a year earlier now could not be sterilized even after hours of boiling. In 1876, the discovery by the German botanist Ferdinand Cohn that certain species of *Bacillus*, especially common in hay and in cheese (and in infusions made from these that showed microbial growth after extended boiling), were capable of producing heat-resistant endospores was taken to explain why Bastian's infusions were full of microbial growth after boiling, and upon being handed a copy of Cohn's article Tyndall immediately adopted this stance to explain his own recent difficulties.

Tyndall struggled for several months and finally discovered that sterility could be achieved by repeated short boilings, followed by a period of allowing the remaining spores to germinate. This process came to be known as 'fractional sterilization' or 'Tyndallization'. This was largely superseded with the development of the autoclave in the 1880s. Equally interesting from a historical point of view is that though this rapid about-face also showed that Tyndall and Huxley had been wrong that Bastian must be a sloppy, incompetent experimenter, that is precisely the version of Bastian (and most spontaneous generation advocates) that

has usually gone into the textbooks until quite recently. The sole exception was Pasteur's student Emile Duclaux, the only contemporary writer among the winners who credited Bastian's perseverance in sticking to his experimental results with the value that it truly had, that is, that without the goad of Bastian and his popularity, Pasteur, Cohn, and Tyndall might never have discovered that they were wrong about the existence of bacteria in boiled hay infusions, and thus been led to realize that heat-resistant spores exist and require autoclaving or fractional sterilization to guarantee their being killed. Pasteur was a devout Catholic and never believed, to his dying day, the viewpoint favored by many modern origin of life hypotheses, that random or chance organization of materials could have formed life, even the very first living organisms.

Twentieth Century Ideas

The origin of life has continued to be a subject of active interest in the twentieth century, and, despite avoidance of the term 'spontaneous generation' since 1880, some modern concepts have resembled older ideas more than superficially. Beginning with Bastian (who revived experiments on spontaneous generation from 1900 to 1915), a number of investigators came to believe that the basic chemistry specific to the living was the chemistry of colloids. A burst of research in colloid chemistry from 1910 until the 1930s included many workers who believed that this avenue would lead to understanding the simplest combination of materials to cross the boundary from nonlife to living matter. Among these were biochemists Benjamin Moore and Albert Mary, bacteriologist Arthur I. Kendall, the Mexican biologist Alfonso Herrera, and medical doctor and researcher George Crile. Geneticist H. J. Muller suggested that the first chemical assembly of a gene should be considered the origin of life, though this still suggested a sudden origin of life from nonlife, perhaps the most central idea implicit in the doctrine of spontaneous generation. The Russian biochemist A. I. Oparin proposed instead a gradual chemical evolution of life, probably proceeding through the intermediate stage of coacervates. Research into life's origin had lost none of its larger associations: the issue still attracted researchers with anticlerical leanings (Herrera was one) as well as affiliations with radical, even Communist political beliefs. J. B. S. Haldane and J. D. Bernal did research in the field and were avowed Communists, as was Muller until the late 1930s. Furthermore, Oparin and Wilhelm Reich explicitly credited the philosophy of dialectical materialism as having been crucial to their origin of life research programs.

Research on viruses, particularly Wendell Stanley's crystallization of tobacco mosaic virus in 1935, lent credence for a time to the idea that a virus might be the simplest 'living molecule'. In addition, after the rise of molecular biology and the discovery of the Watson–Crick DNA structure, many considered Muller's idea of "the gene as the origin of life" more likely. The Urey–Miller experiment, published just 3 weeks after Watson and Crick's paper on DNA, lent further support to the notion that the building blocks of life could have assembled very rapidly. In that experiment, an electrical discharge passed through a mixture of steam, hydrogen, methane, and ammonia in a sealed container produced amino acids and other organic compounds after only a few days. Throughout the 1950s and 1960s, such views were in tension with those of Oparin and his students in the Soviet Union, that the process of chemical evolution was a more gradual one, with no single decisive 'living molecule'. Cold War and anti-Lysenkoist hostilities were involved, as any scientific theory openly lauding dialectical materialism was seen as suspect in the West in the aftermath of Lysenko's takeover of Soviet genetics. The involvement of NASA as a major patron for origin of life research, beginning in 1960, led to the funding of a wide variety of approaches, including gradual evolution models as well as sudden, gene-based models; 'protein-first' or 'metabolism-first' as well as 'nucleic acid-first' or 'information-first' models. This work took place under the rubric of 'exobiology' (broadened and renamed 'astrobiology' in the late 1990s).

Thus, 'spontaneous generation' has been considered a dead-end, disproven belief since 1880 or so. But, despite the dropping of the older term, the conceptual continuities between modern and older origin of life ideas, particularly for abiogenesis in the earth's distant past, are in some ways as interesting as the discontinuities. The continued association of these ideas with larger philosophical and political concerns is also a striking feature of their history well into the twentieth century.

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Staphylococcus☆

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Glossary

Biofilm Sessile microbial communities consisting of an accumulated mass of bacteria, extracellular matrix molecules, and secreted bacterial products that aid in adherence to biopolymers and other solid surfaces.
Capsule Polysaccharide outermost layer of the cell. Production imparts a viscous slimy look to colonies.
cell wall One of the outer layers of the bacterial cell that protects the cell from osmotic perturbations and provides mechanical protection to the fragile cellular membrane.
Endonuclease Enzymes that have specific binding sites of varying complexities at which they sever phosphodiester bonds of DNA.
MSCRAMM (Microbial surface components recognizing adhesive matrix molecules) Proteins involved in the establishment of infection via the initial attachment of bacteria to host molecules such as elastin, collagen, fibrinogen, and fibronectin.
Plasmid Autonomously replicating small extrachromosomal DNA molecules.
Superantigen Any of a number of proteins that elicit a massive T-cell receptor Vβ-restricted primary response.

Nomenclature

CA-MRSA	Community-acquired MRSA
CDC	Centers for Disease Control
CHEF	Clamped homogeneous electrophoretic field
CoNS	Coagulase-negative staphylococci
HA-MRSA	Healthcare-associated MRSA
IFN-γ	Interferon-γ
MHC II	Major histocompatibility complex class II
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
NCBI	National Center for Biotechnology Information
PIA	Production of a slime substance
PVL	Panton-Valentine leukocidin
RFLP	Restriction fragment length polymorphism
SE	Staphylococcal enterotoxin
SSSS	Staphylococcal scalded-skin syndrome

Defining Statement

The staphylococci are important bacterial pathogens that can infect both animals and humans and are responsible for numerous hospital- and community-acquired infections yearly. Staphylococcal infections result in a significant burden both economically and clinically for a variety of reasons, including virulence factors, increasing antibiotic resistance, and lack of effective vaccines.

Taxonomy

The genus *Staphylococcus* is defined in *Bergey's Manual of Determinative Bacteriology* as a member of the family *Micrococcaceae*. According to the most recent approved list from *Bergey's Manual* (9th ed.), there are presently 49 recognized species of staphylococci (Table 1),

☆*Change History:* February 2015. AF Gillaspy, JJ Iandolo, Y-W Tang, and CW Stratton updated Section text and added references.

This article is an update of A.F. Gillaspy, J.J. Iandolo, *Staphylococcus*, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 293–303.

Table 1 Currently recognized staphylococcal species

Indigenous or colonize humans	*Indigenous or colonize non-humans*
S. aureus	*S. agnetis*
S. auricularis	*S. arlettae*
S. capitis	*S. carnosus*
S. caprae	*S. caseolyticus*
S. cohnii	*S. chromogenes*
S. epidermidis	*S. condimenti*
S. haemoyticus	*S. delphini*
S. hominis	*S. equorum*
S. jettensis	*S. felis*
S. lugdunensis	*S. fleurettii*
S. massiliensis	*S. gallinarum*
S. pasteuri	*S. hyicus*
S. petrasii	*S. intermedius*
S. pettenkoferi	*S. kloosii*
S. pseudolugdunensis	*S. lentus*
S. saccharolyticus	*S. lutrae*
S. schleiferi	*S. microti*
S. sciuri	*S. muscae*
S. simulans	*S. nepalensis*
S. vitulinus	*S. piscifermentans*
S. warneri	*S. pseudintermedius*
S. xylosus	*S. pulvereri*
	S. saprophyticus
	S. rostra
	S. simiae
	S. stepanovicii
	S. succinus

22 of which are indigenous to or have been shown to colonize humans; the remaining species have been isolated from various animal (including one primate species), plant, or food specimens. Staphylococci are Gram-positive cocci 0.7–1.2 μm in size that occur singly and in pairs in liquid media and in tetrads and clusters when grown on solid media. Over the years, they have been characterized by their variety of colonial, morphological, and biochemical activities that have resulted in description of several biotypes of variable stability. They are aerobic or facultatively anaerobic, catalase-positive, and capable of generating energy by respiratory and fermentative pathways. These organisms are nutritionally fastidious with complex nitrogen requirements. Most species require several amino acids, vitamins (thiamine and niacin), and uracil (to grow anaerobically) for growth. In complex, nutritionally complete growth media, the organism is able to grow at generation times of $\leq$20 min, a rate comparable to that of *E. coli*.

At the molecular level, the genus can be distinguished from other members of the *Micrococcaceae* by the low GC content of its DNA, which ranges from 30% to 38%, the presence of teichoic acid in their cell wall, and their ability to tolerate extremely low water activities. *Staphyloccocus aureus* is routinely cultured at a_w of 0.88 (15% NaCl) and has been reported to grow at water activity levels as low as 0.83 (saturated NaCl solution). Anaerobically, tolerance to low a_w is less with the limit at 0.90. The normal habitat of these organisms is the skin, skin glands, and mucous membranes of warm-blooded animals. However, staphylococci can be isolated from a variety of sources that include soil, dust, air, water, and food and dairy products. As a result they present a food and water public health hazard as well as an infection risk.

Cellular Structure

Cell Wall

The cell wall of *Staphylococcus* is a thick, electron-dense structure that provides great mechanical support to the cell. It is composed of a giant polymer consisting of peptidoglycan complexed with teichoic acid and other surface proteins described elsewhere in this article. The cell wall is a heteropolymer consisting of glycan chains cross-linked through short peptides. The repeating unit in the glycan backbone is β-1,4-*N*-acetylglucosamine and *N*-acetylmuramic acid (muramic acid). About 60% of the *N*-acetylmuramic acid residues are O-acetylated. The high level of O-acetylation makes the staphylococcal cell wall resistant to lysozyme digestion, therefore making this genus unique from most other bacteria. In fact, lysis of the cell wall under laboratory conditions is only efficient when the staphylococcal endopeptidase, lysostaphin, is added to cultured cells. Lysostaphin is produced by *S. simulans* and specifically cleaves the pentaglycine crossbridges found in the staphylococcal peptidoglycan. In *S. aureus*, adjacent polypeptides are cross-linked by pentaglycine crossbridges between the ε-amino of lysine and C-terminal D-alanine, whereas in other species, the composition of the crossbridge is variable. The carboxy group of muramic acid is substituted by an oligopeptide that contains

alternating L- and D-amino acids (L-alanine, D-glutamine, L-lysine, D-alanine, D-alanine). The teichoic acid component is linked to the D-alanine component of the mucopeptide by α- or β-glycosidic linkage through *N*-acetyl-D-glucosamine. In *S. aureus*, the teichoic acid backbone is ribitol based whereas in *S. epidermidis* it is glycerol based. In other species, glycerol teichoic acids are more common than their ribitol counterparts. These highly charged immunogenic cell wall components have been found to play a role in *S. aureus* nasal colonization, biofilm formation, and susceptibility to vancomycin and other glycopeptides.

Capsule

Capsule production has been shown to occur *in vivo* and *in vitro* for both *S. epidermidis* and *S. aureus*. For *S. epidermidis*, there have been at least three different capsular polysaccharide types identified in the literature. In contrast, 11 capsular polysaccharide serotypes have been described for the highly virulent species, *S. aureus*. The most common occurring serotypes among clinical isolates are types 5 and 8. The main components of the capsules are *N*-acetylaminouronic acids and *N*-acetylfucosamine. The genes for capsule production have been identified and are located in a single operon. The production of a capsule renders the staphylococci resistant to host defenses such as opsonization and phagocytosis. However, antibodies to capsular polysaccharides can neutralize the antiphagocytic properties of the capsule and opsonize the cell for phagocytosis. Opsonization has made the capsule a prime target in the search for an effective staphylococcal vaccine.

Several staphylococcal species, particularly *S. epidermidis* and *S. aureus*, have been shown to produce biofilms, and capsule production has been shown to be of particular importance during biofilm formation by aiding in adherence to biomaterials such as indwelling medical devices. A trademark of staphylococcal biofilm formation is the production of a slime substance (PIA), which is a polysaccharide composed of β-1,6-linked *N*-acetylglucosamines with partially deacetylated residues giving them a positive charge. Once bacterial cells are within this slime, they are protected from the host's immune defenses and are resistant to other treatments such as antibiotics. PIA is not the only component that has been shown to contribute to biofilm production under certain conditions, and research in this area continues. Recent investigations have shown that the presence of staphylococcal biofim actively shifts host immunity towards an anti-inflammatory, pro-fibrotic response that favors staphylococcal persistence. *S. epidermidis* is the primary cause of catheter-associated staphylococcal infections and is a primary producer of biofilms. *S. aureus* biofilms have also been shown in patients with diseases such as osteomyelitis and endocarditis. Biofilm production in the staphylococci has been shown to be multifactorial and appears to be a bacterial survival mechanism when cells are exposed to sublethal levels of antibiotics and/or other stressful environmental conditions (i.e., limited nutrients, changes in temperature, oxygen limitation). It is also important to note that large phenotypic variations in biofilm production exist, with some isolates incapable of biofilm formation regardless of culture condition and others being hyperproducers under a specific set of conditions. Continued focus on understanding all of the components involved will give us more insight into chronic staphylococcal infections, especially those involving medical devices, and may lead to more efficient treatment of these diseases.

Molecular Structure

Genome

Presently, the genomes of multiple members of the genus *Staphylococcus* have been completely sequenced. These sequenced genomes include strains of *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*. The genomic data for each of these projects are freely available on the World Wide Web at the National Center for Biotechnology Information (NCBI) website.

In all cases, the genome is circular and ranges from 2.49 Mb (*S. epidermidis*) to 2.9 Mb pairs (*S. aureus* strains). Table 2 shows a summary of the information obtained from the first 18 completed staphylococcal genome projects. Consistent with the *Micrococcaceae* family, all 18 of the isolates have a low GC content (~33%). The sequenced strains also exhibit a high amount of diversity within this genus, with some species and strains having acquired resistance to the antibiotics methicillin and/or vancomycin and some containing extrachromosomal elements called plasmids that most often contain additional genes that can contribute to pathogenesis. Among the sequenced isolates, the number of plasmids present varies from 0 to 3 and the size of these elements varies as well. Genetic and sequence data available indicate that in addition to the normal complement of housekeeping genes, the chromosome contains many accessory genetic elements that are not necessary for growth under laboratory conditions.

In *S. aureus* strains, pathogenicity islands SaPI1 and SaPI2 have been described and have been shown to encode virulence factors such as the toxic shock syndrome toxin gene, staphylococcal enterotoxin B, and as well as resistance to methicillin. In strains that do not contain SaPI1 or SaPI2 there are no allelic counterparts for these genes. The genes and overall genomic organization of *S. aureus* bear a striking resemblance to the genome of *Bacillus subtilis* such that the organism has been called a morphologically degenerate form of *Bacillus*. In addition, the genomic organization of the *S. aureus* strains sequenced thus far has been shown to be highly similar. Specifically, there is a genomic 'backbone' common to all *S. aureus* strains containing varying numbers of genes that may contribute to antibiotic resistance, tissue tropism, and virulence. Comparison of the two sequenced *S. epidermidis* strains showed that the overall genomic organization is similar, with two relatively small areas of inversion apparent when the two genomes are aligned to each other at the nucleotide level. Figure 1 shows alignments at the nucleotide level for two representative *S. aureus* strains as well as the two *S. epidermidis* strains. Alignment of *S. haemolyticus* and *S. saprophyticus* to *S. aureus*, *S. epidermidis*, or to one another showed little or no conservation of gene order. However, at the level of protein content, members of the genus are highly similar and this is illustrated graphically in Figure 2. Specifically, when the translated sequences for several of the sequenced staphylococcal

Table 2 Completed staphylococcal genome project

Organism	*Genome size (Mb)*	*Plasmid(s) size (kb)*	*% GC*	*Number of predicted genes*	*MRSA/VRSA*
S. aureus strain MW2	2.82	N/A	32.82	2632	MRSA
S. aureus strain Mu50	2.9	25.1	32.84	2748	MRSA
S. aureus strain N315	2.84	24.6	32.8	2623	MRSA
S. aureus strain NCTC8325	2.82	N/A	32.86	2892	N/A
S. aureus strain RF122	2.74	N/A	32.77	2589	MRSA
S. aureus strain COL	2.81	4.44	32.81	2787	MRSA
S. aureus strain MRSA252	2.9	N/A	32.8	2744	MRSA
S. aureus strain MSSA476	2.79	N/A	32.85	2619	N/A
S. aureus strain USA300 – FPR3757	2.91	37, 4.4, 3.13	32.69	2691	MRSA
S. aureus strain JH1	2.9	30.4	32.0	2870	MRSA, VRSA
S. aureus strain JH9	2.9	30.4	32.9	2816	MRSA, VRSA
S. aureus strain Newman	2.9	N/A	32	2687	N/A
S. aureus strain Mu3	2.9	N/A	32	2776	MRSA, VRSA
S. aureus strain USA300 TCH1516	2.9	27.0	32	2802	MRSA
S. epidermidis strain ATCC12228	2.49	N/A	32.09	2419	MRSA
S. epidermidis strain RF62A	2.64	27.3	32.14	2665	MRSA
S. haemolyticus strain JCSC1435	2.68	N/A	32.79	2678	MRSA
S. saprophyticus strain ATCC15305	2.57	38.5, 16.3	33.18	2514	MRSA

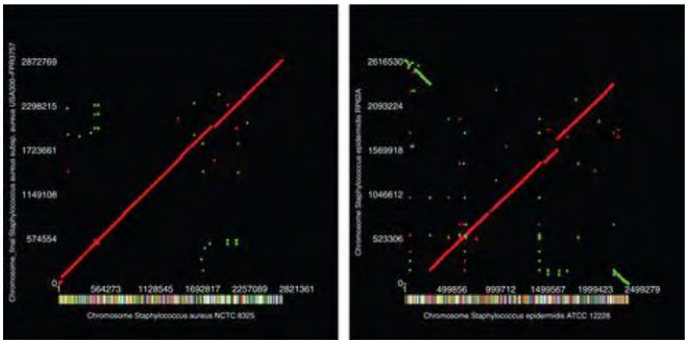

Figure 1 The left panel shows alignment of *S. aureus* strains NCTC8325 and USA300 at the nucleotide level. The solid red line indicates that the two strains are virtually identical in genomic organization. Right panel shows the alignment of *S. epidermidis* strains ATCC12228 and RP62A. The break in the red line and the shorter green lines show that although the two strains are overall highly similar in organization, there are some differences. Differences are believed to be due to the presence or absence of such things as bacteriophage and pathogenecity islands.

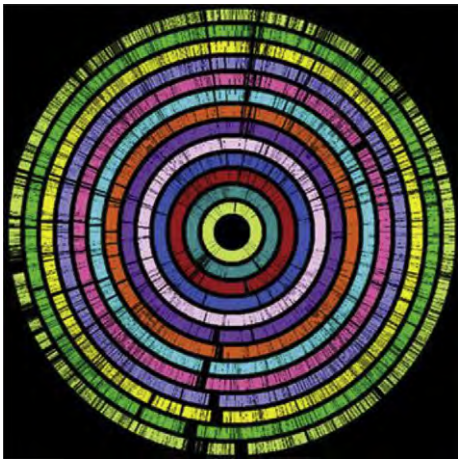

Figure 2 Comparison of proteins encoded within 13 members of the genus *Staphylococcus*. Completed genomes used in the above comparison are listed starting with the outermost circle: *S. aureus* strain NCTC8325, *S. aureus* strain COL, *S. aureus* strain USA300-FPR3757, *S. aureus* strain MW2, *S. aureus* strain Mu50, *S. aureus* strain MSSA476, *S. aureus* strain N315, *S. aureus* strain MRSA252, *S. aureus* strain RF122, *S. haemolyticus* strain JCSC1435, *S. epidermidis* strain ATCC12228 and *S. epidermidis* strain RP62A. Using NCTC8325 as the reference strain resulted in a total number of 2892 predicted proteins used for the comparision. Of the 2892 proteins used, 1775 were found to be present in all other genomes, with 2756 present in at least another genome and only 136 not found in any other of the comparison genomes.

genomes are compared (at a minimum cutoff level of $\geq$40% similarity) to one another using *S. aureus* NCTC8325 as the reference strain (since this strain has the largest number of predicted open reading frames), the level of protein similarity is 61.4% across the entire group. Of course, the level of similarity is slightly higher when the same type of comparison is done within species (85–92%) but the overall similarity at the genus level is still highly significant. For perspective, the same comparison done with *S. aureus* NCTC8325 and *B. subtilis* showed only 59% overall protein similarity.

In addition to large pathogenicity islands the staphylococcal genome (especially *S. aureus*) typically contains multiple instances of transposable elements such as *Tn551*, insertion sequences IS256, IS257, IS1181, and others. *S. aureus Tn551* and its close relative *Tn917* have been extensively utilized to generate knockout mutations in staphylococci, many of which have been mapped and localized near other genes. The primary strain of *S. aureus* used for genetic manipulation and gene discovery is NCTC8325 and its derivatives. Also known as PS47, this is the propagating strain for the typing bacteriophage ø47 and is a member of phage group III. It is routinely used to generate batches of bacteriophage used for typing purposes and is lysogenized by three temperate phage: ø11, ø12, and ø13. A derivative (8325-4) that has been cured of all demonstrable phage was originally developed as the prototype strain whose genome was used to build a circular map based on genetic and physical parameters. With the advancements in whole-genome sequencing technology this map is no longer the primary resource used for mapping and typing experiments. However, in the absence of sufficient genomic information, this type of mapping is still used to examine new *S. aureus* isolates and to determine basic lineage information for these isolates.

Plasmids

As mentioned above, the staphylococci are also endowed with a generous array of plasmids ranging in size from a few kilobases to 40–50 kb. In fact, typically one or more plasmids are found in most staphylococcal clinical isolates, including both *S. aureus* and the coagulase-negative staphylococci. The inability of two plasamids to coexist in a single host indicates that they have the same replication control functions and are incompatible and assigned to the same incompatibility group (*inc* group). Thus far, there are 15 recognized incompatibility groups (*inc* 1–15), but the replication characteristics of some staphylococcal plasmids remain undetermined and they are still unclassified. The 15 *inc* groups have been further divided into three main classes (I, II, and III) with a fourth class that contains the pSK639 family of plasmids. The four groups are based on both physical and genetic organization as well as functional characteristics. Some staphylococcal plasmids are cryptic but most carry resistance determinants and some carry other virulence factors such as toxins. Group I plasmids are relatively small plasmids (typically $<$5 kb) that replicate by the rolling circle mechanism. They are of a low copy number (10–60 copies per cell) and usually do not contain any transposons or mobility factors. The pSK639-type plasmids (group IV) are of intermediate size (8 kb range) in comparison to group I and II plasmids. The prototype plasmid for this group, pSK639, was originally isolated from *S. epidermidis* and was later found to be in some strains of *S. aureus*. pSK639-like plasmids carry only one resistance determinant and may harbor IS elements and/or transposons as well. The origin of replication appears to be similar to the theta replication system and unlike the group I plasmids they typically contain mobility factors. Group II plasmids are larger in size than the pSK639 group and are also known to carry multiple resistance factors. They range in size from 15 to 40 kb and have low copy number ($\sim$5 copies per cell). Along with the capability of encoding for several resistance factors on a single plasmid, they also contain transposable elements and like the pSK group replicate via theta mode. Group II plasmids generally confer resistance to β-lactam antibiotics and heavy metals and are also the group responsible for conferring methicillin resistance. Plasmids in group III are typically of the largest size (30–60 kb) and are conjugative in nature. They are found in both coagulase-negative staphylococci and *S. aureus* and tend to carry a number of resistance determinants. The replication region is similar in sequence to the group II plasmids and similarly they contain multiple copies of IS elements and transposons. Although conjugation of these staphylococcal plasmids is not completely understood, it is known to require cell-to-cell contact on a solid surface; also, there is no pilus as described in conjugation of *E. coli* plasmids. Vancomycin resistance at both high and intermediate levels has been shown to be encoded on group III plasmids, and there is evidence suggesting that the resistance marker and the plasmid have been acquired through transfer from the enterococci. It is widely held that plasmids are exchanged among staphylococci, enterococci, streptococci, and bacilli, accounting for the presence of the same or similar plasmids in each genus.

Staphylococcal Bacteriophages

S. aureus was among the first organisms used to demonstrate the existence of bacteriophages. Bacteriophages have also been found in coagulase-negative staphylococci. The vast majority of phages in coagulase-positive and coagulase-negative staphylococci are double-stranded DNA phages belonging to the *Siphoviridae* family of the *Caudovirales* order. I Intensive study of staphylococcal bacteriophages has led to the establishment of a complex method of bacteriophage typing of disease isolates identified in epidemiological investigation. A panel of 21 bacteriophages called the International Typing Series of Bacteriophages is used to define strain types by specific patterns of susceptibility to these typing phages. Phage typing led to the identification of five strain groups (I–IV and miscellaneous). Most pathogenic strains belong to groups II and III, but all phage groups are capable of causing disease. More recently, phage typing has lost favor because of the finding that the standard bacteriophage-propagating strains carry temperate bacteriophages that are distinct from the typing phage they propagate. The phage preparations derived from these strains are therefore mixtures of several bacteriophages. In fact, virtually all strains of *S. aureus* are lysogenized by at least one bacteriophage and many carry multiple temperate phage in their genome. Some of the lysogenizing phage carry additional genes that they bring in to the recipient strain and alter its phenotype by inserting into the genome at specific attachment sites located within genes.

Both negative conversion (the inactivation of expressed genes (e.g., lipase and β-toxin)) and positive conversion (the introduction of newly expressed genes (staphylococcal enterotoxin A and staphylokinase)) are common occurrences in staphylococci. Temperate bacteriophage have been shown to alter the restriction pattern of the genome by introducing additional restriction sites that produce restriction fragment length polymorphisms (RFLPs) when analyzed by clamped homogeneous electrophoretic field (CHEF) gel electrophoresis. The creation of additional sites has caused ambiguities and difficulties in establishing clonal derivations in many epidemiological investigations of disease outbreaks. Phage typing of the staphylococci has also been made more difficult because not all methicillin-resistant *S. aureus* (MRSA) isolates come under the defined phage categories. Recently, studies have been performed to identify new phage to recognize this group as well as to identify alternative typing methods that may be more reliable for all kinds of isolates.

Bacteriophage are also thought to play a major role in the transmission of virulence factors and in the occurrence of genetic rearrangements. They are responsible for the only naturally occurring means of genetic exchange in the staphylococci. The first reports of transduction in staphylococci occurred in 1960 and concerned the transmission of resistance to penicillin. Since then many detailed studies of the transductional transmission of genes have been published. Most staphylococcal bacteriophage are generalized transducers, capable of transferring ~40–45 kb of DNA (a headfull) to a suitable recipient strain. DNA is packaged from PAC sites that are randomly dispersed around the genome.

Bacteriophage also play a role in transformation after induction of competence by Ca^{2+} treatment. Both plasmid and chromosomal DNA can be transferred by transformation, albeit at low frequency. In order for the transforming DNA to be recombined, it is necessary that the recipient strain be lysogenized by a serogroup B bacteriophage (there are 12 serogroupings). Other procedures, such as electroporation, enjoy limited effectiveness for plasmid transfer, but are not useful for transfer of chromosomal genes.

Coagulase-Negative Versus Coagulase-Positive Staphylococci

The primary players in human infection are actually *S. aureus*, *S. lugdunesis*, *S. epidermidis*, and, to a lesser degree, *S. saprophyticus*. These species are basically identical except for the ability of *S. aureus* to produce the fibrinogen clotting substance coagulase. The coagulase-negative species, *S. lugdunesis* and *S. epidermidis*, are largely commensal and can be isolated in high numbers from all areas of human skin. Likewise, *S. aureus* can be a colonizer of human skin and nares, but is more capable of causing infection if the microorganism should gain entrance to subcutaneous human tissues. It was proposed by Baird–Parker that coagulase distinguished pathogenic from nonpathogenic species and this differentiation was used until the mid-1970s. However, recent evidence has shown that coagulase-negative staphylococci (CoNS) are a major cause of wound infections and infections caused by foreign bodies including intravascular catheters, peritoneal dialysis catheters, prosthetic heart valves, joint prostheses, pacemaker electrodes, and fluid shunt systems. Therefore, coagulase is no longer considered an exclusive indicator of pathogenicity but is still used as a method for species identification.

S. lugdunesis, *S. epidermidis*, and *S. saprophyticus* are ultimately distinguished from *S. aureus* based on the lack of coagulase activity. Of the staphylococci that are of medical importance, *S. aureus* is the only one that is coagulase-positive. Two other staphylococcal species (*S. hyicus* and *S. intermedius*) are also coagulase-positive but are rarely isolated from human infections. *S. aureus* also differs from the other clinically relevant members of this genus in that it is β-hemolytic on blood agar and forms large, yellow-pigmented colonies upon growth.

In a clinical setting it is imperative that the particular species involved is correctly identified as this plays a role in treatment options. All staphylococci are Gram-positive, coccoid, and grow as clusters in culture. *S. saprophyticus* grow as small, nonhemolytic, white colonies on blood agar and are isolated almost exclusively from urinary tract infections in young, sexually active females. *S. epidermidis* is the most abundant organism found on the skin of humans and is characteristically seen as nonhemolytic, white colonies on blood agar plates. *S. lugdunesis* is also found on the skin of humans and can cause infections, including endocarditis on a native valve, that are identical to those caused by *S. aureus*. Only half of the 32 CoNS have been found in humans and this group can be further divided based on whether they are resistant or sensitive to novobiocin. *S. epidermidis* is novobiocin-susceptible while *S. saprophyticus* is novobiocin-resistant. Clinical manifestations of coagulase-negative staphylococci are more subtle and nonspecific as compared to *S. aureus* and are also less likely to be fatal. However, with *S. epidermidis* in particular, these infections typically involve indwelling medical devices and are most often chronic. Indeed, the large majority of CoNS infections are nosocomial and strains isolated from humans typically produce an inducible β-lactamase, with 60–80% of them resistant to methicillin.

Pathogenesis and Disease

Before discussing staphylococcal pathogenesis it is important to note the difference between colonization and infection. Colonization can be defined as the presence of an organism in or on a particular body site in the absence of signs and symptoms of disease. Colonizing organisms can typically cause disease if they spread to a different site on the same individual (i.e., from skin to bloodstream) or are transferred to a more susceptible host. Infection is defined as the ability to isolate a replicating organism from a disease site within the host. Infection may result in immediate signs and symptoms or may be clinically unapparent. However,

infection by an organism does eventually result in some level of inflammation within the host. Interestingly, although a host can become infected as soon as a pathogen invades, in many cases colonization precedes infection.

Carriage

Staphylococci are one of the major groups of organisms that inhabit the skin of mammals. Usually, several different strains are found on the same host. They may be present as transient contaminants, short-term replicating residents, or as long-term colonizers that persist for long periods. The majority of species are opportunistic pathogens that become infectious when the skin or mucous membranes are compromised by trauma, inoculation by needle, or direct implantation of medical devices (foreign bodies). Staphylococci are then able to attach, colonize, and produce toxic substances that destroy host tissues.

As stated previously, the majority of staphylococcal disease is due to infection with the coagulase-positive species *S. aureus*, which is considered an opportunistic pathogen because it is not uncommon for healthy individuals to carry the organism either persistently (10–35% of population) or intermittently (20–75%) in the anterior nares. Although colonization is not uncommon, it is also not ubiquitous among humans. In fact, recent studies have shown that 5–50% of people never carry *S. aureus*. In addition, the higher carriage rates are often related to the level of exposure; with healthcare workers often more likely to be colonized than individuals in the general population. Most often, carriers of *S. aureus* show no signs of disease; however, this organism can cause severe, even life-threatening disease under certain circumstances.

Disease Types

Species of *Staphylococcus* are the leading cause of nosocomial infection. Each year about 2 million hospitalizations result in nosocomial infection and ~50% are due to *S. aureus* and *S. epidermidis*. Community-acquired infections have a similar incidence rate. A predisposing condition has been the acquisition of multiple drug resistance by *S. aureus* (MRSA), which has caused the incidence of nosocomial and community-acquired infection to increase steadily since the 1960s. Prior to the 1990s, MRSA infections were associated with hospitals or other healthcare facilities; such MRSA isolates were usually resistant to multiple antibiotics and susceptible only to vancomycin and rifampin. These MRSA strains have been characterized as healthcare-associated MRSA (HA-MRSA). Beginning in the early 1990s, infections due to MRSA was seen in patients who had no previous healthcare exposure; these strains were resistant to beta-lactam agents but were often susceptible to other antibiotics such as clindamycin and trimethoprim-sulfanethoxazole. These MRSA strains have been characterized as community-acquired MRSA (CA-MRSA). Infections initially seen in the United States were due to CA-MRSA strains of ST1 lineage, which were also characterized as USA400 based on PFGE typing. CA-MRSA strains were found to possess an SCC*mecA* element (SCC*mec* IV) that was distinct from the elements I – III seen in most healthcare-associated MRSA. These CA-MRSA strains also contained genes encoding the Panton-Valentine leucocidin (PVL); PVL damages polymorphonuclear leukocyte membranes via a beta-barrel pore-forming mechanism. Subsequently the USA400 strain of CA-MRSA was replaced by another clone, ST8 (USA300); the USA300 strain now accounts for almost 90% of the CA-MRSA isolates in the United States. Of note is that the USA300 strain contains an SCC*mec* element that is smaller than SCC*mec* I – III and possesses fewer resistance genes.

The primary mode of pathogenesis for *S. epidermidis* is via colonization of biomedical devices such as indwelling catheters as a result of biofilm formation on the surface of these biomaterials. Although *S. aureus* causes a wide variety of pyogenic and toxin-mediated diseases, *S. epidermidis* rarely causes pyogenic disease in the immunocompetent host and there is very little evidence to suggest that it is responsible for toxin-mediated disease. Outside of indwelling medical device infection, *S. epidermidis* is primarily seen in intravenous drug users and immunocompromised patients such as premature newborns and individuals undergoing immunosuppressive therapy. The clinical manifestations associated with these groups are right-sided endocarditis and septicemia, respectively. Unlike *S. aureus* infections, there are typically no fulminant signs of infection and the clinical course is therefore more chronic in nature.

S. lugdunensis is part of the normal human skin flora and is an infrequent, but not rare, cause of human infections. *S. lugdunensis* is an unusually virulent strain of coagulase-negative staphylococci and can cause aggressive infections similar to those caused by *S. aureus*. Indeed, these human infections include native valve endocarditis. The most common infections caused by *S. lugdunesis* are skin and soft tissue infections; such infections are often seen in the perianal, inguinal, or pelvic region. *S. lugdunensis* has also been associated with orthopedic infections involving prosthetic joints as well as osteomyelitis.

S. aureus is the most highly pathogenic organism of the staphylococci and has been isolated from infection sites involving virtually every tissue of the body. In general, three types of disease are usually associated with infection by *S. aureus*. These are characterized as superficial or cutaneous infections such as pimples, boils, and toxic epidermal necrolysis; systemic infections such as heart valve disease, bacteremia, and osteomyelitis; and toxinoses such as food poisoning and toxic shock syndrome.

Immunity

Immunity to staphylococcal infections is poorly understood. Normal healthy humans have a high degree of innate resistance to invasive infections. Experimental infections are difficult to establish in animals and require large inocula containing millions of organisms. In humans, the organism is able to colonize mucosal and epidermal surfaces with little resistance, and as long as they remain intact, these barriers are the main source of natural immunity to infection. After invasion, however, phagocytosis by polymorphonuclear leukocytes is the main humoral defense. Because of repeated exposure to *S. aureus* and *S. epidermidis* in natural

settings, antibodies to various components of the cell and its products (both cell surface and soluble) are prevalent in animals. Nevertheless, with the exception of toxic shock syndrome where antibody is an important factor in immunity, serological studies have not successfully related immunity and antibody titer. Moreover, prior infection fails to elicit immunity to reinfection. In spite of these drawbacks, vaccine research is being pursued strongly. Although there is currently no vaccine that stimulates active immunity in humans, a vaccine based on fibronectin-binding protein has been shown to confer protective immunity against mastitis in cattle. Among the vaccines being studied for use in humans, the most promising is a polysaccharide conjugate vaccine that was given Fast Track Status by the FDA in 2004 for the prevention of bacteremia in certain at-risk patient populations; however, the vaccine failed to reduce the incidence of *S. aureus* infections when used in clinical trials.

Identification

The clinical laboratory must be able to identify *S. aureus* quickly and accurately. Many types of clinical samples have been utilized for detection. Samples that are heavily contaminated with other bacterial flora (i.e., nasal, skin, or wound specimens) can be grown on solid media or liquid media containing various selective agents that exploit the resistance of staphylococci to NaCl, chemicals such as potassium tellurite and lithium chloride, or antibiotics. Suspect colonies from the primary isolation are then subjected to antibiotic sensitivity determination to provide treatment alternatives. As mentioned above, coagulase determination, an important consideration for treatment decisions regarding CoNS strains, is usually carried out. A variety of other phenotypic markers can be tested if further characterization is desired. These include α- and β-hemolysins, nuclease, proteases, lipase, protein A, and the determination of specific toxins.

Treatment

More than 95% of patients with *S. aureus* infections worldwide do not respond to first-line antibiotics such as penicillin and ampicillin. These strains have been routinely treated with methicillin, but resistance appears in a large number of infections (HA-MRSA and CA-MRSA). Vancomycin is the most effective treatment for multiresistant strains, but the recent emergence of vancomycin resistance in staphylococci poses a critical problem to effective treatment of these infections. The emergence of strains that are resistant to multiple antibiotics, especially the recent appearance of vancomycin resistance, has spurred renewed interest in antibiotic discovery and vaccine therapy. CA-MRSA strains are often treated with clindamycin, doxycycline, or trimethoprim-sulfamethoxazole. New drug targets are being investigated to devise novel families of antibiotics. Methicillin resistance is not limited to *S. aureus* and all of the other staphylococcal species that have been sequenced to date are isolates that carry the genes conferring resistance to this antibiotic and some contain plasmids that confer resistance to other compounds used for treatment.

Antibiotic Resistance and MRSA

Nosocomial infections with HA-MRSA have been identified as a problem since the 1960s, with ~20% of bloodstream infections being due to *S. aureus*. As many as 64.4% of hospital onset *S. aureus* infections reported in US intensive care units were due to HA-MRSA. These types of infections are not only important due to the danger they pose to the patient but they also result in longer hospital stays, higher mortality, and increased costs for alternative treatments. Until recently, the focus of MRSA infections was limited to those that were nosocomial (hospital onset) in nature, with little or no surveillance activity for community-acquired MRSA (CA-MRSA). This was due in large part to a much lower incidence of CA-MRSA in relation to hospital-acquired infections. However, in a study carried out by the US Centers for Disease Control (CDC) to estimate the burden of invasive MRSA infections in the United States in 2005, CA-MRSA was identified as a major source of infection. Of the almost 8987 cases of invasive MRSA that were reported, ~85% were related to recent hospitalization or were of hospital onset and 13.7% were classified as CA-MRSA. The study also showed that infection rates were highest in older patients (≥65 years), African Americans, and males. Interestingly, the group at lowest risk included young people ranging from 5 to 17 years of age. The study concluded that invasive MRSA affects certain populations disproportionately and that it is a major public health problem primarily related to health care. The researchers also stress the point that MRSA should no longer be confined to healthcare institutions, and that the incidence of CA-MRSA will most likely continue to increase.

There are multiple reasons that the staphylococci, especially *S. aureus*, have become antibiotic-resistant including overuse of antibiotics in humans, the presence of antibiotics in food and water supplies, and mutation and/or exchange of genes within the genus. Unnecessary prescriptions for antibiotics is one of the main sources contributing to staphylococcal (and other microorganisms) developing resistance. Decades of excessive antibiotic use for colds, flu, and other viral infections that do not respond to these drugs results in low-level exposure to these compounds by normal bacterial flora found in the host. This repeated exposure to sublethal concentrations results in the elimination of the majority of the resident bacteria and selects for spontaneously occurring mutants that are resistant to the antibiotic. Over time, the presence of the antibiotic has no effect on the organism and therefore they are resistant to killing by the drug when it is prescribed for actual treatment. Prescription drugs are not the only source of antibiotics. In the United States, antibiotics can be found in animal feeds especially for beef cattle, pigs, and chickens. The same antibiotics then find their way into municipal water systems when the runoff from feedlots contaminates streams and groundwater. However, antibiotics given in the proper doses to sick animals do not appear to produce resistant bacteria. Even appropriate antibiotic use can contribute to the increase in drug-resistant bacteria because they may not destroy every organism within the population. Bacteria

evolve rapidly; so those that survive treatment with one antibiotic are soon capable of resisting others. Bacteria mutate much more quickly than new drugs can be produced, which makes it possible for a given organism to become resistant to all available treatment options. In addition to mutation of genes, the staphylococci are adept at gene transfer via mobile genetic elements such as plasmids, phage, and transposons. By carrying and transferring antibiotic-resistant genes via these mechanisms, antibiotic resistance has become rampant within this group. Although there has not yet been a strain of *S. aureus* identified that is resistant to all of the available antibiotics, there are many that are resistant to the majority of drugs currently available. In fact, it is relatively uncommon for any given clinical isolate to be resistant to only one drug and almost all that are isolated are resistant to penicillin, which was the original drug used to treat most bacterial infections. More importantly, there are now multiple strains that have acquired resistance to what used to be the drug of last resort (vancomycin). Using vancomycin and other more aggressive forms of treatment contribute to the economic burden endured by the patient and hospital, and these types of treatments are also more physically demanding for the patient. For example, vancomycin is generally given in multiple intravenous doses over several weeks, which requires additional specialized care and a longer stay within the hospital. The drug itself often makes the individual feel even worse than before treatment began, with potential side effects including, but not limited to, kidney failure, temporary or permanent hearing loss, neutropenia, anaphylaxis, pain and inflammation at the injection site, severe stomach pain, diarrhea, and fever or chills.

Virulence Factors

Staphylococci produce a wide array of extracellular and cell surface proteins with a large number of these encoded on plasmids and other accessory elements. Because of this, different strains have been shown to exhibit a variable array of toxins, enzymes, and other factors. A list of some of these exoproteins is presented in Table 3. The extracellular proteins are subdivided into cell surface-oriented proteins and soluble proteins. All the exoproteins are translated as precursor proteins with signal peptides, which are removed at secretion. In addition, the cell surface proteins also possess a characteristic amino sequence motif (LPXTG) at the C-terminus, which precedes the membrane spanning region and serves as an anchor, linking the protein to the cell wall peptidoglycan. Both types of exoproteins are secreted by Type II secretory mechanisms involving the SecYEG pathway. With the exception of certain bacteriocins, there is no evidence to date that extracellular proteins are secreted by other secretory pathways.

Soluble Exoproteins

These include a wide array of toxins and enzymes such as food poisoning enterotoxins, exfoliative toxins, toxic shock syndrome toxin, hemolysins, coagulase proteases, lipases, and other enzymes. The exfoliative toxins are proteolytic and attack the epidermis of susceptible animals. Exfoliative toxins cause blistering skin diseases known as bullous impetigo and staphylococcal scalded-skin syndrome (SSSS). Three isoforms of exfoliative toxins (ETA, ETB, and ETD) have been identified in virulent strains of *S. aureus* and four isoforms have been shown to exist in the animal pathogen *S. hyicus*. Clinical manifestation of SSSS is typically seen in neonates and has been termed scalded skin syndrome due to the symptoms that occur culminating in areas of raw, red skin that resembles that of a first-degree burn. The enterotoxins have been shown to act at the interface between the stratum granulosum and stratum spinosum of the epidermis, thereby resulting in characteristic exfoliation of the skin. In other words, there is a loss of keratinocyte cell–cell adhesion in the epidermis. It was initially difficult to determine the mechanism of action for these toxins because purified forms of both ETA and ETB showed no direct protease activity toward multiple targets. However, recent studies have shown that the three isoforms of exfoliative toxins, ETA, ETB, and ETD, are glutamate-specific serine proteases and that ETA and ETB specifically cleave a protein in the epidermal interface called desmogelin 1. ETD has been shown to be encoded within a pathogenecity island and to play a slightly different role in the development of bullous impetigo and scalded skin syndrome disease.

The hemolysins are membrane-damaging proteins whose activity is mediated through pore formation or lipolytic action. The most studied of the hemolysins is α-toxin, which is toxic to a wide range of mammalian cells and is highly hemolytic for rabbit erythrocytes. It is also dermonecrotic and neurotoxic and is produced by almost all strains of *S. aureus*. β-toxin is made in high concentrations, particularly by animal strains of *S. aureus*, and is highly hemolytic for sheep erythrocytes but not rabbit red blood cells. This toxin exhibits phosphorylase C activity that requires magnesium but it has a limited range of activity due to specificity for sphingomyelin and lysophosphatidyl choline. The role of β-toxin in disease is not well understood but the fact that it is made at much higher levels in animal strains suggests that it may provide an advantage in animal hosts as opposed to humans. Another example of an *S. aureus* pore-forming toxin is the Panton–Valentine leukocidin (PVL), which is a cytotoxin that causes leukocyte destruction and tissue necrosis. PVL is encoded on a bacteriophage and has been associated with both staphylococcal skin and pulmonary infections. PVL-containing strains have also been isolated at a higher frequency from patients with severe cMRSA pneumonia and the presence of the toxin is much higher in strains that carry the SCCmecIV cassette than in strains that do not.

Another group of toxins that play a significant role in staphylococcal disease are the enterotoxins. The enterotoxins are responsible for the clinical manifestations of staphylococcal food poisoning and a septic shock-like illness. There are five major classical types of staphylococcal enterotoxins (SEs), and several new SEs or SE-like toxins have recently been identified. Ingestion of these toxins leads to severe gastroenteritis with emesis, nausea, and diarrhea. In addition, the SEs are resistant to extreme heat and are stable over a wide pH range besides being resistant to degradation by a variety of proteases. They are also classified as superantigens, a characteristic that is described in more detail below.

Table 3 Extracellular proteins of *Staphylococcus aureus*

Protein	*Gene locus*
Hemolysins	
Alpha toxin	Chromosome
Beta toxin[a]	Chromosome
Gamma toxin	Chromosome
Delta toxin[a]	Chromosome
Panton-Valentine leukocidin	Chromosome/pathogenicity island
Enterotoxins	
SEA	Bacteriophage/chromosome
SEB	Chromosome/pathogenicity island
SEC	Plasmid
SED	Plasmid
SEE	Chromosome
SEG	Chromosome
SEI	Chromosome
Enzymes and other toxins	
Lipase[a]	Chromosome
Nuclease[a]	Chromosome
V8 Protease[a]	Chromosome
Esterase	Chromosome
Coagulase	Chromosome
Cell wall hydrolase	Chromosome
Hyaluronadase	Chromosome
Staphylokinase	Bacteriophage/chromosome
Protein A	Chromosome
Serine proteases[a]	Chromosome/genomic island
Zinc metalloproteinase aureolysin[a]	Chromosome
Phospholipase C	Chromosome
Leukotoxins	Chromosome/genomic island
Leukocidins	Chromosome/pathogenecity island
Exfoliative toxin A	Chromosome
Exfoliative toxin B	Plasmid
Toxic shock syndrome toxin-1	Chromosome/pathogenicity island
MSCRAAMs	
Clumping factors	Chromosome
Fibronectin-binding proteins A/B	Chromosome
Fibrinogen-binding protein	Chromosome
Collagen-binding protein	Chromosome
Elastin binding protein[a]	Chromosome
Extracellular matrix-binding proteins[a]	Chromosome
Intercellular adhesion proteins[a]	Chromosome

[a]Denotes virulence factors that are also present in *S. epidermidis*.

Several of the soluble exoproteins including enterotoxins, exfoliative toxins, and toxic shock toxin are superantigens due to their ability to stimulate mitogenic activity and cytokine production for a wide array of T-lymphocyte haplotypes. They are able to activate specific sets of T-lymphocytes by binding to major histocompatibility complex class II (MHC II) proteins. They bind to the variable region of the T-cell receptor β-chain. The activated cells proliferate and release cytokines/lymphokines, interferon-γ (IFN-γ), and interleukins. Because they exhibit this broad-based activity, they have been called superantigens. This activity is suspected to enhance virulence by suppressing the hosts response to staphylococcal antigens produced during infection. In addition to the factors discussed above, there are many staphylococcal enzymes such as lipase, nuclease, and proteases, and all are presumed to enhance invasiveness through tissue destruction.

Cell Surface Proteins

A major class of cell surface proteins are adhesins termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). These molecules comprise the main adhesins of the organism and include collagen-binding protein, fibronectin-binding proteins, fibrinogen-binding protein, elastin-binding protein, clumping factor, and the matrix adhesin factor. There may be as many as 12 other surface proteins that contain membrane anchor domains and potentially qualify as MSCRAMMs. A second

group of cell surface proteins includes nuclease and protein A. Staphylococcal nuclease is a thermally stable endonuclease able to withstand boiling for 30 min without significant loss of activity. Protein A is able to bind to the nonantigenic F_c fragment of immunoglobulin G, causing the complex to precipitate. Its role in virulence is believed to be in escape from immune surveillance.

Regulatory Mechanisms

The expression of extracellular proteins is largely under the influence of a master genetic circuit called *agr* (accessory gene regulator). This signaling arm of the operon (AgrBDCA) is activated by a quorum-sensing mechanism that depends upon the accumulation of an activating octomeric peptide (processed from the AgrD precursor by AgrB). The peptide triggers increased expression of the entire operon via an integral signal transduction pathway (AgrC, AgrA), upregulating production of the octomer and activating a second promoter that produces an unique regulatory molecule, RNAIII. RNAIII is the effector molecule for regulated protein expression. It is neither translated nor does it bind to the promoter regions of regulated genes. It presumably interacts with other genes, in an unknown way, as both a positive and negative regulator of exoprotein gene expression. RNAIII is required for the expression of soluble exoproteins and represses the expression of cell surface proteins. Because a threshold level of octapeptide is required for activation, RNAIII is not expressed until late in growth. Therefore, cell surface proteins are produced early, presumably to allow the organism to attach and colonize. The RNAIII-induced activation of soluble proteins genes results in a necrotic effect, allowing the organism to invade deeper tissues and become bacteremic. A second locus, *sarA*, modulates expression of the *agr* locus by binding to the promoter region of AgrBDCA. The *sarA* locus is transcribed from three different promoters (sarP1, P2, and P3) that are active at different times during growth. The major regulatory molecule encoded by *sarA* is a 14.5 kDa protein that has been shown to bind to the promoter regions for fibronectin-binding protein A, the collagen adhesion, protein A, and *agr*. It is reported that the *sarA* locus plays a role in transcription of over 100 different gene targets either by direct binding of upstream promoter regions or indirectly due to its effects on other regulatory loci including *agr*. Several *sar* homologues have been identified including SarR, SarS, SarU, and SarY that play a role in regulation of SarA and other factors. With the continued efforts aimed at whole-genome sequencing of staphylococcal isolates, many other regulatory systems that are not discussed in detail here have been identified and characterized. These include Rot, SaeRS, SrrAB, ArlRS, and LytRS.

Another important level of regulation of virulence is regulation of gene expression in response to environmental factors. It has been demonstrated that depending on the environment that the staphylococci encounter they can adapt by altering gene expression for a variety of systems. This is not unusual for bacteria and in fact the main environmental response system that has been described for *S. aureus* and *S. epidermidis* is the sigma B pathway. Sigma factors are bacterial proteins that enable specific binding of RNA polymerase to promoter regions within the DNA. The staphylococcal sigma B response is similar to that described for other Gram-positive pathogens such as *B. subtilis*. Sigma B is an alternative sigma factor that is activated under environmentally stressful conditions such as high salt levels, presence of ethanol, energy depletion, and low pH. Comprehensive studies in *S. aureus* have shown that sigma B regulates gene expression of some factors by directly binding to a specific site upstream of promoters and also by indirectly affecting upstream factors to gene expression. Some of the virulence factors that have been shown to be affected due to sigma B expression include coagulase, fibronectin-binding protein B, biofilm formation, and α-toxin. A change in resistance levels to some antibiotics that affect the bacterial cell wall has also contributed to overexpression of sigma B. It is important to note that some strains of *S. aureus* have a mutation in the sigma B activator (RsbU) that renders them sigma B defective. These strains are also capable of growing under environmentally stressful conditions, which suggests that there are additional systems that have yet to be identified and that must play a role in virulence factor expression.

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Streptococcus Pneumoniae Evolving – Impact of Antibiotics and Vaccines[☆]

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Abbreviations

CbpA	Choline-binding protein A
CibABC	Competence induced bacteriocin
CSP	Competent stimulating peptide
DCC	Day care center
EUROPNEUMO	European Meeting on the Molecular Biology of the Pneumococcus
IPD	Invasive pneumococcal disease
ISPPD	International Symposium on Pneumococci and Pneumococcal Disease
LytA	Autolysin A
MLST	Multilocus sequence typing
NanA	Neuraminidase
NET	Neutrophil extracellular trap
Pal	Pneumococcal bacteriophage lytic enzyme
PBP	Penicillin-binding protein
PFGE	Pulsed-field gel electrophoresis
Ply	Pneumolysin
PMN	Polymorphonuclear leucocytes
PspA	Pneumococcal surface protein A
SrtA	Sortase A

Defining Statement

The primary natural habitat of *Streptococcus pneumoniae* on this planet is the nasopharynx of preschool age children, and antibiotics and vaccines not only combat pneumococcal disease but also drive the evolution of drug resistant and novel capsular types of this species. In this sense, humans are not only targets but also evolutionary partners of *S. pneumoniae* as well.

Guides to the *S. pneumoniae* Literature

Useful guides to the rapidly expanding literature on various aspects of the microbiology and infectious diseases of *Streptococcus pneumoniae* may be found in books listed at the end of this article which cover contributions to the field in the early and the more recent era.

Periodic updates on progress are also available through informal meetings of two groups of scientists: those interested primarily in pneumococcal molecular biology (EUROPNEUMO: European Meeting on the Molecular Biology of the Pneumococcus) and those interested in pneumococcal disease (ISPPD: International Symposium on Pneumococci and Pneumococcal Diseases). An international workshop that brought together scientists interested in the molecular biology and infectious diseases aspects of *S. pneumoniae* was held between September 23–29, 1996 in Portugal – organized by Herminia de Lencastre and Alexander Tomasz. The proceedings of this meeting were published in book form (In *Streptococcus pneumoniae* - Molecular Biology and Mechanisms of Disease - Update for the 1990s. A. Tomasz, editor. Mary Ann Liebert, Inc. publishers, New York). This ground-breaking meeting led to the foundation of the ISPPD meetings.

The first meeting of the EUROPNEUMO took place in 1993 and the first meeting of ISPPD was held in 1998. The two groups meet on a bi-annual basis.

[☆]Change History: July 2014: A Tomasz and R Sá-Leão updated the text and further readings to this entire article.

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Pneumococcus as a Pathogen and as a Model Microbe for Molecular Biology

> Pneumococcus is altogether an amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs biochemical feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death
>
> Benjamin White, 1938 in. The Biology of Pneumococcus

Streptococcus pneumoniae was first described in 1881 by Pasteur and Sternberg in independent observations. In the same decade, this Gram-positive pathogen with lancet-shaped cells which grow in most media in pairs or short chains of 'diplococci' was recognized as a major cause of infections that included pneumonia, meningitis, otitis media and endocarditis. *S. pneumoniae* routine identification is done through the alpha haemolysis surrounding colonies obtained on blood agar, negative reaction with catalase, susceptibility to optochin and solubility of the bacteria in bile salts. Most *S. pneumoniae* isolates are shielded by a polysaccharide capsule which hinders phagocytosis. Over 95 different capsules have been described to date and serologic typing (serotyping) remains one of the most frequently used methods for the characterization of pneumococcal isolates.

Since its discovery, this bacterium has been the subject of intensive studies as a cause of major and often life threatening human infections. While the primary aim of these studies was the control of pneumococcal disease, the same efforts have also led to seminal scientific discoveries in the laboratory which included the identification of the pneumococcal polysaccharide antigens as vaccines, the ability of capsular polysaccharides to induce antibodies, the discovery of bacterial gene transfer which led to the identification of the 'transforming principle' (later named DNA) as the genetic material (Avery et al, 1944). Efforts to degrade the capsular polysaccharide surrounding the pneumococcus have led to the first use of an 'enrichment culture'. The remarkable and rapid dissolution of pneumococci by bile led to the identification of the first bacterial autolytic enzyme. The pneumococcus was among the first pathogens in which the therapeutic efficacy of the newly discovered penicillin was tested. The role of the polysaccharide capsule providing resistance against phagocytosis was identified and studies on the rapid fluctuations in the pneumococcal capacity to take up DNA from the medium and undergo genetic change has led to the identification of the first bacterial quorum sensing factor (Tomasz, 1965).

Thus, efforts to understand and control pneumococcal disease went hand in hand with some of the fundamental discoveries of molecular biology. Interestingly, many of these phenomena first discovered in the in vitro world of the microbiology laboratory were subsequently identified as major factors driving the evolution of new pneumococcal lineages in the real life world of pneumococcal colonization, infection and disease.

The most promising – and ambitious – current efforts to understand the impact of antibiotics and vaccines on the pneumococcal colonization, infection and disease in humans are combining carefully designed epidemiological studies with the characterization of pneumococcal isolates by high resolution molecular techniques developed in the molecular biology laboratory.

Burden of Pneumococcal Disease

In the pre-antibiotic era, pneumococcal pneumonia was so common and fatal that it was termed as the 'old man's friend' and the 'captain of the men of death' by William Osler.

In the late 1990s, before the introduction of the first pneumococcal seven-valent conjugate vaccine in the US, data from the Center for Disease Control and Prevention estimated the annual frequency of pneumococcal infections as 3000 cases of meningitis, 50 000 cases of bacteremia, 500 000 cases of pneumonia, and 7 million cases of otitis media with an estimated mortality of about 40 000 deaths per year.

While no similar dependable estimates are available from the less developed countries in the world, evaluation of the impact of a nine-valent conjugate vaccine in one randomized control trial conducted in Africa clearly indicated that pneumococcal disease is a major contributor to the mortality of children in African countries.

In the Gambia, 77% (95%CI, 51-90%) efficacy against vaccine type invasive pneumococcal disease was found with a 50% (95% CI, 21-69%) efficacy against all types of invasive pneumococcal disease. Furthermore, a 16% (95%CI, 3-28%) decrease in all-cause mortality was found among vaccinated children. In South Africa, the efficacy of the nine-valent pneumococcal conjugate vaccine against invasive pneumococcal disease among HIV-negative children was 83% (95%CI, 39-97%) and corresponding figures were 65% (95%CI, 24-86%) among HIV-positive children.

A report in 2007 from the WHO estimated that 1.6 million people continue to die every year due to pneumococcal disease including 0.7-1 million children aged less than 5 years, the majority living in developing countries where the pneumococcal conjugate vaccine is not available. The burden of disease associated with the elderly in these countries remains to be defined.

In addition to young children and the elderly, individuals of all ages infected with HIV are at a substantial higher risk of serious pneumococcal infection. For example, the risk of pneumococcal pneumonia is 25-fold higher among HIV-infected people compared to HIV-uninfected people. The incidence of pneumococcal invasive disease among HIV-positive children is nine to 43 times higher than among HIV-negative children and the rates of IPD among HIV-positive adults are 6 to 343 times higher than among HIV-negative adults.

Pneumococcal Infection and Viral Disease

In the era of preparedness for an anticipated new influenza or bird flu pandemic the well-documented contribution of pneumococci in the mortality associated with flu becomes increasingly important.

Several lines of evidence have highlighted that secondary infections by pneumococci in patients with viral respiratory disease can have devastating consequences. Studies conducted during the 1918 influenza epidemic which is estimated to have led to at least 40–50 million deaths, demonstrated that an important fraction of the deaths took place 2 weeks after the onset of influenza symptoms suggesting that superinfection by a common bacterial respiratory pathogen had occurred. Direct evidence supporting the role of pneumococcal secondary infection leading to fatal pneumonia has been described.

In a double-blind, randomized, placebo-controlled trial of a nine-valent pneumococcal conjugate vaccine in South Africa, it was found that the vaccine prevented 31% of virus-associated pneumonia in hospitalized children, suggesting that an important fraction of virus-associated pneumonia among hospitalized children was attributable to bacterial co-infection which could be prevented by bacterial vaccines.

Experiments on modeling viral-bacterial infection in animals showed that if a mouse model was challenged with a non-lethal dose of influenza virus and approximately seven days later was challenged with pneumococcus, 100% mortality occurred. This effect was specific for viral infection preceding bacterial infection.

Altogether these data strongly suggest that pneumococcal vaccination could have a beneficial role in preventing influenza-associated mortality in the advent of a new influenza pandemic.

The Natural Reservoir of *S. pneumoniae*

Humans are not only the target of diseases caused by *S. pneumoniae* but are also the primary ecological reservoir of this bacterial pathogen - although anecdotic studies have found carriage of pneumococci by horses and other human-handled mammals, and isolation from wild deceased chimpanzees. Thus, interventions to combat pneumococcal disease such as the introduction of antibiotics or vaccines also impact on the human nasopharyngeal flora of pneumococci selecting for drug resistant lineages and strains with less common capsular types. In this sense, Man is also an evolutionary partner of this microbe and many – if not all – of the genetic events that allow these bacteria to borrow pieces of foreign DNA to remodel their penicillin sensitive enzymes, acquire mobile elements that confer antimicrobial resistance to different classes of antibiotics, or undergo capsular switches to evade the action of vaccines targeting the capsule most likely occur in pneumococcal populations that inhabit the human nasopharynx, more specifically, the nasopharynx of preschool age children. The latter, for reasons not fully understood, are the primary carriers of this bacterial species. For these reasons the day care centers in which preschool age children are now recruited in many of the countries of the developed world have become major foci of epidemiological studies of pneumococci.

Indeed, several studies have shown that children of preschool age are the major reservoir of pneumococci and by school time a spontaneous decrease in carriage (or, at least, in carriage density) occurs. The mechanism of extensive colonization in infancy and the loss of carriage with age are not well understood. It is also well established that in infancy, in the absence of multivalent pneumococcal conjugate vaccines, the most dominant capsular types colonizing the nasopharynx are typically of the serogroups 6, 9, 14, 19 and 23 in countries across the world. This commonality of serotypes colonizing the young host contrasts with the well documented and sometimes extensive differences in the serotypes of pneumococci that most frequently cause invasive disease in various parts of the world at a given time. The mechanism of geographic variation in serotype abundance and their change in time is not known.

An important source of problems contributing to the difficulties of interpretation of many aspects of pneumococcal epidemiology is the way pneumococcal colonization has been routinely assayed in the overwhelming majority of the studies conducted so far. In most studies, a single colony recovered on blood agar plates from the nasopharyngeal swabs is assumed to represent the entire colonizing flora. However, simultaneous carriage of multiple strains of pneumococci in the nasopharynx has been known for several decades and was documented in early studies conducted in the 1930s and 1940s which used mouse inoculation assays to detect the strains. As these methods were very labor-intensive and expensive they have been abandoned. More recent studies in which a number of colonies were picked from the primary blood agar plates and were characterized clearly showed that the nasopharyngeal flora is heterogeneous: it may be composed of more than one strain of pneumococci; some representing the majority, others – often present with lower frequencies – may be of completely different serotypes and molecular types (Table 1). Recent studies based on DNA-based technologies highly sensitive to detect multiple serotypes in carriage samples are being developed and shedding light on this phenomena (Valente et al., 2012). Certain pneumococcal serotypes such as serotypes 1 and 5 that can be recovered from disease sites but have seldom been seen in the nasopharynx may represent such minority residents in the nasopharyngeal flora. The same minority clones may be the source of the novel pneumococci emerging after the introduction of the conjugate pneumococcal vaccine.

Multiple pneumococcal carriage is apparently more abundant among populations with high pneumococcal carriage rates such as children from Papua New Guinea, Gambia or Australian Aborigines. Multiple carriage rates in the range of 20–30% have been reported among these populations. Among other children typical rates of multiple carriage have been in the range of 10–20%.

Table 1 Properties of multiple isolates obtained from nasopharyngeal samples containing two strains of *S. pneumoniae*.

Sample	*Isolate code*	*Serotype*	*MIC (microgram/ml) to penicillin*	*Antibiotype*[a] *(resistant to)*	*PFGE type*	*PFGE - lytA (kb)*	*Addition mitomycin C*	*Phage DNA*	*comC allele*
A	106	11	0.016	E, Cc	SSS	85	no lysis		comC 1
A	106-1	6B	0.023	E, Cc, Te	M	280, 110, 90	lysis	yes	comC 1
A	106-2	11	0.006	E, Cc	SSS	85			
A	106-3	11	0.008	E, Cc	SSS	85			
A	106-4	11	0.008	E, Cc	SSS	85			
A	106-5	11	0.008	E, Cc	SSS	85			
A	106-6	6B	0.016	E, Cc, Te	M	280, 110, 90			
B	325	19 F	0.047	-	H	90, 35	no lysis		comC 2.1
B	325-1	NT	0.5	-	TTT	170, 90, 40, 30	ND		comC 2.1
B	325-2	NT	0.75	-	TTT	170, 90, 40, 30			
B	325-3	NT	1	-	TTT	170, 90, 40, 30			
C	448	19A	0.094	SXT	D	235, 90, 80	lysis	yes	comC 1
C	448-1	19A	0.064	SXT	D	235, 90, 80			
C	448-2	23F	2	C, Te, SXT	A	230, 100, 40	lysis	yes	comC 2.2
C	448-3	19A	0.094	SXT	D	235, 90, 80			
C	448-4	19A	0.094	SXT	D	235, 90, 80			
C	448-5	19A	0.094	SXT	D	235, 90, 80			
C	448-6	19A	0.064	SXT	D	235, 90, 80			
D	541	6B	0.023	Te, SXT	M	280, 90, 60	lysis	yes	comC 1
D	541-1	19A	0.023	SXT	UUU	90	no lysis		comC 1
D	541-2	19A	0.023	SXT	UUU	90			
D	541-3	6B	0.016	Te, SXT	M	280, 90, 60			
D	541-4	6B	0.012	Te, SXT	M	280, 90, 60			
D	541-5	6B	0.008	Te, SXT	M	280, 90, 60			

E - erythromycin, Cc - clindamycin, Te - tetracycline, SXT - sulfamethoxazole-trimethoprim

[a]C - chloramphenicol

Taken from Sá-Leão et al. 2002. *J Clin Microbiol* 40:3577–3585. For full details check original reference.

Stages in Pneumococcal Pathogenesis: Virulence Factors and Host Defense

Virulence in pneumococci is multifactorial and involves complex and multiple interactions with the host. Well over 300 genes have been implicated in virulence in at least one animal model of pneumococcal infection. A genome wide screen – by signature tagged mutagenesis – of the reference strain TIGR4 for genes essential for lung infection in an animal model identified close to 400 genes. Individual putative virulence factors appear to play a variety of roles – both 'aggressive' as well as 'defensive' at various stages of pneumococcal infections. Some of the pneumococcal virulence factors are present in virtually all pneumococcal isolates such as the capsular polysaccharide, the major autolysin LytA, the intracellular toxin pneumolysin (Ply), and sortase A (SrtA). Other important virulence determinants such as the pilus gene also play important roles in virulence but these determinants seem to be associated with specific clones of pneumococci. Secreted DNAase appears to have a role in escaping the neutrophil extracellular traps (NETs) and pneumococcal resistance to the widely spread cationic antimicrobial peptides appears to involve incorporation of D-alanine esters into the pneumococcal cell wall teichoic acids.

Host factors 'matching' in numbers the virulence genes of the pneumococcus have also been identified using a variety of models. These factors cover a wide range of functions: pattern recognition elements that are components of the innate immune system as well as host defense factors more specific for the invading pneumococcus such as the scavenger receptor involved with lung defense or the generation of adaptive immunity directed primarily against the capsular polysaccharide.

Capsular polysaccharides. While *S. pneumoniae* isolates free of the capsule do exist the overwhelming majority of clinical strains express one capsular polysaccharide attached to the pneumococcal cell wall. Non-encapsulated strains, for instance, strains R6 or R36A which are most frequently used in laboratory studies on *S. pneumoniae* are completely avirulent in animal models of disease. The role of the capsular polysaccharide in pneumococcal virulence appears to be the inhibition of complement-mediated opsonophagocytosis by macrophages or polymorphonuclear leucocytes (PMN). Except for the capsular polysaccharide type 3, all the other polysaccharide capsules appear to be covalently linked to the pneumococcal cell wall.

Pneumococci have an enormous genetic repertoire to produce – potentially – as many as 95 chemically different polysaccharide chains. A unique feature of the structure of capsular loci is that type specific genes are flanked by common determinants, thus allowing for a relatively easy exchange of genetic determinants within this region of the pneumococcal chromosome. The frequent 'capsular switch' that can appear spontaneously or driven by vaccine pressure among clinical isolates appears to be the consequence of a relatively easy genetic change at the capsular loci. In such cases, isolates that share common genetic backgrounds (defined by multilocus sequence type or pulsed-field gel electrophoresis profile) appear to differ only in the type of capsule at their surface. Whole genome sequencing, however, has revealed other more or less subtle changes in addition to the capsular operon.

Cell walls in virulence. The unique choline component of the wall and membrane teichoic acid appears to perform multiple roles both in the physiology of the pneumococcus and also as an interactive component with the host. The species of *S. pneumoniae* is unique in that it requires as an essential nutrient choline for growth. Recently, pneumococcal strains have been isolated in which this auxotrophic requirement for choline is lost. In one of these isolates point mutations in one of the choline utilization genes – *tacF* – have been identified as the mechanism responsible for the choline independent growth. TacF, a teichoic acid flippase was proposed to catalyze the transfer of teichoic acid chains across the pneumococcal plasma membrane. A second choline independent strain was recovered from a heterologous genetic cross in which *S. oralis*, a bacterial species that contains choline in its teichoic acid but does not require choline for growth, served as DNA donor and the recipient was the R6 strain of *S. pneumoniae*. The mechanism of choline independence in this strain called R6Cho$^-$ appears to be different from the mechanism identified in the other choline independent mutants.

Recent studies have shown that pneumococcal constructs capable of growing without choline and expressing the capsular polysaccharide type 2 on their surface have severely reduced virulence potential in several animal models of pneumococcal disease and are also inhibited from colonizing the nasal epithelium of mice. The mechanism of this striking impact of the teichoic acid choline units on pneumococcal virulence is not understood at the present time.

Pneumococcal cell wall components were shown to induce the production of preinflammatory cytokines both in the murine intraperitoneal models as well as in the rat and rabbit models of meningeal disease. Structural features of the peptidoglycan involved with the recognition by the innate immunity system have been identified in the Drosophila model.

At least two pneumococcal cell wall modifying enzymes: PGDA, a peptidoglycan glucosamine deacetylase and PCE, a phosphoryl choline esterase, were shown to play roles in virulence as indicated by the impact of inactivation of the corresponding genetic determinant on virulence.

Regulation of Virulence

The expression of capsular polysaccharides appears to be regulated: contact with epithelial cells was shown to cause suppression in the amounts of capsule which can subsequently increase once the bacteria have reached the blood stream.

Different modalities of growth. Recent studies have shown that pneumococci can grow in two different modalities: as planktonic cells typical of bacteremic infections and as biofilms typical of colonization. Different sets of genetic determinants are expressed matching these two different growth styles of pneumococci.

Opaque versus transparent phenotype. In 1994, Weiser et al described that pneumococci could undergo phase variation, that is changes in properties of the cell surface could lead to two different colony phenotypes: opaque and transparent. Spontaneous, reversible variation between the two colony phenotypes with a frequency ranging from 10^{-3} to 10^{-6} per generation has been described. This frequency appears to be independent of in vitro growth conditions.

The same authors showed that the opaque phenotype was associated with increase amounts of capsular polysaccharide and pneumococcal surface protein A (PspA) while transparent variants were associated with increased amounts of choline binding protein A (CbpA) and autolysin A (LytA). Opaque colonies were described as dome shaped and large contrasting with transparent colonies which were umbilicated, suffered quicker autolysis and had a higher efficiency of natural transformation. Cultures obtained from opaque colonies had decreased binding of serum C-reactive protein, decreased opsonophagocytosis and increased virulence in systemic infections. By contrast, transparent colonies had increased adherence, and colonized more efficiently in an animal model of carriage.

More recently, DNA microarray strategies have identified an additional number of genes, which appear to have differential regulation between isogenic pairs of strains displaying opacity or transparent phenotypes. In particular, the transparent phenotype was associated with increased production of neuraminidase (NanA).

Studies on the fatty acid composition of pairs of opaque and transparent colonies found a lower degree of unsaturated fatty acids in the opaque variants.

A single large study has attempted to examine the prediction that opaque variants are associated with increased pathogenesis by looking at the colony phenotypes displayed by a large collection of invasive disease isolates with a low number of in vitro passages. This collection of 304 isolates included representatives of 10 serotypes displaying genetically diverse backgrounds (Serrano et al., 2006). The authors confirmed that the opaque phenotype dominated among invasive disease isolates but also noted a previously unreported association between serotype and colony phenotype. This observation led them to suggest that the association between the opaque phenotype with particular serotypes might contribute in part to explain observed differences in the invasive disease potential of certain serotypes.

Contribution of Genotype and Serotype to the 'Invasive Disease Potential'

The introduction of molecular typing techniques for the characterization of *S. pneumoniae* recovered from disease and from colonization sites combined with extensive epidemiological studies has initiated efforts to better define the contribution of serotype versus molecular type to the 'invasive' potential of a pneumococcal strain. The frequent representation of the so-called pediatric serotypes (primarily 6B, 14, 19F and 23F) in the nasopharyngeal flora of children clearly provides an increased opportunity for strains expressing these serotypes to invade during periods of decreased host defense. Thus, assignment of a true 'invasive disease potential' to a strain must take into account the odds ratios expressing the frequency of recovering the particular strain from colonization versus infection sites.

The relative contribution of serotype versus genotype to the invasive disease potential is a currently unsettled issue. Although it is relatively consensual that the capsular type expressed is extremely important for the disease potential of a particular strain, there are studies suggesting that the genetic background of that strain is also important, which, after all, is in agreement with the finding of several genetic determinants essential for full virulence of pneumococci.

A further potential problem contributing to the difficulties of interpretation of these empirical estimates of invasive disease potential of serotypes and clones of pneumococci is the way pneumococcal colonization has been traditionally studied ignoring multiple colonization (discussed above).

The relative contribution of the host versus the pathogen for the occurrence of pneumococcal infection is debatable but clearly the host plays a very important role. Recent studies have started to identify genetic polymorphisms in determinants implicated in the host immune response including some that are associated with susceptibility to invasive pneumococcal disease and others that confer a protective effect to it.

Human Intervention

There have been at least three important interventions by man that radically altered the in vivo landscape and epidemiology of *Streptococcus pneumoniae*: (i) the introduction of antibiotics into therapeutic practice; (ii) the introduction of the conjugate anti-pneumococcal vaccine and (iii) the massive emergence of day care centers in the developed part of the world.

The introduction of antibiotics did not change the incidence of pneumococcal infections; it contributed significantly to the control and outcome of pneumococcal disease but has also led to the emergence of antibiotic resistant strains.

The introduction of multivalent conjugate anti-pneumococcal vaccines in several countries worldwide is having a major impact on the incidence of invasive pneumococcal disease. Although these vaccines prevent colonization by capsular types targeted by them, the rate of pneumococcal carriage does not decrease. Furthermore, as these vaccines target serotypes frequently associated with antibiotic resistance, the frequency of antibiotic resistant strains among colonizing pneumococci has initially decreased (following introduction of these vaccines), but is now bouncing back due to expansion of pneumococcal non-vaccine capsular serotypes also resistant to antibiotics (Simões et al, 2011).

The institution of day care has emerged as a unique epidemiological entity in which many children of preschool age are cohorted. The high carriage rate of respiratory pathogens, immunological status and typical child behavior, together with the frequent occurrence of viral respiratory diseases and the extensive and often imprudent use of antimicrobial agents among this age group are the most likely reasons why attendance at day care centers has become a risk factor for both carriage and infection by antibiotic resistant strains of pneumococci.

Antibiotic Resistance and Insights Provided by Molecular Typing

The first penicillin resistant strain of *S. pneumoniae* that appeared in the clinical environment and invoked comments in the infectious diseases literature was the strain isolated from the throat of a healthy child in the mid 1960's in a remote village, Agunganak in Papa New Guinea. Although initially the possibility of geographic spread of the penicillin resistant pneumococcus was judged remote, this prediction was soon contradicted by the massive outbreak of pneumococcal disease in South African hospitals in 1977, which was caused by multidrug resistant strains of this bacterium. Between the early 1980s and the late 1990s reports on the detection and increase both in frequency and in antibiotic resistance level of drug resistant strains of *S. pneumoniae* have appeared in increasing numbers and the antibiotic resistant pneumococcus has become a global phenomenon that began to make effective chemotherapy sometimes problematic.

Global Spread of a few Pandemic Clones of Penicillin Resistant Pneumococci

Introduction of pneumococcal typing techniques such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) has clearly shown that among the very large number of lineages of resistant pneumococci a handful of highly epidemic clones emerged and achieved massive and often pandemic spread. The most outstanding of these is Spain^{23F} ST81 originally called the 'Spanish/USA clone' (now called 'PMEN-1' clone – for Pneumococcal Molecular Epidemiology Network 1) usually expressing serotype 23F and carrying resistance to penicillin, tetracycline and chloramphenicol and often to erythromycin and sulfamethoxazole-trimethoprim.

An apparent intercontinental transfer of this clone from Southern Europe to the United States was demonstrated. This clone was subsequently identified in numerous national and international surveillance studies, both as a powerful colonizer and also as a strain capable of causing the entire spectrum of pneumococcal diseases among both adults and children.

Similar importation of a multidrug resistant pneumococcal clone, the penicillin resistant clone Spain^{6B} ST90 (now called 'PMEN-2' clone) presumably from Southern Europe to Iceland was demonstrated in the early 1990s. This so-called 'Icelandic' clone expressing serotype 6B and resistance to penicillin, tetracycline, chloramphenicol, erythromycin, sulfamethoxazole-trimethoprim and occasionally to third generation cephalosporins as well, quickly spread in Iceland and within three years of its detection was shown to be responsible for close to 20% of all pneumococcal disease in that country.

A third genetic lineage France^{9V} ST156 originally referred to as the 'French/Spanish' clone carries resistance to penicillin and tetracycline and occasionally to sulfamethoxazole-trimethoprim and typically exists in two capsular serotypes: 9V or 14. This clone (PMEN-3) was shown to have spread in Europe, Latin America, USA, Canada and Asian countries.

The introduction and widespread use of molecular typing techniques has led to the establishment of an international depository and 'clearing' house for characterized *S. pneumoniae* clones the so called Pneumococcal Molecular Epidemiology Network (PMEN, for more information visit www.sph.emory.edu/PMEN/). This platform has become also useful in providing a uniform set of rules to name new clonal lineages, register both their molecular as well as epidemiological characteristics, serotypes, isolation dates and sites and clinical sources as well.

Despite the fact that penicillin resistant strains have been isolated from countries all over the world, their incidence varies widely from country to country and from one geographic site to another.

The impact of antibiotic resistance on chemotherapy varies with the particular infection. Even low-level resistance to antibiotics requires change in chemotherapy in meningitis because of the low penetration of the cerebral spinal fluid by this class of antibiotics and because of the need for bactericidal concentrations. On the other hand, penicillin therapy was shown to remain effective in pneumococcal pneumonia caused by penicillin resistant strains with MIC values as high as 1–2 μg ml^{-1}.

Penicillin Resistance: Genes and Phenotypes

The mechanism of penicillin resistance in pneumococcal clinical isolates is based on the remodeling of several of the genetic determinants – *pbp* genes that encode for proteins PBPs (penicillin binding proteins) that catalyze various stages in the pneumococcal cell wall synthesis. The process of remodeling involves recombinational events with fragments of *pbp* genes imported from heterologous species most often from *S. mitis*. These 'mosaic' *pbp* genes produce PBPs with decreased affinity for penicillin which is the ultimate basis of the increased penicillin MIC values.

Examination of the cell wall chemical structure in penicillin resistant clinical isolates showed additional profound abnormalities in these bacteria, specifically the increased representation of branched muropeptide components in their cell wall peptidoglycan. A genetic follow-up of these studies identified the new determinants *murM* and *murN*, responsible for the biosynthesis and attachment of the two amino acid components that form the muropeptide branches. The *murM* and *murN* genes of resistant strains showed clear evidence of mosaicism, indicating the presence of DNA sequences of heterologous origin. Most interestingly, inactivation of *murM* caused a complete loss of penicillin resistance which could be recovered in appropriate complementation experiments. A detailed biochemical mechanism of the synthesis of pneumococcal muropeptides and the mode of action of MurM and MurN proteins was recently described.

The critical nature of cell wall chemistry for the penicillin resistant phenotype was further documented by the recent identification of yet another cell wall modifying enzyme: a muramic acid O-acetylase. Inactivation of the structural gene named *adr* caused loss of penicillin MIC value, similarly to the case of strains in which MurM was inactivated.

It seems that genetic determinants in addition to the mosaic *pbp* genes producing the low affinity penicillin targets are also essential for optimizing the resistant phenotype. This scenario is reminiscent of the mechanism identified in methicillin resistant *Staphylococcus aureus*, in which high level resistance requires not only the central resistance determinant *mecA*, but a number of additional 'auxiliary determinants' as well in the genetic background of the bacteria.

Genetic Diversity Among Penicillin Resistant and Penicillin Susceptible Pneumococci Causing Invasive Disease

Several studies have compared the genotypes of penicillin resistant (MIC higher than 1 μg ml^{-1}) versus penicillin susceptible isolates of pneumococci expressing the same serotypes and recovered from invasive disease. Studies with strains isolated from children in South American countries are illustrative of the common findings of such studies. The major and striking difference among the resistant and susceptible isolates was the relative genetic homogeneity of most resistant isolates, over 80% of which were shown to belong to one of two pandemic penicillin resistant clones: Spain^{23F}-ST81 and/or France$^{9V/14}$ ST156 - Figure 1. In contrast, penicillin susceptible isolates expressing the same serotypes and recovered from similar disease sites showed great genetic diversity – Figure 2. A similar – inverse – relationship between genetic diversity and penicillin MIC value has already been described in other epidemiological studies. These observations may reflect the expensive 'fitness' cost associated with the mechanism of pneumococcal penicillin resistance which may only be compatible with a limited number of genetic backgrounds available in this bacterial species.

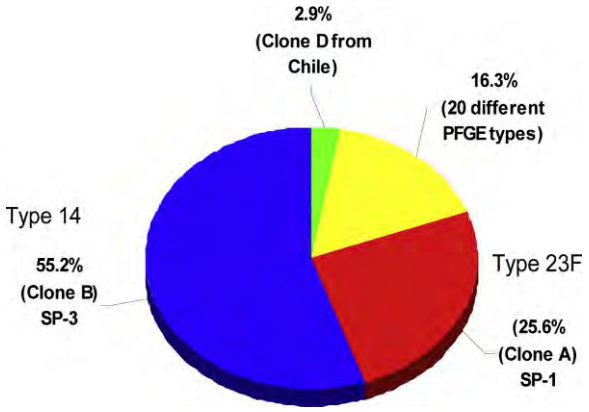

Figure 1 Pie chart showing the isolation percentages in Latin America of two penicillin resistant pandemic clones versus all other clones from among 172 penicillin resistant invasive isolates. Note that the two clone types (A and B) are responsible for ~81% of all pneumococcal infections. Clone A (serotype 23F) refers to the Spain^{23F} ST81 clone and clone B refers to the France^{9V} ST156 clone. Adapted from Tomasz et al. 1998 *Microb Drug Resist* 4:195–207.

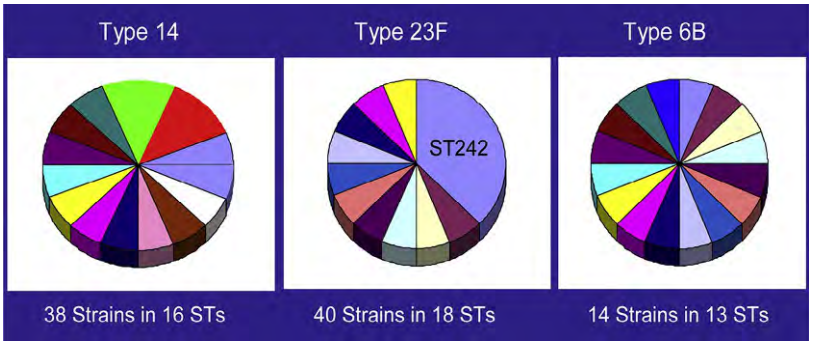

Figure 2 Pie charts (from left to right) showing the large number of different MLST types (ST's) for each of the three most prevalent capsular types (serotypes) 14, 23F and 6B that were associated with clinically invasive penicillin-sensitive pneumococcal disease in Latin America. Figure taken from Zemlickova et al. 2005. *Microb Drug Resist* 11:195–204.

Impact of the Seven-Valent Pneumococcal Conjugate Vaccine on Pneumococcal Disease and Carriage

In 2000, a seven-valent pneumococcal conjugate vaccine (PCV7 targeting serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) was licensed in the US and soon after in many other countries worldwide. The vaccine was intended for children younger than two years old. This vaccine was formulated to target the serotypes that cover over 80% of the cases of invasive pneumococcal disease among children younger than 5 years old in the US. Expected coverage rates in other countries, particularly in developing countries, are lower.

Following introduction of PCV7 in the US, a reduction in incidence of invasive pneumococcal disease (IPD) occurred not only among young children but also in all other age groups due to a substantial indirect herd effect. In particular, a sharp decrease in IPD caused by vaccine types was observed which was accompanied by a modest increase in IPD caused by non-vaccine types. By 2003 the total incidence of IPD among children <5 years of age had declined 75% from 96.7 cases per 100 000 population to 23.9 and among those aged 65 or older a 29% decline was observed from 60.1 cases per 100 000 population to 41.7 cases.

The observed indirect effect of the vaccine is best explained taking into account that the vaccine decreases carriage of vaccine-types among vaccinees and these are the major reservoirs of pneumococci. By vaccinating young children, transmission of vaccine types has decreased substantially. An earlier study from Israel had demonstrated an indirect beneficial effect of PCV7 among children too young to be vaccinated whose siblings had received PCV7. The same studies showed that pneumococcal colonization levels remain stable after vaccination due to a serotype replacement effect that results in the nasopharynx becoming mostly colonized by non-vaccine types.

An added bonus of the vaccine relies on the fact that most antimicrobial-resistant pneumococci isolated worldwide including those non-susceptible to penicillin, and/or resistant to macrolides, or multidrug-resistant express mostly serotypes targeted by PCV7 (i.e. 6B, 9V, 14, 19F, and 23F). In agreement with these observations, a decline in antimicrobial-resistant pneumococci has been observed in the US among isolates causing invasive pneumococcal disease. However, recent reports from the US and elsewhere found that among colonizing isolates as well as those causing respiratory tract infections, levels of antimicrobial resistance among non-vaccine types are increasing suggesting that the impact of PCV7 on antimicrobial resistance may be short lived.

The phenomenon of serotype replacement and the short-lived effect of PCV7 on antimicrobial resistance are also beginning to be noticed in other countries that are using PCV7. For example, a study on the impact of PCV7 on pneumococci carried by Portuguese day-care center attendees observed that serotype replacement has occurred among attendees regardless of their vaccination status

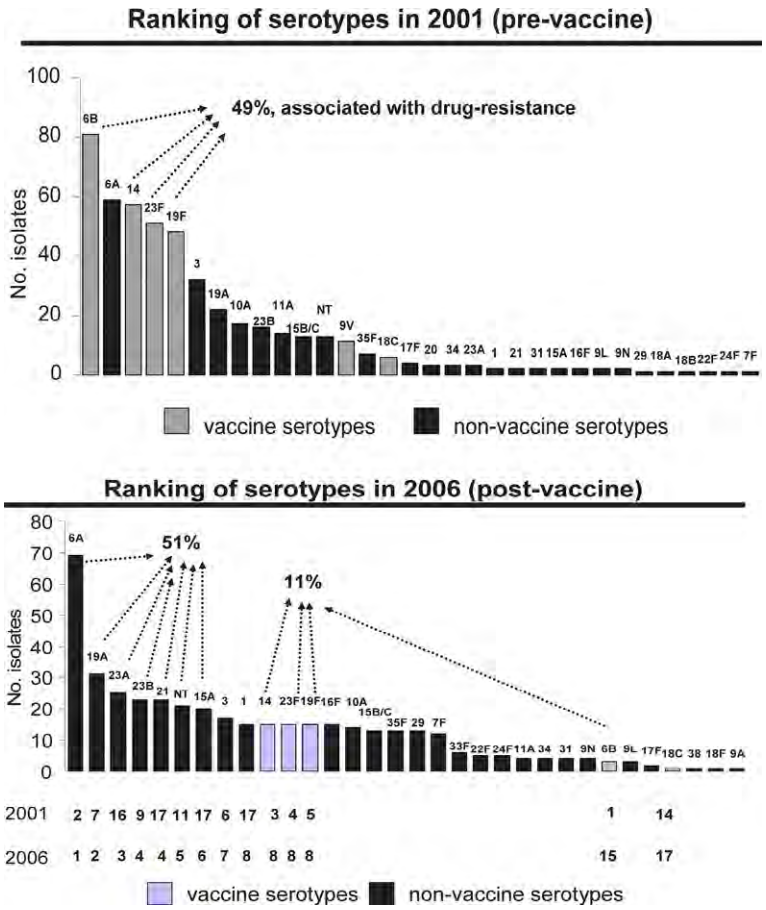

Figure 3 Bar charts showing the number of clinical isolates recovered of each pneumococcal capsule type (serotype) before introduction (2001) of PCV7 (top graph) and after vaccine introduction (2006) in Portugal (bottom graph). All capsular types targeted by the vaccine dropped in recovery and most non-vaccine types increased in abundance. Adapted from Sá-Leão et al., 2008.

(57% of the children had been vaccinated) (Figure 3). In other words, unvaccinated children were benefiting from being in contact with vaccinated children. Interestingly, rates of antibiotic resistance remained unchanged due to a balance between reduction of vaccine types and an increase of antimicrobial resistance among non-vaccine types.

Novel vaccines with greater valency - 10-valent (PCV7 serotypes plus 1, 5, 7F) and 13-valent (PCV10 serotypes plus 3, 6A, and 19A) have now been introduced in the market. These expanded valency vaccines include serotypes which are major causes of IPD in developing countries such as serotypes 1 and 5. The 13-valent conjugate vaccine also targets serotype 19A which, it has become in the last decade an important cause of IPD in some countries such as the US and is often multidrug resistant.

Universal protein vaccines – which should have broad coverage and low price - are currently in different stages of development.

Day-Care Center Studies

Day-care centers, have become increasingly frequent in the last decades in the developed world and their impact on infectious diseases has been discussed. In particular, several studies have focused on pneumococcal carriage among day care center attendees. Molecular epidemiological studies have shown that these 'novel' institutions play a crucial role in the transmission and amplification of pneumococcal lineages. Furthermore, marked differences in pneumococcal carriage rates across communities have been explained by differences in the proportion of children who attend day-care centers in those communities.

Perhaps one of the largest surveillance initiatives of pneumococcal carriage among day-care center attendees has been the one conducted in Lisbon and Oeiras, Portugal. The project began in 1996 and through the years, an ever-growing collection of isolates has been obtained. As of February 2014, over 14 000 nasopharyngeal samples have been collected, resulting in the isolation of over 9500 pneumococci. Approximately 40% of the isolates were resistant to at least one commonly used antimicrobial agent. All drug-resistant isolates have been characterized by capsular type and antimicrobial susceptibility pattern, and several by molecular typing either by pulsed-field gel electrophoresis (PFGE) and/or multilocus sequence typing (MLST). Over 40% of the fully susceptible pneumococci have also been characterized by the same combination of techniques (Sá-Leão and de Lencastre personal

communication). These and other studies have provided insights on the natural biology and population structure of pneumococci, on the dynamics of the carrier state, and on the properties of day-care centers as epidemiological entities.

It was observed, for example, that day-care centers (DCC's) are autonomous epidemiological units, which, at a given time, differ from each other in the pneumococcal population circulating among its attendees, even if the units are geographically close.

A longitudinal study showed that introduction of novel pneumococcal lineages occurred all year long in the DCCs and the fittest lineages were transmitted among attendees for several months resulting in their amplification and providing ample opportunities for their spread in the open community as well.

Portuguese DCC attendees were found to be frequently colonized by pneumococci: in winter months an average of 65% of children were found to be carriers and virtually all children (98%) carried pneumococci at least once during a one-year longitudinal study. It was also shown that the nasopharyngeal flora of DCC attendees was a reservoir of internationally disseminated drug resistant lineages capable of causing the entire spectrum of pneumococcal diseases, such as the successful clones Spain^{23F} ST81, Spain^{6B} ST9, and France^{9V} ST156.

Genetic Exchange in Vivo

Several of the intriguing phenomena first observed during studies of pneumococci in the microbiology laboratory also play important roles in the in vivo environment of this bacterium. DNA mediated genetic transformation appears to be a major mechanism for acquiring antibiotic resistance genes and also for switching capsular type – as observed in epidemiological studies. The release of DNA through the activity of autolytic enzymes also appears to play an important role in the in vivo evolution of pneumococcal lineages.

Competence

Competence is a transitory state in which bacterial cells are able to take up exogenous DNA. This process may lead to genetic transformation if during the competent state there is exogenous DNA available in the environment – particularly if the DNA has high degree of homology to the DNA of the competent recipient cell. Nevertheless, heterologous DNA from other streptococcal species can also serve as 'donor' in recombinational events.

Pneumococcal competence has been studied for over four decades. However, it was during the last decade that major breakthroughs have been made in the elucidation of the molecular mechanisms leading to competence, the genes implicated in the competence regulon, and its role in pneumococci.

As of today most studies in this area are done based on a few well-characterized laboratory strains. Nonetheless, competence appears to be widely disseminated in pneumococci: a study on the distribution of two competence genes among a diverse collection of 214 clinical isolates found that both genes were ubiquitous in the collection.

The competent state is controlled by the early *com* genes *comABCDEWX*. Competence is triggered by accumulating exogenous levels of the 17 amino acids long pheromone CSP (competent stimulating peptide). The pre-CSP is 24 aa long encoded by *comC*, which is cleaved to CSP during export across the cytoplasmic membrane. This process uses a dedicated secretion apparatus coded by *comAB*. Competence is triggered by CSP stimulation of its receptor comD, a membrane-bound histidine kinase which autophosphorylates and transphosphorylates its cognate response regulator comE. ComE binds to a specific site in the DNA activating the early competence genes, including *comABCDE*, leading to further accumulation of CSP, and *comX*. This latter gene encodes a sigma-X factor which activates the transcription of the late competent genes.

Two transcriptomic studies using DNA microarrays have identified over one hundred genes induced by CSP. Ninety-one genes were common to both studies and included 17 early *com* genes and 60 late *com* genes. Of significant interest, it was shown that only 22 genes of the *com* regulon are required for transformation suggesting that the role of competence extends well beyond genetic transformation.

In the past decade it was discovered that competent cells have the capacity to trigger the lysis of genetically identical as well as non-identical non-competent cells. This phenomenon of 'fratricide' predation has been termed allolysis. A bacteriocin system named CibABC (competence induced bacteriocin) consisting of a two-peptide CibAB bacteriocin and its immunity factor CibC has been identified. It was shown that CibAB is absolutely required for allolysis but cannot promote lysis per se. Gene *cibC*, encodes a 65 aa peptide with two predicted transmembrane segment. Evidence suggests that cell-to-cell contact is necessary for allolysis to occur. It was proposed that CibAB may insert into the membrane of noncompetent cells (which lack CibC) and deplete them of energy. Cell lysis of noncompetent cells would then occur as a consequence of the action of three amidases - LytA, LytC, and CbpD.

The observation that allolysis occurs during the transient state of competence (*cibABC*, *lytA* and *cbpD* are late genes of the *com* regulon) is probably intimately related to its biological significance. Current hypotheses on the contribution of allolysis to the biology of pneumococci include not only DNA release for genetic transformation but also a role in colonization and/or virulence, competition between strains and release of virulence factors. The elucidation of the mechanism of competence has recently seen several important advances several of which have been reviewed recently (Johnston et al, 2014).

Bacteriophages of Pneumococci

Pneumococcal bacteriophages of the lytic and temperate types were first described in 1970s. Subsequent work on pneumococcal phages found that the lytic enzymes of many of them shared a high identity (at the protein and DNA level) to the major pneumococcal autolysin (LytA).

The incidence of prophages among a large collection of pneumococcal clinical isolates was high - 76% - and multiple carriage was frequent. The initial observations were based on the fact that multiple bands of SmaI-digested total DNA separated by PFGE hybridized with a *lytA* probe. Further assays on a subset sample of 17 isolates showed that the majority of the isolates tested (11 of 17) contained functional prophages that entered the lytic cycle following mitomycin induction. Some PFGE subtypes of SmaI-digested total DNA could be explained by changes in the number and chromosomal localization of prophages suggesting that prophage carriage could be used as a molecular epidemiological marker (Ramirez et al., 1999).

In recent years, the use of pneumococcal phage lytic enzymes as anti-infective agents has been explored. It was shown that a purified pneumococcal bacteriophage lytic enzyme (Pal) was able to rapidly kill pneumococci of common serotypes including highly penicillin-resistant strains. In an animal model of colonization, pneumococci were eradicated without disturbing the normal flora. Following these initial studies, the therapeutic use of bacteriophage enzymes has been tested with success in different animal models of disease (bacteremia, endocarditis, and otitis media).

More recently, the pneumococcal LytA autolysin was found to be a potent therapeutic agent in experimental peritonitis-sepsis caused by highly beta-lactam-resistant *Streptococcus pneumoniae*.

Genome Sequencing

The first three pneumococcal genomes sequenced were TIGR4 (serotype 4 invasive disease isolate from Norway), R6 (avirulent, non-capsulated derivative of the serotype 2 strain D39), and G54 (serotype 19F derivative of a serotype 15 clone from Spain). All were published in 2001. Since then an increasing number of strains have been sequenced as novel and affordable sequencing technologies and more powerful bioinformatic tools became available, lowering dramatically the costs and time needed for such projects. As of 2014, high quality sequence information is available for numerous *S. pneumoniae* isolates. Full genome sequencing was used to characterize 240 isolates of the PMEN-1 clone – collected from several countries over an extended time period (Croucher et al., 2011). Other genome sequencing projects are ongoing. Among the genome sequences available are representatives of the most successful pandemic drug-resistant clones as well as representatives of susceptible strains that are frequent causes of invasive pneumococcal disease in countries of the developing world. In addition, the capsular operons of all capsular types described to date have been sequenced.

Pan-Genome of *S. pneumoniae*

The size of the pneumococcal genome depends on the strain analyzed. Among strains sequenced so far it is generally in the range of 2.0–2.2 Mb. Evidence obtained up to now supports the hypothesis that pneumococci (as other highly recombinogenic bacteria) contain a supragenome that is much larger than the genome of any individual strain. Each pneumococcus genome contains a core set of genes (common to all pneumococci) and a non-core set of genes that are part of a large pool of genes.

Comparative genomic analyses published so far, used the combined analyses of the genome sequences of numerous strains, to estimate the number of orthologous clusters in the pneumococcal supragenome based on a finite-supragenome model. The model predicted that the pneumococcal supragenome should contain approximately 5100 orthologous clusters of which 27% are core, 41% are unique, and the remaining 32% are distributed (i.e., present in more than one strain but not common to all).

Pneumococcal Cell Wall: Composition, Structure and Mechanisms of Replication During Cell Division

The cell wall is composed in approximately equal proportions of a peptidoglycan and a teichoic acid of unusual chemical composition, the components of which include galactosamine phosphate, 2,4,6-trideoxy-2,4-diaminohexose, ribitol phosphate and phosphocholine. A membrane teichoic acid of identical primary structure is also present at the cell surface.

High resolution chemical analysis of the peptidoglycan performed on many clinical isolates demonstrated that the composition of the pneumococcal peptidoglycan was specific for the species. The muropeptide units of the peptidoglycan of pneumococci are either directly crosslinked to one another or are crosslinked via short dipeptide branches composed of either seryl alanine or alanyl alanine units which are attached to the free amino group of the stempeptide lysine residues. While direct crosslinks are the dominant mode of crosslinking in penicillin susceptible pneumococci, the branched peptides become the prominent mode of crosslinking in penicillin resistant strains.

Genetic determinants of the dipeptide branches have been acquired by pneumococci from an as yet unidentified extra species source. The genetic determinants *murM* and *murN* are essential for the expression of high level penicillin resistance.

The choline residues of the teichoic acid polymers were shown to perform critical and multiple physiological functions in *S. pneumoniae* and most recent studies also identified choline residues as major modulators of pneumococcal virulence in animal models of pneumococcal disease. The presence of choline residues was shown to be essential for a wide range of physiological phenomena such as separation of daughter cells at the end of cell division (LytB), sensitivity of pneumococci and pneumococcal cell walls to degradation by the pneumococcal autolysin (LytA); choline residues were shown to be essential for genetic transformation and the cell wall choline units were shown to serve as non-covalent attachment sites for a group of proteins, the so-called choline binding proteins, which serve multiple functions in the interaction of pneumococci with their eukaryotic hosts. The choline residues are also sites for the attachment of the C-reactive protein and for the large family of myeloma proteins.

Fine Structure of Cell Wall

The shape and size of microbial cells is imprinted in their cell walls which – in the case of *S. pneumoniae* – resembles an American 'football' with diameters of 1.0 – 1.5 micrometer from tip to tip and with a 'waistline' of 0.5 – 0.8 micrometer. The cell wall of *S. pneumoniae* appears in electron microscopic thin sections as a tri-laminar layer directly underneath the somewhat amorphous polysaccharide capsule.

Fine structure studies were done on a 'rough' (non-encapsulated) *S. pneumoniae* strain R6 which is a derivative of strain R36A. The overwhelming majority of laboratory studies that have been done on pneumococci over the past several decades all used either R36A, R6 or some simple mutant derivatives of these two strains. R36A was derived from strain D39 a clinical isolate expressing a serotype 2 capsular polysaccharide on its surface. R36A was selected after 36 serial passages of D39 in medium containing anti-capsular type II antibody. Strain R36A was shown to carry a 7510 base pair deletion involving five of the genetic determinants of the capsular type II genetic locus. The fully sequenced strain R6 is not isogenic with R36A: R6, unlike it's 'parent' strain, carries an inactivating point mutation in the *dlt* gene and it also has a muropeptide composition in which branched peptides typical of penicillin resistant isolates are frequent.

Replication During Cell Division

Similarly to the case of other streptococci, the pneumococcal surface is inherited in a conservative fashion during growth: old hemispheres of the cell surface assembled during the previous cell division are passed on intact to the daughter cells in each subsequent cell generation (Figure 4(a)).

The various polymeric components of the pneumococcal cell wall – the peptidoglycan, teichoic acid and capsular polysaccharide chains – all appear to enter the pneumococcal surface at a single centrally located growth zone. The wall teichoic acid chains are attached by covalent bonds to the peptidoglycan – presumably to the muramic acid C-6 hydroxyl residues which are also the attachment sites for the capsular polysaccharide chains and the recently discovered O-acetyl groups as well. The enzymes catalyzing these three types of reactions competing for the same peptidoglycan attachment sites are not known at the present time, neither is the physiological control of these reactions well understood.

Replication of the cell wall seems to occur through the carefully coordinated functioning of two synthetic systems: one that appears to catalyze a peripheral cell wall synthesis (see P in Figure 4(b)), and a second one which catalyzes septal growth of the cell wall (see S in Figure 4(b)). In a newly born cell the future site of cell division is indicated by a 'raised cell wall band' located at the perfect center of the coccoid shaped cell. The next step in cell division is an apparent splitting of the raised cell wall band into two ring-like structures which proceed to 'move' in a symmetrical fashion to the left and to the right of their initial position. Thus, as cell division progresses, the distance between the two rings increases (see P1, P2, P3 in Figure 4(b)) In parallel with this movement is the activity of the second – septally located – cell wall synthetic system which produces cell wall along the newly developing septum (see S1, S2, S3 in Figure 4(b)).

Studies by Morlot and colleagues indicate that the three bifunctional PBPs of pneumococci (PBP1A, PBP1B and PBP2A) as well as the two monofunctional penicillin binding proteins (PBP2B and PBP2X) are all colocalized to the center of the newly born pneumococcus and seem to remain there during the entire course of cell division. The localization of PBP3, a D-D-carboxypeptidase

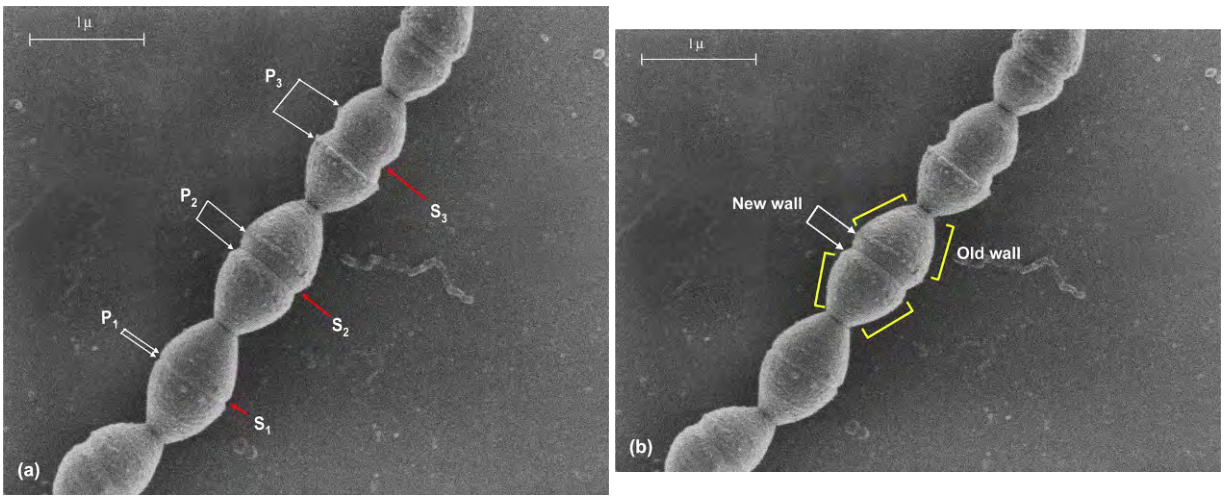

Figure 4 4(a) Symmetrical movement of the cell surface during cell division of *S. pneumoniae*. Scanning electron micrograph of a chain forming pneumococcus shows cells in different stages of cell division. In the cell at the beginning of a division cycle the 'raised cell wall band' is shown just after separation to two ring-like structures catalyzed by enzymes participating in the peripheral cell wall synthesis (P1). Also shown is the beginning of septal wall synthesis (S1). During subsequent stages the distance between the two ring-like structures associated with peripheral wall synthesis (P2 and P3) increases gradually and so does the advance of the septal wall synthesis ring (S2 and S3). 4(b). Conservation of 'old' hemispheres of the cell wall and insertion of 'new' wall material in the vicinity of the new cellular septum.

seems to be unique in that PBP3 is present along the entire pneumococcal surface except the septal zone suggesting an abundance of donor muropeptides available at the site of active cell wall growth. A model to explain the coordination of septal versus peripheral cell wall growth has been proposed recently. The exact functioning and localization of other cell division related proteins possibly involved with cell division such as FtsA, the gene products of DivIVA and the newly described putative murein hydrolase PcsB is not clear at the present time.

Postscript

Writing an 'Encyclopedia' chapter on the Pneumococcus is a risky undertaking in the era of Pubmed and Google search: highly specific and detailed information fitting a reader's particular curiosity is instantly available upon pushing a few buttons on the computer. Also, the number of contributors and contributions to the microbiology of *Streptococcus pneumoniae* has increased substantially since the last edition of the Encyclopedia in the Year 2000. A Pubmed search of all papers with *S. pneumoniae* in their titles and/or abstracts published between January 2000 and January 2014 identified more than 15 000 publications with topics about equally divided between pneumococcal disease, epidemiology and drug resistance plus a number of papers on clinical trials. Even a comprehensive listing of these is clearly impossible.

With these caveats in mind, we shall provide the reader with an admittedly subjective sampling of the more recent activities and publications of the field, a kind of updated 'subject index with a narrative'. Using this treatise the interested reader may then launch his/her more detailed search of the literature.

Acknowledgments

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Further Reading

Suggested Books and Articles

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Streptomyces☆

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Introduction

Streptomyces spp. are Gram-positive bacteria that typically colonize terrestrial and marine soils as free-living saprophytes. In addition, some species also colonize the rhizosphere of plant roots and even plant tissues. They have evolved complex morphological and physiological responses enabling them to adapt to large changes in their environments, for example adjusting to climatic variation or competition from other organisms inhabiting the same niche. In a similar manner to a filamentous fungus, they colonize the particulate environment of the soil by growing branching multigenomic hyphae that form a ramifying network in order to exploit a localized nutrient source. As saprophytes, they are responsible for the breakdown of complex biological polymers and, consequently, for carbon and nitrogen turnover. Being non-motile, they achieve dispersal by means of unigenomic spores borne on specialized non-feeding aerial hyphae (Figure 1). The latter grow out of the semi-aqueous environment inhabited by the feeding vegetative hyphae and ultimately undergo multiple coordinated cell division, generating chains of spores. The spores can then be dispersed by physical agents or the activities of motile animals inhabiting the same niche. The morphological differentiation leading to sporulation is fuelled by cannibalization of parts of the vegetative mycelium, while other parts exhibit physiological differentiation, leading to the synthesis of an elaborate array of secondary metabolites. These complex organic molecules are a significant reason why streptomycetes have achieved their status of industrial microorganisms of major importance. The streptomycetes produce an unparalleled diversity of bioactive secondary metabolites that have been exploited in medicine and agriculture as antibiotics, anti-cancer agents, immunosuppressants and pesticides.

These fascinating attributes have stimulated intensive research worldwide. Moreover, they are not only model systems to investigate microbial differentiation, but are also members of the same taxonomic order (Actinomycetales) as the causative agents of tuberculosis, leprosy and diphtheria (*Mycobacterium tuberculosis*, *M. leprae* and *Corynebacterium diphtheriae*, respectively). Investigating the biology of experimentally more tractable *Streptomyces* can inform critical aspects of the biology of these pathogens. This article reviews many of the fascinating and novel features of streptomycete biology, with particular focus on *Streptomyces coelicolor*. This is considered by many as the model species for which we have a profound although by no means complete understanding, helped in recent years by researchers having access to its annotated genome sequence. Researchers were originally attracted to this species because of its ability to synthesise a striking pigment, later shown to be a mixture of the blue antibiotic actinorhodin (Act) and the red undecylprodigiosin (Red). The annotated genome sequences of several other species have also been completed more recently: notably that of *S. avermitilis*, producer of the potent anthelminthic secondary metabolite avermectin; *S. scabies*, a plant pathogen, *S. clavuligerus*, a β-lactam producer, *S. venezualae*, producer of chloramphenicol, and *S. griseus*, a producer of streptomycin (information on these genomes can be accessed at http://streptomyces.org.uk). These add to an impressive array of different actinomycete genomes that have been fully sequenced and which can be openly accessed. Comparative genomics offers unprecedented insight into both the shared aspects of actinomycete biology, and also the plethora of conditionally adaptive functions that *Streptomyces* in particular possess.

This article reviews many intriguing aspects of streptomycete biology. More detailed coverage of the historical perspectives and recent advances in *Streptomyces* research can be found in two books (Dyson, 2011; Hopwood, 2007); in addition, recent reviews on certain topics are cited in this article.

The Genome

Base Composition

The actinomycetes in general, and *Streptomyces* in particular, have DNA with an unusually high G + C content. For streptomycetes, with 73% G + C, this is close to the theoretical limit for coding conventional proteins. One consequence is that certain AT-rich codons are very rare, which can have implications for gene expression. In particular, there is a biological relevance for expression of genes involved in differentiation: genes necessary for vegetative growth lack the rare TTA leucine codon. Mutation of the cognate tRNA gene, *bldA*, prevents progress from the vegetative stage of the life cycle, blocking aerial development and antibiotic production. This is a consequence of the inability to translate the mRNAs of key developmental genes containing the rare codon.

Host DNA modification enzymes equivalent to the Dam and Dcm methylases of *Escherichia coli* do not appear to have a significant role in *Streptomyces*. Site-specific methylation of adenine or cytosine residues is related to the various restriction-modification systems found in members of the genus. A novel sulfur-containing base modification, due to phosphorothioation of guanine, has been

☆*Change History:* July 2014. P Dyson modified the Abstract, made changes in Sections 'Plasmids'; 'Hyphal Growth and Cell Division'; 'Primary Metabolism'; 'Regulation of Secondary Metabolism'; 'Secondary Metabolism'; and 'Regulation of Secondary Metabolism', and revised Figure 2.

This article is an update of P. Dyson, *Streptomyces*, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 318–332.

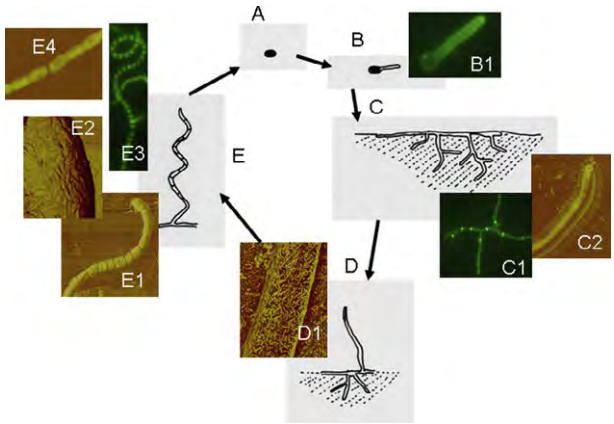

Figure 1 The streptomycete life cycle illustrated by that of *S. coelicolor*. After dispersal, in favorable conditions, a spore (A) can germinate (B). Fluo-vancomycin staining of an emerging germ-tube (B1) reveals that synthesis of new peptidoglycan, and hence cell growth, occurs primarily at the tip. A substrate mycelium grows by tip extension and sub-apical branching (C): fluo-WGA (wheat germ agglutinin) staining reveals infrequent cross-wall formation in the substrate mycelium (C1). Atomic-force microscopy (AFM) reveals that the surface of substrate hyphae is relatively smooth (C2), without the decoration that is characteristic of aerial hyphae. Moreover an extracellular matrix is deposited as the tip grows, allowing the hyphae to adhere to the substrate. Growth of an aerial hypha is away from the nutrient source (D), fuelled in part by cannibalization of the substrate hyphae; antibiotic biosynthesis at this stage in development is believed to protect the nutrients released by breakdown of the substrate mycelium from other competing microorganisms. Young aerial hyphae are decorated with an irregular fibrous layer (AFM image, D1) specified by chaplin and rodlin genes. After growth cessation, the aerial hyphae differentiate to form spore chains (E). This involves highly coordinated multiple septation, revealed by fluo-WGA staining (E3), and by the appearance of regular-spaced spore compartments revealed by AFM (E1). The surface of the spore-compartments consists of a dense layer of well-organized fibers specified by the chaplin and rodlin genes (AFM image, E2). Older spores have characteristic surface concavities (AFM image, E4), indicating dehydration and loss of turgor.

discovered in a few members of the genus, including *S. lividans* and *S. avermitilis*. Subsequent to its discovery in these species, it has also been found in the DNA of several other non-streptomycete bacteria from diverse habitats and appears to be specified as part of a genetic island that has spread between these species. The biological significance of this modification is at present unknown.

The Chromosome

Streptomyces spp. characteristically possess a single large linear chromosome. The linear topology is unusual even among the actinomycetes, and is discussed in more detail below. The chromosome of *S. coelicolor* is 8.7 Mb, that of *S. avermitilis* 9.0 Mb, and *S. scabies* 10.1 Mb in size. The origin of chromosomal replication, *oriC*, and linked *dnaA* gene, are located in a central position. The coding density is largely uniform across the chromosome, with only a slight decrease in the distal regions, or chromosome arms. A central 'core,' comprising approximately half the total size of the chromosome, contains nearly all the genes that are likely to be unconditionally essential for growth. These include genes for cell division, DNA replication, transcription, translation and amino acid biosynthesis. This core region shares considerable synteny with the whole (circular) genomes of other actinomycetes such as those of *M. tuberculosis* (4.4 Mb) and *C. diphtheriae* (2.5 Mb), suggesting a common ancestry of primary cellular functions among actinomycetes. In contrast, the chromosome arms of *Streptomyces* genomes, consisting largely of 'conditionally adaptive' loci coding for probable non-essential functions such as an extensive array of hydrolytic enzymes and, to some extent, secondary metabolism, have either been acquired subsequent to divergence of *Streptomyces* from a common ancestor, or have been lost in actinomycetes with more specialized lifestyles. Closer examination of elements within the arms is consistent with an acquisition model, with evidence of integration of mobile DNA (transposable elements and plasmids) in these regions. In addition, corresponding genes shared between streptomycete genomes tend to occupy the core, whereas the arms contain non-orthologous genes, again consistent with gene acquisition, with the arms a potential 'playground' for evolution of novel attributes permitting a particular species to compete in its ecological niche.

The two ends of the chromosome consist of terminal inverted repeats (TIRs) that, depending on the species, can vary from less than 1 kb to more than 550 kb in size. A 170-bp region at the extremity of each TIR contains an array of tightly organized palindromic sequences that could permit the adoption of secondary structure in a single-stranded molecule. While this may have functional significance, in double-stranded DNA these palindromes are bound by a Telomere-associated protein (Tap) that is necessary for recruitment of Terminal Protein (TP), forming a complex needed for 'end-patching': limited DNA synthesis that completes chromosome replication.

Chromosome circularity is the norm among prokaryotes and it avoids the problem of replicating the ends of a linear molecule. This arises as DNA polymerases can extend a new DNA strand only in the 5′ to 3′ direction by adding nucleotides one at a time to an existing 3′ –OH end. This end is provided by a short RNA primer that is subsequently removed, leaving a gap that is then filled in. Gap filling is primed from the 3′-end of an adjacent DNA strand. The problem with linear molecules is that an RNA primer cannot be synthesized opposite the extreme 3′-end of the template strand, and, even if a suitable complementary RNA primer could be synthesized from an internal template and then base-pair with the 3′–end, its subsequent removal would leave a gap which could not be filled as there is no priming strand. To account for this, small linear genomes such as that of *Bacillus* phage Φ29 and adenoviruses initiate replication from either end using a TP bound to their free 5′-ends to prime synthesis. *Streptomyces* combine typical prokaryotic bidirectional chromosomal replication initiated from *oriC*, together with end-patching initiated from their TPs.

With a virtual absence of essential cellular functions coded by the chromosome arms, these extremities can in certain circumstances be dispensed with. Survival of spontaneous mutants with extensive deletions of terminal sequences may be a particular feature of laboratory grown cultures nurtured in a non-hostile environment. Progressively large deletions (hundreds of kilobases) extend into the arms from the ends. In a well-documented example, approximately 0.5% of spores of *S. lividans* give rise to variants lacking one chromosomal end, including a gene conferring resistance to the antibiotic chloramphenicol. This instability may be due to problems of orchestrating segregation of chromosomes when a multigenomic aerial hypha differentiates into a chain of unigenomic spores. The frequency of this genetic instability in the wild-type can be enhanced to 20% in *ftsK* mutants, probably due to incomplete segregation of chromosomes during sporulation septation: replication-terminal sequences may be trapped by in-growing septa in a compartment adjacent to that containing the chromosome core (see Sections 'Plasmids' and 'Hyphal Growth and Cell Division' for accounts of FtsK function and cell division, respectively). Initial spontaneous mutants also exhibit enhanced instability, possibly due to loss of *cis*-acting terminal sequences that are necessary for correct chromosome segregation, and up to 25% of segregants have an even larger chromosomal deletion spanning the *argG* gene (one of the few essential genes located in the arms). The deletion end-point is often a 5.7-kb tandemly amplified DNA element that exists as a duplicated element in the progenitor genome. In unstable mutants, this element exists as several hundred copies, maintained in the absence of any overt selection pressure, perhaps buffering any further deletions that could affect the integrity of the shortened chromosome. The relatively high instability of genes in the chromosome arms could provide a selection pressure to maintain essential genes within the core region, and, at the same time, contribute to rapid evolution of new functions encoded by the arms.

Plasmids

Scrutiny of *Streptomyces* genome sequences indicates that plasmids have played an important role in chromosome evolution, especially of the arms, with evidence of past integration events. In addition, there is a body of experimental evidence pointing to dynamic interactions between autonomous plasmids and the chromosome. *Streptomyces* plasmids are unusually diverse entities: they differ in terms of size, copy-number, topology and autonomy. There are small, covalently closed circular (ccc) high copy-number plasmids, larger ccc low copy-number plasmids, ccc plasmids that arise by reversible site-specific recombination from the chromosome, and linear plasmids that share features of the chromosome: a centrally-located origin of replication and TIRs with bound TPs to prime end-patching. A property common to maybe all is their self-transmissibility and ability to mobilize chromosomal DNA, making them supremely important in promoting genetic exchange and streptomycete evolution. The frequency of plasmid transfer between strains can approach 100%, and is apparent in laboratory conditions by the formation of 'pocks' on a lawn of recipient bacteria. These pocks are zones of (so far unexplained) retarded development surrounding the primary site of plasmid transfer: the retarded development is a consequence of intrahyphal spread of copies of the newly transferred plasmid.

The conjugation mechanism is quite unlike that of enteric bacteria such as *E. coli*. For the latter, conjugation depends on the function of more than 30 genes (e.g., the transfer region of the F-plasmid). In contrast, a single *tra* gene is responsible for the transfer of circular streptomycete plasmids (e.g., pIJ101, pSN22, pSG5, SCP2 and pSAM2). In addition, 'spread' genes (*spd*) promote subsequent intrahyphal spread, leading to the appearance of pocks. The F-plasmid *E. coli* paradigm involves transfer of a single DNA strand from donor to recipient after the two strains have been temporarily united via the sex-pilus, processes specified by the more than 30 F-plasmid *tra* genes. In *Streptomyces*, plasmid transfer is likely to be initiated by the fusion of donor and recipient hyphae growing together, perhaps promoted by plasmid functions: certain plasmids, such as the linear SLP2 of *S. lividans*, encode a muramidase-like function that could remodel the peptidoglycan of the fusing hyphae. For other plasmids this may be unnecessary: the TraB protein of pSG5 localizes to growing hyphal tips, where peptidoglycan is constantly being remodeled as a part of the growth process of hyphal extension (Section 'Cell Biology'). The streptomycete plasmid TraB proteins belong to the same family as FtsK, first characterized in *E. coli* as being involved in chromosomal DNA segregation at cell division. FtsK is assembled as a multimeric ring with a hole as an integral part of the septum formed during cytokinesis. Two DNA double helices are accommodated in the hole as FtsK pumps a looped circular double-stranded chromosomal DNA molecule between compartments in an ATP-dependent manner. The emerging evidence is that *Streptomyces* plasmid TraB proteins also hydrolyse ATP as they transfer double-stranded DNA between hyphae. Similar to FtsK, the TraB proteins also have a membrane-spanning domain that could promote the fusion of membranes necessary for interhyphal DNA transfer. The purified TraB protein of pSVH1 binds non-covalently, probably as multimers, to 8 bp repeats of a specific plasmid sequence, *clt*: this binding presumably permits specific transfer of the plasmid, but there is no DNA processing (e.g., nicking) during conjugal transfer as is necessary for single-stranded F-plasmid transfer between *E. coli* strains.

The mobilization of chromosomal DNA during the transfer of plasmids is thought to depend on a given TraB protein recognizing 8 bp sequences in the chromosome that are similar to the respective plasmid *clt* sequences: bioinformatic analysis indicates 23 regions containing repeats of the *clt* sequence recognized TraB encoded by pSVH1 in the *S. coelicolor* chromosome.

Cell Biology

Hyphal Growth and Cell Division

Streptomyces grow by hyphal tip extension and sub-apical branching (Figure 1; reviewed by Flardh, 2003). Most *de novo* cell wall synthesis is at the tips, rather than by insertion of new murein into the lateral walls. Cross-wall formation in the feeding substrate

hyphae is relatively infrequent and a sub-apical cell separated from the tip compartment by such a cross-wall can only grow once it has created a new tip by lateral branching. Tip growth contrasts markedly to the paradigm in rod-shaped bacteria such as *E. coli* and *B. subtilis*, which grow by intercalation of new murein into lateral walls, not into the cell wall surrounding the poles: the cell wall material of polar caps is inert after its initial synthesis during cell division. Apical extension in *S. coelicolor* involves assembly of a multiprotein 'tip-organizing center' involving Scy (*Streptomyces* cytoskeletal element), a unique bacterial coiled-coil protein with an unusual repeat periodicity, and DivIVA. The latter is essential for growth and its over-expression in preformed hyphae induces multiple branch-like lateral outgrowths, consistent with a role in stimulating tip growth. Interestingly, rod-shaped actinomycetes such as corynebacteria grow by apical extension at both poles, suggesting that apical growth may be conserved among members of the taxon. Again, in these bacteria, DivIVA localizes to the poles and is implicated in control of apical growth. In *S. coelicolor*, another cytoskeletal coiled-coil protein, FilP, also interacts with both Scy and DivIVA. FilP forms filaments along the hyphae and is considered to organize cell shape. Tip growth is regulated by a serine/threonine kinase, AfsK, that phosphorylates DivIVA causing it to disassemble form the tip-organizing center. This same kinase is also involved in the regulation of antibiotic production (Section 'Regulation of Secondary Metabolism').

In a non-actinomycete such as *B. subtilis*, DivIVA localizes to the poles but has an entirely different function in that it sequesters two proteins, MinC and MinD, which function as cell division inhibitors. Largely due to the localisation of these proteins to the pole regions, vegetative septation is confined to the mid-cell in *Bacillus*. These cell division inhibitors and their mode of action appear to be ubiquitous among bacteria, with a notable exception being the actinomycetes that lack a bona fide Min system. This introduces the intriguing question of how septa are positioned in actinomycetes in general and in *Streptomyces* in particular, in which different mechanisms may function in the substrate and aerial hyphae. A typical prokaryote mechanism for septum formation in *Streptomyces* (and other actinomycetes) is apparent from conservation of both the tubulin homologue FtsZ, which polymerises into the cytokinetic Z-ring, and several membrane proteins involved in linking the Z-ring to synthesis of septal peptidoglycan. A single *ftsZ* gene is required for two types of septation in *S. coelicolor*: infrequent cross wall formation that separates non-detached syncytial cells in the vegetative mycelium, and the multiple synchronous septation of the apical compartments of aerial hyphae that leads to the formation of unigenomic spores that ultimately separate from each other (Figure 1, panels C1 and E3). These developmentally distinct types of cell division are in part a consequence of how the *ftsZ* gene is regulated: there are two promoters, one of which is specifically activated in the aerial hyphae. In addition, the isolation of a missense mutation in *ftsZ* that preferentially affects sporulation septation indicates that there are differences in Z-ring assembly between vegetative and sporogenic hyphae. The synchronous formation of regularly spaced septa in the latter is particularly intriguing: not only is there an absence of the Min system to guide Z-ring formation, but also nucleoid occlusion, which affects the formation of Z-rings in *E. coli* and vegetative cells of *B. subtilis*, appears to have little influence in *Streptomyces*. The early stages of sporulation septation in *Streptomyces* often occur over non-segregated chromosomes, and separation of nucleoids is not observed until septal constriction has started. Emerging evidence points to novel ways by which cytokinesis in the aerial hyphae is regulated. A new family of SsgA-like proteins (SALPs), unique to sporulating actinomycetes, plays an important role in coordinating sporulation-specific cell division after growth arrest of the aerial hyphae. *S. coelicolor* possesses seven different SALPs, of which four are common to other streptomycetes. Unusually, these proteins have no motifs resembling those of known proteins. Each SALP is believed to have a specific role in the sequence of events from septum site selection to eventual spore separation, influencing peptidoglycan remodeling at the various stages, perhaps by interaction with penicillin binding proteins and autolysins. For example, SsgG is required for regular septum formation while chromosomes are being segregated into individual pre-spore compartments.

During hyphal growth, replication of the chromosome occurs asynchronously in many compartments. The highest replication activity occurs in the apical compartments of the presporogenic aerial hyphae. Orchestrating DNA segregation during sporulation appears to be an active process involving FtsK (Section 'The Chromosome'), ParA, a cytoskeletal protein that forms ATP-dependent polymers, and ParB, a DNA-binding protein. The latter binds to a set of 20 *parS* sites within 200 kb of the *S. coelicolor oriC* to form a massive nucleoprotein complex. These complexes are regularly spaced along the aerial hyphae immediately prior to multiple septum formation, indicating a role in accurate partitioning of chromosomes into eventual spore compartments. Aberrant DNA segregation is apparent in more than 10% of the spores of *S. coelicolor par* mutants. ATP-dependent polymerization of the cytoskeletal protein, ParA, is required to position the newly replicated origin-proximal region of the chromosome via interaction with ParB complexes assembled on *parS* sites. This positioning helps to ensure the accurate and synchronized segregation a copy of the chromosome into individual prespore compartments. Another important player in this active segregation of DNA is the actin-like protein MreB that is localized to the inner face of developing spore walls. Among actinomycetes, *mreB* occurs only in genera that grow sporulating aerial hyphae and is specifically required for formation of normal spores and DNA segregation in *S. coelicolor*. The same protein also has a role in DNA segregation in some other non-actinomycetes such as *B. subtilis* and *Caulobacter crescentus*, which also divide to produce daughter cells with different developmental prospects, but not in *E. coli*, where all daughter cells are equivalent.

ParB chromosome complexes are also observed in the tips of the vegetative hyphae, suggesting that active partitioning may be important for DNA segregation into newly formed hyphal branches. Moreover, the tip-organizing center protein Scy recruits ParA to the hyphal tips and regulates ParA polymerization. However, no abnormalities in vegetative growth of *S. coelicolor par* mutants have been observed, perhaps indicating that in the absence of an active partitioning mechanism a passive process can substitute, especially given the relatively infrequent formation of cross-walls in the feeding hyphae. This is itself in part due to the low level of *ftsZ* expression. Frequent septation in sub-apical compartments of aerial hyphae is inhibited by expression of a small membrane protein, CrgA, that has a predicted 'hairpin' topology and functions to prevent Z-ring formation. *crgA* homologues

are ubiquitous but unique to the genomes of actinomycetes (sporulating and non-sporulating), where the gene is located in a conserved morphogenetic gene cluster. During aerial hyphal growth, when *ftsZ* expression (and possibly expression of other cell division genes) is developmentally up-regulated, Z-ring formation extends into sub-apical compartments of the aerial hyphae of a *S. coelicolor crgA* mutant. Conversely, when *crgA* is ectopically expressed in the aerial hyphae, Z-ring formation is inhibited and FtsZ is degraded: the resulting hyphae remain syncitial and are unable to produce spores. The conserved *crgA* gene cluster contains an essential *rodA* gene, probably involved in controlling hyphal extension. It is notable that while many cell division genes are essential in unicellular bacteria, they are dispensable in *Streptomyces* (although cell division mutants are often defective in sporulation). As a consequence, streptomycetes are attractive model systems to dissect the actinomycete cell division apparatus in more detail.

Morphological Differentiation

The erection of spore-bearing aerial hyphae is a way for non-motile streptomycetes to meet the challenges of localized nutrient depletion or other types of deterioration of their immediate environment: the spores can be dispersed to more favorable locations (reviewed by Flardh & Buttner, 2009). The aerial hyphae are quite distinct from the substrate feeding hyphae: they grow away from any remaining nutrient source and whereas the surface of substrate hyphae is smooth and hydrophilic, the aerial hyphae are coated with a hydrophobic fibrous layer (Figure 1, panels D1 and E2). Elaboration of this layer is believed to help the hyphae breach surface tension at the aqueous-air interface, presumably repelling the hyphae from growing back into an aqueous phase once they have emerged into an air pocket in the soil, and provide the aerial filaments a measure of resistance to desiccation in the gaseous phase. In *S. coelicolor* at least two types of hydrophobic surface-active proteins contribute to formation of the layer: the chaplins consist of eight members, ChpA-H, that self-assemble into amyloid fibrils, and the rodlin proteins, RdlA and RdlB, are believed to organize chaplin fibrils into a stable rodlet layer. Recent atomic force microscopy studies have documented changes to the cell surface as aerial hyphae develop. ChpD-H are short proteins made up of a single chaplin domain but with no anchor to the cell wall. These form unstable fibers coating young aerial hyphae and are continuously shed as the hyphae grow. A more stable layer, believed to be comprised of fibers of the longer chaplins, ChpA-C, which have cell-wall anchors, and organized by the Rdl proteins, is apparent on the surface of older hyphae. This layer is transiently disrupted and then reformed during cell division and the formation of spore chains. Rodlin genes have been identified in a number of different *Streptomyces* species but are absent in *S. avermitilis* that produces longer fibers that coat its aerial hyphae, whereas its spores are relatively smooth. The chaplin genes are also characteristic of sporulating actinomycetes, but not non-filamentous species such as *M. tuberculosis*.

Streptomyces spp. employ another strategy to ensure the release of nascent aerial hyphae into the gaseous phase by synthesizing and secreting surfactant molecules. *S. coelicolor*, for example, produces the surfactant peptide SapB during growth and development on glucose-containing rich medium in the laboratory (although not when grown on other media). SapB is a lantibiotic-like peptide that can restore the ability of 'bald' mutants, blocked in the ability to initiate sporulation, to form aerial hyphae. It is derived from the *ramS* gene, itself part of the *ram* gene cluster. A 42-amino acid peptide product of translation of *ramS* is modified by dehydration and thioether formation, probably by RamC, which is similar to other lantibiotic modification enzymes, to generate preSapB. The *ramAB* genes, which encode components of an ABC transporter, are required for export of this molecule, during which the pre-leader sequence is removed to yield mature SapB. Expression of the *ramCSAB* operon is controlled by a response regulator, RamR, the product of the divergently transcribed *ramR* gene. Similar lantibiotic-like surfactant molecules are produced by other *Streptomyces* species.

A surprising revelation of actinomycete genome sequencing projects has been that many species inhabiting the soil possess gas vesicle (*gvp*) genes. These organelles have been studied extensively in planktonic cyanobacteria and halophilic archaea where they function to enable these bacteria to position themselves optimally in the water column. Although homologues of the eight essential *gvp* genes occur in duplicate in certain *Streptomyces* genomes (e.g., in *S. coelicolor* and *S. scabies*), or even triplicate in *S. avermitilis*, there is no evidence to suggest a similar function in actinomycetes; indeed typical gas vesicles have not been detected in these bacteria. A possibly significant difference in structure and function may be attributable to the major envelope protein GvpA of actinomycetes that has a long C-terminal extension. It has been suggested that a novel role of the gas vesicle proteins in *Streptomyces* may be to aid the aerial hyphae in breaking through the water-air interface in certain conditions, based on the 'normal' activity of GvpA in creating a stable gas-water interface. Alternatively, novel actinomycete gas vesicles may function as turgor sensors. Maintaining turgor pressure may be vital for the erection of aerial hyphae; a *S. coelicolor* double *gvp* mutant is compromised in its ability to grow aerial hyphae in high salt conditions. Much has yet to be learnt about the function of these genes in actinomycetes as there does not appear to be a correlation between their presence and the life-style of the organism: they are present in the unicellular animal pathogen *Rhodococcus equi* but not in the filamentous saprophyte *Thermobifida fusca*, nor in unicellular pathogens such as *M. tuberculosis* and *C. diphtheriae*.

Metabolism

Primary Metabolism

Soil nutrient levels tend to be carbon-rich but nitrogen- and phosphate-poor, reflecting that the principle nutrient input is plant matter. The variety and multiplicity of carbohydrate catabolic pathways in streptomycetes reflects the diversity of soil-derived

carbohydrates. In their natural environment, streptomycetes feed by secreting complexes of hydrolytic enzymes. *S. coelicolor* has genes for 60 proteases/peptidases, 13 chitinases/chitosanases, 8 cellulases/endoglucanases, 3 amylases and 2 pectate lyases. Often, induction of a hydrolytic enzyme complex is coordinated by an inducer that is unrelated to the substrate or product of any particular enzyme in the complex; for example, a product of xylan catabolism can induce cellulase production. The converse to induction is carbon catabolite repression whereby specific metabolic processes are down-regulated, exemplified by glucose repression, in which the readily utilized glucose represses synthesis of enzymes needed to break down harder-to-use carbon sources. This is of particular interest in *Streptomyces* as it not only affects the utilization of various carbon sources, but also the synthesis of secondary metabolites (see Section 'Secondary Metabolism'). The mechanisms of glucose repression are different from the paradigm in enteric bacteria. For example, an ATP-dependent glucose kinase activity is required for glucose repression of many genes, and it has been suggested that glucose catabolites (e.g., fructose 1,6-biphosphate) mediate this effect. Glucose repression of other genes, for example, *chi63* encoding a chitinase in *S. coelicolor*, is independent of glucose kinase activity.

In *Streptomyces*, many steps in the glycolytic, pentose phosphate and TCA cycle pathways are represented by two or more forms, isoenzymes, possibly under differential regulation dependent on temporal and spatial cues and the nature of the primary carbon source. Most analysis has focussed on glucose metabolism as this is the common carbon source used in antibiotic fermentations. The Embden-Meyerhof pathway is the most important glycolytic pathway; it is used to generate ATP and also provide precursors for secondary metabolism. It is regulated primarily at the level of phosphofructokinase; in *S. coelicolor* there are three phosphofructokinase isoenzymes, although only one has a major role in glycolysis. The function of the other two isoenzymes is as yet unclear.

Given the variability of their natural habitat, which can include periods of flooding, it is perhaps not surprising that *Streptomyces* spp. can adapt to growth in anoxic conditions. Indeed, in standing liquid culture they form floating, differentiating colonies (although flotation is not dependent on functional *gvp* genes). In these conditions a steep oxygen gradient can result so that at a depth of approximately 1 mm the hyphae are growing under near anaerobic conditions. The genome sequence of *S. coelicolor* contains three operons that putatively each encode a typical four-subunit respiratory nitrate reductase. This protein is involved in anaerobic metabolism, indicating that *S. coelicolor* possesses enzymes to accommodate metabolism under anoxic or microaerobic conditions, as do pathogenic actinomycetes such as corynebacteria and mycobacteria. There is also evidence to indicate that *Streptomyces* spp. are capable of a mixed-acid type of fermentation in oxygen-limiting conditions: they have had to evolve a broad range of metabolic pathways to deliver sufficient energy to maintain the membrane potential, or to sustain growth, when oxygen gets depleted.

The soil is typically a nitrogen-poor environment and consequently many nitrogen catabolic pathways are constitutively expressed. In addition, there are few examples of feedback repression of amino acid biosynthesis, reflecting the nutrient-poor nature of soil. Amino acids are key precursors for the biosynthesis of many secondary metabolites and in some cases a duplicate set of biosynthetic genes is expressed specifically to provide substrates for secondary metabolism. An example is a second partial set of tryptophan biosynthetic genes located as part of the gene cluster for synthesis of calcium-dependent antibiotic (CDA) in *S. coelicolor*. In virtually all bacteria glutamine synthetase (GS) is a key enzyme in nitrogen metabolism and has dual functions in L-glutamine biosynthesis and nitrogen assimilation. In the biosynthetic reaction, glutamine synthetase catalyzes the ATP-dependent formation of L-glutamine from L-glutamic acid and ammonia. Assimilation of ammonium into cellular metabolism is governed by glutamine synthetase in concert with glutamate synthase in the GS/GOGAT pathway, which is ubiquitous in bacteria, yielding L-glutamine and L-glutamate, the key nitrogen donors for biosynthetic reactions. *S. coelicolor* possesses two types of glutamine synthetase: a prokaryotic GSI-beta-subtype enzyme (encoded by *glnA*) and a eukaryote-like glutamine synthetase II (encoded by *glnII*). This appears to be the case for other streptomycetes, but not mycobacteria. In *S. coelicolor* GSI and GSII are differentially regulated. In conditions of nitrogen limitation, *glnA* is constitutively expressed throughout the developmental cycle, whereas *glnII* transcription increases with the onset of morphological differentiation, and significant GSII activity is only detected in morphologically differentiating cells. Two unique OmpR-like regulators with disparate functional properties (GlnR and GlnRII) are involved in transcriptional control of GSI, GSII, and other nitrogen metabolism genes. GlnR is an activator of *glnA* transcription, whereas GlnRII may modulate expression of both genes (GlnR is also directly involved in regulation of antibiotic production; Section 'Regulation of Secondary Metabolism'). In nitrogen-rich conditions, GSI is post-translationally controlled via modification by an adenylyltransferase homologous to GlnE from enteric bacteria. However, whereas this post-translational control in enterobacteria is dependent on components of the nitrogen-sensing Ntr system, in particular GlnK and GlnD, the orthologous proteins in *S. coelicolor*, exert no regulatory influence on GSI. Although the roles of GlnK and GlnD in the latter remain to be elucidated, it is noteworthy that in *S. coelicolor* GlnK is post-translationally adenylated by GlnD. In enterobacteria, GlnD modifies GlnK by uridylylation. Low GSII activity in *S. coelicolor* is in contrast to GSII activity in *S. viridochromogenes*, in which it constitutes up to 30% of the overall activity. This active GSII in *S. viridochromogenes* may have a special function, as this species produces the antibiotic phosphinothricin-tripeptide (PTT), an inhibitor of type I and type II glutamine synthetases. The PTT biosynthesis cluster includes the *pat* gene encoding an acetyltransferase, which inactivates the antibiotic, but additional resistance mechanisms may be advantageous to the producer. Resistance against PTT can be achieved by elevated levels of GS, which protect bacteria from the action of PTT: the increased activity of GSII in *S. viridochromogenes* may be part of the self-protection mechanism against PTT. It is thought that isoenzymes of primary metabolic enzymes are very often involved in antibiotic autoimmunity.

The importance of understanding nitrogen regulation in *Streptomyces* in more detail is underlined by the general repressive affects on antibiotic biosynthesis of having high ammonium concentrations in the growth medium. High inorganic phosphate (P_i) also negatively impacts on antibiotic biosynthesis: indeed nutritional limitation of P_i triggers antibiotic production, a condition that is

likely to equate to the soil environment. Our understanding of the physiological response to P_i limitation points to a critical role of intracellular ATP/ADP ratios. Phosphate metabolism is controlled by elements of the *pho* regulon, itself regulated by a two-component signal transduction system, PhoR-PhoP, that functions to promote adaptation to low P_i by increasing phosphate uptake and/or utilization of cellular polyphosphate reserves. The latter is mediated by polyphosphate kinase (PPK): expression of the *ppk* gene is up-regulated by the PhoP response regulator in P_i-limiting conditions. When the ATP/ADP ratio is high (P_i-sufficient conditions), PPK catalyzes the reversible polymerisation of the γ phosphate of ATP into polyphosphate. ATP is regenerated from polyphosphate by PPK when the ATP/ADP ratio falls. It has been suggested that in the absence or depletion of polyphosphate, central carbon metabolism is up-regulated in response to a low ATP/ADP ratio as another way to generate ATP: as this may coincide with the end of exponential growth, the products (reduced cofactors, ATP, carbon catabolites) will not be consumed in general anabolic reactions but can instead be utilized for the biosynthesis of secondary metabolites. The PhoR-PhoP system also regulates nitrogen assimilation as phosphorylated PhoP represses expression of *glnR* (PhoP also regulates antibiotic production; Section 'Regulation of Secondary Metabolism').

Secondary Metabolism

Secondary or specialized metabolites are defined as being produced by narrow taxonomic groups of organisms. They are typically non-essential for the producing organism and produced after the main phase of growth (although some are continuously produced during growth, for example chloramphenicol by *S. venezualae*). They have diverse, unusual and often complex chemical structures. Usually, production of a given secondary metabolite is determined by a set of genes clustered together, arranged as a number of operons and single genes; these gene clusters range from a few to over 100 kb. The range of biological activities of streptomycete secondary metabolites is broad: some have inhibitory or microcidal activities against microorganisms (i.e., true antibiotics), others are toxic against metazoans, others have a hormone-like role in microbial differentiation, whereas some have a role in metal transport (blurring the distinction between primary and secondary metabolism). There are also many secondary metabolites with either no known biological activity or an activity demonstrated in the laboratory or clinic that is probably incidental to their function in the environment (e.g., vertebrate immunomodulators). The actinomycetes produce the greatest diversity, about two-thirds, of secondary metabolites, and *Streptomyces* spp. account for more than 70% of the actinomycete compounds discovered to date: consequently they are undeniably model systems to understand this aspect of biology.

To some extent the building blocks for assembling secondary metabolites are the products of primary metabolism (e.g., acetyl CoA, some amino acids). As noted above, however, with the example of the second *trpCDGE* operon located within the *cda* cluster, dedicated isoenzymes are also utilized to produce specific precursors. *S. coelicolor* possesses several *fabH* paralogs. One of these is essential and is part of the main fatty acid biosynthetic operon, coding for a dedicated ketosynthase required for the first step in fatty acid synthesis (primary metabolism). Two of the other *fabH* paralogs are in gene clusters for known secondary metabolites: the *red* and *cda* clusters. These two clusters determine production of antibiotics with fatty acid components: the presence of *fabH* homologues in these clusters indicates that rather than 'borrowing' the product of the ketosynthase from primary metabolism, each antibiotic gene cluster specifies a ketosynthase dedicated to production of the respective secondary metabolite.

The genome sequence of *S. coelicolor* has revealed 23 clusters of genes, representing about 4.5% of the genome, predicted to encode biosynthetic enzymes for secondary metabolites, only six of which had been identified pre-sequencing. The genome of *S. avermitilis*, previously known to produce melanin, oligomycin and avermectin, has 30 gene clusters (~6% of the genome) dedicated to biosynthesis of secondary metabolites. In both organisms, many of these clusters lie in the chromosome arms, consistent with a concept that the compounds may only be synthesized under certain growth conditions, as an adaptive response. Although at least three of the *S. coelicolor* clusters specify antibiotics, most of the others are probably responsible for products with different functions. For example, hopanoids may protect against water loss through the plasma membrane of aerial hyphae, and eicosapentaenoic acid may help to maintain membrane fluidity at low temperature. Three clusters code for siderophore biosynthesis, enabling the organism to scavenge iron from its extremely insoluble FeIII form in the environment. Consequently, many of the secondary metabolites may be termed 'stress metabolites,' enabling adaptation to physical and chemical stresses, and, in the case of antibiotics, permitting competition against rival soil microorganisms. A current challenge is to understand more about the regulation of biosynthesis of these products (see Section 'Regulation'), in particular how to turn on production of 'cryptic' pathways that could potentially augment the number of metabolites with useful pharmacological activities.

Another artificial strategy to extend the variety of secondary metabolites produced by the genus is to employ 'combinatorial biosynthesis' (reviewed by Baltz, 2006). Essentially this means genetically manipulating biosynthetic pathways, based on precise knowledge of the roles of individual enzymes or active sites within these proteins. This strategy has been applied to change the aglycone skeleton of metabolites produced by an 'assembly-line' process – non-ribosomally synthesized peptides and type I polyketides – both amenable to redesign, or to alter the pattern of glycosylation subsequent to synthesis of an aglycone. The polyketides encompass a very large and diverse class of streptomycete secondary metabolites. Indeed *Streptomyces* spp. have evolved three types of polyketide synthase (PKS): a type I PKS consists of multifunctional polypeptides that include different catalytic functions as separate domains, similar to vertebrate fatty acid synthases, whereas a type II PKS is a complex of several separate polypeptides, related in function to the fatty acid synthases of other bacteria and of plants. In bacteria, type III PKSs typically catalyze iterative decarboxylation and condensation reactions of malonyl-CoA building blocks in the biosynthesis of polyhydroxyaromatic products. The concept of combinatorial biosynthesis, first demonstrated with the genes for the Type II PKSs of actinorhodin and related compounds, was exploited for practical purposes by manipulating synthesis of the aglycone of erythromycin, a type

I polyketide produced by the actinomycete *Saccharopolyspora erythrea* (formerly *Streptomyces erythreus*). The aglycone macrolide is synthesized by sequential condensation of propionate units in a process involving 28 active sites arranged along three giant proteins that comprise the PKS: as the polyketide chain grows it is passed processively along the assembly-line PKS. At the end of the assembly line, the polyketide is lactonised and the aglycone is then hydroxylated and glycosylated, involving 18 further proteins. Different aglycones can be derived by genetic manipulation to change individual steps along the assembly-line. These modifications can alter, for example, the number of propionate units assembled in the polyketide chain, substitution of other building units for propionate, or change the degree of reduction of a specific carbonyl group formed at a given condensation reaction.

These types of engineered changes to secondary metabolite structure supplement the natural variability in biosynthetic pathways that often result in several related molecules being determined by a single gene cluster. In combination with the presence in a single species of multiple gene clusters specifying the synthesis a variety of secondary metabolites of quite distinct chemical structures, the biosynthetic capacity of a streptomycete is remarkable. As indicated previously, a driving force for the evolution of this capacity is the diversity of function of different metabolites. In addition, there are examples of two structurally distinct secondary metabolites produced by a single species that function synergistically. An example is the coproduction by several species, the best studied of which is *S. clavuligerus*, of the β-lactam cephamycin C and the β-lactamase inhibitor clavulanic acid. This combination of a β-lactam antibiotic and a β-lactamase inhibitor is effective against β-lactam-resistant bacteria that could compete for the same ecological niche as *Streptomyces*. Another example concerning increased effectiveness of antibiotic activity is provided by producers of the streptogramins that inhibit bacterial protein synthesis by binding to the ribosome. All streptogramin producers, for example *S. pristinaespiralis*, produce two structurally distinct molecules: a type A and a type B streptogramin, determined by two different gene clusters. Whereas type A or type B streptogramins alone are bacteriostatic, the combination is bacteriocidal. This is due to synergistic binding to the ribosome: binding of the type A antibiotic is thought to change the conformation of the ribosomal RNA to expose a high-affinity binding site for the type B compound. Both these examples of synergy have been clinically exploited in the formulation of effective combination drugs: Augmentin is a combination of the unnatural β-lactam amoxicillin with natural clavulanic acid, and Synercid is a combination of semi-synthetic type A and type B streptogramins.

Many of the gene clusters determining antibiotic production contain one or more cluster-specific regulatory (CSR) genes encoding transcriptional activators. Expression of these genes is itself coordinated by pleiotropic regulatory networks (see Section 'Regulation') that integrate information about cell physiology and environmental conditions to ensure that an antibiotic is produced in a highly regulated manner. The cluster-specific regulators activate expression of the cognate biosynthetic genes and also antibiotic self-resistance genes contained within the clusters. The products of these resistance genes can protect antibiotic producers by a variety of mechanisms, including modification of the antibiotic target and by active efflux of the drug. Interestingly, the primary transcripts of several self-resistance genes lack untranslated leader sequences – the start-points for transcription and translation are coincident; the significance of this in relation to ribosome recognition and control of translation is as yet unclear. The architecture of antibiotic biosynthetic gene clusters allows for coordinated regulation of biosynthesis and resistance, and this can clearly facilitate horizontal transfer of entire clusters to other species via mechanisms such as conjugal transfer (Section 'Plasmids'). An example of this coordinated regulation is the induction of resistance involving active efflux of Act by *S. coelicolor*: the inducer is an inactive precursor of the antibiotic, ensuring that resistance is established before the antibiotic accumulates to a toxic level.

Due to the extensive background knowledge concerning antibiotic production by *S. coelicolor*, it is an attractive 'chassis' for expressing heterologous secondary metabolite gene clusters. With this in mind, strains have been specifically developed for this purpose. As existing endogenous pathways can compete with introduced pathways for the precursor compounds supplied by primary metabolism, genome-reduced strains have been derived lacking the linear plasmid SCP1 that carries the methylenomycin biosynthetic gene cluster and also chromosomal Act, Red, CDA and CPK clusters. Deletion of these pathways also facilitates identification of a new product as the strain has a much simplified extracellular metabolome. In addition, antibiotic production-promoting *rpoB* (RNA polymerase) and *rpsL* (ribosomal protein S12) mutations have been introduced. These strains support high levels of production of a wide range of secondary metabolites.

Transport in and out of the Cell

Transport is an important aspect of streptomycete biology, enabling many interactions between the bacteria and their complex soil environment. *S. coelicolor* has 614 proteins with predicted transport function. The largest number is of the ABC transporter type predicted to be involved in either import or export of molecules, including sugars, amino acids, oligopeptides and ions. Import is believed to be facilitated by 75 putative surface-anchored substrate-binding proteins. Several ABC transporters that function as specific antibiotic efflux pumps have been identified in a number of species. For example, secretion and self-resistance to oleandomycin by *S. antibioticus* is mediated by the OleB ABC transporter, and in *S. argillaceous* an ABC transporter and an integral membrane protein encoded by the *mtrA* and *mtrB* genes are responsible for export and self-resistance to the anthracycline antitumour antibiotic mithramycin. Whereas the ABC transporters are multicomponent proteins that bind and facilitate transport by ATP hydrolysis, other single component transporters effect transport of small solutes in response to chemiosmotic ion gradients. The latter are also employed for self-resistance and efflux of antibiotics, examples being the product of the *mmr* gene that is part of the methylenomycin biosynthetic gene cluster encoded by a large linear plasmid of *S. coelicolor*, and the multidrug efflux pump protein encoded by the *cmcT* gene in the cephamycin cluster of *S. clavuligerus*.

The saprophytic lifestyle of *Streptomyces* depends on secretion of a plethora of hydrolases: *S. coelicolor* has 100 predicted secreted hydrolases out of a total of 819 potentially secreted proteins. As in most bacteria, the general secretory pathway (Sec) is the

predominant route for protein export, whereby proteins are translocated across the membrane in an unfolded state through a membrane-embedded translocon, to which they are targeted by cleavable N-terminal signal peptides. However, an additional protein export pathway, designated Tat (for twin-arginine translocation), also functions in *Streptomyces*: indeed *in silico* predictions suggest that of all prokaryotes, *Streptomyces* spp. possess by far the largest number of Tat substrates (up to 189 of the 819 predicted secreted proteins of *S. coelicolor*). Proteins are targeted to the Tat pathway by tripartite N-terminal signal peptides that contain a conserved twin-arginine motif. Importantly, the Tat pathway transports only prefolded protein substrates and in *E. coli* the system is used to secrete prefolded proteins associated with cofactors. Of the 189 predicted Tat substrates of *S. coelicolor*, so far 27 have been experimentally verified (comprising 30% of all detectable exported proteins). Of these, several are predicted to be involved in phosphate and carbohydrate metabolism, nutrient transport, and lipid metabolism. In contrast to the paradigm established with Tat substrates in *E. coli*, only 3 of the 27 *S. coelicolor* Tat-dependent proteins are likely to be cofactor-containing. Consequently, it appears that the Tat pathway in *Streptomyces* is a general protein export system responsible for secretion of a diverse range of proteins.

Compared to unicellular pathogenic actinomycetes such as *M. tuberculosis* and *C. diphtheriae*, *Streptomyces* spp. have a far greater capacity for transport, both in terms of the numbers of predicted secreted proteins and the numbers of transporters, undoubtedly reflecting their free-living lifestyle and the importance of interactions with a highly variable environment.

Regulation

The proportion of regulatory genes increases in relation to bacterial genome size. *Streptomyces* spp, with some of the largest prokaryote genomes, combined with their need to adapt their physiology and cell biology to varying environmental conditions, possess an enormous capacity for regulation. A total of 965 proteins (12.3%) encoded by the *S. coelicolor* genome are predicted to have regulatory function. There are 65 predicted sigma factors, 85 sensor kinases and 79 response regulators (including 53 sensor-regulator pairs), 44 serine/threonine protein kinases, and >100 DNA binding proteins belonging to different regulator families. Recent evidence suggests that *Streptomyces* spp. also have the ability to modify local chromatin structure according to their growth conditions, although it is too early to predict whether these dynamic changes to nucleoid structure influence or reflect patterns of gene expression.

Alternative Sigma Factors

Sigma factors act by binding to and influencing the promoter specificity of the RNA polymerase core enzyme, thereby directing selective transcription of different gene sets, coordinating gene expression in response to various environmental and endogenous cues. Phylogenetic comparison of streptomycete sigmas indicates that they belong to 4 groups. *S. coelicolor* possesses a single group 1 'housekeeping' sigma (HrdB), 3 group 2 sigmas (HrdA, C, D), 10 group 3 sigmas, and 45 group 4 (extracytoplasmic function, or ECF) sigmas. As a *hrdACD* triple mutant lacks any obvious phenotype, the function of the group 2 sigmas is unknown. At least 2 group 3 sigmas regulate developmental events after emergence of aerial hyphae, culminating in spore formation (Sections 'Hyphal Growth and Cell Division' and 'Regulation of Development'). This sporulation programme begins, before sporulation septation, with σ^{WhiG}, and ultimately leads to expression of the late spore-specific sigma, σ^{F} (Figure 2). The *sigF* gene is not transcribed until after the sporulation septa are formed. σ^{F} is required for expression of the *whiE* gene cluster specifying a type II polyketide synthase responsible for synthesis of the gray spore pigment. Two of the other group 3 sigmas, σ^{H} and σ^{B}, have dual roles: they have been implicated in controlling proper differentiation and, together with another group 3 sigma, σ^{I}, they are also involved in osmotic stress responses (Figure 2). Indeed, σ^{H} is also involved in a temperature stress response. As sporulation is itself a stress response by non-motile streptomycetes, this overlap in function is perhaps not unexpected. The activities of some of these group 3 sigmas are regulated by interaction with anti-sigma factors. For example, σ^{B} is activated by interaction of its anti-sigma factor RsbA with an anti-anti-sigma factor RsbV.

Only a few of the 45 group 4 (ECF) sigmas have been studied thus far. σ^{BldN} is required for formation of aerial hyphae (Figure 2). Transcription of its gene is repressed by the transcription factor BldD, which also represses transcription of *whiG*. In addition, activation of σ^{BldN} is believed to require proteolytic cleavage of a ~90 amino acid N-terminal extension that may anchor the inactive sigma factor to the membrane. σ^{U} indirectly affects aerial development as well. Overexpression of σ^{U} or eliminating its cognate antisigma factor RsuA causes a delay in the growth of aerial hyphae. A mutant with both the sigma factor and anti-sigma factor genes deleted develops normally, indicating that uncontrolled activity of σ^{U} causes the developmental phenotype. The group 4 σ^{E} is activated by cell wall stress and regulates, in turn, genes involved in cell wall integrity. The sigma factor gene is part of an operon that also specifies a two-component sensor-regulator gene pair. When activated, the histidine protein kinase, CseC, phosphorylates the response regulator, CseB, which in turn activates transcription of the operon.

At least one other group 4 sigma, σ^{R}, and possibly σ^{T} as well, respond to cytoplasmic oxidative stress. σ^{R} transcribes an operon encoding both thioredoxin and thioredoxin oxidase: together, these proteins reduce disulphide bonds formed in proteins under oxidizing conditions. In reducing conditions, σ^{R} is bound by its anti-sigma factor, RsrA. This is relieved in oxidizing conditions because of disulphide bonds formed between several cysteine residues in RsrA, causing release of the sigma factor. RsrA was the first example of a new family of zinc binding anti-sigma factors subsequently discovered in many other bacteria. In *M. tuberculosis*, the corresponding proteins, σ^{H} and RshA, regulate responses to both oxidative stress and heat shock.

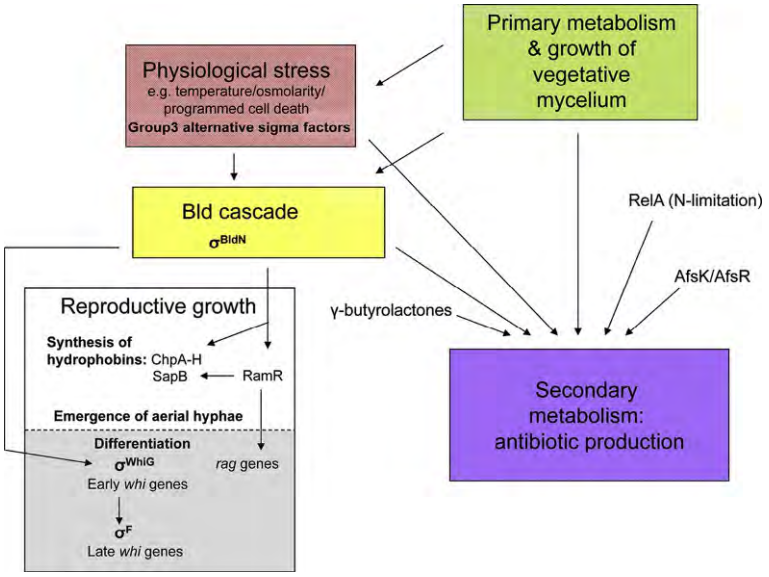

Figure 2 An overview of the regulation of morphological differentiation and secondary metabolism in *S. coelicolor*. This simplified scheme illustrates the complexity of regulatory networks, highlighting the role of some of the critical alternative sigma factors and other regulatory molecules involved in the control of reproductive growth (sporulation) and secondary metabolism (antibiotic production) – see Section 'Regulation' for details. It should be noted that the central role of the Bld cascade is conditional on glucose being the carbon-source in the growth medium, implying the existence of alternative regulatory pathways that function during growth in other conditions. The indicated regulatory dependencies are not necessarily direct and, in some cases, can also operate in reverse: for example, BldD represses *sigH*. In *S. griseus*, the γ-butyrolactone, A-factor, regulates both sporulation and secondary metabolism.

Regulation of Development

As a consequence of many years' analysis of mutants affected in morphological differentiation, *S. coelicolor* has proved a useful model to study streptomycete development (reviewed by Flardh and Buttner, 2009). Sporulation of this species occurs only during surface culture. In contrast, *S. griseus* can be induced to sporulate in submerged culture, and this has extended our understanding of the control of development. For surface grown cultures, mutants blocked in the initiation of aerial hyphae formation, resulting in a smooth colony surface, have a bald (Bld) phenotype: many *bld* mutants are unable to make the hydrophobic extracellular proteins involved in morphogenesis (see Section 'Morphological Differentiation'), including chaplins and SapB. This phenotype is conditional on the medium and, in particular, the carbon source, used to support growth. For example, on a glucose-containing rich medium, *bld* mutants cannot produce SapB or develop aerial hyphae. When purified SapB is applied exogenously to these mutants (with the exception of the *bldF* mutant), hyphae can emerge from the substrate, although they do not differentiate into morphologically recognizable aerial hyphae. When pairs of *bld* mutants are grown close to each other, one may act as a donor of a diffusible activity that induces SapB production and hence the emergence of aerial hyphae. These extracellular complementation data have suggested a model in which each *bld* gene is directly or indirectly involved in the synthesis and response to a specific extracellular signaling molecule, defining a cascade where SapB is the final product (Figure 2). Many of these *bld* genes encode regulatory proteins. For example, *bldA* encodes a leucyl-tRNA (Section 'Base Composition'). The *bldD* gene encodes a small DNA binding protein that represses the promoters of two developmental sigma factor genes, *bldN* and *whiG*, and one promoter of the stress-response sigma factor gene *sigH* (Section 'Alternative Sigma Factors'). σ^{BldN} itself is responsible for transcription of the *chp* genes, encoding the hydrophobic proteins that contribute to the fibrous layer coating aerial hyphae (Section 'Morphological Differentiation'). *bldK* is an exception in that it is not a regulatory gene: it encodes an oligopeptide transporter that imports a 655 Da peptide encoded, indirectly, by the *bldJ* locus. The *bld* cascade that commits the bacterium to aerial hyphal growth is believed to integrate factors like the energy balance and hyphal density with environmental conditions like nutrient availability and stress. Indeed, mutations in genes outside the cascade also result in a *bld* phenotype. For example, mutations in genes involved in primary metabolism such as *citA*, encoding citrate synthase, *cya*, encoding adenylate cyclase, and *acoA*, encoding aconitate citrase, result in a failure to form aerial hyphae because of an accumulation of organic acids. The group 3 sigma factor σ^{B} (Section 'Alternative Sigma Factors') and an atypical response regulator OsaB are both required for aerial hyphal growth on medium supplemented with ionic or non-ionic osmolytes.

The *bld* cascade activates expression of *ramR*, encoding the response regulator that in turn controls expression of the *ramCSAB* operon, specifying synthesis and export of SapB (Figure 2; see Section 'Morphological Differentiation'). Mutation of *ramR* itself delays aerial growth, while its overexpression restores SapB synthesis and aerial growth in *bld* mutants and the *citA* mutant. For the latter, metabolic defects are not suppressed, indicating that RamR can uncouple morphological differentiation from metabolic signals. This is a consequence of RamR functioning as a critical regulator of the events leading to sporulation, and, in particular, because of its regulatory role in the commitment of emerging aerial hyphae to undergo sporulation. This pathway involves

transcriptional activation by RamR of the *ragABKR* operon encoding two subunits of an ABC transporter, a histidine kinase and a response regulator. A *ΔragABKR* mutant develops defective branching aerial hyphae (branching is normally suppressed during aerial growth), reminiscent of those of a *whiG* mutant (σ^{WhiG} is the first group 3 sigma factor involved in the sporulation pathway; Section 'Alternative Sigma Factors'). Several regulatory pathways may modulate activation of σ^{WhiG}, representing an early checkpoint for commitment to sporulation. The RagK histidine kinase may sense the radically different environment encountered by the hyphae as they emerge into the air, activating the response regulator RagR that may, in turn, influence activation of σ^{WhiG}. In addition, the *rag* operon may be necessary for export of an as yet unidentified morphogenetic compound that influences differentiation of aerial hyphae. Examples of compounds that influence aerial development identified in other *Streptomyces* spp. are the γ-butyrolactone A-factor in *S. griseus* (see Section 'Regulation of Secondary Metabolism') and the antibiotic pamamycin in *S. alboniger*.

Mutants affected in subsequent differentiation of aerial hyphae do not generate normal mature spores and stay white instead of developing the normal gray color conferred b a spore-wall-associated polyketide pigment. These *whi* mutants have been grouped as 'early' or 'late' (Figure 2), early mutants failing to achieve full sporulation septation. σ^{WhiG}, encoded by the early *whiG* gene, transcribes two further early sporulation genes: *whiH* and *whiI*. The WhiH transcription factor belongs to the GntR family that, in addition to a DNA binding domain, possess a signal-sensing domain that typically responds to an acidic carbon metabolite. Despite its overall homology with response regulators, the WhiI protein lacks two key conserved residues in its putative phophorylation pocket. A plausible model posits that these two transcription factors normally repress transcription of their own genes, but respond to signals at the cessation of aerial growth to become activators of late sporulation promoters.

In addition to the σ^{WhiG}-dependent early *whi* genes, the expression of two other early σ^{WhiG}-independent *whi* genes, *whiA* and *whiB*, is upregulated during aerial growth. Both the WhiB protein and the late sporulation protein WhiD belong to a protein family unique (and abundant – *S. coelicolor* encodes 10 such proteins) to actinomycetes. They contain four conserved cysteine residues, suggesting that they may respond to intracellular redox changes by disulphide bond formation or by chelation of a redox-active metal atom. By sensing redox changes at the end of aerial growth, they could coordinate orderly growth cessation: a characteristic of *whiA* or *whiB* mutants is formation of unusually long aerial hyphae. After growth cessation, all the early *whi* genes are needed for proper sporulation septation and transcription of late sporulation genes and operons. This includes regulation of *sigF* (-Section 'Alternative Sigma Factors') and the *ftsZ2p* promoter that is activated to provide an increased amount of the FtsZ protein required for sporulation septation (Section 'Hyphal Growth and Cell Division').

Regulation of Secondary Metabolism

Many secondary metabolite biosynthetic gene clusters contain at least one cluster-specific regulatory (CSR) gene, often encoding a *Streptomyces* Antibiotic Regulatory Protein (SARP) or a transcriptional regulator of the LAL family (Large ATP-binding regulators of the LuxR family). The former are transcriptional activators containing a winged helix-turn-helix motif towards their N-termini that is also found in the OmpR family of proteins. The SARP family of proteins have been found only in actinomycetes, and mostly within the streptomycetes. A very complex set of regulatory cascades link physiological, environmental and developmental signals to expression of pleiotropic regulatory genes and ultimately these pathway-specific regulators, thereby controlling secondary metabolism after the main growth phase of the bacterium (Figure 2; reviewed by Liu et al, 2013; van Wezel & McDowall, 2011). Examples of the pleiotropic regulators are the products of the *bld* genes that also regulate the growth of aerial hyphae (Section 'Regulation of Development'). As discussed previously (Section 'Primary Metabolism'), high concentrations of both ammonium and inorganic phosphate in the growth medium negatively impact antibiotic biosynthesis. In nitrogen–limiting conditions, antibiotic biosynthesis is triggered by the highly phosphorylated guanosine nucleotide (p)ppGpp. Consequently, in these conditions, antibiotic production depends on the ribosome-associated ppGpp synthetase (RelA). This can be by-passed by mutations in the β-subunit of RNA polymerase of the kind that confer resistance to rifampicin: it is possible that these mutations mimic the effect of ppGpp binding to RNA polymerase and hence influence its activity for transcription after the main growth phase. Mutations that affect translation during this part of the life-cycle also have a significant impact on antibiotic biosynthesis. A mutation conferring streptomycin resistance within *rpsL*, encoding the S12 ribosomal protein, results in increased expression of ribosome recycling factor and this, in turn, increases gene expression during stationary phase.

The streptomycetes typically produce extracellular signals that regulate antibiotic biosynthesis. These signals are γ-butyrolactones. They are effective in nanomolar concentrations, and the best characterized is A-factor produced by *S. griseus*. Unusually, A-factor is required for both secondary metabolism (streptomycin and grixazone biosynthesis) and morphological development. A-factor, specified by the *afsA* gene, binds to its cytoplasmic receptor protein ArpA, thereby preventing the latter from binding and repressing the *adpA* promoter. AdpA is required for activation of transcription of both *strR*, the pathway-specific regulatory gene for streptomycin production, and other members of the *adpA* regulon, some of which are required for morphological differentiation. γ-butyrolactones produced by other species specifically regulate secondary metabolism. Examples are the virginiae butanolides controlling virginiamycin biosynthesis in *S. virginiae* and SCB1 that regulates production (possibly indirectly) of the pigmented antibiotics of *S. coelicolor*. For these examples, the receptor protein is also required for normal production of the cognate γ-butyrolactone and is encoded by a gene adjacent to that specifying production of the signaling molecule. Moreover, both genes are adjacent to secondary metabolite gene clusters whose regulation is controlled by the γ-butyrolactone. For example, in *S. coelicolor*, *scbA*, specifying SCB1 synthesis, and *scbR*, encoding the receptor protein, both lie next to a cluster of genes, the *cpk* genes, that specify synthesis of a 'cryptic' Type I polyketide. In the absence of SCB1, ScbR binds to the promoter of the pathway-specific

regulatory gene *cpkO* and represses its transcription. A second ScbR-like repressor, ScbR2, also controls *cpkO* expression. The *scbR2* gene is close to *cpkO* and the repressor protein, unlike ScbR, does not bind gamma-butyrolactones. The products of the *cpk* cluster were revealed when *scbR2* was deleted, as this effectively upregulates expression of both the CSR *cpkO* and, consequently, the CPK biosynthetic genes. This example of manipulating expression of a CSR of a given cryptic gene cluster to activate biosynthesis has also been achieved for another cryptic Type I polyketide synthase gene cluster in *S. ambofaciens* and could be applied to other examples of 'cryptic' biosynthetic pathways as part of drug-discovery programmes to extend the range of bioactive compounds produce by streptomycetes.

A pleiotropic regulator of antibiotic production in *S. coelicolor*, AfsR, shares in its N-terminal region significant amino acid homology with the SARP family of proteins, while containing ATP-binding sequences in its central region. AfsR plays a role in a signal transduction phosphorelay that regulates synthesis of the pigmented antibiotics Act and Red, and also the calcium-dependent antibiotic (CDA) specified by the *cda* cluster. A membrane-associated serine/threonine protein kinase, AfsK, when activated by an as yet unknown environmental cue, autophosphorylates and in turn phosphorylates cytoplasmic AfsR (and also DivIVA, Section 'Hyphal Growth and Cell Division'). AfsR-P then activates transcription of *afsS*, encoding a 63-amino acid protein that functions in an unknown way to enhance production of Act, Red and CDA. Other kinases (PkaG and AfsL) can phosphorylate AfsR, suggesting that it can integrate a variety of environmental signals.

A paradigm for understanding the complexity of regulation of synthesis of a given secondary metabolite concerns Act biosynthesis in *S. coelicolor*. In this example the CSR is the ActII-ORF4 regulator that controls Act biosynthesis. This CSR acts as a critical node in the regulatory network controlling Act biosynthesis, integrating information on nutritional status, growth and development among other factors. This is achieved largely at the transcriptional level as the *actII*-ORF4 promoter is extraordinarily complex (for a bacterial promoter region), in that it is itself a target for at least 10 regulatory proteins. These include AdpA, itself repressed both by a γ-butyrolactone-binding transcription factor (see above) and by BldD (linking morphological development with regulation of secondary metabolism). AdpA binds to the *actII*-ORF4 promoter and helps to recruit RNA polymerase thereby activating transcription and Act biosynthesis. Another AdpA-activated target gene is *bldA* (Section 'Base Composition'), so establishing a developmental positive-feedback loop, and this impinges on Act biosynthesis as the *actII*-ORF4 mRNA also contains a rare *bldA*-dependent UUA codon. A repressor of *actII*-ORF4 is DasR that regulates the global response to N-acetylglucosamine released on programmed cell death and autolysis of parts of the vegetative hyphae at the end of growth. This product is released from degrading cell walls and, once assimilated, is deacylated and phosphorylated. The resulting metabolite binds to DasR, relieving its repression of *actII*-ORF4.

The cyclic AMP receptor protein, Crp, involved in glucose repression in other bacteria, is an activator of *actII*-ORF4 expression as well as the CSRs for CDA and Red. Other regulators acting directly on *actII*-ORF4 and involved in nutritional responses are the xylose operon repressor; AfsQ1, an activator that responds to nitrogen excess; GlnR (Section 'Primary Metabolism'); DraR, part of a two-component system that responds to nitrogen excess; PhoP (Section 'Primary Metabolism'); and AtrA, itself regulated by PhoP. In addition, the *actII*-ORF4 promoter is a target for the two-component response regulators AbrC3 and AbsA2.

Regulation of secondary metabolism in streptomycetes includes other diverse mechanisms; this complexity is to be expected as the different bioactive products have a wide range of adaptive functions, many of which we do not yet understand. Consequently, several parallel and overlapping regulatory pathways can exist within a single species, coordinating production of one or more secondary metabolites in response to different environmental cues, and often linking production with developmental signals that trigger morphological differentiation.

Conclusion

Although more complex than most prokaryotes, and consequently often less amenable to research, *Streptomyces* spp. are continuing to offer exciting new paradigms as model systems in microbiology. As the genomes of more members of the genus are sequenced, and this information is assessed using functional genomic tools, a lot more information about this important family of bacteria will be added to that summarized in this article. This will contribute to increasing our knowledge of fundamental aspects of biology as well as offer insights into related, less experimentally tractable bacteria such as *M. tuberculosis*, and potentially provide vital new drugs to extend the 'antibiotic era' of medicine that is currently being seriously challenged by the evolution of multi-drug resistant pathogens.

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Stress, Bacterial: General and Specific☆

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Glossary

Adapter and anti-adapter proteins Proteins that regulate protein degradation by the ClpXP protease.
Ancillary factors Proteins or other molecules that influence RNA polymerase activity.
Antiporter A protein in cytoplasmic membrane that brings about exchange of external protons and a cellular ion/compound.
Electrophiles Compounds that accept electrons.
Eutrophic environments Environments made nutrient-rich primarily through human activity.
Inclusion bodies Precipitated and denatured proteins inside a cell. These are usually formed in bacteria when a heterologous protein is overproduced.
Periplasm Space between outer and cytoplasmic membranes in Gram-negative bacteria.
Pex proteins The core set of proteins induced in response to diverse stresses.
Porins Proteins in the bacterial outer membrane that form water-filled pores, permitting transport.
Proteome Complete protein profile of a cell.
Redox cycle A reduction reaction that generates unstable radicals. These give their electrons to oxygen generating reactive oxygen species (ROS). The radical is changed back to the original compound and becomes available for further ROS generation.
Sigma factors Small proteins that combine with the RNA polymerase core enzyme. The resulting RNA polymerase holoenzyme can transcribe various genes. Each species of RNA polymerase generally recognizes specific promoter sequences.
Transcriptome Complete gene transcription profile of a cell.

Abbreviation

cAMP	Cyclic AMP
GSR	General stress response
HARVs	High aspect to ratio vessels
HGH	Human growth hormone
HPK	Histidine protein kinase, the sensor kinase of thetwo component systems
IHF	Integration host factor
LpDH	Lipoyldehydrogenase
OMPs	Outer-membrane proteins
QH_2	Hydroquinone
RNAP	RNA polymerase
ROS	Reactive oxygen species
RR	Response regulator
RR	Response regulator protein, also, a part of the two component systems.
rRNA	Ribosomal RNA
sRNAs	Small RNA
TIR	Translational initiation region
UTR	Untranslated region

Defining Statement

Bacteria counter stress at two levels, specific and general, to escape a given stress and to acquire greater robustness. I will discuss here the mechanisms of escape, increased cellular robustness, and the molecular mechanisms that enable a bacterium to shift from rapid growth mode to stasis and enhanced resistance.

☆*Change History:* August 2014. AC Matin added abbreviations, references, and further reading. The author made changes in sections 'General Stress Response' and 'Regulation of Stress Response.'

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Introduction

Bacteria, like other living things, require certain physicochemical conditions in order to thrive. Usable nutrients need to be sufficiently available, temperature and pH maintained within specific limits, and toxic influences absent. Under such optimal conditions, bacteria grow at maximal rates of which they are genetically capable. The animal gut flora encounters such conditions after the host has taken a meal, intracellular pathogens often immediately after invasion, and environmental bacteria in, for example, eutrophic environments. But such conditions are rare and fleeting, and as a rule, bacteria in nature exist under conditions that are not only suboptimal but can be outright hostile to their survival, exposing them to diverse kinds of stresses.

A common stress is lack of food. Thus, the gut flora by its rapid growth soon exhausts the nutrients passed on to the host intestine and progresses from feast to famine, and the same is likely true of an intracellular pathogen. While eutrophic environments are on the rise due to human activities, much of the natural environment nevertheless remains severely nutrient-poor. Oceans are estimated to have 0.8 mg carbon nutrients per liter, and the concentration of individual carbon compounds in fresh water is often as low as 6–10 $\mu g\, l^{-1}$. Similarly, soils as rule possess little usable nutrients, as most of the 0.8–2.0% carbon in this environment is humus, which bacteria for the most part cannot use. In other natural environments, bacterial growth is restricted by the scarcity of other nutrients, such as nitrogen, phosphorus, and/or iron (Ghiorse and Wilson, 1988; Kaiser et al., 2014; Matin et al., 1989; Rohmer et al., 2011).

The fluctuating conditions in nature expose bacteria to additional stresses. Diurnal and seasonal changes in temperature can be significant, and a host of abiological and biological factors can result in exposure to a variety of insults, such as pH, osmotic, shear, and oxidative stresses. The pathogenic bacteria have not only to be adept at surviving these stresses during their extra-host existence but also to be able to cope with deleterious influences as they attempt to survive in the host in disease initiation. For example, to infect a host, *Salmonella enterica* serovar Typhimurium, which causes a typhoid-like disease in mice, has to survive passage through the stomach where the average pH over a 24-h period is as low as 1.5. It then invades the interior of the host by infecting the microvilli of the gastrointestinal tract, which are low-shear environments (Guo et al., 2000), and it is then ingested by the host macrophage, where additional insults await – oxidative stress, nutrient deprivation, and low pH. To meet such threats to survival, bacteria have evolved elaborate adaptive responses; these are the subject of this article with special emphasis on starvation, although other stresses are also considered.

The Stress Response is Two-Pronged

Bacteria meet the challenge to survival posed by stresses using a two-pronged strategy. One is aimed at neutralizing and escaping the specific stress that is encountered. This response tends to be unique to each stress; thus the proteins a bacterium needs to escape, for instance, oxidative stress are different from those it utilizes to escape starvation. This is termed the specific stress response. The second component of the stress response is aimed at preventing and repairing the damage that the stress might cause and is activated as an insurance policy, since there is no guarantee that the first response will succeed in preventing the deleterious effects of the stress. All stresses, if not neutralized, lead to a common outcome, namely damage to the cell biomolecules, and the second tier of the stress response is aimed at preventing and repairing this damage. Thus, this facet of the stress response results in making bacteria resistant not only to the stress that is experienced but also to others, and is thus termed the general stress response (GSR).

Specific Stress Response

Starvation

The first definitive indication that bacteria respond to stresses by a two-pronged strategy came when the proteomes of bacteria subjected to different stresses were examined. For example, starvation for carbon, nitrogen, or phosphorus resulted in the induction not only of proteins unique to that starvation condition but also to that of a core set of proteins that was common to all the starvation conditions (referred to as Pex proteins) (Groat et al., 1986). Exposure to stresses mechanistically different from starvation, *viz.*, oxidative, osmotic, pH, and others, also led to the induction of unique and common proteins, many of the latter being the same as the core starvation (Pex) proteins (Jenkins et al., 1988a). Based on these findings, it was proposed that the proteins unique to a specific stress were concerned in enabling the bacteria to neutralize that particular stress, while the core set of proteins was concerned with conferring resistance to stresses in general (Matin, 1991a; Matin et al., 1989). This has been found to be the case (Peterson et al., 2005a). In this section, I will discuss the physiological role of selected proteins that are concerned with the escape response; the function of the Pex proteins that confer general resistance is discussed in subsequent sections.

Examples of proteins concerned with escaping stresses are provided in Table 1. Starvation-escape response consists in the synthesis by bacteria of enzymes that amplify their capacity to obtain the scarce nutrient (Harder et al., 1977). This is accomplished either by increasing the concentration of the relevant enzymes or by synthesizing a new set that possess a higher affinity for the nutrient. Either way, a superior capacity is acquired to scavenge the scarce nutrient. The proteins that are induced can concern every metabolic feature: transport through the outer and cytoplasmic membranes, enzymes involved in substrate capture, and those responsible for subsequent flux through the metabolic pathways. Thus, when phosphate concentration falls below some 1 $mmol\, l^{-1}$ in the environment, cells increase the protein PhoE, which is a porin facilitating the passage of phosphate compounds

Table 1 Selected escape-response proteins

Protein	*Function*
Phosphorous starvation	
Pst	High-affinity phosphate transport system
PstS (also called PhoS)	Periplasmic Pi-binding protein required for PstS function
PhoE	Porin that facilitates Pi transport through the outer membrane
PsiB and PsiC	Glycerol phosphate transport systems
Bacterial alkaline phosphatase	Carbon–phosphorus bond lyase
Carbon starvation	
Periplasmic-binding proteins (e.g., MalE)	Enhanced transport (e.g., maltose)
Glucokinase	Substrate capture (glucose)
Lactate dehydrogenase	Substrate capture (lactate)
β – Galactosidase	Substrate capture (lactose)/metabolic potential amplification
CstA	Substrate capture (peptides)/metabolic potential amplification
Glycerol kinase	Substrate capture (glycerol)
Glucose-6-phosphate dehydrogenase	Enhanced flux through catabolic pathways
Phosphofructokinase	Enhanced flux through catabolic pathways
Pyruvate kinase	Enhanced flux through catabolic pathways
Aconitase	Enhanced flux through catabolic pathways
Isocitrate dehydrogenase	Enhanced flux through catabolic pathways
Malate dehydrogenase	Enhanced flux through catabolic pathways
Other stresses[a]	
Aerobactin (iron starvation)	Iron chelator
Glutamine synthetase (nitrogen starvation)	Substrate capture
Kdp (potassium starvation)	High affinity K^+ transport
Superoxide dismutase (oxidative stress)	Decomposes superoxide
KatE (oxidative stress)	Catalase
KatG (oxidative stress)	Catalase
Thiol peroxidase (oxidative stress)	Thiol-dependent hydroperoxidase
Sulfate adenylyltransferase (oxidative stress)	Cysteine biosynthesis
Cysteine synthase (oxidative stress)	Cysteine biosynthesis
ChrR (oxidative stress)	H_2O_2 quencher
Lysine decarboxylase (acid stress)	Generates cadaverine that buffers the cytoplasm
CadB (acid stress)	Brings about exchanges of cellular cadaverine for medium lysine
UreI (acid stress)	Increases membrane permeability to urea which, through urease activity,buffers the cytoplasm

[a] Text in parentheses indicates the stress.

through the outer membrane into the periplasmic space of *Escherichia coli*. Here, it interacts with a high affinity-binding protein (PstS), also induced under these conditions, promoting efficient functioning of PhoE. The compounds thus transported to the periplasm are hydrolyzed by another protein induced by phosphate starvation, the bacterial alkaline phosphatase, generating Pi. Rapid transport of the latter across the cytoplasmic membrane is ensured by the fact that a high affinity Pi transport system, Pst (energized by ATP; K_m for Pi, 0.16 μmol l^{-1}), is concomitantly induced under these conditions, replacing the low affinity Pit system (energized by proton motive force; K_m for Pi, 25 μmol l^{-1}) that operates under phosphate-sufficient conditions (Harris et al., 2001; Matin et al., 1989).

This pattern has been demonstrated in several bacteria also when limitation for other nutrients is encountered. Carbon-scarce cells often also synthesize high affinity-binding proteins, for example, MalE, which binds maltose facilitating its transport into the cell. When *Pseudomonas* or enteric bacteria utilizing lactate or glucose as carbon source were subjected to the limitation of these substrates, they greatly increased the synthesis of lactate dehydrogenase or glucokinase, respectively. Concomitantly, there was a marked induction of several enzymes of glycolysis and tricarboxylic acid cycle, ensuring effective channeling of low levels of catabolites through them (Matin et al., 1976). Large amounts of glutamine synthetase, which catalyzes the first step in ammonium assimilation, are synthesized during ammonium limitation (Reitzer and Magasanik, 1986), and induction of high affinity substrate-capturing proteins occurs also during potassium and glycerol scarcity. In the former case, the cells shift to the Kdp system (high affinity; energized by ATP) from the Trk transport system (low affinity; energized by proton motive force) that is used when potassium is plentiful (Rhoads et al., 1976). Cells grown on nonlimiting concentrations of glycerol utilize a low affinity pathway for its catabolism whose initial step is catalyzed by glycerol dehydrogenase; under glycerol scarcity on the contrary, a high affinity pathway initiating with glycerol kinase is utilized (Martinez-Gomez et al., 2012). Iron-challenged cells increase the synthesis of the iron siderophore, aerobactin (Hantke, 1981). Thus, a combination of the synthesis of high affinity transport and other proteins coupled with a general increase in the level of metabolic enzymes ensures that the cells can effectively scavenge and utilize the scarce nutrient from the environment.

These measures can of course not always succeed in alleviating starvation. For instance, cells growing on glucose can synthesize any amount of enzymes to facilitate its utilization, but this would not help if this substrate becomes completely absent from the environment. An additional measure is therefore employed, which is to de-repress the synthesis of enzymes for substrates other than glucose counting on the chance that the constantly fluctuating conditions might promote their appearance in the environment. Thus, cells subjected, for instance, to glucose starvation also synthesize enzymes such as β-galactosidase and CstA, which confer on them the capacity to utilize lactose and peptides, respectively, thereby acquiring the capacity to cast a wider net for alleviating carbon starvation (Schultz and Matin, 1991).

Oxidative Stress

Ground state oxygen has two unpaired spins, and the constraints of quantum mechanics, and the resulting spin restriction, hinder its divalent reduction. This favors the univalent pathway that generates highly reactive (and toxic) oxygen species (ROS). Consequently, oxidative stress from ROS is a constant threat to bacteria and other living entities. Bacterial respiratory chains (like those of mitochondria) leak ROS (Imlay, 2008). Phagocytes possess a membrane-bound NADPH reductase, whose function is to catalyze one-electron reduction of O_2 to generate ROS so as to kill the invading bacteria (Babior, 1984). When plant cells come in contact with soil-dwelling bacteria, such as *Pseudomonas putida*, they release an immediate burst of H_2O_2. Many electrophiles generated internally by bacteria or those found in the environment are also a source of oxidative stress. Examples are quinones, nitro-compounds, chromate, and several dyes; quinones such as plumagin and juglone are secreted by plants as defense mechanisms against bacteria (Lin et al., 2010). These compounds are vicariously attacked by cellular metabolic enzymes such as glutathione and cytochrome *c* reductases, and lipoyl dehydrogenase (LpDH), which reduce them by one-electron transfer. The result is the generation of reactive radicals, such as semiquinones and Cr(V), which set up a redox cycle. In this process, the radical (e.g., semiquinone) transfers its electron to O_2 or, depending on the conditions to another molecule (e.g., NO_3), regenerating quinone and producing ROS or other equally destructive oxidizing agents (e.g., nitrosative radicals). With the continued activity of one-electron reducers, the quinone (or other such electrophiles) shuttles back and forth between its quinone and semiquinone valence states, producing large quantities of ROS. These compounds are referred to from here on as 'univalent reduction-prone' electrophiles.

That bacteria do indeed experience severe oxidative stress when exposed to univalent reduction-prone compounds was demonstrated by the use of the intracellular oxidative stress sensor 2′, 7′-dihydrodichlorofluorescein (H_2DCFDA), which is taken up by the cells and emits green fluorescence in the presence of ROS. For instance, *E. coli* cells exposed to chromate do indeed emit green fluorescence (Figure 1). Proteome analysis showed that these cells induced several proteins concerned with combating oxidative stress, for example, superoxide dismutase, which decomposes the superoxide radical, and those concerned with cysteine and thiol biosynthesis, which are ROS quenchers. Mutants unable to synthesize these proteins proved more sensitive to chromate killing, and strains with bolstered capacity to synthesize antioxidant defense proteins, such as ChrR (Table 1; see below) less so compared to the wild type (Ackerley et al., 2006). Other examples of proteins that permit escape from oxidative stress are given in Table 1.

ChrR, mentioned above, belongs to a recently discovered class of enzymes that combat oxidative stress in a variety of ways. These enzymes bring about a simultaneous two-electron reduction of univalent reduction-prone electrophiles. Thus, for example, they convert in one step quinone into fully reduced and stable hydroquinone (QH_2), bypassing semiquinone formation. The experimental approach to determine if an enzyme reduces the univalent reduction-prone electrophiles by one- or two-electron pathway utilizes pure proteins and a source of electrons, namely NADH or NADPH. It takes advantage of the fact that cytochrome *c* is reduced by semiquinones but not by hydroquinones, and since reduced cytochrome *c* absorbs light of 550 nm wavelength, its reduction can

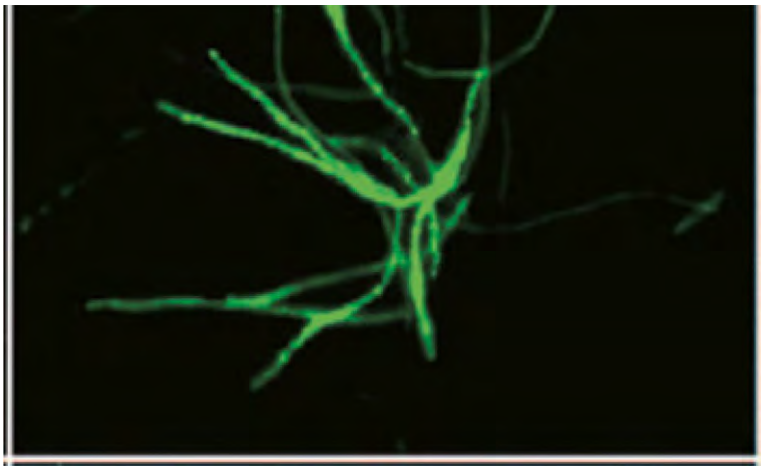

Figure 1 *Escherichia coli* cells exposed to 250 μmol l^{-1} chromate and treated with intracellular ROS sensor 2′, 7′-dihydrodichlorofluorescein. Cells were examined at ×1000 magnification with an Olympus BX60 upright fluorescence microscope. Note that the cells form snakes and fluoresce green; both are indicative of oxidative stress. Reproduced from Ackerley DF, Barak Y, Lynch SV, et al. (2006) Effect of chromate stress on *Escherichia coli* K12. *Journal of Bacteriology* 188: 3371–3381.

easily be monitored in a spectrophotometer, serving as a facile probe for semiquinone formation. It was found that when quinone was reduced by a number of different cellular enzymes, such as LpDH, large amounts of reduced cytochrome *c* were generated, indicating that the quinone was reduced by one-electron transfer and generated semiquinone. However, when the reduction was catalyzed by the enzyme ChrR, no reduction of the cytochrome was seen (Figure 2(a)). Thus, the latter enzyme bypassed semiquinone formation resulting in direct conversion of the quinone to QH_2 (Gonzalez et al., 2005).

In an extension of this experimental approach, limiting concentrations of quinone were used, which ensured that the reaction ceased because all the available quinone in the reaction mix was exhausted. Figure 2(b) shows that in such a situation when ChrR is added to an in-progress LpDH-catalyzed quinone reduction, cytochrome reduction is swiftly halted, indicating that the LpDH is no longer generating semiquinone. Addition of further quinone to the reaction mix reinitiated cytochrome *c* reduction but at a very low rate and this too was soon halted. The experiment thus indicated that when ChrR is present, quinone is made largely nonavailable to LpDH, so semiquinone formation ceases. Experiments using other single-electron reducing enzymes have given similar results. Thus, not only ChrR constitutes a safe pathway for the reduction of univalent reduction-prone electrophiles, such as quinones, it is also effective in preempting their reduction by the one-electron reducers, thereby affording a two-way protection to the cell exposed to such electrophiles (Gonzalez et al., 2005).

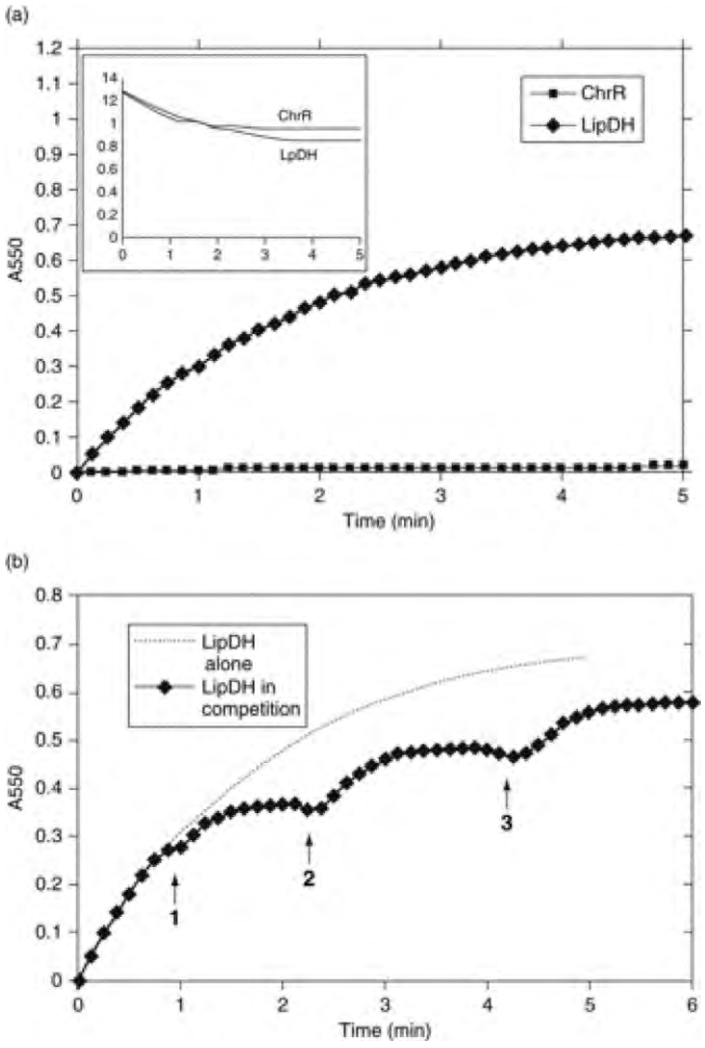

Figure 2 (a) Reduction of cytochrome *c* monitored spectrophotometrically at 550 nm during LpDH- or ChrR-catalyzed reduction of 50 μmol l^{-1} of a quinone species, benzoquinone. The appearance of reduced cytochrome *c* during the LpDH-catalyzed reaction indicates one electron transfer and generation of semiquinone, whereas the lack of this species in the ChrR-catalyzed reaction signifies a divalent mode of quinone reduction that generates QH_2 bypassing semiquinone generation. (b) Addition of ChrR to an LpDH-catalyzed reduction of limiting benzoquinone, at the point marked by *arrow* 1, rapidly arrested the reduction of cytochrome *c* relative to LpDH alone (dashed line). The addition of fresh benzoquinone (arrows 2 and 3) reinitiated cytochrome *c* reduction, but with ChrR now present, only little semiquinone is generated as indicated by very limited cytochrome *c* reduction. This indicates that the presence of the two-electron reducer, ChrR, preempts quinone reduction by the one-electron reducer, LpDH. Reproduced from Gonzalez CF, Ackerley DF, Lynch SV, et al. (2005) ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against H_2O_2. *Journal of Biological Chemistry* 280: 22590–22595.

There is in fact another level at which ChrR protects the cell against oxidative stress and that is by virtue of the fact that QH_2, which it generates, is an effective quencher of ROS, such as H_2O_2. Strains of *P. putida* devoid of ChrR and those overproducing this enzyme were grown in the presence of 3 mmol l^{-1} H_2O_2. The different cell cultures exhibited lag phases of varying duration, following which normal growth was seen (Figure 3). The ChrR overproducing strain was the first to recover, followed by the wild type, and finally the ChrR mutant. The recovery correlated with the ability of each strain to remove H_2O_2 from the medium, indicating that the cellular ChrR bolsters this capacity. Protein carbonylation, which is an indication of oxidative damage, was greatest in the strain devoid of ChrR and least in the one overproducing this enzyme (Gonzalez et al., 2005).

Acid Stress

Escape from acid stress involves a combination of physicochemical processes as well as the use of special enzymes to ensure that the cytoplasm is not acidified. The former mechanisms include making the cytoplasmic electric potential ($\Delta\psi$) positive, so as to oppose the entry of protons that, of course, are positively charged. It also includes changes in the composition of cytoplasmic membrane so as to render it less permeant to protons. In *Clostridium acetobutylicum*, for example, exposure to low pH results in a decrease in the ratio of unsaturated to saturated fatty acids and an increase in cyclopropane fatty acid content. An increase in phospholipids with amino acid head groups is another measure that appears to be aimed at decreasing proton permeability of the cytoplasmic membrane (Baumeister and Lembcke, 1992; Matin, 1999; Sunamoto et al., 1982).

The enzymes involved are amino acid decarboxylases. A well-studied system involves lysine decarboxylation, which removes CO_2 from lysine and generates cadaverine. Cadaverine picks up a proton, thereby contributing to the de-acidification of the cytoplasm (Merrell and Camilli, 1999). The protonated cadaverine is exchanged for external lysine by the antiporter CadB. Another enzyme involved in the buffering of the cytoplasm is urease, which is thought to be critically important in the ability of the gastric ulcer/carcinoma-causing bacterium *Helicobacter pylori* to colonize the stomach. This bacterium synthesizes a special membrane protein called UreI that enhances urea transport into the cell. Urea is present in the gastric juice, but its permeation into the cell without UreI is too slow to be effective in enabling *H. pylori* to keep a neutral cytoplasm (Sachs et al., 2000).

General Stress Response

Cross-Protection

As mentioned above, cells respond to different insults not only by measures aimed at escaping a particular stress, but also by bolstering the cellular machinery meant to prevent and repair damage to biomolecules that may result if the escape response fails. The evolutionary basis for this is obvious: the external environment is often so unforgiving that the escape response strategies can often at best have only a partial success and survival necessitates that measures be activated to deal with the damaging effect of stresses. This is the function of the (Pex) core set of proteins that are synthesized regardless of the nature of stress, and they confer on the cell a robustness enabling it to withstand stresses in general.

Proteome analysis of cultures starved for glucose or other nutrients showed that the proteins synthesized fall into different temporal classes and that this synthesis program is essentially complete in 4 h after the onset of starvation (Schultz et al., 1988). The Pex proteins for the most part exhibit a sustained pattern of synthesis through this period, leveling off at its end. Consistent with their role in enhancing cellular robustness, it was found that inhibition of protein synthesis in a starving culture had a time-dependent effect on starvation survival, with maximum resistance developing after 4 h of protein synthesis during starvation (Reeve et al., 1984). That the core proteins are involved in conferring general resistance on the cell is further indicated by the fact that the cross-protection that starvation confers on cells against unrelated stresses, for example, heat, oxidation, hyperosmosis, and others

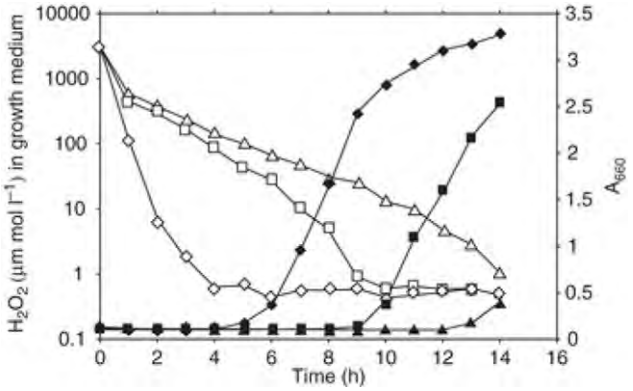

Figure 3 H_2O_2 scavenging (open symbols) and growth (as measured by increase in absorbance at 660 nm, solid symbols) of ChrR-overproducing (♦), wild-type (▪), and ChrR-deficient (▲) strains of *P. putida*. Note that the overproducing strain is most efficient in decomposing H_2O_2. Reproduced from Gonzalez CF, Ackerley DF, Lynch SV, et al. (2005) ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against H_2O_2. *Journal of Biological Chemistry* 280: 22590–22595.

Table 2 Stress-induced resistances

Starvation
Heat
Cold
pH extremes
Oxidation
Hyperosmosis
Cl_2
ClO_2
Ethanol
Acetone
Deoxycholate
Toluene
Irradiation
Antibiotics and other antimicrobials

Source: Reproduced from Matin A (2001). Stress response in bacteria. In: Bolton S (ed.) *Encyclopedia of Environmental Microbiology*, vol. 6, pp. 3034–3046. New York: John Wiley and Sons.

(Table 2), is also dependent on the time, up to 4 h, for which they have been starved. This phenomenon is illustrated in Figure 4(a) for the starvation-mediated cross-protection against heat, involving exposure to the normally lethal temperature of 57 °C. For the first 4 h after the onset of starvation, increasing resistance to heat is exhibited the longer the cells are starved, with maximal resistance being acquired within this period. The phenomenon is completely dependent on protein synthesis during starvation, since its inhibition by inclusion in the starvation regime of chloramphenicol or by other means prevents resistance development (Jenkins et al., 1988b).

Since the core protein set is synthesized regardless of the nature of stress, it follows that exposure to any stress and not just starvation should confer general resistance. This is indeed the case as is illustrated in Figure 4(b), which shows that cells exposed to adaptive doses of a variety of mechanistically unrelated stresses become more resistant to lethal concentrations of H_2O_2.

Role of σ^s in Resistance to Bactericidal Antibiotics

Recent studies have shown that the loss of σ^s renders stationary phase *E. coli* highly sensitive to the bactericidal antibiotics, gentamicin, ciprofloxacin, and ampicillin. The *rpoS* mutant experienced greater oxidative stress than the wild type as determined by the activation of the SOS response, and measurement of O●, and OH● by appropriate dyes. Further investigation showed that

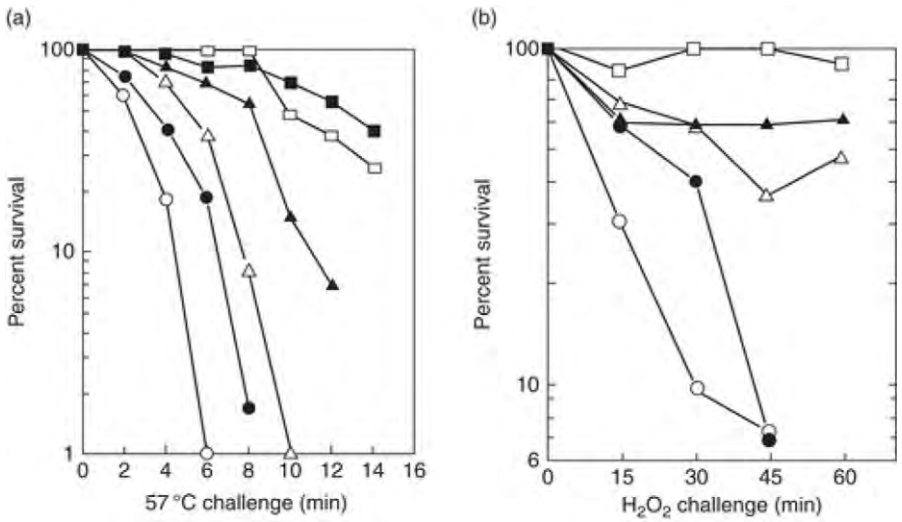

Figure 4 (a) Induction of thermal resistance in *Escherichia coli*. Cells grown at 37 °C were exposed to 57 °C during exponential growth (o), or at 1 h (Δ), 2 h (▲), 4 h (□), or 24 h (▪) after glucose exhaustion from the medium. (●) Represents culture starved in the presence of chloramphenicol. (b) Comparison of the H_2O_2 resistance of glucose-starved *E. coli* cultures to growing cultures adapted by heat, H_2O_2, or ethanol. Symbols: (o) untreated; (●) ethanol-adapted; (Δ) heat-adapted; (▲) H_2O_2-adapted; (□) glucose-starved. Reproduced from Jenkins DE, Schultz JE, and Matin A (1988) Starvation-induced cross protection against heat or H_2O_2 peroxide challenge in *Escherichia coli*. *Journal of Bacteriology* 170: 3910–3914.

mutational loss of antioxidant proteins, those that decompose ROS or supply NADPH for their activity namely the pentose pathway proteins (Grant, 2008; Ralser et al., 2007) – also results in the generation of greater oxidative stress in the mutants and heightened sensitivity to these drugs (Wang JH, Singh R, Benoit M, Keyhan M, Sylvester M, et al. (2014)). These findings promise to lead to measures for increasing the effectiveness of these antibiotics, an important result, given the serious threat posed by increased bacterial antibiotic resistance.

Biochemical Basis

The comprehensive resistance that stresses confer on cells is due to the fact that the core set of proteins are concerned with protecting vital cell biomolecules – proteins, DNA, cell envelope – from damage as well as to bring about repair of any damage that may still result. Envelope protection and reinforcement is afforded by proteins such as D-alanine carboxypeptidase, which likely increases peptidoglycan cross-linkage (Ghosh et al., 2008), and the products of the *otsBA* (*pexA*) genes which protect the cell membrane by promoting trehalose biosynthesis (Hengge-Aronis, 2002a). Furthermore, several periplasmic proteins concerned with the proper folding of proteins in this cell compartment are upregulated by stress; these include Dsb proteins that play a role in the formation or isomerization of disulfide bonds in proteins secreted into the periplasm, and peptidyl-prolyl isomerases concerned with the proper folding of proline-containing substrates. A consequence of stress is the accumulation in the periplasm of misfolded outer-membrane proteins (OMPs) due to the stress and excessive OMP synthesis. The OMP mRNAs are unusually stable. Two small noncoding RNAs, RybB and MicA, are induced under stress. These function as global mRNA repressors (Gogol et al., 2011a), and accelerate the decay of OMP mRNAs, thereby minimizing stress-induced damage by preventing excessive OMP production (Papenfort et al., 2006).

Protein repair

This is brought about by proteins called chaperones, which are a large and diverse group with indispensable physiological roles under all growth conditions, but which become more important under stress. Apart from conferring stress resistance, the chaperones are responsible for proper folding of nascent proteins and protein translocation across membranes. The chaperones DnaK, DnaJ, and GrpE, as well as GroEL and GroES are among the most extensively studied. These proteins are widely conserved through evolution: hsp70 is the eukaryotic homologue of the bacterial chaperone DnaK and hsp60 that of GroEL (Hendrick and Hartl, 1993).

It is thought that the nascent polypeptide chains or denatured proteins (referred to from here on as 'substrate proteins') bind DnaK and DnaJ (Figure 5). Interaction between the chaperones in the presence of ATP results in the formation of a ternary complex consisting of the substrate protein, DnaK–ADP, and DnaJ. Dissociation of this complex is mediated by interaction with GrpE and by binding of ATP. The final stages of folding/repair in most cases involve GroEL and GroES. This model is supported by several lines of evidence. For example, the denatured enzyme rhodanese aggregates in a buffer solution, but not in the presence of DnaK, DnaJ, and ATP, as the protein is protected by the ternary complex formation. Addition of GrpE, GroEL, and GroES results in efficient refolding and activation of the enzyme. In bacteria lacking these chaperones, newly synthesized proteins aggregate *in vivo*. However, this aggregation is prevented if the chaperone production is restored. Similarly, proteins imported into the yeast mitochondria from the cytosol show defective assembly in mutants missing hsp60 (GroEL homologue), and most soluble denatured proteins of *E. coli* form complexes with GroEL as a prelude to their repair. Strikingly, proteins in their native state do not interact with the chaperones. Exposure to stresses results in association of a large number of proteins *in vivo* with chaperones presumably to escape damage. In essence, chaperones are slow ATPases, which, when bound to ADP, have a high affinity for denatured proteins, but a low affinity for them when bound to ATP. These characteristics determine the duration of their action on an unfolded part of a protein and ensure the continuation of the process until renaturation is complete.

Bacteria are often used in industry and laboratory to overproduce heterologous proteins as the process is fast and economical. However, often the overproduced protein is denatured within the cell and precipitates, resulting in the formation of inclusion bodies. A protective role against this denaturation for DnaK was demonstrated by its overproduction in the cells. Human growth hormone (HGH) is produced industrially using *E. coli* transformed with a high copy number plasmid containing the *hgh* gene that encodes this hormone. In control cells producing normal levels of DnaK, the HGH produced in the cell formed massive inclusion bodies, but in cells overproducing this chaperone there was marked breakup of these bodies (Figure 6) and a corresponding increase in the soluble hormone (Blum et al., 1992). Recently, CysQ, another stress response protein has been shown to play a similar role (Lee et al., 2014).

DNA repair

Several enzymes induced by stresses are concerned with DNA repair. Examples are endonuclease III and IV, Dps (PexB), and AidB, which reverse DNA methylation. A role for DnaK in DNA repair has also been reported. A major mechanism for DNA repair is the SOS response, which is activated by many different stresses, such as starvation, oxidative stress, irradiation, and treatment with antibiotics that damage DNA. This response promotes various kinds of DNA repair such as excision repair. This is aimed at excising pyrimidine dimers and other bulky lesions found in damaged DNA. The enzymes involved are UvrABC endonuclease, which is made up of proteins encoded by the *uvrA*, *uvrB*, and *uvrC* genes, helicase II (encoded by *uvrD* gene), DNA polymerase I, and DNA ligase. The UvrABC endonuclease makes incisions on each side of the lesion, generating a 12 to 13 base pair oligonucleotide. Different components of the enzyme act separately in this process. UvrA and UvrB interact to form a $UvrA_2UvrB$ complex, which identifies the DNA lesion and locally unwinds it, producing a kink in the DNA of 130°. This is followed by

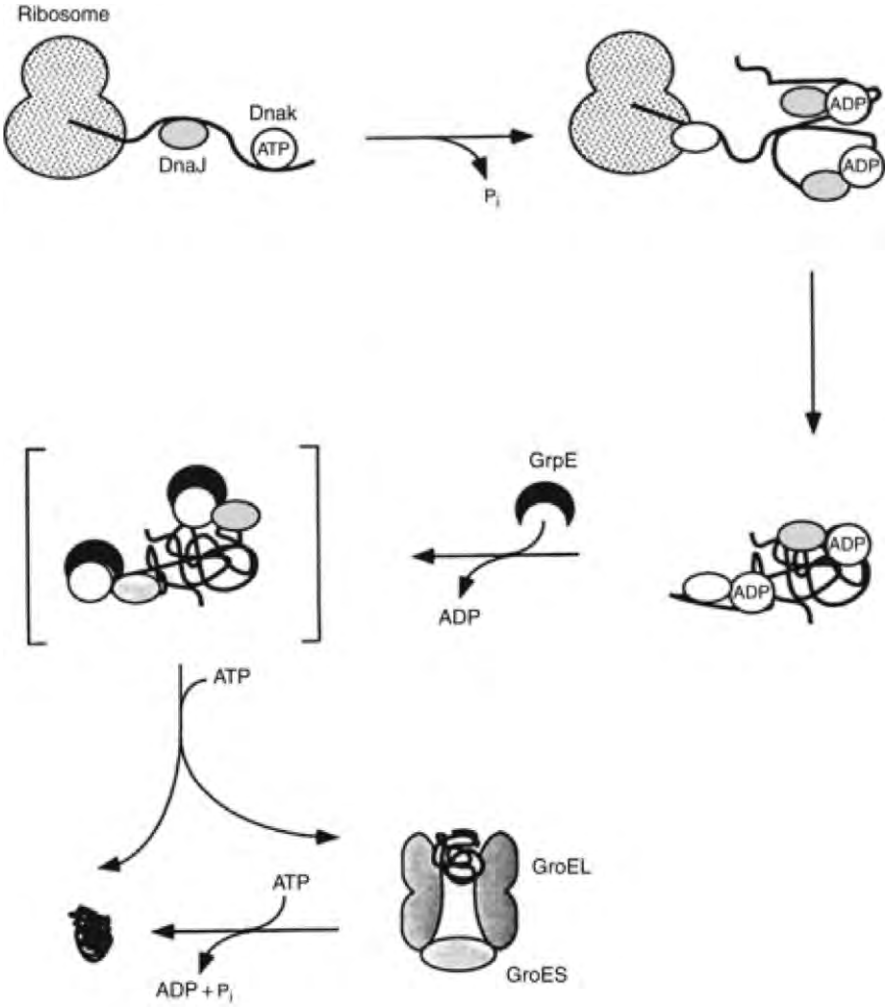

Figure 5 Schematic of the two-step pathway involved in the folding of nascent proteins and repair of damaged proteins. Reproduced from Mayhew M and Hartl F (1996) Molecular chaperone proteins. In: Neidhardt F et al. (eds.) *Escherichia coli and Salmonella typhimurium*: *Cellular and Molecular Biology*, pp. 922–937. Washington, DC: American Society for Microbiology.

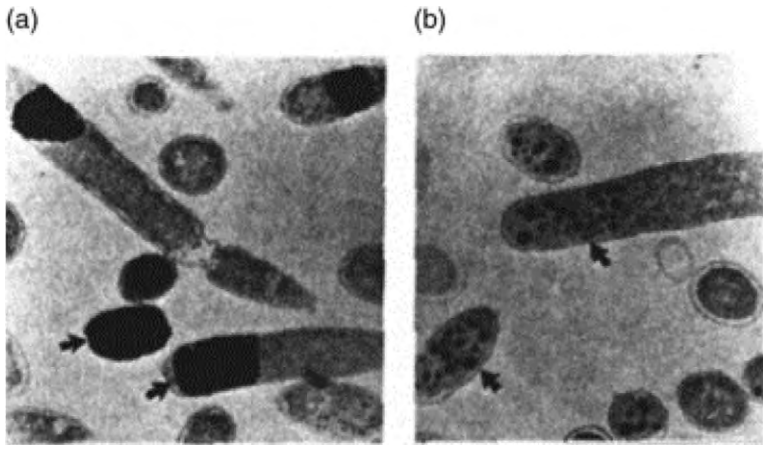

Figure 6 Transmission electron micrographs of *Escherichia coli* cells fixed in late exponential phase growth from cultures overproducing HGH protein. (a) Overproduction of HGH alone; (b) HGH overproduction along with that of DnaK. Note that in the latter, the HGH inclusion bodies are much smaller; and that a corresponding increase in soluble HGH was seen. Magnification, $\times$26000. Reproduced from Blum P, Velligan M, Lin N, et al. (1992) DnaK-mediated alterations in human growth hormone protein inclusion bodies. *Biotechnology* 10: 301–303.

dissociation of the UvrA protein and formation of a stable UvrB–DNA complex, which is acted upon by UvrC to make the incision. The function of helicase II is to release the oligonucleotide and to free UvrC after the excision of the nucleotide. The gap generated by the incision is filled by DNA polymerase I, which carries out the repair synthesis, and DNA ligase, which fills the remaining nick (Hoeijmakers, 1991).

Regulation of Stress Response

Shift in the cellular gene expression and protein synthesis profile under stressful conditions involves several factors, *viz.*, changes in the concentration of sigma factors, ancillary regulatory molecules, and chemical alteration in certain proteins. Salient examples of each will be discussed.

Sigma Factors

Sigma (σ) factors are small proteins that associate with the RNA polymerase (RNAP) 'core' enzyme and determine what promoter the resulting 'holoenzyme' will recognize (Figure 7). The core RNAP (abbreviated as E) is made up of four polypeptides, $\alpha_2\beta\beta'$. Examples of sigma factors that play a role in stress response are σ^{70}, σ^{s}, σ^{32}, σ^{E} and σ^{54}; their holoenzymes recognize specific DNA sequences present in a region called the promoter that is located, as a rule, 10 and 35 nucleotides upstream of the transcriptional start site. The σ^{70} holoenzyme $E\sigma^{70}$ is indispensable under all growth conditions and is referred to as the vegetative sigma factor. The consensus promoter sequences recognized by three of these holoenzymes are $E\sigma^{70}$: −10: TATAAT, −35: TTGACA; $E\sigma^{32}$: −10: CATNTA, −35: CTTGAA; and $E\sigma^{54}$: GG-N_{10}GC. ($E\sigma^{s}$-recognized promoters are discussed below.) It should be noted that considerable variations from these sequences are tolerated by different species of RNAP, the enzyme species differ in their promiscuity in this respect, and a given promoter sequence can be recognized by different RNAP depending on specific conditions. For example, during starvation or osmotic stress, the transcription of the gene encoding an oxidative stress protection protein, Dps (also known as PexB), depends upon increased cellular levels of $E\sigma^{s}$ (Lomovskaya et al., 1994). However, under oxidative stress, $E\sigma^{70}$ with the help of the ancillary factor, called the integration host factor (IHF), allows transcription of *pexB* without $E\sigma^{s}$ (Kolter et al., 1993). Other genes are also transcribed by different RNAP species depending upon the presence of modifying conditions.

While all of these holoenzymes have a role in different stresses, their major role is concentrated on particular conditions. Thus, $E\sigma^{70}$ primarily transcribes the exponential phase genes and those concerned with the stress-escape response; $E\sigma^{32}$, the heat shock and starvation genes; $E\sigma^{s}$, the genes that are commonly expressed under stresses in general; and $E\sigma^{54}$, genes of diverse functions

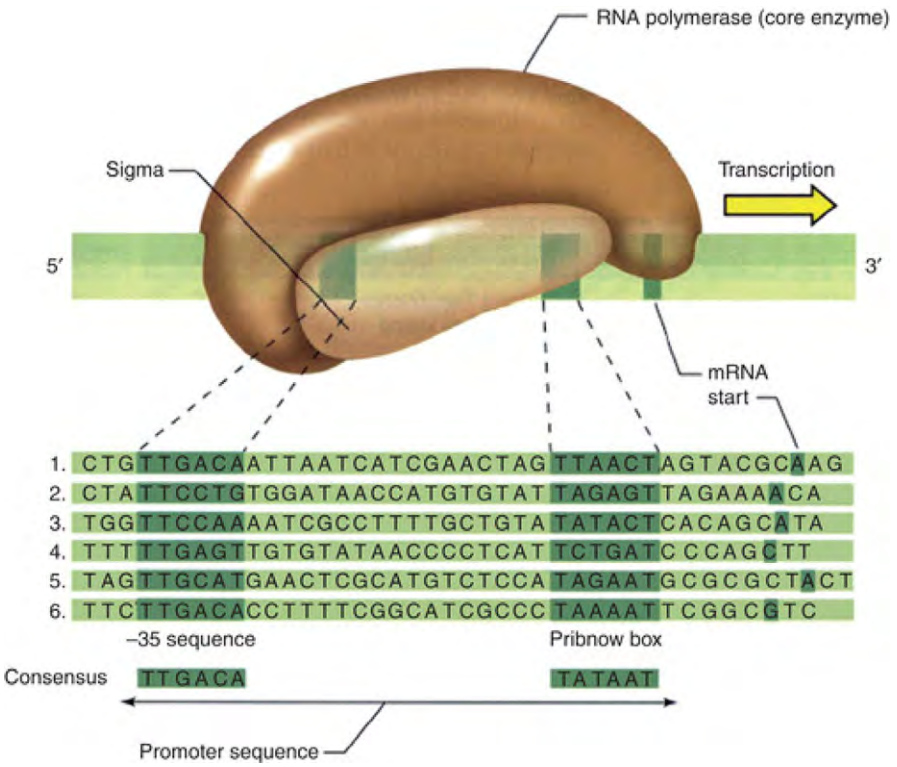

Figure 7 Schematic representation of RNA polymerase holoenzyme showing the 2.4 and 4.2 regions, which recognize respectively the −10 and −35 promoter elements. Reproduced from Madigan MT and Martinko JM (2006) *Brock Biology of Microorganisms*, p. vi. Upper Saddle River, NJ: Prentice Hall.

including those involved in starvation, flagellar synthesis, and in cell growth on nonpreferred substrates, such as environmental pollutants.

The RNAP holoenzyme most important in inducing the GSR in bacteria is $E\sigma^s$, as it controls the expression of some 140 core stress genes that are induced by diverse stresses and are responsible for this response. σ^s bears close homology with σ^{70} in critical regions of the sigma protein referred to as regions 2.4 and 4.2, which recognize respectively the −10 and −35 promoter elements. Indeed, $E\sigma^{70}$ and $E\sigma^s$ recognize many of the same promoters *in vitro*. *In vivo* however, under stresses such as starvation, despite the fact that σ^{70} is more abundant in the cells than σ^s, $E\sigma^s$ specifically targets the stress genes. Subtle differences in the promoter sequences and the role of ancillary factors account for this specificity.

Specific features of σ^s-recognized promoters

$E\sigma^s$-recognized promoters differ from those that $E\sigma^{70}$ recognizes in following respects. (1) They possess special features around their −10 region. Thus, a cytosine (C) at −13 position (i.e., 13 nucleotides upstream of the transcriptional start site) and a thymidine (T) at −14 facilitate $E\sigma^s$ binding to the promoter. Indeed, the −13 C may antagonize $E\sigma^{70}$ binding due to the differences in charged amino acids in the two sigma factors. In one instance, introduction of C at this position in a $E\sigma^{70}$ promoter improved its recognition by $E\sigma^s$. Adenine (A)/T-rich stretch is also involved, TAA at positions −6 to −4 being a common feature of $E\sigma^s$-recognized promoters; this feature may allow easier promoter melting (i.e., unwinding of the DNA strands to permit transcription). (2) $E\sigma^s$ can tolerate much wider deviations from consensus promoter sequences than $E\sigma^{70}$ and can, for example, recognize promoters with degenerate −35 sequences, possibly because it does not need such a sequence *in vivo*, or is able to recognize other sequences in place of this sequence. (3) While the requirement of a 17 base pair space between the −10 and −35 region is a strong preference of $E\sigma^{70}$, $E\sigma^s$ is more relaxed in this requirement. Indeed, many $E\sigma^s$-recognized promoters exhibit −35 like elements at other locations. (4) Certain AT-rich sequences present upstream of the −35 region favor $E\sigma^s$ binding to the promoter; the C-terminal domains of the RNAP α subunit play a role in this. (5) Both $E\sigma^{70}$- and $E\sigma^s$-recognized promoters tend to possess −10-like elements downstream of the transcriptional start site. Since early transcript complexes retain the sigma factors, these sequences cause the transcription to pause. σ^s is released more rapidly than σ^{70} from these complexes; thus the pause is shorter when $E\sigma^s$ is the transcriber, and this may facilitate $E\sigma^s$-mediated transcription of promoters that are recognized by both $E\sigma^{70}$ and $E\sigma^s$ (Hengge-Aronis, 2002b).

Other factors involved in favoring $E\sigma^s$-mediated transcription

Several *trans*-acting proteins seem to favor $E\sigma^s$-mediated transcription over that of $E\sigma^{70}$. Examples are H-NS, IHF, and Lrp. The mechanisms are not understood. In the case of H-NS, one possible mechanism is that the binding of this protein to a promoter interacting with $E\sigma^{70}$, but not $E\sigma^s$, renders the promoter unavailable for transcription. Changes in core RNAP, cytoplasmic ionic composition, as well as DNA supercoiling can also influence what RNAP species will transcribe a given gene.

A major factor responsible for a shift to different RNAP species under stress is competition for the RNAP core enzyme. The core RNAP concentration in bacterial cell is limiting and different sigma factors have to compete for it. σ^{70} possesses highest affinity for the core enzyme of all sigma factors and is present in excess; this accounts for the predominance of $E\sigma^{70}$ in unstressed cells. In stressed cells, even though σ^{70} retains its quantitative dominance, the balance shifts to RNAP species containing the alternate sigmas. Several factors account for this. $E\sigma^{70}$ dissociates so that core RNAP concentration goes up. The effectiveness of σ^{70} to bind to core RNAP is impaired due to the activity of the stationary phase-specific protein Rsd, and the small 6S RNA. σ^s has the lowest affinity of all sigma factors for RNAP and its increased synthesis under stress notwithstanding, it never attains more than one-third the level of σ^{70}. Nevertheless, it becomes the most active sigma factor in stressed cells because proteins like Crl, by binding to $E\sigma^s$, greatly enhance its activity. The small nucleotide, guanosine tetraphosphate (ppGpp), has a similar role; this is discussed further below. Certain cell metabolites such as glutamate and acetate may also have a role in stimulating $E\sigma^s$ efficiency (Foster, 2007; Paul et al., 2004). The mechanism by which σ^s concentration increases under stress has received a lot of attention and is discussed below.

Ancillary Regulatory Molecules

Cyclic AMP (cAMP)

As stated above, the core stress genes responsible for general resistance are transcribed mainly by $E\sigma^s$ and other species of RNAP bound to alternate sigma factors. However, $E\sigma^{70}$ does have a role in stress gene expression. The stress genes that this polymerase species transcribes tend to have weak promoters, that is, they deviate from the canonical promoter sequence that $E\sigma^{70}$ recognizes. Consequently, the transcription of these genes depends on the availability of ancillary transcriptional factors. This is the case with several starvation genes concerned with uptake of different compounds, and their efficient metabolism when they are present at low concentration. These genes are transcribed if cAMP is available. cAMP binds a protein called CRP, and the resulting complex binds to a specific sequence ($AGTGAN_6TAACA$) present upstream of the promoters of these genes, facilitating transcription by $E\sigma^{70}$. cAMP is present in cells at low concentration under nutrient-sufficient conditions but is increased dramatically during starvation, thereby promoting the transcription of these genes by $E\sigma^{70}$. The cAMP-dependent stress genes, however, play no role in enhanced general resistance, since starved cAMP-deficient strains exhibit the same degree of cross-protection against stresses in general as do cAMP-proficient strains. The role of these genes appears to be confined to the escape response by encoding proteins that enhance the cellular scavenging capacity by improving cellular uptake and metabolic functions.

Given the similarity between the $E\sigma^{70}$and $E\sigma^{s}$ promoters, the following finding is of interest: changing the position of the CRP-binding site in certain genes can alter promoter preference from $E\sigma^{s}$ to $E\sigma^{70}$ and vice versa.

Guanosine tetraphosphate (ppGpp)

The small nucleotide ppGpp has been studied intensively in the context of the stringent response, which refers to the phenomenon whereby amino acid starvation results in rapid downregulation of ribosomal RNA (rRNA) biosynthesis and ribosomes. It is now known that the concentration of this nucleotide goes up also in response to starvation for other nutrients as well as in stresses. Its synthesis, initially as pppGpp (which is later dephosphorylated to ppGpp), involves two pathways in *E. coli*: by the ribosome-associated protein RelA, when the ribosome A-site contains an uncharged tRNA during amino acid starvation, and by the protein SpoT, which is responsible for ppGpp synthesis in most other stresses. SpoT can also degrade ppGpp and thus has a dual role. A strain of *E. coli* missing both RelA and SpoT (referred to as ppGpp° strain) cannot synthesize this nucleotide and fails to lower its ribosome production under starvation conditions; such strains are referred to as relaxed strains. In other bacteria, for example, *Streptococcus mutans*, additional enzymes appear to be involved in ppGpp synthesis, such as RelP and RelQ.

In general, ppGpp positively affects the transcription of stress-related genes and negatively those related to growth. It exerts its regulation by binding to ββ′ subunits of RNAP near its active site, as has recently been confirmed by crystal structure. This regulation is affected by several mechanisms, such as direct effect on the rate of formation and stability of the open complex, interference with promoter clearance (which obstructs further rounds of transcription), and competition with nucleotide triphosphates used in mRNA synthesis.

A major role of ppGpp in the stress response is that it increases the ability of σ^{s} (and that of other minor sigma factors) to compete with σ^{70} for binding to the core enzyme. This has been shown in *in vitro* transcriptional assays and is supported by the finding that ppGpp-deficient cells exhibit decreased fractions of both σ^{s} and σ^{54} bound to the core polymerase. The protein DksA may have a role in augmenting this effect. As can be expected from these findings, absence of ppGpp greatly compromises starvation survival, and proteome and transcriptome analyses have shown that this is because of the lack of stress protein synthesis; instead, the cells continue to express growth-specific proteins. Thus, ppGpp is a necessary adjunct to σ^{s} for stress survival, and although much of this effect is likely to be affected by ensuring σ^{s} function, some are likely to be directly due to ppGpp activity.

ppGpp has important roles also in growing cells, where it is required for amino acid synthesis – a deficient strain cannot grow in the absence of exogenously provided amino acids. Further, ppGpp deficiency affects bacterial virulence, for example, expression of genes of pathogenicity islands (Magnusson et al., 2005).

Chemical Alteration in Proteins

Protein phosphorylation

An important mechanism in bacteria for sensing starvation and other stresses, which involves chemical alteration of proteins, is the so-called two-component system. One component of this pair is a histidine protein kinase (HPK) that autophosphorylates at a conserved histidine residue. In response to specific stimuli, the phosphorylated form is stabilized; for this reason, it is also called the 'sensor kinase.' In turn, the HPK phosphorylates the response regulator (RR) protein at a conserved aspartic acid residue. This phosphorylated form of the protein then activates transcription of the target loci. Several pairs of such proteins have been found; these initiate special adaptive strategies in response to specific environmental cues. The HPKs of different systems share homology of about 100 amino acids at their C-terminus; the RRs share homology in the 130 amino acid segments of their N-terminal ends. Among the environmental stimuli sensed by the different two-component systems are phosphate and nitrogen starvations, osmotic changes, and chemotactic stimuli. Here, the phenomenon is illustrated in the context of sensing phosphate starvation.

As stated above (Table 1), several genes are induced in response to phosphate starvation; together these genes are referred to as the phosphate regulon. This regulon is under the control of the *phoBR* operon encoding the PhoB and PhoR proteins. The PhoB protein is a positive regulator of this regulon, since:

1. Mutations in *phoB*, which inactivate the protein, or deletion of this gene, render the phosphate regulon noninducible.
2. Sequence analysis shows that upstream of the *phoA*, *phoBR*, *phoE*, and *pstS* (*phoS*) promoters is a highly conserved 18-bp region (CTNTCATANANCTGTCAN) called the phosphate box. *In vitro* studies demonstrate that purified PhoB protein binds to the phosphate box and that this binding is required for the transcription of the phosphate regulon genes.
3. PhoB bears close homology to the RRs in other systems, such as NtrC (involved in sensing nitrogen starvation) and OmpR (involved in sensing osmotic stress).

The *phoR* gene has a hydropathy profile typical of a membrane protein, and it shows homology to the HPK family of proteins. Like other sensor kinases, it autophosphorylates, a condition that is stabilized by phosphate starvation. It then phosphorylates PhoB, which activates the transcription of the phosphate regulon as discussed above.

Protein oxidation

This type of chemical alteration is involved in activating genes that protect against oxidative stress specifically in response to the ROS, H_2O_2, and O_2^-. A more general mechanism that activates many of the same genes in response to diverse stresses is controlled by σ^{s}, as discussed above.

H_2O_2 is generally sensed by the transcriptional factor OxyR and O_2^-, by the SoxR/Sox S proteins, although the two systems probably overlap. H_2O_2 directly oxidizes OxyR. The conserved cysteines, at positions 199 and 208, are in free thiol form in OxyR; H_2O_2 converts them to disulfide form. The resulting conformational change, which has been documented by crystal structure, enables OxyR to activate the transcription of genes involved in escape from oxidative stress (Table 1). Upon removal of the H_2O_2 stress, OxyR is reduced by glutaredoxin 1.

The SoxR protein is constitutively synthesized and also becomes activated by direct oxidation, in this case by O_2^-. The protein is a homodimer with two [2Fe-2S] centers per dimer; these centers are the loci of redox changes, that is $[2Fe\text{-}2S]^{1+} \rightleftarrows [2Fe\text{-}2S]^{2+}$ conversion. The oxidized SoxR activates *soxS* gene transcription, which in turn induces a collection of genes called the *soxRS* regulon (Figure 8). These genes encode enzymes that can decompose O_2^- (Table 1) as well as repair the damage to DNA that may result from oxidative stress, such as the endonuclease IV, mentioned above. At the termination of the stress, SoxR is reduced by an NADPH-dependent SoxR reductase (Storz and Imlay, 1999).

Regulation of σ^S Synthesis

As stated above, σ^s is the most important regulatory element in the GSR. Its cellular levels and/or activity increase in response to starvation for diverse individual nutrients as well as other stresses, and how this is accomplished is now understood in some detail at all three levels of control – transcriptional, translational, and posttranslational. I will discuss the results mainly in the context of starvation stress, unless the available information is confined to another stress.

Transcriptional control

The *rpoS* gene is located in an operon downstream of the *nlpD* gene and is transcribed from two promoters, one within the *nlpD* gene and the other upstream of this gene. Use of transcriptional fusions suggested regulation in *E. coli* at this level under starvation, and by ppGpp. However, direct measurement of *rpoS* transcription in *E. coli*, by quantifying the *rpoS* mRNA levels and determination of its half-life, indicated that enhanced transcription has no role in the observed increased levels of this sigma factor under carbon starvation. More recently, the BarA-UvrY two-component system has been found to stimulate *rpoS* transcription (Mukhopadhyay et al., 2000). Also, proteins have been identified that negatively regulate the transcription of this gene; these include the Fis and the ArcA-P proteins; cyclic AMP too has a similar role (Hirsch and Elliott, 2005). K173 of the RpoS protein may also play a role in regulating *rpoS* transcription.

Translational control

The main *rpoS* transcript contains an unusually long untranslated region (UTR), which is central to its translational control. The UTR may form two types of hairpin structures. One of these sequesters the translational initiation region (TIR) by pairing with a complementary sequence present within the coding region of the *rpoS* mRNA (called the antisense element), thereby making it unavailable to the ribosomes for translation. Other hairpins may form due to complementary sequences within the UTR. It is possible that both types of secondary structures have a role in regulating *rpoS* mRNA translation, although the involvement of the antisense element-mediated secondary structure in this regulation has not been documented yet. But considerable evidence is available indicating that secondary structures within the UTR minimize *rpoS* translation in unstressed cells and that their relaxation under certain stresses is the major reason for increased cellular σ^s concentration (Figure 9). Small noncoding RNAs (sRNAs) and the RNA-binding protein, Hfq, play a role in this phenomenon. For example, the sRNA, RprA, possesses a complementary sequence to

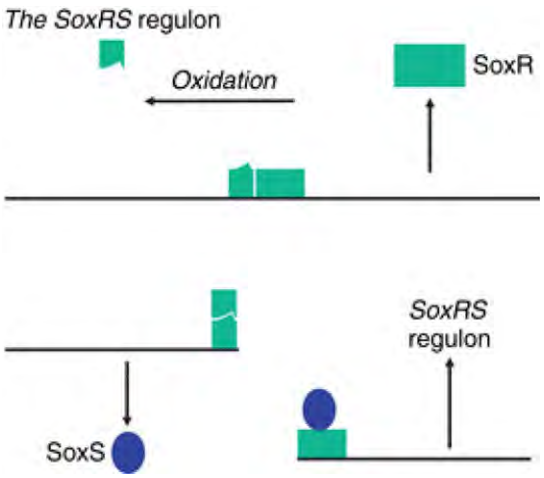

Figure 8 Schematic of SoxRS regulation of the genes involved in defense against O_2^- radical. The change in the configuration of the SoxR protein upon oxidation by O_2^- is schematically represented to show that in its altered configuration, it can activate SoxS transcription, which in turn activates the individual genes of the SoxRS regulon. Reproduced from Matin A (2001) Stress response in bacteria. In: Bolton S (ed.) *Encyclopedia of Environmental Microbiology*, vol. 6, pp. 3034–3046. New York: John Wiley and Sons.

the UTR stretch of *rpoS* mRNA, which is involved in hairpin formation. Base pairing and hydrogen bonding by this sRNA is able to unfold the hairpin, free TIR, and permit translation to proceed. Another sRNA, DsRA, is induced under cold stress and promotes *rpoS* translation by a similar mechanism. ArcZ and GcvB sRNAs also perform similar roles; the latter is encoded by the intergenic region of the *pst* operon. The CsdA protein and the CspA family of RNA-binding proteins likely assist in the DsrA annealing with the UTR while also stabilizing the *rpoS* mRNA; the CspC and CspE proteins stimulate *rpoS* translation possibly by affecting the mRNA structure, and the histone-like protein, HU, likewise stimulates *rpoS* translation. In contrast, the OxyS sRNA, down-regulates *rpoS* translation, and so do a number of proteins, e.g., the LrhA protein, and RNase III, which degrades the *rpoS* mRNA (Battesti et al., 2011; Hussein and Lim, 2011).

Under phosphate starvation, the synthesis of σ^s is thought to be regulated at the translational level (Mandel and Silhavy, 2005), but its mechanism is not known. Some five other sRNAs are known to affect *rpoS* translation, but none of these appears to have a role under these starvation conditions. It is possible that an as yet undiscovered sRNA is involved or that the control is exerted through modulation of the antisense element-mediated hairpin. Additional possibilities involve regulation through a variety of proteins that are known to regulate *rpoS* translation. These include the nucleoid protein HU that binds two regions in the *rpoS* mRNA and may influence its secondary structure; the histone-like protein StpA; the cold shock proteins CspC and CspE; a PTS protein; and DnaK. It is known that PhoB-P stimulates translation of *rpoS* mRNA, and it is surmised that the former may activate the transcription of an as yet unknown sRNA that is responsible for facilitating *rpoS* mRNA translation. An additional reason for the increase in RpoS levels under phosphate starvation is stabilization of this protein as is discussed below.

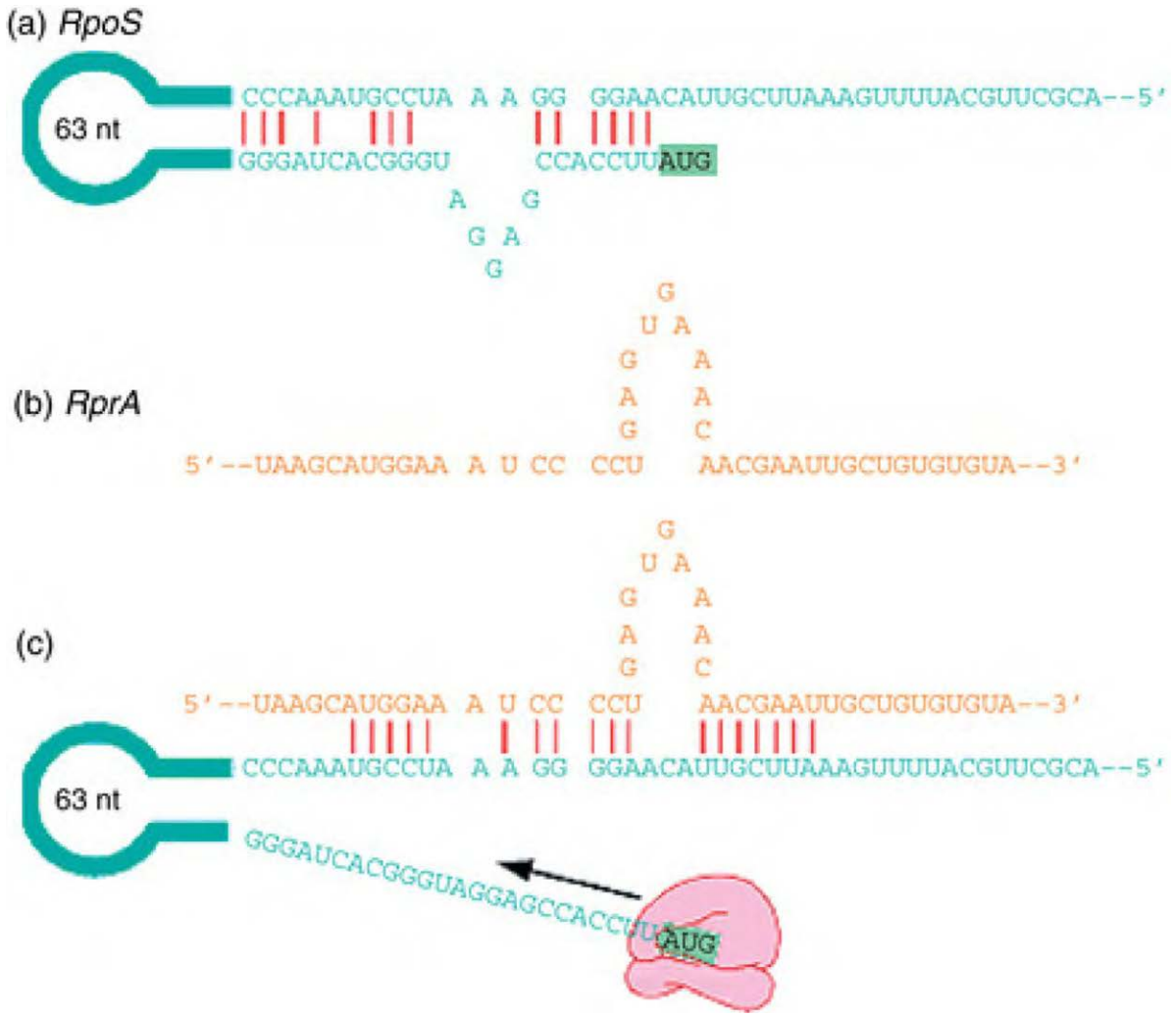

Figure 9 The untranslated region (UTR) of the *rpoS* mRNA that encodes σ^s. Note that the sequences upstream of the translational initiation codon (ATG) of the RNA includes regions of internal complementarity that result in the formation of a hairpin structure. This prevents the availability of the initiation codon. The small noncoding RNA, RprA, has regions of homology to the UTR of the *rpoS* mRNA (shown in red; B). Hydrogen bonding between the homologous regions of RprA and *rpoS* mRNA opens the hairpin, permitting translation (C). Reproduced from Matin A and Lynch SV (2005) Investigating the threat of bacteria in space. *ASM News* 71(5): 235–240. Washington, DC: American Society for Microbiology.

Posttranslational control-increased RpoS stability

It was thought that the control of σ^s synthesis in carbon starvation in *E. coli* occurred at the translational level. Direct measurements of *rpoS* mRNA translational efficiency, however, disproved this notion and showed that the increase under these conditions is solely due to enhanced stability of the sigma protein (Figure 10). The experimental results shown in Table 3 indicate this fact. In this experiment, the rates of *rpoS* mRNA and σ^s synthesis and their half-lives were measured, which permitted calculation of the *rpoS* mRNA translational efficiency, that is, the σ^s protein synthesized per unit of its mRNA. *E. coli* cells were cultured in a glucose-limited chemostat in order to precisely establish the relationship between dwindling glucose concentration in the medium (with decreasing dilution rate) and the above mentioned parameters (Table 3). As the available glucose diminished, both σ^s synthesis rate and *rpoS* mRNA translational efficiency declined. Meanwhile, however, the stability of the sigma protein increased from 7- to 16-fold, accounting for the observed overall increase in the cellular levels of σ^s (Zgurskaya et al., 1997); this was subsequently conformed (Mandel and Silhavy, 2005).

What accounts for the instability of the σ^s protein under carbon-sufficient or other non-starvation/stress conditions? The answer to this question was provided by the discovery that a specific protease, called ClpXP, which is composed of two proteins, ClpX and ClpP, is involved in this regulation (Schweder et al., 1996). It rapidly degrades σ^s in unstressed cells, but not in those experiencing starvation (Figure 10). ClpP is a tetradecameric peptidase and has 14 active sties. ClpX is a hexameric ATPase, which binds to one or both ends of the ClpP chamber (Figure 11). ClpX binds directly or indirectly to proteins to be degraded and, using the energy provided by its ATPase activity, feeds them into the ClpP chamber. ClpX belongs to a family of proteins referred to as AAA+ATPase (ATPases associated with diverse cellular activities), and directly binds to proteins containing a short C-terminal sequence ('tagon'). The tagon is added by SsrA, or tmRNA to, for example, proteins with defects due to stalled ribosomes or other factors, causing these proteins to be rapidly degraded by the ClpXP protease. (It is estimated that some 5 proteins per thousand that *E. coli* synthesizes are destroyed by this quality control mechanism (Baker and Sauer, 2012; Moore and Sauer, 2007a).)

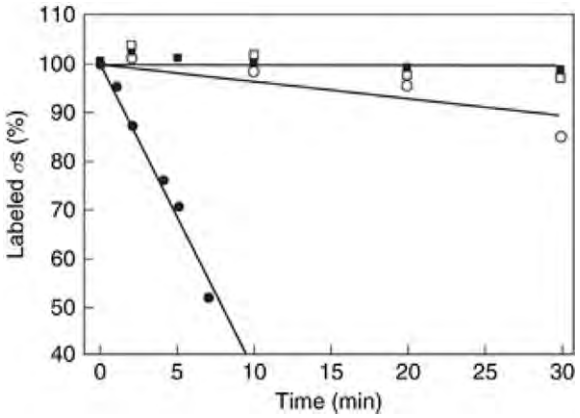

Figure 10 Comparison of σ^s stability in exponential phase (solid symbols) and stationary phase (open symbols) cultures in *clpP*-proficient (circles) and *clpP*-deficient (squares) backgrounds. Note that in a wild-type background, σ^s is stable only in the stationary phase, but in a mutant missing the Clp protein, it is stable in both the phases of growth. Reproduced from Schweder T, Kyu-ho L, Lomovskaya O, et al. (1996) Regulation of *Escherichia coli* starvation sigma factor (σ^2) by ClpPX protease. *Journal of Bacteriology* 178(2): 470–476.

Table 3 σ^s synthesis rate and *rpoS* mRNA translational efficiency in glucose-sufficient cells and those subjected to increasing degree of glucose starvation (last three rows)

Glucose concentration (M)	*σ^s concentration*[a]	*σ^s half-life (min)*	*σ^s synthesis rate*[b]	rpoS *mRNA concentration*[c]	rpoS *translational efficiency*[d]
10^3 (glucose sufficiency)	190	5	55	1.0	1.0
2.2×10^6	270	11	34	0.75	0.75
1.3×10^6	300	34	13	0.52	0.52
1.2×10^6	570	>60	ND	0.5	0.5

ND, not determined.
[a]pmol mg^{-1} cell protein.
[b]pmol mg^{-1} cell protein per min.
[c]Relative units.
[d]σ^s synthesis rate/*rpoS* mRNA concentration.
Source: Reproduced from Zgurskaya HI, Keyhan M, and Matin A (1997). The σ^s level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. *Molecular Microbiology* 24(3): 643–651.

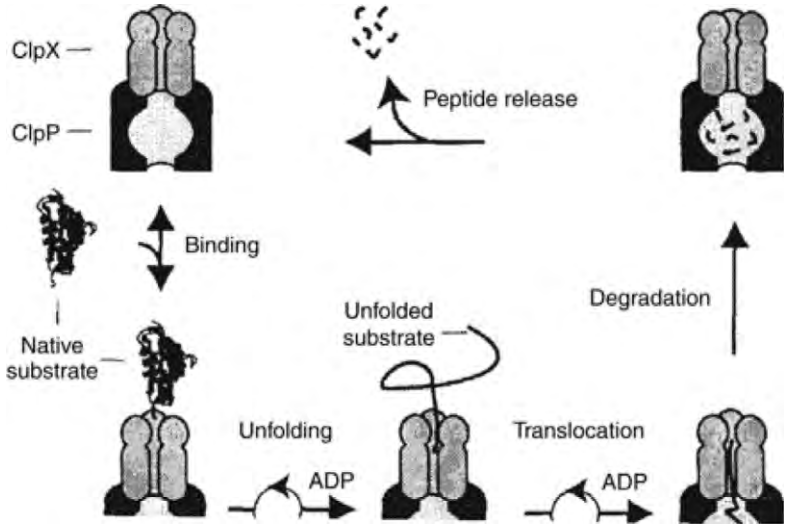

Figure 11 Schematic representation of native protein degradation by ClpXP protease. The ClpX component of the protease binds the substrate protein and unfolds it by its ATPase activity. The unfolded protein is translocated through the ClpP chamber, a process that also requires ATP, and is degraded; the resulting peptide fragments are released. Reproduced from Kenniston JA, Burton RE, Siddique SM, et al. (2004) Effect of local protein stability and the geometric position of the substrate degradation tag on the efficiency of ClpXP denaturation and degradation. *Journal of Structural Biology* 146: 130–140.

If ClpXP protease can degrade σ^s in exponential phase cells, why does this protein become resistant to this protease in the stationary phase? The answer to this and related questions has revealed involvement of a control mechanism involving adapter and anti-adapter proteins. While, as mentioned above, ClpXP can recognize proteins that are candidates for degradation, it does not recognize RpoS and requires the adapter protein RSSB for gaining access to it. Schweder et al. (1996) showed that a stretch of amino acids spanning K173 to K188 residues in RpoS protein was required for its cleavage by the ClpXP protease. Subsequent work has shown that K173 is essential for RpoS cleavage: K173/E mutation abolishes RpoS proteolysis. Also, E174 and V177, although not absolutely required, facilitate this degradation (Becker et al., 1999). It is likely that RssB binding alters the conformation of RpoS, thereby exposing the site situated close to the N-terminus of this protein that binds to ClpX.

Under non-starvation conditions RSSB maintains an active configuration promoting RpoS degradation, and its inactivation under starvation accounts for the increased stability of σ^s. RssB is a homologue of RR proteins, mentioned above, but is unique in its C-terminal output domain. By analogy to other RRs, its activation may require phosphorylation, and it may be dephosphorylated, for instance, in starving cells due to the ArcB/ArcA two component system. ArcB senses the redox status of quinones; when these are oxidized, disulfide bond formation within ArcB results in its inability to autophosphorylate (Malpica et al., 2004) resulting in dephosphorylation of ArcA, which is thought to donate the phosphate group to RssB. Thus, RssB would be primarily dephosphorylated and may be inactive for this reason in starving cells leading to increased RpoS stability. However, it is not certain that phosphorylation of RssB is essential for its activity, since removal of the aspartate residue from RssB, which is thought to be the phosphorylation site, does not inactivate it. Another explanation for RSSB inactivation involving anti-adapter proteins has emerged. Three such proteins are known, IraP, IraM and IraD, which are induced in response to different stresses. Thus, IraP is induced under phosphate starvation, and IraD under conditions leading to DNA damage (Battesti and Gottesman, 2013). RSSB has low cellular levels and the antiadapters are able to saturate it. Despite this shared activity, they do not bear structural homology, although several contain conserved RR domains, and their activity involves interaction with the flexible N-terminal domains of the ATPase components of the proteases. ppGpp, a global regulator of stringent response (see above), is a positive regulator of the *ira*P promoter; this may further explain the observed up-regulation of σ^s by ppGpp (Gentry et al., 1993). Additionally, H-NS, a histone-like protein, negatively regulates σ^s by modulating the translational efficiency of *rpo*S mRNA and the stability of newly synthesized σ^s. H-NS is a DNA-binding protein known to regulate genes at transcriptional level. The precise molecular mechanisms by which it controls σ^s at the post-transcriptional level remain to be fully elucidated (Barth et al., 1995; Yamashino et al., 1995). Its negative influence on σ^s stability is likely mediated by the suppression of *ira*M and *ira*D expression.

It is interesting to note that the K173-K188 region corresponding to 519–564 nucleotides of the *rpoS* mRNA constitutes the antisense element, which as mentioned above, may also have a role in translational regulation of Sigma S synthesis, and that, as already mentioned, K173 may be important also in transcriptional control. Thus, the K173-K188 stretch of the RpoS protein could be involved in regulating Sigma S synthesis at all three levels of regulation.

Activity control

Control at the level of activity of σ^s evidently operates in nitrogen starvation. Under these conditions, the core set of proteins are still synthesized even though σ^s levels show only a very modest increase. Thus, it is thought that the sigma protein is more active under these conditions. The factors that may account for this are hypothesized to be those that increase the competitiveness of σ^s for RNAP. These have been discussed above.

Regulation under low-shear/simulated microgravity conditions

Low-shear environments, such as brush border microvilli of the gastrointestinal, respiratory, and urogenital tracts (Guo et al., 2000), are common routes of microbial infection. Low shear environments closely resemble microgravity conditions experienced by astronauts during space flight. There has therefore been considerable interest in studying the biological effects of these conditions. On Earth, the effects of such environments are simulated by the use of a special cultivation equipment that utilizes high aspect to ratio vessels (HARVs). There is strong evidence that these conditions weaken the human immune response (Stowe et al., 2011) and make bacteria more virulent and stress-tolerant (Lynch et al., 2004; Wilson et al., 2002); these have obvious implications for the control of disease on Earth and on astronauts' health. Studies on the regulation of this phenomenon have resulted in some intriguing findings. Thus, the increased bacterial resistance that low-shear environments confer on bacteria appears to be independent of σ^s in exponential but not in stationary phase. Further, these environments markedly enhance *rpoS* translational efficiency regardless of the growth phase and promote σ^s instability, especially in the exponential phase. Since both these regulatory phenomena involve macromolecular folding pattern, the findings raise the possibility that low-shear/microgravity environments can influence these patterns.

Sensing starvation

Given that the regulation of the starvation response differs depending on the missing nutrient, it seems likely that the dearth of different nutrients is sensed by different mechanisms. The sensing mechanism in the case of carbon starvation could be an effector that inactivates RssB or ClpXP. Recent reports indicate that an increase in denatured proteins may have a role. Starvation affects fidelity of ribosomes, resulting in the synthesis of abnormal proteins with a proclivity for oxidation. The latter sequester Clp, impairing ClpXP activity, resulting in the stabilization of σ^s. In this view, starvation is sensed by the increase in aberrant proteins. Phosphate and nitrogen starvations may involve the PhoBR- and NtrBC-sensing systems mentioned above (Battesti et al., 2011). In *P. putida*, a G-protein, called FlhF, which is situated at the cell pole and controls flagellar localization on the cell, may be involved in sensing stress, as its absence robs the cell of the capacity to develop the general stress resistance (Pandza et al., 2000).

Concluding Remarks

It is evident that in response to hostile and frequently fluctuating conditions in nature, bacteria have evolved highly sophisticated mechanisms that permit them to swiftly shift between rapid growth and static survival modes. Our understanding of this phenomenon has enhanced greatly in the last two decades, and further progress is likely to yield information that will permit better control of bacterial growth – its enhancement toward beneficial ends, such as ecosystem management, industrial processes, and bioremediation, as well as its mitigation, as in disease.

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Stress Responses: Heat☆

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Glossary

Homologues Genes sharing common ancestry, often determined by structural comparison.
HSE Heat shock element, it is a sequence in the promoter region of heat shock genes to which HSF binds.
HSF Heat shock transcription factor.
HSP Heat shock protein, chaperones, proteases, and regulatory proteins produced in response to temperature changes and other stresses.
MDR Multidrug resistant microorganism.
Operon Genes within the same transcriptional unit under the control of a common promoter element.
Regulon A set of genes under the control of a common regulator.
Repressor A protein or a regulatory element that inhibits transcription or translation, respectively.
Sigma factor It is the subunit of bacterial RNA polymerase needed for initiation, it is the major influence on selection of promoters.

Abbreviation

CIRCE	controlling inverted repeat of chaperone expression
ECF	extracytoplasmic function
ER	endoplasmic reticulum
HAIR	HspR-associated inverted repeat
HS	heat shock
HSE	heat shock element
HSF	heat shock transcription factor
HSPs	heat shock proteins
RNAP	RNA polymerase
ROSE	repression of heat-shock gene expression
SD	Shine–Dalgarno
TBP	TATA element-binding protein
TFB	transcription factor B

Defining Statement

All living cells, from microorganisms to human cells, respond to a temperature upshift by transiently increasing the rate of synthesis of a set of proteins denominated heat shock proteins (HSPs), which maintain protein homeostasis by alleviating protein misfolding defects and aggregation, thus protecting the cells from damage. The increase in HSP levels is mainly due to the induction of transcription of the corresponding genes. Although the HSPs are among the most conserved proteins in nature, the mechanisms responsible for the transcriptional control of heat shock (HS) genes vary greatly. Bacteria, for instance, can control HS gene expression either negatively or positively, using either repressors that act by inhibiting HS gene expression at normal temperatures being inactivated at high temperatures, or alternative sigma factors that once activated induce transcription during heat shock, or in some bacteria both mechanisms take place. HS response has also been linked to antimicrobial resistance in pathogenic bacteria. In eukaryotic microorganisms like *Saccharomyces cerevisiae* induction of the HS response occurs by the activation of a heat shock transcription factor (HSF1) which exists constitutively as a trimer bound to a specific DNA element, the heat shock element (HSE), present in multiple copies in HS gene promoters. In higher eukaryotes a family of HSFs is observed, with mammalian cells containing four members: HSF1, HSF2, HSF3 and HSF4. Each of them possesses unique and overlapping functions, exhibiting

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tissue-specific patterns of expression, multiple post-translational modifications and interacting protein partners. These HSFs are present as monomers under physiological conditions, differently from *S. cerevisiae*, with HSF1 being the most potent and dominant. Activation of HSF1 occurs by the formation of trimers and extensive post-translational modifications, followed by its binding to the HSEs in the HS gene promoters. The control of HS gene expression in Archaea is not well understood, but a repressor has been characterized which is inactivated by a temperature upshift. HSPs have shown to be involved in addition to the HS response in the control of the cell cycle. The evidence being that accumulation of misfolded proteins in both bacteria and yeast, for instance, has been observed to cause G1 arrest. HSPs have also been strongly associated with microbial infections and with human diseases such as cancer and neurodegenerative diseases making the study of these proteins of even greater importance.

Introduction

The viability of all organisms crucially depends on the integrity of its constituent components, correctly folded proteins being one of the most important actors in all cellular processes. The folding state of proteins within cells is monitored by an energy-dependent quality control network of ATP-dependent proteases and molecular chaperones, which operates under all growth conditions but becomes extremely important during stress. In order to maintain homeostasis for protein folding, cells tightly regulate the expression of chaperones and proteases to compensate for environmental perturbations. Exposure of cells and organisms to high temperatures can cause misfolding and aggregation of proteins, which if not acted upon can lead to cell death.

The heat shock (HS) response is the way by which cells counteract to prevent the potential damages due to temperature upshift and consists in the induction of almost all the universally conserved HS genes. These genes encode chaperones, proteases, and other stress-related proteins, which play important roles in helping cells cope with the toxic effects due not only to high temperature exposure but also to several other environmental stresses, such as starvation for carbon or amino acids, DNA-damaging agents, oxidative stress, and heavy metals.

The first report of the HS response was by Ritossa (1962) who discovered the heat inducible chromosome puffs in the salivary glands of *Drosophila melanogaster* larvae. Subsequently, the puff-associated genes and proteins were identified initiating a rapidly expanding research field on HS. In bacteria, the HS response of *Escherichia coli* was the first to be characterized and the master regulator, the HS sigma factor σ^{32}, has been the first alternative sigma factor to be discovered in this organism. Later, many other bacteria have had their responses to high temperature investigated, and different control mechanisms have been revealed for the induction of HS genes, showing that even though the response is almost universally conserved, the molecular solutions for triggering gene induction can be quite distinct.

The sequential molecular events in the HS response include drastic repression of normal transcription and translation pathways, and activation of transcription of HS genes. The increased expression of HS genes is transient, and as the heat shock proteins (HSPs) accumulate and repair or prevent the damages caused by temperature stress through their chaperone and protease activities, the cells can return to nearly normal growth conditions, downregulating HSP gene expression. Both prokaryotic and eukaryotic cells have established a number of complex strategies to tightly control the transcription of HS genes under any given condition and here we will show some of these mechanisms of control. In addition, the function of HSPs will also be discussed.

Control of Heat Stress in Bacteria

Positive Control by Alternative Sigma Factors

The HS response in the Gram-negative bacterium *E. coli* is the best-characterized stress response in bacteria, and its regulation involves the use of minor sigma factors. The HS sigma factor σ^{32}, also called σ^{H}, was the first alternative sigma factor to be uncovered in *E. coli* and it controls the major HS regulon in this bacterium, coping with cytoplasmic protein damage. The second HS regulon is controlled by sigma factor σ^{E} and protects cells against extracytoplasmic stress or extreme HS in *E. coli*. A third regulon of the stress response has been identified more recently and is under the control of σ^{N} or σ^{54}. This regulon includes the phage shock operon (*pspABCDE*) and the small HSP encoded by the *ibpB* gene. Its regulation is not well understood and will not be discussed further.

The E. coli σ^{32} regulon

The σ^{32} regulon includes the major HSPs, encoded in operons or single genes, such as DnaK, DnaJ, GrpE, GroES, GroEL, ClpB, ClpX, ClpP, Lon, FtsH, and several other proteins. The most important chaperone machines such as DnaK/DnaJ/GrpE, ClpB, and GroES/EL, which protect the cell against cytoplasmic stress, belong to this regulon, as well as the proteases ClpXP, Lon, and FtsH, which degrade the irreversibly denatured proteins unable to be recovered.

Expression of these HSPs is rapidly enhanced upon temperature upshift due to the transient increase in the levels of σ^{32}, which occurs mainly by a higher rate of synthesis and temporary stabilization and activation of the HS sigma factor. The increased σ^{32} levels leads to the formation of σ^{32}-RNA polymerase (RNAP) holoenzyme, which transcribes the HS genes. The higher rate of synthesis of σ^{32} during HS is not due to increased transcription of the *rpoH* gene, which encodes the HS sigma factor, since its mRNA levels show only a small increase during temperature upshift. Even though the regulatory region of *E. coli rpoH* is rather complex, containing several distinct promoters, none of them is dependent on σ^{32} itself.

An mRNA thermosensor

The translational control of σ^{32} is mediated by the secondary structure of the *rpoH* mRNA. Deletion analysis of a translational fusion *rpoH–lacZ* revealed two regions near the 5′-end portion of the mRNA (regions A and B) involved in thermoregulation. Region A (6–20 nt from the AUG initiation codon) and region B (112–208 nt) form a secondary structure by base pairing, which includes the AUG preventing ribosome binding and translation of the mRNA at normal temperatures (Figure 1). At HS temperatures, the base pairing is destabilized enhancing the translation of *rpoH* mRNA.

Experimental evidences for such an RNA structure were generated by point mutations, deletion analysis, and structure probing correlating the RNA thermostability with the corresponding expression levels of σ^{32}. Furthermore, no evidence for the contribution of additional cellular factors was found suggesting the RNA structure itself as the molecular thermometer.

Control of σ^{32} stability

During normal growth temperatures σ^{32} is quite unstable, with a half-life of less than 1 min. However, a remarkable (at least eightfold) transient stabilization of the protein occurs immediately upon temperature upshift, which is responsible at least in part for the practically instantaneous induction of HSP synthesis. Degradation of σ^{32} can be carried out by several proteases, however, FtsH, an ATP-dependent metalloprotease located in the cytoplasmic membrane, was the first to be implicated in this process. In addition, the absence of FtsH causes complete stabilization of σ^{32} *in vivo*. Furthermore, the DnaK/DnaJ/GrpE chaperone machine seems to be involved in the turnover of σ^{32}, as absence of any of these proteins causes stabilization of the sigma factor at normal temperatures. However, *in vitro* degradation of σ^{32} by FtsH is not affected by any of the members of DnaK chaperone system, indicating that the effect may be indirect.

Role of DnaK in the turnoff of the HS response

Changes in the levels of DnaK affect not only the induction of the HS response in *E. coli*, but also the downregulation of the response. Cells with low levels of DnaK have a prolonged shutoff phase, whereas cells with high levels of this chaperone turn off the response more rapidly. Negative regulation of HSP gene induction by DnaK is mediated primarily by its direct association with σ^{32}, which leads to sigma factor inactivation and degradation by FtsH. This effect has been explained by the titration of DnaK by unfolded proteins during temperature upshift. When unfolded proteins are in excess relative to DnaK, DnaK dissociates from σ^{32} to

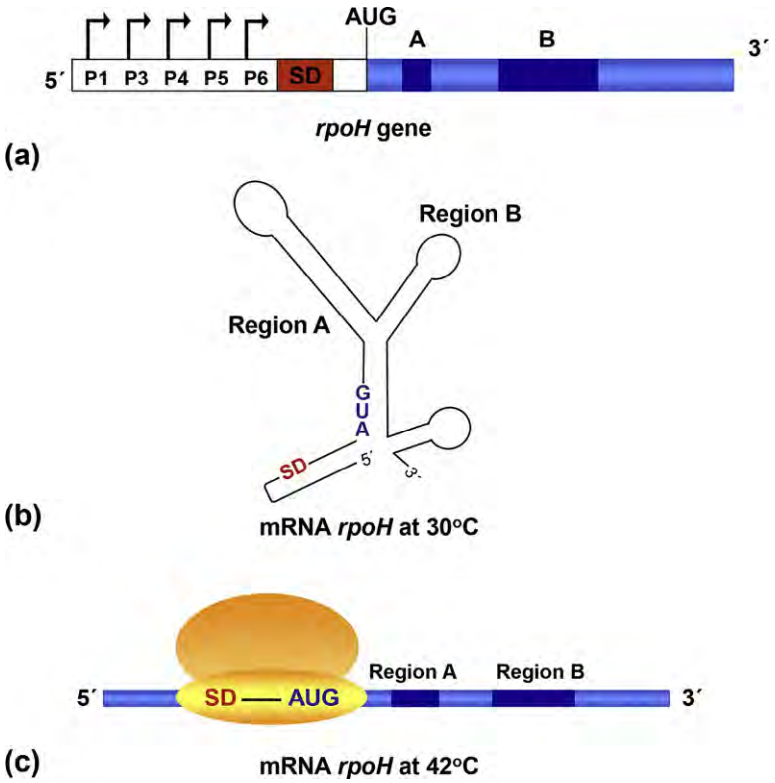

Figure 1 RNA thermometer controls the expression of *rpoH* gene. (a, b) Expression of *Escherichia coli* heat shock transcription factor σ^{32}, encoded by the *rpoH* gene, depends on five promoters differently modulated by three sigma factors (σ^{70}, σ^{E}, and σ^{54}) and the secondary mRNA structure is involved in its translational control. (b) At physiological temperatures (30 °C), the Shine–Dalgarno (SD) sequence, the AUG start codon, and two specific regions (A and B) in the coding region of the *rpoH* gene generate a secondary structure that represses the translation process. (c) Melting of the secondary structure at high temperatures (42 °C) permits ribosome access and translation initiation.

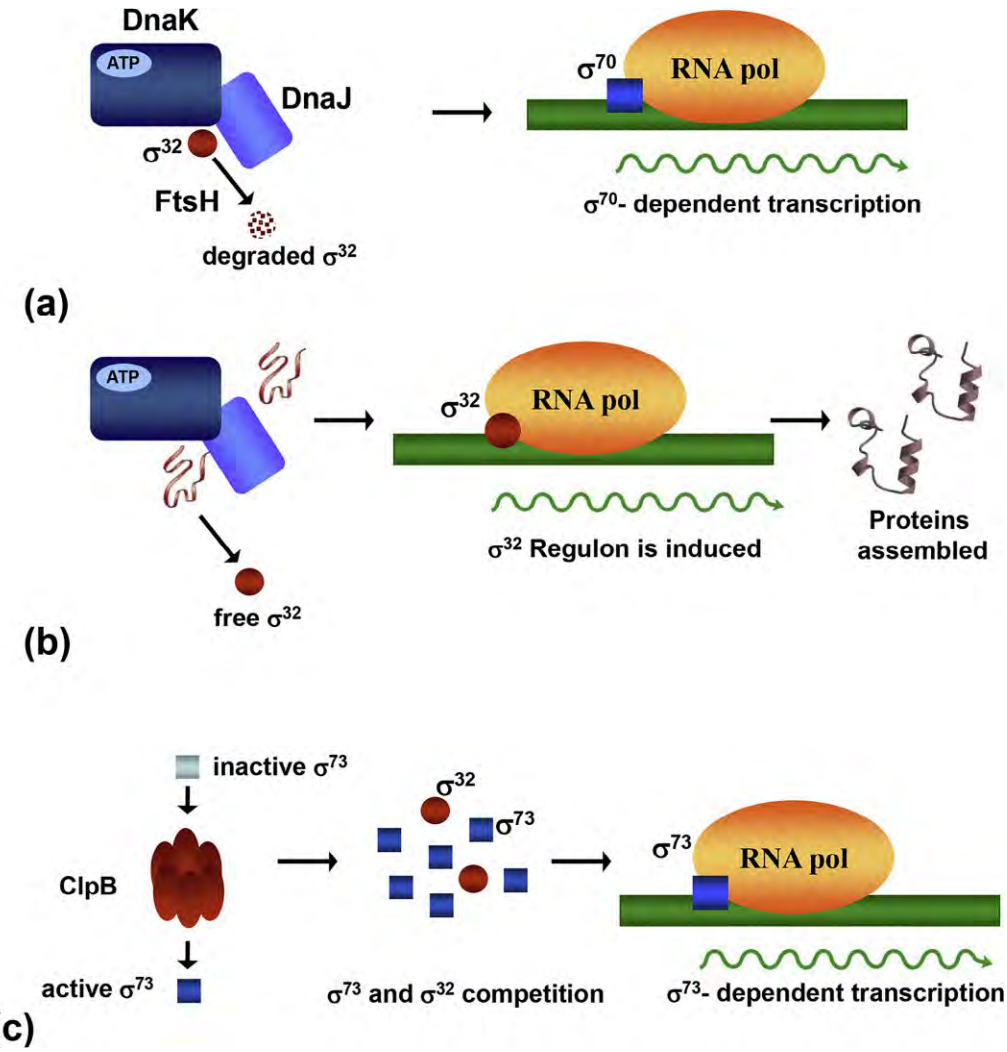

Figure 2 Induction and downregulation of the heat shock (HS) response in bacteria. (a) The direct DnaK association with σ^{32} leads to heat shock transcription factor inactivation and degradation by FtsH at normothermic temperatures. (b) Under HS stress, unassembled proteins are in excess, DnaK dissociates from σ^{32} and exerts its chaperone activity. The transcription complex RNA polymerase (RNAP)–σ^{32} is formed and transcription of HS genes increases. In *Escherichia coli*, the accumulation of chaperones, including DnaK, causes denatured proteins to acquire a correct folding, liberating DnaK to promote the negative regulation of σ^{32} and downregulation of the HS response. (c) In *Caulobacter crescentus*, changes in the amount of DnaK/DnaJ only affect the induction phase of the HS response. Reactivation of heat-inactivated σ^{73} (σ^{70} homologue) in this bacterium is dependent on ClpB chaperone activity, and the competition between σ^{32} and σ^{73} for the RNAP core seems to be the most important factor during the recovery phase.

exert its chaperone activity. Free σ^{32} then binds the RNAP core and transcription of the HS genes increases drastically, the *dnaKJ* genes among them. With the accumulation of DnaK/DnaJ and other HSPs, unfolded proteins are correctly folded, liberating DnaK to associate again with σ^{32} inactivating it, and leading to sigma factor degradation and the turn off of the HS response (Figure 2). Recently, the involvement of the chaperones GroES/EL together with the DnaK chaperone system in the regulation of the HS response in *E. coli* has been described, indicating that a cellular network regulates the activity of the HS sigma factor σ^{32}.

rpoH orthologues in other Gram-negative bacteria

Orthologues of *rpoH* have been described in many other proteobacteria of α, β, and γ subgroups, but not in the subgroups δ and ε or in Gram-positive bacteria. In most cases, only one *rpoH* gene has been found, but in some two or even three genes were characterized. In *Agrobacterium tumefaciens* and *Caulobacter crescentus*, bacteria from the α group, a single *rpoH* gene has been found. Interestingly, a marked increase in transcription is observed for the *rpoH* gene during HS in these bacteria, and the 5′ portion of its mRNA is not predicted to form a secondary structure, unlike the situation observed in *E. coli* and other bacteria of the γ subgroup. The increased transcription observed for the *rpoH* gene in *Caulobacter* and *Agrobacterium* is due to the presence of an RpoH-dependent promoter. However, an essentially normal induction of HSPs was observed in *Agrobacterium* even with mutant strains unable to increase RpoH levels upon HS. These data indicate that controlling the activity rather than the amount of σ^{32} is the most important mechanism for the induction of the HS response in this bacterium. Furthermore, even though σ^{32} levels increase

transiently during HS, DnaK-mediated control of σ^{32} activity was demonstrated to play a primary role in the induction of the HSP synthesis. In addition, in *Caulobacter*, it was shown that downregulation of the response is independent of DnaK levels, whereas the chaperone ClpB is necessary for normal turnoff of HSP synthesis. This important role of ClpB in the shutoff of the HS response was suggested to be related to the capacity of this chaperone to renature the major *Caulobacter* sigma factor, names σ^{73}, in conjunction with the DnaK chaperone machine, which would then compete with σ^{32} for the RNAP core, leading to the recovery of normal protein synthesis (Figure 2(c)).

The root-nodulating bacteria *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* contain three and two *rpoH* genes, respectively, which are regulated by distinct mechanisms. In *B. japonicum*, each *rpoH* gene is regulated differently. Transcription of *rpoH1* is HS inducible by an unknown mechanism, and *rpoH2* transcription decreases during HS, with both genes being transcribed from sigma 70-dependent promoters. The *rpoH3* gene is in a probable operon with two genes coding for a classical two-component regulatory system, with transcription initiating from a promoter similar to σ^{32}-dependent promoters. In addition, the distinct RpoH proteins transcribe different sets of genes. $RpoH_2$ seems to be essential for the synthesis of cellular proteins under physiological growth conditions, whereas $RpoH_1$, and probably also $RpoH_3$, are involved in their synthesis during the stress response. In *S. meliloti*, cells containing an *rpoH1* gene mutation are impaired in growth at 37 °C under free-living conditions and are defective in nitrogen fixation during symbiosis with alfalfa. In addition, expression of *rpoH1* increases upon culture entry into the stationary phase of growth, but not under HS conditions. Cells containing an *rpoH2* mutation have no apparent phenotype, and its expression increases later in stationary phase. Since both *rpoH1* and *rpoH2* are induced in stationary phase, the proteins they encode could play roles in the general stress tolerance that develops in starved cells.

The σ^E regulon

The second HS sigma factor of *E. coli* was identified from studies of *rpoH* transcription at extreme temperatures (45–50 °C). One of its promoters (P3) is activated at high temperatures and is dependent on σ^E, which places the σ^{32} regulon under the control of σ^E under extreme heat stress. The σ^E regulon is involved in protection against extracytoplasmic stress, with its members including a periplasmic protease DegP and a periplasmic peptidyl prolyl isomerase FkpA, among others. Even though σ^{32} is essential for *E. coli* growth only at high temperatures, σ^E is necessary at all temperatures. Besides heat and ethanol exposure, unfolded outer membrane or periplasmic proteins can induce the σ^E regulon. During normal growth conditions, activity of σ^E is inhibited due to its association with a membrane-bound anti-sigma factor, called RseA. Another periplasmic protein, called RseB, is also bound to RseA stabilizing the complex. Under extracytoplasmic stress, misfolded proteins bind to RseB, which releases RseA to be cleaved in its periplasmic domain by DegS. Additional proteolysis of transmembrane and cytoplasmic portions of RseA then frees the σ^E transcription factor, which directs the transcriptional response to extracytoplasmic stress. Transcription of *rpoE* is autoregulated, as the operon *rpoE-rseA-rseB* has a σ^E-dependent promoter. Thus, once the stress conditions are no longer present, σ^E is again bound to RseA and inactivated, and the response is turned off (Figure 3).

Orthologues of rpoE

Homologues of σ^E have been classified as extracytoplasmic function (ECF) sigma factors and are present in different numbers in distinct bacteria. Similarly to σ^E, they are usually found in an operon with the anti-sigma factor gene, which inactivates the ECF sigma factor by binding to it. Under stress conditions, the anti-sigma factor is inactivated by different mechanisms, liberating the sigma factor to bind the RNAP initiating transcription of their regulons. The ECF sigma factors are involved not only in HS but also in a variety of types of environmental stresses, including saline and osmotic stress, oxidative stress, antimicrobial biosynthesis, heavy metal exposure, and bacterial virulence. Interestingly, in alpha-proteobacteria certain ECF sigma factors, whose activation is regulated by a two-component system (sensor histidine kinase/ response regulator), have been shown to control the general stress response. In *C.crescentus*, for instance, the sensor histidine kinase PhyK is autophosphorylated in response to stress then transferring this phosphate group to the response regulator PhyR, which binds the anti-sigma factor NepR liberating the ECF sigma factor σ^T to transcribe the general stress response regulon.

Negative Regulation of HS Genes

In contrast to the positive regulation by alternative sigma factors observed only in proteobacteria, negative regulatory systems are more widespread in prokaryotes, typically involving repressor proteins that bind to *cis*-acting regulatory sites located in the 5′ region of the genes encoding chaperones and proteases. Evidence for additional regulatory mechanisms transpired from the finding that several stress genes in *Bacillus subtilis*, a Gram-positive bacterium, including the *dnaK* and *groE* chaperone operons, remained heat inducible in a *sigB* mutant – the gene encoding an alternative sigma factor involved in stress responses. In addition, inspection of the corresponding promoter sequences revealed typical housekeeping promoters (SigA-dependent promoters) for the HS operons, and analysis of a temperature-sensitive *sigA* mutant revealed that *groESL* transcription is SigA-dependent. Transcription from vegetative promoters was subsequently demonstrated in a number of bacteria, and regulatory DNA elements were found in the promoter regions, being proposed as putative repressor-binding sites responsible for transcriptional repression under normal growth conditions. Presently, it appears as if negative control of HS genes is more prominent in eubacteria than positive regulation by alternative sigma factors. HS regulation by HrcA and CIRCE (controlling inverted repeat of chaperone expression), the repressor and

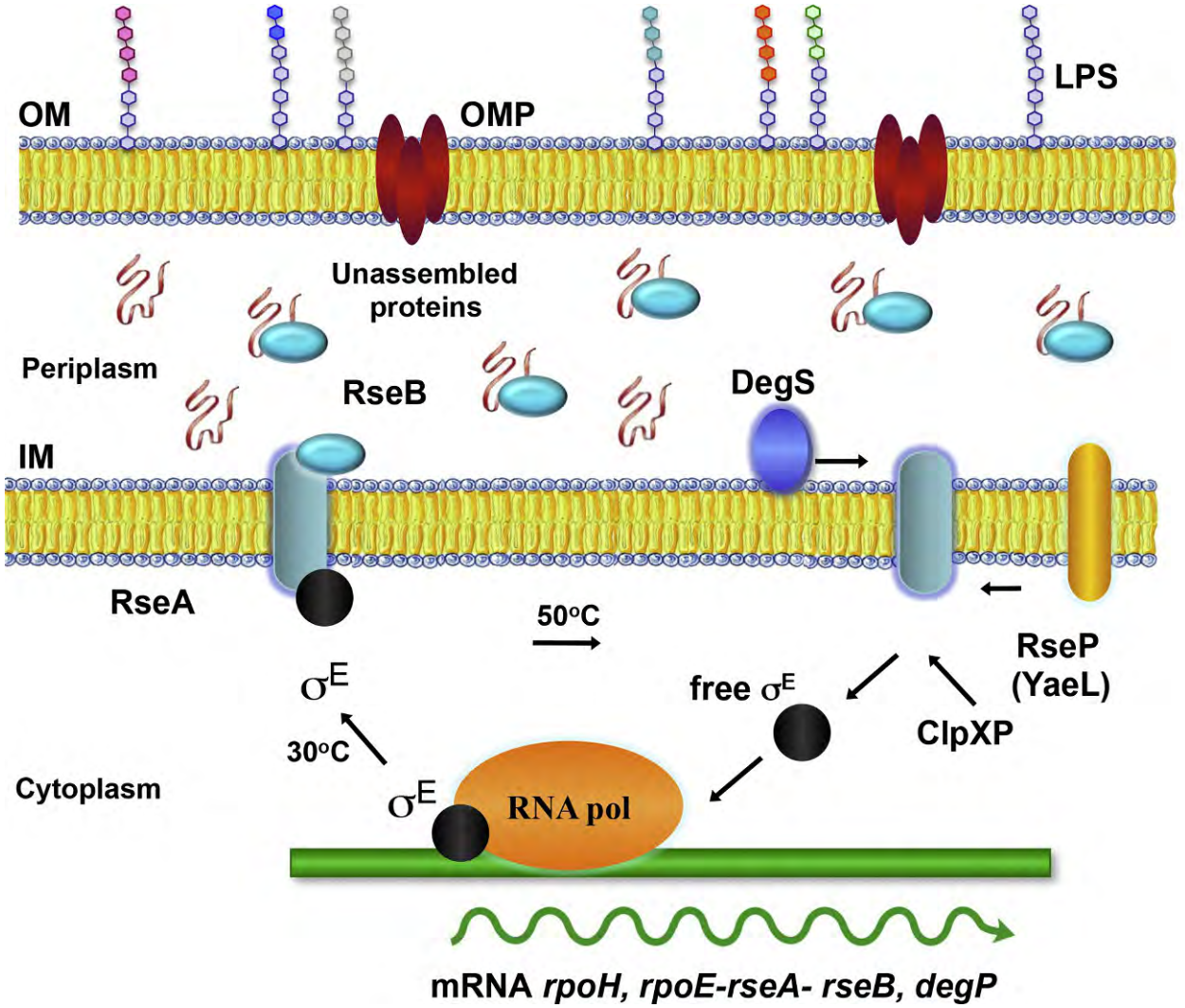

Figure 3 The periplasmic response to heat is regulated by σ^E. Under normothermic growth conditions, σ^E is inhibited by the anti-sigma factor RseA. RseB stabilizes the RsaA–σ^E complex in the absence of heat stress. Perturbations in OMP folding due to extreme heat stress lead to disruption of the RseA–RseB interaction. The inner membrane-anchored DegS protease cleaves RseA in its periplasmic domain, the membrane-embedded protease RseP (YaeL) cleaves RseA near the inner membrane, and the released cytoplasmic RseA fragment is further degraded by protease ClpXP. The transcription factor σ^E is released in the cytoplasm and interacts with RNA polymerase core enzyme to promote transcription of genes involved in OMP assembly and degradation.

its binding site, was the first to be characterized and this regulatory system is widespread in Gram-positive bacteria, proteobacteria, and cyanobacteria.

CIRCE/HrcA

A 9-base inverted repeat with a consensus sequence of TTAGCACTC-N9-GAGTGCTAA was first noticed upstream of *groESL* and/or *dnaK* operons of mycobacteria, *Clostridium acetobutylicum* and *B. subtilis*. Initially thought to be exclusive to Gram-positive bacteria, this regulatory element in now believed to be one of the most conserved in nature. As it had been reported exclusively in association with *groE* and *dnaK* operons, it was named CIRCE. However, its presence was more recently detected upstream of some other HS genes indicating that this element can have a greater regulatory potential than initially expected.

CIRCE is frequently located in the DNA region corresponding to the 5′-untranslated region of the mRNA, but it can also be positioned upstream or overlapping the transcription start site. The variability in the position of CIRCE in conjunction with the observation that this element is able to heat-control transcription from a position upstream of the promoter indicates a role mainly of the DNA rather than of the mRNA level. A negative role for CIRCE in the expression of HS genes was proposed based on the observation that mutations in this element resulted in elevated transcription of the genes located downstream from it.

HrcA acts as a repressor

The elucidation of CIRCE as a potential repressor-binding site began with a search for the cognate repressor. Evidences showing that a deletion mutant in the first gene of the *B. subtilis* dnaK operon (*orf39*) resulted in elevated levels of *groE* mRNA and that regulatory mutants affecting *B. subtilis groE* and *dnaK* expression were mapped in *orf39* strongly suggested this gene as encoding the CIRCE cognate repressor, which was named HrcA for heat regulation at CIRCE. Subsequently, the binding of HrcA to CIRCE was investigated by gel shift assays, and it was shown that the presence of GroEL is important for HrcA to acquire its active repressor conformation, both *in vitro* and *in vivo*. These results led to the proposal of a GroEL titration model for the CIRCE/HrcA regulatory system. In this model, HS leads to depletion of the GroEL pool due to the necessity of refolding denatured proteins. This depletion would cause inactivation of HrcA and derepression of transcription of the chaperone genes regulated by the CIRCE/HrcA system. Once the levels of the chaperones increase, sufficient amounts of GroEL would be present in the cell and HrcA would be activated and capable of binding to CIRCE again, turning off transcription of *groE* and *dnaK* operons (Figure 4).

Interestingly, in certain proteobacteria including *A. tumefaciens*, *B. japonicum*, *C. crescentus*, and *Xylella fastidiosa*, both *rpoH* and CIRCE/HrcA are present regulating *groESL* operons, but in no other *HSP* gene. The *groE* genes are negatively controlled by HrcA at normal temperatures and positively regulated by *rpoH* during HS.

The repressor HspR

HspR is an autoregulatory repressor protein discovered initially in *Streptomyces*, a genus of the high G+C actinomycete bacterial group. The HspR repressor/operator system was later found in other actinomycetes including *Mycobacterium tuberculosis* and in *Helicobacter pylori* and *Aquifex aeolicus*. However, HspR is much less widespread than HrcA, in particular it is not found in any low G+C Gram positive as *B. subtilis*. HspR acts as a repressor by binding at the HspR-associated inverted repeat (HAIR) site (consensus sequence: 5′-TTGAY-N_7-ACTCAA-3′) within the promoter regions of the genes and operons it controls. In *Streptomyces coelicolor*, studies *in vitro* and *in vivo* have provided evidences the DnaK functions as a corepressor of the HspR regulon, which is shown to include *lon* and the *dnaK* and *clpB* operons. Thus, DnaK negatively regulates its own expression. Although 17 genes were shown to be upregulated more than twofold in a *S. coelicolor hspR* disruption mutant, transcriptome and genome sequence analyses indicated

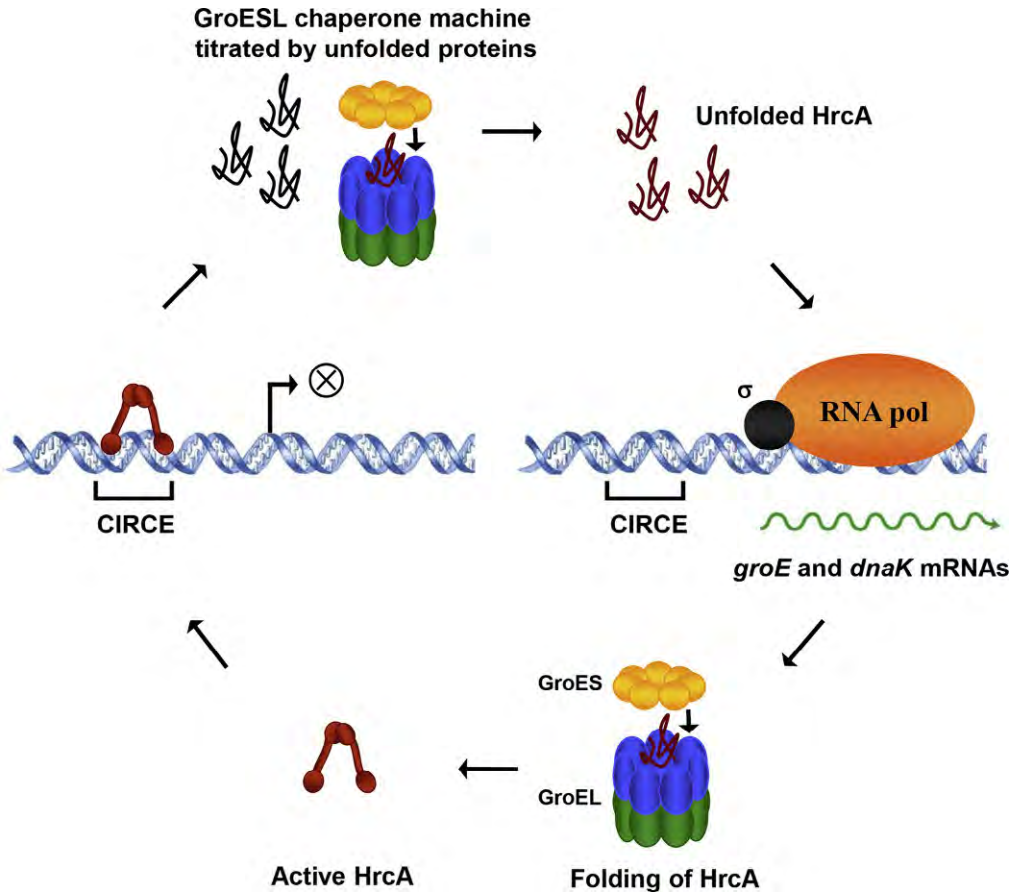

Figure 4 The activity of the repressor HrcA depends on the GroEL system. In the absence of heat stress, GroESL converts the HrcA protein into its active form. HrcA binds controlling inverted repeat of chaperone expression (CIRCE) regions on heat shock (HS) genes inhibiting the RNA polymerase holoenzyme access. During temperature upshift, unfolded proteins titrate the GroESL chaperone system, leading to an increase in the amount of inactive HrcA. HS gene transcription is induced leading to activation of repressor HrcA that binds to CIRCE turning off transcription of the HS operons.

that the above three transcription units are probably the only direct targets of HspR in this bacterium. Interestingly, in the genus *Streptomyces* besides the HspR/HAIR regulatory system, the HrcA/CIRCE system has also been described regulating the *groEL1/ES* operon and the *groEL2* gene, and the RheA repressor protein (which will be described below) negatively regulating the transcription of *hsp18* in *Streptomyces albus*. This is a clear example of multiple regulatory systems acting in conjunction to control HSP gene expression in bacteria.

The regulatory element ROSE

The *cis*-acting regulatory element repression of heat shock gene expression (ROSE) contains a conserved sequence of about 100 nucleotides in the 5′-untranslated region of multiple small HS genes including *rpoH* in several bacteria. The inhibitory effect of ROSE is based on experimental evidences that indicated this regulatory element as a putative site to bind to a repressor protein. Extracts from non-HS cells of *B. japonicum* retarded full-length ROSE in gel shift assays. However, a regulatory protein with a potential ability of binding DNA containing ROSE element was never isolated. In fact, computer calculations indicated that ROSE predicted to fold into a complex structure occluding both the Shine–Dalgarno (SD) sequence and the translation start site AUG, suggesting the possibility of ROSE acting as a RNA thermosensor similar to the *rpoH* thermometer. The thermoresponsive structures folded in the 5′-UTR region of mRNAs are known to control translation of HS and virulence genes in bacteria. The potential role of this RNA structure was determined by mutational studies with ROSE cloned upstream the *hspA–rpoH* operon from *B. japonicum*. Mismatches introduced into unpaired regions of the stem-loop IV of the thermometer structure were completely neutral, but when mismatches were introduced in the paired regions of the same loop, an increased expression of a translational *lacZ* fusion was observed. Besides this, compensatory mutations restored repression of translation. The correct function of ROSE thermometer depends on the presence of a conserved G residue opposite to the SD, its elimination made the thermosensor irresponsive to high temperatures. The G residue closes the loop of the thermometer by a weak GG base pair that breaks at elevated temperatures making the ribosome-binding site accessible to initiation of the translation process.

The repressor RheA

An open reading frame (*rhea*) located 150 bp uspstream in the opposite orientation of the small HS gene *hsp18* participates in negative regulation in the Gram-positive bacterium *S. albus*. The small HSP, Hsp18, is involved with acquisition of thermotolerance in the bacteria of *Streptomyces* genera.

The *S. albus rheA*-null mutant displays high *hsp18* mRNA levels at physiological temperature. On the other hand, overexpression of the *rheA* gene restores repression of *hsp18* in *rheA* mutant cells. Curiously, the *hsp18* mRNA is translated only after temperature upshift, suggesting a posttranscriptional control to *hsp18* gene expression in this bacterium. One possible model for this posttranscriptional control involves changes in mRNA conformation as described for the ROSE element and the *rpoH* gene. Denaturation of the secondary structure of the mRNA would therefore lead to an increase in ribosome binding and translation initiation.

Two similar inverted–repeated sequences are present in the promoter region of the *hsp18* gene (GTCATC-N_5-GATGAC) and in the 5′untranslated region of the *rheA* gene (GTCGTC-N_5-GATGAC), indicating that both the genes are controlled in a coordinated way and probably by the same regulator. In gel shift assays, when DNA/RheA sample was incubated under normothermic temperature, a shift was observed in the mobility of the DNA fragment. However, no complex formation was observed at HS temperatures. In fact, RheA is an autoregulatory protein and its activity is inhibited at elevated temperatures, but the temperature-induced derepression by RheA is reversible. At low temperature, RheA binds its target in the promoter region, preventing *hsp18* expression. At high temperature, RheA is inactivated derepressing the *hsp18* gene. Then, RheA acts as a cellular thermometer in *hsp18* expression, sensing increases in temperature and derepressing *hsp18* gene transcription.

CtsR

The CtsR regulon consists of three transcriptional units: the tetracistronic *clpC* operon containing *ctsR*, *mcsA*, *mcsB*, and *clpC* genes and two monocistronic *clpP* and *clpE* operons. The *clpC* and *clpE* genes code for ATPase subunits and *clpP* codes for a proteolytic subunit of the two ClpCP and potential ClpEP ATP-dependent proteases. McsA and McsB are kinases that act as modulators of CtsR. In Gram-positive bacteria, CtsR is a repressor that controls the class III HS genes, class I genes are regulated primarily by the repressor HrcA, class II genes are regulated by the alternative sigma factor σ^B, and the two-component system CssRs controls the class V genes.

The repressor CtsR is a dimeric protein that contains a classical helix-turn-helix DNA-binding motif, which binds to a highly conserved heptanucleotide direct repeat (A/GGTCAAAnAnA/GGTCAAA), located upstream of *clpP*, *clpE*, and the *clpC* operons. Besides this, CtsR contains two other functional domains: a dimerization domain (the first 24 domains) and the central glycine-rich region (aa 64–67) involved in heat sensing.

All three transcriptional units regulated by CtsR are preceded by two promoters each, clpE by two σ^A-dependent promoters and clpC and clpP operons by σ^B- and σ^A-type promoters. The dimeric protein CtsR binds the −35 and −10 regions of the σ^A promoter preventing the association of the σ^A RNAP holoenzyme to it.

At physiological temperatures (37 °C), the expression of CtsR maintains a certain steady-state level, which leads to a low expression of the genes from CtsR regulon. Upon exposure to heat stress, not only a rapid degradation of CtsR by ClpCP proteases occurs but also an induction in the expression of HS genes. ClpE was reported as a new independent type of the HSP100 ATPase belonging to the CtsR regulon in Gram-positive bacteria. ClpEP was found to destabilize CtsR after HS *in vivo*. Coherent with this, it was recently reported that a *clpE*-null mutant displays a significant delay of protein disaggregation and retardation of CtsR-dependent gene induction.

All the genes of *clpC* operon are involved in the regulation of the activity of CtsR. McsB is at the center of a network interacting with the McsA, ClpC, and CtsR. McsB is a tyrosine kinase, which utilizes a guanidine kinase domain, known only from eukaryotic kinases. McsB needs to be activated by McsA, which becomes concurrently phosphorylated by McsB. McsA activates the kinase activity of McsB that when phosphorylated causes the phosphorylation of CtsR and a stronger CtsR–DNA-binding inhibiting activity. On the other hand, ClpC inhibits the kinase activity of McsB. Upon HS *in vivo*, CtsR is degraded and this HS-induced degradation depends on the presence of McsA.

Bacterial HS Response Induced by Antibiotic-Stress

Pathogenic bacteria have the ability to persist in the human host and medical environment where they encounter an excess of stressors like heat shock and antimicrobials. Heat shock response activation by antimicrobials has been reported in different *Gram* negative multi drug resistant bacteria (MDR) such as *E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Burkholderia cenocepacia* and in *Gram* positive bacteria such as *Staphylococcus aureus* and *Streptomyces coelicolor*. A large amount of recent data have shown that molecular chaperones clearly play important roles in bacterial stress tolerance, but their role in antibiotic tolerance is still starting to be clarified.

HS response induced by antibiotic in Gram Positive Bacteria

In *S. aureus*, a knockout in the *dnaK* gene increased the susceptibility of the meticillin-resistant strain COL to the antibiotics oxacillin and methicillin. Chaperones are known to be involved in responses to antibiotics because they are induced when the cell wall is subjected to antibiotic stress in *S. aureus*. On the other hand, ciprofloxacin treatment in *S. coelicolor* caused reduced levels of the chaperones GroESL and DnaK at both, transcript and protein levels. In a transcriptomic study of *S. coelicolor* (strain A3-2) in the presence of ciprofloxacin the *htpG* gene was up regulated. This increase is supposed to occur in compensation of the decrease in GroESL and DnaK proteins levels. The role of HtpG chaperone has not been clearly defined in bacteria yet. However, it is suggested that HtpG could become the primary folding mechanism under stress caused by antimicrobials, when other chaperones are down regulated. Despite the data mentioned above, recent proteomic studies for *S. coelicolor* indicated the down regulation of the chaperone HtpG and ClpB upon exposure to ciprofloxacin. In this bacterium, ClpB and DnaK belong to the same regulon, reflecting their functional cooperation.

HS response induced by antibiotic in Gram Negative Bacteria

Antimicrobial peptides (AMPs) are antibiotic agents commonly found in animals, plants and microorganisms, and they have been suggested as the future of antimicrobial chemotherapies. Resistant and susceptible strains of *E. coli* ATCC 8739 to the AMP named magainin I, were compared by proteomic analysis. The authors report the observation that alterations of DnaK and GroEL expression in the metabolism of resistant strains occurred only in a non-stress environment. The arousing decrease in the levels of the intact forms of the chaperones DnaK and GroEL and the increase of their forms with reduced molecular masses were also observed only in the magainin-resistant strains, showing that individual metabolic alterations could be occurring among the resistant strain group suggesting that in *E. coli* could take place posttranslational modifications induced by AMPs in the HSP group.

Quantitative proteomic was also used to compare the expression programs of two clonal isolates of *B. cenocepacia* retrieved from patient presenting chronic respiratory infection. The isolate IST439 was the first bacterium recovered while the clonal variant IST4113 was obtained after three years of persistent infection and intravenous therapy with ceftazidime/gentamicin antimicrobials. However, both isolates exhibited a MDR profile towards different classes of drugs. A higher content of the molecular chaperones ClpB, DnaK and GroEL was observed in the isolate IST4113 compared with IST439 and it is also consistent with higher robustness of isolate IST4113 against environmental stresses and in particular antibiotic-induced stress.

In the case of *A. baumannii* (ATCC 19606), cells pretreated for 30 min at heat shock temperatures (45 °C) proved to be more thermotolerant and resistant to aminoglycoside streptomycin than bacteria pretreated at physiological temperatures (37 °C). In addition, DnaK levels increased more than 4-fold after 1 hour of exposure to a subinhibitory concentration of streptomycin, and GroEL levels doubled. Incubation of a MDR *A. baumanii* RS4 strain in the presence of different antibiotics resulted in a significant increase in the production of DnaK mRNA and protein. A similar result was observed by comparative proteomics for isolates of *A. baumannii* under carbapenen stress, which increased the synthesis of three different chaperonins.

Multidrug-resistant *A. baumannii* strains have been considerably studied at nucleic acid sequence and proteomic level. The proteome of the multidrug resistant strain BAA-1605 was compared with the proteome of the drug-sensitive strain ATCC 17978. Not all stress-related proteins are more abundant in the MDR strain, but ClpA/B chaperone and DnaK were 7-fold and 2-fold higher in abundance in the MDR strain than in the drug-sensitive strain, respectively. In *P. aeruginosa*, for instance, both the aminoglycoside tobramycin and heat shock induce the expression of Lon protease AsrA mediated by the heat shock sigma factor σ^{32}. However, overexpression of the *asrA* transcript makes *P. aeruginosa* cells just moderately resistant to the aminoglycoside.

The reason for the connection between the heat shock response and aminoglycosides is the fact that this antimicrobial impacts cytosolic protein folding leading to accumulation of insoluble proteins in the cell. Overexpression of GroESL from *E. coli* (strain MG1655) reduced protein misfolding and rescued the membrane potential, cell growth and survival. In contrast, inhibition of GroESL expression increased aminoglycoside sensitivity allowing the collapse of the membrane potential abridging cell life. Overexpression of the DnaK/DnaJ/GrpE chaperone system increases survival, but less efficiently than the GroES/EL system because it does not promote growth of aminoglycoside-exposed bacteria. Thus, it is supposed that HSPs can eliminate unfolded proteins

targeting the atypical mistranslated polypeptides that are produced by aminoglycoside-disrupted ribosomes and that insert into and disturb bacterial membranes. Aminoglycoside-mediated membrane damage has been described and is supposed to be a key-step in the lethal activity of these agents. Then, elimination of atypical polypeptides would therefore reduce their toxicity to bacterial cells. In fact, HSPs help bacteria cope with early exposure to aminoglycosides (Figure 5).

HS Response in Archaea

HSPs

The three domains of life on Earth include the two prokaryotic groups, Archaea and Bacteria. The Archaea domain presents three phyla, Crenarchaeota, Euryarchaeota, and Korarchaeota. All of them are distinguished from Bacteria based on differences in tRNA and rRNA sequences, in the cytoplasmic membrane and cell wall composition, in the transcription and translation apparatus, and their restriction to unusual habitats. In addition, members of archaea present a general capacity to dominate or out-compete bacteria in niches in which chronic energy stress is a characteristic, including environmental stresses (temperature, acidity, and salinity) and low energy availability.

The ability to cope with environmental stress is essential to all microorganisms, including those thriving in extreme temperatures like hyperthermophiles archaea, they also have thermal limits and display a classic heat shock-response when thermally stressed. However, genome sequence information indicates that some archaea lack HSPs that were previously known to be ubiquitous in Eucarya and Bacteria. Hyperthermophiles archaea lack several HSPs, to name a few, HSP90, HSP70/DnaK, DnaJ, GrpE, HSP33, and HSP10 homologues. The minimal protein-folding machinery present in these organisms may represent a prototype of antistress systems in early life. However, it remains unclear whether archaea members use other proteins or proteases associated to resolubilize and eliminate the aggregates formed following stress by high temperature.

Despite the fact that not all archaea have DnaK or the other component of the KJE (DnaK-DnaJ-GrpE) chaperone system, chaperonins, also known as Hsp60 or GroEL, are present, in the genome of all archaea and their up-regulation is part of multiprotein complexes known as thermosomes during stress by heat shock. Thermosomes are constituted by large oligomeric ring containing 7–9 subunits that assist in the protein folding and other functions. These proteins are more similar to the type II chaperonins found in the eukaryotic cytosol than to the type I chaperonins found in chloroplasts, mitochondria and bacteria. However, some archaea species also possess type I chaperonin homologues

Genome analysis in the archaea species has revealed that these organisms do not always contain *hsp70* or the other two genes of the chaperone machine triad, *hsp40* and *grpE*. Nevertheless, the full genome sequence of *Methanobacterium thermoautotrophicum* and *Methanosarcina mazei* S-6 demonstrates the presence of the genes from the Hsp70 chaperone machinery.

The amino acid sequence of the archaeal HSP70 resembles more the bacterial than the eukaryal counterpart. In addition, the activity of *M. mazei* HSP70 is enhanced by interaction with bacterial cochaperone DnaJ, but not by the eukaryotic homologue, indicating that archaeal *hsp70* gene may have been received from bacteria by lateral gene transfer. In addition, *in vitro* experiments demonstrated functional similarities between the *M. mazei* HSP70 and *E. coli* DnaK, but they do not present complete functional resemblance. *M. mazei* HSP70 functions as a chaperone in luciferase renaturation *in vitro*, and it requires bacterial-type chaperones like DnaJ and GrpE to perform its function. However, *E. coli dnaK* mutants were not complemented by the *hsp70 M. mazei* gene.

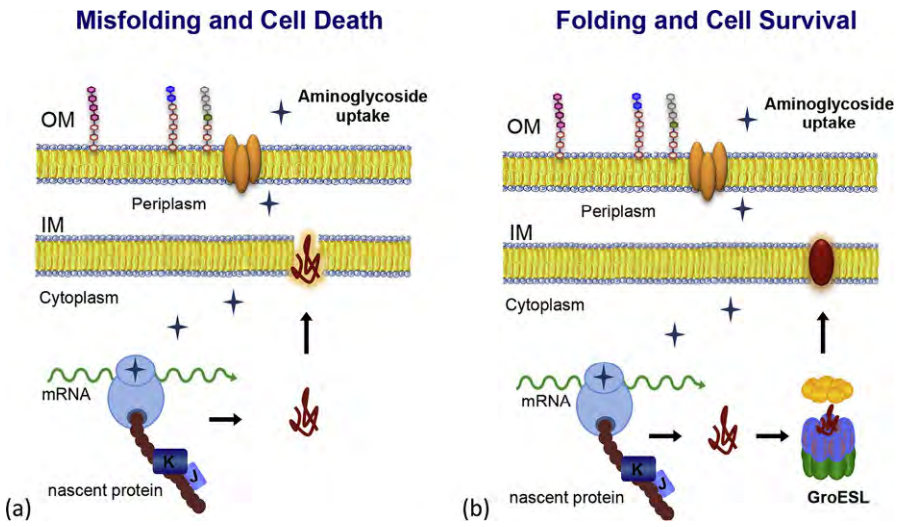

Figure 5 Chaperone effects on bacterial survival during early aminoglycoside exposure. (a) Aminoglycoside uptake and binding to ribosome causing translational misreading. Misread polypeptides are encountered by chaperones acting in succession. Limiting chaperone capacity causes protein misfolding, membrane permeabilization, depletion of essential cytosolic functions and cell killing. (b) In contrast, chaperone availability (mainly GroESL) ensures protein folding and increased bacterial survival (KJ: component of DnaK-DnaJ-GrpE chaperone system).

In the thermoacidophilic archaeon *Sulfolobus sulfataricus,* Hsp20 gene is heat-inducible and the protein possesses chaperone activity and ability to recognize and bind unfolded proteins to prevent their aggregation in vitro. In addition, *S. fulfataricus* Hsp20 expression in an *E. coli* system protects the cells from thermic stress.

The *Crenarchaeota* species has a separate class of HSP60 chaperonins related to the eukaryotic protein and only distantly related to the highly conserved bacterial GroEL. As mentioned above chaperonins are organized into two groups: class I and class II. Class I is found in Eubacteria, mitochondria, and chloroplasts. Class II is found in Archaea and the eukaryotic cytosol. Class II chaperonins are oligomeric proteins that have two rings containing eight or nine subunits each. Some chaperonins are heat inducible, but others are regulated at low temperatures.

A Lon protease gene homologous to the bacterial *lon* gene was also identified in the Crenarchaeota *Archaeoglobus fulgidus* by sequence analysis, but the ATP-binding domain was absent in the predicted protein. In general, some HSPs are absent in members of the Crenarchaeota, although they are found in some members of the Euryarchaeota. Furthermore, transcription factors that control thermotolerance are missing in crenarchaeotal genomes

HS Control in Archaea

Archaeal *hsp* genes respond to HS by an increase in the production of their monocistronic transcripts, as one would expect for stress genes. Maximum transcript levels are reached after longer exposures to high temperature than those that would induce a peak response in bacteria, in agreement with what is observed in eukaryotes.

The mechanism of transcription initiation for archaeal genes differs from those known to operate in bacteria and must involve factors that are not of a bacterial type, thus different from σ factors. In fact, transcription initiation in archaea closely resembles eukaryal class II transcription initiation and is mediated by a single RNAP and two general transcription factors, TATA element-binding protein (TBP) and transcription factor B (TFB). Archaeal cells do not have sigma factors or homologues of eukaryotic heat shock transcription factors (HSFs) or sequences similar to heat shock elements (HSEs), which will be described below, and this suggests that Archaea have evolved a unique mechanism to control the HS response, but the components involved in this process are not obvious until the present time.

The archaeal repressor Phr

Interestingly, in the hyperthermophiles *Pyrococcus furiosus* there is the first transcriptional repressor (Phr) characterized until now in Archaea that shows little similarity to eukaryotic or bacterial regulators. *P. furiosus* grows optimally near 100 °C and undergoes a heat shock response at 105 °C mediated in part by the repressor Phr. The N- and C-terminal domains in Phr exhibit bacterial and eukaryal features, respectively. In addition, Phr amino acid sequence displays 23% of identity to the human BAG domain. The conserved eukaryotic BAG proteins bind to the ATPase domain of HSP70 and regulate the chaperone activity. In spite the fact that no homologue to HSP70 is encoded in *Pyrococcus*, HSP60-like chaperonins and AAA^+ chaperones are detected and could be potential binding targets of the BAG-like domain in Phr.

Phr exists as a homodimer and recognizes a nucleotide sequence overlapping the transcription start site of some HS genes (sequence consensus: 5´TTTAnnnACnnnnnGTnAnnAAAA3´), thereby inhibiting RNAP recruitment. Phr does not affect binding of TBP and TFB to the promoter, but abrogates RNAP recruitment to the TBP/TFB complex at optimal temperature.

Three of the nine genes described to be regulated by Phr in *P. furiosus* are annotated as encoding conserved hypothetical proteins. Two of them have homologues in bacteria and archaea and only one has homologues just in bacteria. This finding suggests a lateral gene transfer from bacteria to *P. furiosus* genome. Another Phr target, is the gene encoding the enzyme myo-inositol-phosphate synthase that is involved in the production of the compatible solute di-myo-inositol-1,1´-phosphate (DIP). This protein has been shown to accumulate to high concentrations in the cytoplasm of *P. furiosus* at suboptimal growth temperatures and to stabilize proteins at extreme temperatures. The other proteins regulated by the repressor include a ferritin/ribonucleotide-like family and a heat shock inducible phosphoesterase that is supposed is involved in the synthesis of organic phosphates. In addition, Phr inhibits specifically cell-free transcription of its own gene, HSP20, and of an AAA^+ ATPase. The *aaa*$^+$*atpase* and *phr* mRNA levels are induced after HS and during stationary growth phase in *P. furiosus*, indicating that the transcription of these genes is also affected by general stress and starvation. By contrast, the levels of the protein Phr are only slightly elevated during heat stress. *In vitro* experiments have shown that at high temperature (103 °C) Phr loses its functional conformation. The dissociation of the protein from its operator sequence may account for the high increase of *phr* mRNA levels detected after temperature upshift. Then, in *Pyrococcus* there is a simple model for HS regulation: Phr binds promoter regions of HS genes at normothermic temperature inhibiting transcription by blocking RNAP recruitment. Subsequent release of Phr along with elevated temperatures leads to activation of HS genes.

VapBC in Archaea

VapBC (virulence-associated protein BC) proteins belong to toxin-antitoxin (TA) system and are thought to promote survival during environmental stress. TA system consists of an intracellular protein toxin co-expressed with a cognate protein antitoxin. The two component system genes are usually contiguous and encoded on the genome or plasmids. Cognate toxins are inactivated by antitoxin that is susceptible to cleavage by proteases due to its natively unfolded C-terminal domain. Free-toxins are able to modulate protein synthesis acting as ribonucleases. Protein expression in the cell is controlled by the concentration of stable toxin and unstable antitoxin. Three TA families are described in archaea: RebBE, Phd/Doc and VapBC. The VapBC family is the most abundant TA system among prokaryotic genomes and is present in high numbers in the archaea including hyperthermophiles and

thermoacidophiles. VapB is the unstable antitoxin and VapC is the stable toxin. VapC toxin has a PIN domain (PitN-terminal) which is suggestive of RNase activity. In the archaea *S. solfataricus*, for instance, VapC toxin appears to regulate abundance of its cognate antitoxin. In contrast, VapC transcript abundance is reduced when VapB is deleted. Apart from the above, the significance of the TA loci in the Archaea remains unknown at the moment.

Heat Stress in Eukaryotic Cells

In eukaryotes, many of the HSPs and molecular chaperones are represented as large gene families with functional homologues in each cellular compartment, including cytosol, nucleus, and specialized organelles, such as endoplasmic reticulum (ER), mitochondria, and chloroplasts. There are families of proteins for HSP100, HSP90, HSP70, HSP60 (which are restricted to mitochondria and chloroplasts), and HSP27/28. Among the questions still outstanding concerning this great number of proteins is to which extent the different homologues vary in their ability to carry out their various chores, such as preventing protein aggregation, maintaining the unfolded state necessary for protein transport among different cellular compartments, disaggregating protein aggregates, and regulation of the HS response.

HSP Families

The HSP100 family

The HSP100 protein family includes the HSP104 from yeast and ClpB from bacteria, which are required for induced thermotolerance to extreme temperatures. HSP104/ClpB belongs to the AAA+protein superfamily, which includes the 'ATPase associated with a variety of cellular activities' (AAA) proteins and Clp/HSP100 proteins. These two protein classes exhibit considerable sequence homology in their 'AAA' domains, which are important for ATP hydrolysis and oligomerization. Usually AAA+proteins form ring-shaped homohexamers, which drive the assembly and disassembly of macromolecular complexes by ATP-dependent remodeling of their substrates.

The main target of the proteins from the HSP100 family is protein aggregates, and they act together with HSP70 proteins as a bi-chaperone system (HSP104/HSP70 or ClpB/DnaK) in the refolding of aggregated proteins. The cooperation DnaK/HSP70 chaperone system in the disaggregation process was proposed to be either by acting sequentially after ClpB/HSP104 or simultaneously. Protein aggregates of reduced size, generated by ClpB/HSP104, could serve as substrates for DnaK/HSP70, which has a disaggregation activity toward small protein aggregates.

The HSP90 family

Concerning the HSP90 family, the abundance and essentiality for cell growth of HSP90 proteins, found in the cytoplasm and ER, suggests that they perform important biological functions in eukaryotes. However, the bacterial homologue HtpG is not found in many bacteria analyzed, and when present it is not essential for growth. Among the major HSPs, HSP90 is unique in its functions, since it is not required for the maturation or maintenance of most proteins *in vivo*. The majority of its identified cellular targets are signal transducers and cell cycle and developmental regulators whose conformational instability is relevant to their roles as molecular switches. Through low-affinity interactions characterized by repeated cycles of binding and release, HSP90 keeps these unstable signaling proteins poised for activation until they are stabilized by conformational changes associated with signal transduction. Studies of yeast illustrate the specificity of HSP90: at normal temperatures, reduction on HSP90 levels that have no apparent effects on cell growth or metabolism, but can completely abolish signaling through HSP90-dependent pathways. Conditions that cause general protein damage can divert HSP90 from its normal targets to other partially denatured proteins, and due to its dual involvement, HSP90 may link developmental programs to environmental contingency.

The HSP70 family

The HSP70 protein family is the best characterized and contains the largest number of members localized in many different compartments of the cell, including cytosol, mitochondria, and ER. The HSP70 is, by far, the most conserved protein in evolution. It is found in all organisms from archaea and plants to humans, and the prokaryotic HSP70 protein DnaK shares approximately 50% amino acid identity with eukaryotic HSP70 proteins. Functionally, the organellar HSP70s are better understood, having roles in protein translocation and folding. They are constituents of what is called the HSP70 (DnaK) chaperone machine, together with the co-chaperones HSP40 (DnaJ) and GrpE. The DnaK and DnaJ members appear to be bona fide chaperones: DnaK prefers to bind unfolded polypeptides and DnaJ binds to the more compact intermediate, termed molten globule. In some instances, the DnaK/DnaJ proteins appear to bind synergistically to an unfolded substrate and then hand it off to the second most important chaperone system formed by GroEL/GroES (HSP60/HSP10). The DnaJ and GrpE proteins dramatically regulate the otherwise weak ATPase activity of the DnaK partner, with DnaJ specifically accelerating the rate of hydrolysis of the DnaK-bound ATP and GrpE accelerating the ADP/ATP exchange by causing the release of DnaK-bound nucleotide.

The HSP60 family

The HSP60/GroEL chaperone machine consists of the GroEL (HSP60) and GroES (HSP10) family members, also called chaperonins. This chaperonin system is restricted to eubacteria, mitochondria, and chloroplasts. The GroEL protein appears to bind unfolded polypeptide substrates when present in the compact molten globule state, which corresponds to a proposed folding intermediate that still exhibits hydrophobic groups. The GroES protein has a pivotal role in substrate maturation by coordinating the ATPase activity of the GroEL subunits. The GroEL subunits are arranged as two stacked heptameric rings with a central cavity where folding takes place. The GroES subunits form a single heptameric ring that binds to one of the GroEL rings regulating its function. ADP is tightly bound to the subunits in the ring adjacent to GroES and with lower affinity in the opposite ring. Unfolded protein binds to GroEL ring opposite to GroES and triggers ADP dissociation from GroEL subunits. This in turn results in the release of GroES. ADP–ATP exchange weakens the affinity for the bound protein. GroES rebinds GroEL in the ATP state and may cover the ring that contains the bound substrate. Cooperative ATP hydrolysis releases the substrate protein for folding in the ring cavity. GroES binding becomes stabilized in the regained ADP state, and partially folded protein may reassociate for another round of interaction. Even though no direct homologues of HSP60 exist in the cytosol, a different class of chaperonins was described in thermophilic archaebacteria (TF55) and found to have homology with the TCP-1 protein present in the cytosol of most eukaryotic cells forming a heterooligomeric ring structure complex named TriC. Although initially proposed as specialized for the folding and assembly of cytoskeletal proteins, it is now becoming clear that TriC has a more general chaperonin function.

The HSP27/28 family

An interesting aspect of the HSP27/28 class of proteins, also called small HSP (sHSP), is its structural and functional similarity to that of α-crystallins. They encompass a large number of related proteins that share some structural features common to the lens protein α-crystallin and are present in virtually all organisms. Analyses of several members of this family of proteins have led to the conclusion that in unstressed cells each sHSP has its own tissue-specific expression and particular intracellular localization. In contrast, during stress, most of the sHSPs analyzed are expressed in every cell and at least some of them were demonstrated to have protective functions. This indicates that they belong to the stress-induced machinery that protects against and/or repairs cellular damages. In fact, it has been shown that sHSPs of *E. coli* cooperate with ClpB and the DnaK system in reversing protein aggregation.

Proteases induced by HS

Many HSPs are proteases or makeup components of a protease system. This indicates that when the chaperone activities of the HSPs are not sufficient to renature the heat-denatured proteins, the proteolytic systems of the cells act by degrading them. In eukaryotes, the bulk of the ATP-dependent proteolysis is carried out by the ubiquitin system. In this system, polypeptides to be degraded are covalently attached to ubiquitin, which is an extremely conserved HSP. In addition, many of the ubiquitin-conjugating enzymes are also under HS or stress regulation. The degradation is mediated by the 26S proteasome, a large machine composed of two different complexes. The 20S core complex forms a hollow cylinder that is composed of four heptameric rings, which harbor the proteolytic active sites in its interior chamber. The substrates need to be unfolded and translocated before they can reach the proteolytic chamber. This task is executed by the 19S complex, which is located at either end of the 20S proteolytic complex. The base of each 19S complex contains AAA+ proteins that promote the ATP-dependent unfolding and threading of substrates into the proteolytic chamber of the 20S complex.

In prokaryotes, proteolysis of misfolded proteins is mediated by proteasome-like machines, which also consist of an ATPase module (i.e., ClpA and ClpX), and a proteolytic component, which is either covalently attached (i.e., Lon) or diffusible (i.e., ClpP). The peptidase ClpP of *E. coli* does not exhibit sequence homology to the subunits of the eukaryotic 20S complex. However, it forms a structure of similar architecture, consisting of two heptameric rings that generate a barrel-shaped proteolytic core with active sites hidden in an interior chamber. Access to these sites is controlled by narrow pores, which do not allow the passage of folded polypeptides. Substrate unfolding and translocation is mediated by various HSP100 proteins (i.e., ClpA), which associate with either end of the ClpP core. Recently, it has been observed that eukaryotic cells exhibit homologues of the Lon protease mitochondrially located.

Control of HSP Gene Expression in Eukarya

The HS response in eukaryotic cells is positively controlled at the transcriptional level by the ubiquitous HSFs that bind to arrays of 5-bp DNA consensus element nGAAn (HSEs) in the HSP gene promoter regions. A single gene (*HSF1*) encodes the HSF in the yeast *Saccharomyces cerevisiae* and in other eukaryotic microorganisms, whereas three to four different HSFs can coexist in plant, avian, and mammalian cells. However, HSF1 is the master regulator in vertebrates. *Hsf1*-knockout mouse and cell models have revealed that HSF1 is a prerequisite for the transactivation of HSP genes, maintenance of cellular integrity during stress and development of thermotolerance. All HSFs share common structural motifs, including a conserved DNA-binding domain, a trimerization domain, and a C-terminal transactivation domain, but are quite variable between species. Nevertheless, examples of functional interchangeability of HSFs from organisms as diverse as yeast, fruit, fly, and man have been demonstrated.

The HSF is synthesized constitutively in all eukaryotic organisms, its activity, however, is regulated post-translationally. Furthermore, there is diversity in the mechanisms of regulation of HSF activity. For example, under nonstressed conditions,

metazoans sequester the majority of HSF as chaperone-bound monomers within the cytoplasm. Upon HS, HSF monomers undergo an intramolecular rearrangement that allows HSF subunits to trimerize. HSF trimers then enter the nucleus and bind DNA at the HSEs, activating transcription of the HSP genes. Despite the apparent simplicity, transcriptional activation is not a necessary consequence of DNA binding. Treatment of human cells with salicylate induces HSF to trimerize and bind DNA but not to activate transcription. This suggests that there are at least two activation steps for metazoan HSF, one of which occurs subsequent to DNA binding. In fact, the DNA-binding and transactivation capacity of HSF1 are coordinately regulated through multiple post-translational modifications (including phosphorylation, sumoylation and acetylation), protein-protein interactions and subcellular localization. HSF1 also has an intrinsic stress sensing capacity, as both *D. melanogaster* and mammalian HSF1 can be converted from a monomer to a trimer *in vitro* in response to thermal or oxidative stress. There is strong evidence that HSF1 interacts with multiple HSPs at different phases of its activation. Trimeric HSF1 can be kept inactive when its regulatory domain is bound by a multi-chaperone complex containing HSP90. Elevated levels of both HSP90 and HSP70 negatively regulate HSF1 and prevent trimer formation upon heat shock. The negative feedback from the end products of HSF1-dependent transcription (the HSPs) provides an important control step in adjusting the duration and intensity of HSF1 activation according to the levels of chaperones and presumably the levels of nascent and misfolded peptides. Furthermore, evidence is accumulating that HSFs are very versatile transcription factors that, in addition to protecting cells against proteotoxic stress, are vital for many physiological functions, especially during development. Genome-wide gene expression studies have revealed that numerous genes not classified as HSP genes or molecular chaperones are under HSF1-dependent control. Although mice lacking HSF1 can survive to adulthood, they exhibit multiple defects, such as increased prenatal lethality, growth retardation and female infertility.

HSF1 from *S. cerevisiae* and *Kluyveromyces cerevisiae* (but not HSF1 from *Schizosaccharomyces pombe*) behaves differently from metazoan HSFs. Budding yeast HSF1 trimerizes and binds to promoters before stress. Nonetheless, upon HS, the level of HSF1-dependent transcription is enhanced. This suggests that yeast HSF1 can exist in two conformations – an unstressed 'low-activity' conformation and a stressed 'high-activity' conformation. *S. cerevisiae* HSF1 regulates transcription under normal physiological conditions as well as under different stress conditions, including heat, oxidative stress, glucose starvation, and pH, and it is essential for cell viability. The genes targeted by HSF1 encode proteins that function in a broad range of biological processes, including protein folding and degradation, detoxification, energy generation, carbohydrate metabolism, and cell wall organization. HSF1 is phosphorylated concomitant with its heat shock-induced activation, and this covalent modification is intra-molecularly controlled by two domains of the protein: CE2 and CTM. The CE2 region functions negatively to restrict the activity of HSF1 at low temperature and/or to convert the protein from an active to an inactive form. The CTM domain is required for extensive phosphorylation, but this requirement is bypassed in an *hsf1* mutant that lacks CE2, suggesting that the CTM domain alleviates the repressive effect of CE2 with respect to hyperphosphorylation. In addition, CTM regulates the activating ability of HSF1 and thereby affects recruitment of the transcription machinery. Cooperative protein–DNA and protein–protein interactions are important for fine-tuning transcriptional levels. The cooperative binding of HSF1 molecules to the HSEs is connected with the binding affinity to DNA and the strength of transcriptional activation.

Sensing the stress

In metazoans, HSPs and co-chaperones are assembled into multi-chaperone complexes to regulate HSF1 activity. HSP90-containing multi-chaperone complexes appear to be the most relevant repressors of HSF1 activity. Because HSP90-containing multi-chaperone complexes interact not only with HSF1 but also with nonnative proteins, the concentration of nonnative proteins can influence the assembly on HSF1 of HSP90-containing multi-chaperone complexes that repress activation, and may play a role in inactivation of HSF1.

Since yeast HSF1 is under negative regulatory control in the absence of stress, a model similar to the one in metazoans, where in the basal state HSF1 would be tied up with HSPs in a regulatory loop was investigated. This model however has been challenged by the observation that overexpression of Ssa2, one of the HSP70 homologues highly induced by HS in yeast, and thus a prominent candidate molecule in this feedback regulation, did not inhibit the HS response. Nevertheless, a role for HSP70 proteins in the down-regulation of the response cannot be discarded. In contrast, evidences were provided indicating that in *S. cerevisiae* and in *D. melanogaster* HS and oxidative stress can act directly on HSF1 by causing conformational changes capable of inducing its

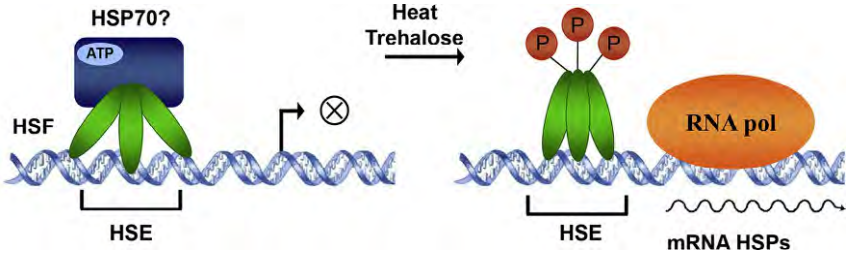

Figure 6 Activation of HSF1 during heat stress in budding yeast. In yeast, HSF1 trimerizes and binds to HSE in the HS gene promoters before stress, but it remains in a low-activity form, probably inhibited by HSP70 or other chaperones at normal temperatures. During heat shock and in the presence of high levels of the chemical cochaperone trehalose, HSF1 undergoes hyperphosphorylation and conformational changes and becomes active producing a dramatic increase in the expression of HSP genes.

activation. In addition, it has been shown that the transcriptional activity of HSF1 during the HS response depends on trehalose levels in *S. cerevisiae*. Strains with low levels of the disaccharide trehalose have a diminished transcriptional response to HS, while strains with high levels of trehalose have an enhanced transcriptional response to HS. The enhanced transcriptional response does not require the other heat-responsive transcription factors present only in yeast, Msn2 and Msn4, but is dependent upon heat and HSF1. In addition, the phosphorylation levels of HSF1 correlate with both transcriptional activity and the presence of trehalose. These *in vivo* results support a new role for trehalose (which was previously known to be correlated with tolerance to adverse conditions in yeast, and suggested to act as a chemical co-chaperone), where trehalose directly modifies the dynamic range of HSF1 activity and therefore influences HSP mRNA levels in response to stress (Figure 6).

Even though the HS response has been better characterized in *S. cerevisiae*, many studies with heat stress have been carried out with other fungi and a number of different eukaryotic microbes. Presently, genomic and postgenomic tools, which have become available for high-throughput studies in several microorganisms, will certainly improve significantly our knowledge of HSP function and control in these organisms.

Final Remarks

For a variety of microbial pathogens, the expression of HS genes has been observed to be upregulated following host cell infection. Concurrent with this fact is the observation that HSPs are immunodominant antigens for a number of infectious organisms including bacteria, fungi, parasites, and protozoa. However, the exact role of HSPs during infection has not been clearly demonstrated yet.

For instance, studies with parasites and protozoa revealed that surface-expressed HSP90 is immunogenic in Chagas' disease, ascariasis, leishmaniasis, toxoplasmosis, *Trichinella spiralis*, and infection due to *Schistosoma mansoni*. In addition, *Trypanosoma cruzi* HSP90 can functionally complement yeasts, and its inhibition by the HSP90 inhibitor geldanamycin demonstrated that it was essential for cell division, as epimastigotes were arrested in the G1 phase of cell cycle. Recombinant *Leishmania* HSP90 and HSP70 were recognized by sera from patients with visceral leishmaniasis but not by Chagas' disease patients. HSP90 inhibitors led to growth arrest and the differentiation from the promastigote to amastigote. HSP70 and HSP83 have been suggested as distracting the immune system to a nonspecific activation of immune cells leading to immunosuppression.

In pathogenic fungi, similar results were found and this knowledge has been used in novel therapies against these microorganisms. For instance, the immunodominance of HSP90 in patients who survived disseminated candidiasis and invasive aspergillosis made this molecule a natural target for immunotherapy. In addition, immunization with a membrane fraction from *Candida albicans* containing HSP90 in combination with fluconazole protected mice from infection.

Finally, the involvement of HSPs in a number of human diseases including cancer and neurodegenerative disorders, as well as aging, gives further importance for the studies of these universally conserved proteins.

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Swimming and Swarming Motility

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Glossary

Chemotaxis Chemotaxis serves as a navigation system for bacteria allowing them to temporally detect chemical gradients and respond. It works by biasing the direction of rotation of the flagellar motor. Additional forms of taxis – towards light and oxygen – work similarly.

Flagellar filament The semi-rigid helical flagellar filament (10–15 μm in length) serves as a propeller. It is polymerized from subunits called flagellins.

Flagellum The rotating bacterial propulsive organelle called the flagellum is composed of the flagellar filament, the hook basal body, and the motor. Cells can have a single flagellum or multiple flagella, inserted at one or both ends of a cell (polar) or around the cell body (peritrichous or lateral).

Hook basal body This structure, composed of rings and rods, serves as the rotating central drive shaft.

Motor The reversible motor transduces the chemical energy of membrane potential to rotate the hook basal body-propeller in a clockwise or counterclockwise direction via stator-rotor interactions.

MS ring The foundational ring for the flagellar structure, the MS ring, serves as the mounting plate in the cytoplasmic membrane for basal body and C ring assembly.

Rotor The core of the rotor is the switch complex and MS ring.

Stator (or torque generator) A multimeric complex of two proteins form the ion-conducting stator, which is anchored to cell-wall peptidoglycan. The passage of ions through a channel in the stator is coupled to generation of torque through electrostatic interactions of the stator with the switch complex to drive rotation. The best characterized stators, $MotA_4B_2$ and $PomA_4B_2$, use protons or sodium ions, respectively.

Surface sensing and mechanosensing The flagellum is an organelle of locomotion and also serves as a sensor of environmental conditions, including external load such as viscosity and contact with a surface, sodium concentration, ion motive force, and pH. Signal reception by the flagellum can be transduced to remodel motor configuration and/or gene expression.

Surfactants or wetting agents Molecules can be secreted or extracted from the surface by the organism to aid swarming motility by reducing surface tension or increasing wetness.

Swarming Flagellar propelled translocation on surfaces.

Swimming Flagellar propelled translocation in aqueous environments.

Switch complex (or C ring) Mounted at the cytoplasmic face of the basal body on the MS ring, the switch complex interacts with the stator to participate in torque generation. In addition, the chemotaxis system interacts with the switch complex to modulate the direction of motor rotation.

Flagella and Motility

Motility is a successful survival strategy for many organisms, and flagellar-propelled motility is a particularly outstanding mode of locomotion for a bacterium. Flagella allow bacteria to swim through dilute aqueous environments or to swarm in highly viscous conditions and over surfaces. The flagellum, which acts like a propeller, is powered by a rotary motor capable of turning the flagellum at very fast speeds. Rotation speed in liquid has been clocked at ~300 revolutions per second for *Escherichia coli* and >1700 revolutions per second for *Vibrio alginolyticus*. Such speeds produce swimming velocities of ~30 microns per second for *E. coli* and more than 100 microns per second for *Vibrio* species. The predatory *Bdellovibrio bacteriovorus* attacks other bacteria using high-speed collisions and swims at ~160 microns per second. On surfaces, flagellar-assisted motility allows bacteria to rapidly swarm in groups, sometimes achieving motility as fast as 10 mm per hour on agar medium. When coupled with a central navigation system (i.e., chemotaxis), flagellar rotation can be adapted to allow bacteria to move towards favorable environments and away from toxic conditions.

Considering that a bacterium is ~2 μm in length, flagellar-assisted movement is very fast. To achieve these speeds, the architecture of the flagellum is highly complex (Fig. 1). It contains about 30 different proteins, and the amount of each protein varies from ~10 to more than 20,000 molecules. Assembly of the flagellum involves a dedicated type III secretion export apparatus. Proteins are secreted through a hollow, central axial structure and assembled in an ordered fashion beginning with the MS ring in the cytoplasmic membrane, followed by other components of the basal body, the hook, and finally filament subunits to make the propeller.

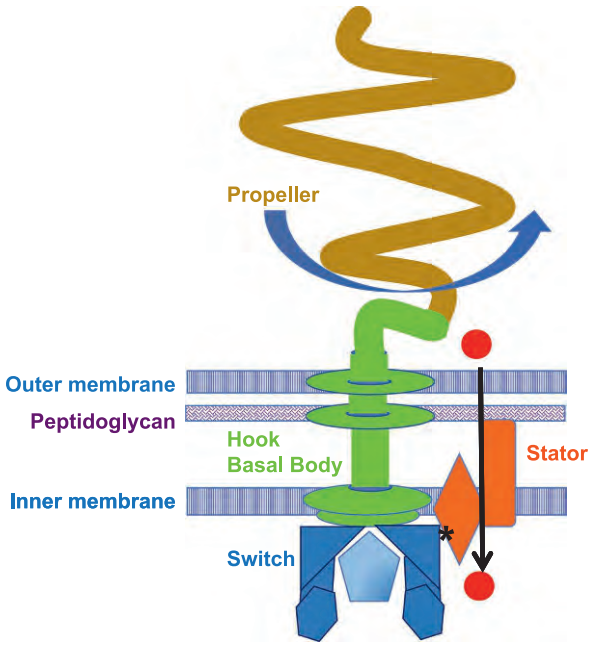

Fig. 1 Key parts of the flagellum. The flagellum works like a propeller (brown) that is turned by a central drive shaft, which is made up of the hook basal body (green) and the switch complex (blue). The type III secretion export/assembly apparatus (light blue pentagon) processively transports flagellar components, which polymerize at the growing distal end. Multiple stators encircle the rotor (one shown in orange). Ion flux (red circles) through the stator is coupled to rotation via electrostatic interactions between the stator and the switch (asterisk). The stators are stabilized via dynamic interaction with peptidoglycan. For some types of flagella, additional stabilization of the rotor can be provided by auxiliary proteins such as MotX and MotY (not shown).

Here, we briefly describe the working parts of the flagellar machinery, torque generation, and accessory elements adjusting movement. In addition to acting as propellers, flagella serve as environmental sensors, informing bacteria of surface contact. The underlying themes of flagellar-assisted propulsion are great structural diversity and superb adaptability. Swimming and swarming motility allow bacteria to navigate varied and changing environments; thus, these forms of locomotion play key roles in bacterial survival and colonization.

The Propellers

The propellers are hollow, semi-rigid helical filaments (~20 nm diameter and 10–15 μm or about 3–10 times the cell body in length). The filaments are composed of single subunits (called flagellins) or mixtures of subunits that self-assemble; these flagellins can be modified by phosphorylation or glycosylation. The flagellar filaments can be naked or protected by an outer membrane sheath, which may provide immune evasion and enhance adhesion. Flagella can be located at one or both poles, occurring as a single, polar flagellum or multiple polar tufts of flagella. They can be placed singly at the side of a uniflagellated cell. Or, peritrichous (also called "lateral") flagella can be multiply arranged around the cell body. Sometimes the filaments are located in the periplasm, which is the case for the Spirochetes: Rotation of these periplasmic flagella turns the spirochete cell body into a screw. Thus, the structural design and arrangement of flagella on the cell body are tremendously varied. Additionally, elegant regulation links flagellar gene expression to morphogenesis and precisely controls flagellar filament assembly, length, number and placement on the cell body. These elements are described in more fascinating detail elsewhere in this series.

The Hook Basal Body (HBB)

The hook basal body serves as the central drive shaft connected to the propeller. The HBB is embedded in the cell membrane and consists of rods and rings (the basal body) and a flexible linker (the hook) to which the propeller is attached. Some of the rings serve as bushings for the rods to pass through the peptidoglycan layer and outer membrane (the P and L rings); other rings are integrated structurally into the rotor, e.g., the MS ring, which is the first element to be assembled. The C ring and the flagellar export apparatus are recruited by the MS ring, at which time the structure becomes competent for sequential secretion and assembly of the remainder of the basal body, hook, and filament.

The Stators

First detected in the cytoplasmic membrane by freeze-fracture electron microscopy, the stators encircle the flagellar basal body like studs (Fig. 2(A)). Each stator contains four copies of MotA and two of MotB (or their homologs); they form the ion-conducting channel complex ($MotA_4MotB_2$) that utilizes ion flux to induce the conformational changes that drive flagellar rotation. During force generation, the stators are transiently anchored to the cell wall via a peptidoglycan-binding domain in the C-terminus of MotB. Each stator functions independently to generate force. For *E. coli*, a single torque generator is sufficient to rotate the flagellum under conditions of low load, and the number of stators surrounding the rotor increases with increasing load on the flagellum to a

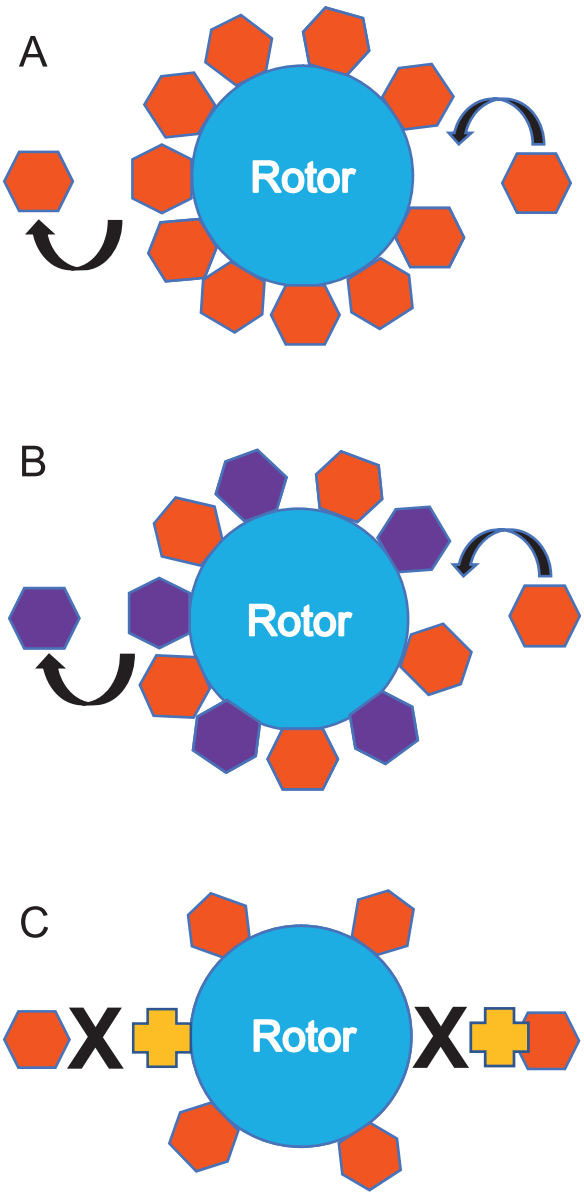

Fig. 2 The diversity of the flagellar torque generators. (A) $MotA_4B_2$ complexes work as stators (red hexagons) surrounding the rotor and dynamically exchange (indicated by arrows) with a pool in the membrane. Each stator complex works independently to conduct ions (usually sodium ions or protons) through a channel to generate force. Although a single stator is sufficient to rotate the flagellum in liquid, increasing stator number increases torque delivered at the rotor to drive greater speed and/or rotation under conditions of higher load (e.g., viscosity). The *E. coli* rotor accommodates ~11 stators, whereas the high-torque rotors of some bacteria have wider scaffolding rings allowing more stators to be docked. (B) Some flagella are powered by dual motors that use different energy sources, e.g., H^+ (purple) or Na^+ (red) powered torque generators. The number and kinds of stators docking at the rotor can be affected by environmental conditions such as viscosity, pH, and sodium concentration. Thus, environmental conditions influence motor configuration and performance. (C) Accessory proteins (gold) can alter rotation by changing direction of rotation (chemotaxis), preventing rotation (braking), or by interfering with stator-rotor interaction (clutching). These features can bind to the switch complex in the rotor (e.g., CheY or EpsS) or to the stator (e.g., MotI or FlgZ). The activity of some of these proteins is controlled by phosphorylation or the second messenger c-di-GMP.

maximum of ~11. Dynamic assembly of the stators at the rotor results in rapid turnover and exchange of stators at the rotor through diffusion with a pool in the cytoplasmic membrane.

Fuel Sources

The power that fuels rotation is derived from the transmembrane potential of specific ions. The proton motive force is used by *E. coli* and *Salmonella enterica* to power MotAB-type torque generators; whereas the sodium motive force drives the PomAB torque generators of the marine *Vibrio* and the MotPS units of *Bacillus* species. More exotic motors can use K^+, Li^+, or Rb^+ as the motor driving force.

Torque Generation by Stator-Rotor Interactions

The rotary motor turns the helical flagellar filament by coupling ion flux through the $MotA_4B_2$ complex to rotation via specific electrostatic interactions between charged amino acids in the MotA protein of the stator and the FliG protein in the switch complex of the rotor. The switch complex is mounted on the base of the MS-ring of the basal body in the cytoplasm. It contains multiple copies of three proteins (FliG, FliM, and FliN) and is essential for torque generation and motor directional switching, which is the basis of chemotaxis.

The generation of torque for rotation involves stator docking at the rotor, stator anchoring to peptidoglycan, and opening of the ion channel in the stator to permit ion flux. The sequence of these events is currently unclear – e.g., whether channel opening is predicated by stator docking at the rotor and/or by peptidoglycan (PG) binding. Atomic force microscopy has shown that sodium flux induces structural transitions in the PG-binding domain of *B. subtilis* MotPS, and this conformational change results in docking of the stator with FliG in the switch complex, ion channel opening, and torque generation. However, the sequence of these events may differ for different kinds of motors. Crystal structures, nuclear magnetic resonance, and small angle X-ray scattering studies are providing insight into these steps.

The structure of some motors is more complex than the MotAB-type. The epsilon proteobacteria like *Campylobacter jejuni*, *Wolinella succinogenes*, and *B. bacteriovorus* have high-torque motors with ~17 stator complexes surrounding the rotor. These large numbers of stators are accommodated by wide-diameter, disk-like structures with periplasmic rings. Thus, additional power is achieved by more stators at a greater distance from the driveshaft. Stator occupancy in these bacteria is normally high (i.e., not responsive to load as for the *E. coli* motor). Since power output is not dependent on stator recruitment, this configuration allows for very fast swimming speed in liquid and immediately available torque to power motility in viscous environs.

PomAB motors, which power the high-speed rotation of the *Vibrio* polar flagellum, require auxiliary parts (MotX and MotY) that are essential for torque generation. MotX and MotY form an extra ring structure (T ring) around the basal body below the peptidoglycan and are required for proper stator rotor engagement. Another ring structure (H ring) aids MotX and MotY assembly. MotX interacts with PomB and MotY; MotY interacts with the rod of the basal body and peptidoglycan. These motor proteins are not specific to sodium-type motors. *Shewanella oneidensis* MR-1 uses MotX and MotY for both Na^+ and H^+ driven motility. In addition to aiding high speed rotation in low load situations, MotY plays a critical role in some types of motors under conditions of high load such as for the swarming motility of *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*. Thus, by contributing an additional peptidoglycan anchoring domain to the motor structure, MotY (by itself or along with MotX in some motors) plays a key role in stator stabilization or stator/rotor docking under conditions when more torque is required to power rotation.

Dual Motors

Some bacteria have multiple kinds of stators that work at the same rotor (Fig. 2(B)). For example, *Bacillus subtilis* and alkaliphilic *Bacillus* species possess dual stators that operate over a wide pH range and using different power sources. In most conditions, the H^+-type MotAB motor of *B. subtilis* drives motor performance and enables fast swimming speeds, but input by its Na^+-type MotPS stator contributes to motility at high pH, elevated salt, and high viscosity. Similarly, *S. oneidensis* uses its Na^+-type PomAB stators to power rotation when Na^+ is high; whereas, MotAB-type stators aid its motility in low Na^+ conditions. *P. aeruginosa* has dual motors and these seem to work optimally under conditions of different load (liquid versus surface translocation). Artificial hybrid fuel motors have been constructed in *E. coli* by co-expressing proton and sodium-driven motors. This hybrid motor adapts to the prevalent ion gradient and removal of that gradient leads to dissociation from the rotor and stator swapping.

Widespread genome sequencing has revealed that many bacteria possess multiple MotAB homologs. Although it is mostly unknown whether all these motor genes are functional, it seems likely that the torque generators they encode function under different environmental conditions. Thus, dual motors powering a single rotor can each have particular contributions under different conditions. Stator selection at the rotor can be the result of abundance of motor parts created by transcriptional control. But also, the type or number of stators docking at the rotor appears linked to the capacity to generate torque. For example, the number of rotor-docked stators is dependent on sodium concentration and ion flux for the *Vibrio* sodium-driven $PomA_4B_2$ torque generators.

Performance Enhancing Features: Dual Flagellar Systems, Flagellar Number Regulation, and Lubricants

In addition to bacteria that possess dual motors powering the same flagella, bacteria like *Aeromonas*, *Azospirillum*, *Photobacterium*, *Shewanella*, and *Vibrio* species have two flagellar systems with dedicated motors for each system. Marine *Vibrio* use sodium-powered polar flagella for swimming and proton-driven lateral flagella for swarming (Fig. 3). *Aeromonas hydrophila* is particularly well equipped for motility in differing conditions: One type of stator complex drives its lateral flagella used for swarming while two other types of stator complexes utilize sodium to power the polar flagellum under high and low sodium ion concentrations. Each of the dual flagellar systems is often superior for movement under specific conditions and used exclusively for that purpose (i.e., polar organelles for swimming and lateral organelles for swarming).

Alternatively, the secondary system can aid navigation. *Shewanella putrefaciens* produces two kinds of flagella: A single polar and a 1–2 lateral flagella. The polar system is the main propulsive organelle for this organism; however, the lateral flagella aid movement in soft agar by decreasing the tumbling turning angle, which results in directional persistence of the cell trajectory and increased spreading into the environment.

Simply having more of the same kind of flagella to power movement facilitates swarming. *Proteus mirabilis* greatly increases flagellar number (>100-fold) to improve performance and rapidly propel swarming translocation over a surface. Some other bacteria more modestly increase flagella production during swarming (including *Bacillus, Pseudomonas, Salmonella* and *Serratia* strains).

Lubricants such as slime and wetting agents can play a role promoting flagellar-assisted motility on surfaces. Many swarming-proficient bacteria secrete biosurfactant-like molecules that aid motility by reducing surface tension. These include the glycolipid rhamnolipids of *P. aeruginosa* or the lipopeptides surfactin and serrawettin produced by *Bacillus* and *Serratia* species, respectively. Flagellar rotation and directional switching of the flagella may also mechanically extract liquid from agar to facilitate swarming.

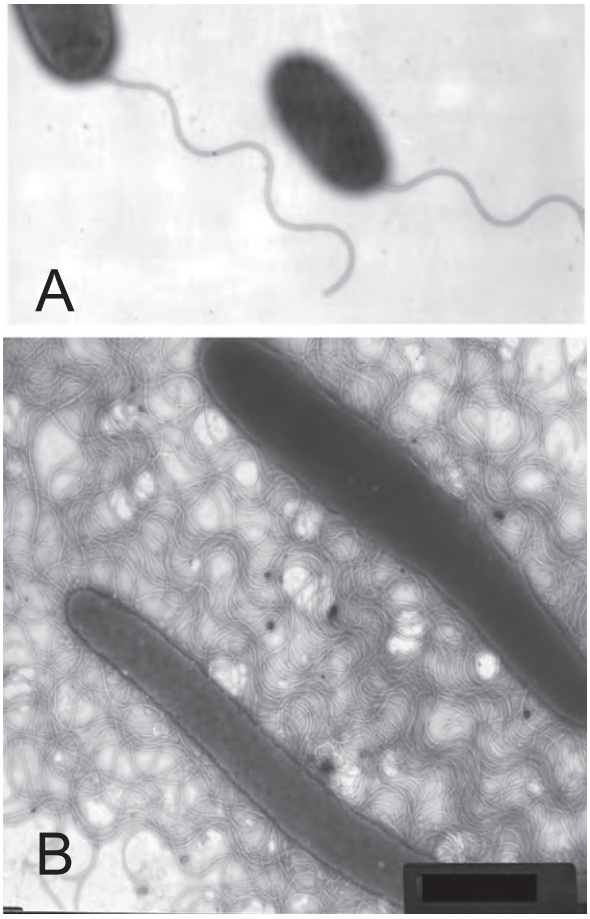

Fig. 3 The dual flagellar systems of *Vibrio parahaemolyticus*. Some bacteria possess two flagellar systems dedicated to swimming or swarming. (A) A single, sodium-powered sheathed polar flagellum propels *V. parahaemolyticus* in liquid; (B) whereas on surfaces, cells differentiate into long, swarmer cells with numerous lateral flagella and move in groups. These flagella are powered by proton-driven motors. Cell width in each micrograph is ~0.5 μm. Note that cell body elongation or hyperflagellation are not required characteristics of swarming for many bacteria. The electron micrograph in (A) is of cells grown in liquid and stained with 1% uranyl acetate; image is from McCarter. The micrograph in panel B is of cells grown on surfaces and stained with 0.5% phosphotungstic acid; image is from McCarter LL. "Multiple modes of motility: a second flagellar system in *Escherichia coli*." J Bacteriol. 2005 Feb;187(4):1207–9. PubMed PMID: 15687183; PubMed Central PMCID: PMC545644.

Accessory Motor Parts: Clutches and Brakes

Motility without maneuverability is not a highly effective way to get around, and numerous accessory proteins influence directional and gear changes (Fig. 2(C)). The switch component proteins (FliG, FliM, and FliN) assemble dynamically and their configuration is influenced by the chemotaxis signaling protein CheY, whose phosphorylation status is controlled by the chemosensory system. Binding of CheY∼P to FliM and FliN induces cooperative remodeling of the switch complex by inducing dissociation of some FliM/N complexes, and this determines directionality of motor rotation. For many bacteria, this induces reversal of the direction of rotation, although for some organisms, like *Rhodobacter sphareoides*, CheY∼P works like a brake to halt rotation.

Other proteins impact motor function. In *E. coli*, YcgR represents a class of proteins used by many bacteria to control flagellar rotation. These proteins contain a c-di-GMP-binding domain, PilZ. For most bacteria, high concentrations of this second messenger diminishes motility. In the presence of c-di-GMP, YcgR interacts with FliG in the rotor to prevent rotation. Other c-di-GMP-binding proteins like *B. subtilis* MotI and *P. aeruginosa* FlgZ bind to MotA homologs; this interaction disengages or blocks engagement of the torque generator at the rotor. Another motor-binding protein that seems to work via interference to decrease power applied to the rotor is *B. subtilis* EspE, which is a glycosyl transferase that has been shown to interact with FliG. Additionally, extracellular polymers can act to directly encumber flagellar rotation, e.g., cellulose production by *S. enteriditis* jams flagellar action.

Working as gear shifting mechanisms to alter direction of motor rotation, like brakes on the motor to impede rotation, or as clutches to disengage or prevent engagement of the stators at the rotor, these features offer simplicity and speed of action. A molecule interacting with the complex, spinning flagellum can bring its action immediately to a halt or reverse its direction of rotation. These strategies allow bacteria to rapidly and reversibly shift between being motile or sessile. Flagella, which are energetically costly to make and operate, propel bacteria towards optimal environments. Upon reaching an ideal niche, flagellar activity can be efficiently and quickly dampened, held in check by mechanisms such as these. When needed again, motility can be rapidly resumed without the expense of producing new flagella.

Feats of Navigation

Flagellar arrangement on the cell body, motor rotation, and the chemotactic signals that control direction of flagellar rotation produce distinctive modes of locomotion. Most well studied is the free-swimming movement of *E. coli* and *S. enterica*. The swimming pattern of these peritrichously flagellated bacteria, which have ∼6 flagella around their rod-shaped cell bodies, is characterized by alternating periods of "runs and tumbles" that are the product of counterclockwise (CCW) or clockwise (CW) motor rotation, respectively. CCW turning motors result in flagellar bundling and smooth propulsion, while a motor that switches to a CW direction will cause the bundle to fly apart and the bacteria will tumble and reorient. Since bacteria are too small to sense spatial changes in concentration, they use a temporal mode of chemical detection. In the absence of sensory input, the swimming trajectories are described as "random walks" that result from alternating periods of smooth runs and tumbles. Chemotaxis biases the movement in a favored direction by controlling the frequency spent in CCW and CW modes of motor rotation, allowing the bacterium to make net progress towards or away from chemical gradients in their environment.

Polarly flagellated bacteria behave somewhat differently. When the motor changes direction, the bacteria reverse direction and back up. However, the reversal of the propeller causes a subsequent buckling deformation of the flexible hook that links the propeller to the rotor. This results in reorientation of the direction of cell movement. This pattern is called a "run-reverse-flick".

Uniflagellated *Rhodobacter sphaeroides* does not have a bidirectional motor. Its swimming pattern alternates between "runs and stops." This strategy of using a motor that only turns in one direction achieves much the same consequence as tumbling. Due to conformational changes induced in the flagellar filament, stopping causes cell reorientation.

Swarming Behavior

Swarming is flagellar-assisted movement on surfaces. Like swimming, swarming requires a fluid environment. However, this is movement in a structured environment, and it often results in complex and beautiful pattern formation. Swarming colonies can be smooth films resulting from rapid radially expanding surface colonization, or they have complex architectures with terracing or fractal patterns. Some examples of swarming pattern formation are shown in Fig. 4(A and C); however, the diversity of the swarm architecture is astonishing (e.g., see "Relevant Websites section"). The colony geometries produced by this form of collective behavior is dependent on many conditions, including the type of surface (e.g., agar concentration and type), nutrient composition of the growth medium, temperature and humidity, surfactant production, cell-cell signaling, and features unique to the organism (e.g., cell body shape and flagellation pattern).

The advancing edge of a swarm is a layer of cells that move in packs (Fig. 4(B and D)). A variety of maneuvers can be observed during swarming: Straight movement, stalls which occur at the advancing edge, lateral movement, and reversals. Movement of the swarm often looks like streaming swirls, and the pack moves forward for longer times than single cells. Much of the analysis of bacterial acrobatics on a surface has been done using *E. coli* on 0.45% agar medium; however, there are significant differences between *E. coli* and some of the more robust swarmers such as *P. mirabilis*, *Rhodospirillum centenum*, and *V. parahaemolyticus*. These bacteria swarm proficiently under restrictive conditions (e.g., 1.5% agar medium). During swarming they can differentiate to

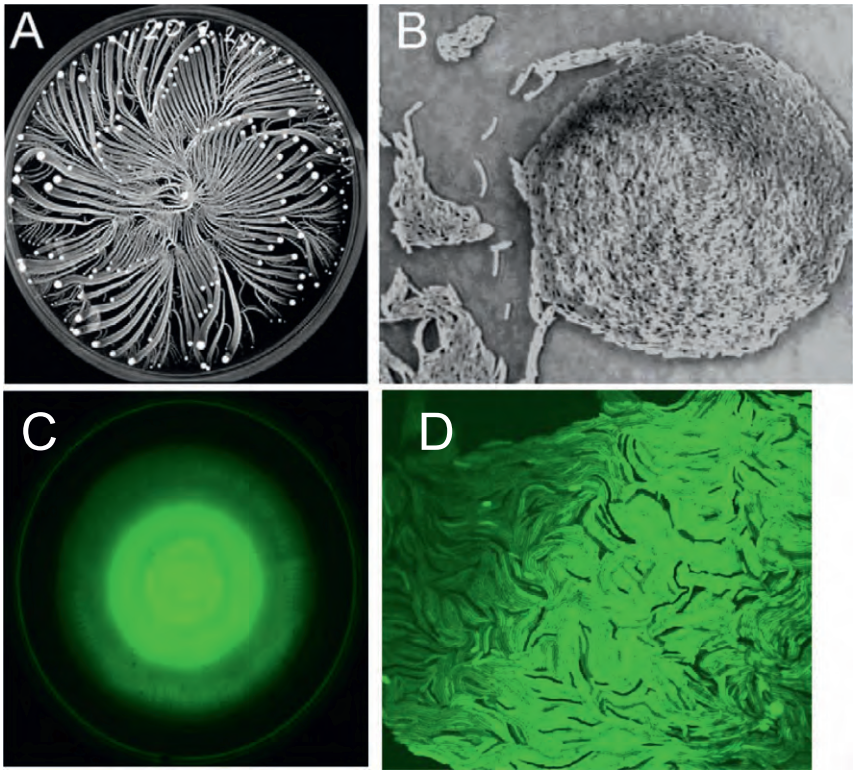

Fig. 4 Swarming can be a social activity resulting in complex pattern formation. Whole colony views of petri dishes with swarming colonies of *Paenibacillus vortex* on 2.25% agar medium (A) and *Vibrio parahaemolyticus* on 1.5 % agar medium (C). (B) Zoomed images at the edge of one of the condensed vortexes (bright spots in panel A). (D) Zoomed images of swarmer cells at the edge of the swarming colony in panel B. A and B are images from Ingham, C.J., Jacob, E.B., 2008. Swarming and complex pattern formation in *Paenibacillus vortex* studied by imaging and tracking cells. BMC Microbiol. 8, 36. https://doi.org/10.1186/1471-2180-8-36. PubMed PMID: 18298829; PubMed Central PMCID: PMC2268691. C and D are fluorescent images of a *V. parahaemolyticus* strain carrying a GFP plasmid.

become very long swarmer cells (~20–40 μm) with hundreds of peritrichously arranged flagella (Fig. 3(B)); moreover, the robust swarmers demonstrate directed movement (e.g., chemotaxis and phototaxis) on a surface, which *E. coli* does not. Thus, it will be interesting to learn how elongation and hyperflagellation as well as chemotaxis affect swarming behavior.

Reversals on a surface occur when the motor changes rotational direction, and the flagellar filament undergoes polymorphic shape transitions. When this happens for *E. coli*, the cell body can back up through its "tail." The flagellar bundle, which has pushed the cell body, then becomes a screw that pulls the cell body. Motor reversal can also result in flagella that wrap around the cell: The flagella of polarly flagellated bacteria like *S. putrefaciens* can coil around the cell body and to propel the bacteria backwards in a screw-like fashion. This kind of flagellar wrapping effectively promotes movement through restricted, mucus-filled passageways, e.g., when *Burkholderia* species infect the bean bug and while *Vibrio fischeri* travels to the squid symbiotic organelle. The ability for the organism to dexterously extract itself from a tight spot or dead-end is probably of great advantage in structured environments like the soil, tissue, mucus, or biofilms.

Movement on a surface involves population behavior. Cells tend to move in coordinated packs, e.g., resulting in the striking "bulls-eye" terracing signature of *P. mirabilis* or the beautiful fractal colony patterns captured in the photography of Eshel Ben-Jacob. Since swarming motility is promoted by self-production of wetting agents, population density can be important in establishing the optimal conditions conducive to swarming. Cell-cell communication plays a role during swarming in some bacteria. Quorum sensing and the accumulation of N acyl-homoserine lactone autoinducers induces the production of the surface wetting agent serrawettin by *Serratia liquefaciens* and rhamnolipids by *Burkholderia* and *Pseudomonas* species. Another kind of autoinducer, the S signal produced by *V. parahaemolyticus*, modulates induction of the swarming gene expression program through control of c-di-GMP production. Thus, swarming motility is an important model for social behavior in bacteria.

The Flagellum as a Sensor Signaling Environmental and Lifestyle Switches

Not only is the flagellum a propulsive organelle, but it also acts as a sensor allowing bacteria to discriminate and adapt to their environment, e.g., between life in liquid or on a surface or viscous environment. As previously discussed, one response to mechanical impedance of flagellar rotation involves loading more stators at the rotor to increase the torque for rotation. Although

the molecular mechanism of motor remodeling is unclear, surface sensing may involve detecting the capacity to generate torque and be responsive to load as well as ion availability or ion flux through the stator.

Impedance of flagellar rotation modulates additional cellular activities. Often this involves reprogramming gene expression or activation of enzyme activity. For example, viscous drag on the flagella of *B. subtilis* increases production of DegU∼P, a regulator that induces transcription of genes important for biofilm formation, transformation, and persistence. For *Caulobacter crescentus*, inhibition of rotation of its polar flagellum stimulates production of the second messenger c-di-GMP and synthesis of the holdfast polysaccharide adhesion. For *Vibrio parahaemolyticus*, impedance of polar flagellar function – physically by growth on a surface or in high viscosity, genetically by mutation, or by using the sodium channel blocker phenamil to interfere with torque generation by the polar motor – leads to profound differentiation to the swarmer, an elongated, hyperflagellated cell. Differentiation is the result of induction of a program of gene expression called the surface sensing regulon. Containing ∼70 genes, the regulon includes genes involved in motility, chemotaxis, c-di-GMP metabolism, cell-cell communication, and virulence. Reducing polar flagellar function of *V. cholerae* increases virulence gene expression. Correlation with pathogenesis is a common theme: Virulence gene expression and, in some cases, antibiotic resistance are co-regulated with swarming in many organisms, including *P. mirabilis*, *P. aeruginosa*, and *S. enterica*. Thus, the flagellum serves as a mechanosensor to inform many other cellular activities, aiding adaptation to viscosity and a surface-adapted lifestyle in the environment and the host.

The Important Roles of Swimming and Swarming

Flagellar propelled motility works robustly under a variety of conditions to get bacteria where they need to go. This can be swimming up a gradient of attractant towards a more favorable environment or away from a toxic chemical. The purple photosynthetic *Rhodospirillum centenum* forms swarm colonies that migrate towards light. The nitrogen-fixing soil bacterium *Azospirillum brasilense* swims to oxygen concentrations that are optimal for energy production. Polar and lateral flagella of the deep-sea bacterium *Photobacterium profundum* work to promote swimming and swarming at high pressure.

Flagella are important for multiple steps in surface colonization and biofilm development on abiotic surfaces and in hosts. Flagella and chemotaxis promote arrival at a surface, and swimming propulsion may overcome some of the physiochemical forces that serve as barriers at interfaces. Studies have shown that flagellar motility is critical for initial stages of biofilm formation and in fact, swimming speed correlates positively with attachment rate to glass. Moreover, the flagellar filament itself may be decorated with molecules that aid adhesion – via charged or glycosylated features of the flagellin subunit itself or by adhesins decorating the membrane sheath of the flagellum. Although swimming into the proximity of a surface plays a critical role in initiation of biofilm formation, flagellar motility also contributes to the timing of development and architecture of the biofilm structure. *Pseudomonas aeruginosa* mutants that do not make flagella or have chemotaxis defects do not form the typical mushroom-shaped biofilms.

Flagellar motility plays key roles in host colonization, and for some bacteria it is required for productive infection. Non-motile or non-chemotactic *H. pylori* and *C. jejuni* are unable to colonize the gastrointestinal epithelium. Swimming motility aids penetration of the host mucosal layer in *V. cholerae* infections. In many bacteria – and not only during swarming as mentioned above – flagellar and virulence gene expression are co-regulated. Flagella and swimming elicit host immune responses; they can activate phagocytosis to allow entry into host cells and play roles in macrophage escape. Swimming *P. aeruginosa* promotes the formation of extracellular traps by neutrophils (NETs). And for some bacteria, the flagellar export apparatus secretes not only flagellar proteins, but also virulence factors, e.g., the phospholipase of *Yersinia enterocolitica* or secreted invasion factors of *C. jejuni*. Motility is also important during infection for dispersion to new sites of infection and for allowing new rounds of infection.

Motility can promote or be the consequence of social interactions among bacteria, fostering cooperative or competing interactions or even division of labor. The cooperative swarming motility of *Paenibacillus vortex* results in complex colony development allowing invasion of toxic environments and sometimes, the transport of helper, non-motile cargo bacteria. Swarming can result in group warfare: The social interactions of swarming colonies of *P. mirabilis* or *P. aeruginosa* result in sharp territorial lines (called Dienes lines) that are the consequence of one group inhibiting growth or killing another group. In addition, rapid movement in a pack can provide some protection from antibiotics.

Motility is critical for establishing many symbiotic relationships. It is essential for the marine luminescent *Vibrio fischeri* to establish its symbiotic relationship with the Hawaiian bobtail squid, in part because motility allows the bacterium to navigate its approach to the light crypt. Furthermore, rotation of the membrane-sheathed flagella of V. *fischeri* produces lipopolysaccharide-rich outer membrane vesicles that elicit host immune responses. Many of the microbes that associate with plant roots in the rhizosphere require flagellar-based motility for establishing their interaction, including *Rhizobium*, *Azospirillum*, and *Pseudomonas* species.

Summary

Thus, the extremely powerful and adaptable mechanisms of flagellar motility allow bacteria to exploit and colonize diverse environments. Fuel source, motor and propeller design, speed, and flexibility are all motility parameters that vary greatly among flagellated bacteria. The motors are often complex and sophisticated machines that can be adjusted under changing demands for increased torque. Some bacteria possess dual flagellar systems for fast swimming and powerful swarming. Others have multiple types of torque generators that differentially engage to accommodate fluctuating conditions such as pH, salt concentration or

second messenger activity. Alternately, other bacteria have a single flagellar system, which is used for swimming and is greatly upregulated to produce more flagella during swarming. Social interactions also factor into modulating bacterial behavior during swarming. Studying the complexity and underlying mechanisms of this robust form of locomotion is crucial for understanding bacterial migration and survival strategies, and may provide insight for promoting or paralyzing bacterial colonization.

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Syphilis, Historical

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Glossary

magic bullet The name given to Paul Ehrlich's Salvarsan, the first specific antibiotic, representing the hope that use of a specific drug to kill the specific bacterial cause of syphilis would control the disease.
morbus gallicus (the French disease) The most common name given to a new disease which appeared in Europe in the late fifteenth century; many physicians and historians identify morbus gallicus as syphilis.
paleopathology The technique of examining ancient skeletal remains to identify the diseases suffered by historical populations.
Treponema pallidum The bacteria that causes syphilis. Other subspecies, all extremely similar to each other, cause endemic syphilis (bejel), yaws, and pinta.

Abbreviation

CDC Centers for Disease Control and Prevention

Defining Statement

Despite centuries of study by historians and scientists, using the latest techniques of molecular biology, details of the origins and spread of syphilis remain unclear. Moreover, despite the existence of decisive treatment, syphilis has not been eradicated and instead thrives wherever social and political conditions allow.

Introduction

The history of syphilis has long been one of the most popular topics in the history of medicine. Despite 500 years of research and debate by historians, physicians, and anthropologists, many questions about the origins of the disease remain unresolved. The evolution of syphilis since the sixteenth century demonstrates remarkable changes in both its medical symptoms and the cultural meanings of those symptoms. The history of efforts to control syphilis reveals the inevitable moral judgments about syphilis and other sexually transmitted infections, the balance between disease control and individual rights, and the limited ability of even powerful medical remedies to control the disease. These lessons take on new relevance as fears of HIV/AIDS motivate efforts to eradicate syphilis.

The Origins of Syphilis

The recorded history of syphilis began in the late fifteenth century with the appearance of a new disease, widely called 'morbus gallicus', the French Disease. The earliest cases were described in 1495 as the mercenary army of French King Charles VIII retreated from its siege of Naples. Victims suffered from fevers, open sores, disfiguring scars, and disabling pains; many were consumed by the disease and met gruesome deaths. As the French army disbanded, infected soldiers carried the disease throughout Europe, to Germany in 1495, and to Holland, England, and Greece by 1496. The voyage of Vasco da Gama took it to India in 1498. By 1505 it had reached Japan. Witnesses described the disease with horror. Joseph Gru¨nbeck (1473–1532) left a typical account: 'In recent times I have seen scourges, horrible sicknesses and many infirmities affect mankind from all corners of the earth. Amongst them has crept in, from the western shores of Gaul, a disease which is so cruel, so distressing, so appalling that until now nothing so horrifying, nothing more terrible or disgusting, has even been known on this earth'. This dramatic appearance has puzzled observers for centuries. The 1490s witnessed great transformations in Europe: Columbus encountered the Americas; France invaded Italy; the Spanish government expelled Jews and Moors from Spain; the Pope abolished leper hospitals. Each of these factors might have contributed to the emergence of a new disease. Spanish witnesses traced the disease to Columbus's voyages to

This article is a reprint of D.S. Jones, Syphilis, Historical, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 91–96.

the Americas: they heard from natives that the disease had long been endemic in Hispaniola, and they witnessed unchaste Spaniards acquiring the infection while there. Details of the timing of the early Spanish voyages suggest that syphilis could have been carried back from the New World to Spain in time to appear in Italy by 1495. This would make syphilis the counterpart to the many diseases, notably smallpox, which the Spaniards carried from Europe to America. However, definitive evidence does not exist.

The traditional alternative to this theory asserts that syphilis always existed in Europe and Asia, but was not recognized before the 1490s. Detailed analyses of Egyptian papyri, the Hebrew Bible, Hippocratic writings, and medical texts from India and China contain abundant evidence of the prevalence of sexually transmitted infections. However, these records do not provide clear evidence of the specific presence of syphilis. Ambiguous medieval descriptions of leprosy, which observers believed could be transmitted sexually, have caused particular confusion.

Several novel explanations have been suggested in recent decades. Four distinct diseases – venereal syphilis, endemic syphilis (bejel), yaws, and pinta – are all caused by the same species of bacteria, *Treponema pallidum*. Scientists have long been unable to find histological or immunological differences between the subspecies that cause these different diseases. This suggested that the four diseases might simply be different manifestations of infection with the same microbe, with the results of infection depending on social and environmental conditions. Yaws and endemic syphilis, long present in Europe, transformed into venereal syphilis in the fifteenth century when changes in sanitation, personal hygiene, and sexual behavior produced new conditions favorable for the venereal transmission and manifestations of the disease. A competing theory argued that differences did exist and that venereal syphilis emerged as a result of new and virulent mutations in existing subspecies of *T. pallidum*. This theory has won support from recent genetic analyses. In 1998 scientists sequenced the 1 138 006 bp of the treponema genome. Subtle variation, at only 1000 bp, distinguishes the subspecies that cause venereal syphilis, endemic syphilis, and yaws. When and where did these subspecies emerge? Immunologists looking for the origins of syphilis have sought treponemal antigens in mummies and skeletal remains, finding the oldest in a Pleistocene bear from Indiana. Paleopathological evidence, from thousands of bones from pre-Columbian America, Europe, Africa, and Asia, confirms the presence of treponemal infections in each location. Although interpretation has been controversial, researchers are now confident in their ability to distinguish the bony lesions caused by the different subspecies. Such analyses suggest that yaws has long existed in Africa, Europe, Asia, and America. Syphilis, in contrast, first appeared in the Americas 2000 years ago, returned to Europe with Columbus's crews, thrived in its new environs, and produced the outbreak of morbus gallicus. This story, however, is not fully supported by genetic analyses, which confirm neither the status of the yaws subspecies as the ancestral form nor the status of the syphilis subspecies as a recent arrival. Recognition of the existence of lateral gene transfers between the subspecies further confuses these analyses. As a result, the hopes that state-of-the-art science would finally resolve this historical question remain unfulfilled.

The History of a Disease

The new disease had many dramatic impacts. Observers were horrified by its emergence and manifestations. Everyone blamed someone else. The Italians called it the 'morbus gallicus' or the 'mal francese'. The French named it the 'mal de Naples'. Others labeled it 'scabies hispanicus', the 'American disease', or – in Japan and the East Indies – the 'Portuguese disease'. Some names reflected its manifestations: the 'Great pox', 'fire-piss', 'gangrene grossa', the 'Neapolitan itch', and 'plum-blossom sores'. 'Syphilis' first appeared in the 1530 Latin poem of humanist and physician Girolamo Fracastoro (1483–1553), but this name was not widely used until the eighteenth century.

Early observers traced syphilis to astrological origins, noting the ominous conjunction in 1484 of Mars, Jupiter, and Saturn in Scorpio, the constellation most closely associated with genital affairs. By the 1520s, the disease had been connected to sexual transmission and given a new name: 'lues venerea', the venereal disease. Many people saw the disease as a punishment from God for sexual debauchery (**Figure 1**). Public bath houses closed, distrust divided friends and lovers, and Platonic love emerged as a vibrant social cult.

Physicians struggled to understand the disease for centuries. Many perceived the varied symptoms, including urethral discharges, penile chancres, and skin rashes as a single phenomena, all caused by a single poison. However, over the eighteenth century, the concept of a single disease became increasingly contested and physicians increasingly argued for the existence of many different 'morbi venerei'. This debate drove English physician John Hunter (1728–93) to his famous 1767 experiment. He reportedly inoculated his own penis with pus from a patient with gonorrhea; when he developed a characteristic syphilitic chancre, he concluded that the existence of a single lues venerea had been proven.

Careful work over the nineteenth century settled the controversy. In 1838, Phillipe Record (1799–1889) reported the results of over 2500 experimental inoculations performed at a Paris hospital. He demonstrated that the primary, secondary, and tertiary stages of syphilis all represented a single disease, distinct from other venereal infections. Rudolf Virchow (1821–1902) and Alfred Fournier (1832–1913) extended this work, describing the characteristic pathological lesions of syphilis and the consequences of congenital infection. In 1879, Albert Neisser (1855–1916) settled lingering doubts by isolating the gonococcus, the causative agent of gonorrhea. The characterization of syphilis, in its modern form, was completed in the early twentieth century. In 1905, protozoologist Fritz Schaudinn (1871–1906) and syphilologist Erich Hoffman (1869–1959) described the slender, spiral bacteria, *Spriochaeta pallidum*, later renamed *T. pallidum*. In August 1906, Wassermann (1866–1925), working with Neisser and Carl Bruck (1879–1944), developed the Wasserman test, a complement fixation reaction which became the definitive serological test for syphilis. The disease had been defined, the agent identified, and an objective diagnostic test developed.

Figure 1 Illustration from Joseph Grunpeck's 'Tractatus de pestilentiali scorra, sive mala de Franzos' (1496). This image is traditionally interpreted as showing rays of divine wrath striking sinners with syphilitic lesions. It illustrates theological interpretations of the appearance of the new epidemic.

Managing Syphilis

When morbus gallicus first appeared, physicians treated its victims by purging their bodies of its poison. They recommend hot houses and extreme exercise (ball games, running, boar-hunting, or farming) to induce sweating. They practiced blood-letting and prescribed purgatives. Mercury, given topically or orally to induce sweating and salivation, became popular. Its severe side effects – loss of teeth, gum ulcerations, skeletal deterioration, and gastrointestinal problems – matched the severity of the disease. Such punitive treatment seemed appropriate for a disease attributed to venery. However, patients and physicians soon became confused about which symptoms came from the disease, and which from the treatment.

In the 1510s, guaiac, a resin extracted from a West Indian tree, arrived in Europe. Although expensive, and not explained by medical theories, guaiac quickly became popular as rumors credited it with miraculous cures. It satisfied a popular theory that treatments for a disease ought to come from the same place (the Americas) as the disease itself. In addition, its side effects were much less severe than those of mercury. Desperation led physicians to attempt many other treatments: massage, tobacco smoking, abstinence from pork and peas, and decoctions of vulture broth with sarsaparilla. Hospitals and hospices appeared throughout Europe. Most were established as hospitals for the incurable, but as the effects of the Great Pox moderated over the century, they increasingly became places for cure and care. The Catholic Church even developed a special mass, 'Missa contra morbum gallicum'.

Mercury and guaiac dominated syphilis treatment for centuries. Surgeons developed ways to excise or cauterize the sores. Medical entrepreneurs produced and marketed secret remedies. But by the late nineteenth century, some physicians had begun to suspect that their treatments provided no benefit. This suspicion was confirmed by the famous Oslo Study, in which 2000 patients, between 1890 and 1910, received no treatment, and fared just as well (or poorly) as those receiving mercurial ointments and other treatments. The infamous Tuskegee Syphilis Experiment sought to extend the results of this study by describing the 'natural history' of syphilis among African-American men in the rural south. Public Health Service officers deceptively promised treatment, but then provided none, from 1932 until 1972. But in contrast to the Oslo Study, these physicians withheld treatments – including penicillin – which they believed to be effective.

As physicians and scientists struggled to develop effective treatments of syphilis, its prevalence steadily increased. In the nineteenth century, syphilis fueled widespread fear of cultural decay and the breakdown of social values. It was identified as a

family poison which spread from profligate men to their innocent wives and children. Fears of casual transmission – through pens, pencils, drinking fountains, toilet seats, and doorknobs – proliferated. In the United States, immigrants were stigmatized as a source of infection. These fears, which reveal deep cultural anxieties about disease and sexuality, motivated many attempts at social engineering. Shocked to learn that as many as one-third of its troops were infected with syphilis, the British government passed the Contagious Disease Acts of 1864, 1866, and 1869. The laws acknowledged that sexual continence was an unrealistic goal. Syphilis could only be contained by minimizing the consequences of the inevitable transgressions: prostitutes would be inspected and, if infected, detained until cured. These laws produced an outcry from purity reformers, who argued that prostitution needed to be criminalized and repressed, not acknowledged and regulated. The laws were repealed in 1886.

Similar calls for social hygiene dominated syphilis control in the United States from the 1890s through the 1920s. Education and moral encouragement beseeched men to be disciplined and restrained, faithful to their wives and families. During World War I, the US military, which saw syphilis as a substantial threat to its effectiveness, conducted extensive campaigns against the disease. Just as they drained swamps to prevent malaria, public health officials shut down red light districts and detained 20 000 suspected prostitutes. The Training Camp Commission distracted soldiers with organized sports and educated them with unambiguous messages: 'A German bullet is cleaner than a whore'. Faced with the French system of regulated prostitution, the Army refused to distribute condoms. Instead, officials established prophylaxis stations to provide treatment if exposure occurred. They hoped that the painful urethral irrigations would be a disincentive to sex. The Navy contributed to the campaign by removing doorknobs from battleships to prevent transmission of syphilis among sailors. Despite all of this work, syphilis rates remained high.

After the war, interest in venereal disease control diminished. Prudish censorship blocked public education in magazines or on the radio. Surgeon General Thomas Parran (1892–1968) led a campaign to reverse this. He decried the 'conspiracy of silence' that surrounded venereal diseases. He argued that sexual continence remained an impossible ideal and that victims of syphilis were victims of a disease and not criminals. His efforts led to the 1938 National Venereal Disease Control Act, which provided federal funding for diagnosis and treatment. States began requiring premarital serological tests (**Figure 2**). This new pragmatic attitude dominated efforts during World War II. Moralistic campaigns against venereal diseases did continue (**Figure 3**). But instead of repressing sexual behavior, officers of the Social Protection Division sought to modify it: instead of providing moral education, they distributed condoms. These efforts substantially reduced the incidence of syphilis among military personnel.

The contrast between the efforts during World War I and World War II illustrates the two extreme options of social management of syphilis. Social hygienists asserted a moral ideal and believed that abstinence was the only way to prevent infection. Pragmatists, in contrast, acknowledged that pre- and extramarital intercourse were inevitable; they sought to prevent infection by encouraging safer sexual practices and by providing unstigmatized treatment.

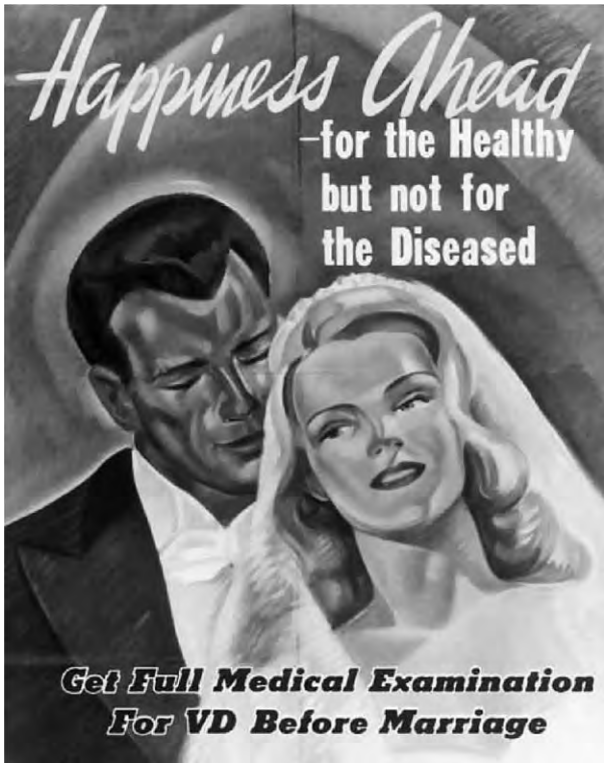

Figure 2 'Happiness Ahead – for the Healthy but not for the Diseased'. During the 1930s, many states began to require premarital screening for venereal diseases. In this poster from a public health campaign, syphilis was portrayed as poison that would destroy marriages and families.

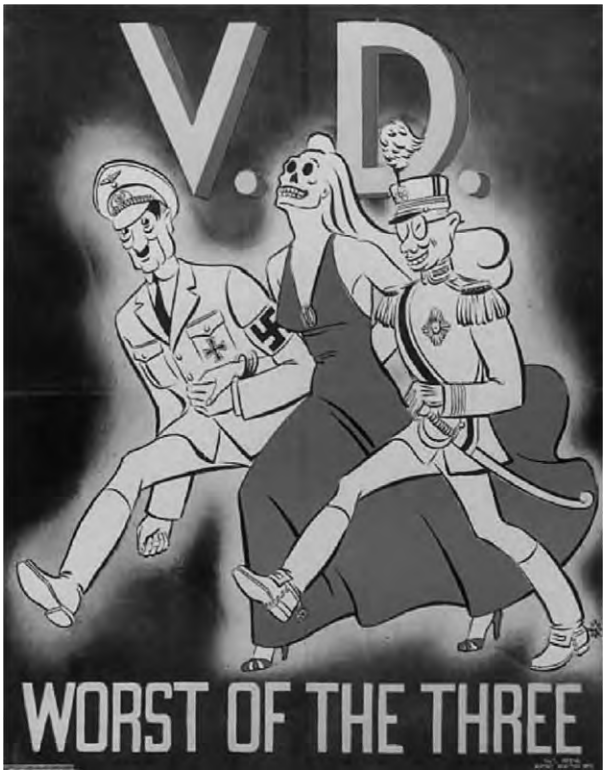

Figure 3 'V.D.: Worst of the Three'. During World War II, the United States military waged a campaign against syphilis that combined clearly moral messages, as seen in this poster, with condom distribution campaigns. Note that in this image, venereal disease is personified as an evil, dangerous woman, and not as a promiscuous soldier.

The Limits of Biomedicine

While public health officials swung between these two extremes of social management, medical researchers gradually produced powerful methods of medical management. In 1909, Paul Ehrlich (1854–1915) announced that his new drug, Salvarsan, had specific activity against syphilis. It was the first time anyone had found a specific drug that killed a specific microbe. Ehrlich called it a 'magic bullet'. With this drug the promise of modern medicine seemed fulfilled: medical scientists had established the specific cause of syphilis, they had developed a specific diagnostic test, and they had a specific treatment. The control of syphilis seemed at hand. Hopes for easy control went unrealized. Salvarsan was toxic and difficult to administer, with some patients requiring two years of treatment; only 25% of patients received the full series of infections. Hope was restored in 1943 when John F. Mahoney (1889–1957) demonstrated that syphilis could be cured with a single injection of penicillin. The miracle drug had been found. Incidence fell rapidly, from 94 957 cases in 1946 to only 6392 cases 10 years later. Syphilis seemed vanquished.

But again, syphilis defied expectations. Despite the power of penicillin, syphilis began a slow recovery during the 1960s. Many factors have been implicated: the promiscuity of the sexual revolution, the availability of oral contraceptives, and decreased funding for education, case tracing, and other disease control programs. Some physicians even refused to prescribe penicillin, fearing that easy treatment only encouraged illicit sexual activity. Whatever the cause, syphilis rates began to rebound. Rising and falling in 10-year cycles, with each peak higher than the last, by 1990 the incidence of syphilis had risen to 50 578, the highest number of cases seen since 1948, with most of the burden falling on impoverished, minority populations. How could syphilis continue to spread despite the existence of penicillin, an affordable and decisive treatment? 'Magic bullets', even those as powerful as penicillin, are never panaceas. The history of syphilis shows the range of scientific, social, political, and cultural factors which contribute to the prevalence of the disease. A single intervention cannot treat the many different problems which become embodied as syphilis. Successful management requires concerted efforts against all of the causes of the disease.

Interest in the history of syphilis took on new urgency in the 1980s with the appearance of HIV and AIDS. Syphilis became both a significant facilitator of the transmission of HIV and a dangerous infection in people with AIDS. In addition, public health officials and policy makers turned to the history of syphilis to guide their efforts against HIV. Many lessons were suggested. Just as penicillin had not solved syphilis, effective treatments for HIV would not end the epidemic. Educational programs, whether advocating abstinence or safe sex, would not cause people to avoid high risk sexual behaviors. Compulsory measures, whether quarantine or serological screening, would bring few results, at high costs to civil liberties. Fear, blame, and stigmatization would shape the development of public health policy.

This new urgency, combined with growing awareness of the risks of sex, fueled more aggressive public health efforts that, combined with periodic ebbing of the prevalence of disease, reversed the slow rise of syphilis. By 2000 the Centers for Disease Control and Prevention (CDC) reported only 5979 new cases, the lowest number reported since it began keeping data in 1941. This reversal triggered optimism that syphilis could be contained. The CDC launched a National Plan to Eliminate Syphilis, which initially made substantial progress. Cases fell most dramatically among African Americans, women, and newborns; the black: white disparity in incidence dropped from 28.6:1 to 5.6:1. However, despite this progress, the absolute number of cases has increased since 2000, reaching 8724 by 2005. Most of the growth has been among men, but rates have also begun to increase among women. Proponents of elimination have known that the most important barriers to eradication would not be scientific, but rather the considerable social and economic obstacles that exist. Syphilis continues to be seen as a moral problem, reducing the willingness of victims to seek treatment. It flourishes in communities which lack basic financial and social resources, which perceive more serious threats from education, unemployment, crime, and other health issues. The shadow of the Tuskegee study has also left many afflicted communities suspicious of public health campaigns. The situation is much worse in developing countries, with roughly 12 000 000 cases worldwide each year. This represents an enormous toll of preventable morbidity, especially in light of the synergy of syphilis and HIV.

The history of syphilis, therefore, demonstrates many important characteristics of diseases. Syphilis is not simply a biological phenomena. Instead, its incidence reflects political and economic structures and cultural beliefs and practices. Its manifestations and meanings change over time, shaping efforts to control the disease. Isolated medical remedies, even those as powerful as penicillin, cannot be the only solution. Successful control requires comprehensive programs, which acknowledge the cultural and social dynamics of syphilis.

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Teaching Resources, Microbiology☆

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Glossary

Archaea A domain of prokaryotic cells with no nucleus, that are distinct from bacteria in that they have evolutionarily unique ribosomal RNA, cell membrane and metabolic components; 'archaeal': pertaining to the archaea.
Aseptic free of microorganisms; not-infected; not-contaminated.
Astrobiology Also called 'exobiology' and 'space biology'; a branch of biology that searches for living organisms (in microbial form) on other planets and their satellites.
Bacteriophage Viruses that infect bacterial cells in order to reproduce and propagate; named so because certain of them, when plated on a lawn of bacteria, form plaques or holes in the lawn, hence 'bacterio-phage' which means 'bacteria eater'.
Bioinformatics A field that combines biology, computer science, and statistics to analyze the large datasets that result from studying an entire genome's worth of information, including the genes that are expressed and the proteins that result from the expression.
Bioterror Terrorism or threats that employ the use of biological agents, organisms or products that they synthesize, to harm individuals or a community.
Competent cells Cells that are able to take up exogenously supplied DNA.
Genomic Having to do with the complete DNA composition of an organism; usually referring to the complete sequence of the chromosomal DNA of an organism.
Immunochemistry The use of antibodies to detect the presence of another molecule in a sample, usually a protein or glycoprotein.
Nanotechnology A field of science that seeks to understand and build structures using atoms and molecules as components. Bacteria are natural nanotechnologists and provide model products.
Polymerase chain reaction An in vitro reaction that uses DNA polymerase from thermophilic bacteria to amplify, by repeated cycles, a specific sequence of DNA, targeted by special primers.
Restriction endonuclease Any of a group of bacterial enzymes that may be used to cleave DNA into discrete fragments; usually via recognition of palindromic sequences.
Transformation The process by which the genetic composition of a cell is enhanced by DNA that has entered the cell and has been expressed.
Winogradsky column An in vitro rendition of an ecosystem containing (usually) a source of water, mud/soil, organic carbon (e.g., newspapers), inorganic sulfur compounds, or other source of electrons – that, over time, will become enriched for self-sustaining, interdependent microbial communities and from which bacterial species can be tracked and isolated.

Abbreviations

AAAS	American Association for the Advancement of Science
ASM	American Society for Microbiology
ASMCUE	American Society for Microbiology Conference for Undergraduate Educators
CDC	Centers for Disease Control and Prevention

☆*Change History*: December 2016. N. Jandu performed major revisions to all the sections: 'Abstract,' 'Defining Statement,' 'Introduction,' 'Sources of Material,' 'Diverse Audiences,' 'Categories of Resources,' 'Scholarship of Teaching and Learning,' and 'Conclusion.' 'Further Reading' and 'Relevant Websites' sections were also updated.

This article is an update of A.C. Vollmer, Teaching Resources, Microbiology, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 299–305.

HHMI	Howard Hughes Medical Institute
NSF	National Science Foundation
PKAL	Project Kaleidoscope
PUI	Primarily undergraduate institution
SoTL	Scholarship of Teaching and Learning
STEM	Science, Technology, Engineering, and Mathematics
WHO	World Health Organization

Defining Statement

This chapter provides a description of the diverse roles, audiences, resources and techniques utilized in the area of microbiology education. This includes the impact and importance of different teaching methods, listings of professional societies and their contributions, as well as a limited inventory of websites that represent online resources.

Introduction

There are multiple resources that support faculty and instructors that are involved with microbiology education and teaching. Some of these resources include peers and colleagues, professional organizations, textbook and publishing companies, and other online resources. This article will describe some of these materials. The spectrum of resources for the teaching of microbiology is appreciated by understanding the context in which microbiology is taught: microbiology is not a stand-alone disciplinary subject. Rather, it is often taught within a wider context that may include general biology, cellular biology, molecular biology, biochemistry, immunology, environmental sciences, or astrobiology.

Sources of Material

Textbook Publishers

Textbook publishers provide new, updated editions of microbiology textbooks every few years. With these textbooks there is a wealth of supplementary material for both the student and the educator. Publishing companies have created complementary online learning platforms. Within these platforms, students can access electronic versions of their textbooks (i.e. e-books), which are easily accessible using a variety of electronic devices (i.e. smartphones, tablets and laptops). These online learning platforms may also provide online quizzes and other review questions, short videos and animations to explain concepts, as well as links to other websites and resources. For those faculty and instructors that are teaching microbiology, publishers provide a variety of 'instructor resources'. These resources typically include electronic files of all textbook figures, images and tables, electronic files of suggested power-point slides that can be modified and incorporated into lectures, as well as test-banks. Most test-banks are often only filled with multiple choice questions, but some test-banks will also include extended-response questions. The most effective test-banks include Bloom's taxonomy classifications of test-questions. All of these instructor resources are typically only accessible by instructors with approved accounts with logins and passwords. Account approval usually follows once the faculty member has committed to adopting a particular textbook for use by the students in their microbiology course. Instructors also have access to electronic versions of the textbook content, videos and animations, as well as links to other online resources and websites. Some faculty and instructors may select a textbook for use by the students in their courses solely for the content in the textbook, while other faculty and instructors may select a textbook based on the combination of textbook content and supplementary resources available for both the student and educator. As well, some publishers will provide microbiology textbooks at varying levels (i.e. for courses for introductory lower-level undergraduate students versus advanced upper-level undergraduates) and for various specialized audiences (i.e. general biology undergraduate students, microbiology major's undergraduate students or for students in health-related programs such as nursing, physician's assistant or medical doctorate). In addition, some textbook publishing companies will also publish a hands-on laboratory manual to complement the microbiology textbook.

Professional Societies

The leading professional society for microbiology content in the United States of America is the American Society for Microbiology (ASM). In a similar way, the Canadian Society for Microbiologists (CSM), the European Society in Clinical Microbiology and Infectious Diseases (ESCMID) and the Australian Society for Microbiology (ASM) provide excellent resources for general microbiology content. But more importantly, these societies provide microbiology information relevant to these countries and regions. For example, country-specific outbreaks and policies could be gathered from these resources. Even more importantly, the names and contact information of country and region specific experts in microbiology can be obtained from these resources. While these general societies all serve as resources for microbiology information, several education-specific societies serve as the ultimate microbiology resources for those involved with microbiology education and teaching. Here again, the American Society for

Microbiology is the leading resource in this area with their annual Conference for Undergraduate Educators (ASMCUE). This conference provides an excellent opportunity for those teaching microbiology to exchange ideas related to teaching undergraduate students, creating assessments and how to actively engage students in the microbiology classroom and laboratories. This conference also provides an opportunity for networking, collaboration and, in general, promotes collegiality. Other organizations, such as Society for the Advancement of Biology Education Research (SABER), the National Association of Biology Teachers (NABT) and the National Science Teachers Association (NSTA) serve a broader membership, but are also useful resources for those involved in microbiology education.

Beyond general microbiology and microbiology education, other professional organizations also serve as resources. Some of these US-based organizations include: the Society for Industrial Microbiology and Biotechnology (SIMB), and the American Biological Safety Association (ABSA).

International-based organizations include: the Microbiology Society, the Society for Applied Microbiology (SFAM) and the Microbiology in Schools Advisory Committee (MISAC), which is a consortium that includes the British Mycological Society, consortium of Local Education Authorities for the Provision of Science Equipment (CLEAPSS), National Centre for Biotechnology Education (NCBE), the The Quekett Microscopical Club (QUEKETT), Society for Applied Microbiology (SFAM), Society of Biology and Scottish Schools Equipment Research Centre (SSERC). All of these websites serve as extensive resources for microbiology education at all levels. All society names and acronyms were up-to-date at the writing of this edition.

Individual Educators

Microbiology educators can be as generous as they are creative – most of them freely share their resources with colleagues and those who seek their advice. Such individuals can be found at a variety of institutions. Sometimes, the materials to be shared are available on the individual faculty members' institutional website. More importantly, a faculty members contact information can be found on their institutional website. This information can be used to request materials or to communicate requests for the use of their materials. When using others resources, be sure to cite the original author and give them credit. A few have published their lessons in publications supported by professional societies.

Other Groups, Organizations and Online Resources

Multiple online resources provide ample current and up-to-date material for those teaching microbiology.

1. The *American Association for the Advancement of Science* (AAAS) is committed to the advancement of science for all individuals. Part of their mission is to promote science education and foster innovative teaching of all disciplines of science at all levels, including microbiology instruction.
2. *BioQuest* was founded in the mid-1980s. With a '3P's' approach (problem posing, problem solving, and peer persuasion), it maintains a library of learning modules, tools, databases, curricular activities, and simulations. In particular, the part of their curriculum that focuses on microbiology is called Microbes Count! BioQuest also sponsors an annual summer workshop.
3. The *Centers for Disease Control and Prevention* (CDC) is the leading organization in the United States for the control and prevention of numerous bacterial and viral agents. The local and regional information that is provided is useful for understanding transmission patterns, conducting outbreak investigations and preventing future cases of illnesses. Information is available in-text, as images, figures and charts, as well as hand-outs, all of which provide excellent resources for both faculty and students in microbiology courses.
4. The *Howard Hughes Medical Institute* (HHMI) sponsors a competition to award university faculty grants under the 'HHMI University Professorship' program in an effort to highlight and promote effective and creative teaching initiatives at institutions that are often better known for their research. In each cohort of these professor awards, there are numerous microbiologists who have contributed new ideas in the area of teaching microbiology and biology in general. These microbiologists have formed an informal network and support the training of graduate students and postdoctoral fellows who are interested in career trajectories that combine teaching and research in a more integrated manner.
5. *MicrobeWiki* is useful resource on the biology of microorganisms. This resource is a collaborative project with articles and entries authored by multiple users. It provides relevant information on the taxonomic classification of select microorganisms, as well as the genomics, cell biology, metabolism and ecological information of the microorganism in question. The content on this page directly relates to the content covered in an undergraduate microbiology course, which makes it a relevant resource for both faculty and students.
6. The *National Science Foundation's* (NSF) mission is to support the advancement of all fields of science, technology, engineering and mathematics (STEM). By supporting scientists and educators, including microbiologists, NSF aims to encourage scientific inquiry and discovery.
7. The *NIH Human Microbiome Project* website provides current information on the microbiome project. This content can be integrated into microbiology courses when discussing the interaction of microbes with humans, when discussing biotechnology, as well as microbial genomics, applied microbiology and, of course, human health and disease.
8. *Project Kaleidoscope (PKAL)* was founded in the late 1980s with a long-term mission of supporting and advocating for strong Science, Technology, Engineering, and Mathematics (STEM) curricula for undergraduate institutions. Since then, there have been strong initiatives to invigorate K-12 programs involving teacher training and a connection to national and state education

standards in science and mathematics. PKAL has resources such as devoted training institutions, designing facilities, as well as a curriculum design for introducing microbiology as part of a robust elementary and secondary science program. science, technology, engineering and mathematics (STEM).

9. *Waksman Foundation for Microbiology* was founded in 1953 by Nobel Laureate Selman Waksman, who was recognized for his discovery of the antibiotic streptomycin. The Foundation has supported many initiatives in microbiology education and research. It has a library of educational resources and activities. At the writing of this edition, it was unclear whether or not the foundation remains active.
10. The *Wellcome Trust* is an international organization that promotes the advancement of science, research and knowledge. The efforts of this organization aim to improve the health of individuals through the sponsorship and funding of a variety of projects. Some of these projects have involved the development of vaccines against bacterial or viral infectious disease agents.
11. *Woods Hole* (Massachusetts) Marine Biological Laboratory's summer course in Microbial Diversity is an unparalleled resource that has trained several generations of microbiologists in the ecological, metabolic, physiological, and, more recently, genomic diversity represented by bacterial and archaeal species.
12. The *World Health Organization* (WHO) is the global leader dedicated to the health and well-being of all global citizens. Much of the work by the WHO has focused on reducing or eliminating global cases of select infectious disease agents, such as Malaria, Polio, HIV and Smallpox. Based on this, the WHO website provides excellent resources on numerous microbial infectious diseases agents. These resources include information on transmission, spread, growth control, risk reduction, immunization and preventative measures of bacterial, viral and parasitic microorganisms. Such content is integral to microbiology instruction, especially those courses directed towards students in pre-medical sciences or allied health programs.

Diverse Audiences

The diverse audiences of microbiology education predominantly include undergraduate students and students in professional-degree programs. Microbiology education is also delivered at community colleges and in the K-12 curriculum.

Institutions and Curricula

1. *K-12*: The concepts of microbiology are introduced into the K-12 curriculum through topics in general science, personal hygiene, recycling and evolution, to name a few. Microbiology educators have provided resources for K-12 teachers in several ways: laboratory manuals with hands-on activities directed toward middle school science teachers and educational workshops at professional meetings such as the ones sponsored by ASM. In turn, K-12 educators have shared their expertise in assessment and outcome measurement with their counterparts in higher education.
2. *Community Colleges*: Microbiology courses are available to students who are preparing for a transition from secondary school to a four-year college or university. These institutions often provide evening and weekend courses with lecture and laboratory courses are often offered back-to-back in one 4–5-h evening session per week. The greater availability of evening and weekend courses at community colleges allows students to enroll in part-time studies. This is an ideal option for students who have jobs during weekdays and for students wanting to transition or enhance their training in the allied health professions.
3. *Primarily Undergraduate Institutions* (PUIs): Smaller universities (also referred to as Liberal Arts Colleges) will predominantly only offer undergraduate degree courses and programs. Within these institutions, the biology departments will typically have 6–12 faculty members. At least one of these faculty members will have the primary responsibility of teaching microbiology. The microbiology courses offered at these institutions might include introductory microbiology, microbiology for majors and microbiology for pre-health professionals. These courses are often accompanied by hands-on laboratory sessions. These institutions are often committed to offer laboratory sessions, which is especially important in microbiology education. The hands-on microbiology laboratory component is often the most valued by graduate and professional students. The students enrolled within these courses are typically majoring in biology or biochemistry.
4. *Universities*: At larger intuitions with undergraduate, graduate and professional-degree programs, microbiology courses can be found in numerous different departments and programs (e.g., biology, cellular biology, molecular biology, biochemistry, immunology or biotechnology departments, as well as environmental science departments and agriculture/agronomy departments). In addition, the study of genomics has placed the study of microbes at the forefront of the evolutionary context of genes and their products. Hence, microbiology courses and the professors who teach them can also be members of departments such as evolutionary biology, geology, astrobiology, nanotechnology, and industrial microbiology.

   At all of these undergraduate institutions, microbiology coursework may be required for a variety of majors, including biology, microbiology, biochemistry, cellular biology, molecular biology, biotechnology, public health and other science/engineering majors and non-majors, and for students preparing for professional training in medical and allied health fields.
5. *Professional schools*: Clinical microbiology, medical technology programs, medical, dental, and veterinary schools all include microbiology courses in the core curriculum. Microbes in the biotechnology industry have now found their way into some business schools that offer joint business and biotechnology degrees. While law schools do not have any formal science coursework, high value is placed on lawyers who have training in the sciences, especially in the area where biotechnology, intellectual property, and patents involve genetically modified organisms.

Categories of Resources

Pedagogy

1. *Lectures*: Microbiology courses are predominantly delivered in lecture format. At larger institutions, the lectures are often delivered in a large auditorium space compared to the smaller classrooms of smaller, primarily undergraduate, institutions. Within these lectures, faculty and instructors aim to utilize creative methods of content delivery to better engage students.
2. *Active Learning*: Creative formats of content delivery can involve asking the students to engage in short discussions with their peers on content-related questions or students may be asked to use an electronic response system (also referred to as clickers) to submit their responses to multiple-choice type questions. These active learning techniques were first pioneered in the undergraduate physics curriculum and have expanded across multiple disciplines of science, including microbiology instruction.
3. *Problem-Based Learning and the Flipped Classroom*: Collaborative exercises, cooperative learning, and case studies can place learning more actively in the students' arena. Some of these activities take place within a scheduled class period. Other activities require students to meet and complete course-work outside of the class time. This method of instruction is also referred to as a 'flipped classroom'. Microbiology is well suited for these exercises since case studies that relate to real-world experiences are available in abundance in everyday new stories, which should be of interest to students. Another useful online resource for case-studies is the 'National Centre for Case Study Teaching in Science' supported by the National Science Foundation.
4. *Inquiry Based Learning*: This learning format aims to more actively engage students by posing questions to students that they are instructed to research and solve instead of just delivering content. As students conduct their research to formulate a response, they will engage in collaborative exchange, may pose their own questions and challenge current dogma.
5. *Videos and animations*: Numerous short videos and animations are made available to those teaching microbiology through their chosen textbook publishers' website (see section on 'Sources of Material – Textbook Publishers'). Another resource for content-related videos and animations includes the iBiology website. In recent years, Ted Talks have proven to be a very useful resource on real-world microbiology content. As well, an archived library of images is available through ASM's MicrobeLibrary. In addition, an endless supply of short videos and clips are available on Youtube. All of these resources can be used to supplement lecture content to enhance student engagement and learning.
6. *Laboratories and Investigative Learning*: Hands-on laboratories sessions are instrumental to the instruction of microbiology content. In these sessions, students learn fundamental techniques in aseptic transfer, growth of microorganisms, enumeration of colonies, unknown identification, enrichment for environmental microbes (and Winogradsky columns), isolation and characterization of bacteriophage, as well as molecular techniques including polymerase chain reaction (PCR), cloning, transformation of competent cells, and restriction endonuclease analysis. Other activities might include immunohistochemistry techniques. Published microbiology laboratory manuals are an excellent resource for routine protocols and procedures that are regularly used in microbiology laboratory activities. Some advantages of utilizing published manuals include detailed descriptions of standard protocols, high-quality photographic images, lab questions for students to assess and review their understanding, and accompanying online resources. A significant advantage to using a published microbiology laboratory manual is the fact that most of these manuals also include a complementary preparatory manual. Preparatory manuals are a vital resource for microbiology laboratory sessions as there are numerous different microorganisms to grow and prepare for each laboratory session. Many of these microorganisms require their own distinct growth medium and conditions. Organizing all of this information in a complementary preparatory manual allows microbiology faculty to easily instruct others (i.e. lab technicians, lab assistants or teaching assistants) on how to prepare for an upcoming microbiology laboratory session. Many microbiology faculty may also develop projects in their laboratory courses that are open-ended investigations, which can span the entire semester. Students are motivated by being actively involved in the study-design, preparation, execution, analysis, and reporting of the experiments. Such projects can be used as a tool to recruit and pre-select students to conduct research with the microbiology faculty member.
7. *Safety*: The microbiology laboratory is the place to teach safety in several ways: working with potential pathogens can provide a context for students to be attentive, keep careful records, perform risk assessment, and practice important containment and disposal techniques. Alternatively, safety can be integrated into the microbiology laboratory session with laboratory exercises designed to emulate water quality, food safety, or other measures that are imperative to public health and safety.
8. *Online courses*: Some microbiology courses may be offered as online courses through the same institution and faculty member that teaches the in-person / in-lecture course. Often the advantage of the online course is that it can be offered in the summer when students may not be on campus. As well, online courses can attract students from other institutions, allow students to improve their grade or prepare for the in-semester course. Online courses are also valuable for those working in the allied health professions that are contemplating returning to school to improve their skills. Websites of individual institutions should be consulted for specific courses. The challenge with online microbiology courses is the administration of hands-on laboratory sessions. As a result, some online courses might only be lecture-only courses. In addition to institution-specific online courses, microbiology courses can also be offered as massive open online courses (MOOC). Leading platforms that may offer microbiology MOOCs include: Coursera, edX, MIT Open Course Ware, Udacity and Khan Academy, as only a few examples of the pioneers in massive open online education.

Assessment and Evaluation

1. *Evaluating Student Learning*: Content-based questions are one certain way to assess student-learning. These questions are often administered as mid-term examinations and final-term examinations. Intermittent quizzes, however, can also be included as a way for both students and faculty to assess learning and misconceptions of microbiology content throughout the semester. Student-learning can also be measured by the student's ability to apply, integrate, and synthesize content through course projects, reports, research papers, presentations or through the design of an experiment.
2. *Surveys*: Surveys have been developed by educators in an effort to determine whether a course has achieved its student learning objectives in communicating content, meeting goals, piquing student interest, teaching skills and motivating students to engage in further study. Some instructors may choose to use pre-tests and post-tests of student knowledge to evaluate student gains in knowledge. Interim assessments or mid-course evaluations can also be utilized so that students and faculty can implement mid-course adjustments toward their learning and teaching goals. Microbiology course assessment usually includes probing whether the students have gained an appreciation and understanding of historic contributions of microbiologists as well as the impact of microbes on human history, bacterial physiology and genetics, diversity and evolution, microbial ecology, and the impact of microbes in the composition of the planet. Medically oriented microbiology courses include aspects of microbial pathogenesis, host–pathogen interactions and coevolution, innate and adaptive immunity, as well as growth control and prevention. Environmental and industrial microbiology courses emphasize the learning and retention of processes and application.
3. *Evaluating Faculty Teaching*: These tools allow for the determination of whether the goals articulated and set by the microbiology faculty or instructor have been achieved. Achievement can be measured objectively in a survey format or subjectively in a narrative format. The former allows for statistical analysis via comparative responses to a graded numerical scale. The latter provides important critiques and testimonials that are equally useful.

Scholarship of Teaching and Learning

The scholarship of teaching and learning (SoTL) includes microbiology teaching, but also encompasses an area of professional development and academic contribution for microbiology faculty and instructors. SoTL emphasizes an understanding of the different modalities of student learning with the utilization of comparably varied techniques in teaching. The latter might include diverse methods of assessment, analysis of knowledge acquisition, retention and application of content and integration of new knowledge with prior knowledge. There are numerous SoTL scholars in microbiology, some of whom have been fellows in the Carnegie Academy for the Scholarship of Teaching and Learning. Microbiology faculty engaged in SoTL have graduate training in microbiology and are regularly involved in microbiology education. As a result, some of these individuals will choose to conduct hypothesis-based research on student learning outcomes in the microbiology classroom and laboratory. Some may be involved with SoTL as their main form of scholarship and professional development, while others may engage in SoTL in addition to hypothesis-driven bench research. In both cases, these scholarship and research activities are recognized as hypothesis-based research, evaluation of prior studies in the primary literature, study designs, research execution, data collection and analysis, as well as communication at conferences and in peer-reviewed journals.

Journals

In microbiology, the *Journal of Microbiology and Biology Education* (JMBE) is the leading peer-reviewed publication that publishes original contributions of microbiology faculty conducting research in the area of microbiology education, teaching and learning. Other journals might also contain publications in related topics. Some of these journals include: *Journal of Biology Education, Cell Biology Education-Life Sciences Education, Journal of Biochemistry and Molecular Biology Education, and Journal of Chemical Education*. The latter provide pertinent articles on information related to some techniques that may be practiced in the microbiology laboratory. Each of these journals is supported by the corresponding American professional society. On occasion, the journals *Nature*, *Cell* and *Science* will publish highly relevant articles on science education, pedagogy and teaching at institutions of higher education.

Conferences

The American Society for Microbiology Conference for Undergraduate Educators (ASMCUE) is an annual conference specifically targeted to those involved in undergraduate teaching and education in microbiology. This conference is an excellent resource for understanding and applying new and innovative teaching techniques, for showcasing faculty scholarship and research in teaching and learning, and for networking and exchanging ideas with peers that are equally focused and dedicated to student learning. The value of ASMCUE was recognized by ASM's Education Board (which organizes ASMCUE), which made a strategic planning decision to expand its breadth to include all biology educators. The ASM also sponsors the Biology Scholars programs, which are aimed at advancing scholarly activities in microbiology education. The Society for the Advancement of Biology Education Research (SABER) is another excellent resource for those conducting research on enhancing biology education within undergraduate courses. The Lilly series of conferences on College and University Teaching and Learning also provide useful insight in the scholarship of

evidence-based teaching and learning amongst undergraduate students. The National Academy of Sciences also sponsors an annual summer workshop on undergraduate education in biology. Other professional societies might also sponsor workshops for educators within the purview of their more general professional meeting.

Professional Networks

Conferences and meetings provide a natural outgrowth of networking, as well other group formats can provide support for those teaching in microbiology and related areas. ASM, for instance, also supports a number of regional branch groups and meetings. There are seven distinct regions which include multiple sub-branches. An example of an ASM branch from each of the seven regions include: Eastern New York, Eastern Pennsylvania, Ohio, Southeastern, North Central, Rocky Mountain and Intermountain. Each branch typically hosts their own regional meeting with microbiology faculty from surrounding schools. Often these meetings include a keynote speaker, student poster presentations and a business meeting. These meetings allow attendees to meet local microbiologists, stay current with topics important for lectures, discussions, and laboratory activities. This support of local and regional networks allows faculty to more easily form collaborations in the scholarship of teaching, meet more frequently and discuss region specific student-populations. In addition to annual regional meetings, branch groups often maintain a website, which allows members to quickly extract the contact information of other local microbiologists. Microbiology faculty can also build and extend their professional networks by maintaining active accounts on Research Gate and LinkedIn.

Awards and Recognition

Many colleges and universities recognize teaching excellence among their faculty; such award conferments are posted on the institutional websites. In many cases, the laureates are microbiologists. Wider recognition of excellence in the teaching of microbiology is afforded at several levels. The Society for Industrial Microbiology annually gives the Waksman Outstanding Teaching Award, naming a recipient who 'shall have been an active full time professor at a recognized institution of higher education for a minimum of 10 years or has attained emeritus status. He/she shall have an active involvement in research in his/her teaching field while carrying a teaching load, and involvement in or contributing to research that leads to advances in his/her career of industrial or applied microbiology or biotechnology'. The ASM Graduate Microbiology Teaching Award honors the 'exemplary teaching and recognizes an individual for distinguished teaching of microbiology and mentoring of students at the graduate and postgraduate levels (e.g., graduate school, medical school, or other health professional schools) and for encouraging students to subsequent achievement'. In addition, since 1968, ASM has recognized an undergraduate educator with the Carski Foundation Distinguished Undergraduate Teaching Award for 'outstanding teaching of microbiology to undergraduate students and for encouraging them to subsequent achievement'.

Conclusion

In summary, the field of microbiology is as diverse and interesting as are the students and faculty who explore the discipline. Emerging areas of inquiry that highlight the interaction of microbes with other organisms, such as their commensal hosts, and the impact of global climate change on surges of infectious agents have grasped the attention of microbiologists. Educators have the privilege and challenge to provide a historical backdrop of microbiology (which paved the way for cellular biology, molecular biology, biochemistry and genetics), important general principles of biology illuminated by bacterial model systems, distinctive perspectives such as biofilms and consortia as well as headline-snaring topics like mysterious viruses, drug-resistance bacterial infections (i.e. superbugs), bioremediation and even bioterrorism. Just as new techniques and resources propel investigators to the frontiers of microbiology research, new methods and strategies in science education and teaching motivate microbiology educators to engage students in active learning and inquisitive discoveries.

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www.asmcue.org—American Society for Microbiology Conference for Undergraduate Educators (ASMCUE).
http://www.asm.org—American Society for Microbiology.
http://www.atcc.org—American Type Culture Collection.
http://www.learner.org/index.html—Annenberg Media Learner.
http://www.ableweb.org—Association for Biology Laboratory Education.
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https://cft.vanderbilt.edu/guides-sub-pages/blooms-taxonomy/—Bloom's taxonomy.
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www.csm-scm.org—Canadian Society for Microbiologists (CSM).
https://www.cdc.gov—Centers for Disease Control and Prevention (CDC).
www.cleapss.org.uk—Consortium of Local Education Authorities for the Provision of Science Equipment (CLEAPSS).
https://www.coursera.org/)—Coursera.
http://eric.ed.gov/—Education Resource Information Center.
https://www.edx.org/)—edX.
www.escmid.org—European Society in Clinical Microbiology and Infectious Diseases (ESCMID).
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https://saber-biologyeducationresearch.wikispaces.com—Society for the Advancement of Biology Education Research (SABER).
www.societyofbiology.org—Society of Biology.
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http://www.aai.org—The American Association of Immunologists.

http://www.the-aps.org—The American Physiological Society.
http://www.ascb.org—The American Society for Cell Biology.
http://www.sicb.org—The American Society for Integrative & Comparative Biology.
http://www.mcw.edu—The American Society for Virology.
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http://www.who.int/en/—World Health Organization (WHO).

Technology Advances in Medical Microbiology☆

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"So many of the things I've predicted were technologies that were just sitting right in front of us".
Joshua Lederberg

Introduction

Two Elsevier encyclopedia books formed the root of the Medical Microbiology subject: (1) *Encyclopedia of Microbiology*, a "bible" recognized nationally and internationally in the field for decades, was firstly edited by Joshua Lederberg and subsequently by Moselio Schaechter (Lederberg, 1992, Lederberg et al., 2000, Schaechter, 2009), and (2) *Encyclopedia of Virology*, on the other side, was firstly edited by Robert Webster and Allan Granoff and subsequently by Brian Mahy and Mac van Regenmortel (Granoff and Webster, 1999, Mahy and Van Regenmortel, 2008, Webster and Granoff, 1994). Medical microbiology is the branch of microbiology devoted to the study of the physiological and pathogenic processes that underlie microbial infections in human and animal hosts. Medical microbiology covers not only basic components on the biology of pathogens in the context of pathogenicity, but clinical components in the context of epidemiology, diagnosis and treatment as well. The roots of medical microbiology trace back to 1676 when Anton van Leeuwenhoek first observed bacteria and protozoa using a single-lens microscope of his own design. However, it was not until the late 1800s that the work of Pasteur, Koch, and others ushered in the modern era of germ theory as well as the use of isolation techniques on artificial growth media (Maccallum and Hastings, 1899). Since then, the capabilities of modern medical microbiology have expanded and improved rapidly, thanks to technological revolutions in microbiology, immunology, chemistry, physics and molecular biology (Persing et al., 2016, Tang and Stratton, 2013).

During the past decades, technical advances in the field of medical microbiology have made constant and enormous progress in various areas, including etiology, pathogenesis, epidemiology, diagnosis and treatment (Tang et al., 2014, Persing et al., 2016). A major milestone in medical microbiology was the Gram stain. In 1884, Hans Christian Gram developed the method of staining bacteria, to make them more visible and differentiable under a microscope (Gram, 1884). Robert Koch's work led to discovery or development of bacterial culture by using potato slices, agar (with the help of Walther and Angelina Hesse) and petri dish (developed by Richard Petri) for growth and isolation of microorganisms. In the 1980s and 1990s, immunoassays came into wider usage in both medical microbiology research and practice with the development of radioimmunoassay and immunofluorescence, and then enzyme immunoassay (EIA) and immunoblotting. Application of monoclonal antibodies and antigen-recognizing probes, such as peptides, has significantly increased the sensitivity and specificity of antigen detection and characterization. DNA sequencing, a method developed by Walter Gilbert and Frederick Sanger in 1977 (Sanger et al., 1977, Maxam and Gilbert, 1977), caused a rapid change in the development of vaccines, medical treatments, and diagnostic methods. Subsequently in 1995, a team at the Institute for Genomic Research sequenced the first bacterial genome; *Haemophilus influenzae* (Fleischmann et al., 1995).

Probably the greatest advance in the field of diagnostic microbiology has been in the area of nucleic acid detection and characterization. The polymerase chain reaction (PCR) invented by Kary Mullis (Mullis and Faloona, 1987, Saiki et al., 1985) and other more recently-developed amplification techniques have simplified and accelerated the in vitro process of nucleic acid amplification. Rapid techniques of nucleic acid amplification and characterization, along with the increased use of automation and user-friendly software, have significantly broadened microbiologists' research and development arsenal. New real-time PCR, multiplex PCR, and microarray techniques provide relevant and accurate microbial quantification and identification, which play important roles in the selection and monitoring of antimicrobial therapy (Tang and Stratton, 2013, Persing et al., 2016). Since 1995, the whole genomes of an increasing number of bacterial species has been described and this has opened up the so-called "omics" technologies (Lederberg and Mccray, 2001), which cover the areas of genomics, transcriptomics, proteomics and metabolomics (Kiechle and Holland-Staley, 2003) (Table 1). These panomic techniques, along with microarray and sequencing, can be used to generate microbial pathogen profiling for management and surveillance (Sintchenko et al., 2007, Goh and Knight, 2017) that play roles in classification, metabolism, pathogenesis, genetics, host responses, epidemiology, diagnosis and treatment of medical microbiology (Fig. 1).

Microbial genomics dissect and analyze the function and structure of genomes, the complete set of nucleic acids within a single microorganism. Ramet et al. devised a functional genomic analysis of phagocytosis and identification of a Drosophila receptor for *E. coli* and demonstrated that peptidoglycan recognition protein LC was an essential component for recognition and signaling of Gram-negative bacteria (Ramet et al., 2002). Bioinformatic analyses of bacterial 16S rRNA gene data revealed *Schistosoma mansoni* infection is associated with quantitative and qualitative modifications of the mammalian intestinal microbiota (Jenkins et al., 2018).

☆*Change History*: April 2019. Yi-Wei Tang update the text throughout the article.

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Table 1 Panomic techniques in medical microbiology

Panomic technique	*Question addressed*	*Target molecule*	*Large-scale data*	*Main method(s)*
Genomics	What can happen? (potential)	DNA	DNA sequences	DNA sequencing
Transcriptomics	What is the plan? (strategy)	RNA	RNA transcript profile	RNA sequencing; real-time RT-PCR
Proteomics	What is happening? (process)	Protein	Protein profiling	MALDI-TOF MS
Metabolomics	What happened? (products)	Metabolites	Metabolite profiling	GC–MS

Abbreviations: *MALDI-TOF MS*, matrix-assisted laser desorption/ionization time of flight mass spectroscopy; *RT*, reverse transcriptase; *GC–MS*, gas chromatography–mass spectrometry.

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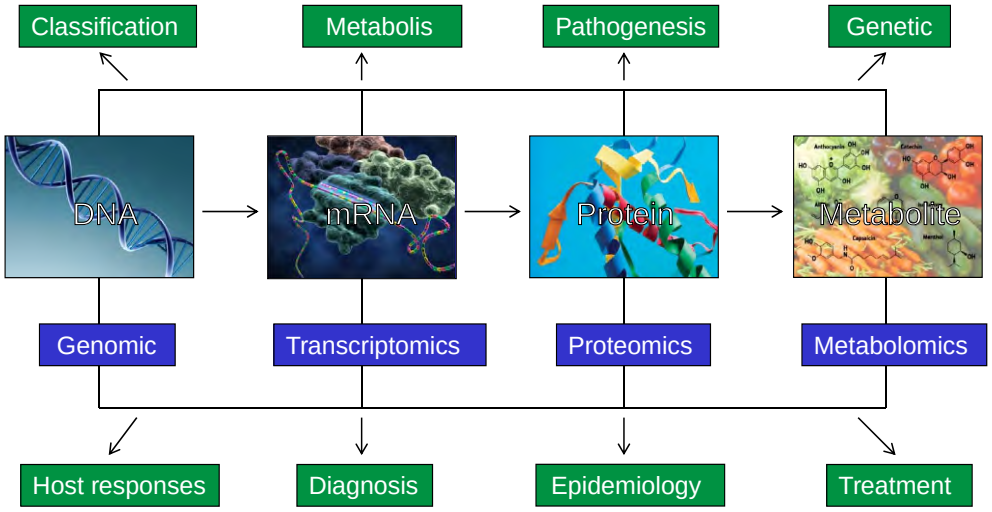

Fig. 1 Panomic techniques and their application niches in medical microbiology.

Diverse sources of *Clostridium difficile* infections were explored by comparing single-nucleotide variants between isolates which were determined by whole-genome sequencing (WGS). The data indicated genetically diverse sources, in addition to symptomatic patients, play a major part in *C. difficile* transmission (Eyre et al., 2013). Using bacterial WGS, Rossi et al. reported a case of a patient from Brazil with a bloodstream infection caused by a strain of methicillin-resistant *Staphylococcus aureus* (MRSA) that was initially susceptible to vancomycin but then acquired the *vanA* gene cluster during antibiotic therapy and became resistant (Rossi et al., 2014). Technological improvements in metagenomic deep sequencing have led to its potential application not only for basic research but also for clinical science and practice (Houldcroft et al., 2017). Metagenomics was successfully used to establish a timely diagnosis of neuroleptospirosis in a 14-year-old boy with severe combined immunodeficiency who suffered from recurrent bouts of fever, headache and coma (Wilson et al., 2014). Further study on a series of seven patients indicated that prioritizing metagenomic data with a scoring algorithm greatly clarified data interpretation and highlighted the problem of attributing biological significance to organisms present in control samples used for metagenomic sequencing studies (Wilson et al., 2018). WGS and computational analysis have been used to compare the taxonomic and functional profiles of microbial communities. Leveraging this approach to understand roles of microbes in human biology and other environments requires quantitative data summaries whose values are comparable across samples and studies. It envisioned a future in which microbiome studies are replicable and new metagenomes are easily and rapidly integrated with existing data (Nayfach and Pollard, 2016).

Transcriptomics studies the set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA, including their structures and functions (Allert et al., 2016, Holcomb et al., 2017). In *Mycobacterium tuberculosis* infections, mycobacterial mRNA better reflects viability, thereby being valuable as a reliable marker of bacteriologic clearance in response to antituberculosis therapies (Mdivani et al., 2009, Li et al., 2010). The transcriptome also includes small, noncoding RNAs, which have emerged as key regulators of gene expression, genome stability and chromatin modification (Moazed, 2009, Moller et al., 2018). High-throughput sequencing of RNA transcripts (RNA-seq), which uses next-generation sequencing technologies to generate transcriptome profiling in both pathogen and hosts (Nagalakshmi et al., 2008, Westermann et al., 2012), gradually comes into the medical microbiology research and practice. RNA-seq allows the investigation of the diverse physiologies from uncultured microorganisms in their natural habitat (Frias-Lopez et al., 2008, Bomar et al., 2011). Bomar et al. reported the use of RNA-seq for characterizing the metatranscriptome of the simple gut microbiome from the medicinal leech *Hirudo verbana* and for utilizing this information to design a medium for cultivating members of the microbiome (Bomar et al., 2011). Using a series of RNA arrays on RNA extracted from pediatric patients with sepsis, Herberg et al. identified two host transcript RNA signatures which can distinguishing bacterial

from viral infection (Herberg et al., 2016). A further study demonstrated that the 2-transcript host cell signature can distinguish viral from bacterial diarrhea and it was influenced by the severity of symptoms (Barral-Arca et al., 2018).

Proteomics is a large-scale study of the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or system (Tyers and Mann, 2003). The application of proteomic technology for the identification of biomarkers, altered pathways, functional alternations and mechanisms has recently been extensively reviewed (Fournier and Raoult, 2011, Meissner and Mann, 2014). Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid and powerful tool for microbial species identification in the diagnostic arena (Welker, 2011, Doern and Butler-Wu, 2016). In addition, MALDI-TOF MS has been used directly to determine mechanisms of antibiotic resistance (Doern and Butler-Wu, 2016, Hrabak et al., 2013). In recent years, proteomics has emerged as a powerful tool to evaluate the complex host-response to sepsis (Sharma and Salomao, 2017). Proteomics coupled with mass spectrometry made possible to identify differentially expressed proteins in clinical samples. In recent years, a number of proteomic studies has been done under sepsis and/or in response to endotoxin and showed various signaling pathways, functions, and biomarkers (Karvunidis et al., 2009, Ludwig and Hummon, 2017). A recent study used quantitative proteomics to evaluate the host proteome response in septic patients secondary to community-acquired pneumonia. Bioinformatic analyses of differentially expressed proteins showed alteration in the cytoskeleton, cellular assembly, movement, lipid metabolism and immune responses in septic patients (Sharma et al., 2017). The proteomic results stressed important changes in cellular structure and metabolism, which are possible targets for future interventions of sepsis.

Metabolomics provides the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification. Over the last decades, metabolic profiling has been increasingly applied to classify diseases and diagnose onset of disease (Goodacre et al., 2004). Using metabolomic approaches, Kashyap et al. investigated how host mucus glycan composition interacted with dietary carbohydrate content to influence the composition and expressed functions of a human gut microbiome (Kashyap et al., 2013). Seymour et al. determined the global metabolomic profile in circulating plasma to differentiate between surviving and non-surviving patients with community-acquired pneumonia and sepsis (Seymour et al., 2013). Metabolomic profiles determined in serum have been reported as a novel approach for early diagnosis of pediatric septic shock and its mortality (Mickiewicz et al., 2013). Sweeney et al. presented prognostic models for 30-day mortality generated independently by three scientific groups by using 12 discovery cohorts containing transcriptomic data collected from primarily community-onset sepsis patients. Combining the new gene-expression-based prognostic models with prior clinical severity scores leads to significant improvement in prediction of 30-day mortality (Sweeney et al., 2018). Similarly, breath fungal secondary metabolite signature, determined by thermal desorption-gas chromatography/mass spectrometry, was used to diagnose invasive aspergillosis (Koo et al., 2014). When these metabolomic biomarkers are used in the clinical practice, we must remember the extreme heterogeneity of clinical illness, and strive for generalizable disease-defining diagnostics (Sweeney and Khatri, 2017). While detection of exogenous microbial volatile metabolites in the breath has opened up a new and exciting dimension of diagnostic research and development in infectious diseases, the daunting challenges to volatile diagnostic biomarker discovery and clinical development have to be kept in mind (Acharige et al., 2018).

The availability of sophisticated and increasingly inexpensive nucleic acid sequencing techniques has revolutionized the fields of genomics resulting in unprecedented advances in medical microbiology. Use of non-genomic technologies such as transcriptomics, proteomics and metabolomics allows full inventories to be made of bacterial genes, their time- and place-dependent expression and the resulting proteins as well as their outcome metabolites, thus further substantiating and broadening the medical microbiology armamentarium. What seems certain is that the advanced techniques will increasingly change and enhance the research and practice of medical microbiology.

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The Bacterial Glycome: From Monomers to Complex Carbohydrate Polymers

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Introduction

The microbial and especially the bacterial glycome is defined by a vast amount of different carbohydrates and carbohydrate derivatives. These include carbohydrate monomers such as sugars, sugar alcohols, nucleotide sugars as well as keto and deoxy sugars, followed by di- to oligosaccharides, lipopolysaccharides and polysaccharides. Additionally, the peptidoglycan layer of bacterial cell walls as well as the murein sacculus are part of the bacterial glycome which also includes glycosylated proteins. Based on these facts, it is obvious that research and analysis of the bacterial glycome (glycomics) represent one of the highest complex omics sections of bacterial systems and is also demonstrated by the versatile functions of the different carbohydrate-based and glycosylated molecules. Glycomics, therefore, are defined here as the study of glycoconjugate assembly and their functions in biological systems.

Sugar nucleotides are the most essential precursors for the biosynthesis of various carbohydrate-containing bacterial cell structures, which are important virulence factors of pathogenic bacteria. In the majority of the cases, they function in the evasion of the bacteria from the host immune system. Other sugar-containing structures, like peptidoglycan, have important roles in bacterial viability. In addition, some of these structures and their biosynthetic pathways are being regarded as attractive targets for the development of new antimicrobial drugs. Some sugar polymers are applied in the food, pharmaceutical, textile and paper industries, thus having important economical significance. Therefore, the study of metabolic pathways responsible for polysaccharide and precursor synthesis, as well as their regulation, is of critical importance for the optimization of microbial production processes of carbohydrate-containing polymers.

Monosaccharides and Carbohydrate Derivatives

For the monosaccharides, the main representatives in the glycome are the aldoses glucose, galactose and mannose (Fig. 1A) as well as ribose and glyceraldehyde but also ketoses such as fructose and erythrose are of importance. Next to the monosaccharides, sugar alcohols play an essential role in cellular metabolism and the glycome and comprise molecules such as glycerol, mannitol or sorbitol (Fig. 1B). From an industrial point of view, sugar alcohols are an interesting alternative to sugars, due to their usually incomplete absorption into the blood stream from the small intestine which generally results in a smaller change in blood glucose than "regular" sugar (sucrose). Thus, they are often used as sweeteners in low-carb and diabetic diets.

Deoxy sugars are characterized by replacement of a hydroxyl group with a hydrogen atom and are a constituent of DNA in the case of deoxyribose but can also be part of complex polysaccharides such as fucoidan which is synthesized by polymerization of fucose, which as a monomer can also participate in protein N-glycosylation. Another deoxy sugar is rhamnose (Fig. 1D) which can be found in many plant glycosides or bacterial polysaccharides. Additional acidic variants of monosaccharides exist in the form of uronic acids, which are a class of sugar acids with both carbonyl and carboxylic acid functional groups (Fig. 1C). For them, the terminal carbon hydroxyl group has been oxidized to a carboxylic acid and they function as main constituents of bacterial exopolysaccharides such as alginate or several further heteropolysaccharides.

Another piece of the glycome are the nucleotide sugars which are the activated forms of monosaccharides and play a crucial role in cell metabolism and regulation and represent the glycosyl donors in glycosylation reactions. Their highly energetic form makes them the perfect glycosyl donor and is realized by the result of a reaction between nucleoside triphosphate (NTP) and glycosyl monophosphate (Fig. 2). In the bacterial glycome nearly all kinds of nucleotide sugars appear and are involved in several cellular reactions and are required for the synthesis of nearly all carbohydrate containing oligomers and polymers.

GDP-mannose for example is the donor of D-mannose which is a sugar residue found in many bacterial polysaccharides such as Xanthan or sphingans (Fig. 3). GDP-D-rhamnose is a more rare deoxyhexose and is mainly found in the lipopolysaccharides of pathogenic bacteria, but also some exopolysaccharides such as the different sphingans. Additionally, rhamnose can be found in the S-layer of some thermophilic Gram-positive bacteria. GDP-L-fucose can be found in many glycoconjugates of microbes, but also plants and animals. Fucose often represents a part of complex cell-wall structures, lipopolysaccharides and like rhamnose its function is related to interactions of the bacteria with host tissue, and to mask the bacterial cells from the host immune system. GDP-mannuronic acid is the precursor for mannuronic acid and guluronic acid since it can be easily epimerized by specific epimerases and is the main constituent of the polysaccharide alginate. Its acidic character makes it interesting for various applications, such as biomedical wound healing in the form of hydrogels.

dTDP-L-rhamnose is mainly found as precursor for O-antigens of lipopolysaccharides in many Gram-negative bacteria, where the O-antigen repeating units is mainly composed of rhamnose. The synthesis of L-rhamnose needs four enzymatic steps, and the genes encoding these enzymes can often be found clustered in the genome. The intermediate dTDP-4-keto-6-deoxy-D-glucose is the

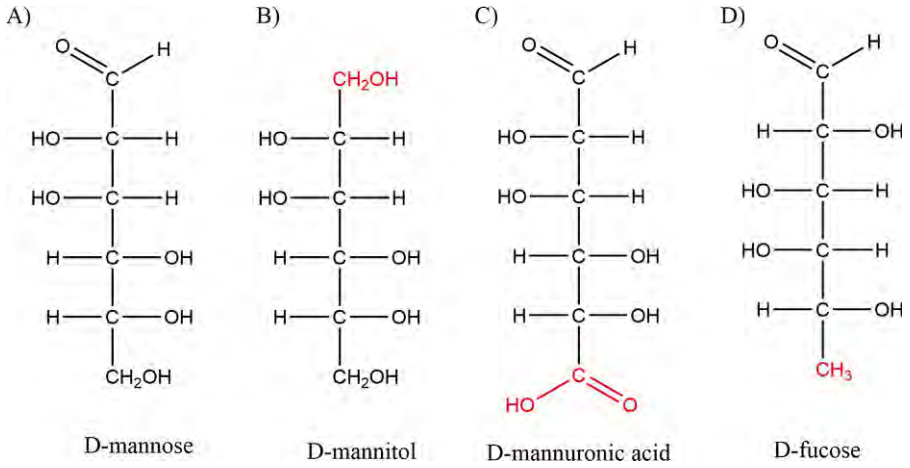

Fig. 1 Examples of carbohydrate monomers and their derivatives. (A) The sugar monomer of D-mannose; (B) The sugar alcohol D-mannitol; (C) The uronic acid D-mannuronic acid; (D) The deoxy sugar D-rhamnose (deoxy-mannose).

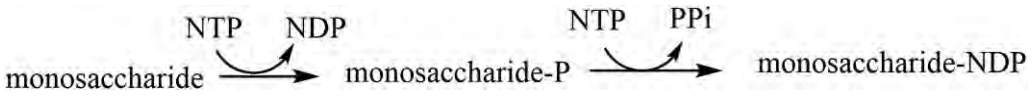

Fig. 2 Mechanism of the synthesis of activated nucleotide sugars. A monosaccharide reacts with a nucleotide triphosphate by release of a diphosphate, resulting in the high energetic nucleotide sugar.

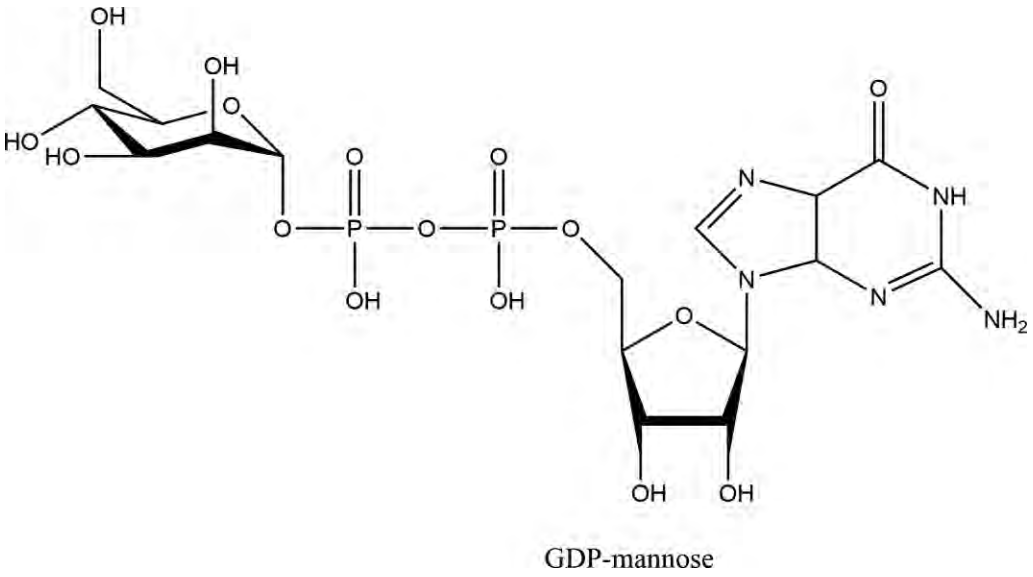

Fig. 3 The activated form of mannose as presented by GDP-mannose. The C1 of the mannose monomer is linked to the nucleotide diphosphate.

precursor for many compounds that are part of antibiotic glycosides. This biosynthesis pathway for dTDP-rhamnose is unique in bacteria and does not exist in humans, thus offering a potential target for antimicrobial agents.

UDP-D-glucose is the main constituent of various bacterial sugar containing bacterial structures, such as peptidoglycan, lipopolysaccharides and exopolysaccharides. Additionally, UDP-D-glucose is the substrate for glycosylation of teichoic acid in Gram-positive bacteria as well as the membrane anchor of lipoteichoic acids, which is made of the glycolipid diglucosyldiaglycerol. It serves as the direct precursor for (bacterial) cellulose and as initial precursor for other exopolysaccharides such as hyaluronic acid by its further conversion to UDP-glucuronic acid. D-glucuronic acid might play an important role in bacterial resistance against antibiotics, since the enzyme converting UDP-glucose towards UDP-glucuronic acid seems to be essential for the resistance. UDP-D-galactose is the substrate for many galactose containing exopolysaccharides and glycoproteins. UDP-N-acetylglucosamine is used for the assembly of the cell wall peptidoglycan, lipopolysaccharides as well as teichoic acid and can be further converted

towards UDP-N-acetyl-D-mannosamine which serves as precursor for Sialic acid biosynthesis which plays an essential role in many capsular polysaccharides of pathogenic bacteria by masking the bacterial cells from the host immune system. An overview of the different nucleotide sugars and their biosynthesis routes is given in Fig. 4(A). Next to the monosaccharides and derivatives thereof, oligosaccharides exist in the bacterial glycome, which mainly are part of protein glycosylation.

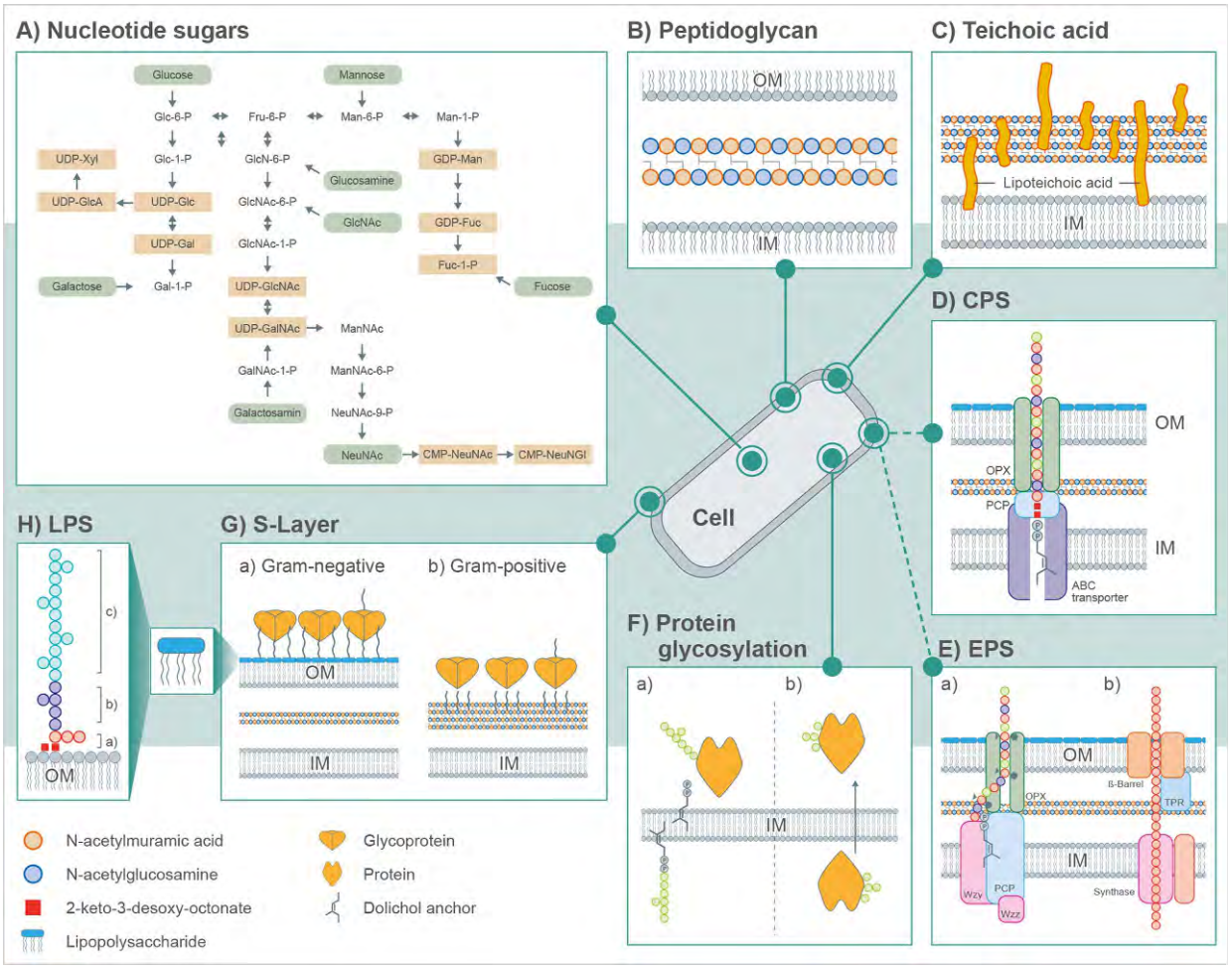

Fig. 4 A schematic overview of the parts of the glycome including their localization within the cell. (A) Schematic biosynthesis pathways of the different nucleotide sugars (orange rectangles) and their precursor carbohydrates (green elapses). The different conversion steps are performed by a huge variety of highly specific enzymes. (B) scheme of the peptidoglycan which is located in the periplasmic space in between the inner membrane (IM) and the outer membrane (OM). The peptidoglycan consists of N-acetylglucosamine (dark blue) and N-acetylmuramic acid (orange). Each N-acetylmuramic acid is linked to another N-acetylmuramic acid in the attached layer via a peptide chain of three – five amino acids (grey bonds). (C) schematic presentation of the teichoic acids which are vertically incorporated into the thick peptidoglycan (yellow) of Gram-positive bacteria. They are covalently linked to N-acetylmuramic acid, or to a terminal alanine of the peptide crosslink. When they are anchored in the inner membrane via a lipid linker, they are called lipoteichoic acids. The most common structures of teichoic acid are disaccharide repeating units of N-acetylmannosamine and N-acetylglucosamine. (D) schematic biosynthesis machinery of capsular exopolysaccharides (CPS) which spans the whole cell envelope from inner to outer membrane. The CPS is anchored to the lipid linker via 2-keto-3-desoxy-octonate (red squares) and is synthesized at the cytoplasm and then transported via the ABC-transporter towards the environment assisted by polysaccharide co-polymerase (PCP) and outer membrane proteins (OPX). CPS remain linked to the cell and are heteropolysaccharides as schematically represented by the different colours of the sugar monomers. (E) schematic biosynthesis of the Wzx/Wzy dependent pathway which is characterized by the assembly of the repeating units at the inner leaflet of the inner membrane via different glycosyltransferases and transport to the periplasmic space via the flippase protein Wzx (not shown). Assembly of the repeating units occurs via the polymerase (Wzy) and polysaccharide co-polymerase protein (PCP) and following transport of the heteropolysaccharide to the environment via OPX proteins (a). The synthase dependent pathway (b) is characterized by assembly of homopolysaccharides via the synthase complex and assisted transport via the tetratricopeptide repeat proteins (TPR) spanning the cell envelope. (F) protein glycosylation via transfer of repeating units assembled at the inner membrane (at a dolichol anchor) and flipped towards the periplasm where the repeating unit is attached to the specific amino acid sequence of the protein (a). Sequential glycosylation at the cytoplasm followed by transport of the glycosylated protein to the periplasmic space (b). (G) S-layer of Gram-negative (a) and Gram-positive bacteria (b). The proteins (orange) are linked to lipopolysaccharides (a) of the outer membrane, or directly to the thicker peptidoglycan layers of Gram-positive bacteria (b). (H) lipopolysaccharides (LPS) consist of the Lipid A, which is part of the outer leaflet of the outer membrane, the inner core region (a) and the outer core region (b), which consist of KDO and heptoses and hexoses (purple) of non-homogenous variation. The O-antigen (c) consists of different carbohydrate monomers (glucose, rhamnose...) as well as dideoxy sugars.

The Bacterial Cell-wall

Additionally, all Gram-negative bacteria exhibit a cell envelope of the same general architecture which is schematically depicted in Fig. 4(B). The periplasmic "space" is the space between the inner and outer membrane and contains peptidoglycan, also known as murein, as a major constituent. This macromolecular sacculus is composed of sugar chains built from alternating 2-acetamido-2-deoxy-D-glucopyranose (GlcpNAc) and 2-acetamido-3-O-[(R)-1-carboxyethyl]-2-deoxy-D-glucopyranose, also termed N-acetylmuramic acid (MurNAc). These carbohydrates represent residues which carry and can be cross-linked by smaller peptides – The amino acid composition of which varies in different species. The peptidoglycan sheet represents a rigid layer that determines the form of the bacterial cell, reinforces the osmotic stability and acts as a protective barrier. However, the murein sacculus is not a completely closed barrier, since it still enables the transport of small molecules, such as nutrients or excretion of metabolites via for e.g., primary and secondary transporters. Only a very limited number of bacteria, mainly belonging to the genera *Mycoplasma* and *Spiroplasma*, do not possess a murein sacculus.

Lipopolysaccharides

Lipopolysaccharides are a combination of lipids and polysaccharides at the outer membrane of Gram-negative bacteria. They are recognized as antigens and can be used for the serological characterization of bacteria. In the case of cell death, the lipopolysaccharides fall apart and show their toxic function.

Lipid A is at the outer leaflet of the outer membrane and functions as endotoxin. The fatty acid is linked by ester bonds to a disaccharide which consists of N-acetylymuraminephosphate. Main fatty acids are capron, lurin, myristin, palmitin and stearin acid. The core region of LPS consists of the 2-keto-3-desoxy-octonate (KDO) linker and heptoses, glucose, galactose and N-acetylglucosamine (Fig. 4(Ha+b)). The sugar pattern of the core region can remarkably differ within the various bacterial species. The O-antigen polysaccharides consist of several hexoses, such as rhamnose, galactose, glucose and mannose, as well as rare dideoxy sugars such as abequose, colitose, paratose or tyvelose which can carry additional modifications and decorations and can contain several branches. The sequence is made from repeating units which are form the polysaccharide. This polysaccharide is named O-antigen and can be used to distinguish between pathogenic and non-pathogenic strains by serotyping. Due to its localization on the cell surface, the O-antigen is a major target for both the immune system as well as bacteriophages and represents one of the most variable cell constituents (Fig. 4(Hc)). Very interestingly, the biosynthesis machinery of O-antigen encoding genes are clustered in the genome and are highly conserved within a species. The O-antigen carbohydrate part mainly consists of oligosaccharide repeating units in the range of 2–8 sugars from a highly broad monomer composition. The lipopolysaccharides also present an important virulence factor which can be serotyped by its specific monomer composition. One important aspect of the O-antigens of lipopolysaccharides is their heat stability compared to other O-antigens, which are mainly heat-labile. The serotyping based on lipopolysaccharides was already performed in-between 1945 and 1950 for various *Escherichia coli* strains and was one of the mostly applied techniques in the identification of pathogens. The K-antigens in contrast are mainly composed of polysaccharides and stuck to the cell envelope, thus hindering the agglutination of the cells. The K-antigens are a synonym for capsular polysaccharides and have an even higher diversity than the O-antigens and are the major virulent determinants of Gram-negative bacteria. Based on this fact structural databases for the 3-D structures exist e.g., for *E. coli*, but also pathogens such as *Klebsiella pneumonia* and are in the focus for enabling a targeted identification of pathogenic strains. Taking all antigens of *E. coli* together, there will be around 50,000–100,000 serotypes, showing the immense diversity, realized by sugar composition and glycosidic linkages. The capsules are molecules of 100–1000 kD multimers, which are assembled by repeating units, and thus enables the prediction of the 3-D structures as well as the monomer composition.

Gram-positive bacteria are defined by their thicker murein sacculus compared to Gram-negative bacteria but lack the outer membrane (Fig. 4(C)). An additional reinforcement of the cell wall is realized by incorporation of teichoic acid, which is unique to Gram-positive bacteria. Teichoic acid is composed of mainly N-acetylmannosamine (ManNac) and N-acetylglucosamine (GlcNac) directly linked to glycerol- or ribitol phosphate. Teichoic acid can attract cations and thus has the function of providing rigidity to the cell wall as a co-polymer and as part of the bacterial glycome.

The so-called S-layer represents the outer layer of the cell wall of many bacteria and the only one in the case of certain archaea. It consists of layered (glyco)proteins which are organized by a self-assembly like process (Fig. 4(G)). The S-layer contains glycosylated as well as non-glycosylated proteins and can act as virulence factor in some species, such as *Campylobacter*. Thus, it is obvious that the cell wall structure is highly complex and can vary massively between different genera and species. But for all of them, carbohydrates and their derivatives are the most abundant compounds of the cell wall and play essential roles in cell communication and pathogenicity of microorganisms.

Protein Glycosylation

Protein glycosylation was first demonstrated in the late 1930s and was long thought to exist only in eukaryotes, but glycosylation is the most abundant polypeptide chain modification in nature, also in bacteria. By that approach glycans can be covalently attached to the amide nitrogen of asparagine residues (N-glycosylation), to the hydroxyl oxygen of, typically, serine or threonine residues (O-glycosylation), or, in rare cases, to the indole C2 carbon of tryptophan through a C–C linkage (C-mannosylation).

The first bacterial and archaeal glycoproteins were discovered in the 1970s on the S-layers of *Halobacterium salinarum* and *Clostridium* sp. by O-glycosylation. It took more than 20 more years to identify the first N-linked protein glycosylation in *Campylobacter jejuni* and step by step more glycosylating pathways were identified, based on the development of more sensitive detection methods, such as next generation sequencing and sophisticated mass spectrometry techniques. The N-glycosylation of *C. jejuni* follows the heptasaccharide formation on an undecaprenyl phosphate anchor at the cytoplasmic side of the inner membrane. This heptasaccharide unit is then flipped towards the periplasmic space by an ATP-dependent flippase and transferred to asparagine residue of the targeted protein by an oligosaccharyltransferase Fig. 4(Fa)). The bacterial N-glycosylation uses a different consensus sequence (Glu-X-Asn-X-Ser/Thr, with X can be any amino acid except proline) of the protein, to specifically find the right asparagine, than the eukaryotic system (Asn-X-Ser/Thr, with X can be any amino acid except proline). Next to the transfer of repeating units, glycosylation can also occur by a sequential transfer of single sugar monomers in the form of their activated nucleotide sugars (Fig. 4(Fb)). The functions of bacterial protein N-glycosylation are not completely understood but might range from stabilizing the enzyme to the modulation of immune response in pathogenic microbes. O-glycosylation can be often found in modification of bacterial flagella and pili, in that the glycosylation can constitute up to 10% of the protein mass. Bacterial O-glycosylation is of main importance for flagellum assembly, secretion of virulence modulating proteins, bacterial colonization of the gastrointernal tract, auto-agglutination as well as biofilm formation. The O-glycans of the *Campylobacter* flagellum are mainly C9 sugars, such as pseudoaminic or legionaminic acid and use mainly GDP-linked intermediates in contrast to the UDP-linked intermediates for the N-glycosylation. As for N-glycosylation, the sequential transfer as well as the block transfer are described in different bacteria. The glycosylation pathways are encoded in genomic islands and more and more sequences get identified by massive genome sequencing approaches. The main targets for O-glycosylation are serine or threonine residues with various consensus sequences. Another widespread protein glycosylation pattern is the O-mannosylation in Gram-positive actinomycetes, which is found in several bioactive products and secreted antigens.

Exopolysaccharides

Exopolysaccharides can occur as homopolysaccharides, by polymerizing only one type of activated sugar monomer such as bacterial cellulose, or in the form of heteropolysaccharides, with alternating sugars or sugar derivatives in their chemical structure. They are assembled by different mechanisms which will be explained in brief here.

In the case of homopolysaccharides, most of the bacterial exopolysaccharides are produced by a synthase complex, which starts from the activated form of the sugar or sugar derivatives at the cytoplasm, by transferring one monomer towards a nascent growing chain of the polymer (Fig. 4(Eb)). This type of biosynthesis is used for cellulose, by forming a beta-1,4-linked glucose-containing polymer, and is also responsible for the production of alginate by firstly assembling a pure mannuronic acid homopolymer by utilization of GDP-mannuronic acid as precursor. By the transport across the cell wall towards the extracellular environment, various epimerases which are integral part of the biosynthesis machinery can specifically convert mannuronic acid (M) towards glucuronic acid (G) in the polymer strand. One famous exception of a synthase-based biosynthesis pathway encoded polysaccharide is hyaluronic acid. This polysaccharide, which has a high importance in cosmetic applications due to its enormous water binding capacity, consists of the repeating disaccharide unit of D-glucuronic acid and N-acetyl-D-glucosamine. The biosynthetic pathway is encoded by five genes, the products of which assemble the final polymer strands, resulting in a heteropolysaccharide based on a synthase based biosynthetic pathway.

In the case of heteropolysaccharide production, two different pathways are mainly found. These are the so-called ABC transporter pathway, as well as the so-called Wzx/Wzy-pathway. Since it is already seen by the examples for lipopolysaccharides and capsular polysaccharides, the insights are not always clearly understood, and the definition of CPS and EPS might vary within different disciplines. For the capsular polysaccharides, mainly the ABC transporter dependent pathway is described, which uses the KDO-linker to produce a growing polymer chain by adding specific repeat units (Fig. 4(D)). The activated monomers are transferred by specific glycosyltransferases in the cytoplasm and the chain is than actively transported over the cell wall via an ABC transporter by utilization of ATP. The second, and main abundant biosynthesis pathway for heteroexopolysaccharides is the Wzx/Wzy-pathway. This pathway uses an inwards-faced polyprenol (dolichol) anchor at the inner membrane, to sequentially transfer activated sugar monomers by the action of various specific glycosyltransferases. This anchor will finally carry the completed repeating unit and is then flipped by the Wzx protein towards the periplasmic space. The next steps are the polymerization of the repeating units by the polymerase protein Wzy followed by transport across the cell wall towards the environment (Fig. 4(Ea)). This biosynthetic pathway also includes co-polymerases which are involved in chain length determination of the final exopolysaccharide strands, which are not covalently linked to the cell. All three pathways as described here are highly specific for the monomer pattern of the polysaccharides, but the chain length of the polysaccharides can vary by the cultivation conditions. For alginate it is also described that the cultivation conditions, such as mixing speed and dissolved oxygen content can influence the M:G ration of the final products. For some exopolysaccharides it is also described that the C- or N- source can influence the monomer pattern of the polysaccharide. But it must kept in mind that around one fifth of bacteria and archaea encode more than one specific pathway for the biosynthesis of capsular polysaccharides or exopolysaccharides, thus the cultivation conditions might more influence the transcription of different polysaccharide encoding operons than the specific biosynthesis itself. This was clearly demonstrated in *Sinorhizobium meliloti*, which encodes biosynthesis pathways for two different exopolysaccharides, EPS I (succinoglycan) and EPS II (galactomannan) in its genome, and EPS II is just produced under phosphate limitation, whereas EPS I is produced also at high

phosphate concentrations. The choice of C-source can also influence the type of polysaccharide which is produced, since there exists another biosynthesis pathway which was not discussed here yet. This pathway uses a single enzyme, which is located at the S-layer of the cell wall and can cleave disaccharides to produce mainly homopolysaccharides by utilizing the energy obtained by cleaving the glycosidic bond. These enzymes are the so-called levan- or dextran sucrase enzymes and can produce levan or dextran respectively. Thus, this biosynthesis is the sole extracellular polysaccharide biosynthesis found in bacteria. Dextrans and levans are mainly produced under stress conditions of the microbes and solely in the presence of sucrose or other di- or trisaccharide's. These polysaccharides are branched and can be produced of different molecular weights. In general, the regulation of microbial exopolysaccharide production is not fully understood and can be of high complexity by recruiting networks and cascades of integrated regulatory processes, such as two-component signal transduction systems, transcriptional as well as posttranscriptional regulation as proven for the well examined biosynthesis of alginate in *Pseudomonas fluorescens*. Further regulation, which seems to be widespread amongst many bacterial exopolysaccharide producers is positively linked to enhanced c-di-GMP levels, which promotes exopolysaccharide production and more and more insights are obtained to evoke silent gene clusters for exopolysaccharide production by enhanced c-di-GMP levels.

Analytics of the Glycome

Novel analytic techniques to analyse the bacterial glycome have massively improved our understanding of the carbohydrate-based world of bacteria. Sugar analysis can be done by high-performance liquid chromatography (HPLC) or gas chromatography (GC), whereas HPLC is mainly used to distinguish the single monosaccharides and GC is used to unravel specific glycosidic bonds via methylation experiments. Very often both methods are coupled to a mass spectrometer which additionally allows the differentiation of overlapping peaks via mass analysis. Highly sophisticated methods, in combination with optimized protocols led to shortened and high-throughput capable methods for the analysis of monosaccharides, oligosaccharides as well as polysaccharides within the last years. Also, the analysis of sugar derivatives is realized via HPLC and GC. Both methods enable the analysis of monomers or dimers and sometimes oligomers, when reliable standards are available, but mostly come to their limits when the complete chemical structure of highly complex polysaccharides must be elucidated. Nuclear magnetic resonance (NMR) analysis can help to obtain insights into the chemical structure by analysing the resonance frequencies of the neighbouring nuclei present in the polymers, but this method is sometimes limited by the high viscosity of microbial polysaccharides and hindered by the high concentrations as necessary for NMR analysis. Another possibility to identify specific antigens, lipopolysaccharides or polysaccharides is the utilization of lectins. This class of carbohydrate-binding proteins is highly specific for a defined sugar moiety and perform recognition on cellular and molecular level. Several techniques have been established for using lectins in microarray based-approaches for the targeted identification of glycans and glycoproteins, which massively improved our understanding of the bacterial glycome. For the identification of glycosylation patterns of proteins, specific proteases are used to cleave the glycosylation from the protein and analyse it by mass spectrometry or other techniques. These sophisticated methods require advanced computational and database infrastructures to analyse and elucidate large-scale data. Therefore, the field of glycoinformatics is rapidly developing and has advanced scientific inquiry in the field of glycome analysis.

Outlook

As described in this article, the bacterial glycome is a highly complex system, which is based on carbohydrate chemistry. Our insights are still limited but with the application of novel techniques, mostly in the field of genome sequencing, metabolic engineering in close connection with highly sensitive analytical methods, we are on a good way towards a better understanding of the carbohydrate-based world of bacteria. These insights might bring new products on the market, such as natural noncaloric sweeteners, beneficial sugar alcohols, oligosaccharides with pre-biotic activity, polysaccharides with a broad range of medical as well as technical applications and a huge amount of targeted stabilized glycosylated enzymes which can be applied in industrial processes, based on their enhanced thermostability or pH-acceptance. These are only some examples of how the bacterial glycome can contribute to a biobased industry. Further, also new production strains can be developed by a better understanding of the glycome.

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The Evolutionary Ecology of Microbes

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Introduction

Antonie van Leeuwenhoek was the first to report on observing microbes. His writings describe many different "sorts" of "little living animalcules" that he observed in dental plaque. The recent revolution in sequencing technology has confirmed that indeed, most sampled environments whether the petals of a flower, a drain pipe or human skin, contain hundreds of co-existing species. Nevertheless, most of our current understanding of microbiology has come from studying clonal cell cultures in laboratory test tubes. Microbial ecology, and even more so, the evolutionary ecology of microbes is a relatively young field.

There are three main approaches to studying how evolutionary and co-evolutionary processes shape microbial communities: the first is through DNA sequencing and genomic analysis of environmental samples, possibly sampled over time; second, simulating community evolution in the lab using synthetic microbial communities composed of few, known species under controlled conditions; and finally, through *in silico* modelling of these processes using digital organisms. Sequencing microbial DNA extracted from their natural habitats does not just reveal their current ecology, but also the outcome of their evolutionary history: the individual species and the community as a whole have been shaped by many years of natural selection. The environment selects for species that are able to survive in it, adapt to it and outcompete their neighbours. In turn, any newly arrived species may alter the environment and interact with and affect their neighbours, thereby triggering new rounds of selection and adaptation. It is the result of this co-evolutionary process that we capture in a snap shot of an environmental sample. In contrast, while synthetic microbial communities may differ radically from natural communities, controlling their environment allows researchers to follow individual species, their abundances and their mutations over time. In addition, since samples from different time-points can be frozen and revived, the approach allows for comparing geno- and phenotypes along their evolutionary history. Finally, mathematical and computational models of microbial life allow to test general theories and hypotheses, while compromising further on realism.

All of these approaches have answered important research questions according to which the article is organized: first, I describe what we know about microbial species diversity in natural environments and how we define microbial species; second, I discuss mechanisms by which microbes can adapt to the selection pressures they are subjected to; third, I outline what we know about how microbes interact with one another, imposing a different kind of selection pressure and why this is difficult to quantify; and finally, I discuss how we can explain the diversity that we observe and how it is maintained. Throughout the article, I take a biased view of microbes and focus mostly on bacteria, although archaea, eukaryotic microbes and viruses are also mentioned. This is not a comprehensive review, but rather a discussion of the main ideas in the field.

Microbial Populations are Highly Diverse

Species Diversity in Natural Microbial Populations

In some environments, microbes live within clonal groups of cells. This is the case for some host-associated bacteria, such as *Vibrio fischeri*, a bacterium that grows inside a specialised organ of the Hawaiian bobtail squid, *Euprymna scolopes*. By producing luciferase, the bacteria illuminate the organ of the squid, camouflaging it from its predators. Since squid and bacteria appear to have co-evolved for this function, the squid tightly monitors access to its light organ, only letting in bacteria that perform that specific function. Similar systems have evolved elsewhere, such as nitrogen-fixing bacteria growing in legume nodules, or fungi farmed by ants as an antibacterial. Another common clonal lifestyle are bacteria that live inside eukaryotic cells, as is common in insects. While these are fascinating examples of cross-kingdom cooperation, single microbial species that are tightly controlled by a host to perform a specific function are probably the exception.

For the vast majority of microbes, life is spent within highly diverse populations that cover virtually every surface on earth. This includes our own – and other mammals' – bodies: *e.g.*, skin, intestines, oral cavity, and lungs; plant leaves, flower petals, and roots; man-made artefacts, unfortunately including medical equipment; soil consists largely of microbes; and we find microbes growing on particles in lakes and oceans, and even in raindrops. Our knowledge of this diversity comes from our ability to extract DNA from these environments, to sequence it at ever decreasing costs, and to identify microbial DNA. Carl Woese and colleagues revolutionized microbial ecology by developing methods to sequence 16S ribosomal RNA genes that are present in bacteria and archaea – which they discovered to be the third branch of the tree of life – and 18S genes in eukaryotic microbes. Because these genes are well-conserved, they are well-suited to inferring phylogenetic relationships between microbial strains. Compared to the naturalist's approach of classifying species by phenotypes, this approach of classifying microbes into "operational taxonomic units" (OTUs) based on their similarity at one locus has accelerated and scaled up identification enormously. Today, at very low effort and cost, one can quickly get a broad idea of the hundreds of microbial OTUs present within a sample. Nevertheless, defining microbial species is by no means straight-forward.

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The Species Concept in Microbes

Species classification based on 16S genes is flawed for one main reason: Even though microbes divide clonally, not all their genetic material is passed on between parent and offspring cells. Instead, a large portion of their genome is acquired "horizontally", from non-clonal cells. So-called Horizontal Gene Transfer (HGT) or Lateral Gene Transfer (LGT) occurs through three general mechanisms: direct passing of genetic material between two connected cells (conjugation), transfer through a third party such as a virus (transduction), or by taking up DNA from the environment (transformation). Current estimates of the average number of genes in a bacterium that have been horizontally transferred at some point in its evolutionary history approximate to 80%. This means that even if two bacteria have very similar 16S genes, they may differ at many other loci, and exhibit completely different phenotypes. A good example is *Escherichia coli*, a model bacterial species that can live in various different environments, and from a human perspective, can be either a harmless commensal or a deadly pathogen. This stands in contrast to the eukaryotes, where species identity correlates closely with an organism's phenotype, and reduces the utility of bacterial species classification by 16S. In this article, I therefore refer to "genotypes" rather than "species" to avoid the somewhat arbitrary cut-offs of 16S gene similarity. Nevertheless, because genetic inheritance still mostly occurs vertically, and because HGT is more likely to occur between closely related species, 16S sequencing remains the most practical and informative approach for describing multi-species microbial communities.

Whole genome sequencing and analysis of bacterial genomes has revealed much about their structure and how they have been shaped by ecology and evolution. In particular, it has allowed for the identification of areas of the genome that are more static and others that evolve more quickly, through mutation or HGT. Genes that are present in all genomes of a particular species (identified based on 16S gene similarity) are classified as "core", while those that vary in at least one sequenced strain of that species are "accessory". The totality of core and accessory genes present in a species or clade is its "pan-genome". The goal of this approach is to predict which core genes are absolutely necessary for survival and cell division, and which accessory genes are rather environment-specific. Genes for antibiotic resistance, for example, are likely to be part of the accessory genome, since they are only needed when antibiotics are present. A useful analogy that has been proposed to help think about microbial genomes, is that the core genes represent the operating system of a smart phone, while the accessory genes represent the applications that can be downloaded onto the phone as needed.

Microbial Adaption to the Environment

Mechanisms for Generating Variation

There are two ways in which microbes can generate genetic diversity, allowing them to adapt to their local environment: through mutation or horizontal gene transfer (HGT). Mutations include single nucleotide modifications, as well as larger changes, such as deletions, duplications and movements of large sequences within the genome. The rate at which mutations occur can vary widely. While in most cases, mutations are quite rare, bacterial response to stress can trigger the emergence of "hypermutators" that have a mutation rate that is orders of magnitude higher than the wildtype. This increases the chances of acquiring a mutation that will allow them to survive in a new environment, while simultaneously being prone to mutations with negative fitness effects that may lead to death. Indeed, experiments using *Escherichia coli* have shown that of the mutations that occur in hypermutators, the majority appear to be neutral, with more deleterious than beneficial mutations. This gambling strategy at the individual level may pay off at the population level: from an initially clonal group of cells, only few need to acquire the beneficial mutations required in the new environment, resulting in successful colonization. Alternatively, adaptation to novel environments often occurs through gene duplication, thereby conserving the original function of the gene and allowing mutations to accumulate on the new copy, leading to additional functions. This process is called "neofunctionalization".

HGT is the second important mechanism by which innovation can occur on arrival in a new environment. A well-known example is the acquisition of antibiotic resistance genes on arrival in an environment containing antibiotics. These newly acquired genes may of course have detrimental effects on their host, and in particular, often carry a fitness cost by slowing down growth due to the expression of additional genes. To avoid this cost, genes on horizontally transferred elements can further adapt within the organism through mutation to be more compatible with its expression profile and reduce their cost.

Finally, migration of genotypes into novel environments provides new variants for selection to act on. Microbes often migrate through flowing liquids, or aerosols, but can also form resistant spores that can persist in dry environments and experience revival when returned to a liquid environment. One of the canonical ideas in microbial ecology and biogeography highlights the role of migration: "everything is everywhere, but the environment selects". Although this idea is frequently cited, infinite dispersal of all species across the globe is clearly an oversimplification. Nevertheless, the principle is useful in highlighting the importance of dispersal and environmental selection on observed genotypes.

Selection by the Environment

Variation generated through the above-listed mechanisms is then purged through selection, since genotypes that are able to survive and replicate in an environment are more likely to become its long-term residents. Nevertheless, the extent to which microbial

communities are shaped by selection compared to drift remains an open question. This question can be asked at two levels: to what extent selection shapes species composition on the one hand, and the genomes of individual microbes on the other.

First, then, does selection or drift shape species composition? Are species found in a particular environment the ones best adapted to it, or did they land there by random chance and are barely surviving? Indeed, surviving in a new environment is likely to be extremely challenging. An incoming species must compete with resident microbes, it must resist predation by protists, bacteriophage (viruses), and macrophages in the case of a mammalian host, and it must survive other environmental conditions, such as salinity, pH, humidity, etc. The habitat filtering model – a classical concept in ecology – proposes that such harsh environmental conditions impose "filters" for incoming genotypes. Those that are unable to survive in it would either quickly die out or adapt. This view proposes selection as the dominant force shaping communities. If this is indeed the case, one would expect to mostly find similar "core species" in each type of environment, with few "accessory species" that are unique to some samples but not found in other environmental samples of the same type.

Consistent with the importance of selection in shaping communities, microbial species identity in human body sites of different individuals (*e.g.*, bacteria in the oral cavity), are more similar to each other than body sites within the same individual (*e.g.*, bacteria in the oral cavity versus the skin of the same person). Similar analyses have been conducted on the microbiomes of different organisms, which were found to differ in the similarity of their communities. The honeybee gut, for example, typically contains the same handful of species across the globe, while the microbiome of the fruit fly *Drosophila melanogaster* varies widely depending on their diet.

The second part of the question focuses on the genomes of these surviving organisms: to what extent are their accessory genes (that differ from the same species in a different environment) adaptive or the consequence of neutral drift? Some genes may be subject to millions of years of selection, whereas others may have hitchhiked along. As a rule of thumb, genetic drift is stronger for small population sizes and weaker selection. One can even measure which genes are important for a given organism in a given environment by mapping out its fitness landscape, *i.e.*, the contribution of different genetic changes to its fitness. This can be achieved by a recently developed technique called TnSeq, where random mutations are applied all over the genome and the surviving genotypes sequenced to assess which genes are important. Gene importance can then be compared across environments to distinguish environmentally specific genes. Other methods include comparing gene expression or protein production in different environments or analysing mutational patterns.

A clear signal of how microbial genomes have evolved as a consequence of their ecology is genome size. It is now well-established that free-living microbes have significantly larger genomes than intracellular microbes. Indeed, versatile microbes capable of surviving in several different environments tend to have larger genomes than specialized microbes, such as the marine cyanobacterium *Prochlorococcus*, which is known to have a particularly small genome. At the extreme end of small genomes lie bacteria living inside a host's cells. These have very minimal accessory genomes and are subject to fewer HGT events compared to their free-living relatives. The reduction in genome size is largely due to their small effective population sizes, which lowers the efficiency of natural selection.

Competition and Cooperation Between Microbes

An integral part of the environment in which a microbe arrives are its resident species. The way in which it interacts with these residents is paramount to its survival in its new community. Such interactions are of three broad types: positive, negative and neutral, describing how the presence of one genotype affects the growth and survival of another. One proposal has been that microbial interactions are typically weak or neutral, which is hypothesized to stabilize communities.

Are Interactions Accidental or Adaptive?

When species do affect each other, these effects may occur accidentally, for example, where two species requiring the same nutrient sources to grow compete with one another. Positive accidental effects may also occur if the waste products of one species provide nutrients to another – known as "cross-feeding" – or improve the environment, for example, by altering the pH to be more favourable for its neighbour. On the other hand, some phenotypes appear to have been selected because of their effect on others. A good example is the secretion of antibiotics or other toxins that inhibit the growth of another or even kill it. Similarly, type VI secretion systems are used in lethal competition with other genotypes. Many cooperative phenotypes have also been observed that are likely to have evolved for that purpose. A classic example is the secretion of siderophores into the environment, allowing the secreting cells as well as their neighbours to harvest insoluble iron that is necessary for growth. Such cooperation typically occurs within genotypes, although some examples of between-species cooperation have also been observed.

The Challenge of Classifying and Quantifying Interactions

Measuring and classifying interactions between microbes is a big challenge, particularly in complex communities. Two approaches have been taken to measure interactions in genomic samples extracted from natural microbial communities: the first is to search for patterns of co-occurrence between species, with the assumption that if two species never co-occur, this may be because they outcompete each other, while co-occurring species may be candidates for positive interactions. Unfortunately, however, this does

not allow one to distinguish interactions from a habitat filtering model. The second approach is to follow the dynamics of a single community over time and search for correlations in growth patterns of the different genotypes. By fitting models to these co-varying population densities, one can estimate interaction coefficients. This approach similarly suffers from the inability to distinguish between-genotype interactions from a scenario where multiple species respond simultaneously to changes in the environment. Measuring interactions in a complex community remains a major challenge.

Smaller communities made up of few genotypes that can be cultured in the lab are instead more amenable to interaction classification. In particular, growing species alone and with a given partner genotype and comparing growth patterns is a reliable way to quantify interactions. However, these may change with environmental conditions, particularly when the species are no longer in their artificial laboratory habitat. Moreover, distinguishing accidental effects on another versus effects that have evolved for that purpose is also challenging. In sum, we are still far from understanding interaction patterns in microbial communities.

The Maintenance of Species Diversity

A long-standing question in microbial ecology is why we observe so many species and strains in the majority of sampled environments. Despite predictions that competing genotypes should drive each other extinct, a single gram of soil is estimated to contain thousands of species, suggesting that there must be mechanisms preventing such competitive exclusion. Indeed, different mechanisms by which diversity can be maintained in microbial populations have been proposed.

The Ecotype Model, Niche Partitioning and Clonal Interference

The canonical model for microbial species distribution is Cohan's ecotype model. It is based on the periodic selection model, which proposes the following: Mutations with large fitness benefits and recombination are sufficiently rare, such that when they do arise, the mutants have a greater fitness than the resident strains. They will therefore quickly sweep to fixation in the population, eliminating all other existing genotypes. The ecotype model applies this idea to individual niches. It predicts that as species adapt to a given niche, they will compete with other genotypes for the available resources in the niche. In the long-term, the genotype best adapted to the niche will competitively exclude all others and dominate through periodic selection. This would result in a one-genotype to one-niche scenario. In other words, this model proposes that many species co-exist together because the sampled environment contains a large number of available niches.

Of course, genotypes that are excluded through competition with others need not go extinct. Instead, they may evolve to specialize on a different available niche in the same environment. This idea is called "niche partitioning" or "niche differentiation": if many nutrient sources or different spatial areas are available in an environment, different genotypes may coexist, if each requires a unique subset of these nutrients to survive and grow or inhabits a different zone in space. In other words, different genotypes can occupy the available niches in a given environment with minimal competition between them. This can occur more easily if the genotypes are distantly related, since they are more likely to differ in their metabolism and thereby occupy different niches. In contrast, because related strains, which have descended from the same genotype will tend to consume the same resources, competition between them should be particularly strong. They are therefore expected to exhibit "clonal interference": as beneficial mutations arise in multiple cells in the population on a similar time-scale, these lineages of clonal cells compete, eventually resulting in a single lineage dominating the population.

One experiment in which these ideas are interesting to test is Richard Lenski's long-term evolutionary experiment, which began in 1988. In it, 12 replicate populations of *E. coli* have been evolving in a defined minimal medium for over 60,000 generations at the time of writing. In three of the 12 replicate populations, mutants that were fitter than their predecessors arose to fixation in a pattern predicted by models of clonal interference. In the remaining nine populations, multiple genotypes co-existed over large time-scales, spanning more than 10,000 generations. In one population, a mutant arose that could grow on citrate, a carbon source that *E. coli* normally cannot metabolize, thus exploiting an untapped niche in the environment. In some of these nine populations, coexisting strains were found to have evolved to depend on each other (Fig. 1).

Having started from a single clonal population, the long-term evolutionary experiment shows evidence of clonal interference, but also of niche partitioning and niche construction. Some strains evolved to survive in uninhabited niches in the same environment (growth on citrate), while in other lineages new niches were created that could then be occupied by emerging strains. These results, then, are in line with the ecotype model. But can genotypes co-exist in the same niche?

Resource Ratio Theory

Resource ratio theory (RRT), originally proposed by David Tilman in the 1980s, suggests that coexistence under competitive conditions is also possible. The theory considers how one or more competing genotypes may respond to the presence of one or more nutrient sources, and under what conditions two or more genotypes can co-exist stably even if they compete for all available nutrients. What RRT predicts is that such stable coexistence is possible if each genotype is limited by a different nutrient source, and preferentially consumes the nutrient source that limits its own growth rate. Although RRT has not been fully tested in microbes, it is a good candidate to explain microbial diversity in environments consisting of few niches where competitive exclusion may be expected.

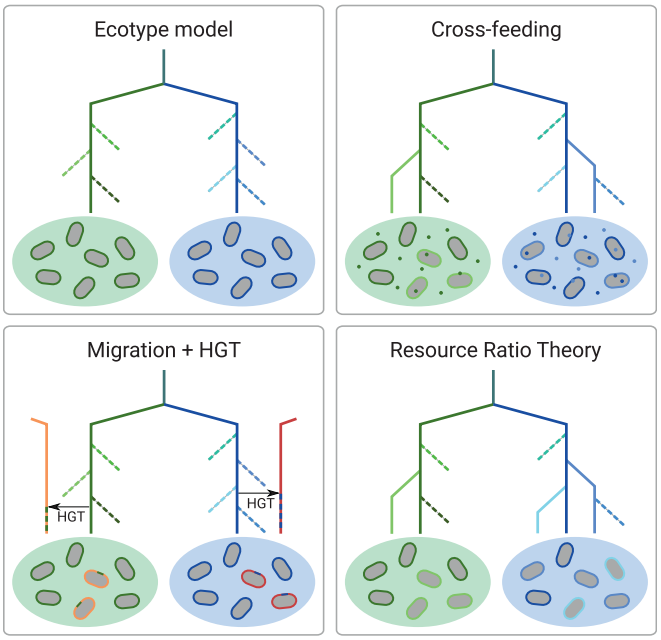

Fig. 1 Different models explaining diversity in microbial communities. Each oval represents a niche that is inhabited by bacteria whose genotype is represented by the color of their border, matching their line in the phylogenetic tree above. The ecotype model proposes that each niche will be filled by single genotypes. In cross-feeding, genotypes can survive on metabolites secreted by others. Another possibility is that genotypes migrate into the community and pick up genetic material allowing them to survive in the niche. Finally, RRT suggests that different genotypes can preferentially take up resources in the niche that do not limit coexisting genotypes' growth.

Cross-Feeding and Parasitism

Another idea of how genotypes can coexist is if they depend on each other through cross-feeding, whereby they consume and grow on each other's waste products. It is still somewhat unclear how common cross-feeding is in natural populations. However, it may explain why it is so difficult to culture certain microbial species in the lab (around 1% of environmental isolates): if they depend on others for survival, they will not grow as long as their partners are absent. This principle can be extended to larger communities: if species depend on each other for nutrients, or to adjust environmental conditions, they will cease to eliminate each other and instead converge to fixed frequencies that maintain both types. This can explain the co-occurrence patterns observed in the long-term evolutionary experiments, where newly emerging mutants were able to make use of a niche constructed by the waste-products of the wildtype, thereby cross-feeding off it. Cross-feeding is expected to be even easier to achieve in communities made up of different species, since their metabolic capabilities are more likely to differ and take on complementary roles.

In cross-feeding, one genotype benefits from the otherwise useless secretions of another. Coexistence can also occur if costly molecules secreted by one genotype for its own benefit (within-genotype cooperation), "leak" and simultaneously provide an advantage for others. The non-producing genotype can then be considered to be a parasite, benefiting from a costly molecule produced by another. At an extreme, the non-producing genotype may depend on the other for survival, creating a uni-directional dependence. Such dynamics are described by the Black Queen Hypothesis, which proposes that many genotypes can stably co-exist if one genotype is providing the resources not only for its own type, but also for other neighbouring genotypes that have all lost the ability to produce this resource themselves. Indeed, this has been observed in some natural communities, such as in wild populations of marine bacteria.

Spatial Structure and Biofilms

An important source of different niches is space. Rather than growing in well-mixed liquid cultures as they do in the lab, microbes are typically embedded within dense, sticky aggregates of microbial cells called biofilms. This lifestyle applies to environments ranging from the surface of a leaf to cells growing in mucus in the human gut or lung, or even cells attached to sand particles or forming marine snow in the ocean. Two things change for a cell in a biofilm compared to when it is living in well-mixed liquid: first, it is physically closer to its neighbours, enhancing their effects, and second, if the cell group is large, substrates will not be able to diffuse through the cell mass, leading to differential access to nutrients or toxic compounds depending on a cell's position within the group. Because of these two effects, the position of a cell within these structures and the identity of its neighbours is extremely important for determining its fate in the community.

In addition, if communities are spatially organized, competition between genotypes can be reduced and promote co-existence. Indeed, spatial organisation is a known mechanism by which diversity can be maintained. This is firstly because different genotypes

will only meet and compete at boundaries of their individual patches, and secondly because it provides many niches, allowing each genotype to adapt to the environmental conditions in its local patch. Because we now think that most microbes live in such spatially structured environments, this may be an extremely common mechanism to reduce competition and promote coexistence. Spatial structure can, however, be easily destroyed due to natural phenomena, such as liquid flow or environmental disturbances. In addition, the microbes themselves will tend to migrate in space to find favourable environments or to escape from predators. In many environments, then, competition may increase due to migration or natural environmental disturbances leading to genotypic mixing.

A classic example of how spatial organisation could lead to co-existence comes from another evolutionary experiment using *Pseudomonas fluorescens* in the simple environment of a standing culture. After just three days of growth, the initially clonal population had evolved into three co-existing genotypes. This co-existence depended on spatial structure in the environment and disappeared if the cultures were shaken.

Migration

Finally, diversity can be maintained through frequent migration of genotypes. In most cases, these migrants will not adapt fast enough and will soon be outcompeted in their new habitat. However, one mechanism that may allow for their stable coexistence is horizontal gene transfer, allowing migrants to acquire the necessary genetic material to survive. In such a scenario, the resulting community will be composed of diverse genotypes that all pass through the habitat filter because they have acquired the genes necessary to survive in this habitat. Whether these communities remain stable will then depend on the extent to which the niches of its genotypes overlap, potentially resulting in rising competition. However, if new genotypes continue to migrate into the community, then diversity could be maintained, even if sampled states represent highly transient ones. In support of this idea, genomic data from environmental samples has shown that rather than whole genomes sweeping to fixation, sweeps appear to occur in particular genomic regions independently of the rest of the genome.

The models proposed to explain the maintenance of diversity, then, posit that arising mutants can occupy unused niches, whether they consist of unused nutrients or spatial niches that already exist in the environment. Alternatively, original resident strains may create niches that can then be filled by future mutant strains, leading to co-existence and co-dependence. A third mechanism of co-existence is if the mutants cease to produce the necessary compounds to survive in the environment, but instead rely on the original strains that cooperatively produce these compounds. Although these strains can be considered to be parasitic cheats, they have been shown to co-exist stably with the original strains in a so-called "negative frequency-dependent" manner: Cheats that are very rare can benefit the most, but if they become too common, they can no longer survive and therefore drop in frequency once again.

Taken from the microbial perspective, a gram of soil may contain considerable amounts of space with different niches, each with its own specific conditions, allowing for large microbial populations consisting of hundreds of species to coexist while never even encountering one another. Indeed, a recent study estimated that even though a gram of soil contains more than 10^8 cells of thousands of species, because of spatial organisation at the scale relevant to the microbes, a given focal cell probably only interacts with around 100 others. This would correspond perfectly to the ecotype model. However, models explaining co-existence within niches may still hold true, thereby predicting an even higher genotypic diversity than the number of available niches.

Conclusions

We have come a long way in understanding what Antonie van Leeuwenhoek first saw under his lens. We have uncovered the challenges microbes face in their environment and how they adapt to them. We have established the mechanisms by which microbes evolve and how their genomes are shaped. We have simulated their evolution in very simple conditions and have generated hypotheses as to why microbial populations are so diverse. Despite these developments, we are now at an exciting point in this field, where ecology and evolution are increasingly being integrated. Many open questions still remain: How do species adapt to the presence of competition in their habitat? To what extent do different environments drive genome evolution? Do certain environments favour horizontal gene transfer over others? And to what extent can microbes shape their environment to promote their own survival over others? We already have preliminary answers to these questions, but there still remains much to discover before we properly understand the dynamics of microbial communities as they adapt to one another and their constantly changing environments.

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The Social Evolution of Bacterial Quorum Sensing

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Introduction

Quorum sensing (QS) is a form of cell-to-cell signaling in bacteria, which regulates the expression of genes and QS-controlled phenotypes in a population-dependent manner. QS involves the production of small diffusible signaling molecules that are released into the surrounding environment. These are taken up actively or passively by neighboring cells where they have two major consequences. First, the uptake of signal molecules stimulates the production and release of a number of QS-regulated public goods. These goods have a variety of uses, which directly or indirectly influence bacterial fitness. Second, the uptake of signaling molecules stimulates the production of more of the signal itself. This leads to autoinduction, positive feedback at high cell densities (when a quorum is reached), which results in an exponential increase in the production of both signal and public goods. A key function of QS is that it regulates behaviors at high cell densities, when the fitness benefits to producing and responding cells are greatest.

One of the first documented QS systems was described in the early 1970s in the marine bacterium *Vibrio fischeri*, a symbiont of the Hawaiian bobtail squid *Euprymna scolopes*. This species of squid has a ventral light organ which luminesces at night, masking shadows it casts by moonlight and allowing it to evade detection by predators. *V. fisheri* cells colonize the crypts of the squid light organ and produce light. In return, the bacteria get to thrive in a much richer and safer growth environment than the surrounding seawater. *V. fisheri* populations do not luminesce at low population densities, but produce light as a coordinated response to growth at high densities (10^{10}–10^{11} cells/mL in the light organ), and this is achieved through the production and sensing of QS signals. At low cell densities, signal diffuses away from the producing cell but at high cell densities, it accumulates, binds to an intracellular receptor and activates production of a light-producing luciferase (Fig. 1A). We now know that QS systems are both widespread and diverse, and control a multitude of cooperative behaviors in many bacterial species that aid in growth, motility, biofilm formation, virulence and bacterial warfare.

The Diversity of QS Systems

The first chemical class of QS signaling molecules fully described in Gram-negative bacteria were the *N*-acyl homoserine lactones (AHLs), and these have been particularly well characterized in *Vibrio* spp. and *Pseudomonas aeruginosa*. AHL structure varies depending on the producing species, but all AHLs possess a homoserine lactone ring connected to an acyl side chain of varying length and branch structure (Fig. 1B). As they are amphipathic molecules, they are able to diffuse across cell membranes. When they bind to cognate receptor proteins within a cell, they induce a conformational change that allows the receptor to bind DNA and modulate expression of QS-controlled genes. AHL-bound receptors also increase expression of their own synthase and receptor in a positive feedback loop called autoinduction, allowing for a rapid and coordinated population response to signals (Fig. 1A).

In contrast, Gram-positive bacterial species communicate using signal peptides called autoinducing peptides (AIPs, Fig. 1B). Examples include the competence-sporulation signaling factor (CSF) of *Bacillus subtilis* and the Agr system of *Staphylococcus aureus*. These peptides vary widely in length, sequence and in post-translational modifications and can be linear or cyclic. Signaling peptides are processed during export and the processed form either binds to a membrane receptor or is actively imported and binds a cytoplasmic receiver where they trigger a QS response.

Although AHLs and AIPs are the most extensively studied signaling molecules, bacteria use an array of mechanisms for communication that vary widely in their structures and their mode of delivery. For example, the hydrophobic *Pseudomonas* quinolone signal (PQS), can be packaged into vesicles that aid its dissemination. *Myxococcus xanthus* produces and responds to a surface bound signal in order to coordinate aggregation and differentiation into a multicellular fruiting body. A furanosyl borate diester called autoinducer-2 (AI-2) is secreted by many Gram-positive and Gram-negative species and has been called a 'universal signaling molecule' and which we will describe further below (Fig. 1B).

Evolutionary Considerations of Quorum Sensing

Whilst a huge amount of mechanistic understanding has been gained in QS over the last 40 years, relatively little attention has been given to the evolutionary implications of QS. This has changed in the last 10 years. Traditionally it was assumed by microbiologists that QS is selected for because it benefits the local group or population, perhaps by providing individuals with information about diffusivity of the local environment. In contrast, evolutionary theory suggests that cooperative communication is only maintained by selection under fairly restrictive conditions. These conflicting perspectives led to investigations into whether QS in microbes is truly a cooperative behavior.

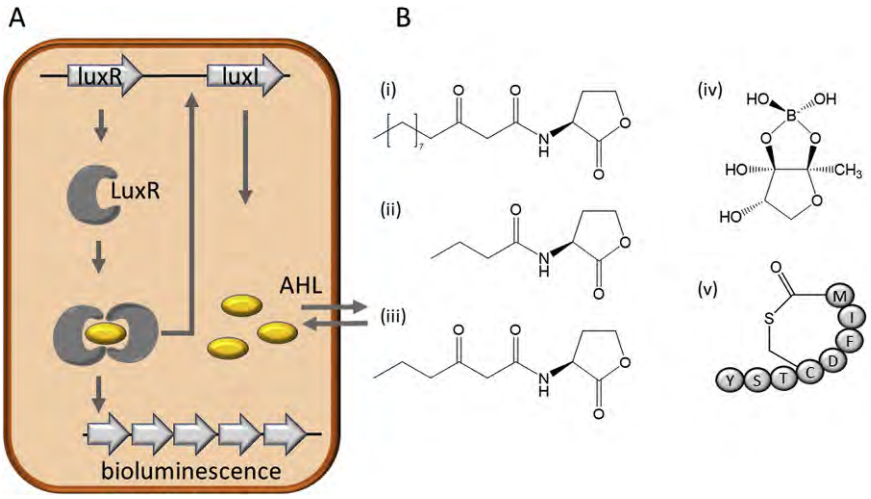

Fig. 1 QS signaling and examples of QS molecules. (A) The LuxI/R QS circuit of *V. fischeri*. The *N*-acyl homoserine lactone (AHL) synthase LuxI produces AHLs (yellow ovals) which freely diffuse across the cell membrane. At sufficiently high concentrations, they bind the receptor LuxR, which then activates transcription of bioluminescence genes and its own promoter (autoinduction). (B) Examples of QS signaling molecules: (i) N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) of the *P. aeruginosa* LasI/R circuit; (ii) N-butanoyl-L-homoserine lactone (C4-HSL) of the *P. aeruginosa* RhlI/R circuit; (iii) N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) of the *V. fischeri* LuxI/R circuit; (iv) *V. harveyi* autoinducer 2 (AI-2) furanosyl borate ester; (v) Autoinducing peptide 1 (AIP-1) of *S. aureus*, amino acid residues indicated by letters in circles.

A key question for the field of bacterial communication is what defines a signal? This can be largely informed from a large body of evolutionary theory on animal communication. A *signal* can be defined as a behavior (or structure) which elicits a response from a receiver and the signal and response have evolved in the sender and receiver respectively because of that response. Critically, the signal and response must benefit both sender and receiver or the signaling behavior breaks down over evolutionary time. True signals should be distinguished from behaviors that only benefit one party. A *cue* is a feature that elicits a response from a receiver, which evolved because of the benefit to the receiver and does not benefit the sender; a behavior that has been called eavesdropping in a microbial context. Examples of cues in microbes could be metabolic waste products produced by one cell that are then imported and utilized by a receiver. *Coercion* is an activity that evolved in the sender that forces a costly response from the receiver, but that response does not benefit and did not evolve for that purpose in the receiver.

It is not always straightforward to categorize an interaction as a signal, cue or coercion without experimental verification. In the lungs of cystic fibrosis patients, *Burkholderia cenocepacia* responds to AHLs produced by *P. aeruginosa* and upregulates virulence genes. This may be a response to a cue rather than a cooperative signaling interaction, since the benefit to *P. aeruginosa* is unclear. Competent *Streptococcus pneumoniae* cells trigger the fratricide of non-competent kin so they can take up their DNA. It is uncertain whether this is cooperative signaling or an example of coercion by the competent population. With the advent of inexpensive sequencing, incomplete parts of QS pathways are often found in bacterial genomes, calling into question whether they represent true signaling mechanisms. The 'universal signal molecule', AI-2, is produced by many different Gram-negative and positive bacterial species, but a response to AI-2 has only been demonstrated in a few examples. Further, AI-2 has been shown to be a product of S-adenosylmethionine metabolism turnover and might, in many cases, simply be a metabolic byproduct that is recognized as a cue (and did not co-evolve in producer and responder). In another example of a disparity between signal producers and corresponding responders, many orphan AHL receptors (LuxR) unaccompanied by their cognate synthase machinery have been cataloged by genome sequencing.

As signal production, and to a greater extent, response to that signal, incurs a fitness cost, differentiating a signal from a cue or coercion allows us to make general predictions about bacterial signaling. First, if the signal has evolved to benefit both sender and receiver, it should usually convey reliable information to be evolutionarily stable. Second, it might be possible for individuals to undermine the reliability of the signal or 'cheat' by (i) not producing signal (or engaging in the cooperative behavior controlled by the signal), avoiding the costs of both signaling and cooperation (Fig. 2) (ii) producing increasing amounts of signal without engaging in cooperation, coercing a cooperative response from receivers. How does a quorum sensing population avoid invasion by these cheats and subsequent collapse? Evolutionary theory provides a solution to the problem of how to maintain a reliable signal through the principle of kin selection.

Kin Selection in the Maintenance of Cooperative Signaling

QS and the behaviors it regulates are cooperative and a cooperative behavior by definition benefits the members in a group or population. If a cooperative behavior benefits both the individual performing the action and other individuals, it can be maintained if the *direct fitness benefit*, i.e the fitness benefit to the producer, outweighs the metabolic cost of the action. When a cooperative

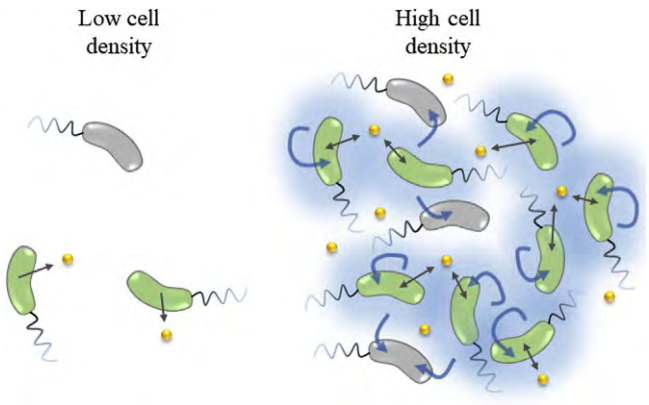

Fig. 2 QS-induction of cooperation and cheating. At low cell densities, green producers produce QS signal (yellow balls) which do not accumulate to high enough concentrations to trigger a response. Gray cheaters avoid the cost of QS signal production. At high cell densities, accumulation of QS signal triggers the production of public goods by producers (blue halos). Cheaters receive the benefit of these public goods (blue arrows) without paying the metabolic cost.

action indirectly benefits another individual at the cost of the actor, this poses a problem since this decreases the fitness of the actor who can then be outcompeted by non-cooperating 'cheats'.

Kin selection theory proposes a solution to the problem of cheating: if the benefit of cooperation is directed towards a closely-related individual, or kin, this stabilizes the cooperative genes in the population. This is encapsulated in Hamilton's rule which states that altruism is favored when $rb - c > 0$, where c is the fitness cost to the actor, b is the fitness benefit to the beneficiary and r is their genetic relatedness. When relatedness is high or when the cost of cooperation is low, altruistic behavior can be maintained. Cooperation can therefore provide an *indirect fitness benefit* to individuals that share cooperative genes and the relative importance of direct and indirect fitness benefits will determine whether the cooperative behavior is mutually beneficial or altruistic. A consequence of Hamilton's rule is that the indirect benefits of cooperation diminishes in populations where the relatedness of its members is low.

In 2001, Brown and Johnstone proposed a kin selection model for the evolutionary stability of QS. They made the assumptions that (1) signals are costly, (2) the cooperative behavior controlled by signaling is costly, (3) the cooperative behavior provides an indirect fitness benefit to group members, and (4) the benefits of both signaling and cooperation increase at high cell densities. They defined an evolutionarily stable strategy as one which, when adopted by the majority of individuals, cannot be displaced or invaded by cheats adopting another strategy. They came to two major conclusions: (1) Cooperation and signaling were both stabilized with increasing cell density. At low cell densities, the costs outweighed the benefits. (2) Cooperation as a strategy was stabilized with increasing relatedness of members, as this increased the indirect fitness benefit of cooperation for the entire population. However, even populations with relatively low levels of relatedness could cooperate to an appreciable level.

Predictions of the Brown and Johnstone model have since been tested experimentally. In 2007, we and others demonstrated that under selective *in vitro* conditions, a QS-controlled protease provides a benefit to *P. aeruginosa* at the population level and that QS signaling is costly to the cell. Cheating signal-blind (defective in the AHL response regulator LasR) or signal non-producer (defective in the AHL synthase LasI) mutants were able to invade a population of wildtype producers. We also found that fitness of cheats was inversely proportionate to their starting frequency in the population and that high relatedness favored the maintenance of QS. *In vivo*, in mouse models of burn and chronic wounds, QS *lasR* mutants were able to invade population of producers even though they were less virulent by themselves than the QS-positive strains. The fitness of these cheats was negatively dependent on their starting frequency in the population, because at low starting frequencies, there are more cooperators for cheats to exploit.

The QS kin selection model made further predictions distinguishing signaling from the signaling-controlled cooperative behavior: unlike cooperation (which is stabilized monotonically with increasing relatedness) the stability of signaling increases with relatedness up to a point and then decreases, reaching a maximum at intermediate relatedness. The authors proposed that at high relatedness, kin selection favors cooperation and an inexpensive signal will suffice. At low relatedness, cooperation is not selected for, and so there is no selection for signaling. At intermediate relatedness however, there is selection for cooperation but the population is vulnerable to cheating. This was predicted to promote higher levels of signaling to force individuals to cooperate but at the same time, selection forces cheaters to ignore higher and higher levels of signal (competitive devaluation of signal). A later theoretical study proposed that this last prediction only holds true if the cost of cooperation is low and only a few cooperating members are required to bring the benefit of cooperation to the entire group. When Popat and coworkers explicitly tested this prediction in *P. aeruginosa*, they confirmed that at low relatedness, both signaling and cooperation broke down as the population was invaded by cheats. This occurred through a variety of mutations in both signaling and response pathways. However, they did not observe maximal selection for signaling at intermediate relatedness. Instead, they found that the stability of signaling monotonically decreased with decreasing relatedness. They suggested that this was because the initial model assumed cooperation and signaling to evolve independently, but autoinduction provides positive regulatory coupling between the two processes, limiting the rapid evolution of coercive high-signal low-cooperation mutants.

Strategies to Promote Kin Selection

In order for kin selection to be an effective stabilizing force on cooperation, cooperators must solve the problem of directing cooperative benefits primarily to kin and not to competitors. This can occur in different ways, both passive and active.

Segregation

Hamilton described one solution in the form of limited mixing or population viscosity. Applied to a microbial context, when individuals reproduce asexually, local areas are likely to be colonized by one clone (relatedness: $r = 1$) and if cooperators indiscriminately distribute public goods to neighbors, those neighbors are likely to be highly related and the indirect fitness benefits will be high. Conditions of high assortment (low mixing) are also favored in structured spatial environments and in bacterial biofilms. Darch and coworkers showed that *Pseudomonas aeruginosa* formed primarily clonal aggregates in an environment that simulated the sputum of a cystic fibrosis lung and that QS signaling and therefore QS-controlled cooperation was largely confined within aggregates. In *V. cholerae* and *P. aeruginosa* biofilms, the structural properties of extracellular matrix exclude invading non-cooperators, making the matrix a relatively non-cheatable public good.

Segregation can also be achieved by direct antagonism. Many Gram-negative bacteria use a type six secretion system that penetrates the cell wall and directly injects toxins into neighbors. In *Vibrio cholerae* and *Burkholderia thailandensis*, this system can be QS-controlled and fires at high cell densities. This has the effect of segregating populations that would otherwise be mixed into clonal patches and modeling and genomic analysis shows that this can promote the evolution of cooperative behaviors. *V. fischeri* strains use this strategy to eliminate competing strains in crypts of squid light organs. Bacteria possess a diverse arsenal of antagonistic strategies including contact-dependent growth inhibition (Cdi) systems and diffusible bacteriocins, all of which in theory would work to segregate populations.

Kin Discrimination

Another strategy for stabilizing cooperation via kin selection works by identifying related individuals and preferentially directing the benefits of cooperative actions toward them, a term called *kin discrimination*. *Myxococcus xanthus* preferentially cooperates with related individuals to form multicellular fruiting bodies by recognizing a polymorphic surface protein TraA. In *Staphylococcus aureus*, QS autoinducing peptides and their receptors are classified into four known kin recognition groups; the AIP of one group can block the receptors of other groups, preventing expression of virulence genes and limiting the ability of potential non-cooperators from establishing an infection.

Repression of Competition

Signaling and cooperation in bacteria can also be stabilized by coupling it to another process required for bacterial fitness. If the opportunities for cheating come with large fitness costs, individuals can be constrained to only increase their fitness by contributing to the productivity of the entire group. This removes the opportunity for cheating and selects for increased cooperation. One study reported that control of a type VI secretion system in *B. thailandensis* by QS led to targeting and killing of QS-cheats in co-culture on solid surfaces, an example of *policing*. In *P. aeruginosa*, QS controls the production of hydrogen cyanide, a cooperative behavior that can inhibit competition by other species and aid in virulence. QS mutants become sensitized to cyanide and so their growth is inhibited in a population of cooperators. *Pleiotropy*, the ability of a single gene to affect multiple phenotypes can also be used as a tool to repress competition. In *P. aeruginosa*, quorum sensing controls expression of the cytoplasmic nucleoside hydrolase, a private good that allows use of adenosine as a carbon source. When adenosine is present in the environment, cheats that are defective in QS cannot utilize it as a carbon source and pay a fitness cost.

It should be noted that strategies for stabilizing cooperation are not exclusive, often overlap, and are sometimes ambiguous to interpret. For example, in *B. thailandensis*, type VI secretion could act both as a mode of policing and as a method for spatial structuring that aids in kin selection.

Complexities of QS

The majority of QS studies have been performed *in vitro*, where cells are allowed to grow to high densities in batch conditions. In these conditions, QS provides a greater benefit at higher densities because QS controlled public goods are more efficiently utilized. However, what constitutes a quorum and to what extent quorum sensing occurs in natural environments are important considerations that have been largely ignored. Local rates of mass transfer and QS signal degradation by both biological and abiotic factors determine the amounts of signal available to a population; a single cell can respond to its own QS signal if mass transfer is sufficiently low and signal stability is high. Redfield proposed that individual bacteria primarily used signaling molecules to determine the local diffusion rate to decide whether to produce costly extracellular factors, a term called 'diffusion sensing'. In this study, she argued that population level selection had not been experimentally demonstrated and that bacterial signaling is an

individual trait, subject only to individual selection and is misinterpreted as a social behavior. However, cheating, a consequence of sociality, has since been documented in a number of studies in both *in vitro* and *in vivo*. We and others have proposed that the two terms are not mutually incompatible; the local rate of diffusion and mass transfer is an important environmental variable that greatly affects both the direct and general fitness benefit of a secreted product. Many bacteria including *Pseudomonas* and *Vibrio spp.* simultaneously use two or more QS signals, to solve the problem of distinguishing environmental from social information. *P. aeruginosa* integrates signals from two AHLs, the more stable C4-HSL and the less stable 3-oxo-C12-HSL, to tailor exofactor secretion in response to cell density and local mass transfer rates. Recent studies have shown that these connected AHL-responsive circuits can be uncoupled both in clinical strains and *in-vitro* evolved populations, allowing *P. aeruginosa* some plasticity in QS control of behaviors.

The Future of QS Research

The majority of studies that have moved our understanding of QS forward, have taken place *in vitro*, in growth conditions not intended to mimic the natural environment. *In vitro* experiments are often designed to exaggerate a selective condition, in order to more easily identify social behaviors and test the predictions of evolutionary theory. While this approach has been extremely useful in building a foundation of fundamental research, we have much to learn about microbial communication and sociality in natural settings. Complex microbial communities can involve many interacting species and are impacted by spatial structuring, ecological settings and host interactions. Progress is being made in increasingly sophisticated experiments that add these layers of complexity to *in vitro* conditions as well as in deciphering the behavior of communities using genomic and transcriptomic approaches. As researchers improve upon and continue to apply the fundamental principles of sociomicrobiology to living systems, this will advance our knowledge of communities in natural and clinical systems in ways that may greatly impact applications for human health, industry and the environment.

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Toxoplasmosis☆

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Glossary

Apicoplast A plastid-like organelle just anterior to the nucleus delimited by four membranes and containing its own genome. Named for its ubiquitous presence in Apicomplexa.
bradyzoite The slowly dividing, infectious, asexual stage found within tissue cysts in the intermediate host. From the Greek for 'slow' (*brady*) 'little animal' (zoite).
endodyogeny Process by which tachyzoites and bradyzoites divide involving creation of two daughter cells wholly within a mother cell. From the Greek for 'gives rise to two' (dyogeny) 'inside' (*endo*).
micronemes Small, thin, anterior organelles that secrete their contents onto the parasite's surface at the start of the invasion process. From the Greek for 'small' (*micro*) 'thread' (*nema*).
micropore A small, single invagination anterior to the nucleus with an apparent clathrin coat presumed but not yet demonstrated to be involved in endocytosis.
oocyst The mature, environmentally robust product of the sexual cycle shed in its immature form in cat feces and comprising a cyst wall and two sporocysts, each of which contains four sporozoites.
paralogous The state of being derived from the same ancestral gene.
rhoptries Club-shaped, apical organelles that release their contents into the nascent parasitophorous vacuole. From the Greek for 'club' (*rhoptry*).
sporozoite The product of the sexual cycle, found as two sets of four within the oocyst.
tachyzoite The rapidly dividing, asexual stage. From the Greek for 'fast' (*tachy*) little animal (zoite).
tissue cyst The protective structure containing the asexual bradyzoites in the intermediate host. Also known as the pseudocyst.
zoonosis A disease of humans that derives from a reservoir of nonhuman animal infection. From the Greek for 'disease' (*nosos*) originating from 'animals' (*zoo*).

Abbreviations

DHFR	dihydrofolate reductase
GAG	glycosaminoglycan
GRA	dense granule protein
HXGPRTase	hypoxanthine/xanthine/guanine phosphoribosyl transferase
IFN	interferon
IL12	interleukin 12
IPN	intraparasitophorous vacuolar network
MIC	microneme protein
PVM	parasitophorous vacuole membrane
RFLP	restriction-fragment-length-polymorphism
RON	rhoptry neck protein
ROP	rhoptry protein
STAT	signal transducers and activators of transcription
TRAP	thrombospondin-related anonymous protein
UPRTase	uracil phosphoribosyl transferase

Defining Statement

Toxoplasma is an obligate, intracellular parasite that causes a range of symptoms in its exceptionally broad host range.

☆*Change History:* September 2014. JC Boothroyd introduced small edits in the text of the article including citations, added the sections 'Applications' and 'References', and added Figures

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Introduction

Toxoplasmosis is caused by the protozoan *Toxoplasma gondii*, an obligate, intracellular microbe whose definitive host is the family Felidae (cats). *T. gondii* has an exceptionally broad range of intermediate hosts, including humans, in which the asexual cycle can occur resulting in serious disease. It is found throughout the world and, in terms of the number and range of hosts infected, *Toxoplasma* may be the most common protozoan infectious agent of warm-blooded animals.

The genus *Toxoplasma* falls within the phylum Apicomplexa and is a close relative of the nonhuman pathogens, *Neospora* and *Sarcocystis*. It is more distantly related to *Eimeria*, the causative agent of coccidiosis in birds and more distant still to *Plasmodium* and *Cryptosporidium*, the etiologic agents of malaria and cryptosporidiosis, respectively. At the still more distant level, *Toxoplasma* is also related to free-living dynoflagellates but the evolutionary timescales involved are enormous (e.g., humans and flies are relatively close cousins by comparison).

Infection of healthy humans by *Toxoplasma* is usually of little consequence but human disease will often occur in either of two scenarios: in the child of a woman infected for the first time during pregnancy and in individuals who are immunocompromised. Occasionally, however, serious eye infections can occur even in people who are otherwise healthy. This unusual occurrence may be the result of an unusually large inoculum, the particular genetic makeup of the person infected or, perhaps, the strain of parasite responsible for the infection.

In animals, toxoplasmosis is also generally self-limiting with the most serious consequences (abortion) being from acute infection during pregnancy. This is a particularly serious problem in the raising of sheep.

The relative ease of handling this haploid agent in the laboratory and the wide range of natural hosts have made *T. gondii* a popular model for experimental study of intracellular infection. Through such studies, much has been learned about the way in which this common microbe interacts with its host, about its population biology, and about the molecular details behind its obligate intracellular life style.

Classification

T. gondii is a protozoan within the phylum Apicomplexa, Class Conoidasida, order Eucoccidiorida, suborder Eimeriorina, family Sarcocystidae. The genus name derives from the Greek word *toxon* meaning bow and *plasma* meaning form and refers to the bow-shaped tachyzoite. The species name derives from the name of the North African rodent from which the parasite was first isolated in 1908 by Nicolle and Manceaux. It was simultaneously described by Splendore in Brazilian rabbits.

Life Cycle

The complete life cycle of *T. gondii* was not established until about 1970 when four groups independently identified the cat as the definitive host, meaning this is the host in which *Toxoplasma* enters into its sexual cycle. Figure 1 depicts the complete life cycle as consisting of two, potentially independent cycles, one asexual and the other sexual. This has been done in order to emphasize that, at least on paper, the parasite appears fully capable of propagating itself through either cycle. The degree to which these two cycles intermix (arrows 4 and 8) is not known. In practical terms, this uncertainty means that it is not clear what fraction of infection in the

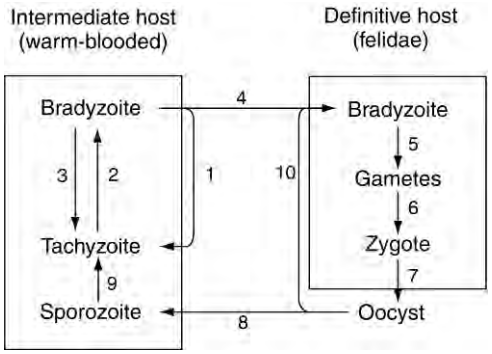

Figure 1 The life cycles of *Toxoplasma gondii*. The asexual cycle can occur in a large number of warm-blooded animals and is shown on the left. It involves an equilibrium between the rapidly dividing tachyzoite ('tachy' means fast in Greek) and the more slowly dividing bradyzoite (arrows 2 and 3; 'brady' means slow in Greek). Transmission in the asexual cycle is through the ingestion (carnivorism) of meat and other tissue containing the infectious encysted bradyzoites (arrow 1; e.g., rat to pig or pig to human). Scavenging allows the parasite to cycle back down the food chain (e.g., pig to rat). The sexual cycle is shown on the right and involves schizogony, gametogenesis, fertilization and development into oocysts (arrows 5, 6, and 7) in the gut epithelium of felines. Cat-to-cat transmission through the sexual cycle is theoretically possible by ingestion of oocysts in fecal contamination (arrow 10). Crossover between the two cycles is represented by arrows 4 and 8, but the extent of this in nature is not known.

intermediate host (e.g., humans) is a result of ingesting meat containing tissue cysts (arrow 1) versus accidental ingestion of oocysts in the environment (arrow 8).

The tachyzoite stage is by far the best studied, mainly because it is easy to grow this form *in vitro*. The other asexual stage, the slow-growing, encysted bradyzoite is poorly understood. Fortunately, however, protocols have recently been developed that can cause tachyzoites to efficiently differentiate to the bradyzoite stage *in vitro*. These protocols rely on a number of stresses (certain drugs, heat, pH) that stimulate the differentiation pathway. Although the differentiation *in vitro* may not be complete, the availability of these protocols has opened up the way for more detailed studies of this critically important stage, which previously could only be obtained in small amounts from the tissue of infected animals.

The two asexual stages are similar in overall morphology with mostly the same complement of organelles. The major differences are in the presence of many amylopectin granules in the bradyzoite and a cyst wall that encloses the entire complement of bradyzoites in any given vacuole. The metabolic differences are not clear, although the existence of stage-specific isoforms of some glycolytic enzymes (e.g., enolase and lactate dehydrogenase) suggests the possibility of a fundamental difference in sugar metabolism. Such differences might be related to the presence of the amylopectin granules and/or the polysaccharide-containing cyst wall.

The sexual stages have only been described morphologically in the gut of infected cats (it has not yet been possible to grow any of these stages *in vitro*). The process of gametogenesis, which occurs in the epithelium of the small intestine, conforms to classic coccidian rules including schizogony where a multinucleated state is reached followed by segmentation to yield multiple uninucleated merozoites. Mating between micro- and macrogametes has never been directly observed but is presumed to occur within infected epithelial cells. The process results in a zygote, which then encysts yielding an immature oocyst. This is released into the environment where, after approximately 2-days of exposure to air, it develops into a mature, infectious oocyst comprising two sporocysts, each with four sporozoites. The genetic relationship of the eight sporozoites within a single oocyst has not yet been determined; that is it is not known when the three divisions occur to take the oocyst from one to eight organisms and which of those divisions are mitotic vs. meiotic.

Clinical Aspects and Public Health

Symptoms

Toxoplasma causes disease in humans primarily under two circumstances: the developing fetus of a woman who acquires her first infection during pregnancy and persons who are immunocompromised. The disease that results varies from mild to severe and sometimes fatal. Note, however, that in persons who are infected *in utero* the disease may not appear until many years later, when the individual is in the second decade of life or older.

The prevalence of seropositive adults ranges from approximately 5% to 20% in the United States to as high as 50–85% in France and many developing countries. Historically, severe disease has not been common because of the low number of persons who fall within the two disease-susceptible groups mentioned above: the children of acutely infected pregnant women and people who are immunocompromised. The AIDS epidemic obviously produced a huge increase in the number of people in the immunocompromised category. Initial results indicated that, if left untreated, about one-fourth of AIDS patients who harbored a chronic infection of *Toxoplasma* (most cases of toxoplasmosis in AIDS patients are thought to be due to reactivation of historic, quiescent infections) were liable to develop the potentially fatal condition of toxoplasmic encephalitis. Within the United States, this proportion has dropped in the last decade, probably as a result of three factors. (1) Improved anti-HIV therapies (with a resulting improvement in general immune competence). (2) Prophylactic treatment with trimethoprim/sulfamethoxazole for another opportunistic infection, *Pneumocystis*: such treatment may have the added benefit of controlling *Toxoplasma* infection, if present. (3) Deliberate prophylaxis for *Toxoplasma* in patients who are seropositive and whose CD4 T-cell counts are low. Data on toxoplasmosis in AIDS patients in the developing world are scant but it appears that toxoplasmic encephalitis may be a significant problem in the AIDS populations of those countries just as it was in the United States prior to the aggressive treatment protocols that are in place today.

Congenital infection rates vary by country, in proportion to the seropositivity of the adult population; in the United States, there are no exact numbers but current estimates are that approximately 400–4000 congenitally infected children are born each year. The probability of transmission from an acutely infected mother to the developing fetus varies with the stage of the pregnancy: acute infections in the first trimester are the least likely to be transmitted, whereas third trimester infections are the most likely to be transmitted. As for many congenital diseases, however, the outcome is worse when the infection occurs earlier in the pregnancy: first trimester infections can lead to severe neurological disease (blindness and retardation) or even death, whereas third trimester infections are more mild and may even yield no symptoms at birth. This is not to say that no disease will occur in this latter group: it appears that the parasite can lie essentially dormant and erupt in early adulthood leading to various symptoms, most notably sudden blindness in one eye due to severe retinal destruction. Drug treatment of acutely infected pregnant women can reduce the probability of congenital transmission but, as discussed below, such treatment is not without significant risk to the fetus.

Diagnosis

Serology is the most common method for diagnosing infection with *Toxoplasma*. Acute infection in pregnant women is usually diagnosed from a high IgM or IgA antibody titer and/or a rise in IgG in successive serological samples taken at multiweek intervals

during the pregnancy. Antibody avidity is also used to discriminate between an acute and chronic infection. In countries like France where the incidence is high, strict monitoring is in place: women are tested for anti-*Toxoplasma* titers at the time of marriage and those who are seronegative and later become pregnant are monitored repeatedly for signs of seroconversion (equating to an acute infection). If an acute infection is detected, it is now possible to monitor for transplacental transmission to the fetus (which is the real concern) by amniocentesis and polymerase chain reaction to detect the parasite (or, rather, its deoxyribo nucleic acid (DNA)) directly.

Treatment

Current treatment of acute infection in nonpregnant humans relies on the synergistic action of pyrimethamine plus sulfadiazine, which combine to block folate metabolism through inhibition of dihydrofolate reductase (DHFR) and dihydropteroate synthase, respectively. Unfortunately, even this powerful combination has little effect on the chronic form of the parasite (the bradyzoite), and thus treatment does not completely eliminate the infection. As a result, in immunocompromised individuals the drug therapy must be maintained for long periods to prevent recrudescence of the infection. This is not a simple option as sulfadiazine is poorly tolerated by many patients and its use often must be stopped due to side-effects. Other therapies, while showing some promise, have yet to prove as effective as pyrimethamine plus sulfadiazine (e.g., pyrimethamine plus clindamycin and the newer drug, atovaquone), although better formulations and treatment protocols could dramatically improve their efficacy.

Because of drug toxicity, treatment of pregnant women is always a clinically complicated decision. In countries like France, which have the most experience of managing such patients, acute infection in the mother is treated with spiromycin although the benefit of such treatment is not firmly established. If transmission to the fetus has been demonstrated, then the more potent but more toxic combination of pyrimethamine plus sulfadiazine is used because the risk of serious disease outweighs the possible harmful effects of the drugs. Therapeutic abortion is also sometimes chosen.

Public Health

In the absence of a vaccine and given the short-comings of the drugs mentioned above, the best approach to controlling human toxoplasmosis is avoidance. This is easier said than done, however, as evidenced by the fact that infection with this parasite is so prevalent worldwide.

Meat-eaters are especially at-risk from undercooked lamb and pork, although the latter may not be a major source because of centuries of knowledge that severe disease can result from eating undercooked pork and the resulting common knowledge to avoid consuming such meat. Lamb, on the other hand, is frequently eaten extremely rare in many cultures. (Note that beef, while also often eaten rare or raw, is not such a problem because, for unknown reasons, bovines appear to harbor fewer numbers of tissue cysts in their muscle or other tissue.) It is thus critical that persons at-risk, for example, persons who are immunocompromised and seronegative women who might become pregnant, avoid eating meat that is not thoroughly cooked. Fortunately, freezing also kills tissue cysts and thus this simple preventative measure can also be used to reduce the risk of infection.

Toxoplasma's unusual life cycle means that simple vegetarianism will not enable someone to avoid infection. The oocysts shed by cats are extremely stable in the environment and cats are particularly fond of defecating in loose soil such as that found in vegetable gardens. It is thus fairly simple for vegetables to be contaminated with minute amounts of oocysts and so at-risk persons should take special care to eat only cooked or thoroughly washed fresh vegetables.

Finally, the stability of the oocysts also means that they can be ingested through daily activities as innocuous as gardening or playing in a dusty field or sandbox. Obviously, cleaning a pet cat's litter tray is not appropriate for at-risk persons although because oocysts take approximately 48 h to become infectious after shedding, regular daily removal of the feces can reduce the risk of infection. Even better, keeping cats strictly indoors and feeding them only on tinned or dried foods can virtually eliminate the chance of their acquiring an infection.

One of the key deficiencies in our understanding of toxoplasmosis is what fraction of human infection comes from ingestion of tissue cysts in undercooked meat products versus oocysts in environmental contamination. This point is discussed further below.

Molecular Biology and Genetics

Genome and Gene Expression

Except for the diploid zygote, all stages of *Toxoplasma* are haploid with a genome of approximately 6.5×10^7 bp per nucleus. Using a combination of physical and genetic means, it has been shown that *Toxoplasma* has 14 chromosomes in its nucleus. The genomic sequences for Type I, II, and III strains have been essentially completed, as have now over 60 additional strains. These genome sequences are all available at www.toxodb.org/toxo/. This is an extraordinary advance and has opened up many new areas of investigation. Unlike some other parasitic protozoa, gene expression in *Toxoplasma* appears to be similar to that seen in other model eukaryotes such as yeast or humans. For better or worse, this has allowed investigators to focus on the coding function of the genes rather than any subtleties about how they are regulated.

In addition to the nuclear DNA, two other genomes are present in *Toxoplasma*: the approximately 35 kb genome of the plastid-like structure known as the apicoplast (see below) and as yet incompletely characterized mitochondrial DNA.

Genetics

Because of its haploid nuclear genome, it is relatively easy to select phenotypic mutants in *Toxoplasma*. A large number of such mutants have been described including lines that are temperature-sensitive for growth, deficient in development, disrupted in cell cycle, or resistant to one or more drugs. Mutant libraries have been made using chemicals or random insertion of DNA sequences carrying selectable markers. The latter has also been done with plasmids carrying a unique sequence that serves as a 'signature' or bar code allowing pools of mutants to be analyzed as a group.

Such mutants have been used as an unbiased way to identify important genes, either through genetic mapping, complementation or sequencing. The latter is an especially exciting development because of 'next generation' sequencing which allows the entire genome of multiple mutants to be analyzed in a single day, thereby revealing mutations responsible for mutant phenotypes. This promises to be a highly productive approach to dissecting key functions in Toxoplasma in the near future and has already yielded interesting results on DOC2, a protein involved in membrane fusion, and CDPK3, a calcium-dependent protein kinase involved in signaling related to invasion and egress.

Molecular Genetic Tools Available for the Study of Toxoplasma

In the past decade or so, there has been a substantial effort invested into the development of molecular genetic techniques for the study and manipulation of *Toxoplasma*. The large number of such techniques now available makes *Toxoplasma* one of the most easily studied and 'engineered' of any protozoan parasite.

Initially, transient expression of suitably engineered DNA plasmids was achieved using electroporation. There are now several selectable markers reported for stable transformation of *Toxoplasma*: bacterial chloramphenicol acetyl transferase (conferring resistance to chloramphenicol); pyrimethamine-resistant DHFR; bacterial tryptophan synthase (*trpB*, conferring tryptophan prototrophy); bacterial *ble*, conferring bleomycin and phleomycin resistance; bacterial β-galactosidase (*lacZ*); jellyfish green fluorescent protein and, in the appropriate null backgrounds, *Toxoplasma* hypoxanthine/xanthine/guanine phosphoribosyl transferase (HXGPRTase); and uracil phosphoribosyl transferase (UPRTase). All have been successfully used to introduce exogenous or engineered genes into the parasite for expression.

This large collection of selectable markers has made reverse genetics a staple of the *Toxoplasma* field. Many genes have been specifically disrupted through targeted insertion/deletion using one or other of the selectable markers listed above. This has allowed the function of many loci to be investigated. Of course, essential genes in a haploid organism cannot be deleted, by definition, unless a backup copy is provided. This has been done using the popular tetracycline-regulated expression system that allows the backup copy to be specifically turned on or off by adding/subtracting the drug, as well as more recent methods involving destabilization domains fused to proteins of interest and methods using engineered forms of Cre recombinase.

Tachyzoites engineered to inject Cre recombinase have also been developed and used to probe 'Cre reporter' mice where the presence of injected Cre turns on a fluorescent marker like green fluorescent protein (GFP). This has allowed infected cells to be readily identified *in vitro* and, more interestingly, has revealed that many cells that have received the injected Cre are not infected. *In vitro* such cells include some that appear to have never been infected, suggesting that the parasites can inject into cells they do not then invade. Whether such is a chance, abortive invasion event or a selected trait that enables the parasite to co-opt cells it has no intention of infecting is not yet clear. Strikingly, such uninfected/injected cells are also seen *in vivo* but there it is not possible to say if such are cells that were infected and the invading parasite was subsequently cleared or if they are cells that were injected into but never invaded. The implications of such cells for the overall interaction of Toxoplasma with its host, especially neurons in the brains of chronically infected animals, could be profound.

Cell Biology

Organelles

As an obligate, intracellular parasite, *Toxoplasma* has many specialized organelles dedicated to this lifestyle. Some of these are shown in Figure 2 and each will now be briefly described.

Apicoplast: This is a spherical, plastid-like organelle just anterior to the nucleus. Its importance to *Toxoplasma* and related parasites is evident from the fact that it is found in Apicomplexan parasites ranging from *Toxoplasma* to *Eimeria* and *Plasmodium*. Its existence has been known for decades, going by various names (e.g., Golgi adjunct, and spherical body). It is delimited by four membranes and contains its own genome whose sequence revealed its relatedness to plastids of algae. The data further argue that it was acquired by the ancestor of *Toxoplasma* (and other Apicomplexa) through a secondary endosymbiosis of an algal-like eukaryote. The function of this organelle is not entirely known and its small genome has provided few clues. A thorough study, however, of the proteins encoded in the nucleus that ultimately are targeted to the apicoplast argue that it has an important role in fatty acid metabolism among other functions. As mentioned below, its conservation throughout the Apicomplexa and the complete lack of a similar organelle in the animal hosts of *Toxoplasma* make it an extremely attractive drug target. Already, one drug that had been empirically determined to be very effective against Apicomplexan parasites, clindamycin, has been shown to act through interfering with translation in the apicoplast. It is likely that this organelle will provide rich pickings for future drug-discovery programs.

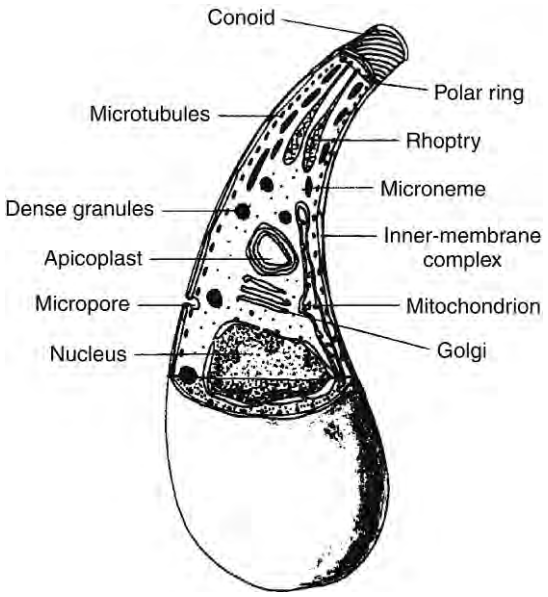

Figure 2 Ultrastructure and morphology of the tachyzoite. This schematic shows a cut-away of the tachyzoite stage of the parasite. The overall morphology is very similar for the bradyzoite and sporozoite. Note that the microtubules gently spiral down the body, starting from the anterior polar ring and ending about two-thirds along the length of the parasite. This is why they have an oval appearance in the cross-section shown.

Dense granules: These are small, spherical, secretory organelles distributed throughout the tachyzoite. They release their contents ('GRAs') into the parasitophorous vacuole upon invasion into a host cell and are believed to help modify the vacuole to make it better suited for growth of the parasites within. One of the most abundant constituents of these granules is a potent NTPase that may have a role in scavenging nucleosides from the host cell although this has yet to be directly demonstrated. At least one dense granule protein, GRA7, is present on the surface of the infected host cell although its function there is unknown. GRAs can also somehow cross the parasitophorous vacuole membrane (PVM) and interact directly with cytosolic and nuclear components of the host cell, as discussed further below.

Inner-membrane complex: This is a set of collapsed vesicles almost completely enveloping the parasite and closely apposed to the parasite's plasma membrane. Together with the latter, they make the parasite surface appear as if it consists of a triple layer of membranes. It is one of the first organelles to appear *de novo* during endodyogeny and behaves as if it were the nucleating point for formation of the two daughter parasites. The inner-membrane complex likely plays many roles, including an anchor for some of the machinery involved in 'gliding motility' of the parasite. Many components of the inner-membrane complex have been identified and shown to play key roles in endodyogeny and invasion.

Micronemes: These are numerous, thin, anterior organelles that secrete their contents ('MICs') onto the parasite's surface at the start of the invasion process. Their components appear key to motility and invasion. One microneme protein, MIC2, has been directly implicated as a way that the parasite attaches to an exterior substrate like a host cell and then pulls itself in. Another, AMA1 (the Toxoplasma orthologue of Apical Membrane Antigen 1 first identified in *Plasmodium*), has been found at the so-called 'moving junction' that forms as the parasite enters a host cell (see the section on 'invasion', below). This moving junction is a ring-shaped entity that represents the point of contact between the parasite and host cell surfaces as the parasite invades into the nascent parasitophorous vacuole. The moving junction also comprises several proteins from another secretory compartment, the rhoptries, as discussed next.

Rhoptries: These are club-shaped, apical organelles that release their contents early in the invasion process. The functions of their protein contents are just beginning to be understood and it is looking like they play several crucial roles in the parasite's interaction with the host. First, as just mentioned, the rhoptries release proteins that collaborate with AMA1 to form the moving junction. Interestingly, these rhoptry proteins all emanate from the more apical, neck-like region of the organelles, rather than the bulbous base. On the basis of this Rhoptry Neck origin, they have been designated 'RON' proteins. It appears that some RONs interact with the host cytoskeleton while at least one, RON2, protrudes out of the host cell where it can be bound by the transmembrane AMA1 embedded in the parasite surface. This interaction likely represents the means by which the parasite and host plasma membranes are maintained in such close apposition at the moving junction. Tachyzoites rely on one RON2/AMA1 pairing while sporozoites use a paralogous pair known as sporoRON2/sporoAMA1. This latter pair are encoded by distinct but paralogous genes that are turned on specifically as sporozoites develop in the oocyst. Interestingly, recent results suggest the moving junction can form without AMA1 being present indicating that the picture at the moving junction is more complex than currently understood.

Rhoptry bulb proteins ('ROPs') also appear to be injected into the host cell during invasion. Some ROP proteins are found associated with the parasitophorous vacuole membrane (PVM), some are found associated with the intravacuolar network while others are found within the host nuclei! The best studied of the ROPs are key 'effectors' that influence the interaction of the parasite with the host cell and are discussed further below.

Micropore This small, single invagination anterior to the nucleus has an apparent clathrin coat and is presumed but not yet demonstrated to be involved in endocytosis.

Acidocalcisomes These are small, membrane-limited vesicles that can be difficult to visualize but that appear to play an important role in maintaining calcium homeostasis. They are rich in polyphosphates and, as the name implies, they are acidic compartments that use proton pumps to maintain their pH.

The Lytic Cycle

Toxoplasma is an obligate intracellular parasite. As such, and given that it causes lysis of the host cell after going through several rounds of replication, the classic virology term, 'lytic cycle' is perfectly suited to describing the various stages of intracellular growth. The cycle begins with attachment followed by invasion, replication (including vacuolar modification), and egress (Figure 3).

Attachment

Taking all the data together, the most likely model for attachment involves at least two steps. First, a resident surface molecule, perhaps one or more of the glycolipid-anchored surface antigens, may interact with as yet unidentified host ligands. This part of the model is based on studies showing that antibodies to the major surface antigen, P30 or SAG1, can block binding of parasites to host cells (fixed or live) and thus prevent invasion. The putative ligand on the host cell side is likely to be something that is nearly ubiquitous as *Toxoplasma* is remarkable for its ability to invade almost any vertebrate cell it encounters, at least *in vitro*.

Following such initial interactions, a signal is sent which results in release of additional molecules involved in invasion, including the micronemal MIC2 and AMA1 proteins, mentioned above. MIC2 is a homologue of the better studied 'thrombospondin-related anonymous protein' (TRAP) molecule of *Plasmodium* sp. TRAP and MIC2 have domains whose sequence suggests an ability to bind glycosaminoglycans (GAGs). GAGs and other proteoglycans have been implicated as key ligands on the host side.

Electrophysiological studies have provided further insight into this initial attachment stage. They show that there is a brief, transient spike of conductance across the host cell plasma membrane suggesting that there is a profound perturbation of the integrity of the host cell plasma membrane associated with the initial attachment. The significance and role of this event are not yet known, although it is tempting to speculate that it somehow is connected to the process of introducing ROP proteins into the host cell; the perturbation seems too brief to reflect the introduction itself but it could be related to the process that does this.

Invasion

Invasion is an active process involving many parasite functions and is accompanied by the sequential discharge of several distinct intracellular compartments including the micronemes, rhoptries, and dense granules. The contents of these compartments serve many functions. Among these, it is presumed that some number of molecules facilitate the invasion process itself, perhaps providing the link between the host cell surface and the parasite's actin/myosin-based motors that provide the driving force of invasion. Thus, molecules such as MIC2 or AMA1 might enable the parasite to literally pull itself into the host cell, creating the moving junction described above and a nascent parasitophorous vacuole as it proceeds. Physiology studies have clearly demonstrated that the PVM is largely derived

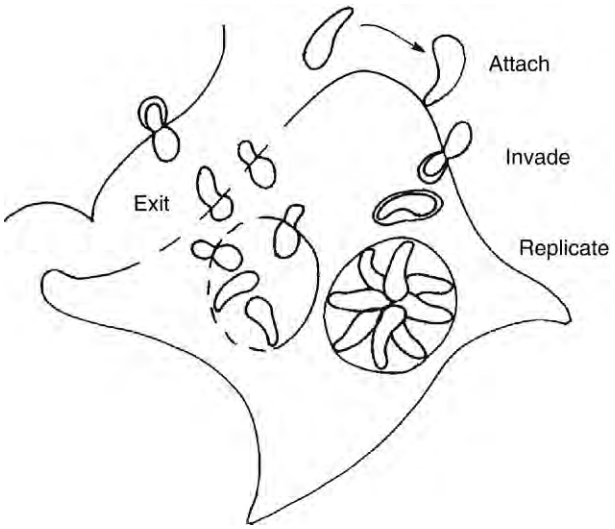

Figure 3 The lytic cycle of asexual reproduction. In this cartoon, the stages of attachment, invasion, replication, and egress are shown. Note that the host cell may have multiple vacuoles. Egress is shown here as involving both membrane destruction and active egress through semi-intact membranes. This is the least well-studied part of the lytic cycle.

from the host cell's plasma membrane. Lipid-labeling studies have shown that subsequent elaboration of the PVM and intravacuolar network also involves 'theft' of host membranes, likely endoplasmic reticulum for the most part.

Replication

The first challenge to the intracellular parasite is to create the necessary niche for optimal growth. For Toxoplasma, this requires modifying the vacuole created on invasion. The PVM appears largely devoid of host markers suggesting that during its formation, most resident proteins are removed from the invaginating host plasma membrane through some sort of molecular sieve that must exist at the circular moving junction that migrates down the parasite surface creating the PVM in its wake. Very quickly, however, parasite proteins, particularly the ROPs, begin to be detectable on the PVM. Few host proteins are ever detected, suggesting that no fusion with lysosomes or other compartments of the host endocytic pathway occurs. This is consistent with ultrastructure studies on infected cells and the fact that there is no acidification of the vacuole.

The major features of the PVM are as follows:

1. Pores arise in the PVM that are permeable to molecules of up to about 1300 Da without regard to charge. These structures, which have yet to be seen and are of unknown composition, are presumed necessary to allow the parasite to feed freely on host cell nutrients. They also result in the lack of a physiological barrier between the vacuolar space and the host cell cytosol. Thus aspects like pH, osmolarity, concentration of specific ions, and so on, should be similar if not identical between these two compartments.
2. An extensive intravacuolar network forms inside the parasitophorous vacuole (PV). This is a loose meshwork of tubules of unknown composition and function. The IPN has been reported to be continuous with the PVM suggesting it may be used to increase the effective surface area of the PVM, although there are no data to show that this is physiologically the case. It is rich with dense granule proteins.
3. Host cell organelles such as mitochondria and endoplasmic reticulum are recruited around the PVM with extraordinary efficiency and uniformity. The protein responsible for mitochondrial association has been identified and dubbed MAF1 for 'mitochondrial association factor 1'. This is a dense granule protein that is somehow presented on the host cytosolic face of the PVM where it can bind to host mitochondria. Remarkably, this protein differs between strains and, as a result, not all strains show the mitochondrial association. This difference appears to have an important effect on the host cell's response to the infection, in terms of cytokines produced.

Thus, through the PV, the parasite is able to create the ideal environment for its own growth, which then occurs with considerable efficiency (e.g., a doubling time of as little as 6 h for some strains under ideal conditions *in vitro*). Division is not by simple binary fission, however. Instead, the parasite undergoes a complex process known as endodyogeny (Figure 4). This term was chosen to convey the fact that two daughter parasites develop within the mother cell, dividing up the organelles between them or creating sets of entirely new ones. Midway through endodyogeny, some organelles that are made *de novo* are found as three distinct sets, being one new set for each of the daughter cells and one old set still in the mother cell. The inner-membrane complex and the rhoptries are examples of this situation. Most organelles, however, are only ever present with a complement of at most two, for example, the nucleus, the mitochondrion, the Golgi apparatus, and the plastid, all of which divide conventionally and flow into the two daughter cells as they develop.

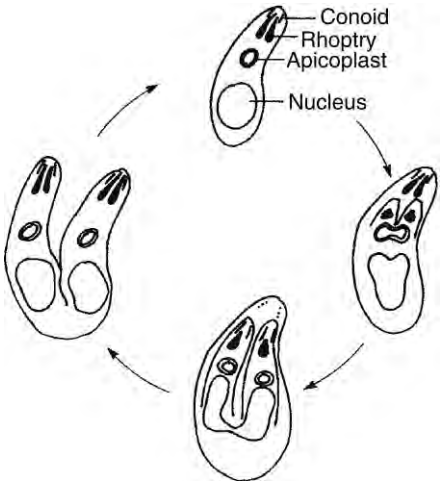

Figure 4 Endodyogeny during asexual reproduction. Endodyogeny involves the creation of two daughter cells within a mother cell. As the two daughters develop from the anterior end backward, the apicoplast and the nucleus divide, and new rhoptries, and inner-membrane and apical complexes, appear. Eventually, the mother cell's organelles, including her plasma membrane, are fully co-opted by the daughters, except for the mother's inner-membrane complex, which mysteriously disappears.

Egress

The final stage of the lytic cycle, egress, is also the least studied. The intuitively appealing notion that the parasite's replication simply fills the host cell to the bursting point is argued against by the following data:

1. Video microscopy clearly shows that the parasites become highly motile just prior to egress suggesting a signal is delivered that initiates the exit process.
2. Egress can be artificially stimulated by various treatments including adding calcium ionophores to the medium, such as A23187, or strong reducing agents, such as dithiothreitol. This effect can be achieved as soon as 30 min after invasion (i.e., when there is only a single parasite per vacuole).

The simplest explanation is that in the natural cycle, as the parasite numbers grow, a critical concentration of a key molecule in the vacuolar space is reached, which then sends a signal to initiate the cascade of events leading to egress. This molecule would likely be freely diffusible (through the pores described above), thus explaining why the total number of parasites within a given cell is important rather than the number within a given vacuole. Hence, an infected cell harboring four vacuoles each with eight parasites will be lysed at roughly the same time as one infected with a single vacuole containing approximately 32 parasites.

Population Biology

Major Genotypes

In recent years, the population biology of *T. gondii* has come under intense scrutiny through studies of isoenyzmes, restriction-fragment-length-polymorphisms (RFLP), microsatellite analysis, and direct genome sequencing. Out of these studies a very clear, consistent, but surprising picture has emerged. First, despite the existence of the well-described sexual cycle in cats, the parasite appears to be reproducing largely clonally. That is, a large fraction of strains come under one of only a few distinct genotypes. In fact, looking at samples from humans and domestic animals/livestock in Europe and North America, reveals the vast majority of strains come under one of just three genotypes (referred to as 'Type' I, II, or III, respectively).

Because *T. gondii* is haploid, this does not necessarily mean that the sexual cycle is not occurring. Instead, it could mean that the mating that does occur is almost entirely between individuals of the same genotype ('selfing'). This is not unreasonable, given that a single haploid tachyzoite can ultimately (via an infected mouse) give rise to a full sexual cycle in the cat, including genetically identical micro- and macrogametes, and thus the parasite does not have fixed mating types or sexes. Moreover, it is known that once a cat is infected, oocysts appear in the feces for about 1–2 weeks but thereafter, the cat is relatively immune to further infection and oocyst shedding decreases precipitously. Whether a cat infected with strain A and subsequently some weeks later with strain B will yield A/B recombinant progeny has not been directly examined but all available data suggest the answer will be no. These factors alone could account for the rarity of recombinants.

There is a second possible explanation for the clonal propagation: cats may play little if any role in the transmission of some strains. For example, it has been noted that Type I strains grown in the laboratory are difficult to pass in cats. As cats are the only known host in which the sexual cycle occurs, a loss of this function would require exclusively asexual expansion with transmission through carnivorism/scavenging or, perhaps, vertically in some host species.

The above could explain why most strains appear to be expanding clonally but why do most strain infecting domestic animals in North America and Europe belong to one of only three genotypes? For one of the most common protozoan parasites on earth (at least in terms of warm-blooded animals), it is hard to imagine that a major 'bottle-neck' in the population has ever occurred; that is, it seems doubtful that virtually all individuals in the *Toxoplasma* population were eliminated and only a few survivors went on to reestablish the species with their specific (chance) genotype. Instead, it appears that the three 'types' that have arisen are remarkably successful recombinants that have been selected for their ability to thrive in today's environment and that there is also a small but important population of recombinant parasites that collectively possess tremendous genetic diversity. Support for this hypothesis comes from the fact that when strains are isolated from other regions or from other species (e.g., South American wild monkeys or North American sea otters), very different genotypes can be found. Such studies have indicated the existence of at least 12 discrete lineages that represent a large pool of genetic diversity upon which the species can draw.

Strain-Specific Virulence

The three major types of this parasite found in Europe and North America are not just minor or random polymorphic states. Indeed, one of the first clues that the population biology of *T. gondii* was unusual was that the virulence of multiple different strains, when measured as the dose that resulted in a lethal infection in mice (LD_{100}) was either a single parasite or more than a thousand without a substantial middle ground. We now know that in susceptible mice, the so-called Type I parasites are highly virulent whereas Types II and III are relatively avirulent.

Recently, crosses between the three types have been used to identify some of the genes responsible for the differences in virulence. Several such genes have so far been identified and found to encode a number of polymorphic secreted proteins, including four paralogous ROPs, ROP5, ROP16, ROP18 and ROP38. ROP18 is a serine/threonine kinase that phosphorylates a set of host proteins in mice, including a group known as immunity-related GTPases or IRGs. IRGs are induced by IFNγ and normally attack the vacuole containing intracellular parasites resulting in death of the parasites within; phosphorylation, however, results in their

inactivation. ROP5 helps ROP18 by binding the IRGs, thereby maintaining them in a monomeric state and making them available for phosphorylation. There are major differences in ROP5 and ROP18 in the three canonical types of Toxoplasma and these differences account for a significant amount of their difference in virulence. ROP5 is actually encoded by a set of 4–10 tandemly repeated genes; some of these genes appear to encode the same protein, while others encode distinct versions. The result is a very diverse set of proteins within and between strains, perhaps to match the diversity of the IRGs seen in rodents (curiously, humans are among the many species that do not have IRGs of the sort discussed here).

ROP16 is a polymorphic tyrosine kinase that is also injected into a host cell. In this case the targets are STATs ('signal transducers and activators of transcription') which are key to turning the immune response up or down. Again, the three canonical types differ in the ROP16 allele they carry with a resulting difference in how the host cell responds to infection. ROP38 is another serine/threonine kinase encoded by another tandemly repeated set of genes. In this case, the target appears to be mitogen-activated protein (MAP) kinase pathways.

Dense granules also release several polymorphic 'effectors' into the host cell during and after invasion. GRA15 differs between strains and somehow influences a potent host transcription factor known as NFκB. GRA16 binds host HAUSP deubiquitinase and PP2A phosphatase to influence the key tumor suppressor p53. GRA24 binds host p38 MAP kinase, another key signaler in the host cell. These latter two GRAs do not seem to be measurably different in their effect between strains, although that could be because the experiments were not done in a host species or cell type where the differences would manifest.

The above results, especially with the strain-specific effectors, beg the question of what evolutionary pressures have led to these three canonical strains of Toxoplasma differing in virulence, at least in laboratory mice. One obvious possibility is that each strain evolved in association with a specific intermediate host (e.g., mice or sparrows) and is optimized for infection/transmission in that host species. When changes in the environment present such a strain with the opportunity to infect a new host species, it may do so but with a very different outcome from infection in its natural host. It seems unlikely, for example, that the Type I strain could have evolved as a natural infection of mice, given its extreme virulence in that species. Instead, it may have evolved with birds as its natural host and be very unsuited to infect mice. A corollary of this is that the serious consequences that result from such infections are not the result of selection because this combination of parasite strain and host species is a dead end (just as essentially all infections with *Toxoplasma* are in humans).

There is very little information on how differences in mouse virulence correlate with disease outcome in humans and livestock. The reason is that the knowledge that there are a limited number of strains is still relatively new and methods to distinguish them have been tedious and/or only recently applied. Nevertheless, some studies have been done and the data suggest that Type I and some non-canonical strains may be more virulent in otherwise healthy humans as well. In the absence of information on what strains are present in asymptomatic infections, however, and on what is the relative frequency of different types in the various animal reservoirs of human infection, it is impossible to draw any definitive conclusions about relative virulence. Determining whether strain type is an important factor in the severity of human infection is an important future goal of *Toxoplasma* researchers.

Host Immune Response

Nature of the Host Response

The innate immune system is well able to recognize Toxoplasma, at least certainly tachyzoites (on which virtually all such experiments have been performed) and presumably bradyzoites and sporozoites, as well (the latter being the first form seen by a naïve host). This recognition occurs within the first immune cells to be infected (e.g., dendritic cells and macrophages) and may be mediated by members of the toll-like receptor family such as TLR2, TLR4, TLR7 and TLR9. In mice, TLR11 and TLR12 (missing in humans) are especially important in this response. Interestingly, the TLR11 gene is defective in humans, and it has been speculated that this state is a result of evolutionary selection against an over-reaction to a Toxoplasma infection that is usually not serious. These TLR responses lead to generation of IL12 which, in turn, causes production of IFNγ from neutrophils, natural killer (NK) cells and T cells. The IFNγ is key to generating the inflammatory response necessary to contain the infection. Based on the use of antibodies that selectively deplete different T-cell subsets and/or mice that are deficient in these cells, it is clear that CD8 + cytotoxic T cells are among the most important effectors. Their action is augmented by a potent Th1-type response facilitated by CD4 + T cells. Serum antibodies likely also play some role in protecting against infection but these appear to be less important than T-cell responses, as might be expected for an infectious agent that grows exclusively within host cells.

Recent results have indicated that inflammasome activation downstream of Nod-like receptors (NLRs) is also key to the innate immune response to Toxoplasma, especially NLRP1 which is activated directly in infected cells and NLRP3 which may be a more general response to parasite-induced damage. Once triggered, the inflammasome generates IL-1β, a key cytokine that plays a central role in the inflammatory response. In addition, inflammasome activation also can drive cells into pyroptosis, a form of inflammatory death and this could be an important protective mechanism as death of the infected host cells results in death of the parasite within.

Despite these robust immune responses that effectively control the acute (tachyzoite) stage of the infection, most animals and humans appear unable to clear the infection completely. Instead, tissue cysts containing bradyzoites persist for the life of the host in many parts of the body but most notably the brain, perhaps due to the very different nature of the immune response in this organ as well as the fact that the bradyzoites are within a cyst within a neuron and are antigenically distinct from tachyzoites. All these factors likely explain their refractoriness to immune clearance.

Genetics of Host Susceptibility

There is some information to indicate that in mice, at least, there is a genetic component that influences the outcome of infection. C57Black/6 mice are especially prone to an overexuberant immune response to oral infections by Type II parasites. The reasons why this particular combination of route of infection and host and parasite genotype results in such severe disease are not known but other experiments have revealed that the key T-cell epitope in Type II parasites comes from a particular allele of GRA6 that is only recognized by major histocompatibility complex (MHC) class I H2-d whereas C57Black/6 mice are H2-b haplotype. Thus, in addition to the strain-specific, biochemical impact of the polymorphic effectors described above (ROP16, GRA15, etc.), particular pairings of polymorphic parasite antigens and host antigen-presentation molecules also influence the progress of an infection.

In humans, there are also data to suggest that MHC plays a role in infection, at least in the context of toxoplasmosis in AIDS patients, but the effect is relatively small and the basis of the effect is unknown.

Immunization Studies

Immunization with crude or purified antigens (e.g., SAG1) or with attenuated strains (e.g., the temperature-sensitive ts4 mutant) can elicit a protective response. Efforts have also been made to use a mutant strain that is unable to complete the sexual cycle in cats. It generates a good immunity in the cat while not producing oocysts and would thus be relatively safe. The best studied attenuated strain was generated by deletion of the gene for carbamoyl phosphate synthase (*CPS*), resulting in uracil auxotrophy (i.e., *cps* mutants cannot grow unless excess exogenous uracil is added to the medium and so, while they can be grown *in vitro*, they will not productively infect an animal). None of these approaches or strains, however, has yet been developed for commercial use.

A commercial vaccine does exist based on the S48 strain, which was serially passed 3000 times through mice. This strain will produce an infection in sheep, which, once cleared, results in an immunity that reduces the problems associated with subsequent infection during pregnancy of the ewes (i.e., abortion). The strain apparently is completely cleared from the infected animals with no tissue cysts developing. Thus vaccination with this strain should not represent a problem if the sheep is subsequently slaughtered for consumption as no transmission to humans should be possible. Likewise, death of the vaccinated sheep in the field should not result in the strain entering the food chain through being ingested by scavengers.

Effect on Behavior

Experiments in rodents have clearly established that a chronic infection, which includes development of tissue cysts within the brain, can result in a marked change in behavior. These changes include increased activity that may make the animals more likely prey for cats because felines are attracted by motion of the prey. Even more intriguingly, several laboratories have reported that infected rats lose their innate fear of cats as demonstrated by their willingness to go where cats have urinated. This too would have obvious evolutionary benefit to the parasite in terms of increasing the probability of transmission. Unfortunately, it is impossible to test experimentally whether this is the real reason for these phenomena but such is an enticing possibility.

The notion that human behavior might be affected in people harboring a chronic infection has not been carefully examined but there are some epidemiological hints that this might be the case. As with most human studies, however, it is extremely difficult to tease out causation from correlation and confounding variables abound in most of the studies. Hence, while an intriguing and important possibility, existing data in support of an effect of *Toxoplasma* infection on human behavior are not yet compelling.

Prospects for Future Improvements in Controlling Toxoplasmosis

Public Health

Educating the public about this disease and ways to avoid it continue to be crucial goals. The clarity of the message would be hugely helped if we better understood the relative contribution to human infection of the two different routes of transmission (undercooked meat containing bradyzoites vs. accidental ingestion of oocysts contaminating water, garden vegetables, hands, etc.). Such information would allow the public health message and control measures to be focused on whichever route proves to be the more serious risk.

Information about which *Toxoplasma* strains cause the most serious disease would also be a huge help in the clinical management of the patient (e.g., in balancing between the use of potentially toxic drugs and the likely disease outcome if the infection is left untreated). Such improvements will depend on the development of diagnostic methods that distinguish between strains and source of the infection. Serological tests that distinguish between people infected with the different 'types' of *Toxoplasma* have begun to be developed but are not yet at the point where they have proven to be clinically useful.

Vaccination

Although there can be little doubt that a human vaccine could be developed, the relative rarity of serious human disease (especially compared to the number of people infected) and the fact that such disease is largely restricted to pregnancy or AIDS make testing and use of such a vaccine extremely difficult. Nevertheless, vaccines could have a tremendously important role to play because we are dealing with a zoonosis: *Toxoplasma* is not passed from human to human but instead is acquired from animals. Thus successful vaccination of these animals would be very effective in preventing human infection. The fact that *Toxoplasma* infection in sheep is a major cause of abortion in these animals adds a commercial incentive for the farmer, which provides further motivation for development of an animal vaccine. This could take the form of a recombinant subunit vaccine based on heterologous expression of the genes for one or more of Toxoplasma's major antigens. Alternatively, the relative ease with which the parasite can be genetically manipulated makes possible the creation of a specifically engineered, attenuated mutant that would give rise to a high level of immunity without being pathogenic or transmissible from one host to another. The more traditional approach of serial passaging a strain to attenuate it has been used to create the S48 strain of *Toxoplasma* mentioned above. In the future, specifically engineered strains are likely to replace S48 because of the greater confidence that comes with knowing the attenuation is due to specific deletions that are non-reverting and can be confidently predicted to confer an attenuated state in any host.

Chemotherapy

Existing drugs are far from benign and/or are limited in their efficacy, especially against the chronic bradyzoite stage. The recent explosion in our understanding of the parasite's metabolism and cell biology, combined with concomitant improvements in the area of drug design, bode extremely well for the development of safer and more effective drugs. The fact that such drugs might also work on related Apicomplexan parasites such as *Eimeria* (the cause of chicken coccidiosis) and *Plasmodium* (the cause of human malaria) provides further incentive for efforts in this direction.

Further Reading

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Relevant Website

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Transcription Regulation in Bacteria☆

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Glossary

−10 element A consensus sequence centered about 10 bp before the start point of transcription that is involved in the initial melting of DNA by RNA polymerase.
−35 element A consensus sequence centered about 35 bp before the start point of transcription that is involved in the initial recognition by RNA polymerase.
promoter A sequence of DNA whose function is to be recognized by RNA polymerase to initiate transcription. A typical *Escherichia coli* promoter contains two conserved elements, a −10 element and a −35 element (see above).
RNA polymerase The enzyme that synthesizes RNA using a DNA template (also termed DNA-dependent RNA polymerase).
sigma (σ) factor A subunit of bacterial RNA polymerase needed for initiation. The σ factor has major influence on the selection of promoters.
start point The position on the DNA that corresponds to the first base transcribed into RNA.
terminator A DNA sequence that causes RNA polymerase to terminate transcription and to dissociate from the DNA template.
transcription The synthesis of RNA on a DNA template.
transcription factor A protein needed to activate or repress transcription but is not part of the RNA polymerase enzyme.
transcription unit The DNA sequence that extends from the promoter to the terminator; it may include more than one gene

Abbreviations

asRNA	Antisense RNA;
CAP	Catabolite gene-activator protein;
H-NS	Histone-like nucleoid structuring protein;
IHF	Integration host factor;
mRNA	Messenger RNA;
NAPs	Nucleoid-associated proteins
Nus	N-utilization substance
RBS	Ribosome binding site
RNAP	RNA polymerase
rRNA	Ribosomal RNA
sRNA	Small RNA
tRNA	Transfer RNA

Defining Statement

The first step in gene expression is the transcription of the coding DNA sequences to discrete RNA molecules. Specific DNA regions, defined as promoters, are recognized by the transcribing enzyme, a DNA-dependent RNA polymerase (RNAP). The RNAP binds to the promoter and initiates the synthesis of the RNA transcript. The enzyme catalyzes the sequential addition of ribonucleotides to the growing RNA chain in a template-dependent manner until it comes to a termination signal ('terminator'). The DNA sequence between the start point and the termination point defines a 'transcription unit'. An RNA transcript can include one gene or more. Its sequence is identical to one strand of the DNA, the coding strand, and complementary to the other, which provides the template. The base at the start point is defined as +1 and the one before that as −1. Positive numbers are increased going downstream (into the transcribed region), whereas negative numbers increase going upstream. The immediate product of transcription, which extends from the promoter to the terminator, is termed as 'primary transcript'. In prokaryotes, messenger RNA (mRNA) is usually translated concomitantly while being transcribed and is rapidly degraded when not protected by the ribosomes, whereas ribosomal RNA (rRNA) and transfer RNA (tRNA) are cleaved to give mature products and are stable.

☆*Change History:* July 2014. Govindarajan S and Amster-Choder O included an Abstract, added and updated paragraphs, which review recent findings, throughout all the sections, added and updated Further Reading and Relevant Websites, and updated Key words and Abbreviations.

This article is an update of O. Amster-Choder, Transcriptional Regulation, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 501–516.

Transcription is the principal step at which gene expression is controlled. Many factors, such as DNA signals, regulatory proteins, noncoding RNAs, and small ligands (nucleotides, metabolites), determine whether the polymerase will choose to transcribe a certain gene and whether the whole process of transcription will be accomplished successfully. The timing of transcription of specific genes is influenced by environmental conditions and by the growth cycle phase.

The molecular picture of how genes are transcribed and the nature of the regulatory mechanisms that control transcription are far from being complete, but lots of progress has been made. Work on relatively simple organisms, bacteriophage, and bacteria has provided new insights into the mechanisms that are involved in the regulation of gene expression, transcriptional control mechanisms being among them. Although there are significant differences in the organization of individual genes and in the details and the complexity of the regulatory mechanisms among prokaryotes and eukaryotes, it is clear that basic principles are shared among all organisms. Due to the relative simplicity of prokaryotic biochemical pathways, their easy manipulation in the laboratory, and the advanced tools that are available for changing their genotype and for testing the resulting phenotype, it is easier to infer these basic principles by studying prokaryotes.

The Transcription Machinery

RNA Polymerase Catalyzes Transcription

RNA synthesis is catalyzed by the enzyme RNA polymerase (RNAP). The reaction involves the incorporation of ribonucleoside 5′ triphosphate precursors into an oligoribonucleotide transcript, based on their complementarity to bases on a DNA template. RNAP catalyzes the formation of phosphodiester bonds between the ribonucleotides. The formation of a phosphodiester bond involves a hydrophilic attack by the 3′-OH group of the last ribonucleotide in the chain on the 5′ triphosphate of the incoming ribonucleotide. The incoming ribonucleotide loses its terminal two phosphate groups, which are released in the form of pyrophosphate. In this manner, the RNA chain is synthesized from the 5′ end toward the 3′ end.

Multisubunit RNAPs are remarkably conserved in fundamental structure and mechanism. The best characterized RNAPs are those of eubacteria, for which *Escherichia coli* is a prototype. Unlike eukaryotic cells, in which various types of polymerases are dedicated to the synthesis of the various types of RNA, in eubacteria a single type of polymerase appears to be responsible for the synthesis of messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA).

The dimensions of bacterial RNAP are approximately 90–95–160 Å. The molecular weight of the complete *E. coli* enzyme is approximately 465 kDa. About 7000 molecules of RNAP are present in an *E. coli* cell, but the number of molecules engaged in transcription at any given time varies from 2000 to 5000, depending on the growth conditions.

The DNA sequence that is being transcribed by RNAP is transiently separated into its single strands, with one of the strands serving as a template for the synthesis of the RNA strand. This region is therefore defined as the 'transcription bubble'. As the RNAP moves along the DNA, it unwinds the duplex at the front of the bubble and rewinds the DNA at the back, so that the duplex behind the transcription bubble reforms. Thus, the bubble moves with the RNAP, and the RNA chain is elongated. The length of the transcription bubble varies from 12 to 20 bp. The length of the transient hybrid between the DNA and the newly synthesized RNA sequence within the transcription bubble is a matter of controversy and the estimates range from 2 to 12 nt. Beyond the growing point, the newly synthesized RNA chain enters a high-affinity binding site within the RNAP.

Bacterial RNA Polymerase Consists of Multiple Subunits

The RNAPs of certain phage consist of single polypeptide chains. These polymerases recognize a very limited number of promoters and they lack the ability to change the set of promoters from which they initiate transcription. In contrast, bacterial RNAPs consist of several subunits. The most studied bacterial RNAP, the one in *E. coli*, exists in two forms, an holoenzyme and a core enzyme. The holoenzyme is capable of selective initiation at promoter regions, whereas the core RNAP is capable of elongation and termination, but not selective initiation. The two forms of the polymerase consist of two identical α-subunits, one β-subunit, and one β′-subunit, but only the holoenzyme contains an additional subunit, one of the several σ proteins. The subunit composition of the holoenzyme is summarized in Figure 1. The α-subunit plays an important role in RNAP assembly, which proceeds in the pathway $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta\beta' \rightarrow \alpha_2\beta\beta'\sigma$. The α-subunit plays some role in promoter recognition and in the interaction of RNAP with transcriptional activators (see later). The β and β′ subunits together constitute the catalytic center of RNAP. The β-subunit was demonstrated to contact the template DNA, the newly synthesized RNA, and the substrate ribonucleotides. The β′-subunit contacts the RNA chain as well. Mutations in the genes that encode β and β′ show that both subunits are involved in all stages of transcription. The sequences of β and β′ show homology to the sequences of the largest subunits of eukaryotic RNAPs. This conservation through the evolution hints that the mechanisms by which all RNAPs catalyze transcription share common features. In accord, high-resolution structural studies on the core enzyme show that it adopts a certain structure, which is similar to the structure that is found in the yeast RNAP II. The assignment of individual functions to the different subunits of the core polymerase is only a rough estimation because most probably each subunit contributes to the activity of the enzyme as a whole. In addition, a small 91-residue protein, termed the ω subunit, associates with the polymerase. It has no direct role in transcription, but seems to function as a chaperone to assist the folding of the β′ subunit.

The σ subunit has three main functions: to ensure the recognition of specific promoter sequences; to position the RNAP holoenzyme at a target promoter; and to facilitate unwinding of the DNA duplex near the transcript start site. Based on recent studies, 'σ' subunits are involved also in other aspects of transcription initiation, such as abortive initiation and promoter escape.

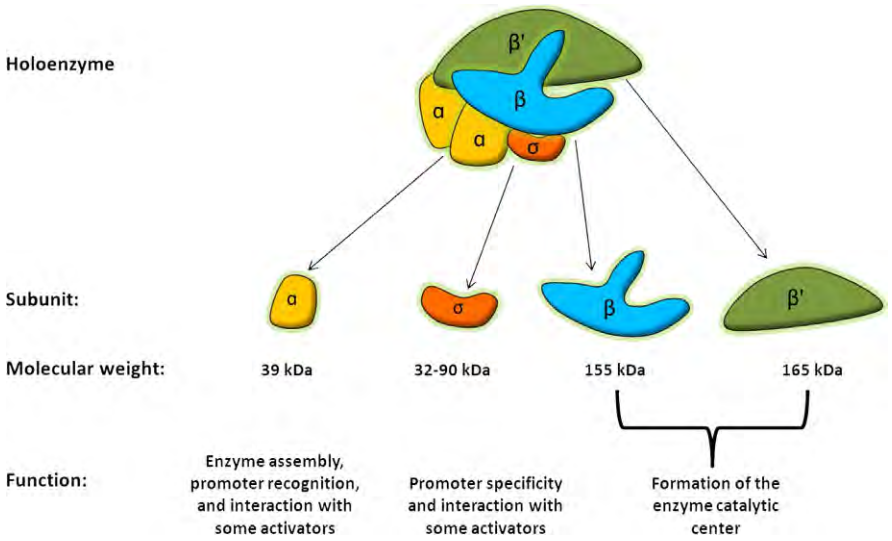

Figure 1 *E. coli* RNA polymerase holoenzyme consists of four types of subunits.

There are several types of σ factors in the bacterial cell (see later). The major σ factor in *E. coli*, which is required for most transcription reactions, is σ^{70}. Although σ^{70} has domains that recognize the promoter DNA sequence, as an independent protein, it does not bind to DNA, perhaps because its DNA-binding domain is sequestered by another domain of the σ^{70} molecule (when σ^{70} is shortened from its N-terminus, it is able to bind to DNA, suggesting that the N-terminal region has an inhibitory effect on the ability of σ^{70} to bind to DNA). However, upon binding to the core enzyme and the formation of the holoenzyme complex, σ^{70} undergoes a conformational change and it now contacts the region upstream of the start point. The dogma is that the σ factor discharges from the core enzyme when abortive initiation is concluded and RNA synthesis is successfully initiated (although findings suggest that, at least in some cases, σ can remain associated with the polymerase during postinitiation steps). The released σ factor becomes immediately available for use by another core enzyme, although the activity of many σ factors is controlled by an anti-σ factor, which sequesters it away from the RNAP. *E. coli* cells contain about 3000 molecules of σ^{70}, enough to bind about one-third of the intracellular core RNAP.

The Ability of RNA Polymerase to Selectively Initiate Transcription is Dependent on the Presence of σ Factor

Bacterial polymerases have to recognize a wide range of promoters and to transcribe different genes on different occasions. Specificity of gene expression is in part modulated by substituting one species of σ for another, each specific for a different class of promoters. In *Bacillus subtilis*, σ factors are implicated in the temporal regulation of sporulation. In *E. coli*, alternative σ factors are used to respond to general environmental changes. The σ factors are named either by their molecular weight (e.g., σ^{70}) or after their genes, which are usually termed 'rpo' (e.g., RpoD for σ^{70}). Sigma factors are subdivided into σ^{70} class or σ^{54} class on the basis of homology as well as the mechanism of action. Members of the σ^{70} class are active immediately upon binding to the core RNAP and to the DNA, and are regulated either by modulating the amount of expression or by the action of anti-sigma factors. Members of the σ^{54} class require an additional ATP-dependent activation event provided by one of several AAA^+ ATPase transcriptional activators and are, therefore, subjected to an additional level of regulation.

When cells are shifted from low to high temperature, the synthesis of a small number of proteins, the heat-shock proteins, transiently increases. The σ^{32} protein (RpoH) is responsible for the transcription of the heat-shock genes. The basic signal that induces the production of σ^{32} is the accumulation of unfolded proteins that results from the increase in temperature. The heat-shock proteins play a role in protecting the cell against environmental stress. Several of them act like chaperones, preventing the unfolding (denaturation) of proteins. The heat-shock proteins are synthesized in response to other conditions of stress, implying that the production of σ^{32} is induced also by conditions other than elevation in temperature. The level of σ^{32} is strictly controlled by various mechanisms. First, σ^{32} protein is unstable and is rapidly degraded when it is not needed. Second, the mRNA of σ^{32} forms a secondary structure that represses translation, but at higher temperatures, that is during heat shock, the secondary structure of the mRNA is relieved resulting in translation of σ^{32}. Third, σ^{32} is regulated by phosphorylation. Finally, σ^{32} was recently shown to localize to the inner membrane of *E. coli* cells, and this localization was found to be important for its proper regulation in response to the protein folding status in the cell during heat shock conditions. Another σ factor, σ^{E}, appears to respond to more extreme temperature shifts that lead to the accumulation of unfolded proteins, which are usually found in the periplasmic space or the outer membrane. Less is known about this σ factor and about the genes it controls.

When *E. coli* and other enteric bacteria are nitrogen-limited, the synthesis of a number of proteins is dramatically induced. The increased production of these proteins enlarges the capacity of cells to produce nitrogen-containing compounds, and to use nitrogen sources other than ammonia. The transcription of the genes that encode these proteins is dependent on σ^{54} (also known as σ^{N}). σ^{N}

is also involved in nitric oxide stress response. Expression of the flagellar genes under normal conditions depends on σ^{28} (also known as σ^{F}). σ^{19} (FecI) is a minor sigma factor that regulates expression of genes involved in transport of ferric citrate.

When *E. coli* cells are starved, they shift from the exponential-growth phase to the stationary phase. The ability of the cells to cope with starvation depends on the production of many proteins. This is enabled due to the synthesis of σ^{S} (RpoS), which transcribes the relevant genes. Similar to σ^{32}, σ^{S} is also subjected to multiple levels of regulation, which involve proteolysis, small RNAs and small molecules. Unlike *E. coli*, when *B. subtilis* cells are starved, they can form spores. Sporulation involves the differentiation of a vegetative bacterium into a mother cell and a forespore; the mother cell lyses and a spore is released. The process of sporulation involves a drastic change in the biosynthetic activities of the bacterium, in which many genes are involved. This complex process is concerted at the level of transcription. The principle is that in each compartment (the mother cell and the forespore) the existing σ factor is successively displaced by a new σ factor that causes the transcription of a new set of genes. Communication between the compartments occurs in order to coordinate the timing of the changes in the mother cell and the forespore.

The promoters identified by the various σ factors are organized similarly, having their important elements centered around 10 and 35 nt upstream from the start point (see later), except for σ^{54}, whose promoters have slightly different characteristics. However, the ability of different σ factors to cause RNAP to initiate at different sets of promoters stems from the fact that each type of σ factor recognizes promoter elements with unique sequences. σ^{54} differs from the other σ factors also in its ability to bind to DNA independently, and by the influence of sites that are distant from the promoter on its activity. In many aspects, σ^{54} resembles eukaryotic regulators.

A comparison of the sequences of the different σ factors identifies four regions that have been conserved. Several sequences in these regions were identified with individual functions, such as interaction with core RNAP or contacting the various promoter elements. Recent structural information shows that two key σ domains are structurally conserved, even among diverse family members.

Template Recognition: Promoters

Promoter Recognition Depends on Conserved Elements

Template recognition begins with the binding of RNAP to the promoter. Promoter is a sequence of DNA whose function is to be recognized by RNAP to initiate transcription. The information for promoter function is provided directly by the DNA sequence, unlike expressed regions, which require that the information be transferred into RNA or protein to exercise their function. Two main approaches were used to identify the DNA features that characterize a promoter. The first is comparative sequence analysis and the second is the identification of mutations that alter the recognition of promoters by RNAP. Comparison of the sequence of many *E. coli* promoters recognized by the major RNAP species, σ^{70} holoenzyme, revealed an overall lack of extensive conservation of sequence over the 60 bp associated with RNAP. Nevertheless, statistical analysis revealed some commonalities. A typical *E. coli* promoter that is recognized by σ^{70} contains four conserved features: the start point, the −10 region, the −35 region, and the distance between the −10 and −35 regions. The start point is usually a purine. It is often the central base in the sequence CAT, but this is not a mandatory rule. The −10 region is a hexanucleotide that centers approximately 10 bp before the start point, although this distance is somewhat variable. Its consensus sequence is TATAAT (in the antisense strand). The conservation is T80 A95 T45 A60 A50 T96, where the numbers refer to the percent occurrence of the most frequently found base at each position. The −35 region is a hexanucleotide sequence that centers approximately 35 bp upstream of the start point. Its consensus is TTGACA and the conservation is T82 T84 G78 A65 C54 A45. The favored spacing between the −10 and the −35 sequences is 17 bp.

For most promoters, there is a good correlation between promoter strength and the degree to which the −10 and −35 elements agree with the consensus sequences. The significance of the conserved promoter features was further emphasized by the finding that most mutations that alter promoter activity (i.e., affect the level of expression of the gene(s) under the control of this promoter) change the sequence of the particular promoter in an expected fashion. Mutations that increase the similarity to the proposed conserved −10 and −35 sequences or bring the spacing between them closer to 17 bp, usually, enhance the promoter activity ('up mutations'); mutations that decrease the similarity to the conserved sequences or bring the spacing between them more distant than 17 bp, usually, reduce the promoter activity ('down mutations'). The nature of down mutations in the −35 and −10 regions of various promoters led to the conclusion that the −35 region is implicated in the recognition of the promoter by RNAP and the formation of a closed transcription complex, whereas the −10 region is implicated in the shift of the closed complex to the open form (see later). The fact that the −10 region is composed of AT base pairs that require low energy for melting makes it suitable to assist in unwinding and, thus, in converting the transcription complex into its open form.

There are several exceptions to the proposed generalized pattern. For example, some promoters lack one of the conserved sequences, the −10 or the −35 region, without a corresponding effect on promoter activity. In some cases, it was proposed that another sequence compensates for the lack of a consensus sequence. In still other cases, it was concluded that the promoter cannot be recognized by RNAP alone, and the involvement of additional proteins that overcome the deficiency in intrinsic interaction between RNAP and the promoter is required. Other exceptional promoters were discovered due to the isolation of promoter mutations that do not affect any of the conserved promoter features described so far. Rather, they are the outcome of base substitutions in other sequences in the vicinity of the conserved sequences or the start point. One explanation for these findings is that the analysis that generated the sequence characteristics of a typical promoter may have missed some sites that contribute to the transcription initiation process, possibly because it included too many promoters, both weak and strong. Alternatively, other

base pairs in the promoter could be recognized by RNAP, but might become significant only if canonical recognition sites are absent.

The isolation of deletions that progressively approach specific promoters from the upstream region demonstrated the involvement of specific upstream sites in the recognition of RNAP in some cases. This led to the discovery that some *E. coli* promoters contain a third important element in addition to the −10 and −35 sequences. This element was named the upstream element, or 'UP element', because it is located approximately 20 bp upstream of the −35 region. Its sequence is AT-rich and it was first identified in the strong promoters of the *rrn* genes, which encode rRNA. It is believed now that promoter strength is a function of all three elements, −10, −35, and UP, with very strong promoters, such as the *rrn* promoters, having all three elements with near-consensus sequences and with weaker promoters having one, two, or three nonconsensus promoter elements. It has been found that, whereas −70 is responsible for the recognition of the −10 and −35 regions, the UP element interacts with the α-subunit of RNAP.

Finally, the activity of some promoters is affected by sequences downstream to the −10 region, or even downstream to the transcription start point. The sequences immediately around the start point seem to influence the initiation event. The effect of the initial transcribed region (from +1 to +30) on promoter strength is explained by the influence of this region on the rate at which RNAP clears the promoter. Unlike the promoters described so far, which are recognized by holoenzymes that contain σ^{70} or a close homologue, a minority of the cellular holoenzymes use σ^{54} and have different basal elements located at −12 and −24. Transcription initiation from these promoters relies also on enhancer-like elements that are remote (upstream) from the promoters (see later).

Possible Mechanisms of Promoter Recognition

A typical *E. coli* cell contains approximately 3000 promoters and, as mentioned above, the number of RNAP molecules engaged in transcription at any given time varies from 2000 to 5000. How does RNAP find the promoter sequences? How does it identify a stretch of approximately 60 bp that defines a promoter in the context of 4×10^6 bp that make up the *E. coli* genome? Several models were suggested to explain the ability of RNAP to find promoters. The first model assumes that RNAP moves in the cell by random diffusion. It associates and dissociates from loose binding sites on the DNA until, by chance, it encounters a promoter sequence that allows tight binding to occur. According to this model, movement of an RNAP molecule from one site on the DNA to another is limited by the speed of diffusion through the medium. However, this parameter might be too low to account for the rate at which RNAP finds promoters. The second model addresses this problem by assuming that once an RNAP molecule binds to a DNA sequence, the bound sequence is directly displaced by another sequence; the enzyme exchanges sequences very rapidly, until it binds to a promoter that allows an open complex to form and transcription initiation to occur. According to this model, the association of the polymerase with DNA sequences and their dissociation are essentially simultaneous. Thus, the time spent on site exchange is minimal and the search process is much faster in comparison to the speed calculated based on the first model. This model fits the accepted notion that core polymerases that are not busy in transcription are stored by binding to loose sites on the DNA. The third model assumes that RNAP binds to a random site on the DNA and starts sliding along the DNA molecule until it encounters a promoter. Recently promoter recognition by RNAP in *E. coli* was suggested to involve a mechanism called 'three dimensional (3D) diffusion or jumping'. According to this model, free RNAP molecules in the solution search the promoter by 3D collisions with the DNA. This model takes into consideration the folding and compaction of bacterial chromosomes, which bring different genomic regions into close proximity. Of note, the presence of ample DNA-bound proteins might limit the 1D movement of RNAP in search for promoters, but not the 3D diffusion, making the 3D diffusion model an attractive one. However, the actual mechanism by which RNAP finds promoters might combine features of the various models.

Transcription Initiation

The activities of genes are frequently regulated at the initiation step of transcription. Therefore, the initiation of transcription is a very precise event that is tightly controlled by various regulatory mechanisms. Studying transcription initiation in *E. coli* has served as a model for understanding transcriptional control throughout all kingdoms of life.

Stages of the Transcription Initiation Process

Transcription initiation is the phase during which the first nucleotides in the RNA chain are synthesized. It is a multistep process that starts when the RNAP holoenzyme binds to the DNA template and ends when the core polymerase escapes from the promoter after the synthesis of approximately the first nine nucleotides.

The stages of the transcription initiation process, which are summarized in Figure 2, can be described in terms of the types of interaction between the RNAP and the nucleic acids that are involved. The first stage in transcription initiation is the formation of a complex between the holoenzyme and the DNA sequence at the promoter, which is in the form of a double-stranded DNA. This complex is termed a closed binary complex or *closed complex*. The second stage is the unwinding of a short region of DNA within the sequence that is bound to the RNAP. The complex between the polymerase and the partially melted DNA is termed an open binary complex or *open complex*. The conversion of the closed complex into the open complex leads to the establishment of tight binding between the RNAP and the promoter sequence. For strong promoters, the conversion into an open complex is irreversible. The third

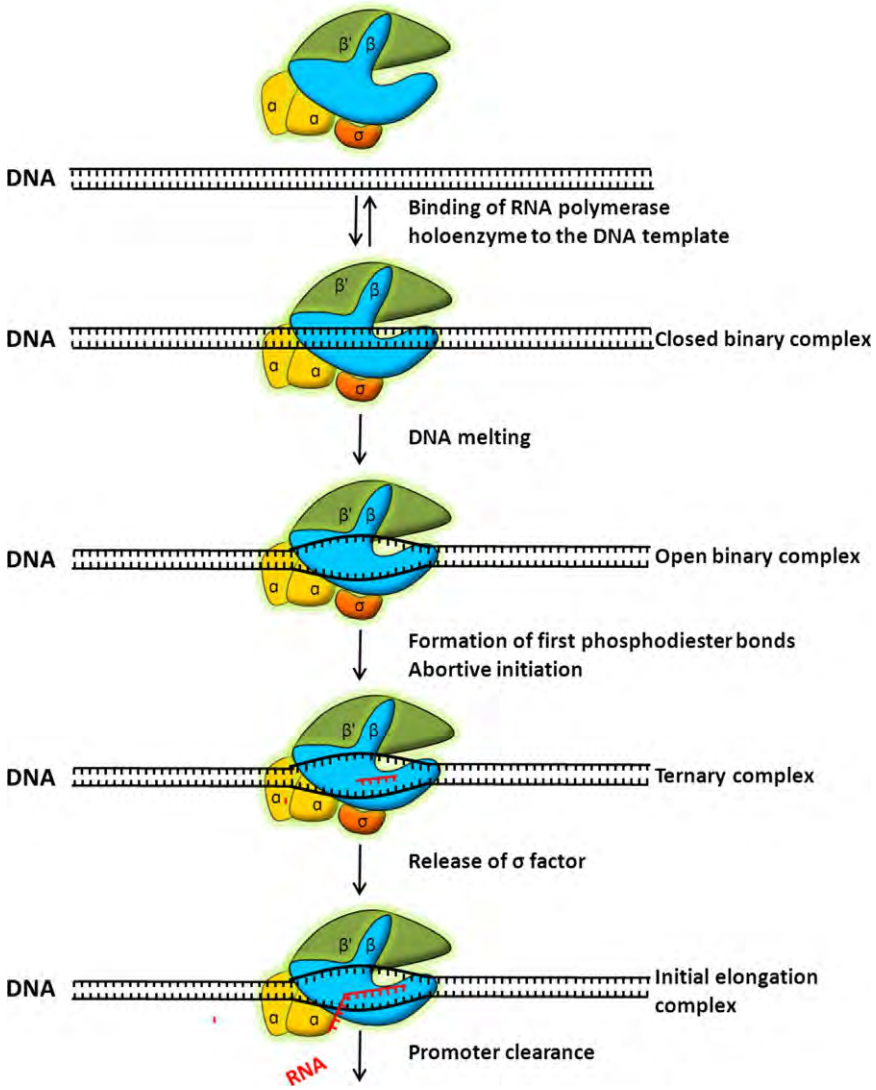

Figure 2 Stages of the transcription initiation process.

stage is the incorporation of two ribonucleotides and the formation of a phosphodiester bond between them. Because the complex at that stage contains an RNA, as well as DNA, it is called an initiation *ternary complex*. Up to seven additional ribonucleotides can be added to the RNA chain without any movement of the polymerase. After the addition of each base, there is a certain probability that the enzyme will release the short (up to nine bases long) RNA chain. Such an unsuccessful initiation event is termed an 'abortive initiation'. Following an abortive initiation, RNAP begins again to incorporate the first base. Several rounds of abortive initiations usually occur and the result is the formation of short RNA chains that are 2–7 bases long. When initiation succeeds, that is, a nine-base-long RNA chain is formed and is not released, the last stage in transcription initiation occurs. At that stage the σ factor is released from the polymerase. As a consequence, a complex containing core polymerase, DNA, and RNA is formed. This complex is called an elongation ternary complex. The departure of the polymerase from the promoter to resume elongation is termed promoter escape or *promoter clearance*. Recently, nanoRNAs, which are 2 to 4 nucleotide long, were found to be important for priming of transcription initiation in vivo. These RNAs provide a basis for transcription start site selection and gene expression.

Transcription Factors

Regulation of transcription involves a complex network, where DNA-binding proteins, termed transcription factors, are a key component. Transcription factor is a protein needed to activate or repress the transcription of a gene, but is not itself a part of the enzyme. Some transcription factors bind to *cis*-acting DNA sequences only; some bind to each other; others bind to DNA as well as to other transcription factors. When a transcription factor binds to a specific promoter, it can either activate or repress transcription

initiation. Some transcription factors function solely as activators or repressors, whereas others can function as either according to the target promoter.

The *E. coli* genome contains more than 300 genes that encode proteins that are predicted to bind to promoters, and to either up- or down-regulate transcription. So far, about half of these have had their functions verified experimentally. Some of these proteins control large numbers of genes, whereas others control just one or two genes. It has been estimated that seven transcription factors (CRP, FNR, IHF, Fis, ArcA, NarL, and Lrp) control 50% of all regulated genes, whereas ~60 transcription factors control only a single promoter. Bacterial transcription factors can be grouped into different families on the basis of sequence analysis. So far, a dozen families have been identified, the best characterized of these being the LacI, AraC, LysR, CRP, and OmpR families. Because a large percentage of the polymerase molecules are occupied with transcription of ribosomal genes, transcriptional regulation of these genes is intensively studied. It has been shown that, in addition to σ^{70}-RNAP, the Fis and H-NS proteins bind near the upstream P1 promoter of the seven ribosomal transcription units and regulate transcription.

The activity of most bacterial promoters is dependent on multiple environmental cues, rather than just one signal. In most cases, for a promoter to respond to multiple signals, multiple transcription factors are required. Accordingly, many promoters are controlled by two or more transcription factors, with each factor relaying one environmental signal. However, in some cases a regulatory protein can 'integrate' multiple signals, like the NifL–NifA system in *Azotobacter vinelandii*, which controls the genes that are involved in nitrogen fixation in response to oxygen levels and the availability of carbon and nitrogen. There are a few examples of integrated regulation that are solely dependent on repressors, but, in most examples studied so far, complex regulation depends on combinations of repressors and activators or codependence on more than one activator.

Repression of Transcription Initiation

Gene expression is sometimes negatively regulated by a repressor protein that, when bound to DNA, inhibits transcription initiation. The ability of the repressor to bind to DNA is in turn modulated by the binding of an effector molecule to the repressor. The regulation of the *lac* operon expression in *E. coli* is a paradigm for this type of transcriptional control. LacI is a repressor protein that blocks the initiation of transcription from the promoter of the *lac* operon. LacI binds to a site on the DNA, termed an *operator*, that overlaps with the promoter. Because of this overlap, the binding of the LacI repressor and of RNAP are competitive events. That is, RNAP cannot bind to the promoter until the repressor is removed from the operator. The binding of one of several β-galactoside compounds to the repressor destabilizes the repressor–operator complex and allows RNAP to bind to the promoter to initiate transcription. Interestingly, the *lac* operon has two additional binding sites for LacI, an upstream site and a downstream site, located in the first gene of the operon. Compared to the operator, the additional sites have a lower affinity for the repressor protein and it was suggested that they do not directly participate in the inhibition of transcription initiation. Rather, the secondary binding sites seem to stabilize the repressor–operator complex.

There are important exceptions to the *lac* repression paradigm. An example is the repression of the *gal* operon transcription initiation by a different and less understood mechanism. The *gal* operon contains two repressor-binding sites, both required for maximum efficacy of the *gal* repressor, yet neither of these operators overlaps with the promoter sequences. Thus, the binding of the *gal* repressor to its operators does not seem to compete directly with binding of RNAP to the *gal* promoter. It was suggested that the *gal* repressor, when bound to both binding sites, holds the DNA in a conformation that is unfavorable for binding to RNAP.

Activation of Transcription Initiation

The frequency of transcription initiation from many promoters is enhanced by activator proteins. Activators improve the performance of a promoter by improving its affinity for RNAP. In most cases, they bind within or upstream from the promoter and make a direct contact with RNAP. The activators that interact with the most abundant form of RNAP involved in transcription initiation in *E. coli*, the σ^{70} holoenzyme, can be roughly divided into two groups, those that interact with the α-subunit of RNAP and those that interact with the σ^{70} subunit.

The best characterized activator from the first group is the catabolite gene-activator protein (CAP). The CAP target site on the DNA was determined in a number of systems. Comparative sequence analyses led to the definition of a consensus sequence for CAP binding. CAP-binding sites are found at various locations relative to the transcription start point in different systems. The most studied case is the activation of transcription initiation from the *lac* promoter by CAP. The regions that are required for the activation on both CAP and the α-subunit of RNAP were defined. CAP acts as a dimer, and although the activating region is present in both subunits of the CAP dimer, transcription activation at the *lac* promoter requires only the activating region of the promoter-proximal subunit. CAP interacts with the C-terminal domain of the α-subunit (α CTD). The α CTD constitutes an independently folded domain, which is connected to the remainder of α by a flexible linker. This allows α CTD to make different interactions in different promoters. The simplest model for transcription activation by CAP is that CAP binds to the DNA and recruits the α CTD, and thus the RNAP holoenzyme, to the promoter.

The best characterized activator from the second group is the cI protein of bacteriophage λ (λcI). λcI binds to a site on the DNA that overlaps the −35 element of the λP_{RM} promoter. The activating region in λcI was defined and was demonstrated to directly contact a specific region in σ^{70}.

The existence of at least two groups of activators that bind to separate targets on the DNA and to different components of RNAP raised the possibility, which was later proven, that, at some promoters RNAP might be contacted simultaneously by two or more

activators. Generally, where two transcription factors are involved, one factor interprets a global metabolic signal, whereas the other responds to a specific metabolic signal. The best illustration of this is the *E. coli lac* promoter, which is regulated by CRP that depends on a global signal, glucose starvation, and by the Lac repressor, which is controlled by a specific metabolite, allolactose. In most cases, multiple activators bind independently to their target promoters. However, there are a few promoters at which activators bind cooperatively.

In contrast to the copious σ^{70} promoters, the rare σ^{54} promoters, which contain -12 and -24 basal elements instead of the well-known -10 and -35 elements, seem to be regulated solely by activation rather than by repression. σ^{54} activators (the most studied is NtrC) bind to enhancer-like sites on the DNA; that is, the sites are remote from the promoters (upstream) and their precise location is not critical for transcriptional activation. In fact, these sites can be moved kilobases in *cis* and retain their residual function. Thus, unlike σ^{70} activators that bind to sites that enable direct communication with RNAP, σ^{54} activators, once bound to their DNA target sites, cannot touch the polymerase without looping out the intervening DNA. This seems to be the reason why σ^{54} promoters frequently require the help of integration host factor (IHF), which enhances the bending of the DNA, as a cofactor. It is accepted that σ^{54} polymerase can bind to its promoters to form a closed complex. However, this polymerase cannot transcribe because it cannot melt the DNA. Once the upstream activator binds to its target site upstream of the promoter, it loops out of the sequence between its binding site and the promoter and touches the complex. This interaction triggers the melting of DNA (with the help of a helicase activity) and the creation of a transcription bubble. Thus, σ^{54} activators catalyze the conversion of the polymerase–promoter complex from a closed state to a transcription-ready open state, rather than tethering the RNAP to the promoter.

One conclusion from studies with various types of activators is that many activators seem to function by helping recruit DNA-binding domains of RNAP to DNA, thus supplementing suboptimal RNAP–DNA interactions with protein–RNAP interactions. Apparently, most activators function by binding to target promoters before acting on RNAP. However, an alternative mechanism has been proposed for the MarA and SoxS regulators, in which they interact with free RNAP before binding to promoter DNA. In most cases, multiple activators bind independently at their target promoters. However, there are a few promoters at which activators bind cooperatively.

Small Ligands

Small ligands provide an alternative mechanism by which RNAP can respond quickly and efficiently to the environment. The best example is guanosine 3′,5′ bisphosphate, ppGpp, and also pppGpp, which are synthesized when amino acid availability is restricted to the extent that translation is also limited. Transcription of the seven transcription units that encode the rRNAs is mostly affected by the levels of ppGpp. A crystal structure shows that ppGpp binds near the catalytic centre of RNAP. This location might allow it to alter interactions with the incoming nucleoside triphosphates, with the catalytic magnesium and perhaps with the non-template DNA strand to destabilize open complexes. ppGpp has a co-regulator, the polymerase-binding DksA protein, which lowers the concentration of ppGpp needed to inhibit transcription. DskA exaggerates the effects of ppGpp by adapting the polymerase for regulation at promoters that are affected by the nucleotide. ppGpp also favors the association of other sigma factors with core polymerase at the expense of the σ^{70} holooenzyme that transcribes the ribosomal promoters. It has been proposed that ppGpp controls expression of the translation machinery in response to sudden starvation, whereas ATP availability controls expression in response to growth rate.

For obvious reasons, rRNA transcription is subject to complex regulation. Hence, in addition to the levels of ppGpp, the promoters of these genes respond to other factors, for example, they show an unusually high affinity for the first two nucleotides that form the 5′ end of the ribosomal transcript. This property, which can be explained by the unusual features of the ribosomal promoters, allows selective inhibition of rRNA transcription when nucleotide concentrations decrease, as is the case in stationary phase. The net amount of rRNA transcription is a function of the ratio of inhibitors to activators – H-NS/Fis (see section 'Transcription factors') and ppGpp/NTP. Both of these ratios are usually highest during slow growth and lowest during rapid growth.

Small RNAs

A subset of small RNAs has been found to regulate transcription in bacteria. One example is the abundant 6S RNA that inhibits transcription at many σ^{70}-dependent promoters during stationary phase by binding to the active site of σ^{70}-RNAP and competing for DNA binding. The conserved secondary structure of 6S RNA, a single-stranded central bulge within a highly double-stranded molecule that is essential for 6S RNA function, has led to the proposal that 6S RNA mimics the open conformation of promoter DNA. Not only does 6S RNA block access to promoter DNA, but, surprisingly, it is used as a template for RNA synthesis. Synthesis of the templated RNA relieves the inhibitory effect of 6S RNA when cells encounter new nutrient sources and resume growth.

Recently, a new subset of RNAs, termed transcription start site-associated RNAs (tssRNAs), was identified in *Mycoplasma*, as well as in *E. coli*. The average size of tssRNAs is approximately 45 bp and they map to the beginning of certain full length transcripts. The tssRNAs appear to be synthesized through cryptic promoters and are not formed as a result of RNA degradation or abortive transcription. Although the regulatory function of tssRNAs is currently not known, their increased synthesis during stationary phase,

a phenomenon not observed for their cognate full length mRNAs, suggests a potential role for tssRNAs in transcriptional reprogramming.

Antisense RNAs

Antisense RNAs (asRNAs) are complementary to mRNAs that are encoded by the opposite DNA strand, thus providing means for regulation. Using ultra-sequencing technologies, it was found that in *E. coli* nearly 20% of genes encode asRNAs. These RNAs are implicated in transcription initiation through a process called transcription interference, which is a phenomenon whereby transcription at one strand affects or suppresses transcription from the opposite strand. This can occur if the promoters of the sense and antisense strands face each other and the regions that encode the transcripts on the two strands overlap. Thus, when asRNA is transcribed from a strong promoter on one strand, transcription is prevented from the promoter on the opposite strand. In such a scenario, the transcription process itself acts as a regulator and the asRNA is formed as a by-product. An example for this mode of regulation is provided by the convergent lytic promoter (pR) and lysogenic promoter (pL) of coliphage 186. Recently, dual purpose asRNAs were identified, which can function both as mRNAs and as asRNA. These RNAs, termed "excludons" are unusually long, spanning the length of an entire operon and extending into neighboring operons encoded by the opposite strand, thus regulating the expression of these opposite strand-encoded operons.

Regulation of Transcription Initiation via Changes in DNA Topology and Nucleoid-Associated Proteins (NAPs)

The template for transcription is a negatively supercoiled DNA. Because the formation of an open transcription complex requires DNA melting, and because the degree of superhelicity affects the energy needed for the melting, it was anticipated that the superhelical character of a template would affect the properties of this template. Indeed, the efficiency of some promoters is influenced by the degree of supercoiling. Most of these promoters are stimulated by negative supercoiling, although few are inhibited. The effects of superhelicity on the process of transcription initiation have been shown in vitro in numerous studies and in vivo by the use of inhibitors of gyrase, which introduces negative supercoils. The reason why some promoters are sensitive to the degree of supercoiling, whereas others are not, might have to do with the fact that the sequence of some promoters is easier to melt and is therefore less dependent on supercoiling. Alternatively, because various regions on the bacterial chromosome are believed to have different degrees of supercoiling, the location of the promoter might determine whether it is sensitive to changes in superhelicity.

An additional level of transcription regulation is achieved by the organization of the nucleoid. A collection of non-specific DNA binding proteins, termed Nucleoid Associated Proteins (NAPs), organize the bacterial nucleoid into higher order conformations that are reminiscent of the eukaryotic nucleosomes. Major proteins of this category include histone-like nucleoid structuring protein (H-NS), HU and Fis. Binding of NAPs induces conformational changes in the DNA, including bending, bridging and wrapping, which are known to affect transcription. HNS, an extensively studied NAP, which is specifically involved in silencing of horizontally acquired genes, binds to AT-rich sequences and affects transcription of nearly 5% of *E. coli* genes. The interplay between the NAPs is implicated in growth phase-dependent global transcription changes. Thus, during the exponential growth phase, ribosomal genes are brought together by NAPs to form looped DNA domains. This higher order nucleoid conformation is called 'transcription factories' since actively transcribing RNAP are concentrated at these loci.

Stochasticity and Bursting Behavior of Transcription Initiation

Monitoring single cells by advanced microscopy techniques have uncovered that transcription is a stochastic process, which is subject to random fluctuations between active and inactive states. As a result, the mRNA molecules are not produced at a constant rate, but are rather randomly produced during the active state periods. Furthermore, in the active state, the initiation event happens in a burst, leading to production of several mRNA molecules and then, for unknown reasons, a switch to a quiescence inactive mode occurs. The time it takes for two consecutive bursting events, as well as the number of mRNA molecules produced from each burst are also subjected to stochasticity. Thus, stochasticity in transcription creates heterogeneity in bacterial populations, which are otherwise considered homogenous. This heterogeneity in the number of mRNA molecules in different bacterial cells within a population is further exaggerated by the non-uniform distribution of mRNA molecules during cell division. Despite these observations, the physiological significance of transcription heterogeneity is currently not understood. Also, it is not known why the transcription apparatus chooses the quiescence or inactive mode at times, given that all the factors required for transcription are constantly present in the cell.

Promoter Occlusion in Transcription Initiation

In *E. coli*, the promoter region of certain genes contains sequences specific for more than one sigma factors. Such promoters are called overlapping promoters and the regulation of transcription initiation at these promoters are poorly understood. A recent study on the regulation of transcription of the gene for the σ^S activator Crl, which contains overlapping promoter sequences specific for σ^{70} and σ^{54}, revealed a previously unappreciated mechanism for regulating transcription initiation termed 'promoter occlusion'.

In this process, transcription initiation from the σ^{70} promoter, which is the first of the overlapping promoters, results in formation of a functional transcript with proper ribosome binding sequence. Thus, translation of the transcript formed from the σ^{70} promoter produces the *crl* gene product. In contrast, under conditions of nitrogen limitation, σ^{54} is activated and transcription of the *crl* gene initiates at the σ^{54} promoter, which is the second of the overlapping promoters. Unlike the σ^{70} promoter, transcription of the *crl* gene from the σ^{54} promoter results in synthesis of a long non-coding RNA. This transcript lacks the ribosome binding sequence and, hence, is not translated. Summarily, promoter occlusion provides a novel regulatory strategy, whereby initiation from one promoter occludes initiation from the other promoter, thus down-regulating expression of the gene simply by producing a non-functional transcript.

Transcription Elongation

The initiation phase ends when RNAP succeeds in extending the RNA chain beyond the first nine nucleotides and escapes from the promoter. At that stage the elongation process begins and the enzyme starts moving along the DNA, extending the growing RNA chain. During the transition from initiation to elongation, the size and shape of the RNAP undergoes successive changes. The first change is the loss of the σ factor. Whereas the holoenzyme covers approximately 75 bp (from −55 to +20), after the loss of σ, the polymerase covers approximately 55 bp (from −35 to +20). At that stage the polymerase is displaced from the promoter (promoter clearance or escape) and undergoes a further transition to form the elongation complex, which covers only 35–40 bp, depending on the stage during elongation. The polymerase now becomes tightly bound to both the nascent transcript and the DNA template, making it very stable.

The average rate of transcript elongation by the various RNAPs is 40 nt s^{-1}. However, this rate varies dramatically among RNAP and loosely correlates with the subunit complexity of the enzyme. Thus, the simple single-subunit bacteriophage RNAPs are the most rapid of all DNA-dependent RNAPs (several hundred nt s^{-1}), bacterial RNAPs transcribe at an intermediate rate (50–100 nt s^{-1}), and eukaryotic polymerases, although diversified, appear to be the slowest (20–30 nt s^{-1}).

Recent studies of both prokaryotic and eukaryotic transcription have yielded an increasing appreciation of the extent to which gene regulation is accomplished during the elongation phase of transcription. Nevertheless, RNAPs are not as accurate as DNA polymerases. The difference in fidelity between RNA and DNA polymerases is apparently due to the higher robustness of the proofreading mechanism that characterizes DNA polymerases than that of RNAPs. Of course, the fidelity of DNA replication is of greater importance than that of transcription because, unlike replication errors, misincorporation during transcription does not result in permanent and inherited genetic changes.

Blocks to Transcription Elongation

Transcript elongation does not occur at a constant rate. Throughout the elongation phase, RNAP can be paused, arrested, or terminated. These are important events that underlie many regulatory mechanisms that govern gene expression. During a 'transcriptional pause', the polymerase temporarily stops RNA synthesis for a certain amount of time, after which it can resume the elongation process. Thus, pausing can be described as transcriptional hesitation. In contrast, during a 'transcriptional arrest' the polymerase stops RNA synthesis and cannot resume it without the aid of accessory proteins. Throughout both pauses and arrests, RNAP remains stably bound to the DNA template and to the nascent transcript. These features distinguish paused and arrested polymerases from those that have terminated and thus detached from the DNA. Pausing and termination are sometimes related because pausing is a prerequisite for termination (see below). However, not all pauses are termination precursors. The time it takes a stalled polymerase to resume elongation varies among pause sites from very short periods of time, which cannot be accurately measured, to several minutes. The fraction of RNAP molecules that respond to an elongation block is also variable because ternary complexes differ in their ability to recognize pausing signals. Elongation is controlled by transcription factors, such as GreA, NusA, NusG and Mfd, that affect RNAP processivity by modulating transcription pausing, arrest, termination or antitermination.

Transcriptional pause and arrest signals can be intrinsic; that is, sequences in the nascent transcript or in the DNA template whose interaction with RNAP can inhibit the progression of the ternary complex, such as RNA regions, which have the propensity to form a stable secondary structure. In addition, extrinsic factors may obstruct the progress of RNAP during transcript elongation. There are numerous examples of RNAPs from various organisms being physically blocked by DNA-binding proteins during RNA synthesis in natural and artificial systems. An example is the purine repressor, which binds well downstream from the *purB* operon transcriptional start point and blocks the polymerase during elongation (it should be noted that in many cases RNAP is able to transcribe beyond the DNA-binding proteins in its path by either displacing them or bypassing them). In addition to DNA-binding proteins, which are the most obvious obstacles for RNAP, factors that perturb the structure of DNA can also inhibit the progression of RNAP and thus interfere with transcript elongation, for instance, extreme positive or negative supercoiling, unusual DNA structures such as Z-DNA, and DNA lesions. The efficiency of such potential impediments to block RNAP from elongating depends on various local factors and on the type of the RNAP. For example, T7 RNAP can efficiently bypass gaps in the DNA template strand that are 1–5 nt, and less efficiently gaps as large as 24 nt.

Pausing appears to occur by a two-tiered mechanism. An initial rearrangement of the RNAP active site interrupts elongation and puts the enzyme in an off-line state, called the elemental pause; additional rearrangements or interactions with regulatory proteins or downstream DNA sequences can create long-lived pauses. Transcriptional pausing is involved in various regulatory mechanisms.

It plays a fundamental role in coupling transcription and translation by halting RNAP to allow a translating ribosome to catch up to polymerase. Given that most RNA exhibits significant secondary structure, even low-efficiency pausing that occurs with sufficient frequency will maintain coupling. Pause sites in the leader regions of operons regulated by attenuation are thought to halt RNAP to ensure that ribosomes are properly located to control the termination decision. The discovery of any attenuation mechanisms that are coupled with small molecule–RNA interactions (riboswitches) highlights the importance of pausing to proper regulation. Pausing also appears critical to ensure binding of elongation regulators to RNAP, for instance of RfaH to polymerase molecules paused at *ops* sites in the leader regions of RfaH-regulated operons. Furthermore, pausing plays a key role as the first step in both ρ-dependent termination and intrinsic termination of transcription (transcription termination is discussed in the section titled 'Transcription termination'). Pausing halts RNAP at terminators until ρ factor interacts with the transcription elongation complex. Additionally, specific pause sites have been found to play important roles in the proper folding of the nascent RNA. The combined effects of pausing in maintaining transcription–translocation coupling and in facilitating ρ-dependent termination when translation fails creates a prokaryotic version of an mRNA surveillance pathway, in which RNA damage or mutation that would lead to defective protein synthesis causes termination of transcription.

Although transcriptional arrest has been characterized in vitro and the evidence for its occurrence in the cell is only circumstantial, it is also believed to be implicated in the regulation of many genes. These predictions are based on the recognition that if an arrest occurs within the coding region of a gene, the arrested complex would block subsequently initiated RNAPs, thereby effectively repressing RNA synthesis from the affected gene.

Transcript Cleavage during Elongation

When RNAP encounters a roadblock during elongation, it backtracks and the 3′-end of the nascent RNA is cleaved to generate a new 3′-terminus. Transcript cleavage serves to rescue RNAPs that are arrested during elongation and gives the enzyme a second chance to transcribe over the roadblock and resume elongation. Following the cleavage, RNAP can correctly resynthesize the discarded RNA segment and continue with the elongation. The cleavage reaction involves accessory proteins in addition to RNAP. In *E. coli*, the GreA and GreB proteins serve as cleavage-stimulatory factors. GreA and GreB also affect the size of the released 3′-fragment. GreA-induced hydrolysis generates mostly di- and trinucleotides, while GreB-induced hydrolysis generates fragments of up to 18 nt long. GreA can only prevent transcription arrest, whereas GreB can reactivate prearrested transcription complexes as well.

Besides antipausing and antiarresting function, the factor-induced endonucleolytic reaction may enhance transcription fidelity by inducing the excision of misincorporated nucleotides, and by facilitating transition of RNAP from the initiation to the elongation stage of transcription. Molecular models for the mechanism of Gre proteins action were recently proposed based on biochemical, mutational, and structural analyses. Other factors, such as NusA, can regulate the cleavage properties induced by GreA and GreB. NusA is a multifunctional transcription factor that may elicit opposite effects on transcription elongation and termination. Its activity is discussed in the section titled 'Transcription termination'.

The Inchworm Model for Transcription Elongation

The old-fashioned view of the elongation process as a smooth forward motion during which RNAP moves 1 bp along the DNA template for every base added to the newly synthesized RNA chain might still hold for some regions of DNA. However, evidence has accumulated for a different type of movement of the polymerase during elongation. Thus, a new model for RNAP translocation has evolved: the 'inchworm model'. This model describes the movement of RNAP on the DNA template in a discontinuous inchworm-like fashion. The model predicts that the process of RNA chain elongation is a cyclic process that consists of discrete translocation cycles. Each cycle involves the steady compression of the RNAP on the DNA template followed by a sudden expansion. According to this model, the upstream (back) boundary of the enzyme moves steadily during elongation, as the RNA chain is extended. However, the downstream (front) boundary of the enzyme does not move while several nucleotides are added; it then 'jumps', that is, it moves 7–8 bp along the DNA. At the beginning of each cycle, RNAP stretches across ~35 nt of template DNA; it gradually compresses from the back end till it covers only ~27 nt; it then releases from the front end and stretches again to cover ~35 nt. As the RNAP compresses, the nascent RNA chain of the complex becomes longer and the single-stranded transcription bubble enlarges as well. The internal tension that these changes probably create in the enzyme is released when the front end expands discontinuously.

The inchworm model for transcription elongation postulates that RNAP binds to the DNA template at two separate sites, one downstream in the direction of transcription and one upstream, which can move independently of each other. This permits the polymerase to move in an inchworm-like manner, so one DNA-binding site on the polymerase remains fixed to the DNA, whereas the other moves along the DNA. There is now both direct and indirect evidence that validate this assumption. The downstream DNA-binding site in the *E. coli* polymerase was found to be double-strand-specific, whereas the upstream is single-strand-specific and interacts with the template strand. The model also assumes that the catalytic site of RNAP is linked to the movement of the upstream DNA site, but can move independently of the downstream DNA-binding site. The inchworm model also makes predictions about the existence of more than one RNA-binding site on the ternary complex. It has been shown that nascent RNAs interact with at least three sites on the *E. coli* polymerase, two on the β-subunit, and one on the β′-subunit. It is believed that together the DNA and RNA sites account for the remarkable stability and flexibility of ternary complexes. However, the precise size, placement, and strand specificity of these nucleic acid-binding sites are currently being elucidated.

Transcriptional Slippage

RNAP usually synthesizes RNA transcripts that are precisely complementary to the DNA template. However, in rare circumstances, RNAP can undergo transcriptional slippage that results in the synthesis of a transcript that is either longer or shorter than the sequence encoded by the DNA template. Such a slippage appears to occur when the polymerase transcribes homopolymeric runs. It has been proposed that the generation of transcripts that are shorter than the encoding template is due to translocation of RNAP without the incorporation of nucleotides, whereas the longer products are due to RNAP-incorporating nucleotides without translocation. Transcriptional slippage can occur during both the initiation and the elongation phases. However, the minimal length of the consecutive template nucleotides that can promote slippage in the two phases is different. During initiation, homopolymeric runs as short as 2 or 3 nt can be reiteratively transcribed by RNAP. During elongation, RNAP tends to slip only on longer runs, but the precise requirements have not been elucidated. In one case, slippage by the *E. coli* RNAP during elongation was reported to require runs of at least 10 dA or dT nucleotides, whereas runs of dG at the same length did not result in slippage. In some cases, the ability to slip seems to require a transcriptional pause in addition to the homopolymeric run. Transcriptional slippage is sometimes an important means of regulating transcription. It has been reported to play an important role in the regulation of transcription initiation at several bacterial operons, for example, *pyrBI*.

Implications of DNA Topology on Transcription Elongation

Because DNA has a helical secondary structure, a rotation about its axis is necessary to accomplish transcription elongation. This requires either that the entire transcription complex rotates about the DNA or that the DNA itself rotates about its helical axis. Under conditions in which the RNAP rotation is constrained, for example, due to the presence of ribosomes attached to the nascent RNA chain (which is often the case in bacteria), the DNA will rotate through the enzyme. Consequently, the process of transcription will tend to generate positive supercoils in the DNA ahead of the advancing RNAP and negative supercoils behind it. Excessive torsional stress in the DNA will arise if the DNA is anchored at various points (as is the case for circular DNA, such as the bacterial chromosome) or from the movement of RNAP in opposite directions along the DNA. DNA topoisomerases are the natural candidates to remove this tension. It was suggested that gyrase, which can relieve positive supercoils, and topoisomerase I, which removes negative supercoils, amend the situation in front of and behind the RNAP, respectively. This model is supported by the finding that when the activities of gyrase and topoisomerase I are inhibited or otherwise defective, transcription causes major changes in DNA supercoiling. A possible implication of this is that transcription, in addition to having a significant effect on the local structure of DNA, is responsible for generating a significant proportion of supercoiling that occurs in the cell.

Implications of DNA Replication and RNA Polymerase backtracking on Transcription Elongation

To prevent collision between the transcribing RNAP and the replicating DNA polymease, transcription regulation is carefully coordinated with DNA replication and chromosome segregation. In *E. coli* and in other bacteria and bacteriophages, heavily transcribed genes are oriented such that replication and transcription occur in the same direction. Despite this arrangement, because DNA replication occurs 10–20 times faster than transcription, RNAP and DNA polymerases do collide. Although the outcome of such an encounter is hard to predict, in several instances the replication-transcription conflict was shown to result in genome instability, leading to formation of chromosomal deletions and rearrangements. Importantly, such an encounter often results in RNAP backtracking, which involves sliding of RNAP along the DNA and RNA within the transcription bubble and stalling of the elongation complex. In addition to RNAP backtracking, transcription-replication collision also produces DNA lesions, which by themselves act as factors promoting elongation stalling. To deal with such conflicts, bacteria have evolved molecular machineries that cooperatively act at the collision site in order to resume DNA replication and transcription elongation. In a process termed 'transcription-coupled repair' (TCR), certain transcription-associated proteins recruit DNA repair factors to the conflict site and correct the mistakes that have been made in the DNA. An important protein that orchestrates TCR is the DNA translocase protein, Mfd. When RNAP stalls at a DNA lesion, the nucleotide excision repair (NER) proteins, which are necessary for repairing the lesion, cannot access the site due to the occupancy of the stalled RNAP. Under such conditions, Mfd binds to the stalled elongation complex by interacting with the β subunit of RNAP and dislodges the stalled complex by pushing it forward i.e., reverse backtracking. It then recruits UvrA to the DNA lesion site and, following repair by the NER system, elongation of the stalled transcription complex is resumed.

In *E. coli*, replication-transcription conflicts are counteracted also by other transcription elongation factors, including DksA, GreA and GreB, which are involved in modulation of RNAP backtracking. Furthermore, co-transcriptional translation, i.e., simultaneous translation of a nascent transcript, was suggested to regulate the rate of transcription elongation by preventing backtracking of RNAP. This notion is supported by the finding that NusG physically links the transcribing RNAP to the translating ribosome, and this coupling was observed to have an effect on transcription elongation and prevention of backtracking. Finally, a recent study identified UvrD, a member of the NER system, as a transcription elongation factor that physically binds to RNAP, thus promoting RNAP backtracking under conditions of replication-transcription collision. In a process independent of Mfd, UvrD was found to induce RNAP backtracking on stalled elongation complexes, using its helicase activity. By inducing backtracking, UvrD exposes the DNA lesion site to repair, which is necessary for resuming elongation of the stalled RNAP. The presence of multiple mechanisms that

function to relieve the arrested elongation complex suggests that transcription stalling, RNAP backtracking and replication-transcription collisions are unavoidable processes, which need to be rectified for efficient transcription.

In contrast to *E. coli*, in which there is evidence suggesting that the replication fork can displace the elongation complex, in bacteriophage T4, the movement of the replication apparatus does not seem to disrupt the elongation complexes, regardless of the direction of their motion relative to the replication fork. Interestingly, when direct collisions occur between the DNA and RNAPs of T4, the RNAP switches from the original DNA template strand to the newly synthesized daughter strand. The mechanism that allows the strand exchange without the dissociation of the elongation complex is not known, but probably relies on the various contacts with the DNA and RNA. Whatever the mechanism, the cell needs to coordinate the replication and transcription processes carefully.

Transcription Termination

Transcriptional elongation is highly processive and can lead to the production of RNA transcripts that are thousands of nucleotides long. The processivity is due to the high stability of the complex between the RNAP and the nucleic acids during elongation. It is this stability that necessitates the involvement of specific signals and factors to implement termination of transcription. To enable efficient termination, the termination signals or factors should cause drastic alterations of the interactions that are responsible for the stable elongation. At termination, RNAP stops adding nucleotides to the RNA chain, all the hydrogen bonds that hold the RNA–DNA hybrid together break leading to the release of the transcript, the DNA duplex reforms, and the enzyme dissociates from the DNA template. The sequence of these events is still not clear because attempts to determine whether the release of the RNAP is simultaneous with the transcript release or occurs subsequently have given ambiguous results. Once the transcript is released from the complex, it is unable to reattach in a way that allows transcriptional elongation to resume. Therefore, the transcript release is the commitment step that makes the termination process irreversible. On one hand, this mechanism ensures the termination at the end of genes and prevents the expression of adjacent distinct genetic units; on the other hand, this mechanism provides an opportunity to control gene expression.

The exact point at which termination of an RNA molecule occurs in the living cell is difficult to define. The 3′-end of an RNA transcript looks the same whether it is generated by termination or by cleavage of the primary transcript. Therefore, the best identification of termination sites is provided by systems in which RNAP terminates in vitro. An authentic 3′-end can be identified when the same end is generated in vitro and in vivo. In *E. coli*, two types of terminators were discovered, intrinsic terminators that do not require ancillary proteins and terminators that require the involvement of termination factors.

Intrinsic Terminators

Intrinsic terminators are sites at which core polymerase can terminate transcription in vitro in the absence of any other factor. The best characterized intrinsic terminators are the ones recognized by *E. coli* RNAP. Intrinsic terminators are characterized by a GC-rich sequence with an interrupted dyad symmetry followed by a run of about 6–8 dA residues on the template strand.

The transcription of the GC-rich sequence with the interrupted inverted repeats will give rise to an RNA segment that has the potential to fold into a stable stem-and-loop secondary structure (sometimes described as a hairpin structure). There is much indirect evidence that this structure is indeed formed in the nascent RNA. For example, mutations that interrupt the pairing in the stem part decrease the efficiency of termination, and compensatory mutations that restore the pairing recover the efficiency. There is also a strong correlation between the predicted stability of the structure and the termination efficiency. DNA oligonucleotides that are complementary to one arm of the stem in the stem-loop structure effectively reduce the efficiency of termination, presumably by annealing to the RNA sequence, and thus prevent the RNA from folding into the stem-loop structure. The sequence of the loop in the stem-loop structure also influences the stability of the RNA secondary structure, but the rules for contributing to loop stability have not been fully elucidated. How does the stem-loop structure contribute to termination? It is suggested that the formation of this structure in the newly synthesized RNA sequence, which is still in contact with the polymerase, causes the polymerase to pause, and thus destabilizes the ternary complex.

The other structural feature of an intrinsic terminator, the run of the dA residues (which is sometimes interrupted) in the template strand, is located at the very end of the transcription unit. The transcription of this sequence will generate a run of rU residues at the 3′-end of the RNA transcript. The hybrid between the dA and the rU residues is significantly less stable than most other hybrids, due to weak base-pairing, and it thus requires the least energy to break the association between the strands. This poor base-pairing is assumed to unwind the DNA–RNA hybrid and destabilize the interaction of the nucleic acids with the paused polymerase. The importance of the dA run has been established by mutational analysis. The importance of the length of the dA stretch was confirmed by introducing deletions that shortened this element; although the polymerase could still pause at the stem-loop, it no longer terminated. Interestingly, the actual termination can occur at any one of several positions toward the end of the dA run.

The DNA sequence within 30 bp downstream to the transcription stop point, which does not reveal an obvious consensus sequence, is also important for termination in certain cases. For example, changes in the sequence 3–5 bp downstream to the stop point of T7 early-gene terminator can reduce the efficiency of termination from 65 to 10%. Although these sequences are not transcribed, they are near or within the contact point between the RNAP and the DNA in the transcription complex. The way these

sequences can affect transcription is by influencing the unwinding of the DNA or the progression of the polymerase along the DNA. Alternatively, the stability of the binding of the polymerase could vary depending on the sequence at the contact points.

Recently, a distinct class of branched intrinsic terminators was identified. Anti-Q is a small RNA encoded from a plasmid of *Enterococcus faecalis* that confers antibiotic resistance. Relative to the larger transcript that is transcribed from the same operon, anti-Q is produced via factor-independent termination. Unlike the known terminator structures, which are typified by a G + C stem loop followed by a poly-uridine tract, the terminator sequence of anti-Q folds into an unusual branched structure with a basal stem, followed by a poly-uridine tract. The branched structure, which resembles a Y-shape, was found to be essential for transcription termination and for subsequent release of the RNA polymerase. Importantly, a bioinformatic search for similar sequences in bacterial genomes identified several genes with potential anti-Q-like terminator structures. Termination of at least one of the studied genes via an anti-Q-like terminator structure, albeit at a low efficiency, was experimentally validated. This newly discovered flexibility in the structure of factor-independent terminators calls for reassessment of bacterial genomes coding capacity and annotation.

Rho-Dependent Termination

The best characterized termination factor is the bacterial ρ protein. ρ is a classic termination factor in the sense that it provides a mechanism for dissociating nascent transcripts at sites that lack intrinsic terminators. Rho is essential for the survival of most bacteria, although in some prokaryotes Rho is dispensable, or absent altogether. Rho binds to the nascent RNA chain. The sequences that form a ρ-dependent terminator extend from at least 60 bp upstream to about 20 bp downstream of the actual stop point. ρ-binding sites show a highly inconsistent sequence homology and their only common feature is a relatively high cytosine content. In addition, ρ has a strong preference for sufficiently long segments of unstructured RNA (lacking base-pairing).

ρ causes RNAP to terminate preferentially at points that are natural pause sites. These pause signals often encode U-rich RNA–DNA hybrids, which have the added consequence of destabilizing the transcription elongation complex. There is no evidence that ρ affects the elongation-pausing specificity of the polymerase, but several lines of evidence point to a highly specific conformation of the elongation complex preferred by ρ. In addition to ρ being an RNA-binding protein, ρ contains an RNA–DNA helicase activity; it hydrolyzes ATP to energize the separation of an RNA–DNA hybrid termed R-loop. Recent studies have found that an essential function of Rho is to prevent R-loop formation, which are formed across the genome not only by transcription read-through, but also by antisense RNAs. In agreement with its role in R-loop prevention, Rho was found to be non-essential in cells expressing the R-loop helicase UvsW. Thus, ρ is acting primarily as an RNA-release factor. The recently solved crystal structure of ρ could explain many of its activities, although many questions pertaining to ρ-recognition sites, enzymatic activities, and interactions with the elongation complex remain obscure. The current model for ρ action is that it binds to the RNA transcript at sites that are unstructured and rich in C residues; it then translocates along the RNA until it catches up with the polymerase at sites where the enzyme pauses; ρ unwinds the RNA–DNA hybrid in the transcription bubble; termination is completed by the release of ρ and RNAP from the nucleic acids. Some ρ mutations can be suppressed by mutations in the genes that encode the β- and β′-subunits of RNAP, implying that in addition to interacting with the nascent RNA chain, ρ also interacts with the polymerase.

The lack of stringent sequence requirements for a ρ-dependent transcription terminator raises the possibility that such terminators might be fairly frequent in DNA sequences, not only at the ends of operons but also within genes. What prevents ρ from terminating within genes? Because transcription and translation are coupled in prokaryotes, the mRNA chain that emerges from the transcription complex is protected by ribosomes, probably preventing ρ from gaining access to the RNA. The phenomenon of polarity (a nonsense mutation in one gene prevents the expression of subsequent genes in the operon) can be explained by the release of ribosomes from the transcript at the nonsense-mutation site, so that ρ is free to attach to and move along the mRNA; when it catches up with RNAP, it terminates transcription, thus preventing the expression of distal parts of the transcription unit. What prevents ρ from acting on transcripts that are not translated, such as rRNAs and tRNAs? One reason seems to be the lack of ρ-binding sites on these RNAs because they are highly structured. rRNA molecules are further protected by the binding of ribosomal proteins. Another mechanism that protects rRNA operons against ρ-dependent termination relies on sequences near the start of the rRNA genes that dictate antitermination. It was suggested that this mechanism increases the rate of transcriptional elongation of rRNA operons by preventing pausing.

Auxiliary Termination Factors

Although some polymerases can spontaneously terminate transcription at intrinsic terminators, the efficiency of termination in vitro is often enhanced significantly by the presence of additional factors. It therefore seems that the DNA signals that characterize intrinsic terminators are necessary, but sometimes not sufficient. The best characterized of these auxiliary termination proteins is NusA. A less-studied factor named τ (tau) is known to enhance and modify recognition of some strong intrinsic terminators for *E. coli* RNAP. ρ-dependent termination can also be enhanced by an auxiliary factor, the NusG protein. Small RNAs were also shown to affect transcription termination via base-pairing interactions with sequences in the mRNA.

The ability of NusA to increase the efficiency of termination at some intrinsic terminators might be attributed to its capability to increase the rate of pausing at certain sites. Enhanced pausing would allow more time for the conformational change that leads to the release of the nascent transcript. Some intrinsic terminators that are predicted to form not a very stable secondary structure, such as the one in the ribosomal protein S10 operon leader, depend on NusA for their operation, and can therefore be defined as

NusA-dependent terminators. In the case of the intrinsic terminator in the attenuator region preceding the gene for the β-subunit of *E. coli* RNAP, NusA, rather than enhancing termination, reduces termination efficiency. Indeed, as mentioned before, NusA may elicit opposite effects on transcription, depending on the RNA–DNA sequence context and the presence or absence of auxiliary factors. By itself, NusA stimulates certain types of pausing and ρ-independent intrinsic transcription termination. However, NusA can induce anti-termination at ρ-dependent terminators with RNA sequences containing 'nut' or 'nut'-like elements (see below). In complex with other Nus factors (NusG, NusB, NusE) or λ phage proteins N and Q, it stimulates antitermination at both ρ-dependent and ρ-independent terminators (see below). NusA antitermination function plays an important role in the expression of ribosomal genes. During transcription of many other genes, NusA-induced RNAP pausing provides a mechanism for synchronizing transcription and translation.

NusG has a minor effect on termination at some intrinsic terminators. However, it plays a significant role in the functioning of some ρ-dependent terminators. This role was deduced from the strong effect of NusG loss on the activity of some ρ-dependent terminators in vivo and from the effect of NusG on ρ termination efficiency and pattern across a terminator in a purified in vitro system. The role of NusG in ρ-dependent termination is not conclusive, but the evidence hints that NusG might increase the retention of ρ on the nascent transcript, thereby increasing its local concentration, and/or that NusG increases the rate of RNA release at termination sites. It is also possible that NusG acts directly to enhance ρ activity or indirectly by slowing the dissociation of ribosomes from the RNA, thus preventing ρ from binding to the transcript. Recent studies have shown that Rho and NusG cooperate to prevent generation of antisense RNA across the genome.

The nucleoid-associated (H-NS) family of proteins was recently found to modulate ρ-dependent termination. Genome-wide analysis of H-NS and ρ-binding sites revealed an enrichment of H-NS at sites that are prone to ρ-dependent termination. Further support for the cooperativity between H-NS and ρ was provided by their apparent genetic interaction. Interestingly, cells expressing a truncated form of H-NS, lacking its C-terminus, or YdgT, which resembles the N-terminal of H-NS, can suppress defects in ρ-dependent termination caused by mutations in *rho*. This suggests that the H-NS family of proteins cooperate with ρ in terminating transcription. A clue to how H-NS affects ρ-dependent termination is offered by its enrichment in coding regions, which suggests that H-NS acts as a roadblock for the transcribing RNAP. Thus, by slowing the elongation rate, H-NS may provide Rho with a window of time during which it can catch up with the transcribing RNAP, a requirement for ρ-dependent termination.

Two proteins that antagonize ρ-dependent transcription termination have been identified: the Psu protein encoded by bacteriophage T4 and YaeO from *E. coli*. Psu seems to inhibit ρ-dependent termination by slowing down the translocation of ρ along the RNA, as this protein was shown to negatively affect the ATPase activity of ρ. A model of the YaeO–ρ complex, which is based on the solved structure of the two proteins, proposes that YaeO binds to ρ, acting as a competitive inhibitor of RNA binding.

Interestingly, the presence of the σ factor in excess significantly increases the rate of RNAP recycling. Hence, the σ factor can also be considered a termination factor. This activity supports the notion that σ can remain associated with the polymerase at postinitiation steps.

Antitermination

Antitermination is used as a control mechanism in phages to regulate the progression from one stage of gene expression to the next, and in bacteria to regulate expression of some operons. Antitermination occurs when RNAP reads through a terminator into the genes lying beyond. The terminators that are bypassed can therefore be defined as conditional terminators. Antitermination is not a general mechanism that can occur in all terminators, but is, rather, dependent on the recognition of specific sites in the nucleic acids. Many mechanisms involve choosing between two alternative hairpin structures in an RNA transcript, with the decision dependent on interactions between ribosome and transcript, tRNA and transcript, or protein and transcript. In other examples, modification of the transcription elongation complex is crucial to make it bypass certain terminators.

The N protein of bacteriophage λ mediates antitermination necessary to allow RNAP to read through the terminators located at the end of the immediate early genes in order to express the delayed early genes. The recognition site needed for antitermination by N, termed *nut* (for N utilization), lies upstream from the terminator at which the action is eventually accomplished. The *nut* site consists of two sequence elements, a conserved 9-nt sequence called BoxA, which is also an antiterminator signal in the operons encoding ribosomal transcripts, and a 15-nt sequence called BoxB, which encodes an RNA that would form a short stem structure with an A-rich loop. A number of host proteins, including NusA, NusG, ribosomal protein S10 (NusE), and NusB, participate in the N-mediated antitermination process (Nus stands for N utilization substance). A model for N-mediated antitermination at ρ-dependent terminators proposes that N recognizes and binds to the BoxB stem-loop structure formed on the nascent transcript, whereas NusB and S10 bind to the BoxA sequence on the RNA. These proteins are held together through interactions with core RNAP that are stabilized by NusA and NusG. Hence, a ribonucleoprotein complex is formed at the *nut* site and stays attached to the elongating RNAP. This complex prevents RNAP from pausing, thus denying the ρ factor the opportunity to cause termination, and the polymerase continues past the terminator. N also suppresses termination at intrinsic terminators; however, NusA suffices for N to prevent termination at these sites. Other phage related to λ have different N proteins and different antitermination specificities. Each phage has a characteristic *nut* site recognized specifically by its N-like protein. All these N-like proteins seem to have the same general ability to interact with the transcription apparatus in an antitermination capacity.

The Q protein is required later in bacteriophage λ infection. It allows RNAP to read through the terminators located at the end of the immediate early genes, to express the late genes of bacteriophage λ. Q has a different mode of action than N. It recognizes and binds to a site on the DNA, called *qut*. The upstream part of *qut* lies within the λ-promoter P_R, whereas the downstream part lies at

the beginning of the transcribed region. Thus, Q antitermination is specific for RNAP molecules that have initiated at the P_R promoter. The part of *qut* that lies within the transcribed region includes a signal that causes RNAP to pause just after initiation. This pause apparently allows Q to interact with the polymerase. Once bound, the Q-modified enzyme is released from the pause and is able to read through most transcription terminators, both intrinsic and ρ-dependent. It seems that the modification of the polymerase by Q increases the overall rate of transcription elongation and permits the polymerase to hurry past the terminators. Interestingly, the pause of the polymerase early in the transcription unit, which is a prerequisite for Q-mediated antitermination, involves the binding of the σ subunit of the RNAP holoenzyme to the nontemplate strand of DNA in the transcription bubble up to 15 nt downstream from the start point of transcription. Thus, an initiation factor acts in concert with a DNA-binding termination factor to modify the elongation properties of RNAP. Once again, it is shown that σ can remain associated with the polymerase and play a role in postinitiation steps.

RNAP molecules that are engaged in transcribing the rRNA (*rrn*) operons are modified in a way that makes them bypass certain terminators within the rRNA genes. The modification is established by the recognition of a sequence signal that is nearly identical to the BoxA sequence involved in N-mediated antitermination. It has been shown that a heterodimer of NusB and S10 protein binds to the BoxA sequence on the RNA of one of the *rrn* operon. It was therefore proposed that the mechanism of transcriptional antitermination in the *rrn* operons, similar to the mechanism mediated by N, involves formation of a ribonucleoprotein complex on the BoxA complex that is carried along with the elongating polymerase. The probable purpose of this mechanism is to ensure that transcription of the rRNAs is immune from ρ action.

Some bacterial proteins regulate gene expression by binding to specific RNA sequences and alter the structure of the leader RNA to promote or prevent transcription termination. The TRAP protein from *B. subtilis* and the BglG protein from *E. coli* represent two families of proteins that promote termination and antitermination, respectively. The BglG protein, encoded by the β-glucoside utilization operon (*bgl*) in *E. coli*, prevents the termination of transcription at two intrinsic terminators. The first terminator is in the 5′ untranslated leader of the *bgl* transcript and the second is in the intergenic region between the first and second genes of the operon. BglG is an RNA-binding protein that recognizes and binds to a specific sequence partially overlapping the sequence of both terminators. By binding to its RNA target site, BglG stabilizes a secondary structure, which is an alternative to the terminator structure. Thus, BglG binding to the *bgl* transcript prevents the formation of the terminators and the polymerase can read through them. BglG exerts its effect as a transcriptional antiterminator only when the expression of the operon is required, that is, when β-glucosides are present in the growth medium. The activity of BglG as an antiterminator depends on its phosphorylation state, which affects its oligomeric form. BglG-like antiterminators were shown to control the expression of sugar utilization genes in various organisms, both Gram-negative and Gram-positive.

Unlike BglG, the TRAP protein from *B. subtilis* promotes termination of *trp* operon transcription. The transcript of the leader region of the *trp* operon can fold to form mutually exclusive antiterminator and terminator structures. When the TRAP protein is activated by tryptophan, it binds to the 5′ segment of the antiterminator hairpin, freeing its 3′ segment to pair with the adjacent 3′ RNA segment to form a terminator structure. Since the stability of the antiterminator is higher than that of the terminator, TRAP binding is required to prevent antiterminator formation. Thus, when cells have adequate levels of tryptophan, activated TRAP binds to the antiterminator region, the terminator formed, and transcription is terminated. When the level of tryptophan drops, an anti-TRAP protein is synthesized. This protein binds to tryptophan-activated TRAP and inhibits its ability to bind to *trp* leader RNA; this results in TRAP inactivation, leading to increased expression of all the genes required for tryptophan biosynthesis.

Transcriptional regulation of operons that are concerned with amino acid synthesis and utilization by ribosome-mediated transcription termination and tRNA-mediated transcription antitermination are described in a different article.

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Transduction: The Transfer of Host DNA by Bacteriophages☆

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Glossary

Abortive transductant A bacterium that has acquired transduced DNA, which has not been degraded or stably integrated into the bacterial DNA. Abortive transductant DNA can be expressed, but cannot be replicated.
Generalized transduction The phage-mediated transfer of any region of bacterial DNA from one bacterium to another. Generalized transduction can be mediated by temperate or virulent phages.
Lysogen A bacterium that harbors a prophage.
Prophage A phage in lysogeny. Prophages are replicated as part of the bacterial chromosome or as a plasmid-like element.
Specialized transduction The phage-mediated transfer of regions of bacterial DNA, located adjacent to the site of prophage insertion, from one bacterium to another. Specialized transduction is mediated by temperate phages upon their incorrect excision from the chromosome when exiting the prophage state.
Temperate phage A phage that is capable of entering either the lytic or lysogenic life cycles.
Transducing particle A phage capsid that has packaged bacterial DNA and hence is proficient for transduction.
Transducing phage A phage capable of mediating transduction.
Transductant A recipient bacterium that has stably acquired the transduced DNA.
Transduction The phage-mediated transfer of bacterial DNA from one bacterium to another.
Virulent phage A phage that is able to replicate only via the lytic cycle.

Abbreviations

cos site	Cohesive end site
HFT	High-frequency transducing
HGT	Horizontal gene transfer
HT	High-transducing

Introduction

Bacteria can transfer genes vertically to daughter cells as well as by horizontal gene transfer (HGT). There are three main classes of HGT, transformation (uptake of free DNA), conjugation (DNA uptake by cell-cell contact) and transduction. Transformation and conjugation are being covered in other articles. This article will focus on transduction, which is the bacteriophage (phage)-mediated transfer of DNA from donor to recipient bacteria. Whilst studying genetic recombination in *Salmonella enterica* serovar Typhimurium in 1952, Zinder and Lederberg observed uptake and recombination of bacterial DNA that was not susceptible to DNase treatment, and that was independent of cell-cell contact. They concluded that this was due to a DNA transfer mechanism distinct from transformation and conjugation. The authors found that the temperate phage P22 was the agent responsible, and they coined the term transduction to describe this new, phage-dependent, form of gene transfer. Even though much less studied, virus-mediated transduction has also been seen to occur in methanogenic archaea. This article however will focus on transduction in bacteria.

There are two major classes of transduction, specialized transduction and generalized transduction (see the following sections). In brief, a prophage can excise incorrectly from the host bacterial chromosome, ending up also packaging some of the flanking regions of host DNA. These genetic regions can be recombined with the homologous region in the chromosome following infection of a subsequent bacterium. This process is called specialized transduction, and requires a temperate phage. In contrast, generalized transduction can be mediated by either temperate or virulent phages. During the lytic mode of phage growth, segments of the bacterial DNA can occasionally be packaged erroneously into phage heads and these particles are released during lysis, together with

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wild type functional phages. The phage particles packed with bacterial DNA are called transducing particles and have the ability to adsorb to bacterial surface receptors and to inject their DNA. However, as they lack phage genes, they cannot go through a lytic cycle. Instead, the injected bacterial DNA might be incorporated into the recipient chromosome by homologous recombination, resulting in stable bacterial transductants. A special case of transducing particles, called "Gene Transfer Agents" are nonreplicative phage-like particles able to move bacterial genes. These are covered in a separate article.

Studies of transduction have enabled an understanding of the nature of phages, their interaction with bacteria and mechanisms of gene regulation. Generalized transduction is an important and powerful tool in bacterial genetics, being used for genetic mapping, strain construction, mutagenesis, etc. The basis and applications of this will be further discussed in the section Transduction as a Genetic Tool. Transduction is also important in natural environments, for bacterial evolution and for the spread of antibiotic resistance genes. The formation of transducing particles is generally considered an accidental process. However this view might be questioned, as a phage's capacity for transduction may benefit its bacterial host, and therefore may also benefit the phage (see section Transduction in the Environment).

Specialized Transduction

Definition and Discovery

The process of specialized transduction was first observed using *Escherichia coli* and phage lambda (λ) in 1956, and has since been shown for many other temperate phages. The fact that λ could only mediate the transfer of a limited number of *E. coli* genes gave rise to the terms "specialized" (or "restricted") transduction. The limited gene transfer also highlights the main difference between generalized and specialized transduction; what DNA is packaged into the phage capsid and thereby can be transferred to other bacteria. In short, a specialized transducing particle arises from an incorrectly excised prophage, which packages all or parts of its own viral genome together with some of the flanking host DNA. A generalized transducer on the other hand, can package bacterial DNA without phage DNA.

Bacteriophage lambda (λ) is one of the most thoroughly studied and well understood biological entities on the planet and our understanding of many of the known gene regulation mechanisms derives from ingenious experiments using this model phage. Not surprisingly, the most complete picture of the steps involved in specialized transduction has been elucidated using λ. Therefore, λ will be discussed below to illustrate the general mechanism of specialized transduction.

Replication of Phage λ

To understand the mechanism of specialized transduction, it is also necessary to discuss the process of λ replication. As λ is a temperate phage, it is able to undergo two alternative life cycles: lytic (where phage particles are assembled and released by the lysed host cell) and lysogenic (during which the phage genome is incorporated as a prophage into the host chromosome). As mentioned, specialized transduction relies on incorrect excision of a prophage. Therefore, the life cycle of λ, with the emphasis on the lysogenic process, will be described here, and is depicted in Fig. 1.

Lambda recognizes host cells via the LamB outer membrane protein and, upon binding to this receptor, injects its linear double-stranded DNA (dsDNA) into the cell. The ends of the linear dsDNA contain 12 nucleotide single-stranded overhangs. These sites at the ends are called cos (cohesive end) sites, and allow the linear dsDNA to cyclize via complementary base pairing of the cos sites. The DNA is then ligated and has thereby formed a stable circular molecule of dsDNA. By a well-characterized regulatory mechanism, the circular λ genome now "decides" whether to enter the lytic or the lysogenic life cycle. Under conditions of the lytic cycle, new phage particles are formed and released from the cell following bacterial lysis. Alternatively, by entering the lysogenic cycle, λ can integrate into the host chromosome and thus replicate as a prophage. Because λ circularizes upon injection into the bacterial cell and integration occurs at *attP* and not the cos site, site-specific recombination into the bacterial chromosome results in a different linear gene order in the prophage than in the mature capsids, as illustrated by the *int* and *xis* genes in Fig. 1.

The insertion of λ into the bacterial genome requires the phage attachment site (*attP*) on the λ DNA, and the bacterial attachment site (*attB*) located between the galactose (*gal*) and biotin (*bio*) operons on the *E. coli* chromosome (Fig. 1). The site-specific recombination between *attP* and *attB* is catalyzed by the phage-encoded Int protein, a site-specific recombinase that recognizes these short and relatively dissimilar sequences, to promote recombination. Int is a member of the family of tyrosine recombinases that have an active site tyrosine residue, which forms a covalent link to the 3′ phosphate after cleavage. The integration host factor (IHF) encoded by the *E. coli* chromosome is also required during integration. During the establishment of lysogeny, *int* alone is transcribed from a promoter located inside the gene neighboring *xis* (illustrated with a *green arrow* in Fig. 1).

Once λ DNA has been inserted into the chromosome, the prophage state is maintained by the cI repressor and the prophage can be stably propagated upon bacterial DNA replication and cell division of these λ-lysogenic bacteria. A regulatory switch from lysogeny to the lytic pathway can be triggered by factors causing cellular stress (such as UV light), and induction of the SOS response. This ultimately leads to the generation of mature phages and cell lysis. The first step in this process is the excision of the prophage and regeneration of the circular phage DNA. During the prophage state, the *attP* and *attB* sites are hybrid sequences (Fig. 1), and cannot therefore be recognized by Int alone. Because of this, an additional phage-encoded excisionase, called Xis, is required. As a response to environmental cues for excision, the *int* and *xis* genes are cotranscribed from a second promoter upstream of *xis* (illustrated with a *blue arrow* in Fig. 1), enabling equal concentrations of the two proteins. The combined action of Int and Xis

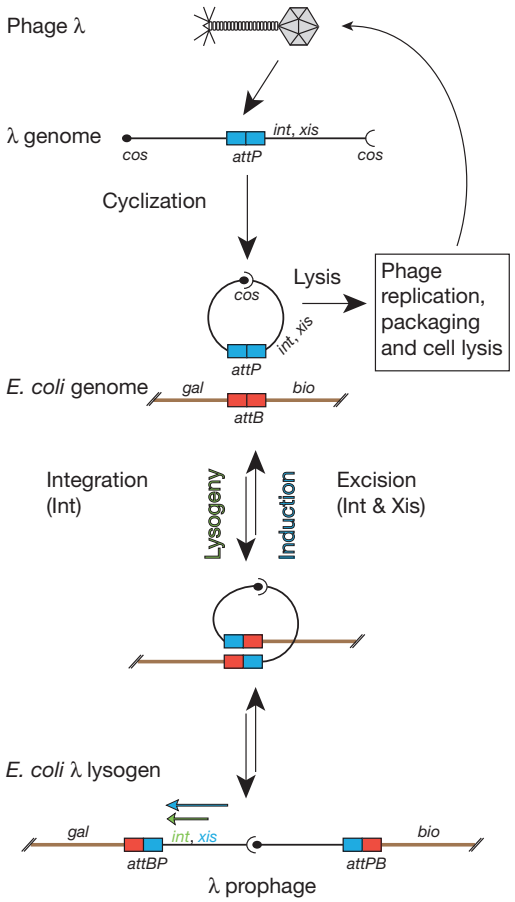

Fig. 1 The lytic and lysogenic lifecycles of phage Lambda (λ). Following binding to the bacterial LamB receptor, λ injects its dsDNA genome (depicted in *black*) into *Escherichia coli*. The linear λ chromosome is then circularized by the single-stranded complementary cos-regions at each end of the λ genome. The cyclized chromosome is then ligated to covalently close the circular structure. After this, λ can enter ether the lytic or the lysogenic lifecycle. In the lytic pathway, the λ genome is replicated and λ proteins are expressed, leading to assembly of new phage particles, genome packaging, lysis of the host cell and release of the new phages. As an alternative, the λ-encoded protein Int can promote site-specific recombination between the λ *attP* site and the *E. coli attB* site, leading to the establishment of the λ prophage in the *E. coli* chromosome (depicted in *brown*). If the *E. coli* λ lysogen is subjected to cellular stress, the prophage can be induced and the λ genome is excised from the *E. coli* chromosome. This process is catalyzed by the λ proteins Int and Xis that together can recombine the hybrid *attBP* and *attPB* sites. After this excision and recyclization, the λ genome is replicated, packaged and released during lysis of the host cell.

catalyzes the site-specific recombination of the hybrid *att* sites on either side of the prophage and enables its excision, leading to the formation of the circular λ genome. Lambda then begins DNA replication to generate long concatemers of λ DNA, interspersed with double-stranded cos sites, as well as transcription and translation of genes needed for production of phage particles. Once phage heads are assembled, the DNA is packaged into the heads and cleaved into genome length units in a single-stranded staggered manner, regenerating each of the single-stranded cos site overhangs at the ends of the linear dsDNA. This process of packaging involves numerous phage and host proteins. Phage tail proteins are then added to the heads and the completed phage particles are released from the host following lysis. These can then initiate another infective cycle.

Incorrect Prophage Excision Creates Specialized Transducing Particles

Occasionally, the λ prophage recombines in a nonspecific manner (in a process called illegitimate recombination) instead of excision by the *att* site-specific Int/Xis system. The schematics of illegitimate recombination are illustrated in Fig. 2. These rare excision events (approximately 10^{-6} frequency compared to excision by Int/Xis) result in circular phage DNA molecules that also contain bacterial genes. If these circular hybrid DNA molecules are approximately the size of the λ genome and if they still contain the cos sites, they can be replicated, cleaved at the *cos* sites and packaged into phage capsids. These phage capsids with hybrid chromosomes are now called specialized transducing particles.

Because the *E. coli attB* is situated between the *gal* and the *bio* operons, and this is where the λ prophage is located, genes in these operons were the first shown to be transduced, as they flank the prophage. A number of methods were developed to use λ to transduce genes that are not normally located adjacent to *attB*. Initially, strains that contained large genetic deletions or rearrangements were utilized because they had genes other that *gal* and *bio* closer to *attB*, which enabled their excision and packaging by λ.

By another strategy, the *attB* site was deleted and λ lysogens were isolated where the prophage had inserted with reduced specificity into the genome at alternative (secondary) sites. As well as allowing the transduction of genes located near these secondary integration points, these experiments also provided information about the sequence specificity and efficiency of the Int protein. Finally, λ can be engineered to contain transposons. If the same transposon is used to mutagenize the bacterial host, the transposon-containing λ can be inserted as a prophage at the bacterial transposon sites, by homologous recombination. Using this technique it is theoretically possible to insert λ in any nonessential gene in the *E. coli* genome. The flanking bacterial genes can be excised together with the prophage by illegitimate recombination. Once the specialized transducing particles have been packaged they are capable of initiating infection of a new recipient bacterium, and thereby bring the prophage-flanking genes with them.

Formation of Transductants

The dsDNA of the infecting transducing particle is injected into the new host and cyclized as described above. To be packaged into a phage head, the λ genome has a size-restriction. Because of this, specialized transducing particles usually have lost some phage genes (Fig. 2). This can have deleterious effects on λ function, especially in terms of integration and generation of new particles. For example, a *gal* transducing phage, λd*gal* (the "d" means *d*efective), which carries the *gal* genes will have lost the phage head and tail genes and, therefore, cannot undergo fully productive lytic growth, but can still lysogenize the host. Alternatively, a *bio* transducer, λp*bio* (the "p" is for *p*laque forming), carries the *bio* genes but lacks the *int* and *xis* genes and subsequently is unable to become a prophage, but can replicate lytically.

For the specialized transductants to be formed, the horizontally acquired DNA must be maintained stably in the recipient cell. This can happen via one of a number of possible routes:

- *Homologous recombination* can occur between sequences common to both the donor and recipient bacterial genomes. This can provide stable transductants that have incorporated the transducing phage genome (Fig. 3A). These lineages will have two copies of the transduced DNA segment (merodiploid) and are useful for complementation analyses. Infrequently, however, a second event of recombination can occur, exchanging the host locus for the homologous transduced locus, resulting in a haploid transductant (Fig. 3A).
- *Lambda integration with the aid of a helper phage* may also be used for introduction of the transduced DNA. In this case the helper phage provides the functions missing from the specialized transducing particle, *in trans* or *in cis* via recombination (Fig. 3B). Coinfection with a wild-type λ and the transducing particle can result in recombination at sites shared by these phages, which can then integrate at *attB* via the *attP* and Int functions supplied by the helper phage. The resulting lysogens will harbor the genomes of both the transducing phage and the helper phage and are hence called double lysogens (or dilysogens). A single excision of both prophages yields a hybrid circular DNA that is replicated and packaged according to the cos sites, giving rise to alternate transducing and wild-type λ particles. The lysate will be a mixture of transducing particles and helper phages, where approximately half will be specialized transducing particles. The frequency of specialized transducing particles in this lysate is thereby a lot higher than in a lysate where the aberrant excision originally took place, and hence dilysogens are useful for the generation of high-frequency transducing (HFT) lysates upon prophage induction.
- *Phage λ can sometimes replicate as a plasmid* in the recipient cell. Mutation of the λ *N* gene or the host *nusA* gene causes reduced expression of the λ *O* and *P* genes, which are required for replication. Expression of these proteins at low levels allows maintenance of λ, essentially as a plasmid. Alternatively, acquisition of a plasmid origin of replication also provides λ DNA with stable replication and maintenance. Therefore, any one of these mechanisms can produce heritable transductants.

Other Specialized Transducing Phages

The λ phage has provided an excellent system for unraveling the mechanisms of specialized transduction. The model based on experiments on this phage predicts that aberrant excision of any prophage that packages its DNA based with phage-specific sequences (analogous to the λ cos sites) could lead to production of specialized transducing particles. Indeed, many specialized transducers have been identified, including *Pseudomonas aeruginosa* phage D3 and *Bacillus subtilis* phage SPβ. Further information on the frequency of specialized transduction may be provided from comparative genomics, since it is also possible that some of the apparently degenerate prophages present in many sequenced bacterial genomes may have resulted from past events of specialized transduction.

Generalized Transduction

Unlike specialized transduction, where (normally) only genes flanking the prophage insertion site can be transduced, generalized transduction is a process whereby any section of the bacterial DNA can be transferred from one bacterium to another via a phage particle. This was first identified by Zinder and Lederberg, during studies of the *Salmonella*-infecting phage P22 in 1952. They showed that this temperate phage was able to transfer chromosomal DNA from several loci, from one strain of *Salmonella* to another. In 1955, Lennox identified phage P1 as a generalized transducing phage of *E. coli*. Early studies of P22 transduction in *Salmonella* and P1 transduction in *E. coli* have been reviewed elsewhere (see "Further Reading"). These two "model" phages have been used in extensive studies about transducing phages and the mechanisms of generalized transduction. The findings from these

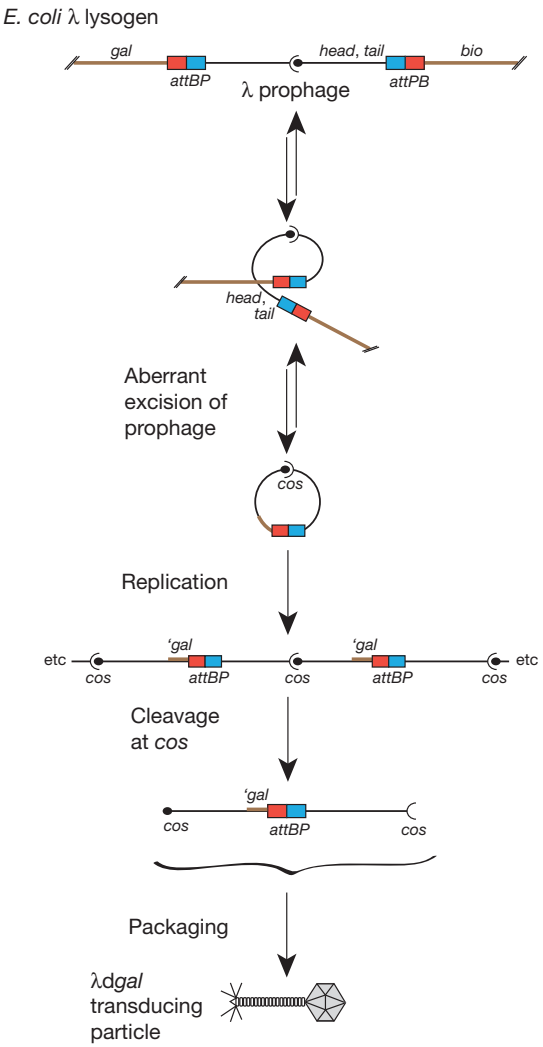

Fig. 2 Formation of specialized transducing particle by aberrant prophage excision. The λ prophage (in *black*) can be imprecisely excised from the *E. coli* chromosome (in *brown*) by infrequent events of illegitimate recombination. As a result, the cyclized λ chromosome might lack some phage genes (in the case depicted; the genes for phage head and tail proteins), but instead carry some bacterial genes that were flanking the prophage (here; part of the *gal* operon). By the events represented in the figure, the formation of a *gal*-transducing λ particle is shown. This λd*gal* transducing particle will be deficient in formation of new phage particles upon infection, but is capable of lysogeny and thereby transduction of the *gal* genes.

studies, as well as studies of other transducing phages, have contributed to current knowledge on generalized transduction, which is summarized below.

Properties of Generalized Transducing Phage

The known, naturally occurring generalized transducing phages are tailed phages (*Caudovirales*, including members of the *Myoviridae*, *Siphoviridae* and *Podoviridae*) and store their genomes as dsDNA. Generalized transducing phages can be either virulent or temperate and, importantly, utilize a sequential headful DNA packaging mechanism. Occasionally during DNA packaging, bacterial DNA, instead of phage DNA, is packaged in error into a structurally unaltered phage head, resulting in a transducing particle instead of a fully functional phage. For this to occur, the phage must not degrade the host DNA completely upon infection. The life cycle of a generalized transducing phage is summarized in Fig. 4.

Headful Packaging

As part of the lytic cycle of phage growth, the phage structural proteins are made. Among these, the phage capsid proteins assemble into the virion head, or prohead. The proteins forming the phage tails are assembled separately. The phage DNA is then replicated and forms concatemers of phage genomes, arranged after one another in tandem. These concatemers are usually around four

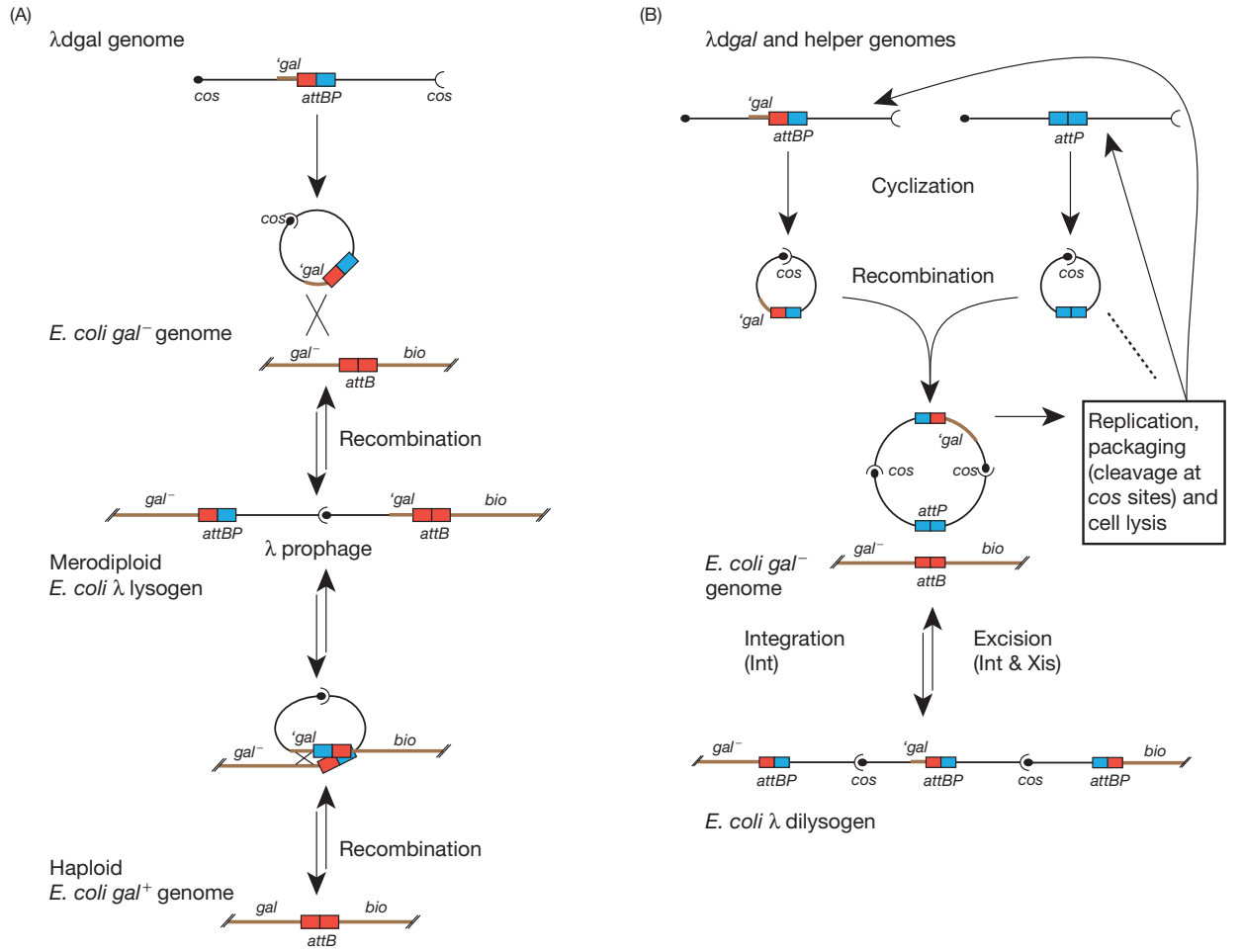

Fig. 3 Transductant formation (merodiploid and haploid) and the formation of high-frequency specialized transducing lysates. (A) Merodiploid and haploid transductants are formed by Rec-dependent recombination between a λ transducing particle and the *E. coli* genome. Here a λd*gal* transducing particle is used for illustration, where '*gal* represents a partial but wild-type gene sequence, and *gal*⁻ represents a complete but mutant *gal* gene sequence. The merodiploid lysogen is formed by a single event of homologous recombination between the shared *E. coli gal* sequences. Alternatively, the '*gal* allele can also end up in the prophage (not shown). By a second event of recombination, the *gal*⁻ allele can be "repaired" by the '*gal* allele, resulting in a haploid *gal*⁺ *E. coli* genome. (B) An *E. coli* coinfected with a λ specialized transducing particle and a wild type λ can form a dilysogen. Here the example of an *E. coli gal*⁻ mutant being coinfected with a λd*gal* and a wild type λ is shown. After the phage genomes have cyclized, they can recombine at shared sequences and integrate together into the host chromosome, in a process catalyzed by Int, resulting in the illustrated dilysogenic state. Prophage induction can lead to excision of the entire dilysogen, giving rise to a high-frequency transducing lysate.

genomes long, depending on the phage. This newly replicated DNA must then be packaged into the phage proheads, which for both P1 and P22 is initiated by the recognition of a specific sequence of the phage DNA, called the *pac* site. The phage terminase protein recognizes the *pac* site and the phage DNA is cleaved at or near this site. This cleaved *pac* end is bound to the large terminase subunit, which is attached to the prohead. This initiates the packaging of the linear DNA concatemer into the prohead. The DNA packaging limit is determined by the size of the phage head; hence the term "headful packaging." Usually a little more than the size of the phage genome is packaged into each prohead. For example, the genome of P1 is 95 kb, but up to 115 kb of DNA is usually packaged per phage head. Therefore, in addition to a single copy of the phage genome, there will also be some extra phage DNA packaged, which becomes terminally redundant. Once the prohead is full, the DNA is cleaved and the cut end of the remaining concatemer is recognized by packaging proteins of the next empty prohead, which starts the next round of headful DNA packaging. By this mechanism, several proheads are filled sequentially from the remaining DNA, with 3–4 headfuls produced on average from one phage DNA concatemer. When the heads have been packaged with DNA, the tails are attached to complete the virions. After this, the host bacterium is lysed by phage-encoded lysis proteins and the newly formed phage particles are released. When they adsorb to a susceptible host bacterial cell, they will inject their DNA into the cell. Because of the terminal redundancy, the ends of the DNA can recombine, and the linear phage DNA undergoes circularization, which protects it from host cell nucleases. The next step will again be the decision between the lysogenic life cycle, with the phage DNA either inserting into the bacterial chromosome (P22) or remaining as a plasmid (P1), or the lytic life cycle and the initiation of the construction of new phage particles.

DNA Packaging in Transducing Particles

As described above, generalized transducing particles arise through a mistake in the packaging of DNA into phage heads, so that the host chromosomal or plasmid DNA is packaged into some phage heads instead of the phage DNA. How often generalized transducing particles are formed depends on the frequency of the DNA mispackaging; normally an infrequent event. Experiments with P22 showed that the transducing particles in a lysate account for approximately 2% of all phage particles in the lysate, and a new analysis of next generation sequencing data from a P1 lysate suggested that 3.8% of the P1 particles carry genomic material from the host, rather than its own phage DNA.

There are two main ways for transducing particles to arise. For P22, the phage DNA packaging apparatus is able to also recognize sequences on the bacterial DNA, which are sufficiently similar to the phage *pac*-sequences. Binding to these *pac*-like sequences, a phage head can be packed with host DNA instead of phage DNA. For P1 however, this phage seems to be able to initiate mispacking of host bacterial DNA from nicks or ends of the DNA. Regardless of the mechanism of initial host DNA recognition, the generalized transducing particles are then filled in the same manner: by headful packaging, followed by DNA cleavage and the filling of the next phage heads from the bacterial chromosome or plasmid DNA. Just as for the packaging of phage DNA, it is the size of the phage head that determines how much host DNA can be packaged, and thereby transduced. Because of different head sizes, P22 can package and transduce approximately 44 kb (around 1% of the host genome) per transducing particle, whereas the larger P1 can transduce around 115 kb (around 2% of the host genome). The *B. subtilis* phage PBS1 has an even larger head and is capable of packaging up to 300 kb (around 8% of the host genome).

Any region of the host DNA can be packaged into a prohead in this way, as long as the DNA fulfills the required criteria (contains a *pac*-like site for packaging by P22, or is cut or nicked if packaged by P1). The frequency of transduction of different regions of the genome can however vary. Because *pac*-like sites might be unevenly distributed along the host DNA, different transduction frequency for different loci is particularly noticeable for generalized transducing particles arising from mispackaging from *pac*-like sites. For this reason, wild type P22 will transduce bacterial loci that are located close to *pac*-like sites with a higher frequency. High-transducing (HT) mutants of P22 display increased and host chromosome-wide efficiency of transduction, which appears to be due to a reduced specificity in *pac*-site recognition. Because of this, the resulting P22 HT particles can transduce different regions of the host chromosome with a similar frequency. Interestingly, the mutations responsible for the HT phenotype of P22 map to gene 3, encoding the small unit of the terminase required for *pac*-site recognition and subsequent initiation of DNA packaging. This shows that mutations affecting packaging specificity can improve and enhance the transducing ability of a phage. These observations support the model for P22 packaging host DNA at sites related to *pac*. Once the DNA has been packaged into a phage head, the only difference between a phage and a transducing particle is the origin of the DNA in the head. Since a transducing particle looks like a regular phage from the outside and is able to adsorb to a new host cell by its tail proteins, subsequent infection occurs as for a normal phage, up to the point of DNA injection into a susceptible host (Fig. 4).

Fate of Transduced DNA

Once the DNA has been injected into a new bacterium, the fate of the transduced bacterial DNA will differ from that of phage DNA. The possible outcomes are listed below and are summarized in Fig. 5.

Abortive transduction

Following DNA injection, the DNA from the majority of transducing particles (90%) will remain extrachromosomal. In this case, the linear DNA is hypothesized to be injected into the cell together with a phage-encoded protein from the prohead. This protein is believed to bind to the ends of the transduced DNA and circularizes it, thus protecting the DNA ends from host nucleases. Studies of phage P1 indicate that the injected DNA in this case is circular in the recipient bacterium, and that the circles can be disrupted by protease treatments, suggesting that one or more proteins are involved in the circularization of the transduced DNA. Genetic studies of P1 and P22 phage mutants with decreased frequencies of abortive transduction have identified phage head proteins to possibly be involved. In the case of phage P1, it has been suggested that the identified protein might also play a role in joining phage heads and tails. For phage P22, a mutation causing a decrease in abortive transduction mapped to a genetic region encoding several proteins that are injected during infection. However, the exact identity and function of these proteins are still unknown.

The circularity of the DNA prevents recombination with the recipient bacterial chromosome, but this DNA may remain stable in this way for several generations and genes that are present on the DNA can even be transcribed. However, since this DNA has no origin of replication, it is unable to replicate, and is therefore inherited by only one single daughter cell following division. It is therefore called abortively transduced DNA. Because of this, any phenotype encoded by this transduced segment of DNA will be apparent in only a small minority of the offspring, giving rise to minute transductant colonies.

Abortive transductant DNA is rarely able to recombine with the chromosome in the recipient cell. However, damage to the transduced DNA may stimulate recombination. Nicks that have formed in the circularized DNA can promote the action of the host DNA repair system, resulting in recombination. For example, it has been shown that UV irradiation of generalized transducing phage lysates achieves a higher rate of stable transductants, by stimulating recombination and by reducing superinfection killing.

Recycling of nucleotides

The DNA injected by a minority of the transducing particles will be left unprotected by the phage proteins that are involved in abortive transduction. This unprotected DNA will face either of two fates. It may be degraded by host cell nucleases into component

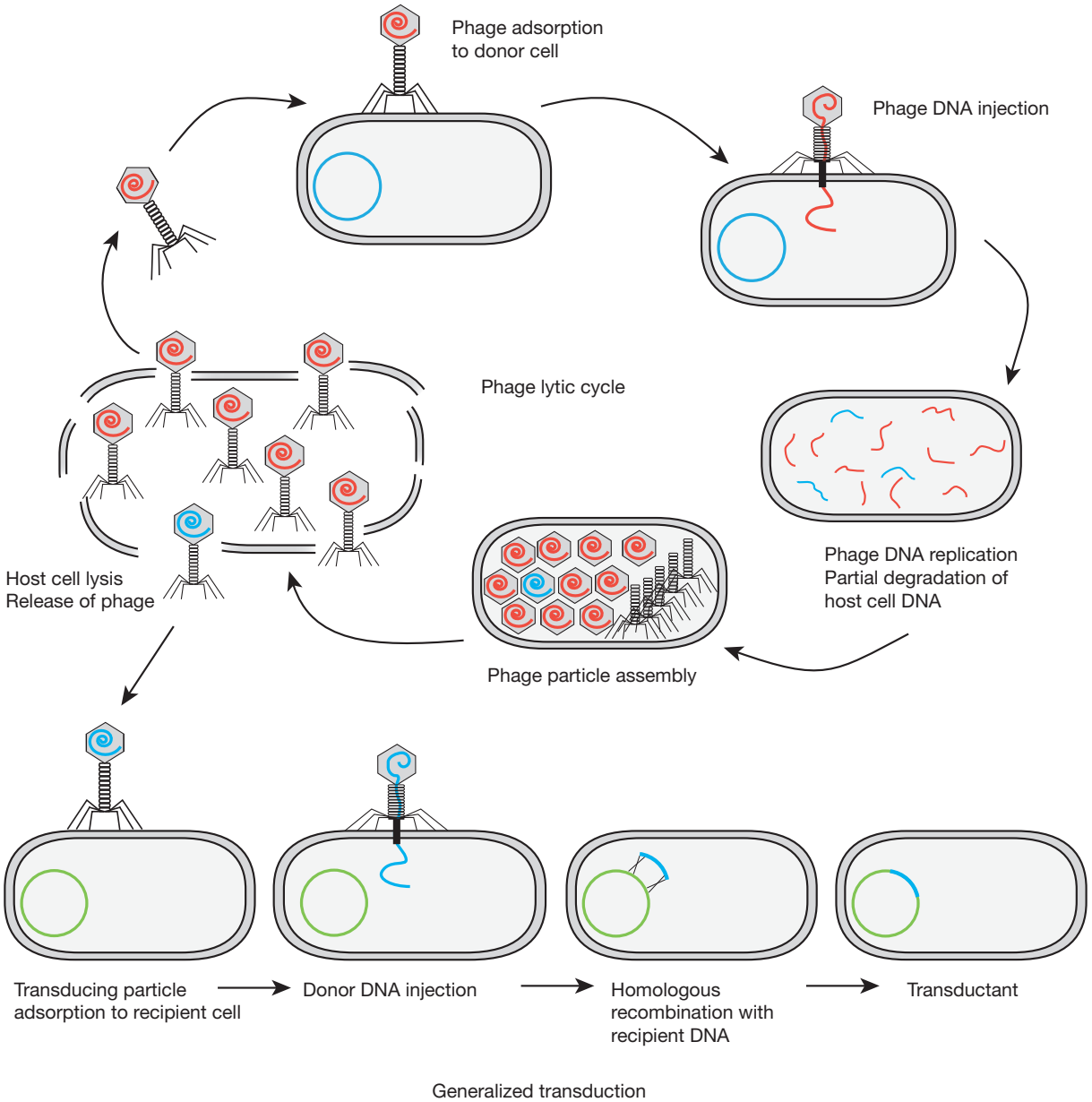

Fig. 4 The life cycle of a generalized transducing phage. During the lytic cycle, the phage adsorbs to the donor bacterium and injects its DNA. The phage DNA (in *red*) is replicated, and the DNA of the donor host (in *blue*) is partially degraded. Phage proteins are expressed, which leads to the assembly of new phage particles and packaging of phage DNA. Occasionally pieces of bacterial DNA can be mispackaged into a phage head. Phage tails are added to the DNA-filled phage heads and the new virion particles are released by cell lysis. Because of the occasional mispackaging, some of the released phage particles will contain host DNA, and are therefore referred to as generalized transducing particles. These transducing particles are still capable of adsorbing to and injecting their DNA into susceptible host cells (the recipients). Since no phage genes are present, the recipient cells will not be lysed. Given sufficient levels of homology, the injected donor DNA can be recombined into the recipient chromosome (in *green*), giving rise to a transductant cell. It is also possible for the transduced DNA to be stably maintained in the recipient cell by mechanisms other than homologous chromosomal recombination, such as the homology-independent insertion of transposons, and various mechanisms of plasmid selection.

nucleotides, for the cell to use for bacterial genome DNA repair. It has been estimated that only 8%–15% of total transduced DNA undergoes this process. The other possible fate of nonprotected transduced DNA is to become stably incorporated into the host genome, which is how transductant bacteria are formed.

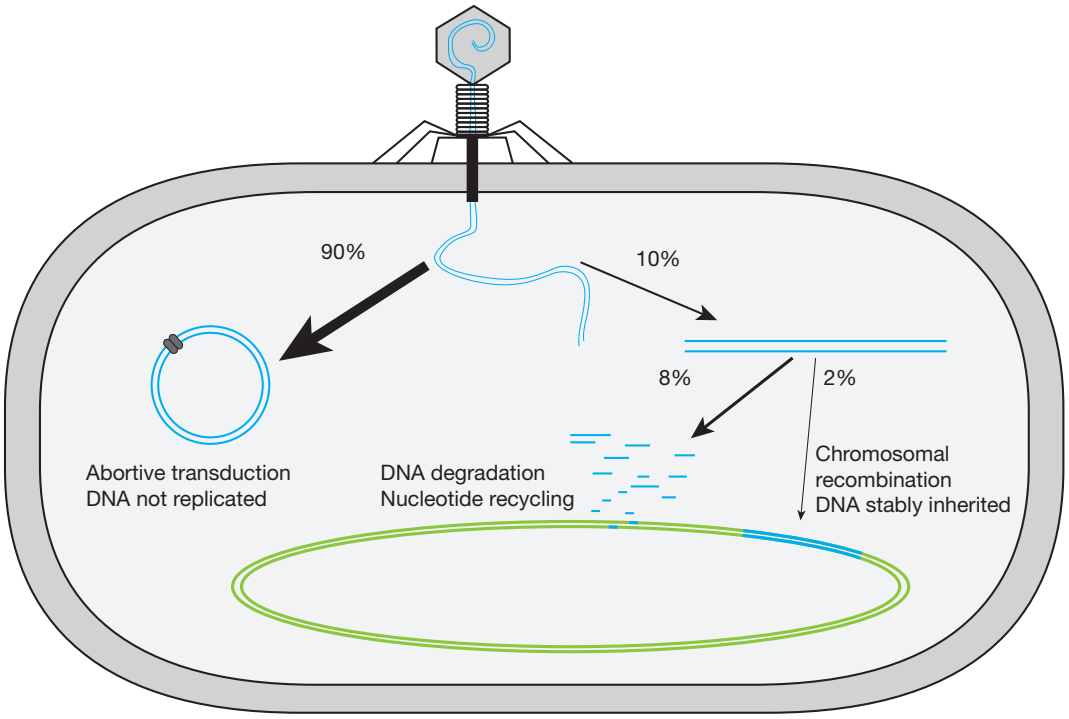

Fig. 5 Possible fates of transferred DNA during generalized transduction. The injected donor DNA can undergo either of three possible fates. The vast majority (estimated to 90%) of the injected DNA will be circularized with the help of phage proteins, thereby being protected from degradation and also from recombination. However, this DNA cannot be replicated and this process is therefore called abortive transduction. Approximately 8% of the injected DNA will be degraded into single nucleotides that will be incorporated into the host chromosome. A small minority (about 2%) of injected DNA will have long (at least 500 bp) stretches of sufficient homology to the recipient genome, and can thereby be recombined into the recipient chromosome.

Chromosomal recombination

The most common way for transduced DNA to be stably incorporated, and thereby inherited, is by recombination into the host chromosome. If the recipient and donor bacterial chromosomes are sufficiently sequence related, the transduced donor DNA can be incorporated into the recipient chromosome by homologous recombination. Usually this process happens within an hour of transducing particle infection of *E. coli* and *Salmonella*. After this time any remaining transduced DNA is usually degraded to nucleotides and recycled, and recombination is unlikely to happen. Homologous recombination and the subsequent formation of stable transductants requires the bacterial Rec proteins to act on the homologous pieces of DNA and replace the equivalent DNA on the recipient chromosome by the donor DNA. This leads to expression of the genes encoded on this stretch of DNA and manifestation of any corresponding phenotypes. Since the transduced DNA is incorporated into the bacterial chromosome, it will be replicated and inherited by all daughter cells, unlike in abortive transduction. Stable recombination into the chromosome is a rare event. For transduced segments of DNA with continuous stretches of at least 500 bp homology to the host chromosome, it has been shown that only about 2% of the DNA injected by transducing particles normally undergoes homologous recombination into the recipient genome.

Transduction of plasmids

Some generalized transducing phage, but not all, are able to transduce plasmids between host bacteria. During this process, the plasmid DNA is packaged into the phage head by normal headful packaging and is injected into the new host by the transducing particle. Once intracellular, the plasmid recircularizes, replicates in the new host and becomes inherited by the daughter cells after cell division. Broad host range phages, which can adsorb to and inject their DNA into even distantly related bacteria, may not be able to successfully transduce chromosomal DNA into recipient bacteria with low genetic identity. However, these phages may still be able to transduce plasmids between genetically diverse bacteria, as this does not rely on homologous recombination and any genetic identity between donor and recipient DNA (see also Transduction as a Genetic Tool).

Development of Other Generalized Transduction Systems

Not all phages that package their DNA by the headful packaging mechanism are natural transducers. As mentioned above, the host genome must not be too highly degraded in order for it to be packaged into transducing particles. For example, the well-characterized *E. coli* phage T4 packages its DNA by the headful mechanism but is not capable of efficient transduction in its

wild-type state. However, this and other virulent phage that use the headful packaging method are obvious targets for engineering into generalized transducing phages. Phage T4 has been modified by mutagenesis of endonuclease genes, thereby preventing the degradation of host DNA upon T4 infection. This results in bacterial DNA being left sufficiently intact to allow mispackaging to occur. As T4 does not require *pac* sites in order to package its DNA, mutant T4 (i.e. T4GT7) is able to package both bacterial and phage DNA equally well, making it an extremely efficient generalized transducer.

Even the temperate phage λ, the classical model for specialized transduction, can be adapted into a generalized transducer, albeit an inefficient one. Still, the necessary adaptations are interesting from a mechanistic point of view. As discussed above, λ does not use the headful packaging mechanism, but instead cleaves the DNA concatemers at specific cos sites found at either end of the phage genome. To initiate headful packaging and DNA cleavage, only one *pac* site (or *pac*-like site) is needed, whereas two cos sites, one at either end of the DNA, are required for DNA packaging in λ. In this system, packaging is initiated by recognition of the first cos site. The second *cos* site is the signal to end the packaging and to cleave the DNA. As with phages dependent on *pac* sites, mispackaging of host DNA can occur if DNA sequences resembling *cos* sites are found on the bacterial chromosome. However, the chances of two *cos* sites being found on the host DNA, exactly the required length apart, are minimal. If the signal to cleave the mispackaged host DNA, the second *cos* site, is absent, a length of DNA will protrude from the prohead. This prevents the tail from binding and functioning transducing particles cannot be formed. However, it is possible to treat a lysate like this with DNase to cleave the excess DNA (which is not being protected by the prohead). After this in vitro treatment, the phage tails are able to spontaneously attach to the proheads, which completes the formation of the generalized transducing particles. However, even with manipulations and treatments, λ is a poor generalized transducer and is mainly used in the laboratory as a specialized transducer.

To search for novel generalized transducers for bacterial strains, a combination of experimental and bioinformatic approaches may be used. For this purpose, headful packaging phages can be enriched for, in an environmental sample, by using their reduced sensitivity to chelating agents. As an example, sodium pyrophosphate has been used to isolate transducing phages for *Streptomyces venezuelae*. As mentioned, the phage terminase protein is involved in the recognition of phage *pac* sequences, initiating the series of packaging events that result in mature phage particles. Therefore, phages with terminases that have reduced *pac*-specificity are predicted to be better transducers, as demonstrated by the observation that P22 HT gene 3 terminase mutant phage is a more efficient transducer compared to the parental P22. With increasing numbers of phage genomes being sequenced, genomic information also could be used in the searches for generalized transducers. There are efforts to use data from next generation sequencing, including the relative frequencies of reads from different genomic regions, to identify phage termini sequences and thereby determine the packaging mechanism of the phage. Using the same sequence data, it has also been shown to be possible to measure the amount of host bacterial DNA in the sample, giving an estimated frequency of transducing particles in a phage lysate. Using genome data and comparative genomics may thus help narrow the search for generalized transducers. To develop and improve generalized transducers in the future, it may be worthwhile to use both rational and randomized mutagenesis approaches to alter terminases (e.g. P22) or genes involved in host DNA degradation (e.g. T4).

Methods independent of genomics have also been devised to increase the frequency of transduction for phages that are poor transducers, or for the transduction of bacterial loci that are normally transduced at low frequencies. As already mentioned, UV irradiation of the donor lysate can increase the frequency of generalized transduction greatly as this treatment leads to the introduction of DNA breaks and by that stimulates recombination between the transduced DNA and the recipient's chromosome. Also, synthetic insertion of a phage *pac* site into the bacterial DNA will greatly increase the transduction efficiency of that region. This is particularly useful for transduction of plasmids, as these are often transduced at lower frequencies than chromosomal markers.

Phage Mu—A Special Case

The highly studied phage Mu is most known for its properties as a transposable element. However, it can also operate as a generalized transducing phage, as well as having some features of a specialized transducing phage. Packaging of Mu DNA into proheads is dependent on a *pac* site, and the DNA is packaged by the headful mechanism. When the genome of phage Mu is packaged it is flanked by host DNA; a short region of up to 150 bp at the left-hand end and a larger region of up to 3 kb on the right-hand end. Since Mu replicates by replicative transposition in the host chromosome, these flanking host sequences are variable. As for other headful packagers, Mu-transducing particles are believed to arise primarily due to the mispackaging of host DNA. However, it cannot be ruled out that at least some transduction events observed for Mu are derived from recombination with host DNA present at the right-hand end, as a version of specialized transduction. It is also possible to perform transduction using mini-Mu, a Mu derivative with the central region of the phage genome deleted, leaving only the ends intact plus the transposase *A* gene (essentially the Mu sequences important for transposition). If the host cell is infected with a helper Mu, the chromosomally integrated mini-Mu can be induced. It will thereby be packaged into a helper Mu-encoded prohead together with parts of the adjacent bacterial chromosome, to a total length of 39 kb of DNA. The vast majority, 90%, of the transductants that arise following infection with this mini-Mu/donor DNA transducing particle, are the result of RecA-dependent homologous recombination with the recipient DNA. The remaining 10% of the transductants result from "mini-Muduction." In this case the donor DNA has a copy of mini-Mu at each end. This Mu-host-Mu DNA can insert anywhere into the recipient DNA, effectively acting as a random insertion of a transposon. Because of this, homology between the donor and recipient DNA is not required for mini-Muduction. This allows transduction/mini-Muduction of DNA between any species of bacteria that are susceptible to Mu

infection. Mu has a relatively broad enterobacterial host range, meaning that it is able to infect and replicate in many different bacteria. This makes it a useful genetic tool, especially for bacterial strains without any other identified generalized transducing phages. Clearly, phage Mu not only functions as a transposable element, but also displays properties of both generalized and specialized transducing phage.

Variations on Transduction

Lysogenic Conversion and "Cargo" Genes

Analyses of the increasing numbers of fully sequenced bacteria (including their prophages) and free phages have revealed that many harbor nonessential genes of putative bacterial origin. Genes on prophages often give an advantage to the bacterial lysogens by the expression of toxins or virulence factors that may aid bacterial pathogenesis. Since the bacterial host is "converted" upon lysogeny, this phenomenon is called lysogenic conversion. Two classical examples are the prophage-encoded shiga toxin produced by certain pathogenic *E. coli* lysogens, and the cholera toxin expressed by *Vibrio cholerae* lysogenized by the CTX phage. There are also examples of phage-encoded gene products, of bacterial origin, that can aid virulent phages during lytic infection. As an example, many cyanophages express photosynthetic proteins during infection of cyanobacteria. These phage-encoded systems have been shown to maintain bacterial photosynthesis during phage infection. This increases biosynthesis in the infected host cell, thereby aiding phage replication. These photosystem genes are bacterial in origin, acquired by phages and then shuffled among the phages by coinfection. In some cases, these genes have been recombined back into some cyanobacteria. The generation of a bacterial recombinant following infection with a virulent phage can only arise when the infection is nonproductive and the phage failed to lyse the host cell. Therefore, it is theoretically possible to define both virulent and temperate phages that carry these "cargo" genes as a form of potential, and environmentally-important, transducing phages, since these genes may be acquired from, and maintained in, infected bacteria. It is interesting to consider how these "cargo" genes might have been acquired by the phages (e.g. by illegitimate recombination and/or incorrect prophage excision). This sort of genetic promiscuity and modularity in phage genomes illuminates the dynamic evolution of phages and their hosts, resulting in a "web of life," rather than a "tree of life" (see also Transduction in the Environment).

Auto-Transduction

Even in bacterial populations without free phages initially, transduction can occur. In a mixed community, where one subpopulation carries prophages and one does not, the prophage-free subpopulation will be susceptible to phages released as a result of prophage excision from the other subpopulation. This can lead to lysis of the prophage-free subpopulation and the mobilization of its genes. The resulting transducing particles might then re-infect the lysogenic subpopulation and insert their DNA, which will include some genes from the competing bacterial population. Since the lysogenic subpopulation is resistant to lysis by what is effectively their own prophage, this results in the prophage-carrying subpopulation ending up with newly acquired genes, which may encode resistance to antimicrobials. Furthermore, the competing prophage-free subpopulation ends up decimated by lysis, which is a further advantage for the lysogenic population. This has been demonstrated in mixed populations of *Staphylococcus aureus*, both in vitro and in vivo in a wax moth larvae system.

Clearly, phages and prophages are constant sources of genetic mixing that often blurs the boundaries between what are considered phage and bacterial genes. When reassessed in this broader context of general phage evolution, many more phages might be considered as transducers, albeit at a very low frequency compared to the well-characterized classes of specialized and generalized transducing phage.

The Influence of CRISPR-Cas Adaptive Immunity on Generalized Transduction

Bacteria have a range of mechanisms to resist phage infection and one of these is the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associate systems. These provide a sequence-specific memory of past viral invaders and, through the use of small guide CRISPR RNAs (crRNAs), they use nucleases to destroy the invading phage nucleic acids. CRISPR-Cas also limits horizontal gene transfer by impeding transformation and conjugation. Recently, it has been shown that by providing resistance against wild-type phages, CRISPR-Cas increases the success of transductants and therefore can positively influence generalized transduction. In rarer cases when spacers are acquired that match the transduced bacterial DNA (instead of the phage genome itself), gene transfer is inhibited. Generalized transduction has also been observed to mobilize the CRISPR sequences and *cas* genes and thus, can lead to the spread of phage resistance. Given the widespread distribution of CRISPR-Cas immune systems in approximately half of bacteria, these systems will likely be having a significant role in generalized transduction and the dissemination of genetic material in global ecosystems.

Transduction as a Genetic Tool

Ever since the discovery of phages, and continuously with the growing knowledge of phage biology, researchers have developed the potential for practical use of phages (and their products), to aid molecular biology and its applications in biotechnology and

synthetic biology. Examples of these tools and applications include phages as antibacterial agents (e.g. for selection of bacterial mutants in the laboratory, or as agents for biocontrol and for phage therapy), as DNA vectors for expression of particular genes in host bacteria (e.g. for transposon delivery or reporter gene expression), and as components of DNA cloning, integration, and expression systems. These cases represent just a handful of phage uses, with those related to their transducing (especially generalized) properties being covered here. In addition, the understanding of various bacterial antiphage mechanisms have been exploited for practical use (such as restriction enzymes and the CRISPR-Cas technology), which however are beyond the scope of this article.

Isolation and Characterization of Generalized Transducing Phage

In order to use a generalized transducing phage for any bacterium, the first requirement is to isolate a phage with transduction capacities, for the bacterium of interest. There is extensive evidence of the ubiquitous and vast distribution of phage in the natural environment, and estimates suggest that there are approximately 10 phages for each bacterium on Earth. To isolate phages, it is therefore a good starting point to use material from where the host bacterium was found. For enteric bacteria for example, sewage water and sludge are good sources of phages. By sampling several sources, using the target bacteria as an indicator, phages infecting many bacterial genera have been isolated. The phages are then screened for transducing ability. To do this, a phage lysate is prepared on a donor strain of the susceptible bacterium that harbors a selectable marker, for example an antibiotic resistance-encoding transposon. Transduction is then performed into a wild-type recipient (near-isogenic to the donor), selecting for the marker. Any putative transductant colonies can then be screened for, for example, by PCR if the exact genetic insertion site of the selectable marker is known. If the selectable marker is inserted in a gene encoding a known phenotype (e.g. an auxotrophy), coinheritance of the expected phenotype can be screened for directly by growth on minimal media. As a complement to the transduction of chromosomal markers, the ability to transduce plasmids can be tested. This is done much in the same way, using selectable markers, with additional screening for physical evidence of the plasmid DNA in the putative transductants, for example by PCR or restriction digestion of isolated plasmid DNA. Transduction of multiple loci is required to confirm isolation of a generalized transducing phage.

Using similar strategies, generalized transducing phages have been isolated for species and strains of many different bacterial genera, including *Bacillus*, *Caulobacter*, *Citrobacter*, *Dickeya*, *Lactobacillus*, *Myxococcus*, *Pantoea*, *Pectobacterium*, *Pseudomonas*, *Salmonella*, *Serratia*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*. The isolation and use of generalized transducing phages have rendered the respective host organisms more genetically amenable, and therefore easier to study. Below, the most common uses of generalized transducing phage are discussed.

Strain Construction

To perform genetic manipulations and genetic analyses of bacteria, the ability to transduce loci using selectable markers is useful. The power of generalized transduction can be seen particularly by its use in combination with transposon mutagenesis or the localized insertion of selectable markers (by e.g. recombineering). Typically, following transposon or recombineering mutagenesis, it is necessary to confirm the presence of the insertion, which can be done by PCR analysis. However, it is also formally possible that secondary mutations may have arisen in the mutant strain during the insertion of the marker. To obtain a strain with the selectable insertion in a background free from any unwanted mutations present in the original strain, generalized transduction can be used to move the insertion of interest. Coinheritance of the marker and the associated phenotype studied confirms that the appropriate selection cassette responsible for the phenotype in question. Furthermore, with the use of a variety of selectable markers, the construction of double and multiple mutant strains by generalized transduction is comparatively facile.

Localized Mutagenesis

To examine gene function in detail it is necessary to analyze the effects of mutations, by comparing the phenotype of the mutant to that of the corresponding wild type. There is often value in studying more subtle mutations than those generated by knockout or transposon disruption, such as point mutations or small insertions or deletions. It is possible to perform localized mutagenesis by error-prone PCR of the locus of interest, followed by a strategy to recombine the mutated sequences into the bacterial chromosome. For some bacterial species it is also possible to directly introduce the desired small or large mutations by the use of lambda red-based recombineering (another phage-based cloning strategy). These are powerful and "clean" techniques, but do rely on suitable delivery (e.g. transformation and/or conjugation) systems for the organism studied. To avoid this potential problem, it is also possible to subject the bacterium to mutagenesis by UV irradiation or chemical treatments, followed by a screen for mutants. However, after a general mutagenesis, any mutants with interesting phenotypes may also contain other mutations elsewhere in the genome, complicating further analysis. If the gene of interest is located in the vicinity of a selectable marker, it can be transduced from the mutated strain into an unmutagenized background. These transductants can be selected for using the resistance marker and, by linkage, screened for the phenotype attributable to nearby mutations in the gene of interest. Similarly, a transducing lysate, prepared on a strain with a selectable marker close to the gene of interest, can itself be exposed to mutagenic agents such as hydroxylamine or nitrous acid. By this method, only the DNA packaged within the transducing particles is mutated, which means that any mutations introduced will be linked to the selectable marker.

After the transduction, and with the help of cotransduction frequency, interesting mutations can be identified (e.g. by sequencing or phenotyping), as the stretches of DNA transferred by transduction are relatively short, thereby enabling a "localized" mutagenesis.

Genetic Mapping

Genetic mapping by transduction has been used historically in *E. coli*, *Salmonella* and in other bacteria to determine the fine genetic structure of closely linked genes and mutations within genes. With the now cost-effectiveness and ease of whole genome sequencing, there is now little need for these transduction-based genetic mapping experiments. However, the basic principle of transductional mapping is the measurement of the genetic linkage of loci, which can be assessed by the cotransduction frequency. Simply, if two mutations are linked (both exist within the DNA packaged in the phage capsid), they can both be recombined into the recipient chromosome (cotransduced) at a particular frequency reflecting linkage. The linkage depends on the distance between the loci and the number of recombination events required to isolate cotransductants. Likewise, an approximate distance (in genetic base pairs) can be calculated if the linkage is measured and the size of the DNA packaged into the heads of the specific phage is known.

Plasmid Transduction

For many genetic modifications and analyses in bacteria, it is necessary to introduce plasmids into recipient cells. For plasmid transfer, conjugation and transformation are usually the systems of choice. However, phage-mediated plasmid transduction can also be useful, especially for bacterial strains that have no conjugal plasmids or that have poor transformation efficiency. It can also be efficient in practical terms, as one lysate can be used to transduce the same plasmid into several recipient strains, without the need to prepare competent cells. Many generalized transducing phages are proficient in plasmid transduction. However, the efficiency varies depending on the phage and on the plasmid to be transduced. The currently accepted model is that long stretches of double-stranded plasmid DNA, formed by rolling circle replication, can be accidentally packaged into the phage proheads. After injection of the plasmid DNA into the new host, the recipient bacterium uses homologous recombination to regenerate the circular plasmid. For wild-type phage P22 to efficiently transduce pBR322, it is necessary to first introduce a *pac* sequence to the plasmid. If no *pac* sequences are present on the plasmid, pBR322 requires cloned P22 DNA sequences for efficient transduction by wild-type P22. For this, an initial homologous recombination step between P22 and the plasmid in the donor is required prior to transduction into the recipient. The dramatic increase in transduction efficiency, seen when a portion of phage DNA is cloned into plasmids, has also been observed for other bacteria and their phages, including species of *Bacillus*, *Lactobacillus*, *Staphylococcus*, and *Streptomyces*. A commonly used P22 HT derivative on the other hand, which has a reduced packaging specificity, can transduce pBR322 without any added *pac* sequences.

The plasmid transduction mechanism has also been analyzed for a T4 phage modified to be proficient in generalized transduction (see Generalized Transduction). In order to transduce pBR322, this modified T4 packages plasmid multimers equivalent of 38 monomers. To establish in the recipient host, the plasmids are separated by homologous recombination. Phage P1 is able to package plasmids that contain a P1 *inc* site. Because P1 has a broad host range (see below), it can transduce packaged plasmids into a variety of strains.

Intergeneric Gene Transfer

Some phage–host receptor interactions are highly specific, allowing a phage to only recognize a single strain of a given bacterial species. Other phages have broad host ranges, with P1 and Mu being examples. There are obvious benefits of isolating a broad host range transducing phage, where it may enable transduction within and between multiple bacterial isolates. Phage P1 has a genetic region that, when inverted, can switch expression between two alternative tail fiber proteins, a major determinant in phage–host interactions and specificity. Even though P1 is commonly used as an *E. coli* phage, depending on what tail fiber form is expressed, it can adsorb to, and replicate in, strains of *Citrobacter*, *Enterobacter*, *E. coli*, *Pectobacterium*, *Klebsiella*, *Pseudomonas*, and *Salmonella*. P1 is also able to inject its DNA into strains of *Agrobacterium*, *Flavobacterium*, *Myxococcus*, and *Vibrio* but cannot produce phage progeny in these hosts. Plasmids have been introduced into *Myxococcus* by P1 transduction, where they are unable to replicate (unless also carrying a *Myxococcus* plasmid origin of replication). The fact that the host range of P1 for adsorption is broader than the range of species where an *E. coli* plasmid origin is functional for replication is the basis of a suicide vector delivery system that is used for transposon mutagenesis and targeted gene disruptions in these bacteria.

Phage P1 has also been used to perform intergeneric gene transfers between *E. coli* and *Salmonella*. Historically, some researchers used transduction efficiency as an approximate indicator of the level of DNA homology between donor and recipient. However, due to the large genomic differences between these genera and because long stretches of near-identical sequence are needed for efficient homologous recombination, these intergeneric transductions are often unsuccessful. The ribosomal RNA loci (*rrn*) are genomic regions where such long stretches of homology can be found. Therefore, analysis of intergeneric transductants frequently showed that recombination occurred at these conserved loci, often resulting in large genome alterations. It is now known that the recipient's methyl-directed mismatch repair system is responsible for some of the stringency of recombination and, for that reason, mismatch repair mutants are more efficient recipients of donor DNA from other genera. Because of the inefficiency and possible off-target effects, intergeneric transduction in a laboratory setting is not widely used outside of *E. coli* and *Salmonella*. However, intergeneric

transduction does have important roles in the ecology and evolution of bacteria in their natural environments, something further covered in the section Transduction in the Environment.

Lambda as a DNA Delivery Vehicle

As previously discussed in this article, phage lambda is an efficient specialized transducer. It has also been developed to function as an efficient tool for the introduction of DNA into host cells. Methods developed include the packaging and transduction of cosmids and transposons by λ into target cells. Cosmids are plasmid cloning vectors that contain λ *cos* sites (giving them their name). By in vitro ligation of large chromosomal DNA fragments (up to 47 kb) into cosmids, genomic libraries can be generated, which are useful for studies of complementation and overexpression, for example. The cosmids are packaged into λ capsids in vitro, by the recognition of, and cleavage at, the consecutive *cos* sites. Phage tails are then attached, making the completed particles able to transduce the cosmids into a suitable recipient. In the recipient cell the cosmid is replicated by virtue of a plasmid origin of replication. If these cells are infected with λ, the cosmids will be further packaged in vivo, which bulks up a lysate of λ particles harboring the cosmid library.

By a similar mechanism, λ particles modified to also carry a transposon (e.g. Tn5, Tn10, Tn*phoA* or Tn*blaM*) can be used to deliver the transposon into a target bacterium. The resistance encoded on the transposon can be used to screen for mutagenesis events by transposition. For the use of this method in *E. coli*, λ replication is inhibited by amber mutations in the phage morphogenesis genes, whereas in some other genera (see below) wild-type λ is unable to replicate and can therefore be used for transposon delivery. Compared to plasmid-based mutagenesis procedures, aided by conjugation, λ-based systems are faster and have no requirement for counterselection of donor bacteria.

Lambda requires its outer membrane receptor, the maltose transporter protein LamB, for adsorption to the bacterial surface, something that originally restricted its host range and use as a genetic tool. However, by expressing the *E. coli lamB* gene from a plasmid in other Gram-negative bacteria, a wider range of hosts for λ adsorption and DNA injection has been generated. Lambda is able to infect bacteria that are capable of expressing, transporting and assembling the LamB protein in the outer membrane. The expression in *trans* of *E. coli lamB* has enabled λ to infect several strains of various genera, including *Agrobacterium*, *Pectobacterium*, *Klebsiella*, *Mesorhizobium*, *Pseudomonas*, *Salmonella*, *Serratia*, *Vibrio* and *Yersinia*. This makes the delivery of cosmids and transposons relatively straightforward in these systems, without lytic replication of λ. Interestingly, it is also possible to extend the λ host range so that λ virions can be taken up by certain eukaryotic cells, in culture as well as by antigen-presenting cells in vivo. This makes it possible to deliver cosmids and other vectors into eukaryotic cells for expression studies and vaccine delivery.

Transduction in the Environment

By comparative genomics analyses of data from the increasing numbers of sequenced bacterial genomes, it is now clear that horizontal gene transfer (HGT) accounts for a large degree of genetic diversity found within bacterial species. Phages are believed to be the most abundant biological entities on Earth, with estimates of 10^{31} phages on the planet, 10 times more than the number of bacteria. It has been estimated that the number of transduction events per year is 10^{14} in the Tampa Bay Estuary and 10^{13} in the Mediterranean Sea, demonstrating how important phage-mediated transduction might be for HGT in natural environments. Indeed, nearly all sequenced bacteria have prophages and prophage-like elements in their genomes, and these regions are often associated with adjacent horizontally acquired regions and the previously mentioned "cargo" genes of bacterial origin. Some of these phage-transferred regions encode bacterial virulence factors that can convert the host into a pathogenic strain, in the process of lysogenic conversion. For example, it is well known that the cholera toxin is encoded on a phage, CTXΦ, and infection of a nonpathogenic *Vibrio cholerae* strain with this phage can render it virulent, increasing the spread and fitness of the bacterium. Additionally, a generalized transducing phage has been shown to transduce the *Vibrio* pathogenicity island, encoding the receptor for the CTXΦ, the toxin-coregulated pilus. This allows CTXΦ to adsorb to and infect strains that normally are resistant to this phage, illustrating how HGT promoted by phage transduction plays an important role in forming the genetic diversity that is key to evolution.

Since transduction frequently relies on homologous recombination between highly related sequences, it has been difficult to determine its exact impact on bacterial genomes. With the recent advances in analyses of whole genome information, there are now a number of softwares available for comparing closely related bacterial strains, and to identify recombination events that have occurred between them. However, since recombination can result from either of the mechanisms for horizontal gene transfer, the specific importance of transduction in bacterial evolution is mainly inferred from a combination of laboratory experiments, an understanding of the global abundance of phages, and the detection of transduction in the natural environment. Some examples of this are described below.

Several studies have demonstrated that transduction of both chromosomal and plasmid DNA can occur in a variety of natural environments. Transduction of both plasmid and chromosomal markers between strains of *P. aeruginosa* has been shown in freshwater environments, as well as between bacteria on leaf surfaces, even when the donor and recipient were originally on different plants. Phages with broad host ranges have been shown to transduce plasmids among diverse bacteria in natural populations of both fresh and marine water environments. The term "silent transfer" has been suggested to describe the

phenomenon where a phage is able to transduce chromosomal or plasmid genetic information from a donor to a recipient, but is unable to plaque on the recipient. This has been seen for example in phages capable of a variant of generalized transduction of *S. aureus* pathogenicity islands (SaPIs) to other nonaureus *Staphylococcus* strains as well as to *Listeria monocytogenes*, where the phages were only seen to form plaques on *S. aureus*. The transduction of pathogenicity islands into *L. monocytogenes* could also take place in raw milk, environmentally and economically important as both *S. aureus* and *L. monocytogenes* can cause bovine mastitis. It is known that SaPIs and other "phage-inducible chromosomal islands" (PICIs) can be transferred between bacterial strains and sometimes across species barriers, by the exploitation of certain temperate helper phages. PICIs can be induced by the presence of active phages, leading to the PICI DNA being excised, replicated and packaged into phage capsids encoded by the active "helper" phage. After host cell lysis and virion release, the PICIs can be transferred with high frequencies to new hosts, much like in the case of generalized transduction. Pathogenicity islands like this often encodes genes that are nonessential for PICI transfer, but that may increase the fitness of the host; such as toxins, iron transport proteins, biofilm-associated proteins and antibiotic resistance. These examples therefore highlight that transduction in the environment might directly influence human and animal health.

A recent study showed that infection and lysis by phages also could promote plasmid spread by transformation, in addition to by transduction. So-called "superspreader" phages were shown to lack genes encoding hydrolytic endonucleases, resulting in them not degrading host cell DNA during the infection. After cell lysis, naturally competent neighboring bacteria could take up the released plasmids. By this mechanism, phages are hypothesized to promote the spread of plasmids to bacteria beyond their host range, adding to the intergeneric transfer of genetic material. Instead, the host range of the released plasmids and the occurrence of naturally competent bacteria would be the limiting factors for this version of phage-promoted horizontal gene transfer.

Transduction of Antibiotic Resistance Genes

Antibiotic resistant bacteria are an increasing problem, today causing approximately 700,000 deaths per year globally, something that has been projected to increase to 10 million deaths per year in 2050. Genes for antimicrobial resistance can be spread between bacterial strains and over species borders, on plasmids and as chromosomal cassettes. Some of this spread is due to conjugation of mobilizable plasmids, but recently the importance of transduction as a driving force for the spread of antibiotic resistance has gained attention.

For the clinically relevant species *S. aureus*, phage-mediated transduction has been suggested as the main contributor to horizontal transfer of resistance genes. Prophages are very common in populations of *S. aureus*, with cells typically harboring one to four prophages. Subinhibitory concentrations of some classes of antibiotics can induce the bacterial SOS response and thus trigger the release of prophages (which in turn might trigger the mobilization of PICIs) and increase the rate of transduction within the community. Such subinhibitory concentrations of antibiotics could be found in less accessible parts of the human body, as well as in aquatic environments. Transduction of plasmids, encoding resistance to β-lactam antibiotics and to tetracycline, has under laboratory conditions been detected at high frequencies in a methicillin resistant *S. aureus* (MRSA) strain, leaving the strain resistant to multiple drugs.

Similarly, clinical strains of the opportunistic pathogen *Enterococcus faecalis* frequently carry prophages that can be activated and isolated. These phages are capable of transducing chromosomal markers and plasmids between different strains of *E. faecalis*. At least one lytic phage isolated from natural environments has been reported to be capable of transducing antibiotic resistance between different species of *Enterococcus*, including *E. faecium*, a species termed "highly important" by the World Health Organisation.

A more sequence-based approach showed that the metagenome of the fecal phage population in antibiotic-treated mice showed elevated resistance-coding genes, compared to the non-treated control. Naïve bacteria exposed to the phage population from the treated mice were also shown to acquire an increased frequency of resistance to the relevant antibiotic, compared to naïve bacteria exposed to phages of untreated mice. These experiments suggest that not only are resistant gut bacteria selected for upon exposure to antibiotics, but also the fraction of phage-carried genes encoding resistance is increased. Hence the resistance genes are both selected for as well as mobilized during the course of antibiotic treatment.

Many phages are stable and resistant to degradation in the environment, particularly in comparison to naked DNA. Therefore, transducing particles are a comparatively stable reservoir for bacteria DNA. Given these observations, and considering the vast global abundance of phages, it seems possible that phage-mediated transduction events in the environment are likely to be a major force driving HGT and adaptive evolution of bacteria.

Conclusions

The phage-mediated transfer of bacterial DNA between donor and recipient cells is called transduction. This is a form of horizontal gene transfer with implications for bacterial evolution in the natural environment. Studies of transduction have also furthered our knowledge of phage genetics and the molecular biology of phages and their hosts, as well as providing the basis for extremely useful laboratory tools. The current understanding of the molecular mechanisms of transduction has been driven by studies of a small number of "model" phage–host systems. With advances in molecular biological techniques (many of which are themselves derived from the products of phage research), it is important not to forget the power of simple genetic techniques that exploit phages. Indeed, development of a generalized transducer is still an extremely useful tool for genetic analysis of any bacterial strain. A recent

stimulation in phage research and the birth of the "-omics era" has provided major advances in knowledge of the genomes, evolution, ecology, and diversity of phages. It is anticipated that the recent rejuvenation in phage biology research will continue to further our appreciation and understanding of transduction as a tool for bacterial geneticists, the direct impacts of transduction on infections and human health, and transduction as an important evolutionary force in the adaptation of phage and their bacterial hosts.

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Translational Control and Fidelity☆

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Glossary

Ribosome A molecular machine composed of multiple RNAs (ribosomal RNAs) and proteins that consists of two subunits, the small and large subunits each of which catalyze one of the two enzymatic functions of the ribosome: decoding in the small subunit and peptide synthesis in the large subunit.

Scanning The most common mechanism of translational initiation in eukarya in which a pre-initiation complex of the small ribosomal subunit bound to the initiator tRNA and translation factors binds to the 5′ end of the mRNA and moves in a 5′ to 3′ direction until it encounters an AUG initiation codon, where it initiates translation.

Internal initiation Initiation by binding to a site in the middle of an mRNA molecule; the common mechanism of initiation in bacteria and archaea but an alternative mode in eukarya requiring specialized mRNA sequences.

Translational repression Regulation of gene expression by blocking translation of an mRNA, which commonly involves binding of a protein factor to a site overlapping or upstream of an initiation site.

Translational coupling A mechanism of translational initiation common in bacteria in which a ribosome terminates translation and reinitiates translation at a nearby initiation codon.

Recoding A non-canonical translation event involving readthrough of a nonsense codon, frameshifting or bypassing of an mRNA segment during translation elongation.

StopGo A mechanism that occurs at sequences encoding proline-glycine-proline where translation terminates at the glycine codon, with release of the protein product, and then immediately reinitiates at the adjacent proline codon to continue and produce a second protein in approximately equimolar amounts.

Introduction

A molecular machine governs the process of transfer of genetic information from nucleic acids to proteins, termed translation. This machine, the ribosome, is a ribonucleoprotein complex composed of several RNA molecules and several dozen associated proteins. The ribosome choreographs the movements of a large assembly of other proteins and RNA molecules using an RNA template, the messenger RNA (mRNA), as a guide for the order of insertion of amino acids into a growing polypeptide chain. The process is complex and living cells depend absolutely on its speed and accuracy. Control mechanisms have evolved that manipulate the process of translation to modulate the production of specific protein products, or of generic classes of proteins.

Translational control of gene expression falls into here are two general categories: regulating the amount of protein expressed from a particular gene, or regulating the structure of the protein product. The first has to do with the speed of translation. Gene output responds to changes in either the rate that mRNAs are recruited to the ribosome (regulation of initiation) or the rate of completion of nascent polypeptide chains (regulation of elongation). Translational control may act globally, for example by activating or inactivating accessory proteins (translation factors) required for efficient translation, or locally, by stimulating or inhibiting translation of single mRNAs. Some genes have evolved processes that allow the expression of alternative protein products by programming a non-standard translational event. Some genes specify decoding of a termination codon as an amino acid with continued decoding past the stop (readthrough), while others allow translation to shift into an alternative reading frame (frameshifting); in either case the result is expression of an alternative protein product. These processes are not 100% efficient so a given mRNA can program the expression of one protein by the canonical translational pathway while encoding an alternative structural form of the protein using a non-canonical translation event.

Mechanism of Translation

The mechanism of translation is conserved throughout all living things, though it differs in various details between the three domains—bacteria, archaea and eukarya. In general, translation in bacteria and archaea is somewhat simpler than in eukarya. In all three domains the ribosome is divided into two dissociable subunits. The large subunit in bacteria and archaea include two ribosomal RNAs (rRNAs), the 23S and 5S, while eukarya encode three: 26-28S, 5.8S and 5S. In all three the small subunit includes a

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single rRNA, 16S in bacteria and archaea and 17-18S in eukarya. All ribosomes include many ribosomal proteins with bacteria having about 57, archaea about 68 and eukarya about 78 ribosomal proteins. Translation in each domain requires a different number of translation factors, accessory factors that facilitate the function of the ribosome. Bacteria require only three initiation factors, three elongation factors and four factors at termination. Eukarya and archaea have similar numbers of elongation and termination factors but require far more initiation factors, with eukarya requiring the most. Thus, translation in eukarya appears to be the most complex. This may partly reflect the greater physiological complexity of eukarya, especially metazoans, in general, but in addition the process of translation in eukarya is intrinsically more complex.

Translation Initiation

Initiation is the step of translation that differs the most markedly between the three domains. The mechanism used to specify the site of translation initiation has profound consequences for initiation control mechanisms. Bacteria and archaea use a different mechanism of initiation from both eukarya. In bacteria and archaea the ribosome can identify and directly bind to a site of translation initiation while in eukarya the ribosome generally identifies the initiation codon by binding to the 5′ end of an mRNA and moving along it in a 5′ to 3′ manner, called scanning, until it encounters an initiation codon. Given the very different mechanisms employed, it should not be surprising to find that regulation of translational initiation also differs markedly. For example, mRNAs in bacteria and archaea are polycistronic because the ribosome can bind directly to internal initiation sites in the mRNA. Because eukarya scan for initiation sites, nearly all eukaryotic mRNAs are monocistronic. Eukarya have membrane-bound nuclei that physically separate the processes of transcription and translation while in the other two domains the processes occur in one compartment. This fact means that cross-talk between transcription and translation occurs in bacteria and archaea but not in eukarya. The possible mechanisms of translational control in the three domains are thus restricted by differences in structure and function.

Ribosomes use transfer RNAs (tRNAs) to perform the decoding of mRNA sequences into a polypeptide product. Each tRNA can have a single amino acid covalently added to its 3′ end in a process called charging or aminoacylation. Each tRNA also includes a three nucleotide sequence (anticodon) that base pairs with one or more complementary sequences in the mRNA (codons). The ribosome insures that the correct tRNA is recruited to each codon and therefore that the correct amino acid is used in the process of protein elongation. The ribosome has three binding sites for tRNAs and each tRNA successively recruited to the ribosome proceeds through each of these three sites to perform distinct functions before it dissociates to be recycled and used again. The first site is the acceptor site (A-site) to which aminoacyl-tRNAs (aa-tRNAs) are recruited. The second is the peptide site (P site) to which the tRNA carrying the growing peptide chain is bound. The third is the exit site (E site) from which tRNA that have donated their amino acid to the growing peptide chain can dissociate to be re-aminoacylated so that they can be reused in translation.

Ribosomes cannot autonomously identify initiation codons, but rather require accessory initiation factors. Bacteria have three initiation factors, IF-1, IF-2 and IF-3, that interact with the 30S ribosomal subunit during initiation. IF-1 and IF-3 prepare the 30S subunit to initiate translation. IF-1 binds to A site of the ribosome and blocks premature association of aa-tRNAs to the A. IF-3 binds to the 30S subunit and blocks it from associating with the 50S subunit prematurely. IF-2 forms a ternary complex with the initiator tRNA (f-Met-tRNA) and GTP to promote its association with the P site of the 30S subunit. This ternary complex also directs tRNA binding to an initiation codon, most often AUG or GUG. GTP hydrolysis by IF-2 provides an error-correction function; hydrolysis only occurs when tRNA binds to a correct initiation codon. After GTP is hydrolyzed to GDP, the initiation factors dissociate and the 50S ribosomal subunit joins with it to form a complete ribosome. This allows the start of the elongation phase of translation by recruitment of another aa-tRNA to the A site.

No codons are dedicated exclusively to initiation. Instead, codons used during initiation are also used in the middle of structural genes. In addition, these codons may occur in untranslated regions or out of the normal reading frame used in translation. The ribosome must identify the small proportion of these codons at the beginning of structural genes, and ignore all others. Prokaryotic ribosomes directly participate in start site selection using a sequence near the 3′ end of the 16S rRNA that is complementary to a sequence immediately upstream of true initiation codons. The upstream sequence is termed the Shine-Dalgarno (SD) box, commemorating the pair or researchers who first identified it. A correct initiation complex requires both tRNA to bind to an initiation codon and Shine-Dalgarno pairing to form immediately upstream. The distance between the two sequences is critical. Increasing or decreasing the distance between the initiation codon and the SD-box can drastically reduce translational initiation. This interaction is a potent target for translational regulation since blocking the SD-interaction can reduce initiation efficiency.

Because eukaryotic ribosomes generally bind scan to and initiate the first AUG codon in an mRNA, they need no further sequence information to identify initiation codons, and therefore do not require a Shine-Dalgarno interaction. It is critical, however, that eukaryotic ribosomes correctly identify the 5′ ends of true mRNAs. To allow their identification, eukaryotic mRNAs have diagnostic structures at their 5′ ends, a 5′-5′ triphosphate linked guanosine, termed the 5′ Cap. This structure is unique to eukaryotic mRNAs and is added post-transcriptionally to them but not structural RNAs. An elongation factor, a multi-protein cap recognition complex, eIF4F, binds to the structure and directs the binding to the mRNA of a eukaryotic initiation complex consisting of the small ribosomal subunit bound by a large number of protein factors. One of these factors is eIF2. Like IF-2, it forms a GTP-containing ternary complex with the initiator tRNA, called Met-tRNA. Hydrolysis of GTP is again coupled to dissociation of the bound initiation factors, recruitment of the large subunit, and the start of elongation. A second important initiation factor, eIF3, like IF-3, probably facilitates binding of the eIF2 ternary complex to the ribosome and insures the dissociation of the subunits, and is therefore critical to efficient translational initiation. In addition to these factors, eukarya employ many other initiation factors to facilitate scanning, initiation codon recognition, GTPase activation, factor release, and regeneration of the GTP form of eIF2.

Despite the fact that archaea initiate more like bacteria, archaeal initiation factors are more closely related to the eukaryotic counterparts. Because archaea do not have 5′ Caps on their mRNAs or employ scanning, they lack the eIF4F factor. They do have homologues of the eIF2 factor and several other eukaryotic factors involved in initiation codon recognition, GTPase activation and factor release. These facts imply that archaeal initiation employs features of initiation in each of the other two domains, and that these mechanisms must have been present in the last common ancestor of the three domains of life. Bacteria and eukarya have since diverged to have very different initiation mechanisms while archaea have retained elements of each.

Elongation and Termination

The elongation phase of translation is universally conserved. Elongation involves the repeated addition of amino acids to a growing polypeptide chain. This addition occurs in the context of the three tRNA binding sites of the ribosome, the A, P and E sites. Amino acids to be added to the growing peptide chain are recruited to the ribosomal A site bound covalently to a tRNA. The covalent linkage, between the carboxyl-group of the amino acid and the hydroxyl group at the 3′ end of the tRNA, is called an acyl linkage. After aa-tRNA is recruited to the ribosomal A site, an intrinsic activity of the ribosome catalyzes the transfer of the nascent peptide chain to the amino acid on the A site tRNA forming a peptide bond between the terminal carboxyl group of the nascent peptide and the free amino group of the amino acid on the A site tRNA. After peptide transfer the newly deacyl-tRNA (tRNA lacking any acylated amino acid) in the P site moves to a third site, termed E for exit, concomitantly with movement (translocation) of the new peptidyl-tRNA (pep-tRNA) into the P site. The cycle then repeats with recruitment of an aa-tRNA to the 3 nt codon immediately downstream of the codon bound to the pep-tRNA. It is important that mRNAs have no internal punctuation that enables the ribosome to identify the correct reading frame. The ribosome appears constrained to read only successive adjacent codons. How this is accomplished is, remarkably, still unknown.

Termination is really a specialized version of a normal elongation cycle which occurs when a special termination codon (UAG, UAA or UGA) occupies the A site. At that point the nascent peptide is released from the ribosome, and the two ribosomal subunits dissociate from the mRNA.

Most of the steps in the elongation cycle require the aid of elongation factors. Most organisms have only two elongation factors, EF1A and EF2 (eEF1A and eEF2 in eukarya), although fungi encode a third elongation factor, eEF3. A ternary complex of aa-tRNA bound to EF1A and GTP binds to the ribosomal A site. GTP hydrolysis provides a proofreading function thought to reduce translational errors. It separates the process into two steps, before and after GTP hydrolysis. Before GTP is hydrolyzed correct (cognate) ternary complexes bind essentially irreversibly while incorrect (near-cognate) ternary complexes tend to dissociate. After GTP hydrolysis, cognate aa-tRNAs essentially quantitatively undergo peptide transfer while near-cognate aa-tRNAs again tend to dissociate before peptide transfer. Until recently, error correction was thought to involve a kinetic proofreading mechanism where a difference in the rate of dissociation of correct (cognate) and incorrect (non-cognate) aa-tRNAs is used to eliminate errors. The two step process was thought to amplify error correction by providing two chances for incorrect, near-cognate aa-tRNAs to be rejected; this double sieve mechanism was used to explain how the ribosome achieves its the low frequency of translational errors. More recently, the process of error correction was shown to involve an induced fit model in which cognate tRNAs induce a conformation change in the ribosome that increase the rates of two steps necessary to peptide bond formation: activation of the EF1A GTPase and accommodation of the aa-tRNA into the peptidyltransferase center, which is responsible for catalyzing peptide transfer to the aa-tRNA. Under this new model, increased accuracy results from near-cognate aa-tRNAs being unable to accelerate these processes resulting in fewer incorrect amino acids being incorporated.

Translocation of the pep-tRNA●mRNA complex appears to be an intrinsic function of the ribosome since it occurs in the absence of EF2. The rate of translocation is extremely slow, however. Recent kinetic and structural studies suggest the new pep-tRNA created after peptide transfer undergoes rapid structural fluctuations that EF2 can bias in favor of movement into the P site of the ribosome. In its GDP-bound form, EF2 is a structural mimic of the GTP-bound form of EF1A ternary complex and, like that complex, has high affinity for the empty A site. GTP hydrolysis causes a structural change to EF2 bound to the ribosome into a form that can fill the ribosomal A site when the pep-tRNA moves into the P site. Thus, GTP hydrolysis is essential to maximal rates of translocation.

Translational termination occurs when the ribosome encounters a termination codon (UAA, UAG or UGA) and is promoted by peptide release factors. The enzymology of termination differs between bacteria and eukarya. Bacteria have two peptide release factors, RF-1 and RF-2. The factors are codon specific, so that RF-1 recognizes the codons UAG and UAA and RF-2 recognizes UAA and UGA. A third factor, RF-3, has no codon specificity. It is a GTP-binding protein that may have a role in regulating the accuracy of codon recognition by the other two factors. In eukarya there are only two factors, eRF-1, the cognate of RF-1 and RF-2, and eRF-3, the cognate of RF-3. The termination factors bind to a ribosomal A site occupied by a termination codon and stimulate the ribosome's peptidyltransferase center to hydrolyze the acyl-bond between the nascent peptide and the tRNA, releasing the peptide from the ribosome. Another factor, ribosome recycling factor, or RRF, facilitates the dissociation of the ribosomal subunits from the mRNA.

Structural Basis of Translation

The most significant advance in decades in understanding of the process of translation came with the solution of a molecular structure of the ribosome and of complexes between the ribosome and various translation factors. We now have available models of the ribosome that provide atomic details of its structure and by extension, its function. Where once the translation system was

basically a black box, we now have atomic explanations for mechanisms underlying many of the steps in protein synthesis and insights into some forms of genetic control.

Structural models of the ribosome have greatly increased our understanding of the reactions that take place at the two active sites of the ribosome: the decoding center, which screens the mRNA-tRNA complex for accuracy, and the peptidyltransferase center, which catalyzes polymerization of the growing polypeptide. Both of these sites are composed largely or exclusively of RNA; this is consistent with a view in which the ribosomal proteins largely modulate the activity of RNA rather than participating in catalysis. The decoding center interactions play a direct role in maintaining translational accuracy. Decoding involves formation of base paired structures between a 3 nucleotide codon in the mRNA and a complementary anticodon in a decoding tRNA. The first and second positions of the codon must form Watson-Crick (WC) base pairs (G-C or A-U) with the anticodon; the third, or wobble position can make either WC or certain non-WC pairs. The non-WC pairs include U-G, U-I (Inosine), C-I or A-I. The structure of the decoding center mRNA●tRNA complex reveals the basis of this specificity. Elements of the ribosomal A site make multiple contacts with the codon and anticodon; these elements mainly consist of nucleotides of the small subunit rRNA but also include amino acid side chains from ribosomal protein S12. The interactions with the first and second base pairs require WC geometry and therefore non-WC base pairs could not form these interactions. Interactions with the third base pair do not require WC geometry, which allows acceptance of interactions using specific non-WC wobble base pairs.

Our understanding of how the ribosome insures accurate decoding has changed recently. The discovery of the structures in the ribosomal A and P sites that can interact with WC base pairs suggested a model in which the ribosome monitors the structure formed by the incoming aa-tRNA, recognizing when a cognate interaction has been formed (involving WC base pairs) and stimulating a conformational change in the ribosome that traps the aa-tRNA on the ribosome. Under this model, because near-cognate complexes (involving one non-WC interaction) would not be recognized, they would not be as efficiently recruited to the A site. This model would require the A and P sites to undergo rapid conformational changes to recognize a cognate complex but not a near-cognate complex. More recent data has shown that the A and P sites are much more rigid than previously thought and that they force both cognate and near-cognate complexes to bind in WC geometry. Cognates easily adopt this geometry but near-cognates forced into WC geometry create structural clashes that cause dissociation of the aa-tRNA from the A site.

Gene-Specific Translational Control of Gene Output

Translation is a complex process. The amount of protein expressed from a given gene, the gene output, can be regulated at multiple steps of translation. The rate of production of protein, in the absence of some other effect, reflects the frequency that ribosomes begin translating. As a result, many genes have evolved mechanisms to modulate this frequency. Other mechanisms indirectly regulate gene output without affecting initiation rate. For example, blocking translational elongation would also reduce gene output. Few systems have evolved that use such a block, and where they have the elongation block effects gene output indirectly by blocking downstream translational initiation by another ribosome (translational attenuation), causing early termination of transcription (transcriptional attenuation) or inducing mRNA degradation.

Alternative Pathways of Translational Initiation in Bacteria

Although translational initiation in bacteria normally occurs by direct binding of the initiation complex to the site of initiation, some genes have evolved a different mode of initiation, translational coupling. In translationally coupled systems ribosomes that initiate translation in an upstream gene continue translating one or more downstream genes by reinitiating at initiation codons overlapping or immediately adjacent to translational termination codons. At the same time, the RNA structure surrounding the downstream initiation codons precludes direct binding of ribosomes from solution. For example, the end of a gene may have the sequence AUGA, where the termination codon of the upstream gene (UGA) overlaps the initiation codon of the downstream gene (AUG). A translating ribosome reaches the end of the upstream gene, recognizes the UGA codon, and terminates translation by releasing the nascent peptide, allowing the last elongator tRNA to dissociate, and releasing the large ribosomal subunit. The initiation region of the downstream gene, which includes the AUG and upstream Shine-Dalgarno site, lies immediately adjacent to the termination site. Unlike eukaryotic ribosomes, the small subunit of prokaryotic ribosomes can bind to an initiation site ('ribosome binding site' or RBS) in the absence of either initiator tRNA or any initiation factor. Thus, the small subunit can be trapped on the mRNA after termination. Translation reinitiates when initiation factors and f-met-tRNA base paired to the AUG codon join the bound ribosome. This process theoretically can 100% efficient. The efficiency of reading the downstream gene depends on the frequency of initiation at the first gene so that a mechanism regulating the efficiency of translation of that gene can coordinately control expression of multiple downstream genes. To make the system entirely dependent on upstream translation, the RBS of the downstream gene is sequestered in a secondary structure; this structure blocks direct binding of initiation complexes but it can be opened up by the ribosome that translates through the upstream gene to allow initiation by coupling.

An alternative form of this type of control occurs when a ribosome translating through an upstream gene disrupts a secondary structure that sequesters the RBS of a downstream gene to block its recognition by initiation complexes. A ribosome translating the upstream gene may pause during elongation at a point where the secondary structure is unwound to release the RBS of the downstream gene so it can be recognized by a 40S subunit that would then initiate translation of the downstream gene. This system also allows translational co-regulation, but without reusing the ribosome.

The consistent feature of these systems is that upstream translation unmasks sites to make them available for translation initiation. Many mechanisms can accomplish this control, not all of which are strictly translational coupling. The use of upstream open reading frames (uORFs), as described below for translational attenuation, is a special case of this type of control.

Alternative Pathways of Translational Initiation in Eukarya

Translation of most eukaryotic mRNAs begins at the first AUG downstream of the 5′ end. In some genes, however, the first AUG is not used and in some genes the 5′ non-translated region includes many AUG codons not used for initiation. Translation initiation occurs in these genes by alternative mechanisms. There are three classes of alternative initiation mechanisms: leaky scanning, ribosomal shunting and internal initiation. In leaky scanning the ribosome ignores the AUGs that it encounters before the true initiation codon. The sequence context around these upstream AUGs tends to be different than that around bona fide initiation codons. This fact suggests that the ribosome scans for both the AUG and its surrounding sequence context, though the molecular mechanism of scanning for the context is not understood.

Ribosome shunting occurs in a very small minority of mRNAs. The mechanism is distinct from scanning despite the fact that the initiation complex binds to the 5′ end of the mRNA, recognizing the eIF4 complex as in normal initiation. The complex begins scanning down the mRNA, but then bypasses a large segment of the 5′ non-translated region ('shunts') before reinitiating scanning and initiating at the first AUG it then encounters. The first example of shunting came from the plant virus, cauliflower mosaic virus, but there are several other examples.

Internal initiation is a much more common mechanism than shunting, but still occurs on a small minority of mRNAs. Unlike either scanning or shunting, in this mechanism the initiation complex binds directly to a site within the body of the mRNA. Since the complex does not bind to the 5′ end of the mRNA this form of initiation does not necessarily require the 5′ cap. In fact, eukaryotic viruses have evolved systems that block normal translational initiation dependent on the 5′ cap. This block strongly decreases translation of endogenous cellular mRNAs that use translational scanning. Normally internal initiation is rather inefficient but when scanning is blocked translation of the viral mRNAs by internal initiation becomes much more efficient because the endogenous transcripts no longer compete for the translational machinery. Internal binding of the initiation complex is directed by a highly-structured region in the 5′ non-translated region called an internal ribosome entry site, or IRES. Some IRESs stimulate initiation by binding translation initiation factors that in turn recruit the preinitiation complex. Other IRESs directly bind to the ribosome to recruit it without the aid of any initiation factor. An extreme case is the IRES from the cricket paralysis virus (CrPV), which requires no initiation factors or even the initiator tRNA. For the CrPV IRES a secondary structure that mimics the tRNA base paired to an initiation codon occupies the ribosome P site. This structure then recruits an EF1A ternary complex to the next codon to initiate protein synthesis.

Co-Translational Control of Gene Transcription in Bacteria: Transcriptional and Translational Attenuation

Transcription and translation in bacteria are not restricted to physically separated sub-cellular compartments, as they are in eukarya. This provides the possibility of a form of genetic control in which transcription is coupled to continued translation of the nascent mRNA. Certain bacterial operons are controlled at the level of transcriptional termination in the leader region of the operon. Termination occurs when transcription and translation of the leader region becomes uncoupled. This process, termed transcriptional attenuation, governs expression of several amino acid biosynthetic genes and was first recognized in the *trp* and *his* operons. Ribosomes bind to and begin translating an upstream open reading frame (uORF) in the leader region of the nascent transcripts of these genes. In the *trp* and *his* systems, the uORFs contain several tryptophan or histidine codons. In addition to this uORF, the leaders contain RNA sequences that can form two alternative structures: a hairpin structure located downstream of the uORF that when formed in the nascent mRNA signals termination of translation (terminator hairpin) and an alternative hairpin located between the terminator hairpin and the uORF that cannot signal termination (attenuator hairpin). When the amino acids are present in sufficient amounts, the ribosome reads through the uORF on the nascent mRNA, blocking formation of the attenuator hairpin. The terminator hairpin forms and transcription terminates without continuing into the downstream amino acid biosynthetic genes. During starvation for these amino acids, however, ribosomes reading the uORF stall at these codons because of lack of sufficient cognate EF1A ternary complexes. The stalled ribosomes allow formation of the attenuator hairpin, so the terminator hairpin is not formed and the polymerase continues on, transcribing the biosynthetic genes ultimately leading to increased synthesis of the corresponding amino acid. Thus, the lack of continuing translation is transduced to a signal to continued transcription of the biosynthetic genes as a feedback control mechanism.

Other similar systems modulate expression using a uORF, but the regulation is at the level of translational initiation. A dramatic example comes from the *cat86* gene of *Bacillus subtilis*. This gene encodes an enzyme that acetylates the antibiotic chloramphenicol, a protein synthesis inhibitor that blocks peptidyltransferase. The gene has a uORF that is translated co-transcriptionally. The nascent polypeptide is itself a peptidyltransferase inhibitor, so that ribosomes translating the uORF become paused because the nascent peptide blocks further extension of the protein. This pause is normally transient but if chloramphenicol is present it binds to the stalled nascent peptide and the stall becomes extremely prolonged. The paused ribosome disrupts a mRNA secondary structure that when formed sequesters the RBS of the *cat86* gene. Pausing thus makes the RBS available for ribosomes to bind and initiate translation of *cat86*, ultimately leading to inactivation of chloramphenicol. In this case a gene exquisitely sensitive to inhibition by chloramphenicol transduces blockage of translation into stimulation of translation. This type of control has been observed in multiple other drug resistance genes in bacteria.

Control of Translational Reinitiation

Ribosomes on some uORF-containing eukaryotic mRNAs scan as normal but initiate translation on the upstream AUG codon rather than at the initiation codon of a downstream structural gene. Ribosomes can translate the uORF into a short oligopeptide and then reinitiate scanning, eventually recognizing the downstream initiation codon and translating the structural gene. This process is called translational reinitiation and constitutes another form of alternative translational initiation. Translational reinitiation occurs on a small minority of mRNAs and has been best studied in the *GCN4* gene of the yeast *Saccharomyces cerevisiae*.

The *GCN4* mRNA has four uORFs. Ribosomes scanning from the 5′ end initiate translation at either the first or second uORF after which the ribosomes reinitiate scanning. During the scanning they must re-stock themselves with initiation factors, including methionyl-tRNA^{Met}●eIF2●GTP ternary complex, in order to be able to reinitiate at a downstream AUG. If they do so and become competent to reinitiate before scanning past the second pair of uORFs the ribosomes will initiate on them, but if they become competent only after scanning past the uORFs they may initiate on the *GCN4* gene. Yeast cells starved for amino acids have a lower concentration of eIF2 ternary complex, so that during starvation it would take longer for the reinitiating ribosomes to become competent for translation. In this case, more ribosomes bypass the second set of uORFs and translate the *GCN4* gene. The Gcn4 protein is a transcriptional activator of amino acid biosynthetic genes, so this system is used to turn on expression of *GCN4* during amino acid starvation by increasing expression of Gcn4 resulting in higher expression of amino acid biosynthetic genes and increased synthesis of the amino acid responsible for the starvation. Genes homologous with *GCN4* exist in metazoan species and use similar mechanisms to regulate translation.

Translational Repression

Translational repression is a ubiquitous and common form of translational control. The best-known example comes from ribosomal proteins in bacteria. During balanced growth, the amount of each ribosomal protein and the rRNA components of the ribosome are equimolar and all ribosomal proteins produced rapidly bind to pre-ribosomes during ribosome assembly. When this balance is disturbed and the ribosomal proteins are present in excess, certain of the ribosomal proteins bind to their own mRNAs inhibiting translation initiation of entire operons comprised largely of genes for ribosomal proteins including the one that binds the mRNA. This mechanism achieves feedback control of ribosomal proteins, reducing their expression when it is in excess, and contributes to homeostasis in ribosome biogenesis. The binding site for the proteins lie within the 5′ non-coding region and block access by ribosomes to the ribosome binding site of the first controlled protein. Through translational coupling, down regulation of the first protein can proceed down the mRNA to other genes located on the same mRNA.

A second well-known example is the gene *32* protein of bacteriophage T4, which is a DNA binding protein that binds randomly to single stranded DNA. It can also bind specifically to a pseudoknot structure in the the 5′ untranslated region of its own mRNA blocks ribosome binding at an adjacent RBS.

One eukaryotic repressor, IRE-BP, works by a very similar mechanism. The protein is the cytosolic isozyme of the mitochondrial enzyme aconitase, but is also a translational repressor that is responsive to the concentration of ferric iron (Fe^{3+}). IRE-BP has an iron-sulfur cluster that loses a single Fe^{3+} when iron concentration is low. The region of the protein normally bound by this Fe^{3+} can then bind to an iron response element (IRE) in the 5′ non-coding region of the ferritin mRNA. When bound, IRE-BP blocks access by the initiation complex to the mRNA. When Fe^{3+} concentration is high another Fe^{3+} can bind to IRE-BP, reducing its affinity for the IRE, and eliminating its repression of ferritin. Because ferritin protects the cell from oxidation by chelating intracellular Fe^{3+} the increased expression of ferritin when Fe^{3+} concentration insures cell viability.

Other eukaryotic translational repressors function differently by binding to sites in the 3′-noncoding regions of mRNAs, which would not directly block access by the scanning ribosome to the mRNA. Instead their effect must be more long-ranged. This long range effect involves the fact that mRNAs in eukarya adopt a circular structure in which the 5′ and 3′ ends are held in close contact by specific RNA binding proteins that recognize the 5′ cap (eIF4F) and 3′ poly(A) tails (poly(A)-binding protein or PABP). It appears that the initiation complex recognizes this PABP●eIF4F complex to bind the mRNA and initiate scanning. The translational repressors bound to the 3′-noncoding region block ribosome binding by interfering with recognition or formation of the eIF4F●PABP complex.

Control by blocking Cap recognition is especially common in maternal mRNAs during the early stages of development. There are two main ways that this is accomplished. First, mRNAs intended to be stored, untranslated in oocytes frequently have very short poly(A) tails, too short to recruit PABP. In the absence of PABP the mRNAs are not actively recruited to translation. During early development, these mRNAs can be translationally activated by extending their poly(A) tails, which then bind PABP and are then translated. On the other hand, some mRNAs that had been translationally active are repressed by deadenylation, which can block their translation and often lead the the degradation of the mRNA. A second mechanism involves proteins like Cup and Maskin, two proteins that bind brought to the 3′ non-coding regions of target mRNAs by their affinity for site specific RNA binding proteins bound there. Cup and Maskin are mimics of a translation factor eIF4E-BP that binds to the eIF4E cap binding protein of the eIF4F complex. When eIF4E is bound by eIF4E-BP or one of the mimics it cannot associate the eIF4F protein that forms the bridge to PABP (eIF4G); inability to form that circularized complex blocks translation of the proteins. The eIF4E-BP is a global regulatory factor, which we will return to below, but Cup and Maskin function only on mRNAs to which they are targeted by associating with their 3′ non-coding regions.

Control of mRNA Degradation

While at first the process of mRNA degradation would seem unrelated to the issue of translational control, much degradation actually occurs either in the context of ongoing translation, or is actually coupled to the process of translation. Especially in eukarya, where mRNAs have half-lives very much longer than in bacteria, gene output can often be profoundly affected by a change in mRNA stability.

A classic example of co-translational degradation comes from mammalian tubulin genes. In addition to long term transcriptional control, both α and β tubulin are subject to very rapid co-translational control by mRNA degradation. The mechanism of this control is better understood for β-tubulin, though regulation of α-tubulin appears quite similar. When the concentration of free β-tubulin subunits is high the mRNA for β-tubulin is rapidly and specifically degraded. The signal for this degradation is surprisingly the first four amino acids of the nascent polypeptide. As this peptide, Met-Arg-Glu-Ile, emerges from the ribosome some cellular factor (perhaps β-tubulin itself) recognizes it and activates an unknown endonuclease which cleaves the mRNA, leading to its ultimate degradation. This system provides a very rapid response to the presence of excess subunits as, for example, when microtubules depolymerize into tubulin monomers, or to adjust an excess of β over α-subunits.

A second example of co-translational control concerns degradation of mRNAs containing premature termination codons (nonsense mediated decay or NMD). The mRNA of a nonsense mutant gene can be much less stable than the identical mRNA lacking the nonsense mutation. When a translating ribosome encounters a premature termination codon there are a group of ribosome-associated proteins that sense the presence of the nonsense codon and induce NMD. Exactly how that signal is sent remains somewhat controversial. In the simple eukaryote, *S. cerevisiae*, the sensitivity to degradation appears to derive from the fact that premature termination codons can create extremely elongated 3′ non-coding regions consisting of all of the normally coding region distal to the mutation and the normal, usually short, normal 3′ non-coding region. The NMD system seems to stimulate degradation if the 3′ non-coding region is much longer than normal. In higher eukaryotes the signal appears to be the presence of a splice junction—a position in the mRNA from which an intron has been excised—downstream of the termination codon. Splice junctions normally do not occur distal to a true termination codon, so finding one downstream identifies the terminator as premature. Premature termination codons could occur because of a genetic mutation but probably much more frequently they result from incorrect splicing in which an intron remains in the mRNA; a ribosome reading such an mRNAs should rapidly encounter an in-frame termination codon in the intron. NMD, by inducing degradation of the mRNA prevents accumulation of an abnormal protein that could be detrimental to the cell.

For some mRNAs sequence-specific RNA binding proteins recognize special sites to activate mRNA degradation. Interestingly, the same IRE-BP protein that regulates translation of ferritin mRNA in response to Fe^{3+} also regulates transferrin, the protein responsible for importing iron into eukaryotic cells. Transferrin must be regulated oppositely to ferritin since iron is limiting in the growth medium transferrin must be abundant to allow recruitment of the diminished supply; when iron is abundant transferrin must be down regulated so that the cell is not oversupplied with the potentially damaging ion. When Fe^{3+} concentration is low, IRE-BP binds to multiple IREs in the 3′ non-coding region of the transferrin mRNA. The IREs are interspersed with instability elements, and bound IRE-BP interferes with their function to stabilize the mRNA. In high Fe^{3+}, IRE-BP dissociates from the IREs and the instability elements then promote rapid degradation of the mRNA, reducing the concentration of transferrin in the cell and slow import of Fe^{3+}.

There are many other examples of genes with instability elements in their 3′ non-coding region and many of them function constitutively to maintain a short half-life for the mRNA. These elements allow for rapid readjustment of mRNA pools by either increasing or decreasing the rate of transcription of the mRNA. These elements are common in mRNAs encoding regulatory proteins, such as transcription factors.

Global Control of Translation

In addition to the many forms of gene-specific regulation, translation is often regulated globally by mechanisms that modulate the activity of translation factors. Cells employ global regulatory processes themselves to regulate translation efficiency by modulating the activity of translation factors. One purpose of this regulation is to diminish expression of proteins encoded by invading viruses by partially inactivating the translational machinery. In addition, cytotoxic viruses upon infection commonly take over the genetic machinery of the infected cell, directing much of the synthetic capacity of the cell to production of virus-encoded proteins. This shift usually involves inhibition of cellular translation through covalent modification (phosphorylation or proteolysis) of cellular translation factors. Viral genes have evolved a lower dependence on these factors for their expression so that they compete more effectively for the translational machinery under these circumstances than do cellular genes.

Phosphorylation of translation factors is the most common form of global translational control and the commonest targets are the elongation factors eIF2 and eIF4E. Phosphorylation of eIF2 reduces its activity while phosphorylation of eIF4E stimulates its activity.

Reticulocytes dedicate virtually their entire protein synthetic machinery to the production of the protein hemoglobin. The rate of synthesis of the apoprotein and synthesis of its iron-containing heme cofactor must be carefully co-regulated. Reticulocytes express an eIF2-specific protein kinase, heme regulated inhibitor (HRI), that phosphorylates the α-subunit of eIF2 in conditions of low heme. When phosphorylated, eIF2 binds much more tightly to its recycling factor, eIF2B, causing eIF2B to become sequestered and

unable to recycle other eIF2●GDP to the active form of eIF2●GTP. This in turn reduces the availability of eIF2●GTP and thus of met-tRNA●eIF2●GTP ternary complex. Translation initiation declines as the concentration of ternary complex falls, so that the ultimate effect of eIF2 phosphorylation is reduced translation. This reduction is used to equalize the concentration of apohemoglobin and the heme cofactor, whose concentration falls in low iron conditions.

A similar mechanism of phosphorylation inhibits protein synthesis in virus-infected cells. A homolog of HRI, the dsRNA-dependent protein kinase (PKR), phosphorylates eIF2α in virus infected cells. This reduces protein synthesis which reduces virus yield from the infected cells. A third homolog, the product of the *GCN2* gene in the yeast *S. cerevisiae* is the sensor of amino acid starvation in the *GCN4* translational reinitiation scheme described above. Cells starved for amino acids accumulate deacylated tRNAs which bind to Gcn2p activating its eIF2α kinase activity. This leads to a reduction in eIF2 ternary complex. The reduction is not enough to affect bulk protein synthesis, as PKR and HRI do, but enough to reduce the efficiency that ribosomes scanning past the first of the *GCN4* uORFs regain competence to reinitiate translation. In starved cells the ribosomes tend to become competent after moving farther down the mRNA, and so tend to initiate translation on the *GCN4* gene rather than on the two inhibitory uORFs, causing an increase in translation of *GCN4*. This system is an unusual case of a global change in the translation system (reduction in eIF2B activity) that has evolved to regulate a single gene (*GCN4*) because of its exquisite sensitivity to reductions in eIF2 ternary complex.

Phosphorylation of eIF4E, the cap-binding factor, and of its antagonist, eIF4E-BP, are examples of the opposite form of global control. Phosphorylation of eIF4E or eIF4E-BP is correlated with increased translation, rather than diminished; the mechanism of this eIF4E effect is not understood but eIF4E-BP is much better understood. In higher eukaryotic systems, various mitogens stimulate phosphorylation. Presumably cells stimulated to proliferate or initiate developmental programs require an increase in translation. Modulating eIF4E activity is especially effective because of its very low-abundance relative to other initiation factors, and because it is critical to the first step in recruitment of ribosomes to the mRNA. Phosphorylation of eIF4E-BP decreases the strength of its association with eIF4E. Since eIF4E-BP competes with the interaction between eIF4E and the PABP-binding factor, eIF4G, decreased binding of eIF4E by eIF4E-BP leads to more efficient formation of circular mRNAs and therefore more efficient translation initiation. This effect is used to modulate translation in response to external nutrients. When cells are deprived of nutrients or growth factors, eIF4E-BP is unphosphorylated, binds to eIF4E and reduces translation globally. When cells are stimulated by addition of nutrients of growth factors, eIF4E-BP is phosphorylated by the TOR kinase pathway, reducing its affinity for eIF4E and stimulating higher translation. Misregulation of this pathway can result in uncontrolled growth and cancer.

The cap-recognition complex is also subject to the opposite type of control by specific degradation. During picornavirus infection eIF4G is specifically cleaved by a protease encoded by gene 2A of the virus. This cleavage reduces translation, though not completely. Since picornaviruses use a cap-independent method of initiation reducing efficiency of cap-recognition would tend to increase the ability of the picornavirus mRNA to compete for the limiting supply of ribosomes in the cell, thus increasing its relative efficiency. The point of this control may not be to completely suppress cellular mRNA translation, but only to maximize viral expression.

Translational Accuracy

Control of the primary structure of proteins is a second ubiquitous, but perhaps not as prevalent, form of translational control. This type of control has been called 'recoding' to suggest that the rules of translational coding are changed to result in an unconventional or non-canonical event. In most cases this type of control is used to allow a single gene to specify two structurally related, yet distinct primary gene products. Termination codon readthrough, the simplest of these events, results in the insertion of an amino acid at a termination codon. Readthrough is a stochastic event, usually much less than 100% efficient. As a result, readthrough allows expression of two forms of a protein: a protein resulting from normal termination and a second consisting of the first protein fused to the product of the reading frame downstream of the terminator. Other events call for the ribosome to shift its reading frame either by reading a codon overlapping one in the previous frame, or by causing the ribosome to bypass a short stretch of codons. These types of events resemble superficially the kinds of errors that occur during translation, though the efficiency of the events during recoding is several orders of magnitude higher. Understanding recoding requires first a consideration of the mechanism of translational errors and of the mechanisms used by the ribosome to forestall them.

Types of Translational Errors

Missense errors, the simplest type of translational error, occur when one amino acid is inserted in the place of another. Missense errors result from either incorrect tRNA aminoacylation or recruitment of an incorrect tRNA to the ribosome. They are the most frequent type of translational error. The translational machinery has to compromise the cost of translational inaccuracy and the advantage of increased translation speed. To the extent that translation rate is increased the ribosome will tend to accept a greater proportion of erroneous, non-cognate tRNAs. To reduce translation errors below the observed frequency would require a significant reduction in translation elongation rates. The observed rate of errors probably optimizes these antagonistic negative and positive effects.

Studies of several different translational misrsense errors suggested that they occur at a rate of about 5×10^{-4} per codon. Although a rate of 5×10^{-4} per codon seems low frequency it results in inaccurate expression of a significant proportion of proteins.

For example, the protein β-galactosidase is 1000 amino acids in length. Errors occurring at 5×10^{-4} per codon would result in 40% of proteins having at least one missense error! Yet missense errors tend to have a relatively low phenotypic effect since most errant proteins retain full activity, and few have seriously lowered activity. More recently, attempts to comprehensively measure all possible missense errors by single tRNAs have revealed a more complex phenomenology of error. First, missense errors essentially cannot be measured on non-cognate codons (those that form fewer than two base pairs with the misreading tRNA). This is consistent with an abundance of biochemical data showing that non-cognate tRNAs are recruited extremely slowly to the ribosomal A site, essentially never getting to even the first step of codon recognition. The surprising result of this work, however, was the finding that most near-cognate codons (those making two base pairs with the codon) are misread extremely infrequently. Errors at most near-cognate codons occur at frequencies of about 2×10^{-5} per amino acid incorporated. Some codons, however, experience much more frequent errors. Those codons are read by the misreading tRNA using a mismatched base pair that appears to have similar geometry to a normal, Watson-Crick pair (A-U or G-C) suggesting that higher frequency errors occur when a mismatched base pair cannot be distinguished from a correctly matched pair. These errors only occur at frequencies between about 10^{-4} and 3×10^{-3}; this low frequency results from the fact that the pairs require a nucleobase to be in a non-physiological tautomeric form (enol rather than keto or imino rather than amino), which occur at frequencies near 10^{-4}, similar to the error rate observed, suggesting that ability to tautomerize may have a large effect on the error rate.

The second general class of errors are those that cause premature termination of translation. These are termed processivity errors since they interfere with the processive nature of translation. Processivity errors occur on average at approximately the frequency of missense errors. Again, that would imply a significant loss in terms of gene output because of these errors. At a rate of 5×10^{-4} terminations per codon, only about 60% of ribosomes that begin translating β-galactosidase would actually produce a full-length product. These errors generally have a much more serious effect on the activity of the gene product. The vast majority of C-terminally truncated products would retain no enzymatic activity, and would indeed be subjected to rapid degradation in vivo. Usually only those proteins retaining all but the most C-terminal portion of the protein retain significant activity. It is perhaps surprising that the translational machinery has not evolved a more efficient correction mechanism for these errors given their seriousness. This failure implies that the compromise in terms of the rate of translation elongation would be too drastic to reduce these errors further.

The simplest kind of processivity failures are those in which the process of elongation is aborted prematurely. Surprisingly, few of these errors are caused by mistaken recognition of a sense codon by peptide release factor, so these errors are not improper termination events per se. Instead, the vast majority occurs when pep-tRNA spontaneously dissociates from the translating ribosome ('ribosome drop-off'). Cells have a special enzyme that removes nascent peptides from these tRNAs, peptidyl-tRNA hydrolase. When this enzyme is inactivated cells rapidly fill with pep-tRNA, underscoring the frequency of the error. Treatments or genetic states that favor missense translation also stimulate this error, suggesting that incorrect pep-tRNAs may tend to dissociate from the ribosome.

Translational frameshifting is the second type of processivity error since a ribosome that changes reading frame cannot complete the intended translational product, and in fact should rapidly encounter a termination codon in the shifted frame. Perhaps significantly, frameshifting errors occur at least ten-fold less frequently than either missense errors or ribosome drop-off. Since these errors should be no more serious than other processivity failures in terms of enzyme activity, their lower occurrence cannot reflect a greater selection against them. Rather it may reflect a fundamental feature of the error mechanism.

Spontaneous frameshifting normally occurs by changes of single nucleotides either in the 5′ or leftward direction (−1), or the 3′ or rightward direction (+1). Frameshifting has been studied as phenotypic suppression of single base pair insertion or deletion mutations. Judged by the level of suppression, +1 frameshifting appears to occur much more frequently. Sequence contexts greatly affect the efficiency of frameshifting because suppression can vary strikingly among various frameshift mutations. This implies that there are specific signals that stimulate frameshifting. The nature of the signals was made clearer from analysis of programmed frameshift sites, sequences in genes that program highly-efficient frameshifting (see below).

Genetics of Translational Accuracy

The classical approach to genetic analysis of translational accuracy was to identify suppressor mutations that restore normal activity to missense or frameshift mutant forms of a reporter gene. For example, using a gene that had suffered a 1 bp insertion and therefore was non-functional, one could identify second-site suppressors that restored normal expression of the gene. Such frameshift suppressor mutations affect tRNAs, ribosomal proteins, rRNA, and translation factors. Similarly, suppressors could be identified that promoted readthrough of premature termination codons or allowed bypass of missense mutations. These experiments have identified factors responsible for maintaining translational accuracy.

tRNA mutations

Seemingly the easiest of the suppressors to understand were the tRNA suppressors. Nonsense, frameshift and missense suppressors all seem to work in the same manner. By changing the sequence of the anticodon the decoding properties of a tRNA are changed. Nonsense suppressors have an anticodon complementary to one of the nonsense codons. Most +1 frameshift suppressors have anticodons expanded from three to four or more. Similarly, missense suppressors have anticodons corresponding to the codon that must be misread. Creating these suppressors is complicated by the fact that most aminoacyl-tRNA synthetases contact bases in anticodons to discriminate among possible substrates. Changing the anticodon can also change recognition by the synthetase. This is most clearly an issue for the missense suppressors, but inefficient aminoacylation would reduce the function of any of the suppressors. As a result, only a few of each class of suppressor have been identified.

Recent work has shown that the accepted model for +1 frameshift suppressors is incorrect for a major class of mutations, and might be invalid in general. The Quadruplet-Decoding model proposed that the expanded anticodon of +1 frameshift suppressors allowed a 4 bp codon●anticodon interaction in the ribosomal A site, resulting in 4 nt translocation catalyzed by EF2. This model cannot explain frameshifting by the $tRNA^{Pro}$ family of suppressors since post-transcriptional modification of the anticodon loops of these tRNAs preclude the putative 4 bp codon●anticodon interaction. For these tRNAs, and probably for all others, it appears that the suppressing tRNA causes a normal 3 nt translocation, but that the pep-tRNA slips +1 on the mRNA while in the ribosomal P site. This Peptidyl-tRNA Slippage model can explain suppression by all of the classes of frameshift suppressing tRNA mutations though direct evidence supporting the model for many of them is not yet available.

Ribosomal mutations

Since translational errors occur in the context of the ribosomal decoding sites one might expect that some suppressors would affect ribosomal components. In fact, some of the earliest suppressor mutations affected the ribosomal proteins S4, S5 and S12 of *E. coli*. Mutations in S12 cause resistance to the antibiotic streptomycin concomitant with reduced error frequency, while those in S4 or S5, called *ram*, for 'ribosomal ambiguity', increase the error frequency. These data suggest that the three ribosomal proteins may form the core of an accuracy center on the ribosome. Significantly, the three are proteins of the small subunit of the ribosome, the site of codon●anticodon pairing, suggesting that they may directly modulate the interaction.

Mutations affecting the rRNA in both subunits of the ribosome can stimulate translational errors. Three regions of the single rRNA of the small subunit, within which the codon●anticodon pair forms, can mutate to suppress either frameshift or nonsense mutations. Frameshift suppressing mutations fall in the part of the rRNA in the decoding center of the ribosome, which is composed of nucleotides from several regions of the small rRNA. These regions are in direct contact with the codon●anticodon complex in the A and P sites as described above. Other frameshift suppressing rRNA mutations affect the large subunit rRNA, 23S rRNA in bacteria. These fall within the α-sarcin loop, a region so-named because it is cleaved by the cytotoxic nuclease α-sarcin.

Translation factor mutations

The final class of suppressors affect the translation elongation factors EF1A, and its eukaryotic homologue, eEF1A, and the peptide release factors. Mutant forms of bacterial EF1A and yeast (*S. cerevisiae*) eEF1A have the same effect of suppressing nonsense or frameshift mutations, or both. The mutations are not clustered in the molecule, and in fact affect either of its two functionally distinct regions, the GTP-binding and tRNA-binding domains. The exact mechanism of suppression is unclear. Those affecting the GTP-binding domain may affect the kinetics of GTP hydrolysis, which would be expected to perturb discrimination between correct and incorrect tRNAs. Those in the tRNA-binding domain may change the interaction between the EF1A ternary complex and the ribosome, perhaps stabilizing the interaction of non-cognate tRNAs, which again under that model would tend to reduce discrimination.

Availability of peptide release factors influence the frequency of nonsense codon readthrough both in vivo and in vitro. Reduction in the effective concentration of release factors tends to allow readthrough, to varying degrees, of all three types of nonsense codons. The amino acid inserted tends to be one that is near in sequence to the nonsense codon. This implies that when the rate of recognition of a nonsense codon is limiting, the ribosome will accept incorrect tRNAs. This is consistent with the idea that ribosomal accuracy is kinetically controlled. In yeast, an epigenetic state can arise in which peptide release factor is limiting. The state is brought on by aggregation of the factor and results in increased nonsense codon readthrough. This situation resembles a prion infection since the condition is heritable but not genetically encoded. This is an active area of investigation given its clinical significance as a model system.

Programmed Alternative Translation

Genetic control by induced alteration of the rules of translation occurs ubiquitously, but infrequently. The majority of programmed alternative translation systems occur in viruses or virus-like genetic elements. A few cellular genes use this form of control and the number is slowly growing. This form of control is expected to remain rare. In most cases the alternative translation system provides the capacity to express a C-terminally extended form of a protein. This extended form is usually required for a morphogenetic process related to the lifecycle of the virus or virus-like element. A minority of genes use this system to express alternative proteins with different enzymatic features, or as a system of autogenous or feedback control.

Programmed Readthrough of Termination Codons

Readthrough of termination codons is arguably the simplest form of alternative decoding. The sequence context surrounding the termination codon probably acts to reduce the efficiency of translational termination and thus increase the probability that a non-cognate tRNA will recognize the nonsense codon. Some programmed readthrough sites are quite simple. In Sindbis virus, a positive-strand RNA animal virus, expression of a full-length nonstructural polyprotein requires readthrough of a single UGA codon. Readthrough requires only the sequence UGA-C. Peptide release factor recognizes true termination codons using a tetranucleotide signal consisting of the codon and its 3′-neighbor nucleotide. An inappropriate 3′ nucleotide can drastically reduce the rate of termination. This appears to be the mechanism of readthrough suppression in Sindbis virus; slow recognition of this very poor termination sequence allows recognition of the UGA codon, probably by tryptophanyl-$tRNA^{Trp}$.

Other programmed readthrough sites are much more complicated. An example of a complex readthrough site comes from the retrovirus Moloney murine leukemia virus (MoMLV). Expression of the *pol* gene (for polymerase), which encodes the enzymes associated with reverse transcription and integration, are expressed as a translational fusion to the structural proteins encoded by the upstream *gag* gene (for group specific antigen). The fusion occurs by readthrough of a single UAG codon separating the two reading frames. The sequence context following this codon determines the frequency of readthrough, about 5–10%. Maximal readthrough requires a 54 nt downstream region which folds to form a pseudoknot. A pseudoknot is a complex secondary structure in which the loop portion of a stem-loop structure basepairs with an immediately flanking sequence. The readthrough requires this secondary structure but not most of its primary sequence. Only the sequence of two short single-stranded regions are actually required; their function in the mechanism is not understood. The pseudoknot causes misreading of the UAG codon as glutamine, which would require a U●G wobble interaction at the first position of the codon.

Perhaps the most bizarre form of programmed readthrough occurs during co-translational insertion of two unusual amino acids which have been termed the 21st and 22nd amino acids since they are encoded, although in an unusual sense. The 21st amino acid is selenocysteine and the 22nd is pyrrolysine. They are encoded at specialized UGA codons. More is known about the mechanism of selenocysteine incorporation but the same mechanisms appear to be used for pyrrolysine. For selenocysteine, the context surrounding the targeted UGA codons prohibits translation termination and promotes recognition of the codon by selenocysteyl-tRNA^{Sec}. This tRNA has an anticodon complementary to the UGA codon. Because of its unusual structure it cannot bind EF1A, but instead binds to a specialized cognate factor, the SELB protein. SELB in turn binds to a specialized secondary structure called the SECIS (selenocysteine insertion sequence). When bound to the SECIS, SELB delivers selenocysteyl-tRNA^{Sec} into the A site at the UGA codon. In bacteria, the SECIS is located immediately downstream of the UGA, but in eukarya it is in the 3′ non-translated region of the mRNA. In eukarya the SECIS and tRNA recognition are encoded in two interacting proteins. The SECIS recognition protein, SBP2, recruits a ternary complex consisting of the specialized selenocysteine elongation factor, EFsec, bound to selenocysteyl-tRNA^{Sec} and GTP. In a sense, selenocysteine incorporation is an example of specialized nonsense suppression since it employs a dedicated cognate tRNA for the UGA codon.

Programmed Translational Frameshifting

The second form of programmed alternative decoding occurs when a sequence in a gene causes the translating ribosome to shift into a new reading frame while continuing elongation. The most common form of programmed translational frameshifting shifts the ribosome in the −1 direction. -1 frameshifting occurs in many metazoan viruses, including retroviruses, in various plant viruses, lower eukaryotic virus-like elements, and in bacterial insertion sequences; −1 frameshifting is used much less frequently in cellular genes. Frameshifting occurs on a slippery heptameric sequence of the form X-XXY-YYZ, shown in codons of the unshifted frame, by simultaneous slippage of two tRNAs from XXY-YYZ to XXX-YYY. A downstream secondary structure, a pseudoknot in most eukaryotic sites and a stem-loop in most prokaryotic sites, stimulates slippage. How the structure does this is not understood, though it involves more than just pausing the ribosome during translation. In bacteria a second site upstream of the slippery heptamer stimulates slippage. At this site the Shine-Dalgarno sequence of the 16S rRNA engages the mRNA and appears to literally pull the mRNA to stimulate slippage of the tRNAs.

+1 frameshifting is much less common. It was first discovered in a yeast retrotransposon (a transposable genetic element related to retroviruses), but is also found in cellular genes in *E. coli*, yeast, and mammalian cells. In each case frameshifting occurs when a poorly recognized codon occupies the A site while a special codon occupies the P site. In most cases, a tRNA occupies the P site codon that can slip +1 while still maintaining at least 2 bp with the mRNA. In some cases, though, frameshifting appears to occur without the need for slipping. In these cases a special feature of the pep-tRNA stimulates out-of-frame decoding in the A site, shifting reading in the +1 direction. In yeast it is clear that the feature causing this behavior is the fact that the pep-tRNA is a near-cognate tRNA, it is an isoacceptor that cannot form a normal wobble interaction with the codon. A purine●purine clash in the wobble position by the pep-tRNA in some way disrupts recognition in the A site, causing out-of-frame decoding.

Programmed Translational Bypassing

Translational bypassing is the rarest form of programmed alternative decoding. To date it has been demonstrated only in the bacteriophage gene *60*, encoding topoisomerase. This gene is interrupted by a 50 nt insertion that is present in the mRNA. Despite the interruption the ribosome is able to decode the gene, bypassing the 50 nt entirely. Bypassing depends on several stimulatory features: matching 'take-off' and 'landing' codons, an in-frame termination codon immediately after the take-off codon, a stem-loop structure encompassing these two codons and a nascent peptide sequence encoded immediately upstream of the take-off codon. The matching take-off and landing codons reflect the need for the pep-tRNA to dissociate from the mRNA and re-pair after the 50 nt interruption. Slow recognition of the next codon, the in-frame termination codon, probably allows sufficient time for dissociation of the pep-tRNA. The exact function of the other features is still unclear.

The generality of the gene *60* bypass has recently been reinforced by a study showing that inefficient bypassing can be stimulated merely by providing a sufficient translational pause at a poorly recognized codon. During the pause pep-tRNA can dissociate from the mRNA and scan in the 3′ direction in search of a matching codon. The efficiency of the bypass depends on the distance to the next codon since pep-tRNAs tend to spontaneously dissociate from the ribosome during the search.

StopGo: Termination and Reinitiation of Translation both by Non-canonical Means

Arguably, the most bizarre form of translational recoding occurs at the end of a gene encoding the 2A peptides of picornaviruses. These viruses express a polyprotein from a single ORF encoded in their genomic RNA. The various activities in that polyprotein must be separated from each other into individual polypeptides and in most cases that separation involves proteolytic cleavage of the primary translation product. 2A was originally thought to be a protease because it is required for two such events in the region encoding sturctural proteins of the virus capsid. Rather unlike an enzyme, though, the peptide is present at two positions immediately upstream of the site of cleavage. This structure suggested that the peptide acts stoichiometrically to induce a conformation of the polyprotein that destabilizes this bond rather than acting as a true enzyme. The putative cleavage site consists of the sequence N-Pro-Gly-Pro-C with the proposed site of cleavage being between the Gly and next Pro.

However, it now has been shown that in fact no chemical bond is ever created between these two amino acids. Rather, translation of the polyprotein proceeds until the peptidyl-$tRNA^{Gly}$ is bound at the Gly codon. At this point, rather than continuing by transfering the peptide to the $tRNA^{Pro}$ on the next codon, the peptide is released possibly as a result of the action of peptide release factor despite the absence of a termination codon in the A site. After release of the peptide, translation immediately resumes by decoding of the adjacent Pro codon. This reinitiation does not involve the normal machinery and the amino acid inserted is Pro rather than the normal Met. So, both the termination and reinitiation events are non-canonical and, depending on whether peptide release factors are involved, may be independent of the normal translation factors. The 2A peptide encoding gene can be inserted into foreign eukaryotic genes where it induces a StopGo event as it does in picornaviruses. The peptide does not function in bacteria suggesting that the event may require some eukaryote-specific feature of the ribosome or translation factors.

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Trehalose: A Crucial Molecule in the Physiology of Fungi

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Glossary

Anhydrobiotic A state of temporary suspension of vital activities triggered by the lack of water, which allows an organism to resist long periods of drought.
Antifungal A drug that inhibits and/or destroys the growth of fungi.
Antioxidant A molecule that inhibits the oxidation of other molecules.
Appressorium An adhesive structure typical of many fungal plant pathogens, which is used to infect host plants.
Biofilm The term describes a highly organized community of microorganisms, which is associated to surfaces (including medical devices) and is enclosed within a self-produced protective extracellular matrix. They possess complex structural and functional features.
Echinocandins A class of semisynthetic lipopeptide antifungal drugs that act by inhibiting β-1,3-glucan synthesis, thereby damaging fungal cell walls.
Exponential phase The step where the microbial population reaches its highest growth rate, and the generation time is minimal. During this phase microorganisms rapidly consume the corresponding nutrients present in the culture medium.
Free radicals Atoms or group of atoms that have at least one unpaired electron and which, therefore, are unstable and highly reactive.
Fungi Eukaryotic organisms, heterotrophic that lack of chlorophyll, aerobic (rarely anaerobic). They display a mycelial structure (mycelium) with rigid cell walls. Widely distributed in nature, they act essentially as saprophytic organisms. Some fungi are opportunistic pathogens of humans, animals and plants, there are species that produce mycotoxins.
Macrophage A type of white blood cell that engulfs and digests cell debris, foreign substances, microbes, cancer cells, and anything else that does not contain the components specific of healthy body cells on its surface, in a process called phagocytosis.
Nosocomial infections Microbial infections (usually caused by opportunistic pathogens) acquired in hospitals or health care centers.
Oxidative stress The loss of the inner redox balance of an organism, affecting the ratio between the oxidants generated as a result of cellular metabolism and those of antioxidant defense systems.
Phagocytosis A type of innate immune response, during which phagocytes engulf and digest microorganisms and cell debris. It constitutes an important defense against infection.
Stationary phase A growth state in which the number of microorganism (or other mass culture parameters) does not increase. Cells in the stationary phase evolve a different metabolism to those in the exponential phase.
Thermotolerance The ability to withstand relatively warm conditions. Usually an adaptive mechanism that allows the cells to survive a lethal heat-shock, provided they are previously subjected to a high, but otherwise non-lethal growth temperature. This "adaptive tolerance" is extended to other environmental types of stress.
Trehalose A natural alpha-linked disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units. It plays important physiological roles in the biosphere, but is absent in mammals
Virulence The degree of pathogenicity of a microorganism expressed as the rate of morbidity/mortality, or the capacity to cause disease.
Yeasts Unicellular and (generally) uninucleate fungi, endowed with asexual reproduction through budding or fission, as well as sexual reproduction by fusion of compatible cells. Some species are able to carry out dimorphic conversion from individual cells to hyphal structures (mycelia) under certain environmental conditions. Several yeasts possess important biotechnological applications, and a few are pathogenic for humans.

Introduction: Trehalose, an Outstanding Molecule

Trehalose (α-D-glucopyranosil α-D-glucopyranoside) is a nonreducing disaccharide composed of two glucose moieties linked by their anomeric carbons. Although three configurations are chemically feasible, (α,β-1,1-, β,β-1,1- and α,α-1,1-), only the last one has been preserved during evolution. In fact, trehalose is widely present in the biosphere, where it fulfils essential roles in a variety of prokaryotic and eukaryotic organisms, ranging from archaea, bacteria and fungi to plant and invertebrates (Argüelles, 2000; 2014;

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Elbein et al., 2003; Eleutherio et al., 2015). Surprisingly, however, trehalose synthesis is absent in vertebrates, including mammals, despite the fact they have conserved genes coding for trehalose-hydrolyzing enzymes (Elbein et al., 2003; Argüelles, 2014). This raises some interesting questions regarding the distinct evolutionary lineages followed by invertebrates and vertebrates. The molecule was discovered in 1832 by Wigers in rye contaminated by a fungus (very likely ergotism) and the etymology of the term "trehalose" comes from, a sweet substance secreted by several beetle and insect cocoons ("trehala manna"). Curiously, it has been suggested that trehalose might have been the "manna," described in the Biblical *Book of Exodus* as the sweet and nutritious food promised by God to Moses. This miraculous substance was eaten by Israelites, allowing them to survive during their long journey through the desert (Richards et al., 2002).

Why, does trehalose seem to be the "chosen" natural compound, compared to other structurally similar sugars? The biological success of trehalose can be explained by its chemical structure as well as by its exclusive physical properties (summarized in Table 1). The α,α-1,1-glycosidic linkage confers a nonreducing character and makes trehalose highly resistant to both acid hydrolysis and cleavage by nonspecific glycosidases. Together with its great hydrophilicity, trehalose is more stable at a wide range of temperatures and pH (Richards et al., 2002; Argüelles, 2014). In water solutions, trehalose is able to maintain a glass state that allows the formation of permanent nonhygroscopic glasses at high temperatures and upon desiccation. Thus, trehalose can protect membranes and biological structures by replacing the water molecules during periods of dehydration, toxic exposure or freezing (Richards et al., 2002; Elbein et al., 2003; Crowe, 2007).

Metabolism of Trehalose

Several metabolic pathways have been characterized in trehalose among the archetypical organisms so far studied (Fig. 1; Scheme 1; Argüelles, 2000; Elbein et al., 2003). Notably, the prokaryotes contain more developed systems for trehalose metabolism, which would explain the ample variety of roles played by the disaccharide compared to eukaryotes. Thus, in bacteria, trehalose synthesis takes place through at least three routes (termed as OtsAB, TreS and TreYZ) (Fig. 1), all of them rendering free trehalose as end product, but differing in the substrates and enzymes involved. In turn, each pathway seems to be induced in response to specific nutritional or environmental stimuli (Elbein et al., 2003; Avonce et al., 2006; Tournu et al., 2013). However, it is quite common for them to coexist in a single bacterial species, even in several copies. In contrast, the eukaryotes have only conserved the *OtsAB* "orthologous" pathway (named as *TPS/TPP*) (Avonce et al., 2006; Tournu et al., 2013; Argüelles, 2014). The corresponding genes coding for enzymes involved in trehalose metabolism have been cloned in a large number of bacteria and fungi. It is worth noting that the only reported archaeal trehalose synthase complex matches the yeast mechanism (Zaparty et al., 2013).

Biosynthesis

In yeasts and fungi, trehalose is synthesized in two enzymatic sequential steps, for which the genes responsible have been cloned and sequenced in several species (Scheme 1): (1) the transfer of a glucosyl unit from UDP-glucose to glucose-6P leads to the formation of trehalose-6P, a reaction catalyzed by a Mg-dependent trehalose synthase (*TPS1*) (Cabib and Leloir, 1958; Zaragoza et al., 1998; Bell et al., 1998); (2) subsequent dephosphorylation by a specific trehalose phosphatase (*TPS2*) gives rise to free trehalose, the physiological compound stored inside the cells (Van Dijck et al., 2002; Zaragoza et al., 2002). An alternative pathway using ADP-glucose as glucosyl donor has been characterized in some bacteria, but, to our knowledge, not yet in fungi.

Trehalose-biosynthesizing enzymes have been extensively studied in budding yeasts (*Saccharomyces cerevisiae*), where Tps1p and Tps2p are integrated in a single complex with two other proteins, named Tls1 and Tls3, which lack catalytic activity and essentially contribute to stabilizing this complex. Single deletion of either Tls1 or Tps3 has no significant effect on trehalose biosynthesis, but a double disruption causes a 50% decrease in the endogenous content of trehalose and modifies the effect of phosphate on trehalose synthase activity (Bell et al., 1998; Gancedo and Flores, 2004).

Table 1 A survey of the main features, physiological roles and applications displayed by the nonreducing disaccharide trehalose (*O*-α,-D-glucopyranosyl-[1→1]-α-D-glucopyranoside) in the biosphere.

Physical properties	*Physiological roles*	*Biotechnological applications*
Nonreducing sugar	Energy and carbon reserve/source	Therapeutic compound
High hydrophilicity	Cell protectant against stress	Antifungal target
Internal chemical stability	Stabilizer of membranes and proteins	Cryoprotectant
Unable to form hygroscopic glasses	Component of the bacterial cell wall	Prophylactic and stabilizer
Absence of inner hydrogen bonds	Major compatible solute	Industrial fermentation
Resistant to acid hydrolysis	Sensing compound	Food sweetener
Elevated melting point	Metabolic regulator	Nutritional supplement
	Virulence factor	Cosmetic additive

Source: Adapted from Argüelles (2014).
Note: For other specific details, see the text

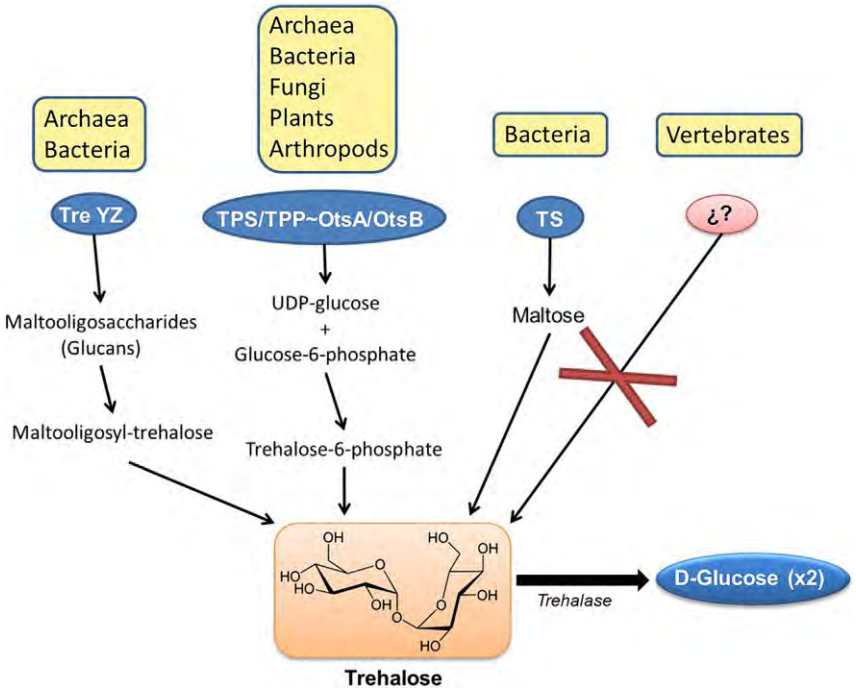


Fig. 1 The established metabolic pathways involved in trehalose biosynthesis in the biosphere. The corresponding organisms are indicated within squares. To date, the annotated genomes of vertebrates lack any functional trehalose synthase/phosphatase gene. Furthermore, no alternative route for trehalose interconversion or isomerization is known. The hydrolysis of trehalose by trehalases releases two molecules of glucose. Partially adapted from Argüelles (2014). For other specific details, see the text.

Hydrolysis

In contrast, trehalose degradation in eukaryotes is essentially confined to the enzyme trehalase (E.C. 3.2.1.28), an α-glucosidase rigorously specific for trehalose that cleaves off the α,α-1,1-glycosidic linkage to yield two molecules of D-glucose (Scheme 1). There are two exceptions to this universal hydrolytic mechanism:

Trehalose phosphorylase: this enzyme carries out the phosphorolysis of trehalose into glucose-1P and glucose, a reversible reaction which, in vivo, tends toward the degradative step (Scheme 1). Among fungi, this activity has mainly been described in basidiomycetes (eg, *Schizophillum*, *Agaricus* or *Flammulina* spp.).

Phosphotrehalase: it catalyzes the conversion of trehalose-6P into glucose and glucose-6P (Scheme 1). Phosphotrehalase has mainly been reported in prokaryotes and in a single strain of the yeast *Pichia fermentans*, which throws doubt on its existence in fungi (Argüelles, 2000; Tournu et al., 2013).

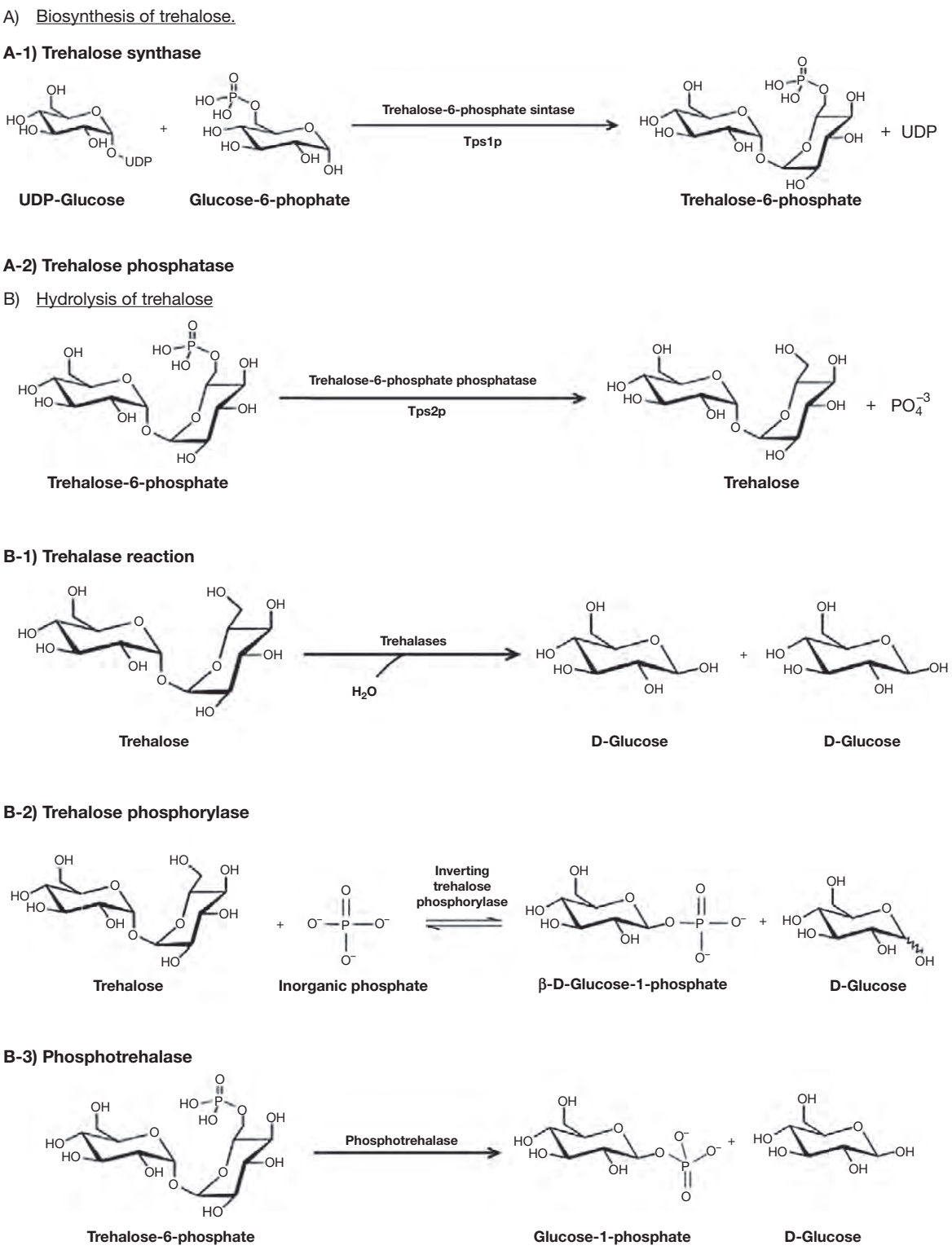
Regarding trehalase activity, yeasts and filamentous fungi usually contain two different types of this α-glycosidase, distinguishable on the basis of their cellular location, amino acid sequence, tridimensional configuration, catalytic parameters, physiological roles and regulatory mechanisms. They are normally called neutral (Nth1p/Ntc1p) and acid (Ath1p/Atc1p) trehalases (Thevelein, 1984, 1996). Despite their very low degree of similarity, the two enzymes are rigorously specific for trehalose as sole substrate, a specificity based on an equivalent folding structure in the active center and an identical catalytic anomeric inversion mechanism. Notably, in the yeast *Candida parapsilosis*, the homozygous disruption of both trehalases does not cause lethality (Sánchez-Fresneda et al., 2015).

On the other hand, although still not fully characterized, another kind of trehalases, identified in some thermophilic fungi, displays a different pattern of biochemical parameters, including an optimum temperature of 50–60°C. Their biological functions remain unknown, but the high thermal stability makes them a promising source of soluble trehalases for both research and biotechnological purposes (Cardello et al., 1994; Bharadwaj and Maheswari, 1999).

Principal Physiological Roles of Trehalose in Fungi

Reserve Carbohydrate and Stress Protective Molecule

For many years, trehalose in fungi has been considered as a carbon and energy reserve compound, because it is largely stored by starved or resting vegetative cells as well as by dormant and reproductive structures (spores, sclerotia, germlings, myxoamoebae) (Wiemken, 1990; Thevelein, 1996; Argüelles, 2000; Elbein et al., 2003), where it may be found in high concentrations (up to 15–20% of dry cell weight) (Fig. 2). The rapid mobilization of trehalose is closely correlated with the resumption of active growth and spore germination. Nevertheless, this role is somewhat controversial (Thevelein, 1984, 1996; Wiemken, 1990; Argüelles, 2000;



Scheme 1 General pathways involved in trehalose biosynthesis (A) and hydrolysis (B) elucidated in prototypical models of fungi.

Elbein et al., 2003), since a "genuine" reserve compound is only synthesized when the exogenous energy exceeds the cellular needs for growth and biosynthesis, and is degraded when cell viability is threatened. However, trehalose biosynthesis contradicts this rule, and is predominantly synthesized at the onset of reduced growth periods, not when there is an excess of exogenous energy. Furthermore, trehalose storage remains intact under resting (ie, spores) or starvation (ie, stationary phase) conditions, and the amount of glucose released from intracellular trehalose mobilization accounts for a minimal percentage of energy compared to that provided by the classical reserve carbohydrate, namely glycogen (Wiemken, 1990; Thevelein, 1996; Argüelles, 2000).

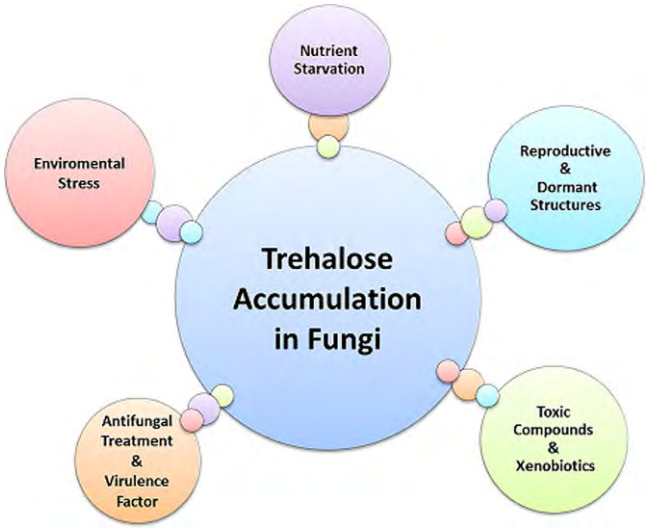


Fig. 2 Main environmental and physiological conditions able to induce trehalose accumulation in Fungi.

More convincing is the evidence supporting trehalose as an efficient protector of cellular integrity against drastic nutritional or environmental stresses (eg, nutrient starvation, high temperatures, osmotic pressures, oxidant agents, heavy metals, anhydrobiosis, xenobiotics, etc., Fig. 2). This phenomenon has been broadly characterized in numerous fungal species, which are able to respond to these sudden perturbations by the rapid synthesis of huge amounts of trehalose, providing them with the potential to withstand these challenges for long periods (Wiemken, 1990; Argüelles, 2000; Richards et al., 2002; Crowe, 2007). The exclusive physical features of trehalose described above (Table 1), together with the absence of internal hydrogen bonds in the molecule, are sufficient to explain the principal role exerted by the disaccharide as stress metabolite (Table 1).

A strong correlation between trehalose content and resistance to osmotic stress was unequivocally demonstrated in many prokaryotic and eukaryotic organisms (Strom and Kaasen, 1993; Thevelein, 1996; Elbein et al., 2003). Indeed, in some bacteria, the enzymes involved in trehalose metabolism are specifically regulated by osmotic shock (eg, the *otsA*, *otsB* and *treA* genes in *Escherichia coli*). In many fungi, large amounts of trehalose are accumulated in response to an increase in external osmolarity (Argüelles, 2000, 2014; Elbein et al., 2003). It is worth mentioning the biological significance of this mechanism in anhydrobiotic organisms, which encompass not only fungi but also plants, insects and microscopic animals (rotifers, nematodes or crustaceans). These life forms are able to survive in virtually dehydrated ecosystems because they accumulate substantial concentrations of endogenous trehalose. Furthermore, during the desiccated state the metabolic activity of these cells is virtually negligible, but they can endure adverse conditions like drought, heat, frost or radiations for many years (Wiemken, 1990; Thevelein, 1996; Eleutherio et al., 2015). The water replacement mechanism and the formation of nonhygroscopic glasses by trehalose, together with its elevated chemical stability may account for this process, since trehalose preserves both membranes and proteins in a physical state resembling that associated with fully hydrated conditions (Elbein et al., 2003; Crowe, 2007). Upon rehydration, the "metabolic resurrection" of anhydrobiotic organisms is accompanied by simultaneous rapid trehalose mobilization.

An important corollary of the above is the pivotal role of trehalose as a molecular chaperone in the efficient refolding of proteins partially denatured after severe heat-shock exposure and stabilizing proteins at high temperatures (Singer and Lindquist, 1998; Crowe, 2007). Later on, trehalose must be quickly hydrolyzed in order to ensure proper thermal-stress recovery (Singer and Lindquist, 1998). The resting states and dormant forms of biological cycles in fungi (ie, stationary phase and spores) are thermotolerant conditions and positively correlate with trehalose storage. These conditions can be mimicked in the laboratory through the "acquired thermotolerance" approach, a mechanism that, in yeasts, allows actively growing cells (25–30°C) to survive subsequent exposure to potentially lethal temperatures (<50°C), provided the cultures are previously subjected to a permissive heat-shock (37–42°C), which induces an enormous accumulation of intracellular trehalose (Singer and Lindquist, 1998). Interestingly, trehalose accumulation is also induced upon cold exposure and is essential for the maintenance of cell viability at low temperatures. This feature explains its useful biotechnological application as cryoprotectant in the food, cosmetics and pharmaceutical industries (see below; Table 1; Ohtake and Wang, 2011; Argüelles, 2014).

The physiological protective function of trehalose can be widened to include the so-called "core stress response," which has been unequivocally demonstrated in yeasts. By means of this mechanism, exponential cells (preferentially) can withstand the exposure to a set of lethal injuries, following stimulation with gentle doses of a non-related stress. For instance, mild upshifts from 28–30 to 37–42°C, increase the degree of cell survival against a further oxidative treatment with 50 mM H_2O_2 (Argüelles, 2000, 2014). Of course, this core response is very complex and trehalose is only part of a global defensive response that includes many components, such as transcription factors, regulatory proteins, detoxifying enzymes and small molecules like glutathione or α-tocopherol. Remarkably, the stress-induced trehalose synthesis in yeasts, as well as its storage as compatible solute in bacteria,

provides consistent support for its protective role in unicellular organisms that must confront the challenging nutritional and environmental conditions (Argüelles, 2014).

Oxidative Stress Protector in Pathogenic Fungi

At this point, we considered convenient to make a few pertinent remarks on the fungal defensive framework against stress. Among potential fungal pathogens in humans and animals, trehalose appears to be mainly related with a specific protection against oxidative stress, a mechanism connected with virulence and essential to withstand the endogenous reactive oxygen species (ROS) produced by phagocytic cells in the course in vivo tissue colonization by the fungus (Martínez-Esparza et al., 2007, 2009). Thus, in *Candida albicans* massive endogenous trehalose synthesis occurs in response to external oxidants or during macrophage ingestion, rather than in the face of other environmental challenges. Furthermore, mutants disrupted in trehalose biosynthesis (*tps1Δ* and *tps2Δ*) show high sensitivity to exogenous oxidants and phagocytosis by murine macrophages (Martínez-Esparza et al., 2007, 2009) In turn, the perturbation of trehalose homeostasis increases the susceptibility of *Aspergillus nidulans* to oxidative stress (Fillinger et al., 2001). In *Cryptococcus* spp., the trehalose metabolism plays a key role in regulating sporulation and further spore viability (*Cryptococcus neoformans*), while trehalose alterations cause defects in melanin synthesis, capsule production, mating and the cell wall architecture (*Cryptococcus gatti*) (Petzold et al., 2006; Botts et al., 2014).

Antifungal Target

Compared with the large number of pathogenic bacteria, infective fungi are scarce and generally opportunistic, most being associated with septicemic mycosis, infections secondary to a previous debilitating illness (Pfaller and Diekema, 2010). For these reasons, antifungal therapy has received less attention than its antibacterial equivalent. However, this picture has dramatically changed in the last two decades, in which the morbidity and mortality caused by opportunistic fungi have strikingly increased, particularly among the ageing and immunocompromised population (including AIDS patients, transplant recipients, neonates or those undergoing intensive chemotherapy). At present, *C. albicans* represents the fourth cause of nosocomial infections recorded in US hospitals.

The biochemical and genetic data available on pathogenic yeasts has led to the enzymes involved in the trehalose metabolism to be proposed as potential targets for the design of new antifungals (Fig. 2). The most important evidence in this respect is: (1) the nonreducing sugar is present in bacteria, fungi, plants and invertebrates, but is absent in mammals, a finding that favours its clinical application with high selective toxicity (Richards et al., 2002; Elbein et al., 2003; Argüelles, 2014); (2) trehalose accumulation seems to be a protective mechanism of pathogenic fungi during in vivo infection (Martínez-Esparza et al., 2007, 2009); and (3) the genes coding for trehalose-6P-synthase (*TPS1*) and trehalose-6P-phosphatase (*TPS2*) are determinants of virulence; and the corresponding *tps1Δ* and *tps2Δ* null mutants show diminished resistance to phagocytosis mediated by macrophages (Zaragoza et al., 1998, 2002; Van Dijck et al., 2002; Martínez-Esparza et al., 2007, 2009). Interestingly, echinochandins, which act as potent inhibitors of cell wall glucan synthases, are widely used as antifungals in clinical practice (Denning, 2003). Accordingly, a *C. albicans tps1Δ* mutant show alterations in the cell wall structure, and the acid trehalase is located in the external cell wall in some pathogenic yeasts (Pedreño et al., 2004; Martínez-Esparza et al., 2007; Sánchez-Fresneda et al., 2015). Furthermore, Amphotericin B significantly induces trehalose synthesis in *C. albicans*, the *tps1Δ* mutant being very sensitivity to the polyene (González-Parraga et al., 2011). Therefore, trehalose might be a promising avenue in the search for new antifungal chemotherapeutic agents.

Virulence Factor

The hypothetical role of trehalose as a determinant of virulence is somewhat interconnected with the rationale documented in the above two sections. Data in favor of this proposal have mainly been obtained using in situ genetic approaches in the prevalent pathogenic yeast *C. albicans*, by means of single disruption of the trehalose-biosynthesizing genes *TPS1* and *TPS2*. To the best of our knowledge, a homozygous double *TPS1/TPS2* disruption has not been achieved yet. In addition to defects in the cell wall architecture, a *C. albicans tps1Δ* mutant displays high vulnerability to oxidative stress and diminished resistance to phagocytosis by macrophages stimulated with interferon-γ (Martínez-Esparza et al., 2007). The *tps2Δ* mutant shares similar sensitivity to oxidants and macrophage killing, but the phagocytosis triggers a proinflammatory response with the release of TNF-α (Martínez-Esparza et al., 2009). Furthermore, *tps2Δ* cells exhibit a pleiotropic phenotype that includes deficient growth at high temperatures (42°C), greater permeability and cell aggregation in resting cultures (Martínez-Esparza et al., 2009).

Regarding trehalose hydrolysis, the lack of a functional neutral trehalase (Ntc1p) does not seem to play any relevant role in dimorphic conversion or pathogenicity (Eck et al., 1997). However, disruption of the *ATC1* gene encoding the acid trehalase activity hinders the cell's capacity to use exogenous trehalase and impairs the infectivity and degree of hypha formation, crucial events during host tissue invasion (Pedreño et al., 2004; Sánchez-Fresneda et al., 2015). Although not yet performed in *C. albicans*, the homozygous deletion of *ATC1* and *NTC1* in *C. parapsilosis* is not lethal (Sánchez-Fresneda et al., 2015).

Trehalose also participates in the pathobiology of other relevant opportunistic fungal pathogens, namely *Aspergillus* spp., which is a frequent cause of pulmonary diseases, and *Cryptococcus* spp., that infects mainly HIV patients and produces a characteristic meningoencephalitis. In *Aspergillus fumigatus*, a mutant deficient in T-6P phosphatase activity shows acute morphological defects, related to asexual reproduction, and is avirulent in two distinct models of invasive pulmonary aspergillosis (Puttikamonkul et al.,

2010). Surprisingly, the double *ΔtpsAB* strain lacking a functional trehalose synthase complex was hypervirulent in a murine test, a perturbation also associated with susceptibility to oxidative stress, alterations in the cell wall and resistance to phagocytosis (Al-Bader et al., 2010). In *C. neoformans*, the orthologous *tps1* mutant unable to synthesize trehalose showed attenuated virulence and sensitivity to exogenous oxidants compared to *tps2* (T-6P phosphatase) and *nth1* (neutral trehalase) mutants (Petzold et al., 2006). In contrast, deletion of *NTH2* gene, which encodes a previously uncharacterized secreted trehalase, caused hypervirulence in a murine model (Botts et al., 2014).

Nevertheless, the strong evidence concerning the crucial implication of trehalose metabolism in fungal virulence was not gathered from human pathogens, but comes from the elegant work performed with the phytopathogenic rice blast fungus *Magnaporthe grisea* (Foster et al., 2003). The inability to synthesize trehalose in a *tps1Δ* mutant gives rise to impaired sporulation and attenuated pathogenicity. In addition, the appressoria developed by the fungus showed poor development and decreased cuticle penetration (Foster et al., 2003). The prevention of trehalose mobilization by disruption of the *NTH1* gene, which encodes neutral trehalase activity, also reduced *M. grisea* infectivity due to the lower capacity to colonize plant tissue (Foster et al., 2003). More relevant, Tps1p is a key regulatory molecule that integrates control of glucose-6P metabolism and nitrogen source utilization, through direct regulation of the pentose phosphate pathway, the generation of NADPH and the nitrate reductase activity in *M. grisea* (Wilson et al., 2010). Together, these results correlate the pivotal role played by Tps1p in fungal virulence and the integration of carbon and nitrogen metabolism.

Regulatory Signal

Research into trehalose in plants reflects the spectacular increase in our knowledge of this singular molecule gained during the last 30 years. For a long time, trehalose was considered a rare sugar confined to the so-termed "resurrection" plants. Nowadays, however, the current picture points to a very complex system that encompasses a large number of genes involved in trehalose metabolism, with distinct degrees of functionality (Eastmond and Graham, 2003). Notably, the intermediate trehalose-6P acts as a key regulator of important physiological and developmental processes in plants, such as carbohydrate utilization, starch mobilization in leaves, embryo maturation, and flower and young tissue development (Eastmond and Graham, 2003).

In the case of yeasts and filamentous fungi, an unexpected regulatory role for glycolysis by trehalose emerged from the unpredictable observation that a set of mutants deficient in *S. cerevisiae* trehalose-6P-synthase activity (*tps1*, *fdp1*, *cif1*, *byp1* or *ggs1*) were unable to grow on glucose, although the glycolytic enzymes assayed in vitro remained fully active (Gancedo and Flores, 2004). In contrast, deletion of the *TPS2* gene did not cause this metabolic defect. When the *tps1* mutants were grown on non-fermentable carbon sources, the accumulation of hexose phosphates was concomitant with a marked drop in the internal ATP content, a defect explained by an imbalance between the rates of the initial ATP-consuming steps of glycolysis and those regenerating it after the glyceraldehyde 3-P dehydrogenase reaction (Gancedo and Flores, 2004). Several hypotheses have been advanced in this respect. (1) Tps1p is a direct regulator of glucose transport and phosphorylation through its alliance with a sugar carrier and hexokinase in a postulated "General glucose-sensing complex" (Thevelein and Hohmann, 1995; Gancedo and Flores, 2004); (2) Trehalose synthesis serves as a metabolic buffer when glycolysis overflows by channeling the excess of glucose-6P (precursor of trehalose synthesis), allowing the recovery of free phosphate, which is required for the mentioned glyceraldehyde-3P dehydrogenase reaction; (3) Biochemical results support the competitive inhibition of hexokinase by T-6P (Blázquez et al., 1993). Thus, this intermediate, whose accumulation might be toxic, has emerged as new key regulator of yeast glycolysis (Gancedo and Flores, 2004).

It is worth noting that in *M. grisea* (see above), *C. albicans* and *Kluyveromyces lactis* a functional Tps1p activity is required for normal glycolytic flux and, accordingly, a *Δtps1* mutant cannot grow on glucose or fermentable sugars, displaying thereby a serious metabolic imbalance. Although the molecular data are still inconclusive, trehalose hydrolysis in fungi may be considered a physiological signal from the transition from resting states to full active growth (in accordance with the abundant evidence documented above).

Final Comments and Perspectives

Since its discovery in 1832, investigation into the biological significance of trehalose has come a long way. Of the wide number of physiological roles played by trehalose, the most relevant appear to be confined to the specific systems analyzed. Thus, in bacteria, trehalose serves as carbon source, compatible solute or structural component (the "cord factor" in mycobacteria). In insects, trehalose is considered the main biofuel present in the circulatory fluid (hemolymph), where it represents up to the 20% of the total carbohydrate pool in certain stages of development. Intriguingly, vertebrates do not have the ability to synthesize trehalose, which raises some interesting questions on its evolutionary phylogeny. Within fungi, yeasts could be considered the simplest eukaryotes and, therefore, a good model for biological studies. The extensive experimental work carried out on trehalose metabolism in several yeasts has revealed the specific functions described here, many of them also present in multicellular organisms. Finally, although beyond the scope of this article, mention must be made of the increasing number of biotechnological, nutritional and therapeutic possibilities of trehalose, some of them already in commercial use. As a conspicuous example, we would like to emphasize the exciting new findings on the important role played by trehalose as an inducer of autophagy via an

mTOR-independent pathway, which is currently used to combat the formation of amyloid plaques, a critical symptom of Hungtinton's, Parkinson's, Alzheimer's diseases and other severe neurodegenerative disorders.

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Trypanosomes☆

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Dedication: We dedicate this chapter to our esteemed colleague Dr. Herbert B. Tanowitz who passed away suddenly on July 17, 2018.

Glossary

African trypanosomiasis (sleeping sickness) An illness endemic in large areas of sub-Saharan Africa, which is transmitted by tsetse flies. *Trypanosoma brucei gambiense* is the cause of West African or gambiense trypanosomiasis, and *Trypanosoma brucei rhodesiense* is the cause of East African or rhodesiense trypanosomiasis.
Amastigote The life-cycle stage of *Trypanosoma cruzi* that multiplies intracellularly.
American trypanosomiasis (Chagas disease) An illness endemic to all non-Caribbean countries in Latin America, which is caused by the protozoan parasite *Trypanosoma cruzi* and is usually transmitted by triatomine insects.
Blood-form trypomastigote The life-cycle stage of both American and African trypanosomiases found in the blood, cerebrospinal fluid, and other extracellular spaces.
Epimastigote The multiplying life-cycle stage of trypanosomes found in insect vectors.
Metacyclic trypomastigote The life-cycle stage of trypanosomes that develops in insect vectors and is infective for humans and other mammals.
Trypanosomiasis A group of diverse diseases caused by members of the genus *Trypanosoma*, which are hemoflagellate protozoa.

Abbreviations

CNS	Central nervous system
DFMO	Difluoromethylornithine
ELISA	Enzyme-linked immunosorbent assay
ES	Expression site
ESAG	ES-associated gene
Hsp70	Heat shock protein 70
ODC	Ornithine decarboxylase
PARP	Procyclic acid-rich protein
PCR	Polymerase chain reaction
RBBB	Right bundle branch block
VSG	Variant surface glycoprotein

Trypanosoma cruzi

Trypanosoma cruzi is the protozoan parasite that causes American trypanosomiasis or Chagas disease. It is epizootic and endemic in all Latin American countries outside the Caribbean, including Mexico, and it is an important cause of chronic heart and digestive diseases in these nations. Studies of autopsied Native American mummies indicate that Chagas disease in humans dates back to the pre-Colombian era, and given the widespread distribution of mammalian reservoirs (e.g., opossums, armadillos, and many dozens of others) it is reasonable to assume that humans have been infected since they first invaded enzootic regions roughly 14,000 years ago. Seroepidemiologic and clinical studies indicate that approximately 8 million persons are chronically infected with *T. cruzi* in endemic countries and roughly 20,000 die annually as a result of the infection. Several autochthonous cases of *T. cruzi* infection have been reported in the United States since 1955, and small numbers of infected blood donors who have not visited endemic countries have been identified since serologic screening of the US blood supply. Naturally infected dogs as well as a wide variety of

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wild mammals have also been described in the southern and southwestern United States, and infected insect vectors have been found in these areas as well.

Transmission

In endemic areas transmission is most common among the rural poor whose dwellings become infested with infected vectors due to the proximity of sylvatic reservoirs. Vector-borne transmission of *T. cruzi* is contaminative, since the infective parasites are in the excreta of the insects. During a blood meal, insect feces containing metacyclic trypomastigotes by chance find their way onto mucosal surfaces, the conjunctivas, or breaks in the skin. There they enter susceptible cells of the new host and establish infection. Control programs directed at decreasing contact with insect vectors through housing improvement, insecticide spraying, and education have met with considerable success in many endemic areas. The transmission of *T. cruzi* by blood transfusion historically has been the second most important mode of spread of the parasite. This was a major public health problem in many endemic regions, but its importance has been reduced markedly in recent years through widespread implementation of serologic screening of donated blood. Several cases of transfusion-associated transmission of *T. cruzi* have been reported in the United States, Canada and European countries, resulting from the transfusion of blood donated by asymptomatic *T. cruzi*-infected immigrants from Latin America. Other modes of transmission include congenital, organ transplantation, laboratory accidents, ingestion of contaminated food or drink, and breast feeding.

Organism and Life Cycle

T. cruzi has a complex life cycle in which four morphologically and biochemically distinct forms exist (Fig. 1). In mammalian hosts, there are two forms: extracellular nondividing trypomastigotes (blood forms) (Figs. 2 and 3), and intracellular dividing amastigotes (Figs. 4 and 5). During a blood meal, the insect vectors ingest blood forms, which then transform into epimastigotes in the midgut. After 3–4 weeks, infective but nondividing metacyclic trypomastigotes are present in the hindgut of the vector. These forms are then deposited with the excreta of the vector during subsequent blood meals. Transmission occurs to a new mammalian host when these infective parasites contaminate susceptible tissues. The infective trypomastigotes parasitize cells by direct penetration or through phagocytosis, and then transform into amastigotes.

The mechanisms by which the trypomastigotes gain entry into host cells are not entirely understood. Although phagocytosis was the first endocytic mechanism described to be used by *T. cruzi*, other mechanisms such as clathrin-mediated endocytosis, caveolar-dependent, and lipid raft-dependent endocytosis macropinocytosis seem to be involved. Studies have implicated the enzyme *trans*-silalidase in this process. Penetrin, another parasite molecule, binds to heparin sulfate receptors on nonphagocytic host cells, thereby promoting adhesion and subsequent parasite invasion. There several host cell receptors that have been described which aide in the entry of the parasite into cells.

Once in the cytoplasm of the host cells, amastigotes (Figs. 4 and 5) undergo binary fission and as sizable numbers accumulate the cell is overwhelmed and transformation to trypomastigotes occurs. Although any nucleated mammalian cell can be invaded by

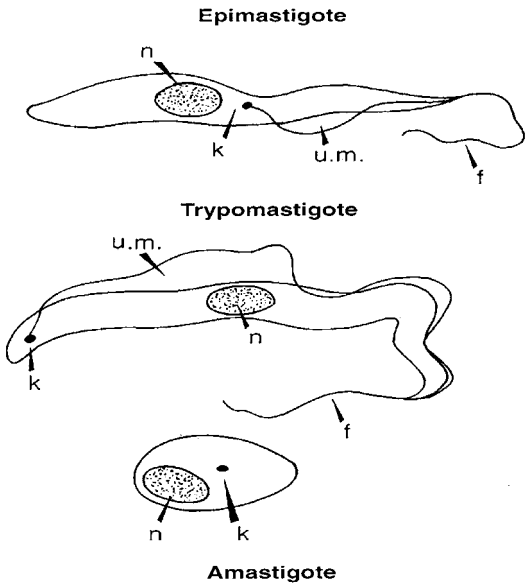

Fig. 1 Life stage forms of *Trypanosoma cruzi*. Note the kinetoplasts; see Fig. 5 for the ultrastructure of this organelle. The trypomastigotes measure 15–20 μm in length, the epimastigotes measure 20 μm length, and the amastigotes measure 3–4 μm in diameter.

Fig. 2 Trypomastigotes of *Trypanosoma cruzi* in the bloodstream. Note the "C" shape. Courtesy of the American Society of Tropical Medicine and Hygiene/Zaiman "A Presentation of Pictorial Parasites."

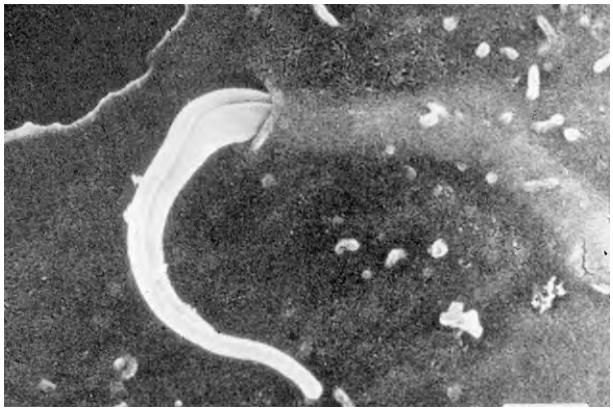

Fig. 3 Scanning electron micrograph of a trypomastigote of *Trypanosoma cruzi* invading a mammalian host cell.

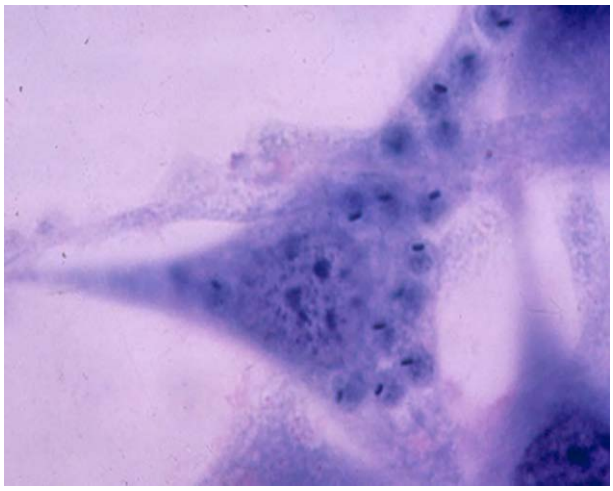

Fig. 4 Giemsa-stained endothelial cells infected with *Trypanosoma cruzi*. Note the numerous amastigotes.

this parasite cells of the reticuloendothelial, nervous and muscle systems appears to be favored. In addition, recent evidence suggests that adipose tissue and adipocytes are readily invaded by trypomastigotes.

The parasitized cells eventually rupture, releasing amastigotes and trypomastigotes that infect adjacent cells and importantly trypomastigotes disseminate through the lymphatics and the bloodstream, ultimately infecting a wide variety of tissues, including cardiac and skeletal muscles, as well as cells of the reticuloendothelial and nervous systems. In the parasitophorous vacuole,

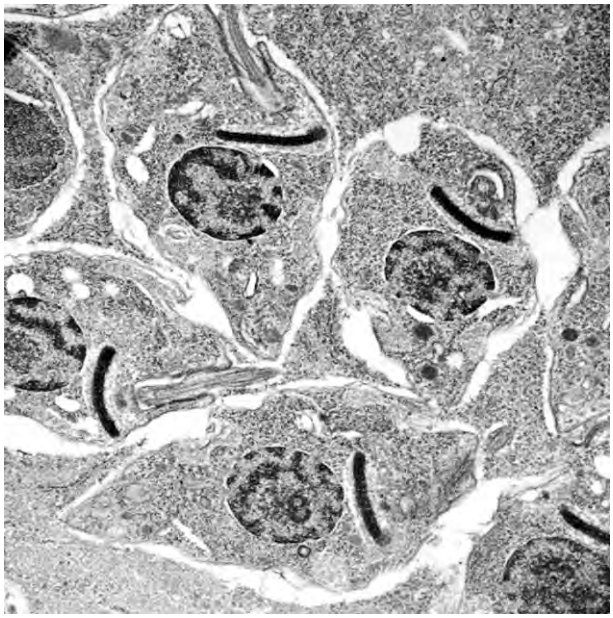

Fig. 5 Transmission electron micrograph of parasitized myoblast cell line. Note the amastigotes in the cytoplasm and their prominent disk-shaped kinetoplasts.

amastigotes synthesize a hemolysin that lyses the vacuolar membrane, thus allowing the parasite to escape the cytocidal mechanism of the cell. Both cell necrosis and apoptosis have been reported to be associated with *T. cruzi* infection.

The chromosomal complement of *T. cruzi* is approximately 50 Mb or roughly 10 times the amount of DNA found in *E. coli*. Pulse-field gel electrophoresis reveals at least 20, and as many as 40, discrete chromosomal bands, ranging in size from 200 to 2000 kb. *T. cruzi* molecular karyotypes are somewhat strain-dependent (i.e., the reference strain CL/Brener contains approximately 60–80 Mb DNA). Among characterized strains, there appears to be only one that contains minichromosomes, in sharp contrast to *T. brucei*. The genome of *T. cruzi* appears to be diploid in all stages. Homologous chromosomes, however, can differ markedly in size, and chromosomes containing conserved linkage groups can vary extensively in size among isolates. *T. cruzi* exhibits significant polymorphism at a number of characterized alleles, far in excess of that seen in higher eukaryotes. It appears that multiplication of *T. cruzi* is exclusively, or nearly exclusively, clonal without any evidence of a sexual stage. Starting back in the 1980s, numerous genes of *T. cruzi* were cloned and characterized, and the complete DNA sequencing of the CL Brener strain of *T. cruzi* and several other strains has been completed (Genome data can be found at http://tritrypdb.org/tritrypdb/).

Many *T. cruzi* genes are arranged in tandem arrays of nearly identical copies, as is the case with GAPDH, histone H1 and heat shock protein (Hsp70). Importantly, *T. cruzi* genes lack introns and all mature mRNAs have a highly conserved 5′ cap (spliced leader) that is added to pre-mRNAs by *trans*-splicing. *T. cruzi* mRNAs contain poly-A tails, but there is no highly conserved poly-A addition site consensus sequence, as is the case with other eukaryotes. The classification of *T. cruzi* strains has been accomplished by a variety of methods including isoenzyme differences (zymodemes), restriction endonuclease digest of kinetoplast DNA (schizodemes), and sequencing of noncoding segments of rRNA genes. There is considerable correlation among the groupings determined by the various approaches. These observations are consistent with the concept that *T. cruzi* consists of many clonal lineages with little sexual recombination. Moreover, the rRNA sequence data also suggest that the clonal lineages fall into two main branches (*T. cruzi* I and *T. cruzi* II) with a very ancient split between them. Assuming a constancy of the molecular clock, the divergence appears to have occurred contemporaneously with the divergence of reptiles and amphibians, which predated the first mammals.

Analyses of the *T. cruzi* CL Brenner strain have revealed that the diploid genome contains a predicted 22,570 proteins, of which 12,570 represent allelic pairs. More than half of the genome consists of repeated sequences, such as retrotransposons and genes for large families of surface molecules, including *trans*-sialidases gp85 super family, mucins, gp63s, and a large novel family (>1300 copies) of mucin-associated surface protein genes. Analyses of the *T. cruzi*, *T. brucei*, and *Leishmania major* genomes imply differences from other eukaryotes in DNA repair and initiation of replication and reflect their unusual mitochondrial DNA, which may offer targets for intervention. Proteomic analysis of the four life-cycle stages of *T. cruzi* discovered that peptide mapping to 2784 proteins in 1168 protein groups from the annotated *T. cruzi* genome were identified across all stages. The different parasite stages use different energy sources depending on the host in which they are found—histidine for stages in the insect vectors and fatty acids by trypomastigotes and amastigotes in the mammalian hosts. With the completion of *T. cruzi* genome, genomic information speeds up researches in drug-discovery, vaccine development, diagnostic markers and pathogenesis.

Epizootiology and Epidemiology

T. cruzi is found only in the southern United States, Mexico, and the nations of Central and South America. There is no Chagas disease on the Caribbean Islands. Infection with this parasite is a zoonosis and the involvement of humans in the life cycle

essentially has nothing to do with the perpetuation of the parasite in nature. The parasite primarily infects both wild and domestic mammals as well as insects. The triatomine insect vectors of *T. cruzi* are distributed from the southern United States to central Argentina. Transmission of the parasite typically occurs among infected vectors and nonhuman mammalian hosts in hollow logs, palm trees, burrows, and other animal shelters. A large number of insect vectors can also be found near houses in piles of roof tiles, wood, and vegetation. *T. cruzi* has been found in more than 100 species of domestic and wild mammals, in a range similar to that of its insect vectors. Opossums, nonhuman primates, wood rats, armadillos, raccoons, dogs, and cats all are typical hosts, but *T. cruzi* is not a problem in livestock.

Nontypical hosts, such as polar bears, can become infected when held in zoos in areas in which *T. cruzi* is enzootic. This lack of species-specificity, combined with the fact that infected mammalian hosts have life-long parasitemias, results in an enormous domestic and sylvatic reservoir in enzootic areas. Humans have become part of the cycle of *T. cruzi* transmission as farmers and ranchers open new lands in enzootic areas. When this development takes place, the vectors, known variously as the "kissing bug" or "assassin bug" (e.g., *Rhodnius prolixus*, *Triatoma infestans*, *Panstrongylus megistus* and many other species) invade the nooks and crannies of the primitive wood, mudwalled, and stone houses that are typical of rural Latin America. In this manner, the vectors become domiciliary, establishing a cycle of transmission involving humans and peri-domestic mammals that is independent of the sylvatic cycle. Historically, Chagas disease has been an infection of poor people living in rural environments. However, over the decades an enormous number of *T. cruzi*-infected people have migrated to cities, thus urbanizing the disease and, prior to the initiation of serologic screening of donated blood, resulting in its frequent transmission by transfusion.

The epidemiology of *T. cruzi* infection has improved markedly in many the endemic countries as vector and blood-bank control programs have been implemented. A major international vector eradication program in the "Southern Cone" countries of South America has provided the framework for much of this progress. To date Uruguay (1997), Chile (1999), and Brazil (2006) have been declared free of transmission. In Argentina the rate of transmission is a fraction of what it was 25–30 years ago, and substantial progress has been achieved in Paraguay and Bolivia. Similar programs have been implemented in the Andean nations and in Central America. The obstacles hindering the elimination of *T. cruzi* transmission to humans are economic and political, and no technological breakthroughs are necessary for overall control of the problem.

Approximately 30% of people who harbor *T. cruzi* chronically will develop associated cardiac or gastrointestinal symptoms. In the past, the high frequency of sudden death among young adults in some areas was attributed to rhythm disturbances due to chronic Chagas disease and in one highly endemic area of Brazil, chagasic cardiac disease was found to be the most frequent cause of death in young adults. There is considerable geographic variation in the relative prevalence of cardiac and gastrointestinal disease in patients with chronic *T. cruzi* infection. It is not known if this variable clinical expression is caused primarily by parasite strain or host factors. In this regard, studies have implicated host genetic factors as important variables in host response to infection.

The number of people in the United States with chronic *T. cruzi* infections has increased substantially in recent decades. Current estimates put the number of immigrants from Chagas-endemic living in the United States at 13 million and at least 300,000 cases of chronic Chagas disease. Their presence creates a risk of transfusion-associated transmission *T. cruzi* here, and seven such cases have been reported in the United States, Canada and European countries. Almost all the reported cases occurred in immunocompromised patients in whom the diagnosis of *T. cruzi* was made because the transfusion recipients became severely ill. This suggests that additional cases have occurred in immunocompetent patients and have gone unnoticed because they were more able to control the acute infection. However, this risk has been essentially eliminated by serologic screening of blood donated here and the confirmed prevalence among donors is about 1 in 29,000. Moreover, transmission of *T. cruzi* through organ transplantation has been reported in the United States. In a series of 32 transplant recipients in the United States who unintentionally received organs from 14 *T. cruzi* seropositive donors from 2001 to 2011, transmission was confirmed in nine recipients.

Clinical Manifestations

Acute Chagas disease

Seroepidemiologic studies in endemic areas indicate that most seropositive patients have no recollection of having had acute Chagas disease and do not carry a specific diagnosis. This results from the generally mild nature of acute Chagas disease, as well as from the fact that the persons most at risk for acquiring *T. cruzi* infection have little access to medical care. Some infected persons may develop severe symptoms after an incubation period of 7–14 days. This has been documented with vector-borne infection as well as infections acquired by blood transfusions or laboratory accidents. Signs and symptoms can include fever, chills, nausea, vomiting, rash, diarrhea, and meningeal irritation. A chagoma, a raised inflammatory lesion at the site of parasite entry, Romaña's sign (unilateral periorbital edema), conjunctivitis, lymphadenopathy, and hepatosplenomegaly have all been described in patients with acute Chagas disease. Laboratory abnormalities can include anemia, thrombocytopenia, and elevated liver and cardiac enzymes. The diagnosis of acute Chagas disease is primarily parasitologic, as motile trypomastigotes often can be found in blood and cerebrospinal fluid. Serologic tests for parasite-specific IgG are often negative during this stage, and assays for IgM have not been standardized and are not widely available. During the acute phase of the illness, asynchronous cycles of parasite multiplication, cell destruction, and infection of new cells occur. Myocarditis and cardiomegaly, sometimes associated with congestive heart failure, are present in some patients and may be more common than was previously appreciated. The appearance of arrhythmias, heart block, or congestive heart failure in the setting of acute. *T. cruzi* infection is poor prognostic indicators. It is not known if the severity of acute Chagas disease is related to the likelihood of development years later of the cardiac and gastrointestinal manifestations of chronic symptomatic Chagas disease. A small percentage of acutely infected patients, often children, die of acute myocarditis or meningoencephalitis. As antibodies appear, the parasitemia wanes, and almost all infected persons resolve the acute phase of the

illness in 2–4 months, but remain infected for life. These patients then enter the indeterminate phase of the illness, which is characterized by a lack of symptoms, easily detectable antibodies to a variety of *T. cruzi* antigens, and low parasitemias. Congenital Chagas disease is another example of acute infection. It occurs in about 5% of infants born to mothers with chronic *T. cruzi* infections.. These babies are most often asymptomatic but can present with fever, jaundice, and occasionally seizures, and may be difficult to distinguish from infants with other congenital infections such as toxoplasmosis. Children with congenital disease also may have cardiac and gastrointestinal manifestations.

Chronic cardiopathy

Chronic cardiac Chagas disease may present insidiously as congestive heart failure or abruptly with arrhythmias and/or thromboembolic events. Dilated congestive cardiomyopathy is an important manifestation of chronic Chagas disease that typically occurs years or even decades after acute infection. Apical aneurysm of the left ventricle is one of the hallmarks of this disease. Chronic chagasic heart disease is associated with myonecrosis and myocytolysis. Contraction-band necrosis occurs after transient hypoperfusion followed by reperfusion, as occurs with local spasm of the coronary microvasculature. Focal and diffuse areas of myocellular hypertrophy are observed with or without inflammatory infiltrates (Fig. 6), and fibrosis replacing previously damaged myocardial tissue is evident. The destruction of conduction tissue results in atrioventricular and intraventricular conduction abnormalities. In areas where the disease is endemic, the presence of right-bundle branch block (RBBB), associated with an anterior fascicular block, is highly suggestive of chagasic cardiopathy. Conduction defects may necessitate the placement of a pacemaker.

Chronic gastrointestinal manifestations

Chagas disease is associated with the loss of neurons of the myenteric plexus. Lesions of the autonomic nervous system produce functional disorders in hollow visceral muscular organs. Initial damage to the nervous system occurs during acute *T. cruzi* infection and may be the consequence of the increase in local inflammatory mediators. Further neuronal loss occurs slowly during the chronic phase of the infection. The development of clinically-manifest chronic gastrointestinal disease may take several decades, and the determinants of this progression are unknown.

The colon is frequently affected in chronic Chagas disease. Although the entire colon may be enlarged, dilatation is often confined to the sigmoid colon. Constipation is a common complaint. Symptomatic patients may have disorders of motility that antedate by years the radiological diagnosis of megacolon. In patients with chagasic megacolon, there is a reduction in the number of ganglia throughout the colon. The esophagus is also frequently affected in patients with long-standing *T. cruzi* infection. Patients with megaesophagus may have <5% of the normal number of ganglia and this loss of neurons is uniform along the length of the organ, including the grossly normal abdominal portion of the esophagus. Typical symptoms of megaesophagus include dysphagia, odynophagia, chest pain, cough, and regurgitation. Mega-gall bladder and mega-ureter also have been reported in patients with chronic *T. cruzi* infections.

Other Clinical Syndromes

Transfusion-associated T. cruzi *infection*

The transmission of *T. cruzi* by transfusion of blood donated by asymptomatic persons who harbor the parasite historically has been a major public health problem in the countries in which Chagas disease is endemic. This mode of transmission of *T. cruzi* has been essentially eliminated in most of the endemic nations as mandatory programs for screening donated blood have been implemented. As noted above, several transfusion-associated instances of *T. cruzi* transmission have been reported in the United

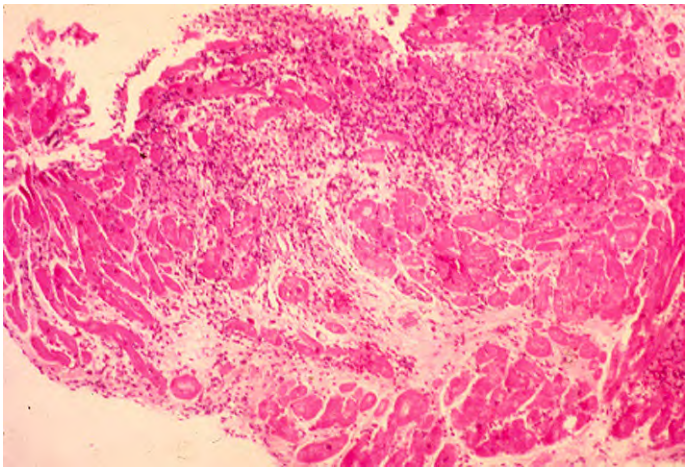

Fig. 6 Endomyocardial biopsy from a patient with chronic Chagas' disease. Note the inflammation and fibrosis. Courtesy of Dr. Alain C. Borczuk, North Shore University Hospital, Manhasset, NY.

States as a consequence of the immigration of infected persons, and widespread screening of donated blood began here in early 2007.

Immunosuppression and transplantation

The incidence of reactivation of *T. cruzi* infection in immunosuppressed people is not known. In view of the possibility of acute reactivation of *T. cruzi*, patients who are at risk of being infected and for whom immunosuppressive treatment is being planned, either as primary or posttransplanation therapy, should be tested serologically and, if positive, should be monitored closely while immunosuppressed for evidence of reactivation. Several dozen HIV-infected patients with reactivation of chronic *T. cruzi* infections have been described. Interestingly, many of these patients developed cerebral lesions radiographically similar to those observed in AIDS patients with cerebral toxoplasmosis. As with the latter, cerebral lesions do not occur in immunocompetent patients chronically infected with *T. cruzi*. Finally, more than a hundred patients with end-stage Chagas heart disease have undergone cardiac transplantation in Brazil, and the procedure has been performed in a couple of dozen *T. cruzi*-infected people in the United States. Because of the intense postoperative immunosuppression used in the early series of patients transplanted in Brazil, life-threatening reactivation of *T. cruzi* infection occurred in some of these patients. However, lower doses of immunosuppressive drugs have been used for many years now and reactivation of *T. cruzi* infection has become much less of a problem. The overall survival of chagasic patients receiving cardiac transplants is better than the total group of patients transplanted for other reasons.

Pathogenesis

Animal models have been used extensively to evaluate the pathogenesis of chagasic heart disease. For example, alterations in cardiac choline acetyltransferase, acetylcholine, norepinephrine, and β-adrenergic adenylate cyclase complex in association with chronic *T. cruzi* infection have been described in experimental animals. The expression of cytokines and NOS2 in the myocardium has been suggested as a possible cause of myocardial dysfunction. Morphological changes during acute *T. cruzi* infection may also be important in the pathogenesis of the myocardial lesions. For example, reduced numbers of autonomic ganglia are associated with clinically-manifest cardiac as well as gastrointestinal Chagas disease, and as noted this may be a direct result of destructive processes that are part of the early phase of the illness. The immunology of *T. cruzi* infection has been studied extensively in animal models and in humans, and a large portion of these efforts have focused on a possible role for autoimmunity in the pathogenesis of chagasic lesions. There is evidence from studies in mice that both humoral and cell-mediated components of the immune system are important in host resistance, as is genetic background. CD8+ T cells are important in the pathogenesis of *T. cruzi* infection. The possible roles of cytokines in the pathogenesis of *T. cruzi* infections also have been the subject of intense investigation. Cytokines are elevated during acute murine infection and may contribute to the pathogenesis of the disease. Increased levels of IL-1β, TNF-α, and IL-6, expressed in infected endothelial cells, result in leukocyte recruitment, coagulation, and smooth muscle cell proliferation. The role of cytokines and nitric oxide in the killing of the parasite has received considerable attention as well. Nitric oxide, IFN-γ, TNF-α, and IL-12 all appear to be involved in intracellular killing. The 21-amino acid peptide endothelin-1 and the eicosanoid thromboxane A_2 have also been implicated in the pathogenesis of *T. cruzi* infection. Trypanosomes contain proteases, gelatinases, and collagenases capable of degrading native type I collagen, heat-denatured type I collagen (gelatin), and native type IV collagen. Proteolytic activities against laminin and fibronectin are also present. These enzymes may play important roles in the degradation of extracellular matrix and the subsequent tissue invasion by *T. cruzi*. It has been proposed that the degradation of the collagen matrix, evident in acute murine Chagas disease, may result in chronic pathology such as apical thinning of the left ventricle. *T. cruzi* uses adipose and intestinal tissues as reservoirs and results in persistent infection of heart or other organs.

Diagnosis

The diagnosis of acute *T. cruzi* infection is generally made by the detection of parasites. Active trypomastigotes frequently can be seen by microscopic examination of fresh anticoagulated blood or buffy coat, and organisms can often be seen in Giemsa stained thin and thick blood smears as well. If the organisms cannot be detected by these approaches, one may inoculate blood specimens or buffy coat into specialized liquid medium or intraperitoneally into mice. The disadvantages of these approaches are their lack of sensitivity and the fact that the parasites are usually not seen in positive cultures or infected mice for several weeks. Assays based on the polymerase chain reaction (PCR) have been described, and the results obtained suggest that this approach may be the most sensitive method for detecting acute *T. cruzi* infections. When acute Chagas disease is suspected in an immunocompromised patient and these methods fail to demonstrate the presence of parasites, additional tissue specimens should be examined. These patients can pose a difficult diagnostic problem because they may present with fulminant clinical disease and low parasitemias that cannot be readily detected. Surprisingly, parasites can sometimes be seen in atypical sites, such as pericardial fluid, bone marrow, brain, skin, and lymph nodes, and thus, these tissues also should be investigated when indicated.

The diagnosis of chronic Chagas disease is generally based on detecting specific antibodies that bind to *T. cruzi* antigens. Several dozen serological assays based on a variety of technologies are used in Latin America for detecting antibodies, such as the indirect immunofluorescence test and the enzyme-linked immunosorbent assay (ELISA). These and other conventional serologic assays are used widely for clinical diagnosis and screening donated blood, as well as in epidemiological studies. False negative and false positive reactions, however, have been a persistent problem with many of these assays. At the present time only two tests available in the United States are approved by the FDA for clinical testing. The first is a lysate-based ELISA (Hemagen Chagas Kit; Hemagen Diagnostics, Inc., Columbia, MD), and the other is an ELISA based on recombinant antigens (Chagatest Elisa Recombinante; Laboratorios Wiener, Rosario, Argentina). Screening of the US blood supply currently is being done with a lysate-based ELISA

(Ortho *T. cruzi* ELISA Test System; Ortho-Clinical Diagnostics, Raritan, NJ), but this test has not been cleared for clinical use. An automated blood screening assay based on four chimeric recombinant antigens is being developed (PRISM Chagas Assay; Abbott Laboratories, Abbott Park, IL) and a test based on a blot format is being developed with the same antigens for confirmatory testing (Abbott Chagas Immunoblot Assay). An immunoprecipitation assay based on iodinated *T. cruzi* proteins (RIPA) has been shown to be highly specific as well as sensitive when used in clinically and geographically diverse groups of infected people. The RIPA currently is being used as the confirmatory assay to test all donor samples that are positive in the Ortho screening assay, and it is also available for clinical testing.

The detection of chronic infection by testing for parasite antigens in blood and urine has been studied, but this approach has not achieved results comparable to those obtained by serologic methods. PCR-based assays for detecting chronic *T. cruzi* infections have been studied extensively, but their usefulness has not been established definitively. It would appear that this approach would be particularly suited for the task of detecting the low number of parasites circulating in the blood of chronically infected patients, but sampling issues and the possibility that parasitemias may in fact be intermittent and thus limit the sensitivity of the assays. Moreover, false positive results have been an issue in some laboratories. The most likely niche for PCR-based assays is in diagnosing congenital *T. cruzi* infections in the days and weeks after birth when serological tests are made useless by the presence of maternal anti-*T. cruzi* antibodies.

An analytical study, using the case-control design, which included 205 people, was performed in Colombia. The comparative analysis among the different methods suggests that the diagnostic strategy of *T. cruzi* infection in patients with chronic Chagas disease can be performed using ELISA assays based on recombinant proteins and/or synthetic peptides, which show higher diagnosis performance and can confirm and exclude the diagnosis of *T. cruzi* infection. The molecular methods show poor performance when used in the diagnosis of patients with chronic Chagas disease.

Treatment

The treatment of *T. cruzi* is unsatisfactory. Nifurtimox (Lampit, Bayer 2502) and benznidazole (Rochagan, Roche 7-1051), the two drugs available for treating this infection, lack efficacy, must be taken for extended periods, and may cause severe side effects. Both drugs reduce the severity of acute Chagas disease, and it is thought that about 70% of acute patients treated with a full course of either nifurtimox or benznidazole are cured parasitologically. This cure rate decreases as a function of the time patients have been infected and may be $< 10\%$ in persons who have harbored the parasite for many years. There are no convincing data from properly controlled trials that treatment with either nifurtimox or benznidazole is beneficial in persons with long-standing infections. Chagas experts in both Brazil and Argentina currently recommend specific treatment only for patients with acute and congenital *T. cruzi* infections, and for chronically infected children. Therapy for adults assumed to have long-standing infections is not recommended, regardless of clinical status. In 2015, a prospective, multicenter, randomized study was completed. Trypanocidal therapy with benznidazole in patients with established Chagasic cardiomyopathy significantly reduced serum parasite detection but did not significantly reduce cardiac clinical deterioration through 5 years of follow-up (the BENEFIT Multicentric Trial).

Allopurinol and several antifungal azoles have been shown to have some anti-*T. cruzi* activity in in vitro experiments and in animal studies, but none has a level of activity that would warrant its use in place of nifurtimox or benznidazole.

Surgery is often necessary for gastrointestinal megasyndromes. As noted, persons with severe Chagas heart disease may benefit from heart transplantation. Stem cell transplantation currently is being evaluated in patients with severe heart failure due to *T. cruzi* infection.

Prevention

No vaccine or chemoprophylactic drug is available for preventing the transmission of *T. cruzi* in any context. The elimination of domiciliary vectors by spraying residual insecticides, improving housing conditions, and educating populations at risk are the linchpins of the widely successful programs for elimination of *T. cruzi* transmission to humans in the endemic countries. Serologic screening of blood donors is practiced throughout most of the endemic range and as a consequence transmission of *T. cruzi* by transfusion has largely been eliminated. The risk faced by short-term travelers to endemic regions of acquiring *T. cruzi* infection is extremely low, as only three such instances have been reported. Nonetheless, persons traveling to rural areas in endemic countries should avoid sleeping in primitive dwellings and should use insect repellent. Laboratorians who work with parasites or infected vectors should use appropriate protective equipment.

Trypanosoma brucei

Human African trypanosomiasis, or "sleeping sickness," is caused by two subspecies of hemoflagellate protozoa; *Trypanosoma brucei gambiense,* and *T. brucei rhodesiense.* The former causes the West African or gambiense form and the latter, the East African or rhodesiense form. Although these subspecies cause similar diseases, the West African form usually evolves over months to years and ends fatally if it is not treated. The East African form usually kills its host in weeks to months. These diseases exist in Africa wherever the various species of tsetse flies belonging to the genus *Glossina* are found.

Organism and Life Cycle

Trypanosoma brucei gambiense and *T. brucei rhodesiense* are pleomorphic flagellates 15–30 μ in length by 1.5–3.5 μ in breadth. The two subspecies are morphologically indistinguishable. There are two forms of trypomastigotes that circulate in the bloodstream, long

slender organisms that are capable of dividing, and short stumpy forms that are thought to be nondividing parasites that are infective for tsetse files. There are no intracellular forms. At various stages of the disease, trypomastigotes may be found in peripheral blood, lymphatics, lymph nodes, cerebrospinal fluid, and neural tissue (Fig. 7). Other than humans, there is no important reservoir host for *T. brucei gambiense,* whereas *T. b. rhodesiense* is primarily a parasite of wild game animals. In the tsetse fly, trypomastigote forms ingested with a blood meal settle in the posterior midgut, where they multiply by binary fission for approximately 7–10 days and then migrate anteriorly to the foregut, where they remain for 2–3 weeks. Finally, they enter the salivary glands, continue to replicate, and after several cycles of division, transform into infective metacyclic trypomastigote forms. These organisms are inoculated the next time a mammalian host is bitten, and once in a human host, trypomastigotes multiply by binary fission in the blood, lymph, and other extracellular spaces. The central nervous system (CNS) eventually is invaded and multiplication continues there as well.

The haploid genome size of *T. brucei* spp. is approximately 35 Mb, although there is up to 14% variation in isolates of the same subspecies and up to 29% between the two subspecies. There is a minimum of seven resolvable chromosome pairs on pulse-field gel electrophoresis in the size range of 1.1–6 Mb. Homologous chromosomes, when probed in Southern blots, can differ in size by up to 20%. In addition to the large chromosome pairs, *T. brucei* contains approximately 100 linear minichromosomes ranging in size from 50 to 150 kb. Minichromosomes contain tandem arrays of 177-bp repeats as well as transcriptionally silent copies of variant surface glycoprotein (*VSG*) genes proximal to their telomeres. *T. brucei* contains approximately 1000 genes capable of coding for *VSG* genes, which are switched at a rate of 102–106 switches per generation. This process serves as the main mechanism of immune evasion for *T. brucei.* Only one *VSG* expression site (ES) is active at any given time. There are 15–20 ESs per genome, all at subtelomeric locations. In addition to the *VSG* genes, several upstream genes, called expression site-associated genes (ESAGs), are also transcribed. Three types of DNA rearrangements are associated with ES switching—duplicative transposition, telomere exchange, and telomere conversion. Transposition involves the 1.6-kb *VSG*, in addition to a 1.5-kb proximal sequence. Thus, a minimum of 8% of the genome is devoted to *VSG* coding and flanking sequences. As mentioned previously, a large fraction of the 25% of the genome found in the minichromosomes also contributes to *VSG* diversity. The modified DNA base "J" (β-D glucosyl-hydroxymethyluracil), unique to organisms in the order Trypanosomatidae, replaces up to 1% of thymidines, and its frequency is higher in repetitive DNA adjacent to transcriptionally silent telomeres, including the $(GGGTTA)_n$ telomeric hexamer.

T. brucei genes are transcribed as large polycistronic units that then undergo *trans*-splicing so that all mature mRNAs contain the same 39-nt sliced leader at their 5′ ends. In contrast, with the exception of an 11-nt intron in a tRNAtyr, *T. brucei* genes contain no introns and, hence, do not undergo *cis* splicing. The spliced leader is also notable for the presence of a 7-methyl-guanosine 5′ cap, a promoter-like region consisting of four methylated nucleotides at the 5′ end, similar to that found upstream from ES regions and from genes that encode procyclic stage-specific coat protein (procyclic acid-rich protein (PARP), also called procyclin). Transcription from the PARP and VSG promoters, similar to that from the trypanosome rRNA promoter, is α-amanitin-resistant, suggesting transcription by RNA Polymerase I. Stage-specific gene expression is also influenced by 3′ untranslated portions of mRNAs, mediated through changes in mRNA stability and in efficiency of mRNA maturation. The sequence influencing stage specificity of *VSG* mRNA abundance is localized to a region 97 nt upstream from the polyadenylation site. Retroposon-like elements are also scattered throughout the genomes of these parasites. The best studied is a 5-kb sequence, designated *ingi* (Swahili for many), that is similar to reverse transcriptase genes in other organisms. There are approximately 400 copies of *ingi*, making up to 5% of the *T. brucei* genome.

Another interesting feature of trypanosome genetics, which is shared with other members of the order Kinetoplastida, is the phenomenon known as RNA editing. The kinetoplast DNA is organized as an interlocking and supercoiled network of approximately 50 maxicircle DNA molecules (20- to 30-kb) and many thousands of minicircle DNA molecules (1.0 kb in *T. brucei* and 1.6 kb in *T. cruzi*). The maxicircle DNAs encode about a dozen mitochondrial proteins. The maxicircles and minicircles both encode small (50–100 bp) guide RNAs that serve as templates for the insertion, and less frequently deletion, of uridines in the primary RNA transcripts of the maxicircle mitochondrial genes. In some cases, nearly 50% of the mature mRNAs consist of uracils inserted

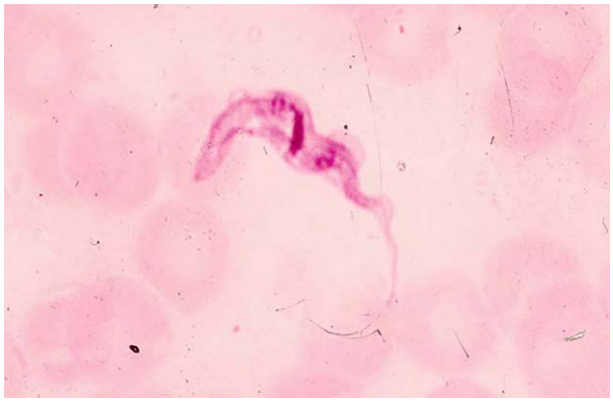

Fig. 7 Trypomastigote of Trypanosoma brucei gambiense in the bloodstream. Courtesy of the American Society of Tropical Medicine and Hygiene/Zaiman "A Presentation of Pictorial Parasites."

posttranscriptionally by the editing process. Studies comparing homologous nuclear genes between *T. brucei* and *T. cruzi* have demonstrated a large evolutionary divergence in codon use. A comparison of the nuclear small and large subunit rRNA gene sequences yields genetic distances comparable to those between plants and animals.

Epidemiology

Sleeping sickness (*Trypanosoma brucei gambiense* and *T. b. rhodesiense*) and veterinary trypanosomiasis caused by *Trypanosoma brucei* subgroup parasites continue to be responsible for much human suffering and economic loss (see Table 1). These agents are endemic and enzootic in an area of sub-Saharan African covering 10 million square kilometers. Approximately 50 million people are at risk for becoming infected with these parasites and tens of thousands of new cases of human African trypanosomiasis occur each year. Exact numbers are not available because the acquisition of reliable health statistics is difficult in the developing countries where sleeping sickness is endemic. Human African trypanosomiasis has undergone resurgence, and in recent years major epidemics have occurred in the Central African Republic, Ivory Coast, Chad, Sudan, and several other endemic countries. Losses of cattle due to trypanosomiasis have had an enormous economic impact in many regions. Human African trypanosomiasis is restricted to those areas south of the Sahara in which the annual rainfall exceeds 500 mm (i.e., 20 in.) because the larval stages of the tsetse fly are vulnerable to desiccation. Thus, the gambiense form occurs in the western portion of tropical Africa and focal incursions eastward all the way over to Lake Victoria and into southern Sudan. The rhodesiense form is found in the southeastern portion of Africa, north of South Africa. There is some overlap in the endemic ranges of these two forms of the disease. Humans are the only important reservoir of *T. b. gambiense*. The cycle of gambiense disease is maintained only where there is a close relationship between humans and tsetse flies that belong to the *G. palpalis* group and preferentially feed on human blood. In contrast to gambiense disease, the cycle of *T. brucei rhodesiense* is maintained in wild mammals, and humans are only incidental hosts. The vectors of *T. b. rhodesiense*, which belong to the *G. morsitans* group, inhabit the relatively dry eastern African savannas and preferentially feed on wild ungulates that are relatively trypanotolerant and maintain infective parasitemias for long periods. Nonetheless, there have been instances in which East African Trypanosomiasis has reached epidemic proportions. Human cases of rhodesiense trypanosomiasis typically occur in young adult men who have occupations in which they are exposed to tsetse flies. In epidemics, however, all age groups are infected, and mechanical transmission is thought to occur in this context. Tourists from nonendemic areas may acquire this infection. This occurs via the blood-filled proboscis of a fly, which may be interrupted while taking a blood meal from an infected individual. When the fly bites an uninfected host within 2–3 h, the blood from the infected host becomes a parasite-bearing inoculum. In contrast to *T. cruzi*, congenital transmission of African trypanosomes is extremely rare. Laboratory-acquired transmission has been reported.

Pathology and Pathogenesis

Metacyclic infective trypomastigote forms are inoculated into the skin by the tsetse fly and multiply there. A characteristic hard and sometimes painful chancre is formed. By about day 10, long slender forms are found in the bloodstream and lymphatics, and for the

Table 1 African animal trypanosomiasis

Parasite	*Host*	*Occurrence*
Trypanosoma vivax	Cattle	Common, mild
	Equines	Rare, mild
	Sheep and goats	Rare, severe
	Camels	Rare mild
	Dogs	Not reported
	Pigs	Not reported
Trypanosoma brucei brucei	Cattle	Common, but most cattle tolerate well
	Equines	Common, severe
	Sheep and goats	Rare
	Camels common	Severe
	Dogs common	Severe
	Pigs	Not reported
Trypanosoma congolense	Cattle	Common, severe
	Equines	Rare, mild disease
	Sheep and goats	Rare
	Camels	Not reported
	Dogs	Not reported
Trypanosoma evansi	Cattle	Mild disease
	Equines	Common, severe
	Sheep and goats	Not reported
	Camels	Common, severe

Animal trypanosomiasis is of great economic importance in Africa and is associated with fever, anemia, thrombocytopenia, wasting, and death. These manifestations are mediated, in part, by cytokines.

next several days their numbers increase exponentially. Soon thereafter, the organisms nearly disappear from the bloodstream, only to reappear later. The interval between waves of parasitemia may vary from 1 to 2 weeks, with clinical symptoms accompanying each bout of parasitemia. Each successive wave of organisms represents a new crop of parasites expressing a *VSG* not previously expressed in that host, and it is through this process of sequential antigenic variation that the parasites stay one step ahead of the host's specific antibody responses. In response to infection, a marked early humoral antibody response, consisting predominantly of IgM, is seen regularly. These macroglobulins consist not only of antitrypanosomal antibodies directed against parasite surface antigens, but also include a variety of other antibodies such as heterophile and rheumatoid factor. As a result of polyclonal B cell activation, there also are many antibodies produced to a wide variety of antigens, including brain-specific auto-antibodies. In addition, antibodies directed against myelin basic protein, gangliosides, and cerebrosides have been found in experimental models. Circulating immune complexes have been reported regularly and these may be responsible for the glomerulonephritis often accompanying acute and chronic disease. Cell mediated immunity also is important in this disease and nitric oxide may be important in the depression of T cell responsiveness and generalized immunosuppression.

Lymphadenopathy usually involves the posterior cervical, submaxillary, supraclavicular, and mesenteric lymph nodes. The advanced disease, often called stage II disease, involves the CNS. The microscopic examination of lymphatic tissues usually reveals generalized hyperplasia with diffuse proliferation of lymphocytes. Initially, affected lymph nodes are markedly hemorrhagic and contain a large number of trypomastigotes; later, the nodes may become small and fibrotic. A progressive, chronic leptomeningitis develops in stage II disease. The brain becomes edematous and there is prominent perivascular cuffing by glial cells, lymphocytes, and plasma cells. Morula cells, reactive astrocytes, and hyperplasia of microglial cells all have been described in brain specimens from patients with CNS disease, and demyelination occurs in chronic cases. Organisms can be found in the brain tissue near vessels and also may be present in the cerebrospinal fluid. There is a striking lymphocytosis in the cerebrospinal fluid and most of these lymphocytes are B cells. Glomerulonephritis, myocarditis, pericardial effusion, pulmonary edema, and hypoplastic bone marrow with an associated anemia may develop in some patients. The pathogenesis of the neuropsychiatric manifestations is poorly understood. Experimental models suggest that a variety of factors may be responsible (e.g., deposition of immune complexes and aberrant levels of brain neurotransmitters, prostaglandins, and cytokines).

Clinical Manifestations

Clinical manifestations of infection with *T. brucei gambiense* and *T. b. rhodesiense* are similar, except that the rhodesiense disease usually runs a much more fulminant course. Untreated patients with the latter typically die in weeks to months, whereas persons infected with *T. b. gambiense* may live for years and have long periods without symptoms. In both forms, the trypanosomal chancre may be evident several days after the bite of an infected tsetse fly. Within a week or two, the lesion becomes a large, red, painful nodule that may reach 5–10 cm in diameter. This nodule, which is reported to be more common in people of European descent, subsides spontaneously in a few weeks. In rhodesiense trypanosomiasis, the incubation period of the systemic disease typically is 2–3 weeks, whereas with the gambiense infection, the first symptoms may be noted many weeks or months after the acquisition of the infection. The early systemic disease is characterized by intermittent fevers, chills, headache, and generalized lymphadenopathy. In gambiense disease, the nodes in the posterior cervical triangle may become enlarged (i.e., Winterbottom sign) and, when present, strongly suggest the diagnosis in a patient with exposure to tsetse flies. Delayed deep hyperesthesia may occur over the tibia and moderate hepatosplenomegaly may be noted. Anemia and thrombocytopenia are frequent as well. Intermittent fevers may last for months to years with gambiense infection. In Europeans, a circinate erythematous rash or erythema multiforme is sometimes present. Untreated patients eventually develop signs of CNS invasion. Severe headaches, loss of nocturnal sleep, and a feeling of impending doom are typical. Following this, there may be progressive mental deterioration, with patients becoming incapable of caring for themselves. Tremors, especially of the tongue, hands, or feet, as well as generalized or focal convulsions may occur. Almost any neurologic and psychiatric manifestation can be seen with progressive mental deterioration until patients finally lapse into coma and die of intercurrent infections.

Diagnosis

A definitive diagnosis of African trypanosomiasis is made by detecting trypanosomes, and this can be done by looking for parasites in fresh and stained specimens of blood, bone marrow, lymph node aspirates, or, in late disease, the cerebrospinal fluid. If parasites are not seen in these specimens obtained from a patient whose history and clinical findings suggest African trypanosomiasis, efforts should be made to concentrate the organisms in blood. This can be done most simply by using commercially available quantitative buffy-coat analysis tubes. The parasites are separated from the blood components by centrifugation in these acridine orange-coated tubes, and are easily seen under light microscopy because of the stain. Alternatively, buffy coat obtained by centrifugation of 10–15 mL of anticoagulated blood can be examined microscopically as a wet preparation and after Giemsa-staining. All of these specimens also can be inoculated into specialized liquid-culture medium. Finally, a highly sensitive method for detecting *T. b. rhodesiense* is the inoculation of small volumes of specimens obtained from the patient into rodents. Patent parasitemias usually develop within a week or two in animals injected with specimens from people infected with *T. b. rhodesiense*. Unfortunately, host specificity precludes the use of this approach for diagnosing *T. b. gambiense*.

Several serologic assays are available to aid in the diagnosis of sleeping sickness, but the variable accuracy of these tests mandate that treatment decisions still be based on detection of the parasite. These assays are useful, none the less, in epidemiologic surveys.

Simple direct agglutination tests for trypanosomes performed on cards (CATT and TrypTect CIATT) are available commercially. A role for PCR-based assays for detecting African trypanosomes has not been defined. In advanced untreated sleeping sickness, the IgM level in the cerebrospinal fluid often is elevated, but it has no relationship to the presence of trypanosomes in the cerebrospinal fluid. After successful treatment, the IgM level declines gradually, disappearing after approximately 1 year and, thus, a persistently elevated level or an abrupt rise in the IgM months after treatment may indicate a relapse. IgM levels, however, should not be used as the sole method of diagnosis or prognosis.

Treatment

The chemotherapy of these diseases, both human and veterinary, has lagged remarkably behind that of other tropical diseases. The main chemotherapeutic agents for human trypanosomiasis remain pentamidine and suramin for the early-stage disease, and melarsoprol (Mel B, Arsobal) for the late-stage (CNS) disease. Difluoromethylornithine (DFMO, Ornidyl) is the only new useful addition to this list since the early 1950s. With the exception of suramin, resistance to the established agents is growing, and the toxicity of these drugs continues to be a problem.

Pentamidine is a water-soluble aromatic diamidine that has been in use since the 1930s. It is effective against early-stage *T. b. gambiense* infection, but is less effective against *T. b. rhodesiense* infection, and is ineffective against late-stage disease. African trypanosomes have a nucleoside transporter (adenine/adenosine: P2) that takes up pentamidine, resulting in the concentration of the agent at levels many times that in plasma. Many studies have focused on the mechanism of pentamidine action; however, none appears to have conclusively defined the target. It is known to bind to the minor groove of kinetoplast (i.e., mitochondrial) DNA, and to promote the cleavage of kinetoplast minicircle DNA, eventually leading to the development of dyskinetoplastic cells. Despite its effects on kinetoplast DNA, pentamidine has no effect on nuclear DNA, and dyskinetoplastic forms can persist in the bloodstream of mammals. Pentamidine also has been found to be a reversible inhibitor of *S*-adenosylmethionine (AdoMet) decarboxylase, an enzyme in the polyamine biosynthetic pathway. Although ki values were in the 200 mM range, we now know that this internal concentration is achievable via uptake through the P2 nucleoside–pentamidine transporter. Other targets studied previously in trypanosomes include the inhibition of glycolysis and lipid metabolism, as well as the effects on amino acid transport and ion exchange. The facts that pentamidine does not kill trypanosomes outright and bloodstream forms persist after treatment argue for a sustained effect more consistent with interference with the nucleic acid metabolism.

Diminazene aceturate (Berenil) is an aromatic diamidine developed as treatment for bovine trypanosomiasis; however, its apparent low incidence of adverse reactions and significant therapeutic activity has led some physicians in endemic countries to use it extensively. It is effective against early-stage *T. b. gambiense* and *T. b. rhodesiense*. Diminazene has also been used in combination with melarsoprol for late-stage disease. Mechanistically, like pentamidine, diminazene has also been linked to kinetoplast DNA binding at the minor groove and cleavage of minicircle DNA. Similar to pentamidine, diminazene may also interfere with RNA editing and *trans*-splicing. Diminazene is also a more effective and noncompetitive inhibitor of AdoMet decarboxylase in trypanosomes, resulting in the reduction of spermidine content and elevating putrescine in the parasite. As with pentamidine, diminazene uptake occurs via the P2 nucleoside transporter, which allows significant accumulation from the external environment. Although diminazene has been used for many years on thousands of sleeping sickness patients, there is little published on its toxicity. This may in part be due to physicians who are unwilling to document human studies with an agent licensed for veterinary use. However, personal accounts of those using diminazene in humans indicate it is well tolerated.

Suramin is a sulfonated naphthylamine, which has been used successfully against early-stage sleeping sickness caused chiefly by *T. b. rhodesiense.* It was first used in 1922, developed from the closely related azo dyes, trypan red and trypan blue. Suramin has an extremely long half-life in humans, 44–54 days, as the result of avid binding to serum proteins. Suramin binds to many plasma proteins including LDL, which trypanosomes avidly bind and endocytose as a result of specific membrane receptors. LDL is a prime source of sterols for bloodstream trypanosomes. The uptake of suramin as a protein complex results in an internal concentration of 100 mM. Suramin has been shown to inhibit all of the glycolytic enzymes in *T. b. brucei* in the range of 10–100 mM, which in most cases is several-fold lower than for the corresponding mammalian enzyme. This specificity for trypanosomal enzymes was attributed to higher (i.e., more basic) isoelectric points for the parasite enzyme than the mammalian enzymes, allowing the negatively charged suramin to bind preferentially to the parasite enzymes. In practice, because most trypanosome glycolytic enzymes are contained in a membrane-bound cytosolic organelle, the glycosome, it is not likely that rapid massive binding occurs. This would rapidly induce lysis in bloodstream forms that depend on glycolysis as the sole energy-generating source. Rather, animals that are heavily infected with trypanosomes and given suramin show a slow decrease in parasite numbers, indicating that enzyme inhibition occurs slowly. Suramin may be affecting newly synthesized enzyme molecules in the cytosol before they are imported into the glycosome. Beyond the inhibition of glycolytic enzymes, suramin has also been found to affect thymidine kinase and dihydrofolate reductase. It is likely that suramin's action may be attributable to the inhibition of several of these enzymes.

Melarsoprol (Mel B, Arsobal) is an arsenical resulting from the efforts of Ernst Freidheim in the late 1940s. His initial compound, melarsen oxide, *p*-(4,6-diamino-*s*-triazinyl-2-*yl*) aminophenylarsenoxide was complexed with dimercaptopropanol (British Anti-Lewisite) to form a less-toxic complex, melarsoprol. Until 1990, this was the only agent available for curing the late-stage (CNS) disease in both of East African and West African infections. It is usually given as two to four series of three daily injections. It is insoluble in water and must be dissolved in propylene glycol, and it must be given intravenously. Toxicity is an important concern with melarsoprol. This takes the form of reactive arsenical-induced encephalopathy, which is often followed by pulmonary edema and death in more than half the cases within 48 h. Although the mechanism of melarsoprol action has been extensively studied, it still

remains unclear. Parasites exposed to low levels (1–10 mM) rapidly lyse. Because the bloodstream forms are intensely glycolytic, any interruption of glycolysis should produce this effect. Thus a series of reports has detailed melarsoprol inhibition of trypanosome pyruvate kinase (ki, 100 mM), phosphofructokinase (ki, <1 mM), and fructose-2,6-bisphosphate (ki, 2 mM). It is likely that the rapid inhibition of fructose 2,6,bis-phosphate production is a key factor in halting glycolysis through down-regulation of pyruvate kinase. Other studies indicated that melarsoprol and melarsen oxide formed adducts with trypanothione (*N*1,*N*8-bisglutathionyl spermidine), a metabolite unique to trypanosomes and believed to be responsible for the redox balance of the cell and detoxification of peroxides. The melarsen–trypanothionine adduct (Mel T) inhibits trypanothione reductase, which has been attributed to the mode of action. However, melarsoprol and related arsenicals may also bind to other sulfhydryl-containing agents in the cell, including dihydrolipoate and the closely adjacent cysteine residues of many proteins. Similar to pentamidine and diminazene, melarsoprol uptake into African trypanosomes has been attributed to entry through the P2 purine nucleoside transporter; thus, significant levels can be concentrated in the cell from a low external (plasma) concentration. Although most laboratory-generated melarsoprol-resistant strains have lost or modified the P2 transporter, clinical isolates appear to have retained uptake capacity.

Eflornithine DFMO (DL-*a*-difluoromethylornithine, Ornidyl) is the most recently developed agent for late-stage *T. b. gambiense* sleeping sickness. After initial testing in model infections, DFMO was studied extensively in human trials. The standard treatment regimen resulting from the trials indicate DFMO is >95% active when given intravenously. DFMO cured children, adults, and patients with melarsoprol-refractory strains, and patients with late-stage disease. The short plasma half-life of DFMO necessitates constant dosing when given as an i.v. drip. The most frequent toxic reaction was reversible bone marrow suppression, which was alleviated upon reduction of the doses. The major drawbacks with respect to DFMO are its cost, the duration of treatment, and its availability. DFMO rapidly and irreversibly binds to the catalytic site [cysteine 360 in mouse ornithine decarboxylase (ODC)], inactivating it. In culture, it blocks division of bloodstream trypanosomes, but it is not trypanocidal. In laboratory infections, DFMO cures when administered continuously in the drinking water as a 2% solution. Within 48 h of administration, DFMO reduces putrescine levels to zero, and reduces spermidine levels by ~75%. Trypanothione levels are also significantly reduced. As noted, DFMO is not trypanocidal and depends on a functional immune system to rid the host of nondividing forms. Morphologically, trypanosomes with multiple kinteoplasts and nuclei are common, as are forms resembling "stumpy" blood forms. DFMO is curative for laboratory infections of *T. b. brucei* and *T. b. gambiense*, but not to all strains of *T. b. rhodesiense*. The reason for this selectivity is not known, although it is not due to uptake of DFMO, because it enters by passive diffusion, not transport. Levels of AdoMet are highly elevated in susceptible strains, but less so in refractory isolates. The elevated levels of AdoMet are due to an AdoMet synthase insensitive to its product. DFMO treatment leads to intracellular concentrations of ~5 mM, an increase of ~50-fold over untreated parasites. Trypanosome ODC is missing the PEST sequence in both procyclic and bloodstream trypanosomes, and this appears to be the major reason for the stability of the trypanosome enzyme. The remainder of the ODC molecule has >60% sequence identity with the mammalian enzyme, including a cysteine 360 residue at the demonstrated DFMO-binding site for the mammalian enzyme. Beyond this, trypanosomes lack a polyamine oxidase, which in mammalian cells converts spermine to the biologically active spermidine. Trypanosomes are also limited in their ability to transport putrescine and spermidine. This treatment has forced retirement of melarsoprol-based therapy for T. gambiense infections, and points to the need for development of an all oral therapy with a minimal dose regimen. New therapy must avoid the toxicity of melarsoprol, penetrate the blood brain barrier and be orally administered.

The usefulness of nifurtimox in combination with DFMO or melarsoprol as treatment for human African trypanosomiasis has been assessed in several recent trials especially with the emergence of resistance to DFMO. Outcomes obtained to date suggest that the addition of nifurtimox to adjusted doses of either of these two drugs results in increased efficacy, while reducing overall toxicity. Clinical studies with DFMO and Nifurtimox led to the development of combination therapy (Nifurtimox-Eflornithine Combination Therapy, NECT) which has become the new standard clinical regimen: Eflornithine 400 mg/kg, i.v. every 12 h per day for 1 week + nifurtimox 15 mg/kg every 8 h for 10 days. This regimen is 96% curative, has very little adverse reaction and reduces the eflornithine dosage from 4/day for 2 weeks to 2/day for 1 week (Priotto et al., 2009). Although this regimen is effective it still has drawbacks because it requires I.V. dosing and a total of 1 week of drug administration.

Recent studies have identified a series of boron-containing benzoxaboroles as having significant antitrypanosomal activity in in vivo animal studies (Fig. 1; Fig. 27.5 in Bacchi et al., 2013). These compounds are orally available and are curative vs. early stage and CNS laboratory model infections (Nare et al., 2010; Jacobs et al., 2011). A series of these benzoxaboroles were initially screened in a high throughput in vitro assay at Scynexis, Inc. Initially, two classes of compounds, AN2920 (6-sulfides) and AN3520 (6-carbamides), were deemed interesting and proceeded to further optimization by Anacor Pharmaceuticals, Scynexis Inc. and Haskins Laboratory of Pace University through a grant from Drugs for Neglected Diseases initiative (DNDi) (Bacchi et al., 2013). These compounds and close analogs were then tested in a range of in vitro ADME (absorption, distribution metabolism and elimination) before undergoing in vivo testing in mice. The animal testing revealed that of approximately 146 compounds tested in an early stage HAT model, 25 were curative. Of these, 12 were also curative in a CNS model (C.J. Bacchi, unpublished) when administered orally, b.i.d. or QD × 4 days at 50 mg/kg. Further study of these analogs included pharmacokinetic testing showing rate of removal from the bloodstream, metabolic stability in the blood, and ability to cure at low dosing. Two analogs, SCYX6759 and SCYX7158, were superior to the other analogs. The differences in structure being a fluorine atom added to the 4 position of the benzamide of AN3520 to yield 6759. The structure was curative for a stage 2 HAT model at 50 mg/kg b.i.d. x 14 days. The 3,3-dimethyl analog added on the borol ring of 6-carboxamide (AN3520) yielded SCYX7158. This compound had impressive efficacy with the CNS model TREU667 curing 100% of mice at 25 mg/kg q.d. × 7 days and an 80% cure at 12.5 mg/kg q.d. (Nare et al., 2010; Jacobs et al., 2011).

Studies on the mechanism of action of SCYX7158 (Wall et al., 2018) using an over expression screening approach has identified cleavage and polyadenylation specificity factor 3 [CPSF3 nuclease] as a potential target of SCYZ7158 and a mechanism by which treatment inhibits mRNA maturation. Molecular docking and CRISPR-Cas9-based editing have also demonstrated how SCYZ7158 can block the active site and mRNA processing by the parasite, but not the host. Another oxaborole (AN3661) was also found to be active against *Plasmodium falciparum* and the target was CPSF3 (Sonoiki et al., 2017, in Wall et al., 2018). Other trypanosomatids besides *T. b. brucei* ssp., such as *T. cruzi* and *Leishmania* spp. have CPSF3 catalytic site residues identical to the *T. b. brucei* sequence (Wall et al., 2018). Thus CPSF3 can now be considered as valid antitrypanosomal drug target and the oxaboroles and similarities in protozoan CPSF3 suggest both clinical and veterinary applications may be valid.

Currently SCYX7158 (acoziborole) is undergoing phase II and III clinical trials after successful phase I testing. In the phase II, III trials, 7158 is proving curative in late stage infections after a single 960 mg oral dose. The half-life is approximately 18 days in humans, which explains the single dose efficacy. There is no reported toxicity. Also interesting is the fact that SCYX7158 is curative in cattle with *T. b. brucei* and *T. congolense* disease (C. Mobray, personal communication).

Prophylaxis and Prevention

Trypanosomes cause complex public health and epizootic problems in many developing countries in Africa. Control programs concentrating on the eradication of vectors and drug treatment of infected people and animals have been in operation in some areas for decades. Considerable progress has been made in a number of regions, but the lack of agreement on the best approach to solving the problem of African trypanosomiasis, combined with a paucity of resources, stands in the way of effective control. Individuals can reduce their risk of becoming infected with trypanosomes by avoiding tsetse fly-infested areas, by wearing clothing that reduces the biting of the flies, and by using insect repellants. Chemoprophylaxis with suramin or pentamidine can be effective, but it is not clear which populations should use this as a preventive measure. No vaccine is available to prevent the transmission of the parasites.

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Type Culture Collections their Databases☆

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Glossary

BLAST search An electronic comparison of a gene sequence with a database of sequences to determine the extent of similarity.
Cryoprotectant A material, such as bovine serum or skim milk, that protects cells from damage during freezing.
Culture In microbiology, the growth of an organism on agar or other nutrient medium.
GenBank A searchable database of gene sequences maintained by the National Center for Biotechnology Information (NCBI). A BLAST search of gene sequences can be conducted at this website.
L-drying A method to preserve microbial cultures that relies on drying unfrozen cells.
Lyophilization A method to preserve microbial cultures that relies on drying frozen cells.
Materials transfer agreement A legal documentbetween parties that governs the transfer of research materials.
Patent culture A culture that is maintained in conjunction with a patent application.
Strain An isolate of a microbial species.

Abbreviations

AHMII	Agent to Help Microbial Information Integration
APHIS	Animal and Plant Health Inspection Service
ARS	Agricultural Research Service
ATCC	American Type Culture Collection
BRC	Biological Resource Centers
CBS	Centraalbureau voor Schimmelcultures
EPC	European Patent Convention
JCM	Japan Collection of Microorganisms
MTA	Materials Transfer Agreement
NCBI	National Center for Biotechnology Information
NRRL	Agricultural Research Service Culture Collections
PCT	Patent Cooperation Treaty
USDA	United States Department of Agriculture
WFCC	World Federation for Culture Collections

Defining Statement

Microbial culture collections, also known as Biological Resource Centers (BRC), are primary suppliers of microbial cultures (germplasm) for medical, agricultural, and biotechnological research and development. A major goal of culture collections is long-term preservation of strains without genetic change. Culture collections are also centers of taxonomic and microbiological expertise.

Microbial Culture Collections – Applications and History

Microbial culture collections, increasingly known as BRC, are primary suppliers of microbial cultures (germplasm) for medical, agricultural, and biotechnological research and development. Culture collections throughout the world have as their primary mission the preservation and distribution of germplasm that has been demonstrated to have significance to the microbiology community. The importance of a particular strain may be as a reference for medical, agricultural, biotechnological, or taxonomic research, use as an assay organism for testing or screening, or in relation to a parent application for a product or process in which it is

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involved. Alternatively, the strain may be placed in a collection in regard to a publication in which it was cited as part of the investigation. The latter deposit is essential so that later investigators may repeat or advance published research, which would be impossible in the absence of the strains involved.

The major culture collections of the world also serve as centers of excellence for research on systematic and taxonomy, partly because the identification and characterization of strains is an integral function of collections and because the availability of a large collection of strains lends itself to this type of research. A major change in methodology for strain identification has been the development of molecular methods that are based on gene sequence comparisons. Much of the molecular work is done in culture collections or in collaboration with collection scientists. The product, species-specific gene sequences, or entire genome sequences, deposited in internationally accessible databases such as GenBank, has allowed even the nontaxonomist to sequence a specific microbial gene, conduct a BLAST search of deposited DNA sequences, and receive an accurate identification. Sequence analysis has also resulted in the recognition that microbial diversity is much greater than initially thought, and thus an enormous number of new microbial species may be available for exploitation in biotechnological and other applications. In this context, culture collection scientists often provide advice on strain and species selection for various aspects of research and development.

Culture collections trace their history to the early beginnings of microbiology, and the first culture collection established specifically to preserve and distribute strains to other researchers was that of Professor Franriek Krái in Prague during the 1880s. Upon the death of Professor Krái in 1911, this collection was acquired by Professor Ernst Pribham who transferred it to the University of Vienna and issued several catalogs listing the collection's holdings. Part of this collection was brought to Loyola University in Chicago by Pribham in the 1930s. Many of these strains were subsequently transferred to the American Type Culture Collection (ATCC) upon Pribham's death, but others remained in the collection at Loyola University. The Vienna portion of the Pribham Collection was apparently lost during World War II. The next oldest culture collection, the Centraalbureau voor Schimmelculrures (CBS), was founded in 1906 and is located in Utrecht, the Netherlands. At about the same time, a large number of filamentous fungi were isolated from spoiled agricultural products by Charles Thom at the US Department of Agriculture (USDA) in Washington, DC, and these well-authenticated cultures formed the nucleus for the fungal collections at both the ATCC and the Agricultural Research Service Culture Collection (NRRL). Both of these collections have served as repositories for main orphaned cultures collections that were previously established in individuals or institutions, such as the N. R. Smith Bacillus Collection or the US Army Quartermaster Collection. Consequently, culture collections receive strains from individual scientists as well as by accessioning other collections that may be abandoned for various reasons. Publicly funded collections have predominated in recent years because many of the large private pharmaceutical collections, with worldwide strain coverage, have either been abandoned or become inactive as emphasis in the pharmaceutical industry has shifted from antibiotic discovery to other types of products.

Types of Culture Collections

General Collections

Large culture collections were often initially established to maintain and provide type cultures and other reference strains, and hence use of the name Type Culture Collection. The type culture is the strain chosen to represent a new species when it is described. Even if other strains are known, it is the type culture that is the primary taxonomic representative of a species. Before strains were characterized from gene sequences, it was not unusual to find misidentified strains attributed to a particular species, and for this reason, gene sequences from type strains form the generic basis for species identification.

Most culture collections, even those that are large, are specialized in some manner. Certain collections might have a preponderance of fungi or bacteria while maintaining fewer species of other microorganisms (Table 1). Consequently, it may be necessary to request strains from several collections if a study requires a diversity of microorganisms. Protocols for obtaining cultures are discussed in a later section.

Patent Collections

With the discovery of microbiologically synthesized antibiotics in the 1940s, a large number of parents were tiled to protect these processes. Since then, many other microbiological processes have also been patented. To accommodate this new technology, the US Patent and Trademark Office implemented in 1949 a new requirement that cultures be deposited in conjunction with parent applications concerning microbiological inventions. The reasoning was that for chemical, electrical, or mechanical patents, a diagram or formula can sufficiently describe the invention, whereas in a microbiological patent, illustrations and narrative descriptions are generally inadequate to define sufficiently the microorganism used and therefore comply with the requirement for a full and complete disclosure of the invention. The Agricultural Research Service (ARS) Culture (Collection was asked by the US Patent Office to assume the responsibility of accepting patent cultures, thereby becoming the first US culture collection, and apparently the first in the world, to accept patent cultures. The first culture was *Streptomyees aureofacients* NRRL 2209, the strain deposited by the American Cyanamid Company of the United States for aureomycin production. Shortly thereafter, the ATCC, as well as several foreign collections, began to accept patent cultures as well.

Cultures may be deposited for US or foreign patent applications, but multicountry filings complicated the issue of patent culture deposits. The Patent Cooperation Treaty (PCT), which first became effective in 1970, and the European Patent Convention (EPC), which was implemented in 1977, allowed for filing a single parent application that included all member countries of the above

Table 1 Some large international culture collections that maintain a diversity of microbial groups

Agricultural Research Service Culture Collection (NRRL) Peoria, Illinois, USA http://nrrl.ncaur.usda.gov
All-Russian Culture Collection (VKM) Moscow Region, Pushchino, Russia http://www.vkm.ru
American Type Culture Collection (ATCC) Manassas, Virginia, USA http://www.atcc.org
Belgian Coordinated Collections of Microorganisms (BCCM) Various cities, Belgium http://bccm.belspo.be/index/php
Centraalbureau voor Schimmelcultures (CBS) Utrecht, The Netherlands http://www.cbs.knaw.nl
German Collection of Microorganisms and Cell Cultures (DSMZ) Braunschweig, Germany http://www.dsmz.de
Japan Collection of Microorganisms (JCM) Saitama, Japan http://www.jcm.riken.jp
National Institute of Technology Evaluation – Biological Resource Center (NBRC) Chiba, Japan http://www.nbrc.nite.go.jp

Many other culture collections can be found at the website of the World Federation for Culture Collections (WFCC) (http://www.wfcc.info).

agreements. However, the issue of patent culture deposits in each country named in a multicountry patent application was not resolved until 1977 with the implementation of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, known simply as the Budapest Treaty. The importance of this treaty is that a single culture deposit satisfies the requirement for a deposit in all countries that are signatories of the Treaty, which includes most industrialized countries. Culture collections in most of these countries have now been certified as International Depositary Authorities under the Budapest Treaty. Cultures deposited under the Budapest Treaty must be maintained for 30 years and for 5 additional years after the last request. The special requirements for distributing patent cultures are discussed below.

Safe Deposit of Cultures

Oftentimes, a laboratory will have an important research culture but does not want to place it in the accessible holdings of a culture collection. In view of this, some culture collections offer a safe deposit option. The culture is not accessioned into the general part of the collection, but is maintained as a private deposit, for which the collection charges a fee.

Other Services

In addition to providing cultures, many culture collections offer related services, some of which require a fee. Among these services are advice on strain selection, advice and training on culture maintenance, microbial strain identification, taxonomy workshops, sale of biological reagents, custom preparation of inoculum for fermentation processes, various types of biological testing, and research and development work.

Databases and Other Informational Services

An essential requirement for all culture collections is the maintenance of records that provide an accession number for strains acquired along with the name of the strain, information regarding the source of the strain, such as where and from what substrate it was isolated, and if the strain has accession numbers in other collections (e.g., cross-referencing). In the early days of culture collections, this catalog might have been a handwritten notebook or card file full of note cards. The World Federation for Culture Collections (WFCC) recognized the importance of documenting strain information and made suggestions regarding the types of data that should be collected and cataloged in its Guidelines for the Operations of Collections of Microorganisms.

With the advent of computers that provided ready access, larger collections were quick to adopt them for management of data relevant to their holdings and as the size, cost, and complexity of computers diminished, virtually every collection of any size currently has catalogs of their holdings maintained electronically in some format. The increasingly stringent regulatory environment of the modern world has made it essential to maintain a growing amount of data on strains in collections. For instance, the Convention on Biodiversity requires collections to track the provenance (or history) of strains hack to the original country of origin, even if the strain has come through many other laboratories during its path to the current collection. Concerns about biosecurity make it crucial that inventory and distribution information on microbes potentially dangerous to animals, humans, or crops be made readily available.

Publicly available catalogs are a requirement in the WFCC Guidelines of Culture Collections, and the advent of the Internet has made it possible for collections to provide online, searchable computer databases as their public access catalogs. These online collection databases can be typically searched for the name of a microorganism, an accession number in the catalog being searched, or for a number in other collections, and in some cases for properties of the strain, such as the ability to make a particular product, such as an enzyme or antibiotic, or for some specific activity, such as the production of yogurt or industrially important organic acids.

The central registry for collections throughout the world can be found at the WFCC website (http://www.wfcc.info/) and there are currently 525 culture collections in 67 different countries. At least 182 of the collections have active websites and many of these have online catalogs of their holdings. Recently, there have been several attempts to integrate the information regarding microbial strains held in many different collections through a Web-based portal at a single site. Examples include the Agent to Help Microbial Information Integration (AHMII) on the aforementioned website and the Strainlnfo.net portal (http://www.straininfo.ugent.be) at the University of Ghent in Belgium.

Preservation of Cultures

A major role of culture collections is to maintain microbial strains for long periods in a genetically unaltered state. Serial transfer on agar or other media is time-consuming and often leads to genetic changes as the organism adapts to its new environment, the culture medium. For this reason, a number of long-term storage procedures have been developed that maintain strains in metabolically and genetically inactive states. Three of these procedures are described here.

Lyophilization

The procedure of lyophilization freeze-dries cultures to an inactive, low-moisture state. The process starts with suspending actively growing cultures in a cryoprotectant such as sterile bovine serum or skim milk. The volume of cryoprotectant needed depends on the size of the ampoule used. Ampoules used in the NRRL are made from 6 mm outside diameter Pyrex glass tubing, which is cut in lengths of 100 mm. One end is fire polished in a gas oxygen flame to remove sharp edges and the other end is sealed by flame. In the ARS process, ~0.1 ml of suspension containing cells and bovine serum is added aseptically to the sterile, labeled lyophil tubes, which have a small cotton plug. After filling, the cotton plug is pushed a short distance into the tube and the excess cotton is burned off in a gas flame. The tube is then inserted into a rubber connector on the manifold of the lyophil machine. After all the tubes are inserted, the manifold is lowered so that the ends of the tubes are immersed in a 50% ethylene glycol bath cooled to approximately −30 °C with dry ice. Once the contents of the tubes are frozen, the bath temperature is raised to approximately −20 to −25 °C with fresh ethylene glycol and the vacuum pump is started. Drying takes about 2 h and is finalized by raising the manifold and allowing further drying at room temperature. Each ampoule is sealed with a gas-oxygen torch and measures about 50 mm in length after sealing (Figures 1 and 2).

Some lyophil machines use larger ampoules that may contain 1 ml of cell suspension. These larger tubes may be initially frozen, but do not need immersion in a freezing bath because evaporation of this larger volume is adequate to keep the preparations frozen. Depending on the design, the larger ampoules may be sealed with a gas-oxygen torch or by inserting a rubber stopper into the ampoule while it is still under vacuum.

An ampoule of each strain lyophilized should be tested for viability within a few days of processing. This is done by suspending the lyophilized pellet in liquid growth medium and by determining it growth occurs. Rough quantitation of viability can be made by sneaking a loopful of the suspension onto an agar plate. Many microorganisms lyophilize well but only a few percent of the original cell population survives the process. Nonetheless, there is little evidence that certain nuclear genotypes preferentially survive, although plasmids may sometimes be lost. Lyophilized strains generally survive for many decades, though there are exceptions and the cultures should be checked periodically for viability. Lyophilized cultures are usually stored in a refrigerator at 4–5 °C, as frozen storage seems unnecessary (Figure 3).

L-drying

A process termed L-drying has been successfully used by several large culture collections in Japan. The process is similar to lyophilization with the exception that cells and cryoprotectant are dried more slowly under vacuum and do not freeze. L-dried ampoules are sealed in the same manner as lyophilized preparations.

Figure 1 Lyophilization of cultures. As described in the text, ampoules containing microbial cells and a cryoprotectant are placed on the manifold of the lyophil apparatus and are freeze-dried. The ampoules are being sealed with a gas-oxygen torch. A dry ice–ethylene glycol bath (covered) for freezing the ampoules is at the base of the manifold. A vacuum pump is on the left. The vertical metal tube behind the manifold on the left is a dry ice moisture trap to prevent contamination of the vacuum pump with water from drying cultures. A vacuum gage is immediately behind the manifold base. Photograph by Don Fraser, National Center for Agricultural Utilization Research.

Figure 2 Lyophilized culture (top). Note the pellet of dried cells at the bottom (left) of the tube. Cryovial (bottom) for storing cultures in liquid nitrogen. Photograph by Don Fraser, National Center for Agricultural Utilization Research.

Figure 3 Refrigerated storage of lyophilized cultures. Ampoules of each strain are placed in plastic boxes arranged in numerical order. Photograph by Don Fraser, National Center for Agricultural Utilization Research.

Liquid Nitrogen Preservation

Some microorganisms do not survive lyophilization or L-drying. The most common alternative, which is generally the most effective, is frozen storage at very low temperatures. While storage in −80 and −125 °C mechanical freezers is often satisfactory, storage at still lower temperatures is preferable. The choice is the vapor phase of liquid nitrogen, which is about −180 °C. Liquid

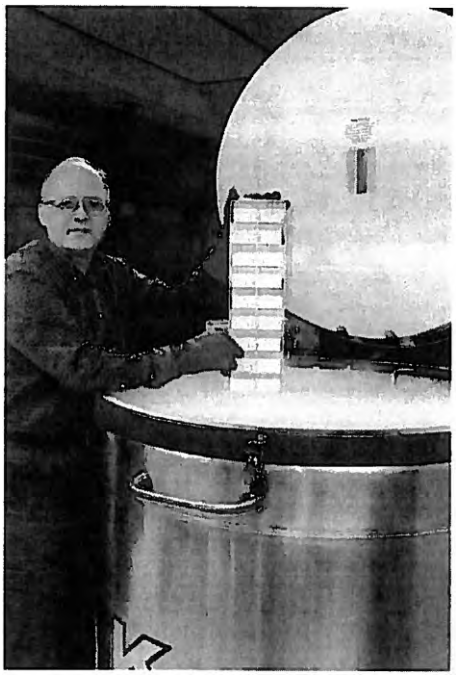

Figure 4 Liquid nitrogen storage of cultures. Ampoules of cultures are arranged in numbered boxes and stored in the vapor phase of liquid nitrogen, which is pooled under a platform in the bottom of the freezer. Photograph by Don Fraser, National Center for Agricultural Utilization Research.

nitrogen at atmospheric pressure has a slightly lower temperature of −196 °C (−320 °F), but a concern with immersion storage is that ampoules might leak and when thawed, the trapped liquid nitrogen will expand to gas so rapidly that the ampoule explodes, potentially causing serious injury to the person thawing the sample.

The general procedure for preparing microorganisms for liquid nitrogen storage is to suspend cells from an actively growing culture in a cryoprotectant such as 10% glycerol. Usually 1 ml of suspension is placed in a 2 ml cryoampoule (Figure 2). Ordinarily, the ampoules are transferred to a storage box in the liquid nitrogen freezer and allowed to undergo uncontrolled freezing (Figure 4). Occasionally, the freeze rate needs to be controlled to ensure high cell viability, which is often greater than 90%. Controlled freezing can be accomplished very simply by putting the ampoule in a plastic foam box, which is then placed in the liquid nitrogen freezer. It this process is not adequate, freezing may be controlled by using commercially available devices that have a programmable freezing rate. To save space, some laboratories use short sections of plastic soda straws as cryoampoules. Straws are cut into sections about 2 cm long, the bottom is heat-sealed with flamed forceps, and the straws are then sterilized by autoclaving. After filling with a cell suspension, the open end of the straw is then heat-sealed with flamed forceps. Microorganisms stored in liquid nitrogen vapor usually remain viable for many decades, and perhaps longer.

Distribution of Cultures

Culture collections almost never charge for depositing strains in their general collections and, in fact, collections often contact persons with new and interesting cultures in order to remain current. Patent culture deposits, however, are another matter, and culture collections charge a fee for patent culture deposits because of the need for more frequent viability determinations and more stringent international administrative requirements.

Many collections charge a fee for distribution of cultures, but some collections, such as the ARS Culture Collection, do not charge for distribution of most of the cultures maintained. Nonetheless, most collections must charge a fee for culture distribution because of the expense of maintaining the strains, replenishment, and shipping. Fees vary among collections, but the fees often do not completely cover operating costs because of the expense of maintaining cultures that are seldom distributed, but which are important for various scientific reasons.

Distribution of cultures from patent collections is governed by a variety of national and international laws and regulations. In the United States, patent cultures cannot be distributed until the subject patents issues or the depositor agrees to distribution, but in Europe, publication of a national patent application can trigger strain release. Requirements of the Budapest Treaty must be taken into consideration, and many collections require that requests for cultures deposited under the Budapest Treaty be received as signed hard copy letters. The collection must then verify that the culture can be distributed, and the depositor must be notified that the culture was requested and by whom.

In recent years, many collections have required that a Materials Transfer Agreement (MTA) be implemented between the collection and the requestor, although some collections do not require an MTA because it is felt that this might in some way limit usage of the strain for scientific and industrial development. Collections that impose an MTA argue that this prevents redistribution of the strain and also ensures that the collection has no liability if the strain is misused. MTAs should not be used to extract revenue from an invention involving the requested strains unless culture collection scientists were active participants in the discovery.

Shipment of cultures is governed by a variety of national and international laws and regulations, and some of these regulations have developed in recent years because of the threat of global bioterrorism. Within the United States, shipment of plant and animal pathogens requires a permit from the USDA Animal and Plant Health Inspection Service (APHIS). Permits for shipment of human pathogens may also be required from the US Public Health Service. Other countries often have their own specific regulations, and shipment of plant and animal pathogens between countries often requires special permits. These requirements should be taken seriously because failure to comply can result in closure of laboratories, large fines, or even incarceration. Shipment of cultures can also be governed by the Convention on Biodiversity, also known as the Rio Treaty. Here the concern is that part of a country's national heritage, that is, unique microbial germplasm, will be exploited by outside parties without due compensation. Consequently, before novel or other strains are shipped between countries, the requirements of the Rio Treaty must be known.

Conclusions

Culture collections play a major role in modern societies. Provision of cultures for medicine, agriculture, and other areas of science has proven essential to disease prevention and control as well as for developments in biotechnology. The taxonomic expertise that is common to culture collections has lead to identification of new pathogens and has promoted advancements in ecology, studies in biodiversity, and other areas of science needing accurate identification of microorganisms.

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Relevant Website

http://www.straininfo.ugent.be—StrainInfo.net.

Typhoid, Historical

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Glossary

carrier An individual who harbors and may excrete virulent bacteria while remaining free of symptoms of the disease.
cyclogeny A theory, widely held in the 1920s and 1930s, that asserted that bacteria in cultures go through stages of a life cycle in which the bacterial cells may undergo changes in shape, size, staining, and biochemical properties.
endotoxin Molecules contained within the bacterial cell, but which when released by lysis of the cell can exert toxic effects on the host cells by a variety of biological mechanisms.
flagellum A flexible fiber-like structure on the surface of some bacteria. Flagellar movement is responsible for bacterial motility. The flagellum is driven by an internal cellular structure, the 'motor', which is powered by a proton gradient across the cell membrane.
Peyer's patches Localized lymphoid tissue in the small intestine of mammals.

Defining Statement

This chapter focuses on the historical aspects of typhoid fever in its clinical manifestations, epidemiology and etiology.

Background and History

Typhoid is the classic 'enteric fever' which begins with malaise, anorexia, and headache, followed by high fever and abdominal tenderness and distention. Rose spots appear on the skin. Patients may become delirious, the 'typhoid-state' for with the disease is named (typhus/ τυ′φ□ς: stupor), have shock from intestinal hemorrhage, and die. It often occurs in epidemics, especially under conditions of poor sanitation such as in military camps and after widespread natural disasters. Its symptoms and epidemiology overlap with that of typhus (camp fever), so typhoid had been confused with this rickettsial disease for much of its history. The name, typhoid, implies a 'typhuslike' condition, and these two diseases have been clearly delineated only since the mid-nineteenth century.

The typhoid organism infects only humans and chimpanzees, entering the body through the alimentary tract. In the sick individual the organisms are usually found in the spleen, bone marrow, lymphoid tissue associated with the gut (Peyer's patches), and almost always in the gall bladder. Much of the pathology and the symptoms of typhoid are the result of endotoxin which is released upon lysis of the bacteria. The typical incubation period is 7–14 days. Mortality in untreated cases is about 10%; 75% of these have intestinal hemorrhage or perforation. About 3% of clinically recovered patients still excrete the typhoid organism in the feces after 1 year and are designated as carriers.

In England, Huxham (1739) in his 'Essay on Fevers' described the Plymouth epidemic of 1737, and he distinguished between putrid typhus (*febris putrida*) and slow nervous fever (*febris nervosa lenta*) that is now recognized as typhoid. As disease nosology evolved, and the concept of specific disease entities developed, by the nineteenth century typhoid and typhus were being recognized as diseases with distinct clinical features. The American physician and medical educator, Nathan Smith, provided the classic description of typhoid in 1824 in his essay 'A Practical Essay on Typhous Fever', in which he recounted his observations of what is clearly typhoid in the Connecticut River Valley in New England. The great Parisian clinician Pierre-Charles-Alexandré Louis named the condition *fièvre typhoïde* in his major work on the disease in 1829. Schönlein (1839) in Germany distinguished *Typhus exanthematicus* (typhus) and *Typhus abdominalis* (typhoid), terms which became established in the German literature.

The English physician William Budd (1811–73), who studied under Louis at the La Pitie´ hospital in the 1830s, saw outbreaks of typhoid fever in his rural general practice and by careful observations on the spread of the disease concluded that typhoid was spread from person to person. This put him in the camp of the 'contagionists' who were vigorously opposed by the 'antic-ontagionists', physicians who favored the miasma theory of disease for diseases such as typhoid. Budd published his first detailed 'natural history' of typhoid (what would now be called an epidemiological study) in 1856. He described similar outbreaks in later publications, and even before the rise of bacteriology, Budd concluded that typhoid was spread by fecal contamination of water and milk. He also proposed that a convalescent patient could still be a source of contagion. Budd was an enthusiastic advocate of

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disinfection, both of drains and of privies as well as water supplies. This work on typhoid and its waterborne dissemination was contemporaneous and apparently independent of parallel work by John Snow on cholera in London.

Carl Joseph Eberth described the typhoid organism in tissues of patients in 1880, and Georg Gaffky isolated and grew this organism in 1884. Since the typhoid bacterium is relatively easy to grow in culture, soon many different bacterial isolates were found in typhoid-like cases, both in humans and in other species. For example, in 1888 Gaerntner isolated what is now designated *Salmonella enteritidis* from a patient who had eaten contaminated meat, and shortly thereafter Durham and de Noeble isolated *Salmonella typhimurium* from gastroenteritis patients who also had eaten contaminated meat. The classification of these early isolates was based on the disease entity (*Salmonella cholerasuis*, i.e., hog cholera), the geographic place of isolation (e.g., *Salmonella montivideo, Salmonella newport*), or the name of the investigator (*Salmonella schottmülleri*). In 1896 Grünbaum and Widal independently observed that serum from infected or recovered patients could agglutinate dead typhoid bacteria, and this test (Widal reaction) became the basis for a serological diagnosis of infection as well as a tool to study *Salmonella* pathogenesis.

Taxonomy and Classification

The early nomenclature of the typhoid bacillus is even more confusing and inconsistent as that of most bacteria. The typhoid organism was variously called *Bacillus typhosa*, *Bacterium typhosum*, *Eberthella typhosa*, *Salmonella typhosa*, and most recently *Salmonella typhi*. The genus is named in honor of Daniel Elmer Salmon (1850–1914), the first director of the Bureau of Animal Industry of the US Department of Agriculture and one of the founders of bacteriology in America. Salmon investigated hog cholera and, along with his prote´ge´ Theobald Smith, identified the hog cholera bacillus (*S. cholerasuis*), long believed to be the cause of hog cholera (later found, however, to be caused by a filterable virus).

When it was found that individual isolates could be distinguished serologically from one another, classification of the typhoid bacillus and its relatives was revised and based on the antigenic reactivities of a particular isolate. Some strains grew in more diffuse colonial form on surface cultures, and this growth form had come to be described as similar to the appearance of 'breath' (perhaps the water condensate) on the agar surface. These strains shared antigenic properties termed H-antigens (*Hauch*: breath). Later it was recognized that this diffuse colony morphology results from the motility of the individual organisms and that the H-antigens are associated with the flagella of these strains. The antigens of the strains without H-antigens were designated as O-antigens (*Ohne*: without), and are now recognized as 'somatic' antigens, belonging to the 'body' of the bacterium. A third category of serological reactions was believed to be related to virulence of the organism in experimental infections, and these antigens were designated as the Vi or virulence antigens. The H-antigens were observed to vary with the culture conditions, and this two-state variation was interpreted in terms of the cyclogenic theories of the interwar period as characteristic of different growth phases of the culture, hence the concept of 'phase variation.' Once the H-antigens were understood in terms of the flagellar antigens and structure, phase variation was reinterpreted as a problem in flagellar biosynthesis and regulation.

Fritz Kauffmann (1937) and Philip B. White (1926) studied the antigenic structures of the typhoid and typhoid-like bacteria in detail, and the Kauffmann– White system of classification became standard. This system of classification of the genus *Salmonella* employs the H, O, and Vi antigenic specificities to identify a particular isolate. In 2005, the International Commission on the Nomenclature of Prokaryotes designated the 'type' culture for the genus *Salmonella* as *Salmonella enterica*. The species includes over 2300 serotypes, many of which were formerly designated as different species, for example, *S. cholerasuis*, *S. typhi*, *Salmonella paratyphi* A, and *S. typhimurium*. The variants are termed serovars, for example, *S. enterica* serovar Typhimurium.

Prevention and Treatment

Although vaccines for typhoid have been in use for a long time, it was only in the 1960s that definitive evidence of their effectiveness was obtained through studies on human volunteers. The best vaccine preparations give over 90% protection for at least 3 years against challenges with doses of typhoid similar to that expected in contaminated water supplies. Higher challenge doses, however, can overcome the vaccine-induced immunity and cause disease. Chloramphenicol (discovered in 1948) was the first effective drug to be used in typhoid, and it was followed by the newer penicillin derivatives as treatment for typhoid and other related *Salmonella* infections.

In addition to vaccines and antibiotics, however, the most effective means to prevent and control typhoid have been sanitary treatment of sewage and establishment of clean drinking water supplies.

Epidemiology

The epidemiology of typhoid, as exemplified by the work of William Budd, has been of great interest, both for medical and for sociological reasons. Based on epidemiological evidence, Robert Koch suggested that new cases of typhoid could come from convalescents long after the disease disappeared, and in support of this assertion, Drigalski (1904) isolated typhoid bacteria from individuals with no signs of illness. Observations such as these led to the concept of the healthy carrier of disease. Soon it was recognized that the gall bladder was a common site in which to find bacteria in these carriers, and ways were sought to eradicate the asymptomatic infections in order to eliminate the carrier state. Gall bladder removal (cholecystectomy) was a favored treatment.

Less drastic approaches such as chemotherapy and various disinfectants were tried without success. Food contamination, usually by flies in summer, but sometimes by food handlers, led to the notion of the carrier as a danger to society. Quarantine and sometimes long-term detention of suspected or proven carriers were justified in the cause of protecting the public from contamination. Probably the most notorious and egregious case was that of Mary Mallon, an Irish immigrant domestic worker in New York, who seemed resistant to treatment and who was deemed uncooperative by the New York City Public Health authorities. As 'Typhoid Mary' she was *de facto* incarcerated in Riverside Hospital on North Brother Island in the East River by Manhattan for many years. Indeed, the term 'Typhoid Mary' has become generic for a carrier of a dangerous disease who is uncooperative in preventing its spread.

Typhoid and its agent played another role in the American legal system when the city of Chicago constructed a drainage canal to help channel Chicago sewage from the Chicago river into the Mississippi River watershed. Downstream, the city of Saint Louis, Missouri was the potential recipient of the diverted sewage. Worried about typhoid outbreaks from Chicago, St. Louis went to court to block the Chicago plan. This case was the first in US history in which the new science of bacteriology was accepted as expert testimony in court.

In circumstances of social disruption, such as wars, civil unrest, famine, or natural disasters which affect water and sewage systems, typhoid is still a major health problem. However, immunization, antibiotics, and sanitary measures have reduced the US typhoid mortality from 26 per 100 000 in the period 1906–10, to about 500 in the entire US population in 1967, to a few sporadic cases at present.

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Typhus Fevers and Other Rickettsial Diseases, Historical

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Abbreviations

AFR	Astrachan spotted fever rickettsia
ATBF	African tick bite fever
DDT	Dichloro diphényl trichloroethane
DEBONEL	Dermacentor-borne necrosis–erythema–lymphadenopathy
ISFR	Israeli spotted fever rickettsia
ITTR	Indian tick typhus rickettsia
MLST	multilocus sequence typing
MSF	Mediterranean spotted fever
MST	multi-spacer typing
ORF	open reading frame
PABA	*p*-aminobenzoic acid
PCR	polymerase chain reaction
POB	*p*-hydroxybenzoic acid
RMSF	Rocky Mountain spotted fever
SFG	spotted fever group
TG	typhus group
TIBOLA	Tick-borne lymphadenopathy

Defining Statement

This article focuses on the historical aspects of rickettsial diseases, the causative agents and their arthropod vectors, and the diagnosis of epidemic and murine typhus, Rocky Mountain spotted fever (RMSF), Mediterranean spotted fever (MSF), African tick bite fever (ATBF), and other rickettsioses.

Introduction

Rickettsial diseases existed since antiquity and have had great effects on the history of human populations. Massive population movements, poor hygiene, and famine favored the development of epidemics, and many wars and invasions were associated with or followed by outbreaks that included typhus. Typhus may cause more deaths than weapons during wartime. The Napoleonic wars were examples of the terrible consequences of a combination of war and diseases, and thousands of soldiers succumbed to typhus, as proven recently by molecular biology. Rickettsioses occurred not only in the history of the military, it has been a continuing threat to public health too.

The serious danger of rickettsial diseases stimulated remarkable medical research and clinical observations, which clarified their specific etiology, mode of transmission, pathogenesis, therapy, and means of control. Because of their unique biological characteristics, such as small size, ranging to the limits of microscopic vision and beyond, ability to pass through a filter, inability to grow on bacteriologic media, and their obligate intracellular habitat in vertebrate and arthropod hosts, researchers had serious difficulties in identifying of rickettsial agents. Moreover, the rickettsial agents were treated as dangerous organisms due to their aerosol transmission, low infectious dose, and high risk-associated morbidity and mortality. Several microbiologists in the laboratory and physicians, particularly in the preantibiotic era, died of rickettsial infection like Howard Ricketts and Stanislav von Prowazek (**Table 1**).

A remarkable progress was made in 1904 when Wilson and Chownin proposed related hypothesis on the transmission of rickettsia by tick bite. In 1909, Nicolle showed the louse transmission of typhus. In 1938, Cox demonstrated that rickettsiae could be cultivated in the yolk sacs of chick embryos. In 1948, it was demonstrated that broad-spectrum antibiotics were shown to be specifically and therapeutically effective. Tetracycline and chloramphenicol are the currently used antibiotics of choice in treating rickettsial diseases and have not been challenged since their discovery. The rickettsial scientific field has undergone significant evolution at the epidemiological, microbiological, and molecular levels in the past 20 years. Currently, the *Rickettsia* genus contains 24 recognized species, in contrast to three human pathogens and five endosymbionts as mentioned in the 1922 Brumpt list.

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Table 1 The great scientists who died of typhus fever

1744–1814	Johann Bartholomä us (Barthel) von Siebold, the ancestor of the "Wurzburg family" of physicians and scientists
1784–1850	Zachary Taylor, president of the United States
1871–1910	Howard Taylor Ricketts, researcher
1875–1915	Stanislaus von Prowazek, zoologist and parasitologist
1870–1915	Hugo Lüthje, scientist
1858–1915	Georg Cornet, researcher, scholars of Robert Koch
1874–1915	Georg Jochmann, physician of the Department of Infectious Diseases, Berlin
1870–1916	Artur Pappenheim, Doctor Habilitatus, professor haematologist
1876–1916	Paul Römer, the professor of Hygiene at the University of Halle
1890–1921	Wolfgang Gärtner, lecturer at the University of Kiel
1866–1922	Arthur William Bacot, entomologist
1879–1922	Edmund Weil, researcher

In this article, we review the milestones of historical aspects of rickettsial diseases, discovery of the infectious agents, their vectors and mechanism of transmission and reservoir, and the major technological developments that have facilitated the study, control, and treatment of rickettsiae and rickettsial diseases.

Rickettsia and Human Rickettsioses

The term 'rickettsia' applies only to arthropod-borne bacteria, belonging to the genus *Rickettsia* within the family Rickettsiaceae of the order Rickettsiales. *Rickettsiae* are small Gram-negative microorganisms that grow in association with eukaryotic cells. These bacteria are described as obligate intracellular organisms that retain basic fuschin when stained by the method of Gimenez. Rickettsial cells divide by binary fission for multiplication and can be cultivated in cell cultures, embryonnated eggs, or susceptible animals. Members of the *Rickettsia* genus are closely related phylogenetically and have a high degree of 16S rDNA sequence homology to each other.

Historically, cross reactions of patient's sera with somatic antigens of strains of *Proteus* OX19, OX2, and OXK were used to classify rickettsial isolates into three groups, namely, the spotted fever group (SFG), the typhus group (TG), and the scrub TG. Subsequently, the scrub typhus species *Rickettsia tsutsugamushi* was reclassified within the genus *Orientia*. Other phenotypic criteria that have been used to describe *Rickettsia* species include the geographical distribution of the strain, the arthropod vector, pathogenicity in humans, mice, and guinea pigs, optimal culture temperature, time for plaque formation, size of plaques, growth in embryonated chicken eggs, and hemolytic activity. Recently, guidelines were proposed to classify rickettsial species and the diseases they cause.

Presently, the genus *Rickettsia*, with 24 currently validated species, is divided into two main groups: the TG with *Rickettsia prowazekii* and *Rickettsia typhi* and the SFG that accounts for most tick-borne rickettsiosis and also *Rickettsia felis*, which is the agent of flea-borne spotted fever and *Rickettsia akari* is the agent of Rickettsialpox transmitted by mite (**Table 2**). Rickettsial organisms are mainly transmitted to humans by bites from infected arthropods. However, infections from aerosols of infected insects, feces, and blood transfusions have also been described. Besides the known pathogens, many other rickettsial strains have been found in arthropods, in particular ticks, but their roles as human pathogens have yet to be determined. New developments in the epidemiologic profile of rickettsioses include the emergence of spotted fever rickettsioses due to *Rickettsia rickettsii* in Argentina, Brazil, Columbia, and the United States; identification of other *Rickettsia* species in ticks in South America and Mexico; and outbreaks of louse-borne typhus in Burundi, Russia, Peru, and Algeria.

To date, 16 known rickettsioses are recognized and represent a global problem. Rickettsioses are seasonal outbreaks and the arthropod host determines their epidemiology and geographic distribution. The clinical presentation of rickettsial diseases can vary from mild to very severe, with the case fatality for highly virulent rickettsiae ranging from 2 to >30% (**Table 3**). The main clinical signs include fever, headache, rashes that are maculopapular or sometimes vesicular, inoculation eschars at the site of the arthropod bite, and localized lymphadenopathy. The severity of the diseases varies with the pathogen virulence and the host factors. The main pathologic mechanism in rickettsioses is vasculitis following infection of the endothelial cells.

Rickettsiae represented a paradigm of reductive evolution. The reconstruction of ancestral genomes, using seven *Rickettsia* genomes that inferred the origin and fate of the genes found in today's species, indicates that their last common ancestor contained more genes, but already possessed most traits associated with cellular parasitism. The obligate endocellular microorganisms tend to evolve faster as a consequence of reduced effectiveness of selection, and suggest a major role of enhanced background mutation rates on the fast protein divergence in the obligate intracellular α-proteobacteria.

Epidemic Typhus

Epidemic or louse-borne typhus, caused by *R. prowazekii*, transmit by the human body louse, which lives in clothes and multiplies rapidly during cold weather and in unhygienic places. Its prevalence reflects the low socioeconomic status of certain members of a

Table 2 Historical data on diseases caused by Rickettsial species

Disease and etiological agent	*Event*	*Researcher*
Siberian tick typhus *R. sibirica*	The first account of disease (1930)	Mil
	Description of disease, '*Primary tick borne disease'* or *'far Eastern tick-borne typhus'* (1936–37)	Mil, Antonov, and Naishat
	Discovery of tick vector of the disease and the mammalian reservoir (1938)	Unknown
	Isolation of *R. sibirica* in guinea pig (1938)	Korshunova et al.
	Naming of the disease: Siberian tick typhus (1944)	Pavlovsky
Rickettsial pox *R. acari*	Description of Rickettsial pox and isolation of *R. acari* (1946) Discovery of the role of rodents and mites (1947)	Huebner Pomerantz
Queensland tick typhus *R. australis*	Isolation of *R. australis* from human (1946)	Andrew *et al.*
	Description of diseases (1946)	Plotz and Smadel
	Naming of the rickettsia (1950)	Philip CB
Tick-borne lymphadenopathy (TIBOLA) *R. slovaca*	Isolation of *R. slovaca* from *Dermacentor marginatus* ticks (1968) First documentation of a human case of infection (1997)	Brezina *et al.* Raoult *et al.*
	Naming the clinical syndrome: TIBOLA (1999)	Lakos A
	DEBONEL: Dermacentor-borne necrosis–erythema–lymphadenopathy (2004)	Oteo *et al.*
	Determining the genome sequence (2007)	Raoult D
Japanese spotted fever *R. japonica*	Description of diseases (1984)	Mahara
	First documentation of human cases (1985)	Mahara
	Isolation of *R. japonica* (1985)	Uchida *et al.*
	Naming the rickettsia (1992)	Uchida *et al.*
	First identification in ticks (1996)	Mahara
Far east spotted fever *R. heilongjiangensis*	Isolation of rickettsia and first identification in ticks (1982)	Lov
	Description of rickettsia (2003)	Fournier *et al.*
	Description of diseases (2004)	Mediannikov et al.
Flinders island spotted fever *R. honei*	Description of diseases (1991)	Stewart
	Isolation of *R. honei* from human samples (1992)	Baird
	First identification in the ticks (1993)	Graves *et al.*
	Naming the rickettsia: *R. honei* ep.nov (1998)	Stenos *et al.*
Lymphangitis-associated rickettsioses *R. sibirica mongolitimonae*	First identification in *Hyalomma asiaticum* ticks (1991)	Yu et al.
	Culture and identification of rickettsiae (1993)	Yu and Raoult
	First clinical description (1996)	Raoult et al.
	Naming the rickettsia (2005)	Fournier et al.
Flea-borne spotted fever *R. felis*	First account, detection in *Ctenocephalides felis* (1918)	Sikora H
	Detection in cat fleas (1990)	Adams et al.
	First case of flea-borne spotted fever, the name 'Elb agent' (1994) The name of rickettsia (1996)	Schriefer and Azad
	Culture of *R. felis* (2000)	Higgins *et al.*
	Genome sequence (2005)	Raoult et al. Ogata *et al.*
R. aeschlimannii	Isolated from *H. marginatum marginatum* (1992)	Beati *et al.*
	The name: *R. aeschlimannii* (1997)	Beati and Raoult
	First case of infection (2002)	Raoult et al.
R. helvetica	Isolation of *R. helvetica* from *Ixodes ricinus* (1979)	Burgdorfer and Peter
	The name of rickettsia (1993)	Beati *et al.*
	First case of acute infection (1997)	Fournier et al.
R. parkeri	Isolation of rickettsia from *Amblyomma maculatum* ticks (1939)	Parker
	The name: *R. parkeri* (1965)	Lackman
	First case (2004)	*et al.* Paddock *et al.*
R. massiliae	Isolation of *R. massiliae* from *Rh. sanguineus* tick (1992)	Beati *et al.*
	The name: *R. massiliae* (1993)	Beati *et al.*
	First recognition of infection (2005)	Vitale et al.
	Genome sequence (2007)	Blanc *et al.*
R. raoultii	First detection in *Ixodid* ticks (1999)	Rydkina *et al.*
	Isolation of *Rickettsia* sp. 'DnS14' (2001)	Oteo *et al.*
	The first human case (2007)	Fournier et al.
	The name of rickettsia (2008)	

society and rises during war, in poor countries, and in homeless people in rich countries, including the United States and Europe. The recent outbreak in a refugee camp in Burundi, which involved thousands of cases (mortality rate >10%), and other cases reported in Rwanda, Russia, Peru, the United States, and Algeria are reminder that epidemic typhus can reemerge as a result of a catastrophic breakdown of social conditions.

Table 3 Effect of specific antibiotics in the course of major rickettsiosses

Disease	*Untreated Average duration of the fever (days)*	*Mortality (%)*	*Treated Average duration of the fever after treatment (days)*	*Mortality (%)*
Rocky Mountain spotted fever	16	21	3	0
Epidemic typhus	14	30–40	2	0
Murine typhus	12	2–5	2	0
Scrub typhus	14	15	1	0

The clinical onset of epidemic typhus is usually abrupt after an incubation period of about 14 days. Classical epidemic typhus is the most severe rickettsial disease, with symptoms including a high fever, headache, chills, myalgias, conjunctival injection, and rales. Erythematous macules (2–6 mm) appear most often on the trunk on day 5 and later on the extremities; the face, palms, and soles are usually spared. The characteristic rash of typhus is also the origin of more descriptive names, such as spotted fever in English, Fleckfieber in German, and thyphus exanthématique in French. Myocarditis, pulmonary involvement, severe neurologic complication, and gangrene of the distal extremities may occur in severe cases. Without the availability of antibiotics, the disease is fatal in 13–60% cases (**Table 3**).

Flying squirrel-associated typhus (sylvatic form) has been less severe (fever, headache, maculopapular rash, confusion, and myalgia), with no fatalities records. Once infected with *R. prowazekii*, convalescent patients maintain the infection for all their life, and with weakening immunity recrudescent typhus, Brill–Zinsser disease may occur. The symptoms of recrudescent typhus are less pronounced, and the associated mortality rate is $<1\%$. Patients with this disease could serve as a longterm source of *R. prowazekii*, permitting transmission of rickettsiae via lice to occur months to years after the primary infection, and may play the mechanism for the dissemination in many parts of the world.

Historical Aspects of Epidemic Typhus and Brill–Zinsser Disease

Typhus, the 'spotted fever' of the sixteenth century in England, the 'gaol fever' of the eighteenth, and the 'Irish fever' of the mid-nineteenth centuries, has a long and distinguished history intimately associated with the social upheavals caused by war and famine. Greek, etymology of typhus, meaning smoky or hazy, was originally applied by Hippocrates to the confused states of mind frequently associated with high fevers. Some historians such as Haeser and Hecker contend that the disease was described by Thucydides during the Plague of Athens (430–425 BC), but recently typhoid fever was reported as the causing agent. The first account of typhus was recorded during the civil wars of Grenada 1489–90, when typhus was introduced in Spain by soldiers from the island of Cyprus (**Table 4**). The distribution of typhus from Spain to Italy, France, and then northward continued in an almost uninterrupted succession of small outbreaks. The disease was described by several physicians such as Girolamo Fracastorius in 1546 in *De Contagione et Contagiosis Morbis*, referring to the Italian epidemics of 1505 and 1528 and distinguished it from plague; Girolamo Cardano in his book *De Malo recentiorum medicorum Ursu Libellus* in 1536; and by Von Zavorziz in 1676 in *The Infection of Military Camps*.

Villalba suggests that typhus was transported from Spain to the New World during the first half of the sixteenth century, but historical evidence suggests that typhus fever existed among South American natives in pre-Columbian days and recognizable epidemics occurred in Mexico before the arrival of Cortez at Vera Cruz in 1519. Moreover, there is a legend, credited by Bernal Diaz and by Nicolas Léon, that typhus destroyed the Toltec city of Tollan in 1116 AD. The description of typhus fever in Mexico was done by missionaries in 1545. Toward the end of the sixteenth century, typhus killed more than 2 million native Indians in the Mexico highlands. It is tempting to speculate that typhus was born in the chance meeting of an American rickettsia and a Spanish louse.

A century after the Thirty Years' War (1618–48), when the epidemic typhus spread in Europe (Germany lost three-fourth to one half of her total population), Huxham, in 1739, made the first distinctions between typhus and typhoid (**Table 5**). Boissier de Sauvages confirmed this in the eighteenth-century in France and called it exantematic typhus. In 1836, William Gerhard of Philadelphia clearly distinguished between typhus and typhoid fevers based on postmortem findings of six patients revealing the remarkable absence of ulceration of Peyer's patches, contrary to observation of typhoid. By the 1860s, the physicians distinguished typhus from another louseborne disease, relapsing fever (*Borrelia recurentis*), while problems of diagnosis remained. Later, Bacot established that trench fever and typhus were different diseases; the former was caused by *Bartonella quintana* and the latter by *R. prowazekii*. This was tragically confirmed by a typhus infection acquired by him in 1922.

In 1909, Nicolle showed the transmission of the disease through the blood of a typhus patient to a chimpanzee and from the chimpanzee to a macacus monkey. The disease was then transmitted from macacus to macacus via the louse. In 1911, the typhus patient transmitted the infection to the guinea pig. Although fever was the only sign of the disease, the guinea pig remained for many decades the most useful experimental animal. Later, Wolbach detailed the clinical observations and the human histopathology, and the effect of *R. prowazekii* infection on guinea pigs.

During 1896–1910, Nathan Brill, in New York, studied 221 febrile patients whose illness reminded him of typhus rather than typhoid fever, in spite of protestations of his colleagues. Negative blood cultures and Widal reactions led him to call it an illness of

Table 4 Milestones of epidemic typhus

Discovery of the epidemic typhus Rickettsia prowazekii	*Author*
1489 The first account of typhus	
1739 First distinctions between typhus and typhoid	Huxham
1760 Description of exantematic typhus	Boissier de Sauvage
1810 Description of diseases	Johann V. von Hildebrand
1837 Histological distinction between typhus and typhoid	William Wood Gerhard
1909 Role of body lice in typhus and transmission to chimpanzee and monkeys	Charles Nicolle (Nobel prize)
1910 Description of recrudescent form	Nathan Brill
1910 Serology test based on Proteus for typhus fever	Wilson
1911 Isolation of *R. prowazekii* in guinea pig	Charles Nicolle
1916 Description of bacteria and named *R. prowazekii*, the genus name after H. Ricketts and the species after the Polish researcher Stanislas von Prowazek	Henrique da Rocha Lima
1916 Weil–Felix test for typhus fever	Weil and Felix
1922 Human histopatology	Wolbach
1930 Typhus vaccine	Rudolph Weigl
1934 Tissue cultures	Kligler and Aschner
1937 Cell culture (mouse and chick embryos in Koll flasks)	Zinsser and Schoenbach
1938 Role of feces of the louse	Starzyk
1938 Culture in yolk sacs of embryonated chicken eggs	Cox
1942 The antirickettsial action of *p*-aminobenzoic acid	Snyder
1948 Treatment by tetracycline and chloramphenicol	Woodward
1952 The base ratio of DNA	Wyatt and Cohen
1956 Vaccine Madrid E attenuated	Clavero and Gallardo Fox
1975 Reservoir: Flying squirrels (*Glaucomys volants*)	Bozeman et al.
1998 Genome sequence	Andersson *et al.*

Table 5 Milestones of suspected outbreaks of epidemic typhus

Years	*Country*	*Number of deaths*
1489–90	Civil wars of Grenada, Spain	17 000 Spanish soldiers
1528	Naples, Italy	21 000 French soldiers
1542	Hungary	30 000 German and Italian soldiers
1545	Mexico	2 million native Indians
1552	Metz, France	10 000 soldiers
1557–59		10% of the English population
1618–49	The Thirty Years War, France (1628)	One half of total population of Germany 60 000 deaths in Lyon and 25 000 Limoges
1756–63	The Seven Years War, Europe	Thousands????
1812	Napoleon's invasion of Russia (confirmed by PCR assay)	550 000 French soldiers 61 964 Russian soldiers
1813–14	Europe	2 million people throughout Europe 250 000 people in Germany
1816–19	Ireland	700 000 people
1847	Canada	20 000 Irish immigrants
1854–56	Crimean war	134 000 soldiers
1914	Australian invasion in Serbia	150 000 Serbs and 30 000–60 000 Austrian prisoners
1917–25	World War I and Russian Revolution	3 million Russian people (30 million cases) 150 000 Serbs and 60 000 Australian prisoners
1942	Egypt	23 000 cases
	French North Africa	77 000 cases
1945	World War II	17 000
1989	Russia	29 cases
1997	Burundi	43 345 cases

unknown cause; it was a mild form of typhus fever, later named as Brill's disease. In 1933, Zinsser and Castenada isolated *R. prowazekii* from these cases. Through careful analysis of epidemiological data, Zinsser concluded that it is a recrudescent typhus case and it served to maintain endemic prevalence of *R. prowazekii* by bridging breaks in the chain of man– louse–man propagation. Zinsser's hypothesis was confirmed by Murray in 1950.

Infectious Agent: Rickettsia prowazekii

In 1910, Ricketts, while working on the etiology of typhus fever in Mexico, speculated that the agent of typhus had the same morphology of bubonic plague and Rocky Mountain spotted fever (RMSF) organism. He described bacillary or pleomorphic bodies, with bipolar dark staining and intervening pale areas, in the blood of patients suffering from typhus and in the dejecta of lice that had fed on these patients. However, these descriptions do not fit with our current knowledge and represent something else. In 1911, Charles Nicolle isolated these bacteria from guinea pig. In 1913, Hegler and von Prowazek described appearances of neutrophilic leucocytes in 51 typhus cases and in a smear from louse, which they believed to be microorganism.

In 1914, in Istanbul, and in 1915, in eastern Germany, Rocha Lima and Stanislaus von Prowazek confirmed the observations of previous workers, and together they described *R. prowazekii* (**Figure 1**). In 1916, Rocha Lima grouped the microorganisms in the order Rickettsiales and named the agent of typhus *R. prowazekii* after Ricketts and his deceased friend von Prowazek. In the same year, Edmund Weil and his English associate Arthur Felix showed that a strain of *Proteus* bacillus was agglutinated by the sera of typhus patients. Some European bacteriologists declared that the *Proteus* bacillus was the 'exciting organism' of typhus, while others argued that *Proteus* and the typhus virus were simply variants of the same organism. The debate about infectious agent was resolved in 1937 by Herarld Cox with the development of the tissue culture technique. Later, cross adsorptions showed that there was a common cross-reacting epitope among *Legionella bozemanii* Wiga, *Rickettsia* TG, and Proteus OX19, due to a common lipopolysaccharide antigen.

In 1998, the complete genome sequence (1.1 Mb) of *R. prowazekii* showed 834 protein coding genes, contains the highest proportion of noncoding DNA (24%) detected so far in a microbial genome and the phylogenetic analysis proved new evidence of an evolutionary relationship between rickettsiae and intracellular mitochondria.

Disease Vector: The Human Body Louse (*Pediculus humanus corporis*)

In 1740, James Lind, a physician observed that typhus was carried on the bodies of men, on clothes; but only in 1909, Charles Nicolle discovered the role of lice in transmission of typhus, later receiving the Nobel Prize (**Figure 2**). By 1903, Tunis was full of typhus patients; Nicolle observed that patients infected others on the street and their clothing was infectious. After the patients had a hot bath and were dressed in hospital clothing, they ceased to be infectious. Later, he fed uninfected lice on an experimentally infected bonnet monkeys (*Macacus sinicus*) and then transferred the lice to uninfected monkeys that later developed typhus. This accomplishment became a powerful impetus for the establishment of delousing procedures. In the same year, Ricketts and Wilder confirmed these results in Mexico City and N. F. Gamaleia in Russia.

In 1916, Da Rocha Lima discovered that the *R. prowazekii* multiplied within the epithelial cells of the louse's stomach, which was confirmed by others researchers, such as Nöller; Töpfer, and Schüssler. In 1922, Wolbach observed that *R. prowazekii* escapes from alimentary tract through feces and they supposed that it may be introduced by scratching or by the mouth parts of the louse after becoming soiled with feces. They did not see rickettsia in the salivary glands or in the esophagus of louse. At that time, it was difficult to investigating lice as many were infected by *B. quintana* (*Rickettsia pediculi*). However, only in 1938, Starzyk demonstrated that patients are infected by the faces and not by the bite of the louse, and *R. prowazekii* could be transmitted to man by inhalation or in a conjunctive way. The role of lice as a vector of epidemic typhus has been studied in many countries, and in 2002, by using modern tools, an experimental model was established,which reproduces the natural infection of body louse.

The question of survival for *R. prowazekii* during interepidemic periods has led to several rather controversial hypotheses about reservoir. As suggested by Zinsser, after his study of 538 Brill's disease cases, the main reservoir appears to be in humans, because lice die of the infection and humans who contract typhus retain some rickettsiae for the rest of their life. This finding was confirmed by

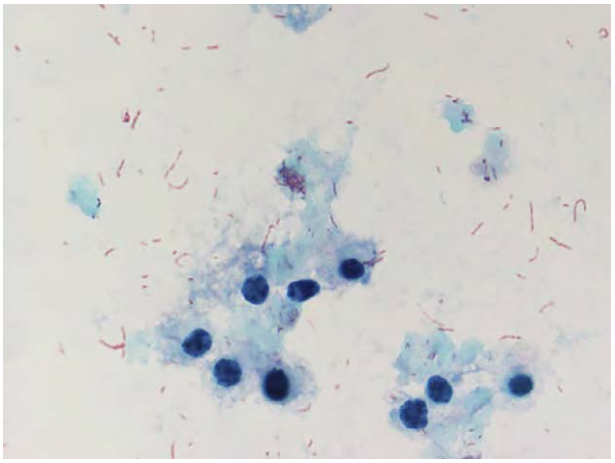

Figure 1 *Rickettsia prowazekii* in the L929 cell culture (day 6) by Gimenez staining.

Figure 2 The human body louse, *Pediculus humanus humanus* (*P. h. corporis*).

Nicolle (1934), Giroud (1948), Mooser (1946), Willsch (1952), Gear (1952), and Murray (1951). In contrast, Reiss Gutfreund in 1956 found, in Ethiopia, *R. prowazekii* in domestic animals and their ticks. *R. prowazekii* was isolated from adult *Amblyomma variegatum* and *Hyalomma marginatum rufipes* and *Hyalomma truncatum* in Ethiopia. Transmission from experimentally infected larvae to nymphs took place in 50% of *A. variegatum* and *Amblyomma lepidum*. *R. prowazekii* was maintained for several years in experimentally infected *Ornithodoros moubata* and in *Ornithodoros papillipes*; however, adverse effect on *Dermacentor marginatus* and *Discoglossus pictus* ticks has been reported. Recently, *R. prowazekii* was detected by molecular detection and isolated by shell vial culture in *Amblyomma* ticks collected in Mexico.

Later, Dyer and Mooser found *R. prowazekii* in the fleas, and several experiments to infect fleas with *R. prowazekii* were reported. The head louse *Pediculus humanus capitis* is capable of maintaining *R. prowazekii* experimentally; its role in the transmission of this rickettsiosis is not well established. An extensive work by Ormsbee and McDade failed to support the hypothesis that an extrahuman reservoir of infection plays an important role in the ecology of epidemic typhus. The search was futher stimulated by the isolation of *Rickettsia canadensis* from a pool of ticks taken from rabbit. While investigating the ecological niche of *R. canadensis*, Bozeman unexpectedly recovered several strains of *R. prowazekii* from flying squirrels (*Glaucomys volans volans*) that had been captured in Virginia and Florida. Later, it was found in their ectoparasites. Thus, the hypothesis of a widespread extrahuman reservoir of epidemic typhus was confirmed.

Diagnosis and Treatment of Epidemic Typhus: Historical Aspects

Serology: During the first two decades of the twentieth century, numerous attempts were made to cultivate the bacteria from the blood, urine, and stools of typhus patients. In 1910, Wilson reported the isolation of a coliform bacterium, designated *Bacillus U*, from stools and rarely from urine, and not from blood. His work demonstrated that the bacterium was agglutinated by sera of typhus patients to a titer 3–10 times greater than by sera from normal individuals or patients with other diseases. Comparable results were obtained in 1916, in Berlin, by Weil and Felix with a *Proteus* isolate from patients' urine. Other agglutination tests were suggested like agglutination with *Vibrio cholerae* by Cantacuzène (1919), with *Pseudomonas aeruginosa*, *V. cholerae* infection, *Bacillus prodigiosus*, and *Bacillus subtilis* by Violle (1920). Epstein and Morawitz named it 'the Weil–Felix test' in honor of the researchers, and it remained for many decades the chief tool for the serological diagnosis of the typhus fevers.

Staining rickettsiae: Rickettsiae stained poorly with the Gram stains, most of early observations were made on smear and sections stained with Giemsa. In 1930, Castenada substituted, to some advantage, the azur II and eosin of Giemsa's with methylene blue and safranin.

More successful was the procedure introduced in 1937 by Macchiavello using basic fuchsin (rickettsiae retain the red strain), following decolorization with citric acid and counterstaining with methylene blue. In 1964, Gimenez changed the methylene blue with malachite green. This procedure has gained wide acceptance until now (**Figure 1**).

Culture: In 1937, Kligler and Aschner proposed the same technique for cultivating *R. prowazekii* such as the one presented earlier by Nigg and Landsteiner for murine typhus using Maitland cultures in tissue cultures. Zinsser found that greater yields of rickettsiae could be obtained on agar cultures with the same constituents. In later experiments, he used Kolle flasks for the mass cultivation of rickettsiae from minced tissues obtained from mouse and chick embryos. Herald R. Cox's discovery of using the yolk sacs of chick embryos for cultivating rickettsiae in 1938 had an enormous impact on rickettsial technology, paved the way for a new generation of vaccines and diagnostic reagents, and facilitated studies of rickettsial susceptibility to chemotherapeutic agents and rickettsial physiology. In the following years, *R. prowazekii* was cultivated in chick fibroblast cell culture, in cotton rat macrophages, in guinea pig macrophages, L929 cell line mouse, vero cells (kidney epithelial cells extracted from African green monkey), and NIAS-AeAl-2 insect cells. Recently, *R. prowazekii* was isolated from a blood sample from a patient by the shell vial cell culture technique using human embryonic lung fibroblasts.

Control and eradication: The first prevention method used for the patients with epidemic typhus was double delousing process, more complete than that practiced on admission to the hospital using kerosene and 'lightwood oil'. This method was used since eighteenth century by James Lind in America and later by all physicians after Nicolle discovery. Dichloro diphényl trichloroéthane (DDT), a poisonous powder for insects, was used in Mexico, Algeria, Italy, and Egypt for delousing. In 1988, Darby proposed a protocol of powder dusting of the entire clothing with 10% DDT, 1% malathion, or 1% permethrin dust, but a resistance of lice to DDT was detected in Korea and Japan. A standard treatment protocol with 1% permethrin has been created by WHO in 1993. Because treatment should be repeated every week, of 3–5 tons of powder is required to treat 100 000 people on one occasion. In 1987, Strong and Brown sowed the therapeutic efficacy of ivermectin to eradicate lice.

Treatment: In 1919, Danielopolu recommended to use strophantin or chlorine injection for typhus treatment. Some investigators employed large doses of the serum of convalescent. Digitalis or strychnia was given for possible supportive effect in most cases, and in case of a collapse camphor or caffeine. Preantibiotic chemotherapy of rickettsial diseases was dominated by *p*-aminobenzoic acid (PABA), discovered in 1942 by Snyder, and separately in 1944 by Greiff. In 1951, Snyder and Davis showed that the growth of rickettsiae is inhibited by PABA and requires *p*-hydroxybenzoic acid (POB); later, Weiss isolated PABA-resistant mutants of *R. prowazekii*, suggesting a role of POB in rickettsial biosynthesis.

The newly discovered antibiotic compounds, namely, the tetracyclines family and chloramphenicol changed the natural history of epidemic typhus. Initial clinical trials, carried out by Payne and Smadel, demonstrated the effect of chloramphenicol on patients ill with epidemic typhus. Chloramphenicol treatment in recrudescent typhus fever (Brill's disease) produced defervescence in approximately 4 days. Now, recommended treatments for epidemic typhus are doxycycline and chloramphenicol. Therapy for a minimum of 5 days and at least 2–4 days after defeverscence should be provided to preclude relapses, especially with early treatment. In case of an outbreak, a single 200-mg oral dose of doxycycline is extremely efficient.

Vaccination and prevention: One of the earliest protective measures for typhus was consuming human lice. The value of this procedure is undocumented. Based on the protocol of RMSF vaccines, Rudolph Weigl developed, in 1930, a similar vaccine against epidemic typhus from the intestine of experimentally infected lice. Initially, more than 100 infected lice were needed to produce a single dose of vaccine; later 90 lice provided three doses of the vaccine. This vaccine was produced on a large scale in Weigl's laboratory before World War II in Poland and was used in China (1936, 1943), Ethiopia (1939), and other countries. After discovery of cultivation of *R. prowazekii* in embryonated chicken eggs, it was possible to produce very large quantities of live Rickettsiae, inactivated by 1.5% solution of phenol. A total of 5–6 millions of individuals were vaccinated against typhus during German occupation in the eastern zone of war operations. Two additional attempts of inactivated vaccine production deserve to be mentioned, one in the guinea pigs on a vitamin-deficient diet or by X-ray radiation made by Zinsser and Castenada in 1932 and the E strain vaccine reported by Fox in 1956, as they provide information on the biology of rickettsiae. The isolation of vaccine strain E, originated from a 1941 typhus case in Madrid, was passed routinely into eggs and reduced virulence for guinea pigs after the 11th passage. Extensive field trials were conducted in Peru and Burundi with strains passed into eggs at least 256 times.

Murine Typhus

Murine typhus, caused by *R. typhi*, was found throughout the world, and found to be prevalent in tropical and subtropical seaboard regions, where the most important rat reservoirs (*Rattus* spp.) and flea vectors (*Xenopsylla cheopis*) (**Figure 3**) are found, during warms months of year. It is difficult to establish the true incidence of the disease, and the number of reported cases does not reflect the true prevalence, because the patients usually mark a rather mild course and present with nonspecific symptoms. During the 1-month attack of Khmers in Thailand, the rate of murine typhus infection was 172 in 100 000 adults. Murine typhus may occur in travelers returning from endemic regions.

After an incubation period ranging from 6 to 14 days, the clinical manifestations were high fever, severe headache, chills, myalgia, weakness, and nausea. The rash described as macular (49%), maculopapular (29%), papular (14%), petechial (6%), and morbiliform (3%) is usually centrally distributed on the trunk, but also found on the extremities. Childhood murine typhus is often mild, with fever (100%) and rash (57%), lymphadenopathy (usually cervical (37%)), and severe headache (29%). Splenomegaly or hepatomegaly was less frequent findings (24 and 10%, respectively). Physicians practicing in or near *R. typhi* endemic areas need to consider murine typhus in the differential diagnosis with a febrile illness without a clear source of infection. Occasionally, patients develop some complications: central nervous system abnormalities (9.6%), renal insufficiency, hepatic insufficiency, respiratory failure are seen in older patients. There was one death in Maxcy's series of 114 cases, no deaths in a series of 180 cases by Stuart and Pullen, and 13 of 345 cases reported by Dumler.

Historical Aspects of Murine Typhus Disease

From the time of Brill's report, a sporadic typhus-like illness was designated throughout the world as 'endemic typhus' to distinguish it from the more serious classic louse typhus. Ricketts and several of the early researchers wondered if Mexican and European forms of typhus were the same diseases. Paullin made the first clinical description of a 'milder form of typhus' without mortality in 1913 in Atlanta (**Table 6**). In 1917, Neil noted that male guinea pigs inoculated with the blood of typhus patients in south Texas often developed a scrotal swelling and inflammation, along with hemorrhage beneath the tunica, similar to the lesions elicited by *R. rickettsii*. His results, confirmed in 1928 by Mooser, showed that the American and the European variety could be clearly

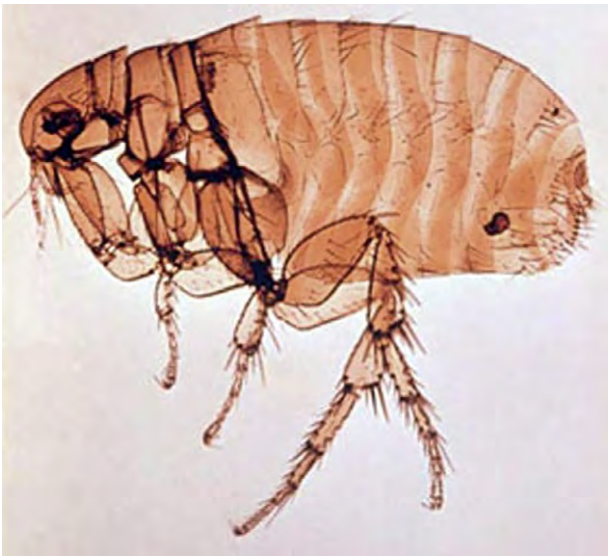

Figure 3 *Xenopsylla cheopis* femelle, the flea vector of endemic typhus.

Table 6 Milestones of murine (endemic) typhus

Discovery on murine (endemic) typhus: ***Rickettsia typhi***	*Authors*
1913 First clinical description	Paullin
1920 The first name: *Dermacentroxenus typhi*	Wolbach and Todd
1921 Identification of *R. typhi* (guinea pig)	Mooser
1924 The first study on typhus	Stuart
1926 Isolation of *R. typhi* from the blood	Maxcy
1926 The first hypothesis of reservoir and vector of diseases	Maxcy
1930 Role of fleas and rat	Dyer and Mooser
1930 Tissue cultures (guinea pig tunica tissue)	Nigg and Landsteiner
1931 Mechanism of transmission	Ceder and Dyer
1940 Distinction of scrub typhus	Lewthwaite and Savoor
2004 Genome sequence	McLeod MP *et al.*

differentiated by their reactions in the guinea pigs. These studies contributed in distinguishing the endemic from the classic typhus. It is difficult to differentiate when the workers were dealing with flea-borne typhus and when with epidemic typhus; both forms were present and both were referred to as Mexican typhus. For several years the murine typhus was misdiagnosed, the cases observed in 1920 in Rome by Carducci would be, according to Pécori, of the cases of murine typhus. In 1929 in Sao Paulo, during an outbreak of *tabardillo*, researchers were able to isolate the bacteria not only from ticks (*Amblyomma* spp.), but also from fleas, urban rats, and small mammals.

In 1923, in the Annual Reports of the Department of Health of Palestine, there was a reference to a mild course of typhus fever, similar to Brill's disease and different from the classic form of typhus. Stuart, in 1924, made the first comprehensive study on typhus in Palestine, and Fletcher and Lesslar in Malaya. In 1925, an outbreak of 200 cases of endemic typhus occurred in Australia. Plazy, Marcandier, and Pirot observed the first Mediterranean cases of murine typhus in sailors on the warships of Toulon (France), Blanc in Morocco, and Nicolle in Tunis (Tunisia). During 1926 and 1932, 135 cases of murine typhus on the warships of Toulon were noted. Lépine in Greece and in Liban observed identical cases, and showed the presence of the microorganism in the brains of infected rats. In 1935–36, Suarez, Palacios, Chavez in Chili, and Sesnic and Giroud in Tunisia identified asymptomatic cases of murine typhus. In the period 1931–46 42 000 cases were reported in the United States. Late reports established the worldwide distribution of endemic typhus. In 1940, Lewthwaite and Savoor reiterated the important distinction between the *X. cheopis*-borne murine typhus ('shop typhus') and the chigger-borne scrub typhus ('tsutsugmushi' or 'rural typhus').

The designation 'murine' typhus was adopted since the infectious agent was well entrenched in the rat population and its flea throughout the world. About the origin of murine typhus, Traub suggested that the *R. typhi* has always associated with the genus or subgenus *Rattus*, and that, in its ancestral homeland, which was probably in southeast Asia, ectoparasites other than *X. cheopis* were the original intramurid vectors. In contrast, murine typhus existed in a sylvan cycle involving other small mammals (theraphions) and ectoparasites, but spilled other into *Rattus*, which proved to be unusually well adapted to maintaining it and disseminating it

over the world and bringing it in contact with man. The nature of such an aboriginal cycle and its geographic location are yet unrecognized, but the data to date indicate that northern Africa, which was believed to be the origin of *X. cheopis*, is unlikely to be involved.

The Infectious Agent: Rickettsia typhi

In the late 1920s and early 1930s, through the efforts of many investigators, the agent of endemic typhus was considered more virulent for the rat than *R. prowazekii*. There was considerable discussion of whether they were variants of the same species or two different species. In 1920, Wolbach and Todd proposed the designation *Dermacentroxenus typhi* for the Mexican variety of typhus. With time *D. typhi* was changed to *R. typhi*, although some rickettsiologists still prefer the designation *R. mooseri* in honor of Herman Mooser. In 1926, Maxcy isolated rickettsia from the blood and noted their antigenic similarity and differences with *R. prowazekii* and *R. rickettsii*. In 1930, Dyer isolated the suggested new 'virus' from fleas collected in Baltimore during an outbreak of three cases of typhus. Mooser recovered the microorganism from brains of rats in a prison of Mexico City, where several immates had presented typhus fever. The studies of Lepine, Lorandos, Lemiere, Combiesco (1934), and Combiesco (1935) led to the isolation of bacteria from cats and dogs. *R. typhi* is an obligate intracellular bacterium that infects endothelial cells in mammalian hosts and midgut epithelial cells in the flea host. In 2004, complete genome (1 111 496 bp) analysis of *R. typhi* revealed 877 genes, 40 pseudogenes, and 838 proteins.

Murine Typhus Vectors

In 1926, Kenneth Maxcy, a wise epidemiologist with a clinical background, observed patients and the environments related to their illness, particularly in Alabama, Georgia, North Carolina, and Virginia. He concluded "that the reservoir is rodents, probably rats or mice, from which the disease is occasionally transmitted to man. The parasitic intermediaries which are first suspected are fleas, mites, or possible ticks". Hone in 1927 postulated the same theory, and the vector other than the louse for endemic typhus was reported in Palestine. Maxcy's theory was validated by two different groups.

In 1930, a physician-pharmacist, Dr. J. Lipsky presented fever, headaches, and a rash. His physicians diagnosed typhoid fever, but blood cultures and the Widal reaction were negative, and the Proteus OX19 reaction reached a titer of 1:320. Endemic typhus fever was the discharge diagnosis. On the day of discharge, Dr. Lipsky's female clerk was hospitalized and placed in the same room with the same illness, as was the male clerk 3 weeks ago. Dyer and his associates, Rumreich and Badger, successfully transmit the rickettsial agent to guinea pigs from rats' brains, and fleas, trapped in the basement of Dr. Lipsky's drugstore, Baltimore. They identified the murine reservoir of the rickettsia and incriminated the flea as a vector, showing that the organisms persisted in rat fleas for at least 9 days and were present in feces of infected fleas. The authors found that the flea *X. cheopis* became highly infectious on the fifth or sixth day, remained infectious for 40 days without evidence of ill effect with an inoculum representing 1/128 000 of a flea.

In the same year, Mooser recovered the microorganism from the brains of rats in a prison in Mexico City, where several inmates had presented typhus fever. The attempt of these two groups elucidates the epidemiological cycle of the disease by revealing the reservoir (rat) and the vector (fleas).

Later, Ceder and Dyer noted that the disease was transmitted not by flea's bite, but by inoculation into the flea-biten part or by skin abrasions with the flea feces or crushed fleas. Mooser and Castenada followed, by cytologic methods, the development and multiplication of the rickettsia of murine typhus in tissues of several species of fleas in epithelial cells of the stomach and in cells of the malpighian tubules. Rickettsiae were not observed in the salivary glands and not in the genitals, thus the fleas were considered inefficient vectors of typhus. Lewthwaite in Malaysia confirmed the findings about transmission via contact with crushed fleas or flea feces, which remains infectious for 8 or 9 years (potential for aerosol transmission) and the absence of transovarian infection.

In 1931, Dove and Shelmire raised a serious doubt as to the exclusive role of flea transmission of endemic typhus, following their studies with the tropical rat mite, *Ornithonyssus bacoti*. They demonstrated the transovarial transmission and the passage of disease from guinea pig to guinea pig and to rat via infected mites. Later, Pang noted natural infection with *O. bacoti* in China. The *Polyplax*, lice of rat, was found infected with murine typhus and was able to transmit the disease experimentally. However, this louse did not bite humans and therefore would not be a direct cause of human infection, but may be important in maintaining enzootic infection. On the contrary, *R. typhi* was isolated from the human lice *P. humanus* from patients in India, Mexico, and Ethiopia. Experimentally infected lice did not transmit *R. typhi* to their progeny. In 1930, Castaneda and Zinsser reported that by experimental model the bedbug *Cimex lectularius* can acquire the rickettsiae of 'Mexican typhus fever', but failed to transmit it to another animal. *R. typhi* was isolated from a pool of Boophilus ticks collected from bushes in India and from *Hyalomma* ticks collected from cattle in Ethiopia. Some ecological and epidemiological studies indicate that the ticks and trombiculidae mites (chiggers) may be of some importance as vectors but they do not challenge seriously the theory that fleas are the principal carriers to man (**Table 7**).

Transmission by aerosols and contamination with urine or feces of infected rats or cats that contaminated foodstuffs might account for some cases. Also, the transmission of the disease by mouth by animal experiments and the possibility of transmitting the disease from human to rabbits and to guinea pigs was proved. Some native wild rodents were susceptible to infection with *R. typhi*: the woodchuck, *Marmota monax monax*; the meadow mouse, *Microtus pennsylvanicus pennsylvanicus*; the white footed mouse,

Table 7 Arthropods implicated as actual or potential vectors of murine typhus

Arthropod	*Distribution*
Fleas	
Xenopsylla cheopis (rat flea)	Almost worldwide
Xenopsylla astia (rat flea)	Southern Asia and coastal East Africa
Xenopsylla brasiliensis	Africa, South America, India
Xenopsylla bantorum	Africa (Ethiopian region)
Ctenocephalide felis (cat flea)	Cosmopolitan
Echidnophaga gallinacea (sticktight flea)	Cosmopolitan
Leptopsylla segnis (mouse flea)	Cosmopolitan
Monopsyllusa nisus (murine flea)	Northern Asia
Nosopsylla fasciatus (ceratophyllid flea)	Cosmopolitan
Pulex irritans (human flea)	Cosmopolitan
Lice	
Hoplopleura oenomydis	Cosmopolitan
Pediculus humanus	Almost worldwide
Polyplax spinulosa	Cosmopolitan
Mites	
Echinolelaps echidninus	Almost worldwide
Ascoschoengastiain dica	Asia
Ornithonyssus bacoti	Almost worldwide

Peromyscus leucopus neveborascensis; the house mouse, *Mus musculus*; and the *spermophiles*, genus *Citillus*. But, the most important components of the murine typhus life cycle are commensal rats of the subgenus *Rattus*, such as *R. rattus* and *R. norvegicus*, and their fleas, in particular the oriental rat flea *X. cheopis*.

Diagnosis and Treatment: Historical Aspects

Diagnosis: The first test for diagnosis of murine typhus was the Weil–Felix test. Weigl reported a more sensitive and specific serological test using suspensions of rickettsia of European typhus and murine typhus grown in the intestine of lice. Today, indirect immunofluorescence assay is the reference method for serodiagnosis of rickettsia in most laboratories, and Western blotting assay associated with the cross-adsorption technique to eliminate the false-positive results through cross reactions between bacteria sharing common antigens, such as *Legionella* and Proteus OX-19. A diagnosis of murine typhus can be confirmed by immunohostological demontration of *R. typhi* in tissues, by polymerase chain reaction (PCR) amplification from blood and by cell culture system.

Treatment: Since 1950, chloramphenicol is the treatment for murine typhus. In the first study, the authors noted that the temperature reached normal levels within 3 days after beginning the therapy. The current recommendation is administration of 100 mg of doxycycline orally twice daily for 2 to 3 days after deffervescence, when tetracycline is contraindicated, chloramphenicol is an alternative treatment.

Vaccine: A vaccine for typhus more unique than Weigl's was developed by Blanc and Baltazar using 'attenuated' *R. typhi*-infected fleas' feces with ox bile/saline diluent, in the Institut Pasteur, Casablanca. A later study of this vaccine in volunteers showed that protection against epidemic typhus occurred only when the flea feces in the vaccine was viable and caused a mild murine typhus infection with serological conversion after vaccination. No effective vaccine is available for murine typhus.

Prevention: Prevention is directed primarily toward the control of flea vectors and potential flea hosts. In 1952, a decline in cases of murine typhus in the United States was reported after DDT dusting campaigns. The rate of infection remained low and no human cases occurred in the area for at least 3 years after cessation of the dusting operations. The recent recommendations are rat trapping and application of rodenticides and dusting harborages with carbaryl or permethrin for flea control.

Rocky Mountain Spotted Fever

RMSF caused by *R. rickettsii* is transmitted by the bite of infected ticks. RMSF has not occurred in epidemic proportions, but causes the most severe clinical manifestations of any rickettsioses in early studies with the virulence to kill before the introduction of effective therapies, 10% of children and 30% of adults (Table 3). Currently, it is not so deadly and has a death rate comparable to that of Mediterranean spotted fever (MSF). The average annual RMSF incidence in the United States during 1997–2002 was 2.2 cases per million persons. The disease also occurs in Central and South America, where it is currently largely unrecognized and possibly misdiagnosed as dengue or other febrile exanthems; hence RMSF has been described as a 'wolf in sheep's clothing' and 'the great imitator'.

Around 1 week following the bite of an infected tick, symptoms of illness begins with fever, chills, myalgia, and headache. During the next few days, these symptoms continue and may be accompanied by anorexia, nausea, vomiting, abdominal pain,

diarrhea, photophobia, and cough. The fever is typically high and associated with a severe frontal headache. Rash, considered as the hallmark feature of RMSF, appears on day 3 of fever as small, pink, blanching macules, typically on the wrists, ankles, and forearms, which evolve into maculopapules. Within 24 h, it spreads centrally to involve the legs, buttocks, arms, axilae, trunk, neck, and face. The entire body may be involved, including the mucous membranes of the palate and pharynx. Characteristic of the rash is petechial lesions, the distribution that includes the palms and soles and occurs in only 36–82% of patients. In some severe cases, petechiae may coalesce to form large ecchimoses. Severe manifestations may include pulmonary edema and hemorrhage, cerebral edema, myocarditis, renal failure, disseminated intravascular coagulopathy, and gangrene. Three factors were independent predictors of failure by the physician to initiate therapy the first time a patient was seen: absence of a rash, presentation between 1 August and 30 April, and presentation within the first 3 days of illness. In untreated patients who survive their illness, the natural course of the fever terminates after 2–3 weeks.

Historical Aspects of Rocky Mountain Spotted Fever Disease

It is not known how long *R. rickettsii* has been indigenous to the Western Hemisphere, a reflection of the 500 years or so since the first explorers, slaves, and immigrants inhabited what is now the United States, Canada, Mexico, and Central and South America. According to the US Public Health Service, the earliest account of RMSF dates to 1873 (**Table 8**). Major W. W. Wood, who collected descriptions of cases from eight Idaho physicians, apparently first recorded the disease in 1896. Edward Maxey published the first report in the medical literature of a case in the Snake River Valley of Idaho in 1899 and he gave a vivid description of the disease known locally as the 'blight on the Bitterroot' or 'black measles'. Its severe dark rash and folk wisdom of the day suggested that infection occurred from drinking the melted snow water that gushed out of Lo Lo Canyon during spring runoff, the notorious site of the infection after Michie and Persons.

In 1902, Wilson and Chowning, both from the University of Minessota, began a significant epidemiological study. Their valid observations included the first description of severe RMSF, findings from eight autopsies, and geographic distribution on the western side of the Bitterroot River. They correctly concluded that 'spotted fever' was a disease of the capillary circulation caused by a noncultivable infectious agent with a wild reservoir with no evidence of transmission of the disease from person to person or by means of a common water or food supply. With Ricketts' findings of 1906, RMSF became a true disease, not a syndrome. From 1904 to 1913, 96 (63%) of 153 patients died of RMSF in Montana.

Clinically and epidemiologically compatible cases of RMSF were described from New York State as early as 1912; however, the first clinically recognized, laboratoryconfirmed case, east of the Rocky Mountains, was documented in an Indiana child in 1925. In 1931, Badger described cases in Delaware, Maryland, North Carolina, Pennsylvania, Virginia, and Washington, establishing the widespread distribution of RMSF in the United States. In 1929 in Sao Paulo, the physicians diagnosed several patients with a febrile exanthematous disease, named 'Sao Paulo exantematic typhus' or 'Brazilian spotted fever' with a high fatality rate (70%), identifying in ticks (*Amblyomma* spp.) and small mammals a rickettsia similar to the RMSF agent. Recently, contemporary diagnostic methods have permitted the retrospective confirmation of fatal case of spotted fever in a Maryland patient that occurred in 1901. During the next several decades, RMSF was confirmed in Columbia (*Toba petechial fever*), Mexico (*Fiebre de Coix* and *Fiebre manchada*), Canada, Panama, Costa Rica, and Argentina.

The Infectious Agent: *Rickettsia rickettsii*

At the beginning of 1900, research of infectious agent was very difficult because the techniques of visualization of bacteria were imperfect. Sometimes, certain works were false tracks like those of Wilson and Chowning (1904) of Fricks, and Noguchi later (1916), which attribute to the parasite *Pyroplasma hominis* identified in the hypochromic and sledged red cells of an afflicted patient

Table 8 Milestones of Rocky Mountain spotted fever

Discovery of the Rocky Mountain spotted fever: ***Rickettsia rickettsii***	*Author*
1873 The earliest records of RMSF	US Public Health
1896 Description of RMSF	Wood WW
1899 The first clinical account	Maxey
1904 Association: tick disease (not proved)	Wilson and Chowning
1905 Transmission from tick to man	McCalla
1906 Isolation of bacteria in guinea pigs	Ricketts
1906 Tick role in RMSF	Ricketts and King
1919 Description of the etiologic agent and proposed the name as *Dermacentroxenus rickettsii*	Wolbach
1922 The name of *R. rickettsii*	Brumpt
1924 Vaccine	Spencer and Parker
1954 Natural reservoir: the meadow vole '*Microtus pennsylvanicus*'	Gould and Miesse
2004 Genome sequence (unpublished)	Madan *et al.*
2007 *R. rickettsii* str. Sheila Smith genome sequence	Eremeeva *et al.*

and in Columbian ground squirrels an etiological role of RMSF. In 1904, Stiles and Craig intensively studied the blood and tissues of spotted fever patients and both showed the absence of protozoan parasites.

The first experimental transmission of infection from the blood of patients to guinea pigs and monkeys was carried out by Rickets in 1906. The maintenance of the agent via serial animal passages alternating between monkeys and guinea pigs was suggested in his laboratory. Ricketts found that the infectious agent was retained by a small Berkefeld filter, was unable to grow in bacteriologic media, and the guinea pigs could also be infected with serum or washed cells. Later, he described "small spherical, ovoid and diplococcoid forms" in the blood of guinea pigs and monkeys infected with RMSF that seemed to be bacteria and "no so frequently in the blood of men". By using Giemsa stain, he saw "two somewhat lanceolate chromatin staining bodies, separated by a slight amount of eosin-staining substance". He wrote, "I have devised no formal name for the organism discussed, but it may be referred to tentatively as the bacillus of Rocky Mountain spotted fever". It is difficult to recognize *rickettsiae* based on this description. In his carefully prepared laboratory notes, Ricketts made drawings of his parasite. Wolbach was the first to demonstrate *R. rickettsii* in human tissues by using a modified Giemsa stain. He felt that the bodies, when present in the circulating blood, were only within phagocytic cells. In this point of identification of the parasite, Wolbach compared one of Ricketts' original preparations with those of his own obtained from eggs of infected ticks. He felt that "while Ricketts may have encountered the true parasite of the disease in ticks, he was led hopelessly astray by the occurrence of bacteria in his infected as well as noninfected ticks".

In the middle of 1916, Wolbach described the morphology of an intracellular bacteria that he called "a wholly new kind of micro-organism", a Gram-negative bacterium measuring from 0.2 to 0.5 mm, and has the capacity to infect and replicate in the cytosol and occasionally in the nucleus of vertebrate and invertebrate cells. This was probably the first description of *Rickettsia*. Later, he reported the experimental infection of primates and the post-mortem findings of five cases of RMSF. His meticulous and detailed descriptions of rickettsiae in the tissues of patients with fatal RMSF established the foundation for the current understanding of the pathogenesis of this disease. The similarity of the agent of typhus and tsutsugamushi fever was remarkable as noted by Wolbach. In 1933, Harris showed rickettsiae in the skin of a patient who contracted RMSF in Tenessee. Wolbach named it *Demacentoxenus rickettsii*, the genus name after its vector ticks and the species name after Howard Ricketts. In 1922, Brumpt proposed the name *R. rickettsii* and Bengston in Bergey's '*Manual of Determinative Bacteriology*' calls it '*Rickettsia rickettsii* (Wolbach)'. The complete genome sequence has not been fully characterized, but contains 1 257 710 bp with an estimated 1365–2849 open reading frames (ORFs) and contains considerable inactivated genetic material in the process of spontaneous degeneration.

Tick Vector

The most brilliant discoveries of the century, quite on par with the great findings of Robert Koch, were the first demonstration of arthropod biological transmission of any disease. The possibility that wood ticks (genus *Dermacentor*) were responsible for transmitting the infection was suggested by an Idaho physician, when a tick was found in the genitals of one of the fever victims. Wilson and Chowning made a crucial association between the discovery of the agent of Texas Fever (*Babesia bigemina*) carried by the cattle tick *Boophilus annulatus* and RMSF. Their acclaimed hypothesis of transmission by tick bite was later rejected when the acknowledged experts – Stiles, Ashburn, and Graig – did not find any protozoa in the patient's blood.

In 1905, McCalla, an Idaho physician, in association with Brereton, placed a tick obtained from the chest of a man who was very ill with spotted fever on the arm of another patient, with the full consent of the participants. The tick remained on the second patient for 48 h and then placed on the leg of a woman, where it remained for at least 10 h. The incubation periods were 9 and 3 days, respectively. McCalla did not publish his finding. Ricketts, during his investigation of RMSF in western Montana, demonstrated that *Dermacentor andersoni* was the principal vector and showed the transovarial transmission, confirmed independently by Walter W. King. Microscopically, Ricketts found the peculiar microorganisms in salivary glands, alimentary sac, and ovaries of infected females as well as in eggs. In collaboration with King, Ricketts showed the ability of a female tick to acquire the infection by feeding on experimentally infected guinea pigs and to subsequently transmit the illness by feeding on another guinea pig. In 1907, Ricketts had shown that Columbian ground squirrels, chipmunks, and woodchucks were susceptible to *R. rickettsii*. Later, Wolbach concluded that there was no cellular reaction in the tick's tissues for the presence of the parasites, 'even when presented in enormous numbers'. Other rarely recognized routes of transmission of *R. rickettsii* include blood transfusion and inoculation of rickettsiae through mucous membranes.

Later, *R. rickettsii* was isolated from other ticks like *Dermacentor variabilis* (**Figure 4**), *Rhipicephalus sanguineus* (**Figure 5**) and *Amblyomma cajenennse*. Burgdorfer (1963) showed 100% transovarial development of rickettsiae through four additional tick generations and filial infection rates in the seventh generation, but the low proportion of infected ticks in nature was explained by negative effect. Transstadial and transovarial transmission of rickettsiae in tick hosts is central to the maintenance of *R. rickettsii* in nature, the same for the horizontal transmission among tick-feeding animals and vertebrates host.

Over many years, extensive research was carried out to find the reservoir of this disease. *R. rickettsii* was first isolated from small mammals such as a meadow vole (*M. pennsylvanicus*) trapped near Alexandria in 1954 by Gould and Miesse. A decade later, it was isolated from eight other mammalian species, including a pine vole (*Pitymys pinetorum*), white-footed mouse (*P. leucopus*), a cotton rat (*Sigmodon hispidus*), cottontail rabbits (*Sylvilagus floridanus*), an opossum (*Didelphis marsupialis virginiana*), chipmunks (*Eutamias amoenus*), a snowshoe hare (*Lepus americanus*), and golden-mantled ground squirrels (*Spermophilus lateralis tescorum*). The significance of birds as reservoir hosts for *R. rickettsii* remains unproven. The rather limited list of animals from which *R. rickettsii* has been isolated, compared to the extensive list of seropositive animals, is not really surprising when one considers the transient level of rickettsemia in animals.

Figure 4 *Dermacentor variabilis adults* (the American dog tick), male (left) and female (right), nonengorged, implicated in the transmission of *Rickettsia rickettsii* collected in Lawrence country, OH, USA.

Figure 5 *Rhipicephalus sanguineus* (the brown dog tick), female (left), male (2nd of left), nymphs, larve, and egg, the main vector of MSF and sometimes vector of RMSF.

Diagnosis and Treatment: Historical Aspects

Diagnosis: For the first time a definitive, objective laboratory method for the diagnosis of RMSF was established after Rickett's discovery in 1907, named 'infection test'. The macroscopic changes in infected guinea pigs yielded a distinctive criterion for the diagnosis of the infection. Later, Ricketts developed an agglutination test using infected ticks, but the Weil–Felix test resides as the most widely used confirmatory procedure. In 1948, the test was completed by the complement fixation test, a specific reaction for the spotted fever strain of rickettsiae. Actually, a diagnosis by serology, by PCR assays, immunohistochemistry, or by the isolation of rickettsiae in cell culture was performed.

Prevention: In 1913, Fricks implemented a program for the Public health Service focused on controlling the tick vector in the Bitterroot Valley. In 1946, Smith showed that spraying an area with DDT or other insecticides was ineffective because of the huge territory and the difficulty in getting under low vegetations. Control of infection in nature is not feasible. The best protection to an individual lies in preventing the attachment of a tick to the skin or in removing it before the rickettsias have become 'activated'.

Vaccination: In 1924, Spencer and Parker developed a vaccine for RMSF from infected tick's intestine. In 1938, they showed that during the period of several years in western Montana there were 38 deaths in 50 nonvaccinated adults (76% fatality rate) and only three deaths in 59 vaccinated persons (9% fatality rate). This vaccine not only gives effective protection, but also produces many local and occasionally severe systemic reactions. It is not certain that vaccines against RMSF developed in 1973 by DuPont and in 1983 by Clement do better. At present, no licensed vaccine exists for RMSF.

Serotherapy: In 1908, Ricketts demonstrated passive serum prophylaxis of experimental disease in guinea pigs, but of nine human patients treated with hyper immune horse serum, six died. Later in 1943, the therapeutic use of an immune rabbit's serum as therapeutic doses administered before the third day of the appearance of the rash diminished mortality around 19%; the initiation after the third day of the rash did not alter the ultimate prognosis, it only reduced the toxemic symptoms. The development of antibiotics relegated serum therapy to use only in extremely toxemic patients or in those who have contraindication to chemotherapeutic agents.

Therapy: The goal of chemotherapy was to develop a rickettsiocidal drug. In 1949, Harrel suggested treatment with 'heavy metals' (arsenic and mercury) and with a solution of neoarsphenamine. After discovery of contraindication of sufonamides, the researcher

proposed the use of PABA as it showed an inhibitory effect on the growth of rickettsiae *in vivo*, but in practice a very large amount of the drug was required. After chloramphenicol discovery, a series of 37 patients with RMSF were successfully treated showing an average on the sixth day of illness, without fatalities. In certain patients with profound toxic syndrome, chloramphenicol with steroids suggested a significant antitoxemic effect. The isolation of viable rickettsiae several years after active infection from RMSF confirmed that the mode of action of this antibiotic is suppressive rather than killing.

In 1949, Harrel reported the first results of aureomycin treatment, highly effective in chick embryos and in guinea pigs, but having a rickettsiostatic effect. After the introduction of broad-spectrum antibiotics in 1948, the incidence of RMSF in the United States dropped to about 250 cases per year (from 500), with only about 24 deaths. In 2007, the recommended therapy for RMSF is doxycycline in a doseage of 100 mg twice daily for adults for 7 days and is continued for 2 days after the patient has become afebrile. Glucocorticoids are sometimes given to severely ill patients, but no documentation of efficacy has occurred. Moreover, they are not recommended, although in experimentally infected dogs treated simultaneously with doxycycline, no detrimental effects of prednisole were noted.

Mediterranean Spotted Fever

Ricketsia conorii, the causative agent of MSF is transmitted to humans by tick bite, particularly by *Rh. sanguineus* ticks. MSF is endemic in the Mediterranean area, including northern Africa and southern Europe, but new cases were reported sporadically in central Europe, Central and South Africa, and India. The role of MSF in history is unknown because the disease has a moderate severity, but it plays an important role as a public health treat. In 2002, in Italy, the national incidence rate is 1.6 cases per 100 000 persons (but, in Sicily 10 cases per 100 000 persons); in Portugal between 1989 and 2003, the annual incidence was 8.9/100 000 inhabitans; and in Spain as well as in Marseilles between 1983 an 1985, the estimated incidence was 23–45 cases per 100 000 persons.

The incubation period from the time of infection to the onset is 6 days. Often, patients present abrupt fever, flu-like symptoms (headache, chills, arthromyalgias), and a *tache noire* at the tick-bitten site. The *tache noire*, the hallmark of the disease, is an inflamed red papule, the center of which becomes necrotic and black, indolent, and usually localized on the trunk and the legs and arms; in infants it is often on the scalp. It is present in the majority of cases; however, in some patients it cannot be found unlike clinics, in 14–28% cases. On the seventh day of the fever, a generalized maculopapular rash erupts first on the extremities and then on the trunk, often involves the palms and soles, and to a lesser extent the face. Other common clinical manifestations were myalgia (73%), headache (69%), conjunctivitis (32%), hepatomegaly (44%), and splenomegaly (19%). Severe forms of the disease have been reported in 6% of patients, especially in adults with one of the following conditions: diabetes, cardiac disease, chronic alcoholism, glucose-6-phosphate dehydrogenase deficiency, end-stage kidney disease. The mortality rate may reach 2.5% among diagnosed cases. In 1997, in Beja, a southern Portuguese district, the case fatality rate of MSF was 32.3% in hospitalized patients, explicated by the authors by the differences in virulence strains.

Historical Aspects of Mediterranean Spotted Fever Disease

It is evidentl the first cases of MSF were observed before the first description by Conor and Brush of the Pasteur Institute in Tunis in 1910. The authors described seven cases of spotted fever; they differentiated the disease from epidemic typhus and other eruptive fevers and compared with RMSF (**Table 9**). Advised by Charles Nicolle, they called the Tunisian spotted fever "*fièvre boutonneuse de Tunisie*". They made the first experimental infection to chimpanzee (cured of typhus since 45 days). After 10 days of fever, the chimpanzee died. In the same year, Conor and Hayat described the clinical features of other four clinical cases (abrupt onset, high fever, headache, chills, arthromyalgias, and cunjunctivitis). The exanthema was papular rather than macular; they named it '*bouton*' and it often turned red-purple in color, involving the palms and soles. The duration of illness was 12–15 days, without fatality cases.

Table 9 Milestones of Mediterranean spotted fever

Discovery: Mediterranean spotted fever: Rickettsia conorii	*Author*
1910 First case reported of MSF	Conor and Bruch
1914 Tick role in MSF: the brown dog tick *Rh. sanguineus*	Wilson
1925 Description of '*tache noire*'	Pieri
1932 Isolation of *R. conorii conorii*	Brumpt
1946 First cases of Israeli spotted fever rickettsia	Valero
1950 Isolation of *R. conorii indica* from tick collected in India	Philip et al.
1972 The first case of a febrile exantema in Astrakhan	Goldwasser
1974 Culture of *R. conorii israelensis*	
1991 Culture and identification of R. conorii caspia	Tarasevitch and Raoult
1991 Description of Astrakhan fever disease	Tarasevich
2001 Description of Indian tick typhus rickettsia	Murali *et al.*
2001 Genome sequence *R. conorii* strain 7	Ogata *et al.*
2005 Subspecies of *R. conorii* based on MLST	Zhu et al.

During the same time, several cases with atypical generalized rash, called '*Marseilles' fever*', was reported in the South France. Later, not only sporadic cases, but small endemic cases of spotted fever in the warm months were mentioned in this region. In 1920, in Italy, Carducci reported 16 cases of spotted fever collected during the last 10 years and distinguished two different diseases by clinical and epidemiological criteria: remittent fever and spotted fever, named after "*Febbre eruttiva, forma speciale descritta dal Pr. Carducci*". In 1925, Jean Pièri and Brugeas described a small lesion '*tache noire*', an eschar of inoculation, the hallmark of diseases. In 1928, Roche, a physician of Sorgues, South of France reported, "During 31 years of my professional work, I carried each year, some clinical cases of one mysterious disease to which I was embarrassed to give a name". The physician named it "*exanthème infectieux épidémique, maladies exanthématique, fièvre exanthématique du littoral*, and *exantheme infectieux méditerranéen*".

In 1925–27, on the Academy of Medicine, Olmer gave an excellent clinical description of the disease and projected new experimental researches on the guinea pigs and monkey for differentiation of epidemic, endemic typhus, and Brill's disease. Boinet and Pieri reported an epidemiological study of disease and insisted that the disease was not very contagious to human, benign, and very different of typhus. Two opposite opinions were projected: Jean Olmer classified this disease in the same group with the typhus. He wrote about several form of typhus: severe winter typhus (epidemic typhus) and benign summer typhus, which consisted of Brill's disease and Marseilles' fever. Several histological studies of MSF between 1932 and 1935 on the skin biopsies showed that *R. conorii* caused vasculitis and was responsible for the clinical anomalies.

The spotted fever disease was clinically described in India by Megaw (1921), who named it Indian tick typhus rickettsia (ITTR); in (1946) Israeli spotted fever rickettsia (ISFR); and in Astrachan, a region of Russia located on the shores of the Caspia Sea (1972), Astrachan spotted fever rickettsia (AFR). Later subspecies of the etiological agent *R. conorii* was isolated from human samples of these diseases.

The Infectious Agent: *Rickettsia conorii*

In 1927, Netter supposed that the agent of spotted fever of Marseille was the same organism associated with Brill's disease, only that it lost some virulence factors by the phenomenon of mutation. In 1932, Blanc and Caminopetros characterized some small bacteria, which seemed probably *Rickettsiae* on the ticks' organ smears using Giemsa staining, and showed that the infectious agent crossed through the filters Chamberland L2 and L3 and remained infectious after centrifugation for humans. P. Durand, Kuszyncki, and Hohenadel showed the same results from the skin biopsies of MSF patients.

Brumpt described the infectious agent in tick samples and gave the name *R. conorii* in honor of the work of Conor (**Figure 6**). *R. conorii* is transmitted by biting, through tick's feces, or by projection of the liquid contents of the ticks on the mucous membrane conjunctivae. The transmission by ingestion as well as the possibility of infection through respiration of environmental pollutants containing Rickettsiae from ticks feces was already noted.

In 1978, Philip by using mouse serotyping demonstrated that Malish, Moroccan, and Kenyan *R. conorii* isolates were closely related antigenically to ITTR and that they belonged to a single serotype. Later, it was demonstrated that AFR differed from both ISFR and *R. conorii* in terms of SDS-PAGE mobility, PFGE profiles, and PCR RFLP, but using a combination of genotypic criteria, the differences were too faint to classify as new species. Among the 39 isolates or tick amplicons studied recently, four multilocus sequence typing (MLST) genotypes were identified; furthermore, they were also classified into four types using multi-spacer typing (MST) genotyping as well as mouse serotyping. Then, four subspecies were reported: *R. conorii* subspecies *conorii* (type strain Malish, ATCC VR-613, formerly MSF), *R. conorii* subspecies *indica* (type strain ATCC VR-597, formerly ITTR), *R. conorii* subspecies *caspia* (type strain A-167, formerly, Astrakhan fever rickettsia), and *R. conorii* subspecies *israelensis* (type strain ISTT CDC1, formerly ISFR). Complete genome sequence determined the 1 268 755 nt of *R. conorii conorii*, containing 1374 ORFs.

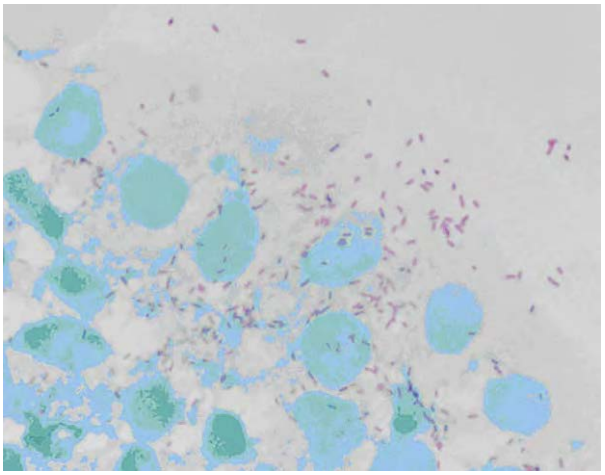

Figure 6 *Rickettsia conorii* detected in hemolymph of infected *Rhipicephalus sanguineus* adult ticks by using Gimenez staining.

Vectors History

Several years after the description of the first case by Conor, the researchers tried to identify the vector of disease. Netter and Olmer proposed a red Japanese mite, *Thrombidium akamushi*. After the discovery of '*tache noire*', an eschar of inoculation of an infectious agent, all researchers believed in the existence of a vector for the disease. Olmer (1928) thought that the vector of the disease was the brown dog tick, a hypothesis confirmed by P. Durand and E. Conseil, who inoculated to humans the crushed infected *Rh. sanguineus* ticks; the patients contracted MSF (**Figure 7**). In 1932, it was shown that the different stages of ticks and over winter unfed males and females could act as vectors of MSF. It was also shown that when eggs or larvae obtained from infected *Rh. sanguineus* females were crushed and used to inoculate humans, the patients contracted MSF. Later, the transtadial passage and transovarial transfer to at least some eggs in the next generation were demonstrated. Ranque supposed that the *Rh. sanguineus* retained the *R. conorii* for at least five or six generations. The reproduction and maintenance of *R. conorii* in tick populations may be related largely to the environment of the tick than of the host. However, the transovarial transmission rate, or the filial infection rate, was not proved yet. Later, a negative effect of *R. conorii* on *Rh. sanguineus* ticks was shown in experimentally infected ticks, which may explain the low prevalence of infected ticks in nature.

No animal reservoir of *R. conorii* has been definitely identified, but A. Raybaud (1929) accused the rabbits, hares, weasels, and fox; Godlewski thought that the herds transhumant that return from the Alps could import in the suburbs of Avignon their ticks and spotted fever; Brumpt suspected the dog, but also various wild animals, cats, rabbits, the rodents, or insectivorous; and Plazy, Marcon, and Carboni suspected the rats. In 1930, Durand infected two 6-week-old puppies with an emulsion of *Rh. sanguineus* ticks collected in Tunis, and no clinical symptoms were observed. The administration of blood collected from these puppies (10 days postinfection) caused typical symptoms of MSF in human subjects. Laboratory experiments found that the dog, rabbit, guinea pig, and rat were not susceptible to infection. Swiss mice, Hartley guinea pigs, spermophile (*Citillus citillus*), and the chimpanzee are susceptible. In 1932, the European rabbit *Oryctolagus cuniculus* was considered a possible reservoir. In 1940, Violle and Joyeux isolated *R. conori* by inoculation of wild rabbit's blood, brain suspension, and crashed ticks into guinea pigs. Furthermore, in 1952, myxomatosis destroyed almost all the rabbits in southern France. Immediately thereafter, there was a spectacular drop in the incidence of MSF, and the reappearance of wild rabbits several years later was followed by an increased incidence of MSF. The role of small rodents such as *Pitymys duodecimcostatus*, living in rabbit's burrows, was suspected to be involved in the life cycle of *R. conorii*; however, this was never confirmed. Later, *R. conorii* was isolated from rats and mice by inoculation of spleen and brain material into guinea pigs. Each of these rodents is a characteristic host of immature *Haemaphysalis leachi* and *Rhipicephalus simus* ticks in Kenya. Recently, some ticks collected on a hedgehog were tested positive for *R. conorii*, but their role in transmission is not proved yet.

Diagnosis and Treatment: Historical Aspects

Diagnosis: The first keys for diagnosis were the clinical presentation with generalized maculopapular rash that often involves the palms and soles and the black eschar '*tache noire*'. The diagnosis may be established by immunofluorescent detection, latex agglutination, enzyme immunoassay, Western blot, compliment fixation, immunohistologic demonstration of *R. conorii* in skin biopsy, isolation in shell vial cell culture, and PCR can be applied to blood or skin biopsy. Recently, diagnostic criteria havè been proposed to help physicians in diagnostic MSF and other rickettsial diseases.

Treatment: In 1949, Janbon and in 1950, Fouquet and Morin investigated chloramphenicol as treatment for MSF. Actually, the treatment of choice for MSF is 200 mg of doxycycline, a single dose, although for severe forms, it should be administered until the patient becomes afebrile.

Figure 7 *Amblyomma variegatum* (female left) and male (right) adults, nonengorged implicated in the transmission of *Rickettsia africae*.

African Tick Bite Fever

African tick bite fever (ATBF) is due to *Rickettsia africae* transmitted by *Amblyomma* ticks in rural sub-Saharan Africa and the French West Indies. Some reports indicate that ATBF pose a significant problem to local populations. During Zimbabwean war of independence in the late 1970s, army medical authorities reported several thousand cases of tick typhus. Both European and African soldiers were affected, although the infection rate seemed to be greater among the former. In Zimbabwe, the incidence rates of ATBF was 60–80 cases per 100 000 patients each year in areas where *Amblyomma* tick was endemic. Whereas reports on ATBF in indigenous populations are scarce, the number of reported cases has recently increased in travelers from Europe and elsewhere.

The clinical course typically comprises an abrupt onset of fever, nausea, headache, and neck myalgia beginning 5–10 days after a tick bite. Most patients develop an inoculation eschar at the site of the tick bite and up to 54% of patients have multiple eschars, a pathognomonical clinical sign. A painful regional lymphangitis is common and may be seen in the absence of a franc eschar. A generalized cutaneous rash, sometimes vesicular and usually best seen close to the eschar, is present in 15–46% of the patients. Less frequent clinical signs of ATBF include aphtous stomatitis and arthralgia. Complications are rarely seen, but it was reported in one patient presenting long lasting fever, reactive arthritis, subacute cranial or peripheral neuropathy, chronic fatigue, neuropsychiatric symptoms, myocarditis, and there are no other known fatal cases. ATBF should be considered along with malaria and other tropical fevers in the differential diagnosis of all febriles returning from the tropics.

Historical Aspects of African Tick Bite Fever Disease

ATBF is probably a very old disease in sub-Saharan Africa, which for several decades has been confused with the MSF. The first human case was described in 1911 by McNaught in Mozambique and South Africa, and by Sant'Anna and Nutall in Angola and Kenya (**Table 10**). They named this disease as tick bite fever because it was transmitted by ticks. In 1929, Béros and Belozet suggested that this disease was the same as MSF. During the 1930s, Pijper, a pathologist working in Pretoria, described the distinct epidemiology and the clinical features of ATBF, isolated a causative organism from ticks, and concluded that ATBF was a separate disease from MSF (which at the time was known to be caused by *R. conorii*). Pijper noted that the vector of the disease was cattle ticks; the clinical manifestation was milder, less illness period with few complications, rare cutaneous rash, and no fatality case. However, subsequent experiments by Gear failed to confirm these findings and he reported the infection of *R. conorii*. For the next 52 years, ATBF were erroneously considered as MSF, which was also present in the same geographic areas, and the observed differences in clinical presentation and epidemiology were attributed to different host factors, such as age and risk behavior.

The final advancement came several decades later; in 1992, a 36-year-old woman presented a history of tick bite behind the right ear, high temperature, and a severe headache. The skin at the bite was erythematous and she had regional lymphadenopathy. The bacterium was isolated and molecular tools confirm that it was distinct of MSF's agent. The authors proposed the name ATBF for the disease. Since then, there have been several reports describing the clinical and epidemiologic features of a number of patients with laboratory-confirmed ATBF.

The Infectious Agent: *Rickettsia africae*

In 1930, Pijper isolated for the first time the causative agent of ATBF in guinea pigs and showed the difference with *R. conorii* and *R. rickettsii* by using cross-protection studies. He described the clinical signs of experimentally infected guinea pigs and the difference with other rickettsial infection. Unfortunately, his isolate was lost. Several years later, different strains from South Africa, Kenya, and Northwest Africa were tested by the toxin-neutralization test in mice, serological and cross-protection tests, but have shown no significant differences with *R. conorii*. Extensive experiences with rickettsial agents and their clinical manifestations have led American and Russian specialists to agree in considering *R. conorii* as a single African rickettsial species and MSF as a single clinical entity. The French school still regards this subject differently.

SFG rickettsiae other than *R. conorii* from Africa were reported from *Amblyomma* ticks in Ethiopia and from *Amblyomma hebraeum* in Zimbabwe. These strains were indistinguishable through serological tools, but distinct from *R. conorii*. Finally, in 1992, one rickettsial agent was isolated from a woman presenting a typical ATBF, and shown to be distinct from *R. conorii* but identical with rickettsial strains isolated in *Amblyomma* ticks collected in Etiopia and Zimbabwe. The authors proposed the name *R. africae*, nomenclatures that were made official in 1996. The genome of *R. africae* consists of a circular chromosome of 1.2 Mb.

Table 10 Milestones of African tick bite fever

Discovery: African tick-bite fever: ***Rickettsia africae***	*Author*
1911 The first human cases	McNaught, Sant'Anna JF
1930 Isolation of *R. africae* and description of the disease	Pijper
1990 Isolation of *R. africae*	Kelly
1992 The name of diseases and of bacteria	Kelly and Raoult
1996 Official nomenclatures	Kelly and Raoult
2007 Genome sequence	Raoult D

Tick Vector

Sant'Anna and Nutall described, in 1911, one new disease transmitted by *A. hebraeum* ticks, especially in places frequented by cattle in Mozambique. Later in South Africa, Pijper described tick bite fever as a rural disease occurring in people having contact with cattle tick. He showed that *A. hebraeum*, *Rhipicephalus appendiculatus*, and *Boofilus decoloratus* ticks are able to transmit the infectious agent. In the following years, Rickettsial agent was reported as *R. conorii* from *Amblyomma* ticks. In the light of actual data, these results are amazing; it would be interesting to test by genotyping the old samples for the presence of *R. conorii* or *R. africae*. In 1990, Kelly isolated rickettsial strains from *A. hebraeum* ticks collected in Zimbabwe and demonstrated the transovarial and transovarial transmission of *R. africae* in this tick. Thus, *A. hebraeum* in southern Africa and *A. variegatum* in West, Central, and East Africa and in the Caribbean are the vectors of ATBF and in the same time the reservoir of *R. africae*.

Diagnosis and Treatment

Diagnosis: In addition to the archaic and poorly specific Weil–Felix test, which continues to be used as a first-line test in many countries, immunofluorescence assay is the most widely used microbiological test. More specific serological tests for ATBF include multiple antigen immunofluorescence assay, Western blotting, and crossadsorption assay. Isolation of *R. africae* from cell cultures and antigen detection by immunohistochemistry or PCR are available to such specialized laboratories, and should be attempted in unusual or complicated cases.

Treatment: The treatment described by Sant'Anna in 1911 for a new tick bite disease with a little effect was the aspirine and alkalies given internally, besides purgatives, and an ointment of potassium iodide and iodine applied externally. Currently, then, doxycycline 100 mg twice daily for 7 days or until 48 h after defervescence can be recommended in severe cases of ATBF. Fluoroquinolones may be also beneficial. Patients with mild symptoms may not require any treatment at all.

Other Rickettsioses

A bacterial species reflects the sum of the biological information on a group of closely related strains isolated in pure culture. It is currently considered that strains with more than 3% divergence belong to different species. Specific rules for recognizing, naming, and classifying species have been established, which avoid redundant descriptions and the use of the same name for more than one species. Several prerequisites are necessary before a new bacterial species is validated. The bacteria should be isolated in pure culture and the description should be based on a minimum of five isolates for environmental bacteria. The new species should exhibit both genomic and phenotypic discriminative characteristics. A type strain should be identified for each new species and available to the scientific community through two independent official culture collections. Finally, the new bacterial name should appear in the approved lists of bacterial names.

With the advent of molecular classification, many of the currently accepted *Rickettsia* species were identified genetically before they were cultured in the laboratory. As is the case with other bacteria, Rickettsia could be given Candidatus status if they fulfill the genomic criteria but have to be cultured. A subspecies is the lowest taxonomic rank that is recognized officially. It contains closely related strains that have distinct phenotypic traits, but do not meet the genomic criteria required for classification as a distinct species. The official validation of a subspecies is comparable to that of species.

The rickettsiae designated as human pathogens have been isolated in cell culture and/or have been detected by molecular methods from blood or tissues of patients presenting illnesses clinically compatible with spotted fever rickettsioses and seroconversion using reference laboratory methods. We have briefly described, in **Table 2**, the first clinical description, rickettsia isolation in cell culture, vector discovery, and other historical aspects of human pathogens *R. sibirica sibirica* (Siberian tick typhus), *R. acari* (Rickettsialpox), *R. australis* (Queensland tick typhus), *R. slovaca* (Tick-borne lymphadenopathy (TIBOLA), Dermacentor-borne necrosis–erythema–lymphadenopathy (DEBONEL)), *R. japonica* (Japanese or oriental spotted fever), *R. heilongjiangensis* (Chinese spotted fever or Far-Eastern tick-borne rickettsiosis), *R. honei* (Flinders Island spotted fever), *R. sibirica mongolitimonae* (Lymphangitis-associated rickettsiosis), *R. felis* (Flea-borne spotted fever), *R. aeschlimannii*, *R. massiliae*, *R. parkeri*, *R. raoultii*, *Candidatus Rickettsia marmionii*, Candidatus *Rickettsia kellyi*, and Candidatus *Rickettsia monacensis*.

Many other rickettsiae, not rewarding the pathogenicity criteria, have been indentifed in ticks and other biological niches. The list of rickettsial species of possible or undetermined pathogenicity in humans is provided in **Table 11** with validated species: *R. asiatica*, *R. canadensis*, *R. montanensis*, *R. peacockii*, *R. rhipicephali*, *R. tamurae*, and the rickettsiae classified as Candidatus species.

Conclusion

During more than a century of microbiological studies of rickettsial infections, our knowledge has fabulously progressed through description of clinical features, discovery of infectious agents and their arthropod vectors, and understanding mechanism of transmission, several pathogenic mechanisms of tissue injury, the modern diagnostic tools and a optimal treatment. These arthropod-associated intracellular bacteria are now being recognized in all parts of world. Rickettsial diseases retain several of its mysteries, and continue to provide countless exciting opportunities for rickettsiologists and rickettsiology. High levels of awareness,

Table 11 Rickettsial species of possible or undetermined pathogenicity in humans

R. asiatica	*1993 First isolation from* Ixodes ovatus *ticks in Japan*	
	2006 The name: *R. asiatica*	Fujita et al.
R. bellii	1966 Isolation in embryonated chicken eggs from *D. variabilis*	Unknown
	1983 The name: *R. bellii*	Philip et al.
	2006 Genome sequence	Ogata et al.
R. canadensis	1967 Isolation of bacteria from the *Haemaphysali leporispalustris tick* and naming of rickettsia	McKiel et al.
	1970 Serological evidence	Bozeman et al.
	2007 Genome sequence	Eremeeva et al.
R. montanensis	1963 First isolation from *D. variabilis* and *D. andersoni*	Bell et al.
R. peacockii	1925 Rickettsia-like organism in *D. andersoni* tick smears	Parker and Spencer
	1997 The name of rickettsia after molecular identification in *D. andersoni ticks*	Niebylski et al.
	2001 Isolation from *D. andersoni tick*	Simser et al.
R. rhipicephali	1975 Isolation from *Rh. sanguineus ticks*	Burgdorfer et al.
	1978 The name of rickettsia	Burgdorfer et al.
R. tamurae	1993 Isolation from *A. testudinarium* ticks	Fujita et al.
	2006 The name of rickettsia	Fournier et al.
	2006 Serological evidence	Phongmany et al.
Candidatus Rickettsia amblyommii	1974 First isolation of *R. ambliomii* but lost	Pretzman et al.
	2000 Serological evidence	Dasch et al.
	2007 Isolation in cell culture	Labruna et al.
Candidatus Rickettsia andeanae	2002 Detection by molecular tools in *A. maculatum* and *I. boliviensis*	Blair et al.
	2005 Phylogenetic analysis	Jiang et al.
Candidatus Rickettsia tarasevichiae	2002 Detection by molecular tools in *I. persulcatus*	Shpynov et al.
	2005 Isolation from ticks	Shpynov et al.

including careful history taking and through physical and laboratory examinations by primary physicians along with the use of contemporary methods based on molecular biology techniques, have facilitated the discovery and description of all emerging human rickettsioses and will undoubtedly lead to the discovery of new tick-borne rickettsial diseases around the world.

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Unusual Infectious Agents

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Introduction

Infections are caused by infectious biological agents spreading between organisms, see Fig. 1. Infections may be harmless, beneficial or cause disease. Cell-based agents can be defined as having their genetic information and ribosomes bounded by a lipid membrane. Cellular agents are either Archaea, Bacteria or Eukaryote, not all are infectious but some of each group are. These would not be described as unusual infectious agents. I will, however, make several observations about them that I think are unusual. Firstly, the Archaea that infect human beings are not yet clearly associated with the development of any disease-why not? This is an observation that may change in the future with the use of enhanced genomic sequencing techniques. Secondly, the process of infection and disease can be complicated. Many infection or disease outcomes are complicated by co-infections, host genetics and environmental factors. Two examples: (1) Epstein Barr virus EBV, infects most humans, sometimes causing disease such as infectious mononucleosis (glandular fever). *Plasmodium falciparum* infection invariably causes malaria. Co-infection with *P. falciparum* and EBV can cause Burkitt's lymphoma. (2) Eukaryotic filarial nematode infections are associated with onchocerciasis or river blindness and lymphatic filariasis or elephantitis. However, much of the inflammatory pathogenesis is probably due to the immune reponse to an intracellular bacteria of the *Wolbachia* genus infecting the nematode rather than to the nematode itself. Finally some cancer cells are infectious and able to spread the cancer between host organisms, a point I will expand on later.

Virus particles are not cell-based, containing neither ribosomes nor necessarily a lipid membrane. The defining feature of a virus is that the genetic information is bounded by a protein layer called a capsid. A virus has to infect a cell that can provide the energy and molecules necessary for the synthesis of new virus particles. Some viruses are associated with unusual infectious agents called satellites or satellite viruses (Fig. 1). These satellite agents require co-infection with a helper virus to facilitate replication. Satellites can have DNA or RNA genomes, the virusoid subgroup have circular single stranded RNA genomes. Viroids are small replicating RNA molecules that directly infect other cells. Prions and prionoids are infectious proteins. Prionoids are able to spread between cells and prions between cells and organisms. This chapter summarizes our understanding as of early 2018 of these various agents.

Sub-Viral Agents: Dependoviruses, Satellites, Satellite-Like Nucleic Acids, Virusoids and Virus-Dependent Nucleic Acids

There is a seeming continuum of infectious agents from viruses through to replicating nucleic acids that all require co-infection with another virus for transmission. Transmission is via a virus-like particle which may contain copies of both the virus and sub-viral genome or just the sub-viral genome (Fig. 1). The virus-like particle may be formed by either the helper virus, the satellite virus or be a chimera of both. The international convention on the taxonomy of viruses or ICTV states that the distinctions between these agents is subtle and not always straightforward. Indeed they were not subject to formal classification in the most recent 2012 classification. It was noted that some satellite agents were also anomalously classified as a virus. For example, virus particles classified in the *Dependovirus* genus and *Deltavirus* genus (discussed later) meet all the definitions of a satellite virus. Thus, the adeno-associated satellite virus AAV, classified as a Dependovirus, require cells to be coinfected with one of several other viruses for replication such as an adenovirus. The co-infecting helper virus is essential for AAV virus particle formation but does not form part of the virus capsid (Fig. 1A).

Satellites are subviral DNA or RNA agents, double and single stranded, that lack gene functions required for their replication and require co-infection with a helper virus for multiplication. Satellite viruses encode structural proteins that package the satellite genome which is then incorporated into the helper virus particle such as chronic bee-paralysis-associated satellite virus (Fig. 1B), the helper virus is CBPV. Satellite nucleic acids do not encode structural proteins, they may not encode any proteins, the genomes are necessarily incorporated into the helper virus particle, for example, artichoke mottled crinkle virus satellite RNA (Fig. 1C), the helper virus belongs to the Tombusviridae. Some viruses are "defective" and replication requires the presence of a small nucleic acid

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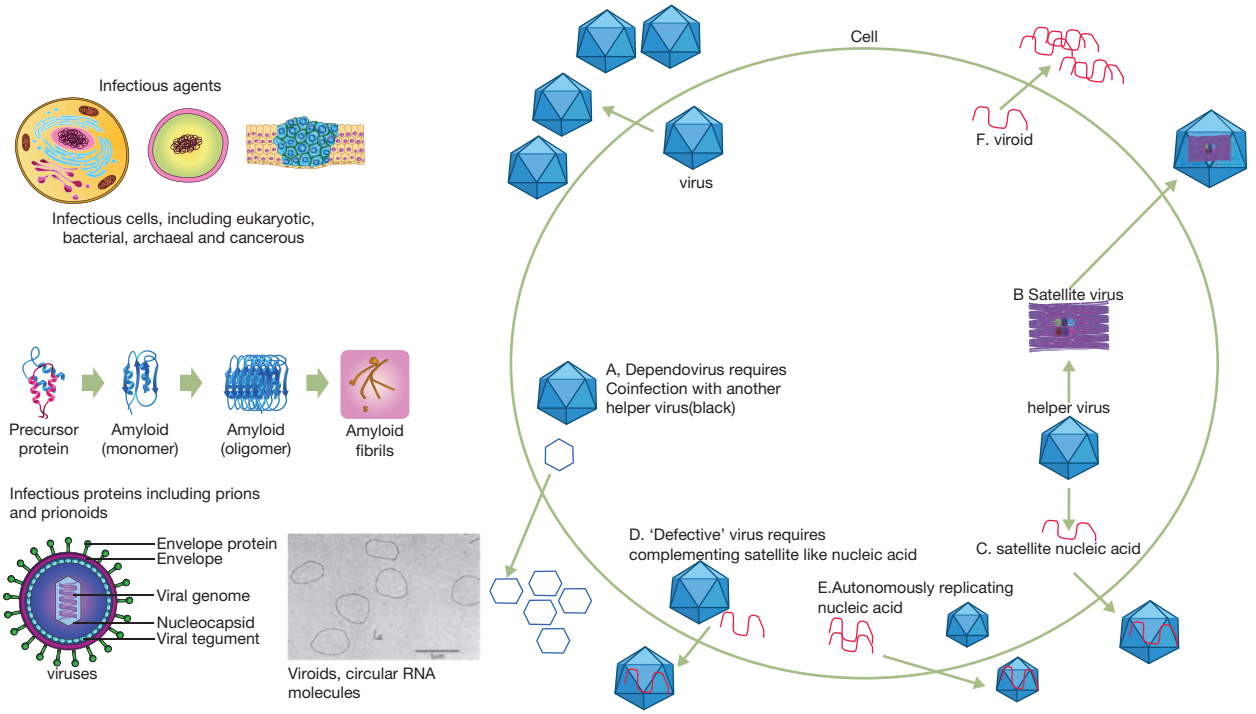

Fig. 1 Left hand panel, infectious cells can be bacterial, archaeal, eukaryotic and even cancerous. Prions, viruses and viroids can also be directly infectious. Right hand panel, shows a cell infected with a replication competent virus and viroid (topLH and RH side, respectively. Also shown are various sub-viral agents: A—dependo virus (red) requiring another virus to replicate. B—Satellite virus encoding a structural capsid protein C—satellite nucleic acid not encoding a structural protein dependent upon virus for replication. D—Defective virus requiring a complementing satellite-like nucleic acid. E—Autonomously replicating nucleic acid or satellite requiring a virus for transmission. A satellite virus encodes proteins that package the genome but still require a helper virus. If the satellite virus or nucleic acid has a circular RNA genome it is called a virusoid. See text for details.

~1–1.5 kb in size that rescues or "complements" the defective virus by supplying an essential biochemical function. These small nucleic acids are called "satellite-like nucleic acids." Examples include RNA associated with members of the *Umbravirus* genus and DNA associated with members of the *Begomovirus* genus (Fig. 1D), which together infect a wide variety of plants. Other nucleic acids are capable of autonomous replication, so strictly speaking are not satellites but they require a helper virus for transmission such as the alphasatellite DNAs also transmitted by members of the Begomovirus and the *Polerovirus* associated RNAs, Fig. 1E.

Virusoids have no formal status in the ICTV classification, but the term is often used. They are satellite nucleic acids with a small, ~220–388 circular single stranded RNA genome (Fig. 1B or C). Replication occurs in the cytoplasm. Many examples infecting plants are known such as tobacco ringspot virus satellite RNA and the required helper tobacco ringspot virus. Coinfection of a cell with virus and satellite agent may increase or decrease pathogenicity or have no effect upon it.

Hepatitis D virus, HDV is a well known human satellite virus (Fig. 1B). HDV is also classified as a *Deltavirus* by the ICTV. The essential helper virus is Hepatitis B, HBV. Coinfections with HDV and HBV are usually resolved quickly. Superinfection, where an HBV-infected individual is then also infected with HDV, often leads to a chronic infection associated with a virulent more aggressive form of hepatitis. The genomic circular single stranded RNA genome of HDV is about 1679 nucleotides in size. A ribozyme is active in replication of both the genome and antigenome strands. Replication may resemble the symmetric pathway for viroids described in Fig. 2 and below. The circular antigenome strand made during replication gives rise to a mRNA, which encodes a protein called the delta antigen. A small, S form of this protein is necessary for genome replication. RNA editing, later in infection, results in a large, L form being made and a shift from genome replication to genome packaging. The L form contains a prenylation site which directs the genome to assembling HBV particles. This ensures the HDV genome associated with about 70 copies of the S and L delta antigen is packaged into a HBV like particles, displacing the HBV genome. HDV replication has features that resemble both a viroid (see below) and a satellite virus.

The term "virophage" has been used (but is not generally accepted) to describe subviral agents *co*-infecting virus infected amoebal cells such as Sputnik virus that infects *Acanthamoeba polyphaga* cells infected with the *Mamavirus*. These agents are currently classified by the ICTV as satellite viruses replicating as shown in 1A or B.

Viroids

Viroids are widely spread infectious agents of many plant species. They were first described in 1971 by T. O. Diener studying an infectious disease of potatoes described some 50 years previously, for which no infectious agent had been identified. Subsequently

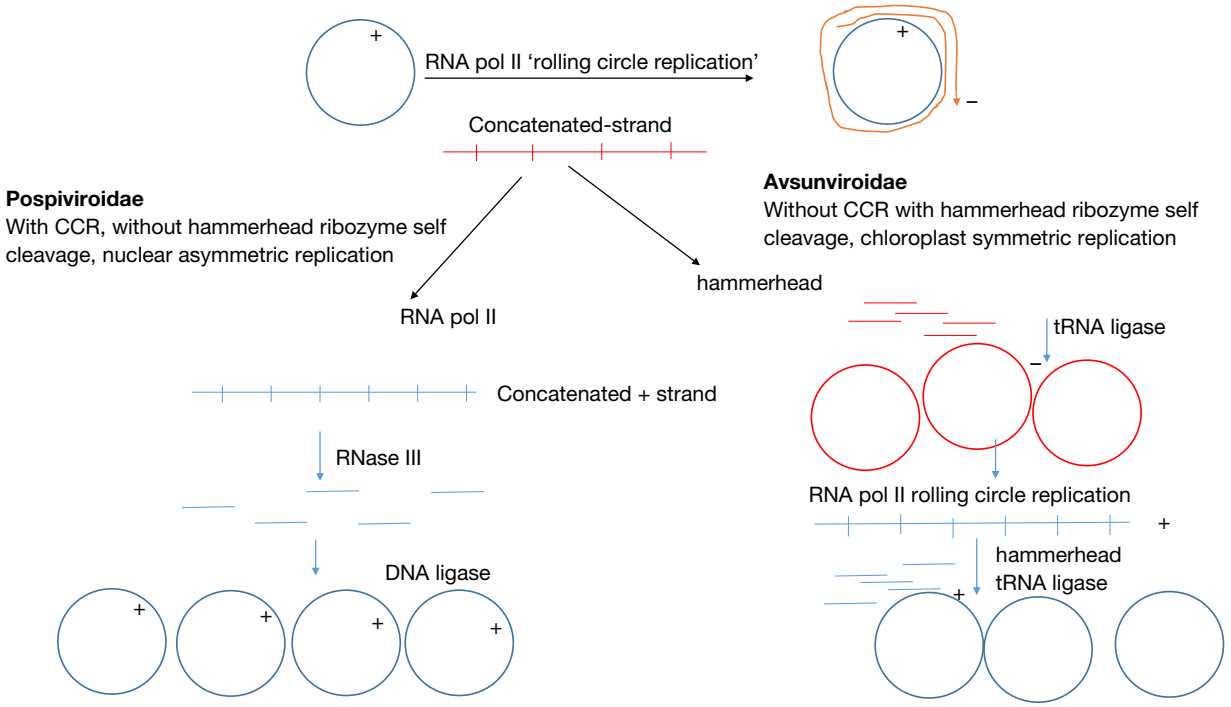

Fig. 2 Viroid replication, see text for more details.

more than 30 different types of viroid have been identified infecting a wide range of other crops including tomatoes, avocados, peach, apple, hops and coconuts. They can impose a significant economic burden on the horticultural and agricultural sectors of the economy. Viroids are composed entirely of RNA, being small, ~246–401 nucleotides in size, and forming closed circular, partially base paired rod-like molecules (Fig. 1). The RNA genome encodes no proteins. Viroids are infectious RNA molecules capable of moving from cell to cell and plant to plant. They move between plant cells via the plasmodesmata and within the plant via the phloem system. Unlike satellite agents they do not require the presence of other infectious agents for their spread. They can be spread via seed, pollen, vegetative propagation, contaminated horticultural implements, water and insects. They can interact with host cell proteins causing cellular pathology such as an RNA-activated protein kinase that phosphorylates other plant proteins disrupting protein synthesis. Cell protein expression can also be disrupted by smaller "silencing" siRNAs formed by cleavage of double stranded regions of the viroid genome.

Viroids are divided into two major groups (Fig. 2). The biggest group are the *Pospiviroidae*, type species Potato spindle tuber viroid. These replicate in the nucleus of the cell. The smaller group are the *Avsunviroidae*, type species Avocado sunblotch viroid. These replicate in the chloroplasts of cells. The pospiviroids contain a conserved central region or CCR, the function of this is not clear but it is presumably essential for the replication of this group. The avsunviroids do not contain the CCR, but contain a particular type of ribozyme called a hammerhead. Ribozymes are catalytic RNA molecules; the hammerhead ribozyme cleaves RNA and is essential for the replication of this class of viroid. Viroids of both classes require three catalytic activities for replication; a polymerase, nuclease and ligase (Fig. 2). A cell RNA polymerase class II activity makes the viroid RNA genomes, this enzyme is located in both the cell nucleus and chloroplast. An RNAse activity generates genome-sized units from a concatenated genome; pospiviroids use a nuclear RNAse III activity, avsunviroids are cleaved by the hammerhead ribozyme activity present in both the positive (+) and negative (−) strands of the genome. Pospiviroids use a nuclear DNA ligase activity and avsunviroids a tRNA ligase activity to ligate and circularize the genomes. Note that the RNA polymerase and DNA ligase enzymes usually function with DNA as a template but are also able to use a viroid RNA template.

The replication of viroid genomes is shown in Fig. 2. By convention the infectious strand is described as positive (+). The + strand genome in blue is copied by RNA polymerase II in a "rolling circle" mechanism resulting in a long strand of RNA in the negative (−) sense having many copies (concatemers) of the genome in red. In the avsunviroidae group the concatemers are self cleaved by the hammerhead ribozyme activity to form monomeric linear negative strands red which are ligated by the tRNA ligase activity to form circular—antisense copies of the genome. These serve as a template for another round of rolling circle replication to form viroid concatemers in the positive sense (blue), which are cleaved by the ribozyme, also active in the + sense genome and ligated by the tRNA ligase activity (blue circles). Because both the negative and positive strands are formed in the same way via a rolling circle this is described as symmetric replication. The pospiviroid group start their replication in exactly the same way as the avsunviroids. The + strand genome is copied by RNA polymerase II in a "rolling circle" mechanism resulting in a long concatemer strand of RNA in the – sense (red). RNA pol II then copies the concatenated—sense RNA to form a concatenated + sense RNA, blue.

These are cleaved to genomic size by RNAse III and ligated to form the infectious circular form using DNA ligase. Because the + strand (blue) concatemers and genomes are not formed by rolling circle replication (unlike the – red strand) this is called asymmetric replication (Fig. 2).

The origins of these various subviral particles is not known with any certainty. One theory postulates that they originate from a prebiotic world populated with replicating RNA molecules. These eventually adapted to replicating in cells, the viroids perhaps? The differences in structure and replication between the two viroid groups indicate different evolutionary origins. It has also been proposed that they have evolved from the diverse cellular pool of small RNA molecules that is produced by normal gene expression and RNA transcription. In the case of the other subviral agents, some replication-competent viruses may have lost functionality and became replication-incompetent. Their continued viability then depended upon co-infection with helper viruses. Most satellites share some common sequences with their helper viruses, particularly at the termini of the genome but lack substantial similarity elsewhere. Other agents may have arisen from the cellular pool of viral and cellular nucleic acids. Complementing RNAs and possibly chimera formation between viral genome fragments and other cellular RNAs with subsequent selection for forms able to replicate and transmit may have resulted in the rich (and perhaps confusing) variety of infectious agents described above.

Prions and Prionoids

In the late 1950s a new disease called kuru was identified in the Fore region of New Guinea. It was characterized by a progressive loss of control of nervous function including movement and mental function resulting in death within a year or so of symptoms appearing. It was quickly realized that ritual cannabilism of dead tribal members was responsible for transmitting the disease. Within a few years it was established that kuru was similar to a number of neurological diseases in humans including Creutzfeld-Jacob disease or CJD, Gurstmann-Sträussler-Scheinker syndrome or GSS and also in animals such as scrapie in sheep. Observation and experiments showed that these diseases could appear sporadically including CJD, or be infectious such as scrapie, CJD, kuru, or be inherited as for GSS and a minority of cases of CJD. More recently other examples of this class of disease including BSE in cows, CWD in mule-deer and elk, FFI and v-CJD in humans have been identified. It has also been recognized that these diseases can be spread iatrogenically, that is, by medical procedures due either to the use of contaminated instruments or the transfer of infected tissue grafts such as cornea, dura mata, blood products and hormones. Together this family of diseases are often called transmissible spongiform encephalopathies.

At first, the transmissible agent was thought to be a virus. It existed as various strains that differed in pathogenic properties. These pathogenic properties were inherited as the strain passed from animal to animal. However, its biological features were very different to any other infectious agent then described. It was extremely resistant to heat, radiation, and other chemical procedures which were known to destroy RNA, DNA, protein and other infectious agents such as bacteria, viruses and viroids. Its resistance to radiation implied that any nucleic acid genome associated with the infectious agent could only be about 60 nucleotides in size. This led to the bold suggestion by J. S Griffith that the infectious agent was a protein, now named a prion. This idea was not immediately accepted because the concept of an infectious protein was new and it was not apparent how an infectious protein could exhibit the strain properties described above. However research by many scientists, especially S. Prusiner, over the following decades proved beyond a reasonable doubt that the the infectious agent comprises a protein made by the host.

The prion was shown to be a form of a cellular (shown as a small superscript^c) protein called PrP^c. PrP^c exists as a protease-sensitive form in normal cells. It is translated into the endoplamic reticulum and undergoes post-translational chemical modifications and transport to the extracellular face of the outer cytoplasmic membrane where it resides. It can also be endocytosed back into the cell. It is not absolutely essential for cell function because *PrP* gene knockout animals which do not make PrP^c are viable. Importantly these animals cannot be infected with or propagate prions, demonstrating the importance of PrP to infectivity. Although nucleic acids were associated with prions purified from animals, none could be shown to be specifically associated

Fig. 3 Tasmanian devil with transmissible facial tumor.

with the prion. Nor could any specific chemical modification be associated with the prion. This led to the suggestion that the difference between normal cellular PrP^c protein and the prion was in their conformation.

PrP^c is known to fold into a structure which is predominantly alpha-helical one clear difference between many uninfected and infected subjects was the appearance of an amyloid deposit of protein in the brain and other tissues. A smaller N-terminal truncated form of PrP is the major component of this amyloid and in this form is known as PrP^{sc} (the superscript^{sc} is for scrapie). Many proteins are known to form amyloid and amyloids are characterized by having a similar β-sheet folded fibrillary structure. This suggests that PrP can adopt two stable conformations, one predominantly α-helical and one predominantly β-sheet. It is this β-sheet form which is the disease-causing prion and a truncated form of it accumulates as the amyloid PrP^{sc}. It is proposed that PrP^c interacts with the infectious prion and this interaction results in PrP^c changing its conformation from the α-helical form to the infectious β-sheet prion form. Prions thus accumulate exponentially in infected tissues due to the continued misfolding of PrP. This eventually leads to pathogenesis, disease manifestation and the appearance of tissue amyloid protein deposit.

In recent years the prion hypothesis- namely that the infectious agent is a distinctly folded form of the PrP^c protein has been proven beyond reasonable doubt. PrP protein expressed in bacteria in association with a chemically synthesized RNA and lipid is able to form a prion and cause transmissible prion disease in animals in strain specific fashions. PrP is thought to fold in a number of subtle distinct conformations that give rise to different strain characteristics. Whether the lipid and RNA molecule form part of the prion or are just required for its formation is not known.

This understanding of the nature of a prion leads to an explanation for inherited, sporadic and transmissible forms of the disease. Sporadic cases may arise because the conformation of PrP^c can change to the prion form spontaneously. If it does so exponential amplification of the prion leads to disease. This happens only rarely and usually manifests itself in older individuals, perhaps because the ability to prevent prion replication naturally declines with age. In transmitted cases the infectious dose of prions is high enough to overcome any normal resistance to prion amplification. In inherited cases, mutations in the PrP gene manifested in an altered protein structure either increase the rate of prion formation or susceptibility to prion infection from dietary or environmental sources, such that disease is an inevitability.

The development of the prion hypothesis has led to the recognition that it may apply to other proteins. Thus prions involving different conformations of a protein with the dominant prion conformation conferring distinct and transmissible traits has been recognized in yeasts, fungi, and other organisms. Amyloid A, AA, amyloidosis is a transmissible disease in captive cheetahs and other animals and birds and can thus be considered a prion disease. AA amyloidosis occurs in humans but it is not known if it is also infectious.

Many other human diseases are associated with the formation of amyloid deposits containing host proteins. The process is thought to be related to the disease manifestation. Examples include Alzheimer's with two associated amyloids, β-amyloid and tau and Parkinson's associated with α-synuclein amyloid. These diseases are not known to be infectious but there is increasing concern that under some circumstances they may be. For instance α-synuclein amyloid and Parkinson's type symptoms has been shown to be transmissible in a mouse model. β-amyloid associated with Alzheimer's can be transmitted from cell to cell. Evidence of the transmission of the β-amyloid pathology due to childhood neurosurgery or the receipt of growth hormone injections has recently been described, and associated with development of early cerebral amyloid angiopathy but not yet with Alzheimer's.

The term prionoid has been coined to describe cellular proteins able to change from one conformation to an amyloid structure and transmit this change in conformation to other homologous proteins and from cell to cell. It has been suggested that prionoid functions may normally exert another level of control in cell physiology which when aberrant may lead to disease. Thus, a prionoid transmits a change in protein conformation from cell to cell within a host leading to disease. A prion acts in exactly the same way but can additionally be transmitted between hosts.

Infectious Cancer Cells

Cancer in humans is associated with mutations in the genome which disrupt cellular growth control mechanisms leading to uncontrolled cell proliferation and the spread of tumor cells throughout the individual. A cancer is usually derived from one ancestral cell and said to be clonal. It is well established that many cancers of humans and animals are caused by infections. Thus, for instance, cervical cancer is associated with the sexual transmission of some strains of the human papilloma virus. More recently, it has been suggested that the bacterium *Helicobacter pylori* may also be implicated in the development of stomach cancers. The infectious agent results in increased cell growth, genomic mutations and eventually a tumor.

Less well known is that cancer cells themselves can also be infectious. Cases of cancer transmission from mother to baby whilst in the uterus and from organ donor to organ recipient are known to occur. There is also the report of the transmission of a cancer from a patient to a surgeon, following a cut with the operating scalpel. The patient sarcoma was shown to be genetically identical to the tumor on the surgeons hand, which was removed. Such cases involve the direct transmission of cancer cells from one individual to another when in intimate contact. These cancer cells then manage to avoid the immune system in the newly inoculated and infected individual and go on to establish new tumors.

However, we now know that cancer cells are more commonly transmissible than had been thought. A canine transmissible venereal tumor, CTVT, not usually fatal and spread during coitus, has been estimated to have been established in the dog population for 11,000 years. It is found on five continents and causes growths on the genitals of affected dogs. Also a recently recognized fatal tumor of the face and mouth of the Tasmanian devil, called devil facial tumor disease, or DFTD, is transmitted during eating and fighting behaviors (see Fig. 3). Indications are that DFTD first occurred in a female in the 1990s. A second distinct

form of DFTD also appears to have recently arisen in a male. The fact that infectious cancers have arisen twice in the Tasmanian devil in such a short period of time may indicate that transmissible cancers frequently occur. CTVT and DFTD cancers are being spread by cells transmitted from one individual to another. It has been shown by genome sequencing that all three tumor types are essentially identical to each other that is, derived from three different clonal tumor cells. Genome sequencing shows that these cells have accumulated many mutations which together allow them to avoid the anti-tumor actions of the host immune system. These mutations include down regulation of the MHC cell surface receptors, which presumably prevent the immune system from destroying the cancer cells. In the case of the Tasmanian devil a lack of genetic diversity in the animals may also have facilitated the spread of the tumor.

Even more recently, five different clonal transmissible cancers have been found to spreading in at least 15 marine bivalve populations species such as soft cell clams *Mya arenaria*, the mussel *Mytilus trossulus* and the clam *Cerastoderma edule*. They have collectively been called bivalve transmissible neoplasias, BTN. Cases have been reported in the Pacific and Atlantic. In one case the neoplasia has spread from one species of clam *Venerupsis corrugata* to another species of clam *Polititatus aureus*. Cancer cells are presumably spreading via the water.

Other metazoan animals and parasites also develop cancers. Parasite infections are often associated with suppression of the host immune system which in turn allows cancer to develop Recently a fatal case of a histologically unusual tumor in a HIV-1 positive Columbian man living in Medellin was described. He was also infected with the tapeworm *Hymenolepsis nana.* His tumor was widely spread, the cells were atypically small and found to be derived from cells of the tapeworm. Further investigation showed that the cancer cells contained mutations commonly associated with development of the disease. It is possible that immunosuppression in this individual allowed *H. nana* growth and genome mutations to occur, which gave rise to the tapeworm tumor which spread to his organs.

Conclusions

Agents responsible for infections are more diverse than is generally realized. Some diseases we don't currently recognize as being transmissible may be under some circumstances. In addition to the widely known bacterial and eukaryotic infectious agents some Archaea are also infectious. I have described a continuum of sub-cellular infectious agents including viruses, defective viruses, virus satellites and viroids. I have referred to strong evidence that some cancer cells including CTVT, DFTD, and BTNs are infectious agents and that some proteins named prions are also infectious such as PrP^{sc} and AA. It is possible that in the future, we may come to realize that infectious cancer cells and prions are more common than described here. I have also described an exceptional case of a human cancer caused by cancerous tapeworm cells. More surprises on the nature of infectious agents and infections will probably be forthcoming.

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V

Viroids/Virusoids☆

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Glossary

Agroinoculation Use of engineered *Agrobacterium tumefaciens,* infecting certain groups of plants, as a vehicle to transfer foreign DNA into plant cell nuclei for transient gene expression.
Biolistic bombardment A technique in which gold particles coated with foreign DNA or RNA are delivered under pressure into cells for expression.
BY2 cell A cell line derived from *Nicotiana tabacum* cv Bright Yellow 2. It can be propagated easily in an appropriate culture medium. The cells are fast-growing and nearly translucent. They have been extremely useful for studying viral and viroid replication and for studying basic plant cell biology and gene expression.
Phloem A type of vascular tissue consisting of several types of living cells that is responsible for the long-distance transport of nutrients and signaling molecules in plants. It is also used by viroids and many viruses to spread infection within a plant.
Plasmodesmata Cytoplasmic channels between plant cells that allow cell-to-cell diffusion of small molecules and selective trafficking of RNAs, proteins, viruses, and viroids.
Protoplast A plant cell with its cell wall removed by treatment with cellulase and pectinase, enzymes that degrade the major cell wall components cellulose and pectin. Protoplasts are prepared fresh for each experiment from plant materials or cultured cells and have been extremely useful for studying viral and viroid replication and for studying basic plant cell biology and gene expression.
RNA silencing A mechanism of transcriptional and post-transcriptional gene regulation operating in many eukaryotic organisms. It is mediated by 20–26 nt small RNAs produced from various RNA and DNA sources and functions in regulating RNA stability and translation as well as chromatin modification underlying numerous developmental processes. It also plays a significant role in microbe–host interactions.
Viroid A non-protein-coding and non-encapsidated circular RNA that replicates autonomously in certain plants without the assistance of helper viruses. Some are the causal agents of important diseases.
Virusoid A specific group of viroid-like satellite RNAs, associated with sobemoviruses, that are circular and assume similar secondary structures with viroids. They have no protein-coding capacity (with one possible exception), replicate by utilizing helper viral- and host-encoded factors, and are encapsidated by the helper viral coat protein.

Abbreviations

ASSVd	Apple scar skin viroid
ASBVd	Avocado sunblotch viroid
CCR	Central conserved región
CChMVd	Chrysanthemum chlorotic mottle viroid
CEVd	Citrus exocortis viroid
CbVd-1	Coleus blumei viroid 1
CsPP2	Phloem protein 2 from cucumber
HPI and HPII	Hairpin I and II
HSVd	Hop stunt viroid

☆*Change History*: August 2016. Ricardo Flores added Section "Do Viroids and Virusoids Emerged in the RNA World?"and Figs. 6 and 7; updated Table 1 and text throughout the article.

This article is an update of B. Ding, X. Zhong, Viroids/Virusoids, *Encyclopedia of Microbiology* (3rd Edn), edited by Moselio Schaechter, Academic Press, 2009, pp. 535–545.

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LTSV	Lucerne transient streak virus
PLMVd	Peach latent mosaic viroid
Pol II	DNA-dependent RNA polymerase II
PSTVd	Potato spindle tuber viroid
RYMV	Rice yellow mottle virus

Defining Statement

Viroids and virusoids, the smallest replicons that infect plants, comprise single-stranded, circular (and linear) RNAs of the same size not encoding (with one possible exception) any protein. They represent simple models to study how an infectious RNA replicates in a host cell and spreads systemically to cause diseases, and also are excellent models to investigate the basic structure–function relationships of RNAs.

Introduction

Theodor O. Diener is credited with the discovery in 1971 of the first viroid, potato spindle tuber viroid (PSTVd). This viroid is the causal agent of potato spindle tuber disease first described in the 1920s. Extensive research over the past four decades has established viroids as the simplest form of RNA-based infectious agents. All viroids are single-stranded, circular RNAs with sizes ranging approximately from 250 to 400 nucleotides (nt) and with a compact secondary structure due to their extensive self-complementarity. Differing from riboviruses, these RNAs do not have protein-coding capacity and are not encapsidated in a protein or membrane shell, and differing from satellite RNAs, they do not require the presence of a helper virus to establish infection. Thus, the viroid genomes and/or their derivatives contain all the genetic information needed for replication in single cells and systemic trafficking throughout the host plant to establish infection.

Depending on viroid–host combinations, an infected plant may or may not develop disease symptoms. Common viroid disease symptoms include growth stunting, leaf epinasty and deformation, fruit distortion, and stem and leaf necrosis, leading in the most dramatic instances to plant death. Since viroids do not encode proteins, viroid diseases must result from direct interactions between viroid genomic RNAs or their derivatives and specific cellular components.

Virusoids are also circular RNAs similar in size and secondary structure to viroids, with the evidence available indicating that, with the possible exception of the virusoid of rice yellow mottle virus (vRYMV), they also lack protein-coding ability as well. However, they rely on protein factors encoded by their helper viruses and hosts for replication and/or encapsidation. When virusoids were initially described it was unclear if they were satellite RNAs. They are now regarded as a special group of satellite RNAs associated with certain plant viruses.

Recent research has contributed significant insights into the sequence/structural elements in viroids that are critical for various aspects of replication, systemic spread, and disease induction in an infected plant. Knowledge of potential host proteins that assist various stages of viroid infection is also emerging. Much less is known about the biological functions of virusoids. It is proposed that continuing studies on these subviral pathogens should yield valuable insights into the simplest mechanisms of infection in eukaryotic cells and help uncover the basic principles of RNA structure–function relationships.

Viroid Classification and Structure

There are over 30 species of viroids according to the Ninth Report of the International Committee on Taxonomy of Viruses and numerous sequence variants in the current database. They belong to two families, Pospiviroidae and Avsunviroidae, with their type species being PSTVd and avocado sunblotch viroid (ASBVd), respectively. All viroids are listed in Table 1 under their respective families and genera, and with their abbreviations and sizes.

The distinguishing features of the two families of viroids are summarized in Table 2. Most members of the Avsunviroidae adopt a highly branched secondary structure (Fig. 1(a)), show limited sequence and secondary structural conservation among them, and replicate in plastids (mostly chloroplasts) through a symmetric rolling-circle mechanism involving hammerhead ribozymes embedded in the strands of both polarities. Members of the Pospiviroidae, which generally adopt a rod-shaped secondary structure (Fig. 1(b)) and display conserved sequences among some species, replicate in the nucleus through an asymmetric rolling-circle mechanism not involving hammerhead ribozymes. Five broad structural domains are defined in the secondary structures of some members of the Pospiviroidae, particularly in those forming the genus *Pospiviroid:* the left-terminal, pathogenicity, central, which contains a central conserved region (CCR), variable, and right-terminal domains (Fig. 1(b)).

Host Range of Viroids

Viroids may infect one or a few plant species in the field, as illustrated by the Avsunviroidae, or display a variable host range as in the Pospiviroidae, with hop stunt viroid (HSVd) having been detected in a wide array of herbaceous and woody species. There is

Table 1 Taxonomy of viroids[a]

Family	*Genus*	*Species*	*Abbreviation*	*Nucleotides*[b]
Pospiviroidae				
	Pospiviroid	*Chrysanthemum stunt*	CSVd	354–356
		Citrus exocortis	CEVd	368–375 (463–467)
		Columnea latent	CLVd	370–373
		Iresine	IrVd	370
		Pepper chat fruit	PCFVd	348
		Potato spindle tuber	PSTVd	356–361 (341)
		Tomato apical stunt	TASVd	360–363
		Tomato chlorotic dwarf	TCDVd	360
		Tomato planta macho	TPMVd	359–360
	Cocadviroid	*Citrus bark cracking*[c]	CBCVd	284
		Coconut cadang-cadang	CCCVd	246–247 (287–301)
		Coconut tinangaja	CTiVd	254
		Hop latent	HLVd	256
	Hostuviroid	*Hop stunt*	HSVd	294–303
		Dahlia latent viroid[d]	DLVd	342
	Apscaviroid	*Apple dimple fruit*	ADFVd	306,307
		Apple scar skin	ASSVd	329–334
		Australian grapevine	AGVd	369
		Citrus bent leaf	CBLVd	315,318
		Citrus dwarfing[d]	CDVd	294,297
		Citrus viroid V	CVd-V	284
		Citrus viroid VI[e]	CVd-VI	330
		Grapevine yellow speckle 1	GVYSVd 1	366–368
		Grapevine yellow speckle 2[f]	GYSVd 2	363
		Pear blister canker	PBCVd	315,316
	Coleviroid	*Coleus blumei 1*	CbVd 1	248–251
		Coleus blumei 2	CbVd 2	301,302
		Coleus blumei 3	CbVd 3	361–364
Avsunviroidae				
	Avsunviroid	*Avocado sunblotch*	ASBVd	246–251
	Pelamoviroid	*Chrysanthemum chlorotic mottle*	CChMVd	398–401
		Peach latent mosaic	PLMVd	335–339 (348–351)
	Elaviroid	*Eggplant latent*	ELVd	332–335

[a]Classification according to the 9th Report of the International Committee on Taxonomy of Viruses (ICTV) with minor modifications.
[b]Sizes of representative variants; those with insertions/deletions arising in vivo are shown in parenthesis.
[c]Formerly *Citrus viroid IV*.
[d]Formerly *Citrus viroid III*.
[e]Formerly *Citrus viroid* original source.
[f]Formerly Grapevine viroid 1B.

Table 2 Distinct features of Pospiviroidae and Avsunviroidae

	Family	
Features	*Pospiviroidae*	*Avsunviroidae*
Secondary structure	Rod-shaped	Branched for most members
Replication site	Nucleus	Chloroplast
Rolling-circle replication mode	Asymmetric	Symmetric
Catalytic activity mediated by hammerhead ribozymes	No	Yes for all current members
Host range	Variable	Mostly restricted to natural hosts

Source: Reproduced, with modifications, from Ding, B., Itaya, A., 2007. Viroid: A useful model for studying the basic principles of infection and RNA biology. Molecular Plant-Microbe Interactions 20, 7–20.

evidence for the recent expansion of host ranges for some viroids. For instance, PSTVd was long known to infect only potato in the field, but more recently it has been reported infecting also avocado, pepper, and tomato. Under experimental conditions, some viroids can infect more species. For example, PSTVd infects *Nicotiana benthamiana* and some specific variants of this viroid also *N. tabacum*. The infected plants may or may not express noticeable symptoms.

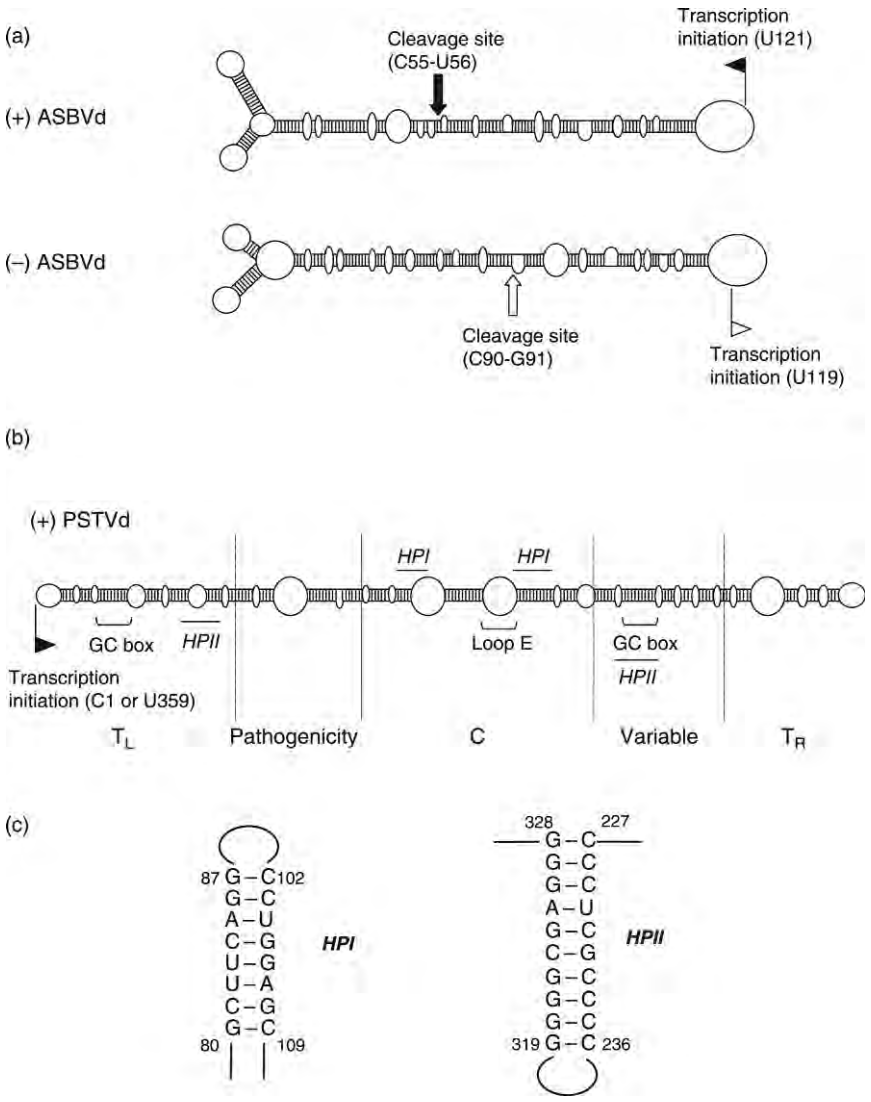

Fig. 1 Secondary structures of ASBVd (a) and PSTVd (b), type members of Avsunviroidae and Pospiviroidae, respectively. Reproduced, with modifications, from Ding, B., Itaya, A., 2007. Viroid: A useful model for studying the basic principles of infection and RNA biology. Molecular Plant-Microbe Interactions 20, 7–20. For PSTVd, the five structural domains are indicated. T_L, left-terminal domain; C, central domain; T_R, right-terminal domain. Arrows indicate the transcription initiation sites and cleavage sites on the viroid genomic RNAs. *HPI* and *HPII* in (b) indicate the positions of nucleotide sequences in PSTVd forming metastable structures hairpin I and hairpin II, which are shown in (c). Reproduced, with modifications, from Zhong, X., Archual, A.J., Amin, A.A., Ding, B., 2008. A genomic map of viroid RNA motifs critical for replication and systemic trafficking. Plant Cell 20, 35–47; www.plantcell.org. Copyright American Society of Plant Biologists.

Recent studies tested if weedy plant species characteristic for potato and hop fields, which are not natural hosts for PSTVd and HSVd, can be potential hosts for transmitting and spreading infection of these viroids, respectively. Indeed, when the leaves of 12 weedy species from a potato field and 14 from a hop field were inoculated with viroid RNAs or cDNAs through biolistic bombardment, many species supported replication of these two viroids. Sequencing revealed the presence of many variants of these viroids in different infected plant species. Therefore, there is always the potential for viroids to invade new species if conditions permit.

The reasons for the narrow host range of a viroid are not clear. Recent studies suggest a possibility that low level of replication and/or inability to traffic between cells contributes to the limited host range for some, if not all, viroids. For instance, none of the known viroids infect *Arabidopsis thaliana* when inoculated into this plant. In transgenic *A. thaliana* plants expressing the dimeric (+)-RNAs of citrus exocortis viroid (CEVd) and HSVd, members of the Pospiviroidae, replication took place. Agroinoculation of *A. thaliana* with CEVd, HSVd, and coleus blumei viroid 1 dimeric cDNAs showed that these viroids did not traffic from the inoculated leaves to distal plant parts. For some viroids, their host range may be underestimated if disease symptom is used as the main detection method, because their infection of certain plants will not necessarily produce visible symptoms.

Viroid Infection

Viroid Transmission

Like viral infection, in many cases viroid infection easily spreads between individual plants through wounding caused by farming tools, human contact, or plant-plant contact. Some viroids can also be transmitted via infected seeds or pollen. Vegetative propagation by grafts or tubers also readily transmits viroids. For instance, PSTVd can be transmitted by infected potato tubers and infected tomato seeds, and ASBVd can be seed-transmitted in avocado. In contrast to the common transmission of viruses, insect transmission of viroids is not common in the field. There is a report of infrequent transmission of PSTVd by the potato aphid *Macrosiphum euphorbiae*. Quarantine and elimination of infected plant/seed stocks are the most effective means currently available to control the spread of viroid infection.

General Scheme of Systemic Infection in a Plant

The establishment of systemic infection by both families of viroids involves the following mechanistic steps (Fig. 2): (1) import into specific subcellular organelles (the nucleus for the Pospiviroidae and the chloroplast for the Avsunviroidae), (2) replication, (3) export out of the organelles, (4) cell-to-cell trafficking, (5) entry into the vascular tissue, (6) long-distance trafficking within the vascular tissue, and (7) exit from the vascular tissue and subsequent invasion of nonvascular cells to reinitiate the cycle. As discussed further below, some steps have been well studied whereas others remain essentially unknown.

Intracellular Localization and Replication

Pospiviroidae

Before replication, members of the family Pospiviroidae must first enter the nucleus. How this is achieved is still poorly understood. The first study to address this question examined nuclear import of fluorescent-labeled in vitro transcripts of PSTVd in protoplasts of tobacco BY2 cells. The protoplasts, prepared by the removal of cell walls via digestion with enzymes such as cellulase and pectinase, were further treated with a detergent such as Triton-X 100 to permeabilize the plasma membrane. When the fluorescent-labeled transcripts were incubated with such protoplasts, they entered the cells through the permeabilized plasma membrane and then accumulated in the nucleus within 15–20 min, which was visualized under a fluorescence microscope. When fluorescent transcripts were mixed with a 10 M excess of non-labeled transcripts, nuclear import of the former was inhibited, suggesting that PSTVd import is a specific and regulated process presumably mediated by a protein carrier that remains to be identified. These findings were confirmed with an independent approach, in which PSTVd could function in *cis* to mediate nuclear import of a large fusion RNA in *N. benthamiana* leaves. Using the latter approach, a subsequent study showed that the upper CCR strand of PSTVd was able to mediate nuclear import of a fusion RNA. The biological significance of this result for viroid infection can now be tested. The cellular factor(s) that recognizes and imports the viroid RNA is not known, and how this RNA exits the nucleus remains to be investigated.

Within the nucleus, viroids replicate via an asymmetric rolling-circle mechanism (Fig. 3(a)). Briefly, the circular (+)-RNA (by convention this polarity is assigned to the most abundant form accumulating in vivo) is first transcribed into concatemeric linear (–)-strand RNA in the nucleoplasm. This long RNA then acts as the replication intermediate for the synthesis, also in the nucleoplasm, of concatemeric, linear (+)-strand RNA. In one possible mechanism the latter is transported into the nucleolus, where it is cleaved into unit-length monomers. Subsequent intramolecular end-to-end ligation of each monomer yields the mature,

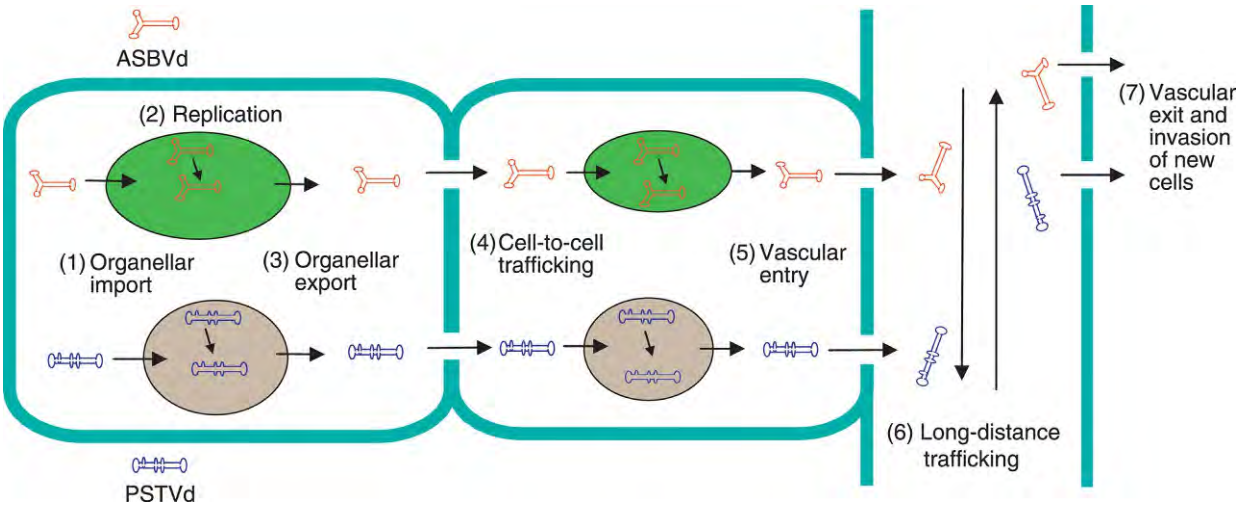

Fig. 2 Distinct steps of systemic infection of ASBVd and PSTVd. Reproduced from Ding, B., Itaya, A., 2007. Viroid: A useful model for studying the basic principles of infection and RNA biology. Molecular Plant-Microbe Interactions 20, 7–20.

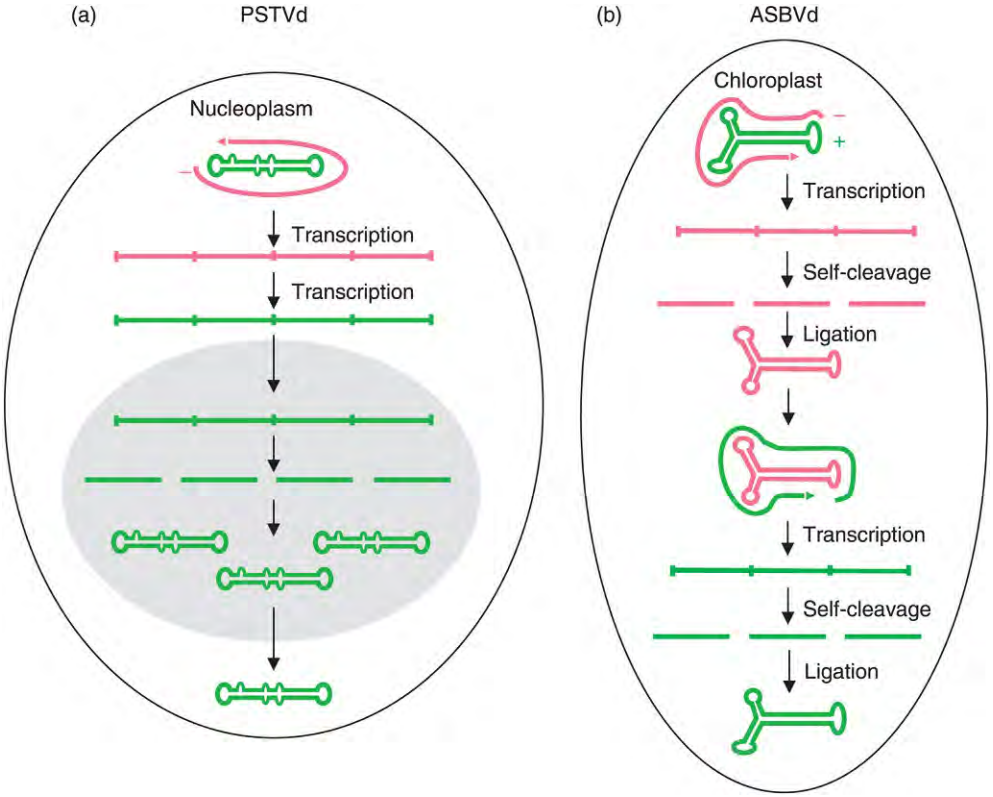

Fig. 3 Rolling-circle replication mechanisms of PSTVd (a) and ASBVd (b). The secondary structure sketches of the genomic RNAs illustrate the approximate transcription initiation sites. Reproduced, with modifications, from Ding, B., Itaya, A., 2007. Viroid: A useful model for studying the basic principles of infection and RNA biology. Molecular Plant-Microbe Interactions 20, 7–20.

circular progeny viroid RNA. Alternatively, the cleavage and ligation occur in the nucleoplasm, and the mature viroid RNA is transported into the nucleolus for storage.

Several lines of evidence suggest that the DNA-dependent RNA polymerase II (Pol II) is redirected to transcribe viroid strands: (1) purified tomato Pol II can transcribe a PSTVd template in vitro, (2) CEVd RNAs are associated with the largest subunit of Pol II in vivo, and (3) treatment of cells or nuclear extracts with Pol II inhibitor α-amanitin impedes replication of CEVd and PSTVd in vivo or transcription in vitro. Recent data support that transcription factor IIIA enhances transcription of PSTVd by Pol II. In the past few years, two plant-specific DNA-dependent RNA polymerases (Pol IV and Pol V) and several RNA-dependent RNA polymerases have been discovered. The role of these enzymes, if any, in viroid transcription remains to be tested.

Where transcription initiates in a viroid RNA of the family Pospiviroidae remains to be fully understood. Studies examining the de novo synthesis of the (−)-strand PSTVd RNAs in potato nuclear extracts mapped the transcription initiation site on the circular (+)-RNA to U359/C1 of the left-terminal loop (Fig. 1(b)). This observation was further tested by loss-of-function genetic experiments in combination with biochemical analysis of where the transcription complex binds on the viroid RNA. The importance of the left-terminal loop for replication was additionally supported by the observation that enlargement of this loop by mutagenesis inhibited replication in protoplasts or infection in *N. benthamiana*. Furthermore, disruption of each of three consecutive loops from the left-terminal domain (loops 2, 3 or 4 in Fig. 4), also inhibited replication in protoplasts. The transcription initiation site of the (+)-strand on the (−)-strand PSTVd template is yet to be identified.

There are two GC boxes in the PSTVd secondary structure (Fig. 1(b)). Mutational studies suggest that they may play a role in transcription; further studies are needed to determine their functions. There is evidence that loop E located in the CCR of PSTVd (see Fig. 1(b)) is critical for replication. Other studies provided evidence that the PSTVd loop E motif exists in vivo, has a defined tertiary structure, and disruption of this structure leads to loss of function in replication. A more recent investigation using whole genome mutational analysis has identified several additional PSTVd loops as motifs critical for replication in single cells. Some loops are located within the central region (loops 13 and 14 in Fig. 4) and others are located in the left-terminal domain (loops 2, 3, and 4 in Fig. 4). The specific roles of these loops in RNA stability, nuclear transport, transcription, cleavage and ligation need to be determined.

A thermodynamically metastable hairpin II (HPII) structure is predicted to form through base interactions involving nucleotide sequences 227–236 and 319–328 (Figs. 1(b) and 1(c)) during thermal denaturation of the PSTVd secondary structure. The HPII structure has been detected in vitro and in vivo in PSTVd, and it is also present in members of the genus *Pospiviroid*, suggesting its importance in viroid infection. Another metastable structure, HPI (Fig. 1(b) and (c)), is important for PSTVd infection of tomato.

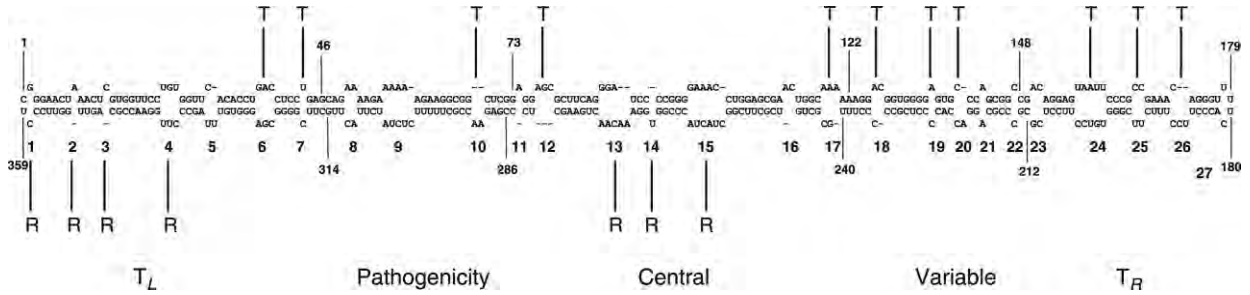

Fig. 4 A genomic map of PSTVd loop motifs critical for replication (R) in single cells or for systemic trafficking (T) in a whole plant. Reproduced, with modifications, from Zhong, X., Archual, A.J., Amin, A.A., Ding, B., 2008. A genomic map of viroid RNA motifs critical for replication and systemic trafficking. Plant Cell 20, 35–47, www.plantcell.org. Copyright American Society of Plant Biologists.

However, recent mutational analyses showed that neither HPII nor HPI is critical for PSTVd replication in *N. benthamiana*. Whether these metastable structures function in PSTVd infection in some plant species, as supported by previous data, but not in others is an important question in further studies.

The sequence and structural conservation of the CCR in members belonging to the same genera of the Pospiviroidae suggests its potential importance in viroid processing. In vitro studies mapped the cleavage and ligation site of PSTVd between G95 and G96 of the CCR. In a more recent work with transgenic *A. thaliana* plants that express dimeric (+)-RNAs of CEVd, HSVd, and apple scar skin viroid (ASSVd), the in vivo processing site for these viroids was mapped at equivalent positions of a putative HPI/double-stranded structure formed by the upper strand and flanking nucleotides of the CCR. More specifically, the substrate for in vivo cleavage is the proposed double-stranded structure, with HPI (conserved in the Pospiviroidae, thus supporting its biological significance) potentially facilitating the adoption of this structure, whereas ligation is determined by loop E and flanking nucleotides of the two CCR strands. Cleavage of the conserved double-stranded structure is proposed to be catalyzed by one or more RNases of class III; enzymes of this class also mediate maturation of highly structured nuclear transcripts. Regarding ligation, it has long been known that wheat germ and *Chlamydomonas reinhardtii* contain RNA ligase activities that circularize PSTVd linear RNAs. However, recent data indicate that PSTVd, and presumably the other members of the Pospiviroidae, divert DNA ligase I to act as an RNA ligase.

Avsunviroidae

Viroids of the family Avsunviroidae replicate in plastids. How they enter and exit the chloroplast is not known. Within the chloroplast, these viroids replicate via a symmetric rolling-circle mechanism (Fig. 3(b)). The circular genomic (+)-RNA is first transcribed into a linear, concatemeric (−)-strand RNA. This RNA is cleaved into unit-length molecules and circularized to serve as the template to generate linear, concatemeric (+)-strand RNAs, which are subsequently cleaved into unit-length monomers and circularized. In vitro studies showed that the *Escherichia coli* RNA polymerase can transcribe peach latent mosaic viroid (PLMVd) RNA templates, suggesting that the plastid-encoded bacterial-like multisubunit RNA polymerase (PEP) may be involved in transcription of PLMVd in vivo. However, sensitivity of ASBVd replication to treatment with tagetoxin, and accumulation of PLMVd strands of both polarities in albino sectors of peach leaves wherein PEP is absent, suggests that a nuclear-encoded and phage-like single-subunit polymerase (NEP) is involved in replication in vivo. Further studies on this question are necessary. In any case, like with Pol II in the Pospiviroidae, the chloroplastic RNA polymerase involved is redirected to transcribe viroid strands instead of its physiological DNA template.

The transcription initiation sites have been determined for ASBVd and PLMVd. For ASBVd, in vitro capping and RNase protection assays mapped U121 as the initiation site on the (+)-RNA and U119 as the site on the (−)-RNA. Both sites are located in the AU-rich terminal loops of the RNA secondary structures (Fig. 1). For PLMVd, studies using an adapted RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) strategy and analysis of a wide repertoire of PLMVd variants have revealed C51 and A286 as the main transcription initiation sites for the (+)- and (−)-strand RNAs, respectively. The possibility that some variants can start transcription in other sites cannot be formally ruled out.

Members of the Avsunviroidae form hammerhead ribozymes in both the (+)- and (−)-RNA strands that catalyze their self-cleavage in vitro. The general thought is that the concatemeric intermediates of these viroids self-cleave during replication in vivo. However, there is evidence that cellular factors may enhance this cleavage. UV-crosslinking of a viroid RNA–protein complex in infected tissues in conjunction with biochemical analyses identified a chloroplast protein, PARBP33, that interacts with ASBVd in vivo. This protein has an RNA-binding motif and accelerates self-cleavage of concatemeric ASBVd RNAs in vitro. The in vivo role of this factor for viroid replication and general RNA processing remains to be further studied.

Although self-cleavage of both the linear concatemeric (+)- and (−)-RNAs is well demonstrated for members of the family Avsunviroidae, less is known about how monomeric molecules are ligated into circles. Nonenzymatic intra- and intermolecular ligation has been demonstrated for PLMVd in vitro, with this self-ligation producing a 2′,5′-phosphodiester bond in vitro. Further studies reported the presence of the 2′,5′-phosphodiester bond at the ligation site of the circular PLMVd RNAs isolated from infected peach plants. It will be important to determine whether this mode of ligation functions during PLMVd replication.

A technical advance may enable genetic investigation of the cellular factors involved in cleavage and ligation. When the dimeric cDNAs of three species of the Avsunviroidae, including ASBVd, chrysanthemum chlorotic mottle viroid (CChMVd), and eggplant latent viroid (ELVd), are expressed in the transformed chloroplasts of *C. reinhardtii*, the dimeric (+)- or (−)-RNA transcripts are correctly cleaved into unit length molecules and circularized. There is no evidence for replication to have taken place. Given the complete genome sequence information and well-established genetic and molecular approaches in *C. reinhardtii*, this model system may prove to be of great utility to identify the protein factors important for viroid RNA processing. More recent data have shown that members of the Avsunviroidae recruit for circularization a plastidic isoform of the tRNA ligase; this enzyme has specificity for the 5′-hydroxyl and 2′,3′ cyclic phosphodiester termini generated by the hammerhead ribozymes and, therefore, appears as an excellent candidate for catalyzing the final step of the replication cycle.

Viroid Cell-to-Cell and Long-Distance Trafficking

To establish a systemic infection, viroid RNAs must traffic from initially infected cells into neighboring cells and distant organs. Studies on PSTVd indicate that cell-to-cell trafficking occurs through cytoplasmic channels (plasmodesmata) and long-distance trafficking occurs through the vascular tissue (phloem). Without encoding proteins, one can assume that viroids either diffuse between cells and through the phloem or they have sequence/structural motifs to mediate the trafficking process. The first clue for motif-mediated trafficking came from studies showing that two mutations in the right-terminal domain of PSTVd do not appear to affect replication in tomato roots, but affect systemic infection. Further studies with microinjection showed that PSTVd could function in *cis* to potentiate cell-to-cell trafficking of a heterologous RNA, suggesting that the viroid RNA has a motif that mediates trafficking. Furthermore, in an infected flower PSTVd traffics into sepals but not into the other floral organs, suggesting that the phloem has a mechanism to recognize and traffic PSTVd RNAs into selective sink organs.

Mutational studies on two PSTVd^{NT} and PSTVd^{NB}, and PSTVd, which differ by 5 nt, identified a bipartite motif that is required for trafficking from bundle sheath to mesophyll, but not in the reverse direction. In addition, this motif is required for trafficking in young, but not in mature leaves. Thus, plant development is also a major factor for the fine-tuning of trafficking controls. Whether the two components of the bipartite motif interact with separate cellular factors for trafficking, or they form a particular tertiary structural motif via conformational changes to interact with a single cellular factor, remains an outstanding question.

A tertiary structural motif, which consists of at least U43/C318 (loop 7 in Fig. 4) that form a *cis*-Watson–Crick base pair with water insertion, is required for PSTVd to traffic from the bundle sheath into the phloem to initiate long-distance transport in *N. benthamiana*. Mechanistically, water insertion distorts the structure of the local helix, with this distortion being necessary for trafficking. This motif recurs in many other RNAs: in rRNAs serves as a binding site for a ribosomal protein. The loop 6 motif of PSTVd is required for trafficking from palisade mesophyll to spongy mesophyll in *N. benthamiana* leaves. This motif has the sequence 5′-CGA-3′...5′-GAC-3′ forming three non-Watson–Crick base pairs.

These studies imply the existence of multiple PSTVd structural motifs to mediate trafficking across various cellular boundaries in an infected plant. Indeed, another study has identified many additional loops in the PSTVd secondary structure that are critical for systemic trafficking in *N. benthamiana* (Fig. 4). Closing of these loops by nucleotide substitutions/deletions to create Watson–Crick base pairing abolishes systemic infection while allowing replication. The tertiary structure of each loop, whether each of these loops function individually or in some combinations to mediate trafficking across specific cellular boundaries, and what host proteins interact with each of these loops for function are outstanding issues for future investigations.

A current hypothesis is that certain cellular proteins recognize specific viroid RNA motifs to potentiate trafficking between cells and among organs. These proteins have not yet been identified. Several promising candidates have been reported but their functions remain to be conclusively tested. These include the mobile phloem lectin PP2 from cucumber (CsPP2) that binds HSVd in vitro and in vivo, as well as two phloem proteins that bind ASBVd. A tomato protein, VIRP1, interacts in vitro with the right-terminal region of PSTVd and HSVd, with further work showing that this protein appears to be important for infection. When VIRP1 expression in transgenic *N. benthamiana* is repressed by antisense methodology, the protoplasts prepared from this transgenic plant fail to support PSTVd replication.

Viroid Pathogenicity

Without encoding proteins, viroid diseases must result from interactions between cellular factors with the viroid genome itself or with genome-derived RNAs. Such interactions disturb the normal course of plant development leading to disease onset. Viroid diseases show great variations, depending on viroid–host combinations: they range from nearly symptomless infections to host lethality. One of the most devastating diseases is the cadang-cadang disease, caused by infection of coconut cadang-cadang viroid (CCCVd), that has killed over 30 million coconut palms. Environmental conditions affect symptom expression. In particular, high temperatures and light intensity enhance disease symptoms. No natural resistance to viroid infection has been reported.

In many cases, small sequence or structural variations in a viroid genome can cause symptoms of different degrees of severity (Fig. 5). The viroid RNA structure–disease relationships have been studied most extensively for members of the family Pospiviroidae. Early studies with PSTVd and CEVd showed that nucleotide changes in association with different degrees of symptom severity occur in the so-called pathogenicity domain (Fig. 1(b) for PSTVd). More recent studies indicate that all five structural domains play a role in pathogenicity.

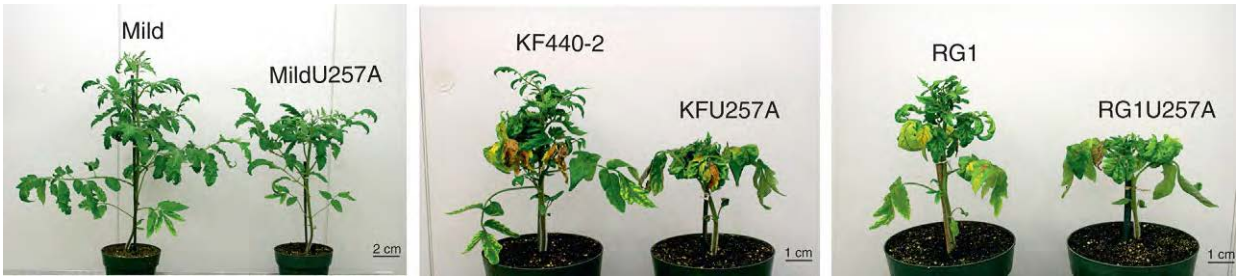

Fig. 5 Mild to lethal disease symptoms caused by infection of several PSTVd variants in Rutgers tomato plants. Reproduced from Qi, Y., Ding, B., 2003. Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. Plant Cell 15, 1360–1374, www.plantcell.org. Copyright American Society of Plant Biologists.

Sequence comparisons among ASBVd variants isolated from diseased and healthy tissues of infected avocado suggest that a "U" insertion between positions 115 and 118 is associated with "bleached" tissues. Studies on symptomatic and non-symptomatic variants of CChMVd identified tetraloop UUUC (positions 82–85) as a major pathogenicity determinant. Conversion of this tetraloop to GAAA in natural variants or by site-directed mutagenesis renders the viroid non-symptomatic. In the case of PLMVd, an albinism termed peach calico (PC) is strictly associated with variants containing a specific 12–14-nt hairpin insertion (Fig. 6).

Other than correlations between viroid sequences and symptom severity, little has been known about the mechanisms of pathogenicity until recently. In general, viroid accumulation levels and tissue localizations are not major the factors for the varying degrees of symptoms, suggesting that specific molecular interactions between viroid sequences/structures with host factors are involved in disease mechanisms. The cellular factors that interact with specific viroid sequences/structures for disease development are not known. Infection of tomato by mild and severe PSTVd strains induced or suppressed expression of common and unique sets of host genes. These include genes involved in general defense/stress responses, cell wall structure and metabolism, and chloroplast functions, among others. Similar alteration of host gene expression has also been reported in Etrog citron leaves infected by citrus dwarfing viroid (CDVd). How the altered expression of any host genes contributes to disease formation is not known. PSTVd infection also causes phosphorylation of a protein kinase that is immunologically related to the mammalian interferon-induced, double-stranded RNA-activated protein kinase. Further studies showed differential in vitro activation of the mammalian protein kinase P68 by PSTVd strains of different pathogenicity. The biological significance of this activation for viroid symptom expression remains to be understood.

Subsequent studies on PSTVd and PLMVd have started to shed light on the molecular mechanisms underlying pathogenicity. A U257A change in the CCR converted several strains of PSTVd into lethal strains that caused severe growth stunting and premature

Fig. 6 Healthy peach leaf (left) and partial (center) and complete (right) leaf albinism, termed peach calico, incited by variants of PLMVd containing a specific 12–14 nucleotide hairpin insertion. Reproduced with modifications from Navarro, B., Gisel, A., Rodio, M.E., *et al.*, 2012. Small RNAs containing the pathogenic determinant of a chloroplast-replicating viroid guide the degradation of a host mRNA as predicted by RNA silencing. Plant Journal 70, 991–1003, www.theplantjournal.org. Copyright Blackwell Publishing Ltd.

death of infected plants (see Fig. 5). The U257A substitution did not alter PSTVd secondary structure, replication levels, or tissue tropism of PSTVd. The stunted growth of infected tomato plants resulted from restricted cell growth, but not cell division or differentiation. This phenotype is correlated positively with downregulated expression of an expansin gene, *LeExp2*, that is known to play an important role in cell expansion in young growing organs. As indicated above, the PC symptomatology, characterized by an extreme chlorosis (albinism) of infected tissues, is associated with the insertion of an extra 12–14 nt sequence that folds into a hairpin in the left-terminal loop of PLMVd. Intriguingly, the insertion occurs sporadically de novo and can be acquired or lost during infection. The presence of this hairpin impairs processing and accumulation of chloroplast rRNAs, eventually affecting the structure and function of the chloroplast translation machinery. Chloroplast development is thus severely disturbed. Still, the underdeveloped chloroplasts retain the capacity to import proteins encoded by nuclear genes, including NEP, and support PLMVd replication. Altogether, these findings sustain the view that specific viroid sequence/structural elements interact with host factors in a highly specific manner to alter host gene expression and developmental processes.

An emerging model for viroid pathogenicity is that small RNAs of 21–24 nt derived from viroid RNA sequences during infection can guide RNA silencing of host genes, thereby leading to development of disease symptoms. Consistent with this hypothesis, there is a positive correlation between the levels of viroid-derived small RNAs (vd-sRNAs) and symptom severity for PSTVd and ASBVd. Moreover, symptom development is correlated with production of vd-sRNAs in some transgenic tomato lines expressing non-replicating, hairpin PSTVd RNAs. However, the correlation between accumulation of vd-sRNAs and symptom expression is not universal. A recent study has provided direct support for the involvement of RNA silencing in PC induction: using deep sequencing, semi-quantitative RT-PCR and RLM-RACE, two PLMVd-sRNAs containing the PC-associated insertion (PC-sRNA8a and PC-sRNA8b) were found to target for cleavage the mRNA encoding the chloroplastic heat-shock protein 90 (Fig. 7). Chloroplast malformations previously reported in PC-expressing tissues are consistent with the down-regulation of cHSP90, which participates in chloroplast biogenesis and plastid-to-nucleus signal transduction in Arabidopsis. Recently, a similar RNA silencing-based mechanism has been proposed for PSTVd and another closely-related viroid: vdsRNAs containing the pathogenic determinants have been involved in symptom induction, although the identity of the primary target is not yet clear.

Virusoids

Viroid-like satellite RNAs are single-stranded molecules found in infected tissues in both circular and linear forms. A singular feature of the viroid-like satellite RNAs of the genus *Sobemovirus* (virusoids) is that the circular form is the predominant RNA encapsidated

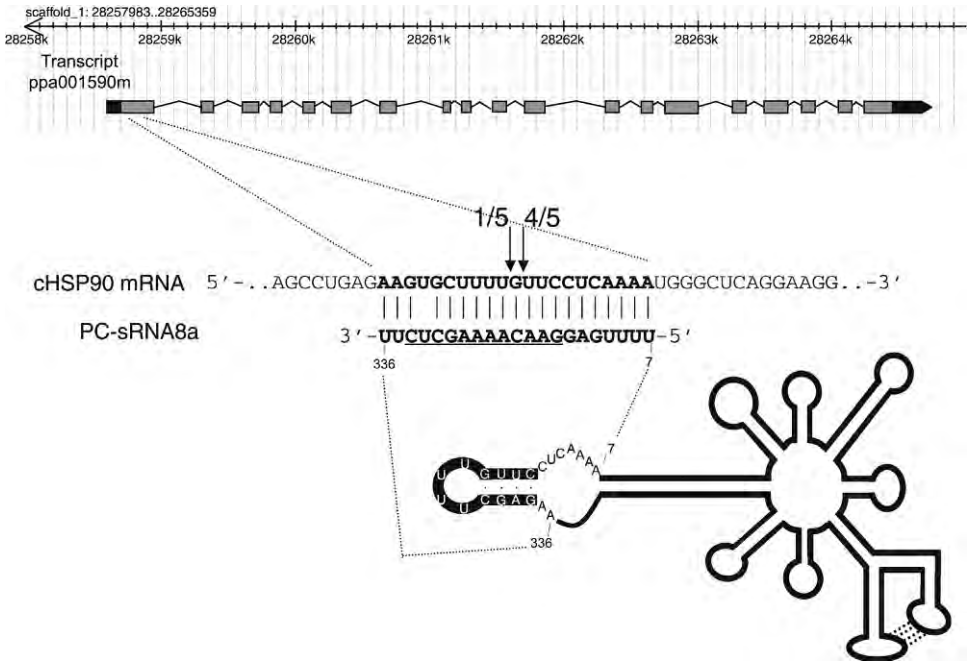

Fig. 7 Specific cleavage of peach cHSP90 mRNA mediated by PC-sRNAs. Upper panel, cHSP90 mRNA:PC-sRNA8a duplex (in bold), with the sequence of PC-sRNA8a corresponding to the PC-associated insertion underlined. Arrows mark the cleavage sites predicted and validated by RLM-RACE from five independent clones (fractions indicate number of clones producing the same results). Lower panel, schematic representation of the secondary structure predicted for PLMVd variant C40, with nucleotides complementary to PC-sRNA8a highlighted, and those forming part of the PC-inducing insertion on a black background. Reproduced with modifications from Navarro, B., Gisel, A., Rodio, M.E., *et al.*, 2012. Small RNAs containing the pathogenic determinant of a chloroplast-replicating viroid guide the degradation of a host mRNA as predicted by RNA silencing. Plant Journal 70, 991–1003, www.theplantjournal.org. Copyright Blackwell Publishing Ltd.

Table 3 Virusoids

Virusoid	*Helper virus*	*Genome size (nt)*	*Accession #*	*(+)-Strand ribozyme*	*(−)-Strand ribozyme*
vRYMV	RYMV	220	AF039909	Yes	No
vLSTV	LSTV	322–324	X01984	Yes	Yes
vSCMoV	SCMoV	332, 388	M33000, M33001	Yes	No
vVTMoV	VTMoV	366	J02439	Yes	No
vSNMV	SNMV	377	J02388	Yes	No

Abbreviations: LSTV, lucerne transient streak virus; RYMV, rice yellow mottle virus; SCMoV, subterranean clover mottle virus; SNMV, solanum nodiflorum mottle virus; VTMoV, velvet tobacco mottle virus

by the viral coat protein, while in viroid-like satellite RNAs associated with members of the genera *Nepovirus* and *Luteovirus* the linear RNA form is predominantly encapsidated. Virusoids bear some structural and functional similarity with viroids but also share biological properties with satellite RNAs. All satellite RNAs must coinfect with a helper virus in order to replicate by utilizing an RNA replicase complex encoded in part by the helper virus and in part by the host. Satellite RNAs have little sequence similarity with the genome of their helper viruses.

Virusoid genomes are 220–388 nt long, assume a rod-shaped secondary structure due to intramolecular base pairing and, with the possible exception of vRYMV, do not encode proteins according to the available evidence. In these aspects virusoids are similar to viroids. However, the thermostability of virusoids is distinct from that of viroids, showing no cooperativity but rather behaving as random base sequences. Virusoids are encapsidated separately from the helper viral RNAs.

Five virusoids are currently known (Table 3). All their helper viruses are members of the genus *Sobemovirus* including RYMV, lucerne transient streak virus (LTSV), subterranean clover mottle virus (SCMoV), velvet tobacco mottle virus (VTMoV), and solanum nodiflorum mottle virus (SNMV). By convention, the encapsidated and infectious form of the RNA is designated as the (+)-strand, which accumulates to higher levels than the (–)-strand that is produced during RNA–RNA transcription. Similarly to viroids, virusoids replicate via a rolling-circle mechanism mediated by hammerhead ribozymes in one or both polarity strands.

There is little understanding of the biology of virusoids, in terms of their interactions with helper viruses, how they initiate replication, how they move between cells and throughout a plant, and how they influence viral disease symptoms. A recent study revealed no involvement of virusoid vRYMV in symptom modulation or ability to break host–plant resistance to the viral disease.

Did Viroids and Virusoids Emerge in the RNA World?

Although in all present cells DNA and proteins store and express the genetic information respectively, with RNA playing just connecting and regulatory roles, the situation differs when descending along the biological scale, with many viruses using RNA as their genetic material. Besides storing genetic information, RNA can express it, as exemplified by the discovery of an increasing number of catalytic RNAs (ribozymes). This dual competence of RNA is the basis for the proposal of an RNA World that preceded the emergence of DNA and proteins. If such a world ever existed, the finding of some sort of survivors in our present world appears as a reasonable assumption. Indeed, viroids (and virusoids) display unique properties consistent with those predicted for the first minimal replicons that might have evolved in the RNA World: (1) small size and high G+C content to cope with error-prone primitive replication, compact folding to enhance survival, and circularity to achieve complete replication without recurring to genomic tags, (2) remnants of structural periodicity suggesting modular assembly into larger genomes, (3) lack of protein-coding ability in agreement with a ribosome-free environment, (4) replication in at least some of them mediated by ribozymes, the key signature of the RNA World and, (5) feasible routes for the transition from the RNA World to a world with proteins and DNA, where the primordial viroids (and virusoids) lost some of their initial abilities and evolved into the plant (and virus) parasites found infecting plants today.

Conclusion

Viroids and virusoids are RNAs small in size, simple in structure, and yet complicated in biological functions. Their replication and systemic trafficking raise the fascinating questions of what RNA structural motifs within the RNA direct all of the biological functions and what host factors are recruited by these motifs to accomplish each function necessary to establish a systemic infection. The viroid disease can be considered as an example of RNA-directed misregulated expression of host genes. Further studies on viroid–host interactions and virusoid–host–helper virus interactions are expected to contribute new and exciting knowledge about the evolution of RNA-based pathogens and about the basic mechanisms of non-coding RNA functions. As compared to viroids, the biology of virusoids has been greatly understudied. It can be anticipated that with appropriate experimental tools developed, virusoids can serve as another powerful set of simple RNA models, like viroids, for fundamental discoveries in biology.

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Relevant Website

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Virus Evolution

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Glossary

Addition mutation Acquisition of one or several nucleotides.
Ambisense coding Said of an RNA genome that can express proteins from either polarity of the same RNA molecule.
Arbovirus Virus transmitted by an arthropod.
Bacteriophage Virus that infect bacteria.
Bottleneck Strong reduction of population size.
Combination therapy Treatment that consists of the administration of two or more drugs.
Complementation Capacity of viral genomes to increase the replicative activity of other genomes through *trans*-acting interactions.
Consensus sequence The sequence that has at each position (nucleotide or amino acid) the most abundant residue at the corresponding position in a set of aligned sequences. The consensus sequence need not coincide with the master sequence, and may not be represented in the population.
Deletion mutation Loss of one or several nucleotides.
Error threshold Average error rate that sets a maximum limit for maintenance of genetic information encoded by a replicating system. Error rates above the error threshold lead to loss of genetic information, also termed error catastrophe.
Evolution Modification of the nucleotide sequence of genetic material as a function of time, independently of the time frame involved.
Frameshift mutation One that changes the reading frame in a coding genomic region.
Genomics The study of genomic nucleotide sequences and their functional and evolutionary inferences.
Homology Quality that defines a common ancestry.
Homopolymer A polymer made of the same monomeric unit. A nucleic acid with a repeated nucleotide.
Hypercycle A system that connects autocatalytic or self-replicative units through a cyclic organization.
Hypermutation Multiple mutations of the same kind, generally introduced in viral genomes by cellular editing activities.
Insertion mutation Gain of one or several nucleotides.
Interference Capacity of viral genomes to reduce the replicative activity of other genomes through *trans*-acting interactions. It can be regarded as the converse of complementation.
Lethal mutagenesis Extinction by an excess of mutations.
LUCA The Last Universal Common Ancestor of present day cellular organisms. It might have been a DNA or a RNA genome with cellular features.
Master sequence The most frequent genomic nucleotide sequence in a mutant distribution because of its higher fitness than the other genomes.
Metagenomics Analysis of the type of genomes that compose a biological community.
Mimivirus A representative of giant viruses, acronym of mimicking microbes viruses.
Mutant spectrum (or mutant cloud, or mutant swarm) The ensemble of mutant genomes that constitute a viral quasispecies.
Mutation (or mutant) frequency The frequency of the total number of mutations or of a specific mutation or mutation type. It differs from the mutation rate in that it is affected by the fitness of the mutated genomes. It is calculated as the total number of mutations counted relative to the consensus sequence, divided by the total number of nucleotides sequenced.
Mutation rate Frequency of occurrence of mutations during genome replication. It is a biochemical event, independent of the fitness of parental and mutated genomes.
Negative selection Process of genome elimination (or maintenance at low frequency) due to low fitness.
Neutral mutation One that does not affect fitness in a given environment. Neutrality is relative to an environment.
Non-synonymous mutation A mutation that gives rise to an amino acid substitution.
Nucleotide diversity Average genetic distance between any two sequences of a population.
Plaque Localized (often circular) region on a cell monolayer made of cells lysed as a result of infection by a single virus particle and virus propagation to neighbor cells.
Plasmid Small circular double stranded DNA that replicates independently of the chromosomal DNA.
Point mutation Replacement of a single nucleotide.
Quasispecies memory Presence in a viral quasispecies of specific subpopulations of genomes that were dominant at an early evolutionary phase.

Rate of fixation (or accumulation) of mutations Number of mutations that become dominant per unit time.
Reassortment In segmented genomes formation of a new genome segment constellation from two different parents.
Replication complex Intracellular site where viral and cellular proteins interact with membrane structures for the replication of viral genomes.
Ribozyme An RNA molecule endowed with catalytic properties.
RNA world It has several meanings in biology. In connection with the present article it may refer to a world that existed prior to the advent of DNA, or to the present day RNA-based part of the biosphere (RNA viruses and other RNA genetic elements).
Synonymous mutation Mutation that does not give rise to an amino acid substitution.
Temperate bacteriophage Bacteriophage whose DNA can integrate into the host DNA.
Transduction Transfer of DNA from one bacterium to another mediated by a bacteriophage.
Transition mutation Replacement of a purine by another purine or of a pyrimidine by another pyrimidine.
Transversion mutation Replacement of a purine by a pyrimidine or vice versa.
Viral fitness Replication capacity of a virus measured relative to a reference isolate or clone of the same virus. A fitness value is relative to an environment.
Virion Viral particle.
Viroid Small circular single-stranded subviral RNA element that infects plants.
Virosphere The part of the biosphere that pertains to viruses.

Abbreviations

DNA	deoxyribonucleic acid
d.str.	double stranded
FMDV	foot-and-mouth disease virus
HDV	hepatitis delta virus
LGT	lateral gene transfer
LUCA	Last Universal Common Ancestor
NCLDV	snucleocytoplasmic large DNA viruses
RNA	ribonucleic acid
s.str.	single stranded

Defining Statement

Metagenomics is providing unprecedented information about viral genomes in different environments, and as viruses replicate in their hosts. The analyses have unveiled a remarkable diversity in genome organization and extreme genetic heterogeneity in the course of infections. Despite accumulating information, it remains challenging to reliably reconstruct the origin of viruses, and their involvement in ancestral evolutionary events. Experimental evolution has contributed insights into the evolutionary mechanisms used by present day viruses that might have been shared by their ancestors. Here we examine observations and proposals.

Early Virus Evolution

Several theories have been proposed for the origin of RNA and DNA viruses, and their connection with the development of the cellular world, beginning with the Last Universal Common Ancestor (LUCA) if a cellular entity with a RNA or DNA genome existed that can be considered the common ancestor of all cells. Two opposite views have been expressed: (i) viruses are descendants of primitive life forms that existed when the cellular world was still under construction, (ii) viruses are late-comers in evolution, and they derived from cells at different points in the process of cellular evolution (Fig. 1). There are arguments in favor and against each of the two assertions.

An Origin Independent of Cells

That the origin of viruses did not require cells as we understand a cellular organization today, even the genetically less complex cells such as mycoplasma, is supported by the presence in viral genomes of genes that do not have an equivalent in present day cells, according to the available data banks. For example, an important group of viruses termed the nucleocytoplasmic large DNA viruses (NCLDVs) [that include the Mimiviruses (a short version of "mimicking microbes viruses" because of their large size) with a 1.2 Mbp genome, that infect some amoebas, and the *Phycodnaviridae* with 160–560 Kbp genomes, that infect algae] share a coat protein

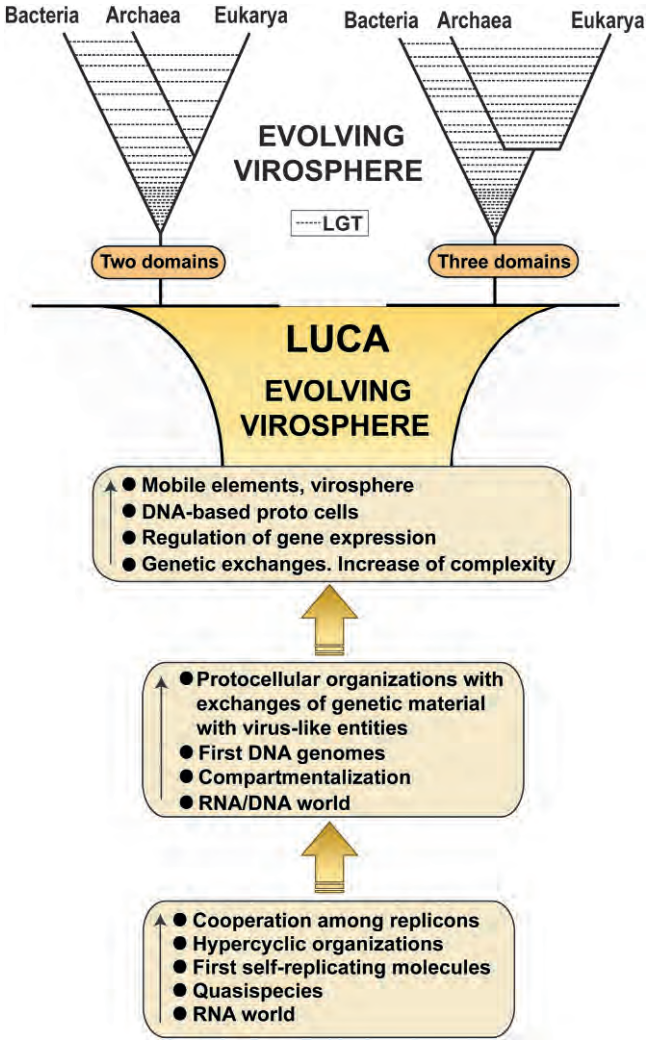

Fig. 1 A possible sequence of major historical occurrences in the origin of viruses and cells. The three large boxes recapitulate several proposals of evolution from a primordial RNA world about 4000 million years ago to the first proto-cells and LUCA. In each box, the left thin arrow suggests a time course of overlapping events. The top schematic trees depict two of several alternatives that have been proposed for the branching of primitive cells into bacteria, archaea and eukarya. In all cases, lateral gene transfers (LGT, horizontal discontinuous lines) are likely to have played an important role. An evolving virosphere is believed to have been involved in the development of the cellular world.

structure characterized by a double jelly-roll fold, similar to a fold found in some viruses that infect the archaeon *Sulfolobus*, and in some single stranded (s.str.) and double stranded (d.str.) RNA viruses that infect eukaryotic cells. This type of coat proteins, together with shared features of other proteins found in viruses but not in cells (termed "virus hallmark proteins"), led to the proposal of "virus self" lineages that would have genome moieties independent from the ones that were the building blocks of cellular genomes. In addition to proteins not represented in cellular data bases, there are other viral genome signatures such as genome size or nucleotide composition that render viruses close to plasmids and distinct from cells. In this scenario, the present day RNA viruses would be the relics of the primitive RNA (or RNA-like) world, while the retroviruses and hepadnaviruses would be remnants of the RNA-DNA transition. The putative virus-like genomes preceded LUCA.

A precellular origin means that viruses were derived from replicating entities that trace back to a primordial RNA world. Prebiological chemical evolution on Earth or elsewhere should have produced molecules that constituted (or evolved into) the building blocks of present day nucleic acids, proteins and lipids. A critical transition should have been primitive RNA structures selected for their capacity of self-replication (Fig. 1). The presence of ribozymes could have favored RNA constructions and phenotypic RNA diversification. Ribozyme activities remain in the present day cellular world (i.e., in ribosomal RNA) and some RNA genetic elements (i.e., in viroids and the hepatitis delta virus (HDV)). The HDV genome appears to have resulted from the conjunction of a viroid-like RNA and RNA with coding capacity. Indeed, in its complementary polarity HDV encodes a protein called "delta antigen". Viroids and HDV could be traces of lineages belonging to a remote pre-cellular RNA world.

Of particular relevance to current virology is the quasispecies theory that proposed that early replicating entities consisted of mutant spectra, as experimentally found with present day RNA viruses (see Section on "Viral Quasispecies"). Hypercyclic organizations were those postulated to couple two classes of replicating cycles (for example, RNA synthesis and RNA translation to produce proteins) so as to sustain primitive life forms. Coupled organizations might have been embodied in virus-like elements before a pre-cellular organization began to be developed.

Viruses From Cells

The complex organization and elaborate gene expression machineries, together with the dependence of present day viruses upon their host cells and organisms, favors the concept that a cellular world should have been in operation before viruses could arise and be established as successful parasites. In this view, viruses could have derived from cells (initially perhaps RNA cells and then DNA cells) via two types of processes that in their extreme versions have been referred to as escape and reduction. *Escape* means that pieces of cellular genetic material, endowed with self-replication capacity (or that acquired self-replicative elements) might have found their way to a partially autonomous existence with a phase of their multiplication cycle inside cells and a phase outside cells, in the form of transmissible particles (termed virions). *Reduction* means that the number of genes of a cell diminished gradually to reach a simplified subset that maintained the capacity to multiply at the expense of other cells. This would imply a gradual transition from cellular autonomy (to the extent that individual cells can be considered autonomous) towards overt dependence on other cells, in particular for the protein synthesis machinery. The large DNA-containing giant viruses have been viewed as a missing link between a primitive DNA cell and a modern DNA virus.

The capacity to spread, essential for the completion of the life cycle of many viruses we study today, might have been attained first by cells, as observed with "infectious cells" that can be disseminated by insects, perhaps a transmission mode that preceded that of arboviruses. Cell-to-cell transmission of viruses (that nowadays coexists with transmission following an extracellular step) may be a sign of a period when the transmitted entities were cells.

A Primitive Proto-Virus, Proto-Cell Soup

A way to reconcile the two extreme views of virus origins "without cells" and "from cells" is to assume a long time of coevolution of primitive viral forms with pre-cellular entities characterized by a fluid variation in terms of mutation, genomic rearrangements, and lateral gene transfers (LGTs). The participation of vesicles (lipid structures that permitted a primitive compartmentalization that separated "internal" from "external" milieu) favored preservation of genetic elements in mobile, virus-like structures that now we perceive as the "virus self". Cells under construction exploited related or different genomic blocks, combined with extensive LGT. Vesicles formed in favorable microenvironments such as hydrothermal vents inaugurated the capacity to exchange with the external medium small molecules for energy production to assist in the increasingly complex coupling of an initial metabolism with genome replication. Budding vesicles should have been positively selected because of their capacity to spread replicating molecules and to acquire metabolites for functional testing. DNA viruses might have had an origin independent of RNA viruses, or may derive from RNA viruses, once reverse transcriptase activities were invented. DNA is a chemically more resistant macromolecule than RNA, and it may have gradually become dominant over RNA as the complexity of the genetic material (length and amount of information) increased. The complexity of chromosomal DNA (Fig. 2) necessitated the presence of proofreading-repair and post-replicative repair activities to preserve the identity (physical integrity and encoded information) of large DNA genomes.

We can safely assume that LGT continued late in cellular evolution and that it involved exchanges among cells, and viral elements (Fig. 1). The reason is that different kinds of transfers have been identified or inferred in our present day biosphere (integration of temperate bacteriophage DNA, bacterial transduction, integration of the DNA versions of retroviruses and retro-elements into chromosomal DNA, molecular fossil evidence of endogenous retroviruses inserted into the genomes of differentiated organisms, etc.). Remarkably, the fusion of cells to form the primate placenta is triggered by syncytin, a protein encoded in endogenous retroviruses of the HERV-W family. Given the evidence of LGT, it is not surprising that in the cellular descendants we study today related genes can be found in representatives of different domains of life. Present day viral proteins involved in viral genome replication are clustered in the phylogenetic trees, but they are as distantly related to their cellular homologues, as are cellular proteins from different domains of life. Two sets of non-homologous proteins involved in DNA replication can be found, one in bacteria and the other common to archaea and eukarya. This is compatible with their having two independent origins, either from evolving primitive cells or viruses.

Signs of complex exchanges of genetic material among viruses and between viruses and cells include the evolutionary connections between the herpesviruses and the tailed bacteriophages, or the evidence that the NCLDVs were built with bacteriophage genes and host cell genes. Different approaches support evolutionary relationships among DNA viruses that infect representatives of the three domains of life. The origin of the eukaryotic cell nucleus has been amply debated, and one of the hypotheses is that it might have derived from a pox-like or Mimivirus-like DNA virus. In fact, the replicative factories of Mimiviruses (the structures where viral DNA replication takes place) have a size comparable to a eukaryotic nucleus. Similar gene expression strategies and regulatory elements such as micro-RNAs are found in cells and viruses. These parallel features speak of common and ancient roots for cells and viruses, and more so considering the geological evidence that several mass extinctions should have eliminated many hosts and their viruses. Present day virosphere is likely to represent a very limited subset of the virus entities that coevolved with cells as they gradually formed the biosphere of differentiated organisms. It is interesting that the term "soup" to designate the

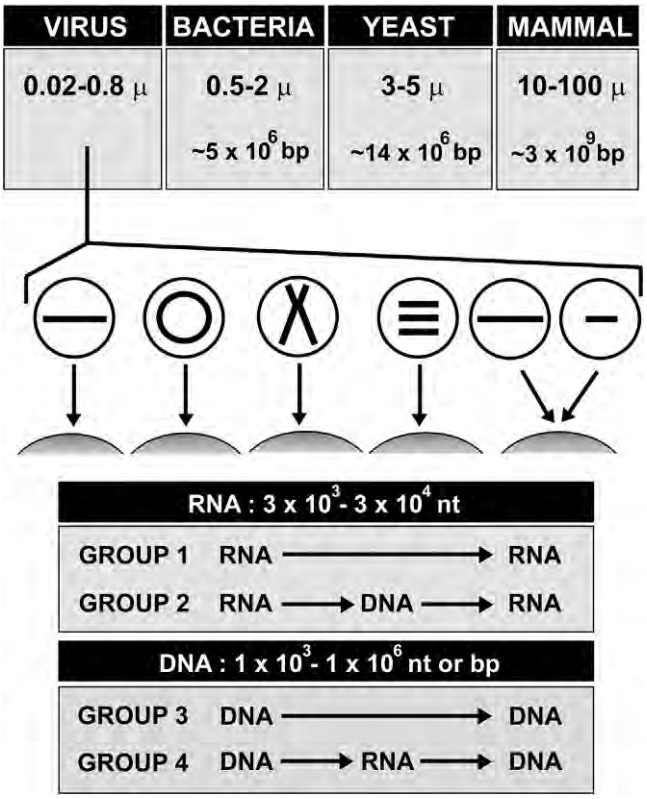

Fig. 2 Representative average diameter values and genome complexity of viruses and some cell types. Viral genomes (depicted as thick outlines inside circles) can be linear, circular, segmented, or multipartite (two or more genome segments encapsidated in separate particles). The bottom boxes describe four groups of viruses according to the type of nucleic acid that acts as replicative intermediate. The figure is modified from Domingo, E., 2016. Virus as Populations. Composition, Complexity, Dynamics, and Biological Implications. Amsterdam: Academic Press Elsevier, with permission from Elsevier.

environment of a cell-virus coevolution coincides with the term used by A.I. Oparin almost a century ago to describe sites where inorganic compounds evolved into organic compounds to produce the building blocks of biological macromolecules and the first living cells. Some would argue that the origin of life and of viruses might in fact be related. Although this is a question unlikely to be settled, it is significant that of the four major features that characterize life, namely replication, evolvability, compartmentalization, and metabolism, viruses display the first two.

Attempts to reconstruct the nature of LUCA are challenging, and suggestions have been put forward that it might have been a RNA or DNA cell, without *bona fide* features of a prokaryotic or eukaryotic cell. Equally uncertain are the reconstructions of phylogenetic branches of different domains of life, at post-LUCA times (Fig. 1). What we have learned from the mechanism involved in the evolutionary dynamics of RNA viruses (see Section on "Diversity and Plasticity of Present Day Viruses") is that the following phenomena might have blurred past phylogenetic relationships: (i) episodes of rapid evolution in primitive cells and viruses; (ii) the availability of multiple molecular pathways for fitness gain: different sets of acquired non-homologous proteins may have achieved comparable functional efficiency; (iii) the occurrence of bottleneck events may have allowed dominance of a molecular solution over others of comparable efficacy; (iv) acquisition of a genomic region from another cell or viral entity may render a prior acquisition useless or even detrimental, and therefore the prior genomic region might be lost, leaving no trace in successful progeny genomes; (v) selective advantages and disadvantages (as a result of point mutations, insertions, deletions, recombination, lateral gene transfers, etc.) are environment-dependent, and the environment changed dramatically over the time periods involved (Fig. 1); (vi) finally we should be aware that distortions of the real events might have been accentuated due to the fact that (i) to (iv) could have happened multiple times over the prolonged time periods involved.

Why Have Viruses Survived to This Date

Viruses may have survived to abound in the present biosphere simply because they parasitized opportunistically cellular organisms, and viruses that encountered a sufficient supply of susceptible hosts were able to persist. In this view, viruses are here merely as selfish elements that persisted blindly, and with no biological function that selected them. A more active role of viruses is implied in an alternative proposal that states that viruses have been positively selected because they promoted variation and diversification of cells, modulation of host population numbers, and they participated in developmental processes. Disease is a secondary consequence of their interactions with their hosts that had a constructive primary function. Expressed as a simple, thought experiment, if

we could compete a biosphere with viruses (as the one we have today) with a biosphere without viruses, for long-term sustainability, the former would be expected to win. Viruses continue performing essential modulatory tasks. To achieve them viruses need diversity commensurate with that of their hosts. There are probably no biological niches devoid of viruses.

Diversity and Plasticity of Present Day Viruses

Present day viruses are diverse regarding genome type (RNA or DNA, single stranded or double stranded, linear or circular, unsegmented or segmented), and in terms of replication strategy (presence or absence of transcription or reverse transcription, ambisense coding, one or multiple messenger RNAs, etc.). According to the nature of the nucleic acid involved in the flow of information during replication (not gene expression) there are four categories of replication cycle, each including important viral pathogens of animals or plants (Fig. 2). Viruses of group 1, those that use only RNA (represented as RNA→RNA), include the RNA bacteriophages MS2 and Qβ, many viruses that infect plants such as tobacco mosaic virus, and important human and animal pathogens such as poliovirus, influenza virus, or the hepatitis A and C, Zika, West Nile and Ebola viruses or the hemorrhagic arenaviruses. Viruses of group 2, those that use a DNA intermediate (RNA→DNA→RNA) include the retroviruses, the best studied being the human immunodeficiency virus type 1, the causative agent of acquired immunodeficiency syndrome (AIDS). Viruses of group 3, those that use only DNA in their replicative cycle (DNA→DNA) comprise the tailed DNA bacteriophages (i.e., T4 and λ) which were historically key for the development of molecular biology, as well as salient pathogens such as papillomaviruses, herpesviruses, poxviruses, iridoviruses or adenoviruses, as well as the insect baculoviruses or giant viruses such as the Mimiviruses. Viruses of group 4, those that use RNA as intermediate (DNA→RNA→DNA) include the plant virus cauliflower mosaic virus and hepatitis B virus, termed the hepadnaviruses.

Diversity is also exuberant regarding the ecological niches where viruses are found: any terrestrial and marine environment, including extreme thermophilic and hyper-saline environments, and infecting representatives of all domains of life. The number of viral particles in the biosphere has been estimated in $\sim 10^{32}$, ten times more than the number of cells. In the Earth oceans there is an impressive rate of 10^{23} new infections per second, so that 10^{8} viral particles can be counted in each ml of sea water. Most viruses are continually replicating and renewing.

Replication is intimately linked to several types of variations of the genetic material that can affect its evolution. Variations include point mutations, insertions, deletions, cell-dependent hypermutation events, recombination and genome segment reassortment, among others (Fig. 3). For many such events we have also approximate numbers for the rate of occurrence. Point mutation rates for RNA viruses are in the range of 10^{-3} to 10^{-5} mutations introduced per nucleotide copied, and similar or lower recombination rates, depending on the virus. Generally, transition mutations are more frequent than transversion mutations, and at least in the viable genomes that we observe, insertions and deletions (also termed *indels*) are less frequent than point mutations. *Indels* occur more frequently in homopolymeric tracts or repeated sequences. The first high mutation rates determined for RNA viruses were unexpected at a time when mutations were considered rare events in the biological world, and procedures for the analysis of genetic material at the molecular level were not available. High mutation rates originate from the absence or low efficiency of proofreading-repair activities in the polymerases that catalyze their replication. These mutation rates are about one million-fold higher than those typically observed during replication of chromosomal DNA, marking a striking difference between the DNA-based cellular world and the RNA virosphere.

Although quantification of many types of genome variations are lacking, current evidence, obtained mainly from deep sequencing, suggests that variations occur exuberantly, and that we observe only a minimal proportion of those that occur during viral genome replication. Many genomes modified by mutation or recombination are eliminated (or kept at low frequency) by negative selection because they inflict a fitness cost. It is expected that similar mechanisms of genome variation operated in the ancestral times when the cellular and virological worlds were under construction (Fig. 1). It can be suspected that early polymerases (be RNA- or protein-based) had a more relaxed nucleotide discrimination than present day enzymes. It is also likely that proofreading-repair and post-replicative repair activities – that preserve the identity of present day large cellular and viral DNA genomes – evolved with the increase of complexity of genetic information. Therefore, we conjecture that at the times of the primitive proto-virus, proto-cell soup, mutation rates were generally high, and that all living matter was much more fluid in terms of genetic instability and genetic exchanges than today. Although it is unlikely than such a proposal can ever be proven in a definitive way, relevant information may come from metagenomics and the use of analytical methods based on computations with increasing power.

Evolutionary Dynamics

Our understanding of virus evolution is founded on two main general approaches: (i) determination and comparison of nucleotide (and deduced protein) sequences, and (ii) design of experiments in which evolutionary events can be tested under controlled laboratory conditions, referred to as experimental evolution. Approach (i) is based upon the determination and comparison of nucleotide sequences of virus from multiple sources: environmental samples (soil, water, cellular organisms without disease symptoms), and infected hosts as they manifest disease symptoms. Sequence information is complemented with virus isolation, purification of viral particles, and functional and structural studies. Comparative structure-function relationships of encoded proteins and viral particles may assist in the establishment of connections among viruses which are distant at the genomic level.

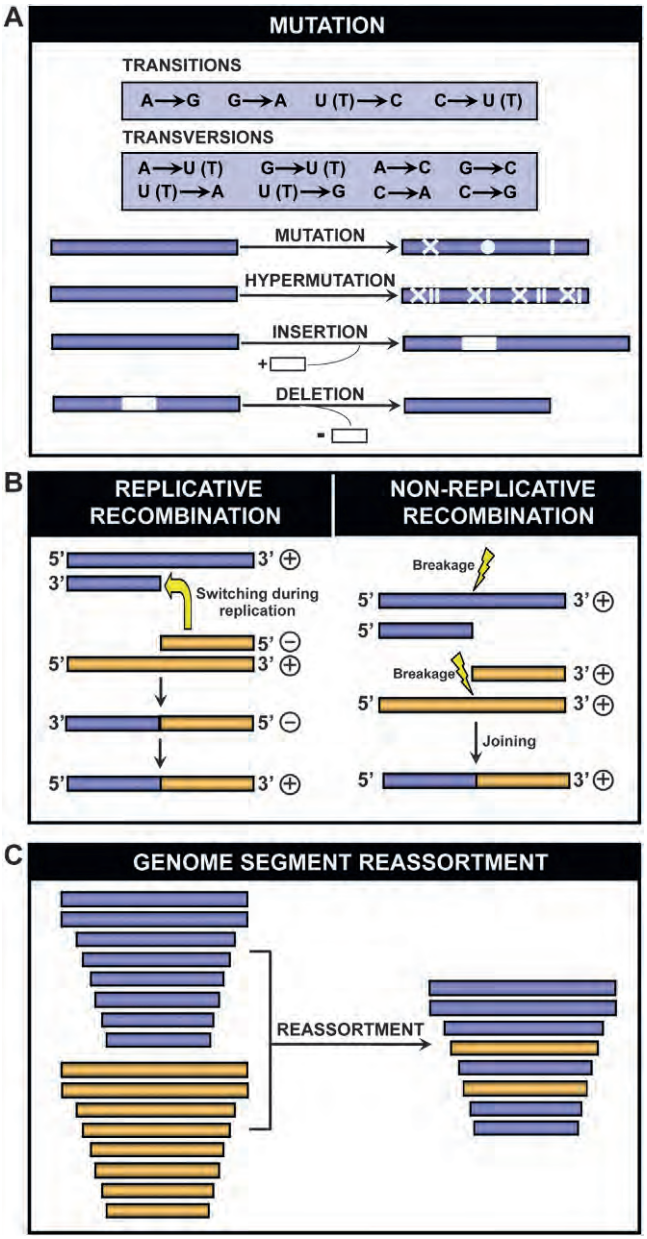

Fig. 3 Representation of genetic modifications that can alter viral genomes. (A) Major types of mutations in RNA (made of the standard nucleotides A, G, C and U as building blocks) and DNA (with T instead of U). A genome is depicted as an elongated rod. Symbols on the rod (cross, circle and line) represent mutations. A fragment inserted or deleted from the genome is depicted as an empty rod. (B) Recombination (formation of mosaic genomes) is found in DNA and RNA genomes. Here, two mechanisms of recombination are shown for RNA. RNA polarity is indicated by + and − symbols. Replicative recombination is the result of template switching during template copying. Nonreplicative recombination is depicted as the outcome of ligation (joining) of RNA fragments. (C) Segment reassortment takes place in segmented genomes. The example shown here refers to human influenza virus type A. In the new segment constellation six genomic segments originate from one parent (blue) and two from the other (gold). The figure is modified from Domingo, E., 2016. Virus as Populations. Composition, Complexity, Dynamics, and Biological Implications. Amsterdam: Academic Press Elsevier, with permission from Elsevier.

Approach (ii) consist of a wide range of experimental designs aimed at interrogating viral populations regarding their evolutionary potential, and response to selective constraints interposed in their infectious cycles.

Inferences From Genomics and Structure

The International Committee on Taxonomy of Viruses (ICTV) in its 2017 report divides known viruses in 10 Orders, and each of them is subdivided in families (in some cases also subfamilies), genera, and species. Unless viruses belong to the same species, nucleotide sequence identities are too tenuous to establish reliable phylogenetic relationships that could relate present day DNA or

RNA viruses. Not only point mutations but also signs of ancestral recombination and lateral gene transfer events render the establishment of phylogenetic relationships for the virosphere extremely challenging. A pan-virus phylogeny is not within reach. However, the fundamental observation that protein structure evolves far more slowly that the corresponding coding sequences, permits the proposal of virus groupings based on the presence of functionally related proteins or in similarity of three dimensional structures of encoded proteins, including the viral capsid. These approaches have grouped large DNA viruses in three distinct categories: the nucleo-cytoplasmic large DNA viruses (NCLDVs), the herpesviruses and the baculoviruses.

It should be emphasized that while phylogenetic relationships inform of the natural history of viruses and their possible ancestors, a given phylogenetic position is not necessarily associated with disease characteristics. This is illustrated by examining the 2017 ICTV report. The order *Picornavirales* includes 7 families, one of them being the *Picornaviridae* that are divided into 40 genera, one of which, the Aphthoviruses, comprises four species: bovine rhinitis A and B viruses, equine rhinitis A virus, and FMDV. It is obvious from their names that the representatives of these viral species do not share host specificity or disease symptoms. The lack of correlation between phylogenetic position and disease potential is part of a general indeterminacy of biological behavior in connection with genomic features. Specifically, regarding viral pathogenesis, one or a few amino acid substitutions have been associated with changes in virus-host interactions (i.e., receptor recognition, capacity to replicate in some cell types, capsid stability, particle transmissibility, etc.) that bear on disease potential. Thus, a limited number of genomic changes can enhance or decrease virulence without modifying the phylogenetic position of a virus.

Inferences From Experimental Evolution

Viruses that can be adapted to cell culture can be used to establish cytolytic or persistent infections in established cell lines, primary cells, organ explants or entire host organisms. These designs have served to understand the response of viral populations to environmental changes (pH, temperature, ionic strength, host cell types, etc. in cell cultures, or host species and their immune status in vivo), or to externally applied selective constraints (drugs, antibodies, etc.). In many cases quantifications of drug and antibody resistance have been performed, with obvious implications in clinical virology, and for the planning of antiviral treatments and vaccine design. Overall, these studies have supported a remarkable capacity of viruses to respond to constraints for survival, in particular the highly variable RNA and single-stranded DNA viruses for which evolutionary events can be recorded in days, and sometimes even in hours. They are excellent systems to establish basic principles of evolution.

Several concepts that originated in population genetics have found support in experimental studies with RNA viruses. An important concept is Muller's ratchet, or fitness decrease by the irreversible accumulation of mutations postulated for asexual organisms. Indeed, in model experiments consisting of repeated plaque-to-plaque transfers (a way to mimic sequential bottleneck events in the laboratory) viral genomes accumulated mutations concomitantly with average fitness decrease. These studies revealed also that multiple mutational pathways are available to a virus for fitness gain, supporting once again a remarkable flexibility for survival. Other important concepts such as the Red Queen hypothesis (parallel fitness gain in competing populations) or the competitive exclusion principle (inability of different evolving populations to coexist for prolonged time periods using the same resources) have found support in experiments with RNA viruses.

Dissection of the composition of viral populations as they replicate in infected hosts was achieved traditionally by molecular cloning (in standard vectors such as bacterial plasmids) of viral genomes (or genes of interest within viral genomes), followed by traditional Sanger sequencing of the individual clones. More recently, application of deep sequencing methodologies has permitted further penetration into the composition of mutant spectra. Despite such procedures analyzing a limited sample of the viral populations under study, they have documented that populations of RNA viruses and some DNA viruses consist of extremely complex collections of mutant genomes as a consequence of high mutation rates, and the capacity of viable genomes to accept part of the newly arising mutations. Mutant distributions are generated rapidly, even following a bottleneck event, during host-to-host transmission (Fig. 4). The mutant spectrum changes displayed in Fig. 4 may explain a frequent observation made with pathogenic viruses, that the rate of virus evolution within individual hosts (intra-host evolution) measured as the number of mutations accumulated per unit time, is significantly higher than the rate calculated for the same virus at the epidemiological level, when multiple transmissions have taken place in the field (inter-host evolution). The fact that viruses replicate as mutant distributions has several implications for clinical virology and the control of virus disease. Also, interestingly, the finding of extreme genome heterogeneity approximates present day viruses to the type of population structure postulated for the primitive replicons at the onset of life (bottom box in Fig. 1). This remarkable connection deserves further discussion.

Viral Quasispecies

Both DNA and RNA viruses are continuously evolving in the present day biosphere as friendly symbiotic partners or as agents of disease. High mutation rates determine that a progeny genome cannot be identical to the corresponding parental genome that served as template for replication. Both in nature and in laboratory preparations RNA viruses and some DNA viruses are found as collections of closely related genomes that may differ in several mutations. The mutant ensembles, mutant spectra or mutant clouds are termed viral quasispecies (Fig. 5), and their complexity (how many mutations distinguish individual genomes) can be quantified by several diversity indices (i.e., mutation frequency or nucleotide diversity). The viral quasispecies concept is an extension to viral genomes of a general theory of molecular evolution formulated by M. Eigen, P. Schuster and their colleagues

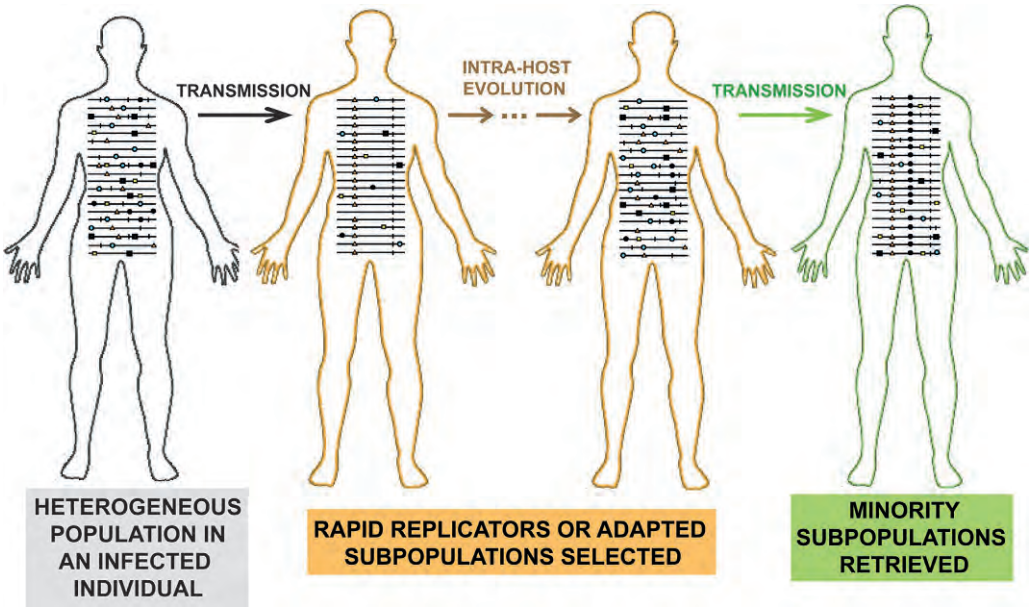

Fig. 4 Mutant distributions during infection and transmission. An infected individual includes a spectrum of mutants, not a repeated genomic sequence, due to high mutation rates during viral replication. Transmission events and intra-host evolution are represented by long and short arrows. Only a subset of the genomes is efficiently transmitted from the donor (individual with black outline) to the recipient (brown outline). The virus in the recipient individual replicates and broadens its mutant spectrum. Again, only a subset of genomes from this mutant spectrum that resemble the ones in the first transmission are efficiently transmitted to the third individual (green outline). The net result is that rates of evolution will appear as slower than those within each host. Taken from Domingo, E., 2016. Virus as Populations. Composition, Complexity, Dynamics, and Biological Implications. Amsterdam: Academic Press Elsevier, with permission from Elsevier.

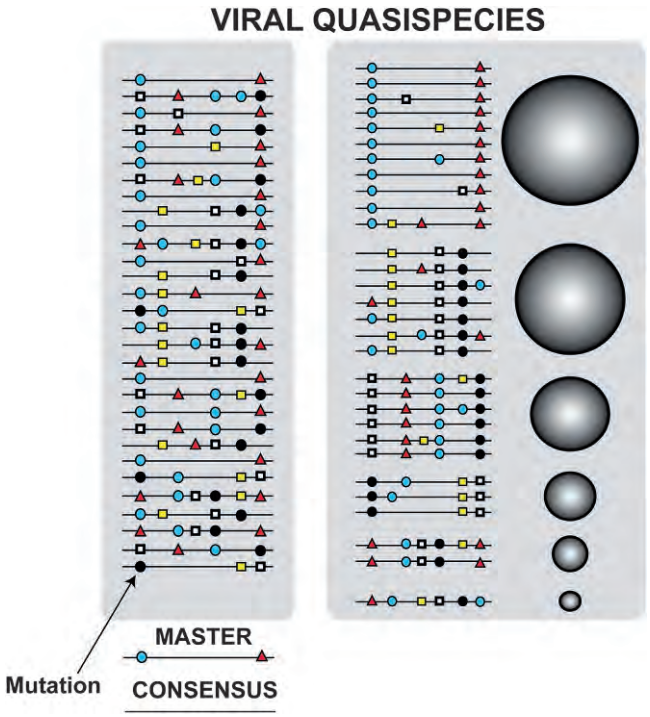

Fig. 5 A representation of viral quasispecies. Genomes (from the same replicative ensemble or the same infected host) are represented as horizontal lines and mutations as symbols on the lines. Note that the master (dominant) sequence in the mutant distribution on the left does not coincide with the consensus sequence. The representation on the right illustrates that a seemingly random distribution of genomes can be divided into six categories of different abundance given by the size of the spheres. The figure is modified from Domingo, E., 2016. Virus as Populations. Composition, Complexity, Dynamics, and Biological Implications. Amsterdam: Academic Press Elsevier, with permission from Elsevier.

to understand self-organization and adaptability of primitive replicons at the early stages of life development prior to the advent of proto-viruses and proto-cells (Fig. 1).

The application of quasispecies theory to virology has helped in the interpretation of many observations in infections both in vivo and in cell culture, regarding the way viruses respond to selective constraints. The biology of RNA viruses is influenced by the dynamic, continuously changing repositories of viral mutants that cannot be captured by studying consensus sequences alone. Examples of mutant spectrum-dependent implications are: (i) change in cell tropism due to mutations that can alter cell receptor specificity, (ii) capacity to respond to external constraints such as antiviral therapies due to the preexistence, or newly generation of viral mutants, (iii) capacity to overcome immune responses, (iv) reversion of attenuated virus vaccine strains to virulence, (v) the presence of a molecular memory in the form of genomes that are present as minorities in a mutant spectrum, but that were dominant at an early phase of evolution of the same lineage. Memory is a reflection of viral quasispecies behaving as complex adaptive systems. A hallmark of quasispecies is that the components of a mutant spectrum may engage in positive (complementation, cooperation) or negative (interference) interactions that affect the behavior of the ensemble. Internal interactions reinforce the notion of mutant distributions acting as "units of selection", as implicit in quasispecies theory.

A corollary of quasispecies theory is the so called error threshold relationship that states that for any replicative system there is a maximum mutation rate compatible with maintenance of genetic information. Exceeding this limit (a transition often termed violation of the error threshold) results in loss of information. Applied to viral populations, this prediction has been experimentally verified by achieving virus extinction with mutagenic nucleotide analogues that elevate the mutation rate above the maximum tolerable limit. This is now an active area of antiviral pharmacology termed lethal mutagenesis. The transition toward virus extinction occurs through an expected increase of mutation frequency, but, most significantly, without any modification of the consensus sequence. In other terms, a profound modification such as loss of infectivity (the ultimate goal of an antiviral intervention) can be achieved without any modification of the consensus sequence. The relevance of mutant spectra for the understanding of viral dynamics is evident.

Quasispecies is an active field of research, both at the theoretical and experimental level. Extensions of quasispecies theory to complex cellular organisms in variable fitness landscapes are compatible with any of the mechanisms of genome variation described for viruses, including recombination and genetic exchanges (Fig. 3). It is tempting to propose that the type of quasispecies dynamics at play with present day viruses might have been the norm in the developing cellular world portrayed in Fig. 1, in epochs still devoid of repair mechanisms for genetic systems, including pre-cellular assemblies. This should have provided a propensity for episodes of error catastrophe, but also extreme plasticity together with complementation and cooperation relationships, to explore molecular pathways for fitness gain. Unfortunately, such flexibility rendered extremely difficult the reconstruction of past events. What we analyze in the present day biosphere and virosphere may be tiny remnant samples of an exuberant complexity that has been lost forever.

Summary and Prospects

Viruses are continuously evolving in the present day biosphere, and there is no reason to suspect that they were not evolving in the past, even at faster rates. The mechanisms of molecular evolution of viruses that we have characterized during the last decades are similar to the mechanisms used by cells. In particular, present day gene transfers and integrations of viral genomes into cellular DNA probably reflect transfers that have occurred among viruses and cells at all times during their generation and gradual increases of complexity. Indications are that fluidity of the living world might have been even more exuberant in the remote past than today.

These arguments can be proposed thanks to results brought about by salient methodological developments. Indeed, the introduction of molecular tools to the study of evolution in the second half of the 20th century changed dramatically the views on the role of genetic change in the present day evolution of organisms. We are aware now that we live in a plastic biosphere that exploits a range of mechanisms of adaptation to different environments, while being subjected also to random events, fundamentally in the form of bottlenecks that accentuate genetic drift. However, the adaptive pluripotency has a cost. Viral disease can be viewed largely as a process of virus adaptation to multiply in tissues and organs of differentiated host organisms, resulting in perturbation of essential and non-essential (referred to as "luxury") cellular functions. The adaptive capacity not only of viruses but also of many cellular types (bacteria, eukaryotic monocellular parasites, tumor cells, etc.) underlies their persistence and disease potential. Mutator bacteria (those that display enhanced mutation rates) pose a specific threat, and both antiviral resistance in viruses and antibiotic resistance in bacteria are major public health concerns. Microbial resistance, genetic disease or cancer are all mediated largely by the same evolutionary mechanisms (mutation, recombination, genetic exchanges, and resulting population heterogeneities) that appear to have presided viral and cellular evolution over eons.

Current evolutionary dynamics for viruses is easier to dissect than the events that took place in the past. However, an expectation is that mounting information from genomics and proteomics of present day organisms, conjointly with bioinformatics tools and increasing computational power may assist in the reconstruction of past events. Improvements may also come from application of deep sequencing technologies that can be focused not only on the most abundant representatives of the biological world but also on viral and cellular minorities that may be hiding in environments still to be explored. Viral or cellular subpopulations that are a minority in one environment might be mobilized and driven towards dominance to face adaptive needs. This is becoming clear for RNA viruses but may be also true of other viruses and cell populations. There is increasing evidence of cellular heterogeneities whose possible biological relevance remains largely unknown.

The present status of knowledge about evolution indicates that the once considered static DNA biosphere (made of DNA cells and DNA viruses) is actually much more variable than thought. Genome rearrangements, recombination, LGT, participation of mobile elements and subcellular entities, render DNA and its environments remarkably dynamic. Expressed in simple terms, an "extremely" dynamic RNA biosphere coexists with a dynamic DNA biosphere. Molecular analyses have also unveiled a difference between the modifications that actually occur in viral and cellular genomes, and those that we observe. As a final summary statement, the study of viral evolution has furnished new insights into the evolutionary history of life on Earth, and it has reinforced the need to consider Darwinian principles for the control of viral disease.

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Vitamins and Vitamin-Like Compounds: Microbial Production☆

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Glossary

BCC Business Communications Company Inc., a leading US information resource producing detailed market research reports, newsletters, and conferences.
FDA Food and Drug Administration, an agency of the United States Department of Health and Human Services responsible for regulating *inter alia* food and dietary supplements.
Oleoresin A coloring or flavoring plant extract obtained by solvent extraction.
PUFAs Polyunsaturated fatty acids, a chemical group of substances containing some essential nutrients originally termed vitamin F.
Retinol Animal form of vitamin A derived from animal retinyl ester precursors or plant provitamin A carotenoids.
Saccharomyces cerevisiae A species of budding yeast traditionally used in baking and brewing and a well-studied eukaryotic model organism.
Sapropel A version of microbial transformation wherein DNA (or RNA) is returned to a species in which it could naturally occur, thereby the nucleic acid used may have been subject to modifications producing a novel order of genes or control elements like promoters or multiple gene copies.
Self-cloning A version of microbial transformation wherein DNA (or RNA) is returned to a species in which it could naturally occur, thereby the nucleic acid used may have been subject to modifications producing a novel order of genes or control elements like promoters or multiple gene copies.

Nomenclature

AMP	adenosine monophosphate
BCC	Business Communications Company Inc.
DHBP	3,4-dihydroxy-2-butanone 4-phosphate
DRL	6,7-dimethyl-8-ribityllumazine
FAD	flavin adenine dinucleotide
FDA	Food and Drug Administration
FMN	flavin mononucleotide
gluc-6p	glucose-6-phosphate
GMP	guanosine monophosphate
GRAS	generally recognized as safe
GTP	guanosine triphosphate
IMP	inosine monophosphate
2KGA	2-keto-L-gluconic acid
PUFA	polyunsaturated fatty acid
ribu-5p	ribulose-5-phosphate
XMP	xanthosine monophosphate

Defining Statement

Studies on the worldwide nutritional status repeatedly reveal vitamin deficiencies and a need for their dietary supplementation. Also, with an increasing number of reported beneficial health effects, the growing vitamin market demands cost-efficient production processes, employing genetically engineered microorganisms as alternatives to chemical synthesis.

Applications and Market

Vitamins, a chemically heterogeneous group of organic compounds essential for an organism, are by definition substances that are not or only inadequately provided by the organism's anabolism. These nutrients fulfill catalytic or hormone-like functions, and thus

☆*Change History*: October 2016. Klaus-Peter Stahmann updated text, some figures in Table 1, and added references.

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a daily dietary intake of small amounts satisfies the requirements. Apart from a catalogue of symptoms caused by vitamin deficiency, nowadays vitamins have gained additional attention due to numerous preventional health benefits, for example, in neoplastic diseases like cancer or degenerative diseases like rheumatism. In developed countries, a more than sufficient and seasonally independent food supply of all vitamins and vitamin-like compounds is available – if only the consumer chooses the right diet. However, studies repeatedly reveal inadequate nutrient intakes, even if situations creating increased requirements like pregnancy, certain pre-existing medical conditions, and an unhealthy lifestyle are made allowances for. A balanced and varied diet should contain nutrient-rich fruit and vegetables, milk and grain products, as well as sea fish and low-fat meat. According to the large-scale eVe Study, nearly 50% of the Spanish population need to adjust their usual food pattern toward a more nutrient-dense, healthier diet. Shortages in single vitamins or combinations of riboflavin, folate, vitamin A, vitamin D, vitamin E, and vitamin C were found in a high proportion of the 10,208 participants. Although the pattern of inadequate vitamin intake varies between populations, it is a worldwide problem causing a demand for additional, industrially produced vitamins.

After the isolation of all 13 vitamin groups by extraction techniques in the 1930s and 1940s, structure determinations of the vitamins led to the development of chemical synthesis procedures, culminating in several Nobel prizes. However, today vitamins are produced by chemical synthesis, extraction chemistry, biotechnical or combined methods, in which biotechnical procedures comprise fermentation or bioconversion processes. Production scales and methods of syntheses of these compounds within the scope of this article are compared in Table 1. Although only the polyunsaturated fatty acid (PUFA) arachidonic acid, vitamin B2, and vitamin B12 are produced exclusively by biotechnical processes, the development of further microbial techniques to replace chemical synthesis is also pursued. Several factors like increasing ecological awareness and environmental constraints, sustainability of renewable resources, and consumer demand for naturally produced products have an impact. However, regarding the latter, no scientific data support a significant difference between chemically and natural derived vitamins, as stated by the Health and Consumer Protection Directorate-General of the European Commission in 2003 for the pigmentation of fish by astaxanthin synthesized chemically or originating from the natural source *Xanthophyllomyces dendrorhous*. But, increasing costs of fossil resources and disposal of chemical waste count in favor of biotechnical processes, since not only is there a need for an efficient process of manufacture but also one that makes a handsome profit for a company in the international market.

This article focuses on vitamins and vitamin-like compounds, whose microbial production already is commercially relevant, with emphasis on the production of vitamin B2. Subsequently, the current state of industrial development of other vitamins is discussed and the impact of the so-called white biotechnology on our daily life and on industry is considered in the Section 'Conclusions and outlook'.

The world market value of vitamins and vitamin-like compounds is estimated at nearly US$2.3 billion by 2007. As a result of the entry of Chinese competitors in the market sales fell from US$2.7 billion in 1999 even though the market was still growing by approximately 4% annually. Interestingly, however, the market volume of vitamins is still a multiple of that of antibiotics. The leading vitamins are E, C, and A, summing up to more than 65% of total sales in 2002. In general, the majority of industrially produced vitamins are used in animal feed, with a share of almost 50%. The supplement and food industries follow with 22 and 17%, respectively, and 11% is used in cosmetic products. The percentage in pharmaceutical and technical applications is marginal.

An increasing demand for feed vitamins is caused by the growing world population going along with factory farming ashore and at sea, in which the fodder, especially conserved feed, needs vitamin supplementation to enable a healthy and competitive livestock. Moreover, people directly consume almost 40% of all vitamins produced either as dietary supplements or as fortified foods. Indeed eating habits, lifestyle, some medical conditions, and food processing or preservation methods render additional vitamin intake useful. Additionally, the positive appeal of vitamin enriched products is boosted by scientific and public reports about their beneficial health effects. Thus nowadays vitamin fortification of industrially produced food to increase nutritional value is very common, with breakfast cereals and fruit juices being typical examples. In parallel, the market for dietary supplements in the form of tablets, powder, or liquids is rapidly growing: according to a Forsa survey in 2000 every third German regularly consumed dietary vitamins with annual costs of €300 on average. The consideration of vitamins as a panacea creates a never-ending debate about the pros and cons of dietary supplementation of vitamins. Pharmaceutical applications like drip-feeding and prescription of vitamins account for less than 1% of the vitamin market.

Table 1 Industrial production of vitamins and vitamin-like compounds showing significant impact by microorganisms

	Production method				
Compound	*Chemical synthesis*	*Extraction (chemical)*	*Microbial*	*Combined*	*World production (tons per year)*
Fat soluble					
Carotenoids	+	+	+	+	>1,000
PUFAs		+	+		>1,000
Water soluble					
Vitamin B2			+		<10,000
Vitamin B12			+		<100
Vitamin C				+	>100,000

Table 2 Microorganisms applied for large-scale production of vitamins and vitamin-like compounds

Compound	Production microorganism			Percentage of world production
	Bacteria	Fungi	Microalgae	
Fat soluble				
Carotenoids				
β-Carotene		*Blakeslea trispora*[a]	*Dunaliella salina*[a]	15
Astaxanthin		*Xanthophyllomyces dendrorhous*[a,b]	*Haematococcus pluvialis*[a]	<10
PUFAs				
Arachidonic acid		*Mortierella alpina*		<10
Docosahexaenoic acid or Eicosapentaenoic acid		*Yarrowia lipolytica*	*Thraustochytrium*	<10
Water soluble				
Vitamin B2	*Bacillus subtilis*	*Ashbya gossypii*[a]		100
Vitamin B12	*Pseudomonas denitrificans*[a]			100
Vitamin C[c]	*Gluconobacter suboxydans*[a] and *Ketogulonicigenium vulgare*[a,d]			50

[a]Natural overproducer modified by strain improvement.
[b]Original name: *Phaffia rhodozyma*.
[c]The bioprocess yields 2-keto-L-gluconate from which L-ascorbic acid, the γ-lactone, is derived by a chemical downstream step.
[d]Original name: Gluconobacter oxidans.

In contrast to the pharmaceutical industry where highly purified vitamins are applied the feed industry, being the principal client, takes an additional advantage of microbial production: biomass residua rich in lipids and proteins represent extra nutrients and thus less purified vitamins have a positive feeding effect. In case of vitamin B2 and the carotenoid astaxanthin, even poor qualities of such vitamins that have residual biomass are being sold, and for which the extraction procedure is simplified and the chemical load is reduced. Nevertheless, although all vitamin productions in microorganisms must be licensed they must also have generally regarded as safe (GRAS) status.

Microorganisms used in industrial scale production of vitamins belong to bacteria, microalgae, or fungi. In classical approaches microbial vitamin production starts with a natural overproducer, such as the fungus *Ashbya gossypii*, rich in vitamin B2. To improve the yield successive rounds of mutagenesis and selection are carried out along with optimization of process parameters. Apart from classical strain improvement, modern biotechnology allows genetic engineering of many microorganisms that are already approved for use in other industrial processes. Their metabolic engineering for application in a vitamin manufacturing process is facilitated by increased knowledge of the biochemical and regulatory pathways in these microorganisms. In-depth analyses of enzymes and their substrates with regard to flux control and genome-wide transcriptional analysis allow further optimization if genetic engineering is feasible and the genomic DNA sequence is known. Nevertheless, most industrial strains still are based on improvement of natural overproducers. Only a few, most impressively Bacillus subtilis in the vitamin B2 production process, are a result of sophisticated genetic engineering (Table 2).

The efficiency of microbial vitamin production is very diverse. Final product titers range from about 100 mg L^{-1}–100 G L^{-1}. The yield, that is, conversion of the carbon source into the final product, ranges from inefficient to highly efficient. Especially when phototrophic microorganisms are used, turnover time and concentrations are low, a large production area is needed, and downstream processing is cost-intensive. Although strains and production processes are continuously optimized, some biotechnical procedures are used only for production of niche products in comparison to chemical methods. Examples include high-quality stereo-specific carotenoids and PUFAs as dietary supplements in infant formulas.

Fat-Soluble Compounds

Carotenoids

Carotenoids are organic pigments naturally produced by microorganisms and plants. They fulfill diverse biological functions like energy transfer in photosynthesis, photoprotection, species-specific coloration, and supply of precursors, for example, in the biosynthesis of vitamin A. They consist of a long carbon backbone derived from isoprenoid moieties. In many cases, both termini form ring systems. The extended system of conjugated carbon–carbon double bonds is responsible for the light absorption and the bright color of the carotenoids. The over 600 different carotenoids described so far fall into two main groups: the carotenes, non-oxidized hydrocarbon carotenoids, and the xanthophylls, oxygenated carotene derivatives. Following the isoprenoid pathway, natural carotenoid synthesis either starts from acetyl-CoA via mevalonate or from deoxyxylulose-5-phosphate, a condensation product of pyruvate and glyceraldehyde-3-phosphate.

Less than a dozen carotenoids are produced industrially from vegetable sources or petals, by fermentation, or by chemical synthesis, the latter providing for the bulk amount of carotenoid demand worldwide. Lutein-containing oleoresins from *Tagetes erecta* grown in large scale mainly in India and China are extensively used for egg yolk and broiler pigmentation. Despite numerous intensive efforts to develop classically improved or genetically engineered carotenoid production strains, microbial carotenoid production is very limited, although the construction of the first recombinant carotenoid-producing *Escherichia coli* strain dates back almost 30 years ago. By contrast a sheer unlimited source for commercial β-carotene production could be crude palm oil, if cheap extraction methods to recover the highly diluted product from the oil could be developed.

Apart from being used as food colorants and animal feed supplements for poultry and aquaculture, carotenoids play an increasing role in cosmetic and pharmaceutical applications due to their antioxidant properties. The pigments are often regarded as the driving force of the nutraceutical boom, since they not only exhibit significant anticarcinogenic activities but also promote ocular health, can improve immune response, and prevent chronic degenerative diseases.

β-Carotene

The nutritional role of carotenoids in human diet is best understood in the context of their provitamin A activity. About 50 carotenoids are known that can function as provitamin A, of which β-carotene is the most efficient one. There are several colorful fruits and vegetables rich in the red-colored β-carotene, examples of vegetables include carrots and sweet potatoes, but the best source is food derived from animals, especially liver and whole milk. A daily intake of 3–6 mg of β-carotene is estimated to ensure a beneficial blood concentration of retinol equivalents, the animal form of vitamin A.

β-Carotene is an important industrially produced carotenoid. As estimated in a Business Communications Company Inc. (BCC) report of 2005, β-carotene accounted for roughly a quarter of the total carotenoid sales in 2004, totaling about US$242 million. DSM Nutritional Products (Switzerland) and BASF (Germany) dominate the market with their chemical synthesis processes, but Chinese competitors are catching up.

Microorganisms account for about 15% of industrial β-carotene production. Vitan Ltd. (Ukraine) and Vitatene (Spain) use the heterothallic zygomycete *Blakeslea trispora*, producing, after intensive classical strain improvement by random mutagenesis and selection, according to patent literature, up to 7 G L^{-1} β-carotene in large-scale fermentations. Pigment production and accumulation is stimulated by several sex-specific pheromones, for example, trisporic acids, the synthesis of which commence after inoculation of the production fermenters with both B. trispora mating partners. The high β-carotene accumulation of 4% or more within the B. trispora biomass might be facilitated by the cell's high triglyceride content. Filtration after the fermentation process, solvent extraction of the dried biomass, and crystallization result in a pure product.

An alternative natural β-carotene producer of commercial interest is the halophilic green microalgae *Dunaliella salina*. It accumulates the pigments in oil globules in the chloroplast interthylakoid spaces, protecting them against photoinhibition and photodestruction. AquaCarotene Ltd. and Cognis take advantage of large, shallow ponds with high salinity in Western Australia that are exposed to intense solar radiation, in which the microalgae are cultivated. Other companies like Nature Beta Technologies with a production site in Israel employ open channels in the form of oblong raceways agitated by paddle wheels. Closed tubular systems are in an experimental stage. Excessive pigment formation in *D. salina* is achieved by numerous stress factors like high temperature, lack of nitrogen and phosphate but excess of carbon, high light intensity, and high salt concentration, the latter two having the highest impact. Whereas in open ponds β-carotene accumulates in the 100 mg m^{-3} range, the obviously more capital-intensive raceway ponds can deliver up to 15 G m^{-3}. An average yearly productivity of around 200 mg m^{-2} day^{-1} has been reported. Harvesting the biomass present at low concentration in the corrosive cultivation brine, for example, by filtration after flocculation, requires special attention. Dried *D. salina* biomass for sale contains 10–16% carotenoids, mainly β-carotene. In addition crystalline material obtained after extraction with edible oil is also sold.

Astaxanthin

Astaxanthin is biosynthesized by providing both β-ionone rings of β-carotene with hydroxyl- and oxo-moieties at C3 and C4, respectively. The intense dark red xanthophyll, which has no provitamin A activity, plays a role in pigmentation of various animals, conspicuous examples include wild and pen-reared salmon and red crabs, which compulsorily require astaxanthin in their diet. Although recent studies point to superior antioxidant properties compared with β-carotene, astaxanthin applications in nutraceuticals, cosmetics, and food industry are still scarce. The predominant use of the xanthophyll is as a pigmentation source, most notably in aquaculture of salmon and trout. Improved fertility and enhanced immune response are also ascribed to astaxanthin supplementation of the fish diet.

A BCC report released in 2017 estimated the astaxanthin market to be US$500 million in 2016. Astaxanthin sales account for 25% of the total carotenoid sales, similar to β-carotene. Chemical synthesis supplies the major part of commercial astaxanthin. A minor amount of astaxanthin originates from solvent extraction of crustacean waste or krill.

Microbial astaxanthin production, occupying a small proportion of the market, is based on the chromophyte microalga *Haematococcus pluvialis* and the basidiomycete yeast *X. dendrorhous*, originally named *Phaffia rhodozyma*.

H. pluvialis is grown heterotrophically under low irradiation in closed systems with an excess of organic carbon source such as acetate to achieve a high yield of green biomass, which is thereafter subjected to environmental and nutrient stress to induce aplanospore formation and astaxanthin accumulation. *H. pluvialis* cultures easily fall prey to contaminating microorganisms and

are sensitive to extreme environmental conditions. A selective cultivation environment is not available. Therefore, the second, reddening cultivation stage can only be operated for periods not exceeding 5–6 days if open systems are used. Closed photobioreactors in the form of transparent tubes or illuminated stirred tanks might be more efficient systems, but the increased investment and operation costs have to be taken into consideration. *H. pluvialis* biomass is harvested by settling and subsequent centrifugation. After drying and fracturing of the thick cell wall to ensure bioavailability of the enclosed pigment, the biomass contains 1.5–3% of astaxanthin as the 3S, 3S′ optical isomer. Several companies with production sites in Hawaii (Mera, Cyanotech), Israel (Algatech), and United States (BioReal AB) offer *H. pluvialis* astaxanthin in the form of a dried algal powder or an oleoresin extract.

X. dendrorhous provides the 3R, 3′R optical isomer of astaxanthin. Mutants selected for increased astaxanthin productivity are cultivated in conventional stirred tank reactors. After harvesting, disintegration, and drying, biomass containing 0.5–1% pigment is marketed by companies like ADM (Ecotone) or Igene (Aquasta) (both in the United States and, since 2013, in Europe). To get about 10 mg per g biomass in shake flasks three steps were performed. First, overexpression of the *X. dendrorhous* genes encoding the enzymes of the biosynthetic pathway. Second, improved precursor supply by an overexpressed 3-hydroxymethyl-3-glutaryl coenzyme A reductase gene. Third, these metabolic engineering steps were performed in a classically selected mutant with a 15-fold production in comparison to the wild-type strain.

Polyunsaturated Fatty Acids

PUFAs are vitamin-like compounds having important structural functions in cell membranes and lipid tissue, and serve as precursors for the biosynthesis of several hormones like leucotrienes, prostaglandins, and thromboxanes. PUFAs are aliphatic monocarboxylic acids with two to six carbon–carbon double bonds in biologically active cis configuration within their C18–C24 carbon backbone. The double bonds are separated by two single carbon–carbon bonds resulting in a nonconjugated polyene system. PUFAs are categorized as ω-3 or ω-6, depending on the position of the first double bond on C3 or C6, respectively, when counted from the terminal carbon atom of the fatty acid. They are biosynthesized starting from monounsaturated C18 oleic acid by a series of desaturase and elongase reactions. The genes of most of the PUFA biosynthetic enzymes have been cloned from various organisms. Since mammals lack the ability to introduce double bonds at the ω-3 or ω-6 position of the fatty acid carbon backbone, linoleic acid (18:2, ω-6) and α-linolenic acid (18:3, ω-3) are essential for them. Since the long chain PUFAs arachidonic acid (20:4, ω-6; ARA) and docosahexaenoic acid (22:6, ω-3; DHA) are incorporated in the developing brain, the neuronal tissue and retina of the human fetus studies with supplemented infant formula were performed. Human breast milk contains these PUFAs in tiny (<1%) but significant amounts. The correlation between long chain PUFAs content in the cerebral cortex of breastfed infants and better cognitive and visual function of infants fed with infant formula supplemented with long chain PUFAs suggested that the activity of the enzyme system of elongases and desaturases is suboptimal. Unfortunately, assays for both types of enzymes are not developed yet.

PUFAs have reached a global market size of >1 billion US$ and are almost exclusively derived from fish oil. In Australia genetic engineering of rape seed has been advanced by Nuseed to produce as much DHA on one hectare as can be extracted from 10,000 fish. Commercial scale microbial processes for the production of DHA and ARA based on natural isolates or classically derived strains are available, which are discussed in some detail here. Attempts to employ molecular engineering techniques in PUFA production have also been published. One example is *S. cerevisiae*, not a natural PUFA producer, that when provided with the appropriate desaturase and elongase genes could perform one or several biosynthetic steps toward eicosapentaenoic acid (20:5, ω-3; EPA) or DHA synthesis starting from the corresponding precursor fatty acids. A series of patent applications from DuPont published in 2004 and 2005 disclosed EPA production in a genetically modified oleogenic yeast, *Yarrowia lipolytica*. In 2013 genetic engineering resulted in about 150 g EPA per kg dry biomass. Analysis of all fatty acids extracted and liberated from glycerol esters more than 50% was found to be EPA.

Docosahexaenoic Acid

DHA, a 22-carbon ω-3 fatty acid with six double bonds, is found in membrane phospholipids of different cell types. For example, in the photosensitive part of the retina DHA accounts for more than 60% of the total fatty acid content. It functions as a signal molecule precursor, and has beneficial effects in prevention of cardiovascular diseases, probably by downregulating intracellular mechanisms leading to expression of proatherogenic genes. Fish, wild catch or from aquaculture, are the most important source of DHA in human diet. Fish cannot synthesize DHA by themselves but marine microorganisms at the lower end of the marine food chain are genuine DHA producers. Aquaculture is often fed with fish oil or fish meal, of which more than 1 million tons and 6 million tons, respectively, were produced in 2005. A negligible proportion of the oil is used for products for the human dietary supplement market. The fish meal industry maintains that fish meal and oil is produced from sustainably managed fish stocks. However, in the general perception, in view of the decade-long over-exploitation of common marine fish resulting in ever-reducing catch size, fish oil is not considered a sustainable source of DHA (and other PUFAs) and microbial alternatives have been developed.

The biomass of the marine microalga *Crypthecodinium cohnii* consists of triglycerides with a high percentage of DHA, which is almost free of other PUFAs. In protracted (200–400 h) lab scale fed-batch fermentations with acetic acid or ethanol as the carbon source, about 100 G L^{-1} biomass accumulates with a triglyceride content between 30 and 50%. DHA represents about a third of the fatty acids of the triglyceride phase obtained by solvent extraction.

The genera *Schizochytrium*, *Thraustochytrium*, and *Ulkenia*, marine heterotrophic protists classified as labyrinthulomycota, are also preferred sources of microbial DHA. In lab scale fermentations with a *Schizochytrium* sp. strain 60 G L^{-1} dry cell mass was obtained with a fatty acid content of 70%, of which 37% was DHA. DHA-containing products obtained from protist fermentations is offered by Martek/DSM United States focusing on the human nutrition and aquaculture market, respectively.

Arachidonic Acid

ARA, a 20-carbon ω-6 fatty acid with four double bonds, is a natural component of breast milk, supporting neonatal eye and brain development. Breastfed infants have higher ARA blood levels than formula-fed infants. Infant formulas fortified with ARA and DHA, sold in Europe for more than 10 years, after permission by the Food and Drug Administration (FDA) were first introduced in the United States in 2002 and since then have become increasingly popular.

In contrast to DHA, which can be obtained from fish oil or microbial processes, industrial ARA production solely relies on fermentation processes. First publications on the zygomycete *Mortierella alpina* as a suitable ARA producer appeared in the late 1980s. With the *M. alpina* strains IS-4 or ATCC 32222 lab scale processes were developed affording about 10 G L^{-1} ARA as triglyceride after 8–10 days fermentation runs in conventional stirred tank reactors. The morphology of the fungal biomass in the reactor is of specific concern. It should appear as small pellets with a diameter of 1–2 mm to allow for sufficient mass transfer and to keep the broth viscosity at an acceptable level. About 70 g L^{-1} biomass and containing 18 g L^{-1} ARA are reported. After fermentation, the *M. alpina* mycelium is harvested by centrifugation or filtration, dried, and solvent-extracted. Alternatively, the mycelium is homogenized as an aqueous suspension and extracted with a water-immiscible organic solvent.

Fungal ARA is produced and marketed as Arasco by Martek /DSM (United States). In 2004, Cargill (United States), a leading supplier of agriculture products and food ingredients, entered into a joint venture with Wuhan Alking Bioengineering Co. Ltd. (Hubei Province, China), an ARA producer serving the Chinese infant formula business. Suntory, a leading Japanese non-alcoholic beverage manufacturer and a pioneer in microbial PUFA production, provides a fungal ARA oil under the trade name Suntga40S. A small volume but premium price application of ARA enriched oil is its use as the basis for cosmetic creams.

Water-Soluble Vitamins

Riboflavin

Riboflavin is the common name of 7,8-dimethyl-10-(D-1′-ribityl)isoalloxazine, also known as vitamin B2, colorant E101, lactoflavin, lactochrome, or ovoflavin. The latter names referring to the source the vitamin was derived from. The compound is naturally synthesized by plants and most microorganisms, but not by higher eukaryotes. Starting from GTP and ribulose 5-phosphate the riboflavin biosynthesis pathways of fungi and bacteria are similar, albeit the order of two consecutive biosynthetic steps, the reductase and deaminase reactions, is inversed. The genes encoding the riboflavin biosynthetic enzymes are well conserved among bacteria and fungi. Vitamin B2 has key functions in energy metabolism, maintenance of healthy skin and muscles, support of immune and nervous system, and promotion of cell growth and division. Riboflavin is the precursor for the coenzymes FMN (flavin mononucleotide) and flavin adenine dinucleotide (FAD), which are both important electron carriers in biological redox reactions. Furthermore, the two flavo-coenzymes participate in nonredox phenomena like bioluminescence, light sensing, phototropism, DNA protection against UV, and in resetting of the circadian clock. Light sensitivity and poor resorption makes riboflavin deficiency recurrent, as suggested by worldwide surveys on nutritional status, and supplementation is often recommended. Overdosing due to dietary supplementation does not occur owing to the direct excretion of riboflavin in the urine. In industrialized countries processed food is often fortified by the use of riboflavin as a colorant or vitamin supplement. The main application (70%) of commercial riboflavin is in animal feed, since productive livestock, especially poultry and pigs, show growth retardation and diarrhea in case of riboflavin deficiency. According to a report by SRIC, a consulting company in Menlo Park (California), in 2005 the need for industrially produced riboflavin was estimated at 6500–7000 t $year^{-1}$.

Industrial riboflavin production, a domain of the chemical industry for a long time, followed a six- to eight-step route starting from 3,4-xylidine, barbituric acid, and D-ribose. The latter is provided by the chemical conversion of glucose or more recently by fermentation from glucose using transketolase-deficient *Bacillus* sp. production strains. The first commercial single-stage riboflavin fermentations came up in the 1940s employing *Clostridium acetobutylicum* and, more efficient, the fungi *Eremothecium ashbyi* and *A. gossypii*. However, all three companies manufacturing riboflavin by fermentation in 1965 shut down their production plants in 1968 as their production processes were competitively disadvantaged to chemical processes. Nevertheless, riboflavin biosynthesis meanwhile has become the paradigm for environmentally superior white biotechnology replacing chemical production processes. Although Merck (Germany) resumed fermentation using *A. gossypii* in 1974, the success story started with an *A. gossypii* production plant by BASF (Germany) in 1990. The market share of riboflavin bioprocesses thereafter increased from 5% in 1990 to 75% in 2002, of which initially, the two naturally vitamin B2-overproducing fungi *A. gossypii* and *Candida famata*, and later, genetically modified strains of the bacterium *B. subtilis* were used. However, *C. famata* is not employed any longer and chemical riboflavin production came to an end, leaving only *A. gossypii*- and *B. subtilis*-based production methods. Both processes are the outcome of classical strain improvement, genetic engineering, and process optimization.

The wild-type strain of the filamentous fungus *A. gossypii*, originally isolated as a severe but today negligible cotton pathogen, produces 2 mg riboflavin per gram of biomass, possibly for UV-light-protection of its spores. Adjustment of process parameters

allows a production above 100 mg G^{-1} biomass even by wild-type strains. However, at the industrial scale the performance of *A. gossypii* production strains was increased by analysis of the pathway from the very first enzyme, an extracellular lipase hydrolyzing plant triglycerides serving as the carbon source, to the carrier systems transporting the vitamin into the vacuole or extracellular space. Within a decade the production process was improved by (1) optimization of culture conditions, that is, different medium compositions for growth and production phases, (2) selection of antimetabolite-resistant mutants, that is, using inhibitors against key enzymes limiting reaction velocity like isocitrate lyase, (3) overexpression of RIB genes, that is, integration of additional copies of RIB3 encoding the enzyme converting ribulose-5-phosphate into 3,4-dihydroxy-2-butanone-4-phosphate (DHBP), and (4) decreasing the flux to unwanted side products by disruption of genes, for example SHM2, encoding cytosolic serine hydroxymethyl transferase that converts glycine, a precursor of riboflavin, into L-serine. Metabolic design of *A. gossypii* is possible by self-cloning, gaining stable genomic integrations targetable by homologous recombination. Large-scale fermentation of *A. gossypii* is advantageous because extracellular hydrolases allow high loads of osmotically inactive carbon sources like triglycerides removing the need for controlled carbon feeding and autolysis of the vitamin-accumulating cells can be controlled by a temperature shift.

B. subtilis, a soil bacterium, strains of which are traditionally used in food preparation in Asia, for example, for the soy bean fermentation product natto, is capable of synthesizing industrially relevant amounts of vitamin B2 only after genetic engineering. In wild-type strains, riboflavin biosynthesis is minimized to the physiological needs of the bacterium by a tight riboswitch transcriptional attenuation mechanism of the rib genes. A suitable *B. subtilis* host strain was obtained by resistance screening against three purine analogues (8-azaguanine (S1), decoyinine (S2), and methionine sulfoxide (S3)) (Fig. 1), yielding mutants with a deregulated purine synthesis pathway that provided an enhanced availability of the riboflavin precursor GTP. Starting from a purine-analogue resistant strain, a mutant resistant against the riboflavin analogue roseoflavin was selected (S4). Later it turned out that flavo-coenzyme-mediated attenuation of rib gene expression was abolished in this mutant, which expressed a flavokinase with drastically reduced activity causing reduced intracellular FMN and FAD levels. A defect in adenylsuccinate synthetase causing a block in the flux from inosine monophosphate (IMP) to adenosine monophosphate (AMP) was advantageous for purin precursor production, but the resulting adenine auxotrophy was detrimental to riboflavin overproducing strains. Overexpression of the B. subtilis rib operon comprising all relevant rib genes (ribA, ribG, ribH, ribB) by use of a phage promoter, multicopy genomic insertion of the engineered operon, and by providing an additional expression cassette for ribA encoding a bifunctional enzyme catalyzing two entrance reactions of the converging riboflavin pathway led to a production strain fit for industrial application (GE1

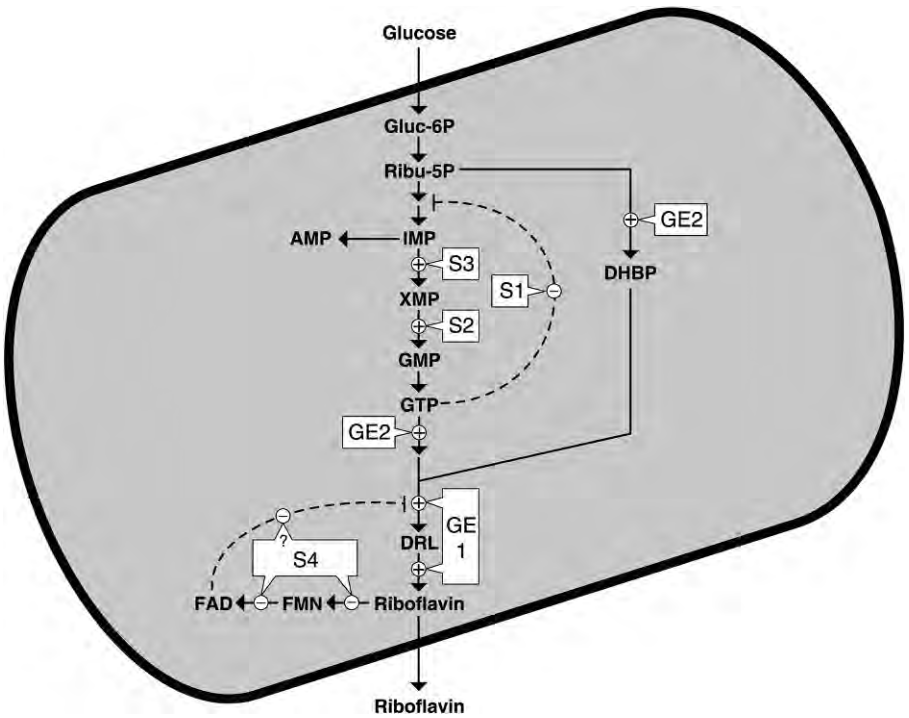

Fig. 1 Important steps in manipulation of biochemical and regulatory pathways to obtain riboflavin overproduction by *Bacillus subtilis*. Simplified scheme of metabolic steps (arrows) and relevant regulations (broken lines) from the main carbon source glucose to the targeted product riboflavin. ⊕ and ⊖ mark increased or decreased fluxes or activities, as appropriate, all with a positive effect on riboflavin production. Labels depict metabolic steps or regulations affected in antimetabolite selected mutants (S) or genetically engineered strains (GE). See text for details. gluc-6p, glucose-6-phosphate; ribu-5p, ribulose-5-phosphate; IMP, inosine monophosphate; AMP, adenosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; DHBP, 3,4-dihydroxy-2-butanone 4-phosphate; DRL, 6,7-dimethyl-8-ribityllumazine; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

and GE2). Meanwhile, a new generation of marker-free, self-cloned B. subtilis production strains have been constructed, achieving high production performance with a single copy of a precisely engineered rib operon. A mutation induced in the tkt gene reduces but not completely abolishes the enzymatic activity of the encoded transketolase enzyme, thus allowing higher intracellular concentrations of the intermediate ribulose-5-phosphate.

Rational design of high performance *A. gossypii* or *B. subtilis* production strains to channel a major metabolic flux toward the target compound and to prevent drainage of resources into side or waste products became possible only after a detailed understanding of riboflavin biosynthesis was available. Construction of the strains depended on a filled tool box for mutant screening, DNA synthesis, and recombination. PCR technologies and synthetic genes including their regulatory DNA components facilitated precision engineering to avoid surplus DNA and antibiotic markers in the final production strains.

During the fermentation process, riboflavin is exported from the cells into the medium and crystallizes in the fermentation broth due to its low solubility in neutral aqueous media. In *A. gossypii* additionally a transport into the vacuoles occurs. The long, needle-shaped crystals can easily be recovered from the fermentation broth and separated from the biomass by several rounds of centrifugation and resuspension in water. Microbial riboflavin production at an industrial scale turned out to be both cost-effective and environmentally friendly compared with conventional chemical synthesis: carbon dioxide emissions and use of nonrenewable resources were reduced by 80% each and water emissions by 66%. Fermentation products for food and pharmaceutical applications offer a higher purity as aniline and other impurities found with chemical synthesis are absent. For feed applications, products with reduced purity are accepted since the impurities consist of residual biomass with additional nutritional value.

Major riboflavin producers are DSM Nutritional Products (Switzerland) and Hubei Guangji (Hubei Province, China), both using genetically engineered *B. subtilis* production strains, and BASF (first in Germany but now in South Korea), employing genetically engineered *A. gossypii*.

In conclusion, the supersession of chemical vitamin B2 synthesis by fermentation aided by classical strain improvement and genetic engineering is an encouraging example of the potentialities of white biotechnology.

Cobalamin

Industrially produced vitamin B12, used as a nutritional supplement, by definition is an artificial cobalamin compound with a cyano ligand derived from natural cobalamins like adenosyl-, methyl-, or hydroxy cobalamins. However, the term vitamin B12 is often also used for the natural cobalamins. Cobalamins are the chemically most complex of all vitamins, consisting of a 15-membered, planar corrin ring with a central cobalt ion (hence the name), a dimethylbenzimidazole group as the lower ligand, and an adenosyl-, methyl-, hydroxy-, or cyano group as the upper ligand, giving rise to the aforementioned four cobalamin compounds. Vitamin B12 is an essential nutrient for plants and animals, since only some bacteria and archaea can synthesize it de novo. Among enteric bacteria two pathways, an aerobic pathway as in *Pseudomonas denitrificans* and an anaerobic pathway as in Propionibacteria or Salmonella typhimurium, have been identified for the initial corrin ring formation and the insertion of cobalt. In the prehistoric environment where the atmosphere lacked oxygen today's corrinoid enzyme originated enabling anaerobic metabolism in which carbon dioxide is reduced to methane. With increasing oxygen in the atmosphere and the evolution of porphyrin compounds like heme or chlorophyll, cobalamin-dependent enzymes gained function in other methyl-dependent reactions. In human physiology, adenosylcobalamin and methylcobalamin are important coenzymes linked to the formation of blood, fat metabolism, and the normal functioning of the nervous system and the brain. Vitamin B12 deficiency causes, among others, several forms of anemia, and has been long used as a diagnostic tool. Recent surveys point to a high proportion of inadequate intake, even in industrial countries, and the need of nutritional supplementation, especially for vegans and elderly people.

Industrial vitamin B12 production has never been carried out by the complex chemical synthesis route, comprising over 70 steps. Instead, cobalamins initially extracted from feces, sapropel, or *Streptomyces* sp. cultures used for antibiotic production were exclusively manufactured by fermentation, employing species of *Bacillus*, *Methanobacterium*, *Propionibacterium*, or *Pseudomonas*. The global production in the range of less than 100 t $year^{-1}$ is low, but a selling price of more than a thousand euros kg^{-1} drew several Chinese producers (e.g. Hebei Huarong Pharmaceutical Co., Ltd., NCPC Victor Co., Ltd., and Hebei Yuxing Bio-Engineering Co., Ltd.) into the market that was dominated by the French Sanofi-Aventis. As a result, the market is currently characterized by severe production overcapacities and concomitant price pressure. Nowadays, the bacterium *P. denitrificans* is almost exclusively used as the production organism. Investigations of the vitamin B12 biosynthesis pathway in *P. denitrificans* and cloning of the 22 cob genes of the organism allowed researchers at Rhone Poulenc to construct genetically engineered production strains presumably providing a technological advantage in terms of productivity and yield on raw materials over strains solely obtained by classical strain improvement. Glycine betaine present in significant amounts in sugar beet molasses or the biosynthetic precursor choline chloride are indispensable for high productivity. The fermentation medium also provides a cobalt salt and dimethylbenzimidazole as components of the cobalamin molecule. During the 7-day fermentation run, adenosylcobalamin is predominantly secreted from the biomass and accumulates in the fermentation broth in milligram amounts. The downstream steps comprise filtration, cyanide treatment, chromatography, extraction, and crystallization yielding vitamin B12 in high purity. However, optimization of recombinant B12 synthesis is still under investigation, wherein the most likely host is *P. denitrificans*. A key limitation is the absence of export systems.

L-Ascorbic Acid

Vitamin C probably has the most interesting history of all vitamins. The famous Ebers papyrus from 1550 BCE already describes the avitaminose symptoms of scurvy, a formerly common disease among mariners and explorers. Scurvy resulting from a lack of vitamin C in the human diet leads to collagen instability causing inter alia loss of teeth and bleeding of all mucous membranes. Vitamin C is chemically defined as L-ascorbic acid, a weak six-carbon sugar acid with a pentagonal ring structure. The exocyclic C5 has L-configuration, and in higher organisms only the L-enantiomer of ascorbic acid is biologically active. The pivotal functions making vitamin C essential are both the high antioxidant capacity and cofactor effects in the synthesis of collagen, carnitine, and hormones like adrenaline. For use as a food additive, several E numbers represent different chemical forms of vitamin C, that is, E300 (free ascorbic acid), E301–303 (ascorbate salts of sodium, calcium or potassium, respectively), E304 (fatty esters), and E315 (erythorbic acid, a stereoisomer). Starting from D-glucose, L-ascorbic acid biosynthesis follows different routes in plants and animals. L-Galactono-γ-lactone, the ultimate precursor of the plant pathway, is reached via an inversion of the D-glucose carbon skeleton. In animals, L-gulono-γ-lactone is obtained by three epimerization reactions. All plants and most animal species are prototrophic for vitamin C. Nevertheless, humans, higher primates, guinea pigs, and a small number of other animals require vitamin C supplementation of their diet due to a defective GULO gene encoding L-gulono-1,4-lactone oxidase, the last enzyme in the synthesis pathway in animals. Fungi of the genera Zygomycetes, Ascomycetes, or Basidiomycetes synthesize D-erythroascorbate, a C5 analogue of ascorbate, from D-arabinose via an inversion pathway.

L-Ascorbic acid and its chemical derivatives are by far the most bulk produced vitamins with more than 100,000 t year^{-1}. Chinese manufacturers entering the vitamin C market in the early 1990s caused a ruinous competition. DSM Nutritional Products Ltd. from Switzerland (formerly Roche Vitamins), the sole Western producer, positioned itself in the premium segment of the market. Chinese companies like Weisheng Pharma, North China Pharmaceutical Company (both in Hebei Province), and Jiangshan Pharmaceutical Co. (Jiangsu Province) became the leading producers for the bulk market. In contrast to other vitamins, feed applications of L-ascorbic acid account for only 10%, whereas the main uses are in the pharmaceutical industry (50%), food (25%), and beverages (15%). Pharmaceutical applications include stimulation of collagen synthesis (especially cosmetic products) and high antioxidant capacity, used for the reported health benefits in the prevention of flu, heart diseases, and cancer, as well as an antidote for poisoning. The food and beverage industry predominantly exploits the antioxidant capacity of L-ascorbic acid to extend durability, prevent discoloration, and to protect flavor and nutrient contents of their products.

Industrial production of vitamin C initially started by extraction from fruit. The first chemical process based on L-xylosone was carried out in 1933. In 1934, the famous Reichstein–Grüssner process was published. The process starts with a chemical hydrogenation of D-glucose to D-sorbitol, followed by the oxidation of the sugar alcohol to L-sorbose using *Gluconobacter* sp. strains as biocatalysts. The subsequent chemical steps including acetonization, oxidation, deacetonization, and rearrangement of the oxidation product 2-keto-L-gluconic acid (2KGA) deliver L-ascorbic acid. Although more than 70 years old, the technique with a typical yield of above 50% on starting glucose is still in use, a testimony to the fact that the advantages of long-term process optimization still allows classical methods to compete with more modern microbial-based production methods.

Chinese vitamin C manufacturers produce 2KGA by a two-stage microbial process developed in China in the late 1960s and 1970s. As in the Reichstein process, L-sorbose is provided by *Gluconobacter* sp. oxidation of D-sorbitol (first stage). In a second fermentation step, replacing the chemical oxidation steps, 2KGA is obtained vial-sorbosone employing Ketogulonicigenium sp. strains, in Chinese literature frequently referred to as *Gluconobacter oxidans*. For efficient oxidation co-cultivation with a helper strain, for example, *Bacillus megaterium*, is mandatory but the underlying molecular mechanism is still unclear. The execution of the process in two steps is necessary to prevent *Ketogulonicigenium* sp. from metabolizing D-sorbitol, which would lead to the production of unwanted D-glucose and its oxidation products. Finally, as in the Reichstein process, 2KGA is chemically converted to L-ascorbic acid. The two-stage process can achieve up to 130 G L^{-1} 2KGA with a yield of above 80% on D-sorbitol. The reduced number of process steps compared to the Reichstein process, less process complexity, lower investment costs, less energy demand, and lower chemical consumption make the two-stage process advantageous.

G. oxidans alone is able to oxidize D-sorbitol to 2KGA, however, even processes based on genetically engineered *G. oxidans* strains cannot match the space–time yield of mixed culture processes.

Alternative microbial routes to 2KGA synthesis starting from D-glucose via 2,5-diketo-D-gluconate have been developed employing *Erwinia* sp. and *Corynebacterium* sp. in a two-step tandem process or by using genetically engineered *Erwinia* sp. expressing a gene for a *Corynebacterium* 2,5-diketo-reductase. These processes have yet to make it to industrial realization.

In summary, all vitamin C bioprocesses developed to an industrial stage so far result in the formation of the precursor compound 2KGA, requiring a cost-driving chemical rearrangement step to achieve the final product. Obviously, numerous attempts have been made to design microbial synthesis routes that result in direct synthesis of vitamin C. One approach tried to utilize the biosynthetic capacity of baker's yeast to synthesize the five-carbon vitamin C analogue D-erythroascorbic acid. Indeed, genetically engineered *S. cerevisiae* strains produced minor amounts of L-ascorbic acid starting from L-galactose, which should be supplied from a combination of epimerase and isomerase reactions starting from the Reichstein intermediate L-sorbose. In a second approach, microalgae synthesizing vitamin C according to the plant biosynthetic pathway vial-galactono-γ-lactone yielded up to 2 G L^{-1}, the synthesized vitamin mainly associated with the biomass. Although cheap D-glucose was the fermentation substrate, the reported low productivity renders a commercial application of the microalgae system rather unlikely. However, a promising direct route to vitamin C might has been opened by the discovery of dehydrogenases present in *Ketogulonicigenium* sp. and *Gluconobacter* sp. that convert L-sorbosone, the partially oxidized biosynthetic intermediate of microbial 2KGA processes, into vitamin C.

Production Efforts for Other Vitamins

R-Pantothenic Acid

Efforts on developing microbial production processes for water-soluble vitamins are not limited to the vitamins B2, B12, and C, as discussed in detail in the previous sections. The next vitamin to be industrially produced by a fermentation process might be R-pantothenic acid, also known as vitamin B5. R-Pantothenic acid plays a vital role for a multitude of metabolic processes in all living cells, serving among others as a building block for the biosynthesis of coenzyme A. Pantothenate is synthesized by bacteria, fungi, and plants, but not by mammals, including livestock and humans. The current worldwide demand of more than 5000 t is produced by chemical synthesis involving a racemic resolution step that can be carried out biocatalytically at the industrial stage.

Biosynthesis of pantothenic acid starting from the common metabolic intermediates α-ketoisovalerate and aspartate is well understood. The products of the panB and panE genes are involved in the conversion of α-ketoisovalerate to R-pantothenate. The panD gene product is a pyruvoyl-dependent decarboxylase converting aspartate to β-alanine. The ATP-dependent panC gene product combines the two pantothenic acid precursors into the final product. Biotechnical approaches based on genetically modified E. coli, *Corynebacterium glutamicum*, and *B. subtilis* have been reported. The *E. coli* process developed by Takeda Chemical Industries Ltd. (Japan) delivers a respectable 66 G L^{-1} of R-pantothenic acid in a 72 h fermentation run, but requires β-alanine as a co-substrate. The fact that in the E. coli process 40% of the molecular mass of pantothenic acid is not provided by the basic carbohydrate fermentation substrate, but originates from the processed chemical β-alanine should not be without disadvantageous economic consequences. The B. subtilis procedure developed by Omnigene Inc. (United States) shows an even higher productivity with 86 G L^{-1} R-pantothenic acid accumulating within 48 h of fermentation. Interestingly, sufficient β-alanine is provided endogenously in this process superseding exogenous β-alanine supply.

D-Biotin

Biotin, also known as vitamin H or B7, is an important catalyst in essential metabolic conversions like gluconeogenesis and fatty acid synthesis. Usually produced in excess by intestinal bacteria, biotin is mainly of cosmetical interest as it strengthens hair and nails. Significant efforts have been made to design a microbial process for D-biotin, which is currently produced in a complex multistep chemical process. Tanabe Seiyaku Ltd. (Japan) in the early 1990s developed a process based on genetically modified bacterium *Serratia marcescens* affording 540 mg L^{-1} of D-biotin during 144 h of fermentation. Biotin-overproducing recombinant Kurthia sp. and B. subtilis were developed at Nippon Roche (Japan) and Omnigene Inc. (United States), respectively.

Biotin biosynthesis involves pimeloyl-CoA, a derivative of the seven-carbon dicarbonic pimelic acid. In B. subtilis pimelic acid and alanine are converted to dethiobiotin via four biosynthetic steps catalyzed by the gene products of bioW, bioF, bioA, and bioD. The subsequent BioB-catalyzed conversion of dethiobiotin to biotin involves the formation of an adenosyl radical. Interestingly, one of the two iron–sulfur clusters of BioB is probably the immediate sulfur source of biotin. This remarkable BioB reaction attracted much scientific attention, but is still incompletely understood. Solving the BioB enigma is regarded as a key milestone toward developing a competitive microbial biotin production process.

Vitamins B1 B6, and Folic Acid

Serious efforts have been devoted to the development of microbial production processes for vitamins B1 (thiamine), B6 (pyridoxol), and folic acid based on various bacterial host strains including E. coli, B. subtilis, and *Rhizobium meliloti*. In all cases, genetic engineering approaches were applied to deregulate and overexpress the genes known to be involved in the respective metabolic pathways. Although the engineered strains produced significantly higher levels of the vitamins compared with their wild-type ancestors, their productivities were far too low for commercial application. As in the case of biotin, it seems that an incomplete understanding of the biochemical fundamentals is a serious impediment for the development of competitive fermentation processes for these vitamins.

Conclusions and Outlook

All microbial processes discussed in this article are part of white biotechnology, which in contrast to red biotechnology (medical sector) and green biotechnology (genetically engineered crops) deals with industrial application of biological systems to produce chemicals, materials, and energy. Several recent studies estimate the current share of biotechnical processes in various chemical productions to increase from about 5–20% by 2010. Fine chemicals with high functionality and a typical annual worldwide demand below 10,000 t $year^{-1}$, for example, vitamins, are expected to be a future market for bioprocesses, with an estimated fivefold increase in their world market value to around US$250 billion within the next 10–20 years. By 2020, it is anticipated that 20% of today's chemically manufactured fine chemicals, bulk products and polymers (production greater than 10,000 t $year^{-1}$) will be biotechnically produced.

Important products of white biotechnical are food ingredients, vitamins, biocolorants, solvents, degradable bioplastics, and biofuels, in which typically microorganisms or isolated enzymes quickly catalyze even crucial chiral products with a remarkable specificity. This goes along with a reduction in emissions, water usage, energy demand, and waste. White biotechnical applications for animal or plant cells are also conceivable, but microorganisms are usually preferred due to their faster growth, easier handling, and often relatively easily modifiable genetic material. As in vitamin B2 production, coexistence of an obviously faster cultivation process with a bacterium and a process applying a more slowly growing filamentous fungus is possible. Hence other advantages like substrate quality or final product concentration have a significant impact. To develop a new bioprocess for vitamin synthesis, in principle two general strategies are feasible, albeit a successful production strain is often the result of both approaches. The first strategy starts with screening for natural overproducers, that is, species already producing the compound of interest in considerable amounts. The yield is then consecutively improved by several rounds of mutagenesis (by chemicals or UV radiation) and selection, for which minimal knowledge about the metabolism and genetic organization of the microorganism employed is sufficient. On the contrary, the second tactic, namely targeted deregulation of metabolism by application of recombinant DNA techniques, is only appropriate for thoroughly studied organisms. Apart from the need for sufficiently effective means of genetic manipulation, pathway elucidation is an important prerequisite for this approach. The economic success story of commercial riboflavin production by B. subtilis is a proof of concept of how satisfactorily the second strategy can work. Moreover, innovative methods like altering metabolic flux and metabolic control studies, as well as transcriptome analyses, advanced screening techniques (such as using novel antimetabolites), and computer simulations further support the re-education of long-time microbial workhorses into production strains of new compounds. However, even for genetically well-known organisms, still some fundamental dilemmas exist. First, elucidation of both the synthesis pathway and the transport processes of product, intermediates, and co-metabolites as yet needs time-consuming and costly basic or pure research. Publication of genomes also is only the beginning of protracted discovery of the function of numerous unknown open reading frames. For instance the genomes of *B. subtilis* and *A. gossypii* have been publicly available for years but only riboflavin importer genes of *B. subtilis* and *S. cerevisiae* were identified recently. Kinetic studies have shown that in *A. gossypii* at least an uptake and a separate export system plus a proton gradient-driven system in the vacuolar membrane must exist. Moreover, metabolic enhancement of several key enzymes in compound synthesis and transport is significantly impeded due to the lack of direct enzyme activity assays, either because the enzyme of interest is membrane-bound or owing to a shortage of sufficient test-substrate availability. As described above for vitamins B12 and B2 production, actual availability for the microbial cell is the crux of highly efficient fermentation, wherein choice of feedstock as well as supplementation with additional precursors and regulative compounds fundamentally affect the financial survival of the microbial fabrication technique versus chemistry. Likewise, as for vitamin C production with yeasts and microbial vitamin B5 synthesis, supplementation costs often render a bioprocess uneconomical until genetic engineering overcomes its limitations or changes in the general framework, such as decreasing prices for the supplements needed or increasing production costs of competing chemical synthesis, become effective. However, some microbial production concepts have no chance of success because of the intrinsic characteristics of the available or preferred microorganisms. An example is the production of fat-soluble vitamins, which not only needs precursors but also a storage depot within the cell, hence excluding bacteria in the production process. Nevertheless, microorganisms have already proven to exhibit an outstanding versatility in metabolism, although as yet only a small fraction of existing species has been discovered and classified. Screening for new isolated strains from seawater, soil, or extreme habitats is thus still a common method in the development of novel bioprocesses, in particular for delicate challenges like synthesizing PUFAs. Moreover, unveiling of vitamin-like functions of novel compounds is expected since, additional to the almost six thousand bioactive metabolites described so far in the literature, the potential industrial capacity of marine microorganisms, to name only one group, is estimated by hundreds of compounds having industrially interesting novel structures and properties.

Given the undisputed beneficial effects of an adequate vitamin supply in food and feed, the question raised now is how this demand can be satisfied. Since fossil and natural resources (e.g., fish oil) are depleting, in the long term a transition from a fossil-based to a bio-based economy and society is needed. Apart from microbial production processes, green chemistry offers a short-term cost-efficient perspective, in which plant products, such as oils or sugars, are microbially converted inter alia into vitamins. A more direct and long-term approach is the metabolic engineering of the plants themselves to increase their nutritional value. A prime example of this strategy is 'golden rice' enriched in provitamin A, manufactured since the late 1990s.

Despite all advantages, there are still many factors that impair the transformation from conventional to biotechnical industry. Many reports conclude that the main barrier in the industry is because of conservative thinking, summing up the lack of knowledge both on bioprocess alternatives and on environmentally crucial steps of the current processes in plant management, as well as a lack of suppliers and advertising of biotechnical intermediate or end products. Moreover, international competition often allows little room and little demand for investments in new technology. However, since fossil resources are diminishing, pollution control becoming increasingly expensive, and customers asking for environmentally friendly products, white biotechnical is boosted. Thus Europe and the United States, the current market leaders, are anticipated to increase state-of-the-art research and industrial implementation of new bioprocesses. Although fermentation processes are already being applied in the synthesis of several vitamins and vitamin-like compounds, often the final aim is the total replacement of chemical synthesis by feasible and economic biocatalytical systems using cost-efficient and sustainable raw materials. It remains to be seen in the following years, whether the above described biotechniques are sufficiently efficient to compete with chemical synthesis, imminent or existing combined methods, or upcoming genetically engineered vitamin-enriched crops.

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Water Treatment, Industrial☆

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Glossary

Activated sludge (AS) An aerobic treatment process with suspended biomass waste.
Biological oxygen demand (BOD) A bulk measurement of the concentration of oxygen consumed during the biodegradation of organic compounds in the wastewater; may be reported after a 5-day measurement period (so-called BOD5) or tested on filtered wastewater (so-called soluble or sBOD).
Chemical oxygen demand (COD) A measurement of the overall oxygen concentration utilized to chemically oxidize all of the organic compounds in a wastewater sample.
Hydraulic residence time (HRT) The average retention time of water in a treatment system.
Membrane bioreactor (MBR) Coupling ultrafiltration or microfiltration membranes with biological treatment reactors (typically an AS reactor) to retain biomass.
Moving bed biofilm reactor (MBBR) A hybrid treatment reactor that is typically aerobic.
Organic loading rate (OLR) The rate at which organics are loaded to a treatment reactor. It is usually calculated as the concentration of organics in the wastewater (typically in kg COD or BOD m^{-3}) multiplied by the flowrate applied to the treatment reactor (in m^3 day^{-1}) divided by the reactor volume (in m^3) or sometimes the surface area (in m^2).
Rotating biological contactor (RBC) An aerobic treatment system with biomass attached on rotating disks.
Sequencing batch biofilm reactor (SBBR) A SBR to which solid media has been added to allow attached biomass to accumulate in the treatment reactor.
Solids residence time (SRT) Average time that the biomass is retained in the biotreatment system.
Surface area loading rates (SALR) Typically reported for organics loading onto filters or wetland treatment systems.
Suspended solids (SS) Solids larger than ~1 μm in size; can include organic solids and bacteria.
Total Kjeldahl nitrogen (TKN) Includes ammonia-N and organic-N.
Total phosphorus (TP) Includes phosphate-P, organic P, and so on.
Upflow anaerobic sludge blanket (UASB) A common anaerobic treatment reactor.

Abbreviations

AF	anaerobic filters
AMW	apparent molecular weights
AS	activated sludge
ASB	aerated stabilization basins
BAFs	biological aerated filters
BES	bioelectrochemical systems
BOD	biological oxygen demand
COD	chemical oxygen demand
CSTR	completely stirred tank reactor
DGGE	denaturing gradient gel electrophoresis
EGSB	expanded granular sludge bed

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EPA	environmental protection agency
FAME	fatty acid methyl ester
FB	fluidized bed
FC	fecal coliforms
FISH	fluorescence in situ hybridization
FWS	free water surface
GSS	gas–solids separators
HRT	hydraulic residence time
IC	internal circulation
IFFAS	integrated fixed film activated sludge
JRLs	jet loop reactors
MBBR	moving bed biofilm reactor
MBR	membrane bioreactors
METs	microbial electrochemical technologies
MF	microfiltration
MFC	microbial fuel cell
NOB	nitrite-oxidizing bacteria
PCR	polymerase chain reaction
RBC	rotating biological contactor
SALR	surface area loading rates
SBBR	sequencing batch biofilm reactor
SBR	sequencing batch reactor
SRT	solids residence time
SSF	subsurface flow
TOC	total organic carbon
TRI	toxics release inventory
UASB	upflow anaerobic sludge blanket
UF	ultrafiltration
WET	whole effluent toxicity

Defining Statement

An overview of microbiological methods to treat industrial wastewaters is provided. The range of characteristics of common industrial wastewaters is provided. Commonly used aerobic, anaerobic, coupled, and wetland treatment processes are described. Case studies from full-scale systems are highlighted, along with key emerging results from laboratory and pilot-scale studies.

Biological Treatment of Industrial Wastewater

Industrial wastewaters are generated from a wide variety of sources and have a broad diversity of chemical properties and constituents. In the United States, the Federal Environmental Protection Agency (EPA) has developed wastewater discharge standards for over 60 industries (40 CFR Chapter 1, Subchapter N Effluent Guidelines and Standards). There are also pretreatment standards that regulate the quality of wastewater discharged from industries into public sewers under the National Pretreatment Program (EPA-833-B-98–002). To achieve the wastewater characteristics required by the standards, more industries are treating their wastewater on-site by a variety of processes. Most of these processes are aimed at reducing the concentrations of total organics, total solids, and/or specific chemicals down to acceptable concentrations. Many treatment processes can be quite expensive, and biological processes are considered to be cost-effective alternatives when the primary contaminants of concern are biodegradable organics or nitrogen compounds. Treatment designs are continually evolving to provide greater treatment efficiency. Microbial methods that are used to treat industrial wastewaters must be more robust than traditional municipal wastewater treatment processes due to the greater variability in flow rates and chemical characteristics, and the potential presence of toxic or inhibitory compounds. More innovative approaches to achieve treatment objectives include coupling anaerobic and aerobic processes, and coupling biological and chemical treatment processes. Molecular methods are beginning to be used to characterize the microorganisms present in the wastewater and present in treatment reactors. Improved capabilities to extract energy and even electricity from industrial wastewater are evolving.

Characteristics of Industrial Wastewater

Unlike municipal wastewater, the wastewater generated from different industrial activities has widely varying composition. Even within a single type of industry, the specific processes and chemicals used to produce similar products can differ resulting in

Table 1 Examples of typical industrial wastewater characteristics

Wastewater type	*Average pH range*	*Suspended solids (mg L^{-1})*	*BOD5 (mg L^{-1})*	*COD (mg L^{-1})*	*TKN (mg N L^{-1})*	*Total P (mg L^{-1})*	*Salt (g L^{-1})*
Brewery	3.3–7.6	500–3000	1400–2000	815–12,500	14–171	16–124	
Dairy milk–cheese plants	5.2–11.3	350–1082	709–10,000	189–20,000	14–450	37–78	0.5
Dairy parlor	2–11	100–300	166–477	470–820	25–45	17–21	0.05–0.7
Dying	8.2–12	56–70	140–840	70–3200	27–42	5–7	
Food pickling	2.6–3	40–110	7000–8000	20,000–22,000	4–6	22–25	30–150
Metal working fluids	9		1500–11,400	5300–40,000	160–440	28–77	
Pulp and paper	6.6–10	21–1120	77–1150	100–3500	1–3	1–3	~0.05
Tannery	8–11	2070–4320	1000–7200	3500–13,500	250–1000	4–107	6–40
Textile mills	4.5–10.1	20–210	700–1650	1900–100,000	14–72	1–18	0.5–0.9
Winery	3.9–5.5	170–1400	210–8000	320–27,200	21–64	16–66	0.1–1
Municipal	6–8	100–350	110–400	250–1000	20–85	4–15	<0.5

significant changes in wastewater characteristics over time. For example, the wastewater stream at a brewery is actually made up of a combination of wastes from different processes, including waste from malting, brewing, fermenting, yeast drying, conditioning, and packaging. Similarly, textile mill effluent may be a combination of bleaching, fiber dyeing, yarn dyeing, and rinsing wastewater streams. A sample of reported wastewater composition is provided in Table 1.

Industrial wastewaters may be caustic, with extreme pH values of less than 2 or greater than 12; these extreme pH values may result from different cleaning agents that are used. Without buffering to more neutral conditions, these extreme pH values would be inhibitory to microorganisms. Some industrial wastewaters contain extremely high concentrations of organic compounds (measured as biological oxygen demand (BOD) or chemical oxygen demand (COD)); these organic concentrations may be more than ten times the concentrations in municipal wastewater. High salt concentrations are also present in some types of industrial wastewaters. Nutrient limitations are a potential problem for biological treatment of some industrial waste streams, including nitrogen (N) limitation in brewery wastewater, phosphorus (P) limitation in pulp and paper wastes including Kraft pulp and cellulose production wastewaters, and N and P limitation in winery and pickling wastewaters. In other cases, a significant excess of nutrients may be present, such as in the case of pectin factory wastewaters with 1280–2990 mg L^{-1} nitrate-N and COD/N ratios of 7 to 12:1.

In addition to bulk chemical constituents, many industrial wastewaters also contain specific organic and inorganic compounds that are toxic. For example, tannery wastewaters may contain 50–100 mg L^{-1} chromium. The US Environmental Protection Agency tracks the industrial use, treatment, and release of more than 600 chemicals via the Toxics Release Inventory (TRI). These chemicals tend to fall into a few main classes: metals or inorganics, aromatic hydrocarbons, or chlorinated hydrocarbons. Microbial-based treatment is only a viable option for organic compounds and nutrients (N, P). Metals may change redox state during biological treatment, but are not destroyed and will be present in the effluent wastewater or associated with the sludge. Therefore, physical and chemical processes are generally used to treat metals in the wastewater. As such, further discussion will be limited to organic compounds. The top chemicals on the TRI list that are amendable to biological treatment, in terms of quantity of treated on- or off-site or released (including air, water, and solids), are summarized in Table 2. Many of the most prevalent TRI compounds are relatively nontoxic. However, the uncontrolled release of these substances into sanitary sewers or the environment can disrupt treatment or ecology. Therefore, pretreatment or permits regulate the quantities of specific chemicals allowable in the waste discharged from an industrial facility, in addition to the more commonly regulated bulk parameters (eg, COD, TSS, and total N). Many industries have modified their processes to use more environmentally compatible (less toxic and more degradable) compounds. In addition to the regulation of the concentration of specific compounds in industrial wastewater effluents, the aggregate toxicity of the wastewater may be regulated. This whole effluent toxicity (WET) is generally tested by short-term and chronic tests on aquatic life such as water fleas, shrimp, and fish.

Regardless of the specific compounds and sources, biological treatment processes typically offer the lowest cost alternative for degrading organic compounds in the wastewater. Biological processes can also be used to remove nitrogen and phosphorus. Nitrogen can be removed from the water via the following processes: organic-N is mineralized by aerobic heterotrophs to ammonia; ammonia-N is aerobically biotransformed to nitrate-N; and under anaerobic conditions, heterotrophic bacteria degrade nitrate-N to N_2, which equilibrates out of the water into the atmosphere. Phosphorus can be removed from the wastewater via accumulation in particular types of bacteria. The processes used to biologically treat industrial wastewaters are often similar to those that are widely used for municipal wastewater. However, the composition range of industrial wastewaters often requires a somewhat more engineered approach to the wastewater treatment. Many industrial wastewater treatment facilities also include pre- and post-treatment by various physical and chemical methods.

In addition to the biodegradation of waste components, a variety of microorganisms may be present in the wastewater itself. Pathogenic species are of concern due to regulatory limits. The removal of pathogens during wastewater treatment is usually tracked using "indicator organisms" such as total coliform or fecal coliform bacteria, which are generally associated with the gut of mammals. Other microorganisms in the wastewater are of interest because they can contribute to or compete against the waste-degrading bacteria. For example, winery and dairy wastewater typically contain significant quantities of yeast and bacteria, which

Table 2 Industrially produced compounds on the US toxics release inventory

Chemical	*TRI rank*[a]	*Class*[b]
Nitrate compounds	3	Inorganic
Ammonia	6	Inorganic
Methanol	9	VOC
n-Hexane	14	VOC
Styrene	15	VOC, AHC
Toluene	19	VOC, AHC
Formaldehyde	20	VOC
Certain glycol ethers	23	Organic
Xylenes	25	VOC, AHC
Ethylene	26	Organic

[a]2014 TRI dataset; rank by total weight on- or off-site disposed or otherwise released. US EPA TRI Explorer. Available at: https://www.epa.gov/toxics-release-inventory-tri-program/tri-data-and-tools
[b]AHC, aromatic hydrocarbon; VOC, volatile organic compound.

have been found as significant populations in the biological treatment units. In addition, there is an emerging concern that microorganisms in the wastewater may carry antibiotic-resistant genes. In particular, antibiotic-resistant bacteria have been found associated with animal wastes, and as such could be present in water used to washout dairy stalls, and so on. Although the animals are fed different antibiotics than are used for humans, the genes associated with resistance to animal antibiotics may also impart resistance to the antibiotics used for humans.

The use of molecular methods to characterize bacterial populations has been gaining popularity, although these methods have not been widely applied to characterize biological treatment processes for industrial wastewaters. These approaches provide tools to better understand the microbiology of a treatment system, particularly under upset conditions. Examples of molecular methods include those targeting bacterial cell membrane components and genetic material. Fatty acid methyl ester (FAME) is a method that extracts and quantifies the fatty acid components of cellular membranes, generating a profile or "fingerprint" of the microbial community. Denaturing gradient gel electrophoresis (DGGE) creates a community fingerprint based on the stage at which DNA melts in the presence of a denaturing agent into fragments; the fragments migrate through an electrophoresis gel to different extents creating different banding patterns. By comparing band location and intensity, variability in the structure of a microbial community can be determined. Further information can be gathered by removing the DNA from specific bands of interest and sequencing it to identify specific bacteria. These methods have been used to characterize microbial diversity in full-scale industrial wastewater treatment plants, and varying levels of species richness and diversity have been found to be associated with different processes.

Genetic methods can target microbial DNA or RNA with probes to identify specific species or groups of bacteria. The so-called full-cycle 16S rRNA approach has the potential to identify previously uncharacterized microbial populations in samples from natural and engineered environments. First, genomic DNA is extracted from all members of the microbial community, followed by 16S rDNA gene amplification by the polymerase chain reaction (PCR), cloning, and finally sequencing of 16S rDNA gene fragments. These sequences are then compared with a database to identify the bacteria and/or their closest relatives. This method is therefore able to determine the diversity of the members of the microbial community in a treatment system. Using the sequence information available from the clone library, 16S rRNA-targeted oligonucleotide hybridization probes can be developed. These probes can be used for fluorescence in situ hybridization (FISH). Examples of studies that have used molecular methods to characterize the microbiology at full-scale industrial wastewater treatment facilities are provided in Table 3.

Table 3 Examples of molecular methods used to characterize microbiology at industrial wastewater treatment plants

Wastewater type; treatment	*Methods used*	*References*
Chemical manufacturing, landfill leachate; activated sludge	PCR-amplified 16s rRNA DGGE	Moretti et al. (2016)
Dairy food processing; activated sludge	Full-cycle 16S rRNA	Oerther et al. (2002)
Dairy; wetland	DGGE, PCR	Ibekwe et al. (2003)
Fibers, plastics, chemical manufacturing; activated sludge	Real-time PCR competitive PCR	Robinson et al. (2003); Dionisi et al. (2002)
Pharmaceutical; activated sludge	DGGE, rDNA sequence analysis	LaPara et al. (2000)
Pulp and paper; upflow anaerobic sludge blanket	454 pyrosequencing, real-time PCR	Shu et al. (2015)
Rendering; activated sludge with nitrification and denitrication (AS-N)	Full-cycle 16S rRNA DGGE	Juretschko et al. (2002); Kreuzinger et al. (2003)
Tannery and fish processing; AS-N	FISH	Moussa et al. (2006)
Slaughterhouse; anaerobic SBR and anaerobic static filter	FAME	Mach and Ellis (2002)

Aerobic

In general, aerobic biodegradation processes for organic compounds are faster than anaerobic. Aerobic treatment processes require the supply of oxygen, and result in oxidation of organics to carbon dioxide and biomass. The biomass or "sludge" is a secondary by-product that may require further treatment. The oxygen supply can be expensive; it is usually supplied by blowers and aeration equipment, involving an energy input. More recently, oxygenation via membranes and co-reactors with algae have also been explored. This article covers typical aerobic treatment reactors used for industrial wastewater treatment, and highlights some specific examples of wastewater treatment by each of the most common reactor types: activated sludge (AS), sequencing batch reactor (SBR), rotating biological contactor (RBC), moving-bed biofilm reactor (MBBR), and submerged membrane bioreactors (MBR). In order for processes using suspended bacterial growth (AS, SBR) to work optimally, the biomass must be "settleable" in the form of flocs so that high biomass concentrations (and long solids retention times) can be maintained in the system. Alternatively, a membrane can be used to retain biomass in the system. Biomass can also grow attached to an inert media, such as in an RBC or biotrickling filter. The wastewater to be treated flows over the biofilms on the media, and leaves the reactor. These reactors therefore have the ability to retain slow growing bacteria. However, too much biomass will eventually clog the media. Newer treatment systems are hybrids of attached and suspended growth, such as MBBR.

Activated Sludge

Activated sludge processes are among the most common biological methods used to treat domestic wastewater in the United States. The process is also frequently used to treat various types of industrial wastewaters. Conventional AS has an aeration basin where oxygen is supplied and the contents of the reactor are mixed by this aeration process, followed by clarifiers where biomass solids are removed by gravity settling. The settled biomass is generally returned to the beginning of the aeration basin as "return activated sludge." This results in an average biomass retention time (so-called solids residence time (SRT)), which is significantly longer than the hydraulic residence time (HRT). Because the treatment performance is dictated by maintaining high biomass concentrations in the aeration basin due to biomass recycle, it is important that the biomass forms aggregates termed "floc" that have good settling properties. When the biomass does not settle well, reactor upsets occur and treatment performance usually decreases. Molecular methods are being used to determine which bacteria might be linked with poor biomass settling.

Another important operational consideration is the amount of sludge wasted from the system; this stream of concentrated biosolids is a waste product that requires further treatment (such as per the Part 503 Biosolids regulations in the United States). Frequently, these solids are treated and used as soil amendments. However, the sorption of toxic compounds, particularly metals, to the biomass can limit the safe uses of these biosolids.

The aerobic bacteria in AS systems biodegrade organic compounds, with a high degree of mineralization to carbon dioxide expected. The amount of oxygen that must be supplied increases with increased organic loading. When the total concentration of carbonaceous chemicals in the wastewater is measured as BOD or COD, the approximate amount of oxygen that must be supplied to the system is known. Oxygen supply involves the bulk of the operational costs in these systems.

Nitrogen and phosphorus must be present at sufficient quantities in wastewater relative to organic loading to allow heterotrophic bacteria to assimilate the carbon and grow in an uninhibited manner. Many industrial wastewaters are deficient in N and P relative to carbon, requiring the addition of supplemental nutrients to meet cellular demand. Examples of nutrient-deficient industrial wastewaters can be found in Table 1; nitrogen will generally be deficient if the COD/TKN ratio is greater than 35:1, and phosphorus limiting when COD/P ratio is greater than 300:1. Thus, the brewery, food pickling, textile, and winery wastewaters are commonly N limited, while the metal working fluids, food pickling wastewater, and winery wastewaters are often P limited.

A suitable aeration system is a key design parameter for AS treatment. Most AS processes are aerated via fine bubble aeration. However, for very deep reactors (>20 m water depth), medium or coarse bubble aeration will be more energy efficient. For very high-strength organic wastewaters, aeration with pure oxygen rather than air may be advantageous. Jet loop systems are another method for supplying oxygen and mixing. Jet loop reactors (JRLs) are vertical systems that claim to achieve higher oxygen transfer at lower energy than conventional aeration. Jet loop reactors have been studied at a pilot scale for treatment of winery wastewater, olive oil wastewater, and brewery wastewater. The systems have high velocity of liquid exiting the venturi nozzle, which could exert selective pressure on the microbiology of the system via a combination of physical shear stress and temperature effects. Specifically, filamentous fungi and bacteria have not been reported in JLRs. However, these systems do not appear widely applied in full-scale industrial wastewater treatment.

In addition to removing carbon compounds efficiently via biodegradation, AS processes have also been used to remove nutrients including nitrogen and phosphorus via sequential processes. Aerobic autotrophic bacteria can nitrify ammonia to nitrite (so-called ammonia-oxidizing bacteria (AOB); typically β-Proteobacteria) to nitrate (so-called nitrite-oxidizing bacteria (NOB); such as *Nitrospira* spp.). Then under anoxic conditions, heterotrophic bacteria can denitrify nitrate to nitrogen gas (N_2). Sequential anoxic – aerobic processes with a "single sludge" circulated/recirculated through the reactors in series are generally used for N-removal from wastewaters, combining the benefits of dentrification in the presence of sufficient organic carbon with nitrification under lower carbon loading. Some biological phosphorus removal is also possible by starting the single sludge system with an anaerobic selector; the sequential anaerobic/aerobic processes encourage intracellular accumulation of P-deposits within the bacteria, such that biosolids waste from the system removes soluble P from the wastewater.

Due to the more complex microbiology that is needed to achieve these multiple goals in wastewater treatment (organic removal by aerobic heterotrophs, nitrification by aerobic autotrophs, and denitrification by anoxic heterotrophs), molecular methods have been used to characterize the microbial populations present in these treatment systems. These methods can be used to help determine the source of treatment problems such as incomplete nitrification due to toxicity or extended anaerobic periods that inhibit nitrifiers (see examples in Table 3).

Aerated Stabilization Basins

Aerated stabilization basins (ASB) are a potentially cost-effective alternative to AS treatment of industrial wastewater. ASB systems have typically lower capital, energy, and operation and maintenance costs than comparable AS systems. In particular, pulp and paper industries appear to be selecting ASBs for wastewater treatment over AS systems. Pulp and paper industry wastewater contains variable-quantity contributions from four to five major process areas with significantly different wastewater chemistries, a wide range of apparent molecular weights (AMW) for organic wastewater components, and a brown color due to high-AMW dissolved lignin. Because AS systems do not handle shock loads efficiently, the installation of flow equalization, neutralization, and sedimentation units prior to the AS unit is common. Mechanically aerated stabilization basins represent about half of the lagoon systems and have the best performance of the lagoon types. There are two classes of mechanically aerated stabilization basins: completely mixed and partially mixed. The large majority of ASBs in the pulp and paper industry are partially mixed. Mixers in the systems also achieve aeration. The mixers used include jet aerators, surface aerators, static aerators, and submerged fine bubble diffusers. In the ASB process, the HRT and SRT are the same at ~5 to 15 days. For pulp and paper wastewater treatment, supplemental nutrient addition is needed, but at lower rates than in AS systems. Properly operated ASB systems can reach the comparable effluent BOD and nutrient concentrations to AS systems. ASB systems are more forgiving in responding to influent quality and flow variations and are simpler to operate. Settled secondary solids in an ASB system reduce in volume through hydrolysis to typically 10% concentrations. At periods up to 20 years the accumulated solids must be dredged, dewatered, and disposed. ASBs, because of their longer hydraulic retention times, tend to have their effluent wastewater temperature approach ambient air conditions. This is desirable during summer conditions, but can be a treatment problem in cold climates. Loss of an insulating foam cover is a greater problem for ASB systems. ASB systems typically require supplemental cooling equipment to attenuate the high influent temperatures of the wastewater. A naturally aerated "polishing" pond sometimes follows ASBs.

Sequencing Batch Reactors

In SBRs the treatment and settling of biomass are done within a single tank, unlike AS reactors. This may result in a smaller footprint overall than the AS process. The technology is becoming more popular in Germany, with 51 SBR plants for industrial wastewater treatment in operation; however SBRs yet represent less than 1% of the German wastewater treatment plants. A review of 29 full-scale SBR plants treating industrial wastewater in Turkey was presented by Artan et al. (2003). SBRs are used for food processing, pharmaceutical, textile, and other wastewaters treating 12–4800 m^3 day^{-1} of flow, inlet COD 600–12,000 mg L^{-1}, cycle times of 6–168 h, HRT 12–200 h, and recycle ratios (carry-over volume to new feed volume) of 0.2–4.8. These plants achieved effluent COD of 50–2000 mg L^{-1}, representing 77–98.5% removal. The advantage of the SBR systems was largely for high strength and variable quality wastewaters. SBRs are generally more automated in their operation than AS systems. SBR effluents generally contain less SS than AS systems. SBRs are somewhat less common at large wastewater plants due to the fairly high area required for the multiple reactors needed to treat continuous inflow. The operation of the SBR can be varied within a treatment cycle to enable different metabolic processes (such as anaerobic or aerobic) to predominate. This makes it particularly suited to simultaneous carbon and nitrogen removal.

Tannery wastewater is an example of a strong wastewater with high carbon and TKN. High chloride, sulfide, and chromium in the wastewater are potentially inhibitory to heterotrophic and nitrifying bacteria. Lab and pilot-scale studies have found comparable SBR performance to the full-scale AS systems on-site. These systems were operated with supplemental P addition, cycle times of 8–24 h, HRT 0.8–3 days, and SRT 15–17 days. Full-scale SBR systems have been used to treat brewery, winery, and pulp mill wastewaters and tanker truck wash water. These systems had 12–24-h cycles and achieved >93% COD removal.

Specific industrial chemicals have been shown to upset biological treatment in SBR systems. This is a disadvantage compared to complete mixed AS systems, which quickly dilute toxic concentrations of inlet spikes, versus the batch treatment where inlet concentrations are only attenuated by the carry-over biomass from the previous treatment cycle. Nitrification and low-temperature conditions seem particularly sensitive to these problems.

Rotating Biological Contactor

RBC treatment uses partially submerged rotating disks on which biomass can grow. As the disks rotate, the attached bacteria are sequentially exposed to the air, where they acquire oxygen and wastewater. Additional bacteria are also suspended in the wastewater treatment reactor. Energy is consumed by the large motor required to turn the biodisks, which can range in size from 3.5–5 m in

diameter. Rotational speeds are slow, at about 1 rpm. RBCs are fairly uncommon in industrial wastewater treatment, and have also largely fallen out of use in municipal wastewater treatment. A search of peer-reviewed literature found only five citations to full-scale RBC and industrial wastewater treatment. All except one citation were RBCs treating combined dairy wastewater with municipal wastewater, and all citations were before 1997. Supplemental aeration in the first and second stages that were often overloaded significantly improved the RBC treatment performance and were more robust against changing organic loading rates due to variable inlet wastewater quality. Winery wastewater during grape harvest has been treated in pilot-scale RBC reactors. Yeast species that are naturally associated with grapes (*Saccharomyces cerevisiae*, *Candida intermedia*, and so on) were found to be co-dominant in the attached biofilms with bacteria, indicating the potential importance of inlet microorganisms on the treatment.

Hybrid Suspended and Attached Growth Systems

Hybrid systems combine some characteristics of both suspended growth and attached growth biotreatment systems. There are three main types of hybrid treatment systems used for industrial wastewater treatment: the MBBR, integrated fixed film activated sludge (IFFAS), and biological aerated filters (BAFs). In each case, a few key companies have patented versions of the technology and market specific varieties. In general, all of the hybrid systems contained a solid media inside the traditional AS basin. This media is retained in the reactor and provides a support matrix on which attached biofilms form. Therefore, higher organic loadings can be treated in the same overall reactor volume due to the higher overall biomass in the reactor. This allows a smaller footprint for the wastewater treatment facility. In addition, the process is less reliant on the performance of the secondary clarifier and upsets in biomass sludge settling, because the attached biomass is retained regardless. In addition, specialized subpopulations of microorganisms can be retained in the fixed films rather than washed out of the reactor, and therefore better able to respond to intermittent loading of various compounds.

Moving-Bed Biofilm Reactor

Since the early 1990s, MBBRs have been in development and use, beginning in Norway and Europe and more recently expanding to applications in the United States. There are more than 400 large full-scale operations in 22 different countries. For industrial wastewater treatment, MBBR systems have been used to treat food industry, dairy, chemical plant (high in aniline, cyanides, and diphenylguanidine), flavor production, slaughterhouse, refinery, and pulp and paper wastewaters. Buoyant plastic media is aerated and suspended in a tank. Polyethylene Kaldnes media (2–50 mm long×9–64 mm diameter) is one example, and has been the most widely reported for use at full-scale industrial treatment facilities. At typical carrier loading, this results in a specific area of biofilm growth of about 350 m^2 m^{-3} reactor volume. A sieve at the reactor outlet (with approximately 5–7 mm openings) is used to retain the media in the reactor. Alternatively, the LINPOR system uses foam cubes (~1.5 cm) as packing media. Circulation in the tanks by aeration or submerged mixers maintains movement of the media, and this agitation dislodges excess biomass from the carrier media and prevents clogging. Waste sludge sloughed from the media is collected in secondary clarifiers, similar to a trickling filter. This sludge is not recirculated. Oxic zones can remove BOD and ammonia, and anoxic zones can remove nitrate. Phosphorus removal will not occur in the bioreactor.

One advantage of these systems is that the media can be added into existing AS basins to increase the biomass in the system. Municipal wastewater treatment systems have increased the total biomass in retrofitted AS basins by as much as a factor of 2. Most of the biomass in an MBBR (>90%) is attached to the media rather than suspended in the liquid. The MBBR system is also less prone to process upsets from poorly settling biomass than AS systems, since much of the total biomass in the system is attached on the retained media rather than being returned to the reactor as underflow from a secondary clarifier. LINPOR reports full-scale use of their technology for primarily municipal wastewaters, with one paper industry installation (9000 m^3 day^{-1}, inlet COD=400 mg L^{-1}, and effluent <70 mg L^{-1}) and a coke over installation (1,25,000 m^3 day^{-1}, COD=280 mg L^{-1} and N=50 mg L^{-1}).

In some cases, MBBRs have been used as roughing filters ahead of downstream treatment processes, including conventional AS. In this configuration, significantly higher loadings can be applied since high removal efficiency of carbon and/or nitrogen is not required. For example, surface area loading rates (SALR) of 20–53 g COD m^{-2} day^{-1} and 10–25 g BOD m^{-2} day^{-1} have been reported (~2–5 kg COD m^{-3} day^{-1}). Given the observed removal efficiency, this translates to removal rates of 17–42 g COD m^{-2} day^{-1} and 8–18 g BOD m^{-2} day^{-1}. These values are expected to vary widely based on the amount of soluble biodegradable organic matter in the total COD. Nitrogen removal up to 0.83 g NH_3-N m^{-2} day^{-1} has been reported.

Another variation on MBBR technology is to add the plastic media into a sequencing batch reactor. This so-called sequencing batch biofilm reactor (SBBR) combines the advantages of an SBR with the biomass retention properties of attached biofilms. Most of the published studies on SBBRs are from laboratory-scale experiments with various synthetic wastewaters. A few pilot-scale studies have been reported with industrial wastewater, with reported removal rates of 0.3–9.1 kg COD m^{-3} day^{-1}.

Integrated Fixed Film Activated Sludge

IFFAS is a hybrid process in which buoyant media is aerated and suspended along with mixed liquor in AS basins. A variety of medium types can be used. Rope-like Ringlace was developed in Japan in the 1970s and has been used in more than 400 locations

worldwide. Biofilms develop on the medium, increasing the overall biomass concentration in the AS basins. Frequently, this medium is added to existing AS basins to retrofit the plant to treat greater flow rates and/or achieve better removal of BOD and nitrification. Incorporation of the medium into an AS basin in sequence with traditional anaerobic and anoxic stages can achieve denitrification and biological phosphorus removal. Minimal peer-reviewed studies on IFFAS have been published. Most of the applications appear to be associated with municipal wastewater treatment.

Biological Aerated Filters

In a BAF, aerated wastewater flows through medium which acts as a filter. Most large systems are for municipal wastewater treatment, although there are at least 18 full-scale systems treating pulp and paper mill effluent and a number of smaller plants in Japan treating various industrial wastewaters. These systems have also been used for pretreatment of industrial wastewaters from food production, carpet dying, and pharmaceuticals to reduce high organic loads, with reported surface loadings of 15–30 g COD m^{-2} day^{-1} and volumetric loadings of ~4.5 kg COD m^{-3} day^{-1}. Higher organic loadings can be applied compared to conventional AS systems. These systems have also been reported to be more robust to changes in influent wastewater characteristics. No secondary clarifier is required. Phosphorus removal cannot be directly incorporated into the bioreactor. Two companies market the majority of the BAF processes.

Kruger/John Meunier, Inc.'s Biostyr uses plastic beads called BioStyrene (3.6 mm diameter; specific biofilm surface area ~276 m^2 m^{-3}). Reported typical nitrification and denitrification rates in municipal wastewater treatment are about 0.9–1.2 kg N m^{-3} day^{-1}, achieving <5–20 mg L^{-1} effluent N. Tertiary denitrification rates are higher at 3 kg N m^{-3} media day^{-1} achieving<2 mg L^{-1} effluent N. In highly loaded facilities with ~0.5 day SRT, effluent BOD is <20 mg L^{-1}. Gravity backwashing about once a day is important due to headloss accumulation. This type of system has been used to treat dairy wastewater at a loading rate of up to 12 kg COD m^{-3} day^{-1}. The Biostyr system was also used to retrofit an AS plant to achieve nitrification at water temperatures as low as 7°C. The wastewater is first treated in AS basins, such that the BAF unit received ~44 mg L^{-1} BOD, 58 mg L^{-1} SS, and 32 mg L^{-1} NH_4-N loadings.

Infilco's BIOFOR BAF uses expanded clay media with a mean diameter of 2.7 mm. In lab- and pilot-scale tests it has been determined that the typical packed-bed macroporosity is ~0.4, with 1333 m^2 of carrier interfacial surface area per m^3 of reactor volume. The media bed depth is typically 3 m. In municipal wastewater treatment applications, the system can achieve effluent inorganic N less than 2 mg L^{-1} and 4–5 mg L^{-1} total N. Backwashing normally occurs once every 24–48 h and can be controlled based on elapsed time and/or accumulated headloss across the filter bed. BIOFOR systems are in operation at over 100 locations worldwide, treating from 380 to 416,000 m^{-3} day^{-1} each. This type of media was used in a pilot study to treat citrus industry wastewater with inlet COD=2400–5800 mg L^{-1}, BOD5=1580–3900 mg L^{-1}, and pH 6–7.2. To reliably meet the effluent standard of 1100 mg L^{-1} effluent COD, volumetric load less than 72 kg COD m^{-3} day^{-1} and hydraulic load less than 0.98 m h^{-1} was recommended. To achieve less than 600 mg L^{-1} effluent COD, a maximum load of 20 kg COD m^{-3} day^{-1} and hydraulic load of 0.36 m h^{-1} was recommended. Cocurrent flow was more effective than countercurrent flow.

Another alternative media used is 2–3 mm diameter sand. This process has been used at a full-scale treatment facility treating up to 654 m^3 day^{-1} of ammonia liquor from a coke plant. The six aerobic upflow filters are each 2.9 m wide, 6.5 m long, and 5.5 m high, with a total of 42.5 m^3 min^{-1} of air added. Removal efficiencies are greater than 99, 78, and 99.9% for thiocyanate, ammonium, and phenols, respectively.

Membrane Bioreactors

MBRs are rapidly gaining popularity for industrial wastewater treatment. In North America, the first full-scale industrial treatment MBRs was implemented in 1991. In Europe, more than 100 full-scale MBR plants for industrial wastewater treatment are in operation, compared with only 39 systems reported in North America. Membrane bioreactors have the advantage of retaining biomass in the treatment reactor via the filtration of the effluent wastewater through the membrane. Most commonly the membranes are in the ultrafiltration (UF) range, with pore sizes 0.1–0.4 μm. In some cases, microfiltration (MF) membranes are used. Originally, the systems were operated with external membranes following the AS basin, replacing conventional secondary clarifiers. Biomass was returned to the reactor using a recirculation loop. In the newer configuration, the membranes are submerged directly in the bioreactor. The biomass is therefore a combination of suspended in the liquid and attached to the surfaces of the membranes. The systems therefore combine the aeration basin and clarifier common to AS systems in a single basin. Very high biomass concentrations can be retained in the system, with 12–15 g L^{-1} typical and as high as 30 g L^{-1}, without worrying about upsets from poorly settling flocs. Very long SRT is usually maintained in the system, and overall sludge wasting is generally less. In North America, GE is a popular manufacturer of submerged hollow-fiber membranes for industrial applications.

The primary disadvantage of the MBR system is the higher overall operating cost due to energy consumption, largely for pulling water through the membranes and the frequent cleaning cycles to minimize membrane fouling. Cornel and Krause (2006) noted that 0.4–4 kW h m^{-3} treated wastewater is typically needed for pump energy. For aerobic treatment systems, oxygen supply is also required. This can be provided via conventional bubble aeration or using submerged membranes for aeration. The energy needed for oxygen supply is typically on the order of 0.3 kW h m^{-3} for municipal wastewater; with higher amounts needed for wastes with greater COD.

A key concern with MBRs is avoiding membrane fouling. Fouling reduces the flux through the membranes and increases the energy consumption. To avoid problems, the influents wastewater is generally screened first, often through a sieve of ~0.5 mm. Common approaches to minimize fouling with submerged hollow fiber membranes include: in situ backwash with permeate every few minutes on an automatic schedule; chemically enhanced backwash on an automatic schedule approximately every day; maintenance cleaning with acids on a weekly basis, and infrequent (1–2x/year) ex situ intensive cleaning.

Most of the industrial wastewater treatment applications for MBRs in Europe are used for pharmaceutical, chemical, and food industry wastewaters, treating 50–1840 m^3 day^{-1}. In North America, MBRs are used primarily for food and beverage processing and chemical plant wastewater, treating 19–18,925 m^3 day^{-1}. Case studies reported include a pharmaceutical/chemical industry wastewater treatment facility with three AS tanks followed by four zenon hollow fiber immersed external membrane units with 15,840 m^2 membrane surface. Inlet average COD and ammonia-N were 3500 and 100 mg L^{-1}, respectively; effluent contained 300 and <1 mg L^{-1}, respectively. The averaged flux was 12.5 L m^{-2} h^{-1} and MLSS was 12,000 mg L^{-1}. Another example is the full-scale MBR plant treating pharmaceutical wastewater in California. The operating liquid volume is approximately 147,000 gallons and the system is designed to treat 5 gallons per minute of wastewater at a feed total organic carbon (TOC) concentration of 14,000 mg L^{-1}. The system contains four Model 500C Zeeweed modules (pore size, 0.03–0.04 μm) with a total membrane area of 186 m^2. The system is designed to operate at 10,000 mg L^{-1} biomass as TSS in the reactor. To avoid fouling that significantly increases the vacuum required to draw the liquid across the membranes, the system automatically backpulses permeate plus 3–5 mg L^{-1} sodium hypochlorite (bleach) for 60 s every 20 min. Once per day, a more vigorous cleaning procedure of maximum 20 min with ~200 mg L^{-1} hypochlorite is conducted.

In a case study treating tannery wastewater, Reemtsma et al. (2002) found that the MBR with UF membranes did not improve the removal of poorly biodegradable polar organic compounds, such as naphthalene disulfonate, and benzothiazoles, compared to conventional AS treatment. Other laboratory studies have explored the suitability of MBR technology for chemical production, food production, paper mill, pharmaceutical, soap production, and various textile wastewaters with varying degrees of success.

Irreversible fouling of membrane surfaces is a significant problem. It is hypothesized that different microbial communities may be associated with irreversible clogging, and that different operating conditions can be used to select for microorganisms less associated with irreversible clogging. This is an active area of research.

Novel types of MBRs are also being explored for industrial wastewater treatment. A recent review article by Yeo et al. (2015) described high-retention MBRs and extractive MBRs. The high-retention MBRs are designed to retain target pollutants, and therefore have pore sizes that are smaller than typical, including nanofiltration MBRs, forward-osmosis MBRs, and membrane distillation bioreactors. Extractive MBRs are used to separate the biodegradable organics that are being targeted for treatment from aqueous conditions that are unsuitable for direct biotreatment, such as extreme pH or high salt concentrations. Unique materials are being tested for these membranes. To date these systems have only been explored in laboratory and pilot scale tests. Further developments are needed before these systems will be viable for full-scale treatment.

Anaerobic

Anaerobic processes degrade organics in the absence of oxygen. A significant advantage is that the high energy required for aeration of aerobic treatment systems is not needed; a potential savings of ~1 kW h kg^{-1} COD removed. Another notable feature is the potential production of methane, which can be captured and used as an energy source, at a theoretical maximum energy yield of 3.8 kW h kg^{-1} COD removed. The biomass yield during organic degradation is also lower than aerobic processes, resulting inasmuch as 90% less by-product sludge. These systems can be used to encourage water reuse in factories. These factors indicate that anaerobic treatment may have significant advantages over aerobic processes and overall may be more sustainable for the environment.

Anaerobic treatment may be better suited to some waste types given the organic and nutrient concentrations present. The C/N/P ratio in typical winery wastewater is unbalanced for aerobic treatment and requires N and P addition; however, the 800:5:1 C/N/P ratio (BOD5/N/P 333:3.3:1) is suitable for anaerobic treatment. The seasonal nature of the wastewater production at small wineries poses an additional challenge for anaerobic systems because they generally have longer start-up times due to lower cell yield. It has also been reported that the presence of a high concentration of sodium (such as 4600–14,000 mg L^{-1} and 200–250 mmol L^{-1}) is inhibitory for anaerobic biological treatment.

VanLier conducted a review of anaerobic technologies and implemented full-scale industrial wastewater treatment. From 1998 to 2004, of 519 systems, the most popular were uplow anaerobic sludge blanket (UASB), internal circulation (IC), expanded granular sludge bed (EGSB), lagoon, hybrid, completely stirred tank reactor (CSTR), and anaerobic filters (AF) or fluidized-bed (FB) technologies at 36, 34, 17, 4, 2, 2, 1, and 1%, respectively. Note that this represented a shift to more granular sludge based and super high rate based systems (FB+EBSG+IC=52%) compared to the 1383 anaerobic treatment technologies implemented from 1981–98 (super high rate=8%). High rate anaerobic treatment was commonly applied to food processing wastewater from industries such as sugar, potato, distilleries, wineries, fruit juice, starch, beer, and soft drinks. Anaerobic biotreatment applications in paper mills and chemical wastewater are also increasing. In another survey paper, Austermann-Haun et al. (1999) noted that of the 125 full-scale methane reactors treating industrial wastewater in Germany; 43 were sludge blanket reactors, 33 fixed-film reactors, 11 CSTRs, and the rest were various hybrid systems. Examples of anaerobic treatment of industrial wastewaters in various anaerobic reactors are provided in Table 4. Note that AF and FB systems are attached growth while the remainder are suspended growth, with hybrid systems containing a combination of attached and suspended biomass.

Table 4 Examples of anaerobic treatment of industrial wastewaters

WW type	*Reactor*	*Scale; volume*	*HRT(days)*	*OLR (g COD L^{-1} day^{-1})*	*Supplements*	*Percent COD removal*	*Notes*	*References*
Brewery	UASB	Full; 500 m^3	1	6	N, P, soda ash for pH	40–75	50% TS removal	Parawira et al. (2005)
Brewery	UASB	Pilot	3.5	7		95	0.30 L Methane yield g^{-1} COD	Oktem and Tufekci (2006)
Brewery	EGSB-AF	Lab; 3.4 L	0.75	4.5		93–95	0.28 L CH_4 g^{-1} COD day^{-1}	Connaughton et al. (2006)
Dairy	AF	Lab; 2–14 L	1–6	3–9		94–98		Omil et al. (2003)
	FB	Lab; 0.6–3 L	0.3–1.3	1–3.8		80–92		
	SBR	3.5 L	3.2	6.25		96		
	UASB	Lab; 1–8 L	0.2–2	9–24		78–98		
Dairy	UASB	Full; 4000 m^3	8	0.55		63		Omil et al. (2003)
Dairy parlor	UASB	Lab; 12 L	3.5	0.2		51–73	10°C Lower COD removal; followed by septic tank	Luostarinen and Rintala (2005)
Distillery	UASB	Full scale; 150 m^3, 300 m^3	~2	15		90	Grain distillation caused floating scum layer problem	Laubscher et al. (2001)
Food processing	MBR	Lab; 0.4 m^3	2–4	4.5		80–85	UF membranes; gas yield 0.14 L g^{-1}COD	He et al. (2005)
Pharmaceutical	CSTR	Lab; 5 L	0.5	13		13	44% Acidification	Oktem et al. (2006)
Potato waste	UASB	Lab; 0.84 L	3.3	6.1		90	0.23 L CH_4 g^{-1} COD	Parawira et al. (2006)
Slaughter-house	MBR	Pilot; 200 L	2–7	3–3.5		95	Biogas circulated to control fouling	Jensen et al. (2015)
Textile sizing	UASB	Lab; 2.5 L	0.5	4.8		85		Spanjers (2006)
Winery	UASB	Lab; 2.6 L	1	7–16		60–85		Kalyuzhnyi et al. (2000)
Winery	UASB	Full; 2 @138 m^3	3	~7	N, P		Prestorage tank with some acidification; postaerobic tmt w/two basins	Moletta (2005)
Winery	AF	Full scale; 4 m^3	1.2	4.6–11		88–98 overall	Pretreat in acidogenic reactor, follow with aerobic tmt	Moletta (2005)
Winery	SBR	Lab; 5 L	2.2	8.6	N, P, NaOH	98 sol		Ruiz et al. (2002)
Synthetic high salt ww	AF	Lab	2	5–6.2		<80	35 g L^{-1} NaCl, 35°C; higher salt or OLR failed	Riffat and Krongthamchat(2002)

Upflow Anaerobic Sludge Blanket

The most widely applied form of anaerobic wastewater treatment is the UASB. In these systems, the biomass tends to form granular agglomerates that have very good settling properties. The systems tend to work best with high strength wastewaters and at higher temperatures. The conventional UASB process can accommodate a fairly high organic loading rate, in the range of 5–15 kg COD m^{-3} day^{-1} for readily biodegradable wastewaters. UASB systems have been used to treat wastewater from alcohol distilleries, fruit juice, meat packing, paper mills, potato processing, slaughterhouses, and sugar processing plants. Problems sometimes noted with UASB systems include difficulties when treating wastewater containing high TSS content, foaming when treating high amounts of fats, lipids, and proteins; clogging of the sludge bed when treating fatty effluents; and biomass washout.

UAB reactors can be operated under a variety of temperature regimes ranging from psychrophilic (<20°C), to mesophilic (35°C), to thermophilic (55°C). Mesophilic is the most common. External heating is generally needed to operate at mesophilic or thermophilic temperatures; this may be supplied by using the methane gas produced in the digestion. The high temperature of effluent water from the pulp and paper industry (50–60°C) may be particularly suited to thermophilic anaerobic digestion, although at present only laboratory studies on these systems are known. Different bacterial species will generally survive at mesophilic versus thermophilic temperatures.

To improve the process performance of UASB reactors, modifications including EGSB and IC have been made. EGSB is designed for a faster upflow velocity of water through the sludge bed (4–10 m h^{-1}) than traditional UASB reactors (0.5–2 m h^{-1}). This increased flux partially fluidizes the granular sludge bed, resulting in improved contact between the wastewater and the sludge. The IC process has two UASB compartments on top of each other. In the first, bottom stage, most of the biogas is produced. This gas is trapped in an internal gas hood, rises through a riser pipe to a gas liquid separator, and then liquid flows down a downcomer pipe to the first state. In this manner, the biomass production drives internal circulation of the wastewater. The second, top stage functions as biomass retention and polishing. The number of full-scale plants using these enhanced processes has been increasing year by year. In these EGSB and IC systems, higher organic loading rates (15–45 kg m^{-3} day^{-1}), tall reactors 12–20 m high, and high upflow velocities of 8–30 m h^{-1} are common. At higher organic loading rates, the higher intensity of biogas production tends to cause sludge washout from the reactor. This problem can be addressed using a compartmentalized UASB reactor, which is equipped with multiple numbers of gas–solids separators (GSS).

Anaerobic Sequencing Batch Reactor

Anaerobic sequencing batch reactors were first developed in the early 1990s to treat landfill leachate and high strength organic wastewater. Like aerobic SBRs, there is a treatment cycle of four discrete steps: fill, react, settle, and withdrawal. These cycles should be as frequent as possible, without idle time. The reactors are amenable to automatic process control, enabling operational flexibility. These systems have been used to treat piggery and slaughterhouse wastewater, winery wastewater, and landfill leachate.

Anaerobic MBR

Most of the literature citations of anaerobic MBRs are for domestic wastewater treatment. Cake formation is noted as a major cause of flux decline. This may result in irreversible clogging and flow membrane flux per headloss. One contributor to this problem may be that the selective pressure to maintain microbial granules is gone so that the biomass quickly degenerates into loose floc even if seeded with granular sludge material. Scaling due to high concentrations of carbonates may also be a problem. Lab-scale studies treating starch wastewater found that a membrane significantly improved the performance of the acid fermentation reactor but had little impact on the efficiency of the methane fermentation tank; other lab-scale studies have been conducted with brewery, fermentation, food, and pulp and paper wastewaters.

Coupled Anaerobic/Aerobic Treatment Processes

By combining anaerobic and aerobic treatment processes, the overall treatment of industrial wastewater may consume less energy, more fully mineralize complex organic contaminants, and enable optimum nutrient removal. Typically, the anaerobic stage is first. This achieves organics removal without requiring energy to supply oxygen and at lower sludge yield. Useful by-products such as methane gas can be generated. The step can generally remove 80–90% of the organics. The second aerobic stage can polish the wastewater to meet stringent discharge limits for organics, and may also remove nitrogen and phosphorus. The specific reactor types selected for the anaerobic and aerobic stages can vary. Some examples are provided in Table 5; Chan et al. (2009) provide about 20 additional examples in their review paper. The UASB-cyclic AS systems for brewery wastewater treatment have been reported to reduce sludge production by a factor of 5 and lower energy consumption by a factor of 3 compared to existing two-stage aerobic treatment systems, with 11 full-scale systems reported.

At full-scale plants in Germany treating wastewater from beet sugar factories, potato processing, breweries, and potato and wheat starch factories, the wastewater is anaerobically pretreated and then followed by an aerobic stage with nitrification and denitrification. In these cases, there is generally a by-pass of some fraction of the wastewater around the methane reactor to supply enough organic carbon for the denitification stage. The authors recommend that 50–70% of the AS tank be used for denitrification.

Table 5 Examples of sequential anaerobic:aerobic treatment of industrial wastewater

Wastewater source	*Scale; Q and OLR*	*Anaerobic stage*	*Aerobic stage*	*References*
Brewery	Full; 24–417 m^3 h^{-1} 2200–32,280 kg COD day^{-1}	UASB ~7 h HRT 9 kg COD m^{-3} day^{-1}	Cyclic AS ~20 h HRT 0.4 kg BOD m^{-3} day^{-1}	Gerards et al. (2005)
Dye containing Remazol Black 5	Lab; 5–25 kg COD m^{-3} day^{-1}	UASB 3–7 kg COD removed m^{-3} day^{-1}; 92–87% color removal	CSTR SRT 1.7 to 11 days resulted in 28–90% COD removal	Sponza and Isik (2002)
Milk quality control lab	Lab; 10.5 g L^{-1} COD; 5–6 kg COD m^{-3} day^{-1}	Anaerobic filter PVC packing, upflow; 2 day HRT; 90% COD removal	SBR, 24 h cycle effluent <200 mg L^{-1} COD, <10 mg L^{-1} N	Omil et al. (2003)
Kraft mill	Full; 5000 m^3 h^{-1} 19,000 kg BOD day^{-1}	Anaerobic basin; 2 km^2; 1.2–3 m deep	Two partial mixed aerobic ponds	Starke (2001)
Pharmaceutical plant	Pilot; 9.7–20 g L^{-1} COD	Baffled 1.25–2.5 day HRT 4–10 kg COD removed m^{-3} day^{-1}	Biofilm airlift suspension 5–12.5 h HRT 3–8 mg COD removed m^{-3} day^{-1}	Zhou et al. (2006)
Slaughter-house	Pilot; 0.14–0.5 m^3 h^{-1}	UASB 2–7 h HRT	RBC 5.3 g sBOD m^{-2} day^{-1}	Torkian et al. (2003)

Some recalcitrant organic compounds can only be mineralized by anaerobic and aerobic biodegradation processes in series. For example, azo dye decolorization can occur via anaerobic reduction or cleavage of the azo bond followed by mineralization of aromatics and amines under aerobic conditions. Therefore, these sequential systems have been studied for treatment of dye water, such as sequential UASB/AS systems.

Natural Treatment: Wetlands

In addition to engineered treatment of industrial wastewaters in tanks and constructed basins, there are various examples of industrial treatment in lagoons and constructed wetlands. These processes are most often used in agricultural areas where large areas of land are available, rather than urban industrial settings. There are two predominant types of constructed wetlands: free water surface (FWS) and subsurface flow (SSF) wetlands. In the FWS wetland, also called surface flow wetlands, there is freestanding water over the soil bedding of the wetland. In contrast, the SSF wetland has a greater depth of granular media into which the plants root, and no water flows above the surface of the media. Typical depths of granular media are 1–1.5 m in SSF wetlands, although the actual water depth flowing through the bed may be much less (as low as 0.15 m reported). In both types of wetlands, good engineering design dictates that the wetland is constructed on a low permeability liner. The liner can be natural material (such as clay) or more commonly a geomembrane material such as high-density polyethylene. The liner prevents wastewater that has yet to be fully treated from leaching out of the bottom of the wetland and contaminating the underlying groundwater.

Both systems achieve removal of organics and nutrients by a combination of plants and associated microorganisms. The types of plants in the wetland are critical to the type of treatment efficiency achieved. The plants evapotranspirate water, uptake nitrogen and phosphorus, and can impact the redox conditions. Common plants in constructed treatment wetlands include bulrush (Typha), grasses, and reeds. Excess plant growth over time may require harvesting. This has been shown to be true in water hyacinth ponds.

The redox conditions in a wetland range from aerobic to anaerobic. Oxygen is available near the free water surface due to dissolution from the bulk atmosphere and near plant roots that excrete oxygen. Anaerobic conditions occur at depth and in the lower soil layers. Pathogens are also attenuated through the treatment process via filtration and natural die-off under environmental conditions and bacterial competition. Removal of fecal coliforms (FC) typically range from one to three orders of magnitude.

Unless a large amount of land is available to allow long residence times, these wetland treatment processes are used for polishing and nutrient removal after the bulk of organics and solids have been removed. Typical natural processes used for wastewater treatment before wetlands include solids settling and various types of lagoons (anaerobic, facultative, and aerobic). Solids removal is particularly important in SSF wetlands, where high solids loading may otherwise clog the porous media. Therefore, presettling is a common pretreatment. Even still, the inlet zone of SSF wetlands often contains coarse gravel to minimize clogging problems. The final effluent from wetlands is often reused for irrigation, agricultural "washing," or other uses. Newman et al. (2000) cite the presence of ten constructed wetlands for agricultural wastewater treatment in North America. Examples of wetland treatment systems are provided in Table 6. Note that residence times are normally determined by nonreactive tracer studies.

Poor treatment efficiency in colder winter months outside the plant growing season may be a problem. However, some treatment occurs even at air temperatures as low at −8°C, water temperatures as low as 1.6°C, and with ice on the top of the wetlands. It has been reported that more TSS, TP, TKN, ammonia, and nitrate/nitrite are removed during the plant growing season than during plant senescence. In fact, effluent ammonia-N may be higher than inlet wastewater during the winter due to plant senescence. Owing to nitrifying bacteria, it is expected that N removal will be more temperature sensitive than carbon removal. Settling and increased storage may account for much of the contaminant removal during winter months.

Table 6 Examples of wetland treatment of industrial wastewaters

Waste-water source	*Location; minimum water temperature*	*HRT, d*	*Pre-treatment*	*Wetland type; total area, m^2; depth, m*	*Plants*	*OLR g/ m^2/day*	*Percent treatment efficiency*	*References*
Dairy	Arizona	4.5	Solid separation, anaer. pond, aerobic pond	SSF; 5000; 1.5	Cattail, bulrush, reeds	12 BOD5	0 BOD5, 30 TSS, 23 TKN	Karpiscak et al. (1999)
Dairy	CA	7	Facultative pond	SSF; 1200; 1	Reeds, bulrush	15 BOD	50 BOD, 40 COD, 87 SS, 26 TKN, 35 PO4-P	Ibekwe et al. (2003)
Dairy	Canada; 1.6°C	45–52	None	FWS; 100; 0.15–1	Cattails, grasses	450 BOD	62–99 BOD5, TSS, TP, NH4-N	Smith et al. (2006)
Dairy	Lab in India	4	None	FWS; not reported; 0.1	Water hyacinth	6–20 BOD	86 BOD, COD 32 TSS; 58 TDS, 67 TN	Trivedy and Pattanshetty (2002)
Milk-house	CT; 2.5°C	>12 (ave 41)	None	FWS; 400; actual ave. 0.3	Cattail or reed or bulrush	15 BOD5	76 BOD5, 90 TSS, 28 TKN, 45 TP, 98 FC	Newman and Clausen (1997); Newman et al. (2000)
Batik Dye	Tanzania	1.2	None	FWS; 3 @ 1.7 ea; 0.6	Cattail or cocoyam	11 COD	70 COD, 56 sulfate, 75 color	Mbuligwe (2005)

Table 7 Examples of coupled oxidation and biological treatment systems

Wastewater source	*Scale; wastewater characteristics*	*Oxidation treatment*	*Biological treatment*	*References*
Dairy and pharmaceutical	Full; COD 3000–11,000 mg L^{-1}; 54 m^3 h^{-1}	Ozone post biotreatment 2 g O_3 g^{-1} COD; 68 kg O_3 day^{-1}, 10 min contact time	Two SBRs, 1.2 MG ea, jet-aeration 77 kg urea day^{-1} added for NSRT 10–20 day; 8 h cycle	Helmig et al. (2002)
Formaldehyde-urea adhesives factory	Bench and full; COD 460–3900 mg L^{-1}, formaldehyde 220–4000 mg L^{-1},TKN 110–805 mg L^{-1}	Post ozonation recycled to aerobic chamber 60 min HRT partially degraded >8000 g/mol^{-1} organics	Single-sludge anoxic-aerobic activated sludge	Garrido et al. (2000)
Olive processing	Lab; phenolic substances up to 500 mg L^{-1}	Pre wet air oxidation w/H_2O_2 6 h at 443–483 K and 3–7 MPa; converted 46–99% COD	AS 2× increase in kinetics with pre-oxidation	Rivas et al. (2001)
Optoelectronic	Lab; contains dimethylsulfoxide (DMSO)	Ozone gas-induced reactor 240 min, complete DMSO removal	SBR achieved effluent limit of <100 mg L^{-1} COD	Lin and Chang (2006)
Pharmaceutical	Lab and full; COD 1000–7000 mg L^{-1}	Fenton's oxidation; H_2O_2/Fe^{+2} ratio 170; 20 L H_2O_2+0.33 kg $FeSO_4$ per m^3 ww; 45–50% COD removal	Two SBR in series overall 98% COD removal	Tekin et al. (2002)
Plastics manufacturing	Full; 5000 m^3 day^{-1}	Ozonation at 6 mgO_3/h post AS reactor	Upflow BAF (six 3.8 m diameter×4.7 m high BAFs with sand media)	Chen et al. (2000)
Tannery	Lab; COD ~3500 mg L^{-1},NH4-N 250 mg L^{-1}	Ozone 18 or 50% of SBBR volume removed, ozonated 1 h at 520 mgO_3/h, returned to the SBBR	SBBR with Kaldnes media 8 h cycles,1 day HRTOLR 3 kg COD m^{-3} day^{-1}, NLR 0.19 kg N m^{-3} day^{-1}, 31–44 g TSS/L filter	Di Iaconi et al. (2004)
Textile wastewater	Full scale; AS lab scale ozone	Ozone pre or post 40 mg O_3/min for 15 min 85–99% color, 14–19% COD removed	AS 7 day SRT and ~20 h HRT	Orhon et al. (2002)
3-Methyl-pyridine	Lab	Pre ozone bubble column produced formate, acetate, NH_3	SBBR	Carini et al. (2001)

Coupled Biological With Chemical or Physical Treatment

The coupling of biological treatment with chemical or chemical treatment may improve the overall treatment of the wastewater. One of the most common approaches is to couple chemical oxidation with biological treatment. The most commonly used oxidant is ozone, with a few examples of Fenton's reagent and hydrogen peroxide use. Examples are summarized in Table 7. Ozone added into water causes both direct oxidation of organics with ozone and indirect oxidation with the hydroxyl radicals that ozone forms. In Fenton's oxidation, iron serves as a catalyst for hydrogen peroxide solution to form hydroxyl radicals. These radicals can oxidize most organic substances. Future ideas under exploration are ways to use solar collectors to power photo-catalysis by TiO_2 or photo–Fenton oxidation. These types of systems would be more environmentally sustainable than traditional oxidant technologies.

Biological treatment processes may also be combined with absorption on activated carbon, to remove recalcitrant organic compounds. The bulk liquid concentration of toxic wastewater components is decreased by adsorption so that inhibitory effects on the bacteria are minimized. Volatile organics may be kept available to the bacteria instead of being stripped. Over time, if the recalcitrant organics are intermittently loaded, biodegradation may regenerate the sorptive capacity of the activated carbon. Kolb and Wilderer (1997) explored this type of system in a lab-scale SBR system treating benzene and 2-chlorophenol or trichloroethene plus phenol wastewater. They found that it was more efficient to sequentially load the activated carbon and then biologically regenerate it, rather than trying to simultaneously sorb and biodegrade the compounds in a single step. Another potential combination is steam stripping followed by biological treatment. This process has been used to treat Kraft Mill wastewater.

Future Outlook: Electricity Production and Other Electrochemical Technologies

High organic-strength industrial wastewaters may be well-suited for various microbial electrochemical technologies (METs) and bioelectrochemical systems (BES). The most widely studied of these processes are microbial fuel cells (MFCs), which produce electricity directly from bioactivity. MFCs have been studied for nearly 20 years, with success achieved in lab-scale systems. However, full-scale MFC treatment systems for industrial wastewater are not known (Logan et al., 2015); the reason for the lack of full-scale adoption is speculated to be largely due to the costs associated with the electrode materials. Types of industrial

wastewaters that have been tested in lab-scale MFCs include cattle manure, liquid cheese production wastes, and wastewater from chemical industries, dyes, glass industries, leather tanning, metal works, textiles, and vegetable oil industries. A different form of MET is a microbial electrolysis cell (MEC), which can produce hydrogen gas. Alternative methods of biohydrogen production were reviewed by Bobescu et al. (2016). With further development and exploration of lower-cost materials, these systems promise electricity and/or net energy production from waste, but even an energy-neutral process whereby the energy production meets the treatment demands for mixing, oxygen supply, etc. would be beneficial over many existing industrial wastewater treatment processes.

Conclusion

Microbiological-based wastewater treatment processes can degrade many of the organic compounds in industrial wastewaters, mineralizing them to carbon dioxide and/or methane. Specific reactor configurations are better suited to some types of wastewaters, although in general all of the main types of industrial wastes have been treated under both aerobic and anaerobic conditions with various reactor configurations for each. Therefore, the optimum waste treatment reactor will depend on site-specific constraints including land availability, operator skill and time, energy, and sludge disposal, just to name a few. Although typical reactor sizing and bulk organic loading rates are available, due to the variability of wastewater characteristics and potential presence of specific target compounds that may or may not be recalcitrant or inhibitory to bacteria, bench scale or pilot scale testing are often conducted prior to sizing the full-scale treatment facility. In general, these smaller scale results seem to be predictive of full-scale performance. If problems arise during full-scale treatment, molecular methods to characterize the microbiology may provide a good tool along with traditional physical (suspended solids) and chemical analyses (pH, nutrients, and organics) to diagnose the source of the problem. Advancements in technology continue, with a goal of improving treatment efficiency at lower cost. In the future, industrial wastewaters may provide an important source of energy, if issues such as material costs for METs and BES can be solved. Owing to the vast breadth of information briefly overviewed in this article, readers are encouraged to read further on the specific technologies outlined in this article.

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Wine☆

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Glossary

Dry wines Wines with residual sugars lower than 10 g/L. Wines lacking sweetness.
Enology The science of winemaking.
Must Grape juice or mashed grape before and during fermentation.
Vinification The technology of wine making.
Viticulture The science of growing of grapes.

Defining Statement

Wine is an alcoholic beverage made by the fermentation of grape juice or other sugar-containing substrates including honey, sugarcane, fruit juices, and other plant juices containing sugars, such as palm tree sap, floral extracts, and *Agave*, the century cactus plant. Wine is distinguished from beer, in which the fermentable sugars are derived principally from starches by means of amylases produced in cereal grains, particularly barley, by germination or malting.

The term "wine" probably should not be applied to beverages that involve the hydrolysis of starch in cereals through ptyalin in saliva, the hydrolysis of starch to sugars via fungal or bacterial amylases, or the hydrolysis of starch to sugars and then fermentation of the sugars to ethanol by microorganisms. Thus, South American chicha, made by chewing maize and subsequently fermenting it, should probably be called a maize beer. Rice wine, such as Japanese sake, made by overgrowing boiled rice with *Aspergillus oryzae* as a source of amylases and then fermenting it with yeast, should probably more correctly be termed rice beer; however, accepted usage may preclude this.

This article will restrict the term "wine" to products made by the fermentation of sugar-rich substrates in which starch hydrolysis is not required.

Antiquity of Wine Fermentation

Wine fermentation is one of the oldest and perhaps the oldest fermentation known to humans. Honey diluted with rainwater ferments spontaneously with yeasts in the environment. Fruit juices ferment spontaneously with yeasts on the fruit. Although the most ancient fermented beverage produced on purpose was most likely beer, wine from grapes is the oldest wine produced. Although some legends may indicate pulque as very old fermented beverage, the absence of evidences cannot sustain those claims.

Wine was truly a gift of God (or nature) to primitive humans. Fruit juices ferment spontaneously, yielding a product that preserves and, in fact, enhances the fruit's nutritive value and retains its essential flavors. The conversion of the sugar to ethanol and ethanol's striking effect on the psyche of the consumer naturally led to the conclusion that the "spirit" of the fruit had been extracted for the benefit of the consumer.

However, the microorganisms responsible of this transformation were present on earth much earlier than humans. Microorganisms have the primary task of recycling organic matter, including sugars; yeasts in the environment ferment the sugars to alcohol as fruits become overripe and fall to the ground. Alcohol is one of the products of recycling reactions. It, in turn, is further fermented to acetic acid or respired to CO_2 and H_2O in continuing recycling reactions.

From the beginnings of civilization, wines have been associated with culture, art, and religion because early humans considered wine a miraculous conversion. For many years, the people responsible for the production of wine were considered as priests or similar, as they were handling the "miracle" of transforming the sugary liquid into a new product with euphoric effects and allowed new social relations.

Alcoholic beverages have always been closely related to politics, and even today governments use alcoholic fermentations as a major method of raising taxes. In the Hammurabi code there are already references to wine and frauds linked to it; also in the Bible there are several references to wine consumption and Noah's drunkenness as result of the celebration after planting a vineyard. In very

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primitive times in South America, emperors could hold office only as long as they provided the people with a sufficient supply of chicha. From ancient times, wines have played a vital role in religious ceremonies, and they continue to do so today. For many centuries, wine and beer had been the safest way to drink water and, thus, being a relevant part of the regular food intake of civilization.

Alcoholic Fermentation

The biochemistry of the alcoholic fermentation is among the most thoroughly studied enzyme–substrate sequences ever investigated. Black, in the 18th century, reported that ethyl alcohol and carbon dioxide were the products of sugar fermentation. Lavoisier, in the same century, reported that 95.9 parts of sugar yielded 57.7% ethyl alcohol, 33.3% carbon dioxide, and 2.5% acetic acid. Gay–Lussac was the first to show that glucose fermentation yielded approximately equal weights of ethanol and carbon dioxide (45 parts glucose yielded 23 parts alcohol and 22 parts carbon dioxide). Pasteur found that 100 parts sucrose yielded 105.4 parts invert sugar, which was fermented to 51.1 parts ethyl alcohol, 49.4 parts carbon dioxide, 3.2 parts glycerol, and 1.7 parts succinic acid. In Neuberg's normal fermentation, 1 mol of glucose yields 2 mol of pyruvic acid, which, in turn, yield 2 mol of carbon dioxide and 2 mol of acetaldehyde, which, in turn, are reduced to 2 mol of ethanol. Detailed research has shown that there are actually 12 intermediate steps between glucose and ethyl alcohol (the Embden–Meyerhof scheme), which account for the production of 2 mol of carbon dioxide, 2 mol of ethanol, and smaller quantities of glycerol from 1 mol of glucose.

Buchner in the 19th century produced ethyl alcohol from sugar using cell-free juice, made by grinding yeast cells with sand under pressure and filtering to remove the whole cells. This demonstrated that it was enzymes that were required for fermentation and not the whole cells themselves.

Yeasts

The principal microorganisms in the alcoholic fermentation of wines are yeasts belonging to genus *Saccharomyces*, in particular *Saccharomyces cerevisiae*. The taxonomy of *Saccharomyces* genus has suffered many revisions and, thus, it is possible to find many different names and species in the literature of wine making. However, nowadays there is a main consensus that *S. cerevisiae* is the main responsible for wine fermentation. Although yeasts belonging to genus *Saccharomyces* are generally present in the grapes or other fruits and they are capable of fermenting the juice to wine, it is a considered a good practice among winemakers, to inoculate pure cultures of selected wine yeasts. The main goal is the control of the alcoholic fermentation, in terms of good fermentation performance as well as a good quality final product. These yeasts are selected basically for the absence of off-flavors, but also for flavor, production of little or no foam, ability to ferment at low temperatures, or flocculation–sedimentation behavior.

Other wild yeast can be present during alcoholic fermentation, especially abundant in grapes and grape juices. Generally regarded as spoilage microorganisms, several efforts are oriented to prevent their growth during early stages of fermentation. Thus, to ensure that the selected wine yeast predominates over those wild yeasts and other microorganisms that may be present, it is also common practice to add 20–50 mg sulfur dioxide/L to the juice, usually in the form of bisulfite, before inoculating the selected yeast, which is generally tolerant or acclimated to these concentrations of sulfite. However, these non-*Saccharomyces* yeasts are also considered to provide some positive attributes to wine, such as more complexity, higher aroma concentration or even reduction of alcohol. Thus, some selected non-*Saccharomyces* yeasts are also available to be inoculated sequentially with *S. cerevisiae*.

S. cerevisiae and other wine yeasts have the ability to grow and metabolize sugars, such as glucose, fructose, and sucrose, aerobically or anaerobically. Aerobic growth yields far more energy and the yeasts multiply vigorously, but little if any ethanol is produced. However, in presence of high concentration of sugar and even in presence of oxygen, *S. cerevisiae* ferments sugar due to a complex inhibitory process of respiration known as Crabtree effect. Anaerobically, yeasts dissimilate glucose according to the Emden–Meyerhof scheme. Theoretically, 1 mol (180 g) of glucose yields 2 mol (92 g) of ethanol and 2 mol (88 g) of carbon dioxide. Actually, yields are slightly less because of the production of yeast biomass, glycerol and other minor compounds; nevertheless, it is practical to consider that 2 g of sugar yields slightly less than 1 g of ethanol. Anaerobic dissimilation of 1 mol of glucose yields 56 Kcal of energy to the yeast, part of which is used in the fermentation itself and a portion in multiplication of the yeast.

Yeasts multiply by budding, and the percentage of yeast cells showing buds when examined microscopically in culture or in a fermenting wine is a measure of the vigor of the culture.

When calculated on an overall fermentation basis, an individual wine yeast cell produces about 30 million molecules of ethanol. In the earlier stages of fermentation, a yeast cell may produce 100 million ethanol molecules. As the ethanol content reaches 12% v/v, the rate of ethanol production may fall to 10 million molecules.

Malolactic Fermentation

Another group of microorganisms accompanying the yeast in wine fermentations is the lactic acid bacteria. Lactic acid bacteria–yeast interactions are rather common in food and beverage fermentations. Grape juice contains organic acids, mainly tartaric acid and

malic acid. If the acidity is too high for the best flavor, it may be desirable to reduce it. Winemakers can take advantage of the spontaneous process led by lactic acid bacteria to ferment the malic to lactic acid, which decreases the total acidity and raises the pH of the wine. It also modifies the aroma, body, and aftertaste. If the grape juice has low acidity, malolactic fermentation may be undesirable because it will decrease the acidity still further, making the wine taste flat. Selected lactic acid bacteria starters are currently available to ensure the malolactic fermentation and the positive aromas that could be associated.

Malolactic fermentation could occur during the alcoholic fermentation, although it is most likely after the alcoholic fermentation is finished and nutrients from yeast lees are released. Spontaneously can also occur during holding before the final clarification and fining because it increases the microbiological stability, decrease fermentable carbohydrates, and produces inhibitory bacteriocins in the wine. If malolactic fermentation occurs in the bottle, it may result in cloudy wine or carbonation of a still wine.

Noble Mold

Under high-humidity conditions, unripened grapes may become infected with *Botrytis cinerea*, the noble rot. If the humidity decreases, the grapes lose moisture and, in fact, become shriveled. Such grapes yield a juice with higher sugar content, the grape flavor may also be concentrated, and the mold may introduce a delicate flavor. Under ideal conditions, very fine flavors can be produced from grapes infected with the noble mold.

Microorganisms Causing Spoilage in Wines

All the microorganisms, even those that are necessary for wine production, can spoil wine. In fact, one of the main characteristics to select a microorganism as starter culture is the ability to ferment without producing any wine spoilage. Thus, yeasts and lactic acid bacteria, fundamental for wine production, together with acetic acid bacteria are the major organisms causing spoilage in wines.

The main spoilage that microorganisms produce on wines are related to the production of acetic acid, which often derives also in the production of acetaldehyde and ethyl acetate, the production of haze or films on the surface and also the production of several phenolic compounds that remind horse sweat or mouse-like odor. Although the main producers of acetic acid are normally the acetic acid bacteria as the name points out, there are not the only ones that can produce it. Several non-*Saccharomyces* yeasts and lactic acid bacteria are also able to produce it in considerable amounts. The species of acetic acid bacteria that produce acetic acid are mostly, but not exclusively, from the genus *Acetobacter*. The process is highly aerobic and, in fact, grows on the surface of the wine if oxygen is present and could lead to the production of vinegar. The acetic acid bacteria are not generally a problem during alcoholic fermentation because carbon dioxide is being rapidly produced, and it displaces oxygen from the must, keeping it anaerobic. However, at the end of fermentation, oxygen exposure may facilitate the activity of these acetic acid bacteria.

One of the worst wine spoilages is produced by *Brettanomyces*, which produces off-flavors if it develops during aging storage. *Brettanomyces* produces of 4-ethyl phenol and 4-ethyl guaiacol, volatile phenols that are defined with descriptors such "mousy," "band-aid," horse sweat or horse stable, etc. and are defined as "*Brett* character" and it is clearly a serious defect of wine.

Microbiology of Sherry Wines

Sherry wines have an oxidized flavor, color, or aroma and are highly acceptable among many wine consumers. The oxidized flavor arises by conversion of a portion of the ethanol to acetaldehyde and other complex reactions. This is normally considered a defect in many wines; however, in sherry wines is its essential characteristic and oxidation is specifically sought. In a true fermented sherry, barrels are half-filled the still wine and the surface of the wine is exposed to air. Specific *S. cerevisiae* yeast strains develop a film, named "velo," which is the origin of the biological oxidation of the wine. Later they follow an aging process that could be "static" (always in the same barrel), or dynamic (more traditional). The traditional dynamic system, with several levels of barrels where the ones at the bottom are named "soleras" and hold the oldest wine, while the ones in the upper levels are increasingly younger wines ("criaderas"). Every year a portion of one third of the barrel is withdrawn from soleras and replaced with the corresponding wine from criaderas. Sherry wines can be aged for many years following this system.

Wine Grapes

Grape wines are made mainly from species *Vitis vinifera*. This is particularly true in Europe, where the major producers are France, Italy, and Spain. The native grapes in the United States belong to the species *Vitis labrusca* and several other species that have a

characteristic fruity, floral ("foxy") aroma, distinctly different from *vinifera* grapes. *Vitis rotundifolia* is an important species in the southeastern United States. However, most American wine is produced in California, based on the cultivation of *V. vinifera*. *V. vinifera* wines are the most appreciated and recognized by the consumers and thus, the most spread throughout the world. *V. vinifera* is sensitive to *Phylloxera*, insect imported from North America, that destroyed all the European vineyards in the late 19th century. Nowadays, with some exceptions, most of the *V. vinifera* is grafted on American cultivars which are resistant to *Phylloxera*.

Wine Classification

Wines are classified according to color (white, red, or rose/pink) and how much ethanol and sugar they contain. The color of the wine depends on two main variables: the color of the grapes (mostly their skins, where the pigments are highly concentrated) and the production system. Red wines are produced from pigmented varieties and produced by maceration of the fermenting must with the skins of the grapes. White wines can be produced from either red or white grapes but without maceration with the skins. Rosé wines are normally wines from red grapes that follow a "white" fermentation after a short maceration with the skins.

Most of the grape juices are fermented to dryness that is until all the sugar is transformed into ethanol. Other wines are partially fermented and according to the sugar content can be defined as semidry, semisweet, or sweet. These wines with sugar left are very unstable microbiologically, unless sulfites or alcohol are added. The wines with added alcohol are named "fortified" wines and several worldwide known wines use this practice (Porto, Sherry, Marsala, Madeira, some sweet muscat, "vins doux naturelles", etc.).

Finally, wines can be also denominated from the grape variety used. There are thousands of cultivars of *Vitis* species. Many yield wines with flavors and aromas reflecting their distinct type. The reader is referred to section "Further reading" for more information. A very wide variety of flavors, aromas, and colors of wines can be produced. The climate and the soil on which the grape variety grows and the particular growing year can have a marked effect on the flavor of the resulting wine. This leads to "vintage" years, in which a particular variety yields unusually high-quality wine. One of the most important wine classifications is the regional of geographical origin of the wines. This derives directly from the soil, climate and varieties that are used in a given area and determine a definite or typical character. This wine typicality is protected by an "Appellation of Origin" label or similar (the names are country specific and also their regulations). Normally all of them have a regulatory board that guarantees the origin of the product and, thus, its typicality.

Steps in Manufacture of Wine

1. Harvesting: grapes should be harvested when they are at their peak of maturity and desired flavor with a high sugar content and desirable acidity. They should be as intact as possible and be processed quickly.
2. Destemming: is the separation of the grapes from the stems. For white wines, the stems are removed and the grapes are crushed. The skins are retained for red wines. Sulfite may be added to inhibit the oxidation and growth of the natural microbiota.
3. Pressing: it is the separation of the juice from the skins, seeds, and pulp – the pomace. Pectinase may be added to facilitate juice extraction. Free-run juice, the juice running from the press before pressure is applied, is considered to be best for the highest quality wines.
4. Fermentation: fermentation can proceed either through the action of the naturally present yeast or by the inoculation of a selected pure yeast culture. In industry, the yeast inoculum may be produced in the company's own laboratory (normally restricted to large companies) or it may be purchased as an active dry yeast from companies specializing in the production of industrial cultures. Fermentation temperature is generally 10–18°C for white wines and somewhat higher (20–28°C) for red wines. The higher the temperature, up to about 30°C, the more rapid the fermentation; however, the slower, lower temperature fermentations are considered to lead to higher-quality wines because less volatile aroma is lost. In red wines, temperature is a compromise between the color extraction from macerated skins and the loss of aromas. As the fermentation proceeds, the actively metabolizing yeasts produce heat and the temperature of the must tends to rise. If not controlled, the temperature can reach a level at which yeast cells are killed or the ethanol becomes so inhibitory that the maximum ethanol content is decreased. The sugar content of the juice must be sufficient to yield a wine with the desired alcohol content. This requires a sugar content of about 13% w/v to yield a 7% v/v alcohol wine or about 22% to yield a 13% v/v alcohol wine. The sugar content of the juice may be raised before or during fermentation to adjust the final ethanol content. Water may be added to lower the acidity where the law allows amelioration. The governing bodies of wine making (Appellations of origin, International Organization of Vine and Wine, national or local governments) normally regulate these practices of water or sugar addition and they are banned in many places were high-quality wines are produced.

   A weight of carbon dioxide gas almost equivalent to the weight of ethanol produced is released during fermentation. This causes the fermentation to "boil" during its most active phases.

   The fermentation gradually ends as the fermentable sugar is consumed. With red wines, the pomace, skins, seeds, and other insoluble matter must be removed. This is usually performed when the color and tannin extraction have reached the maximum toward the end of fermentation or several days after completion of the fermentation. The separation of the pomace is accomplished by draining and pressing. With white wines, the pomace has already been removed during the pressing before fermentation.

5. Racking: as the fermentation nears completion, the yeast cells tend to settle, and after fermentation has been completed, the wine can be decanted or siphoned (racked) as the first step in clarification of the wine. Racking is repeated periodically until most or all the yeast cells (the lees, tartrate crystals, and other insoluble particles) have been separated from the wine. This may be combined with cold stabilization, which is accomplished by storing the wine at about −2°C. Cold stabilization removes excess tartrates and other materials that might cause cloudiness in the bottle later.
6. Clarification: most wine consumers like a crystal-clear wine with no haze or sediment. To accomplish this objective, gelatin, isinglass, or egg white may be added to the wine. These ingredients combine with tannin and form a fine precipitate that removes some of the materials that might cause turbidity. White wines may not contain sufficient tannin. In that case, some added tannin sufficient to react with the added protein may be necessary to produce the precipitate and stabilize the wine. Bentonite, a clay, is also widely used for clarification. Ion exchange may be used to change potassium to sodium tartrate, thus increasing the solubility and reducing the likelihood of its precipitation later.
7. Bottling: wines are generally bottled in dark green or brown bottles to decrease the deleterious effects on quality that can be caused by sunlight. Small amounts of sulfite may be added to inhibit oxidation. Sweet wines may be fortified with ethanol (18–20%) or the wines may be pasteurized or sterile-filtered (the choice for high-quality wines) to prevent the growth of contaminants in the bottle.
8. Aging: aging occurs after fermentation. It can occur in tanks, in barrels, or in the bottle. The flavor may be improved through esterification reactions in which small portions of ethanol combine with organic acids in the wine. Aging improves some wines, but has little effect on others.
9. The great enemy of stability in wine is oxygen and oxidation. Barrels, tanks, or bottles holding the wine must be kept free of oxygen. This is done principally by keeping the containers full or replacing the atmosphere in the container with nitrogen or carbon dioxide. In the case of sherries, however, there is a deliberate oxidation to modify the flavor, as discussed earlier.

Sparkling Wines

Still wines given a secondary fermentation in the bottle or in a tank to produce carbon dioxide under pressure are called "sparkling." When the secondary fermentation is produced in tanks under pressure the method is known as Charmat process, while the others where secondary fermentation occurs in bottles are named "traditional" or "champenoise" method. However, the term "Champagne" has been restricted to the sparkling wines produced in this region of France. The initial sugar content of the grapes should yield a dry still wine with about 10% v/v ethanol. The base wine, cuvee, is stabilized, fined, and filtered. It is often a blend of several wines to obtain the acidity and alcohol content desired with a clean flavor – no off-flavors or off-odors. Special wine yeasts that tend to agglomerate and thus can be more easily removed after the secondary fermentation are generally used. The same yeasts may also be used for the primary fermentation, if desired. It is particularly important that the yeasts do not produce any off-odors or off-flavors, as part of the sparkling wine flavor is derived from the yeast cells as they autolyze after the secondary fermentation. Sugar addition for the secondary fermentation is carefully controlled, as too little added will lead to a flat sparkling wine and too much sugar can lead to dangerous pressures and even explosions of the bottles. A sugar concentration of 2.4% w/v is enough to produce 7 atm pressure in the sparkling wine bottles, which generally will withstand 8 atm pressure.

In the Charmat process, the tanks offer the most efficient, lowest cost method of manufacturing sparkling wines; however, fermentation in the bottle with the longer contact of the wine with the yeast, particularly during autolysis, even though less efficient and more costly, generally yields the highest quality sparkling wines. If fermentation occurs in the bottle, the yeast cells and any precipitates must be removed after the secondary fermentation. This involves the riddling process, in which bottles are placed neck down in racks and turned a quarter turn every day or so until the yeast and all sediment in the bottle is in the neck against the cork or a crown seal. The bottles are then cooled to close to freezing, and the neck of the bottle containing the yeasts and precipitates is frozen. The bottle is then opened and held at such angle that the ice plug is blown out of the bottle. The volume lost is immediately replaced with a small quantity of sugar in wine (sometimes brandy), called "dosage." A dry sparkling wine is called brut and contains 0.5–1.5% sugar; sec indicates 2.5–4.5% sugar; doux contains 10% sugar. The bottles are sealed with a final cork and stored on their sides to age or labeled and sent to the distributor.

Fortified Wines

Although it is possible to ferment wines to ethanol levels of 18% v/v or higher if sufficient sugar is present and a lengthy fermentation is acceptable, wines containing higher concentrations of ethanol (i.e., 17–21%) have been generally fortified to the higher ethanol concentration by the addition of wine spirits produced by distillation of wine. Wine spirits contain about 95% ethanol v/v and are generally manufactured by the distillation of lower-quality wines.

Sweet dessert wines such as port, muscatel, or sherry, with sugar contents of 12%, generally start with grapes containing more than 25% sugar. Such wines are fortified by the addition of wine spirits to an ethanol content of 17–21% v/v. The addition of alcohol stops the alcoholic fermentation and thus, the amount of sugar left can be somehow controlled by the time of alcohol addition. Thus, very sweet wines can be produced with the addition of alcohol to the must or shortly after the start of fermentation.

In other cases the alcoholic fermentation proceeds a little bit longer. Normally high ethanol concentrations do not allow the survival of spoilage microorganisms such other yeasts or acetic acid bacteria.

Flavor and Acceptability

Wine preferences are strictly personal. No other fermented beverages offer so much variety in flavor, aroma, color, sweetness, and carbonation. Consumers should drink what they like within their economic means. There are many excellent values at reasonable cost on the market. Many new wine consumers prefer the sweet wines initially, but, over time, come to prefer dry wines, which provide more of the subtle flavors and aromas characteristic of individual grape varieties and which vary from country to country. Pairing of wine and food is becoming a very attractive social activity, as many wines go along very well with some specific tastes, increasing the appreciation of both the food and wines.

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X

Xylanases☆

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Glossary

Cellulose A complex carbohydrate $(C_6H_{10}O_5)n$ of D-glucose units that constitutes the chief part of the cell walls of plants. It is used in the manufacture of numerous products, such as paper, textiles, pharmaceuticals, and explosives.
Fungi A kingdom of life forms that are eukaryotic, mycelial, or yeast-like, heterotrophic, lacking in chlorophyll, sexually and/or asexually reproductive, and mostly aerobic.
Hemicellulose Hemicellulose is a term used to refer to a wide variety of heteropolysaccharides found in association with cellulose and lignin in both woody and herbaceous plant species. These heteropolymers vary in their monosaccharide composition, glycosidic linkage composition, substitution pattern, and degree of polymerization.
Lignin A highly stable polymer of mostly methoxylated phenyl-propanoic residues, synthesized as part of the cell wall of vascular plants. The monomeric components in lignin are p-coumaryl alcohol (p-hydroxyphenyl unit), coniferyl alcohol (guaiacyl unit), and sinapyl alcohol (syringyl unit).
Lignocellulose Lignocellulosic materials consist mainly of cellulose, lignins, and hemicelluloses. The proportions among these three main components vary considerably in different plants species.
Protein engineering The design, development, and production of new protein products having properties of commercial value.
Xylooligosaccharides Xylooligosaccharides are sugar oligomers made of D-xylose units. They are produced from xylan-rich lignocellulosic materials by chemical methods, autohydrolysis, direct enzymatic hydrolysis of xylan, or a combination of chemical and enzymatic treatments. Xylooligosaccharides have great prebiotic potential and can be incorporated into many food products.

Introduction

Plant cell wall is mainly constituted by cellulose, hemicellulose and lignin. The major constituent of hemicellulose is xylan. The degradation of the xylan polymer occurs by the action of glycosyl hydrolases (GH) and carbohydrate esterases (CE), which hydrolyze glycosidic bonds and ester bonds, respectively. *Endo*-1,4-β-xylanases or merely xylanases (EC 3.2.1.8) are important enzymes for xylan depolymerization, since they catalyze the random hydrolysis of β-1,4 glycosidic bonds inside the xylan chain (Polizeli et al., 2005; Álvarez-Cervantes et al., 2016). Xylanases are mainly produced by microorganisms and have special importance in several industrial sectors, such as supplement in animal feed, manufacture of bread, food and drinks, textile industry, pulp bleaching, ethanol and xylitol production (Polizeli et al., 2005).

The market for xylanases has been growing since the beginning of the 1990s. Because of that, various commercial xylanases, obtained from distinct biological sources, and, consequently, endowed of particular intrinsic properties, are now available for individual use or associated with other enzymes forming enzymatic cocktails. The development and application of molecular biology, structural biology and protein engineering has resulted in significant progress in the research on structures and functions of xylanases with industrial use (Yang et al., 2005). In nature, xylanases are clearly involved in providing sources of carbon and energy for the organisms that produce them, acting likewise for hosts harboring xylanase-producing organisms. Moreover, xylanases seem

☆*Change History*: April 2017. Paulo R. Heinen, Jorge H.A. Betini and Maria L.T.M. Polizeli substituted the old Figs. 1 and 2, as well as Table 1, while Table 2 was deleted. The sections of "Hemicellulose," "Xylanases," "Structure of Xylan," and "References" were added to the article, while the sections of "Introduction," "Properties of Xylanases," "Purification of Xylanases," "Applications" and "Conclusions" were reviewed and updated. The old sections of "Mode of Action of Xylanases" and "Production of Xylanases" were expanded and renamed as "Classification and Mode of Action of Xylanases" and "Production, Cloning and Expression of Xylanases", respectively.

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to be involved in the invasion of plants and fruits by pathogens (Bajpai, 2009). In the present article, we described multiple forms of these enzymes, displaying their mechanism of action, substrate specificities and physicochemical characteristics. The research has mainly focused on two of the xylanases-containing glycoside hydrolase families, namely families 10 and 11, although enzymes with xylanase activity belonging to GH families 5, 7, 8, 9, 12, 16, 26, 30, 43, 44, 51 and 62 have also been identified and studied, albeit to a lesser extent.

Hemicellulose

Schulze (1891) introduced the term "hemicellulose," denoting a substance somewhat similar in character to cellulose and that can be easily extracted, in comparison to cellulose, from plants by aqueous alkaline solutions. Hemicellulose is a class of polymers based on pentose and hexose sugars, or combinations of both, wherein xylan (polymer of D-xylose) is the most abundant polysaccharide among the constituents hemicellulosics. It is known that hemicellulose is found in the axial parenchyma, medullar rays and mainly in the plant secondary cell walls, where it can act as an adhesive by forming covalent and non-covalent bonds with lignin, cellulose and the other polymers essential to the integrity of the cellular wall (Polizeli, 2009).

The classification of the hemicellulose fractions is based on the main sugar in the polysaccharide backbone. The main monomers present in these hemicelluloses are D-xylose, D-mannose, D-glucose, D-galactose and L-arabinose. Hemicellulose includes xylan, xyloglucan, glucomannan, galactoglucomannan and arabinogalactan as its main heteropolymers. Additionally, hemicelluloses can present branched chains with D-glucuronic acid, D-galacturonic acid, 4-O-methyl-D-glucuronic acid, O-acetyl groups, ferulic acid and p-coumaric acid (Polizeli et al., 2005; van den Brink and de Vries, 2011). Hemicelluloses are non-crystalline, have lower molecular weight compared to cellulose and short lateral branches that are easily hydrolyzed (Bajpai, 2016). Furthermore, the precise composition of hemicellulose is highly dependent on the plant species and tissue of origin. Bamboo shoots, barley, oat, rice, rye, sorghum and wheat, for instance, produce satisfactory levels of glucuronoarabinoxylan (Izydorczyk and Biliaderis, 1995). Therefore, the hydrolysis of this polysaccharide produces the monomers corresponding to its classification. For example, the hydrolysis of glucuronoarabinoxylan releases D-glucuronic acid, L-arabinose, and, mainly, D-xylose units.

Hemicellulose may be extracted from plants by alkaline reagents, but autohydrolysis (thermogravimetric treatment) on agro-industrial residues has shown good results (Michelin et al., 2012), avoiding the chemical corrosions in equipment. The pre-treatments for the extraction of hemicellulose and lignin of plant cell wall are important for the separation of cellulose, which is used in the production of writing paper, as it will be reported at the end of this article. Besides, hemicellulose is important in the production of furfural, the raw material in the manufacturing of some plastics (furan resins, Bakelite) with a range of applications (Polizeli, 2009). In addition, monosaccharides obtained from hemicellulose can also be fermented by yeasts in the production of ethanol.

Structure of Xylan

Xylan is the most common hemicellulosic polysaccharide in cell walls of land plants, and is found in larger quantities in hardwoods from angiosperms (15–30% of the cell wall content) and lower abundance in softwoods from gymnosperms (7–12%) (Haltrich et al., 1996). Most xylans occur as a complex polysaccharide comprising a homopolymeric backbone of β-1,4-linked D-xylopyranosyl units substituted in varying degrees for D-glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, L-arabinofuranosyl, acetyl, feruloyl and/or p-coumaroyl side-chain groups. Based on the common substituents found on the backbone, xylans are categorized as arabinoxylan, glucuronoxylan and glucuronoarabinoxylan. In addition, a minority of xylans occurs as linear homoxylan, consisting exclusively of D-xylopyranosyl units. This type of xylan is not widespread in nature and has been isolated from limited sources, such as esparto grass, tobacco stalks and guar seed husks (Motta et al., 2013). Xylans with backbone of β-1,3 linked D-xylopyranosyl units have also been reported in marine algae (Dekker and Richards, 1976). Moreover, the xylan from the marine algae *Rhodymenia palmate* is another exception with an irregular form, consisting of a linear unsubstituted structure with approximately 80% of the D-xylopyranosyl units linked by β-1,4 glycosidic bonds and 20% of β-1,3 glycosidic bonds (Ferreira-Filho, 1994).

The two main types of xylan are represented by glucuronoxylan and glucuronoarabinoxylan. Glucuronoxylan is the main hemicellulose of hardwoods, such as aspen, birch and beech. This polysaccharide generally consists of 150–200 D-xylopyranosyl residues, linked by β-1,4 glycosidic bonds. Every tenth of xylose residue carries a 4-O-methyl-D-glucuronopyranosyl attached to its 2-position, by α-1,2 glycosidic bonds. Furthermore, hardwood xylans have a high rate of substitution (70–80%) for acetyl groups, at the position 2 and/or 3 of the D-xylopyranosyl units, conferring partial solubility for these xylans in water (Coughlan and Hazlewood, 1993). Acetylation occurs more frequently at the O-3 position than at the O-2 position. Birchwood xylan, for example, contains more than 1 mol of acetic acid per 2 mol of D-xylose residue. However, these acetyl groups are readily removed when the xylans are subjected to alkali extraction (Sunna and Antranikian, 1997).

On the other hand, xylans from softwood are composed mainly of glucuronoarabinoxylans. They have a higher 4-O-methyl-D-glucuronopyranosyl content than do hardwood xylans, and are not acetylated. Likewise, D-glucuronopyranosyl units methylated at position O-4 are preferably attached to the O-2 position of D-xylose residues of the xylan backbone. Softwood xylans have L-arabinofuranosyl groups linked with the O-3 position of each tenth D-xylose residue, by α-1,3 glycosidic bonds (Puls, 1997).

The L-arabinofuranosyl substituents occur in almost 12% of the D-xylose units (Wong et al., 1988). Softwood xylans are less branched and shorter than hardwood xylans, with a degree of polymerization between 70 and 130 (Sunna and Antranikian, 1997).

According to Bajpai (2009), there is an intrinsic relationship between the xylan chemical structure versus botanical origin and cellular localization. This fact results in a certain degree of complexity of xylan-containing materials, and various xylan-related structures are described, which may differ by major or minor particulars. In some tissues of cereals and grasses, up to 50% of the biomass corresponds to xylans (Ebringerová, 2005). Generally, this xylan polymer is present in tissue secondary walls having structural functions, but it occurs in some extension in the primary walls of growing cells, seeds and bulbs of certain plant species that have reserve functions.

Xylans are covalently bound with the overlapping sheath of lignin (Biely, 1985). The layers of covalently linked xylans and lignin, and the non-covalent interaction of xylans with cellulose fibers are important for the maintenance of the integrity of the cellulose in situ, and consequently, for the protection of the fibers against the action of cellulases (Uffen, 1997). In this way, pretreatment of lignocellulosic material before enzymatic hydrolysis is an interesting step due to the complex structure of the plant cell wall, increasing the accessibility of the xylanases and other lignocellulolytic enzymes in the microcrystalline lignocellulose fibers. The pretreatment procedures aim to remove lignin and hemicellulose, reducing cellulose crystallinity and increasing the porosity of the lignocellulosic materials. There are various pretreatments described in the literature, with different characteristic for distinct biomass: physical (mechanical comminution and extrusion), chemical (acid and alkaline pretreatments, organosolv and ionic liquids), physicochemical (steam explosion, ammonia fiber explosion and liquid hot water), biological (using microbial biocatalytics, as lignin-degrading enzymes, for example) and integrated methods of these pretreatments, in order to improve the efficiency of fractionating, decrease the formation of inhibitors and shorten process time (Polizeli et al., 2016a).

Conformational analysis of xylans and other component of biomass have been carried out by several techniques. For the purpose of screening, the high throughput analysis of recalcitrance and high throughput analysis of chemical fingerprint may be used, which are a modified pyrolysis molecular bean mass spectrometry method to detect the presence of cellulose, xylan, lignin, syringyl and guaiacyl units. The glycome profiling is also a technique based on the combination of monoclonal antibodies and carbohydrate-binding modules for monitoring epitopes of cell wall glycans. However, downscaled compositional analysis includes, among others, the high-resolution methods, such as (1) sequential extraction with solvents and reagents; (2) gel permeation chromatography coupled to polymer derivatization and light scattering, refractive index and viscometry detectors to provide molecular weights and consequent DP of polymers; (3) spectroscopy, such as liquid-state NMR Spectroscopy coupled with extraction techniques (which have gained attention due to their low destructive characteristic and level of information generated) and solid-state NMR Spectroscopy (which uses the native cell wall to analyze the spatial dimensions between biopolymers, ultra-structures and pore structures), while the Fourier-Transform Infrared Spectroscopy (FTIR) offers functional group and chemical linkage profiles; there is also the X-ray diffraction allowed to determine crystallinity index; and (4) imaging of biopolymers, which includes the scanning electron microscopy, transmission electron microscopy, time-of-flight secondary ion mass spectrometry (ToF-SIMS), MALDI-imaging MS (MALDI-IMS), mode-synthesizing atomic force microscopy (MSAFM), a variant of the atomic force microscopy and the confocal laser scanning microscopy (CLSM) (Polizeli et al., 2016a).

Xylanolytic System

Due to the heterogeneity and complexity of xylan structures, their complete hydrolysis requires several enzyme activities that act cooperatively and synergistically to convert xylan into its monomeric sugars of origin. The degradation of the xylan polymer occurs through the action of *endo*-1,4-β-xylanases, which cleaves the xylan backbone into smaller xylooligosaccharides, and β-xylosidase, which cleaves the xylooligosaccharides into D-xylose residues. In addition, accessory enzymes contribute to the release of all substitutions present on the main backbone and, therefore, achieve the complete degradation of the xylan polymers (Polizeli et al., 2016b).

The xylanolytic system includes the (1) glycosyl hydrolases (GH), which hydrolyze glycosidic bonds, and (2) carbohydrate esterases (CE) which hydrolyze ester bonds of acetate, ferulic and p-coumaric acid side groups. Here, the GHs are described first and, thereafter, the action of CEs.

- *Endo*-1,4-β-xylanases (E.C.3.2.1.8) are the main glycosyl hydrolases of the xylanolytic system, and are commonly named, in the literature, only by "xylanases." These *endo*-enzymes hydrolyze β-1,4 glycosidic bonds inside the xylan chain producing xylooligosaccharides. The rate of the hydrolysis reaction normally decreases with the decrease in the chain length of xylooligosaccharide substrates (Polizeli et al., 2005; Nacke et al., 2012). In general, *endo*-1,4-β-xylanases do not hydrolyze xylobiose, and the hydrolysis of xylotriose is in most cases negligible or at least limited. Both the length and the type of the products released depend on the mode of action of each xylanase (Bajpai, 2009).
- Xylan-1,4-β-xylosidases (E.C.3.2.1.37) have been classified into the GH families 3, 39, 43, 52 and 54 based on the amino acid sequence similarities (Xia et al., 2015). β-xylosidases act cooperatively with *endo*-1,4-β-xylanases, converting the xylooligosaccharides from the non-reducing end until D-xylose units. Some β-xylosidases show transxylosylation at high substrate concentrations, which can be used to produce novel xylose-containing xylooligosaccharides (Kurakake et al., 2005).
- α-glucuronidases (E.C.3.2.1.139) hydrolyze the α-1,2 glycosidic bonds between xylose residues and glucuronic acid or 4-O-methyl-D-glucuronic acid in glucuronoxylan and/or glucuronoarabinoxylan (Polizeli et al., 2005; Gírio et al., 2010).

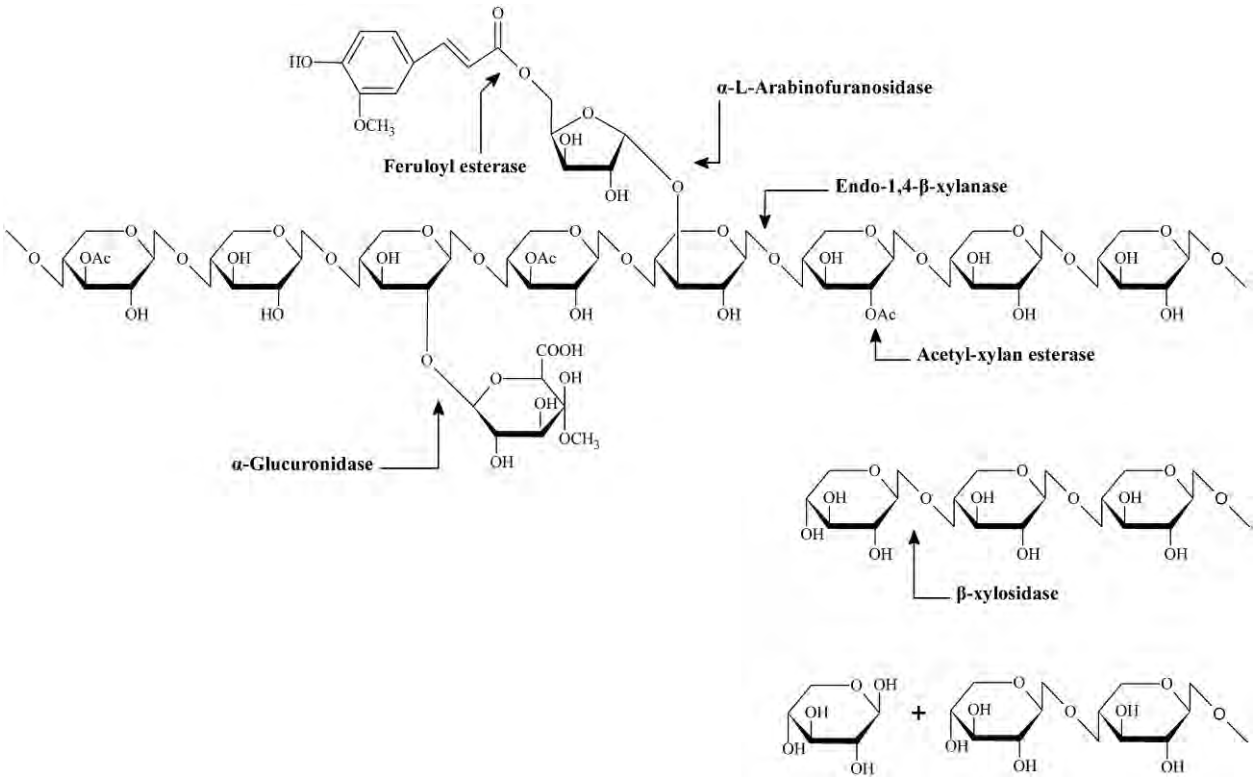

Fig. 1 Schematic representation of the xylan polymer and xylanolytic system enzymes.

- α-L-arabinofuranosidases (E.C.3.2.1.55) are enzymes that hydrolyze the terminal non-reducing L-arabinofuranosyl groups from L-arabinose-containing polysaccharides attached to the O-2 and/or O-3 positions of D-xylose residues (Margolles and de los Reyes-Gavilán, 2003; Polizeli et al., 2005; Burlacu et al., 2016). The removal of L-arabinose side residues allows a better access of *endo*-1,4-β-xylanases and β-xylosidases to the xylosidic linkage in the xylan backbone (Sun et al., 2012).
- Acetyl-xylan esterases (EC 3.1.1.72) are carbohydrate esterases that cleave the ester linkages of the xylan acetyl groups, releasing acetyl substituents from the position O-2 and/or O-3 of D-xylose residues in glucuronoxylan (Polizeli et al., 2005).
- Feruloyl esterases (EC 3.1.1.73) and p-coumaric acid esterases (EC 3.1.1.-) cleave ester bonds between ferulic acid and L-arabinose residues and p-coumaric acid and L-arabinose residues of xylan, respectively (Segato et al., 2014; Burlacu et al., 2016).

Fig. 1 shows a hypothetical plant xylan structure and different substituent groups with the possible sites of attack by microbial xylanolytic enzymes.

Classification and Mode of Action of Xylanases

Endo-1,4-β-xylanases, as GH members, have been classified based on at least three criteria: (1) molecular weight and isoelectric point, (2) kinetic properties or the substrate specificities and (3) similarities in primary structure together with the conservation of the catalytic residues and catalytic mechanism (Collins et al., 2005; Motta et al., 2013). Updated information on the functional characteristics, sequence annotations and family classification of enzymes may be found in the carbohydrate-active enzyme (CAZy) database (Cantarel et al., 2009). According to the search in this database by Motta et al. (2013), *endo*-1,4-β-xylanases (EC 3.2.1.8) are related to GH families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62.

Literature survey shows that only the enzymes classified in families 5, 7, 8, 10, 11 and 43 contain truly distinct catalytic domains with *endo*-xylanase activity, while the enzymes classified in families 16, 51 and 62 seem to be bifunctional enzymes containing two catalytic domains. Members of families 9, 12, 26, 30 and 44 should be considered as having a residual or secondary xylanase activity (Collins et al., 2005; Motta et al., 2013). On the basis of primary sequence homology and hydrophobic cluster analysis, *endo*-1,4-β-xylanases have mainly been grouped into glycoside hydrolase families GH10 and GH11, which correspond to former families F and G, respectively (Wang et al., 2014).

Members of families 5, 7, 8, 10, 11 and 43 differ in their physicochemical properties, structure, substrate specificity and mode of action (Taibi et al., 2012). Generally, their mode of action results in the release of products with either retention (GH families 5, 7, 10 and 11) or inversion (GH families 8 and 43) of the substrate anomeric configuration (Collins et al., 2005; Motta et al., 2013; Álvarez-Cervantes et al., 2016; Heinen et al., 2017). Families 5, 7, 10 and 11 contain enzymes that catalyze the xylan hydrolysis with

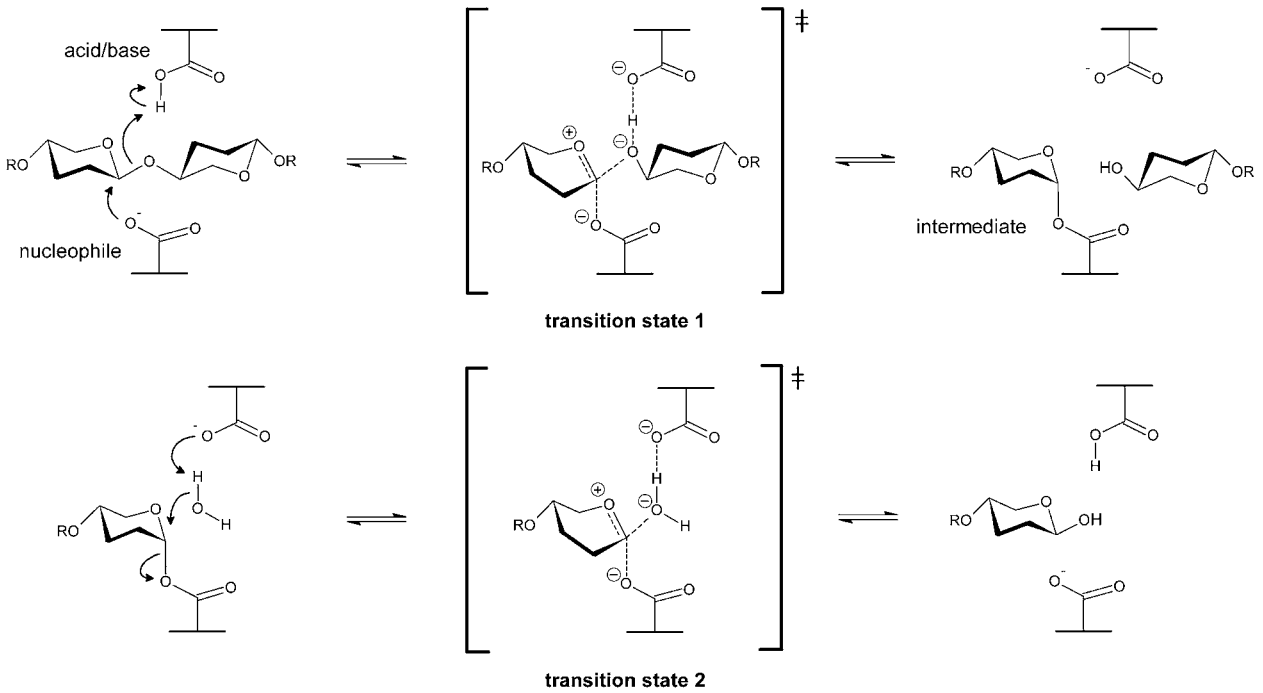

Fig. 2 A hypothetical model of double-displacement reaction mechanism for retaining xylanases. Figure was prepared with ChemSketch version 12.0.

the involvement of a pair of glutamate residues at the active site. That indicates a double-displacement mechanism, in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolyzed via oxocarbenium-ion-like transition state. Primarily, two carboxylic acid groups are involved in the formation of the intermediate, wherein one of them acts as a general acid catalyst by protonating the substrate while the second performs a nucleophilic attack, which results in the departure of the leaving group and the formation of the α-glycosyl enzyme intermediate (β to α inversion). In the second step, the first carboxylate group now functions as a general base, subtracting a proton from a nucleophilic water molecule, which attacks the anomeric carbon. That leads to a second substitution, in which the anomeric carbon again passes through a transient state to give rise to a product with the β configuration (Collins et al., 2005; Motta et al., 2013; Álvarez-Cervantes et al., 2016). Thus, the overall result of this catalytic mechanism is the retention of the configuration in the anomeric center, as shown in Fig. 2.

Xylanases from the GH11 family have low molecular weight, high pI values and a tertiary structure known as β-jelly-roll, while those from the GH10 family have high molecular weight, lower pI values and display an (α/β) 8-barrel fold (Collins et al., 2005; Motta et al., 2013; Álvarez-Cervantes et al., 2016).

The glycoside hydrolase family 10 consists of *endo*-1,4-β-xylanases (EC 3.2.1.8), *endo*-1,3-β-xylanases (EC 3.2.1.32) and cellobiohydrolases (EC 3.2.1.91). Although the main members of this family are *endo*-1,4-β-xylanases, studies about substrate specificities have revealed that these may not be highly specific for xylan and may also be active on cellulose substrates of low molecular weight (Collins et al., 2005; Cheng et al., 2009; Motta et al., 2013). Furthermore, these enzymes are highly active on xylooligosaccharides of low polymerization degree, thereby indicating that their substrate-binding sites are small and apparently not so deep clefts (Biely et al., 1997; Álvarez-Cervantes et al., 2016).

In contrast to all other families, the GH11 family is monospecific since it consists only of *endo*-1,4-β-xylanases (EC3.2.1.8). Its members are considered as true xylanases because they are exclusively active on xylose-containing substrates. Compared to other xylanases, GH11 members display several interesting biochemical properties, such as high catalytic efficiency and substrate selectivity, a small size and a variety of optimum pH and temperature values, making them suitable to various conditions and in many industrial applications (Shrinivas et al., 2010; Motta et al., 2013). These enzymes are most active on xylooligosaccharides with higher degree of polymerization. Indeed, it has been found that they have larger substrate binding clefts than do higher molecular weight xylanases (Biely et al., 1997; Shrinivas et al., 2010; Motta et al., 2013).

Properties of Xylanases

Microbial xylanases have been extensively studied in diverse microorganisms. Table 1 illustrates the intrinsic characteristics of fungal xylanases from some species of *Aspergillus*, *Fusarium*, *Penicillium*, *Scytalidium*, *Talaromyces*, *Thermomyces* and *Trichoderma*. The majority of the xylanases purified from fungi present molecular weight around 20–40 kDa, but xylanases with lower and higher molecular weight have also been found, as the minor xylanase (7.7 kDa) isolated from *Aspergillus caespitosus* (Sandrim et al., 2005) and the xylanase (66 kDa) from *Aspergillus fumigatus* (Thiagarajan et al., 2006), respectively. In general, these enzymes are monomeric

Table 1 Physicochemical characteristics of xylanases from different fungi

Fungus	Molecular weight (kDa)	pI	Optimum pH	Optimum Temperature (°C)	Substrate	Km value (mg/mL)	GH family	References
Aspergillus carneus	18.8	7.7–7.9	6.0	50	Birchwood xylan	–	11	Fang et al. (2008)
Aspergillus niger	25.0	–	5.5	55	Birchwood xylan	0.10	11	Yang et al. (2010)
A. niger	23.0	5.6	5.5	50	Oat spelt xylan	7.10	11	Levasseur et al. (2005)
Aspergillus terreus	19.0	9.0	7.0	50	Birchwood xylan	2.50	11	Kocabas et al. (2011)
Fusarium oxysporum	38.0	9.5	7.0	45	Oat spelt xylan	0.80	10	Christakopoulos et al. (1997)
Fusarium proliferatum	22.4	–	5.0–5.5	55	Oat spelt xylan	8.40	11	Saha (2002)
Penicillium funiculosum	23.6	3.7	5.0	55	Birchwood xylan	4.70	11	Furniss et al. (2002)
P. funiculosum	36.0	4.6	4.2–5.2	–	Birchwood xylan	2.00	10	Furniss et al. (2005)
Penicillium purpurogenum	33.0	8.6	7.0	60	Birchwood xylan	–	10	Belancic et al. (1995)
P. purpurogenum	23.0	5.9	3.5	50	Birchwood xylan	–	11	Belancic et al. (1995)
Scytalidium thermophilum	21.0	8.6	6.5	65	Beechwood xylan	2.40	11	Kocabas et al. (2015)
Talaromyces thermophilus	25.0	–	7.0–8.0	75–80	Birchwood xylan	22.51	11	Maalej et al. (2009)
Thermomyces lanuginosus	23.6	3.8	6.5	70–75	Birchwood xylan	3.26	11	Lin et al. (1999)
Trichoderma sp.	30.1	–	5.5	70	Beechwood xylan	4.47	10	Chen et al. (2009)
Trichoderma sp.	20.1	–	5.0	55	Beechwood xylan	8.33	11	Chen et al. (2009)
Trichoderma reesei	32.0	9.1	6.0	55	Birchwood xylan	2.02	10	Xu et al. (1998)

proteins and display very varied pI values. Some fungal xylanases have acidic pI (3.7–3.8), as in *Penicillium funiculosum* and *Thermomyces lanuginosus*, while others have basic pI (7.7–9.5) (Table 1).

The optimum pH values are generally around 5.0–7.0, for most fungal xylanases. However, there are exceptions with extreme pH values. Al Balaa et al. (2009), for example, reported an acidic pH value of 3.2 to xylanase from *Scytalidium acidophilum*. In contrast, xylanases with alkaline pH values of 8.0 and 8.5 have also been isolated from *Fusarium graminearum* (Beliën et al., 2005) and *Penicillium citrinum* (Dutta et al., 2007), respectively. Moreover, xylanases are normally stable in the range of pH 2–9 (Bajpai, 2009). The optimum temperature range of most fungal xylanases is between 50 and 60°C, while the xylanases from thermophilic fungi produce enzymes with maximum activity at higher temperatures, as reported for *Talaromyces thermophilus* and *T. lanuginosus* (Table 1). Regarding thermal stability, microbial xylanases generally have a wide range of temperature tolerance (20–60°C).

Production, Cloning and Expression of Xylanases

Xylanases are produced by various organisms, such as fungi, bacteria, yeast, protozoa, snails, crustaceans, insect and seeds (Suzuki et al., 1991; Devillard et al., 1999; Suda et al., 2003; Brennan et al., 2004; Crawford et al., 2005). Filamentous fungi are particularly interesting producers of these enzymes from an industrial point of view, due to the fact that they secrete xylanases into the medium. Furthermore, xylanase levels from fungal cultures are generally much higher than those from yeasts or bacteria. In addition, fungi tend to produce mixtures of xylan-degrading enzymes, different xylanases combined with accessory enzymes for debranching substituted xylans (Haltrich et al., 1996).

The cost-effectiveness, ease of use and eco-friendliness of enzymes are factors that justify the use of a biocatalytic process. In order to meet industrial requirements, an ideal xylanase should have specific properties, such as stability over a wide range of pH values and temperatures, high activity, and strong resistance to metal ions and chemicals. Therefore, most of the xylanases reported do not possess all these physicochemical and functional characteristics required by industries. Moreover, the native enzymes are not

sufficient to supply the demand, due principally to low yields and incompatibility of the standard industrial fermentation processes (Motta et al., 2013).

In this context, molecular approaches can be implemented to design xylanases with the characteristics required. Recombinant DNA techniques offer opportunities for the construction of microbial strains with selected enzyme machinery, and also allow large scale expression of these enzymes in both homologous and heterologous protein expression hosts (Juturu and Wu, 2012). In xylan bioconversion, for instance, the main objective for recombinant DNA technology is improvement of the fermentation characteristics of xylose-fermenting industrial strains by introducing xylanase and β-xylosidase genes, so that the direct fermentation of xylan is possible (Bajpai, 2009).

Both the prokaryotic and eukaryotic organisms are commonly used as hosts for the recombinant protein expression. The system selection depends on protein, desired yield and the requirements for its function activity (Chakravorty and Patra, 2013). *Escherichia coli* is the prokaryotic organism most widely used for expressing recombinant proteins. This host, as a recombinant expression platform, has multiple advantages, such as rapid growth on inexpensive media, simple techniques required for transformation, easy isolation and purification of expressed enzymes. Nevertheless, it does not provide efficient and functional heterologous expression of many xylanases. This problem can be due to the repetitive appearance of rare codons and the requirement for specific post translational modifications such as disulfide bond formation and N-glycosylation. *E. coli* can only perform simple O-glycosylation, while the *Lactobacillus* species and *Bacillus subtilis* are able to perform N-glycosylation and possess Generally Regarded As Safe status (GRAS), making them more accepted for use in food and pharmaceutical industries. Moreover, xylanase expression levels are relatively higher in *Lactobacillus* species and *B. subtilis* than in *E. coli* (Juturu and Wu, 2012; Motta et al., 2013).

On the other land, protein expression in yeast and fungal systems are also highly attractive, once they provide additional benefits over bacterial expression systems. Among these benefits are the ability to perform eukaryotic post-translational modifications and the ability to secrete proteins into the fermentation media. Yeast is free of toxins and the majority has GRAS status. Accordingly, *Saccharomyces cerevisiae* for secreting high amounts of enzymes into the culture medium is an ideal microorganism in the large-scale production of xylanases at low costs. Indeed, it has already been established as an industrial microorganism. *Pichia pastoris* has also emerged as an excellent host for the commercial production of xylanases. However, the expression under the methanol-inducible alcohol oxidase promoter, the first enzyme involved in the methanol utilization pathway, has limited the use of this recombinant expression platform in a large scale due to the health and fire hazards of methanol. Recently, fungal expression systems have been preferred for expressing xylanases. Additional advantages of fungal systems are experiences in handling fungal cultivation and feasibility of using native xylanase expressing machinery for functional expression of foreign xylanases from remote sources (Juturu and Wu, 2012; Motta et al., 2013). For instance, the xylanase genes from *Malbranchea pulchella* and *Aspergillus niveus* were cloned and successfully expressed in *Aspergillus nidulans* (Damasio et al., 2011; Ribeiro et al., 2014).

Purification of Xylanases

The commercial and industrial applications usually require the development of cost-effective strategies not only for the large-scale production of xylanases, but also for their purification. Utilization of crude enzyme for some applications might be beneficial, such as bioethanol production and waste treatment, where synergistic effects of glycoside hydrolases are desirable for lignocellulosic biomass deconstruction. On the other hand, a high degree of purity is required for the food and pharmaceutical industries, since the presence of interfering enzyme activities and undesirable secondary metabolites in the preparations prevent their application in such industries (Kumar and Sharma, 2015; Yegin, 2017).

Xylanase purification schemes have generally used different methods including salt fractionation, ion-exchange, gel filtration and hydrophobic interaction chromatography. The low molecular weight of certain xylanases has also enabled their separation from other proteins using ultrafiltration (Bajpai, 2009). However, the rapid and economical purification of microbial xylanases represents a persistent challenge for their industrial applications. Purification of target enzymes from a pool of proteins typically involves several tedious purification steps, and each of which can be costly and time-consuming, decreasing the final yield of the product (Banki et al., 2005; Juturu and Wu, 2012). In this context, the importance of affinity chromatography has increased over the years due to the advantages of recombinant DNA technology. The sequence of protein labels may be incorporated into the gene of interest, which will make purification and isolation of the target protein in a much easier way (Ravindran and Jaiswal, 2016). In particular, the histidine (His) tag has been widely used to facilitate purification of recombinant enzymes. This tag is comprised of a sequence of six consecutive histidine residues which are added to either the N or C terminal of recombinant protein sequence and exhibit high affinity toward metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} (Terpe, 2003; Saraswat et al., 2013). After binding, the tagged enzymes can be eluted from immobilized metal affinity chromatography under mild conditions with a high degree of purity and recovery. This single-step purification strategy helps to decrease the processing cost, thus broadening the scope of xylanse applications (Yegin, 2017).

Applications

Over the last few years, interest in xylanases from microorganisms has rapidly increased because of their potential application in the bioconversion of lignocellulosic materials into value-added commodities, as well as the traditional applications in

food, feed, pharmaceutical and paper industries. These enzymes not only make the process environmentally benign, but also play an important role in improving the productivity and reducing the cost of product formation (Deswal et al., 2012; Motta et al., 2013).

Pulp and Paper Industry

Xylanases are commercially used in the bleaching of the kraft pulp. The use of xylanases is often referred to as a prebleaching process because the nature of its effect, which consists of enhancing the efficiency of bleaching chemicals rather than removing lignin directly (Bajpai, 1999). These enzymes do not attack the lignin-based chromophores but rather the xylan network by which the residual lignin particles are surrounded and trapped (Bajpai et al., 2006). A limited hydrolysis of the xylan network is often enough to ease the subsequent chemical attack on lignin with various bleaching chemicals, without sacrificing the yield (Bajpai, 1999, 2009).

The application of enzymes in pulp bleaching was first published by Viikari et al. (1986). In that study, the results indicated that xylanases are capable of enhancing the effects of bleaching, resulting in savings of 10–20% of chemicals. Subsequent research was directed at enzymes that oxidize lignin such as laccases, but these enzymes have only a limited effect on the pulp bleaching (Viswanath et al., 2014). Following that, an important advance was achieved with the discovered of a mediator called 2,2′-azinobis(3-ethylbenzthiazoline-6 sulfonate) (ABTS), which increases the efficiency of laccases in the bio-bleaching (Bourbonnais et al., 2000). The Finnish forest companies were the first in the world to start mill-scale trials, in 1988. In North America, the first mill trials with xylanases were carried out in Port Alberni, in 1991, and ongoing usage in some mills started in 1992 (Bajpai, 2009). Currently, a significant number of European, North American, South American and Japanese mills are using xylanases as a prebleaching process.

The various pulp and paper industries have different strategies and processes regarding the sequential stages of bleaching cellulose pulp. Basically, the chemical elements most commonly used in traditional bleaching are: sodium hydroxide (NaOH), sodium sulfide (NaH_2S) (high-temperature cooking stage and pressure), chlorine dioxide (ClO_2), hydrogen peroxide (H_2O_2), oxygen (O_2) and ozone (O_3) (bleaching stage). The most commonly used extraction agent is sodium hydroxide (Polizeli et al., 2005; Polizeli, 2009). The companies differ in the bleaching process according to the desired degree of pulp and paper whiteness. The most common sequences are those divided into 4 or 5 stages according to the chemical combination chosen.

The control of bleaching and bleaching reactions is important in the pulp and paper industry to ensure the quality of the final product (de Vaal and Sandrock, 2007). The main variables in each stage of bleaching are: chemical additives, reaction time, pulp consistency, intensity of agitation, pH and reaction temperature. The kappa number and pulp viscosity are control parameters widely used to evaluate the pulp quality during the stages of its production. The first parameter determines the amount of residual lignin in the fibers that make up the cellulosic pulp, while the viscosity measures the degree of degradation these fibers, since the more degraded the fibers are in the cellulosic pulp, the lower its viscosity and whiteness will be.

At present, many sectors of the pulp and paper industries have adhered to the production and processes that no longer use chlorine as an oxidizing element in the bleaching stages of cellulosic pulp. That can be considered an ecological process and is known as EFC (Elementar Free Clorine). However, the use of chlorine dioxide is still quite common. In this context, the use of enzymes such as xylanases at certain stages of the bleaching process allows the decrease in the amount of chemicals, thus reducing the effluents to be treated and, consequently, their environmental impacts (Bajpai, 2009; Gangwar et al., 2014).

Food Industry

Xylanases have applications in different sectors of food industry. Using xylanases in the bread-making industry significantly improves the desirable texture, loaf volume and shelf life of the bread (Dobrev et al., 2007; Heinen et al., 2014). Xylanases, together with pectinases, cellulases and amylases, are used for the clarification of juices (Motta et al., 2013). They are also used in the brewing industry in order to improve filtration efficiency (Wang et al., 2016). In addition, in recent years, there has also been an interest in the production of D-xylose and short-chain xylooligosaccharides by the enzymatic hydrolysis of xylan. Xylooligosaccharides are important ingredients for the development of novel functional foods due to their prebiotic effects, since they increase mineral absorption, induce peristalsis and act as regulatory substrate in the intestinal flora (Heinen et al., 2017; Yegin, 2017). The D-xylose residues, in turn, may be converted to xylitol, a valuable sweetener widely used as a sugar substitute for the diabetic population (Meyrial et al., 1991; Sena et al., 2016).

Pharmaceutical Industry

The features of xylooligosaccharides such as resistance to heat up to about 100°C and stability over a wide range of pH (2.5–8.0) make them good candidates for drug formulation and pharmaceutical applications. XOS, as bioactive components, can thus be used as a preventative agent against oxidative stress, anemia, arteriosclerosis, atherosclerosis, diabetes, osteoporosis and certain types of cancers (Deutschmann and Dekker, 2012). It was also demonstrated that XOS can have immunomodulatory activity, anti-microbial activity, growth regulator activity and other biological activities like anti-allergic, anti-inflammatory,

anti-hyperlipidemic and cosmetics (Aachary and Prapulla, 2011; Deutschmann and Dekker, 2012; Kumar et al., 2012; Gupta et al., 2016).

Animal Feed

Animal feed, including cereals like wheat, triticale, rye, barley and soy-based diet, are rich in biomass composed by cellulose, hemicellulose and lignin. However, the viscous properties of these lignocellulosic materials hinder their digestion (Kalim et al., 2015). The addition of xylanases ensures the utilization of nutrients trapped inside the plant cells, while they also reduce the viscosity created by non-starch polysaccharides (NSPs) in the animal's digestive tract (Pirgozliev et al., 2015). Addition of exogenous xylanases to the animal diets has shown beneficial effects on feed conversion efficiency, growth and weight gain in monogastric animals (Yegin, 2017). Furthermore, specific xylooligosaccharides can be produced through the use of appropriate xylanases in pig and poultry diets to improve their prebiotic action (Choct, 2015).

Xylanases can also be used to improve the nutritional properties of agricultural silage. Treatment of forages with hydrolytic enzymes results in silage of better quality and improves the subsequent rate of plant cell wall digestion by ruminants (Kalim et al., 2015). There is a considerable amount of sugar sequestered in the xylan of plant biomass. In addition to converting hemicellulose into nutritive sugars that the cow or other ruminant can digest, xylanases also produce nutritionally suitable compounds for the desired ruminal microflora, which in turn further assist digestion (Bajpai, 2009; Kalim et al., 2015).

Bioethanol Production

Currently, important variables such as global climate change, scarcity of petroleum reserves, and increase in the costs of fuels have stimulated an unprecedented research into the production of alternative fuels, preferably from renewable energy sources (Polizeli et al., 2016b, Uday et al., 2016). For such reason, bioethanol is gaining great importance as a renewable and sustainable source of fuel (Kanimozhi and Nagalakshmi, 2014). Despite the notorious worldwide production of first-generation ethanol, from sucrose and starch, the production of second-generation ethanol has a greater advantage because it does not compete with food production and can provide environmental, economic and strategic benefits for the production of biofuels (Sarkar et al., 2012; Motta et al., 2013).

In second-generation bioethanol production, the first step is the delignification of lignocellulose, to release cellulose and hemicellulose from their complex with lignin. The second step corresponds to the hydrolysis of carbohydrate polymers to produce reducing sugars, as D-glucose, D-xylose and L-arabinose, followed by the fermentation of mixed pentose and hexose sugars by yeast up to bioethanol (Polizeli et al., 2016b). In this context, xylanases together with other accessory enzymes can efficiently be used to increase the depolymerization of the carbohydrate polymers from lignocellulosic biomass, leading to a more economical and viable production of bioethanol (Motta et al., 2013).

Conclusions

In nature, endo-1,4-β-xylanases are synthesized by diverse organisms, but those with biotechnological applications are obtained from microorganisms, mainly filamentous fungi because they may secrete enzyme to the medium. In general, such enzymes are produced in submerged or solid-state fermentation using xylan extracted from different plants, but in order to reduce costs, agro-industrial residues as sugar cane bagasse, wheat straw and others, may be used as inductor. Innumerous applications are attributed for xylanases, which have special importance in several industrial sectors, such as supplement in animal feed, manufacture of bread, food and drinks, textile industry, pulp bleaching, effluent treatment, agro-waste treatment, ethanol and xylitol production. Currently, this enzyme have been widely used in the cellulose-pulp bleaching with the objective of reducing the large amount of chemical solvents applied in the conventional Kraft process. The use of chlorine dioxide results in the production of organochlorine compounds from the degradation of lignin, and these are highly toxic and mutagenic, requiring effluent treatments from the paper-making plant to make the paper production a less harmful process to nature. Regarding this problem, environmental restrictions about the use of chlorine compounds in the paper industry are being implemented, but a drastic change in the existent mentality from people is necessary.

Another important focus is the prospection of novel xylanases with suitable properties for biorefinery, aiming at biofuel production from biomass. In this context, microorganisms with high xylanase secretion are desirable, and enzymes with high resistance to end-products, as D-xylose. Moreover, for this target to be successful, the prospection of thermophilic microorganisms is necessary, as well as searches for enzymes with high resistance to the temperatures and pH range, development of recombinant DNA technologies for super-expression of these enzymes and yeasts with metabolism regulated adequately for the fermentation of pentasaccharides released after the action of hydrolytic enzymes. Concluding, in order to be effective in the several industrial sectors reported, xylanases must not be used alone, but in combination with other enzymes, forming an enzymatic cocktail for complete degradation of biomass. Currently, the commercial cocktails available for the bioenergy field are still catalytically incomplete (and expensive), and more studies about enzymes structural and functional characterization will provide additional tools to fill the scientific knowledge gap.

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